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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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]. Symbiosis is a general term used to describe two or more different species living in close association with each other [4]. 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 [5]. It is estimated that 5–10% of insect species harbour this symbiont group [6,7]. *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 [8–11]. Several *Spiroplasma* species are known to cause disease in arthropods such as bees and plants [12–14]. On the other hand, some flies infected with *Spiroplasma* can develop resistance to other pathogens [11,15–17]. This characteristic of *Spiroplasma* is not only biologically interesting but also useful for symbiotic control applications among host individuals [18].

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 [19]. Another species, *Spiroplasma ixodetis*, was isolated from *Ixodes pacificus* in the United States in 1981 [20]. 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* [21–28].

In Japan, Taroura et al. first detected *Spiroplasma* DNA in questing *I. ovatus* ticks captured in several prefectures [22]. Subsequently, a microbiome study revealed the presence of *Spiroplasma* in the salivary glands of *I. ovatus* and *I. persulcatus* [21]. More recently, several *Spiroplasma* isolates were obtained by incubating the homogenates of *I. monospinosus*, *I. persulcatus*, and *Haemaphysalis kitaokai* with tick and mosquito cells [29]. 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*.

Chapter I focused on the infection status and genetic diversity of *Spiroplasma* in ticks. 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.

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. Tick species were identified morphologically under a stereomicroscope according to the standard morphological keys [30,31]. A total of 712 adult ticks from four genera were examined in this study. The procedures for DNA extraction from individual ticks have been reported previously [32]. To detect *Spiroplasma* DNA, PCR amplification of a sequence of approximately 1,028 bp in the 16S rDNA was performed. 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 [6,33]. 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. 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 [28,34]. *Spiroplasma* infection in ticks can be affected by various extrinsic and intrinsic factors. 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.

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). A total of 101 amplicons of 16S rDNA were successfully sequenced, resulting in 17 different 16S rDNA alleles (G1–G17). The detected alleles were classified into the Ixodetis or CCM group in a phylogenetic tree based on the sequences of 16S rDNA. To further characterize *Spiroplasma* in ticks, 50 *Spiroplasma*-positive samples were selected based on 16S rDNA

genotyping results. Multi-locus sequence typing using 16S rDNA, ITS region, *dnaA*, and *rpoB* genes showed presence of 31 different haplotypes. 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 ($p \leqq 0.001$). Moreover, when tick species was used as the principal predictor, the model for testing *Spiroplasma* infection in ticks was improved ($p \leqq 1.73 \times 10-75$).

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. In this LMM analysis, the introduction of district as the random effect variable improved the models significantly, indicating that the *Spiroplasma* infection status in ticks may be partially influenced by the sampling location.

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.

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