bioRxiv preprint doi: <https://doi.org/10.1101/2021.08.24.457456>; this version posted October 21, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Conclusion: Our results suggest that the utilization of two *Wolbachia* specific markers, *wsp* and *16S* demonstrated *Wolbachia* presence in individual *Ae. albopictus* and *Ae. aegypti*. The results of the *Wolbachia* infection in *Ae. albopictus* showed that the detected strains were from either supergroups A and B. Despite the low infection rate of *Wolbachia* in *Ae. aegypti*, our results demonstrated the presence of *Wolbachia* in field-collected *Ae. aegypti* supporting previous studies.

Keywords: *Wolbachia*, dengue, *Aedes aegypti*, *Aedes albopictus*, Manila City

Background

Wolbachia is a maternally inherited endosymbiotic bacteria, infecting 40% of arthropod species and some filarial nematodes [1-2]. *Wolbachia* is naturally present in medically important mosquito species, including *Aedes albopictus* [3-5] and *Aedes aegypti* [6-9] inhibiting the replication of arboviral pathogens [10]. Natural populations of *Ae. aegypti* were considered negative for *Wolbachia*, but recent studies have reported both positive [6-9;11-14] and negative [15-16] results for *Wolbachia* in field-collected *Ae. aegypti* among different countries. Due to these varying results, further validation of natural infection of *Wolbachia* in field-collected *Ae. aegypti* is needed.

The presence of natural *Wolbachia* strains in field-collected *Ae. aegypti* is important as it may affect future *Wolbachia* mass release programs, thereby influencing the successful invasion of *Wolbachia* strains from transfected mosquitoes into the natural population. Most studies have found that naturally occurring *Wolbachia* in *Ae. aegypti* are strains that are phylogenetically close to the wAlbB strain, which also infects *Ae. albopictus* [6-8;11]. *Ae. albopictus* is known to be infected with two *Wolbachia* strains, known as wAlbA and wAlbB, which belong to the supergroups A and B, respectively [17]. However, previous studies detecting *Wolbachia* in field-

collected *Ae. aegypti* did not test *Ae. albopictus*, comparing natural Wolbachia strains present in *Ae. aegypti* with *Ae. albopictus* from the same localities at the same time, regarded as naturally infected is important to further characterize and validate natural Wolbachia infection in *Ae. aegypti*.

In this study, we detected *Wolbachia* in field-caught *Ae. albopictus* and *Ae. aegypti* using *Wolbachia*-specific DNA markers (*wsp* and 16S). We also identified and compared the *Wolbachia* strains and supergroups found in *Ae. albopictus* and *Ae. aegypti*. Our study provides further support in the presence of *Wolbachia* in field-collected mosquitoes.

Main Text

Adults of 12 *Ae. albopictus* and 359 *Ae. Aegypti* were collected using commercially available mosquito light traps from 183 of 428 households randomly selected in Manila City, Philippines from June to September 2017. We found 17 water containers with *Aedes* larvae ($n = 509$) in 11 households. The adults ($n = 371$) and larvae ($n = 509$) were morphologically identified as *Aedes* sp. using a stereomicroscope using the keys of [18]. We extracted DNA using Qiagen AllPrep DNA/RNA micro kit and Qiagen DNA Blood and Tissue DNEasy Kits© (Qiagen, Hilden, Germany) in adult ($n = 371$) and larval ($n = 509$) samples, respectively.

Wolbachia was detected using: *wsp* (610 base pairs) with primer pairs *wsp* 81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and *wsp* 691R (5'-AAAAATTAAACGCTACTCCA-3') [16] and 16S specific for *Wolbachia* (850 base pairs) with primer pairs *WolbF* (5'-GAAGATAATGACGGTACTCAC-3') and *Wspecr* (5'-AGCTTC GAGTGAAACCAATTC-3') [19].

For both *wsp* and 16S gene amplification, we followed the PCR 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 MgCl₂, 2.5 mM dNTP, 10 μM forward and reverse primers, 10% dimethyl sulfoxide, and 5 units/μl of Takara Ex Taq™. 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 at 100V for 30 minutes. The criteria for a positive *Wolbachia* test were based on two successful amplifications per marker, *wsp*, and 16S.

We assembled and aligned the sequences for each marker using the CodonCode Aligner version 1.2.4 and MAFFT version 7. We checked all generated sequences for similarities with reference sequences from GenBank (NCBI, 2016) using Basic Local Alignment Search Tool–Nucleotide BLAST. All *wsp* and 16S, sequences were separately analyzed using DNAsp version 6.12.03 [20] 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 [21].

We screened all individual *Aedes sp.* larvae (*n* = 509) and adults (*n* = 371) for *Wolbachia* using the *wsp* and 16S. 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. A total of three (0.84%) were *Wolbachia* positive out of 359 *Ae. aegypti*, based on the *wsp* and 12 (100%) out of 12 *Ae. albopictus* (Figure 1), based on the *wsp* and 16S. The *wsp* detected *Wolbachia* in six *Ae. albopictus*, whereas the 16S detected it in three *Ae. aegypti* and ten *Ae. albopictus*. Among these *Wolbachia*-infected *Ae. albopictus*, 4 (26.67%) out of 15 were positive for both *wsp* and 16S. Confirmation of the species identification using the *cox1* revealed that the 12 adult mosquitoes were *Ae. albopictus*, whereas the remaining three were *Ae. aegypti* based on BLAST results with 100% identity match (Table 1).

The phylogenetic tree built using the *wsp* (Figure 2 A) 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 (wAlbA), identified in an *Ae. albopictus* host in the USA [17]. 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 USA [7].

In the phylogenetic tree constructed using 16S, 11 out of 13 sequences (84.62%) found in *Ae. albopictus* (n = 10) and *Ae. aegypti* (n = 3) were clustered within supergroup A (Figure 2 B). The remaining two (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*.

We found that the *Ae. albopictus* were infected with both supergroups A and B of *Wolbachia* using the *wsp*, widely used for *Wolbachia* strain identification and phylogeny studies [17]. We found more *Wolbachia*-positive *Ae. albopictus* in supergroup B (4/6; 66.66%) than in supergroup A (2/6; 33.33%), as observed in a previous study [22] (China; 631/693; 91.05%). Our results support previous studies, and we observed two *Wolbachia* supergroups in *Ae. albopictus* collected from the same area at the same time.

We found a low prevalence of *Wolbachia* in adult *Ae. aegypti*, which is concordant with the previous results of [8] obtained from field-collected *Ae. aegypti* in Florida, USA and those of [7] conducted in Metro Manila, Philippines. In contrast, a high (>50%) prevalence rate was observed in the studies of [9] conducted in Malaysia and [8] conducted in New Mexico, USA. The low *Wolbachia* prevalence rate in *Ae. aegypti* may be due to the low density of the *Wolbachia*

endosymbiont in *Ae. aegypti* [7]; this is supported by the previous metabarcoding studies of [8;11-12], which all reported a low number of sequence reads in the *Ae. aegypti* midgut, indicating a low density of *Wolbachia*. Although our results are limited since we did not quantitatively measure *Wolbachia* density, our 40-cycle PCR amplification method adapted from [7] 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*.

We acknowledge the uncertainties of *Wolbachia*-positive *Ae. aegypti* due to the 16S and the conventional PCR method, e.g., the false-positive rate. In light of this information, we were careful to ascertain positive *Wolbachia* in adult *Ae. aegypti*. Thus, we used the primers of [19], which are known to produce fewer false-positive results and negative detections of *Wolbachia*. We also performed repeated PCR tests of our *Wolbachia*-positive samples to ensure successful *Wolbachia* detection as defined in the previous study by [7]. In addition to conventional PCR, tests such as fluorescent *in situ* hybridization and whole genome sequencing can be used to provide strong support for the presence of *Wolbachia* in natural *Ae. aegypti* populations. Despite these limitations, our results provide additional evidence for *Wolbachia* detection in field-collected *Ae. aegypti*.

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 offsprings would also be negative for *Wolbachia* if the mother is negative for *Wolbachia*. More *Wolbachia* might be detectable in larval samples if we increase the number of water containers by setting up ovitraps. We also recommend detecting

Wolbachia in representative larval samples from one water container. Future research may also explore the potential horizontal transmission of *Wolbachia* as observed in the study of [23].

Conclusions

Our study showed the detection of *Wolbachia* in field-collected *Ae. aegypti* and *Ae. albopictus* in Manila City, Philippines. For *wsp*, we found six *Wolbachia* positive *Ae. albopictus* while the 16S showed three *Ae. aegypti* and ten *Ae. albopictus* positive for *Wolbachia*. We found 3 out of 359 (0.84%) in adult *Ae. aegypti* and 12 out of 12 (100%) adult *Ae. albopictus* while negative results were found in all larvae (05/509;0%). Our findings may provide additional support for the presence of *Wolbachia* in field-collected *Ae. aegypti* and its ability to invade and persist in the population.

List of Abbreviations

PCR: polymerase chain reaction

DNA: deoxyribonucleic acid

wsp: *Wolbachia* specific primer

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. All newly generated sequences are available in the GenBank database under the Accession Numbers _____. (Accession numbers have not yet been obtained at the time of submission, it will be provided during the review).

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported in part by the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid Fund for the Promotion of Joint International Research [Fostering Joint International Research (B)] under grant number 19KK0107, JSPS Grant-in-Aid for Scientific Research (B) under grant number 21H02206, the JSPS Core-to-Core Program B Asia-Africa Science Platforms under grant number JPJSCCB20190008, and the Endowed Chair Program of the Sumitomo Electric Industries Group Corporate Social Responsibility Foundation.

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 molecular experiments for this study. MAFR, TI and KW performed the data analysis. MAFR and KW wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We are thankful to all the households that participated in our sampling collection of mosquitoes and larvae and to the village officials of Sampaloc, Manila, for their assistance and support. We

appreciate the help of Johanna Beulah T. Sornillo of the Research Institute for Tropical Medicine, Philippines, for her technical assistance in the household sampling design. We want to thank Katherine Viacrusis for her service in the fieldwork and Mr. Micanaldo Francisco for constructing the sampling map. Our deepest thanks to Dr. Thaddeus M. Carvajal, Dr. Divina Amalin, Dr. Mary Jane Flores, Dr. Joeselle M. Serrana, and Jerica Reyes for their valuable suggestions and technical assistance. The authors would like to thank Enago (www.enago.jp) for the English language review. Maria Angenica F. Regilme is a recipient of the Japanese Government (Monbukagakusho) Scholarship from the Ministry of Education, Science, Sport, and Culture of Japan.

References

1. Werren JH, Baldo L, Clark ME. Wolbachia: master manipulators of invertebrate biology. *Nature Reviews Microbiology*. 2008 Oct;6(10):741-51.
2. Zug R, Hammerstein P. Still a host of hosts for Wolbachia: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PloS one*. 2012 Jun 7;7(6):e38544.
3. Albuquerque AL, Magalhães T, Ayres CF. High prevalence and lack of diversity of *Wolbachia pipientis* in *Aedes albopictus* populations from Northeast Brazil. *Memórias do Instituto Oswaldo Cruz*. 2011 Sep;106(6):773-6.
4. Joanne S, Vythilingam I, Yugavathy N, Leong CS, Wong ML, AbuBakar S. Distribution and dynamics of Wolbachia infection in Malaysian *Aedes albopictus*. *Acta tropica*. 2015 Aug 1;148:38-45.

- 198 5. Kitrayapong P, Baimai V, O'Neill SL. Field prevalence of Wolbachia in the mosquito
199 vector *Aedes albopictus*. The American journal of tropical medicine and hygiene. 2002
200 Jan 1;66(1):108-11.

- 201 6. Balaji S, Jayachandran S, Prabakaran SR. Evidence for the natural occurrence of
202 Wolbachia in *Aedes aegypti* mosquitoes. FEMS microbiology letters. 2019
203 Mar;366(6):fnz055.

- 204 7. Carvajal TM, Hashimoto K, Harnandika RK, Amalin DM, Watanabe K. Detection of
205 Wolbachia in field-collected *Aedes aegypti* mosquitoes in metropolitan Manila,
206 Philippines. Parasites & vectors. 2019 Dec;12(1):361.

- 207 8. Kulkarni A, Yu W, Jiang J, Sanchez C, Karna AK, Martinez KJ, Hanley KA, Buenemann M,
208 Hansen IA, Xue RD, Ettestad P. Wolbachia pipientis occurs in *Aedes aegypti*
209 populations in New Mexico and Florida, USA. Ecology and evolution. 2019
210 May;9(10):6148-56.

- 211 9. Teo CH, Lim P, Voon K, Mak JJ. Detection of dengue viruses and Wolbachia in *Aedes*
212 *aegypti* and *Aedes albopictus* larvae from four urban localities in Kuala Lumpur,
213 Malaysia. Trop Biomed. 2017 Sep 1;34:583-97.

- 214 10. Iturbe-Ormaetxe I, Walker T, O'Neill SL. Wolbachia and the biological control of
215 mosquito-borne disease. EMBO reports. 2011 Jun;12(6):508-18.

- 216 11. Coon KL, Brown MR, Strand MR. Mosquitoes host communities of bacteria that are
217 essential for development but vary greatly between local habitats. Molecular ecology.
218 2016 Nov;25(22):5806-26.

12. Thongsripong P, Chandler JA, Green AB, Kittayapong P, Wilcox BA, Kapan DD, Bennett SN. Mosquito vector-associated microbiota: Metabarcoding bacteria and eukaryotic symbionts across habitat types
13. Hegde S, Khanipov K, Albayrak L, Golovko G, Pimenova M, Saldaña MA, Rojas MM, Hornett EA, Motl GC, Fredregill CL, Dennett JA. Microbiome interaction networks and community structure from laboratory-reared and field-collected *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* mosquito vectors. *Frontiers in microbiology*. 2018 Sep 10;9:2160.
14. Bennett KL, Gómez-Martínez C, Chin Y, Saltonstall K, McMillan WO, Rovira JR, Loaiza JR. Dynamics and diversity of bacteria associated with the disease vectors *Aedes aegypti* and *Aedes albopictus*. *Scientific reports*. 2019 Aug 21;9(1):1-2.
15. Gloria-Soria A, Chiodo TG, Powell JR. Lack of evidence for natural Wolbachia infections in *Aedes aegypti* (Diptera: Culicidae). *Journal of medical entomology*. 2018 Aug 29;55(5):1354-6.
16. Ross PA, Ritchie SA, Axford JK, Hoffmann AA. Loss of cytoplasmic incompatibility in Wolbachia-infected *Aedes aegypti* under field conditions. *PLoS neglected tropical diseases*. 2019 Apr 19;13(4):e0007357.
17. Zhou W, Rousset F, O'Neill S. Phylogeny and PCR-based classification of Wolbachia strains using *wsp* gene sequences. *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 1998 Mar 22;265(1395):509-15.
18. Rueda LM. Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with dengue virus transmission. *Walter Reed Army Inst Of Research Washington Dc Department Of Entomology*; 2004 Aug 3.

19. Simoes PM, Mialdea G, Reiss D, Sagot MF, Charlat S. Wolbachia detection: an assessment of standard PCR protocols. *Molecular Ecology Resources*. 2011 May;11(3):567-72.
20. Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A. DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular biology and evolution*. 2017 Dec 1;34(12):3299-302.
21. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic biology*. 2003 Oct 1;52(5):696-704.
22. Hu Y, Xi Z, Liu X, Wang J, Guo Y, Ren D, Wu H, Wang X, Chen B, Liu Q. Identification and molecular characterization of Wolbachia strains in natural populations of *Aedes albopictus* in China. *Parasites & vectors*. 2020 Dec;13(1):1-4.
23. Haine ER, Pickup NJ, Cook JM. Horizontal transmission of Wolbachia in a *Drosophila* community. *Ecological Entomology*. 2005 Aug;30(4):464-72.

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

282

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

	House hold	Molecular species identification	Individual code	Haplotypes			
				<i>co</i>	<i>wsp</i>	16S (n = 13)*	
				<i>x1</i>	Supergroup	<i>wsp</i> (n = 6)*	
1	SH9	<i>Ae. albopictus</i>	40	C1			S7
2	SH27	<i>Ae. albopictus</i>	306	C5			S3
3	SH134	<i>Ae. aegypti</i>	384	C6			S1
4	SH214	<i>Ae. albopictus</i>	425	C1			S6
5	NH43	<i>Ae. albopictus</i>	172	C4			S3
6	NH56	<i>Ae. albopictus</i> (n=3)	095, 177, 178	C1	A (177) and B (178)	W1 (177) and W2 (178)	S8 (095) and S9 (177)
7	NH107	<i>Ae. albopictus</i>	218	C5			S4
8	NH130	<i>Ae. aegypti</i>	116	C2			S1
9	NH131	<i>Ae. albopictus</i>	240	C5	A	W3	S5
1	NH149	<i>Ae. albopictus</i>	258, 259,	C1	B (258, 259,	W2 (258, 259,	S6 (258, 259)
0		(n = 3)	260		260)	260)	
1	NH181	<i>Ae. aegypti</i>	121	C3			S2
1							
Total number of haplotypes				6		3	9

285

286 * total number of *Wolbachia*-positive mosquitoes

287

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.