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 km²). 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

the dispersal patterns and potential pathogen transmission in ticks (Araya-Anchetta et al., 2015). The direction, distance and gene flow patterns between tick populations can be inferred from population genetic studies (Mccoy 2008). 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.

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 (Araya-Anchetta et al., 2015). For example, *Amblyomma americanum* and *A. triste* (Ixodidae, Acari) exhibited high gene flow across various spatial scales (137, 000 km² to 2.78 million km²) and this was attributed to their hosts' dispersal capabilities (large mammals and birds) (Guglielmone et al., 2013; Mixson et al., 2006; Trout et al., 2010), 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) (Lampo et al., 1998). In contrast, some studies have reported low gene flow despite the high mobility of the host, for example in *Ixodes scapularis* (Qiu et al., 2002) and *Ornithodoros coriaceus* (Teglas et al., 2006) which can be attributed to the host mobility.

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

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

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

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

Material and methods

Study site, collection, sampling and identification

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

DNA extraction, PCR amplification and sequencing

Genomic DNA (*I. ovatus* n=320; *H. flava* n=220) from each identified tick was extracted using the Isogenome DNA extraction kits (NIPPON GENE Co. Ltd., Tokyo, Japan) following the manufacturer's recommended protocol. The total number of analyzed samples (n=540) was chosen based on its species identity while the other identified tick species were excluded for this study. Prior to DNA extraction, each tick was washed with alcohol and PBS solution. DNA quality was checked using a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific™). Two mitochondrial genes were amplified by polymerase chain reaction (PCR): Cytochrome Oxidase 1 (*cox1*) (658 base pairs) using the primer pairs LCO-1490 (5'- GGTCACAAATCATAAAGATATTGG -3') and HCO1-2198 (5'- AAACCTCAGGGTGACCAAAAATCA-3') (Folmer et al., 1994) and the 16S rRNA gene (16S) (407 base pairs) with the following primer pairs 16S+1 (5'CTGCTCAATGAATATTAAATTGC-3') and 16S – 1 (5' - CGGTCTAAACTCAGATCATGTAGG-3')(Tian et al., 2011). All PCR amplifications of both *cox1* and 16S were performed with a final volume of 10 µl with 1µl of genomic DNA. The PCR reaction for both markers were composed of the following: 10x Ex Taq buffer, 25mM MgCl₂, 2.5mM dNTP, 10µm of forward and reverse primers and 5 units/µl of TaKaRa Ex Taq™ (Takara Bio Inc.). The *cox1* PCR amplification were as follows: an initial denaturation of 94°C for 2 minutes, denaturation of 94°C for

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

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/>). No ambiguous bases were observed and the low quality bases were removed in the start and end of the reads. Multiple alignment of sequences was performed using the MAFFT alignment online program with default settings (<https://mafft.cbrc.jp/alignment/server/>). To ensure sequence quality and correct species identification, we checked the similarity of the sequences against reference sequences from GenBank using BLASTN (Basic Local Alignment Search Tool – Nucleotide, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The quality of the final aligned sequences (*cox1* = 658bp; 16S = 407bp) was checked in Mesquite version 3.5 (Maddison et al., 2011) wherein the protein coding genes were translated into amino acids to confirm the absence of stop codons.

Population genetic analysis

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

Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010) for the following parameters: number of haplotypes (nh), 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 3.5.2.2. The population genetic structure among and within sampling locations in Niigata Prefecture was calculated by analysis of molecular variance (AMOVA) performed in Arlequin with 1023 permutations. The degree of molecular genetic differentiation between sampling locations defined as populations was assessed by calculation of the pairwise F_{ST} values in Arlequin. 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) and second we compared genetic distance (F_{ST} values) with altitudinal differences (m/a.s.l.). The geographic distances were obtained from the GPS coordinates (latitude and longitude) recorded during the sampling. All Mantel tests were assessed using 9999 permutations for the significance of the correlation.

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

resampling. The upper and lower confidence limits bound the 95% confidence interval of the null hypothesis of no spatial structure in the distance class. The genetic relationships among the sampling locations (i.e., populations) were calculated by unweighted pair group method 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 dendrogram using the genetic distance matrix (pairwise F_{ST} values) generated from GenAEx.

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

Phylogenetic analysis

We constructed maximum likelihood (ML) gene trees for *cox1* and 16S haplotype sequences of *I. ovatus* and *H. flava* using PhyML version 3.1 (Guindon and Gascuel, 2003) under the default settings. We applied HKY and GTR nucleotide substitution models for *cox1* and 16S, respectively, as suggested by jModelTest ver 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) (Mitani et al., 2007; (Norris et al., 1999), Takano et al., 2014)) were included. We used *Ixodes canisuga* an outgroup because it is a closely related species (KY962023, KY962074; (Hornok et al., 2015)).

Results

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

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

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

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

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

AMOVA results revealed a high among-population divergence (41.54%) in *I. ovatus cox1* sequences, whereas both *cox1* and 16S markers of *H. flava* indicated no significant genetic variation among populations (Table 2). Global F_{ST} values were highest in *I. ovatus* with values 0.415 in *cox1* and 0.074 in 16S sequences. Pairwise F_{ST} values (0.500 to 1.00) of *I. ovatus* based on *cox1* showed significant genetic differences between most pairs of populations such as between populations 18 and 25 and between populations 28 and 25 (Additional File 2 Table S2). We also observed significant differences in 16S ranged from (0.0225 to 0.6319) (Additional File 3 Table S3). Pairwise F_{ST} values from the *cox1*

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

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}
<i>cox1</i>	<i>I. ovatus</i>	Among populations	28	190.38	0.5803 Va	41.54
		Within populations	278	227.04	0.8167 Vb	58.46
	<i>H. flava</i>	Among populations	16	12.42	0.0039 Va	0.53
		Within populations	203	148.73	0.7327 Vb	99.47
16S	<i>I. ovatus</i>	Among populations	22	14.04	0.0260 Va	7.39
		Within populations	261	84.87	0.3252 Vb	92.61
	<i>H. flava</i>	Among populations	12	23.6	0.0642 Va	5.21
		Within populations	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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

Species complex formation in *I. ovatus cox1* sequences

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

Conclusions

Two Ixodid ticks, *H. flava* and *I. ovatus* exhibited contrasting patterns of genetic structure in Niigata Prefecture, Japan. We suggest that these differences are due to differences in host mobility of the immature life stages of the ticks. The more pronounced population genetic structure of *I. ovatus* might be influenced by the restricted movement of its small mammalian host in its immature stage, whereas the lack of structure in *H. flava* may be due to its avian-mediated movement in its immature stage. 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 groups and the divergent *cox1* haplotypes in *I. ovatus* emphasizes the need of additional methods in determining the existence of species complex in *I. ovatus* populations in Japan.

List of Abbreviations

H. flava – *Haemaphysalis flava*

I. ovatus – *Ixodes ovatus*

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

355 Not applicable

356 **Availability of data and materials**

357 All data generated or analyzed during the study are included in this published article and its
358 supplementary additional files. All the newly generated sequences are available in the Genbank
359 database under the accession numbers _____.

360 **Declarations of interests**

361 The authors declare that they have no competing interests.

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368 **Authors' contributions**

369 MAFR, MG, MM, KW conceptualized and the designed the experiment. MS, TT, RA, SI and MOS
370 designed the sampling collection, collected and identified the tick samples of this study. MD and KT
371 conducted the molecular analyses. MAFR, MG and KW performed the data analysis and wrote the
372 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

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 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.

Additional File 12 Figure S7 Median-joining haplotype network of *I. ovatus* from *cox1* marker. Each circle represents a unique haplotype and the lines correspond to mutations.

Additional File 13 Figure S8 Median-joining haplotype network of *I. ovatus* from 16S marker. Each circle represents a unique haplotype and the lines correspond to mutations.

Additional File 14 Figure S9 Median-joining haplotype network of *H. flava* from *cox1* marker. Each circle represents a unique haplotype and the lines correspond to mutations.

Additional File 15 Figure S10 Median-joining haplotype network of *H. flava* from 16S marker. Each circle represents a unique haplotype and the lines correspond to mutations.

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.

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.

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