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# 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 timeconsuming. 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

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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 graybrown 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 Wolbachiaspecific, 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  $\approx 600$  bp from *O. nipae* and *B. longissima* DNA templates (Fig. 3). Only a small portion ( $\approx 600$  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

	i	10	20	30	40	50	eò	
Brontispa Octodonta	TTTTTTGG	TCATCCAC ACATCCGC	AAGTCTACH AAGTTTATH	ATTCTAATT ATTTGATT	CTCCCAGGA CTCCCAGGA	TTTGGAATA TTTGG <mark>G</mark> ATA	AATCTCCCAC	АТСАТТА АТСАТТА
	70	80	90	100	11	.0 3	120 :	130
Brontispa Octodonta	GCCAAGAA GCCAAGAA	AAG <mark>A</mark> GGAA AAG <mark>T</mark> GGAA	AAAAGAAAG AAAAGAAAG	CATTC <mark>GGAG</mark> CCTT <mark>TGGAG</mark>	TAT <mark>TG<mark>GGA</mark>A TCC<mark>T</mark>A<mark>GGA</mark>A</mark>	TAATTTATO	SC <mark>T</mark> ATAATAG SC <mark>A</mark> ATAATAG	CA <mark>AT</mark> C <mark>GG</mark> CT <mark>AT</mark> TGG
	140	150	) 16	5 O	170	180	190	200
Brontispa Octodonta	TC <mark>TATTAC</mark> AT <mark>TATTAC</mark>	GATTCGTA	GTTTGAGC	CATCACAT CATCACAT	ATTTACTG ATTTACTG	T <mark>ggaatag</mark> Aggaatag	ATGTTGATAC	CGAGCT
			220	220	240	250	260	
Brontispa Octodonta	TATTT TATTTCAC	ATCAGCCA TCAGCCA	CTATAAT CAATAATCZ	ATTGCTGTT ATCGCAGTA	CCAACAGG CCTACTGG	ATTAAAAT ATCAAAAT	TTCAGATGA TTCAGATGA	ATAGCAA ATAGCAA
	270	280	290	300	310	) 33	20 37	3.0
Brontispa Octodonta	CCTACCAT CTTACCAT	TGGAGTTAZ TGGTGTAAZ	ACTITIATI ACTACAATI	TTAACCCAT CAACCCTC	TAAC <mark>CTTAT</mark> TAAC <mark>TTTAT</mark>	IGATCTTAC	GGATTTGTT GGATTTGT <mark>A</mark> T	CCTTTT CCTTTT
	340	250	360		70	290	290	400
Brontispa Octodonta	TACAGTAG CACAGTAG	GAGGTTT GAGGACTA	ACAGGAATI	TATTTTAGC TGTTCTAGC	TAATTCATC CAATTCATC	TATTGATAT AATTGATAT	TATCCTCCA TTATTCTCCA	GATACA GATACA
						450		
Brontispa Octodonta	TACTATGI TACTATGI	TTGTGGCTC TTGTAGCCC	ATTTTCACT ATTTTCAT	FATGTCCTT FATGTATTG	TCTATGGGA TCAATAGG1	GCTGTCTTC GCAGTATTC	GCTATCATG CGCAATTATA	GC <mark>AGG</mark> TT GG <mark>AGG</mark> AT
	7.0	400	400	500	510	5.27		
" Brontispa Octodonta	TCAGTCAA TTAGACAA	TGATTCCC	TCTAATAAC CCTAATTAC	TGGAACCA CAGGAACAA	CACTTAATO CCCTTAATO	SACAAATTI SAAAAATTI	ATAAAAATTCA ATAAAAATTCA	AATTCAT AATTCAT
Brontispa	CATCACAT	TTATTGG	GTAAACTT7	AACTTTCTT	TCCACAACA	CTTTTAG	SACTTAGAGG	ATACCT
Octodonta	TGTTATAI	TTCATTGGA	GTTAATTTA	AACTTTITT	CCACAACA	AT TTCTTAGE	GATTAAGAGG	ATGCCT
	610	62	20 0	530	640	650	660	670
Brontispa Octodonta	CGACGATA	TCAGATI	ACCCTGAT	CATTTITA FCCTTTACT	AA <mark>ATGAAAO</mark> TTATGAAAO	GCCGTTTC	TCAATTGGA	AGAT <mark>TAA</mark> ICCC <b>TAA</b>
	e	580	690	700	710	720	730	
Brontispa Octodonta	TTTCCCTA TTTCTCT	AG <mark>TAGG</mark> G <mark>A1</mark> TATT <mark>GG</mark> A <mark>A1</mark>	TATTTA <mark>C</mark> TI TATTTA <mark>T</mark> TI	FAATCTTCA FAGTTTTCG	TTATCTGGO TA <mark>AT</mark> TTGAO	SAAAG <mark>a</mark> ttt <i>i</i> Saaag <mark>t</mark> ttt <i>i</i>	ACAGCACAACO ACAGCACAACO	GAAAAAG Gaaaaag
	740	750	760	770	78		790 1	300

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 Journal of Economic Entomology, 2018, Vol. 111, No. 6

 1
 10
 20
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 40
 50
 60

 (R)
 Brontispa
 GAAGGIGGAGAATICATGIGATAAAAATIGAGGGAAGAATITTGGGGTAGAATCGGTTAGAAT
 CAAGGIGGAGAATICATGIGAATAAAAATIGAGGGAAGAATITTGGGGTAGAATCGGTTAGAATCGAATCGGTTAGAATCGGTTAGAATCGGTTAGAATCGGTTAGAATCGAATCGGTTAGAATCGAATCGGTTAGAATCGAATCGAATCGAATCGGTTAGAATCG

(	Octodonta	GAAGCTGCAGAZ	AT <mark>CCATGAAAA</mark> CI	ATTGAGGCAG	ATTTTGCGTT.	ACAT <mark>IGGTTC</mark>	ATGTGAT
	Brontispa	70 GGTGATATGGAS	80	90 TCGCTGTGAT	100 GCAAATGTTT	110 CTGTTCGCCA	
	occouoncu	130	140	150	160	170	180
	Brontispa Octodonta	AGTAGCACATTI Agtagcacatti	IGGCACTCGTTG IGGCACTCGTTG	ТБА <mark>А</mark> АТАААА Тба <mark>с</mark> атаааа	AACTTAAATT AATCT <mark>SAACT</mark>	C <mark>A</mark> ATACGTTA C <mark>C</mark> ATACGTTA	FATTGT <mark>a</mark> Fattgt <mark>c</mark>
	Brontispa Octodonta	190 CAAGCTATAGA CAAGCTATAGA	200 TATGAASCACA TATGAAATACA	210 AAGGCAGATC AAGACAAATI	220 AAAATTTTGG S <mark>AAATTTTA</mark> G	230 AAAGCGGAGG AACGIGGCGA	240 Agaaata Agaaata
	Brontispa	250 AGTCAAGATACO	260 TTATTGTTTGA	270 IGTCACTTTA	280 GGAAAAACAA	290 AAGTGATGAG	300
	-	310	3 Z Ņ	330	340	35 Q	360
	Octodonta	GAAGAT CAAG GAAGAT CAAG	GACTATAGATA GACTATAGATA	TTCCCTGAA TTCCCTGAG	CCTGATTTGC CCTGATTTAT	TACCTGTTGA TACCTGTTGA	HATAAGC
	Brontispa Octodonta	CAAGACAAAATO Cacgacaaaato Cacgacaaaato	SCGTGCAAAAGG SCGCGCAAAAGG	IATGTCCCTC ATGTCATTA	ACTAAGATGC ACTAAGATGC	410 CACTATTTGT CACTATTTGT	TIGGTCT TIGGTCT
	Brontispa Octodonta	430 GTITTACTAACO GTCTTGCTAACO	440 STCATTTATGTT AGCATTTATGTT	450 AATTGTTGCC AATTGTCGCC	460 TTACCGGTAC TTACCAATGC	470 TTGCIGGTGC TTGCCGGTCC	480 FATAACT FATAACT
	Brontispa	490 Atgetgetare	500 GATCGCAATAT	510 TGGTACTTCC	520 TTTTTTGATC	530 CTGC <mark>1</mark> GGTGG	540 T <mark>ggtga</mark> t
	Octodonta	ATGCT CT FAC	GATCGCAATAT:	570	580	SSD	ECD 600
	Brontispa Octodonta	CCTGTGTTATTT CCTGTGTTATTT	CAACACCTOTT CAACAIITATT	TTGGTTTTTT TTGGTTTTTT	GGTCATCCAG. GGTCATCCGG.	AA <mark>GTTTACA</mark> TI A <mark>HGTTTAC</mark> GTI	AATTATT BATTATT
	Brontispa Octodonta	610 TTTCCTGCATT TTTCCTGCATT	620 FGGCAT FGGCAT FGGCAT ATAAG	630 CAAGTCGTG ICACGTTGTA	64D TCAACTTTTT TCAACTTTTT	650 CCCATAGGCC CTCACAGACC	660 A <mark>gtettet</mark> I <mark>gt</mark> att
	Brontispa Octodonta	670 GGTTATAAGGG GGTTACAIAGG0	680 ATGATTTATGC ATGCTTTATGC	690 Catgataggt Aatgataggt	700 ATAGCAGCAT ATAGCAGTAT	710 TTGGITTTAT TTGGCTTTAT	720 GGTTTGG GGTTTGG
	Brontispa Octodonta	730 GCTCA <mark>(CATATO</mark> GCTCA <mark>(CATATO</mark>	740 STT <sup>T</sup> ACTGTTGG STT <mark>C</mark> ACTGTTGG	750 A <mark>CTTAG</mark> CGAA G <mark>CTTAG</mark> TGCT	760 GA <sup>ng</sup> ctgctg GA <mark>cgctgctg</mark>	770 ATTTTTCGA ATTTTTCGA	780 ICCCGAA ICCCGAA
	Brontispa	790 CTCAATCCACG	800	810 TATCTTTGCT	820 GCCCGAAAGG	830 AAAATCTACC	840 AAAAGAT
		85 ņ	<b>១ ៩</b> ពុ	870	eeņ	890	900
	Octodonta	AAAATAGAAACA AAAATAGAAACA	GCAATAAAAAA GCAATAAAAAA	TGCACCTGGT TGCAACTGGT	AACGTTGCTG	GAGAAAGTTA GAGAAA <mark>A</mark> TTA	GAGGAA
	Brontispa Octodonta	ATACAATATGAA ATACAATATGAA	AGGTCAIGGCC	TICIGGTGCT TICIGGTACT	GCACTIATTG GCACTCATTG	TCCATGC CCT TCCATGC CCT	SACAAAT SACTAAT
	Brontispa Octodonta	970 AAT <mark>CGCAACCG</mark> AAC <mark>CGCAACCG</mark>	980 ACTGCTTCTGA ACTGCTTCTGA	990 GATACGITAI GGTACGITAI	1000 ATCTTTCTC ATATTTCTC	1010 GCAAAGGCGG GCAAGGGGGGG	1020 AATTTG AACTTG
	Brontispa Octodonta	1030 GGAGAAACAGGA GGAGAAACAGGA	1040 ATGTGTCAGTTAG AGTGTIAGTTAG	1050 CCTTTTCGAT CCTTTTTGAT	106P CATGTAGGCT CATGTAGGTT	1070 TAATTGTCTA TAATTGTCTA	1080 Faaagca Faaagca
	Brontispa Octodonta	1090 GAGGGTATAAAT GAGGGTCTCAAT	1100 ITTTGA <mark>A</mark> GATTTI ITTTGA <mark>I</mark> GATTTI	<b>1110</b> ATTTAA <mark>C</mark> TAT ATTTAA <mark>T</mark> TAT	1120 GGAATIGAAT GGCATCGACT	1130 TAGAAGTATT TAGAAGTATT	1140 GAATGTT GAATGTT
	Brontispa Octodonta	1150 GAGGAAAATAAG GAGGAAAATGAG	1160 CAAAGAAGAATTI CAAAGAAGGATTI	1170 ATAT <mark>GTTATA</mark> ACACGTTATA	1180 ACTTGTGAA ACTTGTGAAR	1190 TAARAGAUTT TAARAGA <mark>U</mark> TT	1200 Iggtaaa Iggtaaa
	Brontispa Octodonta	1210 GTACG <sup></sup> GA GTACG <sup></sup> GA IGC	1220 TTCGGTGGTAC TTTGGTGGTAC	1230 TGGAAC <mark>A</mark> GGT TGGAAC <mark>A</mark> GGT	1240 GC <sup>TI</sup> GCACCGG GCACCGG	1250 TAATTGCARA TAATTGCARA	1260 Agcagcc Agcagcc

	1270	1280	1280	1300	1310	1320
Brontispa Octodonta	AGAGAAGCAAG Agagaagcaag	AGCA <mark>gt</mark> agtta Agc <mark>ug</mark> cagtta	AAGATAAAGCA Aggatagagcg	CARAAGARA Caraagara	AAAAGATACT AAAAAGATATT	GACTGTT GACTGTT
	1330	1340	1350	1360	137 p	138ņ
Brontispa Octodonta	GGAGTTGTAAC GGAGTTGTAAC	TAA <mark>C</mark> CCGTTCG TAA <mark>A</mark> CCGTTCG	GTTTTGAAGGT GTTTTGAAGGT	GTGCGACGTA GTGCGCCCCCA	ATGCGCATTGC. ATGCGCATTGC.	AGAGCTT AGAGCTT
	1390	1400	1410	1420	1430	1440
Brontispa Octodonta	GGACTTGAAGA GGACTTGAAGA	GT <mark>TGCAAAAAT</mark> RC <mark>TGCAAAAAT</mark>	ACGT <mark>R</mark> GATACA ACGT <mark>G</mark> GATACA	CTTATTGTCA CTTATTGTCA	ATTCC <mark>C</mark> AATCA ATTCC <mark>A</mark> AATCA	AAATTTA Gaattta
	1450	1460	1470	1480	1490	1200
Brontispa Octodonta	TTTAGAATTGC TTTAGAATTGC	IAACGAGAAAA AAATGA <mark>AAAA</mark> A	CTACATTT <mark>6</mark> CT CTACATTT <mark>C</mark> CT	GA <mark>C</mark> GCATTT GA <mark>CGCATTT</mark>	AACTCGCCGA AACTIGCCGA	TAATGTT TAATGTT
	1510	1520	1530	1540	1550	1560
Brontispa Octodonta	CTGCATATTGG CTGCATATTGG	CAT <mark>A</mark> AGAGGAG Cat <mark>c</mark> agaggag	TAACTGA <mark>T</mark> TTG TAACTGACTTG	ATG <mark>A</mark> TCATGO ATG <mark>C</mark> TCATGO	CAGG <mark>ACT</mark> CAT CAGG <mark>CTTAT</mark>	TAATCTT TAATCTT
	1570	1580	1590	1600	1610	1620
Brontispa Octodonta	GAITTIGCTGA GACTT <mark>C</mark> GCTGA	TATAGAAACA <mark>.</mark> TATAGAAACA <mark>A</mark>	TAATGAG <mark>T</mark> GAG TAATGAG <mark>C</mark> GAG	ATGGG <mark>T</mark> AAAG ATGGG <mark>C</mark> AAAG	SCAATGAT <mark>I</mark> GG SC <mark>CATGATC</mark> GG	TACT <mark>GGA</mark> CACC <mark>GGA</mark>
	1630	1640	1650	1660	1670	1680
Brontispa Octodonta	GAGGCAGAAGG GAGGCAGAAGG	AGAAGATAG <mark>G</mark> G AGAAGATAG <mark>A</mark> G	CAATTAG <mark>C</mark> GCT CAATTAG <mark>T</mark> GCT	GGAATGCTAC GGCCTGCTCC	CACTTATTTT CACTTATTTT	ЗААССТТ ЗАА <mark>А</mark> СТТ
	1690	1700	1710	1720	1730	1740
Octodonta	AATAGUUCHAA AATAGUUC <mark>H</mark> AA	CTCTTTACACT CTCTTT <mark>ACA</mark> TT	CAAAAAAGGUTA CAAA <mark>GGAUGTA</mark>	ACTE AGATO	CAAGCAATAAC CA <mark>G</mark> GCAATAAC	TCTTCT
	1750	1760	1770	1780	1790	1800
Octodonta	GT <mark>A</mark> AAAGATGC GT <mark>C</mark> AAAGATGC	CTACGTTTGG CTCCGTTTGG	GCTGCTTAGCT	GTCGGATTTA	CTATATATCC'	TGGTTCT
<b>B</b>	1810	1820	1830	1840	1950	1960
Octodonta	GCTAAGTGTTT GCCAAGTGTTT	TGATATGATGG	AAGAAGCCCGI	CAAATTATAC CAAATCCTCC	CTGAGGCTAA CTGA <mark>A</mark> GC <mark>C</mark> AA	ATCTTAT
Prontima	1870	1880	1890	1900	1910	1920
Octodonta	GGCTTGCAGT	AGTGCTATGGT	CTTATCCACG	GGTGAAGGA	ATTTC <mark>C</mark> AAAGA	AGGTGAA
Brontigna	1930	1940	1950	1960	1970	1980
Octodonta	ATAGCAGTIGA	TGT ATTGC	ATGCTGC	ATGGC <sup>A</sup> GCT1	TACTIGGCGC	TAATATA
Brontiapa	1990 ATAAAAGTAAA	2000	2010 ACCATOTOGAA	2020 AGAGAAAAA	2 TAGA	030 AAATATT
Octodonta	AT <mark>C</mark> AAAGTAAA	ACTTCCAACTA	AATATTTGGAA	AGCGACAAAA	TACAAAC <mark>AGA</mark>	AAATATT
Brontispa	2040 GAATCATTATC	2050 TAAAAGAATTG	2060 2 AATATATATAAA	AAGTCT		
Octodonta	GAATCATTATC	TAAAAGAATTG	AATAT	AGATCT		

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 ( $\leq$ 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 longterm 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 nonhost 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.

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