University of Veterinary Medicine

Doctoral School of Veterinary Science

Eco-epidemiological investigation of blood-sucking ectoparasites of bats

Ph.D. thesis

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Introduction

Bats (Chiroptera) are the second largest order of mammals after rodents, also the most widely distributed land-based mammals. Appearance of men in bat habitats and adaptation of bats to urban areas increased the chances for contact between humans and bats. In the past few decades they are also recognized to be natural hosts of many zoonotic diseases. However, their migratory habit, high population density and roosting behavior increase the transmission of several infections and enable pathogens to spread long distances. Bats host more zoonotic viruses per species than rodents do and most of them show high human pathogenicity. The transmissions of pathogens to humans or other species from bats can occur by direct contact, or through vectors like parasites. Many of bat ectoparasites are highly host-specific, but there are species among them which can infest humans and other mammals.

Hard ticks (Acari: Ixodidae) that are specific to bats are only known to occur in the Old World. Only three species of ixodid ticks were known to infest bats in Eurasia, the long-legged bat tick *(Ixodes vespertilionis), I. simplex* and *I. kopsteini*. However, recently a new long-legged species, *I. ariadnae* has been discovered. Both *I. vespertilionis* and *I. simplex* are among the tick species with the largest known geographical range, encompassing much of the Old World (from Europe to the south in Africa and Australia, and to the east in Asia, including Japan). However, despite this, phylogeographical studies have not yet been conducted to investigate the morphological and/or genetic uniformity of these two tick species throughout their vast range. Only few data are available on their vector potential.

The soft tick (Acari: Argasidae) *Argas (Carios) vespertilionis*, the most common and geographically widespread hematophagous ectoparasite in the Old World, is specialized to bats. It is one of the epidemiologically most important haematophagous ectoparasites of bats, because it was reported to also infest humans and domestic animals. Previous studies showed that soft ticks may carry bacteria (*Rickettsia sp., Elrichial/Anaplasma* sp. and *Bartonella* sp.) and piroplasms (*Babesia* species).

Bats are regarded as the primary (ancestral) hosts of bugs in the Cimicidae, with subsequent switches to other hosts, including birds and humans. The historically and economically most important species in the family is the common bedbug (*Cimex lectularius*), because of its worldwide occurrence and preference of human environment. *Cimex lectularius* is the potential vector of at least 65 pathogens, but its vector competency awaits verification.

Aims of the study

Given the above, the aims of the study were:

1. to evaluate the mitochondrial gene heterogeneity of ixodid ticks from bats over a larger range in the Old World, including ticks that showed the morphological characteristics of *I. vespertilionis*, *I. simplex* and *I. ariadnae*, it was also within the scope of this study to examine the geographical range and host spectrum of these tick species
2. to investigate *A. vespertilionis* in the same context, i.e. its morphology, mitochondrial gene heterogeneity and host range in the Old World
3. to expand the knowledge on the phylogeny of cimicid bugs of bats, by investigating samples from Hungary, Romania (the latter representing the Balkans) and two further countries (South Africa and Vietnam)
4. to ameliorate lack of data on pathogens and/or pathogen DNA carried by ixodid bat ticks, piroplasms were chosen as the target group of analyses
5. to molecularly screen large numbers of soft tick larvae for piroplasms
6. to screen a large sample collection of blood-sucking bat ectoparasites (ixodid and argasid ticks, as well as cimicid bugs) for DNA of kinetoplastids
7. to screen bat faeces for arthropod-borne protozoa (Apicomplexa: Piroplasmida and related groups) and bacteria

Material and methods

Collection and identification of parasites and faecal samples

Ectoparasites (Ixodid ticks: *Ixodes vespertilionis*, *I. ariadnae*, *I. simplex*; soft ticks: *Argas vespertilionis* and bat-associated cimicid bugs: *Cimex* sp. and *Cacodmus* sp.) were collected from bats, caught for monitoring purposes, from 17 countries (Hungary, Romania, Germany, Serbia, Montenegro, Bosnia-Herzegovina, Czech Republic, Italy, France, Spain, Russia, Vietnam, India, Japan, China, Kenya and South-Africa) in Eurasia and Africa from 24 bat species between 1890 and 2016.

The morphology of ticks was compared according to the length/shape of palps, shape and index (length/width) of the scutum, density of alloscutal setae, arrangement of coxal setae. Morphological identification of soft ticks was based on the description of *A. vespertilionis* by Hoogstraal (1957 and 1958). Morphological identification of adult bugs was carried out by using standard keys, focusing on the pronotum, paragenital sinus (*Cimex* spp.) or the paramere (*Cacodmus* spp.). *Cacodmus* sp. females were identified according to the cox1 sequences of morphologically identified males. Structures of representative specimens from each country were measured under a Jenaval light microscope after clearance with lactic acid.

In 2014, between May and September, bat faecal samples were collected in Hungary, and in the Netherlands. The standard sample size was three to five faecal pellets for each bat. The individual faecal pellets were transferred into numbered, screw cap plastic tubes and stored frozen at −20 °C until evaluation.

DNA extraction from bat ectoparasites and faeces

DNA of ixodid ticks (*Ixodes ariadnae*, *I. vespertilionis*, *I. simplex*) and bugs (*Cimex* spp. and *Cacodmus* spp.) were extracted individually or from hind leg, DNA of *A. vespertilionis* larvae individually or in small pools with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and including extraction controls. Ticks were dried, then washed three times (in detergent containing water, in tap water and in distilled water) and minced.

DNA was extracted from bat faeces with the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. All samples were tested for the quantity and quality of DNA contents with a TaqMan real-time PCR specific for the 18S rRNA gene (Thermo Fisher Scientific, Vantaa, Finland).

Phylogenetic analysis of bat ectoparasites

PCRs were used to amplify an approx. 460 bp fragment of the 16S rDNA gene of Ixodidae and to amplify an approx. 420 bp fragment of the 12S rDNA gene of hard ticks. In the

case of Japanese samples, the COI PCR, that amplifies an up to 710 bp long fragment of the gene. Tick from Germany was compared with other tick isolates, of which relevant data are available in the GenBank. The cytochrome oxidase subunit I (COI) gene was chosen as the first target for molecular analysis, on account of its suitability as a DNA-barcode sequence for tick species identification. The PCR was modified and amplifies an approx. 710 bp long fragment of the gene. From *Argas vespertilionis* two mitochondrial markers were amplified: a 710 bp long fragment of the cytochrome c oxidase subunit 1 (cox1) gene, and an approx. 460 bp part of the 16S rRNA gene. The cytochrome c oxidase subunit 1 (cox1) gene was chosen as the primary target for molecular analysis, on account of its suitability as a DNA-barcode sequence for cimicid bug species. The PCR amplifies a 658 bp long fragment of the cox1 gene of various insect orders. In addition, a similar length fragment of the cox1 gene of the sample from Vietnam was amplified. To complement the results obtained with the mitochondrial cox1 gene, 16 samples that showed different cox1 haplotype within a country, were also tested for a nuclear marker, the internal transcribed spacer 2 (ITS2). This PCR amplifies a ~1027 bp fragment of the ITS2 of Hemiptera.

PCR products were visualized in 1.5% agarose gel. Purification and sequencing (twice for each sample) were done by Biomi Inc. (Gödöllő, Hungary). Obtained sequences were manually edited, then aligned and compared to reference GenBank sequences by nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). Representative sequences were submitted to GenBank.

Phylogenetic analyses were conducted according to the Tamura-Nei model and Maximum Composite Likelihood method by using MEGA program. Phylogenetic trees were compared in the R development framework.

Pathogen detection in ectoparasites and faeces

DNA samples were molecularly screened with a conventional PCR that amplifies an approx. 500 bp long part of the 18S rDNA gene of piroplasms (*Babesia/Theileria* spp.), this method also detects other apicomplexan genera, including vector- borne haemogregarines and certain cystogenic coccidia. For trypanosomes and related kinetoplastids an approx. 900-bp-long fragment of the 18S (SSU) rRNA gene were amplified. In the case of *Argas vespertilionis*, four DNA extracts were further tested with a conventional PCR that amplifies an approx. 950-bp fragment of the cytochrome *c* oxidase subunit 1 (*cox1*) gene of Piroplasmida. The presence of hard tick (Acari: Ixodidae) DNA in the bat faeces was evaluated by a conventional PCR that amplifies a 460 bp portion of the mitochondrial 16S rDNA gene of Ixodidae. Faecal samples were screened for the presence of DNA from *Anaplasma phagocytophilum*, *Neorickettsia risticii*, *Rickettsia spp*., *Francisella tularensis*, *Co. burnetii* and Chlamydiales. In addition, DNA extracts were also analyzed for haemoplasmas.

All PCRs were run with appropriate positive and negative controls. During all tests positive controls showed positivity, whereas negative (non-template) controls and extraction controls remained negative (the latter indicating absence of sample contamination).

PCR products were electrophoresed in a 1.5% agarose gel, stained with ethidium-bromide and visualized under ultra-violet light. Purification and sequencing (twice) were done from all PCR positive samples by Biomi Inc. (Gödöllő, Hungary) and by Macrogen Europe (Amsterdam, The Netherlands). Representative sequences were submitted to Gen-Bank. Evolutionary analyses were conducted in MEGA program.

Statistical analysis

Association of tick species with bat families was assessed by Fisher's exact test. Intensities of tick infestation (i.e. number of ticks on a bat individual) were compared between bat species by using Mann-Whitney U-test and Kruskal Wallis H-test in R program. Bat species with small sample size ($n<5$) were excluded from the latter analysis. The COIN (Conditional Inference Procedures in a Permutation Test Framework) package was used to correct P values of linked parameters. Bonferroni-Holm correction was used to correct P-values of multiple comparison. Differences were considered significant when $P<0.05$.

The means of the measurements of *Argas vespertilionis* were compared by using two-tailed Student's *t*-test and were considered significantly different if $P < 0.05$. Bonferroni- Holm correction was used to correct P-values.

Results

Molecular taxonomic investigations of bat ectoparasites in a geographical context

In total, 21 bat ticks (16 specimen of *Ixodes vespertilionis* (6 female, 2 male, 7 nympha, one larvae), two *I. ariadnae*-like specimen (one female and one larvae) and three specimen of *I. simplex* (one female, one nympha and one larvae) have been collected from 10 countries from Eurasia. No morphological differences were noted between specimens of *I. vespertilionis* collected in different countries of Europe. In In the phylogenetic tree these genotypes clustered together (maximum of eight nucleotide difference, 98.7 % identity), but separately from Western and South-Western European *I. vespertilionis* specimens collected in France (15 nucleotides differences, 97.6 % identity) and Spain (34 nucleotide differences, 94.6 % identity). In the phylogenetic analysis the South-Western European isolates formed a distinct cluster, a sister group of all other evaluated European specimens, similarly to genotypes of *R. hipposideros*, the main host of *I. vespertilionis*. Accordingly, based on the amplified part of its 16S rDNA gene, the French isolate (accession number: KR902772) clustered together with CE European isolates, but separately from the Spanish one (KR902773), although the bootstrap support for the latter was low. The *I. vespertilionis* nymph collected in Vietnam had convex posterolateral margin of the scutum, as contrasted to the concave shape in the case of European specimens. The COI sequence of this tick (KR902756) also had the highest level of intraspecific genetic divergence observed in this study: it differed from the CE European *I. vespertilionis* genotypes (101 nucleotides difference, 84.1 % identity) and clustered distantly on the phylogenetic tree. This nymph from Vietnam had the closest sequence identity (88 %) to a formerly published Japanese genotype (AB231667). Another tick was collected from an undescribed species of *Myotis* in Vietnam. This larva showed morphological similarities to *I. ariadnae*. Consistently with this, the COI sequence of this specimen (KR902767) had the highest (89.5 %) identity with the CE European *I. ariadnae* genotype (62 nucleotide differences. This finding was only partly confirmed by the analysis of the 16S gene (although poorly supported by low bootstrap values), because the Vietnamese *I. ariadnae*-like specimen (KR902770) clustered separately from CE European specimens of *I. ariadnae* (12–14 nucleotide differences, 96–96.6 % identity). One female tick collected in Japan also resembled morphologically to *I. ariadnae*. The COI phylogenetic analysis showed that this *I. ariadnae*-like genotype from Japan clustered together with the above *I. ariadnae*-like tick from Vietnam and close to *I. ariadnae* from Hungary. One nymph and one female tick, collected in India and Japan, showed a similar morphology to *I. simplex.* In the phylogenetic analysis these two Asiatic *I. simplex* specimens clustered together (25 nucleotide differences, 96 % identity with), but separately from two European (French and Hungarian) genotypes.

On 6 March 2015 a long-legged tick was removed from a greater mouse-eared bat (*Myotis myotis*). Based on morphological characteristics, i.e. 5 mm size, long legs, short palps, broad and posteriorly rounded scutum and sparse covering with setae, the tick was identified as an engorged female of *I. ariadnae*. The partial COI sequence of the tick (KR093169) showed 100% homology with I*. ariadnae* (KJ490306).

Altogether 329 soft tick larvae were collected from 17 bat species (belonging to five genera) in eight countries. All, except four soft tick larvae, were morphologically identified as *Argas vespertilionis*. The majority of *A. vespertilionis* larvae (59.1%: 188 out of 318, CI: 53.5–64.6%) were found on *Pipistrellus* spp.. Measurements of selected, diagnostically important structures of *A. vespertilionis* larvae revealed no significant differences between specimens from Europe and Vietnam, except for the length and width of the dorsal plate (plate length of ticks from Italy/ Romania *vs* Vietnam: $t = 3.49$, $df = 13$, $P = 0.008$; plate width of ticks from Italy/Romania *vs* Vietnam: $t = 3.21$, $df = 13$, $P = 0.012$). The morphology of serrate setae showed minor difference between geographically distant specimens: larvae from Europe had separated surface protrusions in the upper half of setae, but those from Vietnam had grouped (tuft-like) fragmentation of the setal end. *A. vespertilionis cox*1 sequences showed 0–2 nucleotide (0–0.3%) differences, i.e. 99.7–100% (650–652/652 bp) similarity between isolates from Hungary, Romania and Italy. Haplotypes from Europe had 37–38 nucleotide (5.7–5.8%) differences from an *A. vespertilionis* larva collected in Kenya, meaning 94.2–94.3% (614–615/652 bp) similarity with the latter. There was a more pronounced sequence divergence between specimens of *A. vespertilionis* from Europe and Vietnam, amounting to 46–49 nucleotide (7.1–7.5%) differences, i.e. only 92.5–92.9% (603–606/652 bp) similarity. Clustering of *A. vespertilionis* isolates with two members of Ornithodorinae received moderate (72%) support.

Based on the 16S rRNA phylogenetic tree, the separation of *A. vespertilionis* from Europe *vs* Kenya/Vietnam was highly supported (99%); *A. vespertilionis* was placed outside Argasinae, but its relationships among Ornithodorinae were only weakly supported.

Altogether 216 cimicid bugs were collected from the bodies or roosts of seven bat species of three genera. Bugs morphologically most closely related to *Cimex lectularius* were found both in the environment of bats and on the bat species *Pipistrellus pipistrellus*, *Myotis bechsteinii* and *Hypsugo pulveratus*. On the other hand, *Ci. pipistrelli* occurred only off-host. *Ci. lectularius s*howed similar general morphological characters if collected near bats or from bats in Hungary, however in the case of specimens from Hungary and Vietnam, the paragenital sinus of the female bug from Vietnam was rounded. Bugs morphologically most closely related to *Ci. lectularius* in Hungary showed five nucleotide differences from each other (99.2–100% similarity). A *Cimex* sp. from Hungary showed 46 nucleotide differences from the *Ci. lectularius* reference sequence (MF161520: from Hungary) (92.7% similarity). Another *Cimex* sp. from

Vietnam revealed an even lower (82.7% similarity) with *Ci. lectularius.* Bugs identified as *Ci. pipistrelli* exhibited up to six nucleotide differences from each other (99–100% similarity). *Cacodmus ignotus* from South Africa had two *cox*1 haplotypes, with only one nucleotide difference (99.8–100% similarity). Unexpectedly, samples identified as *Ca. ignotus* and *Ca. sparsilis*, which showed only 93.2% *cox*1 sequence similarity, were identical in their ITS2.

Piroplasm and free-living bodonids DNA detection in ectoparasites

Altogether, 308 ixodid ticks have been collected from 200 individuals of 17 bat species between 2008 and 2015 in Romania and Hungary. *Ixodes ariadnae* was represented by 45, *I*. *vespertilionis* by 124 and *I*. *simplex* by 139 specimens (larvae, nymphs and females). DNA sequences of piroplasms were detected in 20 bat ticks. *Ixodes simplex* carried piroplasm DNA significantly more frequently (13 of 138 specimens), than *I*. *vespertilionis* (3 of 124 specimens) (P = 0.02). The largest variety of *Babesia* and *Theileria* DNA sequences was also shown to be present in *I*. *simplex*. In *I*. *ariadnae* only a DNA sequence of *Ba*. *vesperuginis* (identity: 448/448 bp = 100%) was shown to be present. All four PCR-positive larvae were removed from the same bat. In *I*. *vespertilionis* larvae sequences of *Ba*. *vesperuginis* (identity: 448/448 bp = 100%) and *Ba*. *crassa* (identity: 404/410 bp = 98.5%) were detected. In *I. simplex* the sequence of another genotype of *Ba*. *crassa* (identity: 403/410 = 98.3%), a shorter sequence of the zoonotic *Ba*. *venatorum* and two sequences of *Ba*. *canis* (both identities: 420/420 bp = 100%) was demonstrated. Results of sequencing demonstrated DNA of two *Theileria* spp. exclusively in *I*. *simplex* larvae. These were *T*. *capreoli* (identity: 423/425 bp = 99.5%) and *T*. *orientalis* (identity: 432/432 bp = 100%). In addition, one female *I*. *simplex* carried the sequence of *Theileria* sp. OT3 (identity: 432/432 bp = 100%). Separation of *Babesia* and *Theileria* spp. amplified from bat ticks in the present study from other piroplasms was confirmed by high bootstrap values.

Altogether 321 soft tick larvae were collected from 17 bat species (belonging to five genera) in eight countries (Hungary, Romania, Italy, Kenya, Vietnam, China). Based on the PCR amplifying part of the 18S rRNA gene, 12 samples contained the DNA of piroplasms. Sequencing of all 12 PCR positive samples revealed the exclusive presence of *Ba. vesperuginis*, with 100% identity (448/448 bp) between samples from Hungary and China. Concerning phylogenetic analyses with concatenated *cox1* and 18S rRNA gene sequences performed here, both applied models achieved trees with similar overall topologies. *cox1* sequence of *Ba. vesperuginis* (KY657243) had the highest similarity to that (KC207821) of *Cytauxzoon felis* (79.1%, 709/896 bp), and less similarity to *Babesia* spp. (i.e. 74.9–77.6%) and *Theileria* spp. 18S rRNA sequence had lower than 90% sequence identity with *Babesia* and *Theileria* spp. sensu stricto. In particular, *Ba. vesperuginis* clustered separately from *Babesia* spp., and this received a strong (99% and 88%) support. In addition, *Ba. vesperuginis*

clustered next to *Ba. conradae* and the phylogenetic group of *Theileria* spp. and *Ci. felis*, or together with *Ba. conradae* in a sister group to the clade containing Theileriidae.

Out of 307 ixodid ticks (*I. vespertilionis*, *I. ariadnae* and *I. simplex*), 299 *Argas vespertilionis* larvae 207 cimicid bug (*Cimex lectularius* and *Cimex pipistrelli*) specimens three DNA samples were PCR positive for kinetoplastids. In these samples sequencing revealed the presence of DNA from free-living bodonids, but none from trypanosomes. In particular, one *I. simplex* larva (collected from *Mi. schreibersii* captured in Somova, Romania) contained the DNA of *Bodo saltans*, with 99.7% (754/756 bp) identity to a reference sequence (AY490224). In addition, the DNA of Bodonidae was detected in one *A. vespertilionis* larva and in one *Ci. pipistrelli* nymph. Corresponding sequences were 100% (i.e. 776/776 bp) identical with Bodonidae sp. Pan-2 (AY753625).

Piroplasm and vector-borne bacteria DNA detection in bat faeces

In addition, 196 individual and 25 pooled bat faecal samples were collected in Hungary and in the Netherlands. *Babesia canis canis* (referred to as *Ba. canis* onwards) DNA was shown to be present in five individual samples (prevalence 2.7 %, CI: 0.9-6.2 %), all from Hungary. These bat-derived *Babesia* isolates showed 100 % identity with two *Ba. canis* isolates from dogs in Croatia. All five bats with *Ba. canis* PCR positive faecal samples were caught within 50 km of the two regions in Hungary, where the highest number of *Ba. canis* seropositive dogs were found in a previous countrywide survey. From one pooled faecal sample of a pond bat (*Myotis dasycneme*) colony roost in the Netherlands another sequence was identified, having the highest (99 %) homology with *Besnoitia besnoiti*. The sequence (accession number KP835555) had six nucleotide difference from, but clustered together with *Be. besnoiti* and *Be. tarandi.*

Among bat faecal DNA extracts, 13 were real-time PCR positive for rickettsiae. Three samples (collected in Hungary and the Netherlands) a novel rickettsia genotype was amplified, which had the highest similarity (333/ 341 bp, i.e., 97.7%) to a Rickettsia genotype recently detected in a rodent species (Apodemus flavicollis) in Poland (KY488187) but was also relatively closely related to *Rickettsia felis* (332/341 bp, i.e., 97.4% identity). In addition, *R. helvetica* was identified in one pooled sample collected in Hungary. Four samples of the pond bat (*Myotis dasycneme*, collected in the Netherlands) were positive for *Neorickettsia risticii*. The 16S rRNA gene sequence from these samples was 100% identical (273/273 bp) with horse-derived isolates of *N. risticii* (e.g., AF380258) and closely related *Neorickettsia* genotypes (e.g., KX818101 from bat-associated flukes). Three of these samples also contained haemotropic *Mycoplasma* DNA, the species of which could not be identified with sequencing.

Discussion

Molecular taxonomic investigations of bat ectoparasites in a geographical context

In the case of Ixodid bat ticks the results can be interpreted in the light of evolutionary factors and events, as well as ecological traits that influence the intra- and inter-specific genetic diversity of animal populations, in this case of ticks and their bat hosts. In this context the geographical range of bat species, geographical barriers and glacial periods (that may isolate related bat populations) may be particularly important as driving forces of disruptive selection. During post-glacial periods *Rhinolophus hipposideros* (main host of *I. vespertilionis*) bat species recolonized central and northern Europe from refugia either in the Balkan or the northern Mediterranean (Southern France). This may account for the relative genetic homogeneity of Central European and French *I. vespertilionis* isolates, as reported here. Specimens collected in Spain were shown to differ markedly (and to cluster apart phylogenetically) from other evaluated genotypes. Probably the most important underlying factor of this phenomenon is that bat hosts of *I. vespertilionis* represent isolated populations on the Iberian Peninsula, prevented from mixing with northern populations by the Pyrenees which act as a barrier to gene flow. *I. vespertilionis* genotypes from Europe (KR902757-66), the high (16 %) genetic divergence of *I. vespertilionis* from Vietnam (KR902756) and of a similar genotype in Japan (AB231667), together with their well separated and distant phylogenetic position, suggest that they probably represent a distinct tick species. The host of the *I. vespertilionis* nymph collected in the present study in Vietnam was *R. affinis*, and of that recorded previously in Japan was *R. cornutus*. Based on phylogenetic analysis of mitochondrial (cytochrome *b*) sequences these two bat species clustered separately from other representatives of the genus (that harbored ticks in the present study), similarly to the phylogenetic position of associated ticks. Findings of this study also attest, for the first time, the existence of genetic and morphologic variants of bat ticks in Asia that are most closely related to *I. ariadnae*, hitherto only recognized in Europe. *Myotis* spp. are among the preferred hosts of *I. ariadnae*, and one *I. ariadnae*-like genotype of the present study was collected from a *Myotis* sp. in Vietnam. Murininae, including *Murina leucogaster* from which the *I. ariadnae*-like genotype was collected in Japan, are also closely related to *Myotis* spp.. This lineage clearly shows a separate position on the bat phylogenetic tree. In the present study *I. simplex* was collected from both *Miniopterus magnater* (in India) and *Mi. fuliginosus* (in Japan), allowing phylogeographical comparison with the ticks collected from *Mi. schreibersii* in Europe. In the phylogenetic analysis not only did the four *I. simplex* genotypes cluster separately from other bat tick isolates, but also their three respective bat host species from other bat species involved

in the present study. Again, glaciation and resultant geographic isolation might be considered as major mechanisms underlying the genetic differentiation.

The collection site of *I. ariadnae* in Germany is at least 250 km from the southeastern country borders, and approx. 650 km from the known habitats of this tick species in Hungary. Taking into account that the tick in the present study was removed from a bat species with migration ranges of approx. 100–250 km in Germany and Hungary, these data suggest the autochthonous occurrence of *I. ariadnae* in Germany.

Finding of only larvae of soft ticks on bats is in line with the life cycle of *A. vespertilionis*, i.e. larvae (unlike nymphs and adults) suck blood for several weeks on their bat hosts (14–31 days), therefore almost exclusively these can be collected from bats. Based on its 12S rRNA gene, *A. vespertilionis* was demonstrated to belong to Ornithodorinae. Phylogenetic analyses of the present study also reflected that haplotypes of *A. vespertilionis* clustered outside the Argasinae. In this study the great majority of relevant parameters were not significantly different between *A. vespertilionis* larvae from Europe and Vietnam, although these larvae proved to be well separated based on two mitochondrial genetic markers. Accordingly, morphologically similar, but genetically distinct populations of *A. vespertilionis* exist in Europe and Southeast Asia, suggesting that this soft tick should be regarded as a complex (group) of at least two putative cryptic species. The limited genetic exchange is most likely due to the presence of geographical barriers (high mountain ranges of the Himalayas and the Tibetan plateau), separating these regions and preventing overbridging of *A. vespertilionis* populations by bat hosts. Compared in the same context, the sequence divergence between *A. vespertilionis* from Kenya and Europe was less pronounced than between samples from Europe and Vietnam, suggesting that genetic exchange has been more likely in this direction. The broad host range of *A. vespertilionis* might partly explain its lower degree of mitochondrial gene heterogeneity in comparison with ixodid bat tick species over the same geographical region of Eurasia.

The results in this study provide molecular data of bat-associated cimicid bug species from three distant regions of the Old World. All *Ci. pipistrelli*, and the majority of *Ci. lectularius* were collected in roosting places of *Myotis* spp., which can be regarded as their principal hosts. Only one *Ci. lectularius* (from *My. bechsteinii*), the *Cimex* spp. (from Hungary, Vietnam) and *Cacodmus* spp. were found on hosts, in particular on four bat species, three of which are pipistrelloid bats (including *Hypsugo* [formerly *Pipistrellus*] *pulveratus*). According to literature data, bat species, which roost in narrow spaces (rock crevices or tree holes) and switch these places quite often, are more likely to carry bat-associated bugs on their wing membrane. This is confirmed by the data presented here. In the present study two new genotypes (belonging to the *Ci. lectularius* group, but highly divergent from its other members) were identified. Both of the relevant specimens were collected from pipistrelloid bat hosts. The first of these specimens in Hungary, showed the morphology of *Ci. lectularius* and was different from *Ci.*

emarginatus. The second specimen in Vietnam, was also similar to *Ci. lectularius*. However, the paragenital sinus of the latter female was different from that in *Ci. lectularius*. Sequence comparisons and phylogenetic analyses of *cox*1 and ITS2 sequences of specimens from pipistrelloid bats (collected in Hungary and Vietnam) suggest that they may belong to new species. In the present case, the identity of ITS2 sequences between individuals of two *Cacodmus* spp. could have resulted from genetic introgression or hybridization.

Piroplasm and free-living bodonids DNA detection in ectoparasites

Babesia vesperuginis DNA was molecularly identified here in *I*. *ariadnae* and *I*. *vespertilionis*. This piroplasm is pathogenic to bats. The babesia has also been found in four other countries from central and eastern Europe in heart tissues of bats and reported five new host species. In addition to this*, Babesia canis* DNA were amplified from *I. simplex*. *Babesia canis* is an important parasite of dogs. The known vector of this piroplasm is *Dermacentor reticulatus*, which is sometimes infesting bats, including *Miniopterus schreibersii*. Recently, bats were reported to pass the DNA of *Ba*. *canis* in their faeces. Taking into account that it is very unlikely that relevant lineages of *I*. *simplex* (found to be PCR positive here) had become infected from canids (from which hosts *I*. *simplex* has never been reported) in a previous stage or generation, *Ba*. *canis* or its DNA might have been present in the blood of relevant bats. This possibility is supported by recent finding of *Ba*. *canis* DNA in bat tissues. Interestingly, the DNA of *Ba*. *venatorum* was amplified from one larva of *I*. *simplex* in Romania. Although the sequence was 100% identical with *B*. *venatorum* and differed from other piroplasms, because of its shortness no final conclusion can be drawn on the occurrence of *B*. *venatorum* DNA in bat ticks. DNA of two *Theileria* spp. have been shown here to be present in larvae of *I*. *simplex* from *Mi*. *schreibersii*. Competent vectors of the above piroplasms are *D*. *reticulatus*, *I*. *ricinus* and *Haemaphysalis* spp. These tick species are rarely found on bats, most likely attaching to bats when roosting in nests of small mammals (e.g. in tree holes), or when gleaning bat species feed on insects from the lower vegetation in meadows or forests. Alternatively, blood-sucking flies have the potential to carry and transmit *Babesia* spp. and *Theileria* spp., and flies (Insecta: Diptera) are among the frequent food items of e.g. *Mi*. *schreibersii*. This implies that bats may get into contact with or may have access to piroplasms or piroplasm DNA from their food. Bat ticks are not known to infest dogs or ruminants, i.e. typical hosts and reservoirs of piroplasms molecularly identified in *I*. *vespertilionis* and *I*. *simplex*. Therefore, DNA sequences of piroplasms detected in these bat ticks most likely originated from the blood of their respective bat hosts. This may indicate that either bats are susceptible to a broader range of piroplasms than previously thought, or at least the DNA of piroplasms may pass through the gut barrier of bats during digestion of relevant insect vectors.

In a high number of *Argas vespertilionis* larvae from Vespertilionidae only *B. vesperuginis* was detected. The most likely vector of *B. vesperuginis* is *A. vespertilionis.* Based on the sequence analysis performed here, the 18S rRNA gene of *B. vesperuginis* also appears to be highly conserved over much larger geographical distances (i.e. 5000 km between Hungary in Central Europe and Xinjiang in Central Asia). *Vespertilio murinus*, which carried genetically closely related *A. vespertilionis* larvae in Central Europe and Northwestern China as demonstrated here, has a broad Palearctic range (from Europe to Siberia and the Pacific coast). It shows relative genetic uniformity (below 1% cox1 sequence divergence) across this region and has a parapatric distribution with its eastern congener *V. sinensis*. In addition, *V. murinus* colonies in Asia are frequently associated with human settlements (buildings). These background factors could thus allow gradual gene flow (mixing) between distant European and Central Asian populations of *A. vespertilionis* while associated with this bat host species, as suggested by the present results.

Three DNA extracts from bat ectoparasites were shown to be PCR-positive for kinetoplastids, and in these samples sequencing identified free-living (water associated) bodonids. The arthropod-origin of these bodonids is confirmed by the PCR negativity of extraction controls throughout the study. Furthermore, because prior to DNA extraction the surface of investigated arthropods was decontaminated, the DNA of identified bodonids must have been present within these ectoparasites. All bat species rely on drinking from freshwater bodies. Such occasions may allow bats to ingest bodonids, although this has not been suggested formerly on a molecular basis. Animal and human feeding studies have demonstrated that fragmented dietary DNA may resist the digestive process and even complete genes can transgress the gut barrier. Taking into account this scenario, the present findings might imply that bats have access to free-living bodonids and at least the DNA of bodonids might pass through the oropharyngeal or gastrointestinal mucosa of bats into their circulation. At the same time, it was unexpected that none of the bat ectoparasites were found to contain the DNA of bat-specific trypanosomes.

Piroplasm and vector-borne bacteria DNA detection in bat faeces

Babesia canis canis (referred to as *Ba. canis* onwards) DNA was shown to be present in five individual samples (prevalence 2.7 %, CI: 0.9-6.2 %), all from Hungary. Taken together, this may be the first molecular evidence that both main European genotypes of *Ba. canis* (group A, B:) occur in Hungary. *Dermacentor reticulatus*, the relevant vector of *B. canis*, is known to sometimes infests bats. Alternatively, blood-sucking flies (e.g. *Stomoxys* spp.) are known to be incriminated as mechanical vectors in the transmission of *Babesia* spp.. *Stomoxys calcitrans* (also called “dog fly”) was reported to frequently bite dogs, and to be a predominant species in the diet of some bat species.

To the best of our knowledge, this is the first finding of a *Besnoitia*-like sequence from a non-ungulate mammal in Europe, and from any bat species in a world-wide context. In general, bats frequently use cattle stables for roosting, where they may have access to the mechanical vectors of *Be. besnoiti*, i.e. blood-sucking flies (*S. calcitrans*, *Tabanus* spp.) and mosquitoes. In particular, *Tabanus* spp. and mosquitoes develop in wet soil near water and in water, respectively, corresponding to the main habitat of the pond bat. Therefore, the *Be. besnoiti*-like sequence in the present study might have originated from cattle via blood-sucking dipterans, or represents a novel *Besnoitia* genotype/species closely related to *Be. besnoiti*.

This is the first report of molecular analyses of a broad range of vector-borne bacteria in bat faeces. For the interpretation of the present results it can be hypothesized that the DNA of vector-borne bacteria in bat faeces may originate either from the arthropod food of bats (having passed through the entire gastrointestinal tract), from bat intestinal parasites (such as digenean flukes or their eggs), or from the bats themselves. In conclusion, bats have shown to pass rickettsia and haemoplasma DNA in their faeces. *Neorickettsia* DNA is present in the faeces of the pond bat (*Myotis dasycneme*) in Europe, suggesting that this bat species plays a final host role in the life cycle of flukes harboring neorickettsiae.

Overview of the new scientific results

1. The present research highlights that although all three ixodid bat tick appear to be widespread in Eurasia, they exhibit pronounced genetic differences, showed similar clustering patterns with those of their associated bat host species. *I. vespertilionis* may represent a species complex. We provided first the existence of *I. aridnae* in Germany.
2. In comparison with the ixodid ticks, in the case of *Argas vespertilionis* only minor morphological differences were observed between specimens from Europe and Vietnam, however, phylogenetic analyses suggest that it represents a complex of at least two putative cryptic species.
3. Molecular evidence is provided here on the existence of two new genotypes, most likely new species, within the *Ci. lectularius* species group. *Ca. ignotus* is reported for the first time in South Africa.
4. DNA sequences of piroplasms were detected in bat ticks. In *I*. *ariadnae* only *Babesia vesperuginis* DNA was detected, whereas in *I*. *vespertilionis* sequences of both *B*. *vesperuginis* and *B*. *crassa*. From *I*. *simplex* the DNA of *B*. *canis*, *Theileria capreoli*, *T*. *orientalis, Theileria* sp. OT3 and a shorter sequence of the zoonotic *B*. *venatorum* were amplified.
5. Only *Babesia vesperuginis* has been found in samples of *Argas vespertilionis* with 100% identity between samples from Hungary and China. To the best of our knowledge, this is the first molecular evidence on the occurrence of *Babesia vesperuginis* in Asia. Phylogenetic analyses of *Babesia vesperuginis* from *A. vespertilionis* specimens indicate that *B. vesperuginis* is more closely related to the phylogenetic group of Theileriidae than to *Babesia* s.s.
6. DNA of free living bodonids (*Bodo saltans* and neobodonids) have been identified in three DNA samples of bat ectoparasites. At the same time, it was unexpected that none of the bat ectoparasites were found to contain the DNA of bat-specific trypanosomes.
7. *Babesia canis* DNA was shown to be present in faecal samples from Hungary. This may be the first molecular evidence that both main European genotypes of *Ba. canis* (group A, B) occur in Hungary. To the best of our knowledge, this is the first finding of a *Besnoitia*-like sequence from a non-ungulate mammal in Europe, and from any bat species in a world-wide context. *R. helvetica* DNA was shown to be present in bat faeces. Here *Neorickettsia* DNA was identified in the faeces of bats. This is the first molecular evidence on the occurrence of a *Neorickettsia* risticii in Europe. Haemoplasmas of the haemofelis group (unidentifiable to the species level) were shown to be present in the faeces of the same bat species, it is likely that relevant bats were actually infected with the detected bacteria.

Scientific publications

In peer-reviewed journals

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