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Bat ticks revisited: *Ixodes ariadnae* sp. nov. and allopatric genotypes of *I. vespertilionis* in caves of Hungary

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Abstract

Background: In Europe two ixodid bat tick species, *Ixodes vespertilionis* and *I. simplex* were hitherto known to occur.

Methods: Bat ticks were collected from cave walls and bats in Hungary. Their morphology and genotypes were compared with microscopy and conventional PCR (followed by sequencing), respectively.

Results: A year-round activity of *I. vespertilionis* was observed. Molecular analysis of the cytochrome oxidase subunit I (COI) gene of twenty ticks from different caves showed that the occurrence of the most common genotype was associated with the caves close to each other. A few specimens of a morphologically different tick variant were also found and their COI analysis revealed only 86-88% sequence homology with *I. simplex* and *I. vespertilionis*, respectively.

Conclusions: The microenvironment of caves (well separated from each other) appears to support the existence of allopatric *I. vespertilionis* COI genotypes, most likely related to the distance between caves and to bat migration over-bridging certain caves. The name *I. ariadnae* sp. nov. is given to the new tick species described here for the first time.

Keywords: *Ixodes*, Tick, Cave, Bat, Genotype

Background

In Europe, two ixodid bat tick species are known to occur [1]. The long-legged bat tick (*Ixodes vespertilionis*) has a broad host range and a worldwide distribution; accordingly, it was also reported from most of the European countries [2]. On the other hand, *I. simplex* is highly specialized to the bat *Miniopterus schreibersii*, and (although geographically widespread) it is usually found in low numbers during surveys [3]. This may be partly attributed to the difficulties in its diagnosis, which is not always clear [2]. The taxonomical relationship of the two bat tick species is also a matter of debate, as formerly they were classified as members of separate subgenera (*Eschatocephalus* and *Pomerantzevella*, respectively), which were later judged to be synonymous [4].

Furthermore, *I. vespertilionis* is usually collected from either bats [3] or cave walls [5], but *I. simplex* almost exclusively from bats [2,3,6].

Concerning the epidemiological significance of bat ticks, *I. vespertilionis* was shown to be present outside caves (e.g. cellars or attics of houses, tree holes: [3]). This species may also infest humans [7] and may carry bartonellae [5]. However, *I. simplex* is unlikely to feed on humans, and its vector potential remains to be elucidated.

Recently, during collections of ticks in Hungarian caves [5] it was observed, that some specimens are morphologically different from both *I. vespertilionis*, and *I. simplex*. Arthur [2] also reported the existence of bat ticks, which show intermediate features between *I. vespertilionis* and *I. simplex*. Therefore, the primary aim of the present study was to collect large numbers of ticks from caves, in order to clarify their taxonomical status on both morphological and molecular bases. It was also within the scope of the survey to obtain data

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on the ecology of bat ticks, i.e. their seasonality and the distribution of cave-related (allopatric) genotypes.

The cytochrome oxidase subunit I (COI) gene was chosen for molecular analysis, on account of its suitability as a DNA-barcode sequence for animal/tick species identification [8,9]. The COI gene allows phylogenetic studies, because it is conserved enough within species, shows variability among species, and there are numerous sequences of this gene from ixodid ticks already deposited in the GenBank.

Methods

Tick collections of the present study were carried out in 2012-2013, and consisted of three parts. The main collection site included three caves (Legény Cave, Leány Cave and Ariadne Cave; entrances within 1 km; central coordinate: 47° 41' 57.67" N, 18° 50' 39.24" E) in the Pilis Mountains, that were visited repeatedly during the winter, spring and autumn months. A few ticks were also provided by speleologists from more distant locations selected randomly (i.e. seven caves in the Gerecse, Bükk and Mecsek Mountains: Figure 1).

Additionally, five ticks (three nymphs, one larva with unusual morphology; and one *I. simplex* nymph from *Miniopterus schreibersii*: Figure 1) removed from five bats during their mating period (August-September) were included in the present study. These animals were caught (as part of a monitoring program) at the entrance of caves between sunset and dawn, using standard Ecotone mist-nets (Gdynia, Poland) with 12 m length,

2.5 m height and 14 × 14 mm mesh [10,11]. Ticks were immediately put into and stored in 70% ethanol.

DNA extraction from ticks was carried out as described [12]. For barcoding a portion of the COI gene was amplified from these tick DNA samples with the universal primer pair LCO/HCO [13] in a concentration of 10 pmol/μl. PCR conditions were the following: 94°C, 5 min; then 35 cycles of 94°C, 40 sec, 44°C, 40 sec and 72°C, 1 min; and final extension at 72°C for 5 min. The length of PCR product was approximately 700 bps. After electrophoresis in 1.5% gel the bands were excised and purified by using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Sequencing was done by Macrogen Inc. (Korea). Sequences were submitted to the GenBank (accession numbers KJ490305 for *I. simplex*, and KJ490307-KJ490311 for genotypes of *I. vespertilionis* designated A-E, respectively). Phylogenetic analysis was performed with Mega 5.2 program package, and the tree was constructed by Neighbor-Joining test.

Scanning micrographs were made in the Hungarian Natural History Museum (Budapest) with a HITACHI SN 2600 scanning electron microscope. All investigated specimens were sputter coated by golden-palladium.

Ethical approval

Authorization for bat capture was provided by the National Inspectorate for Environment, Nature and Water (No. 14/2138-7/2011). Bat banding licence numbers are TMF-14/32/2010 and TMF-493/3/2005.

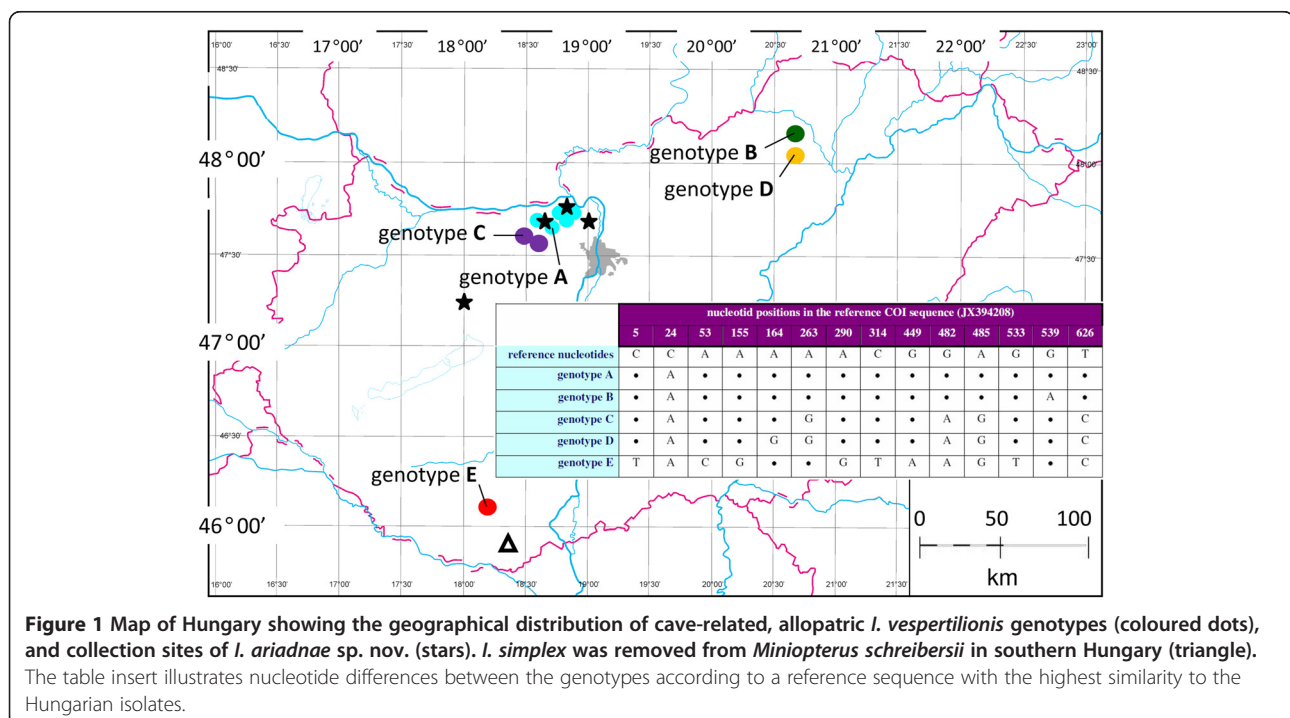


Figure 1 Map of Hungary showing the geographical distribution of cave-related, allopatric *I. vespertilionis* genotypes (coloured dots), and collection sites of *I. ariadnae* sp. nov. (stars). *I. simplex* was removed from *Miniopterus schreibersii* in southern Hungary (triangle). The table insert illustrates nucleotide differences between the genotypes according to a reference sequence with the highest similarity to the Hungarian isolates.

Results

Spatiotemporal distribution of *I. vespertilionis*

In the caves, altogether 535 ticks were collected: 527 *I. vespertilionis* (220 males, 119 females, 171 nymphs and 17 larvae), and eight specimens showing different morphology (only engorged females). Concerning *I. vespertilionis*, except for the summer months (with low tick activity) non-parasitic males outnumbered females in the populations. The presence of questing females on cave walls appeared to be equilibrated between the seasons (Table 1). However, the proportion of nymphs was more pronounced during the spring and autumn. Larvae were observed from March to June.

Twenty ticks were selected for molecular analysis, including 15 specimens of *I. vespertilionis* (eight from the main collection sites, seven from other caves), four specimens with different morphology, and for comparison one *I. simplex* collected from *Miniopterus schreibersii*. In the three caves of the main collection site only genotype A was identified (corresponding to 8 tick specimens). This genotype was also found in two caves within 20 km westward, but not in any other, more distant caves. In two further caves within 40 km southwest genotype C was identified, and in three caves approx. 200 km to the east and to the south three other genotypes (B, D, and E: Figure 1). Genotypes A, B, C, D and E differed from the reference sequence (accession number JX394208) in one, two, five, six or eleven nucleotides, respectively (Figure 1). Their phylogenetic relationships with each other and further ixodid species occurring in Europe are shown on Figure 2.

Description of *Ixodes ariadnae* Hornok sp. nov.

Taxonomic summary

Phylum Arthropoda, class Arachnida, subclass Acari, family Ixodidae, genus *Ixodes*.

Diagnosis

Medium size (engorged females 6-7 mm) prostriate ticks. The legs long, palps short and hypostome medium length. Scutum rounded, broad hexagonal, posteriorly curved, with deep cervical grooves on engorged specimens. Hair covering sparse both dorsally and ventrally. Only engorged females, nymphs and a larva were collected. Male is unknown.

Type material and locality

Holotype (1) female, from Leány Cave, Pilis Mountains, Hungary, (collected by D. Angyal, March 31, 2012).

Paratypes (2) nymph, from *Myotis alcaethoe*, Pisznicse Cave, Gerecse Mountains, Hungary (collected by D. Kováts, August 25, 2012); (3)-(6) three females from Leány Cave and one female from Legény Cave, Pilis Mountains,

Hungary, (collected by D. Angyal, March 31, 2012); (7)-(9) three females from Leány Cave, Pilis Mountains, Hungary, (collected by S. Hornok, March 24, 2013); (10) nymph from *Plecotus auritus* at Szopláki-ördöglyuk Cave, Pilis Mountains, Hungary, (collected by D. Kováts, April 6, 2012); (11) nymph from *Myotis blythii* at Szopláki-ördöglyuk Cave, Pilis Mountains, Hungary, (collected by D. Kováts, September 4, 2012); (12) larva from *Myotis alcaethoe* at Alsópere, Bakony Mountains, Hungary, (collected by D. Kováts, July 27, 2013).

Holotype and paratypes (2), (4)-(6) are stored in alcohol and deposited at the Department of Parasitology and Zoology, Szent István University. Paratypes (8)-(10) are stored in alcohol and deposited in Soil Zoology Collection of the Hungarian Natural History Museum. Paratypes (3), (7), (11) and (12) were used for molecular phylogenetical comparison.

Morphology

Anal groove anterior to the anus (genus *Ixodes*).

Female (engorged): Length 6 mm. Legs long (tarsus I: 1.5 mm). Haller's organ open, long, confluent, with 11 setae in three groups. On the basis capituli posterolaterally blunt flange, posterior edge with dorsal ridge (maximum width 0.5 mm). Palps short (0.44 mm), broad at the distinct joining of segments II-III. Hypostome of medium length (0.35 mm), with 10 rows of broad teeth, situated ventrally in four lines. Scutum rounded, broad hexagonal, with a shape index of 1.2. Idiosoma has sparse hair covering both dorsally and ventrally. Coxae are convex posteroexternally, with rounded surface. Genital pore between coxae III. Anal groove posteriorly divergent. Spiracle openings oval, with irregular outline.

Nymph (engorged): Length 3 mm. Legs moderately long (tarsus I: 0.75 mm). Basis capituli at maximum width 0.35 mm. Palps short (0.2 mm; segments II + III: 0.125 + 0.075 mm), broad at the distinct joining of segments II-III. Hypostome of medium length (0.13 mm). Scutum rounded, broad hexagonal (length: 0.72 mm, width: 0.6 mm, shape index 1.2). Idiosoma has sparse hair covering both dorsally and ventrally. Spiracles oval, with irregular outline.

Differential diagnosis

Characteristics of engorged females in comparison with *I. vespertilionis* and *I. simplex* are shown in Table 2, Figures 3, 4, 5, 6.

Gene sequences

Mitochondrial cytochrome oxidase subunit I (COI) gene sequence deposited in the GenBank is KJ490306. Its phylogenetic relationships are shown on Figure 2.

Table 1 Collection data of *I. vespertilionis* in caves according to seasons

	Winter (6/6)				Spring (18/12)				Summer (3/3)				Autumn (4/4)			
	Larvae	Nymphs	Males	Females	Larvae	Nymphs	Males	Females	Larvae	Nymphs	Males	Females	Larvae	Nymphs	Males	Females
Total number	-	5	23	7	16	114	160	92	1	-	3	3	-	52	34	17
Percentage	-	14%	66%	20%	4%	30%	42%	24%	14%	-	43%	43%	-	50%	33%	17%
Presence of bats		+++				++				+				++		

Next to each season the number of visits per number of caves is shown in brackets.

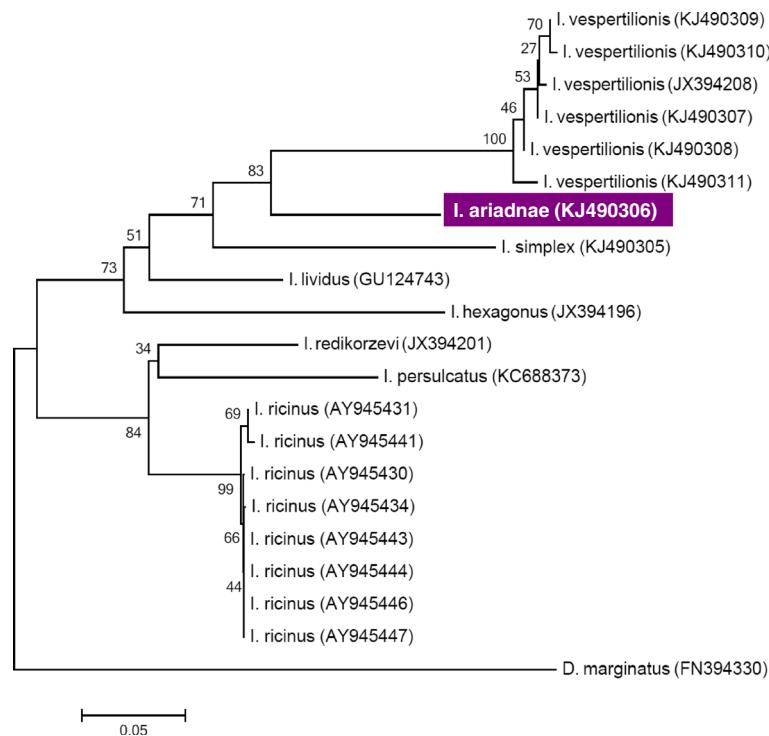


Figure 2 Phylogenetic relationship of *I. ariadnae* sp. nov., *I. vespertilionis* genotypes and *I. simplex* inferred from COI gene analysis in the present study, in comparison with other *Ixodes* spp. and *Dermacentor marginatus* based on sequences deposited in the GenBank.

Table 2 Morphological comparison of *I. vespertilionis*, *I. ariadnae* sp. nov. and *I. simplex* female

Trait	<i>I. vespertilionis</i> *	<i>I. ariadnae</i> sp. nov.	<i>I. simplex</i> *
Max. engorged size	10	7	4.5
Length of legs	long (tarsus I: 1.6)	long (tarsus I: 1.5)	moderately long (tarsus I: 0.73)
Basis capituli	- dorsally posterolaterally flange - posteriorly straight	- dorsally posterolaterally flange - posteriorly slightly curved with dorsal ridge	- dorsally no flange (ventrolaterally triangular ridge) - posteriorly sinuous
Palps (II+III segments)	long, narrow (0.39 + 0.22)	short, broad (0.28 + 0.16)	short (0.16 + 0.14)
Joining of II-III segments	distinct	distinct	indistinct
Hypostome	long, 15 rows of slender teeth, ventrally in 6-8 lines	medium, 10 rows of broad teeth, ventrally in 4 lines	short, 8 rows of broad teeth, ventrally in 4-6 lines
Areae porosae	large, oval, interval broad	large, oval, interval slightly broad	large, oval, interval narrow
Scutal setae	anterolaterally prominent	anterolaterally very few	anterolaterally prominent
Scutal length per width	1.76/1.08 (approx. 1.6)	1.15/0.95 (approx. 1.2)	1.13/0.8 (approx. 1.4)
Idiosoma hair covering	dense	sparse	dense
Coxae	posteroexternally concave, surface flat	posteroexternally convex, surface rounded	posteroexternally concave
Anal groove	parallel	posteriorly slightly divergent	posteriorly markedly divergent
Spiracle opening	oval, regular outline	oval, irregular outline	(sub)circular, broad

*Descriptions according to Arthur (1956), Babos and Janisch (1958), Nosek and Sixl (1972). Sizes are given in mm.

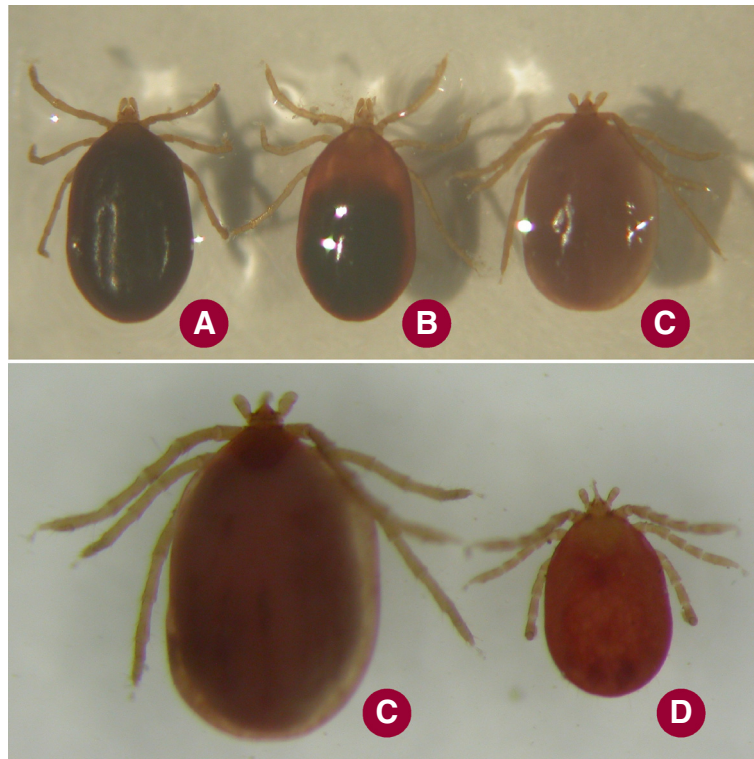


Figure 3 Larva of *I. ariadnae* sp. nov. collected from *Myotis alcaethoe* and showing apolysis (C), in comparison with *I. vespertilionis* larvae from the cave wall without (A) or with apolysis (B); and (D) *I. simplex* larva collected from *Miniopterus schreibersii*.

Host records and distribution

Myotis alcaethoe (of two specimens), *Myotis blythii* and *Plecotus auritus*. Distribution is shown on Figure 1.

Etymology

The name of the new species refers to Ariadne, the Greek mythological heroine associated with labyrinths.

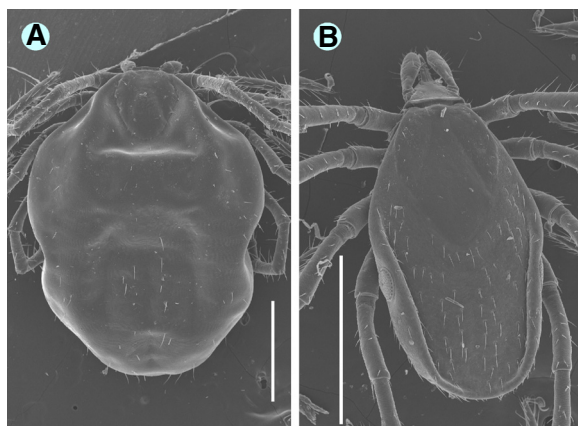


Figure 4 Scanning electron microscopy (SEM) pictures of engorged *I. ariadnae* sp. nov. nymph (A) and unfed *I. vespertilionis* nymph (B) (bars: 1 mm).

Its relevance to the new species is that most specimens were collected at the caves in the Ariadne Cave system.

General

In accordance with section 8.5 of the ICZN's International Code of Zoological Nomenclature, details of the new species have been submitted to ZooBank with the life science identifier (LSID) [zoobank.org/pub: F9432D03-627D-4D86-A307-D9A8E8300361](http://zoobank.org/pub:F9432D03-627D-4D86-A307-D9A8E8300361).

Discussion

Current knowledge on the taxonomy and ecology of *Ixodes* ticks of bats is vague. The review by Arthur [2] notes that descriptions are inadequate in many respects. Data on the seasonal activity of the most widespread species, *I. vespertilionis* have become available only recently [3,14], and these are restricted to ticks collected from bats. At the same time, with growing concern about using molecular methods for the analysis of the taxonomical status of ticks, these were not applied to compare bat ticks, despite the long-known uncertainty in the morphology [2] and nomenclature [15,16] of relevant species. The present study was undertaken to compensate for this lack of information and to contribute to the taxonomy and ecology of ticks that are specialized to

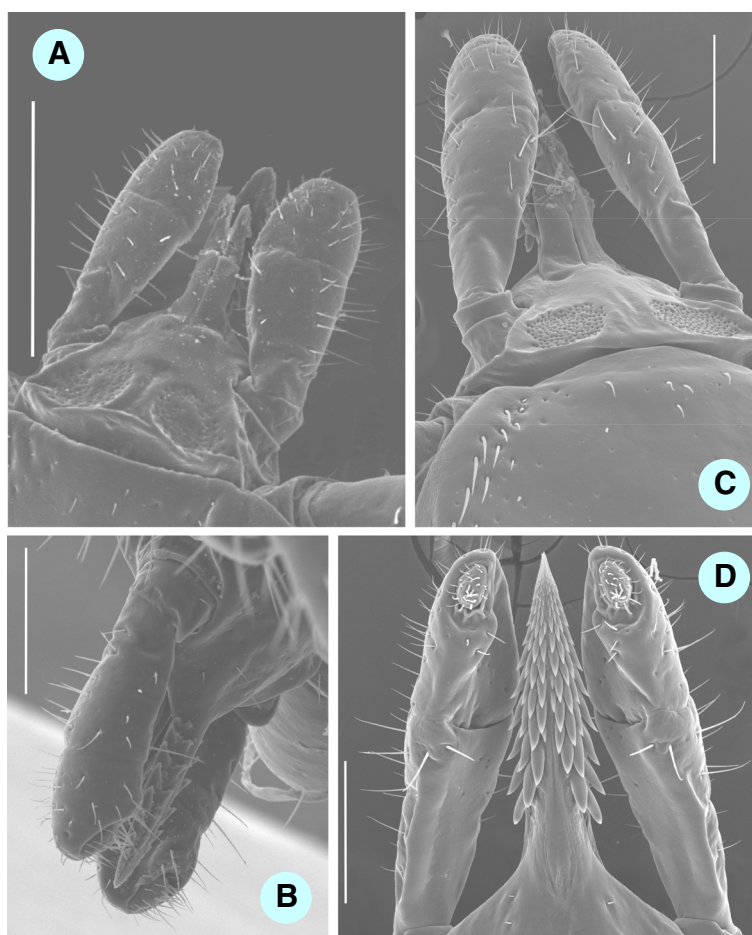


Figure 5 SEM pictures of head parts of female ticks: engorged *I. ariadnae* sp. nov. (A - dorsal, bar = 500 µm; B - ventral, bar = 250 µm) and unengorged *I. vespertilionis* (C - dorsal, bar = 250 µm; D - ventral, bar = 250 µm).

bat hosts and have off-host stages living underground, in caves.

Concerning the seasonality of *I. vespertilionis* in caves, tick numbers (including all stages) appeared to be highest in the spring time, in line with what was reported on ticks collected from bats [3,14]. In the present study immature stages were found in all seasons, and this may indicate a continuous, year-round activity in the protected, underground shelters (meaning less exposure to weather variables), with increase or decrease in tick numbers depending on the presence of hosts. Similarly, the blood-sucking of *I. simplex* larvae and nymphs on bats was observed throughout the year, with some seasonal differences [6]. The low number of ticks during the summer time in this study can be explained by hiding (“over-summering”) of stages in the absence of bat hosts in many of the caves, as some bat colonies use different roosts during the winter (i.e. their hibernation) and summer (June: nursing, August-September: mating). Arthur [2] also noted that during the summer time only a few males were reported in caves by some authors. On

the other hand, female ticks were not observed on bats during the summer and autumn in another survey [14], but in this study were shown to be present in caves. These females may have developed from nymphs that completed their blood meal in the previous season.

A significant part of the life cycle of ixodid ticks is spent off-host, and in case of *Ixodes* spp. even mating can take place in the environment. Unlike overlapping populations of most ixodid ticks that live on large, continuous land surface biotopes, the small populations and habitats of *I. vespertilionis* in caves are physically well separated, only connected by bat migration. However, because the majority of bats prefer to use the same caves for hibernation repeatedly [17], this may constitute further restrictions for genetic exchange between *I. vespertilionis* populations. Correspondingly, this physical and “host behavioural” separation may entail a high degree of reproductive isolation, a prerequisite for the formation and establishment of divergent genotypes (in the long run speciation).

The present data show that divergent COI genotypes of *I. vespertilionis* exist. Only genotype A and C were

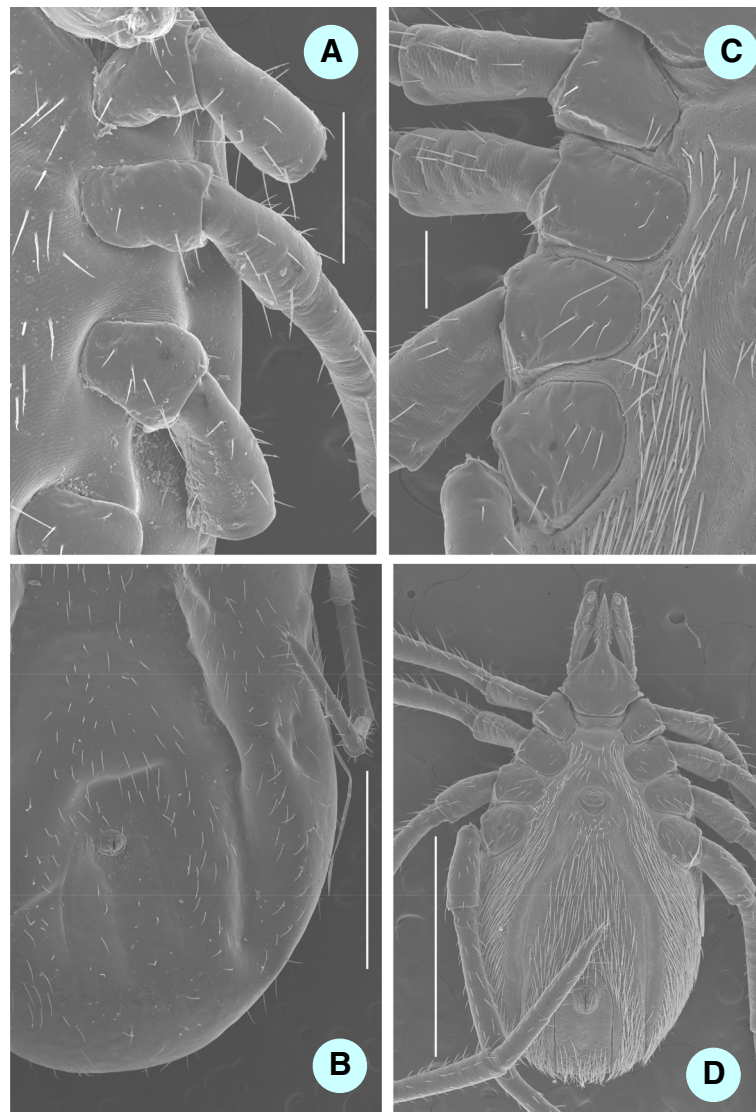


Figure 6 SEM pictures of ventral parts of female ticks: engorged *I. ariadnae* sp. nov. (A - coxae, bar = 500 µm; B - sparse hair covering, bar = 2 mm) and unfed *I. vespertilionis* (C - coxae, bar = 250 µm; D - dense hair covering, bar = 2 mm).

identified repeatedly, and only in caves nearby. As genotype A ticks collected at the main collection site were not found in distant caves (which had further, different genotypes), these preliminary results suggest that the occurrence of *I. vespertilionis* genotypes may be associated with particular cave(s). On the contrary, *I. ricinus* isolates of different origin did not show correlation of genotype and geographical location [18]. As concluded from bat ringing data, the three caves of the main collection site and the two westward (where genotype A was found) are connected by bat migration [10,11], thus making the transport of *I. vespertilionis* possible. Bat recaptures in other countries also reflected, that a small interchange of bats between caves close to each other does occur [17]. On the other hand, large distance,

mountain ranges or other limiting factors of bat migration may prevent contact (genetic exchange of ticks via bats) between more distant caves.

However, the COI sequences of all four analysed specimens of *I. ariadnae* sp. nov. were identical, although one was collected at a more distant location (Figure 1), reflecting that this tick species has different bat hosts (with different migration habits) from those of *I. vespertilionis*. Hosts of the latter species are mainly *Rhinolophus* species [3], whereas in the present study *I. ariadnae* sp. nov. was collected from three species of other bat genera. At the same time *Miniopterus schreibersii* (the specific host of *I. simplex*) is an unlikely host for *I. ariadnae* sp. nov., because this bat species does not occur in any of the caves where engorged female ticks were collected.

The COI sequence of *I. ariadnae* sp. nov. had only 88% and 86% similarities with *I. vespertilionis* and *I. simplex*, respectively (which showed 85% COI sequence homology with each other). The similarities of COI sequences between tick species of the same genera were estimated to range from 70% to 94% [19]. Accordingly, in comparison with the two hitherto described bat tick species in Europe, the 86-88% sequence similarity of *I. ariadnae* sp. nov. supports its taxonomical status as a separate species. Phylogenetically this species is closer to *I. vespertilionis*, but is placed in a separate cluster, supported by high bootstrap value (Figure 2). Confirming this, its morphology and hosts have also been shown to be different from both *I. vespertilionis* and *I. simplex*.

Further molecular taxonomical studies will be undertaken to investigate the ecology and host associations of this novel species in a broader context.

Conclusion

I. vespertilionis shows year-round activity in caves of Hungary, but larger populations during the autumn, winter and spring and “over-summering” in low numbers. The microenvironment of caves (well separated from each other) appears to support the existence of allopatric genotypes, most likely related to the distance between caves and to bat migration over-bridging certain caves. During the present study a morphologically and genetically distinct new bat tick species was found and is described for the first time. The name *I. ariadnae* sp. nov. is given to this species.

Competing interests

No competing interests exist.

Authors' contributions

SH initiated and supervised the study, processed the samples, extracted the DNA and wrote the manuscript. JK made the electron microscopic pictures and performed phylogenetic analysis. DK, RK, DA, TG and ZSP significantly contributed to the sample collection. ZSK performed molecular analysis and ADM arranged, designed and supervised the Romanian part of the study. All authors read and approved the manuscript.

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