Title

Comparative population genetic structure of two Ixodidae ticks (*Ixodes ovatus* and *Haemaphysalis flava*) in Niigata Prefecture, Japan

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Abstract

Ixodid tick species function as important vectors of tick-borne diseases in Japan. In this study, we used genetic structure at two mitochondrial loci (*cox1*, 16S rRNA gene) to infer gene flow patterns of *Ixodes ovatus* and *Haemaphysalis flava* from Niigata Prefecture, Japan. We hypothesized that *I. ovatus* and *H. flava* have different population genetic structure because of their host mobility in different tick life stages despite sharing of hosts. Samples (n = 1 to 77) were collected in 29 (*I. ovatus*) and 17 (*H. flava*) sampling locations across Niigata. For *I. ovatus*, pairwise F_{ST} and analysis of molecular variance (AMOVA) analyses of *cox1* sequences indicated significant among-population differentiation. This was in contrast to *H. flava*, for which there were only two cases of significant pairwise differentiation and no overall structure. A Mantel test revealed isolation by distance and there was positive spatial autocorrelation of haplotypes in *I. ovatus cox1* and 16S sequences, but non-significant results were observed in *H. flava* in both markers. Newly sampled *I. ovatus* grouped together with a published *I. ovatus* sequence from northern Japan and were distinct from two other *I. ovatus* groups that were reported from southern China. The three genetic groups (China 1, China 2, and Japan) in our data set suggest the potential for cryptic species within the lineage. While many factors can potentially account for the observed differences in genetic structure, including population persistence and large-scale patterns of range expansion, we propose that differences in the mobility of hosts of tick immature stages (small mammals in *I. ovatus*; birds in *H. flava*) may be driving the observed patterns.

Keywords: gene flow, host mobility, Mantel test, Spatial autocorrelation, cryptic species

Background

Tick-borne diseases are a public health concern and their control is often challenging due to the fact that it involves a complex transmission chain of vertebrate hosts and ticks interacting in a changing environment [1]. In order to understand the vector-host relationship, studies on the spatial distribution and population genetic structure of ticks are needed. Population genetic studies have helped in understanding the dispersal patterns and potential pathogen transmission in ticks [2]. The direction, distance and gene flow patterns between tick populations can be inferred from population genetic studies. For example, if high gene flow is observed there might be a greater chance of colonizing new areas or of re-colonizing areas following successful vector control programs [3]. Therefore, estimates of genetic structure and gene flow in tick populations may give insights regarding the spread of tick-borne disease.

Host mobility can affect the genetic patterns of tick populations, although its effects are not consistent. Several studies have reported low levels of gene flow in ticks with less mobile hosts (e.g. smaller mammals) and high levels of gene flow in ticks with highly mobile hosts [2]. For example, *Amblyomma americanum* and *A. triste* (Ixodidae, Acari) exhibited high gene flow across various spatial scales (137, 000 km^2 to 2.78 million km^2) and this was attributed to their hosts' dispersal capabilities (large mammals and birds) [4-6], while low gene flow was observed in *Amblyomma dissimile* populations and this was attributed to the low mobility of its hosts (small mammals, reptiles and salamanders) [7]. In contrast, some studies have reported low gene flow despite the high mobility of the host, for example in *Ixodes scapularis* [8] and *Ornithodoros coriaceus* [9] which can be attributed to the host mobility.

In Japan, tick-borne diseases are an increasing public health concern, affecting humans and animals [10]. A total of 8 genera of ticks have been recorded in Japan, composed of 47 species: 43 Ixodidae species and 4 Argasidae species [11]. Out of 47 species, 21 species are known to

62 parasitize humans [12]. Among these 21 species, *Ixodes ovatus*, the main vector of Lyme disease
63 [13] and *Haemaphysalis flava,* a vector of severe fever with thrombocytopenia syndrome (SFTS)
64 and Japanese spotted fever (JSF), were reported in Japan [10,14]. A previous study found that the
65 hosts of adult *I. ovatus* were mainly hares and can also be large mammals (e.g. cows and horses),
66 and the hosts of immature forms were small rodents [15]. *H. flava* adult host preference are cows,
67 dogs, horses, wild boar, bear, and deer, while immature forms only parasitized birds [15]. Despite
68 the similarity of *I. ovatus* and *H. flava* in which blood meal from different vertebrate host is taken
69 in each life stage (larva, nymph and adult) [16], comparative population genetic studies on two
70 Ixodid tick species such as *I. ovatus* and *H. flava* from the same sampling location remain
71 nonexistent. Thus, our study is important because there are no previous studies about dispersal
72 movement of two important tick species: *I. ovatus* and *H. flava*, we conclude that low gene flow
73 might be observed in *I. ovatus* due to the lower mobility of hares in the adult stage and small
74 rodents in its immature stage while *H. flava* will exhibit high gene flow because of its avian
75 mediated dispersal in its immature stage.

76 In addition to tick population structure studies, genetic studies may also reveal the presence of
77 cryptic species, where morphologically identified individuals might represent more than one
78 species [17]. Previous studies on *Rhipecephalus appendiculatus* [18], *I. holocylus* [19] and *I. ovatus*
79 [20] have revealed the presence of cryptic species based on the clustering of haplotypes in a
80 phylogenetic tree, and concluded that morphological criteria for species differentiation alone are
81 equivocal and that genetic analysis is important. A putative *I. ovatus* species complex was
82 identified based on the presence of 3 distinct haplotype clusters in both *cox1* and 16S rRNA genes;
83 2 groups in China and one in Japan that included haplotypes from North America [20].

84 Here, we studied the population genetic structure of *I. ovatus* and *H. flava* and also tested for the
85 presence of cryptic species using mitochondrial DNA sequences of *cox1* and the 16S rRNA gene.

86 We hypothesized that *I. ovatus* and *H. flava* may have differences in their population genetic
87 structure despite some overlap in their adult host preference because of the differences in
88 mobility of the immature tick hosts. We predicted that *H. flava* exhibits high gene flow patterns
89 due to avian-mediated dispersal of immature ticks, in contrast to *I. ovatus* which uses small
90 mammals as hosts during its immature stages.

91 **Methods**

92 **Study site, collection, sampling and identification**

93 From April 2016 until November 2017, ticks were collected by the standard flagging methods [21]
94 across Niigata Prefecture in Japan and a total of 29 sampling locations were surveyed for *H. flava*
95 and *I. ovatus* ticks (Additional File 1. Table S1). Altitude per location ranged from 8 -1402 m/a.s.l.
96 and the geographic distance between sites ranged from 4.28 - 247.65 km. Collected ticks were
97 stored in microcentrifuge tubes with ethanol at 4^0C. A total of 2,103 individual tick samples were
98 collected. Identification of sex, developmental stage and species was performed using a
99 compound microscope and identification keys of [15].

100 **DNA extraction, PCR amplification and sequencing**

101 Genomic DNA (*I. ovatus* n=320*; H. flava* n=220) from each identified tick was extracted using the
102 Isogenome DNA extraction kits (NIPPON GENE Co. Ltd., Tokyo, Japan) following the manufacturer's
103 recommended protocol. Prior to DNA extraction, each tick was washed with alcohol and PBS
104 solution. DNA quality was checked using a NanoDrop™ 2000 Spectrophotometer (Thermo
105 Scientific™). Two mitochondrial genes were amplified by polymerase chain reaction (PCR):
106 Cytochrome Oxidase 1 (*cox1*) (658 base pairs) using the primer pairs LCO-1490 (5'-
107 GGTCAACAAATCATAAAGATATTGG -3') and HCO1-2198 (5'- AAACTTCAGGGTGACCAAAAAATCA-3')
108 [22] and the 16S rRNA gene (16S) (407 base pairs) with the following primer pairs 16S+1

109 (5'CTGCTCAATGAATATTTAAATTGC-3') and 16S – 1 (5' -CGGTCTAAACTCAGATCATGTAGG-3') [23].
110 All PCR amplifications of both *cox1* and 16S were performed with a final volume of 10 µl with 1µl
111 of genomic DNA. The PCR reaction for both markers were composed of the following: 10x Ex Taq
112 buffer, 25mM $MgCl_2$, 2.5mM dNTP, 10µm of forward and reverse primers and 5 units/µl of TaKaRa
113 Ex Taq™ (Takara Bio Inc.). The *cox1* PCR amplification were as follows: an initial denaturation of
114 94^0C for 2 minutes, denaturation of 94^0C for 30 seconds, annealing of 38^0C for 30 seconds,
115 extension of 72^0C for 1 minute for 30 cycles and final extension of 72^0C for 10 minutes. While the
116 16S PCR amplification followed the protocol of [23] with some modifications, initial denaturation
117 of 94^0C for 3 minutes, denaturation of 94^0C for 30 seconds, annealing of 50^0C for 40 seconds,
118 extension of 72^0C for 40 seconds for 30 cycles and final extension of 72^0C for 5 minutes. PCR
119 products were purified using the QIAquick 96 PCR Purification Kit (Qiagen) in accordance with the
120 manufacturer's instructions, and sequenced by Eurofin Genomics, Inc., Tokyo, Japan.

121 **Sequence data analysis**

122 We assembled forward and reverse reads for each individual using CodonCode Aligner ver 1.2.4
123 software (https://www.codoncode.com/aligner/). No ambiguous bases were observed and the
124 low quality bases were removed in the start and end of the reads. Multiple alignment of
125 sequences was performed using the MAFFT alignment online program with default settings
126 (https://mafft.cbrc.jp/alignment/server/). To ensure sequence quality and correct species
127 identification, we checked the similarity of the sequences against reference sequences from
128 GenBank using BLASTN (Basic Local Alignment Search Tool – Nucleotide,
129 https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=bl
130 asthome). The quality of the final aligned sequences was checked in Mesquite ver 3.5 [24] wherein
131 the protein coding genes were translated into amino acids to confirm the absence of stop codons.

132 **Population genetic analysis**

133 We analyzed the final sequences of both *H. flava* and *I. ovatus* per genetic marker separately using
134 DNASp ver 6.12.03 [25] and Arlequin ver 3.5.2.2 [26] for the following parameters: number of
135 haplotypes, average number of polymorphic sites, average number of nucleotide differences,
136 gene/haplotype diversity and nucleotide diversity. The population genetic structure among and
137 within sampling locations in Niigata Prefecture was calculated by analysis of molecular variance
138 (AMOVA) performed in Arlequin with 1023 permutations. The degree of molecular genetic
139 differentiation between sampling locations defined as populations was assessed by calculation of
140 the pairwise F_{ST} values in Arlequin.

141 To determine if the genetic differentiation was influenced by geographical distance or altitudinal
142 differences among populations, we performed Mantel Test in GenAlEx ver 6.51b2 [27]. Two tests
143 per species and marker were conducted. First we compared pairwise genetic (pairwise F_{ST} values)
144 and geographical distances (km) and second we compared genetic distance (F_{ST} values) with
145 altitudinal differences (m/a.s.l.). The geographic distances were obtained from the GPS
146 coordinates (latitude and longitude) recorded during the sampling. All Mantel tests were assessed
147 using 9999 permutations for the significance of the correlation.

148 The spatial component of genetic variation was further assessed by spatial autocorrelation [28]
149 using GenAlEx [27]. The autocorrelation coefficient (r) was computed from the geographic
150 distance between sampling locations and the genetic distance (pairwise F_{ST} values). This measures
151 the genetic similarity between individual pairs within an identified distance class. The size of the
152 distance class influences the estimation of the spatial autocorrelation. We identified the
153 appropriate distance class based on the observed distribution of pairwise geographic distance
154 between the sampling locations (data not shown). The distance class sizes used were the
155 following: 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, 300, and 320 kilometers.
156 Bootstrap estimates of r and random permutations were set at 9999 each for the test for

157 significance. The upper and lower error bars in the correlogram bound the 95% confidence interval
158 of *r* value as identified by bootstrap resampling. The upper and lower confidence limits bound the
159 95% confidence interval of the null hypothesis of no spatial structure in the data set.

160 The genetic relationships among the sampling locations (i.e., populations) were calculated by
161 unweighted pair group method with the arithmetic mean (UPGMA) cluster method using the APE
162 package [29] and R studio [30] to create dendrogram using the genetic distance matrix (pairwise
163 F_{ST} values) generated from GenAlEx.

164 **Phylogenetic analysis**

165 We constructed maximum likelihood (ML) gene trees for *cox1* and 16S haplotype sequences of *I.*
166 *ovatus* and *H. flava* using PhyML ver 3.1 [31] under the default settings. We applied HKY and GTR
167 nucleotide substitution models for *cox1* and 16S, respectively, as suggested by jModelTest ver 2
168 [32]. Additional sequences from China (MH208506, MH208512, MH208514, MH208522,
169 MH208515-19, MH208524, MH208531, MH208574, MH208577, MH208579, MH208681-87,
170 MH208689-93, MH208706, KU664519 [20]), Japan (Hokkaido, Yamanashi and Aomori) (AB231670,
171 AB819241, AB819243, AB819244 [33-34]) and the USA (U95900; [35]) were included. We used
172 *Ixodes canisuga* as an outgroup (KY962023, KY962074; [36]).

173 **Results**

174 The number of successfully sequenced and analyzed *H. flava* ranged from 1 to 36 individuals per
175 study site, with more successfully sequenced for *cox1* (220/223; 98.7%) than for 16S (172/223;
176 77.1%). The number of *I. ovatus* ranged from 1 to 36 individuals per study site and, as with *H. flava*,
177 more were successfully sequenced for *cox1* (307/320; 95.9%) than for 16S (284/320; 88.8%)
178 (Additional File 1. Table S1). There were 63 and 60 *cox1* haplotypes in *H. flava* and *I. ovatus*,

179 respectively, and 40 and 24 16S haplotypes (Table 1). Haplotype diversity ranged from 0.442 to
180 0.852 and nucleotide diversity ranged from 0.001 to 0.006 for both markers and species (Table 1).

181 AMOVA results revealed a high among-population divergence (41.54%) in *I. ovatus cox1* sequences,
182 whereas both *cox1* and 16S markers of *H. flava* indicated no significant genetic variation among
183 populations (Table 2). Global F_{ST} values were highest in *I. ovatus* with values 0.415 in *cox1* and
184 0.074 in 16S sequences. Pairwise F_{ST} values (0.500 to 1.00) of *I. ovatus* based on *cox1* showed
185 significant genetic differences between most pairs of populations such as between locations 18
186 and 25 and between locations 28 and 25 (Additional File 2 Table S2). No significant differences
187 were observed in 16S (Additional File 3 Table S3). Pairwise F_{ST} values from the *cox1* marker of *H.*
188 *flava* showed no significant genetic differences except between locations 18 and 15 and 18 and 25
189 (Additional File 4 Table S4). No significant genetic differentiation was also observed in 16S marker
190 of H. flava except in between locations 3 and 20 and 4 and 20 (Additional File 5 Table S5).

191 The Mantel test of *I. ovatus* showed evidence of isolation by geographic distance in both markers:
192 (*cox1* r = 0.197, p = 0.008; 16S r = 0.293, p = 0.011) (Figures 1a, b). In contrast, there was no
193 evidence for isolation by altitudinal difference (*cox1* r = -0.066, p=0.225; 16S r = -0.023, p = 0.577).
194 Both Mantel tests were not significant for *H. flava* (distance: *cox1* r= -0.145, p= 0.23; 16S r = -0.049,
195 p= 0.33; altitude: r = 0.092, p = 0.30; 16S r = 0.217, p= 0.06). The spatial autocorrelation test in *I.*
196 *ovatus* revealed positive and significant correlation between genetic and geographic distance from
197 20 – 40 km, and 60 – 80 km, and 20 – 50 km and 120 – 160 km from *cox1* and 16S, respectively
198 (Figure 1c, d).

199 The UPGMA cluster dendrogram from the pairwise F_{ST} values from the *cox1*, *I. ovatus* (Figure 2)
200 revealed 2 genetic clusters across the 29 sampling locations, where the cluster 2 included
201 populations from the more western sites including the island (location 24 and 25) while cluster 1
202 mostly included populations in the northern and southern part of Niigata Prefecture (Figure 3). No

203 evident genetic clustering on the dendrogram was observed from the *I. ovatus* 16S sequences and
204 in both *cox1* and 16S *H. flava* sequences (Additional File 6 Figure S1; Additional File 7 Figure S2;
205 Additional File 8 Figure S3).

206 The *cox1* gene tree of 60 *I. ovatus* haplotypes indicated three groups: group 1 of the published
207 sequences from western China, one large group 2 containing the 58 haplotypes and 2 divergent
208 haplotypes (Hap 60 and Hap 59), and group 3 from western China (Figure 4). The 2 divergent
209 haplotypes were singletons. One was found in location 29 and the other in location 7. The
210 published haplotype from Hokkaido [33] occurred within our large group and the published
211 sequences from western China did not. The *I. ovatus* 16S gene tree (24 newly sequenced
212 haplotypes) (Additional File 9 Figure S4) also recovered published haplotypes from Japan
213 (Yamanashi and Aomori; [34]) as well as from North America [35] within the same cluster as our
214 new sequences. The *cox1* haplotype sequences of *H. flava* were similar to reference sequences
215 from China (Additional File 10 Figure S5) [unpublished manuscript] while the 16S haplotype
216 sequences (Additional File 11 Figure S6) showed similarities with Japan (Kagoshima, Fukui Aomori,
217 Fukui, Yamanashi, Kagawa and Ehime [34]) and China [unpublished manuscript] published
218 sequences.

219 **Discussion**

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

221 Our results supported our hypothesis that *H. flava* and *I. ovatus* display contrasting population
222 genetic structure. The noticeable high gene flow in *H. flava* may be a result of the higher level of
223 mobility of its hosts such as birds in tick's immature stage and the more pronounced genetic
224 structure and lower gene flow in *I. ovatus* may be due to the restricted ability of its small
225 mammalian hosts at the immature stage to disperse further. 28 species of birds were previously

226 reported as hosts of *H. flava,* mostly from the order Passeriformes known as migratory birds [37].
227 The host for adults of *I. ovatus* included large mammals such as hares while immature forms are
228 found on small mammals such as small rodents and rats [15]. The movement of small mammals is
229 limited because of environmental and ecological factors such as difficulty in crossing roads and
230 higher possibility of road kill due to vehicle collision and or traffic [38], which may explain the
231 genetic structuring in *I. ovatus*. The host preference of the adults *H. flava* are large mammals (e.g.
232 cows, horses, wild boar, deer, bear) and hares, dogs, rodents while the immature forms parasitize
233 birds [15]. Mammals and birds may have wide habitats ranges that may allow maintaining the
234 gene flow of *H. flava* between the locations in Niigata, as previously observed in *Amblyomma*
235 *americanum* populations [5,6,39], and *Ixodes ricinus* [40]. Birds present the greatest potential for
236 farther dispersal of *H. flava* that could reduce its genetic structuring [41] that supports the results
237 of our study.

238 Several alternative factors such as tick behavior, biology and ecology can also affect the tick
239 dispersal movement. For example, long movement patterns, resistance against unfavorable
240 weather conditions causing high survival in their hosts and low host specificity [4] are possible
241 reasons for the low genetic structuring in *H. flava* populations. It could also be influenced by
242 ecological factors such as changes in land use and cover, wildlife and forestry management as
243 observed in the previous study of [42] in the *Ixodes ricinus* tick populations from Europe. Previous
244 study on two tick species: *Hyalomma rufipes* and *Amblyomma hebraum* also displayed contrasting
245 genetic pattern despite the overlap sharing of highly mobiles hosts such as birds [43] due to the
246 capacity of the immature stages of ticks to survive off the hosts in various habitat conditions [44-
247 46]. Population structure can also be influenced by assortative mating (e.g. *Ixodes ricinus*) wherein
248 genetically similar individuals tend to mate, rather than random mating resulting in increased
249 genetic divergence [47]. Our study does not have supporting data to test these alternative factors

250 thus it is suggested in future studies to further analyze these factors that could play a role in the
251 tick's dispersal movement.

252 Based on previous studies [48-49], isolation by distance suggests that the populations are in
253 equilibrium of migration and genetic drift at the spatial scale of Niigata Prefecture. Our results
254 were congruent with a previous study of *Amblyomma ovale* that showed positive relationship of
255 geographic and genetic distance at the spatial scale of the whole of Brazil whose main hosts for
256 the immature stage are small rodents while tapir and jaguar in its adult stage [50]. In contrast, *H.*
257 *flava* populations showed an absence of isolation by distance. In circumstances when geographic
258 distance can't explain the genetic structure of tick populations, other factor such as ecological
259 variables (e.g. land use) maybe affecting the genetic structuring within tick populations [7, 40, 51].

260 A significant and positive spatial autocorrelation of *I. ovatus* in both *cox1* and 16S sequences was
261 observed within the geographic range of 20 – 40 km and 60- 80 km in *cox1* sequences and 20 – 50
262 km and 120 – 160 km range in the 16S sequences. We can infer that the positive *r* values at the
263 maximum geographic rages of 60-80 km in *cox1* and 120 - 160 km in 16S *I. ovatus* sequences
264 indicate that gene flow is occurring in the wide geographic range. The lack of significance at 40-60
265 km in *cox1* may suggest patch structure indicating genetic similarity or variation [28] as observed
266 on limpet species, *Siphonaria raoulensis* from New Zealand that also displayed recovery of positive
267 *r* values at greater distances [52]. However, no autocorrelation was observed in *H. flava* at any
268 distance class, suggesting that gene flow occurring has no specific pattern favoring certain
269 distances [53-55]. Since the sampling geographic range of our study was within Niigata Prefecture
270 and distances >220 km were not included in our sampling, we suggest that future studies collect
271 samples at a wider geographic scale to more precisely determine the geographic distance at which
272 autocorrelation exists.

273 16S *I. ovatus* sequences showed genetic differentiation (pairwise F_{ST} = 0.5-0.6) in few pairs of
274 sampling locations as compared to the *cox1* sequences which may be due to lower variability of
275 the marker thus providing inadequate genetic variation for population genetic analysis, as
276 previously observed in *Amblyomma ovale* [50] and *Rhipicephalus microplus* [56-57]. The low
277 variability of the 16S in *I. ovatus* may not be sufficient to infer intraspecific relationship and is
278 further supported by its low (*nd* = 0.001) nucleotide diversity as compared in *cox1 I. ovatus* (*nd* =
279 0.004). Thus, it may also explain the grouping observed in the *cox1 I. ovatus* tree which is absent in
280 the 16S *I. ovatus* sequences. Despite of these, we used 16S mitochondrial gene because this gene
281 mutates at a rate that is informative for species-level phylogenetic and broad biogeographic
282 inferences [2]

283 The negative pairwise F_{ST} values obtained in *H. flava* sequences do not have any biological meaning.
284 These results from the unbiased estimator allowing a negative F_{ST} when the sample size is small.
285 [58-60]. It can also be noted that we found significant pairwise F_{ST} values in 2 sampling location in
286 *H. flava cox1* sequences probably due to the fact that both locations have only one tick sample.
287 Although, some sampling sites in this study have small sample size (n < 10), the *cox1* and 16S
288 sequences provided valuable insight into the genetic structure and gene flow patterns of the
289 present-day tick populations. Thus, for future research, we recommend to increase the sample
290 size per sampling site.

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

292 Our *cox1 I. ovatus* gene tree showed Japanese individuals to form a group that was distinct from
293 haplotypes from southern China [20]. Despite the low gene flow we found in *cox1 I. ovatus*,
294 haplotypes found from Niigata were closely related to the published sequence from Hokkaido,
295 Japan which may indicate that these ticks originated from a diverse set of geographical locations in
296 Japan which might be transported by its hosts or is undergoing recent population expansion from

297 northern Japan (Hokkaido) to south (Niigata) or *vice versa*. We found three groups (China 1, Japan
298 and China 2) and 2 slightly divergent *cox1* haplotypes (Hap 60 and 59) in the Japan group of *I.*
299 *ovatus* in Niigata. Considering two or more cryptic species can be concealed in one
300 morphologically described species [59], the occurrence of the 3 groups and the divergent
301 haplotypes suggests that *I. ovatus* may be a species complex. Previous studies have also observed
302 species complexes in *Ixodes* and *Rhipicephalus* [19-20,57;62] suggesting that morphological
303 criteria for species differentiation alone are equivocal and that genetic analysis is important.

304 **Conclusion**

305 Two Ixodid ticks, *H. flava* and *I. ovatus* exhibited contrasting patterns of genetic structure in
306 Niigata Prefecture, Japan. We suggest that these differences are due to differences in host
307 mobility of the immature life stages of the ticks. The more pronounced population genetic
308 structure of *I. ovatus* might be influenced by the restricted movement of its small mammalian host
309 in its immature stage, whereas the lack of structure in *H. flava* may be due to its avian-mediated
310 movement in its immature stage. Even though *I. ovatus* populations were genetically structured
311 within Niigata, a published haplotype from Hokkaido was also found, indicating that widespread
312 dispersal is possible. The occurrence of three groups and the divergent cox1 haplotypes in *I.*
313 *ovatus* emphasizes the need of additional methods in determining the existence of species
314 complex in *I. ovatus* populations in Japan.

315 **List of Abbreviations**

316 *H. flava – Haemaphysalis flava*

317 *I. ovatus – Ixodes ovatus*

318 AMOVA – Analysis of molecular variance

UPGMA - unweighted pair group method with arithmetic mean

ML – maximum likelihood

F_{ST} – fixation index

Declarations

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 supplementary additional files. All the newly generated sequences are available in the Genbank database under the accession numbers _____.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

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

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Additional Files

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

Additional File 2.xls Table S2. Pairwise comparison of genetic differentiation (F_{ST}) calculated for all *I. ovatus* using the *cox1* gene

Additional File 3.xls Table S3. Pairwise comparison of genetic differentiation (F_{ST}) calculated for all *I. ovatus* using the 16S gene

Additional File 4.xls Table S4. Pairwise comparison of genetic differentiation (F_{ST}) calculated for all *H. flava* using the *cox1* gene

Additional File 5.xls Table S5. Pairwise comparison of genetic differentiation (F_{ST}) calculated for all *H. flava* using the 16S gene

Additional File 6.docx Figure S1. An unweighted pair group method with the arithmetic mean (UPGMA) dendrogram of *I. ovatus* based on the pairwise genetic distance (F_{ST}) of 16S among 23 sampling locations across Niigata Prefecture, Japan.

Additional File 7.docx Figure S2 An unweighted pair group method with the arithmetic mean (UPGMA) dendrogram of *H. flava* based on the pairwise genetic distance (F_{ST}) of *cox1* among 17 sampling locations across Niigata Prefecture, Japan.

Additional File 8.docx Figure S3 An unweighted pair group method with the arithmetic mean (UPGMA) dendrogram of *H. flava* based on the pairwise genetic distance (F_{ST}) of 16S among 17 sampling locations across Niigata Prefecture, Japan.

Additional File 9.docx Figure S4 Phylogenetic tree of 16s haplotype sequences of I. ovatus inferred by maximum likelihood analysis with evolutionary model GTR with sequences from China (MH208506, MH208512, MH208514 – MH208519, MH208522, MH208524, MH208531, MH208574, MH208577, MH208579, KU664519), Japan (AB819241, AB819242, AB819243, AB819244) and US (U95900). An outgroup (Ixodes canisuga) was also included.

Additional File 10.docx Figure S5 Phylogenetic tree of *cox1* haplotype sequences of *H. flava* inferred by maximum likelihood analysis with evolutionary model HKY with sequences from China (KY021800- KY021807; KY021810-KY021819, KY003181, JQ62588 - JQ625889; JQ737097; JF758632). An outgroup (*Ixodes canisuga)* was also included.

Additional File 11.docx Figure S6 Phylogenetic tree of 16S haplotype sequences of *H. flava* inferred by maximum likelihood analysis with evolutionary model GTR with sequences from China (KC844858 -KC844867; KC844880-KC844882, M696720, KP324926, KX450279) and Japan (AB19177- AB819192). An outgroup (*Ixodes canisuga)* was also included.

Tables

Table 1. Summary of *cox1* and 16S haplotype distribution, variability, genetic diversity of *Ixodes ovatus* and *Haemaphysalis flava* populations in Niigata Prefecture

Marker	Species	n	h	s	k	hd±SD	nd±SD
cox1	*I. ovatus*	307	60	65	2.728	0.852 ± 0.012	0.004 ± 0.000
	H. flava	220	63	60	1.472	0.789 ± 0.026	0.002 ± 0.000
16S	*I. ovatus*	284	24	22	0.699	0.442 ± 0.037	0.001 ± 0.000
	H. flava	172	40	49	2.447	0.835 ± 0.024	0.006 ± 0.000

Abbreviations: *n* sample size; *h* number of haplotypes; *s* number of polymorphic sites; *k* mean number of nucleotide differences; *hd±SD* haplotype diversity ± Standard Deviation; *nd±SD* nucleotide diversity ± Standard Deviation

Table 2. Analysis of molecular variance (AMOVA) using *cox1* and 16S of *Ixodes ovatus* and *Haemaphysalis flava* populations

Marker	Species		df	ss	vc	pv	F_{ST}
cox1	*I. ovatus*	Among	28	190.38	0.5803 Va	41.54	0.4154**
		Within	278	227.04	0.8167 Vb	58.46	
	H. flava	Among	16	12.42	0.0039 Va	0.53	0.0053
		Within	203	148.73	0.7327 Vb	99.47	
16S	*I. ovatus*	Among	22	14.04	0.0260 Va	7.39	0.0739**
		Within	261	84.87	0.3252 Vb	92.61	
	H. flava	Among	12	23.6	0.0642 Va	5.21	0.0521**
		Within	159	185.58	1.1672 Vb	94.79	

Abbreviations: *df* degrees of freedom; *ss* sum of squares; *Va, Vb* and *Vc* are associate covariance components; *pv* percentage variation; *P<0.05 ** P<0.01

Figures

Figure 1. Plots (a) and (b) show the relationship between the pairwise F_{ST} values and the geographic distances for *I. ovatus* in both *cox1* and 16S markers from the Mantel test analysis. Figures (c) and (d) illustrate the influence of geographic distance in the spatial autocorrelation analysis of *cox1* and 16S *I. ovatus* sequences. The upper and lower 95% confidence intervals are depicted as the red dotted lines (U and L) and the correlation coefficient *r* is plotted. The values above and below the dashed lines indicate spatial structure. The error bars result from the 1000 permutations of individuals within each distance class. Both analyses were done in Genalex software.

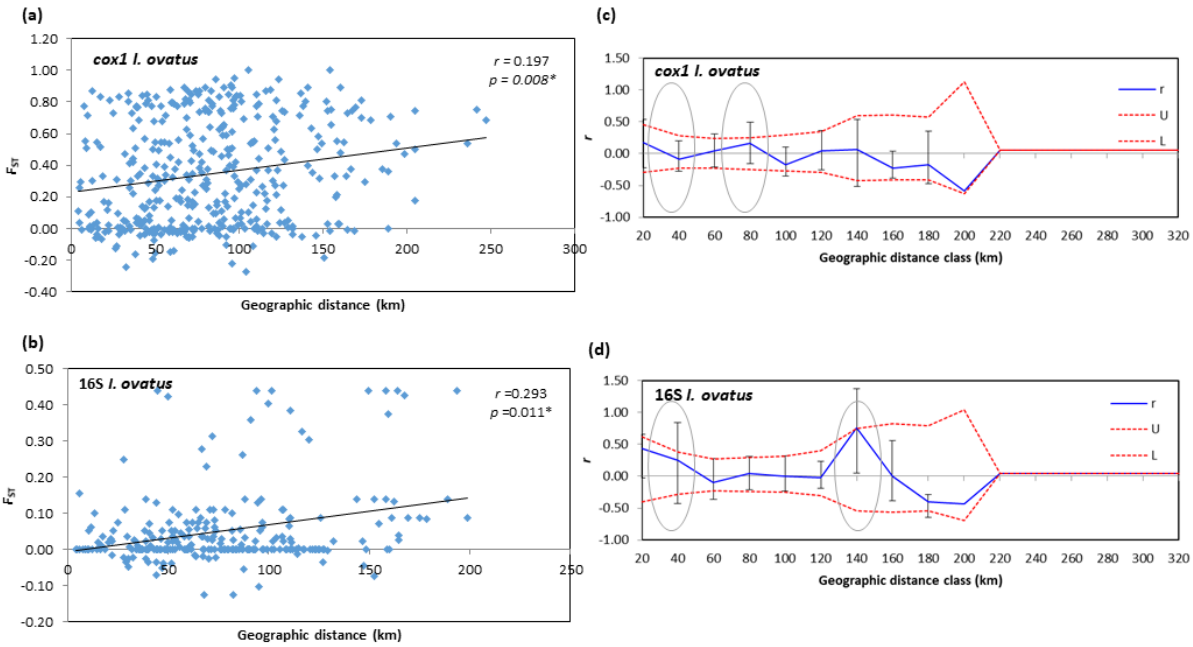


Figure 2. An unweighted pair group method with the arithmetic mean (UPGMA) dendrogram of *I. ovatus* based on the pairwise genetic distance (F_{ST}) of *cox1* among 29 sampling locations across Niigata Prefecture, Japan.

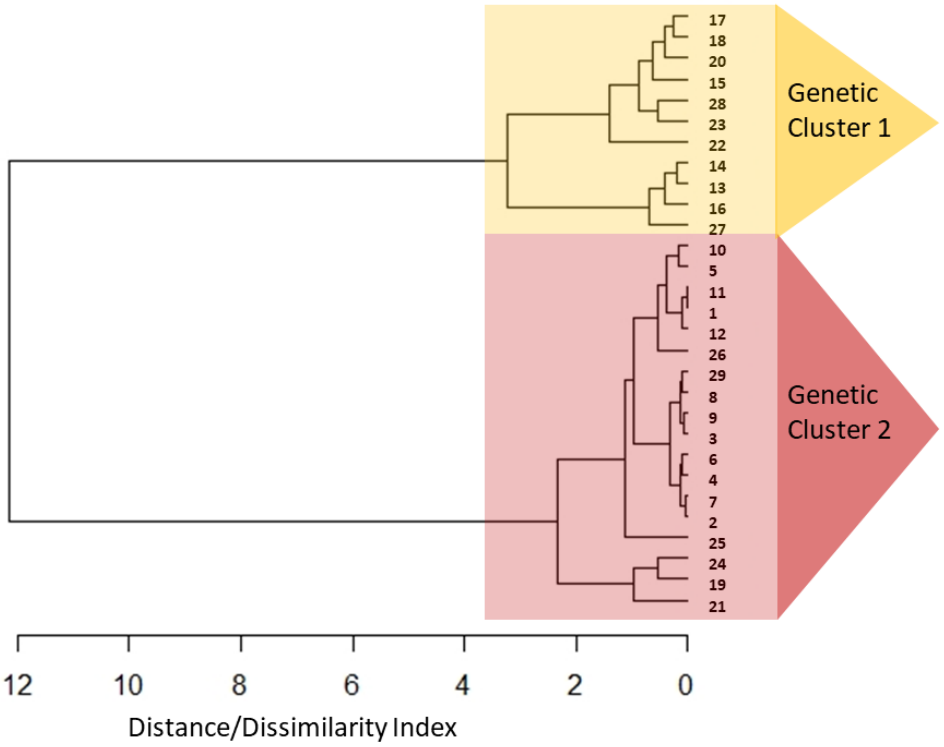


Figure 3. The distribution of the two genetic clusters as observed in the UPGMA cluster dendrogram (Figure 3) of *I. ovatus cox1* sequences from Niigata Prefecture.

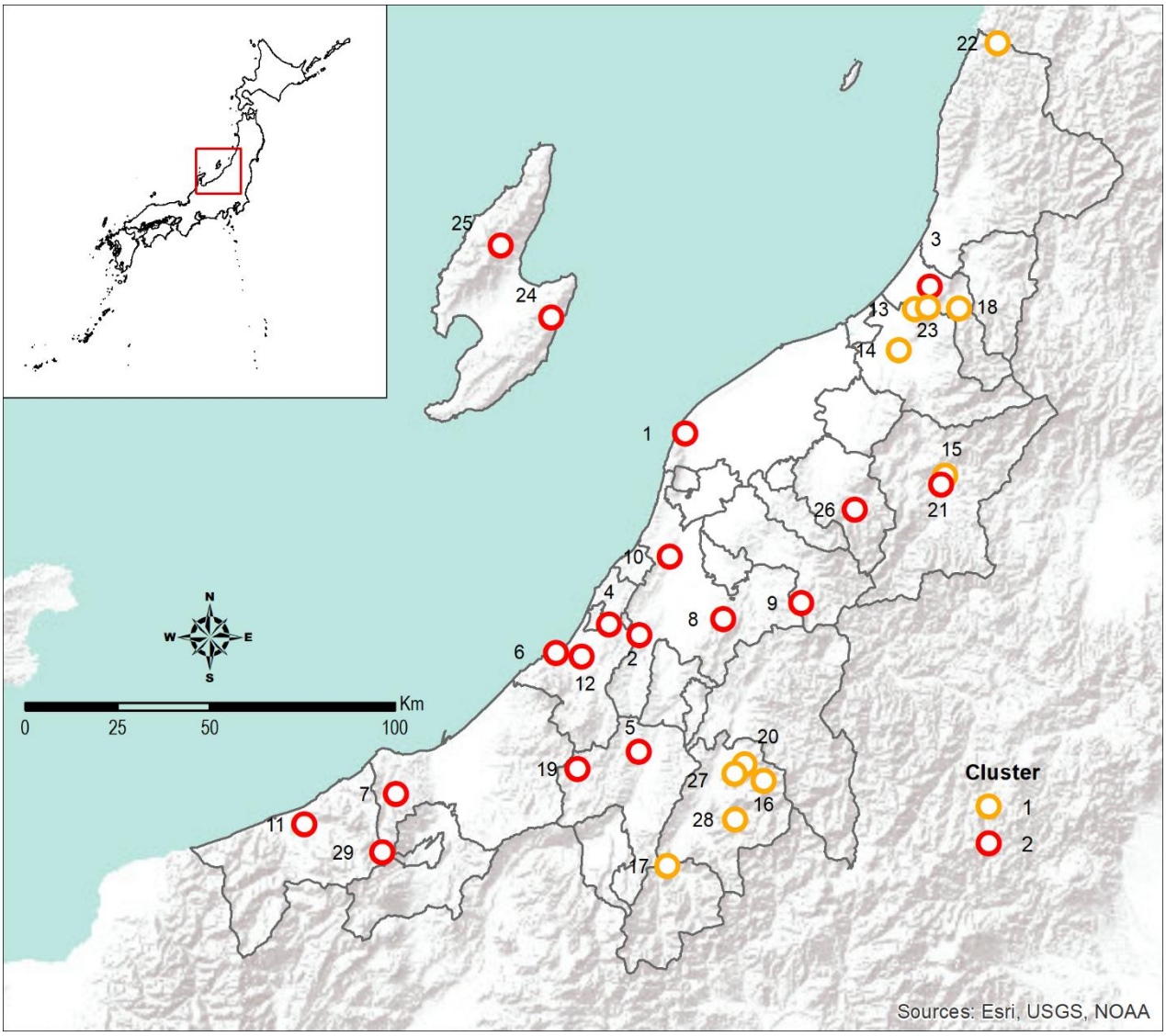
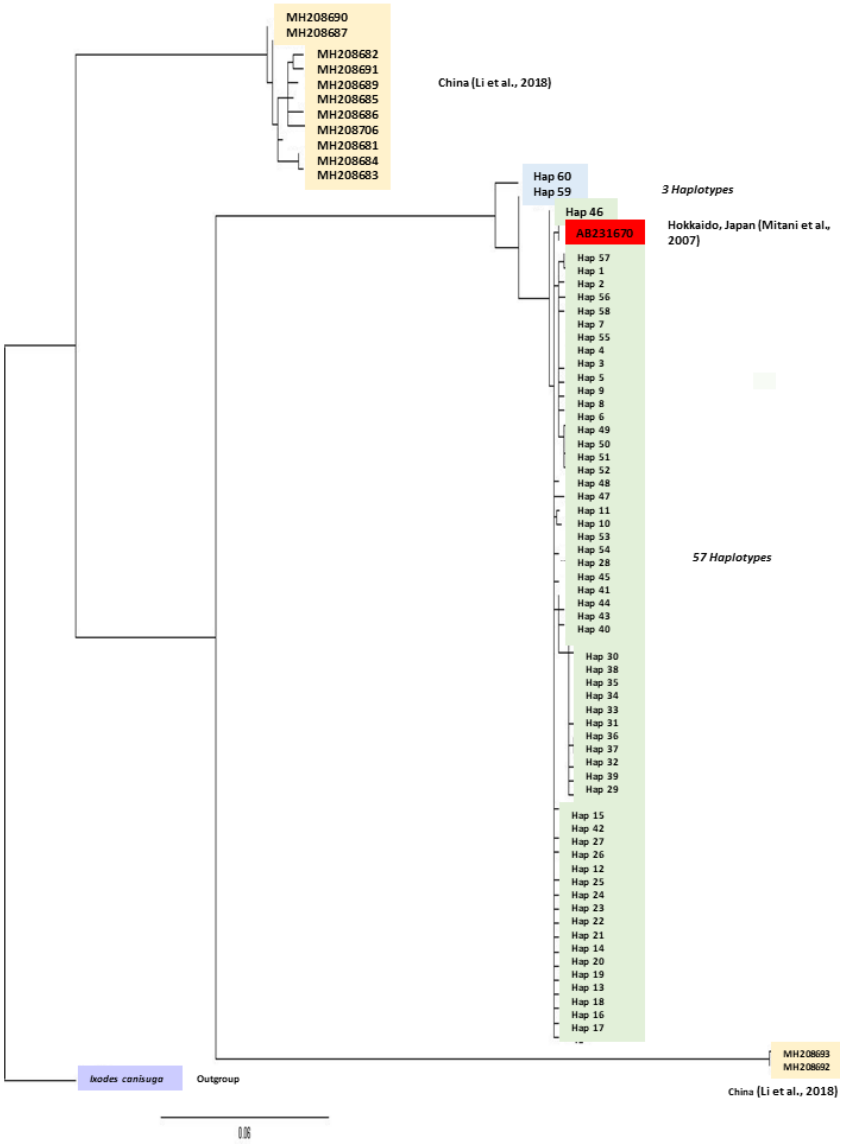


Figure 4. Maximum likelihood gene tree of *cox1* haplotype sequences of *I. ovatus* with sequences from China (MH208681-MH208687, MH208689-MH2086393, MH208706), Japan (AB231670) and an outgroup of *Ixodes canisuga.*



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