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 such as *Ixodes ovatus* and *Haemaphysalis flava* are important vector 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 *I. ovatus* and *H. flava* from Niigata Prefecture, Japan. Samples were collected in 29 (*I. ovatus*) and 17 (*H. flava*) sampling locations across Niigata Prefecture (12,584.18 km^2). 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 few cases of low significant pairwise differentiation. 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. We found three genetic groups (China 1, China 2 and Japan) in the *cox1 I. ovatus* tree. 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 in our data set suggest the potential for cryptic species among the groups. While many factors can potentially account for the observed differences in genetic structure between the two species, including population persistence and large-scale patterns of range expansion, the differences in the mobility of hosts of tick immature stages (small mammals in *I. ovatus*; birds in *H. flava*) is possibly driving the observed patterns.

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

Introduction

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 (Dantas-Torres et al., 2012). Population genetic studies have helped in understanding

45 the dispersal patterns and potential pathogen transmission in ticks (Araya-Anchetta et al., 2015).
46 The direction, distance and gene flow patterns between tick populations can be inferred from
47 population genetic studies (Mccoy 2008). For example, if high gene flow is observed there might be
48 a greater chance of colonizing new areas or of re-colonizing areas following successful vector control
49 programs.

50 Host mobility can affect the genetic patterns of tick populations, although its effects are not
51 consistent. Several studies have reported low levels of gene flow in ticks with less mobile hosts (e.g.
52 smaller mammals) and high levels of gene flow in ticks with highly mobile hosts (Araya-Anchetta et
53 al., 2015). For example, *Amblyomma americanum* and *A. triste* (Ixodidae, Acari) exhibited high gene
54 flow across various spatial scales (137, 000 km^2 to 2.78 million km^2) and this was attributed to their
55 hosts' dispersal capabilities (large mammals and birds) (Guglielmone et al., 2013; Mixson et al.,
56 2006; Trout et al., 2010), while low gene flow was observed in *Amblyomma dissimile* populations
57 and this was attributed to the low mobility of its hosts (small mammals, reptiles and salamanders)
58 (Lampo et al., 1998). In contrast, some studies have reported low gene flow despite the high mobility
59 of the host, for example in *Ixodes scapularis* (Qiu et al., 2002) and *Ornithodoros coriaceus* (Teglas et
60 al., 2006) which can be attributed to the host mobility.

61 In Japan, tick-borne diseases are an increasing public health concern, affecting humans and animals
62 (Yamaji et al., 2018). A total of 8 genera of ticks have been recorded in Japan, composed of 47
63 species: 43 Ixodidae species and 4 Argasidae species (Fujita et al., 2006). Out of 47 species, 21
64 species are known to parasitize humans (Okino et al., 2010). Among these 21 species, *Ixodes ovatus*,
65 the main vector of Lyme disease (Miyamoto et al., 1993) and *Haemaphysalis flava,* a vector of severe
66 fever with thrombocytopenia syndrome (SFTS) and Japanese spotted fever (JSF), were reported in
67 Japan (Yamaji et al., 2018; Yu et al., 2011). A previous study found that the hosts of adult *I. ovatus*
68 were mainly hares and can also be large mammals (e.g. cows and horses), and the hosts of immature

69 forms were small rodents (Yamaguti et al., 1971). *H. flava* adult host preference are cows, dogs,
70 horses, wild boar, bear, and deer, while immature forms only parasitized birds (Yamaguti et al.,
71 1971). Despite the similarity of *I. ovatus* and *H. flava* in which blood meal from different vertebrate
72 host is taken in each life stage (larva, nymph and adult) (Herrmann and Gern, 2015), comparative
73 population genetic studies on two Ixodid tick species such as *I. ovatus* and *H. flava* from the same
74 sampling location remain nonexistent. Thus, our study field the gaps about the dispersal movement
75 of two important tick species: *I. ovatus* and *H. flava.* The low gene flow observed in *I. ovatus* might
76 be due to the lower mobility of hares in the adult stage and small rodents in its immature stage
77 while *H. flava* will exhibit high gene flow because of its avian mediated dispersal in its immature
78 stage.

79 In addition to tick population structure studies, genetic studies may also reveal the presence of
80 cryptic species, where morphologically identified individuals might represent more than one species
81 (Fegan et al., 2005). Previous studies on *Rhipecephalus appendiculatus* (Kanduma et al., 2016) , *I.*
82 *holocylus* (Song et al., 2011) and *I. ovatus* (Li et al., 2018) have revealed the presence of cryptic
83 species based on the clustering of haplotypes in a phylogenetic tree, and concluded that
84 morphological criteria for species differentiation alone are equivocal and that genetic analysis is
85 important.

86 Here, we studied the population genetic structure of *I. ovatus* and *H. flava* and also tested for the
87 presence of cryptic species using mitochondrial DNA sequences of *cox1* and the 16S rRNA gene. We
88 hypothesized that *I. ovatus* and *H. flava* may have differences in their population genetic structure
89 despite some overlap in their adult host preference can be attributed to the differences in mobility
90 of the immature tick hosts.

91 **Material and methods**

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

93 From April 2016 until November 2017, ticks were collected by the standard flagging methods
94 (Ginsberg et al., 1989) across Niigata Prefecture in Japan and a total of 29 sampling locations were
95 surveyed for *H. flava* and *I. ovatus* ticks (Additional File 1. Table S1). Altitude per location ranged
96 from 8 -1402 m/a.s.l. and the geographic distance between sites ranged from 4.28 - 247.65 km.
97 Collected ticks were stored in microcentrifuge tubes with ethanol at 4^0C. A total of 2,103 individual
98 tick samples were collected. Identification of sex, developmental stage and species was performed
99 using a compound microscope and identification keys of (Yamaguti et al., 1971) .

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. The total number of analyzed samples (n=540) was chosen based on its
104 species identity while the other identified tick species were excluded for this study. Prior to DNA
105 extraction, each tick was washed with alcohol and PBS solution. DNA quality was checked using a
106 NanoDrop™ 2000 Spectrophotometer (Thermo Scientific™). Two mitochondrial genes were
107 amplified by polymerase chain reaction (PCR): Cytochrome Oxidase 1 (*cox1*) (658 base pairs) using
108 the primer pairs LCO-1490 (5'- GGTCAACAAATCATAAAGATATTGG -3') and HCO1-2198 (5'-
109 AAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994) and the 16S rRNA gene (16S) (407 base
110 pairs) with the following primer pairs 16S+1 (5'CTGCTCAATGAATATTTAAATTGC-3') and 16S – 1 (5' -
111 CGGTCTAAACTCAGATCATGTAGG-3')(Tian et al., 2011). All PCR amplifications of both *cox1* and 16S
112 were performed with a final volume of 10 µl with 1µl of genomic DNA. The PCR reaction for both
113 markers were composed of the following: 10x Ex Taq buffer, 25mM $MgCl_2$, 2.5mM dNTP, 10µm of
114 forward and reverse primers and 5 units/µl of TaKaRa Ex Taq™ (Takara Bio Inc.). The *cox1* PCR
115 amplification were as follows: an initial denaturation of 94^0C for 2 minutes, denaturation of 94^0C for

116 30 seconds, annealing of 38^{0}C for 30 seconds, extension of 72^{0}C for 1 minute for 30 cycles and final
117 extension of 72^{0}C for 10 minutes. While the 16S PCR amplification followed the protocol of (Tian et
118 al., 2011) with some modifications, initial denaturation of 94^{0}C for 3 minutes, denaturation of 94^{0}C
119 for 30 seconds, annealing of 50^{0}C for 40 seconds, extension of 72^{0}C for 40 seconds for 30 cycles and
120 final extension of 72^{0}C for 5 minutes. PCR products were purified using the QIAquick 96 PCR
121 Purification Kit (Qiagen) in accordance with the manufacturer's instructions, and sequenced by
122 Eurofin Genomics, Inc., Tokyo, Japan.

123 **Sequence data analysis**

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

135 **Population genetic analysis**

136 The group of sequences obtained from ticks collected in the same geographic location was
137 considered as belonging to the same population. We analyzed the final sequences of both *H. flava*
138 and *I. ovatus* per genetic marker separately using DNASp version 6.12.03 (Rozas et al., 2017) and

139 Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010) for the following parameters: number of
140 haplotypes (nh), average number of polymorphic sites (s), and average number of nucleotide
141 differences (k). The haplotype diversity (h) and nucleotide diversity (π) were calculated in Arlequin
142 3.5.2.2. The population genetic structure among and within sampling locations in Niigata Prefecture
143 was calculated by analysis of molecular variance (AMOVA) performed in Arlequin with 1023
144 permutations. The degree of molecular genetic differentiation between sampling locations defined
145 as populations was assessed by calculation of the pairwise F_{ST} values in Arlequin. To determine if the
146 genetic differentiation was influenced by geographical distance or altitudinal differences among
147 populations, we performed Mantel Test in GenAlEx version 6.51b2 (Peakall and Smouse, 2006). Two
148 tests per species and marker were conducted. First we compared pairwise genetic (pairwise F_{ST}
149 values) and geographical distances (km) and second we compared genetic distance (F_{ST} values) with
150 altitudinal differences (m/a.s.l.). The geographic distances were obtained from the GPS coordinates
151 (latitude and longitude) recorded during the sampling. All Mantel tests were assessed using 9999
152 permutations for the significance of the correlation.

153 The spatial component of genetic variation was further assessed by spatial autocorrelation (Smouse
154 and Peakall, 1999) using GenAlEx (Peakall and Smouse, 2006). The autocorrelation coefficient (*r*)
155 was computed from the geographic distance between sampling locations and the genetic distance
156 (pairwise F_{ST} values). This measures the genetic similarity between individual pairs within an
157 identified distance class. The size of the distance class influences the estimation of the spatial
158 autocorrelation. We identified the appropriate distance class based on the observed distribution of
159 pairwise geographic distance between the sampling locations (data not shown). The distance class
160 ranged into 10 classes of 20 kilometers from 20 to 200 kilometers. Bootstrap estimates of *r* and
161 random permutations were set at 9999 each for the test for significance. The upper and lower error
162 bars in the correlogram bound the 95% confidence interval of *r* value as identified by bootstrap

163 resampling. The upper and lower confidence limits bound the 95% confidence interval of the null
164 hypothesis of no spatial structure in the distance class. The genetic relationships among the
165 sampling locations (i.e., populations) were calculated by unweighted pair group method with the
166 arithmetic mean (UPGMA) cluster method using the APE package (Paradis and Schliep., 2018) and
167 R program (R Development core team., 2016) to create dendrogram using the genetic distance
168 matrix (pairwise F_{ST} values) generated from GenAlEx.

169 To evaluate the relationship between the haplotypes, we constructed haplotype network on
170 PopART program version 1.7 per marker (cox1, 16S rRNA gene) and species (*I. ovatus* and *H. flava*
171 (http://popart.otago.ac.nz/index.shtml) using the median-joining (MJ) network algorithm (Bandelth
172 et al., 1999).

173 **Phylogenetic analysis**

174 We constructed maximum likelihood (ML) gene trees for *cox1* and 16S haplotype sequences of *I.*
175 *ovatus* and *H. flava* using PhyML version 3.1 (Guindon and Gascuel, 2003) under the default settings.
176 We applied HKY and GTR nucleotide substitution models for *cox1* and 16S, respectively, as suggested
177 by jModelTest ver 2 (Darriba et al., 2012). Additional sequences from China (MH208506, MH208512,
178 MH208514, MH208522, MH208515-19, MH208524, MH208531, MH208574, MH208577,
179 MH208579, MH208681-87, MH208689-93, MH208706, KU664519 (Li et al., 2018)), Japan (Hokkaido
180 AB231670, U95900; Yamanashi AB819241, AB819243 and Aomori AB819244) (Mitani et al., 2007;
181 (Norris et al., 1999), Takano et al., 2014)) were included. We used *Ixodes canisuga* an outgroup
182 because it is a closely related species (KY962023, KY962074; (Hornok et al., 2015)).

183 **Results**

184 The number of *I. ovatus* ranged from 1 to 36 individuals per study site and, as with *H. flava*, more
185 were successfully sequenced for *cox1* (307/320; 95.9%) than for 16S (284/320; 88.8%) (Additional

186 File 1. Table S1). The number of successfully sequenced and analyzed *H. flava* ranged from 1 to 36
187 individuals per study site, with more successfully sequenced for *cox1* (220/223; 98.7%) than for 16S
188 (172/223; 77.1%). There were 60 and 63 *cox1* haplotypes in *I. ovatus* and *H. flava* respectively, and
189 24 and 40 16S haplotypes (Table 1). Haplotype diversity (h) ranged from 0.442 to 0.852 and
190 nucleotide diversity (π) ranged from 0.001 to 0.006 for both markers and species (Table 1).

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

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

193

194 Abbreviations: n sample size; nh number of haplotypes; s number of polymorphic sites; k mean
195 number of nucleotide differences; h haplotype diversity; π nucleotide diversity

196 AMOVA results revealed a high among-population divergence (41.54%) in *I. ovatus cox1* sequences,
197 whereas both *cox1* and 16S markers of *H. flava* indicated no significant genetic variation among
198 populations (Table 2). Global F_{ST} values were highest in *I. ovatus* with values 0.415 in *cox1* and 0.074
199 in 16S sequences. Pairwise F_{ST} values (0.500 to 1.00) of *I. ovatus* based on *cox1* showed significant
200 genetic differences between most pairs of populations such as between populations18 and 25 and
201 between populations 28 and 25 (Additional File 2 Table S2). We also observed significant differences
202 in 16S ranged from (0.0225 to 0.6319) (Additional File 3 Table S3). Pairwise F_{ST} values from the *cox1*

203 marker of *H. flava* showed no significant genetic differences except in few population pairs such as
204 in 5 and 6 and 6 and 13 (Additional File 4 Table S4). No significant genetic differentiation was also
205 observed in 16S marker of *H. flava* except in few population pairs such as between 3 and 20 and 4
206 and 20 (Additional File 5 Table S5).

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

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

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

211 The Mantel test of *I. ovatus* showed evidence of isolation by geographic distance in both markers:
212 (*cox1 r* = 0.197, *p* = 0.008; 16S *r* = 0.293, *p* = 0.011) (Figures 1a, b). In contrast, there was no evidence
213 for isolation by altitudinal difference (*cox1* r = -0.066, *p*=0.225; 16S r = -0.023, p = 0.577). Both
214 Mantel tests were not significant for *H. flava* (distance: *cox1* r= -0.145, p= 0.23; 16S r = -0.049, p=
215 0.33; altitude: r = 0.092, p = 0.30; 16S r = 0.217, p= 0.06). The spatial autocorrelation test in *I. ovatus*

216 revealed positive and significant correlation between genetic and geographic distance from 20 – 40
217 km, and 60 – 80 km, and 20 – 50 km and 120 – 160 km from *cox1* and 16S, respectively (Figure 1c,
218 d).

219 The UPGMA cluster dendrogram from the pairwise F_{ST} values from the *cox1*, *I. ovatus* (Figure 2)
220 revealed 2 genetic clusters across the 29 populations, where the cluster 2 included populations from
221 the more western sites including the island (location 24 and 25) while cluster 1 mostly included
222 populations in the northern and southern part of Niigata Prefecture (Figure 3). No evident genetic
223 clustering on the dendrogram was observed from the *I. ovatus* 16S sequences and in both *cox1* and
224 16S *H. flava* sequences (Additional File 6 Figure S1; Additional File 7 Figure S2; Additional File 8
225 Figure S3).

226 A putative I. ovatus species complex was identified based on the presence of 3 distinct haplotype
227 clusters in both cox1 and 16S rRNA genes; 2 groups in China and one in Japan. The *cox1* gene tree
228 of 60 *I. ovatus* haplotypes indicated three groups: group 1 of the published sequences from western
229 China, one large group 2 containing the 58 haplotypes and 2 divergent haplotypes (Hap 60 and Hap
230 59), and group 3 from western China (Figure 4). The haplotype network of the *cox1* gene of *I. ovatus*
231 also displayed the 2 divergent haplotypes (Hap 60 and Hap 59) (Additional File 12 Supplementary
232 Figure S7). The 2 divergent haplotypes were only found once in the whole data set. One was found
233 in population 29 and the other in population 7. The published haplotype from Hokkaido (Mitani et
234 al., 2007) occurred within our large group and the published sequences from western China were in
235 a different clade. The *I. ovatus* 16S gene tree (24 newly sequenced haplotypes) (Additional File 9
236 Figure S4) also recovered published haplotypes from Japan (Yamanashi and Aomori; (Takano et al.,
237 2014) and Hokkaido (Norris et al., 1999) within the same cluster as our new sequences. The *cox1*
238 haplotype sequences of *H. flava* were similar to reference sequences from China (Additional File 10
239 Figure S5) [unpublished manuscript] while the 16S haplotype sequences (Additional File 11 Figure

240 S6) showed similarities with Japan (Kagoshima, Fukui Aomori, Fukui, Yamanashi, Kagawa and Ehime
241 (Takano et al., 2014) and China [unpublished manuscript] reference sequences from Genbank.

242 **Discussion**

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

244 Our results supported our hypothesis that *H. flava* and *I. ovatus* display contrasting population
245 genetic structure. The noticeable high gene flow in *H. flava* may be a result of the higher level of
246 mobility of its bird hosts in the tick's immature stage and the more pronounced genetic structure
247 and lower gene flow in *I. ovatus* may be due to the restricted ability of its small mammalian hosts
248 at the immature stage to disperse further. 28 species of birds were previously reported as hosts of
249 *H. flava* from Japan*,* mostly from the order Passeriformes known as migratory birds (Yamauchi and
250 Takeno, 2000). The host for adults of *I*. *ovatus* included large mammals such as hares while
251 immature forms are found on small mammals such as small rodents and rats (Yamaguti et al., 1971).
252 The movement of small mammals is limited because of environmental and anthropogenic factors
253 such as difficulty in crossing roads and higher possibility of road kill due to vehicle collision and or
254 traffic (Haemig et al., 2008), which may explain the genetic structuring in *I. ovatus*. The host
255 preference of the adults *H. flava* are large mammals (e.g. cows, horses, wild boar, deer, bear) and
256 hares, dogs, rodents while the immature forms parasitize birds (Yamaguti et al., 1971). Mammals
257 and birds may have wide habitats ranges that may allow maintaining the gene flow of *H. flava*
258 between the locations in Niigata, as previously observed in *Amblyomma americanum* populations
259 (Mixson et al., 2006; Trout et al., 2010; Reichard et al., 2005) , and *Ixodes ricinus* (Casati et al., 2008).
260 Birds present the greatest potential for farther dispersal of *H. flava* that could reduce its genetic
261 structuring (Mcclain et al., 1995) that supports the results of our study.

262 Several alternative factors such as tick behavior, biology and ecology can also affect the tick dispersal
263 movement. For example, long movement patterns, resistance against unfavorable weather
264 conditions causing high survival in their hosts and low host specificity (Guglielmone et al., 2013) are
265 possible reasons for the low genetic structuring in *H. flava* populations. Previous study on two tick
266 species: *Hyalomma rufipes* and *Amblyomma hebraum* also displayed contrasting genetic patterns
267 despite the overlap sharing of highly mobiles hosts such as birds (Cangi et al., 2013) due to the
268 capacity of the immature stages of ticks to survive off the hosts in various habitat conditions
269 (Needham et al., 1986; Cumming, 1999; Estrada-Peña, 2015). Population structure can also be
270 influenced by assortative mating (e.g. *Ixodes ricinus*) wherein genetically similar individuals tend to
271 mate, rather than random mating resulting in increased genetic divergence (Kempf et al., 2009). Our
272 study does not have supporting data to test these alternative factors thus it is suggested in future
273 studies to further analyze these factors that could play a role in the tick's dispersal movement.

274 Based on previous studies using microsatellites and Random Amplification of Polymorphic DNA
275 (RAPD) markers (Yaegashi et al., 2014; Watanabe et al., 2010), isolation by distance suggests that
276 the populations are in equilibrium of migration and genetic drift at the spatial scale of Niigata
277 Prefecture. Our results were congruent with a previous study of *Amblyomma ovale* that showed
278 positive relationship of geographic and genetic distance at the spatial scale of the whole of Brazil
279 whose main hosts for the immature stage are small rodents while tapir and jaguar in its adult stage
280 (Bitencourth et al., 2019). In contrast, *H. flava* populations showed an absence of isolation by
281 distance. In circumstances when geographic distance can't explain the genetic structure of tick
282 populations, other factor such as ecological variables (e.g. land use) maybe affecting the genetic
283 structuring within tick populations (Casati et al., 2008; Krakowetz et al., 2010; Lampo et al., 2015)
284 which can be inferred when using molecular markers with higher mutation rate such as
285 microsatellites.

286 A significant and positive spatial autocorrelation of *I. ovatus* in both *cox1* and 16S sequences was
287 observed within the geographic range of 20 – 40 km and 60- 80 km in *cox1* sequences and 20 – 50
288 km and 120 – 160 km range in the 16S sequences. We can infer that the positive *r* values at the
289 maximum geographic rages of 60-80 km in *cox1* and 120 - 160 km in 16S *I. ovatus* sequences indicate
290 that gene flow is occurring in the wide geographic range. The lack of significance at 40-60 km in *cox1*
291 may suggest patch structure indicating genetic similarity or variation (Smouse and Peakall, 1999) as
292 observed on limpet species, *Siphonaria raoulensis* from New Zealand that also displayed recovery
293 of positive *r* values at greater distances (Wood and Gardner, 2007). However, no autocorrelation
294 was observed in *H. flava* at any distance class, suggesting that gene flow occurring has no specific
295 pattern favoring certain distances (Pardanani and Mather, 2009; Kitron and Kazmierczak.,
296 1997;Nicholson et al., 2014). Since the sampling geographic range of our study was within Niigata
297 Prefecture and distances >220 km were not included in our sampling, we suggest that future studies
298 collect samples at a wider geographic scale to more precisely determine the geographic distance at
299 which autocorrelation exists.

300 16S *I. ovatus* sequences showed genetic differentiation (pairwise F_{ST} = 0.0532-0.5652) in few pairs
301 of sampling locations as compared to the *cox1* sequences which may be due to lower variability of
302 the marker thus providing inadequate genetic variation for population genetic analysis, as
303 previously observed in *Amblyomma ovale* (Bitencourth et al., 2019) and *Rhipicephalus microplus*
304 (Low et al., 2015;Burger et al., 2014). The low variability of the 16S in *I. ovatus* may not be sufficient
305 to infer intraspecific relationship and is further supported by its low (*nd* = 0.001) nucleotide diversity
306 as compared in *cox1 I. ovatus* (*nd* = 0.004). Thus, it may also explain the grouping observed in the
307 *cox1 I. ovatus* tree which is absent in the 16S *I. ovatus* sequences. Despite of these, we used 16S
308 mitochondrial gene because this gene mutates at a rate that is informative for species-level
309 phylogenetic and broad biogeographic inferences(Araya-Anchetta et al., 2015)

310 The negative pairwise F_{ST} values obtained in *H. flava* sequences do not have any biological meaning.
311 These results from the unbiased estimator allowing a negative F_{ST} when the sample size is small.
312 (Bitencourth et al., 2016; Vial et al., 2006; Weir and Cockerham, 1984) . It can also be noted that
313 we found significant pairwise F_{ST} values in 2 sampling location in *H. flava cox1* sequences probably
314 due to the fact that both locations have only one tick sample. Although, some sampling sites in this
315 study have small sample size (n < 10), the *cox1* and 16S sequences provided valuable insight into the
316 genetic structure and gene flow patterns of the present-day tick populations. Thus, for future
317 research, we recommend to increase the sample size per sampling site.

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

319 Our *cox1 I. ovatus* gene tree showed Japanese individuals to form a group that was distinct from
320 haplotypes from southern China (Li et al., 2018). Despite the low gene flow we found in *cox1 I.*
321 *ovatus*, haplotypes found from Niigata were closely related to the published sequence from
322 Hokkaido, Japan which may indicate that these ticks originated from a diverse set of geographical
323 locations in Japan which might be transported by its hosts or is undergoing recent population
324 expansion from northern Japan (Hokkaido) to south (Niigata) or *vice versa*. We found three groups
325 (China 1, Japan and China 2) and 2 slightly divergent *cox1* haplotypes (Hap 60 and 59) in the Japan
326 group of *I. ovatus* in Niigata. Considering two or more cryptic species can be concealed in one
327 morphologically described species (Bitencourth et al., 2016), the occurrence of the 3 groups and the
328 divergent haplotypes suggests that *I. ovatus* may be a species complex. Previous studies have also
329 observed species complexes in *Ixodes* and *Rhipicephalus* (Li et al., 2018; Song et al., 2011;Burger et
330 al., 2014;Xu et al., 2003) suggesting that morphological criteria for species differentiation alone are
331 equivocal and that genetic analysis is important. An integrative approach of tick species concept
332 that includes the morphology, genetics, biology and ecological trails of ticks must be tackled (Dantas
333 et al., 2019).

334 **Conclusions**

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

344 **List of Abbreviations**

345 *H. flava – Haemaphysalis flava*

346 *I. ovatus – Ixodes ovatus*

347 AMOVA – Analysis of molecular variance

348 UPGMA - unweighted pair group method with arithmetic mean

349 ML – maximum likelihood

350 F_{ST} – fixation index

351 **Declarations**

352 **Ethics approval and consent to participate**

353 Not applicable

354 **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 _____.

Declarations of 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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562

563 **Additional Files**

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

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

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

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

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

574 **Additional File 6.docx Figure S1.** An unweighted pair group method with the arithmetic mean
575 (UPGMA) dendrogram of *I. ovatus* based on the pairwise genetic distance (F_{ST}) of 16S among 23
576 sampling locations across Niigata Prefecture, Japan.
577 **Additional File 7.docx Figure S2** An unweighted pair group method with the arithmetic mean
578 (UPGMA) dendrogram of *H. flava* based on the pairwise genetic distance (F_{ST}) of *cox1* among 17
579 sampling locations across Niigata Prefecture, Japan.
580 **Additional File 8.docx Figure S3** An unweighted pair group method with the arithmetic mean
581 (UPGMA) dendrogram of *H. flava* based on the pairwise genetic distance (F_{ST}) of 16S among 17
582 sampling locations across Niigata Prefecture, Japan.
583 **Additional File 9.docx Figure S4** Phylogenetic tree of 16s haplotype sequences of *I. ovatus* inferred
584 by maximum likelihood analysis with evolutionary model GTR with sequences from China
585 (MH208506, MH208512, MH208514 – MH208519, MH208522, MH208524, MH208531,
586 MH208574, MH208577, MH208579, KU664519), Japan (AB819241, AB819242, AB819243,
587 AB819244, and U95900). An outgroup (*Ixodes canisuga*) was also included.
588 **Additional File 10.docx Figure S5** Phylogenetic tree of *cox1* haplotype sequences of *H. flava*
589 inferred by maximum likelihood analysis with evolutionary model HKY with sequences from China
590 (KY021800- KY021807; KY021810-KY021819, KY003181, JQ62588 - JQ625889; JQ737097;
591 JF758632). An outgroup (*Ixodes canisuga)* was also included.
592 **Additional File 11.docx Figure S6** Phylogenetic tree of 16S haplotype sequences of *H. flava*
593 inferred by maximum likelihood analysis with evolutionary model GTR with sequences from China
594 (KC844858 -KC844867; KC844880-KC844882, M696720, KP324926, KX450279) and Japan
595 (AB19177- AB819192). An outgroup (*Ixodes canisuga)* was also included.
596 **Additional File 12 Figure S7** Median-joining haplotype network of *I. ovatus* from *cox1* marker.
597 Each circle represents a unique haplotype and the lines correspond to mutations.

598 **Additional File 13 Figure S8** Median-joining haplotype network of *I. ovatus* from 16S marker. Each
599 circle represents a unique haplotype and the lines correspond to mutations.
600 **Additional File 14 Figure S9** Median-joining haplotype network of *H. flava* from *cox1* marker. Each
601 circle represents a unique haplotype and the lines correspond to mutations.
602 **Additional File 15 Figure S10** Median-joining haplotype network of *H. flava* from 16S marker. Each
603 circle represents a unique haplotype and the lines correspond to mutations.
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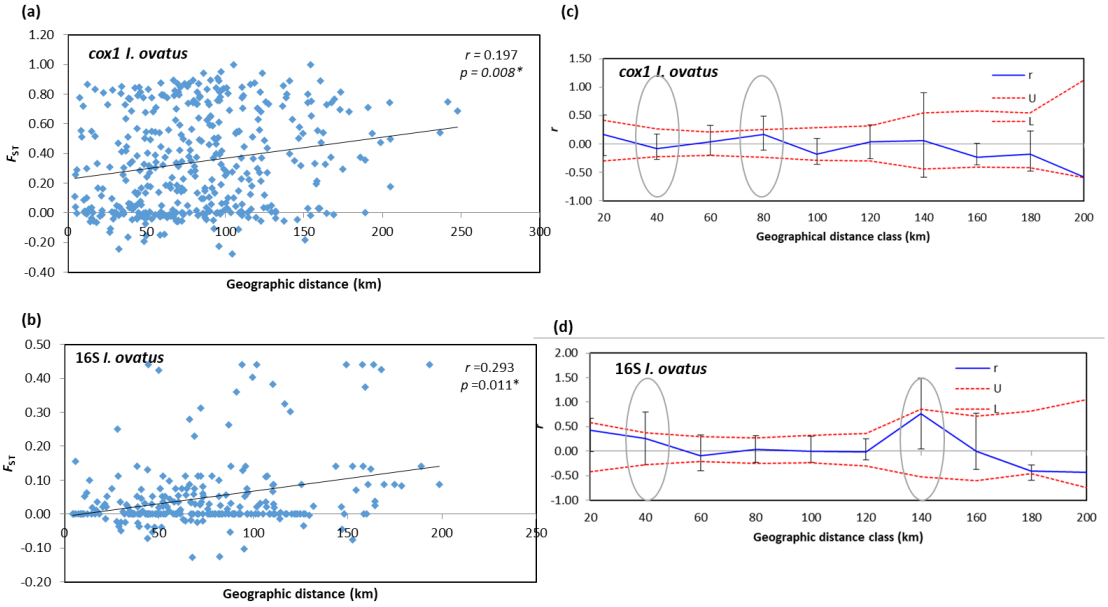
Figures

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.

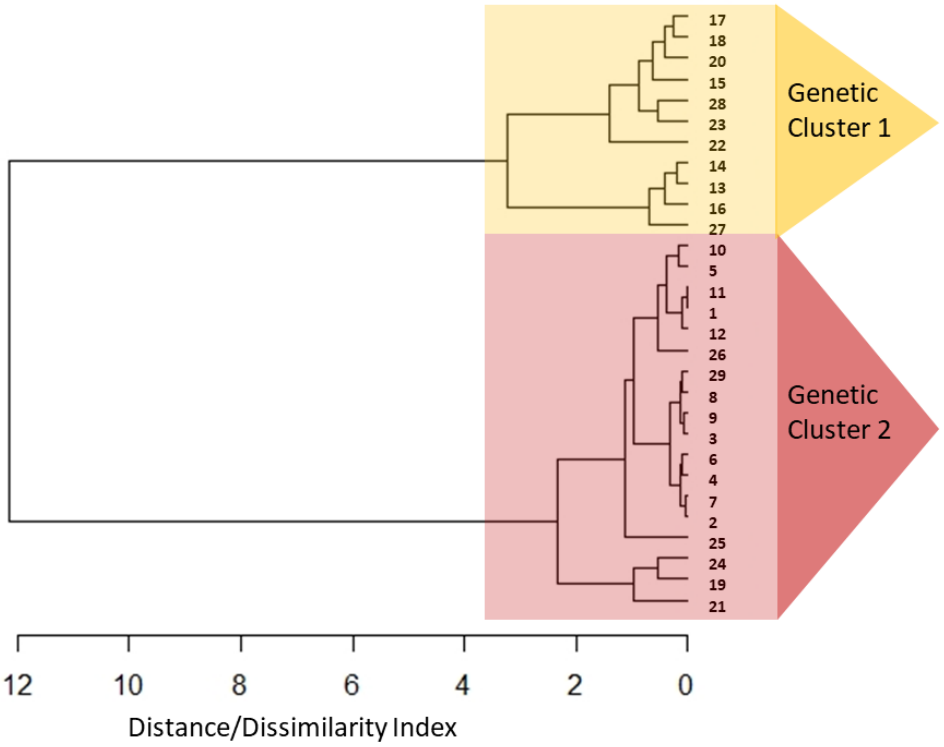


633 **Figure 2.** An unweighted pair group method with the arithmetic mean (UPGMA) dendrogram of *I.*

634 *ovatus* based on the pairwise genetic distance (F_{ST}) of *cox1* among 29 sampling locations across

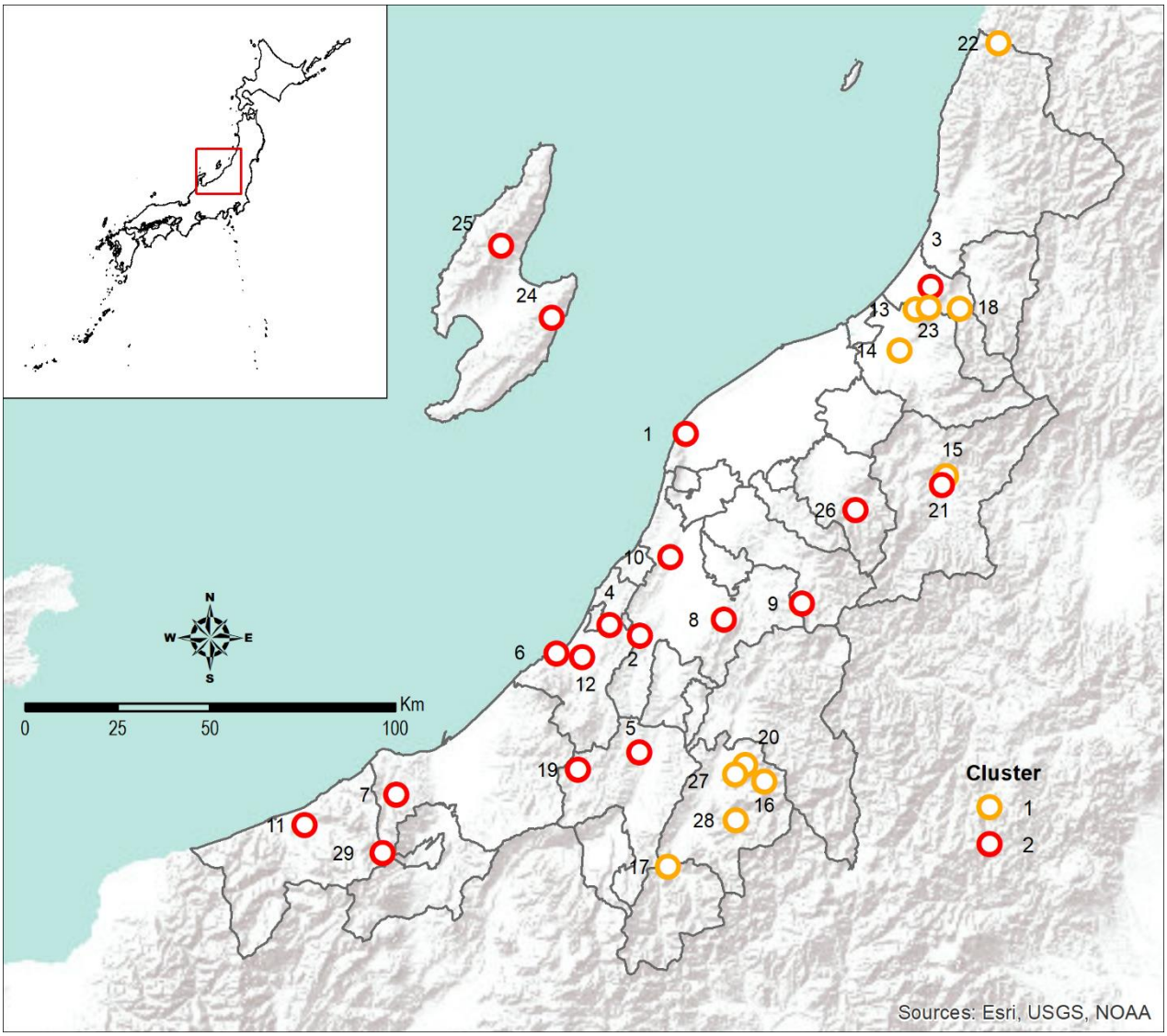
635 Niigata Prefecture, Japan.

636



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

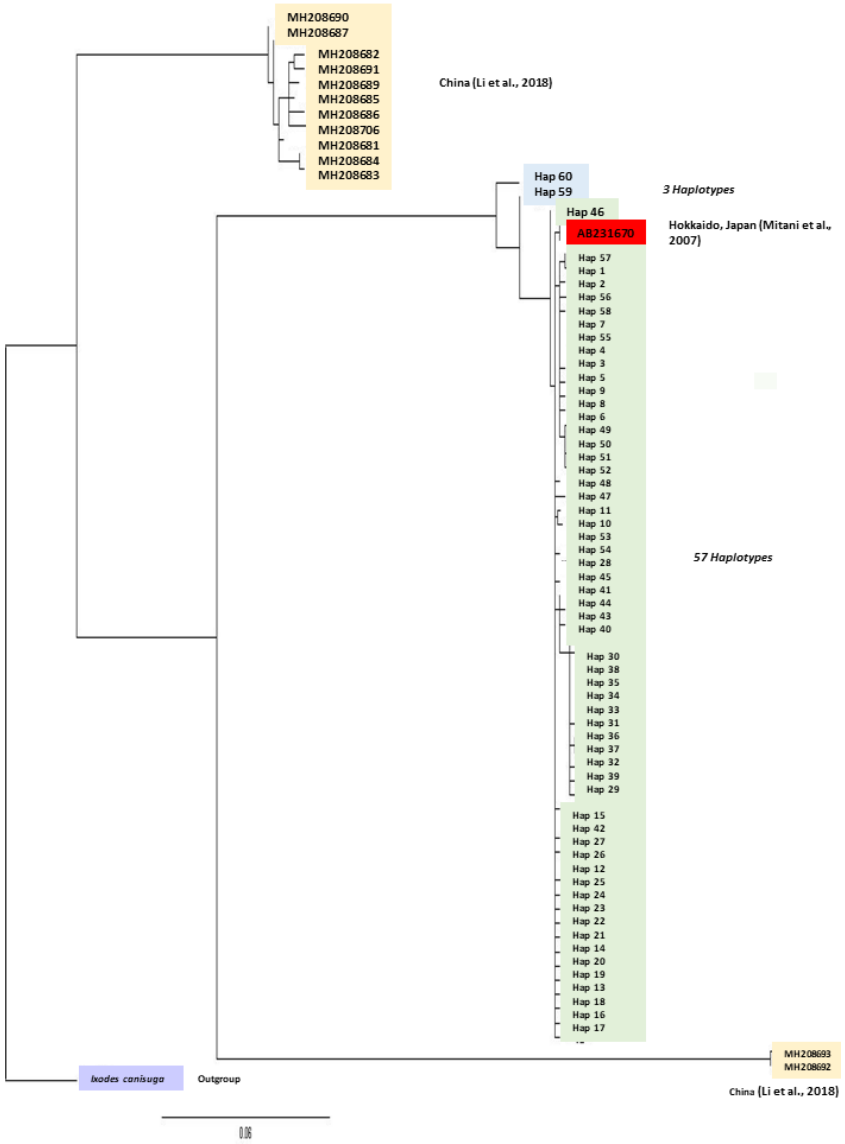
638 (Figure 3) of *I. ovatus cox1* sequences from Niigata Prefecture.



639 **Figure 4.** Maximum likelihood gene tree of *cox1* haplotype sequences of *I. ovatus* with sequences

640 from China (MH208681-MH208687, MH208689-MH2086393, MH208706), Japan (AB231670) and

641 an outgroup of *Ixodes canisuga.*



642 *Yellow* reference sequences from China (Li et al., 2018); *red* reference sequence from Hokkaido,

643 Japan (Mitani et al., 2007); *blue I. ovatus* divergent *cox1* haplotypes; *green I. ovatus cox1*

644 remaining haplotypes; *purple I. canisuga* outgroup