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## **Molecular investigation of hard ticks (Acari: Ixodidae) and fleas (Siphonaptera: Pulicidae) as potential vectors of rickettsial and mycoplasmal agents**

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1           **Molecular investigation of hard ticks (Acari: Ixodidae) and fleas**  
2                           **(Siphonaptera: Pulicidae) as potential vectors of**  
3                                   **rickettsial and mycoplasmal agents**

4  
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19  
20           **ABSTRACT**

21           The aim of the present study was twofold. First, in general, to reveal new aspects of the  
22           potential vector role of ixodid ticks and fleas by screening large numbers of specimens with  
23           recently developed molecular biological methods. Second, to evaluate the occurrence of  
24           vector-borne infectious agents in a geographical context. Altogether 3,442 unfed hard ticks  
25           (*Ixodes ricinus*, *Dermacentor marginatus*, *D. reticulatus*, *Haemaphysalis inermis*, *H.*

26 *concinna*, *H. punctata*) and 939 fleas of cats and dogs (*Ctenocephalides felis*, *C. canis*, *Pulex*  
27 *irritans*) were collected in Hungary. DNA was extracted and analyzed in pools for  
28 representatives of the orders Rickettsiales and Mycoplasmatales.

29 *H. inermis* was newly identified as the most important potential vector for *Rickettsia*  
30 *helvetica* in the study region. A novel *Rickettsia* genotype (designated '*Candidatus* R.  
31 *hungarica*') was also detected in the same tick species, with a maximum of 95.8% *gltA* gene  
32 sequence identity to known rickettsiae. In addition, *P. irritans* tested positive for *Rickettsia*  
33 sp. RF2125, which has not been previously described in Europe. The human pathogen *R. felis*  
34 and the feline pathogen '*Candidatus* Mycoplasma turicensis' were shown for the first time to  
35 occur in Central-Eastern Europe. Further novel findings include the presence of *Spiroplasma*  
36 spp. in *D. marginatus* and fleas.

37 In conclusion, this molecular study extends the geographic range and vector spectrum  
38 of several arthropod-borne agents, some of which have zoonotic potential.

39

40 *Keywords:* rickettsia; *Ehrlichia canis*; haemotropic mycoplasma; spiroplasma; tick; flea

41

## 42 **1. Introduction**

43

44 Blood-sucking arthropod vectors, exemplified by ticks and fleas, are able to transmit  
45 infectious agents between animals and/or humans. Most of the pathogenic bacteria belonging  
46 to the order Rickettsiales are vector-borne (Dumler et al., 2001), and this transmission route  
47 has also been suggested for several representatives of the order Mycoplasmatales (Neimark et  
48 al., 2001).

49 *Rickettsia* spp. (family Rickettsiaceae, order Rickettsiales) are Gram-negative, obligate  
50 intracellular bacteria (Dumler et al., 2001). Although they have been long known for their  
51 public health importance, recently an emerging veterinary significance of various species was

52 also recognized (Hechemy et al., 2006). Similarly, *Ehrlichia* spp. (family Anaplasmataceae,  
53 order Rickettsiales) include both human and animal pathogens (Dumler et al., 2001).

54 Haemotropic mycoplasmas (also known as haemoplasmas; order Mycoplasmales),  
55 formerly assigned to the genera *Haemobartonella* and *Eperythrozoon* (Neimark et al., 2001),  
56 attach to red blood cells. This may lead to haemolytic anaemia in infected domestic and wild  
57 animals. Vectors of haemoplasmas may include fleas, hard ticks, and mosquitoes (Neimark et  
58 al., 2001).

59 *Spiroplasma* spp. are helical mycoplasmas. Although they have been implicated in  
60 spongiform encephalopathies (Bastian et al., 2007), unambiguous information on their exact  
61 role in diseases is still lacking. Various categories of insects and arachnids, including certain  
62 hard ticks, such as *I. ricinus*, have been shown to harbor these bacteria (Carle et al., 1995).

63 In Hungary *Rickettsia slovaca*-like organisms have been found in questing (unfed)  
64 *Dermacentor* spp. (Reháček et al., 1979). More recently engorged ticks were investigated: *R.*  
65 *helvetica* and *R. monacensis* have been detected in *I. ricinus*, *R. raoultii* (formerly *Rickettsia*  
66 sp. RpA4) in *D. reticulatus*, and a distinct rickettsia genotype in *Haemaphysalis concinna*  
67 (Sréter-Lancz et al., 2006). However, no other tick species – particularly not questing ones –  
68 or flea species have been evaluated. Similarly, no data have been published to date on the  
69 occurrence of *Ehrlichia canis*, haemotropic *Mycoplasma*, or *Spiroplasma* spp. in ticks or fleas  
70 of this region in Europe. Therefore, the present study was undertaken to obtain relevant and  
71 updated information on these important vector-borne agents.

72

## 73 **2. Materials and methods**

74

### 75 *2.1. Origin of the samples*

76

77 Between March and July 2007, a total of 3,442 hard ticks (Acari: Ixodidae) were collected  
78 from vegetation at 37 locations in Hungary using the cloth-dragging and flagging method.  
79 The species, stage, and sex of the ticks were determined by microscopic examination.  
80 Subsequently, ticks were allotted into pools (Table 1) taking into account their size, i.e. for  
81 optimal DNA extraction. The last pool of each species contained the remainder of specimens.

82 In addition, 939 fleas (Siphonaptera: Pulicidae) were collected – 420 from cats and 519  
83 from dogs – at 14 small animal clinics in various parts of the country. All fleas of one species  
84 removed from one animal were pooled (Table 1). *Ctenocephalides felis* were obtained from  
85 cats, *C. canis* from cats and dogs, and *Pulex irritans* from dogs. Both tick and flea samples  
86 were stored in 70% ethanol until molecular biological analysis.

87

## 88 2.2. DNA extraction

89

90 Pools of ticks and fleas were air-dried, washed twice in phosphate-buffered saline without  
91 MgCl<sub>2</sub> or CaCl<sub>2</sub> (DPBS; Invitrogen, Basel, Switzerland) and minced with scissors in 100 µl  
92 DPBS at the bottom of 2 ml Eppendorf tubes. Between the preparations of pools, the scissors  
93 were decontaminated by washing in tap water, burning, and cooling in distilled water.

94 From all tick pools and from those of *C. canis* and *P. irritans*, DNA was extracted using  
95 the MagNA Pure LC total nucleic acid isolation kit (Roche Diagnostics, Rotkreuz,  
96 Switzerland). Briefly, 150 µl of DPBS and 375 µl of lysis buffer containing guanidinium  
97 thiocyanate and Triton X-100 were added to each pool, together with a 5 mm stainless steel  
98 bead (Schieritz and Hauenstein AG, Arlesheim, Switzerland). The samples were  
99 homogenized in a Mixer Mill device (Retsch GmbH, Haan, Germany) for 2 min at 30,000 Hz.  
100 After a short centrifugation at 5,000 g, 500 µl of the supernatant from each sample was loaded  
101 onto the MagNA Pure LC Instrument (Roche Diagnostics). Two negative extraction controls

102 containing only DPBS were included per 30 samples in order to monitor for cross-  
103 contamination. DNA from the 187 pools of *C. felis* was extracted using the QIAamp DNA  
104 mini kit (QIAGEN, Hilden, Germany). In both procedures the elution volume was 100 µl.

105 The presence of amplifiable DNA was confirmed for each pool using a real-time PCR  
106 assay to detect the 18S rRNA gene (Applied Biosystems, Rotkreuz, Switzerland). The  
107 concentration of amplifiable DNA was determined as sufficient if the threshold cycle (Ct)  
108 value was less than 25. Samples were stored at -20 °C until analysis.

109

### 110 2.3. Real-time PCRs

111

112 All samples were first screened with real-time PCR. Cycling conditions were: 50 °C for 2 min  
113 and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Ninety  
114 three samples (including three negative extraction controls) were analyzed in one assay,  
115 together with two – external and internal – negative and one positive PCR controls. The tests  
116 were performed using either the ABI Prism 7500 (for *Rickettsia* spp.) or 7700 (for all others)  
117 Sequence Detection Systems (Applied Biosystems).

118

#### 119 2.3.1. TaqMan PCRs for *Rickettsia* spp.

120

121 First an assay that amplifies a 65-bp portion of the 23S rRNA gene of *R. helvetica* was  
122 performed. Then all samples negative in the 23S rRNA PCR (or chosen for confirmatory  
123 analysis) were tested with TaqMan PCR for a 74-bp fragment of the *gltA* gene in spotted fever  
124 and typhus group rickettsiae (Boretto et al., in press).

125

126 2.3.2. *TaqMan PCR for Ehrlichia canis*

127

128 This method to detect a portion of the 16S rRNA gene of *E. canis* was performed as described  
129 previously (Foley et al., 2007).

130

131 2.3.3. *Universal SYBR Green PCR for haemotropic mycoplasmas*

132

133 The assay served to screen all DNA samples for the presence of haemotropic mycoplasmas  
134 (Willi et al., submitted for publication). In brief, the reaction volume was 25 µl, consisting of  
135 12.5 µl of SYBR Green Mastermix (Applied Biosystems), a final concentration of 300 nM of  
136 forward primer (5'-AGC AAT RCC ATG TGA ACG ATG AA- 3') and an equimolar mixture  
137 of two reverse primers (5'-TGG CAC ATA GTT TGC TGT CAC TT-3' and 5'-GCT GGC  
138 ACA TAG TTA GCT GTC ACT-3'), and 5 µl of template DNA. After 40 cycles a  
139 dissociation run was performed: 95 °C for 15 s, 60 °C for 20 s, a temperature increase from 60  
140 °C to 95 °C in 20 min, and finally 95 °C for 15 s.

141

142 2.3.4. *Species-specific TaqMan PCR for haemotropic mycoplasmas*

143

144 Samples that tested positive or gave inconclusive results in the SYBR Green assay were  
145 further analyzed. Real-time TaqMan PCR assays specific for the 16S rRNA gene of feline (*M.*  
146 *haemofelis*, '*Candidatus M. haemominutum*', and '*Candidatus M. turicensis*'), canine (*M.*  
147 *haemocanis*, '*Candidatus M. haematoparvum*'), and murine haemotropic mycoplasmas (*M.*  
148 *coccoides*) were performed as previously described (Wengi et al., 2008; Willi et al., 2006;  
149 Willi et al, 2007). In addition, for bovine/ovine haemoplasmas (*M. wenyonii* and *M. ovis*) a  
150 119-bp long fragment of the 16S rRNA gene was amplified (Meli et al., submitted for

151 publication), involving the forward primer 5'-CCA CGT GAA CGA TGA AGG TCT T-3',  
152 the reverse primer 5'-GGC ACA TAG TTA GCT GTC ACT TAT TCA A-3', and the probe  
153 5'-6FAM-AGT ACC ATC AAC GCG CGC TGA TTT CCT AG-MGB-3'. The 25µl PCR  
154 reaction mixture contained 900 nM of each primer, 0.25 µM of probe, 12.5 µl of 2× qPCR  
155 MasterMix (Eurogentec, Seraing, Belgium), and 5 µl of template DNA.

156

#### 157 2.4.1. Conventional PCRs for *Rickettsia* spp.

158

159 Selected *gltA* TaqMan PCR-positive samples (n = 19, with the lowest Ct values) were further  
160 analyzed by a conventional PCR. A 381-bp portion of the *gltA* gene was amplified using the  
161 primers Rp877p and Rp1258n, as previously described (Boretti et al., in press). Additionally,  
162 in the case of one sample that gave a sequence different from those deposited in GenBank, a  
163 longer *gltA* gene fragment (796 bp) was amplified with the primers CS477f and CS1273r. Part  
164 of the PCR products were stained with ethidium bromide and visualized in agarose gel.

165

#### 166 2.4.2. Conventional PCRs for *Mycoplasmatales*

167

168 Samples positive in the species-specific TaqMan real-time PCR were further analyzed for the  
169 nearly complete 16S rRNA gene as described earlier (Willi et al., 2006). Samples that yielded  
170 inconclusive SYBR Green real-time PCR results, but were negative in all species-specific  
171 TaqMan assays, were subsequently evaluated with a conventional PCR for a shorter,  
172 approximately 200 bp region in the 16S rRNA gene of haemoplasmas (Jensen et al., 2001).

173

#### 174 2.5. Sequencing

175

176 After purification of the conventional PCR product with the GenElute™ PCR Clean-Up Kit  
177 (Sigma-Aldrich) and determination of the DNA concentration with the NanoDrop ND-1000  
178 spectrophotometer (Witec Ag, Littau, Switzerland), cycle sequencing was performed using  
179 the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems).  
180 The cycling conditions were as follows: 1 min at 96 °C, then 25 cycles of 96 °C for 30 sec  
181 and 50 °C for 15 sec, and finally incubation at 60 °C for 4 min. Products were purified with  
182 SigmaSpin™ Post-Reaction Purification Columns (Sigma-Aldrich), and evaluated on the ABI  
183 Prism 310 Genetic Analyzer (Applied Biosystems).

184

185

#### 186 *2.6. Statistical analysis*

187

188 Pool infection prevalence was calculated from the number of positive pools, expressed as the  
189 percentage of the number of evaluated pools. Given that there must have been at least one  
190 infected individual in each positive pool, the minimum prevalence was determined from the  
191 number of positive pools, expressed as the percentage of the number of evaluated arthropod  
192 individuals of the species. Confidence intervals (CI) for the prevalence rates at the 95% level  
193 were calculated according to Sterne's method (Reiczigel, 2003).

194

#### 195 *2.7. Phylogenetic analyses and nucleotide sequence accession numbers*

196

197 Obtained sequences were edited, aligned and compared to reference GenBank sequences as  
198 described previously (Hornok et al., 2009). New sequences were submitted to the GenBank,  
199 with the accession numbers EU170604 for '*Candidatus* Mycoplasma haemominutum',  
200 EU170605-15 for *Spiroplasma* spp., and EU853830-5 plus EU853837-8 for *Rickettsia* spp.

201

202 **3. Results**

203

204 All extraction and PCR negative controls were negative.

205

206 *3.1. Rickettsia spp.*

207

208 Using real-time TaqMan PCR for the 23S rRNA gene, *R. helvetica* was detected in 98.5%  
209 (95% CI: 91.6-100%) of the *H. inermis* pools (minimum prevalence 19.7%; 95% CI: 15.5-  
210 24.5%) (Table 2). A total of 92.6% (95% CI: 86.7-96.4%) of the *I. ricinus* pools were also  
211 shown to be infected (minimum prevalence 7.2%; 95% CI: 6-8.5%). Further two tick species  
212 and one flea species were found to harbor *R. helvetica* (Table 2). The minimum prevalence of  
213 *Rickettsia* spp. in *D. marginatus* and in *D. reticulatus* was 17.5% (95% CI: 13.2-22.5%) and  
214 16% (95% CI: 12.3-20.3%), respectively (Table 2).

215 One pool of *H. inermis* collected in northern Hungary contained a *gltA* sequence, a 712-bp  
216 region of which showed only 95.8% maximal identity to that of any other known *Rickettsia*  
217 spp. available in the GenBank. Hence the name '*Candidatus R. hungarica*' is proposed for this  
218 new isolate. In addition, *R. monacensis* was detected in *I. ricinus*, *R. slovaca* was found in  
219 *D. marginatus*, and *R. raoultii* was identified in *D. reticulatus* (Table 2). Moreover,  
220 '*Candidatus R. kotlanii*' was shown in an *H. concinna* pool, and *R. felis* as well as *Rickettsia*  
221 sp. RF2125 were found in flea pools (Table 2). The phylogenetic relationships of these  
222 specimens are depicted in Fig. 1.

223

224 3.2. *Ehrlichia canis*

225

226 Tick pools were evaluated for the presence of *E. canis*. Two pools of female *I. ricinus*, both  
227 collected in southwestern Hungary, were found to be PCR-positive.

228

229 3.3. *Haemotropic Mycoplasma spp.*

230

231 All tick pools and the *C. canis* and *P. irritans* pools were PCR-negative for haemotropic  
232 *Mycoplasma* spp. Two *C. felis* pools were positive in the assay specific for '*Candidatus M.*  
233 *haemominutum*'; one of them was also found to contain '*Candidatus M. turicensis*'. The  
234 presence of '*Candidatus M. haemominutum*' was also confirmed by sequencing of the 16S  
235 rRNA gene (EU170604). This isolate showed 100% identity to a feline isolate recently  
236 reported in Hungary (EU128752).

237

238 3.4. *Spiroplasma spp.*

239

240 Fifteen tick pools and four flea pools gave inconclusive results in the real-time SYBR Green  
241 assay and were negative in all TaqMan assays designed to detect individual haemoplasma  
242 species. Five of the 15 tick pools and three of the four flea pools were found to be negative by  
243 conventional PCR. In the remaining pools, representatives of the genus *Spiroplasma* were  
244 found. These included six *I. ricinus* pools (five female, one nymph), four *D. marginatus* pools  
245 (three male, one female), and one *C. felis* pool. Sequencing revealed 95-98% identity to  
246 *Spiroplasma* spp. deposited in GenBank (AJ132412).

247

248 **4. Discussion**

249

250 This is the first molecular study of the occurrence of *Rickettsia* spp., *E. canis*, haemotropic  
251 mycoplasmas and *Spiroplasma* spp. in questing ticks and in fleas obtained from cats and dogs  
252 in Hungary. Since fed (engorged) ticks may harbour infectious agents that were acquired  
253 during the blood meal but are irrelevant to their vector potential, only ticks collected from the  
254 vegetation were included in the present survey.

255 Many rickettsiae cause emerging diseases (Hechemy et al., 2006; Brouqui et al., 2007).  
256 The geographical distribution of *Rickettsia* spp. depends on their association with particular  
257 tick vectors and animal reservoirs. *R. helvetica*, a potential human pathogen (Brouqui et al.,  
258 2007), is transmitted by *I. ricinus* in Europe (Parola, 2004) and by other *Ixodes* spp. in Asia  
259 (Fournier et al., 2002). Results of the present study introduce *H. inermis* as a new and  
260 significant potential vector of this rickettsia. *H. inermis* is an emerging species (Hornok and  
261 Farkas, 2009), occurring sporadically in most European countries, as well as in the Middle  
262 East and South Asia. In addition, *H. concinna*, *H. punctata*, and the cat flea *C. felis* were  
263 identified for the first time in this study as possible vectors of *R. helvetica*. However, based on  
264 the low number of infected pools, they appear to be of only minor importance.

265 Remarkably, a novel *Rickettsia* genotype has been found in *H. inermis*. The difference of  
266 at least 4.2% between the *gltA* sequences of the new genotype and the sequences of other  
267 rickettsiae, argue for its status as a candidate new species ('*Candidatus R. hungarica*'). For  
268 comparison, previous work has estimated the *gltA* sequence to diverge by up to 2.3% between  
269 members of the SFG group (Regnery et al., 1991). Furthermore, characterization of a shorter,  
270 381-bp portion of the *gltA* gene has previously been used to describe other new species of the  
271 genus (Higgins et al., 1996), and here it showed a minimum of 4.8% sequence dissimilarity  
272 between the new isolate and relevant GenBank sequences.

273 PCR positivity for *R. slovaca*, another clinically important species (Brouqui et al., 2007),  
274 has already been detected in two engorged *D. marginatus* individuals in Hungary (Raoult et  
275 al., 2002). The present study extends these findings to unfed ticks. Although only five PCR-  
276 positive *D. marginatus* samples were sequenced, *R. slovaca* was identified in all cases. If we  
277 assume that the majority of the PCR-positive *D. marginatus* pools had been infected with *R.*  
278 *slovaca*, the observed minimum prevalence of 17.5% is higher than the prevalence of 7.2%  
279 reported previously in this region (Reháček et al., 1979). Similarly, the sequencing of all five  
280 *D. reticulatus* pools detected *R. raoultii* (formerly *Rickettsia* sp. RpA4). If we extrapolate this  
281 to the remainder of the PCR-positive samples, the high rate of infection in this species is  
282 comparable to previous reports evaluating engorged ticks in Hungary (Sréter-Lancz et al.,  
283 2006) and Germany (Dautel et al., 2006).

284 The identification of *R. monacensis* in questing *I. ricinus* and of '*Candidatus R. kotlanii*' in  
285 *H. concinna* in the present study corroborates their earlier detection in engorged specimens  
286 (Sréter-Lancz et al., 2006). On the other hand, although the human pathogen *R. felis* (Higgins  
287 et al., 1996) had already been found in Western Europe, both in Germany and France (Rolain  
288 et al., 2003), this is the first report of a highly similar sequence from Central-Eastern Europe.  
289 Furthermore, *Rickettsia* sp. RF2125 was shown for the first time in Europe and in the human  
290 flea, *Pulex irritans*; as formerly this species had been identified in Asia, North America, and  
291 Africa in *C. canis* (AF516333), *C. felis* (AY953289), and *Echidnophaga gallinacea* (Loftis et  
292 al., 2006), respectively.

293 In Europe, *E. canis*, the etiological agent of canine monocytic ehrlichiosis, is transmitted  
294 by *Rh. sanguineus* (Groves et al., 1975). This restricts its endemicity to the Mediterranean and  
295 Balkanian countries, with prevalences declining towards the north (Solano-Gallego et al.,  
296 2006). Recently, however, *E. canis* was also detected in *I. ricinus* collected from vegetation in  
297 the Netherlands (Wielinga et al., 2006). Since the method used in the present study is highly

298 specific for *E. canis* (Foley et al., 2007) and cross-contamination of the samples is very  
299 unlikely, our results raise the possibility of the indigenous status of *E. canis* in Hungary.  
300 However, confirmation of this would require sequencing, which could not have been carried  
301 out from the two positive pools due to the low bacterial load.

302 Vectors of haemotropic mycoplasmas, the causative agents of haemolytic anaemia, may  
303 include fleas and hard ticks, as suggested by previous transmission experiments (Seneviratna  
304 et al., 1973; Woods et al., 2005). This is further substantiated by the fact that unfed ixodid  
305 ticks contained '*Candidatus M. haemominutum*' (Taroura et al., 2005). Nevertheless, although  
306 haemoplasma infections have been reported in Hungary (Hornok et al., 2008; Hornok et al.,  
307 2009), the current study failed to detect PCR positivity in tick pools. This may suggest that  
308 ticks do not play a significant role in the transmission of haemotropic mycoplasmas in  
309 Hungary, confirming previous results from Switzerland (Willi et al., 2007). On the other  
310 hand, feline haemoplasmas are frequently detected in fleas (Willi et al., 2007). Accordingly,  
311 '*Candidatus M. haemominutum*' and '*Candidatus M. turicensis*' were found in *C. felis*, the  
312 latter for the first time in Central-Eastern Europe. The 100% identity of the 16S rRNA gene  
313 sequence of the flea isolate with that of one Hungarian cat (EU128752), two Swiss cats  
314 (DQ157141, DQ157147), and one UK cat (AF 271154) is consistent with the small genetic  
315 differences between haemoplasmas from geographically distant places (Tasker et al., 2003).

316 The genus *Spiroplasma* has an enormous diversity of often incompletely characterized or  
317 unnamed species, including non-pathogenic or symbiotic organisms that persist in insects and  
318 arachnids. These bacteria have been associated with transmissible spongiform encephalopathy  
319 in humans and ruminants (Bastian et al., 2007), but how they contribute to these diseases  
320 remains unclear. Spiroplasmas are found not only in horse flies and mosquitoes, but also in  
321 hard ticks (Carle et al., 1995). In particular, *I. ricinus* from Germany (Henning et al., 2006)  
322 and *Haemaphysalis* spp. (Carle et al., 1995) have been reported to be carriers of these agents.

323 However, to the best of our knowledge, this is the first report of a *Spiroplasma* infection in *D.*  
324 *marginatus* (for which ruminants are the most preferred hosts) and in any fleas. Since fleas  
325 are not host-specific, the spiroplasma-carrier status of some *C. felis* shown in the present  
326 study should be taken into account when assessing the risks associated with pet fleas near  
327 human beings.

328 In conclusion, the present study extends the geographic range of important infectious  
329 agents, reports identification of a novel rickettsia sequence, and suggests new potential vector  
330 role for some of the evaluated tick and flea species.

331

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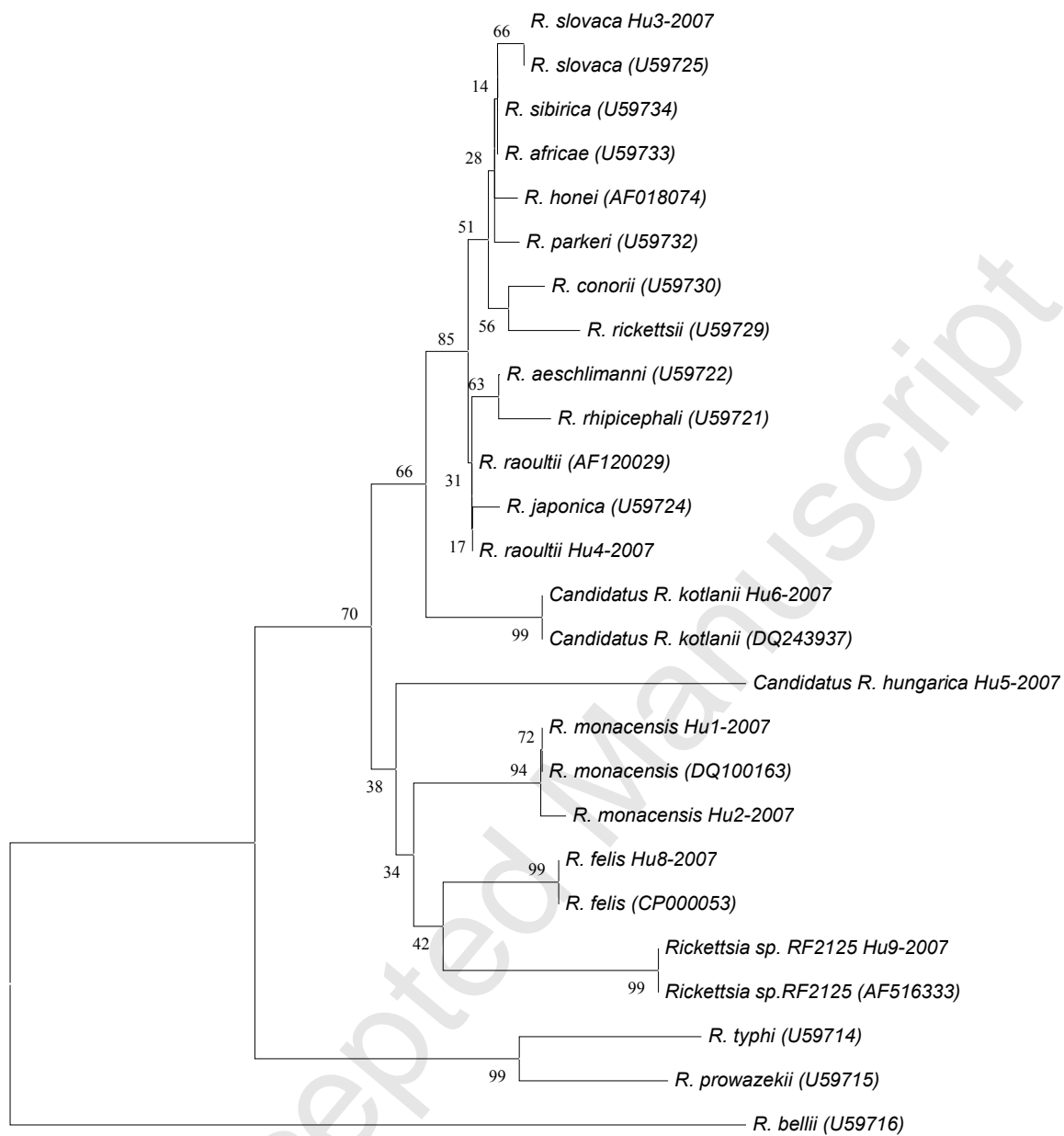
482

483 **Figure caption:**

484

485 **Figure 1.** A bootstrap phylogenetic tree of the *gltA* gene demonstrating the relationship of  
486 Hungarian isolates to other *Rickettsia* spp. using the Neighbor-Joining method. The numbers  
487 at the nodes were generated from 1,000 Bootstrap resamplings. The scale bar represents the  
488 mean number of differences per 50 sites.

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**Table 1.** Pooling criteria for ticks and fleas.

	<b>Total number</b>	<b>Number of adults per pool</b>	<b>Number of immature stages per pool<sup>1</sup></b>	<b>Total number of pools</b>
<b>Ticks</b>	3442			
<i>Ixodes ricinus</i>	2116	20 ♂, 10 ♀	30 n, 24 l	41 ♂, 94 ♀, 9 n, 1 l
<i>Dermacentor marginatus</i>	369	5 ♂, 5 ♀	-	23 ♂, 34 ♀
<i>D. reticulatus</i>	361	5 ♂, 5 ♀	-	25 ♂, 46 ♀
<i>Haemaphysalis inermis</i>	315	5 ♂, 5 ♀	-	24 ♂, 40 ♀
<i>H. concinna</i>	259	3 ♂, 3 ♀	10 n, 52 l	29 ♂, 16 ♀, 7 n, 1 l
<i>H. punctata</i>	22	3 ♂, 3 ♀	1 n	5 ♂, 3 ♀, 1 n
<b>Fleas</b>	939			
<i>Ctenocephalides felis</i>	393	1-11	-	187
<i>C. canis</i>	526	1-12	-	195
<i>Pulex irritans</i>	20	1-4	-	15

<sup>1</sup>Abbreviations: n, nymph; l, larva

**Table 2.** Real-time PCR and sequencing results of *Rickettsia* spp. in hard ticks (Ixodidae) and fleas (Ctenocephalidae, Pulicidae). Except for *Ixodes ricinus* and *Ctenocephalides felis*, only samples negative for the 23S rRNA gene were evaluated in the *gltA* gene TaqMan assay.

	Sample source	TaqMan assay to detect <i>Rickettsia helvetica</i> 23S rRNA gene (positive/tested samples)	TaqMan assay to detect <i>Rickettsia</i> spp. <i>gltA</i> gene (positive/tested samples)	Results of partial sequencing of the <i>gltA</i> gene (sequence identity: number and sex of sample source)
<b>Ticks</b>	<i>Ixodes ricinus</i>	126/136	9/58	<i>R. monacensis</i> (100%: 2♂)
	<i>Dermacentor marginatus</i>	0/55	48/55	<i>R. slovacca</i> (100%: 1♂, 4♀)
	<i>D. reticulatus</i>	0/70	56/70	<i>R. raoultii</i> (99.7%: 1♂, 4♀)
	<i>Haemaphysalis inermis</i>	63/64	1/1	Unknown <i>Rickettsia</i> sp. designated ' <i>Candidatus R. hungarica</i> ' (1♂)
	<i>H. concinna</i>	1/53	1/52	' <i>Candidatus R. kotlanii</i> ' (99.7%: 1♂)
	<i>H. punctata</i>	1/9	0/8	-
<b>Fleas</b>	<i>Ctenocephalides felis</i>	2/20	1/20	<i>R. felis</i> (99.2%: 1)
	<i>Ct. canis</i>	0/17	0/17	-
	<i>Pulex irritans</i>	0/15	1/15	<i>Rickettsia</i> sp. RF2125 (100%: 1)