**Wolbachia** (Rickettsiales: Alphaproteobacteria) Infection in the Leafhopper Vector of Sugarcane White Leaf Disease

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Abstract

*Wolbachia* is a maternally inherited bacterium ubiquitous in insects that has attracted interest as a prospective insect pest-control agent. Here, we detected and characterized *Wolbachia* in the leafhoppers *Matsumuratettix hiroglyphicus* (Matsumura) (Cicadellidae: Hemiptera) and *Yamatotettix flavovittatus* (Matsumura) (Cicadellidae: Hemiptera) in the leafhopper *Y. flavovittatus*. The 16S rRNA and wsp gene markers revealed that *Wolbachia* was not present in the *M. hiroglyphicus* but naturally occurs in *Y. flavovittatus*. Additionally, the infection rates in adult leafhoppers ranged from 0 to 100% depending on geographic location. Moreover, *Wolbachia* was detected in the eggs and first- to fifth-instar nymphs of *Y. flavovittatus*. A phylogenetic tree of *Wolbachia* indicated that it resided in the monophyletic supergroup B clade and clustered in the Ori subgroup. Furthermore, fluorescence in situ hybridization revealed that *Wolbachia* localized to the egg apices, randomly distributed in the egg cytoplasm, and was concentrated in the nymph and adult bacteriomes, as well as occasional detection in the thorax and abdomen. To the best of our knowledge, the present study is the first to demonstrate the prevalence of *Wolbachia* in the leafhopper *Y. flavovittatus*. The obtained results would provide useful information for the future development of *Wolbachia* as a biological control agent for the leafhopper vectors.

Key words: *Wolbachia*, bacterial symbiont, leafhopper vector, phytoplasma vector, sugarcane white leaf disease

The genus *Wolbachia* is an intracellular, maternally inherited bacterium that naturally occurs in a wide range of arthropods and filarial nematodes (Werren 1997, Bandi et al. 1998). Several reports suggest that *Wolbachia* infection exists in 20–80% of all insect species, and it is especially prevalent in the orders Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera (Werren and Windsor 2000, Hilgenboecker et al. 2008, Zug et al. 2012). The taxonomic classification of *Wolbachia* diversity is complex due to its high genetic diversity. Although based on the specific gene analyses, *Wolbachia* strains are clustered into ≥13 major clades known as supergroups (A–F; H–N; Lo et al. 2007, Augustinos et al. 2011). The supergroups A and B are the most widely distributed among insects (Zhou et al. 1998).

Several *Wolbachia* strains are reproductive parasites that cause alterations of their hosts via parthenogenesis, feminization, male killing, and cytoplasmic incompatibility (CI). These phenomena enable *Wolbachia* to spread and maintain the infection within the host populations (Stouthamer et al. 1999, Werren et al. 2008). Moreover, some associated strains exert benefits to the hosts such as protecting its hosts from viral infections (Hedges et al. 2008), or supplement its hosts with essential nutrients (Hosokawa et al. 2010).

*Wolbachia* serve as a novel potential biological control agent for vector and pest insects (Bourouis et al. 2014, Hoffmann et al. 2015). *Wolbachia* from *Drosophila melanogaster* (wMel) transfected to *Aedes aegypti* shortened the mosquito–vector lifespan and reduced dengue virus transmission (McMeniman et al. 2009, Walker et al. 2011). The use of *Wolbachia* to control mosquito vectors has been extensively investigated (Bian et al. 2010, Hoffmann et al. 2011, Ye et al. 2015, Fraser et al. 2017). Moreover, *Wolbachia* has also been explored for plant insect–pest management, with its transfection from *Rhagoletis cerasi* (L.) to *Ceratitis capitata* (Wiedemann) (Tephritidae) inducing CI in the latter host (Zabalou et al. 2007, Augustinos et al. 2011). The leafhoppers *Matsumuratettix hiroglyphicus* (Matsumura) and *Yamatotettix flavovittatus* Matsumura (subfamily Deltocephalinae; family Cicadellidae; suborder Auchenorrhyncha) are vectors of the phytoplasma that causes sugarcane white leaf (SCWL) disease (Hanboonsong et al. 2002, 2006, Thein et al. 2012, Youichi and Hanboonsong 2017). This disease causes substantial losses in sugarcane yield in southeast Asian countries, especially in Thailand, which is the second largest exporter of refined sugar worldwide (FAO 2017). Currently, there are no resistant sugarcane...
cultivars or effective means to control SCWL disease. Measures to limit the spread of the disease have focused on insect-vector control; however, the use of insecticides is unsustainable for large-scale commercial crops. Moreover, there have been no reports on the use of traditional insect pest-control methods or natural enemy insects against the leafhoppers *M. hiroglyphicus* and *Y. flavovittatus*.

Our preliminary data suggested that *Wolbachia* is present in some population of the leafhopper *Y. flavovittatus* (Tewaruxxu et al. 2017); however, more in-depth investigation of *Wolbachia* biology is needed. We attempted to obtain adequate information with potential to be used for future development of control methods of the vectors. Therefore, the aim of the present study was to increase our understanding of natural infections of *Wolbachia* in leafhopper vectors in order to elucidate *Wolbachia* distribution, inheritance, and localization in leafhopper vectors of SCWL disease.

Materials and Methods

Leafhopper Collection DNA Extraction

Adults of leafhoppers *M. hiroglyphicus* and *Y. flavovittatus* were collected from light traps in four different sugarcane fields during August and September of 2016 or 2017. These four sugarcane fields are located in different provinces of Thailand: Khon Kaen and Udon Thani located in the northeastern region, Lopburi located in the central region, and Kanchanaburi located in the western region. Some of the specimens were immediately immersed in absolute ethanol and stored at −20°C until DNA extraction, whereas others were maintained in sugarcane plant cages and transferred to the laboratory for mass rearing.

DNA Extraction

Insect DNA was extracted using the phenol-chloroform method (Ausubel et al. 2008). Briefly, each leafhopper was ground in DNA extraction buffer (200 mM Tris [pH 8.0], 250 mM NaCl, 25 mM ethylene diamine tetraacetic acid [EDTA], 0.5% [v/v] sodium dodecyl sulfate [SDS], and 0.1 mg/ml proteinase K [Invitrogen, Carlsbad, CA]) and incubated at 37°C for 24 h. The DNA was extracted with an equal volumetric ratio of phenol:chloroform:isoamyl alcohol (25:24:1), followed by 5-min centrifugation at 10,000 g and 4°C. The supernatants were transferred to other vials, and an equal volumetric ratio of chloroform:isoamyl alcohol (24:1) was added. After a 5-min centrifugation at 10,000 g and 4°C, the supernatants were precipitated with 3 M sodium acetate and isopropanol. The pellet was washed with 70% (v/v) absolute ethanol, air-dried, and re-suspended in TE buffer (10 mM Tris and 1 mM EDTA) before being stored at −20°C until further use.

*Wolbachia* Detection

The leafhoppers were screened for *Wolbachia* by PCR using two *Wolbachia*-specific primers. The first primer pair was used to amplify 900 bp of the 16S rRNA gene using the forward primer 16SFV1 (′5′-TGTGAGTTGCTGTATTAGTAACT-3′) and the reverse primer 16SRV6 (′5′-GAATACGGATTACTTCTGTA-3′; O’Neill et al. 1992).

The *wsp* was used to amplify a 610-bp *Wolbachia* surface protein using the forward primer 81F (′5′-TGGTCAATAAGTGA TGAAGAAC-3′) and the reverse primer 961R (′5′-AAAAAAATAC CGCTACTCCA-3′; Zhou et al. 1998). The reaction for 16S rRNA was performed in a final volume of 25 µl comprising 2 µl of DNA templates, 1× PCR buffer, 2.5 mM MgCl₂, 0.2 µM primers, 0.2 mM dNTPs, and 1 U Taq DNA polymerase (Invitrogen). Cycling conditions consisted of an initial denaturation (95°C, 5 min), followed by 30 cycles of denaturation at 95°C (1 min), annealing at 55°C (1 min), extension at 72°C (1 min), and a final extension at 72°C (10 min). For *wsp*, the PCR was run in a final volume of 20 µl comprising 2-µl DNA templates, 1× PCR buffer, 2.5 mM MgCl₂, 0.5 µM primers, 0.2 mM dNTPs, and 1 U Taq DNA polymerase (Invitrogen) and using a PCR program similar to that for 16S rRNA gene. The amplicons were analyzed by gel electrophoresis. The total specimens that were tested in this experiment included 200 males and 200 females of the leafhopper *Y. flavovittatus* and 100 males and 100 females of the leafhopper *M. hiroglyphicus*.

*Wolbachia* Detection in the Various Leafhopper Life Stages

*Wolbachia* distribution was determined for various life cycle stages of *Y. flavovittatus* and *M. hiroglyphicus*. Adult leafhoppers from Udon Thani province were maintained in sugarcane plant cages (10 males and 10 females per cage, with a total of three cages for each leafhopper species) and allowed to mate and produce a subsequent generation. The eggs and first- to fifth-instar nymphs were collected and subjected to DNA extraction using the aforementioned protocol. The individual samples (a total of 40 eggs and 200 nymphs for each leafhopper species) were tested for the presence of *Wolbachia* by PCR reaction as described.

Sequence and Phylogenetic Analyses

To validate the PCR products, certain *Wolbachia*-positive samples were selected for nucleotide sequencing. These included 16S rRNA and *wsp* fragments from adult males, adult females, and fifth-instar nymphs of *Y. flavovittatus*. The PCR products were purified and cloned into a TOPO-TA vector (Invitrogen) according to manufacturer instructions. Five recombinant plasmid clones were randomly selected from each leafhopper DNA template. Sequencing was conducted by Macrogen, Inc. (Seoul, Korea), and the 30 sequences obtained were compared with those deposited in the National Center for Biotechnology Information (NCBI; Bethesda, MD) GenBank database using the basic local alignment search tool (BLAST).

Phylogenetic relationships were established between the sequences of each group and closely related and unrelated sequences retrieved from the GenBank database. Multiple alignment was performed with a MAFFT algorithm using the default parameters (Katoh and Standley 2013). Phylogenetic trees were constructed by the maximum likelihood method in the IQ-TREE web server (http://www.iqtree.org/; Nguyen et al. 2015, Trifinopoulos et al. 2016). The automatically selected best-fit substitution models for 16S rRNA and *wsp* were TPM2u+F+I and TPM3+F+G4, respectively. Ultrafast bootstrapping assigned branch-support values with 1,000 replicates (Minh et al. 2013). The phylogenetic tree was viewed and edited in FigTree (v.1.4.3; http://tree.bio.ed.ac.uk/software/figtree/).

Fluorescence In Situ Hybridization

Whole-mount in situ hybridization was performed on the eggs, nymphs, and adults of *Y. flavovittatus* to localize *Wolbachia*. We tested individual specimens in this experiment, including 50 eggs, 30 nymphs, and 20 females. The oligonucleotide probe for hybridization was W2 (′5′-CTTCTGAGAGTCCCTCCTATTCC-3′), which aligned with positions 319 through 336 of Wolbachia 16S rRNA (Shi et al. 2016). This probe was 5′-labeled with Qasar 670 (excitation wavelength: 647 nm; emission wavelength: 670 nm). Fluorescence in situ hybridization (FISH) was conducted following previously reported methods (Sakurai et al. 2005, Gottlieb et al. 2006), with slight modifications. Briefly, fresh samples were collected and
stored in acetone until further use. Nymphs and adults were immersed in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4), and cuticles were pricked with a needle in two or three places under a microscope to facilitate reagent infiltration. All samples were fixed overnight in Carnoy’s solution (ethanol:chloroform:acetic acid, 6:3:1) at room temperature (28–35°C) in a shaker. The samples were decolorized by immersion in 6% (v/v) hydrogen peroxide in 100% ethanol at room temperature (28–35°C) for 2–3 wk to quench the autofluorescence of the insect tissues. After decolorization, the samples were hydrated three times in 100% ethanol and PBS with Tween-20 [PBST; 0.2% (v/v)] for 10 min each time. Before hybridization, the samples were covered with a coverslip. The slides were viewed under a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan) at the Center of Nanoimaging, Mahidol University, Thailand. Reactions without probes or RNase digestion before hybridization were used as negative controls.

Sequence Accession Numbers
Representative 16S rRNA and wsp gene of Wolbachia from Y. flavovittatus was submitted to the GenBank database under accession numbers MN509027 and MN631207, respectively.

Results
Wolbachia Detection by PCR
Wolbachia infection was tested in both leafhopper species with 16S rRNA and wsp specific primers. The results show that Wolbachia was absent (0/200) from M. hiroglyphicus collected at two locations (Table 1); however, it was identified from Y. flavovittatus. The infection rates varied among the four populations collected from different sugarcane fields (Table 1). Although the infection rates within the same location differed slightly depending on the effectiveness of the primers being used. The highest infection rates were obtained for leafhoppers collected in Udonthani province, with levels of 100% (160/160) coverage for 16S rRNA and 95.62% (153/160) for wsp. For the population in Khon Kaen province, the average infection rates were 87.5% (70/80) for 16S rRNA and 77.5% (62/80) for wsp. The specimens collected from two other fields showed low infection rates of 18.75% (15/80) for 16S rRNA and 9.60% (12/120) for wsp (Lopburi province) and 2.5% (2/80) for 16S rRNA and undetected for wsp (Kanchanaburi province).

Prevalence of Wolbachia in Different Leafhopper Life Stages
Wolbachia was detected at various growth stages of both leafhopper species. The samples tested in this study were the progeny of leafhoppers collected in Udonthani province, where the Y. flavovittatus population displayed the highest measured incidence of Wolbachia. The results show that Wolbachia was found in the eggs and the five nymphal instar stages of Y. flavovittatus (Table 2). The 16S rRNA Wolbachia infection rates were 82.5% for the eggs and 92.5, 97.5, 90.0, 87.5, and 92.5% for the first through the fifth nymph stages, respectively. The rates of wsp infection were 60.0% for the eggs and 77.5, 90.0, 90.0, 90.0, and 87.5% for the first through the fifth nymph stages, respectively. Whereas no Wolbachia was found in the eggs and nymphal stages of the leafhopper M. hiroglyphicus.

Nucleotides Analysis and Phylogenetic Analysis
To validate the PCR products, 16S rRNA and wsp amplicons of Wolbachia were cloned from infected males, females, and fifth nymphal instars of Y. flavovittatus. The results of nucleotide sequencing are presented in Table 3. Fifteen clones of 16S rRNA gene showed a sequence identity of 99.88% with Wolbachia of the citrus blackfly Aleurocanthus woglumi (GenBank accession no. AY589872).

| Leafhopper species | Location province (yr) | Sex  | No. tested | 16S rRNA % positive | wsp % positive |
|--------------------|------------------------|------|------------|---------------------|---------------|
| **Y. flavovittatus** | Udonthani (2016) | Male  | 40         | 40 (100)            | 39 (97.50)    |
|                     |                        | Female | 40         | 40 (100)            | 39 (97.50)    |
|                     | Udonthani (2016) | Male  | 40         | 40 (100)            | 37 (92.50)    |
|                     |                        | Female | 40         | 40 (100)            | 38 (95.00)    |
|                     | Khonkaen (2016) | Male  | 40         | 35 (87.50)          | 29 (72.50)    |
|                     |                        | Female | 40         | 35 (87.50)          | 33 (82.50)    |
|                     | Lopburi (2016) | Male  | 40         | 4 (10.00)           | 2 (5.00)      |
|                     |                        | Female | 40         | 11 (27.50)          | 10 (25.00)    |
|                     | Kanchanaburi (2017) | Male | 40         | 1 (2.50)            | 0 (0)         |
|                     |                        | Female | 40         | 1 (2.50)            | 0 (0)         |
|                     | Total                  | Male  | 200        | 120 (60.00)         | 107 (53.50)   |
|                     |                        | Female | 200        | 127 (63.50)         | 120 (60.00)   |
| **M. hiroglyphicus** | Khonkaen (2016) | Male  | 50         | 0 (0)               | 0 (0)         |
|                     |                        | Female | 50         | 0 (0)               | 0 (0)         |
|                     | Udonthani (2016) | Male  | 50         | 0 (0)               | 0 (0)         |
|                     |                        | Female | 50         | 0 (0)               | 0 (0)         |
|                     | Total                  | Male  | 100        | 0 (0)               | 0 (0)         |
|                     |                        | Female | 100        | 0 (0)               | 0 (0)         |
JX281793; Pandey et al. 2013), with multiple alignments of these sequences revealing 100% identity.

A BLAST search for wsp gene disclosed nine samples showing a sequence identity of 99.64% with Wolbachia found in parasitoid wasp Phaenoglyphis villosa (GenBank accession no. MG968806; Ferrer-Suay et al. 2018). The other six samples showed 99.63% identity with Wolbachia found in the true bug Homoeocerus unipunctatus (GenBank accession no. AB109572; Kikuchi and Fukatsu 2003). Multiple sequence alignments for wsp revealed 99–100% identity.

The phylogenetic trees were constructed to confirm systematic affinity of Wolbachia from the leafhopper Y. flavovittatus. The 16S rRNA gene sequence from this study was aligned with representative sequences of the Wolbachia from supergroups A and B. A tree analysis of 16S rRNA gene revealed that the Wolbachia from Y. flavovittatus was positioned in the monophyletic clade of supergroup B (100% support value) and placed in the clade containing Ori subgroups with 99% statistical support. However, the wsp gene sequence in present study formed a distinct evolutionary lineage from other Wolbachia strains (Fig. 2).

Localization of Wolbachia by FISH Assay
To localize the Wolbachia, a FISH assay was conducted on whole mounts of Y. flavovittatus eggs, nymphs, and adults. Wolbachia was detected at all developmental stages, with the signals indicating the presence of Wolbachia in the eggs (Fig. 3A–D). In eggs that were 2–3 d old, the Wolbachia signals concentrated around the apices (Fig. 3A and B), whereas in eggs that were 5–6 d old, the signals were randomly distributed (Fig. 3C and D). The whole mounts of eggs without probes or RNase digestion revealed no signals.

For the Y. flavovittatus nymphs and adults, the Wolbachia signals were concentrated in the bacteriomes along the lateral margins of the anterior abdomen (Fig. 4A–D), with the highest Wolbachia concentrations detected in the adult bacteriomes (Fig. 4D). Moreover, the Wolbachia signals were more highly concentrated in the third instar (Fig. 4C) than the first or second instar (Fig. 4A and B). Additionally, the Wolbachia signals were observed in the thorax (Fig. 4B and D) and the abdomen (Fig. 4E and F). The whole mounts of adults and nymphs without probes or RNase digestion revealed no signals.

### Table 2. Wolbachia infection rates in various developmental stages of Y. flavovittatus and M. hiroglyphicus

| Leafhopper species | Stage          | No. tested | 16S rRNA No. positive (%) | wsp No. positive (%) |
|--------------------|----------------|------------|---------------------------|---------------------|
| Y. flavovittatus   | Egg            | 40         | 33 (82.5)                 | 24 (60.0)           |
|                   | First nymph    | 40         | 37 (92.5)                 | 31 (77.5)           |
|                   | Second nymph   | 40         | 39 (97.5)                 | 36 (90.0)           |
|                   | Third nymph    | 40         | 36 (90.0)                 | 36 (90.0)           |
|                   | Fourth nymph   | 40         | 35 (87.5)                 | 36 (90.0)           |
|                   | Fifth nymph    | 40         | 37 (92.5)                 | 35 (87.5)           |
| M. hiroglyphicus  | Egg            | 40         | 0 (0)                     | 0 (0)               |
|                   | First nymph    | 40         | 0 (0)                     | 0 (0)               |
|                   | Second nymph   | 40         | 0 (0)                     | 0 (0)               |
|                   | Third nymph    | 40         | 0 (0)                     | 0 (0)               |
|                   | Fourth nymph   | 40         | 0 (0)                     | 0 (0)               |
|                   | Fifth nymph    | 40         | 0 (0)                     | 0 (0)               |

### Table 3. BLAST search results for Wolbachia 16S rRNA and wsp genes sequences from Y. flavovittatus

| Gene    | Insect                  | No. clones | Closest match                                      | Identity (%) |
|---------|-------------------------|------------|---------------------------------------------------|--------------|
| 16S rRNA| Male                    | 5          | Wolbachia of Aleurocanthus woglumi (JX281793)     | 99.88        |
|         | Female                  | 5          | Wolbachia of Aleurocanthus woglumi (JX281793)     | 99.88        |
|         | Fifth nymph             | 5          | Wolbachia of Aleurocanthus woglumi (JX281793)     | 99.88        |
| wsp     | Male                    | 1          | Wolbachia of Phaenoglyphis villosa (MG968806)     | 99.64        |
|         | Female                  | 4          | Wolbachia of Homoeocerus unipunctatus (AB109572)  | 99.63        |
|         |                         | 5          | Wolbachia of Phaenoglyphis villosa (MG968806)     | 99.64        |
|         | Fifth nymph             | 2          | Wolbachia of Homoeocerus unipunctatus (AB109572)  | 99.63        |
|         |                         | 3          | Wolbachia of Phaenoglyphis villosa (MG968806)     | 99.64        |
be equally sensitive to all developmental stages (Tables 1 and 2). These primers might not accuracy, even when they were applied to the same specimens across infection rates did not generate results of similar ac-
Wolbachia Y. flavovittatus planthoppers might be affected by ecological factors. (2015) suggested that the Wolbachia the host plants of infected insects. Moreover, Wiwatanaratanabutr et al. 2009), rice leafroller (Sitobion miscanthi) (Nirgianaki et al. 2003), aphid (Sitobion micanthi) (Wang et al. 2009), leafhopper (Cimex lectularius) (2011), and bed bug (C. lectularius) (Akhoundi et al. 2016). The Wolbachia belongs to super-
Wolbachia Y. flavovittatus from the leafhopper Y. flavovittatus. The results were well-structured trees as shown by the bootstrap values and the retrieving sequences consistent with reported groups and strains of Wolbachia. Both phylogenetic trees indicated that Wolbachia from the leafhopper Y. flavovittatus belongs to supergroup B, which is commonly distributed in insects (Zhou et al. 1998). Our usp gene sequence fell into the clade of Ori subgroup, though it revealed a new lineage of Wolbachia strains. Thus, based on classification and the nomenclature system (Zhou et al. 1998), we propose the designation UYfla as the specific strain for Wolbachia from the leafhopper Y. flavovittatus.

However, different patterns of supergroup infection in insect hosts have been reported (Kikuchi and Fukatsu 2003, Ahmed et al. 2010, Wang et al. 2014). In the present study, the number of clones sequencing were quite low; therefore, the obtained data could not support clear inferences of infection by either a single supergroup or other supergroups that remain undetected in the leafhopper Y. flavovittatus. Recently, the analysis of the housekeeping genes (gatB, coxA, hcpA, fbpA, and ftsZ) by multilocus sequence typing (MLST; Baldo et al. 2006) has been widely employed to characterize the genetic diversity of Wolbachia. In future research, Wolbachia
strain genotyping will be performed by means of MLST using a larger number of specimens collected from various locations. Wolbachia was detected in the egg and nymph stages of Y. flavovittatus samples collected from Udon Thani province, where there was a high incidence of Wolbachia infection in adult Y. flavovittatus. Wolbachia was observed in all developmental stages of this leafhopper species. Our data confirmed that Wolbachia is likely vertically transmitted in Y. flavovittatus, as previously reported (Gottlieb et al. 2008, Bing et al. 2014, Guo et al. 2018). Evidence for

Fig. 2. Phylogenetic tree of Wolbachia in Y. flavovittatus (MN631207) and other hosts based on wsp gene sequences. Wolbachia strains are indicated after accession numbers; groups and subgroups are listed on the right side. The numbers at each node indicate clade support based on 1,000 bootstrap replications. The scale bar represents the number of substitutions per site.

Fig. 3. FISH visualization of Wolbachia-specific signal probes (red) in Y. flavovittatus eggs. (A, B) Eggs at 2–3 d. (C, D) Eggs at 5–6 d. Scale bar = 0.5 mm.

Fig. 4. FISH visualization of Wolbachia-specific signal probes (red) in nymph and adult Y. flavovittatus. (A–C) First, second, and third instars, respectively. (D) Adult stage. (E, F) Nymph abdomen. Scale bar = 1 mm. B, bacteriome; T, thorax; A, abdomen.
Wolbachia infection and vertical transmission in Y. flavovittatus was acquired by FISH assays on the eggs and nymphs; however, we did not establish the presence of Wolbachia in the female adult ovaries. FISH analysis of nymph and adult Y. flavovittatus revealed that Wolbachia localized primarily in the bacteriome on both sides of the abdomen (Fig. 4), with negligible detection in the thorax and abdomen. The bacteriomes of Y. flavovittatus harbor the primary symbionts Candidatus Sulcia muelleri and Candidatus Yamatodendromediicicola (Wangkeeree et al. 2019). The coexistence of Wolbachia and primary symbionts has been reported for the aphid Cinara cedri (Gomez-Valero et al. 2010), the bed bug Cimex lectularius (Bing et al. 2010), and abdomen. The bacteriomes of the abdomen (Fig. 4), with negligible detection in the thorax and abdomen. Wolbachia localization and density in different tissues might depend on Wolbachia-host interactions according to coevolution, routes of transmission and functions or effects of Wolbachia (Pietri et al. 2016).

This study of Wolbachia infection in Y. flavovittatus demonstrated that Wolbachia might represent a novel strategy for noninsecticide-based control of economically important plant pathogen vectors. This research is the first step toward providing sufficient information for potential future development; however, further research is required, including elucidation of the effects of Wolbachia on leafhopper reproduction and development, as well as Wolbachia genetic diversity and relationship with pathogen transmission. We found that Wolbachia was not detected in the leafhopper M. hiroglyphicus. One explanation for this might be the coevolutionary history of Wolbachia-host interactions or the host genetic background. Additionally, it is possible that the low infection prevalence and density of Wolbachia played a role. To resolve this or confirm the naturally uninfected status, future research will aim to increase the detection sensitivity through the use of quantitative PCR, and a larger number of specimens from various geographic locations will be processed.

Future development of Wolbachia as a biological control agent for the leafhopper M. hiroglyphicus can be potentially achieved by transinfection with virulent Wolbachia strains.

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