Studies on genetic diversity and transmission dynamics of *Spiroplasma* in ixodid ticks

（マダニにおける *Spiroplasma* の遺伝的多様性と伝播動態に関する研究）

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ABBREVIATIONS

DNA: deoxyribonucleic acid
PCR: polymerase chain reaction
LMM: linear mixed model
rDNA: ribosomal RNA gene
ITS: 16S–23S rRNA internal transcribed spacer
dnaA: chromosomal replication initiator protein dnaA
dnaK: chaperone protein DnaK
gyrA: DNA gyrase subunit A
EpsG: transmembrane protein EpsG
rpoB: RNA polymerase B
TBD: tick-borne disease
SFTS: severe fever with thrombocytopenia syndrome
JSF: Japanese spotted fever
TBE: tick-borne encephalitis
NCBI: National Center for Biotechnology Information
SRA: the sequence read archives
DDBJ: DNA Data Bank of Japan
MEGA: Molecular Evolutionary Genetics Analysis
ML: maximum likelihood
MCA: multiple correspondence analysis
No.: number
sp.: species
PBS: phosphate-buffered solution
Df: the number of degrees of freedom
GVIF: the generalized variance inflation factor

GENERAL INTRODUCTION

Ixodid ticks are blood sucking ectoparasites of vertebrates with about 700 species distributed in the world [1]. They serve as vectors of many pathogens and cause significant public health and veterinary health problems globally [2,3]. Ixodid ticks attach to their hosts for days or weeks while blood feeding. Depending on the number of hosts that they infest during their life cycle, they are divided into one-, two- and three-host ticks. One-host ticks attach a single host during their life cycle, while two- and three-host ticks drop off the host after feeding to molt in the environment between stages [4–8].

Since the 19th century, when the initial description of tick-borne disease (TBD) was made in cattle [9], many tick species are now recognized as vectors and/or reservoirs of the infectious agents [10]. For examples, Crimean-Congo hemorrhagic fever virus, which is one of the tick-borne viral pathogens, is a public health and socio-economic threat due to its high pathogenicity in humans. Protozoan parasites such as *Theileria* and *Babesia* could lead to clinical symptoms in animals with high mortality and morbidity [11–15]. In Japan, forty-two species of ixodid in five genera (*Amblyomma*, *Dermacentor*, *Rhipicephalus*, *Haemaphysalis* and *Ixodes*) have been recorded [16,17]. So far, several human TBDs such as Lyme disease, Q-fever, severe fever with thrombocytopenia syndrome (SFTS), Japanese spotted fever (JSF), tick-borne encephalitis (TBE) and Yezo virus infection have been reported in Japan [18, 19]. Among these TBDs, the highest number of annual cases was observed for JSF (318 cases in 2019) [20]. SFTS and TBE, which are respectively recognized as emerging and re-emerging TBDs, have a public health importance due to their high mortality [21].

The term “symbiosis” was firstly defined in 1879 to describe the condition where different species live together in close association [22]. Generally, the host stands for the larger partner and the symbiont does for the smaller partner [23]. Symbiotic relationships are important in biological processes and the ecological system [23]. In nature, symbiotic relationships between bacteria and arthropods are well known and have been studied extensively. The biochemical capabilities of some symbionts enable the host to adapt in novel environment. For example, the arthropod hosts living in nutritionally strict environment such as plant-sap-sucking aphids [24] and blood-feeding tsetse flies [25] acquire nutrition from their bacterial endosymbionts. Some of the mechanisms behind various symbiotic relationships were reported elsewhere [26–29].

Symbionts employ disparate transmission strategies to allow their survival in hosts [30]. Bacterial symbionts, such as *Wolbachia* and *Arsenophonus*, are transmitted vertically [28]. Other symbionts are transmitted horizontally, for instance, by being acquired from environmental or infected conspecifics or other species [31,32]. In some

cases, a combination of multiple infection routes creates a complex transmission dynamics in nature [33,34]. The dominant transmission form of endosymbionts is vertical transmission and occurs primarily from mother to offspring [35]. Some insect symbionts even display specialized transmission strategies, for instance, via parental postoviposition secretions [36].

Members of the genus *Spiroplasma* are gram-positive bacteria without cell walls. They are known as symbionts of arthropods and plants and are classified into three major clades based on the 16S ribosomal RNA gene (rDNA) sequence: Ixodetis, Cit-ri-Chrysopicola-Mirum (CCM), and Apis [37,38]. *Spiroplasma* is one of the most common endosymbionts with a wide range of hosts, including insects, arachnids, crustaceans, and plants [39]. It is estimated that 5–10% of insect species harbour this symbiont group [38,40].

Spiroplasma has a wide range of fitness effects and transmission strategies [16,17,38,41–51]. Some *Spiroplasma* species affect the sex ratio by inducing male killing in hosts such as flies, butterflies, and ladybird beetles [47–50]. Several *Spiroplasma* species are known to cause disease in arthropods such as bees and plants [44,46,52]. On the other hand, some flies infected with *Spiroplasma* can develop resistance to other pathogens [16,17,45,50]. A wide range of symbiotic relationships involving *Spiroplasma* have been observed [41–43,45,47,48]. The rapid spread of *Spiroplasma* infection in fruit fly natural populations has been reported in some areas of North America, and this phenomenon has been confirmed in laboratory settings [53]. This characteristic of *Spiroplasma* is not only biologically interesting but also useful for symbiotic control applications among host individuals [54].

Ticks have also been shown to harbor *Spiroplasma*, but no comprehensive studies have been done on its role in ticks and to determine the genetic diversity and prevalence of tick-associated *Spiroplasma*. In chapter I of this study, the infection status of *Spiroplasma* in ticks were investigated using samples collected from different geographic regions in Japan. The detected *Spiroplasma* were genetically characterized based on 16S rDNA, 16S–23S rRNA internal transcribed spacer (ITS), chromosomal replication initiator protein dnaA (*dnaA*), and RNA polymerase B (*rpoB*) gene sequences to understand their genetic diversity and transmission cycle in wild tick populations. In chapter II, the vertical transmission potential of *Spiroplasma* was evaluated under the laboratory conditions. Two isolates of *Spiroplasma* were experimentally inoculated into laboratory colonies of female ticks. The presence of *Spiroplasma* in the resulting eggs and larvae were confirmed.

Chapter I

Spiroplasma infection among ixodid ticks exhibits species dependence and suggests a vertical pattern of transmission

1. Introduction

Ticks have long been studied, since they transmit a variety of pathogens to humans and animals. *Spiroplasma mirum* is the first reported tick-associated *Spiroplasma*, which was obtained from *Haemephysalis leporispalustris* in the United States in 1982 during the search for rickettsiae in ticks [55]. Another species, *Spiroplasma ixodetis*, was isolated from *Ixodes pacificus* in the United States in 1981 [56]. Thus far, these two species are the only validated *Spiroplasma* species detected in ticks. Nevertheless, several alleles or putative new species of *Spiroplasma* have been found in various tick species such as *Ixodes arboricola*, *Ixodes frontalis*, *Ixodes ovatus*, *Ixodes persulcatus*, *Ixodes ricinus*, *Ixodes uriae*, *Dermacentor marginatus*, *Rhipicephalus annulatus*, *Rhipicephalus decoloratus*, *Rhipicephalus geigyi*, and *Rhipicephalus pusillus* [57–64].

In Japan, Taroura et al. first detected *Spiroplasma* DNA in questing *I. ovatus* ticks captured in several prefectures [58]. Subsequently, a microbiome study revealed the presence of *Spiroplasma* in the salivary glands of *I. ovatus* and *I. persulcatus* [57]. More recently, several *Spiroplasma* isolates were obtained by incubating the homogenates of *I. monospinosus*, *I. persulcatus*, and *Haemaphysalis kitaokai* with tick and mosquito cells [65]. These studies collectively indicate that there is a close relationship between *Spiroplasma* and ticks in Japan; however, no comprehensive studies have been conducted to determine the genetic diversity and prevalence of tick-associated *Spiroplasma*.

The aim of this study was to identify and genetically characterize *Spiroplasma* in different tick species in Japan. A linear mixed model was developed to resolve the correlation among several extrinsic and intrinsic factors associated with *Spiroplasma* infection in ticks.

2. Materials and methods

2.1. Sample collection

Ticks were collected by flagging the vegetation during the period of tick activity (between April 2013 and August 2018) at 112 different sampling sites in 19 different prefectures in Japan. The sampling sites were classified into nine geographical blocks: Hokkaido (Hokkaido prefecture), Tohoku (Yamagata and Fukushima prefectures), Kanto (Chiba prefecture), Chubu (Nagano and Shizuoka prefectures), Kinki (Mie, Nara, and Wakayama prefectures), Chugoku (Hiroshima and Shimane prefectures), Shikoku (Kagawa, Ehime, and Kochi prefectures), Kyushu (Nagasaki, Kumamoto, Miyazaki, and Kagoshima prefectures), and Okinawa (Okinawa prefecture). All collected ticks were transferred to Petri dishes and maintained in an incubator at 16°C until use.

2.2. Identification of tick species

Tick species were identified morphologically under a stereomicroscope according to the standard morphological keys [66,67]. A total of 712 adult ticks from four genera were examined in this study. These included 2 species in the genus *Amblyomma* (*Amblyomma geoemydae*, *n* = 3; *Amblyomma testudinarium*, *n* = 26), 1 species in the genus *Dermacentor* (*Dermacentor bellulus*, *n* = 9), 10 species in the genus *Haemaphysalis* (*Haemaphysalis concinna*, *n* = 2; *Haemaphysalis cornigera*, *n* = 1; *Haemaphysalis flava*, *n* = 65; *Haemaphysalis formosensis*, *n* = 83; *Haemaphysalis hystricis*, *n* = 60; *Haemaphysalis japonica*, *n* = 20; *H. kitaokai*, *n* = 78; *Haemaphysalis longicornis*, *n* = 106; *Haemaphysalis megaspinosa*, *n* = 66; *Haemaphysalis yeni*, *n* = 1), and 7 species in the genus *Ixodes* (*Ixodes monospinosus*, *n* = 21; *Ixodes nipponensis*, *n* = 3; *I. ovatus*, *n* = 80; *Ixodes pavlovsky*, *n* = 26; *I. persulcatus*, *n* = 55; *Ixodes tanuki*, *n* = 1; *Ixodes turdus*, *n* = 6).

2.3. DNA extraction

The procedures for DNA extraction from individual ticks have been reported previously [68]. In brief, the surface of tick bodies was individually washed with 70% ethanol and sterilised PBS. The whole tick bodies were homogenised in 100 µL of high-glucose Dulbecco's modified Eagle's medium (Gibco, Life Technologies, Grand Island, NY, USA) using Micro SmashTM MS100R (TOMY, Tokyo, Japan) for 30 s at 3,000 rpm. DNA was extracted from 50 µL of the tick homogenate using the blackPREP Tick DNA/RNA Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol.

2.4. Detection of *Spiroplasma* in ticks

To detect *Spiroplasma* DNA, PCR amplification of a sequence of approximately 1,028 bp in the 16S rDNA was performed. The PCR was carried out in a 20 µL reaction mixture containing 10 µL of 2 x Gflex PCR Buffer (Mg^{2+},dNTP plus), 400 nM of Tks Gflex™ DNA Polymerase (Takara Bio, Shiga, Japan), 400 nM of each primer, 1 µL of DNA template, and sterilised water. The reaction was performed at 94°C for 1 min, followed by 45 cycles at 98°C for 10 s, 60°C for 30 s, and 68°C for 45 s and a final step at 68°C for 5 min. PCR products were electrophoresed on a 1.0% agarose gel. The DNA of a *Spiroplasma* species isolated from *I. persulcatus* in the previous study [57] and sterilised water were included in each PCR run as positive and negative controls, respectively. Primer sets used for each assay are shown in Table 1 [51,69]. The amplified PCR products were purified using ExoSAP-IT Express PCR Cleanup Reagent (Thermo Fisher Scientific, Tokyo, Japan). Sanger sequencing was performed using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 3130xl Genetic Analyzer according to the manufacturer' s

instructions. Sequence data were assembled using ATGC software version 6.0.4 (GENETYX, Tokyo, Japan).

2.5. Molecular characterisation of *Spiroplasma*

To further characterise *Spiroplasma* in ticks, additional PCRs based on ITS region (301 bp), *dnaA* (515 bp), and *rpoB* genes (1,703 bp) were performed with primers widely used for the characterisation of *Spiroplasma* in arthropods [38,69]. These PCRs were performed for selected samples using the following criteria: 1) more than three samples (when available) were selected for each 16S rDNA allele; 2) the samples were selected from each tick species when the 16S rDNA allele was from multiple tick species. The PCRs were carried out as described above, except that 56°C and 52°C were used as the annealing temperatures for ITS and *dnaA* PCRs, respectively. The primer sets used for each assay are shown in Table 1. All PCR amplicons were subjected to Sanger sequencing analysis. The sequences obtained were submitted to DDBJ (http://www.ddbj.nig.ac.jp) under specific ac-cession numbers (16S rDNA: LC592079–LC592113; ITS: LC592139–C592161; *dnaA*: LC592127–LC592138; *rpoB*: LC592114–LC592126).

Table 1. Primers for PCR and sequencing for detection of *Spiroplasma*.

Primer	Sequence (5'-3')	Target gene	Annealing temperature (°C)	Purpose	Amplicon size (bp)	Reference
spi_f1	GGGTGAGTAACACGTATCT	16S rDNA	60	PCR	1,028	[51]
spi_r3	CCTTCCTCTAGCTTACACTA					
16S_1	ACCTTACCAGAAAGCCACGG	16S rDNA	NA	Sequencing	NA	This study
16S_2	AGACCTTCATCAGTCACGCG					
16S_3	GTAATATGTGCCAGCAGCCG					
16S_4	ACCGCATTCTCCATCAGCTT					
SP-ITS-JO4	GCCAGAAGTCAGTGTCCTAACCG	ITS1	56	PCR	301	[51]
SP-ITS-N55	ATTCCAAGCCATCCACCATACG					
SRdnaAF1	GGAGAYTCTGGAYTAGGAAA	*dnaA*	52	PCR	515	[69]
SrdnaAR1	CCYTCTAWYTTTCTRACATCA					
RpoBF1	ATGGATCAAACAAATCCATTAGCAGA	*rpoB*	60	PCR	1,703	[69]
RpoBR2	GCATGTAATTTATCATCAACCATGTGTG					
Rpo_1	TGACCATTACTACGAGCAATAACA	*rpoB*	NA	Sequencing	NA	This study
Rpo_2	CCCCTGTTTTTGATGGTGCA					

2.6. Phylogenetic trees

Phylogenetic trees were constructed based on the partial sequences of 16S rDNA, *dnaA*, *rpoB* genes, and ITS region. The nucleotide sequences obtained were aligned with representative sequences of known *Spiroplasma* species available in GenBank as implemented in MEGA7 [64,70]. The reference sequences of ITS region of *S. ixodetis* were obtained by *de novo* assembly of Illumina raw reads of *Spiroplasma*-infected African monarch butterfly *Danaus chrysippus* deposited in the sequence read archives (SRA) of the NCBI with accession numbers of SRX3872086 and SRX3872088-SRX3872090 [71] using CLC Genomics Workbench v 20.0.4 (Qiagen, Hilden, Germany). Phylogenetic trees were constructed using ML method with bootstrap tests of 1,000 replicates. The sequence data of the evolutionary models were determined using the Akaike information criterion with MEGA7 [70].

2.7. Phylogenetic analysis

Spiroplasma infection in ticks can be affected by various extrinsic and intrinsic factors. Here, the extrinsic factors included sampling district, city/town, season, month, and year variations, and the intrinsic factors were tick species and sex. First, multicollinearity among the explanatory variables was examined using pairwise correlations and the "VIF" function in *R* package [72] to determine whether multicollinearity was likely to influence LMM results. A correlation between several variables affecting *Spiroplasma* infection in tsetse flies was reported in a previous study [73]. To identify this possible correlation in ticks, MCA was performed by using the "MCA" and "fviz_mca_var" functions in the *R* packages FactoMineR and Factoextra, respectively [74]. LMM was used to resolve the correlation among the predictor variables associated with *Spiroplasma* infection in ticks. The LMM was fitted with the predictor variables (sampling season, year, tick sex and species) as the fixed effects with and without geographic location (district) as the random effect. This was followed by testing in additional LMMs using combinations of the predictor variables with district as the random effect variable and *Spiroplasma* infection as the response variable. The effectiveness of the tested models was compared with the Chisquare test using the "ANOVA" function in *R* software. Finally, the "lmer" function in the *R* package lme4 [75] was used for the selected LMM, with each detected *Spiroplasma* allele as the response variable. Fisher's exact test was used to confirm the significant differences of *Spiroplasma* infection rates between male and female ticks by Microsoft Excel software.

3. Results

3.1. Infection rate of *Spiroplasma* in different tick species

In this study, 109 of 712 samples (15%) were positive for *Spiroplasma* infection. Among the 20 different tick species, eight species were positive for *Spiroplasma* infection, and the highest infection rate was observed in *I. ovatus* (84%; 67/80), followed by *H. kitaokai* (35%; 27/78), *I. turdus* (17%; 1/6), *I. persulcatus* (16%; 9/55), *D. bellulus* (11%; 1/9), *I. pavlovsky* (8%; 2/26), *A. testudinarium* (4%; 1/26), and *H. flava* (2%; 1/65) (Figure 1). Only female ticks were positive for the infection in *I. turdus*, *D. bellulus*, and *H. flava*, while only one male was positive in *A. testudinarium*. The difference in *Spiroplasma* infection rates between male and female ticks was not statistically significant (Fisher's exact test). *Spiroplasma*-positive ticks were detected from most of the geographic blocks in Japan except for Kanto and Okinawa (Figure 2).

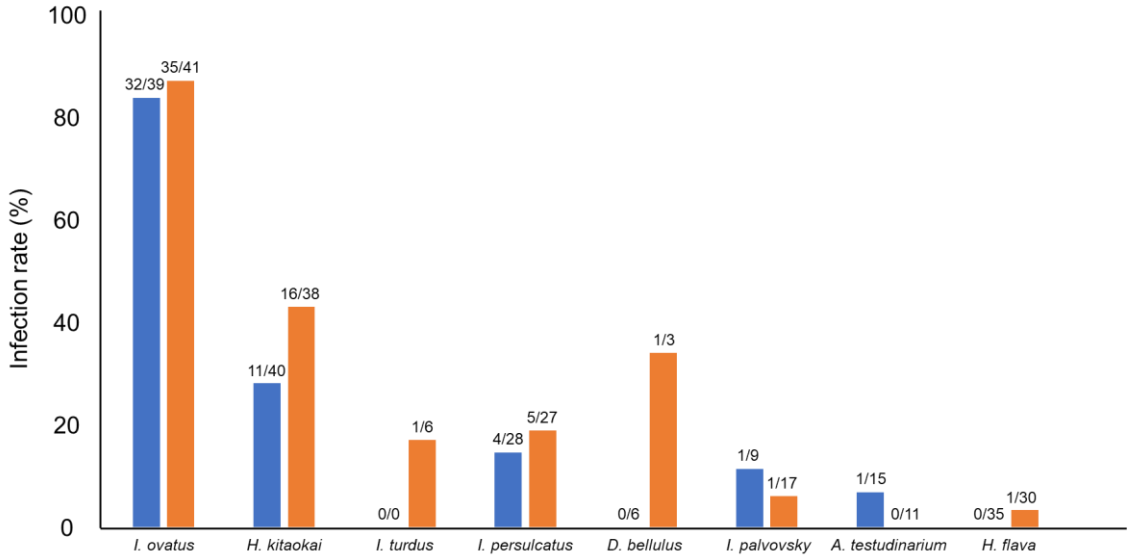

Figure 1. *Spiroplasma*-positive rates of different tick species. Blue and orange bars represent male and female ticks, respectively. The numbers at the top of the bars indicate the number of *Spiroplasma*-positive ticks/number of tested ticks.

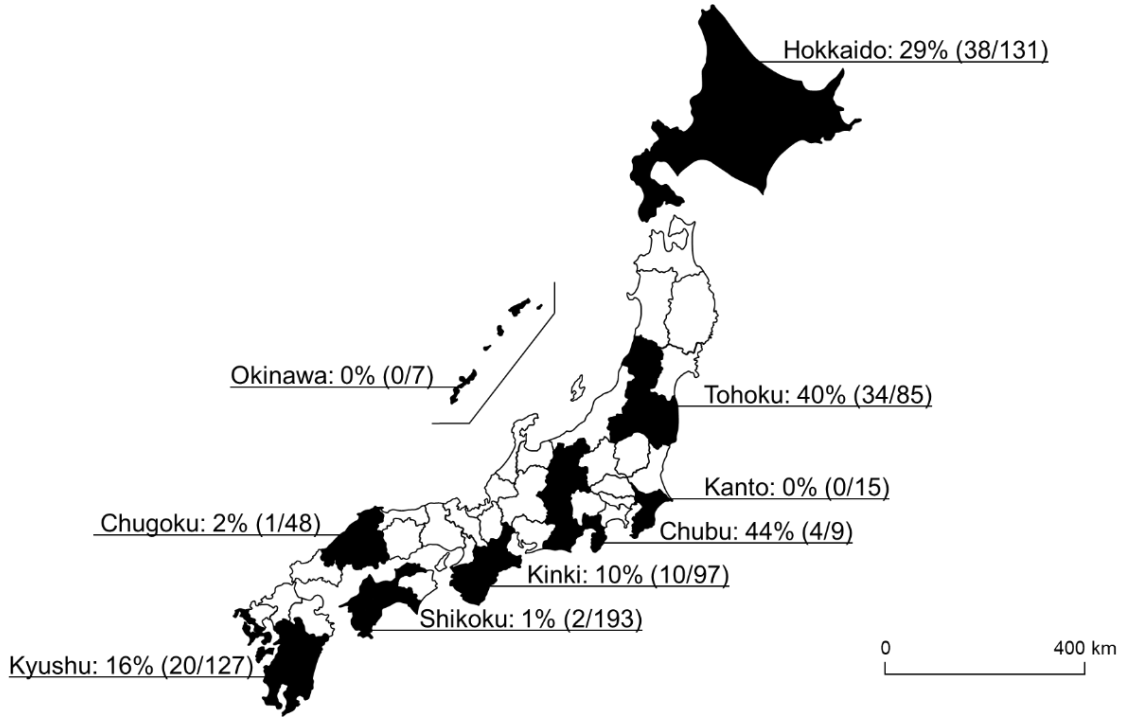

Figure 2. A map of Japan showing the *Spiroplasma*-positive rate of each geographical block. The numbers in the parentheses refer to the number of *Spiroplasma*-positive ticks/number of tested ticks. Prefectures where *Spiroplasma* was detected are filled in black.

3.2. 16S rDNA genotyping of *Spiroplasma* in ticks

A total of 101 amplicons of 16S rDNA were successfully sequenced, resulting in 17 different 16S rDNA alleles (G1–G17) (Table 2). Eight samples failed in sequencing due to mixed signals. Of the 17 alleles, 13 alleles (G3–G8, and G11–G17) were detected in a single tick species (*I. ovatus, I. pavlovsky*, and *H. kitaokai*). Two alleles (G1 and G10) were detected in two different tick species: G1 from *I. ovatus* and *I. persulcatus* and G10 from *A. testudinarium* and *I. persulcatus*. One allele (G2) was detected in three different tick species: *I. ovatus*, *I. persulcatus*, and *H. kitaokai*. Another allele (G9) was observed in four different tick species: *I. turdus*, *I. persulcatus*, *D. bellulus*, and *H. kitaokai*. The detected alleles were classified into the Ixodetis or CCM group in a phylogenetic tree based on the sequences of 16S rDNA (Figure 3). G10 and G17 were clustered with *Spiroplasma* spp. in the CCM group, whereas other alleles were grouped with members in the Ixodetis group. G10 and G17 showed 99.7% and 99.4% sequence identity, respectively, to *S. mirum* (CP006720). Alleles in the Ixodetis group formed a cluster with *S. ixodetis* found in *Ixodes*, *Rhipicephalus*, and *Dermacentor* ticks in other countries and a variety of arthropods such as ladybird, beetle, louse, butterfly, planthopper, and mealybug (Figure 3).

Table 2. *Spiroplasma* 16S rDNA alleles, host tick species and their geographical distribution.

16S rDNA allele	Tick species	No. of positive/no. of tested (%)								
		Hokkaido	Tohoku	Kanto	Chubu	Kinki	Chugoku	Sikoku	Kyushu	Okinawa
G1	*I. ovatus*	21/44 (48)	2/32 (6)	-	0/4 (0)	-	-	-	-	-
G1	*I. persulcatus*	1/39 (3)	0/8 (0)	-	0/4 (0)	0/4 (0)	-	-	-	-
G2	*H. kitaokai*	-	0/5 (0)	-	-	2/12 (17)	-	0/36 (0)	0/45 (0)	-
G2	*I. ovatus*	1/44 (2)	0/32 (0)	-	0/4 (0)	-	-	-	-	-
G2	*I. persulcatus*	3/39 (7)	0/8 (0)	-	0/4 (0)	0/4 (0)	-	-	-	-
G3	*I. ovatus*	3/44 (7)	1/32 (3)	-	3/4 (75)	-	-	-	-	-
G4	*I. ovatus*	1/44 (2)	0/32 (0)	-	0/4 (0)	-	-	-	-	-
G5	*I. ovatus*	1/44 (2)	0/32 (0)	-	0/4 (0)	-	-	-	-	-
G6	*I. ovatus*	3/44 (7)	1/32 (3)	-	1/4 (25)	-	-	-	-	-
G7	*I. ovatus*	1/44 (2)	1/32 (3)	-	0/4 (0)	-	-	-	-	-
G8	*I. ovatus*	1/44 (2)	0/32 (0)	-	0/4 (0)	-	-	-	-	-
G9	*D. bellulus*	-	0/1 (0)	-	-	1/4 (25)	-	-	0/4 (0)	-
G9	*H. kitaokai*	-	0/5 (0)	-	-	3/12 (25)	-	2/36 (6)	18/45 (40)	-
G9	*I. persulcatus*	0/39 (0)	0/8 (0)	-	0/4 (0)	4/4 (100)	-	-	-	-
G9	*I. turdus*	-	-	-	-	0/2 (0)	-	0/2 (0)	1/2 (50)	-
G10	*A. testudinarium*	-	-	-	-	-	1/9 (11)	0/15 (0)	0/2 (0)	-
G10	*I. persulcatus*	0/39 (0)	1/8 (13)	-	0/4 (0)	0/4 (0)	-	-	-	-
G11	*I. ovatus*	0/44 (0)	16/32 (50)	-	0/4 (0)	-	-	-	-	-
G12	*I. ovatus*	0/44 (0)	2/32 (6)	-	0/4 (0)	-	-	-	-	-
G13	*I. pavlovsky*	1/26 (4)	-	-	-	-	-	-	-	-
G14	*H. kitaokai*	-	0/5 (0)	-	-	0/12 (0)	-	0/36 (0)	1/45 (2)	-
G15	*H. kitaokai*	-	1/5 (20)	-	-	0/12 (0)	-	0/36 (0)	0/45 (0)	-
G16	*I. ovatus*	0/44 (0)	1/32 (3)	-	0/4 (0)	-	-	-	-	-
G17	*I. pavlovsky*	1/26 (4)	-	-	-	-	-	-	-	-

'-; *Spiroplasma* negative

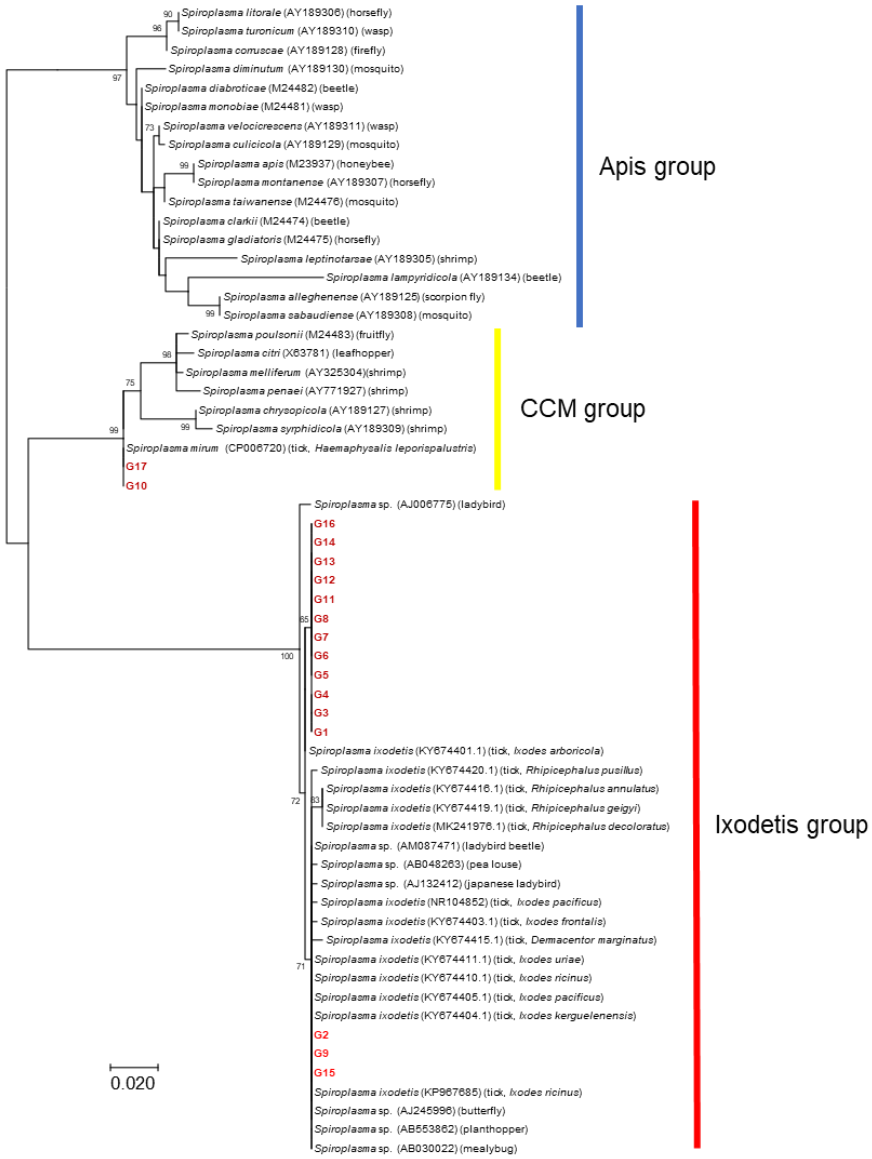

Figure 3. A phylogenetic tree based on the sequences of 16S rDNA of *Spiroplasma*. The analysis was performed using a maximum-likelihood method based on the Hasegawa–Kishino–Yano model with bootstrap tests of 1,000 replicates in MEGA7. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.2496)). The sequences obtained in this study are included with allele names provided in Table 2 and are shown in red. The sequences of other *Spiroplasma* species were retrieved from GenBank. The host is indicated in the parenthesis for each *Spiroplasma* sequence.

3.3. Characterization of *Spiroplasma* based on the sequences of ITS region, *dnaA*, and *rpoB* genes

To further characterize *Spiroplasma* in ticks, 50 *Spiroplasma*-positive samples were selected based on 16S rDNA genotyping results. The ITS region was amplified in all 16S rDNA alleles, resulting in five different alleles (T1–T5) (Table 3). T1 was the most abundant allele detected in the samples of 10 different 16S rDNA alleles (G1, G2, G4, G8–G10, and G12–G15). Multi-locus sequence typing using 16S rDNA, ITS region, *dnaA*, and *rpoB* genes showed presence of 31 different haplotypes (Table 3). Phylogenetic analysis revealed that T4 was clustered with *Spiroplasma* spp. including *S. mirum* in the CCM group, whereas T1-T3 and T5 formed a cluster with *S. ixodetis* reported from butterflies (Figure 4). There was a discrepancy between the 16S rDNA and ITS genotyping results; haplotype SP22 had a 16S rDNA allele (G10) belonging to the CCM group and an ITS allele (T1) belonging to the Ixodetis group. PCR amplification of the *dnaA* and *rpoB* genes were only successful for six and seven 16S rRNA alleles, respectively.

Table 3. Multi-locus sequence typing of *Spiroplasma* in ticks.

***Spiroplasma* haplotype**	**16S rDNA allele**	**ITS allele**	***dnaA* allele**	***rpoB* allele**	**Tick species**
SP1	G1	T3	A1	B1	*I. ovatus*
SP2	G1	T1	-	-	*I. persulcatus*
SP3	G2	T1	A1	B4	*H. kitaokai*
SP4	G2	T1	A1	-	*H. kitaokai*
SP5	G2	T2	-	-	*I. ovatus*
SP6	G2	T1	A2	B1	*I. persulcatus*
SP7	G2	T1	A2	B7	*I. persulcatus*
SP8	G2	T1	-	-	*I. persulcatus*
SP9	G3	T2	-	-	*I. ovatus*
SP10	G4	T1	A2	B3	*I. ovatus*
SP11	G5	T3	A2	B3	*I. ovatus*
SP12	G6	T2	-	-	*I. ovatus*
SP13	G7	T2	A1	-	*I. ovatus*
SP14	G8	T1	A2	B3	*I. ovatus*
SP15	G9	T1	A2	B2	*D. bellulus*
SP16	G9	T1	A2	B4	*H. kitaokai*
SP17	G9	T1	A2	B7	*H. kitaokai*
SP18	G9	-	A2	B7	*H. kitaokai*
SP19	G9	T1	-	-	*I. persulcatus*
SP20	G9	T1	A1	-	*I. persulcatus*
SP21	G9	T1	A1	B7	*I. persulcatus*
SP22	G9	T5	-	B6	*I. persulcatus*
SP23	G9	T1	-	B5	*I. turdus*
SP24	G10	T1	-	-	*A. testudinarium*
	G10	T1	-	-	*I. persulcatus*
SP25	G11	T2	-	-	*I. ovatus*
SP26	G12	T1	-	-	*I. ovatus*
SP27	G13	T1	-	-	*I. pavlovsky*
SP28	G14	T1	-	-	*H. kitaokai*
SP29	G15	T1	-	-	*H. kitaokai*
SP30	G16	T2	-	-	*I. ovatus*
SP31	G17	T4	-	-	*I. pavlovsky*

-, Not amplified.

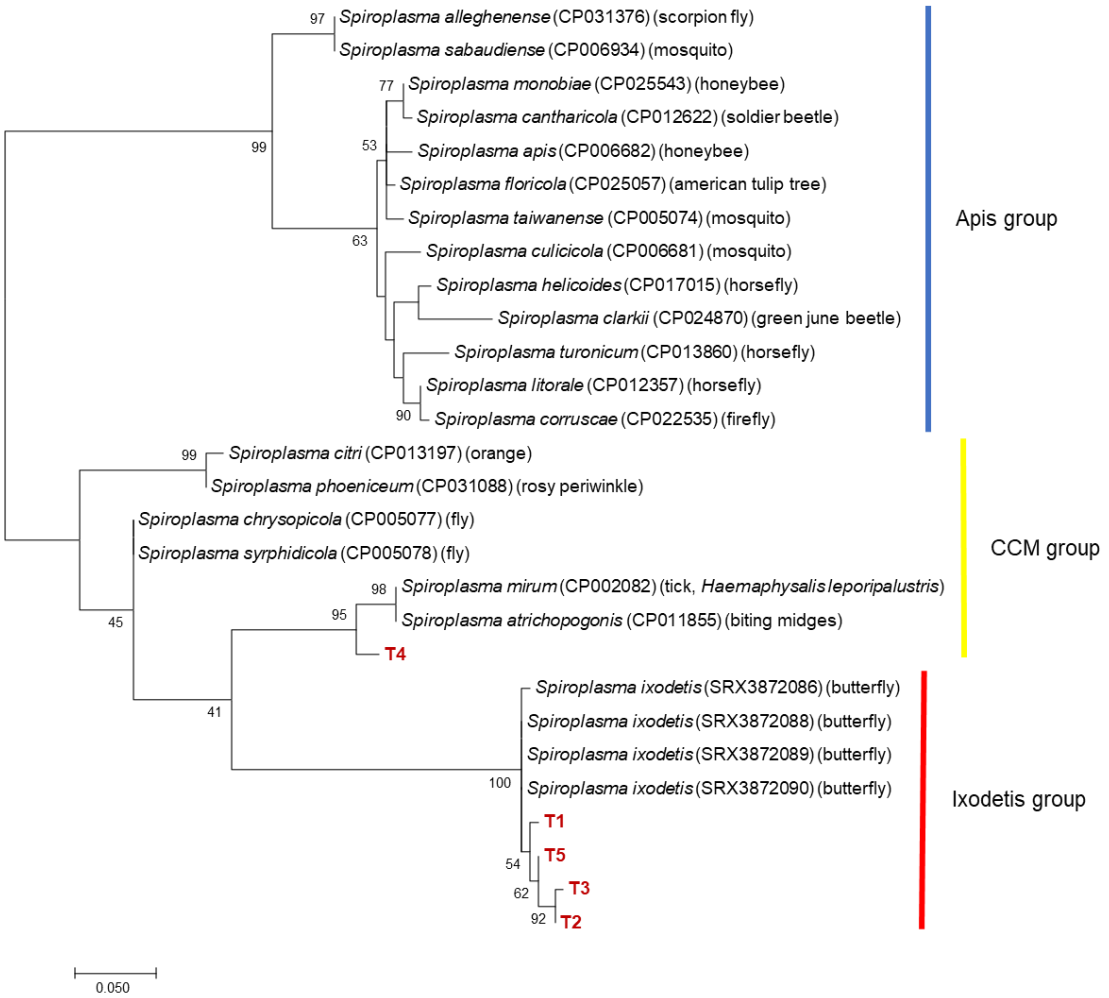

Figure 4. A phylogenetic tree based on the sequences of ITS region of *Spiroplasma*. The analysis was performed using a maximum-likelihood method based on the Tamura 3-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.2599)) with bootstrap tests of 1,000 replicates in MEGA7. The sequences obtained in this study are included with allele names provided in Table 3 and are shown in red. The sequences of other *Spiroplasma* species were retrieved from GenBank.

3.4. Effect of the genetic background on *Spiroplasma* infection

Based on the estimation of multicollinearity using VIF, Df was more than 1 for all variables except the year; thus, GVIFs were calculated. The Df is equal to the number of associated coefficients for a GVIF. Therefore, GVIF1⁄2Df was used to make GVIF values comparable among those with different numbers of Df. High collinearity is usually indicated by VIF >20. However, multicollinearity analysis using VIF indicated low multicollinearity with all variables (VIF < 5), suggesting that linear regression models would not be influenced by a combination of these variables. Multicollinearity analysis showed that there was a moderate correlation between the predictor variables (season and month; district and city/town). Both month and city/town variables were excluded from further analysis. Then, MCA was performed to identify associations between the predictor variables. The strongest association was detected between district, species, and season. LMM analysis using the predictor variables (season, year, sex, and species) revealed that the introduction of district as the random effect variable improved the models significantly ($p \leqq 0.001$) (Table 4). Moreover, when tick species was used as the principal predictor, the model for testing *Spiroplasma* infection in ticks was improved ($p \leqq 1.73 \times 10{-}75$; Table 5).

Table 4. LMM to test the correlation between each predictor with *Spiroplasma* infection using district as the random effect variable.

Model	Predictor variable	Random variable	AIC	BIC	logLik	Dev	Chisq	Df	Pr(>Chisq)	
M1-1	Species	No	99.33	195.26	-28.67	57.33	NA	NA	NA	
M1-2	Species	District	74.43	174.93	-15.22	30.43	425.55	7	8.34E-80	***
M2-1	Year	No	467.30	481.00	-230.65	461.30	NA	NA	NA	
M2-2	Year	District	459.39	477.67	-225.70	451.39	6.82	1	0.00899482	***
M3-1	Sex	No	495.23	513.50	-243.61	487.23	NA	NA	NA	
M3-2	Sex	District	465.98	488.82	-227.99	455.98	0.00	0	NA	
M4-1	Season	No	538.56	556.83	-265.28	530.56	NA	NA	NA	
M4-2	Season	District	451.24	474.08	-220.62	441.24	10.16	1	0.00143586	***

NA, Not applicable; AIC, Akaike information criterion; BIC, Bayesian information cri-terion; logLik, log-likelihood; ChiSq, ANOVA Chi-square value; Dev, Deviance of the model; Df, Chi-square degrees of freedom; Pr(>Chisq), ANOVA p value. The level of significance was marked as *** if $p < 0.0001$ and not marked if $p > 0.05$.

Table 5. Effect of several variables on the probability of *Spiroplasma* infection in the LMM.

Model	**Predictor variable**	**Random variable**	**AIC**	**BIC**	**logLik**	**deviance**	**Chisq**	**Df**	**Pr(>Chisq)**	
M5	NO	District	464.22	477.92	-229.11	458.22	NA	NA	NA	
M7	Year	District	459.39	477.67	-225.70	451.39	6.82	1	0.00899482	
M8	Season	District	451.24	474.08	-220.62	441.24	10.16	1	0.00143586	
M9	Sex	District	465.98	488.82	-227.99	455.98	0.00	0	NA	
M6	Species	District	74.43	174.93	-15.22	30.43	425.55	7	8.34E-80	***
M10	Season + Species	District	71.83	181.47	-11.92	23.83	6.60	2	0.03694614	
M11	Species + Season	District	71.83	181.47	-11.92	23.83	0.00	0	NA	
M12	Species + Season + Sex	District	69.87	188.64	-8.93	17.87	5.97	2	0.05065574	.

NA, Not applicable; AIC, Akaike information criterion; BIC, Bayesian information criterion; log-Lik, log-likelihood; ChiSq, ANOVA Chi-square value; Dev, Deviance of the model; Df, Chi-square degrees of freedom; Pr(>Chisq), ANOVA p value. The level of significance was marked as *** if $p < 0.0001$ and not marked if $p > 0.05$.

Table 6. Association between *Spiroplasma* 16S rDNA alleles and tick species.

16S rDNA allele	Tick species (No. of positive samples)	Significance
G1	*I. ovatus* (*n* = 23), *I. persulcatus* (*n* = 1)	*I. ovatus*
G2	*H. kitaokai* (*n* = 2), *I. ovatus* (*n* = 1), *I. persulcatus* (*n* = 3)	Not significant
G3	*I. ovatus* (*n* = 7)	Not significant
G4	*I. ovatus* (*n* = 1)	NA
G5	*I. ovatus* (*n* = 1)	NA
G6	*I. ovatus* (*n* = 5)	Not significant
G7	*I. ovatus* (*n* = 2)	NA
G8	*I. ovatus* (*n* = 1)	NA
G9	*D. bellulus* (*n* = 1), *H. kitaokai* (*n* = 23), *I. turdus* (*n* = 1), *I. persulcatus* (*n* = 4)	*H. kitaokai*
G10	*A. testudinarium* (*n* = 1), *I. persulcatus* (*n* = 1)	NA
G11	*I. ovatus* (*n* = 16)	*I. ovatus*
G12	*I. ovatus* (*n* = 2)	NA
G13	*I. pavlovsky* (*n* = 1)	NA
G14	*H. kitaokai* (*n* = 1)	NA
G15	*H. kitaokai* (*n* = 1)	NA
G16	*I. ovatus* (*n* = 1)	NA
G17	*I. pavlovsky* (*n* = 1)	NA

NA, not applicable.

4. Discussion

Prior to this study, there was only limited information available on the prevalence and genetic diversity of tick-associated *Spiroplasma* in Japan. In addition to three tick species (*H. kitaokai*, *I. ovatus*, and *I. persulcatus*) that were previously revealed to harbour *Spiroplasma* [58,65], five additional species, i.e., *A. testudinarium*, *D. bellulus*, *H. flava*, *I. pavlovsky*, and *I. turdus*, were found to be infected with *Spiroplasma*, thus expanding knowledge of the host range of tick-associated *Spiroplasma* in Japan.

The infection rate of *Spiroplasma* ranged from 0% to 84% depending on the tick species. To investigate whether this difference in infection rate is determined by the tick species or other factors, LMM analysis was performed. The results indicated that *Spiroplasma* infection was mainly influenced by the species of ticks but less likely to be influenced by temporal and seasonal factors (Table 5). Although the prevalence of *Spiroplasma* in tick populations has not been well understood, several previous studies reported that the *Spiroplasma* infection rates are variable between populations such as in *I. arboricola*, *I. ricinus*, and *R. decoloratus* [62,76]. A study investigating *Spiroplasma* infection rates in natural *Drosophila* populations in the southwestern United States and northwestern Mexico observed varying infection rates depending on the fly species [77]. In the same study, there was a difference in *Spiroplasma* infection rates in two fly species between the two collection sites. Similarly, in this LMM analysis, the introduction of district as the random effect variable improved the models significantly (Table 4), indicating that the *Spiroplasma* infection status in ticks may be partially influenced by the sampling location.

The highest infection rate was observed in *I. ovatus*; 82% (32/39) of males and 85% (35/41) of females were positive based on PCR amplification of *Spiroplasma* 16S rDNA (Figure 1). Sequencing analysis of PCR amplicons identified 11 *Spiroplasma* alleles in this tick species (Table 3). Furthermore, *H. kitaokai*, the second most infected species (28% (11/40) of males and 42% (16/38) of females), had four different *Spiroplasma* alleles. The association between specific 16S rDNA alleles (G1, G9, and G11) and their host tick species was statistically confirmed (Table 6). The presence of these alleles resulted in the high overall infection rates in *I. ovatus* and *H. kitaokai*. These *Spiroplasma* alleles may have adapted to the tick environment, which is important for symbionts [78]. The transmission of symbionts occurs mainly through the vertical or horizontal route. Vertical transmission involves the dispersal of symbionts and occurs primarily from the mother to offspring. Horizontal transmission occurs via host-to-host contact and acquisition from the environment [78]. Finding specific 16S rDNA alleles of *Spirolasma* in *I. ovatus* and *H. kitaokai* indicates that the horizontal transmission of

Spiroplasma may not be frequent in nature since this transmittion mode can contribute to the distribution of the same alleles among different tick species. Therefore, this finding, along with the high infection rates observed in *I. ovatus* and *H. kitaokai*, suggests the vertical transmission of *Spiroplasma* in these tick species. Symbionts can positively affect the nutrition, reproduction, and defence of their hosts. These positive effects may promote the coexistence or coevolution of symbionts and their hosts [78]. Therefore, it is of particular interest to investigate whether *Spiroplasma* affects tick fitness, as it may help understand the close association between *Spiroplasma* and ticks.

Among the three *Spiroplasma* clades, tick-associated *Spiroplasma* has only been identified in the Ixodetis and CCM groups. In the present study, most of the samples were classified as belonging to the Ixodetis group (n = 98), and only three samples were classified as belonging to the CCM group (Figure 3). Considering that most of the *Spiroplasma* species from ticks identified in previous studies belong to the Ixodetis group [55,56,58,59,63,64,76,79], this group of *Spiroplasma* may be widely distributed in the world. On the other hand, there is a lack of information on the geographic distribution and host range of tick-associated *Spiroplasma* in the CCM group. The alleles G10 and G17 obtained in the present study showed high sequence identities (99.7% and 99.4%, respectively) to *S. mirum*, which has been found to cause persistent infection in the mouse brain [80] and neurological deterioration and spongiform encephalopathy in suckling rats [81,82]. Furthermore, several ruminants such as deer, sheep, and goats developed spongiform encephalopathy in a dose-dependent manner when experimentally inoculated with *S. mirum* in their brains [83]. The alleles G10 and G17 were obtained from *A. testudinarium*, *I. pavlovsky*, and *I. persulcatus*, whose primary hosts include domestic and wild ruminants such as cattle and sika deer in Japan [84,85]. Furthermore, *A. testudinarium* and *I. persulcatus* are human-biting species that serve as main vectors for human tick-borne diseases [86,87]. Hence, it is important to investigate the potential of these *Spiroplasma* alleles as agents of human and animal diseases.

The 16S rDNA-based genotyping of 101 *Spiroplasma*-positive samples identified 17 alleles, some of which were observed in more than two different tick species (Table 2). However, further characterization by sequencing additional genes (ITS, *dnaA*, and *rpoB*) divided them into 31 haplotypes, and only one of them (SP24) was observed in two tick species (*A. testudinarium* and *I. persulcatus*) (Table 3). A previous study suggested the possible horizontal transmission of *Spiroplasma* between different ticks and other arthropods, considering that tick-derived *S. ixodetis* did not form a tick species-specific clade [64]. However, this study indicated that horizontal transmission among tick species was not common, at least among the tested tick species. Novertheless, the fact

that certain alleles (G2, G9, and G15) in the Ixodetis group were more related to *Spiroplasma* found in other arthropods than other alleles found in ticks may highlight the important role of horizontal transmission between arthropods in the spread of *Spiroplasma* in ticks, as suggested previously [64].

The genes *dnaA* and *rpoB* are frequently used in the detection and characterization of *Spiroplasma* alleles in various arthropods [63,69,73,79,88,89]. In this study, *dnaA* and *rpoB* were not amplified in nearly half of the haplotypes tested (Table 3). This may be attributed to nucleotide mismatches in the primer annealing sites. To understand the genetic diversity of *Spiroplasma* and clarify the mode of horizontal transmission in ticks, further assays using different gene targets and primer sets are necessary. A previous study developed a multi-locus sequence typing method based on five genes (16S rDNA, *rpoB*, *dnaK*, *gyrA*, and *EpsG*) by referring the daft genome of *S. ixodetis* Y32 type [64]. Considering high PCR success rates reported for ticks and other arthropods, the method might be useful to haplotype *Spiroplasma* in ticks.

Some species of *Spiroplasma* are known to affect host reproductive systems through mechanisms such as male killing [47–50]. For instance, *Spiroplasma* kills *Drosophila* males by inducing male X chromosome-specific DNA damage and activating p53-dependent abnormal apoptosis in male embryos [90]. In this study, 49 male ticks and 60 female ticks were infected with *Spiroplasma*, and there was no statistically significant difference for any of the tested tick species (Figure 1). This result is consistent with that of LLM analysis, where sex was not selected as a variable to improve the model of *Spiroplasma* infection in ticks (Table 4). Similarly, two previous studies targeting wild populations of *R. decoloratus* and wild and laboratory populations of *I. arboricola* did not find any association between sex and *Spiroplasma* infection [61,64].

In a previous study, *Spiroplasma* was highly abundant in the salivary glands of *I. ovatus* [57]. It is known that *Spiroplasma citri*, a plant pathogenic *Spiroplasma*, propagates in the salivary glands of arthropod hosts such as leafhoppers and is released along with the saliva into a new plant during feeding, which leads to transmission from an infected plant to new arthropod hosts [91,92]. Similarly, the presence of *Spiroplasma* in the tick salivary glands may cause horizontal transmission via feeding to unidentified hosts. One recent study reported that the salivary protein components of *Wolbachia*/*Spiroplasma*-infected spider mites differed from those of uninfected mites [93]. Tick saliva is an important biological material for various processes such as combating host defences, accelerating blood-feeding processes, and facilitating the transmission of pathogens to hosts [94]. Therefore, the effects of *Spiroplasma* on tick physiology and pathogen transmission involving the tick salivary glands should be

clarified in future experimental studies.

5. Summary

Members of the genus *Spiroplasma* are Gram-positive bacteria without cell walls. Some *Spiroplasma* species can cause disease in arthropods such as bees, whereas others provide their host with resistance to pathogens. Ticks also harbour *Spiroplasma*, but their role has not been elucidated yet. Here, the infection status and genetic diversity of *Spiroplasma* in ticks were investigated using samples collected from different geographic regions in Japan. A total of 712 ticks were tested for *Spiroplasma* infection by PCR targeting 16S rDNA, and *Spiroplasma* species were genetically characterized based on 16S rDNA, ITS, *dnaA*, and *rpoB* gene sequences. A total of 109 samples originating from eight tick species were positive for *Spiroplasma* infection, with infection rates ranging from 0% to 84% depending on the species. The LMM analysis indicated that tick species was the primary factor associated with *Spiroplasma* infection. Moreover, certain *Spiroplasma* alleles that are highly adapted to specific tick species may explain the high infection rates in *I.ovatus* and *H. kitaokai*. A comparison of the alleles obtained suggests that horizontal transmission between tick species may not be a frequent event. These findings provide clues to understand the transmission cycle of *Spiroplasma* species in wild tick populations and their roles in host ticks.

Chapter II

Analysis of the vertical transmission potential of *Spiroplasma* in ticks

1. Introduction

A number of species of arthropods are infected with maternally transmitted bacterial endosymbionts with diverse transmission modes such as transovarial transmission, transmission via parental postoviposition secretions, the attachment of capsules containing symbionts on the egg, and feeding on mother's excrements [36,95–97]. Some symbionts, such as *Buchnera* and *Candidatus* Sulcia muelleri, are solely transmitted in a vertical manner, resulting in a congruent phylogeny of hosts and their symbionts [98,99]. In contrast, other endosymbionts, such as *Wolbachia* and *Spiroplasma*, are known to have less congruent phylogeneis with those of their hosts [100,101]. This pattern of phylogenetic relationship between hosts and symbionts as well as the occurrence of the same or closely related endosymbionts in distantly related hosts indicates that these symbionts must occasionally colonize new hosts in a horizontal manner. However, the mechanisms by which such horizontal transmission occurs are unknown.

Many species of *Spiroplasma* maintain their infection in hosts through vertical transmission [102]. In *Drosophila*, *Spiroplasma* use the yolk uptake machinery to move the germ line for vertical transmission [103]. In recent years, studies have shown that some of these vertically-transmitted *Spiroplasma* confer protection against nematodes, parasitoid wasps, and fungi to their hosts [102]. Although vertical transmission of *Spiroplasma* is confirmed in some species [102], phylogenetic studies reported poor clustering of *Spiroplasma* from the same host species, suggesting that horizontal transmission between unrelated hosts occurs frequently [64,88,102].

Ticks generally harbor maternally inherited bacterial endosymbionts [62,104]. Some of these endosymbionts such as *Coxiella* and *Francisella* are presumably essential for the life cycle of ticks [105]. How *Spiroplasma* infection spreads among tick population is still unclear despite the fact that the high infection rates observed in some tick species such as *I. ovatus* and *H. kitaokai* [106]. In this chapter, the vertical transmission potential of *Spiroplasma* strains isolated from ticks was analyzed in a laboratory setting.

2. Materials and methods

2.1. *Spiroplasma* islolates

This study employed two species of *Spiroplasma*; *S. ixodetis* (strain 135) and *S. mirum* (strain Q35). *Spirolasma ixodetis* strain 135 was isolated from a male of *I. monospinosus* in a previous study [65]. ISE6 cells, originally reported by Munderloh et al (1994) [107] and received from the CEH Institute of Virology and Environmental Microbiology (Oxford, UK), were grown in L-15B medium supplemented with 10%

foetal bovine serum, and 5% tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO, USA) at 32°C as described previously [108], except that 0.1% bovine lipoprotein concentrate was not included in the culture medium. *Spiroplasma mirum* strain Q35 was obtained by coculturing a homogenate of female of *I. pavlovsky* with ISE6 cells (unpublished). The isolate was thereafter cultured by using modified SP4 medium containing components A and B; A) components to be autoclaved: 1.75 g *Mycoplasma* base broth, 2.65 g peptone, 5 g tryptone, 2.5g Glucose adjust pH to 7.6, and distilled water (total volume to 125 mL), B) components to be filter sterilized: 85 mL heat-inactivated fetal bovine serum, 250 mL CMRL 1066 (1X), 50 mL 2% autoclaved TC Yeastolate, 2.5 ml 200mM L-Glutamine and 0.75 mL 1% phenol red [109]. Culture medium was changed when color changed from red to yellow. Cultured *Spiroplasma* was counted by using dark-field microscope and prepared two concentrations (1×10^9 bacteria/µl and 1×10^{12} bacteria/µl).

2.2. Ticks

The parthenogenetic laboratory colonies of the hard tick *H. longicornis* Okayama strain (Fujisaki, 1978), maintained in Obihiro University of Agriculture and Veterinary Medicine, Japan, were used in all experiments. The strain was established in 1997 and has been maintained by feeding on rabbits and mice.

2.3. Injection of *Spiroplasma*

Ticks were attached to glass slides and injected with 0.5 µl of PBS or *Spiroplasma* solution through the fourth coxae using an IM 300 Microinjector (Narishige, Tokyo, Japan). There were 10 injection groups; PBS groups; only PBS and PBS + antimicrobial penicillin (100 units/ml), *S. ixodetis* inoculation groups; PBS + *S. ixodetis* (5×10^8 bacetria and 5×10^{11} bacetria) and PBS + *S. ixodetis* (5×10^8 bacetria and 1×10^{11} bacetria) + antimicrobial, penicillin (100 units/ml), *S. mirum* inoculation group; PBS + *S. mirum* (5×10^8 bacetria and 1×10^{11} bacetria) and PBS + *S. mirum* (5×10^8 bacetria and 1×10^{11} bacetria) + antimicrobial, penicillin (100 units/ml).The injected ticks were left for 7 days at 25°C in an incubator.

For rabbit infestation, a total of 70 ticks per injected group were attached in separate ears of Japanese white rabbits (Slc:JW/CSK, Japan SLC, 21-40), individually covered with an ear bag. Attached ticks were allowed to feed until they naturally dropped off. After blood-feeding, the fully engorged ticks were incubated in the dark at 25 °C and saturated humidity for oviposition.

2.4. PCR testing for *Spiroplasma* infection

Approximately 10 pools of eggs (n = 100) and larvae (n = 100) were prepared per each tick and homogenized using a BioMasher (Nippi, Tokyo, Japan) as described in

the manufacturer's protocol. Genomic DNA was extracted using the NucleoSpin® DNA Insect Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's guidelines. To detect *Spiroplasma* DNA, PCR amplification targeting 1,028 bp of 16S rDNA was performed. The PCR was carried out in a 20 μL reaction mixture containing 10 μL of 2× Gflex PCR Buffer (Mg^{2+}, dNTP plus), 400 nM of Tks Gflex™ DNA Polymerase, 400 nM of each primer, 1 μL of DNA template, and sterilised water. The reaction was performed at 94°C for 1 min, followed by 45 cycles at 98°C for 10 s, 60°C for 30 s, and 68°C for 45 s and a final step at 68°C for 5 min. PCR products were electrophoresed on a 1.0% agarose gel. The DNA of a *Spiroplasma* species isolated from *I. persulcatus* in the previous study [57] and sterilised water were included in each PCR run as positive and negative controls, respectively.

2.5. Statistical analyses

All statistical analyses were done with Microsoft Excel software. Kruskal-Wallis test was used to confirm the significant differences of engorged tick weight, the egg weight and the rate of egg hatching per injection group.

3. Results

A total of 63 ticks were used for detection of *Spiroplasma*. Seven ticks were dead during incubation after injection. The engorged tick weight, the egg weight and the rate of egg hatching were measured (Table 7). Significant different was not obtained among injection groups by Kruskal-Wallis test.

From eggs, *Spiroplasma* was detected in the groups of *S. ixodetis* (5×10^{11} bacteria) injection and *S. ixodetis* (5×10^{11} bacteria) + penicillin inhection (Table 8). The rest of the groups were all negatve by *Spiroplasma*-specific PCR. *Spiroplasma mirum* was not detected in any of the injection groups.

From larvae, *Spiroplasma* was detected from the groups of *S. ixodetis* (5×10^{11} bacteria) injection and *S. ixodetis* (5×10^{11} bacteria) + penicillin injection (Table 8). The rest of the groups were all negatve by *Spiroplasma*-specific PCR. *Spiroplasma mirum* was not detected in any of the injection groups.

Table 7. The average engorged body weight, the egg weight and the rate of egg hatching per injection group.

Group	Average engorged body weight (mg)	Egg weight/body weight (%)	Hatching rate (%)
PBS	188.2	45.1	84.8
PBS + penicillin	185.2	44.9	76.4
S. mirum (5×10^{8} bacteria)	177.2	51.1	82.4
S. mirum (5×10^{11} bacteria)	172.5	49.2	76.7
S. mirum (5×10^{8} bacteria) + penicillin	177.2	50.3	66.1
S. mirum (5×10^{11} bacteria) + penicillin	201.8	39.3	61.8
S. ixodetis (5×10^{8} bacteria)	178.2	45.9	81.7
S. ixodetis (5×10^{11} bacteria)	172.5	38.3	71.7
S. ixodetis (5×10^{8} bacteria) + penicillin	200.4	46.6	80.4
S. ixodetis (5×10^{11} bacteria) + penicillin	214.5	46.9	86.5

Table 8. Detection of *Spiroplasma* from eggs and larvae per injection group.

Group	**Detection of *Spiroplasma* from egg (No. of positive/no. of tested)**	**Detection of *Spiroplasma* from larvae (No. of positive/no. of tested)**
PBS	0/6	0/6
PBS + penicillin	0/7	0/7
S. mirum (5×10^{8} bacteria)	0/7	0/7
S. mirum (5×10^{11} bacteria)	0/7	0/7
S. mirum (5×10^{8} bacteria) + penicillin	0/6	0/6
S. mirum (5×10^{11} bacteria) + penicillin	0/7	0/7
S. ixodetis (5×10^{8} bacteria)	0/7	0/7
S. ixodetis (5×10^{11} bacteria)	2/7	2/7
S. ixodetis (5×10^{8} bacteria) + penicillin	0/6	0/6
S. ixodetis (5×10^{11} bacteria) + penicillin	1/3	1/3

4. Discussion

This study investigated whether two species of *Spiroplasma* isolated form ticks are transmitted vertically in ticks in a laboratory setting. In a previous study, the vertical transmission rate of *Spiroplasma* in *Drosophila hydei*, a *Spiroplasma* species having exclusive dependence on maternal transmission and no noticeable reproductive manipulations or positive effect on the host flies, was recorded as high as 95–100% at 25°C [43,110]. In the present study, the vertical transmission was confirmed only in two out of seven pools in *S. ixodetis* (5×10^{11} bacteria) injection group (Table 8). The lower frequency of the transmission may be resulting from poor adaptation of the isolates in the new host tick species; that is, *S. ixodetis* and *S. mirum* respectively isolated form *I. monospinosus* and *I. pavlovsky* were experimentaly inoculated into *H. longicornis*. It is reported that *Spiroplasma* strain introduced into other species were often poorly transmitted from mother to offspring in *Drosophila* [111]. Another report revealed that the vertical transmission rate of *Spiroplasma* in *Drosophila* was severely affected by temperature [110], indicating that environmental factors such as temperature might have affected the results.

Spiroplasma colonizes host oocytes at specific stages, coinciding with vitellogenesis and requires the yolk transport and uptake machinery to achieve efficient vertical transmission [103]. *Spiroplasma citri* has been observed to undergo endocytosis for infection into the host [112]. These findings suggest that *Spiroplasma* might have a general capacity to interact with the host endocytic machinery to ensure its transmission. Interactions between *Spiroplasma* and host insects are highly specific in general. *Spiroplasma citri*, which normally infects leafhoppers, grows well in *D. melanogaster* hemolymph but cannot access to the oocyte and consequently is not vertically transmitted [113]. Interestingly, despite that a number of interspecific transfers of *Spiroplasma* between *Drosophila* species are confirmed [114], the transfer of *Spiroplasma* from *Drosophila* to other arthropod species is rarely documented.

Because of its well-explored biology and genome, *H. longicornis* has been particularly exploited as one of the model ticks. The current study assessed the vertical transmission potential of *Spiroplasma* isolaytes using laboratory colonies of *H. longicornis* rather than other tick species such as *I. ovatus* and *H. kitaokai* where *Spiroplasma* infecton is much common. The transmission of *Spiroplasma* between different tick species in nature has not been reported. Moreover, the results of the allele coparison of *Spiroplasma* in Chapter I suggest that the possibility of horizontal transmission of *Spiroplasma* between different tick species is very low. Taken together, the further experiments employing tick colonies of appropriate tick species is essential to

evaluate the vertical transmission rate of *Spiroplasma* in ticks.

In this study, *Spiroplasma* was inoculated with antibiotics in some injection groups. However, significant difference was not observed in tems of engorged weight, egg weight and hatching rate between antibiotics-treated and non-treated groups. It was reported that when the symbiont density in host insects is experimentally decreased using antibiotics, host reproduction ability is reduced because of symbionts sorting upon vertical transmission [115]. In the present experiment, antibiotics treatement had no effect on the vertical transmission rate of *Spiroplasma* in ticks. In general, the antibiotics treatment for ticks has been associated with several reproductive dysfunction such as reduced engorged and egg weight and hatching rate because of dysbiosis of microbiota and reduction of endosymbionts [116,117]. The lack of significant effect of antibiotics treatment in the current experiment may be patly explained by the lower concentration of antibiotics where microbiota was not affected in the antibiotics-treated groups. Therefore, it is necessary to optimize the concentration and type of antibiotics to be administered as well as inoculation routes in future in order to understand the interaction between *Spiroplasma* and tick microbiota.

5. Summary

A number of arthropods are infected with maternally transmitted bacterial endosymbionts such as *Buchnera*, *Wolbachia*, and *Spiroplasa*. Some symbionts are solely transmitted in a vertical manner, while others are maintained by both vertical and horizontal transmissions in nature. However, how *Spiroplasma* infections have spread in tick species is still unclear although the high infection rates were observed in some tick species. In this chapter, the vertical transmission potential of *Spiroplasma* strains isolated from ticks was analyzed by using experimental infection model. Two species of *Spiroplasma*, *S. ixodetis* (strain 135) and *S. mirum* (strain Q35), were experimentaly inoculated into laboratory colonies of *H. longicornis*. *Spiroplasma* infection was examined in the eggs and larvae originating from *Spiropalsma*-inoculated ticks. The results indicated that only *S. ixodetis* was detected in the eggs and larvae when ticks were inoculated with the concentration of 5×10^{11} bacteria per individual. There was no significant difference in engorged weight, egg weight and hatching rate between *Spiroplasma*-inoculated and control groups, indicating that *Spiroplasma* infection does not affect the reproduction of ticks. This study is the first experimental data to suggest the vertical transmission potential of *S. ixodetis* in ticks

GENERAL CONCLUSION

Ixodid ticks are blood sucking ectoparasites of vertebrates with about 700 species distributed in the world. They serve as vectors of many pathogens and cause significant public health and veterinary health problems globally. Symbiosis is a general term used to describe two or more different species living in close association with each other. In nature, symbiotic relationships between bacteria and arthropods are well known and have been studied extensively. Understanding the molecular and biochemical mechanisms that underpin these relationships is a notable focus of symbiont research. Members of the genus *Spiroplasma* are gram-positive bacteria without cell walls. They are known as symbionts of arthropods and plants. *Spiroplasma* is one of the most common endosymbionts with a wide range of hosts, including insects, arachnids, crustaceans, and plants. It is estimated that 5–10% of insect species harbour this symbiont group. *Spiroplasma* has a wide range of fitness effects and transmission strategies. Some *Spiroplasma* species affect the sex ratio by inducing male killing in hosts such as flies, butterflies, and ladybird beetles. Several *Spiroplasma* species are known to cause disease in arthropods such as bees and plants. On the other hand, some flies infected with *Spiroplasma* can develop resistance to other pathogens. This characteristic of *Spiroplasma* is not only biologically interesting but also useful for symbiotic control applications among host individuals.

Chapter I focused on the infection status and genetic diversity of *Spiroplasma* in ticks. A total of 712 ticks were tested for *Spiroplasma* infection by PCR targeting 16S rDNA, and *Spiroplasma* species were genetically characterized based on 16S rDNA, ITS, *dnaA*, and *rpoB* gene sequences. A total of 109 samples originating from eight tick species were positive for *Spiroplasma* infection, with infection rates ranging from 0% to 84% depending on the species. A linear mixed model indicated that tick species was the primary factor associated with *Spiroplasma* infection. Moreover, certain *Spiroplasma* alleles that are highly adapted to specific tick species may explain the high infection rates in *Ixodes ovatus* and *Haemaphysalis kitaokai*. A comparison of the alleles obtained suggests that horizontal transmission between tick species may not be a frequent event. These findings provide clues to understand the transmission cycle of *Spiroplasma* species in wild tick populations and their roles in host ticks.

Chapter II focused on vertical transmission potential of *Spiroplasma* in ticks. Two species of *Siproplasma*, *S. ixodestis* and *S. mirum*, were experimentally inoculated into laboratory colonies of *Haemaphysalis longicornis*. The presence of *Spiroplasma* was examined in the eggs and larvae originating from *Spiropalsma*-inoculated ticks by PCR.

The results indicated that only *S. ixodetis* was transmitted into the eggs and larvae when ticks were inoculated with the concentration of 5×10^{11} bacteria per individual. There was no significant difference in engorged weight, egg weight and hatching rate between *Spiroplasma*-inoculated and control groups, indicating that *Spiroplasma* infection does not affect the reproduction of ticks. The data obtained are the first experimental evidence to demonstrate the vertical transmission potential of *Spiroplasma* in ticks.

日本語要旨

マダニは脊椎動物の吸血外部寄生虫で世界に約 700 種が分布し、多くの病原体の媒介動物となり世界的に公衆衛生や獣医学上の重大な問題を引き起こしている。共生生物とは、1879 年に Bary が定義したもので、2 つ以上の異なる種が互いに密接に関連して生活していることを示す一般的な用語である。自然界では、微生物とマダニを含む節足動物の共生関係がよく知られており、広く研究されている。このような共生関係を支える分子・生化学的なメカニズムを解明することは、外部寄生虫の防除法開発にもつながる点で新たな研究領域として期待されている。

Spiroplasma 属細菌は、細胞壁をもたないグラム陽性細菌である。*Spiroplasma* は、昆虫、クモ類、甲殻類、植物など幅広い宿主に共生する最も一般的な内共生体の 1 つであり、昆虫種の 5〜10%がこの共生生物群を保有していると推定されている。*Spiroplasma* は、幅広い環境適応効果と感染戦略を持っている。いくつかの *Spiroplasma* 種は、ハエ、チョウ、テントウムシなどの宿主のオス殺しを誘発することで、性比に影響を与える。いくつかの *Spiroplasma* 種は、ミツバチや植物などの節足動物に病気を引き起こすことが知られている。一方で、*Spiroplasma* に感染したハエの中には、他の病原体に対する耐性を獲得する

ものもいる。このような *Spiroplasma* の特性は、生物学的に興味深いだけでなく、宿主個体間の共生制御の応用にも有用である。近年マダニにも *Spiroplasma* が共生していることが知られており、マダニと *Spiroplasma* の共生関係による制御機構を明らかにすることは、公衆衛生や獣医学領域に大きく貢献するものと考えられる。

第 1 章では，マダニにおける *Spiroplasma* の感染状況と遺伝的多様性に焦点を当てた。合計 712 匹のマダニを対象に、16S rDNA を標的とした PCR による *Spiroplasma* の検出を行い、16S rDNA、ITS、*dnaA* および *rpoB* 遺伝子の配列に基づいて *Spiroplasma* 種の遺伝的特徴を調べた。8 種のマダニから得られた合計 109 検体が *Spiroplasma* 陽性であり、感染率は種によって 0%から 84%であった。一般線形混合モデルでは、マダニの種が *Spiroplasma* の感染に関連する主要な因子であることが示された。さらに、特定のマダニ種に高度に適応したスピロプラズマ遺伝子型が、*Ixodes ovatus* と *Haemaphysalis kitaokai* の高い感染率を説明している可能性が示された。得られた遺伝子型の比較からはマダニ種間の *Spiroplasma* の水平伝播は頻繁に起こるものではないことが推察された。これらの知見は、自然界におけるマダニ集団内の *Spiroplasma* 種の感染サイクルと宿主マダニにおける役割を理解する手がかりとなる。

第 2 章では、マダニにおける *Spiroplasma* の垂直感染に焦点を当てた。

マダニから分離された *Spiroplasma ixodetis* と *Spiroplasma mirum* を、*Haemaphysalis longicornis* にインジェクション法を用いて実験的に接種した。接種したマダニ個体が産出した卵および、孵化後の幼ダニにおける *Spiroplasma* 感染の有無を PCR 法により検査した。その結果、*S. ixodetis* を 5×10^{11} 菌体/個体で接種したマダニが産出した卵および幼ダニでのみ *Spiroplasma* の感染が確認され、*Spiroplasma* が垂直伝播することを確認した。非接種対象群と比較して、*Spiroplasma* 接種群の飽血時体重、卵重量、孵化率に有意な差が見られなかったことから、*Spiroplasma* のマダニ生殖機能における影響は観察されなかった。本研究結果は、*Spiroplasma* がマダニにおいて垂直伝播することを実験的に証明した最初の報告となる。

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