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Research

The Endosymbiotic *Wolbachia* and Host COI Gene Enables to Distinguish Between Two Invasive Palm Pests; Coconut Leaf Beetle, *Brontispa longissima* and Hispid Leaf Beetle, *Octodonta nipae*

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Abstract

To elucidate taxonomic eminence of identical pest species is essential for many ecological and conservation studies. Without proficient skills, accurate molecular identification and characterization are laborious and time-consuming. The coconut leaf beetle, *Brontispa longissima* (Gestro) (Coleoptera: Chrysomelidae), is biologically and morphologically identical to hispid leaf beetle, *Octodonta nipae* (Maulik) (Coleoptera: Chrysomelidae), and is known as the most harming nuisances of palm cultivation worldwide. The present examination was to establish *Wolbachia* genotyping analysis along with host cytochrome oxidase subunit I (COI) gene for accurate identification between these individuals of the same family (Chrysomelidae). Here, we have cloned and sequenced a gene coding *Wolbachia* surface protein (*wsp*) and COI gene regions amplified from both species by polymerase chain reaction. The nucleotide sequences were directly determined (≈600 bp for *wsp* and ≈804 bp for COI) and aligned using the multiple alignment algorithms in the ESPript3 package and the MEGA5 program. Comparative sequence analysis indicated that the representative of *wsp* and COI sequences from these two beetles were highly variable. To ensure this bacterial variation, multilocus sequence typing (MLST) of bacterial genes was conducted, and the results vindicated the same trend of variations. Furthermore, the phylogenetic analysis also indicates that *B. longissima* and *O. nipae* being the two different species harbors two distinct *Wolbachia* Hertig and Burt (Rickettsiales: Anaplamataceae) supergroups B and A, respectively. The present outcomes quickly discriminate between these two species. Considering its simplicity and cost-effectiveness, it can be used as a diagnostic tool for discriminating such invasive species particularly *B. longissima* and *O. nipae* which has overlapping morphologic characters.

Keywords: *Brontispa longissima*, *Octodonta nipae*, *Wolbachia*, multilocus sequence type, invasive species

Understanding the exact taxonomic status of insect pests is fundamental to devise efficient control or management measures against them (Rossman and Miller 1996). Pest management studies rely on the fact that taxonomically, individuals are correctly identified and having a scientific name, and that their ecology along with other biological features is known. Any misidentification or failure to distinguish between closely related species can obscure and obstruct the management of pests (Miller and Rossman 1995). Recently, morphologically indistinct or fundamentally similar insect group (such as a species, clade, or biotype) can be segregated by utilizing DNA succession information (Yassin et al. 2008, Takano et al. 2011, Zhang et al. 2015). Since the availability of reliable methods based the morphological characters is rare, the molecular methods such as cytochrome oxidase subunit I (COI) and other come into play as more reliable ways for taxonomic classification (Zhang et al. 2015). Another technique such as polymerase chain reaction (PCR) restriction fragment-length polymorphism is also regularly utilized for this purpose (Scheffer et al. 2001, Takano et al. 2013).

Brontispa longissima (Gestro) (Chrysomelidae: Coleoptera) is a natural intrusive nuisance pest on palm cultivations in Southern China and worldwide (Wan et al. 2015, Zhang et al. 2015). Being, among the most severe pest of coconut palm, *Cocos nucifera* L. (Arecales: Arecaceae), *B. longissima*, probably originated from Indonesia and New Guinea and has been reported from many other countries and islands including, Southeast and East Asia and the Pacific region (Nakamura et al. 2006) where the host plant (*C. nucifera*) is cultivated

abundantly (Nakamura et al. 2006). During 2002, *B. longissima* was primarily reported from Haikou (Hainan province, China), where it exhibited symptoms of severe damage to palm plants, specifically on *C. nucifera* (Fu and Xiong 2004). The palm trees from other provinces such as Guangxi, Guangdong, Yunnan, and Fujian provinces of China are also infested (Lu et al. 2004). All developmental stages are found inside the young unopened leaflets, and the larvae and adults can move to the fresh fronds when the leaflets separate. This pest causes overwhelming damage to the commercial coconut industry and the tropical tourism industry, as both the hatchlings and grown-ups feast upon the tender tissues of unopened leaves, which results in brownish leaves and decreased fruit production (Nakamura et al. 2006, Lu et al. 2008).

Octodonta nipae (Maulik) (Chrysomeloidea: Coleoptera) was first reported from Malaysia (Maulik 1937), is also an economical palm pest worldwide. It mostly infests ornamental palm plants and infestations reached to China in 2001 (Sun et al. 2003). After that in 2007, it was discovered in Fujian province (Hou and Weng 2010). *O. nipae* devastations, morphological characters of life stages (larva, pupa, and adult; Fig. 1) and biological traits (size, color, feeding habitat, etc.) are remarkably similar to *B. longissima* (He et al. 2005, Hou and Weng 2010, Vassiliou et al. 2011, Tang and Hou 2017). These beetles can infest around 20 palm species (Sankaran 2006; Hou et al. 2014a,b) including *Phoenix canariensis* Hortulanorum ex Chabaud and *Trachycarpus fortune* (Hooker) H. Wendland (Hou and Weng 2010, Yamashita and Takasu 2010, Xi et al. 2013), *Areca catechu* L. (Hou et al. 2014a,b), *Syagrus romanzoffiana* (Chamisso) Glassman (Wu et al. 2006, Vassiliou et al. 2011), and *Washingtonia filifera* (Linden ex. Andre´) H. Wendland (Sun et al. 2003). The feeding behavior and damage pattern are similar to that of *B. longissima*, and the larvae and adults preferably attack the unopened leaf fronds (He et al. 2005, Vassiliou et al. 2011, Tang et al. 2014a). The damage symptoms appear in the form of gray-brown leaves with rolled edges that affect photosynthesis and plant growth (Hou and Weng 2010, Vassiliou et al. 2011, Li et al. 2014, 2016, Zhang et al. 2017).

Since the beetles can infest the same host and can be easily misjudged, especially when invading a new region. Invasion disarray, pathogenicity assay, and control efficiency of these pests are still in stagnate. Therefore, along with traditional identification based on morphological characters, molecular techniques are also employed to synergize the correct identification and avoid any taxonomic inaccuracy of closely related species. This emphasizes the necessity for the development of accurate and reliable identification techniques to be used in quarantine and biological control to avoid any inappropriate management. Thus, a method should be developed which is less costly, easy, and environment-friendly. Nevertheless, the broad applicability of DNA sequencing for identifying particular species, we herein proposed the development and use of *Wolbachia* genotyping (*wsp: Wolbachia* surface protein and MLST: Multilocus sequence typing) tool to determine demarcation of *Wolbachia* strains along with host COI gene regions to discriminate these two invasive palm species from each other reliably.

Materials and Methods

Insect Collection

In total 150 *B. longissima* specimens and 110 of *O. nipae* specimens were collected during 2016–2017. The *B. longissima* specimens were picked from infested host tree, canary date palm, *P. canariensis* in Zhangzhou city (Fujian province) (24.5130°N, 117.6471°E) and *O. nipae* specimens were collected from coconut tree, *C. nucifera* in Puqing city (Fujian province) (25°43.529°N, 119°20.855°E). Additionally, a detailed description of specimens is provided as Supp Table 1. After capturing, samples were preserved in 100% ethanol. Voucher specimens were dislodged for further experimentation in the laboratory.

Fig. 1. Various life stages (egg to adult) of *Brontispa longissima* and *Octodonta nipae*. Life cycle durations (in days) information of *B. longissima* were presented in detail from Takasu et al. (2010) and information of *O. nipae* are presented from Hou et al. (2014a).

DNA Extraction

The DNA was extracted from randomly selected individuals of both invasive species (*B. longissima* and *O. nipae*) with at least 20 repetitions of each beetle (one adult for each repetition). The entire insect was used for DNA extraction. DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Valencia) as we described previously (Tang et al. 2014b; Meng et al. 2016; Ali et al. 2018a,c). The concentration of the DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Furthermore, to assess DNA integrity, a 3 µl of DNA was run on agarose gel electrophoresis using 0.5X TAE buffer (composition: 0.5 µg/ml ethidium bromide, and TRIS-EDTA-Buffer) and visualized under UV transillumination.

Host COI and *Wolbachia* Genotyping Through PCR Assays

PCR analysis was carried out to determine partial amplifications part of host COI, *wsp* and MLST loci by using specific primers reported elsewhere (Baldo et al. 2006a; Zhang et al. 2015; Ali et al. 2018a,c). PCR assays were carried out in a total reaction volume of 25 µl (contained 2 µl of template DNA, 12.5 µl of 2X Taq PCR Master mix (Tiangen Biotechnology Beijing, China), 1 µl of each primer (10 µM), and 8.5 µl of double distilled water. The thermal cycling profiles were set as: initial denaturation at 94°C for 4 min, followed by 30 cycles for 40 s at 94°C, annealing at 40 s at 55°C, elongation for 1 min at 72°C, and final extension step for 10 min at 72°C for *Wolbachia*-specific, *wsp* gene (81F-691R) and for the bacterial 16S rRNA gene (27F-1492R) the thermal condition were: 94°C for 3 min, 40 s at 94°C, 40 s at 55°C, 1 min at 72°C, and final extension for 5 min at 72°C. For MLST genes, PCR protocols available at http://pubmlst.org/Wolbachia (Baldo et al. 2006) were followed by the modification of annealing temperature (*coxA* and *hcpA* at 50°C, *gatB*, and *fbpA* at 55°C and *ftsZ* at 48°C). PCR temperature profile of host COI gene was adopted as described by Zhang et al. (2015). Negative controls (without DNA) were run along with tested samples to avoid ambiguity. PCR positive clones were cleaned on QIAquick columns (Qiagen, Inc., Hilden, Germany) and send to BioSune commercial sequencing Company (BioSune Biotech. Shanghai, China). The DNA sequences from the company were assembled, examined manually for errors and then searched against BLAST in GenBank to compare with other *Wolbachia* sequences in the database.

Phylogenetic Classification of *Wolbachia* Supergroup in *B. longissima* and *O. nipae*

Phylogenetic analysis was carried out for accurate placement of *wsp* and MLST sequences into *Wolbachia* supergroups. As all of our sequences showed 100% similarity with their corresponding species sequences, only two *Wolbachia wsp* and two concatenated MLST sequences from both study insects were compared to NCBI GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Reference sequences from 20 and 13 insect species (Ali et al. 2018a) for *wsp* and MLST genes, respectively, were used to construct Maximum Likelihood (ML) tree. For computation of tree topology and estimating the ML values, the parameters were set as follows. Codon positions included were first + second + third + noncoding, alignment positions contained gaps and missing information were removed, and evolutionary analyses were calculated in MEGA5 with bootstrap analysis of 1,000 replications (Tamura et al. 2011). All selected sequences belong to different *Wolbachia* supergroups (A, B, F, D, and H).

Sequence Analysis

Sequences were aligned by multiple sequence alignment (MSA) algorithm (either host COI, *wsp*, or MLST loci) through ClustalW in MEGA5 (Tamura et al. 2011) and the evolutionary distances were calculated using the Kimura 2-parameter method (Kimura 1980). Extra sequence length of all subjected genes was trimmed from both sides. Sequence homology was determined by ENDscript package (ESPript3; http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi), and aligned outputs were saved.

Sequence Accession Numbers

All sequence from host COI, *wsp*, and MLST genes were submitted to the National Center for Biotechnology Information (NCBI) GenBank database (https://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) and the MLST database (https://pubmlst.org/).

Results and Discussion

Here, we employed PCR assays to document the host COI gene regions from both beetles through universal primer (C1-J-2195-TL2-N-3014) as documented (Zhang et al. 2015). The analysis results indicated a clear, single band of COI gene regions for both DNA templates of *O. nipae* and *B. longissima* were validating the quality of DNA extracted from both species and suitable for further *Wolbachia*-specific primer analysis. The COI sequence results were similar (Genbank accession number KM186303 and KF939632 for *B. longissima* and *O. nipae* respectively) with previous report (Zhang et al. 2015). After trimming, a ≈804 bp amplification product of COI gene regions of the two beetles were used for comparative analysis and results revealed a reasonable variability (18.65%) that quite enough to distinguish the sequences of both species (Table 1;

Table 1. Host COI gene regions with *Wolbachia* genotyping (*wsp* and MLST loci) analysis indicates the sameness and difference of conserved regions isolated from *Brontispa longissima* and *Octodonta nipae*

Genes	Sameness %	Difference %
Host COI gene	654/804 (81.34)	150/804 (18.65)
MLST loci		
gatB	324/369 (87.80)	45/369 (12.19)
coxA	348/402 (86.56)	54/402 (13.43)
fbpA	366/429 (85.31)	63/429 (14.68)
ftsZ	393/435 (90.34)	42/435 (9.65)
hcpA	395/444 (88.96)	49/444 (11.03)
Concatenated MLST	1829/2079 (87.97)	252/2079 (12.12)
wsp gene	483/605 (79.83)	122/605 (20.16)

Sameness and difference (%) were calculated by MEGA5 software.

Fig. 2), and indicated that both beetles are different from each other. The universal COI primers in this study are used widely to taxonomic classification of various other organisms (Takiya et al. 2006, Uddin et al. 2007, Zhang et al. 2015).

The *Wolbachia* genotyping using *wsp* gene-specific primer as explain earlier (Baldo et al. 2006; Ali et al. 2018a,c) and yielded a predicted band size of ≈600 bp from *O. nipae* and *B. longissima* DNA templates (Fig. 3). Only a small portion (≈600 bp) of the whole *wsp* region was used in this study. The gene *wsp* which encodes a major cell surface protein has confirmed to be the speediest evolving and has been widely utilized for intragroup phylogenetic investigations of *Wolbachia*-mediated species. Despite, mounting studies of *Wolbachia* investigation through *wsp* gene (Werren et al. 1995, Mitsuhashi et al. 2002, Nugapola et al. 2017), yet it is hard to believe that the partial DNA sequences of a single gene can reflect the authentic *Wolbachia* evolutionary information. It is due to high genetic divergence that undergoes extensive intragenic recombination in *Wolbachia* under certain conditions (Vandekerckhove et al. 1999, Baldo et al. 2006, Baldo et al. 2006). Therefore, an advanced, accurate, and universal *Wolbachia* MLST genotyping tool has

Fig. 2. MSA of host COI gene regions of ≈804 bp amplified from *Brontispa longissima* and *Octodonta nipae*. Shaded nucleotides indicate the sequence homology, while no shaded indicates the heterogeneity between the both species.

Fig. 3. MSA of (A)*Wolbachia* outer surface protein (*wsp*) of ≈600 bp and (B) concatenated MLST loci of five conserved regions (2073 or 20179 bp) isolated from *Brontispa longissima* and *Octodonta nipae*. Shaded nucleotides indicate the sequence homology, while no color indicates the heterogeneity between the both species.

been purposed (Baldo et al. 2006). *Wolbachia* MLST utilizes five housekeeping genes (*coxA*, *gatB*, *hcpA*, *fbpA*, and *ftsZ*) that are extensively circulated over the genome as a core set of markers for *Wolbachia* genotyping. Accordingly, in this study, we also performed PCR reactions on *Wolbachia* MLST gene-specific primers (Baldo et al. 2006; Ali et al. 2018a,c) to amplify *coxA*-402, *hcpA*-444, *ftsZ*-435, *gatB*-369, and *fbpA*-429 bp fragments for both beetle species. Furthermore, to determine the quality of bacterial DNA, a universal bacterial 16S rRNA primer (27F-14192R; Ali et al. 2018b) corroborate the quality of extracted DNA which was processed for further analysis. All *wsp* and MLST genotyping sequences from the same species demonstrated exact homology (100%) with *Wolbachia* from that specific host (*B. longissima*) (*wsp*—MG345108 and five MLST locus—MG553911, MG553916, MG553921, MG553926, and MG553931) (Ali et al. 2018a) and *O. nipae* (*wsp*—MG551861 and five MLST locus—MG641073, MG641078, MG641083, MG641088, and MG641093), respectively (Ali et al. 2018c). Concatenated MLST sequences were also identical with the sequence type (ST) 483 for *B. longissima* and ST-484 for *O. nipae*. Identification of *Wolbachia*, using *wsp* and MLST genotyping markers was used successfully for a number (approximately 16–76%) of insects and other arthropods (Werren et al. 1995, Jeyaprakash and Hoy 2000, Hilgenboecker et al. 2008, Dossi et al. 2014, Nugapola et al. 2017).

Although gel electrophoresis analysis revealed similar fragment size (≈600 bp) of gene-specific primers, MSA and Kimura

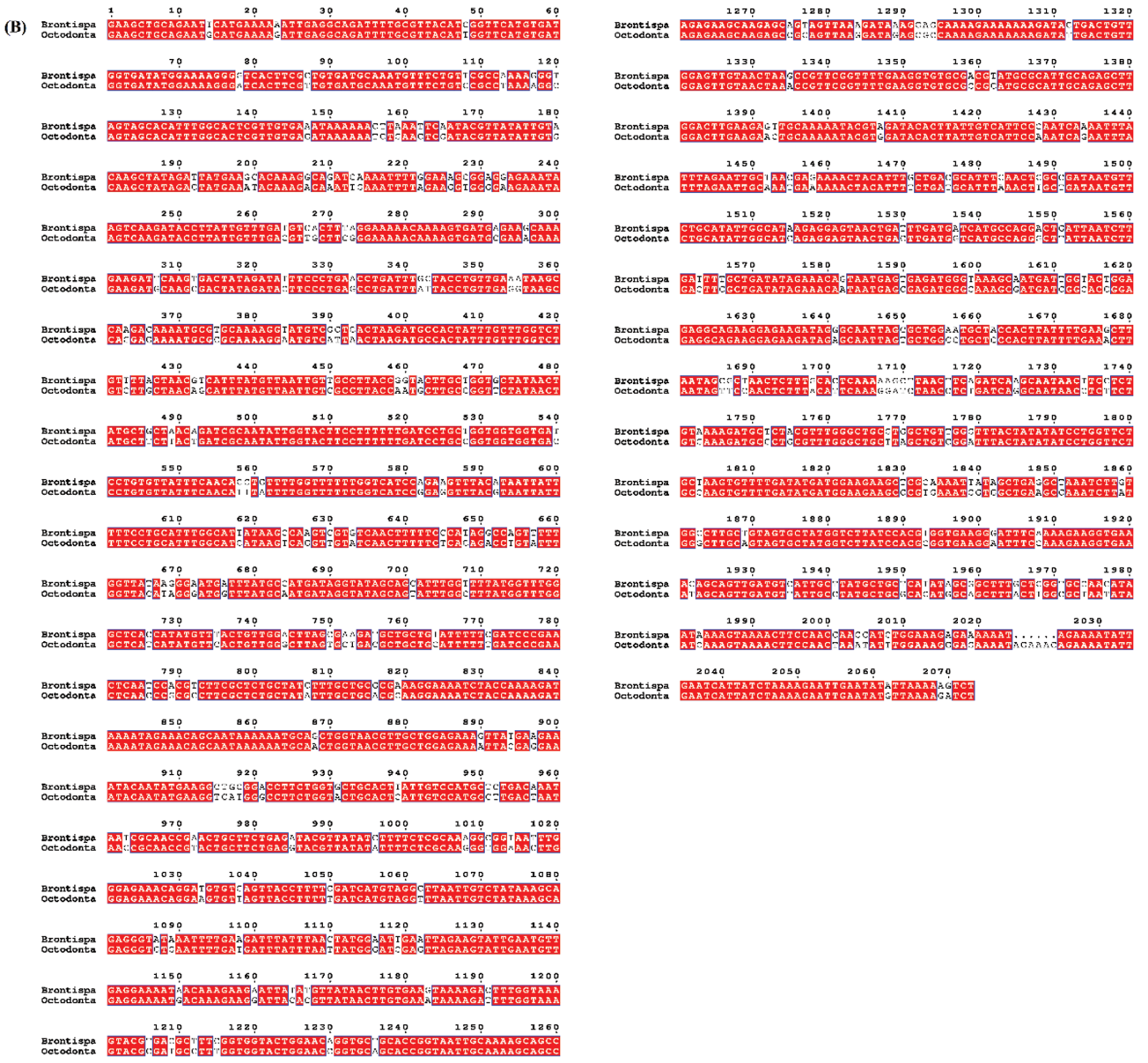

Fig. 3. Continued

2-parameter method accompanied a significant difference between *B. longissima* and *O. nipae wsp* gene (20.16%; Table 1; Fig. 3). Whereas, the MLST dataset analysis also showed higher variations in *fbpA* (14.68%) as compared to other MLST genes (Table 1; Supp Fig. 1). In general, the maximum similarity value or narrow range of 16S rRNA sequences (94.3 to 100%) may forbid discrimination (Kim et al. 1999). On the other hands, in our report, the sequence variation of *wsp* and MLST locus among beetle species were recorded to be moderate (79.80 to 100% and 85.31 to 100% similarity, respectively), which confirm that *B. longissima* and *O. nipae* are two different species of Coleoptera. Moreover, the apparent dissimilarity of gene sequences between these beetle species is substantial evidence of distinct genera (≤94.5%; Stackebrandt 2006, Tindall et al. 2010, Yarza et al. 2014).

In additions, the result of phylogenetic analysis based on *Wolbachia wsp* gene is summarized in Fig. 4A. ML tree revealed that *wsp* clade of *B. longissima* showed relatedness to *Diaphorina citri* and *Apis mellifera capensis* and clad of *O. nipae* showed closeness to *Tetramorium lanuginosum* which ultimately illustrated two distinct *Wolbachia* supergroup B and A, respectively. Likewise, the same trend of *Wolbachia* placement was observed in the phylogenic analysis of concatenated MLST data set as shown in Fig. 4B. Trees generated on *wsp* or concatenated MLST data set by ML algorithms indicated general concordance with one another (Fig. 4A concordance with Fig. 4B). Our phylogenetic results were strongly supported by previous studies on the phylogeny of *Wolbachia* in *B. longissima* (Ali et al. 2018a) and *O. nipae* (Ali et al. 2018c). Two insect species live in such close proximity may share related *Wolbachia* strains due to horizontal transmission or infection of microflora from one host to the other. For instance, infection with parallel *Wolbachia* strains by horizontal transfer was predicted in leafhoppers that assimilated the symbiont by feeding on the shared food resources (Mitsuhashi et al. 2002). Contrary to this assumption, our study insects may share the same host (*P. canariensis* and *C. nucifera*) but progressively occupy two different *Wolbachia* strains which are helpful to discriminate these two beetle species. Due to higher incidence rate of

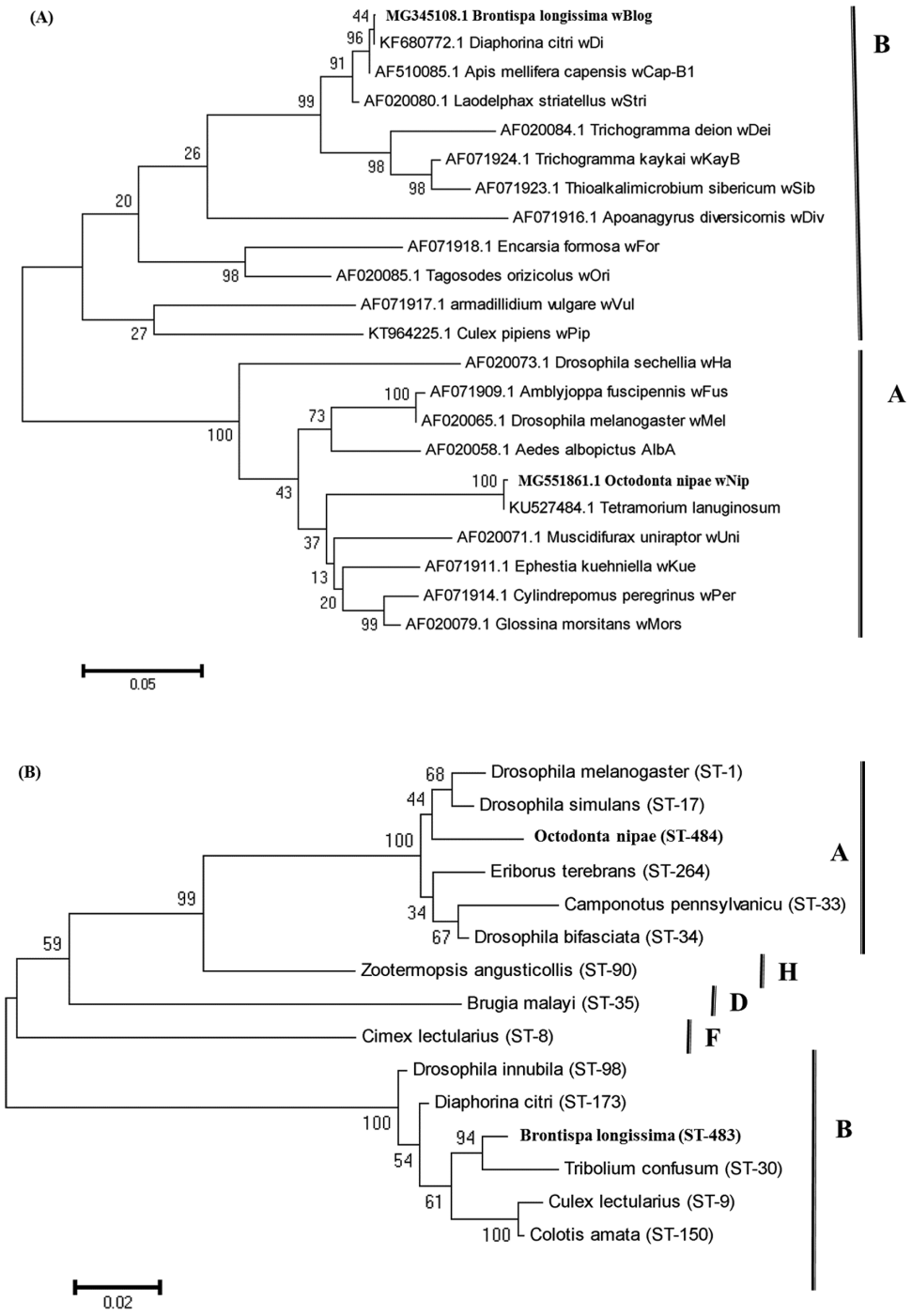

Fig. 4. Phylogenetic placement of *Wolbachia* strains isolated from *Brontispa longissima* and *Octodonta nipae* by ML inference phylogeny using MEGA (v. 5.05). (A) ML tree is constructed based on 2 (one from each beetle) *Wolbachia* outer surface protein (*wsp*) sequences (≈600 bp) with 20 *Wolbachia* strains from various arthropods belong to A, and B *Wolbachia* supergroups were assembled and aligned together for phylogenetic analysis. (B) ML tree based on the 2 (one from each beetle) concatenated MLST loci (2073 or 2079 bp) with 13 closely related sequence type (ST) retrieved from MLST database (http://pubmlst.org/wolbachia/). Sequence from this study is highlighted in bold, while alphabetic letters (A, B, H, F, and D) indicate different *Wolbachia* supergroups.

Wolbachia in weevils (Ali et al. 2016, 2018b) and numerous other insects and arthropods (Werren et al. 1995; Jeyaprakash and Hoy 2000; Hilgenboecker et al. 2008; Ali et al. 2018b), current strategy to discarnate insect species, particularly *B. longissima* and *O. nipae* is valuable for further quarantine and management strategies.

These beetles (*B. longissima* and *O. nipae*) can impersonate and inflict similar damage appearances and devastation posing a significant threat to palm and tourism industry. In additions, they have enormous ability to infect new regions and palm species previously unreported (Staines 2012). Hence, it is critical to gain thorough understating of pest biology and accurately discriminate them for quarantine and bio-control management (Wu et al. 2006, Hou et al. 2011). In the extent of damages, *B. longissima* causes extensive injuries to coconut palm and has become more economically valuable (Wu et al. 2006), while the later (*O. nipae*) preferably infest ornamental palms (Sun et al. 2003, Vassiliou et al. 2011).

Although it is also found on coconut palm, the damage is comparatively less severe (Vassiliou et al. 2011). Therefore, it is important to be able to distinguish between these two beetles for quarantine and to conduct effective research on natural enemies for long-term management. Traditionally, beetle species have been identified by their morphology, but this is often difficult and error-prone. Because morphological approaches often fail and misdirected. Correlate with this study, for further molecular and biological investigations, it is necessary to distinguish them correctly because both shared common host (*P. canariensis* and *C. nucifera*). Thus, the present approach using the molecular tool can be regarded as a feasible method for identification and facilitates rapid discrimination of same family member (Chrysomelidae) and allow timely decisions to prevent the spread of these quarantine pests and efficiently manage their damage.

During the recent years, *Wolbachia* has emerged as a hot topic of extensive research due to its significant impact on their host biology. From a practical perspective, this bacterium can be manipulated in biological control of various insect pests. *Wolbachia*-induced cytoplasmic incompatibility (CI) can be exploited for driving desirable traits such as resistant to the pathogen, into the insect vector of various diseases (Calvitti et al. 2010). Another mechanism analogous to sterile insect technique (SIT), in which the *Wolbachia*-infected males utilize the CI phenotype to control the pest population (Zabalou et al. 2004). Transfection for the naturally infected host to non-host insect species can generate a stable infection which could be engineered desirably. However, certain technical challenges such as unavailability of culture medium, generating a viable infection, and the risk of unintentional negative consequences hamper the exploitation of this bacterium in the field application. Nevertheless, the recent advances would soon enable us to come up with a novel reliable *Wolbachia*-based control strategy.

Conclusions

We, therefore, conclude that the host COI gene regions along with *Wolbachia* genotyping (*wsp* and MLST loci) analysis revealed *B. longissima* and *O. nipae* are two discrete species harbors distinct *Wolbachia* strains and these molecular tools can easily discriminate between them.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

Acknowledgments

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