

**ANALYSES OF SEPARATE AND CONCATENATED *COXI*  
AND 18S rRNA GENE SEQUENCES INDICATE THAT  
THE BAT PIROPLASM *BABESIA VESPERUGINIS* IS  
PHYLOGENETICALLY CLOSE TO *CYTAUXZON FELIS*  
AND THE ‘PROTOTHEILERID’ *BABESIA CONRADE***

Sándor HORNOK<sup>1\*</sup>, Alexandra CORDUNEANU<sup>2</sup>, Jenő KONTSCHÁN<sup>3</sup>, Katinka BEKŐ<sup>4</sup>,  
Krisztina SZŐKE<sup>1</sup>, Tamás GÖRFÖL<sup>5</sup>, Miklós GYURANECZ<sup>4</sup> and Attila D. SÁNDOR<sup>2</sup>

<sup>1</sup>Department of Parasitology and Zoology, University of Veterinary Medicine, István u. 2,  
H-1078 Budapest, Hungary; <sup>2</sup>Department of Parasitology and Parasitic Diseases,  
University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania;  
<sup>3</sup>Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of  
Sciences, Budapest, Hungary; <sup>4</sup>Institute for Veterinary Medical Research, Centre for  
Agricultural Research, Hungarian Academy of Sciences, Budapest, Hungary;  
<sup>5</sup>Department of Zoology, Hungarian Natural History Museum, Budapest, Hungary

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*Babesia vesperuginis* is the only piroplasm known to infect bats. Unlike most members of the genus *Babesia*, it is probably transmitted by a soft tick species (i.e. *Argas vespertilionis*). Recently, two studies have been conducted to clarify the phylogenetic status of this species, and both agreed on placing it into a basal position among *Babesia* sensu stricto (s.s.). However, several important groups of piroplasms were not included in the already reported phylogenetic trees of *B. vesperuginis* isolates. Therefore, the aim of the present study was to amplify an approx. 950-bp fragment of the cytochrome *c* oxidase subunit 1 (*cox1*) gene of *B. vesperuginis* from *A. vespertilionis* specimens, 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.

**Key words:** Apicomplexa, Piroplasmida, Western *Babesia* group, Chiroptera

\*Corresponding author; E-mail: Hornok.Sandor@univet.hu; Phone: 0036 (1) 478-4187

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Bats (forming the second largest order of mammals: Chiroptera) are well known for their widespread occurrence, migratory habit, frequently high population densities (especially during hibernation and nursing) and association with urban habitats (Dietz et al., 2009). In this way they are regarded as epidemiologically important, i.e. incriminated as reservoirs and carriers of viruses, bacteria and parasites of medical and veterinary significance (Klimpel and Mehlhorn, 2014). Accordingly, during the past years studies on bats and the pathogens they may harbour attracted great attention in the scientific world.

*Babesia* species (Apicomplexa: Piroplasmida) are tick-borne, intraerythrocytic protozoan parasites, with more than 100 species described from birds and mammals (Hunfeld et al., 2008). In this genus only *B. vesperuginis* is known to infect bats. *Babesia vesperuginis* elicits pathophysiological changes in bats, justifying its pathogenic nature (Gardner and Molyneux, 1987). The likely vector of *B. vesperuginis* is the soft tick *Argas vespertilionis* (Gardner and Molyneux, 1987).

Recently, the phylogenetic position of *B. vesperuginis* has been discussed, based on molecular analyses of its various isolates by two methods, i.e. PCR amplification and sequencing part of a nuclear (18S rRNA) gene (Hornok et al., 2016) and a mitochondrial marker [cytochrome *c* oxidase subunit 1 (*cox1*)] (Corduneanu et al., 2017). Both studies indicated that phylogenetically *B. vesperuginis* aligns outside theileriae, but (as reflected by its name) within the clade of *Babesia* spp., as a sister group to other members of this genus. However, while results of these studies implied that *B. vesperuginis* is an important and unique species in the phylogeny of Piroplasmida, members of other important piroplasm groups (*Theileria equi*, the genus *Cytauxzoon*, prototheilerids or Western *Babesia* group and *B. microti* group: Schreeg et al., 2016) were not included in the reported phylogenetic trees. Therefore, the aim of this study was to compare the above two genes of *B. vesperuginis* in a broader context, i.e. to phylogenetically analyse new *cox1* sequences of *B. vesperuginis* (amplified from the bat soft tick *A. vespertilionis*) in the context of all five piroplasm groups, and to perform these analyses with concatenated 18S rRNA and *cox1* gene sequences.

### Materials and methods

Four DNA samples were used, which were extracted from bat soft tick (*A. vespertilionis*) larvae and were positive in the PCR, amplifying an approx. 500-bp-long part of the 18S rRNA gene of *Babesia/Theileria* spp. as already reported (Hornok et al., 2017). Relevant soft tick larvae were collected from grey long-eared bat (*Plecotus austriacus*) at the Dráva river, Szent Anna Lake, Hungary, on 20 July 2015. Ethical approval for bat capture was provided by the National Inspectorate for Environment, Nature and Water; the bat banding licence number is TMF-493/3/2005 (TG).

The 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- $\mu$ l reaction mixture contained 2  $\mu$ l DNA template, 12.5  $\mu$ l Master Mix (PCRBIO Taq Mix Red), 1  $\mu$ l of both primers (10 pmol/ $\mu$ l) and 8.5  $\mu$ l 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). BLASTN comparisons between *B. vesperuginis* and other piroplasms, as elaborated below, 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).

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 (Fig. 1).

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 (Fig. 2). 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 MEGA7 (Kumar et al., 2016).

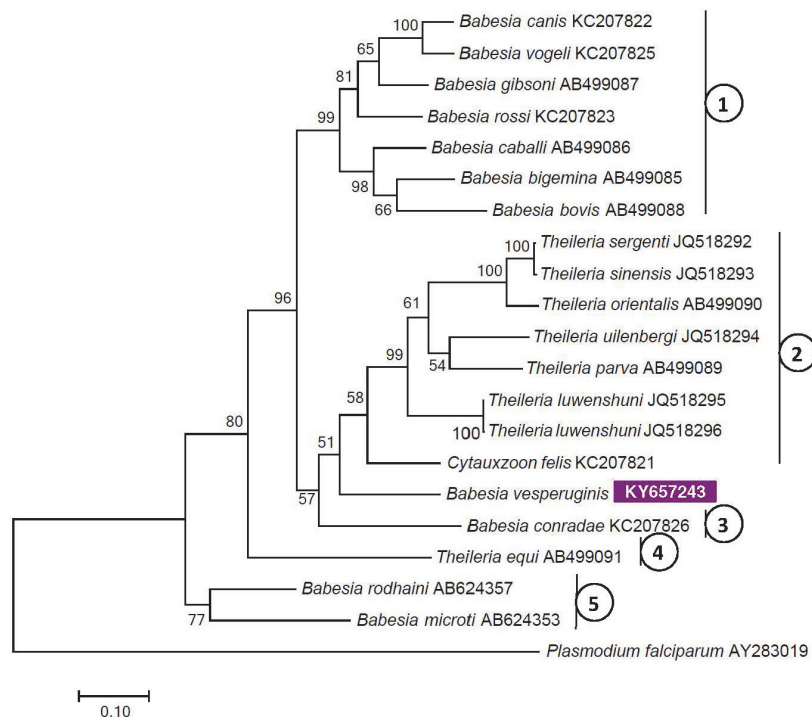


Fig. 1. Maximum likelihood tree of Piroplasmida based on *cox1* gene sequences, obtained with the Hasegawa–Kishino–Yano (HKY) model. Accession number of the *Babesia vesperuginis* sequence from this study is shown in inverse purple colour. The percentage of trees in which the associated taxa clustered 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

An additional analysis was performed on the Phylogeny.fr platform (Dereeper et al., 2008; Dereeper et al., 2010) including the following steps. Sequences were aligned with MUSCLE (v3.8.31) (Edgar, 2004) configured for highest accuracy (MUSCLE with default settings). The phylogenetic tree (Fig. 3) was reconstructed using the Maximum Likelihood method implemented in the PhyML program (v3.1/3.0 aLRT) (Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006). The HKY85 substitution model was selected assuming an estimated proportion of invariant sites (of 0.211) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma = 0.473). Reliability for internal branch was assessed using the aLRT test (SH-Like). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3) (Chevenet et al., 2006).

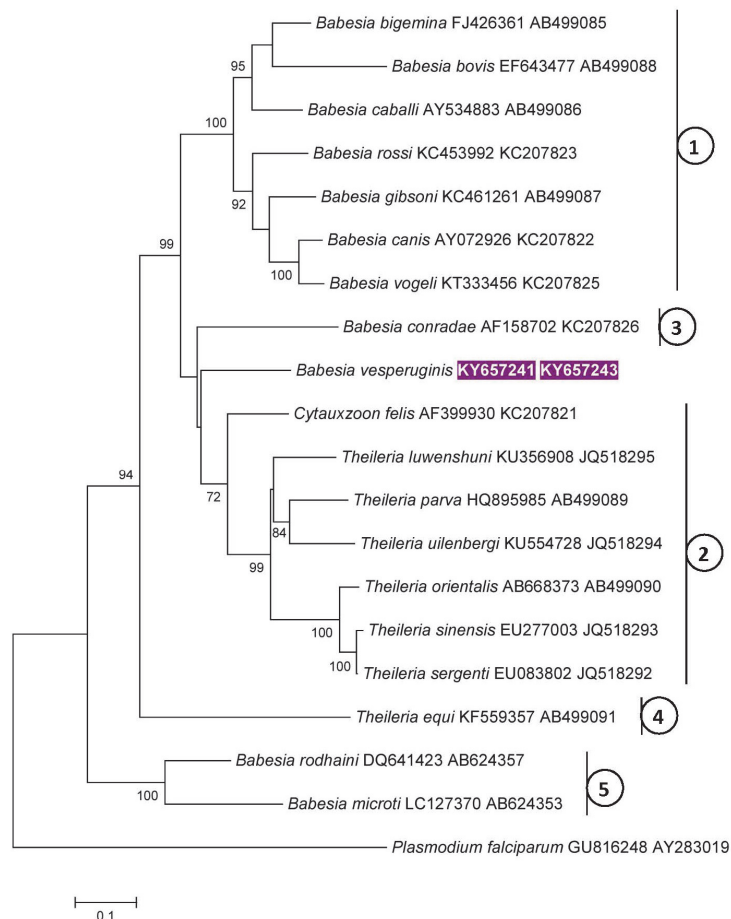


Fig. 2. Maximum likelihood tree of Piroplasmida based on concatenated *cox1* and 18S rRNA gene sequences (the GenBank accession numbers of which are shown in this order after the species name), obtained with the General Time Reversible (GTR) model. Accession number of *Babesia vesperuginis* sequence 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

## Results and discussion

This is the first report on comprehensive phylogenetic analyses of *B. vesperuginis*, involving two genes. In addition, the *cox1* gene of *B. vesperuginis* was amplified for the first time from soft ticks, and also for the first time from a sam-

ple collected in Hungary. 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.e. 74.2–77.5% 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 *B. vesperuginis* and *B. conradae* (Western *Babesia* group) were only 73.2% (659/900 bp) similar, but in this alignment there were only two gaps.

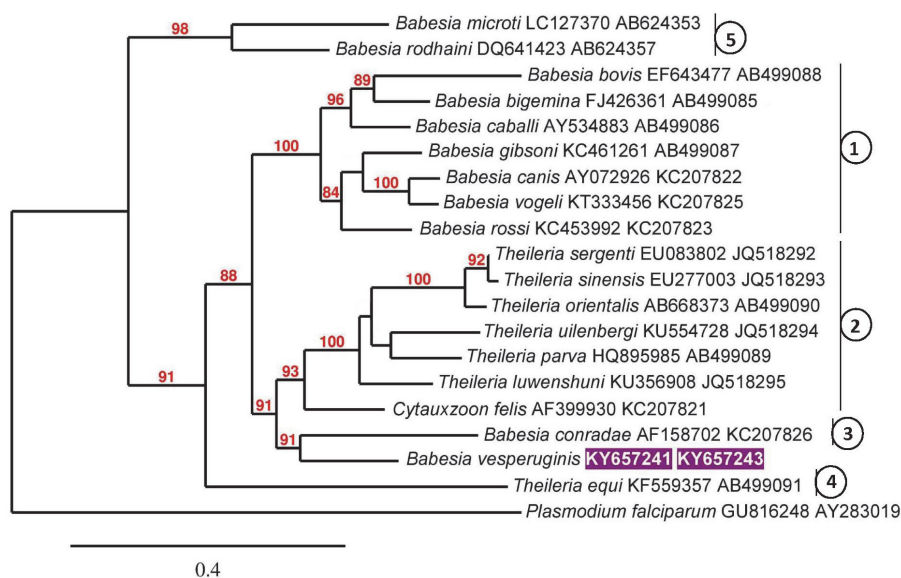


Fig. 3. Maximum likelihood tree of Piroplasmida based on concatenated *cox1* and 18S rRNA gene sequences (the GenBank accession numbers of which are shown in this order after the species name), obtained with the Hasegawa–Kishino–Yano (HKY) model. Accession number of *Babesia vesperuginis* sequence from 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

This is in line with the highest similarity of *B. vesperuginis* 18S rRNA sequence from *A. vespertilionis* (KY657241) with those of an ‘outgroup *Theileria*’, *T. bicornis* (AF499604: 422/455 bp = 92.7% identity); of the ‘prototheilerid’ *B. 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, *B. vesperuginis* clustered outside *Babesia* s.s. (with 96% support) and close to *C. felis* and theileriae (Fig. 1). 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. 2: 99%, Fig. 3: 88%) support. In addition, *B. vesperuginis* clustered next to *B. conradae* and the phylogenetic group of *Theileria* spp. and *C. felis* (Fig. 2), or together with *B. conradae* in a sister group to the clade containing Theileriidae (Fig. 3).

Literature data on the developmental/morphological characteristics of relevant species support the above phylogenetic relationships of *B. vesperuginis*, i.e. its closer association with *Theileria* than with *Babesia* spp., and its closest association with *C. felis* and *B. conradae*. In particular, although the life cycle of *B. vesperuginis* is not completely known, it was reported to have quadrinucleated dividing forms ('tetrads') (Gardner and Molyneux, 1987). This is similar to what was reported in the case of *C. felis* (Glenn et al., 1982) and *B. conradae* (Kjemtrup et al., 2006). Unlike these, members of *Babesia* s.s. divide by binary fission in erythrocytes (Conrad et al., 2006).

The 18S rRNA gene is known to be a suitable marker for the estimation of phylogenetic relationships among Piroplasmida, and was therefore used in the great majority of studies to clarify the taxonomy of this group (Schreeg et al., 2016). However, using this gene alone resulted in some inconsistencies, and phylogenetic analyses do not agree on the taxonomic placement of certain *Babesia/Theileria* spp. Consequently, adding to the 18S rRNA gene, mitochondrial genome sequences [such as the cytochrome *c* oxidase subunit I (*cox1*) gene] and their concatenation with the 18S rRNA sequences have proven to be useful for the elucidation of evolutionary relationships, delineating taxa and resolving Piroplasmida phylogeny (Gou et al., 2012; Schreeg et al., 2016).

This is the first comprehensive analysis of this type, in which *B. vesperuginis*, a unique species was included. Taken together, phylogenetic trees in this study reflect the clustering pattern (separation of the same five piroplasmid groups) as recently reported in the case of phylogenetic trees based on *cox1* amino acid sequences or concatenated mitochondrial and 18S rRNA gene nucleotide sequences (Schreeg et al., 2016).

In conclusion, *cox1* sequence and phylogenetic analyses performed here clearly indicate that *B. vesperuginis* is not a member of the phylogenetic group *Babesia* s.s., but belongs to a separate clade, closely related to *C. felis* and *B. conradae*. Therefore, *B. vesperuginis* is a unique and taxonomically important species, which should be included in future studies serving to resolve the comprehensive phylogeny of Piroplasmida.

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### Competing interests

The authors declare that they have no competing interests.

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