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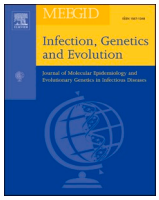

Comparative population genetic structure of two ixodid tick species (Acari: Ixodidae) (*Ixodes ovatus* and *Haemaphysalis flava*) in Niigata prefecture, Japan

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ABSTRACT

Ixodid ticks (Acari:Ixodidae) are essential vectors of tick-borne diseases in Japan. In this study, we characterized the population genetic structure and inferred genetic divergence in two widespread and abundant ixodid species, *Ixodes ovatus* and *Haemaphysalis flava*. Our hypothesis was that genetic divergence would be high in *I. ovatus* because of the low mobility of their small rodent hosts of immature *I. ovatus* would limit their gene flow compared to more mobile avian hosts of immature *H. flava*. We collected 320 adult *I. ovatus* from 29 locations and 223 adult *H. flava* from 17 locations across Niigata Prefecture, Japan, and investigated their genetic structure using DNA sequences from fragments of two mitochondrial gene regions, *cox1* and the 16S rRNA gene. For *I. ovatus*, pairwise F_{ST} and analysis of molecular variance (AMOVA) analyses of *cox1* and 16S sequences indicated significant genetic variation among populations, whereas both markers showed non-significant genetic variation among locations for *H. flava*. A *cox1* gene tree and haplotype network revealed three genetic groups of *I. ovatus*. One of these groups consisted of haplotypes distributed at lower altitudes (251–471 m.a.s.l.). The *cox1* sequences of *I. ovatus* from Japan clustered separately from *I. ovatus* sequences reported from China, suggesting the potential for cryptic species in Japan. Our results support our hypothesis and suggest that the host preference of ticks at the immature stage may influence the genetic structure of the ticks. This information may be important for understanding the tick-host interactions in the field to better understand the tick-borne disease transmission and in designing an effective tick control program.

List of Abbreviations

H. flava	*Haemaphysalis flava*
I. ovatus	*Ixodes ovatus*
bp	base pairs
AMOVA	analysis of molecular variance
UPGMA	unweighted pair group method with arithmetic mean
ML	maximum likelihood
F_{ST}	fixation index
PBS	phosphate-buffered saline
IBD	Isolation by distance

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1. Introduction

Tick-borne diseases are a public health concern, and their control is often challenging because of the complex interactions between ticks and their vertebrate hosts in a changing environment (Dantas-Torres et al., 2012). Population genetic studies can shed light on the dispersal patterns of ticks, including the direction, distance, and potential factors influencing movement, by estimating gene flow between tick populations (McCoy, 2008; Araya-Anchetta et al., 2015). If high levels of gene flow are observed, there might be a greater chance of colonizing new areas or re-colonizing areas once vector control programs have been carried out.

Due to the small size of ticks and their vulnerability to harsh environments while not living on the host, tick dispersal is complex and closely linked to its host movement (Falco and Fish, 1991; McCoy et al., 2001; Oliver et al., 2017). While host mobility can affect the genetic structure of tick populations, its effects are not consistent. Studies of *Ixodes scapularis* (Qiu et al., 2002) and *Ornithodoros coriaceus* (Teglas et al., 2006) have reported low levels of gene flow despite the high mobility of their hosts. In the study of Teglas et al. (2006), ticks were collected in sites that are separated by potential geographic barriers to dispersal. Other 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 (Araya-Anchetta et al., 2015). For example, high levels of gene flow observed in *Amblyomma americanum* (Linnaeus, 1767) and *A. triste* (Koch, 1844) (Acari, Ixodidae) across spatial scales ranging from 137,000 km^2 to 2.78 million km^2 was attributed to their hosts' dispersal capabilities (large mammals and birds) (Mixson et al., 2006; Trout et al., 2010; Guglielmone et al., 2013). Lampo et al. (2015) observed low levels of gene flow in *A. dissimile* (Koch, 1844) and concluded this was the result of its hosts' low mobility (small mammals, reptiles, and salamanders).

Tick-borne disease is an increasing public health concern in Japan, affecting humans and animals (Yamaji et al., 2018). Eight genera of ticks have been recorded from Japan, composed of 47 species: 43 belonging to Ixodidae and four to Argasidae (Fujita et al., 2006). Of these 47 species, 21 parasitize humans (Okino et al., 2010). *Ixodes ovatus* (Neumann, 1899) is the primary vector of the causative agents of Lyme borreliosis (Miyamoto et al., 1993) and *Haemaphysalis flava* (Neumann, 1897) is a vector of the causative agents of severe fever with thrombocytopenia syndrome (SFTS) and Japanese spotted fever (JSF) (Yu et al., 2014; Yamaji et al., 2018; Arai et al., 2021). Yamaguti et al. (1971) observed that hares (Leporidae) were the primary hosts of adult *I. ovatus,* but that larger mammals (e.g., cows and horses) also acted as hosts. The hosts of immature *I. ovatus* were small rodents. Yamaguti et al. (1971) also found that the primary hosts of adult *H. flava* were cows, dogs, horses, wild boar, bear, and deer, while birds were the hosts of immatures. Despite this understanding of host differences, and the potential for host differences to be reflected in tick gene flow, we are aware of no comparative population genetic studies of these two important vectors. In addition to population genetic structure, genetic analysis may also reveal the presence of cryptic species, where morphologically identified individuals might represent more than one species (Fegan and Prior, 2005). Previous studies of *I. holocyclus* (Neumann, 1899) (Song et al., 2011), *Rhipicephalus appendiculatus* (Neumann, 1901) (Kanduma et al., 2016), and *I. ovatus* (Li et al., 2018) have indicated the presence of cryptic species based on haplotype differences observed within morphological species.

Here, we studied the population genetic structure of *I. ovatus* and *H. flava* in Niigata Prefecture, Japan, using DNA sequences from the mitochondrial *cox1* and the 16S rRNA genes. We also examined the potential for the presence of cryptic species using the same data. We hypothesized that *I. ovatus* and *H. flava* would display contrasting population genetic structures based on the species having hosts of different mobility. The relatively low mobility of *I. ovatus* hosts, mainly hares at the adult tick stage and small mammals during the immature stage, could lead to significant genetic divergence among populations at the scale of our study. In contrast, the relatively high mobility of *H. flava* hosts, large mammals at the adult stage and birds at the immature stage, could lead to higher levels of gene flow and, therefore, lower genetic divergence among populations. We chose to examine these species because of the reported differences in host mobility and proposed occurrence of cryptic species. Both *I. ovatus* and *H. flava* have wide geographic distributions and occur throughout our study region.

2. Material and methods

2.1. Study site, collection, sampling, and identification

From April 2016 until November 2017, ticks were collected using standard flagging methods (Ginsberg and Ewing, 1989) at 29 sites (Fig. 1) (Additional File 1. Table S1) across Niigata Prefecture, Japan. Ticks were collected 2 to 14 times in 6 core sites among the 29 sites, other sites were collected only once. (Additional File 2. Table S2). Site altitude ranged from 8 to 1402 m.a.s.l. (mean = 350) and the geographic distance between sites ranged from 8.83 to 247.65 km (mean = 77.36). Collected ticks were stored in microcentrifuge tubes with 70% ethanol at 4 °C. We identified the developmental stage, sex, and morphological species identification using a stereomicroscope and identification keys of Yamaguti et al. (1971).

2.2. DNA extraction, PCR amplification, and sequencing

Genomic DNA (*I. ovatus n* = 320*; H. flava n* = 223) from each identified adult tick was extracted using Isogenome DNA extraction kits (Nippon Gene Co. Ltd., Tokyo, Japan) following the manufacturer's recommended protocol. Other 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™). Fragments of these mitochondrial genes were analyzed by polymerase chain reaction (PCR): *cox1* (658 base pairs) using the primer pairs LCO-1490 (5′ - GGTCAACAAATCATAAAGATATTGG - 3′) and HCO1–2198 (5′ – AAACTTCAGGGTGACCAAAAAATCA - 3′) (Folmer et al., 1994); 16S (407 base pairs) using the primer pairs 16S + 1 (5′ – CTGCTCAATGAATATTTAAATTGC - 3′) and 16S-1 (5′

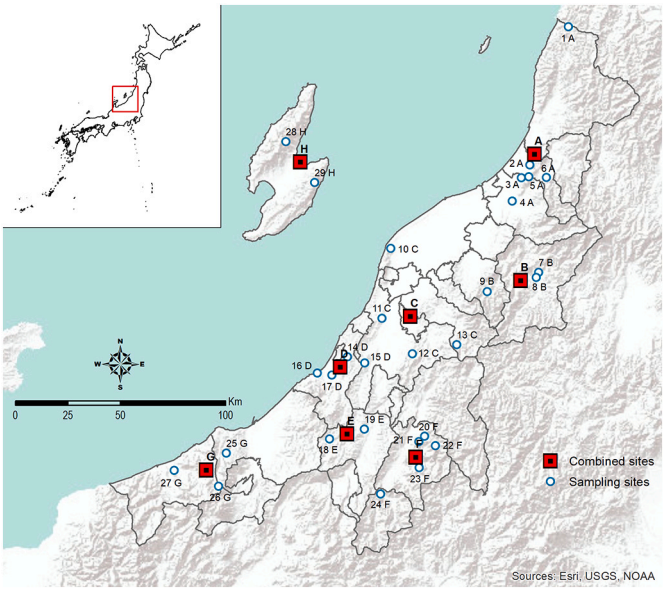

Fig. 1. Map of the 29 sampling sites used for this study. Populations (A to H) were composed of multiple sites (labeled 1 to 29) and used for the population genetic analysis.

-CGGTCTAAACTCAGATCATGTAGG - 3′) (Tian et al., 2011). PCR amplifications were performed in a final volume of 10 μl with 1 μl of genomic DNA. The PCR reaction for both markers was composed of the following: 10× Ex Taq buffer, 25 mM $MgCl_2$, 2.5 mM dNTP, 10 μm of forward and reverse primers, and five U/μl of TaKaRa Ex Taq™ (Takara Bio Inc.). The *cox1* PCR amplification was as follows: an initial denaturation of 94 °C for 2 min, denaturation of 94 °C for 30 s, annealing of 38 °C for 30 s, an extension of 72 °C for 1 min for 30 cycles, and a final extension of 72 °C for 10 min. The 16S amplification followed the protocol of (Tian et al., 2011) with some modifications (94 °C for 3 min; 30 cycles of 94 °C for 30 s, 50 °C for 40 s, 72 °C for 40 s; and 72 °C for 5 min). PCR products were purified using the QIAquick 96 PCR Purification Kit (Qiagen) following the manufacturer's instructions, and sequenced in both directions by Eurofin Genomics, Inc., Tokyo, Japan.

2.3. Sequence data analysis

We assembled forward and reverse reads for each individual using CodonCode Aligner version 1.2.4 software (https://www.codoncode.com/aligner/). We observed no ambiguous bases and manually removed low-quality bases at the start and end of the reads. Multiple sequence alignments were done using the MAFFT online program with default settings (https://mafft.cbrc.jp/alignment/server/). To ensure sequence quality and to verify morphological species identification, we compared our sequences against reference sequences from GenBank using BLASTn. The alignment (*cox*1 = 658 bp; 16S = 407 bp) quality was checked for each fragment and *cox1* sequences were translated into amino acids to confirm the absence of stop codons in Mesquite version 3.5 (Maddison and Maddison, 2011).

2.4. Population genetic analysis

For the population genetic analysis, sites were combined if they had fewer than eight individuals and were separated by a geographic distance of 8.83 to 79.81 km (mean = 44.00 km). This was done because the accurate estimation of allele frequencies is difficult for small populations. This resulted in a total of 8 populations (A to H) (Additional File 1 Table S1). Some sites were excluded from the population genetic analysis because of the small sample size (< 8 individuals) and the fact that there were no close sites.

The size of the aligned data matrix used in analyzing both *I. ovatus* and *H. flava* sequences was 658 bp for *cox1* and 407 bp for 16S. We analyzed the sequences of the two markers separately for both *H. flava* and *I. ovatus* using DNASp version 6.12.03 (Rozas et al., 2017) and calculated the following parameters: number of haplotypes (nh), the average number of polymorphic sites (s), and average number of nucleotide differences (k). The haplotype diversity (h) and nucleotide diversity (π) were calculated in Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010). The population genetic structure within and among populations was assessed by analysis of molecular variance (AMOVA) performed in Arlequin with 9999 permutations. Pairwise genetic differentiation between populations was assessed by calculating the pairwise F_{ST} values using Arlequin. The neutrality test of Tajima's D was carried out using DNAsp software, to determine if the populations have undergone recent expansions or bottlenecks.

To determine if the genetic differentiation was influenced by geographical distance or altitudinal differences among populations, we performed Mantel Test in GenAlEx version 6.51b2 (Peakall and Smouse, 2006). Two tests per species and marker were conducted. First, we compared pairwise genetic (pairwise F_{ST} values) and geographical distances (km). Second, we compared the pairwise genetic distance (F_{ST} values) with altitudinal differences (m.a.s.l.) calculated from GenAlex version 6.51b2. The geographic distances were obtained from the geographic midpoint using the online calculator (http://www.geomidpoint.com/) of the populations using the GPS coordinates (latitude and longitude) of each site recorded during the sampling. Altitude was calculated as the mean altitude of all the sites making up a given population. All Mantel tests were assessed using 9999 permutations for the significance of the correlation.

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

2.5. Haplotype network and phylogenetic analyses

To evaluate the relationship among haplotypes, we constructed a haplotype network on the PopART program version 1.7 for each marker (*cox1* and 16S) and species (*I. ovatus* and *H. flava* (http://popart.otago.ac.nz/index.shtml) using the median-joining (MJ) network algorithm (Bandelt et al., 1999). We performed a Bayesian phylogenetic analysis using BEAST version 1.10.4 (Drummond and Rambaut, 2007) to determine the phylogenetic structure of *I. ovatus cox1* haplotypes within Niigata Prefecture. Additional sequences from China (see below) were also included in the Bayesian analysis. We used the HKY substitution model with the estimated base frequencies. A strict clock model was employed, and a coalescent prior was used as the tree prior. A maximum clade credibility tree was acquired using TreeAnnotator v1.10.4 from the many trees obtained from BEAUti v1.10.4, with 90% of trees used as burn-in. The maximum clade credibility tree was viewed using FigTree v1.4.4.

We constructed maximum likelihood (ML) gene trees for *cox1* and 16S sequences of *I. ovatus* and *H. flava* using PhyML version 3.1 (Guindon and Gascuel, 2003) default settings. We calculated the node support using ML bootstrap. We applied HKY and GTR nucleotide substitution models for *cox1* and 16S, respectively, as suggested by jModelTest version 2 (Darriba et al., 2012). Additional sequences from China (MH208506, MH208512, MH208514, MH208522, MH208515–19, MH208524, MH208531, MH208574, MH208577, MH208579, MH208681–87, MH208689–93, MH208706, KU664519(Li et al., 2018)), Japan (Hokkaido AB231670, U95900; Yamanashi AB819241, AB819243 and Aomori AB819244) (Norris et al., 1999; Mitani et al., 2007; Takano et al., 2014) were included to check for the presence of cryptic species. Some GenBank sequences have a different length as compared to our sequences. We used MAFFT alignment to align the sequences. We then checked the sequences in Mesquite software and trimmed the sequences for them to have the same length and we also checked for the presence of stop codons. The final length of the trimmed matrix used for the ML tree is 658 bp for *cox1* and 407 bp for 16S. We used *Ixodes canisuga* as an outgroup because it is closely related to *I. ovatus* and *H. flava* (KY962023 and KY962074; Hornok et al., 2015).

3. Results

A total of 2374 individual ticks was collected. Adult and immature *Ixodes nipponensis* (Kitaoka and Saito, 1967), *I. persulcatus* (Shulze, 1930), *I. monospinus*, and *Dermacantor taiwanensis* (Sugimoto, 1936) were also identified and used for another research study. The number of *I. ovatus* ranged from 1 to 36 adults per site and were more successfully sequenced for *cox1* (307/320; 95.9%) than for 16S (284/320; 88.8%) (Additional File 1 Table S1). The number of *H. flava* ranged from 1 to 77 adults per site and sequencing was also more successful for *cox1* (220/223; 98.7%) than for 16S (172/223; 77.1%)(Additional File 1 Table S1). For the population genetic analysis, in which populations consisted of combined sites (see above) the number of individuals were as follows: *cox1 I. ovatus* (28 to 62), 16S *I. ovatus* (24 to 66), *cox1 H. flava* (8 to 81) and 16S *H.flava* (8 to 76) (Additional File 1 Table S1). There were 60 and 63 *cox1* haplotypes and 24 and 40 16S haplotypes in *I. ovatus* and *H. flava,* respectively (Table 1). Haplotype diversity (h) per population ranged from 0.582 to 0.964, and nucleotide diversity (π) per population

Table 1
Summary of *cox1* and 16S haplotype diversity of adult *I. ovatus* and adult *H. flava* populations in Niigata Prefecture, Japan.

Marker	Species	n	nh	s	k	h (min – max)	π (min – max)	Tajima's D
cox1	*I. ovatus*	307	60	65	2.728	0.852 (0.582–0.871)	0.004 (0.002–0.004)	−2.598 *
	H. flava	220	63	60	1.472	0.789 (0.718–0.964)	0.002 (0.718–0.964)	−2.193*
16S	*I. ovatus*	284	24	22	0.699	0.442 (0.074–0.712)	0.001 (0.000–0.712)	−2.226*
	H. flava	172	40	49	2.447	0.835 (0.731–0.964)	0.006 (0.005–0.007)	−2.143*

Abbreviations: n sample size; nh number of haplotypes; s number of polymorphic sites; k mean; number of nucleotide differences; h haplotype diversity (minimum-maximum values); π nucleotide diversity (minimum-maximum); * $p < 0.01$.

ranged from 0.000 to 0.096 among markers and species (Additional File 1 Table S1). The demographic inference in both *H. flava* and *I. ovatus* showed statistically significant negative Tajima D's values in both markers (Table 1).

We found significant global F_{ST} values ($p < 0.01$) for *I. ovatus* with values of 0.3801 in *cox1* and 0.0378 in 16S (Table 2). In contrast, both *cox1* and 16S markers of *H. flava* indicated no significant genetic differentiation. AMOVA results revealed a high among-population divergence in *I. ovatus cox1* (38.01%) compared to 16S (3.78%). The AMOVA results in *H. flava* revealed low divergence at both *cox1* (1.81%) and 16S (0.78%) markers. Pairwise F_{ST} values (0.0963 to 0.6808) of *cox1* for *I. ovatus* were significant between most pairs of populations, such as between populations B and D and populations F and D (Additional File 3 Table S3). We also observed significant genetic differences in 16S *I. ovatus* sequences (range 0.0514 to 0.0949)(Additional File 3 Table S3). Pairwise F_{ST} values from the *cox1* marker of *H. flava* showed significant genetic differences among a few population pairs including A and C, C and E, and D and E; 16S was significant between A and C (Additional File 4 Table S4). Mantel tests showed no significant isolation by geographic distance in *I. ovatus* (*cox1* $r = 0.108$, $p = 0.269$; 16S $r = 0.518$, $p = 0.065$) (Additional File 5 Fig. S1, a and b) or isolation by altitudinal difference (*cox1* $r = -0.066$, $p = 0.225$; 1S $r = -0.023$, $p = 0.577$). The same was true for *H. flava* (distance: *cox1* $r = 0.444$, $p = 0.130$; 16S $r = 0.355$, $p = 0.189$; altitude: $r = 0.092$, $p = 0.30$; 16S $r = 0.217$, p = 0.06) (Additional File 5 Fig. S1, c and d).

The UPGMA cluster dendrogram constructed from the pairwise F_{ST} values of *I. ovatus* for the *cox1* marker (Fig. 2) revealed two genetic clusters among the seven populations. Population H is not included in this analysis because the sample number is less than 8 individuals. Cluster 1 (populations A, B, and F) included populations in the northern and southern sites (Fig. 3) and were distributed in mountainous areas with higher elevations. Cluster 2 included populations from the more western sites. We observed no evidence of genetic clustering on the dendrogram of *I. ovatus* using 16S, or for either marker in *H. flava* (Additional File 6 Fig. S2; Additional File 7 Fig. S3; Additional File 8 Fig. S4). The Bayesian tree of Japanese *I. ovatus cox1* haplotypes (Fig. 4) and the haplotype network with the additional sequences from China (Fig. 5) showed similar patterns of 4 genetic groups within Japan. Two haplotypes of *I. ovatus* were distinct from all others (Hap59 and Hap60) (Fig. 5; Additional File 9 Fig. S5) and were found in sampling site 6 (Pop A) and sampling site 26 (Pop G). The Bayesian tree of *I. ovatus cox1* haplotype sequences with reference sequences from China also displayed the four genetic groups (Additional File 10 Fig. S6). The 16S haplotype network of *I. ovatus* (Additional File 11 Fig. S7) and both the *cox1* (Additional File 12 Fig. S8) and 16S (Additional File 13 Fig. S9) networks in *H. flava* did not reveal any distinct genetic groups.

Evidence for a putative *I. ovatus* species complex was identified in the *cox1* ML tree based on the presence of three distinct haplotype groups: group 1 which included published sequences from Southwestern China, group 2 which contained the 58 Japan haplotypes including the two divergent haplotypes (Hap 60 and Hap 59), and group 3 which also included sequences from west China (Fig. 6). The published haplotype from Hokkaido, Northern Japan (Mitani et al., 2007) occurred within group 2. In the *I. ovatus* 16S tree (Additional File 14 Fig. S10), our Niigata sequences were grouped with published haplotypes from Yamanashi Prefecture, located south of the study area, and from Aomori Prefecture (Takano et al., 2014) and from Hokkaido (Norris et al., 1999), both of which are north of the study area. The *cox1* tree for *H. flava* (Additional File 15 Fig. S11) were similar to reference sequences from China KY021800 – KY021807, KY021810 – KY021819 and KY003181 (Li et al., 2018; Unpublished results from NCBI); JQ625688 – JQ625689, JF758632 and JQ737097 (Lu et al., 2013) and JG737097 (Gou, H., Guan, G., Yin, H. and Luo, J.; unpublished results from NCBI). The *H. flava* 16S haplotype sequences (Additional File 16 Fig. S12) were similar to reference sequences from Japan (Kagoshima, Aomori, Fukui, Yamanashi, Kagawa, and Ehime Prefectures (Takano et al., 2014)) and China (KC844858 –KC844867 (Cheng et al., 2013); KX450280 –KX450282 (Zhang, Y., Cui, Y., Peng, Y., Yan, Y., Wang, X. and Ning, C. Liu, Q., Zhang, Y. and Zhu, D.; unpublished results from NCBI); MG696720 (Zheng, W., Chen, S. and Chen, H.; unpublished results from NCBI) and KP324926 (Liu, Q., Zhang, Y. and Zhu, D.; unpublished results from NCBI)).

4. Discussion

4.1. Contrasting population genetic structures between I. ovatus and H. flava

Our results supported our hypothesis that *I. ovatus* may display high levels of genetic divergence among populations because of its low host mobility. The significant global F_{ST} estimates in *I. ovatus cox1* (0.3801) and 16S (0.0378) revealed population differentiation as supported by AMOVA results of high among population variation (38.01%) in *I. ovatus cox1*. Although, there is higher between population variation (61.99%)

Table 2
Analysis of molecular variance (AMOVA) using *cox1* and 16S of adult *I. ovatus* and adult *H. 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	
	H. flava	Among populations	4	5.74	0.0092 Va	0.78	0.0079
		Within populations	164	191.31	1.1665 Vb	99.22	

Abbreviations: df degrees of freedom; ss sum of squares; vc variance component where Va, Vb and Vc are associate covariance components; pv percentage variation; * $p < 0.01$.

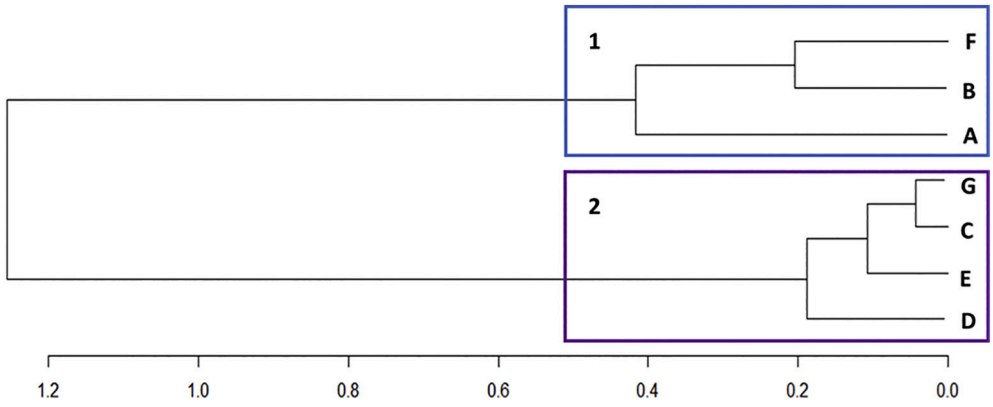

Fig. 2. Clustering of adult *I. ovatus* populations in Niigata Prefecture, Japan, based on an unweighted pair group method with the arithmetic mean (UPGMA) dendrogram of the pairwise genetic distance (F_{ST}) of *cox1* among 7 populations.

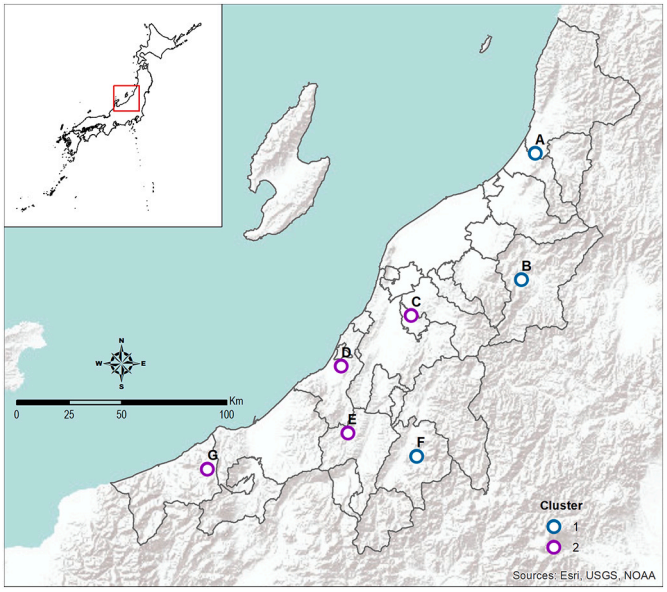

Fig. 3. The distribution of the two genetic clusters of *I. ovatus* as observed in the UPGMA cluster dendrogram (Fig. 2) of *cox1* sequences from Niigata Prefecture.

in AMOVA for *I. ovatus cox1* this is likely to occur because of the large sample size per population. This doesn't suggest that the among population variation in *I. ovatus* is limited since we observe higher among population variation in *I. ovatus cox1* as compared to *H. flava.* The host preference of *I. ovatus* may also contribute to their being separated into four genetic groups in the Bayesian gene tree and the haplotype network. Haplotypes from groups 2 and 3 were primarily from low altitudinal areas, whereas ticks from group 1 were found in high-altitude areas. We assume that the large mammalian hosts of *I. ovatus* such as cows and horses have enabled the group 1 ticks to reach high elevation areas. The distribution of the type of hosts may also affect altitude, influencing the formation of groups 1, 2, and 3 in the *cox1 I.ovatus* dendrogram.

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

In addition to host mobility, several alternative factors can affect tick dispersal patterns, including tick behavior, biology, and ecology. A previous comparison of *Hyalomma rufipes* (Koch, 1844) and *A. hebraeum* (Koch, 1844) also revealed contrasting genetic patterns despite the two species having similar, highly mobile hosts (Cangi et al., 2013). The contrasting genetic patterns might be due to the species-specific survival rates of immature ticks after having detached from their host (Cangi et al., 2013; Cumming, 1999; Estrada-Peña, 2015; Needham and Teel, 1986). Population genetic structure can also be influenced by assortative mating (e.g., *I. ricinus*), wherein mating is not random but is more likely between genetically similar individuals, resulting in increased genetic divergence (Kempf et al., 2009). Our study does not have supporting data to test these alternative factors; thus, we suggest future studies analyze these factors further.

A significant negative Tajima D value means there is an abundance of rare alleles. This can result from selective sweeps or from expansion after a bottleneck. The fact that Tajima D's values was significant and that there was no IBD can be interpreted to mean that populations are not in drift-gene flow equipibrium. This might be because we have cryptic species as seen in *I. ovatus cox1* ML tree.

Only a few population pairs of *I. ovatus* showed significant genetic differentiation at the 16S marker. This is likely due to its low nucleotide diversity ($nd = 0.001$) compared to *cox1* ($nd = 0.004$). Such a pattern has been reported in other studies, where 16S did not provide suitable levels of variation for population-level analysis of *A. ovale* (Koch, 1844) (Bitencourth et al., 2019) and *R. microplus* (Canestrini, 1888) (Burger et al., 2014; Low et al., 2015). Nonetheless, the 16S data did support the finding of genetic variation in *I. ovatus* populations (Table 1), and the inclusion of 16S allowed for the comparison of other published 16S sequences for many more regions of Japan (Takano et al., 2014; Norris et al., 1999) and in China (Cheng et al., 2013). We also observed greater success in the PCR amplification and sequencing of *cox1* compared to 16S. This could be the result of variation at primer binding sites.

Understanding the genetic structure and gene flow of *I. ovatus* and *H. flava* can provide insights in predicting the spread of tick-borne diseases (Sato et al., 2021). In addition, population genetic information can be used to design effective vector control programs and reveal tick dispersal mechanisms. For example, the high levels of gene flow we observed in *H. flava* suggest a higher probability of newly colonizing areas or recolonizing areas that were subject to tick control programs (McCoy, 2008).

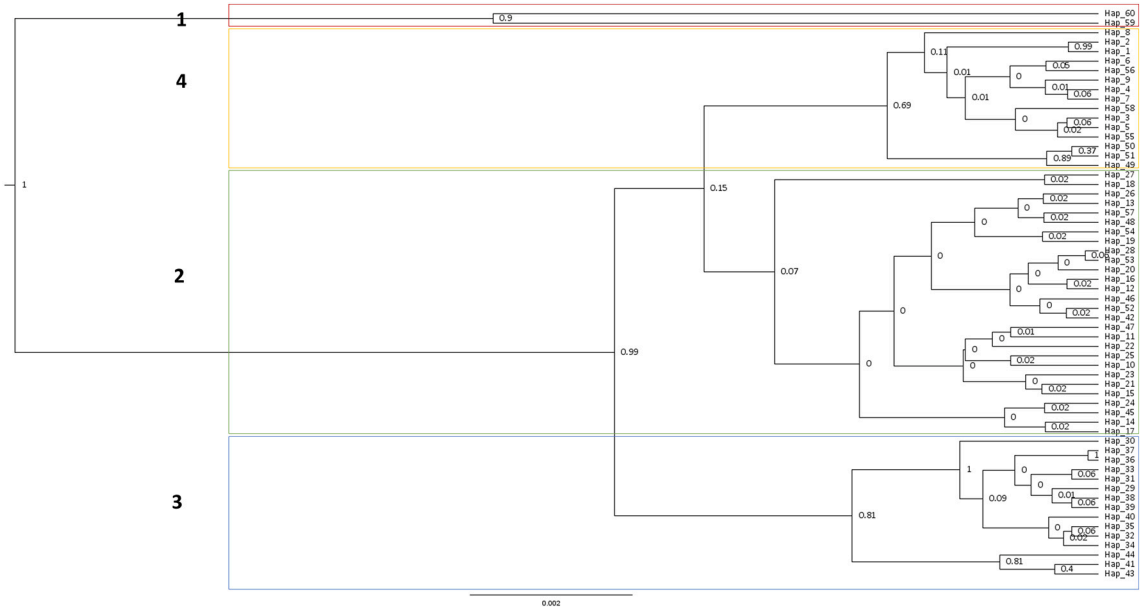

Fig. 4. Bayesian gene tree of *I. ovatus cox1* haplotypes from Japan. Posterior probability values are indicated above the branches.

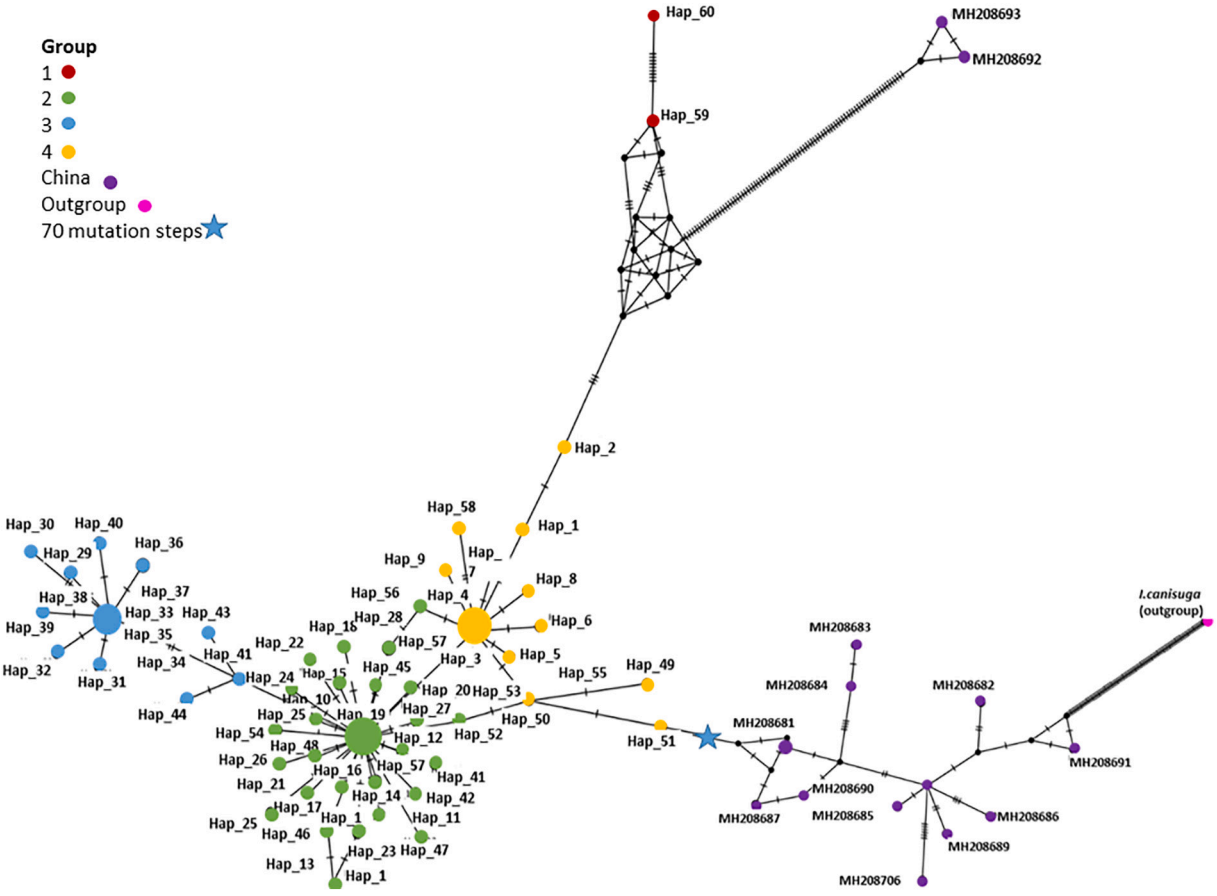

Fig. 5. Median-joining *cox1* haplotype network of *I. ovatus* haplotypes including published sequences from China. Each circle represents a unique haplotype and the lines correspond to mutations. The group number is linked to the groups shown in Fig. 4.
For easier visualization, the mutation steps ($n = 70$) were marked as.

4.2. Species complex formation in I. ovatus cox1 sequences

Our *cox1 I. ovatus* gene tree showed Japanese individuals to form a distinct group from haplotypes from Southwestern China (Li et al., 2018). Despite the high genetic divergence in *I. ovatus* in our data set, we found that the haplotypes from Niigata were very similar to published

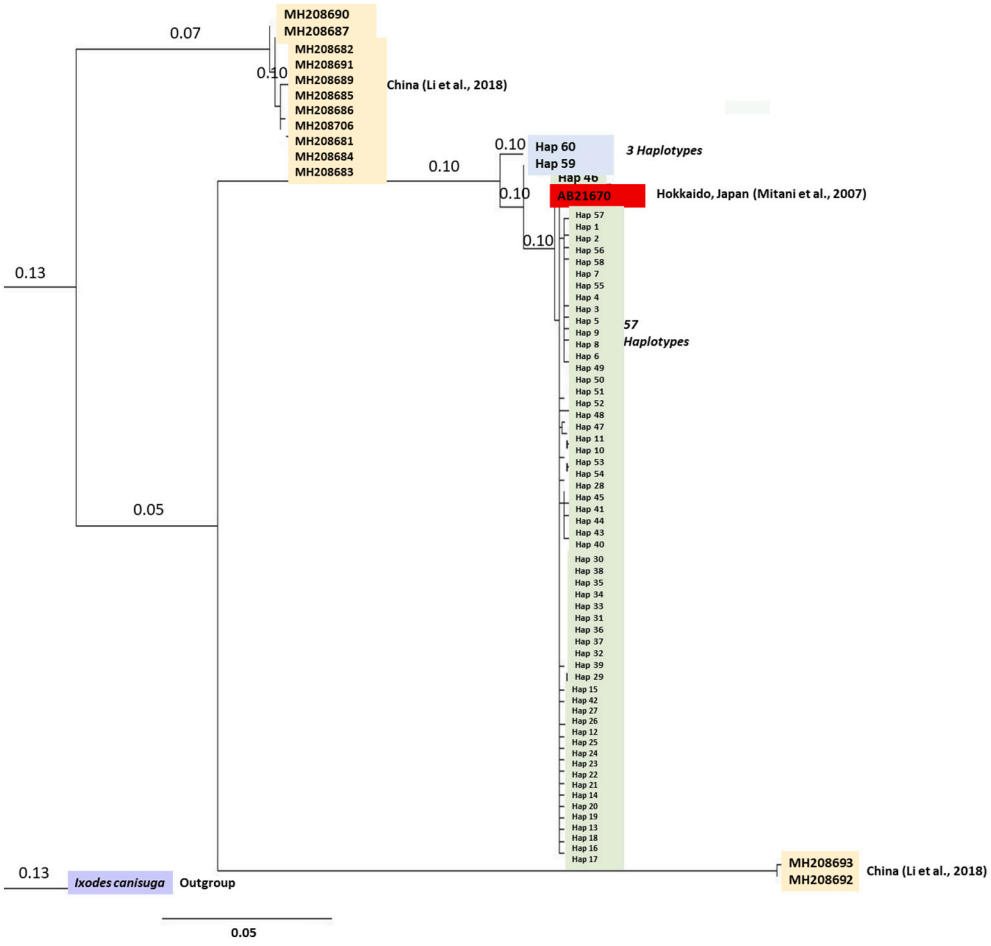

Fig. 6. Maximum likelihood gene tree of *cox1* sequences of *I. ovatus* that includes published sequences from China and Japan, and *I. canisuga* as an outgroup. Support values are indicated above the branches. Green background indicates Japanese haplotypes from this study; yellow indicates published sequences from China (Li et al., 2018); red indicates published sequence from Hokkaido, Japan (Mitani et al., 2007); blue indicates divergent Japanese haplotypes from this study (Hap 59, Hap 60); purple indicates *I. canisuga* outgroup.

sequences from Hokkaido in northern Japan. This may indicate that these ticks originated from a diverse set of geographical locations in Japan, which might be transported by its hosts or are undergoing recent population expansion from northern Japan (Hokkaido) to central Japan (Niigata) vice versa. We found three groups (China 1, Japan, and China 2) and two slightly divergent *cox1* haplotypes (Hap 60 and 59) in the Japan group of *I. ovatus* in Niigata. Considering two or more cryptic species can be concealed in one morphologically described species (Bitencourth et al., 2019), the occurrence of the three groups and the divergent haplotypes suggests that *I. ovatus* may be a species complex. It can be inferred that China 1 and China 2 might have a longer evolutionary time than Japan. The co-existence of a species in the same geographic area may explain the occurrence of the species complex. One of the limitations of this study is that both Chinese and Japanese individuals were collected in a limited geographical area. We suggest that future studies sample and sequence more individuals from other locations. Extensive geographical sampling can lead to improved taxonomic understanding, as indicated in the study of Liu et al. (2013) on *R. sanguineus* (Latreille, 1806). Previous studies have also observed species complexes in *Ixodes* and *Rhipicephalus* (Xu et al., 2003; Song et al., 2011; Burger et al., 2014; Li et al., 2018;), suggesting that morphological criteria for tick species differentiation alone can be equivocal, and that genetic analysis is essential. Future studies should take an integrative approach that includes morphology, genetics, biology, and ecological traits (Dantas-Torres et al., 2012).

5. Conclusions

In summary, our findings revealed contrasting patterns of population genetic structure of *I. ovatus* and *H. flava* in Niigata Prefecture, Japan. The greater genetic divergence among populations in *I. ovatus* might result from the restricted movement of its small mammalian hosts during its development, while the homogenous structure in *H. flava* might be due to the more widespread movement of its avian and large mammalian hosts. Although our results suggest that the host preference of immature ticks may influence the population genetic structure of adult ticks due to their higher ability to survive into adult stage (Needham and Teel, 1986; Cumming, 1999; Estrada-Peña, 2015), we can only infer that the host mobility affects genetic structure. Since the present study only focused on adults of *I. ovatus* and *H. flava,* studying immature ticks and population structuring should be explored by future studies. Understanding the population genetic structure of ticks such as *I. ovatus* and *H. flava* can inform studies of their distribution and the control of tick-borne diseases. Even though *I. ovatus* populations were genetically structured within Niigata, a published haplotype from Hokkaido was also found, indicating that widespread dispersal is possible. The occurrence of three genetic groups and the divergent *cox1* haplotypes in *I. ovatus* emphasizes the need for additional research into the existence of a species complex of *I. ovatus* populations in Japan.

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 additional supplementary files. All the newly generated sequences are available in the GenBank database under the accession numbers MW063669-MW064124 and MW065821 - MW066347.

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, commented on, and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2021.104999.

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