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Molecular investigations of the bat tick *Argas vespertilionis* (Ixodida: Argasidae) and *Babesia vesperuginis* (Apicomplexa: Piroplasmida) reflect "bat connection" between Central Europe and Central Asia

Sándor Hornok[1] · Krisztina Szőke[1] · Tamás Görföl[2] · Gábor Földvári[1] · Vuong Tan Tu[3] · Nóra Takács[1] · Jenő Kontschán[4] · Attila D. Sándor[5] · Péter Estók[6] · Sara Epis[7] · Sándor Boldogh[8] · Dávid Kováts[9] · Yuanzhi Wang[10]

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Abstract *Argas vespertilionis* is a geographically widespread haematophagous ectoparasite species of bats in the Old World, with a suspected role in the transmission of *Babesia vesperuginis*. The aims of the present study were (1) to molecularly screen *A. vespertilionis* larvae (collected in Europe, Africa and Asia) for the presence of piroplasms, and (2) to analyze mitochondrial markers of *A. vespertilionis* larvae from Central Asia (Xinjiang Province, Northwestern China) in a phylogeographical context. Out of the 193 DNA extracts from 321 *A. vespertilionis* larvae, 12 contained piroplasm DNA (10 from Hungary, two from China). Sequencing showed the exclusive presence of *B. vesperuginis*, with 100% sequence identity between samples from Hungary and China. In addition, *A. vespertilionis* cytochrome oxidase *c* subunit 1 (*cox*1) and 16S rRNA gene sequences had

✉ Sándor Hornok
hornok.sandor@univet.hu

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

[2] Department of Zoology, Hungarian Natural History Museum, Budapest, Hungary

[3] Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Hanoi, Vietnam

[4] Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Budapest, Hungary

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

[6] Department of Zoology, Eszterházy Károly University, Eger, Hungary

[7] Department of Biosciences, University of Milan, Milan, Italy

[8] Department of Nature Conservation, Aggtelek National Park Directorate, Jósvafő, Hungary

[9] Department of Evolutionary Zoology and Human Biology, University of Debrecen, Debrecen, Hungary

[10] Department of Pathogenic Biology, School of Medicine, Shihezi University, Shihezi, China

99.1–99.2 and 99.5–100% similarities, respectively, between Hungary and China. Accordingly, in the phylogenetic analyses *A. vespertilionis* from China clustered with haplotypes from Europe, and (with high support) outside the group formed by haplotypes from Southeast Asia. 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.

Keywords Chiroptera · *Argas* · Soft tick · *Babesia* · Phylogeography

Introduction

Bats (Chiroptera) represent the second largest order of mammals, with nearly 20% of living mammalian species (Wilson and Reeder 2005). They can be found all over the globe, except the polar regions (Altringham 1996). In the past few decades the eco-epidemiological importance of bats has become increasingly recognized, because they can be natural reservoirs and host of viruses, bacteria and parasites (Klimpel and Mehlhorn 2014). In this context, particularly zoonotic agents deserved attention, owing to the high population density and roosting behavior of bats, as well as to their occurrence in urban habitats, even in man-made buildings. The migratory habit of certain bat species further contributes to their role in disease transmission, implicating them in the long distance transportation of relevant pathogens. Infections can be contracted from bats in several ways, including direct contact or through blood-sucking arthropod vector species. Considering the latter, one of the epidemiologically most important haematophagous ectoparasites of bats is the bat soft tick *Argas vespertilionis*, because it was also reported to infest humans and domestic animals (Hoogstraal 1956; Jaenson et al. 1994; Manzano-Román et al. 2012).

Several categories of vector-borne protozoa may occur in the blood of bats, including trypanosomes and piroplasms (Gardner et al. 1987). *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. *Babesia vesperuginis* was reported for the first time from *Nyctalus noctula* in Italy (Dionisi 1898), 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 surveys the only ectoparasites found on bats were larvae of *Argas vespertilionis*, therefore the vector role of this soft tick species was postulated in the transmission of *B. vesperuginis*.

Babesia vesperuginis has been recently identified in Central Europe (Hornok et al. 2016). Simultaneously with this species, DNA from a high diversity of piroplasms were identified molecularly in ixodid bat ticks (Hornok et al. 2016). In addition, a high number of argasid soft ticks (*A. vespertilionis*) have been collected in three European countries, and

were used for taxonomic/phylogeographic comparison with specimens from Southeastern Asia (Vietnam) and Africa (Kenya) (Hornok et al. 2017). Therefore, the aim of the present study was to molecularly screen large numbers of soft tick larvae for piroplasms, in order to investigate if they carry a similar diversity of piroplasm DNA as do ixodid bat ticks. In addition, because *A. vespertilionis* larvae from Central Asia were not included in the above phylogeographical study, 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.

Materials and methods

Sample collection

In this study 321 *A. vespertilionis* larvae were examined. The majority (310 larvae) were collected from 15 bat species in Hungary, Romania, Italy, Kenya and Vietnam as recently reported (Hornok et al. 2017). In addition, 11 *A. vespertilionis* larvae collected from *Vespertilio murinus* in Northwestern China (Xinjiang province) in 2016 were also included. Permission for bat capture was provided by the National Inspectorate for Environment, Nature and Water (Hungary), 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-14/32/2010 (DK) and TMF-493/3/2005 (TG).

DNA extraction and molecular analyses

DNA was extracted from *A. vespertilionis* larvae individually or in small pools (i.e. two or three larvae collected from the same host individual) with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction, including an overnight digestion at 56 °C in tissue lysis buffer containing Proteinase-K. In order to monitor cross-contamination of samples, extraction controls (phosphate-buffered saline solution without larvae) were processed parallel with each set of samples.

The resultant 193 DNA extracts (Table 1) were first molecularly screened for the presence of piroplasm DNA with a conventional PCR (Hornok et al. 2016). 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: 5′-GTC TTG TAA TTG GAA TGA TGG-3′) and BN2 (reverse: 5′-TAG TTT ATG GTT AGG ACT ACG-3′). In addition, two soft tick mitochondrial markers were amplified from the DNA of four *A. vespertilionis* larvae collected in China: a 710 bp long fragment of the cytochrome oxidase *c* subunit 1 (*cox*1) gene, and an approx. 460 bp part of the 16S rRNA gene (Hornok et al. 2017).

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 were submitted to GenBank (accession numbers: KY657241-2 for *B. vesperuginis* from *A. vespertilionis* collected in Hungary and China, respectively; KY657239-40 for *A. vespertilionis* collected in China, *cox* 1 and 16S rRNA genes, respectively). Phylogenetic analyses of soft tick mitochondrial

Table 1 Country of origin, bat host species (Chiroptera: Vespertilionidae) and DNA extracts of *Argas vespertilionis* larvae used in this study

Country (location or their number)	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

[a] Locations are listed in Hornok et al. (2017)

markers were conducted with the Maximum Likelihood method and Tamura-Nei model (*cox*1 gene) or Hasegawa-Kishino-Yano model (16S rRNA gene) by using MEGA version 6.0 (Hornok et al. 2017).

Results

Piroplasm DNA in *Argas vespertilionis*

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.

Mitochondrial marker analysis of *Argas vespertilionis* from Northwestern China (Xinjiang)

*Argas vespertilionis cox*1 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

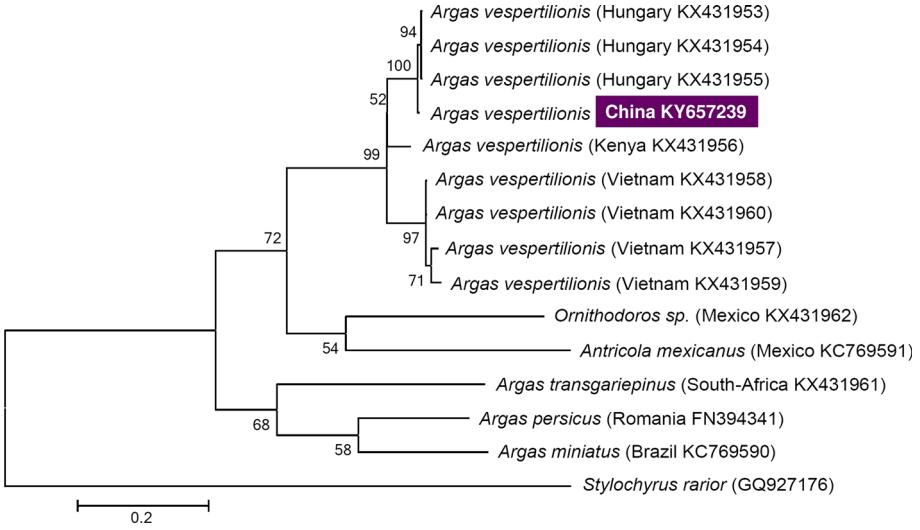

Fig. 1 Phylogenetic tree of cytochrome oxidase *c* subunit 1 (*cox*1) gene of *Argas vespertilionis* based on reference sequences in GenBank. Accession number of the sequence from China (this study) is shown in inverse purple. Branch lengths represent the number of substitutions per site inferred according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

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 *cox*1 phylogenetic tree (Fig. 1) 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. Based on the 16S rRNA phylogenetic tree (Fig. 2), *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%).

Discussion

To the best of the authors' knowledge, this is the first molecular evidence on the occurrence of *Babesia vesperuginis* in Asia. In a previous study, ixodid ticks were collected from bats of three families, out of which *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. 2016). 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 (Table 1), and (2) in a

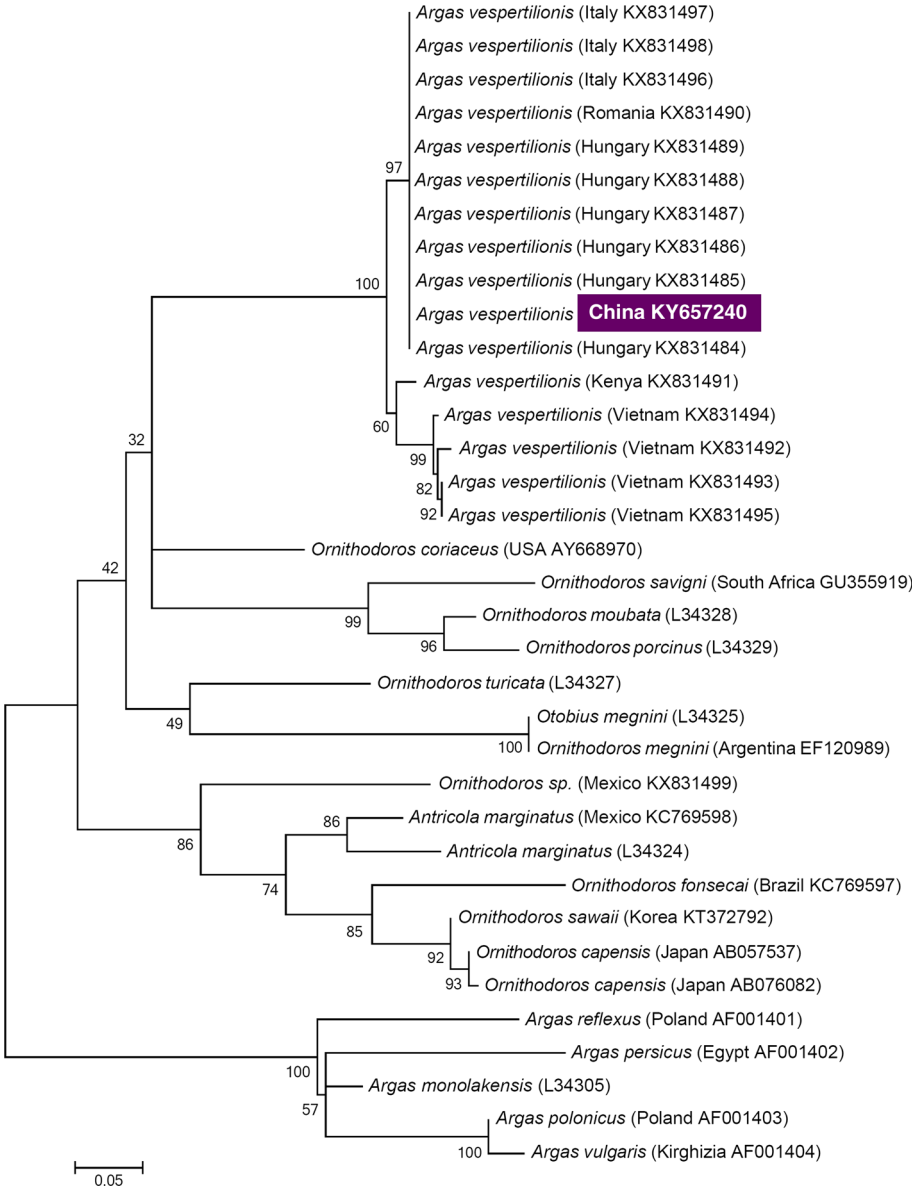

Fig. 2 Phylogenetic tree of 16S rRNA gene of *Argas vespertilionis* based on reference sequences in GenBank. Accession number of the sequence from China (this study) is shown in inverse purple. Branch lengths represent the number of substitutions per site inferred according to the scale shown

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. 2016).

Based on previous results (Hornok et al. 2016), *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. 2015; KU958551-2 in Romania: Hornok et al. 2016).

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 has to be taken into account that postglacial recolonization of Europe by several small *Myotis* spp. (including important hosts of *A. vespertilionis*, e.g. *M. alcathoe*, *M. 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. *M. 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 *M. dasycneme*, *V. murinus* and *E. nilssonii* (Orlova 2014)—the first two being important host species of *A. vespertilionis* (Hornok et al. 2017).

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% *cox*1 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 (*cox*1 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.

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