Verification of Maternal Transmission of Wolbachia in Field-collected Aedes Albopictus and Aedes Aegypti Using a Combination of Wolbachia and Mitochondrial DNA Sequencing

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Abstract

Wolbachia is the most common bacterial endosymbiont of arthropods, such as the medically important *Aedes albopictus* and *Aedes aegypti.* Both *Wolbachia* and mitochondrial DNA are maternally inherited. This study collected 12 adult *Aedes albopictus* and 359 *Aedes aegypti* from 183 households in a dengue-prone area, Manila, Philippines, between June and September 2017. *Aedes* larvae (n = 509) were also collected from 17 water containers from 11 households. The DNA of the *Aedes* larvae and adults were screened for the presence of *Wolbachia* using the *wsp* and *16S* markers, following optimized polymerase chain reaction (PCR) conditions, and sequenced. We also performed PCR using the mitochondrial DNA *cox1* marker and sequenced the *Wolbachia*-positive samples. Our results showed that three out of 359 (0.84%) adult *Ae. aegypti* and 12 out of 12 (100%) adult *Ae. albopictus* were *Wolbachia* positive, while all of the larvae tested negative for *Wolbachia* (0/509; 0%). The *wsp* marker revealed six *Wolbachia*-positive *Ae. albopictus*, while the *16S* marker showed *Wolbachia* in three *Ae. aegypti* and 10 *Ae. albopictus.* Three *Wolbachia*-positive *Ae. albopictus* from one household were found to have a single haplotype for all *Wolbachia* (*wsp* and *16S*) and mitochondrial *cox1* markers. This result indicates maternal transmission of *Wolbachia* in the household because siblings of a *Wolbachia*-infected mother mosquito share the *Wolbachia* and mitochondrial genomes. Our results showed the feasibility of combined sequencing analyses of *Wolbachia* (*wsp* and *16S*) and mitochondrial DNA to test maternal transmission in field-collected mosquitoes.

Introduction

Wolbachia is a maternally inherited endosymbiotic bacteria, infecting 40% of arthropod species and some filarial nematodes [1;2]. It is naturally present in *Culex* and *Aedes* mosquito species, including *Aedes albopictus* [3–5] and *Aedes aegypti* [6–9]. Several studies have detected *Wolbachia* in medically important mosquitoes, such as *Anopheles sp.* and *Aedes sp.* It may have a role in inhibiting the replication of arbovirus pathogens [10]. Maternal transmission permits the rapid spread of *Wolbachia* in mosquito populations [11].

Since *Wolbachia* and mitochondrial genomes are maternally inherited, siblings of a *Wolbachia*-infected mother mosquito will share these genomes, showing close association leading to linkage disequilibrium among *Wolbachia*-infected mosquitoes. Maternal transmission suggests that analysis of both the *Wolbachia* genome and the mitochondrial genome should indicate whether two or more *Wolbachia*-infected mosquitoes living in the same or close localities carried bacteria maternally transmitted from the same mother. To our knowledge, there have been no previous studies that tested the maternal transmission of field-collected mosquitoes using *Wolbachia* markers, such as *wsp* and *16S*, combined with mitochondrial DNA markers. Usually, validation of maternal transmission is carried out by collecting adult mosquitoes from the field [12] or preparing transfected mosquitoes [13] and reared them into the next generation in the laboratory before testing the offspring for the presence of *Wolbachia*, using polymerase chain reaction (PCR). However, *Wolbachia* in mosquitoes may worsen in field conditions that are more stressful than those in the laboratory. For example, mosquitoes infected with the pathogenic *Wolbachia* strain wMelpop failed to successfully persist in release areas because of the huge fitness cost imposed by this strain [14]. Therefore, the results of studies of maternal transmission of *Wolbachia* obtained based on laboratory rearing do not necessarily apply to actual phenomena occurring in the field.

Ae. albopictus and *Ae. aegypti* are vectors of dengue virus. *Ae. albopictus* is known to be infected with two *Wolbachia* strains, known as wAlbA and wAlbB, that belong to the supergroups A and B, respectively [15].

Natural populations of *Ae. aegypti* were considered *Wolbachia* negative, but many recent studies [6–9;16–19] have identified *Wolbachia* in field-collected *Ae. aegypti.* Most of these recent studies observed that the *Wolbachia* infecting *Ae. aegypti* were phylogenetically close to the wAlbA strain, which also infects *Ae. albopictus* [6–8;17]. However, the previous studies detecting *Wolbachia* in *Ae. aegypti* did not test *Ae. albopictus*. To our knowledge, there have been no previous studies that found *Wolbachia* supergroups A and B from *Ae. aegypti* and *Ae. albopictus* samples collected from the same localities at the same time.

In this study, we examined the feasibility of a new approach to verify whether *Wolbachia*, found in multiple field-caught *Ae. albopictus* and *Ae. Aegypti*, is maternally transmitted from a common female ancestor, based on combined information from mitochondrial DNA (*cox1*) and *Wolbachia* marker (*wsp* and *16S*) sequences. We tested the hypothesis that *Wolbachia*-positive *Aedes* mosquitoes collected from same household may have the same haplotype in the mitochondrial DNA (*cox1*) and Wolbachia markers (*wsp* and *16S*), using adult *Aedes* samples collected from Manila City, Philippines. We also identified and compared the *Wolbachia* strains and supergroups found in *Ae. albopictus* and *Ae. aegypti*.

Methods

Study site, adult mosquitoes, larvae collection, and identification

We selected the study site in Manila City, the capital city of the Philippines. It is a highly populated and urbanized area that connects two major cities, Manila City and Quezon City. It consists of residential, commercial, and industrial infrastructure. We collected adults and larvae between June and September 2017, the rainy season and the peak time for dengue cases.

Adults were collected using commercially available mosquito light traps (Jocanima Corporation, Manila, Philippines). We set the mosquito trap for 48 hours either inside or outside the selected household premises (n = 428). The mosquito trap attracts the mosquito to enter a capture net by heat and CO_2 transmitted by a strong current from the ventilator [20;21]. We surveyed water containers in each household (n = 428) and found 17 containers with *Aedes* larvae. The adults (n = 371) and larvae (n = 509) collected were morphologically identified as *Aedes sp.* using the keys published by Rueda et al. [22]. The adult and larval samples were preserved in RNAlater (Ambion, Invitrogen, CA) solution to keep their RNA and DNA intact and stored at −20℃ before nucleic acid extraction.

Using microsatellite data from a recent study [21], we were also able to confirm the species of 359 adult mosquitoes as *Ae. aegypti.* We had already used the adult DNA of 359 *Ae. aegypti* for population genetics study [21] and reused the data in this study. The microsatellite primers were species-specific for *Ae. aegypti* [23;24]. There was PCR amplification observed using the primers on the 12 *Ae. albopictus* samples.

DNA extraction, molecular identification, PCR amplification, and sequencing

We extracted DNA using Qiagen AllPrep DNA/RNA micro kits and Qiagen DNA Blood and Tissue DNEasy Kits© (Qiagen, Hilden, Germany) in adult (n = 371) and larval (n = 509) samples, respectively. DNA concentration and quality were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Wolbachia was detected using two molecular markers: *wsp* (610 base pairs) using the primer pairs *wsp* 81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and *wsp* 691R (5'-AAAAATTAAACGCTACTCCA-3') [15], and *16S* specific for *Wolbachia* (850 base pairs) with the primer pairs *WolbF* (5'-GAAGATAATGACGGTACTCAC-3') and *Wspecr* (5'-AGCTTC GAGTGAAACCAATTC-3') [25].

For both *wsp* and *16S* gene amplification, we followed the protocol published in [7], in a final volume of 10 µl with 1 µl of the genomic DNA. We used the following components for the PCR reaction for both markers: 10x Ex Taq buffer, 25 mM $MgCl_2$, 2.5 mM dNTP, 10 µM forward and reverse primers, 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA), and 5 units/µl of Takara Ex Taq™ (Takara Bio Inc., Shiga, Japan). The *wsp* PCR amplification was as follows: initial denaturation at 95℃ for 3 minutes, denaturation at 95℃ for 1 minute, annealing at 55℃ for 1 minute, an extension at 72℃ for 1 minute for 40 cycles, and a final extension at 72℃ for 3 minutes. The *16S* amplification followed these conditions: initial denaturation at 95℃ for 2 minutes, denaturation at 95℃ for 2 minutes, annealing at 60℃ for 1 minute, an extension at 72℃ for 1 minute for two cycles, another 35 cycles of denaturation at 95℃ for 30 seconds, annealing at 60℃ for 1 minute, an extension at 72℃ for 45 seconds, and a final extension at 72℃ for 10 minutes. We included a positive control of a *Wolbachia*-positive *Culex sp.* and negative control of water in each PCR run.

PCR products were analyzed in 1.5% agarose gel stained with Midori Green Advance DNA stain at 100 V for 30 mins. To validate the presence of *Wolbachia* in each sample, we performed PCR amplification twice per marker. The criteria for a positive *Wolbachia* test were based on two successful amplifications per molecular marker, *wsp,* and *16S*.

We also amplified the *cox1* mitochondrial gene of *Wolbachia*-positive samples using the primer pairs LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO1-2198 (5'-AAACTTCAGGGTGACCAAAAAATCA-3') [26]. We used the following PCR amplification profile: initial denaturation at 95℃ for 5 minutes, denaturation at 95℃ for 30 seconds, annealing at 48℃ for 45 seconds for 35 cycles, and a final extension at 72℃ for 7 minutes. The amplified PCR products were purified using QIAquick (Qiagen, Hilden, Germany) PCR Purification kits and sequenced by Eurofin Genomics Inc. Tokyo, Japan.

Identification of *Wolbachia* strains, haplotypes, and phylogenetic analysis

We assembled the forward and reverse sequences for each marker using the CodonCode Aligner version 1.2.4 software (https://www.codoncode.com/aligner/). We aligned the sequences using the online program MAFFT version 7 with the default settings (https://mafft.cbrc.jp/alignment/software/). We checked all generated sequences d for similarities with reference sequences from GenBank [27] using Basic Local Alignment Search Tool–Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Finally, we checked the sequence quality of the aligned sequences in Mesquite version 3.5 [28] by confirming the absence of stop codons.

All *wsp, 16S*, and *cox1 s*equences were separately analyzed using DNAsp version 6.12.03 [29] to determine the number of haplotypes. We assessed the relationship of the *Wolbachia* strains of our study with representative sequences from different insect hosts by constructing a phylogenetic tree for the *wsp* and *16s* sequences using PhyML 3.1 [30] using the default settings. We applied the GTR + G model for *wsp* and the GTR + G + 1 model for

16S. The model per marker was selected using the SMART model selection method [31]. We used *Brugia pahangi* (AY527207) and *Rickettsia sp.* (U11021) as the outgroups for the *wsp* and *16S*, respectively.

Results

Adult mosquito collection and larval survey

We collected 12 adult *Ae. albopictus* and 359 adult *Ae. Aegypti* from 183 households across the study site. We also found 17 water containers outside the premises of 14 households. As a result, we were able to collect a total of 509 individual *Aedes* larvae. We classified the breeding containers into four general types following the World Health Organization guidelines [32]: water storage containers such as drums, pails, and buckets (*n* = 8), plant pots (*n* = 2), water fountain (n = 2), and others (a discarded toilet bowl and a street canal; *n* = 2). The number of larval samples collected per water container ranged from 2 to 287 (mean = 31) individual larvae.

Detection of Wolbachia infection in adult and larval mosquitoes

We screened all individual *Aedes sp.* larvae (*n* = 509) and adults (*n* = 371) for *Wolbachia* using the *wsp* and *16S* markers. Larval samples showed no evidence of *Wolbachia* from either marker. However, *Wolbachia* was detected in 15 (4.04%) out of 371 *Aedes sp.* adult mosquitoes based on the *wsp* and *16S* markers. Thus, a total of three (0.84%) were *Wolbachia* positive out of 359 *Ae. aegypti*, based on the *wsp* marker and 12 (100%) out of 12 *Ae. albopictus* (Fig. 1), based on the *wsp* and *16S* markers. The *wsp* marker detected *Wolbachia* in six *Ae. albopictus*, while the 16S marker detected it in three *Ae. aegypti* and 10 *Ae. albopictus*. Among these *Wolbachia*-infected *Ae. albopictus*, four (26.67%) out of 15 were positive for both *wsp* and *16S* markers. Confirmation of the species identification using the *cox1* marker revealed that the 12 adult mosquitoes were *Aedes albopictus*, while the remaining three were *Aedes aegypti*, based on BLAST results with 100% identity match (Table 1).

Table 1
Summary of sampling data and Wolbachia detection results

	Total
Surveyed households	428
Households with adult Aedes mosquitoes	183
Households with larval Aedes	11
Collected Aedes mosquitoes	371
Aedes albopictus	12
Aedes aegypti	359
Collected Aedes larvae	509
Wolbachia positive	
Larva	0
Adult Ae. albopictus	12
Adult Ae. aegypti	3
Households with Wolbachia positive	11

The phylogenetic tree built using the *wsp* marker (Fig. 2A) revealed two major clades: supergroup A and supergroup B [15]. Two *Ae. albopictus* were infected with *Wolbachia* belonging to supergroup A and four with *Wolbachia* belonging to supergroup B. The haplotype sequences from supergroup A were grouped with the *Wolbachia* type strain A (wAlb A), identified in an *Ae. albopictus* host in the USA [15]. In contrast, the sequences from supergroup B clustered with the *Wolbachia* type strains from *Ae. aegypti* (*wAegB*) in the Philippines [6;7] and *Ae. albopictus* (*wAlbB*) in the USA [15].

In the phylogenetic tree constructed using *16S* sequences, 11 out of 13 sequences (84.62%) found in *Ae. albopictus* (n = 10) and *Ae. aegypti* (n = 3) were clustered within supergroup A (Fig. 2B). The remaining two sequences (15.38%) found in *Ae. albopictus* were clustered together with the supergroups C, D, E, F, and J from insect hosts such as *Folsomia candida, Mansonella azzardi*, and *Dirofilaria immitis*.

Wolbachia and mitochondrial cox1 haplotypes

We identified six *cox1*, three *wsp*, and nine *16S* haplotypes among the 15 *Wolbachia*-positive sequences (Table 1). We found that three *Ae. albopictus* samples collected from one household (NH149) were *Wolbachia* positive, based on the *wsp* and *16S* markers (Table 1). An identical *cox1* haplotype, C1, was observed among all three *Ae. albopictus*. The same haplotypes of *wsp* (W2) and *16S* (S6) also occurred among these three *Ae. albopictus* (Table 2). Another household (NH56) had three *Wolbachia*-positive *Ae. albopictus* with the same *cox1* haplotype (C1). Two *Ae. albopictus* (Individual codes 177 and 178) were *Wolbachia* positive according to the *wsp* sequences, while two *Ae. albopictus* (Individual codes 95 and 177) were, according to the 16s sequence. We

observed different haplotypes in each *wsp* (W1 and W2) and *16S* (S8 and S9) marker. Other than these two households, no other households had more than one *Wolbachia*-positive mosquito.

Table 2. A detailed summary of the results of the Wolbachia infection in selected *Aedes* mosquitoes using *wsp* and *16S* marker

	Household	*Molecular species identification*	*Individual code*	*Haplotypes*			
				cox1	*wsp*		*16S* (n=13)*
					Supergroup	*wsp* (n=6)*	
1	SH9	*Ae. albopictus*	40	C1			S7
2	SH27	*Ae. albopictus*	306	C5			S3
3	SH134	*Ae. aegypti*	384	C6			S1
4	SH214	*Ae. albopictus*	425	C1			S6
5	NH43	*Ae. albopictus*	172	C4			S3
6	NH56	*Ae. albopictus* (n=3)	095, 177, 178	C1	A (177) and B (178)	W1 (177) and W2 (178)	S8 (095) and S9 (177)
7	NH107	*Ae. albopictus*	218	C5			S4
8	NH130	*Ae. aegypti*	116	C2			S1
9	NH131	*Ae. albopictus*	240	C5	A	W3	S5
10	NH149	*Ae. albopictus* (n=3)	258, 259, 260	C1	B (258,259,260)	W2 (258,259,260)	S6 (258,259)
11	NH181	*Ae. aegypti*	121	C3			S2
Total number of haplotypes				**6**		**3**	**9**

* total number of Wolbachia positive

Discussion

The presence of the same haplotype of *cox1, wsp*, and *16S* among the three *Wolbachia*-positive *Ae. albopictus* collected from household NH149 strongly suggests maternal transmission of the *Wolbachia*. To our knowledge, this is the first study to find evidence for the maternal transmission of *Wolbachia* in the field, using the combined analyses of *Wolbachia* (*wsp* and *16S*) and mitochondrial DNA (*cox1*) sequences. Thus, one of the advantages of the *Wolbachia wsp* and *16S* combined with the mitochondrial DNA sequences is that it allows us to see maternal transmission occurring in the field, different from laboratory-controlled conditions.

We assumed that our approach eliminates the bias of laboratory-controlled conditions, including temperature, light, and feeding times, during mosquito rearing. The maternal transmission of *Wolbachia* in mosquitoes is usually confirmed by detecting *Wolbachia* in the offspring of infected mothers [6;8]. For example, *Wolbachia*-microinfected *Ae. aegypti* embryos were reared into adults and then tested using PCR for the presence of *Wolbachia* [13]. Severe environmental conditions such as high temperature causing heat stress to mosquitoes

may decrease *Wolbachia* density, thus reducing the ability of *Wolbachia* to persist in the mosquito population. The results of a previous study [33] suggest that high temperature affects *Wolbachia*. For example, when breeding containers are exposed to more sunlight than shade, the density of *wMel* declined. Previous sterile insect studies failed to successfully deploy *Wolbachia* because of the poor performance of the mosquitoes released in the field, possibly because of the extreme environmental conditions differing from those of the laboratory [34;35]. Thus, it is important to verify *Wolbachia* transmission in the field. In a previous study [36], the establishment of *Wolbachia* in Australia during and after mass release was determined by collecting mosquitoes in the field and then detecting *Wolbachia* using loop-mediated isothermal amplification. The researchers successfully confirmed maternal transmission because the field-collected mosquitoes had wMel strains, which were considered absent from the natural populations before mass release. However, the verification of maternal transmission of naturally occurring *Wolbachia* strains in mosquito populations is not as simple as the case after mass release. It is difficult to determine whether the strains detected in field-collected mosquitoes resulted from transmission from the mother or the naturally occurring *Wolbachia* populations in the area. Our approach, combining the analysis of *Wolbachia* sequences with mitochondrial DNA sequences, appears to be an effective verification method.

One of the limitations of this study is that we observed an unexpected pattern of *Wolbachia* positivity in one household, NH56, that did not show the same haplotype in Wolbachia *wsp* and *16S* sequences. However, the same mitochondrial DNA haplotype appeared. One of the possible reasons is that the resolution of the *cox1* marker might not be fine enough to tell whether the mosquitoes are from the same mother. Our study detected six *cox1* haplotypes from the 15 *Wolbachia*-positive individuals, and some mosquitoes from the same or different households shared the same *cox1* haplotype. The mitochondrial genome reflects the long-term maternal lineage [26]. Therefore, although the mothers of these three individuals were different, they are considered to have a common ancestor in their maternal lineage, although how many generations ago cannot be estimated.

Our results were negative for *Wolbachia* in all the collected larvae (n = 509). One possible reason is that we found only 17 water containers during our field sampling. Since *Wolbachia* is maternally transmitted, if the mother is negative for *Wolbachia*, all its offspring will also be negative for *Wolbachia*. More *Wolbachia* might be detectable in larval samples if we increase the number of water containers than the number of individuals per container. Although 428 households were surveyed in this study, only 17 water storage containers were found. Thus, we suggest increasing the number of sampled water containers in future studies by setting up ovitraps in households. We also recommend detecting *Wolbachia* in representative larval samples from one water container.

Another possible reason for the negative results for *Wolbachia* in larvae can be linked to a lower *Wolbachia* titer during the larval stage of mosquitoes. Previous studies [17;37] have found that the *Wolbachia* titer is much lower in the larval stage than in the adult. Stevanovic and colleagues [37] found a lower *Wolbachia* load in *Drosophila melanogaster* larvae than in adult females. We presume that this is due to the immature organs of larva, such as the thoracic ganglia and muscles, ovaries, head, and salivary glands. In future studies, we propose that the confirmation of *Wolbachia* in larval samples be done after rearing it into adults.

We also found that the *Ae. albopictus* were infected with both supergroups A and B of *Wolbachia* using the *Wolbachia* surface protein gene (*wsp*), widely used for *Wolbachia* strain identification and phylogeny studies

[15]. A previous study [7] found that field-collected *Ae. aegypti* positive for *Wolbachia* from Manila, Philippines were clustered into two supergroups: A and B. We found more *Wolbachia*-positive *Ae. albopictus* in the *Wolbachia* supergroup B (4/6; 66.66%) than in supergroup A (2/6; 33.33%), as observed in a previous study [38] (China; 631/693; 91.05%). Thus, our research results support previous studies, and we observed two *Wolbachia* supergroups in *Ae. albopictus* collected from the same area at the same time.

In our research, we discovered a higher prevalence of *Wolbachia* in *Ae. albopictus* than in *Ae. aegypti.* A previous study into *Wolbachia* in mosquitoes also found a higher *Wolbachia* prevalence in *Ae. albopictus* than in other mosquito species [39]. Previous studies found a low prevalence rate of *Wolbachia* in *Anopheles sp.* and in *Ae. aegypti* compared with other mosquito species because of the lack of a symbiotic relationship between *Wolbachia* and its hosts [40–43]. Therefore, our results support the findings of these previous studies.

Conclusions

An interesting approach to testing the maternal transmission of *Wolbachia* in field-collected mosquitoes is through the analysis of *Wolbachia wsp* and *16S* sequences combined with mitochondrial sequences. Since *Wolbachia* and mitochondria are both maternally inherited, a non-random association of the mitochondrial genome with the *Wolbachia* endosymbiont is expected. Our strategy of combining mitochondrial and *Wolbachia* sequence information effectively [44] tests for the maternal transmission of *Wolbachia* in field-collected *Ae. albopictus* and *Ae. aegypti.* In this study, we used mosquitoes naturally infected with *Wolbachia* collected from the same households. We confirmed that they were from the same mother through the presence of the same mitochondrial haplotype sequences. Therefore, our proposed *Wolbachia wsp* and *16S* sequences combined with mitochondrial DNA sequences are advantageous because we used field-collected mosquitoes rather than laboratory-reared mosquitoes. Using field-collected mosquitoes allows us to test maternal transmission in the environment, a phenomenon that can be overestimated under laboratory conditions. Our findings can provide a better understanding of the maternal transmission of *Wolbachia* in naturally-infected mosquitoes in the field and its ability to invade and persist in the population.

Abbreviations

PCR – polymerase chain reaction

DNA – deoxyribonucleic acid

Ae. albopictus - *Aedes albopictus*

Ae. aegypti - *Aedes aegypti*

LAMP – loop-mediated isothermal amplification

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated and/or analyzed during this study are included in this published article and its additional files. All newly generated sequences are available in the GenBank database under the Accession Numbers _____.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

MAFR and KW conceptualized and designed the experiment. MAFR and TI collected and identified the adult and larval samples for this study. MAFR and TI performed the Wolbachia detection and all the molecualr experiements for this study. MAFR, TI and KW performed the data analysis. MAFR and KW wrote the manuscript. All authors read and approved the final mansucript.

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Figures

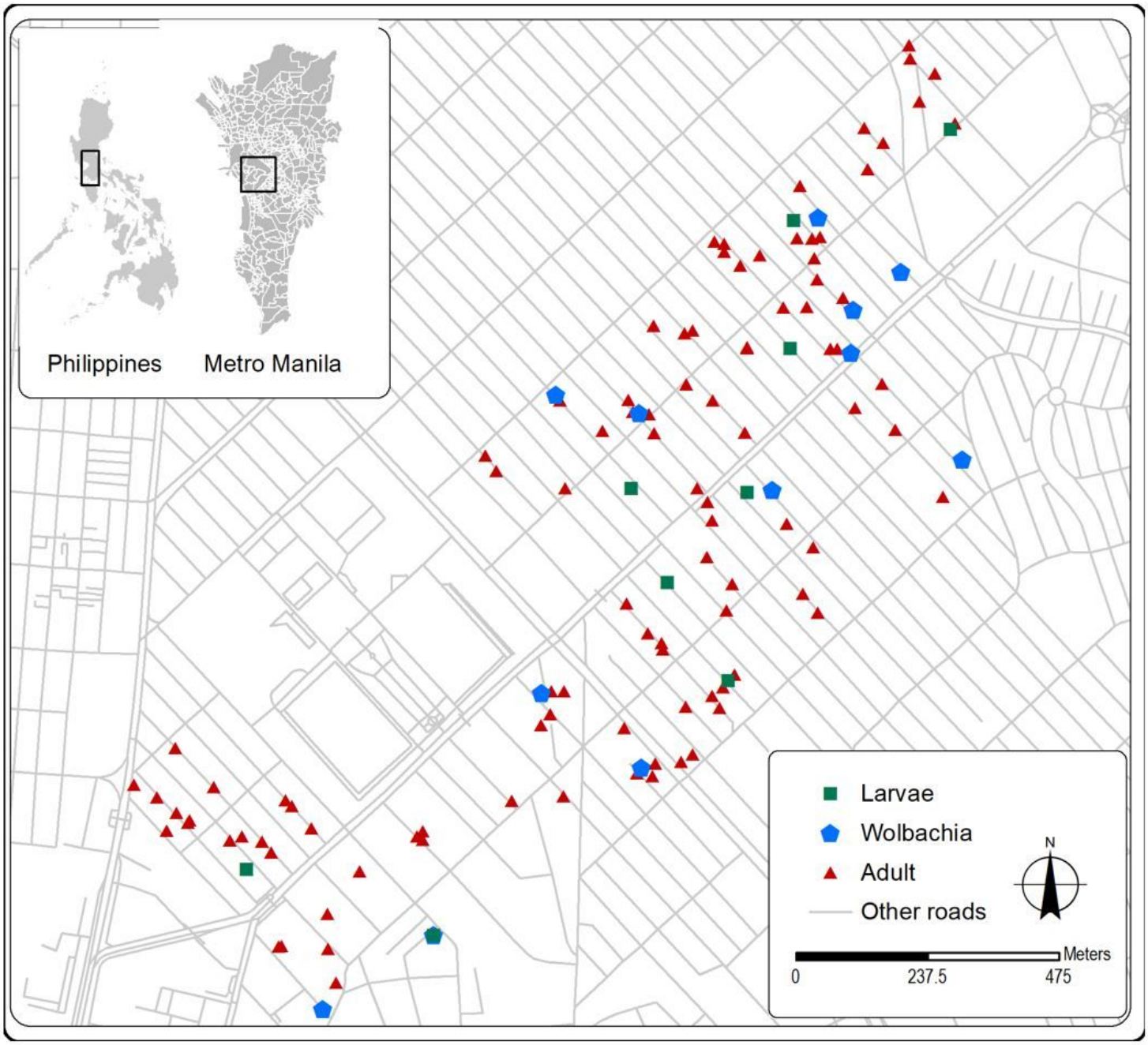

Figure 1

Spatial distribution of Wolbachia positive households (n = 11) out of 428 households surveyed across a 2-kilometer road in highly urbanized area in Manila, Philippines.

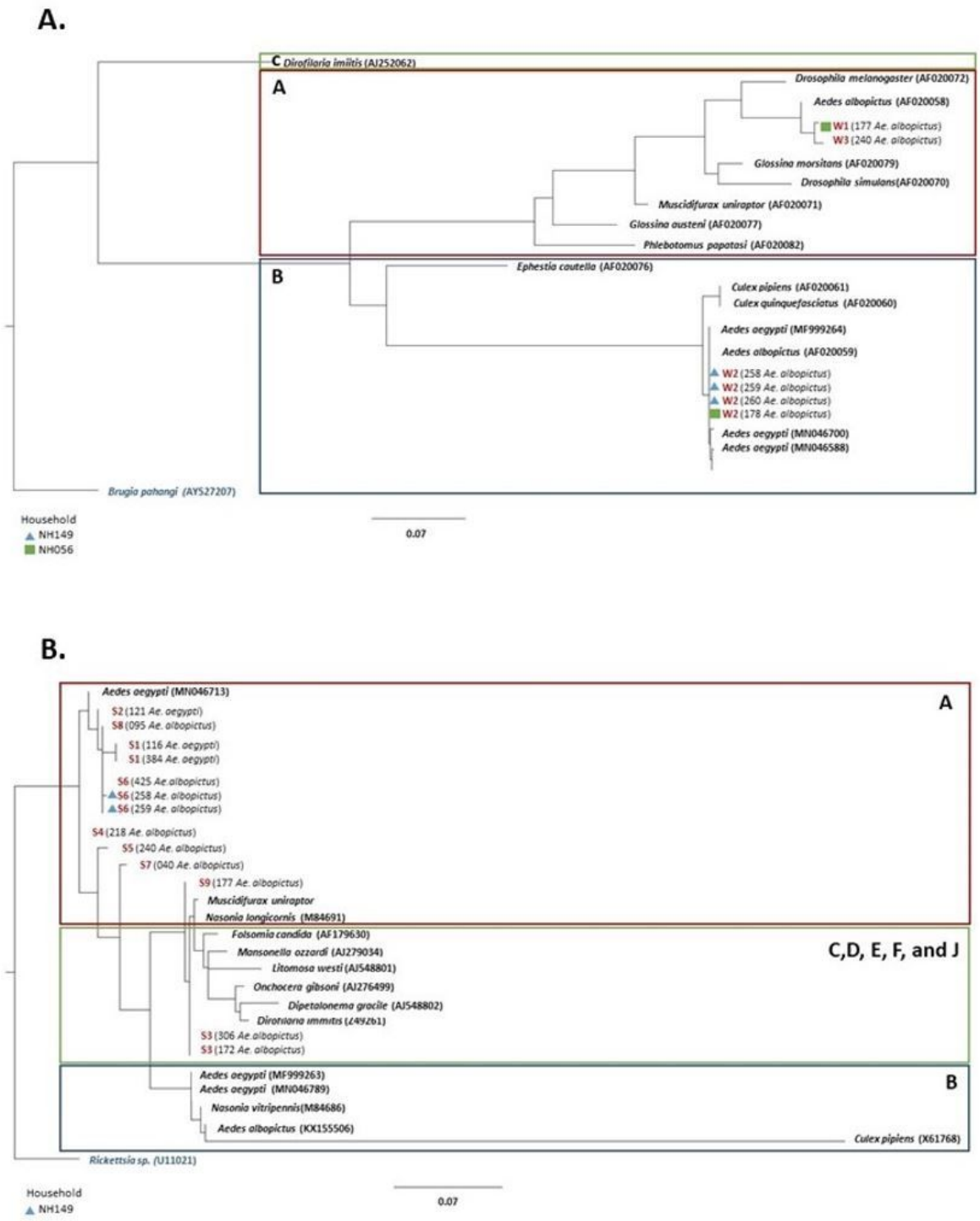

Figure 2

The maximum likelihood phylogenetic tree of Wolbachia infected urban mosquitoes based on the (A) WSP sequences with reference sequences from supergroups A, B, and C and an outgroup of Brugia pahangi (B) 16S sequences with reference sequences from supergroups A, B, C, D, E, F and J and an outgroup of Rickettsia sp.

Supplementary Files

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