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Short communication

Molecular analysis of *Ixodes rugicollis*, *Candidatus* Neoehrlichia sp. (FU98) and a novel *Babesia* genotype from a European badger (*Meles meles*)

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ABSTRACT

The European badger (*Meles meles*) is a widespread mammal in most countries of the European continent, with increasingly recognized veterinary/medical importance owing to its preferred habitats (including pastures and urban environments), broad spectrum of food items, and role as a game hunting target. However, ticks and tick-borne pathogens associated with badgers are only partly known, and most of them have not yet been analysed with molecular biological methods The aim of this study was to perform molecular taxonomic analysis of ticks collected from a road-killed European badger, as well as to molecularly investigate its ticks and blood sample for the presence of Anaplasmataceae and piroplasms.

Ticks from the badger were morphologically identified as females of *Ixodes rugicollis*. Based on its cytochrome oxidase subunit I (COI) and 16S rRNA sequences, *I. rugicollis* phylogenetically clustered together with *I. lividus* and *I. arboricola*, *i.e.* other members of the subgenus *Pholeoixodes*. The blood sample of the badger contained the DNA of *Candidatus* Neoehrlichia sp. (FU98) recently identified in red fox in Austria and the Czech Republic. This genotype is most closely related to *Ca.* N. lotoris (from raccoons in North America), and has lower sequence identity with the *I. ricinus*-transmitted zoonotic agent, *Ca.* N. mikurensis found in Eurasia. In the blood of the badger and in one female *I. rugicollis*, the DNA of a new *Babesia* genotype was also present, which differed from a piroplasm detected in *M. meles* in Spain, and clustered phylogenetically in the *B. microti* clade.

Phylogenetic analysis of *I. rugicollis* (based on two genetic markers) confirms its status in subgenus *Pholeoixodes*. *Ca.* Neoehrlichia sp. (FU98) was identified for the first time in *M. meles* and in Hungary. In addition, a molecularly previously not yet characterized *Babesia* genotype occurs in badgers in Central Europe.

1. Introduction

The European badger (*Meles meles*) is a medium-sized mammal (Carnivora: Mustelidae) which occurs (and is abundant) in almost all countries of Europe, from the British Islands in the west, to River Volga in the east (Piza Roca et al., 2014). It is a species well adapted to various habitats, preferring woodlands and shrubs in combination with pastures (Piza Roca et al., 2014). The European badger may consume a broad spectrum of food items, such as roots and fruits, as well as earthworms, insects, small terrestrial vertebrates (including hedgehogs) and their cadavers. The latter increase the potential epidemiological significance of badgers, because they may get into contact with pathogens from tissues of other terrestrial vertebrates. The epidemiological role of badgers is supported by their susceptibility to infections with viruses, bacteria, endo- and ectoparasites of high veterinary and/or medical significance (Hancox, 1980). For instance, the European badger is highly susceptible to rabies virus, *Mycobacterium bovis*, and may carry the human flea (*Pulex irritans*) as well as the most widespread tick species in Europe, *Ixodes ricinus* (Piza Roca et al., 2014). Badgers can also achieve relatively high population densities in urban environments (Huck et al., 2008), contributing further to their veterinary/medical significance. In the

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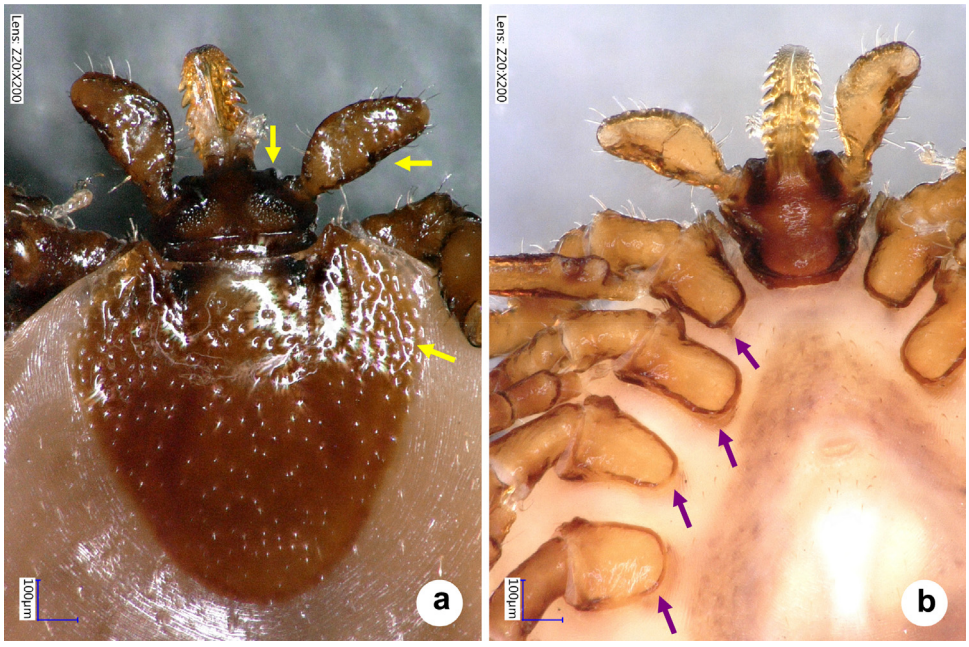

Fig. 1. Morphological characteristics of female *I. rugicollis* identified in the present study: (a) dorsal view (arrows mark the frontal protrusion, laterally straight palps and wavy, porous surface of scutum); (b) ventral view (arrow mark coxae lacking spurs).

present study ticks and a blood sample from a road killed European badger were molecularly investigated.

2. Materials and methods

2.1. Sample collection and DNA extraction

A road-killed European badger was found in eastern Hungary near Hortobágy (47.5868751N, 21.1560332E) on April 11, 2016. A blood sample was collected from the heart into a Vacuette K3-EDTA tube (Greiner Bio-One GmbH, Kremsmünster, Austria), and three ticks were removed from the skin surface. The ticks were stored in 70% ethanol, and identified morphologically based on the descriptions by Morel and Aubert (1975), Filippova (1977; under *Ixodes cornutus*, which is regarded as a synonym of *I. rugicollis* by Camicas et al., 1998) and Siuda et al. (2010). Pictures were made with a VHX-5000 (Keyence Co., Osaka, Japan) digital microscope.

One tick was kept as a voucher specimen of *I. rugicollis*. The DNA was extracted from the two remaining ticks and from 200 µl blood (in duplicate) with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction (for the ticks including an overnight digestion at 56 °C with proteinase-K) as reported (Hornok et al., 2014a).

2.2. Molecular analyses

For the molecular taxonomic analysis of ticks, an approx. 710 bp long fragment of the cytochrome oxidase subunit I (COI) gene, and an approx. 460 bp fragment of the 16S rDNA gene were amplified as reported (Hornok et al., 2015).

In addition, blood and tick DNA extracts were screened for representatives of the family Anaplasmataceae by a conventional PCR amplifying a short, 345 bp portion of the 16S rRNA gene (Hornok et al., 2008). Positive samples were evaluated further by amplifying a longer, 917 bp long fragment of the 16S rRNA gene and a 806 bp fragment of the groEL (chaperone) gene according to Hodžić et al. (2015). The presence of piroplasm DNA was investigated by a conventional PCR detecting an approx. 500 bp part of the 18S rRNA gene of *Babesia*/*Theileria* spp., as described in Hornok et al. (2014b).

Sequences were submitted to GenBank (accession numbers: tick COI gene KX218106, tick 16S rRNA gene KX218233, *Ca.* Neoehrlichia sp. 16S rRNA gene KX231830, *Ca.* Neoehrlichia sp. groEL gene KX245423, *Babesia* sp. 18S rRNA gene KX218234). Phylogenetic analyses were conducted with the p-distance model and Neighbour Joining method by using MEGA version 6.0.

3. Results and discussion

3.1. Molecular analysis of Ixodes rugicollis

The ticks from the badger were morphologically identified as females of *I.* (*Pholeoixodes*) *rugicollis*, based on the conspicuous protrusions between the hypostome and the palps, the wavy surface of scutum, and the nearly perpendicular caudomedial angle of coxa I (Fig. 1). *Ixodes rugicollis* can be regarded as a rare, but widespread species in Europe, with sporadic occurrence reported from Germany, France (Morel and Aubert, 1975), Switzerland (Zimmerli, 1982), Austria (Visser et al., 2011), Poland (Eichler, 1968), Romania (Feider, 1965), but to the best of our knowledge, never before in Hungary. Although the European badger is mentioned as a potential host of *I. rugicollis* (Morel and Aubert, 1975), this tick species mostly infests other mustelids, such as the European pine marten (*Martes martes*) (Morel and Aubert, 1975), beech marten (*Martes foina*), least weasel (*Mustela nivalis*), European polecat (*Mustela putorius*) (Eichler, 1968), and also dogs and cats (Siuda et al., 2010).

The two molecularly analysed females of *I. rugicollis* had identical sequences (COI and 16S rDNA). Concerning the amplified part of its COI gene, *I. rugicollis* was most closely related to another member of the subgenus *Pholeoixodes*, *I. lividus* (583/627 bp = 93% identity). In the amplified part of its 16S rRNA gene, *I. rugicollis* appeared to be closely related to two other members of subgenus *Pholeoixodes*, *i.e. I. arboricola* (382/403 bp = 94.8% identity) and *I. lividus* (379/402 bp = 94.3% identity). At the same time, *I. rugicollis* had similar degree of identity with *I. vespertilionis* (384/406 = 94.6% identity) and *I. ariadnae* (384/409 bp = 93.9% identity).

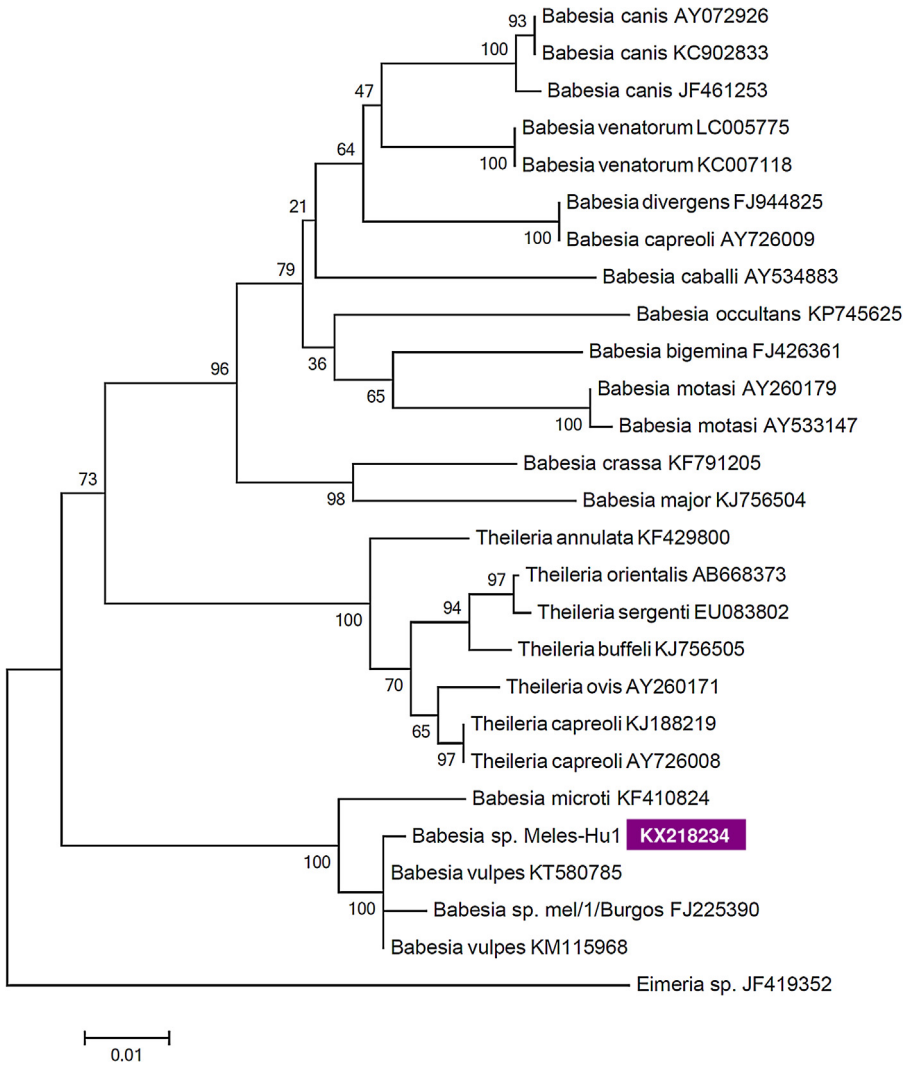

Fig. 2. Phylogenetic comparison of 18S rRNA gene sequence of *Babesia* sp. Meles-Hu1 identified in the present study (accession number in inverse purple colour) and relevant sequences from GenBank. Branch lengths correlate to the number of substitutions inferred according to the scale shown.

3.2. *Molecular identification of* Ca. *Neoehrlichia sp. (FU98)*

Amplification of part of the 16S rRNA and groEL genes of *Ca.* Neoehrlichia sp. (FU98) was only successful from the blood sample of the badger. Corresponding sequences showed 100% identity (868/868 bp with KT833357 and 761/761 bp with KT833358, respectively) with *Ca.* Neoehrlichia sp. (FU98) recently identified in red fox in Austria and the Czech Republic (Hodžić et al., 2015, 2016). This sequence is most closely related to *Ca.* N. lotoris (864/868 bp = 99.5% 16S rRNA gene identity with EF633744; 727/761 = 95.5% groEL gene identity with EF633745), reported from raccoons (*Procyon lotor*) in North America (Yabsley et al., 2008), but has lower sequence identity with the *I. ricinus* transmitted zoonotic agent, *Ca.* N. mikurensis occurring in Eurasia (859/869 bp = 98.8% 16S rRNA gene compared to AB196304; 683/755 = 90.5% groEL gene compared to AB074461).

This is the first finding of the novel *Ca.* Neoehrlichia sp. (FU98) in a European badger (which, based on its PCR positive blood sample, is a new host for this genotype), and in Hungary. The identity of the Hungarian sequences reported here with sequences from Austria and the Czech Republic is not surprising in light of the facts that no sequence variations were found in three gene genes (16S rRNA, groEL, and gltA) between the *Ca.* N. lotoris sequences, and both the 16S rRNA and the more variable groEL sequences of *Ca.* N. mikurensis tend to be highly conserved in Europe (Rar and Golovljova, 2011).

3.3. *Molecular analysis of* Babesia *sp*

In the blood of the badger and in one female *I. rugicollis*, the DNA of a not yet reported *Babesia* genotype (tentatively designated here as *Babesia* sp. Meles-Hu1) was also detected. Its sequence was different from, but showed the highest similarity (442/446 bp = 99.1% identity) to a piroplasm detected in *M. meles* in Spain (FJ225390: Gimenez et al., 2009). Phylogenetically, *Babesia* sp. Meles-Hu1 clustered together with isolates (UK: KT580785, Austria: KM115968) of the recently described *B. vulpes* (syn. *Theileria annae* or *B. cf. microti*) from red foxes, having up to 98% (442/451 bp) identity with this species (Fig. 2). The topology of the phylogenetic tree reflects that *Babesia* sp. Meles-Hu1 belongs to the *B. microti* clade, which is in a basal position to (and differs from) all other piroplasmid groups, in agreement with previous studies (Lack et al., 2012; Schnittger et al., 2012).

The 18S rRNA gene is the most widely used genetic marker for the identification of *Babesia* and *Theileria* spp. (Hunfeld et al., 2008). Taking into account that the 18S rRNA gene may have very few nucleotide substitutions between closely related species (*e.g.* only 0.2% difference delineating *B. divergens* [FJ944825] and *B. capre-*

oli [AY726009]: Malandrin et al., 2010), this new *Babesia* genotype may represent a species different from the one from *M. meles* in Spain (Gimenez et al., 2009).

Previously, a piroplasm named *B. missirolli* was described morphologically from *M. meles* in Italy (Biocca and Corradetti, 1952). Unfortunately, for this species there is no reference sequence available in GenBank for comparison in the present context.

In conclusion, phylogenetic analysis of *I. rugicollis* (based on two genetic markers) confirms its status in subgenus *Pholeoixodes*. *Ca*. Neoehrlichia sp. (FU98) was identified for the first time in *M. meles* and in Hungary. In addition, a molecularly previously not yet characterized *Babesia* genotype occurs in badgers in Central Europe.

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