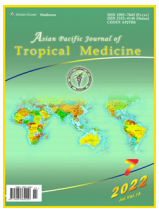

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Mosquito larva distribution and natural *Wolbachia* infection in campus areas of Nakhon Ratchasima, Thailand

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ABSTRACT

Objective: To determine the prevalence of mosquito larvae in campus areas and the infection rate of endosymbiotic bacteria, *Wolbachia* in mosquito larvae.

Method: The mosquito larvae samples were collected in residential areas and academic buildings of Suranaree University of Technology located in Northeastern Thailand during 2017-2018. Mosquito species identification was performed using GLOBE mosquito protocols and Rattanarithikul & Panthusiri's keys. The gene encoding for the surface protein of *Wolbachia* was amplified by PCR and confirmed by DNA sequencing.

Results: *Armigeres* sp. is the highest proportion of mosquito larvae followed by *Culex* spp., *Aedes albopictus*, *Aedes aegypti*, and *Toxorynchites* spp., respectively. *Aedes aegypti* have breeding sites mostly in the containers found indoors, whereas the main breeding sites of *Aedes albopictus* were found in both outdoors and indoors. The House Index and Breteau Index for *Aedes* spp. was more than 5% and 20%, respectively, in both areas, indicating that these areas are dengue sensitive. The highest proportion of *Wolbachia* infection was found in the larvae of *Culex* spp. (86.21%), followed by *Aedes albopictus* (69.23%) and rarely detected in *Aedes aegypti* (9.09%).

Conclusion: The present study reported the first natural infection of *Wolbachia* in mosquito larvae in Thailand. Our result suggested that the mosquito species containing higher proportion of *Wolbachia* are less likely to be vectors for dengue. Therefore, *Wolbachia* transfection in mosquito larvae could be applied as a biocontrol for dengue and other mosquito-borne disease prevention.

KEYWORDS: Mosquito larvae; *Wolbachia*; Breeding sites; House Index; Breteau Index; Campus area; Dengue

1. Introduction

Mosquitoes are extensively distributed worldwide, especially throughout the tropics and temperate regions[1,2]. Mosquitoes are natural vectors that transmit pathogens to infect humans and cause several diseases such as dengue fever, malaria, chikungunya, filariasis, and Japanese encephalitis[3]. The medical important mosquito species belong to the subfamilies of Culicinae (including genera *Aedes*, *Armigeres*, *Culex*, *Haemagogus*, *Mansonia*, *Psorophora*, *Sabethes*, and *Toxorhynchites*), and Anophelinae (includes genus *Anopheles*) which composes of more than 3 000 species[4]. From all mentioned genera, *Aedes* is of most concern because of their

Significance

This study revealed mosquito larvae habitats and the prevalence of mosquito species in the campus areas of Nakhon Ratchasima province. In Thailand, there have been reports on *Wolbachia* infections in insects and mosquito adults but there is no report on *Wolbachia* infection in mosquito larvae. Our study is the first report on *Wolbachia* infection rate in mosquito larvae which has never been reported in Thailand.

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distribution and transmission of many intractable pathogenic organisms. *Aedes* (*Ae.*) *aegypti* is the most important species that transmits dengue, zika, and yellow fever viruses and filaroid helminths worldwide[3]. *Ae. albopictus* is a native mosquito species in tropical and subtropical areas, especially in Southeast Asia that also serves as a vector of dengue fever, yellow fever, and chikungunya[5,6]. Every year, around 390 million dengue infections have been reported worldwide[7] with 96 million of them presented clinical manifestations and 70% was reported in Asia[8]. In addition to *Aedes*, other mosquitoes species are also important, as they act as vectors for many infectious diseases. *Culex* is another Culicinae mosquito that transmits several diseases such as lymphatic filariasis and Japanese encephalitis, while *Mansonia* and *Armigeres* are the vectors of nematode parasites that cause lymphatic filariasis[9]. Apart from the pathogenic vector, *Toxorhynchites* is a beneficial biocontrol since they are predaceous on other mosquito larvae found in the same areas[10].

In the absence of effective vaccines or prophylactic agents against most of the arboviruses and vector-borne parasites, current efforts are mainly based on controlling vector populations by eliminating breeding sites, killing mosquito larvae, and treating with outdoor insecticides or repellents. However, chemical-based control methods may lead to the development of mosquito resistance, as well as environmental contamination and side effects on non-target organisms[11]. Therefore, the safest ways to control the disease are either eliminating breeding sites or using biocontrol methods. Natural mosquito breeding sites could be different among the mosquito species, leading to the risks of each region depending on the characteristics of breeding site containers. Consequently, alternative and innovative vector control strategies have emerged, and one of the most promising methods is based on the use of endosymbiotic bacteria, *Wolbachia*[12–15]. *Wolbachia* has been one of the most studied biocontrol for arboviruses and parasite transmission control. This approach involves the release of mosquitoes transinfected with the vertically transmitted *Wolbachia*, which can suppress arbovirus replication in mosquitoes, so it can be a potentially promising means for controlling dengue transmission in endemic settings[6,16–19].

Thailand is an endemic area for dengue fever with more than 60 000 cases in 2019. In the same year, more than 10 000 cases were reported in Nakhon Ratchasima and surrounding provinces, indicating this area is one of the highest endemic regions of the country[20]. Previous studies have reported that most cases of dengue fever patients were children and teenagers[21,22]. Therefore, we have been interested in studying the distribution of mosquito species and the occurrence of *Wolbachia* in Nakhon Ratchasima province where more than 10 000 students reside. There have been a few studies on the distribution of *Wolbachia* in Thailand but most of them focused on *Wolbachia* in adult insects or mosquitoes[23–27]. Our research aims to study the distribution and breeding sites of mosquito species collected in 2017 and 2018 as well as detect the presence of endosymbiotic bacteria *Wolbachia* from the collected mosquito larvae.

2. Materials and methods

2.1. Study sites

Mosquito larval survey was conducted in two different study sites (residential areas and academic buildings) from August 2017 to November 2018 at Suranaree University of Technology, Nakhon Ratchasima located in Northeastern Thailand (14.881 8° N, 102.020 7° E) where there are natural forests, ponds, and constructed buildings which has an area of 11.2 km^2. Mosquito larvae samples were collected from 13 and 17 buildings from the residential areas and academic buildings, respectively.

2.2. Entomological studies

Larval surveys were conducted in both study areas by using an 11.5 cm diameter fishnet. Mosquito breeding sites were sampled in both indoors and outdoors within 15 meters of the households as suggested by Wongkoon[28]. All breeding larvae found in small containers were filtered through the fishnet into the buckets. The ones in large containers were sampled by dipping the fishnet in the water, starting a swirling motion, and sampling all edges of the containers[29]. All breeding sources of mosquitos were grouped into 13 different container types: flower plastic vases (FPV), flower glass vases (FGV), flower ceramic vases (FCV), plastic tank (PT), plant water pot (PWP), bowl (BO), small earthen jars (SEJ), cement tank (CT), paint bucket (PB), pottery vases (PV), waste containers (WC), coconut shells (CS), and others (OT).

All 5 472 live mosquito larvae were collected in plastic bags and brought to the laboratory for species identification by using GLOBE mosquito protocols[30] and, Rattanarithikul & Panthusiri's keys[31]. After identification, all mosquito larvae samples were fixed in 70% ethanol and stored in the freezer (-80 ℃) for DNA extraction. The number of the larvae was counted and calculated for three larval indices: House Index (HI), Container Index (CI), and Breteau Index (BI) according to the standard WHO guidelines on dengue control (vector surveillance). The BI and HI are commonly used for determination of priority (risk) areas for control measures. The HI and BI of greater than 5% and 20%, respectively, for any locality is indicated that these areas are dengue-sensitive, suggesting a high risk of dengue virus distribution[32].

Table 1. List of the primers used for *wsp* gene, *16s* rDNA, and *28s* rDNA amplification.

Organisms	Primer names	Primer sequences (5´ to 3´)	Expected size (bp)
Universal bacteria[33,34]	*16s* rDNA R	CATACCTATTCGAAGGGATAG	438
	16s rDNA F	AGCTTCGAGTGAAACCAATTC	
Eukaryote cell[33,34]	*28s* rDNA F	TACCGTGAGGGAAAGTTGAAA	443
	28s rDNA R	AGACTCCTTGGTCCGTGTTT	
Wolbachia[47]	*wsp* 81F	TGGTCCAATAAGTGATGAAGAAAC	590-632
	wsp 691R	AAAAATTAAACGCTACTCCA	

Table 2. Different mosquito larval species found in a variety of the water containers.

Container	*Aedes aegypti* (*n*=661)	*Aedes albopictus* (*n*=1 178)	*Culex* spp. (*n*=1 582)	*Armigeres* sp. (*n*=2 043)	*Toxorhynchites* spp. (*n*=8)
FPV	458 (69.29%)	807 (68.51%)	140 (8.85%)	0 (0%)	0 (0%)
PWP	21 (3.18%)	209 (17.74%)	106 (6.70%)	0 (0%)	0 (0%)
PT	100 (15.13%)	14 (1.19%)	98 (6.19%)	3 (0.15%)	0 (0%)
SEJ	1 (0.15%)	0 (0%)	377 (23.83%)	2 (0.10%)	1 (12.50%)
PB	0 (0%)	2 (0.17%)	64 (4.05%)	0 (0%)	5 (62.50%)
CT	0 (0%)	0 (0%)	139 (8.79%)	2 030 (99.36%)	0 (0%)
WC	0 (0%)	0 (0%)	286 (18.08%)	6 (0.29%)	0 (0%)
PV	68 (10.29%)	17 (1.44%)	0 (0%)	0 (0%)	0 (0%)
Others	13 (1.96%)	129 (10.95%)	372 (23.14%)	2 (0.09%)	2 (25.00%)

FPV: flower plastic vases; PWP: plant water pot; PT: plastic tank; SEJ: small earthen jars; PB: paint bucket; CT: cement tank; WC: waste containers; PV: pottery vases; OT: others.

2.3. DNA extraction

The mosquito larvae from 57 containers collected from different areas covering different types of containers were chosen for DNA extraction. The larval samples were homogenized in liquid nitrogen and DNA were then extracted according to the manufacturing by using HiPurA™ Multi-Sample DNA Purification Kit (Himedia, India). The DNA concentration was quantitated *via* NanoDrop™ 2000/2000c spectrophotometers (Thermo Fisher Scientific, USA) before proceeding to polymerase chain reaction (PCR) amplification.

2.4. PCR amplification and DNA sequencing

The larvae samples collected from each location were screened for the presence of *Wolbachia* by PCR amplification as previously described[33,34]. The gene encoding for *Wolbachia* surface protein was amplified with the *wsp* 81F and *wsp* 691R primers. The primer sequences used in this study are shown in Table 1. PCR was conducted in a 25 μL reaction volume using (KOD One™, Toyobo, Japan). The PCR was carried out on C1000 Touch™ Thermal Cycler (Bio-Rad, USA) with the appropriate condition including a pre-denaturation for 5 min at 95 ℃, followed by 35 cycles of denaturation for 30 s at 95 ℃, annealing for 45 s at 55 ℃, and extension for 90 s at 72 ℃, and a final extension for 10 min at 72 ℃. All genomic DNA samples used for *wsp* gene detection were also amplified for *16s* rDNA and *28s* rDNA gene sequence as a positive control for the presence of bacterial and eukaryotic (mosquito) genomic DNA, respectively. The PCR products received from *16s* rDNA detection appeared at 438 bp, whereas *28s* rDNA was at 443 bp in size, and the PCR products of *wsp* gene were ranged in 590-632 bp. These PCR products were proceeded for DNA sequencing (Biobasic, Canada) for *wsp* gene confirmation.

2.5. Bioinformatics analysis

The sequence alignment was generated using 4 peaks program (B.V. Gerberastraat, the Netherlands). Each sequence was checked and edited manually for *16s* rDNA. The modified DNA sequence was submitted to the BLASTN on NCBI database, whereas the reverse DNA sequence was converted to reverse complement sequence before DNA sequencing analysis.

3. Results

3.1. Mosquito breeding sites

The association of the mosquito breeding sites, and the study areas was investigated. The breeding sites in the academic buildings from the most to least were FPV (63.50%, 87/137), PT (8.03%, 11/137), PWP (7.30%, 10/137), OT (5.84%, 8/137), CT (4.38%, 6/137), PV (2.92%, 4/137), SEJ (2.19%, 3/137), PB (2.19%, 3/137), FCV (2.19%, 3/137), FGV (0.73%, 1/137), and WC (0.73%, 1/137), respectively. The breeding sites in the residential areas were PWP (33.33%, 16/48), SEJ (25.00%, 12/48), FPV (22.92%, 11/48), PT (6.25%, 3/48), CS (4.17%, 2/48), FCV (4.17 %, 2/48), PV (2.08%, 1/48), and BO (2.08%, 1/48) respectively. These data revealed that FPV was the main breeding site of mosquitos in both academic buildings and residential areas.

Table 3. The number of households, containers, and larval indices of *Aedes* species during 2017 and 2018.

Year of investigation	2017		2018	
	Aedes aegypti	*Aedes albopictus*	*Aedes aegypti*	*Aedes albopictus*
No. of households	30	30	25	25
No. of positive households	8	11	10	15
No. of containers	1 262	1 262	926	926
No. of positive containers	24	29	65	96
House index (%)	26.67	36.67	40.00	60.00
Container index (%)	1.90	2.30	7.02	10.37
Breteau index (%)	80.00	96.67	260.00	384.00

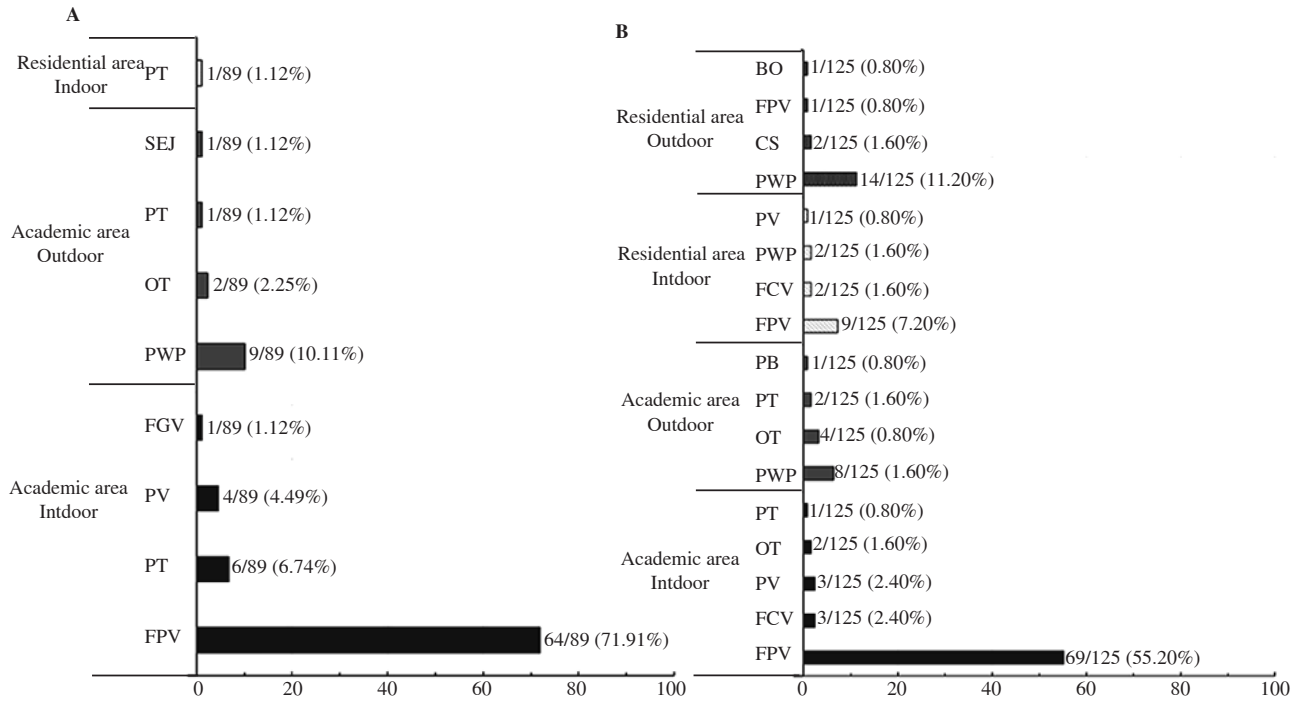

Figure 1. The breeding site distribution of *Aedes* spp. A: the breeding site distribution of *Aedes aegypti*. B: the breeding site distribution of *Aedes albopictus*. FPV: flower plastic vases; PWP: plant water pot; PT: plastic tank; SEJ: small earthen jars; PB: paint bucket; CT: cement tank; WC: waste containers; PV: pottery vases; OT: others.

3.2. The prevalence of mosquito larval species

A total of 5 472 mosquito larvae were collected. Five species were identified in which the abundance from the most to least were *Armigeres* sp. (37.34%, 2 043/5 472), *Culex* spp. (28.91%, 1 582/5 472), *Ae. albopictus* (21.53%, 1 178/5 472), *Ae. aegypti* (12.08%, 661/5 472), and *Toxorhynchites* spp. (0.15%, 8/5 472), respectively.

3.3. The breeding sites specific for each mosquito species

The different mosquito larval species were found in both academic buildings and residential areas in a variety of the water containers as shown in Table 2. The three major breeding sites of *Ae. aegypti* were FPV, PT, and PV. *Ae. albopictus* were also found in both academic buildings and residential areas, and mostly in FPV and PWP whereas *Culex* spp. were randomly distributed in a variety of containers (SEJ, WC, FPV, CT, PWP, PT, PB, and OT). *Armigeres* sp. is the most abundant mosquito larvae found mostly in cement tank. *Toxorhynchites* spp. were rarely seen in all containers.

3.4. Breeding sites for dengue virus vectors

Breeding sites of *Aedes* spp. as the major vectors of the dengue fever illustrated that, *Ae. aegypti* were mainly distributed in academic buildings in which FPV was the most abundant breeding site found indoors and the second most abundant was PWP found outdoors (Figure 1A). *Ae. albopictus* have the major breeding sites in the containers found in FPV located in indoors of the academic buildings (Figure 1B), while *Ae. albopictus* were distributed mainly in both indoors (FPV) and outdoors in the residential areas (PWP).

3.5. Larval indices for dengue fever risk indication

Our results reflected that in the period of 2017, the HI and BI for

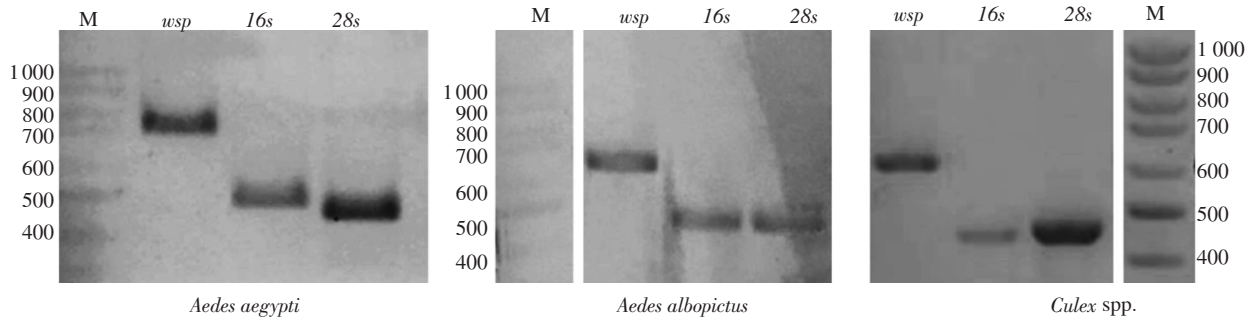

Figure 2. PCR amplification of *wsp* gene, *16s* rDNA, and *28s* rDNA from 57 larvae samples. Agarose gel electrophoresis of PCR amplicons showing detection of *Wolbachia* in *Aedes* spp. and *Culex* spp. in which the expected band of *wsp* gene ranged from 590 to 632 bp, *16s* rDNA was 438 bp, and *28s* rDNA was 443 bp.

Ae. aegypti were 26.67% and 80.00%, respectively, and that for *Ae. albopictus* were 36.67% and 96.67%, respectively. In the period of 2018, the HI and BI for *Ae. aegypti* were 40.00% and 260.00%, respectively and that for *Ae. albopictus* were 60.00% and 384.00%, respectively (Table 3).

3.6. Prevalence of Wolbachia infection in different mosquito larval species

In this study, *wsp* gene which encodes for the *Wolbachia* surface protein was detected from DNA extracted from the mosquito larvae samples performed by PCR. The results showed that the *wsp* gene presented in *Ae. aegypti*, *Ae. albopictus*, and *Culex* spp. ranged in 590-632 bp in size, indicating that *Wolbachia* resided in some of these mosquito larvae (Figure 2). Confirmation of *wsp* gene by sequence-based analysis showed high-scoring alignments (more than 200) and 100% identity with *wsp* gene of *Wolbachia* endosymbiont resided in *Aedes* spp. and *Culex* spp. The abundance of *Wolbachia* in our study revealed that 61.40% of total mosquito larvae were infected with *Wolbachia*. The highest proportion of *Wolbachia* was seen in the larvae of *Culex* spp. (86.21%) when compared to other mosquito larvae, followed by *Ae. albopictus* (69.23%) and rarely found in *Ae. aegypti* (9.09%) (Table 4). *Wolbachia* infection in *Toxorynchites* spp. was not detected.

Table 4. Prevalence of *Wolbachia* infection in different mosquito larval species.

Species	No. of *Wolbachia* positive containers/No. of tested containers (% *Wolbachia* infection)	Proportion of *Wolbachia* infected among all species (%)
Aedes aegypti	1/11 (9.09)	5.53
Aedes albopictus	9/13 (69.23)	42.08
Culex spp.	25/29 (86.21)	52.40
Toxorynchites spp.	0/4 (0.00)	-
Total	35/57 (61.40)	100.00

4. Discussion

Climate change could bring mosquito-borne diseases to the areas where these diseases had previously not seen. Every year, there is a fluctuation of climate and weather that could lead to the evolution or shifts of mosquito species. Some of the mosquito species including *Ae. aegypti* and *Ae. albopictus* are the main vectors for dengue transmission. The high-risk population during primary infection of dengue fever is found in the patient age ranged in 9-20 years old especially children in tropical and subtropical areas who have high chances to expose to these mosquito species[21,22,35].

In our study, we established the distribution of mosquito larvae in containers found in residential areas and academic buildings of Suranaree University of Technology to investigate the association between the areas and container types which could reflect the water conditions whether they are favored for mosquito larvae's survival and breeding. The FPV was the main mosquito breeding site in the academic building areas and the PWP followed by the FPV was the main spots in the residential areas. Mosquitoes that preferred to breed *via* FPV might be due to stagnant water. Previous studies elsewhere have shown faster rates of mosquito evolution when temperature and CO_2 level were higher. In Southeast Asia, *Ae. aegypti* is the main vector for dengue virus breeds in stagnant water and commonly found indoors, while *Ae. albopictus* is commonly found outdoors that could be a result of environmental adaptation[36,37]. However, our study showed that indoors were the main breeding sites for both *Ae. aegypti* and *Ae. albopictus*.

We also reported that *Armigeres* sp. was the most prevalent mosquito species in the campus areas of Suranaree University of Technology. This species could lead to the high infection risk of lymphatic filariasis in these areas. The high breeding rate of this species might be a result of being well-adapted to live in any clogged waterways such as CT and other natural habitats. *Culex* spp., *Ae. albopictus*, and *Ae. aegypti* were also widely distributed in the campus areas which brought the most concern of many

mosquitos-borne diseases including dengue fever. In addition, our result indicated that the period from 2017 to 2018 are dengue-sensitive determined by the HI and BI which were greater than 5% and 20%, respectively. The results reflected that the HI and BI are related with dengue endemic in our study areas, which is agreed with Preechaporn *et al.* who found the *Aedes* larvae in the highest proportion in three topographical areas (mangrove, rice paddy and mountains)[38].

Wolbachia are distributed ranging from 40%-70% in all types of insects[39–41] including butterflies, bees, beetles, and some mosquito species worldwide. *Wolbachia* have been found to mediate dengue virus interference depending on several factors such as elevation of the basal immunity and increase in longevity of mosquitoes[42]. *Wolbachia* alone was found to be able to inhibit viral replication, dissemination, and transmission in transinfected *Ae. aegypti* in experimental studies. Based on the evidence from Cardona-Salgado *et al.*, *Wolbachia* found in *Ae. albopictus* did not affect the replication of dengue virus but was able to reduce the viral infection of mosquito salivary glands and limit viral transmission[43].

Previous studies from Kittayapong *et al*. reported that *Wolbachia* have been found to occur naturally in *Ae. albopictus*[24] but not in *Ae. aegypti* which is the main vector of the dengue virus. Another study on *Wolbachia* distribution in *Ae. albopictus* conducted in Malaysia showed *Wolbachia* infection rate ranging from 60% to 100%[44] and a study of the distribution of *Ae. albopictus* collected from different locations in Peninsular Malaysia reported that *Wolbachia* infection was widespread in *Ae. albopictus* population, both in female and male mosquitos[35]. There is evidence of vertical transmission of *Wolbachia* from mother to offspring of *Ae. albopictus* population[24]. Another study has shown for the first time that *Wolbachia* is present in *Ae. albopictus* and *Ae. aegypti* larvae from Kuala Lumpur, Malaysia. In Thailand, although there are some studies on *Wolbachia* distribution in insects and mosquito adults, but there have been no studies to date on *Wolbachia* in mosquito larvae[24,25,27]. To the best of our knowledge, this study is the first on the detection of *Wolbachia* in the larvae in Thailand.

Our study showed that the *wsp* gene existed in *Ae. aegypti*, *Ae. albopictus*, and *Culex* spp., indicating that *Wolbachia* resided in some of these mosquito larvae. This study revealed that 61.40% of total mosquito larvae were infected with *Wolbachia*. The highest proportion of *Wolbachia* infection was seen in the larvae of *Culex* spp. and the infection rate was found in *Ae. albopictus* more than *Ae. aegypti*. No detection of *Wolbachia* was found in *Toxorynchites* spp. These observations indicated that *Toxorynchites* mosquito larvae may be either physiologically unable to support *Wolbachia* infection or seldom encounter *Wolbachia* horizontal transmission events. However, larger numbers of the samples in these groups may be required.

Wolbachia did not affect the replication of dengue virus in *Ae. albopictus* but was able to reduce the viral infection in the mosquito salivary glands and therefore limit viral transmission, suggesting the role of *Wolbachia* in naturally restricting the transmission of dengue virus in *Ae. albopictus*[45]. Therefore, scientists have attempted to transinfect *Wolbachia* into *Ae. aegypti* and release these mosquitos containing the endosymbiont *Wolbachia* to the field that would be beneficial for control of dengue fever and other vector-borne diseases[46]. Moreover, there is no evidence on the harm of *Wolbachia* to human, animals, or the environment. A previous study showed that *Wolbachia* bacteria did not cause diseases in people or animals (for example, fish, birds, cats, and dogs)[42].

The limitation of our study was that we did not detect *wsp* gene in all larvae samples. We detected approximately 36% from all larvae samples. Therefore, there might be some incomplete data represented in this report. Another limitation was that we did not submit for an ethic approval for animal (mosquito). Therefore, this is our flaw about performing this project.

In conclusion, the campus areas of Suranaree University of Technology located in Northeast of Thailand was found to be at high risk of endemic mosquito-borne diseases, especially dengue fever, with the higher risk found in indoors rather than outdoors of academic buildings. This is the first study on the distribution of endosymbiont bacteria, *Wolbachia* in mosquito larvae in Thailand that we found the highest proportion of *Wolbachia* in *Culex* spp. and *Ae. albopictus* but very few in *Ae. aegypti*. Therefore, transfection of *Wolbachia* in mosquito larvae as a purpose of suppression of viral transmission could be used as a potential strategy for a biocontrol of mosquito-borne diseases in the future.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Authors' contributions

TS performed the experiments and data analysis, reviewed final version to be published. SC developed concept, designed experimental studies, and performed the experiments. PM performed data analysis, wrote manuscript, and designed data visualization, and revised the manuscript. PC performed data analysis and designed data visualization. MJ developed concept, designed experimental studies, wrote and edited manuscript, reviewed final version to be published. All authors read and approved the manuscript.

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