

Closely Related *Wolbachia* Strains within the Pumpkin Arthropod Community and the Potential for Horizontal Transmission via the Plant

S. Sintupachee¹, J. R. Milne¹, S. Poonchaisri², V. Baimai^{1,3} and P. Kittayapong^{1,3}

(1) Department of Biology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand

(2) Entomology and Zoology Group, Plant Protection Research and Development Office, Ministry of Agriculture and Co-operatives, Chatuchak, Bangkok 10900, Thailand

(3) Center for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand

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Abstract

Phylogenetic studies have implicated frequent horizontal transmission of *Wolbachia* among arthropod host lineages. However, the ecological routes for such lateral transfer are poorly known. We surveyed the species of two arthropod communities, one on pumpkin and the other on loofah plants, for *Wolbachia*, constructed *wsp* gene phylogenies of those *Wolbachia* strains found to infect community members, and established ecological links among infected members. Four taxonomically diverse insects in the pumpkin arthropod community contained very closely related *Wolbachia wsp* sequences (<1.5% divergence by Kimura-2-parameter distances). These insects, namely, the whitefly *Bemisia tabaci*, the planthopper *Nisia nervosa*, the flea beetle *Phyllotreta* sp., and the fleahopper *Halticus minutus*, were all collected from pumpkin leaves. They were ecologically linked through feeding on the same leaf substrate. Unlike other infected leaf insects, the whitefly population appeared to have a permanent breeding relationship with pumpkin plants, and high and stable, but not fixed, monthly *Wolbachia* infection rates. Our findings suggest potential roles for the plant in *Wolbachia* transmission and for whiteflies in being an infection source for other pumpkin leaf-feeding insects.

Introduction

Wolbachia are endosymbiotic α -proteobacteria that are extremely widespread among arthropod species [30] and

are well known for their diverse effects on arthropod reproduction [23, 24, 29]. Normally, *Wolbachia* are vertically transmitted from mother to offspring. However, phylogenetic studies have often detected very closely related *Wolbachia* gene sequences in distantly related arthropods. This suggests either that horizontal transmission of whole bacteria between host species had occurred [26, 28, 32] or that genes had transferred between *Wolbachia* bacteria that had come into close proximity within the same host [1, 10, 12, 31]. Whether it be *Wolbachia* bacteria or genes that transfer, an ecological link between species is required that brings *Wolbachia* into novel hosts [9].

Evidence from both phylogenetic [3, 22, 27] and experimental studies [6, 8] that specifically target the parasitoid–host relationship point to this interaction as being a likely transfer route. However, most strains that appear closely related in *Wolbachia* phylogenies occur in arthropods not linked by this interaction and other means of horizontal transfer seem likely.

We took a different approach for determining potential *Wolbachia* horizontal transmission routes among arthropods. Rather than target a specific ecological interaction such as parasitoidism, we collected all available arthropods within two arthropod communities, one on pumpkin (*Cucurbita moschata*) and the other on loofah (*Luffa cylindrica*) plants, without prior knowledge of the interactions among community members. We then constructed *Wolbachia* phylogenies and determined the ecological links among infected species. We found four taxonomically diverse insect species in the pumpkin community that harbor very similar *Wolbachia* strains. All four species feed on pumpkin leaf tissue. We discuss the potential for the plant to be a medium for *Wolbachia* transmission among leaf-feeding insects.

Correspondence to: J. R. Milne; E-mail: frjrm@mahidol.ac.th

Methods

Arthropod Collection and Identification. Arthropods were collected from a 10 × 10 m pumpkin and loofah field plot at Sai Yok (14°00'N, 99°33'E), western Thailand. In many *Wolbachia* surveys, few specimens per species are tested and negative results may reflect low sample size rather than uninfected species. We undertook intensive surveys over more than a year to test as many specimens as possible to improve *Wolbachia* detection. Sampling was conducted monthly from October 1999 to September 2000 and in October and November 2001. All arthropods on arboreal plant parts were collected into labeled vials, taken back live to the laboratory, and frozen (−20°C). Fruits, flowers, and leaves infested internally with arthropods were brought back to an insectary ($T = 26 \pm 2^\circ\text{C}$, $\text{RH} = 65 \pm 10\%$) and placed on moist sawdust in ventilated cylindrical plastic containers. Arthropods that emerged were placed in labeled vials and frozen (−20°C).

Arthropods were sorted into species groups. Some individuals of each species were identified and deposited as voucher specimens in the Insect Museum of the Plant Protection Research and Development Office, Department of Agriculture, Bangkok. The remainder was tested for *Wolbachia*. All except whiteflies (F. Aleyrodidae, O. Homoptera) were identified using specialist morphological keys. Whiteflies were identified molecularly by Dr. Paul DeBarro (CSIRO, Australia).

Polymerase Chain Reaction. DNA was extracted from reproductive tissues removed from large specimens. Whole abdomens or whole individuals were used for small specimens. The STE method [16] was used for 1999/2000 samples and phenol–chloroform extraction [20] for 2001 specimens.

A reaction mix was made for each polymerase chain reaction (PCR) run; 20 µL of the mixture [2 µL 10× buffer (Promega), 2 µL MgCl₂ (Promega), 0.5 µL 100 mM dNTPs, 0.5 µL each of forward and reverse primer, 1 µL *Taq* DNA polymerase, and 13.5 µL double-distilled H₂O] was added to each 0.65-mL microcentrifuge tube. DNA template (1 µL) was added to each reaction volume and topped with 1 drop of sterilized mineral oil. PCR amplification used the following thermal profile: one 3-min cycle at 95°C; 30 cycles of 1 min each at 95, 55, and 72°C; one 10-min cycle at 72°C. PCR products and a 1-kb ladder (Promega) were loaded onto a 1% agarose gel, stained with ethidium bromide, and visualized under a UV transilluminator.

Every month for each species, the first PCR was a batch PCR in which the DNA of up to 10 specimens (0.1 µL template/specimen) was pooled. If a batch was *Wolbachia*-positive, then 1 µL DNA template of each specimen was tested. *ftsZ* primers [7] were used to detect *Wolbachia*. A mosquito from a *Wolbachia*-infected *Aedes*

albopictus culture (Department of Biology, Mahidol University) was used as a positive control in each PCR run. A negative control to check for contamination consisted of the normal DNA extraction procedure but without a specimen. Negative results may have been due to unsuccessful DNA extraction. To check this, 38% of samples negative by *ftsZ* primers were tested for insect DNA using the insect mitochondrial 12S rRNA primers, 12SAI and 12SBI [16].

Cloning and Sequencing. DNA from one *Wolbachia*-positive specimen of each infected species was used for cloning and sequencing. PCR products were obtained from DNA amplification using the general *wsp* primers, *wsp81F* and *wsp691R* [32], and the same thermal profile as for *ftsZ* amplification except for 35 cycles in the

Table 1. *Wolbachia* host species from which *wsp* sequences were derived for phylogenetic analyses

Host species	Accession no.
From this study	
<i>Bemisia tabaci</i>	AY157679
<i>Diachasmimorpha longicaudata</i>	AY157680
<i>Graptomyza brevisostis</i>	AY157681
<i>Halticus minutus</i>	AY157682
<i>Onthophagus vaulongeri</i>	AY157683
<i>Phyllotreta</i> sp.	AY157684
<i>Nisia nervosa</i>	AY157685
Extracted from GenBank	
<i>Acraea encendon</i>	AJ130716
<i>Aedes albopictus</i> A	AF020058
<i>Aedes albopictus</i> B	AF020059
<i>Bemisia afer</i>	AJ291370
<i>Bemisia tabaci</i>	AJ291376
<i>Blastophaga brownii</i>	AF521165
<i>Culex quinquefasciatus</i>	AF020060
<i>Drosophila melanogaster</i> (Aub)	AF020063
<i>Drosophila simulans</i> (Coff)	AF020067
Extracted from GenBank (ctd)	
<i>Drosophila simulans</i> (Riv)	AF020070
<i>Drosophila simulans</i> (Haw)	AF020068
<i>Drosophila simulans</i> (Nou)	AF020074
<i>Ephestia cautella</i> A	AF020075
<i>Ephestia cautella</i> B	AF020076
<i>Glossina austeni</i>	AF020077
<i>Glossina brevipalpis</i>	AF164685
<i>Glossina centralis</i>	AF020078
<i>Glossina morsitans</i>	AF020079
<i>Laodelphax striatellus</i>	AF020080
<i>Muscidifurax uniraptor</i>	AF020071
<i>Nasonia vitripennis</i>	AF020081
<i>Phlebotomus papatasi</i>	AF020082
<i>Rhagoletis cerasi</i>	AF418557
<i>Tagosedes orizicolus</i>	AF020085
<i>Torymus bedeguaris</i>	AF071915
<i>Tribolium confusum</i>	AF020083
<i>Trichogramma deion</i>	AF020084

The *wsp* sequences from this study were lodged in GenBank; other *wsp* sequences were extracted from GenBank. Accession numbers of all sequences are given in the table.

Table 2. PCR detection of *Wolbachia* among pumpkin arthropod species at Sai Yok, western Thailand

Arthropod species	PP	No. +ve/ tested
Class Arachnida		
Order Arachnida		
F. Parasitidae Unid. sp. 1	L	0/23
F. Parasitidae Unid. sp. 2	L	0/1
Class Insecta		
Order Blattaria		
<i>Blattella germanica</i> (Linnaeus)	L Fr	0/4
Order Coleoptera		
<i>Astycus</i> sp.	L	0/1
<i>Aulacophora frontalis</i> Baly	L Fl	0/7
<i>Aulacophora indica</i> Gmelin	L Fr	0/70
<i>Aulacophora similis</i> (Olivier)	Fl	0/1
<i>Blasyrus herthus</i> Hbst.	L	0/1
<i>Calomyeterus</i> sp.	L	0/2
<i>Coccinella transversalis</i> Fabricius	L	0/7
<i>Coelophora bisellata</i> Mulsant	L	0/5
<i>Epilachna indica</i> Mulsant	L	0/1
<i>Epilachna vigintioctopunctata</i> Fabricius	L	0/11
<i>Eugnathus alterans</i> Fabricius	L	0/1
<i>Haptoncus</i> sp.	Fl	0/10
<i>Lema rufotestacea</i> Clark	L	0/4
<i>Lycostomus lateritius</i> Gorham	L	0/1
<i>Lygaria westermanni</i> Stal	L	0/2
<i>Micraspis discolor</i> (Fabricius)	L	0/1
<u><i>Monolepta signata</i> (Olivier)</u>	<u>L</u>	<u>1/19</u>
<u><i>Onthophagus vaulongeri</i> Bouche*</u>	<u>Fr</u>	<u>1/6</u>
<i>Phrixopogen hausti</i> Marshall	L	0/1
<u><i>Phyllotreta</i> sp.*</u>	<u>L</u>	<u>1/4</u>
F. Carabidae Unid. sp.	FL	0/10
F. Staphylinidae Unid. sp.	L	0/2
Order Diptera		
<i>Agromyza</i> sp.	L	0/3
<i>Bactrocera cucurbitae</i> (Coquillett)	Fr	0/1
<i>Bactrocera diversa</i> (Coquillett)	Fr	0/1
<u><i>Bactrocera tau</i> (Walker)</u>	<u>Fr</u>	<u>3/44</u>
<u><i>Graptomyza brevirostris</i> (Wiedemann)*</u>	<u>Fr</u>	<u>3/5</u>
Order Diptera (ctd)		
<u><i>Melanagromyza</i> sp.</u>	<u>Fl</u>	<u>2/2</u>
<i>Sarcrohndendorfia</i> sp.	Fr	0/1
<i>Sympycnus</i> sp.	Fr	0/13
<i>Syrirta ruffifacies</i> Big	L	0/2
F. Agromyzidae Unid. sp.	Fl	0/1
F. Drosophilidae Unid. sp.1	Fr	0/5
F. Drosophilidae Unid. sp.2	Fr	0/1
Order Hemiptera		
<i>Cletus trigonus</i> Thunberg	L	0/6
<i>Dysdercus cingulatus</i> (Fabricius)	L	0/1
<u><i>Halticus minutus</i> Reuter*</u>	<u>L</u>	<u>49/129</u>
<i>Leptocoris oratorius</i> (Fabricius)	L	0/1
<i>Leptoglossus gonagra</i> (Fabricius)	L	0/3
<i>Malcus scutellatus</i> Distant	L	0/5
F. Plataspididae Unid. sp.	L	0/3
Order Homoptera		
<u><i>Bemisia tabaci</i> (Gennadius)*</u>	<u>L</u>	<u>127/172</u>
<i>Botrogonia indistincta</i> Walker	L	0/7
<i>Callitettix versicolor</i> Fabricius	L	0/10
<i>Geocoris</i> sp.	Fr	0/2
<u><i>Nisia nervosa</i> (Motsch)*</u>	<u>L</u>	<u>1/5</u>
F. Aphididae Unid. sp. 1	L	0/2
F. Cicadellidae Unid. sp. 1	L	0/2

Table 2. Continued

Arthropod species	PP	No. +ve/ tested
F. Cicadellidae Unid. sp. 2	L	0/2
Order Hymenoptera		
<i>Apis florea</i> Fabricius	Fl	0/1
<i>Celyphus scutatus</i> Wiedeman	Fl	0/3
<u><i>Diachasmimorpha longicaudata</i> (Ashmead)*</u>	<u>Fr</u>	<u>15/19</u>
F. Apidae Unid. sp.	Fr	0/1
F. Formicidae Unid. sp.	L	0/5
Order Odonata		
<i>Ischnura</i> sp.	L	0/5
Order Thysanoptera		
<u><i>Frankliniella schultzei</i> (Trybom)</u>	<u>L Fl</u>	<u>1/11</u>

Species that tested positive using *ftsZ* primers are underlined; those confirmed by *wsp* sequencing are marked by an asterisk. Some species were identified to family but not to genus or species, and are represented by their family (F.) name followed by "Unid. sp." L: leaf; Fr: fruit; Fl: flower.

last step. One microlitre of each PCR product was used for DNA ligation and cloning using a pGEM-T plasmid vector (TA cloning Kit, Promega) inserted into *Escherichia coli* bacteria following the manufacturer's instructions. Three clones for each *Wolbachia* strain were sequenced using the dideoxy chain termination method [21] on an ABI automated sequencer.

Phylogenetic Analyses. Seven *Wolbachia wsp* sequences from pumpkin insects and 27 from GenBank were used in phylogenetic analyses (Table 1). Sequences were aligned by the clustal algorithm followed by manual modification. The third hypervariable region of the *wsp* gene (positions 519–559) was excluded because it could not be confidently aligned [32]. Phylogenies were generated by maximum parsimony (MP), maximum likelihood (ML), and neighbor-joining (NJ) methods. For MP analysis, heuristic searches with a stepwise addition method were performed and gaps were treated as missing data. For NJ analysis, a tree was generated from Kimura-2-parameter (K-2-p) distances by Modeltest 2.1 [17] using heuristic searches. For ML analysis, substitution (transversion/transition) rates were estimated and the general time reversible model was used; the proportion of invariant sites was 0.418 and the value of the α parameter of the gamma distribution was set at 1.33 as determined by Modeltest 2.1. Phylogenetic trees were generated using PAUP 4.0 b1 [25]. All trees were midpoint-rooted. Bootstrap values were obtained from 1000 replicates each for NJ and MP analyses and 100 replicates for ML analysis.

Results

***Wolbachia* Distribution among Species.** A total of 59 arthropod species (from 930 specimens) collected from

pumpkin and loofah plants during the 2 years of sampling were PCR-tested for *Wolbachia* (Tables 2 and 3). All 59 species were found on pumpkin plants, whereas only nine occurred on loofah plants. Collected arthropods encompassed two arthropod classes, the Arachnida (with two species in the mite Order Acarina) and the Insecta (comprising 57 species).

Positive PCR results were obtained for 11 insect, but no mite, species from pumpkin plants (Table 2): three each in the Orders Coleoptera (*Monolepta signata*, *Onthophagus vaulongeri*, *Phyllotreta* sp.) and Diptera (*Bactrocera tau*, *Graptomyza brevirostris*, *Melanagromyza* sp.), two in the Order Homoptera (*Bemisia tabaci*, *Nisia nervosa*), and one each in the Orders Hymenoptera (*Diachasmimorpha longicaudata*), Hemiptera (*Halticus minutus*), and Thysanoptera (*Frankliniella schultzei*). Only one insect species from loofah in the Order Diptera (*Bactrocera tau*) gave positive PCR results (Table 3). Of those specimens that tested negative using *ftsZ* primers, 274 were PCR-tested using 12S rRNA primers. Of these, 206 or 75.2% were positive, thus indicating that DNA had been successfully extracted from the majority of specimens.

Wolbachia presence was confirmed by *wsp* sequencing in seven of the 11 *ftsZ*-positive species (Tables 2 and 3). Three—the wasp parasitoid, *Diachasmimorpha longicaudata*, the syrphid fly, *Graptomyza brevirostris*, and the scarab beetle, *Onthophagus vaulongeri*—emerged from pumpkin fruit. The remaining four, i.e., the white fly, *Bemisia tabaci*, the fleahopper, *Halticus minutus*, the planthopper, *Nisia nervosa*, and the flea beetle, *Phyllotreta* sp., were found on pumpkin leaves. For the four unconfirmed species (the leaf beetle, *Monolepta signata*, the tephritid fruit fly, *Bactrocera tau*, from both pumpkin and loofah fruit, the stem fly, *Melanagromyza* sp., and the flower thrips, *Frankliniella schultzei*), DNA could not

be amplified using *wsp* primers. This was despite trying common remedies for such PCR problems, such as altering the DNA template concentration, PCR reaction mix, and thermoprofile. We only had one or two positive samples for the leaf beetle, stem fly, and flower thrips, and DNA may have denatured after long-term storage. Attempts to collect more specimens of these three species were unsuccessful. For *B. tau* flies, however, we had numerous fresh *ftsZ*-positive DNA samples but could not amplify any using *wsp* primers. PCR bands using *ftsZ* primers for these flies were slightly lower and much less intense than those for other infected species. In addition, attempts to maintain *Wolbachia* in fly cultures were unsuccessful because PCR did not detect these bacteria in successive generations. We therefore suspect the positive *ftsZ* results for *B. tau* to be a PCR artifact and that it was not infected by *Wolbachia*.

Phylogenetic Relationships among *Wolbachia* Strains. The ML, MP, and NJ trees all showed the division of *Wolbachia* strains into two major clades, which correspond with the A and B groups of previous 16S rRNA [16], *ftsZ* [28] and *wsp* [32] phylogenetic analyses. Within each of the A and B groups, the three trees were almost identical, with minor differences in positions of some internal nodes. Bootstrap support was greater than 50% for the majority of nodes in each tree with many being greater than 70%. For brevity, we show only the NJ tree (Fig. 1).

Within the A group, the only difference among trees was the placement of the *Glossina brevipalpis* *Wolbachia* strain. In both the ML and MP trees, this strain was placed in the same clade as the *Wolbachia* strains of *Drosophila simulans* (Riv), *Diachasmimorpha longicaudata*, and *Blastophaga brownii*. The node for this placement, however, had low (<50%) bootstrap support in both trees. The NJ tree, in contrast, placed the *Wolbachia* strain of *G. brevipalpis* in the same clade, with high (90%) bootstrap support, as those of *Glossina morsitans*, *Nasonia vitripennis*, and *Glossina centralis* (Fig. 1). Within the B group, the only difference was the placement of the clade containing the *Onthophagus vaulongeri* and *Trichogramma deion* *Wolbachia* strains. In the ML tree, this clade was separate from the large clade comprising the *Wolbachia* strains of *Laodelphax striatellus*, *Acraea encedon*, *Bemisia afer*, *Bemisia tabaci*, *Nisia nervosa*, *Phyllotreta* sp., *Halticus minutus*, *Torymus bedeguaris*, and *Tribolium confusum*, whereas in the MP and NJ trees the *O. vaulongeri*–*T. deion* clade was closely related to this large clade with high bootstrap support of 89 and 98%, respectively.

Wolbachia strains in pumpkin arthropods encompassed both A and B groups (Fig. 1). The *Wolbachia* strains of the wasp parasitoid, *Diachasmimorpha longicaudata*, and the syrphid fly, *Graptomyza brevirostris*,

Table 3. PCR detection of *Wolbachia* among loofah insect species at Sai Yok, western Thailand

Arthropod species	PP	No. +ve/tested
Order Blattaria		
<i>Blattella germanica</i> (Linnaeus)	Fl	0/1
Order Coleoptera		
<i>Aulacophora frontalis</i> Baly	L	0/10
<i>Aulacophora indica</i> Gmelin	L Fl	0/4
<i>Epilachna indica</i> Mulsant	L	0/1
<i>Epilachna vigintioctopunctata</i> Fabricius	L	0/5
Order Diptera		
<u><i>Bactrocera tau</i> (Walker)</u>	<u>Fr</u>	<u>13/222</u>
Order Homoptera		
<i>Botrogonia indistincta</i> (Walker)	L	0/2
<i>Callitettix versicolor</i> Fabricius	L	0/7
Order Thysanoptera		
<i>Frankliniella schultzei</i> Trybom	Fl	0/9

Only insects and no other types of arthropod were found. Species that tested positive using *ftsZ* primers are underlined; *Wolbachia* infection was not confirmed by *wsp* sequencing. Plant parts (PP): L: leaf; Fr: fruit; Fl: flower.

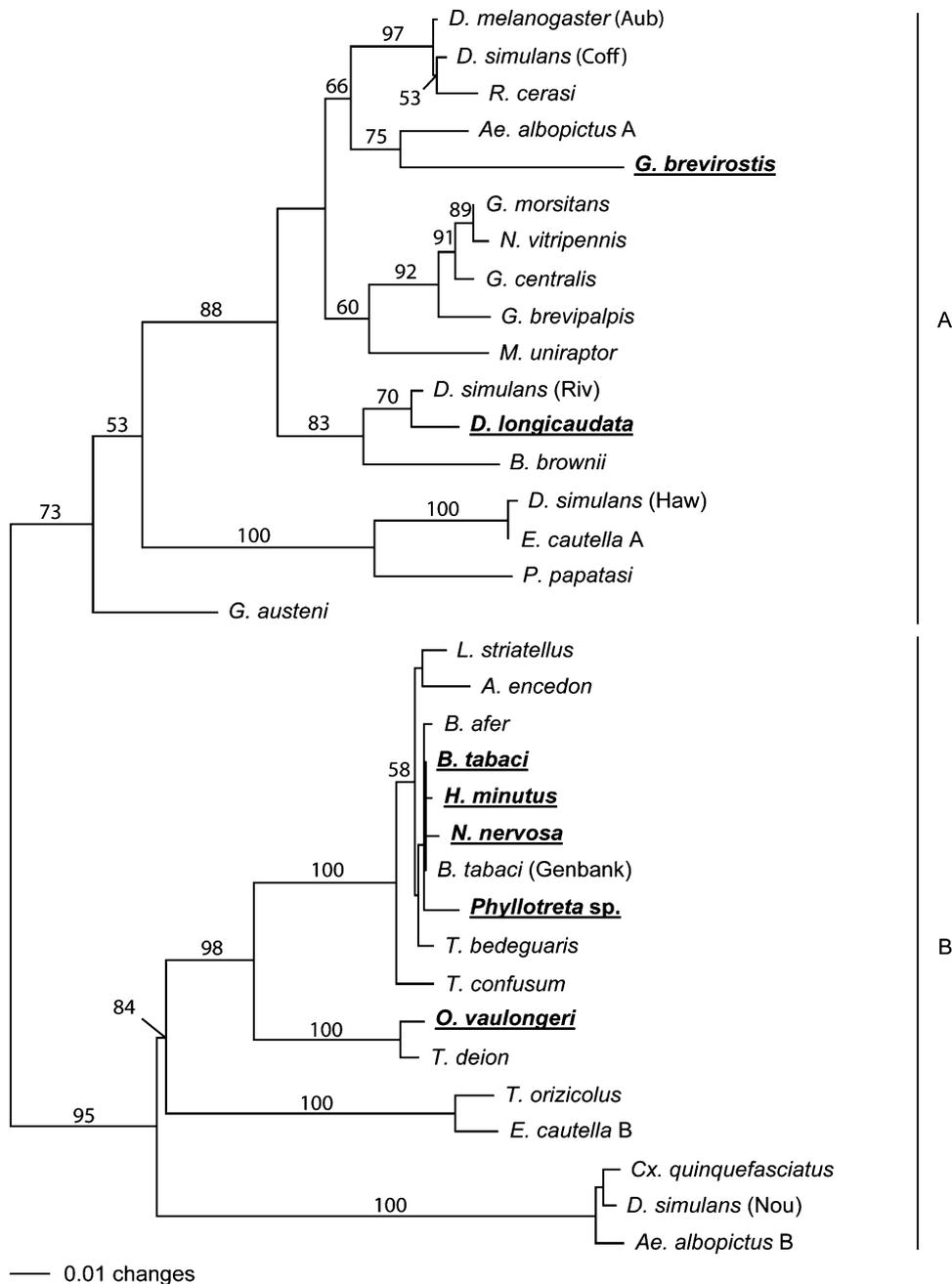


Figure 1. A midpoint rooted neighbor-joining phylogenetic tree of *Wolbachia wsp* sequences including seven sequences from *Wolbachia*-infected pumpkin insects (**bold and underlined**). Numbers at nodes are bootstrap values greater than 50% obtained from 1000 replicates. A and B refer to the major *Wolbachia* divisions.

both from fruit, were placed in the A group, whereas *Wolbachia* strains of the remaining four species, i.e., *Phyllotreta* sp., *Halticus minutus*, *Bemisia tabaci*, and *Nisia nervosa*, collected from leaves and the scarab beetle, *Onthophagus vaulongeri*, from fruit were placed in the B group. The *Wolbachia* A group strains of *D. longicaudata* and *G. brevirostris* were placed into separate clades (Fig. 1); the *G. brevirostris* strain with the *Aedes albopictus* A strain, and the *D. longicaudata* strain in the clade

containing the *D. simulans* (Riv) and *B. brownii* strains. B group *Wolbachia* strains from pumpkin insects were placed into two clades. The four *Wolbachia* strains from pumpkin leaf insects, namely *Bemisia tabaci*, *N. nervosa*, *Phyllotreta* sp., and *H. minutus*, were all placed in the large clade containing the *Laodelphax striatellus*, *A. encedon*, *Tribolium confusum*, *B. afer*, *B. tabaci* (from GenBank), and *Torymus bedeguaris* *Wolbachia* strains (Fig. 1). Bootstrap support was very high for this

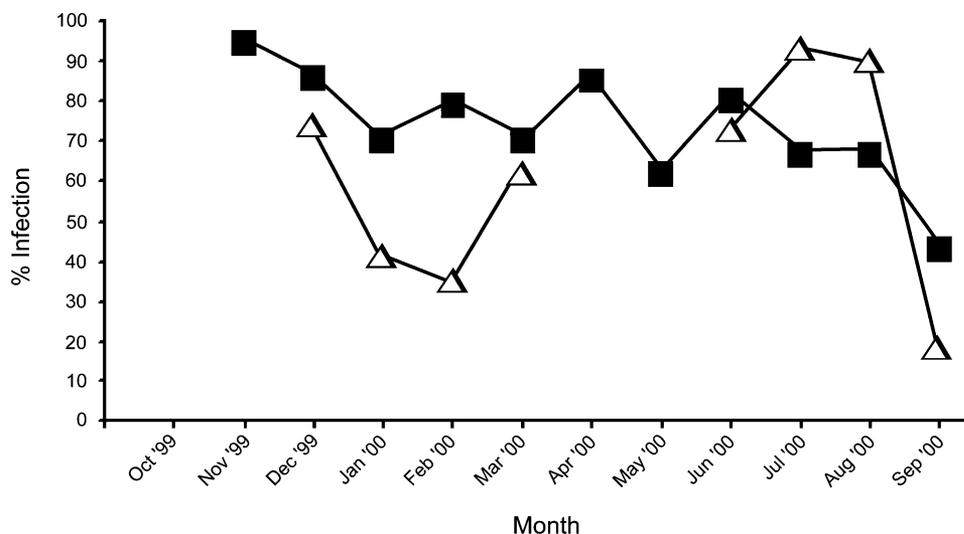


Figure 2. Monthly fluctuations of *Wolbachia* infection in populations of the fleahopper, *Halticus minutus* (Δ), and the whitefly, *Bemisia tabaci* (\blacksquare), on pumpkin plants determined by PCR using *ftsZ* primers at monthly intervals from October 1999 to September 2000 at a single location at Sai Yok, western Thailand. Fleahoppers were absent in October 1999 and April/May 2000; whiteflies were absent in October/November 1999.

placement, with $\geq 99\%$ support in all three trees. Less than 1.5% K-2-p divergence occurred among the four strains from pumpkin leaf insects. The remaining B group *Wolbachia* strain, from *O. vaulongeri* from pumpkin fruit, was placed in the same clade with *Trichogramma deion* with 100% bootstrap support.

***Wolbachia* Seasonal Fluctuations.** Monthly *Wolbachia* infection rates in populations of the whitefly and the fleahopper, both of which occurred in high numbers on pumpkin plants, were determined from October 1999 to September 2000. Whiteflies did not appear on plants for the first 2 months. *Wolbachia* infection rate in the whitefly population ranged from 42.9 to 94.7% (Fig. 2), with a mean \pm SD of $72.9 \pm 14.1\%$ per month, but did not vary significantly among months ($\chi^2 = 13.82$, $df = 10$, $p = 0.181$, $\alpha = 0.05$). Except for the first month, both immature and adult whitefly stages were present on pumpkin leaves all year. The pumpkin fleahopper did not occur all year, being absent in October 1999 and April/May 2000. Only adults, but no nymphs, were found. *Wolbachia* infection rate fluctuated markedly (Fig. 2) with a mean \pm SD of $59.2 \pm 27.0\%$. Statistically, infection rate depended significantly on the month ($\chi^2 = 18.53$, $df = 7$, $p = 0.010$; $\alpha = 0.05$).

Discussion

The total of 59 arthropod species collected from pumpkin plants (Table 2) indicates the many potential interactions within a local community. That seven of these species harbored *Wolbachia* (Table 2) highlights the potential for transmission among interacting species. We found that the degree of genetic similarity among *Wolbachia* strains depended on the plant part from which their insect hosts were collected.

The three species from pumpkin fruit, namely the wasp parasitoid, *Diachasmimorpha longicaudata*, the syrphid fly, *Graptomyza brevivostis*, and the scarab beetle, *Onthophagus vaulongeri*, were taxonomically distantly related and harbored very different *wsp* sequences. The parasitoid–host interaction is the most commonly hypothesized route by which *Wolbachia* [2, 26, 27, 28], or at least *Wolbachia* genetic material [31], may transfer across distantly related host lineages. However, *D. longicaudata* is neither a parasitoid of the syrphid fly nor the scarab beetle; rather, it is a parasitoid of tephritid fruit flies. In addition, the *Wolbachia* strain in *D. longicaudata* was not closely related to those of any other pumpkin arthropod, making horizontal transfer via this route improbable, at least in this community.

Like the *Wolbachia*-infected insects from fruit, the four infected insects collected from pumpkin leaves, namely, the white fly, *Bemisia tabaci*, the fleahopper, *Halticus minutus*, the planthopper, *Nisia nervosa*, and the flea beetle, *Phyllotreta* sp., were very taxonomically diverse, encompassing four families and three orders. In stark contrast to fruit insects, *wsp* sequences from *Wolbachia* strains infecting the four leaf insects were very closely related (<1.5% K-2-p divergence). The clade comprising these sequences (Fig. 1) was very well supported with high bootstrap values ($\geq 99\%$) for all three phylogenetic analyses.

Such a pattern is suggestive of horizontal transmission of *Wolbachia* bacteria [2, 24, 28] in the past among insects that inhabit pumpkin leaves. What do these four insects do that could facilitate such transmission? Haemolymph-to-haemolymph *Wolbachia* transfer by contact between wounded individuals has been demonstrated in isopods [19]. However, during our 2 years of arthropod collection from pumpkin plants, we never observed any kind of close contact between leaf-dwelling insects, and transmission through this route seems un-

likely. Parasitoidism also seems an unlikely means of *Wolbachia* transfer between pumpkin leaf insects, because none of the four infected species we collected are parasitoids of the others. In addition, these four insects encompass four families in three orders. It is therefore extremely unlikely that they share parasitoids and that *Wolbachia* was transmitted among them by this route.

There is one activity that all four leaf insects have in common: they all feed on leaf tissues. This suggests that leaf feeding may be a means by which *Wolbachia* could transfer to new host species. The small differences among *wsp* sequences indicates that any such transmission occurred sufficiently long in the past for some divergence to have occurred and that it is an occasional event. Our results do not form an isolated case among plant-feeding insects. Moths whose larvae feed on rice plants as well as rice planthoppers and leafhoppers also harbor *Wolbachia* strains with very similar *wsp* sequences [11, 15]. Further, identical *Wolbachia wsp* sequences have been reported in two mulberry leaf-feeding hoppers by Mitsuhashi *et al.* [14]. In a novel hypothesis, these authors suggested that *Wolbachia* may have transferred between hopper species through their plant-feeding activities in a manner analogous to that of leafhopper-vectored plant bacterial pathogens. In what follows, we apply this hypothesized process to *Wolbachia* transmission within the pumpkin leaf insect community. By examining each transmission step as well as our *Wolbachia* infection rate data, we show that: (1) two of the four infected insects are unlikely to be *Wolbachia* transmitters, and (2) of the remaining two insects, the whitefly is potentially of central importance to *Wolbachia* transmission and maintenance within the pumpkin insect community.

Wolbachia has been detected in the salivary glands of several insects [4], including those that feed on plants [14]. Insects with piercing–sucking mouthparts, such as the whitefly and planthopper in this study, may be particularly suited to *Wolbachia* transmission via the plant. Such insects typically inject saliva into plant cells to digest their contents before ingesting cell fluids [5, 13]. In so doing, these insects inoculate plants with pathogens and presumably other microorganisms such as *Wolbachia* that occur in saliva. For another insect to acquire *Wolbachia*, these bacteria must survive in the plant environment to be taken up live by plant-feeding insects. Because the structural integrity of the cell is maintained after feeding by piercing–sucking insects [13, 18], the survival and spread within the plant of introduced microorganisms including *Wolbachia* is likely. In contrast, the fleahopper feeds by lacerating parenchyma cells [13], and the flea beetle cuts tissue from leaves with its chewing mouthparts. So, both destroy plant cells and are unlikely to inoculate *Wolbachia* into plants. Nevertheless, they could possibly acquire *Wolbachia* by feeding on leaf tissues previously fed upon by infected whiteflies or planthoppers.

The whitefly and the fleahopper differed considerably in their patterns of monthly *Wolbachia* infection rate (Fig. 2). Two factors are considered important influences on *Wolbachia* infection rates within populations: the rate of maternal transmission to offspring and infection effects on host fitness [24].

In the fleahopper population, infection rate fluctuated greatly from month to month (Fig. 2). This fleahopper appears not to breed on pumpkin leaves because nymphs were never found. Maternal transmission rate is thus not relevant. Effects of *Wolbachia* on fleahopper fitness, however, may be important to host survival. Nevertheless, differential and fluctuating survival rates among *Wolbachia*-infected and uninfected fleahoppers seem unlikely to account for the large monthly fluctuations in *Wolbachia* infection rate. That this fleahopper breeds little on pumpkin indicates that the population is mainly migrant individuals and that *Wolbachia* infection is determined in breeding populations on other plant hosts. Infection rates may differ among these other populations, and their relative contributions to the pumpkin fleahopper population may vary sufficiently to cause the observed major monthly fluctuations in *Wolbachia* infection rate.

In the whitefly population, monthly *Wolbachia* infection rate was relatively high and stable (Fig. 2) although not fixed in the population, with infection rates generally being less than 90%. This population breeds on pumpkin leaves, because both adult and immature stages were observed. Either of the two factors presented earlier, therefore, could account for the observed monthly rates of *Wolbachia* infection. Of all the insects feeding on pumpkin leaves, the whitefly being infected at high and stable rates by *Wolbachia*—having a close, perhaps even permanent, breeding relationship with pumpkin plants, and because of its piercing–sucking feeding manner—may be of central importance to hypothesized transmission and maintenance of *Wolbachia* within the pumpkin arthropod community. Direct experimental tests of whitefly *Wolbachia* transmission via the plant, in which uninfected whiteflies are allowed to feed on pumpkin leaves recently fed on by *Wolbachia*-infected whiteflies and then tested for *Wolbachia*, are now warranted.

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