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Genomic evaluations of *Wolbachia* and *mt*DNA in the population of coconut hispine beetle, *Brontispa longissima* (Coleoptera: Chrysomelidae)

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ABSTRACT

Wolbachia pipientis is a diverse, ubiquitous and most prevalent intracellular bacterial group of alpha-Proteobacteria that is concerned with many biological processes in arthropods. The coconut hispine beetle (CHB), *Brontispa longissima* (Gestro) is an economically important pest of palm cultivation worldwide. In the present study, we comprehensively surveyed the *Wolbachia*-infection prevalence and mitochondrial DNA (*mt*DNA) polymorphism in CHB from five different geographical locations, including China's Mainland and Taiwan, Vietnam, Thailand, Malaysia and Indonesia. A total of 540 sequences were screened in this study through three different genes, i.e., cytochrome oxidase subunit I (COI), *Wolbachia* outer surface protein (*wsp*) and multilocus sequencing type (MLST) genes. The COI genetic divergence ranges from 0.08% to 0.67%, and likewise, a significant genetic diversity ($\pi = 0.00082$; $P = 0.049$) was noted within and between all analyzed samples. In the meantime, ten different haplotypes (H) were characterized (haplotype diversity = 0.4379) from 21 different locations, and among them, H6 (46 individuals) have shown a maximum number of population clusters than others. Subsequently, *Wolbachia*-prevalence results indicated that all tested specimens of CHB were found positive (100%), which suggested that CHB was naturally infected with *Wolbachia*. *Wolbachia* sequence results (*wsp* gene) revealed a high level of nucleotide diversity ($\pi = 0.00047$) under Tajima's *D* test ($P = 0.049$). Meanwhile, the same trend of nucleotide diversity ($\pi = 0.00041$) was observed in *Wolbachia* concatenated MLST locus. Furthermore, phylogenetic analysis (*wsp* and concatenated MLST genes) revealed that all collected samples of CHB attributed to same *Wolbachia* B-supergroup. Our results strongly suggest that *Wolbachia* bacteria and *mt*DNA were highly concordant with each other and *Wolbachia* can affect the genetic structure and diversity within the CHB populations.

1. Introduction

The coconut hispine beetle (CHB), *Brontispa longissima* (Gestro) (Coleoptera: Chrysomelidae), is a devastating insect pest of palm plants (Nakamura et al., 2006), and its larvae and adult damage delicate parts of the plant. After maturity of old fronds, the larvae and adults shift to other young fronds. Severe infestation of beetles leads toward the restricted development of palm cultivations due to browning and curling of leaves. The massive leaf damage retards the growth, production losses and eventually causes the death of palm tree (Nakamura et al., 2006). The CHB has been supposed to be local to Papua New Guinea and Indonesia, and after that, it has spread worldwide (Nakamura et al., 2006; Zhang et al., 2015). During 2002, CHB was first time reported from Hainan province (China), and it caused substantial destructions to palm trees, exclusively the coconut plants (Hou et al., 2011; Wan et al., 2015). Later on, the palm plantations from other provinces of China (Fujian, Yunnan, Guangxi, and Guangdong) were damaged by CHB (Lu et al., 2004; Hou et al., 2011; Chen et al., 2015). Recently, the invasion of CHB is alarming, due to which it is listed in the Global Invasive Species Database (2010).

Wolbachia pipientis is a gram-negative alpha-Proteobacteria (Rickettsiaceae; Rickettsiales), and considered to be the most ubiquitous obligate bacterial genus among other endosymbionts (Cordaux et al., 2001; Rowley et al., 2004; Werren and Windsor, 2000).

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Wolbachia contains a remarkable genetic diversity which was firstly characterized by molecular markers, i.e., 16S rRNA, *ftsZ*, *wsp*, *groEL* and *gltA*. However, more advanced strain genotyping technique was established based on multilocus sequence typing (MLST) system targeted to typing of five different loci (Baldo et al., 2006; Paraskevopoulos et al., 2006). Therefore, based on nucleotide differentiation, *Wolbachia* strains can be divided into eight to eleven supergroups (A–F and H–L) (Ali et al., 2016; Bordenstein and Rosengaus, 2005; Casiraghi et al., 2005; Ros et al., 2009).

Genetic evaluation of invasive species has importance about evolutionary and ecological aspects (Scheffer, 2000; Yassin et al., 2008), in which genetic variations attributed to estimate the location of birth or distribution route of pests. Hence, such information is essential for developing an appropriate pest management strategy (Muraji et al., 2008). For this purpose, the mitochondrial DNA (*mt*DNA) is widely employed tool both in phylogenetic and as a neutral marker in populations genetics. However, such outcomes are confounded because various arthropods are infected with ecto- or endo-symbiotic parasites that are maternally transmitted (inherited from mother to offspring's). Maternal inheritance means that parasites are genetically correlated with the mitochondrial genome and in female heterogametic taxa. Therefore, the variation pattern of *mt*DNA may often modulate the evolutionary history of ecto-or-endo-symbiotic parasites rather than their hosts.

Recently, *Wolbachia* got more focus due to its extensive distributions, biology as well as influence on ecology and their interactions with insect host's physiology (Stouthamer et al., 1993; Werren, 1997). Furthermore, owing to its reproductive phenotypes [Male killing (MK) Cytoplasmic incompatibility (CI) Parthenogenesis inductions (PI) and Feminization (F)], *Wolbachia* has not merely recognized as a manipulator of reproductive schemes but also identified to set a notable impact on the decline of *mt*DNA variation (Galtier et al., 2009). Both *mt*DNA and *Wolbachia* are transferred through parents to offspring and speculated that decreasing the *mt*DNA diversity and hitches alongside as mounting of *Wolbachia* infections (Armbruster et al., 2003). A specific mitochondrial cluster can sweep through a population and minimize the *mt*DNA polymorphism in the *Wolbachia*-infected populations (Ballard, 2000; Jiggins, 2003; Charlat et al., 2009). Therefore, mitochondrial variability pattern can be confounded by the spread of *Wolbachia*. Globally, a small number of reports have been explored the presence of *Wolbachia* in beetles (41 beetle species reported so far) (Ali et al., 2016, 2018a). Although, the absence of *Wolbachia* was also reported from some of the beetle species (Ali et al., 2018b). But, a comprehensive report of *Wolbachia*-presence in CHB was reported by Ali et al., (2018a) which is an addition to previous knowledge on *Wolbachia*-mediated beetles.

In this study, we undertook an extensive screening of *Wolbachia* and *mt*DNA variability in *B. longissima* from various geographical regimes based on the *Wolbachia* specific primers and host COI gene. The present study had been taken as a point of initiation to answer the following questions; (a) scrutinize the prevalence status and diversity of *Wolbachia* in CHB populations, (b) evaluate whether the *Wolbachia*-infection connected with the variability of *mt*DNA and, (c) endeavor the genetic structure, phylogenetic relationship and estimation of the rates of genes flow within this species by *Wolbachia* (based on *wsp* and MLST locus) and COI genes of CHB populations from five countries.

2. Material and methods

2.1. Collections of test specimens

We collected 76 adult specimens (male and female) of CHB from different geographical locations including, China Mainland (15 local locations), Taiwan China, Vietnam, Thailand, Malaysia and Indonesia (Fig. 1 and Table 1) during 2015–2017. Summary of collected specimens is elaborated in Fig. 1 and Table 1. Specimens from the same

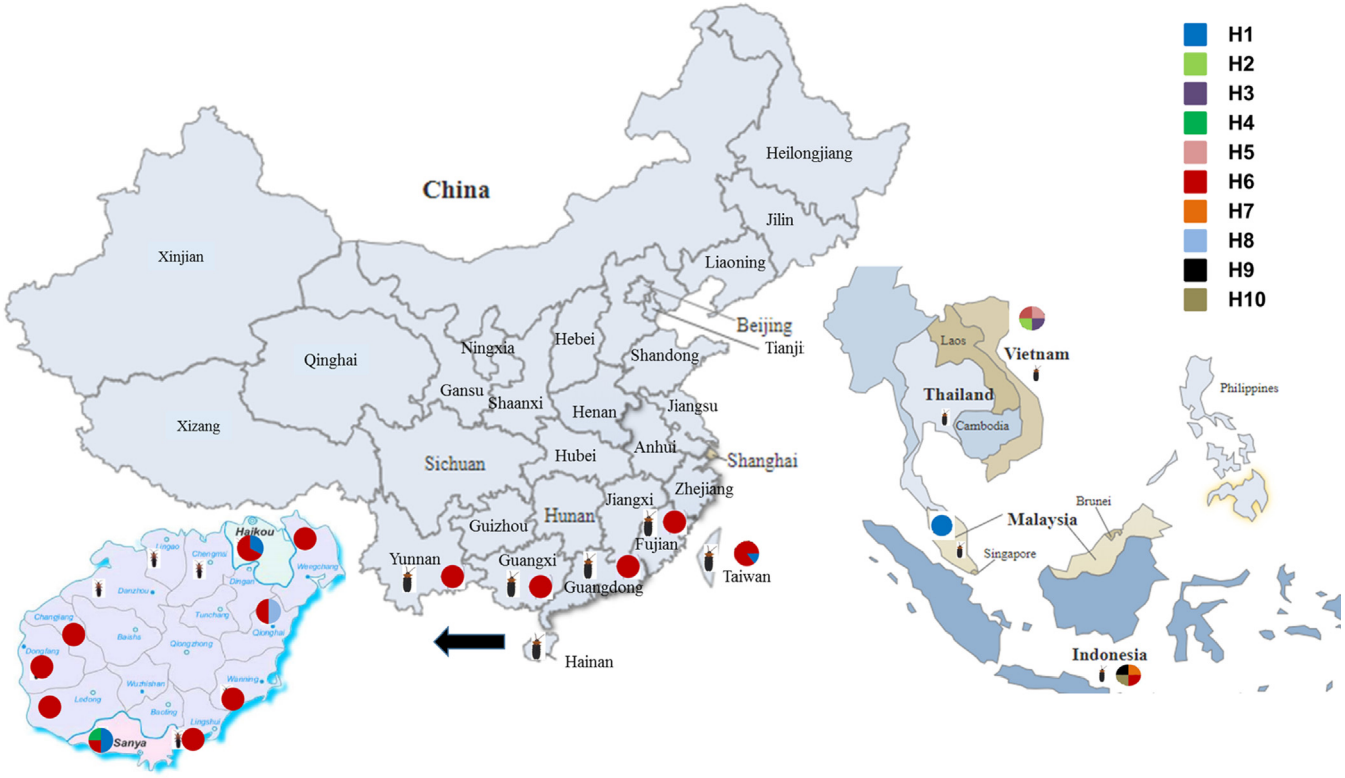

Fig. 1. The map depicting sampling sites of *Brontispa longissima* (Gestro) and *Wolbachia* haplotypes. Different colors within the circles indicate the variable haplotypes, and diameters are proportional to haplotype frequency. The map was created through Smartdraw (https://www.smartdraw.com/) and maximum parsimony networks for all haplotypes was constructed with software NETWORK4.5.

Table 1
Sample collection sites and sequence accession numbers of all targeted genes (Host COI and *Wolbachia* genes i.e. *wsp* and MLST genes) of *Brontispa longissima* (Gestro) from various geographical locations.

Sr. no.	District/province/country		COI gene	*wsp* gene	MLST genes				
					gatB	*coxA*	*hcpA*	*ftsZ*	*fbpA*
1	Nanning, Guangxi, China	GX1-4	MG747081-84	MG747143-46	MG758285-88	MG758150-53	MG758428-31	MG758222- 25	MG758357-60
2	Sanya, Hainan, China	SY1-3	MG747085-87	MG747147-48	MG758289-90	MG758154-56	MG758432-34	MG758226-28	MG758361-63
3	Kunming, Yunnan, China	YN1-3	MG747088-90	MG747149-51	MG758291-93	MG758157-59	MG758435-37	MG758229-31	MG758364-66
4	Guangzhou, Guangdong, China	GD1-3	MG747091-93	MG747152-54	MG758294-96	MG758160-62	MG758438-40	MG758232-34	MG758367-69
5	Zhangzhou, Fujian, China	ZZ1	MG747094	MG747155	MG758297	MG758163	MG758441	MG758235	MG758370
6	Danzhou, Hainan, China	DZ1-2	N/A	MG747156-57	MG758298-99	MG758164-65	MG758442-43	MG758236-37	MG758371-72
7	Haikou, Hainan, China	HK1-3	MG747095-97	MG747158-60	MG758300-302	MG758166-68	MG758444-46	MG758238-40	MG758373-75
8	Wenchang, Hainan, China	WC1-4	MG747098-101	MG747161-64	MG758303-06	MG758169-72	MG758447-50	MG758241-44	MG758376-79
9	Qionghai, Hainan, China	QH1-3	MG747102-03	MG747165-67	MG758307-09	MG758173-75	MG758451-53	MG758245-47	MG758380-82
10	Changjiang, Hainan, China	CJ1-4	MG747104-06	MG747168-71	MG758310-13	MG758176-79	MG758454-57	MG758248-51	MG758383-86
11	Dongfang, Hainan, China	DF1-3	MG747107-09	MG747172-74	MG758314-16	MG758180-82	MG758458-60	MG758252-53	MG758387-89
12	Ledong, Hainan, China	LD1-3	MG747109-11	MG747175-77	MG758317-19	MG758183-85	MG758461-63	MG758254-55	MG758390-92
13	Wanning, Hainan, China	WN1-5	MG747112	MG747178-82	MG758320-22	MG758186-90	MG758464-68	MG758256-59	MG758393-97
14	Sansha, Hainan, China	SS1-3	MG747113-15	MG747183-85	MG758323-25	MG758191-93	MG758469-71	MG758260-62	MG758398-400
15	Lingshui, Hainan, China	LS1-7	MG747116-22	MG747186-92	MG758326-32	MG758194-200	MG758472-77	MG758263-69	MG758401-07
16	Xiamen, Fujian, China	XM1-5	MG747123-27	MG747193-97	MG758333-37	MG758201-05	MG758478-82	MG758270-74	MG758408-11
17	Taipei, Taiwan, China	TW1-3	MG747128-30	MG747198-200	MG758338-40	MG758206-08	MG758483-85	MG758275-77	MG758412-14
18	Ho Chi Minh, Vietnam	VN1-4	MG747139-42	MG747201-03	MG758341-44	MG758209-11	MG758486-87	MG758278-79	MG758415-18
19	Wilayah Persekutuan Kuala Lumpur, Malaysia	MY1-5	MG747135-38	MG747208-12	MG758345-48	MG758212-15	MG758488-90	MG758280-81	MG758420-22
20	Manado, Sulawesi, Indonesia	ID1-4	MG747131-34	MG747204-07	MG758349-52	MG758216-19	MG758491-94	MG758282-84	MG758423-26
21	Suratthani, Thailand	TH1-4	N/A	N/A	MG758353-56	MG758220-21	MG758495-98	N/A	MG758427-28

N/A; Not applicable due to short sequence or low quality DNA.

territory and host plant were recognized as same populations. Collected specimens primarily immersed in absolute ethanol (100%) and soon after dislodged to experimental place and kept at −80 °C till DNA extractions. Geographic coordinate system (GPS) locations of all collected samples were identified through the application of online tool (www.simplemapper.net) with some manual amendments to elaborate the CHB populations (Fig. 1).

2.2. DNA extraction, PCR assays, cloning and sequencing

The whole insect was homogenized for DNA extractions using DNeasy Blood and tissue kit (Qiagen, Valencia CA) with strictly following the manufacturer's directions. CHB samples with weak amplification bands were extracted again or not included in this study. All samples of mitochondrial DNA (*mt*DNA) isolated from CHB were initially amplified ≈1044 bp fragment by Cytochrome Oxidase Subunit I (COI) primer pair (Table 1S), according to the procedure of Takano et al. (2017) with little modification. A total volume of 25 μL PCR assay, (8.5 μL ddH_2O, 12.5 μL Master Mix, 1 μL (10 μM) of each primer and 2 μL of DNA) with denaturation at 95 °C for 5 min followed by 35 cycles for 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C, and final extension step for 10 min at 72 °C, was used for the amplification of COI gene. A particular bacterial PCR reaction was conducted on all specimens of CHB by following standard protocols for the detection of *Wolbachia*-infection to amplify a part of *wsp* gene (≈600 bp) with a primer pair (wsp81F-wsp691R) (Table 1S). PCR reaction and cycling conditions were performed as previously explained by Ali et al. (2018c). A subset of each individual was examined twice to confirm their infection status. Moreover, the *Wolbachia* strains from all adult specimens of CHB were also genotyped by five *Wolbachia* MLST (*gatB*, *coxA*, *ftsZ*, *fbpA*, and *hcpA*) loci that have been utilized as a standard tool for strain composing and evolutionary investigations of *Wolbachia* by following recommendations (https://pubmlst.org/wolbachia/info/amp_seq_single.shtml). PCR conditions were performed as 5 min of DNA denaturation at 95 °C, followed by 35 cycles of 95 °C at 30 s, 30 s at the adjustable temperature cycles for each primer pair (*hcpA* and *coxA* at 50 °C, *gatB* and *fbpA* at 55 °C and *ftsZ* at 48 °C), 1 min at 72 °C and 10 min at 72 °C final extension step for all reactions. For all reactions, negative control (no DNA) were also incorporated. All PCR products were decontaminated by MiniElute Gel-Extraction Kit (Qiagen) and ligated directly into the pGEM T-Easy Cloning Vector (Promega, Madison, WI). Ligation clones were transformed with T1-Competent Cells (Qiagen) following the manufacturer's recommendations. Positive recombinants were sequenced by BioSune commercial sequencing Company (BioSune Biotech. Shanghai, China). After that, to assess DNA integrity, 1 μL and 3 μL of DNA from each sample were processed on NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and agarose gel-electrophoresis, respectively.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.07.003.

2.3. Estimations of COI and Wolbachia loci diversity within the population of CHB

The successfully amplified product sequences through COI (1044 bp), *wsp* (≈600 bp) and five MLST (*coxA*-402, *fbpA*-423, *ftsZ*-435, *gatB*-369, and *hcpA*-444 bp) genes were initially blasted to the National Center for Biotechnology Information (NCBI) to assure the amplification of expected genes. All COI gene was compared with the highest similarity to the NCBI database, while *wsp* and MLST genes fragment length were made according to the template provided by PubMLST database (https://pubmlst.org/wolbachia/info/allele_templates.shtml). To estimate country-wise and overall COI indices for genetic diversity, neutrality tests [Fu's *Fs* (Fu, 1997) and Tajima's *D* (Tajima, 1989)] and *Wolbachia* strains via *wsp*, single as well as concatenated MLST genes alignments were conducted using DnaSPv5 (Librado and Rozas, 2009) through polymorphism data analysis. The MEGA-7 program was used to analyze the nucleotide identity percentage within and among species (Kumar et al., 2016). Moreover, Kimura-2-Parameter (K2P) model was employed through MEGA-7 to estimate pairwise genetic distances of COI and *Wolbachia* strains of CHB populations. Short or incomplete fragment sequence of COI, *wsp* and MLST profile (*coxA*, *fbpA*, *hcpA*, *gatB* and *ftsZ*) were not entertained in this study (Table 1).

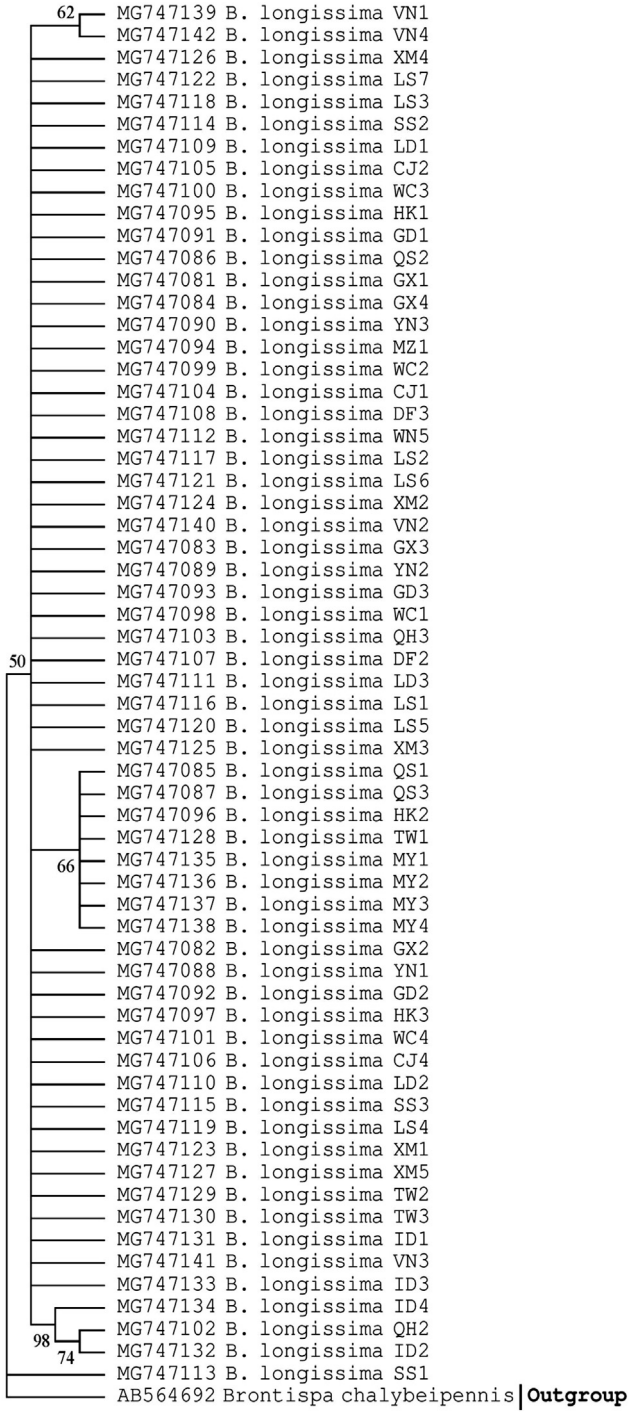

Fig. 2. Phylogenetic tree for COI gene sequences from *Brontispa longissima* (present study). Phylogenetic tree was constructed through Neighbor-Joining method by using Kimura-2-Parameter model. For tree construction, we used 62 COI sequences from our data, and one sequence of *Brontispa chalybeipennis* (AB564692) was used as an outer-group member. All sequence from this study are represented in short names as shown in figure and explained briefly in Table 1. For tree construction, bootstrap value was replicated 1000 times, and tree was condensed with 50% cut off value.

2.4. Phylogenetic analysis

To identify COI sequence relatedness of CHB from 21 different locations, we retrieved around 48 nucleotide sequence (related to our species sequence; Supplementary Fig. 1) from NCBI database were selected in this study. The portion (1044 bp) of the COI-3′ gene sequence utilized in our analysis, existed in between nucleotide 355–1398 bp (COI complete sequence 1543 bp) (KX087251 and KX087334). To better understand the relatedness, only 62 COI sequences (this study) along with *Brontispa chalybeipennis* (AB564692) as an out-group were subjected to final alignment and phylogenetic tree construction (Fig. 2). While for *Wolbachia* sequence relatedness, only 19 *wsp* and 17 concatenated MLST nucleotide sequence collected from NCBI and MLST database, as described previously (Ali et al., 2018c) from different *Wolbachia* supergroups, were used to conduct phylogenetic analysis and nucleotide similarity percentage within and among species of CHB (Figs. 3 and 4). All gene sequences (62 COI, 70 *wsp*, and 59 MLST loci) generated in this study were aligned by ClustalW with MEGA-7 software (Tamura et al., 2011) and was manually corrected through BioEdit software. Phylogenetic trees were constructed using Neighbor-Joining method for all datasets (COI, *wsp* and concatenated dataset of five MLST genes). The final alignment involved ≈2073 bp to 2079 bp for concatenated MLST sequences and ≈600 bp for *wsp* gene fragments. Haplotype number and haplotype diversity (Hd) were computed by the DnaSP5.10 program (Librado and Rozas, 2009). COI haplotypes intraspecific phylogeny was inferred by network algorithm Median-Joining (MJ) in the Network program (Bandelt et al., 1999).

2.5. Nucleotide sequence accession numbers

The sequences obtained from COI, *wsp* and MLST profile genes in this report were directly submitted to the GenBank database (National Center for Biotechnology Information) and accession numbers respectively noted in Table 1.

3. Results

3.1. Phylogenetic analysis

Phylogenetic of 62 CHB COI sequences presented that all populations were closely related to each other, showing > 90% resemblance (Fig. 2). Whereas the phylogenetic relationship of 70 *wsp* sequences (≈600 bp) indicated the highest similarity with *Apis malifera capensis* (AF510085) and *Diaphornia citri* (KF680772) and was belonged to supergroup-B of *Wolbachia* clade (Fig. 3). Likewise, Phylogenetic analysis, based on the concatenated dataset (MLST loci 2073 or 2079 bp), indicated that the *Wolbachia* strains (59 full MLST strains) of CHB populations also showing the same trend of relatedness with supergroup-B (Fig. 4). Both *wsp* and MLST gene-based phylogenetic evaluations exhibited the similar results.

3.2. Genetic divergence

On the basis of our sequenced database results, pairwise distance divergence for an entire COI sequence group (62 species) ranges between 0.09% and 0.67% (Table 3). The highest country wise interspecific distance was observed in China (0.67%), while the lowest interspecific distance (0.00%) was noted in Malaysia (Table 10S). The pairwise distance divergence in *wsp* (70 sequences) showing 0.93% and 0.047% up and down divergence, respectively (Table 3). Between country-wise dataset of *wsp*, Malaysia and Indonesia show a bit divergence (0.74% and 0.18%, respectively) as compared to others (Table 11S). Likewise, among the MLST genes, *hcpA* and *gatB* showing maximum divergence (4.08% and 1.59%, respectively) as compared to other genes (Table 3), whereas country (or district)-wise dataset indicating the higher divergence of *coxA* in Chinese Mainland and Malaysia (0.25% and 0.25%, respectively; Table 13S), *fbpA* in Malaysia and Vietnam (0.24% and 0.24%, respectively; Table 14S), *ftsZ* in China Mainland (0.46%; Table 15S), *gatB* in Malaysia and Thailand (2.01% and 0.91%, respectively; Table 16S) and *hcpA* in Malaysia and china (3.80% and 1.14%, respectively; Table 12S). Additionally, the same trend of divergence was observed in the *Wolbachia* concatenated MLST

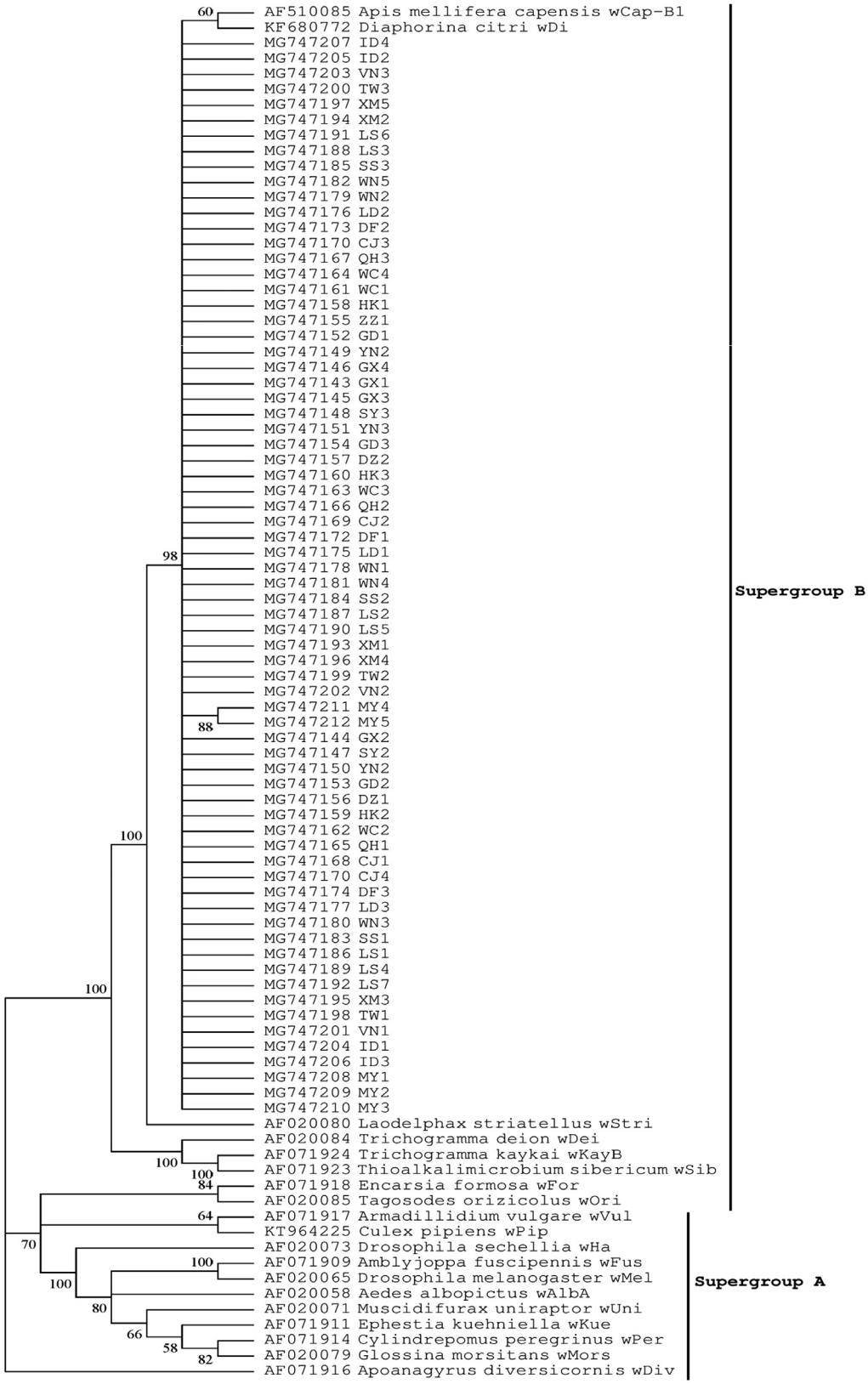

Fig. 3. Phylogenetic tree was constructed based on *wsp* gene sequences of *Brontispa longissima* through Neighbor-Joining method using Kimura-2-Parameter model. For tree construction, we used 70 *wsp* sequences from our data and 19 sequences of other arthropods retrieved from NCBI database. Our entire group of sequences represent Supergroup-B along with other eight sequences, whereas all the other sequences indicates the Supergroup-A, after alignment with our blasted sequences. All sequence from this study are represented in short names as shown in figure and explained briefly in Table 1. For tree construction, bootstrap value was replicated 1000 times, and tree was condensed with 60% cut off value.

dataset (Table 17S).

3.3. Haplotype network of CHB populations

Median-joining (MJ) network assay of COI sequences from all populations (21 sites) indicated ten well-differentiated genetic clusters (Haplotype diversity = 0.4379; Fig. 5). To display the genetic structure projected by the MJ network, different populations were allotted different colors according to the cluster outcomes through STRUCTURE software. The highest level of haplotype was exhibited in H6, where 46

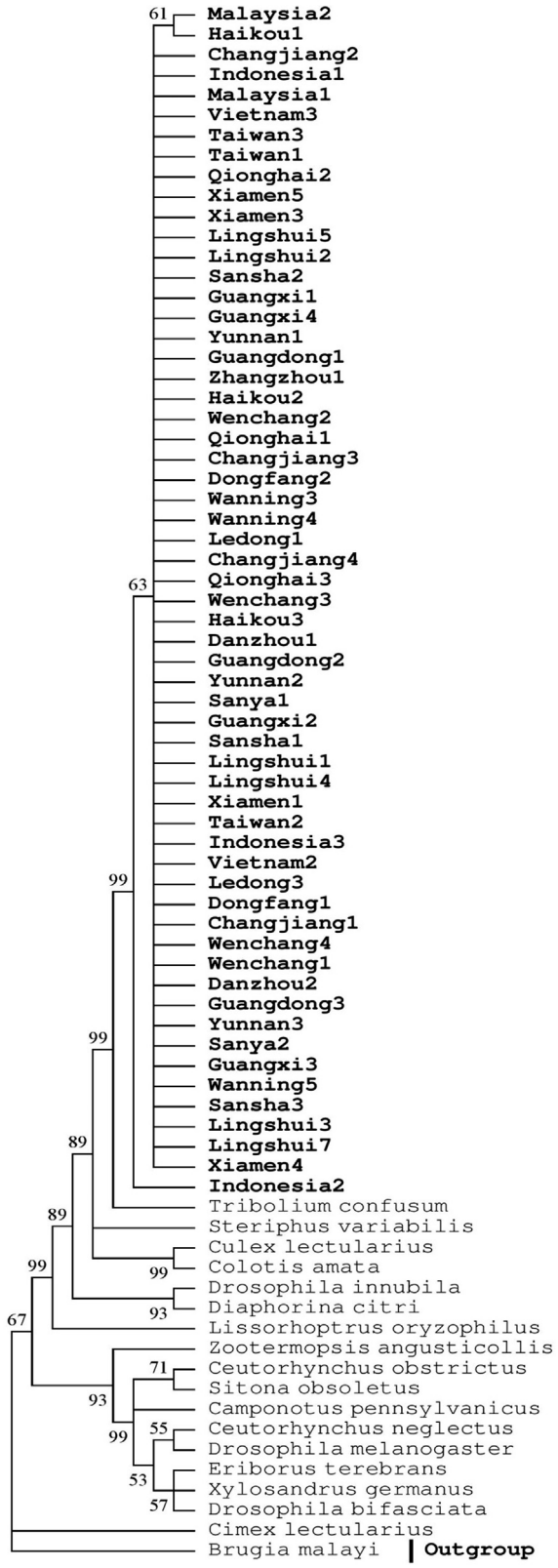

Fig. 4. Maximum Likelihood inference phylogeny based on the concatenated MLST data (2079 or 2073 bp). The 59 *Wolbachia* concatenated MLST strains of *Brontispa longissima* are indicated in bold letters; the other arthropods strains (18 sequences) represent different *Wolbachia* supergroups i.e. A, B, D, F and H. Strains are characterized by the names of their host species. *Wolbachia* supergroups are shown to the right of the host species names. For ML tree construction, bootstrap value was replicated 1000 times, and tree was condensed with 60% cut off value.

individuals were clustered in the same group, showing maximum population interconnections, followed by H1 (8 individuals) that represented the small cluster. All the other haplotypes (H2, H3, H4, H5, H7, H8, H9 and H10) represented a single individual (Fig. 5).

3.4. Genetic variability of CHB

To ascertain the genomic diversity of adult CHB specimens, 76 individuals were collected from different geographical locations, and 62 sequences were screened after amplifying COI gene, which presented 90–100% identity with the NCBI database. After confirming the sequence originality, all genetic diversity indices (Table 2) were measured in the light of pairwise nucleotide alterations as well as nucleotide diversity, and these indices were affirmed by two neutrality tests, like Tajima's *D* and Fu's Fs. Genetic diversity, based on Eta values, was observed in CHB specimens, and overall 10 mutations within all COI sequences from all populations. Likewise, there was a significant genetic diversity ($\pi = 0.00082$) for all COI sequences of CHB populations, under both neutrality test (Tajima's $D = 0.008$, and Fu's $Fs = 0.049$; Table 2). On the other hand, country (or district)-wise genetic diversity was significant for the populations of China Mainland ($\pi = 0.00040$) and Indonesia ($\pi = 0.00319$) under Tajima's *D* test ($P = 0.049$ and $P = 0.05$ respectively; Table 2S). However, there was a non-significant genetic variation within the populations of Taiwan (China), Vietnam, and Malaysia (Table 2S).

3.5. Wolbachia prevalence surveys

To determine the infection prevalence of the bacterial endosymbiont *Wolbachia* from 76 CHB specimens, the total genomic DNA was extracted from each of CHB individuals (Table 1), and major pattern of *Wolbachia* infection was found positive in all field-collected specimens of CHB. Furthermore, we tested *Wolbachia* genotyping differences through *wsp*, single *Wolbachia* MLST (*gatB*, *coxA*, *ftsZ*, *hcpA*, and *fbpA*) and concatenated MLST (2079 bp) genes from each geographic location using nucleotide diversity and neutrality test by DnaSPv5 software. From entire *wsp* (70) sequences, nucleotide diversity indicated a total of five mutations (Eta = 5) and an average number of nucleotide difference ($k = 0.25383$) showed statistically significant variation ($\pi = 0.00047$) under Tajima's *D* test ($P = 0.049$) (Table 2). Whereas neutrality tests were employed separately on each population sequence and outcomes were described in Table 2. In common estimates, Tajima's *D* and Fu's *Fs* produced statically significant and non-significant results respectively. Meanwhile, country (or district) wise population data were also assessed, and results indicate no mutation was observed in all tested populations except Malaysia and Indonesia (Table 3S).

A total of 349 multilocus gene sequences (*gatB*-72, *coxA*-72, *hcpA*-71, *fbpA*-71 and *ftsZ*-63) were assessed from earlier explained locations. Overall 23 mutations were recorded in MLST genotyping, whereas, from single gene assessment, higher mutations were recorded in *hcpA* (Eta = 17) and *gatB* (Eta = 6) followed by other multilocus genes. MLST genes representing significant genetic variations (Table 2), for example, Fu's *Fs* test validated significant results in *coxA* ($P = 0.026$) and *fbpA* ($P = 0.026$), whereas, Tajima's *D* test indicated *gatB* ($P = 0.05$) and *hcpA* ($P = 0.001$) genes. Accordingly, Country (or district)-wise data, only *hcpA* gene showed the genetic difference in China Mainland ($\pi = 0.00041$) through Tajima's *D* ($P = 0.05$) (Table 4S), whereas *coxA*, *fbpA*, *ftsZ* and *gatB* genes showed little or no genetic variations from any other country (or district)-site as verified from both tests (Tables 5S–8S respectively). Likewise, the 59 concatenated MLST dataset shown significant differences ($\pi = 0.00041$) validated through Tajima's *D* ($P = 0.001$; Table 9S), whereas, country (or district)-wise dataset indicated higher significant variation in China Mainland ($\pi = 0.0007$) under Tajima's *D* and Fu's *Fs* test ($P = 0.020$, $P = 0.049$, respectively; Table 9S).

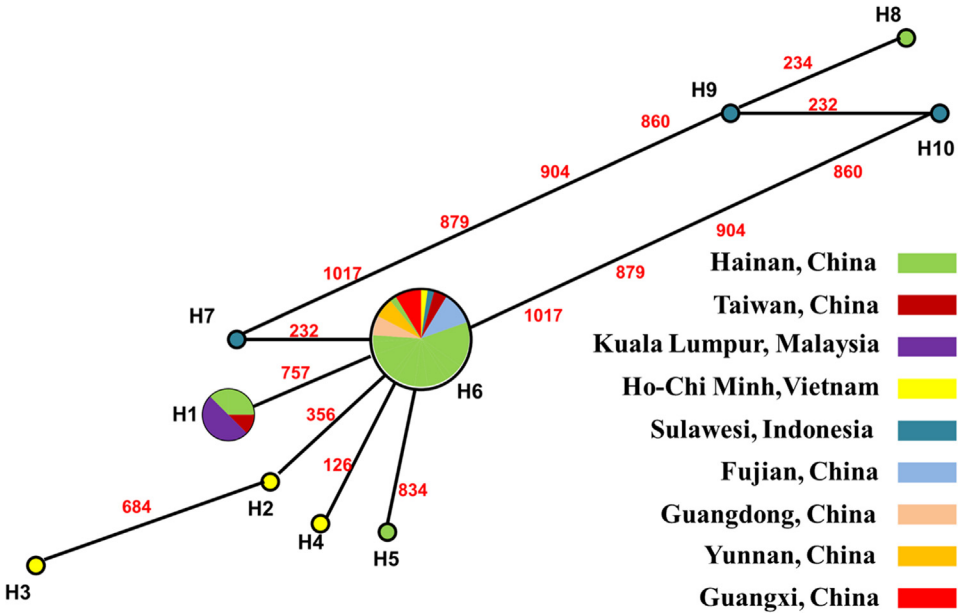

Fig. 5. Median-joining haplotype network infer the intraspecific phylogenies of *Brontispa longissima* (Gestro) generated through sequencing of *mt*DNA (COI) from different geographic locations. Each haplotype is represented by a circle and relative sizes of the circles indicate haplotype frequency. Circles of the same color represent haplotypes from the same population. The maximum parsimony networks for all haplotypes were constructed with software NETWORK4.5.

Table 2
Overall Genetic diversity of mitochondrial cytochrome oxidase subunit I (*mt*COI), *Wolbachia* outer surface protein (*wsp*) and five concatenated MLST genes (*coxA*, *fbpA*, *ftsZ*, *hcpA* and *gatB*) estimated by DnaSP v5 through polymorphism data analysis and MEGA7 by using Kimura 2-parameter model under pairwise deletion for *Brontispa longissima*, respectively.

Gene	n	Nucleotide diversity					Neutrality tests			
		S	k	Eta	Θ	π	Fu's *Fs*	p-value	*D*	p-value
*mt*COI	62	10	0.85669	10	0.0020	0.00082	−4.508	0.008	−1.64600	0.049
gatB	72	6	0.16667	6	0.0039	0.00052	−1.537	0.138	−2.05575	0.05
coxA	72	1	0.05516	2	0.0011	0.00015	−3.561	0.026	−1.42271	0.10
hcpA	71	16	0.47847	17	0.0087	0.00119	−0.705	0.193	−2.55765	0.001
fbpA	71	2	0.05634	2	0.0009	0.00013	−3.528	0.026	−1.42292	0.10
ftsZ	63	1	0.03175	1	0.0005	0.00008	−1.830	0.129	−1.07873	0.10
MLST concatenated	59	22	0.81181	23	0.0025	0.00041	−1.501	0.108	−2.64405	0.001
Wsp	70	5	0.25383	5	0.0019	0.00047	−0.780	0.215	−1.71276	0.049

Abbreviations: Eta = Total number of mutations, n = number of sequences, k = Average number of nucleotide differences, S = Number of segregating sites, Θ = nucleotide substitution rate, π = nucleotide diversity, Fu's *Fs* = A negative value of *Fs* is evidence for an excess number of alleles, whereas a positive is related to deficiency of alleles, and *D* is the Tajima test statistic (both *D* and Fu's *Fs* are used to single nucleotide polymorphism). Neutrality analysis was implemented only in populations that contained more than three individuals, with the following level of significance.

Table 3
Overall Genetic divergence of mitochondrial cytochrome oxidase subunit I (*mt*COI), *Wolbachia* outer surface protein (*wsp*) and five MLST loci of *Wolbachia* estimated using DnaSP v5 through polymorphism data analysis and MEGA7 by using Kimura 2-parameter model under pairwise deletion for *Brontispa longissima* respectively.

Gene	n	Mean distance	Max distance
*mt*COI	62	0.08	0.67
gatB	72	0.052	1.59
coxA	72	0.015	0.27
hcpA	71	0.121	4.08
fbpA	71	0.013	0.24
ftsZ	63	0.008	0.25
MLST concatenated	59	0.041	1.02
Wsp	70	0.047	0.93

3.6. Mismatch distribution

Finally, the mismatch distribution plot of both genotypes (*mt*DNA and *Wolbachia*) isolated from CHB specimens of all locations are considerably different; statistical values are given in Fig. 6. Despite the genetic diversity observed within all populations, (DNA sequences were probed for population size alterations), nucleotide mismatch distribution among all *mt*DNA and *Wolbachia* sequences of CHB were also detected. Analysis results demonstrated a genetic variation among *mt*DNA and *Wolbachia* sequences, but there was less or no population expansion of CHB in all five countries (or district) as shown in Fig. 1S.

4. Discussion

The COI-3′ DNA study was organized to characterize the genetic characterization of CHB from five different locations (or district). We collected 62 sequences of CHB from five variable locations and assessed the phylogenetic relationship among all populations and their genetic diversity. Phylogenetic relationship of CHB has defined the genetic interaction among the populations from various locations as well as diversity, based on COI DNA. Various reports have anticipated that the low genetic diversity of *mt*DNA and diversions from neutral evolutions in insect species can be accompanied with either a selective sweep on *mt*DNA, or genome-wide bottleneck effect (Shoemaker et al., 2004; Sun et al., 2011; Islam et al., 2018a,b,c) and deleterious of mutations during population expansions (Seger et al., 2010). As the *Wolbachia* and the *mt*DNA of the insect hosts contribute the same pattern of transmission (maternal inheritance). Therefore, the potential role of *Wolbachia* in

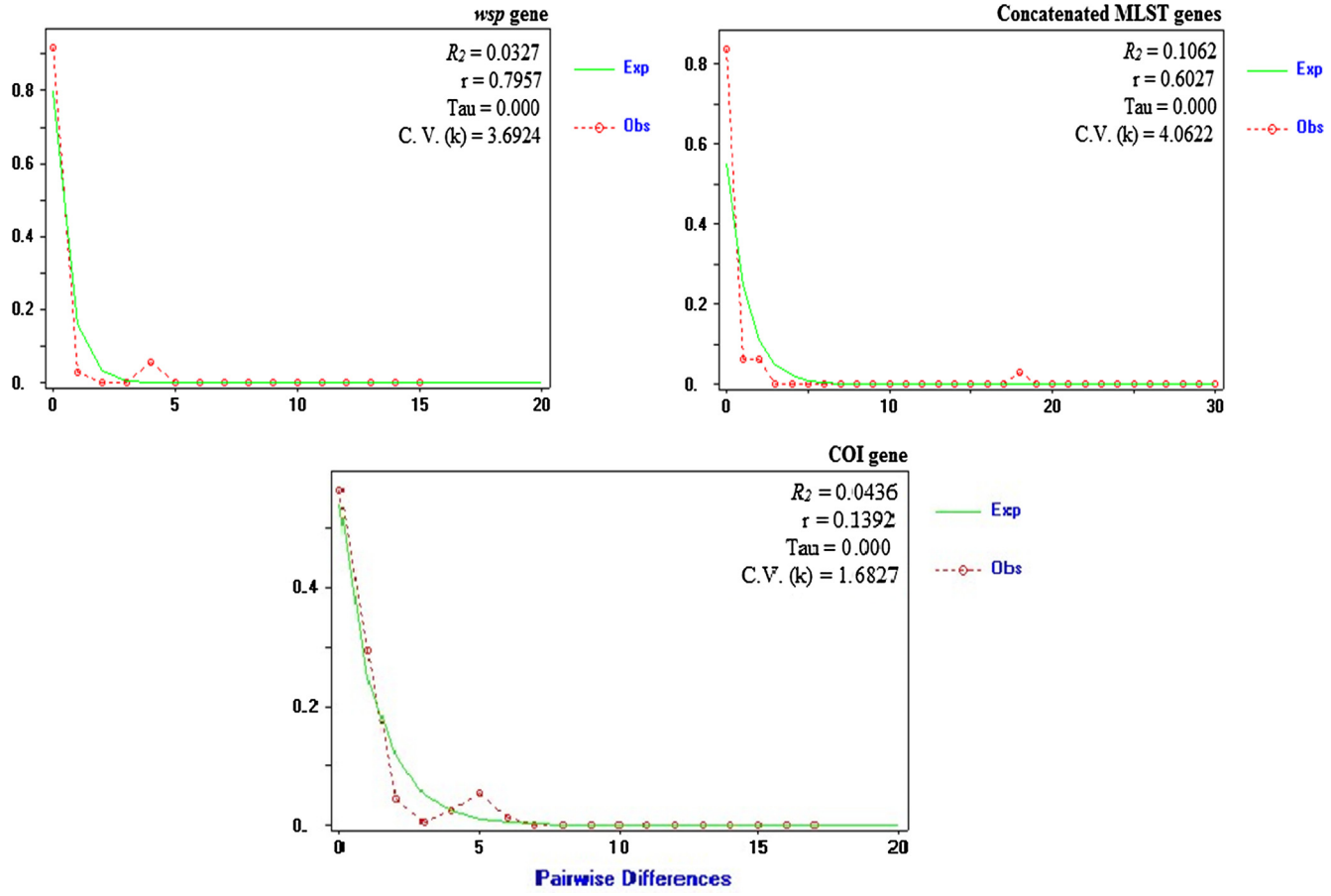

Fig. 6. Overall pairwise mismatch distributions of *Brontispa longissima* based on cytochrome oxidase subunit I (COI), *Wolbachia* outer surface protein (*wsp*) and concatenated MLST loci (2079-bp) sequences using DnaSP v5. The X-axis shows the observed distribution of pairwise genetic variation, and the Y-axis represents the frequencies.

genetic diversity of host *mt*DNA cannot be neglected (Zhang et al., 2013). In this study, overall populations of CHB show less *mt*DNA polymorphism (Eta = 10) than that of *Wolbachia* infection (Eta = 23) (Table 2).

In the present investigation, we demonstrated that the patterns of *mt*DNA deviations connected with variable *Wolbachia* infection prevalence in natural populations of CHB and uncover the impacts of *Wolbachia* on host *mt*DNA differentiation. Our analysis proposed that the prevalence of *Wolbachia* was high in the studied natural populations of CHB with 100% infection rate. The perfect concordance of *mt*DNA polymorphism and *Wolbachia* infection prevalence advocates that the mitochondrial genetic structure of the insect species may be incredibly affected by the *Wolbachia* infection. Various other *Wolbachia*-host-coevolution examples can support our study results, such as, in *Drosophila simulans*, *mt*DNA deviations have spread rapidly as the consequence of CI from *Wolbachia* infection (Turelli and Hoffmann, 1991). A similar trend of associations has been witnessed in *Orseolia oryzae* (Behura et al., 2001), *Solenopsis* spp. (DeWayne Shoemaker et al., 2000), and *Culex pipiens* L. (Rasgon et al., 2006). Reduced *mt*DNA polymorphism as a consequence of *Wolbachia* infection has also been congruent in various other insects (Sun et al., 2011; Jäckel et al., 2013; Jiang et al., 2014). Hereafter, the solid negative relationship between the *Wolbachia*-infection and *mt*DNA diversity reinforce the nation that *Wolbachia* has manipulated intraspecific divergence, consequently diminishing the genetic diversity of CHB populations

Distribution and prevalence of *Wolbachia* are widespread in various groups of invertebrate. The *wsp* gene used abundantly to characterize the *Wolbachia* from various arthropods (Werren and Windsor, 2000; Augustinos et al., 2011; Cordaux et al., 2012). Our study results represent the first comprehensive analysis of *Wolbachia* infections in invasive CHB samples through *wsp* gene. On the other hand, recent detection of recombination both within and between *Wolbachia* genes (Baldo et al., 2005, 2006) suggested that a single-locus method to strain characterization may be misleading. Thus, the patterns observed in the single locus studies may not accurately reveal the true evolutionary and demographic records of *Wolbachia* isolates (Baldo et al., 2007). Therefore, in the present study, PCR with *wsp* and additionally with MLST genes were used for genotyping the *Wolbachia* presence and exploit its variability from different geographical populations of CHB. An MLST pattern for *Wolbachia* was established to overcome the recombination concerns and offer an expanded dataset for comparative analyses (Baldo et al., 2006).

The prevalence level of *Wolbachia* was not homogenous among the different natural populations of invertebrates. For example, *Wolbachia* prevalence has been reported as 100% in *Callosobruchus chinensis* (Kondo et al., 2002) and *Culex quinquefasciatus* specimens (Behbahani, 2012), 94.4% in *Cx. Pipiens* (Rasgon, 2011), 94% in *Drosophila simulans* (Turelli and Hoffmann, 1995) and 20% in *Trichogramma kaykai* (Stouthamer and Kazmer, 1994). Contrary to these variations (20–100%), the overall rate of infection determined in our study was 100%, which indicate that *Wolbachia* in CHB was highly prevalent. There are many reasons which may influenced the infection level, for example high or low temperature (Chen et al., 2009; Qasim et al., 2018), host sex (Correa and Ballard, 2012), life stages (Humphreys and Douglas, 1997), polymorphism (Nishikori et al., 2009), population density (Wiwatanaratanabutr and Kittayapong, 2009), reproductive cycle (Wolschin et al., 2004), geographic location, host immune response (Ratzka et al., 2013), and competition among symbionts (Goto

et al., 2006).

The *Wolbachia* strains characterized in the genus *Brontispa* (70 sequences of *B. longissima*) were genetically very close to each other. Such close relationship of symbionts within the same host genus indicates that the CHB *Wolbachia* strains were identical to the *Wolbachia* clad of supergroup-B. Several arthropods have been identified to be infected by both A and B supergroups representatives (Werren and Windsor, 2000) or possessed bacteria in which part of the DNA originated from one and the rest from another supergroup. Furthermore, phylogenetic analysis (*wsp* and concatenated MLST genes) revealed that all collected samples of CHB attributed to same *Wolbachia* B-supergroup. Our results strongly suggest that *Wolbachia* bacteria and *mt*DNA were highly concordant with each other and *Wolbachia* can affect the genetic structure and diversity within CHB populations-group. Various former studies reinforce our *Wolbachia* phylogenetic results which indicated that weevils mainly infected by *Wolbachia* belonged to supergroup-B (Hsiao, 1996; Son et al., 2008; Ali et al., 2016, 2018a). There are also some exceptional cases such as *Octodonta nipae* hosting *Wolbachia* supergroup-A (Ali et al., 2018c) and *Rhinocyllus conicus* with the distinct supergroup-F (Campbell et al., 1992). Moreover, a recent report by Ali et al. (2018b) integrate all beetle species that were found to be *Wolbachia* positive.

5. Conclusion

We conclude that *Wolbachia* infection was highly prevalent infecting all CHB populations throughout all the tested geographical locations and found a single *Wolbachia* strain belongs to supergroup-B. Moreover, these findings suggest that a reduction in *mt*DNA diversity of CHB resulted from hitchhiking concordance with the spread of *Wolbachia*. However further study needs to define *Wolbachia* inter-population reproductive incompatibility pattern and its usefulness as a bio-agent control measure.

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Conflict of interests

Authors declare no conflict of interests.

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