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Title: Mutualism enhances *Wolbachia* infection rates in ant-attended *Tuberculatus* aphid species (Hemiptera: Aphididae)

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Abstract

Some aphid species form close associations with ants: offering them honeydew and obtaining protection from ants in return. However, mutualistic interactions with ants can also have a negative influence on aphid physiological and morphological traits. *Wolbachia* are intracellular bacteria whose major genotypes are classified into 17 supergroups (A to S except G and R). Aphid species within the genus *Tuberculatus* feed on Fagaceae leaves and exhibit two contrasting ecological characteristics, ant-attendance and non-attendance. Previous work has found that ant-attended species exhibit lower dispersal and are likely to form aggregated colonies. Considering that host-parasitoid interactions may well be one of the most common horizontal transmission routes of *Wolbachia*, it is therefore expected that ant associations will be associated with higher *Wolbachia* infection rate in *Tuberculatus* aphid species. 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 30.2% in ant-attended species and 3.1% in non-attended species. The *Wolbachia* haplotypes detected were classified into supergroups B, M, N, and O. A phylogenetic tree of *Tuberculatus* aphids constructed from a mitochondrial gene of cytochrome oxidase subunit I (*COI*) and nuclear gene of *18S rRNA* was used to examine the correlation between *Wolbachia* infection rates and ant associations. The phylogenetic comparative analysis showed that *Wolbachia* infection rates were significantly higher in ant-attended species. Possible *Wolbachia* infection routes are discussed in terms of the differences in the ecological characteristics between ant-attended and non-attended aphid species. This study shows that the spread of microorganisms is affected by host species interactions, and contributes new insights into the evolution of mutualistic interactions.

Introduction

Aphids (Insecta: Hemiptera: Aphididae) feed on plant phloem sap using their sucking and piercing mouthparts, and excrete honeydew including carbohydrates and amino acids (Yao and Akimoto 2001, 2002; Leroy et al. 2011; Renyard et al. 2021). Some aphid species form close associations with ants by offering them honeydew and obtain protection from ants in return (Way 1963; Yao et al. 2000). However, there is growing evidence that mutualistic interactions with ants can have negative effects on aphid physiological and morphological traits, such as changes in sugar and amino acid composition of honeydew (Fischer and Shingleton 2001; Yao and Akimoto 2001, 2002) and decreases in colony size, body size and embryo numbers (Stadler and Dixon 1998; Flatt and Weisser 2000; Yao et al. 2000; Katayama and Suzuki 2002). These examples show that evolution of ant-aphid interactions has resulted in both benefits and costs to aphids (Stadler and Dixon 2005; Yao 2014).

Recently, a number of studies have raised the possibility that microorganisms are involved in the establishment of aphid-ant mutualisms. For example, the bacterium *Staphylococcus xylosus* in *Aphis fabae* produces a blend of semiochemicals that attracts ant scouts (Fischer et al. 2015). Additionally, it is reported that the ant *Lasius niger* could potentially use cuticular hydrocarbons cues to discriminate among aphid lines (*Aphis fabae*) harbouring different endosymbionts (Hertaeg et al. 2021). Henry et al. (2015) demonstrated that two symbiont species, *Hamiltonella defensa* and *Regiella insecticola*, which protect aphids from natural enemies (Oliver et al. 2003; Scarborough et al. 2005; Vorburger et al. 2010), were more likely to occur in aphid species that are *not* tended by ants.

Wolbachia are intracellular bacteria that occur in arthropods and nematodes (Werren et al. 2008; Kauer et al. 2021). It is suggested that more than half of arthropod species are infected with *Wolbachia* (Hilgenboecker et al. 2008; Weinert et al. 2015). At present, it has been reported that the major genotypes of *Wolbachia* are highly diverse and classified phylogenetically into 17 supergroups (A to S except for G and R) (Glowska et al. 2015; Lefoulon et al. 2020). The roles of *Wolbachia* in hosts range from parasitism to mutualism. *Wolbachia* infection can alter host reproduction by inducing feminization, parthenogenesis, male killing, and cytoplasmic incompatibility (Werren et al. 2008). By contrast, *Wolbachia* has been observed associating mutualistically with the bedbug *Cimex lectularius*, providing

B vitamins to the host (Hosokawa et al. 2010).

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; Yao 2019; 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 aphidicola*, the primary endosymbiont of aphids, while Manzano-Marín (2020) rejected the nutrition provision hypothesis by arguing that it was based on a biased interpretation of antibiotic treatment analyses and incorrect genome-based metabolic inference.

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). In a previous phylogenetic independent contrasts analysis, it was found that ant-attended species have higher wing loading (the ratio of wing area to body size) (Yao 2011), suggesting that ant-attended aphids have allocated more resources to their bodies than to their wings, resulting in lowered dispersal. Lower dispersal is likely to result in the formation of aggregated colonies (Stadler et al. 2003). It has also been demonstrated that ant-attended colonies attract more parasitoid wasps compared to ant-excluded colonies (Völkl 1992; Kaneko 2002, 2003; Sadeghi-Namaghi and Amiri-Jami 2018). Considering that host-parasitoid interactions may well be one of the most common horizontal transmission routes of *Wolbachia* (reviewed by Sanaei et al. 2021), it is expected that ant associations will be associated with higher *Wolbachia* infection rates in *Tuberculatus* aphid species.

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

Materials & Methods

DNA extraction and *Wolbachia* infection rate

Tuberculatus aphids (Aphididae: Calaphidini), 11 ant-attended and 12 non-attended species (Table 1 and Table S1), were collected from regions throughout Japan and around Mt. Kariwangsan of South Korea (Fig. S1 and Table S2). A species was regarded as ant-attended if aphids offered honeydew directly from their anus to attending ants. Because it was difficult to physically identify in the field three of the ant-attended aphid species (*T. fulviabdominalis*, *T. indicus*, and *T. pilosulus*) and seven of the 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 genetic sequencing (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 likely to be clones. Therefore, aphids were collected from more than ten leaves in a tree, to avoid collecting clonal aphids. Individuals were placed into 99.5% ethanol and stored at -20℃. Before DNA extraction, the collected aphids were dissected to check for the presence of parasitoid wasps. Aphids with parasitoid wasps were excluded from DNA extraction. 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 2). 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 because it was able to amplify *Wolbachia* at the maximum number of species (seven species) of the 23 species (Table 3). 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 (site 4 in this study) 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 volumes 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 a Mantel test (Mantel 1967) using the package vegan (Oksanen et al. 2012) in R (R Development Core Team 2021). The values of latitude and longitude of collection sites were obtained from Google Maps and were used for the geographic distance matrix. *Wolbachia* infection rates at the collection sites were used for an environmental parameter distance matrix. Except for exhaustive infection of *T.* sp. B, a Mantel test was applied to the species that was collected from more than a single site.

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 were used, but the annealing temperature was changed to 47 °C. PCR products were purified and sent to a sequencing service (using Sanger sequencing) (Eurofins, Japan). The sequence data of the *18S rRNA* gene (515bp) were deposited in the DDBJ and accession numbers are listed in Table 1. 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* 4.0b10 PPC (Swofford 2002) ($P > 0.05$). Maximum likelihood (ML) analysis was performed using PAUP* 4.0a 169. 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 combined sequence of *COI* and *18S rRNA* genes. 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. Because phylogenetic tree for the comparative analysis of independent contrasts must be fully dichotomous with no gaps in the data, outgroup species were excluded from the analysis.

Phylogenetic independent contrasts

As a consequence of their common ancestry, closely related species share many characteristics, and similarity between lineages is often influenced by relatedness rather than by independent evolution. Most statistical tests assume independence of data points and, therefore, data that are phylogenetically non-independent will tend to inflate the degrees of freedom (Felsenstein 1985; Harvey and Pagel 1991). Comparative analysis by independent contrasts (CAIC) uses independent comparisons of components within a phylogeny, with each comparison being made at a different nodes in the phylogeny (Purvis and Rambaut 1995). To examine the correlation between *Wolbachia* infection rates (continuous data as dependent variables) and ant association (discrete data as independent variables) in *Tuberculatus* species, phylogenetically independent contrasts were calculated using the pic function implemented in the package ape (Paradis and Schliep 2019) in R. Discrete data of ant association were coded as continuous variable using the contr.treatment function in R. The extent of ant association was categorized as either 0 (non-attendance) or

1 (facultative and obligate ant-attendance). *Wolbachia* infection rates were arcsine-square root transformed before analysis. The regression of contrasts between ant association and *Wolbachia* infection rates passes through the origin (the intercept is set to zero) as recommended by Garland et al. (1992).

***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 consisting 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 alignments 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 S3) were processed with Clustal W (Thompson et al. 1994) on the DDBJ. Supergroup P was not included in multiple sequence alignments because it had insufficient sequence length for the lower region of the gene. After multiple sequence alignments, the length of sequences was 471bp. To determine what types of *Wolbachia* supergroup are present in *Tuberculatus* aphids, neighbor joining (NJ) with the BioNJ method was applied to the constructed *Wolbachia* phylogenetic tree. NJ analysis was performed using PAUP* 4.0a 169. The distance matrix was calculated using the Jukes-Cantor substitution model. For the bootstrap test on NJ, 1,000 replicates were performed using fast stepwise addition as a starting option.

Results

Wolbachia infection rate

Wolbachia was detected in eight of 11 ant-attended aphid species (Table 1 and Fig. S1(a-f)) and five of 12 non-attended species (Table 1 and Fig. S1(g-l)), in which at least one individual was detected. Mean *Wolbachia* infection rates were 30.2% 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* (Fig. S1b), *T. pappus* (Fig. S1e), and *T*. sp. E (Fig. S1f); 100% in *T*. sp. B (Fig. S1f). The Mantel test on *T. fulviabdominalis* and *T. macrotuberculatus* showed that *Wolbachia* infection rates was significantly correlated with distance between collection sites (for *T. fulviabdominalis*, Mantel statistic $r = 0.842$, $P = 0.035$ (Table 1 and Fig. S1b); for *T. macrotuberculatus*, Mantel statistic $r = 0.164$, $P = 0.03$ (Table 1 and Fig. S1d).

Phylogenetic independent contrasts

The ML phylogenetic tree based on the combined sequences of *COI* and *18S rRNA* genes showed fully resolved tree topology (Fig. 1). CAIC showed a significant positive correlation between contrasts of *Wolbachia* infection rates and ant association (CAIC, $F_{1,21}$

= 13.7, *P* = 0.00134, Fig. 2); *Wolbachia* infection rates in *Tuberculatus* aphids were significantly higher in ant-attended species compared to non-attended species.

Wolbachia **supergroups**

Because the sequencing for *T. pilosulus* and *T*. sp. D was unsuccesful, only 11 *Wolbachia*-positive were analyzed. The results of sequencing showed that each species harboured one haplotype of *Wolbachia* except for *T. macrotuberculatus* (Fig. 3). *Tuberculatus macrotuberculatus* harboured two haplotypes (Fig. 3): one haplotype was found at nine sites (sites 1 to 8 and site 23), the other at site 22. A NJ tree showed that 12 haplotypes of *Wolbachia* were classified into four supergroups B, M, N, and O (Fig. 3). 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 belonged to supergroup M. *Wolbachia* in *T. macrotuberculatus* collected from the site 22, *T. capitatus*, *T. fulviabdominalis* and *T. japonicus* were placed into supergroup N. *Tuberculatus higuchii* A-type harboured

Wolbachia of supergroup O, which was supported with a high bootstrap value (100%). Twelve DNA sequences of *Wolbachia*'s *16S rRNA* were deposited in the DDBJ and accession numbers are listed in Fig. 3 and Table S3.

Discussion

The phylogenetic comparative analysis showed that *Wolbachia* infection rates were higher in aphid species that have mutualistic associations with ants. One possible infection route of *Wolbachia* to aphids could be horizontal transmission between *Wolbachia*-infected parasitoid wasps and aphids. Regardless of whether aphids are attended by ants, aphid colonies are frequently attacked by parasitoid wasps (Brodeur and Rosenheim 2000). Field experiments on some ant-attended aphid species demonstrated that ant-attended colonies attracted more parasitoid wasps compared to ant-excluded colonies (Völkl 1992; Kaneko 2002, 2003; Sadeghi-Namaghi and Amiri-Jami 2018). These behaviours of parasitoid wasps are thought to be triggered by visual and chemical cues from aphid colonies attended by ants (Mouratidis et al. 2021). Ant-attended species form dense colonies (Stadler et al. 2003) and disperse less than non-attended species (Oliver et al. 2007; Yao 2010), which could by itself an explanation for a higher *Wolbachia* prevalence. 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 *Bemisia 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; Tsutsui et al. 2003; Viljakainen et al. 2008; Frost et al. 2010; Reeves et al. 2020) and thus could be a possible agent to spread *Wolbachia* into aphid populations. In a study of scale insects and their associated groups (ants, wasps, beetles, flies, mites, moths, and thrips), Sanaei et al. (2022) showed that significantly higher *Wolbachia* infection rates in ant-attended scale insects, suggesting a possible horizontal transfer route between ants and scale insects. This study did not aim to identify the *Wolbachia* strains of parasitoid wasps or attending ants. Further studies on *Wolbachia* strains for aphids and their parasitoid wasps or their mutualistic ants are need to elucidate the possible routes by ants.

Although the average *Wolbachia* infection rates was higher in ant-attended species (30.2%) than in non-attended species (3.1%), a wide range of variation was found in the infection rates for ant-attended species (0-100%). The difference in realized infection rate can be attributed to ecological or environmental factors affecting the cost-benefit balance of *Wolbachia* infection to hosts (Gavotte et al. 2010; White et al. 2011; Okayama et al. 2016). Higher infection levels across all populations of *T. capitatus* (on average 94.6% from 15 sites) and *T*. sp. B (100 % from 4 sites) could be responsible for positive selection favouring benefits from *Wolbachia* infection such as nutrition provision (Hosokawa et al. 2010; De Clerck et al. 2015; but see Manzano-Marín 2020) or resistance to parasitoid wasps (Oliver et al. 2003). Hence, it could be possible that *Wolbachia* plays obligate mutualistic roles in *T. capitatus* and *T*. sp. B. On the other hand, for the species with infection rate of between 10 and 52 %, it is difficult to determine whether *Wolbachia* infection is a mutualistic or parasitic interaction with the species. The previous study of seasonal changes in *Wolbachia* density in a population (site 4 in this study) of *T. macrotuberculatus* showed that 315 of 316 (99.7%) of the aphids harboured *Wolbachia* and

Wolbachia density in an individual aphid exhibited no significant fluctuations during the survey period, implying that seasonal deterioration of host plants did not affect *Wolbachia* density, even though host aphids decreased in their body size and embryo numbers (Yao 2019). *Wolbachia* of the aphids in this site seems to give a beneficial effect on the nutritional status of aphids during the harsh summer. However, in this study, no *Wolbachia*-infected aphids were found in 13 of 23 collection sites of *T. macrotuberculatus*. Furthermore, there was a significant correlation between geographical distance and difference in infection rates in two species *T. fulviabdominalis* and *T. macrotuberculatus*. This means that there is an isolation-by-distance effect among the collection sites. Indeed, it has been demonstrated that the genetic structure of *T. macrotuberculatus* in Hokkaido populations shows a higher inbreeding coefficient in each subpopulation and less dispersal due to ant attendance (Yao 2010), suggesting that region-specific patterns as to whether *Wolbachia* infection is costly or beneficial could occur in isolated populations. For the species with less than 5 % infection rate, three of four species (*T. japonicus*, *T. paiki*, and *T.* sp. D) are non-attended species and sometimes have been observed with ant-attended species (*T. fulviabdominalis*, *T. macrotuberculatus*, *T. stigmatus* and *T*. sp. B) on the same host plant (*Quercus dentata*). This sympatric host plant use might provide the non-attended species with an opportunistic infection of *Wolbachia*, such as via plant-mediated horizontal transmission (Li et al. 2017).

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 (100%) will support existence of supergroup O. Given that supergroup O has so far been found only in China, it could have originated in East Asia and spread into Japan. As *Wolbachia* supergroups have evolved independently, infections by different supergroups presumably represent independent gains of the trait even for two species with the same ant-attendance state, but these are ignored in the current analysis. This would be overcome by the comparison of characteristics of hosts infected by different supergroups and distributed in close distance areas. *Tuberculatus macrotuberculatus* harboured two phylogenetically-distant supergroups of M and N as previously seen in Moreira et al. (2019); the two sites of southern island, site 22 and site 23 (apart from approximately 40 km, Fig. S1d), had the supergroup N and supergroup M, respectively. Comparison between the two populations may help to elucidate the difference of independent gains of *Wolbachia* supergroup involving aphid-ant mutualisms.

This study has revealed that the ecological characteristics of aphid hosts have influenced the extent of *Wolbachia* spread in these species. Further studies are needed to clarify what roles *Wolbachia* play in aphids, especially for ant-attended aphid species.

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Authors' contributions IY conceived the study, collected samples, carried out molecular work, analyzed the data and wrote the manuscript.

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

Declarations

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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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Figure legends

Fig. 1. Maximum likelihood phylogenetic tree based on the combined sequences of *COI* and *18S rRNA* genes.

(Note) Operational taxonomic units (OTUs) shown in bold font indicate ant-attended species. The numbers on the branches of phylogenetic tree show bootstrap values (>50%).

Full terms of abbreviations are provided in Table 1.

Fig. 2. Correlation between contrasts of ant association and *Wolbachia* infection rates.

(Note) Contrastant and contrastwol represent independent contrasts that were calculated based on the status of ant association and *Wolbachia* infection rates at each node (n = 22) in the ML phylogenetic tree.

Fig. 3. Bootstrap 50 % majority-rule consensus tree inferred by neighbor-joining (NJ) analysis for 16 *Wolbachia* supergroups

(Note) The labels of operational taxonomic units (OTUs) mean *Wolbachia* sp. (indicated by w) and its host species. Thick vertical lines with alphabets indicate the clades of *Wolbachia* supergroups. See also Table S3. Bootstrap values of more than 50% were shown on branches. Full terms of abbreviations are provided in Table 1.

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

(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*. Except for exhaustive infection of *T*. sp. B, a Mantel test was applied to the species that were collected from more than a single site. Statistics of Mantel test, r, and *P*-values are given. The bold font shows a significant difference below 0.05 of *P*-values. Abbreviated names were used in Table 3, all 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 and Windsor (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 et al. (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 using seven pairs of primers

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.

Fig. 1

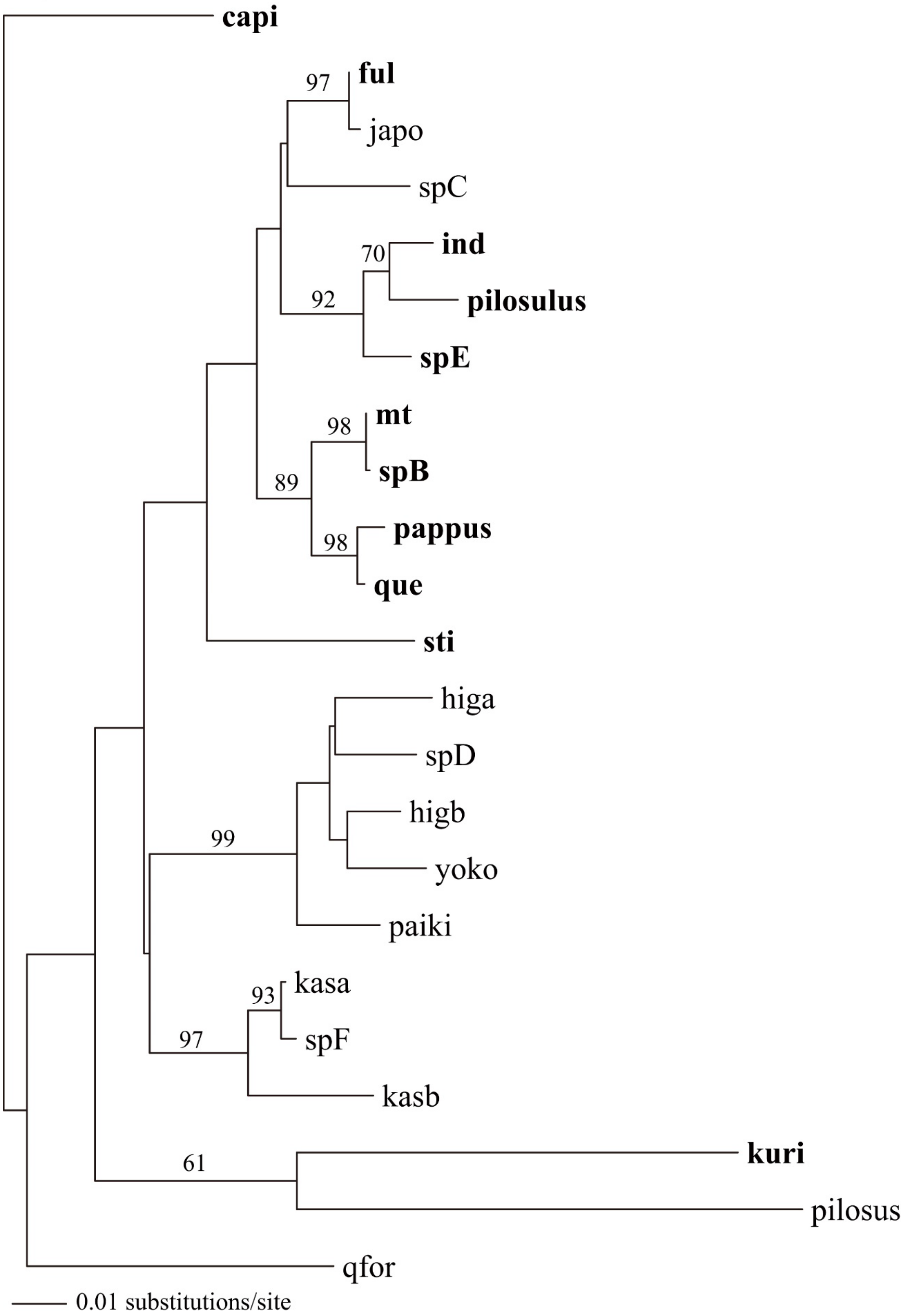
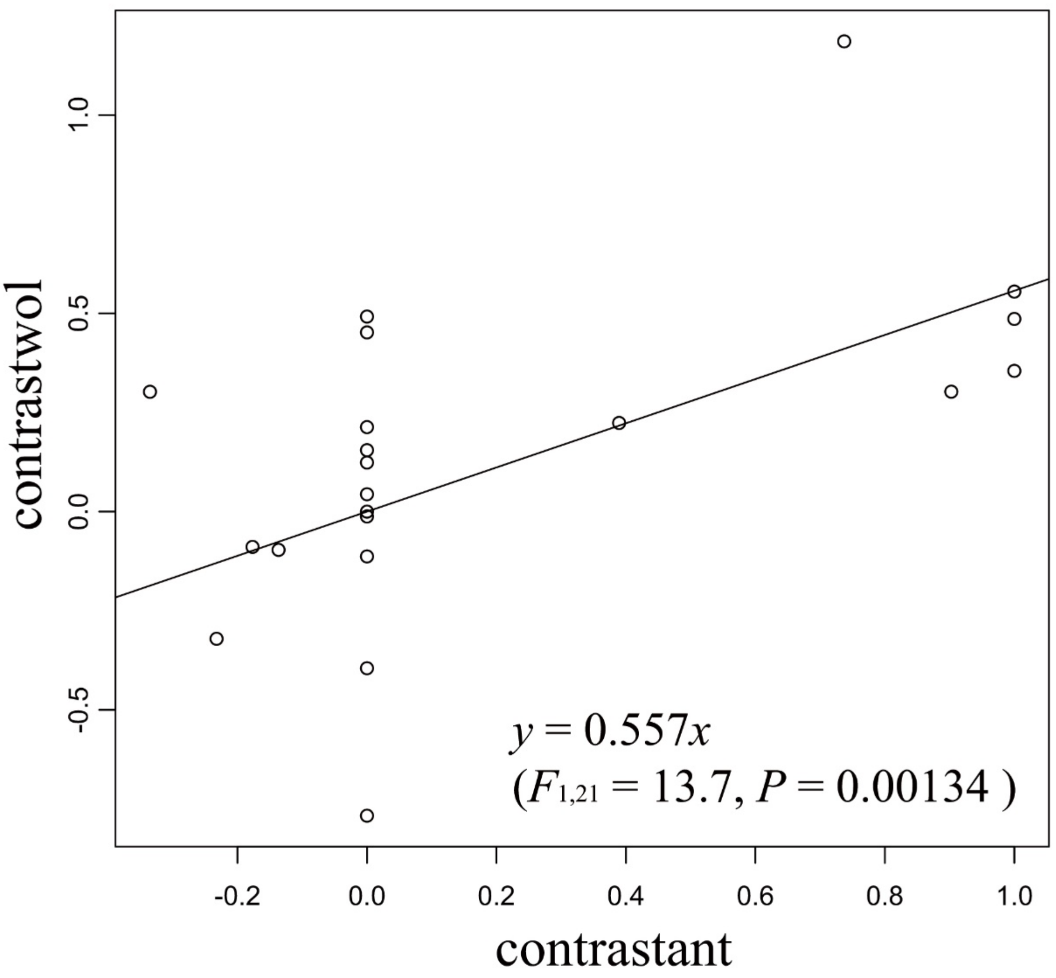
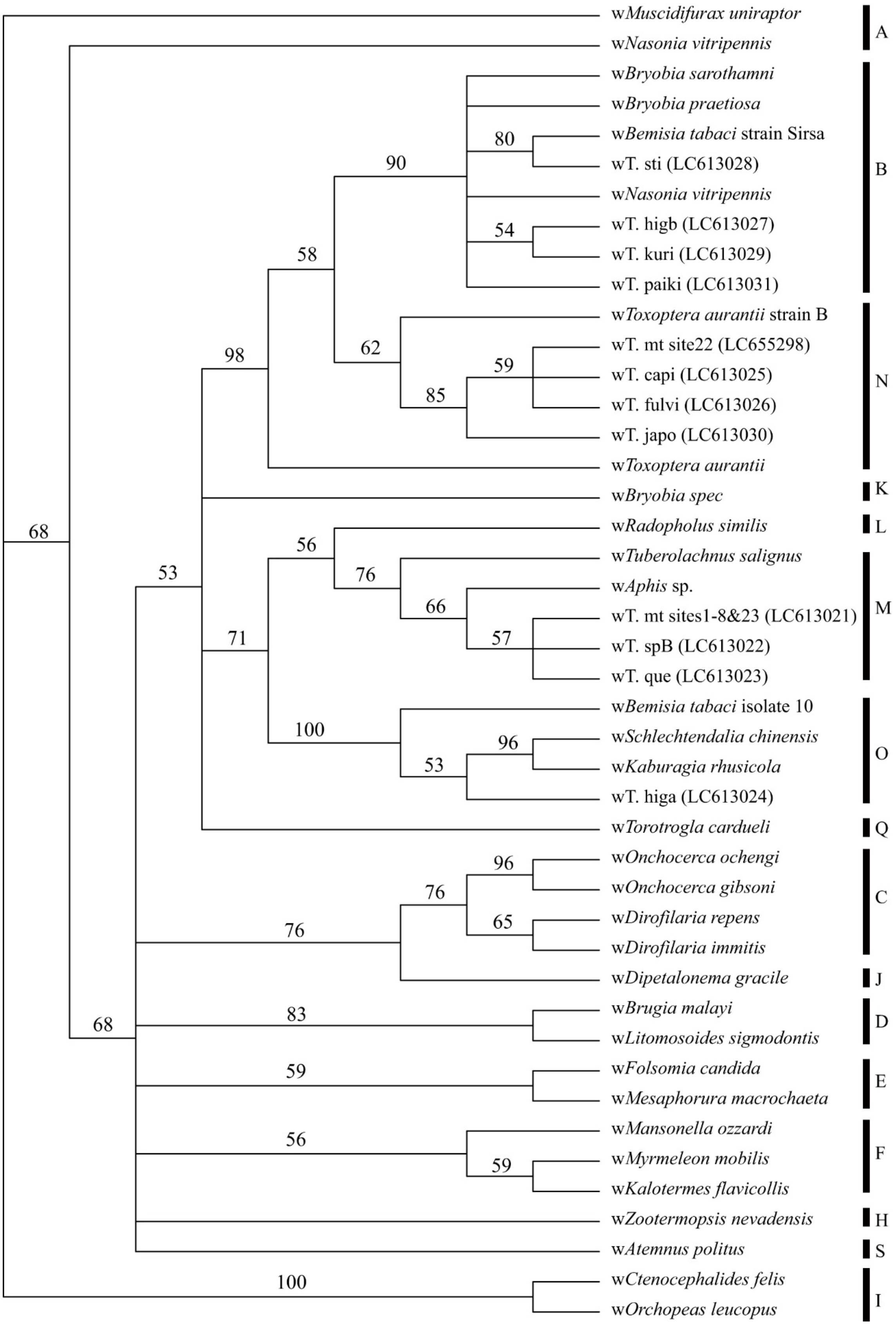

capi
97
ful
japo
spC
70
ind
92
pilosulus
spE
98
mt
89
spB
98
pappus
que
sti
higa
spD
higb
99
yoko
paiki
93
kasa
97
spF
kasb
61
kuri
pilosus
qfor
0.01 substitutions/site

Fig. 2



1.0
0.5
0.0
-0.5
contrastwol
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
contrastant
$y = 0.557x$
$(F_{1,21} = 13.7, P = 0.00134)$

Fig. 3


w*Muscidifurax uniraptor*
w*Nasonia vitripennis*
A
w*Bryobia sarothamni*
w*Bryobia praetiosa*
w*Bemisia tabaci* strain Sirsa
wT. sti (LC613028)
w*Nasonia vitripennis*
wT. higb (LC613027)
wT. kuri (LC613029)
wT. paiki (LC613031)
B
w*Toxoptera aurantii* strain B
wT. mt site22 (LC655298)
wT. capi (LC613025)
wT. fulvi (LC613026)
wT. japo (LC613030)
w*Toxoptera aurantii*
N
w*Bryobia spec*
K
w*Radopholus similis*
L
w*Tuberolachnus salignus*
w*Aphis* sp.
wT. mt sites1-8&23 (LC613021)
wT. spB (LC613022)
wT. que (LC613023)
M
w*Bemisia tabaci* isolate 10
w*Schlechtendalia chinensis*
w*Kaburagia rhusicola*
wT. higa (LC613024)
O
w*Torotrogla cardueli*
Q
w*Onchocerca ochengi*
w*Onchocerca gibsoni*
w*Dirofilaria repens*
w*Dirofilaria immitis*
C
w*Dipetalonema gracile*
J
w*Brugia malayi*
w*Litomosoides sigmodontis*
D
w*Folsomia candida*
w*Mesaphorura macrochaeta*
E
w*Mansonella ozzardi*
w*Myrmeleon mobilis*
w*Kalotermes flavicollis*
F
w*Zootermopsis nevadensis*
H
w*Atemnus politus*
S
w*Ctenocephalides felis*
w*Orchopeas leucopus*
I
80
90
54
58
98
62
59
85
68
53
56
76
66
71
57
100
96
53
96
76
65
76
83
68
59
56
59
100