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Eco-epidemiological investigation of blood-sucking ectoparasites of bats

Ph.D. thesis

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1. Summary

Bats (Chiroptera) are the second largest order of mammals after rodents, also the most widely distributed land-based mammals. They can be found all around the world, except the hot deserts and polar regions. Appearance of men in bat habitats and adaptation of bats to urban areas increased the chances for contact between humans and bats. Phylogeographical studies allow precise genetic comparison of specimens, which were collected over large geographical ranges and belong to the same or closely related animal species. We investigated bat ectoparasites with molecular biological methods in order to make a taxonomic comparison in a geographical context.

In the present work a total 329 Ixodid ticks (*Ixodes vespertilionis*, *I. ariadnae*, *I. simplex*) were collected from bats from 13 countries in Eurasia between 1890 and 2015. The DNA of the ticks were extracted, and PCRs were performed to amplify part of the COI, 16S and 12S rDNA genes, followed by sequencing for identification and molecular-phylogenetic comparison. No morphological differences were observed between *Ixodes vespertilionis* specimens from Spain and from other parts of Europe, but corresponding genotypes had only 94.6 % COI sequence identity. An *I. vespertilionis* specimen collected in Vietnam was different both morphologically and genetically. Two ticks (collected in Vietnam and in Japan) formed a monophyletic clade and shared morphological features with *I. ariadnae*, recently described and hitherto only reported in Europe. In addition, two Asiatic specimens of *I. simplex* were shown to differ markedly from European genotypes of the same species. Phylogenetic relationships of ticks showed similar clustering patterns with those of their associated bat host species.

In comparison with the ixodid ticks, *Argas vespertilionis* showed a lower degree of mitochondrial gene heterogeneity over the same geographical region of Eurasia. Altogether 329 soft tick larvae were collected in seven countries. Based on the general morphology of 314 *A. vespertilionis* larvae, and the detailed measurements of fifteen larvae, only minor morphological differences were observed between specimens from Europe and Vietnam. On the other hand, gene sequence analyses of 17 specimens showed that *A. vespertilionis* from Europen is genetically different from specimens collected in Vietnam, and their phylogenetic separation is well supported. In its evaluated geographical range, no larval phenotypic differences justify the existence of separate species under the name *A. vespertilionis*. However, phylogenetic analyses suggest that it represents a complex of at least two putative cryptic species.

Altogether 216 cimicid bugs were collected and molecularly analyzed. Members of the *Cimex lectularius* species group were found both in the environment of bats (only *Myotis emarginatus*, which is a cave/attic-dwelling species) and on three crevice-dwelling bat species (two pipistrelloid bats and *My. bechsteinii*). On the other hand, *Ci. pipistrelli* always occurred

off-host (near *My. myotis/blythii*, which are cave/attic- dwelling species). In addition, two *Cacodmus* spp. were collected from *Pipistrellus hesperidus*. Analysis of cox1 sequences generated from 38 samples indicated relative genetic homogeneity of *Ci. pipistrelli*, while the *Ci. lectularius* group had two haplotypes (collected from pipistrelloid bats in Hungary and Vietnam) highly divergent from other members of this species group. Bat-associated bugs morphologically identified as *Ca. ignotus* and *Ca. sparsilis* were different in their cox1, but identical in their ITS2 sequences. Molecular evidence is provided here on the existence of two new genotypes, most likely new species, within the *Ci. lectularius* species group. The relevant specimens (unlike the others) were collected from pipistrelloid bats, therefore the association of *Ci. lectularius* with different bat host species (pipistrelloid vs myotine bats) should be evaluated further as a possible background factor of this genetic divergence. In addition, *Ca. ignotus* is reported for the first time in South Africa.

Bats and their blood-sucking ectoparasites are recognized to be natural reservoirs of a large variety of pathogens, among them many with zoonotic potential to infect humans. In this study, 308 bat tick, collected from Hungary and Romania, were molecularly analysed for the presence of piroplasm DNA. Piroplasms DNA were detected in 20 bat ticks. *Ixodes simplex* carried piroplasm DNA sequences significantly more frequently than *I*. *vespertilionis*. 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* and *Theileria* sp. OT3 were amplified, as well as a shorter sequence of the zoonotic *B*. *venatorum*. This may indicate either that 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 arthropod vectors.

193 DNA extracts out of 325 *A. vespertilionis* larvae were screened vector-borne pathogens and 12 contained piroplasm DNA (10 from Hungary, two from China). Sequencing showed the presence of *B. vesperuginis*, with 100% sequence identity between samples from Hungary and China. This is the first molecular evidence on the occurrence of *B. vesperuginis* in Asia. Bat ticks from hosts in Vespertilionidae contained only the DNA of *B. vesperuginis* (in contrast with what was reported on bat ticks from Rhinolophidae and Miniopteridae). Molecular taxonomic analyses of *A. vespertilionis* and *B. vesperuginis* suggest a genetic link of bat parasites between Central Europe and Central Asia, which is epidemiologically relevant in the context of any pathogens associated with bats. Four DNA samples, which were extracted from bat soft tick (*A. vespertilionis*) larvae and were positive in the PCR from Hungary, served to amplify an approx. 950-bp fragment of cox1 gene of *B. vesperuginis* and to compare its sequences with those from other piroplasmid groups in a broader phylogenetic context. Sequence comparisons focusing on either 18S rRNA or cox1 genes, as well as phylogenetic analyses involving separate and concatenated 18S rRNA and cox1 sequences indicate that *B.*

vesperuginis is more closely related to the phylogenetic group of Theileriidae than to *Babesia* s.s. In particular, *B. vesperuginis* clustered closest to *Cytauxzoon felis* and the 'prototheilerid' *B. conradae*. The results of this study highlight that *B. vesperuginis* is a unique and taxonomically important species, which should be included in future studies aimed at resolving the comprehensive phylogeny of Piroplasmida.

Amplification of 18S rRNA gene of kinetoplastids was attempted from 307 ixodid and 299 argasid ticks collected from bats, and from 207 cimicid bugs collected from or near bats in Hungary and Romania. Three samples, one per each bat ectoparasite group, were PCR positive to the DNA of free-living bodonids (*Bodo saltans* and neobodonids), but no trypanosomes were detected. However, how bodonids were acquired by bats, can only be speculated. Bats are known to drink from freshwater bodies, i.e. the natural habitats of *B. saltans* and related species. Consequently, the DNA of bodonids might pass through the alimentary mucosa of bats into their circulation. The above findings highlight the importance of studying bats and other mammals for the occurrence of bodonids in their blood and excreta, with potential relevance to the evolution of free-living kinetoplastids towards parasitism.

Altogether 221 (mostly individual) bat faecal samples were collected in Hungary and the Netherlands. The DNA was extracted, and analysed with PCR and sequencing for the presence of arthropod-borne apicomplexan protozoa and bacteria. *Babesia canis canis* (with 99-100 % homology) was identified in five samples, all from Hungary. Because it was excluded with an Ixodidae-specific PCR that the relevant bats consumed ticks, these sequences derive either from insect carriers of *Ba. canis*, or from the infection of bats. In one bat faecal sample from the Netherlands a sequence having the highest (99 %) homology to *Besnoitia besnoiti* was amplified. Rickettsia DNA was detected in 13 bat faecal DNA extracts, including the sequence of a rickettsial insect endosymbiont, a novel Rickettsia genotype and *Rickettsia helvetica*. Faecal samples of the pond bat (*Myotis dasycneme*) were positive for a *Neorickettsia* sp. and for haemoplasmas of the haemofelis group. In conclusion, bats were shown to pass rickettsia and haemoplasma DNA in their faeces. Molecular evidence is provided for the presence of *Neorickettsia* sp. in bat faeces in Europe.

2. Introduction

Bats (Chiroptera) are the second largest order of mammals after rodents, constitute more than 20% of living mammal species (Simmons 2005). Bats had separated from the mammals probably in the end of the Cretaceous period, the oldest fossils originate from the early Eocene of Europe and North-America (Novacek 1985; Dietz et al. 2009). Their adaptive radiation was probably response to an increase in prey diversity and that the varied echolocation and flight strategies that characterize families may have evolved as a result of differential exploitation of ecological niches available at that time (Teeling et al. 2005). Bats were divided into two subordinal groups: the large, non-echolocating fruit-eating megabats (Megachiroptera) with one family (Pteropodidae) and the smaller, mostly insectivorous and echolocating microbats (Microchiroptera) with 18 families (Dobson 1875), but new molecular genetic studies revealed that the rhinolophid bats are more closely related to the Pteropodidae than to the other microbats. After these findings the order of Chiroptera was divided into two new infraordinal groups: Yinpterochiroptera (Rhinolophoidea and Pteropodidae) and Yangochiroptera (Teeling 2000; Teeling 2005). The two groups were renamed to Pteropodiformes (Yinpterochiroptera) and Vespertilioniformes (Yangochiroptera) (Hutcheon, 2006).

Bats are also the most widely distributed land-based mammals. They can be found all around the world except hot deserts and polar regions (Altringham 1996). The use of caves as daytime roosts probably led to the adaptation to night activity and echolocation. Caves have great advantages for bats to hide. In the tropics, caves offer stable microclimate and provides divers roosting sites for large aggregations. In temperate regions most of them are too cold for summer roosts, they are used mostly for winter hibernation and roosting sites (Dietz et al. 2009).

Bats are the only mammals capable of active flying. This flying ability evolved more than 50 million years ago. Bats developed a variety of styles of flight to adapt to the different ecological niches. The morphology of the wing and the style of the flight differs from species to species because it has been influenced by their foraging habit, roosting style, echolocation type, migration and hibernation behavior (Jepsen 1970, Dietz et al. 2009). There is much variation in flight pattern and foraging habits between different insectivorous species. Bats that inhabit bushy or wooded areas (like *Rhinolophus* sp., *Myotis* sp., *Plecotus* sp., *Pipistrellus* sp.) are well adapted to low-speed flight, usually hunt near vegetation (“gleaning bats”). *Plecotus* and most *Myotis* species pick their prey from leaves, trunks or rocks. Their prey spectrum contains bugs, earwigs (Dermaptera), bush-crickets, spiders and various larvae. *My. myotis* and *My. bechsteinii* fly as low as possible above the forest floor or over a meadow and hunt for flightless arthropods like beetles (*Carabus* and *Melolontha* sp.) or crickets (Tettigoniidae)

or take a short rest on a tree trunk and passively listen to all noises of insects (Russo et al. 2007). Other *Myotis* species like *My*. *daubentonii*, *My*. *dasycneme* and *My*. *capaccinii* hunt for insects over water surfaces. They have large hind feet with long toes and are free from the flight membrane. The perch hunting (flycatcher) method is used by *Rhinolophus* species. They hang on a branch and actively scan the surroundings. *Pipistrellus* species are "aerial hawkers", they mostly hunt above the canopy. Other aerial hawkers like *Nyctalus* species and *Tadarida teniotis* fly high and fast far above the tree canopy or clearings. *Mi. schreibersii* can also fly high and fast, travel long distances, but hunt with a slow flight. Bats in Europe can occur together without mutual competition, because they occupied different food niches in their evolution (Vaughan 1970; Fenton 1982; Dietz et al. 2009).

Some species migrate to distant areas and some of them gather at the near caves in a behavior known as swarming. Up to many hundreds of bats may arrive and depart in caves during a night. This swarming activity acts as a mating behavior, allows the gene flow between the isolate summer colonies. This may also help to the localization and assessment of the potential hibernacula. Within the swarming season, there is a variation in behavior and the timing of swarming activity from species to species (van Schaik et al. 2015). In Europe, depending on the weather, bats move from October to November to their winter roosts. Most of the species choose caves and mines for hibernation, others like *N. leisleri, N. lasiopterus, My. bechsteinii, Pi. pygmaeus, Pi. nathusii* use mostly tree holes, *N. noctule, V. murinus, E. serotnius* hibernate predominantly in buildings, roofs and church towers. During hibernation, bats have been shown to have preferences for certain sites and microclimates. Adequate and stable humidity is one of the most important attributes (van Schaik et al. 2015). Bats are able to choose optimal hibernation conditions by shifting locations in or between the hibernacula. Some bat species such as *My. myotis, My. daubentonii* and *My. dasycneme* constantly moving in caves during the hibernation period (Daan and Wichers 1968). These bats can be found near the entrance of caves. They are migrating from the rear parts of the cave to the entrance during the winter – to a protected/highly protected place from an exposed one (Daan and Wichers 1968; Zukal et al. 2005). *My. daubentonii* often hibernate in large colonies with up to 20,000 animals (Dietz et al. 2009). *My. emarginatus* males and *R. hipposideros* are non-clustering species, they are hanging freely on exposed places as ceilings or rooftops. They are capable for hibernating in higher air temperature but in stable microclimate in the inner parts of the cave (Daan and Wichers 1968, Zukal et al. 2005). *My. emarginatus* have an unusually long hibernation period. Some animals stay in their winter roosts until the middle of May (Dietz et al. 2009). *R. ferrumequinum* in contrast to *R. hipposideros* form large aggregations in warmer and stable places (Borda et al. 2007). *My. capaccinii* and *R. euryale* were found at low elevation at warm temperature in Romania (18.0 °C, thermal conditions created by hot springs) (Nagy and Postawa 2010).

The majority of the bat species are sedentary or occasionally migrant, and only travel from summer roosts to the nearest optimal winter roosts. However, some bats in temperate regions of the world perform regional- and long-distance flight. Typical sedentary species are the *Rhinolophus*, *Plecotus,* and most of the *Myotis* species. Their summer and winter roosts are only a few kilometers apart. Other bats migrate more than some hundreds of kilometers from summer to winter roosts in high lands to find frost-free underground sites. *My. dasycneme* and *My. myotis* considered to be a facultative migrant. They migrate between the lowlands and mountainous regions with no preference for direction. *Nyctalus* species (*N. noctula* and *N. leisleri*), *Pi. nathusii*, *V. murinus* cross large areas of Europe as long-distance migrants. These bats fly up to 3-4,000 km in one return flight. Also *N. lasiopterus* and *Pi. pipistrellus* considered to be long-distance migration bats. *Mi. schreibersii* is an exceptional species. This bat accomplishes seasonal migration in several regions (from a few kilometers, to up to hundreds of kilometers), but most individuals are rather sedentary. Records indicate that majority of the bats in central Europe move southwest, while bats in eastern Europe move into a south-eastern direction (Hutterer 2005).

Migrating capabilities, varied lifestyles, and the diverse diet of bats make them one of the most important providers of the ecosystem of terrestrial biotic communities. They are one of the most important interaction partners of *plants* for *pollination* and *seed dispersal, and* they help to control insects. In the past few decades they are also recognized to be natural hosts of many zoonotic diseases. Myths and misunderstandings about bats lead to a declining population. However, their migratory habit, high population density and roosting behavior increase the transmission of several infections, and enables pathogens to spread long distances. Studies confirmed a phylogenetic relationship between bats and viruses, without demonstrating apparent disease. Bats host more zoonotic viruses per species than do rodents and most of them show high human pathogenicity (Luis et al. 2013) (**Table 1**).

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. In an extended research, a total of 98 ecto- and endoparasite was identified on 14 native *Myotis* species in Europe. 43% (n=42) of the parasites belonged to arachnids and 37% (n=30) to insects. The parasitic fauna of *Myotis myotis* showed the highest diversity (Frank et al. 2015).

Ticks are widely distributed among host taxa, which include mammals, birds, reptiles and amphibians. Hard ticks (**Acari: Ixodidae**) that are specific to bats are only known to occur in the Old World. For more than a century, 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 (Hornok et al. 2014) (**Figure 1.**). These four species appear to differ in their host preferences, i.e. *I.*

vespertilionis occurs predominantly on *Rhinolophus* spp. (Arthur 1956; Piksa et al. 2014*), I. ariadnae* mainly on *Myotis* spp. (Hornok et al. 2015a), whereas *I. simplex* on *Miniopterus schreibersii* (Arthur 1956) and *I. kopsteini* on Molossidae (Robbins and Bangs 2004). *I. vespertilionis* has a worldwide distribution, can be found in Europe, Asia and Africa, primarily found in caves. Two cases exist in which the tick was found feeding on human after caving (Piksa et al. 2013). The pattern of seasonal infestation of *I. vespertilionis* on bats coincides with their seasonal activity (Piksa et al. 2014). *I. simplex* can only occur in bats and highly specialized to *Mi. schreibersii*. Despite the widely distribution *I. simplex* is a relatively rare species because of the close relationship to the main host. Mainly dwells in caves with the summer colonies of *Mi. schreibersii* (Siuda et al. 2009). *I. ariandae* has been found in Hungary (Hornok et al. 2014), Germany (Hornok et al. 2015b) and Belgium (Hornok and Krawczyk 2016b) so far. Thus, the geographical range limit, the migration habit, the various habitat preferences and hibernating behavior of relevant bat species will significantly influence the geographical distribution of their ticks. 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. Formerly, allopatric genotypes of *I. vespertilionis* were shown to exist between distant caves within a country (Hornok et al. 2014). To extend the scope of such observations, the present study was initiated in order to evaluate the mitochondrial gene heterogeneity of ixodid bat ticks over a larger range in the Old World, including ticks that showed the morphological characteristics of *I. vespertilionis*, *I. simplex* and *I. ariadnae*. In Southern and Eastern Asian countries involved in the present study the sample number was limited, because ixodid bat ticks are rare (e.g. Yamauchi and Funakoshi 2000). It was also within the scope of this study to examine the geographical range and host spectrum of these tick species in Hungary and Romania.

I. ricinus, one of the most common tick in West Palearctic, rarely can infest bats. More cases were reported from literature. The host species was *My. myotis* and *My. bechsteinii*. Moreover, two larvae were found in *My. dasycneme* and *My. mystacinus.* Probably the tick attacks the bats while feeding on the ground (Siuda et al. 2009).

The soft tick (**Acari: Argasidae**) *Argas vespertilionis* (syn. *Carios vespertilionis*) (**Figure 2.**), the most common and geographically widespread hematophagous ectoparasite in the Old World, specialized to bats. It was also reported to infest humans and domestic animals (Hoogstraal 1955; Jaenson et al. 1994; Manzano-Román et al. 2012). The taxonomy of the Argasidae is controversial, because the majority of soft tick species can be assigned to

Figure 2. Larva of *Argas vespertilionis* (Photo: Dr. Hornok Sándor)

more than one genus (Burger et al. 2014). Accordingly, the taxonomical status of *A. vespertilionis* also appears to be uncertain. It was originally the type species of the genus *Carios* (Hoogstraal 1958), but in the most recent list of valid tick names it is mentioned as a member of the genus *Argas* (Guglielmone et al. 2010). Recent phylogenetic analyses do not support *A. vespertilionis* as a member of the genus *Argas*, as this species has been misplaced into the subfamily Argasinae (Klompen and Oliver 1993) and based on its 12S rRNA gene it should belong to Ornithodorinae (Burger et al. 2014). In addition, the homogeneity of *A. vespertilionis* on the species level has long been questioned (Hoogstraal 1958). It was also within the scope of this study to molecularly analyze soft tick larvae from Northwestern China in the same context, i.e. to compare their two mitochondrial genetic markers with conspecific larvae from Europe and Vietnam.

Argas transgariepinus parasites bats in Europe, Asia and Africa, only limited information is available about this species. Occasionally can bite humans, the first case was reported in 1913 from Italy when a child was bitten by the parasite (Hoogstraal 1957).

122 species exist of the family **Ischnopsyllidae**, mainly specialized to bats. Members of the *Ischnopsyllus* and *Nycteridopssylla* genus are frequent in Europe. The larvae individuals develop in the roost sides of bats in guano. Human infection has not been known hitherto, however, literature data are also available of human flea infection in the case of other small insectivorous mammals which live close to humans (ex. Pomykal 1985).

Bats are regarded as the primary (ancestral) hosts of bugs in the **Cimicidae**, with subsequent switches to other hosts, including birds and humans. Populations specialized to humans have separate to three species, *Cimex lecturarius*, *Ci. hemipterus* and *Leptocimex boueti* (Balvín et al. 2012a). 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 (Zorrilla-Vaca et al. 2015), but its vector competency awaits verification.

It was suggested that the *Cimex pipistrelli* species group (*Ci. pipistrelli*, *Ci. dissimilis* and *Ci. stadleri*) parasites on bats in the West Palearctic but based on molecular evidences suggests that separate species do not exist in the region. These batbugs are morphologically adapted to a bat host, but not genetically differentiated (Balvín et al. 2013). The morphology of cimicid bugs is frequently a matter of controversy. For instance, in the *Ci. pipistrelli* group, the morphological characters intended to delineate species were shown to vary significantly enough to ascribe progeny of the same female to different species (Balvín et al. 2013; Wendt 1941). Accordingly, the taxonomy of bat-associated bugs is currently in a state of transition. While some formerly distinguished *Cimex* species are suggested to be synonymous (as exemplified by *Ci. pipistrelli* and *Ci. dissimilis*, see Balvín et al. 2013), new species are also discovered/described (Simov et al. 2006). The genus *Oeciacus* (associated with birds) has been transferred to *Cimex* (Balvín et al. 2015). These and other examples highlight the importance of molecular phylogenetic studies focusing on cimicid bugs, which recently have started to expand (Balvín et al. 2013; Booth et al. 2015). Bat-associated bugs, as well as other cimicid bugs, are temporary ectoparasites, which spend most of their life off- host. On the other hand, bat-associated bugs are unable to fly, therefore strictly rely on their hosts for colonization of new habitats, as well as for distribution over large distances (Balvín et al. 2013; Heise 1988). Cimicid bug species associated with bats show different host ranges (Usinger 1966; Balvín et al. 2014). Adaptation to host species has been suggested to be a driver of morphological rather than genetic diversification in the case of *Ci. pipistrelli* (Balvín et al. 2013). At the same time, host preference will also influence the small-scale (habitat-related) as well as large-scale (geographical) distribution of bat-associated bugs (Booth et al. 2015; Balvín et al. 2014; Balvín et al. 2012b). While *Ci. lectularius*, as a man-associated parasite, has a worldwide geographical distribution (Zorrilla-Vaca et al. 2015; Booth et al. 2015), accounts of its bat-related lineages concentrate in the Western Palaearctic (Booth et al. 2015; Balvín et al. 2014). In this region a recent phylogeographic study on *Ci. lectularius* revealed that this species is currently undergoing lineage divergence through host association (Booth et al. 2015). However, in the latter survey Hungary was underrepresented, and in another comprehensive survey on bat-associated bugs (Balvín et al. 2013) the Balkans and other regions of the Old World had not been included. In this thesis, the molecular-phylogenetic research was initiated in order to expand the knowledge on the phylogeny of cimicid bugs of bats.

Bats and their ectoparasites may carry pathogens with high public health impact (**Table 2**). Interestingly, while ixodid ticks of bats are not known to feed on other mammals, except *I. vespertilionis* on humans (Piksa et al. 2013), ixodid ticks that frequently infest domestic animals (e.g. *Ixodes ricinus*, *Dermacentor reticulatus*, *Haemaphysalis* spp.) have also been collected

from bats (Neumann 1911; Sevcik et al. 2010; Estrada-Peña 1989). Only few data are available on their vector potential. Concerning molecular investigations of vector-borne pathogens in ixodid bat ticks, bartonellae were reported from *I. vespertilionis* (Hornok et al. 2012), but to the best of our knowledge none from *I. simplex* or *I. ariadnae*. Therefore, the present study was undertaken to ameliorate this lack of data on pathogens and/or pathogen DNA carried by ixodid bat ticks.

One of the epidemiologically most important haematophagous ectoparasites of bats is the *A. vespertilionis*, because it was also reported to infest humans and domestic animals (Hoogstraal 1956; Jaenson et al. 1994; Manzano-Román et al. 2012). *Borrelia* is a genus of bacteria of the spirochete phylum, the causative agent of *Lyme*- disease and can be transferred primarily by ticks. Among bat ectoparasites soft ticks could be the primary host of this bacteria, causing fatal borreliosis in bats (Evans et al. 2009; Socolovschi et al. 2012). Rickettsiae are other bacteria transferred by arthropods (ticks, fleas, mites), cause acute self-limiting fevers in humans and other animals. *Rickettsia* sp. and other *Elrichia*/*Anaplasma* species, including *Anaplasma phagocytophilum* were found in soft ticks collected from bats (Reeves et al. 2007; Socolovschi et al. 2012). *Rickettsia helvetica* was reported from bat fleas in Hungary (Hornok et al. 2012). *Neorickettsia risticii*, the causative agent of Potomac-fever in horses are also could carry by bats transmitted from trematodes (Gibson et al. 2005). In Buenos Aires, five Tadarida brasilinesis from 61 were positive to the bacteria DNA (Cicuttin et al. 2017). Bats have coevolutionary specificity with *Bartonella* spp. bacteria. *Bartonella* spp. DNA was isolated from bat ticks (hard and soft ticks), fleas, and bed bugs (Reeves et al. 2007; Hornok et al. 2012). Candidatus *Bartonella mayotimonensis* was also found in bats in Finland (Veikkolainen et al. 2014). This can cause endocarditis in humans. *Coxiella burnetii,* that is known to be the main pathogen that causes Q fever in mammals and humans and was detected in urine of bats in Australia and in soft ticks in Algeria (Tozer et al. 2014; Leulmi et al. 2016). Besides bacteria, soft ticks may also carry piroplasms. *Babesia* species (Apicomplexa: Piroplasmida) are intra-erythrocytic piroplasms with more than 100 species described in birds and mammals (Hunfeld et al. 2008), of which only *B. vesperuginis* is known to infect bats. *B. vesperuginis* was reported for the first time from *Nyctalus noctula* in Italy (Dionisi 1899), then from *Pipistrellus pipistrellus*, *Myotis nattereri*, *M. daubentonii*, *M. mystacinus*, *N. noctula* and *Plecotus auritus* in the Netherlands (Goedbloed et al. 1964). British studies found *B. vesperuginis* in the blood of *Pi. pipistrellus* and *M. mystacinus* (Gardner and Molyneux 1987; Concannon et al. 2005). Bats infected with this piroplasm naturally or experimentally showed reduced hemoglobin levels and splenomegaly, justifying the pathogenic nature of *B. vesperuginis* (Gardner and Molyneux 1987). In the latter survey the only ectoparasites found on bats were larvae of *A. vespertilionis*, therefore the vector role of this soft tick species was postulated in the transmission of *B.*

vesperuginis. In this study a large numbers of soft tick larvae were molecularly screen for piroplasms, in order to investigate if they carry a similar diversity of piroplasm DNA.

Bats also demonstrate association with *Trypanosoma cruzi*, that cause trypanosomosis in humans, dourin and surra in horses and brucellosis-like disease in cattle, spread by kissing bugs, evolved from within a broader clade of bat trypanosomes and have successfully switched into other mammalian hosts. More than 30 trypanosome species infect bats including the subgenus *Schizotrypanum* and *T. cruzi*. Bats can get infected by the blood meal of the kissing bugs or through the ingestion of infected arthropods. In Europe, *T. vespertilionis* and *T. dionisii* are the most common in bats. The prevalence of *Schizotrypanum* was 17% across the United Kingdom. *Pi. pipistrellus* was the most infected bat species and the bat bug, *Cimex pipistrelli,* suspected as the vector (Klimpel and Mehlhorn 2014). Bats are known to be infected with the haemosporidians *Polychromophilus melanipherus* and *Po. murinus*. These bat haemosporidia are related to malaria parasites and have no impact to their hosts. In Hungary, bat trypanosomes were found in a former, unpublished case but have never been sought for with molecular methods. Therefore, it was decided to screen a large sample collection of blood-sucking bat ectoparasites (ixodid and argasid ticks, as well as cimicid bugs) for DNA of kinetoplastids. In this context, cimicid bat bugs were the most relevant to test, as they are competent vectors of bat trypanosomes (Gardner and Molyneux, 1988).

Besides the parasites also bat faeces was chosen as sample source for molecular survey, in part because of its non-invasive availability to screen bat samples for arthropod-borne protozoa (Apicomplexa: Piroplasmida and related groups) and bacteria. In addition, molecular investigation of bat faeces proved to be useful in taxonomical identification of macroscopic prey insects (Liu et al. 2010). On the other hand, to the best of our knowledge, this method was hitherto not used to reveal the presence of arthropod-borne pathogens bats may have contact with. Demonstration of microbial/protozoan DNA from bat faeces is not only informative on prey insect (or bat intestinal) pathogens. It may also have relevance to the role bats may play as potential reservoirs of extraintestinal apicomplexans, because invasive stages or intracellular forms of these may cross the gut barrier. In this way the DNA of haemotropic protozoa may pass in detectable amounts with the faeces, as exemplified by *Plasmodium* spp. in primates (Casati et al. 2006).

Table 1. Most common pathogens carried by bats (* vector-mediated pathogens)

Pathogens	Diseases	Can cause disease in	Bat-human transmission	Reference
Viruses				
Rabies virus	Acute fatal encephalitis	human	Yes	Banyard et al. 2011
European Bat Lyssavirus type 1,2	Acute fatal encephalitis	human	Yes	Banyard et al. 2011
Australian Bat Lyssavirus	Acute fatal encephalitis	human	Yes	Banyard et al. 2011
Irkut virus	Acute fatal encephalitis	human	Yes	Banyard et al. 2011
Ebola virus	Haemorrhagic fever	human	Yes	van der Poel et al. 2006
Lloviu virus	Respiratory syndrome	bats	Unclear	Kohl and Kurth 2014
Marburg virus	Haemorrhagic fever	human	Yes	Olival and Hayman 2014
SARS-CoV*	Severe Acute Respiratory Syndrome	human	Yes	Kohl and Kurth 2014
MERS-CoV*	Middle Eastern Respiratory Syndrome	human	Yes	Kohl and Kurth 2014
Nipah virus	severe encephalitis	human, pigs	Yes	Chua 2000
Hendra virus	fatal respiratory disease	human, horses	Yes	Murray 1995
Hantaan virus	Fatal hemorrhagic fever	humans	No	Holmes and Zhang 2015
Mammalian orthoreovirus	Enteric and respiratory infections	mammals	unclear	Wang et al. 2015
Bacteria				
Bartonella spp.*	Endocarditis	humans	unclear	Reeves et al. 2006
Coxiella burnetii	Q fever	humans	unclear	Tozer et al. 2014
Pasteurella spp.	Systemic infections	humans	no	Blehert et al. 2014
Leptospira spp.	Systemic infections	humans	unclear	Vashi et al. 2010
Protozoa				
Babesia spp.*	Babesiosis	bats	unclear	Klimpel and Mehlhorn 2014
Toxoplasma sp.	Toxoplasmosis	humans	unclear	Cabral et al. 2013
Trypanosoma spp.*			unclear	Klimpel and Mehlhorn 2014
Fungi				
Histoplasma capsulatum	Pulmonary and systemic infections	humans	unclear	Klite and Diercks 1965
Pseudogymnoascus destructans	White-nose syndrome	bats	no	Zukal et al. 2016

Table 2. Common vector-borne pathogens carried by bat ectoparasites and their common hosts in Europe.

Vector-borne pathogens	Vector species	Country	Host species	Reference
Bartonella spp.	*Ixodes vespertilionis*	Hungary	cave wall	Hornok et al. 2012
	Ischnopsyllus octactenus	Hungary	*Nyctalus noctula*	Hornok et al. 2012
	Steatonyssus occidentalis	Hungary	*Myotis myotis*	Hornok et al. 2012
	Spinturnix myoti	Hungary	*Myotis myotis*	Hornok et al. 2012
	Nycteribia spp.	Hungary Romania	*Miniopterus schreibersii* *Myotis bechsteinii* *Myotis blythii* *Myotis capaccinii* *Myotis daubentonii* *Myotis myotis* *Rhinolophus ferrumequinum* *Rhinolophus hipposideros* *Rhinolophus mehely*	Hornok et al. 2012 Sándor et al. 2018
	Basilia spp.	Hungary Romania	*Myotis bechsteinii* *Myotis myotis* *Myotis nattereri* *Plecotus auritus* *Plecotus austriacus* *Rhinolophus mehelyi*	Sándor et al. 2018
	Penicillidia spp.	Hungary Romania	*Miniopterus schreibersii* *Myotis blythii* *Myotis capaccinii* *Myotis daubentonii* *Myotis myotis* *Rhinolophus ferrumequinum*	Sándor et al. 2018
	Phthiridium biarticulatum	Hungary Romania	*Myotis capaccinii* *Rhinolophus blasii* *Rhinolophus euryale* *Rhinolophus hipposideros* *Rhinolophus mehely*	Sándor et al. 2018
		England*	*Pipistrellus pipistrellus* *Myotis daubentonii* *Nyctalus noctula*	Concannon et al. 2005
Borrelia sp.	*Carios kelleyi*	England	*Pipistrellus* sp.	Evans et al. 2009
	Argas vespertilionis	France	loft	Socholovschi et al. 2012
	Ixodes ricinus	Poland	*Myotis daubentonii*	Piksa et al. 2016
Ehrlichia sp.	*Argas vespertilionis*	France	loft	Socholovschi et al. 2012
	Argas vespertilionis	England	*Pipistrellus pipistrellus*	Lv et al. 2018
Rickettsia helvetica and other *Rickettsia* sp.	*Argas vespertilionis*	France	loft	Socholovschi et al. 2012
		England	*Plecotus auritus* *Pipistrellus pipistrellus*	Lv et al. 2018
	Ixodes ricinus	Poland	*Rhinolophus hipposideros* *Myotis myotis*	Piksa et al. 2016
	Nycteridopsylla eusarca	Hungary	*Nyctalus noctula*	Hornok et al. 2012
*Babesia vesperuginis**	*Argas vespertilionis*	England	*Pipistrellus pipistrellus*	Lv et al. 2018
Babesia venatorum	*Argas vespertilionis*	England	*Plecotus auritus*	Lv et al. 2018

3. 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* (Chpt: 5.1.1; 5.1.2; 6.1.1; 6.1.2), it was also within the scope of this study to examine the geographical range and host spectrum of these tick species (Chpt: 5.2.1; 6.2.1)
2. to investigate *A. vespertilionis* in the same context, i.e. its morphology, mitochondrial gene heterogeneity and host range in the Old World (Chpt: 5.1.3; 6.1.3)
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) (Chpt: 5.1.4; 6.1.4)
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 (Chpt: 5.2.1; 6.2.1)
5. to molecularly screen large numbers of soft tick larvae for piroplasms (Chpt: 5.2.2; 6.2.2)
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 (Chpt: 5.2.3; 6.2.3)
7. to screen bat faeces for arthropod-borne protozoa (Apicomplexa: Piroplasmida and related groups) and bacteria (Chpt: 5.2.4; 5.3; 6.2.4; 6.3)

4. Materials and Methods

4.1 Collection and identification of parasites and faecal samples

Ixodid ticks (*Ixodes vespertilionis*, *I. ariadnae*, *I. simplex*) were collected from bats, caught for monitoring purposes, from 13 countries (Hungary, Romania, Germany, Serbia, Montenegro, Bosnia- Herzegovina, Czech Republic, France, Spain, Russia, Vietnam, India and Japan) in Eurasia between 1890 and 2015 (**Table 3**). 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.

Table 3. All collection data of bat ticks processed in this thesis.

Tick species	Number of ticks	Place of collection: country
Ixodes vespertilionis	4	Serbia
	1	Czech Republic
	2	Bosnia-Herzegovina
	1	Montenegro
	105	Romania
	19	Hungary
	2	France
	4	Spain
	1	Russia
	1	Vietnam
***Ixodes ariandae/Ixodes ariadnae*- like**	38	Hungary
	1	Germany
	1	Vietnam
	1	Japan
Ixodes simplex	39	Hungary
	100	Romania
	1	France
	1	India
	1	Japan

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 $P<0.05$.

Soft ticks were collected in seven countries, on 13 locations in Hungary, on two locations in Romania, on one location in Italy, on three locations in Vietnam and on one location in Kenya, South-Africa and Mexico. Morphological identification was based on the description of *A. vespertilionis* and *A. transgariepinus* larvae by Hoogstraal (1957 and 1958), and of Ornithodoros larvae (on the genus level) according to Barros-Battesti et al. (2013), and Jones

and Clifford (1972). Structures (**Table 4**) of representative specimens from each country (*A. vespertilionis*: eight larvae from Vietnam, three larvae from Italy, four larvae from Romania; except *A. vespertilionis* from Kenya, which was damaged) were measured under a Jenaval light microscope (Carl Zeiss GmbH, Jena, Germany) after clearance with lactic acid. The means of these data sets 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.

Table 4. Measurements, i.e. size range (mean value) of selected structures with diagnostic importance in the case of *Argas vespertilionis* larvae from three geographical regions.

*Most larvae had broken hypostome; only two specimens from Italy and two from Vietnam allowed measurements

Idiosoma dorsum	Idiosoma venter	Capitulum	Legs
Anterolateral setae (4th)	Sternal setae (3rd)	Post-hypostomal setae	Tarsus I length
Central setae (3rd)	Circumanal setae (1st)	Palpal length	Longest seta of tarsus I (near Haller's organ)
Posterolateral setae (4th)	Circumanal setae (2nd)	Hypostome length*	
Dorsal plate length	Anal valve setae	Hypostome width (anterior)*	
Dorsal plate width	Posteromedian setae	Hypostome width (posterior)*	
Dorsal plate ratio length:width			

Bat-associated cimicid bugs were collected in Hungary (six locations), Romania (two locations), South Africa (one location) and Vietnam (one location) between 2011 and 2016. Concerning study design, because the size of structures important for morphological identification of cimicid bugs was shown to exhibit significant intraspecific variation (Usinger 1966; Balvín et al. 2013), detailed measurements were not taken, and only discrete morphological characters were considered for species identification. Morphological identification of adult bugs was carried out under a stereomicroscope (SMZ-2 T, Nikon Instruments, Japan) by using standard keys (Usinger 1966), focusing on the pronotum, paragenital sinus (*Cimex* spp.) or the paramere (*Cacodmus* spp.). Concerning samples (one from Hungary and two from Vietnam), which showed high degrees of genetic divergences from other members of their phylogenetic group, their conspecificity with *Ci. emarginatus* (reported in Bulgaria) and *Ci. insuetus* (reported in Thailand), respectively, was excluded based on descriptions of the latter species (Simov et al. 2006; Ueshima 1968). *Cacodmus* sp. females were identified according to the *cox1* sequences of morphologically identified males. Pictures were made with a VHX-5000 (Keyence Co., Osaka, Japan) digital microscope.

In 2014, between May and September, bat faecal samples were collected on 38 locations in Hungary, and on 10 locations 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.

4.1.1 Bat capturing and ethical approval

The bats were caught for monitoring and ringing purposes at cave entrances from sunset to dawn, using harp traps or Ecotone mist-nets (Gdynia, Poland) of standard size (12 m length, 2.5 m height and 14 × 14 mm mesh size). Bats were released immediately after parasite removal; their species and sex were recorded. The ectoparasites were immediately put into and stored in 70–95% ethanol. For faecal collection bats were individually held in sterile paper bags (i.e. one bat per one bag) until sufficient defecation.

Authorization for bat capture was provided by the National Inspectorate for Environment, Nature and Water (No. 14/2138-7/2011), the Vietnamese Ministry of Agriculture and Rural Development (Vietnam Administration of Forestry), School of Medicine, Shihezi University (China) and the Underground Heritage Commission (Romania). Bat banding license numbers are 59/2003 (PE), 305/2015 (ADS), TMF-513/1/2004 (SAB), 65/2003 (SAB), TMF-493/3/2005 (TG), TMF-14/32/2010 (DK). Bats were handled according to the current law of animal welfare regulation (1998. XXVIII.). Permission from the Institutional Animal Care and Use Committee (IACUC) was not necessary, because bats were released in the field after ectoparasite removal (none taken to participating Institutes) and defecation.

4.2 Molecular taxonomic analysis

4.2.1 DNA extraction from bat ectoparasites and faeces

DNA of ixodid ticks (*Ixodes ariadnae*, *I. vespertilionis*, *I. simplex*) and bugs (*Cimex*) were extracted individually (except for one *I*. *simplex* larva from *Barbastella barbastellus*) or from hind leg, DNA of *A. vespertilionis* larvae individually or in small pools (i.e. two or three larvae collected from the same host individually) 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 at the bottom of 1.5 ml Eppendorf tubes in 100 µl PBS with pointed scissors. Between each sample the scissors were washed and burned for decontamination. Samples were then incubated overnight at 56°C in tissue lysis buffer containing proteinase-K.

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; Boretti et al. 2009). Samples were consequently used in the dilution (undiluted, 1:10 or 1:100) yielding the lowest Ct value.

4.2.2 Phylogenetic analyses of ixodid ticks based on COI, COX1 (cytochrome oxidase subunit 1) and 12S/16S rDNA genes

In the case of Japanese samples, the COI PCR, that amplifies an up to 710 bp long fragment of the gene with the primers HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'), was performed according to Folmer et al. (1994). Two further PCRs were used to amplify an approx. 460 bp fragment of the 16S rDNA gene of Ixodidae with the primers 16S + 1 (5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3') and 16S - 1 (5'-CCG GTC TGA ACT CAG ATC AAG T-3') (Black and Piesman 1994); and to amplify an approx. 420 bp fragment of the 12S rDNA gene of hard ticks with the primers 12S + 1 (5'-TAC TAT GTT ACG ACT TA-3') and 12S - 1 (5'-AAA CTA GGA TTA GAT ACC C-3') (Norris et al. 1999). For the remaining samples PCR conditions were slightly modified. The COI primers were used in a reaction volume of 25 µl, containing 1 U (0.2 µl) HotStarTaq Plus DNA polymerase, 2.5 µl 10× CoralLoad Reaction buffer (including 15 mM MgCl2), 0.5 µl PCR nucleotid Mix (0.2 mM each), 0.5 µl (1 µM final concentration) of each primer, 15.8 µl ddH2O and 5 µl template DNA. For amplification, an initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 48 °C for 1 min and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 10 min. In the case of selected specimens that were highly divergent according to the COI analysis, the 16S and 12S rDNA gene PCRs were also performed. Reaction components other

than primers, as well as cycling conditions were the same, except for annealing at 51 °C. PCR products were electrophoresed (for visualization), purified and sequenced. The sequences were submitted to GenBank (accession numbers KR902756-77). The partial 12S rDNA gene amplification and sequencing with the above primer pair was not successful in the case of the *I. vespertilionis* nymph from Vietnam and *I. simplex* from India, consistently with the selective success of primers reported by Norris et al. (1999). For bat host species of ticks based on cytochrome b sequences retrieved from GenBank.

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 from Folmer et al. (1994) and amplifies an approx. 710 bp long fragment of the gene. The primers HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') were used in a reaction volume of 25 µl, containing 1 U (0.2 µl) HotStarTaq Plus DNA polymerase, 2.5 µl 10× CoralLoad Reaction buffer (including 15 mM MgCl2), 0.5 µl PCR nucleotide Mix (0.2 mM each), 0.5 µl (1 µM final concentration) of each primer, 15.8 µl ddH2O and 5 µl template DNA. For amplification, an initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 48 °C for 1 min and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 10 min. Another PCR was used to amplify an approx. 460 bp fragment of the 16S rDNA gene of Ixodidae (Black and Piesman 1994), with the primers 16S+1 (5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3') and 16S-1 (5'-CCG GTC TGA ACT CAG ATC AAG T-3'). Other reaction components, as well as cycling conditions were the same as above, except for annealing at 51 °C.
PCR products were electrophoresed in a 1.5% agarose gel (100 V, 60 min), stained with ethidium bromide and visualised under ultraviolet light. Purification and sequencing were done by Biomi Inc. (Gödöllő, Hungary). The sequences were submitted to the GenBank (accession numbers KR093169 and KR093170, respectively).

Phylogenetic analyses were conducted according to the Tamura-Nei model and Maximum Composite Likelihood method by using MEGA version 5.2. Phylogenetic trees were compared in the R development framework.

4.2.3 Phylogenetic analyses of *Argas vespertilionis* ticks based on COX1 (cytochrome oxidase subunit 1) and 16S rRNA genes

From these samples 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. PCR products were visualized in 1.5% agarose gel. Purification and Sanger dideoxy 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. The MEGA model selection method was applied to choose the appropriate model for phylogenetic analyses. In the phylogenetic analyses reference sequences with high coverage (i.e. 98–100% of the region amplified here) were retrieved from GenBank and trimmed to the same start and stop positions (cox1: 652 bp in length, 16S rRNA gene: 439–442 bp in length). This dataset was resampled 1,000 times to generate bootstrap values.

Phylogenetic analyses were conducted with the Maximum Likelihood method and Tamura-Nei model by using MEGA version 6.0.

4.2.4 Phylogenetic analyses of cimicid bugs based on COX1 (cytochrome oxidase subunit 1) gene and ITS2 (internal transcribed spacer 2)

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 (Balvín et al. 2012a). The PCR amplifies a 658 bp long fragment of the cox1 gene of various insect orders. The primers Lep1F (5'-ATT CAA CCA ATC ATA AAG ATA TTG G-3'), Lep1Fdeg (5'-ATT CAA CCA ATC ATA AAG ATA TNG G-3') and Lep3R (5'-TAT ACT TCA GGG TGT CCG AAA AAT CA-3') (Balvín et al. 2015) were used in a reaction volume of 25 µl, containing 1 U (0.2 µl) HotStarTaq Plus DNA polymerase, 2.5 µl 10× CoralLoad Reaction buffer (including 15 mM MgCl2), 0.5 µl PCR nucleotide Mix (0.2 mM each), 0.25 µl (0.5 µM final concentration) of each Lep1F and Lep1Fdeg primers and 0.5 µl (1 µM final concentration) of Lep3R primer, 15.8 µl ddH2O and 5 µl template DNA. For amplification, an initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 53 °C for 1 min and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 10 min. In addition, a similar length fragment of the cox1 gene of the sample from Vietnam was amplified with the primers HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') (Folmer et al. 1994) as reported. 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, with the primers CAS5p8sFc (5'-GCG AAC ATC GAC AAG TCG AAC GCA CAT-3') and CAS28sB1d (5'-TTG TTT TCC TCC GCT TAT TAA TAT GCT TAA-3'). Five µl of template DNA were added to 20 µl reaction mixture, containing 1 U (0.2 µl) HotStarTaq Plus DNA polymerase, 2.5 µl 10× CoralLoad Reaction buffer (including 15 mM MgCl2), 0.5 µl PCR nucleotide Mix (0.2 mM each), 0.5 µl (1 µM final concentration) of each primer and 15.8 µl ddH2O. An initial denaturation step at 95 °C for 5 min was followed by 35 cycles of denaturation

at 95 °C for 30 s, annealing at 59 °C for 40 s and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 7 min. PCR products were visualized in 1.5% agarose gel. Purification and sequencing 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 (including identical haplotypes from different locations) were submitted to GenBank (accession numbers: MF161520–MF161531 for cox1, and MF161532–MF161540 for ITS2). Phylogenetic analyses were conducted by using MEGA version 6.0, with the Maximum Likelihood method and the model (Tamura 3) selected by the program.

4.3 Vector-borne pathogen detection in ectoparasites

4.3.1 Piroplasm DNA detection in ixodid bat ticks based on 18S rDNA gene

DNA samples were molecularly screened with a conventional PCR that amplifies an approx. 500 bp long part of the 18S rDNA gene of piroplasms, modified from Casati et al. (2006). The primers BJ1 (forward: 5'-GTC TTG TAA TTG GAA TGA TGG-3') and BN2 (reverse: 5'-TAG TTT ATG GTT AGG ACT ACG-3') were used in a reaction volume of 25 µl, which included 5 µl of extracted DNA, and 20 µl of reaction mixture containing 0.5 unit HotStarTaq Plus DNA polymerase (5U/ µl), 200 µMPCR nucleotid mix, 1 µMof each primer and 2.5 µl of 10× Coral Load PCR buffer (15mM MgCl2 included). For amplification an initial denaturation step at 95 ˚C for 10 min was followed by 40 cycles of denaturation at 95 ˚C for 30 s, annealing at 54 ˚C for 30 s and extension at 72 ˚C for 40s. Final extension was performed at 72 ˚C for 5 min. 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). Representative sequences were submitted to Gen- Bank (accession numbers KU958544-53).

Phylogenetic analyses were conducted according to the Tamura-Nei model and Maximum Composite Likelihood method by using MEGA version 5.2.

4.3.2 Piroplasm DNA detection in *Argas vespertilionis* based on COX1 (cytochrome oxidase c subunit 1) and 16S/18S rRNA gene

The resultant DNA extracts were first molecularly screened for the presence of piroplasm DNA with a conventional PCR. In brief, this PCR amplifies an approx. 500 bp long part of the 18S rRNA gene of *Babesia/Theileria* spp., using the primers BJ1 (forward: 50-GTC TTG TAA TTG GAA TGA TGG-30) and BN2 (reverse: 50-TAG TTT ATG GTT AGG ACT ACG-30).

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. This method was modified from Gou et al. (2012), with two sets of primers: Bab_For1: (5'-ATW GGA TTY TAT ATG AGT AT-3') and Bab_Rev1: (5'- ATA ATC WGG WAT YCT CCT TGG-3'), then Bab_For2: (5'-TCT CTW CAT GGW TTA ATT ATG ATA T-3') and Bab_Rev2: (5'- TAG CTC CAA TTG AHA RWA CAA AGT G-3'). The 25-µl reaction mixture contained 2 µl DNA template, 12.5 µl Master Mix (PCRBIO Taq Mix Red), 1 µl of both primers (10 pmol/µl) and 8.5 µl water. The thermal profile included 1 min initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 45 °C for 30 s and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 10 min. Purification and sequencing of the PCR products were done by Macrogen Europe (Amsterdam, The Netherlands). The obtained sequences were manually edited, then aligned and compared to GenBank sequences by BLASTN program (https://blast.ncbi.nlm.nih.gov). The consensus sequence was submitted to GenBank (accession number: KY657243).

Phylogenetic analyses of twenty 18S rRNA and *cox1* gene nucleotide sequences (including those of *B. vesperuginis*: 448 bp and 900 bp in length, respectively) were conducted in three steps. First, *cox1* sequences with nearly 100% coverage to that of *B. vesperuginis* were compared with the Maximum Likelihood method [Hasegawa–Kishino–Yano (HKY) model], with 1000 resamplings, by using MEGA version 6.0. Next, for concatenated trees, the evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Nei and Kumar 2000). Initial trees for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree with the highest log likelihood (–13216.2045) was selected. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.8921)]. The rate variation model allowed for some sites to be evolutionarily invariable [(+I), 31.1701% sites]. There were a total of 1433 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7 (Kumar et al. 2016).

BLASTN comparisons between *B. vesperuginis* and other piroplasms, include species shown on the phylogenetic trees, except for *T. bicornis* (for which *cox1* sequence was not available, therefore it had to be omitted from the phylogenetic trees).

4.3.3 Kinetoplastid detection in bat ectoparasites based on 18S rRNA gene

DNA samples were molecularly screened with a conventional PCR that amplifies an approx. 900-bp-long fragment of the 18S (SSU) rRNA gene of trypanosomes and related kinetoplastids. The primers 609F (forward: 5'-CAC CCG CGG TAA TTC CAG C-3') (da Silva

et al. 2004) and 706R (reverse: 5'-CTG AGA CTG TAA CCT CAA-3') (Ramírez et al. 2012) were used in a reaction volume of 25 µl, which included 5 µl of extracted DNA, and 20 µl of reaction mixture containing 0.5 unit HotStarTaq Plus DNA polymerase (5U/µl), 200 µM PCR nucleotide mix, 1 µM each primer and 2.5 µl of 10× Coral Load PCR buffer (15 mM MgCl2 included). For amplification, an initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 49 °C for 1.5 min and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 5 min. All PCRs were run with positive control (sequenceverified *T. avium*) and negative control (non-template reaction mixture). During the tests, positive controls showed positivity, whereas negative (non-template) controls and extraction controls remained negative (the latter indicating the absence of sample contamination). PCR products were electrophoresed and visualized in a 1.5% agarose gel. Purification and sequencing of PCR products were done by Biomi Inc. (Gödöllő, Hungary). The obtained sequences were submitted to GenBank (accession numbers MF000702-4).

4.4 Arthropod-borne apicomplexan protozoa and bacteria DNA detection in bat faeces based on 18S rDNA gene

All samples were molecularly screened with a conventional PCR that amplifies an approx. 500 bp long part of the 18S rDNA gene of piroplasms (Casati et al. 2006). This method also detects other apicomplexan genera, including vector-borne haemogregarines and certain cystogenic coccidia. The primers BJ1 (forward: 5'-GTC TTG TAA TTG GAA TGA TGG-3') and BN2 (reverse: 5'-TAG TTT ATG GTT AGG ACT ACG-3') were used. The reaction volume was 25 µl, i.e. 5 µl of extracted DNA was added to 20 µl of reaction mixture containing 0.5-unit HotStarTaq Plus DNA polymerase (5U/ µl), 200 µM PCR nucleotid mix, 1 µM of each primer and 2.5 µl of 10× Coral Load PCR buffer (15 mM MgCl2 included). For amplification an initial denaturation step at 95 °C for 10 min was followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 40 s. Final extension was performed at 72 °C for 5 min. Electrophoresis and visualization of the PCR product was done in a 1.5 % agarose gel, followed by sequencing (Biomi Inc., Gödöllő, Hungary). Representative sequences were deposited in the GenBank.

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, with the forward primer 16S + 1 (5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3') and reverse primer 16S-1 (5'-CCG GTC TGA ACT CAG ATC AAG T-3'). The original method (Black and Piesman 1994) was slightly modified by using 1.0 unit of HotStartTaq Plus DNA polymerase in a reaction mixture as above, and a thermal profile of initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 51 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. Exact confidence interval (CI) for the prevalence rate was calculated at the 95 % level.

Phylogenetic analyses were conducted according to the Tamura-Nei model and Maximum Composite Likelihood method by using MEGA version 5.2.

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. The presence of tick DNA in the *Rickettsia helvetica*-positive bat faecal sample was excluded by a PCR, which amplifies part of the 18S rRNA gene of Ixodidae (Hornok et al. 2015e). Each PCR was run with positive and negative controls (i.e., sequence-verified DNA extract of the relevant agent and non-template reaction mixture, respectively). Positive controls were always PCR positive, whereas negative controls and extraction controls remained PCR negative. Sanger-dideoxy sequencing was attempted from samples yielding the lowest Ct values. Sequences were aligned and compared to reference GenBank sequences using the nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). Representative sequences were submitted to GenBank (accession numbers: KP862896 [*Neorickettsia* sp.], MF347694 and MF347695 [*Rickettsia* spp.]).

Phylogenetic analysis was conducted according to the Tamura–Nei model and Maximum Likelihood method by using MEGA 6.0.

5. Results

5.1 Molecular taxonomic investigations of bat ectoparasites in a geographical context

5.1.1 Mitochondrial gene heterogeneity of the bat tick species *Ixodes vespertilionis*, *I. ariadnae* and *I. simplex* from Eurasia

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 on larvae) have been collected from 10 countries from Eurasia (**Table 5**).

No morphological differences were noted between specimens of *I. vespertilionis* collected in four different countries of Central-Eastern (CE) Europe. The amplified part of the COI gene showed a maximum of eight nucleotide difference, i.e. 98.7 % identity between isolates. In the phylogenetic tree these genotypes clustered together, but separately from Western European *I. vespertilionis* specimens collected in France (**Fig. 3A**). The latter differed with up to 15 nucleotides, and in that case had only 97.6 % sequence identity with CE European genotypes. The specimens of *I. vespertilionis* from Spain also appeared to be morphologically identical to those from CE Europe. However, the COI genotypes of these South- Western European isolates showed up to 34 nucleotide differences when compared to CE Europe, i.e. only 94.6 % identity between genotypes of the two regions. In the phylogenetic analysis the South-Western European isolates formed a distinct cluster, a sister group of all other evaluated European specimens (**Fig. 3A**), similarly to genotypes of *R. hipposideros*, the main host of *I. vespertilionis* (**Fig. 3B**). 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 (**Fig. 4**). The distinct phylogenetic position of a Spanish *I. vespertilionis* isolate was further confirmed by analysis of part of the 12S rDNA gene, because the genotype from France (KR902776) showed three nucleotide differences from (i.e. 99 % sequence identity to) specimens collected in CE Europe, whereas the Spanish isolate (KR902777) had nine nucleotide differences and thus only 97.5 % sequence identity.

Table 5. Collection data of bat ticks processed in this study. DNA extraction was attempted from all samples, but GenBank accession number is provided only in the case of successful COI gene amplification. 16S and 12S rDNA sequence accession numbers are provided in the text.

*provided by the Natural History Museum of Berlin. Abbreviations: M-male, F-female, N-nymph, L-larva

Tick species	Tick stage/sex	Host species	Date of collection	Place of collection: country (city or region)	GenBank accession number
Ixodes vespertilionis	F	*Myotis mystacinus*	20-05-2010	Serbia (Zlot)	KR902765
	N	*Rhinolophus hipposideros*	03-05-2013	Serbia (Dimitrovgrad)	KR902764
	F, F	(cave wall)	1907*	Serbia (Novi Pazar)	-
	N	*Rhinolophus ferrumequinum*	07-09-2014	Bosnia-Herzegovina (Šipovo)	KR902763
	N	*Rhinolophus* sp.	1899*	Bosnia-Herzegovina (43.2° N, 17.8°E)	-
	N	*Rhinolophus euryale*	22-09-2013	Montenegro (Rijeka Crnojevića)	KR902766
	F	*Rhinolophus hipposideros*	19-09-1913a	Czech Republic (Hranice na Morave)	-
	M, F	near *Rhinolophus hipposideros*	21-02-2008	France (Ruillé en Champagne)	KR902757-8
	F	*Rhinolophus* sp.	ca. 1992	Spain - Vasque Country	KR902759
	N	*Rhinolophus* sp.	ca. 1992	Spain - Vasque Country	KR902760
	N	*Rhinolophus* sp.	ca. 1992	Spain - Vasque Country	KR902761
	L	*Rhinolophus* sp.	ca. 1992	Spain - Vasque Country	KR902762
	M	(cave wall)	1890*	Russia (Caucasus - Labinsk)	-
	N	*Rhinolophus affinis*	20-10-2014	Vietnam (Cao Bang - Phia Oac)	KR902756
Ixodes ariadnae-like	L	*Myotis* n. sp. (undescribed)	17-10-2014	Vietnam (Thanh Hoa - Ban Vin)	KR902767
	F	*Murina leucogaster*	09-03-2013	Japan (Okayama)	LC036330
Ixodes simplex	F	near *Miniopterus schreibersii*	09-07-2008	France (Rancogne)	KR902768
	N	*Miniopterus magnater*	19-02-2015	India (Meghalaya - Jaintia Hills)	KR902769
	L	*Miniopterus fuliginosus*	09-09-2013	Japan (Wakayama)	LC055099

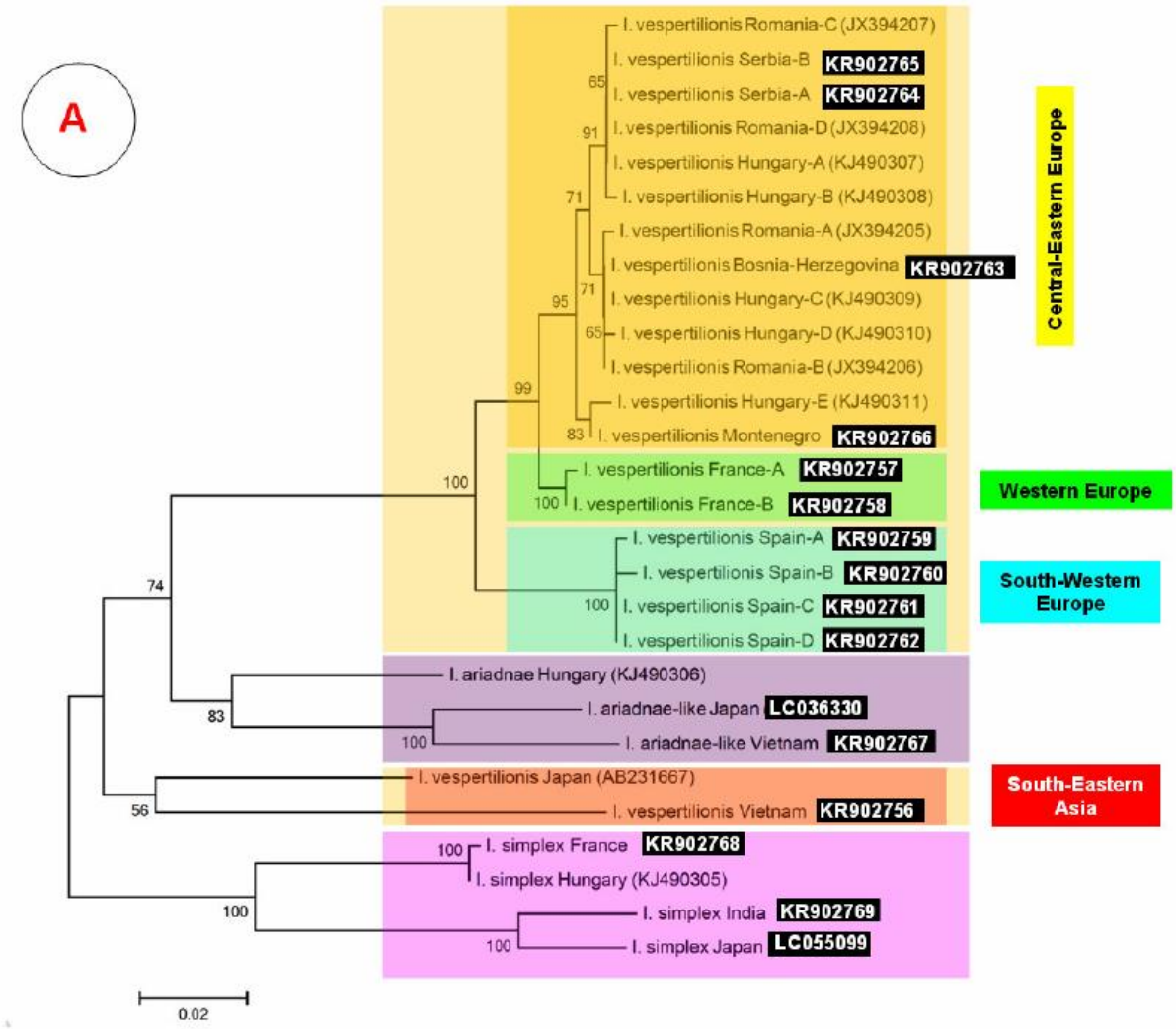

Figure 3A. Phylogenetic relationships of bat ticks collected in the present study (accession number in inverse colour) based on COI gene and related data from the GenBank. Light yellow background indicates isolates of *I. vespertilionis*, with overlaying background colours according to geographical regions as indicated. Purple background colour stands for genotypes of *I. ariadnae*, and pink for those of *I. simplex*.

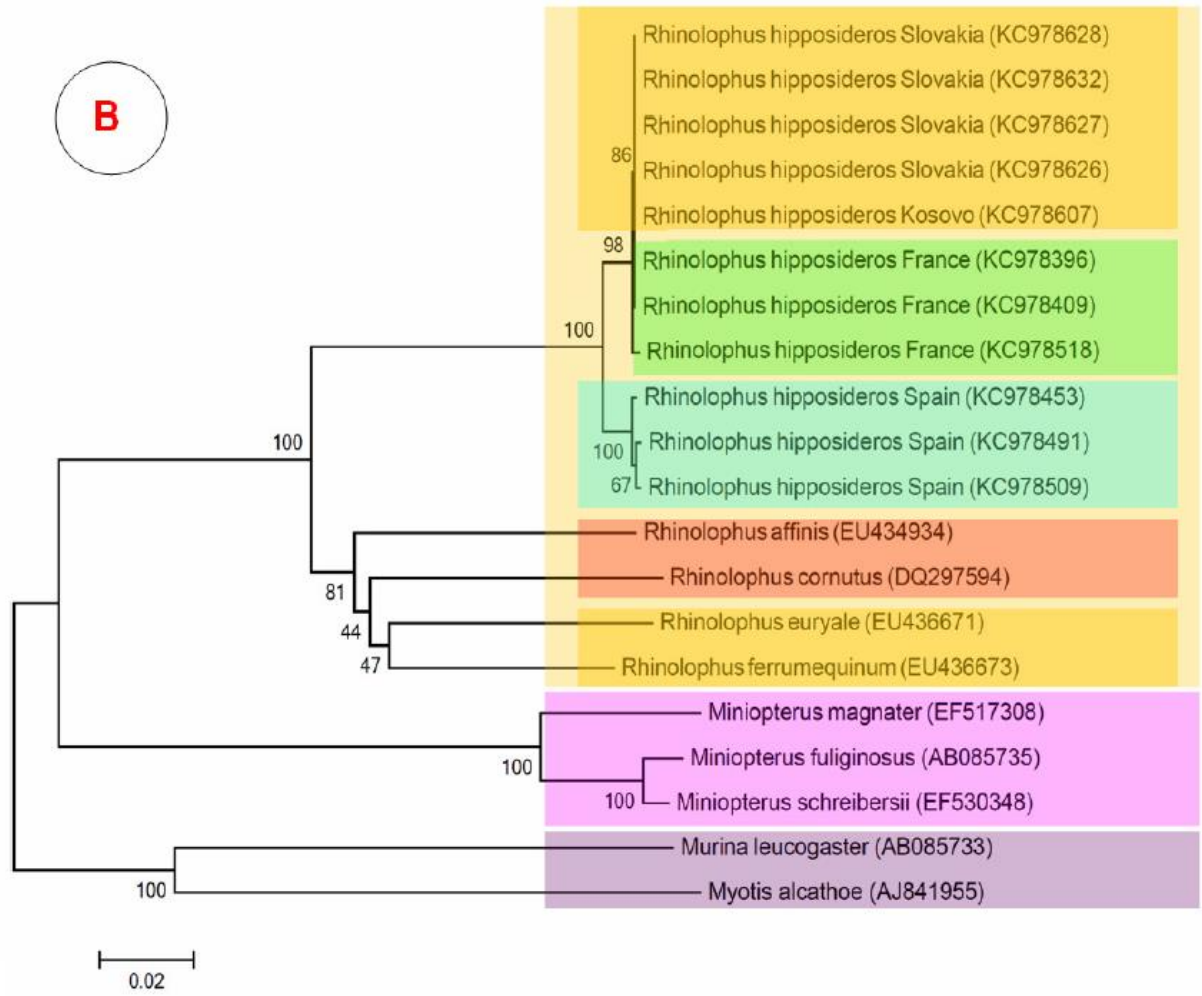

Figure 3B. Phylogenetic relationships of bat species that were hosts of the tick species (according to the colour code of the tick phylogenetic tree) analysed in the present study. Branch lengths correlate to the number of substitutions inferred according to the scale shown

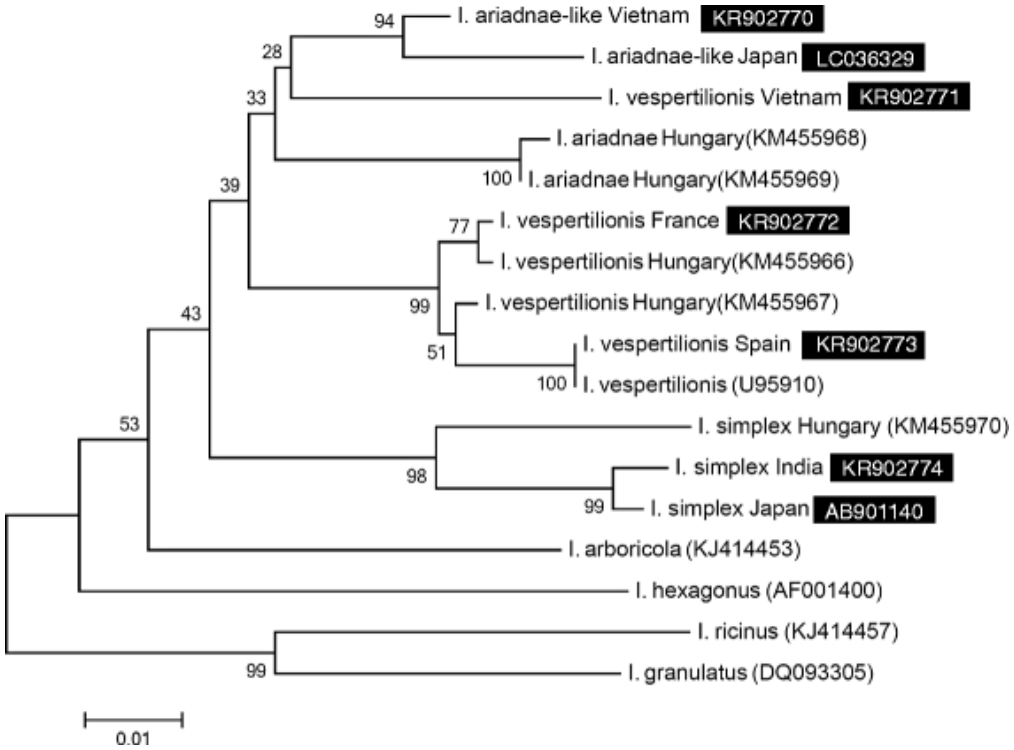

Figure 4. Phylogenetic comparison of 16S rDNA sequences of bat tick genotypes identified in the present study (inverse colour) and other sequences from the GenBank. Branch lengths correlate to the number of substitutions inferred according to the scale shown.

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 with up to 101 nucleotides, i.e. showed only 84.1 % sequence identity and clustered distantly on the phylogenetic tree (**Fig. 3A**). This nymph from Vietnam had the closest sequence identity (88 %) to a formerly published Japanese genotype (AB231667) (**Fig. 5**). Based on its partial 16S rDNA gene sequence (KR902771) this tick clustered separately from all European *I. vespertilionis* specimens and as the sister group of the two Asiatic ticks resembling *I. ariadnae* (**Fig. 4**).

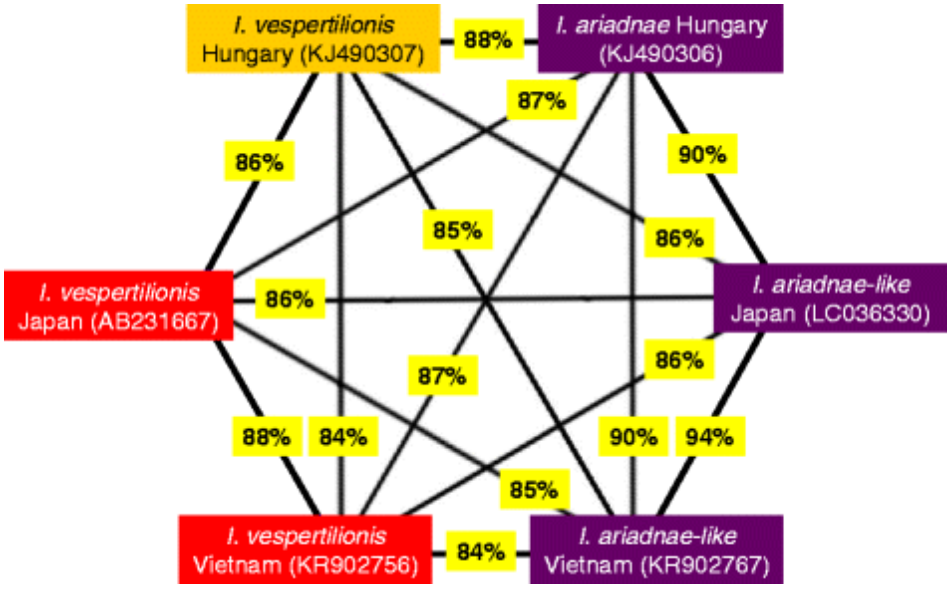

Figure 5. The approximated degree of COI sequence identity between highly divergent genotypes of *I. vespertilionis* and *I. ariadnae*. Background colour of species names corresponds to the one used on Fig. 1a. Three sequences are from other studies (KJ490306-7: (Hornok 2014); AB231667: (Mitani et al. 2007)

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), and only 85.2 % identity with the CE European *I. vespertilionis* genotypes (i.e. up to 87 nucleotide differences). Based on COI phylogenetic analysis this *I. ariadnae*-like genotype from Vietnam clustered close to *I. ariadnae*, but separately from *I. vespertilionis* collected in CE Europe (**Fig. 3A**). 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 both *I. ariadnae* and *I. vespertilionis* (**Fig. 4**). The partial 12S gene sequence of this larva (KR902775) showed only 12–14 nucleotide differences from (96–96.6 % identity with) *I. ariadnae*, whereas 19–20 nucleotide differences from (94.3–94.6 % identity with) *I. vespertilionis*.

One female tick collected from *Murina leucogaster* in Japan also resembled morphologically to *I. ariadnae*. The COI sequence of this specimen (LC036330) differed from the CE European *I. ariadnae* genotype in 65 nucleotides (amounting to 89.7 % identity), but from CE European *I. vespertilionis* genotypes in up to 85 nucleotides (meaning only 86.4 % identity). 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, but separately from *I. vespertilionis* collected in CE Europe (**Fig. 3A**).

This finding was also confirmed by the phylogenetic tree based on the amplified part of the 16S gene (LC036329, **Fig. 4**). The partial 12S gene sequence of this tick (LC036328) showed 15–17 nucleotide differences from (95.4–95.9 % identity with) *I. ariadnae*, whereas 20–21 nucleotide differences from (94.3–94.6 % identity with) *I. vespertilionis*. The genetic distances based on partial COI sequences between highly divergent *I. vespertilionis* and *I. ariadnae* genotypes are summarized in **Fig. 5**.

One nymph, collected from *Miniopterus magnate* in India, showed a similar morphology to *I. simplex*. However, the partial COI sequence of this specimen (KR902769) had 55–58 nucleotide differences from two European genotypes (i.e. 90.8–91 % identity), from which it also clustered apart in the phylogenetic analysis (**Fig. 3A**). Similarly, the separation of this Indian *I. simplex* genotype (KR902774) from the Hungarian one was supported by high bootstrap value in the 16S phylogenetic analysis (**Fig. 4**).

A female *I. simplex*, collected from *Mi. fuliginosus* in Japan, was also included in the present study. The partial COI sequence of this isolate (LC055099) had only 25 nucleotide differences from (i.e. 96 % identity with) the above genotype from India, and in the phylogenetic analysis these two Asiatic *I. simplex* specimens clustered together, but separately from two European (French and Hungarian) genotypes (**Fig. 3A**). The same grouping pattern was seen on the basis of the partial 16S rDNA gene (AB901140) of this isolate (**Fig. 4**). At the same time, its partial 12S rDNA sequence (LC055100) showed 24 nucleotide differences from (93.6 % identity with) *I. simplex* from Hungary.

5.1.2 Identification of *Ixodes ariadnae* in Germany

On 6 March 2015 a long-legged tick was removed from a greater mouse-eared bat (*Myotis myotis*), hibernating in a natural cave at the north rim of the river Bühler valley (49.1550° N and 9.9272° E, ca. 400 m above sea level, northwest of the village Hopfach, Baden-Württemberg, Germany). The collection site is shown in **Fig. 6**. The authorisation number for bat handling is 55-8850.68/SHA (issued by the Regierungspräsidium Stuttgart Abteilung Umwelt).

Figure 6. Map of Europe showing the collection site of Ixodes ariadnae in Germany (red dot) and its formerly reported occurrence in Hungary (black dots)

Based on morphological characteristics, i.e. 5 mm size, long legs, short palps, broad and posteriorly rounded scutum (**Fig. 7**) and sparse covering with setae, the tick was identified as an engorged female of *I. ariadnae*. The greater mouse-eared bat (*My. myotis*), from which the specimen was collected, is a new host record for this tick species.

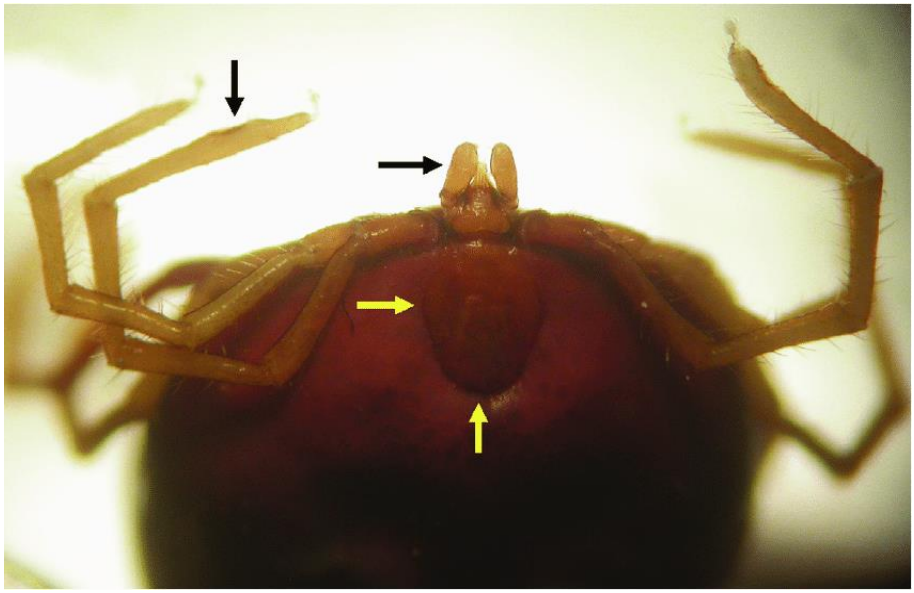

Figure 7. Anteriodorsal view of the bat tick collected in the present study. Arrows indicate the characteristics of *Ixodes ariadnae* mentioned in the text

The partial COI sequence of the tick (KR093169) showed 100% homology with I*. ariadnae* (KJ490306). The partial 16S rDNA gene sequence of the tick (KR093170) was identical with genotype F among Hungarian isolates, collected at the main habitat of *I. ariadnae* in Hungary (Ariadne cave system, Pilis Mountains: Hornok et al. 2015a).

5.1.3 Morphological and mitochondrial gene heterogeneity of the bat soft tick (Ixodida: Argasidae) in the Palaearctic

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 *A. vespertilionis* (**Table 6**). The majority of *A. vespertilionis* larvae (59.1%: 188 out of 318, CI: 53.5–64.6%) were found on *Pipistrellus* spp. (**Table 6**). *Myotis alcathoe* is a new host for this soft tick species.

One specimen from South Africa was identified as *A. transgariepinus* (based on idiosomal setae, palpal articles, coxae and tarsus I). Three larvae from Mexico represented the genus *Ornithodoros* (based on the elongated piriform dorsal plate with non-parallel sides) *but* could not be identified on the species level because of the lack of hypostome.

Measurements of selected, diagnostically important structures of *A. vespertilionis* larvae revealed no significant differences between specimens from Europe and Vietnam (**Table 7**), 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: $t = 3.21$, $df = 13$, $P = 0.012$).

Table 6. Host species, place of collection and GenBank accession numbers for sequences from soft ticks used in this study. n: number of the collected sample from the given stage

Species	Stage (n)	Host species	Country (Locality)	*cox*1	16S rRNA
Argas vespertilionis	larva (58)	*Pipistrellus pygmaeus*	Hungary (Mezőföld)	KX431953	KX831484
	larva (5)	*Myotis alcathoe*	Hungary (Bakony)	KX431955	KX831486
	larva (12)	*Eptesicus serotinus*	Hungary (Béda)	–	–
	larva (15)	*Pipistrellus pygmaeus*	Hungary (Dráva)	–	–
	larva (1)	*Pipistrellus kuhlii*	Hungary (Dráva)	–	–
	larva (27)	*Plecotus austriacus*	Hungary (Dráva)	KX431954	KX831485
	larva (6)	*Myotis dasycneme*	Hungary (Gemenc)	–	–
	larva (58)	*Pipistrellus nathusii*	Hungary (Gemenc)	–	–
	larva (10)	*Pipistrellus pygmaeus*	Hungary (Gemenc)	–	–
	larva (1)	*Pipistrellus pipistrellus*	Hungary (Kecső)	KX431954	KX831489
	larva (2)	*Eptesicus serotinus*	Hungary (Mecsek)	–	–
	larva (1)	*Myotis alcathoe*	Hungary (Mecsek)	–	–
	larva (18)	*Myotis brandtii*	Hungary (Mecsek)	–	–
	larva (27)	*Myotis dasycneme*	Hungary (Mecsek)	–	–
	larva (4)	*Nyctalus noctula*	Hungary (Mecsek)	–	–
	larva (1)	*Plecotus auritus*	Hungary (Mecsek)	–	–
	larva (5)	*Pipistrellus pipistrellus*	Hungary (Mecsek)	–	–
	larva (1)	*Pipistrellus pygmaeus*	Hungary (Mecsek)	–	–
	larva (19)	*Vespertilio murinus*	Hungary (Miskolc)	–	–
	larva (1)	*Myotis alcathoe*	Hungary (Nagyvisnyó)	–	–
	larva (1)	*Pipistrellus pipistrellus*	Hungary (Nagyvisnyó)	–	–
	larva (1)	*Pipistrellus pipistrellus*	Hungary (Noszvaj)	–	–
	larva (1)	*Pipistrellus pipistrellus*	Hungary (Ócsa)	KX431953	KX831488
	larva (2)	*Vespertilio murinus*	Hungary (Sopron)	KX431953	KX831487
	larva (2)	*Eptesicus serotinus*	Romania (Somova)	KX431954	KX831490
	larva (9)	*Pipistrellus pipistrellus*	Romania (Salciua)	–	–
	larva (6)	*Pipistrellus pipistrellus*	Italy (Bergamo)	KX431953–KX431954	KX831496–KX831498
	larva (7)	*Pipistrellus javanicus*	Vietnam (Can Gio)	KX431957	KX831492
	larva (3)	*Pipistrellus cf. abramus*	Vietnam (Thanh Hoa)	KX431958	KX831493
	larva (9)	*Pipistrellus cf. abramus*	Vietnam (Bach Long Vi)	KX431959–KX431960	KX831494–KX831495
	larva (1)	*Pipistrellus cf. rueppellii*	Kenya (South Horr)	KX431956	KX831491
	larva (11)	*Vespertilio murinus*	China (Xinjiang province)	KY657239-KY657240	
Argas transgariepinus	larva (1)	*Pipistrellus hesperidus*	South Africa (Makhado)	KX431961	–
Ornithodoros sp.	larva (3)	*Balantiopteryx plicata*	Mexico (Chiapas)	KX431962	KX831499

Sternal and anal setae were consistently pointed (needle-like), whereas dorsal setae were serrate. The morphology of serrate setae showed minor difference between geographically distant specimens (**Fig. 8**): 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.

Table 7. Measurements, i.e. size range (mean value) of selected structures with diagnostic importance in the case of *Argas vespertilionis* larvae from three geographical regions.
Lengths are provided in µm, rounded to decimals (except for dorsal plate ratio). Values within a row having different superscript letters are significantly different
*Most larvae had broken hypostome; only two specimens from Italy and two from Vietnam allowed measurements

		Italy (*n* = 3)	**Romania (*n* = 4)**	**Vietnam (*n* = 8)**
Idiosoma dorsum	Anterolateral setae (4th)	35–44 (38.3)	31–44 (37.8)	35–48 (42.3)
	Central setae (3rd)	31–45 (37.8)	38–41 (39.5)	33–41 (37.6)
	Posterolateral setae (4th)	51–61 (56.8)	55–65 (61.8)	56–71 (64)
	Dorsal plate length	**200–208 (203.3)$_a$**	**194–211 (204.8)$_a$**	**193–201 (196.3)$_b$**
	Dorsal plate width	**100–111 (106.3)$_a$**	**101–113 (108.3)$_a$**	**99–105 (101)$_b$**
	Dorsal plate ratio length:width	1.85–2 (1.92)	1.87–1.92 (1.89)	1.91–1.98 (1.94)
Idiosoma venter	Sternal setae (3rd)	25–27 (25.8)	23–30 (27.8)	20–35 (25.8)
	Circumanal setae (1st)	30–31 (30.3)	28–30 (29.3)	30–33 (30.8)
	Circumanal setae (2nd)	35–36 (35.3)	34–35 (34.8)	33–37 (35)
	Anal valve setae	32–38 (35)	32–38 (34.8)	35–39 (37)
	Posteromedian setae	23–29 (25.8)	25–28 (27)	25–30 (27.5)
Capitulum	Post-hypostomal setae	10–18 (13.8)	11–14 (12.3)	10–15 (11.9)
	Palpal length	165–180 (173.3)	174–176 (175)	165–176 (170.3)
	Hypostome length*	125–130 (127.5)	–	124–125 (124.5)
	Hypostome width (anterior)*	30–31 (30.5)	–	31–35 (33)
	Hypostome width (posterior)*	36–39 (37.5)	–	39–40 (39.5)
Legs	Tarsus I length	125–135 (129)	124–139 (128.3)	130–137 (132.8)
	Longest seta of tarsus I (near Haller's organ)	36–45 (42.5)	43–47 (44.8)	38–55 (46.5)

Sequencing of the *cox*1 gene fragment was successful in the case of 17 samples (**Table 7**; **Fig. 9**). *Argas 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.

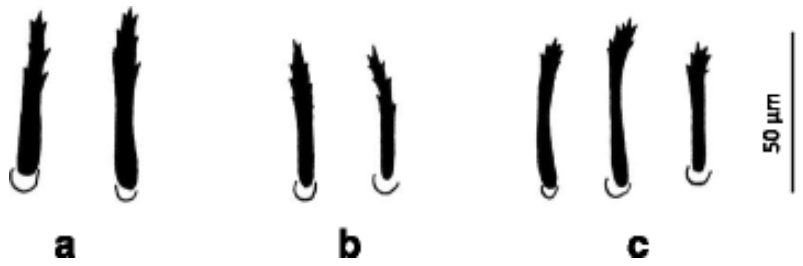

Figure 8. Schematic drawings of differences between serrate setae of *Argas vespertilionis* larvae from Romania (**a**), Italy (**b**) and Vietnam (**c**)

The *cox*1 sequences of *A. vespertilionis* from Vietnam had 2–15 nucleotide (0.3–2.3%) differences from each other, amounting to 97.7–99.7% (637–650/652 bp) similarity, i.e. were more heterogeneous within Vietnam than between samples from three European countries. The topology of the *cox*1 phylogenetic tree reflected the above differences (with high support of separation of *A. vespertilionis* haplotypes both within Vietnam, and between Hungary and Vietnam). Clustering of *A. vespertilionis* isolates with two members of Ornithodorinae received moderate (72%) support (**Fig. 9**).

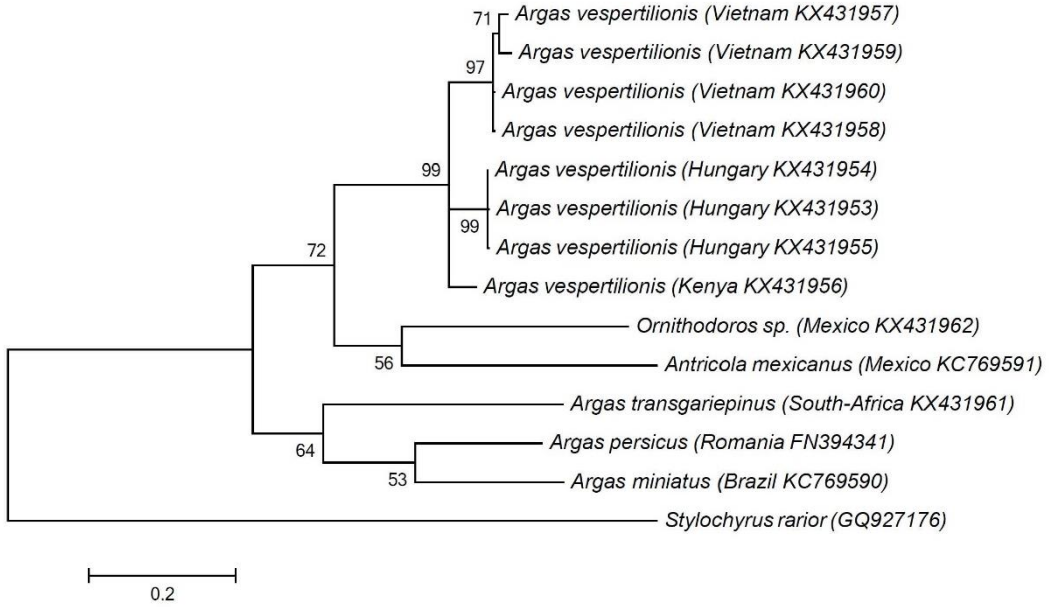

Figure 9. Phylogenetic relationships of *Argas vespertilionis* (collected in Hungary, Kenya and Vietnam) and other soft tick species, based on the cytochrome *c* oxidase subunit 1 (*cox*1) gene. *Cox*1 sequences of *A. vespertilionis* from Romania and Italy were identical with those from Hungary, therefore are not shown. Accession numbers of sequences from this study are highlighted in bold. Branch lengths represent the number of substitutions per site inferred according to the scale shown.

Concerning the amplified part of the 16S rRNA gene, this was successfully sequenced from 16 samples. *Argas vespertilionis* had up to four nucleotide (0.9%) differences (437/441 bp = 99.1% similarity) between European haplotypes, whereas these had 20 nucleotide (4.5%) differences from the *A. vespertilionis* larva collected in Kenya (420/440 bp = 95.5% similarity), and 25 nucleotide (5.7%) differences (416/441 bp = 94.3% similarity) from *A. vespertilionis* larvae from Vietnam. The 16S rRNA gene sequences of *A. vespertilionis* from Vietnam had up to six nucleotide (1.4%) differences from each other, i.e. 98.6% (436/442 bp) similarity. Based on the 16S rRNA phylogenetic tree (**Fig. 10**), 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 **(Fig. 10**).

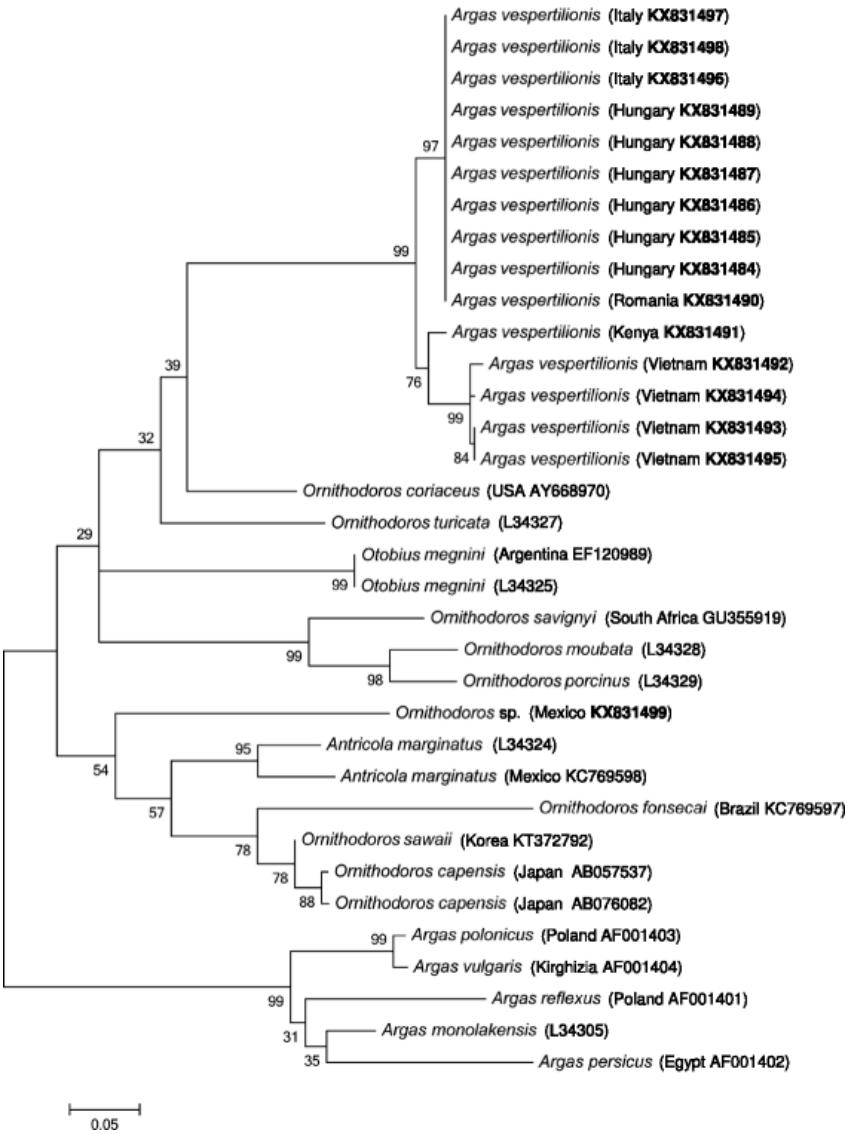

Figure 10. Phylogenetic relationships of *Argas vespertilionis* (collected in five countries), and other soft tick species, based on the 16S rRNA gene. Accession numbers of sequences from this study are highlighted in bold. Branch lengths represent the number of substitutions per site inferred according to the scale shown

Argas vespertilionis cox1 sequences had 5–6 nucleotide (0.8–0.9%) differences, i.e. 99.1–99.2% (646–647/652 bp) similarity between isolates from Hungary and China. On the other hand, haplotypes from China had 45–48 nucleotide (6.9–7.4%) differences from *A. vespertilionis* larvae collected in Vietnam, meaning 92.6–93.1% (604–607/652 bp) similarity with the latter. The cox1 phylogenetic tree (**Fig. 11**) reflected these relationships, i.e. *A. vespertilionis* from Hungary and China clustered together, but separately (with a high, 99% bootstrap support) from those collected in Vietnam. *Argas vespertilionis* 16S rRNA gene sequences had 0–2 nucleotide (up to 0.5%) differences (438–440/440 bp = 99.5–100% similarity) between haplotypes from Hungary and China, whereas these had 23–24 nucleotide

(5.2–5.4%) differences (418/ 441–442 bp = 94.6–94.8% similarity) from *A. vespertilionis* larvae collected in Vietnam.

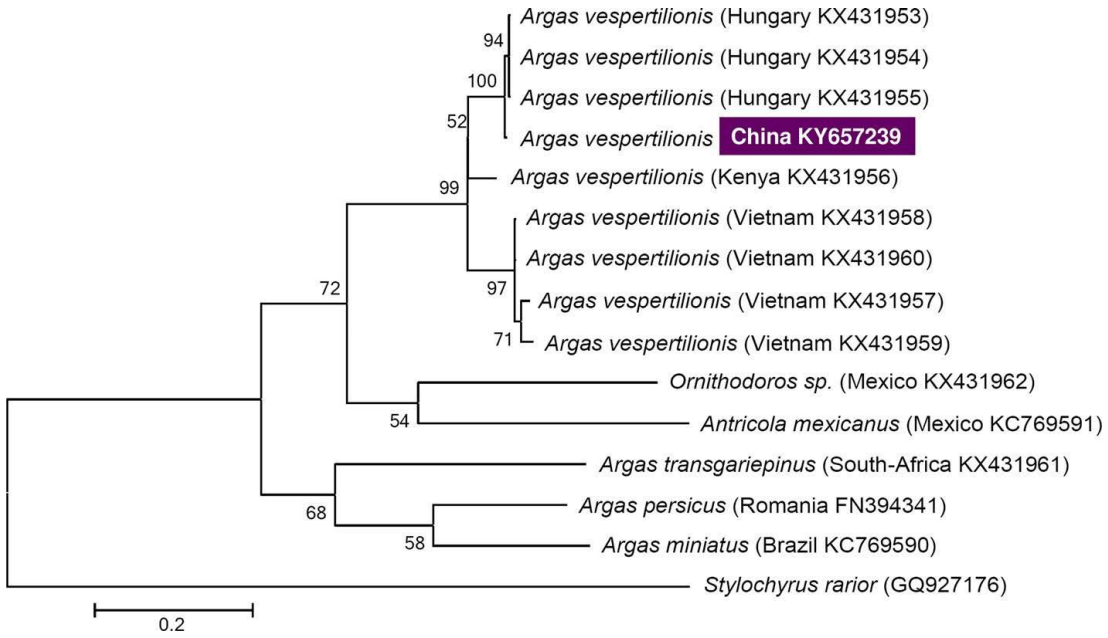

Figure 11. Phylogenetic tree of cytochrome oxidase c subunit 1 (cox1) gene of Argas vespertilionis based on reference sequences in GenBank. Accession number of the sequence from China (this study) is shown in inverse purple. Branch lengths represent the number of substitutions per site inferred according to the scale shown

Based on the 16S rRNA phylogenetic tree (**Fig. 12**), *A. vespertilionis* from China belonged to the group formed by specimens reported from three European countries (Hungary, Romania and Italy), but the separation of *A. vespertilionis* collected in China versus Vietnam was highly supported (100%).

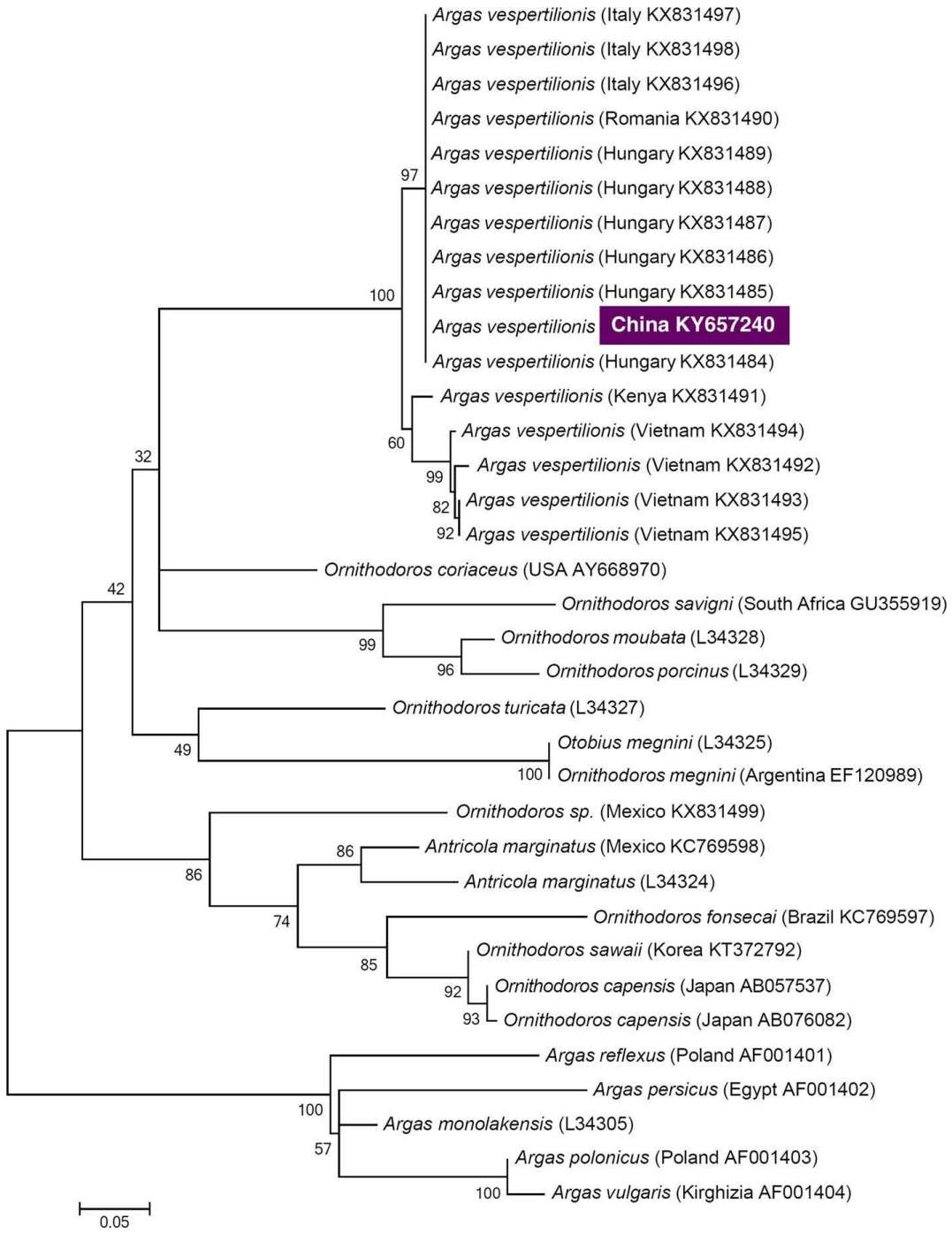

Figure 12. Phylogenetic tree of 16S rRNA gene of Argas vespertilionis based on reference sequences in GenBank. Accession number of the sequence from China (this study) is shown in inverse purple. Branch lengths represent the number of substitutions per site inferred according to the scale shown

5.1.4 Phylogenetic analyses of bat-associated bugs (Hemiptera: Cimicidae: Cimicinae and Cacodminae)

5.1.4.1 General morphology and host species of bat-associated bugs

Altogether 216 cimicid bugs were collected from the bodies or roosts of seven bat species of three genera (**Table 8**). 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 (**Table 8**).

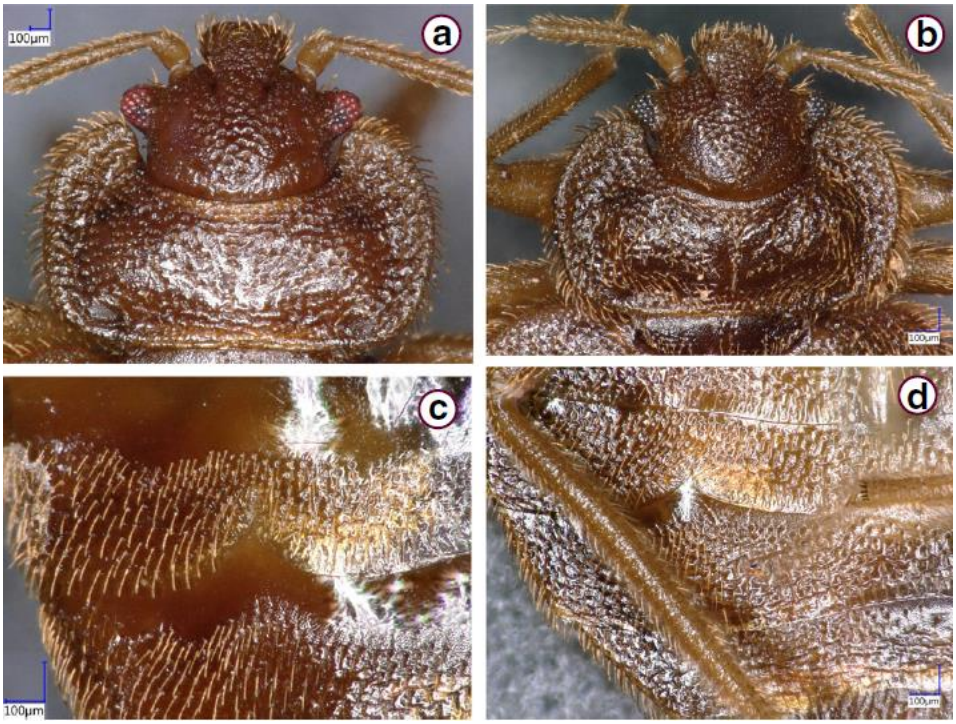

Figure 13. *Cimex lectularius*, females collected near *Myotis emarginatus*, in three locations of Hungary: Dráva (**a, c**); Trizs (**b**); and Ragály (**d**). **a**, **b** Head and pronotum. **c**, **d** Paragenital sinus

The *Cimex lectularius* species group was represented by 73 specimens. These showed similar general morphological characters if collected near bats (**Fig. 13**) or from bats in Hungary (**Figs. 14 and 15b**), including the shape of the pronotum (breadth to length ratio ≥ 2.5, broad lateral lobes), paragenital sinus (cleft with bristles) and external spur on coxa III (with a broad basis). However, while the pronotum and coxal spur were similar in the case of specimens from Hungary (**Fig. 15a-c**) and Vietnam (**Fig. 15d**), the paragenital sinus of the female bug from Vietnam was rounded (**Fig. 16d**).

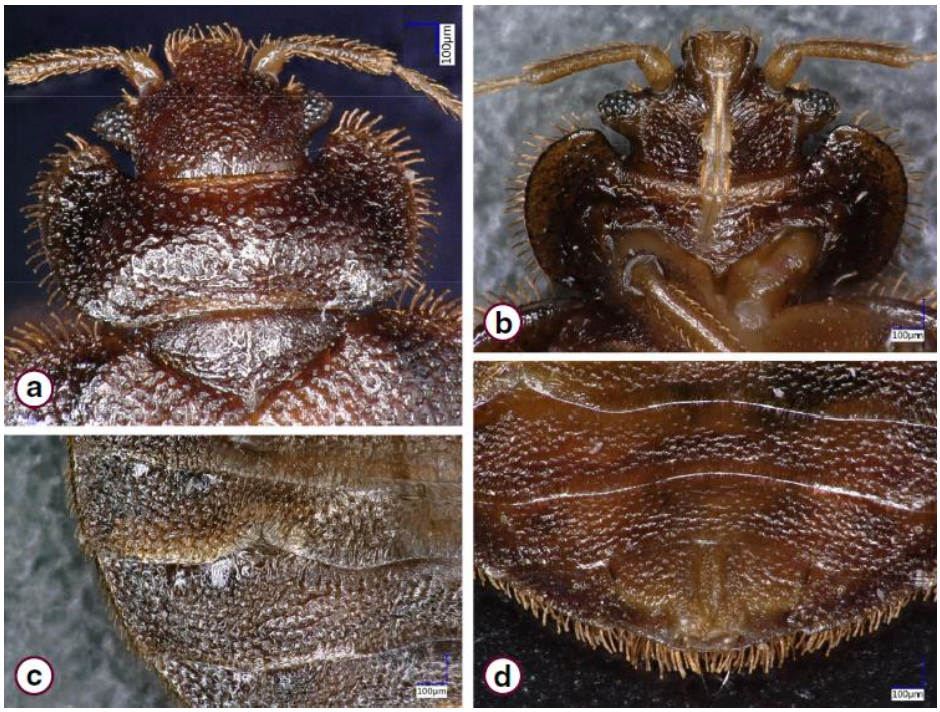

Figure 14. *Cimex* sp., female collected from *Pipistrellus pipistrellus* in Hungary (Nagyvisnyó). **a)** Head and pronotum, dorsal view. **b)** Head and pronotum, ventral view. **c)** Paragenital sinus. **d)** Last two abdominal segments

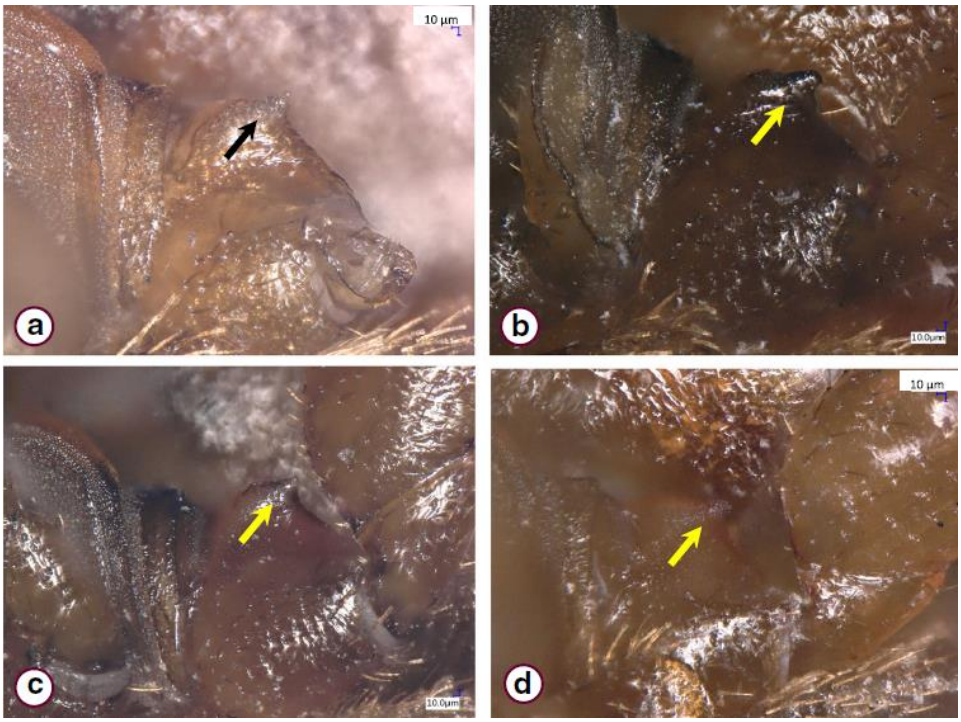

Figure 15. *Cimex lectularius*, broad basis (arrow) of external spur on coxa III. Specimens collected in Hungary from *Pipistrellus pipistrellus* (Nagyvisnyó) (a) and *Myotis bechsteinii* (Noszvaj) (b); in a human dwelling (Budapest, Neptun street) (c); and a specimen collected in Vietnam from *Hypsugo pulveratus* (d)

Table 8. Data for the samples used in this study (*Abbreviations*: *A* attic, *CH* church tower, *LH* lich house, *M* mine, *ns* not successful, *nd* not done)

[a]On host

[b]Used as reference sequence of the given bug species in the text

Bug species or species group	Country	Location (no. of samplings × habitat type)	Host nearby ([a]on host)	Stage			GenBank ID (no. of samples amplified)	
				Nymph	Female	Male	*cox*1	ITS2
Cimex lectularius group	Hungary	Dráva (1 × A)	*Myotis emarginatus*	1	3	4	MF161525 (2×)	ns
		Trizs (3 × CH)		13	19	23	MF161526 (2×); MF161527 (6×)	MF161534[b] (2×); MF161535 (1×)
		Ragály (1 × LH)		1	4	1	MF161522 (3×)	MF161535 (1×)
		Nagyvisnyó (1)	*Pipistrellus pipistrellus* [a]	0	1	0	MF161521 (1×)	MF161532 (1×)
		Noszvaj (1)	*Myotis bechsteinii* [a]	0	0	1	MF161520[b] (1×)	ns
	Vietnam	Thanh Hoa, Ngoc Khe (1)	*Hypsugo pulveratus* [a]	0	1	1	MF415647 (1×)	MF161540 (1×)
Cimex pipistrelli	Hungary	Szőlősardó (2 × CH)	*Myotis myotis/blythii*	3	2	4	MF161523[b] (5×)	MF161533[b] (4×)
	Romania	Sant (1 × M)	*Myotis myotis*	1	0	0	nd	nd
		Leghia (6 × M)	*Myotis blythii*	32	49	42	MF161524 (5×); MF161528 (2×)	MF161536 (2×); MF161537 (1×)
Cacodmus ignotus	South Africa	Makhado (2)	*Pipistrellus hesperidus* [a]	0	2	7	MF161529[b] (8×); MF161530 (1×)	MF161538[b] (2×)
Cacodmus sparsilis				0	0	1	MF161531 (1×)	MF161539 (1×)

Cimex pipistrelli was represented by 133 specimens (**Table 8**). All of these from Hungary and Romania shared the shape of the pronotum (breadth to length ratio < 2.5, narrow lateral lobes) and of the paragenital sinus (cleft and naked) (**Fig. 17**).

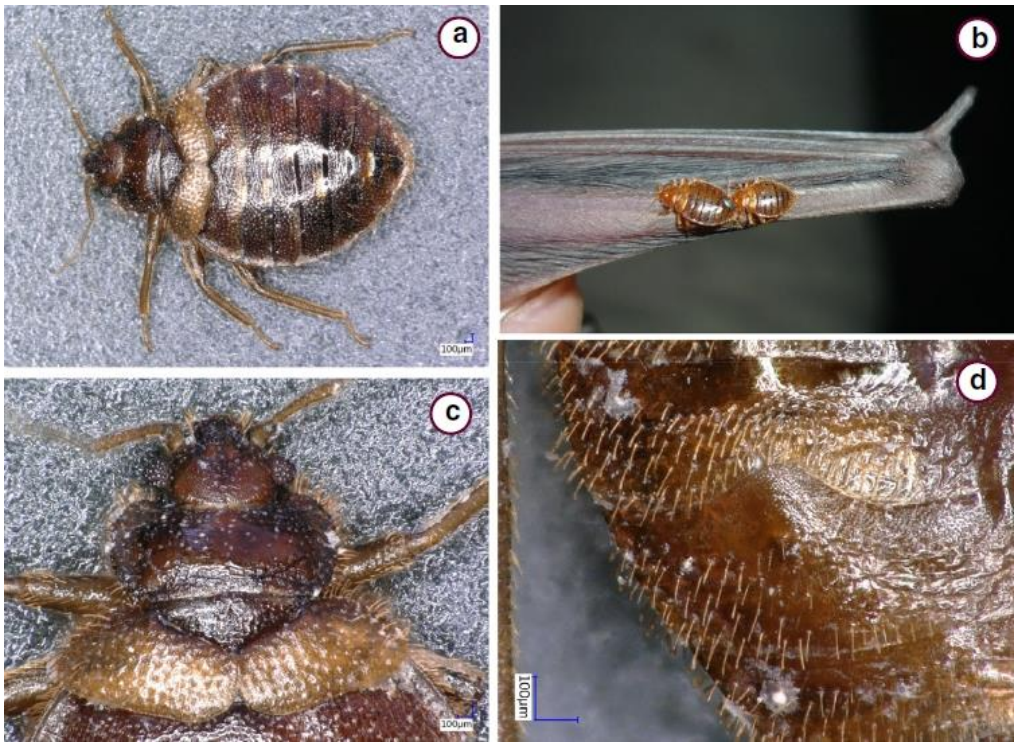

Figure 16. *Cimex* sp., female collected from *Hypsugo pulveratus* in Vietnam. **a)** Habitus. **b)** In situ on bat patagium. **c)** Head and pronotum. **d)** Paragenital sinus

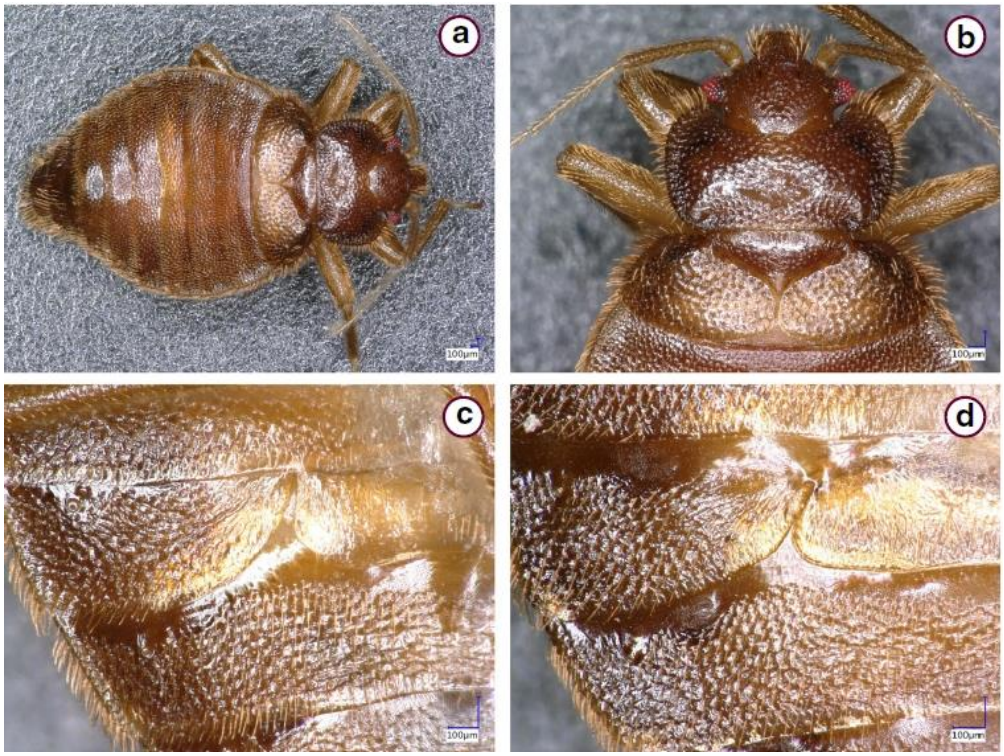

Figure 17. *Cimex pipistrelli*, female collected near *Myotis* spp. **a-c)** Specimen collected in Hungary (Szőlősardó). **a**) Habitus. **b**) Head and pronotum. **c**) Paragenital sinus. **d**) Specimen collected in Romania (Leghia), paragenital sinus.

Cacodmus sp. males from South Africa had either evenly curved and tapering, apically straight, medium to long paramere (**Fig. 18c-d**), or long paramere bent laterally at the tip (almost sinuate at apex) (**Fig. 18b**). On this basis specimens were assigned to *Ca. ignotus* and *Ca. sparsilis*, respectively.

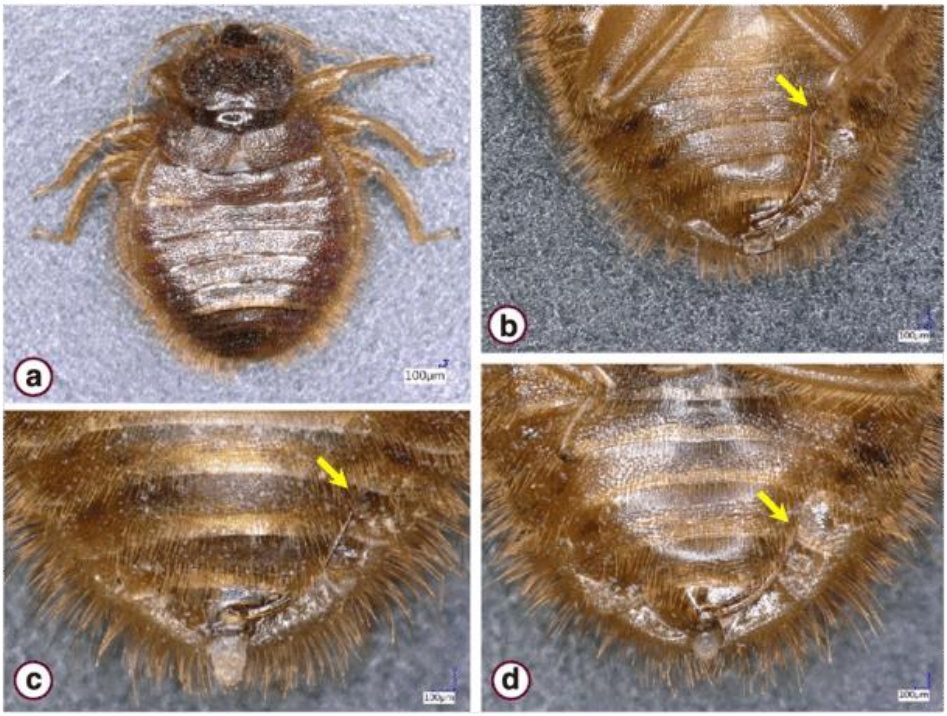

Figure 18. *Cacodmus* spp. collected from *Pipistrellus hesperidus* in South Africa (Makhado). **a**) *Ca. ignotus*, habitus. **b**) *Ca. sparsilis* male with long (> 1000 µm) paramere, curved apically. **c)** *Ca. ignotus* male with medium length paramere (850 µm). **d)** *Ca. ignotus* male with slightly longer paramere (950 µm). Arrows indicate paramere apex.

5.1.4.2 Sequence comparison and phylogeny of bat-associated bugs

The *cox*1 gene fragment was successfully amplified and sequenced from 38 samples (**Table 8**). Bugs morphologically most closely related to *Ci. lectularius* (15 samples) had four *cox*1 haplotypes in Hungary. The majority of these exhibited up to five nucleotide differences from each other, corresponding to 99.2–100% sequence similarity (626–631/631 bp). However, a *Cimex* sp. from *P. pipistrellus* (Hungary) showed 46 nucleotide differences from the *Ci. lectularius* reference sequence (MF161520: from Hungary), i.e. only 585/631 bp (92.7%) sequence similarity. The *cox*1 gene fragment of another *Cimex* sp. from Vietnam revealed an even lower, 522/631 bp (82.7%) sequence similarity with *Ci. lectularius*.

Bugs identified as *Ci. pipistrelli* (represented by 12 samples) had three *cox*1 haplotypes. These exhibited up to six nucleotide differences from each other, amounting to 99–100% sequence similarity (625–631/631 bp). *Cacodmus ignotus* from South Africa had two *cox*1 haplotypes, with only one nucleotide difference (630–631/631 bp, i.e. 99.8–100%

similarity). The bug identified as *Ca. sparsilis* showed 43 nucleotide divergence from *Ca. ignotus* (588/631 bp, i.e. 93.2% similarity).

The ITS2 fragment was successfully amplified and sequenced from 16 samples (**Table 8**). In general, this nuclear marker showed a much lower degree of intraspecific divergence compared to *cox*1. Members of the *Cimex lectularius* group from Hungary had only two different ITS2 haplotypes. However, the *Cimex* sp. from *Pi. pipistrellus* (Hungary) showed only 96.7% (622/643 bp) sequence similarity in its longest region of continuous alignment with the ITS2 reference sequence (MF161534: from Hungary). In addition, the *Cimex* sp. from Vietnam showed even lower, 88.3% (580/657 bp) sequence similarity in its longest region of alignment with the ITS2 reference sequence.

Cimex pipistrelli had two nearly identical ITS2 sequences (941–942/942 bp, i.e. 99.9–100% similarity). Unexpectedly, samples identified as *Ca. ignotus* and *Ca. sparsilis*, which showed only 93.2% *cox*1 sequence similarity, were identical in their ITS2.

The phylogenetic relationships of *cox*1 and ITS2 sequences are shown in **Figs. 19** and **20**, respectively. The separation of the *Cimex* specimen (collected from *Pi. pipistrellus* in Hungary) from other isolates of the *Ci. lectularius* group was highly supported (with 100%) in both the *cox*1 and ITS2 phylogenetic analyses (**Figs. 19 and 20**). Similarly, the within-group separation of *Cimex* sp. from Vietnam received high (99%) support based on its ITS2 haplotype (**Fig. 20**), but only low (59%) support based on its *cox*1 haplotype (**Fig. 19**).

All *Ci. pipistrelli cox*1 haplotypes belonged to the same group with other conspecific isolates (**Fig. 19**), and this was confirmed in the phylogenetic analysis based on ITS2 sequences (**Fig. 20**). Bugs identified morphologically as *Ca. ignotus* and *Ca. sparsilis* were well separated from each other (with moderate, 87% bootstrap value) in the *cox*1 phylogenetic tree (**Fig. 19**).

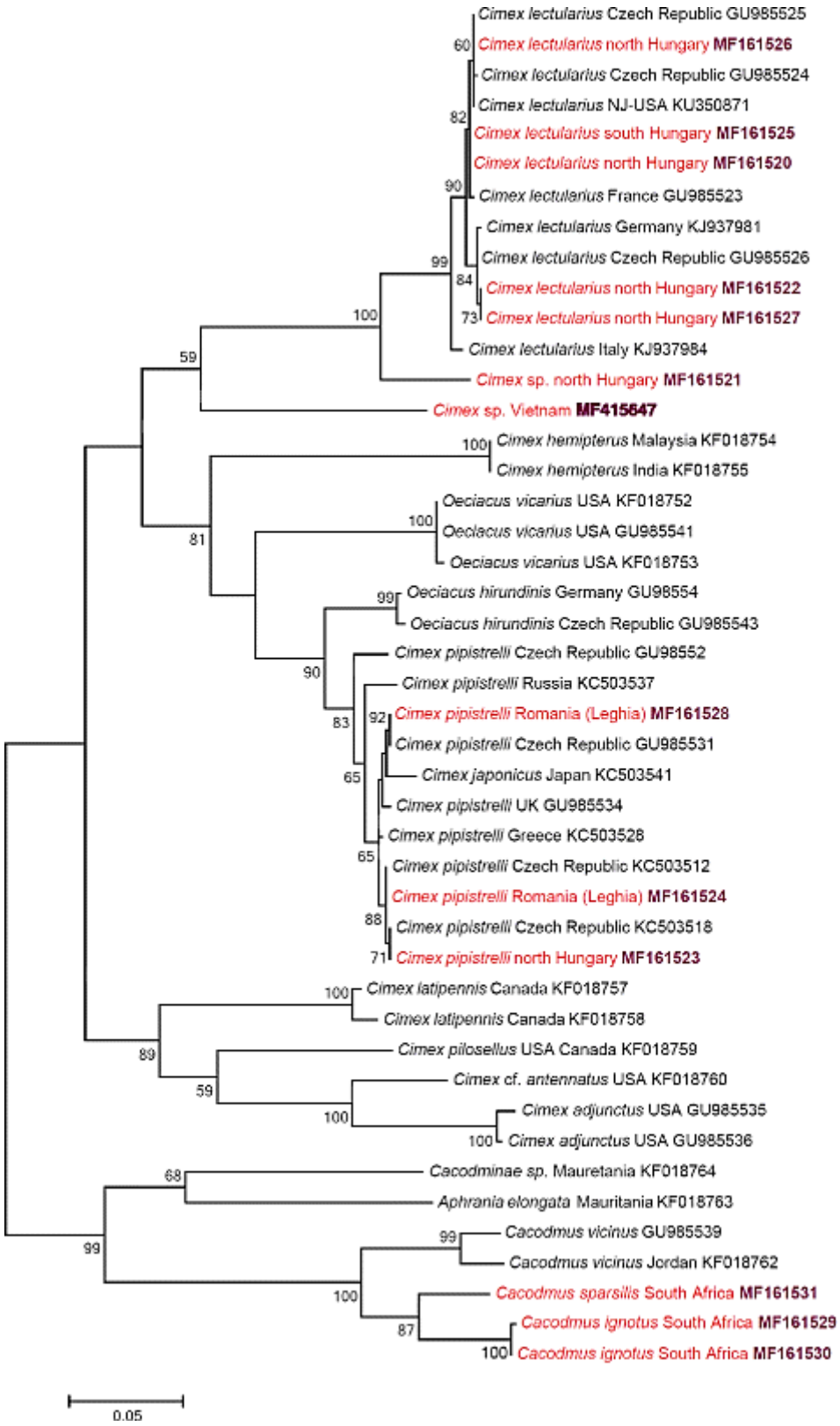

Figure 19. Phylogenetic tree based on the cox1 gene including sequences obtained in this study (indicated in red and with GenBank accession numbers in bold) and representative sequences from GenBank. Species identification is provided as in the GenBank database, although Oeciacus spp. were recently transferred into the genus Cimex (Balvín et al. 2015). Branch lengths represent the number of substitutions per site inferred according to the scale shown

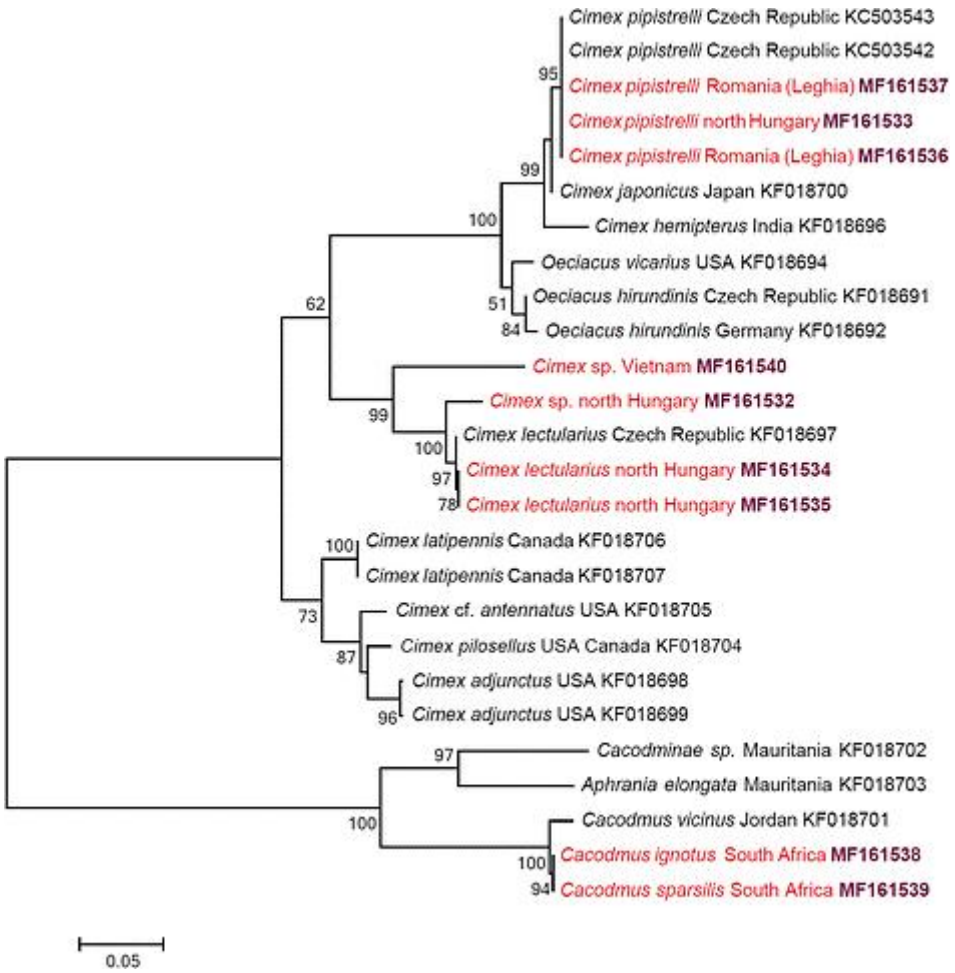

Figure 20. Phylogenetic tree based on the ITS2 including sequences obtained in this study (indicated in red and with GenBank accession numbers in bold) and representative sequences from GenBank. Species identification is provided as in the GenBank database, although *Oeciacus* spp. were recently transferred into the genus *Cimex* (Balvín et al. 2015). Branch lengths represent the number of substitutions per site inferred according to the scale shown

5.2 Parasitic (vector-borne) and free-living protozoa in bat ectoparasites and faeces

5.2.1 DNA of piroplasms of ruminants and dogs in Ixodid bat ticks

On 24 locations in Hungary in 2008–2015, and on seven locations in Romania in 2015 308 ixodid ticks have been collected from 200 individuals of 17 bat species (**Table 9, Table 10**). Because the small sample size, the following bat species (in **Table 9**, **Table 10:** harboring different stages of the same tick species) were analysed: *Myotis bechsteinii* (n=7), *My*. *daubentonii* (n=28), *My*. *emarginatus* (n=9), *Miniopterus schreibersii* (n=95), *Rhinolophus ferrumequinum* (n=16), *R*. *hipposideros* (n=12).

Ixodes ariadnae was represented by 45, *I*. *vespertilionis* by 124 and *I*. *simplex* by 139 specimens (larvae, nymphs and females). In Hungary, *I*. *ariadnae* was significantly more frequently found on bat species in the family Vespertilionidae, whereas *I*. *vespertilionis* was associated with Rhinolophidae (P<0.00001). *Ixodes ariadnae* was not collected in Romania, where *I*. *vespertilionis* occurred usually on representatives of both Vespertilionidae and Rhinolophidae (**Table 10**). Discounting one larva collected from *Barbastella barbastellus*, *I*. *simplex* was exclusively found on *Mi*. *schreibersii*.

In general, there was no significant difference between the intensity of tick infestation between bat species (p=0.4279, df=5, χ^2=4.9026), but there was a significant difference in the intensity of infestation of bats with different tick stages (larva: n=155; nymph: n=79; female: n=13; p=0.0005, df= 2, χ^2=14.924), i.e. ixodid tick larvae occurred in highest individual number on their hosts. In the case of *I*. *ariadnae* or *I*. *vespertilionis* there was no significant difference in the intensity of infestation between bat species, i.e. between *My*. *bechsteinii* and *My*. *emarginatus* (p=0.4497, W=28) or between *My*. *daubentonii*, *R*. *ferrumequinum* and *R*. *hipposideros* (p=0.8719, df= 2, χ^2=0.27423), respectively (**Table 9, Table 10**). Similarly, concerning these five bat species, there was no significant difference between intensities of their infestations with different tick stages, except for *My*. *daubentonii* and *R*. *ferrumequinum* on which larvae occurred significantly more frequently than nymphs/females (p=0.02013, df=2, χ^2=7.8106). Similarly, infestation of *Mi*. *schreibersii* with *I*. *simplex* had the highest intensity when larvae were present on bats (larva: n=79; nymph: n=50; female: n=9; p=0.001674, df=2, χ^2=12.786).

Table 9. Tick species and stages collected in Romania, shown according to their bat hosts.

Tick		Bat (number of ticks per number of bats)								
		Vespertilionidae					Rhinolophidae			Miniopteridae
Species	**Stage**	**MNAT**	**MCAP**	**MDAU**	**MBLY**	**ESER**	**REUR**	**RFER**	**RMEH**	**MSCH**
Ixodes vespertilionis	larva	1/1	9/1	28/16	4/1	2/2	-	26/9	7/4	2/2
	nymph	-	4/2	9/8	2/2	-	-	6/6	-	-
	female	-	1/1	1/1	-	1/1	1/1	1/1	-	-
Ixodes simplex	larva	-		-	-	-	-	-	-	56/33
	nymph	-		-	-	-	-	-	-	39/33
	female	-		-	-	-	-	-	-	5/5

Table 10. Tick species and stages collected in Hungary, shown according to their bat hosts. Five females and two nymphs of *I*. *ariadnae*, which were collected from cave walls (Ariadne Cave System), are not included.

Tick		Bat (number of ticks per number of bats)											
		Vespertilionidae									Rhinolophidae		Miniopteridae
Species	**Stage**	**MALC**	**MBEC**	**MNAT**	**MEMA**	**MDAU**	**MDAS**	**MMYO**	**PAUR**	**BBAR**	**RHIP**	**REUR**	**MSCH**
Ixodes ariadnae	larva	4/2	3/3	1/1	6/6	5/3	5/2	-	1/1	-	1/1	-	-
	nymph	-	4/4	-	4/3	-	-	1/1	3/3	-	-	-	-
	female	-	-	-	-	-	-	-	-	-	-	-	-
Ixodes vespertilionis	larva		1/1	-	-	-	-	-	1/1	-	8/7	-	-
	nymph	-	-	-	-	-	-	-	-	-	6/4	-	-
	female	-	-	-	-	-	-	-	-	-	2/1	1/1	-
Ixodes simplex	larva	-	-	-	-	-	-	-	-	1/1	-	-	23/10
	nymph	-	-	-	-	-	-	-	-	-	-	-	11/10
	female	-	-	-	-	-	-	-	-	-	-	-	4/4

Abbreviations: ESER-*Eptesicus serotinus*, MSCH-*Miniopterus schreibersii*. MCAP-*My*. *capaccinii,* MALC- *Myotis alcathoe*, MBEC- *My*. *bechsteinii*, MNAT- *My*. *nattereri*, MEMA- *My*. *emarginatus*, MDAU-*My*. *daubentonii*, MDAS-*My*. *dasycneme*, MMYO-*My*. *myotis*, MBLY-*My*. *blythii,* PAUR-*Plecotus auritus*, BBAR-*Barbastella barbastellus*, RHIP-*Rhinolophus hipposideros*, REUR-*R*. *euryale*, RFER-*R*. *ferrumequinum*, RMEH-*R*. *mehelyi*, MSCH-*Miniopterus schreibersii*.

DNA sequences of piroplasms were detected in 20 bat ticks (**Table 11**). *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* (**Table 11**).

In *I*. *ariadnae* only a DNA sequence of *B*. *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 *B*. *vesperuginis* (identity: 448/448 bp = 100%) and *B*. *crassa* (identity: 404/410 bp = 98.5%) were detected (**Table 11**).

Adding to the presence of the latter sequence in *I*. *simplex* nymphs, in a larva of this tick species the sequence of another genotype of *B*. *crassa* (identity: 403/410 = 98.3%) was demonstrated (**Table 11**), which was not detected before in Hungary. From *I*. *simplex* a shorter sequence of the zoonotic *B*. *venatorum* (identity: 105/105 bp = 100%) was also amplified, showing less identity with other piroplasms (second closest to *B*. *occultans* and *T*. *equi*, with 103/105 bp = 98.1% identity). In *I*. *simplex* larvae/nymphs two sequences of *B*. *canis* (both identities: 420/420 bp = 100%) were also detected.

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%) (**Table 11**).

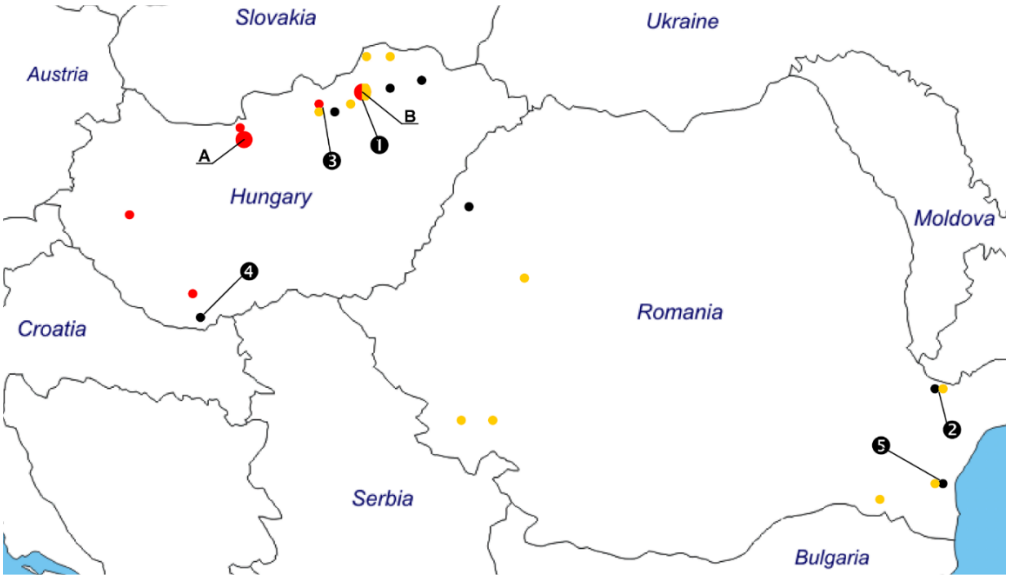

Figure 21. Sampling sites of the present study. Color of collection sites for *Ixodes ariadnae* are marked with red dots, for *I. vespertilionis* with yellow dots, and for *I. simplex* with black dots. Letters: A—Ariadne Cave System and caves in the Pilis Mountains (bats sampled at three caves), B—Bükk Highlands Cave system (bats sampled at nine caves). Numbers in black circles indicate places, where piroplasm-carrier bat ticks were collected (**Table 11**). Small and unseparated dots with different colour indicate the same place with two tick species. Two places close to each other in northeast Hungary (Baradla and Béke Caves) are marked with one dot.

In the phylogenetic analysis, all sequences of *Babesia* and *Theileria* spp. amplified from bat ticks in the present study clustered together with relevant genotypes available in GenBank (and published from previously known "type" hosts of these piroplasms) (**Fig. 22**). Their separation from other piroplasms was confirmed by high bootstrap values (**Fig. 22**). Taken together, piroplasm sequences were demonstrated in bat ticks from three places of Hungary and two places of Romania (**Table 11**, **Fig. 20**); three sequences of piroplasms were detected only in samples from Hungary, three of them only in Romania and three in both countries (**Fig. 22**).

Table 11. Results of molecular analyses of bat ticks for the presence of piroplasms.

Abbreviations: MDAS-*Myotis dasycneme*, ESER-*Eptesicus serotinus*, MDAU-*My*. *daubentonii*, RHIP-*Rhinolophus hipposideros*, MSCH-*Miniopterus schreibersii*

***Ixodes* species**	**Tick stage or sex**	**PCR positive / all analysed ticks**	**Results of sequencing (length, % identity, sample number)**	**Bat host of PCR positive ticks[#]**	**Location(s) of PCR positive ticks in Fig 1**	**Reference sequence**	**Accession number of sequences in this study (name of isolate)**
I. ariadnae	larva	4/26	*Babesia vesperuginis* (448 bp, 100%, 4×)	MDAS	1	AJ871610	KU958544 (Ia-Bv-1)
	nymph	0/14	-	-	-	-	-
	female	0/5	-	-	-	-	-
I. vespertilionis	larva	3/89	*Babesia vesperuginis* (448 bp, 100%, 2×)	ESER, MDAU	2	AJ871610	KU958544 (Ia-Bv-1)
			Babesia crassa (410 bp, 98.5%, 1×)	RHIP	3	KF791205	KU958546 (Iv-Bcr-1)
	nymph	0/27	-		-	-	-
	female	0/8	-		-	-	-
I. simplex	larva	8/79	*Babesia crassa* (410 bp, 98.3%, 1×)	MSCH	4	KF791205	KU958545 (Is-Bcr-1)
			Babesia venatorum-like (105 bp, 100%, 1×)	MSCH	2	KC007118	KU958553 (Is-Bv-1)
			Babesia canis (420 bp, 100%, 1×)	MSCH	2	JF461253	KU958552 (Is-Bca-2)
			Theileria capreoli (425 bp, 99.5%, 1×)	MSCH	4	KJ188219	KU958547 (Is-Tc-1)
			Theileria orientalis (432 bp, 100%, 4×)	MSCH	2, 4, 5	AB668373	KU958549 (Is-To-1)
	nymph	4/50	*Babesia crassa* (410 bp, 98.5%, 1×)	MSCH	2	KF791205	KU958546 (Iv-Bcr-1)
			Babesia canis (420 bp, 100%, 1×)	MSCH	2	KC902833	KU958551 (Is-Bca-1)
			Babesia canis (420 bp, 100%, 2×)	MSCH	2	JF461253	KU958552 (Is-Bca-2)
	female	1/9	*Theileria* sp. OT3 (432 bp, 100%, 1×)	MSCH	4	DQ866839	KU958550 (Is-TOT3-1)

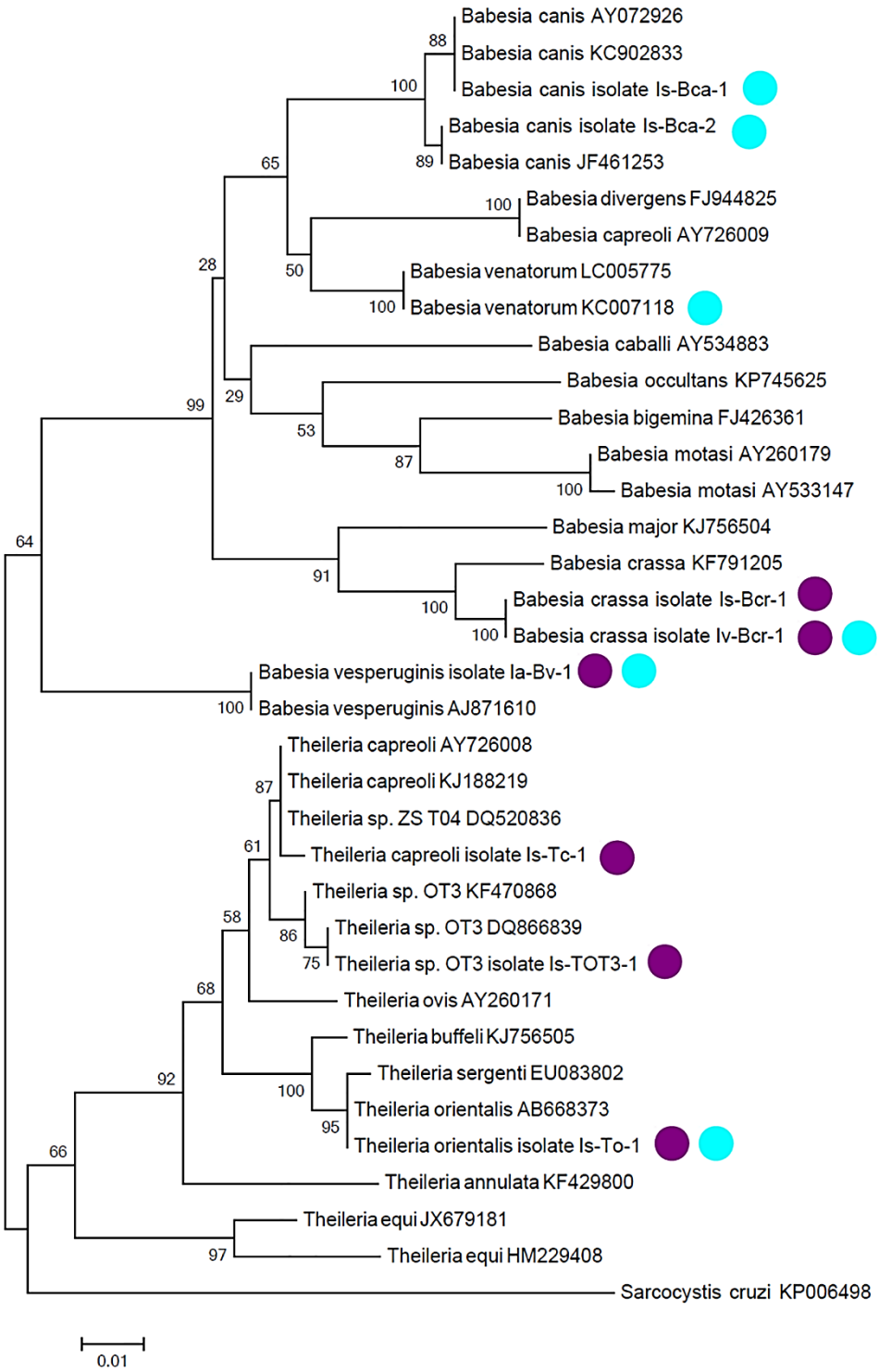

Figure 22. Phylogenetic relationships of 18S rDNA sequences of piroplasms identified in this study and relevant sequences previously deposited in GenBank. Sequences identified in the present study in Hungary and in Romania are highlighted with purple or turquoise dots, respectively. The shorter sequence of *Babesia venatorum* from this study is not included, therefore its reference sequence (to which it showed 100% identity) is marked. Branch lengths correlate to the number of substitutions inferred according to the scale shown.

5.2.2 Piroplasm DNA detection in *Argas vespertilionis*

Altogether 321 soft tick larvae were collected from 17 bat species (belonging to five genera) in eight countries (**Table 6**). DNA was extracted from *A. vespertilionis* larvae individually or in small pools (i.e. two or three larvae collected from the same host individual) and the resultant 193 DNA extracts (**Table 12**) were first molecularly screened for the presence of piroplasm DNA.

Table 12. Country of origin, bat host species (Chiroptera: Vespertilionidae) and DNA extracts of *Argas vespertilionis* larvae used in this study

[a] Locations are listed in **Table 6.**

Country	Host species (number of individuals)	Number of larvae	Number of DNA extracts
Hungary (13)[a]	*Pipistrellus pipistrellus/ pygmaeus/nathusii/kuhlii* (6/14/1/1)	152	81
	Myotis alcathoe/dasycneme/brandtii (4/5/1)	60	32
	Plecotus auritus/austriacus (1/4)	28	18
	Nyctalus noctula (2)	4	3
	Eptesicus serotinus (2)	14	9
	Vespertilio murinus (2)	21	12
Romania (2)[a]	*Pipistrellus pipistrellus* (5)	9	6
	Eptesicus serotinus (1)	2	1
Italy (1)[a]	*Pipistrellus pipistrellus* (1)	3	3
Kenya (1)[a]	*Pipistrellus cf. rueppellii* (1)	1	1
Vietnam (3)[a]	*Pipistrellus cf. javanicus/cf. abramus* (1/2)	16	16
China (Xinjiang)	*Vespertilio murinus* (2)	11	11

Based on the PCR amplifying part of the 18S rRNA gene, 12 samples contained the DNA of piroplasms: 10 from Hungary and two from China. In Hungary, PCR positive *A. vespertilionis* larvae originated from three locations, from three individuals of bats belonging to the following species: (1) *E. serotinus* (yielding 12 larvae, of which three pools of two larvae and two individual samples were positive, amounting to 42–67% prevalence among larvae from this single host); (2) *Pl. austriacus* (yielding six larvae, of which two pools of two larvae and two individual samples were positive, amounting to 67–100% prevalence among larvae from this single host); and (3) *Pi. pipistrellus* (yielding one larva, which was positive). In China, two individual DNA samples from *A. vespertilionis* (collected from two bat hosts, *V. murinus*) contained piroplasm DNA. Sequencing of all 12 PCR positive samples revealed the exclusive presence of *B. vesperuginis*, with 100% identity (448/ 448 bp) between samples from Hungary and China.

Four DNA samples which were extracted from bat soft tick (*A. vespertilionis*) larvae collected from grey longeared bat (*Plecotus austriacus*) 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. All four samples yielded identical *cox1* sequences. This *cox1* sequence of *B. 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% from *B. bigemina* (AB499085) to *B. canis* (KX712138), respectively] and *Theileria* spp. [i.ec from *T. sinensis* (JQ518293) to *T. luwenshuni* (JQ518295), respectively]. The number of gaps was up to 10 and 14 in comparison with *Babesia* and *Theileria* spp., but none in comparison with *C. felis*. The *cox1* sequences of *Ba. vesperuginis* and *Ba. conradae* (Western *Babesia* group) were only 73.2% (659/900 bp) similar, but in this alignment, there were only two gaps. This is in line with the highest similarity of *Ba. vesperuginis* 18S rRNA sequence from *A. vespertilionis* (KY657241) with those of an 'outgroup *Theileria*', *Th. bicornis* (AF499604: 422/455 bp = 92.7% identity); of the 'prototheilerid' *Ba. conradae* (AF158702: 416/450 bp = 92.4% identity); and of *C. felis* (AF399930: 409/450 bp = 90.9% identity) (sequences, category names are from: Criado-Fornelio et al. 2003; Lack et al. 2012; Hornok et al. 2017).

On the other hand, the *B. vesperuginis* 18S rRNA sequence had lower than 90% sequence identity with *Babesia* and *Theileria* spp. sensu stricto. In this decreasing order of sequence similarity, the number of gaps was relatively low in comparison with *T. bicornis*, *B. conradae* and *C. felis* (10, four and eight, respectively), whereas medium level (20–29) or high (28–58) in comparison with *Theileria* and *Babesia* spp., respectively. The above sequence similarities and gap numbers were reflected by the topologies of phylogenetic trees. In particular, based on its *cox1* gene, *Ba. vesperuginis* clustered outside *Babesia* s.s. (with 96% support) and close to *C. felis* and Theileriae (**Fig. 23**).

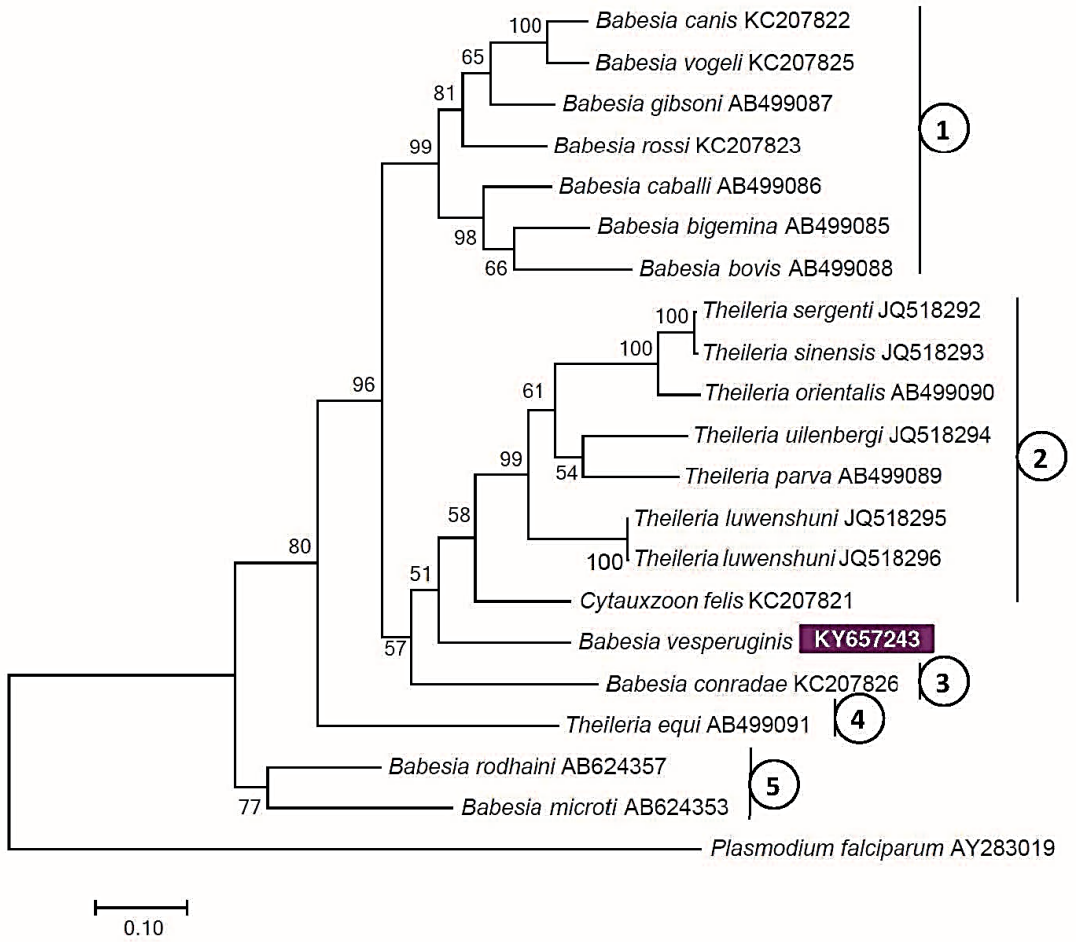

Figure 23. Maximum likelihood tree of Piroplasmida based on *cox1* gene sequences, obtained with the Hasegawa–Kishino–Yano (HKY) model. Accession number of the *Babesia vesperuginis* sequence from this study is shown in inverse purple colour. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values of >50 are shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers connected to vertical lines indicate category names (according to Schreeg et al. 2016) as follows: 1 – *Babesia* sensu stricto; 2 – *Theileria* (s.s.) and *Cytauxzoon* spp.; 3 – Western *Babesia* group; 4 – *Theileria equi*; 5 – *Babesia microti* group

If *C. felis* was omitted from the analysis, *B. vesperuginis* remained a member of the phylogenetic group of *Babesia* s.s. (data not shown). This clearly argues against omitting *C. felis* (or other major piroplasmid categories) from phylogenetic analyses which serve to define the precise taxonomic status of *B. vesperuginis*. Concerning phylogenetic analyses with concatenated *cox1* and 18S rRNA gene sequences performed here, both applied models achieved trees with similar overall topologies. In particular, *B. vesperuginis* clustered separately from *Babesia* spp., and this received a strong (**Fig. 24**: 99%, **Fig. 25**: 88%) support. In addition, *B. vesperuginis* clustered next to *B. conradae* and the phylogenetic group of *Theileria* spp. and *C. felis* (**Fig. 24**), or together with *B. conradae* in a sister group to the clade containing Theileriidae (**Fig. 25**).

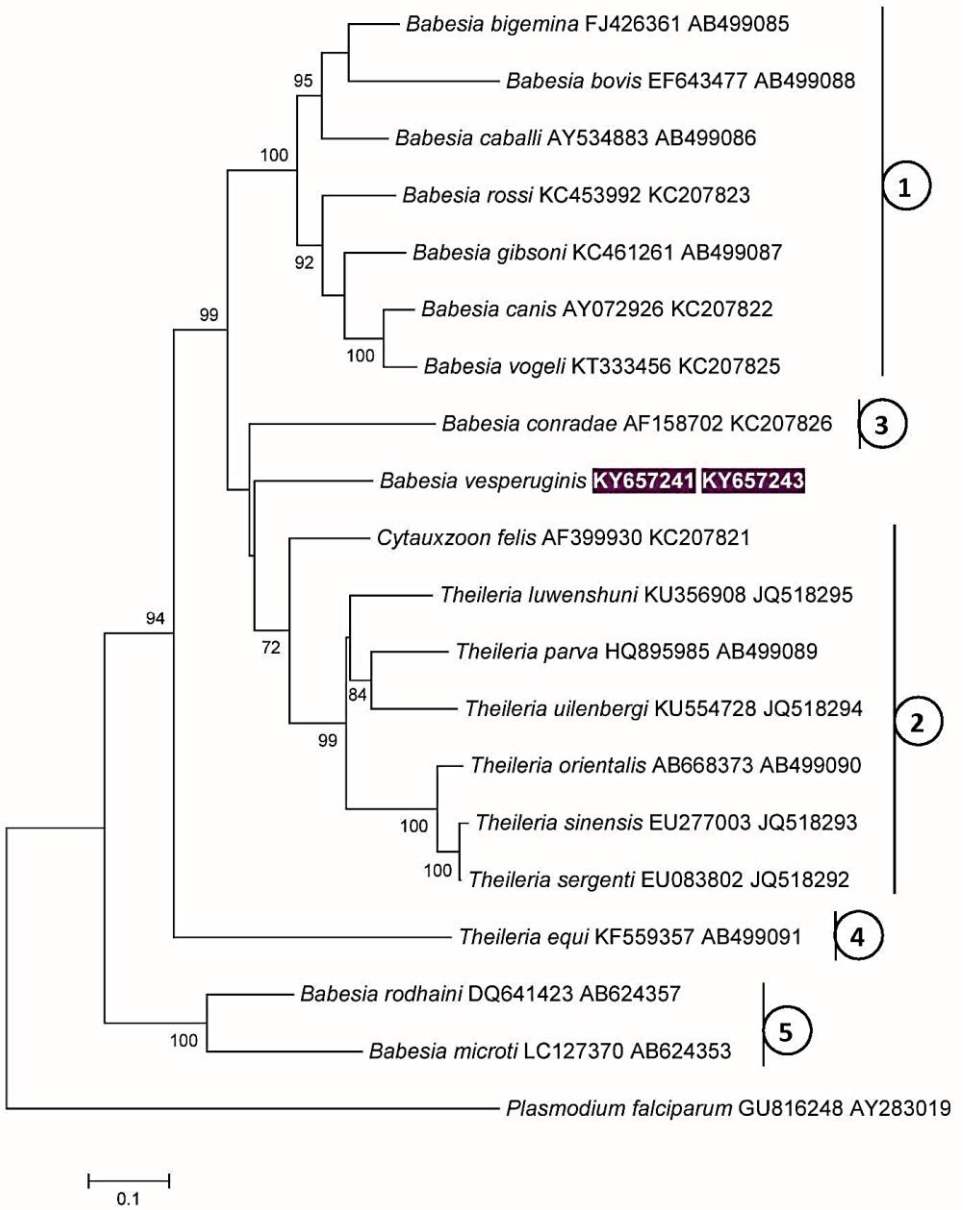

Figure 24. Maximum likelihood tree of Piroplasmida based on concatenated *cox1* and 18S rRNA gene sequences (the GenBank accession numbers of which are shown in this order after the species name), obtained with the General Time Reversible (GTR) model. Accession number of *Babesia vesperuginis* sequence from this study is shown in inverse purple colour. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values of > 70 are shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers connected to vertical lines indicate category names (according to Schreeg et al. 2016) as follows: 1 – *Babesia* sensu stricto; 2 – *Theileria* (s.s.) and *Cytauxzoon* spp.; 3 – Western *Babesia* group; 4 – *Theileria equi*; 5 – *Babesia microti* group

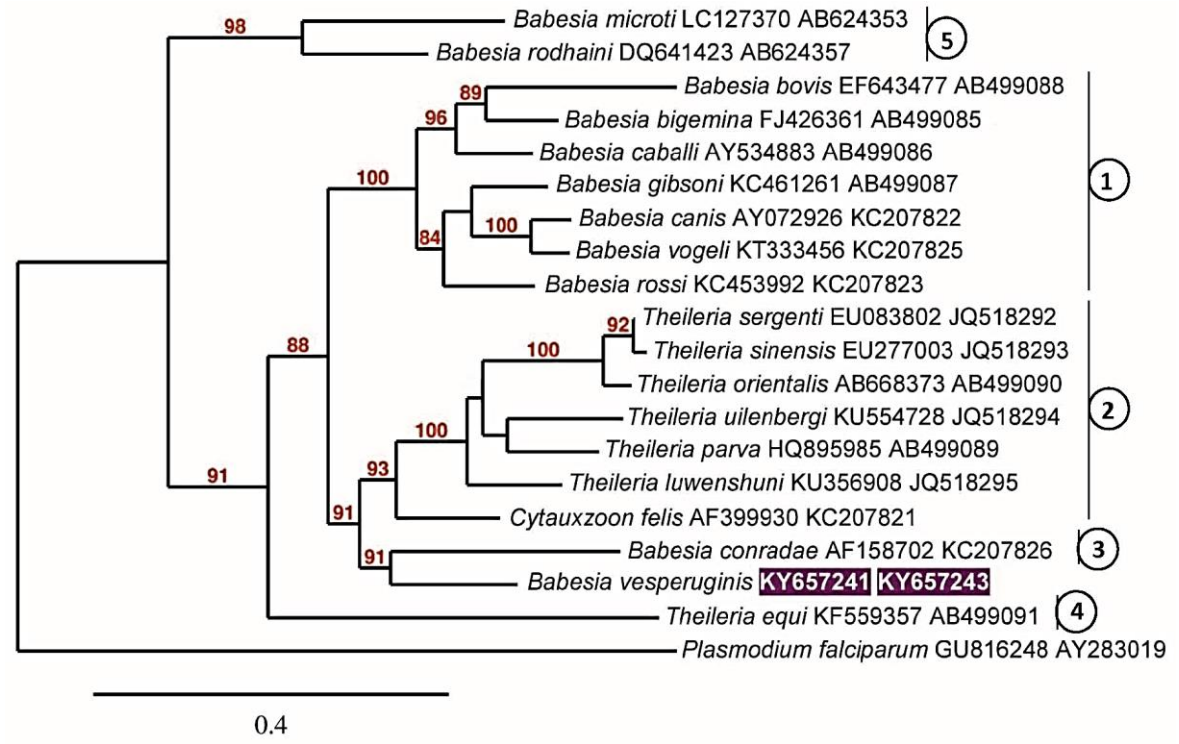

Figure 25. Maximum likelihood tree of Piroplasmida based on concatenated *cox1* and 18S rRNA gene sequences (the GenBank accession numbers of which are shown in this order after the species name), obtained with the Hasegawa–Kishino–Yano (HKY) model. Accession number of *Babesia vesperuginis* sequence from this study is shown in inverse purple colour. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values of > 70 are shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers connected to vertical lines indicate category names (according to Schreeg et al. 2016) as follows: 1 – *Babesia* sensu stricto; 2 – *Theileria* (s.s.) and *Cytauxzoon* spp.; 3 – Western *Babesia* group; 4 – *Theileria equi*; 5 – *Babesia microti* group

5.2.3 DNA of free-living bodonids (Euglenozoa: Kinetoplastea) in bat ectoparasites

Out of 307 ixodid ticks, 45 *I. ariadnae* specimens have been collected from nine bat species (three genera), 124 *I. vespertilionis* specimens from 12 bat species (five genera), and 138 *I. simplex* specimens from *Miniopterus schreibersii*. *Argas vespertilionis* was represented by 299 larvae collected from 15 bat species (six genera), and cimicid bugs by 207 specimens from four bat species (two genera) (**Table 13**).

Table 13. Bat ectoparasites used in this study

*including seven engorged specimens from cave walls

	Hosts		**Number of samples (total)**			
Family	**Genus (number of species)**	***Argas vespertilionis* (299)**	***Ixodes ariadnae* (45) ***	***Ixodes vespertilionis* (124)**	***Ixodes simplex* (138)**	***Cimex* spp. (207)**
Vespertilionidae	*Pipistrellus* (4)	161	–	–	–	1
	Myotis (10)	58	33	60	–	206
	Nyctalus (1)	4	–	–	–	–
	Eptesicus (1)	16	–	3	–	–
	Plecotus (2)	28	4	1	–	–
	Vespertilio (1)	32	–	–	–	–
Rhinolophidae	*Rhinolophus* (4)	–	1	58	–	–
Miniopteridae	*Miniopterus* (1)	–	–	2	138	–

Three DNA samples of bat ectoparasites were PCR positive for kinetoplastids. In these samples sequencing revealed the presence of DNA from freeliving bodonids, but none from trypanosomes. In particular, one *I. simplex* larva (collected from *Mi. schreibersii* captured in Somova, Romania) contained the DNA of *B. 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 (collected from *Myotis brandtii* captured at Mánfa cave, Mecsek Mountains, Hungary) and in one *Ci. pipistrelli* nymph (collected near the *My. blythii*/*myotis* colony in a church at Szőlősardó, Hungary). Corresponding sequences were 100% (i.e. 776/776 bp) identical with Bodonidae sp. Pan-2 (AY753625).

Phylogenetic analysis of these three DNA-positive samples (**Fig. 26**) showed that the sequence of *B. saltans* from the bat tick *I. simplex* aligned closest to other *B. saltans* isolates within the Eubodonida clade, which is a sister group to the Neobodonida clade that includes the two bodonid sequences amplified from the bat tick *A. vespertilionis* and the bat bug *Ci. pipistrelli*.

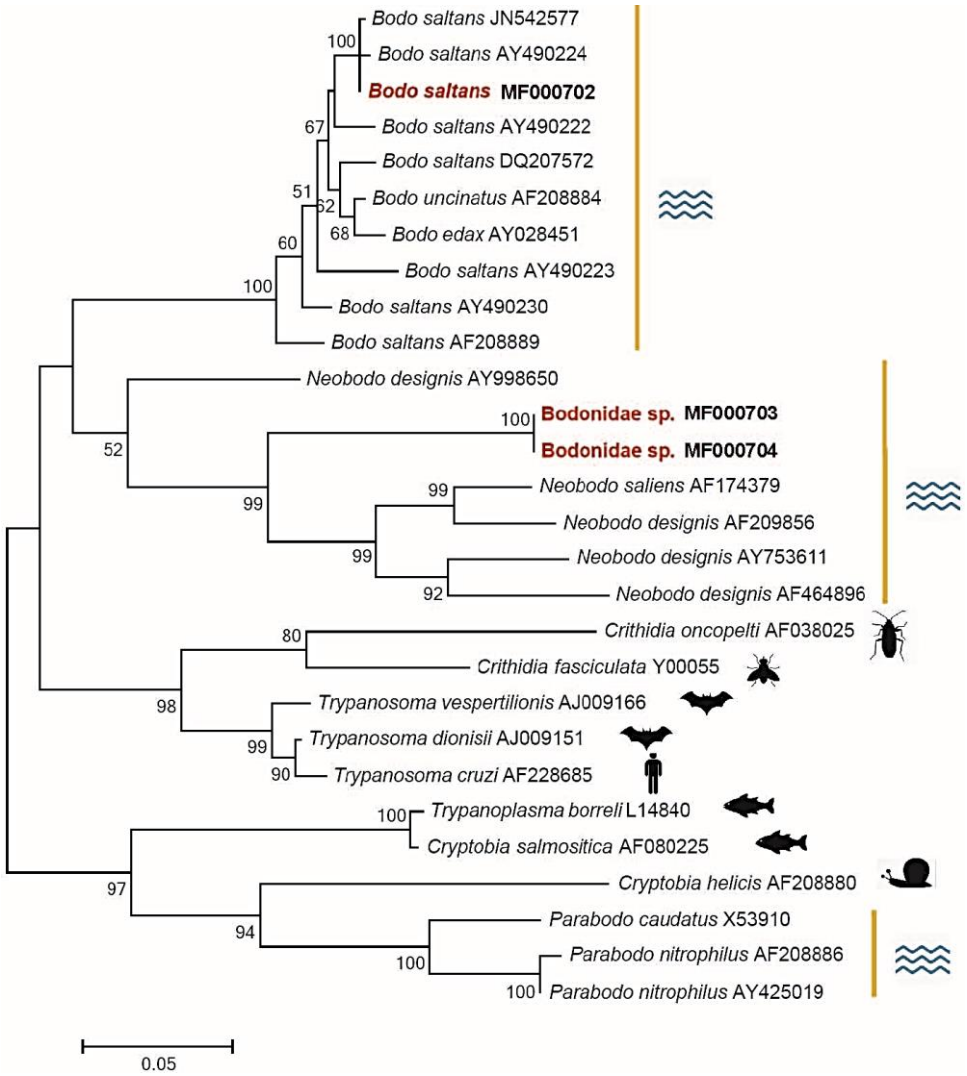

Figure 26. Maximum Likelihood phylogenetic tree of an approx. 900-bp-long fragment of the 18S rRNA gene, including sequences obtained in this study (highlighted by red fonts and bold accession numbers) and representative sequences of other kinetoplastids retrieved from GenBank. The pictographic symbols indicate the typical hosts or water in case of free-living bodonid species. Branch lengths represent the number of substitutions per site inferred according to the scale shown

5.2.4 Screening of bat faeces for arthropod-born apicomplexan protozoa

In addition, 196 individual and 25 pooled bat faecal samples were collected (192 on 38 locations in Hungary, and 29 on 10 locations in the Netherlands: **Fig. 27**).

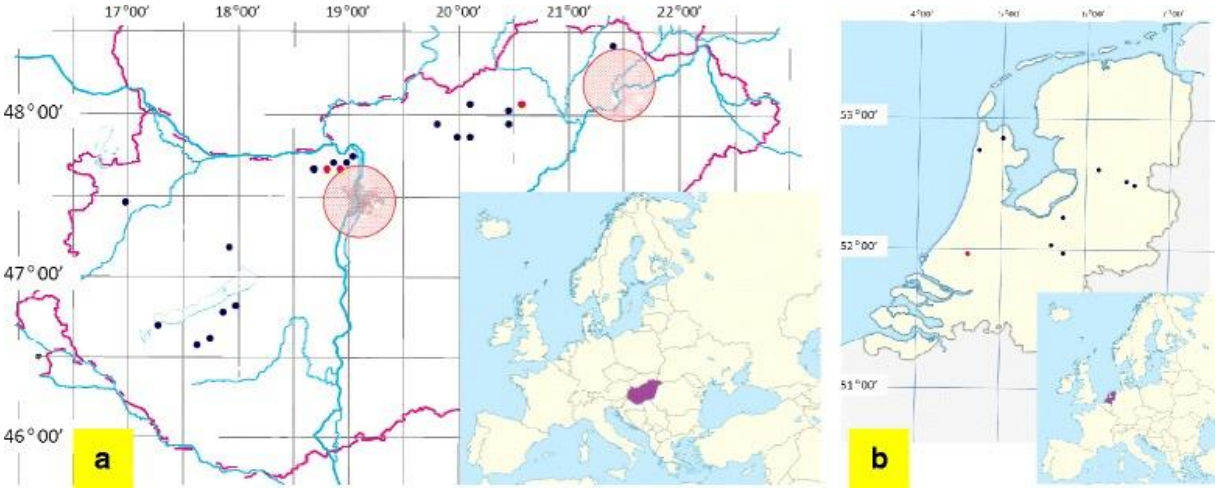

Figure 27. Map of Hungary (**a**) and Netherlands (**b**) showing the sampling sites. Only places at least 10 km apart are shown. The red dots on the map of Hungary (**a**) indicate places, where *Babesia canis* PCR positive bat droppings were collected. The shaded red circles mark the highly endemic regions of *Babesia canis* according to (Hornok et al. 2006). The red dot on the map of Netherlands (**b**) indicates the location, where the *Besnoitia besnoiti*-like sequence originated

The study involved the following 19 bat species (sample number): *Nyctalus noctula* (21), *N. leisleri* (9), *Myotis alcathoe* (23), *My. daubentonii* (49), *My. bechsteinii* (21), *My. emarginatus* (6), *My. myotis* (8), *My. dasycneme* (4), *My. brandtii* (6), *My. nattereri* (13), *My. blythii* (5), *Rhinolophus ferrumequinum* (3), *R. hipposideros* (2), *Pipistrellus nathusii* (3), *Pi. pipistrellus* (14), *Pi. pygmaeus* (1), *Barbastella barbastellus* (6), *Miniopterus schreibersii* (1), *Plecotus auritus* (1).

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 (**Table 14**). Two sequences were identified (accession numbers KP835549-50) with 2 nucleotide differences (inversion of GA to AG at positions 151–152 in the 18S rDNA gene). These bat-derived *Babesia* isolates showed 100 % identity with two *Ba. canis* isolates from dogs in Croatia (FJ209024 and FJ209025: (Beck et al. 2009)), and in phylogenetic comparison they clustered together with other *Ba. canis* isolates (**Fig. 28**). On the other hand, the relevant sequences exhibited only 88 % similarity to *Ba. vesperuginis* (AJ871610) known to infect bats (**Fig. 28**). All five bats with *Ba. canis* PCR positive faecal samples were caught within 50 km of the two regions in Hungary (**Fig. 27**), where the highest number of *Ba. canis* seropositive dogs were found in a previous countrywide survey (Hornok et al. 2016d).

Table 14. Data of sample collections and results of molecular analyses according to country and bat species. All, except one (*) were individual samples. The reference sequences were FJ209024 for *Babesia canis* and KJ746531 for *Besnoitia besnoiti*. The bat ring number is also provided in the case of two samples collected from different individuals of the same bat species caught on the same date and in the same place

Country	Date (2014)	Longitude	Latitude	Bat species (ring No.)	Results of sequencing (homology)	GenBank accession number
Hungary	July 19	20°33'06"	48°06'02"	*Nyctalus noctula*	*Babesia canis* (100 %)	KP835549
	August 29	18°52'30"	47°42'30"	*Myotis daubentonii* (A5783)	*Babesia canis* (100 %)	KP835549
	July 23	20°36'50"	48°06'39"	*Pipistrellus pygmaeus*	*Babesia canis* (99 %)	KP835550
	August 29	18°52'30"	47°42'30"	*Myotis daubentonii* (A5773)	*Babesia canis* (99 %)	KP835550
	August 30	18°50'35"	47°41'58"	*Myotis alcathoe*	*Babesia canis* (99 %)	KP835550
Netherlands	July 28	4°39'05"	52°02'42"	*Myotis dasycneme**	*Besnoitia besnoiti* (99 %)	KP835555

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* (**Table 14**). The sequence (accession number KP835555) had six nucleotide difference from, but clustered together with *Be. besnoiti* and *Be. tarandi* (**Fig. 28**). It showed less (98 %) homology with (i.e. nine nucleotide difference) and clustered separately (**Fig. 28**) from a cystogenic coccidium, *Nephroisospora eptesici* recently identified from New World bats (Wünschmann et al. 2010).

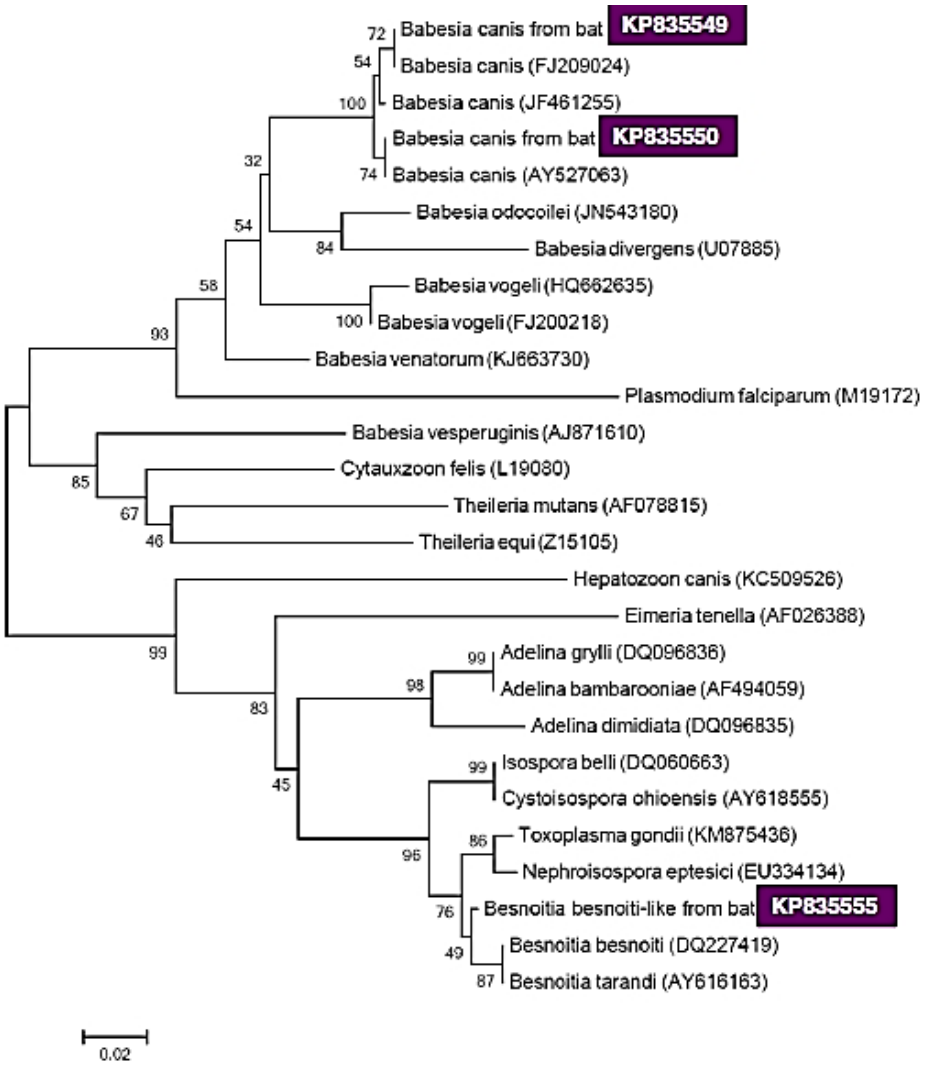

Figure 28. Phylogenetic comparison of 18S rDNA sequences of arthropod-borne apicomplexan protozoa identified in the present study (inverse colour), with related sequences from the GenBank. Branch lengths correlate to the number of substitutions inferred according to the scale shown.

5.3 Vector-borne bacteria in bat faeces

Among bat faecal DNA extracts, 13 were real-time PCR positive for rickettsiae (**Table 15**). In one sample the sequence of a rickettsial endosymbiont (reported from the fly species *Medetera jacula*: JQ925589) was identified (341/341 bp, i.e., 100% identity; **Table 15**). From three further 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 with a species-specific real-time PCR (**Table 15**) in one pooled sample collected in Hungary. All four samples of the pond bat (*Myotis dasycneme*, collected in the Netherlands) were real-time PCR positive for *N. risticii*. The 16S

rRNA gene sequence from these samples, designated *Neorickettsia* sp. BF87, 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), whereas it had one nucleotide difference (272/273 bp, i.e., 99.6 identity) from *N. risticii* reported from bats (e.g., KX986616) and *Neorickettsia* isolates from flukes (e.g., KX818100). These geographically diverse sequences clustered together in the phylogenetic analysis (**Fig. 29**). Three of these *My. dasycneme* samples also contained haemotropic *Mycoplasma* DNA, the species of which could not be identified with sequencing (**Table 15)**. All samples were negative for *F. tularensis*, *C. burnetii* and *Chlamydiales*.

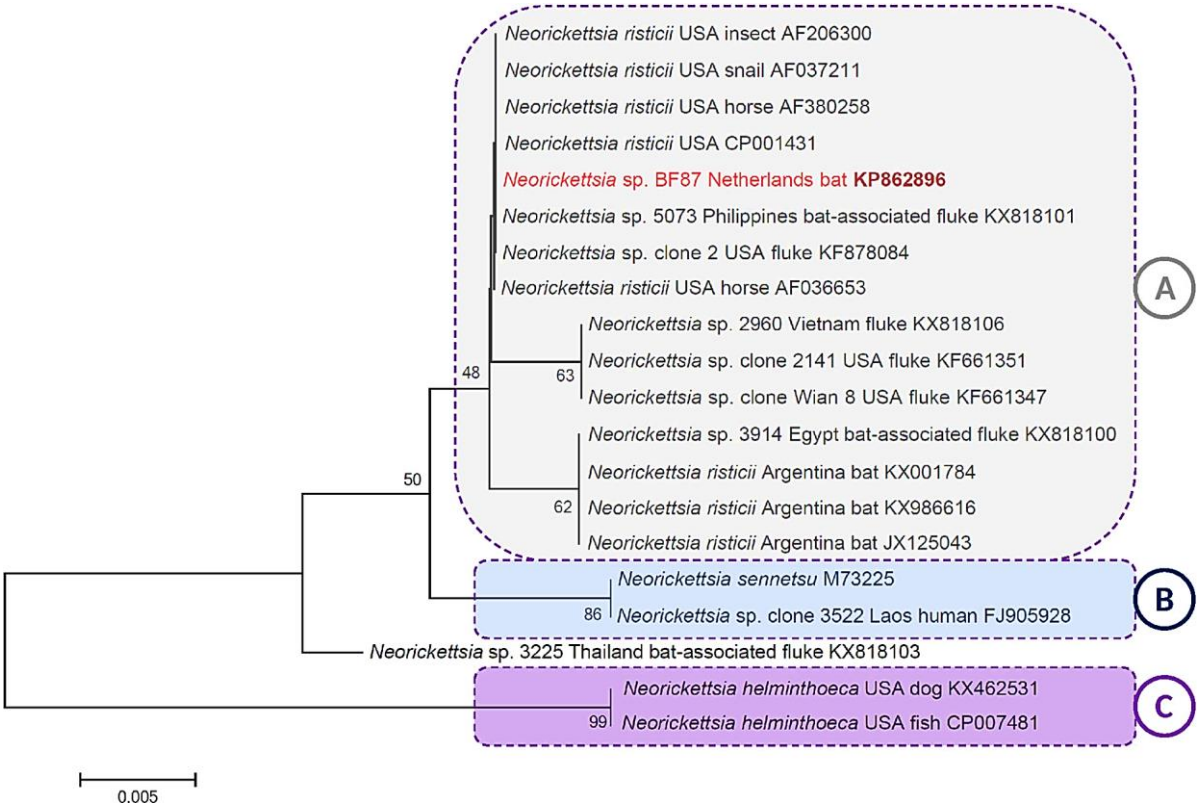

Figure 29. Phylogenetic tree of Neorickettsia spp. and genotypes, based on partial 16S rRNA sequences. For each entry, GenBank data are shown in the following order: species or isolate name, country of origin, generic name of isolation source (when available), finally accession number. The bat-related *Neorickettsia* sp. identified in the present study is highlighted with red fonts and bold accession number. Three phylogenetic groups, which include their ‘‘type species’’ (N. risticii, N. sennetsu and N. heminthoeca), are also indicated (i.e., encircled with dashed line filled with different background colour, and labelled as A, B or C, respectively). Branch lengths represent the number of substitutions per site inferred according to the scale shown.

Table 15. Results of bat faeces analyses. Data of vector-borne bacteria in a field are relevant to a single sample, unless otherwise indicated

Abbreviation: HU Hungary, NL The Netherlands

[a]Sequencing of rickettsiae was performed from samples with the lowest Ct values

[b]Sequencing of haemoplasmas was unsuccessful

Bat genus (number of spp.)	Number of samples	Rickettsia spp.		Neorickettsia risticii		Mycoplasma spp. (haemofelis group)
		Real-time PCR positive bats species (country)	Identified rickettsiaea (accession number)	Real-time PCR positive bat species (country)	Sequence identity (accession number)	Real-time PCR positive bat species (county)b
Myotis (9)	135	2 x *My. daubentonii* (HU)	–	4 x *My. dasycneme* (NL)	100% (KP862896)	3 x *My. dasycneme* (NL)
		My. alcathoe (HU)	Novel genotype (MF347695)			
		My. alcathoe (HU)	Fly endosymbiont (MF347694)			
Pipistrellus (3)	18	*Pi. pipistrellus* (NL)	–	–	–	–
		Pi. pipistrellus (HU)	–	–	–	–
Nyctalus (2)	30	–	–	–	–	–
Barbastella (1)	6	*B. barbastellus* (HU)	–	–	–	–
Plecotus (1)	1	–	–	–	–	–
Rhinolophus (2)	5	–	–	–	–	–
Miniopterus (1)	1	–	–	–	–	–
Unknown (pooled)	25	2 x Unknown (NL)	Novel genotype (MF347695)	–	–	Unknown (NL)
		4 x Unknown (HU)	*R. helvetica*	–	–	–

6. Discussion

6.1 Molecular taxonomic investigations of bat ectoparasites in a geographical context

6.1.1 High degree of mitochondrial gene heterogeneity in the bat tick species *Ixodes vespertilionis*, *I. ariadnae* and *I. simplex* from Eurasia

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. We acknowledge that the reduced number of samples and stages used in the present study prevent conclusions about the precise taxonomic status of collected specimens. However, the low number of samples is compensated by an adequate representation of the territories and a high similarity of the samples within-sites. We could not explicitly calculate the amount of phylogenetic diversity at each node of the phylogenetic tree because the presence of singletons, or isolated taxa that produced only a single edge in the tree. However, the relative distances among the tips of the tree are an indirect measure of the similarity within and between sites.

The main host of *I. vespertilionis* in Europe is *R. hipposideros* (Arthur 1956; Piksa et al. 2014). During post-glacial periods this bat species recolonized central and northern Europe from refugia either in the Balkan or the northern Mediterranean (Southern France) (Dool et al. 2013). This may account for the relative genetic homogeneity of CE European and French *I. vespertilionis* isolates, as reported here.

Considering all *I. vespertilionis* analyzed in the present study from Europe, 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 (Dool et al. 2013). This is also well reflected by the phylogenetic position of *R. hipposideros* from Spain and CE Europe (**Fig. 3b**). The level of sequence divergence between Spanish and CE European genotypes of *I. vespertilionis* (5.4 % in the COI gene) approached the proposed sequence difference as species boundary for ticks (6.1 % COI: (Lv et. al 2014)). In contrast to this, another member of the genus, *I. ricinus* was demonstrated to show only 0.5 % COI sequence divergence between specimens from CE Europe or France and Spain (e.g. GU074908 or GU074941 vs. GU074910: (Noureddine et al. 2011)), explained by gene flows due to dispersal and continuous exchange of ticks between populations connected by migrating hosts.

The homologies of COI sequences were reported to be above 93.9 % within a species, and below 94.4 % between species of ixodid genera (Lu et al. 2013). Similarly, in another study the percentage of species boundary was deemed to be 6.1 % sequence divergence for the COI gene, only in exceptional cases amounting to higher values (Lv et al. 2014). Accordingly, in comparison with *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. This is partly confirmed by the morphological difference observed between the European and the Vietnamese variant. In addition, the degree of partial COI sequence divergence between the European and Vietnamese *I. vespertilionis* genotypes (101 nucleotide differences, i.e. 84.1 % identity) was similar to that observed between the latter and *I. ricinus* (e.g. in comparison with KF197132: 102 nucleotide differences, i.e. 83.9 % identity). At the same time, the two South-East Asiatic genotypes (KR902756, AB231667) clustered together and showed 88 % similarity to each other (**Fig. 5**).

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* (Mitani et al. 2007: AB231667). 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 (**Fig. 3a-b**). These two Asiatic *Rhinolophus* spp. are closely related phylogenetically to *R. ferrumequinum* (**Fig. 3b**). which appears to be the most important host of *I. vespertilionis* in Japan and Eastern Asia (Arthur 1956; Yamauchi and Funakoshi 2000). Although the Palearctic geographical range of *R. ferrumequinum* extends from Western Europe to Eastern Asia, the spatial separation of glacial refugia and temporal shift of recolonization events (in Europe vs. East Asia) may have helped to maintain the genetic diversity in both the bat hosts (as reported by Flanders et al. 2009) and their *I. vespertilionis* ticks (as supported by data of the present study).

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* (Hornok et al. 2014; S. Hornok, unpublished observations), 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. (Piksa et al. 2014). This lineage (consisting of hosts of *I. ariadnae* and similar genotypes) shows clearly a separate position on the bat phylogenetic tree (**Fig. 3b**).

The taxonomy of the preferred host of *I. simplex*, *Miniopterus schreibersii*, has recently undergone revision. While previously it was considered as a uniform species widely distributed across the Old World, it turned out to be a complex comprising several species (Li et al. 2015). Among them, in the present study *I. simplex* was collected from both *Mi. 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 (**Fig. 3a**), but also their three respective bat host species from other bat species involved in the present study (**Fig. 3b**). Again, glaciation and resultant geographic isolation might be considered as major mechanisms underlying the genetic differentiation of these closely related Asiatic *Miniopterus* spp. (Li et al. 2015) and may have also served as the basis for the relative genetic heterogeneity (96 % COI sequence similarity) of their ticks as demonstrated in the present study.

It has been suggested that in the case of an exophilic, generalist tick species, like *I. persulcatus* (which has a similarly broad, but northern Eurasian geographical range), glaciation events may have caused the loss of significant genetic variation because of genetic mixing during refuge formation *(*Kovalev and Mukhacheva 2012*).* On the contrary, populations of bat ticks and their hosts in the southern parts of Asia may have remained relatively unaffected by ice age(s), maintaining drivers in formerly initiated species divergence. Moreover, separation from other parts of Eurasia during the glacial periods might fulfill the role of geographical barrier formation for South-Eastern Asia, where the biodiversity could be maintained or was even enhanced during ice ages (Woodruff 2010*).*

Strict morphological criteria of all tick developmental stages and genotyping from a larger number of samples may help to clarify the precise taxonomical status of highly divergent genetic variants of ixodid bat ticks reported here. This will also compensate for the lack of voucher specimens in the case of some South East Asiatic bat tick genotypes tested molecularly in the present study or reported previously. Data of this study also reflect that *I. vespertilionis* may represent a species complex, given the strong bootstrap support for the exclusion of two specimens from Japan and Vietnam from the clade that contains all specimens from Europe.

6.1.2 Identification of *Ixodes ariadnae* in Germany

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 (Griffin 1970), these data suggest the autochthonous occurrence of *I. ariadnae* in Germany.

This is the first record of *I. ariadnae* in Germany, and in any country other than Hungary, where this species has been recently discovered (Hornok et al. 2014). The tick will be deposited in the Museum für Naturkunde Berlin. In order to ascertain if the present finding represents a unique case, or *I. ariadnae* has a more widespread occurrence in Germany or the surrounding countries, further bat tick monitoring is recommended.

6.1.3 Mitochondrial gene heterogeneity of the bat soft tick *Argas vespertilionis* (Ixodida: Argasidae) in the Palaearctic

A total 314 *A. vespertilionis* larvae from 329 were srcreened and morphologically investigated.

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: (Hoogstraal 1956)), therefore almost exclusively these can be collected from bats. In the present study *A. vespertilionis* was found on 15 bat species, most of which are already reported hosts (including four *Pipistrellus* spp., seven *Myotis* spp. and two *Eptesicus*, as well as two *Nyctalus* spp. (Siuda et al. 2009; Frank et al. 2015)).

While *A. vespertilionis* is mentioned in the most recent list of valid tick names as a member of the genus *Argas* (Guglielmone et al. 2010), morphological and phylogenetic analyses do not support this assumption (Burger et al. 2014; Klompen and Oliver 1993). Instead, based on its 12S rRNA gene, *A. vespertilionis* was demonstrated to belong to Ornithodorinae (Burger et al. 2014). Phylogenetic analyses of the present study also reflected that haplotypes of *A. vespertilionis* clustered outside the Argasinae.

During the past few decades scientific debate tried to establish morphological features suitable to solve the taxonomical uncertainty among the Argasidae. In the larval stage the number of setae according to anatomical location is an important feature to recognize genera (Barros-Battesti et al. 2013) and the shape of dorsal plate, the morphology of hypostome and the length of setae may be used to distinguish closely related species (Jones and Clifford 1972, Venzal et al. 2013).

The possibility that more than one species exist under the name *A. vespertilionis* has already been suggested by Hoogstraal (1958), but this remained hitherto unevaluated. 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. Similarly, in a previous study comparing neotropical bat soft ticks, selected measurements (length of certain setae) differed slightly between larvae from different countries, but these were considered to represent the same species (Venzal et al. 2013). Intraspecific variations in body outline of *A. vespertilionis* have also been reported (Hoogstraal 1958). Furthermore, despite the differences in the mean

length and width of the dorsal plate between *A. vespertilionis* from Europe and Vietnam, as demonstrated here, these alone cannot serve to delineate species, because the shape of the dorsal plate (reflected here by similar length:width ratios) is regarded as more relevant in this context (Jones and Clifford 1972, Venzal et al. 2013).

A minor difference was also observed between the serrate setae of *A. vespertilionis* larvae collected in distant regions of Eurasia. However, while the types of fringed setae were reported to be different between larvae of closely related *Ornithodoros* spp. (Jones and Clifford 1972), the latter were also shown to differ in the ranges of their setal lengths and hypostome (unlike *A. vespertilionis* larvae here). Therefore, in the absence of further distinguishing characteristics, the present data suggest that *A. vespertilionis* in Europe and Vietnam belong to the same species and observed minor differences (i.e. dorsal plate size) should be interpreted as intraspecific variations between populations. The morphology of specimens analyzed in this study also suggests that they are conspecific with *A. vespertilionis* reported from Japan (Yamaguti et al. 1971).

On the other hand, specimens from Europe and Vietnam had *cox*1 sequence divergence (7.1–7.5%) exceeding that proposed for closely related ixodid tick species (6%, see (Lv et al. 2014)). 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. This seems to be justified from the morphology of the larval stage alone (because differences between argasid larvae served to describe new soft tick species, e.g. in (Jones and Clifford 1972)), but morphological investigation of adult specimens from both regions and molecular/phylogenetic analyses of nuclear markers (18S and 28S rRNA genes) should ultimately confirm this conclusion.

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 (although a larger sample size from sub-Saharan Africa is needed to draw final conclusion in this respect). In support of this possibility, some of the main hosts of *A. vespertilionis* in the present study, most notably *Pipistrellus nathusii* is known to migrate long distances (up to 1,900 km) in the north-eastern to south-western direction (Hutterer 2005). Another important host, *P. kuhlii* is widespread in certain regions across Europe, the Middle-East, North Africa and Asia (Bray et al. 2013).

In Eurasia, high degree of mitochondrial gene heterogeneity (i.e. up to 16% *cox*1 sequence divergence) has recently been demonstrated between ixodid bat ticks that had been regarded as conspecific (Hornok et al. 2015c). This was explained by the preference of each tick species for bat hosts from a single genus, as well as by the geographical separation of

relevant bat host species (Hornok et al. 2015c). In comparison with ixodid bat ticks, the less pronounced difference (in terms of both morphology and genetics) between geographically distant isolates of *A. vespertilionis*, as shown here, may root in the fact that this soft tick species has a broad host spectrum (involving vespertilionid bats from at least six genera, as also shown here), thus preventing complete allopatric separation of its populations.

In its evaluated geographical range, no larval phenotypic differences justify the existence of separate species under the name *A. vespertilionis*. However, phylogenetic analyses based on two mitochondrial markers suggest that it represents a complex of at least two putative cryptic species. 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.

6.1.4 Phylogenetic analyses of bat-associated bugs (Hemiptera: Cimicidae: Cimicinae and Cacodminae)

These results provide molecular data of bat-associated cimicid bug species from three distant regions of the Old World (i.e. central-eastern Europe, south-eastern Asia and South Africa). One of the studied bug species, *Ci. lectularius* is the most significant member of Cimicidae, taking into account its association with humans, global distribution, historical and economic impact, recently witnessed emerging character and potential health hazards (Zorrilla– Vaca et al. 2015). In a recent study on *Ci. lectularius* (Booth et al. 2015) mostly central and western Europe were represented by sampling sites, therefore results shown here can be regarded as complementary to that study, introducing samples from more locations in Hungary, as well as samples from Romania and Vietnam into the phylogenetic analysis of this species group. In addition, molecular analyses of *Cacodmus* spp. from South Africa have not yet been reported.

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 (Balvín et al. 2014). 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 (such as *Pipistrellus* and *Nyctalus* spp.), 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 (Balvín et al. 2012b). This is confirmed by the data presented here, taking into account the roosting behavior of the four bat species, which were found bug-infested (**Table 8**). In addition to *Pi. pipistrellus*, *Pi. hesperidus* colonies can also be found in narrow cracks and dead trees (Taylor 2005). *Myotis bechsteinii* is a tree-dwelling bat species; its

females establish their maternity colonies in tree holes and switch their day-roosts regularly (Dietz and Pir 2009, Kerth et al. 2001).

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, collected from *Pi. pipistrellus* in Hungary, showed the morphology of *Ci. lectularius* and was different from *Ci. emarginatus* (e.g. in the parameters of its head, palpal segments, posterior bristles and the shape of paragenital sinus). The second specimen, collected from *H. pulveratus* in Vietnam, was also similar to *Ci. lectularius* based on its coxal spur and some aspects of its pronotum (which was 2.5 times broader than long, unlike that of *Ci. insuetus*). However, the paragenital sinus of the latter female was different from that in *Ci. lectularius*, i.e. it was rounded, which is a character of Neotropical species of the genus *Cimex*, not found in the Old World (Usinger 1966). In addition, taking into account that in case of both of these new variants the *cox*1 genetic difference exceeded 7% in comparison with *Ci. lectularius* (and this value was 5.8–6.4% between *Ci. lectularius* and members of the *Ci. hemipterus* or *Ci. pipistrelli* species groups, see (Balvín et al. 2015)), they probably represent new species. In order to clarify the precise taxonomical status of these new genotypes, they will have to be compared by including more specimens and analyses.

While *Ci. lectularius* from roosts of *Myotis* spp. yielded multiple haplotypes within the same major haplogroup (Booth et al. 2015), in the present study *Ci. lectularius*-related specimens from pipistrelloid bats showed highly divergent *cox*1 and ITS2 haplotypes at both small and large geographical scales (i.e. in Hungary and Vietnam, respectively). This phenomenon is similar to the one suggested in the case of bat-associated bugs of the *Ci. pipistrelli* group, which were also shown to have different host ranges (Usinger 1966), although the association of *Ci. pipistrelli* with different host species is thought to be a driver of morphological (rather than genetic) variability (Balvín et al. 2013).

The present results extend the geographical range of *Ca. ignotus* (hitherto only reported from Uganda, see (Usinger 1966) to South Africa. This can be explained by the occurrence of *Pi. hesperidus* (from which it was collected in the present study) in much of East Africa, from Ethiopia to South Africa (Kearney 2013). However, it was unexpected to find that bat-associated bugs identified here on a morphological basis as *Ca. ignotus* and *Ca. sparsilis* had highly (6.8%) different *cox*1, but identical ITS2 sequences. This observation is similar to that reported previously in the Western Palaearctic region on *Ci. pipistrelli*, which had only limited variability in ITS2 sequences (and none in other nuclear markers), despite the separation of corresponding *cox*1 haplogroups (Balvín et al. 2013).

In general, the resolution of *cox*1 analysis to assess the degree of divergence between closely related species is known to be much higher compared to ITS2 (ticks: 6.1 *vs* 2.3%;

mites: 3.0–4.0% *vs* < 0.5%, respectively) (Lv et al. 2014, Navajas and Boursot 2003).

Nevertheless, in the present case, the identity of ITS2 sequences between individuals of two *Cacodmus* spp. could have resulted from genetic introgression or hybridization.

Bugs of the *Ci. lectularius* group associated with different bat host species (myotines *vs* pipistrelloid bats) were found to belong to different genetic lineages. 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 addition, *Ca. ignotus* is reported for the first time in South Africa.

6.2 Parasitic (vector-borne) and free-living protozoa in bat ectoparasites and faeces

6.2.1 DNA of piroplasms of ruminants and dogs in Ixodid bat ticks

In this study all three ixodid tick species have been collected, which are specialized to bat hosts in Europe. In the present study *I*. *ariadnae* was not found in Romania, suggesting that the bat tick fauna in this country is similar to that in the Balkans, with the predominance of *I*. *simplex* (Burazerović et al. 2015). A plausible explanation for this phenomenon is the absence of regular bat migration between the mountainous regions of Hungary and Romania, which otherwise could have caused the spread of *I*. *ariadnae* towards the southeast (the main route of long-distance bat migration in the region is in the southwestern-northeastern direction: (Hutterer 2005)).

The present results confirmed that the preferred hosts of *I*. *ariadnae* belong to Vespertilionidae (Casati et al. 2006), those of *I*. *vespertilionis* to Rhinolophidae, while *I*. *simplex* is adapted to parasitize *Mi*. *schreibersii* (Miniopteridae) (Arthur 1956). Nevertheless, to the best of our knowledge, several new host associations of ixodid bat ticks are reported here for the first time.

In particular, *I*. *ariadnae* was found newly on four *Myotis* spp., *I*. *vespertilionis* on two *Myotis* spp., as well as on *Eptesicus serotinus* and *Rhinolophus mehelyi*, finally *I*. *simplex* on *Barbastella barbastellus* (**Table 16**).

Table 16. Host associations of ixodid bat ticks reported previously and in this study.

	Host species reported previously	References	New host species in this study
Ixodes ariadnae	*Myotis alcathoe*	Hornok et al. 2014 Hornok et al. 2016b	*Myotis dasycneme*
	Myotis bechsteinii		*Myotis daubentonii*
	Myotis blythii		*Myotis emarginatus*
	Myotis myotis		*Myotis nattererii*
	Plecotus auritus		
Ixodes vespertilionis	*Miniopterus schreibersii*	Ševčík et al. 2010 Burazerović et al. 2015 Arthur 1956 Rupp et al. 2004 Siuda et al. 2009 Mihalca et al. 2012 Frank et al. 2015 Piksa et al. 2016	*Eptesicus serotinus*
	Myotis bechsteinii		*Myotis capaccinii*
	Myotis blythii		*Myotis dasycneme*
	Myotis brandtii		*Rhinolophus mehelyi*
	Myotis daubentonii		
	Myotis emarginatus		
	Myotis myotis		
	Myotis mystacinus		
	Myotis nattererii		
	Plecotus auritus		
	Rhinolophus euryale		
	Rhinolophus ferrumequinum		
	Rhinolophus hipposideros		
Ixodes simplex	*Miniopterus schreibersii*	Burazerović et al. 2015 Arthur 1956 Rupp et al. 2004 Krištofík and Danko 2012	*Barbastella barbastellus*
	Myotis alcathoe		
	Rhinolophus euryale		
	Rhinolophus ferrumequinum		
	Rhinolophus ferrumequinum		

The intensity of tick infestation was not significantly different between small and large size bat species (in the case of *My*. *daubentonii* vs. *R*. *ferrumequinum*, respectively), suggesting that factors depending on body size (such as the body surface area, interrelated with metabolic rate, heat emission: (Hock 1951)) may not be crucial for host finding by bat ticks. Similarly, it has been reported that body size of passeriform bird species did not significantly influence the intensity of their tick infestation (Hornok et al. 2016c). On the other hand, the intensity of infestation with bat tick larvae was significantly higher, than with later stages in the life cycle. This finding is consistent with the significant decrease of individual number of tick stages with the advance of tick developmental cycle (Korotkov 2004).

Taking into account the considerable lack of data in literature on the vector potential of ixodid bat ticks, DNA extracts of 307 specimens were molecularly analysed for the presence of piroplasms (Apicomplexa: Piroplasmida). Among piroplasms, *Babesia* species are known to be transmitted transovarially by female ticks to the next generation (i.e. to larvae prior to their blood meal), whereas *Theileria* species are transmitted transstadially (Fujisaki et al. 1994). The latter implies that there is no other way for tick larvae to harbor theileriae or to contain theileria DNA, then to ingest these with the blood meal from a host/reservoir which is either theileria-infected or at least theileria DNA is present in its blood stream.

Babesia vesperuginis DNA was molecularly identified here in *I*. *ariadnae* and *I*. *vespertilionis*. This piroplasm is pathogenic to bats and was reported to infect *Pipistrellus pipistrellus* (Gardner and Molyneux 1987), several *Myotis* spp. (including *My*. *daubentonii*, on which bat species a PCR positive tick was collected in the present study) as well as *Plecotus auritus* (Sebek et al. 1975). 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 (Corduneanu et al. 2017). In 2018, *Ba. vesperuginis* was molecularly detected in *Pi. pipistrellus*, in *Eptesicus serotinus* and in soft ticks collected from bats in China (Han et al. 2018; Liu et al. 2018). Taking into account that soft ticks (*Argas vespertilionis*) have been incriminated as vectors of *B*. *vesperuginis* (Gardner and Molyneux 1987), the present results suggest that bat ticks carrying this piroplasm (or its DNA) ingested it with the blood meal, i.e. further *Myotis* spp. (exemplified by *My*. *dasycneme*) and *Eptesicus serotinus* might also be susceptible to *B*. *vesperuginis*.

Babesia crassa has low pathogenicity in small ruminants and its vector is unknown (Hashemi-Fesharki and Uilenberg 1981). This piroplasm (or closely related genotypes) were reported to occur only in the Middle-East, but recently one genotype has also been identified in *Haemaphysalis inermis* ticks in Central Europe, Hungary (Hornok et al. 2015d). In the present study two different DNA sequences of *B*. *crassa* were detected in bat ticks (*I*. *vespertilionis*, *I*. *simplex*) in both Hungary and Romania. These two bat tick species have never been reported from small ruminants, and therefore their PCR positivity can be explained by ingesting *B*. *crassa* DNA-containing blood meal from bats. In this context it may be epidemiologically relevant that *B*. *crassa* was reported to be present in *H*. *sulcata* (Aktas 2014), and this tick species was reported to infest bats in the larval stage and small ruminants in the adult stage (Filippova et al. 1976).

Babesia canis is an important parasite of dogs. Wild canids are also susceptible (Kuttler 1988). The known vector of this piroplasm is *Dermacentor reticulatus*, which is a tick species seldom infesting bats, including *Mi*. *schreibersii* (Neumann 1911). Recently, bats were reported to pass the DNA of *B*. *canis* in their faeces (Hornok et al. 2015e). 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, *B*. *canis* or its DNA might have been present in the blood of relevant bats. This possibility is supported by recent finding of *B*. *canis* DNA in bat tissues (Corduneanu et al. 2016).

Interestingly, the DNA of *B*. *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. This piroplasm (associated with cervids as hosts) is zoonotic, with *I*. *ricinus* as its vector. It is noteworthy that *I*. *ricinus* occurs on bats (e.g. Ševčík et al.

2010), and the present results suggest that this may allow bats to become carriers of *B*. *venatorum* or its DNA (taking into account that *I*. *simplex* has never been reported from cervids or from humans). On the other hand, the host of *I*. *simplex*, *Mi*. *schreibersii* may live in large colonies in the human environment (e.g. mines, man made tunnels, ruins: (Hutson et al. 2008)). Therefore, this preliminary finding deserves further molecular epidemiological investigation.

Among *Theileria* spp. and genotypes, the DNA of *Theileria* sp. OT3 has been detected here in a female *I*. *simplex*. This piroplasms (with unknown pathogenicity) was formerly reported to infect small ruminants in Italy (Giangaspero et al. 2015), but recently its DNA has also been reported from *Haemaphysalis punctata* in northern Hungary (Hornok et al. 2015d), and this tick species is known to infest bats (Estrada-Peña 1989).

In addition, the DNA of two *Theileria* spp. have been shown here to be present in larvae of *I*. *simplex* from *Mi*. *schreibersii*. Among them, *T*. *capreoli* is a mildly pathogenic parasite of cervids. The tick species *H*. *concinna*, in which the DNA of *T*. *capreoli* has been recently demonstrated in Hungary (Hornok et al. 2015d), is also known to occasionally infest bats (Lebedeva and Korenberg 1981).

Members of the *T*. *orientalis* complex (*T*. *orientalis*, *T*. *buffeli*) infect cattle in the tropical-subtropical regions of the globe, usually with low pathogenicity. Recently, *T*. *orientalis* has been shown to emerge in Central Europe (Hornok et al. 2015d) and Australia (Kamau et al. 2011), sometimes severely affecting cattle (Izzo et al. 2010). Vectors of the *T*. *orientalis* complex are *Haemaphysalis* spp. (Fujisaki et al. 1994). *Haemaphysalis* spp. may accidentally infest bats, and in particular *H*. *punctata*, the most likely vector of *T*. *orientalis* in Europe, was synonymously called "*H*. *rhinolophi*" (Estrada-Peña 1989). Furthermore, in South-East Asia (where species of the *T*. *orientalis* complex are widespread) at least one *Haemaphysalis* sp. has bats as preferred hosts (Hoogstraal 1964). These literature data attest a possible connection between large ruminants and bats via *Haemaphysalis* sp. ticks. In the present study only bat tick (*I*. *simplex*) larvae were PCR positive for *T*. *capreoli* and *T*. *orientalis*. This means that relevant piroplasms (or their DNA) could have been acquired by the larvae exclusively from the blood of bat hosts, because there is no transovarial, only transstadial transmission in the case of theileriae (Fujisaki et al. 1994). The significance of the potential epidemiological role of bats in bovine theileriosis deserves further attention, as several bat species may use cattle stables for roosting (Dekker et al. 2013).

In summary, competent vectors of the above piroplasms (that have been hitherto reported from hosts other than bats) 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) (Hornok et al. 2015e), or when gleaning bat species feed on insects from the lower vegetation in meadows or forests. However, *Mi*. *schreibersii*, associated with most of the piroplasms identified in the present study, is not known to forage

on the ground level (Vincent et al. 2011). Alternatively, blood-sucking flies have the potential to carry and transmit *Babesia* spp. (Friedhoff 1988) and *Theileria* spp. (Hammer et al. 2016), and flies (Insecta: Diptera) are among the frequent food items of e.g. *Mi*. *schreibersii* (Presetnik and Aulagnier 2013). This implies that bats may get into contact with or may have access to piroplasms or piroplasm DNA from their food.

Thus, there are two plausible explanations for the above, unexpected findings. The first is that bats get into frequent contact with the DNA of vector-borne pathogens contained in their food. During digestion this DNA may pass through the gut wall (barrier) un- or only partly digested, thus appearing in the circulation (or perhaps other tissues) from where bat ticks can take it up with their blood meal. In support of this possibility, it has recently been verified that meal-derived DNA fragments (even long ones) can avoid degradation and through not-yet-known mechanisms enter the circulation, at least in humans (Spisák et al. 2013).

Another, although less likely explanation is that bats are susceptible to a broader range of piroplasms than previously thought. The phylogeny of piroplasms has recently been shown to reflect considerable host diversity and limited host specificity (Lack et al. 2012), suggesting that these tick-borne protozoa have undergone frequent host switches during their evolution. In this context the present results may imply that bats may share piroplasms with a broad range of mammals (from various orders). Similarly, several *Babesia* and *Theileria* spp. are known to infect hosts from different mammalian orders (e.g. *B*. *caballi*, *B*. *canis*, *B*. *divergens*, *B*. *microti*, *T*. *equi*: (Lack et al. 2012)).

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 light of these findings, the role of bats in the epidemiology of piroplasmoses deserves further investigation.

6.2.2 Piroplasm DNA detection in *Argas vespertilionis*

Based on the previous studies, this is the first molecular evidence on the occurrence of *Babesia vesperuginis* in Asia. In a previous, *B. vesperuginis* DNA was only detected in hard ticks (*Ixodes ariadnae* and *I. vespertilionis*) from bats in Vespertilionidae, whereas DNA of other piroplasms (infecting ruminants and dogs) were shown to be present in ixodid ticks (*I. vespertilionis* and *I. simplex*) from Rhinolophidae and Miniopteridae (Hornok et al. 2016d). This is in line with findings of the present study, because (1) up to now, despite extensive examinations of rhinolophid and miniopterid bats in Hungary (data not shown), *A. vespertilionis* was only found on members of Vespertilionidae, and (2) in a high number of soft tick larvae

from Vespertilionidae (investigated here for the presence of piroplasm DNA) only *B. vesperuginis* was detected. The most likely explanation for this phenomenon is (accepting that the most likely vector of *B. vesperuginis* is *A. vespertilionis*) that vespertilionid bats acquire *B. vesperuginis* from its biological soft tick vector, whereas Rhinolophidae/Miniopteridae species have access to a broader range of piroplasms or their DNA from relevant mechanical vectors in their food (Hornok et al. 2016d). Based on the previous results (Hornok et al. 2016d), *B. vesperuginis* 18S rRNA gene sequences were identical within Europe, i.e. between Hungary, Romania (KU958544) and the UK (AJ871610). 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). This is in contrast to several other *Babesia* spp., as exemplified by *B. canis* with different 18S rRNA genotypes within countries (e.g. KP835549-50 in Hungary: Hornok et al. 2015e; KU958551-2 in Romania: Hornok et al. 2016d).

The bat fauna of the Northern Palearctic is characterized by two isolated complexes, the European-Ural and the Siberian-Far Eastern (Orlova 2014). As already suggested, a similar pattern of spatial distribution also exists among ectoparasites found on relevant bat species in Northern Eurasia, and bat ectoparasites found on (common) hosts from these different faunistic complexes may elucidate the possibility of contacts between the European and Siberian parts (Orlova 2014). While studying this concept, it must be taken into account that postglacial recolonization of Europe by several small *Myotis* spp. (including important hosts of *A. vespertilionis*, e.g. *My. alcathoe*, *My. brandtii*) occurred from the eastern direction, from the region of Caucasus (Dietz and Kiefer 2016), and historically this may have also contributed to the observed genetic homogeneity of bat ticks and a tick-borne pathogen between Central Europe and Central Asia. More recently, the West Siberian Plain acted as a barrier (segregating North Palearctic Chiroptera faunae), but during the past decades human settlements became more expanded into this area and non- migratory bat species became provided with increasing numbers of anthropogenic shelters, favoring the spread of bats between the West and East Palearctic (Orlova 2014). Some bat species that migrate middle range distances (e.g. *My. daubentonii*, a common host of *B. vesperuginis*), occur across the Northern Palearctic from Europe to the Far East (Bogdanowicz 1994; Dietz and Kiefer 2016). In addition, there are long-distance migratory (transpalearctic) species which may even cross this vast region, such as *My. dasycneme*, *V. murinus* and *E. nilssonii* (Orlova 2014) the first two being important host species of *A. vespertilionis*. *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 (Kruskop et al. 2012) 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), and their eastward expansion may have been linked historically with the spread of human-altered habitats (Kruskop et al. 2012). This bat species can migrate both in latitudinal and meridional directions (Orlova 2014). 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.

In contrast to this, both mitochondrial markers analyzed here (cox1 and 16S rRNA gene) indicate high genetic difference (i.e. reduced gene flow) between *A. vespertilionis* from Central Asia and Southeastern Asia (represented by Vietnam) over a shorter distance. This 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.

In summary, molecular analyses of *A. vespertilionis* suggest a genetic link of bat parasites (soft ticks and piroplasms) between Central Europe and Central Asia through the North Paleartic. Other flying vertebrates, i.e. birds had already been incriminated in spreading tick-borne pathogens in a similar geographical context, i.e. between the Far East, Siberia and Europe (Moskvitina et al. 2014; Ponomareva et al. 2015).

Therefore, the present results draw the attention to the connectedness of these regions of Eurasia, which is relevant and should be taken into account whenever considering epidemiological scenarios associated with (not only tick-borne) pathogens of bats.

6.2.3 DNA of free-living bodonids (Euglenozoa: Kinetoplastea) in bat ectoparasites

Morphological and molecular-phylogenetic evidences strongly support the origin of parasitic trypanosomatids from free-living bodonids like *B. saltans* (Simpson et al. 2006; Flegontov et al. 2013; Lukeš et al. 2014). 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.

The typical natural habitat of *B. saltans* and related bodonids is freshwater (Mitchell et al. 1988). Taking into account that neither ixodid/argasid bat ticks nor cimicid bat bugs develop in water, and these ectoparasites live in a very close association with their terrestrial bat hosts, it is reasonable to suppose that these arthropods could not ingest bodonids from an aquatic environment. In addition, both PCR-positive ticks were larvae, i.e. did not feed in a previous stage, but were collected during their first blood meal. Therefore, in the absence of known transovarial transmission of Bodonidae in ticks, the most likely source of bodonid DNA detected here in obligatorily haematophagous bat ectoparasites is the blood of their bat hosts. If so, this phenomenon is not completely new, because free-living species of bodonids were reported to occur in the urine and faeces of various mammals other than bats (Das Gupta and Chatterjee 1983; Vandersea et al. 2015). In addition, bodonid DNA was repeatedly detected in blood samples obtained from ungulates (Auty et al. 2012; Jan Votýpka, unpublished data). Results of the present study are in line with the finding of *B. saltans*-like DNA in the blood of bats in South America (Dario et al. 2017). Although in the relevant study (Dario et al. 2017) the authors could not entirely rule out that the detection of *B. saltans*-like DNA in bat blood samples resulted from environmental contamination during sampling, their precautions argued against this.

In the present study environmental contamination related to the sampling procedure can be excluded, because bat ectoparasites were put into ethanol on the premises, and it is very unlikely that *B. saltans* (or its DNA) would withstand ethanol-based disinfection (Dario et al. 2017). In addition, bat ectoparasites of the present study were further surface-disinfected under laboratory conditions, and not only the DNA of *B. saltans* was detected in them (for which the possibility of contamination during sampling was considered on account of its ubiquity: Dario et al. 2017), but also the DNA of a neobodonid. Unfortunately, even when assuming that bodonid DNA amplified in the present study derived from the blood of bats, it can only be speculated how these protists were acquired by bats. Although echolocating bats are predominantly insectivorous and may thus have access to a wide range of insect-parasitic

monoxenous trypanosomatids (e.g. *Crithidia* spp.) in their insect food, these are an unlikely source of bodonids identified in the present study, because Bodonidae includes only a few entomophilic species (Lipa 1963), and in particular *B. saltans* is not known to occur in insects. On the other hand, all bat species rely on drinking from freshwater bodies (Greif and Siemers 2010). 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 (Rizzi et al. 2012) and even complete genes can transgress the gut barrier (Spisák et al. 2013). 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. Similarly, this was the most likely mechanism explaining the presence of DNA from piroplasms of ruminants and dogs in bat ticks (Hornok et al. 2016d). It might be relevant to note in this context that bats are susceptible to *T. cruzi* (which, according to phylogenetic evidence, evolved from bat trypanosomes), and bats may acquire *T. cruzi* infection orally (Klimpel and Mehlhorn 2014). This necessitates the ability of *T. cruzi* to pass through the oral/gastric mucosa (Thomas et al. 2007), similarly to other stercorarian trypanosomes (Lima et al. 2013). However, this has not been demonstrated in case of bodonids.

At the same time, it was unexpected that none of the bat ectoparasites were found to contain the DNA of bat-specific trypanosomes. The quantity and quality of the DNA in the tick extracts, molecularly analysed here for kinetoplastids, were verified in other studies involving the same samples (Hornok et al. 2016d, 2017); therefore, based on their PCR negativity, these ticks did not contain trypanosomes. One plausible explanation is that none of the bats sampled in this study were infected with *Trypanosoma* spp., i.e. their ectoparasites were also trypanosome free. On the other hand, it is also possible that some of the tickinfested bats were infected with trypanosomes, but these bats had low parasitaemias and the few trypanosomes in the tick blood meal became digested after phagocytosis by midgut cells. This latter phenomenon might account for the PCR negativity of non-vector ticks, even when collected from hosts harbouring trypanosomes in their blood, as observed by others (Botero et al. 2016). The above findings highlight the importance of studying bats and other mammals for the occurrence of bodonids in their blood and excreta. This phenomenon should be investigated further as a potential, hitherto missing link in the evolution of free-living kinetoplastids towards parasitism.

6.2.4 Screening of bat faeces for arthropod-born apicomplexan protozoa

6.2.4.1 *Babesia canis* DNA 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: (Adaszek and Winiarczyk 2008)) occur in Hungary.

There are three possible explanations for this unexpected finding. First, relevant bats may have eaten infected tick vectors of *Ba. canis*, i.e. *Dermacentor reticulatus*. To evaluate this possibility, the five *Babesia*-positive faecal DNA samples were molecularly analysed for the presence of tick DNA (mitochondrial 16S rDNA gene). All five samples were PCR negative. If relevant bats (with *Ba. canis* PCR positive faeces) have ingested infected tick vectors, the DNA of *D. reticulatus* should have been detected in their faeces, similarly to that of other prey arthropods (Witsenburg et al. 2014). This is supported by literature data: although bats also feed on arachnids, to the best of our knowledge ticks were never reported to be part of their diet (e.g. (Hope et al. 2014, Pereira et al. 2002)). Alternatively, blood-sucking flies (e.g. *Stomoxys* spp.) are known to be incriminated as mechanical vectors in the transmission of *Babesia* spp. (Friedhoff 1988). *Stomoxys calcitrans* (also called “dog fly”) was reported to frequently bite dogs (Fankhauser et al. 2015), and to be a predominant species in the diet of some bat species (Kervyn et al. 2012). Therefore, *Ba. canis* DNA in bat faeces may have originated from haematophagous flies which had sucked blood on parasitaemic dogs (in an opportunity offered by the two regions highly endemic for *Ba. canis*) and were consequently eaten by the relevant bats. Unfortunately, two factors precluded to test this hypothesis, i.e. (1) the whole faecal sample of relevant bats was used for DNA extraction (thus morphological analysis of fly remnants was not possible), and (2) to the best of our knowledge PCR-based molecular methods specific for *S. calcitrans* are not available.

However, the presence of *Ba. canis* DNA in the faeces may also indicate the infection of relevant bats (i.e. parasitaemia), in which case *Babesia* DNA could get from the circulation into the gut contents (similarly to the DNA of other erythrocyte-infecting protozoa, e.g. *Plasmodium* spp. in primates: (Liu et al. 2010)). In support of this possibility, among the preferred rodent hosts of *D. reticulatus* larvae/nymphs (Nosek 1972) many *Apodemus* spp. are arboreal, i.e. known for their climbing habit on trees (Holišová 1969). *Dermacentor* larvae and nymphs were reported to be present in such arboreal nests (Durden et al. 2000), and in this way may be shared between rodents and bats (Apanaskevich and Bermúdez 2013). All four bat species with *Ba. canis* PCR positive faeces are known for their preference of tree holes as summer roosting places (Dietz et al. 2009, Eurobats 2010), where they could thus have become infested with *Dermacentor* larvae/nymphs (as reported for *Pipistrellus pipistrellus*

sampled in July: (Filippova et al. 1976)). Therefore, it cannot be completely excluded that those bats, which were PCR positive in their faeces, may have actually become infected with *Ba. canis* − a protozoan hitherto reported from two mammalian orders (besides Carnivora also from Perissodactyla: (Hornok et al. 2007)), both taxonomically closely related to Chiroptera (Nishihara et al. 2006).

6.2.4.2 *Besnoitia besnoiti*-like DNA in bat faeces

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.

The source of the *Be. besnoiti*-like sequence in the present study, the pond bat (*Myotis dasycneme*) is known to be a long distant migratory species (up to 300 km seasonal migration: (Limpens et al. 2000)), and the closest endemic focus of bovine besnoitiosis in northern France is situated within 300 km of the relevant sampling site (Alvarez-García et al. 2013). In general, bats frequently use cattle stables for roosting (Dekker et al. 2013), where they may have access to the mechanical vectors of *Be. besnoiti*, i.e. blood-sucking flies (*S. calcitrans*, *Tabanus* spp.) and mosquitoes (Alvarez-García et al. 2013). 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. Blood-sucking flies (especially *S. calcitrans*) were also reported to constitute a significant portion of bat prey insects (Kervyn et al. 2012). 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*.

On the other hand, *Besnoitia* cystozoites (carried by flies) are able to penetrate mucosal surfaces (Njenga et al. 1999). Accordingly, the quest for the final host of *Be. besnoiti* should be extended to include chiropterans, particularly because experimental infection with another *Besnoitia* sp. was shown to establish in bats (Schneider 1966).

These findings suggest that some aspects of the epidemiology of canine babesiosis are underestimated or unknown, i.e. the potential role of insect-borne mechanical transmission and/or the susceptibility of bats to *Ba. canis*. In addition, bats need to be added to future studies in the quest for the final host of *Be. besnoiti*.

In the present study no mixed infections were detected. This can be explained by the relatively low prevalence of those apicomplexans, the DNA of which could be amplified with the applied method (Casati et al. 2006) from bat faeces.

Toxoplasma gondii was reported to infect at least some of the bat species evaluated in the present study (Dodd et al. 2014). This apicomplexan is able to invade most nucleated cells (including cells crossing the gut barrier), and it was shown to be present in bat liver as well (Qin et al. 2014), therefore its DNA is likely to be shed in bat faeces. However, *T. gondii* was not detected in the present study. This can be explained by the inability of the applied method

(Casati et al. 2006) to amplify toxoplasma DNA, because the forward primer BJ1 cannot anneal to the 18S rDNA gene of *T. gondii* with its 3' end, unlike in the case of piroplasms, *Besnoitia* and *Sarcocystis* spp. (Hornok et al. 2016d).

6.3 Vector-borne bacteria in bat faeces

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 hypothesised 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 (Hornok et al. 2015c).

Thus, the DNA of a fly endosymbiont *Rickettsia* sp. in bat droppings can be explained by the presence of those flies as food items in the diet of the relevant bat species (*My. alcathoe*). On the other hand, the novel *Rickettsia* genotype demonstrated here from bats both in Hungary and the Netherlands may as well originate from the tissues of bats, especially taking into account that the *Rickettsia* sp. closest to this genotype was formerly amplified from rodent blood (KY488187). In addition, *R. helvetica* DNA was shown to be present in bat faeces. This most likely means that the relevant bat(s) harboured *R. helvetica*, because bats are not known to feed on the arthropod vector and reservoir of this *Rickettsia* species (i.e., *Ixodes ricinus*) and DNA of *I. ricinus* was not detectable in that particular sample. In support of this assumption, *R. helvetica* was formerly demonstrated from bat flea (Hornok et al. 2012). Therefore, bats should be further evaluated as potential reservoirs of *R. helvetica*.

N. risticii is the causative agent of equine neorickettsiosis (formerly called Potomac horse fever) (Vaughan et al. 2012). In digenean bat flukes this species has an endosymbiotic nature, i.e., it is transmitted vertically from the adult flukes to their eggs (Gibson et al. 2005). Subsequent developmental stages ensure the maintenance of *N. risticii* in the intermediate hosts (aquatic snails, insects) and eventually in the final hosts (insectivorous bats) of flukes (Vaughan et al. 2012). If horses inadvertently take up *Neorickettsia*-carrier insects when grazing, they become dead-end hosts in the life cycle and their infection usually leads to pathological manifestations such as acute diarrhoea, laminitis and abortion, with up to 30% mortality (Vaughan et al. 2012).

Here *Neorickettsia* DNA was identified in the faeces of the pond bat (*My. dasycneme*), which appears to be the most significant finding of the present study. Although the *Neorickettsia* sp. present in bat faeces was identified by sequencing only a short portion of its 16S rRNA gene, the real-time PCR used here for its detection is regarded as highly sensitive and specific for *N. risticii* DNA and is therefore the current standard for the diagnosis of equine neorickettsiosis (Taylor 2018). Coherent results of these two molecular approaches, as well as the phylogenetic clustering of this bat-related *Neorickettsia* sp. with *N. risticii* isolates (**Fig. 29**),

suggest that it may belong to the latter species. However, to confirm this, sequencing of the complete 16S rRNA gene and/or another marker would have been necessary, but this was beyond the scope of the present study.

To the best of our knowledge, this is the first molecular evidence on the occurrence of a *Neorickettsia* sp., phylogenetically clustering with *N. risticii*, in Europe. *N. risticii* and closely related genotypes are geographically widespread. Phylogenetic studies on *Neorickettsia* spp. were hitherto reported from North and South America, Australia, China and South-East Asia, as well as from North Africa, while molecular evidence on their presence in Europe has been lacking (Vaughan et al. 2012; Greiman et al. 2014, 2017). At the same time, isolated cases with seropositivity to *N. risticii* have already been published in France (Vidor et al. 1988) and the Netherlands (van der Kolk et al. 1991), where underlying epidemiological factors remained unelucidated.

Bat fluke species harbouring *N. risticii* and closely related species belong to genera Acanthatrium and Lecithodendrium (Pusterla et al. 2003), which can be found in European *Myotis* bats, including *My. dasycneme* (Frank et al. 2015). PCR positivity in the present case may have originated from fluke eggs containing *Neorickettsia* DNA (while passing with bat faeces) or perhaps from *Neorickettsia* DNA in infected cells crossing the gut barrier of relevant bats, which may also become horizontally infected (Gibson et al. 2005).

Haemoplasmas of the haemofelis group (unidentifiable to the species level) were shown to be present in the faeces of the same bat species, *My. dasycneme*. Although haemoplasmas have been reported in the blood of bats in Spain (Millán et al. 2015), to the best of our knowledge, they have never been reported from the faeces of bats. Haemoplasmas are known to pass detectable DNA in the faeces of their feline host (Willi et al. 2007), thus it is likely that relevant bats were actually infected with the detected bacteria. This implies that when/where blood sampling of bats is not possible, their faecal pellets may also provide useful data on their haemoplasma-carrier status. This is especially important from the point of view of further studies on bat haemoplasmas of the haemofelis group, which were found to be closely related to human haemoplasmas (Millán et al. 2015).

It is relevant in this context that several bat species roost in large colonies, sometimes within buildings (such as steeples), where the droppings of many individuals can accumulate (Klimpel and Mehlhorn 2014). This may increase the epidemiological risks associated with bat faeces. At the same time, in order to assess infection prevalence of bat-borne pathogens, bat dropping have to be sampled individually.

In conclusion, bats were shown to pass rickettsia and haemoplasma DNA in their faeces. Neorickettsia DNA is present in the faeces of the pond bat (*My. dasycneme*) in Europe, suggesting that this bat species plays a final host role in the life cycle of flukes harbouring neorickettsiae.

7. Overview of the new scientific results

1. The present research highlights to although all three ixodid bat tick species evaluated in the present study appear to be widespread in Eurasia, they exhibit pronounced genetic differences. Phylogenetic relationships of ticks showed similar clustering patterns with those of their associated bat host species (Chpt: 5.1.1; 6.1.1). Datas of this study also reflect that *I. vespertilionis* may represent a species complex. The present results confirmed that the preferred hosts of *I*. *ariadnae* belong to Vespertilionidae, those of *I*. *vespertilionis* to Rhinolophidae, while *I*. *simplex* is adapted to parasitize *Mi*. *schreibersii* (Miniopteridae) (Chpt: 5.2.1; 6.2.1). Nevertheless, to the best of our knowledge, several new host associations of ixodid bat ticks are reported here for the first time. We provided first the existence of *I. aridnae* in Germany (Chpt: 5.1.2; 6.1.2).
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. 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 (Chpt: 5.1.3; 6.1.3).
8. Molecular evidence is provided here on the existence of two new genotypes, most likely new species, within the *Ci. lectularius* species group. The relevant specimens were collected from pipistrelloid bats, therefore the association of *Ci. lectularius* with different bat host species (pipistrelloid vs myotine bats) should be evaluated further as a possible background factor of this genetic divergence. *Ca. ignotus* is reported for the first time in South Africa (Chpt: 5.1.4; 6.1.4).
9. The screening of bat ectoparasites for vectore- borne piroplasms highlite that 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 arthropod vectors. DNA sequences of piroplasms were detected in bat ticks. *I*. *simplex* carried piroplasm DNA sequences significantly more frequently than *I*. *vespertilionis*. 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* and *Theileria* sp. OT3 were amplified, as well as a shorter sequence of the zoonotic *B*. *venatorum* (Chpt: 5.2.1; 6.2.1).
3. Only *Babesia vesperuginis* has been found in samples of *Argas vespertilionis* with 100% identity (448/ 448 bp) 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. Molecular analyses of *A. vespertilionis* suggest a genetic link of bat parasites (soft

ticks and piroplasms) between Central Europe and Central Asia through the North Paleartic. Phylogenetic analyses of *Babesia vesperuginis* from *A. vespertilionis* specimens from Hungary indicate that *B. vesperuginis* is more closely related to the phylogenetic group of Theileriidae than to *Babesia* s.s. In particular, *B. vesperuginis* clustered closest to *Cytauxzoon felis* and the 'prototheilerid' *B. conradae* (Chpt: 5.2.2; 6.2.2).

4. DNA of free living bodonids (*Bodo saltans* and neobodonids) have been identified in three DNA samples of bat ectoparasites (*Ixodes. simplex*, *Argas vespertilionis* and *Cimex pipistrelli*). Phylogenetic analysis of these three DNA-positive samples showed that the sequence of *B. saltans* from the bat tick *I. simplex* aligned closest to other *B. saltans* isolates within the Eubodonida clade, which is a sister group to the Neobodonida clade that includes the two bodonid sequences amplified from the bat tick *A. vespertilionis* and the bat bug *Ci. pipistrelli*. At the same time, it was unexpected that none of the bat ectoparasites were found to contain the DNA of bat-specific trypanosomes (Chpt: 5.2.3; 6.2.3).
5. *Babesia canis canis* DNA was shown to be present in five individual 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. This is the first report of molecular analyses of a broad range of vector-borne bacteria in bat faeces. The DNA of a fly endosymbiont *Rickettsia* sp. in bat droppings can be explained by the presence of those flies as food items in the diet of the relevant bat species (*My. alcathoe*). *R. helvetica* DNA was shown to be present in bat faeces. This most likely means that the relevant bat(s) harboured *R. helvetica.* Here *Neorickettsia* DNA was identified in the faeces of the pond bat (*My. dasycneme*). This is the first molecular evidence on the occurrence of a *Neorickettsia* sp., phylogenetically clustering with *N. 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, *My. dasycneme*. Haemoplasmas are known to pass detectable DNA in the faeces of their feline host, thus it is likely that relevant bats were actually infected with the detected bacteria (Chpt: 5.2.4; 5.3; 6.2.4; 6.3).

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10. Author contribution

Krisztina Szőke took part in the collection, identification of ectoparasites, performed the majority of DNA extractions, participated in molecular-phylogenetic examinations, as well as in preparation of manuscripts; wrote the PhD thesis. Dr. Sándor Hornok initiated and organized the studies, did part of the morphological and genetic comparisons, wrote some of the articles. Dr. Tamás Görföl (Departement of Zoology, Hungarian Natural History Museum), Dr. Dávid Kováts (Department of Evolutionary Zoology and Human Biology, Debrecen University), Dr. Sándor A. Boldogh (Department of Nature Conservation, Aggtelek National Park Directorate), Dr. Péter Estók (Department of Zoology, Eszterházy Károly University) collected most of the Hungarian ectoparasite samples. Dr. Attila Sándor, Cordunenanu Alexandra and Dr. Andrei D. Mihalca (Department of Parasitology and Parasitic Diseases, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca) and Levente Barti (Romanian Bat Protection Association, Satu Mare, Romania) provided the ectoparasite samples from Romania and took part in the molecular analyses. Dr. Jason Dunlop at the Natural History Museum of Berlin (Museum für Naturkunde Berlin, Germany) lending old specimens for the study. Dr. Agustín Estrada-Peña (Department of Animal Pathology, University of Zaragoza, Zaragoza, Spain), Dr. Olivier Plantard (LUNAM Université, Oniris, Ecole nationale vétérinaire, agroalimentaire et de l'alimentation Nantes-Atlantique, Nantes, France), Dr. Sara Epis (Department of Biosciences, University of Milan, Milan, Italy), Dr. Vuong Tan Tu (Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Hanoi, Vietnam), Dr. Yuanzhi Wang (Department of Pathogenic Biology, School of Medicine, Shihezi University, Shihezi, China), Dr. Ali Halajian (Department of Biodiversity, School of Molecular and Life Sciences, Faculty of Science and Agriculture, University of Limpopo, Polokwane, South Africa), Dr. Adora Thabah (Solar View Cottage, Upper Mawprem, Shillong, Meghalaya, India), Dr. Snežana Tomanović (Laboratory for Medical Entomology, Centre of Exellence for Food and Vector-Borne Zoonoses, Institute for Medical Research, University of Belgrade, Belgrade, Serbia), Dr. Jelena Burazerović (Chair of Animal Ecology and Zoogeography, Institute of Zoology, Faculty of Biology, University of Belgrade, Belgrade, Serbia), Dr. Isabel G. Fernández de Mera, Dr. José de la Fuente (15SaBio. Instituto de Investigación en Recursos Cinegéticos IREC, Ciudad Real, Spain), Dr. Mamoru Takahashi (Department of Anesthesiology, Saitama Medical University, Iruma-gun, Japan), Dr. Takeo Yamauchi (Toyama Institute of Health, Imizu, Toyama, Japan) and Dr. Ai Takano (Department of Veterinary Medicine, Joint Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan) provided the parasites samples from Eurasia and Africa. Dr. Jenő Kontsán (Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Budapest) helped in phylogenetic analysis. Nóra Takács (Department of Parasitology and Zoology, University of Veterinary

Medicine), Dr. Hein Sprong (Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM)), Dr. Regina Hofmann-Lehmann, Dr. Marina L. Meli (Clinical Laboratory and Center for Clinical Studies, Vetsuisse Faculty, University of Zürich), Dr. Kinga Görföl-Sulyok and Dr. Miklós Gyuranecz (Institute of Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, Budapest) performed the laboratory examinations.

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