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Mutualism Enhances Wolbachia Infection Rates in Ant-attended Tuberculatus Aphid Species (Hemiptera: Aphididae)

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Abstract

Wolbachia are intracellular bacteria, the infection of which alters host reproductive characteristics. *Wolbachia* are estimated to infect more than half of arthropod species and some species of nematodes, and *Wolbachia* major genotypes are classified into 17 supergroups (A to S except G and R). It has been documented that the distribution and infection rate of *Wolbachia* on insects in the wild varies intra- or inter-species through competitions between infected and uninfected hosts. Aphid species within the genus *Tuberculatus* feed on Fagaceae leaves and exhibit two contrasting ecological characteristics, ant-attendance and non-attendance. This study compared *Wolbachia* infection rates between 11 ant-attended and 12 non-attended *Tuberculatus* aphid species, which were collected throughout Japan and around Mt. Kariwangsan in South Korea. Mean infection rates of *Wolbachia* were 30.3% in ant-attended species and 3.1% in non-attended species. *Wolbachia* haplotypes detected were classified into supergroups B, M, N, and O. Phylogenetic trees of *Tuberculatus* aphids constructed from a mitochondrion gene of cytochrome oxidase subunit I (*COI*) and nuclear gene of *18S rRNA* showed that mutualistic interactions between *Tuberculatus* aphids and ants have evolved at least five times. The phylogenetic comparative method showed that *Wolbachia* infection rates were significantly higher in ant-attended species. Possible *Wolbachia* infection routes were discussed in terms of the differences in the ecological characteristics between ant-attended and non-attended aphid species. Based on the phylogenetic comparative method, this study first revealed that the spread of microorganisms was affected by species interactions of hosts, and would contribute a profound understanding of the evolution of mutualistic interactions.

Introduction

Wolbachia are intracellular bacteria that occur in arthropods and nematodes (Werren et al 2008; Kauer et al 2021). One meta-analysis suggests that 66% of arthropod species are infected with *Wolbachia* (Hilgenboecker et al 2008). At present, it has been reported that major genotypes of *Wolbachia* are highly diverse and classified phylogenetically into17 supergroups (A to S except for G and R) (Glowska et al 2015; Lefoulon et al 2020). *Wolbachia* can often alter host reproductive characteristics by inducing feminization, parthenogenesis, male killing, and cytoplasmic incompatibility (Werren 1997). Reproductive manipulation can help *Wolbachia* spread to high frequencies in host populations, indicating the selfish aspects of the bacterium in hosts (Hoffmann et al 1990). On the other hand, costs and benefits of infection to host have bean reported in terms of emerging timing and survival rate of the mosquito *Aedes albopictus* (Gavotte et al 2010) and life history and reproductive traits of the been beetle *Callosobruchus chinensis* (Okayama et al 2016). These empirical studies suggest that *Wolbachia* behaves ranging from obligate mutualist to facultative or parasite bacterium depending on ecological and environmental conditions of host populations (Correa and Ballard 2016).

The distribution of *Wolbachia* on insects in the natural populations has been widely documented (Baudry et al 2003; Tagami and Miura 2004; Augustinos et al 2011; Bing et al 2014; Ren et al 2020), revealing that infection rates varied from zero to 100%, possibly depending on *Wolbachia* strains in the been beetle *C. chinensis* (Kondo et al 2002), two social forms of monogyne or polygyne in the fire ant *Solenopsis invicta* (Shoemaker et al 2003), spread speed estimated from univoltine life cycle and limited dispersal ability in the cherry fruit fly *Rhagoletis cerasi* (Bakovic et al 2018), reproduction mode and trophy (horn) in terrestrial beetles (Kajtoch et al 2019), and biogeographical factors in the common yellow butterfly *Eurema hecabe* (Narita et al 2006). These studies on natural populations exhibit that *Wolbachia* infection spread varies unevenly in intra- or inter-species through competitions between infected and uninfected hosts.

Aphids harbour intracellular bacteria *Buchnera aphidicola* (aγ-proteobacterium, hereafter *Buchnera*), which is the primary symbiont (Munson et al 1991) and provides essential amino acids to the host aphid by synthesizing them from asparagine in phloem sap (Sasaki et al 1990, 1991; Bermingham and Wilkinson 2010). Besides *Buchnera*, *Wolbachia* has been found in some aphid species (Gómez-Valero et al 2004; Wang et al 2009; Augustinos et al 2011; De Clerck et al 2014; Ren et al 2020). However, the roles of *Wolbachia* in host aphids are unknown. De Clerck et al (2015) claimed that *Wolbachia* in the banana black aphid *Pentalonia nigronervosa* could provide nutrition to the host by association with *Buchnera*, while Manzano-Marín (2020) disproved the nutrition provision hypothesis by pointing out the biased interpretation of antibiotic treatment analyses together with an incorrect genome-based metabolic inference. Some aphid species are associated with ants on the balancing of costs and benefits (Stadler and Dixon 2005; Yao 2014). Moreover, inter-species comparisons using molecular phylogenies on ant-

aphid mutualisms revealed that ant attendance has impacted on the morphology of aphids, such as length of mouthparts of *Chaitophorus* aphids (Shingleton et al 2005) and wing loading of *Tuberculatus* aphids (Yao 2011). A number of studies have documented that endosymbionts other than *Buchnera* and *Wolbachia* may have possible effects on ant-attended aphids (Łukasz et al 2020; Hertaeg et al 2021). However, direct relationships between the endosymbionts and ant-aphid mutualisms remain unclear. *Tuberculatus* aphids feed on Fagaceae (oak, chestnut, and beech) leaves and do not alternate host plants during the season (Quednau 1999) (Table S1). This group encompasses species with two contrasting ecological characteristics, ant-attendance and non-attendance (Yao 2011). Thus, phylogenetic comparative methods are applicable to test whether the presence or absence of ant associations are related to morphological or ecological traits of the aphids.

This study examined (1) *Wolbachia* infection rates and the type of *Wolbachia* supergroup in *Tuberculatus* aphid species collected throughout Japan and around Mt. Kariwangsan in South Korea, (2) the loss and gain of mutualistic interactions with ants using molecular phylogenetic trees based on a mitochondrion gene and a nuclear gene, and (3) the correlation with *Wolbachia* infection rates and ant associations using a phylogenetic comparative method. Infection routes of *Wolbachia* to aphids were discussed in terms of horizontal transmission via parasitoid wasps.

Materials & Methods

DNA extraction and Wolbachia **infection rate**

Tuberculatus aphids were collected from regions throughout Japan and around Mt. Kariwangsan of South Korea (Table S2 and Figures 1 and 2). A species was regarded as ant-attended if aphids offered honeydew directly from their anus to attending ants. Eleven ant-attended and 12 non-attended species were obtained (Table 1 and Table S1). Because it was difficult to identify the three ant-attended aphid species (*T. fulviabdominalis*, *T. indicus*, and *T. pilosulus*) and the seven non-attended aphid species (*T. higuchii* A- and B-types, *T. kashiwae* A- and B-types, *T. yokoyamai*, *T*. sp. D, and *T*. sp. F), those species were identified through the sequence (Table S1). Sampling was conducted on viviparous females, which appears from April to September. Since *Tuberculatus* aphid species parthenogenetically produce nymphs in summer, several nymph individuals on a leaf are a high possibility of a clone. Therefore, aphids were collected from several leaves in a tree, to avoid collecting clonal aphids. They were collected in 99.5% ethanol and stored at -20 °C. Before DNA extraction, the collected aphids were dissected to check for the presence of parasitoid wasps. Total DNA was extracted from each dissected aphid (whole body) with the Wizard genomic DNA purification kit (Promega, Tokyo, Japan). Since the *16S rRNA* gene is highly conserved in a wide variety of microorganisms, it was used for polymerase chain reaction (PCR) amplification to determine the presence or absence of *Wolbachia*. In the small-scale experiment, using a gene map of the *16S rRNA* locus of *Wolbachia* (Simões et al 2011), seven pairs of primers were selected and tested for each of 23 *Tuberculatus* species, in which two to three individuals per species were tested (Table 3). One pair of primers, *16SWolbF* (*16S-3f*) (Casiraghi et al 2001) and *WspecR* (*16S-2r*) (Werren and Windsor 2000), was identified as the most appropriate for assessing the 23 species (Table 3) because it was able to amplify *Wolbachia* at the maximum number of species (seven species) of the 23 species. After the small-scale experiment, a full-scale experiment using the pair of primers was conducted on all collected samples (Table 1). To check whether DNA extraction was successful, the barcoding region (in mitochondrion) of primer pairs, *LCO1490* and *HCO2198*, was also used (Table 2). Because more than 90% of individuals of *T. macrotuberculatus* in the Ishikari site harboured *Wolbachia* (Yao 2019), one individual of the species from the site was used for a positive control sample for *Wolbachia* detection. PCR was performed in 10 μL volume which included 2 μL of 5×KAPATaq Extra buffer (Nippon Genetics, Tokyo, Japan), 1 μL 25 mM $MgCl_2$, 0.3 μL dNTP mixture (10 mM of each), 0.5 μL of 10 μM of each primer, 1 μL template DNA, and 0.05 μL KAPATaq Extra DNA polymerase (5 units/ μL). Reaction cycle parameters were: 94°C for 1 min; 40 cycles of 94°C for 20 sec, 50°C for 20 sec, and 68°C for 1 min, followed by a final extension of 68°C for 1 min. When PCR products had faint bands, the samples were rechecked by PCR in 20μL reaction volume. If the bands were false, nothing was amplified in 20μL reaction volume. The PCR product was checked using 1.5% agarose gel electrophoresis with ethidium bromide stain illuminated by UV light. The *Wolbachia* infection rate of each species was defined as the percentage of individuals amplified with the *Wolbachia*-specific primer out of all individuals amplified with the barcoding region primer. The correlation between the *Wolbachia* infection ratio in each collection site and geographical distance was tested by Mantel test (Mantel 1967) using the package vegan in R (R Development Core Team 2008). Mantel test was applied to the species that was collected from more than a single site and showed mean infection rates of less than 100%.

Phylogenetic trees for Tuberculatus **aphids**

A phylogenetic tree of the 23 *Tuberculatus* aphid species was constructed from the nucleotide sequences of a mitochondrion gene of a partial of cytochrome oxidase subunit I (*COI*) (940bp) from DDBJ (DNA Data Bank of Japan) (Table 1). Besides the *COI* gene, a partial of the nuclear gene of *18S rRNA* (approx. 670bp) was amplified and used to construct phylogenetic trees. For reading the sequences of *18S rRNA* gene, PCR was performed in 20μL reaction volume with a pair of primers (*Ns1* and *Ns2a*; Table 2), the same reagents, and reaction cycles, as mentioned in the previous section, but changed annealing temperature to 47°C. PCR products were purified and sent to sequencing service (using Sanger sequencing) (Eurofin, Japan). The sequence data of the *18S rRNA* gene (515bp) were deposited in the DDBJ and accession numbers were listed in Table 1. In addition to sequences of *COI* and *18S rRNA* genes, a combined sequence of *COI* and *18S rRNA* genes (1,455bp) was used for the construction of phylogenetic trees. The appropriateness of the combined sequence was checked by a homogeneity test implemented in PAUP* ($P > 0.05$). Judging from the phylogenetic position of Calaphidini aphid species (Lee et al 2017, 2021), specimens of two aphid species, *Tinocallis zelkowae* and a species of *Takecallis*, were included as outgroups. Neighbor-joining (NJ), most parsimonious (MP), and maximum likelihood (ML) analyses were performed using PAUP* 4.0b10 PPC and 4.0a 169 (Swofford 2002). For MP analysis, all characteristics were equally weighted. MP trees were searched heuristically, with 1,000 random addition replications using tree bisection-reconnection (TBR) branch swapping. To assess confidence in clades, bootstrap tests were performed for NJ and MP trees using a full heuristic search. Replicates with TBR branch swapping were 10,000 and 1,000 for NJ and MP, respectively. For the ML tree, parameters were chosen based on the Akaike Information Criterion, as implemented in Modeltest ver 3.7 (Posada and Crandall 1998). The GTR + I + G model was selected for the sequence of *COI* and the combined sequence of *COI* and *18S rRNA* genes. The Trn +I model was selected for the sequence of the *18S rRNA* gene. ML trees were searched heuristically with TBR branch swapping. For the bootstrap test on ML, 1,000 replicates were performed using fast stepwise addition as a starting option. For Bayesian analysis, MrBayes ver. 3.2.0 (Ronquist and Huelsenbeck 2003) was performed with the GTR + I + G model selected by MrModeltest ver. 2 (Nylander 2004). The sample frequency and burn-in fraction were set to 100 and 25%, respectively. The numbers of generations were 20,000 for the *COI* gene, 1,220,000 for *18S rRNA* gene, and 100,000 for the combined sequence of *COI* and the *18S rRNA* genes.

Phylogenetic Pairwise Comparisons

The extent of ant association was categorized as either 0 (non-attendance) or 1 (facultative and obligate ant-attendance). *Wolbachia* infection rates in some species had a score of zero, and so were log-transformed. To examine the correlation between ant association (binary data) and *Wolbachia* infection rates in *Tuberculatus* species (continuous data), phylogenetically independent contrasts were calculated using pairwise comparisons (Read and Nee 1995; Maddison 2000) implemented in Mesquite ver. 3.61 (Maddison and Maddison 2019). First, taxa were paired as sets of terminal taxa (a set of comparison) with contrasting characteristics (ant-attendance vs. non-attendance). The pair of terminal taxa had to be phylogenetically separate from each other. Then, taxa pairs were counted up to maximal pairings that were equal to the number of parsimony-counted steps in the characteristic treated as unordered in a dichotomous tree. Finally, all maximal pairings were placed on a dichotomous tree, using an algorithm that performed two traversals through the tree. Wilcoxon matched-pairs signed-ranks test was applied on each pair to test whether the second characteristic (*Wolbachia* infection rate) had consistently higher or lower values to the first characteristic (ant-attendance and non-attendance).

Wolbachia **supergroups**

For *Wolbachia* that were detected in aphids (Table S2), the PCR products were sequenced with the same primers (*16S-3f* and *16S-2r*) (Table 2). PCR products were purified with FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The cycle sequencing reaction was performed with a 5 μL volume consisted of 2 μL of Quick Start Mix (Beckman Coulter, Tokyo, Japan), 0.5 μL of 10 μM forward or reverse primers, and 2.5 μL of 10 ng/μL template DNA. The reaction cycle was 40 cycles of 94°C for 20 sec, 50°C for 20 sec, and 60°C for 1 min. DNA sequencing was analyzed using the CEQ2000XL DNA Analysis System (Beckman Coulter, Tokyo, Japan). The length of sequences that were successfully read through the samples were from about 500bp to 900bp. Multiple sequence alignment including the sequences of 16 *Wolbachia* supergroups (A, B, C, D, E, F, H, I, J, K, L,

M, N, O, Q, S) that were cited by Bing et al (2014) (A to O), Ren et al (2020) (O found in aphids), Glowska et al (2015) (Q), and Lefoulon et al (2020) (S) (Table 4) was processed with the Clustal W (Thompson et al 1994) on the DDBJ. Supergroup P was not included in multiple sequence alignment because it was insufficient sequence length for the lower region of the gene. After multiple sequence alignment, the length of sequences was 471bp. Because of the insufficient length of sequences, it was expected that an unreliable model would be selected by the DNA base substitution model test. This study aims to determine what kind of *Wolbachia* supergroup is found in *Tuberculatus* aphids so that the unweighted pair group method with arithmetic mean (UPGMA) emphasized clustering formation was applied to construct *Wolbachia* phylogenetic tree. The bootstrap test was performed using the UPGMA method and 1000 replicates with TBR branch swapping.

Results

Wolbachia **infection rate**

Wolbachia was detected in eight of 11 ant-attended aphid species (Table 1 and Figure 1) and five of 12 non-attended species (Table 1 and Figure 2), in which at least one individual was detected. Mean *Wolbachia* infection rates showed 30.3% in ant-attended species and 3.1% in non-attended species (Table 1). A large variation of *Wolbachia* infection rates was found in ant-attended species (0% in *T. indicus* (Figure 1b), *T. pappus* (Figure 1e), and *T*. sp. E (Figure 1f); 100% in *T*. sp. B (Figure 1f)). Mantel test on *T. fulviabdominalis* and *T. macrotuberculatus* showed that *Wolbachia* infection rates significantly varied between collection sites (for *T. fulviabdominalis*, Mantel statistic $r = 0.842$, $P = 0.035$ (Table 1 and Figure 1b); for *T. macrotuberculatus*, Mantel statistic $r = 0.164$, $P = 0.03$ (Table 1 and Figure 1d)).

Phylogenetic Pairwise Comparisons

In all phylogenetic reconstruction methods, phylogenetic trees based on the sequences of *COI* gene showed fully resolved tree topology, whereas phylogenetic trees from the *18S rRNA* gene resulted in large polytomies (Figure S1). For phylogenetic trees from the combined sequences of both genes, only the Bayesian tree included a polytomy in one clade (Figure S2). Since phylogenetically independent contrasts require fully resolved tree topology, phylogenetic trees with polytomies were not used in calculating pairwise comparisons. Phylogenetic trees showed that mutualistic interactions with ants have evolved at least five times, regardless of phylogenetic reconstruction methods, indicating that the number of maximal pairings was five (Figure 3). Mesquite showed that the number of combinations of a set of five pairings ranged from 570 (on a ML tree of *COI*) to 1,178 (on NJ trees) (Table 5). The Wilcoxon matched-pairs signed-ranks test showed that *P*-values ranged from 0.031 to 0.313 (Table 5), indicating that the positive direction was exhausted in some sets of five pairings, but not others. *Wolbachia* infection rate in *Tuberculatus* aphids was significantly higher in ant-attended species compared to non-attended species ($P = 0.031$).

Wolbachia **supergroups**

Because sequence for *T. pilosulus* and *T*. sp. D was failed, *Wolbachia*-positive 11 species were analyzed. The results of sequencing exhibited that each species harboured one haplotype of *Wolbachia* except for *T. macrotuberculatus*. *Tuberculatus macrotuberculatus* harboured two haplotypes: while one haplotype was found in 22 sites (sites 1 to 21 and site 23), the other in site 22. An UPGMA tree showed that 12 haplotypes of *Wolbachia* were classified into four supergroups B, M, N, and O (Figure 4). The haplotypes of *Wolbachia* in *T. kuricola*, *T. stigmatus*, *T. higuchii* B-type, and *T. paiki*, were placed into supergroup B. *Wolbachia* in *T. macrotuberculatus* collected from all infected sites except for site 22, *T. quercicola*, and *T*. sp. B, were the same haplotype and belonged to supergroup M. *Wolbachia* in *T. macrotuberculatus* collected from site 22, *T. capitatus*, and *T. fulviabdominalis*, which were the same haplotype, and *Wolbachia* in *T. japonicus*, were placed into supergroup N. *Tuberculatus higuchii* A-type harboured *Wolbachia* of supergroup O, which was supported with a high bootstrap value (98%). Twelve DNA sequences of *Wolbachia*'s *16S rRNA* were deposited in the DDBJ and accession numbers were listed in Table 4 and Figure 4.

Discussion

Wolbachia infection rates in *Tuberculatus* aphid species ranged from zero to 100%. Among *Wolbachia*-infected species, four species, *T. pilosulus*, *T. japonicus*, *T. paiki*, and *T*. sp. D showed a quite lower level of infection rates less than 5%, while *T. capitatus* and *T*. sp. B exhibited a much higher infection level more than 90%. Considering the marked difference in infection rates, there could be some sort of differences in infection routes and *Wolbachia*'s role in the host between the species of lower and higher infection rates. Along with the cost-benefit balance on *Wolbachia* infection to hosts (Gavotte et al 2010; Okayama et al 2016), higher infection levels in the populations of *T. capitatus* and *T*. sp. B could be responsible for positive selection favouring benefits from *Wolbachia* infection such as nutrition provision (De Clerck et al 2015; but see Manzano-Marín 2020) or resistance to parasitoid wasps (Oliver et al 2003), while lower infection levels in the four species may incur substantial costs from *Wolbachia* infection.

The phylogenetic comparative method showed that *Wolbachia* infection rates in 23 *Tuberculatus* aphid species were significantly differed by the presence or absence of ant attendance. One possible infection route of *Wolbachia* to aphids could be via parasitoid wasps (Sadeghi-Namaghi and Amiri-Jami 2018). Regardless of whether aphids are attended by ants, aphid colonies are frequently attacked by parasitoid wasps (Brodeur and Rosenheim 2000). Field experiments using single aphid species also demonstrated that ant-attended colonies attracted more parasitoid wasps compared to ant-excluded colonies (Völkl 1992; Kaneko 2002, 2003). These behaviours of parasitoid wasps have been thought to be triggered by visual and chemical cues from aphid colonies attended by ants. Ant-attended aphid species tend to form dense colonies (Stadler et al 2003) and parasitoid wasps could exploit ant semiochemicals (Mouratidis et al 2021). Moreover, ant-attended aphid species tend to disperse less compared to non-attended species (Oliver et al 2007; Yao 2010; Yao and Kanbe 2012), potentially providing greater opportunities for parasitoid wasps to oviposit the former. A study using fluorescence in situ hybridization on the parasitoid wasp *Eretmocerus* sp. showed that *Wolbachia* were present in the mouthparts and ovipositors of wasps feeding on *Wolbachia*-infected whitefly *B. tabaci* (Ahmed et al 2015). Thus, the horizontal transmission of endosymbionts via the parasitoids of insects represents a potential pathway. Besides parasitoid wasps, ants are also known to harbour *Wolbachia* (Keller et al 2001; Shoemaker et al 2003; Frost et al 2010; Reeves et al 2020) and thus could be a possible agent to spread *Wolbachia* into aphid populations. In the study of scale insects and their associated groups (ants, wasps, beetles, flies, mites, moths, and thrips), Sanaei et al (2021) showed that significant differences in *Wolbachia* infection rates in the scale insects were found in ant attendance and the associated groups but wasps, suggesting a possible route of horizontal transfer between ants and scale insects. Nonetheless, it is unlikely that *Wolbachia* was transported by ants into *Tuberculatus* aphids because the two non-attended species, *T. higuchii* A- and B-types, showed over 10% infection rates and a mean infection rate of 30% in ant-attended species seems to be a low score considering constant contact with ants. Further studies on *Wolbachia* strains for both aphids and their mutualistic ants need to elucidate the possible routes by ants.

Wolbachia haplotypes were clustered into the four supergroups B, M, N, and O. Out of the 11 *Wolbachia*-infected *Tuberculatus* species in the phylogenetic tree, *T. higuchii* A-type fell into supergroup O that has been firstly detected in the white fly *Bemisia tabaci* (Bing et al 2014) and recently found in the galling aphid species, *Kaburagia rhusicola* and *Schlechtendalia chinensis* (Ren et al 2020). Detection in the novel host and a monophyletic group with a high bootstrap value (98%) will support existence of supergroup O. Given that supergroup O has so far been found only in China, it could be originated in East Asia and spread in Japan.

Although significant geographic differences in infection rate were found in *T. fulviabdominalis* and *T. macrotuberculatus*, the distribution of infected populations showed distinctive patterns. While *T. fulviabdominalis* harboured *Wolbachia* at the two near sites (sites 7 and 8; Figure 1b), *T. macrotuberculatus* showed drastic differences in infection rate among northern island (sites 1 to 9; infection rate, 89%), mainland (sites 10 to 21; 0%), and southwestern main-island Japan (sites 22 to 23; 100%). As for *T. fulviabdominalis*, it seems that infection event had occurred in the limited area and less spread out due to geographical isolation. In contrast, *T. macrotuberculatus* harboured two supergroups of M and N that were phylogenetically distant clade as have seen in Moreira et al (2019); while only supergroup M was detected in the entire populations infected in northern island, supergroups M and N were solely found in the two near areas of site 23 and site 22 (apart from about 40km) (Figure 1d), respectively. The previous study showed that the phylogenetic tree based on mitochondrion *COI* genotypes of *T. macrotuberculatus* from all over Japan exhibited the three independent clades that almost matched to the geographic regions of northern island (clade 1), mainland (clade 2), and southwestern main-island (clade 3) (Yao and Kanbe 2012). The three clades

also matched well to the areas of presence or absence of *Wolbachia* infection in *T. macrotuberculatus* populations in this study, suggesting that formation process of the Japanese Archipelago could be responsible for *Wolbachia* infection status in *T. macrotuberculatus* populations. This theme is under investigation and will be reported in another journal.

This study first revealed that, based on the species comparisons using molecular phylogenetic trees, ecological characteristic of host organisms influenced the extent of *Wolbachia* spread. Further studies need to clear what roles do play *Wolbachia* in the aphids, especially for ant-attended aphid species.

Declarations

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Authors' contributions IY conceived the study, collected the specimens, analyzed the data and wrote the manuscript.

Availability of data and material Data are available by request to the author.

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Conflicts of interest/Competing interests The author declares no conflicts of interest or competing interests.

Consent to participate N/A.
Consent for publication N/A.

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Tables

Table 1. *Tuberculatus* aphid species used in the study and *Wolbachia* infection rate

Ant-attended	Collection sites	*N*	*wol*$^+$	Infection rate (%)	Mantel statistic r	*P*	Abbreviation	*COI*	*18S rRNA*
T. capitatus	15	56	53	94.6	0.032	0.196	capi	AB592769	LC654240
T. fulviabdominalis	8	55	12	21.8	0.842	**0.035**	fulvi	AB592755	LC654241
T. indicus	11	53	0	0.0	-	-	ind	AB592759	LC654242
T. kuricola	10	54	15	27.8	-0.093	0.719	kuri	AB592750	LC654243
T. macrotuberculatus	23	54	28	51.9	0.164	**0.030**	mt	AB592752	LC654244
T. pappus	1	10	0	0.0	-	-	pap	AB861442	LC654245
T. pilosulus	16	79	1	1.3	0.596	0.059	pilosulus	AB592758	LC654246
T. quercicola	11	54	8	14.8	-0.105	0.551	que	AB592754	LC654247
T. stigmatus	15	56	11	19.6	-0.039	0.415	sti	AB592760	LC654248
T. sp. B	4	31	31	100.0	-	-	spB	AB592753	LC654249
T. sp. E	1	9	0	0.0	-	-	spE	AB861448	LC654250
Average	10.5	46.5	14.5	30.2					
Non-attended									
T. higuchii A-type	14	71	8	11.3	-0.100	0.560	higa	AB592762	LC654251
T. higuchii B-type	7	42	8	19.0	-0.089	0.657	higb	AB592764	LC654252
T. japonicus	7	59	1	1.7	0.814	0.143	japo	AB592756	LC654253
T. kashiwae A-type	5	39	0	0.0	-	-	kasa	AB592765	LC654254
*T. kashiwa*e B-type	4	47	0	0.0	-	-	kasb	AB592766	LC654255
T. paiki	18	51	1	2.0	0.086	0.221	paiki	AB592768	LC654256
T. pilosus	11	52	0	0.0	-	-	pilosus	AB592751	LC654257
T. querciformosanus	9	52	0	0.0	-	-	qfor	AB592761	LC654258
T. yokoyamai	3	18	0	0.0	-	-	yoko	AB592767	LC654259
T. sp.C	1	41	0	0.0	-	-	spC	AB592757	LC654260
T. sp.D	1	29	1	3.4	-	-	spD	AB592763	LC654261
T. sp.F	1	4	0	0.0	-	-	spF	AB861457	LC654262
Average	6.8	42.1	1.6	3.1					
Out group									
Takecallis sp.	-	-	-				Takecallis	AB592749	LC654263
Tinocallis zelkowae	-	-	-				Tinozel	AB592748	LC654264

(Note): Collection sites represent the number of collection sites for aphids (see Table S2 for details). *N* and *wol*$^+$ mean the numbers of aphid individuals amplified with barcoding region primers and those with *Wolbachia* specific primers. Infection rate (%) was defined by the per cent of *wol*$^+$ divided by *N*. To test the difference in infection rate among collection sites, Mantel test

was applied to the species collected from more than a single site and with mean infection rates less than 100%. Statistics of Mantel test, r, and *P*-values were given. The bold font showed a significant difference below 0.05 of *P*-values. Abbreviated names were used in Table 3, Figures, and supplementary files. Accession numbers of *COI* and *18S rRNA* genes from DDBJ were used to create phylogenetic trees of the aphids.

Table 2. Primer set used in the small-scale experiment of *Wolbachia* detection and the amplification of *18S rRNA* gene in host aphids.

Primer name	Primer sequence (5' to 3')	Product size (bp)	References
WspecF (16S-2f)	CATACCTATTCGAAGGGATAG	438	Werren *et al*. (2000)
WspecR (16S-2r)	AGCTTCGAGTGAAACCAATTC		
16SWolbF (16S-3f)	GAAGATAATGACGGTACTCAC	1014	Casiraghi *et al*. (2001)
16SwolbR3 (16S-3r)[*1]	GTCACTGATCCCACTTTAAATAAC		
553F_W (16S-6f)	ATACGGAGAGGGCTAGCGTTA	781	Simões *et al*. (2011)
1334R_W (16S-6r)	CTTCATRYACTCGAGTTGCWGAGT		
16SWup	GCCTAACACATGCAAGTCGAA	1400	Gomez-Valero *et al*. (2004)
16SWlo	AGCTTCGAGTGAAACCAATTCCC		
groEL-F (Wol)	CAACRGTRGSRRYAACTGCDGG	550	Ros *et al*. (2009)
groEL-R (Wol)	GATADCCRCGRTCAAAYTGC		
wsp81F	TGGTCCAATAAGTGATGAAGAAA	610	Zhou, Rousset & O'Neill (1998)
wsp691R	AAAAATTAAACGCTACTCCA		
FbpA_F1	GCTGCTCCRCTTGGYWTGAT	509	Baldo *et al*. (2006)
FbpA_R1	CCRCCAGARAAAAYYACTATTC		
16SWolbF (16S-3f)	GAAGATAATGACGGTACTCAC	972	This study
WspecR (16S-2r)	AGCTTCGAGTGAAACCAATTC		
LCO1490	GGTCAACAAATCATAAAGATATTGG	708	Folmer *et al*. (1994)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA		
Ns1	GTAGTCATATGCTTGTCT C	670 (approximately)	Barker *et al*. (2003)
Ns2a	CGCGGCTGCTGGCACCAGACTTGC		

(Note) *1. Reverse primer was not used in this study.

Table 3. Result of the small-scale experiment

Primer combination	capi	fulvi	ind	kuri	mt	pap	pilosulus	que	sti	spB	spE	
*16S-2f*16S-2r*	+	–	–	–	+	–	–	+	+	+	–	
*16S-6f*16S-6r*	+	+–	–	–	+	–	+–	+	+–	+	–	
*16SWup*16SWlo*	+	–	–	–	+	–	–	+	–	+	–	
*groEL-F*groEL-R*	+	–	–	–	+	–	–	+	–	+	–	
*FbpA_F1*FbpA_R1*	+	–	–	–	+	–	–	+	–	+	–	
*wsp81F*wsp691R*	+	–	–	–	–	–	–	–	–	–	–	
*16S-3f*16S-2r*	+	–	–	–	+	–	–	+	+	+	–	
Primer combination	higa	higb	japo	kasa	kasb	paiki	pilosus	qfor	yoko	spC	spD	spF
*16S-2f*16S-2r*	–	+	–	–	–	–	–	–	–	–	–	+–
*16S-6f*16S-6r*	+–	+	–	–	–	–	–	–	+–	–	+–	–
*16SWup*16SWlo*	–	–	+	–	–	–	–	–	–	–	–	–
*groEL-F*groEL-R*	–	–	+	–	–	–	–	–	–	–	–	–
*FbpA_F1*FbpA_R1*	–	+	–	–	–	–	–	–	–	–	–	–
*wsp81F*wsp691R*	–	–	+	–	–	–	–	–	–	–	–	–
*16S-3f*16S-2r*	–	+	+	–	–	–	–	–	–	–	–	–

(Note) Symbols + and – indicate that a clear band appeared and no band appeared, respectively. Symbols +– mean that a faint band appeared in 10 μL of PCR reaction volume, but disappeared when rechecked with PCR in 20 μL volume. Full terms of abbreviations are provided in Table 1.

Table 4. Host species list used in the determination of *Wolbachia* supergroups

Host species	Generic name	Supergroup	Accession No.
Muscidifurax uniraptor	Wasp	A	L02882
Nasonia vitripennis	Wasp	A	M84688
Bryobia sarothamni	Spider mite	B	EU499315
Bryobia praetiosa	Clover mite	B	EU499317
Nasonia vitripennis	Wasp	B	M84686
Bemisia tabaci	Whitefly	B	JN204507
Onchocerca ochengi	Nematode	C	AJ010276
Onchocerca gibsoni	Nematode	C	AJ276499
Dirofilaria repens	Nematode	C	AJ276500
Dirofilaria immitis	Nematode	C	Z49261
Brugia malayi	Nematode	D	AF051145
Litomosoides sigmodontis	Nematode	D	AF069068
Folsomia candida	Springtail	E	AF179630
Mesaphorura macrochaeta	Springtail	E	AJ422184
Mansonella ozzardi	Nematode	F	AJ279034
Myrmeleon mobilis	Antlion	F	DQ068882
Kalotermes flavicollis	Termite	F	Y11377
Zootermopsis nevadensis	Termite	H	AY764280
Dipetalonema gracile	Nematode	H	AJ548802
Ctenocephalides felis	Cat flea	I	AY335923
Orchopeas leucopus	Rat flea	I	AY335924
Dipetalonema gracile	Nematode	J	AJ548802
Bryobia sp.	Mite	K	EU499316
Radopholus similis	Nematode	L	EU833482
Tuberolachnus salignus	Aphid	M	JN384085
Aphis sp.	Aphid	M	JN384091
Toxoptera aurantii	Aphid	N	JN384094
Toxoptera aurantii strain B	Aphid	N	JN384095
Bemisia tabaci isolate10	Whitefly	O	KF454771
Kaburagia rhusicola	Aphid	O	MT554837
Schlechtendalia chinensis	Aphid	O	MT554838
Torotrogla cardueli strain EG044	Mite	Q	KP114101.1
Atemnus politus strain K5	False scorpion	S	NZ_WQMQ01000035.1
Tuberculatus higuchii B-type	Aphid	B	LC613027
Tuberculatus kuricola	Aphid	B	LC613029

Tuberculatus stigmatus	Aphid	B	LC613028
Tuberculatus paiki	Aphid	B	LC613031
Tuberculatus macrotuberculatus sites1-8 & 23	Aphid	M	LC613021
Tuberculatus sp. B	Aphid	M	LC613022
Tuberculatus quercicola	Aphid	M	LC613023
Tuberculatus macrotuberculatus site 22	Aphid	N	LC655298
Tuberculatus capitatus	Aphid	N	LC613025
Tuberculatus fulviabdominalis	Aphid	N	LC613026
Tuberculatus japonicus	Aphid	N	LC613030
Tuberculatus higuchii A-type	Aphid	O	LC613024

Table 5. The results of pairwise comparisons on the phylogenetic trees reconstructed by the NJ, MP, ML, and Bayesian methods.

Gene	NJ		MP		ML		Bayesian	
	Pairings	*P*	Pairings	*P*	Pairings	*P*	Pairings	*P*
COI (940bp)	1,178	0.031-0.313	722	0.031-0.313	570	0.031-0.125	722	0.031-0.313
COI + 18SrRNA (1455bp)	1,178	0.031-0.313	722	0.031-0.313	722	0.031-0.313	NA	

(Note) Pairings indicate the number of combinations of a set of five pairings found in the analysis in the algorisms of the Mesquite. Wilcoxon matched-pairs signed-ranks test was applied on each pairing. *P*-values showed the range of results of the test on each pairing. NA means that pairwise comparison was not applied to the Bayesian phylogenetic tree from the combined *COI* and *18S rRNA* genes because fully resolved topology was not constructed. Examples of pairings in phylogenetic trees showing significant differences below 0.05 were given in Figure 2.

Figures

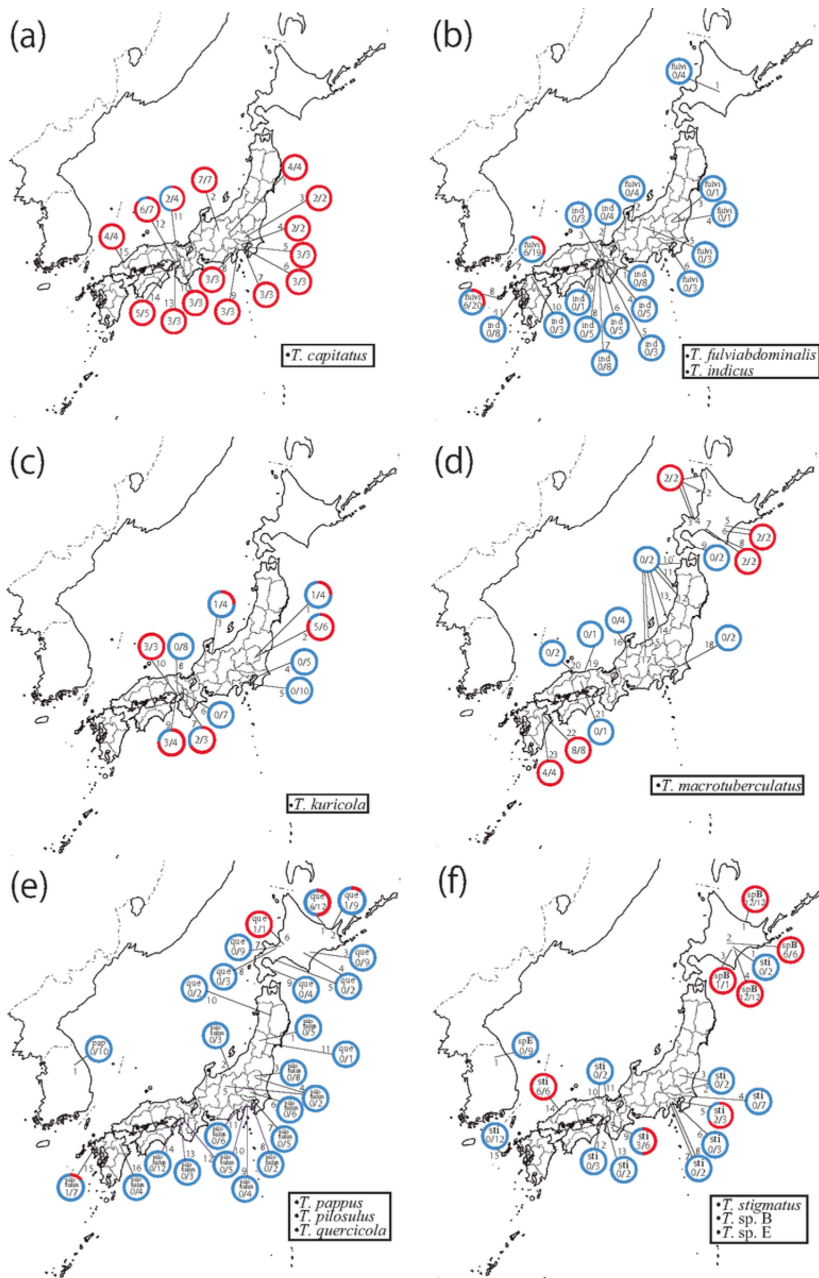

Figure 1

Maps for collection sites and *Wolbachia* infection rates of ant-attended *Tuberculatus* aphid species

(Note) Collection sites and pie charts of *Wolbachia* infection rates in ant-attended species of (a) *T. capitatus*, (b) *T. fulviabdominalis* and *T. indicus*, (c) *T. kuricola*, (d) *T. macrotuberculatus*, (e) *T. pappus*, *T. pilosulus*, and *T. quercicola*, and (f) *T. stigmatus*, *T.* sp. B, and *T.* sp. E. Red and blue in a pie chart indicate the presence and absence of *Wolbachia*, respectively. The numbers in a pie chart divided by "/" mean the number of *Wolbachia*-detected individuals per total individual number collected from the site. The numbers in maps and on/under the lines indicate collection sites listed in Table S2. Full terms of abbreviations in pie charts are provided in Table 1.

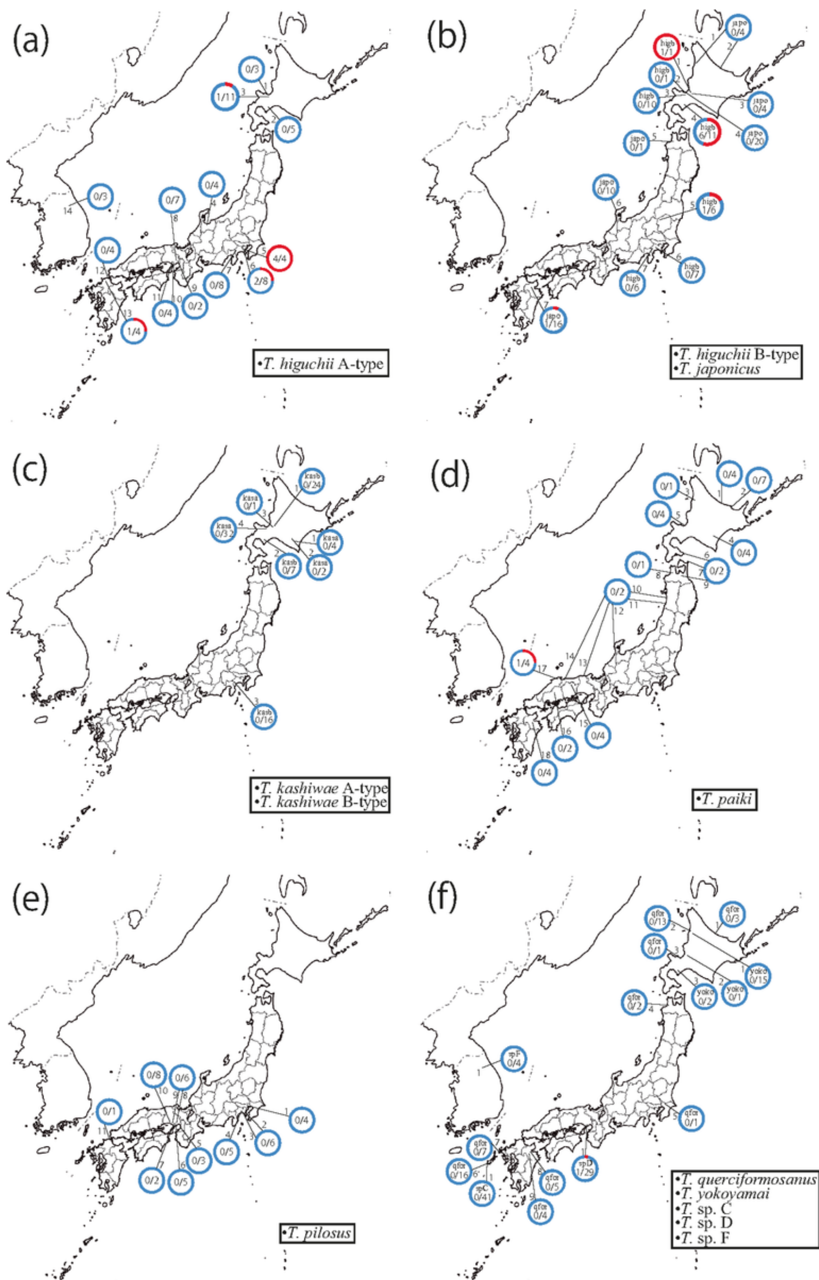

Figure 2

Maps for collection sites and *Wolbachia* infection rates of not-attended *Tuberculatus* aphid species.

(Note) Collection sites and pie charts of *Wolbachia* infection rates in non-attended species of (a) *T. higuchii* A-type, (b) *T. higuchii* B-type and *T. japonicus*, (c) *T. kashiwae* A and B-types, (d) *T. paiki*, (e) *T. pilosus*, and (f) *T. querciformosanus*, *T. yokoyamai*, *T.* sp. C, *T.* sp. D, and *T.* sp. F. Red and blue in a pie chart indicate the presence and absence of *Wolbachia*, respectively. The numbers in a pie chart divided by "/" mean the number of *Wolbachia*-detected individuals per total individual number collected from the site. The numbers in maps and on/under the lines indicate collection sites listed in Table S2. Full terms of abbreviations in pie charts are provided in Table 1.

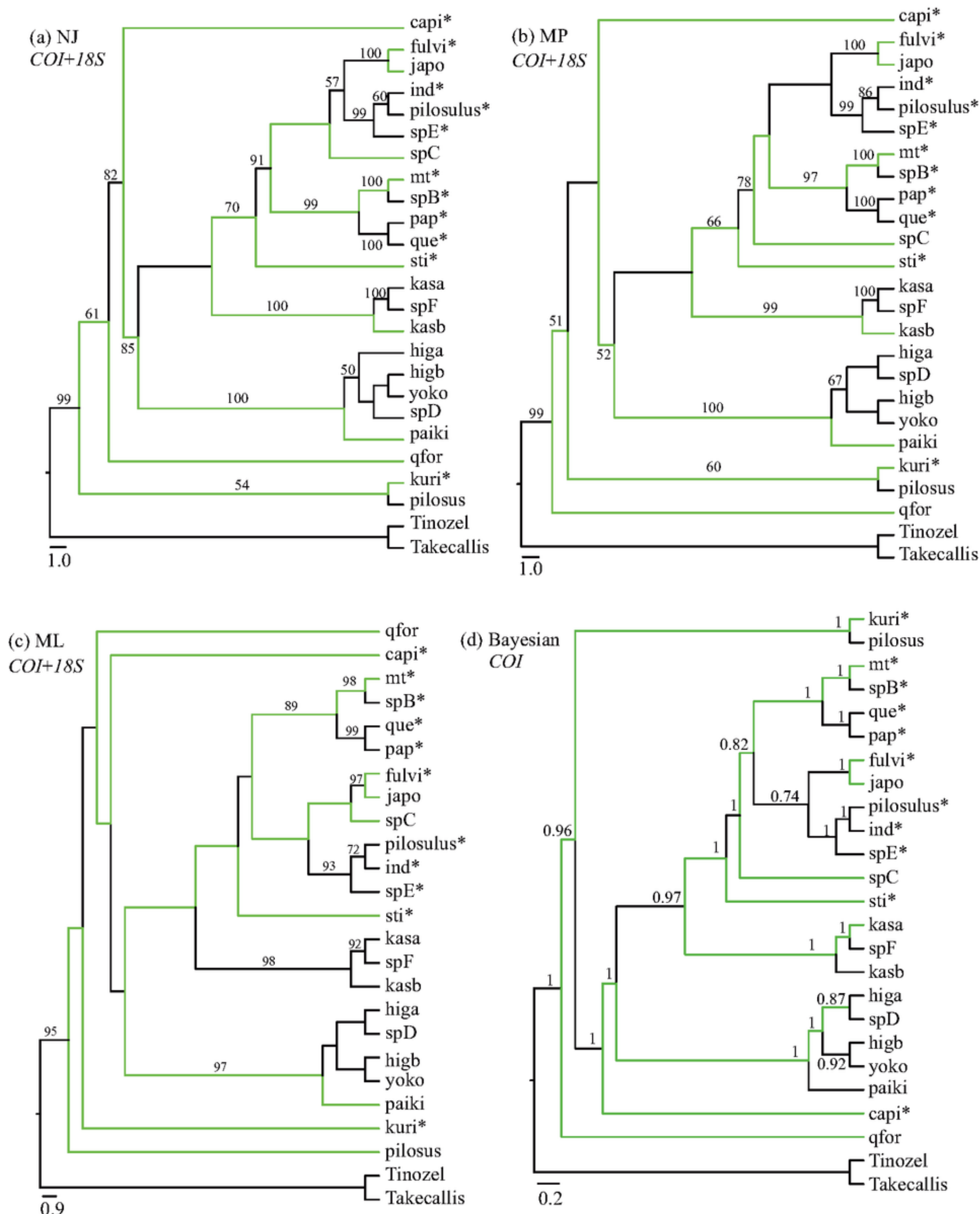

Figure 3

Illustrations of pairwise comparisons on phylogenetic trees

(Note) Examples of pairings in (a) Neighbor-Joining (NJ), (b) Most parsimonious (MP), and (c) Maximum likelihood (ML) phylogenetic trees constructed from the combined sequences of *COI* and *18S rRNA* genes, and in (d) Bayesian phylogenetic tree from *COI* gene. Each of the examples indicates the pairings, in which a *P*-value less than 0.05 was found in pairwise comparisons after the Wilcoxon matched-pairs signed-ranks test. The green-coloured path shows a pairing that had terminal taxa of ant-attended and non-attended aphid species. Each phylogenetic tree contained five sets of pairings. The five sets of pairings were found by moving a tree using the algorithm in Mesquite. Species names with * were ant-attended. The numbers on/under the branches of phylogenetic trees show bootstrap values (>50%) for NJ, MP, and ML. For the Bayesian tree, the numbers on/under branches show the post probability supporting the node in the tree.

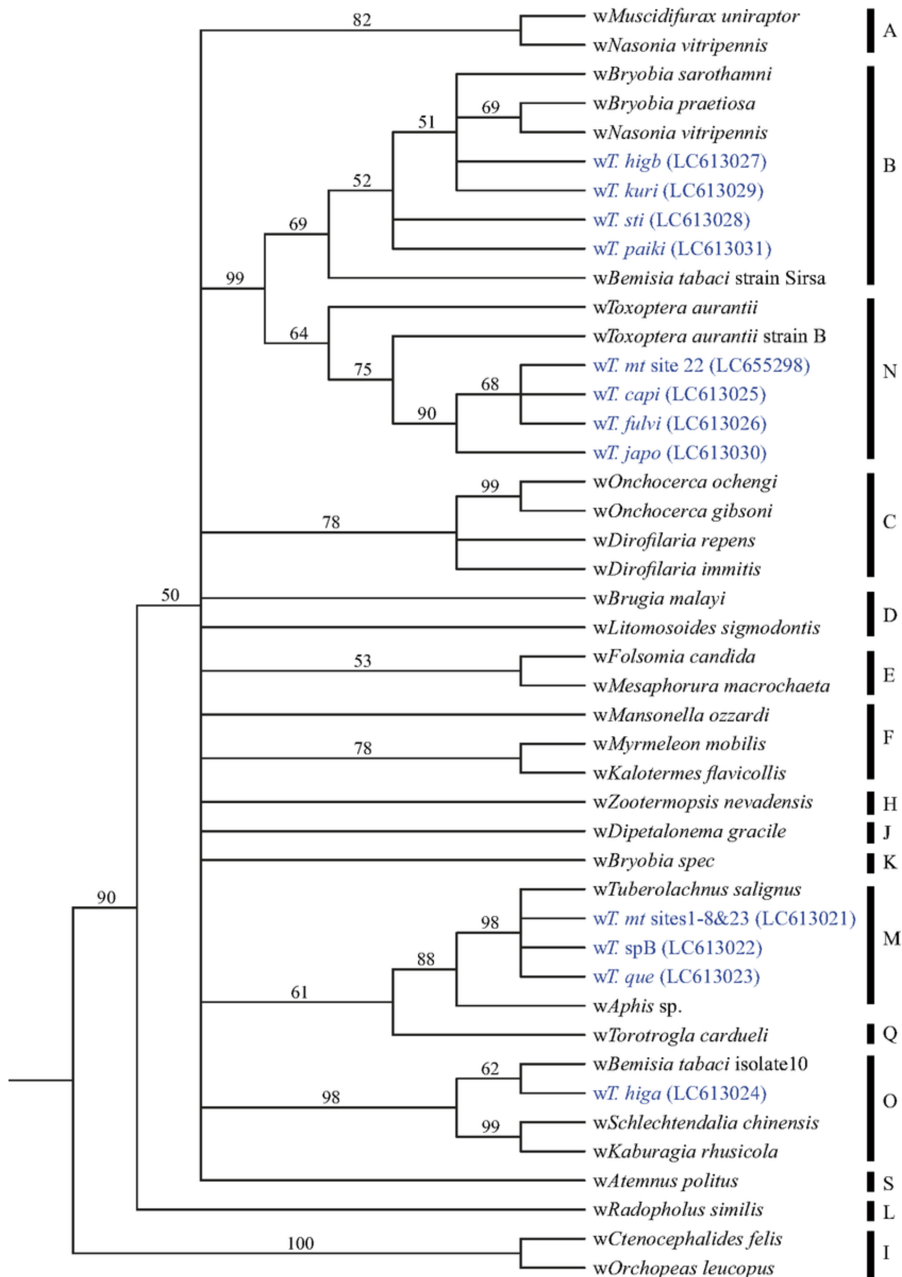

Figure 4

The 50 % majority rule consensus tree inferred by unweighted pair group method with arithmetic mean (UPGMA) analysis for 16 *Wolbachia* supergroups

(Note) The labels of operational taxonomic units (OTU) mean *Wolbachia* sp. (indicated by w) and its host species. Known *Wolbachia* sp. and their host species were in black and examined species in this study were in blue. Thick vertical lines with alphabets indicate the clades of *Wolbachia* supergroups. See also Table 4. Bootstrap values of more than 50% were shown on branches.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaltablesFigures.docx