

Research Article

Wolbachia-like bacteria represent major endosymbionts of *Leptotrombidium* (Acari: Trombiculidae) mites in various areas of Japan

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Mites of the *Leptotrombidium* genus (Acari: Trombiculidae) are important from a public health perspective because certain species can transmit *Orientia tsutsugamushi*, which is the causative agent of scrub typhus. In this study, we evaluated the endosymbiotic bacterial diversity of predominant *Leptotrombidium* mites in certain regions of Japan via next-generation sequencing of 16S rRNA gene amplicons. Members of the genus *Wolbachia* were the most common bacteria found in all surveyed areas of this study and were even found in different species of mites, *Leptotrombidium scutellare* and *Leptotrombidium deliense*. Full-length 16S rRNA sequences were used to cluster two genetically similar groups of *Wolbachia*-like endosymbionts. Phylogenetic analysis using the 16S rRNA sequences showed that the two *Wolbachia*-like endosymbiont groups were classified into a monophyletic cluster, which was phylogenetically distant from a cluster of all previously reported strains. Based on the results, the *Wolbachia*-like endosymbionts were suggested to have diverged from the cluster of previously identified *Wolbachia* a long time ago, even before *L. scutellare* and *L. deliense* were separated. The abundance of *Wolbachia* spp. in the host mites elicits academic interest in their role in the host mites. We speculated that the endosymbionts may enhance the reproduction of host mites.

Keywords: *Leptotrombidium* mites, Microbiome, 16S rRNA, *Wolbachia*, Endosymbionts, Scrub typhus, *Orientia tsutsugamushi*

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INTRODUCTION

Leptotrombidium is a genus of mites in the family Trombiculidae. *Leptotrombidium* mites are important from a public health perspective because certain species can transmit *Orientia tsutsugamushi* (Tamura et al., 1995), an obligate intracellular bacterium, which is the causative agent of scrub typhus. The larval mites feed on rodents and other animals including humans, whereas nymphs and adults mites feed on eggs of other insects. When uninfected larval mites acquire *O. tsutsugamushi* from infected rodents, the bacteria are retained until the adult stage via transstadial transmission; however they are not transmitted from female adults to progeny via transovarial transmission (Takahashi et al., 1988). Therefore, uninfected mites are not important as a reservoir of *O. tsutsugamushi*; however, they are essential for completing the life cycle of mites. *O. tsutsugamushi* is transmitted from generation to generation via transstadial and transovarial transmission (Takahashi et al., 1988). Therefore, only the infected mites which carry *O. tsutsugamushi* are important for transmitting scrub typhus to humans. Regarding this aspect of *Leptotrombidium* mites, the other bacterial endosymbionts of mites have not been extensively studied. We recently found that *Leptotrombidium scutellare* mites collected in Kagoshima prefecture, Kyushu island in the southern region of Japan, harbor certain obligate intracellular bacteria other than *O. tsutsugamushi* (Ogawa et al., 2020). These endosymbiont bacteria have been found to be most similar to *Wolbachia* spp., *Rickettsia* spp., and *Rickettsiella* spp. Certain species of the genus *Wolbachia* have been shown to exert various effects on their hosts including feminization, male-killing, and cytoplasmic incompatibility (Werren et al., 2008). However, the detected *Wolbachia*-like endosymbiont has been classified in a different cluster from all *Wolbachia* spp. described in the database, so it was not possible to determine the role of the novel *Wolbachia*-like endosymbiont. One of the detected *Rickettsia* sp. was identical to the human pathogen, *Rickettsia akari* (Akram et al., 2021) based on comparison of the short-sequence 16S rDNA. The detected *Rickettsiella* spp. are found as endosymbionts of many ticks and insects (Leclercq, 2008). However, the distribution of these novel bacterial endosymbionts in *L. scutellare* mites in different regions in Japan, and in different species of *Leptotrombidium* mites are unknown. Therefore, we investigated the diversity of the obligate intracellular bacteria carried by *L. scutellare* mites collected from Fukui and Saitama prefectures in Honshu, central Japan, and also in a different mite, *Leptotrombidium deliense* collected from Okinawa prefecture, southern Japan; the microbiome was analyzed via next-generation sequencing of 16S rRNA gene amplicons [1].

MATERIALS AND METHODS

Sample collection and DNA extraction Larval trombiculid mites were collected from wild rodents in each survey area as described previously (Takahashi et al., 2004; Takahashi et al., 1988). The survey areas in this study are shown in (Figure 1).

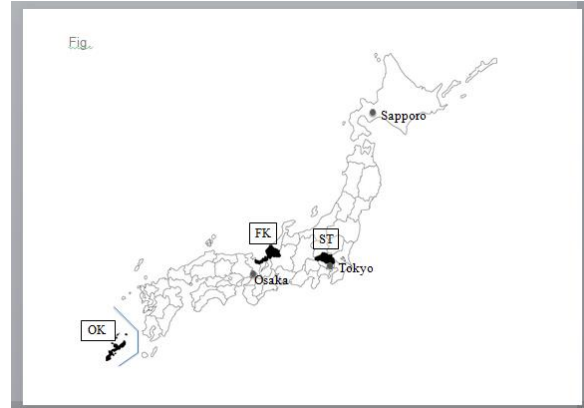


Figure 1: Regions of the collection sites of mites in this study. OK, Okinawa prefecture; FK, Fukui prefecture; ST, Saitama prefecture.

Scrub typhus was prevalent in Fukui and Okinawa prefectures, but not in Saitama prefecture. The collected engorged larval trombiculid mites were morphologically identified using a stereomicroscope. Numbers and species of the mites collected from each region are listed. For DNA extraction, the collected mites were disinfected using a hypochlorous acid solution for at least 15 min, and then washed twice with phosphate-buffered saline. DNA was extracted from the disinfected mites as described previously (Ogawa et al., 2020). [2] Sequence analysis of 16S rRNA amplicons and microbiome analysis The diversity of obligate intracellular bacteria in the mites was investigated using Illumina sequencing of 16S rRNA V3-V4 region amplicons as described previously (Ogawa et al., 2020). Briefly, the V3-V4 region of the 16S rRNA was amplified via polymerase chain reaction (PCR) using the specific primers 341F and 806R, and then the PCR amplicons were applied to an Illumina MiSeq platform to obtain 300 bp paired-end sequence reads (Illumina K.K. Tokyo, Japan). A taxonomic bar-plot was generated using the paired-end sequences as described previously. Sequence analysis of full-length 16S rRNA. To classify the detected major endosymbiotic bacteria, almost full-length 16S rRNA genes were amplified via PCR using the universal forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-GNTACCTTGTTACGACTT-3') (Fredriksson et al., 2013) using EX Taq HS polymerase (Takara Bio Inc., Shiga, Japan). Cycling conditions were as follows: denaturation at 94 °C for 1 s

followed by 30 cycles of amplification (denaturation at 94 °C for 5 s, annealing at 55 °C for 1 s, extension at 68 °C for 40 s) and a final extension at 68 °C for 1 min. The PCR amplicons whose sizes were approximately 1,500 bp were sent to Novogen (Novogene Japan K.K., Tokyo, Japan) for sequencing using PacBio long read technology with PacBio Sequel (Wagner et al., 2016). Sequences with a sampling depth of less than 10 reads were excluded. Homology search and phylogenetic analysis. The obtained full-length sequences of 16S rRNA were analyzed using the Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the entire database for further classification. The 16S rRNA sequences were also subjected to phylogenetic analysis using MEGA6 software (Tamura et al., 2013). Phylogenetic trees were constructed using the neighbour-joining method with bootstrap analysis of 1,000 pseudo-replicates. A bootstrap value $\geq 70\%$ was considered as the threshold for good confidence as described previously (Hillis and Bull, 1993). In the analysis, six major strains of *O. tsutsugamushi* were used as an outer group [3].

RESULTS

Comparison of bacterial diversity of larval trombiculid mites in different regions of Japan via microbiome analysis. We collected *L. scutellare* mites from Fukui and Saitama prefectures and *L. deliense* mites from Okinawa prefecture. Three samples of mites from Fukui prefecture, one sample of Saitama prefecture, and two samples of Okinawa prefecture were analyzed for determining the diversity of obligate intracellular bacteria in the microbiome. After processing the sequence reads to exclude any sequences of non-obligate cellular bacteria, a taxonomic bar-plot was generated (Figure 2).

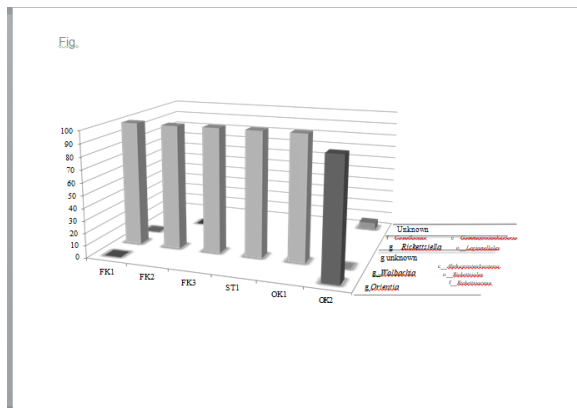


Figure 2: Microbiome analysis of *Leptotrombidium scutellare* (Acari: Trombiculidae) mites. The diversity of obligate intracellular bacteria was evaluated via 16S rRNA amplicon sequencing using an Illumina MiSeq platform. The results are

presented at the genus level. g, genus; f, family; o, order; c, class; OK1, Okinawa prefecture sample 1; FK1, Fukui prefecture sample 1; ST1, Saitama prefecture sample 1.

The genus *Wolbachia* was found in all samples of the three prefectures, even though the three prefectures are geographically separated. Okinawa prefecture is separated by the Pacific Ocean from the Japanese main islands. Furthermore, the types of mites differ among the prefectures; *L. deliense* in Okinawa prefecture and *L. scutellare* in Fukui and Saitama prefectures. Members of the genus *Orientia* were found both in Fukui and Okinawa prefectures where scrub typhus is currently endemic. Notably, in the OK2 sample, bacteria of the genus *Orientia* were dominant whereas the population of *Wolbachia* was scarce. In contrast, *Wolbachia* was the only genus detected in sample OK1, in which no member of *Orientia* was present. Two samples of the same species of mite of the same region displayed different diversities in symbiotic bacteria [4].

The genera *Rickettsia* and *Wolbachia* belong to class Alphaproteobacteria (order, Rickettsiales; family, Rickettsiaceae). Members of the genus *Rickettsiella* belonging to the class Gammaproteobacteria (order, Legionellales; family, Coxiellaceae) were found only in sample FK2. Few unclassified bacteria were found in samples FK2 and OK2. Analysis of regional differences and phylogeny of the detected bacteria via full-length sequencing of 16S rRNA. The full-length 16S rRNA sequences were obtained to classify the detected bacteria more precisely. We obtained the full-length sequences of 16S rRNA of all *Wolbachia* bacteria except for those in samples FK1 and OK1 due to sample shortage. However, the sequences of the minor populations of other bacteria were not obtained. The members of *Wolbachia* were separated into two closely related groups: *Wolbachia_like_endosymbiont_01* and *Wolbachia_like_endosymbiont_02*.

The sequences of both groups were similar to that of an unclassified arthropod-derived *Wolbachia* endosymbiont, although the identity rates were not high (92.5). *Wolbachia_like_endosymbiont_01* members were found in *L. scutellare* mites obtained from Fukui and Saitama prefectures, whereas *Wolbachia_like_endosymbiont_02* members were found in *L. deliense* mites obtained from Okinawa prefecture and also in *L. scutellare* mites from Saitama prefecture. In *L. scutellare* mites from Saitama prefecture, the population of the *Wolbachia_like_endosymbiont_02* group was approximately ten-fold higher than that of the *Wolbachia_like_endosymbiont_01* group. Phylogenetic analysis using the sequences of *Wolbachia_like_endosymbiont_01* and *02*, and those of certain bacteria of *Wolbachia* which were obtained from the BLAST search results and classified into supergroups in a previous study were used (Ros et al.,

2009). The phylogenetic tree branched into two monophyletic clusters, W1 and W2 (Figure. 3).

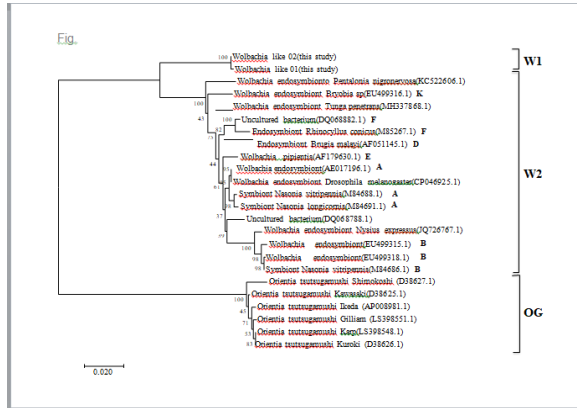


Figure. 3: Phylogenetic analysis of the bacteria detected in this study. Phylogenetic trees were constructed using full-length 16S rRNA sequences via the neighbour-joining. Only known supergroups of Wolbachia described previously (Ros et al., 2009) are shown. OG: an outer group.

All the strains whose sequences were acquired from the database were clustered in W2, whereas the two endosymbiont groups obtained in the present study were clustered in W1. The genetic distance between clusters W1 and W2 was large; therefore, W2 may have diverged from the W2 lineage much earlier than the formation of all the supergroups in W2 [5].

DISCUSSION

In this study, we evaluated the diversity in endosymbiotic bacteria of major mites in certain regions in Japan via microbiome analysis. Members of the Wolbachia genus were the most common bacteria present in all surveyed areas of this study and found even in different species of mites, *L. scutellare* and *L. deliense*. Full-length 16S rRNA sequences were used to classify the members into two groups of Wolbachia-like endosymbionts which were closely related. The group Wolbachia-like endosymbiont_01 has been identically found in *L. scutellare* mites collected from Kagoshima prefecture in our previous study (Ogawa et al., 2020). Phylogenetic analysis using the full-length 16S rRNA sequences showed that the two groups of Wolbachia-like endosymbionts obtained in this study were classified into a monophyletic cluster, which was genetically distant from the cluster of all previously reported strains. Based on these results, the Wolbachia-like endosymbionts isolated in this study were suggested to have diverged from the cluster of previously identified Wolbachia a long time ago, even before

L. scutellare and *L. deliense* were separated. *Orientia* spp. were detected in mites isolated from areas where scrub typhus was currently endemic. Notably, the opposite trend in the proportion of the bacteria of Wolbachia and *Orientia* between samples in the same region indicates that members of each genus may compete in the same host. Both symbiotic bacteria may be maintained in individual lineages of the same host mite for a long period. The population of *Orientia* spp. in mites is generally not high (Kawamura et al., 1995). However, the reasons for why the proportion does not change and the genus Wolbachia remains dominant are not known; it is also not known why the genus *Orientia* is not extinct. These questions may be answered by elucidating the relationship between symbiotic bacteria of the genus Wolbachia and their host mites.

Our interest in the abundance of Wolbachia spp. in host mites elicits speculative curiosity about their role in the host mites. Spider mites (*Bryobia* spp.) carrying symbiotic bacteria of the genus Wolbachia belonging to the supergroup K (Fig. 3) were genetically related the most to the Wolbachia-like endosymbionts identified in this study. The endosymbiotic Wolbachia in spider mites belong to the same family (*Tetranychus* spp.); they induce cytoplasmic incompatibility in their hosts, and then improve the reproductive performance of their host (Bing et al., 2020; Zhu et al., 2012). The Wolbachia-like endosymbionts obtained in this study may play similar roles in host mites to enhance reproductive performance and then expand their population among the host mites. Based on these results, the role of the Wolbachia-like endosymbionts may be associated with the control of scrub typhus whose vectors are *Leptotrombidium* mites. In fact, regulation of host reproduction via Wolbachia using several strategies, such as cytoplasmic incompatibility, has been applied in pest population control (Bourtzis, 2008; Zabalou et al., 2004). *Rickettsiella* spp. were found in Saitama prefecture and in the geographically separated region, Kagoshima prefecture, as described before (Ogawa et al., 2020). Members of *Rickettsiella* including certain human pathogens were not found in any samples in this study; however they were found in the mites obtained from Kagoshima prefecture in the previous study. Therefore, the regional differences associated with endosymbiont diversity should be investigated in future studies.

CONCLUSIONS

The Wolbachia-like endosymbionts detected in this study may be classified into a novel taxon based on the 16S rRNA sequences. The isolation and culturing of the Wolbachia-like endosymbionts should be performed for further classification; however, isolation of intracellular bacteria is generally very difficult. Furthermore, the detected Wolbachia spp. were dominant in the host mites which elicits academic interest regarding their

role in the host mites. Sequencing the whole genomes of the bacteria via large-scale sequencing using a next-generation sequencer should be performed to clarify more precise characteristics including the roles of these bacteria in the host mites.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

MO primarily designed the study and performed most of the analyses. MT, SN, and NT mainly assisted with mite collection and classification. MM primarily assisted with genetic analysis. MT helped write the manuscript.

REFERENCES

1. Akram SM, Radia T J, William GG (2021). "Rickettsia Akari." StatPearls.
2. Bourtzis K (2008). "Wolbachia-based technologies for insect pest population control." *Transgenesis and the management of vector-borne disease*. 104-113.
3. Fredriksson NJ, Malte H, Britt-Marie W. "The choice of PCR primers has great impact on assessments of bacterial community diversity and dynamics in a wastewater treatment plant." *PloS one* 8: e76431.
4. Hillis DM, James JB (1993). "An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis." *Systematic biology* 42:182-192.
5. Leclerque A (2008). "Reorganization and monophyly of the genus *Rickettsiella*: All in good time." *Applied and environmental microbiology*. 74: 5263-5264.