ECOLOGICAL AND EVOLUTIONARY ASPECTS OF COMPLEX RELATIONS BETWEEN MICRO- AND MACROPARASITES AND THEIR WILD ANIMAL HOSTS

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ECOLOGICAL AND EVOLUTIONARY ASPECTS OF COMPLEX RELATIONS BETWEEN MICRO- AND MACROPARASITES AND THEIR WILD ANIMAL HOSTS

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frontiers in Veterinary Science

EDITORIAL
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Editorial: Ecological and Evolutionary Aspects of Complex Relations Between Micro- and Macroparasites and Their Wild Animal Hosts

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Keywords: one health, wildlife, zoonotic disease, evolutionary ecology, disease ecology, conservation medicine

Editorial on the Research Topic

Ecological and Evolutionary Aspects of Complex Relations Between Micro- and Macroparasites and Their Wild Animal Hosts

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Disease ecology emphasizes how ecological interactions between microparasites (pathogenic microbes), macroparasites (helminths, protists), and animal hosts help understanding transmission of diseases and parasites within the epidemiological landscape. Diseases and parasites are ecological and evolutionary forces at all biological levels of organizations from organisms, populations, communities to ecosystems and there is an increasing interest to investigate them in an evolutionary ecological perspective.

Integrating evolution, co-evolution into the ecology of transmission in a spatial context poses many challenges. To tackle these challenges disease ecologists use a wide varieties of methods and tools such as molecular approaches developed from pathogens screening, high-throughput technologies, population genetics, phylogenetics and phylogeography, quantitative epidemiology, population dynamics, theoretical epidemiology, spatial analyses, and landscape ecology.

This special issue calls into presenting advances and identifying gaps in the disease ecology and evolutionary ecology of diseases, using wild mammals and their pathogenic bacteria, viruses, parasites, and vectors as so many models. This special issue is a collection of studies in disease ecology that contribute to Conservation Medicine (1) and One Health approaches (2).

In two studies presented in this special issue, bats and their parasites, vectors, and microbes were investigated. Information on bats, bat flies (obligate hematophagous ectoparasites of bats) and their microparasites was synthesized by Szentiványi et al. Viruses, bacteria, blood protists, and fungi have been detected in bat flies that show physiological consequences on bats and their ectoparasites. The authors recommended additional studies to understand the interlinkages between bat hosts, ectoparasites, and their associated microparasites. McKee et al. examined *Bartonella* from European bats and their ectoparasites using network analysis, Bayesian phylogenetics, and tests on co-phylogenetic association. The authors were able to disentangle the processes, ecological, or evolutionary, that contribute to shape the interactive communities. Bat phylogeny and bat roost sharing help to explain the evolutionary patterns of vector-borne diseases.

Carnivores and bacterial diseases were the topic of two studies. Kosoy and Goodrich reviewed published studies on the phylogenetic sister clades *Bartonella* and *Brucella* that infect wild carnivores to analyse and compare the ecology of these two clades of bacteria in closely related host species. *Bartonella* species were much reported in every sampled wild felid species, whereas

among *Brucella* studies only few of them have reported *Brucella* in felids by detection of antibodies. The authors stressed that wild carnivores often carry the same microparasites as the domesticated cats and dogs, merely exposure is related to differences in biology, distribution, and historical interactions. In a comprehensive review, André synthesized the actual knowledge on the diversity of the tick-borne bacteria of species from *Ehrlichia, Anaplasma* and "*Candidatus Neoehrlichia* sp." in wild carnivores worldwide and discussed consequences for human and animal health as well as wildlife conservation. The author emphasized the importance of Whole Genome Sequencing and Next Generation Sequencing (NGS) technologies to better understand the importance of wild carnivores in the transmission of several agents such as Anaplasmataceae.

Flea-borne rickettsial disease was further explored by Maina et al. who summarized and discussed the actual knowledge of the epidemiology and distribution of *Rickettsia asembonensis*, a well-characterized rickettsia of the *Rickettsia felis*-like organisms, worldwide, as well as its arthropod hosts. The authors emphasized the need to conduct further analyses, functional and structural, to find out differences and/or similarities between *R. asembonensis* and other rickettsial species, and to better characterize the current/potential arthropod vectors with other flea-borne rickettsial species (*R. felis* and *R. typhi)*, but also non-rickettsial pathogens such as *Yersinia pestis*, the agent of the plague.

Sariyeva et al. investigated the role of gray marmots in the maintenance of highly virulent strains of *Y. pestis* in endemic foci of the Tien Shan Mountains, Kyrgyzstan. Plague circulates incessantly in populations of gray marmots, their fleas and other rodent species, stressing the importance of significant changes in rodent communities during the previous two decades. Biggins and Eads reviewed hypotheses regarding the epidemiology of *Y. pestis* using recent data from North America supporting maintenance of *Y. pestis* by persistent transmission. They proposed a maintenance mechanism, the Synergistic Positive Feedback cycles, that facilitates periodic epizootic eruptions "in place" resulting in sudden outbreaks that spread rapidly in time and space involving flea vectors, hosts, and the plague bacterium. The authors stressed that the absence of plague epizootics may reduce public health risk, but may still have ecologic impact on wild mammalian populations.

Studies on the parasites and diseases find application in conservation medicine as exemplified by Tangtrongsup et al. who investigated the prevalence of intestinal parasites of *Giardia duodenalis* and *Cryptosporidium spp.* in captive agile gibbons (*Hylobates agilis*), lar gibbons (*H. lar*), and pileated gibbons (*H. pileatus*) at the Krabokkoo Wildlife Breeding Center, Thailand. The authors stressed the improvement of hygiene management to prevent potential transmission between gibbon and human.

Finally, two studies contributed to the One Health approach. Using a metagenomic approach, Takhampunya et al. conducted an intensive study in populations of humans, animals, and vectors in Northern Thailand where scrub typhus is highly endemic. *Leptospira* spp., *Bartonella* spp., *Rickettsia* spp., and *Orientia tsutsugamushi* were detected using NGS in the studied populations. The authors confirmed the transmission of several bacterial diseases in the area, some of which are known to cause severe illness in human populations. Ruiz-Arrondo et al. outlined the benefits and the limitation of the entomological surveillance programme of mosquitoes implemented by the Government of La Rioja (Northern Spain). In order to implement a One Health approach, the surveillance programme should screen wild birds for flaviviruses and sentinel horses. Better coordinating efforts from biologists, epidemiologists, and veterinarians would be an added value to enable ecological data to be operationalised to inform human, animal, and ecosystem health.

AUTHOR CONTRIBUTIONS

SM and MK have served as editors of the Research Topic and have co-written the editorial.

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Conflict of Interest: MK was employed by company KB One Health LLC.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Bat Flies and Their Microparasites: Current Knowledge and Distribution

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Bats are the second most diverse mammalian group, playing keystone roles in ecosystems but also act as reservoir hosts for numerous pathogens. Due to their colonial habits which implies close contacts between individuals, bats are often parasitized by multiple species of micro- and macroparasites. The particular ecology, behavior, and environment of bat species may shape patterns of intra- and interspecific pathogen transmission, as well as the presence of specific vectorial organisms. This review synthetizes information on a multi-level parasitic system: bats, bat flies and their microparasites. Bat flies (Diptera: Nycteribiidae and Streblidae) are obligate, hematophagous ectoparasites of bats consisting of ∼500 described species. Diverse parasitic organisms have been detected in bat flies including bacteria, blood parasites, fungi, and viruses, which suggest their vectorial potential. We discuss the ecological epidemiology of microparasites, their potential physiological effects on both bats and bat flies, and potential research perspectives in the domain of bat pathogens. For simplicity, we use the term microparasite throughout this review, yet it remains unclear whether some bacteria are parasites or symbionts of their bat fly hosts.

Keywords: bat flies, microparasite, chiroptera, pathogen, distribution

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INTRODUCTION

Bats are the second most diverse mammalian group after rodents, with ∼1,390 recognized species across 227 genera (1). Many bat species play keystone roles in ecosystems, where they are essential to pollination, seed dispersal, and pest control (2). Several studies have also highlighted their prominent role as pathogen-reservoirs (3, 4); viruses being the best studied due to their potential as human pathogens (3, 5–8). Bats host more viruses per species than rodents, making them an interesting system for both disease ecology and public health research (4, 9).

Bacteria (such as *Bartonella* spp. and *Borrelia* spp.) and protozoans (such as *Trypanosoma* spp. and *Plasmodium* spp.) have also been detected in bats (8, 10, 11). In recent years, bat-associated *Bartonella* genotypes have been found in humans, indicating the public health importance of this parasite in bats (12–14). *Bartonella* and other pathogen transmission from bats to humans may occur through religious activities in caves, bat consumption or contact with contaminated products (12, 15). There are documented cases of bat-specific ectoparasites biting humans (16, 17), increasing the potential of bat-born pathogen transmission. Additionally, bat-associated pathogen, such as *Trypanosoma cruzi* genotype has also been found in humans (18).

Bats host numerous ectoparasitic groups, such as bat flies (Diptera: Nycteribiidae and Streblidae), bugs (Hemiptera: Cimicidae and Polyctenidae), fleas (Siphonaptera: Ischnopsyllidae), and several bat specialized arachnids, such as mites (Mesostigmata: Spinturnicidae and Macronyssidae) and ticks (e.g., *Argas* spp., *Carios* spp., *Ixodes* spp., and *Ornithodoros* spp.) (19–25).

Bat flies (Nycteribiidae and Streblidae) are the most common bat ectoparasites (**Figure 1**). Both families, along with Hippoboscidae (louse and ked flies) and Glossinidae (tsetse flies) belong to the Hippoboscoidea superfamily. Currently 275 species across 21 genera of nycteribiids and 227 species across 31 genera of streblids are recognized. Nycteribiids have a higher diversity in the Eastern Hemisphere, while streblids are mainly found in the Western Hemisphere (17).

Members of Hippoboscoidea have developed a unique reproductive strategy. A single larva develops within a female, feeding on the secretion of the so-called milk glands. Larviposition occurs at the third instar stage and the larva immediately pupates. The four families have thus been previously referred as "Pupipara" (an obsolete clade). This unique reproductive strategy necessitates milk gland secretion transfer for larval development (26–28), which may shape the community of certain bacteria such as *Arsenophonus*, *Bartonella,* or *Wolbachia* by vertical transmission (26, 27, 29, 30). Horizontal transmission may occur through parasitoids or individuals contacting contaminated saliva, as in plant consuming insect communities (31, 32).

Bat flies deposit their larva on substrates such as the host roost wall. After larviposition, females return to their host. When the offspring emerge, they actively search for bat hosts. Emergence time depends on several factors including temperature and host presence (33, 34). Regarding their reproductive strategy, bat flies also show strong morphological adaptations to their parasitic life style. Some species are eyeless or have reduced facets (35). Nycteribiids are wingless, while most streblid species have partly or fully developed wings.

Early studies assumed that bat flies show no strong host specificity (36, 37); nevertheless more comprehensive recent works showed that the majority of bat fly species exhibit high specificity to a single or closely related bat species when collection is controlled and contamination avoided (25, 38–41).

Bats' ectoparasites may have vectorial potential. For example, *Polychromophilus* spp. are transmitted by nycteribiids (42) and *Trypanosoma* spp. by cimicids (43). Although, the transmission route of *Bartonella* has not been experimentally tested, this bacteria has been detected in a wide range of bat ectoparasites, such as bat flies (44–46), tick, and mites (47–51). In a recent study, ectoparasite burden was shown to positively correlate with *Bartonella* infection, suggesting their potential role as vectors (52). Furthermore, *Bartonella* was detected in bat flies and their host in the Madagascan fruit bat (*Eidolon dupreanum*), but not in fleas, indicating the potentially crucial role of bat flies in *Bartonella* transmission (53). Additionally, ectoparasite and virus species richness positively correlate, suggesting a vectorial role of ectoparasites for viruses (54).

In this review we focus on bat flies, the most diverse and prevalent group of bat ectoparasites. Bat flies are common on most species and since they are obligate hematophagous dipterans, they may play an important role in the transmission and maintenance of bat pathogens. The exact nature of the interaction between some bacteria and their bat fly hosts is unknown: *Wolbachia* and *Arsenophonus* may act as parasites and/or as mutualists (55, 56) (we consider them as potential microparasites in this review).

Here we review the presence of microparasites in bat flies and their geographical distribution. We consider the following organisms as microparasites: blood parasites, represented by *Polychromophilus* spp. and the extinct genus *Vetufebrus* sp. (Haemosporidia: Plasmodiidae); bacteria, such as *Arsenophonus* and *Providencia* (Enterobacteriales: Enterobacteriaceae), *Bartonella* (Rhizobiales: Bartonellaceae), *Wolbachia* and *Rickettsia* (Rickettsiales: Anaplasmataceae and Rickettsiaceae); viruses, such as Kanyawara virus (Mononegavirales: Rhabdoviridae), Mahlapitsi virus (Reoviridae), Wolkberg virus and Kaeng Khoi virus (Bunyavirales: Bunyaviridae and Peribunyaviridae), dengue virus (Flaviviridae); hyperparasites, such as fungi (Ascomycota: Laboulbeniaceae) and finally parasitoids (Hymenoptera: Eupelmidae). We test whether bat host phylogenetic origin effects the presence of different microparasitic groups of bat flies. We discuss the potential physiological effects of microparasites on both bats and bat flies, and future research perspectives related to bat-associated ectoparasites and microparasites.

MATERIALS AND METHODS

We present microparasite data collected from various literature source (**Supplementary Data Sheet 1**). We searched Google Scholar and ISI Web of Science, using all combinations of the following terms in English and French: Chiroptera or bat*; ectoparasite, bat fly, Nycteribiidae, Streblidae or Hippoboscidae*; and pathogen, parasitoid, parasite, microparasite, fungi, protozoa, haemosporidians, bacteria or virus.

Each bat fly—microparasite association (genus or species, depending on the taxonomic level provided by the authors) is an entry of the dataset, and is characterized by its geographical origin and bat host species.

We use currently valid taxonomical names for both bats and bat flies in our database (57–59). Statistics are conducted using R 3.5.1 (60). Bat fly-microparasite networks were visualized using the R package bipartite (61). Map of reported bat fly-microparasite associations were made in QGIS 2.16 (62).

RESULTS

Effect of Bat Host Family on Detected Microparasite Distribution in Bat Flies

Bat flies infected with microparasites were observed on 75 bat species comprising 33 bat genera, with most in Vespertilionidae (16/505 known species), Phyllostomidae (21/216), Pteropodidae (13/196), Miniopteridae (10/38, the highest observed ratio), and Rhinolophidae (8/103). Bat flies with microparasite observations were also found in only a few species of Emballonuridae, Hipposideridae, Noctilionidae, and Mormoopidae.

Microparasite distribution in bat flies is dominated by bacterial and fungal parasites (**Figure 2**). Viruses detected in bat flies are only known from the family Phyllostomidae ($n = 2$) and Pteropodidae ($n = 4$). Blood parasites were mostly in flies from Miniopteridae ($n = 7$), but were also found in Pteropodidae ($n = 1$) and Vespertilionidae ($n = 2$) (**Figure 2**).

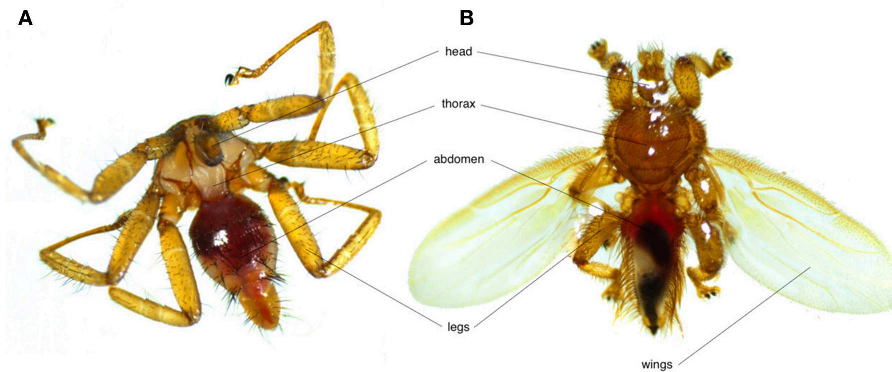

FIGURE 1 | Photos showing the morphological differences between **(A)** a wingless nycteribiid and **(B)** a streblid bat fly.

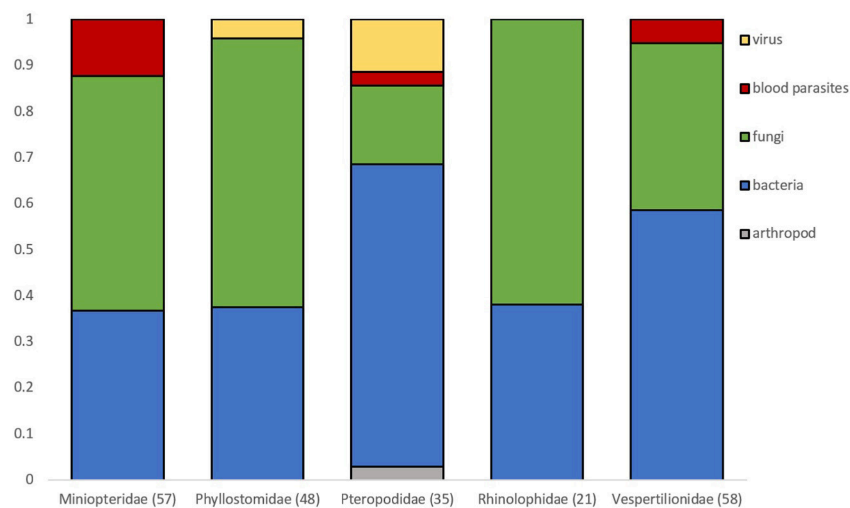

FIGURE 2 | Proportion of microparasite groups observed in bat flies collected from different bat host families. Numbers in brackets are sample sizes. Families with <20 observations are not represented.

Diversity Within Nycteribiidae and Streblidae

A total of 188 and 101 microparasite observations are reported in bat fly families Nycteribiidae and Streblidae respectively, belonging to 27 bat fly genera (**Figure 3**). The most frequently reported infected bat fly genera are *Penicillidia* (n = 67), *Nycteribia* (n = 51), *Trichobius* (n = 44), *Eucampsipoda* (n = 20), and *Basilia* (n = 15); all of them Nycteribiidae, with the exception of the streblid genus *Trichobius*. Both host fly families displayed a similar distribution of microparasite taxa (**Figure 4**).

The most commonly reported microparasites in bat flies are bacteria (n = 149), followed by fungi (n = 118), blood parasites (n = 15), viruses (n = 6), and arthropods (n = 1) (**Table 1**). Within bacteria, the three most frequently detected microparasites are *Bartonella* sp. (Alphaproteobacteria: Bartonellaceae) (n = 91, 61%), *Arsenophonus* sp. (Gammaproteobacteria: Enterobacteriaceae) (n = 30, 20.1%) and *Wolbachia* sp. (Alphaproteobacteria: Anaplasmataceae) (n = 8, 5.4%). All observed fungi are Laboulbeniaceae (Ascomycota: Laboulbeniales) and belong to three genera, *Arthrorhynchus* (n = 80, 67.8%), *Gloeandromyces* (n = 16, 13.6%), and *Nycteromyces* (n = 5, 4.2%), as well as 17 (14.4%) unidentified or undescribed observations. *Polychromophilus* species (Haemosporida: Plasmodiidae) represent 93.3% (n = 14) of blood parasite observations in bat flies. Virus and parasitoid arthropod represent a much smaller proportion of all microparasitic observations in bat flies, with only six and one published record, respectively.

Global Geographical Distribution of Bat Fly—Microparasite Associations

Bat fly -microparasite associations originated from 61 countries (**Figure 5**) with a total of 269 reports (excluding those with unspecified or unknown geographical locations). Associations reported from countries were most commonly from Europe (n = 89, 33%), North America (n = 69, 25.7%), and Africa (n = 61, 22.7%). Observations in Asia (n = 33, 12.3%), South America (n = 21, 7.8%), and Oceania (n = 5, 1.9%) were represented less frequently. The highest number of microparasite—bat fly species associations are reported from Madagascar (n = 33).

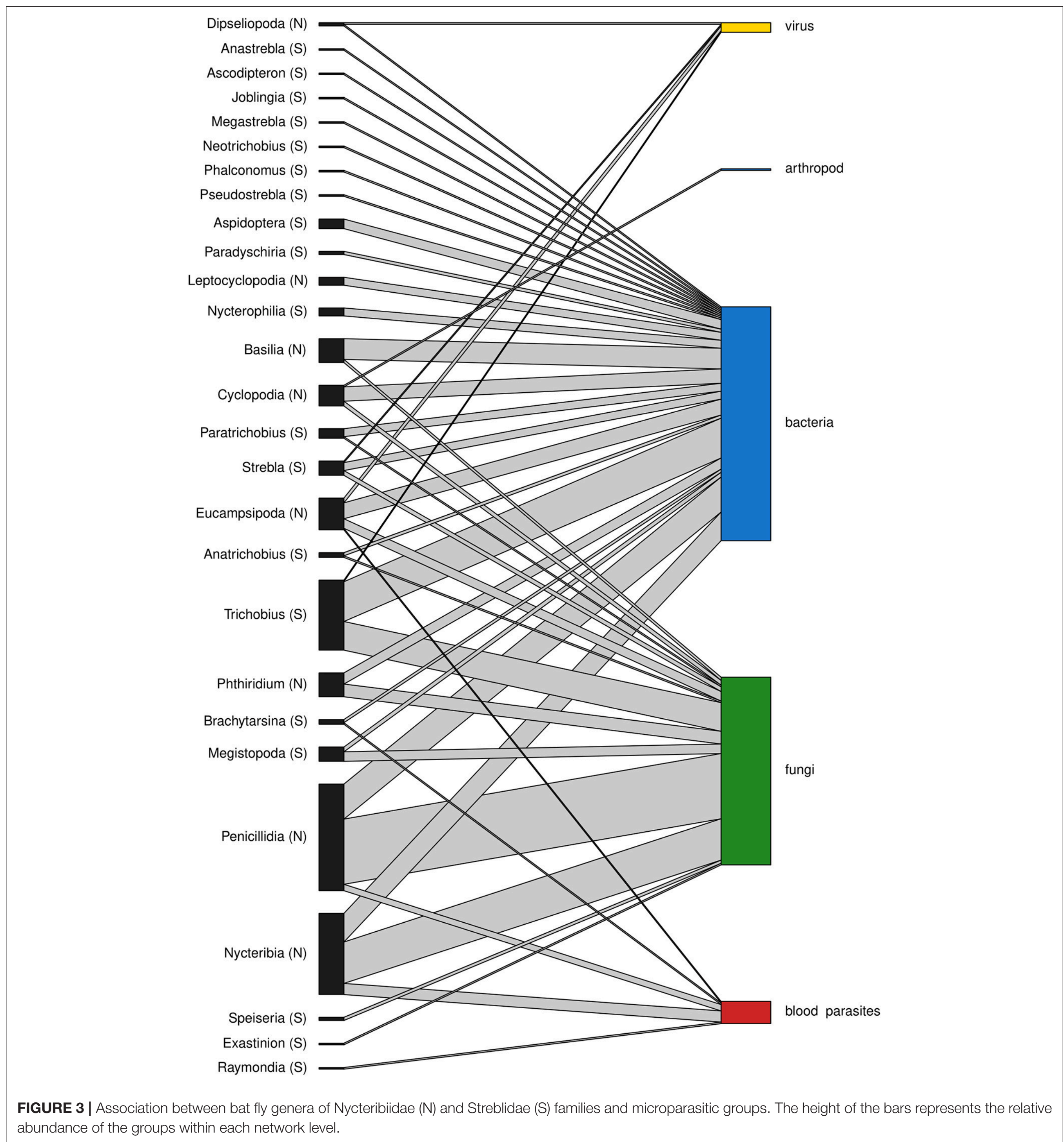

FIGURE 3 | Association between bat fly genera of Nycteribiidae (N) and Streblidae (S) families and microparasitic groups. The height of the bars represents the relative abundance of the groups within each network level.

Sampling Effort on Microparasite Diversity in Bat Flies

We tested the number of published studies by bat fly genera and number of microparasite associations reported (including same species associations but different bat hosts and countries). Spearman rank correlation showed that sampling effort strongly predicts the number of detected microparasites in different bat fly genera ($n = 27$, $r = 0.68$, $p = 0.0001$; **Figure 6**).

DISCUSSION

Microparasite Diversity in Bat Flies

Based on literature data, we have identified five main groups of microparasites in parasitic bat flies. Bacteria are the most frequently observed group in both Nycteribiidae and Streblidae and within bacteria, *Bartonella* is the most prevalent microorganism. Some species of *Bartonella* are blood-borne parasites, transmitted by blood-sucking arthropods (104) found

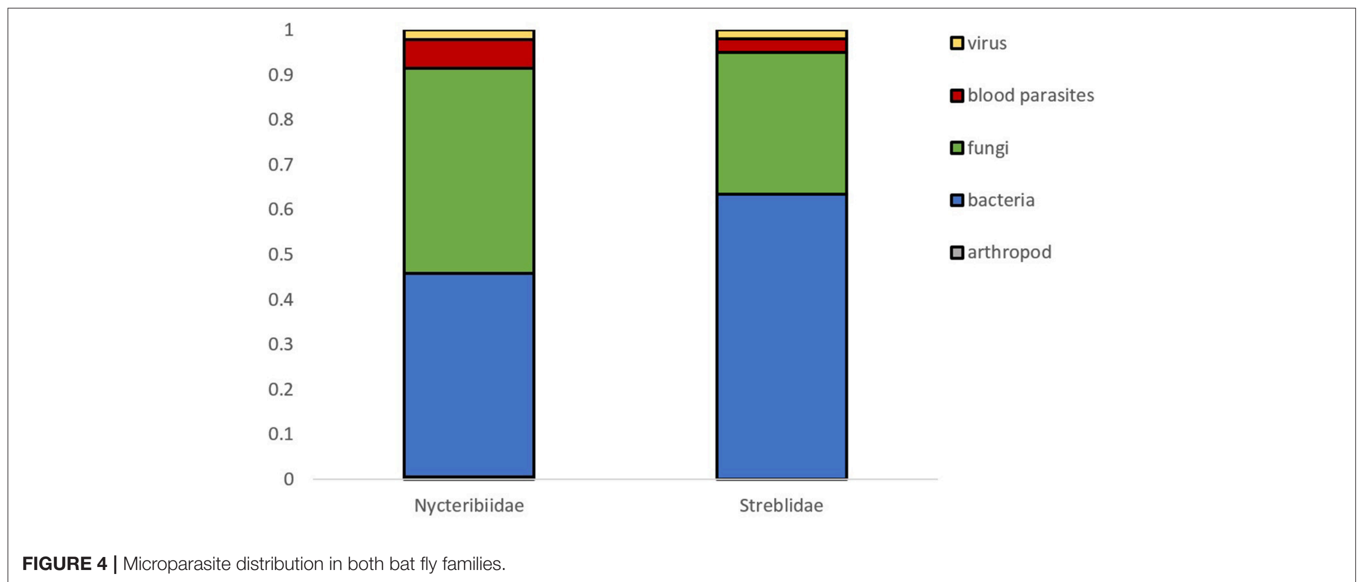

FIGURE 4 | Microparasite distribution in both bat fly families.

in a wide range of mammalian groups and several arthropod ectoparasites (14). For example, *Bartonella quintana*, a louse-borne parasite, was responsible for trench fever, which affected over one million soldiers during World War 1 (105). The presence of identical *Bartonella* genotypes in bats and bat flies suggests that bat flies may serve as vectors (44, 53, 80, 81). Host specific bat flies show higher prevalence but lower diversity of *Bartonella* infection than polyxenous species (46). However, the generally high prevalence and diversity of *Bartonella* suggests their long co-evolutionary history with bats.

The second most frequently observed microparasites in bat flies are fungi. All species recognized here belong to the order Laboulbeniales. Three genera of Laboulbeniales are known to parasite bat flies, *Arthrorhynchus* spp. (the most frequently reported genus), *Gloeandromyces* spp., and *Nycteromyces* spp. The distribution, specificity and diversity of these microparasites have recently been uncovered. Locally (e.g., in Europe) these species show some degree of high specificity (with occasional "accidental" transfers) (64, 69), although at a larger geographical scale, they do not show strict specificity to host species or genera (65).

While blood parasites are frequently found in bats (77, 106–108), observations in bat flies are much less common. *Polychromophilus* species are vectored by nycteribiids (102), and one haemosporidian report is known from a single fossil streblid specimen but observations from extant streblids are still missing (109). Other blood parasites, such as *Trypanosoma* is transmitted to bats by hemipterans including *Cimex* species (42). *Trypanosoma cruzi cruzi*, the causative agent of Chagas disease in humans and other mammal species, is transmitted by triatomine bugs (110). Bat flies have not yet been reported as vectors of *Trypanosoma* species. Nevertheless, *Glossina* tsetse flies (members of the Hippoboscoidea superfamily along with bat flies) are known to transmit *T. brucei*. Therefore, it remains possible that bat flies transmit other blood parasites besides *Polychromophilus* (e.g., trypanosomatids). Additionally, nycteribiids may serve as vectors in the transmission of other protozoans, such as *Nycteria* spp. (Haemosporida: Plasmodiidae), infecting Afrotropical insectivorous bats; but their vectorial potential has not yet been clarified (107). More work is needed to address these questions.

Most of the reports on viruses in bat flies are relatively recent (87, 92–96). As such, it is possible that the number of isolated viruses in bat ectoparasites might thus rise in the future with improvement in diagnostic methods.

There is only one report of a parasitoid wasp using nycteribiids as host (88). Parasitoid wasps are extremely diverse groups with about 100,000 described species. However, host species information is missing for many species. We expected that other parasitoids use bat flies as hosts during their development, but data collection is challenging due to the ecology of these flies. Furthermore, it has been observed that mite species can have phoretic relationships with bat flies (111–113), but their effect on bat flies is not clear. Nonetheless, some phoretic mites which were previously assumed to have no effect on their invertebrate hosts, have now been shown to negatively affect their fecundity and/or survival rate (114, 115).

Studies have previously suggested that microfilaria might be transmitted by hippoboscid louse flies to their vertebrate hosts, such as dogs (116). Filarial nematode DNA has also been observed in streblid bat flies and bat mites (117). It is not clear if these microfilaria are transmitted by bat flies or if the detected microfilaria DNA was only present in the last blood meal (117).

Microparasite diversity is similar between nycteribiids and streblids flies, although nycteribiids have 2.5 times more reported cases of microparasites. The reason behind this is more likely due to biased sampling efforts in different geographical regions. For example, in Europe where most of the studies were performed, 16 species of nycteribiids are present, whereas only one streblid species have been recorded.

TABLE 1 | Microparasite groups found in bat flies and their associated bat families.

Bat host family	*N* of observation	*N* of bat fly species with microparasites	Microparasites detected from flies	*N* of observation	Location	References
Emballonuridae	1	1	Blood parasites	1	Gabon	(63)
Hipposideridae	7	6	Bacteria	2	Gabon, Malaysia	(30, 44)
			Fungi	3	Sri Lanka, Zambia	(64–67)
			Blood parasites	2	Gabon	(63)
Miniopteridae	57	14	Bacteria	21	Hungary, Japan, Madagascar, Romania	(26, 46, 68)
			Fungi	29	Australia, Bulgaria, Croatia, France, Hungary, India, Kenya, Portugal, Romania, Slovakia, Spain, Sri Lanka, Switzerland, Taiwan	(64, 66, 69–76)
			Blood parasites	7	Gabon, Madagascar	(63, 77, 78)
Mormoopidae	3	2	Bacteria	1	Mexico	(44)
			Fungi	2	Costa Rica, Panama	(79)
Noctilionidae	2	2	Bacteria	2	Dominican Republic, Panama	(28, 44)
Phyllostomidae	48	18	Bacteria	18	Brazil, Costa Rica, Dominican Republic, French Guyana, Mexico, Panama, Peru	(27, 45, 80, 81)
			Fungi	28	Brazil, Costa Rica, Grenada, Panama, Venezuela	(64, 74, 79, 82–86)
			Virus	2	Mexico	(87)
Pteropodidae	35	17	Arthropod	1	São Tomé Island	(88)
			Bacteria	23	China, Gabon, Ghana, Kenya, Madagascar, Malaysia, Philippines, Union of the Comoros	(12, 28, 30, 44, 53, 68, 89)
			Fungi	6	Egypt, Gabon, Israel, Malaysia, New Guinea, Sierra Leone	(66, 71, 90, 91)
			Blood parasites	1	Gabon	(63)
			Virus	4	China, South Africa, Uganda	(92–96)
Rhinolophidae	21	7	Bacteria	8	China, Hungary, Laos, Philippines, Romania	(28, 44, 46)
			Fungi	13	Croatia, France, Hungary, Italy, Kenya, Romania, Serbia, Sri Lanka	(64, 66, 67, 97)
Vespertilionidae	58	19	Bacteria	34	Costa Rica, Hungary, Madagascar, Malaysia, Peru, Romania, Slovenia, United States	(28, 30, 44, 46, 47, 50, 68, 81, 98)
			Fungi	21	Austria, Brazil, Czech Republic, England, France, India, Italy, Poland, Portugal, Romania, Spain, Tunisia	(64, 66, 71, 75, 76, 83, 99–101)
			Blood parasites	3	England/Scotland, Switzerland	(102, 103)

The number of microparasite and host species associations (both bat species and bat fly species) are given, as well as the country of observation. See references and additional details in ***Supplementary Data Sheet 1.***

Geographical Distribution

All major groups of microparasites have been reported widely, though our knowledge of the diversity and distribution of many groups remains scarce. Bacteria such as *Bartonella* show a high molecular and geographic diversity in bats and bat flies, at global and regional scales (44, 46, 118). Six major bat associated *Bartonella* clades have been reported so far from bats and bat flies (118). Clade I, II, IV, and V are represented in both Old and New World areas while clade III seems to be restricted to the Old World (Africa, Asia, and Europe) and clade VI to some parts of the New World (Central America) (118).

Fungal microparasites (Laboulbeniales) show a rather divided Eastern (*Arthrorhynchus* spp.) and Western Hemisphere (*Nycteromyces* spp. *Gloeandromyces* spp.) distribution and diversity (65). Similar patterns have been demonstrated regarding nycteribiids (Eastern) and streblids (Western) (17). These diversity and distribution patterns suggest a long evolutionary history between bat flies and these fungal microparasites.

It is important to highlight that these distribution patterns might be strongly influenced by biased sampling efforts rather than actual geographical patterns. Therefore, the distribution map helps to recognize well studied areas on a global scale, however it does not necessarily reflects

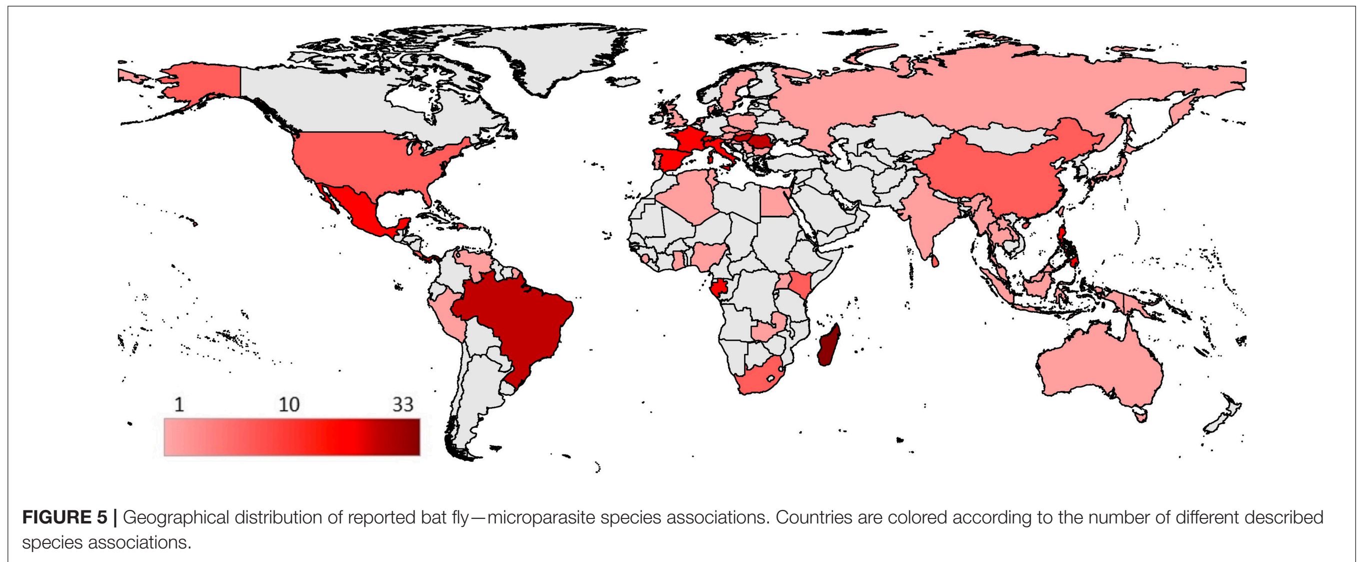

FIGURE 5 | Geographical distribution of reported bat fly—microparasite species associations. Countries are colored according to the number of different described species associations.

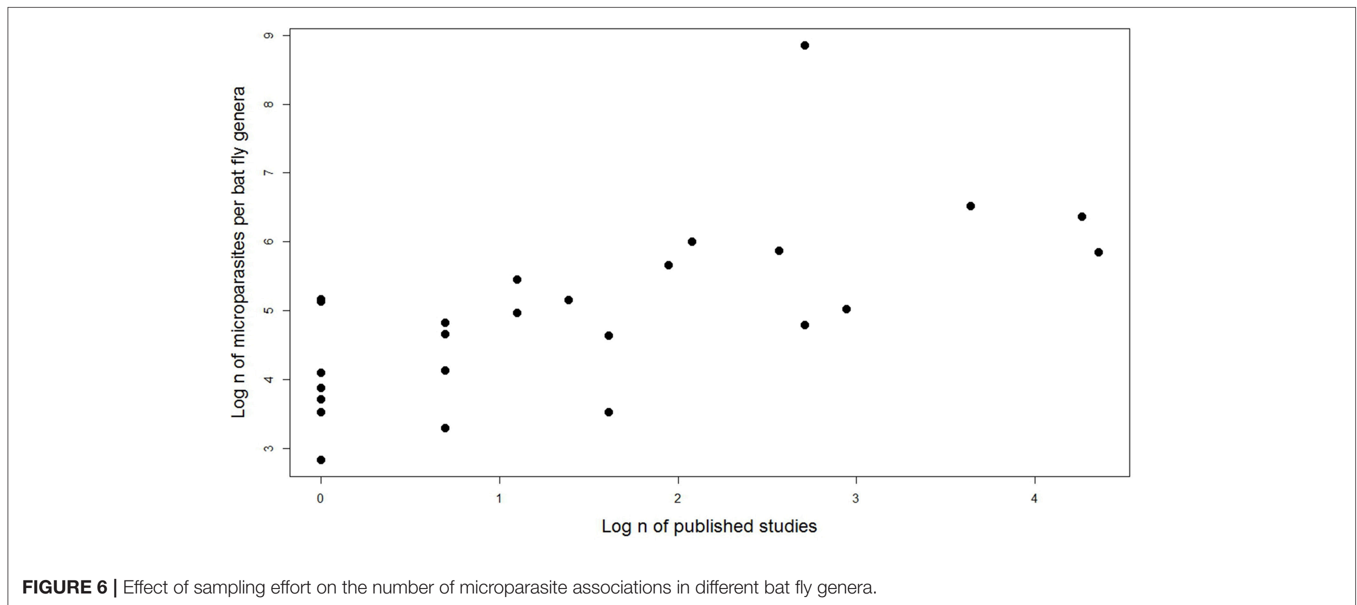

FIGURE 6 | Effect of sampling effort on the number of microparasite associations in different bat fly genera.

actual distributional patterns of these microparasites detected in bat flies. It is our hope that it will be useful for further studies.

Effects of Bat Host Ecology on Microparasites

Previous work showed that viral richness in bats correlates with IUCN threat status, with near-threatened and vulnerable hosts having higher viral richness. In addition, population genetic structure positively correlates with viral richness (119). Host longevity, reproductive strategy and distribution pattern may also play an important role in viral richness (9, 54, 120).

In general, the bat host family does not affect the distribution of microparasites in their bat flies. The bent-winged bats, family Miniopteridae, have the highest observed ratio of bat species infected by bat flies parasitized by microparasites. Miniopteridae are insectivorous, cave-dwelling species occurring in dense and multi-species colonies. From a disease ecology and parasitology point of view, it is a unique family hosting many highly specific ecto- and endoparasites such as mites, bat flies and malarial parasites (21, 121, 122). It is still unclear whether the ecology and/or the immune system of Miniopteridae species is responsible for such a high parasite diversity compared to other bat families. Moreover, Miniopteridae is considered as underrepresented in viral research so more parasites and pathogens likely remain undiscovered in these species (123).

Bacteria and fungi are the most abundant group of microparasites in all bat flies from different host families. The occurrence of *Bartonella* infection in bats is associated with host diet; hematophagous and carnivorous species are

more frequently infected than species with other diets (124). Hematophagous and carnivorous bat species also show higher white-blood cell count, suggesting a higher risk of pathogen exposure, probably due to the fact that these bat species are more exposed to vertebrate specific pathogens (125). Therefore, we might expect a higher microparasite occurrence in bat flies collected from bat species that feed on vertebrates or blood. Nevertheless, there are only a few studies that have focused on microparasites in parasitic bat flies collected from these host species (44, 80, 87).

Viruses are only known from bat flies infecting the New World leaf-nosed bats Phyllostomidae and the Old World fruit bats Pteropodidae, but observations are still scarce. These observed viruses represent distant groups, such as Dengue virus (family *Flaviviridae*) isolated from the bat flies of the common vampire bat, *Desmodus rotundus* (87); Kaeng Khoi virus (*Peribunyaviridae*), Kanyawara virus (*Rhabdoviridae*), Mahlapitsi virus (*Reoviridae*), and Wolkberg virus (*Bunyaviridae*), isolated from *Myonycteris* and *Rousettus* species (92–96).

There are great ecological differences between bat families. Bat host ecology and physiology, such as roosting habits, body size, and sex can affect bat fly burden and species richness (126–129). More studies are again needed to clarify how host traits affect the distribution of microparasite communities of bat flies.

Potential Physiological Effects on Flies and Bats

We still know little about the physiological effects of microparasites on bat flies and on their bat host. Viruses such as *Lyssavirus* spp. are known to cause mortality in bats (130, 131). The bacterial parasite *Borellia* sp. (from the relapsing fever group) has been documented causing fatal borreliosis in a single bat individual (*Pipistrellus* sp.) (132). The haemosporidian parasite *Polychromophilus murinus* has a well-documented impact on both bat and bat fly life-history traits (103, 106). In the Daubenton's bats (*Myotis daubentonii*), it has a strong negative effect on the body condition of subadults (106). Additionally, it negatively affects the life span of infected bat flies (103).

The relationship between bat flies and some bacterial species such as *Wolbachia* and *Arsenophonus* has not yet been clarified. It is suspected that they are either parasitic and/or symbiotic of bat flies. In some cases, *Wolbachia* is considered as a nutritional mutualist, due to its ability to produce vitamin B in certain hematophagous arthropod species, such as *Cimex* spp. (133). *Arsenophonus* is a highly diverse group of bacteria found mainly in insects, including bat flies (134–138). *Arsenophonus* species have been suggested to be primary or secondary symbionts in other taxa (134, 138, 139). Here, we categorize *Arsenophonus* and *Wolbachia* as microparasitic organisms in bat flies, since it is unclear how they affect their hosts (35). Furthermore, *Wolbachia* DNA has been also detected in mammalian blood due to the presence of infected nematodes in host blood (140). It has been observed once in an avian blood system, with the strain being more closely related to the arthropod-associated *Wolbachia* group (141), and likely having no direct effect on their vertebrate hosts.

The presence of the fungal parasite Laboulbeniales has an effect on bat fly mortality in some species (Szentiványi et al., Unpublished), as an arthropod specialized microparasite. Nevertheless, it is unclear if it has any direct or indirect effect on the bat host.

Additionally, and as mentioned above, the potential effect of phoretic mite infestation on bat flies has never been tested. Therefore, it remains possible that these mites have direct or indirect negative effects on host behavior, survival rate, and/or fecundity.

Perspectives for Additional Research, Sampling Effort

Our knowledge of the microparasites of bat flies is strongly biased by sampling effort, which may also strongly reflect the currently known geographical distribution patterns of these parasites. We suggest to balance these biases by increasing sampling effort in less prospected countries as well as areas where human exposure to pathogen transmission is more likely to occur, due to cultural or touristic reasons (e.g., visiting caves) (15, 142). Additionally, we have little knowledge on the microparasites of other bat ectoparasitic groups, such as fleas, bugs, and mites. Future studies should focus on how microparasite and pathogen communities interact on the intra- and interspecific levels. For example, *Wolbachia* infection is known to inhibit malarial infection in mosquitos (143). Additionally, it is important to understand how bat host traits such as sex, geographical distribution and/or host group size [which are known to shape the distribution of bat fly populations (17, 128, 129)] may affect the occurrence of microparasitic communities in these ectoparasites. Lastly, experimental studies are needed to understand the relationship between bat hosts and ectoparasites, including the transmission and the distribution of microparasites.

AUTHOR CONTRIBUTIONS

PC and OG initiated the study. TS performed data collection and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2019.00115/full#supplementary-material

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Host Phylogeny, Geographic Overlap, and Roost Sharing Shape Parasite Communities in European Bats

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How multitrophic relationships between wildlife communities and their ectoparasitic vectors interact to shape the diversity of vector-borne microorganisms is poorly understood. Nested levels of dependence among microbes, vectors, and vertebrate hosts may have complicated effects on both microbial community assembly and evolution. We examined *Bartonella* sequences from European bats and their ectoparasites with a combination of network analysis, Bayesian phylogenetics, tip-association and cophylogeny tests, and linear regression to understand the ecological and evolutionary processes that shape parasite communities. We detected seven bat–ectoparasite–*Bartonella* communities that can be differentiated based on bat families and roosting patterns. Tips of the *Bartonella* tree were significantly clustered by host taxonomy and geography. We also found significant evidence of evolutionary congruence between bat host and *Bartonella* phylogenies, indicating that bacterial species have evolved to infect related bat species. Exploring these ecological and evolutionary associations further, we found that sharing of *Bartonella* species among bat hosts was strongly associated with host phylogenetic distance and roost sharing and less strongly with geographic range overlap. Ectoparasite sharing between hosts was strongly predicted by host phylogenetic distance, roost sharing, and geographic overlap but had no additive effect on *Bartonella* sharing. Finally, historical *Bartonella* host-switching was more frequent for closely related bats after accounting for sampling bias among bat species. This study helps to disentangle the complex ecology and evolution of *Bartonella* bacteria in bat species and their arthropod vectors. Our work provides insight into the important mechanisms that partition parasite communities among hosts, particularly the effect of host phylogeny and roost sharing, and could help to elucidate the evolutionary patterns of other diverse vector-borne microorganisms.

Keywords: ***Bartonella*, ectoparasites, disease ecology, parasite communities, host-switching, network analysis**

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INTRODUCTION

The enormous complexity of natural communities results from the large number of coexisting species and the diverse and unequal strengths of their interactions. Parasites, including macroparasites (e.g., worms and arthropods) and microparasites (e.g., bacteria and viruses), are an integral component of natural communities. Parasitism is a widespread life history strategy used by approximately one-third to over one-half of all species (Poulin, 2014; Morand, 2015). These parasitic organisms are under selective pressure to optimize their life history traits to efficiently colonize and reproduce in or on their hosts. This process of developing host specificity can be complicated, however, when there are several layers of parasitism. Such is the case for vector-borne microorganisms. For these organisms, selection can occur in both the host and the vector. When combined with host-associated selection on vectors, vector-borne microbes may exhibit complicated patterns of host and vector associations and phylogenetic differentiation. In light of this, classic models of parasite cospeciation and host-switching (de Vienne et al., 2013) must give way to novel approaches that examine the contributions of both hosts and vectors to the evolution and community assembly of vector-borne microorganisms.

Examining these processes is fundamental to understanding microbial diversity and surveillance of vector-borne microorganisms (Braks et al., 2011). Vectors vary in their host specificity, potentially leading to transmission of microorganisms to atypical hosts including humans. Vector-borne microorganisms account for a substantial proportion of emerging infectious diseases worldwide (Jones et al., 2008), and the zoonotic potential of mammalian viruses has been positively linked with being vector-borne (Olival et al., 2017). Knowledge of associations between microbes, hosts, and vectors will help to understand how humans become exposed to zoonotic agents and mitigate these risks. Thus, disentangling the ecological and evolutionary relationships between microorganisms and their hosts and vectors is important for managing the potential spillover of zoonotic agents to humans.

Disentangling these complex ecological and evolutionary processes requires sampling and analytical methods that integrate across trophic levels. If sampling is done across multiple ectoparasites and hosts, we can characterize the strength of host–parasite associations and identify host–vector–microbe communities using network-based approaches. Knowledge of these communities would directly facilitate disease management and the prevention of spillover events. For instance, hosts or vectors that have high infection prevalence or are connected with a large number of other nodes in the tripartite host–vector–microbe network may be targeted for pathogen surveillance or vector control. Looking at patterns of microbe sharing among hosts, we can highlight factors that constrain microbial host range using multiple regression, including host phylogenetic distance, vector sharing, geographic range overlap, and roost sharing as covariates (Streicker et al., 2010; Willoughby et al., 2017). Finally, we can examine how biases in historical microbial host-switching result in the observed congruence between host and microbial phylogenies (Charleston and Robertson, 2002).

To understand how complex host–vector–microbe communities are assembled and maintained in nature, we examined the associations of *Bartonella* spp. bacteria and ectoparasitic arthropods with their bat hosts using compiled data from nine European countries. We argue that *Bartonella* infections in bats and their ectoparasites represent an ideal system for understanding these complexities, first because *Bartonella* infections are prevalent and genetically diverse in many bat species studied to date (McKee et al., 2016; Stuckey et al., 2017b), providing rich data with which to analyze complex patterns. Second, bats are present on all continents except Antarctica and have traits that favor parasite transmission and geographic spread, including flight and long life spans. Many bat species are highly social and may form large colonies (Kerth, 2008), frequently co-roosting with other species, which could facilitate cross-species parasite transmission. Third, bats are a phylogenetically ancient lineage (Shi and Rabosky, 2015; Foley et al., 2016), allowing extended time for microbes and ectoparasites to develop host specificity. Finally, bats have many ectoparasites that vary in host specificity, ranging from highly specific wing mites (Bruyndonckx et al., 2009) to more generalist vectors like ticks (Hornok et al., 2016, 2017), which could have opposing effects on the evolution of host specificity in microorganisms they transmit. Such ectoparasite life history traits can interact with bat social systems in shaping microbial transmission (van Schaik et al., 2015). All these forces may combine to generate complex host–vector–microbe communities over evolutionary time but may be predictable given sufficient data and appropriate analytical methods. Moreover, bats are a highly threatened group of wildlife species, play central roles in ecosystems, and deliver valuable ecosystem services such as pollination and pest control (Boyles et al., 2011; Kunz et al., 2011); thus, ecological and evolutionary information on parasites could be informative for bat conservation and ecosystem sustainability (Whiteman and Parker, 2005; van Schaik et al., 2018). In addition to these factors, there are outstanding questions regarding the forces that drive *Bartonella* evolution in bats. Previous work has shown that the phylogeny of bat-associated *Bartonella* lineages is congruent with the bat host phylogeny (Lei and Olival, 2014), and *Bartonella* lineages tend to cluster by bat suborders and families (McKee et al., 2016). This previous work indicates that *Bartonella* species have developed some level of host specificity; however, the relative influence of ectoparasites and the biogeography of bat hosts on bat–*Bartonella* associations remain unclear.

Lastly, recent studies have highlighted the zoonotic potential of bat-associated *Bartonella* species. *Bartonella* spp. infections in humans and domestic animals can lead to symptoms ranging from mild fever to potentially life-threatening endocarditis (Chomel and Kasten, 2010). In one case of human endocarditis, the etiological agent was identified as a novel pathogenic species, *Candidatus* Bartonella mayotimonensis (Lin et al., 2010). This species and related strains have been identified in European and North American bat species (Veikkolainen et al., 2014; Lilley et al., 2017; Stuckey et al., 2017a; Urushadze et al., 2017). *Bartonella* spp. have been detected in numerous bat ectoparasites (Stuckey et al., 2017b; Hornok et al., 2019), some of which are known to occasionally attack humans (Jaenson et al., 1994;

Estrada-Peña and Jongejan, 1999). A recent study also found serological evidence of a *Bartonella* species specific to fruit bats in humans in Nigeria where members of the community capture and sometimes eat bats (Bai et al., 2018). Given these emerging patterns, knowledge of the host and vector associations of bat-associated *Bartonella* species could have implications for managing spillover risk.

Our strategy to investigate how bat–ectoparasite–*Bartonella* communities are assembled and how they evolve involves a multifaceted analytical approach (**Figure 1**) that splits this problem into three fronts: (1) assessing the diversity of *Bartonella* species in European bats and their ectoparasites and the structure of bat–ectoparasite–*Bartonella* communities using network analysis, (2) understanding the evolutionary implications of these ecological patterns using tip-association and cophylogeny tests and phylogenetic measures of historical host-switching rates, and (3) linking patterns of *Bartonella* host specificity to ecological and evolutionary covariates using linear regression. We hypothesized that associations between bats, ectoparasites, and bacteria can be resolved into identifiable communities that separate by bat phylogeny, geographic overlap, and roosting patterns. Second, we expected that the phylogeny of *Bartonella* species will exhibit significant clustering by bat taxonomy and will have significant congruence with the phylogeny of bat species. Linking these patterns together, we hypothesized that host phylogenetic distance, ectoparasite sharing, geographic range overlap, and summer roost sharing are predictors of bacterial species assemblages and host-switching rates among bat species. This multifaceted approach aims to bridge ecological processes to observed evolutionary patterns to better understand the diversity and epizootiology of bartonellae in bats. Such an approach could be generalized to study and manage other microorganisms with complex, multihost dynamics.

MATERIALS AND METHODS

Study Sites and Specimen Collection

Bat ectoparasites were collected in the Netherlands, Belgium, Hungary, and Romania between 1993 and 2015. Sampling sites included roosting, swarming, and foraging areas, and all sampling occurred during the summer maternity and autumn mating phases when ectoparasites are more active (van Schaik and Kerth, 2017). In the Netherlands and Belgium, ectoparasite specimens were collected with forceps either directly from bats during inspections of bat boxes and night mist netting or from their roosts. In addition, ectoparasites were sampled during inspection of dead bats collected in the Netherlands between 1993 and 2011 and stored in the Naturalis Biodiversity Center, Leiden, the Netherlands. Bat flies from Hungary and Romania derived from a study by Sándor et al. (2018). All bats were morphologically identified to the species level. Initial identification of bat flies was based on morphological characteristics (Theodor and Moscona, 1954; Theodor, 1967). Ectoparasites were stored in 70% ethanol in separate vials prior to further analysis. The distribution of sampling sites is mapped in **Figure 2**, and the coordinates of sampling sites are listed in **Table S13**.

Bat Species Tree, Geographic Range Overlap, and Roosting and Mating Data

A phylogenetic tree of bats (**Figure S1**) was obtained from the Open Tree of Life (http://www.opentreeoflife.org) from a previous study of bat taxonomy using multiple mitochondrial and nuclear loci (Shi and Rabosky, 2015). The tree was pruned to the 21 species in **Table 1**. *Myotis oxygnathus* was considered a synonym for *My. blythii* (Agnarsson et al., 2011; Balvín and Bartonička, 2014; Wilson and Reeder, 2015).

The geographic ranges of each bat species were downloaded from the International Union for Conservation of Nature (IUCN) Red List website (http://www.iucnredlist.org) (IUCN, 2014). IUCN ranges are convenient data that are available for all the bat species in this study, and previous studies have successfully used these data for understanding the determinants of viral diversity in bats (Luis et al., 2013, 2015; Maganga et al., 2014; Webber et al., 2017; Willoughby et al., 2017). Shape files were imported into R using the "readShapeSpatial" function in the "maptools" package (Bivand et al., 2017; R Core Team, 2018). Individual range maps (**Figure S2**) and a map of overlapping ranges (**Figure 3**) were generated by drawing shape files over the "worldHires" map from the "maps" package (Becker et al., 2016). We then calculated pairwise percent geographic range overlap between each bat species as described previously (McKee et al., 2016); see the **Supplementary Material** for more details. Data on roosting patterns and mating systems of bats (**Tables S1** and **S2**) were collected from books by Dietz and Kiefer (2014) and Niethammer and Krapp (2001, 2004).

Ectoparasite DNA Extraction and Barcoding

DNA from bat flies, mites, fleas, and bat bugs was extracted with ammonium hydroxide as described previously (Wielinga et al., 2006). DNA from bat ticks was extracted using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's protocol for the purification of total DNA from ticks (Qiagen, Venlo, the Netherlands). Confirmation of ectoparasite identification was performed by sequencing a 658-base-pair fragment of the cytochrome oxidase subunit I (COI) using primers LCO1490 and HCO2198 (Folmer et al., 1994); see the **Supplementary Material** for details. For species identification, both strands of PCR products were Sanger sequenced (BaseClear, Leiden, the Netherlands) using the same forward and reverse primers as in the conventional PCR. Trimming and manual cleaning of COI sequences were performed in BioNumerics v7.1 (Applied Math, Belgium). A phylogeny was inferred using the GTR+Γ model with 25 distinct rate categories with 1,000 bootstrap replicates using RAxML (Stamatakis, 2014). Based on this phylogeny, individual associations between a *Bartonella* sequence and a vector species were corrected if the phylogenetic position of the COI sequence conflicted with the morphological identification, replacing the morphological identification with the phylogenetic identification.

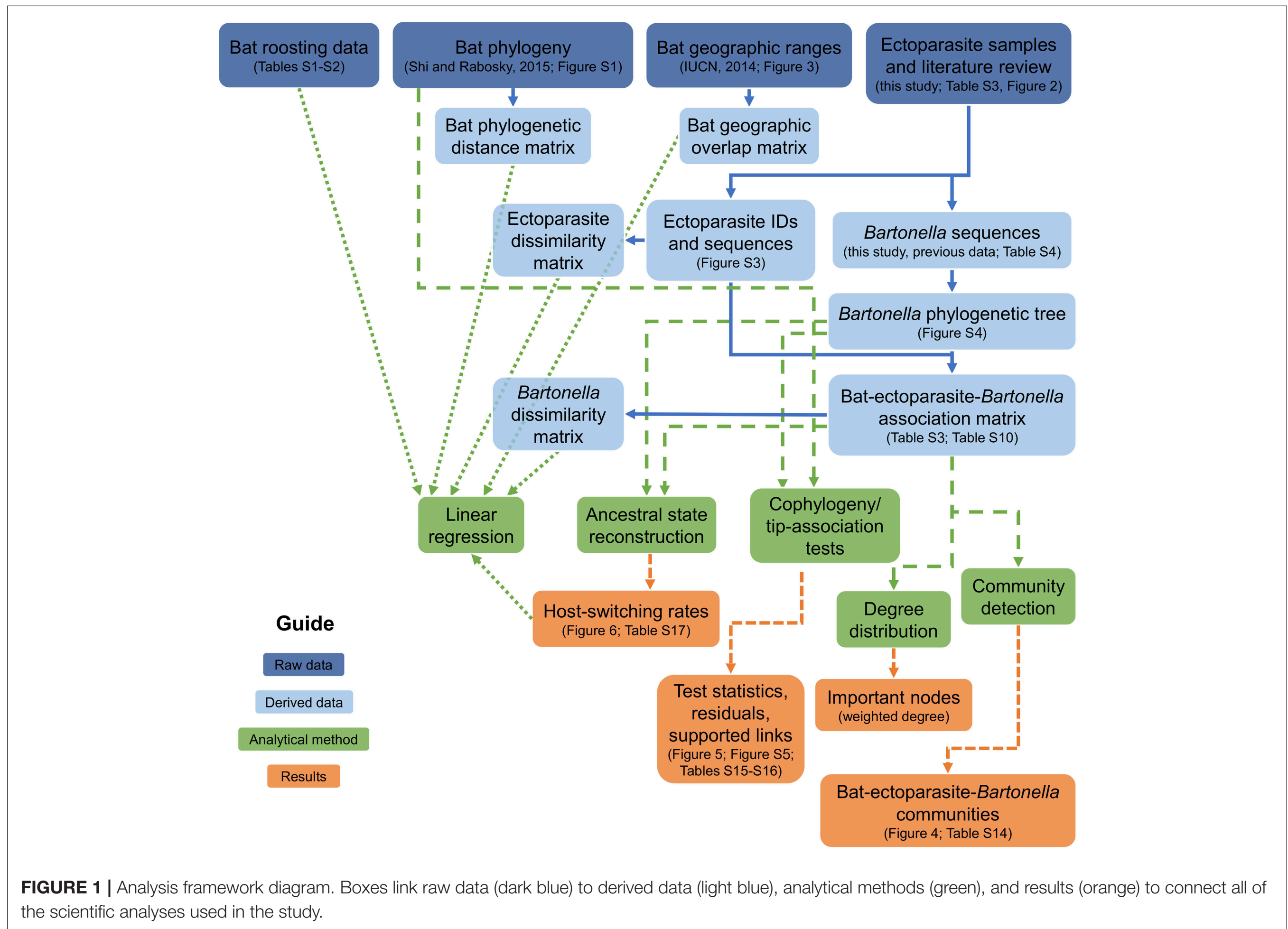


FIGURE 1 | Analysis framework diagram. Boxes link raw data (dark blue) to derived data (light blue), analytical methods (green), and results (orange) to connect all of the scientific analyses used in the study.

Review of Ectoparasite Host Range

We recognize that the relationships between bat hosts and ectoparasites in our dataset may not capture the full ectoparasite host range due to infrequent associations between ectoparasites and some bat hosts. To capture some of this additional variation, we performed a literature review (**Table S3**) of the host range of the 17 ectoparasite species for which we had associated *Bartonella* data. The search was implemented in Google Scholar, Web of Science, and GenBank using the ectoparasite species epithet in the search terms. Additional publications were obtained based on citations therein and from previous reviews of bat fly host associations (Szentiványi et al., 2016). This review gathered a total of 302 publications from 1835 to 2018, 212 of which yielded information relevant to the ectoparasite and bat species in the current study. We counted the number of publications for which an ectoparasite species was noted as occurring on a bat species, adding the current study toward each total only if an ectoparasite was sampled from a bat species in our collection. Bat–ectoparasite associations noted in the studies that did not record the full species epithet for both ectoparasite or host species were excluded.

Bartonella Amplification and Sequencing

Ectoparasites were tested individually for the presence of *Bartonella* spp. with a conventional PCR assay targeting the citrate synthase gene (*gltA*) using primers designed by Norman et al. (1995); the **Supplementary Material** contains additional details on *Bartonella* detection protocols. Previous studies have found *gltA* sequences to be sufficiently diverse to distinguish among *Bartonella* species and some subspecies (La Scola et al., 2003). Additionally, *gltA* is the most common marker for genotyping *Bartonella* species (Kosoy et al., 2018); hence, it is useful for comparing *Bartonella* diversity across studies. For *Bartonella* species identification, both strands of PCR products were Sanger sequenced (BaseClear, Leiden, the Netherlands) using the same forward and reverse primers as in the conventional PCR. To minimize cross-contamination and false-positive results, positive (pool of *Bartonella*-positive ticks; Tijsse-Klasen et al., 2011) and negative (water only) controls were included in each batch tested by PCR. Furthermore, DNA extraction, PCR mix preparation, sample addition, and PCR-product analysis were performed in assigned separate labs. Trimming and manual cleaning of *Bartonella* sequences were performed in BioNumerics v7.1 (Applied Math,

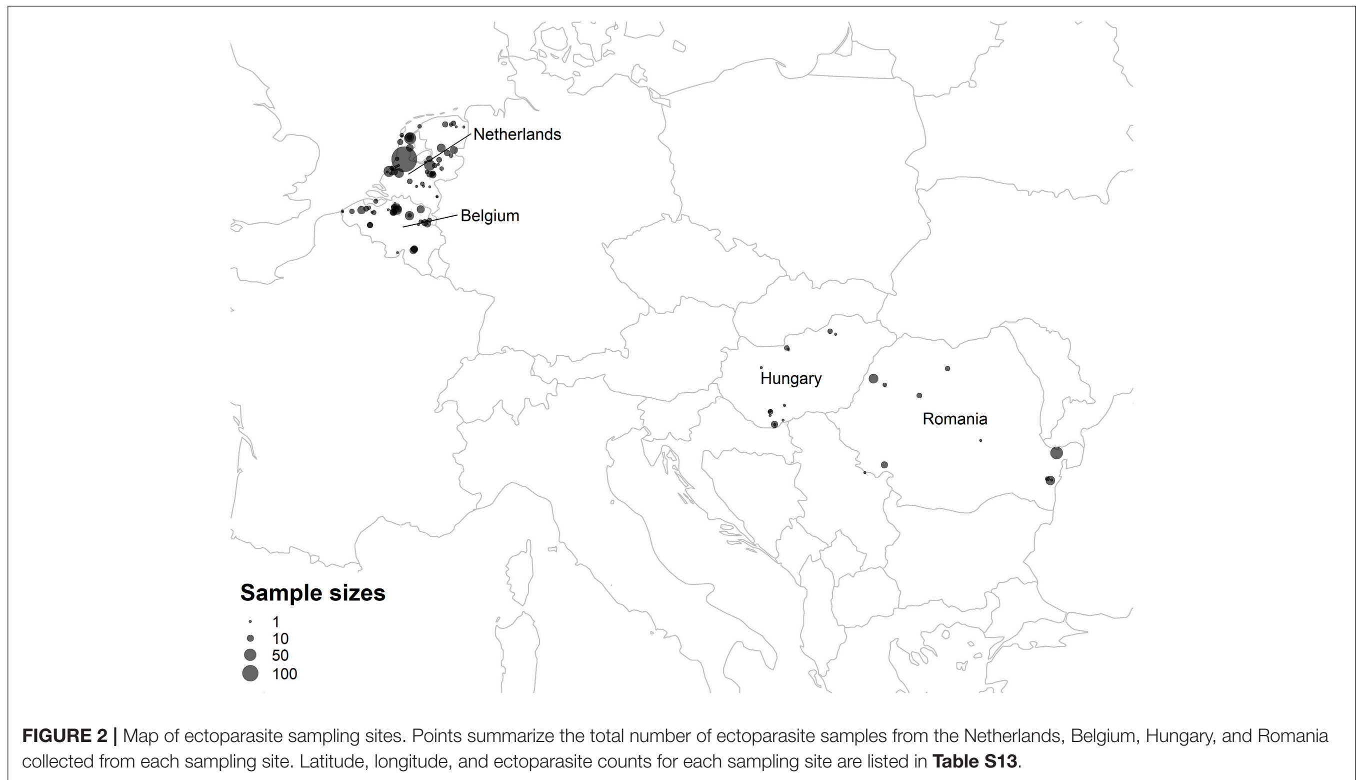


FIGURE 2 | Map of ectoparasite sampling sites. Points summarize the total number of ectoparasite samples from the Netherlands, Belgium, Hungary, and Romania collected from each sampling site. Latitude, longitude, and ectoparasite counts for each sampling site are listed in **Table S13**.

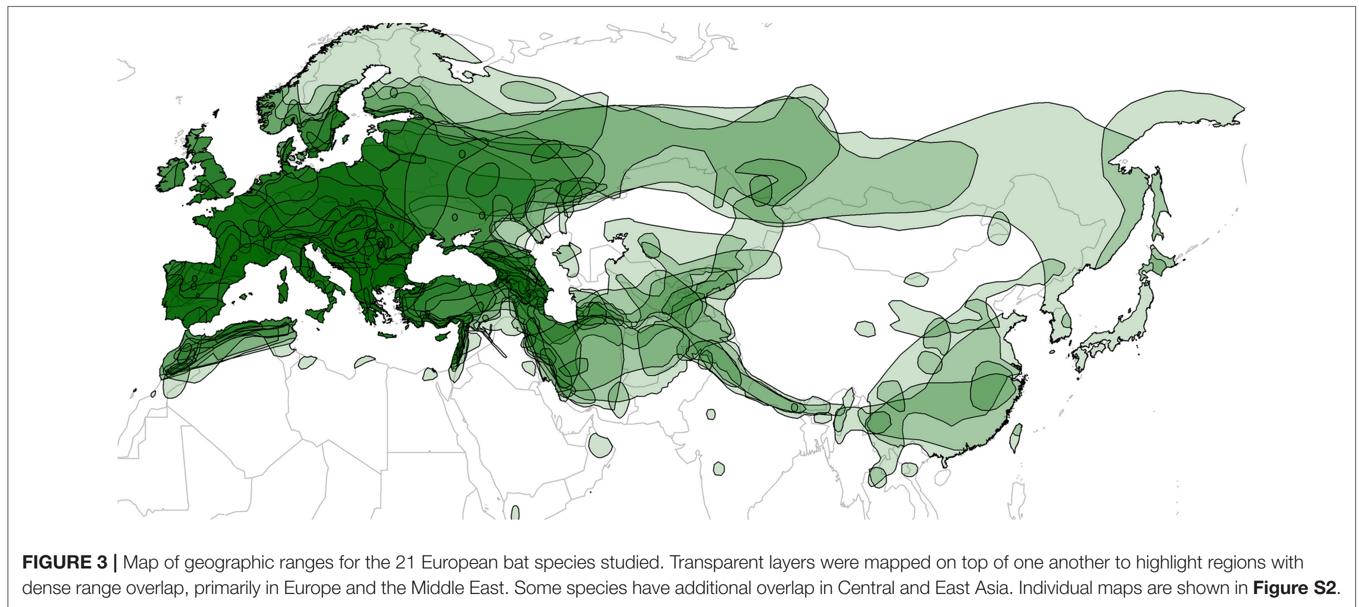


FIGURE 3 | Map of geographic ranges for the 21 European bat species studied. Transparent layers were mapped on top of one another to highlight regions with dense range overlap, primarily in Europe and the Middle East. Some species have additional overlap in Central and East Asia. Individual maps are shown in **Figure S2**.

Belgium) together with *Bartonella* reference sequences available in GenBank.

Sequences were further confirmed as *Bartonella* through Basic Local Alignment Search Tool (BLAST) searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on this initial screening, some sequences were identified as originating from *Bartonella* species not associated with bats or from bacteria of other genera and were removed before further analysis (**Supplementary Material**).

For the purposes of ecological and evolutionary analysis, we have assumed that a *Bartonella* strain amplified from an ectoparasite species may also be carried by the bat species on which that ectoparasite was found. We argue that even incidental ectoparasitism on an atypical bat host may lead to transmission of bacteria and is thus important for understanding available parasite host range. The host associations of ectoparasite-derived sequences were validated for the subset of *Bartonella*

TABLE 1 | Summary of *Bartonella* phylogenetic diversity for 21 European bat species included in our study.

Bat species	Species abbreviation	Median Faith's PD [95% CI]	Number of *gltA* sequences	Number of *Bartonella* OTUs	Number of host–ectoparasite linkages	Number of unique publications reviewed
Eptesicus nilssonii	Ept.nil	0.44 [0.36, 0.65]	1	1	5	20
Eptesicus serotinus	Ept.ser	1.06 [0.93, 1.29]	10	5	11	34
Miniopterus schreibersii	Min.sch	4.05 [3.7, 4.57]	111	9	7	45
Myotis bechsteinii	Myo.bec	1.34 [1.2, 1.61]	13	4	8	36
Myotis blythii	Myo.bly	2.68 [2.44, 3.08]	42	9	12	49
Myotis capaccinii	Myo.cap	0.44 [0.36, 0.65]	1	1	6	29
Myotis dasycneme	Myo.das	2.76 [2.48, 3.12]	80	4	11	24
Myotis daubentonii	Myo.dau	3.03 [2.79, 3.44]	57	8	15	81
Myotis emarginatus	Myo.ema	1.51 [1.36, 1.79]	18	5	8	17
Myotis myotis	Myo.myo	2.05 [1.87, 2.4]	26	5	14	85
Myotis mystacinus	Myo.mys	0.44 [0.36, 0.65]	1	1	12	23
Myotis nattereri	Myo.nat	0.93 [0.79, 1.14]	4	2	11	52
Nyctalus noctula	Nyc.noc	0.76 [0.62, 0.96]	4	3	9	24
Pipistrellus nathusii	Pip.nat	1.05 [0.89, 1.28]	26	3	7	15
Pipistrellus pipistrellus	Pip.pip	0.77 [0.65, 0.97]	4	2	9	48
Pipistrellus pygmaeus	Pip.pyg	0.6 [0.5, 0.8]	2	2	3	13
Plecotus auritus	Ple.aur	0.63 [0.52, 0.83]	5	1	13	42
Rhinolophus blasii	Rhi.bla	0.76 [0.67, 0.99]	3	2	4	14
Rhinolophus euryale	Rhi.eur	1.29 [1.14, 1.52]	30	4	6	23
Rhinolophus ferrumequinum	Rhi.fer	1.66 [1.54, 2.01]	18	6	10	38
Rhinolophus mehelyi	Rhi.meh	0.61 [0.51, 0.83]	5	4	4	12

*Median Faith's phylogenetic diversity (PD) and 95% confidence intervals were calculated from 100 posterior samples of the Bartonella gltA tree in **Figure S4**, pruned to the sequences found in the 21 species of European bats. The number of host–ectoparasite linkages observed is summarized from the literature review in **Table S3**, including new specimens collected during this study. Species abbreviations are used in **Figures 5**, **6** and **Figure S5**.*

species that have been characterized from bats in previous studies (**Supplementary Material**).

To aid in phylogenetic inference and the delineation of novel *Bartonella* species, additional *Bartonella gltA* sequences amplified from bats and ectoparasites were compiled from published articles and records in GenBank. This search was implemented in Google Scholar, Web of Science, and GenBank. Initial BLAST screening also indicated that some *Bartonella* sequences had close similarity to *Bartonella* sequences found in humans (Podsiadly et al., 2010; Lin et al., 2012) and stray dogs (Bai et al., 2010); thus, representative sequences from these studies were also included. Details on the origin of *Bartonella* sequences are listed in **Table S4**. Sequences were trimmed to a common length of 337 base pairs and aligned using the local and accurate L-INS-i method in MAFFT v7.187 (Katoh and Standley, 2013). The sequences were inspected for

gaps and misalignments and were removed if they contained obvious errors.

Phylogenetic Analysis of *Bartonella* Sequences

Two datasets were created for phylogenetic analyses. First, we compiled the full set of *Bartonella* sequences from bats and their ectoparasites from **Table S4** plus sequences from the current study (full dataset, $n = 754$), using three sequences from *Brucella* spp. as the outgroup. Second, this full set was restricted to *Bartonella* sequences from bats and their ectoparasites in Europe (European bat dataset, $n = 456$). While ectoparasites collected from roosts and bat boxes were tested for *Bartonella* spp., the DNA sequences derived from these samples were only used in the phylogenetic analysis of the full dataset. Since they did not contain information on the host species, they were excluded from the European dataset and were thus not used to assess *Bartonella* diversity among bat species or in the other tests detailed below (network analysis, cophylogeny, and regression). This restricted set contained sequences from the 21 bat species represented in **Figure S1** and the 17 ectoparasites represented in our literature review. Sequences from *My. oxygnathus* were combined with those from *My. blythii*. Due to the potential confounding factor of recombination in phylogenetic inference (Posada and Crandall, 2002), we performed the pairwise homoplasy index test (Bruen et al., 2005) in SplitsTree v4.13.1 (Huson, 2005). Tests using both the full and European bat datasets indicated no significant evidence that recombination affects our phylogenetic inference ($P = 0.41$ and $P = 0.25$, respectively).

We selected models derived from both datasets to determine the best sequence evolution, speciation, and codon partitioning models. Following this procedure, a phylogenetic tree was generated for the full dataset in BEAST using the GTR+Γ+I sequence evolution model and the birth–death speciation model with incomplete sampling and rate partitions for each codon position. See the **Supplementary Material** for additional details on model selection and phylogeny generation.

Branches of the phylogenetic tree were collapsed according to probable *Bartonella* species. Classification of bacterial species is challenging and may not conform well to species concepts developed for eukaryotes (Konstantinidis et al., 2006; Fraser et al., 2007). La Scola et al. (2003) proposed that *Bartonella* species could be distinguished if *gltA* sequences differed by >4% identity. Konstantinidis et al. (2006) advocated for a more stringent approach wherein bacterial species are demarcated by >5% difference in sequence identity, which corresponds well with another standard of bacterial species, 70% DNA–DNA hybridization. We chose to follow this more conservative approach and collapsed branches into operational taxonomic units (OTUs) based on ≤5% identity among sequences. Using these *Bartonella* OTUs, we can understand their ecology, specifically their host and ectoparasite associations, factors that can aid in demarcating *Bartonella* species or species complexes (Kosoy, 2010; Kosoy et al., 2012). Additional *post hoc* comparisons of OTUs with recently published *Bartonella* sequences from insectivorous bats in China and western, central, and eastern Europe were performed (Han et al., 2017; Stuckey et al., 2017a; Corduneanu et al., 2018). Phylogenetic diversity (PD) of *Bartonella* sequences from each bat species was assessed by the number of OTUs found in the species and (Faith, 1992) PD index based on branch lengths in 100 posterior samples of the *gltA* tree. Faith's PD was calculated in R using the "picante" package (Kembel et al., 2014).

Network Analysis and Community Detection

Weights for edges linking bat, ectoparasite, and *Bartonella* nodes were initially assigned based on the number of citations linking ectoparasite species to bat species in the literature review (**Table S3**) or the number of *Bartonella gltA* sequences for a given *Bartonella* OTU linked to a host bat or host ectoparasite (**Table S10**). To account for sampling intensity on edge weights, we adjusted bat–ectoparasite edge weights, w_{ab}, by dividing the number of citations linking ectoparasite b to bat a, n_{ab}, by the sum of the total unique publications surveyed for bat a, x_a, and the total unique publications surveyed for ectoparasite b, x_b; thus, $w_{ab} = n_{ab}/(x_a + x_b)$. Similarly, we adjusted weights for edges linking *Bartonella* OTUs to hosts (either bat species or ectoparasite species), w_{cd}, by dividing the number of *gltA* sequences linking OTU d to host c, n_{cd}, by the sum of the total *gltA* sequences obtained from host c, y_c, and the total *gltA* sequences obtained for OTU d, y_d; thus, $w_{cd} = n_{cd}/(y_c + y_d)$. Therefore, edge weights were constrained to be between 0 and 0.5, with an actual range of 0.00431 to 0.429.

We performed community detection on the tripartite network using three algorithms available in the R "igraph" package: the information map method (Rosvall and Bergstrom, 2008), the Louvain method (Blondel et al., 2008), and the spin glass method (Reichardt and Bornholdt, 2006). The purpose of using multiple algorithms was to account for some uncertainty in the identification of communities (see the **Supplementary Material** for details on the algorithms). Communities were visualized using the "HiveR" package in R (Hanson et al., 2016).

To identify bat, ectoparasite, and *Bartonella* species that might be highly influential in the network, we examined nodes that were highly connected in the tripartite network based on calculation of their weighted degree. A node's weighted degree represents the sum of the edge weights connecting a node to other nodes. Nodes were selected as influential if they were in the top 25th percentile of weighted degree. We then examined how the selected nodes were connected to other nodes within the communities detected by the community detection algorithms.

Cophylogeny and Tip-Association Tests for Bats and *Bartonella*

Clustering of traits among tips of the *Bartonella* tree (European bat dataset) was tested using the Bayesian Tip-association Significance Testing (BaTS) program (Parker et al., 2008). We assessed the clustering of bat taxonomic traits (species, genera, families, and suborders) and countries sampled. Significance of clustering for each trait was assessed by comparing the calculated

association index (AI) and parsimony score (PS) for 100 posterior samples of the *Bartonella* tree against null distributions generated from 1,000 randomizations of traits to tips along each sampled *Bartonella* tree.

Phylogenetic trees of bat species and *Bartonella* OTUs were assessed for evidence of evolutionary codivergence using two algorithms: the Procrustean Approach to Cophylogeny (PACo; Balbuena et al., 2013) and the ParaFit method (Legendre et al., 2002). Trees were imported into R using the "ape" package (Paradis et al., 2004, 2016) and then rescaled to have a maximum branch length of one by dividing all branch lengths by the longest branch in each tree. A binary association matrix linking bat species to *Bartonella* OTUs was assembled based on **Table S10**. We tested the pattern of codivergence using ParaFit using the "ParaFit" function in the "ape" package with 999 permutations and stored the *P*-values for the contributions of individual linkages (known as ParaFitLink1 or F1 statistics). Codivergence was tested in PACo using the "paco" package in R (Balbuena et al., 2016) with 1,000 permutations. PACo residuals and mean jackknife contributions for individual linkages were stored to compare with results from ParaFit. We performed the tests on the maximum clade credibility *Bartonella* tree and 100 sampled posterior trees to assess the effect of phylogenetic uncertainty in the global fit tests.

Bayesian Prediction of *Bartonella* Host-Switching Rates

Historical rates of host-switching by *Bartonella* lineages among bat host species were predicted using Bayesian ancestral state reconstruction in BEAST. The host-switching rates discussed here represent a latent biological process (movement of agents between species) that was not observed but can be estimated based on observed host-switching events in the phylogenetic tree. Since we are interested primarily in the process, we chose to look at estimated host-switching rates rather than observed events. With the European bat *Bartonella* sequence dataset, we used the GTR+Γ+I sequence evolution model and the birth–death speciation model with incomplete sampling and rate partitions for each codon position (see the **Supplementary Material** for more details). Rates with Bayes factors (BF) > 3 after the stochastic search variable selection were considered well-supported (Lemey et al., 2009). A graph representing *Bartonella* transitions among bat species was drawn based on well-supported rates using the "arcdiagram" package in R (Sanchez, 2013).

Regression Analyses

Regression analyses centered around two primary response datasets: a dissimilarity matrix calculated from the counts of *Bartonella* OTUs found in each of the 21 bat species or their associated ectoparasites and the predicted host-switching rates from the ancestral state reconstruction analysis (**Figure 1**). *Bartonella* and ectoparasite dissimilarity were calculated using the binomial and Cao indices (Cao et al., 1997; Anderson and Millar, 2004), which can handle variable sample sizes and can calculate dissimilarity between species that have no shared parasites (Oksanen et al., 2015), and the Spearman rank correlation, which was subtracted from one to transform it into a dissimilarity measure. These same indices were used to measure ectoparasite dissimilarity. Other indices, specifically Pearson correlation and Bray–Curtis dissimilarity, were explored but were not chosen because they violated assumptions of normality in residuals. Only those host-switching rates with BF > 3 were kept for this analysis to be confident in the estimated rate values. Additional details on data selection for regression can be found in the **Supplementary Material**.

There were five primary predictors considered in the regression analyses: dissimilarity in ectoparasite sharing between bat species, phylogenetic distance between bat species, bat geographic range overlap, summer roost sharing of bats, and a vector of the least sampled species from the bat–*Bartonella* association matrix (**Figure 1**). The phylogenetic distance matrix from Shi and Rabosky (2015) was initially scaled in terms of branch ages (in millions of years); hence, we rescaled the branch lengths to be between zero and one by dividing all branch lengths by the maximum length. The summer roosting patterns of bats are a binary variable indicating whether or not bats share roosts during the summer months. The vector of least sampled species was selected from the bat–*Bartonella* association matrix as the minimum row sum for each species pair; this was then log-transformed. We only used the vector of least sampled species in the regression of *Bartonella* host-switching rates because there appeared to be a sampling bias in the predicted rates such that better sampled species tended to have higher median rates (Pearson's $R = 0.62$, $t = 4.15$, df $= 28$, $P = 0.00028$). Our dissimilarity measures did not appear to have such a bias.

Before performing regressions, we rescaled all the data and predictors to standard normal distributions with the exception of roost sharing, which was retained as a binary predictor. We performed separate model selection procedures on three global candidate model sets including a global model with all predictors and all subsets of the global model. The first global model set used ectoparasite dissimilarity, host phylogenetic distance, geographic range overlap, and roost sharing to predict *Bartonella* dissimilarity. The second set used host phylogenetic distance, geographic range overlap, and roost sharing to predict ectoparasite dissimilarity. The third set used the samples from the least sampled host species, ectoparasite dissimilarity, host phylogenetic distance, geographic range overlap, and roost sharing to predict *Bartonella* host-switching rates. For all models containing *Bartonella* or ectoparasite dissimilarity as data or predictors, we performed model selection based on regressions using all three dissimilarity indices (Spearman correlation, binomial, and Cao indices). For the host-switching models, we performed model selection on both median and mean host-switching rates.

Models were fit using linear regression with normally distributed errors. We selected models based on iterative testing of predictors in the full model and ranked them according to the Akaike information criterion with a correction for finite sample sizes (AICc) using the "dredge" function in the "MuMIn" package in R (Barton, 2016). We chose the model with the smallest AICc unless another model was less than

two AICc away from the top model (Burnham and Anderson, 2004), in which case we chose the simplest model based on the principle of parsimony. For both the global and the top models, we recorded adjusted R^2, inspected residual plots and quantile–quantile plots, and performed a Shapiro–Wilk test (Shapiro and Wilk, 1965) to confirm normality of residuals. We recorded standardized main effect coefficients, *t* statistics, *F* statistics, and associated *P*-values for regression parameters. To assess model fit, we performed *k*-fold cross-validation with 10-folds using the "cv.lm" function in the "DAAG" package in R (Maindonald and Braun, 2015). To assess the relative importance of parameters in models, we recorded adjusted partial R^2 and relative importance values. Relative importance was calculated with the "calc.relimp" function using the (Lindeman et al., 1980)method in the R package "relimpo" (Groemping and Matthias, 2013). Bootstrap confidence intervals for relative importance values were estimated from 1,000 replicates. Due to potential nonindependence of comparisons between pairs of species, we also used Mantel tests (Mantel, 1967) to examine correlations between responses and predictors. We calculated the Pearson correlation and compared it to a null distribution generated from 999 random combinations of cells from the two matrices.

RESULTS

Bat Ectoparasites

Ectoparasites ($n = 903$) were collected in the Netherlands and Belgium from 268 individual bats belonging to 11 species (*E. serotinus*, *Ny. noctula*, *Pi. nathusii*, *Pi. pipistrellus*, *Pi. pygmaeus*, *Pl. auritus*, *My. bechsteinii*, *My. dasycneme*, *My. daubentonii*, *My. mystacinus*, and *My. nattereri*). In addition, 170 nycteribiid flies from 169 individual bats (*Mn. schreibersii*, *My. bechsteinii*, *My. blythii*, *My. capaccinii*, *My. daubentonii*, *My. myotis*, *My. nattereri*, *R. blasii*, *R. euryale*, *R. ferrumequinum*, and *R. mehelyi*) derived from a study by Sándor et al. (2018) in Hungary and Romania were included.

A total of 1,073 ectoparasites were collected across the four countries (**Figure 2**). Morphological and molecular identification revealed 15 ectoparasite species from 7 families: two ticks, *Argas vespertilionis* and *Ixodes ariadnae* (Ixodida: Argasidae, Ixodidae); one bat bug, *Cimex pipistrelli* (Hemiptera: Cimicidae); one bat flea, *Ischnopsyllus variabilis* (Siphonaptera: Ischnopsyllidae); seven bat flies, *Basilia nana*, *B. nattereri*, *Nycteribia kolenatii*, *Nb. schmidlii*, *Penicillidia conspicua*, *Pn. dufourii*, and *Phthiridium biarticulatum* (Diptera: Hippoboscoidea: Nycteribiidae); and two bat mites, *Spinturnix andegavinus* and *S. plecotina* (Mesostigmata: Spinturnicidae). Additional mite specimens (Mesostigmata: Macronyssidae, Spinturnicidae) that could not be identified to the species level by morphology were delineated as two distinct taxa by COI sequences (**Supplementary Material**; **Figure S3**). Ectoparasite specimen counts collected in the Netherlands, Belgium, Hungary, and Romania from each bat species are summarized in **Table S11**. **Table S12** records individual ectoparasite species identifications, *Bartonella* testing results, and sampling sites.

Phylogenetic Relationships Between *Bartonella* Sequences

In total, 412 *gltA* sequences were obtained from the 1,073 ectoparasites (38%) collected from European bats in Belgium, Hungary, the Netherlands, and Romania (**Table S12**). After filtering out sequences that were not *Bartonella*, these 316 sequences were combined with the 438 reference sequences listed in **Table S4**, resulting in the full dataset of 754 *Bartonella gltA* sequences used for phylogenetic analysis and delineation of *Bartonella* OTUs. The subset of 456 *gltA* sequences comprising the European bat dataset represents data from nine countries: Belgium, Finland, Georgia, Hungary, the Netherlands, Poland, Romania, Slovenia, and the United Kingdom. The sequences from Finland, Georgia, Poland, Slovenia, and the United Kingdom derive from past observational studies of *Bartonella* infections in bats and their ectoparasites (Concannon et al., 2005; Morse et al., 2012; Veikkolainen et al., 2014; Lilley et al., 2015; Urushadze et al., 2017; Szubert-Kruszynska et al., 2018).

Based on our demarcation of 5% sequence divergence for separating *Bartonella* species, we observed 49 monophyletic clusters of *gltA* sequences identified as OTUs and 39 individual *gltA* sequences that are distinct from these OTUs out of the 754 *gltA* sequences analyzed, resulting in an estimate of at least 88 distinct *Bartonella* species found in bats worldwide, 20 of which are found in European bats and ectoparasites (**Figure S4**). All OTUs had strong posterior support (posterior node probability > 0.9); however, support for nodes connecting OTUs into larger clades decreased significantly for deeper nodes. A general pattern of separation between *Bartonella* OTUs and sequences found in New World bats (colored green in **Figure S4**) and Old World or European bats (colored blue and red in **Figure S4**, respectively) was observed, as in a previous analysis by McKee et al. (2016).

Bartonella diversity varied across European bat species, with a range of one to nine OTUs and a range of 0.44–4.05 for Faith's PD for *E. nilssonii* and *Mn. schreibersii*, respectively (**Table 1**). Faith's PD per species was significantly positively correlated with the log number of *gltA* sequences obtained for that species (Pearson's $R = 0.88$, $t = 8.04$, df $= 19$, $P < 0.0001$) and with the number of *Bartonella* OTUs observed per species (Pearson's $R = 0.88$, $t = 7.96$, df $= 19$, $P < 0.0001$). After accounting for this significant sampling effect on *Bartonella* diversity, there was no significant correlation between Faith's PD and the number of ectoparasites associated with each bat species ($t = 0.6$, df $= 18$, $P = 0.56$) or the number of OTUs and ectoparasites ($t = 0.17$, df $= 18$, $P = 0.87$).

Network Analysis and Community Assignment

The three community detection algorithms consistently identified seven communities: Min/Myo, VespA–VespE, and Rhi (**Figure 4** and **Table S14**). There were only minor inconsistencies in the community assignment of a few species. Two algorithms (information map and spin glass) lumped *Pl. auritus* and *S. plecotina* into a distinct community, but the Louvain algorithm placed them with community VespC. The Louvain and spin glass algorithms grouped *I. ariadnae* with community VespB,

whereas the information map algorithm grouped this species with community VespD.

The seven communities are broadly organized based on host phylogeny, geographic overlap, and roost sharing (**Figures S1, S2** and **Tables S1**, **S2**). Min/Myo contains species from two related families of bats (Vespertilionidae and Miniopteridae) that roost together in caves or other cave-like structures predominantly in southern Europe (with the exception of *My. myotis*, which is more widespread in Europe; **Figure S2**). Rhi contains only *Rhinolophus* species that roost together in caves mostly in southern Europe, North Africa, and the Middle East (**Figure S2** and **Table S2**). VespA contains three closely related *Myotis* species that roost in tree cavities during the summer and swarm at underground sites during autumn and hibernate there in winter. VespB contains vespertilionid bats that roost in tree cavities, buildings, and caves during the summer and winter, although with little overlap in roosting patterns among species. However, these species do share some other traits in common, including long-distance migration (*My. dasycneme*, *Ny. noctula*, and *Pi. nathusii*) and a relatively northern distribution within Europe. VespC contains two *Eptesicus* species that roost in buildings during the summer and winter. Communities VespD and VespE contained single bat species that had highly specific ectoparasite or *Bartonella* species and were thus segregated from other communities despite having phylogenetic similarity or similar roosting habits to other species in these communities.

For 11 of the *Bartonella* OTUs in this study, community assignments corresponded well with other sequence data collected from related bats in Africa, Asia, and Europe (Kosoy et al., 2010a; Lin et al., 2012; Morse et al., 2012; Anh et al., 2015; Wilkinson et al., 2016; Han et al., 2017; Lilley et al., 2017; Stuckey et al., 2017a; Corduneanu et al., 2018). Further details on comparisons between OTUs and other sequences from previous studies can be found in the **Supplementary Material**.

Fifteen nodes were identified as being influential based on their weighted degree in the bat–ectoparasite–*Bartonella* association network. Five bats (*Mn. schreibersii*, *My. blythii*, *My. daubentonii*, *My. myotis*, and *R. ferrumequinum*), nine ectoparasites (*A. vespertilionis*, *B. nana*, *C. pipistrelli*, *Nb. kolenatii*, *Nb. schmidlii*, *Pn. conspicua*, *Pn. dufourii*, *Ph. biarticulatum*, and *S. myoti*), and one *Bartonella* OTU (OTU19) were identified as influential because they fell in the top 25th percentile for weighted degree. The ecology of these species may explain their influence in the network. *My. daubentonii* males are known to form social colonies and therefore high ectoparasite densities have been observed on both sexes (Encarnação et al., 2012). *Mn. schreibersii*, *My. blythii*, *My. myotis*, and *R. ferrumequinum* form mixed roosts in caves (**Table S2**). All nine ectoparasite species are very promiscuous in their host associations (**Table S3**). We note that *C. pipistrelli* and *A. vespertilionis* are known to bite humans (Jaenson et al., 1994; Estrada-Peña and Jongejan, 1999; Whyte et al., 2001) and were grouped in the same community as OTU19 and OTU26, which have both been found to infect humans (Veikkolainen et al., 2014; Urushadze et al., 2017). For additional details on the identification of highly influential nodes and their community assignments, see the **Supplementary Material**.

Cophylogeny and Tip-Association Tests for Bats and *Bartonella*

Tip-association tests showed significant clustering for all taxonomic levels (species, genera, families, and suborders) and sampled countries with none of the observed AI or PS distributions overlapping with the null distributions (**Table S15**). Mean AI and PS values were smaller for genus, family, and suborder compared to the AI and PS values for sampled countries, indicating that the clustering of tips of the tree is better explained by host phylogeny than geography. The cophylogeny global fit analyses both found significant evidence of evolutionary congruence between the bat and *Bartonella* phylogenies (PACo sum of squared residuals = 21.69, $P < 0.0001$; ParaFit sum of squared residuals = 21.97, $P = 0.001$). All PACo and ParaFit tests using sampled posterior *Bartonella* trees showed significant congruence, demonstrating that the results are robust with respect to phylogenetic uncertainty in the *Bartonella* tree.

Many of the supported links from the PACo and ParaFit tests are between bat species and *Bartonella* OTUs in the same community (indicated by line colors in **Figure 5**) and had high network edge weights (indicated by line width in **Figure 5**). Thirty-one out of the 81 (38%) bat–*Bartonella* links were highly supported by either PACo or ParaFit (**Figure S5** and **Table S16**). These links were considered highly supported if the upper limit of the PACo jackknife 95% confidence interval was below the mean of all the squared residuals or if the ParaFit F1 statistic was assigned a P-value < 0.01. Of these 31 highly supported links, 22 (71%) were between bat species and *Bartonella* OTUs identified as being in the same community (**Table S16**). This proportion of highly supported links in the same community was higher than the proportion of less supported or unsupported links in the same community ($\chi^2 = 3.23$, df = 1, $P = 0.036$). Additional details on individual host–parasite links can be found in the **Supplementary Material**.

Bayesian Prediction of *Bartonella* Host Transition Rates

Using the symmetrical rate partition model, only 30 (14%) out of the possible 210 bat host species combinations had significant Bayes factors (BF > 3) from the stochastic search variable selection procedure (**Figure 6** and **Table S17**). The median host-switching rates varied from 0.37 for *E. nilssonii* and *E. serotinus* to 1.83 for *My. blythii* and *My. myotis*. Large host-switching rates tended to have higher BF support (Pearson's $R = 0.52$, $t = 3.23$, df = 28, $P = 0.0032$), and rates tended to be biased toward bat species in the same family and subfamily (**Figure 6**). Of the 30 rates, 25 (83%) were between bat species in the same family. This is significantly higher than the expected proportion (126/210, 60%) based on the number of possible species combinations that are in the same family ($\chi^2 = 5.17$, df = 1, $P = 0.012$). The majority of host-switching rates (25/30, 90%) have species pairs in the same identified community (11/30, 37%) or in a community containing bats from the same family (16/30, 53%). Fourteen of the 30 host-switching rates (47%) had species pairs that co-roost during summer months (**Table S2**).

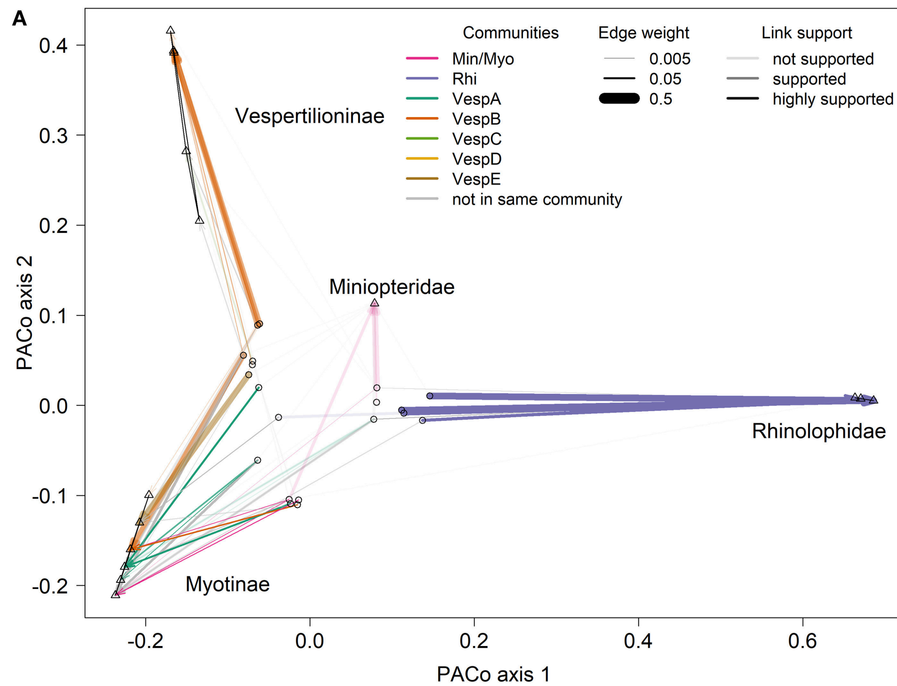
FIGURE 4 | Communities of *Bartonella* operational taxonomic units (OTUs), ectoparasite species, and bat species. Tripartite networks were drawn using separate axes for *Bartonella*, bats, and ectoparasite nodes. Edges connecting nodes were drawn based on **Tables S3** and **S10**, with edge weights adjusted for sampling intensity. Communities were identified by three detection algorithms (information map, Louvain, and spin glass). Species membership in each community is recorded in **Table S14**.

Regression Analysis

The top regression model according to AICc (**Table S18**) for Spearman *Bartonella* dissimilarity was the model with host phylogenetic distance, geographic range overlap, and roost sharing covariates (AICc = 496). The top model for the binomial *Bartonella* dissimilarity was the model containing only the host phylogenetic distance covariate (AICc = 562). The top model for the Cao dissimilarity contained the host phylogenetic distance and roost sharing covariates (AICc = 565).

All top models were statistically significant (**Table S18**) and estimated a statistically significant positive effect of host phylogenetic distance on *Bartonella* dissimilarity among bat species (**Table S19**). Since the three candidate models (Spearman, binomial, and Cao) all use different data for regression, we cannot compare them *via* AICc. Instead, we compared them according to the proportion of variance in *Bartonella* dissimilarity explained (adjusted R^2), the regression mean squared error, and the cross-validation mean squared error. The model using the Spearman correlation explained more variation in *Bartonella* dissimilarity and had lower mean squared error than the binomial and Cao indices (**Table S18**). From this model, we can infer that bats are more likely to have dissimilar *Bartonella* assemblages if they are more distantly related to each other (t = 8.6, df = 206, P < 0.0001), bats have little overlap in their geographic ranges (t = −2.43, df = 206, P = 0.016), and bats do not roost together during the summer (t = −3.81, df = 206, P = 0.00019). Between these three covariates, host phylogenetic distance explains more variation (adjusted partial R^2 = 0.26) than geographic range overlap (adjusted partial R^2 = 0.023) or roost sharing (adjusted partial R^2 = 0.061). This difference in explanatory power is significant because the 95% bootstrap confidence intervals for their relative importance do not overlap (**Table S19**). Individual Mantel tests confirmed that phylogenetic distance had the strongest correlation with *Bartonella* dissimilarity (**Table S20**).

The second candidate model set used host phylogenetic distance, geographic range overlap, and roost sharing to predict ectoparasite dissimilarity. The top model chosen for all dissimilarity indices (Spearman, binomial, and Cao) included all covariates. All three models were statistically significant (**Table S18**) and estimated the effect of host phylogenetic distance to be positive and the effects of geographic range overlap and roost sharing to be negative (**Table S19**). According to the Spearman model, bats are more likely to have more dissimilar ectoparasite assemblages if they are more distantly related to one another (t = 3.85, df = 206, P = 0.00016), they have less geographic range overlap (t = 3.04, df = 206, P = 0.0026), and they do not roost together in the summer (t = −3.98, df = 206, P < 0.0001). All three covariates explain similar amounts of variation, and they do not significantly differ in their explanatory power since their relative importance confidence intervals overlap (**Table S19**). Mantel tests confirmed these findings with correlations of similar magnitude across all the three predictors (**Table S20**). Therefore, since ectoparasite dissimilarity is explained by host phylogenetic distance, geographic range overlap, and roost sharing, we observe no additional effect



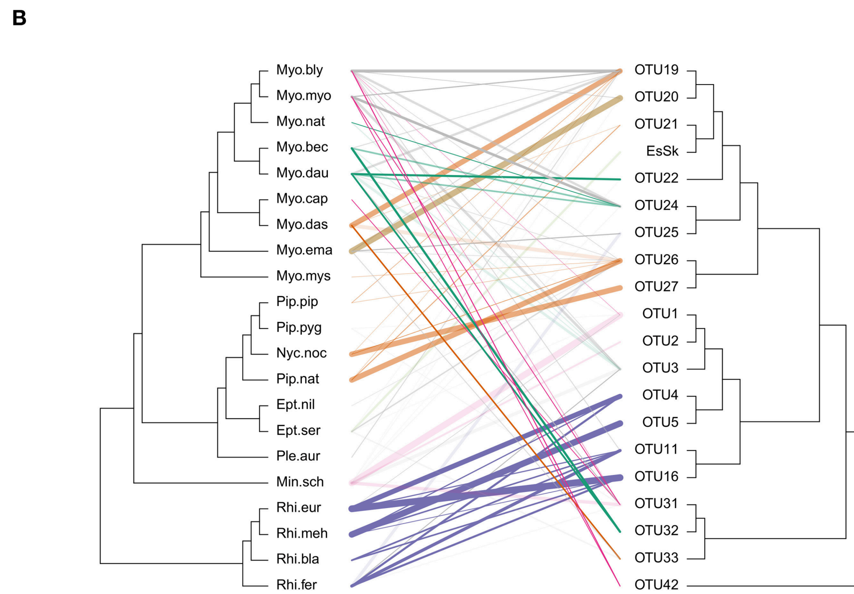


FIGURE 5 | Comparisons of bat species and Bartonella OTU phylogenies. **(A)** Procrustes superimposition plot with *Bartonella* OTUs (open circles) and bat species (open triangles). In this plot, the axes represent the principle components of the bat species phylogeny. Bat species are projected onto the two main axes explaining the most variation. Names of bat families and subfamilies are indicated for clusters of species. The *Bartonella* OTUs are then projected and rotated to fit the bat phylogeny by minimizing the residual distances for each bat-Bartonella association (connected by lines). **(B)** Cophylogeny plot with the bat species phylogeny on the left side and the *Bartonella* OTU phylogeny on the right side. Lines in the middle connect bat species to *Bartonella* OTUs based on sequence data collected from bats or associated ectoparasites. Species abbreviations for bat species are listed in **Table 1**. In both **(A)** and **(B)**, bat-*Bartonella* links colored by the community if both the bat species and *Bartonella* were placed in the same community (as in **Table S14**). Line widths in both **(A)** and **(B)** are proportional to network edge weight and the line transparency depend on the link support from PACo and ParaFit analyses.

of ectoparasite dissimilarity on *Bartonella* dissimilarity after accounting for these effects.

Finally, the third set of candidate models used the number of *gltA* sequences from the least sampled bat species plus all four other covariates. The first covariate was used to account for the observed sampling bias in host-switching rates discussed above. We evaluated models using both mean and median host-switching rates taken from the posterior distributions of

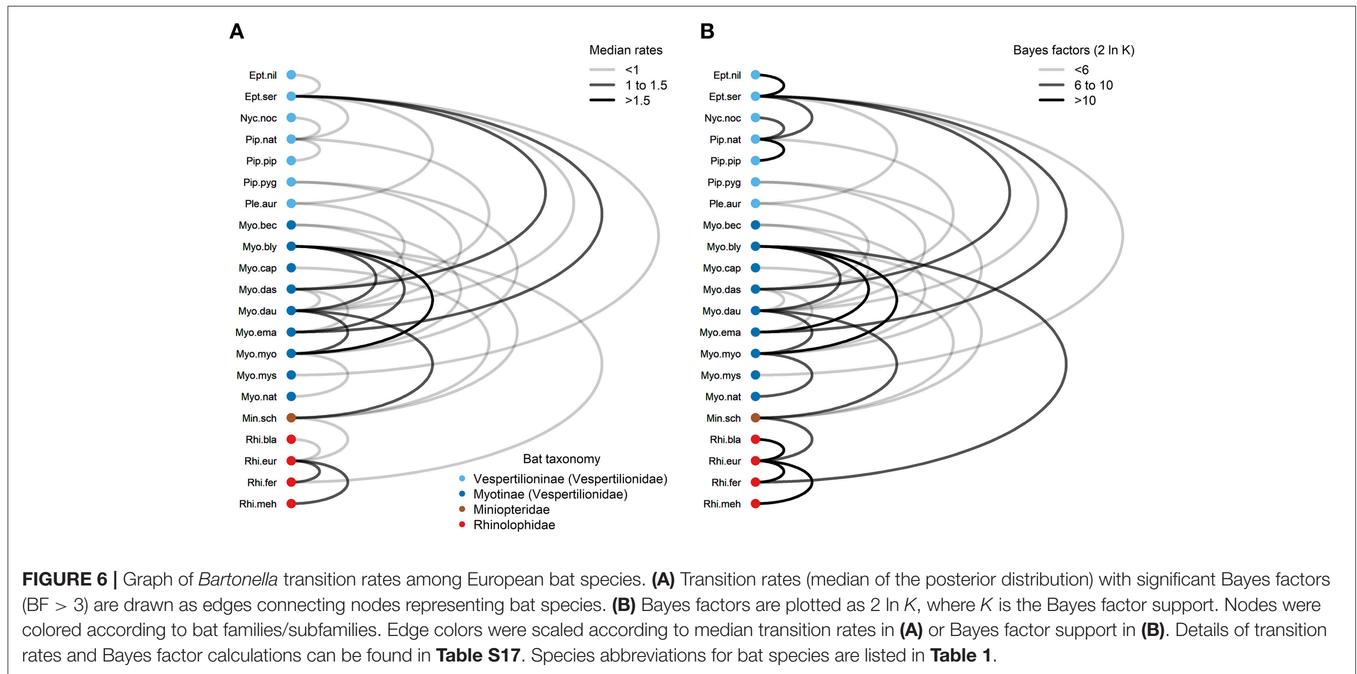


FIGURE 6 | Graph of *Bartonella* transition rates among European bat species. **(A)** Transition rates (median of the posterior distribution) with significant Bayes factors (BF > 3) are drawn as edges connecting nodes representing bat species. **(B)** Bayes factors are plotted as 2 ln *K*, where *K* is the Bayes factor support. Nodes were colored according to bat families/subfamilies. Edge colors were scaled according to median transition rates in **(A)** or Bayes factor support in **(B)**. Details of transition rates and Bayes factor calculations can be found in **Table S17**. Species abbreviations for bat species are listed in **Table 1**.

rates from the Bayesian ancestral state reconstruction analysis. Following model selection by AICc, both the mean and median host-switching models contained covariates for the least sampled bat species and host phylogenetic distance. Therefore, all the top Spearman, binomial, and Cao models were identical. Both the mean and median models were significant (**Table S18**) and estimated a positive effect for sampling and a negative effect for host phylogenetic distance (**Table S19**). The median host-switching model explained more variation and had lower mean squared error than the mean model (**Table S19**). This model indicates that after accounting for a positive sampling bias ($t = 4.59$, df $= 27$, $P < 0.0001$), *Bartonella* infections are more likely to switch between bat species that are more phylogenetically related ($t = -2.32$, df $= 27$, $P = 0.028$).

For all top model sets (Spearman *Bartonella* dissimilarity, Spearman ectoparasite dissimilarity, and median host-switching rates), we confirmed the fit of regression models visually by plotting the standardized data against the standardized partial regression coefficients. The plots for Spearman dissimilarity (**Figure S6**) confirm the positive effect of host phylogenetic distance and the negative effects of geographic range overlap and roost sharing on both *Bartonella* dissimilarity and ectoparasite dissimilarity. We also confirm the negative effect of host phylogenetic distance and positive sampling effect on *Bartonella* host-switching rates (**Figure S6**).

DISCUSSION

Diversity and Structure of Bat–Ectoparasite–*Bartonella* Communities

Using a multifaceted analytical approach (**Figure 1**), we explored the complex nature of *Bartonella* associations with bats and their ectoparasites in nine European countries. Our first objective was to measure the diversity of *Bartonella* infections in European bats and their ectoparasites and to analyze the structure of bat–ectoparasite–*Bartonella* communities. We observed high PD among *Bartonella* infections in our samples (**Table 1**), identifying 20 *Bartonella* OTUs that likely represent distinct species (**Figure S4**). Network analysis provided support for our hypothesis that associations between bats, ectoparasites, and bacteria could be resolved into identifiable communities. We detected seven distinct bat–ectoparasite–*Bartonella* communities (**Figure 4**) that separate by bat phylogeny, geographic ranges, and roosting patterns.

However, the separation of parasites among *Myotis* spp. bats in our study illustrates the complexity of ecological factors that shape host–parasite associations. *Bartonella* species found in *Myotis* spp. bats and their ectoparasites were partitioned into four distinct communities: Min/Myo, VespA, VespB, and VespE (**Figure 4** and **Table S14**). All of these communities were separate from the *Rhinolophus*-associated Rhi community. This is notable especially for the Min/Myo community because there is overlap in the geographic range and habitat usage (caves) among the bat species in the Rhi and Min/Myo communities. This suggests that distinct host–parasite associations can form despite hosts living in sympatry. Community VespA separates from Min/Myo because the hosts roost mainly in trees during the summer and only use caves during the winter. Species in community VespB use a variety of roosts with little overlap; some are long-distance migrants, and others have relatively northern distribution within Europe. The parasite communities of *Myotis* spp. bats have thus been shaped by a mixture of different factors that reduce exchange of parasites among hosts, providing motivation for analyzing patterns of

Bartonella sharing using a linear regression approach as we have done.

Evolutionary Patterns of Bat–*Bartonella* Associations

The second objective of our study was to understand the phylogenetic patterns generated from host–parasite associations. We expected that the phylogeny of *Bartonella* species would exhibit significant clustering by bat taxonomy and would have significant congruence with the phylogeny of bat species. As predicted, our tip-association and cophylogeny tests demonstrated that *Bartonella* lineages strongly cluster by host taxonomy, and the structure of the *Bartonella* phylogeny is congruent with the host phylogeny. However, these patterns are not entirely consistent with a pattern of strict cospeciation with bats. Rather, *Bartonella* lineages infecting bats appear to be polyphyletic, suggesting a more complex history of host-switching and possibly multiple introductions from other mammals deeper in the evolutionary tree (McKee et al., 2017; Urushadze et al., 2017; Frank et al., 2018). Cospeciation of hosts and parasites is a rare phenomenon in general (de Vienne et al., 2013), and several studies of bat viruses have shown that host-switching is the more dominant macroevolutionary force shaping microbial evolution than cospeciation (Cui et al., 2007; Mélade et al., 2016; Anthony et al., 2017). Previous research on *Bartonella* associations in bats and rodents showed that host-switching is more common than cospeciation (Lei and Olival, 2014). As we will discuss more below, it is more likely that the observed congruence of *Bartonella* and bat phylogenies is driven by phylogenetic bias in microbial host-switching, such that historical host shifts are more likely to happen between closely related species.

Predictors of *Bartonella* Sharing and Host-Switching Among Bat Species

Our last objective was to identify the ecological and evolutionary constraints that lead to *Bartonella* host specificity. We hypothesized that host phylogenetic distance, ectoparasite sharing, geographic range overlap, and roost sharing would be predictors of *Bartonella* sharing and host-switching rates among bat species. Our regression analysis (**Figure S6**; **Table S18**, **Table S19**) demonstrated that bats that are more phylogenetically related, overlap more in their geographic ranges, and share roosts are more likely to share *Bartonella* species, with phylogenetic distance being the most important predictor. Ectoparasite sharing between bats had no significant effect on *Bartonella* sharing after accounting for its own correlation with phylogenetic distance, geographic range overlap, and roost sharing. Finally, our analysis of historical host-switching rates showed that *Bartonella* lineages are biased to switching between phylogenetically related hosts.

Our regression results explaining variation in *Bartonella* sharing among bats are in agreement with previous work on bat viruses and other systems. Longdon et al. (2011) demonstrated using *Drosophila* sigma viruses that the host phylogeny explains most of the variation in viral replication among host species. Primates have more similar parasite communities if they are phylogenetically closely related and inhabit the same region (Davies and Pedersen, 2008). Streicker et al. (2010) found that the frequency of cross-species transmission (CST) of rabies virus between bat species (similar to our measure of *Bartonella* sharing) increases with decreasing phylogenetic distance and increasing geographic overlap. A later study analyzing these same data confirmed that host phylogenetic distance is a key determinant of rabies CST while other ecological covariates including roost structures, wing aspect ratio, wing loading, and body size were poor predictors (Faria et al., 2013). Luis et al. (2015) found that bat phylogeny and sympatry explained viral sharing in bats, with sympatry being the more important predictor. In addition, viral sharing communities of bats segregated by geographic regions. A recent global analysis of virus sharing in bats showed that after accounting for publication bias, bat species are more likely to share viruses if they have more geographic overlap and they roost in caves (Willoughby et al., 2017). Among cave-roosting bats, species shared viruses more frequently if they overlapped geographically and were documented as sharing roosts. These patterns indicate that host phylogeny and geographic overlap are general predictors of parasite communities in bats. These apparent biases in the arrangement of *Bartonella* and ectoparasite species among bat species likely contributed to our ability to identify communities of highly interacting bat, ectoparasite, and *Bartonella* species that tend to cluster by bat family and roosting patterns.

These results demonstrate how key ecological factors constrain the host range of *Bartonella* species in bats. Yet how do we explain the observed congruence between bat and *Bartonella* phylogenies? As noted above, we believe that this pattern is best explained by a phylogenetic bias in microbial host-switching, where host shifts occur more frequently between closely related species. This bias, if persistent over the evolution of *Bartonella* lineages, could produce a *Bartonella* tree that is largely congruent with the host tree without the need for strict cospeciation (Charleston and Robertson, 2002; de Vienne et al., 2013). This is in line with ecological fitting, which allows host colonization for ecological specialists prior to the evolution of novel capabilities for host exploitation (Araujo et al., 2015). We observed a negative relationship between historical *Bartonella* host-switching rates and host phylogenetic distance, but no significant relationship with ectoparasite sharing, geographic overlap, or roost sharing. These findings are similar to previous research on bat rabies showing that the host phylogeny is the strongest predictor of rabies host shifts (Streicker et al., 2010; Faria et al., 2013). Thus, biological constraints on parasite shifts among hosts are expected to be the dominant force that shapes host specificity over evolutionary time.

Influence of Ectoparasites on *Bartonella* Host Specificity

We noted above that ectoparasite sharing failed to explain additional variation in *Bartonella* sharing between bat species after accounting for the effects of host phylogenetic distance,

geographic overlap, and roost sharing. In addition, ectoparasite sharing, geographic overlap, and roost sharing were not included as significant predictors of historical *Bartonella* host-switching rates. This suggests that the forces of host phylogenetic distance, geographic overlap, and ecological interactions may act on the assembly of ectoparasite and *Bartonella* communities independently and that host-associated, vector-borne microorganisms come to be vectored by the available ectoparasite communities associated with each host. This is supported by the fact that *Bartonella* lineages in European bats appear to be associated with a polyphyletic assemblage of arthropods, including ticks, mites, hemipteran bugs, fleas, and flies. Since ectoparasites exhibit differences in life history traits and among-host dispersal mechanisms (Giorgi et al., 2004; Dick and Patterson, 2006; Reckardt and Kerth, 2009), generalist ectoparasites may be more influential in spreading microorganisms among species inhabiting the same environment while specialist ectoparasites are important for the maintenance of microorganisms in separate host species. This broad vector usage could explain why *Bartonella* infections are so prevalent in bats.

The variation in ectoparasite life history traits and host specificity would also be expected to influence microbial host specificity, though in contrasting ways. While ectoparasites may develop their own specificity for particular host species, as observed in bat wing mites and bat flies (Bruyndonckx et al., 2009; Sándor et al., 2018), these associations between hosts and vectors will predominantly either compound or counteract the isolation already occurring as microbes develop associations with host species. In the first case, the effects of ectoparasite host specificity on microbial evolution may not be statistically separable from the overriding effect of microbial host specificity, as we noted above. On the other hand, generalist vectors could be seen as simply adding noise to the associations of specialist microorganisms through accidental associations with atypical hosts. As long as noise does not totally obscure the predominant host–microbe associations, then it will be possible to measure the host-specific signal, either statistically or through genetic data. For example, Withenshaw et al. (2016) were able to show that distinct genetic variants of *Bartonella* species separately infect two sympatric rodent species despite the presence of generalist flea vectors. Since the two host species have differences in microhabitat usage and activity patterns, they would rarely have opportunity to exchange fleas. This can lead to covert microbial host specificity even when vectors are host generalists. These patterns suggest that what separates vector-borne microorganisms from directly or environmentally transmitted microorganisms in terms of their host specificity is the added layer of vector host specificity, which will either inflate host specificity already present in the microbe or dilute host-specific patterns through noisy associations. In the case of *Bartonella* communities in bats, host specificity is clear despite the presence of generalist or polyxenous vectors (e.g., *A. vespertilionis*, *C. pipistrelli*, and *S. myoti*).

Despite the patterns noted above, we should not rule out the possibility that ectoparasites may contribute to the evolution of vector-borne microorganisms like *Bartonella*. The genus *Bartonella* appears to have evolved from insect gut symbionts that transitioned to a parasitic lifestyle after adapting to blood-feeding arthropods (Segers et al., 2017). Coevolutionary processes in early *Bartonella* lineages associated with blood-feeding arthropods may have influenced later patterns in *Bartonella* associations with mammalian groups. Gene exchange between *Bartonella* and other arthropod symbionts could also influence the formation of distinct phylogenetic lineages deep in the evolutionary tree (Zhu et al., 2014). The phylogeny of ectoparasite groups may help to understand these processes more fully, but considering the polyphyly of arthropod groups carrying *Bartonella* observed in our study, it may be more practical to study the influence of ectoparasite host specificity within particular arthropod groups (e.g., bat flies) on *Bartonella* host specificity and macroevolution. Future studies could use sequences from multiple genetic loci in hosts, vectors, and *Bartonella* to generate time-calibrated phylogenies and compare the influence of evolutionary processes on structural and temporal patterns within trees across trophic scales.

It is also possible that host and ectoparasite phylogenetic structure in geographically separate populations may influence microevolutionary patterns in associated microbes. Historical processes, such as the postglacial recolonization of regions of Europe by bats (Flanders et al., 2009; Dool et al., 2013), or patterns of host and ectoparasite dispersal across distant locations (Bruyndonckx et al., 2009; Witsenburg et al., 2015; van Schaik et al., 2018) may lead to the formation of distinct microbial lineages. These types of analyses demand additional genetic data to detect fine distinctions between related lineages and are thus beyond the scope of this current work but would be fruitful avenues for future research on vector-borne microorganisms like *Bartonella*.

Based on our results and previous work, we suggest that while geographic overlap, ecological interactions (e.g., roost sharing), and ectoparasite sharing provide the necessary conditions for *Bartonella* transmission between hosts, the success of transmission and perhaps an eventual host shift will ultimately depend on biological compatibility between the host and the microbe, which can be predicted by the phylogenetic distance between hosts (Pedersen and Davies, 2009). Strong patterns of host specificity in microbial communities can still be observed even when generalist ectoparasites are present, and while specialist vectors may be present in the community, their effects on microbial host specificity would be expected to be more pronounced for a generalist microorganism and not a specialist, wherein host specificity of vector and microorganism would not be statistically independent.

Bat-Associated *Bartonella* Species as Zoonoses

Beyond the scientific insights produced by this study, there may be additional practical value in our results. Several *Bartonella* species are known to be human pathogens, and new cases of zoonotic bartonellosis are consistently being described (Roux et al., 2000; Kosoy et al., 2003, 2010b; Iralu et al., 2006; Chomel and Kasten, 2010; Bai et al., 2012; Kandelaki et al., 2016;

Vayssier-Taussat et al., 2016). While we recognize that not all of the 20 putative *Bartonella* species described in this study may have the potential to infect humans, OTU19 and OTU26 have been previously found to infect humans (Veikkolainen et al., 2014; Urushadze et al., 2017). These particular OTUs are also strongly linked with two ectoparasite species, *C. pipistrelli* and *A. vespertilionis*, which are known to sporadically bite humans (Estrada-Peña and Jongejan, 1999; Whyte et al., 2001).

A recent report also detected antibodies to a *Bartonella* strain specific to Egyptian fruit bats (*Rousettus aegyptiacus*) in eight people from a community in Nigeria (Bai et al., 2018). Members of the community enter caves and capture bats for consumption or sale as part of an annual festival. Thus, in addition to exposure to bat ectoparasites that may bite humans, these practices may provide alternative routes for human exposure to bat-associated *Bartonella* species. These include direct exposure through handling of bats (including possible bites or scratches), contamination of open wounds with blood or bat excreta during the capture process, or contamination of wounds from the excreta of bat ectoparasites. *Bartonella* DNA has been previously detected in bat guano, urine, and saliva (Veikkolainen et al., 2014; Banskar et al., 2016; Dietrich et al., 2017; Becker et al., 2018); however, more studies are needed to confirm that viable bacteria are present in the excreta of bats or their ectoparasites. Nonetheless, there is accumulating evidence that bat-associated *Bartonella* species may present an infection risk to human populations.

While assessing the zoonotic risks from the environment aids the prevention and control of human diseases, loss and disturbance of roost sites, among other reasons out of sensationalized fear of zoonotic diseases, is one of the threats for endangered bat species (López-Baucells et al., 2018). Disturbance of bat roosts in such sites could even be counterproductive if bat ectoparasites seek alternative food sources, potentially leading to infections of atypical host species such as humans or pets. Therefore, humans should avoid unnecessary contact with bats or their ectoparasites. Bat colonies in attics or walls of buildings do not pose a health risk as long as they are excluded from parts of the house occupied by humans (Tuttle, 2005). For individuals who may encounter bats within caves or other habitats, including tourists, scientists, and bat or guano harvesters, it is advisable to minimize their disturbance of the animals and to limit contact with bats, bat ectoparasites, and their excreta through protective equipment.

Study Limitations

The analyses we have performed greatly expand our understanding of the complex ecology and evolution of *Bartonella* in bats. Our approach, which integrates across levels of parasitism and explores ecological and evolutionary patterns, could be applied to *Bartonella* in bats outside of Europe, to other *Bartonella* species associated with different mammalian orders, and possibly to other complex vector-borne diseases. However, we recognize that this study is observational and correlative and has limitations to the data that must be acknowledged.

Regarding our phylogenetic analysis, we needed to confirm that the *gltA* gene serves as an accurate marker for assessing the evolutionary history of the *Bartonella* lineages being studied. As detailed in the **Supplementary Material**, we are satisfied that the lineages identified in the *gltA* tree have a similar topology to a tree that uses additional markers, that recombination within and among loci has not significantly distorted evolutionary patterns, and that the topology of our tree is similar to trees assembled using another approach, neighbor-joining. Furthermore, by using 100 posterior sampled *Bartonella* trees in our tip-association and global fit tests, we demonstrated that our results are robust with respect to phylogenetic uncertainty in the parasite tree.

We recognize that our estimates of *Bartonella* diversity (number of OTUs and Faith's index) in European bats are limited by the extent of current sampling, with a significant positive correlation between these measures and the number of *gltA* sequences obtained per species. Thus, we probably do not yet have an accurate survey of *Bartonella* diversity in some of our bat species with few *gltA* sequences. Additional sequencing of *Bartonella* strains from some poorly sampled host species (e.g., *E. nilssonii*, *My. capaccinii*, *My. mystacinus, Pi. pygmaeus*) could clarify their membership within communities. Additional sampling of these species would also allow researchers to perform rarefaction analyses to determine if bat species actually differ in the number of *Bartonella* species that infect them. After accounting for this sampling effect, correlational analyses could attempt to explain this variation in *Bartonella* diversity using bat traits, as has been done successfully for viral diversity (Luis et al., 2013; Gay et al., 2014; Maganga et al., 2014; Webber et al., 2017; Willoughby et al., 2017).

Moreover, the *Bartonella* sequence data we compiled for this study were derived from numerous studies that relied on convenience sampling from numerous sources (mist netting, bat boxes, roosts, and dead bats) over varying time periods. No information on host species richness, host density, or community dynamics of host species within roosts was collected. Better structured and controlled sampling strategies could capture such data and would broaden our understanding of parasite diversity and persistence within bat populations.

We also acknowledge that phylogenetic distance only approximates the process of microbial host adaptation. *Bartonella* traits like the presence of particular secretion system genes and effector proteins (Harms et al., 2017) or bat traits linked to immune function, such as major histocompatibility complex or toll-like receptor alleles (Baker et al., 2013), may be better at predicting whether bats will share *Bartonella* species. Some bat species may also be more tolerant or resistant to certain *Bartonella* species, as has been shown for the fungus that causes white nose syndrome (Frank et al., 2014). This could help explain additional variation in *Bartonella* communities among phylogenetically related and sympatric bat species. Such traits have not been explored in the *Bartonella* or bat species from our study, but would be useful for understanding how bat species differ in their *Bartonella* prevalence and diversity.

Finally, we used geographic range overlap as a proxy for spatiotemporal proximity in our regression analysis. This assumes that if species share a roosting preference, the

species will interact in a way that could lead to parasite exchange. Some species may interact more frequently within roosts than others, such as by using the same microhabitat or by overlapping within roosts during the same season; thus, there would be a greater potential for ectoparasite and microbial transmission. Fluctuations in colony size and bat community composition between summer maternity colonies and winter roosts would also be expected to influence parasite exchange (Dietrich et al., 2018). Our sampling approach did not capture these important variables and should be considered in future studies.

CONCLUSION

Our analysis of *Bartonella* infections in European bats and their ectoparasites has increased our understanding of how *Bartonella* species are segregated among sympatric hosts and how these ecological associations influence the evolution of *Bartonella* lineages. We find that while the host phylogeny primarily explains how bats share *Bartonella* and how *Bartonella* lineages evolve, the complete evolutionary history of *Bartonella* in bats may involve additional processes, including multiple introductions from other mammalian orders and biogeographic separation of hosts. Additional sampling and phylogenetic analysis of *Bartonella* from bats and other mammals and their ectoparasites could help to shed more light on these processes. As we have demonstrated, *Bartonella* is a productive system for studying complex host–parasite associations. The methods that we have used in this study and our findings regarding the important processes that constrain parasite evolution may be applicable to other vector-borne microorganisms, such as those carried by ticks. For example, studying *Anaplasma* spp. infections in different carnivore or ungulate species taking into account their phylogeny, geographic overlap, and tick associations could provide information about spillover potential to humans and domestic animals (Dugat et al., 2015; André, 2018). Investigating multitrophic interactions would also help to understand the processes behind the segregation of *Borrelia* genospecies among vertebrate hosts (Estrada-Peña et al., 2016). Comparison among these various systems may reveal general patterns in the ecology and evolution of host–vector–parasite associations.

DATA AVAILABILITY

Representative sequences from all ectoparasites and *Bartonella* OTUs have been submitted to GenBank. Accession numbers for *Bartonella gltA* sequences are MK140184–MK140336 and accession numbers for ectoparasites are MK140016–MK140183. Phylogenetic trees, R code, and additional data sheets are available on GitHub (https://github.com/clifmckee/eurobats).

ETHICS STATEMENT

No live bat was harmed for this study. Authorization for bat capture was provided by the Netherlands Enterprise Agency over the entire sampling period (ontheffing Flora en Faunawet permits FF75a/2008/033, FF75a2012/37a, and FF75a/2016/048 assigned to the Dutch Mammal Society) with permissions of all site owners; the National Inspectorate for Environment, Nature and Water (Hungary); the Underground Heritage Commission (Romania); the Flemish Forest and Nature Agency (ANB/BL-FF/V15-00095); and the Wallonian Department of Nature and Forests (SPW-DNF-DGo3 permit réf déro 2013/RS/n°15 and dero 2014/RS/n°12). Bat banding license numbers are 59/2003, 305/2015, 46/2016, and TMF-493/3/2005. Bats were handled according to the current laws of animal welfare regulation (L206/2004), and the Research Bioethics Commission of USAMV CN approved all working protocols for data collection in Romania.

AUTHOR CONTRIBUTIONS

HS and AK conceived the study. AK, AS, and TG performed the sample collection in the field. AS and GF organized parts of the sample collection and contributed to the study design. DD, A-JH, MF, GF, and TG contributed important samples to the study. MF identified bat flies. AK performed laboratory and molecular analyses. CM performed the phylogenetic and statistical analyses. CM and AK wrote the first draft of the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00069/full#supplementary-material

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Comparative Ecology of *Bartonella* and *Brucella* Infections in Wild Carnivores

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Phylogenetic sister clades *Bartonella* and *Brucella* within the order Rhizobiales present some common biological characteristics as well as evident differences in adaptations to their mammalian reservoirs. We reviewed published data on *Bartonella* and *Brucella* infections in wild carnivores to compare the ecology of these bacteria in relatively similar host environments. Arthropod vectors are the main mechanism for *Bartonella* species transmission between mammalian hosts. The role of arthropods in transmission of *Brucella* remains disputed, however experimental studies and reported detection of *Brucella* in arthropods indicate potential vector transmission. More commonly, transmission of *Brucella* occurs via contact exposure to infected animals or the environment contaminated with their discharges. Of 26 species of carnivores tested for both *Bartonella* and *Brucella*, 58% harbored either. Among them were bobcats, African lions, golden jackals, coyotes, wolves, foxes, striped skunks, sea otters, raccoons, and harbor seals. The most common species of *Bartonella* in wild carnivores was *B. henselae*, found in 23 species, followed by *B. rochalimae* in 12, *B. clarridgeiae* in ten, and *B. vinsonii* subsp. *berkhoffii* in seven. Among *Brucella* species, *Br. abortus* was reported in over 30 terrestrial carnivore species, followed by *Br. canis* in seven. Marine carnivores, such as seals and sea lions, can host *Br. pinnipedialis*. In contrast, there is no evidence of a *Bartonella* strain specific for marine mammals. *Bartonella* species are present practically in every sampled species of wild felids, but of 14 *Brucella* studies of felids, only five reported *Brucella* and those were limited to detection of antibodies. We found no reports of *Bartonella* in bears while *Brucella* was detected in these animals. There is evident host-specificity of *Bartonella* species in wild carnivores (e.g., *B. henselae* in felids and *B. vinsonii* subsp. *berkhoffii* in canids). A co-adaptation of *Brucella* with terrestrial wild carnivore hosts is not as straightforward as in domestic animals. Wild carnivores often carry the same pathogens as their domesticated relatives (cats and dogs), but the risk of exposure varies widely because of differences in biology, distribution, and historical interactions.

Keywords: *Bartonella*, *Brucella*, carnivores, disease ecology, wildlife disease

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INTRODUCTION

Sixty percent of emerging infectious diseases are zoonoses and majority of these (71.8%) originate from wildlife (1). Among pathogens, *Bartonella* species might represent an underappreciated danger for human and animal health (2) and human brucellosis remains one of the most common zoonotic diseases worldwide, with more than 500,000 new cases every year (3).

Bartonella and *Brucella* are phylogenetic sister clades in the order Rhizobiales (4, 5). The genus *Brucella* is composed of 12 recognized species defined to their preferential hosts (6, 7). The more diverse genus *Bartonella* includes over 33 validated species exhibiting extremely high genetic diversity (8). Genome analyses of representative species of these bacterial genera have confirmed their shared ancestry. Alsmark et al. (9) identified 760 *Bartonella henselae* genes, for which homologs are present in one of chromosomes of *Brucella suis.* In addition to their genetic proximity, the *Bartonella* and *Brucella* genera present analogies in their life history and ecology that are even more important for our analysis. Whereas, most closely related species of the order Rhizobiales are symbiotic on plant roots, both *Bartonella* and *Brucella* are adapted to diverse mammalian hosts. Each *Bartonella* and *Brucella* species has one or a few closely related mammal reservoir hosts (5).

Investigations of wild animals, including predators, for *Brucella* infections started much earlier and were more intensive compared to studies of *Bartonella* infections. Research of *Brucella* infections in animals has been dominated by studies of domestic animals and, to a lesser degree, of wild ruminants. Although *Brucella canis* was identified in domestic dogs more than 50 years ago and is well known to veterinary community as a causative agent of canine abortion (10), investigations of *Brucella* in dogs are much fewer than those of *Brucella* in cattle, sheep, goats, and pigs. This is mainly due to lack of good tests for rough *Brucella* species, not because of lack of interest. Publications on the distribution of *Brucella* species among wild canids, as well as among other wild carnivores, are even more limited in the western literature. At the same time, a good number of reports on this topic are scattered across Russian literature. Most of these were published during Soviet times, sometimes in classified proceedings, and are not easily available (11–13). Identification of novel species and genotypes of *Brucella* in rodents, bats, marine mammals, and amphibians stimulated epidemiological research of diverse animal species, including wildlife (7).

Extensive investigations of animals for *Bartonella* species started in the early 1990s after the discovery that one or more *Bartonella* species could cause cat scratch disease in people. For this reason, most studies targeted domestic cats and dogs with limited investigations of stray dogs and feral cats (14, 15). Studies on detection, identification, and characterization of *Bartonella* species in wild animals usually targeted small mammals: rodents (8) and bats (16). Chomel et al. (17) pioneered *Bartonella* research in wild carnivores and ruminants. Since then, numerous wildlife studies have been conducted in various parts of the world. However, a comprehensive analysis of the available data on prevalence and diversity of *Bartonella* and *Brucella* infections in wild carnivores has yet to be published. Such an analysis would allow comparisons to be made between the ecologies of these two bacterial groups living in similar host environments and identify possible directions for future research.

In this review we undertook such an analysis through an extensive literature review. We examined the similarities and differences in the *Bartonella* and *Brucella* ecology and, more importantly, analyzed biological features that may reveal ways of these phylogenetically close bacterial genera exhibit evolutionary adaptations to the same or related mammalian hosts, presumably during the long periods over which they have co-occurred. Considering the differences in the genera's life history, we paid special attention to possible arthropod-mediated transmission of these bacteria between mammalian hosts.

For this review, we followed the more accepted taxonomic division of the order Carnivora into suborders Feliformia ("cat-like") and Caniformia ("dog-like"), with pinnipeds included as a separate superfamily level clade (Pinnipedia). We chose these divisions not for preference for a specific taxonomic scheme, but as a convenient basis for analysis of available data on *Bartonella* and *Brucella* infections in 12 families: suborder Feliformia (Felidae, Herpestidae, Hyaenidae, and Viverridae), suborder Caniformia (Canidae, Mephitidae, Mustelidae, Procyonidae, and Ursidae), and clade Pinnipedia (Odobenidae, Otariidae, and Phocidae).

We conducted a thorough literature search by using PubMed, Scopus, OVID Medline, BioOne, Crossref, WorldCat, Web of Science, Google Scholar, and other databases. In the search we used keywords: "*Bartonella* ecology," "*Brucella* ecology," "*Bartonella* AND wild animals," "*Brucella* AND wild animals," "*Bartonella* AND carnivores," "*Brucella* AND carnivores," "*Bartonella* AND predators," "*Brucella* AND predators," "*Bartonella* AND marine mammals," "*Brucella* AND marine mammals," "Bacteria AND wild carnivores," "*Bartonella* AND fleas AND mammals," "*Brucella* AND arthropods," and their variations. We realized that all these search engines had missed numerous reports on detection of *Brucella* in wild animals in the Russian language literature and we conducted our own search of such sources in the Russian Internet and search engines as well as by working through the references in related articles and reviews in the Russian language.

We used the word "wild" in the meaning of "free-ranging" and apart from a few publications of particular interest, we excluded reports of *Bartonella* and *Brucella* in captive and zoo animals as the composition of bacterial communities in such animals could have been modified by separation from the natural environment or via acquisition of bacterial infections from the surrounding environment (e.g., from urban rats). The literature on *Bartonella* and *Brucella* infections in domestic carnivores (cats and dogs) is abundant, so we limited inclusion for comparative purposes only.

We collected data from serological, bacteriological, and molecular investigations of *Bartonella* and *Brucella* infections in all families of wild terrestrial and marine carnivores worldwide. Providing data from various techniques, we need to acknowledge that discrimination power of characterization of pure cultures and sequence analyses for identification of *Bartonella* and

Brucella species is greater than that of serological procedures. However, serological methods remain an important tool in detection and identification of these infections in animals and should be taken in consideration with full awareness of their limitations.

Then we collated the obtained information in *Bartonella*, *Brucella* and combined tables by carnivore species divided into their respective families listed in alphabetical order of their Latin names. We listed information on location where the samples were collected, investigation method, prevalence and bacterial species, and reference to the study. Both positive and negative results of investigations were included. The combined table shows only references listed by carnivore species in their respective families.

FEATURES OF *BARTONELLA* AND *BRUCELLA* BACTERIA RELATED TO THEIR ECOLOGY IN WILD ANIMALS

Biological Characteristics

Bartonella and *Brucella* bacteria share some biological characteristics, yet there are evident differences in their adaptations to their animal reservoirs. Infections caused by bacteria of both taxonomic groups can lead to a long-lasting bacteremia with ability to invade specific mammalian cells and survive inside them. Via analogous mechanisms, the specialized secretion system (T4SS) works as a molecular syringe to inject effector molecules into their target cells (18, 19). *Bartonella* and *Brucella* modulate their gene expression to adapt to the different environments during the infectious process (20, 21). The VirB systems of *Bartonella* and *Brucella* are associated with distinct groups of effector proteins that collectively mediate interactions with host cells (19).

Bartonella bacteria infect endothelial cells and seed into the bloodstream, colonizing erythrocytes which provide a persistence niche for the bacteria. The ability of these bacteria to exploit their reservoir hosts with diminished morbidity and to cause a high level of bacteremia justifies the definition of "elegant hemotrophic parasites" given by Birtles (22) to bartonellae. In incidental hosts, *Bartonella* infections can cause various clinical manifestations commonly without high-level bacteremias (21). In contrast to *Bartonella*, *Brucella* bacteria invade and multiply within mammalian host's macrophages and placental trophoblasts (18, 19, 23). Although bacteremia is common during brucellosis, data on duration and mechanisms of *Brucella* persistence in animal blood are limited.

Transmission of *Bartonella* and *Brucella* Bacteria

The persistence of *Bartonella* bacteria in red blood cells optimizes transmission of these bacteria by blood-sucking arthropods. High prevalence and long-term bacteremia in reservoir mammals and adaptation to specific vectors seem to be the common strategy of bartonellae for transmission and host diversity (24). Many described *Bartonella* species are vector-borne bacteria transmitted by fleas, sand flies, lice, and biting flies depending on the bacteria species involved and their vertebrate reservoirs (24, 25). Experimental studies demonstrated louse and flea transmission of *B. henselae* and *B. quintana* (26–28). Some investigators provided evidence of potential role of ticks and mites in transmission of *Bartonella* species, but debates continue on their role of as vectors (25, 29). A 2008 study by Cotté et al. (30) showed that *Ixodes* spp. ticks are capable of transmitting *B. henselae* via salivary contents, but Telford and Wormser (31) found no convincing evidence that ticks were vectors of *Bartonella* species. Molecular detection of *Bartonella* spp. in terrestrial leeches (*Haemadipsa rjukjuana*) by Kang et al. (32) opens up a discussion of the pathogen transmission by land leeches.

Transcutaneous transmission of *Bartonella* via animal bites and scratches during hunting, as well as through butchering or handling wild meat is another possibility (33). Cat scratch disease, caused by *B. henselae* is the best-documented example of direct animal-to-human transmission of a *Bartonella* species by scratch or bite inoculation (14). Finkelstein et al. (27) showed that *B. henselae* can remain viable in flea feces for over 72 hours. Therefore, transmission potentially can occur via inoculation of *B. henselae* from infected flea feces into the skin via open wounds. Suspected *Bartonella alsatica* transmission from wild rabbits to humans, presumably occurring during hunting and butchering, was reported in patients with endocarditis or lymphadenitis in France (34, 35). Suspected dog bite transmission of *B. vinsonii* to a human was reported based upon serological evidence (36). There is little information on possible vertical transmission of bartonellae in animals. However, *Bartonella* species were isolated from the embryos and neonates of naturally infected cotton rats (*Sigmodon hispidus*) and white-footed mice (*Peromyscus leucopus*) (37). Experimental inoculation of *B. henselae* to adult female cats was accompanied by decreased conception or failure to maintain pregnancy (38). Considering the extensive animal reservoirs and the large number of insects that have been implicated in the transmission of *Bartonella* species, animal exposure to these organisms may be more substantial than is currently believed.

Transmission of *Brucella* occurs mainly via close contact with placenta, aborted fetuses, fetal fluids, reproductive tract discharges, and secretions (7). Infected dogs intermittently shed low concentrations of bacteria in seminal fluids and nonestrus vaginal secretions. Postabortion vaginal fluids contain a high level of bacteria and are a source of infection for other dogs (39). In addition, dogs can shed the bacteria in the saliva, nasal secretions, and urine (40). Studies suggest that the concentration of *Br. canis* in urine is higher in male than female dogs; this difference is attributed to urine contamination with seminal fluid (41). However, the role of urine as a source of infection is not fully understood (39).

There is a widely accepted perception that absence of transmission of *Brucella* species via arthropod vectors is the most essential difference in ecology of these bacteria compared to the *Bartonella* species. We found a number of rarely cited publications on either detection of *Brucella* species in arthropods or experimental studies designed to verify a possibility of vector transmission of these bacteria. Although detection of

Brucella in arthropods collected from different sources does not often directly relate to carnivores, such information can help interpret potential mechanisms of bacterial transmission. The invasion of *Brucella* into erythrocytes and its persistence in blood suggest a possibility for transmission by bloodsucking arthropods in nature (42). Although *Brucella* may be found in erythrocytes, these bacteria exhibit strong tissue tropism and replicate within vacuoles in macrophages, dendritic cells, and placental trophoblasts. Evidence that *Brucella* species can be spread among animals by arthropods is very limited. Some Russian authors argued that parasitic arthropods, especially ticks, could preserve *Brucella* in nature and transmit them within a population from one animal to another (43, 44). Rementsova (43) listed 20 observations of *Brucella* detection in ticks. Experiments in Russia reported that both ixodid and argasid ticks were infected with *Brucella* at different phases of their development and could transmit the pathogen to uninfected animals during bloodsucking (43). *Brucella* in ticks retained their virulence even after 2 years (43). More recently, Neglia et al. (45) detected *Br. abortus* DNA and RNA in different stages of development of the sucking louse (*Haematopinus tuberculatus*).

Alimentary transmission is important for *Brucella* as proved by experimental studies in wild carnivores. Scanlan et al. (46) infected gray foxes with *Br. abortus* in dog food. Seven of eight foxes became seropositive. Neiland and Miller (47) infected six beagle dogs, two wolves (*Canis lupus*), one black bear (*Ursus americanus*), and two grizzly bears (*Ursus arctos horribilis*) with a strain of *Br. suis* biovar 4 isolated from a sled dog from Alaska. Their experiments demonstrated that canids and ursids are susceptible to the infection via intraperitoneal inoculation and through oral mucous membranes. During acute stages of the infection, *Brucella* congregated in these species in high numbers in lymph nodes and distributed throughout the body. Importantly, *Brucella* invaded salivary glands and probably also mammary glands and kidney, thus providing conditions for shedding the bacteria in saliva, milk, and urine. The authors reported reproductive failure during infection in wolves, but were not confident that the failure was a consequence of the infection (47). Morton (48) experimentally infected foxes with *Br. suis* biovar 4 and observed that the incidence of positive titers, positive cultures, and shedding of bacteria was related to the number of *Brucella* organisms experimentally fed to the animals. Lowest doses did not produce infection. Highest doses produced positive titers and cultures.

Tests on rats showed transmission of *Br. abortus* biovar 1 from infected male to uninfected female rats resulted from sexual intercourse (49). Vertical transmission of *Br. abortus* caused sterility in pregnant mice (50); Wang et al. (51) documented vertical transmission of *Br. melitensis* on a pregnant mouse model. Guzman-Verri et al. (52) cited the more likely modes of transmission of *Br. ceti* to be through sexual intercourse, maternal feeding, aborted fetuses, placental tissues, vertical transmission from mother to the fetus or through fish or helminth reservoirs.

Brucellae have high viability and can survive in the environment for 3–21 days in spring-summer and for 151–233 days in winter-fall seasons. Brucellae maintain viability in carcasses (muscles, internal organs, and lymph nodes) at −7.2° to 38.4°C for 1–12 months (53). Long-term survival of *Br. microti* in soil was described and, thus, soil might act as a reservoir of infection (54).

PREVALENCE OF *BARTONELLA* INFECTIONS IN WILD CARNIVORES

General Prevalence Pattern

Overall, prevalence of *Bartonella* infections in carnivores was higher compared to *Brucella* infections. In the studies of over two Feliformia animals, the highest prevalence was registered by culture in bobcats [37%, 7/19, (55)], by IFA in bobcats again [74%, (56)], and by PCR of blood in Iberian lynx [33.3%, 10/30, (57)]. In studies of over two Caniformia animals, the highest *Bartonella* prevalence was registered by culture in gray foxes [49%, 26/53, (58)], by IFA in coyotes [89%, 48/53, (58)], and by PCR of blood in raccoons [43%, 16/37, (59)]. A very high overall prevalence of antibodies to *B. henselae* (95%) was detected among Brazilian free-ranging felids (60) (**Table 1**). As expected, the number of seropositive animals was usually higher than the numbers of culture or PCR positive individuals from the same study. Thus, of the 54 lions from South Africa, 5.2% were positive by culture, 3.7% were PCR positive for *Bartonella* DNA, and 17% had *Bartonella* antibodies (62). A study on golden jackals in Iraq found 14.5% of animals positive by PCR and 40.4% (23/57) by IFA (86).

Age and Gender Pattern

Prior studies usually show no statistical difference in prevalence by age or gender in felines (61, 69, 79). However, Chomel et al. (17) found antibody prevalence for *B. henselae* to increase with age in pumas in California. In contrast, Rotstein et al. (79) found antibody prevalence higher in Florida panthers under 2 years of age (40%) compared to panthers over 2 years of age (13%).

Geographic Pattern

Prevalence of *B. henselae* antibodies in mountain lions and bobcats varied significantly between different states of the U.S. (17). Mountain lions from Arizona, California, and Texas were more likely to be seropositive for *B. henselae* (26.7–40.0%) than pumas from the Northwest and Mountain states (0–11.8%) (17). In California, the highest prevalence in bobcats was from the coastal range (37.5%), while the highest prevalence in pumas was from Southern California and Sierra Nevada (17). The reported pattern was similar to the geographic distribution of *Bartonella* infection in domestic cats. It has been demonstrated that in cat populations (stray or pets), prevalence of infection was demonstrated to vary considerably with an increasing gradient from cold climates (0% in Norway) to warm and humid climates (68% in the Philippines) (14). In the U.S., prevalence of *B. henselae* antibodies in pet cats varied significantly with the highest average prevalence in the southeastern United States, Hawaii, coastal California, the Pacific Northwest, and low prevalence in Alaska, the Rocky Mountain-Great Plains region, and the Midwest (113). Comparing wild felids at four sites in California and Colorado, Bevins et al. (68) noted that seroprevalence varied considerably, but in almost all cases, it

TABLE 1 | *Bartonella* studies in wild carnivores by species.

Species	Location	Method	Prevalence/*Bartonella* spp.	References
SUBORDER FELIFORMIA				
Felidae family				
Cheetah (*Acinonyx jubatus*)	Namibia	Culture, PCR (*16S rRNA, gltA, ribC, groEL, ftsZ*, ITS)	*Culture: 5.9% (1/17) new Bartonella strain between Bh and Bk*	(61)
	Africa	Culture, PCR (*gltA*), IFA	*Culture: 5.9% (1/17) unid'd Bartonella* sp. *close to Bk; PCR (blood): 23.3% (17/73); IFA 31.1% (23/74) B. henselae*	(62)
	Zimbabwe	Culture, PCR	*Culture: 33.3% (1/3) B. henselae*	(63)
Wildcat (*Felis silvestris*)	Spain	PCR (*gltA*, ITS)	*PCR (tissue): 16.7%(1/6) B. henselae*	(64)
		PCR (ITS)	*PCR (fleas): 16.7% (1/6 pools) B. alsatica*	(65)
Ocelot	Brazil	PCR	*PCR (blood): 0/7*	(66)
(*Leopardus pardalis*)		IFA	*IFA: 100% (1/1) B. henselae*	(60)
Little spotted cat (*Leopardus tigrinus*)	Brazil	IFA	*IFA: 100% (2/2) B. henselae*	(60)
Iberian lynx (*Lynx pardinus*)	Spain	PCR (ITS)	*PCR (fleas): 0% (0/5 pools)*	(65)
		PCR (*gltA*)	*PCR (blood): 13.3% (6/45) & 33.3% (10/30) B. henselae*	(57)
Bobcat (*Lynx rufus*)	Mexico	Culture, PCR (*gltA*, ITS)	*Culture: 0/5; PCR (blood): 0/5; PCR (fleas): 5.6% (1/18)* *Bartonella* sp.	(67)
	CA, USA	Culture, PCR (*16S rRNA, gltA, ribC, rpoB, ftsZ, groEL*, ITS), IFA	*Culture: 37% (7/19); Bh II and Bk* subsp. *bothieri; IFA: 13/19 Bh II*	(55)
	CO, CA	ELISA	*ELISA: 31% Bartonella* sp.	(68)
	CA, USA	IFA	*IFA 74% (n=25) B. henselae*	(56)
	USA	IFA	*IFA: 22.4% (19/85) B. henselae*	(17)
	Mexico		*IFA: 33.3% (2/6) B. henselae*	
	CA, USA	IFA	*IFA: 53% (33/62) B. henselae*	(69)
African lion (*Panthera leo*)	South Africa	Culture, PCR (*16S rRNA, gltA, ribC, groEL, ftsZ*, ITS)	*Culture: 5.2% (3/58) (2 Bh & 1 Bk subsp. koehlerae)*	(61)
	Zambia	PCR (ITS)	*PCR: 0% (0/24)*	(70)
	Africa	Culture, PCR (*gltA*), IFA	*Culture: 5.2% (3/58) 2 Bh & 1 unid'd Bartonella sp. close to Bk; PCR (blood): 3.7% (2/54); IFA: 16.8% (19/113) B. henselae*	(62)
	Africa	Culture, ELISA	*Culture: 1/65 (1.5%); B. henselae II; ELISA: 29% (18/62)*	(71)
	Zimbabwe	Culture	*Culture: 0%*	(63)
Far Eastern leopard	Russia	Western Blot	*WB: 0% (0/4) B. henselae*	(72)
(*Panthera pardus orientalis*)		Western Blot	*WB: 40% (2/5) B. henselae*	(73)
Amur tiger	Russia	Western Blot	*WB: 0% (0/17) B. henselae*	(72)
(*Panthera tigris altaica*)		Western Blot	*WB: 0% (0/17) B. henselae*	(73)
Iriomote cat (*Prionailurus*	Japan	PCR (ITS)	*PCR (ticks): 0% (0/13 pools), PCR (blood): 0% (0/11)*	(74)
bengalensis iriomotensis)		PCR (ITS)	*PCR (blood): 6% (2/33) B. henselae*	(75)
Tsushima leopard cat	Japan	PCR (ITS)	*PCR (ticks): 37.5% (3/8 cats), Bh; PCR (blood): 0% (0/6).*	(74)
(*Prionailurus bengalensis*)		PCR (ITS)	*PCR (blood): 8% (1/13) Bc*	(75)
Mountain lion (*Puma concolor*)	FL, USA	PCR (ITS, *pap31, rpoB*)	*PCR: 100% (3/3) B. henselae*	(76)
	CA, USA	Culture, IFA, PCR (*16S rRNA, gltA, ribC, rpoB, ftsZ, groEL*, ITS)	*Culture: 29% (4/14) Bh II & Bk* subsp. *boulouisii; IFA: 8/14 Bh II*	(55)
	CO, USA	PCR (ITS, *gltA, ftsZ*)	*PCR (tissue): 0% (0/3)*	(77)
	CA, USA	IFA	*IFA: 37.1% (164/442) B. henselae I*	(78)
	CO, CA	ELISA	*ELISA: 16% Bartonella* sp.	(68)
	Brazil	IFA	*IFA: 88.9% (16/18) B. henselae*	(60)
	USA	IFA	*IFA: 20.2% (73/361) B. henselae total; 37.5% in coastal CA*	(17)
	Canada		*IFA: 0% (0/23) B. henselae*	
	Mexico		*IFA: 8.3% (1/12) B. henselae*	
	Central America, Venezuela		*IFA: 33.3% (8/24) B. henselae*	

(Continued)

TABLE 1 | Continued

Species	Location	Method	Prevalence/*Bartonella* spp.	References
	S. America		*IFA: 22.4% (11/49) B. henselae*	
	Andean countries		*IFA: 0% (0/10) B. henselae*	
	FL, USA	IFA	*IFA: 20% (7/35) B. henselae*	(79)
	CA, USA	IFA	*IFA: 35% (26/74) B. henselae*	(69)
Herpestidae family				
Egyptian mongoose (*Herpestes ichneumon*)	Algeria	PCR (ITS)	*PCR (tissue): 0% (0/1)*	(80)
Small Asian mongoose (*Herpestes javanicus*)	Grenada	IFA, PCR (*gltA, rpoB, 16S rRNA*)	*IFA: 32.3% (54/167); PCR (blood): 35.3% (18/51) B. henselae I*	(81)
	Japan	Culture, PCR (*16S rRNA, ftsZ, gltA, groEL, ribC,rpoB*)	*Culture: 15.9% (10/63) B. henselae*	(82)
Hyaenidae family				
Spotted hyena (*Crocuta crocuta*)	Zambia	PCR (ITS)	*PCR (blood): 0% (0/19)*	(70)
Viverridae family				
Common genet	Spain	PCR (ITS)	*PCR (blood): 5.9% (2/34) 2 Bc; PCR (ticks): 0% (0/15 pools)*	(83)
(Genetta genetta)		PCR (*gltA*, ITS)	*PCR (tissue): 0% (0/13)*	(64)
		PCR (ITS)	*PCR (fleas): 0% (0/10 pools)*	(65)
Masked palm civet (*Paguma larvata*)	Japan	Culture, PCR (*16S rRNA, ftsZ, gltA, groEL, ribC,rpoB*)	*Culture: 2.0% (1/50) B. henselae*	(82)
SUBORDER CANIFORMIA				
Canidae family				
Golden jackal (*Canis aureus*)	Serbia	PCR (ITS)	*PCR: 0% (0/216)*	(84)
	Israel	PCR (ITS, *ssrA, rpoB, gltA*)	*PCR: 13% (9/70) 5/9 Br, 3/9 related to Candidatus B. merieuxii, 1/9 between Bvb & B. merieuxii*	(85)
	Algeria	PCR (ITS)	*PCR (tissue): 0% (0/2)*	(80)
	Iraq	*PCR (ITS, rpoB, gltA), IFA*	*PCR: 12.3% (7/57) Candidatus B. merieuxii, 2% Bvb; IFA: 40.4% (23/57) any Bartonella spp., Bh 35% (20/57), Bc 37% (21/57), Bvb 33% (19/57), B. bovis 35% (20/57).*	(86)
Coyote (*Canis latrans*)	Mexico	Culture, PCR (*gltA*, ITS)	*Culture: 1/18; PCR (blood): 5.6% (1/18) Br; 5.6% (1/18) Bvb; PCR (fleas): 15.1% (8/53) Bvb*	(67)
	CO, USA	PCR (ITS, *gltA, ftsZ*)	*PCR (tissue): 28% (7/25); 5/7 Bvb, 2/7 Br*	(77)
	CA, USA	PCR (*gltA*)	*PCR (valves, spleen): 21% (15/70); Bvb, Bh, Br*	(87)
	CA, USA	Culture, PCR (ITS, *gltA, rpoB, ftsZ, groEL*)	*Culture: 9.5% (2/21) Br*	(88)
	CA, USA	Culture, IFA, PCR (ITS, *gltA*)	*Culture: 42% (22/53) novel B. clarridgeiae-like; 9.4% (5/53) Bvb; IFA: 89% (48/53)*	(58)
	CA, USA	ELISA	*ELISA: 28% (n = 239) Bvb*	(89)
	CA, USA	IFA, PCR (*gltA, 16S rRNA*)	*IFA 76% (83/109) Bvb; PCR: 28% (31/109) Bvb*	(90)
	CA, USA	ELISA	*ELISA: 35% (306/869) Bvb (7–51% in CA)*	(91)
Wolf (*Canis lupus*)	Spain	PCR (*gltA*, ITS)	*PCR (tissue): 33.3% (1/3) Br*	(64)
Crab-eating fox	Brazil	PCR	*PCR (blood): 0/78*	(66)
(*Cerdocyon thous*)		PCR (*gltA, ribC*)	*PCR (fleas): 100% (9/9 fleas from the only fox), Br*	(92)
Darwin's fox (*Lycalopex fulvipes*)	Chile	PCR (ITS)	*PCR (blood): 0% (0/24)*	(93)
Wild dog (*Lycaon pictus*)	Zambia	PCR (ITS)	*PCR (blood): 0% (0/11)*	(70)
Raccoon dog	Korea	PCR (ITS, *groEL, rpoB*)	*PCR: 1.3% (2/152 spleen samples) B. henselae*	(94)
(*Nyctereutes procyonoides*)	Japan	PCR (ITS, *16SrRNA, ftsZ, gltA, groEL, ribC, rpoB*)	*PCR (blood): 0% (0/171)*	(95)
Gray fox	Mexico	Culture, PCR (*gltA*, ITS)	*Culture: 0/7; PCR (fleas): 9.7% (3/31) Br, 3.2% (1/31) Bvb*	(67)
(Urocyon cinereoargenteus)	CO, USA	PCR (ITS, *gltA, ftsZ*)	*PCR: 0/1*	(77)
	TX, USA	IFA	*IFA: 50% (66/132), 22Bc, 8 Bvb, 36 Bc+Bvb*	(96)

(Continued)

TABLE 1 | Continued

Species	Location	Method	Prevalence/*Bartonella* spp.	References
	CA, USA	PCR (ITS, *ftsZ*)	*PCR (fleas): 39% (42/108) (78.5% Br, 19% Bvb)*	(97)
		Culture, PCR (ITS, *gltA*), IFA	*Culture: 49% (26/53) (22/53 B. clarridgeiae-like, 5/53 Bvb); IFA: 89% (48/53) Bartonella spp.*	(58)
Island fox *(Urocyon littoralis)*	CA, USA	Culture, IFA, PCR (ITS, *pap31*)	*IFA: 62.7% (31.4% (16/51) Bc; 9.8% (5/51) Bvb; 21.6% (11/51) both); Culture: 11.8% (6/51) Bvb; PCR: 1 Bvb type III, 3 Br*	(98)
		IFA	*IFA: 25.8% (68/263) Bvb, 27.7% (73/263) Bc*	(99)
Arctic fox (*Vulpes lagopus*)	Canada	PCR	*PCR (blood): 15% (3/20) Bh*	(100)
Kit fox (*Vulpes macrotis*)	Mexico	Culture, PCR (*gltA*, ITS)	*Culture: 0/15; PCR (blood): 13.3% (2/15) Br; PCR (fleas): 5.0% (4/80) Br*	(67)
Red fox (*Vulpes vulpes*)	Slovakia	PCR	*PCR: 4.7% (19/407) fleas, Bartonella* spp.	(101)
	Romania	PCR (*ssrA*)	*PCR: 0/56*	(102)
	Austria	PCR (ITS)	*PCR (blood): 0% (0/351); PCR (spleen): 0.2% (1/506) Br*	(103)
	CO, USA	PCR (ITS, *gltA, ftsZ*)	*PCR: 27% (7/26) 2/7 Bvb, 5/7 Br*	(77)
	Israel	PCR (ITS, *ssrA, rpoB, gltA*)	*PCR: 18% (2/11) 1/2 Br, 1/2 related to B. merieuxii*	(85)
	Bosnia and Herzegovina	PCR (ITS)	*PCR: 0% (0/119)*	(104)
	Spain	PCR (ITS)	*PCR (blood): 25% (3/12) 3 Br; PCR (ticks): 0% (0/52 pools)*	(83)
		PCR (*gltA*, ITS)	*PCR: 1.6% (1/62) Br*	(64)
	Iraq	*PCR (ITS, rpoB, gltA), IFA*	*PCR: 0% (0/39); IFA: 13% (5/39) any Bartonella spp., Bh 5% (2/39), Bc 3% (1/39), Bvb 5% (2/39), B. bovis 13% (5/39).*	(86)
	Australia	PCR (ITS, *gltA, 16S rRNA, ftsZ, rpoB*)	*PCR (fleas): 70.5% (24/34) (20/24 Bc, 4/24 Bh); PCR (blood): 1/14 Bc*	(105)
	France	Culture, PCR (ITS, *gltA, rpoB, ftsZ, groEL*)	*PCR: 100% (1/1) Br*	(88)
	Spain	PCR (ITS)	*PCR (fleas): 31.8% (7/22 pools), related to Br*	(65)
	Hungary	PCR (*groEL, pap31*)	*PCR (ticks): 0%; PCR (fleas): 4.2% (4/95 pools) Bartonella* spp.	(106)
Mephitidae family				
Hooded skunk (*Mephitis macroura*)	Mexico	Culture, PCR (ITS, *gltA*)	*Culture: 0/3; PCR (blood): 33.3% (1/3) Br; PCR (fleas): 26.7% (4/15) Br*	(67)
Striped skunk *(Mephitis mephitis)*	Mexico	Culture, PCR (ITS, *gltA*)	*Culture: 25% (2/8); PCR (blood): 12.5% (1/8) Br; 12.5% (1/8) Bvb; PCR (fleas): 5.4% (2/37) Br; 2.7% (1/37) Bvb*	(67)
	CO, USA	PCR (ITS, *gltA, ftsZ*)	*PCR: 23% (10/44) Br*	(77)
Mustelidae family				
Northern sea otter (*Enhydra lutris keyoni*)	AK, USA	IFA	*IFA: 34% (15/44) of live animals (27% Bw, 2.2% Bc, 4.5% Bc & Bw) and 50% of necropsied animals (14% Bw, 25% Bh & Bw, 2% Bh & Bc, 2% Bc & Bw, 6.2% Bh, Bc, & Bw)*	(107)
	AK, USA	Culture, PCR (ITS, *pap31, rpoB*)	*Culture: 0/9; PCR (valves): 45% (23/51); Bh I, B. bacilliformis, Bartonella* spp.	(108)
Southern sea otter (*Enhydra lutris nereis*)	CA, USA	IFA	*IFA: 16% (24/148) of necropsied animals (4.7% Bw, 1.3% Bc, 2% Bh, 5.4% Bh & Bw, 1.3% Bc & Bw, 1.3% Bh, Bc, & Bw)*	(107)
	CA, USA	Culture, PCR (ITS, *pap31, rpoB*)	*PCR (valves): 10% (3/30) B. spp, B. bacilliformis*	(108)
River otter (*Lontra canadensis*)	NC, USA	Culture, PCR (ITS)	*PCR: 15.2% (19/65), novel B. volans-like; culture: 1*	(109)
Beech marten	Spain	PCR (ITS)	*PCR (blood): 10% (1/10) 1 Bc; PCR (ticks): 0% (0/146 pools)*	(83)
(*Martes foina*)		PCR (*gltA*, ITS)	*PCR: 0% (0/26)*	(64)
Pine marten (*Martes martes*)	Spain	PCR (*gltA*, ITS)	*PCR: 0% (0/14)*	(64)
Japanese marten (*Martes melampus*)	Japan	PCR (ITS, *16SrRNA, ftsZ, gltA, groEL, ribC, rpoB*)	*PCR (blood): 12.5% (1/8) close to B. washoensis*	(95)
Japanese badger (*Meles anakuma*)	Japan	PCR (ITS, *16SrRNA, ftsZ, gltA, groEL, ribC, rpoB*)	*PCR (blood): 6.7% (1/15) novel Bartonella species*	(95)

(Continued)

TABLE 1 | Continued

Species	Location	Method	Prevalence/*Bartonella* spp.	References
European badger	Spain	PCR (*gltA*, ITS)	*PCR: 12% (9/75), B. clarridgeiae-like* sp.	(64)
(*Meles meles*)		PCR (ITS) (ITS, *16SrRNA, ftsZ, gltA, groEL, ribC, rpoB*)	*PCR (blood): 0% (0/3); PCR (ticks): 0% (0/2 pools)*	(83)
		PCR (ITS)	*PCR (fleas): 0% (0/3 pools)*	(65)
Stoat (*Mustela erminea*)	New Zealand	Culture, PCR (*gltA*)	*Culture (blood): 0% (0/47); PCR (blood): 0% (0/94)*	(110)
Japanese weasel *(Mustela itatsi)*	Japan	PCR (ITS, *16SrRNA, ftsZ, gltA, groEL, ribC, rpoB*)	*PCR (blood): 0% (0/2)*	(95)
Least weasel	Spain	PCR (*gltA*, ITS)	*PCR: 0% (0/5)*	(64)
(*Mustela nivalis*)	New Zealand	PCR (*gltA*)	*PCR (blood): 0% (0/2)*	(110)
European polecat (*Mustela putorius*)	Spain	PCR (*gltA*, ITS)	*PCR: 0% (0/5)*	(64)
Ferret (*Mustela putorius furo*)	New Zealand	Culture, PCR (*gltA*)	*Culture (blood): 0% (0/1); PCR (blood): 0% (0/25)*	(110)
Siberian weasel (*Mustela sibirica*)	Japan	Blood, PCR (ITS, *16SrRNA, ftsZ, gltA, groEL, ribC, rpoB*)	*PCR: 0% (0/1)*	(95)
American mink (*Mustela vison*)	Spain	PCR (*gltA*, ITS)	*PCR: 0% (0/3)*	(64)
American badger (*Taxidea taxus*)	Mexico	Culture, PCR (ITS, *gltA*)	*Culture: 0/6; PCR (blood): 0/6;* *PCR (fleas): 5.9% (2/34) Br; 2.9% (1/34) Bvb*	(67)
	CA, USA	IFA	*IFA: 10% (1/10) Bh; 10% (1/10) Bvb; 10% (1/10) Bh + Bc*	(111)
Phocidae family				
Harbor seal (*Phoca vitulina*)	The Netherlands	PCR (ITS, *rpoB*)	*PCR (spleen): 2.1% (1/48); PCR (lice): 16.7% (1/6 pools); 100% Bh / 97% B. grahamii*	(112)
Procyonidae family				
Ring-tailed coati (*Nasua nasua*)	Brazil	PCR	*PCR: 0/31*	(66)
Raccoon (*Procyon lotor*)	Mexico	Culture, PCR (ITS, *gltA*)	*Culture: 0/4; PCR (blood): 0/4; PCR (fleas): 0/17*	(67)
	CO, USA	PCR (ITS, *gltA, ftsZ*)	*PCR: 8% (14/186) Bartonella* spp. (21% Bvb, 79% Br)	(77)
	GA, USA	PCR (ITS)	*PCR (blood): 43% (16/37) Bartonella spp.: Bh (12/37), Bk (1/37)*	(59)
	Japan	PCR (ITS, *16SrRNA, ftsZ, gltA, groEL, ribC, rpoB*)	*PCR (blood): 0% (0/977)*	(95)
	CA, USA	Culture, PCR (ITS, *gltA, rpoB, ftsZ, groEL*)	*Culture: 26% (11/42) Br*	(88)
Ursidae family				
Black bear (*Ursus americanus*)	CO, USA	PCR (ITS, *gltA, ftsZ*)	*PCR: 0% (0/7)*	(77)

Bc, B. clarridgeiae; Bh, B. henselae; Bk, B. koehlerae; Br, B. rochalimae; Bvb, B. vinsonii subsp. berkhoffii; Bw, B washoensis.

was higher in warmer and more humid California than in Colorado. For mountain lions, suburban land use predicted increased exposure to *Bartonella* species in southern California (114).

Seasonal Pattern

Studies have yielded conflicting evidence about the seasonality of *B. vinsonii* subsp. *berkhoffii* infection in coyotes. First, Chang et al. (91) reported that the prevalence of *Bartonella* antibodies was highest in summer (42%) and lowest in spring (29%), whereas a geographically more restricted study conducted in coastal central California, U.S., by the same authors found the highest seroprevalence in winter (100%) and the lowest in summer (62%) (90). Investigating antibody prevalence in 239 coyotes from northern California, Beldomenico et al. (89) identified some environmental factors associated with the seropositivity. In that study, prevalence of antibodies against *B. vinsinii* subsp. *berkhoffii* was 44% in the summer, 40% in the spring, 27% in the winter, and 19% in the fall. The authors noticed that *Bartonella* seropositivity was associated with higher precipitation and proximity to the coast. In addition, coyotes seropositive for *B. vinsonii* subsp. *berkhoffii* were more likely to be seropositive for tick-borne agents *Anaplasma phagocytophilum* and mosquito-vectored *Dirofilaria immitis* (89). Interestingly, California Zoo felids of the genus *Felis* were found almost three times more likely to be seropositive for *B. henselae* than animals belonging to the genera *Panthera* and *Acinonyx* (69).

PREVALENCE OF *BRUCELLA* INFECTIONS IN WILD CARNIVORES

General Pattern

Eighty-nine percent of *Brucella* studies of wild carnivores were conducted by serological and bacteriological methods, but no reports were found on culturing *Brucella* from representatives of suborder *Feliformia*. Only a few wild felid species (lion, jaguar, and bobcat), mongooses, and spotted hyena were serologically positive. Apart from one bobcat that had antibodies against *Br. canis* (115), the rest of seropositive Feliformia animals had antibodies against *Br. abortus.* We have to be cautious with the claim about presence of specific antibodies in this paper, as well in many other reports, because *Br. abortus* suspensions can also detect *Br. melitensis*. The highest seroprevalence was registered in white-tailed mongoose [33.3%, 1/3, (116)]. In evident contrast to Feliformia animals, prevalence of *Brucella* in various Caniformia species varied greatly, with many reporting high prevalences of positive antibody titeres. Antibodies to *Brucella* species were recorded in 40% of coyotes (117), 42% of wolves (118), 43% of black-backed jackals (116), 50% of Arctic foxes and 40% of red foxes (48), 64% of grizzly bears (119), 28% of Asian sea otters (120), 23% of California sea lions (121), and 74% of Australian seals (122). *Brucella* was cultured from 30.8% of wolves (123) (**Table 2**).

Age Pattern

We could find information on age dependence only in marine *Brucella*. In the 2018 study on gray and harbor seals, Kroese et al. (212) noted remarkable age-dependent prevalence of *Br. pinnipedialis* in both serology and in the investigation of the tissues from stranded animals. The PCR positivity was 84% (26/31) in juveniles compared to 57% (4/7) in adults and *Br. pinnipedialis* was cultured only from juveniles and not from adults in that study. Similar age dependence was shown in harbor seals by Miller et al. (169) and Ewalt et al. (229). Nymo et al. (206) noted the age-dependent prevalence of anti-*Brucella* antibodies in hooded seals. Pups (<1 mo old) had a substantially lower probability of being seropositive (4/159, 2.5%) than yearlings (6/17, 35.3%), suggesting that exposure may occur post-weaning, during the first year of life. For seals over 1 year old, the mean probability of being seropositive decreased with age, with no seropositives older than 5 years, indicating loss of antibody titer with either chronicity or clearance of infection (206).

BARTONELLA SPECIES IDENTIFIED IN WILD CARNIVORES

Bartonella Species in Wild Feliformia Animals

Wild Feliformia animals mostly carry the same *Bartonella* species as domestic cats, namely *B. henselae* (types I and II), *B. koehlerae*, and *B. clarridgeiae* (234). The same species were detected in feral cats from Georgia, U.S. (59). In Africa, free-ranging lions were found infected with *B. henselae* type II and *B. koehlerae* subsp. *koehlerae* and Namibian cheetah with a strain that clustered between *B. henselae* and *B. koehlerae* and was considered a new subspecies of *B. koehlerae* (61, 63). In Japan, *B. henselae* was found in Iriomote leopard cats and *B. clarridgeiae* DNA was detected in Tsushima leopard cats (74, 82).

In a study on free-ranging mountain lions and bobcats from California, U.S., Chomel et al. (55) described new *Bartonella* strains, which were similar to but different from *B. henselae* and *B. koehlerae,* and named them *B. koehlerae* subsp. *boulouisii* and *B. koehlerae* subsp. *bothieri*. Phylogenetic analysis based on comparison of four genetic markers revealed two clusters: one with five strains obtained from bobcats and another with three strains obtained from mountain lions indicating a degree of host-speciation of these strains (55). In Brazil, sequencing analysis revealed a *Bartonella* strain close to but different from *B. henselae* and *B. koehlerae* in wild-born captive margay (*Leopardus wiedii*) (235).

Other *Bartonella* species were detected in fleas collected from wild felids. For example, *Bartonella alsatica* was found in one of six rabbit fleas *Spilopsyllus cuniculi* collected from a European wildcat (*F. silvestris)* in Spain (65). This *Bartonella* species is usually associated with rabbits and possibly fleas were infected or they contained blood meal from infected rabbits, as *S. cuniculi* is normally found on European rabbits (*Oryctolagus cuniculus*). A different situation has been reported by López-Pérez et al. (67) regarding a genetic variant obtained from a flea (*Pulex simulans*) collected from a bobcat (*L. rufus)* in northwestern Mexico. This variant had ITS sequence 99.1% similar to a strain previously isolated from another bobcat from California, U.S., but distant from all other *Bartonella* genotypes.

Bartonella Species in Wild Caniformia Animals

In the studies, Caniformia animals were found to carry *B. henselae, B. clarridgeiae, B. vinsonii* subsp. *berkhoffii, B.rochalimae, B. washoensis,* and *B. bacilliformis*. In an investigation of wild carnivores from Colorado, U.S., Bai et al. (77) identified two *Bartonella* species, *B. vinsonii* subsp. *berkhoffii* and *B. rochalimae*. Striped skunks exclusively carried *B. rochalimae,* while coyotes, red foxes, and raccoons were infected with either or both *Bartonella* species. *Bartonella rochalimae* DNA was found in a wolf *(C. lupus*) in northern Spain (64). Investigating wild canids along with stray dogs throughout Iraq, Chomel et al. (86) identified a novel strain of *Bartonella*, which was named *Candidatus* B. merieuxii, in six jackals (*Canis aureus*). By three genetic markers, the "jackal" strain was aligned most closely with *B. bovis* and the other ruminant *Bartonella* species. Sequences closely related to *Candidatus* Bartonella merieuxii later were found in three jackals and one red fox (*V. vulpes*) in Israel (85). Besides this strain, *B. rochalimae* and *B. rochalimae*-like were found in five jackals and one fox, and one jackal harbored *B. vinsonii* subsp. *berkhoffii* (85).

Kehoe et al. (87) documented the presence of three *Bartonella* species in heart valves and/or spleen of free-ranging coyotes from northern California, U.S. Partial DNA sequencing showed that aortic valves from 8 (53%) of 15 coyotes were *B. vinsonii* subsp. *berkhoffii* positive, *B. rochalimae* DNA was amplified from the

TABLE 2 | *Brucella* studies in wild Carnivores by species.

Species	Location	Method	Prevalence/*Brucella* spp.	References
SUBORDER FELIFORMIA				
Felidae family				
Wildcat *(Felis silvestris)*	Russia	Serology	*Serology: 0/6*	(124)
Lynx *(Lynx canadensis)*	Canada	Serology, culture	*Serology/culture: 0*	(125)
Bobcat *(Lynx rufus)*	AL, USA	Culture	*Culture: 0/3*	(126)
	CA, USA	RPA	*Serology: 6.6% (5/75) B. abortus*	(127)
	TX, USA	RSA, SMTA	*Serology: 0 B. canis*	(128)
	USA	Tube agglutination	*Serology: 14% (1/7) B. canis (1/3 in TX, 0/1 in FL, 0/3 in SC)*	(115)
	UT, USA	Tube agglutination	*Serology: 0/3 B. abortus*	(129)
African lion (*Panthera leo*)	Tanzania	RBPT, BAPA, Riv	*Serology: 50% (1/2) Brucella* sp.	(130)
		Tube agglutination	*Serology: 15.4% (2/13) B. abortus*	(116)
	SAR	Agglutination	*Serology: 0/4 Brucella* sp.	(131)
Jaguar *(Panthera onca)*	Brazil	RBPT	*Serology: 3.2% (1/31) B. abortus*	(132)
		RBPT, 2-ME	*Serology: 0/11 B. abortus*	(133)
Leopard *(Panthera pardus)*	Tanzania	Tube agglutination	*Serology: 0/1 B. abortus*	(116)
Florida panther (*Puma concolor coryi*)	FL, USA	Plate agglutination	*Serology: 0/24 B. abortus*	(134)
Herpestidae family				
White-tailed mongoose (*Ichneumia albicauda*)	Tanzania	Tube agglutination	*Serology: 33.3% (1/3) B. abortus*	(116)
Banded mongoose (*Mungos mungo*)	Tanzania	Tube agglutination	*Serology: 1/1 B. abortus*	(116)
Hyaenidae family				
Spotted hyena *(Crocuta crocuta)*	SAR	Agglutination	*Serology: 0/2 Brucella* sp.	(131)
	Tanzania	Tube agglutination	*Serology: 26.7% (4/15) B. abortus*	(116)
	Tanzania	Agglutination	*Serology: 50% (2/4) Brucella* sp.	(135)
Viverridae family				
Genet *(Genetta genetta)*	Rhodesia	Tube agglutination	*Serology: 0/2 B. abortus*	(136)
Cape genet *(Genetta tigrina)*	Tanzania	Tube agglutination	*Serology: 0/3 B. abortus*	(116)
SUBORDER CANIFORMIA				
Family Canidae				
Golden jackal (*Canis aureus*)	Serbia	qPCR (*bcsp31, alkB, BMEI1162*)	qPCR: 1.9% (4/216) *B. canis*	(137)
Coyote (*Canis latrans*)	NC, USA	Card, RIV, IFA, agglutination	*Serology: 0/28 B. abortus/suis; 0/30 B. canis*	(138)
	NE, USA	Rapid slide agglutination	*Serology: 0/67 B. canis*	(139)
	WY, USA	Standard plate test	*Serology: 0/70 B. abortus and B. canis*	(140)
	GA, USA	Tube test	*Serology: 0/17 B. canis*	(141)
	TX, USA	Card, RIV, SAT,CF, ELISA	*Serology: CARD: 40.4% (38/94); RIV: 21.3% (20/94); CF: 22.3% (21/94); SAT: 18.1% (17.94); ELISA: 30.9% (29/94) B. abortus*	(117)
	AL, USA	Culture	*Culture: 0/2*	(126)
	TX, USA	BBA, RIV, SAT, CFT, culture	Serology: 18% (9/51) by 2+ tests. Culture: 16.3% (7/43) *B. abortus biovar 1.*	(142)
	CA, USA	Plate agglutination	*Serology: 6% (9/148); B. abortus*	(127)
	TX, USA	RSA, SMTA *B. canis*	Serology: Card: 5.6% (11/198); RSA: 6.6% (13/198); SMTA: 8.1% (16/198) ≥1:50 *B. canis*	(128)
	USA	Tube agglutination	*Serology: 2% (2/103) B. canis (2/86 in TX, 0/1 - NY, 0/16 - ND)*	(115)
	TX, USA	Plate agglutination	*Serology: 0/33 B. abortus*	(143)
	UT, USA	Tube agglutination test	*Serology: 0/6 B. abortus*	(129)
Wolf (*Canis lupus*)	AK, USA	BBA, STT& SPT, CAR	*Serology: 0–25% B. suis biovar 4*	(144)
	Russia	Culture	*Culture: 11.8% (30/254) B. suis biovar 4*	(53)
	AK, USA	BAPA	*Serology: 1% (1/76) B. suis biovar 4*	(145)
	Canada	Culture	*Culture: 31% (4/13) B. abortus 1* [From (123)]	(146)

(Continued)

TABLE 2 | Continued

Species	Location	Method	Prevalence/*Brucella* spp.	References
	Canada	Culture	*Culture: B. abortus biovar 1 isolated from a wolf. New strain of biovar 1 isolated from another wolf.*	(125)
	Canada	CF, rapid slide agglutination	*Serology: 0/3 B. abortus*	(147)
	AK, USA	SAT, CFT	*Serology: CF: 39% (11/28), agglutination: 26% (7/27) B. suis biovar 4*	(148)
	NY, USA	Tube agglutination	*Serology: 0/4 B. canis*	(115)
	Russia	Culture	*Culture: 15 Brucella* sp. *isolates*	(11)
	AK, USA	SAT, CFT	*Serology: 42.9% (3/7) B. suis biovar 4*	(118)
	Russia	Culture	*Culture: 10.9% (12/110) B. suis biovar 4*	(13)
	Russia	Serology	Serology: 0/56	(124)
Black-backed jackal *(Canis mesomelas)*	Tanzania	Tube agglutination test	*Serology: 43% (3/7) B. abortus*	(116)
Crab-eating fox	Brazil	RBPT, FPA	*Serology: 13.2% (5/38) smooth Brucella*	(149)
(*Cerdocyon thous*)	Brazil	RBPT, CFT	*Serology: 0/7 B. abortus*	(150)
	Bolivia	Slide agglutination/ AGID	*Serology: 0/5 B. canis*	(151)
		Serology	*Serology: 0/55 B. canis*	(152)
Maned wolf *(Chrysocyon brachyurus)*	Brazil	RBPT, CFT	*Serology: 0/3 B. abortus*	(150)
Foxes?	Argentina	Characterization	*B. abortus*	(153)
Patagonian gray fox *(Dusicyon griseus griseus)*	Argentina	Plate agglutination test	*Serology: 21.7% of 318 (11.3% of these ≥1:100); B. abortus*	(154)
Pampas gray fox *(Dusicyon gymnocercus antiquus)*	Argentina	Plate agglutination test, culture	*Serology: 25.4% of 410 (13.9% of these ≥1:100); Culture: 16.1% (5/31 pools of 77 foxes), B. abortus biovar 1.*	(154)
Pampas fox (*Lycalopex gymnocercus*)	Bolivia	Slide agglutination/ AGID	*Serology: 0/9 B. canis*	(151)
Hoary fox (*Lycalopex vetulus*)	Brazil	BPAT, AGID, MAT, SMTA	*Serology: BPAT: 26.6% (16/60) B. abortus; SMTA: 6.7% (4/60); AGID: 0/60 B. canis*	(155)
Wild dog *(Lycaon pictus)*	Tanzania	Tube agglutination test	*Serology: 33.3% (1/3) at 1:160 B. abortus*	(116)
Bat-eared fox (*Otocyon megalotis*)	Tanzania	Tube agglutination test	*Serology: 0/1 B. abortus*	(116)
Gray fox (*Urocyon cinereoargenteus)*	AL, USA	CARD, STA, 2-ME, RIV	*Serology: 14.3% (1/7) during exposure, or 5.6% (1/18) total; B. abortus biovar 1*	(126)
	FL, SC, USA	Tube agglutination	*Serology: 0/15 (0/10 in FL, 0/5 in SC) B. canis*	(115)
	AR, USA	Culture	*Culture: 0/14*	(156)
Arctic fox (*Vulpes lagopus*)	Russia	Culture	*Culture: 2.3% (18/777) B. suis biovar 4*	(53)
	AK, USA	SP, BBA, Riv, ST, ME, CF, culture	*Serology: 50% (2/4), culture: 25% (1/4) B. suis biovar 4*	(48)
	Russia	Culture	*Culture: 10 Brucella isolates*	(11)
	Russia	Culture	*Culture: 1.1% (4/370) B. suis biovar 4*	(13)
	Russia	Culture	*Culture: 1.7% (9/530) B. suis biovar 4*	(157)
	Russia	Culture, serology	*Culture: 4% (5/128); Serology: 3% (58/1,890)*	(124)
Kit fox *(Vulpes macrotis)*	UT, USA	Tube agglutination test	*Serology: 0/5 B. abortus*	(129)
San Joaquin kit fox	CA, USA	Serology	*Serology: 0/46 B. canis*	(152)
(Vulpes macrotis mutica)	CA, USA	CF, BBA, SAT, 2-ME	*Serology: B. abortus CF: 8% (3/23) in 1981/2, card test 3% (1/29) in 1984; B. canis CF: 14% (5/23) in 1981/2, ME: 0% (0/20) in 1984.*	(158)
Red fox (*Vulpes vulpes*)	Austria	Characterization	*B. vulpis* sp. nov.	(159)
	Austria	Culture	*Two novel Brucella strains*	(160)
	Austria	Culture	*Culture: B. microti*	(161)
	AK, USA	SP, BBA, Riv, ST, ME, CF, culture	*Serology: 39.5% (15/38), culture: 8% (3/38) B. suis biovar 4*	(48)
	Canada	Culture	*Culture: 2.7% (1/37) B. abortus biovar 1* [from (123)]	(146)
	Canada	Culture	*Culture: B. abortus biovar 1*	(125)
	AK, USA	STT, CFT	*Serology: CF: 18.2% (2/11); agglutination: 9.1% (1/11); B. suis biovar 4*	(148)
	NY, USA	Tube agglutination	*Serology: 1.5% (1/68) B. canis*	(115)
	Wales, UK	RBPT, SAT, CFT, AGT	*Serology: 9% (8/87); culture: B. abortus biovar 1*	(162)
	Ireland, UK	SAT, CF, culture	*Serology: SAT: 12.5% (4/32) B. abortus; Culture: 0/2*	(163)
	AR, USA	Culture	*Culture: 0/9*	(156)
	Russia	Serology, culture	Serology: 8.5% (374/4,380); culture: 7.8% (13/166);	(124)
	Bulgaria	Serology, culture	Serology: 3.6% (16/440); culture: 3.5% (1/29) *B. suis*	(164)

(Continued)

TABLE 2 | Continued

Species	Location	Method	Prevalence/*Brucella* spp.	References
Family Mephitidae				
Striped skunk *(Mephitis mephitis)*	CA, USA	Plate agglutination	*Serology: 8.7% (2/23) ≥ 1:100 B. abortus*	(127)
	TX, USA	RSA, SMTA	*Serology: 0 B. canis*	(128)
	USA	Tube agglutination ≥1:200	*Serology: 0/17 B. canis*	(115)
	AR, USA	Culture	*Culture: 0/18*	(156)
Western spotted skunk (*Spilogale gracilis*)	CA, USA	Plate agglutination	*Serology: 3.85% (1/26) ≥ 1:100 B. abortus*	(127)
Family Mustelidae				
Northern sea otter	WA, USA	BAPA, ELISA, FPA	*Serology: 10% (3/30) B. abortus*	(165)
(*Enhydra lutris keyoni*)	AK, USA	cELISA	*Serology: 2.7% (1/72) marine Brucella* sp.	(120)
	WA, USA	Card, BAPA, rivanol, CF	*Serology: 0/30 B. abortus*	(166)
	AK, USA	RBT	*Serology: 7.7% (5/65) B. abortus*	(167)
Asian sea otter (*Enhydra lutris lutris)*	Russia	PCR (*IS711*)	*PCR (rectal swabs): 4% (3/78) B. abortus, B. melitensis, B. pinnipedialis*	(168)
		ELISA	*Serology: 28.1% (25/89) marine Brucella* sp.	(120)
Southern sea otter *(Enhydra lutris nereis)*	CA, USA	Culture, ELISA, FPA, PCR (*16S rDNA, bp26*)	*1/1 marine Brucella* sp.	(169)
	CA, USA	RBT	*Serology: 5.9% (4/68) B. abortus*	(167)
Wolverine *(Gulo gulo)*	Russia	Culture	*Culture: 10.2% (4/39) B. suis biovar 4*	(53)
		Culture	*Culture: 1 B. suis biovar 4*	(11)
		Culture	*Culture: 11.1% (1/9) B. suis biovar 4*	(13)
Eurasian otter *(Lutra lutra)*	UK	ELISA, culture	*Serology: 10.8% (8/74) B. abortus; culture: 0.6% (1/160) marine Brucella* sp.	(170)
	UK	Culture	*Culture: 1/1 Brucella* sp.	(171)
American pine marten (*Martes americana*)	Canada	Serology, culture	*Serology/culture: 0*	(125)
Asian badger (*Meles leucurus)*	South Korea	PCR, culture	*PCR (tissue): 100% (1/1) Brucella* sp.; *culture: 0/1*	(172)
Stoat	Russia	Culture	*Culture: 1.2% (6/484) B. suis biovar 4*	(53)
(*Mustela erminea*)		Serology, culture	*Serology: 0/7; culture: 0/3*	(124)
Steppe polecat (*Mustela eversmanii*)	Russia	Serology, culture	*Serology: 0/30; culture: 0/15*	(124)
European mink (*Mustela lutreola*)	Russia	HT, AT, CFT	*Serology: 7.2% (108/1,506); culture: 10.4% (11/106)*	(124)
Least weasel (*Mustela nivalis*)	France	Culture	*Culture: 0/10*	(173)
American mink (*Neovison vison)*	Argentina	ELISA, CFT	*Serology: 9.2% (8/87) B. abortus*	(174)
Fisher (*Pekania pennanti*)	Canada	Serology and culture	*Serology/culture: 0*	(125)
American badger *(Taxidea taxus)*	CA, USA	Plate agglutination	*Serology: 50% (2/4) B. abortus*	(127)
	TX, USA	RSA, SMTA	*Serology: B. canis*	(128)
	UT, USA	Tube agglutination	*Serology: 0/5 B. abortus*	(129)
	UT, USA	CF; agglutination test	*Serology: 0/1 B. canis*	(175)
Family Procyonidae				
Brown-nosed coati (*Nasua nasua)*	Brazil	RBPT, FPA	*Serology: 8.8% (3/34) smooth Brucella*	(149)
Raccoon (*Procyon lotor*)	S Korea	ELISA, PCR, culture	*Serology: 0/32; PCR (blood): 11.1% (1/9); culture: 0/9; PCR (tissue): 40% (2/5); culture: 0/5. B. abortus*	(172)
	NE, USA	Rapid slide agglutination	*Serology: 0% (0/63) B. canis*	(139)
	AL, USA	Culture, CARD, STA, 2-ME, RIV	*Culture: 16.7% (1/6) B. abortus biovar 1 during exposure, or 4.2% (1/24) total; Serology: 25% (1/4) during exposure, 8.3% (1/12) post exposure, or 9.5% (2/21) total*	(126)
	TX, USA	SAT, card, CF	*Serology: 0/3 B. abortus*	(176)
	AL, USA	Culture, card, tube agglutination test	*Culture: B. abortus biovar 1 from spleen & lymph node; Serology: Card: trace; tube: 1:200*	(177)
	CA, USA	Plate agglutination	*Serology: 6.25% (1/16) ≥ 1:100 B. abortus*	(127)
	TX, USA	RSA ≥1:2, SMTA	Serology: Card: 9.1% (1/11); RSA: 27.3% (3/11); SMTA: 9.1% (1/11)≥1:50, 9.1% (1/11)≥1:100; 0% (0/11)≥1:200 *B. canis*	(128)
	FL, USA	Tube agglutination	*Serology: 0.3% (1/360 (0.4% (1/269) in FL, 0/87 in TX, 0/4 in SC)) B. canis*	(115)
	FL, USA	Agglutination test	*Serology: 1.8% (4/222) at two counties (0.7 and 3.9%), B. canis*	(115)
	AR, USA	Culture	*Culture: 0/25*	(156)
Family Ursidae				
Black bear (*Ursus americanus*)	MD, USA	BAPA/card	*Serology: 0% (0/61) B. canis*	(178)

(Continued)

TABLE 2 | Continued

Species	Location	Method	Prevalence/*Brucella* spp.	References
	AK, USA	BBA, SPT	*Serology: 0.8% (1/92)*	(179)
	Canada	CF, rapid slide agglutination	*Serology: 0.4% (1/283) B. abortus*	(147)
	ID, USA	Tube agglutination	*Serology: 5% (18/332) ≥1:20 B. abortus*	(180)
Brown bear *(Ursus arctos)*	AK, USA	BBA, SPT	*Serology: 14% (13/92)*	(179)
Alaska peninsula brown bear *(Ursus arctos gyas)*	AK, USA	ELISA, RBPT, ELISA+, RBPT+	*Serology: ELISA: 0/6; RBPT: 83.3% (5/6); ELISA+: 0/6; RBPT+: 33.3% (1/6); B. abortus*	(119)
Grizzly bear *(Ursus arctos horriblis)*	AK, USA	ELISA, RBPT, ELISA+, RBPT+, *B. abortus*	*Serology: ELISA: 62.1% (36/58); RBPT: 70.7% (41/58); ELISA+: 63.8% (37/58); RBPT+: 69% (40/58); B. abortus*	(119)
		BBA, STT& SPT ≥1:50, CAR	*Serology: 0–24% B. abortus*	(144)
		SP, BBA, Riv, ST, ME, CF	*Serology: 25% (2/8)*	(48)
		SPT, card	*Serology: 5% (6/122)*	(181)
		SAT, CFT	*CF: 29% (6/21); SAT: 43% (9/21) at Porcupine caribou herd; CF: 94% (15/16); SAT: 82% (14/17) at Arctic caribou herd.*	(148)
Kodiak brown bear *(Ursus arctos middendorffi)*	AK, USA	ELISA, RBPT, ELISA+, RBPT+	*Serology: ELISA: 75% (6/8); RBPT: 87.5% (7/8); ELISA+: 75% (6/8); RBPT+: 75% (6/8) B. abortus*	(119)
Marsican brown bear (*Ursus arctos marsicanus)*	Italy	Rapid serum agglutination	*Serology: 10% (2/22) B. abortus / B. melitensis*	(182)
Polar bear	AK, USA	BBA, SPT	*Serology: 13% (18/138)*	(183)
(Ursus maritimus)		BBA, SPT, cELISA	*Serology: 10.2% (28/275) (6.8% –18.5% over 2003–2006)*	(179)
		BACA, rapid automated presumptive test	*Serology: 5% (25/500) B. abortus*	(184)
		SAW, SAW-EDTA, RBT, Protein-A ELISA	*Serology: 5.4% (16/297) by all tests; SAW: 6% (18/297); SAW-EDTA: 5.4% (16/297); RB: 7% (21/297); Protein-A ELISA 53% (157/297)*	(185)
SUPERFAMILY PINNIPEDIA				
Family Odobenidae (Walruses)				
Pacific walrus (*Odobenus rosmarus divergens*)	AK, USA	Card, tube agglutination	*Serology: 0/40 B. abortus*	(186)
Atlantic walrus	Canada	ELISA	*Serology: 2.9% (5/170) B. abortus*	(187)
(Odobenus rosmarus rosmarus)	Canada	ELISA, tube agglutination	*ELISA: 12% (7/59); tube test: 5/5 of ELISA positive*	(188)
Family Otariidae (Fur seals & sea lions)				
South American fur seal (*Arctocephalus australis*)	Peru	ELISA, PCR	*Card: 0/29 B. canis; 3.5% (1/29) B. abortus; ELISA: marine Brucella 53.7% (15/28)*	(189)
New Zealand fur seal (*Arctocephalus forsteri)*	New Zealand	ELISA	*Serology: 0/101 (pre-weaned pups) B. abortus*	(190)
Antarctic fur seal	Antarctica	RBT, ELISA	*Serology: 0% (0/21) B. abortus*	(191)
(*Arctocephalus gazella*)		RBT, ELISA	*Serology: 0/64 B. abortus*	(192)
		ELISA	*Serology: 7.7% (4/52)*	(193)
		RBT, ELISA, COMPELISA	*Serology: RBT: 1.2% (1/86); ELISA: 5.8% (5/86)*	(194)
		RBT, CFT, AGID, ELISA	*Serology: 0–31% (AGID 0/16; RBT 1/16; CFT 2/16; ELISA 5/16) B. abortus*	(195)
Australian fur seal (*Arctocephalus pusillus doriferus*)	Australia	ELISA, FPA	*ELISA: 57% (71/125) adult females; 74% (32/43) in 2007; 53% (32/61) in 2008; 33% (7/21) in 2009.*	(122, 196)
		ELISA	*Serology: 7% (1/15)*	(197)
Guadalupe fur seal (*Arctocephalus townsendi)*	Mexico	RBT, RIV, FPA	*Serology: 0/46 (pups 1-2 mo old) B. abortus*	(198)
Northern fur seal	AK, USA	ELISA	*Serology: 0/107*	(199)
(Callorhinus ursinus)		qPCR (*IS711*), BMAT	*qPCR (placentas): 5% (6/119); PCR (sera): 1/40; BMAT: 1/40 positive, 12/40 borderline*	(200)
Steller sea lion	AK, USA	ELISA	*Serology: 1.6% (2/124)*	(199)
(*Eumetopias jubatus*)		ELISA	*Serology: 0.5% (1/197)*	(201)
Western Steller's sea lion (*Eumetopias jubatus jubatus*)	Japan	ELISA, *Western blot*	*Serology: 18% (3/17) B. abortus; 18% (3/17) B. canis*	(202)
Australian sea lion (*Neophoca cinerea*)		ELISA	*Serology: 75% (9/12)*	(197)
New Zealand sea lion (*Phocarctos hookeri*)	New Zealand	ELISA	*Serology: 0.7% (1/147) B. abortus*	(203)
California sea lion (*Zalophus californianus*)	CA, USA	RBT, AGID *B. abortus*, FPA, PCR (*bp26*), culture	*Serology: 22.7% (5/22) Brucella spp.; culture: 0%; 2/5 strains of terrestrial origin*	(121)
		Culture, PCR (*omp2, bcsp31*)	*PCR: 5.1% (3/59) placentae, culture: 3.4% (2/59)*	(204)

(Continued)

TABLE 2 | Continued

Species	Location	Method	Prevalence/*Brucella* spp.	References
Family Phocidae (True seals)				
Hooded seal *(Cystophora cristata)*	Norway	RBT, ELISA	*Serology: 0/3*	(205)
	Norway	ELISA	*Culture: 5% (1/21) B. pinnipedialis from lymph node; Serology: overall 15.6% (59/379) (pups 2.5%, yearlings 35.3%)*	(206)
	Norway	SAW-EDTA, RB, CFT, ELISA, PCR, culture	*Serology: 31% (9/29), culture: 38% (11/29) B. pinnipedialis; highest in spleen (9/29) and lung lymph nodes (9/24)*	(207)
	UK	Culture	*Culture: from 3 seals from lung, spleen, lymph nodes etc.*	(208)
	Canada	ELISA	*Serology: 4.9% (10/204) B. abortus*	(187)
	UK	ELISA, culture	*Serology: 50% (1/2); culture: 60% (3/5) B. abortus*	(170)
		ELISA, SAT, SAT-EDTA, RBT, CFT	*Serology: 35% (48/137) B. abortus*	(209)
	UK	Culture	*Culture: 1/1*	(171)
Bearded seal		Culture, ELISA	*Culture: B. pinnipedialis; serology: 11% (22/200)*	(210)
(*Erignathus barbatus*)	AK, USA	Card, tube agglutination	*Serology: 0/6 B. abortus*	(211)
		ELISA, SAT, SAT-EDTA, RBT	*Serology: 0/16 B. abortus*	(209)
Ribbon seal	AK, USA	*ELISA*	*Serology: 16.4% (9/55)*	(199)
(*Histriophoca fasciata*)	Japan	ELISA *B. abortus, B. canis, Western blot*	*Serology: 15% (3/20) B. abortus; 5% (1/20) B. canis*	(202)
Gray seal (*Halichoerus grypus*)	The Netherlands	RBT, SAT, ELISA	*Serology: SAT 9% (1/11), ELISA 36% (4/11) B. abortus*	(212)
	Finland	Culture, PCR	*Culture: 2.5% (3/122 livers) B. pinnipedialis; PCR: Brucella DNA in liver flukes 1/4 seals*	(213)
	Germany	Culture, PCR	*Culture: 3% (1/34 lungs)*	(214)
	UK	Culture	*Culture: 3/3 from lungs, testes, spleen*	(208)
	Canada	ELISA	*Serology: 3.9% (10/255) B. abortus*	(187)
	UK	ELISA, culture	*Serology: 19% (24/125) B. abortus; culture: 3% (2/66)*	(170)
	UK	ELISA	*Serology: 10% (6/62)* marine *Brucella*	(215)
	UK	Culture	*Culture: 6.3% (1/16 testes)*	(171)
	UK	RBPT, SAT, ELISA	*Serology: RBPT: 32% (10/31), SAT: 13% (4/31); ELISA: 23% (7/31) B. abortus*	(216)
Leopard seal (*Hydrurga leptonyx*)	Australia	ELISA	*Serology: 33% (1/3)*	(197)
Weddell seal	Antarctica	ELISA, RBT	*Serology: ELISA: 24.2% (8/33); RBT 65.6% (21/33) B. abortus*	(191)
(*Leptonychotes weddellii*)		RBT, ELISA	*Serology: 37% (7/19) B. abortus*	(192)
		RBPT, CFT, SAT, ELISA, culture	*Serology: RBPT: 62.9% (22/35); SAT: 68.6% (24/35); CFT: 98.3% (56/57); ELISA: 96.5% (55/57); culture: 0*	(217)
		Unspecified	*Serology: 0/81*	(218)
		RBT, ELISA, COMPELISA	*Serology: 42% (5/12)*	(219)
		RBT, CFT, AGID, ELISA	*Serology: 0–100% (RBT 0/1; AGID 0/1; CFT 0/1; ELISA 1/1) B. abortus*	(195)
Crab-eater seal (*Lobodon carcinophaga*)		RBT, ELISA	*Serology: 11% (1/9) B. abortus*	(192)
Southern elephant seal	Antarctica	ELISA, RBT	*Serology: 4.7% (2/48) B. abortus*	(191)
(*Mirounga leonina*)		ELISA	*Serology: 0/13*	(193)
Hawaiian monk seal (*Neomonachus schauinslandi*)	HI, USA	qPCR (*IS711*)	*PCR (placenta): 0/50*	(220)
	HI, USA	SCA, PCFIA, BAPA, CF, SPT, RIV	*Serology: B. canis: 0/111; B. abortus: SCA: 5–33%*	(221)
		BPAT, ELISA, FPA	*Serology: all tests: 17.1% (28/164); BPAT: 17.1% (28/144), cELISA: 15.2% (25/144), iELISA and FPA: 11.6% (19/144).*	(222)
Ross seal (*Ommatophoca rossii*)		RBT, ELISA	*Serology: 5% (1/20) B. abortus*	(192)
Harp seal	Norway	RBT, C-ELISA	*Serology: 0/6*	(205)
(*Pagophilus groenlandicus*)	NE, USA	Culture, card, BAPA, RIV	*Serology: 8% (4/53); culture: 33.3% (3/9) from lungs and lymph nodes, B. abortus*	(223)
	Canada	ELISA	*2% (8/453) (1.8% (8/453), 1.8% (5/269), 3.1% (3/95)) B. abortus*	(187)
	UK	ELISA	*Serology: 50% (1/2) B. abortus*	(170)
	Canada	Culture	*Culture: 1/1 from lymph nodes - novel Brucella* sp.	(224)
		ELISA, SAT, SAT-EDTA, RBT, CFT	*Serology: 2% (15/811)*	(209)
Ringed seal *(Phoca hispida)*	Sweden	RBT, C-ELISA	*Serology: 16.7% (2/12)*	(205)
	AK, USA	ELISA	*Serology: 14% (21/150)*	(199)
	Norway	SAW-EDTA, RB, CFT, ELISA	*Serology: 0/20 B. abortus*	(207)

(Continued)

TABLE 2 | Continued

Species	Location	Method	Prevalence/*Brucella* spp.	References
	Canada	ELISA	*Serology: 1.1% (7/628) B. abortus*	(187)
	UK	ELISA	*Serology: 0/1 B. abortus*	(170)
	Canada	Culture	*Culture: 66.7% (4/6) from lymph nodes - novel Bartonella* sp.	(224)
		ELISA, SAT, SAT-EDTA, RBT, CFT	*Serology: 10% (5/49) B. abortus*	(209)
	Canada	ELISA	*Serology: 4% (10/248)*	(188)
Spotted seal (*Phoca largha)*	AK, USA	ELISA	*Serology: 18.8% (16/85)*	(199)
	Japan	ELISA, *Western blot*	*Serology: 66% (27/41) B. abortus; 32% (13/41) B. canis*	(202)
Baikal seal *(Phoca sibirica)*	Russia	RBPT, SAT, iELISA	*Serology: 0/45*	(216)
Harbor seal *(Phoca vitulina)*	The Netherlands	Culture, RBT, SAT, ELISA, qPCR (*IS711*)	*Serology: RBT 53% (21/40), SAT 40% (16/40), ELISA 60% (24/40); qPCR (tissue) 77% (30/39); culture: 31% (12/39)*	(212)
	AK, USA	ELISA	*Serology: 24.6% (276/1,122)*	(199)
	Germany	Culture	*Culture: 17% (0–25% in different years)*	(225)
	UK	RBT, ELISA, culture	*Serology: RBT: 15.9% B. abortus, cELISA: 25.4% (n=343) B.melitensis; culture: 16% (24/150)*	(226)
	AK, USA	cELISA	*Serology: 52% of 152 adults, 53% of 110 subadults, 77% of 93 yearlings, 26% pups <5 mo old (n=554), from 29% to 64% in different populations; marine Brucella* sp.	(227)
	AK, USA	Card, plate, ELISA, RSAT	*Serology: 16–74% (plate B. abortus: 74%; card B. abortus: 16%; cELISA marine Brucella: 37%; ELISA B. ovis and RSAT: 0)*	(228)
	Germany	Culture	*Culture: 11% (47/426) from lungs and lung lymph nodes*	(214)
	WA, USA	Serology, PCR, culture	*Serology: 7% pups, 8% adults, 34% subadults, 54% weaned pups/yearlings*	(229)
	USA	Card, BAPA, RIV, culture	*Serology: 14% (3/21) B. abortus; culture: 2/4 from lungs and lymph nodes*	(223)
	UK	Culture	*Culture: from 11 animals from lung, spleen, lymph nodes*	(208)
	Canada, USA	ELISA	*Serology: 12.9% (21/163) (US Atlantic coast 50% (4/8)) B. abortus*	(187)
	UK	ELISA, culture	*Serology: 49% (147/297) B. abortus; culture: 10/117*	(170)
	UK	ELISA	*Serology: 8% (1/12) B. melitensis*	(215)
	UK	Culture	*Culture: 14.3% (4/28)*	(171)
	UK	Culture, RBPT, SAT, ELISA	*Culture: 4 (2 spleens, 2 lymph nodes) Brucella* spp.; *Serology: RBPT: 49% (69/140), SAT: 18% (25/140), iELISA: 32% (45/140)*	(216, 230)
Western Pacific harbor seal *(Phoca vitulina stejnegeri)*	Japan	ELISA	*Serology: 24% (13/55) B. abortus; 11% (6/55) B. canis*	(202)
Pacific harbor seal *(Phoca vitulina richardsi)*	WA, USA	BAPA, BBA, qPCR (*bcsp31*), CF, RIV, culture	*Culture: 17.7% (18/102); qPCR: 1.2% (4/336); Serology: 7.6% (100/1314 live healthy seals)*	(231)
	AK, USA	ELISA	*Serology: 46% (46/100) Brucella* spp.	(232)
		BAPA, BBA, CF, culture	*BAPA, BBA, CF: 1/1; Culture: 1/1 from lung and lymph nodes. Brucella* spp. *in lungworms.*	(233)

AGID, Agar gel immunodiffusion; BACA, buffered acidified card antigen; BAPA, buffered acidified plate antigen; BBA, buffered Brucella antigen test; BMAT, Brucella microagglutination test; BPAT, Buffered antigen plate agglutination test; CF, Complement fixation; CFT, the cold complement fixation tube test; RAS, Rapid slide agglutination; RBPT, Rose Bengal Plate test; RIV, the rivanol precipitation test; RPA, Rapid Plate Agglutination; RSA, Rapid Slide Agglutination; SAT, standard agglutination tube test; SAW, Slow Agglutination of Wright; SCA, Standard Card Agglutination test; SMTA, salt 2-mercaptoethanol tube agglutination test; SPT, Standard plate test; STT, Standard test tube.

spleen of one coyote, and *B. henselae* DNA was amplified from the mitral valve of another coyote. By sequence analyses, four coyotes were infected with *B. vinsonii* subsp. *berkhoffii* genotype I, three with genotype II, and one with genotype III (87).

Two species of *Bartonella*, a novel *Bartonella clarridgeiae*-like bacterium and *B. vinsonii* subsp. *berkhoffii*, were isolated from rural dogs and gray foxes in northern California (58). Two *B. henselae* sequences detected in the spleen of raccoon dogs in Korea matched the strain Houston-1 and by ITS sequences were 99.8% similar to a strain found in dogs in China (94). Northern and Southern sea otters were found IFA positive for *B. washoensis* (107, 108). The authors also detected *B. bacilliformis* by PCR in heart valves of both species. A strain close to *B. washoensis* was detected by PCR in Japanese marten (95). Chinnadurai et al. (109) detected a novel strain in river otters by PCR with a sequence matched a strain previously described in Southern flying squirrel. In the Netherlands, harbor seals were found to carry a strain 97% similar to *B. grahamii* (112).

BRUCELLA SPECIES IDENTIFIED IN WILD CARNIVORES

There are no reports on identification of *Brucella* species by culture or by sequence analysis in animals belonging to Felidae, Herpestidae, and Hyaenidae families. Except for one bobcat that had antibodies against *Br. canis* (115), the other few seropositive Feliformia animals had antibodies against *Br. abortus* (116, 132). Since the authors did not use specific tests that identify rough *Brucella* species, they were not able to find antibodies.

In contrast, multiple *Brucella* species can infect Caniformia animals. *Brucella* species identified by culture or PCR/sequencing in terrestrial carnivores included *Br. canis* in coyotes (137); *Br. abortus* in wolves, red foxes, gray foxes, pampas gray foxes, and raccoons (123, 126, 154, 177); *Br. suis* biovar 4 in wolves, arctic foxes, and red foxes (11, 157, 164); *Br. microti* and *Br. vulpis* in red foxes (159, 161). One red fox species, *V. vulpes,* can carry four different *Brucella* species (*Br. abortus, Br. vulpis, Br. microti,* and *Br. canis*) (115, 125, 159–161). All isolates obtained from arctic foxes were identified as *Br. suis* biovar 4 (11, 13, 48). This is not surprising as reindeer are common hosts of *Br. suis* biovar 4, and arctic foxes often scavenge dead reindeer.

Various aquatic carnivores carry a different species, *Brucella pinnipedialis.* It was identified in the harbor seal (*Phoca vitulina*), the ringed seal (*P. hispida*), the harp seal (*Pagophilus groenlandicus*), the gray seal (*Halichoerus grypus*), the hooded seal (*Cystophora cristata*), Asian sea otter (*Enhydra lutris*), and European river otter (*Lutra lutra*) (168, 171, 207, 208, 214). Characterization of the isolates belonging to this species indicated that *Br. pinipedialis* may contain different biovars (208).

BARTONELLA AND *BRUCELLA* INFECTIONS IN WILD CARNIVORES BY FAMILY

Family Felidae

Genus *Panthera*

Bartonella infection was reported in three big cats species: African lion (*P. leo*), jaguar (*P. onca*), and Far Eastern leopard (*P. pardus orientalis*). *B. henselae* and *B. koehlerae* subsp. *koehlerae* were cultured from the blood of three (5.2%) of 58 lions from Kruger National Park in South Africa (61, 62). The level of bacteremia in the culture-positive lions varied from 35 to 2,000 bacteria per 1 ml of blood. *Bartonella* culture- and antibody-positive lions were found among semi-captive lions from three ranches in South Africa (71). Interestingly, all studied lions from Zambia and Zimbabwe were negative for *Bartonella* by culture and PCR (63, 70). A wild-caught jaguar in Brazil, which was maintained in captivity for only a week, was found *B. henselae* positive (236). This finding led the authors to believe that the animal had been infected in the wild. In the Russian Far East, wild Amur tigers (*P. tigris altaica*) tested negative for antibodies to *B. henselae* (72, 73), but two of five Far Eastern leopards from that area had antibodies against *B. henselae* (73).

Limited information exists about *Brucella* in the wild cats of the genus *Panthera.* During the investigation of *Brucella* infection in the human, livestock and wildlife interface in the Katavi-Rukwa ecosystem in Tanzania, Assenga et al. (130) found one of the two tested lions serologically positive for *Brucella* at a titer 1:200 by three different tests (RBPT, BAPA, and Riv.T). In a 1968 study in Tanzania, Sachs et al. (116) found two of 13 lions had antibodies to *Brucella* species by tube agglutination test. De Vos and Van Niekerk (131) were not able to detect *Brucella* antibodies in four lions from the Kruger National Park, South Africa. Furtado et al. (132) tested serum samples from 31 free-ranging jaguars (*P. onca*) from Brazil using *Br. abortus* as antigen and reported antibodies in one jaguar.

Genus *Puma*

Two *Bartonella* species were cultured from mountain lions (*P. concolor*) (55). *Bartonella* antibodies were found in mountain lions from Arizona, California, Idaho, Oregon, Texas, Wyoming, and Florida in the U.S. (17, 68, 69, 78, 79). No *Bartonella* DNA was detected in spleen samples of three mountain lions from Colorado, U.S. (77). *B. henselae* antibodies were found in pumas from Bolivia, Peru, and Venezuela (17). Filoni et al. (60) reported 16 out of 18 pumas serologically positive to *B. henselae* in Brazil. *B. henselae* DNA was detected in lung tissues of three Florida pumas with the first and only up to date reported association of *B. henselae* infection with a fatal disease syndrome of necrotizing interstitial pneumonia and suppurative myocarditis in pumas (76).

All 24 free-ranging Florida panthers (*P. c. coryi*) were seronegative for *Brucella* (134). Reports of *Brucella* in populations of pumas from elsewhere in the Americas were unavailable.

Genus *Acinonyx*

The cheetah (*A. jubatus*) is only member of its genus. Kelly et al. (63) reported isolation of *B. henselae* genotype II from an African pet cheetah from Zimbabwe. In 2016, Molia and colleagues (61) isolated *Bartonella* bacteria from blood of 5.9% (1/17) Namibian cheetahs, and the cheetah was infected with a previously unidentified *Bartonella* strain. The Namibian cheetah strain was close but distinct from isolates from North American wild felids and clustered between *B. henselae* and *B. koehlerae;* it was claimed to be a new subspecies of *B. koehlerae* (61). The same study documented that 23% of the 73 animals were positive for *Bartonella* DNA by PCR and 31% (23/74) of cheetahs had antibodies to *B. henselae*. No reports on *Brucella* infections in the cheetah were found.

Genus *Lynx*

Those are medium-sized cats represented by four species: Canadian lynx (*Lynx canadensis*), Eurasian lynx (*L. lynx*), Iberian lynx (*L. pardinus*), and bobcat (*L. rufus*). Chomel et al. (55) isolated two *Bartonella* species (*B. henselae* and *B. koehlerae* subsp. *bothieri*) from bobcats in California, U.S. A high prevalence of *Bartonella* antibodies (22.4–74.0%) was reported in bobcats from California, Colorado, Florida, Nevada, and Oregon in the U.S. and from Mexico (17, 56, 68, 69). In northwestern Mexico, a *Bartonella* genotype was found in a flea *P. simulans* collected from a bobcat, but not in the blood of that animal (67). *B. henselae* DNA was found in 16 of 75 (21.3%) blood samples of Iberian lynx from southern Spain (57).

Antibodies against *Brucella* species in bobcats were reported in two studies: *Br. abortus* at 6.6% (5/75) in California (127) and *Br. canis* at 33% (1/3) in Texas (115). Serological investigations of bobcats from Alabama, Texas, and Utah in the U.S. did not result in identification of antibodies to *Brucella* (126, 128, 129). Tessaro

(125) reported the absence of *Brucella* bacteria and anibodies in Canadian lynx.

Genus *Leopardus*

These are small spotted cats mostly native to Middle and South America. Representatives are the ocelot (*L. pardalis*), the little spotted cat (*L. tigrinus),* Geoffroy's cat (*L. geoffroyi*), and the margay *(L. wiedii*). Antibodies to *B. henselae* were reported in the ocelot (1/1) and the little spotted cat (2/2) in Brazil (60). A *Bartonella* sequence similar to *B. koehlerae* and *B. henselae* was detected in the captive margay in Brazil (235). The animal was born in the wild and lived in captivity prior to sampling, thus it is not possible to ascertain if the infection was acquired in the wild or in captivity. The authors claimed this animal exhibited clinical signs of bartonellosis: episodes of accentuated weight loss, dullness, dehydration, and anemia (235). The main reason why we have included the case of captive margay into our review is that the identified strain was different from all strains described in domestic and wild felines.

Genus *Prionailurus*

This is a genus of small spotted wild cats native to Asia. The genus includes the Iriomote cat (*P. iriomotensis*) and the Tsushima leopard cat (*P. bengalensis euptilura*), both endangered in Japan. A molecular epidemiologic survey in Japan resulted in identification of *B. henselae* in 6% (2/33) of Iriomote leopard cats and *B. clarridgeiae* in 8% (1/13) of Tsushima leopard cats (75). In the following study, four ixodid ticks collected from Tsushima leopard cats were PCR positive for *B. henselae* (74).

Genus *Felis*

The European wildcat (*F. silvestris silvestris*) is a subspecies of the same species that includes domestic cats (*F. s. catus*). This species is found in forest habitats of Europe. There are two reports of the presence of *Bartonella* in wildcats from Spain. First, Márquez et al. (65) identified *B. alsatica*, strain associated with rabbits, in a flea *Spilopsyllus cuniculi* collected from a wildcat in Spain. Then, Gerrikagoitia et al. (64) detected *B. henselae* DNA in a carcass of a wildcat. A study of feral cats in the U.S. state of Georgia by Hwang and Gottdenker (59) also reported that 48% of feral cats were PCR positive for three *Bartonella* species: *B. henselae*, *B. koehlerae*, and *B. clarridgeiae*.

Family Viverridae

The most common species are civets and genets widely distributed in South and Southeast Asia, Africa, and Southern Europe. The first evidence suggesting that civets can host *Bartonella* came from a description of a human cat scratch disease case reported in 2001 in Japan. In the case, a patient scratched by a masked palm civet (*Paguma larvata*) developed fever and inguinal lymphadenopathy with a high antibody titer (1:1,024) to *B. henselae* (237). Later, Sato et al. (82) cultured *B. henselae* from blood of one of 50 masked palm civets collected in Chiba Prefecture of Japan. The level of bacteremia was high (7,000 bacteria per 1 mL of blood). Importantly, the multi-locus sequence type detected from the isolated strain revealed a unique genotype. Though the prevalence of *Bartonella* in cats in Chiba prefecture was 5%, the same genotype had never been found in any *B. henselae* strains from cats from the same and other prefectures (82). *Bartonella* DNA was detected in another Viverridae species, the common genet (*Genetta genetta*). Conducting molecular detection of vector-borne pathogens in wild carnivores in natural parks and adjacent residential areas in Barcelona, Spain, Millán et al. (83) identified *B. clarridgeiae* in tissues of two of 34 (6%) common genets, but ticks collected from genets were free of *Bartonella* DNA. In another study conducted in Northern Spain (Basque County), Gerrikagoitia et al. (64) did not detect *Bartonella* DNA in 13 common genets tested. Márquez et al. (65) also found no *Bartonella* DNA in 18 fleas *S. cuniculi* collected from 10 common genets in Andalusia, Spain.

Reports of *Brucella* testing among viverrids are nearly nonexistent. No *Brucella* antibodies were found in two common genets (*G. genetta*) and three Cape genets (*G. tigrina*) from eastern Africa tested by tube agglutination test (116, 136).

Family Herpestidae

Mongooses is the common name for the weasel-like small carnivores that live in southern Asia, Africa, and southern Europe, and are introduced to some other areas. We have information about *Bartonella* in one species of this genus—the small Asian mongoose (*Herpestes javanicus*). Sato et al. (82) isolated *B. henselae* from 15.9% (10/63) of small Asian mongooses from Okinawa prefecture, Japan. Based on multi-locus sequence analysis, they identified four types of *B. henselae* strains cultured from mongooses (82). Jaffe et al. (81) tested small Asian mongooses in Grenada and found 32% (54/167) of the animals IFA positive and 35% (18/51) PCR positive for *B. henselae*. The only additional report of investigation of mongooses was from testing a single Egyptian mongoose (*Herpestes ichneumon)* in Algeria and the PCR test was negative (80).

There is a report of antibodies against *Br. abortus* in one white-tailed mongoose (*Ichneumia albicauda*) (33%, 1/3) and one banded mongoose (*Mungos mungo*) (100%, 1/1) in Tanzania (116).

Family Hyaenidae

The family contains four species of hyenas and phylogenetically belongs to the suborder *Feliformia* despite the dog-like appearance of these animals. The only available report on testing hyenas for *Bartonella* is from a molecular survey of 19 spotted hyenas (*Crocuta crocuta*) from two sites in Zambia with no positive results (70).

Serological observation of 15 spotted hyenas from Tanzania resulted in detection of antibodies against *Brucella* in four out of 15 (27%) animals (116). In the prior study, Sachs and Staak (135) found *Brucella* species exposure in two out of four hyenas in Tanzania. Another serological study did not detect *Brucella* antibodies in two spotted hyenas from the Kruger National Park in South Africa (131).

Family Canidae

Genus *Canis*

Multiple wild species, including coyotes, jackals, and wolves belong to this genus. The golden jackal (*C. aureus*) is a species experiencing rapid geographic expansion with significant public health impacts (238). Of 57 golden jackals sampled from four sites in Iraq, seven (12.3%) were PCR positive for *Candidatus* B. merieuxii and one (2%) for *B. vinsonii* subsp. *berkhoffii* (86). In Israel, Marciano et al. (85) found nine out of 70 (13%) golden jackals PCR positive for *Bartonella* species: 5/9 *B. rochalimae*, 3/9 close to *Candidatus* B. merieuxii, and 1/9 between *B. vinsonii* subsp. *berkhoffii* and *Candidatus* B. merieuxii. A search for *Bartonella* in coyotes (*C. latrans*) from California and Colorado in the U.S. and from Mexico demonstrated a high prevalence of up to 89% by IFA, 42% by culture, and 28% by PCR (58, 67, 77, 87–91). There is one report of PCR detection of *Bartonella* DNA in a wolf (*C. lupus*) from northern Spain (64).

Most reports of *Brucella* infections in canids are based on detection of antibodies. Serologically positive coyotes were identified from California and Texas, U.S. (115, 117, 127, 128, 142). In wolves, evidence of *Brucella* infections also included *Brucella* isolations in Canada and Russia (13, 125). *Brucella* was found in two jackal species: 1.9% (4/216) of golden jackals (*C. aureus*) in Serbia were positive for *Br. canis* by PCR (137) and 43% (3/7) of black-backed jackals (*C. mesomelas*) in Tanzania were seropositive for *Br. abortus* by tube agglutination test (116).

Genus *Vulpes*

There are more reports on detection of *Bartonella* in red foxes (*V. vulpes*) than in any other species of wild carnivores. *Bartonella* DNA was identified in red fox tissues from Australia, Austria, France, Israel, Spain, and U.S. (64, 77, 83, 85, 88, 103, 105). Most sequences were identified as *B. vinsonii* subsp. *berkhoffii* and *B. rochalimae.* Out of two red foxes positive for *Bartonella* DNA in Israel, one harbored DNA sequences that were 100% identical to *B. rochalimae* and the other was positive for *Candidatus* B. merieuxii (85). Hodžić et al. (104) did not detect *Bartonella* DNA in 119 fox spleen samples from Bosnia and Herzegovina. Blood samples from 39 red foxes from Iraq were also negative for *Bartonella* by PCR; however, 12.8% of these foxes were serologically positive for *Bartonella* antibodies (86). Mascarelli et al. (100) detected *B. henselae* DNA in three out of 20 tested arctic foxes (*V. lagopus*) from Canada and López-Pérez et al. (67) identified *B. rochalimae* DNA in two out of 15 kit foxes (*V. macrotis*) tested.

There are several reports about screening of ectoparasites from red foxes. DNA of a *Bartonella* strain, closely related to *B. rochalimae*, was found in fleas (*Pulex irritans*) from red foxes in Andalusia, Spain (65). PCR tests detected *B. clarridgeiae* and *B. henselae* in 20/34 and 4/34 fleas (*Ctenocephalides felis*), respectively, from red foxes in Australia, where it is an introduced species (105). Sréter-Lancz et al. (106) found *Bartonella* DNA in 4.2% (4/95) pools of fleas (*P. irritans*) from red foxes in Hungary, but all ticks from foxes were negative.

Similarly, there are numerous reports of *Brucella* infections in red foxes in Austria, Canada, Ireland, Russia, the U.S., and the UK (115, 125, 159–161). Tessaro (125) cultured *Br. abortus* from red foxes in Canada. Morton (48) cultured *Br. suis* biovar 4 from three out of 38 red foxes from Alaska. Scholtz et al. (161) cultured *Br. microti* and the proposed novel species *Br. vulpis* from red foxes in Austria in 2016. *Br. suis* biovar 4 cultures were obtained from arctic foxes from Alaska and Russia (11, 13, 48). McCue and O'Farrell (158) conducted a serological survey of San Joaquin kit foxes in California, U.S. and reported antibodies to *Br. abortus* in 8% in 1981–1982 and 3% in 1984 and to *Br. canis* in 14% in 1981–1982 and none in 1984.

Genus *Cerdocyon*

Investigators tested another fox species, the crab-eating fox (*Cerdocyon thous*), in Brazil and found *B. rochalimae* DNA in all nine *P. irritans* fleas collected from one animal (92). In another study by De Sousa et al. (66), none of the 78 sampled crab-eating foxes showed presence of *Bartonella* DNA in blood samples by qPCR.

Genus *Urocyon*

This genus contains two species of Western Hemisphere foxes: the gray fox (*U. cinereoargenteus*) and closely related island fox (*U. littoralis*), which is a dwarf cousin of the gray fox (239).There is a comprehensive study of *Bartonella* in gray foxes in northern California, U.S., conducted by Henn et al. (58). A novel *B. clarridgeiae*-like bacterium was isolated from 22 (42%) of 53 gray foxes and *B. vinsonii* subsp. *berkhoffii* from five gray foxes (9.4%). Serology showed that 48 gray foxes (89%) had detectable antibodies against *Bartonella*. The authors made the conclusion that the high prevalence of bacteremia and seroreactivity in gray foxes suggests that they may act as a reservoir species for the *B. clarridgeiae*-like species in this region. In another study of gray foxes in northern California, 14 (64%) of 22 foxes were infected with *Bartonella* species at one or more of the capture dates (97). Fleas collected from gray foxes in the study were identified as *P. simulans*, and 39% of the fleas were PCR positive for *Bartonella*, with *B. rochalimae* and *B. vinsonii* subsp. *berkhoffii* identified in 81% and 19% of the PCR positive fleas, respectively.

A serological survey of 132 gray foxes from Texas, U.S., demonstrated an antibody prevalence of 50% (66/132), with 22 (33.3%) individuals seropositive for *B. clarridgeiae*, eight (12.2%) for *B. vinsonii* subsp. *berkhoffii*, and 36 (54.5%) for both *B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii* (96). In gray foxes from Colorado, U.S., and northern Mexico *Bartonella* DNA was not detected (67, 77). Serological survey of the endangered island foxes (*U. littoralis*) conducted on several islands near the Californian coast by Namekata et al. (99) demonstrated a wide range of seroprevalence for *B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii* from 0% on San Nicolas Island to 86% on Santa Cruz Island. The following serological survey of 51 island foxes on Santa Rosa Island identified the overall antibody prevalence of 62.7% with 16 (31.4%) foxes seropositive for *B. clarridgeiae* only, five (9.8%) for *B. vinsonii* subsp. *berkhoffii* only, and 11 (21.6%) for both antigens (98). Importantly, *B. vinsonii* subsp. *berkhoffii* was isolated from six (11.8%) foxes using blood culture medium. All of the isolated *B. vinsonii* subsp. *berkhoffii* belonged to type III, the same type found in mainland gray foxes (98).

A culture of *Br. abortus* was obtained from one gray fox (*U. cinereoargenteus*) from Alabama, U.S. (126) while there were no positive results in foxes of this species in Arkansas, Florida, and South Carolina, U.S. (115, 156).

Genus *Lycalopex*

Several investigations of the South American foxes for *Brucella* infection have been published, including those investigating the pampas gray fox (*L. gymnocercus*) and Patagonian gray fox (*L. griseus*). Szyfres and González Tomé (154) found evidence of *Brucella* in both species from Argentina and isolated *B. abortus* biovar 1 from a pampas gray fox.

Genus *Nyctereutes*

The DNA identified as *B. henselae* was detected in spleens of two out of 142 raccoon dogs (*N. procyonoides*) in Korea, but not in any of 51 blood samples tested (94).

Family Ursidae

We found research on *Bartonella* and *Brucella* in three bear species, namely black bear (*U. americanus*), brown bear (*U. arctos*), and polar bear (*U. maritimus*). *Bartonella* DNA was not detected in seven black bears from Colorado, U.S. (77). All other research was focused on *Brucella* in bears (119, 147, 148, 179, 180, 182–185). Despite high seroprevalence levels for *Br. abortus* antibodies in all investigated bear species, we could not find any report on successful isolation of *Brucella* from these animals. Serological tests of 61 black bears for *Br. canis* by Bronson et al. (178) were negative.

Family Mephitidae

Twelve skunks of two species, the hooded skunk (*Mephitis macroura*) and the striped skunk (*M. mephitis*), from Colorado, U.S., and Mexico were found infected with *B. rochalimae* and one skunk from Mexico was infected with *B. vinsonii* subsp. *berkhoffii* (67, 77).

Antibodies against *B. abortus* were found in 8.7% of striped skunks and 3.9% of western spotted skunks (*Spilogale gracilis*) from California, U.S. (127).

Family Procyonidae

The common raccoon (*Procyon lotor*) has a natural range from southern Canada to Panama. Of 37 raccoons trapped on St. Simon Island in Georgia, U.S., 12 were positive for *B. henselae* and one for *B. koehlerae* (59). Interestingly, raccoons from the western regions of the U.S. carried different species of *Bartonella*. Henn et al. (88) isolated *B. rochalimae* from 11 of 42 raccoons from California, and Bai et al. (77) found 11 of 186 raccoons from Colorado PCR positive for *B. rochalimae* and three for *B. vinsinii* subsp. *berkhoffii*. All 977 raccoons from Japan, where it is an introduced species, were PCR negative for *Bartonella* (95).

Two *Brucella* strains cultured from raccoons from Alabama were identified as *Br. abortus* biovar 1 (126, 177). Raccoons seropositive to *Brucella* species were found in California, Alabama, Florida, and Texas in the U.S. (115, 126–128). None of 63 raccoons from Nebraska, U.S., had antibodies to *Br. canis* (139). In South Korea, *Brucella* DNA was found in blood (1/9) and tissues (2/5) of introduced raccoons (172). Three (8.8%) of 34 brown-nosed coatis (*Nasua nasua*), which also belong to family Procyonidae, were serologically positive for *Brucella* in the Brazilian Pantanal (149).

Family Mustelidae

Mustelidae is the largest family in the order Carnivora. Many terrestrial species of this genus were tested for *Bartonella,* including the beech marten (*Martes foina*), pine marten (*M. martes*), Japanese marten (*M. melampus*), American badger (*Taxidea taxus*), stoat (*Mustela erminea*), Japanese weasel (*M. itatsi*), least weasel (*M. nivalis*), Siberian weasel (*M. sibirica*), American mink (*M. vison*), European polecat (*M. putorius*), and ferret (*M. putorius furo*) (**Table 1**). However, out of 16 mustelid species tested for *Bartonella* DNA, only two cultures were obtained: one from a Japanese badger (*Meles anakuma*) and another from a Japanese marten (95). The isolate from the marten was close to *Bartonella washoensis*, a species typically found in squirrels, suggesting that it could have potentially "jumped" from a squirrel to its natural predator. The isolate from the Japanese badger was unique, with the closest match being to *B. clarridgeiae* and *B. rochalimae* (95). *Bartonella clarridgeiae* or related sequences were also detected in a beech marten and in European badgers, all from Spain (64, 83).

In North Carolina, U.S., Chinnadurai et al. (109) revealed a novel *Bartonella* species in 19 (29%) of 65 tested river otters (*Lontra canadensis*). *Bartonella* infection was detected in 45% (23/51) and 10% (3/30) of heart valves of northern and southern sea otters (*Enhydra lutris kenyoni* and *E. l. nereis*), respectively, by PCR (108). Analysis of the *Bartonella* ITS region identified two *Bartonella* species in those animals: a novel species closely related to *Bartonella washoensis* and *Candidatus* B. volans, whereas another genotype was molecularly identical to *B. henselae.* Sera from 50% of necropsied and 34% of presumed healthy, live-captured northern sea otters and in 16% of necropsied southern sea otters contained antibodies against *Bartonella* species (107).

Antibodies against *Brucella* species were detected in the American badger (*Taxidea taxus*), American mink (*Neovison vison*), European mink (*Mustela lutreola*), Eurasian otter (*Lutra lutra*), wolverine *(Gulo gulo)*, and northern, southern and Asian sea otters (*Enhydra lutris keyoni, lutris, nereis*) from Europe, Asia, North and South Americas (11, 124, 125, 127, 174). We found only one report of successful culturing of *Brucella* (*Br. abortus*) from terrestrial mustelids (farmed European mink) and only one report of PCR detection of *Brucella* DNA in tissues of Asian badger (*Meles leucurus*) (172, 240). Similar to *Bartonella*, sea otters carry different species of *Brucella* than terrestrial mustelids. Investigating rectal swab samples of Asian sea otters (*E. l. lutris)* from Russia (168) found DNA of three *Brucella* species (*Br. abortus, Br. melitensis,* and *Br. pinnipedialis*). Miller et al. (169) isolated marine *Brucella* from a southern sea otter (*E. l. nereis*) with osteolytic lesions that was stranded on the central California coast. Antibodies to *Brucella* were detected in Northern sea otters (*E. l. keyoni*) from Alaska in the U.S. and Russia (120).

Families Phocidae, Otariidae, and Odobenodae

There is only one report on the identification of *Bartonella* in any of the pinnipeds, including walruses, eared seals, and true seals. Morick et al. (112) tested spleen samples and seal lice (*Echinophtirius horridus*) collected from seven harbor seals (*Phoca vitulina*). One spleen of 48 tissue samples and one of six lice pools were positive. The *Bartonella* species identified in the spleen and lice were found to be identical to each other by two genetic loci. One genetic marker identified the genotype as *B. henselae,* while another marker indicated 97% sequence similarity with *B. grahamii*.

In contrast to *Bartonella*, there is abundant evidence of *Brucella* infections in various species of the clade Pinnipedia. In family Phocidae (true seals), cultures of *Brucella* species were obtained from hooded seals (*Cystophora cristata)*, gray seals (*Halichoerus grypus*), ringed seal *(Phoca hispida)*, harp seal (*Pagophilus groenlandicus*), and harbor seal *(Ph. vitulina)* (170, 171, 206, 208, 214, 223, 224, 226, 231, 233). All identified cultures from true seals were *Br. pinnipedialis*. Serological evidence of *Brucella* was reported from investigation of even more species of true seals, including the bearded seal (*Erignathus barbatus*), ribbon seal (*Histriophoca fasciata)*, leopard seal (*Hydrurga leptonyx*), Weddell seal (*Leptonychotes weddellii*), crab-eater seal (*Lobodon carcinophaga*), southern elephant seal (*Mirounga leonina*), Hawaiian monk seal (*Neomonachus schauinslandi*), Ross seal (*Ommatophoca rossii*), and several species of the genus *Phoca*.

In the family Odobenidae (walruses), Nielsen et al. reported serological prevalence of 12% (7/59) in 1996 and 3% (5/170) in 2001 in Atlantic walrus (*Odobenus rosmarus rosmarus*) from Canada; however serological tests of 40 Pacific walruses (*O. r. divergens*) from Alaska by Calle et al. (211) showed no antibodies to *Brucella* species

There are multiple reports of *Brucella* antibodies in fur seals and sea lions of the family Otariidae—nine species of the genera (*Arctocephalus, Callorhinus, Eumetopias, Neophoca, Phocarctos,* and *Zalophus*) (**Table 2**).

DIFFERENCES IN DISTRIBUTION OF *BARTONELLA* AND *BRUCELLA* SPECIES IN WILD CARNIVORES

Carnivores have regular exposure to both *Bartonella* and *Brucella* bacteria through predation on pathogen hosts, scavenging, and arthropod vectors. As with plague caused by *Yersinia pestis* (241), testing one carnivore for *Bartonella* and *Brucella* species could be equivalent to sampling a large number of its prey animals and give an idea of the epidemiological situation in the local environment. Overall, both *Bartonella* and *Brucella* are common in wildlife. Our review demonstrated numerous reports of infections caused by bacteria of both taxa in wild carnivores. We analyzed over 170 *Bartonella* and *Brucella* studies covering 109 species and subspecies of carnivores (**Table 3**). Eighty-four species of carnivores were tested for *Brucella* and 79% of these species were found positive by serological, bacteriological, or molecular methods. Out of 51 species examined in *Bartonella* studies, 71% tested positive.

Although no species of wild carnivores were tested for both pathogens in a single study, 26 species were tested for both pathogens in different studies. Of those, 15 (58%) species were positive for both *Bartonella* and *Brucella* (among them bobcat, African lion, golden jackal, coyote, wolf, foxes, striped skunk, sea otters, raccoon, and harbor seal), meaning these carnivores can harbor either pathogen or potentially both. We know that other mammalian groups [bats for example, (242)] can be co-infected with *Bartonella* and *Brucella* species, and we speculate that this is also possible in carnivores, a hypothesis that definitely needs more investigation.

The most commonly identified *Bartonella* species was *B. henselae*, which was found in at least 23 species of wild carnivores, followed by *B. rochalimae* in 12, *B. clarridgeiae* in ten, and *B. vinsinii* subsp. *berkhofii* in seven species. Similarly, *Br. abortus* led the list of *Brucella* species, being identified in 36 terrestrial carnivore species, followed by *Br. canis* in eight. However, most of the reports are based on serology that cannot reliably discriminate these species until there are bacteriological data or sequences of PCR amplicons. *Br. pinnipedialis* is prevalent in marine carnivores, and some of the early reports of antibodies to *Br. abortus* in marine animals probably can be attributed to *Br. pinnipedialis* as well.

The analysis revealed some striking differences in distributions of these infectious agents in wild populations belonging to different carnivore families. One of the evident differences is abundance of several species of *Bartonella* practically in every explored species of wild felids. In contrast, very few reports of *Brucella* in the same species are available and those are limited to detection of antibodies that may indicate an exposure to the agent rather than direct involvement of these animals in the circulation of *Brucella*. At the same time, we could not find any report of *Bartonella* in bears while the presence of *Brucella* in these animals was well documented. An even more evident difference was found in marine carnivores, such as seals and sea lions, with practically every species reported infected with a specific species of *Brucella* (*Br. pinnipedialis*). In contrast, there is only one report of detection of *Bartonella* DNA in one tissue sample of a seal and there is no evidence of a *Bartonella* strain specific to marine mammals. A comparison with other marine mammals, such as dolphins, porpoises, and whales, which were not the subjects of our paper, also indicated a presence of specific *Brucella* species in blood of these animals, known as *Brucella ceti.* Whereas, the cat pathogen *B. henselae* was found in cetaceans, albeit less commonly than species of *Brucella* (243, 244).

Prevalence and the spectrum of bacterial species present depends on a potential exchange of bacteria between domestic and wild terrestrial carnivores. Wild carnivores are often infected with the same pathogens as their domesticated relatives (cats and dogs) though the risk of exposure varies widely because of differences in biology, distribution, and historical interactions. Confirmation of the identity of the bacterial species, however, remains critical for making such a statement regarding host specificity. Using a rapid test for differentiation of *Bartonella*

TABLE 3 | *Bartonella* and *Brucella* studies in wild carnivores by species.

Host species	+/–	*Bartonella* ref.	*Brucella* ref.	+/–
SUBORDER FELIFORMIA				
Family Felidae				
Cheetah (*Acinonyx jubatus*)	+	(61–63)		
Wildcat *Felis silvestris*)	+	(64, 65)		
			(124)	–
Ocelot (*Leopardus pardalis*)	+	(60)		
	–	(66)		
Little spotted cat (*Leopardus tigrinus*)	+	(60)		
Iberian lynx	+	(57)		
(*Lynx pardinus*)	–	(65)		
Lynx (*Lynx canadensis*)			(125)	–
Bobcat *(Lynx rufus)*	+	(17, 55, 56, 67–69)	(115, 127)	+
			(126, 128, 129)	–
African lion *(Panthera leo*)	+	(61, 62, 71)	(116, 130)	+
	–	(63, 70)	(131)	–
Jaguar (*Panthera onca*)			(132)	+
			(133)	–
Leopard (*Panthera pardus*)			(116)	–
Far Eastern leopard	+	(73)		
(*Panthera pardus orientalis*)	–	(72)		
Amur tiger (*Panthera tigris altaica*)	–	(72, 73)		
Iriomote cat (*Prionailurus bengalensis*	+	(75)		
iriomotensis)	–	(74)		
Tsushima leopard cat (*Prionailurus bengalensis)*	+	(74, 75)		
Mountain lion (*Puma concolor*)	+	(17, 55, 60, 68, 69, 76, 78, 79)		
	–	(77)	(134)	–
Family Herpestidae				
Small Indian mongoose *(Herpestes javanicus)*	+	(81, 82)		
Egyptian mongoose *(Herpeses ichneumon)*	–	(80)		
White-tailed mongoose (*Ichneumia albicauda*)			(116)	+
Banded mongoose (*Mungos mungo*)			(116)	+
Family Hyaenidae				
Spotted hyena (*Crocuta crocuta*)			(116, 135)	+
	–	(70)	(131)	–
Family Viverridae				
Common genet (*Genetta genetta*)	+	(83)		
	–	(64, 169)	(136)	–
Cape genet (*Genetta tigrina*)			(116)	–
Masked palm civet *(Paguma larvata)*	+	(82)		
SUBORDER CANIFORMIA				
Family Candiae				
Golden jackal *(Canis aureus)*	+	(85, 86)	(137)	+
	–	(80, 84)		
Coyote *(Canis latrans)*	+	(58, 67, 77, 87–91)	(115, 117, 127, 128, 142)	+
			(126, 129, 138–141, 143)	–

(Continued)

TABLE 3 | Continued

Host species	+/−	*Bartonella* ref.	*Brucella* ref.	+/−
Wolf (*Canis lupus*)	+	(64)	(11, 13, 53, 118, 125, 144–146, 148)	+
			(115, 124, 147)	−
Black-backed jackal (*Canis mesomelas*)			(116)	+
Crab-eating fox (*Cerdocyon thous*)	+	(92)	(149)	+
	−	(66)	(150–152)	−
Maned wolf (*Chrysocyon brachyurus*)			(150)	−
Patagonian gray fox (*Dusicyon griseus griseus*)			(153, 154)	+
Pampas gray fox (*Dusicyon gumnocercus antiquus*)			(153, 154)	+
Darwin's fox (*Lycalopex fulvipes*)	−	(93)		
Pampas fox (*Lycalopex gymnocercus*)			(151)	−
Hoary fox (*Lycalopex vetulus*)			(155)	+
Wild dog (*Lycalopex pictus*)			(116)	+
	−	(70)		
Raccoon dog	+	(94)		
(*Nyctereutes procyonoides*)	−	(95)		
Bat-eared fox (*Otocyon megalotis*)			(116)	−
Gray fox *(Urocyon cinereoargenteus)*	+	(58, 67, 96, 97)	(126)	+
	−	(77)	(115, 156)	−
Island fox *(Urocyon littoralis)*	+	(98, 99)		
Arctic fox (*Vulpes lagopes*)	+	(100)	(11, 13, 48, 53, 124, 157)	+
Kit fox *(Vulpes microtis)*	+	(47)		
			(129)	−
San Joaquin kit fox			(158)	+
(*Vulpes macrotis mutica)*			(152)	−
Red fox (*Vulpes vulpes*)	+	(64, 65, 77, 83, 85, 86, 88, 103, 105, 106)	(48, 115, 124, 125, 146, 148, 159–164)	+
	−	(102, 104)	(156)	−
Family Mephitidae				
Hooded skunk (*Mephitis macroura*)	+	(67)		
Striped skunk *(Mephitis mephitis)*	+	(67, 77)	(127)	+
			(115, 128, 156)	−
Western spotted skunk (*Spilogale gracilis*)			(127)	+
Family Mustelidae				
Northern sea otter (*Enhydra lutris keyoni*)	+	(107, 108)	(120, 165, 167)	+
			(166)	−
Asian sea otter *(Enhydra lutris lutris)*			(120, 168)	+
Southern sea otter (*Enhydra lutris nereis*)	+	(107, 108)	(167, 169)	+
Steppe polecat (*Mustela eversmanii)*			(124)	−
Japanese weasel *(Mustela itatsi)*	−	(95)		
European mink (*Mustela lutreola)*			(124)	+
Least weasel *(Mustela nivalis)*	−	(64, 110)	(173)	−
European polecat (*Mustela putorius*)	−	(64)		
Ferret (*Mustela putorius furo*)	−	(110)		
Siberian weasel *(Mustela sibirica)*	−	(95)		
American mink *(Mustela vison)*			(174)	+
	−	(64)		
Fisher (*Pekania pennant*)			(125)	−
American badger (*Taxidea taxus*)	+	(67, 111)	(127, 128)	+
			(129, 175)	−

(Continued)

TABLE 3 | Continued

Host species	+/−	*Bartonella* ref.	*Brucella* ref.	+/−
Family Procyonidae				
Brown-nosed coati *(Nasua nasua)*			(149)	+
	−	(66)		
Raccoon *(Procyon lotor)*	+	(59, 77, 88)	(115, 126–128, 172, 177)	+
	−	(67, 95)	(139, 156, 176)	−
Family Ursidae				
Black bear (*Ursus americanus*)			(147, 179, 180)	+
	−	(77)	(178)	−
Brown bear *(Ursus arctos)*			(179)	+
Alaska peninsula brown bear *(Ursus arctos gyas)*			(119)	+
Grizzly bear *(Ursus arctos horriblis)*			(48, 119, 144, 148, 181)	+
Kodiak brown bear *(Ursus arctos middendorffi)*			(119)	+
Marsican brown bear (*Ursus arctos marsicanus)*			(182)	+
Polar bear (*Ursus maritimus)*			(179, 183–185)	+
SUPERFAMILY PINNIPEDIA				
Family Odobenidae *(Walruses)*				
Pacific walrus (*Odobenus rosmarus divergens*)			(186)	−
Atlantic walrus (Odobenus rosmarus rosmarus)			(187, 188)	+
Family Otariidae *(fur seals and sea lions)*				
South American fur seal (*Arctocephalus australis*)			(189)	+
New Zealand fur seal *(Arctocephalus forsteri)*			(190)	−
Antarctic fur seal (*Arctocephalus gazella*)			(193–195)	+
			(191, 192)	−
Australian fur seal (*Arctocephalus pusillus doriferus*)			(122, 196, 197)	+
Guadalupe fur seal (*Arctocephalus townsendi)*			(198)	−
Northern fur seal *(Callorhinus ursinus)*			(200)	+
			(199)	−
Steller sea lion (*Eumetopias jubatus*)			(199, 201)	+
Western Steller's sea lion (*Eumetopias jubatus jubatus*)			(202)	+
Australian sea lion (*Neophoca cinerea*)			(197)	+
New Zealand sea lion (*Phocarctos hookeri*)			(203)	+
California sea lion (*Zalophus californianus)*			(121, 204)	+
Family Phocidae (True seals)				
Hooded seal *(Cystophora cristata)*			(170, 171, 187, 206–209)	+
			(205)	−
Bearded seal			(210)	+
(Erignathus barbatus)			(209, 211)	−
Ribbon seal (*Histriophoca fasciata)*			(199, 202)	+
Gray seal (*Halichoerus grypus*)			(170, 171, 187, 208, 212–216)	+
Leopard seal (*Hydrurga leptonyx*)			(197)	+
Weddell seal (*Leptonychotes weddellii)*			(191, 192, 195, 217, 219)	+
			(218)	−
Crab-eater seal (*Lobodon carcinophaga*)			(192)	+

(Continued)

TABLE 3 | Continued

Host species	+/−	*Bartonella* ref.	*Brucella* ref.	+/−
Southern elephant seal (*Mirounga leonina*)			(191)	+
			(193)	−
Hawaiian monk seal (*Neomonachus schauinslandi)*			(221, 222)	+
			(220)	−
Ross seal (*Ommatophoca rossii*)			(192)	+
Harp seal (*Pagophilus groenlandicus*)			(170, 187, 209, 223, 224)	+
Ringed seal *(Phoca hispida)*			(187, 188, 199, 205, 209, 224)	+
			(170, 207)	−
Spotted seal (*Phoca largha)*			(199, 202)	+
Baikal seal (*Phoca sibirica*)			(216)	−
Harbor seal *(Phoca vitulina)*	+	(112)	(169–171, 187, 199, 208, 212, 214–216, 223, 225, 226, 228–230)	+
Western Pacific harbor seal *(Phoca vitulina stejnegeri)*			(202)	+
Pacific harbor seal *(Phoca vitulina richardsi)*			(231–233)	+

species without sequencing amplicons, Carver et al. (114) came to the conclusion that free-ranging felids (pumas and bobcats) could be infected with *Bartonella* species that are generally considered to cross felid species barriers from domestic cats. Sequence analysis of some cultures and PCR amplicons has challenged such a conclusion. For example, in Californian mountain lions and bobcats Chomel et al. (55) found *Bartonella* species, typical for domestic cats (*B. henselae* and *B. koehlerae*); however, their detailed analysis demonstrated that these strains were sufficiently different for them to propose new subspecies of *B. koehlerae* (55). The authors who described the novel strains noted that these strains appear highly adapted to their particular species of wild cats and likely originated from a common ancestor.

There are some limitations in the analysis provided herein on the distribution of *Bartonella* and *Brucella* species in wild carnivores. The timing of samples collection for the animals listed in our review varied among studies and this factor could influence prevalence of infections. Differences in diagnostic methods used for identification can significantly affect comparison of the results. For a number of reasons, the number of *Brucella* studies relying on detection of antibodies in wild carnivores was much higher compared to the number of *Bartonella* studies in the same species that included either culturing or molecular detection. Several species of *Brucella (Br. suis, Br. abortus,* and *Br. melitensis)* are select agents and culturing of these species requires BSL-3 level capacity. Investigations of *Brucella* in wildlife started much earlier than similar investigations of *Bartonella* when DNA amplification techniques were not available. We should be carefull with interpretation of *Brucella* antibodies since available serological tests cannot identify all species of *Brucella*. There are separate tests for rough *Brucella* species (*Br. canis*) and for smooth *Brucella* species (*Br. abortus, Br. melitensis*, and *Br. suis*), and reported serology depends on the used tests.There are more described species of *Bartonella* (>35) and multiple diverse strains exist within this genus than for *Brucella* species. For many decades, the genus *Brucella* included six species, with some experts arguing that this genus is monospecific. In the past decade, new and more diverse *Brucella* species have been described (7). Recognition of the ubiquitous presence of *Brucella* in the environment will most likely continue (6). Nevertheless, reports of *Brucella* in wildlife without discrimination between species and biovars are still common, whereas future studies of *Bartonella* infections are more likely to be accompanied by proper identification down to species or subspecies level. Clearly, serological investigations are less informative for identification of bacterial species because of possible cross-reactivity between different antigens. The analysis presented in this review demonstrates the need for more information on genetic polymorphism of bacterial pathogens for the purposes of making comparison of strains from domestic and wild carnivores.

EVOLUTIONARY ASPECTS

Another issue that may influence the choice of methods for discriminating among *Bartonella* species is the effective level of association between these bacteria and their mammalian hosts, ranging from host species to host genus (245). Presumably, such a close bacteria-host association relates to the long-history of co-adaptation between *Bartonella* and their mammalian hosts and possibly arthropod vectors (245). An association of these bacteria with rodents, bats, and ruminants is described elsewhere, but analysis of the literature on *Bartonella* in wild carnivores also supports some degree of host-specificity (e.g., *B. henselae* in felids and *B. vinsonii* subsp. *berkhoffii* in canids).

A co-adaptation of *Brucella* with terrestrial wild carnivore hosts is not as straightforward as in domestic animals. A clear exception to this observation is *Br. pinnipedialis,* a species found

in true seals only. Typical for domestic dogs, *Br. canis* may be expected to be commonly shared with wild canids, such as wolves and coyotes. However, this bacterial species has not been cultured from these predators and only few serological findings are available (115, 128, 139). Noticing the absence of *Br. canis* in wolves and coyotes, Moreno (7) proposed that this bacterial species evolved in the dog's ancestor after its predation on *Br. suis* biovar 4 infected animals (e.g., caribou/raindeer). This can be also explained by lack of specific serological tests available and low yield of culture.

Recent phylogenetic reconstructions and diversification analyses of prokaryotes have led to a better understanding of patterns of bacterial macroevolution. According to the analysis of prokaryote evolution based on the 16S rRNA gene (246), the common ancestor between the *Brucella* and the *Bartonella* genera split from the common ancestor with Phyllobacteriaceae in the order Rhizobiales about 567 million years ago and diverged about 507.4 million years ago (247) around the time of the Cambrian explosion and diversification of life during the Paleozoic Era, still on the giant supercontinent Pangea. As the species of the order Rhizobiales most closely related to *Bartonella* and *Brucella* are symbionts on plant roots, we can speculate that the ancestor of the two genera may have been a plant symbiont as well.

Bartonella evolved around 134 million years ago during Early Cretaceous Period around the time the flowering plants appeared in the middle of the dinosaur era (247). Segers et al. (248) suggest that the last common ancestor of the *Bartonella* was a gut symbiont of insects that produced its own amino acids and vitamins and that the adaptation to blood-feeding insects facilitated colonization of the mammalian bloodstream. Indeed, Bartonellaceae species were identified in honeybees (248, 249) and ants (250) filling the gap between the pathogenic *Bartonella* clade and more ancient bacterial symbionts. The honeybee strains of *B. apis* form a clade basal to species of the genus *Bartonella* (249). However, the *B. apis* genomes are almost twice as large (2.6 to 2.9 Mb) as the ant symbionts, suggesting that the association with the bee is more recent or that the association is less intimate (251). The phylogenetic trees show that the ant-related bacterial clade is a sister group to bee-related clade and other mammal-related *Bartonella* species (249, 252). Ants predate bees by some 35 million years in the order Hymenoptera which is 325 million years old itself (246). We can only speculate how the *Bartonella* ancestor adapted from a plant symbiont to gut symbiont through possible consumption routes and suggest looking into other "ancient" insect orders, like Archaeognata, or the orders that have maintained connection with water in their metamorphosis, like mayflies or dragonflies; and the ones that include sap-sucking insects.

Genomic and functional similarities between *Br. suis* and organisms from the *Rhizobium Agrobacterium* group suggest that the *Brucella* may have evolved from a soil plant-associated ancestral bacteria and speculatively, it may be metabolically active outside of a mammalian host (253). According to the analysis of prokaryote evolution based on the ribosomal gene, the genus *Brucella* is much younger than *Bartonella* and diverged about 230 thousand years ago (247) during Middle Pleistocene epoch. Previously it was hypothesized that *Brucella* species diverged roughly 20 million years ago following the divergence of their bovine and goat hosts (254). However, whole-genome-based phylogeny (255) supports the ribosomal gene analysis suggesting a much younger age for *Brucella* than previously estimated. Their rooted phylogeny suggests that brucellosis in various mammalian species emerged from infected sheep roughly in the past 86,000 to 296,000 years. This analysis has also suggested that transmittal of *Brucella* from pigs to canids likely happened within the past 22,500 years from infection of wolves or other canids feeding on pigs that were themselves infected (255). So, while possible paleo-brucellosis cases in the Bronze Age and later (256) fit perfectly within the timeframe, the possibility of brucellosis in a 2.5-million-year old hominid (257) brings an exciting prospect of an ancestral *Brucella*-like strain that either became extinct or has not been detected yet.

CONCLUSION

We can only speculate that a longer period of evolution of *Bartonella* has resulted in higher diversity and better co-adaptation to specific mammalian hosts compared to *Brucella*. Asymptomatic persistence of *Bartonella* bacteria in their natural reservoir animals contrasts with the well-documented pathological manifestations of *Brucella* in host animals. The only until the present time association of *Bartonella* infection with fatal cases of clinical disease in wild carnivores was reported in Florida pumas (76). Three diseased pumas had spent time in captivity prior to being released in the wild and were found later exhibiting respiratory signs and reluctance to move. Autopsy findings included necrotizing interstitial pneumonia and suppurative myocarditis associated with *B. henselae* infection (76). There is much more information on pathology caused by *Brucella* in domestic animals than in wildlife in general and even less in wild carnivores. Describing a range of pathologies caused by *Brucella* in sea mammals, Foster et al. (208) listed sub-blubber abscesses, hepatic and splenic necrosis, macrophage infiltration in liver and spleen, possible abortion, epididymitis, and meningitis.

In spite of shared mammalian reservoirs, the difference in transmission cycles presents distinct ecological traits. While *Bartonella* species use arthropod vectors as a main mechanism for transmission between mammalian hosts, the role of arthropod vectors in transmission of *Brucella* remains disputed. In our review, we provided some data, mostly from Russian sources, which support a potential role of ticks and other arthropods in transmission of *Brucella*. Nevertheless, it is hard to argue that such means of transmission are significant, let alone dominant, in transmission of these bacteria. Commonly, wild terrestrial predators contract brucellosis through consumption of infective tissues during predation and scavenging (258). Considering potential modes of *Brucella* transmission between marine mammals, Foster et al. (208)

also included social interactions, sexual activity, maternal transmission, physical trauma, ingestion during feeding, and carriage by parasites.

We realize that our analyses create more questions than answers; the current review brought up significant parallels and differences in *Bartonella* and *Brucella* ecologies in wild carnivores and we hope it will prove to be useful for a wide range of specialists and can stimulate interest in comparing the ecologies of *Bartonella* and *Brucella* in wildlife and, at a larger scale, in investigating ecological trends of phylogenetically related zoonotic agents; benefitting epidemiological research and wildlife conservation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Diversity of *Anaplasma* and *Ehrlichia/Neoehrlichia* Agents in Terrestrial Wild Carnivores Worldwide: Implications for Human and Domestic Animal Health and Wildlife Conservation

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Recently, the incidence and awareness of tick-borne diseases in humans and animals have increased due to several factors, which in association favor the chances of contact among wild animals and their ectoparasites, domestic animals and humans. Wild and domestic carnivores are considered the primary source of tick-borne zoonotic agents to humans. Among emergent tick-borne pathogens, agents belonging to family Anaplasmataceae (Order Rickettsiales) agents stand out due their worldwide distribution and zoonotic potential. In this review we aimed to review the genetic diversity of the tick-transmitted genera *Ehrlichia*, *Anaplasma and "Candidatus* Neoehrlichia sp." in wild carnivores Caniformia (Canidae, Mustelidae and Ursidae) and Feliformia (Felidae, Hyanidae, Procyonidae and Viverridae) worldwide, discussing the implications for human and domestic animal health and wildlife conservation. Red foxes (*Vulpes vulpes*) have been identified as hosts for *Anaplasma* spp. (*A. phagocytophilum, Anaplasma ovis, A. platys*), *Ehrlichia canis* and "*Candidatus* Neoehrlichia sp." (FU98 strain) and may contribute to the maintaenance of *A. phagocytophilum* in Europe. Raccoons (*Procyon lotor*) have been reported as hosts for *E. canis, A. bovis*, "*Candidatus* Neoehrlichia lotoris" and *A. phagocytophilum*, and play a role in the maintenance of *A. phagocytophilum* in the USA. Raccoon dogs (*Nyctereutes procyonoides*) may play a role as hosts for *A. bovis* and *A. phagocytophilum*. New *Ehrlichia* and/or *Anaplasma* genotypes circulate in wild canids and felids from South America and Africa. While *Ehrlichia* sp. closely related to *E. canis* has been reported in wild felids from Brazil and Japan, *Anaplasma* sp. closely related to *A. phagocytophilum* has been detected in wild felids from Brazil and Africa. Red foxes and mustelids (otters) are exposed to *E. canis* in countries located in the Mediaterranean basin, probably as a consequence of spillover from domestic dogs. Similarly, *E. canis* occurs in procyonids in North (raccoons in USA, Spain) and South (*Nasua nasua* in Brazil) Hemispheres, in areas where *E. canis* is frequent in dogs. While "*Candidatus* Neoehrlichia lotoris" seems to be a common and specific agent of raccoons in the USA, "*Candidatus* Neoehrlichia sp." (FU98 strain) seems to show a broader range of hosts, since it has been

detected in red fox, golden jackal (*Canis aureus*) and badger (*Meles meles*) in Europe so far. Brown (*Ursus arctos*) and black (*Ursus americanus*) bears seem to play a role as hosts for *A. phagocytophilum* in the North Hemisphere. *Anaplasma bovis* has been detected in wild Procyonidae, Canidae and Felidae in Asia and Brazil. In order to assess the real identity of the involved agents, future works should benefit from the application of MLST (Multi Locus Sequence Typing), WGS (Whole Genome Sequencing) and NGS (Next Generation Sequencing) technologies aiming at shedding some light on the role of wild carnivores in the epidemiology of Anaplasmataceae agents.

Keywords: *Anaplasma, Ehrlichia*, "*Candidatus* Neoehrlichia sp.", carnivora, genetic diversity, ticks

INTRODUCTION

Recently, the incidence of tick-borne diseases in humans and animals have increased due to several factors, which in association favor the chances of contact among wild animals and associated ectoparasites, domestic animals and humans. The bi-directional flow of tick-borne parasites may occur from wildlife to domestic carnivores and vice-versa (1). Among the main factors associated with the emergence or re-emergence of vector-borne diseases, we can name: climate change, including global warming (for instance, shorter winters have been reported in continental areas of Europe, which impact the development and activity of ticks); "outdoor" activities, global traveling, urbanization, changes in land use, deforestation, habitat fragmentation, natural environment encroachment, which together predispose to a higher contact among wildlife, humans and domestic animals; the employment and easier access to molecular tools, favoring the diagnosis and identification of vector-borne agents; and the increase of awareness of tick-borne agents by veterinarians, physicians, scientists, and public health authorities. Regarding the latter, the veterinary practitioner play a central role and acts as a sentinel to alert epidemiologists, since they are the first one to notice the emergence of clinical cases (1–3).

Wild and domestic carnivores are considered the primary source of tick-borne zoonotic agents to humans. The dynamic of tick-borne agents transmission has been driven by different vertebrate host species living in sympatry. In this scenario, the overlapping of different species' ecological niches creates opportunities to parasites spread their geographical distribution, abundance and host range. As a consequence, several newly discovered arthropod-borne pathogens originated from wildlife has emerged, or reemerged [when a sudden peak of a certain disease occurs after a silent period (1)].

Among emergent tick-borne pathogens, agents belonging to family Anaplasmataceae (Order Rickettsiales) agents stand out due their worldwide distribution and zoonotic potential. Anaplasmataceae family comprises the genera *Ehrlichia, Anaplasma, Neorickettsia,* and *Wolbachia*. These agents are Gram-negative, small, most frequently pleomorphic, coccoid to ellipsoidal bacteria that reside within cytoplasmic vacuoles of the host cells (erythrocytes, reticuloendothelial cells, bone marrow-derived phagocytic cells, endothelial cells and cells of insect, helminth and arthropod reproductive tissues), either singly or, more frequently, in compact inclusions called morulae (4).

In this review we aimed to review the genetic diversity of the tick-transmitted genera *Ehrlichia*, *Anaplasma and "Candidatus* Neoehrlichia sp." in terrrestrial wild carnivores worldwide, discussing the implications for human and domestic animal health and wildlife conservation. In the first section, we presented the molecular prevalence and diversity of Anaplasmataceae agents in wild carnivores Caniformia (Canidae, Mustelidae and Ursidae) and Feliformia (Felidae, Hyanidae, Procyonidae and Viverridae) around the world, including: (i) the previously recognized agents, namely *Anaplasma bovis* and *Anaplasma ovis*, *Anaplasma platys, Anaplasma phagocytophilum, Ehrlichia canis, Ehrlichia chaffeensis;* (ii) new *Candidatus* species ["*Candidatus* Neoerlichia lotoris" and "*Candidatus* Neoehrlichia sp." (FU98) in the United States and Europe, respectively]; (iii) new genotypes of *Ehrlichia* and *Anaplasma* in Brazil and South Africa. Second, we presented previously reported findings related to the consequences associated to contact among wildlife-domestic animals-humans, such as: the effect of the infection by *Ehrlichia* and *Anaplasma* agents on wild carnivores health; the consequences of the contact between domestic dogs and wild canids on the exposure to *E. canis* in wild carnivores and vice-versa; the implications of *Ehrlichia* and *Anaplasma* agents infection in wild carnivores on human and domestic animals health; the role of coyotes (*Canis latrans*) in the epidemiological cycles of *Ehrlichia chaffeensis* in the USA; and the role of raccoons (*Procyon lotor*) in the epidemiological cycles of *Anaplasma phagocytophilum* in the USA and Europe. Finally, we presented the final remarks, highlighting the future directions of the research on the diversity of Anaplasmataceae agents in wild carnivores, emphasizing the need for the application of MLST (Multi Locus Sequence Typing), WGS (Whole Genome Sequencing) and NGS (Next Generation Sequencing) technologies in order to assess the real role of wild carnivores in the epidemiology of these group of α-proteobacteria worldwide.

Considering that interpretation of *Ehrlichia* spp. and *Anaplasma* spp.-reactive antibody titers detected in indirect fluorescent antibody (IFA) surveys among wild vertebrate hosts can be complicated by serological cross-reactions (5), we focused on studies that employed molecular techniques in order to confirm the identity of a certain Anaplasmataceae agent. Results of serological assays were only referenced when a lack of

molecular studies precluded inferences related to the interaction between wild and domestic carnivores in the epidemiology of *Ehrlichia* and *Anaplasma* infections. Therefore, for the purpose of the present study, we search for the following index terms in Medline database: "wild carnivores," "wild canids," "wild felids," "procyonids," "mustelids," "Hyaenidae," "Ursidae," "Viverridae" in association with "*Ehrlichia,*" "*Anaplasma,*" "*Neoehrlichia,*" and "Anaplasmataceae."

MOLECULAR PREVALENCE AND DIVERSITY OF TICK-BORNE ANAPLASMATACEAE AGENTS IN TERRESTRIAL FREE-RANGING AND CAPTIVE CARNIVORES WORLDWIDE

Anaplasma bovis

A molecular occurrence of 5.15% (36/699) for *A. bovis,* an Anaplasmataceae agent that parasitizes monocytes and macrophages (4), has been reported among raccoons in Hokkaido, Japan (6) (**Table 1**). Indeed, raccoons were imported as pets from North America to Japan due to the influence of the popular cartoon "Rascal Raccoon" on the television in 1977. However, when they eventually manifested their wild nature and became aggressive, these animals were intentionally released or run away from their homes, spreading through several areas of Japan (6). These medium-sized carnivores have been incriminated as potential reservoirs for *A. bovis* in Japan, playing a role in the maintenance of this agent in the environment. A statistical association between PCR-positivity for *A. bovis* and infestation by *Haemaphysalis* spp. ticks has been reported in raccoons from Japan (6). This agent was also recently detected in raccons dogs (*Nyctereutes procyonoides*) from Korea (2.1–6.6%) (14, 16). In South America, *A. bovis* 16S rDNA gene sequences (*rrs*) were detected in 4/31 (13%) coatis (*Nasua nasua*), a procyonid species in the Brazilian Pantanal (12). In Brazilian Pantanal, De Sousa et al. (12) detected *Anaplasma rrs* closely related to *A. bovis* in *Amblyomma* ticks collected from coatis (one *A. ovale* adult and *A. sculptum* nymphs).

Besides raccoons and raccons dogs, *A. bovis rrs* has also been detected in Tsushima leopard cats (*Prionailurus bengalensis euptilura*) sampled in Japan (15%) (47, 53) and Korea (6.9%) (48), and in *Haemaphysalis longicornis* ticks associated to these wild felids (47, 53). *A. bovis rrs* were also recently detected in ocelots (*Leopardus pardalis*) (14%) and crab-eating foxes (*Cerdocyon thous*) (1.3%) in the Brazilian Pantanal (12) (**Table 1**). This agent was also detected in a bush-dog (*Speothos venaticus*) maintained in captivity in a Brazilian zoo based on a PCR assay targeting *groEL* gene (9) (**Table 1**). In Thailand, *Anaplasma rrs* closely related to *A. bovis* was detected in three *Haemaphysalis lagrangei* ticks collected from a specimen of Malayan sun bear (*Helarctos malayanus*) (54).

Anaplasma ovis

Anaplasma ovis was molecularly detected (based on *rrs* and *msp4* genes) in foxes (3.3%; 1/13) from Palermo and Ragusa provinces of Sicily, Italy. Out of 110 fleas collected from foxes, *Anaplasma* sp. was molecularly detected in 30% of them. Interesting, while one *Xenopsylla cheopis* flea showed copositivity for *A. ovis* (*msp4*) and *A. phagocytophilum (msp5)*, another one, from the same species, showed copositivity for *A. ovis (msp4)* and *A. marginale (msp4)*. These intriguing findings could be explained considering the fact that foxes in Sicily, Italy, are frequently found surrounding sheep farms. According to the authors, fleas may have acquired the found *Anaplasma* species from sheep, which served as prey for foxes. Although these findings do not incriminate fleas as vectors for *Anaplasma,* they suggest that siphonapterans may maintain these organisms (19).

Anaplasma platys

Anaplasma platys, an Anaplasmataceae agent that parasitizes platelets (4), has been molecularly detected in a moderate rate (14.5%; 10/69) in foxes from Portugal (**Table 1**). This moderate proportion of positive foxes both in northern/central and southern Portugal suggests the existence of a sylvatic cycle of *A. platys* in this country, driven by the homogeneous distribution of this agent in the tick vectors. Foxes are incriminated as possible reservoirs for this agent for domestic dogs in Portugal. Indeed, the infection with *A. platys* seems to be more prevalent than that observed for *E. canis* in red fox populations in Portugal (23). Molecular evidence of the occurrence *A. platys* in humans has been reported in Venezuela (55) and in the USA (56), suggesting the possible zoonotic potential of this agent.

Anaplasma phagocytophilum

Anaplasma phagocytophilum, a zoonotic agent that parasitizes neutrophils, is transmitted by ticks belonging to the *Ixodes persulcatus* complex, which are mainly found in the Northern hemisphere. This agent is mainly transmitted by *Ixodes persulcatus* in Asia and *Ixodes ricinus* in Europe, although *Ixodes trianguliceps* may also play an important role in the transmission of *A. phagocytophilum* among rodents. While *I. scapularis* is the vector of *A. phagocytophilum* in the eastern USA, *I. pacificus* is the main vector of this agent in the western USA [reviewed by (57)]. In addition to *I. pacificus*, the nidicolous tick species *I. angustus, I. ochotonae, I. spinipalpis,* and *I. woodi* may act as vectors for *A. phagocytophilum* in California, USA (58). This agent is responsible for causing the human granulocytic anaplasmosis (HGA) in northern hemisphere, equine and canine granulocytic anaplasmosis in the USA, and tick-borne fever in cattle and sheep in Europe [reviewed by (57)].

Since transstadial transmission of *A. phagocytophilum* in its vector ticks is well known, but transovarial transmission has not been demonstrated so far, vertebrate reservoir hosts are responsible for the maintenance of this agent in the environment (57).

Among wild canids, *A. phagocytophilum* has been molecularly detected in gray foxes (*Urocyon cinereoargenteus*) (9%) from northern California, USA (59), red foxes from Italy (0.5–16.6%) (18, 30), Germany (8.2%) (21), the Netherlands (9.9%) (22), Romania (2.55%) (24), Hungary (12.5%) (26), Switzerland (2.4%) (27), Czech Republic (0.8%) (7), Austria (0.6%) (32), raccoon dogs from Germany (23%) (21), golden jackals (*Canis*

TABLE 1 | Molecular detection of *Anaplasma* spp. in free-ranging and captive terrestrial wild carnivores (Canidae, Mustelidae, Ursidae, Felidae, Hyaenidae, Procyonidae and Viverridae) around the world.

Host	Technique (Target genes)	Result	Sample origin	References
Suborder Caniformia				
Family Canidae				
Canis aureus (golden jackal)	cPCR (*rrs*)	0/1 – *A. phagocytophilum*	Czech Republic (F)	(7)
	cPCR (*rrs/msp2*) qPCR/ RFLP *Anaplasma* spp. (*A. ovis, A. marginale, A. centrale, A. phagocytophilum*) (*msp4*)	2/216 (0.9%) – *A. phagocytophilum*	Serbia (F)	(8)
Canis lupus (gray wolf)	cPCR (*rrs*)	0/3	Brazil (C)	(9)
	qPCR: *Anaplasma* sp. (rrs) *A. marginale/A. ovis* (*msp-4*) *A. hagocytophilum* (*msp-2*) cPCR (*rrs*)	0/2	Spain (F)	(10)
Canis mesomelas (black backed jackal)	RLB (*rrs*) for *A. bovis, A. centrale, A. marginale, A. phagocytophilum, Anaplasma sp. Omatjenne, A. platys* cPCR (*rrs*)	82/142 (57.7%) *Anaplasma* sp. showing identity to A. *phagocytophilum* and *Anaplasma* sp. South African Dog	South Africa (F/C)	(11)
Cerdocyon thous (crab-eating fox)	cPCR (*rrs*)	0/39	Brazil (C)	(9)
	cPCR (*groEL*)	1/78 (1.2%) – *Anaplasma* sp. related to *A. bovis*	Brazil (F)	(12)
Chrysocyon brachyurus (maned wolf)	cPCR (*rrs*)	0/23	Brazil (C)	(9)
Lycaon pictus (African wild dog)	cPCR (*rrs*) RLB (*A. centrale A. marginale A. ovis , A. phagocytophilum 1, A. phagocytophilum 3, A. phagocytophilum 5, A. phagocytophilum* 7) (*rrs*)	0/301	South Africa (F)	(13)
Nyctereutes procyonoides (raccoon dog)	cPCR (*rrs*)	1/15 (6.6%) – *A. bovis*	Korea (F)	(14)
	cPCR *A. phagocytophilum* (*rrs*)	0/7	Czech Republic (F)	(7)
	cPCR *A. phagocytophilum* (*rrs/groEL*)	0/10	Poland (F)	(15)
	cPCR (*rrs/groEL/ankA/msp-2*)	2/193 (1%) – *A. phagocytophilum* 4/193 (2.1%) – *A. bovis*	Korea (F)	(16)
Pseudalopex vetulus (hoary fox)	cPCR (*rrs*)	0/8	Brazil (C)	(9)
Speothos venaticus (bush dog)	cPCR (*rrs*)	0/27	Brazil (C)	(9)
Vulpes lagopus (arctic foxes)	cPCR (*rrs*)	1/28 (3.6%) *Anaplasma* sp.	Canada (F)	(17)
Vulpes vulpes (red fox)	cPCR (*rrs*)	25/150 (16.6%) – *A. phagocytophilum*	Italy (F)	(18)
	cPCR (*msp4*)	1/13 (7.7%) – *A. ovis*	Italy (F)	(19)
	cPCR (*rrs*)	0/36 – *Anaplasma* sp.	Austria (F)	(20)
	qPCR (*msp-2*) cPCR (*rrs/ankA*)	10/122 (8.2%) – *A. phagocytophilum*	Germany (F)	(21)
	qPCR (*msp-2*) cPCR (*groEL*)	8/81 (9.9%) – *A. phagocytophilum*	The Netherlands (F)	(22)
	cPCR/qPCR (*rrs*)	10/69 (14.5%) – *A. platys*	Portugal (F)	(23)
	cPCR (*rrs, ankA*)	9/353 (2.55 %) – *A. phagocytophilum* 0/353 – *A. platys*	Romania (F)	(24)
	cPCR (*rrs*)	0/119 – *Anaplasma* sp.	Bosnia and Herzegovina (F)	(25)

(Continued)

TABLE 1 | Continued

Host	Technique (Target genes)	Result	Sample origin	References
	qPCR/cPCR (*rrs*)	51/415 (12.5%) – *A. phagocytophilum* 0/415 – *A. platys*	Hungary (F)	(26)
	qPCR for *Anaplasma* sp. (rrs) *A. marginale/A. ovis* (*msp-4*) *A. phagocytophilum* (*msp-2*) cPCR (*rrs*)	0/54	Spain (F)	(10)
	qPCR for *A. phagocytophilum/A. platys* (*rrs*)	4/162 (2.4%) – *A. phagocytophilum*	Switzerland (F)	(27)
	HRM for A. *phagocytophilum (rrs)*	0/195	Germany (F)	(28)
	cPCR (*rrs*)	0/12 – *Anaplasma* sp.	Spain (F)	(29)
	cPCR (*rrs*)	1/151 (0.65%) – *A. phagocytophilum*	Italy (F)	(30)
	cPCR (*rrs*)	1/114 (0.8%) – *A. phagocytophilum*	Czech Republic (F)	(7)
	qPCR – A. *phagocytophilum (msp-2)*	0/97	Italy (F)	(31)
	cPCR *(rrs/groEL)*	3/506 (0.6%) – *A. phagocytophilum*	Austria (F)	(32)
Family Mustelidae				
Lutra lutra (otter)	qPCR: *Anaplasma* sp. (rrs) *A. marginale/A. ovis* (*msp4*) *A. phagocytophilum* (*msp-2*) cPCR (*rrs*)	0/2	Spain (F)	(10)
	cPCR (*rrs*)	0/1	Czech Republic (F)	(7)
Meles meles (Eurasian badger)	qPCR *A. phagocytophilum* (*msp-2*)	0/40	The Netherlands (F)	(22)
	qPCR: *Anaplasma* sp. (rrs) *A. marginale/A. ovis* (*msp4*) *A. phagocytophilum* (*msp-2*) cPCR (*rrs*)	2/130 (1.5%) – *Anaplasma* sp.	Spain (F)	(10)
	cPCR (*rrs*)	0/3	Spain (F)	(29)
	cPCR (*rrs*)	0/3	Czech Republic (F)	(7)
Martes foina (stone marten)	qPCR: *Anaplasma* sp. (rrs) *A. marginale/A. ovis* (*msp4*) *A. phagocytophilum* (*msp-2*) cPCR (*rrs*)	0/22	Spain (F)	(10)
	cPCR (*rrs*)	0/10	Spain (F)	(29)
	cPCR (*rrs*)	0/4	Czech Republic (F)	(7)
	qPCR (*msp2*) – *A. phagocytophilum*	0/2	Hungary (F)	(33)
Martes martes (pine marten)	qPCR: *Anaplasma* sp. (rrs) *A. marginale/A. ovis* (*msp4*) *A. phagocytophilum* (*msp-2*) cPCR (*rrs*)	0/14	Spain (F)	(10)
Mustela erminea (stoat)	qPCR: *Anaplasma* sp. (rrs) *A. marginale/A. ovis* (*msp4*) *A. phagocytophilum* (*msp-2*) cPCR (*rrs*)	0/1	Spain (F)	(10)
Mustela nivalis (weasel)	qPCR: *Anaplasma* sp. (rrs) *A. marginale/A. ovis* (*msp4*) *A. phagocytophilum* (*msp-2*) cPCR (*rrs*)	0/6	Spain (F)	(10)
	qPCR (*msp2*) – *A. phagocytophilum*	0/2	Hungary (F)	(33)
Mustela putorius (polecat)	qPCR: *Anaplasma* sp. (rrs) *A. marginale/A. ovis* (*msp4*) *A. phagocytophilum* (*msp-2*) cPCR (*rrs*)	0/6	Spain (F)	(10)

(Continued)

TABLE 1 | Continued

Host	Technique (Target genes)	Result	Sample origin	References
	cPCR (*rrs*)	0/4	Czech Republic (F)	(7)
	cPCR (*rrs*)	0/3	Spain (F)	(29)
Mustela sibirica (weasel)	qPCR (*rrs*) cPCR (*rrs*)	½*	Korea (F)	(34)
	qPCR (*rrs*)	½*	Korea (F)	(35)
Neovison vison (American mink)	qPCR: *Anaplasma* sp. (rrs) *A. marginale/A. ovis* (*msp4*) *A. phagocytophilum* (*msp-2*) cPCR (*rrs*)	½ (50%) – *Anaplasma* sp.	Spain (F)	(10)
	qPCR (*E. canis rrs*)	0/3	Spain (F)	(36)
Family Ursidae				
Ursus americanus (American black bear)	qPCR (*msp-2*) cPCR (*rrs*)	3/80 (4%) – *A. phagocytophilum*	USA (F)	(37)
	qPCR (*msp-2*)	30/288 (10%) – *A. phagocytophilum*	USA (F)	(38)
	cPCR (*rrs*)	2/68 (3%) – *A. phagocytophilum*	USA (F)	(39)
Ursus arctos (brown bear)	cPCR (*rrs*)	18/74 (24.3%) – *A. phagocytophilum*	Slovakia (F)	(40)
Ursus arctos yesoensis (Hokkaido brown bear)	cPCR (*rrs/gltA*) RLB (*rrs*)	2/13 (15%) – *Anaplasma* sp. (AP-sd)	Japan (F)	(41)
Suborder Feliformia				
Family Felidae				
Acinonyx jubatus (cheetah)	cPCR (*rrs*)	0/4	Zimbabwe (C)	(42)
Caracal caracal (caracal)	cPCR (*rrs*)	0/1	Brazil (C)	(9)
Felis silvestris (wildcat)	qPCR/cPCR *Anaplasma* sp. (*rrs*) *A. marginale/A. ovis* (*msp-4*) *A. phagocytophilum* (*msp-2*)	0/8	Spain (F)	(10)
Felis lybica cafra (South African wildcat)	cPCR (*rrs*)	1/6 (13%) – *A. phagocytophilum*	Zimbabwe (C)	(42)
Herpailurus yagouaroundi (jaguarondi)	cPCR (*rrs*)	0/19	Brazil (C)	(9)
Leopardus pardalis (ocelot)	cPCR (*rrs*)	0/15	Brazil (C)	(9)
	cPCR (*rrs*)	1/7 (14.2%) – *Anaplasma* sp. related to *A. bovis*	Brazil (F)	(12)
Leopardus tigrinus (little spotted cat)	cPCR (*rrs*)	4/25 (16%) – *Anaplasma* sp. related to *A. phagocytophilum* 4/25	Brazil (C)	(9)
Leopardus wiedii (margay)	cPCR (*rrs*)	0/2	Brazil (C)	(9)
Leptailurus serval (serval)	cPCR (*rrs*)	0/1	Brazil (C)	(9)
	cPCR (*rrs*)	½(50%) – *A. phagocytophilum*	Zimbabwe (C)	(42)
Lynx lynx (Eurasian lynx)	qPCR (*rrs*)	0/22 – *A. phagocytophilum*	Sweden (F)	(43)
Oncifelis colocolo (pampas cat)	cPCR (*rrs*)	0/1	Brazil (C)	(9)
Panthera leo (lion)	cPCR (*rrs*)	1/10 (10%) – *A. phagocytophilum*	Italy (C)	(44)
	cPCR (*rrs*)	0/12	Brazil (C)	(9)
	cPCR (*rrs*)	6/86 (7%) – *A. phagocytophilum*	Zimbabwe (C)	(42)
	cPCR/RLBH (*rrs*)	0/13	Botswana (F)	(45)
Panthera onca (jaguar)	cPCR (*rrs*)	0/6	Brazil (C)	(9)

(Continued)

TABLE 1 | Continued

Host	Technique (Target genes)	Result	Sample origin	References
Puma concolor (puma)	cPCR (*rrs*)	7/47 (16%) – *A. phagocytophilum*	USA (F)	(46)
[-1pt]	cPCR (*rrs*)	0/8	Brazil (C)	(9)
[-1pt] *Panthera tigris (tiger)*	cPCR (*rrs*)	0/8	Brazil (C)	(9)
*Prionailurus bengalensis euptilura (*Tsushima leopard cat)	cPCR (*rrs*)	2/13 (15%) – *A. bovis*	Japan (F)	(47)
*Prionailurus bengalensis euptilura (*Tsushima leopard cat)	cPCR (*rrs*)	2/29 (6.9%) – *A. bovis*	Korea (F)	(48)
Prionailurus iriomotensis (Iriomote cat)	cPCR (*rrs*)	0/33	Japan (F)	(47)
Prionailurus viverrinus (fishing cat)	cPCR (*rrs*)	0/1	Brazil (C)	(9)
Family Hyaenidae				
Crocuta crocuta (spotted hyaena)	RLB *Anaplasma* (*rrs*)	0/47	Namibia and South Africa (F/C)	(49)
Parahyaena brunnea (brown hyaena)	RLB *Anaplasma* (*rrs*)	0/15	Namibia and South Africa (F/C)	(49)
Procyonidae				
Nasua nasua (coati)	cPCR (*rrs*)	7/31 (22.5%) – *Anaplasma* sp. 4/7 (*Anaplasma* sp. closely related to *A. bovis*) 1/7 (*Anaplasma* sp. closely related to *A. phagocytophilum)*	Brazil (F)	(12)
Procyon lotor (raccoon)	cPCR (*rrs*, *groEL* *p44*)	14/57 (24.6%) – *A. phagocytophilum*	USA (F)	(50)
	cPCR (*rrs*)	0/187	Japan (F)	(51)
	cPCR (*rrs*) for *A. phagocytophilum*	0/169	USA (F)	(52)
	cPCR (*rrs*)	36/699 (5.15%) – *A. bovis*	Japan (F)	(6)
	cPCR (*rrs*)	0/15	Czech Republic (F)	(7)
	cPCR (*rrs*)	0/15	Czech Republic (F)	(7)
	cPCR (*rrs/groEL*)	1/78 (1.3%) – *A. phagocytophilum*	Poland (F)	(15)
	cPCR (*rrs/groEL*)	0/40	Germany (F)	(15)
Family Viverrridae				
Genetta genetta (common genet)	qPCR: *Anaplasma* sp. (rrs) *A. marginale/A. ovis* (*msp4*) *A. phagocytophilum* (*msp-2*) cPCR (*rrs*)	0/14	Spain (F)	(10)
	cPCR (*rrs*)	0/34	Spain (F)	(29)

*C, captive; F, free-ranging; *Not sequenced.*

aureus) from Serbia (0.9%) (8), and raccons dogs (*Nyctereutes procyonoides*) from Korea (1%) (16). In Africa, *Anaplasma* sp. *rrs* closely related to *A. phagocytophilum* was detected in black backed jackals in South Africa (57.7%) (11) (**Table 1**). Recently, *A. phagocytophilum rrs* was detected in *Ixodes ricinus* collected from two foxes in Romania (60).

Among wild felids, *A. phagocytophilum rrs* has been molecularly detected in free-ranging mountain lions (*Puma concolor*) from California, USA (16%) (46), captive lions in Italy (10%) (44), captive little spotted cats (16%) in Brazil (9), and captive lions (7%), Southern Africa wild cats (13%), and servals (50%) (*Leptailurus serval*) in Zimbabwe (42) (**Table 1**).

Among procyonids, *A. phagocytophilum rrs* have been molecularly detected in raccoons (24.6%) from Connecticut, USA (50) and Poland (1.3%) (15), and coatis (3.2%) from Pantanal, Brazil (12) (**Table 1**). In Brazilian Pantanal, an *Anaplasma rrs* closely related to *A. phagocytophilum* was detected in *A. sculptum* nymphs collected from one coati (12).

Finally, *A. phagocytophilum* (*rrs* or *msp-2*) have been molecularly detected in free-ranging American black bears (*Ursus americanus*) (3–10%) in the USA (37–39) and free-ranging brown bears (*Ursus arctos*) in Slovakia (24.3%) (40) was reported in Slovakia (40) (**Table 1**).

Although *A. phagocytophilum rrs* have been detected in little spotted cats and coatis in Brazil (9), these animals were negative in qPCR assays targeting specific *msp-2* of *A. phagocytophilum* and conventional PCR directed to *groEL* gene, indicating that an *Anaplasma* genotype closely related to *A. phagocytophilum* circulate among wild carnivores in South America (**Table 1**). In Africa, although *A. phagocytophilum rrs* were detected in black backed jackals by reverse line blotting (RLB) (11), and lions, Southern African wild cats and servals by conventional PCR assays (**Table 1**), additional molecular characterization based on other genes was not performed (42). Therefore, the real identity of these *Anaplasma* genotypes circulating in wild carnivores in southern hemisphere, where recognized vectors of *A. phagocytophilum* don't occur, should be evaluated with caution. Future works aiming at isolating these new genotypes in order to carry out more accurate molecular characterization is much needed.

While serological and molecular prevalence rates for *A. phagocytophilum* of 89.5 and 24.6% have been reported among raccoons from Connecticut, USA (50), all 169 raccoons sampled in peridomestic areas in the states of Florida and Georgia showed to be negative in PCR assays for *A. phagocytophilum* (52). In another study, only one out of 156 raccoons from five populations sampled in the states of Georgia and Florida showed to be seropositive to *A. phagocytophilum* (61). Raccoons showed to be susceptible to experimental infection with a human-originated *A. phagocytophilum* strain (52). In Japan, one out 187 feral raccoons (0.5%) sampled in Kanagawa Prefecture, Japan, showed to be seropositive to *A. phagocytophilum* (51). Recently, *A. phagocytophilum* was detected in a raccoon (1.2%) from northwestern Poland (15). The *groEL* sequence analysis showed that the found *A. phagocytophilum* belongs to the European zoonotic ecotype I previously reported by Jahfari et al. (22) and Hildebrand et al. (15).

Other *Anaplasma* Genotypes

Non-characterized *Anaplasma groEL* sequences were detected in *Amblyomma* ticks from crab-eating foxes (*A. sculptum* adults and nymphs, *A. parvum* adult, *A. ovale* adult and *Amblyomma* larvae) and coatis (*A. sculptum* numphs and *A. ovale* adult) in Brazilian Pantanal (12). In Japan, *Anaplasma* sp. (AP-sd), previously reported in ticks, cattle, sika deer (*Cervus nippon yesoensis*) and rodents, was detected in wild Hokkaido brown bears (*Ursus arctos yesoensis*) (15%) (41).

Ehrlichia canis

Ehrlichia canis, the agent of canine monocytic ehrlichiosis, infects monocytes and macrophages of domestic dogs and wild carnivores (62). *Rhipicephalus sanguineus* sensu lato (s.l.) (63) and *Dermacentor variabilis* (64) are the recognized vectors for *E. canis*. Although the brown dog tick *Rhipicephalus sanguineus* s.l. has been considered the primary vector of *E. canis* (65) showed, using experimental trials, that while the "tropical lineage" of *R. sanguineus* (populations from the state of São Paulo) showed vectorial competence for *E. canis*, the "temperate lineage" of this tick species (populations from Argentina, Uruguay, and southern Brazil) was not able to transmit this *Ehrlichia* species. In addition to *R. sanguineus* s.l. and *Dermacentor variabilis* (62), *Dermacentor marginatus* and *Ixodes canisuga* have been suggested as possible vectors of *E. canis* (66).

Molecular evidence of the occurrence *E. canis* in humans has been reported in Venezuela (67, 68) and Costa Rica (69), suggesting the zoonotic potential of this agent. Based on the amino acid tandem repeat sequence of the TRP36 protein, a novel genotype of *E. canis* was described in blood donors from Costa Rica, grouping within a single clade closely related to the Brazilian genogroup of *E. canis* detected in dogs (69).

Among wild canids, *E. canis rrs* has been detected in bush dogs (*Speothos venaticus*) (11.1%) and crab-eating foxes (*Cerdocyon thous*) (2.6–10.2%) from Brazil (9, 12), artic foxes (*Vulpes lagopus*) from Canada (3.6%) (17), red foxes (*Vulpes vulpes*) from Italy (31–52%) (19, 30, 70), Portugal (2.29%) (23) and Spain (16.6%) (29), gray wolves (*Canis lupus*) from Italy (50%) (70). An *E. canis dsb* sequence has been detected in one crab-eating fox maintained in captivity in a Brazilian zoo (9) (**Table 2**). In Brazilian Pantanal, (12) detected *Ehrlichia rrs* in *Amblyomma* ticks collected from crab-eating foxes (*A. parvum* adults, *A. sculptum* adults and nymphs, and *Amblyomma* larvae) and coatis (*A. sculptum* adults and nymphs and *Amblyomma* larvae). The *Ehrlichia rrs* detected in one *A. sculptum* adult and nymph, one *A. parvum* and one *Amblyomma* larvae pool were closely related to *E. canis* (12). In the USA, specific antibodies to *E. canis* were detected in 18% (9/50) coyotes sampled in Texas and Oklahoma, USA, using a p16 peptide-based microtiter plate ELISA (79).

Among wild felids, *E. canis rrs* has been detected in ocelots (*Leopardus pardalis*) (13.3–17.2%), jaguarondis (*Herpailurus yagouaroundi*) (10.5–16.6%), little spotted cats (*Leopardus tigrinus*) (8.0–14.3%), pumas (*Puma concolor*) (1.1–25%), jaguars (*Panthera onca*) (22.2%), lion (*Panthera leo*) (16.6%) maintained in captivity in zoos in Brazil (9, 75), free-ranging Iriomote cats (12%) (*Prionailurus iriomotensis*) and Tsushima leopard cats (*Prionailurus bengalensis euptilura*) (8%) in Japan (47), and lions (1%) maintained in captivity in Zimbabwe (42) (**Table 2**). Among procyonids and mustelids, *E. canis rrs* has been detected in raccoons (*Procyon lotor*) (1.7%) from the USA (78) and Spain (2.6%) (36), coatis (3.2%) from Brazil (12), and Eurasian otters (50%) from Italy (73) (**Table 2**). Although *E. canis rrs* has been detected in several wild captive felid species in Brazil (75), phylogenetic analysis based on *omp-1* gene positioned *Ehrlichia* sp. *omp-1* sequences obtained from three ocelots and one jaguar in a separated clade from *E. canis* and *E. chaffeensis* sequences, suggesting the occurrence of a new *Ehrlichia* species in wild felids in Brazil (75). Similarly, even though *E. canis rrs* was detected in crab-eating foxes and coatis in Brazilian Pantanal, these samples showed negative results in specific qPCR assays targeting *E. canis*-dsb gene (12). Considering that the majority of the *E. canis* sequences obtained from wild carnivores worldwide are based only on short *rrs*, future studies regarding the genetic diversity of *Ehrlichia* spp. in wild animals should focus on different target genes other than *rrs*, in order to assess the real identity of these newly reported Anaplasmataceae genotypes as discussed in section Final remarks and future directions.

TABLE 2 | Molecular detection of *Ehrlichia* spp. in free-ranging and captive terrestrial wild carnivores (Canidae, Mustelidae, Ursidae, Felidae, Hyaenidae, Procyonidae, and Viverridae) around the world.

Host	Technique (Target genes)	Result	Sample origin	References
Suborder Caniformia				
Family Canidae				
Canis aureus (golden jackal)	cPCR (*rrs/groEL*)	0/1 – "*Candidatus* Neoehrlichia sp." (FU98)	Czech Republic (F)	(7)
Canis lupus (gray wolf)	cPCR (*rrs*)	1/3 (33.3%) – *Ehrlichia* sp. related to *E. chaffeensis*	Brazil (C)	(9)
Canis lupus (gray wolf)	cPCR for *Ehrlichia* sp. (*rrs*)	0/2	Spain (F)	(10)
Canis mesomelas (black backed jackal)	RLB (*rrs*) for *E. canis, E. chaffeensis, E. ruminantium,* cPCR (*rrs*)	0/142	South Africa (F/C)	(11)
Cerdocyon thous (crab-eating fox)	cPCR (*rrs/dsb*)	4/39 (*rrs*: 10.2%); 1/39 (*dsb*: 2.5%) *Ehrlichia* sp. related to *E. canis* 4/39 (10.2%) – *Ehrlichia* sp. related to *E. chaffeensis*	Brazil (C)	(9)
	cPCR (*rrs/dsb*)	6/58 (10.3%) – *Ehrlichia* sp. related to *E. ruminantium*	Brazil (F)	(71)
	cPCR (*rrs*)	2/78 (7.6%) – *Ehrlichia* sp. related to *E. canis*	Brazil (F)	(12)
Chrysocyon brachyurus (maned wolf)	cPCR (*rrs*)	0/23	Brazil (C)	(9)
Lycaon pictus (African wild dog)	cPCR (*rrs*) RLB for *Ehrlichia* spp. (*Ehrlichia/Anaplasma* catch-all, *E. canis/E. ovina E. chaffeensis*) *E. ruminantium Ehrlichia* sp. (Omatjenne)	0/301	South Africa (F)	(13)
	cPCR *E. canis/E. ewingii* (*rrs*)	0/11	Zambia (F)	(72)
Nyctereutes procyonoides (raccoon dog)	cPCR (*rrs*)	0/15 – *Ehrlichia* sp.	Korea (F)	(14)
	cPCR (*rrs/groEL*)	0/7 – "*Candidatus* Neoehrlichia sp." (FU98)	Czech Republic (F)	(7)
	cPCR (*rrs/groEL*)	3/10 (30%) – "*Candidatus* Neoehrlichia sp." (FU98)	Poland (F)	(15)
Pseudalopex vetulus (hoary fox)	cPCR (*rrs*)	0/8	Brazil (C)	(9)
Speothos venaticus (bush dog)	cPCR (*rrs*)	3/27 (11.1%) – *E. canis*	Brazil (C)	(9)
Vulpes lagopus (arctic foxes)	cPCR (*rrs*)	1/28 (3.6%) – *E. canis*	Canada (F)	(17)
Vulpes vulpes (red fox)	cPCR (*rrs*)	4/13 (31%) – *E. canis*	Italy (F)	(19)
	cPCR (*rrs*)	0/36 – *E. canis*	Austria (F)	(20)
	cPCR/qPCR (*rrs*)	2/69 (2.9%) – *E. canis*	Portugal (F)	(23)
	cPCR (*rrs*)	0/353 – *E. canis*	Romania (F)	(24)
	cPCR (*rrs /groESL*)	1/164 (0.6%) – "*Candidatus* Neoehrlichia sp." (FU98)	Austria (F)	(25)
	cPCR (*rrs*)	0/119 – *Ehrlichia* sp.	Bosnia and Herzegovina (F)	(25)
	qPCR/cPCR (*rrs*)	0/415 – *E. canis*	Hungary (F)	(26)
	cPCR for *Ehrlichia* sp. (*rrs*)	0/54	Spain (F)	(10)
	qPCR multiplex for "*Ca*. Neoehrlichia mikurensis," "*Ca*. Neoehrlichia", and Anaplasmataceae (*rrs*) qPCR for *E. canis* (*rrs*)	0/162	Switzerland (F)	(27)
	HRM for *E. canis* and "*Candidatus* Neoehrlichia mikurensis" (*rrs*)	0/195	Germany (F)	(28)

(Continued)

TABLE 2 | Continued

Host	Technique (Target genes)	Result	Sample origin	References
	cPCR (*rrs*)	2/12 (16.6%) – *E. canis*	Spain (F)	(29)
	cPCR (*rrs*)	55/105 (52%) – *E. canis*	Italy (F)	(70)
	cPCR (*rrs*)	68/151 (44.44%) – *E. canis*	Italy (F)	(30)
	cPCR (*rrs/groEL*)	1/114 (0.8%) -*"Candidatus* Neoehrlichia sp." (FU98)	Czech Republic (F)	(7)
	qPCR (*rrs-E. canis*)	0/3	Spain (F)	(36)
	qPCR – "*Candidatus* Neoehrlichia mikurensis"(*groEL*)	0/97	Italy (F)	(31)
	cPCR (*rrs*)	2/506 (0.6%) *"Candidatus* Neoehrlichia sp."(FU98)	Austria (F)	(32)
Family Mustelidae				
Lutra lutra (otter)	cPCR (*rrs*)	0/2	Spain (F)	(10)
	cPCR (*rrs*)	0/1	Czech Republic (F)	(7)
	qPCR/cPCR (*E. canis rrs*)	3/6 (50%)	Italy (F)	(73)
Martes foina (stone marten)	cPCR (*rrs*)	0/22	Spain (F)	(10)
	cPCR (*rrs*)	0/10	Spain (F)	(29)
	cPCR (*rrs*)	0/4	Czech Republic (F)	(7)
Meles meles (Eurasian badger)	cPCR (*rrs*)	1/130 (0.7%) *Ehrlichia* sp. related to *E. chaffeensis*	Spain (F)	(10)
	cPCR (*rrs*)	0/3	Czech Republic (F)	(7)
Mustela erminea (stoat)	cPCR (*rrs*)	0/1	Spain (F)	(10)
Mustela nivalis (weasel)	cPCR (*rrs*)	0/6	Spain (F)	(10)
Mustela putorius (polecat)	cPCR (*rrs*)	0/6	Spain (F)	(10)
	cPCR (*rrs*)	0/4	Czech Republic (F)	(7)
Mustela sibirica (weasel)	qPCR (*rrs*) cPCR (*rrs/nadA*)	½*	Korea (F)	(34)
Neovison vison (American mink)	cPCR (*rrs*)	0/2	Spain (F)	(10)
	qPCR (*E. canis rrs*)	0/3	Spain (F)	(36)
Family Ursidae				
Ursus americanus (American black bear)	cPCR (*rrs/gltA*)	0/49	USA (F)	(74)
Suborder Feliformia				
Acinonyx jubatus (cheetah)	cPCR (*rrs*)	0/4	Zimbabwe (C)	(42)
Caracal caracal (caracal)	cPCR (*rrs*)	0/1	Brazil (C)	(9)
Felis silvestris (wildcat)	cPCR (*rrs*)	0/8	Spain (F)	(10)
Felis lybica cafra (South African wildcat)	cPCR (*rrs*)	0/1	Zimbabwe (C)	(42)
Herpailurus yagouaroundi (jaguarondi)	cPCR (*rrs, omp1*)	1/6 (16.6%) – *Ehrlichia* sp. related to *E. canis*	Brazil (C)	(75)
Herpailurus yagouaroundi (jaguarondi)	cPCR (*rrs*)	2/19 (10.5%) – *Ehrlichia* sp. related to *E. canis* 1/19 (5.3%) – *Ehrlichia* sp. related to *E. chaffeensis*	Brazil (C)	(9)
Leopardus pardalis (ocelot)	cPCR (*rrs, omp1*)	5/29 (17.2%) – *Ehrlichia* sp. related to *E. canis*	Brazil (C)	(75)
	cPCR (*rrs*)	2/15 (13.3%) – *Ehrlichia* sp. related to *E. canis* 2/15 (13.3%) – *Ehrlichia* sp. related to *E. chaffeensis*	Brazil (C)	(9)
	cPCR (*rrs*)	0/7	Brazil (F)	(12)

(Continued)

TABLE 2 | Continued

Host	Technique (Target genes)	Result	Sample origin	References
Leopardus tigrinus (little spotted cat)	cPCR (*rrs*)	2/14 (14.3%) – *Ehrlichia* sp. related to *E. canis*	Brazil (C)	(75)
	cPCR (*rrs*)	2/25 (8%) – *Ehrlichia* sp. related to *E. canis* 3/25 (12%) – *Ehrlichia* sp. related to *E. chaffeensis*	Brazil (C)	(9)
Leopardus wiedii (margay)	cPCR (*rrs, omp1*)	0/2	Brazil (C)	(75)
	cPCR (*rrs*)	0/2	Brazil (C)	(9)
Leptailurus serval (serval)	cPCR (*rrs*)	0/1	Brazil (C)	(9)
	cPCR (*rrs*)	0/2	Zimbabwe (C)	(42)
Oncifelis colocolo (pampas cat)	cPCR (*rrs, omp1*)	0/3	Brazil (C)	(75)
	cPCR (*rrs*)	0/1	Brazil (C)	(9)
Panthera leo (lion)	cPCR (*rrs*)	2/12 (16.6%) – *Ehrlichia* sp. related to *E. canis* 1/12 (8.3%) – *Ehrlichia* sp. related to *E. chaffeensis*	Brazil (C)	(9)
	cPCR (*rrs*)	1/86 (1%) – *E. canis*	Zimbabwe (C)	(42)
	cPCR/RLBH (*rrs*)	0/13	Botswana (F)	(45)
	cPCR (*rrs*)	0/24	Zambia (F)	(72)
Panthera onca (jaguar)	cPCR (*rrs, omp1*)	2/9 (22.2%) – *Ehrlichia* sp. related to *E. canis*	Brazil (C)	(75)
	cPCR (*rrs, dsb*)	2/10 (20%) – *Ehrlichia* sp. related to *E. ruminantium*	Brazil (F)	(76)
	cPCR (*rrs*)	0/6	Brazil (C)	(9)
Puma concolor (puma)	cPCR (*rrs, omp1*)	1/9 (11.1%)	Brazil (C)	(75)
	cPCR (*rrs*)	2/8 (25%) – *Ehrlichia* sp. related to *E. canis* 2/8 (25%) – *Ehrlichia* sp. related to *E. chaffeensis*	Brazil (C)	(9)
Panthera tigris (tiger)	cPCR (*rrs*)	2/8 (25%) – *Ehrlichia* sp. related to *E. chaffeensis*	Brazil (C)	(9)
Prionailurus bengalensis euptilura (Tsushima leopard cat)	cPCR (*rrs*)	1/8 (8%) – *E. canis*	Japan (F)	(47)
Prionailurus iriomotensis (Iriomote cat)	cPCR (*rrs*)	4/33 (12%) – *E. canis*	Japan (F)	(47)
Prionailurus viverrinus (fishing cat)	cPCR (*rrs*)	0/1	Brazil (C)	(9)
Family Hyaenidae				
Crocuta crocuta (spotted hyaena)	cPCR *E. canis/E. ewingii* (*rrs*)	0/19	Zambia (F)	(72)
	RLB *Ehrlichia* (*rrs*)	0/47	Namibia and South Africa (F/C)	(49)
Parahyaena brunnea (brown hyaena)	RLB *Ehrlichia* (*rrs*)	0/15	Namibia and South Africa (F/C)	(49)
Family Procyonidae				
Nasua narica (white-nose coati)	cPCR (*rrs/dsb*)	0/20 – *Ehrlichia* sp. 0/20 – "*Candidatus* Neoehrlichia lotoris"	Costa Rica (F)	(77)
Nasua nasua (coati)	cPCR (*rrs*)	1/31 (3.8%) – *Ehrlichia* sp. related to *E. canis*	Brazil (F)	(12)
Procyon lotor (raccoon)	cPCR (*rrs*)	1/60 (1.7%) – *E. canis* 32/60 (53.3%) – "*Candidatus* Neoehrlichia lotoris"	USA (F)	(78)
	cPCR (*rrs*)	0/187	Japan (F)	(51)
	cPCR (*rrs*) for *E. canis, E. ewingii, E. chaffeensis*	0/169	USA (F)	(52)

(Continued)

TABLE 2 | Continued

Host	Technique (Target genes)	Result	Sample origin	References
	cPCR (*rrs*)	131/197 (67%) – "*Candidatus* Neoehrlichia lotoris"	USA (F)	(61)
	cPCR (*rrs*)	0/15	Czech Republic (F)	(7)
	qPCR (*E. canis rrs*)	5/194 (2.57%)	Spain (F)	(36)
	cPCR *Neoehrlichia* sp. (*rrs/groEL*)	0/78	Poland (F)	(15)
	cPCR *Neoehrlichia* sp. (*rrs/groEL*)	0/40	Germany (F)	(15)
Family Viverridae				
Genetta genetta (common genet)	cPCR (*rrs*)	0/14	Spain (F)	(10)
	cPCR (*rrs*)	0/34	Spain (F)	(29)

*C, captive; F, free-ranging; *Not sequenced.*

Red and gray foxes (80) showed to be susceptible to experimental infection by *E. canis* from dog-infected blood. Additionally, the gray fox was able of providing an infectious blood meal for *R. sanguineus* larvae (80). Millán et al. (29) observed that foxes inhabiting natural areas in periurban Barcelona, Spain, showed a high frequency of infection by *E. canis* when compared to dogs from surrounding residential areas. Likewise, *E. canis* seems to be frequent among wild canids (foxes and gray wolves) in Italy, suggesting that a sylvatic life cycle of this pathogen may occur in that country (70). On the other hand, *E. canis* (2.9%) showed to be less prevalent than *A. platys* (14.5%) in red foxes from Portugal. These findings may be related to a heterogeneous distribution of the agents within the vector populations in Europe (23). Recently, *R. sanguineus* s.l. ticks collected from vegetation (questing) or from domestic and wild animals (including three red foxes) from 18 administrative regions of mainland Portugal, showed to belong to the "temperate lineage" and were negative in PCR assays for *E. canis* (81). In southern Brazil, where a "temperate lineage" of *R. sanguineus* is present, sampled dogs showed positive results in PCR assays for *A. platys* but not for *E. canis* (82). Further studies are needed in order to investigate the role of other tick species in *E. canis* transmission cycles among dogs and foxes in Portugal.

In Palermo and Ragusa provinces of Sicily, Italy, prevalence rates for *E. canis* (*rrs*) of 31% (4/13) and 3% (3/110) were reported among foxes and associated fleas. While 2 positive fleas were collected from foxes that were also positive for *E. canis*, a third positive flea was collected from a fox that was negative for this pathogen. Although fleas have not been recognized as vectors for Anaplasmataceae agents so far, the detection of *E. canis* in the third flea may suggest that this insect might be involved in the maintenance of *E. canis* (19). Similarly, *Ehrlichia* sp. *rrs*, closely related to *E. canis*, was detected in two *Amblyomma sculptum* ticks, non-recognized vectors for *E. canis*, collected from *Ehrlichia* sp.-PCR positive wild crab-eating foxes in Brazilian Pantanal (12). In this case, PCR-positivity in ticks could be related to the remnant of infected host blood meal (12).

In North America, the detection of *E. canis* and *Anaplasma* sp. in arctic foxes in Canada provides evidence that these tick-borne pathogens, which has not been frequently associated with the arctic ecosystem, may circulate, even at low levels, in that sampled *Vulpes lagopus* population. Even though arctic foxes can occasionally interact with foxes and domestic dogs, these findings may reflect the consequences of direct or indirect human activity on natural environments, such as the global warming that may contribute to the spread of ticks and associated pathogens to this unique ecosystem. Therefore, arctic foxes may act as sentinels for the assessment of climate change on the emergence and eco-epidemiology of tick-borne zoonotic agents (17).

In the African continent, canine monocytic ehrlichiosis was incriminated as cause of death of several wild dogs (*Lycaon pictus*) in Kruger National Park, South Africa, although the confirmation of the aetiological agent was not performed at that time (83). When a black-backed jackal (*Canis mesomelas*) was experimentally infected with *E. canis*, it did not develop clinical signs, but remain a chronic carrier up to 112 days (83). Later, a case of fatal ehrlichiosis in a black-backed jackal following the exposure to ticks was reported in a kennel at the Onderstepoort Veterinary Research Institute, South Africa (84). Wild dogs and black-backed jackals showed to be susceptible to experimental infection with *E. canis*. Even though all experimental infected wild canids showed the presence of morulae in stained blood smears, while the first showed clinical and hematological abnormalities compatible with canine monocytic ehrlichiosis (depression, anorexia, pancytopaenia), the latter showed to be asymptomatic. Wild dogs appeared to be more resistant than domestic dogs, with a longer incubation period, despite higher levels of bacteremia. Moreover, clinical and haemotological abnormalities showed to be less severe and intensive treatment was not required. Besides, the disease was then successfully transmitted from experimentally infected black-backed jackals to domestic dogs (85), confirming the findings described by Neitz and Thomas (83). Then, *E. canis* was detected in eight out of 15 free-living jackals (*Canis mesomelas*) in Kenya, using a modified cell culture test (86). Therefore, jackals have been incriminated as reservoirs for *E. canis* (83, 85). The presence of antibodies to *E. canis* was detected in 34% (14/55) of free-ranging black backed jackals sampled in Kenya (87). Despite these findings, *E. canis* has not been molecularly detected in spotted (*Crocuta crocuta*) and brown (*Parahyaena brunnea*) hyenas, wild dogs, and black backed jackals in Zambia, Namibia and South Africa so far (11, 13, 49, 72). Interestingly, *E. canis rrs* was detected in 1 out of 86 captive lions in Zimbabwe (42).

Although *E. canis* has been molecularly detected in free-ranging raccoons from the USA (1.7%) (78) and Spain (2.6%) (36), Yabsley et al. (52) showed that *Procyon lotor* was not

susceptible to experimental infection with *E. canis*. In the USA, although *D. variabilis*, a recognized vector for *E. canis* (64), has been found parasitizing dogs and medium-sized wild mammals, such as raccoons (52, 61, 78), allowing for *E. canis* inter-species transmission in suburban areas, raccoons seem to play a limited role as a vertebrate reservoir for this agent in the USA (78). Among 60 raccoons sampled in the state of Georgia, molecular and serological evidence of exposure to *E. canis* of 1.7 ands 21.7% were reported (78). Later, all 169 raccoons sampled in peridomestic areas from counties located in the states of Florida and Georgia, USA, showed to be negative in PCR assays for *E. canis*, despite seropositivity rates of 17.4 and 6.9% in the aforementioned states, respectively (52). In Japan, all 187 raccoons sampled in Kanagawa Prefecture showed to be PCR-negative for *E. canis*, despite the serological evidence of exposure to this agent in one animal (51) (**Table 2**). According to Criado-Fornelio et al. (36), *E. canis* may be tick-transmitted between domestic dogs and wild carnivores, including raccoons, and vice-versa in central Spain. In Brazil, among 31 coatis sampled in the Pantanal wetland, 3.2% showed to be seropositive to *E. canis* and presented *E. canis rrs* in blood sample (12). Therefore, the real role of procyonids in the epidemiological cycles of *E. canis* in nature should be further investigated.

Ehrlichia chaffeensis

In the USA, *Ehrlichia chaffeensis*, the causative agent of human monocytotropic ehrlichiosis (HME), is maintained in a complex cycle involving the white-tailed deer (*Odocoileus virginianus*) and the lone star tick (*Amblyomma americanum*), which play a role as its primary reservoir and vector, respectively [reviewed by (88)]. Even though wild tailed deer act as the main host for *E. chaffeensis*, serological and molecular evidence of infection by this agent has been reported in wild carnivores.

Among wild carnivores, *E. chaffeensis rrs* has been detected in free-ranging coyotes (*Canis latrans*) (71%) from the USA, crab-eating foxes (10.2%), and gray wolves (33.3%), little spotted cats (12%), ocelots (13.3%), pumas (25%), jaguarondis (5.3%), lion (8.3%), and tigers (25%) maintained in captivity in Brazilian zoos (9). A genotype showing 97.3% identity to *E. chaffeensis* was detected in badgers (*Meles meles*) (1.5%) and in one (1/2) American mink (*Neovison vison*) from Spain (10) (**Table 2**). Although *Ehrlichia rrs* closely related to *E. chaffeensis* was detected in wild captive carnivores in Brazil (9), additional specific qPCR assays targeting *vlpt* gene of *E. chaffeensis* yielded negative results, emphasizing the possible occurrence of a genotype closely related to, albeit distinct from, *E. chaffeensis* in other regions of the world outside the USA. Considering that additional molecular characterization targeting other genes of these genotypes detected in mustelids from Spain was not performed, the real identity of these agents remains unknown. Considering that badgers' food habit can range from roots and fruits, earthworms, insects to small terrestrial vertebrates (including hedgehogs) and their cadavers, these wild animals may get infected with pathogens circulating in vertebrate tissues. Moreover, since badger populations can reach high numbers in urban environments, the role of these wild carnivores in the epidemiology of tick-borne diseases should be further addressed (10).

Although serological evidence of exposure to *E. chaffeensis* associated to parasitism by ticks including *A. americanum*, the recognized arthropod vector for this agent, has been reported among raccoons from Georgia, USA (28.7–38.3%) (52, 78) and Florida (34.8%) (52), when these wild carnivores were experimentally infected with *E. chaffeensis*, the infection course showed to be transient (52). While *E. chaffeensis* was isolated in cell culture from one experimentally infected raccoon, molecular and serological evidence of infection was reported in two out of the five experimentally infected raccoons (52). Indeed, all 169 raccoons sampled in peridomestic areas from counties located in the states of Florida and Georgia showed to be negative in PCR assays for *E. chaffeensis* (52) (**Table 2**). Therefore, they seem not to play a role as important reservoirs in the enzootic cycles of *E. chaffeensis* due to the transient or lacking rickettsemia observed during the experimentally course of infection (52).

Red foxes (*Vulpes vulpes*), but not gray foxes (*Urocyon cinereoargenteus*), showed to be susceptible to experimental infection with *E. chaffeensis* based on isolation of the agent from blood, seroconversion, and positive PCR results from spleen and lymph nodes samples. However, neither morulae in stained blood smears nor clinical signs were observed in experimentally infected animals. Considering that red foxes are also susceptible to *E. canis* infection (80) and keeping in mind the occurrence of serological cross-reactions among *Ehrlichia* species in the IFA, antibodies to *E. chaffeensis* or other *Ehrlichia* species in foxes should be interpreted with caution.

Ehrlichia chaffeensis vlpt was detected in in 1 of 23 (4.3%) *A. americanum* collected from black bears (*Ursus americanus floridanus*) in Georgia, USA (89). Recently, *E. chaffeensis rrs* was detected in 2/46 (4%) adult *A. americanum* ticks removed from brown bears (*Ursus americanus*) in Oklahoma, southcentral USA. Despite the negative results in PCR assays for *Ehrlichia* spp., all sampled brown bears ($n = 49$) showed to be seropositive to *E. chaffeensis* by IFAT (74).

Ehrlichia ewingii

Although *E. ewingii* DNA has not been detected in wild canids so far, specific antibodies to this agent were detected in 46% (23/50) coyotes sampled in Texas and Oklahoma, USA, using a p28 peptide-based microtiter plate ELISA (79). Indeed, this agent seems to be the most common *Ehrlichia* species in coyotes from areas where *A. americanum* is prevalent (79).

Genotypes Related to *Ehrlichia ruminantium*

A genotype (Strain Jaguar) related to, albeit distantly from, *E. ruminantium* was detected in jaguars (2/10; 20%) and in associated tick species (*A. triste*, *A. sculptum*, and *Amblyomma* sp.) sampled in Pantanal wetland, central-western Brazil, based on *rrs* and *dsb* sequences (76) (**Table 2**). Later, a similar genotype (fox-ES-1) was detected in crab-eating foxes (6/58; 10.3%) in southeastern Brazil (71) (**Table 2**). These two genotypes formed a sister group of the *E. ruminantium* group (71, 76).

Other *Ehrlichia* Genotypes

Recently, *Ehrlichia* sp. HF *rrs* has been detected in specimens of *Ixodes apronophorus* collected from dogs and foxes in Romania (90). This *Ehrlichia* strain has been already detected in *I. ricinus* ticks in France (91) and in dogs, rodents and *Ixodes ovatus* ticks in Japan (92–94). Further studies aiming at investigating the role of foxes as linking mammals carrying ticks from infected rodents in the wild to dogs in suburban areas are necessary in Europe. An *Ehrlichia* sp. *rrs* genotype closely related to *E. chaffeensis/E. muris* was recently detected in an *Ixodes ricinus* female tick collected from a fox in Romania (60).

"Candidatus Neoehrlichia lotoris" (CNL)

Based on a partial *rrs* gene fragment, an Anaplasmataceae agent closely related to "*Candidatus* Neoehrlichia mikurensis" was detected in 53% of tested raccoons (*Procyon lotor*) sampled in the Piedmont region of Georgia, USA (78). This agent was then isolated in ISE6 tick cell culture (95). Even though the culture was able to infect raccoons, detectable infection was not seen in laboratory mice, rats and rabbits (61). Yabsley et al. (96) showed, using phylogenetic analyses based on three target genes (*rrs*, *groEL,* and *gltA*), that this new agent (CNL) isolated from a raccoon in a tick cell line (95) was closely positioned to, but distinct from, TK4456^R and IS58 strains of *"Candidatus* Neoehrlichia mikurensis." Differences in sequences of the three target genes were not found between the strain RAC413^R of CNL and samples obtained from naturally infected raccoons from three states in the USA. No serological cross-reactivity with *E. chaffeensis, E. canis, E. ewingii, A. marginale* and *A. phagocytophilum* antigens was noted in four raccoons experimentally infected with CNL (RAC413^R strain). According to the authors, the lack of cross-reactivity between CNL and other Anaplasmataceae agents might be due to a weak antibody response developed by raccons to CNL antigens.

The fact that neither CNL nor "*Candidatus* Neoehrlichia sp." (FU98) (see below) have been confirmed in populations of raccoons in Europe (15) and Japan (6) support the hypothesis that the narrow specificity of CNL to native populations of raccoons in North American presumably relates to a vector (15).

"Candidatus Neoehrlichia sp." (FU98)

A new agent, so called "*Candidatus* Neoehrlichia sp." (FU98), was detected in a fox spleen sample (0.6%) from Austria. The obtained *rrs* and *groEL* sequences showed to be closely related to the raccoon associated "*Candidatus* Neoehrlichia lotoris" from North America but clearly distinct from the *Ixodes ricinus* transmitted zoonotic "*Candidatus* Neoehrlichia mikurensis" found in Eurasia (97). Later, this agent was also detected in foxes (0.8%) from Czech Republic (7), in an European badger (*Meles meles*) from Hungary (98) and, more recently, in foxes (0.4%) in Western Austria (32) and in raccoon dogs (30%) from Poland (15) (**Table 2**). More studies are necessary in order to isolate this new agent as well as investigate its geographic distribution and host range.

IMPACT OF ANAPLASMATACEAE AGENTS ON WILD CARNIVORES HEALTH AND CONSERVATION

Although *Ehrlichia* and *Anaplasma* infections have been reported in wild carnivores worldwide, few clinical cases of erhlichiosis and anaplasmosis have been described so far in this group of mammals. For instance, an outbreak of canine monocytic ehrlichiosis with high mortality and associated to a high *R. sanguineus* s.l. infestation was reported among wolves, dogs and wolf–dog hybrids in a zoo in Florida, USA (99).

In experimental studies carried out in South Africa, *E. canis* was successfully transmitted from domestic dogs to African wild dogs (*Lycaon pictus*) and black-backed jackals (*Canis mesomelas*). While the latter showed no clinical signs of infection, wild dogs showed clinical (anorexia and depression) and hematological (anemia, leucopaenia and thrombocytopaenia) signs of canine monocytic ehrlichiosis. The success of experimental infection trails was confirmed by the presence of morulae in leucocytes from experimentally infected wild carnivores. Besides, blood samples from jackals showed to be infective to domestic dogs (85). Moreover, coyotes, gray and red foxes showed to be susceptible to experimental infection with *E. canis* (80, 100).

In Italy, foxes naturally infected by *A. phagocytophilum* presented nonspecific microscopic alterations, such as mild lymphoreticular hyperplasia of the splenic follicles primarily localized to cortical areas (18).

A male timber wolf (*Canis lupus occidentalis*) maintained in captivity in an outdoor enclosure in Austria and naturally infected by *A. phagocytophilum* showed clinical (tick infestation, anorexia, depression) and hematological (thrombocytopenia, lymphopenia, mild anemia) signs of canine granulocytic anaplasmosis (101).

Indeed, the real impact of these pathogens on wild carnivores health has been seldom investigated, mainly because these animals have been incriminated as potential reservoirs for these agents. As a consequence, little effort has been made in order to deeply investigate the clinical course of the diseases caused by *Ehrlichia* and *Anaplasma* agents in wild carnivores. However, such sort of studies is much needed before assuming the role of these mammals as reservoirs (29).

COULD THE CONTACT BETWEEN DOMESTIC DOGS AND WILD CANIDS DRIVE A HIGHER EXPOSURE TO *E. CANIS* IN WILD CARNIVORES AND VICE-VERSA?

Susceptible animal population may suffer from generalist pathogens "spill over" from abundant domestic reservoir hosts. In this specific case, control strategies should be directed to the domestic reservoir hosts. Alternatively, when a certain pathogen is transmitted within a threatened population, the effort of control measures over the wild population will favor the health management of the susceptible population rather than taking actions on another sympatric host species (102). Aiming at investigating interspecific and intraspecific transmission routes

of exposure of South African wild dogs to *E. canis* and other selected canine pathogens, (102) analyzed behavioral measures of opportunities for contact between domestic dogs and other wild dogs. As a result, wild dogs presenting higher contact with domestic dogs were at higher risk of exposure to *E. canis*. On the other hand, contact with other wild dogs did not increase their exposure to *E. canis*. Indeed, exposure to *E. canis* was associated with small instead of large pack size. According to the authors, the lack of evidence of higher risk of acquiring *E. canis* within large packs might be explained by the deleterious effect of canine monocytic ehrlichiosis in wild dog puppies, which will promote high mortality rates and, consequently, diminishes the pack size. Similarly, both the increase of wild dog density over time and inter-pack contact was not associated to higher *E. canis* exposure. Therefore, domestic dogs may play a role as reservoir hosts for *E. canis*, since higher exposure to this pathogen was observed when wild dogs were in contact with domestic dogs. Alternatively, *E. canis* might be maintained in low-density wild dog populations, without the involvement of another host species. Keeping in mind that *E. canis* infection does not require direct contact between hosts since it is transmitted by ticks, the contact with domestic dogs would drive an elevation of the prevalence of this agent among wild dog populations rather than being necessary for the pathogen persistence in wild dog packs (102).

Although not confirmed, a decline in wild dog populations in South Africa was suspected to being caused by ehrlichiosis (83). However, despite the report of *E. canis* detection in 8 of 15 free-living jackals (*Canis mesomelas*) in Kenya using a modified cell culture test (86), this agent has not been molecularly detected in wild canids in Africa so far (11, 13, 49, 72). In the presence of higher contact with domestic dogs, and consequently, higher exposure to *E. canis*, a weaknesses status of the wild dog population analyzed in the studied performed by (102) would be expected. In fact, the wild dog packs sampled in the abovementioned study was growing and healthy. According to the authors, the level of contact between wild dogs and domestic dogs might have contributed to the development of immunity in the studied wild dog packs, preventing canine monocytic ehrlichiosis outbreaks and mortality. On the other hand, habitat fragmentation might favor a more frequent contact between both populations and, as consequence, promote higher exposure to *E. canis* and mortality (102).

In another scenario, red foxes *(Vulpes vulpes)* are suspected to play a role as a reservoir in the epidemiology of canine ehrlichiosis in Europe (66). In Hungary, domestic dogs, red foxes and golden jackals (*Canis aureus*) share the same tick species. Due to the spread of its geographic occurrence, golden jackals may act as carriers of Mediterranean ticks toward north Europe. The detection of *E. canis* DNA in *I. canisuga* larvae collected from red foxes in Hungary may represent the missing link between the domestic and sylvatic cycles of *E. canis*, involving foxes as reservoirs and dogs as susceptible hosts (66).

In Israel, similar seroprevalence rates to *E. canis* were reported among free-ranging golden jacks (35.8–54-3%) and stray dogs (37.5%) (103–105). The high seropositivity rates to *E. canis* found among jackals may be due to the ubiquitous presence of *R. sanguineus* s.l. ticks in Israel. Besides, the frequent incursions of golden jackals into urban areas in order to find food leftovers, associated to the close phylogenetic relation between jackals and dogs, may facilitate the spread of some pathogens to domestic dogs (104, 105). Among sampled jackals, three (9.7%) showed thrombocytopenia and, of these, two were seropositive to *E. canis*. Besides, three out of jackals presenting low haematocrit were seropositive to the studied agent. These findings indicated that free-ranging jackals in Israel might act as subclinical carriers of the pathogen (105).

IMPLICATIONS OF ANAPLASMATACEAE INFECTION IN WILD CARNIVORES ON HUMAN AND DOMESTIC ANIMALS HEALTH

Since wild carnivores are free ranging, the odds for being exposed to arthropod-vectors carrying pathogens are expected to be higher than those found among humans and domestic dogs. Therefore, the exposure rate to Anaplasmataceae agents is expected to be higher in wild carnivores than for domestic dogs and cats and humans. In fact, wild sentinels are more likely to reflect changes in ecological patterns of disease occurrence when compared to humans and domestic animals. While the reporting of tick-borne diseases in humans depends mostly on the definition of case reports, the prevalence of such diseases in domestic dogs and cats is more prone to be affected by factors that may mask the real circulation of vector-borne agents, such as the regular application of ectoparasiticides, preventive use of endoparasiticides, vaccination and antibiotic therapy. Moreover, wild carnivore surveillance may better define areas at higher risk for exposure to tick-borne agents, mainly because its wide geographic living range. On the other hand, domestic dogs and cats living area are mostly restricted to areas surrounding the activity zones of their owners (106).

The understanding about the exposure dynamics to tick-borne agents in wild carnivores is a crucial step in order to conduct accurate surveillance programs aiming at defining areas at higher risk of exposure to vector-borne pathogens. For instance, Jara et al. (106) investigated the exposure of gray wolves (*Canis lupus*) from different age groups (puppies, yearlings and adults) to two important tick-borne pathogens for humans and domestic dogs, namely *A. phagocytophilum* and *E. canis*, respectively, in the state of Wisconsin, USA. According to the authors, wolf seroprevalence is higher in adults than puppies for both studied tick-borne pathogens. On the other hand, seroprevalence in yearlings is similar to that one found among adults. Considering that antibodies can last long periods of time since the first exposure, it is more likely that adults show a higher chance to be exposed to certain pathogen during their life span. Besides, keeping in mind that the process of acquisition of Ixodidae ticks is a passive phenomenon, i.e, ticks that will transmit pathogens are sedentary and can be found waiting for vertebrate hosts on grasses and bushes, for example, the exposure to these arthropod vectors are associated to the movement rates of wild carnivores. While puppies are more likely to stay near the den sites, yearlings and adults are more prone to explore

wider geographic areas and, consequently, being exposed to tick infested areas (106). Although white-tailed deer (*Odocoileus virginianus*) and white footed mice (*Peromyscus leucopus*) act as the main hosts for the black-legged tick *Ixodes scapularis* (the vector for *A. phagocytophilum*) and the main reservoirs for *A. phagocytophilum* in the Midwestern and Northeastern USA, respectively [reviewed by (57)], gray wolves can also play a role as hosts for both tick vectors and *A. phagocytophilum* (106).

In order to deal with habitat fragmentation and degradation, several wild carnivore species have developed the ability to adapt themselves to periurban/urban environments. As a consequence, the chances for contact among wild carnivores, domestic animals and humans have risen, facilitating the transmission of vector-borne agents. Because of that, the role that carnivores may play in the epidemiology of tick-borne pathogens of public health and veterinary importance has been investigated worldwide (29).

Foxes are the most widespread and abundant wild carnivores in Europe (1, 24). The red foxes has adapted and become a successful species in the urban environment, mainly due to the availability of food and resting places, lacking of predators, and human tolerance (1). During their excursions into suburban and urban environments looking for food, these wild carnivores are responsible for several troubles, such as predation of chickens and rabbits, fossicking trashcans and damaging gardens (19). As a result, the red fox plays a role as a linking between wild and urban environments. Indeed, its huge population size and widespread abundance make this wild carnivore species an important reservoir for pathogens that infect vertebrates sharing the same areas, including humans (1). Similarly to the red fox, the golden jackal (*Canis aureus*) is a generalist predator that is expanding its geographical distribution, being adaptable to any sort of environment and showing impressive capacity of colonizing different habitats (1).

These wild carnivores may be infested with tick and flea species acquired from associated prey or from other animals sharing the same environment (19). Considering that foxes may carry Anaplasmataceae-infected ticks, they may act as infection source for both domestic dogs and humans (30). Their adaptation to urban environment and human presence make them an important key in the ecoepidemiology of VBP in synanthropic environments (19, 24). Keeping that in mind, carrier wild carnivores may represent a potential source of Anaplasmataceae infection for hunters, professionals working with wildlife (veterinarians, zoo keepers), people residing and working in rural settlements, and people living in urban areas invaded by these animals (30).

Gabriel et al. (59) listed gray foxes (*Urocyon cinereoargenteus*) as good sentinels for *A. phagocytophilum* infections in northwestern California. Gray fox populations can be found at high densities in areas where HGA cases are reported in the USA. Moreover, these wild carnivores can share areas with domestic animals and humans. In a surveillance study conducted in Hoopa Valley Indian Reservation in Humboldt County, northern California, USA, Gabriel et al. (59) observed that the seroprevalence (70%) to *A. phagocytophilum* was higher in backcountry foxes than in urban-zone foxes (39%), which, in turn, showed a similar seroprevalence to that one found among domestic dogs (31%). Therefore, periurban foxes could transport *A. phagocytophilum*-infected ticks from wild environments to urban areas, contributing to tick exposure to domesticated animals and humans. Despite the seroprevalence rates, only 9% out of the 70 fox samples were PCR-positive for *A. phagocytophilum*, including four (13%) of the 30 urban and two (6%) of the 34 backcountry foxes (59).

In the USA, 47 mountain lions (*Puma concolor*) were sampled in Sierra Nevada foothills, northern coast range, and Monterey County in California. Among them, 17 and 16% showed positive results in serology and PCR, respectively, to *A. phagocytophilum* (46). According to the authors, even though wild canids and mountain lions do not play a role as important reservoirs of *A. phagocytophilum,* due to infrequently found PCR positive results, these mammals may be competent sentinels for the detection of this agent. This epidemiological role as sentinel is favored by their relatively large home ranges, long life spans, and common exposure to tick-vectors (46, 59).

Wild carnivores inhabiting natural areas in periurban Barcelona showed to be infested and infected, respectively, by ticks and fleas and vector-borne pathogens that infect dogs and cats, albeit with a higher frequency of infestation/infection. The contact between wild carnivores and domestic dogs may occur during scent communication behavior or during prey sharing. When it comes to transmission of vector-borne pathogens, the lack of necessity for direct contact between infected carrier wild carnivores and domestic dogs draw a favorable scenario for this group of pathogens (29).

Based on *groEL* sequences, (22) grouped *A. phagocytophilum* isolates from Europe in four ecotypes. Although ecotype I showed the widest host range, birds and rodents isolates were not found circulating in this ecotype. Considering that the *A. phagocytophilum* sequences isolated from red foxes and humans were found in this ecotype highlight the fact that some genotypes in this cluster are zoonotic. According to the authors, the generalist feeding behavior of *I. ricinus* nymphs and adults, a recognized vector for *A. phagocytophilum* in Europe, may facilitate the spread of ecotype I among different vertebrate host species, including humans. In Germany, *A. phagocytophilum ankA* sequences obtained from raccoons and red foxes clustered in *ankA* gene cluster I (107), which contains *A. phagocytophilum* strains from humans, dogs, and horses (21). Recently, *A. phagocytophilum groEL* sequences detected in red foxes in Austria showed to belong to a G-variant previously detected in humans, and domestic and wild animals (32).

Although foxes may play a limited impact on the circulation of emerging zoonotic *A. phagocytophilum* due to low to moderate infection rates (ranging from 0.5 to 16.6%), these wild carnivores may still represent a potential source of human infections (21, 24, 32) and a risk for the urbanization of the *A. phagocytophilum* life cycle (26). Molecular phylogenetic assessments from previous studies conducted in Europe evidenced that foxes were infected with the same strains of *A. phagocytophilum* previously reported in human patients (21, 22, 32).

Even though wild carnivores have been incriminated as sentinels for Anaplasmataceae agents in natural environments,

as well as in degraded and periurban areas (29, 31) emphasized that the molecular screening of TBPs in vector ticks represents a more efficient system than the screening of foxes as sentinel animals in the specific epidemiological context of northeastern Italy (Belluno Province). Despite the detection of *A. phagocytophilum* and "*Candidatus* Neoehrlichia mikurensis" in *Ixodes ricinus* nymphs and adults, all sampled foxes ($n = 97$) were negative to Anaplasmataceae agents. Recently, *A. phagocytophilum msp-2* sequence was detected in one (2.3%) out 43 *Ixodes ricinus* collected from 90 foxes in Slovakia (108). Besides, *A. phagocytophilum rrs* was detected in *I. ricinus* ticks collected from two foxes in Romania (60, 90).

A positive role of golden jackals (*Canis aureus*) in the ecosystems has been reported based on the services these animals provide by removing a substantial amount of animal waste through their diet. However, the recent detection of *A. phagocytophilum* (0.2%) in these animals in Serbia brought up the fact that these wild carnivores may play a role as potential carriers of vector-borne zoonotic pathogens (8). In Israel, a seroprevalence of 26% to *A. phagocytophilum* was detected among a population of 53 free-ranging golden jackals; 5.7% of which were only seropositive to *A. phagocytophilum,* without any seroreactivity to either *E. canis* or *E. chaffeensis* (104). Considering that jackals are scavengers and usually are seen invading urban areas and feeding on garbage, there is a real risk of these wild canids carry *A. phagocytophilum*-infected ticks to the urban area, favoring the transmission of this agent to domestic dogs and human beings (104).

Similarly, bears may share living areas with wild and domestic canids. Likewise wild canids and raccoons, black bears have adapted to living in proximity to humans, which can result in trash cans rummage, car strikes and higher probability of pathogens transmission. In fact, molecular prevalence rates for *A. phagocytophilum* ranging from 3 to 10% in black bears in the USA (37–39) to 24% in brown bears in Europe (40) have been reported. On the other hand, this agent was not detected in 86 ticks collected from 17 black bears in the state of Lousiania, USA (109). The low levels of infection in bears in the USA may indicate a spillover phenomenon for this pathogen (39). While a seroprevalence rate of 26% (54/210) to *A. phagocytophilum* was reported among a black bears population in California, USA, a higher seroprevalence rate (65.2%) was reported among brown bears in Slovenia, central Europe (110). These results emphasize the need of a better surveillance of black and brown bears as additional potential reservoirs for this zoonotic agent in the USA and Europe (38, 40).

The Role of Coyotes and Raccoons in the Epidemiological Cycles of *Ehrlichia chaffeensis* in the USA

In the USA, although white-tailed deer (*Odocoileus virginianus*) and *Amblyomma americanum* are incriminated as the main reservoir and vector hosts, respectively, for *E. chaffeensis*, coyotes (*Canis latrans*) can play a role as bridge mammals in spreading this tick-borne agent. This key position in the epidemiological cycle of human monocytic ehrlichiosis in the USA is due to their following biological features: they act as vertebrate hosts for all *A. americanum* stages, show a wider geographic home range (31 km) when compared to WTD (1.6 km), and are susceptible to *E. chaffeensis* infection. Therefore, these wild canids may contribute to the dispersion of *E. chaffeensis*-infected ticks in the environment, which may subsequently feed on domestic animals and humans (111).

Raccoons (*Procyon lotor*), besides being abundant, show a wide geographical range in the USA. Indeed, they can be found in distinct ecologic niches. Considering the high numbers of raccoons are usually found in urban and suburban areas, they can easily may be in contact with domestic animals and humans (52). Although raccoons have been found naturally infected by *E. canis* (78), they seemed not to be susceptible to experimental infection with *E. canis* and *E. ewingii* (52). On the other hand, these mammals were susceptive to *E. chaffeensis* in experimental trials. *E. chaffeensis*-experimentally infected raccoons showed a transient pattern of infection: only two of three exposed raccoons became infected. *A. americanum* nymphs did not acquire the pathogen when fed on a single infected raccoon (52).

The Role of Raccoons in the Epidemiological Cycles of *Anaplasma phagocytophilum* in the USA and Europe

Raccoons have been incriminated as an important reservoir for *A. phagocytophilum* in the eastern United States. A dual pattern of infection was observed when raccoons were experimentally infected by two different *A. phagocytophilum* strains: while a long-lasting infection (at least 76 days) was observed in raccoons infected by a human isolate of *A. phagocytophilum*, a transient infection was found in raccoons experimentally infected by a white-tailed deer isolate of *A. phagocytophilum.* Despite the source of *A. phagocytophilum* strains, both group of experimentally infected raccoons seroconverted (52).

Besides being usually exposed to *A. phagocytophilum* in the environment, raccoons are highly susceptible to and proved to be able to transmit the agent to competent ticks. In fact, when compared to white-footed mice (*Peromyscus leucopus*), which also acts as a remarkable reservoir host for *A. phagocytophilum* in the USA, raccoons play an important role in the amplification of *A. phagocytophilum* infection in engorged nymphs (50). A plethora of reasons supports the hypothesis that raccoons contribute to the maintenance of *A. phagocytophilum* in the environment (50):

1. Raccoons have been found more parasitized by *A. phagocytophilum*-infected ticks when compared to white-footed mice. This observation is mainly due to a higher infestation density in this procyonid species, which comprises medium-sized mammals, comparatively to mice that encompass small-sized mammal specimens.
2. Higher seropositivity rates to *A. phagocytophilum* were observed in raccoons when compared to those found among white-footed mice. This difference in *A. phagocytophilum* exposure rate can be mainly attributable to a higher infestation of *A. phagocytophilum*-infected ticks in raccoons. In the

second, the higher replacement rate in the mouse population can also affect the rates of seropositivity among white-footed mice.
3. Higher chances (almost twice) of capturing *A. phagocytophilum*-bacteremic raccoons were observed in field works when compared to those found for white-footed mice. This finding is mainly due to higher exposure rates to the agent, and/or to higher levels of bacteremia in raccoons.
4. Raccoons seem to amplify the *A. phagocytophilum* infection in the tick population. The prevalence of infection in ticks feeding on raccoons was higher than in those fed on white-footed mice and in questing ticks collected at the same location.
5. *A. phagocytophilum* DNA was detected in nymphs fed as larvae under similar proportions of tested raccoons and white-footed mice.
6. Nymphal ticks that fed as larvae upon raccoons transmitted *A. phagocytophilum* to naive mice.

Raccoons, originally from North America, are considered invasive species in Europe. Their expansion and harm effect on native fauna have raised fervent discussions surrounding this issue, mainly because of the lack of natural enemies and their fast pacing growing, making their control virtually impossible. Although little is known about the role of raccoons in the *A. phagocytophilum*-epidemiological cycles in Europe, a low frequency (0.8%) of infection has been recently reported among a population of raccoons sampled in Hungary (15).

FINAL REMARKS AND FUTURE DIRECTIONS

As shown in this work, the majority of molecular studies performed around the world aiming at detecting and characterizing Anaplasmataceae agents in wild carnivores used *rrs* as the only target gene for both screening and/or molecular characterization. The referred gene has been extensively used for molecular investigations on Anaplasmataceae agents due to its high conservation. Whether, on one hand, it allows the detection of new genotypes in wildlife, on the other hand, the phylogenetic assessment based on short *rrs* gene fragments does not provide sufficient genetic discrimination to allow the identification of *Ehrlichia* and *Anaplasma* species (9, 12). Moreover, previously described PCR protocols used for amplification of target genes other than *rrs* have been proven unsuitable for amplification of variants of *Anaplasma* and *Ehrlichia* species infecting wild mammals in Brazil, as previously reported (9, 12, 112).

The low bacteremia level in non-reservoirs wild carnivore blood or spleen samples often results in variable amplification of different target genes, precluding accurate prevalence investigations and phylogenetic assessments (12). Moreover, the primer sequences designed for more variable genes can be too dissimilar to anneal properly in the genic regions from newly discovered genotypes (113). Regarding the screening of wild carnivores populations for Anaplasmataceae agents, sensitive and specific broad range quantitative real-time PCR assays are desirable, instead of species-specific qPCR assays for an individual *Ehrlichia* or *Anaplasma* species. Recently, a sensitive and specific duplex qPCR assay targeting *groEL* sequences of *Anaplasma* and *Ehrlichia* species was designed (112) and used in the molecular screening for this group of agents in wild carnivores in Brazil (12). The performance of such assay in catching different variants of *Ehrlichia* and *Anaplasma* in other geographical regions should be further addressed.

Attempts to molecularly characterize new Anaplasmataceae genotypes in wild carnivores based on more evolving target genes are imperative in order to assess the real identity of the new strains. For instance, while *rrs*-based phylogenetic inference grouped the *Ehrlichia* sequences found in wild felids in Brazil together with the *E. canis* clade, the analysis based on *omp-1* gene positioned the same samples in a different and unique clade, indicating the circulation of another species/genotype in these wild carnivores in South America (75).

In order to deal with low bacteremia in biological samples, the isolation of new *Ehrlichia* and *Anaplasma* strains in mammal or tick-derived cell lines is needed aiming at describing a new species. For instance, the ultrastructure and an accurate molecular characterization and phylogenetic positioning of "*Candidatus* Neoerlichia lotoris" from raccoons from the USA (based on *rrs*, *groEL* and *gltA* genes) (95, 96) and *Ehrlichia minasensis* nov. sp. (*rrs*, *groEL*, *dsb*, *gltA* and *trp36* genes) (114) from a *Rhipicephalus microplus* tick from Brazil was only possible after the isolation of these agents in ISE6 and IDE8 cell lines, respectively. As far as we are concerned, no *Ehrlichia* and *Anaplasma* strain has been isolated from wild carnivores around the world so far. Moreover, the isolation of "*Candidatus* Neoerlichia lotoris" in tick cells lines provided enough amount of the pathogen to assess the susceptibility of raccoons and rodents to this new described agent (52). The isolation of new species/genotypes of *Ehrlichia* and *Anaplasma* would also provide substantial amount of antigen, allowing the standardization of serological assays for investigating the exposure of wild and domestic animals and humans to these new agents. In parallel, the isolation of new Anaplasmataceae agents from wild carnivores followed by Whole Genome Sequencing (WGS) will contribute to decrypting the complexity of α-Proteobacteria in wildlife.

The understanding of the epidemiology of canine monocytic ehrlichiosis would benefit from the genotyping of *E. canis* strains found in wild canids and domestic dogs by sequencing the TRP36, a major immunoreactive protein used for investigating the genetic diversity of *E. canis* strains based on differences in tandem repeat number or sequences (115). Would circulate the same TRP-36 genotypes in red foxes (or jackals) and domestic dogs in periurban areas where an overlap of the ecological niches of these canid species is observed? Would there be a predisposition of certain *E. canis* genotypes for species of wild carnivores? *E. canis* genotypes found in humans would be more related to those found in wild or domestic canids? Recently, De Sousa et al. (12) failed to detect the *trp36* sequence in *rrs*-*E. canis* positive blood samples from domestic dogs, crab-eating foxes and coatis from Brazilian Pantanal, which precluded additional genotyping. On the other hand, a new *E. canis*-TRP36 genotype

detected in blood donors in Costa Rica showed to be closely related to those detected in Brazilian domestic dogs (69).

Although *E. canis*-like *rrs* and/or *dsb* sequences have been detected in domestic cats from the USA (116), Brazil (117, 118), Portugal (119), and Angola (120), and wild felids from Brazil (9, 75) and Zimbabwe (42), the agent has not been isolated yet, hampering a more deep genetic and antigenic characterization of this agent in felids. The isolation and TRP36-genotyping would shed some light to the genetic diversity of these *E. canis*-like strains, aiming at clarifying if the occurrence of *E. canis* in domestic and wild felids would represent a "spill-over" from infected dogs in endemic areas for canine monocytic ehrlichiosis.

Similarly, the genotyping of *A. phagocytophilum* strains circulating in wild carnivores based on different genetic markers (*rrs*, *groEL,* and *ankA* genes and 23S-5S rRNA intergenic spacer) (113) will contribute to the assessment of the evolutionary distance among *A. phagocytophilum* clusters formed by strains found in wild carnivores, domestic and wild mammals, ticks and humans, contributing to the understanding of the role of wild carnivores in the epidemiology of human granulocytic anaplasmosis. In this respect, Stephenson et al. (121) designed a qPCR assay targeting the *ank* gene of *A. phagocytophilum*, which showed sensitivity and specificity for the p-Ap genospecies (pathogenic *A. phagocytophilum* strains detected in dogs, horses and humans in the USA), differentiating them from the apparently non-pathogenic DU1 genospecies (found in woodrats [*Neotoma fuscipes*] and bears from California). However, this assay showed cross-reaction with Ap-Variant 1, which is found in deer and goats in the USA. The designed assay will contribute to the screening of wild animals, including carnivores, and vectors in the USA. In areas where an overlap of DU1 and Ap-Variant 1 genospecies is expected, an additional PCR assay to differentiate the latter from p-Aph (122) is needed (121). The "distantly related to human marker" (*drhm*) gene locus has been proposed as a virulence marker for *A. phagocytophilum* isolates. This association was mainly due since this locus was not found in human and dog isolates (123); besides, canine granulocytic anaplasmosis resembles most of the disease aspects of the human anaplasmosis. In western USA, carnivores (dogs, bears and gray foxes) showed both *drhm*-positive and negative strains. On the other hand, virulent strains detected in dogs, humans and horses in eastern USA lacked this locus. Although *drhm* did not seem to indicate host-tropism of *A. phagocytophilum* strains, it may be used as a phylogeographic marker in association with other genes (124).

The expanding universe of Anaplasmataceae agents in wild carnivores and their associate ticks will benefit from the use of next-generation sequencing (NGS). This technology has been used for both detecting tick-borne pathogens and understanding the interactions between pathogenic and non-pathogenic microorganisms (commensals and symbionts) associated to ticks (125) and vertebrate hosts (126). Based on these approaches (which can be performed using several platforms, such as Sanger sequencing of full-length *rrs*, 454-pyrosequencing, Ion torrent, Illumina-sequencing of *rrs* hypervariable regions, etc.), an astonishing diversity of microorganisms has been identified in ticks (125). The main advantage of NGS over PCR-based methods is the use of a detection system that is not biased toward specific microorganisms. Therefore, NGS in association with network analysis will shed some light into the composition of microbial communities associated to ticks and wild carnivores, contributing to the understanding of the role of these hosts in the epidemiology of anaplasmosis, ehrlichiosis and other tick-borne diseases. For instance, Ge et al. (126), when exploring the composition of bacterial community in wild mice and shrews' spleen tissues from Chongming Island, China, found that *Anaplasma*, *Rickettsia* and *Coxiella* were adjacently clustered by hierarchical analysis. Besides, *Anaplasma*-infection was associated with a specific composition of microorganisms in rodents' spleen tissues.

In this review, molecular and serological evidence of infection by Anaplasmataceae agents was reported in wild carnivores around the world, including in even unexpected areas, such as in the arctic ecosystem (17). While abundant wild carnivores (foxes and golden jackals) may act as reservoirs for these agents, those life-threatened (wolves and wild cats) or presenting limited population size (arctic foxes and Iberian lynxes) and are less prone to play important role in the maintenance of endemic sylvatic life cycles (1). Due to increase in numbers and expansion of geographical ranges, the first group of carnivores, which can be represented by red foxes, may be responsible for the transmission for tick-borne agents to domestic animais and humans, mainly in periurban and urban areas (3).

Since a plethora of *Ehrlichia* and *Anaplasma* agents has been detected in both captive and free-ranging wild carnivores, zoos and conservation institutions should be aware of the circulation of these pathogens in these mammals. Even though free-ranging wild carnivores would likely have a much greater risk of exposure to potentially different pathogens than captive animals, the later are potentially exposed to different pathogens due to proximity to other captive and commensal species that may not be encountered in the wild, despite the use prophylactic measures aiming at avoiding ectoparasites infestation. Therefore, surveillance studies in zoos and safari parks, which comprise important piece in conservation, reproduction and recovery strategies, should be also stimulated. The knowledge of the circulation of Anaplasmatceae agents in wild carnivores should be in the biosecurity list of zoos and conservationist institutions, aiming at achieving their goals of preservation and recovery programs. Special attention should be directed to old or immunologically compromised wild carnivores, mainly those whose population has declined (44). Translocation procedures aiming at establishing new populations or reinforcing existing ones in certain areas, release of captive carnivores into the wild, transference of animals between zoos and maintenance of carnivores in rehabilitation centers can favor the spread of tick-borne *Ehrlichia* and *Anaplasma* agents in non-endemic areas. The real consequence of the introduction of new species/strains into naïve populations is still unknown. Management procedures involving wild carnivores can also predispose to a recrudescence of subclinical Anaplasmataceae infection due to stress-mediated immunosuppression (3).

Despite the considerable progress in the identification of tick-borne Anaplasmataceae agents in wild carnivores, a lot

of "gaps" still needs attention in order to draw a clearer picture of the epidemiology of these α-Proteobacteria in wildlife, such as: the real identity of the newly *Anaplasma* and *Ehrlichia* genotypes described in wild carnivores in South America and Africa; the identification of vectors and reservoirs involved in the transmission cycles of "*Candidatus* Neoehrlichia sp." (FU-98) in Europe and those associated to the new genotypes of Anaplasmataceae agents in South America and Africa; the vectors involved in the transmission cycles of "*Candidatus* Neoehrlichia lotoris" among raccoons in North America; the search for additional transmission routes (transplacental, mechanic, ingestion of infected tissues or ticks, etc.) of these agents among wild carnivores; the confirmation of a certain wild carnivore species as "true" reservoir for a selected Anaplasmataceae agent by carrying out experimental infection followed by xenodiagnosis with known competent vectors; studies aiming at investigating the pathogenicity of new species/*Candidatus* agents; the development of serological assays using the new species/*Candidatus* of *Ehrlichia* and *Anaplasma* antigens in order to perform serosurveillance studies on these agents in humans and wild and domestic animal populations as well as investigating the occurrence of serological cross reactions with other Anaplasmataceae agents; the standardization of MLST (*Multi Locus Sequence Typing*) for *Anaplasma* and *Ehrlichia* aiming at improving the phylogenetic assessment of these agents detected in wild carnivores; an extending use of WGS of *Anaplasma* and *Ehrlichia* strains circulating in wild carnivores; the understanding of the pathobiome associated to the community of Anaplasmataceae agents in both ticks and wild carnivores tissues using NGS.

CONCLUSIONS

- Red foxes are hosts for *Anaplasma* spp. (*A. phagocytophilum, A. ovis, A. platys*), *E. canis* and "*Candidatus* Neoehrlichia sp." (FU98 strain) and may contribute to the maintaenance of *A. phagocytophilum* in Europe;
- Raccoons are hosts for *E. canis, A. bovis*, "*Candidatus* Neoehrlichia lotoris'e" *A. phagocytophilum*, and play a role in the maintenance of *A. phagocytophilum* in the USA;
- Raccoon dogs may play a role as hosts for *A. bovis* and *A. phagocytophilum*;
- New *Ehrlichia* and/or *Anaplasma* genotypes circulate in wild canids and felids from South America and Africa;
- While *Ehrlichia* sp. closely related to *E. canis* has been reported in wild felids from Brazil and Japan, *Anaplasma* sp. closely related to *A. phagocytophilum* has been detected in wild felids from Brazil and Africa;
- Red foxes and mustelids (otters) are exposed to *E. canis* in countries located in the Mediaterranean basin (Portugal, Spain and Italy), probably as a consequence of spillover from domestic dogs. Similarly, *E. canis* occurs in procyonids in North (raccoons in USA, Spain) and South (coatis in Brazil) Hemispheres, in areas where *E. canis* is frequent in dogs;
- While "*Candidatus* Neoehrlichia lotoris" seems to be a common and specific agent of raccoons in the USA, "*Candidatus* Neoehrlichia sp." (FU98 strain) seems to show a broader range of hosts, since it has been detected in red fox, golden jackal and badger in Europe so far;
- Brown and black bears seem to play a role as hosts for *A. phagocytophilum* in the North Hemisphere.
- *bovis* has been detected in wild Procyonidae, Canidae and Felidae in Asia and Brazil.

AUTHOR CONTRIBUTIONS

MA designed the conceptualization and write, review and edited the original draft of the manuscript.

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MINI REVIEW
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Worldwide Presence and Features of Flea-Borne *Rickettsia asembonensis*

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Rickettsia asembonensis, the most well-characterized rickettsia of the *Rickettsia felis*-like organisms (RFLO), is relatively unknown within the vector-borne diseases research community. The agent was initially identified in peri-domestic fleas from Asembo, Kenya in an area in which *R. felis* was associated with fever patients. Local fleas collected from domestic animals and within homes were predominately infected with *R. asembonensis* with <10% infected with *R. felis*. Since the identification of *R. asembonensis* in Kenya, it has been reported in other locations within Africa, Asia, the Middle East, Europe, North America, and South America. With the description of *R. asembonensis*-like genotypes across the globe, a need exists to isolate these *R. asembonensis* genotypes in cell culture, conduct microscopic, and biological analysis, as well as whole genome sequencing to ascertain whether they are the same species. Additionally, interest has been building on the potential of *R. asembonensis* in infecting vertebrate hosts including humans, non-human primates, dogs, and other animals. The current knowledge of the presence, prevalence, and distribution of *R. asembonensis* worldwide, as well as its arthropod hosts and potential as a pathogen are discussed in this manuscript.

Keywords: *Rickettsia*, *Rickettsia asembonensis*, flea-borne, worldwide distribution, arthropod hosts, *Rickettsia felis*-like organisms

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INTRODUCTION

Rickettsia asembonensis is a Gram negative, obligate intracellular bacteria of the order Rickettsiales and family Rickettsiaceae (1). Among *Rickettsia* spp. with validly published names, it is most closely related to *R. felis* (**Table 1**) (4–6, 8, 9, 11, 13–15, 17–19, 30). However, among incompletely characterize rickettsiae, *R. asembonensis* genetically groups with other *R. felis*-like organisms (RFLO). The RFLOs are genetically related to *R. felis* but consist of a unique group of rickettsiae that are associated with various arthropods including fleas, ticks, mites, and tsetse flies for which limited knowledge of their biology and pathogenicity is available (3, 16, 31). Unfortunately, the genetic information of the majority of RFLOs in the GenBank database is fragmentary. Of the RFLOs described, only *R. asembonensis* (32) and "*Candidatus* Rickettsia senegalensis" (3) have been cultured (from *C. felis*) and characterized.

Other flea-borne rickettsiae include, besides the aforementioned *R. felis* and "*Ca.* R. senegalensis," *Rickettsia typhi*, a member of the typhus group of rickettsiae (TGR). *R. typhi* is the causative agent of murine typhus, a febrile disease that is found throughout the world. *R. typhi* is vectored by various flea species-especially *X. cheopis*, but also other *Xenopsylla*

TABLE 1 | Worldwide distribution of *Rickettsia asembonensis* and closely related, incompletely characterized rickettsiae.

Rickettsial agents	Source	Country	Location	Sequence comparison with R. *asembonensis* NMRCii (%)						Year in GenBank	References
				rrs	gltA	om pA	om pB	sca4	17kDa		
Rickettsia felis URRWXCal2	*Ctenocephalides felis*	USA	California. El Labs at Soquel	99.5	98	92.5	94.7	95.7	97.3	1999	(2)
"*Candidatus* Rickettsia senegalensis"	*Ctenocephalides felis*	Senegal	Dakar	99.4	98	-	94	94.7	-	2013	(3)
Rickettsia sp. RF2125	*Ctenocephalides canis*	Thailand	Sangkhlaburi District, Kanchanaburi province	–	99.3	–	99.7	–	–	2002	(4)
Rickettsia sp. cf1and5	*Ctenocephalides felis*	USA	Greenville County, South Carolina	–	99.5	–	–	–	100	2005	(5)
Rickettsia sp. SE313	*Echidnophaga gallinacea*	Egypt	Mansoura, Zagazig	–	99.7	–	–	–	100	2005	(6)
Rickettsia sp. cf9	*Ctenocephalides felis*	USA	Not provided	–	–	–	99.9	99.8	–	2006	Reeves et al., (Unpubl.)
Rickettsia sp. FS27	*Orchopeas horwadi*	USA	not provided	–	99.7	–	–	–	99.7	2006	Reeves et al., (Unpubl.)
Rickettsia sp.	*Ornithonyssus bacoti*	Egypt	Ebshaway, El Quseir, Qara Oasis, Zagazig, Arab El Maamal	–	–	–	–	–	100	#	(7)
Uncultured R. sp. Clone Hf56–2	*Archaeopsylla erinacei*	Germany	Bavaria	–	–	–	100	–	–	2008	(8)
Uncultured R. sp. Clone ARV5606	*Ctenocephalides felis*	Peru	Iquitos	–	99.7	–	–	–	99.7	2009	(9)
Rickettsia sp. RF2125	*Pulex irritans*	Hungary	Various parts of the country, specific information not provided	–	>99.3	–	–	–	–	#	(10)
Rickettsia sp. R14	*Ceratophylus fasciatus*	India	Not provided	–	99.7	–	99.9	–	–	2010	Chahota et al., (Unpubl.)
R. endosymbiont of *C. felis* isolate F143	*Ctenocephalides felis*	Thailand	No specific information provided (45 Districts)	–	99.7	–	–	–	100	2011	(11)
R. endosymbiont of *C. felis* isolate F144	*Ctenocephalides felis*	Thailand	No specific information provided (45 Districts)	–	99.7	–	–	–	100	2011	(11)
Rickettsia sp. clone 4-G/G/JP-10-2	*Ctenocephalides felis*	Costa Rica	Limon (Guacimo)	–	99	–	–	–	–	2011	(12)
Rickettsia sp. 'Synosternus'	*Synosternus pallidus*	Senegal	Dielmo	–	100	–	100	–	–	2011	(13)
Rickettsia asembonensis F30	*Ctenocephalides canis*	Kenya	Nyanza	100	100	99.8	99.9	100	100	2011	(14)
Rickettsia asembonensis F82	*Ctenocephalides felis*	Kenya	Nyanza	100	–	99.9	–	100	100	2011	(14)
Uncultured *R.* sp. Clone HL2a	*Ctenocephalides felis*	Malaysia	Kuala Lumpar, Selangor	–	99.7	–	–	–	–	2013	(15)
Rickettsia sp. RFLO-18	*Ctenocephalides felis*	Thailand	Was not deposited in the GenBank	–	–	–	99.7	–	–	#	(16)

(Continued)

TABLE 1 | Continued

Rickettsial agents	Source	Country	Location	Sequence comparison with R. *asembonensis* NMRCii (%)						Year in GenBank	References
				rrs	gltA	om pA	om pB	sca4	17kDa		
Rickettsia sp. J28p	*Ctenocephalides felis*	Peru	Not provided	–	99.7	–	–	–	–	2015	Palacios-Salvatiera et al., (Unpubl.)
Rickettsial strain from *C. felis*	*Ctenocephalides felis*	Ecuador	Pastaza	99.9	99.7	–	100	100	100	#	(17)
Rickettsia sp. Clone Xr	*Xenopsylla ramesis*	Israel	Negev	100	99.7	100	100	–	100	2014	(18)
Rickettsia sp. 9AL	*Ctenocephalides felis*	Colombia	Villeta	100	100	–	100	–	–	2014	(19)
Rickettsia sp. 0095	*Macaca fascicularis*	Malaysia	Not provided	–	100	–	99.9	–	–	2014	(20)
Rickettsia sp. Clone Mal	*Homo sapiens*	Malaysia	University Malaya Medical Center	–	99	–	99.9	–	–	2015	(21)
Uncultured *Rickettsia* sp. Isolate F1	*Ctenocephalides felis*	South Africa	Mpumalanga Province	100	–	–	–	–	–	2015	(22)
Rickettsia asembonensis 0-TP-1	*Ctenocephalides felis*	Costa Rica	Cahuita, La Virgen, Limon, Tulialba, Guapiles	–	99.7	–	–	–	–	2016	(23)
Rickettsia asembonensis 6-CP-4-3	*Pulex simulans*	Costa Rica	Cahuita, La Virgen, Limon, Tulialba, Guapiles	–	99.7	–	–	–	–	2016	(23)
Rickettsia asembonensis 6-CP-4-4	*Ambylyomma ovale*	Costa Rica	Cahuita, La Virgen, Limon, Tulialba, Guapiles	–	99.7	–	–	–	–	2016	(23)
Rickettsia asembonensis CF26B/US	*Ctenocephalides felis*	USA	Orange County, California	99.9	99.7	99.9	99.9	100	–	2016	(24)
Rickettsia asembonensis Tapes	*Rhipicephalus sanguineus*	Brazil	Tapes	–	99.6	–	–	–	100	2016	(25)
Rickettsia sp. Clone SP003-M	*Ctenocephalides orientis*	Malaysia	Kuala Lumpur, Perak, Johore, Kelantan, Pahang, Negeri Sembilan	–	99.2	–	–	–	–	2016	(26)
Rickettsia asembonensis DB32B	*Rhipicephalus sanguineus*	Malaysia	Kuala Lumpur, Selangor, Pahang	–	99.6	–	–	–	–	2017	(27)
Rickettsia asembonensis CF#68	*Ctenocephalides felis*	Brazil	Maranhao State	–	99.6	–	99.9	–	100	2017	(28)
Rickettsia asembonensis F30	*Ctenocephalides felis*	Uganda	Southwestern Uganda	–	100	–	99.6–100	–	–	#	(29)
Rickettsia asembonensis 7.2	*Ctenocephalides felis*	USA	Galveston, Texas	–	–	–	–	–	100	2018	Quade et al., (Unpubl.)
Rickettsia asembonensis VGD7	*Ctenocephalides felis*	Peru	Peruvian Amazon	–	99.8	99.8	100	99.8	100	2017	(30)

Rickettsia felis and Candidatus Rickettsia senegalensis are provided as reference rickettsiae that are closely related to but distinct from Rickettsia asembonensis.

species such as *X. astia* and *X. brazilliensis* (33, 34), *Synosternus pallidus,* and rarely, but importantly, *Ctenocephalides felis* the common cat flea that readily parasitizes cats, opossums, and other domestic, peri-domestic, and wild animals. *C. felis* is believed to be capable of hosting *R. typhi* and to vector murine typhus in areas outside the traditional range of rat fleas and rats (35, 36).

R. felis, R. asembonensis, and "*Ca.* R. senegalensis" fall within the spotted fever group rickettsiae (SFGR) that genetically clusters within the transitional group of rickettsiae (37). *R. felis* is associated with flea-borne spotted fever (38, 39) and the pathogenicity of *R. asembonensis* and "*Ca.* R. senegalensis" is currently unknown. These three agents have worldwide distribution, are often sympatric and most often found parasitizing cat and dog fleas (3, 4, 14, 38, 40, 41).

"*Candidatus* R. senegalensis" was first described in *C. felis* fleas from Senegal (3) and an agent believed to be "*Ca.* R. senegalensis"-like (*Rickettsia* sp. RF31) had been detected previously in *C. felis* near the Thailand-Myanmar border (4). A very close genetic relationship (99.9% based on *gltA* gene sequence) between *Rickettsia* sp. RF31 and the latter is notable (3). "*Ca.* R. senegalensis" is distinct from, but can be sympatric with, *R. felis* and *R. asembonensis* (40). It has worldwide distribution but is not reported as often as *R. felis* or *R. asembonensis*. Reports of its molecular presence in cat tissues suggests it may be able to infect vertebrate animals (41).

HISTORY OF *RICKETTSIA ASEMBONENSIS*

Incompletely characterized rickettsiae with various identities most closely related to *R. asembonensis* populated the literature in the early 2000s (**Table 1**). These agents were detected by molecular techniques [i.e., PCR, nested PCR (nPCR), and/or quantitative real-time PCR (qPCR)] and then characterized by sequencing different size fragments of one or more commonly used gene targets (*rrs, gltA, ompA, ompB, sca4*, or the 17 kDa antigen gene). The first agent, referred to as *Rickettsia* sp. RF2125, was detected in *Ctenocephalides canis* in western Thailand near the Myanmar border (4). The agent was characterized by the sequence of a 1,171 bp fragment of the *gltA* that showed the rickettsial agent to be unique but most closely related to *R. felis* (4). The sequence of a 790 bp fragment of *ompB* (JX183538) from the original *Rickettsia* sp. RF2125 DNA preparation was obtained at that same time as the *gltA* but was not reported in the original article (4). It was reported in 2013 (14). We believe that RF2125 may have been the first detection of *R. asembonensis* or a very similar agent. Additional reports of *R. asembonensis* or an agent closely related to it continued to occur worldwide (**Figure 1**) shortly thereafter including: *Rickettsia* sp. cf1 and 5, USA (5); *Rickettsia* sp. SE313, Egypt (6); *Rickettsia* sp. Hf56-2, Germany (8); *Rickettsia* sp. ARV5606, Peru (9); and *Rickettsia* sp. Synosternus, Senegal (13). These partially characterized agents were described prior to our complete characterization of *R. asembonensis* (1). These agents are summarized along with *R. asembonensis* to include their distribution, vector hosts, and genetic characterization (see **Table 1**).

R. asembonensis was initially described as an unknown *Rickettsia* sp. detected in various flea species (i.e., *C. felis, C. canis, Echidnophaga gallinacean, X. cheopis,* and *Pulex irritans*) collected from various domestic animals (i.e., dogs, cats, and rodents) and houses (by light traps) in Asembo, Kisumu, in western Kenya during an epidemiologic surveillance study (14). This study was conducted concurrently with a fever study in which the presence of *R. felis* was identified in 7.2% of febrile patients (42). The initial molecular characterization of the *R. asembonensis* agent was accomplished utilizing a multilocus sequence typing (MLST) algorithm (43). Prevalence of this new agent (~91.7%) in collected fleas was found to be distinctly different from that of *R. felis* (8.3%) (14).

Subsequently, additional fleas collected from the same hosts and locations within the livestock-owning compounds in Asembo were processed for rickettsial culture. The new agent, *Rickettsia asembonensis* NMRCii, was successfully cultured from a pool of five individual flea triturate cultures isolated from *C. canis* and *C. felis* fleas obtained from domestic dogs. The cultures were initially grown in S2 and subsequently in C6/36 cell lines at 25°C (32), but not in Vero and L929 cell lines or embryonated chicken eggs incubated at 37°C (1).

The culture of *R. asembonensis* NMRCii was analyzed by microscopy, including Diff-Quik/acridine orange staining and transmission electron microscopy (32). The *R. asembonensis* were observed in the *Drosophila* S2 and *Aedes albopictus* C6/36 cells lines as early as 3 days post-infection, and could be observed at multiple time points throughout the average culture time of 40–45 days (32). Rickettsiae were observed both intra- and extracellularly at time points ranging from 15 to 30 days throughout the course of the continuous culture (32). The new agent was observed by acridine orange staining in singlets, doublets, and during heavy parasitization of host cells, in long chains (32). Transmission electron microscopy of the *R. asembonensis* revealed multiple free rickettsiae (round to elongated morphology) in the cytoplasm of the host cells, with normal rickettsial size [diameter 0.375–0.5 µm (round morphology), length 0.5–0.625 µm, width/diameter 0.25–0.375 µm (elongated morphology)]. A cell wall membrane, defined periplasmic space, and cytoplasmic membrane were observed, as well as the electron lucent "halo" (rickettsial slime layer) (32). Intranuclear localization/growth of the agent was not detected by acridine orange or by transmission electron microscopy (32).

Genetic characterization of the cultured *R. asembonensis* NMRCii by MLST using rickettsial genes *rrs, gltA, ompA, ompB,* and *sca4*; plasmid analysis; and whole genome sequencing confirmed that the new agent was indeed a unique *Rickettsia* species (1, 44). *R. asembonensis* NMRCii was shown to have an estimated genome size of 1.40 Mb, possessed a 21,692 bp circular plasmid and had a G+C content of 32.2%. The *R. asembonensis* plasmid, pRAS01, was discovered to be unique as it only shared 89% homology with that of *R. africae* ESF5 and only 84% homology with that of *R. felis*. The *R. asembonensis* genome has 1,147 predicted protein-coding genes, 33 tRNA genes, and three

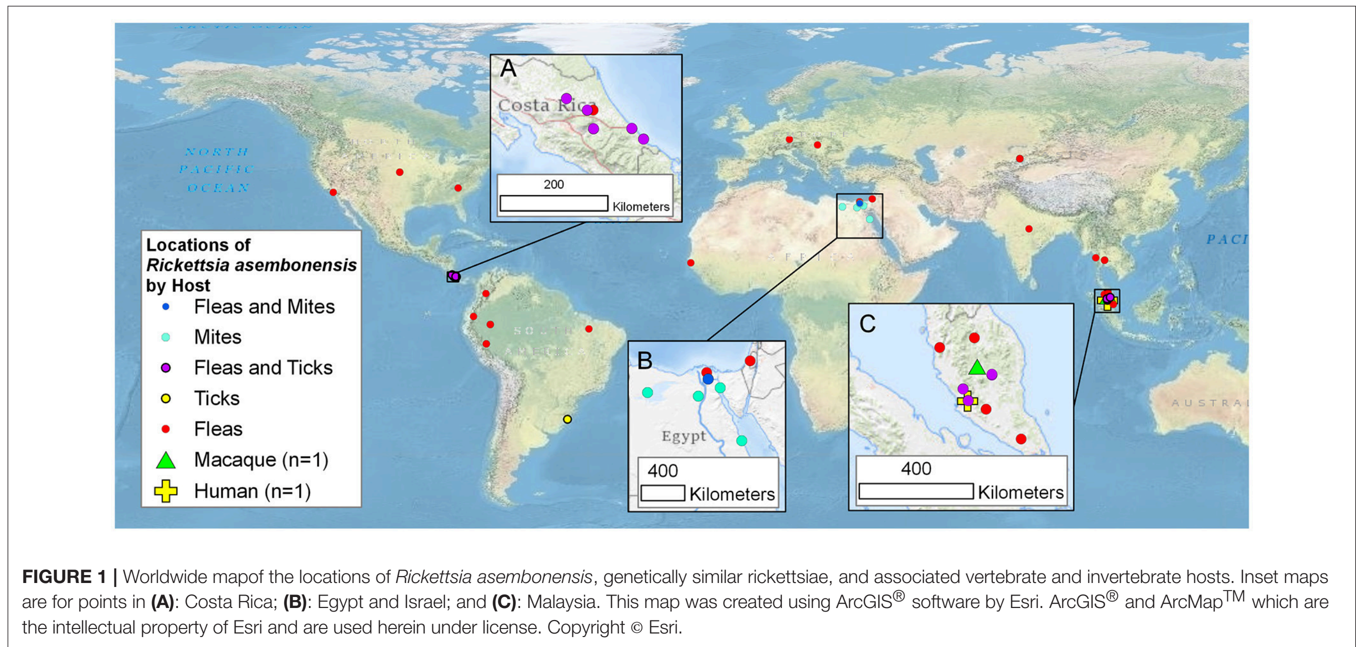

FIGURE 1 | Worldwide mapof the locations of *Rickettsia asembonensis*, genetically similar rickettsiae, and associated vertebrate and invertebrate hosts. Inset maps are for points in **(A)**: Costa Rica; **(B)**: Egypt and Israel; and **(C)**: Malaysia. This map was created using ArcGIS® software by Esri. ArcGIS® and ArcMap™ which are the intellectual property of Esri and are used herein under license. Copyright © Esri.

rrn operons. These characteristics are similar with those found within the genome of *R. felis* (NC_007109), which is 1.49 Mb in size and contains 1,400 protein-coding genes, 33 tRNA genes, and three rrn operons. Of the *R. felis* proteins, 1,157 (83%) have homologs in *R. asembonensis* (1, 44).

The sequences of *R. asembonensis* NMRCii, were 100% identical to those previously described for "*Ca.* R. asemboensis" isolates F30 and F82 for the following genes: *rrs*, *gltA*, *sca4,* and the 17kD antigen gene. For the *ompA* and *ompB* genes, the *R. asembonensis* NMRCii shared 99.86 and 99.98% similarity respectively, with the "*Ca.* R. asemboensis" isolates F30 and F82. The differences observed were as a result of nucleotide substitutions in two positions for the *ompA* gene and in one position for the *ompB* gene. A molecular phylogenetic analysis using 4,130 bp sequence of the variable gene-*ompB* open reading frame was conducted and the phylogenetic relationship between *Rickettsia asembonensis* NMRCii with *R. felis*, *Rickettsia* sp. PU01-02 ("*Ca.* R. senegalensis") and other recognized *Rickettsia* species was determined (**Figure 2**).

Rickettsia asembonensis NMRCii was deposited in two separate culture collections (=DSM 100172^T and =CDC CRIRC RAS001^T) and the name officially changed (according to the rules of the International Journal of Systematics and Evolutionary Biology) from "*Candidatus* Rickettsia asemboensis" to *Rickettsia asembonensis* (1).

ARTHROPODS ASSOCIATED WITH *RICKETTSIA ASEMBONENSIS*

R. asembonensis DNA has been detected in various arthropods, but most commonly in fleas (**Table 1**). It has been identified in fleas from three families namely the *Pulicidae*, *Ceratophyllidae* and *Coptopsyllidae*. In the cosmopolitan *Pulicidae* family it has been associated with seven genera: *Ctenocephalides* (*C. felis, C. canis*, and *C. orientis*); *Xenopsylla* (*X. cheopis*, *X. ramesis*, and *X. gerbilli*); *Archaeopsylla* (*A. erinacei*); *Echidnophaga* (*E. gallinacea*); *Pulex* (*P. irritans*); and *Synosternus* (*S. pallidus*). In the family *Ceratophyllidae, R. asembonensis* has been detected in three genera: *Ceratopsyllus* (*C. fasciatus); Orchopeas* (*O. howardi*); and *Nosopsyllus* (*N. laeviceps*) and in one genus in the family *Coptopsyllidae*: *Coptopsylla* (*C. lamellifer*) (45).

High prevalence rates of *R. asembonensis* have been reported in *C. felis* and *C. canis* (sympatric species), *S. pallidus, X. ramesis*, and *X. gerbilli* with up to 95, 95, 91.4, 100, and 33.3% of the fleas positive for *R. asembonensis*, respectively (13, 14, 18, 40, 46). Similar results in Costa Rica and Brazil confirm the high prevalence of *R. asembonensis* in *C. felis* (23, 28). In addition, *R. asembonensis* has been associated with other fleas, usually in much lower prevalence than in the aforementioned fleas. These include *E. gallinacea*, *P. irritans*, *C. lamellifer, X. hirtipes*, and *N. laeviceps*. Often these fleas are positive for *R. asembonensis* in the same areas as fleas highly infected with *R. asembonensis* (14, 46). The presence of the *R. asembonensis* in minimally infected flea species may be due to co-feeding and not that these fleas are reservoir hosts for *R. asembonensis*. Other arthropods in which evidence of *R. asembonensis* has been found include the tropical rat mites (*Ornithonysus bacoti*) in Egypt (7) and ticks (*Amblyomma ovale* and *Rhipicephalus sanguineus*) (23, 25–27).

PATHOGENICITY

In limited laboratory studies no marked cytopathic effects were observed in S2 and C6/36 cells, beyond lysis of overly parasitized host cells (32). Additionally, no growth was observed in embyronated chicken eggs (1). Moreover, in two febrile studies conducted in Kenya no molecular evidence of this agent in

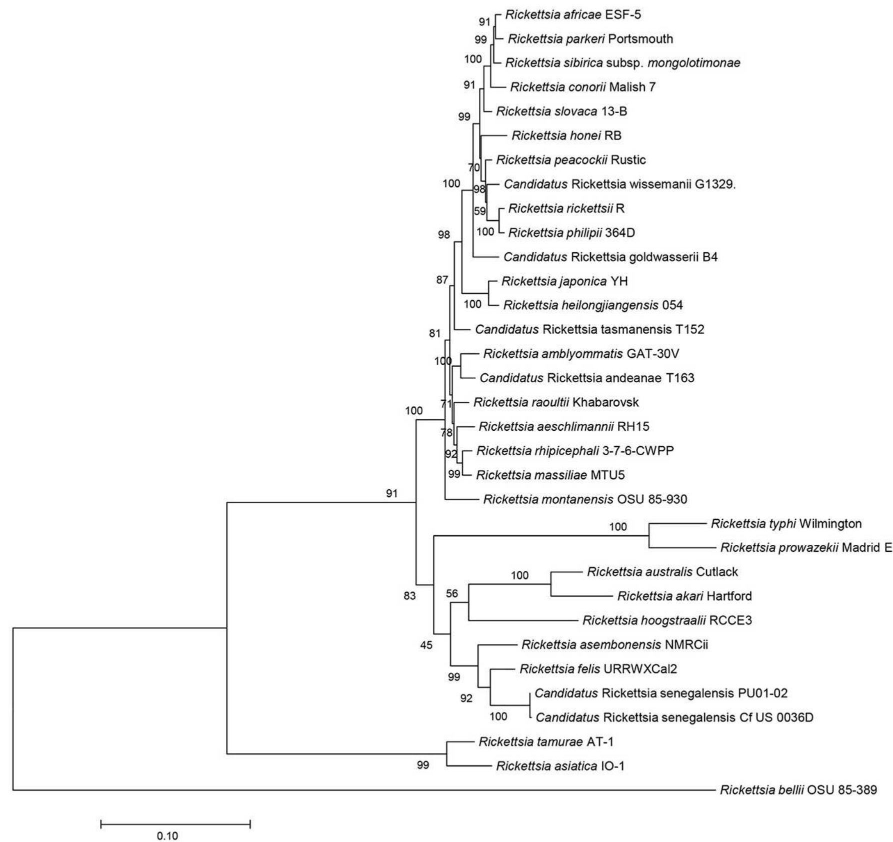

FIGURE 2 | Molecular phylogenetic analysis using *ompB* open reading frame (4,130 bp). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Evolutionary analyses were conducted with MEGA7.

patients' blood was seen whereas *R. felis* DNA was detected in 3.7 and 7.2% of fever patients' blood (42, 47). However, there is molecular evidence of *R. asembonensis* in a patient from Malaysia with fever, myalgia, arthralgia, mild headache, conjunctival suffusion, and the presence of petechiae noted on his limbs. Molecular analysis (*gltA* and *ompB* sequences) of the patient's blood identified *R.* sp. RF2125 (21). In addition, in the blood from a healthy free range domestic dog from Mnisi community situated in the northeastern corner of the Bushbuckridge Municipal Area, Mpumalanga Province, South Africa *R. asembonensis* was detected by NGS (22). Lastly, 12 of 50 healthy monkeys from Peninsular Malaysia had molecular evidence (100% *gltA* sequence similarity) of *R.* sp. RF2125/"*Ca.* R. asemboensis" (20). Thus, from the mixed results presented, the question of pathogenicity for humans and other animals is not yet resolved and requires more investigation.

FUTURE RESEARCH DIRECTION

R. asembonensis-genotypes have been described in various biting and non-biting arthropods. Apart from *R. asembonensis* NMRCii that has been isolated in cell culture and whose full genome sequence is available in the GenBank Database, many of the others are just molecular isolates derived from arthropods with very limited sequence data for comparison. Functional and structural analysis of *R. asembonensis* is needed to ascertain differences and/or similarities between it and other rickettsial species. Moreover, research concerning the known/potential hosts of *R. asembonensis*, its current/potential arthropod vectors (both common and non-common), and its potential for interference with other rickettisal flea-borne pathogens (*R. felis* and *R. typhi*), as well as non-rickettsial pathogens such as *Yersinia pestis*, will be crucial to fully defining its pathogenicity and probability as a public health concern/nuisance across the world.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the review. AM wrote the first draft of the manuscript. JJ, AL-F, HS, CF, and AR wrote revisions of the manuscript. All authors contributed to the manuscript's final version, and read and approved the submitted version.

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Marmots and *Yersinia pestis* Strains in Two Plague Endemic Areas of Tien Shan Mountains

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The main purpose of this study was to clarify the role of gray marmots (*Marmota baibacina*) in the long-term maintenance of highly virulent strains of *Yersinia pestis* in two plague endemic foci of the Tien Shan Mountains in Kyrgyzstan. We present data from regular observations of populations of *M. baibacina* and small rodents cohabiting with marmots in the mountainous grasslands of the Sari-Dzhas (east of Issyk-Kul Lake) and the Upper-Naryn (south of Issyk-Kul Lake) natural foci. During 2012–2017, an abundance of marmots and their ectoparasites (fleas and ticks) was significantly higher in Upper-Naryn comparing to Sari-Dzhas, although there were no differences in a number and diversity of small rodents cohabiting with marmots. The plague bacterium was detected either in marmots or in their ectoparasites collected during 4 of 6 years of observation in Sari-Dzhas and during 2 of 4 years of observation in Upper-Naryn. Plague was found in three sectors situated closely to each other in Sari-Dzhas and in 1 of 8 repeatedly surveyed sectors in Upper-Naryn. During 6 years, we isolated 9 strains of *Y. pestis* from marmots, two from their fleas *Oropsylla silantiewi*, one from an unidentified tick, and one from the gray hamster (*Cricetulus migratorius*). All plague strains isolated from the rodents and their ectoparasites in this study were similar to *Antiqua* biovar specific for marmots. The results indicate that plague can circulate continuously in the Tien Shan Mountains in populations of gray marmots and their ectoparasites with a facultative involvement of other rodent species after significant changes in rodent communities that happened in Kyrgyzstan during the previous two decades. The simultaneous field survey of two natural foci of plague, Sari-Dzhas, and Upper-Naryn, would be important for further analysis of circulation of *Y. pestis* strains belonging to *Antiqua* biovar in the Tien Shan Mountains.

Keywords: grey marmot, ectoparasites, plague, rodent, Kyrgyzstan, *Yersinia pestis*

INTRODUCTION

One of the most active plague endemic areas in the world is located in Kyrgyzstan, Central Asia. There are two independent foci of plague in the Issyk-Kul province: the Sari-Dzhas (east of Issyk-Kul Lake) and the Upper-Naryn (south of Issyk-Kul Lake) sub-regions of the Tien Shan natural focus of plague (1, 2). Several plague outbreaks involving natural vectors, including the gray marmots (*Marmota baibacina*) and other rodents, have been registered there since 1907 (3). Most of the 5,000 km^2 Sari-Dzhas natural plague focus is located in Kyrgyzstan (4,250 km^2), with a part extending to Kazakhstan (750 km^2). The 8,000 km^2 Upper-Naryn area is located in the Naryn and Issyk-Kul regions of Kyrgyzstan.

The first plague outbreak with high human mortality was recorded in the Kyrgyzstan part of Tien Shan focus in 1907, and the last big outbreak of plague occurred in 1928 in the Bash-Kaindi settlement of Atbashi district resulting in 54 deaths (Upper-Naryn mezofocus) (4). Sporadic human cases were also seen in 1965 and 1982. In 2013, a 15-year-old boy who ate marmot meat died from plague in Kyrgyzstan. The Kyrgyz Ministry of Health established a temporal quarantine in parts of the country's mountainous northeast (5), where the risk of plague is well-known for a century. The endemic areas were investigated previously starting from 1942, and the last activity of plague was reported there in 1983 (4, 6, 7).

Gray marmots (*M. baibacina*) are well-known hosts of multiple zoonotic diseases, out of which plague presents the greatest danger to people (8–10). The gray marmots prefer grasslands and shrubs and avoid woodlands, including even the forest edges and forest-steppe areas with a tree cover <10% (11). Marmot fleas are actively involved in transmission of plague pathogen between animals (12–14). Marmots can host the following species of fleas: *Oropsylla silantiewi*, *Rhadinopsylla li ventricosa*, *Ceratophyllus lebedewi*, and *Pulex irritans*; the first two flea species are host-specific for marmots (15, 16).

Human activities, such as agriculture, hunting, recreation, and degradation of natural habitats, may dramatically influence plague manifestations (17). Changes in landscape potentially can lead to altering the rodent and flea communities that in turn affect plague transmission cycle (18). Plague in Asian natural systems is commonly spatially stable and corresponds with distribution of primary rodent hosts that can support a strong association of specific strains of *Yersinia pestis* in mammalian communities (19). Marmots in mountainous plague foci of Central Asia carry a specific strain of *Y. pestis* (20). Disturbance of evolutionary relations between plague pathogen and their rodent hosts, such as marmots, may result in unpredictable dynamics of the infection.

The collapse of the Soviet Union and subsequent separation of anti-plague station in Kyrgyzstan from the federal anti-plague system led to dramatic reduction of plague investigations and plague control measures. During the last couple of decades, the density of marmots was significantly reduced as a result of chemical suppression in the 1960s, increased hunting of marmots, habitat destruction, and climate changes (6–8, 21). This raises the question whether the changes in rodent communities in these areas affected marmots playing a leading role in circulation of plague pathogen. Thus, the present study aimed to clarify the role of gray marmots in the long-term maintenance of the highly virulent strains of *Y. pestis* in two plague foci of the Tien Shan Mountains in Kyrgyzstan. The human case in 2013 attracted attention to the situation that plague is endemic to the territory of Kyrgyzstan and the urgent need to obtain new information about the status of the epidemiological situation in these plague foci.

MATERIALS AND METHODS

Fieldwork

In June–August 2012–2017, the Karakol Anti-Plague Department (KAPD) of the Republic Center for Quarantine and Dangerous Infections (RCQDI) organized field surveys within the Sari-Dzhas and Upper-Naryn foci. The fieldwork was organized as described by Weaver et al. (22) and Sariyeva et al. (6, 7). All work with wild and laboratory animals and plague strains was conducted in accordance with the regulations and protocols approved by the Ministry of Health of Kyrgyzstan (23) in 2015. The procedures were similar to those described by Aytkuluyev (24) and Ezhlova et al. (25). The animal work in the field was performed according to the Regulations approved by the State Agency for Environmental and Forest Protection of the Kyrgyz Republic (details of permits are presented in **Table 1**). Each permit was issued for collecting a certain number of animals (400 marmots, 300–400 rodents of other species, and excavating 10 nests of marmots and rodents to look for nest parasites) and used during a fixed period of time (from June 1 to August 30) in specific areas within plague foci.

TABLE 1 | Numbers and dates of permits for trapping of animals for epizootological research in 2012–2017.

Year	Sari-Dzhas			Upper Naryn		
	Number of license	Data of issue	Permitted number of animals	Number of license	Data of issue	Permitted number of animals
2012	000078-KC	26.04.2012	400 marmots, 400 small rodents	–	–	–
2013	000110-KC	06.05.2013	400 marmots, 300 s.r.	000111-KC	06.05.2013	300 marmots, 300 small rodents
2014	000144-KC	06.05.2014	400 marmots, 300 s.r.	000145-KC	06.05.2014	400 marmots, 300 s.r.
2015	000168-KC	22.05.2015	400 marmots, 300 s.r.	000169-KC	22.05.2015	400 marmots, 300 s.r.
2016	000173-KC	17.05.2016	400 marmots, 300 s.r.	000174-KC	17.05.2016	400 marmots, 300 s.r.
2017	000003-KC	05.03.2017	400 marmots, 300 s.r.	000004-KC	05.03.2017	400 marmots, 300 s.r.

TABLE 2 | Strains of *Y. pestis* isolated in the Sari-Dzhas and Upper-Naryn plague foci used for DNA analysis.

Strain code	Object, site, and period of strain isolation
KG-1	Human outbreak, the Sari-Dzhas, 2013
KG-2	Human outbreak, the Sari-Dzhas, 2013
KG-3	Gray marmot, the Sari-Dzhas, 2014
KG-4	Gray marmot, the Upper-Naryn natural plague focus, Ishtyk-Akshiyrak site, 2015
KG-5	Gray marmot, the Upper-Naryn natural plague focus, Ishtyk-Akshiyrak site, 1963
KG-6	Ectoparasites, there was a suspicion that this strain was *Y. pseudotuberculosis*, 1962
KG-7	Gray marmot carcass, the Sari-Dzhas natural plague focus, Enylchek-Kaindy site, 2016
KG-8	Ectoparasites—ticks from the carcass of gray marmot, the Sari-Dzhas natural plague focus, Enylchek-Kaindy site, 2016
KG-9	Ectoparasites—fleas from the carcass of gray marmot, the Sari-Dzhas natural plague focus, Enylchek-Kaindy site, 2016
KG-10	Gray marmot, the Sari-Dzhas natural plague focus, Enylchek-Kaindy site, 2016
KG-11	Gray marmot, the Upper-Naryn natural plague focus, Ishtyk-Akshiyrak site, 1957
KG-12	Gray marmot, the Upper-Naryn natural plague area, Ishtyk-Akshiyrak site, 1962
KG-13	Gray marmot, the Upper-Naryn natural plague area, Ishtyk-Akshiyrak site, 1960
KG-14	Gray marmot, the Upper-Naryn natural plague area, Ishtyk-Akshiyrak site, 1983

Trapping Rodents

At the beginning of the annual anti-plague field work, zoologists visually estimated a number of marmots within study sites and accordingly planned trapping efforts in each sector (10 km^2). The number of installed traps depends on the average density of marmot population in each sector. Around 8–10 traps were set next to marmot burrows each day at early morning. As the weather in May–June in high-altitude areas of Tien Shan is highly variable with snow, rains, and cold weather, the trapping was conducted during each sunny day. The marmot traps were set at the entrance of burrows and removed by evening. One trap remained at each burrow for half a day. The burrows for trapping were chosen based on external signs such as presence of fresh litter at the entrance, presence of freshly excavated and well-rammed entrances, grass coverage, presence of marmot footprints, and paths around the burrow. The traps used for capturing marmots were 20 cm wide and 8 cm high (**Figure 5**). The traps were attached to 50-cm-long metal rods with a strong metal chain. The rods are driven into the rocky soil to their full depth to prevent marmots or their predators from dragging the traps away. The metal parts were masked by horse manure to reduce smell of metal.

A number of small rodents, such as mice, voles, and hamsters, were estimated by counting animals captured by handmade snap traps set in transects (1, 25). The snap traps were also sprinkled with soil and baited with dry bread soaked in vegetable oil,

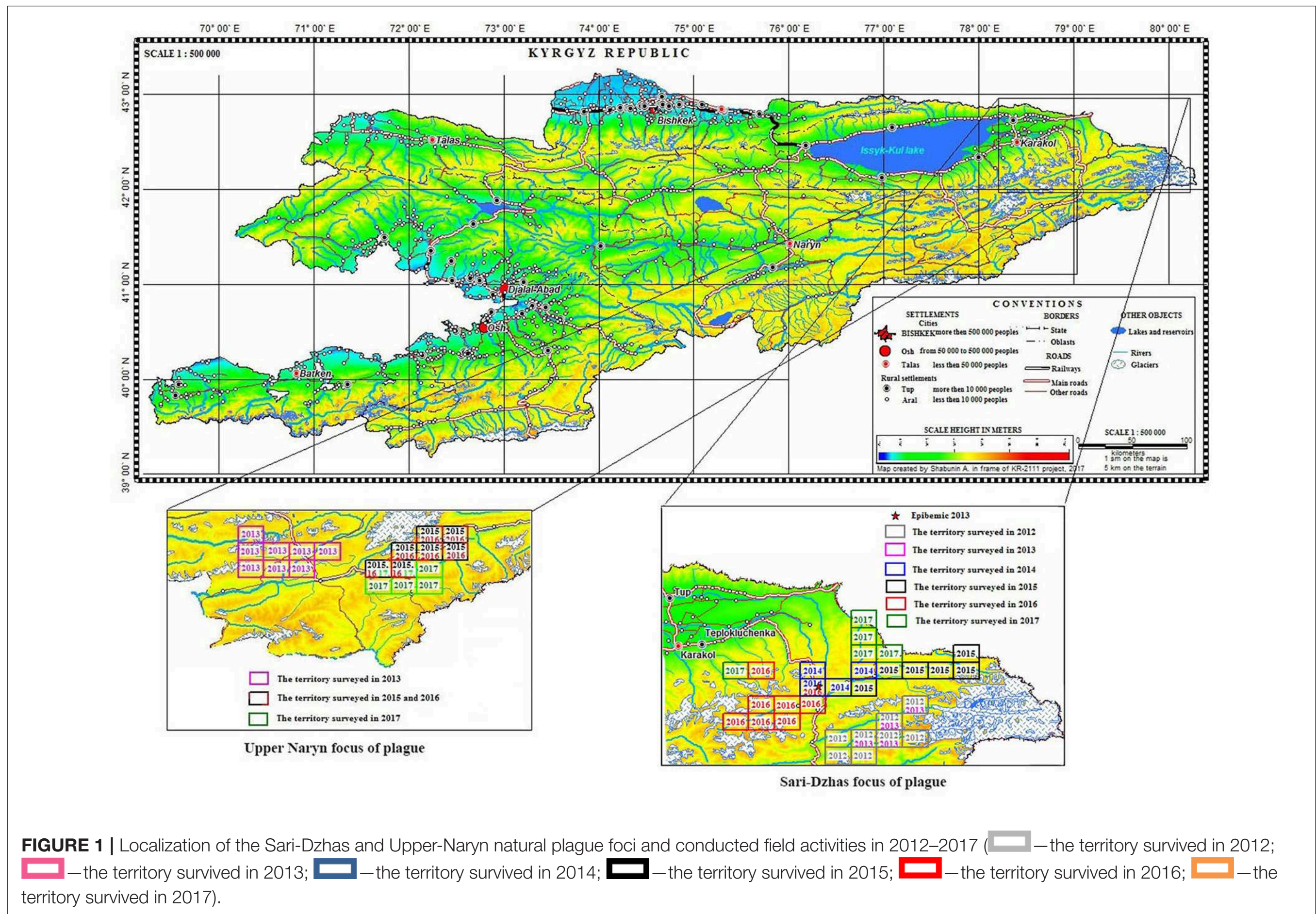

FIGURE 1 | Localization of the Sari-Dzhas and Upper-Naryn natural plague foci and conducted field activities in 2012–2017 (—the territory survived in 2012; —the territory survived in 2013; —the territory survived in 2014; —the territory survived in 2015; —the territory survived in 2016; —the territory survived in 2017).

vegetables, or fruit. We placed traps in the evening and checked them in the early morning. If there was a captured rodent, we transferred the trap to another spot. The predators were estimated by trapping and visual observations. Each trapped animal was morphologically identified by species. The captured marmots were euthanized by cervical dislocation (25). Then, the animal was wrapped in two linen sacs with label (date, place, number of sector, and name of catcher), the inlet was tightly wrapped so that the ectoparasites could not escape, and then the animal was placed in a plastic bag, in a canvas bag, and finally brought to camp.

Parasitological Analysis

Ectoparasites of marmots were collected by combing the captured animals ("body" ectoparasites) and by collecting "off-host" ectoparasites from rodent burrows and nests (25). Ectoparasites were then identified using entomological keys (26) and placed in labeled glass tubes with ether. Inside burrows, dry grass bedding from the nesting chamber was carefully removed with a wire and was searched for fleas and other ectoparasites. Fleas of one species from a single animal were pooled and triturated in saline solution.

Bacteriological Analysis

The triturated flea suspension was inoculated on Hottinger agar with pH 7.2 (27) (bacteriological approach). The inoculation pool consisted of 20 ectoparasites of one species, collected from the same sector during several days and stored before inoculation in glass tubes without ether. If the animal exhibited any pathological manifestation, the ectoparasites from the pool were inoculated individually. Identification of the isolated bacteria was carried out by standard microbiological microscopy of

TABLE 3 | Field survey of the Sari-Dzhas and Upper Naryn sites of the Tien Shan high-altitude focus of plague in 2012–2017.

Focus	Year	Month	Area and number of sectors	Number of caught marmots	Number of other mammals trapped	Number of collected ectoparasites	*Y. pestis* isolated from marmots	*Y. pestis* isolated from ectoparasites	*Y. pestis* isolated from other rodents
Sari-Dzhas	2012	June–July	800 km^2, 8 sectors	218	*Microtus gregalis*—42, *Apodemus uralensis*—47, *Cricetulus migratorius*—12 + carcass, *Lepus tolai*—2 (total 103)	240 fleas, 459 ticks	5	–	1 (gray hamster)
	2013	June	400, 4 sectors	70	*Microtus gregalis*—30, *Apodemus uralensis*—6, *Cricetulus migratorius*—10 (total 46)	10 fleas, 137 ticks	–	–	–
	2014	July	400, 4 sectors	197	*Microtus gregalis*—42, *Ochotona macrotis*—1, *Apodemus uralensis*—1, *Cricetulus migratorius*—11 (total 55)	84 fleas, 155 ticks, 6 lice	–	1 from fleas	–
	2015	July–August	600, 6 sectors	177	*Microtus gregalis*—129, *Martes foina*—5 (total 134)	175 fleas, 42 ticks, 16 lice	–	–	–
	2016	June–July	800, 8 sectors	180	*Microtus gregalis*—124, *Apodemus uralensis*—3, *Cricetulus migratorius*—1 (total 128)	145 fleas, 344 ticks, 9 lice	2	1 from ticks	–
	2017	June–July	700, 7 sectors	130	*Microtus gregalis*—75, *Apodemus uralensis*—4 (total 79)	124 fleas, 15 ticks, 125 lice	–	–	–
Upper Naryn	2013	June–August	800, 8 sectors	154	*Microtus gregalis*—47, *Cricetulus migratorius*—13, *Mustela eversmanni*—4 (total 64)	268 fleas, 1045 ticks, 127 lice	–	–	–
	2015	June–July	800, 8 sectors	260	*Microtus gregalis*—107, *Lepus tolai*—2, *Cuon alpinus*—1, *Mustela eversmanni*—2 (total 112)	489 fleas, 113 ticks, 52 lice	2	–	–
	2016	June–August	700, 7 sectors	190	*Microtus gregalis*—150, *Alticola argentatus*—1, *Vulpes vulpes*—1 (total 152)	261 fleas, 233 ticks, 2 lice	–	1 from fleas	–
	2017	June–July	600, 6 sectors	224	*Microtus gregalis*—38, *Lepus tolai*—2, *Vulpes vulpes*—2 (total 42)	311 fleas, 593 ticks, 38 lice	–	–	–

TABLE 4 | The number of trapped mammals in open stations of the Sari-Dzhas natural plague focus in 2012–2017.

Years of observation	#sector	Time period of observation	*Marmota baibacina* (trapped)	*Marmota baibacina* (carcass)	*Microtusgregalis*	*Apodemus uralensis*	*Ochotona macrotis*	*Cricetulus migratorius*	*Lepus tolai*	*Martes foina*	Isolated strain of *Y. pestis*
2012	3224406334	29 May–3 June	28		1	1			1	–	
	3224407512	29 May–2 June	18		1	3		1			
	3124407521	6–11 June	43		–	14		3			
	3124406343	6–11 June	26	1	–	6		4 + carcass			
	3124406344	13–15 June	18		–	6		3			
	3124406433	14–15 June	7		–	–					
	3124406342	18–29 June	53	2*	33	12		1*	1		**5**
	3124406413	19–26 June	25		7	5		–			
	Average:		**27.25 ± 10**		**Total 42**	**47**		**Total 12 + carcass**	**2**		
2013 (human case)	3124406222	22 August									**1**
2013	3124406342	4–11 June	28		21			4			
	3124406413	8–10 June	13		9			6			
	3124406344	11–18 June	14								
	3124406343	10–17 June	15			6					
	Average:		**17.5 ± 5**		**Total 30**	**6**		**Total 10**			
2014	3124406311	9–20 July	58	2*	23	1	1	6		–	**3**
	3124406312	16–21 July	88		19	–	–	5			
	3124405133	19–21 July	29		–	–	–	–			
	3124405143	19–21 July	22		–	–	–	–			
	Average:		**49.25 ± 23**		**Total 42**	**1**	**1**	**Total 11**			
2015	3124405233	27 July–5 August	54		20	–	–	–		3	
	3124405234	27 July–16 August	52		76					1	
	3124405243	8–11 August	30		13					1	
	3124405241	9–11 August	12		0					–	
	3124405144	5–6 August	6		11					–	
	3124406321	13–16 August	23		9					–	
	Average:		**29.5 ± 15**		**Total 129**					**5**	
2016	3124406242	28 June–6 July	39		82	3		1			
	3124406241	30 June–2 July	16		0						
	3124406313	30 June–5 July	25		26						
	3124406223	2–9 July	25		0						
	3124406224	5–6 July	6		0						
	3124406232	8–9 July	29	1*	16						3
	3124406311	15–19 July	31		–						
	3124405043	18–22 July	9		–						
	Average:		**22.5 ± 9**		**Total 124**	**3**	**–**	**1**			

(Continued)

TABLE 4 | Continued

Years of observation	#sector	Time period of observation	*Marmota baibacina* (trapped)	*Marmota baibacina* (carcass)	*Microtusgregalis*	*Apodemus uralensis*	*Ochotona macrotis*	*Cricetulus migratorius*	*Lepus tolai*	*Martes foina*	Isolated strain of *Y. pestis*
2017	3124205123	15–29 June	19		7						
	3124405141	16–19 June	18		6						
	3124405052	17–21 June	13		11	2					
	3124405034	23–27 June	27		23	1					
	3124405121	27 June–6 July	34		21	1					
	3124405142	2–4 July	19		7						
	Average:		**21.67 ± 5**		**Total 75**	**4**					

*Object of isolation of Y. pestis. Bold values indicate average or total meanings.

smears and by Pokrovsky's test for *Y. pestis* using both pseudotuberculosis bacterial phage and bacteriophage for *Y. pestis* (L-413-C) produced by the Kazakh Scientific Center for Quarantine and Zoonotic Diseases (KSCQZD). Additional diagnostic methods included serological indirect hemagglutination assay with both erythrocyte–immunoglobulin and erythrocyte–antigen diagnostic reagents produced by KSCQZD. The tissues of internal organs of sampled marmots (liver, lung, spleen, lymph nodes, and blood from the heart) were sterilely taken with forceps and immediately placed on an agar plate by directly touch. A Petri dish was divided into three segments to separately plate lungs, livers, and spleens. Each plate was marked and placed in an incubator at 37°C. We used individual and pool methods of inoculation. The individual method was used for ectoparasites collected from carcass of marmot or marmot with visual pathological abnormalities relevant to plague (pathology of inner organs, sick marmots). For inoculation of pooled tissue suspensions, we combined tissue pieces from five marmots collected on the same day from the same sector. Then, the organ suspension was inoculated on agar plate with a bacterial inoculating loop. After inoculation, tissue samples were placed in liquid nitrogen (Dewar flask) and transported to the laboratory in Karakol for further confirmation.

Genetic Analysis

The genetic analysis was performed using two types of material: whole-genome DNA samples and MLVA fragments (**Table 2**). The 14 studied *Y. pestis* strains were obtained from the RCQDI collection. The strains were isolated during the last several years in the territory of Sari-Dzhas and Upper-Naryn plague foci (**Table 2**). The suspension of *Y. pestis* cultures was heated at 100°C for 20 min and centrifuged at 12,000 rpm for 2 min. This allowed the inactivation of the pathogens and the release of the DNA in the cells. The DNA samples were further used for genotyping. The control MLVA amplification fragments of the reference strains CO92, Pestoides F, KIM10+, and Nepal 516 of *Y. pestis* were obtained from the University of Texas Medical Branch, Texas, USA. In addition, nucleotide sequences of nine *Y. pestis* species (GenBank accession numbers: CP010023, CP010247, CP006751, CP009935, AE017042, CP006806, CP000308, CP006794, and CP002956) and three *Yersinia pseudotuberculosis* strains (GenBank accession numbers: CP009712, CP008943, and CP001048) were used for phylogenetic analysis. The studied strains belonging to the *Y. pestis* species was confirmed using the "Pest-Quest" PCR assay ("Master-Gene" Limited Liability Partnership, Almaty, Kazakhstan). The genotyping of several *Y. pestis* strains was performed by the methods of Multi-Locus VNTR Analysis (MLVA) and Melt Analysis of Mismatch Amplification Mutation Assay (Melt-MAMA). For MLVA assay, seven VNTR (Variable Number Tandem Repeats) loci were studied using conventional PCR and agarose gel electrophoresis as described by Le Flèche et al. (28). For Melt-MAMA assay, three sets of PCR primers designed and produced by KSCQZD were used to identify three SNP loci, described by Morelli et al. (29). The selected SNP loci allowed differentiation of four branches of *Y. pestis* bv. *Antiqua* (0.ANT1, 0.ANT2, 0.ANT3, and 3.ANT). The

Melt-MAMA analysis was performed as described by Birdsell et al. (30). Each primer set consisted of two forward primers ("ancestral" and "derived") and a universal reverse primer. One of the forward primers was linked to the so-called GC-clamp, three to four repeats of a GGGGC motif, which increased the melting temperature of the corresponding amplicons and made it possible to differentiate the alleles. Phylogenetic analysis of the studied strains was carried out using the PAUP 4.0 software and the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm.

Mapping

The map of Sari-Dzhas and Upper-Naryn areas of plague was prepared using GIS MapInfo Professional 7.8. For their construction, we used the geographic objects digitized from topographic maps of a scale of 1:100,000, as well as the Digital Elevation Model GDEM2. The maps are constructed in UTM—the Mercater projection (WGS 84). The boundaries of the foci are plotted along the boundaries of the sectors (21).

RESULTS

Sari-Dzhas Plague Focus

Rodents

Overall, in the Sari-Dzhas plague focus for the period 2012 to 2017, we surveyed a total of 3,700 km^2, out of which 500 km^2 were surveyed repeatedly in 2012, 2013, 2014, and 2016 (**Figure 1**; **Table 4**). In total, we trapped and analyzed 972 marmots (*M. baibacina*), 442 narrow-headed voles (*Microtus gregalis*), 61 wood mouse (*Apodemus uralensis*), 40 gray dwarf hamsters (*Cricetulus migratorius*), and a small number of other mammals: the large-eared pika (*Ochotona macrotis*), the hare-tolai (*Lepus tolai*), and the beech marten (*Martes*

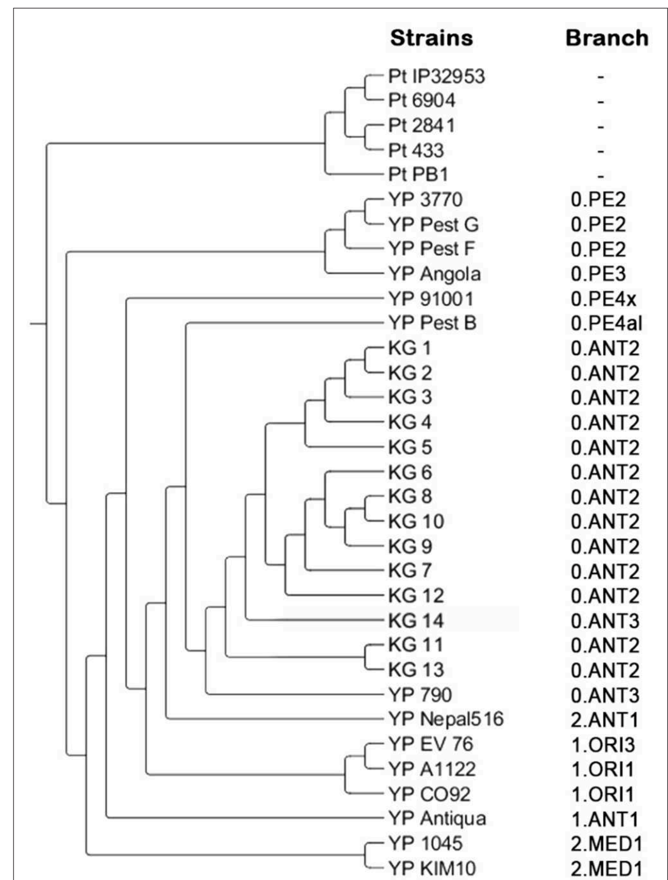

FIGURE 3 | Phylogenetic tree created based on the results of MLVA and SNP analysis. Pt, *Y. pseudotuberculosis* control strains; YP, *Y. pestis* control strains; KG, *Y. pestis* strains isolated on the Kyrgyz territory of Sari-Dzhas and Upper-Naryn plague foci.

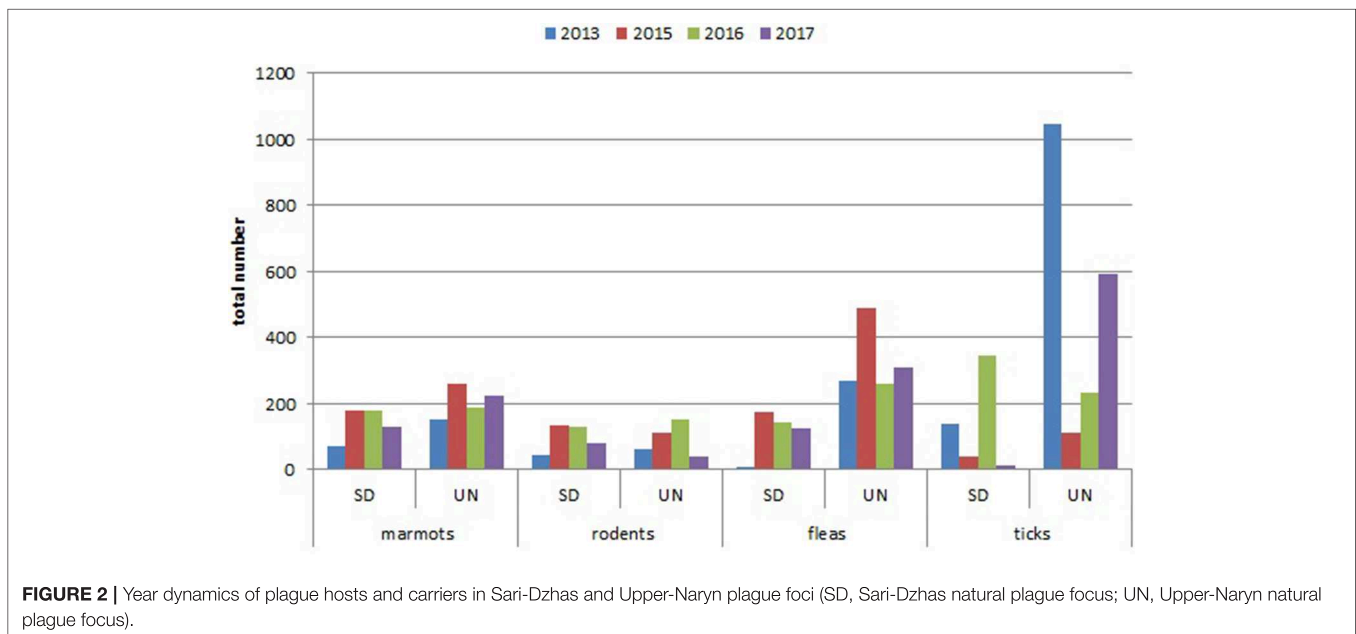

FIGURE 2 | Year dynamics of plague hosts and carriers in Sari-Dzhas and Upper-Naryn plague foci (SD, Sari-Dzhas natural plague focus; UN, Upper-Naryn natural plague focus).

foina) (**Table 3**). Additionally, we found carcasses of six dead rodents.

From all sampled marmots, we collected and tested 778 fleas, 1,152 ticks, and 156 lice (**Table 3**). The average number of sampled marmots ranged from 17 to 27 animals per sector in 2012 and 2013 (**Table 4**). The highest number of trapped marmots (49 ± 7.62) was recorded in 2014 in a forested area with preferable conditions such as vegetation and humidity. In 2015–2017, the number of collected marmots was similar to 2012. Among small rodents, the most common species was *M. gregalis*, which is widely distributed across all observed territories (**Tables 3**, **4**). The density of population of *M. gregalis* and *A. uralensis* varied significantly. A reverse correlation between abundance of these two species was observed. A

TABLE 5 | The number of "body-ectoparasites" collected from trapped marmots in open stations of the Sari-Dzhas natural plague focus in 2012–2017.

Years of observation	#sector	Number of analyzed animal (*M. baibacina*)	Number of fleas	*O. silantiewi*	*Rh. li ventricosa*	Number of ticks	Number of lice
2012	3224406334	39	19	19	–	95	–
	3224407512	18	43	43		60	
	3124407521	43	35	35		92	
	3124406343	26	44	44		62	
	3124406344	18	23	23		7	
	3124406433	7	22	22		1	
	3124406342	53	12	12		95	
	3124406413	25	21	19	2	36	
	Total:	**229**	**219**	**217**	**2**	**459**	
2013	3124406342	28	5	5	–	70	–
	3124406413	13	4	4	–	10	–
	3124406344	14	1	1	–	46	–
	3124406343	15				11	
	Total	**70**	**10**	**10**		**137**	**–**
2014	3124406311	58	15	13	2	62	2
	3124406312	88	18	16	2	81	4
	3124405133	29	1	1	–		
	3124405143	22	8	8	–	12	
	Total:	**197**	**42**	**38**	**4**	**155**	**6**
2015	3124405233	54	43	42	1	7	–
	3124405234	52	36	35	1	6	
	3124405243	30	7	7			
	3124405241	12	11	11			
	3124405144	6	6	4	2	5	
	3124406321	23	28	28		24	
	Total:	**177**	**131**	**127**	**4**	**42**	
2016	3124406242	39	90	90		91	
	3124406241	16	1	1		13	
	3124406313	25	4	2	2	105	
	3124406223	25	5	5		89	
	3124406224	6	8		8	8	
	3124406232	29	5	5		37	
	3124406311	31					
	3124405043	9					
	Total:	**180**	**113**	**103**	**10**	**343**	
2017	3124205123	19	12	12			
	3124405141	18	6	6			
	3124405052	13	6	6			
	3124405034	27	7	7			
	3124405121	34	8	8			
	3124405142	19	45	35	10	15	
	Total:	**130**	**84**	**74**	**10**	**15**	

TABLE 6 | The number of trapped mammals in open stations of the Upper Naryn natural plague focus in 2013–2017.

Years of observation	# sector	Time period of observation	*Marmota baibacina*	*Microtusgregalis*	*Apodemus uralensis*	*Ochotona roley*	*Cricetulus migratorius*	*Lepus tolai*	*Cuon alpinus*	*Mustela eversmanni*	*Alticola argentatus*	*Vulpes vulpes*	Isolated strain of *Y. pestis*
2013	3224308443	10–15 June	10	12									
	3224308434	12–17 June	28	11						1			
	3224308444	14–18 June	14										
	3224308621	19–24 June	16				4						
	3224309612	22–29 June	18				3			1			
	3224308433	28 June–2 July	16	5									
	3224309611	3–6 July	29	6			4						
	3224308431	7–12 July	23	8			2			2			
	Total:		**154**	**42**			**13**			**4**			
2015	3224407343	15–21 June	38	75	–	–	–	2					
	3224408513	22–28 June	62	9									
	3224408512	29 June–4 July	49	4						1			
	3224407344	5–9 July	88	19					1	1			2 from marmots
	3224407431	10–13 July	9	0									
	3224407342	11–13 July	9	0									
	3224407433	12–13 July	5	0									
	Total:		**260**	**107**				**2**	**1**	**2**			
2016	3224407344	7–10 June	99	112							1		1 from fleas
	3224407431	11–15 June	30	9									
	3224407342	12–17 June	10	8								1 (carcass)	
	3224407343	16–20 June	23	10									
	3224407433	20–25 June	9										
	3224408513	26–30 June	11	11									
	3224408512	30 June–5 July	8										
	Total:		**190**	**150**								**1**	
2017	3224408514	28 May–6 June	58	17									
	3224408522	30 May–6 June	52	15				1				1	
	3224408512	2–7 June	36	3									
	3224408523	3–10 June	51	2				1					
	3224408522	10–15 June	16	1								1	
	3224408524	16–25 June	11	–									
	Total:		**224**	**38**				**2**				**2**	

high number of *M. gregalis* was associated with limited or no *A. uralensis* (**Table 4**).

Comparing the total number of collected marmots in Sari-Dzhas for 2013 and 2015–2017 with that in Upper-Naryn, the last one has much higher value on the whole area observed in this period (**Figure 2**). At the same time, there were no differences in the total number of trapped rodents and other mammals in these periods (2013, 2015–2017) between the two plague foci (**Figure 2**).

Ectoparasites

The number of ticks collected from marmots was three to five times higher than fleas collected within all sectors of both investigated plague foci (**Tables 5**, **7**; **Figure 2**). In the Upper-Naryn natural plague area, the average number of fleas and ticks was significantly higher than in the Sari-Dzhas in all years, except ticks in 2016 (**Figure 2**).

Isolation of *Y. pestis*

In 2012 after the 29-year period between outbreaks, one observed an acute epizootic of plague in its primary (*M. baibacina*) and secondary carriers (*C. migratorius*). In total, five strains of *Y. pestis* were isolated during the current study: two strains were from found carcasses of marmots, two were from trapped marmots, and one strain was from *C. migratorius* (**Tables 3**, **4**). All strains were isolated from marmots trapped or found in one sector from eight studied in 2012 within the southeast part of the Sari-Dzhas focus (**Figure 4**).

In 2013, the human case of plague was registered on the north-west part of the Sari-Dzhas focus, sector #3124406222 (**Table 4**; **Figure 4**). In 2014, the plague epizootic in marmots with involved host-specific fleas (*O. silantiewi*) was in the neighbor sector #3124406311. Three strains of *Y. pestis* were isolated from found carcasses of marmots and fleas, collected from them, and confirmed serologically.

TABLE 7 | The number of "body-ectoparasites" collected from trapped marmots in open stations of the Upper-Naryn natural plague focus in 2013–2017.

Years of observation	#sector	Number of analyzed animal	Number of fleas	*O. silantiewi*	*Rh. li ventricosa*	Number of ticks	Number of lice
2013	3224308443	10	8	4	4	45	40
	3224308434	28	8	8	–	238	43
	3224308444	14	–	–	–	76	–
	3224308621	16	4	4	–	161	44
	3224309612	18	50	39	11	159	–
	3224308433	16		5	–	95	–
	3224309611	29	39	35	4	167	–
	3224308431	23	42	19	23	99	–
	Total:	**154**	**151**	**114**	**42**	**1040**	**127**
2015	3224407343	38	21	21	–	1	19
	3224408513	62	99	99	–	13	–
	3224408512	49	53	51	2	60	–
	3224407344	88	62	54	8	33	13
	3224407431	9	5	5	–	–	–
	3224407342	9	2	2	–	1	–
	3224407433	5	3	3	–	4	–
	Total:	**260**	**245**	**235**	**10**	**112**	**32**
2016	3224407344	99	40	36	4	57	2
	3224407431	30	22	12	10	128	–
	3224407342	10	22	12	10	1	–
	3224407343	23	13	7	6	23	–
	3224407433	9	–	–	–	–	
	3224408513	11	19	9	10	13	–
	3224408512	8	10	7	3	9	–
	Total:	**190**	**126**	**83**	**43**	**231**	**2**
2017	3224408514	58	182	182	–	146	17
	3224408522	52	25	25	–	247	5
	3224408512	36	35	35	–	104	3
	3224408523	51	12	12	–	18	–
	3224408522	16	13	13	–	51	4
	3224408524	11	3	3	–	15	–
	Total:	**224**	**270**	**270**	**–**	**581**	**29**

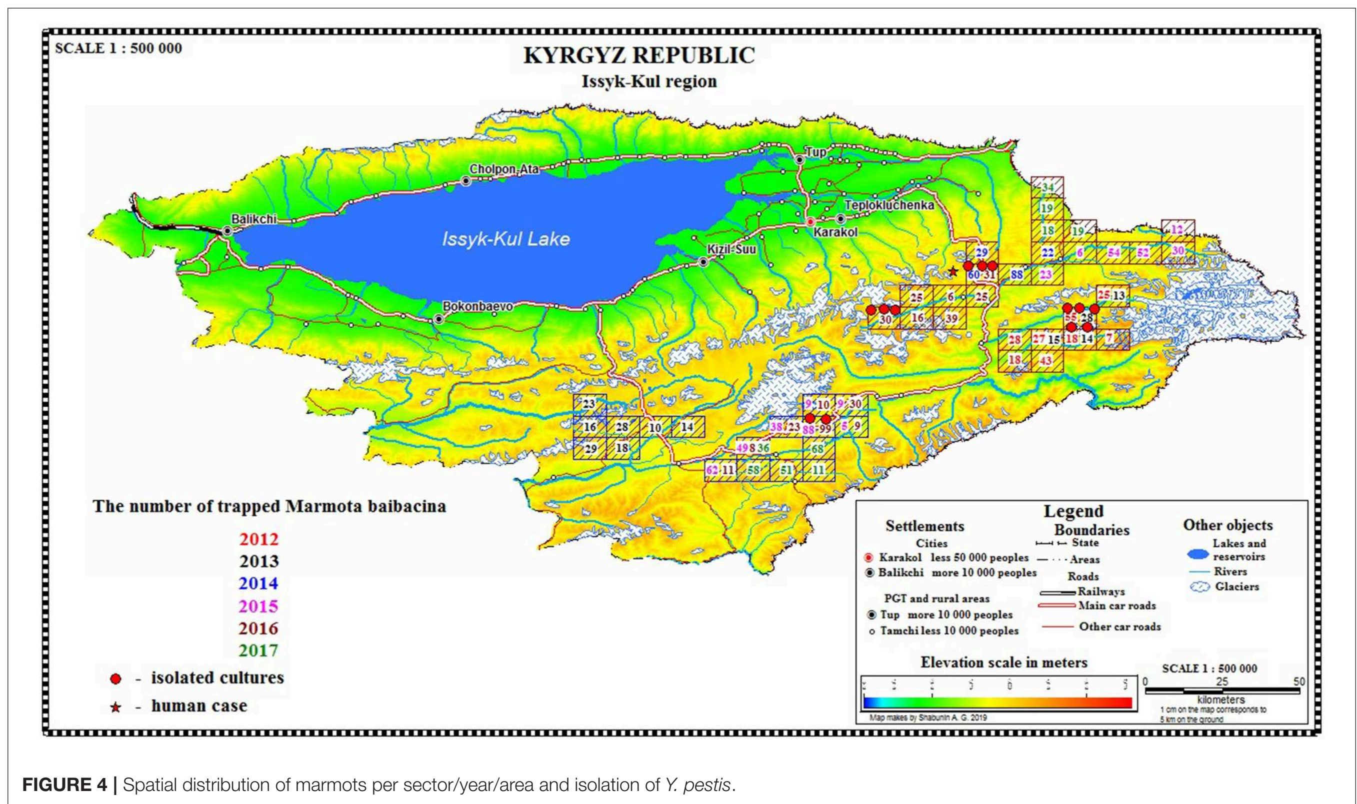

FIGURE 4 | Spatial distribution of marmots per sector/year/area and isolation of *Y. pestis*.

FIGURE 5 | Metallic trap #3 used for trapping of marmots with chain and peg.

In 2016, the field study was conducted near the area observed in 2014–2015, with one sector overlapping between surveys (**Figures 1**, **4**). As a result of this investigation, three strains of plague pathogen were isolated—two from *M. baibacina* (one strain from the pool inoculation of marmots captured in this area and second from a carcass of marmot found in another sector, **Table 4**). The third strain was isolated from pooled unidentified mites collected from a plague-positive carcass.

In 2013 and 2015, different areas of the Sari-Dzhas plague focus were surveyed with plague-negative result (**Figure 1**).

Three strains were isolated in the Upper-Naryn: two from marmots in 2015 and one from ectoparasites collected from marmots in 2016. All three strains were isolated in the same sector #3224407344 (**Figure 4**; **Table 6**).

The Upper-Naryn Plague Focus

During 2013–2017, a total of 2,900 km^2 were investigated in the Upper-Naryn focus, out of which we surveyed 700 km^2 repeatedly in 2015, 2016, and 2017 (**Figure 1**; **Table 3**). In total, 828 marmots, 342 narrow-headed voles (*M. gregalis*), 13 gray dwarf hamsters (*C. migratorius*), and a small number of other mammals: six steppe polecats (*Mustela eversmanni*), four tolai hares (*L. tolai*), one red fox (*Vulpes vulpes*) were trapped and screened (**Table 6**). Additionally, we found the carcass of a dead fox.

From all sampled marmots, we collected and tested 1,329 fleas, 1,984 ticks, and 219 lice (**Table 3**). As a result, in 2015–2016, a plague epizootic was recorded in the same sector (**Figure 4**; **Table 6**). *Y. pestis* strains were isolated from *M. baibacina* and their specific fleas—*O. silantiewi*.

Results of the Genetic Analysis

The application of the "Pest-Quest" PCR assay confirmed that all the studied strains from Sari-Dzhas plague focus belonged to the *Y. pestis* species. Based on the results of the MLVA-7 genotyping, all the studied *Y. pestis* isolates were presented by nine genotypes differing in one or several VNTR loci. Five

strains (KG-1–KG-5) shared the same MLVA-7 profile (probably clones of the same strain). The software we used for phylogenetic analysis showed them not as one branch, but as a bunch of sub-branches (**Table 8**). All 14 strains were assigned to biovar *Antiqua* that was considered to be typical for marmot strains (**Figure 3**). According to the Melt-MAMA analysis, most of the strains belonged to the branch 0.ANT2, while one strain (KG-14) was apparently a member of the branch 0.ANT3 (**Table 9**). These two branches were among those reported by Eroshenko et al. (31) in Kyrgyzstan (along with branches 0.ANT5, 0.PE4t, and 2.MED1).

DISCUSSION

The greater part of both Sari-Dzhas and Upper-Naryn plague foci were studied simultaneously in 2013 and 2015–2017. If an epizootic of plague was observed once, the area was repeatedly examined in the succeeding years. This allows us to compare two independent plague origin areas for its epizootological status during the same time period. In the Tien Shan mountain focus of plague, the gray marmot is known as the main carrier of plague pathogen, with other rodents serving as secondary hosts (2, 8, 32, 33). The spatial distribution of marmots within plague focus is related mainly to local landscape and climatic conditions, as well as to different human activity–animal grazing, hunting, and tourism that are intensively developing during the last decade, but unequally presented in different sectors (21). Actually, the high-altitude pastures for horses and sheep (so called "syrts") are more distant from human settlements in the Upper-Naryn area than in Sari-Dzhas. A higher distance to humans may favor the increase in density of marmots in Upper-Naryn compared to Sari-Dzhas where most of the syrts are tightly used for summer seasonal pastures and for international tourism. The disturbance of the environment may affect the marmots leading to a decrease in their population sizes. The number of other small rodents and their variability and density are approximately equal in simultaneously studied years in both plague foci. Prevalence of *M. gregalis* confirms the role of this species as a potential secondary host of plague pathogen in mixed ecosystems of high-altitude Tien Shan focus. Isolation of one *Y. pestis* strain from *C. migratorius* in the Sari-Dzhas focus confirms the role of small mice-like rodents in the epidemiology of plague. Previously, plague strains were isolated in the neighboring Aksay focus from *M. gregalis* (1968) and *C. migratorius* (1983–1984) and in the Upper-Naryn focus from *Alticola argentatus* and *C. migratorius* (3). Similar observations were reported in the high altitude of Altay and Tuva plague foci of Russia (34) and the North Aral sandy plague focus of Kazakhstan (35). Increasing density of rodents co-inhabiting with the marmots altogether with an increased number of their specific ectoparasites as observed in the Upper-Naryn focus in 2013–2017 could be a sign of potential activity of plague epizootics in this area of Kyrgyzstan. The isolation of 9 of 13 strains from marmots in 2012–2017, 1 strain from other rodents, and 3 strains from their ectoparasites is in favor of this hypothesis. Both independent plague foci (Sari-Dzhas and Upper-Naryn), located on the border of Kyrgyzstan, Kazakhstan, and China, are among the most active natural high-altitude foci of Central Asia. This territory is characterized by special climatic conditions, different relief with many heavily rugged canyons, mountain river valleys, and patches with specific flora and fauna with a high degree of biological diversity (36). Apparently, these factors give optimal conditions for the long-term circulation of *Y. pestis* biovar *Antiqua* (0.ANT2, 0.ANT3) in the populations

TABLE 8 | Amplicon sizes of *Y. pestis* strains on 7 VNTR loci.

Strain of *Y. pestis*	MLVA−7							Genotypes
	ms01	ms04	ms06	ms07	ms46	ms62	ms70	
KG_1	264	213	547	174	259	276	164	Genotype 1
KG_2	264	213	547	174	259	276	164	Genotype 1
KG_3	264	213	547	174	259	276	164	Genotype 1
KG_4	264	213	547	174	259	276	164	Genotype 1
KG_5	264	213	547	174	259	276	164	Genotype 1
KG_6	246	230	547	174	259	294	146	Genotype 2
KG_7	246	196	546	174	252	294	146	Genotype 3
KG_8	246	213	546	174	259	294	146	Genotype 4
KG_9	246	213	546	174	259	303	146	Genotype 5
KG_10	246	213	546	174	259	294	146	Genotype 4
KG_11	246	179	0	174	259	276	137	Genotype 6
KG_12	246	196	546	174	259	294	146	Genotype 7
KG_13	246	179	546	184	259	276	137	Genotype 8
KG_14	228	213	546	174	293	240	155	Genotype 9
YP_EV_76	210	230	606	184	252	222	155	Genotype 10
YP_3770	210	179	547	174	245	285	119	Genotype 11
YP_Angola	210	162	362	134	259	276	137	Genotype 12
YP_91001	210	196	303	184	252	258	128	Genotype 13
YP_Antiqua	192	179	487	164	238	231	209	Genotype 14
YP_1045	210	196	305	174	322	303	155	Genotype 15
YP_790	192	213	545	174	322	249	137	Genotype 16
YP_A1122	210	230	606	184	252	294	146	Genotype 17
YP_Pest_G	210	179	303	174	245	312	119	Genotype 18
YP_Pest_B	192	179	547	174	252	240	137	Genotype 19
YP_KIM10	192	196	305	164	378	276	146	Genotype 20
YP_Nepal516	192	196	185	174	259	222	137	Genotype 21
YP_Pest_F	210	179	487	194	245	294	119	Genotype 22
YP_CO92	228	230	606	184	252	240	146	Genotype 23
Pt_IP32953	192	145	1088	144	266	330	155	Genotype 24
Pt_6904	192	145	786	144	0	285	155	Genotype 25
Pt_PB1	192	164	606	154	301	267	200	Genotype 26
Pt_2841	174	213	0	154	252	240	128	Genotype 27
Pt_433	174	213	0	154	259	231	137	Genotype 28

TABLE 9 | Results of Melt-MAMA analysis.

Strains	Locus s87 (T/G)	Locus s332 (G/T)	Locus s645 (G/T)	Biovar	Estimated branch
KG-1–KG-13	G	G	G	*Antiqua*	0.ANT2
KG-14	G	T	G	*Antiqua*	0.ANT3

of its natural host (31, 37). In total, 462 strains of *Y. pestis* were registered in the Sari-Dzhas area from 1944 to 1976 (4, 6, 7). Ectoparasites are actively involved in the epizootic process, in particular *O. silantiewi* and *Rh. li ventricosa* fleas specific to *M. baibacina*, as well as ixodes mites. The Sari-Dzhas area was very active in the 1940s to 1980s, and then mass disinsection of marmot populations with dichlorodiphenyltrichloroethane (DDT) insecticide and the decrease in the number of animals as a result of human activities (special extermination conducted in 1960s, hunting) led to a significant decrease in epizootic activity. Whereas, human activity (agriculture development, tourism, and hunting) changed environment during this period significantly, an obvious activation of epizootic plague activity within the area was observed after 30 years. Overall, the obtained results confirm ongoing epidemiological risk and vulnerability of the territory to plague. In such situation, it is necessary to strengthen ecological and epidemiological monitoring and control over the entire endemic area in order to preserve safety of local populations.

ETHICS STATEMENT

All manipulations with wild and laboratory animals and handling plague strains were conducted according to the protocols approved by the Kyrgyz government regulations that are related to guidelines of Russia, Kazakhstan (1, 25). The animal work was performed according to the Regulation of the State Agency for Environmental and Forest Protection Facilities at the Government of the Kyrgyz Republic. Animal work in the field was conducted according to the following protocols: protocol 000144-KC for trapping marmots, other rodents, and analysis of their nests approved on May 6, 2014; protocol # 000168-KC approved on May 22, 2015; protocol #000173-KC approved on May 17, 2016; and protocol #000003-KC approved on March 5, 2017. Each permit to collecting a certain number of animals (400 marmots, 400 rodents of other species, and 10 nests of marmots and rodents) was issued and used during the fixed period of time (from June 1 to August 30).

AUTHOR CONTRIBUTIONS

GS initiated the study and wrote the first draft. GB organized and conducted the field studies and performed zoological and entomological analysis. RMu performed agreement with the state agencies for animal catching and analysis. SA provided strains for DNA analysis and genotyping. BK and AA performed molecular-genetic analysis of strains. AS performed GIS-mapping and database design. ZS performed bacteriological analysis and spatial epizootical analysis. AD performed the bacteriological analysis. ZA analyzed the environmental effect on population structure. RMa provided consultative assistance. SM provided collaborative efforts. VM edited the draft and provided reference strains. MK edited the draft and provided collaborative efforts. All authors read and approved the final manuscript.

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Prairie Dogs, Persistent Plague, Flocking Fleas, and Pernicious Positive Feedback

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Plague (caused by the bacterium *Yersinia pestis*) is a deadly flea-borne disease that remains a threat to public health nearly worldwide and is particularly disruptive ecologically where it has been introduced. We review hypotheses regarding maintenance and transmission of *Y. pestis*, emphasizing recent data from North America supporting maintenance by persistent transmission that results in sustained non-epizootic (but variable) rates of mortality in hosts. This maintenance mechanism may facilitate periodic epizootic eruptions "in place" because the need for repeated reinvasion from disjunct sources is eliminated. Resulting explosive outbreaks that spread rapidly in time and space are likely enhanced by synergistic positive feedback (PFB) cycles involving flea vectors, hosts, and the plague bacterium itself. Although PFB has been implied in plague literature for at least 50 years, we propose this mechanism, particularly with regard to flea responses, as central to epizootic plague rather than a phenomenon worthy of just peripheral mention. We also present new data on increases in flea:host ratios resulting from recreational shooting and poisoning as possible triggers for the transition from enzootic maintenance to PFB cycles and epizootic explosions. Although plague outbreaks have received much historic attention, PFB cycles that result in decimation of host populations lead to speculation that epizootic eruptions might not be part of the adaptive evolutionary strategy of *Y. pestis* but might instead be a tolerated intermittent cost of its *modus operandi*. We also speculate that there may be mammal communities where epizootics, as we define them, are rare or absent. Absence of plague epizootics might translate into reduced public health risk but does not necessarily equate to inconsequential ecologic impact.

Keywords: plague, flea, *Yersinia pestis*, feedback, epizootic, rodent, enzootic

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INTRODUCTION

Plague is a zoonotic disease (caused by the bacterium *Yersinia pestis*) that has a long history of causing human suffering and massive death rates. *Y. pestis* is a generalist vectored by a wide range of fleas (Siphonaptera) (1) and infecting a wide range of mammalian species. The impact of plague on humans has motivated much research, but the complexities caused by the array of hosts and fleas as they interact with each other and their environments have left many ecological questions unanswered (2). Plague has colonized North America, South America, and portions of Africa and southeast Asia, at least, but relatively little attention has been devoted to plague

as an invasive disruptor of ecosystems or its effect on species of conservation concern (3–5). Recent studies of plague in the prairie dogs (*Cynomys* spp.) of North America and their critically endangered associated predator, the black-footed ferret (*Mustela nigripes*), have suggested that this disease played a pivotal role in the decline of these mammals and continues to heavily influence conservation activities for them, and associated species (6–8). More than half the species of rodents of conservation concern in North America occur within regions where plague is present (9); perhaps the plight of ferrets and prairie dogs (PDs hereafter) represents a phenomenon that is more common than has been recognized. Thus, broad conservation and public health issues associated with plague make this disease a prime candidate for consideration within a One Health framework (10).

Two questions that are highly relevant to One Health objectives of understanding and managing plague risk are (1) how is plague maintained as a sylvatic disease and (2) what factors lead to epizootic outbreaks? Gage and Kosoy (2, 11) summarized 4 hypotheses for plague maintenance in communities of free-ranging mammals and their fleas: (1) continued enzootic transmission within populations of susceptible hosts and fleas, (2) chronic infection of partially resistant hosts, (3) prolonged survival in fleas, and (4) prolonged survival in soil. Experimental and field evidence has not been able to eliminate any of these hypotheses from consideration, and the 4 ecological mechanisms are not mutually exclusive (2, 11).

Highly susceptible species such as PDs have traditionally escaped notice as potential reservoirs for plague. The logic was, because PDs are "highly vulnerable to plague, they should not be long-term reservoirs of the disease" (12), more dramatically stated as Gunnison's PDs (*C. gunnisoni*) "are clearly not the maintenance species for plague" (13). Historically, partially resistant species were thought to be probable reservoirs or maintenance hosts for *Y. pestis* (14), with microtine or cricetid mice listed as candidates (15–17). Barnes (18) implied that plague was maintained in foothill foci in Colorado, only periodically expanding onto the plains of eastern Colorado and causing epizootics in PDs.

The presumption that PDs and other highly susceptible rodents (19) are not long-term reservoirs of plague implies *Y. pestis* is a periodic invader from residency elsewhere. Recent field studies support the hypothesis of maintenance by susceptible species whose populations often suffer moderate and varying levels of plague-caused mortality during the process and may be periodically decimated by epizootic eruptions. A 5-year controlled study employing flea-control as the treatment to impede plague transmission in 3 PD species implied that there was persistent plague circulation at sub-epizootic levels (20), although vector control effects cannot be unerringly equated to plague effects. In similar studies using vector control, but with experimental plague vaccines added as a second treatment, woodrat (*Neotoma mexicana*) survival in Colorado (2-year study) (Biggins et al. submitted manuscript) and New Mexico (3-year study) (21) was significantly improved by the plague management tools. Unlike vector control, plague vaccine is thought to be specific in its protective effect. In another multi-year study of woodrats (*N. albigula*) in New Mexico, Kosoy et al. (22) collected nest occupancy evidence suggesting maintenance of plague by localized die-offs that shifted over space and time. Finally, either vector control or a plague vaccine improved black-footed ferret survival by $> 200\%$ despite lack of epizootic plague during the 4-year experiment in Montana (23). Studies of the genetics of *Y. pestis* and detection of the bacterium during sub-epizootic periods provide additional support for the hypothesis that PDs help to maintain plague or that *Y. pestis* is otherwise maintained locally in or near PD colonies (23–27). New invasions and colonization events may characteristically begin with epizootic plague and later subside into enzootic plague (28) and disease maintenance.

The notion that plague is resident in a geographic area allows for epizootic eruptions in place, without the need for invasion or reinvasion by the bacterium or its resurrection from a quiescent state in soil or elsewhere. Thus, the discussion should be about the scales of eruptions in place vs. movement and the relative importance of each. The parsimonious hypothesis that plague "circulates at much reduced rates among most, if not all, of the same hosts that commonly become infected during epizootics" (2) facilitates a discussion of factors that might promote the transition from enzootic to epizootic transmission rates. One goal in the discussion that follows is to review the roles of flea density, host density, and *Y. pestis* density in that transition, and to propose positive feedback (PFB, hereafter) cycles as definitive elements of epizootic plague. We define PFB as an exponential increase in an effect resulting when the cause is cyclically amplified by the effect such that cause and effect labels become interchangeable. A second goal is to introduce the concept of triggering mechanisms that might initiate runaway PFB.

In addition to the concept of local enzootic plague maintenance by highly susceptible mammalian hosts or their associates, a second influential factor facilitating the PFB cycle might be early phase transmission (EPT) by fleas. Recent evidence on EPT (29–31) is compelling. The speed of the PFB cycle might be dramatically enhanced if infected fleas can immediately transmit *Y. pestis* rather than being delayed 5 days to months while the biofilm-mediated blockage of the proventriculus develops. Also, most fleas die of starvation shortly after becoming fully blocked, ending their ability to contribute to a PFB cycle. These attributes build a strong case for considering EPT as an important contributor to epizootic plague. However, epizootics (as we define them—see below) may last up to several months, thus allowing for blocked fleas to contribute to plague transmission. Another consideration might be the seemingly more efficient transmission reported for blocked fleas (32). Proventricular blockage is not thought to occur in *Oropsylla hirsuta* and *O. tuberculata cynomuris* (33), two important PD fleas, but contradictory results from studies of flea blockage raise questions (32) about the relative involvement of the two forms of transmission in free-ranging rodents.

Definitions

Before delving into the details of transitions from enzootic plague maintenance to epizootic eruptions, it seems essential to discuss and explicitly define the terms. If plague circulates within a host species at rates that vary along a continuum (2),

binomial classification of those rates into epizootic and enzootic is artifactual. Nevertheless, at least two arguments support continued use of these terms. First, the terms and concepts have a long history and, at least at the both ends of the spectrum, convey a sense of real and observable phenomena. When one observes the nearly complete collapse of a PD colony in just a few weeks due to plague, the term epizootic seems intuitively apt. Second, and within the context of this paper, we might give more refined meaning to epizootic if we can associate it with runaway PFB.

Epizootic has been defined as "Pertaining to an epidemic in animals" and epidemic as "a disease affecting a high proportion of the population over a wide area" (34). There is no temporal component to this definition, and the vagueness of "high proportion" and "wide area" render such definitions inadequate for our purposes. Because the definitions might vary somewhat when considering different species and contexts, it is useful for authors to define these terms in each individual report. For example, Biggins et al. (20) described epizootics as resulting in the deaths of >90% of a PD population and enzootic plague as affecting lesser proportions, but they did not provide temporal or spatial criteria. Ramakrishnan (21) used the 90% mortality cutoff but required the episode to occur within 3 months and over at least 10 ha of habitat. In both examples, "affecting" animals is narrowed to considering plague-caused deaths, which seems appropriate given the lethality of plague and the need for a metric that estimates demographic attributes of populations relevant to conservation. For this paper, we adopt the criteria of Ramakrishnan (21) to distinguish between epizootic and enzootic transmission, with further discussion below about the relationship to PFB cycles.

What if an outbreak takes several years to decimate the population of hosts (a phenomenon we have observed)? Under our definition of enzootic plague, populations can either decline or grow over long periods. What about deaths of just a few PDs that comprise a territorial harem polygynous family, or so-called "coterie" occupying a few hectares? At some point on the scale of individual organism to sub-population to population to range-wide distribution of a species we must pick a defining limit for clarity of communication. Clearly, death of an individual PD cannot define an epizootic, nor should we need extinction of a PD species to define it. Coining phrases like "mini-epizootic" or "small-scale epizootic" captures a sense of the mechanism working at small spatial scales but are semantically inarticulate oxymorons because epizootic and epidemic are defined as large scale phenomena.

The term enzootic may be used in a broad context that considers all forms of plague maintenance, not just the transmission of plague at sub-epizootic rates. It can include *Y. pestis* residing in micro-organisms (35), soil (1), or fleas (2, 36). Here, however, we limit our discussion to active enzootic plague transmission. If epizootic defines only one end of a broad spectrum, enzootic must encompass a truly large range of transmission rates and host mortality. The concept of plague maintenance by low rates of transmission dates back almost to the discovery of *Y. pestis* by Yersin in 1894. Low (37) and Elton (38) used the term "smoldering" plague to describe what we might think of as the slow transmission end of the spectrum. That term has been more recently resurrected (39, 40), but it connotes a rather benign manifestation of the disease that does not seem to accurately depict the moderate rates of transmission and mortality that are common and can have substantial impacts on host populations (20, 21, 23, 41).

PFB Cycle Components of Epizootic Plague

Fleas are a vital component of the PFB cycles discussed herein. An increase in flea parasitism accompanying epizootic plague was observed at least a half century ago when Shchekunova et al. (42) noted "The dying out of the original inhabitants of burrows was accompanied by a migration of fleas onto surviving rodents and onto new settlers. As a result the index of the abundance of fleas on *O. mongolica* here in the beginning of summer amounted to 3.2 and in the autumn—to 8.5…" Pauli et al. (43) uses the term "swarming" of fleas onto hosts during epizootics. Tripp et al. (44) suggests "Concentration of infected fleas on surviving animals may account for the rapid spread of plague during epizootics." Salkeld et al. (40) mentions that "transmission rates snowball" due to "increased abundance of fleas searching for meals" [see also (45)]. These descriptions seem to infer PFB cycles. The graphics and notes on feedbacks from Ray and Collinge (46), the graphic of Reijniers et al. (47), and the discussion on "vicious circles" of disease transmission by Beldomenico and Begon (48) articulate parts of the PFB cycles we emphasize herein.

Disruptive effects of plague on PD social systems may fortify the flocking of infectious fleas to PD hosts. The presence of kin within PD coteries encourages PDs to remain in coterie territories, affording them fitness benefits such as cooperative predator detection and allogrooming to remove ectoparasites (49). As plague transmission increases and kin disappear, PDs likely inspect vacated burrows (e.g., to entomb dead PDs) and risk acquiring infectious fleas (50). Moreover, as PD coteries become vacated, opportunities for cooperation are diminished or eliminated, and PDs can move among former territories (49, 51), allowing them to acquire and ferry infectious fleas (40).

These two mutually reinforcing PFB loops were encapsulated in a general description by Gage (52):

> "The rate of plague transmission by fleas also could be influenced by increased contact rates between infectious vectors and susceptible host individuals, with increased contact resulting in a concomitant increase in secondary infections as the disease spreads from an initial focal infection… Transmission rates also have been suggested to increase during epizootics as a result of infectious fleas becoming more and more concentrated on the decreasing number of surviving hosts…"

We summarize these PFB cycles during an epizootic in PDs (**Figure 1**) as juxtaposed loops of increasing flea:host ratios (Cycle A) joining increasing host and flea contact due to altered PD social systems and behaviors (Cycle B). The interaction is critical; the 2 loops must be considered together. A triggering event might initiate the primary PFB loop involving altered flea:host ratios. In the short term, the population of plague

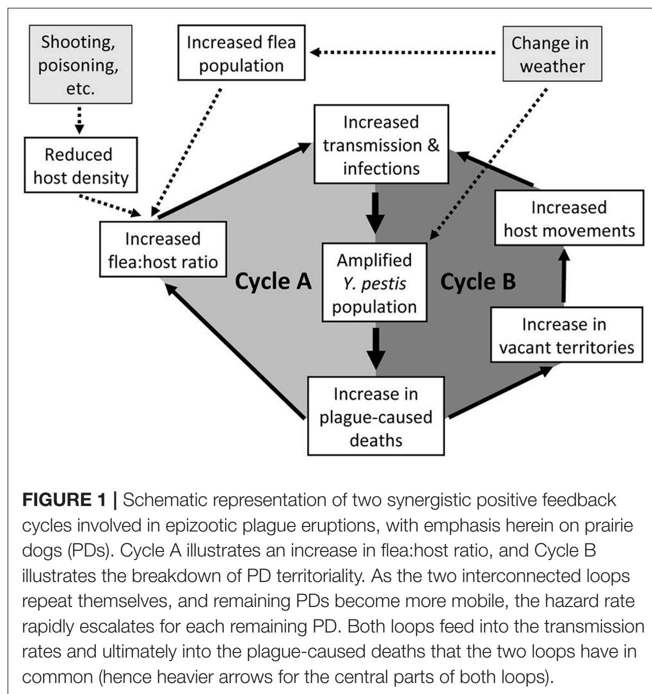

FIGURE 1 | Schematic representation of two synergistic positive feedback cycles involved in epizootic plague eruptions, with emphasis herein on prairie dogs (PDs). Cycle A illustrates an increase in flea:host ratio, and Cycle B illustrates the breakdown of PD territoriality. As the two interconnected loops repeat themselves, and remaining PDs become more mobile, the hazard rate rapidly escalates for each remaining PD. Both loops feed into the transmission rates and ultimately into the plague-caused deaths that the two loops have in common (hence heavier arrows for the central parts of both loops).

bacteria rapidly increases, the host population declines, vector numbers remain high, and infections increase. This is soon followed by initiation of the secondary PFB loop as sufficient deaths within coteries cause territorial vacancies that enhance unimpeded PD movements. At that point, both feedback loops operate together to synergistically magnify the overall PFB cycle. As the two connected loops repeat themselves, and remaining PDs become more mobile, the hazard rate rapidly escalates for each remaining PD. Both loops feed into ever higher transmission rates and ultimately into plague-caused deaths that both loops have in common (hence heavier arrows for the central parts of both loops). Triggers are exemplified (**Figure 1**). A dramatic trigger may initiate an epizootic under less than optimal conditions, or the PFB cycle might spontaneously ignite without any trigger when host and flea densities are high and *Y. pestis* is enzootically abundant in the focal host or associated species.

Weather and habitat conditions doubtless influence hosts, fleas, and *Y. pestis* (53, 54), which we simplistically represent with a single input block (**Figure 1**). Changes in temperature can influence flea reproduction and survival (55), replication rates of *Y. pestis* (56), and proventricular blockage in fleas (57), thereby influencing transmission rates. Although trophic responses of hosts to weather are likely over longer terms (46), we consider only short-term changes; host populations respond more slowly than populations of fleas or *Y. pestis.* Barnes (18) captured the oversimplification risks of such conceptual models by saying “In this complex and shifting milieu, it is often difficult to determine if fleas or rodents are most important because their roles may change with time, space, and circumstance.” Regardless, an external trigger causing substantial mortality of a subpopulation of hosts, or otherwise optimal conditions for transmission, might initiate an explosive PFB-mediated plague epizootic.

The illustration of the two PFB cycles is representative of early to mid-stages. At some point, there are few PD movements because most PDs are dead, fleas perish from starvation (33), and populations of live *Y. pestis* likely diminish as host carcasses deteriorate or are consumed by scavengers (58, 59). Little is known about demographics and plague-caused mortality of PD fleas (60, 61). However, the primary fleas that seem to be central to *Y. pestis* transmission in PDs (*Oropsylla hirsuta and O. tuberculata cynomuris*) are able to transmit *Y. pestis* before blocking occurs (EPT) (31, 32), are perhaps highly capable of blocked flea transmission (Hinnebusch, personal communication), and might clear some infections but become infected once again when feeding on an infectious host, the latter of which helps to perpetuate plague transmission until the density of hosts is insufficient to support fleas (30, 62).

The combined PFB loops (**Figure 1**) are described in a temporal context but also have an implied spatial component. As with a metaphoric forest fire PFB cycle, this PFB of fleas and *Y. pestis* cannot erupt for long in one place without running out of PD fuel. It must keep moving. However, unlike fire which moves primarily with the wind, it can move equally well in all directions. In fact, maximizing the area affected per unit of time would involve a feedback cycle that gets triggered in the middle of suitable space, where the movement can be envisioned as expanding circles of impact. This dynamic of *Y. pestis* over time and space may reflect how it maintains itself in an enzootic state (22).

The potentially destructive nature of PFB is commonly illustrated by reference to nuclear weapons. The self-accelerating chain reaction of an atomic bomb releases enormous energy, but the system needs a trigger of conventional explosives (which themselves involve PFB) for activation. Similarly, but at a smaller scale, the bullet from a rifle is propelled down the bore by the self-amplifying explosion of gunpowder, also ignited by a chain of triggering actions. The first of these actions is the shooter physically pulling the rifle’s trigger that slams its firing pin into a small, pressure sensitive primer; these actions are analogous to any sudden and localized reduction of PD hosts that increases the flea:host ratio. The primer explodes, triggering the larger PFB explosion in the gunpowder within the cartridge casing and unleashing the destructive power of a speeding bullet, which is analogous to the destructive power of an expanding PFB-powered plague epizootic. Ironically, the rifle and shooter exemplified above could serve as a trigger in our PFB example involving PDs, fleas, and plague.

TWO FIELD EXPERIMENTS ON POTENTIAL PFB TRIGGERS

The Role of Fleas

Foundational to the PFB hypothesis is the assumption that fleas are critical to plague transmission. Substantial evidence of this has accumulated for >100 years and remains basically unchallenged (2, 11, 32, 36, 58, 63, 64). That said, fleas may not be particularly efficient at transmitting *Y. pestis*, providing an explanation for evolution of high virulence of this pathogen

(65); probability of transmission is positively correlated with high levels of host bacteremia that often become lethal. Importantly for the PFB hypothesis, flea inefficiency leads to the need for large numbers of fleas to further increase the probability of transmission and infection (65). Field evidence regarding flea abundance and plague transmission includes flea control experiments that increased rodent survival rates (20, 21) and halted the progression of epizootic plague (66–68). Although less dramatic variation in flea densities may be more difficult to link to plague transmission rates (69), flea parasitism in one study was negatively correlated with PD survival (*Cynomys parvidens*; Eads and Biggins in preparation).

Below, we provide experimental evidence regarding the plausibility of recreational shooting and poisoning of PDs as potential triggers for the flea-plague PFB cycle. Recreational shooting (70) and poisoning (71) are episodic and cause high localized mortality in PD populations. These types of events occur at scales that would seem relevant for PFB triggering. For example, we observed > 97 PDs shot during one morning on a colony of about 300 PDs in Montana (not the colony sampled for study below), and the rodenticide in our South Dakota study was distributed over a 20.6-ha portion of a 70.4-ha colony. Under the PFB hypothesis, episodic host mortality should cause fleas to abandon PD carcasses and flock to living hosts. If so, large numbers of fleas should be collected from burrows near PDs killed by recreational shooters and from burrows in portions of PD colonies that are poisoned.

Flea Sampling and Data Analyses

On 22 June 2006, we conducted flea sampling in "active" burrows of a black-tailed PD (*Cynomys ludovicianus*) colony in Phillips County, Montana (Colony B-100) on which recreational shooting had occurred within the previous few days (judging from the condition of the PD carcasses found). Burrow activity was classified using the presence of fresh scat (72). Sampling consisted of inserting a plumber's snake tipped with a 15 × 15 cm flannel cloth into each active burrow opening as far as possible for about 30 s and removing the cloth for flea collection and counting (66). The flannel is a crude surrogate for a PD that is investigating the burrow. The insertion technique was done twice at each burrow with a delay between insertions to allow counting and removing fleas from the cloth. Total number of fleas was recorded for each burrow, along with the presence or absence of a dead PD within 1 m of the burrow opening. We graphically presented the data as prevalence (frequencies of burrows from which fleas were collected and not collected), but we used a non-parametric Mann-Whitney test on numbers of fleas collected from each burrow to evaluate the influence of presence or absence of a dead PD at or near the burrow.

Zinc phosphide rodenticide was applied to a portion of a black-tailed PD colony (Cutbank) on the Buffalo Gap National Grassland in South Dakota as part of a "boundary control" effort on 12 December 2017. We sampled active burrows and recorded data as described above, except each burrow (of at least 0.5 m depth) was sampled three times (instead of twice). Sampling was conducted before (5 October) and after application of the rodenticide (13–14 December) on poisoned and non-poisoned portions of the colony. This before-after-control-impact design allowed assessment of treatment effect while controlling for the effect of time, a desirable feature when measuring flea abundance which can vary considerably from month to month (33, 44, 73–76). We evaluated flea abundance using logistic regression models that had time (before or after) and treatment (poisoned or non-poisoned) as predictor variables. A significant ($\alpha = 0.05$) treatment by time interaction would suggest a treatment-related disproportionate change in fleas over time. Because fleas were much more abundant on this South Dakota colony than on the Montana colony, we used a binomial response variable that considered 6 fleas as the cutoff point ($\leq$ 6 fleas = 0, > 6 fleas = 1) rather than simple presence or absence (prevalence, as used to graphically illustrate the Montana data).

RESULTS

In Montana, we collected 5 fleas from 8 sampled burrows associated with shot PDs and we collected 8 fleas from 25 burrows without a carcass. Average penetration of the sampling apparatus was 2.70 m (range 1–4.5 m). We found 2 additional burrows that contained dead PDs that were visible below the surface. Those 2 burrows were not sampled but suggest there may have been dead PDs present deeper within burrows that were categorized as lacking a PD carcass. Burrow openings accompanied by a dead PD had significantly more fleas than openings without visible carcasses (Mann-Whiney $U = 52.500$, $P = 0.013$) and had higher flea prevalence (**Figure 2**). Of the 8 burrows with a carcass, 1 had 2 fleas and 1 had 3 fleas; no more than a single flea was collected from any burrow without a carcass.

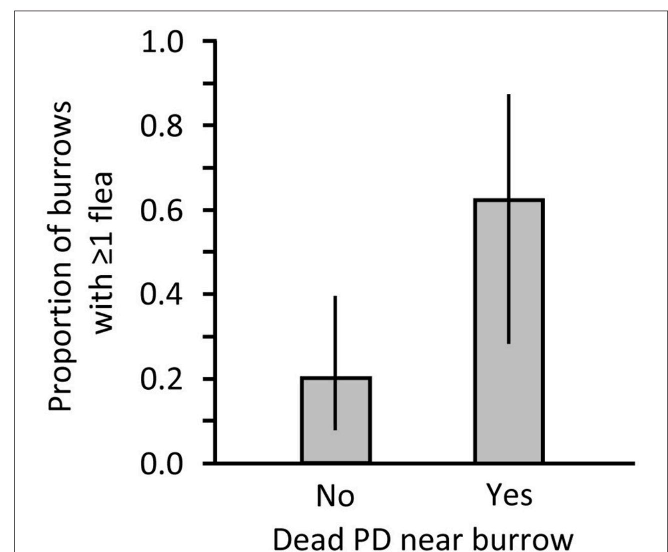

FIGURE 2 | Proportion of burrows on a black-tailed prairie dog (PD) colony in Montana where 1 or more fleas were collected after a recreational shooting event in Montana. Data are presented for burrows at which a dead PD was not (No) or was (Yes) found nearby.

Before application of zinc phosphide on the South Dakota colony, no dead PDs were found in the non-poisoned or poisoned areas. In contrast, after application, no dead PDs were found in the non-poisoned area but 3 dead PDs and 1 dead PD were found in the poisoned area on 13 and 14 December, respectively. We collected 474 and 390 fleas from 50 swabbed burrows in the non-poisoned and poisoned areas, respectively, before the poisoning event, and we collected 363 and 852 fleas from 50 burrows in non-poisoned and poisoned areas, respectively, after the event. In the logistic regression model, the interaction of time and treatment was significant (Likelihood Ratio $X^2 = 4.486$, $df = 1$, $P = 0.034$). There was little difference in proportions of burrows with >6 fleas between the poisoned and non-poisoned portions of the colony pre-treatment, but there were nearly twice as many burrows with >6 fleas on the poisoned portion than on the non-poisoned portion following application of the rodenticide (**Figure 3**; $X^2 = 7.955$, $df = 1$, $P = 0.005$).

DISCUSSION

If our inserted flannel swabs were indeed reasonable surrogates for burrow investigations by PDs that explore newly unoccupied territories, they illustrate how flea loads could rapidly increase on PDs due to PFB triggering events (**Figure 1**). But do flannel swabs provide reasonable indices for flea-host encounters? Perhaps host deaths alter flea behaviors. "During and following an epizootic, fleas migrate to burrow entrances and can be captured in large numbers. When prairie dogs are alive and healthy, fleas tend to remain in the nest where they are not reachable" (18). Questions about detection probabilities (77, 78) within our simple field experiments raise additional uncertainties about measuring flea abundance in burrows. Nevertheless, increased collection of fleas after shooting and poisoning is consistent with the hypothesis that PFB cycles are sometimes triggered by episodic events causing high mortality in a host subpopulation. We might have underestimated the importance of this phenomenon in the poisoning experiment; subsequent observations suggest the zinc phosphide treatment was less effective than expected (causing about 75–80% mortality instead of >90%), meaning the flocking of fleas (which was dramatic) may have been dampened.

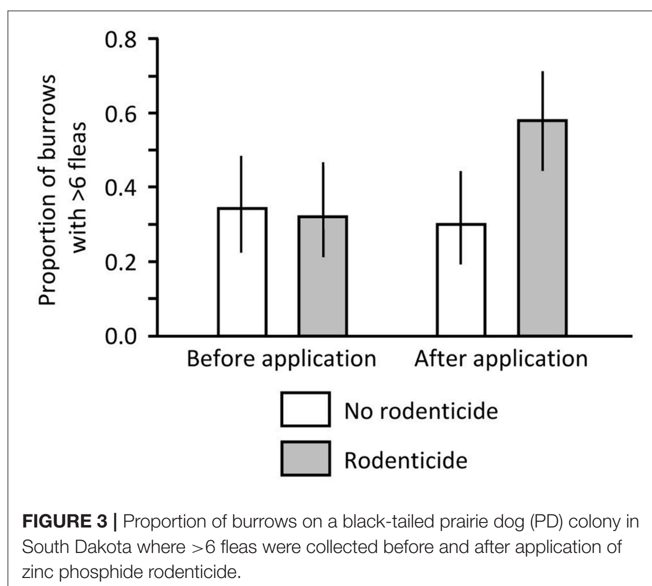

FIGURE 3 | Proportion of burrows on a black-tailed prairie dog (PD) colony in South Dakota where >6 fleas were collected before and after application of zinc phosphide rodenticide.

Ramifications of PFB Cycles for Plague Transmission

Intuitively, high host death rates will initiate an increase in flea densities beyond the threshold for epizootic plague (65). Rates of flea collection [e.g., (43, 77)] and flea infection [e.g., (26, 44, 79)] are commonly higher from PD burrows after or during an epizootic than under non-epizootic. Some investigators emphasize the increase in flea abundance and infection as predictors or causes of epizootic plague [e.g., (40, 44, 65)] and others as responses to epizootics [e.g., (42, 63)]. Under the PFB hypothesis, burgeoning flea numbers and infections are both cause and consequence after a cycle begins (**Figure 1**).

Due to PD social structure and territoriality, flea-plague PFB cycles may occur in a patchy manner (i.e., multiple "explosions" of feedback at the coterie level and slower transmission of *Y. pestis* between coteries). Group deaths within coteries seem likely because coterie members may share burrows as nesting environments (51) and probably share the same sub-population of fleas in their burrows (similar to great gerbils, *Rhombomys opimus*, in Central Asia) (80). After death of the primary coterie defenders, adjustment by members of adjacent coteries is likely (51) and exposure rates of neighbors would be enhanced (40). As epizootic activity increases and plague spreads among coteries, PFB cycles can become self-amplifying until nearly all hosts are parasitized by fleas and succumb to the disease.

Our representation of change in flea:host ratio (**Figure 1**) as hosts die may be oversimplified due to unequal susceptibility of individuals to flea parasitism and interactions among biotic and abiotic factors. For example, as the flea:host ratio increases during rapid plague transmission, the most susceptible individuals may take the initial brunt of the parasite shift and plague mortality. Adult male PDs could play an especially important role (44); they have much higher flea loads than adult females just after breeding season and often harbor the most fleas in summer and fall (44, 81, 82). Adult male PDs might be the primary initiators of the PRB cycle because of their higher flea loads and because they are the primary coterie defenders (51) and may be the first PDs to explore newly unoccupied territories and thus the first to accumulate newly questing, plague-positive fleas.

Another potential PFB cycle that is intertwined with the flea-plague PFB is mediated by drought. It can be simplistically described as: poor forage (due to drought) leads to water balance and/or energetic limitations (83) that lead to poor host body condition (81) that leads to increased flea loads (74, 81, 84) that lead to even poorer host body condition (55), and so on. This cycle might promote increased circulation of plague due to higher flea loads and perhaps initiate a flea-plague PFB eruption (85). Under sub-optimal conditions for transmission, the developing

epizootic may be self-limiting at the point where relatively flea-resistant individuals (e.g., PDs in good body condition) are remaining. Lending support to this hypothesis, Pauli et al. (43) found that PDs surviving an epizootic exhibited improved body condition compared to PDs before the epizootic. In many cases, however, a rapid change in flea:host ratio might overwhelm the entire population, resistant and susceptible alike.

Recreational shooting of PDs might trigger PFB cycles by shifting fleas to the dwindling number of PDs and by affecting PD body condition. During a before-after-control-impact experiment involving shooting of PDs in Wyoming, surviving PDs on shot colonies increased vigilance 8-fold and reduced time spent foraging by 66% relative to PDs on control colonies protected from shooting (86), contributing to a 35% reduction in PD condition. Reductions in PD condition and hypothesized (81) increases in flea parasitism may trigger PFB. Moreover, the significant stresses of recreational shooting (86) may compromise the immune systems of some PDs, causing increases in flea parasitism (55) and mortality in PDs that fail to overwinter, thereby further concentrating fleas on PDs. Although recreational shooting could potentially trigger several types of deleterious PFB cycles, a short-term epizootic cycle (if it developed) would overpower other cycles.

Interactions among a wide array of variables could influence the change in flea:host ratio of the proposed PFB cycles. Flea populations are influenced by many factors that are beyond the scope of detailed discussion here (74, 81, 84, 87–89). Weather and climate at spatial and temporal scales from microsites to El Niño patterns are influential (**Figure 1**) (90–92) and, as noted above, recent studies suggest precipitation lag effects and host body condition further increase the complexity. Recreational shooting, poisoning, and other sources of host mortality may interact with weather and season. For example, shooting or poisoning after optimal weather conditions for plague transmission may be more likely to trigger an epizootic than shooting or poisoning that follows moisture and temperature conditions that are less favorable for fleas or *Y. pestis*.

Several factors might serve to counter the initiation of epizootic PFB cycles. First, the flea density threshold concept of Lorange et al. (65) is assumed to be critical, although the necessary levels of flea parasitism are unknown for wild, free-ranging hosts such as PDs. Second, intraspecific and interspecific competition among both fleas and hosts could provide negative feedback that impedes the initiation of the flea-plague PFB cycle (55, 93). These phenomena could become interactively complex in systems involving multiple hosts and multiple flea species, but in situations where a single host is primarily responsible for plague circulation, host territoriality could limit transmission to enzootic rates (22). Third, disease transmission rates in general are assumed to be at least somewhat dependent on host densities (94). However, for PDs, it seems that flea densities are more important. An epizootic eruption of plague occurred in Utah PDs when densities (from adjusted visual counts) (72) were just 2.3 PDs ha^{-1} (Biggins unpublished data). Flea parasitism was an important predictor of Utah PD annual survival during a 4-year study; epizootic plague was suspected in many cases, despite low PD densities (Eads and Biggins unpublished data). Thus, it is unsurprising that large rodent control campaigns have failed to eradicate plague in sylvatic systems and that tactic has been abandoned in Russia (95). More localized control of peridomestic rodents, however, can reduce risk of plague exposure in humans (96). We emphasize highly plague-susceptible North American PDs in this treatise, but other species with proportions of populations that are immune to plague would be expected to exhibit much different population dynamics when challenged by plague.

In keeping with the idea that any significant cause of mortality might initiate a PFB cycle (increasing ectoparasite:host ratios), other vector-borne diseases (e.g., tularemia) should also be considered. Triggers might result in secondary interactions between diseases, transforming diseases that might characteristically have a moderate effect (which probably include some diseases native to North America, like tularemia) into triggers for the flea-plague PFB cycle. Conversely, we might consider that plague, operating within its own PFB cycle, might exacerbate the effect of native disease by altering the parasite:host ratio.

PFB Cycles, Balancing Negative Feedback, and Source-Sink Dynamics

There are examples of PFB that build and sustain ecological systems (97, 98). Nevertheless, "Positive feedback mechanisms are usually associated with instability in a system" (99) and are often considered to be destabilizing and deleterious. Examples are the self-reinforcing nitrogen dynamics of invasive cheat grass (*Bromus tectorum*) in the western U.S (100), the human-triggered algal and microbial feedback loops that threaten coral reefs (101), and even the postulated runaway greenhouse involved in the massive Permian extinction (102). PFB can be facilitative or disruptive (34), depending in part on the status of a process over time, and on the scale of assessment. Taken alone, a PFB seems to be ultimately destructive, but working in concert with negative feedback and other complex interactions, it can contribute to overall stability (103).

Not all PFB cycles are destructive in PDs. One of the more interesting aspects of these tradeoff phenomena in PDs is the balancing of negative feedback and PFB cycles prior to invasion of plague. Over much longer time spans than those for the flea-plague PFB of epizootics, PFB has been discussed in PDs in the context of Allee effect (a positive correlation between population density and average individual fitness) resulting from increased effectiveness of predator warning communications and higher individual survival rates at higher population densities (104). PDs clip grasses and forbs seasonally to maintain unrestricted vision, and repeated clipping of shrubs results in declining shrub densities over periods of years to decades; increased PD densities facilitate this PFB loop (105). In addition to the increased survival rates accompanying this PFB, PDs might have higher birth rates at higher population densities (106). Historically, the slow process of PFB in shrub reduction and increasingly efficient anti-predator behaviors with PD population growth may have

gradually come into balance with the negative feedback of coterie territoriality and limiting resources. However, the plague-flea PFB cycle is explosive, and runaway flea-plague PFB will curb other processes.

Plague epizootics may occur in multispecies communities of hosts because *Y. pestis* is a generalist parasite. It might be a mistake to single out a particular host species as the driver of these phenomena, although outbreaks are characteristic in various species of ground squirrels (including PDs). Even within the *Cynomys* genus, manifestations of plague epizootics appear to vary among species. White-tailed PDs (*C. leucurus*) and other species within the *Leucocrossuromys* subgenus may no longer reach peak densities in preferred habitat (grass-dominated sites with few shrubs) because epizootics repeatedly decimate populations that reach reasonable densities (107). From a source-sink perspective, the source has become the poor, shrub-dominated, habitats that maintain enzootic plague, which may have been considered the sinks for these PDs historically. An indirect effect of this phenomenon is failure of white-tailed PDs to create optimum habitat by clipping shrubs and killing them. This is not a true source-sink reversal. A source (prime PD grassland patch) can become a sink because of plague, but the sink (in this case shrubby habitat) was probably not a true sink in the sense of PD mortality exceeding natality. Nevertheless, this reversal in the overall flow of dispersing animals again illustrates the potential for *Y. pestis* to be a "transformer species" in the western U.S. (5).

Epizootics of Plague as an Adaptation of *Yersinia pestis*?

Epizootics have been identified as a manifestation that "amplifies" *Y. pestis* [e.g., (18, 108)]. The term amplification might imply that epizootics are adaptive, for example by facilitating population growth and expansion of *Y. pestis*. Instead, these epizootic events might be considered as anomalies, triggered by factors that favor PFB cycles. The ecological results of PFB cycles are sometimes destabilizing and can be devastating (109). The explosiveness of PFB epizootics might be a cost of the evolved life history of *Y. pestis* rather than an adaptation; it seems maladaptive for an organism to destroy and sometimes eliminate essential habitat (herein, hosts and fleas).

Perhaps plague epizootic events played little role in the evolution of *Y. pestis*, fleas, and mammalian hosts in Asia where *Y. pestis* originated (110) and these coevolutionary processes had their origins. Plague cycles in Asia are often measured as the prevalence of detected infections in hosts. In populations of great gerbils, plague prevalence is reportedly "always low" (47). In North America, host mortality is pervasive at enzootic and epizootic levels [e.g., (20, 21, 23)]. As an invader in North America, *Y. pestis* may be subject to accidental juxtaposition of conditions favorable to a non-adaptive outcome for all players. Nevertheless, runaway PFB-driven outbreaks might have resulted in evolutionary consequences for *Y. pestis*. For example, periodically destroying its own habitat might have favored mechanisms for *Y. pestis* survival under hostile conditions, such as ability to colonize protozoa or survive in soil, fleas, or elsewhere (1, 35, 36, 111).

Plague, PFB Cycles, Conservation, and One Health

In this paper, we emphasize the transition of plague activity from enzootic to epizootic explosions due to PFB. Our intent herein has been to focus primarily on the PFB loops that likely occur during an epizootic outbreak of plague, and to propose that those expanding cycles are a central element of epizootics as we narrowly define them (**Figure 1**). For an epizootic with PFB to occur, there must be adequate (although sometimes relatively low) densities of PDs distributed sufficiently uniformly to allow the rapid expansion of PFB to occur. There also needs to be adequate densities of fleas at the starting point.

The change in flea:host ratios during epizootics have been recognized and repeatedly mentioned for more than 50 years, and the recognition of plague as an enzootic phenomenon (smoldering), as well as exploding into epizootics, is also historic. We suggest these phenomena, coupled with the relatively inefficient transmission of *Y. pestis* by fleas, as pivotal to understanding both the evolution of *Y. pestis* and the ecological manifestations of plague. In PDs at least, the breakdown of territories during epizootics likely contributes substantially as a second reinforcing PFB loop. Our synthesis is a recasting of earlier discussions and observations into a theme that emphasizes sustained transmission and mortality caused by enzootic plague as a common starting point for epizootics, and centering on PFB as the amplifying centerpiece. PFB loops might be initiated by triggers; we speculated on anthropogenic triggers for the plague-flea epizootic loop and provided some supporting evidence.

This reevaluation seemed useful because the history surrounding plague has tended to dampen such thinking. Plague initially received most attention as a series of human epidemics, and public health investigators later recognized epizootic outbreaks of sylvatic plague as elevating the risk to human health (2). The focus on epizootics and epidemics motivated conversations (at least) about how such cycles could be adaptive and diverted attention from thinking about the more common conditions under which natural selection likely molded the life history attributes of *Y. pestis*.

If epizootics are not a necessary component of plague maintenance, and *Y. pestis* evolved a lifestyle that requires high vector loads and high levels of bacteremia to persist (65), we might expect host mortalities to be chronically high even without epizootics, especially in ecosystems where plague is not native. Mammalian species that can persist with sustained high population losses (e.g., PDs) may serve as reservoirs for *Y. pestis*, but plague spillover into associated bystander species, even during enzootic periods, might result in their extirpation or extinction (e.g., as exemplified by black-footed ferrets). There may be mammal communities where epizootics, as we define them, are rare or absent (e.g., due to consistently low flea parasitism or intense territoriality). Lack of noticeable epizootic outbreaks should not be equated with lack of ecological impact of plague.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/ or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

DB accumulated notes on this PFB hypothesis over the past decade and collected data on PD shooting in Montana. DE added new ideas on PFB and collected data on PD poisoning in South Dakota. Both authors performed data analyses. DB drafted the original manuscript. DE made additions and substantial organizational revisions. Both authors approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2019.00075/full#supplementary-material

Table S1 | Fleas Collected after Recreational Shooting on a Prairie Dog Colony in Montana.

Table S2 | Fleas Collected Before and After Poisoning on a Prairie Dog Colony in South Dakota.

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Intestinal Parasites and the Occurrence of Zoonotic *Giardia duodenalis* Genotype in Captive Gibbons at Krabokkoo Wildlife Breeding Center, Thailand

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Intestinal parasitic infections can have an impact on health and growth of wildlife. The current study aims were to determine the prevalence of intestinal parasites and to molecular characterize *Giardia duodenalis* and *Cryptosporidium* spp. in captive gibbons at Krabokkoo Wildlife Breeding Center, Thailand. Fifty-five gibbons, 2 agile- (*Hylobates agilis*), 38 lar- (*Hylobates lar*) and 15 pileated gibbons (*Hylobates pileatus*) were included in this study. Fecal samples were collected individually at Krabokkoo Wildlife Breeding Center, Chachoengsao province, eastern Thailand, in November 2013. Intestinal parasitic infections were examined by zinc sulfate centrifugation flotation and by a commercially available immunofluorescent assay (IFA) for detection of *G. duodenalis* and *Cryptosporidium* spp.. Polymerase chain reaction targeting the *Giardia* glutamate dehydrogenase (gdh), beta- giardin (bg), triose phosphate isomerase (tpi) genes, and the *Cryptosporidium* small subunit-rRNA and heat-shock protein (hsp70) following by DNA sequencing were performed on the IFA positive samples. The overall prevalence of intestinal parasitic infection in gibbons at Krabokkoo Wildlife Breeding Center was 12.7% (95%CI: 5.3–24.5), *Strongyloides* spp. eggs or larvae were present in all positive samples. Co-infections with *G. duodenalis* were detected in 1.8% (95%CI: 0.1–9.7) of the samples. Based on the sequencing results of the three genes, the IFA *Giardia* positive isolate typed as the zoonotic genotype B. Since the data reveals the occurrence of zoonotic *Giardia* genotype, good hygiene management is suggested to prevent the transmission of this pathogen from gibbon to human, and vice versa.

Keywords: intestinal parasites, *Giardia duodenalis*, captive, gibbons, Thailand

INTRODUCTION

Intestinal parasitic infections are the most common causes of gastrointestinal diseases in captive wildlife. These infections can cause a wide range of clinical signs, from subclinical infections to malabsorption, abdominal pain, diarrhea, vomiting, anemia, severe dehydration, and death (1–3). As the living area is limited, stress and other factors such as artificial environment, poor diet or the presence of humans lead to the high risk of infection and weaken the natural resistance of the host, making the clinical illness possible (4). The weakened health condition of these captive animals can have a negative impact on their reproduction which is of major concern in the zoos and wildlife breeding facilities of captive or endangered species (3, 5).

Several studies on helminthic parasites in the free-ranging (5–10) and captive populations (4, 11–15) of non-human primates (NHP) have been conducted worldwide and they reported a high prevalence of intestinal parasites. For example, the prevalence of endoparasites in western lowland gorillas at Bai Hokou, Dzangha-Ndoki National Park, Central African Republic has been reported to be up to 100% (7). Of all intestinal parasites detected in NHP, *Strongyloides* spp., *Oesophagostomum* spp., *Trichuris* spp., *Ascaris* spp., and hookworms were the most common intestinal parasites.

Eight assemblages (A-H) of *Giardia duodenalis* and at least 27 *Cryptosporidium* spp. have been described (16, 17). Infection with *G. duodenalis* and *Cryptosporidium* ssp. in NHP are common (18–21). In wild and captive NHP, prevalence rates of these infections range from undetectable level to as high as 70% (20, 22–26). In several studies on NHP, zoonotic assemblages of *G. duodenalis*, assemblage A and B, were identified and the assemblage B was more prevalent in both captive and free-range animals (18, 23, 27, 28). *Cryptosporidium hominis* and *C. parvum* were commonly identified in *Cryptosporidium*-infected primates (29–31).

Currently, there is no information available regarding intestinal parasitic infection in captive gibbons in breeding facilities in Thailand. Knowing background prevalence of gastrointestinal parasites can be beneficial in the health management program in gibbons for the reproduction at the Krabokkoo Wildlife Breeding Center. The aims of this study were, therefore, to determine the prevalence of intestinal parasites and to molecular characterize *Giardia duodenalis* and *Cryptosporidium* spp. isolates to determine the potential of zoonotic transmissions of these pathogens from captive gibbons at Krabokkoo Wildlife Breeding Center, Thailand.

MATERIALS AND METHODS

Study Area

Krabokkoo Wildlife Breeding Center is located in Chachoengsao province, eastern Thailand, at the coordinates of 13°28′5.05″N, 101°35′37.30″E (**Figure 1**) and at 47 meters above the sea level. In November 2013, this facility accommodated four species of gibbons, 65 white-handed (*Hylobates lar*), 15 pileated (*Hylobates pileatus*), 2 agile (*Hylobates agilis*), and 2 crown (*Nomascus* spp.) gibbons. The gibbons were separated among species and were housed individually or in groups. Siblings or a family were housed together. They were fed with vegetable- and fruit-based diet and water was supplied in a bowl. Drinking water was replaced on a daily basis. All gibbons were dewormed every 3 months. The temperature are 23–27°C in winter (mid-October–mid-February), 35–40°C in summer (mid-February–mid-May), and 28–35°C in rainy season (mid-May–mid-October) (32).

Fecal Sample and Data Collection

Fifty-five fecal samples were collected from white-handed (*H. lar*) ($n = 38$), pileated (*H. pileatus*) ($n = 15$), and agile (*H. agilis*) ($n = 2$) gibbons during the November of 2013. The samples used in the study were part of the study on genetic diversity of macaques and Hylobatidae gibbons in Thailand. Each fecal sample was collected from the ground (care was taken not to have soil contamination), kept in a labeled plastic bag and stored at 4°C until examination. Sex, age, cage and identification number were recorded at the time of collection. Fecal samples were shipped on ice to the Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand within a week and the fecal consistencies were determined upon arrival.

DIAGNOSTIC PROCEDURES

Microscopic Examination of Fecal Samples After Zinc Sulfate Centrifugal Flotation and IFA for *Giardia duodenalis* and *Cryptosporidium* spp. Detection

Fecal samples were examined for the presence of intestinal parasitic eggs, larvae, protozoal cysts and oocysts using microscopic examination after zinc sulfate centrifugal flotation (33). *Giardia duodenalis* and *Cryptosporidium* spp. infections were determined using a commercially available direct immunofluorescent assay (IFA) (MeriFluor® *Cryptosporidium/Giardia* Test Kit, Meridian Diagnostic Corporation, Cincinnati, OH). Prior IFA, the fecal samples (3 grams) were concentrated using sucrose gradient centrifugation technique as previously described (34). IFA was carried out according to the manufacturer's instruction.

DNA Isolation and Molecular Detection of *Giardia duodenalis* Infection

Three hundred microliters of each *Giardia* or *Cryptosporidium* IFA positive fecal concentrate were subjected to DNA extraction using the FastDNA® kit (MP Biomedicals, Solon, OH, USA) following an established protocol (35).

PCR assays targeting *Giardia* glutamate dehydrogenase (gdh), beta-giardin (bg), and triosephosphate isomerase (tpi) genes, and *Cryptosporidium* heat-shocked protein (hsp70), and small subunit ribosomal RNA (SSU-rRNA) were used for molecular characterization of the respective organisms in the IFA positive samples. Previously described PCR protocols were used (36–42).

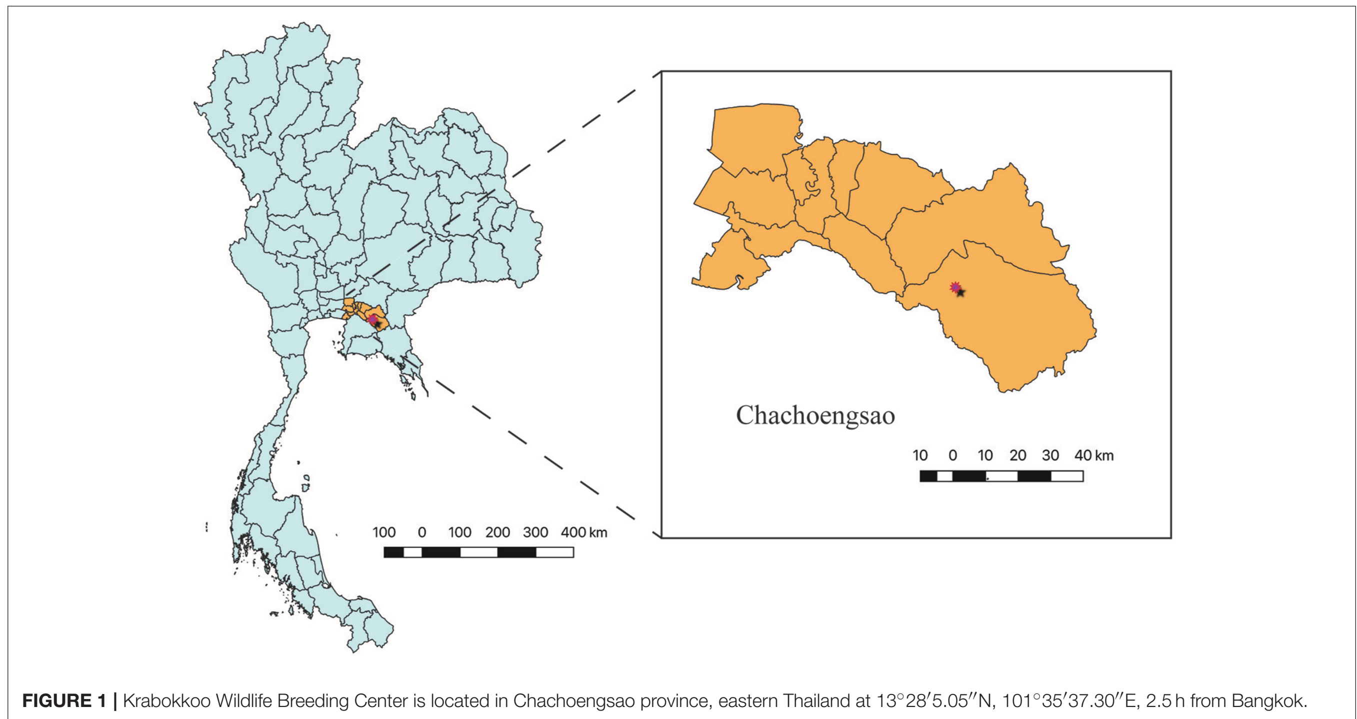

FIGURE 1 | Krabokkoo Wildlife Breeding Center is located in Chachoengsao province, eastern Thailand at 13°28′5.05″N, 101°35′37.30″E, 2.5 h from Bangkok.

DNA Sequencing and Phylogenetic Analysis

The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, OH, USA) and purified PCR product was evaluated by nucleotide sequencing using a commercially available service (1st Base Laboratory, Selangor, Malaysia). For each target gene, the obtained sequences from both directions were aligned and a consensus sequence was generated and compared with nucleotide sequences from the nucleotide database from the GenBank using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic and molecular analyses were conducted using the MEGA 6.06 program (43). Multiple sequence alignments were performed using MUSCLE (44), and the phylogenetic analyses were performed by the Maximum Likelihood method based on the Kimura 2-parameter model. The consensus tree was obtained after bootstrap analysis with 500 replications. Reference strains of the different assemblages were retrieved from the GenBank and included for comparative phylogenetic analyses.

Statistical Analyses

A sample was considered positive for gastrointestinal parasites if parasitic eggs or larvae were detected by light microscopic examination after zinc sulfate centrifugal flotation. A sample was considered positive for *Giardia* and *Cryptosporidium* if at least one (oo)cyst was detected by either microscopic examination or IFA. Gibbons were grouped by species, age (<10 years, ≥10 years), and sex. Overall prevalence and 95% confidence interval (95%CI) were calculated. Associations of age category, sex, fecal consistency, gibbon species, and parasitic infestation results were analyzed using Fisher's Exact test. A $P < 0.05$ was considered statistically significant. All statistical analyses were performed using STATA statistical software release 10.1 (Stata Corp., College Station, Texas, USA).

TABLE 1 | Prevalence of intestinal parasitic infection by gibbon species, age, sex, and fecal consistency.

	***Strongyloides* spp. % (95%CI*)**	***Giardia duodenalis* % (95%CI*)**
Overall (55)	12.73 (5.27–24.48)	1.80 (0.05–9.71)
SPECIES		
Hylobates agilis (2)	0.00 (0.00–84.19)†	0.00 (0.00–84.19)†
Hylobates lar (38)	15.79 (6.02–31.25)	2.63 (0.07–13.81)
Hylobates pileatus (15)	6.67 (0.17–31.95)	0.00 (0.00–21.80)†
AGE		
<10 years (6)	0.00 (0.00–19.51)†	0.00 (0.00–45.93)†
≥ 10 years (17)	16.67 (0.42–64.12)	0.00 (0.00–19.51)†
Unknown (32)	18.75 (7.21–36.44)	3.13 (0.08–16.22)
SEX		
Female (28)	17.86 (6.06–36.89)	3.57 (0.09–18.34)
Male (27)	7.41 (0.91–24.29)	0.00 (0.00–12.77)†
FECAL CONSISTENCY		
Formed or soft (49)	12.24 (4.63–24.77)	2.04 (0.05–10.85)
Diarrhea (6)	16.67 (0.42–64.12)	0.00 (0.00–45.93)†

*A number in parentheses represents the number of samples in each category. *95% Confidence Interval †One sided 97.5%CI.*

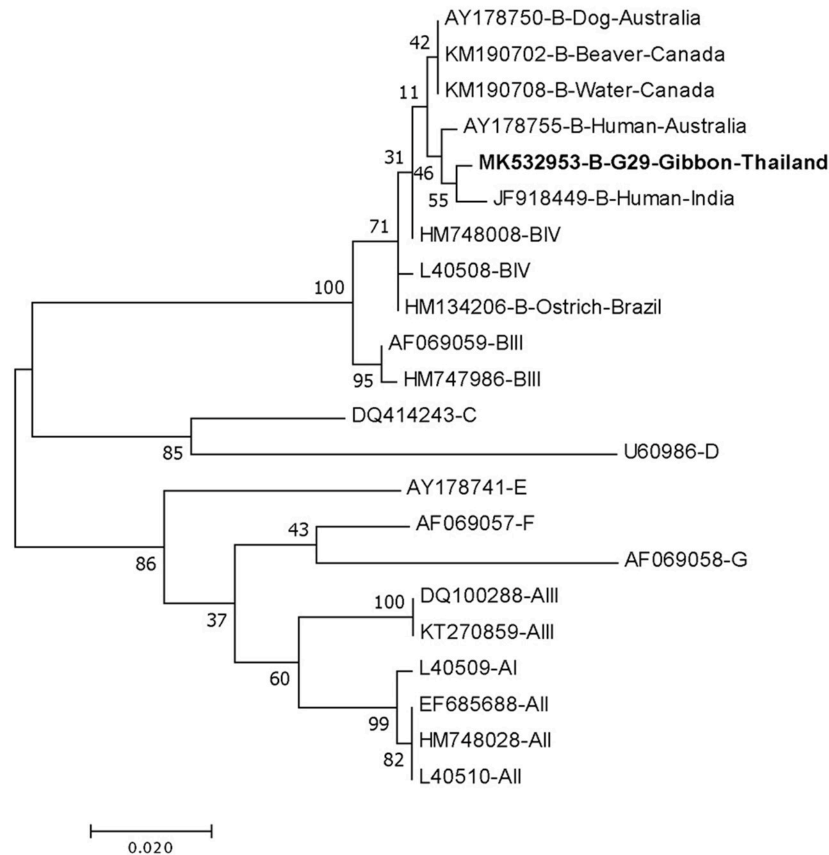

FIGURE 2 | Phylogenetic tree of *Giardia* isolate based on the sequence of glutamate dehydrogenase (gdh) gene from a gibbon in this study by the Maximum Likelihood algorithm using the MEGA 6.06 program. Sequences obtained from GenBank are indicated by their accession numbers. Percentage bootstrap supports (500 replicates) are shown by numbers at the respective nodes. Bold texts represent the *Giardia* detected in this study.

RESULTS

Microscopic Examination of Fecal Samples After Zinc Sulfate Centrifugal Flotation and IFA for *Giardia duodenalis* and *Cryptosporidium* spp. Infections

Characteristics of gibbons and samples and the descriptive statistics are shown in **Table 1**. Of 55 fecal samples, *Strongyloides* spp. eggs or larvae were detected in 7 samples by microscopic examination after zinc sulfate centrifugal flotation. *Giardia* cysts were detected in one fecal sample by IFA. *Cryptosporidium* oocysts were not detected by IFA in any fecal samples, therefore, PCR assays were not performed.

Giardia duodenalis Sequences and Phylogenetic Analyses

DNA fragments of the only IFA *Giardia* positive sample (G29) were successfully amplified and typed as assemblage B by the three genes (gdh, bg, and tpi). The gdh sequence of G29 showed 99% homology to the assemblage B gdh sequences recovered from a water sample and a beaver in Canada, an ostrich in Brazil, a human and a dog in Australia, and a human from India (**Figure 2**). The G29 gdh sequence has 3 SNPs (single-nucleotide polymorphism) at position 12 (A vs. T), 93 (A vs. G), and 199 (A vs. G), when compared to those sequences mentioned previously; however, neither of these SNPs resulted in amino acid change. The beta-giardin sequence of G29 showed 100% homology to the assemblage B from human from Thailand and India and 99.9% homology to assemblage B human isolates from Kenya, Egypt, Brazil, and Ethiopia (**Figure 3**). Sequences from tpi gene contained ambiguous nucleotides at position 108 (T or G) and 443 (A or T). When translating the G29 tpi sequence to amino acids, substitution of T with G at position 108 did not cause amino acid change, whilst substitution of A with T at position 443 resulted in an amino acid change from Valine to Aspartic acid. Variants of tpi sequences showed 99–100% homology to the tpi sequences recovered from rhesus macaque, long-tailed macaque, and gibbons from China, Sumatran Orangutan from Indonesia, beaver from Canada, cat from Japan, rabbit from Nigeria, and humans from Canada, Malaysia, and Spain (**Figure 4**). From the phylogenetic analyses of gdh, bg and tpi genes, the *G. duodenalis* isolate in this study was placed into BIV, BI, and BIV branch, respectively (**Figures 2–4**).

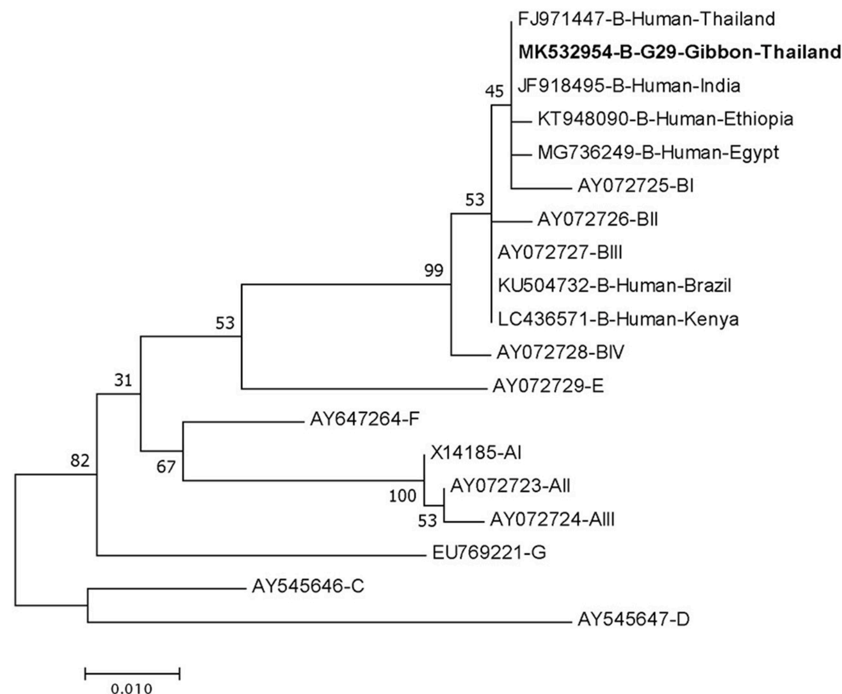

FIGURE 3 | Phylogenetic tree of *Giardia* isolate based on the sequence of beta-giardin (bg) gene from a gibbon in this study by the Maximum Likelihood algorithm using the MEGA 6.06 program. Sequences obtained from GenBank are indicated by their accession numbers. Percentage bootstrap supports (500 replicates) are shown by numbers at the respective nodes. Bold texts represent the *Giardia* detected in this study.

Statistical Analyses

Due to the low detection of the parasites and the small sample size, the power to detect associations between any risk factors and infections was insufficient.

DISCUSSION

The current study represents the first report of the intestinal parasites as well as *G. duodenalis* and *Cryptosporidium* spp. prevalence rates and *Giardia* genotypes in captive gibbons in Thailand. The prevalence of these infections were commonly high and ranged from 25 to 100% in either free-ranging (5, 7–10, 22, 28, 31, 45, 46) or captive non-human primates (4, 7, 11, 13–15, 20, 23–26, 46, 47). In the current study, overall prevalence rates of nematodes, *G. duodenalis*, and *Cryptosporidium* spp. in gibbons were 12.7, 1.8, and 0%, respectively. These prevalence rates, however, were comparable to the previous report of 0–16.4% in gibbons in zoological parks in China (48). The low prevalence in this study may be from collecting a single fecal sample from each animal. Parasitic eggs, *Giardia* cysts and *Cryptosporidium* oocysts are intermittent shed in the feces, therefore, three or more fecal samples from animals can increase the sensitivity of intestinal parasites (33). In addition, the low detection rate of *Giardia* and the lack of *Cryptosporidium* spp. were also because of the number of cysts/oocysts were below the detection limit of the diagnostic tests used in the study (49).

The most common helminthic species detected in NHP were *Strongyloides* spp., *Trichuris* spp., *Oesophagostomum* spp., *Ascaris* spp. and hookworms (5, 7, 22, 45, 46). A similar pattern of gastrointestinal parasites was also observed in captive gibbons in a zoo in China (13). In a study in 23 wild white-handed gibbons at Khao Yai National Park, Thailand, *Trichuris* spp. and *Ternidens* spp. were the most prevalent helminthic parasites detected (91.3%), followed by *Strongyloides fuelleborni* (56.5%) (9). However, in the current study, only *Strongyloides* spp. eggs or larvae were detected in the fecal samples. *Strongyloides* spp. are soil-transmitted nematodes with an estimated 370 million people infected worldwide (50). In this study, the detection of *Strongyloides* spp. in feces is less likely to be from contaminated soil as fecal samples were carefully collected not be contaminated with soil before the storage in a plastic bag. These nematodes can cause a chronic and persistent strongyloidiasis in the infected host because of the autoinfective life cycle (51) and cause diarrhea, hyperinfection syndrome, dissemination, and death in immunocompromised hosts. *Strongyloides stercoralis* is a primary species infecting human; however, the infections of primates' parasites *S. fuelleborni fuelleborni and S. fuelleborni kellyi* have also been reported (8). The molecular characterization of *Strongyloides* positive samples is suggested since microscopic identification is insufficient for species identification and determination of its zoonotic potential. In this study, the species identification was not performed but this finding has raised concerns regarding the zoonotic potential. Since the

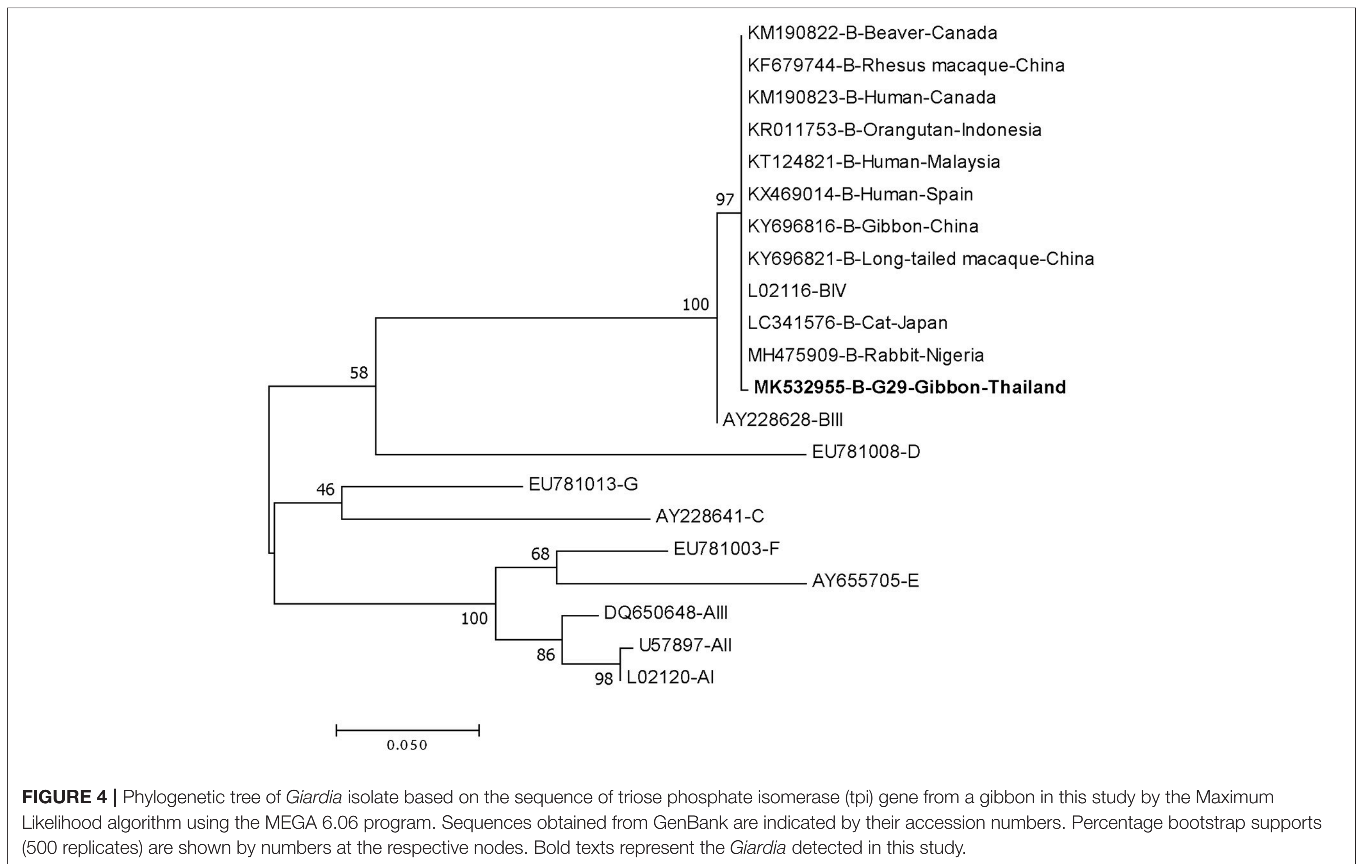

FIGURE 4 | Phylogenetic tree of *Giardia* isolate based on the sequence of triose phosphate isomerase (tpi) gene from a gibbon in this study by the Maximum Likelihood algorithm using the MEGA 6.06 program. Sequences obtained from GenBank are indicated by their accession numbers. Percentage bootstrap supports (500 replicates) are shown by numbers at the respective nodes. Bold texts represent the *Giardia* detected in this study.

fatal strongyloidiasis cases of gibbons in Thailand has been reported (1) and *Strongyloides* spp. is also an important parasitic helminth of humans (8), an effective anthelminthic program is recommended.

Giardia duodenalis and *Cryptosporidium* spp. are important intestinal protozoans in non-human primates. These pathogens can cause a wide range of clinical signs, from subclinical to malabsorption, abdominal pain, failure to thrive, acute or chronic diarrhea especially in young, old and immune-compromised animals (3, 52, 53). The organisms are commonly found in both free-ranging and captive non-human primates with the prevalence from 0–70% to 0–48%, for *Giardia* and *Cryptosporidium* infections, respectively (19, 21, 22, 24, 31, 47, 54). In Thailand, a low prevalence rate (1/23, 4.35%) of *Cryptosporidium* spp. infection has been previously reported in wild white-handed gibbons at Khao Yai National Park in Thailand. IFA was used for the detection of *Giardia* cysts or *Cryptosporidium* oocysts in repeatedly collected fecal samples that ranged from 3 to 25 samples per gibbon, resulting in a total of 324 samples (9). In that study, there was no *Giardia* detected. In this study, in contrast, no *Cryptosporidium* oocysts were detected in all fecal samples and *Giardia* cysts were detected in only one fecal sample of 55 samples. These findings could be due to that the numbers of cysts or oocysts of these pathogens were low and were below the detection limit of the IFA tests. The *Giardia* positive sample, in this study, typed as assemblage BIV and BI by gdh and tpi and bg genes, respectively. This finding is in agreement with previous reports that assemblage B was predominant in NHP (18, 23, 27, 28). Although the prevalence of *Giardia* infection in this study is low, the identification of *G. duodenalis* assemblage B may suggest the potential of zoonotic or anthroponotic transmissions in this area.

The limitations of this study are the small sample size and the nature of single sample collected from each animal; selection bias may have led to an underestimation of the prevalence rates. A larger sample size or more frequent collection of gibbon's feces are needed for further studies. This study had inadequate power to detect associations between any risk factors and infections. In addition, we analyzed gibbons' species, age, sex, and diarrhea status; however, other important risk factors, e.g., season, diet, or water source could be suggested for future study to help in prevention and control of intestinal parasitic infection in this population.

AUTHOR CONTRIBUTIONS

ST, SM, and ML designed the study. SM and RA performed fecal sample collection. DS performed the microscopic fecal examination, ST performed IFA and molecular analyses. ST analyzed sequences. ML provided laboratory supplies. ST interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

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Metagenomic Approach to Characterizing Disease Epidemiology in a Disease-Endemic Environment in Northern Thailand

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In this study, we used a metagenomic approach to analyze bacterial communities from diverse populations (humans, animals, and vectors) to investigate the role of these microorganisms as causative agents of disease in human and animal populations. Wild rodents and ectoparasites were collected from 2014 to 2018 in Nan province, Thailand where scrub typhus is highly endemic. Samples from undifferentiated febrile illness (UFI) patients were obtained from a local hospital. A total of 200 UFI patient samples were obtained and 309 rodents and 420 pools of ectoparasites were collected from rodents (n = 285) and domestic animals (n = 135). The bacterial 16S rRNA gene was amplified and sequenced with the Illumina. Real-time PCR and Sanger sequencing were used to confirm the next-generation sequencing (NGS) results and to characterize pathogen species. Several pathogens were detected by NGS in all populations studied and the most common pathogens identified included *Bartonella* spp., *Rickettsia* spp., *Leptospira* spp., and *Orientia tsutsugamushi*. Interestingly, *Anaplasma* spp. was detected in patient, rodent and tick populations, although they were not previously known to cause human disease from this region. *Candidatus* Neoehrlichia, *Neorickettsia* spp., *Borrelia* spp., and *Ehrlichia* spp. were detected in rodents and their associated ectoparasites. The same *O. tsutsugamushi* genotypes were shared among UFI patients, rodents, and chiggers in a single district indicating that the chiggers found on rodents were also likely responsible for transmitting to people. Serological testing using immunofluorescence assays in UFI samples showed high prevalence (IgM/IgG) of *Rickettsia* and *Orientia* pathogens, most notably among samples collected during September–November. Additionally, a higher number of seropositive samples belonged to patients in the working age population (20–60 years old). The results presented in this study demonstrate that the increased risk of human infection or exposure to chiggers and their associated pathogen (*O. tsutsugamushi*) resulted in part from two important factors; working age group and seasons for rice cultivation and harvesting. Evidence of pathogen exposure was shown

to occur as there was seropositivity (IgG) in UFI patients for bartonellosis as well as for anaplasmosis. Using a metagenomic approach, this study demonstrated the circulation and transmission of several pathogens in the environment, some of which are known causative agents of illness in human populations.

Keywords: metagenomic, bacterial community, disease epidemiology, disease transmission, scrub typhus, undifferentiated febrile illness

INTRODUCTION

Most public health surveillance systems and laboratories rely on serological and molecular assays that were developed to detect specific pathogens. However, conventional laboratory assays are often ineffective at detecting all causative agents of disease. Studies have shown that 40% of gastroenteritis cases (Finkbeiner et al., 2008) and as many as 60% of encephalitis cases (Ambrose et al., 2011) went undetected by conventional laboratory testing. Pathogens can go undetected if they are novel or are not known to previously occur in an area. There are many examples of the emergence of novel pathogens or reemergence of known organisms in new places where the available surveillance systems were inadequate, such as occurred with outbreaks of H7N9 influenza (Gao et al., 2013), Middle East respiratory syndrome coronavirus (MERS-CoV) (van Boheemen et al., 2012; Kindler et al., 2013), and the severe acute respiratory syndrome (SARS) outbreak in 2003 (Wang and Jolly, 2004).

Conventional diagnostic tests used by most reference laboratories require culture, microscopy, serology, and polymerase chain reaction (PCR). Such tools are useful for pathogen detection but only if culture conditions, test sensitivity, and primers are compatible and suitable for the microbial target. Other molecular approaches can be used to capture a wider range of pathogenic species such as multiplex PCR that targets highly conserved DNA regions or multiplex assays that target many of the most common pathogens known to cause similar symptoms. However, it is worth noting that even when multiplex assays are used, pathogens not included in the multiplexing may go undetected. The use of 16S rDNA was first proposed by Woese and Fox (1977) and Woese et al. (1990) as a tool for the molecular identification and characterization of microorganisms. The 16S rDNA gene is highly conserved among prokaryotes and some parts of its sequence are hypervariable between species, which makes it an ideal marker for species identification and for understanding evolutionary relationships (Gill et al., 2006; Sogin et al., 2006; Dethlefsen et al., 2008; McInerney et al., 2008; Tringe and Hugenholtz, 2008; Sunagawa et al., 2009). Metagenomics allows for comparisons of genetic material from multiple samples. One of the most common metagenomic approaches is deep amplicon sequencing (DAS), which employs universal primer to amplify parts of the 16S rRNA gene from specimens. A major benefit of metagenomics is the simultaneous detection of all microorganisms in clinical samples without prior knowledge of their identities. In addition, metagenomics has the potential to detect rare and novel pathogens. Current surveillance assays are limited in their ability to detect the emergence of novel pathogens or ones not previously known to be present in a given region. Metagenomic approaches can fulfill such gaps by identifying unknown etiological agents and assisting in the development of a new test for pathogen detection (Miller et al., 2013; Mokili et al., 2013; Wan et al., 2013).

Metagenomic approaches are especially suitable for zoonotic diseases. It is estimated that more than 60% of human pathogens are of animal origin (Taylor et al., 2001). Rodents are major reservoirs that account for a wide range of emerging zoonotic diseases in humans and livestock (Jones et al., 2008; Meerburg et al., 2009). Co-infection of multiple pathogens within individual rodents is frequently observed and the interaction between pathogens can have significant effects (Cox, 2001). Such co-infections can cause rodents to be more or less susceptible to other microparasites (Tadin et al., 2012). Generally, multiple infections in wildlife can increase disease severity in a host (Lello et al., 2005), affecting the survival and reproduction of animal hosts (Davidar and Morton, 2006; Holmstad et al., 2008). Disease surveillance in rodents and other wildlife can provide important information for public health preparedness. Surveillance can also be used to measure biodiversity and disease emergence which are both directly linked to the stability of ecosystems (Keesing et al., 2010; Grogan et al., 2014). Metagenomic approaches combined with NGS can be powerful tools to disentangle complex patterns of pathogen transmission among ectoparasites, animal reservoirs, and humans. For example, NGS has been used to perform blood meal analysis to determine the wide-range of animals that vectors feed on and possible reservoirs (Alcaide et al., 2009). NGS has also been useful in finding unexpected pathogens not normally associated with particular vectors (Vayssier-Taussat et al., 2013) and has been used to show the genetic diversity of bacteria that are specific to certain animal hosts and vectors (Pierlé et al., 2014; Swei et al., 2015). Such information can be used to correlate infections in people with important vectors and reservoir hosts.

In this study, metagenomics and NGS technology were used to characterize human (patients with undifferentiated febrile illness (UFI)), reservoir host (rodents and small mammals), and ectoparasite (chiggers, ticks, fleas, and lice) populations for bacterial pathogens. All samples were collected from Nan province in northern Thailand. Since all samples were from the same sites, bacteria could be compared from different populations to determine potential vectors and reservoirs. Nan province is highly endemic for scrub typhus, caused by the agent *Orientia tsutsugamushi*, and one of the major goals of this study was to determine the etiology and transmission dynamics of scrub typhus in the area. Another goal was to identify other bacterial pathogens that were under-reported or not previously known from this region. NGS results were verified by conventional methods such as real-time PCR, PCR, and

DNA sequencing to confirm the pathogenic potential of detected bacteria and to better characterize those important pathogens to the species level. In addition, serological tests were performed to determine the seroprevalence and the history of human exposure to the pathogens detected by the NGS approach. The in-depth characterization of bacteria performed in this study from humans, animal hosts, and ectoparasites allowed us to determine the transmission dynamics of pathogens and identify several new and previously unreported pathogens from this area.

MATERIALS AND METHODS

Ethics Statement

Rodents were trapped according to the institutional animal collection protocol entitled "Field Sampling of Small Mammal (Orders: Erinaceomorpha, Soricomorpha, Scandentia, Macroscelidea, and Rodentia) Populations to Support Zoonotic Disease Surveillance and Ectoparasite Collection" (PN# 12–06), reviewed and approved by the USAMC-AFRIMS Institutional Animal Care and Use Committee (IACUC). All sampling procedures and experimental manipulations were reviewed and approved as part of the animal collection protocol (PN# 12–06). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations related to animals and experiments involving animals, and adhered to principles outlined in the "Guide for the Care and Use of Laboratory Animals," NRC Publication, 2011 edition.

Undifferentiated Febrile Illness (UFI) Patients

A total of 200 individual UFI patients' coded specimens were received from Bo Kluea hospital, Nan province, Thailand. Samples were from outpatients and inpatients presenting to Bo Kluea hospital with UFI and suspicion of scrub typhus infection during February–November 2017. Residual whole blood and serum samples from routine laboratory testing were coded and sent to the Department of Entomology to be tested for the possible causative agent of UFI as well as for scrub typhus or murine typhus infection, caused by *Rickettsia typhi*. The protocol was determined on February 01, 2016 by WRAIR Human Subjects Protection Branch (HSPB) to be research not involving human subjects for this investigation, since the work described herein involves the use of existing, coded specimens wherein investigators will not receive associated identifiable data, the project did not require a review by the Institutional Review Board (IRB) and 45 CFR 46 and 32 CFR 219 does not apply.

Study Locations

Rodents and ectoparasites were collected during the wet (June–September) and dry (November–April) seasons in Bo Kluea, Mae Charim, and Phu Phiang districts of Nan province, Thailand in 2014–2018 (**Figure 1** and **Table 1**). Ectoparasites were also collected from domesticated mammals (dogs, cats, and cattle). All study sites were on private land, and permission was obtained from each of the owners to conduct research on their land. None of the field studies involved endangered or protected species. Rodents were captured using live traps baited with bananas, palm fruit, or dried fish, and were collected from orchards, palm and rubber plantations, cultivated rice-fields, grassland areas, edges of dense forest, stream margins, and around dwellings. Traps were set for 3–5 nights and were checked early in the morning. Captured rodents were removed from the traps, euthanized using carbon dioxide, and processed immediately at the site of collection. Blood, serum, and tissue samples (liver, spleen, kidney, and lung) were collected and stored on dry ice. Ears were removed and stored in 70% ethanol for chigger collection. All tissues were then transported to the AFRIMS laboratory for further processing. All rodents were later identified to the species level as described previously (Muul, 1979).

Genomic DNA Extraction

UFI Whole Blood

Genomic DNA was extracted from whole blood samples by automated extraction machine, a QIAsymphony® SP instrument (Qiagen, Hombrechtikon, Switzerland) with QIAsymphony® DNA Mini Kit (Qiagen, Germany). For each patient, 250 μl of whole blood was used for the DNA extraction with DNA Blood 200 DSP protocol. DNA was eluted in 50 μl and stored at −20°C until use. Ultrapure DNA/RNA-free distilled water was also included in every extraction procedure as an extraction control.

Rodent Tissue

Spleen and kidney tissues from each rodent were cut into pieces (∼3 mm in diameter) and added to 230 μl of ATL Tissue Lysis Buffer and 20 μl of Proteinase K solution (20 mg/ml), then incubated at 55°C for 1 h or until the tissues were homogenized. A total volume of 250 μl homogenized solution was then used for DNA extraction on the QIAsymphony® SP with QIAsymphony® DNA Mini Kit and Tissue HC 200 DSP protocol. The DNA was eluted in 200 μl and stored at −20°C until use. Ultrapure DNA/RNA-free distilled water was also included as an extraction control.

Ectoparasite Morphological Identification and DNA Extraction

Ectoparasites (chiggers, ticks, fleas, and lice) collected from rodents and small mammals were morphologically identified and pooled by genus, the host species they were collected from, and ectoparasitic stage. Chiggers were identified to genus level using a taxonomic key (Nadchatram, 1974). Other ectoparasites (fleas, ticks, and lice) were identified morphologically (Hopkins and Miriam, 1953; Tanskull and Inlao, 1989; Durden and Musser, 1994) and pooled by the host species they were collected from, type, stage, and gender of ectoparasites. Each pool was subjected to genomic DNA extraction using a modified protocol of QIAamp DNA Mini Kit (Qiagen). Briefly, ectoparasites in 180 μl of ATL buffer were punctured with a fine needle under a stereomicroscope to release the tissue from the hard chitin exoskeleton prior to adding 20 μl of Proteinase K solution (20 mg/ml). Samples were then incubated at 55°C for 1 h or until the ectoparasites were homogenized. A volume of 200 μl

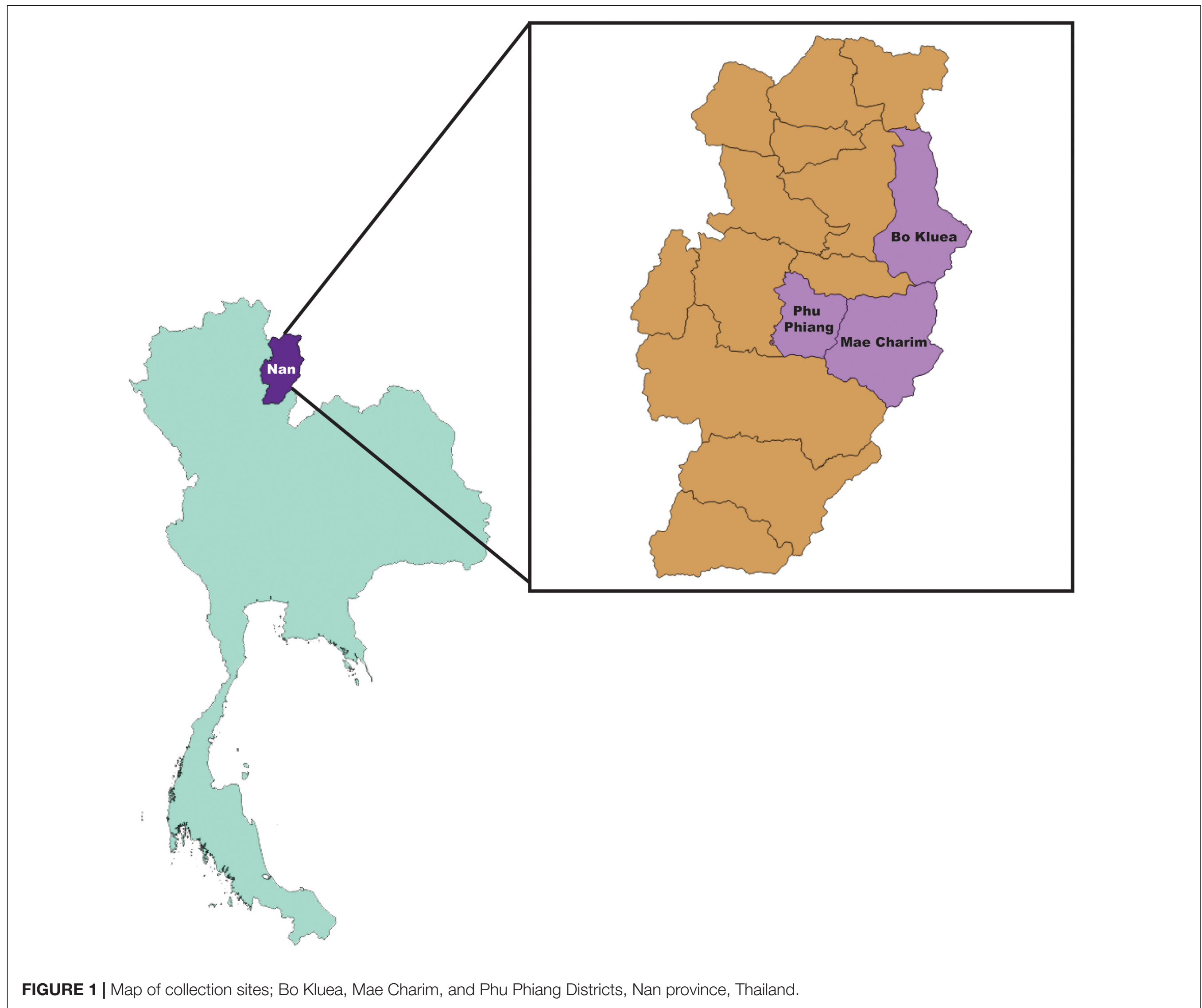


FIGURE 1 | Map of collection sites; Bo Kluea, Mae Charim, and Phu Phiang Districts, Nan province, Thailand.

of AL buffer was added to the sample and the sample mixed and incubated at 70°C for 10 min. Then 100 μl of absolute ethanol was added to precipitate DNA. The solution was transferred to a QIAamp DNA column then centrifuged at 8,000 rpm for 1 min. The supernatant was discarded. DNA was washed twice with 500 μl of AW1 and AW2, respectively. The DNA was eluted at 50 μl of AE buffer and stored at −20°C until used. Ultrapure DNA/RNA-free distilled water was also included as an extraction control.

Amplification of Bacterial 16S DNA

Following DNA extraction of patient whole blood and rodent tissue, bacterial-specific 16S rDNA (V3–V4, a 550 bp fragment) was amplified in three replicates using the universal bacterial primer set; 16S amplicon PCR Forward primer (TCGTCGGCA GCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCW GCAG) and Reverse primer (GTCTCGTGGGCTCGGAG ATGTGTATAAGAGACAG GACTACHVGGGTATCTAATCC) (gene-specific sequences are underlined). PCRs were performed in a 20-μl volume containing 5 μl (1–100 ng/μL) of DNA template, 400 nM each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, and 0.4 U of iProof High-Fidelity DNA Polymerase (Bio-Rad, Hercules, CA, United States). Amplification was performed using a T100 DNA thermal cycler (Bio-Rad) under the following conditions: initial denaturation at 98°C for 3 min; 40 cycles of 98°C for 10 s, 60°C for 20 s, and 72°C for 30 s; and a final extension at 72°C for 5 min.

For DNA from all ectoparasites, a fragment of 16S rDNA (V1–V6) region (1,016 bp) was amplified in triplicate in a first-round PCR using primers; 27F-Y (5′-GAGTTTGATCCTGGCTYAG-3′), 1061R (5′-CRRCACGAGCTGACGAC-3′) (Ong et al., 2013), and 2.5 μM MidBlocker oligonucleotide to inhibit 16S *Candidatus* Midichloria mitochondrii amplification (Gofton et al., 2015b). The reaction was performed in a 20-μl volume containing 3 μl of ectoparasite DNA, 400 nM each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, and 0.4 U of

TABLE 1 | Summary of sample description, sample size, pooling, and NGS coverage.

Sample types	Host	Number of samples studied	Number of NGS pool	Number of sample(s) per NGS pool	Collection sites in Nan province, Thailand	Year of collection	Number of reads in OTUs (minimum–maximum)	Mean number of read ± SD
UFI patients	N/A	200	23	4–14	Bo Kluea Hospital	2017	17,879–123,232	69,493.39 ± 37,001.57
Rodents	N/A	309	64	1–13	Bo Kluea, Mae Charim, Phu Phiang	2014, 2017, 2018	1,583–133,878	67,858.53 ± 32,222.56
Chiggers	Rodents	199	43	1–12	Bo Kluea, Mae Charim, Phu Phiang	2014, 2017, 2018	2,810–56,552	30,355.47 ± 15,008.78
Ticks	Rodents	59	17	1–11	Bo Kluea, Mae Charim, Phu Phiang	2014, 2017, 2018	30,968–130,783	56,570.71 ± 21,921.34
Fleas	Rodents	23	8	1–8	Bo Kluea, Mae Charim	2014, 2018	24,890–131,129	75,680.25 ± 35,071.38
Lice	Rodents	4	4	1	Bo Kluea, Mae Charim, Phu Phiang	2014, 2017, 2018	55,352–93,336	74,310.25 ± 16102.85
Ticks	Domesticated mammals	35	8	1–13	Mae Charim	2014	30,026–70,664	45,411.88 ± 12,203.16
Fleas	Domesticated mammals	88	12	1–11	Mae Charim	2014	19,163–106,928	49,278.42 ± 25,610.98
Lice	Domesticated mammals	12	4	2–4	Mae Charim	2014	6,773–160,184	59,614.00 ± 68,479.14

N/A, not available.

iProof High-Fidelity DNA Polymerase (Bio-Rad). Amplification was performed using a T100 DNA thermal cycler (Bio-Rad) under the following conditions: initial denaturation at 98°C for 3 min; 40 cycles of 98°C for 10 s, 60°C for 20 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. The second amplification was performed as described above for human and rodent samples. Negative control PCR reactions were included in every experimental run using Ultrapure DNA/RNA-free distilled water in place of DNA template. PCR reactions were also performed with eluates from mock DNA extractions.

Three PCR products from each sample were pooled and cleaned using AMPure magnetic bead-based purification system (Beckman Coulter, United Kingdom) following the manufacturer's instructions. Purified PCR products were eluted and quantified using the Quant-iT PicoGreen dsDNA assay (Invitrogen Life Technologies, MA) according to the manufacturer's protocol. Each purified PCR was normalized and then pooled again with other purified PCR products from other samples by; (i) gender and age group for UFI patients, (ii) season of collection, location (sub-district/district), and rodent genera/species for rodents and rodent chiggers, and (iii) the host type they were collected from, genus/species and stages of ectoparasites for all other ectoparasites (ticks, fleas, and lice) collected from rodents and domesticated mammals (dogs and cows). Additional details on sample pooling for NGS are provided in **Supplementary Table S2**.

Library Preparation and High Throughput Sequencing

Indexing Samples

The dual indices and Illumina sequencing adapters were attached to pooled, purified PCR products using the Nextera XT Index Kit following the manufacturer's protocol (Illumina). Index control reaction: combination of index primers that were not used with samples, was also included with PCR grade water as template. The number of reads recovered from these particular index combinations should be used to filter the cross-contaminations between indexed PCR primers and to identify errors in an Illumina sample sheet.

Library Clean Up, Normalization and Pooling

The final products were cleaned using Agencourt AMPure XP beads. The purity of the libraries was checked on the QIAxcel Advanced System (Qiagen) with a QIAxcel DNA High Resolution Cartridge. Purified amplicon libraries were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen). DNA concentration was calculated and normalized to reach 4.0 nM for each library. Five microliters of DNA from each library were pooled (each NGS pool had 29–78 DNA libraries) for a NGS run (1–5 runs in total). Pooled libraries were denatured and diluted to a final concentration of 8 pM with a 10% PhiX (Illumina) control. Sequencing was performed using the MiSeq Reagent Kit V3 on the Illumina MiSeq System.

Data Analysis

The sequence reads generated by the 16S rRNA on MiSeq sequencers were processed on the CLC Genomics workbench v 11.0.1 (Qiagen, Aarhus A/S[1]). High-throughput sequences were imported into CLC Genomics Workbench according to quality scores of Illumina pipeline 1.8. In order to achieve the highest quality sequences for clustering, paired reads were merged in CLC microbial genomics module v3.0 using default settings (mismatch cost = 1; minimum score = 40; gap cost = 4 and maximum unaligned end mismatch = 5). Primer sequences were trimmed from merged reads using parameters (trim using quality

[1] http://www.clcbio.com

scores = 0.01, trim ambiguous nucleotides = 2, and discard read length shorter than 150 bp). Samples were removed from analysis if the number of reads was less than 100 or less than 25% from the median (the median number of reads across all samples).of minimum read from the median. Chimeric sequences were detected and removed. Filtered sequences were clustered into operational taxonomic units (OTUs) according to a threshold of 97% sequence identity. All such processes were performed using CLC microbial genomics module v3.0. Reference OTU data used in the present study were downloaded from the Greengenes database V13.8 (DeSantis et al., 2006) and SILVA 16S V132 (Quast et al., 2013). Alpha rarefaction curve plots were generated among samples using CLC Microbial Genomics Module v3.0 with default parameter settings (minimum depth = 1, maximum depth = 100,000 and number of point = 20).

Pathogen Characterization by PCR Amplification and Sanger Sequencing

Real-time PCR and PCR assays were performed on positive NGS pools to confirm the detection of pathogen and the taxonomic species assignment generated by NGS analysis. The detail of assays and target gene(s) for selected pathogens was provided as online **Supplementary Data** (**Supplementary Table S1**) (Norman et al., 1995; Barbour et al., 1996; Horinouchi et al., 1996; Schwaiger et al., 2001; Scoles et al., 2001; Smythe et al., 2002; Park et al., 2004; Klee et al., 2006; Chmielewski et al., 2009; Colborn et al., 2010; Ganoza et al., 2010; Billeter et al., 2011; Parola et al., 2011; Diaz et al., 2012; Lalzar et al., 2012; Shakya et al., 2013; Gofton et al., 2015a; Pereira et al., 2018). For all real-time PCR, the reaction consisted of 1X Platinum quantitative PCR SuperMix-UDG (Invitrogen) using standard real-time PCR conditions with primer/probe concentrations and annealing temperatures as indicated in **Supplementary Table S1**. For conventional PCR, the assay was carried out in a 50 μl reaction volume containing 0.5 U of iProof High-Fidelity DNA Polymerase, 200 μM dNTPs, $MgCl_2$ and primer concentration as indicated (**Supplementary Table S1**). The PCR conditions consisted of 98°C for 3 min, followed by 40 cycles of 98°C for 10 s, annealing temperature for 30 s, and 72°C for 45 s.

Estimating the Prevalence of Each Pathogen in Samples Studied

After performing NGS analysis of pooled samples, all samples in each NGS-positive pool for potential pathogenic bacteria were individually tested by their respective confirmatory assays using either real-time PCR or conventional PCR as indicated in **Supplementary Table S1**. Any positive signal was then confirmed by DNA sequencing by the Sanger method for species characterization. The prevalence rate for each pathogen was calculated based on the number of positive samples verified by confirmatory assays in the total number of samples studied. For some pathogens including *O. tsutsugamushi* and *Bartonella* spp., all samples were screened as routine tests. Therefore, the prevalence rate was calculated based on the number of combined positive samples detected by the NGS analysis then confirmatory assays and routine screening tests.

DNA Sequence and Phylogenetic Analysis

PCR amplicons were purified using the QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The PCR products were cycle-sequenced using an ABI BigDye™ Terminator v3.1 Cycle Sequencing Kit, ethanol precipitated, and run on a SeqStudio Genetic Analyzer (Applied Biosystems Thermo Fisher, Thailand). Sequences of each sample and pathogen were assembled using Sequencher™ ver. 5.1 (Gene Codes Corp., Ann Arbor, MI, United States). The pathogen sequences were aligned with reference sequences retrieved from the GenBank database using the MUSCLE codon alignment algorithm (Edgar, 2004). A maximum likelihood phylogenetic tree was then constructed from bacterial target gene(s) (**Supplementary Table S1**) using the best fit model of nucleotide substitution with bootstrapping (1000 replicates) in MEGA 6 (Tamura et al., 2013).

Serological Tests

Immunofluorescence Assay (IFA)

Scrub typhus and typhus group and spotted fever group of rickettsial diseases IFA tests were used to detect group-specific IgM antibodies against scrub typhus orientiae, and murine typhus and the spotted fever group of rickettsiae. The *Rickettsia* Screen IFA IgM Antibody Kit was used following the manufacturers' instruction (Fuller Laboratories, Fullerton, CA, United States). The assay is intended for the simultaneous detection and semi-quantitation of IgM human antibody to both typhus group (TG) and spotted fever group (SFG) rickettsiae. An *O. tsutsugamushi* IFA IgM Antibody Kit (Fuller) included 4 strains (Boryong, Gilliam, Karp, and Kato) in one well. Positive reaction appears as bright staining (at least 1+) of positive control cut-off level in any of the four antigens areas. *Bartonella henselae* and *Anaplasma phagocytophilum* IFA tests were conducted for the detection of human IgG antibodies from serum using commercial kits [*B. henselae* IFA Human IgG Antibody Kit, *A. phagocytophilum* (HGA) IFA IgG Antibody Kit, Fuller]. Serum screening dilutions for *B. henselae* and *A. phagocytophilum* were 1:64 and 1:80, respectively.

In-house *B. quintana* IFA assay was prepared by CDC, Fort Collins (CO, United States) for testing the presence of human IgG against *B. quintana*. The protocol has been published previously (Iralu et al., 2006; Myint et al., 2011). Briefly, the human serum (1:32 dilution) was added to a slide fixed with *B. quintana* antigen, prepared by infecting Vero E6 cells with the bacteria. The slide was then incubated in a moist chamber at 35°C for 30 min, washed with PBS for 10 min, rinsed with distilled water, and air dried. Anti-human fluorescein isothiocyanate-labeled IgG conjugate was added to the slide which was processed as before. The slide was then mounted and read on a fluorescent microscope. All positive samples were then serially diluted in PBS, and an IFA-endpoint titer was determined using the same procedure; the end cut-off value for *B. quintana* was a titer greater than 1:200.

Enzyme-Linked Immunosorbent Assay (ELISA)

The determination of serological reactivity to *O. tsutsugamushi* 56-kDa recombinant protein was performed by in-house ELISA as previously described (Jiang et al., 2004). The ELISA plates were coated with 4 recombinants of *O. tsutsugamushi* 56-kDa protein from Karp, Gilliam, Kato, and TA763 genotypes. Patient sera were diluted at 1:100 with PBS for screening procedure. Samples considered positive (>0.5 OD) were further titered to determine their endpoint. The titer procedure was performed by diluting the positive sera by a factor of 4 (1:100, 1:400, 1:1,600, and 1:6,400) and tested again with the same procedure. If the sample had a total absorbance for all 4 dilutions of 1.00 or greater for the net OD, then the sample was considered reactive and the titer value was the inverse of the highest dilution with the OD of 0.2 or greater.

Enzyme-linked immunosorbent assay for the detection of IgG class antibody against *R. typhi* and spotted fever group *Rickettsia* in human serum was performed using commercial kits (*R. typhi* EIA IgG Antibody Kit, Spotted Fever *Rickettsia* IgG EIA Antibody Kit, Fuller). The kits utilized a group-specific lipopolysaccharide (rLPS) antigen extracted from spotted fever group *Rickettsia* species and a species-specific protein (r*Omp*B) purified from *R. typhi*.

Statistical Analysis and Data Visualization

All statistical analyses (linear regression and two-way ANOVA tests) were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, United States[2]). Some graphical illustrations presented in this study were performed in the R environment for statistical computing (Wickham, 2009). A nucleotide distance matrix was generated using "DNADist DNA Distance Matrix" in BioEdit (Hall, 1999). Maps used in this study were created by QGIS software (QGIS Development Team, 2009. QGIS Geographic Information System. Open Source Geospatial Foundation[3]).

RESULTS

Sample Collection and NGS Procedure

Samples included in this study were from UFI patients (n = 200), rodents and small mammals (n = 309), rodent-associated ectoparasites (chiggers = 199 pools, ticks = 59 pools, fleas = 23 pools, and lice = 4 pools), and ectoparasites collected from larger animals including dogs, cats, and cattle (ticks = 35 pools, fleas = 88 pools and lice = 12 pools) (**Table 1**). Samples were collected mainly from Bo Kluea and from nearby districts of Nan province (**Figure 1**), Thailand. UFI samples were from inpatients and outpatients visiting the hospital with symptoms similar to scrub typhus infection or fever of unknown origin throughout the year 2017. The sampling of rodents and ectoparasites took place twice each year (wet and dry seasons) in 2014 and 2017, and only once in 2018 (dry season). Each sample was amplified in triplicate reactions to minimize PCR bias (Acinas et al., 2005; Sipos et al., 2007; Aird et al., 2011) and PCR products from the three reactions were pooled for each sample and purified before pooling with other samples for library preparation before NGS. All totaled, 929 samples were pooled according to their sample type, area of collection, season of collection, and host species into 183 NGS pools (**Table 2**). After NGS quality control procedures, 13,225,584 16S sequences from the field-collected samples and 38 control samples (6 extraction controls, 25 PCR controls, and 7 index controls) were used for analysis. From the 38 control samples, 153 bacterial genera were detected and then subtracted from the field samples' 16S sequence dataset before conducting downstream analysis. These bacterial genera were considered to be contaminants from molecular reagents, the environment (water), or from cross-contamination during sample processing and between NGS runs (Salter et al., 2014). The number of pass-filtered raw reads per NGS pool ranged from 24,939 to 627,297, with the highest read (1,625,690) belonging to an ectoparasite pool collected from domesticated mammals (mean ± *SD* = 294,518 ± 152,314). The number of reads per NGS pool used in OTU assignment ranged from 1,583 to 160,184 (mean ± *SD* = 110,802 ± 34,095) (**Table 1** and **Figure 2A**). The majority of samples with low numbers of reads were from chigger samples. Overall, 7.8% of OTUs (1,032,389 reads) were unclassifiable at the phylum level. Rarefaction curves demonstrated that sequence data for all samples approached completeness as indicated by the curve plateaus (**Figure 2B**). These data suggest that most bacterial profiles of all samples studied were nearly complete.

Microbial Profiling in Human, Rodent, and Vector Populations

The classification of OTUs from each sample were made against Greengenes reference databases, with SILVA reference databases as a secondary database, and the similarity confidence threshold for each taxonomic level was set at 0.97 in CLC microbial genomic module. There were 19 recorded phyla found among all sample types and the ten most abundant phyla were seen in all sample types (Proteobacteria, Firmicutes, Actinobacteria, Spirochaetes, Tenericutes, Bacteroidetes, [Thermi], Planctomycetes, Fusobacteria, Cyanobacteria) (**Figure 3A**). The most prevalent phylum in all sample types was Proteobacteria and it was most abundant in ectoparasites from rodents (88.0%), domesticated mammals (83.0%), and UFI patients (70.0%) (**Figure 3A**). In rodent populations, the phyla Proteobacteria, Tenericutes, and Firmicutes were most abundant (38.0, 31.0, and 27.0%, respectively). In chiggers, the highest abundance was of Actinobacteria (43.0%), followed by Firmicutes (32.0%), and Proteobacteria (9.0%). Other interesting phyla include Spirochaetes found in UFI patients (5%), chiggers (5%), and rodents (1%); and two phyla, Thermi and Plantomycetes, that were found most commonly in UFI patients and chigger samples, respectively.

At the genus level, as many as 651 genera were found among sample populations with the greatest diversity of taxa belonging to the phylum Proteobacteria (n = 254 genera), followed by

[2]www.graphpad.com
[3]https://www.qgis.org/en/site/

TABLE 2 | Eleven pathogenic bacterial genera detected in samples.

Sample types	Number of samples	Number of pools	Number of NGS-positive pools (number of reads)										
			***Anaplasma* spp.**	***Bartonella* spp.**	***Borrelia* spp.**	***Coxiella* spp.**	***Ehrlichia* spp.**	***Ca.* Neoehrlichia spp.**	***Francisella* spp.**	***Leptospira* spp.**	***O. tsutsugamushi***	***Rickettsia* spp.**	***Neorickettsia* spp.**
UFI patients	200	23	8 (14–13,226)	2 (239–7,797)	0	4 (18–347)	0	0	0	3 (10–4,393)	9 (224–7,347)	0	0
Rodents	309	64	20 (13–14,357)	47 (18–60,351)	10 (11–1,384)	0	9 (21–53,134)	11 (17–5,141)	0	3 (1,771–11,216)	2 (92–212)	3 (31–150)	2 (51–2,803)
Rodent chiggers	199	43	0	2 (23–808)	8 (26–2,965)	1 (15)	0	0	4 (28–1,713)	2 (19–135)	8 (10–974)	0	0
Rodent ticks	59	17	5 (13–15,237)	6 (68–7,624)	2 (90–264)	7 (30–65,264)	1 (14,725)	0	6 (16–13,144)	1 (36)	0	10 (20–23,924)	0
Rodent fleas	23	8	0	5 (35–119,256)	0	0	0	0	0	0	0	0	0
Rodent lice	4	4	0	4 (3,834–90,081)	0	0	0	0	0	0	0	1 (1,366)	0
Mammal ticks	35	8	4 (367–10,042)	0	0	7 (264–35,079)	0	0	1 (6,960)	0	0	4 (12–2,074)	0
Mammal fleas	88	12	0	3 (19–1,001)	0	2 (6,053–39,729)	0	0	0	0	0	12 (11–22,961)	0
Mammal lice	12	4	0	0	0	0	0	0	0	0	0	0	0

Numbers of NGS-positive pools in each sample type are shown for 11 bacterial genera and the number of reads indicated by range are provided in parentheses. The pooling strategy for each sample type was provided in ***Supplementary Table S2****.*

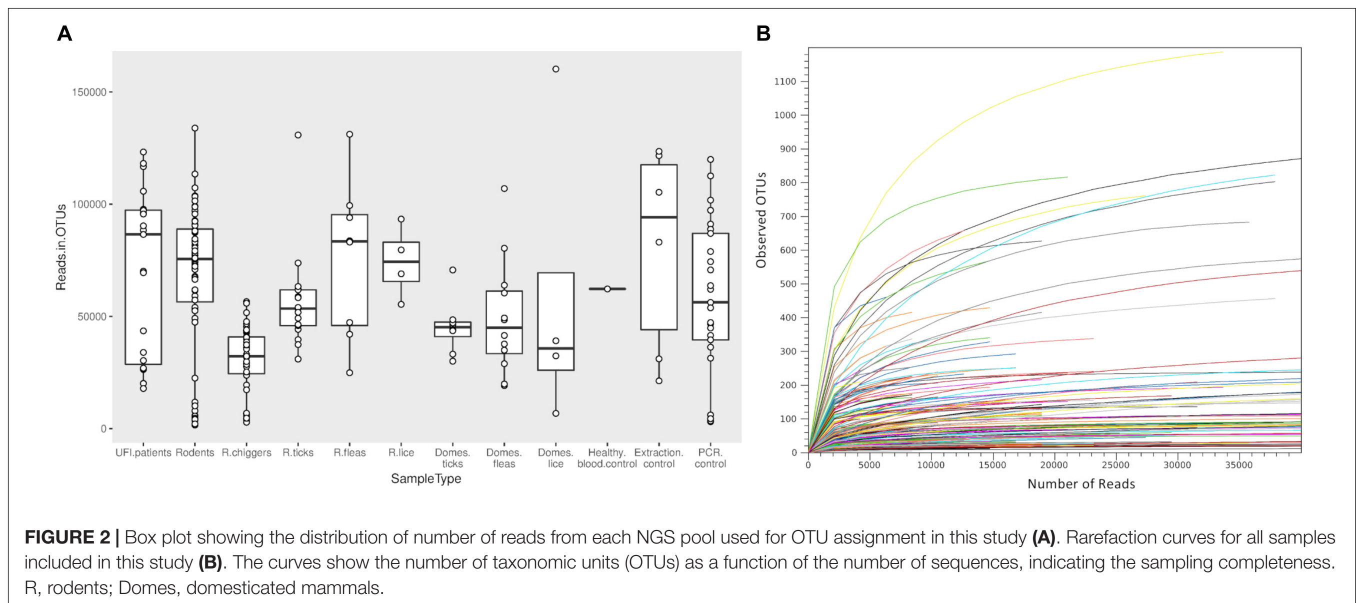


FIGURE 2 | Box plot showing the distribution of number of reads from each NGS pool used for OTU assignment in this study **(A)**. Rarefaction curves for all samples included in this study **(B)**. The curves show the number of taxonomic units (OTUs) as a function of the number of sequences, indicating the sampling completeness. R, rodents; Domes, domesticated mammals.

Actinobacteria (n = 152), Firmicutes (n = 122), and Bacteroidetes (n = 57). The most abundant reads belonged to genera in Proteobacteria from UFI patients (*Methylobacterium* = 18%, *Orientia* = 14%, *Anaplasma* = 10%), rodent associated ectoparasites (*Bartonella* = 50%, *Wolbachia* = 14%, *Coxiella* = 8%, and *Rickettsia* = 7%), and ectoparasites collected from domesticated mammals (*Wolbachia* = 46%, *Coxiella* = 21%, and *Rickettsia* = 9%) (**Figure 3B**). In rodent samples, the most abundant bacterial genera were distributed among three major phyla; Tenericutes (*Mycoplasma* = 31%), Proteobacteria (*Bartonella* = 28%), and Firmicutes (*Streptococcus* = 23%). With the chigger samples, the majority of reads belonged to *Corynebacterium* spp. in the Actinobacteria phylum (41%), followed by *Bacillus* spp. and *Staphylococcus* spp. in the Firmicutes phylum (both with 11% abundance).

Bacterial Endosymbionts in Ectoparasites From Rodents and Domesticated Mammals

Wolbachia spp. was found mainly in mammal fleas (n = 10 NGS pools) with one pool each in rodents and ticks, lice, and fleas collected from rodents. The data of endosymbionts detected in NGS pools were provided as online **Supplementary Data** (**Supplementary Figure S1**). Few reads were detected in UFI patients (273 reads). This may have been due to cross-contamination during the sample preparation process. *Coxiella* endosymbionts were equally found in ticks collected from rodents and domesticated mammals. Few (n = 2) were associated with *Francisella* spp., and they were suspected as endosymbionts since they tested negative by a confirmatory assay (qPCR and PCR). Only one NGS pool of chiggers was found to carry a *Coxiella* endosymbiont. *Francisella* endosymbiont was mainly found in chiggers and ticks collected from rodents but one pool was also found in ticks from a dog. *Candidatus* Cardinium was mostly detected in chiggers; however, it was also detected in one pool of rodent fleas and in one pool of rodent ticks. *Rickettsia* spp. were mainly detected in ticks and lice from rodents and fleas and ticks from domesticated mammals. However, some *Rickettsia* were pathogenic species as confirmed by real-time PCR and DNA sequencing. This phenomenon was mostly observed in *Rickettsia* from flea pools (mostly from domesticated mammals) where *Rickettsia* could be identified to species and excluded from being identified as endosymbiont bacteria. Therefore, *Rickettsia* endosymbiont was not discussed here. When considering only known and confirmed endosymbionts (*Wolbachia*, *Coxiella*, and *Ca. Cardinium*) found in vectors, there was usually one or two predominant endosymbionts harbored by each vector type.

Detection of Pathogenic Bacteria in all Populations by NGS Results and Prevalence Rate After Verification by Confirmatory Assays

After NGS analysis, eleven potential pathogenic bacteria were detected among samples. **Table 2** shows details about positive NGS pools and the number of reads indicated by range for each potential pathogen detected among sample populations. *Bartonella* spp. were the most prevalent and detectable bacteria among samples studied and the highest infection rate was found in rodent population (47/64 NGS pools). *Bartonella* spp. were found in most sample types with the exception of ticks and lice from domesticated mammals (69/183 NGS pools). The second most prevalent bacteria was *Anaplasma* spp. (37/183 NGS pools) which was found in UFI patients (8/23), rodents (20/64) and rodent-associated ticks (5/17), and ticks collected from domesticated mammals (4/8). *Rickettsia* spp. were also found in high prevalence as well (30/183), mostly in pools of fleas collected from domesticated mammals (12/12), 10 out of 17 pools of rodent ticks, 4/8 pools of ticks from domesticated mammals, 3/64 pools of rodents, and 1/4 pools of rodent lice. *Borrelia* spp.,

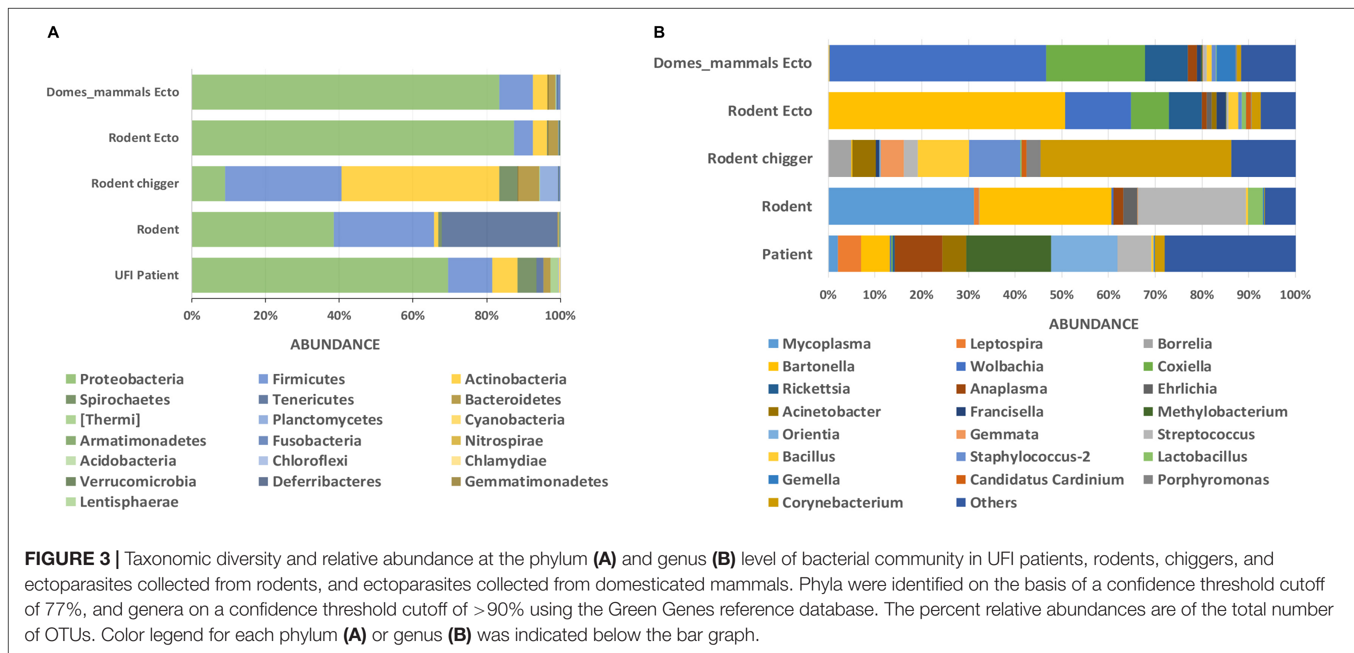


FIGURE 3 | Taxonomic diversity and relative abundance at the phylum **(A)** and genus **(B)** level of bacterial community in UFI patients, rodents, chiggers, and ectoparasites collected from rodents, and ectoparasites collected from domesticated mammals. Phyla were identified on the basis of a confidence threshold cutoff of 77%, and genera on a confidence threshold cutoff of >90% using the Green Genes reference database. The percent relative abundances are of the total number of OTUs. Color legend for each phylum **(A)** or genus **(B)** was indicated below the bar graph.

Coxiella spp., and *Orientia* spp. were equally detected; however, the distribution among populations was less similar. *Borrelia* spp. was found among rodents and its ectoparasites (chiggers and ticks), while *Orientia* spp. was detected among UFI patients, rodents, and chigger populations. *Coxiella* spp. were detected in a wide range of populations such as UFI patients, chiggers and ticks collected from rodents, and ticks and fleas collected from domesticated mammals. Other less prevalent pathogens such as *Ehrlichia* spp. (Rodents and their ticks), *Candidatus* Neoehrlichia spp. (rodents), *Francisella* spp. (chiggers and ticks from rodents, ticks from domesticated mammals), *Leptospira* spp. (UFI patients, rodents and their associated chiggers and ticks), and *Neorickettsia* spp. (rodents) were also detected in 2–11 out of 183 pools. Rodents carried the most diverse and wide-range of potential pathogenic bacteria and as many as 9 genera were detected. Likewise, ticks collected from rodents had the greatest pathogenic bacterial diversity of any vectors sampled (8 genera).

In this study, all samples present in each positive NGS pool were individually tested by confirmatory assays using either real-time PCR or conventional PCR (**Supplementary Table S1**). All amplified products were sequenced by the Sanger method. The correlation between the number of NGS-positive pools and the number of positive pools verified by confirmatory assays was determined using linear regression analysis (**Figure 4**). The results from both assays were positively correlated with R^2 = 0.8968 (95% confidence interval = 0.7440–0.9368) or R^2 = 0.6004 (95% confidence interval = 0.3950–0.7024) when the far point was removed. Prevalence rates for each pathogen were calculated based on NGS results verified by confirmatory assays or a combination of both NGS results and routine screening tests as mentioned earlier. **Table 3** shows the prevalence rate for each pathogen detected among samples. A high prevalence of *Bartonella* spp. was seen in rodents, rodent fleas, and rodent lice populations (41.1, 65.2, and 75.0%, respectively). The prevalence of *Rickettsia* spp. was highest in fleas collected from domesticated mammals, mostly from dogs (84.1%), followed by rodent lice (25.0%), and rodent ticks (6.8%). *Coxiella* spp. was detected at highest prevalence in ticks collected from rodents and domesticated mammals (32.2 and 71.4%), but later was identified as a *Coxiella* endosymbiont (**Table 4**). Other highly pathogenic species known to cause disease in humans and animals were detected among vectors, rodents, and UFI patient samples, although at low prevalence. These included *O. tsutsugamushi*, *Anaplasma* spp., *Borrelia* spp., and *Leptospira* spp.

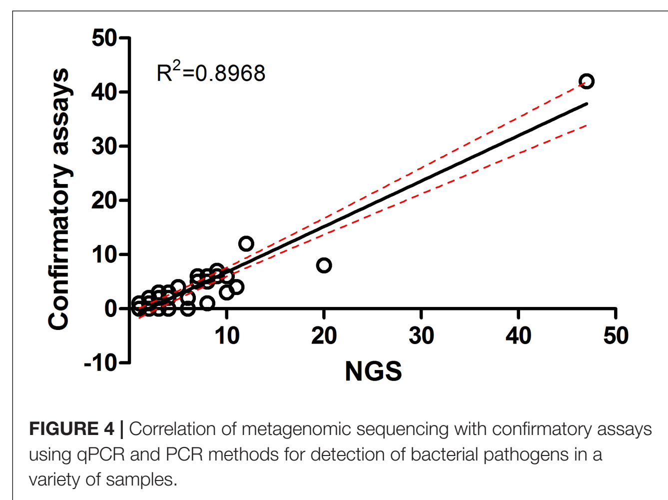


FIGURE 4 | Correlation of metagenomic sequencing with confirmatory assays using qPCR and PCR methods for detection of bacterial pathogens in a variety of samples.

Orientia tsutsugamushi was found among UFI patients (11/200, 5.5%), chiggers (6/199, 3.0%) (a well-known scrub typhus vector), and rodents (3/309, 1.0%). *Anaplasma* spp.

TABLE 3 | Prevalence of pathogenic bacteria detected in sample populations.

		NGS analysis	Confirmatory assays	
Sample types	**Detected pathogens (genera)**	**Number of NGS-positive pools (number of samples tested)**	**Number of positive pools (number positive samples)**	**Number positive/total number of samples studied (% prevalence)**
UFI patients	*Anaplasma* spp.	8 (66)	1 (1)	1/200 (0.5)
	Bartonella spp.	2 (21)	1 (1)	1/200 (0.5)
	Coxiella spp.	4 (41)	0 (0)	0/200 (0)
	Leptospira spp.	3 (32)	0 (0)	0/200 (0)
	Orientia spp.	9 (78)	7 (11)	11/200 (5.5)
Rodents	*Anaplasma* spp.	20 (99)	8 (9)	9/309 (2.9)
	Bartonella spp.	47 (259)	42 (127)	127/309 (41.1)
	Borrelia spp.	10 (64)	6 (10)	10/309 (3.2)
	Ehrlichia spp.	9 (47)	6 (6)	6/309 (1.9)
	Candidatus Neoehrlichia spp.	11 (64)	4 (4)	4/309 (1.3)
	Leptospira spp.	3 (16)	3 (4)	4/309 (1.3)
	Orientia spp.	2 (5)	0	3/309 (1.0)*
	Rickettsia spp.	3 (11)	0 (0)	0/309 (0)
	Neorickettsia spp.	2 (17)	2 (2)	2/309 (0.7)
Rodent chiggers	*Bartonella* spp.	2 (20)	0 (0)	0/199 (0)
	Borrelia spp.	8 (40)	5 (7)	7/199 (3.6)
	Coxiella spp.	1 (4)	1 (1)	1/199 (0.5)
	Francisella spp.	4 (18)	0 (0)	0/199 (0)
	Leptospira spp.	2 (16)	0 (0)	0/199 (0)
	Orientia spp.	8 (32)	6 (6)	6/199 (3.0)
Rodent ticks	*Anaplasma* spp.	5 (12)	4 (4)	4/59 (6.8)
	Bartonella spp.	6 (18)	2 (2)	2/59 (3.4)
	Borrelia spp.	2 (2)	2 (2)	2/59 (3.4)
	Coxiella spp.	7 (40)	5 (19)	19/59 (32.2)
	Ehrlichia spp.	1 (1)	1 (1)	1/59 (1.7)
	Francisella spp.	6 (26)	0 (0)	0/59 (0)
	Leptospira spp.	1 (1)	0 (0)	0/59 (0)
	Rickettsia spp.	10 (43)	3 (4)	4/59 (6.8)
Rodent fleas	*Bartonella* spp.	5 (16)	4 (15)	15/23 (65.2)
Rodent lice	*Bartonella* spp.	4 (4)	3 (3)	3/4 (75.0)
	Rickettsia spp.	1 (1)	1 (1)	1/4 (25.0)
Tick	*Anaplasma* spp.	4 (21)	2 (4)	4/35 (11.4)
	Coxiella spp.	7 (34)	6 (25)	25/35 (71.4)
	Francisella spp.	1 (4)	0 (0)	0/35 (0)
	Rickettsia spp.	4 (30)	0 (0)	0/35 (0)
Flea	*Bartonella* spp.	3 (21)	2 (5)	7/88 (7.9)*
	Coxiella spp.	2 (2)	0 (0)	0/88 (0)
	Rickettsia spp.	12 (88)	12 (74)	74/88 (84.1)

The NGS results verified by conventional methods (confirmatory assays) are shown as well as the comparison of the number of positive pools performed by both assays.
*Numbers of positive samples are from the results of both NGS and routine screening in which all samples were screened by real-time PCR or conventional PCR.

were also found in one UFI patient (1/200, 0.5%), rodents (9/309, 2.9%) and ticks collected from rodents (4/59, 6.8%) and domesticated mammals (4/35, 11.4%), while *Borrelia* spp. were found only in rodents and their associated ticks and chiggers with prevalence rates ranging from 3.2 to 3.6%. Other bacteria such as *Leptospira* spp., *Ehrlichia* spp., *Candidatus* Neoehrlichia spp., and *Neorickettsia* spp. were found in rodents and their associated ticks with prevalence rates in the range of 0.7–1.7%.

Characterization of Bacterial Species by DNA Sequence (Sanger Sequencing) and Phylogenetic Analyses

Several bacterial species were identified based on their highest similarity to reference sequences (GenBank database) as shown in **Table 4**, as well as their identity (%) corresponding to highly matched reference sequences as indicated in parenthesis after each pathogen. Only some important pathogenic bacteria

TABLE 4 | Pathogen characterization by DNA sequence and phylogenetic analyses.

Sample type	Host	Pathogens	Number of positive	Number of characterization	Target gene(s)	Number of sequences match (% identity)
UFI patient	N/A	*Anaplasma* spp.	1	1	groEL	1× *Anaplasma* spp. (96.8)
		Bartonella spp.	1	1	gltA	1× *B. quintana* (100)
		Orientia spp.	11	11	56 kDa TSA	2× *O. tsutsugamushi*_Karp A genotype (99.6), 7× *O. tsutsugamushi*_JG-C genotype (98.7–99.1), 1x *O. tsutsugamushi*_Kato B genotype (90.1), 1x *O. tsutsugamushi*_unknown genotype (73.2)
Rodents	N/A	*Anaplasma* spp.	9	9	16S	6× *A. bovis* (97.3–100), 3× *A. phagocytophilum* (100)
		Bartonella spp.	127	62	ssrA/gltA/nuoG	6× *B. elizabethae* (ssrA:99.1–99.5), 3× *B. japonica* (ssrA:99.5), 3× *B. queenslandensis* (gltA: 97.2), 1× *B. silvatica* (ssrA: 99.1), 1× *Candidatus* B. thailandensis (gltA: 98.1), 49× *Bartonella* spp. (ssrA: 96.4–98.6/nuoG: 90.2–98.1/gltA:96.3)
		Borrelia spp.	10	2	fla/16S	1× *Bor. yangtzensis* (16S: 98.4), 1× *Bor. miyamotoi* (flaB: 99.6/16S: 99.7)
		Ehrlichia/Ca. Neoehrlichia spp.	10	6	16S	2× *Ehrlichia* spp. (97.9–100), 4× *Candidatus* Neoehrlichia mikurensis (96.4–99.2),
		Leptospira spp.	4	4	16S/secY	4× *L. interrogans* (16S: 99.8–100/secY: 98.0)
		Neorickettsia spp.	2	2	16S	2× *Neorickettsia* spp. (85.7–100)
		Orientia spp.	0	3*	56 kDa TSA	1× *O. tsutsugamushi*_Saitama-karp genotype (91.6), 1× *O. tsutsugamushi*_JG-C genotype (98.7), 1× *O. tsutsugamushi*_TA763 B genotype (99.3)
Chiggers	Rodents	*Borrelia* spp.	7	5	16S/flaB	2× *Borrelia* spp. (16S: 97.4/flaB: 68.0–68.3)
					flaB	3× *Borrelia* spp. (68.3–79.2)
		Coxiella spp.	1	1	16S	1× *Coxiella* endosymbiont (98.2)
		Orientia spp.	6	6	56 kDa TSA	1× *O. tsutsugamushi*_TA763 B genotypes (97.9), 1× *O. tsutsugamushi*_Kato B genotype (76.7), 3× *O. tsutsugamushi*_Saitama-Karp genotype (92.1–93.1), 1× *O. tsutsugamushi*_Karp A (99.6)
Ticks	Rodents	*Anaplasma* spp.	4	4	16S	2× *A. bovis* (100), 2× *A. phagocytophilum* (100)
		Ehrlichia spp.	1	1	16S/groEL	1× *Ehrlichia* spp. (16S: 99.6, groEL: 87.7)
		Borrelia spp.	2	2	flaB	2× *Bor. yangtzensis* (97.6–99.3)
		Coxiella spp.	19	19	16S	19× *Coxiella* endosymbiont (97.4–100)
		Rickettsia spp.	4	4	gltA	3× *Candidatus* Rickettsia jingxinensis (99.6), 1× *Rickettsia* spp. (99.4)
Fleas	Rodents	*Bartonella* spp.	15	3	ssrA/nuoG	1× *B. silvatica* (ssrA: 99.1), 2× *Bartonella* spp. (nuoG: 77.5–91.4)
Lice	Rodents	*Bartonella* spp.	3	2	gltA/nuoG	1× *B. queenslandensis* (gltA: 97.2), 1× *Bartonella* spp. (nuoG: 91.1)
		Rickettsia spp.	1	1	gltA	1× *R. asemboensis* (100)
Ticks	Domesticated mammals	*Anaplasma* spp.	4	3	16S/groEL	2× *A. platys* (16S/groEL: 100), 1× *A. bovis* (16S: 99.2)
		Coxiella spp.	25	25	16S	25× *Coxiella* endosymbionts (93.9–99.4)
Fleas	Domesticated mammals	*Bartonella* spp.	5	7*	ssrA/gltA	5× *B. clarridgeiae* (gltA/ssrA: 100), 2× *B. elizabethae* (gltA: 96.4)
		Rickettsia spp.	74	8	gltA	7× *R. asemboensis* (100), 1× *Candidatus* Rickettsia senegalensis (100)

**Number of positive samples is from the results of both NGS and routine screening; N/A, not available.*

are discussed here. *O. tsutsugamushi* and *Leptospira* spp. are well-known pathogenic bacteria endemic to Thailand and were frequently detected in the samples studied. *O. tsutsugamushi* was detected among UFI patients, rodents, and chiggers and it was observed that some populations shared the same genotypes as demonstrated by the phylogenetic analysis present in **Figure 5**. Although *L. interrogans* was confirmed to be present in rodent population, the pathogenic status of *Leptospira* spp. (NGS reads in the range 36–4,393) belonging to other populations could not be verified (**Tables 3**, **4**). *B. quintana*, the causative agent of trench fever, was detected in one UFI patient but not in other populations. However, other common rodent-associated *Bartonella* species were found in rodents and their associated fleas and lice. Interestingly, *B. clarridgeiae*, a possible causative agent of cat-scratch disease, was found in 2 pools of fleas (*Ctenocephalides felis*) collected from domesticated mammals

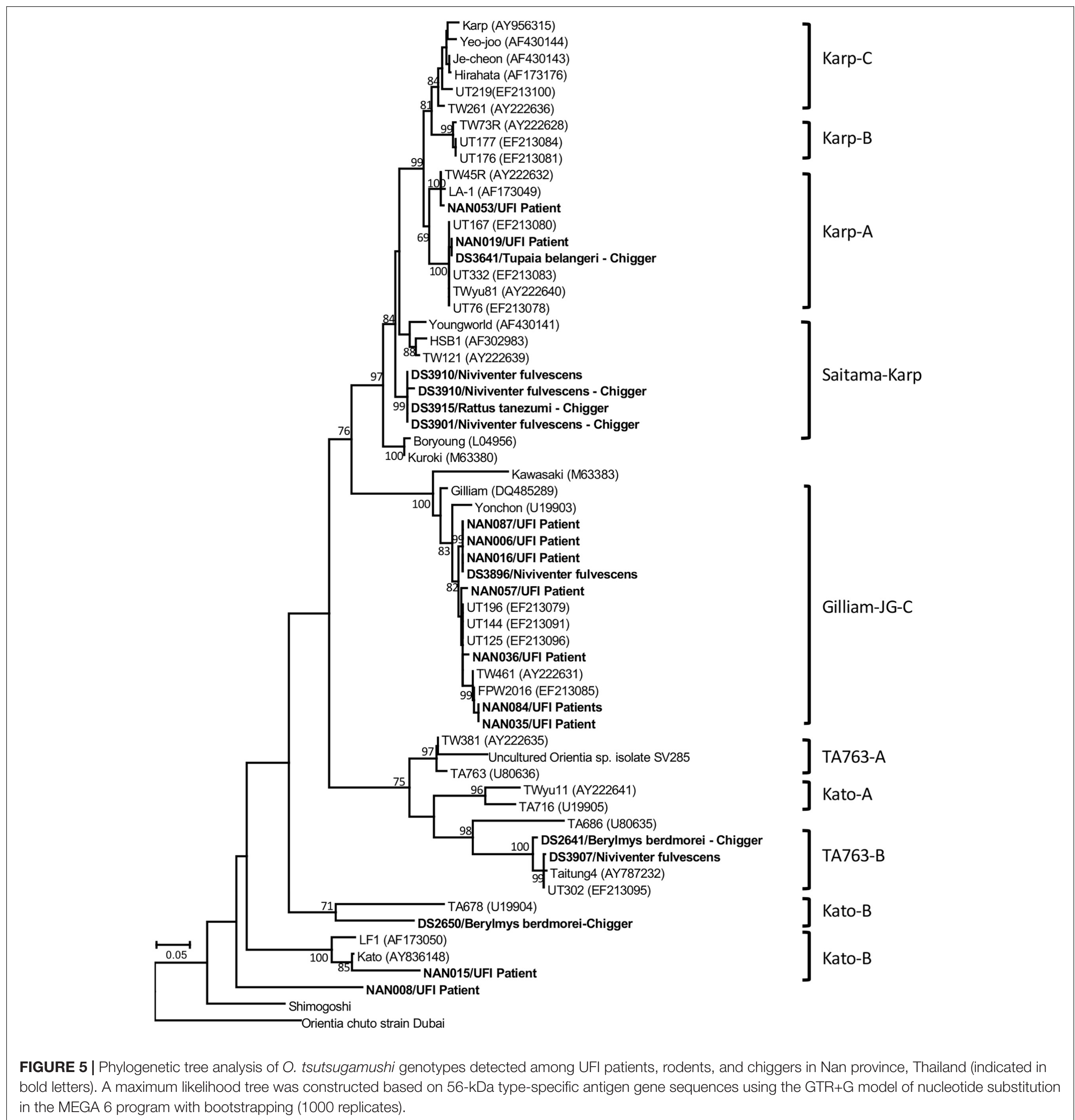


FIGURE 5 | Phylogenetic tree analysis of *O. tsutsugamushi* genotypes detected among UFI patients, rodents, and chiggers in Nan province, Thailand (indicated in bold letters). A maximum likelihood tree was constructed based on 56-kDa type-specific antigen gene sequences using the GTR+G model of nucleotide substitution in the MEGA 6 program with bootstrapping (1000 replicates).

(dogs). Human granulocytic anaplasmosis (*A. phagocytophilum*) was also detected in rodents and their associated *Ixodes* ticks. Other anaplasmosis causative agents, *A. bovis* and *A. platys* were also detected from rodents and their ticks, and ticks of domesticated mammals. Interestingly, one UFI patient was positive for *Anaplasma* spp. with 96.8% identity to Uncultured *Anaplasma* spp. detected in a tick from China (GenBank accession No. KF728361.1). Even though its identity to pathogenic species, *A. phagocytophilum* and *A. bovis,* was 92.3–92.7%, the phylogenetic relationship of its *gro*EL sequence with these pathogenic species was relatively closer than other known species in the tree (online **Supplementary Data**). *Borrelia miyamotoi* was detected in one rodent (*Niviventer* spp.) with 99.6% identity to reference sequence of flagellin and 16S rRNA genes. *Borrelia yangtzensis*, a newly recognized *B. valaisiana*-like strain, was also found in one rodent and 2 tick pools collected from rodents (*Ixodes* spp.). *Borrelia* spp. detected in chiggers were sequenced and the results showed

that only 2 sequences (16S rRNA gene) were highly similar to *Borrelia* species (97.4%) which were not grouped in any of 2 *Borrelia* groups; relapsing fever and Lyme. All five *fla*B sequences had very low identity to *Borrelia* species (68.0–79.2%) and were very distantly related to the genus of *Borrelia* as demonstrated by the phylogenetic tree analysis. The phylogenetic trees for most of pathogens detected were provided as online **Supplementary Data**.

Seroprevalence in UFI Patients for Selected Pathogens With Highest Prevalence in Rodent and Vector Populations

The seroprevalence of the most prevalent pathogenic bacteria was determined in UFI patient sera (n = 200). IFA and ELISA assays were performed to examine the presence of IgM or IgG antibodies and to measure the titer of IgG antibody in UFI patients (**Table 5**). The results of IFA assays testing for IgM antibodies against scrub typhus, murine typhus and spotted fever group *Rickettsia* (SFGR) showed relatively high numbers of patients with past infection. Patients with IgM antibody against murine typhus had the highest number accounting for 33.0% (66/200), followed by SFGR (28.0%, 56/200), and scrub typhus (20.0%, 40/200). However, when the same set of sera (n = 200) were tested for their IgG titer, scrub typhus seems to dominate the other two diseases with 154 patients having higher titers (1,600 and >6,400), compared to SFGR (n = 26) or murine typhus (n = 0) (**Table 5** and **Figure 6A**). Cross-reactivity between scrub typhus and rickettsiosis was determined using positive controls from commercial kits and the results showed no cross-reactivity was found among these pathogens for IgG and IgM.

Patients were grouped into four age groups; <19, 20–40, 41–60, and >60 years old, and by sex; male and female, to determine how seroprevalence to rickettsiosis and scrub typhus differed among the groups. The data showed higher seroprevalence (both IgM and IgG) of the two diseases in two age groups (20–40 and 41–60 years old) compared to the other two age groups (<19 and >60) with statistical significance (**Figures 6B,C**). However, the overall prevalence was not different between male and female patients. In addition, lower seroprevalence (IgG) was observed for other pathogens such as *B. quintana* (9.5%, 19/200), *B. henselae* (8.0%, 16/200), and *A. phagocytophilum* (0.5%, 1/200) (**Table 5**).

Circulation of Pathogenic Bacteria Among Human, Reservoir Host, and Vector Populations

A Venn diagram illustrates the shared bacterial species among samples (**Figure 7A**). Samples were grouped into four groups [UFI patients, rodents, rodent-associated ectoparasites (including chiggers)], and ectoparasites collected from domesticated mammals. The diagram illustrates the sharing of *O. tsutsugamushi* genotypes among UFI patients, chiggers, and rodent populations. Coordinates of all positive samples for *O. tsutsugamushi* were mapped and it was shown that almost all positive samples clustered together in Bo Kluea district (**Figure 7B**). *A. bovis* was another pathogen found circulating among animal reservoirs and ticks. The pathogen was detected in *Rattus* rats and *Tupaia glis* (common treeshrew) as well as in *Rhipicephalus sanguineus* and *Haemaphysalis bandictota* ticks where both tick species were known to share common hosts (*Bandicota indica* and *Rattus* rats) (Tanskul et al., 1983). Other pathogens such as *A. phagocytophilum*, *Borrelia yanzensis*, and various *O. tsutsugamushi* genotypes were shared between rodents and their associated vectors and are shown in **Figure 7A**. Pathogens solely detected in each population are also indicated in the figure, especially *Borrelia miyamotoi*, *B. clarridgeiae*, and *Leptospirosis interrogans* which are known to cause infections in humans. *A. platys* was found from *R. sanguineus* ticks collected from a dog and is the causative agent of canine ehrlichiosis.

The effect of environmental factors on the prevalence and transmission of scrub typhus among populations studied was also evaluated. Rainfall (mm) and temperature in 2017 from Nan province was acquired from the Thai Meteorological Department[4]. The rainy season started from late April through September corresponding to increased rainfall (mm) recorded during this period of the year (**Figure 8A**). The temperature was relatively constant throughout the year except a slight decrease at the end and the beginning of the year (October–March). The chigger index (no. of chigger/number of hosts collected) and the *O. tsutsugamushi* infection rates in rodents and chiggers were examined each month (**Figure 8B**). The chigger index slightly increased at the beginning of the year and peaked around June–September. *O. tsutsugamushi* in chigger could be found almost every month and the highest infection rates were in March. On the other hand, the number of UFI patients with IgM antibody against scrub typhus slowly increased from April to October and peaked at the end through the beginning of the year (November–February) (**Figure 8C**). *O. tsutsugamushi* was also detected by PCR from patient whole blood samples but the number did not correlate well with the number of patients having IgM seroactivity. Interestingly, the number of patients with rickettsiosis IgM increased sharply from April to September and this high number continued through the rest of the year. Likewise, the same pattern was observed with their corresponding IgGs although the number of patients with scrub typhus IgG and its titer seemed to be higher than that observed for rickettsiosis (TG and SFG) (**Figure 8C** and **Table 5**).

DISCUSSION

Scrub typhus is a major public health problem in Nan province with the highest cases of scrub typhus infection (152.64 per 100,000 population) reported to Bureau of Epidemiology, Department of Disease Control, the Ministry of Public Health (MoPH), Thailand in 2017[5]. Samples analyzed in this study included all factors/populations that are involved in disease transmission. Most samples from UFI patients were collected from Bo Kluea hospital in 2017, while rodents and ectoparasites

[4]http://climate.tmd.go.th/content/category/17

[5]http://www.boe.moph.go.th/boedb/surdata/index.php

TABLE 5 | Seroprevalence of scrub typhus, *Rickettsia* typhus group, and *Rickettsia* spotted fever group in UFI patients, Nan province, Thailand.

Pathogens	Assay types	Antibody types	Screening titer	Number of positive (% prevalence)	Antibody titer			
					ELISA/IFA (100/32)	ELISA/IFA (400/64)	ELISA/IFA (1600/128)	ELISA/IFA (>6400/256)
Orientia tsutsugamushi	IFA	IgM	1:64	40 (20.0)	–	–	–	–
Orientia tsutsugamushi	ELISA	IgG	1:100	161 (80.5)	0	7	49	105
Rickettsia spp., typhus group	IFA	IgM	1:64	66 (33.0)	–	–	–	–
Rickettsia spp., typhus group	ELISA	IgG	1:100	108 (54.0)	91	17	0	0
Rickettsia spp., spotted fever group	IFA	IgM	1:64	56 (28.0)	–	–	–	–
Rickettsia spp., spotted fever group	ELISA	IgG	1:100	125 (62.5)	63	36	24	2
Bartonella quintana	IFA	IgG	1:32	19 (9.5)	2	12	3	2
Bartonella henselae	IFA	IgG	1:64	16 (8.0)	–	–	–	–
Anaplasma phagocytophilum	IFA	IgG	1:80	1 (0.5)	–	–	–	–

Seroprevalence of other pathogens (cat-scratch disease, trench Fever, anaplasmosis) detected by NGS approach are also shown.

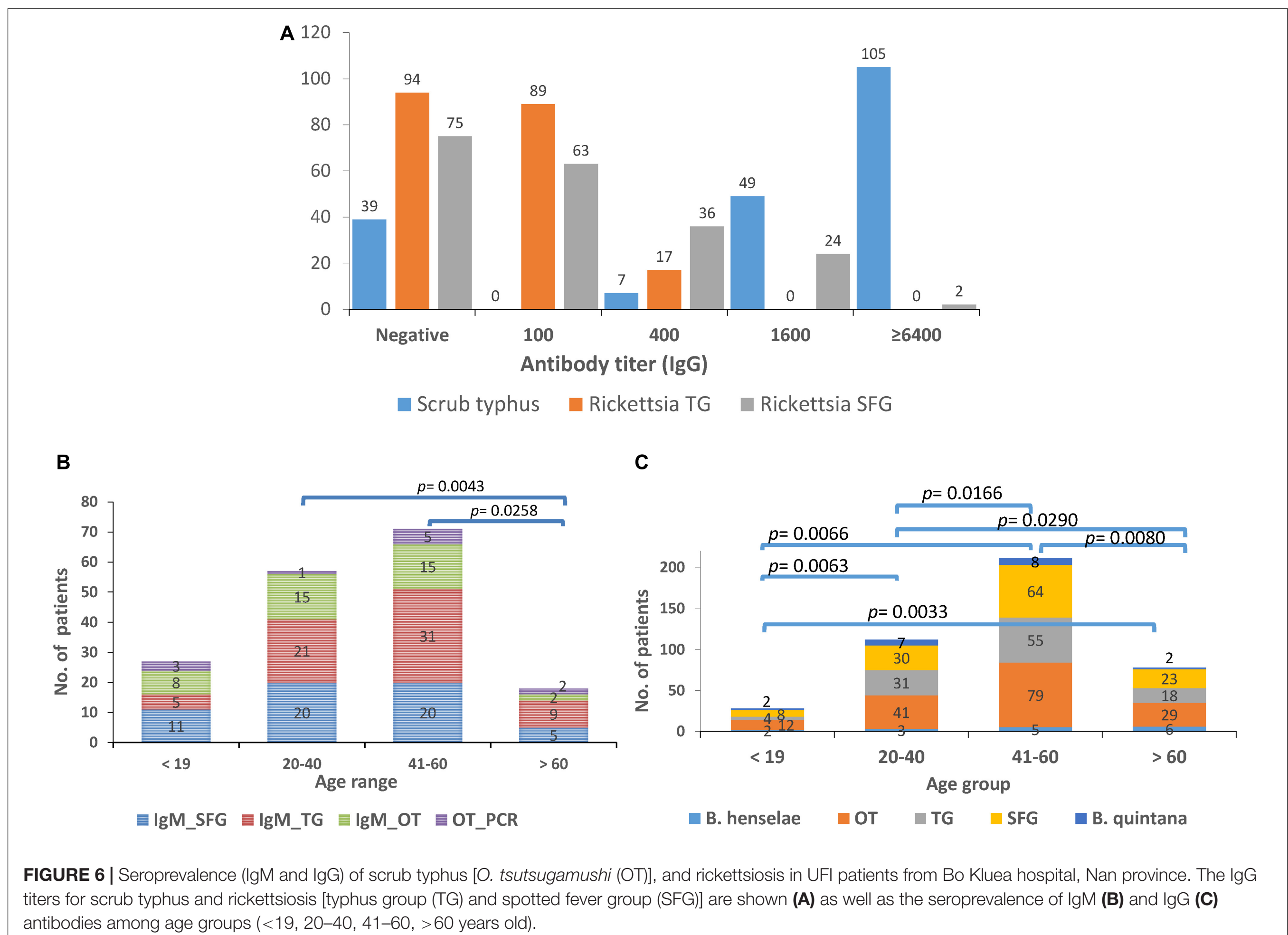
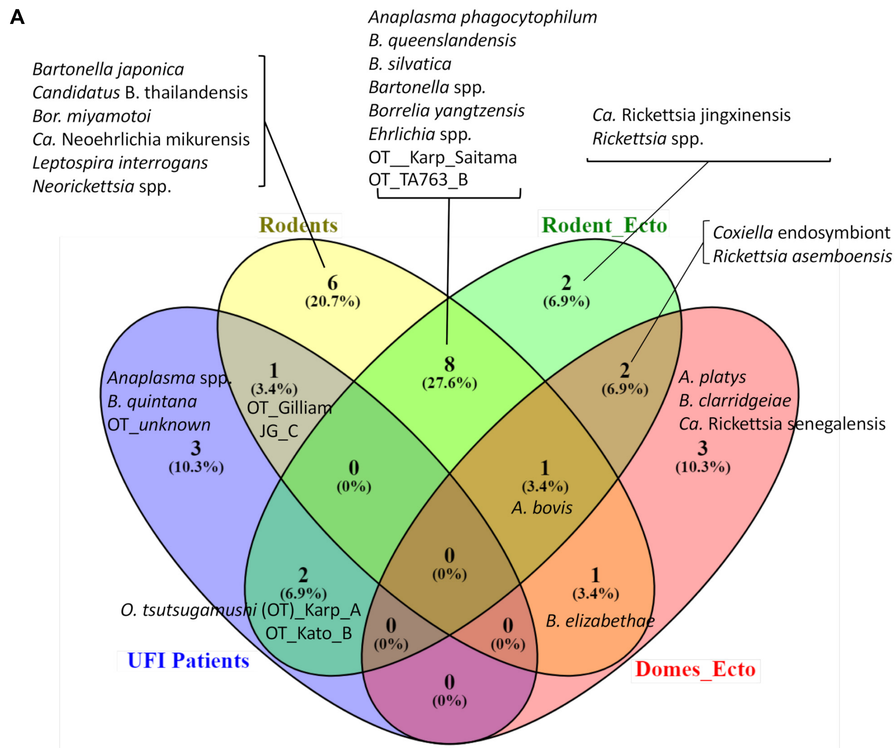


FIGURE 6 | Seroprevalence (IgM and IgG) of scrub typhus [*O. tsutsugamushi* (OT)], and rickettsiosis in UFI patients from Bo Kluea hospital, Nan province. The IgG titers for scrub typhus and rickettsiosis [typhus group (TG) and spotted fever group (SFG)] are shown **(A)** as well as the seroprevalence of IgM **(B)** and IgG **(C)** antibodies among age groups (<19, 20–40, 41–60, >60 years old).



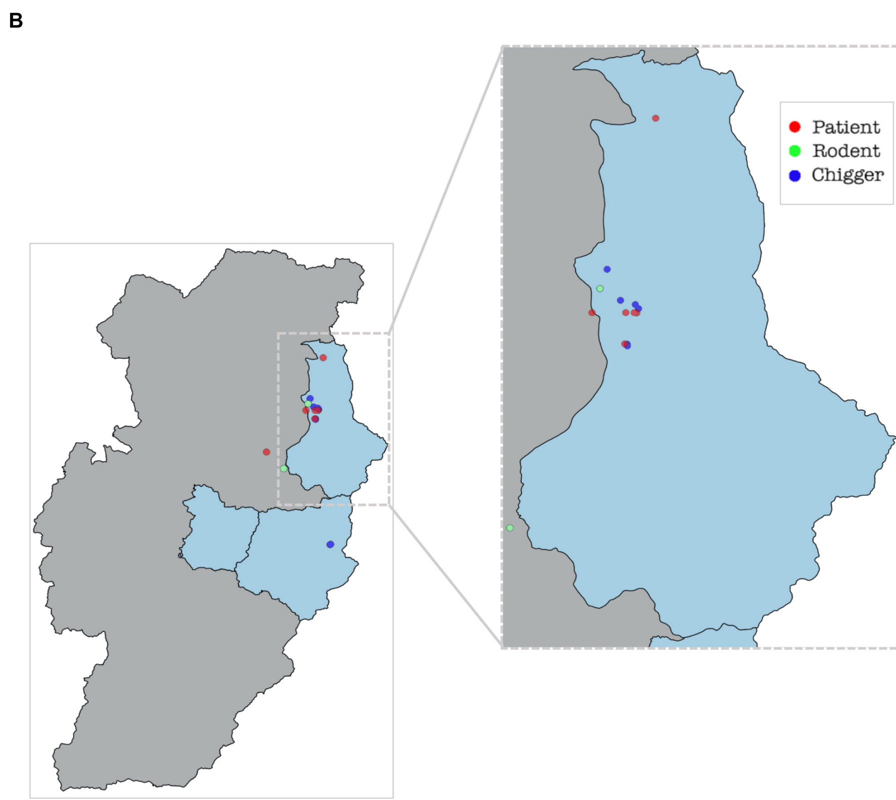


FIGURE 7 | Venn diagram (Oliveros et al., 2007–2015) indicates the bacteria species shared between populations or unique to each of them **(A)**. Bacterial species were identified on the basis of DNA sequence and phylogenetic analyses of their target genes (**Supplementary Table S1**). Rodent_ecto = ectoparasites (chiggers, ticks, fleas, and lice) collected from rodents, Domes_ecto = ectoparasites collected from domesticated mammals. Map of *O. tsutsugamushi*-positive samples in Bo Kluea district, Nan **(B)**. Each dot represents only positive samples found among UFI patients (red), chigger pools (blue), and rodent populations (green).

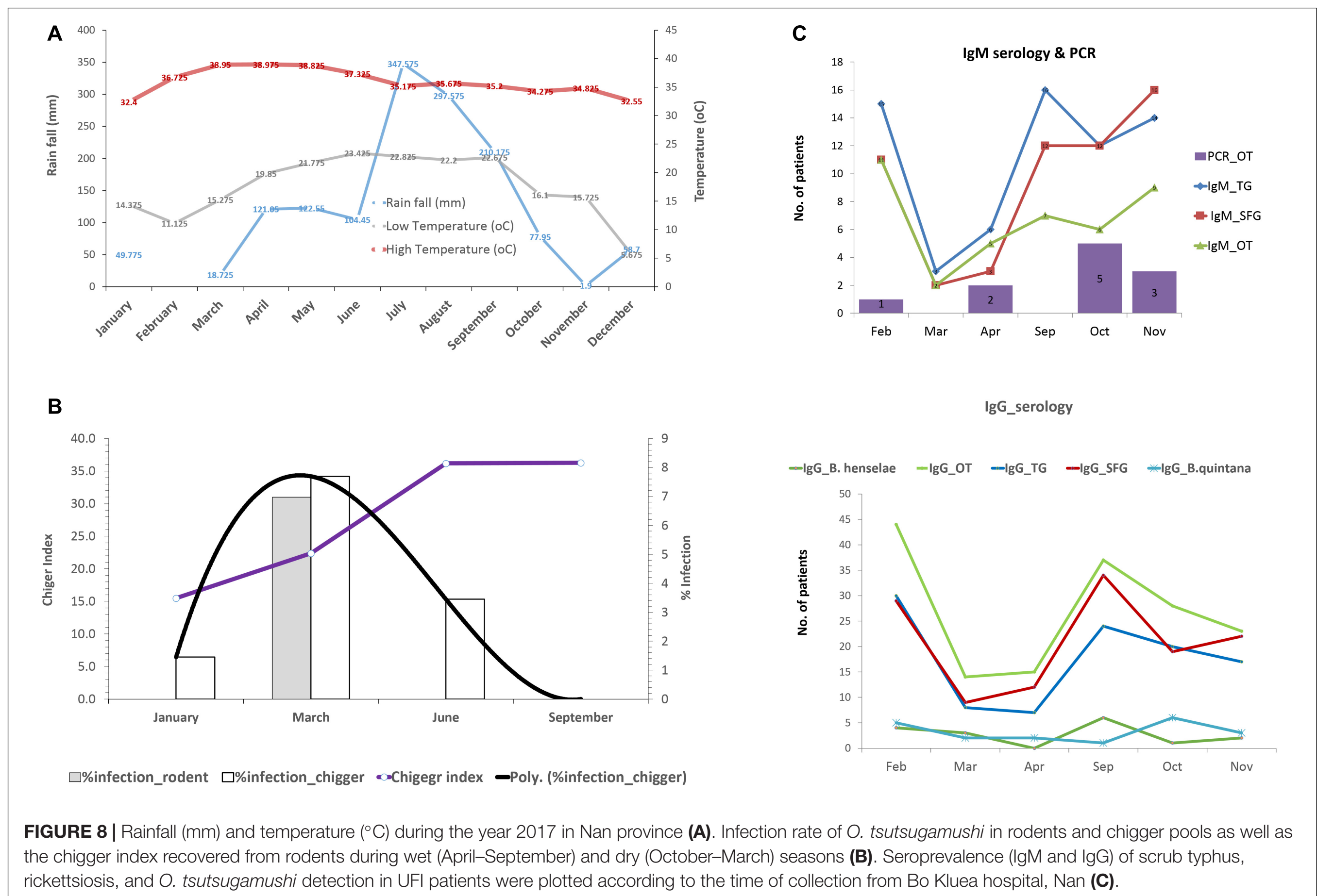


FIGURE 8 | Rainfall (mm) and temperature (°C) during the year 2017 in Nan province **(A)**. Infection rate of *O. tsutsugamushi* in rodents and chigger pools as well as the chigger index recovered from rodents during wet (April–September) and dry (October–March) seasons **(B)**. Seroprevalence (IgM and IgG) of scrub typhus, rickettsiosis, and *O. tsutsugamushi* detection in UFI patients were plotted according to the time of collection from Bo Kluea hospital, Nan **(C)**.

were collected twice a year (dry and wet seasons) from 2014 until the beginning of 2018 at field collection sites in Mae Charim, Phu Phiang, and Bo Kluea districts.

In this study, extraction and PCR controls were included in each NGS run to exclude bacteria genera commonly found in molecular reagents, water, and other environments (Tanner et al., 1998; Goodrich et al., 2014). Several common bacterial genera were found in controls similar to previous studies (Salter et al., 2014; Razzauti et al., 2015). Some potential zoonotic and pathogenic bacteria were also detected in controls such as *Bartonella* spp., *Leptospira* spp., and *Rickettsia* spp. albeit at relatively low numbers of reads. Therefore, we applied cut-off values (number of reads detected in controls) for those bacteria detected in controls and applied these numbers to all samples in our study. Contamination likely came from cross-contamination during sample processing and carry-over between sequencing runs (Swei et al., 2013). The NGS technique has many benefits over conventional tests since it does not require prior knowledge of the target pathogens which conventional tests most often rely upon. However, there is no standardized protocol for all laboratories and contamination from reagents and the environment can complicate the analysis. Therefore, conventional methods were also employed as confirmatory assays in this study. The 16S sequence can only discriminate pathogens to the genus level with confidence. However, one genus may consist of multiple species, some of which may not be pathogenic to humans or animals. Therefore, it is necessary to further identify the bacteria genera detected by NGS to the species level using conventional PCR or Sanger sequencing.

Several bacterial genera are saprophytes and commensals or can be found as contaminants in reagents and the environment. Therefore, they are considered non-pathogenic bacteria and were not considered in our analysis (Razzauti et al., 2015). A list of bacteria commonly detected in reagents and laboratory contamination was previously published by Salter et al. (2014). In this study, most of the bacterial genera found in sample populations were commensals or saprophytes such as *Methylobacterium* in UFI patients, *Mycoplasma* and *Streptococcus* in rodents, and *Corynebacterium* in chiggers. These bacteria comprised 18–41% of the total OTU reads in each population. Detection of bacterial DNA in human blood was not unexpected since healthy blood donors also contain bacterial DNA such as Proteobacteria (>80%), Actinobacteria, Firmicutes, and Bacteroidetes (Paisse et al., 2016) which is similar to what we found in UFI patients. Bacterial endosymbionts were highly abundant in ectoparasites such as *Wolbachia* (46% in fleas from dogs) or *Coxiella* endosymbionts in ticks from rodents and dogs. What we found in this study is that fleas and ticks collected from domesticated mammals

harbored one predominant endosymbiont such as *Wolbachia* in fleas (*Ctenocephalides felis*) and *Coxiella* endosymbiont in *R. sanguineus* ticks collected from dogs. Chiggers and ticks collected from rodents had two predominant endosymbionts such as *Haemaphysalis* ticks which carried both *Coxiella* and *Francisella* endosymbionts, while chiggers had both *Candidatus* Cardinium and *Francisella* endosymbionts. However, since ectoparasites were pooled before the NGS procedure, the number of endosymbionts or co-infection of endosymbionts in single vectors could not be determined.

In this study, a high prevalence from NGS results was observed for few bacterial genera; however, some genera could not be verified with conventional assays such as real-time PCR, PCR, or DNA sequencing. The main reason was likely that the number of reads was too low and below the limit of detection for the conventional test, or they could have been non-pathogenic strains or species and were not picked up by confirmatory assays. For example, *Leptospira* spp. comprise both pathogenic, intermediate, and saprophytic (non-pathogenic) species that can be introduced as contaminants from the environment into samples. Here *Leptospira* spp. were only detected in the rodent population, although NGS analysis showed reads were also detected in UFI patients, chiggers, and ticks at lower levels. However, testing with confirmatory assay resulted in no signal or PCR product using a genus-based assay (Ahmed et al., 2009) with primer sets targeting house-keeping genes such as *gyr*B or *Sec*Y (Slack et al., 2006; Victoria et al., 2008). In some cases, PCR assays targeting *Leptospira* 16S rRNA genes showed some positive bands for UFI patients but the product size was shorter than expected and DNA sequences from these products matched only human DNA (100%). *Rickettsia* spp. and *Francisella* spp. could not be verified in some sample types such as ticks, chiggers, and rodents. These could be endosymbiont bacteria which our assays could not detect (Wright et al., 2011; Takhampunya et al., 2017).

Originally, the confirmatory assays did not verify the NGS results (2 pools) of *O. tsutsugamushi* detected in rodents. However, since scrub typhus detection has been run in our lab as part of routine surveillance assays, three *O. tsutsugamushi*-positive rodents were verified by a routine real-time PCR test (**Table 4**). Similarly, four *Bartonella* species detected in fleas from domesticated mammals were also verified by a routine real-time PCR test and they were included in **Table 4**. NGS seems to have less sensitivity than the conventional method. In support of this observation, a previous study has compared MiSeq and RNA-seq, and found that MiSeq cannot detect bacteria at a value lower than 4% prevalence in the population and thus RNA-seq is better in terms of sensitivity (Razzauti et al., 2015). In all likelihood, this is due to differences in sequencing depth for each of the techniques used. The detection of *B. quintana* in one UFI patient and *B. clarridgeiae* in *Ctenocephalides felis* fleas provides significant evidence that Trench fever and cat-scratch disease-causing bacteria are present in the study area. The seroprevalence data (IgG) of *B. quintana* and *B. henselae* also confirmed previous human exposure to these bacteria. Although we detected *A. phagocytophilum* (anaplasmosis) in rodents and ticks, only one UFI patient was seropositive (IgG) to anaplasmosis. Further characterization of *Anaplasma* species detected in the UFI patient is required to identify this pathogenic species causing human infection. Given the fact that a few *Anaplasma* species were detected from rodents and ticks in this study area such as *A. phagocytophilum*, *A. bovis*, and *A. platys*, knowing what species caused infection in humans would lead to a better understanding of the transmission dynamics among the vector, host, and reservoir enable to and to better understand its transmission in the area and reservoir host and the vector involved. Other bovine and canine ehrlichiosis were also detected in ticks. Interestingly, *Bor. miyamotoi* and *Bor. yangtzensis* were detected in rodents and *Ixodes* ticks which marks the first detection of these human pathogenic species in Thailand. More research and surveillance is needed to further characterize their prevalence and distribution in the country. *Borrelia* spp. detected in chiggers could be some other unidentified bacteria since their sequence identity to most *Borrelia* species were quite low based on *fla*B gene sequences (64.0–70.2%), while the percent identity among all reference sequences used in the alignment ranges from 69.6 to100%. While the 16S rRNA sequences of two chigger pools were 97.4% identical to some unknown *Borrelia* species and *Candidatus* Borrelia africana (Accession No. KT364339), additional analysis such as multilocus sequence typing (MLST) should be performed in order to determine whether *Borrelia* spp. detected in chiggers are new *Borrelia* species or some other bacterial genus. Since some sample types included in this study were not collected across all years of sampling (2014, 2017, and 2018) such as ectoparasites collected from domesticated mammals (2014) and UFI patients (2017), the observed pathogens reported here might not represent the true picture of pathogens shared among the vectors, reservoirs, and hosts in Bo Kluea district, Nan province. In this study, co-infection between *Anaplasma* spp. (*A. phagocytophilum, A. bovis*) and *Bartonella* spp. was observed in *Rattus* and *Bandicota* rats (2/309, 0.65%). It is unfortunate that the co-infection/co-occurrence patterns in ectoparasites could not be examined in this study due to our pooling procedure for ectoparasites which was performed immediately after they were collected from animal hosts.

Seroprevalence (IgM and IgG) for scrub typhus and rickettsiosis in UFI patients confirmed that the two diseases are highly endemic to the region, especially for scrub typhus. *O. tsutsugamushi* was present in all related samples studied and human exposure was clearly observed with high prevalence and titers (n = 154 with 1600, >6400 titers). Although human rickettsiosis was not detected in rodents or vectors, the levels of IgM and IgG seroprevalence for TGR and SFGR indicate the circulation of these pathogens in the area as well. Some pathogens were detected in animals and vectors but not in humans; however, seroprevalence (IgG) of the pathogens in patients indicated previous exposure in humans, such as *B. henselae*. It is worth noting that serological differentiation between *B. henselae* and *B. quintana* IgG antibody might not be possible since there could be some cross-reactivity between the two species. With *O. tsutsugamushi*, when age and sex of patients were considered, prevalence was significantly higher in 20–40 and 41–60 year-old groups, which shows the working age population having increased risk of contracting the diseases. The incidence of scrub

typhus infections in humans seems to occur at higher rates during the rainy season corresponding to the time when local people start rice/corn cultivation and continues throughout the year until the harvesting season ends in October/November as the high seroprevalence in working age groups (20–60 years old) strongly supports this speculation. Moreover, the infection rate of *O. tsutsugamushi* in rodents and chiggers was highest in March just before the rainy season, followed by the increase of chigger indexes (number of chiggers per host) possibly leading to increased potential for disease transmission.

The discovery of certain bacterial pathogens was expected based on previous surveillance data and reported cases from the Ministry of Public Health. Additionally, other unexpected pathogens such as *Anaplasma* spp. and *B. quintana* (an agent causing Trench fever) were detected among UFI patients as well as *Bor. miyamotoi* in rodent populations. However, to date in-depth analyses as to how, when and where transmission occurs are lacking. Human, animal, and vector interactions play a major role in disease transmission and form a dynamic transmission cycle. Pathogens can spread from animal-to-animal or animal-to-human by several modes of transmission. Probably the most important method of transmission occurs during feeding by parasitic arthropods. This study employed NGS and metagenomics to characterize bacterial pathogens and understand their transmission in animal, human, and vector populations. Several pathogens were detected in rodent and vector populations indicating the complex ecology of bacterial pathogens and their reservoir hosts and vectors in the area close to where human activities occur which increase the risk of human–animal interface. The most apparent example is scrub typhus where *O. tsutsugamushi* was found in UFI patients, chiggers, and rodent populations. These data clearly illustrate the complex picture of pathogen transmission from animal reservoir hosts to humans via arthropod vectors. Local public health officials can effectively use the data to assist in understanding the seasonality of diseases such as scrub typhus and the populations most at risk. Information can be shared locally and preventive measures, such as repellents, can be used when appropriate. From this study, multiple bacterial pathogens known to cause human diseases in other locations were identified for the first time in Nan province. Such information is useful for local medical providers as they try to diagnose and treat patients with undifferentiated fevers. Finally, the data presented in this study effectively illustrate the utility of metagenomics in future epidemiological surveys involving multiple types of samples.

AUTHOR CONTRIBUTIONS

RT and AK conceived and designed the experiments. AR, NC, SP, TM, ST, and BT performed the experiments. CP, KY, and NK were the hospital staff involved in this study. RT, AK, and SD analyzed the data. RT, SD, and AK wrote the manuscript. ALR provided reagents and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00319/full#supplementary-material

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Surveillance of Mosquitoes (Diptera, Culicidae) in a Northern Central Region of Spain: Implications for the Medical Community

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Mosquitoes are important to public and animal health due to their capacity to transmit diseases. Since the Zika virus was declared a pandemic by the WHO in 2016, and it has been recorded in different regions of Mediterranean Area (included Spain), the Government of La Rioja (Northern Spain) through the Center of Rickettsiosis and Arthropod-Borne Diseases, implemented an entomological surveillance programme of mosquitoes in La Rioja and in a close area of Navarra. This surveillance extended to some of the pathogens that they can transmit. Here we describe the framework of the initial surveillance programme for the detection of mosquitoes and associated human pathogens. We outline the benefits and the limitation of the programme to date, and explore how greater benefits can be achieved, for example using a One Health approach. Entomological surveillance has been carried out with BG-Sentinel traps, human bait technique and other methods such as collecting adults in resting places or immature stages by dipping in several wetlands. Since *Aedes albopictus*, vector of arbovirus such as Dengue, Chikungunya, and Zika, has not been detected yet in the region, the entomological programme included the surveillance of this exotic species using ovitraps in the most important cities. Morphological identification was supported using the mitochondrial cytochrome C oxidase subunit I and the internal transcribed spacer 2 genes analysis. In 2016 and 2017, more than 6,000 mosquitoes were collected. The mosquito's community included 21 species associated with six genera: *Anopheles* ($n = 4$), *Aedes* ($n = 5$), *Culex* ($n = 6$), *Culiseta* ($n = 4$), *Uranotaenia* ($n = 1$) and *Coquillettidia* ($n = 1$). Eleven species represent new records for La Rioja and Navarra regions. Several species were collected biting humans and a great proportion of the sampled mosquito population are competent vectors of several pathogens, such as West Nile virus. Sequences closely related to mosquito–only flavivirus have been detected in 0.34% of analysed pools. At the same time, the epidemiological surveillance emphasis is placed in the early detection of mosquito-borne diseases in primary health and emergency services. The surveillance programme represents a relevant and necessary assessment of the risk of pathogen transmission in a region, and it allows for the establishment of the appropriate preventive measures.

Keywords: surveillance, mosquito, One Health, flavivirus, molecular identification, La Rioja, Northern Spain

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INTRODUCTION

Mosquitoes are considered the most important arthropod vectors in the world (1, 2). Globalization in conjunction with climate change, landscape change and the capacity of mosquitoes to adapt to a changing world favour the emergence and re-emergence of numerous mosquito-borne diseases (3, 4).

Vector-borne diseases are increasing in Europe with the presence of alien and native species of mosquitoes. Thus, the invasive tiger mosquito (*Aedes albopictus*) has been involved in the transmission of Chinkungunya virus (CHIKV), and autochthonous cases of CHIKV have been reported in France and Italy from 2007 to 2017 (5, 6). *Aedes albopictus* has been also related to cases of Dengue virus (DENV) reported in France from 2010 to 2015 (7). More recently, DENV has been recorded from Spain, and again in France (8, 9). Moreover, native species such as *Culex pipiens* s.l. or *Anopheles atroparvus*, could play a prominent role in the transmission of pathogens, such as the West Nile virus (WNV) (10) or malaria, respectively (11, 12).

The emergence and resurgence of some mosquito-borne diseases has led to the implementation of mosquitoes and arboviruses surveillance programs in some European countries, in an effort to reduce the impact of these infections on public health (13). Arboviruses surveillance requires a One Health approach that integrates the health of humans, animals (livestock and wildlife), and the ecosystems to prevent disease outbreaks (14). This includes the surveillance of mosquitoes. Research on the distribution, abundance and species composition of mosquitoes in a region is vital in order to estimate the risk of incidence of vector-borne diseases (15–17).

Mosquito-borne disease surveillance programs vary among European countries, according to different environmental and socio-economic scenarios (18) and, to a greater or lesser extent, within the One Health perspective. This is, for instance, the case of West Nile disease (WND) surveillance program. WNV remains in an enzootic cycle among birds, and it does not easily adapt to urban spaces (19). Mosquitoes of the genus *Culex* are the main vectors in Europe (18), and humans and equids are accidental hosts. WNV is continuously circulating in Europe with a recent increasing trend of incidence in several European countries (20). In Spain, a country where WNV is endemic (21), a specific national surveillance plan for WNV has been carried out since 2007 in high risk areas, located mainly in southern Spain. Nevertheless, WNV screening in mosquitoes had been previously done in wetlands in western Andalucía (2001–2013) and Catalonia (2001–2009) (18). In addition to the entomological surveillance, both passive and active surveillance were carried out on birds and horses (22).

In February 2016, WHO declared Zika virus (ZIKV) infection as a public health emergency of international importance due to its rapid expansion over-wide and severe complications, including congenital microcephaly and Guillain-Barré syndrome (23). In Spain, a National Plan of preparedness and response against CHIKV, DENV, and ZIKV was then developed (23) due to the presence of *Ae. albopictus* in several regions of the country (24). At the same time, the 17 Autonomous Communities from Spain were urged to make their own plans against this mosquito threat. The latter and the lack of knowledge about the circulation of mosquitoes in La Rioja (northern central of Spain), urged La Rioja Government to implement a mosquito (and their related microorganisms) surveillance program in the region in 2016, through the Center of Rickettsiosis and Arthropod-Borne Diseases (CRETAV).

CRETAV is a reference centre for arthropod-borne diseases in Spain. A multidisciplinary team (physicians, biologists, veterinarians, entomologists, biochemists and pharmacists) works in coordination dedicated to the study of these zoonosis, focused on the One Health concept. In addition, physicians who treat patients with febrile syndromes are sensitized with the emergence and re-emergence of diseases transmitted by arthropod vectors (25–27).

Within the regional plan for surveillance of arboviruses in La Rioja, a coordinated group among the different sectors involved was formed to follow up on imported cases and adequately respond to risk situations. The key elements within this plan were: epidemiological, entomological and microbiological surveillance, entomological response, individual protection, training and information, and coordination and communication (28); thus requiring a multidisciplinary team which needs to understand the ecology of the mosquitoes. Specifically, the main measures were focused on entomological surveillance as well as epidemiological surveillance of imported cases in case of *Ae. albopictus* (and/or other competent vector) detection. The aim of this manuscript was to describe initial surveillance programme for the detection of mosquitoes and associated human pathogens. The study involved not only mapping the mosquito species distribution, but also investigating their abundance, phenology and preference for hosts. It was focused on the collection of ecological data to inform about the epidemiology of mosquito-borne diseases. We outline the benefits and the limitation of the programme to date, and explore how greater benefits can be achieved, for example using a One Health approach.

METHODOLOGY

Study Area

The study area is located in the Autonomous Community of La Rioja (northern Spain) and a close area of Navarra region (**Figure 1**). La Rioja is a small region (5,034 km² and 312,830 inhabitants) in Spain. It has different habitats with great biodiversity. Its territory expands between the plain in the North, with the Ebro river Valley, with altitudes between 300 and 400 metres above sea level (m.a.s.l.), and the mountains in the South, with the presence of several valleys with North-South direction, with maximum altitude of 2,271 m.a.s.l. (29). The climate is temperate with variations according to altitude.

The entomological surveillance encompasses areas placed in Iregua river (in Logroño) as well as La Grajera and Las Cañas wetlands (the last one in Navarra region), both located very close to Logroño (red points, **Figure 1**). These areas were

Abbreviations: CHIKV, Chinkungunya virus; DENV, Dengue virus; USUV, Usutu virus; WNV, West Nile virus; ZIKV, Zika virus.

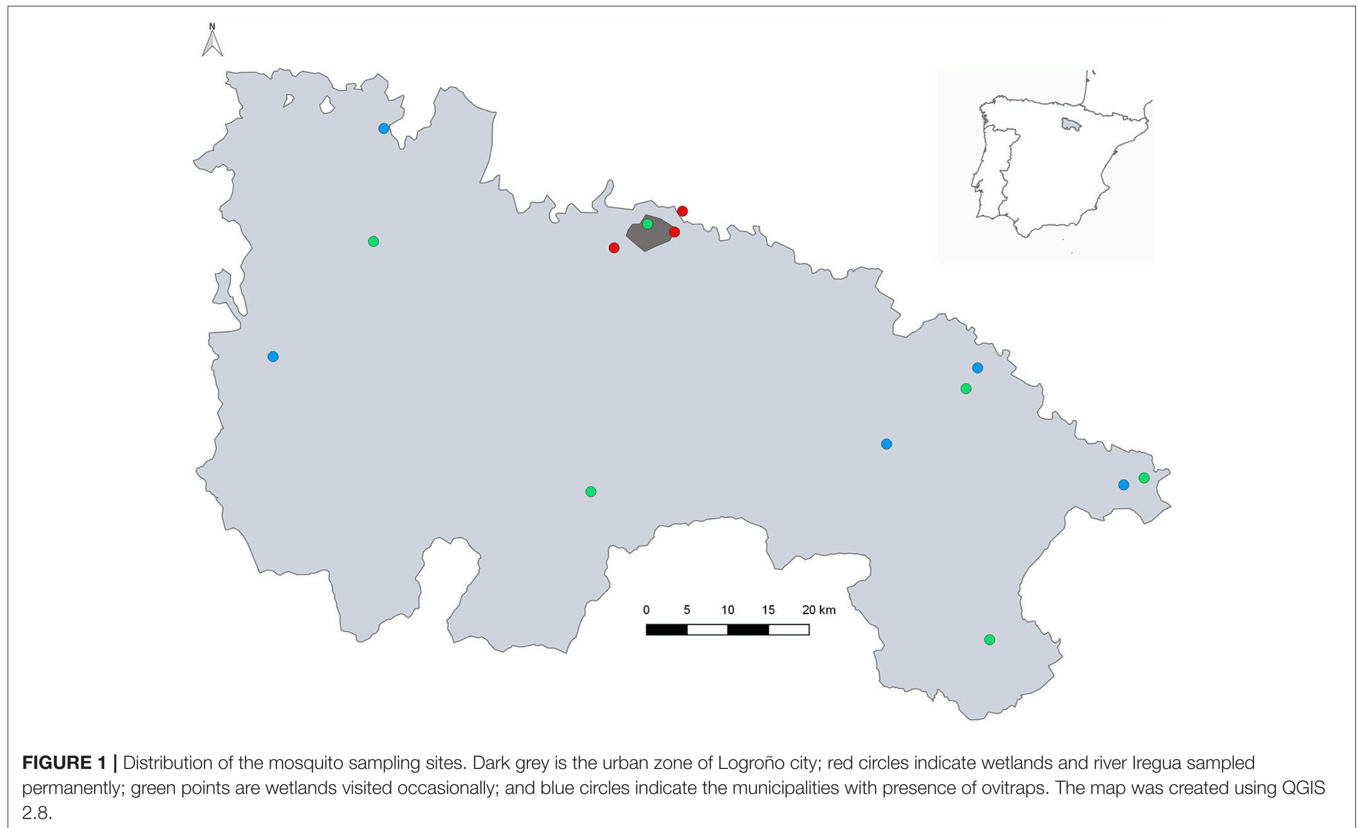

FIGURE 1 | Distribution of the mosquito sampling sites. Dark grey is the urban zone of Logroño city; red circles indicate wetlands and river Iregua sampled permanently; green points are wetlands visited occasionally; and blue circles indicate the municipalities with presence of ovitraps. The map was created using QGIS 2.8.

chosen according to the presence of mosquitoes, waterfowl and migratory bird species, and because they were regularly visited by the public. This situation makes these wetlands points of specific interest for monitoring arboviruses. The entomological surveillance also included sporadic visits to other wetlands (green points, **Figure 1**) in order to investigate the mosquito fauna through the entire region of La Rioja. The surveillance of alien species was carried out in six municipalities (blue points and Logroño city, **Figure 1**). Geolocation of sampling sites are included in **Table S1**.

Mosquito Collection

Entomological surveillance was carried out using different collecting techniques: Trapping devices, human landing technique and other methods, such as collecting adults in resting places or catching immature stages by dipping.

Mosquitoes were collected from July to September 2016, and from May to September 2017. A total of 16 BG-1 Sentinel^{TM} traps (BioGents GmbH, Regensburg, Germany) baited with BG-Lure$^{®}$ and CO_2 were set once every 2 weeks in wetlands.

Traps were placed at dusk and checked the following morning and, at the same time, the mosquitoes were captured by human landing technique during 10 min per trap using mouth aspirators (30). Resting adults were captured from natural and artificial hiding places and the surrounding vegetation in breeding sites by vacuuming (31) using an InsectaZooka and an AC/DC aspirators (Bioquip Products, Rancho Dominguez, CA, USA).

The entomological programme also included the surveillance of *Ae. albopictus* using ovitraps (31) in the most important cities because it was known to be present in three border regions, the Basque country, Aragón (32), and in Navarra (unpublished data). A total of 80 ovitraps locations were chosen in selected municipalities (blue points and Logroño city, **Figure 1**). The ovitraps were checked every 2 weeks for *Ae. albopictus* eggs from July to October 2016–2017.

Mosquito Identification and Viruses Screening

Collected adult specimens were placed into a cooler containing dry ice and transported to the laboratory for storage at −80°C until processing. Larval specimens were preserved in 80% ethanol until mounted on slides and pupae were conserved with water from breeding place to obtain link-reared adults. Adults were separated on a chill table, according to their gender and their engorged status. Wooden sticks of the ovitraps were checked under a stereoscope in the search of eggs. If present, they were introduced into water for hatching, following the protocol of Alarcón-Elbal et al. (33). All specimens were morphologically classified using taxonomic keys (34, 35).

Molecular identification was carried out in selected adults and in unhatched eggs using PCR assays targeting the mitochondrial cytochrome C oxidase subunit I (COI) and the internal transcribed spacer 2 (ITS2) (36). A modified hotshot technique was used for DNA extraction using only leg(s) from the adult specimen (37). Eggs collected on every wooden stick were pooled, and DNA was extracted using

the kit DNeasy Blood and Tissue (Qiagen, Hilden, Germany). PCR products were sequenced in both senses using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Forest City, CA, USA) at the Sequencing Unit, Center for Biomedical Research of La Rioja (CIBIR), Spain. Nucleotide sequences were compared with those deposited in GenBank using BLAST tool (www.ncbi.nlm.nih.gov/genbank), and in BOLD Systems (http://www.boldsystems.org/index.php/IDS_IdentificationRequest). Neighbour joining analyses were conducted in MEGA4. Detailed specimen records and sequence information (including trace files) are available on Barcode of Life Database (BOLD) (see http://www.boldsystems.org) and Genbank.

After identification, unfed female mosquitoes were pooled (a maximum of 50 individuals/pool) by wetland, collection date and species. The RNA was extracted from the homogenates and reverse-transcribed using RNeasy Mini Kit and Omniscript RT kit (Qiagen, Hilden, Germany), respectively, following the manufacturer's instructions and tested for flavivirus using a generic nested PCR assay (38). Japanese Encephalitis virus was used as positive control. The strains-14 was obtained through the European Virus Archive (EVAg) consortium and passed three times in Vero Cells. All procedures were carried out under sterile conditions in a Class II biosafety cabinet in a biosafety level 2 laboratory at CIBIR. PCR products were sequenced and analyzed as explained above.

RESULTS

Identification of Mosquitoes

In the studied period 2016–2017, a total of 6,658 mosquitoes were collected by traps in permanently sampled wetlands. The community composition of the samples included 21 species belonging to six genera: *Anopheles* ($n = 4$), *Aedes* ($n = 5$), *Culex* ($n = 6$), *Culiseta* ($n = 4$), *Uranotaenia* ($n = 1$) and *Coquillettidia* ($n = 1$) (**Table 1**). Eleven species represented new records for La Rioja (*Anopheles algeriensis, Anopheles plumbeus, Aedes berlandi, Aedes cantans, Aedes vexans, Aedes detritus, Coquillettidia richiardii, Culex theileri, Culiseta litorea, Culiseta subochrea,* and *Uranotaenia unguiculata)* added to the fourteen species previously described in the region (43, 44). Five species were new records for Navarra (region nearby of La Rioja) (*An. algeriensis, An. plumbeus, Ae. detritus, Cs. litorea* and *Cs. subochrea*) along with the fourteen species previously reported (45, 46). During the surveillance in 2016, eggs that morphologically seemed compatible with those from *Ae. albopictus* were detected, although the identification could not be confirmed by molecular methods. To date, *Ae. albopictus* has not been detected in La Rioja or in the studied area of Navarra.

Table 1 shows the capture methods for each species with six species that were collected biting humans: *An. plumbeus, Ae. cantans, Aedes caspius, Ae. detritus, Ae. vexans, and Cq. richiardii*. All identified species have been molecularly confirmed.

TABLE 1 | Mosquito species captured with different collection methods and vector competence for humans (34, 35, 39–42).

Species	Collection method				Vector competence (confirmed in laboratory)
	T	HB	R	D	
Anopheles algeriensis Theobald, 1903	x		x		*Plasmodium* sp.
Anopheles atroparvus Van Thiel, 1927	x		x	x	*Plasmodium* sp. WNV
Anopheles claviger s.l. (Meigen, 1804)	x		x		*Plasmodium* sp.
Anopheles plumbeus Stephens, 1828	x	x			*Plasmodium* sp. WNV
Aedes berlandi Seguy, 1921	x				-
Aedes cantans (Meigen, 1818)		x			Tahyna virus WNV
Aedes caspius (Pallas, 1771)	x	x	x		Tahyna virus WNV
Aedes detritus (Haliday, 1833)	x	x			JE virus WNV
Aedes vexans (Meigen, 1830)	x	x			EEE virus RVF virus Tahyna virus WNV
Culex hortensis Ficalbi, 1889	x			x	-
Culex impudicus Ficalbi, 1890	x		x	x	-
Culex mimeticus Noè, 1899	x				WNV
Culex modestus Ficalbi, 1889	x			x	Lednice virus Tahyna virus WNV
Culex pipiens s.l. Linnaeus, 1758	x		x	x	Sindbis virus Usutu virus JE virus SLE virus RVF virus WNV
Culex theileri Theobald, 1903	x		x	x	Sindbis virus RVF virus WNV
Culiseta annulata (Schrank, 1776)	x		x	x	WNV
Culiseta longiareolata (Macquart, 1838)	x		x	x	-
Culiseta litorea (Theobald, 1901)	x			x	-
Culiseta subochrea (Edwards, 1921)	x		x	x	-
Coquillettidia richiardii (Ficalbi, 1889)	x	x	x		WNV
Uranotaenia unguiculata Edwards, 1913	x		x	x	-

T, BG-1 Sentinel^{TM} trap; HB, Human-bait; R, Rest; D, Dipping; EEE, Easter equine encephalitis; JE, Japanese encephalitis; SLE, Sant Louis encephalitis; RVF, Rift Valley fever.

In total, we obtained 262 full length 658 bp barcodes for COI and 47 barcodes for ITS2. The neighbour joining (NJ) trees show that all specimens belonging to the same species based upon morphological characters grouped together in the tree (**Figure 2**). *Anopheles claviger* s.l. was not included in the ITS2 NJ tree because of failure of DNA amplification. In addition, for those samples that showed PCR products, the obtained sequences were too short to be included in the dataset.

Regarding blood-fed, 341 female specimens were collected. Forty per cent of the samples were caught in the BG-1 Sentinel^{TM} traps, and the remaining 60% in resting places.

The composition and the abundance of the species varied depending on the wetland (see **Table 2**). In La Grajera wetland, *Cq. richiardii* (43.7%), *Cx. pipiens* s.l. (16.9%), *An. algeriensis* (15.9%) and *An. claviger* s.l. (11.5%) were the most abundant species, whereas in Las Cañas wetland, *Ae. caspius* (47%) and *Cx. pipiens* s.l. (23%) were the main collected species. In Iregua river, *Cx. pipiens* s.l. (64.4%) and *Cx. modestus* (12.5%) were the most common species. In occasionally sampled wetlands, all but *Aedes cantans* species were the same as those found in permanently sampled sites. Phenology and ecological data have been obtained and those from 2017 in La Grajera wetland have been already published (30).

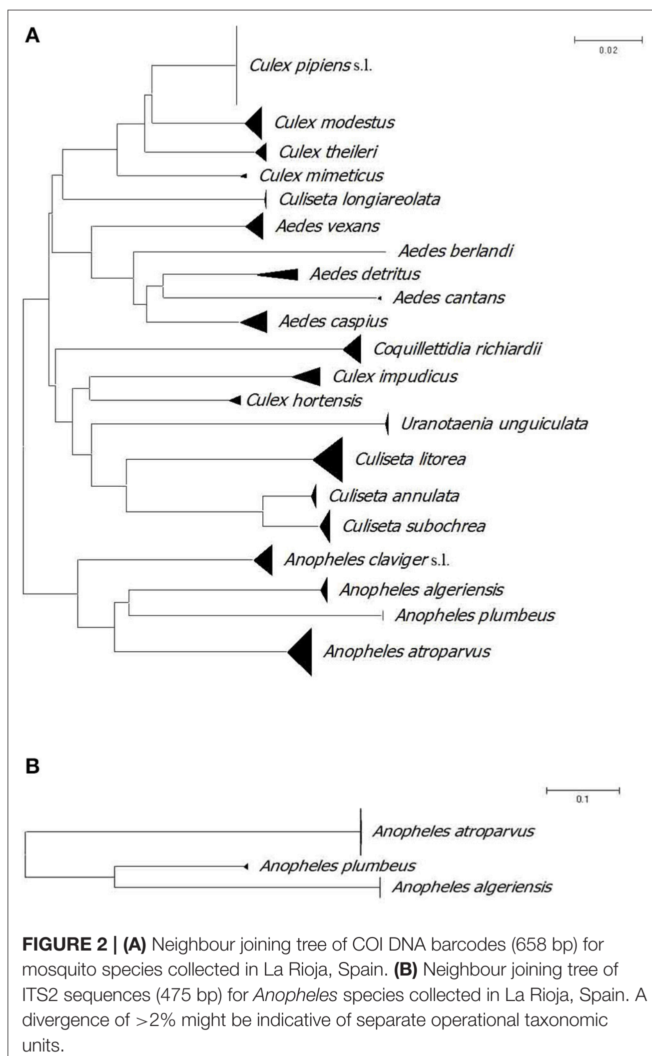

FIGURE 2 | (A) Neighbour joining tree of COI DNA barcodes (658 bp) for mosquito species collected in La Rioja, Spain. **(B)** Neighbour joining tree of ITS2 sequences (475 bp) for *Anopheles* species collected in La Rioja, Spain. A divergence of >2% might be indicative of separate operational taxonomic units.

Screening for Flaviviruses

Up to date, four pools (0.34%), three from La Grajera wetland and one from Las Cañas wetland, from *Ae. vexans* captured in 2016 and 2017, have yielded positive results for flavivirus PCR screening (**Table 3**). They showed maximum identity (97-99%) with the sequences of *Aedes vexans* flavivirus (AeveFV) group deposited in GenBank (GQ476996- GQ476998, GQ477000 and JN802280).

DISCUSSION

This is the first study performed in northern central Spain focused on the investigation of the mosquito species and their potential infections with flaviviruses. In Spain, mosquito screening for arboviruses had been previously performed in other regions, like Catalonia and Western Andalusia (47–49). The study has allowed us to identify numerous mosquito species with vector capacity as well as providing an insight into the ecology of these vectors. Sequences closely related to mosquito–only flavivirus have been detected in the analysed samples. The surveillance of mosquito's circulation is very important for the clinical practice since travellers affected by DENV, CHIKV and ZIKV have been diagnosed in the country, and because the first autochthonous cases of Dengue have been reported in Spain as well as in neighbouring countries (8).

Since *Ae. albopictus* has not been detected in the region, the risk of autochtonous transmission of arbovirus like DENV, CHIKV, and ZIKV remains very low. This fact means that the next level of action within the regional plan for surveillance of arboviruses in La Rioja should not be extended and, among other measures, epidemiological surveillance and control of the vector have not been necessary in the case of imported cases of arboviruses.

In this study, six mosquito species were found biting humans. Some species, like *Ae. caspius* or *Cq. richiardii*, are very abundant in the studied area and could act as bridge vectors for pathogens such as WNV, a virus that is endemic in Europe (41). *Anopheles plumbeus* is the only human-biting anopheline species out of four anopheline species identified herein. This finding suggests that despite its scarcity in the studied area, this species could be the responsible one for the case of autochthonous malaria by *Plasmodium vivax* that occurred in 2014 in Viana (Navarra), just a few kilometres away from Logroño (50). *Anopheles plumbeus* is considered a secondary vector of malaria in Europe, but it was implicated as potentially responsible for the transmission of *Plasmodium falciparum* in Germany (51). Nevertheless, *An. atroparvus* should not be ruled out as the causative agent. This species, which is more frequent in our area (**Table 2**), is the recognized main vector of malaria in Europe and it was involved in the transmission of the autochthonous malaria case occurred in Spain in 2010 (11). In addition, *An. atroparvus* has a wider distribution and activity range in La Rioja since their breeding sites are not restricted to water-filled holes of trees, and it can

TABLE 2 | Number of mosquitoes (per species and sex), percentage of relative abundance and distribution in the permanently sampled wetlands during 2016–2017.

Species	*n*	%	F	M	Iregua		La Grajera		Las Cañas	
An. algeriensis	617	9.3%	613	4	0	0.0%	587	15.9%	30	1.2%
An. claviger s.l.	441	6.6%	440	1	9	2.1%	427	11.5%	5	0.2%
An. maculipennis s.l.	36	0.5%	19	17	2	0.5%	6	0.2%	28	1.1%
An. plumbeus	5	0.1%	4	1	4	0.9%	0	0.0%	1	0.0%
An. spp.	33	0.5%	33	-	0	0.0%	32	0.9%	1	0.0%
Ae. berlandi	1	0.0%	1	0	0	0.0%	1	0.0%	0	0.0%
Ae. caspius	1,245	18.7%	1,245	0	6	1.4%	53	1.4%	1,186	47.0%
Ae. detritus	10	0.2%	10	0	0	0.0%	2	0.1%	8	0.3%
Ae. vexans	64	1.0%	44	20	0	0.0%	60	1.6%	4	0.2%
Ae. spp.	40	0.6%	40	-	0	0.0%	0	0.0%	40	1.6%
Cq. richiardii	1,857	27.9%	1,814	43	8	1.9%	1,616	43.7%	233	9.2%
Cx. pipiens s.l.	1,504	22.6%	1,350	154	299	69.4%	625	16.9%	580	23.0%
Cx. modestus	348	5.2%	348	0	54	12.5%	77	2.1%	217	8.6%
Cx. mimeticus	4	0.1%	1	3	3	0.7%	1	0.0%	0	0.0%
Cx. theileri	160	2.4%	158	2	2	0.5%	77	2.1%	81	3.2%
Cx. impudicus	4	0.0%	4	0	0	0.0%	4	0.2%	0	0.0%
Cx. spp.	96	1.4%	96	-	21	4.9%	33	0.9%	42	1.7%
Cs. annulata	40	0.6%	40	0	5	1.2%	27	0.7%	8	0.3%
Cs. longiareolata	29	0.4%	22	7	11	2.6%	18	0.5%	0	0.0%
Cs. litorea	30	0.5%	27	3	4	0.9%	17	0.5%	9	0.3%
Cs. subochrea	88	1.3%	88	0	3	0.7%	37	1.0%	48	1.9%
Cs. spp.	1	0.0%	1	-	0	0.0%	1	0.0%	0	0.0%
Ur. unguiculata	5	0.1%	4	1	0	0.0%	1	0.0%	4	0.2%
Total	6,658		6,369	256	431		3,702		2,525	

Aedes cantans and Cx. hortensis are not included in the table because they were identified during occasional samplings in 2018. F, Female; M, Male.

breed in a large collection of water bodies such as lagoons, irrigation channels, etc. Several studies have demonstrated that *P. vivax* is well-adapted to European populations of both *Anopheles* species (52–54). The establishment of the mosquito surveillance programme in La Rioja has contributed to increase the knowledge about the diversity, distribution abundance and ecology of species that are present in the region. These factors may determine the incidence of vector-borne pathogens in vertebrate hosts (55). In the "Big-Data era," the generation of data about the geographic distribution will be useful to recognize possible hotspots for an outbreak and then to start the implementation of preventive measures.

In order to expand the diversity of identified species, different methodologies for mosquito collection were used. Adult trapping is most commonly used to capture flying mosquitoes (31). There are species (e.g. *An. atroparvus*, *Cx. impudicus,* and *Ur. unguiculata*) that have barely been detected using the BG-1 Sentinel™ traps. This could explain their scarcity in the area. However, the capture of resting mosquitoes has shown that these species mentioned above are more abundant in the sampled wetlands than previously thought. In addition, this technique made possible the capture of numerous engorged females (31).

The molecular identification of mosquitoes proved to be a useful tool to support the morphological identification. Correct identification of mosquito vectors is critical to define pathogen transmission pathways and it is the first step for preventing arboviruses transmission. The use of two genetic markers increased our taxonomic resolution (36). This molecular approach, not only helped us to identify damaged specimens and to distinguish species within a complex, but also allowed us to detect taxonomic errors based on morphological identification alone (36). Nevertheless, we could not obtain the complete fragment ITS2 gene (species-specific for *Anopheles*) studied for *An. claviger* sibling species. Kampen et al. (56) previously described also lower ITS2 region lengths for the *An. claviger* s.l. members than for other species of *Anopheles*. Both species of the complex, *Anopheles claviger* s.s. and *Anopheles petragnani* had been previously reported for several breeding sites in La Rioja region; although their morphological identification was based on preimaginal stages (44). In our study, adult specimens from these two sibling species were morphologically indistinguishable. A deeper study on the molecular identification of this anopheline mosquito complex is required. Molecular identification of all the captured individuals is unsustainable from a cost-effective point of view. However, this tool is highly recommended in groups of species very similar each other that are difficult to identify by classical morphologic and morphometric parameters, such as *Cx. impudicus-Cx. hortensis-Cx. territans,* and *Cs. litorea-Cs. morsitans-Cs. fumipennis* in our case. This approach has also been developed in other vector surveillance programs in a number of European countries [e.g., (16, 57, 58)].

The screening for flaviviruses allowed the detection of four genomic sequences closely related to mosquito-only flavivirus group. The sequences showed the highest similarity to flavivirus amplicons of AeveFV group detected in *Ae. vexans* in Italy

TABLE 3 | Pools of unfed female specimens from each mosquito species tested up to the moment for this project.

Mosquito species	Number of tested mosquitoes	Number of tested pools	PCR results for flavivirus screening
Anopheles algeriensis	307	15	-
Anopheles claviger s.l.	36	3	-
Anopheles maculipennis s.l.	14	3	-
Anopheles plumbeus	-	-	
Aedes berlandi	-	-	
Aedes cantans	1	1	-
Aedes caspius	135	9	-
Aedes detritus	3	1	-
Aedes vexans	18	4	4
Culex hortensis	-	-	
Culex impudicus	-	-	
Culex mimeticus	1	1	-
Culex modestus	106	8	-
Culex pipiens s.l.	573	24	-
Culex theileri	94	9	-
Culex spp.	11	2	-
Culiseta annulata	3	1	-
Culiseta longiareolata	9	2	-
Culiseta litorea	6	5	-
Culiseta subochrea	26	7	-
Coquillettidia richiardii	523	21	-
Uranotaenia unguiculata	-	-	
Total	1,866	116	4

and Czech Republic (59, 60). This is the first report of this AeveFV group in mosquitoes in Spain, although it has been detected previously by another group (Ana Vázquez, personal communication). However, the length of the obtained amplicons did not allow complete phylogenetic characterization. All *Ae. vexans* pools tested from 2016 to 2017 were positive for RNA flavivirus detection, suggesting active circulation of this flavivirus in this species. Other mosquito-only flaviviruses had been previously detected in Spain in several mosquito species including *Ae. vexans* (47, 61, 62). Further analyses of these results are necessary to characterize this flavivirus. The low number of specimens screened for flaviviruses (screening is on-going) does not allow to obtain further conclusions, specially taking into account the low prevalence of pathogenic WNV and Usutu virus (USUV) found in *Cx. perexiguus* (1.5%) and *Cx. pipiens* s.l. (0.05%) in Spain (18).

The number of mosquitoes captured in this project is lower compared to other regions from Spain where mosquitoes have been monitored (18). To date, no cases of WNV and USUV have been reported in humans, equids or in birds in northern central Spain, suggesting that there is no circulation of these viruses or, at least, their prevalence is low. In addition, this study adds new species for flaviviruses screening in Spain (e.g., *Ae. cantans*, *Cs. litorea,* and *Cx. mimeticus*) and significantly increases the number of specimens of certain species such as *An. claviger* s.l. and *Cq. richiardii*.

A surveillance of emerging vector-borne infections integrating the animal-human-vector approach is costly to maintain on a long-term basis (49, 63, 64). Therefore, surveillance have to adapt to the existing reality and cost-effective use of resources at the national and regional levels (14, 18, 65). The entomological surveillance started in La Rioja represents a good approach to the diagnosis of the situation of possible arboviruses in the region and may provide insights into the change in the force of infection (66) before there is an ecological alteration that may impact on human or animal health. To implement a One Health approach, it would be interesting to complete this surveillance with the screening for flaviviruses in wild birds or other potential sentinel animal species, from wetlands of interest and to include serological testing of sentinel horses. The coordinating efforts from biologists and veterinarians (18, 65, 67) would be an added value to the ongoing efforts to be aware of medical records and reports of imported mosquito-borne arbovirus human cases in this area. This approach would enable the ecological data to be operationalised to inform human, animal and ecosystem health.

DATA AVAILABILITY

Detailed mosquito records and sequence information and can be found on BOLD within the Working Group 1.4 Initiative Human Pathogens and Zoonoses, in the project entitled "Surveillance of Mosquitoes in La Rioja, Spain [LRMQS, MLQSR, MQSLR]." The Digital Object Identifier (DOI) for the BOLD project is: dx.doi.org/10.5883/DS-MQSVLR. Generated sequences were also deposited in GenBank database under accession numbers MK402666 - MK402927 for COI and MK412721 - MK412767 for ITS2. Flavivirus sequence identified was also deposited in GenBank database under under accession number MK501751.

AUTHOR CONTRIBUTIONS

IR-A and JO designed the study. IR-A conducted the surveys and the morphological and molecular identification. IR-A and LH-T performed the molecular analysis. IR-A and PS conducted the screening for flavivirus. IR-A, BM, AP, and JO outlined the structure of the manuscript. IR-A compiled the main information and wrote the first draft of the manuscript. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2019.00086/full#supplementary-material

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