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Diversity of bartonellae in mites (Acari: Mesostigmata: Macronyssidae and Spinturnicidae) of boreal forest bats: Association of host specificity of mites and habitat selection of hosts with vector potential

Attila D. Sándor[1,2,3] | Alexandra Corduneanu[3,4] | Maria Orlova[5,6,7] | Sándor Hornok[1,2] | Alejandro Cabezas-Cruz[8] | Angélique Foucault-Simonin[8] | Joanna Kulisz[9] | Zbigniew Zając[9] | Mihai Borzan[4]

[1]HUN-REN-UVMB Climate Change: New Blood-sucking Parasites and Vector-borne Pathogens Research Group, Budapest, Hungary

[2]Department of Parasitology and Zoology, University of Veterinary Medicine, Budapest, Hungary

[3]Department of Parasitology and Parasitic Diseases, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania

[4]Department of Animal Breeding and Animal Production, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania

[5]Department of Mobilization Training of Health Care and Disaster Medicine, Tyumen State Medical University, Tyumen, Russia

[6]Department of Research and Production Laboratory of Engineering Surveys and Environmental Technologies, National Research Tomsk State University, Tomsk, Russia

[7]Laboratory of Transmissible Viral Infections and Tick-Borne Encephalitis, Federal Scientific Research Institute of Viral Infections 'Virome', Yekaterinburg, Russia

[8]ANSES, INRAE, Ecole Nationale Vétérinaire d'Alfort, UMR BIPAR, Laboratoire de Santé Animale, Maisons-Alfort, France

[9]Department of Biology and Parasitology, Medical University of Lublin, Lublin, Poland

Correspondence
Alexandra Corduneanu, Department of Parasitology and Parasitic Diseases, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania.
Email: alexandra.corduneanu@usamvcluj.ro

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Abstract

Research into various bacterial pathogens that can be transmitted between different animals and may have zoonotic potential has led to the discovery of different strains of *Bartonella* sp. in bats and their associated ectoparasites. Despite their enormous species diversity, only a few studies have focussed on the detection of bacterial pathogens in insectivorous bats of boreal forests and their associated Macronyssidae and Spinturnicidae mites. We collected and molecularly analysed mite samples from forest-dwelling bat species distributed all along the boreal belt of the Palearctic, from Central Europe to Far East. Ectoparasitic mites were pooled for DNA extraction and DNA amplification polymerase chain reaction (PCRs) were conducted to detect the presence of various bacterial (Anaplasmataceae, *Bartonella* sp., *Rickettsia* sp., *Mycoplasma* sp.) and protozoal (*Hepatozoon* sp.) pathogens. *Bartonella* sp. DNA was detected in four different mite species (Macronyssidae: *Steatonyssus periblepharus* and Spinturnicidae: *Spinturnix acuminata*, Sp. *myoti* and Sp. *mystacinus*), with different prevalences of the targeted gene (*glt*A, 16-23S ribosomal RNA intergenic spacer and *fts*Z). Larger pools (>5 samples pooled) were more likely to harbour *Bartonella* sp. DNA, than smaller ones. In addition, cave-

dwelling bat hosts and host generalist mite species are more associated with *Bartonella* spp. presence. Spinturnicidae mites may transmit several distinct *Bartonella* strains, which cluster phylogenetically close to *Bartonella* species known to cause diseases in humans and livestock. Mites with ubiquitous presence may facilitate the long-term maintenance (and even local recurrence) of *Bartonella*-infestations inside local bat populations, thus acting as continuous reservoirs for *Bartonella* spp in bats.

KEYWORDS
Chiroptera, generalist parasite, host ecology, pathogens, vector-borne disease, zoonotic

INTRODUCTION

Human intrusion into natural habitats, coupled with encroachment and frequent contacts with wildlife, poses a global risk of spillover for various zoonotic diseases (Glidden et al., 2021). As shown in several studies (Openshaw et al., 2017; Van Brussel & Holmes, 2022), mammals with regular synanthropic populations, like bats (Chiroptera), play a major role in the circulation of zoonotic pathogens. Recent disease outbreaks with significant public health implications have prompted increased research on wildlife pathogens, particularly those associated with bats (Field, 2009; Olivero et al., 2020; Zhou et al., 2020). Bats have high taxonomic diversity, being divided into 20 families, with the number of species ranging from 1–2 up to 400 per family (Dietz et al., 2009; Taylor, 2019). As other mammals (Light et al., 2010; Petersen et al., 2007; Tsai et al., 2011), bats, often host specialist or generalist ectoparasites, which on their turn are capable of maintaining and transmitting various vector-borne pathogens (Bruyndonckx et al., 2009; Dick & Patterson, 2006; McKee et al., 2018; Reeves et al., 2007; Sándor et al., 2019; Zahn & Rupp, 2004). These ectoparasites, including ticks, bat flies, bat bugs, fleas and mites, are mostly obligate haematophagous arthropods (Bruyndonckx et al., 2009; Dick & Patterson, 2006; McKee et al., 2018; Reeves et al., 2007; Sándor et al., 2019; Zahn & Rupp, 2004). Through their feeding behaviour, they can acquire and transmit various pathogens (Brook et al., 2015; Reeves et al., 2007).

While larger and more conspicuous bat ectoparasites like bat flies (Diptera: Nycteribiidae) and ticks (Acari: Ixodidae, Argasidae) have well-documented vectorial capacity for several bacterial and protozoal pathogens (Hornok et al., 2016; Hornok et al., 2019; Sándor et al., 2021; Szentiványi et al., 2023), research on the role of bat mites as vectors is limited. Bat-ectoparasitic mites (Acari: Mesostigmata) are obligate parasites that complete their entire life cycle on the host. They undergo multiple development stages, can feed several times in each life stage and may exhibit varying levels of host specificity, ranging from a single host to several host species (Christe et al., 2003; Deunff, 1977; Rudnick, 1960). These mites display high species diversity, with two groups, Spinturnicidae and Macronyssidae, commonly found in most Western Palearctic bats (Stanyukovich, 1997).

Certain groups of mesostigmatid mites are known for their vectorial role, for example, dermanyssoid mites (Dermanyssidae) are suspected to transmit several microbes causing viral, bacterial, and parasitic diseases (Moro et al., 2005). Dermanyssoid mites are typically parasitizing birds, mammals or reptiles, but sometimes also bite humans or other animals (George et al., 2015; McClain et al., 2009). Another group, the chiggers (Trombiculidae), is infamous for vectoring scrub-typhus causing *Orientia* spp. (Xu et al., 2017). Studies on pathogen-detection in bat-related acarians are rare, with a few notable exceptions being the detection of *Bartonella* spp. in mites from China (Han et al., 2002; Kuang et al., 2022), Poland (Szubert-Kruszyńska et al., 2019), Hungary (Hornok et al., 2012) and Brazil (Ikeda et al., 2021). Bartonellaceae are a group of Gram-negative, facultative intracellular bacteria, which infest mammalian erythrocytes. Several *Bartonella* species hold important epidemiologic role, causing diseases like cats-scratch disease (*B. henselae*), trench-fever (*B. quintana*) or Carrion's disease (*B. bacilliformis*), to name just a few. In addition to those *Bartonella* species for which humans are the main reservoir, there is a much larger group with zoonotic origin. The validation of bats as natural reservoirs of a *Bartonella* sp. causing disease in humans (*B. mayotimonensis*, see Veikkolainen et al., 2014) launched a surge in bat-related *Bartonella* studies. Several taxonomic groups of bats were investigated, resulting in the establishment of *Bartonella*-presence in many bat species inhabiting all continents (Corduneanu et al., 2018). Studies quickly were expanded to bat-related ectoparasites, too, as bartonellae are transmitted by blood-feeding arthropods (Stuckey et al., 2017; Tsai et al., 2011). In addition, *Borrelia* spp. (uncategorized strain and *B. afzelii*) was detected in south-eastern corner of European Russia (Zabashta et al., 2019), while *Anaplasma* spp. were recorded in several regions including Central America (US Virgin Islands, Puerto Rico and the Dominican Republic), Africa (Madagascar and Tanzania), as well southeast Asia (The Philippines). All these were detected in mites belonging to Macronyssidae and Spinturnicidae (Reeves et al., 2006). While cases of transfer of bat-specialist acarians to human hosts are rare (Mullen, & OConnor, 2019), increasingly regular contacts with bats and bat roosts in anthropic areas (Davy et al., 2023; Jackson et al., 2023; Jung & Threlfall, 2016; Lunn et al., 2023) may facilitate such transfers in the future, as demonstrated by recent cases of other exclusively bat-specialist parasites (Péter et al., 2021; Piksa et al., 2013).

Though ticks and nycteribiid flies are occasional parasites of bats, with variable prevalence and low intensity (Postawa & Furman, 2014; Sándor et al., 2019; Sándor et al., 2021), parasitic mites are extremely common (Zahn & Rupp, 2004), and may occur in high numbers on most bat hosts (Lourenço & Palmeirim, 2007; Postawa & Furman, 2014). Consequently, they may act as continuous mechanical

or biological vector for any pathogen they are able to vector, providing opportunity for the maintenance of long-term, host-pathogen cycles in bat populations (Szubert-Kruszyńska et al., 2019).

The aim of the present study was to investigate the presence of various bacterial and parasitic pathogens in mites from a wide range of bat-specific mites collected from bats inhabiting the boreal forests of Eurasia and to assess the potential role of Macronyssidae and Spinturnicidae mites in maintaining host-pathogen cycles in bat populations.

MATERIALS AND METHODS

Sample collection

The samples analysed in this work were collected from bats caught in surveys organised for biodiversity monitoring and faunistical studies. They were captured using mist nets and were morphologically identified following Dietz et al., 2009. Each captured bat underwent a thorough examination of its pelage, ears, propatagium, plagiopatagium, dactylopatagium and uropatagium to search for ectoparasites including spinturnicid and macronyssid mites, bat flies, bat fleas and bat bugs, using a multifocal LED headlamp. Only spinturnicid and macronyssid mites were targeted by this survey.

Mites were meticulously collected from each bat using forceps and placed into vials filled with 95% ethanol. Subsequently, they were transferred to new vials with 70% ethanol in the laboratory for identification and long-term preservation. After identifying and collecting ectoparasites, all captured bats were released at the study sites. All samples were stored at room temperature until morphological identification and DNA extraction.

Mites were identified using dichotomic keys, while their development stage and sex was recorded. Voucher specimens of each mite species were mounted on permanent slides with Faure-Berlese's mounting medium. They were morphologically identified by one of the authors (MO), following Rudnick (1960), Stanyukovich (1997), Radovsky (1967, 2010). Photographs were taken using a digital camera AxioCam ICc5 (Zeiss, Oberkochen, Germany) attached to a compound microscope AxioImager A2 (Zeiss, Oberkochen, Germany). Slide-mounted voucher specimens were deposited in the collection of the Museum of medical zoology (Tyumen State Medical University, Tyumen, Russia).

MOLECULAR DETECTION OF PATHOGENS

DNA extraction

For DNA extraction, mites belonging to the same species, collected from a single site and host were pooled. Each mite from the pool was cut in half with a sterile surgical blade, then immersed in proteinase K and the initial buffer solution. The mixture was incubated overnight at 56°C to ensure complete lysis. DNA extraction from the mites was performed using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, Spain). The extracted DNA was stored at −20°C until further analysis.

DNA amplification PCRs

We targeted pathogen groups that are vector-borne and are regularly occurring in bats. These were Anaplasmatacee (Ikeda et al., 2021), *Bartonella* spp. (Corduneanu et al., 2018), *Rickettsia* spp. (Matei et al., 2021), *Mycoplasma* spp. (Fritschi et al., 2020) and the apicomplexan *Hepatozoon* spp. (Perles et al., 2020). DNA amplification PCRs were conducted to detect the presence of pathogens employing the primers and protocols outlined in Table 1. Each PCR reaction was carried out in a 25-μL mixture composed of 6.5 μL H_2O (for Anaplasmataceae [cPCR], *Bartonella* sp., *Rickettsia* sp., *Mycoplasma* sp.—first PCR) or 9.5 μL H_2O (for *Hepatozoon* sp.), 1 μL each of forward and reverse primers, 12.5 μL Master Mix (My Red Taq Mix, Meridian Bioscience, London, UK) and 4 μL DNA (for Anaplasmataceae, *Bartonella* sp., *Rickettsia* sp., *Mycoplasma* sp.—first PCR) or 1 μL DNA (for *Hepatozoon* sp.) and 1 μL aliquot for the second PCR for *Mycoplasma* sp.

For quality control, two samples of nuclease-free water served as negative controls (one at the beginning and one at the end) for each PCR assay. Additionally, one positive control for each pathogen was used, sourced from different samples and confirmed by sequencing. These include Anaplasmataceae (blood of *Canis familiaris* from Romania—Matei et al., 2018), *Bartonella* sp. (*B. henselae* blood of *Felis catus* from Israel—Corduneanu et al., 2018), *Hepatozoon* sp. (isolated from *Felis silvestris*, Romania—Panait et al., 2023), *Rickettsia* sp. (tick-derived *Rickettsia africae* from Benin—Matei et al., 2021), and *Mycoplasma* sp. (isolated from blood of *Bison bison*—Corduneanu et al., 2023). The resulting PCR products were visualised by electrophoresis with a 1.5% agarose gel. Positive samples, exhibiting the expected amplicon size for each gene fragment, were purified using the Gel/PCR DNA fragment kit (Geneaid, Taipei, Taiwan), and sent to Macrogen (Macrogen Europe, Amsterdam, The Netherlands) for sequencing.

Obtained sequences of targeted gene fragments were deposited in the NCBI GenBank database with the following accession numbers: *glt*A gene, 800 bp (Supporting Information File S1), OR813951.1 and OR813952.1 (16-23S rRNA ITS, 600 bp) and OR885847–OR885854 (*fts*Z gene, 700 bp).

Community analysis

To evaluate the possible links between pathogens, mites and vertebrate hosts as well their epidemiologic risk posed, we grouped our samples into ecological groups, according to the roost type (*cave-dwellers*—bat species roosting underground vs. *crevice-dwellers*—bats roosting in rock crevices/tree holes) and capture location in relation to human inhabitations (*urban*—inside human habitations or less than 1 km from these, vs. *natural*—any location more than 1 km from human settlements, see Table S1 for further details). To compare

TABLE 1 Oligonucleotide sequences and cycle parameters of PCR-s targeting pathogen identification.

PCR type	Target taxa	Gene targeted	Primers (5′–3′)	Initial denaturation	Cycle denaturation	Cycle annealing	Cycle extension	Final extension	Cycle (*n*)
cPCR	Anaplasmataceae	16S rRNA (Parola et al., 2000)	EHR16SD (3′-GGTACCYACAGAAGAAGTCC-5′) EHR16SR (3′-TAGCACTCATCGTTTACAGC-5′)	95°C, 5 m	94°C, 30 s	45°C, 30 s	72°C, 30s	72°C, 5 m	35
nPCR	*Bartonella* spp.	*glt*A (Birtles & Raoult, 1996)	CS443f (3′-GCTATGTCTGCATTCTATCA-5′) CS1210r (3′-GATCYTCAATCATTTCTTTCCA-5′)	95°C, 2 m	95°C, 30 s	48°C, 30 s	72°C, 2 m	72°C, 7 m	40
			BhCS781.p (3′-GGGGACCCAGCTCATGGTGG-5′) BhCS1137.n (3′-AATGCAAAAAGAACAGTAAACA-5′)	95°C, 3 m	95°C, 30 s	55°C, 30 s	72°C, 30s	72°C, 7 m	40
cPCR	*Bartonella* spp.	16-23S ribosomal RNA intergenic spacer (600 bp) (de Paiva Diniz et al., 2007)	325s (3′-CTTCAGATGATGATCCCAAGCCTTCTGGCG-5′) 1100as (3′-GAACCGACGACCCCCTGCTTGCAAAGA-5′)	95°C, 3 m	95°C, 30s	66°C, 30 s	72°C, 30s	72°C, 5 m	55
nPCR	*Bartonella* spp.	*fts*Z (Colborn et al., 2010; Zeaiter et al., 2002)	Bfp1 (3′-ATTAATCTGCAYCGGCCAGA-5′) Bfp2 (3′-ACVGADACACGAATAACACC- 5′)	95°C, 4 m	95°C, 30 s	55°C, 30 s	72°C, 1 m	72°C, 10 m	40
			ftsZR83 (3′-ATATCGCGGAATTGAAGCC-5′) ftsZL83(3′-CGCATAGAAGTATCATCCA-5′)	95°C, 4 m	95°C, 30 s	55°C, 30 s	72°C, 1 m	72°C, 10 m	40
cPCR	*Hepatozoon* spp.	18S rRNA (Hrazdilová et al., 2021)	EF_M (3′-AAAACTGCAAATGGCTCATT-5′) Hep1615R (3′-AAAGGGCAGGGACGTAATC-5′)	95°C, 1 m	95°C, 15 s	55°C, 15 s	72°C, 30 s	72°C, 5 m	35
cPCR	Rickettsiaceae	*glt*A (Regnery et al., 1991)	RpCS.877p (3′-GGGGGCCTGCTCACGGCGG-5′) RpCS.1258n (3′-ATTGCAAAAAGTACAGTGAACA-5′)	95°C, 2 m	95°C, 30 s	58°C, 30 s	72°C, 30s	72°C, 2 m	35
nPCR	*Mycoplasma* spp.	16S rRNA (Kaewmongkol et al., 2017)	Myco 184-F1 (3′-ACCAAGSCRATGATRGRTAGCTGG) Myco 1310-R1 (3′-ACRGGATTACTAGTGATTCCAACTTCAA-5′)	95°C, 5 m	95°C, 45 s	55°C, 45 s	72°C, 90 s	72°C, 5 m	35
			Myco 322-F2 (3′-GCCCATATTCCTACGGGAAGCAGCAGT-5′) Myco 538-R2 (3′-CTCCACCACTTGTTGTTCAGGTCCCCGTC-5′)	95°C, 5 m	95°C, 45 s	60°C, 45 s	72°C, 45 s	72°C, 5 m	35

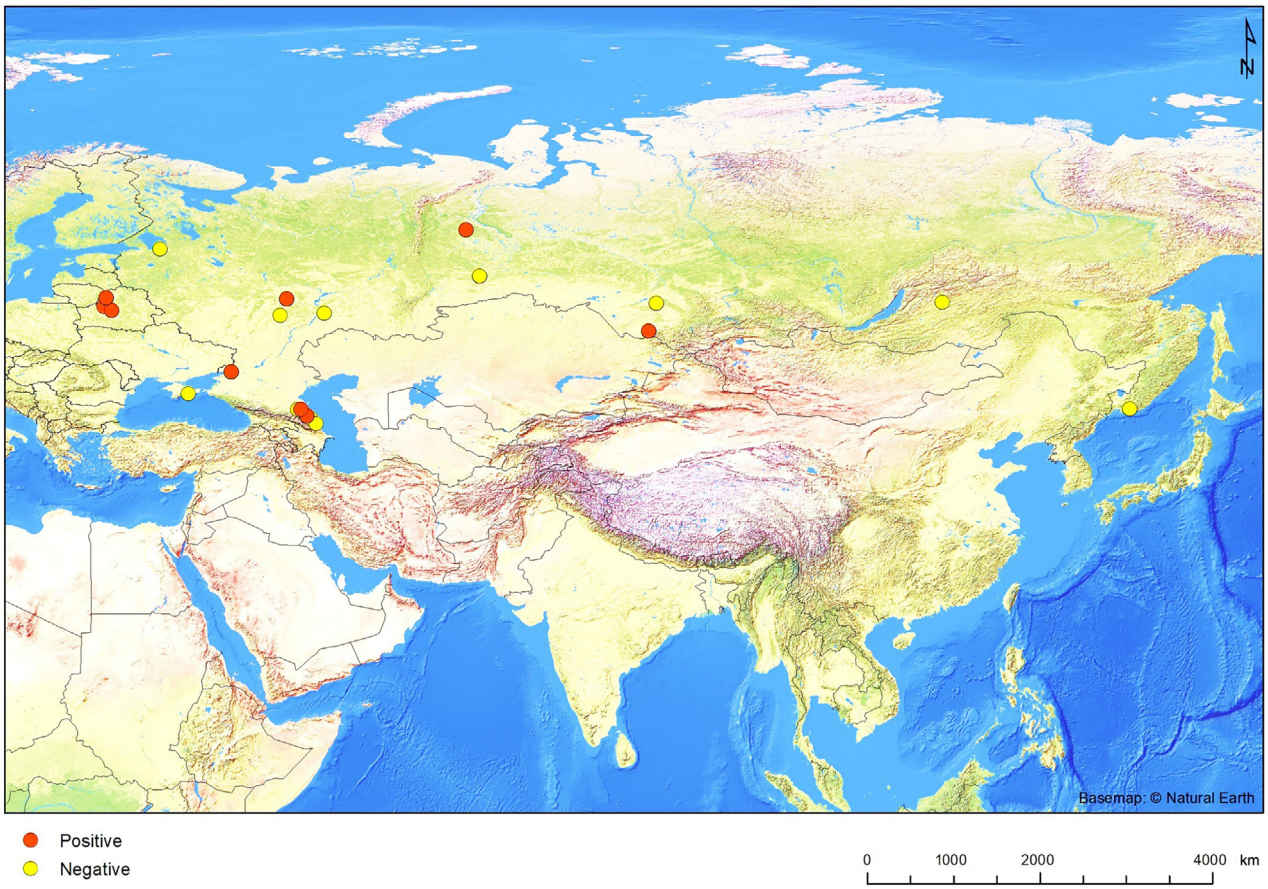

FIGURE 1 Map with the distribution of collecting sites of Mesostigmatid mites (Acari: Meostigmata: Macronyssidae, Spinturnicidae).

pathogen prevalence rates, vector and host categories we used Chi-square or Fisher's exact tests. Differences were considered significant when $p < 0.05$.

In order to determine the prevalence of *Bartonella* in mites collected from bats, estimated pooled prevalence method was used with option for variable pool size and perfect tests (available at https://epitools.ausvet.com.au/, see also Sergeant, 2018). The prevalence of *Bartonella* in mites was calculated for individual mite-bat 'pairs' found in particular study sites with a 95% confidence interval.

Phylogenetic analysis

Sequences obtained in the current study were individually compared to voucher sequences using Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) to assess their specific status. Next, the GenBank database (https://www.ncbi.nlm.nih.gov) was searched for sequences of target gene that have been previously identified. Collected sequences were then independently aligned using MAFFT (https://www.mafft.cbrc.jp) configured for the maximum accuracy. Redundant ones were excluded from further analysis. The best-fit models of *glt*A, *fts*Z, and 16S-23S rRNA ITS sequences were selected based on the lowest values of corrected Akaike information criterion (cAIC) and Bayesian information criterion (BIC), as implemented in MEGA X (Kumar et al., 2018). The Tamura 3-parameter (T92) showed the lowest cAIC and BIC values in case of *glt*A and *fts*Z, while Kimura 2-parameter (K2) in case of 16S-23S rRNA ITS gene. Selected models were applied for phylogenetic tree reconstruction. The phylograms were built using the maximum likelihood with option 'complete deletion' (i.e., removal of unaligned positions). Reliability of internal branches was assessed using the bootstrapping method (1000 bootstrap replicates) as implemented in MEGA X.

RESULTS

Numbers and details of captured bats and ectoparasites

Bat ectoparasites used in this study were collected from bats sampled at 21 different locations across the whole Palearctic, from Eastern Europe (Narochanskiy National Park, Western Belarus) to the Far East region of Asia (Primorski Velikan cave, Primorskiy District, Russia), during 2017–2021 (Figure 1).

TABLE 2 Mesostigmatid mites (Acari: Meostigmata: Macronyssidae, Spinturnicidae) collected in this study (with host species) and estimated pooled prevalence of *Bartonella* in positive pools (EPP—estimated pooled prevalence with 95% confidence interval).

Mite species	Host species	Sampling location	Number of pools	Number of positive pools	EPP (%)
Spinturnix kolenatii	*Eptesicus serotinus*	Khasavyurt vicinity, Republic of Dagestan, Russian Federation	1	0	0.00
		Samurskiy Sanctuary, Republic of Dagestan, Russian Federation	1	0	0.00
		Kayakentskiy district, Republic of Dagestan, Russian Federation	1	0	0.00
Spinturnix maedai	*Murina hilgendorfi*	Barsukovskaya cave, Novosibirsk Oblast, Russian Federation	1	0	0.00
Spinturnix myoti	*Myotis blythii*	Letuchikh Myshey Cave, Altai Republic, Russian Federation	4	2	1.67
		Karabudakhkentskaya Cave, Republic of Dagestan, Russian Federation	8	5	7.06
Macronyssus granulosus		Karadag Natur Reserve, Crimea, Ukraine	1	0	0.00
Spinturnix mystacinus	*Myotis brandtii*	Malaya Sos'va Nature Reserve, Khanty-Mansi Autonomous Okrug, Russian Federation	2	1	29.29
		Penza Oblast, Russian Federation	1	0	0.00
Macronyssus corethroproctus	*Myotis dasycneme*	Staraya Ladoga gallery, Leningrad Oblast, Russian Federation	1	0	0.00
Spinturnix myoti		Samarskaya Luka Natur Reserve, Samara Oblast, Russian Federation	1	0	0.00
		Staraya Ladoga gallery, Sanktpetersbur O., Russian Federation	1	0	0.00
Spinturnix myoti	*Myotis daubentonii*	Starinky, Dzerghinskiy district, Republic of Belarus	2	1	10.81
		Nalibokskiy Reserve, Republic of Belarus	1	1	99.95
		Narochanskiy Park, Republic of Belarus	4	3	20.84
		Samurskiy Sanctuary, Republic of Dagestan, Russian Federation	2	0	0.00
		Smol'niy National Park, Republic of Mordovia, Russian Federation	6	4	8.00
Spinturnix uchikawai	*Myotis macrodactylus*	Primorskiy Velikan Cave, Primorsky Krai, Russian Federation	2	0	0.00
Macronyssus charusnurensis	*Myotis petax*	Barsukovskaya cave, Novosibirsk Oblast, Russian Federation	1	0	0.00
Spinturnix myoti		Barsukovskaya cave, Novosibirsk Oblast, Russian Federation	1	0	0.00
Spinturnix mystacinus	*Myotis sibiricus*	Dolganskaya Yama cave, Buryatia, Russian Federation	1	0	0.00
Macronyssus flavus	*Nyctalus noctula*	Shaytan Kazak Lake, Republic of Dagestan, Russian Federation	1	0	0.00
		Rostov on Don, Rostov Oblast, Russian Federation	3	0	0.00
Spinturnix acuminata		Shaytan Kazak Lake, Republic of Dagestan, Russian Federation	1	1	99.95
		Smol'niy National Park, Republic of Mordovia, Russian Federation	1	0	0.00
		Rostov on Don, Rostov Oblast, Russian Federation	3	1	25.00
		Samarskaya Luka Natur Reserve, Samara Oblast, Russian Federation	1	0	0.00
Steatonyssus noctulus		Starinky, Dzerghinskiy district, Republic of Belarus	1	0	0.00
		Rostov on Don, Rostov Oblast, Russian Federation	3	0	0.00
Steatonyssus periblepharus	*Pipistrellus nathusii*	Narochanskiy Park, Republic of Belarus	2	2	94.33
Steatonyssus periblepharus	*Pipistrellus pygmaeus*	Rostov on Don, Rostov Oblast, Russian Federation	1	1	100.00
Steatonyssus spinosus	*Vespertilio murinus*	Tyumen, Tyumen Oblast, Russian Federation	5	0	0.00

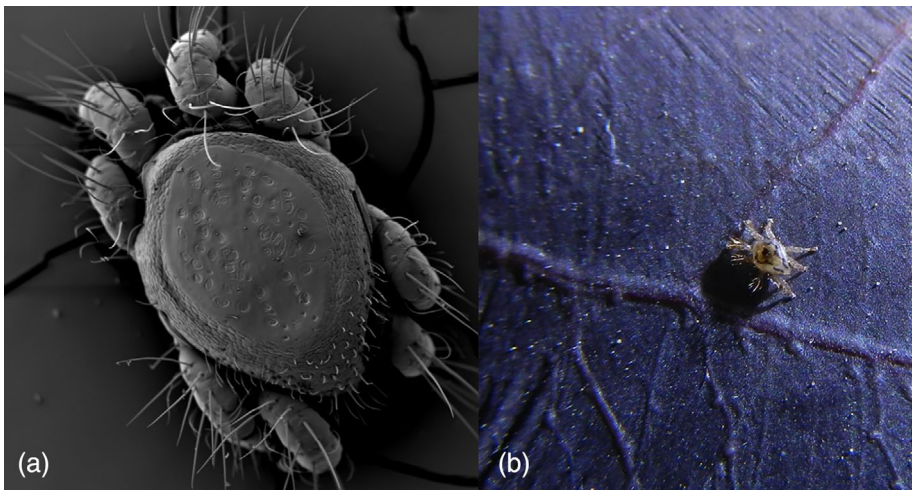

FIGURE 2 Images of bat-associated mites. (a) SEM image of *Spinturnix uchikawai* Orlova, Zhigalin, Zhigalina, 2015, adult female collected on *Myotis macrodatylus* Temminck, 1840, Primorskiy Velikan Cave, Primorsky Krai, Russian Federation, 3 April 2018; (b) Adult female *Spinturnix acuminatus* (C.L. Koch, 1836), on wing membrane of *Nyctalus noctula* (Schreber, 1774), Shaytan-Kazak Lake, Republic of Dagestan, Russian Federation, 19 May 2019.

These mites were collected from 13 bat species, all belonging to Vespertilionidae family (see Table S1 for all collection details). Altogether 535 individual mites were collected and grouped into 65 pools, according to mite species, host species and collection location (Table 2 and Figure 2). On average, each pool contained 8.23 mites, ranging from 1 to 55 individuals per pool.

PCR results of pathogen screening

We were unable to detect any DNA from Anaplasmataceae, *Rickettsia* sp., *Mycoplasma* sp. or *Hepatozoon* sp. in the DNA pools generated from mites. The only identified pathogen in the mite samples was *Bartonella*.

Of the 65 pools, 22 pools (32%) tested positive for *Bartonella* spp. Four different mite species (Macronyssidae: *Steatonyssus periblepharus* and Spinturnicidae: *Spinturnix acuminata*, Sp. *myoti* and Sp. *mystacinus*) harboured *Bartonella* DNA. The prevalence of *Bartonella* varied among mite species, reaching a maximum of 100% in case of *S. periblepharus* collected from *P. pygmaeus* (Table 2). Pools of Sp. *myoti* were positive for all three target genes (*glt*A, 16-23S ribosomal RNA intergenic spacer and *fts*Z) of these specific bacteria, while Sp. *acuminatus*, Sp. *mystacinus* and *St. periblepharus* were positive by targeting the *fts*Z gene only. *Bartonella* spp. occurrence was more likely in larger pools, with significantly more pools containing >5 mites being positive than the ones with less than five mites ($\chi^2 = 9.51$, $p < 0.01$). Samples of mite species with a generalist host selection (Sp. *myoti* and *St. periblepharus*) had significantly higher *Bartonella* prevalence than samples of the more host-specific mite species ($\chi^2 = 19.57$, $p < 0.001$). A higher proportion of cave-dweller bats harboured *Bartonella*-infected mites, than bats that inhabit crevices ($\chi^2 = 13.87$, $p < 0.001$), while we found no difference in *Bartonella* spp. prevalence between the two types of capture habitats.

Genetic analysis of *Bartonella*

The *Bartonella* sequences obtained were compared with those from the NCBI GenBank database for each individual gene of *Bartonella* spp. Our unique mite-derived *glt*A sequence was nearly identical (99% identity) with uncultured *Bartonella* spp. from blood of *Myotis blythii*, while also clustered together with different sequences of *B. hemsundetensis* isolated from bats and their ectoparasites (KR822802.1—from bat's blood, Finland, Lilley et al. 2015, MH578453.1—from a bat tick, Romania, Hornok et al., 2019, see also Figure 3b). Sequences targeting *fts*Z gene clustered together with other bat- or bat-parasite-derived sequences reported from various geographical regions and formed three distinct clades of *Bartonella* spp. (Figure 3c). Among these, Clade I clustered together with several samples of *B. vinsonii* from Chile, France, and the USA, while Clade III clustered together with *B. tribocorum* from China and France. Samples amplified on 16S-23S rRNA ITS gene phylogenetic analysis did not show high similarity with any GenBank deposited sequence, but clustered together with *B. henselae* from Australia, Brazil, and North Korea (Figure 3a). Thus, all analysed sequences were recognised as *Bartonella* spp.

DISCUSSION

Our study analysed mites sampled from a wide geographic range, chiefly covering the boreal forested belt of the whole Palearctic realm (24° to 165° latitudes, ca. 7500 km W to E), targeting the mite species collected on 13 mostly forest-inhabiting bat species. Our results showed the presence of *Bartonella* DNA in 32% of all pools of individual mite species, with likely infection in four different mite species (first-ever records in Sp. *acuminata*, Sp. *mystacinus* and *St. periblepharus*) collected from six different host species. This prevalence is

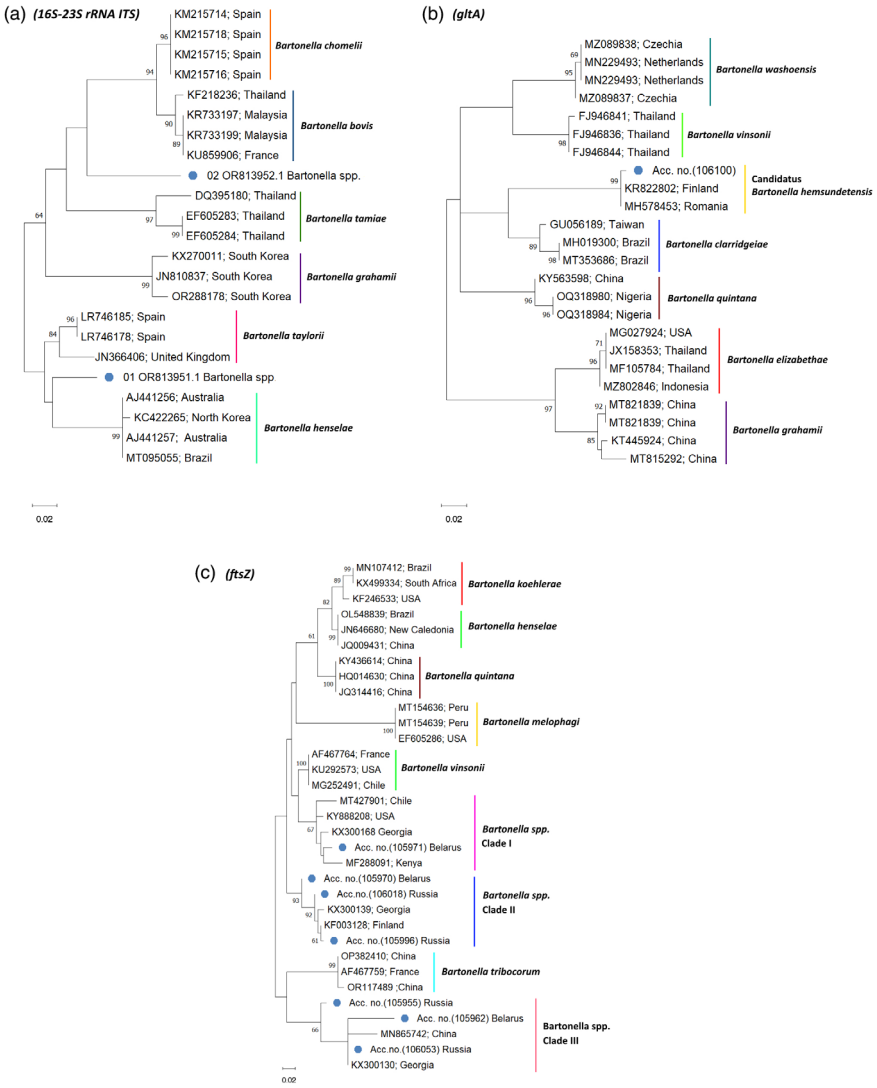

FIGURE 3 Phylogenetic relationships of the detected *Bartonella* sequences. (a) shows position of the sequences obtained in the current study targeted at 16S-23S rRNA ITS gene (marked blue) and other previously reported sequences available in GenBank (accession numbers and country of origin are displayed). The phylogenetic tree was inferred using the maximum likelihood method and the Kimura 2-parameter evolutionary model with a discrete Gamma distribution (K2 + G) (5 categories; parameter = 0.4147). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 23 nucleotide sequences with a total of 152 positions in the final dataset. (b) shows position of the sequences obtained in the current study targeted at *glt*A gene (marked blue) and other previously reported sequences available in GenBank (accession numbers and country of origin are displayed). The phylogenetic tree was inferred using the maximum likelihood method and the Tamura 3-parameter evolutionary model with a discrete Gamma distribution (T92 + G) (5 categories, parameter = 0.3117). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 24 nucleotide sequences with a total of 218 positions in the final dataset. (c) shows position of the sequences obtained in the current study targeted at *fts*Z gene (marked blue) and other previously reported sequences available in GenBank (accession numbers and country of origin are displayed). The phylogenetic tree was inferred using the maximum likelihood method and the Tamura 3-parameter evolutionary model with a discrete Gamma distribution and invariable sites (T92 + G + I) (5 categories, G parameter = 0.3117; *I* = 45.67% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 33 nucleotide sequences with a total of 233 positions in the final dataset. Each analysis was conducted with the use of complete deletion option. In each phylograms bootstrap values are represented as percent of internal branches (1000 replicates), only values ≥60% are shown.

also much higher than any previous mite-related analysis, overpassing by far all the European records (i.e., from 5% to 28.4%, see Hornok et al., 2012; McKee et al., 2019; Szubert-Kruszyńska et al., 2019). Our results show that *Bartonella* presence in bat-infesting mites is a general phenomenon among mites infesting boreal forest bats and has a wide geographical distribution (Figure 1).

In the temperate region of the Palearctic realm, studies suggested several arthropod groups as potential *Bartonella*-vectors among bat ectoparasites. Flightless dipterans (Nycteribiidae and Streblidae) were identified in most cases (Brook et al., 2015; Han et al., 2002; McKee et al., 2019; Sándor et al., 2018; Stuckey et al., 2017), with suggested instances of ticks (both Ixodidae and Argasidae: Hornok et al., 2012;

Hornok et al., 2019; McKee et al., 2019), and also records of fleas (Hornok et al. 2012) and cimicid bugs (Kejíková et al., 2022; McKee et al., 2018, 2019). Studies targeting mesostigmatid mites are only a handful worldwide, with mixed results reported. *Bartonella* DNA was identified in Sp. *myoti* pools in Hungary (Hornok et al. 2012), and in Poland (Szubert-Kruszyńska et al., 2019). A more targeted sampling of bat-related mites collected in Belgium and in the Netherlands identified *Bartonella* DNA in three Spinturnicidae species (Sp. *andegavinus*, Sp. *kolenati* and Sp. *plecotina*) and in pools of unidentified macronyssids and spinturnicids (McKee et al., 2019). There are two studies of mixed pools of unidentified spinturnicid mites in the Carribean (St Kitts and Navis, Reeves et al., 2016) and China (Han et al., 2002). A common characteristic of all these studies is the highly localised sampling (usually single or very few sites) and the narrow taxonomic focus both in bat host, as well mite species numbers (usually single or a few host/mite species).

Host generalist mite species showed a significantly higher prevalence, suggesting a higher vectorial capacity for these mites (Erazo et al., 2019; Graham et al., 2009; McCoy et al., 2013; McKee et al., 2016). Mite species collected from hosts inhabiting underground roosts were more prone to infection, than mites of crevice dweller hosts. This may be caused by the fact that cave-dweller bats constantly roost in stable, larger clusters (possibility for more frequent transfer for mites among individual hosts and more chances for being infected by *Bartonella* spp.), in contrast to crevice-dwellers roosting individually or in ever-changing small groups (Dietz et al., 2009).

Phylogenetic analyses of our positive samples showed that most sequences are well embedded into other bat derived *Bartonella* groups. Our samples showed high identity scores primarily with bat- and bat fly derived *Bartonella* sequences, from Europe, Asia, Africa and Central and South America (Figure 3b,c). They also clustered together with small mammal- (e.g., *B. henselae*) or ungulate-derived sequences (e.g., *B. bovis*, *B. chomelii*) (see Figure 3a). Analysis of *ft*ZA sequences showed an interesting clustering, with three well-separated groups, well embedded in the general *Bartonella*-tree, likely suggesting the presence of three different bat-specialist, yet undescribed *Bartonella* species. This amazing diversity should not surprise us, as former surveys already established high genetic diversity among bat ectoparasite-derived *Bartonella* sequences (McKee et al., 2019; Stuckey et al., 2017), even at small geographical scales (Sándor et al., 2018). Moreover, *Bartonella* spp. shows intimate relationships with bats all over the world (Corduneanu et al., 2018). Recently it was suggested that early evolution of *Bartonella* spp. is closely linked to Chiroptera in general and New World-inhibiting Phyllostomatidae in particular (McKee et al., 2021).

Bats (and their associated ectoparasites) may harbour *Bartonella* spp. of zoonotic concern, as it was already proven (Bai et al., 2018; Lin et al., 2010; Veikkolainen et al., 2014) and the increasing frequency of human-bat encounters (Davy et al., 2023; Jackson et al., 2023; Lunn et al., 2023) may facilitate the transfer of such pathogens between bats/ectoparasites and humans. While records of bat-specialist acarians on humans are rare (Mullen, & OConnor, 2019), these mites may pose a distant, but also long-term risk. With their ubiquitous presence and large numbers on most individual hosts (Lourenço & Palmeirim, 2007; Ter Hofstede & Fenton, 2005; Zahn & Rupp, 2004), these arthropods may facilitate the long-term maintenance (and even local recurrence) of such *Bartonella*-infestations inside local bat populations, thus acting as continuous reservoirs for *Bartonella* spp.

CONCLUSIONS

Palearctic forest bats are infested by blood-feeding Macronyssidae and Spinturnicidae mites, regularly harbouring *Bartonella* spp. DNA. Cave-dwelling bat hosts and host generalist mite species are more associated with *Bartonella* spp. presence. Spinturnicidae mites may transmit several distinct *Bartonella* strains, which cluster phylogenetically close to *Bartonella* species known to cause diseases in humans and livestock.

AUTHOR CONTRIBUTIONS

Attila D. Sándor: Conceptualization; writing – original draft; writing – review and editing; resources; data curation; validation; supervision; funding acquisition; project administration. **Alexandra Corduneanu:** Conceptualization; methodology; investigation; writing – original draft; writing – review and editing; software; validation; project administration. **Maria Orlova:** Writing – review and editing; validation; visualization. **Sándor Hornok:** Conceptualization; writing – review and editing; validation; supervision; visualization. **Alejandro CABEZAS-CRUZ:** Writing – review and editing; validation; supervision; visualization. **Angélique FOUCAULT-SIMONIN:** Formal analysis. **Joanna Kulisz:** Writing – review and editing; formal analysis. **Zbigniew Zając:** Writing – review and editing; formal analysis. **Mihai Borzan:** Formal analysis; visualization.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets supporting this paper are included within the paper and its supplementary material.

ETHICS STATEMENT

All applicable international, national and institutional guidelines for the care and use of animals were followed.

ORCID

Alexandra Corduneanu https://orcid.org/0000-0002-6682-1827
Maria Orlova https://orcid.org/0000-0001-7811-4364

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

File S1. Details of pools, with mite species, bat host species, collection location, date of sampling, type of roost, siteing of roost and Bartonella DNA presence, with the indication of sequence type and Acccession number.

Table S1. Collection details of the samples used in this study including sampling location, host species, host roost-type, mite species and number of mite individuals in the sample/pool.

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