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Experimental demonstration of the transmission of *Spiroplasma* between different arthropod taxa

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Abstract

Spiroplasma is one of the most widely distributed symbionts of arthropods. *Spiroplasma* species can infect their hosts via vertical or horizontal transmission. However, the mode of transmission of *Spiroplasma* between different arthropod taxa has not been elucidated. In this study, we investigated the potential for the transmission of *Spiroplasma* to non-native arthropod species, using two *Spiroplasma* spp. isolated from ticks, namely *Spiroplasma ixodetis* and *Spiroplasma mirum*, and three species of mosquito laboratory colonies, namely *Aedes albopictus*, *Aedes aegypti*, and *Culex pipiens pallens*. After feeding the adult mosquitoes with *Spiroplasma*-containing artificial meals, they were kept at 25°C for 10 d. Homogenates prepared from *Spiroplasma*-fed mosquitoes were used to re-isolate *Spiroplasma* using the *in vitro* culture method. Nine weeks after culture initiation, the presence of *Spiroplasma* was tested using PCR. The results revealed that only *S. ixodetis* was detected from all three species of mosquitoes and re-isolated from two of them. The differences in the infection ability of different *Spirolasma* species could be attributed to several factors, including environmental effects. Nevertheless, this is the first experimental demonstration of *Spiroplasma* transmission among different arthropod taxa. Further studies are needed to elucidate the evolutionary mechanism that supports the survival of *Spiroplasma* in nature.

Keywords

Spiroplasma, horizontal transmission, mosquito, symbiont, tick

Introduction

Spiroplasma is one of the most widely distributed symbionts and is present in approximately 5–10% of arthropod species (Duron et al. 2008). Among the several *Spiroplasma* spp. identified in arthropods, some confer the host with the ability to resist various pathogens, including parasites and fungi (Ballinger and Perlman 2019). For instance, tsetse flies infected with *Spiroplasma* have a significantly lower infection rate for parasitic trypanosomes (Schneider et al. 2019). Another study reported that *Spiroplasma* in *Drosophila* protects them against infection by parasitoid wasps (Xie et al. 2010). *Spiroplasma ixodetis* has been shown to significantly reduce the mortality rate of pea aphids caused by fungal pathogens (Łukasik et al. 2013). Another significant effect of *Spiroplasma* infection in arthropod hosts is male killing, in which male-specific death is induced during development. *Spiroplasma ixodetis* induces male killing in ladybugs, butterflies, and moths (Jiggins et al. 2010, Ballinger and Perlman 2019, Arai et al. 2022).

Symbionts have two different modes of transmission, i.e. vertical and horizontal (Bright and Bulgheresi 2010). *Spiroplasma* spp. can infect their hosts by vertical transmission, and some of the underlying molecular mechanisms of vertical transmission have been experimentally demonstrated; for e.g. in *Drosophila*, *Spiroplasma poulsonii* uses the yolk protein uptake mechanism to transfer the germline (Herren et al. 2013).

63 More recently, a single membrane lectin, Spiralin B, was identified as a responsible
64 protein for *S. poulsonii* to enter host oocytes during vitellogenesis (Masson et al. 2022).
65 Horizontal transmission of *Spiroplasma* is well studied in plant pathogenic *Spiroplasma*
66 species. *Spiroplasma citri*, the causative agent of citrus stubborn disease, is transmitted
67 between plants by the leafhoppers *Circulifer haematoceps* (Fos et al., 1986). After being
68 ingested by leafhoppers feeding on the infected plant, *Spiroplasma* multiplies in the insect
69 haemocoel, invades the insect organs, and is finally released along with saliva into a new
70 plant vis feeding. Efforts have been made to clarify the molecular mechanisms to explain
71 how *Spiroplasma* overcome two physical barriers, the midgut epithelial and the salivary
72 gland cell membranes, to complete its lifecycle in insects. An earlier study by Fletcher et
73 al. suggested that adhesion of *Spiroplasma* to host cell membrane is mediated by a
74 receptor-mediated endocytosis (Fletcher et al. 1998). Thereafter, several proteins such as
75 phosphoglycerate kinase (Labroussaa et al. 2010), adhesins (Béven et al. 2012), and
76 lipoproteins (Duret et al. 2014), have been identified as possible molecules associated
77 with adhesion to and/or invasion of insect cells.

78 Molecular studies have shown that the phylogenetic relationship between
79 different *Spiroplasma* spp. or genotypes does not always correlate with the lineage of their
80 host (Gasparich 2002). For instance, *S. citri* is transmitted persistently and propagatively

by leafhoppers, but other *Spiroplasma* species genetically related to *S. citri* have been found in phylogenetically distinct arthropods, such as ants and flies (Ballinger et al. 2018, Ballinger and Perlman 2019, Rattner et al. 2021). This discrepancy in the phylogenetic relationship between the host and the symbiont and the occurrence of identical or genetically related symbionts in distantly related hosts suggest that some *Spiroplasma* spp. sustain transmissions between different arthropod taxa (Gasparich 2002).

Vector arthropods with medical and veterinary importance also harbour *Spiroplasma* spp. (Henning et al. 2006, Chang et al. 2014). Ixodid ticks are blood-sucking parasitic arthropods with approximately 700 species identified worldwide (Guglielmone et al. 2010). Ticks are important from both veterinary and public health perspectives because they transmit various pathogens to humans and animals through blood feeding (Brites-neto et al. 2015). Ticks harbour *Spiroplasma ixodetis* and *Spiroplasma mirum* (Tully et al. 1981, Tully et al. 1982). *Spiroplasma ixodetis* has been reported worldwide from 21 different species of five tick genera, namely *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes*, and *Rhipicephalus*; *Amblyomma sculptum* (Brazil), *Dermacentor bellulus* (Japan), *Dermacentor marginatus* (Hungary, France, and Spain), *Dermacentor nitens* (Brazil), *Dermacentor reticulatus* (The Netherlands and Poland), *Haemaphysalis kitaokai* (Japan), *Ixodes arboricola* (Belgium), *Ixodes frontalis* (Belgium), *Ixodes*

monospinosus (Japan), *Ixodes ovatus* (Japan), *Ixodes pacificus* (US), *Ixodes pavlovsky* (Japan), *Ixodes persulcatus* (Russia and Japan), *Ixodes ricinus* (Slovakia, The Netherlands, and UK), *Ixodes* sp. (Germany), *Ixodes turdus* (Japan), *Ixodes uriae* (Russia), *Rhipicephalus annulatus* (Benin), *Rhipicephalus decoloratus* (South Africa), *Rhipicephalus geigyi* (Burkina Faso), and *Rhipicephalus pusillus* (France) (Tully et al. 1981, Tully et al. 1995, Henning et al. 2006, Hornok et al. 2010, Bell-Sakyi et al. 2015, Van Oosten et al. 2018, Binetruy et al. 2019, Palomar et al. 2019, Thu et al. 2019, Beliavskaia et al. 2021, Ogata et al. 2021, Olsthoorn et al.2021, Da Silva et al. 2022) (Table 1). *Spiroplasma mirum* has been reported in four species of the following three tick genera: *Amblyomma*, *Haemaphysalis*, and *Ixodes*; *Amblyomma testudinarium* (Japan), *Haemaphysalis leporispalustris* (US), *I. persulcatus* (Japan), and *I. pavlovsky* (Japan) (Tully et al. 1982, Ogata et al. 2021) (Table 1).

Mosquitoes are another group of arthropods that are globally distributed and transmit various pathogens during their blood-feeding behaviour. Several *Spiroplasma* spp. have been reported in mosquitoes, including *Spiroplasma culicola*, *Spiroplasma taiwanense*, *Spiroplasma sabaudiense*, *Spiroplasma diminutum*, *Spiroplasma cantharicola*, and *Spiroplasma insolitum* (Chang et al. 2014). *Spiroplasma taiwanense* is pathogenic to *Aedes aegypti* and induces significant reduction

in larval survival (Humphery-Smith et al. 1991). Moreover, female *Anopheles stephensi* infected with *S. taiwanense* exhibit loss-of-flight owing to the degradation of their thoracic flight muscles (Phillips and Humphery-Smith 1995). Female of *Ae. aegypti* infected with *S. sabaudiense* exhibit significantly increased male-to-progeny ratio, and those infected with *S. culicicola* exhibit significantly reduced reproductive ability (Vazeille-Falcoz et al. 1994). Futhermore, a previous study confirmed that *S. insolitum* is vertically transmitted in *Anopheles gambiae* (Chepkemoi et al. 2017).

In this study, we assessed the potential of the transmission of *Spiroplasma* to non-native arthropod species, using two different *Spiroplasma* spp. isolated from ticks and three lines of laboratory mosquito colonies. The results of this study indicate that *S. ixodetis* has the potential to survive in non-native host species.

Materials and Methods

Mosquitoes: source and rearing conditions

Laboratory colonies of adult fertilised female mosquitoes (*Culex pipiens pallens* Osaka strain, *Ae. aegypti* SMK strain, and *Aedes albopictus* Hatsudai strain) were maintained at insectary room of the International Institute for Zoonosis Control, Hokkaido University, Japan, and used in the infection experiments. All mosquitoes were

bred at 25°C, 75% relative humidity (RH), and a 16:8 light/dark photoperiod. Paper cups (500-mL capacity) were covered with a mesh lid, and 40 female mosquitoes were transferred into each cup (Figure 1). Cotton (1.5 cm^2) moistened with 4% sucrose was placed on the mesh lid of the cups, and they were maintained in an incubator (25°C, RH 75%).

Spiroplasma: source and growth conditions

For this study, we used two species of *Spiroplasma*, namely *S. ixodetis* (strain 135) and *S. mirum* (strain Q35). *Spiroplasma ixodetis* strain 135 was isolated from a male *I. monospinosus* in a previous study (Thu et al. 2019). *Spiroplasma mirum* strain Q35 was obtained by co-culturing the homogenate of female of *I. pavlovsky* with ISE6 cells (Ogata et al. 2023). ISE6 cells, reported by Munderloh et al (1994) 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 previously described (Munderloh and Kurtti 1989), but 0.1% bovine lipoprotein concentrate was not included in the culture medium. *Spiroplasma mirum* was cultured using modified SP4 medium without any

151 antimicrobial agents (Whitcomb 1983). The culture medium was replaced with fresh
152 medium when its colour changed from red to yellow. The number of *Spiroplasma* cells in
153 culture media was determined using a dark-field microscope.
154
155 **Oral feeding of mosquitoes with *Spiroplasma***
156 Artificial meals containing *Spiroplasma* (1 mL) were prepared, which comprised
157 *Spiroplasma* (1×10^{12} bacteria/mL), 10% sucrose, and 0.1 M ATP (Nacalai Tesque, Kyoto,
158 Japan). The control meal contained PBS, 10% sucrose, and 0.1 M ATP. Each meal was
159 kept at 31.5°C, and the mosquitoes (40 individuals per mosquito species and *Spiroplasma*
160 species) were allowed to feed for 60 min in an artificial feeding device (Hemotek
161 membrane feeding systems, Hemotek Ltd, England). The number of feeding and non-
162 feeding female mosquitoes in the cups were recorded. To prevent mosquitoes from
163 escaping, the cages containing the feeding mosquitoes were kept sealed until the end of
164 the experiment, and non-feeding female mosquitoes were not removed. After
165 *Spiroplasma* feeding, the mosquitoes were kept in an incubator at 25°C (RH 80%), 10%
166 sucrose cotton was changed daily, and the number of deaths was recorded. The
167 mosquitoes were harvested 10 d after the *Spiroplasma* feeding and frozen to death (30 to

60 min) at -80°C (Figure 1).

Isolation of *Spiroplasma* from mosquito homogenate

Frozen mosquitoes were sorted into surviving and dead mosquitoes on aluminum plates at 4°C. Surviving female mosquitoes (10 individuals for *Spiroplasma*-feeding groups and 3 individuals for control groups) were placed in individual microtubes. L-15B medium or SP4 medium (40 μL) was added to the microtubes, and a homogenate solution (40 μL) was prepared using BioMasher (Nippi, Tokyo, Japan). Ten microlitres of the supernatant (centrifuged at 4,126 g for 5 min) was inoculated in a 48-well plate for *Spiroplasma* isolation, and 10 μL of the supernatant was stored at 4°C in a new microtube for DNA extraction. The remaining 20 μL supernatant containing the crushed mosquito sediment was stored at -80°C. *Spiroplasma ixodetis* was cultured in 48-well plates containing L-15B medium supplemented with penicillin/streptomycin (100 U/mL), amphotericin B (2.5 μg/mL), and *S. mirum* was cultured in 48-well plates containing SP4 medium supplemented with the same antibiotics mentioned above. The culture medium was changed twice weekly for ISE6 cells, whereas the SP4 medium was changed when the colour of the culture medium became yellow. At 9 weeks, the experiment was terminated, and 250 μL of the cell suspension or culture medium was collected and used

for DNA extraction.

Detection of *Spiroplasma* using PCR

Genomic DNA from the mosquito homogenates was extracted using the NucleoSpin® DNA Insect Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's guidelines. Genomic DNA was extracted from the cell suspension or culture medium using the Qiagen DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany). To confirm the presence of *Spiroplasma* DNA, we performed PCR targeting 1,028 bp of *Spiroplasma* 16S rDNA using the following primers spi_f1 (5′-GGGTGAGTAACACGTATCT-3′) and spi_r3 (5′-CCTTCCTCTAGCTTACACTA-3′) (Ogata et al. 2021). PCR was performed in a 20-μL reaction mixture containing 10 μL of 2× Gflex PCR Buffer (Mg^{2+}, dNTP plus), 400 nM Tks Gflex ™ DNA Polymerase, 400 nM of each primer, 1 μL of DNA template, and sterilised water. The reaction conditions were as follows: 94°C for 1 min; followed by 40-45 cycles of 98°C for 10 s, 60°C for 45 s, and 68°C for 60 s; and a final step at 68°C for 5 min. The PCR products were electrophoresed on a 1.5% agarose gel. The *Spiroplasma* DNA isolated from *I. persulcatus* in a previous study (Qiu et al. 2014) and sterilised water were used as the positive and negative controls, respectively.

The amplified PCR products were purified using the ExoSAP-IT Express PCR Cleanup Reagent (Thermo Fisher Scientific, Tokyo, Japan). Sanger sequencing was performed using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing data were assembled using the ATGC software version 6.0.4 (GENETYX, Tokyo, Japan).

Results

Number of mosquitoes that fed *Spiroplasma*-containing artificial meals and survived

The mosquitoes were allowed to feed on artificial meals containing *Spiroplasma* or PBS in a feeding chamber for 60 min. Feeding success was visually assessed, and the mosquitoes with fully enlarged abdomen were considered fed. A total of 36–39 *Cx. p. pallens*, 30–33 *Ae. Aegypti*, and 14–21 *Ae. albopictus* individuals were confirmed to have consumed the artificial meals. Feeding rates of *Ae. albopictus* were lower than those of the other two species, with only 14 fed individuals in the *S. mirum*-inoculated group; both species of *Spiroplasma* were consumed at the same feeding rate (Table 2).

Ten days after feeding, the number of live mosquitoes was counted; 36–38, 17–20, and 18–20 individuals of *Cx. p. pallens*, *Ae. aegypti*, and *Ae. Albopictus*, respectively, were found to be alive. The survival rates of *Cx. p. pallens* were higher than those of the other

two species, and difference in the survival rates was not observed between the different artificial meals (Table 2).

Detection of *Spiroplasma* in mosquitoes and cultures

DNA was extracted from each mosquito (ten mosquitoes were selected from each infection group, and three mosquitoes were selected from the control group), and PCR and Sanger sequencing were performed targeting the 16S rDNA of *Spiroplasma.* *Spiroplasma* was detected only in the *S. ixodetis*-fed group from four, five, and five *Cx. p. pallens*, *Ae. Aegypti*, and *Ae. Albopictus* individuals, respectively (Table 3).

Spiroplasma re-isolation experiments were performed using ten mosquitoes from each infection group, and three mosquitoes from the control group. The mosquitoes used were the same as those used for the PCR-based detection of *Spiroplasma.* During the incubation period of nine weeks, 42 of the 69 wells were contaminated with fungi or bacteria, thus their medium was not processed further. DNA was extracted from the culture medium present in the remaining 27 wells and subjected to PCR and sequencing to confirm the re-isolation of *Spiroplasma*. Among the 13 wells that contained the homogenate of *S. ixodetis*-fed mosquitoes, one well each of the *Cx. p. pallens* and *Ae. albopictus* inoculation groups tested positive for *Spiroplasma*. All seven wells containing

240 the homogenate of *S. mirum*-fed mosquitoes tested negative for *Spiroplasma* (Table 3).

241

242 **Discussion**

243 In this study, we investigated the possibility of the transmission of *Spiroplasma*
244 between different arthropod taxa under experimental conditions. Previous studies on the
245 molecular phylogenetic analysis of *Spiroplasma* have suggested that the genus is
246 transmitted both vertically and horizontally, based on the phylogenetic divergence
247 between *Spiroplasma* and its hosts (Haselkorn et al. 2009, Ballinger and Perlman 2019,
248 Binetruy et al. 2019). The findings of this study revealed that *Spiroplasma* ingested by
249 non-host arthropod species can survive in the recipient hosts, which may support the
250 intraspecies transmission potential of *Spiroplasma* in nature.

251 In our study, two different species of *Spiroplasma* isolated from ticks were fed
252 to three different species of mosquitoes. *Spiroplasma ixodetis* DNA was detected in all
253 tested mosquito species, and the bacterium was successfully isolated from two mosquito
254 species using cell culture (Table 3), indicating that the *S. ixodetis* ingested via the artificial
255 meals survived in the mosquitoes. This finding is in accordance with the results of a
256 previous study, wherein *S. ixodetis* was isolated from field-collected ticks by co-culturing
257 with *Ae. albopictus* mosquito-derived cells (C6/36) (Thu et al. 2019). Another study

reported that *S. ixodetis* isolated from moths (*Homona magnanima*) could be cultured in mosquito (*Ae. albopictus*-derived NIAS-AeAl-2) and silkworm (*Bombyx mori*-derived aff3) cell lines (Arai et al. 2022), suggesting that *S. ixodetis* has a broad host range among arthropods. In contrast, *S. mirum* DNA was not detected in any mosquito species (Table 3). Although *S. mirum* has been detected in freshwater crustaceans, such as crabs, crayfish, and shrimp (Wang et al. 2005, Huang et al. 2008, Sánchez-Salgado et al. 2021), among terrestrial arthropods, it is only reported from ticks. Thus, *S. mirum* is not prevalent among terrestrial arthropods.

The fluid consumed by mosquitoes firstly reaches their midgut; the mosquito midgut has a midgut infection barrier that prevents pathogens from infecting and replicating in the mosquito midgut cells and acts as a wall that separates the body cavity from the internal midgut environment (Kumar et al. 2018). We hypothesized that *S. ixodetis* passed through the midgut membrane and spread to the haemocoel, whereas *S. mirum* did not. Several bacterial surface proteins have been associated with insect cell invasion in *Spiroplasma* (Labroussaa et al. 2010, Béven et al. 2012, Duret et al. 2014 Berho et al. 2016). Of note, spiralin is the most abundant surface lipoprotein of *S. citri* required for adhesion and entry into insect cells (Duret et al. 2014). It has been demonstrated that the genes encoding lipoproteins including spiralin are differentially

276 expressed in the different hosts of *S. citri* (Dubrana et al. 2016). Moreover, comparative
277 genome analysis of different *Spiroplasma* species revealed that the repertoires of the
278 genes encoding lipoproteins and their expression levels vary depending on the species
279 (Masson et al. 2018). Therefore, the absence of *S. mirum* in the mosquitoes 10 d after the
280 feeding may be attributed to a lack or deficiency of lipoproteins required for adhesion to
281 mosquito cells. The interactions among the midgut microbiota of arthropods have a
282 significant impact on vector competence (Zindel et al. 2011, Gao et al. 2020). For example,
283 *Spiroplasma* and *Wolbachia* are popular endosymbionts among arthropods, and they
284 compete with small brown planthoppers and mites (Yoshida et al. 2019, Yang et al. 2020).
285 This may partly explain the differential response to the *Spiroplasma* infection in
286 individual mosquitoes.

287 Transmission routes of *S. ixodetis* between different arthropod taxa in the
288 natural setting is unclear. Though the high abundance of *S. ixodestis* was observed in
289 salivary glands of ticks (Qiu et al., 2014), none of the studies revealed the presence of *S.*
290 *ixodetis* in the blood of tick-infested animals, implying that *S. ixodetis* may not be released
291 into the host blood during feeding at least with high concentration. The current study fed
292 mosquitoes with artificial meals containing *Spiroplasma* at a conc entration of 1×10^{12}
293 bacteria/mL, which is much higher concentration than those observed in *Drosophila*

294 (ranging from 2.8 x 10^7 to 4.0 x 10^8 copies per mg of fly) (Haselkorn et al. 2013).
295 Therefore, the present experimental conditions have little relevance to the actual
296 transmission system of *Spiroplasma* in nature. It is known that ectoparasitic mites can
297 facilitate interspecies transmission of *Spiroplasma* in *Drosophila* (Jaenike et al. 2007).
298 Similarly, parasitoid wasps are responsible for transmitting *Wolbachia* between
299 individuals in whiteflies (Ahmed et al. 2015). There are several parasitoid wasp species
300 that are known to infest ticks (Ramos et al. 2023). Such ectoparasites may have potential
301 to mediate the intraspecies transmission of *Spiroplasma* though the frequency of such
302 events in the natural setting should be much lower than that observed in the current study.
303 Another possible interpretation is that ticks are the dead-end hosts for *S. ixodetis* in nature.
304 In this scenario, *S. ixodetis* has been maintained in tick populations via vertical
305 transmission after the introduction from other arthropod taxa during the evolutionary
306 process and not been transferred from ticks to other arthropod taxa in nature despite
307 having the ability to survive in other arthropods.

308 Recently, several studies have suggested that *S. ixodetis* can cause various
309 diseases in humans. Two cases of *S. ixodetis* infection in immunocompromised
310 individuals have been reported in Sweden (Eimer et al. 2022). As both patients had a
311 history of frequent exposure to ticks before the manifestation of symptoms, the most

312 likely infection route was suspected to be through tick bites. Congenital cataracts and
313 concomitant intraocular inflammation have also been associated with *S. ixodetis* infection,
314 especially in newborn babies (Matet et al. 2020, Farassat et al. 2021). However, the
315 transmission route of *S. ixodetis* in infants is unknown, as they are unlikely to have come
316 contact with ticks. The results of this study reveal that *S. ixodetis* infection is transmitted
317 by ticks as well as other arthropods that could serve as a vector for or a source of
318 spiroplasmosis in anthropogenic environments. Further studies are needed to elucidate
319 the relationship between *Spiroplasma*, arthropods, and mammals, including humans, and
320 to understand the routes of infection in clinical spiroplasmosis.

321 This study has several limitations. First, the *Spiroplasma* transmission rate was
322 not calculated owing to technical difficulties and small sample size. For instance, the
323 amount of meal consumed could vary between individual mosquitoes; moreover, the
324 mosquitoes that did not feed on the *Spiroplasma*-containing meal could not be
325 distinguished from those that consumed the *Spiroplasma*-containing meal. These
326 drawbacks may have led to an underestimation of transmission efficacy. Second, we could
327 not investigate the localisation of the injected *Spiroplasma* in the mosquito body.
328 Understanding the dynamics of *Spiroplasma* is crucial for evaluating its transmission
329 potential to other host animals and vertical transmission to their offspring. Third, several

culture wells were contaminated with fungi during the experiment. This may have led to successful re-isolation of *Spiroplasma* only in a small number of wells.

In the present study, *S. ixodetis* isolated from ticks was transmitted to the following three mosquito species via an oral infection: *Cx. p. pallens*, *Ae. aegypti*, and *Ae. albopictus*. However, *S. mirum* infection was not observed in any mosquito species. Differences in the infection ability of mosquitoes could be attributed to several factors, including environmental effects. Nevertheless, this is the first experimental demonstration of *Spiroplasma* transmission among different arthropod taxa. Further studies are needed to elucidate the evolutionary mechanism behind the survival of *Spiroplasma* in nature.

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Author Contributions

Shohei Ogata: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing – original draft; Writing – review & editing, Naoki Hayashi: Investigation; Validation; Writing – review & editing, Yuki

Eshita: Conceptualization; Investigation; Methodology; Resources; Writing – review & editing, Yasuha Nagasawa: Investigation; Validation; Writing – review & editing, Nariaki Nonaka: Project administration; Supervision; Writing – review & editing – Preparation, Ryo Nakao: Conceptualization; Data curation; Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing – review & editing – Preparation

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649 **Tables**

650 **Table 1. *Spiroplasma* species reported from ticks.**

Spiroplasma species	Host tick species	Country	Reference
Spiroplasma ixodetis	*Amblyomma sculptum*	Brazil	Da Silva et al. 2022
	Dermacentor bellulus	Japan	Ogata et al. 2021
	Dermacentor marginatus	Hungary, France, Spain	Hornok et al. 2010, Binetruy et al. 2019, Palomar et al. 2019
	Dermacentor nitens	Brazil	Da Silva et al. 2022
	Dermacentor reticulatus	The Netherlands, Poland	Palomar et al. 2019
	Haemaphysalis kitaokai	Japan	Thu et al. 2019, Ogata et al. 2021
	Ixodes arboricola	Belgium	Van Oosten et al. 2018
	Ixodes frontalis	Belgium	Binetruy et al. 2019
	Ixodes monospinosus	Japan	Thu et al. 2019
	Ixodes ovatus	Japan	Ogata et al. 2021
	Ixodes pacificus	US	Tully et al. 1981, Tully et al. 1995
	Ixodes pavlovsky	Japan	Ogata et al. 2021
	Ixodes persulcatus	Russia, Japan	Thu et al. 2019, Beliavskaia et al. 2021, Ogata et al. 2021
	Ixodes ricinus	Slovakia, The Netherlands, UK	Bell-Sakyi et al. 2015, Olsthoorn et al.2021, Palomar et al. 2019
	Ixodes turdus	Japan	Ogata et al. 2021
	Ixodes uriae	Russia	Binetruy et al. 2019
	Ixodes sp.	Germany	Henning et al. 2006
	Rhipicephalus annulatus	Benin	Binetruy et al. 2019

	Rhipicephalus decoloratus	South Africa	Binetruy et al. 2019
	Rhipicephalus geigyi	Burkina Faso	Binetruy et al. 2019
	Rhipicephalus pusillus	France	Binetruy et al. 2019
Spiroplasma mirum	*Amblyomma testudinarium*	Japan	Ogata et al. 2021
	Haemaphysalis leporispalustris	US	Tully et al. 1982
	Ixodes persulcatus	Japan	Ogata et al. 2021
	Ixodes pavlovsky	Japan	Ogata et al. 2021

659 **Table 2. Number of mosquitoes that consumed *Spiroplasma*-containing meals and survived for 10 days after feeding.**

	Number of fed mosquitoes (Feeding rate)			Number of live mosquitoes (Survival rate)			Total number of mosquitoes used		
Inoculation group	*Cx. p. pallens**	*Ae. aegypti*†	*Ae. albopictus*‡	*Cx. p. pallens*	*Ae. aegypti*	*Ae. albopictus*	*Cx. p. pallens*	*Ae. aegypti*	*Ae. albopictus*
S. mirum§	38 (95%)	30 (75%)	14 (35%)	37 (93%)	17 (43%)	20 (50%)	40	40	40
S. ixodetis¶	36 (90%)	32 (80%)	16 (40%)	38 (95%)	18 (45%)	18 (45%)	40	40	40
PBS control	39 (98%)	33 (83%)	21 (53%)	36 (90%)	20 (50%)	18 (45%)	40	40	40

* *Culex pipiens pallens,* † *Aedes aegypti,* ‡ *Aedes albopictus,* § *Spiroplasma mirum,* ¶ *Spiroplasma ixodetis*

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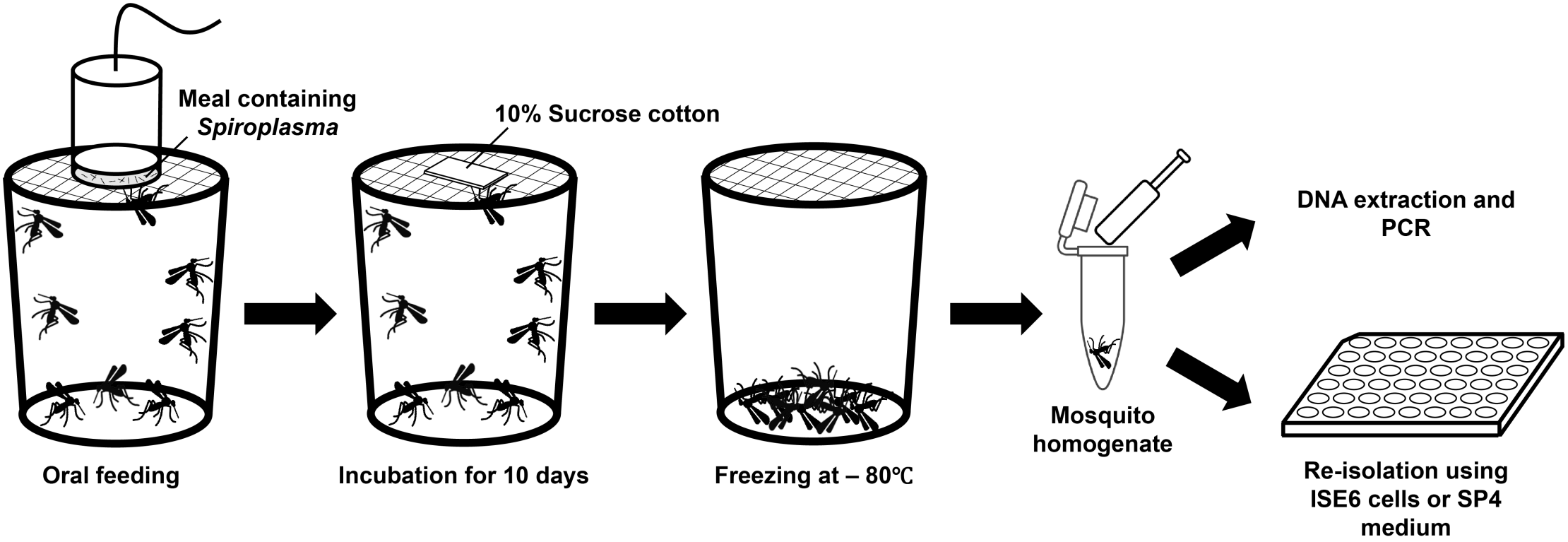
Table 3. Number of mosquitoes and wells tested for *Spiroplasma* infection.

	No. of mosquitoes positive for *Spiroplasma*/no. of mosquitoes tested			No. of wells positive for *Spiroplasma*/no. of wells without contamination		
Inoculation group	*Cx. p. pallens**	*Ae. aegypti*†	*Ae. albopictus*‡	*Cx. p. pallens*	*Ae. aegypti*	*Ae. albopictus*
S. mirum§	0/10	0/10	0/10	0/6	0/1	-/0
S. ixodetis¶	4/10	5/10	5/10	1/10	0/2	1/1
PBS control	0/3	0/3	0/3	0/3	0/1	-/0

- not applicable, * *Culex pipiens pallens*, † *Aedes aegypti*, ‡ *Aedes albopictus*, § *Spiroplasma mirum*, ¶ *Spiroplasma ixodetis*

Figures

Figure 1. Experimental workflow of *Spiroplasma* feeding and re-isolation from mosquitoes.


Meal containing
Spiroplasma
10% Sucrose cotton
Oral feeding
Incubation for 10 days
Freezing at – 80℃
Mosquito
homogenate
DNA extraction and
PCR
Re-isolation using
ISE6 cells or SP4
medium