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Detection and phylogeny of *Wolbachia* in field-collected *Aedes albopictus* and *Aedes aegypti* from Manila City, Philippines

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Abstract: *Wolbachia* is the most common bacterial endosymbiont of arthropods, such as the medically important *Aedes albopictus.* Recent reports also detected in *Aedes aegypti.* This study collected 12 adults *Ae. albopictus* and 359 *Ae. 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. Our results showed that 12 out of 12 (100%) adult *Ae. albopictus* and3 out of 359 (0.84%) adult *Ae. aegypti* were *Wolbachia* positive, whereas all larvae tested negative for *Wolbachia* (0/509; 0%). The *wsp* marker revealed six *Wolbachia*-positive *Ae. albopictus* belonging to supergroups A (*n* = 2) and B (*n* = 4). The 16S marker showed *Wolbachia* in ten *Ae. albopictus* and 3 *Ae. albipictus*,most sequences were in supergroup A (*n* = 11) and two in supergroup B. Our results revealed *Wolbachia* supergroups in field-collected *Ae. albopictus* and *Ae. aegypti* with implications for a successful *Wolbachia* mass release programs.

Keywords: *Wolbachia*, dengue, *Aedesaegypti, Aedes albopictus*

1. Introduction

Wolbachia is a maternally inherited endosymbiotic bacteria, infecting 40% of arthropod species (Werren et al. 2008; Zug et al. 2012). *Wolbachia* can alter the reproductive mechanisms of its hosts, such as cytoplasmic incompatibility, feminization, parthenogenesis, and male-killing (Fraser et al. 2017). Maternal transmission permits the rapid spread of *Wolbachia* in mosquito populations (Fraser et al. 2017). At present, the identified supergroups or major clades are 17 (A-Q) that infects arthropods such as insects (Kaur et al. 2021). *Wolbachia* is naturally present in medically important mosquito species, including *Aedes albopictus* (Albuquerque et al. 2011; Joanne et al. 2015; Kitrayapong et al. 2002) and *Aedes aegypti*(Balaji et al. 2019; Carvajal et al. 2019; Kulkarni et al. 2019 and Teo et al. 2017) that may inhibit the replication of arboviral pathogens (Iturbe-Ormaetxe et al. 2011). Natural populations of *Ae. aegypti* were considered negative for *Wolbachia*, but recent studies have reported both positive (Balaji et al. 2019; Carvajal et al. 2019; Kulkarni et al. 2019; Teo et al. 2017; Coon et al. 2016; Thongsripong et al. 2017; Hegde et al. 2018; Bennett et al. 2019) and negative (Gloria-Soria et al. 2018 and Ross et al. 2019) results for *Wolbachia* in field-collected *Ae. aegypti* among different countries. Due to these varying results, further studies of natural infection of *Wolbachia* in field-collected *Ae. aegypti* is needed.

The presence of natural *Wolbachia strains* in field-collected *Ae. aegypti* may affect the current release programs. For example, mating of a transfected *Wolbachia* infected *Ae. aegypti* with naturally occurring *Wolbachia* infected *Ae. aegypti* may cause reduced or no viable offspring due to the different Wolbachia strains of the parents thereby, halting the successful invasion of *Wolbachia* strains from transfected mosquitoes into the natural population. Most studies have found that naturally occurring *Wolbachia* in *Ae. aegypti* were strains that were phylogenetically close to the *w*AlbB strain, which also infects *Ae. albopictus*(Balaeji et al. 2019;Carvajal et al. 2019;Kulkarni et al. 2019; and Coon et al. 2016). In the study of (Coon et al. 2016) *w*AlbA and *w*AlbB strains were found in *Ae. aegypti.* Thus, additional *Wolbachia* detection studies on natural populations of *Ae. aegypti* are needed to support the presence of *w*AlbA strongly. *Ae. albopictus* is known to be infected with both *w*AlbA and *w*AlbB strains, which belong to the supergroups A and B, respectively (Zhou et al. 1998).The phylogenetic relationship of the *Wolbachia* strains of Ae. *albopictus* and *Ae. aegypti* may suggest the possibility of post-lateral transfer between these two species.

A high *Wolbachia* detection rate has always been reported in *Ae. albopictus*(Joanne et al. 2015 and Kitrayapong et al. 2002)because of the reproductive manipulations of *Ae. albopictus* that have enabled *Wolbachia* to spread within the mosquito populations (Nugapola et al. 2017).*Wolbachia* has been detected in *Ae. aegypti* natural populations (Balaji et al. 2019;Carvajal et al. 2019; Kulkarni et al 2019, Teo et al. 2017; Coon et al. 2016 Thongsripong et al. 2017; Hegde et al. 2018 and Bennett et al 2019), but some studies showed negative for *Wolbachia* in *Ae. aegypti* (Gloria-Soria et al. 2018 and Ross et al. 2019). The absence of *Wolbachia* in *Ae. aegypti* and the low detection rate might be because *Wolbachia* is not well adapted to *Ae. aegypti* that makes it difficult to have stable *Wolbachia* infection and proliferation in the *Ae. aegypti* populations. In the study of Teo et al. (2017) they compared the *Wolbachia* detection rate in larval *Ae. albopictus* and *Ae. aegypti* from Malaysia. Their results revealed 71/284 (25%) *Wolbachia* infected *Ae. albopictus* and 4/16 (25%) in *Ae. aegypti.* However, the sample size for *Ae. aegypti* was small to have a solid validation about the comparison of the detection rate of these two species. Other adult studies also found a high *Wolbachia* detection rate in *Ae. albopictus* (Wiwatanaratanabutr et al. 2013; Nugapola et al. 2017 and Kittayapong et al. 2000) but did not detect *Wolbachia* in *Ae. aegypti*.

Wolbachia load in adult mosquitoes is commonly found in the thoracic muscles, malphigian tubules, thoracic ganglia, head, ovaries, and salivary glands (Moreira et al. 2009). However, these organs are still not yet fully developed during the immature stage of mosquitoes which may cause a lower *Wolbachia* detection rate of larval *Aedes sp.* as compared to adult mosquitoes. Previous studies also revealed that mosquitoes' larval stage has lower *Wolbachia* density than the adult stage (Coon et al. 2016 and Tolley et al. 2019) because of the influence of the larval crowding and temperature changes. The reduced *Wolbachia* density during the immature stage can cause failure in the maternal transmission in *Wolbachia* (Zhou et al. 1998). Thus, *Wolbachia* detection rate might be lower in larval stage than the adult mosquitoes.

Here, we studied the *Wolbachia* supergroups found in field-collected *Ae. albopictus* and *Ae. aegypti* simultaneously collected in the same site using phylogenetic analysis of *wsp* and 16S sequences. We tested three hypotheses. The first hypothesis was that both supergroups A and B can be observed in both *Ae. albopictus* and *Ae. aegypti.* The second hypothesis was that *Ae. albopictus* has a higher *Wolbachia* detection rate than *Ae. aegypti.* The third hypothesis was that

larval *Aedes sp.* mosquitoes have a lower *Wolbachia* detection rate than adult*Aedes sp.* mosquitoes.

2. Materials and Methods

2.1. 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.

Adult mosquitoes were collected using commercially available mosquito light traps (Jocanima Corporation, Manila, Philippines) placed inside or outside each randomly selected household. The target sample size was calculated based on the two-stage cluster systematic sampling design. An estimate of p (0.23) was used with the maximum tolerable error of 10%. An additional 15% allowance was added as a buffer resulting in 472 households as summarized in the recent study of (Regilme et al. 2021). We set the mosquito trap for 48 hours inside or outside the surveyed households. The mosquito trap attracts the host-seeking and blood-fed mosquitoes to enter a capture net via the heat and CO_2 transmitted by a strong current from the ventilator (Regilme et al. 2021 and Balingit et al. 2020). We also surveyed water containers in each household (n = 428). The collected adult and immature mosquitoes were morphologically identified as *Aedes sp.* using a stereomicroscope using the keys published by (Rueda et al. 2004). The larval stages (L1-L4) of the collected larvae were not identified. The adult and larval samples were preserved in RNAlater (Ambion, Invitrogen, CA) solution to keep their RNA and DNA intact and stored at -20^0C before nucleic acid extraction.

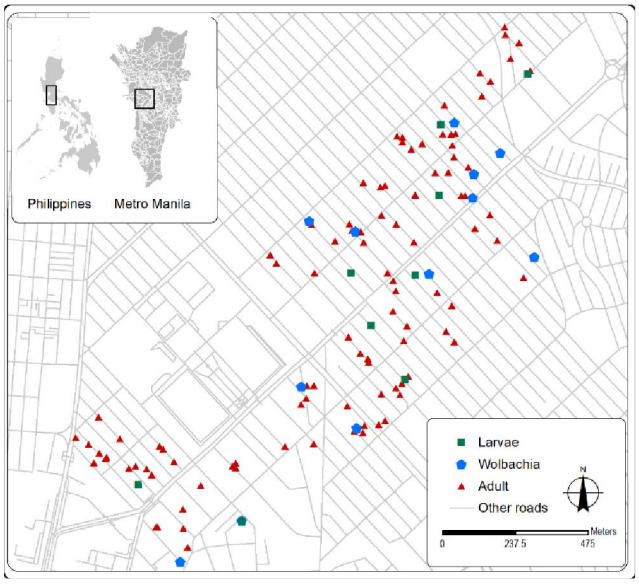

Figure 1. Spatial distribution of *Wolbachia*-positive households (n = 11) out of 428 households surveyed in a highly urbanized area in Manila, Philippines

To confirm the species of the collected adult and immature mosquitoes, we used microsatellite data from a recent study of Regilme et al. (2021) that used species-specific primers for *Ae.*

aegypti(Chambers et al. 2007 and Slotman et al. 2007). One representative immature mosquito from water containers was tested for species identification using microsatellite primers. The DNA of 359 adult *Ae. aegypti* were also used for the population genetics analysis (Regilme et al. 2021) and the remaining DNA of the 359 adult *Ae. aegypti* and 12 adult *Ae. albopictus* were used for this study of *Wolbachia* detection. Using *cox1* sequences compared with reference sequences from GenBank using BLASTn, we confirmed the species identification of the *Wolbachia* positive adult mosquitoes as *Ae. albopictus* or *Ae. aegypti.*

2.2. DNA extraction, molecular identification, PCR amplification, and sequencing

We extracted DNA using Qiagen AllPrep DNA/RNA micro kit and Qiagen DNA Blood and Tissue DNeasy Kits© (Qiagen, Hilden, Germany) from adult (n = 371) and larval (n = 509) samples, respectively. In this study, the extracted DNA was only used while the extracted RNA was stored in -80^{0}C for future studies. 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′) (Zhou et al. 1998) and 16S specific for *Wolbachia* (850 base pairs) with the primer pairs *WolbF* (5′-GAAGATAATGACGGTACTCAC-3′) and *Wspecr* (5′-AGCTTC GAGTGAAACCAATTC-3′) (Folmer et al. 1994).

For both *wsp* and 16Sgene amplification, we followed the protocol published in (Carvajal et al. 2019), 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 five 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 16Samplification 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 minutes. 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′) (Folmer et al. 1991). 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.

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 the sequence quality of the aligned sequences in Mesquite version 3.5 (Maddison et al. 2019) by confirming the absence of stop codons. Finally, we checked all generated sequences for similarities with reference sequences from GenBank (NCBI, 2016) using Basic Local Alignment Search Tool–Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

2.3. Identification of Wolbachia strains, haplotypes, and phylogenetic analysis

All *wsp,* 16S, and *cox1 s*equences were separately analyzed using DnaSP version 6.12.03 (Rozas et al. 2017) 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 (Lefort et al. 2017) 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 (Guindon et al. 2003). We used *Brugia pahangi* (AY527207) and *Rickettsia sp.* (U11021)as the outgroups for the *wsp* and 16S, respectively.

We acknowledge the uncertainties of *Wolbachia*-positive *Ae. aegypti* due to the 16S (Ma et al. 2017) and the conventional PCR method, e.g., the false-positive rate. We were careful to ascertain positive Wolbachia in adult *Ae. aegypti* in light of this information. Thus, we used the primers of Simoes et al. (2011) which are known to produce fewer false-positive results and negative detections of *Wolbachia*. PCR amplication of individual samples were performed twice per marker. We also performed repeated PCR tests of our *Wolbachia*-positive samples to ensure successful *Wolbachia* detection as defined in the previous study by (Carvajal et al.2019). To address the issue of the sensitivity of the conventional PCR method, such as the false-positive detection, we combined the sequencing analysis of the PCR amplicon.

3. Results

3.1. Detection of Wolbachia infection in adult and larval mosquitoes

A total of ten (10/12; 83.33%) adult *Ae. albopictus* were *Wolbachia* positive based on the 16S marker while six adult *Ae. albopictus* (6/12; 50.00%) were positive based on *wsp* marker. Four (4/12; 33.33%) adult *Ae. albopictus* were *Wolbachia* positive both in *wsp* and 16S markers. A total of three (3/359; 0.83%) were positive out of the 359 adult *Ae. aegypti* based on the 16S marker. However, we did not detect *Wolbachia* using *wsp* marker in the 359 adults *Ae. aegypti*. Larval *Aedes* samples (n = 509) showed no evidence of *Wolbachia* from either marker. The species identification of 12 *Ae. albopictus* and 3 *Ae. aegypti* adults with *Wolbachia* infection detected by *wsp* or 16S markers or both markers was confirmed by BLAST analysis using *cox1* marker. It was confirmed that there was no error in species identification with 100% identity match.

The phylogenetic tree built using the *wsp* marker(Figure 2 A) revealed two major clades: supergroup A and supergroup B (Zhou et al. 1998). Two *Ae. albopictus* were infected with *Wolbachia* belonging to supergroup A and four with *Wolbachia* belonging to supergroupB. The haplotype sequences from supergroup A were grouped with the *Wolbachia* type strain A (*w*Alb A), identified in an *Ae. albopictus* host in the USA (Zhou et al. 1998). In contrast, the sequences from supergroup B clustered with the *Wolbachia*-type strains from *Ae. aegypti* (*w*AegB) in the Philippines (Carvajal et al. 2019) and *Ae. albopictus* (*w*AlbB) in the USA (Zhou et al. 1998).

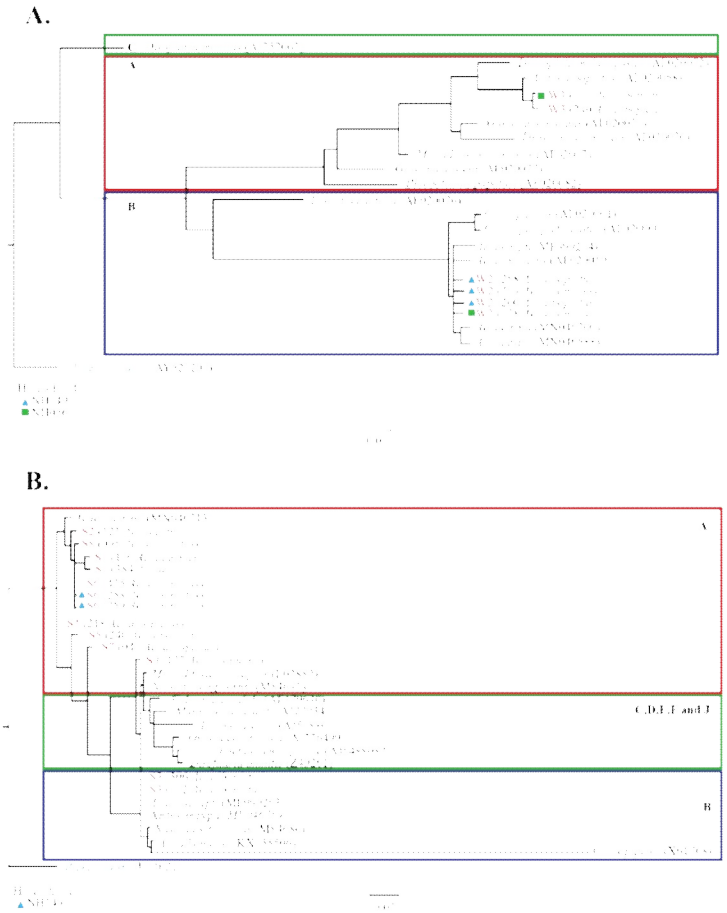

Figure 2. The maximum likelihood phylogenetic tree of *Wolbachia* sequences found from mosquitoes based on the **(A)** *wsp* sequences with reference sequences from supergroups A, B, and C and an outgroup of *Brugia pahangi***(B)** 16S sequences with reference sequences from supergroups A, B, C, D, E, F, and J and an outgroup of *Rickettsia* sp. The colored boxes represent the *Wolbachia* supergroups of the sequences, red for supergroup A, green for supergroups C, D,E, F and J and blue represents supergroup B. The codes in parenthesis are the GenBank Accession number. The phylogenetic trees are redrawn for better resolution in Adobe InDesign.

In the phylogenetic tree constructed using 16Ssequences, 8 out of 10 (80%) *Ae. albopictus* and three *Ae. aegypti* (100%)in this study were clustered within supergroup A (Figure 2 B). The remaining two *Ae. albopictus* sequences (20%) in this study were clustered together with the supergroup B with reference sequences of *Ae. albopictus* and *Ae. aegypti.*

3.2. Wolbachia and mitochondrial cox1 haplotypes

We identified six *cox1,* three *wsp,* and nine 16S haplotypes among the 15 adult samples positive with *Wolbachia* (Table 1). We found that three *Ae. albopictus* samplescollected from one household (NH149) were all *Wolbachia* positive, based on the *wsp* and 16S markers (Table 1). An identical *cox1* haplotype (sequence), C1, was observed among all these three *Ae. albopictus*. The identical 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 out of these three *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 positive,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 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 mosquitoes	11

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

	HH	**MSI**	**IC**	***cox*1 (H)**	***wsp(*S)**	***wsp(*H)**	**16S(H)**
1	SH9	*Ae.*	40	C1			S7
2	SH27	*Ae.*	306	C5			S3
3	SH134	*Ae. aegypti*	384	C6			S1
4	SH214	*Ae.*	425	C1			S6
5	NH43	*Ae.*	172	C4			S3
6	NH56	*Ae.albopictus* (n=3)	095, 177, 178	C1	A (177); B (178)	W1 (177); W2 (178)	S8 (095); S9 (177)
7	NH107	*Ae.*	218	C5			S4
8	NH130	*Ae. aegypti*	116	C2			S1
9	NH131	*Ae.*	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**

Abbreviations: HH households; MSI molecular species identification; IC individual code; H haplotype; S supergroup;

4. Discussion

We found that the adult *Ae. albopictus* were infected with both supergroups A and B of *Wolbachia* using the *wsp*, widely used for *Wolbachia* strain identification and phylogeny studies (Zhou et al. 1998). A previous study (Carvajal et al. 2019) found that field-collected *Ae. aegypti* positive for *Wolbachia* from Manila, Philippines were clustered into A and B supergroups. However, *Ae. albopictus* were not collected in the study of (Carvajal et al. 2019).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 (Hu et al. 2020) (China; 631/693; 91.05%). In this study, we observed two *Wolbachia* supergroups (A and B) in *wsp* sequences of *Ae. albopictus* and supergroups A and B in the 16S phylogenetic tree of *Ae. albopictus* and *Ae. aegypti.* Our results were consistent with the previous studies of (Zhou et al. 1998) in *Ae. albopictus* that found both *Wolbachia* supergroups A and B while the clustering of *Wolbachia* infected *Ae. aegypti* in supergroup A supports the study of (Coon et al. 2016). The presence of two *Wolbachia* supergroups is common in *Ae. albopictus* populations (Zhou et al. 1998 and Kittayapong et al. 2002) but the observance of two supergroups in two co-occurring adult mosquito species of *Ae. albopictus* and *Ae. aegypti* from the same site is not yet studied to the best of our knowledge. Our result is significant because it can give information about the phylogenetic relationship of *Wolbachia* and the supergroups present in the *Ae.albopictus* and *Ae. aegypti* collected from Manila, Philippines. However, the limited number of *Wolbachia* infected *Ae. aegypti* in this study may not strongly show the relationship of *Wolbachia* found in both *Aedes* species from the same site. Despite that, our results can be a starting information to know the phylogenetic relationship between *Wolbachia* supergroups A and B of both *Aedes* species.

The molecular phylogeny based on the 16S marker revealed that most of the *Wolbachia* positive *Ae. albopictus* (n=8) and three *Wolbachia Ae. aegypti* were clustered in *Wolbachia* supergroup A. Most studies found that *Wolbachia* in *Ae. aegypti* were usually closely related to *w*AlbB infection of supergroup B that is also common to *Ae. albopictus*(Balaji et al. 2019; Carvajal et al 2019 and Kulkarni e al. 2019). However, in the study of (Coon et al. 2016) both supergroups A and B were present in the *Wolbachia* infected *Ae. aegypti*. The presence of supergroup A in *Ae. aegypti* in our study supports the previous study of (Coon et al. 2016). Our results show that supergroup A detected in *Ae. aegypti* are related to *Wolbachia* we identified in the *Ae. albopictus* samples, which might suggest the possibility of post-lateral transfer because of the sharing of habitat ranges between *Ae. albopictus* and *Ae. aegypti* (Coon et al. 2016). At present, there are no studies about the transfer of *Wolbachia* between hosts in the field, thus in future studies we recommend investigaiting the possible post-lateral transfer of *Wolbachia* from *Ae. albopictus* to *Ae. aegypti* in field populations.

We found a low detection rate of *Wolbachia* in adult *Ae. aegypti*, which isconcordant with the previous results of Kulkarni et al. (2019) obtained from field-collected *Ae. aegypti* in Florida, USA, and Carvajal et al. (2019) conducted in Metro Manila, Philippines. In contrast, a high (>50%) detection rate was observed in the studies of Teo et al. (2017) conductedin Malaysia and Kulkarni et al. (2019) conductedin New Mexico, USA. The low *Wolbachia* detection rate in *Ae.*

aegypti in our study and Carvajal et al. (2019).might be due to the low density of *Wolbachia* that cannot be detected by conventional PCR (Iturbe-Ormaetxe et al. 2011). Previous metabarcoding studies on *Ae. aegypti* found a low number of *Wolbachia* sequence reads in the *Ae. aegypti* midgut (Kulkarni et al. 2019; Thongsripong et al. 2017 and Hegde et al. 2018), implying low *Wolbachia* density. Although our results are limited since we did not quantitatively measure *Wolbachia* density, our 40-cycle PCR amplification method adapted from Carvajal et al. (2019) could infer that *Wolbachia*-positive *Ae. aegypti* might be present in Metro Manila, Philippines. Further *Wolbachia* detection studies are thus needed to affirm natural *Wolbachia* infection in field *Ae. aegypti.*

Our research discovered a higher detection rate of *Wolbachia* in *Ae. albopictus* (12/12; 100%) than in *Ae. aegypti* (3/359; 0.84%). A previous study of *Wolbachia* detection in field mosquitoes also found a higher Wolbachia detection rate in *Ae. albopictus* than in other mosquito species (Wiwataratanabutr et al. 2013). Previous studies found a low detection rate of *Wolbachia* in *Anopheles sp*. and *Ae. aegypti* compared with *Ae. albopictus* because of the lack of a symbiotic relationship between *Wolbachia* and its hosts (Baldini et al. 2018; Jeffries et al. 2018 and Sawasdichai et al. 2019). Therefore, *Wolbachia* and *Ae. aegypti* might have a weak stable symbiotic relationship that makes it difficult for *Wolbachia* to spread in the *Ae. aegypti* populations. In contrast, *Ae. albopictus* displayed a higher Wolbachia detection rate because of the host's ability to utilize better reproductive manipulations, enabling the *Wolbachia* to spread further in the mosquito populations.

Our study found eight out of 10 (80.00%) *Wolbachia* infected *Ae. albopictus* in the 16S marker, but no bands were amplified using the *wsp* marker. On the other hand, three *Ae. aegypti* were positive using the 16S marker but negative for *wsp* marker. In the study of (Wolfgang et al. 2009), repeated failures in *Wolbachia* detection using the standard PCR amplifying the *wsp* gene were encountered due to the mismatch in the primer and DNA template which may also explain our negative results in the *wsp* marker.

Our results were negative for *Wolbachia* in all the collected *Aedes* larvae(n = 509). One possible reason is that we found only 17 water containers during our field sampling. Since *Wolbachia* is maternally transmitted, all of its offspring would also be negative for *Wolbachia* if the mother is negative. *Wolbachia* might be detectable in larval samples if we increase the number of water containers by setting up ovitraps. Another possible reason for obtaining negative results for *Wolbachia* in larvae can be linked to a lower *Wolbachia* density during the larval stage of mosquitoes. Previous studies (Coon et al. 2016 and Stevanovic et al. 2015) have found that the *Wolbachia* density is much lower in the larval stage than in the adult stage. Wiwatanaratanbutr et al. (2013) discovered that temperature and larval crowding may affect the relative *Wolbachia* densities in *Ae. aelbopictus*. Stevanovic et al. (2015) found a lower *Wolbachia* density 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 the present study, we could not quantify the *Wolbachia* density in *Wolbachia*-positive individuals because of low DNA quantity.

The same haplotype of *Wolbachia* (*wsp* and 16S)and mitochondrial DNA (*cox1*) among the three *Wolbachia*-positive *Ae. albopictus* collected from household NH149 may infer the maternal transmission of the *Wolbachia* occurring in the field, which may be different from laboratory-controlled conditions. The maternal transmission of *Wolbachia* in mosquitoes is usually confirmed by detecting *Wolbachia* in the offspring of infected mothers(Balaji et al. 2019 and

Kulkarni et al. 2019). For example, *Wolbachia*-microinjected *Ae. aegypti* embryos were reared into adults and then tested using PCR for the presence of *Wolbachia*(McMeniman et al. 2009). We assumed that using haplotype inference of *cox1* sequences of field-collected *Wolbachia* infected mosquitoes may eliminate the bias of laboratory-controlled conditions, including temperature, light, and feeding times, during mosquito rearing.

However, in another household (NH56), an unexpected pattern was also observed: although three Wolbachia-infected mosquitoes had an identical *cox1* haplotype, the haplotypes of Wolbachia *wsp* and 16S sequences were not identical among the three individuals. Based on the number of *cox1* haplotypes [i.e., 4 (C1–C4)] found in the 12 *Wolbachia*-positive *Ae. albopictus*, we calculated a Poisson probability of 0.149 for obtaining the same *cox1* haplotype among 3 randomly selected individuals. The mitochondrial genome reflects the long-term maternal lineage (Folmer et al. 1994). Therefore, although the mothers of these three individuals were different, they can have a common ancestor in their maternal lineage, although how many generations ago cannot be estimated. One of the limitations of this study is the few number of *Wolbachia*-positive mosquitoes. In future studies, we suggest increasing the sample size to obtain more *Wolbachia*-positive mosquitoes from one household for a stochastically strong validation of the maternal transmission through whole genome sequencing or double-digest restriction site-associated DNA.

5. Conclusions

Overall, our study displayed *Wolbachia* supergroups A and B in the *wsp* sequences of *Ae. albopictus* and in the 16S sequences of *Ae. albopictus* and *Ae. aegypti.* The result suggests that *Wolbachia* supergroups A and B are currently infecting the natural populations of *Ae. albopcitus* and *Ae. aegypti* in Manila, Philippines. Understanding the *Wolbachia* supergroups of these two species collected simultaneously in the same site can give insights into their phylogenetic relationship in the Culicidae phylogenetic tree and can give information about the possibility of post-lateral transfer of *Wolbachia*. A higher *Wolbachia* detection rate was observed in the adult *Ae. albopictus* (12/12;10%) than the *Ae. aegypti* (3/349; 0.84%). This result may suggest the higher reproductive manipulations ability of *Ae. albopictus* that may have contributed to the higher *Wolbachia* detection rate. We found no *Wolbachia*-infected larval samples in our study, thus supporting our hypothesis of a lower *Wolbachia* detection rate in larva than in adults. This might be due to the lower *Wolbachia* density during the immature stages of *Aedes sp.* than the adult stage. Our findings emphasize the need to further detect *Wolbachia* infection in field-collected *Ae. albopictus* and *Ae. aegypti* and to characterize the existing *Wolbachia* strains present. This information is vital in the successful *Wolbachia* deployment programs to ensure the compatibility of the *Wolbachia* strain that may already be present in the natural mosquito populations.

Reproducibility: 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 OM131675-OM131689, OM169215 – OM169223 and OM304377 – OM304379.

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