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Sharing roosts but not ectoparasites: high host-specificity in bat flies and wing mites of *Miniopterus schreibersii* and *Rhinolophus ferrumequinum* (Mammalia: Chiroptera)

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Abstract

Schreiber's bent-winged bat *Miniopterus schreibersii* and the greater horseshoe bat *Rhinolophus ferrumequinum* are widespread and common cavernicolous species across southern Europe that host numerous specialized ectoparasite species. The objective of this study was to characterize the species assemblage, genetic diversity, and host specificity of bat flies (Nycteribiidae, Diptera) and wing mites (Spinturnicidae, Acari) found on these bat hosts in Serbia and Bosnia and Herzegovina. Notably, while bat flies lay puparia on the cave walls and can thus be transmitted indirectly, wing mites require direct body contact for transmission. Morphological identification and sequencing of a 710-bp fragment of cytochrome oxidase I gene of 207 bat flies yielded 4 species, 3 on *M. schreibersii* and 1 on *R. ferrumequinum*. Sequencing of a 460-bp small subunit ribosomal RNA fragment, in all 190 collected wing mites revealed 2 species, 1 per host. In no case was a parasite associated with 1 host found on the other host. Species and genetic diversity of flies were higher in *M. schreibersii*, likely reflecting their host's larger colony sizes and migratory potential. Mite species of both hosts showed similarly low diversity, likely due to their faster life history and lower winter survival. Our findings highlight a remarkably high host-specificity and segregation of ectoparasite species despite direct contact among their hosts in the roost, suggesting a defined host preference in the investigated ectoparasite species. Furthermore, the differences in ectoparasite genetic diversity exemplify the interplay between host and parasite life histories in shaping parasite population genetic structure.

Key words: barcoding, bats, mtDNA, Nycteribiidae, parasite, Spinturnicidae.

Parasite transmission and host–parasite evolutionary dynamics are shaped by the biotic and abiotic environment (Sorci and Garnier 2018), life histories of involved species (Dick and Patterson 2007; Barrett et al. 2009), as well as the host's social system. When multiple host species are in close contact, that can lead to spillover and homogenization of parasite assemblages (Dick and Patterson 2007;

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Fagundes et al. 2017), which can have significant consequences for the host's health (Melaun et al. 2014), considering that parasites are often vectors of pathogens. Spatial and temporal co-occurrence of different bat species in the same shelter promotes interactions between them and increases the probability of exchanging parasites (Dittmar et al. 2006; Dick et al. 2009). Host characteristics such as abundance, roosting ecology, and social organization can be expected to affect parasite diversity, population size, transmission dynamics, and host specialization (Patterson et al. 2007; Presley 2011). Here, we investigate species diversity, host specificity, and genetic diversity of 2 obligate ectoparasite groups in 2 bat species that often roost together, sometimes even in mixed clusters.

One host species, Schreiber's bent-winged bat *Miniopterus schreibersii* (Kuhl, 1817) forms large, densely packed colonies of up to 70,000 animals (Dietz and Kiefer 2016; Gazaryan et al. 2020) and regularly travels 40–100 km between summer and winter underground roosts (Hutterer et al. 2005; Rodrigues et al. 2010). Migratory movements of several hundred kilometers are occasionally recorded in this bat species (Ramos Pereira et al. 2009). As in most temperate bat species, natal philopatry is pronounced in females, while gene flow is male-mediated (Moussy et al. 2012). No genetic structuring among populations was found in the entire range of *M. schreibersii*, suggesting that dispersing males may cover large distances, and consequently vector parasites across wide geographic ranges (Rodrigues and Palmeirim 2008; Rodrigues et al. 2010; Bilgin et al. 2016; Gürün et al. 2019; Wright et al. 2020).

The second host species, the greater horseshoe bat *Rhinolophus ferrumequinum* (Schreber 1774) in southern Europe primarily uses caves as roosts throughout the year, colonies are less densely clustered, with less physical contact between conspecifics, and maximally consist of a few thousand animals (Dietz and Kiefer 2016). It is considered a sedentary species, only moving 10–60 km between summer and winter roosts (Hutterer et al. 2005). Females of *R. ferrumequinum* exhibit strong natal philopatry to their maternity roost over many years (Rossiter et al. 2002), and dispersal is male-biased (Jang et al. 2021). Taken together, these characteristics likely lead to lower parasite transmission and stronger sub-structuring of *R. ferrumequinum* populations. Notably, these 2 cavernicolous bat species are often found sharing roosts in Serbia, sometimes with individuals in close physical proximity or even in mixed clusters (Figure 1). Differences in their mobility, as well as differences in group sizes and spacing of individuals within roosts, can serve as predictors of parasite infestation (Webber and Willis 2016; Patterson and Ruckstuhl 2021).

Bats are hosts to several types of blood-feeding ectoparasites, including bat flies (Nycteribiidae) and wing-mites (Spinturnicidae). Both groups live obligately on the bat host and cannot spread between roost sites on their own, but differ in several key phenological and life-history traits (van Schaik et al. 2015). Nycteribiidae (Diptera, Insecta) are wingless flies, living on bats' pelage and feeding on blood (Hutson 1984; Dittmar et al. 2015). Female flies leave the host and deposit a third instar larva, which immediately pupates on the roost wall (Patterson et al. 2007). Each decoupling from the host hypothetically provides an opportunity to colonize a different individual or host species if present (Reckardt and Kerth 2009; Szentiványi et al. 2017). Despite that, many bat flies species are remarkably host-specific and have only 1 or 2 main hosts (Dick and Patterson 2007; Lourenço and Palmeirim 2008; Seneviratne et al. 2009), and numerous incidental hosts (Szentiványi et al. 2016; Burazerović et al. 2018).

Wing mites from the Spinturnicidae family (Acari, Mesostigmata) live on the bat patagium surfaces (Rudnick 1961) and contrary to the bat flies, never leave their host. Larval development happens internally and females give birth to live offspring, not needing to decouple from the host at any point (Giorgi et al. 2004). A comprehensive study on the co-phylogeny of European bat species and their mites (Bruyndonckx et al. 2009a) confirms that "relatively high, but not strict" host specificity exists in Spinturnicid mites in western Europe.

The distribution, diversity, and primary host–parasite associations of European bat ectoparasites are comparatively well documented in several checklists (Baker and Craven 2003; Szentiványi et al. 2016). In Europe, *M. schreibersii* is the main host for 2 fly species: *Nycteribia schmidlii* Schiner, 1853 and *Penicillidia conspicua* Speiser, 1901, and *R. ferrumequinum*, together with other European horseshoe bats, is the primary host of the fly species *Phthiridium biarticulatum* Hermann, 1804 (Szentiványi et al. 2016). For the mites, *M. schreibersii* is the primary host of *Spinturnix psi*, and *R. ferrumequinum*, along with other European horseshoe bats, is the primary host of *Eyndhovenia euryalis* (Canestrini, 1884) (Baker and Craven 2003). Nevertheless, all of the above-mentioned fly and mite species have been collected from the other investigated bat host as well, albeit often at unknown or very low prevalence (Szentiványi et al. 2016; Burazerovic et al. 2018).

Critically, genetic reference sequences and within-species genetic diversity information are scarce for both wing mites and bat flies. Genetic markers can be used to evaluate the accuracy of morphological identification in closely related species (Tahir et al. 2018), to explore overall genetic diversity, and to assess population differentiation in ectoparasites. In permanent ectoparasites, where transmission between hosts is largely through direct contact and cohabitation, host specificity may strongly affect parasite population genetic structure by affecting the dispersal opportunities afforded to the parasite (Nadler 1995). For example, in *Polyplax* lice infecting *Apodemus* mice, strictly host-specific parasites possess a lower level of genetic diversity and more structured populations, due to limited dispersal and smaller effective population size (Martinů et al. 2018). In addition, the host specificity and transmission dynamics of ectoparasites may also affect their role as pathogen vectors (Witsenburg et al. 2015).

We investigate the bat fly and wing mite assemblages of *M. schreibersii* and *R. ferrumequinum* from 9 underground roosts, some of which were shared between 2 host species. We aimed to 1) identify parasite species morphologically and/or genetically, and to determine host specificity in a mixed colony setting; 2) analyze the genetic diversity based on mtDNA obtained from collected bat flies and wing mites, and compare it with previously published data for those species. We hypothesized that 1) considering their regular contact, the parasite assemblages of the 2 host species would overlap, with infections of the non-primary host occurring at low prevalence; 2) parasites found on *M. schreibersii* will have a higher intra-specific genetic diversity due to their host's larger colony sizes and migratory potential.

Materials and Methods

Sampling

Samples of bat ectoparasites were collected from 168 *M. schreibersii* and 73 *R. ferrumequinum* at 8 roosts in Serbia and 1 in Bosnia and Herzegovina (Table 1 and Figure 2) during the 2017 and 2018 summer and autumn seasons. Four sites out of 9 (no. 4, 5, 7, and 8 in

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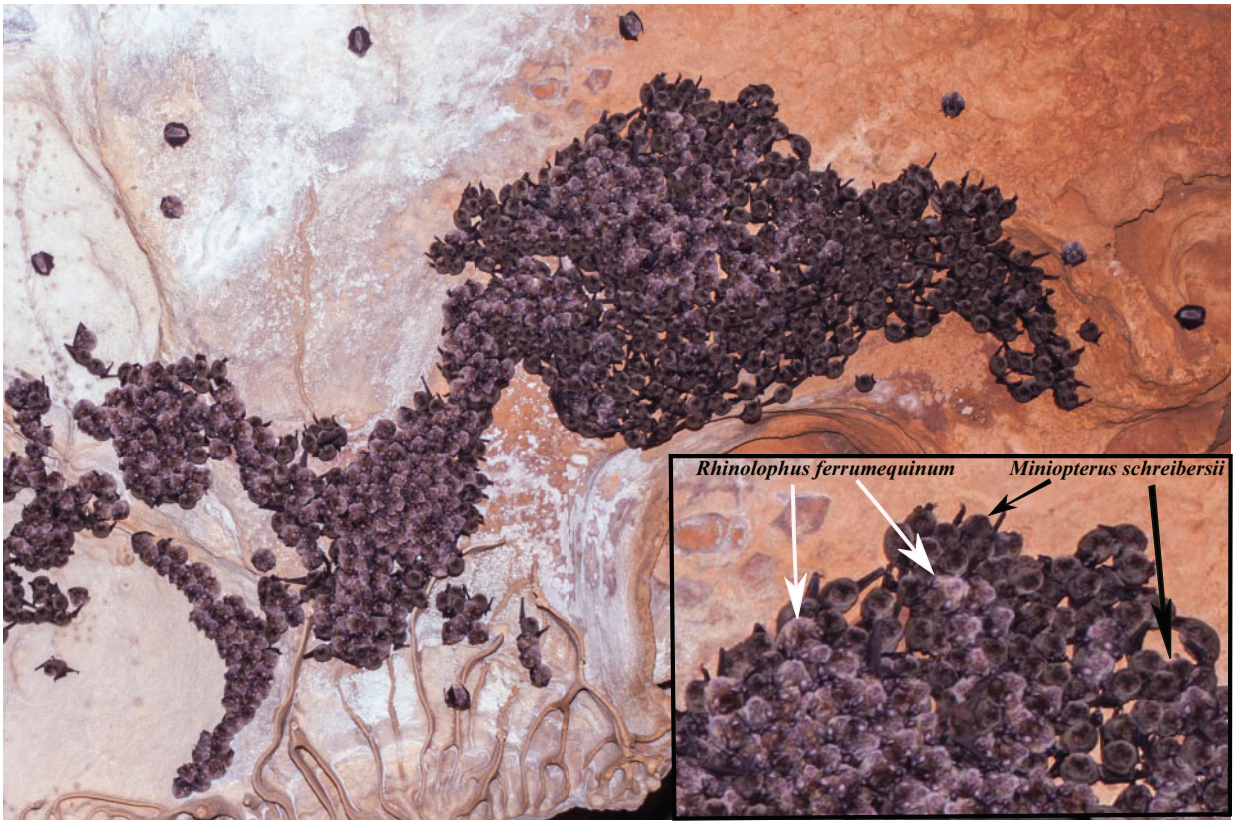

Figure 1. A large mixed colony of Schreiber's bent-winged bat *M. schreibersii* and the greater horseshoe bat *R. ferrumequinum* in close physical contact. Zoomed in section of the photograph in the lower right corner shows a clear distinction of the two host species.

Table 1. Sampling: number of bat host individuals (*M. schreibersii* and *R. ferrumequinum* depicted by bat silhouettes) caught at 8 sites in Serbia and 1 in Bosnia and Herzegovina (B&H), and the number of ectoparasite specimens collected from them (bat fly species: *N. schmidlii*, *P. conspicua*, *P. dufourii*, and *Ph. biarticulatum*; wing mite species: *S. psi* and *E. euryalis*)

Sampling site	*M. schreibersii*	*N. schmidlii*	*P. conspicua*	*P. dufourii*	*S. psi*	*R. ferrumequinum*	*Ph. biarticulatum*	*E. euryalis*
1 Mali kamenolom	51	45(14)	21(6)	1(1)	18	NA	NA	NA
2 Petrovaradin	7	10(9)	4(4)	0	9	NA	NA	NA
3 Dardagani (B&H)	11	13(12)	5(4)	3(3)	20	NA	NA	NA
4 Drenajicka	17	15(11)	8(4)	0	18	18	4(4)	19
5 Petnicka	19	19(12)	14(5)	0	2	5	7(7)	13
6 Bela sala	32	48(16)	10(4)	1(1)	20	NA	NA	NA
7 Toplik	29	32(15)	2(2)	0	17	16	38(20)	19
8 Temska	2	28(17)	7(4)	0	7	19	19(19)	20
9 Baloj	NA	NA	NA	NA	NA	15	15(13)	8
∑	168	210(106)	71(33)	5(5)	111	73	83(63)	79

All mite specimens and a subset of flies (numbers in parentheses) were selected for sequencing. NA, sites where neither host species nor ectoparasite species were found. Value 0, sites where host species was present, but specific ectoparasite species was not found.

Table 1 and Figure 2) had both target host species present at the time of sampling. At the other 4 sampling sites (no. 1, 2, 3, and 6 in Table 1 and Figure 2), both species have been found to share the roost in the past but were not both present at the time of sampling for this study. Site no. 9 (Table 1 and Figure 2) was used only by *R. ferrumequinum*. Bats were captured using mist-nets at each roost entrance during emergence, or by hand-net inside the roost. Each bat was placed in a separate, clean cotton bag to avoid parasite cross-contamination. Bat flies and wing mites were collected with forceps and each specimen was stored in a separate vial with 99% ethanol.

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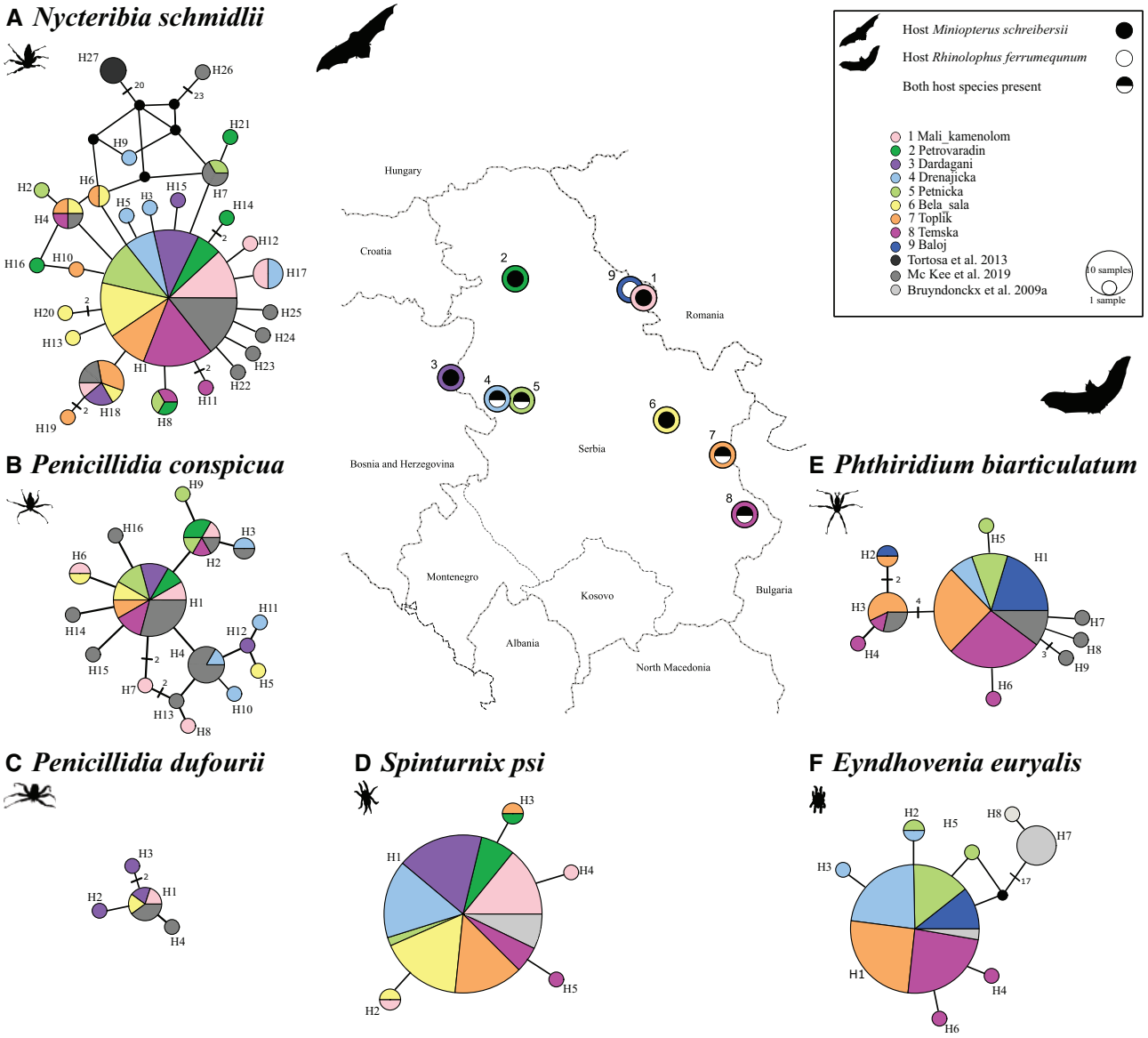

Figure 2. Map of sampling sites where host species *M. schreibersii* (black-filled circles), *R. ferrumequinum* (empty circles), or both species (half-filled circles) were sampled. (**A–F**) Median-joining haplotype network for each ectoparasite species. Circle sizes correspond to the number of individuals having each haplotype; dashes on the branches carry the number representing mutational steps between haplotypes; gray haplotypes represent those recovered in 3 previous studies (see legend inset for citations). A, bat fly *N. schmidlii*; B, bat fly *P. conspicua*; C, bat fly *P. dufourii*; D, wing mite *S. psi*; E, bat fly *Ph. biarticulatum*; and F, wing mite *E. euryalis*. Parasite haplotype networks on the left (A–D) represent those found on the host *M. schreibersii*; those on the right (E and F) parasites recovered from *R. ferrumequinum*.

All bats were released immediately after processing. Capturing and handling of bats were conducted under the license of responsible authorities of both countries, in compliance with ethical and safety guidelines (Supplementary File S1). Flies were morphologically identified to species level (Theodor 1967) prior to genetic analysis, while mites were identified solely by comparing DNA sequences with other voucher material in GenBank. All mites and a subset of flies selected to yield a representative sample of each species at each site (in parentheses; Table 1) were selected for sequencing.

DNA extraction and sequencing

DNA was extracted from the whole fly specimens using Mag-Bind Blood&Tissue DNA HDQ extraction kit (Omega) according to the manufacturer's protocols. A 710 bp segment of cytochrome oxidase subunit I (COI) was amplified using the primer pairs LCO1490 and HCO2198 (Folmer et al. 1994), and sequenced in one direction only using primer LCO1490.

Whole wing mite specimens were crushed with a disposable plastic pestle and incubated overnight in digestion buffer containing proteinase K (Strauss 1993), after which DNA was extracted using Quick-DNA Miniprep extraction kit (Zymo Research), following the manufacturer's instructions. A 460 bp fragment of the mitochondrial gene for small subunit ribosomal RNA (16S rRNA) was amplified using primers 16S + 1/16S-1 as in Mangold et al. (1998). Markers were selected to maximize the amount of reference material available for both species groups.

All PCR reactions were performed in a 10-μL volume, containing 1 μL of DNA, 0.2 μM each primer, 5 μL of Qiagen multiplex PCR

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master mix (Qiagen), and 3 µL of double-distilled water. PCR conditions for both reactions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation for 30 s at 95°C, primer annealing for 60 s at 47°C, and elongation for 30 s at 72°C, and then final elongation for 30 min at 60°C. All PCR amplifications were conducted using 2720 Thermal Cycler (Applied Biosystems). For both fly and mite DNA, PCR products were visualized using 1.5% agarose gel electrophoresis with Gel Red (Biotium). Clean amplicons were purified with Exo SAP (New England BioLabs) following the manufacturer's protocol and sequenced on an ABI Prism 3130 genetic analyzer (Applied Biosystems).

Data analysis

Sequence data were aligned and edited using CodonCodeAligner 4.2.7 (www.codoncode.com) or ClustalW algorithm implemented in MEGA v.6 (Tamura et al. 2013) and manually adjusted where needed. Ends were trimmed, with the final length given for every gene sequence/species in Table 2. Sequences were collapsed into haplotypes using DnaSP v.6 (Rozas et al. 2017). Molecular diversity indices (number of haplotypes, haplotype diversity [Hd], number of polymorphic sites, number of nucleotide differences, and nucleotide diversity [π]) were calculated in DnaSP v.6 (Rozas et al. 2017) and ARLEQUIN v.3.5.2.2 (Excoffier and Lischer 2010). Median-joining haplotype networks were visualized in POPART v.1.7 (Bandelt et al. 1999) applying default settings ($\varepsilon = 0$). Additionally, using previously published sequences from GenBank (Bruyndonckx et al. 2009b; Tortosa et al. 2013; McKee et al. 2019; Supplementary File S3), expanded datasets were analyzed and a median-joining network was constructed in POPART v.1.7 (Bandelt et al. 1999) for each species. Analyses of evolutionary divergence between sequences of the expanded dataset were conducted in MEGA v.6 (Tamura et al. 2013), using the Maximum Composite Likelihood model (Tamura et al. 2004).

Results

On *M. schreibersii* 3 bat fly species were found: *N. schmidlii* ($N = 210$), *P. conspicua* ($N = 71$), and *P. dufourii* ($N = 5$), whereas all wing mites belonged to only a single species: *S. psi* ($N = 111$). On *R. ferrumequinum* only a single bat fly species—*Ph. biarticulatum* ($N = 83$), and mite species—*E. euryalis* ($N = 79$) were found. No parasite species were detected on both hosts. The morphological identification of all flies corresponded to the molecular species identification.

Molecular DNA polymorphism indices for COI and 16S rRNA with the number of sequences used for analyses and their length in each species are given in Table 2. Unique sequences were uploaded to GenBank (accession numbers: *N. schmidlii*: MZ380293-MZ380313; *P. conspicua*: MZ396953-MZ396964; *P. dufourii*: MZ389896-MZ389898; *S. psi*: MZ390121-MZ390126; *Ph. biarticulatum*: MZ396965-MZ396970; *E. euryalis*: MZ389890-MZ389895; see Supplementary File S2 file for details). In flies, the number of recovered haplotypes ranged from 21 in *N. schmidlii* to 3 in *P. dufourii* (Figure 2 and Table 2). The highest haplotype diversity (Hd) and nucleotide diversity (π) values were observed in *P. conspicua* (Hd = 0.722; $\pi = 0.00251$), and lowest in *Ph. biarticulatum* (Hd = 0.289; $\pi = 0.00179$). Six haplotypes were detected in both wing mites species (Figure 2D and F), of which 5 haplotypes in each species were newly reported. Haplotype and nucleotide diversity values were higher in *E. euryalis* (Hd = 0.147; $\pi = 0.00044$, respectively) than in *S. psi* (Hd = 0.122; $\pi = 0.00035$; Table 2). In both flies and wing mites, all haplotype networks show a star-like topology, radiating from a single common haplotype (H1), accounting for a majority of the sample (e.g., 51.5% in *P. conspicua* to 93.7% in *S. psi*).

For each ectoparasite species, recovered sequences were compared with those available on GenBank, originating from 3 previous studies: Bruyndonckx et al. (2009a); Tortosa et al. (2013); McKee et al. (2019) (see Supplementary File S3 file for a complete overview). In *N. schmidlii*, collected from *M. schreibersii*, 21 haplotypes were found ($N = 106$; Table 2 and Figure 2A), of which 4 (H1, H4, H7, and H18) corresponded to previously reported haplotypes from Romania and Hungary (McKee et al. 2019). Two haplotypes from the previous studies, H26 from Romania, and H27 from Kenya (Tortosa et al. 2013) differed considerably from all others (27 and 20 bp from the hypothetical median vector, respectively; Figure 2A). The divergence between the sequence representing H26 and the rest of the sequences ranged from 4.4% to 5.1% (Supplementary File S4), and the divergence between the sequence of H27 and the rest of the sequences was 3.9–4.6% (Supplementary File S4). H26 and H27 differed from each other by 5.1%. The range of divergence between the rest of the sequences was 0.2–0.9% (Supplementary File S4).

Table 2. Molecular diversity indices of bat ectoparasite species from Serbia and Bosnia and Herzegovina

Host species	*M. schreibersii*				*R. ferrumequinum*	
Ectoparasite species	*N. schmidlii*	*P. conspicua*	*P. dufourii*	*S. psi*	*Ph. biarticulatum*	*E. euryalis*
						
Number of sampling sites	8	8	8	8	5	5
Gene	COI	COI	COI	16S	COI	16S
Sequence length (bp)	585	589	579	354	626	341
Number of sequences	106	33	5	111	63	79
Number of haplotypes	21	12	3	6	6	6
Haplotype diversity—Hd (±SD)	0.535 (0.059)	0.722 (0.080)	0.700 (0.218)	0.122 (0.043)	0.289 (0.073)	0.147 (0.054)
Nucleotide diversity—π (±SD)	0.00132 (0.0002)	0.00251 (0.00047)	0.00207 (0.00084)	0.00035 (0.00013)	0.00179 (0.00049)	0.00044 (0.00017)
Average number of nucleotide diff.	0.77233	1.473	1.20000	0.125	1.12135	0.151
Number of polymorphic sites	21	10	3	5	9	5
Parsimony informative sites	7	7	0	2	6	1

Standard deviation values (±SD) for haplotype diversity (Hd) and nucleotide diversity (π) are given in parentheses.

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In *P. conspicua* flies ($N = 33$), collected from *M. schreibersii*, 12 haplotypes were detected (Figure 2B), of which 4 (H1, H2, H3, and H4) were previously reported from localities in Romania and Hungary. The divergence between all the sequences of the expanded dataset was 0.2–0.9% (Supplementary File S4).

In *P. dufourii* ($N = 5$), collected from *M. schreibersii*, 3 haplotypes were found (Table 2 and Figure 2C), of which the most common, H1, was previously reported in Romania and Hungary (McKee et al. 2019). The divergence between all the sequences of the expanded dataset was 0.2–0.5% (Supplementary File S4).

In the mite *S. psi* ($N = 111$), collected from *M. schreibersii*, 6 haplotypes were detected (Table 2), of which one matched the only previously reported haplotype for this species (from France, Italy, and Switzerland). In order to merge the haplotypes with those available on GenBank, the sequence was trimmed from 354 bp to 313 bp, which resulted in the loss of H6, thus Figure 2D shows only 5 haplotypes. The divergence between all the sequences of the expanded dataset was 0.3–0.6% (Supplementary File S4).

In *Ph. biarticulatum* ($N = 63$), collected from *R. ferrumequinum*, 6 haplotypes were detected, of which 2 (H1 and H3) matched haplotypes previously reported from Romania. The divergence between all the sequences of the expanded dataset was 0.2–1.5% (Supplementary File S4, S4.E).

Finally, in *E. euryalis* ($N = 79$), collected from *R. ferrumequinum*, 6 haplotypes were detected (Figure 2F), of which one (H1) had previously been found in France. The range of divergence between these sequences was 0.3–0.6% (Supplementary File S4). Two additional haplotypes from France, but not observed in the current study (H7 and H8; Figure 2F), were both 17 mutational steps away (divergence 5.7–6.0% and 5.7–6.3%, respectively) from the median vector (unsampled haplotype), arising from H5 or H1.

Discussion

In this study, we recorded 4 ectoparasite species on the bat *M. schreibersii* (*N. schmidlii*, *P. conspicua*, *P. dufourii*, and *S. psi*) and 2 on *R. ferrumequinum* (*Ph. biarticulatum* and *E. euryalis*). All ectoparasite species were found on their primary hosts and there were no cases of cross-infection between the 2 bat host species, despite their direct association within the roost or use of the same roost at different times of the year. For all Nycteribiid species, morphological identification matched the genetic identification for all sequenced samples. However, it must be noted that for many of the genera in this study, closely related sister species were not present in the sample. Overall results from this study are surprising given the extensive reports of species being found on secondary or incidental hosts (Estrada-Peña and Serra-Cobo 1991; Lanza 1999; Krištofik and Danko 2012; Postawa and Furman 2014), including reports of all 6 of the investigated ectoparasites on both host species (comprehensively reviewed in Szentiványi et al. 2016; Burazerović et al. 2018). Even though the overall proportion of individuals found on non-primary hosts is often likely exceedingly low, at least some cross-infection events were expected, considering 2 hosts' close association. In a study quantifying host-specificity across bat ectoparasites in the Central Balkans, Burazerović et al. (2018) found high specificities for all of the species recorded here (>90% when the greater horseshoe bat *R. ferrumequinum* and the Mediterranean horseshoe bat *Rhinolophus euryale* are pooled) with the exception of *P. dufourii*. Looking specifically at *M. schreibersii* and *R. ferrumequinum*, very low cross-infection rates are found (e.g., 1205 *S. psi* wing mites on *M. schreibersii* versus 2 on *R. ferrumequinum*). Nevertheless, other authors noted a pronounced increase in cross-infection when colonies of both species were present in the roost (Estrada-Peña and Serra-Cobo 1991; Krištofik and Danko 2012). There is a possible additional parasite sharing between the species investigated herein and other bat species using the same caves. For example, *P. dufourii*, the fly species represented in the fewest numbers of our sample, is considered to be specialized for Mouse-eared *Myotis* bat species—*Myotis myotis* and *Myotis blythii*, or even nonspecific according to some authors (Imaz et al. 1999). It often appears on a range of cavernicolous bat species, including *M. schreibersii* (Postawa and Furman 2014), as observed here. Similarly, *R. ferrumequinum* shares many of its parasites, including both flies and wing mites found in this study, with most other European *Rhinolophus* species (Hutson 1984; Imaz et al. 1999). The absence of cross-infection observed here may serve as a cautionary note that many of these parasites show some morphological variation and that studies identifying parasites solely using morphology may overestimate incidental infection rates through misidentification. Many Nycteribiid species possess great plasticity in morphological characters, and in some cases, closely related species are difficult to distinguish (Theodor 1967). In cases where molecular reference sequences are available, simple barcoding methods can be applied, as performed here, for species identification confirmation.

Different parasite specialization strategies and affinities toward host species are displayed even in closely related species of the same genera (Christe et al. 2003). In bats with similar ecological characteristics, such as *M. schreibersii* and *R. ferrumequinum*, the chances for sharing ectoparasites are increased, regardless of their phylogenetical relatedness (Estrada-Peña and Serra-Cobo 1991; Bruyndonckx et al. 2009a). Caves used as underground roosts are places where individual bat flies and bat fly species accumulate over time (Patterson et al. 2007). In addition to transfer by direct host contact, indirect transmission via puparia deposited on the roost walls enables flies to be transferred between host individuals and species that use the same roost, even at different times of the year. This trait was expected to increase the number of findings of fly species on the non-native host, yet in this study we found none. Likewise, although mites require close physical contact between hosts for transmission, we observed several mixed clusters with tight contact between *M. schreibersii* and *R. ferrumequinum* at our study sites, and yet no mites were found on their non-primary host. The lack of more frequent (accidental) cross-infection in this study might suggest that the species of both ectoparasite clades are highly adapted and have strong preferences for their primary host despite the roost circumstances enabling them to change hosts. Experimental evidence for the strong preference and advantage of the primary host species has been found in several *Spinturnix* species (Giorgi et al. 2004), and competitive exclusion has been suggested as an explanation for why different wing-mite species were never observed to co-occur within individual host colonies (Bruyndonckx et al. 2009a).

The general readiness of many cave-dwelling species to cluster together may more broadly suggest that these species are able to cohabit because their parasites are sufficiently specialized so that they do not increase their host's risk of exposure to new parasites by associating. Forming such clusters can be advantageous because it provides social thermoregulatory benefits (Kerth 2008). On the other hand, precisely because their parasites are specialized and probably highly immunocompatible with the primary host species

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(Dick and Patterson 2007), any new parasite might impose a higher risk by, for example, bringing them in contact with different infectious bacteria, fungi, viruses, and blood parasites (Witsenburg et al. 2015; Szentiványi et al. 2019; McKee et al. 2021; Sándor et al. 2021). Thus, accidental cross-infection, however rare, might not be important from the aspect of establishing new viable populations of that ectoparasite on a new host species but from the aspect of the host's health and immunological status (Alcala et al. 2017).

The population genetics and molecular diversity of bat flies and wing mites have been studied to a limited degree so far, and rarely in the Balkan Peninsula, the important center of European biodiversity (Kryštufek and Reed 2004). In our study, all 3 bat fly species collected from *M. schreibersii* exhibited relatively high genetic diversity, while in the fly *Ph. biarticulatum* collected from the bat host *R. ferrumequinum*, it was notably lower (Table 2).

Group size of the host populations is positively correlated with the prevalence and intensity of parasites (Patterson and Ruckstuhl 2021), and the assumption that *M. schreibersii* will be more heavily parasitized and with higher diversity of species than *R. ferrumequinum* was supported by the results of our study. The total number of fly individuals found on *M. schreibersii* was higher, and it hosted 3 fly species, when compared with 1 fly species found on *R. ferrumequinum*. In the larger host colonies, the probability of ectoparasite encountering a mate increases, thus enhancing reproduction. Host species that form dense clusters and aggregate in large colonies are expected to be the most infected ones (Orlova et al. 2018; Szentiványi et al. 2019). The migratory potential of a host species can also contribute to ectoparasite exchange. *Miniopterus schreibersii*, being capable of long-distance flights, simply by covering wider geographical areas and coming in contact with other bats on the way, is expected to encounter a broader parasites diversity than it is the case for sedentary species, like *R. ferrumequinum* (Presley 2011; Wright et al. 2020). A high level of migration and a gene flow between distinct parasite populations coming in contact through their host maintains high levels of genetic diversity (Matthee 2020).

Between mites, the genetic diversity of the investigated 16S rRNA gene was similarly low in the species from both hosts (Table 2, *S. psi* and *E. euryalis*). However, the highly conserved nature of this sequence fragment makes comparing diversity within species at such scales difficult (De Rojas et al. 2002). Mite infection intensity fluctuates substantially throughout the annual cycle of their hosts, with a peak at host parturition and strong reductions during winter hibernation. This dynamic strongly shapes the population genetic structure, by effectively bottlenecking mite populations of smaller bat colonies each winter (van Schaik et al. 2014). Such population dynamics also reduce the chance of new rare haplotypes remaining in the population and thus may partially explain the low overall genetic diversity observed here in both species.

The star-like topology of all haplotype networks suggests that in each species, the sampled populations originated from single glacial refugia. The comparison of our sequences to previously published records revealed several interesting open questions. In the bat fly *N. schmidlii*, most haplotypes were closely related (H1–H25; sequence divergence 0.2–0.9% [Supplementary Files S4, S4.A]). Given the confirmed long-distance migrations of its host *M. schreibersii* in Europe (Wright et al. 2020), it is not surprising that COI haplotypes from 4 sites in Romania and 2 in Hungary were also found in this study. Two of the previously published sequences, however, gave haplotypes that deviated substantially from all others (H26: 4.4–5.1%, from *M. schreibersii* in Romania [McKee et al. 2019]; H27: 3.9–4.6% from 2 other bat species in the genus *Miniopterus*, *M. africanus*, and *M. inflatus* in Kenya [Tortosa et al. 2013]). Intraspecific sequence divergences in COI genes of animals are rarely greater than 2% (Hebert et al. 2003), suggesting there may be considerable unreported diversity or cryptic lineages in this fly species across its broad geographic range, warranting further investigation.

In the wing mite *E. euryalis*, our 16S rRNA H1 haplotype matched the only other sequenced sample from a *R. ferrumequinum* host (from Corsica). The other previously published haplotypes for this species (H7, H8) were collected from 2 other bat species, the Mediterranean horseshoe bat (*R. euryale*) and Geoffroy's bat (*Myotis emarginatus*), and differed substantially from haplotypes obtained in this study (5.7–6.3%). Sequences of 16S rRNA gene have been previously used to discriminate between *Ixodes* tick species (Caporale et al. 1995). In these species, species differed up to 12.8% of the sequence (6% on average), but intraspecific variation averaged less than 0.3%. Similar levels of divergence were found in other invertebrate groups (Kornobis et al. 2010). The divergence of sequences representing H7 and H8 and the rest of the sequences in our study were much higher than expected within the same species. Interestingly, *E. euryalis* mite is represented by 2 morphologically described subspecies, *E. e. euryalis* (Canestrini, 1884) that primarily parasitizes *R. euryale*, and *E. e. oudemansi* (Eyndhoven, 1941), primarily parasitizing on *R. ferrumequinum* (Imaz et al. 1999). Therefore, as noted by the authors of the original study that described these haplotypes (Bruyndonckx et al. 2009a), further exploration of the cryptic lineage sorting occurring in this species could be undertaken through a methodical sampling of wing mites from horseshoe bat species from sites where they are found living in sympatry.

Taken together, our morphological and molecular characterization of the parasite assemblages of 2 co-roosting bat host species, *M. schreibersii* and *R. ferrumequinum*, found no cases of cross-infection in either bat flies or wing mites between the 2 hosts. Our results point to a surprisingly high host-specificity in these ectoparasites, providing further evidence for the specialization of species in both parasite clades to their native host. Broader studies and literature reviews have noted considerably more overlap in parasite assemblages between species, thus the use of molecular barcoding to confirm species identity should be expanded in these taxa to better characterize these secondary infection rates. In this context, we report a comprehensive set of new haplotypes for each of the ectoparasite species analyzed in the study, contributing to the reference database for bat flies and wing mites of European bats.

In addition, higher genetic diversity was observed in flies from the more numerous and mobile host, *M. schreibersii*, as predicted. This pattern was not observed in the mites, although the limited power of the single sequence fragment used in this study may partially explain the difference. Though host group size and mobility both positively affect parasite intensity and diversity, other factors such as host specialization, parasite life history, interspecific competition among parasites, time spent on host and landscape also influence gene flow, and consequently genetic diversity in ectoparasites (Matthee 2020). Given their close host association, species diversity, and differences in reproductive life history, Nycteribiid flies and Spiturnicid mites provide a promising comparative framework to explore these dynamics in further detail.

Author Contributions

B.P. wrote the first draft of the manuscript. All authors contributed to the conception and design of the work. B.P. and I.B. collected samples in the field. B.P. and J.B. did laboratory analyses. J.v.S.

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contributed to the analysis and interpretation of data. I.B., J.B., and J.v.S. substantively revised the manuscript.

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Conflict of interest

Authors report no conflict of interest.

Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz.

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