Comparative population genetic structure of two ixodid tick species (*Ixodes ovatus* and *Haemaphysalis flava*) in Niigata Prefecture, Japan

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Abstract

Ixodid tick species such as *Ixodes ovatus* and *Haemaphysalis flava* are essential vectors of tick-borne diseases in Japan. In this study, we investigated the population genetic structures and gene flow of *I. ovatus* and *H. flava* as affected by the tick host mobility. We hypothesized that *I. ovatus* and *H. flava* may have differences in their genetic structure due to the low mobility of small rodent hosts of *I. ovatus* at the immature stage in contrast to the mediated dispersal of avian hosts for immature *H. flava.* We collected 307 adult *I. ovatus* and 220 adult *H. flava* from 29 and 17 locations across Niigata Prefecture, Japan. We investigated the genetic structure at two mitochondrial loci (*cox1*, 16S rRNA gene). For *I. ovatus*, pairwise F_{ST} and analysis of molecular variance (AMOVA) analyses of *cox1* sequences indicated significant genetic variation among populations. Both *cox1* and 16S rRNA markers showed non-significant genetic variation among locations for *H. flava*. The Bayesian tree and haplotype network of *cox1* marker for *I. ovatus* samples in Niigata Prefecture found 3 genetic groups wherein most haplotypes in group 2 were distributed in low altitudinal areas. When we added *cox1* sequences of *I. ovatus* from China to the phylogenetic analysis, three genetic groups (China 1, China 2, and Niigata and Hokkaido, Japan) were formed in the tree suggesting the potential for cryptic species in the genetic group in Japan. Our results support our hypothesis and suggest that the host preference of ticks at the immature stage may influence the genetic structure and gene flow of the ticks. This information is vital in understanding the tick-host interactions in the field to better understand the tick-borne disease transmission and in designing an effective tick control program.

Keywords: genetic structure, gene flow, host mobility, cryptic species, haplotypes

43 **Introduction**

44 Tick-borne diseases are a public health concern, and their control is often challenging
45 because of the complex transmission chain of vertebrate hosts and ticks interacting in a changing
46 environment (Dantas-Torres et al., 2012). Population genetic studies helped understand the
47 dispersal patterns and potential pathogen transmission in ticks (Araya-Anchetta et al., 2015).
48 Population genetic studies can elucidate the direction, distance, and gene flow patterns between
49 tick populations (McCoy, 2008). For example, if a high gene flow is observed, there might be a
50 greater chance of colonizing new areas or re-colonizing areas following successful vector control
51 programs.

52 Due to the small size of ticks and their vulnerability to harsh environments during off-host,
53 tick dispersal is complex and is linked to its host movement (Falco and Fish, 1991). Host mobility can
54 affect the genetic patterns of tick populations, although its effects are not consistent. Several studies
55 have reported low gene flow in ticks with less mobile hosts (e.g., smaller mammals) and a high gene
56 flow in ticks with highly mobile hosts (Araya-Anchetta et al., 2015). For example, *Amblyomma*
57 *americanum* and *Amblyomma triste* (Acari, Ixodidae) exhibited a high gene flow across various
58 spatial scales (137,000 km^2 to 2.78 million km^2) due to their hosts' dispersal capabilities (large
59 mammals and birds) (Guglielmone et al., 2013; Mixson et al., 2006; Trout et al., 2010). Lampo et al.,
60 (1998) observed low gene flow in *Amblyomma dissimile* populations due to its hosts' low mobility
61 (small mammals, reptiles, and salamanders).

62 In Japan, tick-borne diseases are an increasing public health concern, affecting humans and
63 animals (Yamaji et al., 2018). A total of 8 genera of ticks have been recorded in Japan, composed of
64 47 species: 43 ixodid tick species and four argasid species (Fujita et al., 2006). Out of these 47 species,
65 21 species parasitize humans (Okino et al., 2010). Among these 21 species, *Ixodes ovatus*, the

66 primary vector of the causative agents of Lyme borreliosis (Miyamoto et al., 1993), and
67 *Haemaphysalis flava,* a vector of the causative agents of severe fever with thrombocytopenia
68 syndrome (SFTS) and Japanese spotted fever (JSF), was reported in Japan (Yamaji et al., 2018; Yu et
69 al., 2014). Yamaguti et al., (1971) observed that the hosts of adult *I. ovatus* were mainly hares but
70 could also be large mammals (e.g., cows and horses) and that the hosts of immature forms were
71 small rodents. They also found that the host preferences of adult *H. flava* were cows, dogs, horses,
72 wild boar, bear, and deer, while the immatures parasitized birds. Comparative population genetic
73 studies on two essential ixodid ticks: *I. ovatus* and *H. flava* are not existing.

74 In addition to population genetic structure, genetic analysis may also reveal the
75 phylogenetic pattern and the presence of cryptic species, where morphologically identified
76 individuals might represent more than one species (Fegan et al., 2005). Previous studies on
77 *Rhipicephalus appendiculatus* (Kanduma et al., 2016), *Ixodes holocyclus* (Song et al., 2011), and *I.*
78 *ovatus* (Li et al., 2018) have revealed the presence of cryptic species based on the clustering of
79 haplotypes in a phylogenetic tree. Based on these previous studies, morphological criteria for
80 species differentiation alone are equivocal and that genetic analysis is essential.

81 Here, we studied the population genetic structure of *I. ovatus* and *H. flava* and also tested
82 for the presence of cryptic species using mitochondrial DNA sequences of *cox1* and the 16S rRNA
83 markers. We hypothesized that *I. ovatus* and *H. flava* would display contrasting population genetic
84 structures despite the overlap sharing of large mammalian hosts at the ticks' adult stage. The low
85 mobility of *I. ovatus'* host, mainly hares at the adult tick stage and small mammals during the
86 immature stage may contribute to the ticks' high genetic divergence. On the other hand, the
87 combined high mobility of large mammalian host at *H. flava* adult stage and avian mediated
88 dispersal at the immature stage may be affecting the ticks' homogenized population genetic
89 structure.

Material and methods

Study site, collection, sampling, and identification

From April 2016 until November 2017, ticks were collected by the standard flagging methods (Ginsberg et al., 1989) in 29 sites (Additional File 1. Table S1) across Niigata Prefecture in Japan. Ticks were collected 2 to 14 times in 6 core sites among the 29 sites (Additional File 2. Table S2). Altitude per location ranged from 8 from 1402 m/a.s.l (mean=350). The geographic distance between sites ranged from 4.28 to 247.65 km (mean=77.36). Collected ticks were stored in microcentrifuge tubes with 70% ethanol at 4^0C. We identified the sex, developmental stage, and species using a compound microscope and identification keys of Yamaguti et al., (1971).

DNA extraction, PCR amplification, and sequencing

Genomic DNA (*I. ovatus* n=320*; H. flava* n=220) from each identified adult tick individual was extracted using the Isogenome DNA extraction kits (NIPPON GENE Co. Ltd., Tokyo, Japan) following the manufacturer's recommended protocol. The total number of analyzed samples (n=540) was chosen based on its species identity, while the other identified tick species were excluded from this study. Before DNA extraction, each tick was washed with alcohol and a PBS solution. DNA concentration and quality were checked using a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific™). Two mitochondrial genes were amplified by polymerase chain reaction (PCR): Cytochrome Oxidase 1 (*cox1*) (658 base pairs) using the primer pairs LCO-1490 (5'- GGTCAACAAATCATAAAGATATTGG -3') and HCO1-2198 (5'- AAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994) and the 16S rRNA gene (16S) (407 base pairs) with the following primer pairs 16S+1 (5'CTGCTCAATGAATATTTAAATTGC-3') and 16S – 1 (5' -CGGTCTAAACTCAGATCATGTAGG-3') (Tian et al., 2011). All PCR amplifications of both *cox1* and 16S were performed with a final volume of 10 µl with 1 µl of genomic DNA. The PCR reaction for both markers was composed of the

113 following: 10x Ex Taq buffer, 25 mM $MgCl_2$, 2.5 mM dNTP, 10 µm of forward and reverse primers,
114 and five units/µl of TaKaRa Ex Taq™ (Takara Bio Inc.). The *cox1* PCR amplification was as follows: an
115 initial denaturation of 94^0C for 2 min, denaturation of 94^0C for 30 s, annealing of 38^0C for 30 s, an
116 extension of 72^0C for 1 min for 30 cycles, and a final extension of 72^0C for 10 min. In contrast, the
117 16S PCR amplification followed the protocol of (Tian et al., 2011) with some modifications, initial
118 denaturation of 94^0C for 3 min, denaturation of 94^0C for 30 s, annealing of 50^0C for 40 s, an extension
119 of 72^0C for 40 s for 30 cycles and final extension of 72^0C for 5 min. PCR products were purified using
120 the QIAquick 96 PCR Purification Kit (Qiagen) following the manufacturer's instructions and
121 sequenced by Eurofin Genomics, Inc., Tokyo, Japan.

122 **Sequence data analysis**

123 We assembled forward and reverse reads for each individual using CodonCode Aligner
124 version 1.2.4 software (https://www.codoncode.com/aligner/). No ambiguous bases were observed,
125 and the low-quality bases were removed at the start and end of the reads. Multiple alignments of
126 sequences were performed using the MAFFT alignment online program with default settings
127 (https://mafft.cbrc.jp/alignment/server/). To ensure sequence quality and correct species
128 identification, we checked the sequences' similarity against reference sequences from GenBank
129 using BLASTN (Basic Local Alignment Search Tool – Nucleotide,
130 https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=bl
131 asthome). We checked the quality of the final aligned sequences (*cox*1 = 658bp; 16S = 407bp) in
132 Mesquite version 3.5 (Maddison et al., 2011) protein-coding genes were translated into amino acids
133 to confirm the absence of stop codons.

134 **Population genetic analysis**

135 We combined the nearby sites with a geographic distance range of 8.83 km to 79.81
136 (mean=44.00) with less than 8 individuals for the population genetic analysis because the accurate
137 estimation of allele frequencies is difficult for small size population. A total of 8 populations (A to H)
138 (Additional File 1 Table S1) were formed. Some populations were not included in the population
139 genetic analysis because of the small sample size (< 8 individuals) and can't be combined with any
140 nearby sites.

141 We analyzed the sequences of both *H. flava* and *I. ovatus* per genetic marker separately
142 using DNASp version 6.12.03 (Rozas et al., 2017) and Arlequin version 3.5.2.2 (Excoffier and Lischer,
143 2010) for the following parameters: number of haplotypes (nh), the average number of polymorphic
144 sites (s), and average number of nucleotide differences (k). The haplotype diversity (h) and
145 nucleotide diversity (π) were calculated in Arlequin version 3.5.2.2. The population genetic structure
146 among and within populations in Niigata Prefecture was assessed by analysis of molecular variance
147 (AMOVA) performed in Arlequin with 9999 permutations. The degree of molecular genetic
148 differentiation between populations was assessed by calculating the pairwise F_{ST} values using
149 Arlequin.

150 To determine if the genetic differentiation was influenced by geographical distance or
151 altitudinal differences among populations, we performed Mantel Test in GenAlEx version 6.51b2
152 (Peakall and Smouse, 2006). Two tests per species and marker were conducted. First, we compared
153 pairwise genetic (pairwise F_{ST} values) and geographical distances (km), and second, we analyzed the
154 genetic distance (F_{ST} values) with altitudinal differences (m/a.s.l.) calculated from GenAlex version
155 6.51b2. The geographic distances were obtained from the geographic midpoint of the populations
156 using the GPS coordinates (latitude and longitude) of each site recorded during the sampling. The
157 altitudinal differences are the calculated mean value per population. All Mantel tests were assessed
158 using 9999 permutations for the significance of the correlation.

159 The genetic relationships among the populations were calculated by the unweighted pair
160 group method with the arithmetic mean (UPGMA) cluster method using the APE package (Paradis
161 and Schliep, 2018) and R program (R Development Core team., 2016). To create a dendrogram, we
162 used the genetic distance matrix (pairwise F_{ST} values) generated from GenAlEx.

163 **Haplotype network and Phylogenetic analysis**

164 To evaluate the haplotypes' relationship, we constructed a haplotype network on the
165 PopART program version 1.7 per marker (*cox1* and 16S) and species (*I. ovatus* and *H. flava*
166 (http://popart.otago.ac.nz/index.shtml) using the median-joining (MJ) network algorithm (Bandelth
167 et al., 1999).

168 We performed a Bayesian phylogenetic analysis using BEAST v1.10.4 (Drummond and
169 Rambaut, 2007) to determine the phylogenetic structure of *I. ovatus* haplotypes within Niigata
170 Prefecture using the *cox1* marker. Additional sequences from China were not included in the
171 Bayesian analysis. We used the HKY substitution model with the estimated base frequencies. The
172 strict clock model was employed and the speciation yule process as the tree prior. A maximum clade
173 credibility tree was acquired using the TreeAnnotator v1.10.4 from the many trees obtained from
174 BEAUti v1.10.4 with 10% of trees used during burn-in. The maximum clade credibility tree was
175 viewed using FigTree v1.4.4.

176 We constructed maximum likelihood (ML) gene trees for *cox1* and 16S haplotype sequences
177 of *I. ovatus* and *H. flava* using PhyML version 3.1 (Guindon and Gascuel, 2003) default settings. We
178 applied HKY and GTR nucleotide substitution models for *cox1* and 16S, respectively, as suggested by
179 jModelTest version 2 (Darriba et al., 2012). Additional sequences from China (MH208506,
180 MH208512, MH208514, MH208522, MH208515-19, MH208524, MH208531, MH208574,
181 MH208577, MH208579, MH208681-87, MH208689-93, MH208706, KU664519 (Li et al., 2018),

182 Japan (Hokkaido AB231670, U95900; Yamanashi AB819241, AB819243 and Aomori AB819244)
183 (Mitani et al., 2007; Norris et al., 1999; Takano et al., 2014) were included to know the presence of
184 cryptic species. We used *Ixodes canisuga* as an outgroup because it is closely related to *I. ovatus* and
185 *H. flava* (KY962023 and KY962074; Hornok et al., 2015).

186 **Results**

187 A total of 2,374 individual ticks was collected. Adult and immature *Ixodes nipponensis*,
188 *Dermacantor taiwanensis*, *Ixodes persulcatus*, and *Ixodes monospinus* were also identified and used
189 for another research study. In this study, adult *I. ovatus* and *H. flava* were only analyzed because
190 these two species were the most abundant. Larval ticks were used in another study. The number
191 of *I. ovatus* ranged from 1 to 36 adult individuals per site and were more successfully sequenced for
192 *cox1* (307/320; 95.9%) than for 16S (284/320; 88.8%). The analyzed *H. flava* ranged from 1 to 77
193 adult individuals per site and were also more successfully sequenced for *cox1* (220/223; 98.7%) than
194 for 16S (172/223; 77.1%). On the other hand, for the population genetic analysis, each population
195 (i.e., combined nearby sites) had the following tick individual range and mean per markers: *cox1 I.*
196 *ovatus* (28 to 62; mean=43), 16S *I. ovatus* (24 to 66; mean=40), *cox1 H. flava* (8 to 81; mean=43) and
197 16S *H.flava* (8 to 76; mean=34). There were 60 and 63 *cox1* haplotypes and 24 and 40 16S haplotypes
198 in *I. ovatus* and *H. flava,* respectively (Table 1). Haplotype diversity (h) ranged from 0.442 to 0.852,
199 and nucleotide diversity (π) ranged from 0.001 to 0.006 for both markers and species.

200 AMOVA results revealed a high among-population divergence (61.99%) in *I. ovatus cox1*
201 sequences, whereas both *cox1* and 16S markers of *H. flava* indicated no significant genetic variation
202 among populations (Table 2). Significant global F_{ST} values ($P<0.01$) were observed for *I. ovatus* with
203 significant values 0.3801 in *cox1* and 0.0378 in 16S markers. Pairwise F_{ST} values (0.0963 to 0.6808)
204 of *cox1* marker for *I. ovatus* showed significant genetic differences between most pairs of
205 populations such as between populations B and D and populations F and D (Additional File 3 Table

206 S3). We also observed significant genetic differences in 16S *I. ovatus* sequences ranged from 0.0514
207 to 0.0949 (Additional File 4 Table S4). Pairwise F_{ST} values from the *cox1* marker of *H. flava* showed
208 no significant genetic differences except in few population pairs such as A and C, C and E and D and
209 E (Additional File 5 Table S5). No significant genetic differentiation was also observed in 16S marker
210 of *H. flava* except between A and C (Additional File 6 Table S6).

211 The Mantel test of *I. ovatus* showed no significant isolation by geographic distance in both
212 markers: (*cox1* r = 0.108, p = 0.269; 16S r = 0.518, p = 0.065) (Additional File 7 Figure S1, a and b)
213 and no evidence for isolation by altitudinal difference (*cox1* r = -0.066, p=0.225; 1S r = -0.023, p =
214 0.577). Both Mantel tests were also not significant for *H. flava* (distance: *cox1* r= 0.444, p= 0.130;
215 16S r = 0.355, p= 0.189; altitude: r = 0.092, p = 0.30; 16S r = 0.217, p= 0.06) (Additional File 7 Figure
216 S1, c and d).

217 The UPGMA cluster dendrogram constructed from the pairwise F_{ST} values of *I. ovatus* for the
218 *cox1* marker (Figure 2) revealed two genetic clusters among the 7 populations, where cluster 2
219 included populations from the more western sites. In contrast, cluster 1 (A, B, and F) mostly had
220 populations in the northern and southern parts of Niigata Prefecture (Figure 3) and are distributed
221 in mountainous areas with high elevation. We observed no evident genetic clustering on the
222 dendrogram of *I. ovatus* for 16S marker and those of *H. flava* for *cox1* and 16S makers, respectively
223 (Additional File 8 Figure S2; Additional File 9 Figure S3; Additional File 10 Figure S4).

224 The haplotype network with the reference sequences from China and the Bayesian tree of
225 the *I. ovatus cox1* haplotypes showed similar patterns (Figure 5 and 6) of 4 genetic groups. The
226 haplotype network of the *cox1* marker of *I. ovatus* also displayed the two distinct haplotypes (Hap
227 60 and Hap 59) (Figure 6; Additional File 11 Figure S5). These two haplotypes were found from
228 sampling site 6 (Pop A) and sampling site 26 (Pop 6). The Bayesian tree of *I. ovatus cox1* haplotype
229 sequences with reference sequences from China also displayed the 4 genetic groups (Additional File

230 12 Figure S6). The haplotype network of *I. ovatus* from 16S marker (Additional File 13 Figure S7), *H.*
231 *flava* in both cox1 (Additional File 14 Figure S8) and 16S markers (Additional File 15 Figure S9) did
232 not display any distinct haplotypes and genetic groups.

233 A putative *I. ovatus* species complex was identified in the phylogenetic tree based on three
234 distinct haplotype groups found with both *cox1* and 16S rRNA markers; 2 groups in China and one
235 in Japan. The *cox1* phylogenetic tree of 60 *I. ovatus* haplotypes indicated three groups: group 1 of
236 the published sequences from western China, one large group 2 containing the 58 haplotypes and
237 two divergent haplotypes (Hap 60 and Hap 59), and group 3 from west China (Figure 4). The
238 published haplotype from Hokkaido, Northern Japan (Mitani et al., 2007) occurred within our large
239 group, and the published sequences from western China were in a different group.

240 The *I. ovatus* haplotypes in the 16S phylogenetic tree (Additional File 16 Figure S10) were
241 grouped with the published haplotypes from Japan (Yamanashi and Aomori Prefectures; Takano et
242 al., 2014) and Hokkaido (Norris et al., 1999). The *cox1* haplotype sequences of *H. flava* phylogenetic
243 tree (Additional File 17 Figure S11) were similar to reference sequences from China KY021800 –
244 KY021807, KY021810 – KY021819 and KY003181 (Li, Z., Cheng, T. and Liu, G.; Unpublished results
245 from NCBI); JQ625688 – JQ625689, JF758632 and JQ737097 (Lu et al. 2013) and JG737097 (Gou, H.,
246 Guan, G., Yin, H. and Luo, J.; unpublished results from NCBI). The *H. flava* 16S haplotype sequences
247 (Additional File 18 Figure S12) showed high similarities with reference sequences from Japan
248 (Kagoshima, Fukui Aomori, Fukui, Yamanashi, Kagawa, and Ehime Prefectures (Takano et al., 2014)
249 and China KC844858 –KC844867 (Cheng et al., 2013); KX450280 –KX450282 (Zhang, Y., Cui, Y., Peng,
250 Y., Yan, Y., Wang, X. and Ning, C. Liu, Q., Zhang, Y. and Zhu, D.; unpublished results from NCBI);
251 MG696720 (Zheng, W., Chen, S. and Chen, H.; unpublished results from NCBI) and KP324926 (Liu,
252 Q., Zhang, Y. and Zhu, D.; unpublished results from NCBI) sequences from GenBank.

253 **Discussion**

254 **Contrasting population genetic structures between *I. ovatus* and *H. flava***

255 Our results supported our hypothesis that *I. ovatus* may display high genetic divergence
256 because of its low host mobility in contrast to the homogenized population genetic structure in *H.*
257 *flava* due to the combined large mammalian hosts at the ticks' adult stage and avian mediated
258 dispersal during the ticks' immature stage. The significant F_{ST} estimates in *I. ovatus cox1* (0.3801)
259 and 16S (0.0378) revealed population differentiation as supported by the high among populations
260 percentage variation (61.99) in the AMOVA analysis. The high genetic divergence in *I. ovatus* can be
261 due to the low mobility of its hosts, for example during the ticks' adult stage wherein they utilized
262 mostly hares while small rodents at the immature stage of *I. ovatus.* The host preference of *I. ovatus*
263 may also contribute to the observed 4 genetic groups in the Bayesian tree and the haplotype
264 network analysis. The haplotypes from groups 2 and 3 were mostly from low altitudinal areas as
265 compared to the haplotypes from group 1 with ticks from the high altitudinal area such as in
266 mountain areas. We assume that the large mammalian hosts such as cows and horses of *I. ovatus*
267 have enabled the ticks to reach high mountain ranges from group 1. The distribution of the type of
268 hosts may also affect altitude affecting also the formation of groups 1, 2, and 3 in the *cox1 I.ovatus*
269 dendrogram.

270 On the other hand, the homogenized population genetic structure observed in *H. flava*
271 might be because of the combined high host mobility of large mammals at the ticks' adult stage and
272 the avian mediated dispersal at the immature stage. Twenty-eight species of birds were previously
273 reported as hosts of immature *H. flava* from Japan, mostly from the order Passeriformes (Yamauchi
274 and Takeno, 2000). Large mammals and birds may have expansive habitats ranges that may allow
275 high gene flow of *H. flava* between the locations in Niigata, as previously observed in *Amblyomma*
276 *americanum* populations (Mixson et al., 2006; Reichard et al., 2005; Trout et al., 2010) and *I. ricinus*
277 (Casati et al.,2008).

278 Several alternative factors such as tick behavior, biology, and ecology can also affect tick's
279 dispersal patterns. A previous study on two tick species: *Hyalomma rufipes* and *Amblyomma*
280 *hebraeum,* also displayed contrasting genetic patterns despite the overlap sharing of highly mobile
281 hosts such as birds (Cangi et al., 2013). This might be due to the species specific capacity of the
282 immature stages of ticks to survive even after host detachment in various habitat conditions
283 (Cumming, 1999; Estrada-Peña, 2015; Needham et al., 1986). Population genetic structure can also
284 be influenced by assortative mating (e.g., *I. ricinus*) wherein genetically similar individuals tend to
285 mate rather than random mating resulting in decreased genetic divergence (Kempf et al., 2009). Our
286 study does not have supporting data to test these alternative factors; thus, it is suggested in future
287 studies to analyze further these factors that could play a role in the tick's dispersal movement.

288 16S marker showed significant genetic differentiation of *I. ovatus* (pairwise F_{ST} =0.0514 to
289 0.0949) in few pairs of sampling locations as compared to the *cox1* marker, which may be due to
290 lower variability of the marker thus providing inadequate genetic variation for population genetic
291 analysis, as previously observed in *Amblyomma ovale* (Bitencourth et al., 2019) and *Rhipicephalus*
292 *microplus* (Burger et al., 2014; Low et al., 2015). The low variability of 16S marker in *I. ovatus* may
293 not be sufficient to infer an intraspecific relationship and is further supported by its low (*nd* = 0.001)
294 nucleotide diversity as compared to *cox1* marker (*nd* = 0.004). Thus, it may also explain the grouping
295 observed in the *cox1* phylogenetic tree of *I. ovatus* was absent in the 16S tree. Despite these, we
296 used the 16S marker because this gene mutates at a rate that is informative for inter-species level
297 phylogenetic analysis (Araya-Anchetta et al., 2015). The negative pairwise F_{ST} values observed in *H.*
298 *flava* do not have any biological meaning. These results from the unbiased estimator allowing a
299 negative F_{ST} when the sample size is small. (Bitencourth et al., 2016; Vial et al., 2006; Weir and
300 Cockerham, 1984) .

301 **Species complex formation in *I. ovatus cox1* sequences**

302 Our *cox1 I. ovatus* gene tree showed Japanese individuals to form a distinct group from
303 haplotypes from southern China (Li et al., 2018). Despite the low gene flow of *I. ovatus* in *cox1*
304 marker, we found that Niigata's haplotypes were near related to the published sequence from
305 Hokkaido in northern Japan. The result indicates that these ticks originated from a diverse set of
306 geographical locations in Japan, which might be transported by its hosts or are undergoing recent
307 population expansion from northern Japan (Hokkaido) to central Japan (Niigata) *vice versa*. We
308 found three groups (China 1, Japan, and China 2) and two slightly divergent *cox1* haplotypes (Hap
309 60 and 59) in the Japan group of *I. ovatus* in Niigata. Considering two or more cryptic species can be
310 concealed in one morphologically described species (Bitencourth et al., 2016), the occurrence of the
311 three groups and the divergent haplotypes suggests that *I. ovatus* may be a species complex. It can
312 be inferred that China 1 and China 2 might have a longer evolutionary time as compared to Japan.
313 The co-existence of a species in the same geographic area may explain the occurrence of the species
314 complex. One of the limitations of this study is that both Chinese and Japanese individuals were
315 collected in a limited geographical area. We suggest that future studies sample and sequence more
316 individuals from other locations. Extensive geographical sampling can explain better the taxonomy
317 and the genetic structure of species as suggested in the study of Liu et al., (2013) on *Rhipicephalus*
318 *sanguineus*. Previous studies have also observed species complexes in *Ixodes* and *Rhipicephalus*
319 (Burger et al., 2014; Li et al., 2018; Song et al., 2011; Xu et al., 2003), suggesting that morphological
320 criteria for species differentiation alone are equivocal and that genetic analysis is essential. Future
321 studies must tackle an integrative approach of the tick species concept that includes the morphology,
322 genetics, biology, and ecological trails of ticks (Dantas-Torres et al., 2012).

323 **Conclusions**

324 In summary, our findings revealed the contrasting patterns of the genetic structure of *I.*
325 *ovatus* and *H. flava* from Niigata Prefecture, Japan. The high genetic divergence in *I. ovatus* might

326 be influenced by the restricted movement of its small mammalian hosts during the ticks' immature
327 stage and hares at the adult stage. On the other hand, the homogenized genetic structure in *H. flava*
328 might be due to the avian mediated movement during the ticks' immature stage coupled with the
329 expansive movement of the large mammalian host at the ticks' adult stage. Our results suggest that
330 the host preference of ticks during its immature stage may strongly influence the population genetic
331 structure of ticks due to its higher ability to survive in an environment. Understanding the
332 population genetic structure of ticks such as *I. ovatus* and *H. flava* can give information about the
333 distribution and control of tick-borne diseases. Even though *I. ovatus* populations were genetically
334 structured within Niigata, a published haplotype from Hokkaido was also found, indicating that
335 widespread dispersal is possible. The occurrence of three groups and the divergent *cox1* haplotypes
336 in *I. ovatus* emphasizes the need for additional methods in determining the existence of a species
337 complex in *I. ovatus* populations in Japan.

338 **List of Abbreviations**

339 *H. flava – Haemaphysalis flava*

340 *I. ovatus – Ixodes ovatus*

341 AMOVA – analysis of molecular variance

342 UPGMA - unweighted pair group method with arithmetic mean

343 ML – maximum likelihood

344 F_{ST} – fixation index

345 PBS – phosphate-buffered saline

346 **Declarations**

347 **Ethics approval and consent to participate**

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during the study are included in this published article and its additional supplementary files. All the newly generated sequences are available in the GenBank database under the accession numbers MW063669-MW064124 and MW065821 - MW066347.

Declarations of interests

The authors declare that they have no competing interests.

Authors' contributions

MAFR, MS, and KW conceptualized and designed the experiment. MS, TT, RA, SI, and MOS designed the sampling collection, collected and identified the tick samples. MAFR and MG conducted the molecular analyses. MAFR, MM, and KW performed the data analysis. MAFR and KW wrote the manuscript. All authors read and approve the manuscript.

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541 **Tables**

542 **Table 1.** Summary of *cox1* and 16S haplotype, variability, genetic diversity of adult *I. ovatus* and

543 adult *H. flava* populations in Niigata Prefecture, Japan

Marker	Species	n	nh	s	k	h	π
cox1	*I. ovatus*	307	60	65	2.728	0.852	0.004
	H. flava	220	63	60	1.472	0.789	0.002
16S	*I. ovatus*	284	24	22	0.699	0.442	0.001
	H. flava	172	40	49	2.447	0.835	0.006

544

545 Abbreviations: n sample size; nh number of haplotypes; s number of polymorphic sites; k mean

546 number of nucleotide differences; h haplotype diversity; π nucleotide diversity

547 **Table 2.** Analysis of molecular variance (AMOVA) using *cox1* and 16S of adult *I. ovatus* and adult *H.*

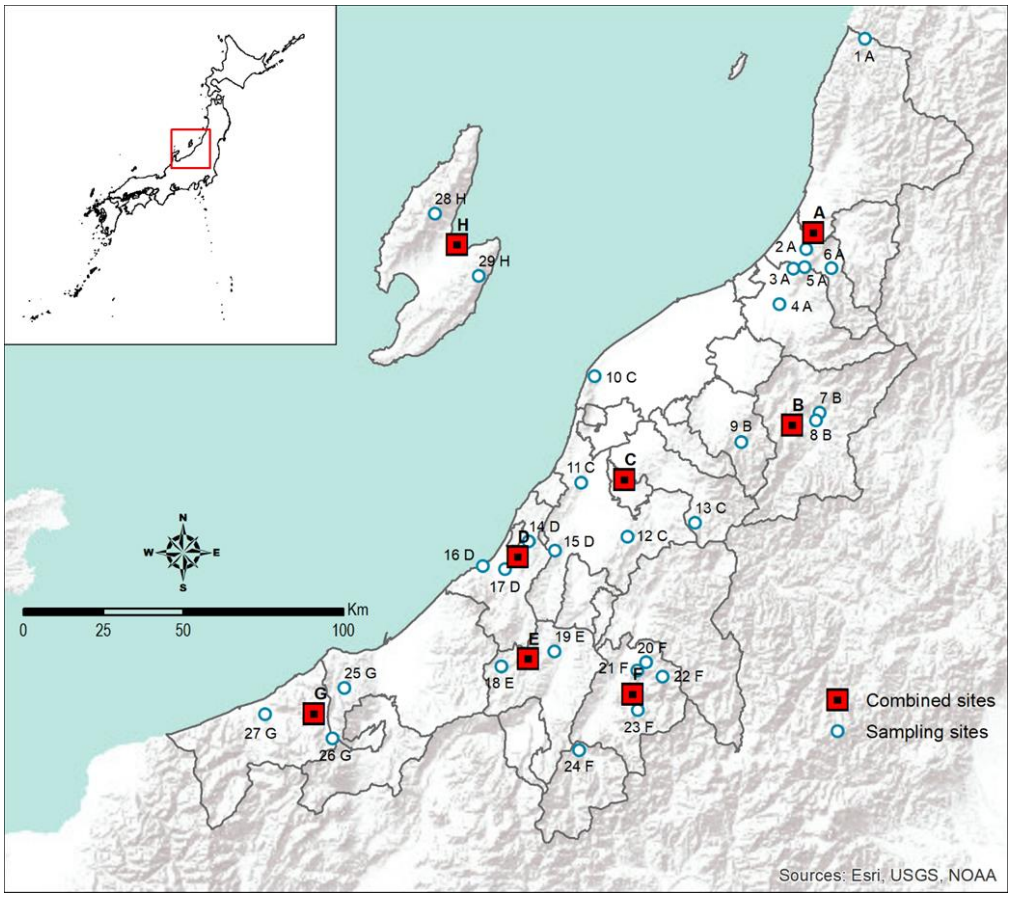
548 *flava* populations

Marker	Species		df	ss	vc	pv	F_{ST}
cox1	*I. ovatus*	Among populations	6	144.45	0.5562 Va	38.01	0.3801*
		Within populations	229	262.17	0.9071 Vb	61.99	
	H. flava	Among populations	4	4.9	0.0135 Va	1.81	0.0181
		Within populations	213	156.12	0.7321 Vb	98.19	
16S	*I. ovatus*	Among populations	6	5.12	0.0133 Va	3.78	0.0378*
		Within populations	272	91.83	0.3376 Vb	96.22	

	Among populations	4	5.74	0.0092 Va	0.78	
H. flava						0.0079
	Within populations	164	191.31	1.1665 Vb	99.22	

Abbreviations: df degrees of freedom; ss sum of squares; Va, Vb and Vc are associate covariance components; pv percentage variation; * P<0.01

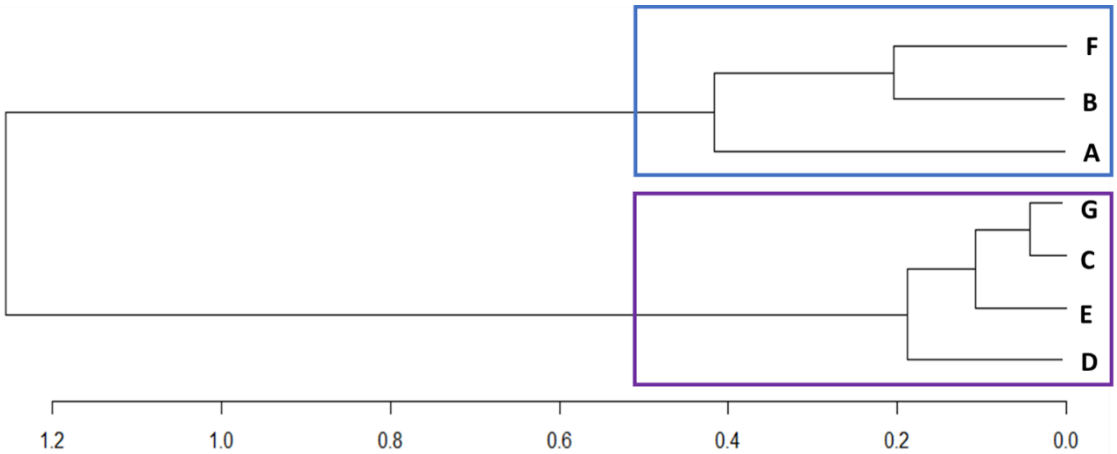
Figure 1. Map of the 29 sampling sites used for this study. The populations labeled A to H are composed of the nearby sites labeled 1 to 29 used for the population genetic analysis.



563 **Figure 2.** An unweighted pair group method with the arithmetic mean (UPGMA) dendrogram of

564 adult *I. ovatus* based on the pairwise genetic distance (F_{ST}) of *cox1* among the 7 populations across

565 Niigata Prefecture, Japan.



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571 **Figure 3.** The distribution of the two genetic clusters as observed in the UPGMA cluster dendrogram

572 (Figure 2) of *I. ovatus cox1* sequences from Niigata Prefecture.

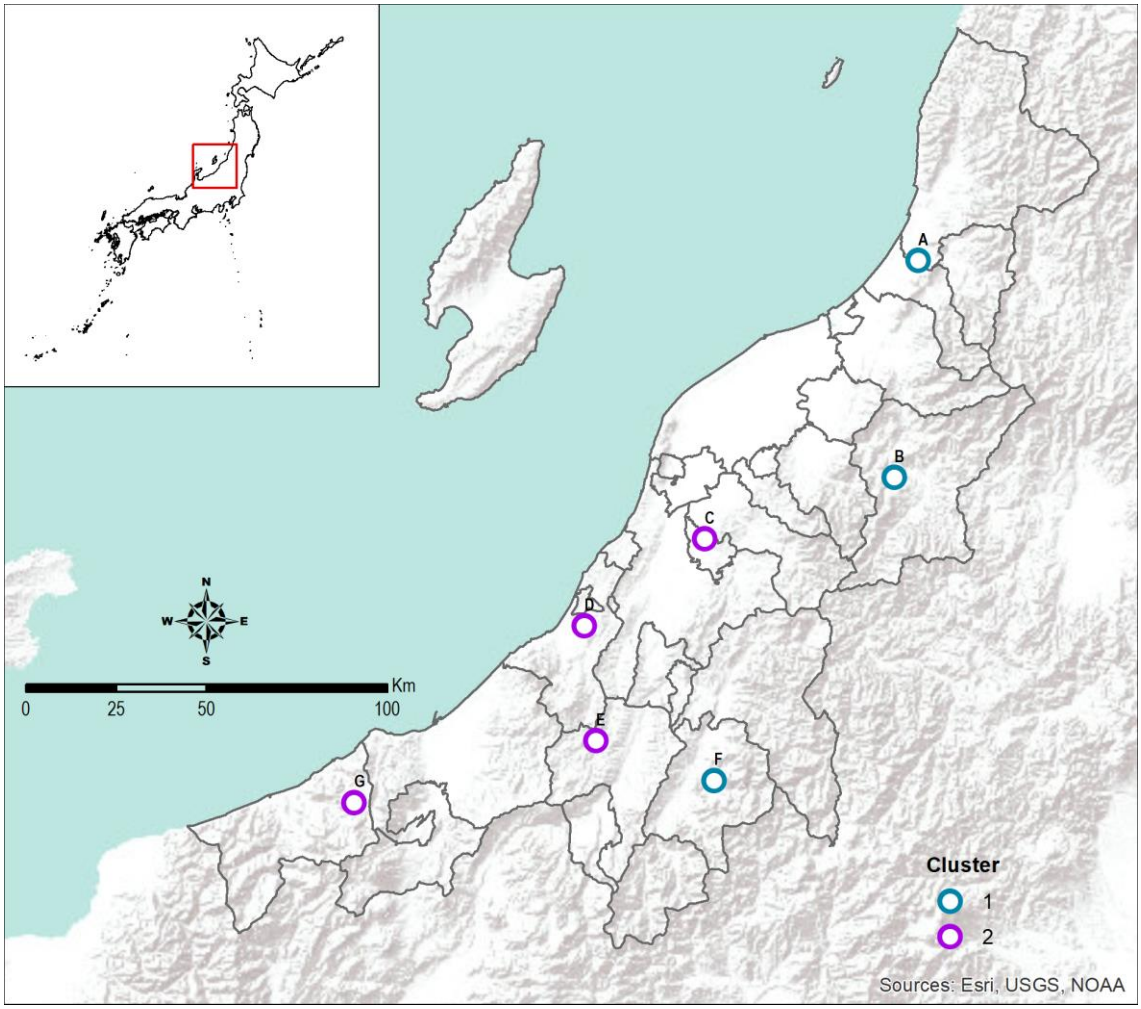
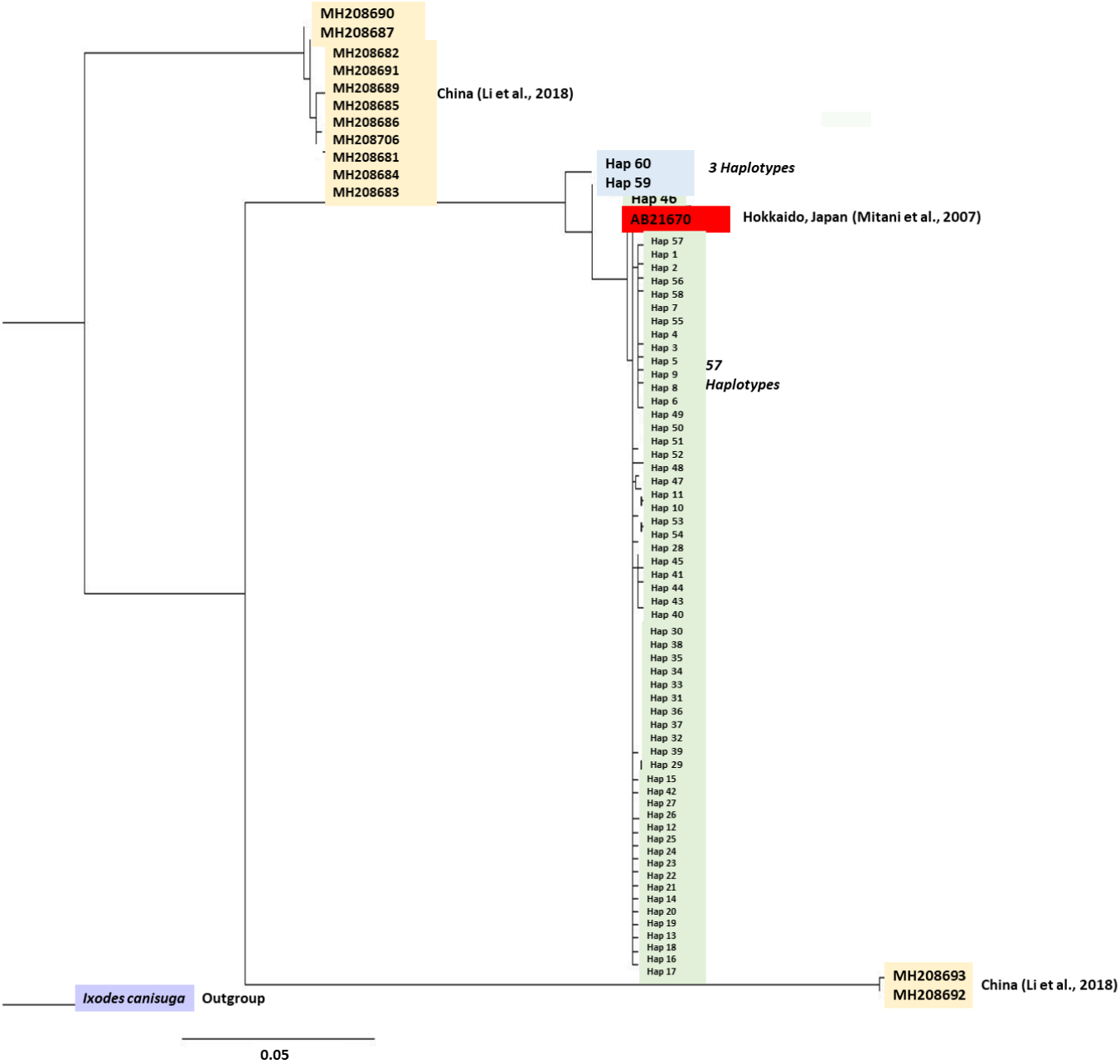
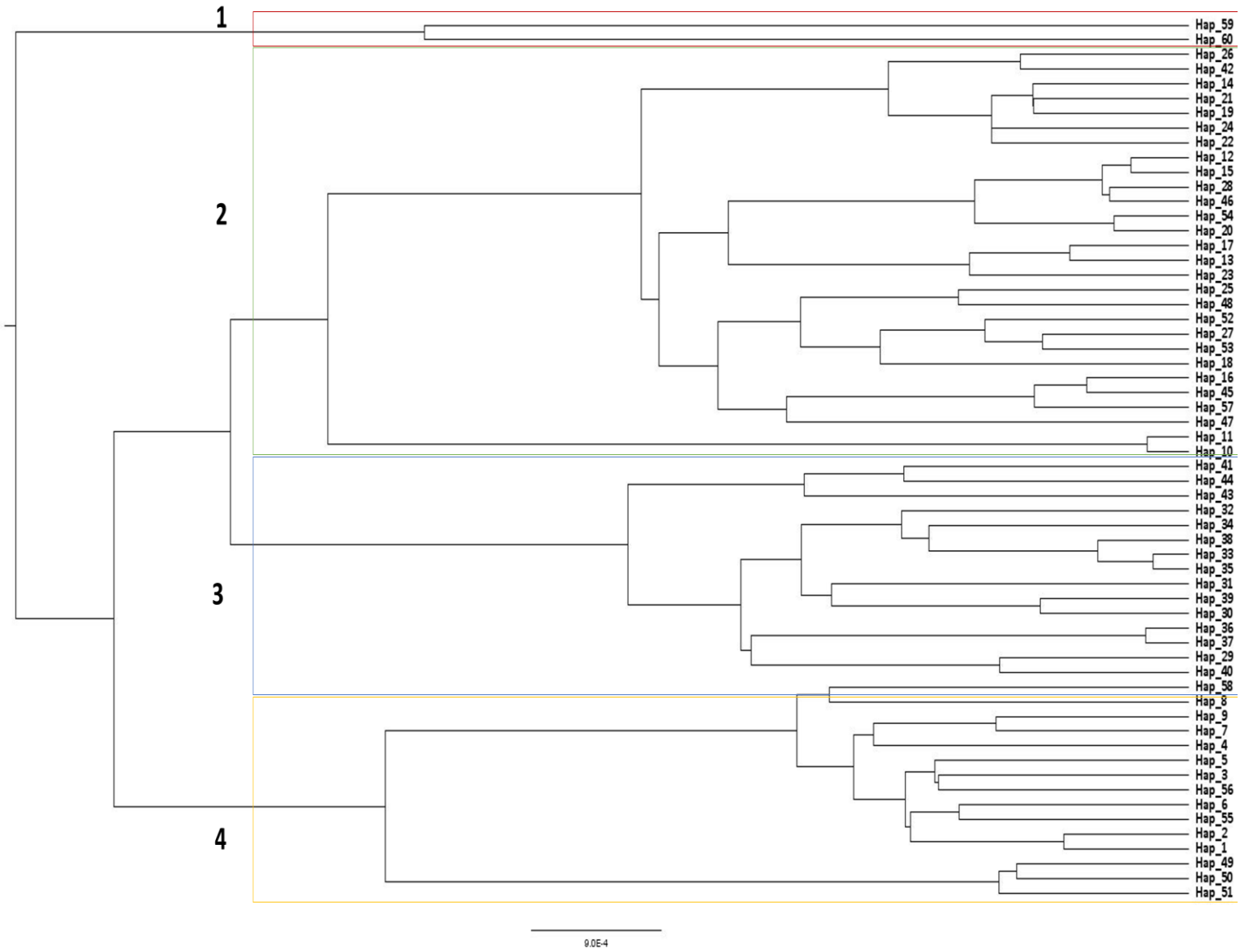


Figure 4. Maximum likelihood gene tree of *cox1* haplotype sequences of *I. ovatus* with sequences from China (MH208681-MH208687, MH208689-MH2086393, MH208706), Japan (AB231670), and an outgroup of *I. canisuga.*



Yellow reference sequences from China (Li et al., 2018); *red* reference sequence from Hokkaido, Japan (Mitani et al., 2007); *blue I. ovatus* divergent *cox1* haplotypes; *green I. ovatus cox1* remaining haplotypes; *purple I. canisuga* outgroup

579 **Figure 5.** Phylogenetic tree from Bayesian analysis of the *cox1 I. ovatus* haplotype sequences



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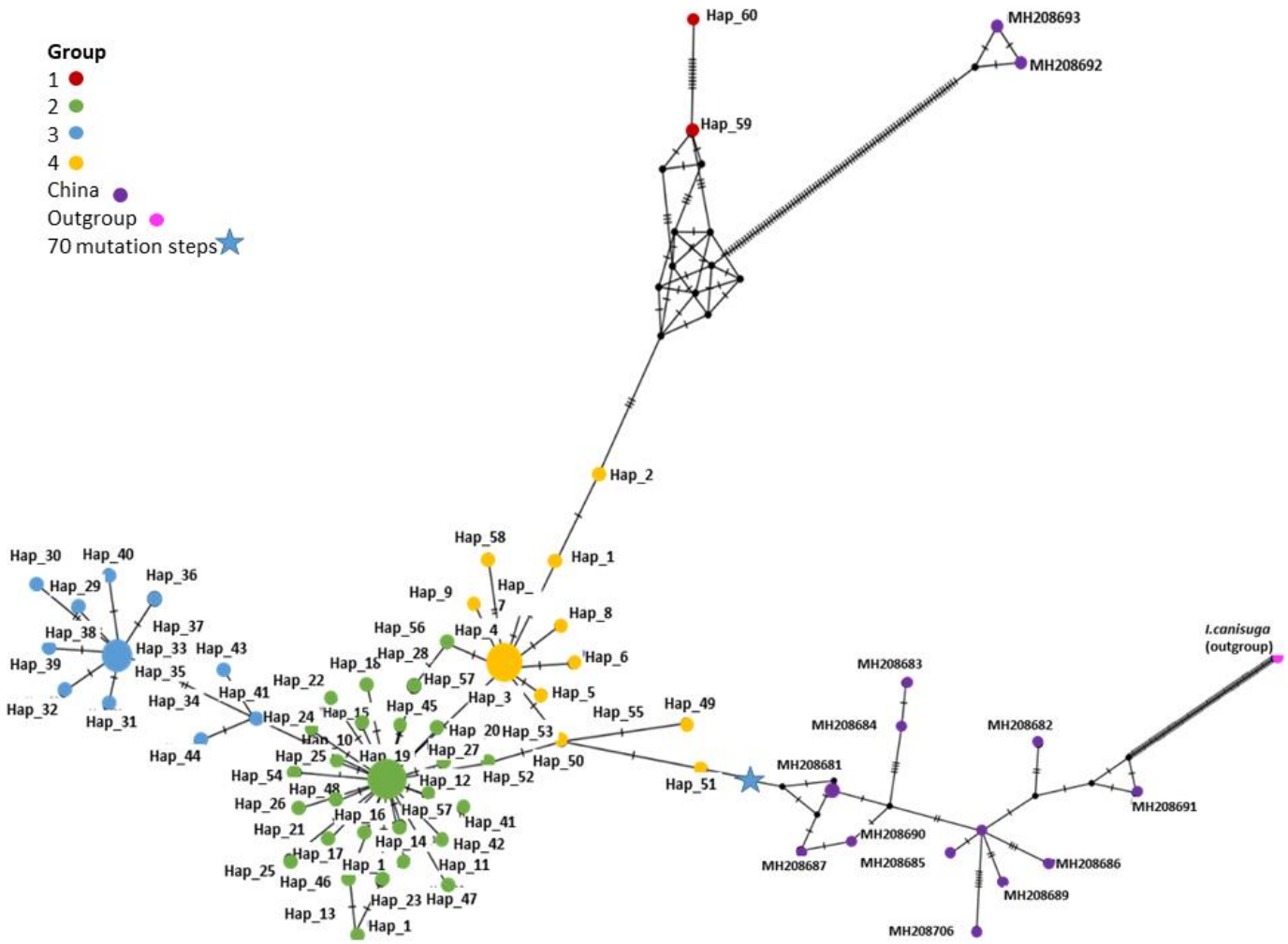
586 **Figure 6.** Median-joining haplotype network of *I. ovatus* haplotypes with reference sequences

587 from China using the *cox1* marker. Each circle represents a unique haplotype and the lines

588 correspond to mutations.

589



590 For easier visualization, the mutation steps (n=70) were marked as ★.

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596 **Additional Files**

597 **Additional File 1.xls Table S1**. Summary of adult *Haemaphysalis flava* and *Ixodes ovatus* collected
598 from the different locations of Niigata Prefecture and its corresponding sample number

599 **Additional File 2.xls Table S2**. Summary of tick collection per species, life stage, and
600 locations

601 **Additional File 3.xls Table S3**. Pairwise comparison of genetic differentiation (F_{ST}) calculated for all
602 *I. ovatus* using the *cox1* gene

603 **Additional File 4.xls Table S4.** Pairwise comparison of genetic differentiation (F_{ST}) calculated for all
604 *I. ovatus* using the 16S gene

605 **Additional File 5.xls Table S5.** Pairwise comparison of genetic differentiation (F_{ST}) calculated for all
606 *H. flava* using the *cox1* gene

607 **Additional File 6.xls Table S6**. Pairwise comparison of genetic differentiation (F_{ST}) calculated for all
608 *H. flava* using the 16S gene

609 **Additional File 7.docx Figure S1.** Plots a to d show the relationship between the pairwise F_{ST}
610 values and the geographic distances for *I. ovatus* and *H. flava* in both *cox1* and 16S markers from
611 the Mantel test analysis.

612 **Additional File 8.docx Figure S2.** An unweighted pair group method with the arithmetic mean
613 (UPGMA) dendrogram of *I. ovatus* based on the pairwise genetic distance (F_{ST}) of 16S among the 7
614 populations across Niigata Prefecture, Japan.

615 **Additional File 9.docx Figure S3** An unweighted pair group method with the arithmetic mean
616 (UPGMA) dendrogram of *H. flava* based on the pairwise genetic distance (F_{ST}) of *cox1* among the 5
617 populations across Niigata Prefecture, Japan.

618 **Additional File 10.docx Figure S4** An unweighted pair group method with the arithmetic mean
619 (UPGMA) dendrogram of *H. flava* based on the pairwise genetic distance (F_{ST}) of 16S among the 5
620 populations across Niigata Prefecture, Japan.

621 **Additional File 11.docx Figure S5** Median-joining haplotype network of *I. ovatus* from *cox1*
622 marker. Each circle represents a unique haplotype and the lines correspond to mutations.
623 **Additional File 12.docx Figure S6** Phylogenetic tree from Bayesian analysis of the *cox1 I. ovatus*
624 haplotype sequences with reference sequences from China
625 **Additional File 13.docx Figure S7** Median-joining haplotype network of *I. ovatus* from 16S marker.
626 Each circle represents a unique haplotype and the lines correspond to mutations.
627 **Additional File 14.docx Figure S8** Median-joining haplotype network of *H. flava* from *cox1* marker.
628 Each circle represents a unique haplotype and the lines correspond to mutations.
629 **Additional File 15.docx Figure S9** Median-joining haplotype network of *H. flava* from 16S marker.
630 Each circle represents a unique haplotype, and the lines correspond to mutations.
631 **Additional File 16.docx Figure S10** Phylogenetic tree of 16S haplotype sequences of *I. ovatus*
632 inferred by maximum likelihood analysis with evolutionary model GTR with sequences from China
633 (MH208506, MH208512, MH208514 – MH208519, MH208522, MH208524, MH208531,
634 MH208574, MH208577, MH208579, KU664519), Japan (AB819241, AB819242, AB819243,
635 AB819244, and U95900). An outgroup (*Ixodes canisuga*) was also included.
636 **Additional File 17.docx Figure S11** Phylogenetic tree of *cox1* haplotype sequences of *H. flava*
637 inferred by maximum likelihood analysis with evolutionary model HKY with sequences from China
638 (KY021800- KY021807; KY021810-KY021819, KY003181, JQ62588 - JQ625889; JQ737097;
639 JF758632). An outgroup (*Ixodes canisuga)* was also included.
640 **Additional File 18.docx Figure S12** Phylogenetic tree of 16S haplotype sequences of *H. flava*
641 inferred by maximum likelihood analysis with evolutionary model GTR with sequences from China
642 (KC844858 -KC844867; KC844880-KC844882, M696720, KP324926, KX450279) and Japan
643 (AB19177- AB819192). An outgroup (*Ixodes canisuga)* was also included.
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