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# Large-scale field sampling reveals Spiroplasma infection in Lymantria dispar japonica and Lymantria umbrosa in Hokkaido, Japan

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# Abstract

The spongy moth (*Lymantria dispar*) exhibits significant sex ratio distortion owing to interspecific hybridization with its closely related species, *Lymantria umbrosa*, in Hokkaido. This study investigated the involvement of sex-manipulating endosymbiotic bacteria by screening 268 egg masses collected from multiple sites in Hokkaido for infection with *Wolbachia* and *Spiroplasma*. PCR analysis amplified *Spiroplasma* 16S rRNA genes from 40 egg masses, and phylogenetic analyses revealed that the identified *Spiroplasma* belonged to the *Spiroplasma ixodetis* clade. Vertical transmission was confirmed in a subset of infected lineages, but no significant sex ratio distortion was observed in *L. umbrosa*-derived infected egg masses. Although one egg mass exclusively produced female adults, nuclear DNA polymorphism analysis and mitochondrial DNA haplotyping revealed that this lineage originated from backcrosses between *L. dispar japonica* females and *L. umbrosa* males. All-female broods were reproduced in experimentally generated *Spiroplasma*-negative backcrosses, indicating that the observed sex-ratio bias could be attributed to genetic effects of interspecific hybridization rather than to endosymbiont infection. These findings provide the first evidence of *Spiroplasma* infection in *L. dispar japonica* and *L. umbrosa* populations in Hokkaido, and indicate that infection with this bacterium does not necessarily induce male killing.


# 1. Introduction

Sex-manipulating endosymbiotic bacteria distort the sex ratio of the next generation by interfering with insect sex determination mechanisms. These endosymbiotic bacteria are vertically transmitted from mothers to their offspring, allowing them to be inherited across generations. Representative examples include *Wolbachia* and *Spiroplasma*. *Wolbachia* is a bacterium belonging to the class Alphaproteobacteria and is estimated to infect approximately 40% of terrestrial arthropod species (Zug and Hammerstein, 2012). It is an endosymbiotic bacterium that manipulates sex and reproduction in four ways: male killing, feminization, parthenogenesis, and cytoplasmic incompatibility. These infections have been confirmed in multiple lepidopteran species (Charlat et al., 2005; Arai et al., 2019). In *Wolbachia*, the male lethality factor *Oscar* has already been identified, targeting the male sex-determining gene *Masculinizer* (*Masc*) of *Ostrinia furnacalis* to induce male-specific death (Katsuma et al., 2022). Similarly, the endosymbiotic bacterium, *Spiroplasma*, causes male lethality in multiple lepidopteran species. *Spiroplasma poulsonii*, which infects *Drosophila*, expresses the male killing factor Spaid protein. It kills host males by targeting the male-specific lethal complex, which is essential for male gene dosage compensation and causes DNA damage (Harumoto and Lemaitre, 2018). The male killing *Spiroplasma* infecting *Homona magnanima* (*Hm*), a lepidopteran insect, does not encode the Spaid protein. It is thought to induce male killing by inducing abnormal *Hm doublesex* (*dsx*) expression and apoptosis in the host via a mechanism different from that in *S. poulsonii* (Arai et al., 2023). It has been revealed that endosymbiotic bacterial infection not only causes host sex ratio distortion but also has evolutionary and ecological impacts on the host, such as the acquisition of resistance mechanisms against reproductive manipulation, reduced effects from other viral infections, suppression of harmful host alleles, and provision of metabolic products (Hayashi et al., 2018; Arai et al., 2025).

The spongy moth, *Lymantria dispar*, is a lepidopteran insect belonging to the genus *Lymantria* within the family Lymantriidae. It is widely distributed across Asia, Europe, North Africa, the Americas, and Japan (Liebhold et al., 1989). The spongy moth is listed among the world's 100 worst invasive alien species by the International Union for Conservation of Nature (IUCN) and is a forest pest whose larvae hatch from spring to early summer, causing extensive defoliation (Lowe et al., 2000). Adult males are attracted to pheromones released by females and fly long distances. Furthermore, the subspecies *L. dispar asiatica*, which is widely distributed across Asia, China, and Korea, is considered more invasive because it includes populations in which females can fly long distances (Keena et al., 2014).

In Japan, spongy moths and closely related species have been found throughout the mainland (Higashiura and Kamijo, 1978; Matsuura and Nakajima, 2016; Jikumaru, 2008; Sawahata and Kochi, 2015). Major outbreaks have been reported in various regions and have occurred cyclically every few years for decades (Inoue et al., 2019). In recent years, satellite image-based prediction methods for *L. dispar* defoliation damage have been developed, with reports indicating that its severity often exceeds that caused by other animals, such as deer (Mori et al., 2024a; Mori et al., 2024b).

Many lepidopteran insects, including spongy moths, exhibit ZZ/ZW or ZZ/Z0 sex determination systems (Hof et al., 2025). In *L. dispar*, the male-determination gene *LdMasc* (*L. dispar Masc*), an ortholog of *Masc*, is located on the Z chromosome. The female-determination gene *Fet-W* (*female-expressed transcripts of the W chromosome*) is located on the W chromosome (Moronuki et al., 2025). *LdMasc* induces $Lddsx^{M}$ expression in males, whereas *Fet-W* induces expression of $Lddsx^{F}$ in females by suppressing *LdMasc* mRNA expression.

The genus *Lymantria* includes 31 species, and *L. dispar* is divided into three subspecies: *L. dispar dispar* (Europe), *L. dispar asiatica* (Asia), and *L. dispar japonica* (Japan) (Goldschmidt, 1940; Schintlmeister, 2004; Pogue and Schaefer, 2007; Kishida, 2011). Among these, 12 species and subspecies have been reported to inhabit Japan (Pogue and Schaefer, 2007; Kishida, 2011). Goldschmidt reported that crossing Hokkaido-type spongy moths with Honshu-type spongy moths in Japan produced an all-male population (Goldschmidt, 1934a, b). This phenomenon was also reported by Saito et al. (Saito, 1954; Clarke and Ford, 1980, 1982), who revealed that mating between females possessing the Hokkaido-type mitochondrial DNA (mtDNA) haplotype of the closely related species *L. umbrosa* (Pogue and Schaefer, 2007) and males possessing the Honshu-type mtDNA haplotype resulted in all-male broods. Furthermore, backcrossing females with the Honshu-type mtDNA haplotype and males with the Hokkaido-type mtDNA haplotype caused male-specific lethality, resulting in all-female broods (Higashiura et al., 1999; 2011).

It is known that sex ratio distortion in spongy moths occurs through interbreeding between populations inhabiting different regions, but it is possible that endosymbiotic bacterial infections may have some effect on the sex determination mechanism of spongy moths as in *O. furnacalis* and *H. magnanima*. In previous studies, *Wolbachia* infection was investigated in *L. dispar asiatica* from Western Siberia, and *Wolbachia* and *Spiroplasma* infections were investigated in *L. dispar* from Honshu and Hokkaido, and in

the closely related species *L. postalba*. However, except for two adult *L. dispar* individuals from Fukushima, no other endosymbiotic bacterial infections have been reported (Martemyaanov et al., 2014; Higashiura et al., 2011; Ilinsky et al., 2017). The two Fukushima adults were infected with a novel *Spiroplasma*, which was identified from its 16S rRNA sequence as belonging to the *Spiroplasma ixodetis* clade (Ilinsky et al., 2017). The closely related species *L. umbrosa* is also distributed in the northern and eastern parts of Hokkaido (Kishida, 2011). However, no studies have reported infections with endosymbiotic bacteria, such as *Wolbachia* or *Spiroplasma*, in *L. umbrosa*.

The objectives of this study were to investigate the presence of *Wolbachia* and *Spiroplasma* in natural populations of *L. dispar japonica* and *L. umbrosa* in Hokkaido, where hybridication causes sex ratio bias, and also evaluate its potential effects on host reproduction. To achieve these objectives, we collected egg masses of *L. dispar japonica* and *L. umbrosa* from Hokkaido on an unprecedented scale. As a result, egg masses infected with *Spiroplasma* were discovered at multiple locations in Hokkaido, derived from both *L. umbrosa* and *L. dispar japonica*. Molecular phylogenetic analysis indicated that the *Spiroplasma* identified in this study belongs to the *S. ixodetis* clade, which includes several male-killing *Spiroplasma* species. The effects of *Spiroplasma* on its hosts were examined by investigating the hatching rate of *Spiroplasma*-infected egg masses collected in the field, the sex ratio of adults, and bacterial density in the next generation. This study represents the first report of *Spiroplasma* infection in *L. umbrosa*.

# 2. Materials and Methods

Test Insects

To detect endosymbiotic bacteria, *L. dispar japonica* and *L. umbrosa* egg masses were collected from the following locations in Hokkaido and Honshu between 2024 and 2025 :Kitami (KIT), Kenbuchi (KB), Pippu (PP), Sapporo-1 (SP), Sapporo-2 (SP-G), Sapporo-3 (KG), Bibai (BB), Shibetsu (SB), Nayoro (NY), Bifuka (BF), Asahikawa (AS), Kamui (KM), Furano (FR), Biei-1 (BE-t), Biei-2 (BE-b), Yubari-1 (YU), Yubari-2 (YU-h), Mukawa (HB), Shimukappu (SM), Shimizu (OB), Memuro (MM), Nakasatsunai (NK), Nobechi (NOB), Nasushiobara (NAS), Fukushima (FUK), and Fukuoka (KS) (Fig. 1-A, B). Detailed information on collection sites is listed in Table S1. In this study, mtDNA haplotypes were identified using the methodology described by Arimoto and Iwaizumi (2014). We classified specimens inhabiting Hokkaido with mtDNA haplotypes identical to those of the Honshu species as *L. dispar japonica* and those with Hokkaido-specific mtDNA haplotypes as the closely related species *L. umbrosa*, treating them as two distinct species (Pogue and Schaefer, 2007). The collected egg masses were stored at 4°C in a refrigerator.

**(A)** Sites where egg masses were sampled in Hokkaido, Japan. Sapporo-1 (SP), Sapporo-2 (SP-G), Sapporo-3 (KG), Bibai (BB), Mikasa (MK), Bifuka (BF), Nayoro (NY), Shibetsu (SB), Asahikawa (AS), Kamui (KM), Biei-1 (BE-t), Biei-2 (BE-b), Furano (FR), Kenbuchi (KB), Pippu (PP), Kitami (KIT), Yubari-1 (YU), Yubari-2 (YU-h), Mukawa (HB), Shimukappu (SM), Shimizutyo (OB), Memuro (MM), Nakasatsunai (NK).

**(B)** Collection sites within Japan, excluding Hokkaido. Nobechi (NOB), Fukushima (FUK), Nasu (NAS), Kyushu (KS).

Rearing

F1 egg masses were collected by rearing and mating wild egg masses in the laboratory. Diapause breaking and rearing were performed using the same methods as described in the previous study (Moronuki et al., 2025). The photoperiod was set to 14 h of light/10 h of darkness. During summer, Wisteria (*Wisteria floribunda*) leaves were used as food, whereas Silk Mate (NOSAN) was used during winter. Emerged adults were placed on the day of emergence into mating nets (made using cylindrical laundry nets; 42 cm diameter × 54 cm length) and propagated through free mating.

Extraction of genomic DNA from eggs

Genomic DNA was extracted from eggs collected in the field by phenol-chloroform extraction and an alkaline method. The phenol-chloroform extraction method followed that of previous studies (Moronuki et al., 2025). Finally, DNA was dissolved in 30 µl of 1×TE (Nippon Gene) and stored at -30°C. For the alkaline method, 50 µl of 50 mM NaOH (Fujifilm Wako) was added to the sample tube, which was then homogenized and incubated at 95°C for 15 min. After incubation, 200 mM Tris-HCl (pH 8.0) (Nippon Gene) was added and vortexed to neutralize the mixture.

The sample conditions for DNA extraction were as follows. First-generation field eggs were collected in batches of 10 eggs from egg masses stored at 4°C for approximately one month after collection, and DNA was extracted collectively. Passaged eggs were collected in batches of five eggs from egg masses stored at room temperature for less than 1 month after laying, and DNA was extracted collectively. Pooled samples were used for PCR and quantitative PCR (qPCR) to detect infection and estimate bacterial density per egg mass.

Detection of genes derived from endosymbiotic bacteria

The presence or absence of infection by the endosymbiotic bacteria *Wolbachia* and *Spiroplasma* was determined by detecting *Wolbachia wsp, Spiroplasma* 16S rRNA, and *RpoB*, following previous studies (Zhou et al., 1998; Tabata et al., 2011; Ilinsky et al., 2017). The primers and PCR conditions used for the detection are listed in Tables S2 and S3.

Species Identification of Infected Egg Clusters

To determine whether egg masses infected with endosymbiotic bacteria originated from female parents of *L. dispar japonica* or its close relative, *L. umbrosa*, mtDNA haplotypes were examined using the PCR and Restriction Fragment Length Polymorphism (PCR-RFLP) method. The primers and experimental methods used for PCR-RFLP were based on those described in previous studies (Tables S2 and S3) (Bogdanowicz et al., 2000; Arimoto and Iwaizumi, 2014).

# 16S rRNA and sequences

16S rRNA and *RpoB* sequences

*Spiroplasma*-derived 16S rRNA and *RpoB* detected in the eggs of *L. umbrosa* in Nayoro (NY) were determined by TA cloning and sequencing. TA cloning was performed using the pGEM®-T Easy Vector System I (Promega) following the attached protocol. The cloned products were sequenced. Direct sequencing was also performed on the PCR products detected in eggs of *L. dispar japonica* collected in NY and Kamui (KM), where the infection rates were similarly high. Sequencing services were provided by Eurofins DNA Sequencing Service (https://eurofinsgenomics.jp/jp/service/dnasequence/order-start.aspx). The primers used for sequencing are listed in Tables S2 and S3.

Bacterial Density Quantification

The density of the endosymbiotic bacterial infection was quantified by qPCR using pooled samples as templates. The procedure was performed using TB Green Premix Ex Taq II (Takara Bio) and the Thermal Cycler Dice Real-Time System II (Takara Bio Inc.) following the manufacturer's recommendations. Based on previous studies, the *L. dispar cad* gene, an autosomal single-copy gene encoding *carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase*, was used as the reference (REF) gene. Bacterial density was determined by estimating the copy number of the target gene *RpoB* (TOI) (Belousova et al., 2019). The cycle threshold (CT) value of the TOI gene was normalized to the CT value of the REF gene using the Multiple RQ software (TaKaRa Bio, Inc.). For each sample, qPCR was performed on the REF and TOI gene in three replicates, and the relative expression levels were calculated. The primers used and qPCR conditions are listed in Tables S2 and S4, respectively.

Identification of the Nuclear DNA Polymorphism of *L. dispar japonica* and *L. umbrosa*

To distinguish between the nuclear DNA polymorphisms of *L. umbrosa* and *L. dispar japonica*, primers were designed to detect polymorphisms in the sequence upstream of *LdMasc* gene on the Z chromosome. In *L. umbrosa*, a 733 bp insertion sequence exists, resulting in an expected amplicon size of 1128 bp. In contrast, *L. dispar japonica* from Hokkaido lacked this insertion sequence and yielded an amplification product of 391 bp. Using pooled DNA samples extracted from 10 eggs per egg mass or DNA extracted from an egg as a template, we estimated the Z chromosome combinations of individuals originating from each egg mass. Egg masses were classified as hybrids if two distinct bands were detected or if the species inferred from mtDNA and nuclear polymorphism type did not match; all others were classified as pure egg masses. The primers used and PCR conditions are shown in Tables S2 and S4.

Phylogenetic and statistical analyses

Based on the nucleotide sequences of *Spiroplasma* 16S rRNA and *RpoB* registered in GenBank (GenBank accession numbers are shown in Fig. 3), alignment was performed using MAFFT (Katoh et al.,

2002), followed by phylogenetic tree construction. A phylogenetic tree was constructed using the maximum likelihood (ML) method in RAxML (Stamatakis, 2014), with 1000 bootstrap replicates.

Statistical differences in *Spiroplasma* bacterial density (*RpoB* copy number relative to *cad* copy number) between infected and uninfected samples, obtained by qPCR, were assessed using one-way ANOVA and the Wilcoxon test. The Games-Howell test was used for multiple comparisons. All analyses were performed using R ver. 4.4.1 (R Core Team, 2024).

# 3. Results

Detection of endosymbiotic bacteria in both species of *L. dispar japonica* and *L. umbrosa*

To date, there have been no reports of *Wolbachia* or *Spiroplasma* infections in *L. dispar japonica* or *L. umbrosa* in Hokkaido, Japan. Therefore, in this study, we first performed PCR targeting *Wolbachia wsp* gene and *Spiropalsma* 16S rRNA gene. No bands derived from *Wolbachia wsp* were detected in any samples. In contrast, PCR targeting the *Spiroplasma* 16S rRNA yielded an amplification product that matched the expected size (725 bp), as shown in Fig. 2A. In this study, these egg masses were considered infected (*Spiroplasma*-positive). As a result, among the 268 egg masses subjected to PCR analysis, 40 egg masses collected from KG, MK, AS, KM, NY, BF, FR, BE-t, BE-b, KB, PP, YU, MM, and NK were determined to be PCR positive (Table 1). The percentage of egg masses showing 16S rRNA amplification ranged from 3.4% to 62.5% across all sites, with the highest percentage observed in NK (Table 1).

To identify the species of the female parent that laid the infected egg mass, mtDNA haplotypes were identified using PCR-RFLP (Arimoto and Iwaizumi, 2014). Based on a previous study by Arimoto and Iwaizumi (2014), individuals whose PCR products were digested by restriction enzyme treatment with *Dra* I were considered *L. umbrosa*, whereas those whose products were not cleaved were considered *L. dispar. japonica* (Fig. 2B). Among the egg masses in which *Spiroplasma* 16S rRNA amplification was confirmed, those from KG, NY, KM, YU, and OB contained eggs derived from female *L. dispar japonica*, whereas the remaining egg masses were derived from female *L. umbrosa* (Table 1). These results support the possibility that both *L. dispar japonica* and *L. umbrosa* collected from multiple sites in Hokkaido were infected with *Spiroplasma*.

**(A)** Representative PCR results for 16S rRNA, *RpoB*, and *EF1α* detection. “C” in the figure indicates positive control (*Spiroplasma*-infected *Homona magnanima*). Specimens showing a clear band at the same position as the positive control were indicated as “P” (*Spiroplasma* positive), while those without a band were indicated as “N” (*Spiroplasma* negative). Small band indicated by a * was a non-specific amplification product arising from autosomal DNA. **(B)** An example of *Lymantria* species identification results using the PCR-RFLP method reported by Arimoto & Iwaizumi (2014). Lane numbers indicate separate egg masses. PCR products digested by *Dra* I were identified as *L. umbrosa* (lanes 1, 3, 5, 6, 7, 8), while those not digested were identified as *L. dispar japonica* (lanes 2, 4).

Table 1
Number of endosymbiotic bacterial-infected egg masses at various locations in Hokkaido

| Location | Egg masses surveyed | *Spiroplasma*-positive | Derived from *L. dispar japonica* | *Spiroplasma*-positivity rate (%) |
|---|---|---|---|---|
| SP | 8 | 0 | 0 | 0 |
| SP-G | 1 | 0 | 0 | 0 |
| KG | 8 | 2 | 2 | 0 |
| BB | 11 | 0 | 0 | 0 |
| MK | 18 | 1 | 0 | 5.6 |
| BF | 20 | 1 | 0 | 5 |
| NY | 20 | 2 | 1 | 10 |
| SB | 20 | 0 | 0 | 0 |
| AS | 20 | 2 | 0 | 10 |
| KM | 20 | 1 | 1 | 5 |
| BE-t | 40 | 2 | 0 | 5 |
| BE-b | 40 | 8 | 2 | 40 |
| FR | 20 | 1 | 0 | 5 |
| KB | 29 | 1 | 0 | 3.4 |
| PP | 38 | 5 | 0 | 13.1 |
| YU | 20 | 5 | 2 | 25 |
| YU-h | 3 | 0 | 0 | 0 |
| HB | 10 | 0 | 0 | 0 |
| SM | 16 | 0 | 0 | 0 |
| OB | 4 | 1 | 1 | 0 |
| MM | 20 | 3 | 1 | 15 |
| NK | 8 | 5 | 0 | 62.5 |

Molecular phylogenetic analysis of *Spiroplasma* identified in Hokkaido *L. dispar japonica* and *L. umbrosa*

Our findings suggest that *L. dispar japonica* and *L. umbrosa* inhabiting Hokkaido were infected with *Spiroplasma*. To analyze this point in greater detail, gene fragments were cloned, and their nucleotide sequences were determined for samples in which the target 16S rRNA band was detected by PCR. For

cloning, PCR products from *L. umbrosa* eggs collected in PP, where the infection frequency was relatively high, and from *L. dispar japonica* eggs collected in KM were used. Sequencing results for 29 clones revealed that the 16S rRNA gene fragment could be classified into two types (Type A and Type B), regardless of whether it was detected in eggs from *L. umbrosa* or *L. dispar japonica*. Type A appeared in 16 clones and was 1726 bp long. Type B appeared in 13 clones and was either 1726 or 1725 bp in length. Five nucleotide substitutions were observed between the two types. Phylogenetic analysis of these sequences revealed that they belonged to the *S. ixodetis* clade (Fig. 3A). Notably, it was closely related to *Spiroplasma* sp., which infects *Laodelphax striatellus*, *Antonina crawii*, and other lepidopteran species. The same clade also included *Spiroplasma* sp. infecting *Homona magnanima*, *Danaus chrysippus*, and *Ostrinia zaguliaevi*, and functioned as a male-killing *Spiroplasma* (marked * in Fig. 3). To further verify these results, PCR was performed using DNA extracted from the eggs of *L. umbrosa* and *L. dispar japonica* as templates and primers specific for *RpoB*. Amplification products matching the expected size (approximately 1443 bp) were detected in all samples, where 16S rRNA amplification was confirmed (Fig. 2A). The sequence length of the amplified product was 1354 bp, and the sequences of both PCR products derived from *L. umbrosa* and *L. dispar japonica* eggs were identical. Phylogenetic analysis using the *RpoB* sequence revealed that, similar to the 16S rRNA results, *Spiroplasma* infecting *L. umbrosa* and *L. dispar japonica* belonged to the *S. ixodetis* clade and were closely related to *Spiroplasma* sp. infecting *Ostrinia zaguliaevi* and *Drosophila ananassae* (Fig. 3B). As mentioned above, while two types of sequences were amplified in the 16S rRNA gene, no common substitution patterns were observed among clones in the *RpoB* gene. This suggests that the PCR results reflect the detection of multiple copies of the rRNA gene within a single *Spiroplasma* genome, rather than the outcome of multiple infections by different *Spiroplasma* strains.

Previous studies have shown that *Spiroplasma* detected in *L. dispar japonica* inhabiting Fukushima belongs to the *S. ixodetis* clade (Ilinsky et al., 2017). The region amplified for *Spiroplasma* 16S rRNA detection in that study (415 bp, GenBank [PP990208.1, PP990209.1]) was present within the 16S rRNA sequence of *Spiroplasma* identified here, and their nucleotide sequences matched. These results indicate that *Spiroplasma* sp., identical to the *Spiroplasma* previously infecting *L. dispar japonica* in Fukushima, also infects *L. dispar japonica* and *L. umbrosa* in Hokkaido. Furthermore, since the *Spiroplasma* identified in this study belongs to the *S. ixodetis* clade, it may possess male-killing ability.

Phylogenetic tree inferred from 16S rRNA and *RpoB* genes of *Spiroplasma* identified in *L. dispar japonica* and *L. umbrosa*. A tree was constructed using the maximum-likelihood (ML) method. Analysis using the neighbor-joining (NJ) method yielded essentially the same results. Bootstrap values obtained from 1000 re-samplings are shown at the nodes. Numbers in parentheses indicate GenBank accession numbers, and * denotes strains previously shown to kill males.

*Spiroplasma* bacterial density and transmission to the next generation

Many endosymbiotic bacteria, such as *Spiroplasma* and *Wolbachia*, infect the next generation of offspring via vertical transmission from infected female hosts. Therefore, we verified whether the

*Spiroplasma* identified in this study infected the next generation of offspring through vertical transmission from infected females. First, we quantified bacterial density in the field among first-generation individuals. Among egg masses determined to be infected by 16S rRNA PCR (confirmed amplification of 16S rRNA-derived DNA), three egg masses with sufficiently large sizes, NY#6 (derived from *L. dispar japonica*), NY#12 (derived from *L. umbrosa*), and KM#4 (derived from *L. dispar japonica*) (numbers indicate egg mass serial numbers), were analyzed. For each egg mass, DNA samples extracted from 10 randomly selected eggs were pooled and used as a template to quantify bacterial density using qPCR. The bacterial density in eggs from NY#12 was $2.93 \times 10^4$ times higher than that in eggs determined to be non-infected by PCR diagnosis (Fig. 4A). In contrast, the bacterial density in NY#6 was 78.2 times higher than that in non-infected individuals, and the bacterial density in KM#4 was $6.3 \times 10^2$ times higher. Next, the individuals that hatched from these three egg masses were reared until adulthood. The hatching rate of the NY#12 egg mass (derived from a female *L. umbrosa*) was 0.88 (Table 2). A total of 219 adults emerged, of whom 127 were females. The female-to-male adult ratio, calculated as the female population divided by the total population, was 0.58, indicating no significant sex ratio bias (Fig. 4B, Table 2). Mating the female adults with male adults derived from NY#12 and uninfected male adults resulted in 47 egg masses. Detection of *Spiroplasma* infection in these egg masses by 16S rRNA PCR revealed that 12 egg masses showed bands of the target size (= *Spiroplasma* positive), indicating an infection rate of 26% (Fig. 4C). Quantitative analysis by qPCR revealed that the bacterial density in these *Spiroplasma*-positive egg masses was $4.97 \times 10^3$ times higher than in egg masses determined to be non-infected (Fig. 4D, $p < 0.01$, Mann-Whitney U test). A similar analysis performed on NY#6 egg masses (derived from female *L. dispar japonica*) yielded a hatching rate of 0.27 (Table 2). A total of 52 emerged adults were obtained, all of whom were female (Fig. 4B). Mating these adult females with male *L. umbrosa* derived from non-infected eggs resulted in 11 egg masses (Fig. 4C). However, the 16S rRNA detection PCR failed to detect bands of the expected size in any egg mass. The larvae hatched from the KM#4 egg mass died due to a viral disease outbreak and could not be propagated.

These results suggest that male killing may have occurred in the *Spiroplasma*-infected egg mass NY#6. Furthermore, they indicated that *Spiroplasma* infecting *L. umbrosa* could be vertically transmitted to the next generation when bacterial density was high in the female parent.

**(A)** *Spiroplasma* bacterial density in first-generation field egg masses. KM#4, NY#12, NY#6 are egg masses determined *Spiroplasma*-positive by PCR; Negative shows data from egg masses determined *Spiroplasma*-negative (four egg masses). The X-axis indicates sample type, and the Y-axis shows the relative value of *Spiroplasma RpoB* relative to the host *cad*. Bar graphs represent the mean, and error bars indicate the standard error between replicates. Samples were tested using the Games-Howell method (*: $p < 0.05$). Note that the right panel shows only data for KM#4, NY#6, and Negative, where bacterial density was particularly low. **(B)** Number of adult females and males in NY#12 and NY#6. The Y-axis shows the number of individuals, color-coded by sex. **(C)** Number of *Spiroplasma*-infected egg masses of the next generation obtained by rearing. The X-axis shows the sample name of the female parent for each egg mass, and the Y-axis shows the number of egg masses. “N” means *Spiroplasma*

Negative, and “P” means *Spiroplasma* Positive. Data for KM#4 are not shown because no next generation was obtained. **(D)** Bacterial density in egg masses of the next generation derived from females of NY#12. “N” indicates *Spiroplasma* Negative, and “P” means *Spiroplasma* Positive. Five samples corresponding to N and 12 samples corresponding to P were tested. The X-axis indicates whether the sample was infected or not, and the Y-axis shows the relative value of *Spiroplasma RpoB* relative to the host *cad*. The Mann-Whitney U test was used to test for significant differences between the two groups. Error bars indicate the standard error between samples.

Table 2
Hatch rate and adult sex ratio in rearing experiments

| Egg mass | Hatched eggs | Unhatched eggs | Hatching rate | Emerged female adults | Emerged male adults | Sex ratio of adults (Females/Total) |
|---|---|---|---|---|---|---|
| NY#12 | 393 | 55 | 0.88 | 127 | 92 | 0.58 |
| NY#6 | 84 | 232 | 0.27 | 52 | 0 | 1.00 |
| KM#4 | 75 | 266 | 0.22 | N. A. | N. A. | N. A. |
| Backcross | 86 | 159 | 0.35 | 36 | 0 | 1.00 |
| Control | 142 | 208 | 0.41 | 28 | 15 | 0.65 |

N. A., Not acquired.

Combinations of sex chromosomes and mtDNA in all-female broods

Since all adults derived from the NY#6 egg mass were females, the *Spiroplasma* identified in this study was suggested to potentially induce male killing. However, no noticeable sex ratio bias was observed in the NY#12 egg mass, which showed a bacterial density of *Spiroplasma* dozens of times higher than the NY#6 egg mass (Fig. 4A, 4B). How can we explain these contradictions? Male embryonic lethality can occur in the second generation and beyond the hybrids produced by backcrossing *L. umbrosa* males with F1 hybrid individuals derived from *L. dispar japonica* females and *L. umbrosa* males (Higashiura et al., 1999; 2011). Therefore, to determine whether the NY#6 egg mass originated from backcrossing *L. dispar japonica* and *L. umbrosa*, PCR was performed to detect polymorphisms in the nuclear genes. Primers were used to detect polymorphisms in the sequence upstream of *LdMasc* on the Z chromosome (Fig. 5B). As expected, PCR using these primers confirmed that purebred *L. dispar japonica* produced a 391 bp band, purebred *L. umbrosa* produced a 1128 bp band, and hybrids of both species produced bands of both sizes (Fig. 5B). When the same PCR was performed using a pooled sample extracted from 10 eggs randomly selected from the NY#6 egg mass, only the *L. umbrosa*-type band (1128 bp) was detected (Fig. 5B). PCR-RFLP confirmed that NY#6 exhibits an *L. dispar japonica*-type mtDNA haplotype (Fig. 5B). These results clearly indicate that NY#6 was an egg mass laid by a backcrossing female *L. dispar japonica* and male *L. umbrosa*. Next, to verify whether male-specific lethality occurs in individuals

resulting from backcrossing between female *Spiroplasma*-infected *L. dispar japonica* and male *L. umbrosa*, *Spiroplasma*-negative backcross egg masses (Backcross) were produced by mating *Spiroplasma*-negative females derived from NY#6 with purebred male *L. umbrosa* (Fig. 5C). PCR-based sexing of 12 randomly selected eggs from the obtained egg masses and polymorphism identification by PCR confirmed that both sexes possessed the *L. umbrosa*-type polymorphism (Fig. 5C). When individuals hatched from the egg masses were reared, all emerged adults were female (Fig. 5D, Table 2). In contrast, in the *Spiroplasma*-negative *L. umbrosa*-derived egg masses used as controls (both mtDNA haplotype and Z chromosome were *L. umbrosa*-type), the female-to-male adult ratio was 0.65, showing no significant bias. These results indicated that egg masses produced by backcrossing *L. dispar japonica* and *L. umbrosa* exhibited a female-biased sex ratio, regardless of *Spiroplasma* infection status.

**(A)** Approximate positions of primers identifying polymorphisms on the Z chromosome of *L. dispar japonica* and *L. umbrosa.* **(B)** The upper panel shows PCR results using the primers indicated in (A). Amplified products for the *L. umbrosa* and *L. dispar. japonica* types are indicated by arrows. The lower panel shows mtDNA haplotype identification results by PCR-RFLP (*Dra* I digestion). a: purebred *L. dispar. japonica* propagated in the laboratory; b: purebred *L. umbrosa* propagated in the laboratory; c, d: hybrids produced by crossing both species; e: DNA from pooled sample (10 individuals) of NY#12; f: DNA from pooled sample (10 individuals) of NY#6. **(C)** Left panel: Schematic diagram of the backcross of female NY#6 and male *L. umbrosa*. The diagram shows mtDNA and sex chromosome combinations. Long blocks represent the Z chromosome; short blocks represent the W chromosome. Right panel: Results of PCR-based sexing (upper panel) and Z chromosome polymorphism identification PCR (lower panel). DNA extracted individually from 12 eggs derived from the Backcross egg mass was used as the PCR template. **(D)** Number of adult females and males in the Backcross and Control lines. The Y-axis shows the number of individuals, color-coded by sex.

# 4. Discussion

Currently, various bacterial taxa, including *Wolbachia* and *Spiroplasma*, have been reported to kill male insects and non-insect arthropods (Arai et al., 2025). The *Spiroplasma* strain discovered in this study, which infects *L. dispar japonica* and *L. umbrosa* in Hokkaido, is an endosymbiotic bacterium that was transmitted vertically within the genus *Lymantria*. This was closely related to the *S. ixodetis* strain infecting *Laodelphax striatellus*, *Antonina crawii*, and other lepidopteran species (Fig. 3A, 3B). Partial nucleotide sequences of the 16S rRNA gene from *Spiroplasma* identified in this study matched those reported for *Spiroplasma* isolated from *L. dispar japonica* in Fukushima in previous research. This suggests that the same *Spiroplasma* strain may have infected hosts across Hokkaido and Honshu, Japan.

Higashiura et al. previously reported that endosymbiotic bacterial infections were not confirmed in *L. dispar japonica* and *L. umbrosa* inhabiting Hokkaido (Higashiura et al., 2011). However, in the study conducted in Bibai, endosymbiotic bacterial infections were examined in only a few egg masses, comprising 25 larvae in total. In this study, we sampled over 20 sites, collected at least eight egg masses

per site, and examined 268 egg masses for infection (Table 1). Given the substantial variation in infection rates observed across our study sites, the sampling scale of the previous study appears insufficient to determine the presence or absence of endosymbiotic bacterial infection. Furthermore, more than 20 years have passed since sampling was conducted in a previous study, raising the possibility that the infection status of endosymbiotic bacteria in wild populations in Hokkaido has changed significantly (Miyata et al., 2024).

In Fukushima Prefecture, Honshu, *Spiroplasma*-infected individuals were found in two of 50 *L. dispar japonica* specimens collected in 2014–2015 (Ilinsky et al.,2017). However, this study used pupae, and it was unclear whether they originated from the same parent. If individuals from the same female parent were examined, the phenotypes would be expected to be identical. Consequently, the sample size would be smaller than expected, making accurate estimation of the infection rate difficult. Based on the above, an extensive sampling of a sufficient number of individuals across a wide range is essential for discussing the presence or absence of endosymbiotic bacterial infections.

*Spiroplasma* sp., identified in previous studies, are described solely by their 16S rRNA gene nucleotide sequences, with no mention of their effects on hosts (Ilinsky et al., 2017). In contrast, this study revealed that *Spiroplasma*, which shares the same 16S rRNA sequence as *Spiroplasma* sp. identified by Ilinsky et al. (2017), infects eggs derived from *L. dispar japonica* and *L. umbrosa* across a wide area of Hokkaido and is transmitted to the next generation. Although *Spiroplasma poulsonii* infecting *Drosophila* has been shown to specifically kill male hosts (Harumoto and Lemaitre, 2018), the causative factors for male killing in other male-killing *Spiroplasma* species (e.g., *S. ixodetis*) remain largely unknown (Arai et al., 2025).

The newly discovered *Spiroplasma* belongs to the *S. ixodetis* clade and is closely related to the known *H. magnanima*-infecting *Spiroplasma* (Hm) (Arai et al., 2022), for which male-killing research is well advanced. However, even in the egg mass with the highest bacterial density (NY#12) among the three field-derived egg masses selected for analysis, no tendency toward male killing was observed (Fig. 4A, 4B). Three possible reasons were identified. First, the density of the endosymbiotic bacteria may have been insufficient to induce male killing. In this study, bacterial density was quantified only for individuals from the three egg clusters; however, bacterial density differed significantly among the clusters. Previous studies on *Drosophila* have shown that temperature and the associated changes in bacterial density significantly influence the male-killing ability of endosymbiotic bacteria and their transmission to the next generation (Anbutsu et al., 2008; Osaka et al., 2008). The *Spiroplasma* bacterial density in NY#12 eggs was comparable to that observed in the early developmental stages of *Drosophila*, where male killing has been reported (Anbutsu and Fukatsu, 2008), yet the transmission rate to the next generation was low (Fig. 4C). This suggests that *Spiroplasma* may not have reached a bacterial density sufficient to kill males under the rearing conditions used in the present study.

Second, *Spiroplasma* infecting Hokkaido *L. dispar japonica* and *L. umbrosa* may not kill males. Among *Spiroplasma* species identified to date, only a small number have been shown to kill males (Fig. 3A, 3B)

(Arai et al., 2025). In addition to male killing, other phenotypes observed following *Spiroplasma* infection include nutrient supply to the host and enhanced host defense (Jaenike et al., 2010; Ballinger and Perlman, 2019). Newly discovered *Spiroplasma* species may also induce host phenotypes beyond male killing. However, because all adults emerging from the NY#6 egg mass discovered in this study were females, it appears that *Spiroplasma*-induced male killing was triggered. However, this egg mass was produced using the same mating system (backcrossing between *L. dispar japonica* and *L. umbrosa*) as the all-female broods described in a previous study (Fig. 5A, 5B) (Higashiura et al., 2011). Similarly, all adults derived from *Spiroplasma*-uninfected egg masses produced by the same backcrossing method in this study were female (Fig. 5C, 5D, Table 2). This suggests that if endosymbiotic bacteria infect individuals produced by backcrossing Hokkaido *L. dispar japonica* and *L. umbrosa*, the endosymbiotic bacteria can be efficiently transmitted to the next generation in a manner similar to the male-killing *Spiroplasma*, regardless of whether they possess male-killing ability. It is also possible that the *Spiroplasma* infecting *L. dispar japonica* lost its male-killing ability through co-evolution with its host.

Third, the host may have developed resistance to male killing, which has been shown to evolve over a relatively short period in multiple field studies (Hornett et al., 2014; Hayashi et al., 2018). Therefore, it is conceivable that male-killing resistance genes have already been acquired by *L. dispar japonica* and *L. umbrosa*, thereby masking the male-killing phenotype.

To investigate these possibilities, future work should include *Spiroplasma* infection experiments, cell culture studies, and genome analysis to identify phenotypic changes associated with bacterial density, as well as the genes and genomic structures underlying these phenotypes.

Goldschmidt (1934a, b), who studied sex determination in *L. dispar*, stated that the sex-determination factor exhibits regional variation in its ability to determine sex and that the sex of the next generation is determined by the balance of the sex-determination factor (Goldschmidt, 1934a, b). His findings were later replicated in several studies. Mating between individuals with different strengths of the sex-determination factor, as proposed by Goldschmidt, reproduced sex-ratio abnormalities, although this definition is not universally accepted (Saito, 1954; 1956; Clarke and Ford, 1980; 1982). Since endosymbiotic bacterial infections were not examined in these studies, the possibility that infection by sex-manipulating endosymbiotic bacteria influenced the results cannot be ruled out. However, as demonstrated in this study, endosymbiotic bacterial infection rates varied widely across regions, and bacterial density differed among egg masses. Therefore, it is unlikely that all individuals used for mating were infected with high-density endosymbiotic bacteria. Furthermore, the confirmation that all-female broods can arise even under low bacterial density conditions (Fig. 4A, 4B, Fig. 5C, Table 2) suggests the existence of the sex-determination factor and diversity proposed by Goldschmidt.

Multiple examples of resistance (suppressors) to sex manipulation by endosymbionts have been observed in organisms infected with sex-manipulating endosymbionts (Hornett et al., 2014; Hayashi et al., 2018). Although the mechanisms of resistance remain unclear in many cases, it has been shown in *Hypolimnas bolina* infected with male-killing *Wolbachia* that the host acquired a genomic region that

suppresses male killing (Hornett et al., 2014; Reynolds et al., 2019). Furthermore, male-killing by *Wolbachia* and *Spiroplasma* in lepidopterans targets sex-determination genes such as *Masc*, downstream genes in the sex-determination cascade, and gene-dosage compensation mechanisms. This suggests that the sex-determining pathway may diversify to acquire resistance (Katsuma et al., 2022; Arai et al., 2023; Arai et al., 2025). Based on the above, it is conceivable that in the genus *Lymantria*, suppressors of sex-manipulating endosymbiotic bacteria were acquired in the past, potentially generating regional variation in the strength of sex-determination factors. To verify this, it is necessary to elucidate the diversity in the strength of sex determination factors in *L. dispar japonica* and *L. umbrosa* at the molecular level and to investigate their relationship with the infection dynamics of endosymbiotic bacteria in the field.

# 5. Conclusions

This study revealed the presence of *Spiroplasma* infection in natural populations of *L. dispar japonica* and *L. umbrosa* in Hokkaido, Japan, based on a large-scale screening of 268 egg masses. Phylogenetic analyses showed that the detected bacteria belonged to the *Spiroplasma ixodetis* clade. This study represents the first report of *Spiroplasma* infection in *L. umbrosa*. Although no consistent male-killing phenotype was observed, bacteria were vertically transmitted to the next generation. On the other hand, female-biased broods were associated with hybrid backcross lineages between *L. dispar japonica* and *L. umbrosa*, suggesting that hybrid incompatibility rather than endosymbiont infection underlies the observed sex ratio distortion. These findings reveal previously unrecognized endosymbiotic bacterial infections in northern Japanese *Lymantria* populations and provide a basis for future studies on the interactions between endosymbionts and host sex-determination systems.

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The authors declare that they have no competing interests.

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# Figures

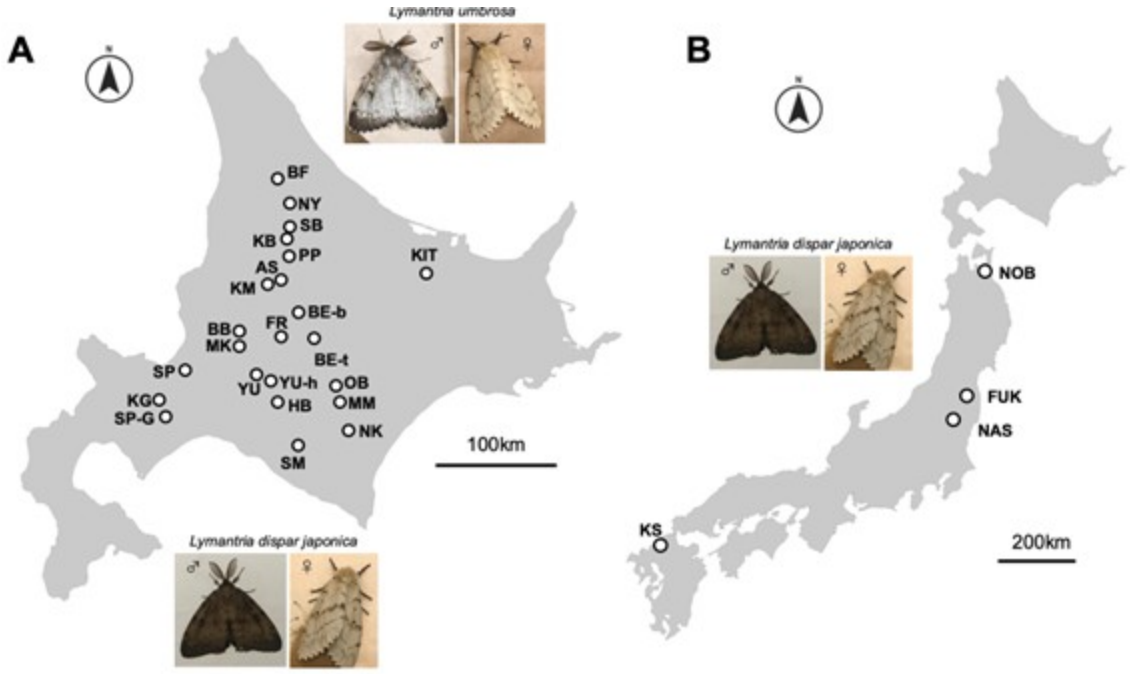
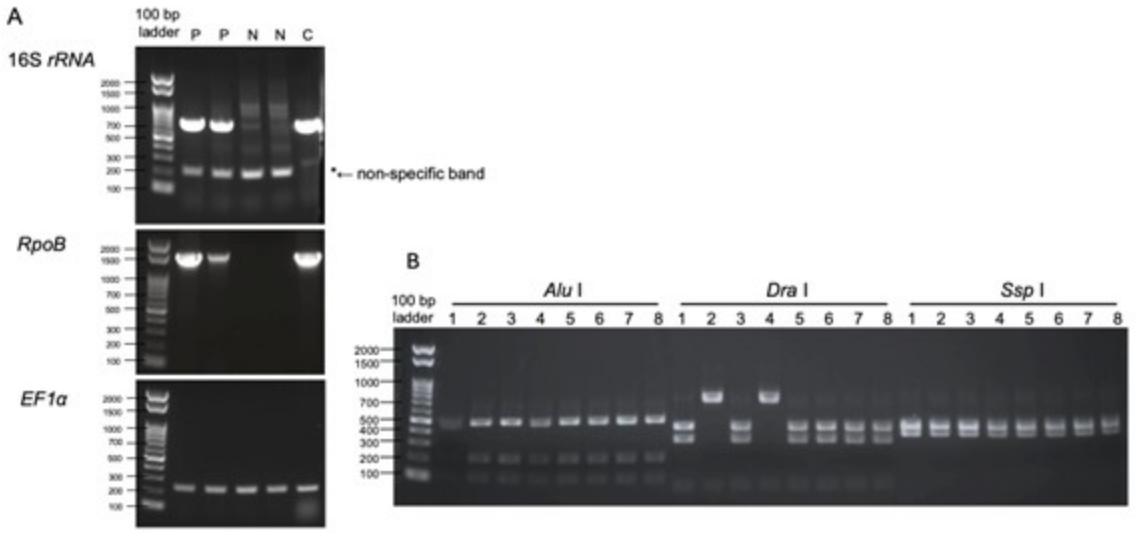



**Figure 1**

Egg mass collection sites

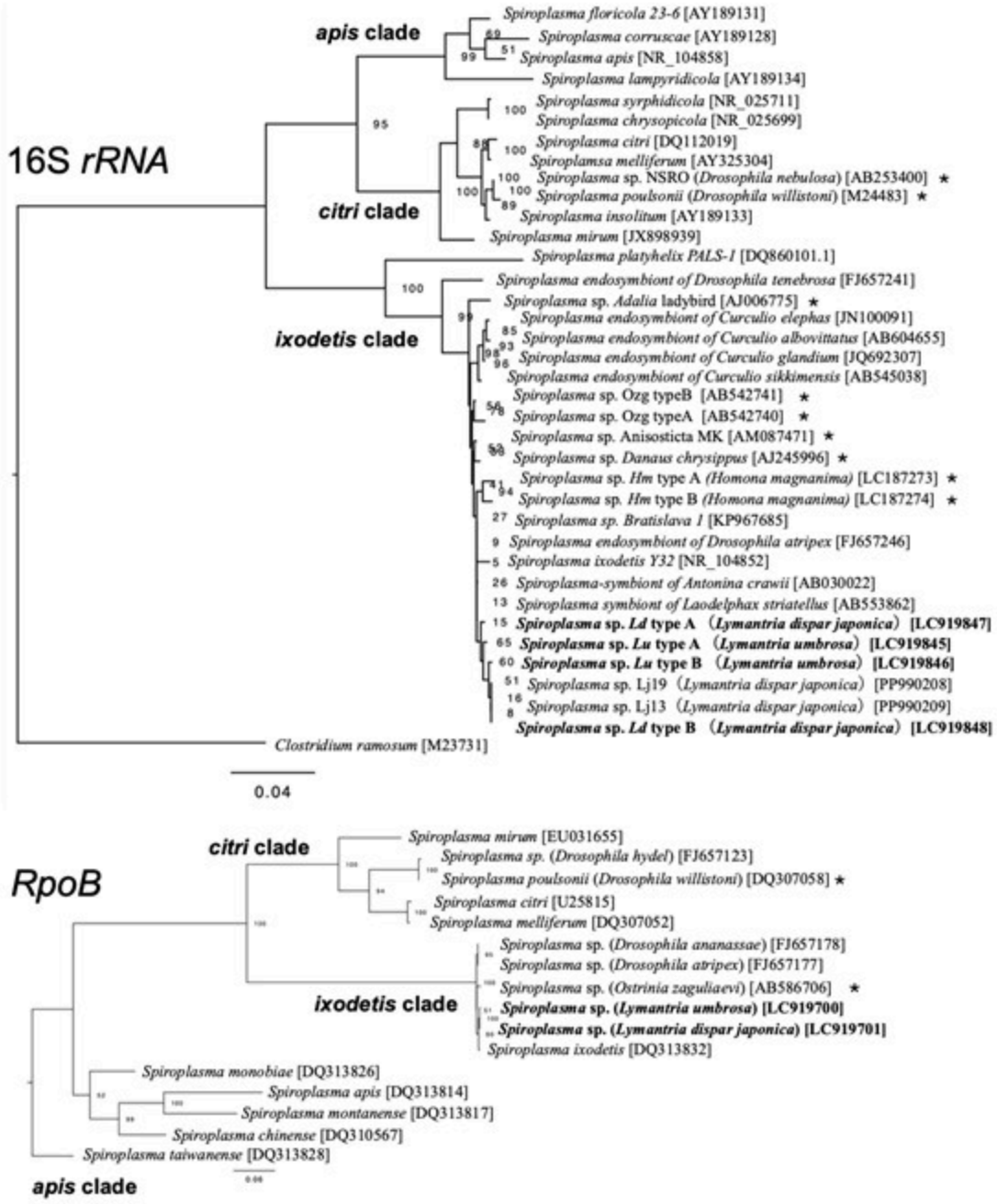
**(A)** Sites where egg masses were sampled in Hokkaido, Japan. Sapporo-1 (SP), Sapporo-2 (SP-G), Sapporo-3 (KG), Bibai (BB), Mikasa (MK), Bifuka (BF), Nayoro (NY), Shibetsu (SB), Asahikawa (AS), Kamui (KM), Biei-1 (BE-t), Biei-2 (BE-b), Furano (FR), Kenbuchi (KB), Pippu (PP), Kitami (KIT), Yubari-1 (YU), Yubari-2 (YU-h), Mukawa (HB), Shimukappu (SM), Shimizutyo (OB), Memuro (MM), Nakasatsunai (NK). **(B)** Collection sites within Japan, excluding Hokkaido. Nobechi (NOB), Fukushima (FUK), Nasu (NAS), Kyushu (KS).




## Figure 2

Identification of *Spiroplasma*-infected egg masses by PCR

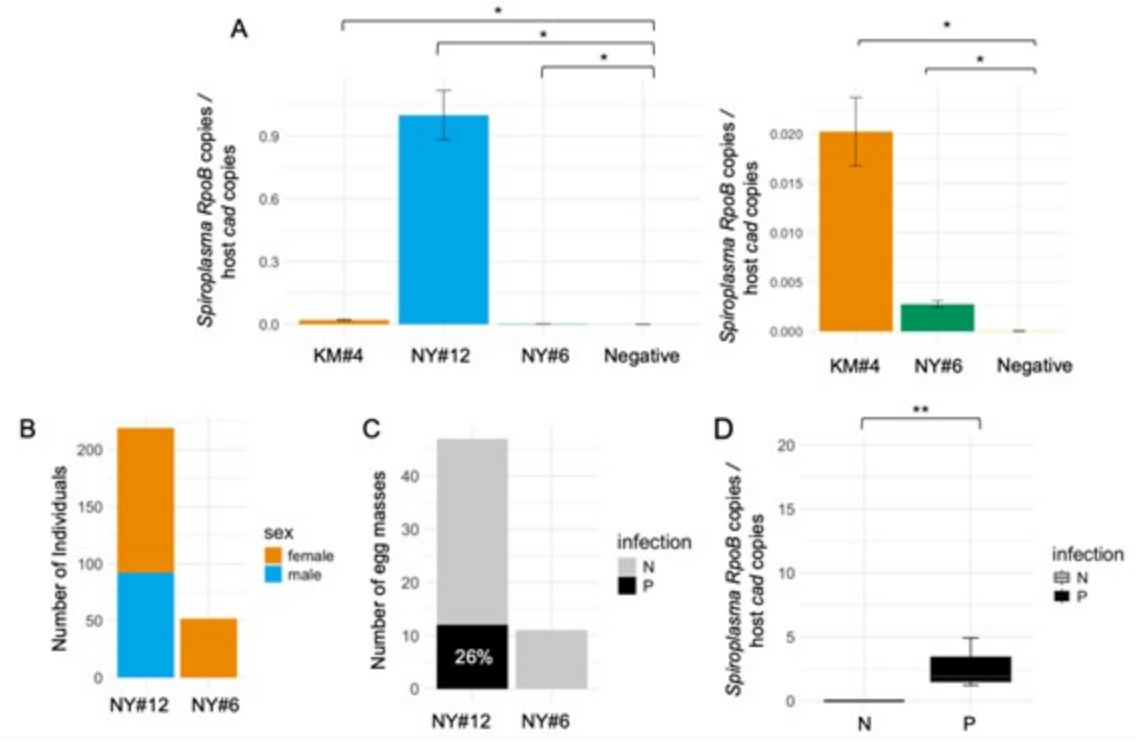
**(A)** Representative PCR results for 16S rRNA*, RpoB*, and *EF1α* detection. “C” in the figure indicates positive control (*Spiroplasma*-infected *Homona magnanima*). Specimens showing a clear band at the same position as the positive control were indicated as “P” (*Spiroplasma* positive), while those without a band were indicated as “N” (*Spiroplasma* negative). Small band indicated by a * was a non-specific amplification product arising from autosomal DNA. **(B)**An example of *Lymantria* species identification results using the PCR-RFLP method reported by Arimoto & Iwaizumi (2014). Lane numbers indicate separate egg masses. PCR products digested by *Dra* I were identified as *L. umbrosa* (lanes 1, 3, 5, 6, 7, 8), while those not digested were identified as *L. dispar japonica* (lanes 2, 4).




## Figure 3

Phylogenetic analysis based on *Spiroplasma* 16S rRNA and *RpoB* gene nucleotide sequences

Phylogenetic tree inferred from 16S rRNA and *RpoB* genes of *Spiroplasma* identified in *L. dispar japonica* and *L. umbrosa*. A tree was constructed using the maximum-likelihood (ML) method. Analysis using the neighbor-joining (NJ) method yielded essentially the same results. Bootstrap values obtained from 1000 re-samplings are shown at the nodes. Numbers in parentheses indicate GenBank accession numbers, and * denotes strains previously shown to kill males.




## Figure 4

*Spiroplasma* bacterial density in *Spiroplasma*-positive egg masses, adult sex ratio, and infection efficiency

**(A)** *Spiroplasma*bacterial density in first-generation field egg masses. KM#4, NY#12, NY#6 are egg masses determined *Spiroplasma*-positive by PCR; Negative shows data from egg masses determined *Spiroplasma*-negative (four egg masses). The X-axis indicates sample type, and the Y-axis shows the relative value of *Spiroplasma RpoB* relative to the host *cad*. Bar graphs represent the mean, and error bars indicate the standard error between replicates. Samples were tested using the Games-Howell method (*: $p < 0.05$). Note that the right panel shows only data for KM#4, NY#6, and Negative, where bacterial density was particularly low. **(B)** Number of adult females and males in NY#12 and NY#6. The Y-axis shows the number of individuals, color-coded by sex. **(C)**Number of *Spiroplasma*-infected egg masses of the next generation obtained by rearing. The X-axis shows the sample name of the female parent for each egg mass, and the Y-axis shows the number of egg masses. “N” means *Spiroplasma*Negative, and “P” means *Spiroplasma* Positive. Data for KM#4 are not shown because no next generation was obtained. **(D)** Bacterial density in egg masses of the next generation derived from females of NY#12. “N” indicates *Spiroplasma*Negative, and “P” means *Spiroplasma* Positive. Five samples corresponding to N and 12 samples corresponding to P were tested. The X-axis indicates whether the sample was infected or not, and the Y-axis shows the relative value of *Spiroplasma RpoB* relative to the host *cad*. The Mann-Whitney U test was used to test for significant differences between the two groups. Error bars indicate the standard error between samples.

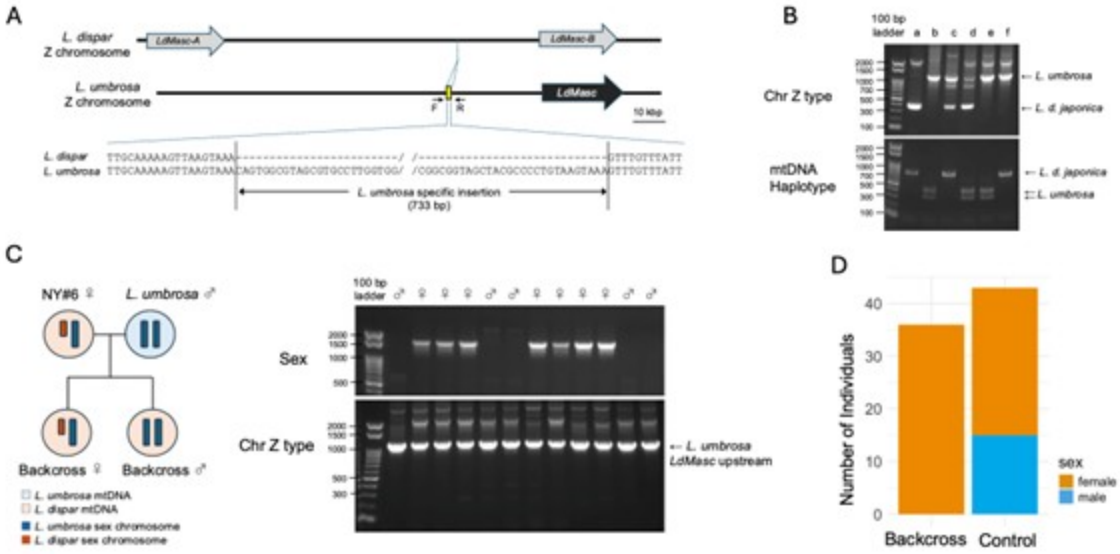



**Figure 5**

Sex ratio bias in adults from egg masses obtained by backcrossing

**(A)** Approximate positions of primers identifying polymorphisms on the Z chromosome of *L. dispar japonica* and *L. umbrosa.* **(B)** The upper panel shows PCR results using the primers indicated in (A). Amplified products for the *L. umbrosa* and *L. dispar. japonica* types are indicated by arrows. The lower panel shows mtDNA haplotype identification results by PCR-RFLP (*Dra* I digestion). a: purebred *L. dispar. japonica* propagated in the laboratory; b: purebred *L. umbrosa* propagated in the laboratory; c, d: hybrids produced by crossing both species; e: DNA from pooled sample (10 individuals) of NY#12; f: DNA from pooled sample (10 individuals) of NY#6. **(C)**Left panel: Schematic diagram of the backcross of female NY#6 and male *L. umbrosa*. The diagram shows mtDNA and sex chromosome combinations. Long blocks represent the Z chromosome; short blocks represent the W chromosome. Right panel: Results of PCR-based sexing (upper panel) and Z chromosome polymorphism identification PCR (lower panel). DNA extracted individually from 12 eggs derived from the Backcross egg mass was used as the PCR template. **(D)**Number of adult females and males in the Backcross and Control lines. The Y-axis shows the number of individuals, color-coded by sex.

# Supplementary Files

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