Title

1 # Relationship of *Rickettsia* infection rates and population genetic structure

2 # of ixodid ticks along an altitudinal gradient

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Abstract

Ixodid ticks, such as *Ixodes ovatus* and *Haemaphysalis flava,* are important vectors of tick-borne diseases in Japan, such as Japanese spotted fever caused by *Rickettsia sp* (i.e., the pathogen). This study describes the population genetic structure and gene flow of *I. ovatus* and *H. flava* and their *Rickettsia* infection rates along an altitudinal gradient. A total of 346 adult *I. ovatus* and 243 *H. flava* were analyzed for the presence of *Rickettsia* using the primers 17kDA, *gltA, rOmpA,* and *rOmpB*. Population genetic structure was performed using the mitochondrial cytochrome oxidase 1 (*cox1*) marker. The *Rickettsia* infection rates were 13.26% in *I. ovatus* and 6.17% in *H. flava.* For *I. ovatus,* the global F_{ST} value revealed significant genetic differentiation among the different populations, whereas *H. flava* showed non-significant genetic differentiation. The *cox1 I. ovatus* cluster dendrogram showed two cluster groups, while the haplotype network and phylogenetic tree showed three genetic groups. The two cluster groups and the three genetic groups found in *I. ovatus* showed a significant difference in their *Rickettsia* infection rates and mean altitude per group. No significant differences were found in the mean altitude or *Rickettsia* infection rates of *H. flava*. Our results suggest that low gene flow in the *I. ovatus* populations have caused spatially heterogenous *Rickettsia* infection rates along the altitudinal gradient. This information is essential in understanding the relationship between the tick vector, its pathogen, and environmental factors, such as altitude.

Keywords: environment, haplotype, hosts, pathogen, dispersal, vector

1. Introduction

Tick-borne diseases are a public health concern in Japan and are transmitted by a wide range of tick species, such as *Ixodes ovatus*, the primary vector of *Lyme borealis* and *Haemaphysalis flava*, a known vector of severe fever thrombocytopenia syndrome and Japanese spotted fever caused by the pathogen *Rickettsia japonica* (Mahara et al., 1997; Miyamoto et al., 1993; Yu et al., 2014, Yamaji et al., 2018, Arai et al., 2021). The dynamics of tick-borne pathogens are influenced by the habitat distribution and dispersal behaviors of vectors and hosts along environmental gradients (Agany et al., 2020; Jongejan et al., 2007).

Tick population genetic analysis provides dispersal pattern data based on gene flow between local populations (Arraya-Anchetta et al., 2015). Tick dispersal primarily depends on the movements of their vertebrate hosts, which influences each tick's potential to spread its pathogen (Falco and Fish, 1991; McCoy et al., 2001, Oliver et al., 2007). For example, contrasting patterns in the population genetic structures of *I. ovatus* and *H. flava* in the Niigata Prefecture of Japan indicate that host mobility during the immature stages of tick development may influence the genetic structure of adult ticks by affecting survivability into their adult stages (Regilme et al., 2021; Estrada-Pena et al., 2015). *I. ovatus* populations had greater genetic divergence due to the limited dispersal of their small mammalian hosts during the immature development stage; whereas *H. flava* populations showed a more homogenized structure due to the higher mobility of their large mammalian hosts and avian-mediated dispersal (Regilme et al., 2021). Other studies have also revealed low gene flow in ticks with low mobility hosts (e.g., small mammals) and higher gene flow in ticks with highly mobile hosts (e.g., large mammals and birds; Arraya-ancheta et al., 2015; Mixson et al., 2006; Trout et al., 2010; Guglielmone et al., 2013; Lampo et al., 2015).

46 The spatial distribution and movement of the vector (i.e., ticks) may determine the spatial
47 distribution of the pathogen's (i.e., *Rickettsia*) infection rate (Dougherty et al., 2018). Previous
48 studies have shown that pathogen infection rate can be influenced by many factors, such as vector
49 host genetic diversity, gene flow, and spatial structure (Regoes et al., 2000; Boots et al., 2007; Lion
50 et al., 2015; Chin et al., 1984, Thrall et al., 2003; White et al., 2020; Gibson et al., 2020; Bono et al.,
51 2012). For example, previous studies have shown that strong gene flow between local host
52 populations tend to reduce the spatial heterogeneity of pathogen infection rates between
53 populations (Streicker et al., 2016; Fountain-Jones et al., 2018). Thus, the exchange of infected and
54 uninfected individuals across a spatial scale is expected to spatially homogenize pathogen
55 infection rates. To our knowledge, no previous studies have examined the relationship between
56 the spatial heterogeneity of *Rickettsia* infection rates and population genetic structure in ticks.

57 Environmental factors, such as altitude, may relate to the population genetic structure of
58 ticks (Daniel et al., 2015; Eisen et al., 2016), with limited gene flow increasing genetic variation
59 between populations along an altitudinal gradient, as reported in several species (Hofreiter et al.,
60 2004; Chapman et al., 2013; Osborne et al., 2013, Polato et al., 2017). Thus, in this study, we
61 expected to see a highly divergent population genetic structure in *I. ovatus* along an altitudinal
62 gradient due to the limited movement of their mammalian hosts along that gradient; whereas *H.*
63 *flava* should show a less divergent structure along the altitudinal gradient due to the higher
64 mobility of its hosts. To our knowledge, no studies have focused on tick gene flow along an
65 altitudinal gradient.

66 In the study by Regilme et al. (2021), no significant influence of environmental factors,
67 including altitude, were observed in the genetic structures of *I. ovatus* and *H. flava* based on the
68 mantel test, but the study did not use any other robust analytical methods to more thoroughly
69 examine the influence of altitude on tick genetic structure. In another study, the relationship

70 between the tick vector and its pathogen (*Rickettsia* sp.) were analyzed in a total of 3,336
71 immature and adult ticks across the Niigata Prefecture, Japan by analyzing *Rickettsia* infection
72 rates per species (Arai et al., 2021). Although *Rickettsia* infections were found in *H. flava,*
73 *Haemaphysalis longicornis, Ixodes monospinus, Ixodes nipponensis*, and *Ixodes ovatus*, no spatial
74 distribution of *Rickettsia* infection rates were found among the local populations. To our
75 knowledge, no previous studies have considered the influence of environmental factors on tick
76 population genetic structure or the spatial distribution of *Rickettsia* infection rates along an
77 altitudinal gradient in local tick populations.

78 Here, we determine the relationship between the vector tick population genetic structure
79 and *Rickettsia* infection rates as influenced by an altitudinal gradient. Based on the isolation by
80 environment (IBE) theory, genetic differentiation increases with environmental variation,
81 regardless of geographic distance (Wang and Summers et al., 2010; Bradburd et al., 2013; Sexton
82 et al., 2014). Thus, we hypothesized that limited gene flow among tick populations along an
83 altitudinal gradient might cause spatially heterogenous *Rickettsia* infection rates in ticks. The
84 present study used cytochrome oxidase 1 (*cox1*) mitochondrial gene sequences and *Rickettsia*-
85 infected/uninfected data for *I. ovatus* (n = 307) and *H. flava* (n = 220) individuals collected in
86 2016–2017 as reported in Regilme et al. (2021) and Arai et al. (2021). Additionally, we added new
87 *cox1* sequences and *Rickettsia* infection/noninfection data from *I. ovatus* and *H. flava* individuals
88 and five other species collected in 2018.

89

2. Materials and Method

Published data of Regilme et al. (2021) and Arai et al. (2021)

In this study, we used *cox1* sequence data from Regilme et al. (2021) for *I. ovatus* (n = 307) and *H. flava* (n = 220) ticks collected from April 2016 to November 2017 from 29 sites across the Niigata Prefecture, Japan. *Rickettsia* infection rate data were obtained from Arai et al. (2021) from *I. ovatus* (n = 29), *H. flava* (n = 2), *I. monospinus* (n = 4), and *I. nipponensis* (n = 2) from 38 sites. Please refer to Regilme et al. (2021) and Arai et al. (2021) for more information about the study sites, collection, sampling identification, DNA extraction, PCR amplification, and sequencing methods used in each study.

To strengthen our analysis, we also added data collected from April to October 2018 from additional individuals sampled at 31 sites across the Niigata Prefecture, including two sites not previously sampled by Regilme et al. (2021). At these sites, ticks were collected 2–14 times from six core sites among the 31 sites while the remaining sites, ticks were collected once. The altitude at each site ranged from 8 to 1402 m.a.s.l., with a mean altitude of 348 m.a.s.l. Multiple sites were combined for population genetic analysis if less than eight individuals were obtained per site, which resulted in 8 populations labeled A to H. Three sites were excluded from the population genetic analysis because of the limited number of obtained individuals (<8) and the lack of a nearby site to combine into a single population.

Unpublished data from 2018 collection

Ticks collected in 2018 were stored at 4 °C in microcentrifuge tubes with 70% ethanol. Each collected tick was morphologically identified using a stereo microscope following the

112 identification keys of Yamaguti et al. (1971). Genomic DNA was extracted using Isogenome DNA
113 extraction kits (Nippon Gene Co. Ltd. Tokyo, Japan) following the manufacturer's recommended
114 protocol.

115 In this study, we combined previously published data from Arai et al. (2021) with our
116 newly collected data to calculate the *Rickettsia* infection rate, which is the percentage of
117 *Rickettsia*-infected ticks from each obtained population. We analyzed the obtained tick DNA for
118 spotted fever group *Rickettsia* (SFGR) detection and host identification, and amplified the
119 mitochondrial gene *cox1* for population genetic analysis. We performed nested PCR targeting the
120 following genes for the detection of *Rickettsia* sp.: 17-kDA antigen gene (17-kDA); citrate synthase
121 gene (gltA); spotted fever group (SFG)-specific outer membrane protein A gene (rOmpA); and
122 outer membrane protein B gene (rOmpB) as described and analyzed in Arai et al. (2021). Briefly,
123 we first amplified the 17-kDa protein. If the results were positive, then PCR was performed to
124 target gltA. Samples that were positive with both 17-kDA and gltA were regarded as positive for
125 SFGR. PCR was also performed to target the rOmpA and rOmpB genes to identify *Rickettsia* species.
126 The amplified PCR products were purified using AMPure XP (Beckman Coulter Co., Japan) and
127 sequenced using the Big Dye Terminator Cycle Sequence Kit (Thermo Fisher Scientific).

128 The *cox1* mitochondrial gene was amplified by PCR for *cox1* (658 base pairs) using the
129 primer pairs LCO-1490 (5′- AAACTTCAGGGTGACCAAAAAATCA-3') and HCO1−2198 (5′–
130 AAACTTCAGGGTGACCAAAAAATCA - 3) for phylogenetic analysis and tick species identification
131 (Folmer et al., 1994). The PCR amplification profile included an initial denaturation of 94 °C for
132 2 min, followed by denaturation at 94 °C for 30 s, then annealing at 38 °C for 30 s, followed by an
133 extension of 72 °C for 1 min for 30 cycles, and a final extension of 72 °C for 10 min. The obtained
134 PCR products were purified using the QIAquick 96 PCR Purification Kit (Qiagen, Germany) following
135 the manufacturer's instructions and were sequenced by Eurofin Genomics, Inc. (Tokyo, Japan).

136 Each forward and reverse read was assembled using CodonCode Aligner version 1.2.4
137 software (https://www.codoncode.com/aligner/). Low-quality bases were removed in the aligned
138 sequences, and no ambiguous bases were detected. We used the MAFFT alignment online
139 program (https://mafft.cbrc.jp/alignment/server/) to perform multiple alignments using the
140 default settings. The sequences were checked for similarities with the deposited reference
141 sequences from GenBank for sequence quality and tick species confirmation using BLAST
142 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All final aligned sequences were checked in Mesquite
143 version 3.5 (Maddison et al., 2011). The protein-coding genes were translated to amino acids to
144 confirm the absence of stop codons.

145

146 ***Population genetic analysis***

147 The final *cox1* sequences from all seven tick species were individually analyzed using
148 DNASp version 6.12.03 to determine the haplotype diversity per species (Rozas et al., 2017). The
149 level of genetic divergence between each population was quantified per species using global F_{ST}.
150 Significance was tested using Arlequin software version 3.5.2.2 (Excoffier and Lischer, 2010) with
151 9999 permutations.

152 The genetic relationship between the *I. ovatus* populations was visualized using the
153 unweighted pair group with the arithmetic mean (UPGMA) cluster method using the APE package
154 (Paradis and Schliep et al., 2018) for the RStudio software (R Development Core Team, 2016). A
155 cluster dendrogram was created using pairwise F_{ST} values genetic distance matrix from GenAlEx.

156

157 ***Haplotype network and phylogenetic analyses***

158 We constructed a haplotype network analysis using PopART program version 1.7
159 (http://popart.otago.ac.nz/index.shtml) on *cox1 I. ovatus* and *H. flava* sequences to assess
160 haplotype relationships using the median-joining network algorithm (Bandeth et al., 1999). Briefly,
161 we constructed a Bayesian phylogenetic tree of *cox1* haplotypes for *I. ovatus* and *H. flava,*
162 respectively, using BEAST version 1.10.14 (Drummond and Rambaut, 2007). We used the
163 Hasegawa-Kishino-Yano substitution model with estimated base frequencies. We employed a
164 strict clock model and used the coalescent prior as the tree prior. A maximum clade credibility tree
165 was acquired using TreeAnnotator version 1.10.14 using trees from BEAUti version1.10.14 with
166 90% of the trees as the burn-in. We viewed the constructed maximum clade credibility tree using
167 FigTree version1.4.4.

168

169 ***Statistical analysis***

170 To determine whether there was a significant difference in the *Rickettsia* infection rate
171 between haplotype groups for *I. ovatus* and *H. flava),* we performed a z-score test at $p < 0.05$. To
172 determine whether there were differences in the mean altitude between the haplotype groups,
173 we used the Welch t-test at $p < 0.05$.

174

175 **3. Results**

176 The total number of positive (pos) and negative (neg) ticks for *Rickettsia* infections from
177 the ticks collected in 2018 were: *I. ovatus* (neg = 22, pos = 17); *H. flava* (neg = 10, pos = 13); *I.*
178 *monospinus* (neg = 11, pos = 6); *I. asanumai* (neg = 4, pos=5); *I. nipponensis* (pos = 4); *I. persulcatus*

179 (neg = 4); and *H. japonica* (pos = 2). In this study, the total number of samples from the previously
180 published data (Regilme et al., 2021; Arai et al., 2021) and the unpublished data from 2018 were: *I.*
181 *ovatus* (n = 347); *H. flava* (n = 243); *I. monospinus* (n = 19); *I. asanumai* (n = 9); *I. nipponensis*
182 (n = 4); *I. persulcatus* (n = 4); and *H. japonica* (n = 2). **T**he number of adult ticks whose *cox1* was
183 successfully sequenced per species were: *I. ovatus* (346); *H. flava* (243); *I. monospinus* (19); *I.*
184 *asanumai* (9); *I. nipponensis* (4); *I. persulcatus* (4); and *H. japonica* (2) (Table 1).

185 We detected SFGR in 78 (12.44%) out of 627 ixodid ticks, with the highest detected in *I.*
186 *ovatus* (46/346; 13.29%), *H. flava* (15/243; 6.17%), and *I. monospinus* (6/19; 31.58%) as
187 summarized in Table 1. Out of the 46 *Rickettsia*-infected *I. ovatus* ticks, 25 displayed a 100%
188 identity-match with *Rickettsia asiatica* in the 17kDA, *gltA,* and *rOmpB* markers (Arai et al., 2021),
189 while an additional 19 adult *I. ovatus* from the 2018 collection were also positive with *R. asiatica.*
190 Two haplotypes were found in the rOmpB and 17kDA markers, respectively. One haplotype was
191 found in only one individual (17369). Two out of the 15 *Rickettsia*-infected *H. flava* ticks were
192 found to have the same haplotypes in the 17Kda, gltA,and rOmpA markers, and were identified as
193 *Rickettsia sp.* (LC461063). The remaining 13 *Rickettsia*-infected *H. flava* ticks were identified as
194 *Rickettsia sp.* Four out of the six *Rickettsia*-infected *I. monospinus* were identified as *Rickettsia*
195 *helvetica* with the same haplotype found in the 17kDA, gltA, and rOmpB markers.

196 Based on the population genetic analysis of the *cox1* sequences, we found a significant
197 global F_{ST} of 0.4154 at $p < 0.05$ for *I. ovatus* (Table 1). In contrast, no significant global F_{ST} values
198 were observed in the *H. flava, I. monospinus, I. asanumai, I. nipponensis* or *I. persulcatus* samples
199 (Table 1). There were 59 and 66 *cox1* haplotypes found among the 346 *I. ovatus* and 243 *H. flava*
200 individuals, respectively. We found the following number of *cox1* haplotypes in the remaining
201 species: *I. monospinus* (n = 9), *I. asanumai* (n = 4), *I. nipponensis* (n = 4), *I. persulcatus* (n = 3), and
202 *H. japonica* (n = 2).

203 The *cox1* haplotype network of *I. ovatus* (Figure 1) revealed four genetic groups, wherein
204 three genetic groups (1, 2, and 3) were distributed along different altitudinal gradients, as shown
205 in Figure 2. These four genetic groups were concordant with the four clusters found in the *I.*
206 *ovatus* phylogenetic tree (Supplementary Figure 1). The habitat distribution of genetic group 3 was
207 limited to high altitude sites only (range = 16–912 m.a.s.l.), whereas genetic groups 1 (255–
208 471 m.a.s.l.) and 2 (84–1354 m.a.s.l.) were distributed at lower altitudes (Figure 2). The Welch t-
209 test revealed a significant difference between the mean altitudes of genetic groups 1 and 3 at
210 $p < 0.05$ (Table 2); however, no significant difference was observed between genetic groups 1 and
211 2 or groups 2 and 3. We found a significant difference in the *Rickettsia* infection rates between *I.*
212 *ovatus* genetic groups 1 and 2 based on the z-score test, but no significant difference between
213 groups 1 and 3 or groups 2 and 3 (Table 2). The mean altitude between the *Rickettsia*-infected
214 (=273.72 m.a.s.l.) and non-infected *I. ovatus* (=369.61 m.a.s.l.) revealed a significant difference
215 based on the Welch t-test at $p < 0.05$ (Figure 3). The UPGMA dendrogram of *I. ovatus* revealed two
216 genetic clusters, 1 and 2 using the genetic distance among the seven populations excluding one
217 population due to the limited number of samples (Figure 4).

218 The *cox1* haplotype network of *H. flava* displayed two genetic groups (Supplementary
219 Figure 2) consistent with the *H. flava* phylogenetic tree (Supplementary Figure 3). Based on the
220 Welch t-test, we found no significant differences in mean altitude (205 and 165 m.a.s.l.) at $p < 0.05$
221 between the two *H. flava* genetic groups (Supplementary Table 1). No significant difference
222 between the *Rickettsia* infection rates of *H. flava* genetic groups 1 and 2 were observed using the
223 z-score test at $p < 0.05$ (Supplementary Table 1).

224

225 ## 4. Discussion

226 Our findings support our hypothesis that a genetically structured tick population, such as *I.*
227 *ovatus*, can cause the *Rickettsia* infection rate to be spatially heterogenous due to limited gene
228 flow along an altitudinal gradient. The significant global F_{ST} estimate of 0.4154 among the *I. ovatus*
229 populations revealed genetic differentiation between the populations as supported by the
230 occurrence of two genetic clusters in the cluster dendrogram. Our results were consistent with our
231 previous study (Regilme et al., 2021) that suggested that the low mobility of the host species for
232 immature *I. ovatus* contributed to low gene flow in the tick populations. The low *I. ovatus* gene
233 flow along the altitudinal gradient might have caused the spatial heterogeneity of *Rickettsia*
234 infection rates among these populations, which is supported by the significant difference found in
235 *Rickettsia* infection rates between genetic clusters 1 and 2. A similar pattern was observed in the
236 studies of Burri et al. (2007), Jouda et al. (2004), and Moran et al. (2007), which found that the
237 infection rate of *Borrelia burgdorferi*, the causative agent of Lyme disease, decreased in ticks along
238 the altitudinal gradient. Low gene flow can cause infected and uninfected ticks to have limited
239 opportunities to traverse a wider spatial area thus causing heterogeneous *Rickettsia* infection
240 rates (Gooding et al., 1996; Paez-Triana et al., 2021).

241 We found two genetic groups in the *H. flava* haplotype network, but no significant
242 difference in the *Rickettsia* infection rates between the two groups. These results might be due to
243 the high gene flow observed in the *H. flava* populations, which enable *Rickettsia*-infected and
244 uninfected *H. flava* individuals to traverse between the study sites. The high mobility of the large
245 mammalian hosts used by adult *H. flava* and avian-mediated dispersal during their immature stage
246 probably contributed to their homogenized population genetic structure (Regilme et al., 2021),
247 and the resulting homogenized *Rickettsia* infection rates. Large mammalian hosts and birds have a
248 wide dispersal range that enable the broader movement of *Rickettsia*-infected ticks, as observed

249 in previous studies of *Amblyomma americanum* (Reichard et al., 2005; Trout et al., 2010; Mixson
250 et al., 2006), *H. flava* (Regilme et al., 2021), and *I. ricinus* (Casati et al., 2008). Birds are especially
251 good at dispersing over large areas, since they can easily traverse landscape barriers such as
252 mountains, fences, glaciers, and oceans that would be difficult for mammals to cross (Hasle et al.,
253 2013).

254 In addition to host mobility, environmental factors can influence the population genetic
255 structure of ticks (Eremeeva et al., 2015). Each tick species has preferred environmental conditions
256 that are conducive to completing the tick life cycle, thus influencing the geographical distribution
257 of ticks and the risk areas for tick-borne diseases (Gage et al., 2008; Parola et al., 2001). Altitude
258 may influence the population genetic structure of ticks through the effect of altitude on the
259 distribution and abundance of ticks and/or their hosts (Van Treuren et al., 2015; Randolph et al.,
260 1999; Macdonald et al., 2017). Altitudinal differences between populations can affect genetic
261 divergence (Funk et al., 2015), through, for example, ecological isolation, which causes natural
262 selection against maladapted immigrants and limits gene flow (Coyne and Orr, 2004; Nosil, 2012).
263 For example, organisms adapted to low altitudinal sites that cannot tolerate the lower
264 temperatures at higher altitudes would not survive if they dispersed to those higher altitude sites,
265 thus restricting gene flow across the altitudinal gradient (Funk et al., 2015)

266 The difference in mean altitude between *I. ovatus* cluster groups 1 and 2 might be due to
267 adaptive divergence along the altitudinal gradient. Individuals from cluster group 1 were
268 distributed in higher altitude areas of the northern mountainous area of Niigata Prefecture, while
269 cluster group 2 individuals were found in the lower altitude regions of northern Niigata Prefecture.
270 Populations along an altitudinal gradient are prone to differentiating selection pressures, which
271 result in local adaptation (Lim et al., 2021). Altitudinal gradients may also cause the varying
272 ambient temperature, precipitation and humidity levels that are essential to the development and

273 survival of ticks (Juvenal and Edward, 2010). These varying environmental factors may cause ticks
274 to have difficulty dispersing over a wide habitat range. Thus, ticks may need to adapt to extreme
275 habitats, such as extreme altitude or precipitation levels, for survival. For example, *Rhipicephalus*
276 *compositus* were found in altitudes of 1000–2500 m, but optimal conditions were between 1200–
277 2500 m (Juvenal and Edward, 2010; Nzige et al., 1978; Morel, 1981).

278 The different *Rickettsia* infection rates and altitudinal ranges between the *I. ovatus*
279 phylogenetic groups may be explained by adaptive evolutionary theory, which states that
280 organisms adjust to new or severe changes in their environment to become better suited to their
281 habitat (Kawecki and Ebert, 2004; Gregory, 2009). Based on the relationship between the *I. ovatus*
282 phylogenetic groups and their mean attitudes*, I. ovatus* might be undergoing local adaptation
283 along the altitudinal gradient due to the higher genetic differentiation between populations as
284 supported by the significant global F_{ST} (0.4154) found in *I. ovatus*. Based on isolation by
285 environment (IBE), genetic differentiation will increase with increased environmental differences
286 independent of geographic distances (Wang and Summers, 2010; Bradburd et al., 2013; Wang and
287 Bradburd, 2014). When environmental conditions differ, the success of immigrations in a new
288 habitat is reduced, which may increase the genetic fixation rate due to a lower chance of
289 outcrossing; thereby enhancing genetic isolation (Jiang et al., 2019). Thus, the lower gene flow
290 along the altitudinal gradient reduced the spatial homogeneity of *Rickettsia* infection rates among
291 the *I. ovatus* tick populations, thus causing the different *Rickettsia* infection rates obtained.

292 In this study, only a few (n < 20) *I. monospinus, I. asanumai, I. nipponensis* and *H. japonica*
293 were collected; however a high *Rickettsia* infection rate (31.58%–100%) was found despite these
294 limited numbers. In previous studies, high *Rickettsia* infection rates despite few individuals were
295 also observed in *I. monospinus* in several prefectures in Japan (Yamauchi et al., 2009; Ishiguro et
296 al., 2008; Sato et al., 2021). The extent of tick habitat distribution may vary between species,

297 which may have influenced the widely different *Rickettsia* infection rates observed among the
298 species in this study. The high Rickettsia infection rate in these ticks is probably due to effective
299 transovarial transmission (Parola et al., 2013). Additionally, the few collected individuals of these
300 species may be habitat specialists that thrive in a narrow habitat range in contrast to *I. ovatus* and
301 *H. flava*, which may be habitat generalists capable of thriving in a wide habitat range (Richmond et
302 al., 2005). The narrow habitat range of habitat specialist ticks might have enabled increased
303 interactions between infected and uninfected *Rickettsia* ticks within the small spatial scale
304 compared to the habitat generalist ticks, thus causing their high *Rickettsia* infection rates. This
305 conclusion should be considered with caution, since the limited number of individual ticks
306 collected across the Niigata Prefecture may have caused a bias in the estimated *Rickettsia*
307 infection rates determined in this study. We suggest conducting future research of *Rickettsia*
308 infection rates in a wider sampling range with an increased number of *I. monospinus, I. asanumai,*
309 *I. nipponensis*, and *H. japonica* individuals to further increase our understanding of the disease-
310 carrying potential of these species.

311 The occurrence of local adaptation in tick populations could affect the future of the tick-
312 borne disease landscape. Environmental factors, such as precipitation, temperature, and altitude,
313 have been shown to drive population differentiation in insects, such as *Anopheles* mosquitoes and
314 *Drosophila* flies (Cheng et al., 2012; Kapun et al., 2016; Love et al., 2016), but studies on the
315 environmental adaptation of ixodid ticks, such as *I. ovatus*, and its *Rickettsia* infection
316 susceptibility, have not yet been performed. McCoy et al. (2001) suggested that environmental
317 conditions that affect bird hosts can also affect the local adaptation of ticks. Few studies have
318 assessed such local adaptation in multiple organisms with varying dispersal abilities (Exposito et al.,
319 2018, Hancock et al., 2011, Oliviera et al., 2018, Zhen et al., 2017, Ahrens et al., 2019 Miller et al.,
320 2019) and is an area in need of future research.

321 Since ticks are obligate vectors, they directly influence their mammalian hosts and the
322 pathogens they transmit (Anderson and Magnarelli, 2008; Hill and Wikel, 2005; Van Zee et al.,
323 2007). The interaction between the vector (tick), host, and pathogen (*Rickettsia*) is essential in
324 understanding and predicting the risk and transmission of tick-borne diseases (Jongejan et al.,
325 2007). Understanding the genetic structure of ticks can serve as an alternative indicator to infer
326 the potential spread of pathogens, such as *Rickettsia* (Gray, 2002). Our study found relationships
327 between (1) the population genetic structure of ticks and the corresponding *Rickettsia* infection
328 rates, (2) altitude and the population genetic structure of ticks, and (3) altitude and *Rickettsia*
329 infection rates. We found that host mobility may influence the genetic structure of ixodid ticks.
330 This information can be used to design more effective tick-borne disease control programs that
331 focus on screening and detecting pathogens found in ticks and their mammalian hosts. For
332 example, patterns of disease transmission from ticks with a high genetic divergence and less
333 mobile hosts, such as *I. ovatus*, are likely due to the movement of infected hosts rather than
334 infected ticks. Thus, screening prospective tick hosts for *Rickettsia* infection would be more
335 suitable in this example. We suggest screening the hosts of immature *I. ovatus*, such as small
336 rodents, instead of screening ticks. The *Rickettsia* infection rate in tick genetic groups can predict
337 the spread of tick-borne diseases caused by *Rickettsia,* such as the Japanese spotted fever. We
338 also found that altitude may influence the *Rickettsia* infection rate of *I. ovatus* genetic groups. This
339 information can be used to determine the high-risk areas (e.g., lowland, mountains etc.) of tick-
340 borne diseases along an altitudinal gradient. Genetically structured arthropod vectors, such as
341 ticks, can have different vector competencies, and environmental factors, such as altitudinal
342 gradients, can influence the vector's ability to acquire, transmit, and maintain the pathogen
343 infection (Mclain et al., 1995).

344

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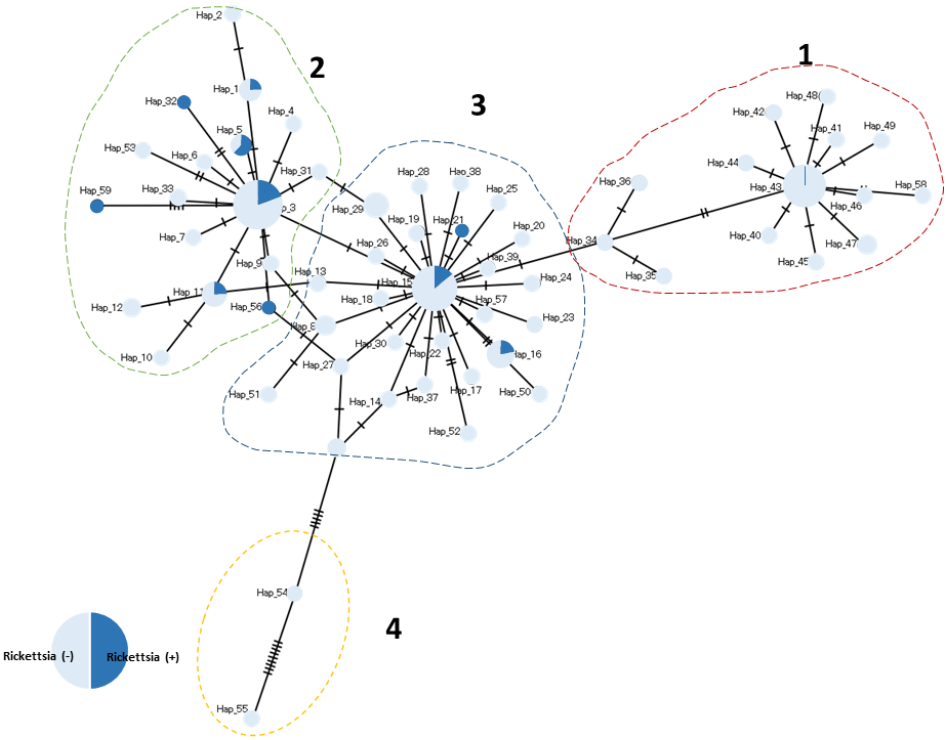
605 **Figure Captions**

606 **Figure 1.** Median-joining network of the 59 *cox1* haplotype sequences of *Rickettsia* positive and

607 negative *I. ovatus.* Haplotype groups are indicated by numbers (1 to 4).



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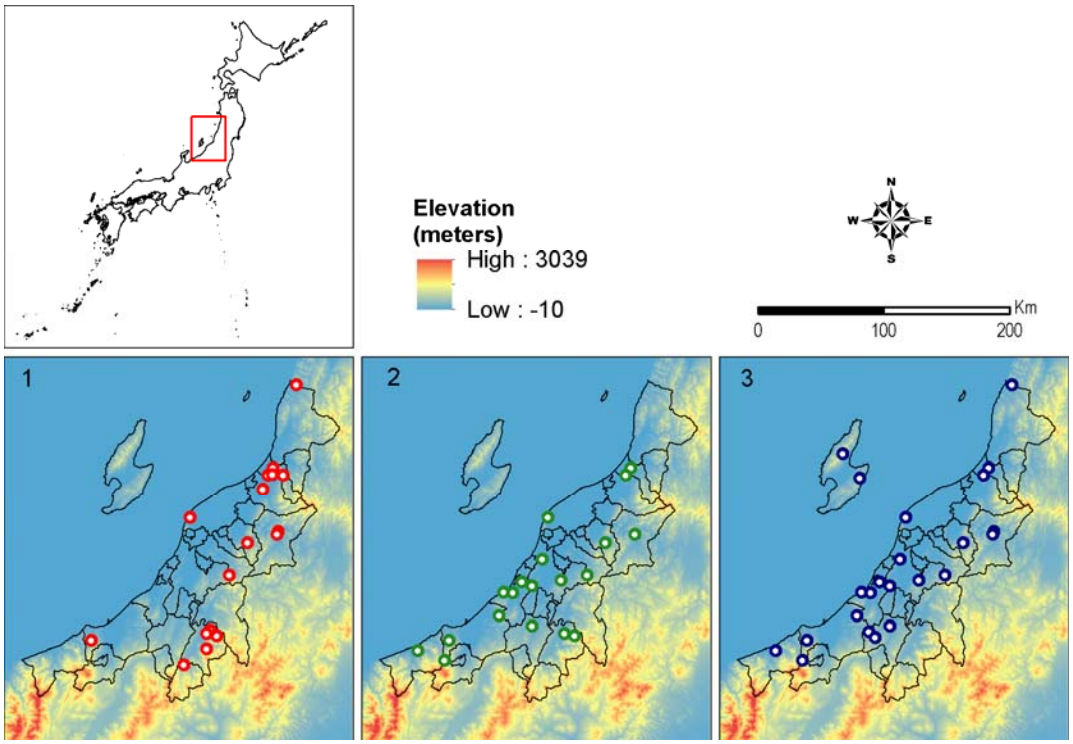
615 **Figure 2.** The influence of altitude on the habitat distribution of *I. ovatus*. The points indicate the
616 sampling sites of the collected individuals per *I. ovatus* haplotype group (n = 3) in the haplotype
617 network (Figure 1). The map shows the elevation level across the sampling area, with Niigata
618 Prefecture Japan depicted as a color gradation. Group 4, not shown in this figure, with haplotypes
619 54 and 55 were found on Sado island, which is encircled in black in box 3.

620 

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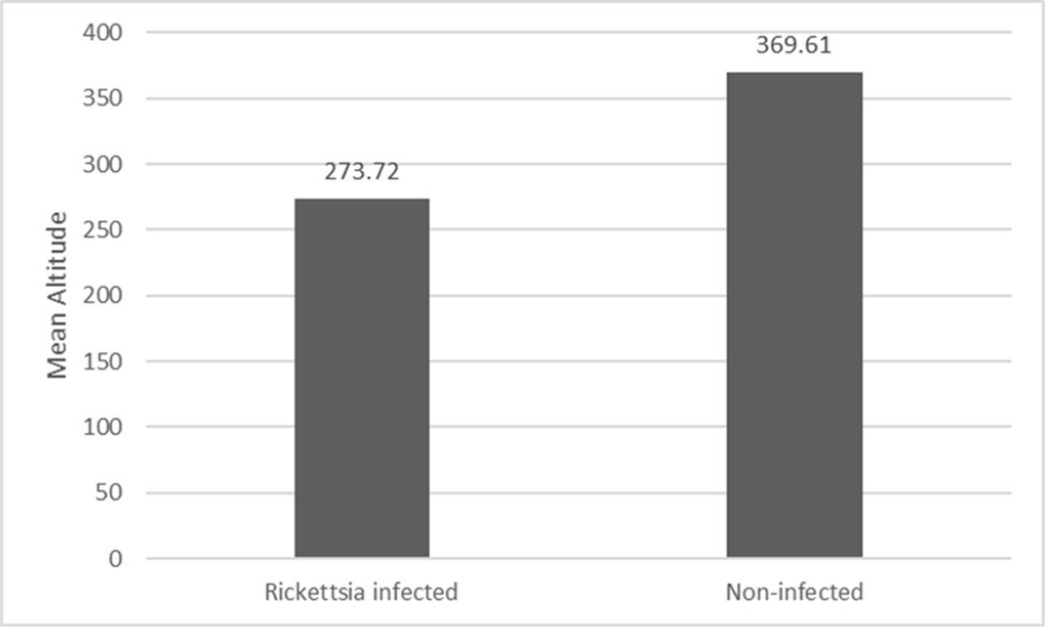
626 **Figure 3.** The relationship between the mean altitude of *Rickettsia-infected* (n = 46) and non-
627 infected (n = 300) *I. ovatus.* Welch t-tests revealed a significant difference in the mean altitude
628 between *Rickettsia-infected* and non-infected *I. ovatus* at $p < 0.05$. Populations with combined
629 sites are labeled A to H.



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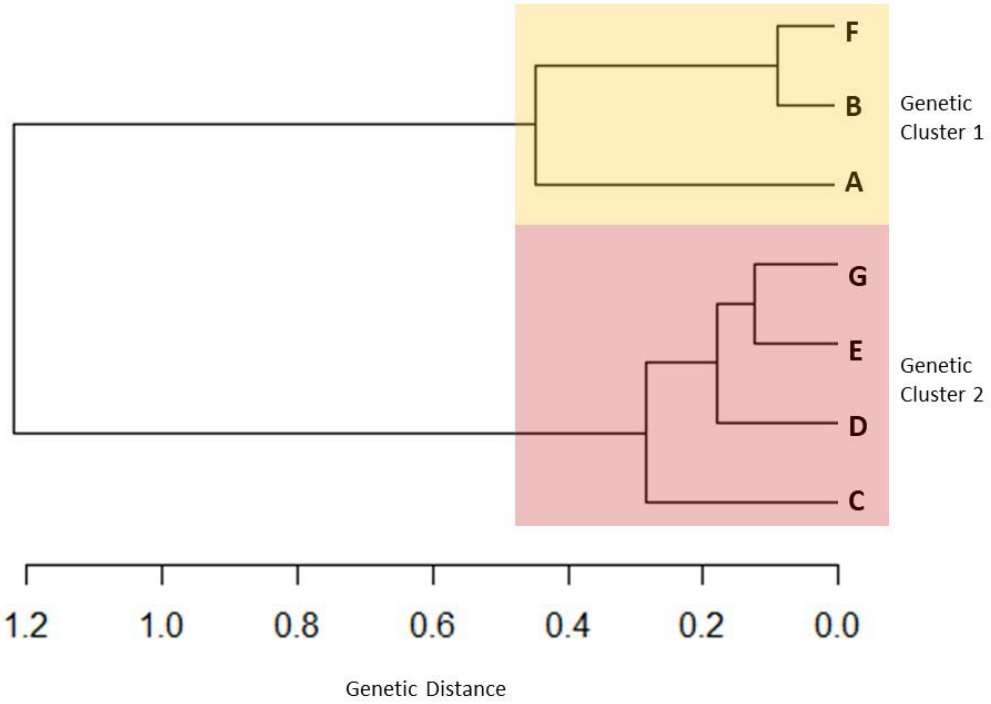
636 **Figure 4.** An unweighted pair group method with the arithmetic mean (UPGMA) dendrogram of *I.*

637 *ovatus* based on the pairwise genetic distance (FST) of *cox1* among the 7 populations across

638 Niigata Prefecture, Japan. We excluded one population due to limited number of samples.



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641 **Tables**

642 **Table 1**. Summary of the haplotype and *Rickettsia* infection rates among the 7 ixodid tick species

643 obtained in the

644 Niigata

Tick species ***	ns	n	nh	r	Global F_{ST}
1. *Ixodes ovatus*	29	346	59	46 (13.26%)	0.4154*
2. *Haemaphysalis flava*	18	243	66	15 (6.17%)	0.3597
3. *Ixodes monospinus*	6	19	9	6 (31.58%)	0.9034
4. *Ixodes asanumai*	7	9	4	5 (55.55%)	1.0000
5. *Ixodes nipponensis*	2	4	4	4 (100%)	0.3345
6. *Ixodes persulcatus*	3	4	3	0 (0%)	1.0000
7. *Haemaphysalis japonica*	2	2	2	2 (100%)	**
Total		628		78	

645 Prefecture, Japan

646 Abbreviations: ns no. of sampling sites; n sample size; nh no of haplotypes; r rickettsia infection rate per species; *p < 0.05** not

647 enough samples for analysis***tick species identification is based on molecular identification using the *cox1* marker and BLAST results

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649 **Table 2**. The differences in *Rickettsia* infection rates and mean altitude in *I. ovatus* haplotype
650 groups and cluster dendrogram groups. The table shows the distribution of *Rickettsia*-infected and
651 uninfected *I. ovatus* and the mean altitude in each of the haplotype groups as shown in Figure 1 *(I.*
652 *ovatus* haplotype network) and the cluster dendrogram in Figure 4 (*I. ovatus* cluster dendrogram).
653 The z-score test showed a significant difference at $p < 0.05$ between the *Rickettsia* detection rates
654 in haplotype groups 1 and 2 (indicated by [ab)] and in cluster dendrogram groups 1 and 2.he Welch t-
655 test at $p < 0.05$ revealed a significant difference in the mean altitude of haplotype groups 1 and 3
656 and cluster dendrogram groups 1 and 2 (indicated by [ab]). Haplotype group 4 was not included in
657 the analysis due to its low sample size.

658

Haplotype group	**Rickettsia infection rate**			**Mean altitude**
	Positive	Negative	Detection rate	
1	1	92	1.07%[ab]	294[ab]
2	28	99	22.04%[ab]	341[a]
3	16	110	12.70%[a]	374[ab]
Dendrogram Group				
1	4	120	3.23%[ab]	30
2	40	227	14.98%[ab]	345

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661 **Supplementary Tables and Figures**

662 **Supplementary Table 1.** The difference in *Rickettsia* infection rates in *H. flava* haplotype groups
663 and a table showing the distribution of *Rickettsia*-infected and uninfected *H. flava* in each genetic
664 groups as shown in Supplementary Figure 2 *(H. flava* median-joining network). The results of the z-
665 score test for two populations proportions at $p < 0.05$ showed no significant difference between
666 the *Rickettsia* detection rate in haplotype groups 1 and 2. The Welch t-test at $p < 0.05$ revealed no
667 significant difference in the mean altitude of the two groups.

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Haplotype group	*Rickettsia* infection			Mean altitude
	Positive	Negative	Detection rate	
1	1	45	2.17%[ab]	205[ab]
2	14	183	7.11%[ab]	165[ab]

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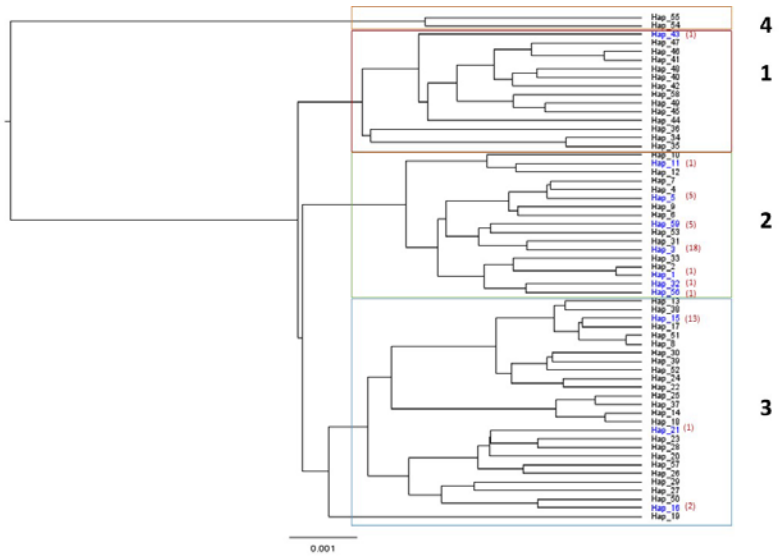

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675 **Supplementary Figure 1.** Phylogenetic tree from the BEAST analysis of 59 haplotype *cox1*

676 sequences of *I. ovatus.* The blue labeled haplotypes indicate the presence of *Rickettsia* infection.

677 The red parentheses provide the number of *Rickettsia* positive individuals per haplotype. The black

678 labeled haplotypes are negative for *Rickettsia* infection.

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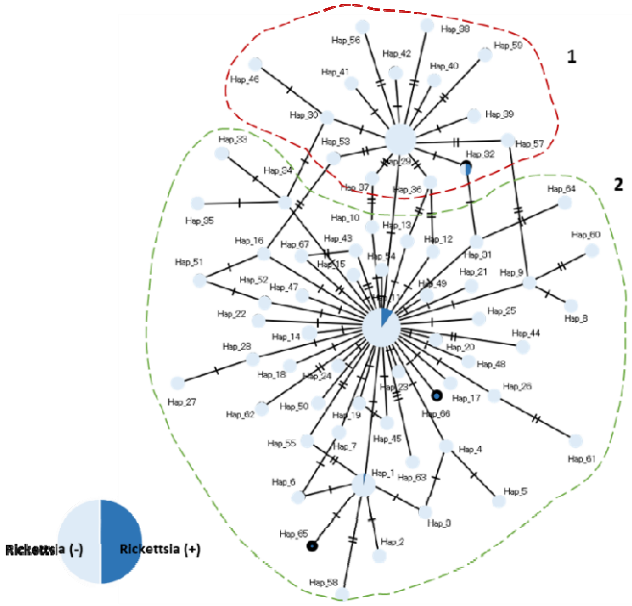

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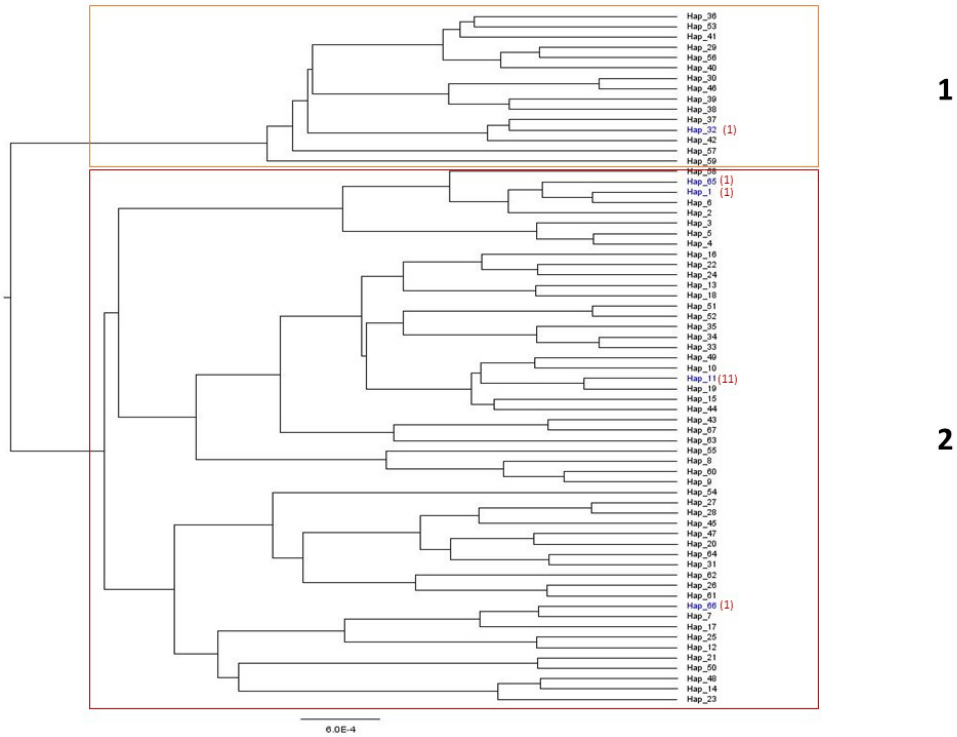
681 **Supplementary Figure 2.** Median-joining network of the 66 *cox1* haplotype sequences of

682 *Rickettsia* positive and negative *H. flava.* Haplotype groups are indicated as 1 and 2.



Supplementary Figure 3. Phylogenetic tree from the BEAST analysis of 66 haplotype *cox1* sequences of *H. flava.* The blue labeled haplotypes indicate *Rickettsia* infection in individual samples. The parentheses in red provide the number of *Rickettsia*-infected ticks. The black labeled haplotypes are negative for *Rickettsia* infection.