1 **DETECTION OF Wolbachia IN FIELD-COLLECTED MOSQUITO VECTOR, Aedes**

2 **aegypti**

3 Thaddeus M. Carvajal[1,2,3*], Kazuki Hashimoto[1], Reza Kurniawan Harnandika[1], Divina M

4 Amalin[2,3] and Kozo Watanabe[1,2,3]

5 [1] Department of Civil and Environmental Engineering - Ehime University, Matsuyama,

6 Japan

7 [2] Biology Department – De La Salle University, Taft Ave Manila, Philippines

8 [3] Biological Control Research Unit, Center for Natural Science and Environmental Research

9 - De La Salle University, Taft Ave Manila, Philippines

10 *corresponding author: tads.carvajal@gmail.com

11 **ABSTRACT**

12 It was the impression from past literature that Wolbachia is not naturally found in Ae. aegypti.

13 However, there are have been reports that recently reveals the presence of this endosymbiont

14 in this mosquito vector. With this, our study presents additional support of Wolbachia

15 infection in Ae. aegypti by screening field-collected adult mosquitoes using Wolbachia-

16 specific 16S rDNA and its surface protein (wsp) makers under optimized PCR conditions.

17 From a total of 672 Ae. aegpyti adult mosquito samples collected in Metropolitan Manila,

18 Philippines, 113 (16.8%) and 89 (13.2%) individual mosquito samples were determined to

19 be Wolbachia infected using the wsp and 16S rDNA markers, respectively. The Ae. aegpyti

20 wsp sample sequences were similar or identical to five known Wolbachia strains belonging

21 to supergroups A or B while majority of 16S rDNA sample sequences were similar to strains

22 belonging to supergroup B. Overall, 80 (11.90%) individual mosquito samples revealed to

23 show positive amplifications in both markers and 69.0% showed congruence in supergroup

24 identification (supergroup B). Our findings illustrate that the infection status of Wolbachia

25 in Ae. aegypti may appear common than previously recognized.

INTRODUCTION

Mosquitoes are considered to be medically important insects because of their capacity to carry notable human disease pathogens[1]. Among the known mosquito vectors, *Aedes aegypti* is an efficient and dangerous mosquito vector because of its ability to carry significant arboviral diseases such as Dengue, Chikungunya, Yellow Fever and Zika[2,3]. Despite the development of vaccines, these arboviral diseases are considered to be the leading cause of global disease burden[4] and thus, targeting the mosquito vector is deemed to be the primary control and prevention. A considerable number of vector control strategies had been implemented, but the disease burden continues to increase. Novel and newer approaches are being developed that shows promising outcomes in vector and disease control and one of which is the utilization of the intracellular bacterial endosymbiont, *Wolbachia*[7-9].

Wolbachia is a naturally occurring endosymbiont which can be maternally inherited and cause different reproductive alterations in its host to increase their transmission to the next generation[10-12]. In insects, it is estimated to be naturally present in 60-65% of known species[13]. As to date, there are 17 identified major clades or supergroups (A-Q) where a majority are known to infect arthropods such as insects, arachnids, and crustaceans[14]. The pathogenic effects of *Wolbachia* in its host are well-studied and determined to cause sperm-egg incompatibility, parthenogenesis, cytoplasmic incompatibility, and feminization[11,15]. Therefore, utilizing these effects towards medically-important mosquito vectors, such as *Ae. aegypti*, has taken great research strides in the past two decades. The discovery of a virulent *Wolbachia* strain (*w*MelPop) in Drosophila melanogaster was successfully transferred to *Ae. aegypti* where it reduced the lifespan of the mosquito vector[16-18]. In addition to this, *w*MelPop and other *Wolbachia* strains (e.g., *w*Mel) were able to demonstrate conferring resistance on a wide range of insect viruses, especially to human viral pathogens, such as dengue and chikungunya[19-22]. The life-shortening capability plus pathogen interference of this *Wolbachia* strain opened an avenue for its potential use as a biological control agent approach against mosquito-borne diseases. The World Mosquito Program (https://www.worldmosquitoprogram.org), formerly known as the Eliminate Dengue Project,

54   was able to generate stable *Wolbachia*-infected *Ae. aegypti* lines that possessed the ability of

55   pathogen interference from dengue viruses under laboratory conditions. These *Wolbachia*

56   strains showed maternal transmission rates close to 100% and induced high levels of

57   cytoplasmic incompatibility to *Ae. aegypti*[16]. Semi-field cage experiments were also

58   conducted to assess the fitness cost effect of the discovered strain towards the mosquito

59   vector and its ability of these strains to invade the mosquito population. These experiments

60   demonstrated the true potential of the endosymbiont because of the reduced fecundity of

61   *Wolbachia*-infected *Ae. aegypti* as compared to the uninfected wildtype[22]. Australia became

62   the first country to release these *Wolbachia*-infected *Ae. aegypti* into the wild population

63   where it exhibited promising results[23,24]. As to date, this methodological strategy against the

64   mosquito vector, *Ae. aegypti*, is now being tested in eight dengue-endemic countries such as

65   Indonesia, Vietnam, Colombia, and Brazil (https://www.worldmosquitoprogram.org). It

66   claimed this approach is considered to be cost-effective and safer for the environment than

67   conventional insecticide-based measures[19,25].

68   With the recognition of about 65% of known insects to be naturally infected with

69   *Wolbachia* including those mosquito species from the genera of *Aedes, Culex, Mansonia*,

70   major mosquito vectors of diseases such as *Ae. aegypti* and Anopheline mosquitoes were

71   reported not to possess this endosymbiont[26-31]. It led to the belief that the presence of

72   *Wolbachia* endosymbiont could be the reason why many of the mosquito species are

73   considered to be weak vectors[23]. Nonetheless, more recent studies show evidence that

74   *Wolbachia* infection in *Ae. aegypti* and *Anopheles gambiae* may appear to be more common

75   than it was previously recognized. Natural *Wolbachia* infections have now been reported in

76   adult, larvae and egg populations of *An. gambiae*[32-34].

77   Lately, studies have reported detecting *Wolbachia* from field-collected *Ae. aegypti*

78   samples using either *wsp* marker[35] or 16S metabarcoding[36-37]. Though these studies are

79   commendable, there were still uncertainties in establishing whether the mosquito vector does

80   harbor naturally the endosymbiont. Although metabarcoding studies had a substantial sample

81   size (n=85-270), there were unable to report an accurate estimate of the infection rate because

82   mosquito adult or larval samples were pooled from each location. In contrast, *wsp* detection

83   in *Ae. aegypti* larval samples[35] were screened individually, thus, was able to report the

84   infection rate (50.0%). However, it was difficult to affirm or ascertain its true prevalence

85   since the sample size was small (n=16 individuals). Moreover, there is possibility of a

86   potential bias in reporting a high infection rate if larval samples were collected from the same

87   water container due to the sampling of mosquito siblings from the same female mosquito.

88   Nevertheless, these studies further suggest the likelihood of *Wolbachia* to be naturally

89   associated with *Ae. aegypti*, thus, opening an avenue to re-visit or re-examine its infection

90   status.

91   Our study aims to present additional support of *Wolbachia* infection found from field-

92   collected *Ae. aegypti* adult mosquitoes using *Wolbachia*-specific 16S rDNA and the

93   *Wolbachia* surface protein (*wsp*) markers. Based on the limitations presented from previous

94   studies, two considerations were applied in addressing these gaps. First, *Wolbachia* screening

95   was done over a large sample size (n=672) and used an individual-based detection of adult

96   *Ae. aegypti* mosquitoes to gain a better estimate of its prevalence in this mosquito vector.

97   Secondly, two molecular markers were used to confirm the detection status and infer the type

98   of *Wolbachia* strains found in *Ae aegypti*.

99   **METHODS**

100   *Study area and Mosquito collection*

101   The study area is the National Capital Region of the Philippines or also known as

102   Metropolitan Manila. Located on the Eastern shore of Manila Bay in Southwestern Luzon

103   Island (14°50′ N Latitude, 121°E Longitude), it is considered to be one of the highly

104   urbanized and densely populated areas in the Philippines. Dengue disease is endemic in this

105   region where it accounts for 15%-25% of the total number of reported Dengue cases annually

106   in 2009 - 2014[38]. Vector control programs are being implemented in various localities of the

107   region. Insecticide application and cleaning of the surroundings have been extensively used

108   however its effectiveness is in question because of the constant and unchanging burden of

109   the disease. As to date, the Philippines, especially Metropolitan Manila, has never conducted

110   any *Wolbachia*-based program against *Ae. aegypti*.

111   Adult mosquito samples were collected using a commercial branded mosquito UV-

112   light trap (Jocanima®) installed in the outdoor premises of 138 residential households

113   (sampling sites) from May 2014 – January 2015 (Figure 1a). Collected samples were then

114   sorted and identified as *Ae. aegypti* using available keys[39]. Each sample was then placed in a

115   tube with 99.5% ethanol for preservation. A total of 672 *Ae. aegypti* adult mosquito samples

116   were collected, identified, labeled accordingly (See Supplementary Table 1) and stored at -

117   20°C for subsequent processing.

118   *DNA Extraction, Polymerase Chain Reaction, and Sequencing*

119   Total genomic DNA of each mosquito individual was extracted using the QIAGEN

120   Blood and Tissue DNEasy Kit© following a modified protocol[40]. Our study used two

121   molecular markers for detecting *Wolbachia* infection namely; *wsp*[41] and 16S rDNA[42]. The

122   primer sequences are as follows: *wsp* 81F (5'TGG TCC AAT AAG TGA TGA AGA AAC)

123   and *wsp* 691R (5' AAA AAT TAA ACG CTA CTC CA) for *wsp* marker while *Wspecf* (AGC

124   TTC GAG TGA AAC CAA TTC) and *Wspecr* (GAA GAT AAT GAC GGT ACT CAC) for

125   16S rDNA.

126   For *wsp* gene amplification, we followed the standard *wsp* protocol[30] where the

127   suggested annealing temperature and number of cycles were 55 °C and 30 cycles respectively.

128   In order to conduct an individual-based detection, we initially performed this protocol in

129   *Culex quinquefasciatus* as our positive control. Certain modifications were made in the

130   standard protocol based on the results where the annealing temperature was set to 57 °C and

131   the number of cycles increased to 35 cycles. This initial modified protocol was performed in

132   individual *Ae. aegypti* samples where it yielded positive faint bands. It prompted us to re-

133   modify again the protocol where the annealing temperature is set at 59 °C with 40 cycles and

134   the addition of 10% DMSO (Sigma-Aldrich®) that led to desirable results necessary for

135   sequencing. Therefore, a 10 μl final reaction volume was used and composed of 10X buffer

136   (TAKARA®), 25 mM MgCl$_2$, 10 mM of each dNTPs, 10 μM forward and reverse primers,

137 10% DMSO (Sigma-Aldrich®) and 5.0U/ μl of *Taq* DNA polymerase (TAKARA®). The

138 final thermal profile consisted an initial denaturation of 95℃ for 3 minutes, followed by

139 another denaturation temperature of 95℃ for 1 minute, an annealing temperature of 59℃ for

140 1 minute and an extension temperature of 72℃ for 1 minute for 40 cycles, and accompanied

141 by a final extension temperature at 72℃ for 3 minutes.

142 On the other hand, 16S rDNA gene amplification used a 10 μl final reaction volume

143 and composed of 10X buffer (TAKARA), 25 mM MgCl$_2$, 10 mM of each dNTPs, 10 μM

144 forward and reverse primers, 10% DMSO (Sigma-Aldrich®) and 5.0U/ μL of *Taq* DNA

145 polymerase (TAKARA®). Thermal profiles follow the protocol of Simões et al[42] with initial

146 denaturation temperature at 95℃ for 2 minutes, followed by two cycles of 95℃ for 2 minutes

147 of denaturation, annealing temperature of 60℃ for 1 minutes and extension temperature of

148 72℃ for 1 minute, afterwards 35 cycles of denaturation of 95℃ for 30 seconds, annealing

149 temperature of 60℃ for 1 minute and extension temperature of 72℃ for45 seconds and final

150 extension at 72℃ for 10 minutes.

151 All PCR amplification experiments included positive and negative controls. The

152 positive control is a *Wolbachia*-infected *Cu. quinquefasciatus* sample while the negative

153 control consisted of water as the template. The product size of each molecular marker was

154 checked through electrophoresis with 1.5% agarose gel set at 100 volts for 30 minutes. The

155 size of the amplified *wsp* gene is 610 bp while the 16S rDNA gene is 438 bp. PCR

156 amplification process underwent two replicates to validate the results obtained (See

157 Supplementary Table 1). A third screening was performed for selected individual samples

158 that had conflicting results based on the two prior replicates. Therefore, the criteria set in

159 reporting the certainty for *Wolbachia* infection is based on two successful amplification of

160 the molecular markers. Amplified PCR products from each molecular marker were sent for

161 sequencing to Eurofins, Operon – Tokyo.

162   *Identity of Wolbachia strains and their positions in phylogroups*

163   All sequences were subjected to the Nucleotide Basic Local Alignment Search Tool

164   (BLAST) and compared to deposited *Wolbachia* sequences in GENBANK. Next, selected

165   sequences of *Wolbachia* strains (Table 1) and those obtained in the study underwent multiple

166   alignment using Clustal W in MEGA 6[43]. After editing, the final length used for phylogenetic

167   inference analyses was 398 bp and 732 bp for *wsp* and 16S rDNA respectively. The identities

168   and relationships of the *Wolbachia* strains obtained in our study were determined by

169   performing the Bayesian method in PhyML 3.0 software with 1000 bootstrap replicates[44].

170   The Smart Model Selection[45] was also utilized to set the parameters for *wsp* as GTR+G

171   (number of estimated parameters k = 232, Akaike Information Criterion (AIC) = 4897.31702)

172   and 16S rDNA as GTR+G+1 (number of estimated parameters k = 207, Akaike Information

173   Criterion (AIC) = 5332.88688). All sample sequences were submitted to GENBANK with

174   Accession numbers _____ - _____.

175   *Statistical Analysis*

176   A Clark-Evans test was performed to determine if the spatial distribution of

177   *Wolbachia*-positive mosquito samples from each molecular marker have a pattern of

178   complete spatial randomness. The test uses the aggregation index (R) where a value of > 1

179   suggests an ordered distribution and a value of < 1 suggests clustering. This analysis was

180   performed using R program version 3.3.5[46] under package *spatstat* [46]

181   **Table 1.** Representative *Wolbachia* type sequences from different insect hosts in *wsp* and

182   16S rDNA molecular markers.

| Molecular Marker	Host	*Wolbachia* supergroup	Accession Number
wsp	*Drosophila melanogaster*	A	AF020072
	Aedes albopictus	A	AF020058
	Glossina morsitans	A	AF020079
	Drosophila simulans (Riverside)	A	AF020070
	Muscidifurax uniraptor	A	AF020071
	Phlebotomus papatasi	A	AF020082
	Glossina austeni	A	AF020077
	Culex pipiens	B	AF020061
	Culex quinquefasciatus	B	AF020060
	Aedes albopictus	B	AF020059
	Ephestia cautella	B	AF020076
	Dirofilaria immitis	C (Outgroup)	AJ252062
16S	*Nasonia longicornis*	A	M84691
	Muscidifurax uniraptor	A	L02882
	Aedes albopictus	B	KX155506
	Culex pipiens	B	X61768
	Nasonia vitripennis	B	M84686
	Onchocera volvulus	C	AF069069
	Dirofilaria immitis	C	Z49261
	Litomosa westi	D	AJ548801
	Folsomia candida	E	AF179630
	Mansonella ozzardi	F	AJ279034
	Dipetalonema gracile	J	AJ548802
	Rickettsia sp.	Outgroup	U11021
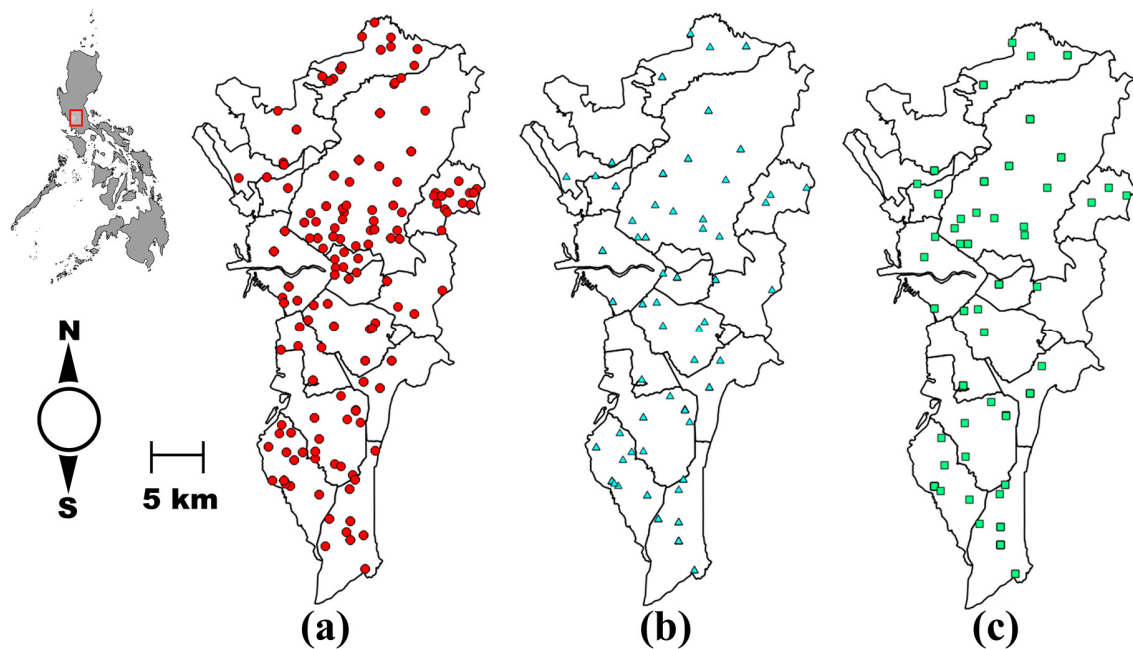
Fig 1. (a) Spatial distribution of the sampling sites (n=138) for collecting adult *Ae. aegypti*. *Wolbachia*-positive sampling sites based on *wsp* (b) and 16S rDNA (c). Details of the number of *Wolbachia*-positive mosquitoes per sampling site is found in the Table S1.

RESULTS

Detection of Wolbachia through wsp and its phylogeny

From a total of 672 adult *Ae. aegypti* screened, 113 (16.8%) individual adult mosquito samples are infected with *Wolbachia* using the *wsp* marker (Table 2). Based on the study's criterion (See methods), only 17 samples demonstrated one successful amplification, thus excluding them for further analysis. In addition, female/male ratio is 0.82 (Table 2). All sequenced amplicons resulted in a high degree of similarity (>98.0%) with deposited *wsp* sequences in GENBANK. The spatial distribution showed that 60 (43.0%) sampling sites (Figure 1b) contained *Wolbachia* positive mosquitoes with 1 – 8 individuals. Further analysis showed that the distribution of *wsp*-positive mosquito samples was significantly clustered (R = 0.003, p < 0.001). Figure 2 and Figure S1 show the phylogeny of *Wolbachia* sequences based on *wsp* sequences. Majority of the sequences were found in supergroup B (n=84) while

198   the remaining were clustered in supergroup A (n=29). Based on descending order of sample

199   sizes, sample sequences in supergroup B were identical (>99.0%) to *Wolbachia* type strains

200   from selected hosts of *Ae. albopictus* (*wAlbB*) (n= 51), *Cu. quinquefasciatus*, *Cu. pipiens*

201   (*wPip*), *Ae. aegypti wMel* strain (n= 23) and *Ephestia cautella* (*wCau*) (n= 10). The sample

202   sequences from supergroup A were either similar (98.0-99.0%) (n = 8) or identical (>99.0%)

203   (n= 21) to the type strain (*wAlbA*) from host *Ae. albopictus*.
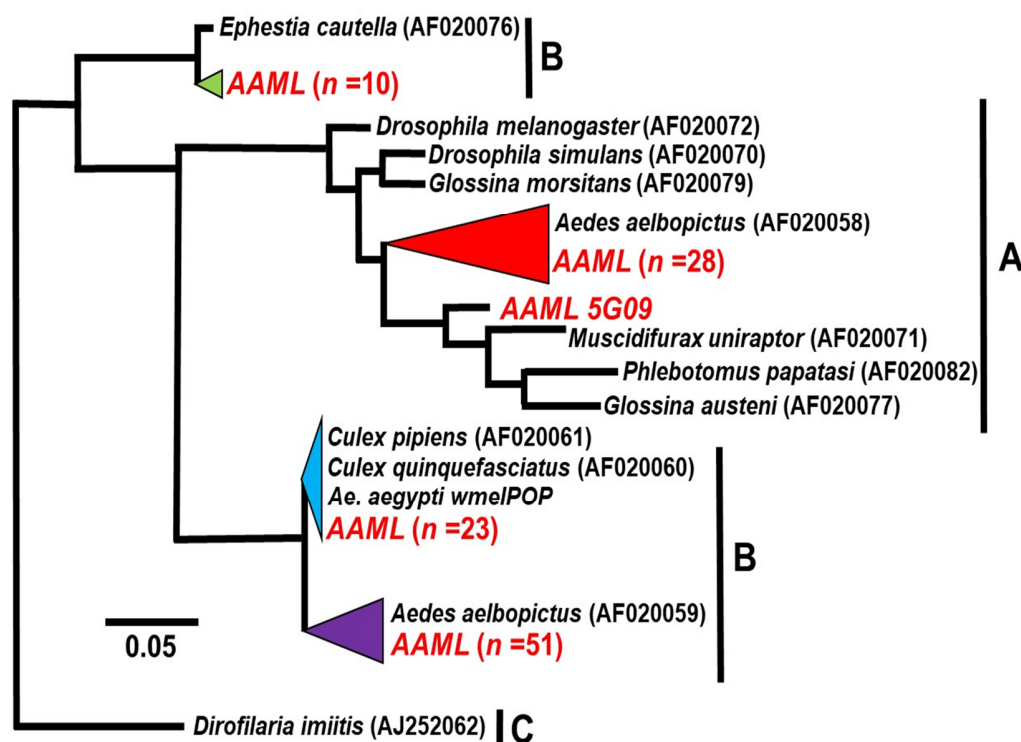

204   **Fig 2.** Phylogenic analysis of *wsp*. The alignment was analyzed in PhyML. Sample sequences

205   of *Ae.aegypti* collected in Metropolitan Manila are in red, labeled as AAML (**A**e. **a**egypti

206   **M**etropolitan Mani**L**a) and alphanumeric values indicate the unique code assigned to each

207   *Ae. aegypti* individual sample. Merging (triangles) of sample and representative *Wolbachia*

208   sequences was done to show degree of similarity (98-100%). Supergroups were indicated as

209   A – C depending on the representative sequences used. The phylogenetic trees are re-drawn

210   for better visualization, thus an expanded version can be viewed in Supplemental Figure S1.

211   Please refer to Table 1 for the *Wolbachia* type sequences (ingroup and outgroup) for both

212   markers.

213   *Detection of Wolbachia through 16S rDNA and its phylogeny*

214   For 16S rDNA, 89 (13.2%) individual adult mosquito samples were infected with

215   *Wolbachia* (Table 2). 20 individual mosquito samples generated one successful 16S rDNA

216   amplification, thus, excluding them for further analysis. Furthermore, female/male ratio is

217   0.85 (Table 2). 50 (36.0%) sampling sites (Figure 1c) contained *Wolbachia*-positive

218   mosquitoes ranging from 1-8 individuals and the distribution of 16SrDNA-positive

219   individuals revealed to be clustered or aggregated ($R = 0.001, p < 0.001$). All sequenced

220   amplicons resulted in a high degree of similarity (>98%) with deposited 16S rDNA

221   Wolbachia sequences in GENBANK. Nearly all 16S rDNA sample sequences (n=85) (Figure

222   3, Figure S2) were grouped in supergroup B. Only one sample sequence was identical to

223   *Nasonia vitripennis* while the remaining sample sequences were up to 99% similar from the

224   selected hosts of the supergroup. The remaining sample sequences (n=4) were grouped in

225   supergroup C & J. One sample sequence was highly similar (>99%) with *Dirofilaria immitis*

226   while the remaining were 98-99% similar from the selected hosts of the supergroup.

227   *Comparison of 16S rDNA and wsp for Wolbachia detection and phylogeny*

228   From the 113 and 89 positively detected mosquito individuals from *wsp* and 16S

229   rDNA respectively, 80 (11.90%) individual samples yielded positive amplification in both

230   markers (Table 2). In *wsp* positive detection (n=113), 80 had two successful amplification of

231   the 16S rDNA marker while 27 had only one amplification of 16S rDNA and the remaining

232   6 had no successful amplification on 16S rDNA marker. On the other hand, the 89 individual

233   samples deemed 16S rDNA positive for *Wolbachia* showed 80 individuals had two

234   successful amplification of the *wsp* marker while 9 had only one successful amplification on

235   the said marker. Next, we focus on the supergroup classification of the 80 individual samples

236   based on the *wsp* and 16S rDNA phylogeny. It was shown that 55 (69%) had the same

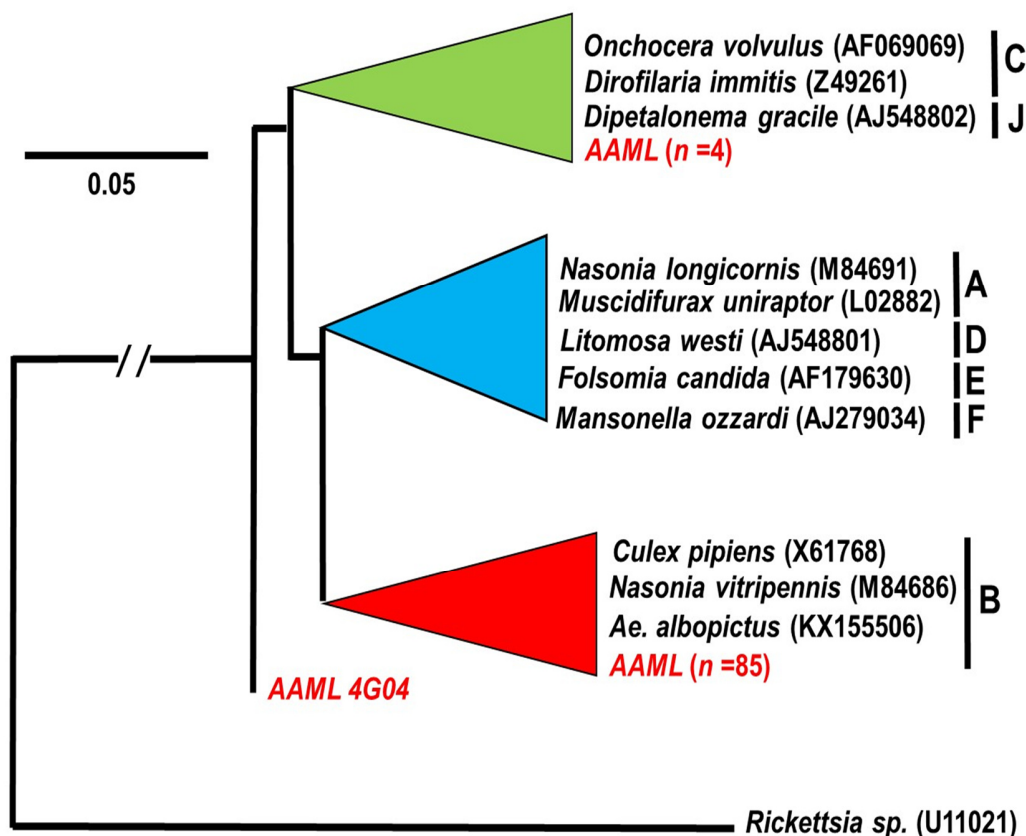237   classification in supergroup B while the remaining 25 (31%) showed a disparity in

238   supergroup classification. Such difference, for example, showed that *wsp* identified the
239   individual sample as supergroup A, but 16S rDNA reveals to be either supergroup B or C &
240   J.

241   **Fig 3.** Phylogenic analysis of 16S rDNA. The alignment was analyzed in PhyML. Sample
242   sequences of *Ae.aegypti* collected in Metropolitan Manila are in red, labeled as AAML (*Ae.*
243   *aegypti* **M**etropolitan Mani**L**a) and alphanumeric values indicate the unique code assigned to
244   each *Ae. aegypti* individual sample. Merging (triangles) of sample and representative
245   *Wolbachia* sequences was done to show degree of similarity (98-100%). Supergroups were
246   indicated as A – J depending on the representative sequences used. The phylogenetic trees
247   are re-drawn for better visualization, thus an expanded version can be viewed in
248   Supplemental Figure S2. Please refer to Table 1 for the *Wolbachia* type sequences (ingroup
249   and outgroup) for both markers.

250 **Table 2.** Summary *of wsp and 16S rDNA* detection results in *Ae. aegypti*

Molecular markers	Number of individuals detected (n=672)	Female (n=379)	Male (n= 293)	Female/Male ratio
wsp	113 (16.82%)	52	61	0.82
16S rDNA	89 (13.24%)	41	48	0.85
wsp+16S rDNA	80 (11.90%)	36	44	0.82

251

252 **DISCUSSION**

253   Our study was able to demonstrate the detection of the endosymbiont *Wolbachia* in

254 field-caught adult *Ae. aegypti*. Notably, the main reason for the positive detection, especially

255 in *wsp*, is because of the procedural modifications or optimization in the amplification of the

256 said marker. A case in point, for example, why optimization is necessary is the evidence

257 presented in the malaria mosquito vector, *An. gambiae*. Previous studies had reported no

258 observed natural *Wolbachia* infection in this mosquito vector[26-31]; however, the

259 endosymbiont was successfully detected in *An. gambiae* from Burkina Faso, West Africa

260 using an optimized *wsp* protocol[32,33]. Another potential reason for a positive detection was

261 the study's sample size. Based on several literature on assessing the prevalence of *Wolbachia*

262 in different mosquito species, the highest number of *Ae. aegypti* individuals screened was

263 119[30] which resulted in non-detection of the endosymbiont. As compared to the actual study

264 (n= 672), the sample sizes from previous studies were low; thus, larger sample size would

265 provide a more accurate estimate of the prevalence of *Wolbachia* infection. Similarly, these

266 reasons were clearly emphasized by recent studies on why earlier investigations may have

267 underestimated the actual incidence of *Wolbachia* infection from different insect hosts[48,49].

268   Our study acknowledges the uncertainties associated with conventional PCR

269 detection such as high false positive detection rates. With this in mind, the study was cautious

270 in affirming a positive infection in each *Ae. aegypti* adult sample. First, the selection of

271 markers is based on the recommendation of Simoes et al.[42] that two of its preferred primer

272   sets (e.g. *Wspecf* and *Wspecr*) was determined to produce the lowest false positive and false

273   negative rates. Secondly, our study performed replications with a stringent criterion for a

274   successful *Wolbachia* infection on each mosquito sample. Although there are several genetic

275   markers (e.g. MLST genes) and techniques (e.g. IFA, FISH or whole-genome sequencing)

276   available, the primary intention of this study is to detect *Wolbachia* infection in *Ae. aegypti*

277   initially using this PCR-Based approach.

278   Linking our findings with the previous studies[35-37] which reported *Wolbachia* in *Ae.*

279   *aegypti* may incidentally provide a clear picture of its infection status. First, the probable

280   density of the endosymbiont found in this mosquito vector may be low. Even though our

281   study did not measure the actual density, a 40-cycle PCR amplification procedure or a long

282   PCR run[50] may detect a small amount of *Wolbachia* present. It partly supports the results

283   presented from metabarcoding studies[36,37] where a low number (2-4) of *Wolbachia* sequence

284   reads were detected in both the larvae and adult *Ae. aegypti* mosquito. These can be another

285   potential reason why earlier prevalence studies were not able to detect *Wolbachia* in *Ae.*

286   *aegypti* samples. Moreover, the low probable density of the endosymbiont may also translate

287   to the observed low infection rate (13-16%) found in our study. This again partly supports

288   metabarcoding studies[36,37] where only two *Ae. aegypti* mosquito pools had the presence of

289   these low number *Wolbachia* sequences. On the other hand, our results are in contrast with

290   the report from *Ae. aegypti* larvae (n=16 individuals) in Malaysia which resulted in a 50%

291   infection rate[35]. However, there could be some uncertainties to this estimate because of its

292   small sample size and, more importantly, the collected larval samples may be siblings from

293   the same female *Ae. aegypti* mosquito. The limitation as mentioned earlier prompted us to

294   conduct an individual-based adult mosquito detection so that it can present a better and

295   explicit estimation of the infection rate. Secondly, we assume that the *Wolbachia* strain/s

296   found in *Ae. aegypti* can be maternally-inherited due to the following reasons: (a) reported

297   positive infections in larval samples from the previous studies[35-37] and (b) detecting positive

298   infections in male *Ae. aegypti* mosquitoes (our study, Table 2). However, there is still a need

299   to present direct evidence of maternal transmission of this endosymbiont during the

300 developmental stages of *Ae. aegypti* since all studies, including ours, were performed
301 independently.

302 Lastly, the *Wolbachia* strains infecting *Ae. aegypti* have been shown in our study
303 belong to supergroups A and B. Both *wsp* and 16S rDNA phylogeny showed that majority
304 of the individual samples belong to supergroup B while a small number of individual samples
305 were found in supergroup A (based on *wsp*). Detecting different *Wolbachia* strains in a single
306 mosquito species is relatively common especially in medically important mosquitoes, *Ae.*
307 *albopictus*[51,52] and *An. gambiae*[32], and other insect host species (e.g. *Drosophila* species[51]).
308 Since our study presented a majority of our sample sequences belonging to supergroup B,
309 this was also the same observation reported by previous studies[35-37]. Dipterans, especially
310 mosquitoes, are commonly infected by these *Wolbachia* strains from supergroups, A and B.
311 It has been shown to cause parasitism towards its insect host by producing phenotype effects
312 such as cytoplasmic incompatibility, male killing, and feminization[11,53]. Nevertheless,
313 whether the identified *Wolbachia* strains in *Ae. aegypti* possess these phenotypic effects
314 remains unclear. Also, further studies are needed to ascertain the pathogenic impact of this
315 local endosymbiont to the mosquito vector. More importantly, it is very essential to determine
316 whether these identified *Wolbachia* strains could render *Ae. aegypti* a less effective vector by
317 blocking key arboviruses such as dengue. It is also worth mentioning that some individual
318 samples have shown to be similar with *Wolbachia* strains found in supergroups C and J based
319 on 16S rDNA. These two supergroups are not generally found in dipterans especially in
320 mosquitoes. It is likely that our 16S rDNA amplified the *Wolbachia* strain residing in the
321 roundworm, *Dirofilaria immitis*. *Ae. aegpyti* mosquitoes are also known to carry this parasitic
322 nematode to certain mammals, such as dogs[54]. This observation was also reported in one of
323 the metabarcoding studies[37] that showed sequences of *Wolbachia* from *Dirofilaria immitis*.
324 However, when these 16S rDNA results were compared to the *wsp* results in our study, it
325 showed the *Wolbachia wsp* sample sequence of the same mosquito individuals belong to
326 supergroup B. We can only infer that the inconsistent results observed in our study may stem
327 towards the sensitivity and specificity of the markers used. The *wsp* gene marker has been
328 likened to antigen protein typing in screening pathogenic bacteria where it can be a perfect

329   diagnostic tool for detecting *Wolbachia* infection[55,56]. However, it is unsuitable for

330   phylogenetic analysis or deeper taxonomic relationship because of its extensive

331   recombination and strong diversifying selection[11,57,58]. 16S rDNA, on the other hand, is

332   known to be a conserved gene highly suited in bacterial identification and phylogeny, but its

333   use in detecting *Wolbachia* infection has demonstrated varying results depending on the

334   specific 16S rDNA primers[42]. It was emphasized that "no single protocol" can ultimately

335   ensure the specificity and accuracy of 16S rDNA to detect *Wolbachia* infection[56]. Thus,

336   further claiming that 16S rDNA markers in *Wolbachia* detection may be far from optimal[56].

337   We consider our findings to be crucially important especially if the Philippines would

338   implement or approve two scenarios in the release of: (a) *Wolbachia*-infected (e.g. *w*MelPop

339   or *w*Mel) mosquitoes or (b) local *Wolbachia* strains found by our study in dengue-endemic

340   areas. In the first scenario, a vital consideration is the presence of "bidirectional

341   incompatibility" mechanism between the intended *Wolbachia* strain (e.g. *w*MelPop or *w*Mel)

342   to be released and the present local strain found in the mosquito. There are instances that two

343   strains in one host cannot stably coexist with each other because the naturally occurring strain

344   is preventing the intended strain to reach fixation or establishment[59-61]. It would serve as an

345   impediment to the intentional spread of *Wolbachia* strain to the mosquito population. It was

346   suggested that to overcome this incompatibility is to remove the existing natural strain

347   inhabiting the mosquito vector or to perform a "superinfection" where the intended

348   *Wolbachia* strain induces unidirectional incompatibility with the natural strain[62].

349   Nevertheless, it very important to re-examine the infection status of *Wolbachia* in *Ae. aegypti*

350   mosquitoes in intended areas prior a mass release program. If the second scenario, utilizing

351   the release of local *Wolbachia* strains, is implemented, there are specific considerations that

352   should be addressed for a successful population replacement. The first and most important

353   consideration is to determine whether these local strains may exhibit the same phenotypic

354   effects and pathogen blocking of *w*Mel strain to *Ae. aegypti*. Currently, these characteristics

355   are still unknown and therefore crucial if utilized for mass release. Another consideration is

356   endosymbiont's density in the mosquito vector. Mosquito species naturally infected with

357   *Wolbachia* are not ideal candidates due to the changing molecular interactions between

358 *Wolbachia* and the host over time[63]. The result of this symbiosis is the amount of bacterial

359 density found in the mosquito host where it can influence the intensity of *Wolbachia*-induced

360 phenotypic or anti-viral effects[22,62,64,65]. Newer infections (e.g. tansinfections) are shown to

361 produce high bacterial density while natural infections lead to lower bacterial density due to

362 the adaptation of the host to the endosymbiont infection over time. In our study, we infer that

363 the local *Wolbachia* strains are in low density inside its host, *Ae. aegypti*. If this is the case,

364 it will result in a reduced physiological and anti-viral impact of the strain to the mosquito

365 vector. However, high *Wolbachia* density which also possesses strong inhibitory effects

366 against insect viruses had been observed from natural *Wolbachia* strains with a long-term

367 association from its host[66,67]. The last consideration is the low infection rate. It raises the

368 question, more importantly to the population replacement approach, if any of the local

369 *Wolbachia* strains could be sustained for an extended period or possess the ability to infect

370 the mosquito population thoroughly. Studies had suggested that a successful strain used in

371 population replacement or invasion should reach an infection rate of >90% and should remain

372 at this rate over an extended period of time[68-70]. Thus, utmost consideration in the infection

373 status of *Wolbachia* and its role in *Ae. aegypti* is necessary for a *Wolbachia*-based vector

374 control program to be successful, efficient and, as well as, effective.

375 **ACKNOWLEDGEMENTS**

AUTHOR CONTRIBUTIONS

T.M.C., D.M.A. and K.W. designed the experiments. T.M.C., K.H., and R.K.H. performed the experiments. T.M.C., K.H., and R.K.H. performed the sequencing while T.M.C., K.W. and D.M.A. accomplished the phylogenetic analysis. T.M.C. wrote the manuscript along with D.M.A and K.W. All authors reviewed the manuscript and approved on its submission.

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601 **COMPETING INTEREST**

602 The authors declare no competing interest

DATA AVAILABILITY

Demographic profiles (location and sex) and detection status from each individual *Ae. aegypti* adult mosquito used in the study are presented in the Supplementary. Accession numbers of Nucleotide sequences of PCR-amplified fragments of *wsp* and *16S* have been deposited in the GENBANK nucleotide database under accession numbers _____to _____ and _____ to _____ respectively.

SUPPLMENTAL MATERIAL

Table S1. Demographic profile (Sex, Sampling Site Code, Location), Detection status (*wsp* and 16S rDNA) of all individual adult *Aedes aegypti* mosquitoes used in the study. Positive *Wolbachia* infection in mosquito samples presents the supergroup classification and GENBANK accession number.

Figure S1. Complete *wsp* phylogeny of *Wolbachia* from *Ae. aegypti* (n=113). The alignment was analyzed in the program PHYML and *Wolbachia* host *Dirofilaria immitis* was selected as an outgroup. All sample sequences are indicated in red dots. The condensed version of this tree is presented as Figure 1.

Figure S2. Complete *16S* rDNA phylogeny of *Wolbachia* from *Ae. aegypti* (n=85). The alignment was analyzed in the program PHYML and *Rickettsia sp.* was selected as an outgroup. All sample sequences are indicated in red dots. The condensed version of this tree is presented as Figure 2