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Short communication

First record of *Ixodes* (*Scaphixodes*) *caledonicus* in the Carpathian Basin and first time molecular-phylogenetic analysis of this tick species with updated host records and geographical range

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ABSTRACT

Four *Ixodes* species represent the subgenus *Scaphixodes* Schulze, 1941 in Europe, but none of them were reported to be compared in a molecular-phylogenetic context. This study compensates for this lack of data. A tick larva, morphologically identified as *Ixodes* (*Scaphixodes*) *caledonicus* Nuttall, 1910, was collected from an Alpine swift (*Tachymarptis melba*) during its nesting period in Transylvania, Romania. Following DNA extraction, PCR analyses and sequencing in part with newly designed primers, three genetic markers of this specimen were amplified and compared to GenBank data, and two were analyzed phylogenetically. Based on sequence comparisons of its mitochondrial cytochrome *c* oxidase subunit I (*cox*1) and nuclear 28S rRNA genes *I. caledonicus* appeared to be closely related to members of the subgenus *Pholeoixodes*. However, the topology of the concatenated *cox*1 and 16S rRNA gene phylogenetic tree clearly showed its clustering with *Ixodes* (*Scaphixodes*) *philipi*. In conclusion, *I. caledonicus* is part of the tick fauna of Romania and is expected to occur also in other countries of the Carpathian Basin where rocky cliffs are available for nesting of swifts and other birds. This is the first species of the subgenus *Scaphixodes* in Europe, for which the traditional (morphology-based) taxonomic assignment is confirmed by molecular-phylogenetic analyses.

1. Introduction

More than 760 extant species of hard ticks (Acari: Ixodidae) are known to science and a large number, i.e., 266 species belong to the genus *Ixodes* (Guglielmone et al., 2023). Traditionally, this genus was divided into 14 subgenera (Clifford et al., 1973). Among these, the subgenus *Scaphixodes* Schulze, 1941 traditionally includes eight valid species (Clifford et al., 1973). However, further tick species with uncertain taxonomic status are also associated with this group, although assigned to the closely related subgenus *Multidentatus* (Camicas et al., 1998). Importantly, while at least four *Ixodes* (*Scaphixodes*) species are indigenous in Europe (i.e., *I. berlesei, I. unicavatus, I. rothschildi, I. caledonicus*: Estrada-Peña et al., 2017), none of them were analyzed and reported as a target of sequencing or phylogenetic analyses.

On a morphological basis, *Ixodes caledonicus* Nuttall, 1910 has a long-known and well-established taxonomic status among members of the subgenus *Scaphixodes* (Clifford et al., 1973; Camicas et al., 1998). Nevertheless, it was also allocated with members of subgenus *Pholeoixodes* (Nosek and Sixl, 1972). All developmental stages of *I. caledonicus* are associated with birds from various orders/families, typically nesting at high structures, such as cliffs, church towers, roofs and pigeon lofts (Hillyard, 1996; Estrada-Peña, 2017). It is a Palearctic tick species with a broad geographical range including western Europe (France, UK, Scotland, Ireland) northern Europe (Denmark, Norway, Sweden, Iceland), central Europe (Poland, Germany, Switzerland, Slovenia), with fewer reports from southern Europe (Slovenia, Italy), eastern Europe (Ukraine), as well as northern Africa (Morocco) and some regions of Asia (Russia, Azerbaijan, Kyrgyzstan, Tajikistan) (Guglielmone et al., 2023). Importantly, reports of *I. caledonicus* are scarce throughout its vast geographical range, and this may explain why

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no molecular-phylogenetic data of this species have been reported.

In this study, we report the autochthonous occurrence of *I. caledonicus* in the Carpathian Basin, where this species was hitherto unknown as a member of the tick fauna. In addition, we also present the first molecular-phylogenetic analysis of this tick species. The significance of the latter is shown by the fact that out of the well-established members of subgenus *Scaphixodes*, only one species, *Ixodes philipi* (occurring in Japan) was ever reported in a similar context (Mitani et al., 2007).

2. Materials and methods

2.1. Sample collection and morphological identification

The tick larva examined in the present study was collected from an adult female Alpine swift (*Tachymarptis melba*, formerly *Apus melba*), caught alive at the entrance of the Unguru Mare cave, Șuncuiuș, Romania (46.932382N; 22.547736E) on 03.07.2019 (Fig. 1). The tick was found on the right, upper eyebrow of the bird. The host was captured using elevated mist nets in addition to a group of other six adult birds caught on the same site and date (neither of these were infested by ticks). The species is breeding in the area, using natural crevices of the cave's ceiling as nesting burrows. All adults showed signs of active nesting (visible brood patches).

The tick was stored in 96 % ethanol, and its species was morphologically identified according to standard keys (Nuttall, 1910; Filippova and Panova, 1975). Pictures and measurements were made with a VHX-5000 digital microscope (Keyence Co., Osaka, Japan).

2.2. DNA extraction

The tick was disinfected on its surface with sequential washing in 10 % sodium-hypochlorite, tap water and distilled water. DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction, including an overnight digestion in tissue lysis buffer and Proteinase K at 56 °C. An extraction control (tissue lysis buffer) was also processed with the tick sample to monitor cross-contamination.

2.3. Molecular taxonomic analyses

PCR amplification of part of the *cytochrome c oxidase subunit* 1 *(cox*1*)* gene was not successful with the primers LCO1490 and HCO2198 which are most widely used for barcoding ticks (Folmer et al., 1994). Therefore, new primers were manually designed based on the corresponding gene of the closely related species *Ixodes* (*Scaphixodes*) *philipi* (AB231663), available in GenBank. A conventional PCR reaction was then run with the new primer pair ICAL-fw: 5'-GAT CAA ATT TAT AAT GTT ATT G -3' and ICAL-rev1: 5' - GAT CAT ACA AAA AGG GGT A - 3' to amplify an approx. 400-bp long fragment of the *cox*1 gene. The PCR was performed with the following conditions: 5 µl of extracted DNA were added to 20 µl of reaction mixture containing 1 U HotStar Taq Plus DNA Polymerase (5U/µl) (QIAGEN, Hilden, Germany), 0.5 µl dNTP Mix (10 mM), 0.5 µl of each primer (50 µM), 2.5 µl of 10x Coral Load PCR buffer

Fig. 1. Map of Europe, North Africa and Western Asia showing reported occurrence of *Ixodes caledonicus* according to taxonomic order of hosts. The location of the present finding is marked with a star.

(15 mM $MgCl_2$ included), and 15.8 μl DW. 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 42 °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'). In addition, a nuclear genetic marker was also amplified: an approximately 330-bp-long fragment of the 28S rRNA gene with the primers Tick-28S-C2-F (5′-GCG GCG AGT AGG TCG GTA ACC - 3′) and Tick-d9-D3-R (5′- ACG TCA GAA TCG CTT CGG A - 3′) (Anstead et al., 2011). In the latter two PCRs reaction components and cycling conditions were the same as above, except for annealing at 51 °C and at 60 °C, respectively. Amplifications of longer portions of the 18S and 28S rRNA nuclear genes were also attempted with previously established protocols (Hornok et al., 2023)

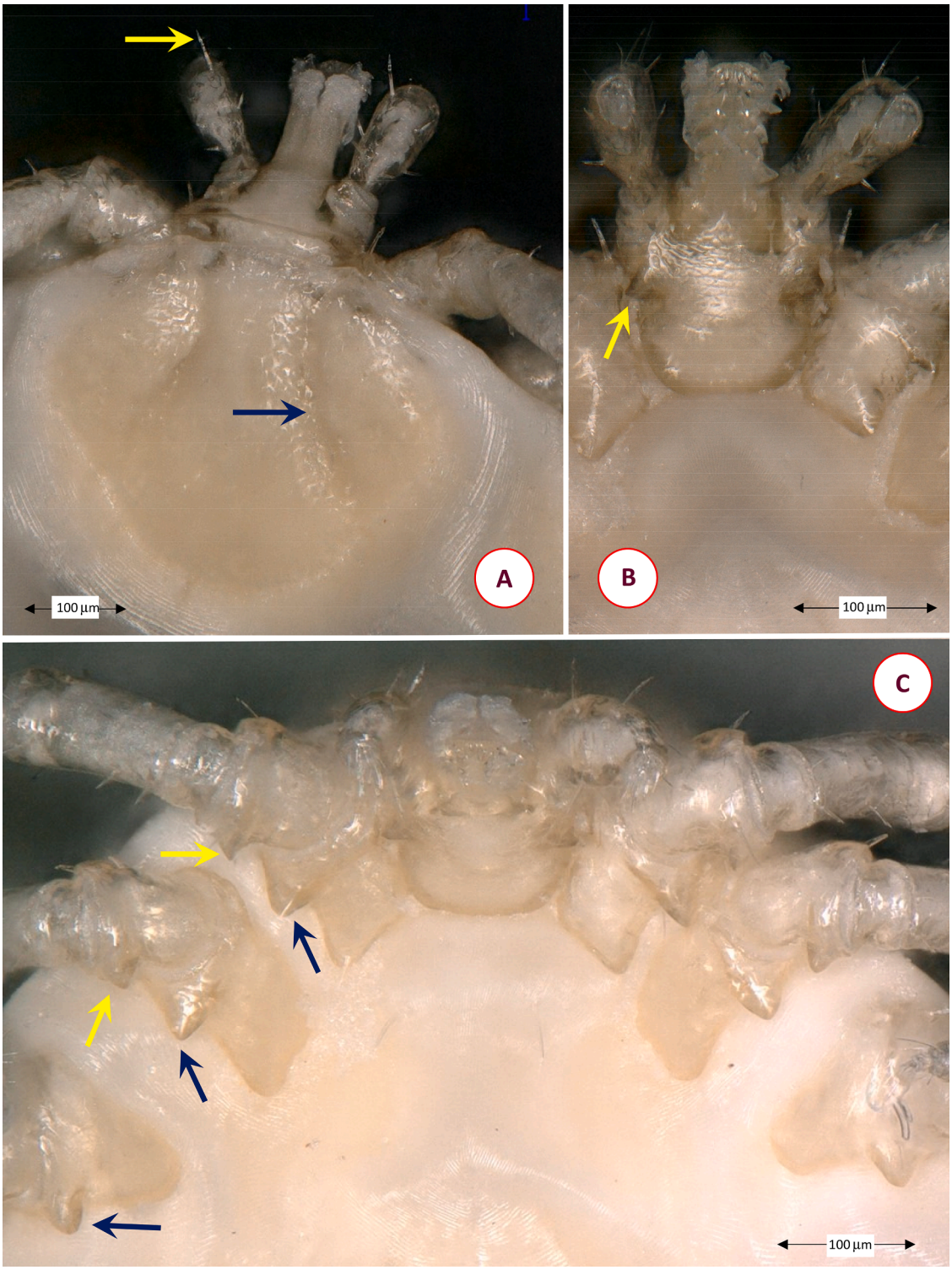

Fig. 2. Morphology of the *Ixodes caledonicus* larva collected in this study: (A) scutum and basis capituli (yellow arrow: long seta on palpal segment III; blue arrow: deep cervical groove); (B) ventral basis (arrow: laterally projected auriculae); (C) trochanters and coxae (spurs marked with yellow or blue arrows, respectively).

but resulted in nonspecific products (data not shown). PCR products were electrophoresed in 1.5 % agarose gel, stained with ethidium-bromide and visualized under ultra-violet light.

2.4. PCR controls, sequencing and phylogenetic analyses

In all PCRs, non-template reaction mixture served as negative control. The extraction control and negative controls remained PCR negative in all tests. Purification and sequencing of the PCR products were done by Eurofins Biomi Ltd. (Gödöllő, Hungary). Quality control and trimming of sequences were performed with the BioEdit program. Obtained sequences were compared to GenBank data by the BLASTN program (https://blast.ncbi.nlm.nih.gov). New sequences were submitted to GenBank (*cox*1 gene: OR296291, 16S rRNA gene: OR294986, 28S rRNA gene: OR294985). Sequences from other studies (retrieved from GenBank) included in the phylogenetic analyses had nearly 100 % coverage with sequences from this study. Sequence datasets were resampled 1,000 times to generate bootstrap values. Phylogenetic analyses were conducted with the Maximum Likelihood method and General Time Reversible model by the program MEGA version 7.0 (Kumar et al., 2016).

2.5. Ethical approval

The study was carried out according to the national wildlife welfare regulation OUG57/2007 and National Directives Ord. 28/31-08-2011. License for bird ringing was provided by the Romanian Ornithological Centre (No 23/2002).

3. Results

3.1. Morphological identification

The tick larva collected from the Alpine swift (*T. melba*) was identified as *I. caledonicus* based on the following characters: shape and width-to-length ratio (1.2) of the scutum; the presence of deep and curving cervical grooves; anteriorly broadening basis capituli with triangular, pointed lateral extensions dorsally; short and broad palps (length-to-width ratio 2); long (40 μm) anterior seta on palpal segment III (Fig. 2 A); laterally directed, acute-angled auriculae (Fig. 2 B); broad internal spur on coxa I; all coxae with prominent and broad external spur; presence of posterior spur on trochanters including the first one (Fig. 2 C).

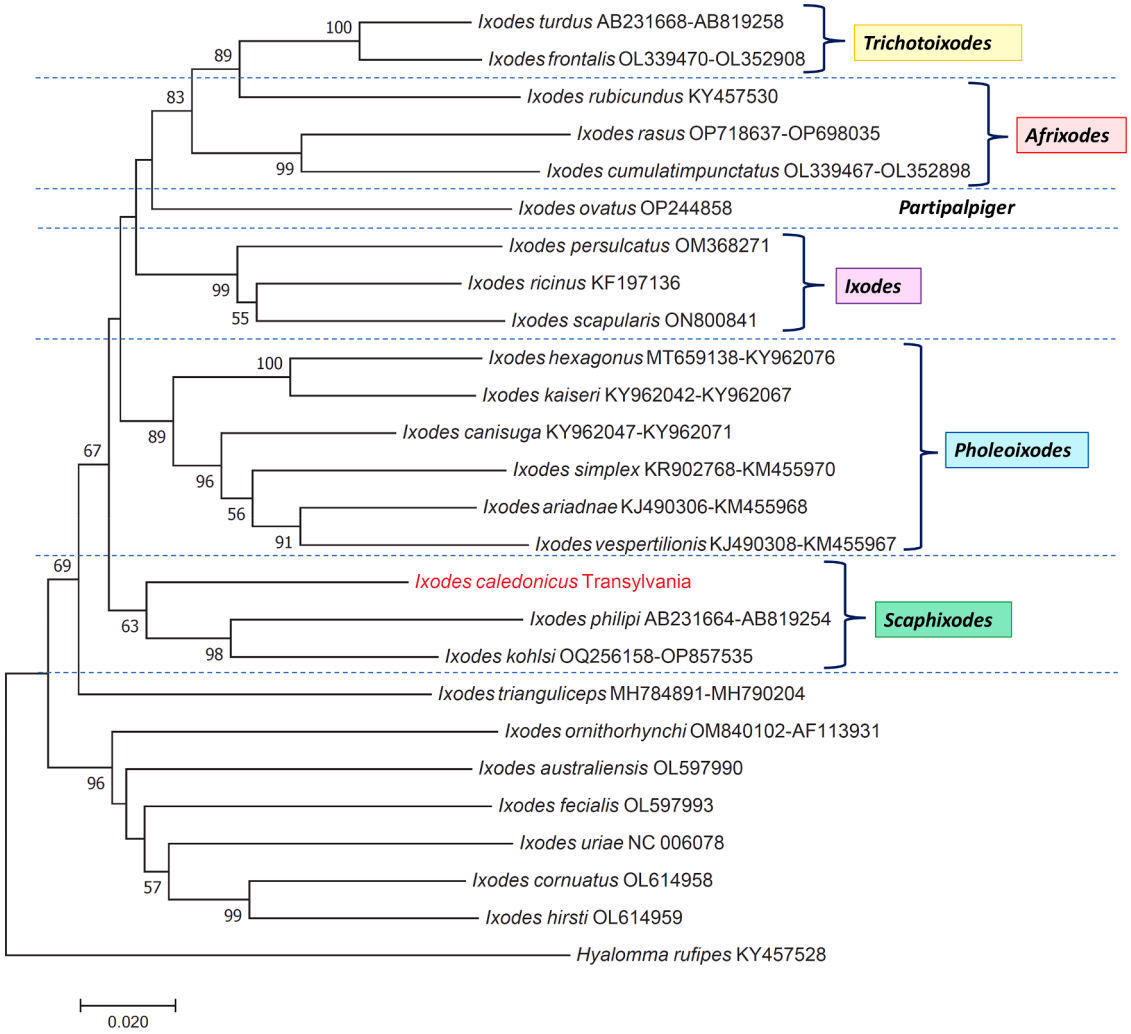

Fig. 3. Phylogenetic tree of ixodid ticks based on concatenated partial *cox*1 and 16S gene sequences. *Ixodes caledonicus* collected in this study is marked with red fonts. Most relevant subgenera that are represented by multiple species are shown on the right and are separated with blue dashed line. Note that the subgenus *Eschatocephalus* is merged with *Pholeoixodes* based on our previous and recent results (Hornok et al., 2023). The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 26 nucleotide sequences. There were a total of 698 positions in the final dataset. Evolutionary analyses were conducted in MEGA version 7.0.

3.2. Molecular-phylogenetic analyses

The *cox*1 sequence of *I. caledonicus* showed the highest, 87.7-87.8 % (329-330/375-376-bp) identity to those of two *Pholeoixodes* species, *Ixodes canisuga* (KY962025) and *Ixodes arboricola* (OR139935). However, the 16S rRNA sequence of *I. caledonicus* appeared to be the most closely related to those of two *Scaphixodes* species, *Ixodes signatus* (AB819255) and *Ixodes auritulus* (MH183252), with similarly low, 91.3 % (337/369-bp) and 90.6 % (336/371-bp) identities, respectively. The nuclear 28S rRNA gene sequence had 99.6 % (263/264-bp) identity with tick species from the subgenera *Afrixodes* (*Ixodes rubicundus*: KY457497) and *Pholeoixodes* (*Ixodes angustus*: FR874099 and *Ixodes cookei*: AY626631) for which corresponding data were available in GenBank.

Phylogenetically, based on its concatenated *cox*1 and 16S rRNA gene sequences, *I. caledonicus* clustered with members of the subgenus *Scaphixodes*, although this received a moderate bootstrap support (Fig. 3). These species, i.e., *I. caledonicus, I. philipi* and *Ixodes kohlsi* together formed a sister group to the clade comprising subgenera *Trichotoixodes, Afrixodes, Partipalpiger, Ixodes* and *Pholeoixodes* (Fig. 3).

4. Discussion

In the present study, the *I. caledonicus* larva was collected from an Alpine swift in Transylvania, for the first time in any countries associated with the Carpathian Basin. It is noteworthy that prior to this study this bird species had only one other tick record in Europe: a nymph of *Haemaphysalis erinacei* was collected from it in Dubrovnik, Croatia (Tovornik and Cerný, 1974). The exceptionally rare tick-infestation among swifts is probably a consequence of their unique habit and anatomy, i.e., they are unable to settle and feed on the ground but spend their lives as aerial hawkers, continuously flying in the air; except for the nesting period when they locate themselves on cliffs and in tree holes.

The tick from the Alpine swift was morphologically identified as *I. caledonicus*. Taking into account that the tick was a larva, collected in the nesting period of its avian host, this implies the first evidence for the indigenous status of this tick species in Transylvania, Romania (Mihalca et al., 2012). All developmental stages of *I. caledonicus* feed on birds from various orders (Hillyard, 1996; Estrada-Peña, 2017). Larvae, nymphs as well as adults were reported from passeriform and falconiform birds, pigeons but only females from seabirds (Charadriiformes: *Rissa tridactyla* and Procellariiformes: *Fulmarius glacialis*). In each taxonomic order of its bird hosts, *I. caledonicus* is mostly reported from species that are known to nest in or to frequently visit rock cliffs, as exemplified by *Apus apus* and *T. melba* as shown in this study (Apodiformes); *Columba livia* (Columbiformes); *Loxia curvirostra, Sturnus vulgaris, Corvus* and *Phoenicurus* spp. (Passeriformes), as well as *Falco* spp. (Falconiformes).

Summarizing the host records of *I. caledonicus* in Eurasia, it was reported from 22 bird species of six avian orders (Table 1; Supplementary Table 1). A broad range of passeriform birds appear to be the predominant hosts of this tick species throughout its geographical range in North Africa and Eurasia (Fig. 1), implying mostly isolated reports of single-tick infestations (Table 1). On the other hand, rock pigeons (*C. livia*) usually have higher intensities of infestations with *I. caledonicus* (Table 1) as reported from the UK (Scotland), France, Italy and Ukraine (Fig. 1). Swifts (Apodiformes) have been sporadically documented hosts of single specimens of this tick species in the middle of its geographical range (Fig. 1). However, falcons/kestrels (Falconiformes) may carry high numbers of *I. caledonicus* larvae (Table 1) as reported exclusively in its northwestern range (Fig. 1). Importantly, based on its overall geographic distribution, *I. caledonicus* occurs either along coastal waters and on islands, or in and near rocky areas of high mountain ranges on continental mainland of Eurasia (Fig. 1).

Pomerantzev stated that *I. caledonicus* is a junior synonym of *Ixodes berlesei* (Pomerantzev, 1950). However, based on the illustrations and description of the latter species (Filippova, 1977) the larva in the present study had several morphological characters different from those of *I. berlesei*, including the laterally directed and acute-angled auriculae (which are caudolaterally directed and perpendicular-angled in *I. berlesei*) and the uniform presence of trochanteral spurs (*vs* no spur on trochanter I of *I. berlesei*). Thus, morphological characters of its larva attest the taxonomic status of *I. caledonicus* as a valid species. At the same time, it should be noted that the host spectra of both species show considerable overlapping (Filippova, 1977). Therefore, results of the present study in this respect should be ultimately confirmed by examining the nymphs and adults of both species in a similar context.

In a broader taxonomic context, this study provides the first molecular and phylogenetic data on *I. caledonicus*. Interestingly, comparison of its *cox*1 sequence (i.e., the most important marker used for barcoding tick species) revealed the close relationship of *I. caledonicus* with

Table 1
Host records according to developmental stages of *Ixodes caledonicus* in North Africa and Eurasia.

Avian order	Bird species	Larva	Nymph	Female	Male	Refs.
Passeriformes	*Corvus corax*	unknown				Martyn (1988)
	Corvus cornix	unknown				Martyn (1988)
	Corvus monedula	×	×	×		Keirans (1984)
	Monticola solitarius	unknown				Filippova and Panova (1975)
	Oenanthe oenanthe			×		Filippova and Panova (1975)
	Petronia petronia	×				Filippova and Panova (1975)
	Phoenicurus ochruros		×			Arthur and Thompson (1953), Schulze (1930)
	Phoenicurus phoenicurus		×			Arthur and Thompson (1953)
	Prunella collaris		×	×		Morel and Aeschlimann (1983)
	Pyrrhocorax pyrrhocorax		×			Bailly-Choumara et al. (1980)
	Rissa tridactyla			×		Richter et al. (2013)
	Sturnus vulgaris		×	×		Nutall (1916), Mehl (1970), Keirans (1984)
	Tichodroma muraria			×		Papadopoulos et al. (2001)
	Loxia curvirostra	unknown				Martyn (1988)
Falconiformes	*Falco peregrinus*		×			Schulze (1930)
	Falco rusticolus	×	×	×	×	Nutall (1916), Richter et al. (2013), Christensen et al. (2015)
	Falco tinnunculus	×	×	×		Keirans (1984)
Apodiformes	*Apus apus*	unknown				Arthur (1955), Schulze (1930)
	Tachymarptis melba	×				**this study**
Columbiformes	*Columba livia*	×	×	×	×	Nutall (1910, 1914), Schulze (1930), Filippova and Panova (1975), Morel and Aeschlimann (1983), Keirans (1984), Manilla (1995)
Charadriiformes	*Rissa tridactyla*			×		Richter et al. (2013)
Procellariiformes	*Fulmarius glacialis*			×		Martyn (1988), Jaenson and Jensen (2007)

Pholeoixodes species, as previously suggested on a morphological basis (Nosek and Sixl, 1972; Pérez-Eid, 2007). This was partly confirmed by the sequence of the nuclear 28S rRNA gene and seems to be ecologically reasonable because pholeophilous tick species also frequently associate with rocks, e.g., in caves. Nevertheless, in the phylogenetic analysis of two mitochondrial (*cox*1, 16S rRNA) genes, *I. caledonicus* belonged to a monophyletic group including other species of the subgenus *Scaphixodes*, confirming its traditional assignment.

In conclusion, *I. caledonicus* is part of the tick fauna of Romania and is expected to occur in other countries of the Carpathian Basin where rock cliffs are available for nesting of swifts. This is the first species of the subgenus *Scaphixodes* in Europe, for which the traditional (morphology-based) taxonomic assignment is confirmed by molecular-phylogenetic analyses.

Data accessibility

All data are available in the main text, or the electronic supplementary material.

Ethics approval and consent to participate

Permission for bird capture was provided by the management authority of the Peştera Ungura Mare Protected Cave. Bird capture was organised as part of scientific bird ringing activity. The study was carried out according to the national wildlife welfare regulation OUG57/2007 and National Directives Ord. 28/31-08-2011. License for bird ringing was provided by the Romanian Ornithological Centre (No 23/2002). Birds were handled according to the current law of animal welfare regulation (L206/2004), and the Research Bioethics Commission of USAMV CN approved the used methodology of bird handling. Permission from the Institutional Animal Care and Use Committee (IACUC) was not necessary, because birds were released in the field after tick removal (none taken to participating Institutes). No live bird was harmed for this study.

Consent for publication

Not applicable.

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CRediT authorship contribution statement

Sándor Hornok: Conceptualization, Writing – original draft, Writing – review & editing. **Jenő Kontschán:** Investigation, Writing – review & editing. **Nóra Takács:** Investigation, Writing – review & editing. **Péter L. Pap:** Methodology, Writing – review & editing. **Attila D. Sándor:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

We declare we have no competing interests.

Data availability

All data used is listed in the paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2023.102280.

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