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Title	Isolation of Rickettsia, Rickettsiella, and Spiroplasma from Questing Ticks in Japan Using Arthropod Cells
Author(s)	Thu, May June; Qiu, Yongjin; Kataoka-Nakamura, Chikako et al.
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26 Hokkaido University,
27 Sapporo 060-0818, Japan
28 Tel: +81-(0)11-706-5196
29 Fax: +81-(0)11-706-5196
30 *E-mail address:* ryo.nakao@vetmed.hokudai.ac.jp
31
32 MJT: winterzun@czc.hokudai.ac.jp
33 YQ: yongjin_qiu@czc.hokudai.ac.jp
34 CK: cnakamura@mail.biken.or.jp
35 CS: sugimoto@czc.hokudai.ac.jp
36 KK: kenkata@vetmed.hokudai.ac.jp
37 NI: isoda@czc.hokudai.ac.jp
38 RN: ryo.nakao@vetmed.hokudai.ac.jp
39
40 **Running title: Bacterial isolation using arthropod cells**
41

42 **Abstract**

43 Ticks are blood sucking ectoparasites that transmit zoonotic pathogens to
44 humans and animals. Ticks harbour not only pathogenic microorganisms, but also
45 endosymbionts. Although some tick endosymbionts are known to be essential for the
46 survival of ticks, their roles in ticks remain poorly understood. The main aim of this
47 study was to isolate and characterise tick-borne microorganisms from field-collected
48 ticks using two arthropod cell lines derived from *Ixodes scapularis* embryos (ISE6)
49 and *Aedes albopictus* larvae (C6/36). A total of 170 tick homogenates originating from
50 15 different tick species collected in Japan were inoculated into each cell line. Bacterial
51 growth was confirmed by PCR amplification of 16S ribosomal DNA (rDNA) of
52 eubacteria. During the 8 weeks observation period, bacterial isolation was confirmed
53 in 14 and 4 samples using ISE6 and C6/36 cells, respectively. The sequencing analysis
54 of the 16S rDNA PCR products indicated that they were previously known tick-borne
55 pathogens/endosymbionts in three different genera; *Rickettsia*, *Rickettsiella*, and
56 *Spiroplasma*. These included 4 previously validated rickettsial species namely
57 *Rickettsia asiatica* (n = 2), *Rickettsia helvetica* (n = 3), *Rickettsia monacensis* (n = 2),
58 and *Rickettsia tamurae* (n = 3) and one uncharacterised genotype *Rickettsia* sp. LON
59 (n = 2). Four isolates of *Spiroplasma* had the highest similarity with previously
60 reported *Spiroplasma* isolates; *Spiroplasma ixodetis* obtained from ticks in North
61 America and *Spiroplasma* sp. Bratislava 1 obtained from *Ixodes ricinus* in Europe,
62 while two isolates of *Rickettsiella* showed 100% identity with *Rickettsiella* sp. detected
63 from *Ixodes uriae* at Grimsey Island in Iceland. To the best of our knowledge, this is
64 the first report on successful isolation of *Rickettsiella* from ticks. The isolates obtained
65 in the present study can be further analysed to evaluate their pathogenic potential in
66 animals and their roles as symbionts in ticks.

67 *Keywords:* Arthropods, Isolation, *Rickettsia*, *Rickettsiella*, *Spiroplasma*, Symbionts

Introduction

Ticks are important vectors among blood sucking ectoparasites that transmit various zoonotic pathogens to humans and animals through their bite. Ticks harbour not only pathogenic microorganisms of veterinary and medical importance (Jongejan and Uilenberg 2004) but also several endosymbionts of the genera *Coxiella*, *Francisella*, and *Rickettsia* (Paddock et al. 2004, Ahantarig et al. 2013). The recent development of deep sequencing technologies has enabled high-throughput screening of pathogens and symbionts in ticks and expanded our knowledge on the diversity of microorganisms harboured by ticks (Nakao et al. 2013, Qiu et al. 2014, Kurilshikov et al. 2015). Although these studies have led to the discovery of several previously unexpected or poorly characterised microorganisms, it is challenging to evaluate their roles in ticks and their pathogenic potential to animals solely based on their partial genome sequences.

In Japan, several tick-borne human diseases have been recognised. Until the recent emergence of severe fever with thrombocytopenia syndrome (Takahashi et al. 2014) and the re-emergence of tick-borne encephalitis (Yoshii et al. 2017), most cases of tick-borne human diseases have been associated with bacterial infections. In particular, Japanese spotted fever caused by *Rickettsia japonica* is the most common tick-borne human diseases with hundreds of cases reported annually (National Institute of Infectious Diseases 2017). Several other rickettsioses caused by *Rickettsia heilongjiangensis*, *Rickettsia helvetica*, and *Rickettsia tamurae* have also been reported to date (Noji et al. 2005, Ando et al. 2010, Imaoka et al. 2011). The etiological agents of these rickettsial diseases were isolated from patients and ticks primarily using L929 mouse fibroblast cells, (Uchida et al. 1992, Fournier et al. 2002, Fujita et al. 2006,

92 Mahara 2006, Ando et al. 2010, Andoh et al. 2014). However, none of these studies
93 have used arthropod cells for isolation of tick-borne pathogens in Japan.

94 At present, tick cell lines are indispensable tools to study the interaction
95 between ticks and tick-borne microorganisms including pathogens and symbionts *in*
96 *vitro* (Bell-Sakyi et al. 2007, Bell-Sakyi et al. 2012). The cells have also been
97 successfully used for isolating and propagating a number of tick-borne
98 microorganisms (Bell-Sakyi et al. 2007, Bell-Sakyi et al. 2015). For example, the
99 previously unculturable *Borrelia lonestari* was isolated and propagated for the first
100 time in a tick cell line (Varela et al. 2004). Similarly, other tick-borne pathogens from
101 genera such as *Anaplasma* and *Ehrlichia* have been successfully cultivated and
102 maintained in tick cell lines (Munderloh et al. 2003, Zweygarth et al. 2013).

103 The main aim of this study was to isolate and characterise tick-borne
104 microorganisms from field-collected ticks using two arthropod cell lines derived from
105 *Ixodes scapularis* embryo (ISE6) and *Aedes albopictus* larvae (C6/36). Our approach
106 led to the isolation of four previously validated rickettsial species, one uncharacterised
107 rickettsial genotype and two tick endosymbionts, *Rickettsiella* and *Spiroplasma*.

108

109 **Materials and Methods**

110 ***Tick samples***

111 This study employed unfed ticks collected in 11 different prefectures by a
112 flagging method between 2013 and 2015 (Table 1). Tick species were identified
113 morphologically under a stereomicroscope using standard keys (Yamaguti et al. 1971,
114 Nakao et al. 1992). The samples included fifteen different tick species; *Amblyomma*
115 *testudinarium* (n = 10), *Dermacentor taiwanensis* (n = 3), *Haemaphysalis concinna* (n
116 = 3), *Haemaphysalis flava* (n = 8), *Haemaphysalis formosensis* (n = 15),

117 *Haemaphysalis hystricis* (n = 24), *Haemaphysalis japonica* (n = 9), *Haemaphysalis*
118 *kitaokai* (n = 3), *Haemaphysalis longicornis* (n = 18), *Haemaphysalis megaspinosa* (n
119 = 27), *Ixodes monospinosus* (n = 7), *Ixodes nipponensis* (n = 2), *Ixodes ovatus* (n = 9),
120 *Ixodes pavlovskyi* (n = 2) and *Ixodes persulcatus* (n = 30). After identifying tick species,
121 ticks were washed with 70% ethanol and sterile PBS, then homogenised in 100 µl of
122 high-glucose Dulbecco's Modified Eagle medium (DMEM Gibco, Life Technologies,
123 Carlsbad, CA, USA) using a Micro Smash™ MS100R (TOMY, Tokyo, Japan). Half
124 of the homogenate was subjected to DNA extraction using a blackPREP Tick
125 DNA/RNA Kit (Analytikjena, Germany), while the other half was kept at -80°C and
126 used for this study. A total of 170 tick homogenates including 158 from single unfed
127 ticks (adult or nymph) and 12 from nymphal pools (5 to 24 nymphs/pool) were
128 included in the present study.

129

130 ***Maintenance of cell lines***

131 ISE6 cells, originally reported by Kurtti et al (1996) and received from the
132 CEH Institute of Virology and Environmental Microbiology (Oxford, UK), were
133 grown in L-15B medium supplemented with 10% foetal bovine serum, and 5%
134 tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO, USA) at 32°C as described
135 previously (Munderloh and Kurtti 1989), except that 0.1% bovine lipoprotein
136 concentrate was not included in the culture medium. C6/36 cells, purchased from the
137 American Type Culture Collection (No. CRL-1660), were grown in Minimum
138 Essential Medium (MEM, Gibco) supplemented with 10% foetal bovine serum, 2%
139 MEM Non-essential amino acids (Gibco), 1% Sodium Pyruvate 100 mM (Gibco), and
140 1% L-glutamine (Gibco) at 28°C in a humidified atmosphere of 5% CO₂ in air.

141

142 ***Co-culture with tick homogenates***

143 ISE6 and C6/36 cells were seeded in 24-well culture plates and incubated
144 overnight. On the following day, 5 µl of each tick homogenate was inoculated into
145 separate wells of both cell lines. Culture medium was changed every three days for
146 C6/36 cells and once a week for ISE6 cells. At 2 weeks post-inoculation (pi), 100 µl
147 of culture suspension was passaged into new wells containing uninfected cells. Second
148 and third passages were conducted in the same way as first at 4 and 6 weeks pi,
149 respectively. At 8 weeks pi, the experiment was terminated. All the bacterial isolates
150 obtained in this study were preserved at -80°C for downstream analysis. Cell
151 morphology was observed daily under an inverted microscope to detect cytopathic
152 effects presumably caused by bacterial infections. When contamination of fungi or
153 environmental bacteria was observed, the contaminated wells were sterilised with 10%
154 hypochlorous acid for more than 10 min to prevent the spread of contamination to the
155 neighbouring wells.

156

157 ***PCR***

158 When the cells showed sign of bacterial infection, DNA was extracted from
159 100 µl of parent culture suspension and/or first subculture using a Wizard Genomic
160 DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's
161 instructions. In addition, cell suspensions from all wells at 4 and 8 weeks pi (from first
162 and third subcultures, respectively) were subjected to DNA extraction to detect
163 possible bacterial infection whether or not morphological changes were seen in cells.
164 PCR was conducted using the primers, fD1 and Rp2 to amplify eubacterial 16S
165 ribosomal DNA (rDNA) (Weisburg et al. 1991). In order to characterise rickettsial
166 isolates, three additional genes were amplified: citrate synthase gene (*gltA*) (Regnery

et al. 1991), 190-kDa outer membrane gene (*ompA*) (Roux and Raoult, 2000), and 120-kDa outer membrane protein gene (*ompB*) (Gaowa et al. 2013). All PCR reactions were conducted in a 25 µl-reaction mixture containing 2.5 µl of 10 × KOD Plus Neo PCR Buffer, 0.5 µl of a high-fidelity KOD-Plus-Neo DNA polymerase (Toyobo), 200 nM of each primer, and 1.0 µl of template DNA. PCR conditions were as follows: 40 cycles of denaturation (94°C, 15 sec), annealing (55°C for *gltA*, *ompA*, and 16S rDNA and 48°C for *ompB*, 30 sec) and extension (68°C, 30 sec for *gltA*, *ompA*, and *ompB* and 90 sec for 16S rDNA). For some samples, we conducted TA-cloning using the pGEM-T vector (Promega, Madison, WI) as described previously (Nakao et al. 2013). The experimental procedures were approved by the Hokkaido University Safety Committee on Genetic Recombination Experiments (No. 2017-046). All the primer information is available in Table 2. All negative control containing sterile water instead of template DNA was included in all PCR assays.

180

181 ***Real-time PCR***

Real-time PCR to detect the *gltA* gene of spotted fever group and typhus group rickettsiae was conducted using the primers and probe shown in Table 2. Reactions mixtures were prepared using THUNDERBIRD Probe qPCR Mix (Toyobo, Osaka, Japan) and the reactions were carried out in a C1000 Thermal Cycler with a CFX96 Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA) as described previously (Nakao et al. 2013).

188

189 ***Sequencing and data analysis***

All amplified PCR products were purified using a NucleoSpin Gel and PCR Clean Up Kit (Takara Bio Inc.) and sequenced using the BigDye Terminator version

192 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). To sequence
193 16S rDNA PCR products, sequencing primers were newly designed in the present
194 study (Table 2). The purified sequencing products were analysed on an ABI Prism
195 3130xl Genetic Analyzer Kit (Applied Biosystems) according to the manufacturers'
196 instructions. The sequences from this study were submitted to the DNA Data Bank of
197 Japan (<http://www.ddbj.nig.ac.jp>) under accession numbers (16S rDNA: LC388759-
198 LC388776; *gltA*: LC388777-LC388788; *ompA*: LC388789-LC388795; and *ompB*:
199 LC388796-LC388807). Sequenced data were aligned using ATGC software version
200 6.0.4. Phylogenetic analyses were conducted by a maximum likelihood method using
201 MEGA7 version 7.0.18 (Kumar et al. 2016). Bootstrap values were obtained with
202 1,000 replicates.

203

204 **Results**

205 ***Real-time PCR***

206 Among 170 tick homogenates examined, 114 tested positive for *Rickettsia*
207 infections by *gltA* real-time PCR (Table 1). We used both negative and *Rickettsia*-
208 positive samples for the cell culture isolation experiments.

209

210 ***Isolation results***

211 During the 8 weeks observation period, we confirmed bacterial isolation in
212 14 and 4 samples using ISE6 and C6/36 cells, respectively (Table 3). Ten isolates (9
213 from ISE6 and 1 from C6/36) were obtained in the parent cultures, while 8 isolates (5
214 from ISE6 and 3 from C6/36) were obtained in the first subcultures (Figure 1). The
215 sequencing analysis of the 16S rDNA PCR products indicated that they were
216 previously known tick-borne bacteria in three different genera; *Rickettsia*, *Rickettsiella*,

217 and *Spiroplasma* (Table 4). Although a further 11 and 4 samples in ISE6 and C6/36
218 cells, respectively, also showed bacterial growth in the well, sequencing analysis
219 indicated the growth of environmental bacteria such as *Bacillus* spp., *Pseudomonas*
220 spp., and *Mycobacterium* spp. (data not shown). Fungal infections developed in 75
221 ISE6 and 57 C6/36 wells; the remaining 77 and 98 wells respectively did not yield any
222 isolates (Table 3). Although 4 rickettsial and 3 spiroplasmal isolates did not show any
223 cytopathic effects in the infected cells, their infections were detected by PCR
224 conducted at 28 days pi (Figure 1).

225

226 ***Rickettsia* isolation**

227 Twelve isolates of *Rickettsia* (11 in ISE6 and one in C6/36) were obtained
228 from different tick homogenates (Table 3). The amplification of *gltA* and *ompB* genes
229 was successful in all rickettsial isolates, while the *ompA* gene was amplified only from
230 seven isolates (Table 4). Based on the phylogenetic analysis of each rickettsial gene,
231 the 12 isolates were identified as 4 previously validated species, *Rickettsia asiatica* (n
232 = 2), *R. helvetica* (n = 3), *Rickettsia monacensis* (n = 2), *R. tamurae* (n = 3), and
233 *Rickettsia* sp. LON, an unnamed rickettsial agent previously isolated from *H.*
234 *longicornis* in Japan (Fujita 2008), (n = 2) (Figure 2 and Supplementary Table S1).
235 There was a complete correspondence between rickettsial species/genotype and tick
236 species of origin; *R. asiatica*, *R. helvetica*, *R. monacensis*, *R. tamurae*, and *Rickettsia*
237 sp. LON were isolated from *I. ovatus*, *I. persulcatus*, *I. nipponensis*, *A. testudinarium*,
238 and *H. longicornis*, respectively. A cytopathic effect was observed in *R. helvetica*-, *R.*
239 *monacensis*-, and *R. tamurae*-infected cells at 6, 14 and 10 days pi, while there was no
240 obvious morphological damage observed in *R. asiatica*- and *Rickettsia* sp. LON-
241 infected cells (Figure 1).

242

243 ***Rickettsiella* isolation**

244 *Rickettsiella* was isolated from a homogenate of *H. concinna* collected in
245 Hokkaido using both ISE6 and C6/36 cells (Table 1). A cytopathic effect was observed
246 at 6 and 13 days pi in ISE6 and C6/36 cells, respectively (Figure 1). The sequences of
247 16S rDNA of *Rickettsiella* obtained from two cell lines were identical and showed
248 100% identity with *Rickettsiella* sp. detected in *Ixodes uriae* from Grimsey Island in
249 Iceland (GenBank No. KT697673). A phylogenetic analysis showed that our isolates
250 formed a cluster with *Rickettsiella* spp. detected in pea aphids (Figure 3 and
251 Supplementary Table S1).

252

253 ***Spiroplasma* isolation**

254 Four *Spiroplasma* isolates were obtained (2 each from ISE6 and C6/36)
255 (Table 3). Since two isolates were obtained from the same tick homogenate using
256 different cell lines, four isolates originated from three tick homogenates: *I.*
257 *monospinosus*, *I. persulcatus*, and *H. kitaokai* collected from Yamagata, Hokkaido and
258 Fukushima prefectures, respectively (Table 1). Only one isolate (147_ISE6) showed a
259 cytopathic effect in ISE6 cells at 23 days pi (Figure 1). Among 4 isolates, two sequence
260 types of 16S rDNA had two base pair (bp) differences in their sequences. One sequence
261 type was obtained from two isolates (135_ISE6 and 135_C6/36) from *I. monospinosus*
262 and one isolate (1033_ISE6) from *H. kitaokai*, while the other was from *I. persulcatus*
263 (147_ISE6). Both sequences showed the highest identities (1443/1444 bp and
264 1441/1444 bp) with 16S rDNA from *Spiroplasma* sp. detected from *Fannia manicata*
265 (little housefly) (GenBank No. AY569829). In a phylogenetic analysis, our isolates
266 were clustered together with 16S rDNA of two tick-derived *Spiroplasma* isolates;

267 *Spiroplasma ixodetis* (GenBank No. NR104852) obtained from *Ixodes pacificus* and
268 *Spiroplasma* sp. Bratislava 1 (GenBank No. KP967685) obtained from *I. ricinus*, and
269 several *Spiroplasma* spp. detected from various arthropods (Figure 4 and
270 Supplementary Table S1).

271

272 **Discussion**

273 We achieved the first isolation and propagation of *R. monacensis* from Japan
274 using ISE6 cells. This rickettsial agent has not been officially reported in Japan; in a
275 recent nationwide survey we found sequences of *R. monacensis* in *I. nipponensis* with
276 high infection rates (Thu et al. submitted). *R. monacensis* was first isolated from *Ixodes*
277 *ricinus* collected in Germany using ISE6 cells (Simser et al. 2002), where the authors
278 detected a cytopathic effect after the third passage (5 months). In our experiment, we
279 observed a cytopathic effect as early as 14 days after the inoculation in both ISE6 and
280 C6/36 cells (Figure 1). Although the reason for this difference is not clear, it may
281 suggest that phenotypes under *in vitro* culture conditions differ between *R. monacensis*
282 strains. *R. monacensis* has been associated with a human rickettsiosis presenting
283 Mediterranean spotted fever-like symptoms (Jado et al. 2007, Madeddu et al. 2012,
284 Kim et al. 2017). The present study reconfirmed the presence of this rickettsial agent
285 in Japan and highlighted the necessity for further investigation of the clinical cases it
286 may cause.

287 In addition to *R. monacensis*, we isolated 3 previously validated rickettsial
288 species, *R. asiatica*, *R. helvetica*, *R. tamurae*, and one uncharacterised genotype
289 *Rickettsia* sp. LON using ISE6 cells, among which only *R. asiatica* was also isolated
290 in C6/36 cells (Table 4). Although the C6/36 cells inoculated with the tick
291 homogenates from which *Rickettsia* were isolated using ISE6 cells were tested for

ricketsial infections by PCR at 8 weeks pi, there were no positive amplicons. However, when the isolates of *R. monacensis* and *R. helvetica* obtained from ISE6 culture were inoculated into C6/36 cells in our preliminary experiments, their persistent growth in the cells was observed (data not shown). In fact, *R. monacensis* and *R. helvetica* were isolated from *I. ricinus* in Portugal using C6/36 cells in a previous study (Milhano et al. 2010). These results may indicate that lower success rates of rickettial isolation using C6/36 cells is partly attributed to low bacterial burden in the inocula. It is also possible that propagation in ISE6 cells might help the rickettsial isolates adapt to *in vitro* culture conditions using C6/36 cells.

There are only a few reports on the *in vitro* propagation of *Rickettsiella*. For example, *Rickettsiella grylli* isolated from the variegated grasshopper, *Zonocerus variegatus*, was cultured in several cell lines derived from different arthropods (Henry et al. 1986). To the best of our knowledge, this is the first report of the successful isolation of *Rickettsiella* spp. from ticks using ISE6 cells and C6/36 cells. Bacteria within the genus *Rickettsiella* are known to be symbionts of many arthropods (Tsuchida et al. 2010, Leclercq et al. 2011, Iasur-Kruh et al. 2013, Łukasik et al. 2013). In ticks, *Rickettsiella* species have been reported in the genera *Ixodes* and *Ornithodoros* (Duron et al. 2016, Duron et al. 2015, Vilcins et al. 2009, Kurtti et al. 2002). A recent metagenomic approach based on 16S rDNA amplicons also showed the presence of *Rickettsiella* in the genus *Haemaphysalis* (Khoo et al. 2016). The sequence analysis of 16S rDNA revealed that the *Rickettsiella* sp. isolated from *H. concinna* showed 100% identity with a *Rickettsiella* endosymbiont detected in *I. uriae* collected from a seabird in Iceland (Figure 3). These facts may support the hypothesis that *Rickettsiella* has a wide geographical distribution in ticks and is maintained by horizontal transfer between arthropod species as previously suggested (Duron et al.

2016). However, a more detailed analysis such as whole genome comparison between *Rickettsiella* spp. found in different arthropod hosts is essential to prove this hypothesis. The role of *Rickettsiella* in ticks is totally unknown; however, the lines of evidence from other arthropods indicated an effect on the survival of their host arthropods (Tsuchida et al. 2010, Łukasik et al. 2013). The isolate obtained in the present study might be useful to further investigate potential roles of *Rickettsiella* in ticks.

We obtained four spiroplasmal isolates from three tick species: *I. monospinosus*, *I. persulcatus*, and *H. kitaokai*. The isolates obtained from *I. monospinosus* and *H. kitaokai* had completely identical 16S rDNA sequences and all spiroplasmal isolates in the present study made one clade with previously reported *Spiroplasma* species which were detected in a variety of arthropods including ticks, ladybirds, plant hoppers and mealybugs (Figure 4). These findings may suggest that *Spiroplasma* is maintained by horizontal transfer between different arthropod species as suggested for *Rickettsiella*. This hypothesis should be explored in future studies. Most members of *Spiroplasma* are symbionts in arthropods and some of them are known to be beneficial to their hosts for example, by protecting from fungal or parasitic infections (Łukasik et al. 2013, Yadav et al. 2018, Xie et al. 2014). In some arthropods, *Spiroplasma* species are pathogenic and cause gender-ratio distortions known as a male-killing effect in *Drosophila* (Harumoto et al. 2014). However, a recent study conducted on a nidicolous tick *Ixodes arboricola* did not find any association between female-biased sex ratios and infections of six maternally inherited bacteria including *Spiroplasma* (Van Oosten et al. 2018). Moreover, *Spiroplasma mirum*, an isolate obtained from the rabbit tick *Haemaphysalis leporispalustris* in the USA (Tully et al. 1982), was shown to have potentially pathogenic properties; for example, *S. mirum* was virulent for chick embryos and induced cataracts or lethal brain infections when

introduced intracerebrally into experimental animals such as suckling rats and rabbits (Tully et al. 1977). Collectively, further biological characterisation of *Spiroplasma* detected in the present study is necessary to understand their roles in ticks and potential risks for human and animal health.

In the present study, a number of wells were contaminated with bacterial or fungal infections (79 wells of ISE6 culture and 78 wells of C6/36 culture) (Table 3), despite the fact that tick surface was washed with 70% ethanol and sterile PBS. This result highlights the necessity of using additional chemicals to sterilize the tick surface, especially those effective against fungal infections. Another possible option might be to use only internal organs for bacterial isolation by dissecting ticks.

Conclusion

In conclusion, the present study employed two arthropod cell lines to isolate pathogenic and symbiotic microorganisms from questing ticks collected in the field. Although the technique needs to be improved to reduce contaminations by fungal infections, the use of arthropod cell lines seems promising to expand our knowledge on microorganisms in ticks. The isolates obtained in the present study are useful materials to further analyze their pathogenic potential in vertebrate animals and their roles as symbionts in ticks.

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369

370 **Disclosure Statement**

371 The authors declare no conflict of interest.

372

373 **References**

374 Ahantarig A, Trinachartvanit W, Baimai V, Grubhoffer L. Hard ticks and their
375 bacterial endosymbionts (or would be pathogens). *Folia Microbiol* 2013;58:419-
376 428.

377 Ando S, Kurosawa M, Sakata A, Fujita H, et al. Human *Rickettsia heilongjiangensis*
378 infection, Japan. *Emerging Infect Dis* 2010;16:1306-1308.

379 Andoh M, Ogasawara Y, Sakata A, Ito T, et al. Isolation of the rickettsial agent
380 genetically similar to *Candidatus Rickettsia kotlanii*, from *Haemaphysalis*
381 *megaspinosus* in Japan. *Vector Borne Zoonotic Dis* 2014;14:681-684.

382 Bell-Sakyi L, Kohl A, Bente DA, Fazakerley JK. Tick cell lines for study of Crimean-
383 Congo hemorrhagic fever virus and other arboviruses. *Vector Borne Zoonotic Dis*
384 2012;12:769-781.

385 Bell-Sakyi L, Palomar AM, Kazimirova M. Isolation and propagation of a
386 *Spiroplasma* sp. from Slovakian *Ixodes ricinus* ticks in *Ixodes* spp. cell lines. *Ticks*
387 *Tick Borne Dis* 2015;6: 601-606.

388 Bell-Sakyi L, Zweggarth E, Blouin EF, Gould EA, et al. Tick cell lines: tools for tick
389 and tick-borne disease research. *Trends Parasitol* 2007;23:450-457.

390 Duron O, Noël V, McCoy KD, Bonazzi M, et al. The recent evolution of a maternally-
 391 inherited endosymbiont of ticks led to the emergence of the Q fever pathogen,
 392 *Coxiella burnetii*. PLoS Pathog 2015;11:e1004892.

393 Duron O, Cremaschi J, McCoy KD. 2016. The high diversity and global distribution
 394 of the intracellular bacterium *Rickettsiella* in the polar seabird tick *Ixodes uriae*.
 395 Microb Ecol 2016;71:761-770.

396 Fournier PE, Fujita H, Takada N, Raoult D. Genetic identification of rickettsiae
 397 isolated from ticks in Japan. J Clin Microbiol 2002;40:2176-2181.

398 Fujita H, Fournier PE, Takada N, Saito T, et al. *Rickettsia asiatica* sp. nov., isolated in
 399 Japan. Int J Syst Evol Microbiol 2006;56:2365-2368.

400 Fujita H. Cell culture system for isolation of disease agents: 15 years of experience in
 401 Ohara Research Laboratory. Annu Rep Ohara Hosp. 2008;48:21-42.

402 Gaowa, Ohashi N, Aochi M, Wuritu D, et al. Rickettsiae in ticks, Japan, 2007–2011.
 403 Emerg Infect Dis 2013;19:338-340.

404 Harumoto T, Anbutsu H, Fukatsu T. Male-killing *Spiroplasma* induces sex-specific
 405 cell death via host apoptotic pathway. PLoS Pathog 2014;10:e1003956.

406 Henry JE, Streett DA, Oma EA, Goodwin RH. Ultrastructure of an isolate of
 407 *Rickettsiella* from the African grasshopper *Zonocerus variegatus*. J Invertebr Pathol
 408 1986;47:203-213.

409 Iasur-Kruh L, Weintraub PG, Mozes-Daube N, Robinson WE, et al. Novel
 410 *Rickettsiella* bacterium in the leafhopper *Orosius albicinctus* (Hemiptera:
 411 Cicadellidae). Appl Environ Microbiol 2013;79:4246-4252.

412 Imaoka K, Kaneko S, Tabara K, Kusatake K, et al. The first human case of *Rickettsia*
413 *tamurae* infection in Japan. *Case Rep Dermatol* 2011;3:68-73.

414 Jado I, Oteo JA, Aldámiz M, Gil H, et al. *Rickettsia monacensis* and human disease,
415 Spain. *Emerging Infect Dis* 2007;13:1405-1407.

416 Jongejan F, Uilenberg G. The global importance of ticks. *Parasitology* 2004;129:S3-
417 S14.

418 Khoo JJ, Chen F, Kho KL, Shanizza AIA, et al. Bacterial community in
419 *Haemaphysalis* ticks of domesticated animals from the Orang Asli communities in
420 Malaysia. *Ticks Tick Borne Dis* 2016;7:929-937.

421 Kim YS, Choi YJ, Lee KM, Ahn KJ, et al. First isolation of *Rickettsia monacensis*
422 from a patient in South Korea. *Microbiol Immunol* 2017;61:258-263.

423 Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis
424 version 7.0 for bigger datasets. *Mol Biol Evol* 2016;33:1870-1874.

425 Kurilshikov A, Livanova NN, Fomenko NV, Tupikin AE, et al. Comparative
426 metagenomic profiling of symbiotic bacterial communities associated with *Ixodes*
427 *persulcatus*, *Ixodes pavlovskyi* and *Dermacentor reticulatus* ticks. *PLoS ONE*
428 2015;10:e0131413.

429 Kurti TJ, Munderloh UG, Andreadis TG, Magnarelli LA, et al. Tick cell culture
430 isolation of an intracellular prokaryote from the tick *Ixodes scapularis*. *J Inv Path*
431 1996;67:318-321.

432 Kurti TJ, Palmer AT, Oliver JH. *Rickettsiella*-like bacteria in *Ixodes woodi* (Acari:
433 Ixodidae). *J Med Entomol* 2002;39:534-540.

434 Leclerque A, Kleespies RG, Ritter C, Schuster C, et al. Genetic and electron-
 435 microscopic characterization of '*Rickettsiella agriotidis*', a new *Rickettsiella*
 436 pathotype associated with wireworm, *Agriotes* sp. (Coleoptera: Elateridae). Curr
 437 Microbiol 2011;63:158-163.

438 Łukasik P, Asch MV, Guo H, Ferrari J. Unrelated facultative endosymbionts protect
 439 aphids against a fungal pathogen. Ecol Lett 2013;16:214-218.

440 Madeddu G, Mancini F, Caddeo AC, Babudieri AS, et al. *Rickettsia monacensis* as
 441 cause of Mediterranean spotted fever-like illness, Italy. Emerging Infect Dis
 442 2012;18:702-704.

443 Mahara F. Rickettsioses in Japan and the Far East. Ann N Y Acad Sci 2006;1078:60-
 444 73.

445 Milhano N, de Carvalho IL, Alves AS, Arroube S, et al. Coinfections of *Rickettsia*
 446 *slovaca* and *Rickettsia helvetica* with *Borrelia lusitaniae* in ticks collected in a
 447 Safari Park, Portugal. Ticks Tick Borne Dis 2010;1:172-177.

448 Munderloh UG, Kurtti TJ. Formulation of medium for tick cell culture. Exp Appl
 449 Acarol 1989;7:219-229.

450 Munderloh UG, Tate CM, Lynch MJ, Howerth EW, et al. Isolation of an *Anaplasma*
 451 sp. organism from white-tailed deer by tick cell culture. J Clin Microbiol
 452 2003;41:4328-4335.

453 National Institute of Infectious Diseases. Scrub typhus and Japanese spotted fever in
 454 Japan 2007–2016. IASR (Infectious Agents Surveillance Report) 2017; 38:109-112.
 455 [accessed 2018 June 4]. [https://www.niid.go.jp/niid/ja/tsutsugamushi-](https://www.niid.go.jp/niid/ja/tsutsugamushi-m/tsutsugamushi-iasrtpc/7324-448t.html)
 456 [m/tsutsugamushi-iasrtpc/7324-448t.html](https://www.niid.go.jp/niid/ja/tsutsugamushi-m/tsutsugamushi-iasrtpc/7324-448t.html) (in Japanese).

457 Nakao M, Miyamoto K, Kitaoka S. A new record of *Ixodes pavlovskgi* Pomerantzev
458 from Hokkaido, Japan (Acari: Ixodidae). Eisei Dobutsu 1992;43:229-234.

459 Nakao R, Qiu Y, Igarashi M, Magona JW, et al. High prevalence of spotted fever group
460 rickettsiae in *Amblyomma variegatum* from Uganda and their identification using
461 sizes of intergenic spacers. Ticks Tick Borne Dis 2013;4:506-512.

462 Noji Y, Takada N, Ishiguro F, Fujino S, et al. The first reported case of spotted fever
463 in Fukui Prefecture, the Northern part of Central Japan. Jpn J Infect Dis
464 2005;58:112-114.

465 Paddock CD, Sumner JW, Comer JA, Zaki SR, et al. *Rickettsia parkeri*: a newly
466 recognized cause of spotted fever rickettsiosis in the United States. Clin Infect Dis.
467 2004;38:805-811.

468 Qiu Y, Nakao R, Ohnuma A, Kawamori F, et al. Microbial population analysis of the
469 salivary glands of ticks; A possible strategy for the surveillance of bacterial
470 pathogens. PLoS ONE 2014;9:e103961.

471 Regnery RL, Spruill CL, Plikaytis BD. Genotypic identification of rickettsiae and
472 estimation of intraspecies sequence divergence for portions of two rickettsial genes.
473 J Bacteriol 1991;173:1576-1589.

474 Roux V, Fournier PE, Raoult D. Differentiation of spotted fever group rickettsiae by
475 sequencing analysis of restriction fragment length polymorphism of PCR-amplified
476 DNA of the gene encoding the protein rOmpA. J Clin Microbiology 1996;34:2058-
477 2065.

478 Roux V, Raoult D. Phylogenetic analysis of members of the genus *Rickettsia* using the
479 gene encoding the outer-membrane protein rOmpB (*ompB*). Int J Syst Evol
480 Microbiol 2000;50:1449-1455.

481 Simser JA, Palmer AT, Fingerle V, Wilske B, et al. *Rickettsia monacensis* sp. nov., a
482 spotted fever group *Rickettsia*, from ticks (*Ixodes ricinus*) collected in a European
483 City Park. Appl Environ Microbiol 2002;68:4559-4566.

484 Stenos J, Graves SR, Unsworth NB. A highly sensitive and specific real-time PCR
485 assay for the detection of spotted fever and typhus group rickettsiae. Am J Trop
486 Med Hyg 2005;73:1083-1085.

487 Takahashi T, Maeda K, Suzuki T, Ishido A, et al. The first identification and
488 retrospective study of severe fever with thrombocytopenia syndrome in Japan. J
489 Infect Dis 2014;209:816-827.

490 Tsuchida T, Koga R, Horikawa M, Tsunoda T, et al. Symbiotic bacterium modifies
491 aphid body color. Science 2010;330:1102-1104.

492 Tully JG, Whitcomb RF, Clark HF, Williamson DL. Pathogenic mycoplasmas:
493 cultivation and vertebrate pathogenicity of a new *Spiroplasma*. Science
494 1977;195:892-894.

495 Tully JG, Whitcomb RF, Rose DL, Bové JM. *Spiroplasma mirum*, a new species from
496 the rabbit tick (*Haemaphysalis leporispalustris*). Int J Syst Bacteriol 1982;32:92-
497 100.

498 Uchida T, Uchiyama T, Kumano K, Walker DH. *Rickettsia japonica* sp. nov., the
499 etiological agent of spotted fever group rickettsiosis in Japan. Int J Syst Bacteriol
500 1992;42:303-305.

501 Van Oosten RA, Duron O, Heylen DJA. Sex ratios of the tick *Ixodes arboricola* are
502 strongly female-biased, but there are no indications of sex-distorting bacteria. Ticks
503 Tick Borne Dis 2018;9:307-313.

504 Varela AS, Luttrell MP, Howerth EW, Moore VA, et al. First culture isolation of
505 *Borrelia lonestari*, putative agent of southern tick associated rash illness. J Clin
506 Microbiol 2004;42:1163-1169.

507 Vilcins IM, Old JM, Deane E. Molecular detection of *Rickettsia*, *Coxiella* and
508 *Rickettsiella* DNA in three native Australian tick species. Exp Appl Acarol
509 2009;49:229-242.

510 Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification
511 for phylogenetic study. J Bacteriol 1991;173:697-703.

512 Yamaguti N, Tipton VJ, Keegan HL, Toshioka S. Ticks of Japan, Korea and the
513 Ryushu islands. Brigham Young University Science Bulletin, Biological Series 15,
514 1971.

515 Yoshii K, Song JY, Park SB, Yang J, et al. Tick-borne encephalitis in Japan, Republic
516 of Korea and China. Emerg Microbes Infect 2017;6:e82.

517 Yadav S, Frazer J, Banga A, Pruitt K, et al. Endosymbiont-based immunity in
518 *Drosophila melanogaster* against parasitic nematode infection. PLoS One
519 2018;13:e0192183.

520 Xie J, Butler S, Sanchez G, Mateos M. Male killing *Spiroplasma* protects *Drosophila*
521 *melanogaster* against two parasitoid wasps. Heredity 2014;11:399-408.

522 Zwegarth E, Schol H, Lis K, Cabezas CA, et al. In vitro culture of a novel genotype
523 of *Ehrlichia* sp. from Brazil. Transbound Emerg Dis 2013;60:86-92.

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527 ***Correspondence address.***

528 Ryo NAKAO

529 Laboratory of Parasitology,

530 Graduate School of Veterinary Medicine,

531 Hokkaido University,

532 Sapporo 060-0818, Japan

533 Tel: +81-(0)11-706-5196

534 Fax: +81-(0)11-706-5196

535 *E-mail address:* ryo.nakao@vetmed.hokudai.ac.jp

**TABLE 1. COLLECTION DETAILS OF TICKS USED FOR BACTERIAL ISOLATION
USING ARTHROPOD CELLS**

Prefecture	Collected year	Tick species	Female	Male	Nymph	Nymphal pool ^a
Hokkaido	2013	<i>H. concinna</i>	-	3(0) ^b	-	-
	2013	<i>H. japonica</i>	5 (0)	1 (1)	-	-
	2013 & 2015	<i>H. megaspinososa</i>	3 (1)	10 (4)	-	5 (5)
	2013	<i>I. ovatus</i>	1(0)	5 (1)	-	-
	2015	<i>I. pavlovskyi</i>	-	2(0)	-	-
	2013, 2014 & 2015	<i>I. persulcatus</i>	20 (18)	8 (5)	-	-
Fukushima	2014	<i>D. taiwanensis</i>	-	1(0)	-	-
	2013	<i>H. flava</i>	-	3 (2)	-	1 (1)
	2014	<i>H. japonica</i>	1 (1)	1 (1)	-	1 (1)
	2014	<i>H. kitaokai</i>	2(0)	1(0)	-	-
	2014	<i>H. megaspinososa</i>	-	-	-	1 (1)
	2014	<i>I. monospinosus</i>	-	1 (1)	-	-
	2014	<i>I. nipponensis</i>	-	1 (1)	-	-
	2014	<i>I. persulcatus</i>	1 (1)	1(0)	-	-
Yamagata	2014	<i>H. flava</i>	1 (1)	-	-	1 (1)
	2013 & 2014	<i>I. monospinosus</i>	5 (5)	1 (1)	-	-
	2013	<i>I. nipponensis</i>	-	1 (1)	-	-
	2014	<i>I. ovatus</i>	1 (1)	1 (1)	-	-
Tochigi	2014	<i>H. flava</i>	-	-	-	1 (1)
Shizuoka	2013	<i>H. longicornis</i>	5 (5)	-	-	-
Nara	2014	<i>H. longicornis</i>	-	-	-	1 (1)
	2014	<i>H. megaspinososa</i>	-	1 (1)	-	1 (1)
Wakayama	2015	<i>A. testudinarium</i>	1 (1)	-	-	-

	2015	<i>D. taiwanensis</i>	1(0)	1(0)	-	-
	2015	<i>H. formosensis</i>	1(0)	-	-	-
	2013	<i>H. hystricis</i>	4 (3)	-	-	-
	2013 & 2015	<i>H. longicornis</i>	3 (2)	8 (8)	1 (1)	-
	2015	<i>H. megaspinosa</i>	1 (1)	-	-	-
	2015	<i>I. ovatus</i>	1(0)	-	-	-
Kumamoto	2015	<i>H. formosensis</i>	4 (1)	3 (2)	-	-
	2015	<i>H. hystricis</i>	1 (1)	2 (1)	-	-
Miyazaki	2013	<i>A. testudinarium</i>	-	-	6 (3)	-
	2013	<i>H. flava</i>	1 (1)	-	-	-
	2013	<i>H. formosensis</i>	1 (1)	2(0)	-	-
	2013	<i>H. megaspinosa</i>	2 (2)	1 (1)	-	-
Kagoshima	2015	<i>A. testudinarium</i>	-	-	2 (1)	-
	2015	<i>H. formosensis</i>	-	4 (1)	-	-
	2015	<i>H. hystricis</i>	5 (4)	12 (11)	-	-
	2013 & 2015	<i>H. megaspinosa</i>	1 (1)	1 (1)	-	-
Okinawa	2014	<i>A. testudinarium</i>	-	-	1 (1)	-
Total			72 (51)	76 (45)	10 (6)	12 (12)

-, this tick species was not collected in the sample year.

^aNymphal pool samples were prepared from a pool of 5 to 24 nymphs.

^bThe number in parentheses indicates the number of samples positive for rickettsiae by *gltA* real-time PCR.

TABLE 2. OLIGONUCLEOTIDE PRIMERS AND PROBE USED IN REAL-TIME AND CONVENTIONAL POLYMERASE CHAIN REACTIONS AND SEQUENCING

Primer	Primer sequence (5'-3')	Target gene	Target organism(s)	Annealing temperature (°C)	Amplicon size (bp)	Reference
CS-F	TCGCAAATGTTACGGTACTTT	Citrate synthase gene (<i>gltA</i>)	Rickettsiae spotted fever group and typhus group	60	74	Stenos et al. (2005)
CS-R	TCGTGCATTTCTTTCCATTGTG					
CS-P	TGCAATAGCAAGAACCGTAGGCTGGATG					
<i>gltA</i> _Fc	CGAACTTACCGCTATTAGAATG	Citrate synthase gene (<i>gltA</i>)	<i>Rickettsia</i> spp.	55	580	Gaowa et al. (2013)
<i>gltA</i> _Rc	CTTTAAGAGCGATAGCTTCAAG					
Rr.190.70p	ATGGCGAATATTTCTCCAAAA	Outer membrane A gene (<i>ompA</i>)	<i>Rickettsia</i> spp.	55	632	Roux et al. (1996)
Rr.190.701n	GTTCCGTTAATGGCAGCATCT					
120-2788	AAACAATAATCAAGGTACTGT	Outer membrane B gene (<i>ompB</i>)	<i>Rickettsia</i> spp.	48	816	Roux and Raoult (2000)
120-3599	TACTTCCGGTTACAGCAAAGT					
fD1	AGAGTTTGATCCTGGCTCAG	16S ribosomal RNA gene (16S rRNA)	Eubacteria	55	about 1500	Weisburg et al. (1991)
Rp2	ACGGCTACCTTGTTACGACTT					
<i>rrs</i> _seq1	AGGCCTTCATCACTCACTCG	16S rRNA	<i>Rickettsia</i> spp.			This study
<i>rrs</i> _seq2	CTACACGCGTGCTACAATGG	16S rRNA	<i>Rickettsia</i> spp., <i>Spiroplasma</i> spp. and <i>Rickettsiella</i> spp.			This study
<i>rrs</i> _seq3	CGTGTCTCAGTCCCAATGTG	16S rRNA	<i>Spiroplasma</i> spp.			This study

TABLE 3. SUMMARY OF BACTERIAL ISOLATION FROM HOMOGENATES OF TICKS COLLECTED IN JAPAN USING TICK (ISE6) AND MOSQUITO (C6/36) CELL LINES

Isolation result	Cell line	
	ISE6	C6/36
<i>Rickettsia</i> and symbionts	14	4
Bacterial contamination	4	11
Fungal contamination	75	57
No isolate	77	98
Total	170	170

TABLE 4. DETAILS OF TICKS FROM WHICH BACTERIAL ISOLATES WERE OBTAINED AND THE RESULTS OF REAL-TIME AND CONVENTIONAL PCRS AMPLIFYING BACTERIAL ISOLATES

Tick ID	Tick species	Stage /Sex	Prefecture	Arthropod cell line		Real-time PCR result ^a		PCR result ^b		
				ISE6	C6/36		16S rDNA	Rickettsial <i>gltA</i>	Rickettsial <i>ompA</i>	Rickettsial <i>ompB</i>
135	<i>I. monospinosus</i>	M	Yamagata	Isolated (<i>Spiroplasma</i>)	Isolated (<i>Spiroplasma</i>)	+	+	NA	NA	NA
141	<i>I. nipponensis</i>	M	Yamagata	Isolated (<i>Rickettsia</i>)	No isolate	+	+	+	+	+
147	<i>I. persulcatus</i>	F	Hokkaido	Isolated (<i>Spiroplasma</i>)	Contaminated (<i>Mycobacterium</i>)	+	+	NA	NA	NA
202	<i>I. persulcatus</i>	M	Hokkaido	Isolated (<i>Rickettsia</i>)	No isolate	+	+	+	-	+
309	<i>I. persulcatus</i>	M	Hokkaido	Isolated (<i>Rickettsia</i>)	No isolate	+	+	+	-	+
318	<i>I. persulcatus</i>	M	Hokkaido	Isolated (<i>Rickettsia</i>)	No isolate	+	+	+	-	+
412	<i>H. concinna</i>	M	Hokkaido	Isolated (<i>Rickettsiella</i>)	Isolated (<i>Rickettsiella</i>)	-	+	NA	NA	NA
772	<i>A. testudinarium</i>	N	Miyazaki	Isolated (<i>Rickettsia</i>)	Contaminated (7 dpi)	+	+	+	+	+
774	<i>A. testudinarium</i>	N	Miyazaki	Isolated (<i>Rickettsia</i>)	No isolate	+	+	+	+	+
1033	<i>H. kitaokai</i>	F	Fukushima	No isolate	Isolated (<i>Spiroplasma</i>)	-	+	NA	NA	NA
1187	<i>I. nipponensis</i>	M	Fukushima	Isolated (<i>Rickettsia</i>)	No isolate	+	+	+	+	+
1284	<i>I. ovatus</i>	F	Yamagata	Isolated (<i>Rickettsia</i>)	Contaminated (<i>Bacillus</i>)	+	+	+	-	+
1328	<i>I. ovatus</i>	M	Yamagata	Contaminated (2 dpi)	Isolated (<i>Rickettsia</i>)	+	+	+	-	+
1994	<i>A. testudinarium</i>	F	Wakayama	Isolated (<i>Rickettsia</i>)	Contaminated (1 dpi)	+	+	+	+	+
2014	<i>H. longicornis</i>	M	Wakayama	Isolated (<i>Rickettsia</i>)	No isolate	+	+	+	+	+
2019	<i>H. longicornis</i>	M	Wakayama	Isolated (<i>Rickettsia</i>)	Contaminated (<i>Williamsia</i>)	+	+	+	+	+

a, Screened by *gltA* real-time PCR using DNA extracted from tick homogenates. +, positive; -, negative.

b, PCR using DNA extracted from ISE6 and C6/36 cells.

NA, not applicable; dpi, day post inoculation.

SUPPLEMENTARY TABLE S1. CHARACTERIZATION OF CLOSEST RICKETTSIA, RICKETTSIELLA AND SPIROPLASMA SPECIES WITH BLAST ANALYSIS

Tick ID	Tick species	16S rRNA		<i>gltA</i>		<i>ompA</i>		<i>ompB</i>	
		% Sequence similarity	Closest <i>Rickettsia</i> species (Accession number)	% Sequence similarity	Closest <i>Rickettsia</i> species (Accession number)				
Ini-141I	<i>I. nipponensis</i>	99% (1417/1422)	<i>R. monacensis</i> (LN794217)	100 % (537/537)	<i>R. monacensis</i> CN45kr (KC993860)	99% (588/592)	<i>R. monacensis</i> (EU665232)	99% (768/770)	<i>R. monacensis</i> Crimea-3 (KU961543)
		99% (1417/1422)	<i>R. monacensis</i> (NR115686)	100 % (537/537)	Uncultured <i>Rickettsia</i> sp. (LC060716)	99% (588/592)	<i>Rickettsiasp.</i> ZJ42/2007 (EU258734)	99% (768/770)	<i>R. monacensis</i> IrR/Munich (KC137254)
		99% (1416/1422)	<i>Rickettsia</i> sp. IRS4 (AF141908)	100 % (537/537)	<i>R. monacensis</i> (EU665235)	99% (584/588)	<i>R. monacensis</i> MT34 (JX972178)	99% (768/770)	<i>R. monacensis</i> A3-264 (EU330639)
		99% (1416/1423)	<i>Rickettsia</i> sp. IRS3 (AF141907)	100 % (537/537)	<i>Rickettsia</i> sp. POTiR5dt (EF501755)	99% (578/582)	<i>R. monacensis</i> CHO11 (KJ588273)	99% (767/770)	<i>R. monacensis</i> D-2 (EU330640)
		99% (1414/1422)	<i>Rickettsia</i> endosymbiont of <i>I. pacificus</i> (KP276591)	100 % (537/537)	<i>Rickettsia</i> sp. IRS3 (AF140706)	98% (585/598)	<i>R. monacensis</i> (EU665233)	99% (766/770)	<i>R. monacensis</i> MT34 (JX625150)
Ipe-202I	<i>I. persulcatus</i>	99% (1417/1421)	<i>R. helvetica</i> st. C9P9 (L36212)	100% (537/537)	<i>R. helvetica</i> (KY488349)	NA		100% (773/773)	<i>R. helvetica</i> Mu10/2166 (MG242313)
		98% (1406/1421)	<i>R. raoultii</i> (KY474575)	100% (537/537)	<i>C. R. mendelii</i> (KY678089)			100% (773/773)	<i>R. helvetica</i> KS14/0497 (MG242307)
		98% (1406/1421)	<i>R. raoultii</i> (CP010969)	100% (537/537)	<i>C. R. mendelii</i> (KY678088)			100% (773/773)	<i>R. helvetica</i> AS819 (MF163037)
		98% (1406/1421)	Uncultured <i>Rickettsia</i> sp. (JX432017)	100% (537/537)	<i>C. R. mendelii</i> (KY678087)			100% (773/773)	<i>R. helvetica</i> Suedafrika 1544 (KT835126)
		98% (1406/1421)	<i>R. raoultii</i> (NR043755)	100% (537/537)	<i>C. R. mendelii</i> (KY678086)			100% (773/773)	<i>R. helvetica</i> (KU310591)
Ipe-309I	<i>I. persulcatus</i>	99% (1417/1421)	<i>R. helvetica</i> st. C9P9 (L36212)	100% (537/537)	<i>R. helvetica</i> (KY488349)	NA		100% (773/773)	<i>R. helvetica</i> Mu10/2166 (MG242313)
		98% (1406/1421)	<i>R. raoultii</i> (KY474575)	100% (537/537)	<i>C. R. mendelii</i> (KY678089)			100% (773/773)	<i>R. helvetica</i> KS14/0497 (MG242307)

		98% (1406/1421)	<i>R. raoultii</i> (CP010969)	100% (537/537)	<i>C. R. mendelii</i> (KY678088)			100% (773/773)	<i>R. helvetica</i> AS819 (MF163037)
		98% (1406/1421)	Uncultured <i>Rickettsia</i> sp. (JX432017)	100% (537/537)	<i>C. R. mendelii</i> (KY678087)			100% (773/773)	<i>R. helvetica</i> Suedafrika 1544 (KT835126)
		98% (1406/1421)	<i>R. raoultii</i> (NR043755)	100% (537/537)	<i>C. R. mendelii</i> (KY678086)			100% (773/773)	<i>R. helvetica</i> (KU310591)
Ipe-318I	<i>I. persulcatus</i>	99% (1417/1421)	<i>R. helvetica</i> st. C9P9 (L36212)	100% (537/537)	<i>R. helvetica</i> (KY488349)	NA		100% (773/773)	<i>R. helvetica</i> Mu10/2166 (MG242313)
		98% (1406/1421)	<i>R. raoultii</i> (KY474575)	100% (537/537)	<i>C. R. mendelii</i> (KY678089)			100% (773/773)	<i>R. helvetica</i> KS14/0497 (MG242307)
		98% (1406/1421)	<i>R. raoultii</i> (CP010969)	100% (537/537)	<i>C. R. mendelii</i> (KY678088)			100% (773/773)	<i>R. helvetica</i> AS819 (MF163037)
		98% (1406/1421)	Uncultured <i>Rickettsia</i> sp. (JX432017)	100% (537/537)	<i>C. R. mendelii</i> (KY678087)			100% (773/773)	<i>R. helvetica</i> Suedafrika 1544 (KT835126)
		98% (1406/1421)	<i>R. raoultii</i> (NR043755)	100% (537/537)	<i>C. R. mendelii</i> (KY678086)			100% (773/773)	<i>R. helvetica</i> (KU310591)
Ate-772I	<i>A. testudinarium</i>	99% (1415/1422)	Uncultured <i>Rickettsia</i> sp. (KF981786)	100% (537/537)	<i>R. tamurae</i> (AB812551)	99% (573/576)	<i>R. tamurae</i> AT-1 (DQ103259)	98% (755/771)	<i>R. tamurae</i> AT-1 (DQ113910)
		99% (1415/1422)	<i>R. tamurae</i> AT-1 (NR042727)	100% (537/537)	<i>R. tamurae</i> (AF394896)	99% (568/576)	<i>Rickettsia</i> sp. Ae-8 (DQ365985)	98% (752/770)	<i>R. monacensis</i> Crimea-3 (KU961543)
		99% (1414/1422)	Uncultured <i>Rickettsia</i> sp. (KF981787)	99% (536/537)	<i>R. tamurae</i> (KT753273)	96% (554/576)	<i>Rickettsia</i> sp. ARAGAOI (KX077194)	98% (752/770)	<i>R. monacensis</i> IrR/Munich (KC137254)
		99% (1409/1422)	<i>Rickettsia</i> endosymbiont of <i>I.</i> <i>pacificus</i> (KP276591)	99% (536/537)	<i>R. tamurae</i> (KT753265)	96% (554/576)	Uncultured <i>Rickettsia</i> sp. (KU001175)	98% (752/770)	<i>R. monacensis</i> A3-264 (EU330639)
		99% (1409/1422)	<i>Rickettsia</i> endosymbiont of <i>I.</i> <i>pacificus</i> (KP276590)	99% (534/537)	<i>Rickettsia</i> endosymbiont of <i>A. dubitatum</i> (JN676158)	96% (553/576)	<i>Rickettsia</i> endosymbiont of <i>I.</i> <i>pararicinus</i> (KU744414)	98% (751/770)	<i>R. monacensis</i> D- 2 (EU330640)
Ate-774I	<i>A. testudinarium</i>	99% (1415/1422)	Uncultured <i>Rickettsia</i> sp. (KF981786)	100% (537/537)	<i>R. tamurae</i> (AB812551)	99% (573/576)	<i>R. tamurae</i> AT-1 (DQ103259)	98% (755/771)	<i>R. tamurae</i> AT-1 (DQ113910)
		99% (1415/1422)	<i>R. tamurae</i> AT-1 (NR042727)	100% (537/537)	<i>R. tamurae</i> (AF394896)	99% (568/576)	<i>Rickettsia</i> sp. Ae-8 (DQ365985)	98% (752/770)	<i>R. monacensis</i> Crimea-3 (KU961543)
		99% (1414/1422)	Uncultured <i>Rickettsia</i> sp. (KF981787)	99% (536/537)	<i>R. tamurae</i> (KT753273)	96% (554/576)	<i>Rickettsia</i> sp. ARAGAOI (KX077194)	98% (752/770)	<i>R. monacensis</i> IrR/Munich (KC137254)

		99% (1409/1422)	<i>Rickettsia</i> endosymbiont of <i>I.</i> <i>pacificus</i> (KP276591)	99% (536/537)	<i>R. tamurae</i> (KT753265)	96% (554/576)	Uncultured <i>Rickettsia</i> sp. (KU001175)	98% (752/770)	<i>R. monacensis</i> A3-264 (EU330639)
		99% (1409/1422)	<i>Rickettsia</i> endosymbiont of <i>I.</i> <i>pacificus</i> (KP276590)	99% (534/537)	<i>Rickettsia</i> endosymbiont of <i>A. dubitatum</i> (JN676158)	96% (553/576)	<i>Rickettsia</i> endosymbiont of <i>I.</i> <i>pararicinus</i> (KU744414)	98% (751/770)	<i>R. monacensis</i> D- 2 (EU330640)
Ini-11871	<i>I. nipponensis</i>	99% (1417/1422)	<i>R.</i> <i>monacensis</i> (LN794217)	100 % (537/537)	<i>R. monacensis</i> (KC993860)	99% (578/582)	<i>R. monacensis</i> CHO11 (KJ588273)	99% (768/770)	<i>R. monacensis</i> Crimea-3 (KU961543)
		99% (1417/1422)	<i>R.</i> <i>monacensis</i> (NR115686)	100 % (537/537)	Uncultured <i>Rickettsia</i> sp. (LC060716)	99% (578/582)	<i>R. monacensis</i> MT34 (JX972178)	99% (768/770)	<i>R. monacensis</i> IrR/Munich (KC137254)
		99% (1416/1422)	<i>Rickettsia</i> sp. IRS4 (AF141908)	100 % (537/537)	<i>R. monacensis</i> (EU665235)	99% (578/582)	<i>R. monacensis</i> (EU665232)	99% (768/770)	<i>R. monacensis</i> A3-264 (EU330639)
		99% (1416/1423)	<i>Rickettsia</i> sp. IRS3 (AF141907)	100 % (537/537)	<i>Rickettsia</i> sp POTiR5dt (EF501755)	99% (578/582)	<i>Rickettsia</i> sp. ZJ42/2007 (EU258734)	99% (767/770)	<i>R. monacensis</i> D- 2 (EU330640)
		99% (1414/1422)	<i>Rickettsia</i> endosymbiont of <i>I.</i> <i>pacificus</i> (KP276591)	100 % (537/537)	<i>Rickettsia</i> sp. IRS3 (AF140706)	98% (575/588)	<i>R.</i> <i>monacensis</i> (EU665233)	99% (766/770)	<i>R. monacensis</i> MT34 (JX625150)
Iov-1284I	<i>I. ovatus</i>	99% (1403/1423)	<i>R. asiatica</i> IO-1 (NR041840)	100 % (537/537)	<i>R. asiatica</i> (AF394901)	NA		99% (767/773)	<i>R.helvetica</i> Mu10/2166 (MG242313)
		98% (1401/1423)	<i>R. helvetica</i> st. C9P9 (L36212)	99% (535/537)	<i>R. asiatica</i> (AB297808)			99% (767/773)	<i>R. helvetica</i> KS14/0497 (MG242307)
		98% (1400/1423)	<i>Rickettsia</i> sp. (L36102)	99% (533/538)	<i>R. asiatica</i> (AB297810)			99% (767/773)	<i>R. helvetica</i> AS819 (MF163037)
		98% (1399/1423)	<i>R. massiliae</i> st. AZT80 (CP003319)	99% (532/539)	<i>R. helvetica</i> (KY488349)			99% (767/773)	<i>R. helvetica</i> Suedafrika 1544 (KT835126)
		98% (1399/1423)	<i>R. massiliae</i> MTU5 (CP000683)	99% (532/540)	<i>C. R. mendelii</i> (KY678089)			99% (767/773)	<i>R. helvetica</i> (KU310591)
Iov-1328C	<i>I. ovatus</i>	99% (1403/1423)	<i>R. asiatica</i> IO-1 (NR041840)	100 % (537/537)	<i>R. asiatica</i> (AF394901)	NA		99% (767/773)	<i>R.helvetica</i> Mu10/2166 (MG242313)

		98% (1401/1423)	<i>R. helvetica</i> st. C9P9 (L36212)	99% (535/537)	<i>R. asistica</i> (AB297808)		99% (767/773)	<i>R. helvetica</i> KS14/0497 (MG242307)
		98% (1400/1423)	<i>Rickettsia</i> sp. (L36102)	99% (533/538)	<i>R. asistica</i> (AB297810)		99% (767/773)	<i>R. helvetica</i> AS819 (MF163037)
		98% (1399/1423)	<i>R. massiliae</i> st. AZT80 (CP003319)	99% (532/539)	<i>R. helvetica</i> (KY488349)		99% (767/773)	<i>R. helvetica</i> Suedafrika 1544 (KT835126)
		98% (1399/1423)	<i>R. massiliae</i> MTU5 (CP000683)	99% (532/540)	<i>C. R. mendelii</i> (KY678089)		99% (767/773)	<i>R. helvetica</i> (KU310591)
Ate-1994I	<i>A. testudinarium</i>	99% (1415/1422)	Uncultured <i>Rickettsia</i> sp. (KF981786)	100% (537/537)	<i>R. tamurae</i> (AB812551)	99% (575/578)	<i>R. tamurae</i> AT-1 (DQ103259)	<i>R. tamurae</i> AT-1 (DQ113910)
		99% (1415/1422)	<i>R. tamurae</i> AT-1 (NR042727)	100% (537/537)	<i>R. tamurae</i> (AF394896)	99% (570/578)	<i>Rickettsia</i> sp. Ae-8 (DQ365985)	<i>R. monacensis</i> Crimea-3 (KU961543)
		99% (1414/1422)	Uncultured <i>Rickettsia</i> sp. (KF981787)	99% (536/537)	<i>R. tamurae</i> (KT753273)	96% (556/578)	<i>Rickettsia</i> sp. ARAGAOI (KX077194)	<i>R. monacensis</i> IrR/Munich (KC137254)
		99% (1409/1422)	<i>Rickettsia</i> endosymbiont of <i>I.</i> <i>pacificus</i> (KP276591)	99% (536/537)	<i>R. tamurae</i> (KT753265)	96% (556/578)	Uncultured <i>Rickettsia</i> sp. (KU001175)	<i>R. monacensis</i> A3-264 (EU330639)
		99% (1409/1422)	<i>Rickettsia</i> endosymbiont of <i>I.</i> <i>pacificus</i> (KP276590)	99% (534/537)	<i>Rickettsia</i> endosymbiont of <i>A. dubitatum</i> (JN676158)	96% (555/578)	<i>Rickettsia</i> endosymbiont of <i>I.</i> <i>pararicinus</i> (KU744414)	<i>R. monacensis</i> D- 2 (EU330640)
Hlo-2014I	<i>H. longicornis</i>	99% (1419/1421)	Uncultured <i>Rickettsia</i> sp. HtM69 (KU758904)	100% (537/537)	<i>Rickettsia</i> sp. LON-13 (AB516964)	99% (586/587)	<i>Rickettsia</i> sp. HIR/D91 (KC888951)	<i>Rickettsia</i> sp. AUS118 (KF666469)
		99% (1419/1421)	Uncultured <i>Rickettsia</i> sp. HtFM4 (KU758903)	100% (537/537)	<i>C. R. jingxinensis</i> (KT899089)	99% (585/587)	<i>Rickettsia</i> sp. FYJ98 (AF169629)	<i>Rickettsia</i> sp. A598 (KJ619632)
		99% (1417/1421)	<i>Rickettsia</i> YN03 (KY433580)	100% (537/537)	<i>C. R. jingxinensis</i> (KT899088)	99% (577/587)	Uncultured <i>Rickettsia</i> sp. (MG228270)	<i>R. japonica</i> HH- 18 (AP017588)
		99% (1416/1421)	<i>R. japonica</i> st. YH (NR074459)	100% (537/537)	<i>Rickettsia</i> sp. st WHBMXZ-80 (KX987339)	99% (576/587)	Uncultured <i>Rickettsia</i> sp. (JN943296)	<i>R. japonica</i> HH- 17 (AP017587)
		99% (1416/1421)	<i>R. japonica</i> (AP017602)	100% (537/537)	Uncultured <i>Rickettsia</i> sp. (KF728367)	99% (555/556)	<i>Rickettsia</i> endosymbiont of <i>H.</i> <i>longicornis</i> (MF590726)	<i>R. japonica</i> HH- 16 (AP017586)
Hlo-2019I	<i>H. longicornis</i>	99% (1419/1421)	Uncultured <i>Rickettsia</i> sp. HtM69 (KU758904)	100% (537/537)	<i>Rickettsia</i> sp. LON-13 (AB516964)	99% (586/587)	<i>Rickettsia</i> sp. HIR/D91 (KC888951)	<i>Rickettsia</i> sp. AUS118 (KF666469)

		99% (1419/1421)	Uncultured <i>Rickettsia</i> sp. HtFM4 (KU758903)	100% (537/537)	<i>C. R. jingxinensis</i> (KT899089)	99% (585/587)	<i>Rickettsia</i> sp. FYJ98 (AF169629)	99% (750/752)	<i>Rickettsia</i> sp. A598 (KJ619632)
		99% (1417/1421)	<i>Rickettsia</i> YN03 (KY433580)	100% (537/537)	<i>C. R. jingxinensis</i> (KT899088)	99% (577/587)	Uncultured <i>Rickettsia</i> sp. (MG228270)	99% (762/770)	<i>R. japonica</i> HH-18 (AP017588)
		99% (1416/1421)	<i>R. japonica</i> st. YH (NR074459)	100% (537/537)	<i>Rickettsia</i> sp. st WHBMXZ-80 (KX987339)	99% (576/587)	Uncultured <i>Rickettsia</i> sp. (JN943296)	99% (762/770)	<i>R. japonica</i> HH-17 (AP017587)
		99% (1416/1421)	<i>R. japonica</i> (AP017602)	100% (537/537)	Uncultured <i>Rickettsia</i> sp. (KF728367)	99% (555/556)	<i>Rickettsia</i> endosymbiont of <i>H. longicornis</i> (MF590726)	99% (762/770)	<i>R. japonica</i> HH-16 (AP017586)
Imo-135I	<i>I. monospinosus</i>	99% (1443/1444)	<i>Spiroplasma</i> sp. 'Gent' (AY569829)						
		99% (1441/1444)	<i>Spiroplasma</i> -symbiont of <i>Antonina crawii</i> (AB030022)						
		99% (1439/1444)	<i>Spiroplasma</i> sp. Anisosticta MK (AM087471)						
		99% (1440/1444)	Uncultured bacterium isolate RFLP (EF121346)						
		99% (1438/1444)	<i>Spiroplasma</i> symbiont of <i>Laodelphax striatellus</i> (AB553862)						
Imo-135C	<i>I. monospinosus</i>	99% (1442/1444)	<i>Spiroplasma</i> sp. 'Gent' (AY569829)						
		99% (1440/1444)	<i>Spiroplasma</i> -symbiont of <i>Antonina crawii</i> (AB030022)						
		99% (1438/1444)	<i>Spiroplasma</i> sp. Anisosticta MK (AM087471)						
		99% (1439/1444)	Uncultured bacterium isolate RFLP (EF121346)						
		99% (1436/1444)	<i>Spiroplasma</i> symbiont of <i>Laodelphax striatellus</i> (AB553862)						
Ipe-147I	<i>I. persulcatus</i>	99% (1441/1444)	<i>Spiroplasma</i> sp. 'Gent' (AY569829)						
		99% (1439/1444)	<i>Spiroplasma</i> -symbiont of <i>Antonina crawii</i> (AB030022)						
		99% (1437/1444)	<i>Spiroplasma</i> sp. Anisosticta MK (AM087471)						

		99% (1438/1444)	Uncultured bacterium isolate RFLP (EF121346)
		99% (1435/1444)	<i>Spiroplasma</i> symbiont of <i>Laodelphax</i> <i>striatellus</i> (AB553862)
Hki-1033C	<i>H. kitaokai</i>	99% (1442/1444)	<i>Spiroplasma</i> sp. 'Gent' (AY569829)
		99% (1440/1444)	<i>Spiroplasma</i> -symbiont of <i>Antonina</i> <i>crawii</i> (AB030022)
		99% (1438/1444)	<i>Spiroplasma</i> sp. Anisosticta MK (AM087471)
		99% (1439/1444)	Uncultured bacterium isolate RFLP (EF121346)
		99% (1436/1444)	<i>Spiroplasma</i> symbiont of <i>Laodelphax</i> <i>striatellus</i> (AB553862)
Hcn-412I	<i>H. concinna</i>	99% (1470/1480)	Uncultured bacterium clone (MF086655)
		99% (1388/1408)	<i>Rickettsiella</i> endosymbiont of <i>Acyrtosiphon pisum</i> (AB522697)
		98% (1387/1408)	<i>Candidatus</i> <i>Rickettsiella</i> viridis (AB780468)
		98% (1387/1408)	<i>Rickettsiella</i> endosymbiont of <i>Acyrtosiphon pisum</i> (AB522705)
		98% (1352/1372)	<i>Rickettsiella</i> endosymbiont of <i>Acyrtosiphon pisum</i> (JX943560)
Hcn-412C	<i>H. concinna</i>	99% (1470/1480)	Uncultured bacterium clone (MF086655)
		99% (1388/1408)	<i>Rickettsiella</i> endosymbiont of <i>Acyrtosiphon pisum</i> (AB522697)
		98% (1387/1408)	<i>Candidatus</i> <i>Rickettsiella</i> viridis (AB780468)
		98% (1387/1408)	<i>Rickettsiella</i> endosymbiont of <i>Acyrtosiphon pisum</i> (AB522705)
		98% (1352/1372)	<i>Rickettsiella</i> endosymbiont of <i>Acyrtosiphon pisum</i> (JX943560)

NA, Not amplified

Figure Legends

FIG. 1. The time of onset of cytopathic effect observed in each bacterial isolate in ISE6 and C6/36 cells.

FIG. 2. Phylogenetic tree of (A) 16S rDNA, (B) *gltA*, (C) *ompA* and (D) *ompB* sequences of *Rickettsia* species isolated from ticks in Japan and representative rickettsiae using maximum likelihood method. The sequences obtained in this study are shown in bold. GenBank accession numbers are given after species name.

FIG. 3. Phylogenetic tree of 16S rDNA of *Rickettsiella* endosymbionts isolated from ticks in Japan using maximum likelihood method. The sequences obtained in this study are shown in bold. GenBank accession numbers are given after species name.

FIG. 4. Phylogenetic tree of 16S rDNA of *Spiroplasma* endosymbionts isolated from ticks in Japan using maximum likelihood method. The sequences obtained in this study are shown in bold. GenBank accession numbers are given after species name.

FIG. 1

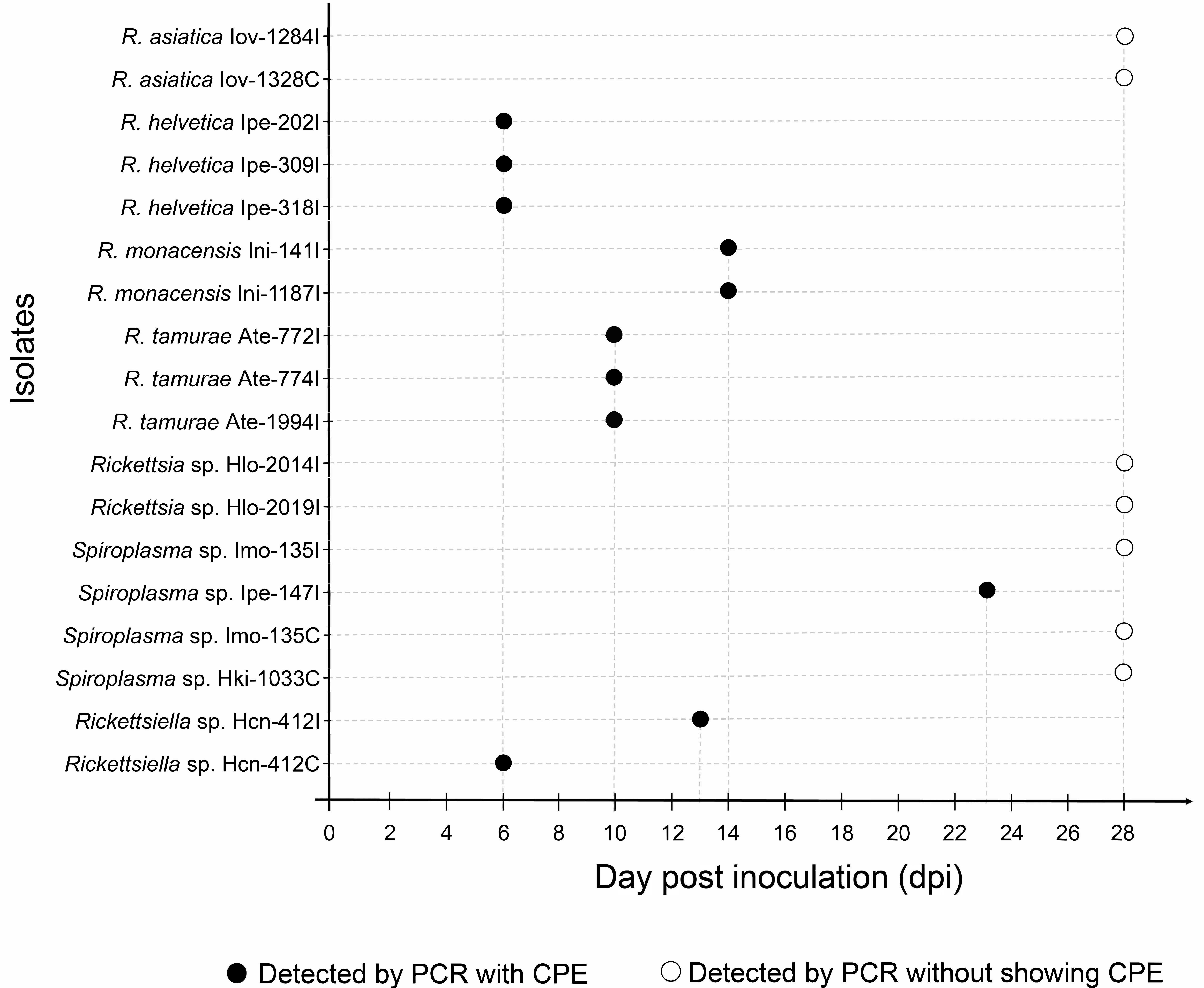
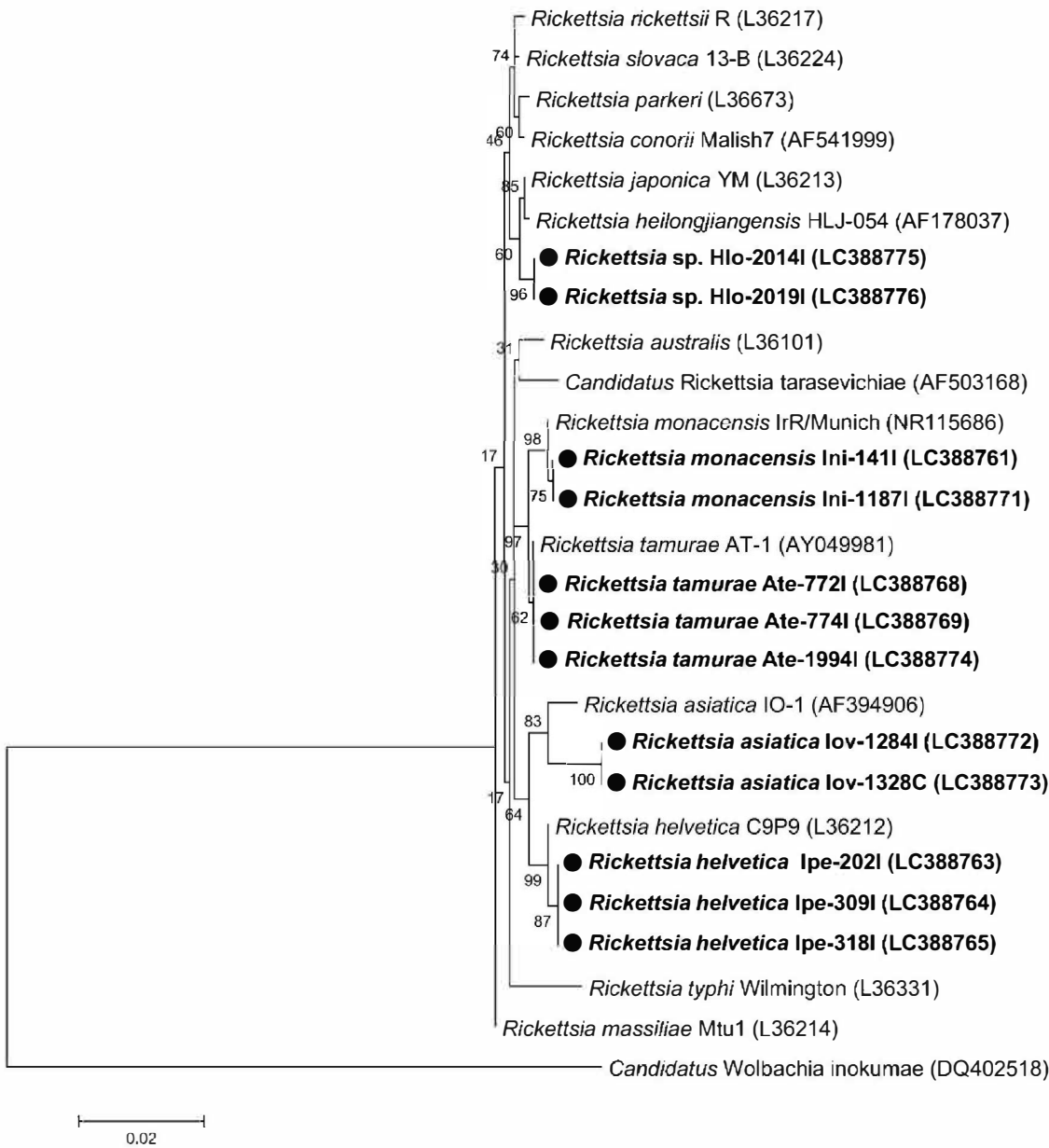
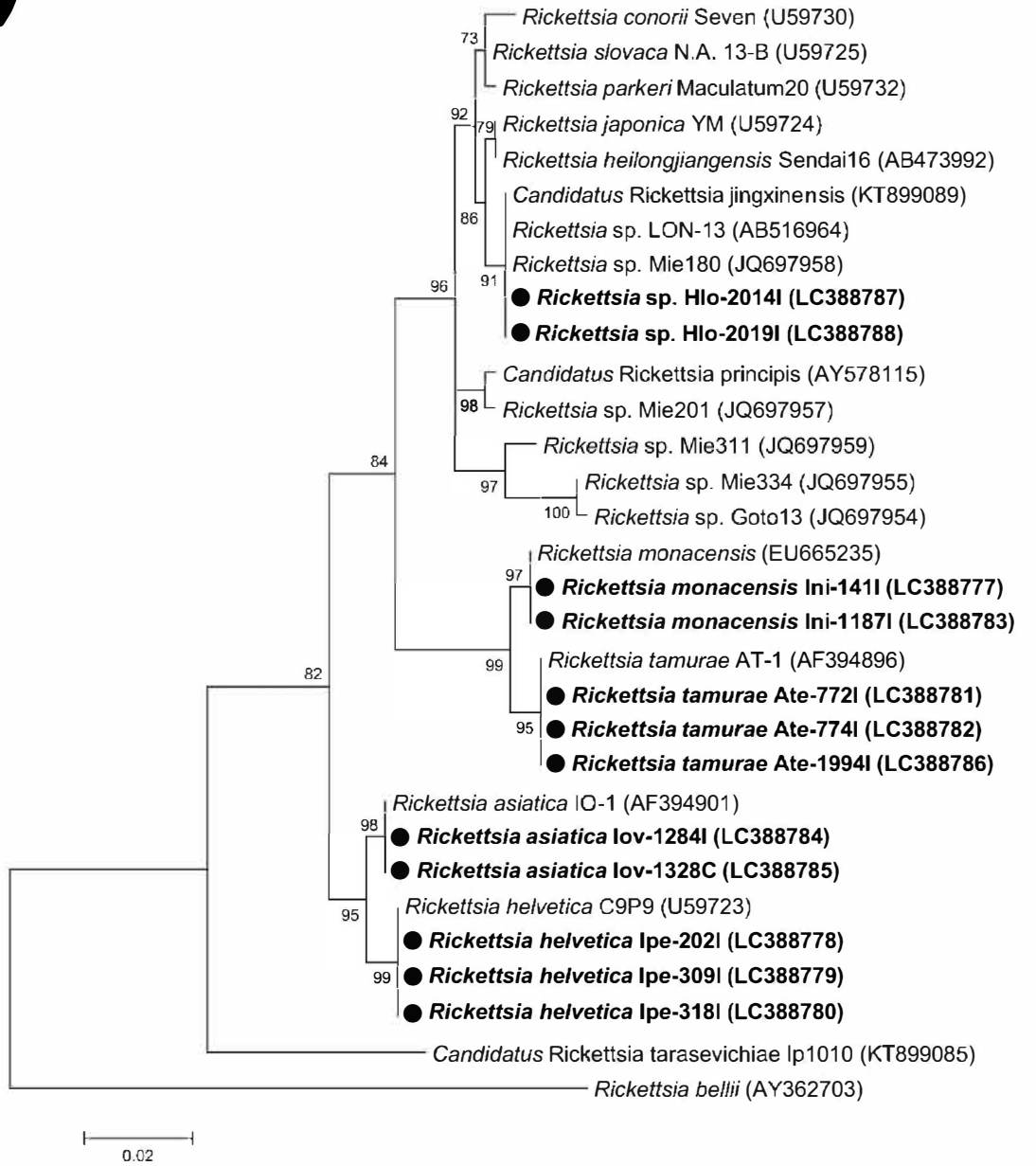


FIG. 2

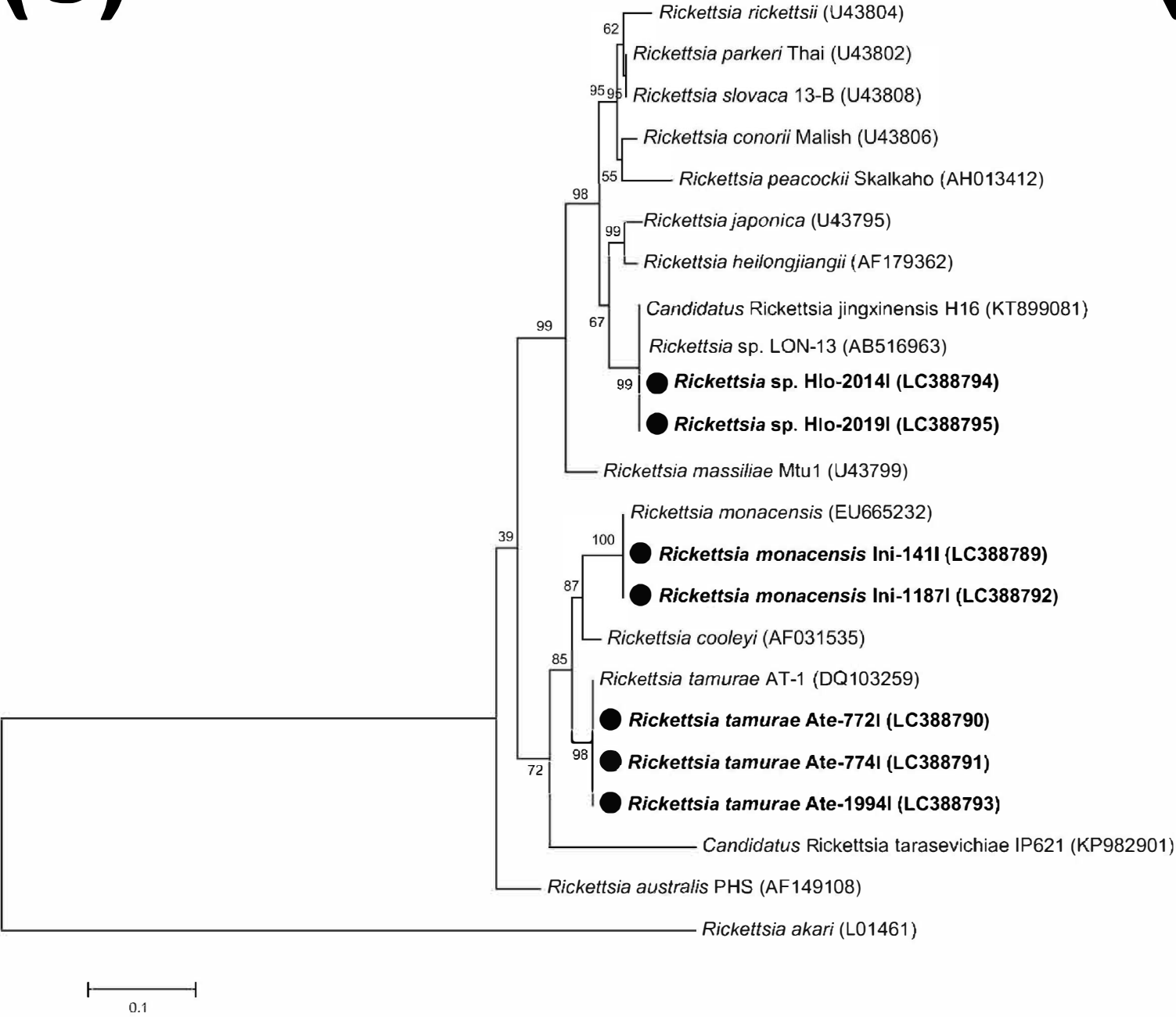
(A)



(B)



(C)



(D)

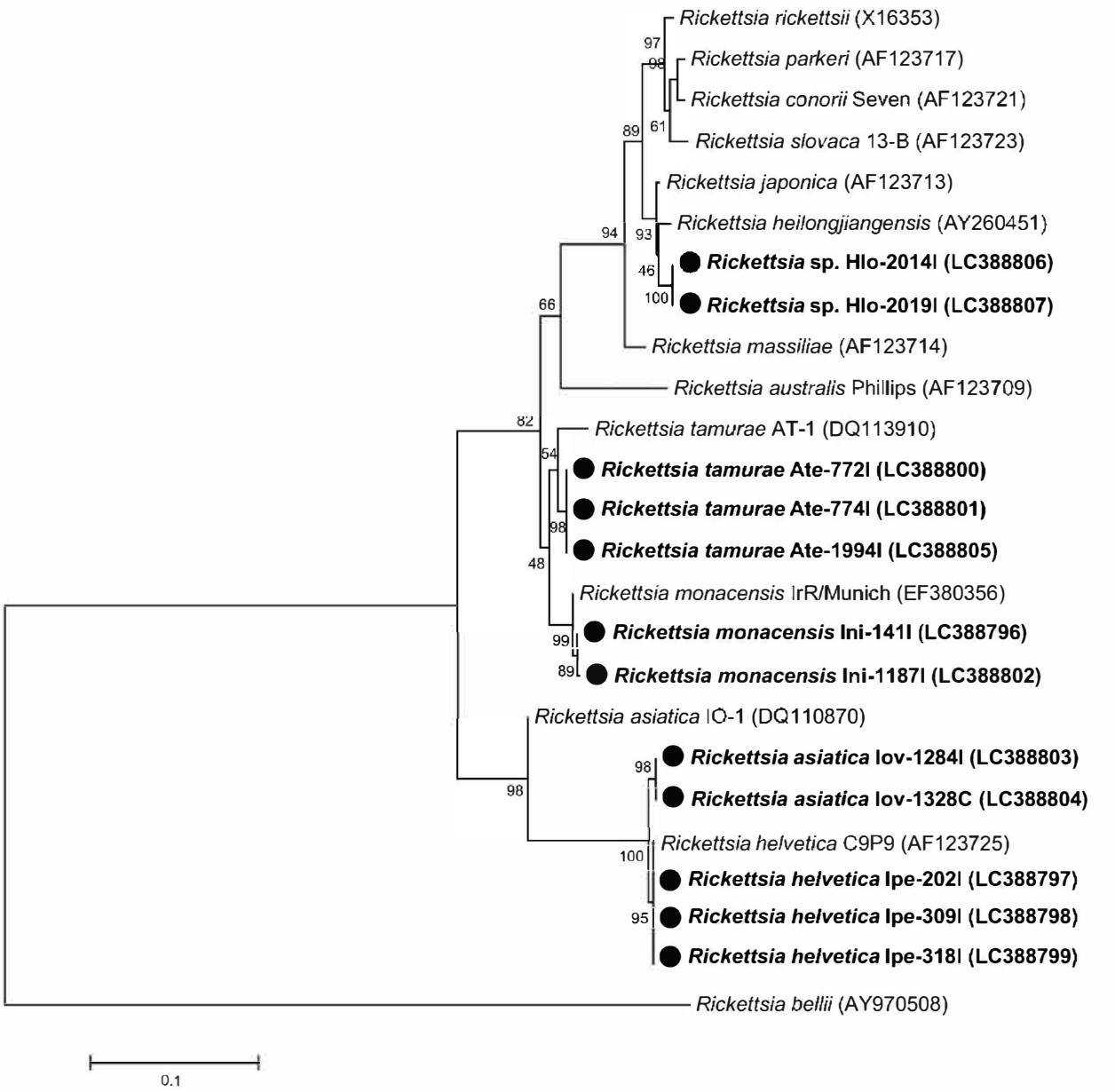
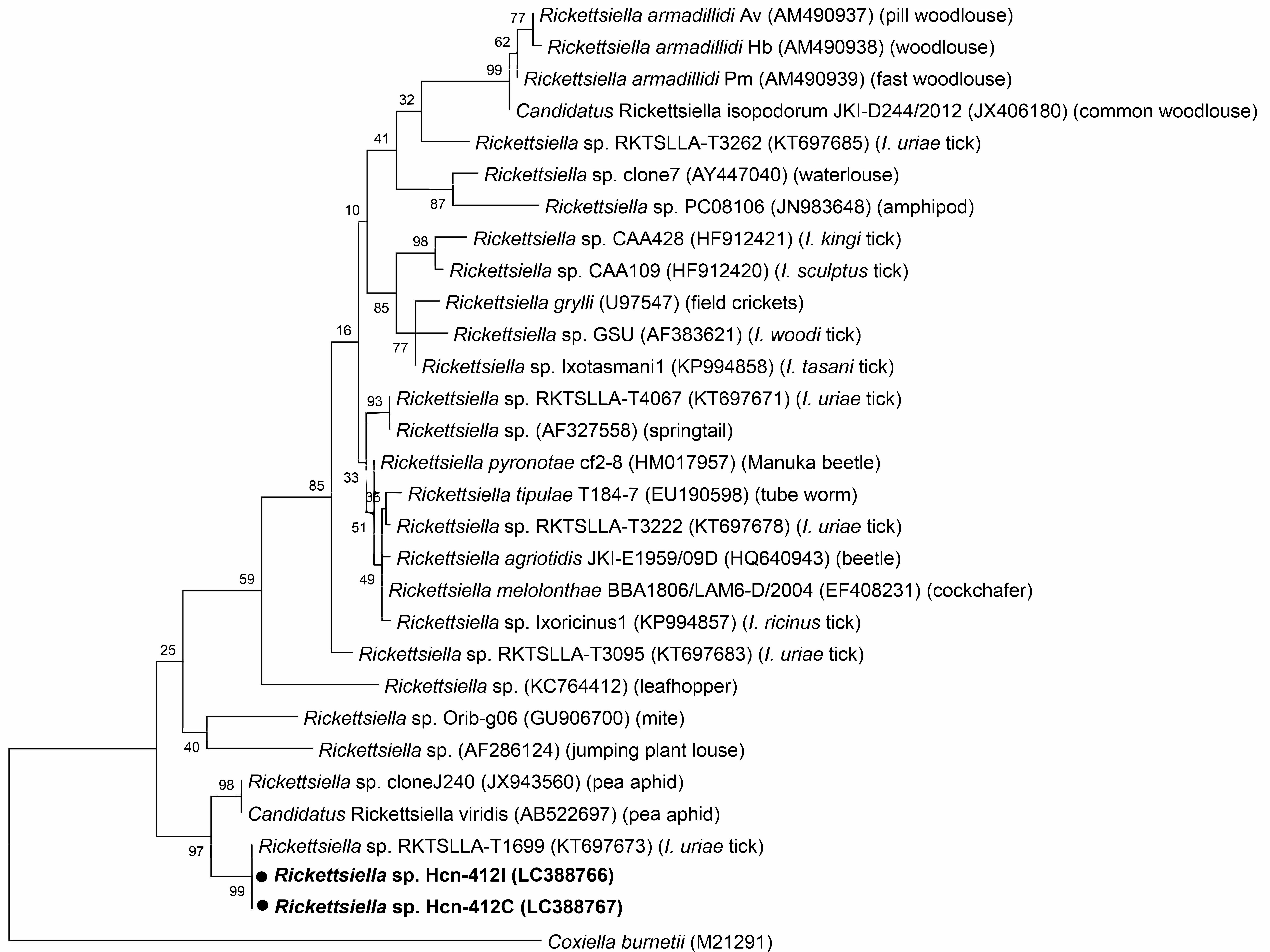
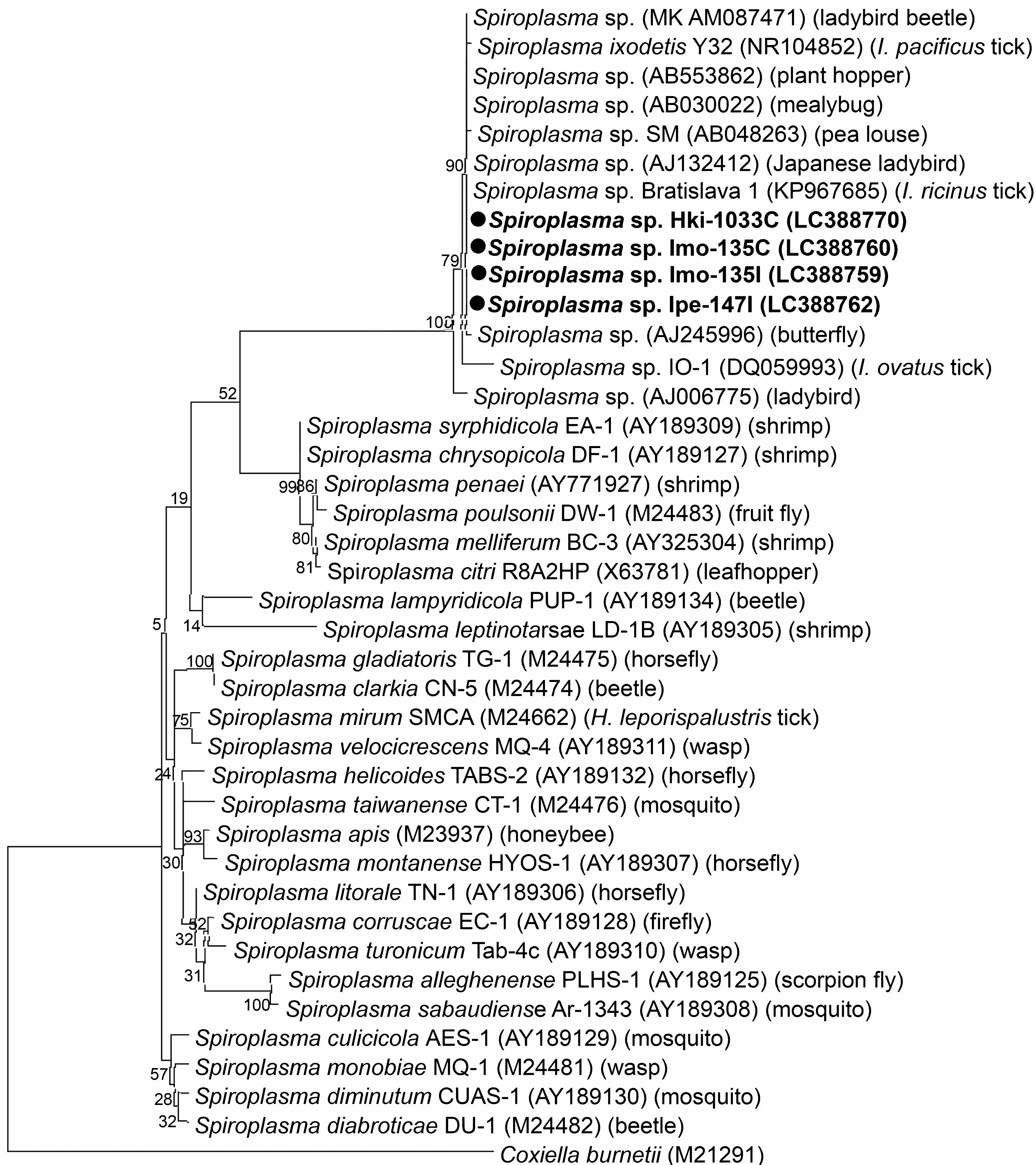


FIG. 3



0.020

FIG. 4



0.05