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HOST MICROBE INTERACTIONS

Eco-epidemiology of Novel *Bartonella* Genotypes from Parasitic Flies of Insectivorous Bats

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Abstract

Bats are important zoonotic reservoirs for many pathogens worldwide. Although their highly specialized ectoparasites, bat flies (Diptera: Hippoboscoidea), can transmit *Bartonella* bacteria including human pathogens, their eco-epidemiology is unexplored. Here, we analyzed the prevalence and diversity of *Bartonella* strains sampled from 10 bat fly species from 14 European bat species. We found high prevalence of *Bartonella* spp. in most bat fly species with wide geographical distribution. Bat species explained most of the variance in *Bartonella* distribution with the highest prevalence of infected flies recorded in species living in dense groups exclusively in caves. Bat gender but not bat fly gender was also an important factor with the more mobile male bats giving more opportunity for the ectoparasites to access several host individuals. We detected high diversity of *Bartonella* strains (18 sequences, 7 genotypes, in 9 bat fly species) comparable with tropical assemblages of bat-bat fly association. Most genotypes are novel (15 out of 18 recorded strains have a similarity of 92–99%, with three sequences having 100% similarity to *Bartonella* spp. sequences deposited in GenBank) with currently unknown pathogenicity; however, 4 of these sequences are similar (up to 92% sequence similarity) to *Bartonella* spp. with known zoonotic potential. The high prevalence and diversity of *Bartonella* spp. suggests a long shared evolution of these bacteria with bat flies and bats providing excellent study targets for the eco-epidemiology of host-vector-pathogen cycles.

Keywords Chiroptera · Bartonella · Bat Fly · Host-parasite Coevolution · Nycteribiidae · Pathogen Diversity

Background

Bats (Chiroptera) are the second largest order of mammals, with the number of extant species over 1250 [1]. The order Chiroptera contains two suborders Yinpterochiroptera and Yangochiroptera; these include biologically and ecologically diverse species [2]. Both suborders have wide distribution, with a primarily tropical and temperate (Yinpterochiroptera),

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but even arctic, representatives (Yangochiroptera, [2]). Bats host a number of arthropod ectoparasites, like mites (Acari), ticks (Ixodida), fleas (Siphonaptera), and flies (Diptera). One of their specialized ectoparasites are the hippoboscoid flies (Hippoboscoidea: Nycteribiidae and Streblidae [3]), mostly flightless flies occurring only on bats [3, 4]. Hippoboscoid flies are semi-permanent parasites, spending their entire life on the host's body, exclusively females leaving the host for a short time when depositing the third stage larvae ready to pupate in the environment. These flies are considered to act as vectors of pathogens [5]. Bats themselves are important zoonotic reservoirs for a number of pathogens. Among these, viruses are especially important and well documented [6], while our knowledge on bacteria [7] and piroplasms [8–10] are limited. This is even more pronounced for arthropod-borne bacteria, for which there are only a handful of studies, reporting primarily pathogens from *Rickettsia* [11], *Borrelia* [12], and *Bartonella* genera [13, 14]. While the incidence of *Bartonella* in bat species has been known from some time [15], it has only recently attracted research interest. As such, recent surveys linked the presence of pathogenic *Bartonella* spp. in bats to molecular detection of this pathogen in ectoparasitic Nycteribiidae flies [16, 17], suggesting the importance of these hippoboscoid flies as vectors of *Bartonella* spp. [5].

Bartonella spp. are facultative intracellular parasites, which are developing in erythrocytes and endothelial cells of a number of mammalian species. These bacteria may cause chronic intra-erythrocyte infections, with a complex of humoral, neurologic, and ocular manifestations in humans and several domestic mammals. Infection in reservoir hosts is usually without clinical signs [18]. Recent studies suggest that *Bartonella* evolved in mammals in such a way that they became highly adapted to their host species or group, currently most known *Bartonella* spp. being host restricted [14, 19]. With the rapid emergence of newly described *Bartonella* infections worldwide [20], knowledge on the reservoirs, vectors, host-ranges, and transmission dynamics is needed for adequate surveillance of possible zoonotic *Bartonella* strains. Bats and their associated Nycteribiidae flies are suggested to be important reservoirs for diverse *Bartonella* spp. in Africa [21–23], Asia [24, 25], and the New World [26, 27]. *Bartonella* strains were already indicated in bat ectoparasites from the region [28], but taking into account the overall scarcity of studies in Europe [16, 29], as well the continuous increase of cave-inhabiting bat populations all over Europe [30], this study intends to widen our knowledge on *Bartonella* spp. related to insectivorous bats in Central and Eastern Europe.

The aim of the present work was to assess, by PCR and sequencing, the prevalence and diversity of *Bartonella* strains in nycteribiid flies collected from bats occurring naturally in Hungary and Romania, and to study the diversity of *Bartonella* spp., while using characteristics of host and vector ecology to explain this diversity among hosts and habitats. In order to reach this, we molecularly identified *Bartonella* sequences from parasitic flies and compared them to sequences deposited in GenBank and evaluated the importance of vertebrate host and insect vector ecology for the presence and prevalence of these bacteria.

Methods

Sampling Sites

Samples analyzed in this study were collected in Central and Eastern Europe, in Hungary and Romania. Hippoboscoid flies (Hippoboscoidea: Nycteribiidae) were collected from bats at 38 different bat capture sites distributed in the Carpathians and the Dobrogean Plateau (Romania) and in various, mainly mountainous parts of Hungary (Fig. 1). Study areas included roosting sites localized in buildings, caves and mine galleries, drinking and foraging areas, as well sites used for mating (swarming sites). Dates for bat captures ranged from 2007 to 2015.

Collection of Bat Flies

Bat flies were collected from individual bats, using forceps or with the help of a Fair Isle Apparatus [3]. Bats were identified to species based on morphological keys, with sex and age identified (based on tooth-wear and metacarpal joint ossification) for all specimens [31]. All ectoparasites from each individual bat were collected to allow prevalence data to be calculated. Preservation and long-term storage of bat flies was in 70 or 87% ethanol in separate vials (one vial per bat host). Identification of bat flies was based on morphological characteristics [32, 33].

To assess the importance of vector ecology, we assigned each bat fly species to one group (either mono-, oligo-, or polyxenous), based on the host specificity of the particular Nycteribiidae species [3]. Bat species were also grouped according to their affinity to a particular roost type in the non-hibernating period, thus creating three groups: (1) cave (including mines), (2) building, or (3) tree specialists [31].

DNA Extraction, PCR, and Sequencing

DNA from bat flies was extracted with ammonium hydroxide as described previously [34]. Bat flies were tested individually for presence of *Bartonella* spp. using a conventional PCR assay, targeting the citrate synthase gene *gltA* using primer sequences BhCS.781p (5'-GGGGACCAGCTCAtGGTGG) and BhCS.1137n (5'-AATGCAAAAAGAACAGTAAACA), yielding amplification products of approximately 380 base pairs [35]. For amplification, an initial denaturation step at 95 °C for 20 s (3 cycles) was followed by 3 cycles of annealing at 55 °C for 20 s, 3 cycles at 53 °C for 20 s, and 35 cycles of

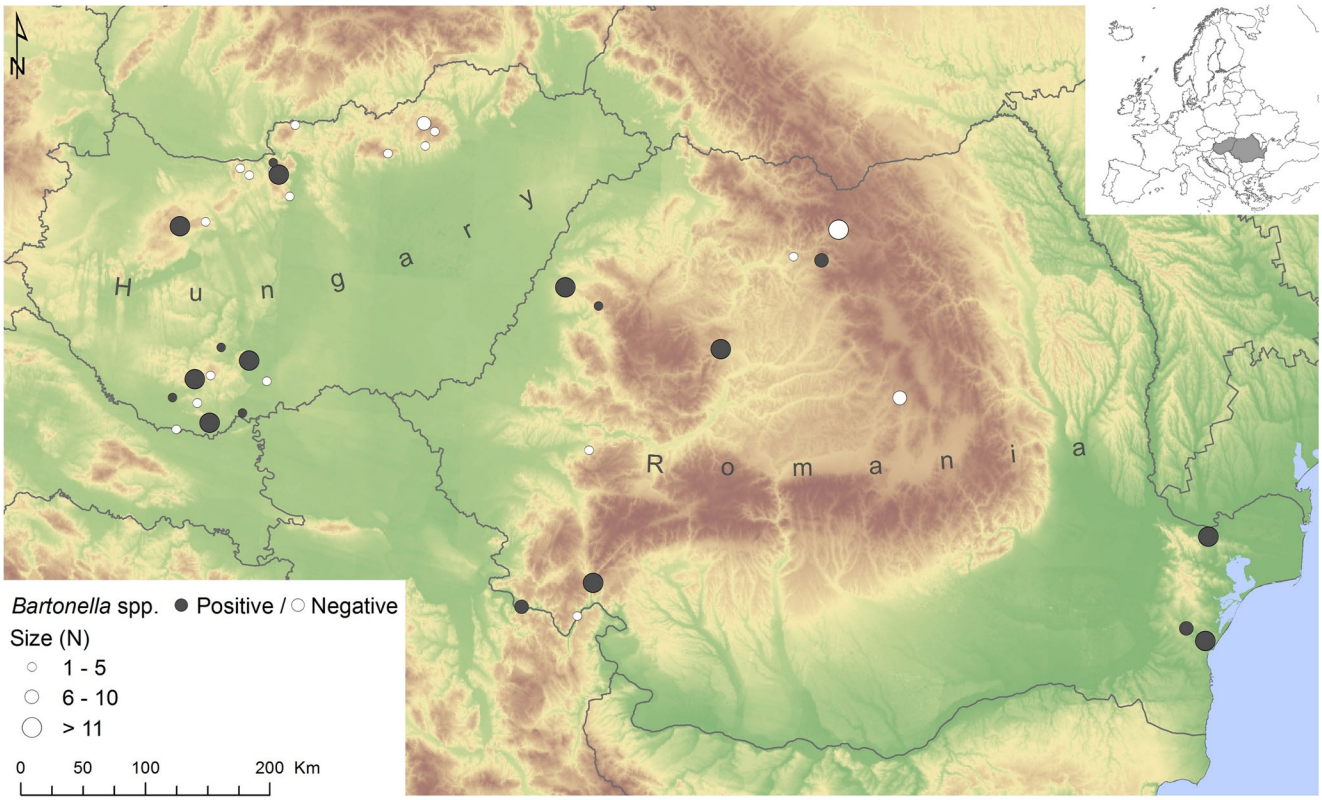

Fig. 1 Geographical distribution of sampling locations for Nycteribiidae flies used in this study

51 °C for 20 s. The final extension was performed at 72 °C for 1 min. PCR products were electrophoresed and visualized in a 1.5% agarose gel. Both strands of PCR products were sequenced with the Sanger method (BaseClear, Leiden, the Netherlands), using the same forward and reverse primers as in the conventional PCR. Trimming, manual editing, aligning, and cluster analyses of *Bartonella* sequences were performed in Bionumerics 7.1. (Applied Math, Belgium) together with *Bartonella* reference sequences available in GenBank.

Phylogenetic Analyses and the Visualization of the Host-Parasite-Pathogen Network

DNA sequences from this study and from the GenBank were aligned and clustered using pairwise and multiple alignments applying Neighbor-joining method in Bionumerics 7.1. The Jukes and Cantor model was used for the rate of nucleotide substitution. Bootstrap values were calculated by the analysis of 1000 replicates. We delineated seven *Bartonella* clusters by visually inspecting a phylogenetic tree (supplementary Fig. S1. For presentation of the parasite network, we used the "bipartite" package in R, function "plotweb" [36].

Statistical Analyses

For assessing microparasite (*Bartonella* spp.) species richness, we calculated the Shannon index (*H*) for each bat and bat fly species, as well as for the ecologic group of bat flies (e.g., groups made by mono-, oligo-, or polyxenous species) or bats (cave-, tree-, or building-dwellers).

We fitted GLMM's in the statistical computing environment R version 3. 3. 2 [37]. We used binomial generalized linear mixed models (GLMM) with the package "*lme4*" [38], using the function "glmer." Output variable was *Bartonella* spp. presence/absence. Random variables were bat specimens, since some of them provided multiple samples. In the model with bat species as input variable, we used roosting places, bat id's, and bat fly species as nested random variables. In the model with bat fly species as input variable, we used the following nested random variables: roosting places, bat species, and individual bat id's. In the model with bat- and bat fly gender and roosting places as input variables, we used bat species, bat id's, and bat fly species as nested random variables.

We assume that one *Bartonella* genotype might be widespread among samples from a particular condition, e.g., a specific combination of a bat species and one ectoparasite species. We tested this possibility using a multinomial model in which the fractions of the seven genotypes are proportional to the total sample sizes per genotype. We then calculated the probability: observing a specific genotype as frequently as or more frequently than actually observed in the sample. The probability (i.e., *p* values) less than 0.05 were considered significant support for selective distribution. In addition, rarity of

a genotype is evaluated by calculating the probability: observing a specific genotype as frequently as or less frequently than actually observed.

Next, likely explanations for the excess or deficit of a genotype involve the bat family, bat species, ectoparasite, and the country. We evaluated these possibilities, by fitting a multinomial model to the dataset in which the fractions of the seven genotypes are specific to the bat family, bat species, ectoparasite, or country. Fit was performed by maximizing the log-likelihood, and the model for which the Akaike Information Criterion is the lowest is designated to be the best-fit model. All computation was performed using the software Mathematica version 11.0.1 (Wolfram Research Inc., Champaign, IL).

Availability of Data and Material All the data from this study is freely available (upon registration) on the www.geo-parasite.orgwebpage.

Results

Bats, Bat Flies, and *Bartonella* Prevalences

Altogether, 544 individual bat flies belonging to 10 species were analyzed. These were collected from 305 individuals of 14 bat species (Hungary, 197 individuals of 10 bat fly species; Romania, 346 individuals of 7 bat fly species, Table 1). A number of 158 bat flies were positive for *Bartonella* spp. DNA (29.1%; CI 25.3–33.1), from which 148 samples were successfully characterized representing 18 unique *Bartonella* sequences. *Bartonella* spp. was detected and sequenced in 9 out of 10 Nycteribiidae fly species, collected from 11 out of the 14 bat species studied (Supplementary Table S1).

Association of *Bartonella* Strains with Bat Flies

Bartonella spp. sequences were obtained from nine nycteribiid species: *Basilia nana*, *B. nattereri*, *Nycteribia kolenatii*, *N. pedicularia*, *N. schmidlii*, *N. vexata*, *Penicillidia conspicua*, *Pe. dufouri*, and *Phthiridium biarticulatum*, while we were unable to detect any in *N. latreillii*. The distribution of *Bartonella* spp. in individual bat fly species was not linear, with certain nycteribiids hosting higher prevalence or diversity of individual *Bartonella* sequences. Polyxenous bat flies had the lowest prevalence (18/130; 13.84%), while either oligoxenous (40/143; 27.97%) or monoxenous species (100/269; 37.17%) had significantly higher prevalence (the latter two did not show significant differences between each other). Both mono- and oligoxenous flies hosted high diversities of individual *Bartonella* sequences (see Fig. 2.); however, the highest diversity of *Bartonella* strains was found in individual polyxenous species ($H_{polyx} = 1.831$ vs. $H_{monox} = 1.779$; $H_{oligox} = 1.556$). Individual Nycteribiid fly species explained the distribution of *Bartonella* spp. only marginally, with only four species (*N. vexata*, *Pe. conspicua*, *Pe. dufouri*, *Ph. biarticulatum*) contributing significantly to the observed pattern (Table 2, and Fig. 3). Although these species define the probability of *Bartonella* occurrences in our sample, among these, high prevalence was established only in the case of *N. vexata* (18.18%) (Table 3). Other species with high prevalence were *B. nattereri* (9.53%) and *N. pedicularia* (9.09%). High prevalence was not associated with high diversity in case of *Bartonella* sequences, as different bat fly species hosted the highest number of individual *Bartonella* sequences (e.g., *N. schmidlii* (9), *Pe. conspicua* (7), and *N. kolenatii* (7), see also Fig. 3.).

Association of *Bartonella* Strains with Bat Species

Bartonella-positive sequences were obtained from bat flies parasitizing 11 bat species (*Miniopterus schreibersii*, *Myotis bechsteinii*, *My. blythii*, *My. capaccinii*, *My. daubentonii*, *My. myotis*, *My. nattereri*, *Rhinolophus blasii*, *R. euryale*, *R. ferrumequinum*, and *R. mehelyi*), with only 3 bat species (*Plecotus auritus*, *Pl. austriacus*, and *R. hipposideros*) hosting only *Bartonella*-negative bat flies. The highest prevalence of *Bartonella*-positive bat flies were collected from *R. euryale* (5/7; 71.42%) and *My. capaccinii* (1/6; 33.33%), followed by *Mi. schreibersii* (78/240; 32.50%; see also Supplementary Table S1). Diversity of different *Bartonella* sequences was uneven among bat species, with the highest number of individual *Bartonella* sequences being recorded in bat flies hosted by *Mi. schreibersii* (11), followed by *My. daubentonii* (8) and *My. blythii* (6, see also Fig. 4). Bat species explained most of the variance found in *Bartonella* spp. distribution, with 6 individual species (*Mi. schreibersii*, *My. blythii*, *My. capaccinii*, *My. daubentonii*, *My. myotis*, and *R. ferrumequinum*) significantly contributing to the modeled distribution (Supplementary Table S3). Bat gender was another significant factor, with males holding more than twice as many *Bartonella*-positive bat flies as females (Table 4). Bat shelter also had an important contribution, as a significantly higher number of *Bartonella*-infected bat flies were collected on bats using underground shelters (Tables 4 and5), than from bats roosting either in buildings or in trees (no significant differences between the latter two groups).

Genetic Heterogeneity and Sequence Clustering

All sequences were confirmed genetically by amplification and sequencing of the gltA gene (sequences are listed as Supplementary Table S2). We identified 18 unique *Bartonella* sequences, which showed 95–100% similarity to *Bartonella* spp. sequences collected from bats (KF003129.1; KX300112)

Table 1 Number and species of bats (Chiroptera: Miniopteridae, Rhinolophidae, and Vespertilionidae) and bat flies (Hippoboscoidea: Nycteribiidae) analyzed

	Bat fly species										
Bat species (*n*)	*Basilia nana*	*Basilia nattereri*	*Nycteribia kolenatii*	*Nycteribia pedicularia*	*Nycteribia latreillii*	*Nycteribia schmidlii*	*Nycteribia vexata*	*Penicillidia conspicua*	*Penicillidia dufourii*	*Phthiridium biarticulatum*	Total no of bat flies
Miniopterus schreibersii (136)			3			110		119	8		240
Myotis bechsteinii (9)	1	8	1								10
Myotis blythii (17)			2		2	1	5	2	20		32
Myotis capaccinii (2)			3						1	2	6
Myotis daubentonii (63)			59	20		9		7	4		99
Myotis myotis (35)	1	1	31	2			4		47		86
Myotis nattereri (10)	3	8									11
Plecotus auritus (3)		3									3
Plecotus austriacus (1)		1									1
Rhinolophus blasii (3)										7	7
Rhinolophus euryale (3)										7	7
Rhinolophus ferrumequinum (15)			6				1			24	31
Rhinolophus hipposideros (1)							1				1
Rhinolophus mehelyi (7)										10	10
	5	21	105	22	2	120	11	128	80	50	544

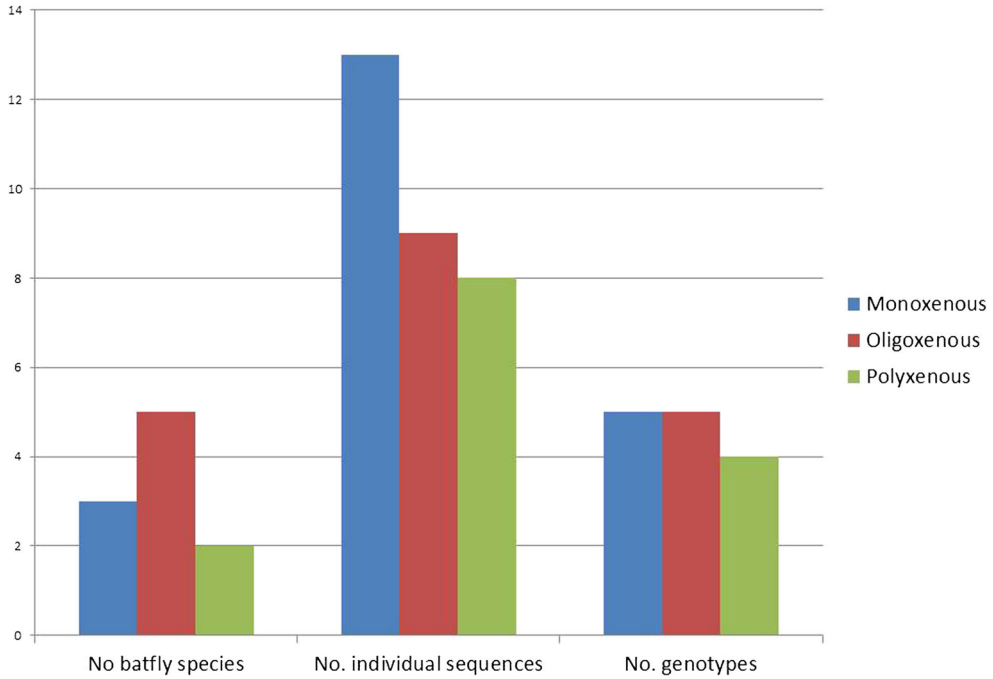

Fig. 2 The relationship between bat fly host specificity, bat fly species numbers, and individual *Bartonella* sequences, as well as genotypes recorded in bat flies in Central Europe

and bat flies (KT751145) (see also Table 3). As most of these sequences seem novel, they most likely represent undescribed *Bartonella* spp. associated with bats and their parasites, while they show similarity with pathogenic *Bartonella* spp., too (see Supplementary Table S4 and Supplementary Fig. S2 for a phylogenetic tree based on pairwise alignments with all the genotypes recorded together with similar *Bartonella* spp. genotypes from GenBank). These sequences clustered together into seven well-defined genotype groups (Genotypes 1 to 7, Figs. 4 and 5). Certain clusters showed wide geographical distribution (Genotypes 3 and 7, each with 10 different occurrences, found in both countries), while Genotypes 4 and 5 were restricted to southern Romania (Fig. 6). Most genotypes were shared by more than one bat fly species, while Genotypes 4 and 5 showed highly conservative distribution, being found only in parasites of the bats of the *Rhinolophus* genus (Fig. 7).

Table 2 Effect of bat fly species on the presence of *Bartonella* spp. (logistic GLMM)

Bat fly species	Estimate	Std. error	z value	Sign
Basilia nana	− 13.5184	197.7710	− 0.068	
Basilia nattereri	− 0.9480	0.8888	− 1.067	
Nycteribia kolenatii	− 1.1359	0.5890	− 1.929	
Nycteribia latreillii	− 13.8761	126.9073	− 0.109	
Nycteribia pedicularia	− 1.5158	0.9551	− 1.587	
Nycteribia schmidlii	− 0.7260	0.6200	− 1.171	
Nycteribia vexata	− 2.1440	0.9785	− 2.191	*
Penicillidia conspicua	− 1.7446	0.6287	− 2.775	**
Penicillidia dufouri	− 4.0185	0.8130	− 4.943	***
Phthiridium biarticulatum	− 1.6938	0.6816	− 2.485	*

Significance levels: $p <$ '***' 0.001 '**' 0.01 '*' 0.05

Discussion

Our study detected a total of 18 unique *Bartonella* strains clustered in 7 genotypes, found in 9 out of the 10 bat fly species studied. The detection of *Bartonella* spp. DNA in 9/10 bat fly species (every third individual being positive) suggests that infection is highly prevalent in most bat fly populations, implying probable vectorial competence for these dipterans. Nycteribiidae bat flies were already suggested as vectors of different *Bartonella* spp. Several studies highlighted the importance of these flies as possible vectors for a series of *Bartonella* spp. [5, 25, 39, 40], while certain studies also suggest that the bat flies themselves are the reservoir hosts for these bacteria [23, 26]. We found high prevalence of *Bartonella* spp. in most bat fly species (overall prevalence 29.1%, range 6.2–66.0%), and also a wide range of geographical distribution (47.3%; 18 out of 39 collecting sites provided *Bartonella*-positive bat flies, with 100% prevalence for sites, where more than 10 flies were collected, $n = 12$ sites). This is in line with previous studies performed in tropical areas, where bat fly-*Bartonella* studies reported prevalences even higher than these figures (e.g., 87% for *Cyclopodia dubia* in Madagascar, [41]; 66% in Western Africa, [42]; up to 100% of Nycteribiidae in Costa Rica [43]) and fairly similar to data from temperate regions (38%, South Africa [44]). Our results are unique in terms of bat-associated *Bartonella* spp. sequence diversity detected in any temperate region. Previous studies in

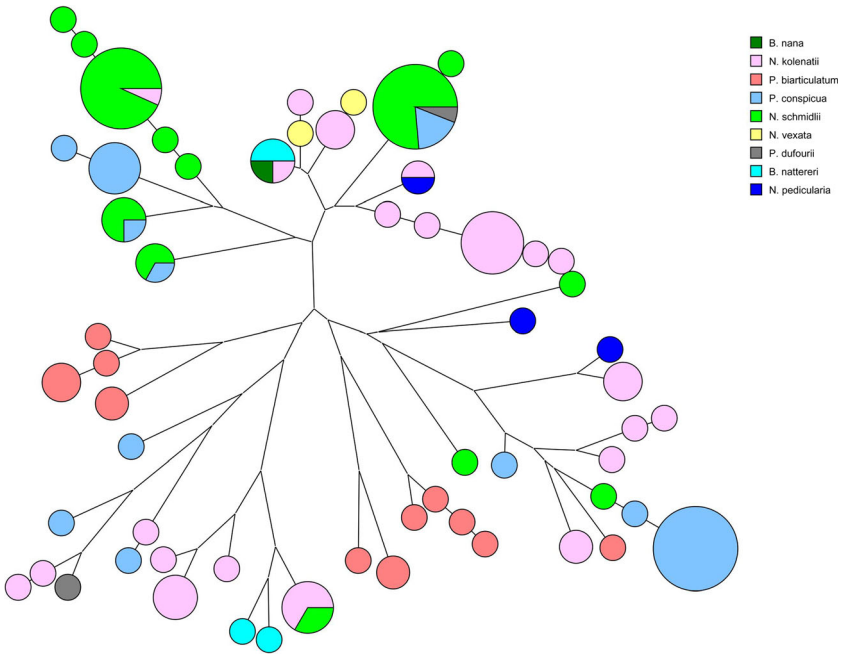

Fig. 3 Unrooted tree representing phylogenetic relationships between bat fly species and *Bartonella* genotypes recorded

Europe recorded 12 sequences in France [45], four in Finland [29] while in UK only three sequences were detected [16].

The diversity of *Bartonella* genotypes found in the studied region is high, comparable only with tropical assemblages of bat-bat fly associations (Costa Rica, 34 strains in bats/bat flies [43]; Madagascar, 21 strains in bat flies [23]; or Western Africa, 39 strains in bats/bat flies [42]) and it was considered primarily a result of high vertebrate host- and ectoparasite diversity characterizing the tropical regions [23, 27, 40]. This is not the case for Central Europe, with its relatively small number of bat species (32, [31]) or Nycteribiidae bat flies (12 species, [3, 46].

The prevalence of *Bartonella* spp. bacteria in nycteribiid flies varied between the individual fly species and collection locations, with high prevalences noted especially in the case of *N. kolenatii*, *N. schmidlii*, and *Pe. conspicua*. Significantly higher prevalences were noted among oligoxenous and monoxenous bat fly species compared to polyxenous species. We hypothesize that this trend is maintained by the recurrent infection of individuals of mono- and oligoxenous bat flies feeding on a few infected individuals of their respective host species in contrast to polyxenous bat fly individuals which have access to more host individuals belonging to more bat host species due to their more promiscuous feeding regime [47]. Due to this habit, their feeding incidence on *Bartonella*-reservoir host individuals or species is likely more reduced. This is supported by our results of *Bartonella* spp. sequence diversity detected in individual polyxenous bat flies. As these fly species are promiscuous in host selection, they may acquire different *Bartonella* genotypes but since they do not always feed on the reservoir host (or they are not reservoir host themselves) they fail to maintain all these genotypes in the cycle for longer periods, causing the low prevalence detected in these flies. Individual Nycteribiidae fly species explained the distribution of *Bartonella* spp. only marginally, as only a few species contributed significantly to the observed pattern (Table 5). The reduced host species selection habit of mono- and oligoxenous parasites (Fig. 8) may favor the high prevalence of the detected *Bartonella* (these flies have to feed on the same infected host individuals of a selected number of species), while maintaining a reduced spectrum of *Bartonella* strain diversity in these host-vector cycles. Our study provides the first

Table 3 Effect of bat species on the presence of *Bartonella* spp. (logistic GLMM)

Bat species	Estimate	Std. error	z value	Sign
Miniopterus schreibersii	− 1.3677	0.5490	− 2.491	*
Myotis bechsteinii	− 0.7561	1.0335	− 0.732	
Myotis blythii	− 2.3330	0.7155	− 3.261	**
Myotis capaccinii	− 2.5477	1.2988	− 1.962	*
Myotis daubentonii	− 1.6233	0.5445	− 2.981	**
Myotis myotis	− 2.1857	0.6002	− 3.642	***
Myotis nattereri	− 1.7322	1.2060	− 1.436	
Plecotus auritus	− 14.7834	128.0378	− 0.115	
Plecotus austriacus	− 13.7995	161.9648	− 0.085	
Rhinolophus blasii	− 1.0248	1.0731	− 0.955	
Rhinolophus euryale	0.1163	1.1325	0.103	
Rhinolophus ferrumequinum	− 2.8974	0.8022	− 3.612	***
Rhinolophus hipposideros	− 15.1181	109.1857	− 0.138	
Rhinolophus mehelyi	− 0.8301	0.9037	− 0.919	

Significance levels: $p <$ '***' 0.001 '**' 0.01 '*' 0.05

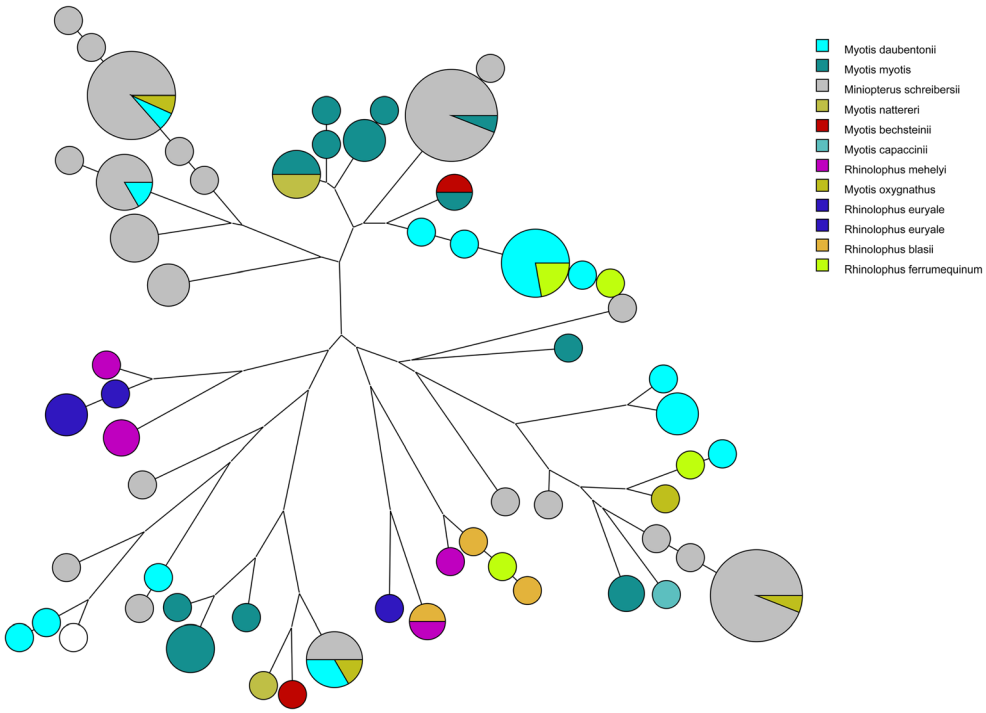

Fig. 4 Unrooted tree representing phylogenetic relationships between bat host species and *Bartonella* genotypes recorded

evidence of *Bartonella* spp. in these bat flies; thus, we lack any comparison with similar findings. We found no effect of bat fly gender on the distribution and diversity of *Bartonella* spp. DNA detection (although bat flies showed a highly skewed sex ratio, data not shown). We therefore hypothesize that this is caused by the large-scale movement of both genders of these flies among host individuals [48], thus providing equal chances of infection for flies of any gender.

The occurrence of bat flies holding *Bartonella* spp. sequences show an uneven distribution among different bat species. While most bat species studied (11 out of 14) hosted infected bat flies, certain bat species were more prone to hold bacteria-positive flies. The highest prevalence of infected flies was recorded among bat species exclusively using caves (*R. euryale*, *My. capaccini*, and *Mi. schreibersii*). This is in line with their roosting ecology, as these species are spending the daylight hours in dense groups in traditional deep-cave roosts [31], thus providing easy access for flies to switch hosts among roosting bat individuals. This phenomenon is reassured by the low *Bartonella* spp. prevalence among flies hosted by bat species roosting in small groups (0 for both *My. bechsteinii* and *My. natterreri*) or individually inside caves (none in case of *Plecotus* spp., 1.6% for *Rh. ferrumequinum*). Thus, bat host species itself explained most of the variance found among *Bartonella* strain distribution (Table 3, Fig. 4). Bat gender is also an important factor regulating the distribution of *Bartonella*-positive bat flies. Male bats were hosting more than twice as many flies as females. While female bats spend most of their time in high cohesion social groups (both hibernating in winter, as well as in female-only reproductive colonies in summer), most males show a higher mobility during the year (Altringham and Senior 2005). As such, males of

Table 4 Bat gender, bat fly gender and roost type effect on the presence of *Bartonella* spp. in bat flies (logistic GLMM)

		Estimate	Std. error	z value	Sign
	(Intercept)	− 0.93673	0.72535	− 1.291	
Gender	Bat	− 0.67422	0.24385	− 2.765	**
	Bat fly	− 0.19017	0.23036	− 0.826	
Roost	Cave	1.49195	0.54205	2.752	**
	Tree	0.02632	0.72715	0.036	

Significance levels: $p <$ '***' 0.001 '**' 0.01 '*' 0.05

Table 5 Effect of bat fly species on the presence of *Bartonella* spp. (logistic GLMM)

Bat fly species	Estimate	Std. error	z value	Sign
Basilia nana	− 13.5184	197.7710	− 0.068	
Basilia nattereri	− 0.9480	0.8888	− 1.067	
Nycteribia kolenatii	− 1.1359	0.5890	− 1.929	
Nycteribia latreillii	− 13.8761	126.9073	− 0.109	
Nycteribia pedicularia	− 1.5158	0.9551	− 1.587	
Nycteribia schmidlii	− 0.7260	0.6200	− 1.171	
Nycteribia vexata	− 2.1440	0.9785	− 2.191	*
Penicillidia conspicua	− 1.7446	0.6287	− 2.775	**
Penicillidia dufouri	− 4.0185	0.8130	− 4.943	***
Phthiridium biarticulatum	− 1.6938	0.6816	− 2.485	*

Significance levels: $p <$ '***' 0.001 '**' 0.01 '*' 0.05

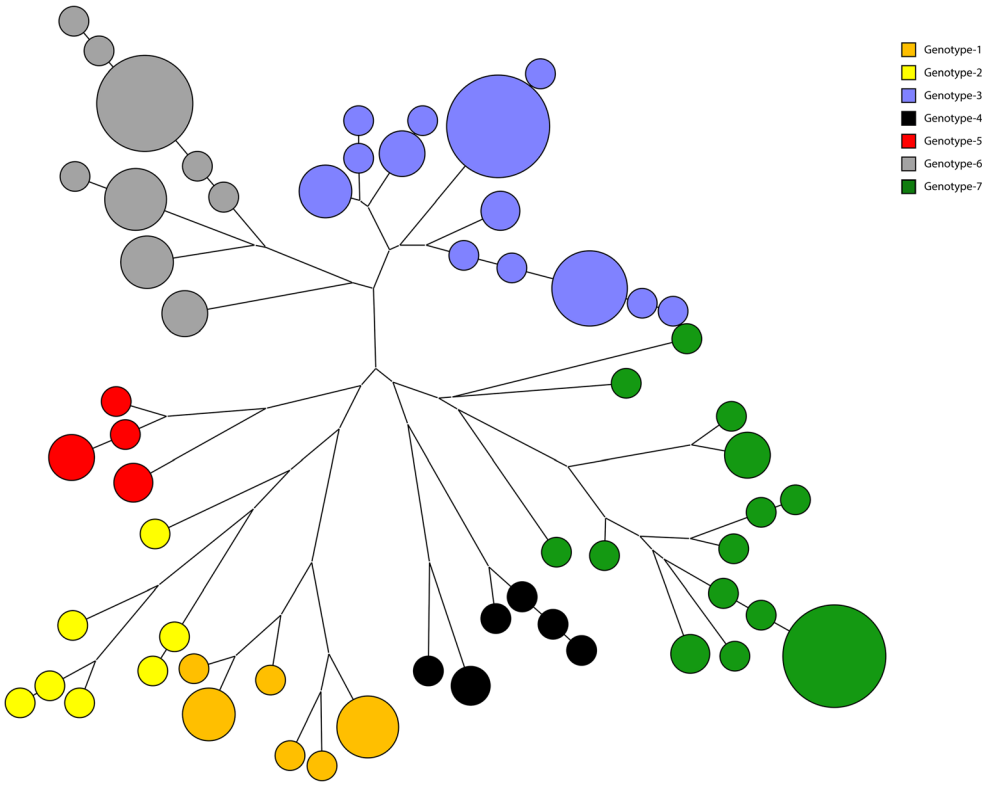

Fig. 5 Unrooted tree representing phylogenetic relationships and the distribution of the seven recorded clusters of *Bartonella* sequences

most tree roosting species switch roosts regularly in summer—some even at a daily basis [49, 50], and even cave or building-dwelling species are highly mobile both in summer [51] and in winter [52], seeking fertile females or visiting multiple lekking sites in swarming periods [53]. This high mobility presents wide opportunities for ectoparasites of these animals to access a large number of different host individuals. Bat gender was not an important factor in case of only one species, *Mi. schreibersii*, for which both genders roost together in colonies located solely in caves [31]. For this species, we

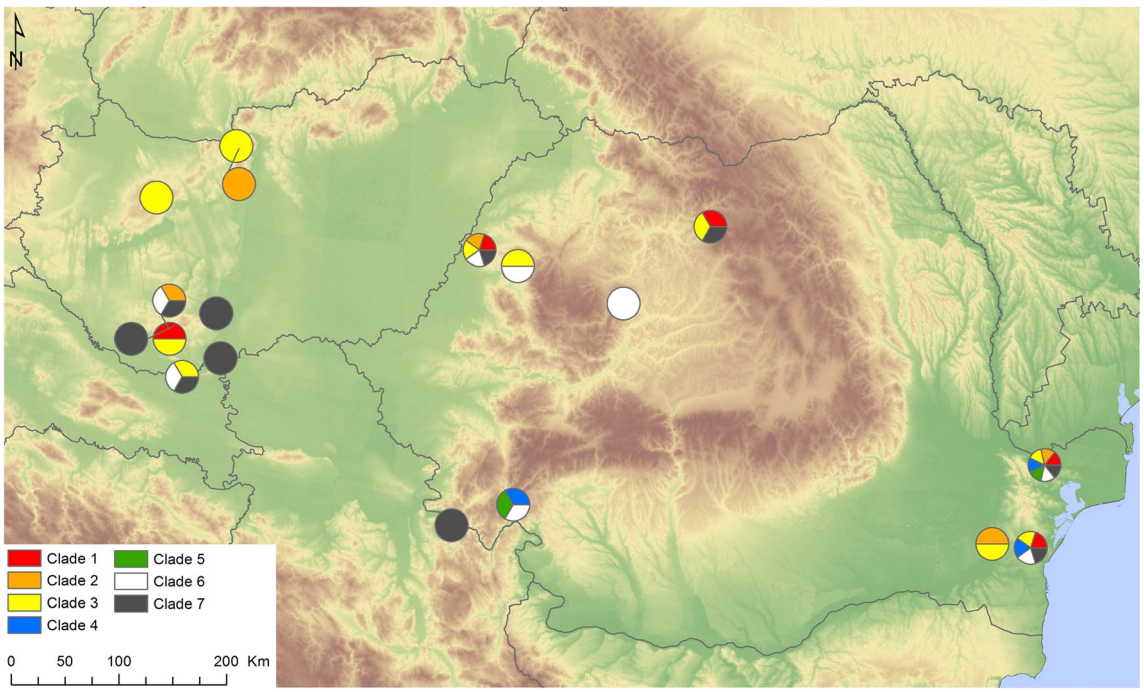

Fig. 6 Geographical distribution of the different *Bartonella* sequences clustered into the seven genospecies

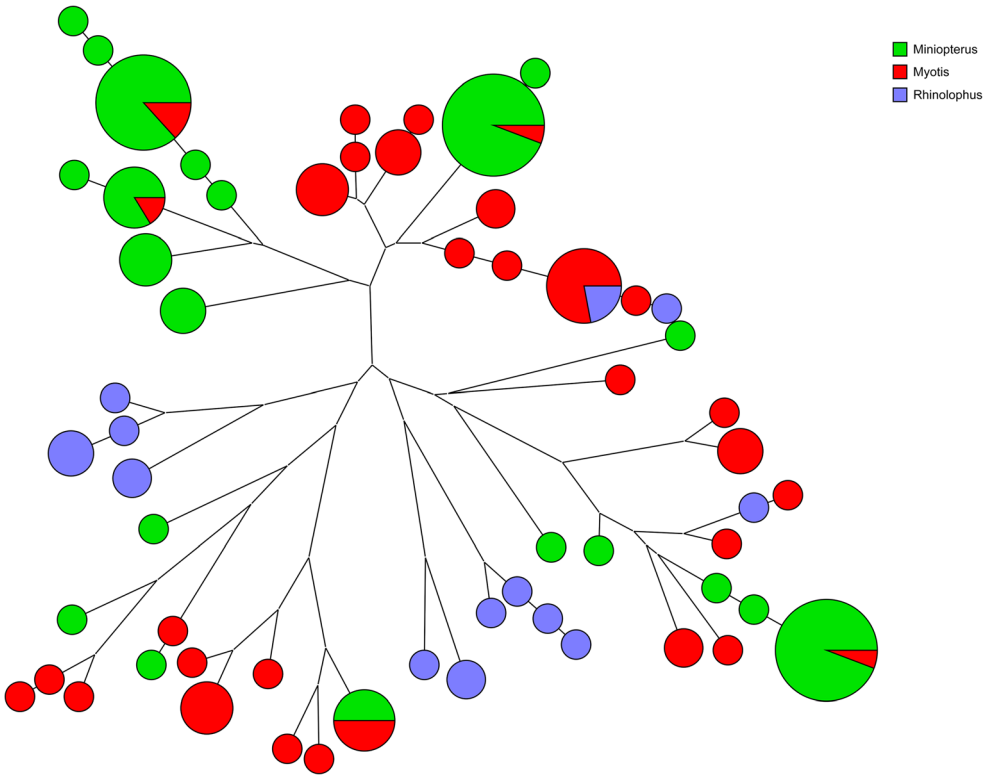

Fig. 7 Unrooted tree representing phylogenetic relationships between *Bartonella* genotypes recorded and bat-host families

found no difference among sexes, neither in prevalence nor in diversity of *Bartonella*-positive bat flies hosted. Bent-winged bats are long-distance migrants, with a number of stop-over sites along the migratory route [54]. This habit, coupled with their roosting preference of forming dense groups in high-number colonies (reaching up to thousand individuals all year long), may have favored the high diversity of *Bartonella* sequences (11 out of 18 molecularly identified) recorded in bat flies hosted by this bat species (see also Fig. 4).

In this study, we generated sequence data for 18 individual *Bartonella* strains from a number of nine bat fly species, which were collected from bats in Central Europe. These *Bartonella* sequences show 95–100% similarity to other *Bartonella* spp. reference sequences collected mostly from bats. However, none of the sequences from the present study are fully similar to any already known sequence collected from bat flies (the highest similarity is 99% with *Bartonella* spp. identified in bat flies from Madagascar, online Supplementary Table S5). All seven

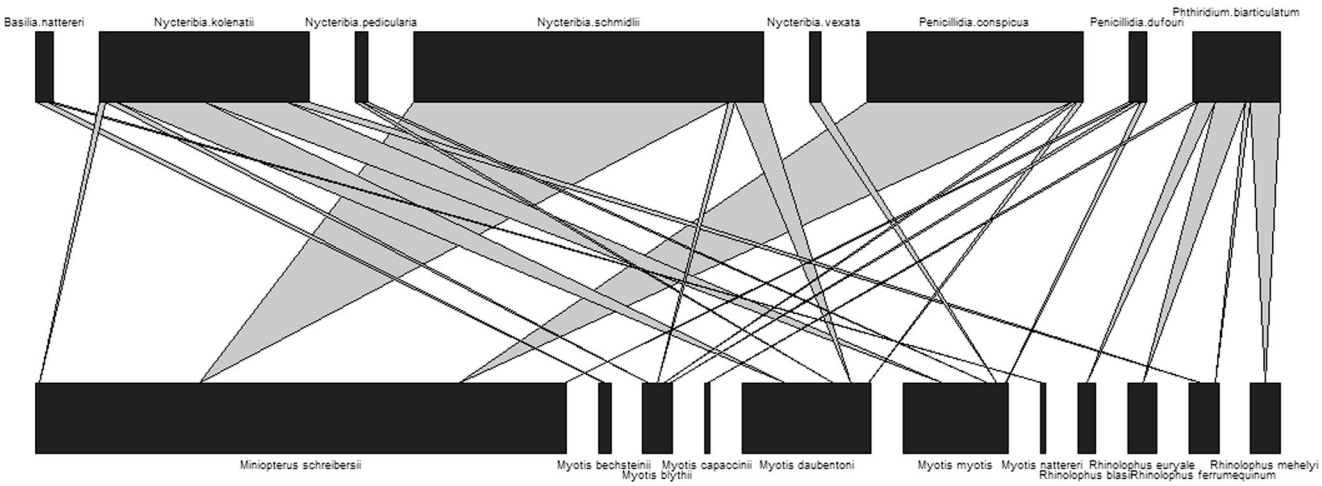

Fig. 8 Quantitative interaction web based on *Bartonella* genotype presence in samples of bat flies and their respective bat hosts. Links between nodes represent the sum of individual *Bartonella* genotype occurrences for a given bat and bat fly species couple

genotypes identified in our study are novel among bat fly-related *Bartonella* spp. and only show distant relationship to any previously described *Bartonella* species present in bat flies. This is in line with the wide diversity of *Bartonella* spp. identified from bats [21, 22, 24–27] or their parasites [28, 40–44]. The sequence characterization of a singular house-keeping gene (gltA) is not enough for establishing species boundaries among possible new *Bartonella* species; thus, we suggest that further characterization (culturing, multi-locus sequences) is necessary to verify whether the identified DNA sequences represent new *Bartonella* species or just variants of one or a few species [55]. Still, the identified genotypes seem generally distributed among a number of parasites (9 bat fly species) and bat species (11 bat species were hosting *Bartonella*-positive flies), with reduced specificity and a wide range of geographical records. While most sequences found show the highest similarity of bat-related *Bartonella* spp., several of the sequences identified are related to *Bartonella* spp. with known zoonotic potential (*Bartonella washoensis*, AF050108 or *Bartonella koehlerae* KX499329; 92% similarity for both). As most *Bartonella*-related diseases show a constantly emerging pattern [18, 20], with a number of known human cases caused by *Bartonella* spp., for which we lack any information regarding reservoir hosts or vectors [56], there is a need to establish the role of bats (and associated parasites) in the circulation of these bacteria. Moreover, the high diversity and prevalence detected in Central Europe suggest a wide and long-standing coevolution of these bacteria with Nycteribiidae and their insectivorous bat hosts, thus providing excellent study targets for close inspection of these host-vector-pathogen cycles (Fig. 8).

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Authors' Contributions ADS initiated the study, did part of the sample collection, and wrote the manuscript. LB, AC, SH, TG, PE, ZL, and DK contributed important samples to the study. MF identified all bat flies. AK, SSz, and HS performed the molecular and phylogenetic analyses. GF organized part of the sample collection and contributed to the study design and manuscript preparation. All authors read and approved the final version of the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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