

# Infection Density Dynamics and Phylogeny of *Wolbachia* Associated with Coconut Hispine Beetle, *Brontispa longissima* (Gestro) (Coleoptera: Chrysomelidae), by Multilocus Sequence Type (MLST) Genotyping <sup>S</sup>

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The intracellular bacterium *Wolbachia pipientis* is widespread in arthropods. Recently, possibilities of novel *Wolbachia*-mediated hosts, their distribution, and natural rate have been anticipated, and the coconut leaf beetle *Brontispa longissima* (Gestro) (Coleoptera: Chrysomelidae), which has garnered attention as a serious pest of palms, was subjected to this interrogation. By adopting *Wolbachia* surface protein (*wsp*) and multilocus sequence type (MLST) genotypic systems, we determined the *Wolbachia* infection density within host developmental stages, body parts, and tissues, and the results revealed that all the tested samples of *B. longissima* were infected with the same *Wolbachia* strain (*wLog*), suggesting complete vertical transmission. The MLST profile elucidated two new alleles (*ftsZ*-234 and *coxA*-266) that define a new sequence type (ST-483), which indicates the particular genotypic association of *B. longissima* and *Wolbachia*. The quantitative real-time PCR analysis revealed a higher infection density in the eggs and adult stage, followed by the abdomen and reproductive tissues, respectively. However, no significant differences were observed in the infection density between sexes. Moreover, the *wsp* and concatenated MLST alignment analysis of this study with other known *Wolbachia*-mediated arthropods revealed similar clustering with distinct monophyletic supergroup B. This is the first comprehensive report on the prevalence, infection dynamics, and phylogeny of the *Wolbachia* endosymbiont in *B. longissima*, which demonstrated that *Wolbachia* is ubiquitous across all developmental stages and distributed in the entire body of *B. longissima*. Understanding the *Wolbachia* infection dynamics would provide useful insight to build a framework for future investigations, understand its impacts on host physiology, and exploit it as a potential biocontrol agent.

**Keywords:** *Wolbachia*, *Brontispa longissima*, quantitative PCR, MLST, biocontrol agent, symbiont-host interactions

## Introduction

Members of the family Chrysomelidae (Insecta: Coleoptera), also known as leaf beetles, are one of the most plentiful, diverse, and successful phytophagous Coleopteran insects, encompassing more than 35,000 species reported worldwide [1]. Owing to its devastating impacts on commercial and

ornamental palm cultivation, the pest has garnered a lot of attention [2]. Among the Chrysomelidae family, the genus *Brontispa* has 22 described species, out of which 17 species have been reported causing considerable damage to various palm species [3]. *Brontispa longissima* (Gestro) (Coleoptera: Chrysomelidae), commonly known as coconut hispine beetle, is a notorious pest and key defoliator of the

coconut palm, *Cocos nucifera* L. (Arecales: Areaceae) [4]. This pest was originally documented from Papua New Guinea and Indonesia; however, owing to international trade of infested plant materials, the beetle has spread to several other countries, including Tahiti Vanuatu, Samoa, and Southeast and East Asian countries such as the Solomon Islands, Maldives, Thailand, Vietnam, Indonesia, and China, and is rapidly infecting other *C. nucifera*-growing regions of the world [5]. Recently, the invasion of *B. longissima* in the newly introduced areas is a matter of great concern and was listed in the Global Invasive-Species Database in 2010. It is also a quarantine pest in China, being first reported on *C. nucifera* in 2002 from Hainan province (Haiku city) [6]. The dispersal rate of *B. longissima* is rapid and has already invaded Guangdong, Guangxi, Yunnan, and Fujian provinces ( $\approx 30,000$  palm trees are infested), and 17 other cities of China are threatened [6, 7]. It can also severely damage several other palm species, particularly *Phoenix canariensis* Hortulanorum ex Chabaud [8], *Areca catechu* L. [9], *Washingtonia filifera* (Linden ex. Andre) H. Wendland, and *Syagrus romanzoffiana* (Chamisso) Glassman [10]. They produce 4 to 5 successive generations in a year [11], where the larvae and adults favorably attack the folded fronds of palms, feeding on the tender leaf tissues, which causes severe damage to leaves that results in stunted growth and fruit production and in some cases can cause tree death [4].

Symbiotic associations among the metazoans are prevalent in nature. Insects, being the most diverse, successful, and plenteous animals on Earth have established a robust relationship with microbial symbionts [12]. Insects acquire their symbiotic microflora through maternal transmission (vertical transmission) or environmental transmission (horizontal transmission) [13]. Microbial symbionts play many critical roles in their host's biology and evolution, ranging from food absorption, production of important nutrients [14] such as vitamins, amino acids, and cofactors [15], assistance to adopt new niches [16] or new host plants [17], manipulation of host reproduction (e.g., *Cardinium* and *Wolbachia*) [16, 18], and protection of the host against natural competitors (parasite, predator, or pathogens) [19]. The composition and infection density of these symbionts vary greatly across the members of the same species, sex, and developmental stages, depending on the physiological conditions of the host and genetics, geographic location, food, and age [20, 21]. It is of prime importance to know the infection density of host symbionts to decipher their biological effects and functions. Imperfect vertical transmissions may occur owing to low infection density, whereas high infection

frequencies may lead to pathogenesis and therefore negatively impact the fitness of their subject. The host must have evolved some sort of mechanism to control the titer of microbial symbionts and keep them in an appropriate range. One possible mechanism to regulate the infection density is the host and symbiont genotypes. Additionally, symbiont-symbiont interactions can also influence the infection density [22]. The symbionts may be in competition for shared resources, space, or niche and hence regulate their own exploitation to avoid the net damage of the symbiotic system and host fitness [23].

The cytoplasmic inherited bacterium *Wolbachia pipientis* (Rickettsiales, Rickettsiaceae) is a gram-negative Proteobacteria, naturally inducing dramatic reproductive abnormalities in various hosts through a number of phenotypic behaviors typified as cytoplasmic incompatibility (CI) in various insects, isopods, and mites [24], feminization (F) in isopods and moths [25], killing of male (MK) embryo in weevils, fruit flies, and butterflies [26], and induction of parthenogenesis (IP) in thrips and parasitoid wasps [27]. Generally, *Wolbachia*-infected hosts are benefited from these phenotypes as they guarantee complete vertical transmission of this symbiont to their offspring. From these above mechanisms, CI is the most exceptional phenotype of *Wolbachia*. Although the molecular methodology is unknown, CI can be elucidated by a "modification-rescue" scheme, in which *Wolbachia* "modifies" sperm inside the testes, and the sperm develops immaturely, resulting in demise of the embryo when rightful *Wolbachia* is not available for the eggs to "rescue" the embryo from the modification [28]. This aspect is critical in biological control; however, the use of *Wolbachia*-induced CI for pest management is still in its infancy.

The prevalence of *Wolbachia* infection in arthropod fauna is striking [29] and about 20–80% of arthropods harbor *Wolbachia* endosymbionts [30, 31]. Studies on the genus *Wolbachia* are mainly focused on phylum Arthropoda owing to the higher prevalence (over 90 arthropods including 5 orders of insects, isopods, spiders, and mites) [32, 33], differences of accessibility, successful transfection in naturally uninfected individuals, and its role in rapid speciation [34]. Phylogenetic studies have indicated that these infectious strains are divided into eight (A–H) discrete supergroups [35]. Nevertheless, more recent literature has documented some more supergroups designated as A–K super orders [36, 37]. Among these taxonomic supergroups, A and B are most likely to be documented in arthropods [38], whereas filarial nematodes, springtails, spiders, and termites are infected by distinct *Wolbachia* clades (C–D, E, F, G, and H) [39–42]. Estimating actual phylogenetic relationships

for each supergroup will provide valuable insight on the evolution and biology of this symbiont. Although the majority of arthropods are discrete in A and B supergroups, in this study we examined whether *Wolbachia* from *B. longissima* (*wLog*) does actually represent a divergent supergroup A or B on the basis of MLST and *Wolbachia wsp* genotype sequence. The MLST scheme (*gatB*, *ftsZ*, *hcpA*, *fbpA*, and *coxA*) is advocating the basis to establish accurate characterization of *Wolbachia* strains and generate phenomenal information about the genetic makeup, distributions, and host range of these ubiquitous bacteria [43]. The *wsp* gene is also a marker to resolve the phylogenetic relationship of variable *Wolbachia* strains [44].

In the present study, we quantified the *Wolbachia* density dynamics from different developmental stages, body parts, and tissues of *B. longissima* using quantitative real-time PCR (qPCR) assay to normalize with 5.8S ribosomal RNA as the housekeeping gene (HkG) for the first time against this beetle. Additionally, by phylogenetic analysis on the basis of MLST and *wsp* genotyping, we established the phylogenetic relatedness of this interrogation with known *Wolbachia*-associated arthropod lineages attributed to supergroups A and B to determine the actual position of this fastidious endosymbiont. Here, we attempted to obtain adequate information with the potential to be used for future pest management strategies. Indeed, the genus *Wolbachia* is opportunistic (parasitic or mutualistic) in nature but needs its status to be confirmed in *B. longissima*.

## Materials and Methods

### Test Specimens

The specimens used in this report were collected from infested coconut palm trees (*Cocos nucifera*) from Zhangzhou city (24.5130° N, 117.6471° E), Fujian Province of China and dislodged alive into the laboratory with a natural food source (fresh leaves of *C. nucifera*). The collected population was bred in the laboratory under optimum controlled conditions (temp. 25–28°C, RH 65–70%, light-dark cycle 12:12) [45] for successive generations as previously described [9]. Specimen identification between males and females of *B. longissima* (Gestro) was made on the basis of size (male usually smaller than female) and modifications of the last abdominal sternum of the adult, using a stereomicroscope under the supervision of Professor Hou Youming (Fujian Agriculture and Forestry University, China). The experiment was carried out on the F2 or F3 laboratory generations.

### DNA Extraction

Individuals from each mature larvae, 4–6 days old pupae, and 7–9 days old adults (female and male) were randomly selected

from F2 or F3 generations, whereas a bunch of newly hatched eggs ( $\approx 50$  eggs) were used for DNA extractions. Prior to DNA extractions, samples (3 individuals/sample) were washed three times with 75% alcohol and autoclaved double-distilled water. Adult beetles (1-week-old virgin male and female) were dissected for their body parts (head, thorax, and whole abdomen), gut, and reproductive tissues (ovary for female and testes for male) using sterile dissecting equipment, under the microscope, with phosphate buffer saline (PBS) (NaCl 8 g, KCl 0.2 g, Na<sub>2</sub>HPO<sub>4</sub> 1.42 g, KH<sub>2</sub>PO<sub>4</sub> 0.27 g, ultrapure water, pH 7.4). After dissection, samples were carefully transferred to new sterilized 1.5 ml tubes and homogenized in 180  $\mu$ l of ATL (animal tissue lysis) buffer using high-throughput TissueLyser II (Qiagen: Cat No./ID: 85300 at 60 Hz for 5 min) homogenizer. Whole genomic DNA from the samples (eggs, larvae, pupae, and adult females and males) was extracted using a DNeasy Blood and Tissue Kit (Qiagen, USA) following the manufacturer's recommendations with appropriate modifications. Briefly, 20  $\mu$ l of proteinase K was added to the suspensions and incubated for more than 1 h at 56°C. The final elution step was repeated two times in 50  $\mu$ l of AE buffer. The purity and concentration of the DNA were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and then run on agarose gel electrophoresis to assess its integrity.

### Screening of *Wolbachia* by Diagnostic PCR

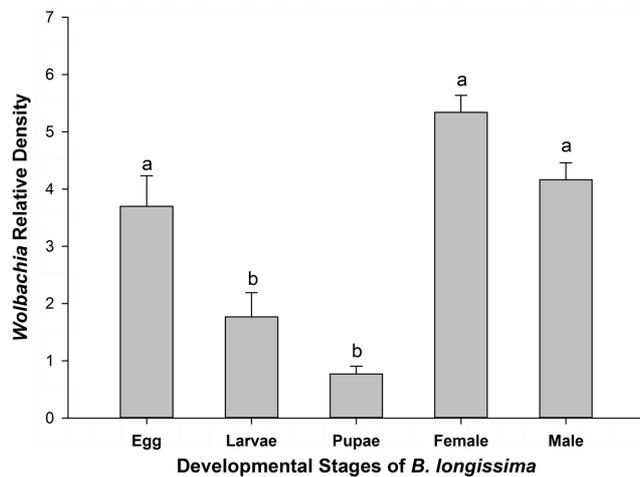
To interrogate the presence of *Wolbachia* infection across various developmental stages, body parts, and tissues of *B. longissima*, diagnostic PCR was conducted with *Wolbachia*-specific primers targeting specific regions of *Wolbachia wsp* and MLST genotype (Table 1). Around a 0.6-kb fragment length of *wsp* and variable length of MLST genes (*gatB*-369, *coxA*-402, *hcpA*-444, *ftsZ*-435, and *fbpA*-423) from all the samples were used (Table 1, Figs. S1A and S1B) [44]. Additionally, the 16S rRNA bacterial gene sequence with  $\approx 1.5$  kb size was also amplified using a universal bacterial primer pair (Table 1 and Fig. S1A) (Sangon Biotech (Shanghai) Co., Ltd., China). Amplification reactions were carried out in a total volume of 25  $\mu$ l that comprised 2  $\mu$ l of template DNA, 12.5  $\mu$ l of 2X Taq PCR, Master mix (Tiangen Biotechnology Beijing, China), 1  $\mu$ l of each primer (10  $\mu$ M), and 8.5  $\mu$ l of double-distilled water. The thermal cycling profile was 94°C for 4 min, followed by 30 cycles of 40 sec at 94°C, 40 sec at 55°C, 1 min at 72°C, and a final extension step for 10 min at 72°C for *wsp* (81F-691R), and 94°C for 3 min, 40 sec at 94°C, 40 sec at 55°C, 1 min at 72°C, and a final extension 5 min at 72°C for the 16S rRNA gene (27F-1492R). For the MLST genes, PCR protocols available at <http://pubmlst.org/Wolbachia/> [43] with modification of the temperature profiles (*gatB* and *fbpA* at 55°C, *coxA* and *hcpA* at 50°C and *ftsZ* at 48°C) were used. Furthermore, for the clarification of single or double *Wolbachia* infection, we used a previously described protocol ([https://pubmlst.org/wolbachia/info/amp\\_seq\\_double.shtml](https://pubmlst.org/wolbachia/info/amp_seq_double.shtml)) (Table S1). MLST analysis was carried out on three DNA samples for each developmental stage, body part, or tissue.

**Table 1.** Primer pairs used in this study

Purpose	Primer descriptions	Primer code	Primer Sequence 5'-3'	Fragment length (bp)	Ref.
Universal bacterial primer	16S ribosomal RNA gene	27F	AGAGTTTGATCCTGGCTCAG	≈ 1,400	[9]
		1492R	GGTACCTTGTTACGACTT		
Specific for <i>Wolbachia</i>	<i>Wolbachia</i> surface protein	wsp81F	TGGTCCAATAAGTGATGAAGAAAC	≈ 600	[44]
		wsp691R	AAAAATTAACGCTACTCCA		
qPCR genes	5.8S ribosomal RNA	5.8S R-F	AACGGTGGATCACTTGGTTC	≈ 151	This study
		5.8S R-R	ATACGACCTCAGCCAGGAG		
MLST genes	<i>Wolbachia</i> surface protein	wspDi_qF3	AGGGCTTTACTCAAAATTGG	≈ 149	[48]
		wspDi_qR3	CACCAACGTATGGAGTGATAGG		
	Glutamyl-tRNA amidotransferase, subunit B	gatB_F	GAKTTAAAYCGYGCAGGBGTT	≈ 369	[43]
		gatB_R	TGGYAAAYTCRGGYAAAGATGA		
	Cytochrome <i>c</i> oxidase, subunit I	coxA_F	TGGRRGRATYAACITTTATAG	≈ 402	
		coxA_R	CTAAAGACTTTKACRCCAGT		
	Conserved hypothetical protein	hcpA_F	GAAATARCAGTTGCTGCAA	≈ 444	
		hcpA_R	GAAAGTYRAGCAAGYTCTG		
	Cell division protein	ftsZ_F	ATYATGGARCATATAAARGATAG	≈ 435	
		ftsZ_R	TCRAGYAATGGATTRGATAT		
	Fructose-bisphosphate aldolase	fbpA_F	GCTGCTCCRCTTGGYWTGAT	≈ 429	
		fbpA_R	CCRCCAGARAAAAYYACTATTC		

### Cloning and Transformations of *wsp* and MLST Genes

PCRs were carried out to amplify the targeted regions of the *wsp* (Fig. S1) and MLST genes (Table 1). The PCR cycling conditions remained the same as described above. The PCR products, after



**Fig. 1.** Variations of *Wolbachia* density in *Brontispa longissima* across developmental stages.

The density of *Wolbachia* is calculated by the ratio between the number of *Wolbachia* genome relative to that of *B. longissima* (evaluated by qPCR). At least three biological replicates were run for each developmental stage to quantify the infection density. Tukey's HSD post hoc test was used for multiple comparison at the  $p < 0.05$  level; the different alphabets indicate the significance level of all tested developmental stages.

evaluation for positive amplification verified through gel electrophoresis, were excised and subjected to cloning and transformations. The DNA from the gel was purified with a MiniElute Gel Extraction Kit (Qiagen, USA) and 2  $\mu$ l of DNA was directly ligated into the pGEM T-Easy Cloning Vector (Promega, USA). After ligation, the products were transformed with T1 Competent Cells (Qiagen, USA) following the manufacturer's guidelines. Positive recombinants were sequenced by a commercial sequencing company (BioSune Biotech, Shanghai, China).

### Analysis of Sequences Attained from MLST and *wsp* Genes

To estimate the origin and closest strains of *Wolbachia* infections in *B. longissima*, the homology of all representative sequences (*wsp*) of this study of *Wolbachia* was checked using the NCBI BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and their closest sequences were computed (Table S2). Multiple sequence alignment was performed within subjected life stages of *B. longissima* using the Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) program (Fig. S4). All *wsp* sequence were analyzed in the *wsp* database (<http://pubmlst.org/wolbachia/wsp/>) to define the hypervariable regions (HVRs) that are used for *Wolbachia* strain characterization. Subsequently, for MLST evaluation, five ubiquitous genotype patterns (*gatB*, *coxA*, *ftsZ*, *fbpA*, and *hcpA*) were separately assessed through the data deposited in the *Wolbachia* MLST database (<http://pubmlst.org/wolbachia/>) for the aim to verify the allelic combination that elucidate the sequence types (STs) of *Wolbachia* mediated with *B. longissima*. All unwanted sequences from both ends were removed as compared with allelic template provided in the MLST

database and specified with targeted fragment length.

### Assemblage and Phylogenetic Analysis

Phylogenetic analyses were performed on *wsp* gene sequences ( $\approx 0.6$  kb) obtained from different life stages of *B. longissima* (5 sequences) along with 20 *Wolbachia*-infected arthropod species [44] from different orders (Table 1) retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/>). Meanwhile, 13 concatenated *Wolbachia* STs associated with different insect species selected from published data [46] and MLST database used for evaluation with ST-483 isolated from *B. longissima* were aligned using ClustalW in MEGA 5.05 [47]. After alignment, the sequences were manually corrected when and where necessary to produce an unambiguous multiple sequence alignment. Maximum likelihood (ML) tree topology was computed to develop the phylogenetic relationship. Analysis preference parameters were set and bootstrap analysis was performed with 1,000 pseudo replications. The CTR+G model with the lowest Bayesian Information Criterion scores is considered to describe the substitution pattern as the best fit model. Gap spaces were removed manually if necessary.

### Measuring the *Wolbachia* Density Dynamics through qPCR

qPCR was carried out to measure the relative density of *Wolbachia* across the tested life stages, body parts, and different tissues of *B. longissima*. Two forward and reverse primer pairs were designed using Primer3 ver. 0.4.0 software ([http://biotools.umassmed.edu/bioapps/primer3\\_www.cgi](http://biotools.umassmed.edu/bioapps/primer3_www.cgi)) and re-evaluated through Beacon Designer Free Edition - PREMIER Biosoft (<http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1>). The analysis was conducted on three DNA samples for each stage, body part, or tissue, targeting the *Wolbachia wsp* gene and normalized with 5.8S ribosomal RNA host gene retrieved from the European Nucleotide Archive database (ENA) (<http://www.ebi.ac.uk/ena/data/view/Noncoding:KM186304.1:1232.1416:rRNA&display=fasta>). Fragment lengths of 149 bp for *wsp* and 151 bp for host 5.8S ribosomal RNA genes were amplified in this study, evaluated from the short sequence (185 bp) [48] of *B. longissima* (KM186304.1) retrieved from ENA by following the above protocols. Prior to qPCR, conventional PCR was carried out for screening the specificity and accuracy of the primer pairs (*wsp*Di\_qR3, *wsp*Di\_F3 and 5.8S R-F, 5.8SR-R). Amplified PCR products were visualized (1% electrophoresis gel), excised from the gel (Fig. S2), cloned into the T1 cloning vector, and transformed with T1 Competent Cells (Qiagen, USA). The BLASTn search for the sequenced fragments showed 100% homology to *Wolbachia* isolated from *Diaphorina citri* and *B. longissima* short sequence (GenBank KF680772.1 and KM186304.1 respectively). After preliminary selection and verification, gene expression through qPCRs (20  $\mu$ l) were conducted in triplicates using the AB 7500 Fast Real-Time PCR System (Applied Biosystems, USA) containing 10  $\mu$ l of 2 $\times$  Power SYBR Green Real-Time PCR Master Mix (Applied Biosystems, USA), 1  $\mu$ l of final 5 ng template DNA, and 0.4  $\mu$ l (5  $\mu$ M) of each primer. Amplification conditions consisted of 94°C for 30 sec, 94°C for

5 sec, and 60°C for 34 sec. Primer efficacies were tested through a standard curve ( $\approx 90$  ng/ $\mu$ l) with 5-fold serial dilutions. The relative *Wolbachia* density, which is synonymous to the number of *Wolbachia* per host cell, was estimated on the basis of the ratio of *wsp* and 5.8S rRNA gene Cq values.

### Statistical Analysis

*Wolbachia* infection densities among all tested developmental stages, body parts, and different tissues of *B. longissima* were measure with one-way ANOVA, and differences among average means were compared with Tukey's HSD (honest significant difference) test. Estimation of difference within development stages, body parts, and tissues was evaluated by the independent sample *t* test. All results were statistically analyzed by SPSS software (ver. 21, SPSS Inc., USA).

### Nucleotide Accession Numbers

All sequences from this study have been deposited to the NCBI GeneBank database under the accession numbers of MG345105 to MG345109 (*wsp* Accession No.) and MG553908 to MG553932 (MLST Accession No.).

## Results

### Validation of *Wolbachia* Prevalence in the Life Stages, Body Parts, and Tissues of *B. longissima*

By mean of the diagnostic PCR approach with *wsp* gene (*wsp*-81F, *wsp*-691R) specific primers, we ascertained *Wolbachia* infection from all tested developmental stages (*i.e.*, eggs, larvae, pupae, and adult female and male), and designated them as *wLogE*, *wLogL*, *wLogP*, *wLogF*, and *wLogM*, respectively (Fig. S1A). *Wolbachia* infection was also found as positive from the tested body parts and tissues of *B. longissima* (Fig. S1B). Our initial analysis in this study showed that all subjected samples of *B. longissima* were fixed ( $\approx 0.6$  kb) and the fidelity of vertical transmission of this endosymbiont to offsprings was complete, with every specimen being infected (Figs. S1A and S1B). The sequencing results from subjected samples were identical (*wLog*), and the un-ambiguous peaks indicated the presence of single *Wolbachia* strains in any stage. Furthermore, the GenBank homology sequence of every life stage, searched against the NCBI database, showed >99% nucleotide BLAST identity with *wsp* encoded with the European honeybee *Apis mellifera capensis* (Apidae/Hymenoptera) and Asian citrus psyllid *Diaphorina citri* (Liviidae/Hemiptera) (Table S2). Multiple sequence alignment with Crustal Omega also showed 99% identity (Fig. S4). Consequently, these results revealed that a single clad (*wLog*) of *Wolbachia* endosymbiont is present across different life stages, with

**Table 2.** Infection clads, supergroups, and phenotypic behaviors of *Wolbachia* associated with various arthropod species based on the *wsp* gene sequence derived for phylogenetic analysis.

Arthropod hosts with <i>Wolbachia</i> -associated strains	Infection clads	<i>Wolbachia</i> supergroup	Phenotypic actions	<i>wsp</i> fragment length	NCBI GenBank Acc. No.
<i>Aedes albopictus</i> wAlbA	Mel	A	CI	655 bp	AF020058.1
<i>Drosophila melanogaster</i> wMel	Mel	A	CI	674 bp	AF020065.1
<i>Amblyjoppa fuscipennis</i> wFus	Fus	A	T	589 bp	AF071909.1
<i>Drosophila sechellia</i> wHa	Ha	A	T	576 bp	AF020073.1
<i>Glossina morsitans</i> wMors	Mors	A	NK	564 bp	AF020079.1
<i>Cylindrepomus peregrinus</i> wPer	Mors	A	T	564 bp	AF071914.1
<i>Ephestia kuehniella</i> wKue	Kue	A	NK	561 bp	AF071911.1
<i>Muscidifurax uniraptor</i> wUni	Uni	A	T	644 bp	AF020071.1
<i>Apis mellifera capensis</i> wCap-B1	Cap-B1	B	T	599 bp	AF510085.1
<i>Tribolium confusum</i> wCon	Con	B	CI	555 bp	AF020083
<i>Laodelphax striatellus</i> wStri	Con	B	CI	555 bp	AF020080.1
<i>Apoanagyrus diversicornis</i> wDiv	Div	B	T	543 bp	AF071916.1
<i>Trichogramma deion</i> wDei	Dei	B	T	555 bp	AF020084.1
<i>Thioalkalimicrobium sibericum</i> wSib	Sib	B	T	555 bp	AF071923.1
<i>Trichogramma kaykai</i> wKayB	Kay	B	T	555 bp	AF071924.1
<i>Culex pipiens</i> wPip	Pip	B	CI	603 bp	KT964225.1
<i>Diaphorina citri</i> wDi	Di	B	NK	600 bp	KF680772.1
<i>Armadillidium vulgare</i> wVul	Vul	B	F	596 bp	AF071917.1
<i>Tagosodes orizicolus</i> wOri	Ori	B	CI	552 bp	AF020085.1
<i>Encarsia formosa</i> wFor	For	B	T	546 bp	AF071918.1

NCBI, National Center for Biotechnology Information; *wsp*, *Wolbachia* outer surface protein; NK, not known; T, Thelytoky; MK, Male killing; CI, cytoplasmic incompatibility; F, Feminization.

widespread distribution in the body parts and tissues of *B. longissima*. Moreover, MLST genotypic evaluations yielded a new distinct strain, ST-483, which was characterized by explanation of two new alleles (*coxA*-234 and *ftsZ*-266), followed by already defined alleles (*i.e.*, *gatB*-158, *fbpA*-302, and *hcpA*-6) assessed on the basis of available data in the *Wolbachia* MLST database (Table 2). Meanwhile, HVRs analysis of *wsp* did not show any difference except HVR4, which showed identical HVRs to the database (Table 3), suggesting that this analysis is less sensitive than MLST. Thus, this study clarified *Wolbachia* infection mediated with *B. longissima* based on two new alleles and new ST. The same allelic profile and HVRs across the life stages indicates that this bacterium has no or little genetic variability from mother to offsprings.

#### *Wolbachia* Infection Dynamics through qPCR Assay

The qPCR primer pairs (*wsp*Di\_qF3 and *wsp*Di\_qR3 primers) specific for the *Wolbachia* *wsp* gene was recruited as previously described [48], whereas primer pairs of the

HkG (5.8S rRNA) with 149 bp product length (Fig. S3) was developed from the ribosomal gene sequence of *B. longissima* (ENA: sequence KM186304.1). The amplification efficiencies of the linearized plasmids were in line with those of *B. longissima* DNA samples, with efficiency rates of  $\approx 90\%$  generated for the *wsp* and 5.8S ribosomal RNA genes. The qPCR results indicated that *Wolbachia* infection densities varied significantly across different developmental stages ( $F_{4,10} = 26.24$ ,  $p < 0.001$ ) and was higher in eggs (average mean = 3.69) and adults (average mean of female and male, 5.34 and 4.16, respectively) as compared with larvae and pupae as shown in Fig. 1. Furthermore, the infection density within the different host body parts and tissues also varied significantly in both females ( $F_{4,10} = 10.175$ ,  $p = 0.001$ ) and males ( $F_{4,10} = 8.41$ ,  $p = 0.003$ ), where the infection density was the highest in the abdomen (average mean = 2.37), followed by reproductive tissues (average mean of female ovary and testes of male, 1.84 and 1.43, respectively), head, thorax, and gut, respectively (Fig. 2). However, in the parallel analysis to compare the infection

**Table 3.** Allelic compositions and accession numbers of *Wolbachia* MLST genotyping (ST-483) and identification of hypervariable regions (HVRs) of the *Wolbachia* surface protein (WSP) profile from various developmental stages of *B. longissima*.

Strain name <sup>a</sup>	Strain ID <sup>b</sup>	<i>wsp</i> (Accession No.)	WSP profile <sup>c</sup>				<i>wsp</i>	MLST profile (Accession no.)					ST <sup>d</sup>
			HVR1	HVR2	HVR3	HVR4		<i>gatB</i>	<i>coxA</i>	<i>hcpA</i>	<i>ftsZ</i>	<i>fbpA</i>	
<i>wLogE</i>	1842	MG345105	2	142	143	23	720	(158) MG553923	(266) MG553908	(6) MG553928	(234) MG553918	(302) MG553913	483
<i>wLogL</i>	1843	MG345106	2	142	143	23	720	(158) MG553924	(266) MG553909	(6) MG553929	(234) MG553919	(302) MG553914	483
<i>wLogP</i>	1844	MG345107	2	142	143	23	720	(158) MG553925	(266) MG553910	(6) MG553930	(234) MG553920	(302) MG553915	483
<i>wLogF</i>	1845	MG345108	2	142	143	23	720	(158) MG553926	(266) MG553911	(6) MG553931	(234) MG553921	(302) MG553916	483
<i>wLogM</i>	1846	MG345109	2	142	143	23	720	(158) MG553927	(266) MG553912	(6) MG553932	(234) MG553922	(302) MG553917	483

<sup>a</sup>*Wolbachia* strain name assigned according to developmental stages of *B. longissima* (*wLogE* for eggs, *wLogL* for larvae, *wLogP* for pupae, *wLogF* for female, and *wLogM* for male).

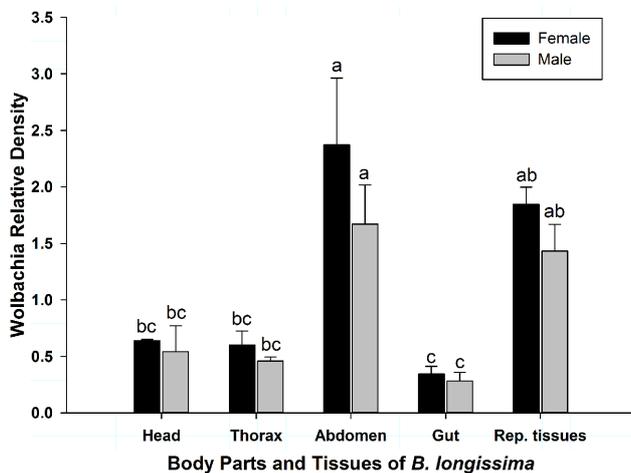
<sup>b</sup>*Wolbachia* strain identifier given by the MLST database.

<sup>c</sup>Peptide haplotypes of four consecutive sections of WSP, each section including a hypervariable regions.

<sup>d</sup>Sequencing type (ST) recognized as a unique allelic profile assigned through the MLST database.

densities between female and male body parts and tissues, the independent sample *t*-test results revealed no significant differences in the regions of head (separate difference  $t = 0.424$ ,  $df = 4$ ,  $p = 0.693$ ), thorax (separate difference

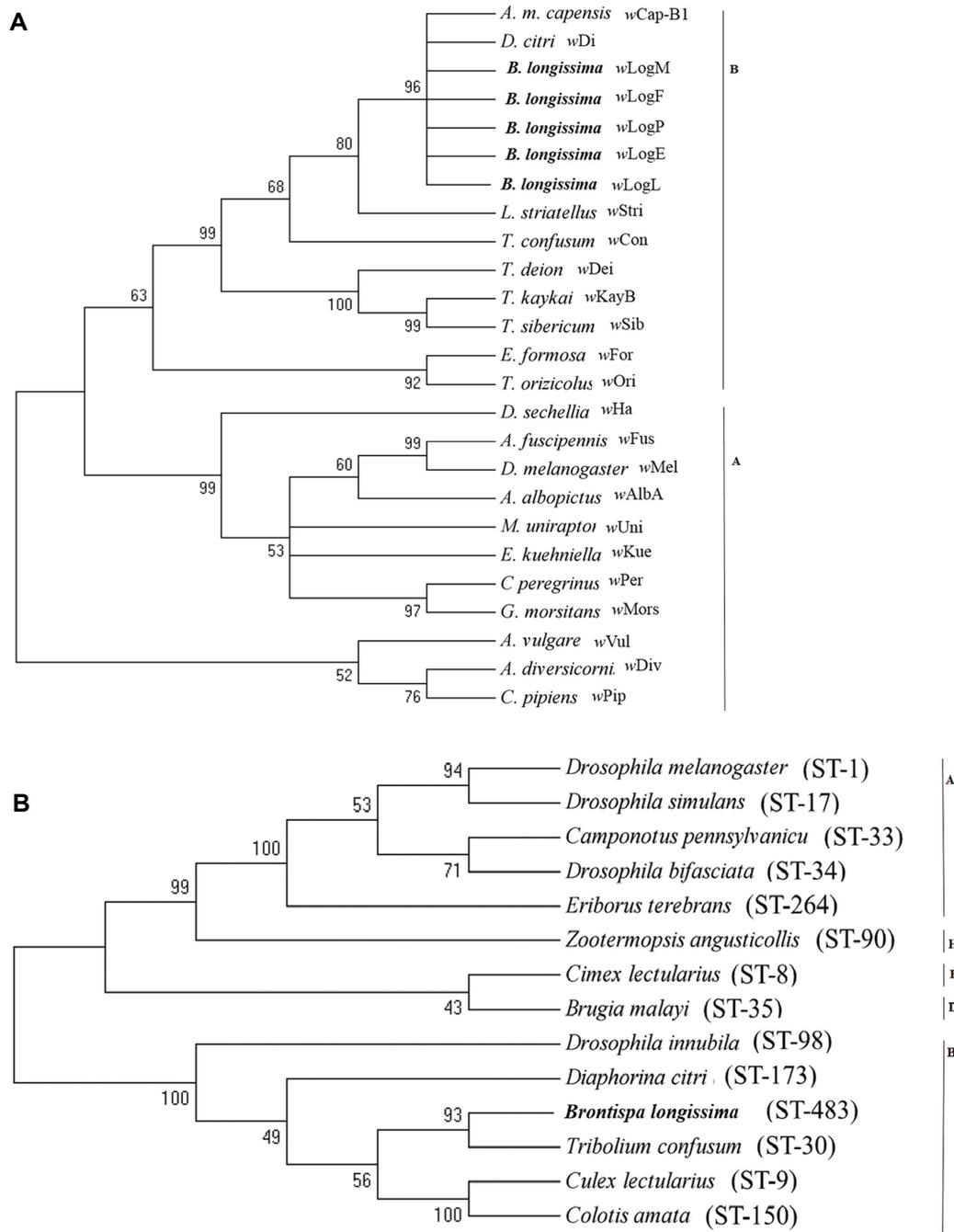
$t = 1.092$ ,  $df = 4$ ,  $p = 0.336$ ), abdomen (separate difference  $t = 1.022$ ,  $df = 4$ ,  $p = 0.365$ ), gut (separate difference  $t = 0.622$ ,  $df = 4$ ,  $p = 0.568$ ), and reproductive tissues (separate difference  $t = 1.483$ ,  $df = 4$ ,  $p = 0.212$ ). These analyses confirmed that the *Wolbachia* infection density in this beetle (*B. longissima*) was influenced by the host developmental stages and body parts or tissues, but not by the gender (male and female).

**Fig. 2.** Variations of *Wolbachia* density in different body parts and tissues of *B. longissima*.

The density of *Wolbachia* is calculated by the ratio between the number of *Wolbachia* genome relative to that of the *B. longissima* (evaluated by qPCR). At least three biological replicates were run for each developmental stage to quantify the infection density. Tukey's HSD post hoc test was used for multiple comparison at the  $p < 0.05$  level; the different alphabets indicate the significance level of all tested body parts.

### Phylogenetic Analysis and Group Interference

The supergroup A or B specific primers (Table S1) inferred the single infection of *Wolbachia* supergroup B in *B. longissima*. To evaluate the phylogenetic relationship between the *Wolbachia* strains isolated from *B. longissima* across various developmental stages, we constructed phylogenetic trees (Figs. 3A and 3B) of a total of 20 arthropod nucleotide sequences using the ML program (Table 1), and the tree topology of all subjected sequences ( $\approx 0.6$  kb) confirmed the partition of *Wolbachia* entailments into two supergroups (A and B). The *wsp* gene sequence of this study showed monophyletic similarity to that of *Wolbachia* in the sister group from *A. m. capensis* (Apidae: Hymenoptera) and *D. citri* (Kuwayama) (Psyllidae: Hemiptera) and attributed to lineages from *Wolbachia* (Con and Di subgroup) *wCap-B1* and *wDi* strains, which were conclusively classified into supergroup B (Fig. 3A). Similarly, the concatenated MLST sequence (2,073 bp) from *B. longissima* and those of *Wolbachia* infecting other organisms from different supergroups (A, B, D, F, and H) were aligned



**Fig. 3.** Phylogenetic placement of *Wolbachia* strains from *B. longissima* (*wLogE* for eggs, *wLogL* for larvae, *wLogP* for pupae, *wLogF* for female, and *wLogM* for male) (bold) with known *Wolbachia* symbionts from various arthropods, as constructed by the maximum likelihood (ML) program using MEGA (ver. 5.05).

(A) ML phylogenetic tree of the *wsp* gene ( $\approx$  600 bp) with (20 nucleotide sequence) 8/20 and 12/20 sequence of supergroup A and B, respectively, from various arthropods assembled and aligned together for phylogenetic analysis. Nomenclature of *Wolbachia* strains and groups are according to their names of host species from which they were identified. The letters A and B indicate the *Wolbachia* supergroups. (B) ML phylogenetic tree of concatenated MLST data (2,073 or 2,079 bp). Phylogeny showing relatedness of *B. longissima* (ST-483) (bold) with *Wolbachia*-mediated arthropods (a total of 13 sequence) 5/13, 5/13, 1/13, 1/13, and 1/13 nucleotide sequence belonging to *Wolbachia* supergroups A, B, H, F, and D, respectively. Alphabet letters (A, B, H, F, and D) indicate different *Wolbachia* supergroups. All *Wolbachia* MLST strains were retrieved from the MLST database (<http://pubmlst.org/wolbachia/>).

together, and ML analysis revealed higher monophyletic relatedness to confused flour beetle, *Tribolium confusum* (Jacquelin du Val) (Tenebrionidae: Coleoptera) belonging to the same supergroup B (Fig. 3B). Considering the homology in *Wolbachia* sequences across different developmental stages, only one MLST locus was used in strain-based ML chromatogram evaluation analysis. Therefore, on the basis of both phylogenetic analysis to *Wolbachia* infecting *B. longissima* individuals with other arthropods, the incidence of *Wolbachia* in *B. longissima* belongs to supergroup B.

## Discussions

*Wolbachia* infection is widespread among the natural populations of various insects. Detection of *Wolbachia* using diagnostic PCR-based approach targeting the *wsp* gene regions confirmed the *Wolbachia* infection from various developmental stages (Fig. S1A), body parts, and reproductive tissue (Fig. S1B) of *B. longissima*, which are the novel findings reported here. The *wsp* gene is commonly used as a strain typing marker [49] and taxonomical sequence tool for micro- and macro classification, as well as identification of new lineages of the genus *Wolbachia* [41, 50]. Previously, *Wolbachia* infection and classification using diagnostic PCR assays have been reported by Zhou *et al.* [44]. The ubiquitous presence of *Wolbachia* infection in our study is analogous with the interpretation of cytoplasmic-inherited bacteria, mainly transfers from mother to offsprings but may also have undergone broadly inter-taxon transmission even within or between different orders of insects. These cytoplasmic endosymbionts presumably conflict the expressions and elicit reproductive modification of various insect communities through a number of phenotypic behaviors, such as cytoplasmic incompatibility, parthenogenesis, feminization, and male killing. Nonetheless, phenotypic expressions (cytoplasmic incompatibility, parthenogenesis, feminization, male killing, manipulating effects, or resistance against pathogens) [51] of *Wolbachia* are the possibilities having no report from *B. longissima*, and therefore, further studies are needed to investigate these biological roles.

Here, we have provided the first comprehensive quantification analysis of *Wolbachia* infection densities across different life stages, body parts, and tissues of *B. longissima* by qPCR, normalized with a component of the large ribosomal subunit, 5.8S rRNA, as the endogenous control gene. Quantitative PCR is commonly employed in gene expression analysis. Nevertheless, assortment of suitable HkGs for normalization is crucial to achieve

accurate and reliable results. Published literature has indicated that the 5.8S rRNA gene represents a uniform expression, more stable (in contrast with 6 commonly used reference genes:  $\beta$ -2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH),  $\beta$ -glucuronidase (GUSB), peptidylprolyl isomerase A (PPIA), phosphoglycerate kinase 1 (PGK1), and TATA-box binding protein (TBP) in collapsed lung tissues of neonatal somatic cell nuclear transfer-derived cattle), conserved, and broadly used as a HkG [52]. A similar study was conducted to compare the 5.8S rRNA gene in the carmine spider mite, *Tetranychus cinnabarinus* (Boisduval) (Acarina: Tetranychidae), with other commonly used HkGs and validated its stability of expression under specific experimental conditions [53]. Another study rectified the stability evaluated from miRNAs and non-coding small RNAs [54] and thus showed uniformity among all cell types and experimental systems. Furthermore, the linearized plasmids of *wsp* and 5.8S rRNA and genomic DNA of *B. longissima* were amplified within the acceptable range of efficiency [55].

Numerous studies have put forward explanations for the observed variations in the infection density of *Wolbachia* across diverse taxonomic categories or ecological guilds [31]. In the present study, detection of *Wolbachia* using qPCR approaches suggested that the endosymbiont is present in all observed samples of *B. longissima*, although there is evident variations across developmental life stages and was the highest in the eggs and adults as compared with others. In line with this, a recent study to measure the *Wolbachia* infection dynamics has reported the highest *Wolbachia* density in the eggs and adult stage as compared with the larvae and pupae, which suggested that the percentage of *Wolbachia* density increases with the proceeding of developmental stage [56]. In the study of *D. citri* endosymbionts, a similar trend of positive correlation was observed between the growth pattern of symbionts and host development [57]. The fluctuations in the *Wolbachia* infection within the adult stage might have been due to the relative size variation of host tissues. This suggests that *Wolbachia* evolutionary consequences change with proceeding developmental stages and ultimately should exhibit the complex *Wolbachia*-host interactions. Additionally, the higher infection density in the abdomen and reproductive tissues of female as compared with male adults in *B. longissima* indicates that *Wolbachia* preferably colonized the abdomen and reproductive tissues of females, consistent with similar observations reported in fruit fly *Drosophila simulans* [58], two plant-hoppers species, *Laodelphax striatellus* and *Sogatella furcifera* [59], and *Aedes*

*albopictus* [60]. Moreover, the higher infection frequencies of *Wolbachia* in females also may be consequently higher than males due to a much greater size of the ovaries relative to the testes, while overall high frequencies in the abdomen and reproductive tissues compared with other body parts and tissues from both adults are a reason to improve successive transmission of the bacterium to next generations. *Wolbachia* is mostly present in somatic and germline tissue [60] and vertically transmitted into various hosts to modify their reproduction; hence, it has been supposed that they just infect the reproductive tissues of the host. Conflicting with this assumption, we showed that *Wolbachia* is abundantly distributed in the developmental stages (Fig. S1A) and whole body, including head, thorax, abdomen, and gut and reproductive tissues (Fig. S1B), which explains their wide distribution within invertebrate animals. In fact, the reason for the high abundance within germline cells and efficient transmission to the various insect species is just because of the usage of this bacterium as a spindle apparatus during cell partitioning [61] and effective travel to the kinesin and dynein motors within the host cells [62]. The findings of *Wolbachia* in *B. longissima* are also similar to previous studies on the prevalence of *Wolbachia* in various insects [63, 64].

In the recent few years, most of the research focus has increased regarding *Wolbachia* infection in weevils [65] and more than 40 weevil species are reported for *Wolbachia* infection, which is arguably a larger number than from any other insect group [37, 66]. This is contrary to the assumption that closely related species should trade off *Wolbachia* strains repeatedly and thus are expected to be associated with similar strains. A broad majority of similar *Wolbachia* strains have been identified in weevils with unknown ecological interactions. The maximum intimate associations of *B. longissima* via *wsp* analysis were found from *Wolbachia* strains in *A. m. carpensis* and *D. citri* (Kuwayama) and *T. confusum* (Jacquelin du Val) through concatenated MLST analysis, which clearly indicates the horizontal transmission of *Wolbachia* infections evaluated through ML analysis. All discovered *Wolbachia* strains in weevils belong to supergroup A or B, except *Rhinocyllus conicus* (Curculionidae: Coleoptera) that is infected by a strain from supergroup F, which is usually found in true bugs, termites, and nematodes [50, 67]. A study conducted to investigate the *Wolbachia* infection status from 40 weevil species from central Europe rectified that around 15% weevils infected with *Wolbachia* belong to supergroup A, 10% belong to supergroup B, and 15% belong to both A and B, while the remaining 60% were uninfected [68]. Similarly,

our phylogenetic analysis from both datasets of endosymbiotic *Wolbachia* in *B. longissima* clearly indicates the monophyletic supergroup B (Figs. 3A and 3B). Thus, irrespective of that case (F supergroup), most of the weevil species infections are classified into supergroups A and B. The apparent discrepancies in these analyses may lead to the variable impact of *Wolbachia* with host resulted from different evolutionary consequences, which reflect complex *Wolbachia*-host interactions but yet need to be determined in *B. longissima*.

Evidence is mounting on the various phenotypic behaviors of *Wolbachia* (CI, IP, F, and MK) inducing dramatic reproductive abnormalities that greatly influence the population demographics of host insect species [24, 27], which makes it a potentially useful tool for efficient pest management [69, 70]. Various effects from *Wolbachia* induced CI may contribute to pest control. Analogous to the sterile insect technique (SIT), *Wolbachia*-infected males in biological control programs when released in the field may suppress the pest population by infecting them with CI-inducing *Wolbachia*, which decreases host fecundity [69, 71]. Indeed, a recent literature rectified that *Wolbachia*-transfected mosquitoes, *Aedes aegypti*, were used successfully to block dengue transmission in Australia with no adverse effect on host physiology [72]. In addition, *Wolbachia* can be used as a potential tool for the introduction of favorable genes into insect population that can suppress the pathogen transmission by insect vectors, such as mosquito (genes that reduce the transmission of human pathogens) [72] and plant-hoppers [73]. In different host species, the *Wolbachia*-induced CI can vary from 0%–100% [74]. In broad spectrum, the palm pests (*B. longissima*) contaminate foods (coconut oil, date palm, etc.) and therefore, potential effects of *Wolbachia* on human fitness may require consideration. Until now, nothing was known about the effects of oral administration of *Wolbachia* endosymbionts on human health. However, some *Wolbachia* strains that are indispensable endosymbionts of the pathogenic nematodes causing lymphatic filariasis and river blindness induce severe inflammatory problems in humans when they are released into the blood [75, 76]. In this case, the present study on *Wolbachia* prevalence and quantification will help us unravel the nature of this symbiont-host interaction and propose the future prospects of *Wolbachia*-mediated insect pest management strategies. However, further studies are needed to explore the capacities of the *Wolbachia*-arthropod relationships and their implementation against vector and disease management. In conclusion, this study is the first report of the prevalence, infection status, and phylogeny of

*Wolbachia* endosymbionts in *B. longissima* and demonstrated that *Wolbachia* is ubiquitous across all developmental stages and distributed in the entire body of *B. longissima*, which provides a roadmap to find out its biological impacts and symbiont host associations.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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