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Original article

Molecular evidence of a badger-associated *Ehrlichia* sp., a *Candidatus* Neoehrlichia lotoris-like genotype and *Anaplasma marginale* in dogs

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ABSTRACT

The family Anaplasmataceae contains pathogenic and endosymbiotic bacteria of veterinary-medical importance. In this study, 90 blood samples from rural dogs, five blood samples from road-killed European badgers and 34 ticks, i.e. 27 *Ixodes (Pholeoixodes) canisuga*, six *I. (Ph.) hexagonus* and one *Haemaphysalis concinna* collected from the badgers were molecularly analysed for members of Anaplasmataceae. Apart from the molecular evidence of *Anaplasma phagocytophilum* in one dog and *Wolbachia* sp. associated with *Dirofilaria repens* in five dogs, four species/genotypes not yet known to occur in canine hosts have also been found. These included *A. marginale* in two dogs, a badger-associated *Ehrlichia* sp. in one dog, a *Candidatus* Neoehrlichia lotoris-like genotype in six dogs and the DNA of arthropod-associated wolbachiae in three dogs. In two badgers the DNA from the *Candidatus* N. lotoris-like genotype was identified. Among ticks, four *I. canisuga* carried the DNA of the above badger-associated *Ehrlichia* sp., one *I. canisuga* contained the *Candidatus* N. lotoris-like genotype, and in *H. concinna* *Wolbachia* DNA was present. In conclusion, results shown here should be interpreted as the first molecular evidence for exposure of dogs to three members of Anaplasmataceae, i.e. *A. marginale*, a badger-associated *Ehrlichia* sp. and a *Candidatus* N. lotoris-like agent. The presence of DNA in the blood of relevant animals may also indicate susceptibility to these bacteria, but in support of this, further studies are needed.

1. Introduction

The family Anaplasmataceae (Alphaproteobacteria: Rickettsiales) includes several groups of bacteria, which are of high veterinary and/or medical importance (Dumler et al., 2001). The genera *Anaplasma*, *Ehrlichia*, *Neoehrlichia* and *Neorickettsia* contain pathogenic species, which infect mostly blood cells (erythrocytes, platelets, neutrophils, macrophages and monocytes) of mammals (Dumler, 2012), whereas the genus *Wolbachia* includes endosymbionts of arthropods and filarial nematodes (Casiraghi et al., 2001). Four of these taxa (except *Neorickettsia*) include tick-borne bacteria. In particular, *Anaplasma*, *Ehrlichia* and *Neoehrlichia* spp. are transmitted to their mammalian host by hard ticks (Acari: Ixodidae) as biological vectors (Dumler et al., 2001). Recently, soft ticks (Acari: Argasidae) have also been shown to have the potential to carry Anaplasmataceae (Passos, 2012; Lafri et al., 2017).

In Europe, dogs can be hosts of Anaplasmataceae from at least three genera. Among them, *Anaplasma platys*, *A. phagocytophilum* and *Ehrlichia canis* affect dog platelets, neutrophils and monocytes, respectively, and consequently are regarded as important canine pathogens (Dumler et al., 2001; Dumler, 2012). Infection with *Candidatus* Neoehrlichia mikurensis has also been reported from dogs (Diniz et al., 2011; Hofmann-Lehmann et al., 2016), similarly to *Neorickettsia risticii* (Amusategui et al., 2008). In addition, dogs with dirofilariosis were shown to have *Wolbachia* DNA in their blood (Rossi et al., 2010). On the other hand, lack of PCR-positivity of dogs for Anaplasmataceae has also been reported (e.g. Dahmani et al., 2017).

Recently, molecular detection of badger-associated *Babesia* sp. in rural dogs highlighted the possibility that rural dogs, particularly those used for hunting or regularly taken on walks into forests, may get tick-borne infections associated with wild carnivores (Hornok et al., 2018a). Therefore, the aim of the present study was to test blood samples of rural dogs for the presence of Anaplasmataceae with molecular biological tools.

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Table 1
Technical data of conventional PCR methods used for sequencing (molecular identification) in this study. Results of the screening assay (PCR "A") were confirmed by additional analyses (PCRs "B" to "G").

PCR code	Target group in this study (family/*genus*)	Gene (~amplicon length)	Forward and reverse primers (5′–3′) (Reference)	Temperature and duration of:					Number of cycles
				Initial denaturation	Denaturation	Annealing	Extension	Final extension	
A	Anaplasmataceae	16S rRNA (350 bp)	EHR16SD (GGTACCYACAGAAGAAGTCC) EHR16SR (TAGCACTCATCGTTTACAGC) (Brown et al., 2001)	95 °C, 10m	95 °C, 30s	55 °C, 30s	72 °C, 45s	72 °C, 5m	40
B	Ruminant-associated *Anaplasma*	msp4 (850 bp)	MSP45 (GGGAGCTCCTATGAATTACAGAGAATTGTTTAC) MSP43 (CCGGATCCTTAGCTGAACAGGAATCTTGC) (de la Fuente et al., 2005)	95 °C, 5m	95 °C, 30s	59 °C, 30s	72 °C, 45s	72 °C, 5m	40
C	*Ehrlichia*	16S rRNA (1400 bp)	EE-1 (TCCTGGCTCAGAACGAACGCTGGCGGC) EE-2 (AGTCACTGACCCAACCTTAAATGGCTG) (Pusterla et al., 2000)	95 °C, 5m	95 °C, 30s	69 °C, 30s	72 °C, 45s	72 °C, 5m	40
D	*Neoehrlichia*	16S rRNA (1050 bp)	16SCNM_for (GTGGCAGACGGGTGAGTAAT) 16SCNM_rev (TGCAGCACCTGTGTAAGGTC) (Hodžić et al., 2015)	95 °C, 5m	95 °C, 30s	60 °C, 1m	72 °C, 50s	72 °C, 7m	35
E	*Neoehrlichia*	groEL (800 bp)	NeoeGroELFw (CAGGTGAAGCACTAGATAAGTCCA) NeoeGroELRv (ACAGCAGCAACATGCAATCCA) (Hodžić et al., 2015)	95 °C, 5m	95 °C, 30s	54 °C, 1m	72 °C, 50s	72 °C, 7m	35
F	*Wolbachia*	16S rRNA (650 bp)	WOLB-F (TATAGGAATCTACCTAGTAG) (Hornok et al., 2018b) EHR16SR (TAGCACTCATCGTTTACAGC) (Brown et al., 2001)	95 °C, 5m	95 °C, 30s	48 °C, 40s	72 °C, 1m	72 °C, 7m	35
G	Anaplasmataceae	16S rRNA (1200 bp)	Ana16SF (TTAGTGGCAGACGGGTGAGTAATG) with Ana16SMR (CTACCAGGGTATCTAATCCTGTTTGC); and Ana16SM (GCAAACAGGATTAGATACCCTGGTAG) with Ana16SRR (TGACGGGCAGTGTGTACAAGACCCGAG) (this study)	95 °C, 5m; and 95 °C, 5m	95 °C, 30s; and 95 °C, 30s	57 °C, 30s; and 58 °C, 30s	72 °C, 1m; and 72 °C, 1m	72 °C, 5m; and 72 °C, 5m	40; and 40

Abbreviations: m = minutes, s = seconds.

Table 2
Results of molecular analyses and amplicon lengths, reference sequences used for comparison. The length of sequences used for phylogenetic analyses, relevant to the genotype or species, is highlighted with bold numbers.

Genotype/species	Dogs: positive/all	Badgers: positive/all	Ticks: positive/all	Highest sequence similarity – Gene: bp/bp (percentage)	Reference accession number	Accession number in this study
Anaplasma marginale	2/90	–	–	16S rRNA: 1212/1212 (100%)	KT264188	MH020201
				*msp*4: 798/**798** (100%)	HM063432	MH020208
Anaplasma phagocytophilum	1/90	–	–	16S rRNA: 306/**306** (100%)	EU982548	MH020202
Ehrlichia sp.	1/90	–	4/27 (Ic)	16S rRNA: 1308/1310 (99.8%)	KR262717	MH020203
Candidatus Neoehrlichia lotoris-like	6/90	2/5	1/27 (Ic)	16S rRNA: 868/**868** (100%)	KX231830	MH020204
				groEL: 758/**761** (99.6%)	KX245423	MH020209
Wolbachia of arthropods	3/90	–	–	16S rRNA: 1211/1216 (99.6%)	DQ115537	MH020205
	–	–	1/1 (Hc)	16S rRNA: 627/**627** (100%)	MF461482	MH020207
Wolbachia of nematodes	5/90	–	–	16S rRNA: 1216/1216 (100%)	KY114937	MH020206

Abbreviations: Ic – *Ixodes canisuga*; Hc – *Haemaphysalis concinna*.

2. Materials and methods

Samples used in this study were collected at 20 locations in south-western Hungary (Somogy-county), within a radius of 20 km around Csurgó (46°15′23.6″N, 17°6′1″E) in 2017. The majority of samples were collected from 90 dogs, including six hunting dogs and six additional dogs frequently taken to forests, as well as 78 dogs (randomly chosen from the patients at the Veterinary Clinic, Csurgó) kept at houses without a history of visiting forests. From these 90 dogs EDTA-anticoagulated blood samples were drawn under clinical conditions (after skin surface sterilization, with sterile instruments) by cephalic venipuncture. From five (No. 1–5) freshly road-killed European badgers (*Meles meles*) EDTA-anticoagulated blood samples were collected (with sterile needle and syringe) from the heart, shortly after being reported to the Veterinary Authority. EDTA blood samples were frozen at −20 °C until further processing. In addition, ixodid ticks were removed from the dogs and badgers with pointed tweezers, then transferred into 96% ethanol for storage in separate vials according to host individuals. Ticks were found on four badgers (No. 1, 2, 3 and 5) and twelve dogs (data not shown). Tick species were identified according to standard keys (Babos, 1965; Hornok et al., 2017a), and their species have already been reported (Hornok et al., 2018a).

DNA was obtained using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction and using extraction controls to monitor cross contamination of samples in each run. DNA was extracted from 200 µl blood of 90 dogs and five badgers, as well as from 34 ticks collected from badgers. These ticks belonged to three species, i.e. *Ixodes (Pholeoixodes) canisuga* (n = 27 females: 8 from badger No. 2, 19 from badger No. 3), *I. (Ph.) hexagonus* (n = 6 females: 2 from badger No. 2, 4 from badger No. 3) and *Haemaphysalis concinna* (n = 1 nymph, from badger No. 5). DNA was extracted from the ticks individually, including an overnight digestion in tissue lysis buffer and proteinase-K at 56 °C, and incubation in lysis buffer for 10 min at 70 °C.

In total, 95 blood and 34 tick DNA extracts were screened for the presence of bacteria from the family Anaplasmataceae by a conventional PCR (Table 1: PCR "A"), followed by sequencing and phylogenetic analysis (see below). PCR-positive samples were further tested with conventional PCRs according to the results, amplifying either a longer portion of the 16S rRNA gene or another genetic marker (Table 1: PCRs "B-G"). The reaction mixtures (25 µl) contained 1 U (0.2 µl) HotStarTaq Plus DNA polymerase, 2.5 µl 10 × CoralLoad Reaction buffer (including 15 mM $MgCl_2$), 0.5 µl PCR nucleotide Mix (0.2 mM each), 0.5 µl (1 µM final concentration) of each primer, 15.8 µl ddH_2O and 5 µl template DNA (except for the PCR amplifying *Wolbachia*, where an extra 1 µl of $MgCl_2$ was added and ddH_2O reduced accordingly). In addition, for *A. phagocytophilum*, a real-time PCR which amplifies part of the gene encoding a major surface protein (*msp2*) was used to confirm the results (Courtney et al., 2004; modified as in Hornok et al., 2014).

Each PCR was run with two sequence-verified positive controls, and one non-template negative control (reaction mixture). PCR products were visualized in 1.5% agarose gel. Negative controls and extraction controls remained PCR negative in all tests. Purification and sequencing (directly from the PCR product, generating the sequence twice per sample) were performed from all PCR positive samples at Biomi Inc. (Gödöllő, Hungary). Sequences were aligned and compared to reference GenBank sequences by nucleotide BLASTn program (https://blast.ncbi.nlm.nih.gov). The *msp*4 sequence of *A. marginale*, the *groEL* sequence of *Candidatus* Neoehrlichia lotoris-like agent, and for each species or genotype the longest obtained 16S rRNA sequences were submitted to GenBank (Table 2).

Phylogenetic analyses were performed with the Neighbor-Joining method, *p*-distance model on short (306–307 bp) sequences obtained from the screening assay (PCR "A") (Fig. 1), and with the Maximum Likelihood method, Juke-Cantor model in the case of other tests amplifying longer gene fragments according to various groups in Anaplasmataceae (Table 1; Figs. 2–4). In the latter (group level) phylogenetic analyses only those members of Anaplasmataceae have been included, which have been detected for the first time in dogs during this study (thus, excluding *A. phagocytophilum*). In the dataset used for the group-level phylogenetic trees, those longer sequences were selected from this study, for which sequences with nearly 100% coverage were available in GenBank from a representative spectrum of taxonomically related species (Table 2). Outgroup was not used in phylogenetic analyses, which only served to observe clustering patterns (genera *Ehrlichia*, *Neoehrlichia*), but an outgroup was included for the genus *Anaplasma*, where evolutionary distances were also taken into account (Kinene et al., 2016). Phylogenetic analyses were done using MEGA 6.0 (Kumar et al., 2016).

Rates of PCR-positivity were compared with the Fisher's exact test and differences were considered significant when $P < 0.05$.

3. Results

Among blood DNA extracts, 18 of 90 samples from dogs, and two of the five samples from badgers were PCR-positive for Anaplasmataceae (Table 2). In addition, six tick DNA extracts (five from *I. canisuga*, the one from *H. concinna*, but none from *I. hexagonus*) were PCR positive. In these samples, six members of Anaplasmataceae were identified with further PCRs and sequencing, as described below. Ticks were not found on dogs with PCR-positive blood samples.

3.1. Anaplasma marginale

The short 16S rRNA sequence of a ruminant-associated *Anaplasma* species was detected in two dogs (Fig. 1). The longer 16S rRNA gene fragment from these samples was identical with several *A. marginale* isolates from both the Old World and the New World (for an example see Table 2). Sequencing of the major surface protein 4 (*msp*4) gene from these samples identified an *A. marginale* genotype, which had

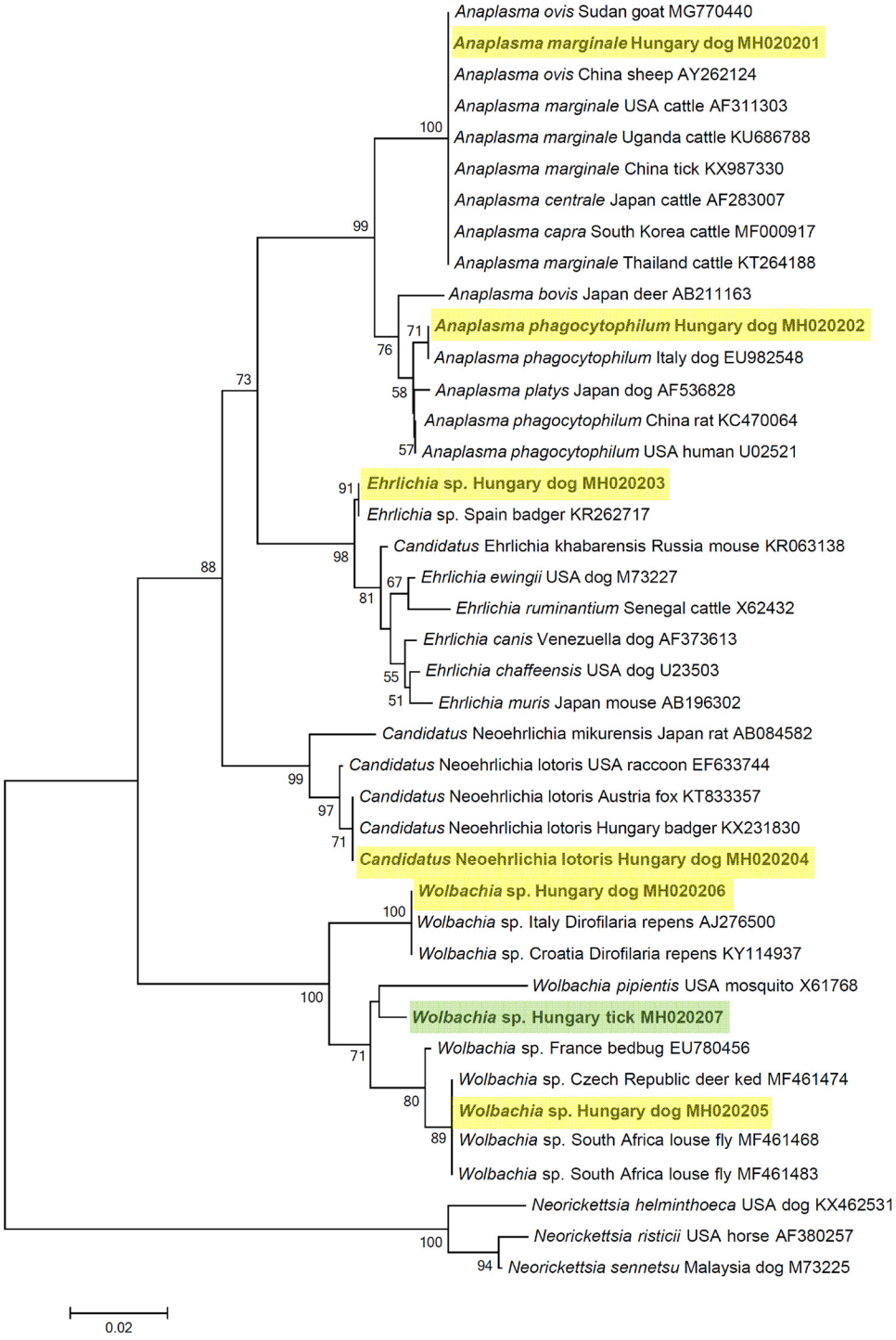

Fig. 1. Phylogenetic overview of PCR-positive samples. The Neighbor-Joining tree is based on the short (306 bp) 16S rRNA gene sequences amplified in the screening assay (PCR "A"). In each row, the species/genus name from Anaplasmataceae is followed by the country of origin, generic name of isolation source and GenBank accession number. Sequences from this study are highlighted with bold characters and yellow or green background according to canine or tick origin, respectively. Branch length correlate to the number of substitutions per site inferred according to the scale shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

100% identity with only one sample in GenBank (Table 2), reported previously from cattle and ticks (*Dermacentor reticulatus* and *I. ricinus*) in Hungary. Phylogenetically, this *A. marginale* genotype did not cluster within any of the two major groups of conspecific sequences retrieved from GenBank (Fig. 2). Its short evolutionary distance (the absence of branch length) was also reflected by the phylogenetic tree (Fig. 2).

3.2. *Anaplasma phagocytophilum*

One blood sample from a dog contained the DNA of *A. phagocytophilum*, with 100% identity (among the others) to an isolate from a dog in Italy (Fig. 1; Table 2). The major surface protein 2 (*msp*2) gene of *A. phagocytophilum* was also successfully detected in this sample, with moderately low Ct value (33.7).

3.3. *Badger-associated Ehrlichia sp.*

Based on the shorter fragment of the 16S rRNA gene, five DNA samples (one from a dog, which was in the group (n = 6) frequently taken to forests; and four from *I. canisuga* ticks collected from badger No. 3) contained DNA with the closest (306/307 bp = 99.7%) sequence identity to an *Ehrlichia* species reported from European badger in Spain (KR262717; Fig. 1). The longer 16S rRNA fragment of this genotype had 99.8% identity to the relevant isolate (Table 2), and was only 98.1%

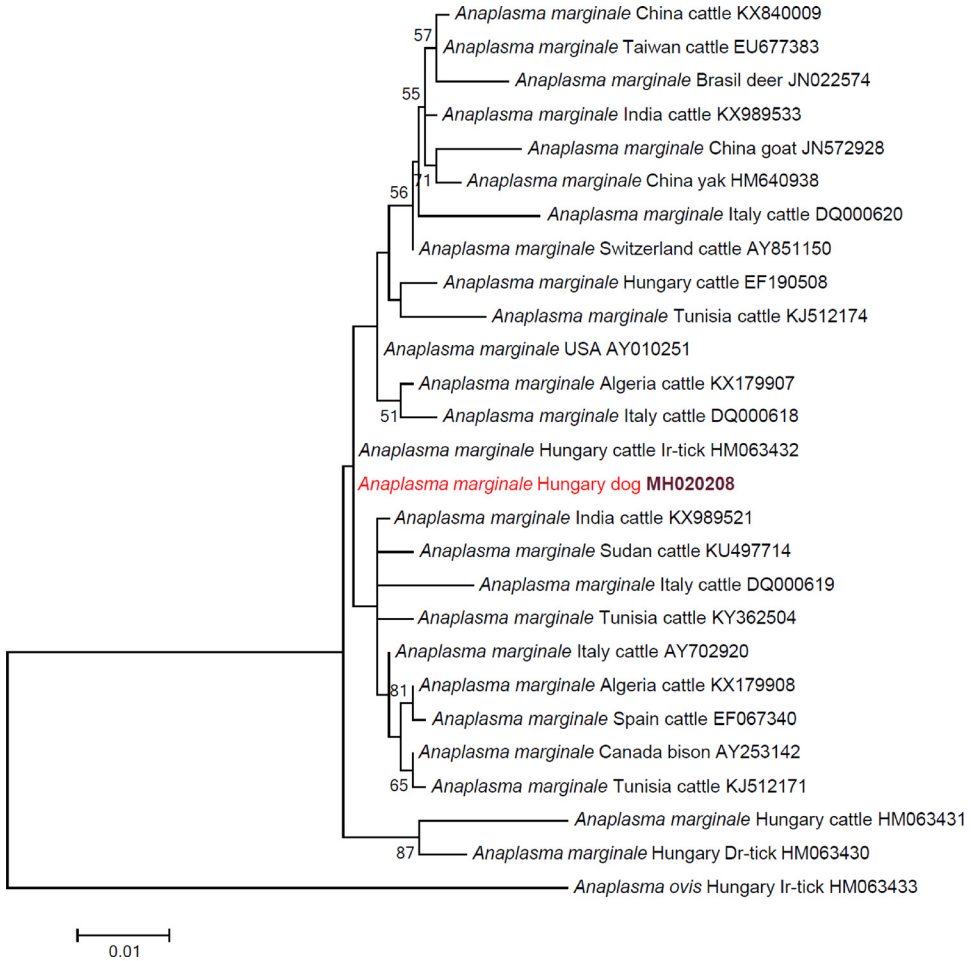

Fig. 2. Maximum Likelihood phylogenetic tree of *Anaplasma marginale msp*4 sequences, including that obtained from dogs in this study (indicated with red color and bold accession number). In each row, the species name is followed by the country of origin, generic name of isolation source and GenBank accession number. Branch length correlate to the number of substitutions per site inferred according to the scale shown. Abbreviations: Ir – *Ixodes ricinus*; Dr- *Dermacentor reticulatus*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identical to *Candidatus* E. khabarensis (KR063138). Phylogenetic analysis confirmed the separation of this dog *Ehrlichia* isolate from both *Candidatus* E. khabarensis and the *Ehrlichia* sp. from badger in Spain, the latter with moderately high (91%) bootstrap support (Fig. 3a).

3.4. *Candidatus Neoehrlichia lotoris-like agent*

The short 16S rRNA sequence of a *Neoehrlichia* species (designated here as *Candidatus* Neoehrlichia lotoris Hu-2) was detected in six dogs, two badgers (No. 3, 5) and one *I. canisuga* tick from badger No. 3 (Fig. 1). Sequencing of a longer 16S rRNA gene fragment indicated 100% identity with *Candidatus* N. lotoris-like strains Hu-1 (KX231830) and FU98 (KT833357) recently reported from European badger (eastern Hungary) and red fox (Austria), respectively (Table 2). In this study the overall length of amplified part of the 16S rRNA gene was longer than those previously reported from Europe, and it showed 99.3% (1214/1222 bp) identity to *Candidatus* N. lotoris detected in raccoon in the USA (EF633744: Fig. 3a).

The phylogenetic clustering of the three isolates of *Candidatus* N. lotoris-like bacteria from Europe confirmed their 16S rRNA gene uniformity (Fig. 3a). However, the chaperone (heat shock) protein gene (*groEL*) sequence of *Candidatus* N. lotoris Hu-2 isolate was different, with only 99.6% sequence identity (Table 2) to *Candidatus* N. lotoris strains Hu-1 (KX245423) and FU98 (KT833358). In the *groEL* phylogenetic analysis, the separation of canine isolate from those of wild carnivores was strongly (100%) supported (Fig. 3b).

Importantly, the DNA of *Candidatus* N. lotoris Hu-2 occurred significantly (P = .0001) more frequently in dogs often taken to forests (5 of 12) than in dogs without this characteristic (1 of 78). Moreover, the presence of DNA from this *Neoehrlichia* genotype was significantly (P = .00008) associated with hunting dogs (4 of 6 were infected) in comparison with dogs not used for hunting (2 of 84).

3.5. *Wolbachia genotypes*

Short 16S rRNA gene sequences of wolbachiae have been detected in eight dogs and in one *H. concinna* tick from a badger (Table 2). Four of these aligned with arthropod-associated *Wolbachia* sp. isolates, while five with nematode-associated *Wolbachia* sequences from GenBank (Fig. 1). In particular, the longer 16S rRNA sequence of *Wolbachia* sp. from three dogs had the highest similarity to different *Wolbachia* isolates from ectoparasites (louse flies) in the genus *Pseudolynchia* (family Hippoboscidae), both with longer (1216 bp) and a slightly shorter (1062 bp) sequence coverage (in comparison with DQ115537 and MF461468: 99.6-99.8% identities, respectively) (Table 2). This *Wolbachia* genotype of canine origin was phylogenetically also closely related to wolbachiae from further louse fly species, i.e. *Lipoptena cervi* and *Icosta* sp. (family Hippoboscidae) (Fig. 4). The 16S rRNA sequence of *Wolbachia* sp. from the badger tick showed the highest (100%) sequence identity to the endosymbiont of the latter louse fly genus (Table 2). The above four wolbachia genotypes (three from canine blood and one from *H. concinna*) belonged to the phylogenetic group of

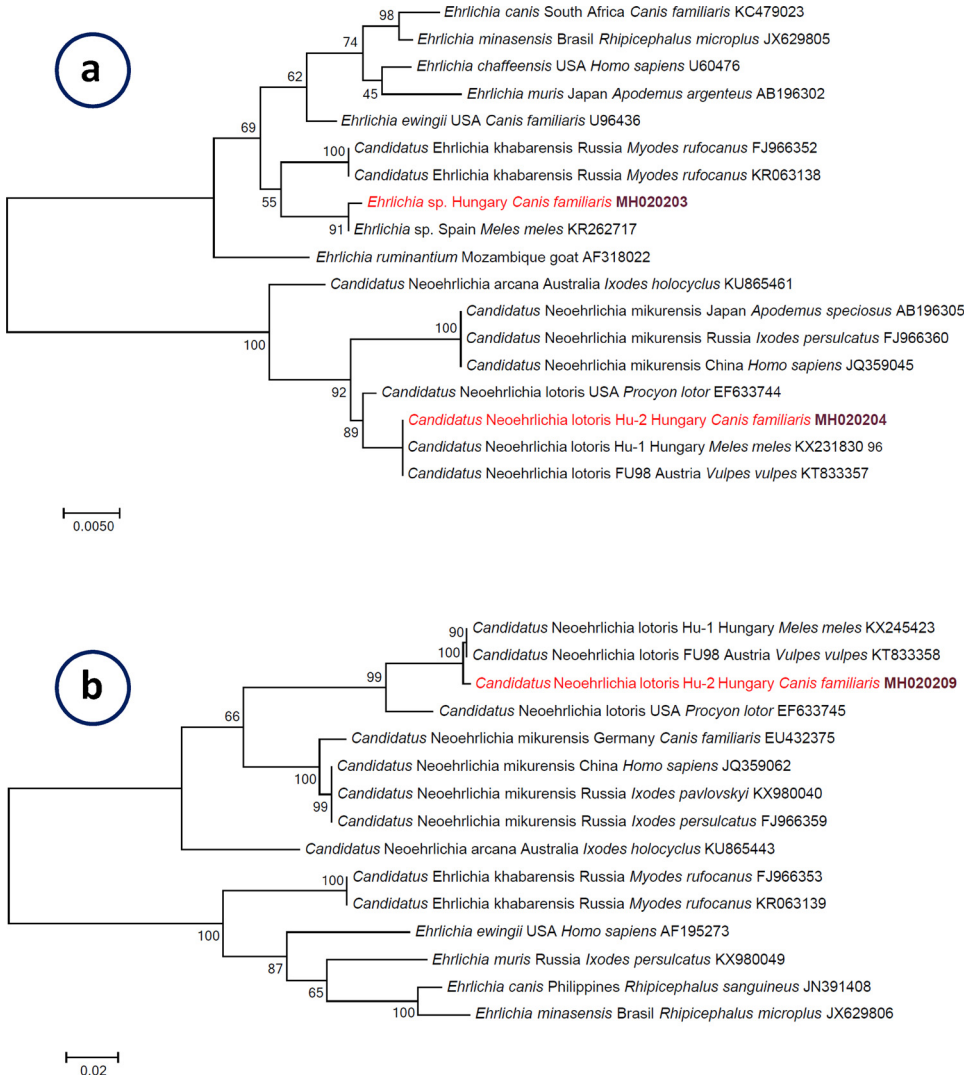

Fig. 3. Maximum Likelihood phylogenetic tree of *Ehrlichia* and *Neoehrlichia* species, based on (a) 16S rRNA gene and (b) *groEL* gene sequences. In each row, the species name is followed by the country of origin, scientific name of isolation source and GenBank accession number. Sequences obtained from dogs in this study are indicated with red color and bold accession numbers. Branch length correlate to the number of substitutions per site inferred according to the scale shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

arthropod-associated wolbachiae (Fig. 4).

In contrast to this, the longer 16S rRNA sequence of a further *Wolbachia* sp. (detected in five dogs) showed 100% sequence identity with the endosymbiont of the filarial nematode *Dirofilaria repens* (under KY114937 in Table 2). These also clustered together, within the clade of nematode-associated wolbachiae (Fig. 4).

4. Discussion

The above results provide molecular evidence for the presence of DNA from several members of Anaplasmataceae in dogs. The majority of these (except one: *A. phagocytophilum*) have not been hitherto reported from canine hosts. During the present study rural dogs were evaluated, which live in an environment different from that of urban pet dogs. Rural dogs are more likely to interact with livestock and wildlife in the context of tick-borne bacteria, and these "connectivities" may entail higher chances of finding regionally new pathogens, including members of Anaplasmataceae (Proboste et al., 2015). Nevertheless, because PCR-positive dogs in this study were not tick-infested at the time of sampling, the present results on tick-borne agents suggest previous exposure to ticks.

Considering *Anaplasma*, *Ehrlichia* and *Neoehrlichia* species in general, the predominant mode of transmission between their hosts is tick-borne (Dumler et al., 2001). In particular, *A. marginale* is known to be transmitted by males of *D. reticulatus* (Zivkovic et al., 2007), which is a tick species common on dogs in Hungary (Földvári and Farkas, 2005). Other *D. reticulatus*-borne pathogens (i.e. *Babesia caballi*, *Theileria equi*) have also been reported to be transmitted from ungulates (horses) to dogs in Croatia, neighboring the study site in southwestern Hungary (Beck et al., 2009). Furthermore, this unusual finding (*A. marginale* in dogs) is not unique in the context of ruminant-associated *Anaplasma* species in dogs, because *A. bovis* (typically infecting cattle, pathogenic to monocytes, and closely related to *A. phagocytophilum*: Fig. 1), has also been reported in canine host (Sakamoto et al., 2010).

According to GenBank data, this *A. marginale* genotype (found in cattle and dogs in Hungary) has not been reported from any other countries. Importantly, as reported in the context of emerging clinical bovine anaplasmosis in Hungary, this was the most frequently detected new genotype in cattle, and was also found in *I. ricinus* ticks (Hornok et al., 2012). The pathogenicity of this *A. marginale* genotype observed in cattle in a region of endemic stability (Hornok et al., 2012), and its phylogenetic relationships shown here suggest its evolutionarily recent emergence.

Another member of the genus, *A. phagocytophilum* was also shown to

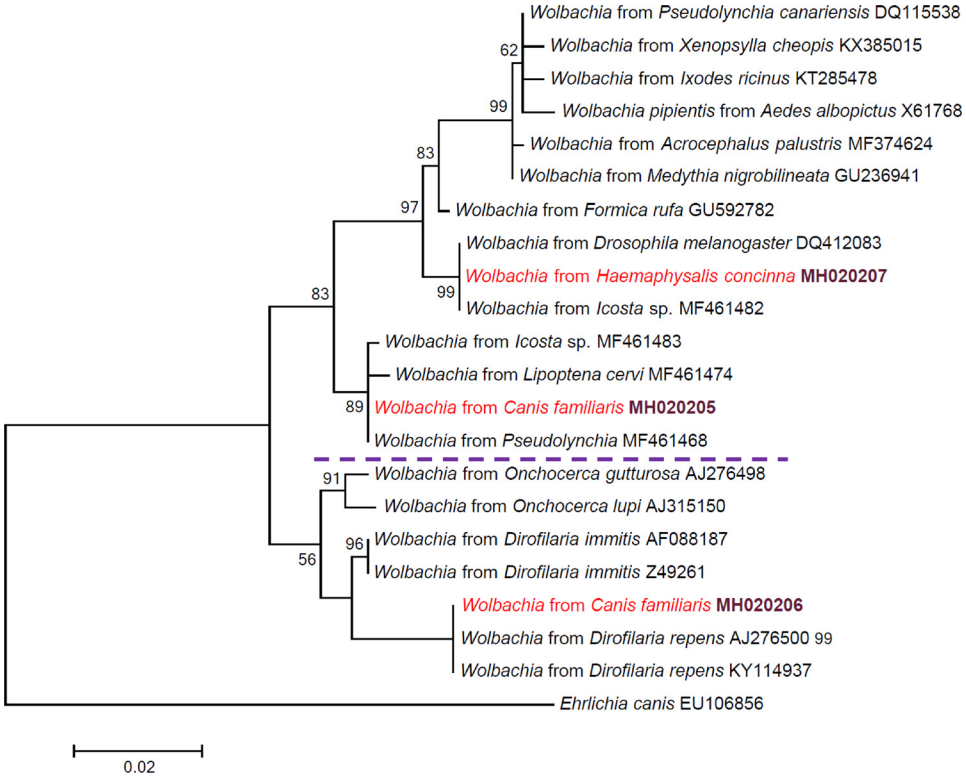

Fig. 4. Maximum Likelihood phylogenetic tree of 16S rRNA gene *Wolbachia* sequences, including those obtained from dogs and one tick in this study (indicated with red color and bold accession numbers). The two main clusters of wolbachiae (upper: arthropod-associated, lower: nematode-associated) are separated with dashed line. In each row, the genus name is followed by the scientific name of isolation source and GenBank accession number. Branch length correlate to the number of substitutions per site inferred according to the scale shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

be present in one dog of this survey. This finding is not new, since dogs are known hosts of this pathogen, as also reported formerly in Hungary (Hornok et al., 2013). Nevertheless, two implications increase the merit of this result. First, domestic and wild carnivores appear to have a role as reservoirs of the zoonotic ecotype of *A. phagocytophilum* (Jahfari et al., 2014), and therefore deserve special attention in the epidemiology of granulocytic anaplasmosis. Second, during the present study it is an important circumstance that a pathogen, for which dogs are known hosts, has also been detected together with bacteria unusual in dogs (i.e. with similar methodology), supporting that dogs sampled and evaluated here might also be susceptible to some of the newly detected agents among Anaplasmataceae.

In one dog and four *I. canisuga* ticks the sequence of an *Ehrlichia* species closely related to a badger-associated member of the genus (reported in Spain: García-Pérez et al., 2016) was identified. The first time detection of this agent of unknown pathogenicity in dogs, as reported here, is a similarly rare hit (taking into account only one PCR-positive dog out of 90), as during its original description from a European badger (in one out of 183 mustelids: García-Pérez et al., 2016). However, the genotype shown here to be present in a dog in Hungary differed in two nucleotides in its 16S rRNA gene (which is regarded as highly conserved within species of Anaplasmataceae: Rar and Golovljova, 2011) from that in Spain, suggesting genetic diversity of this *Ehrlichia* sp. according to geographical regions of Europe. The present findings suggest further evaluation if *I. canisuga* is the vector of this genotype.

The first molecular evidence is also provided here for the occurrence of a species most closely related to *Candidatus* N. lotoris in dogs. In this study, *Candidatus* N. lotoris-like sequences have been successfully amplified from six dogs, two badgers and one tick (*I. canisuga*). The PCR-positivity of badgers is consistent with previous findings in eastern Hungary (Hornok et al., 2017b). However, the difference of the southwestern Hungarian *groEL* sequence reported here from sequences from eastern Hungary, Austria and the Czech Republic (Hodžić et al., 2015, 2016) is also new in light of the fact that no sequence variations were found previously between the *Candidatus* N. lotoris sequences in the USA (Yabsley et al., 2008a). In addition, both the 16S rRNA and the more variable *groEL* sequences of the closely related species, *Candidatus* N. mikurensis tend to be highly conserved in Europe (Rar and Golovljova, 2011).

The likely vectors of *Candidatus* N. lotoris are *Pholeoixodes* species (Yabsley et al., 2008b). However, in the present study only one out of 19 *I. (Ph.) canisuga* ticks (all collected from a bacteraemic badger) were PCR-positive, suggesting that this tick species is either not a vector of *Candidatus* N. lotoris Hu-2, or it has low efficiency in acquiring it. Nevertheless, members of the subgenus *Pholeoixodes* are often associated with burrow-dwelling mammals (Hornok et al., 2017a), and the possibility of contact with such shelters could explain the significantly higher prevalence of *Candidatus* N. lotoris Hu-2 in hunting and forest-visiting dogs of the present study.

During the present study, *Wolbachia* DNA was demonstrated in the blood of eight dogs and one tick. Dogs with dirofilariosis were shown to have *Wolbachia* DNA in their blood (Rossi et al., 2010). However, wolbachiae from three dogs aligned in the group of arthropod-associated genotypes. Although *Wolbachia* DNA of arthropod origin has recently been detected in avian blood (Hornok et al., 2018b), similar findings in canine blood (during this study) cannot be interpreted with certainty. Even the tick-origin of *Wolbachia* DNA found here in *H. concinna* is uncertain, taking into account that *Wolbachia* endosymbionts in *I. ricinus* were reported to derive from parasitoid wasps (Plantard et al., 2012).

5. Conclusions

In this study, the DNA from a hitherto unknown diversity of Anaplasmataceae has been detected in rural dogs. Results shown here should be interpreted as first time molecular evidence for the exposure of dogs to the three well-established or potential pathogens (*A. marginale*, badger-associated *Ehrlichia* sp. and a *Candidatus* N. lotoris-like agent). The presence of DNA in the blood of relevant animals may also indicate susceptibility to and (at least a transient) infection with Anaplasmataceae new in canine hosts, but ultimate verification of this

awaits further studies.

Availability of data and materials

The sequences obtained and/or analyzed during the current study are deposited in GenBank under accession numbers (MH020201–MH020209). Other relevant data are included in the manuscript, or will be provided by the corresponding author upon request.

Ethical approval

Dogs were sampled during regular veterinary care and all badgers were road-killed animals, therefore no ethical permission was needed.

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