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ORIGINAL PAPER

Distribution of mosquito (Diptera: Culicidae) species and *Wolbachia* (Rickettsiales: Rickettsiaceae) infections during the bird immigration season in Pathumthani province, central Thailand

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Abstract Mosquito distribution in the immigration bird-nested area, Pathumthani province, was investigated from August to December in 2006. Mosquitoes were collected by using CO_2-baited Centers for Disease Control light traps in which dry ice was used as a source of CO_2 to attract mosquitoes. Six traps were operated from 4 p.m. until 7 a.m. on each study day. Four genera, which were *Anopheles*, *Armigeres*, *Culex*, and *Mansonia* with 14 species of mosquitoes were collected. *Culex gelidus* (13.94–59.41%) and *Culex tritaeniorhynchus* (32.87–70.30%) were most collected species in this area for every month. Other two species with moderate distribution in this area were *Anopheles barbirostris* (0.76–3.30%) and *Mansonia uniformis* (1.55–11.36%). Polymerase Chain Reactions were performed for testing *Wolbachia* infection in *Cx. gelidus* and *Cx. tritaeniorhynchus* only. Fifty-four percent (15/28 pools) of *Cx. gelidus* and none (0/20 pools) of *Cx. tritaeniorhynchus* were positive for *Wolbachia* infection. *Wolbachia* infection in other mosquito species collected in this and other areas need to be investigated to understand species and geographic variation of *Wolbachia* infection in mosquitoes in nature.

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Introduction

Thailand is one of the tropical countries that have many places for immigration birds to escape from cold weather during the winter season. Immigrated birds can be found in the North, Northeast, South, and also central of Thailand, approximately from November to May every year. Each area usually has different majority of immigration bird species. These areas eventually become popular places for bird watchers. However, since emerging and re-emerging diseases are more concerned in every country including Thailand, therefore immigration birds are concerned as reservoir hosts for infectious agents (Liu et al. 2004; Gilbert et al. 2006; Jourdain et al. 2007a, b). These birds might carry some pathogens with them during the immigration season to Thailand, particularly pathogens that cause vector borne diseases (Ratho et al. 1999; Paramasivan et al. 2003; Lahariya and Pradhan 2006). These pathogens can be dispersed rapidly by blood-sucking insects including mosquitoes.

Phailom temple is one of the famous places for Asian or white open-billed storks (*Ciconia ciconia*) to emigrate from India to Thailand during the immigration season. This temple is located on the side of Chao Phraya River in Pathumthani province, central Thailand. This province is a neighboring province of Bangkok which is only 46 km

from Bangkok. Since this area is located closely to Bangkok, it is concerned that vector borne pathogens can reach Bangkok in a short period of time.

Wolbachia are maternally inherited bacterial symbionts found in many species of insects, isopods, mites, and nematodes. These bacteria live inside the reproductive cells and cause reproductive alterations in their host, including cytoplasmic incompatibility, parthenogenesis induction, and feminization of genetic males, which result in few or no offspring (O'Neill and Karr 1990; Rousset et al. 1992; Stouthamer et al. 1993). Previous study by Kittayapong et al. (2000) indicated *Wolbachia* infections in Thailand mosquitoes including some species in genus *Aedes*, *Armigeres*, *Coquillettidia*, *Culex*, *Hodgesia, Mansonia*, *Tripteroides*, and *Uranotaenia*. However, percentage infection with *Wolbachia* within species did not point out.

This study was conducted to investigate the distribution of mosquito species in the nested area of immigrated white, open-billed storks, and also *Wolbachia* infection in some selected mosquitoes collected in this area. This study data would be useful for the future works on epidemiological study, prevention, and control of emerging diseases from immigration birds in Thailand.

Materials and methods

Collected mosquitoes

Mosquitoes were collected from the Phailom temple (14° 14′35″ N, 100°31′35″ E), Pathumthani province, Thailand from August to December, 2006, using CO_2-baited Centers for Disease Control (CDC) light traps. Dry ice was used as a source of CO_2 to attract mosquitoes. They were collected one time in August, and at the first and third week in October, November, and December. Mosquitoes were transferred to the Parasitology Laboratory, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Mosquito species were identified using morphological characteristics (Rattanarithikul and Panthusiri 1994). Detection of *Wolbachia* in *Culex gelidus* and *Culex tritaeniorhynchus* female mosquitoes were performed by using Polymerase Chain Reaction (PCR).

Laboratory rearing mosquitoes

Thailand strain *Aedes albopictus* from laboratory rearing were used as a positive control for the *Wolbachia* detection (Kitrayapong et al. 2002). These mosquito eggs were kindly provided by Dr. Padet Siriyasatien, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Mosquito DNA extraction

Each mosquito species was pooled as a group of ten mosquitoes. DNA was extracted from each mosquito pool using DNeasy® Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's recommendation with slightly modification. Briefly, pool of mosquitoes was homogenized with 180 µl of Dulbecco's modified Eagle's medium (GIBCO®, Invitrogen Corp., Carlsbad, CA, USA) by using a disposable microtube pestle. Twenty microliters of proteinase K and 200 µl of lysis buffer were added, mixed thoroughly, and incubated at 56°C for 10 min. Two hundred microliters of absolute ethanol was added to the sample, mixed thoroughly, and transferred into the DNeasy Mini spin column. The column then was centrifuged and washed twice with washing buffer. One hundred microliters of elution buffer was added into the column at the final step to elute the DNA. DNA samples were kept at −20°C until tested.

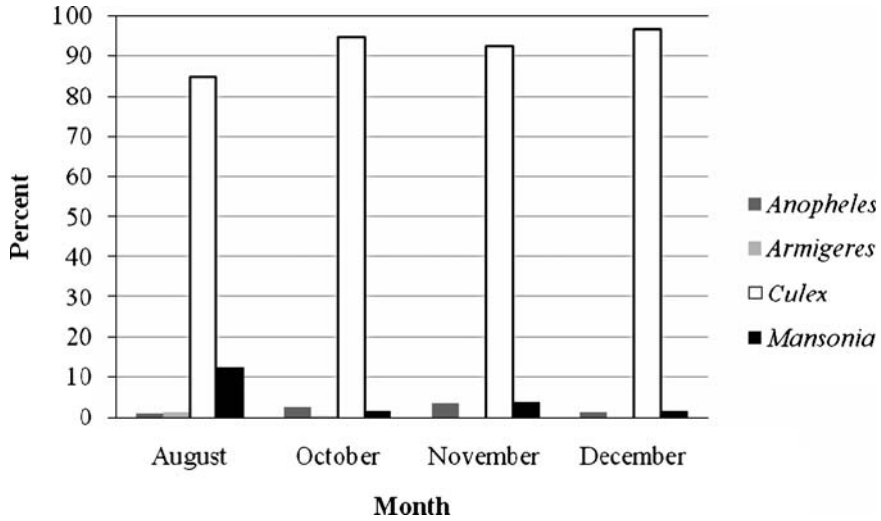
Wolbachia detection

Two diagnostic PCRs were performed to amplify a fragment of the 16s ribosomal DNA (rDNA) gene of *Wolbachia* and a fragment of the 28s rDNA gene of mosquitoes (Werren et al. 1995; Werren and Windsor 2000). The 16s rDNA *Wolbachia* primers, which were forward (5′CAT ACC TAT TCG AAG GGA TAG) and reverse (5′AGC TTC GAG TGA AAC CAA TTC) were used in this study. PCR cycling conditions were 15 min at 95°C for the initial activation step followed by 38 cycles of 30 sec at 94°C (denaturation), 45 sec at 55°C (annealing), 90 sec at 72°C (extension) and 10 min at 72°C for the final extension.

The 28s rDNA universal arthropod primers were used as control for PCR. The primers were forward (5′TAC CGT GAG GGA AAG TTG AAA) and reverse (5′AGA CTC CTT GGT CCG TGT TT). PCR cycling conditions were 15 min at 95°C for the initial activation step followed by 38 cycles of 30 sec at 94°C (denaturation), 50 sec at 58°C (annealing), 90 sec at 72°C (extension) and 10 min at 72°C for the final extension.

PCRs were performed in 25 µl-reaction using Hot Star Taq DNA polymerase (QIAGEN, Valencia, CA, USA). PCR reaction is composed of 2.5 µl of 10× buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7), 1 unit of Taq DNA polymerase, 100 µM Deoxynucleotide Solution Mix (dNTP; New England Biolabs Inc., Ipswich, MA, USA), 300 µM of forward primer, 300 µM of reverse primer, 17.3 µl of ultra pure water (Invitrogen Corp., Carlsbad, CA, USA), and 2 µl of DNA template. PCR product was mixed with loading buffer (10X BlueJuice™, Invitrogen Corp., Carlsbad, CA, USA), analyzed in 1.2% agarose gel

Table 1 Percentage of mosquito genera distribution in the immigration bird-nested area at Phailom temple, Pathumthani province, central Thailand during August–December 2006



(Ultrapure™ Agarose, Invitrogen Corp., Carlsbad, CA, USA) in TAE buffer, and stained with SYBR safe™ DNA gel staining (Invitrogen Corp., Carlsbad, CA, USA; 20 µl per 150 ml of gel).

If *Wolbachia* 16s rDNA was positive, the sample was scored as a positive for *Wolbachia* infection, and if *Wolbachia* 16s rDNA was negative and arthropod 28s rDNA was positive, the sample was scored as a negative for *Wolbachia* infection. If *Wolbachia* 16s rDNA was negative and arthropod 28s rDNA was negative, then the DNA concentration in the PCR reaction would be adjusted. If it was still yield a negative result for the 28s rDNA, then the sample was discarded from the analysis.

Results

Mosquito identification

A total of 30,112 mosquitoes were collected. There were four genera of mosquitoes identified, which were *Anopheles*, *Armigeres*, *Culex*, and *Mansonia* (1.11–3.80%, 0.01–1.47%, 84.94–96.70%, and 1.66–12.48%, respectively) with total of 14 species (Tables 1 and 2). *Cx. gelidus* and *Cx. tritaeniorhynchus* were most collected species in this area for every month (13.94–59.41% and 32.87–70.30%, respectively). Other two species with moderate distribution in this area were *Anopheles barbirostris* and *Mansonia*

Table 2 Percentage of mosquito species distribution in the immigration bird-nested area at Phailom temple, Pathumthani province, central Thailand during August–December 2006

Species	Percent				
	August	October	November	December	Average
Anopheles barbirostris	0.76	1.66	3.30	1.45	2.36
Anopheles peditaeniatus	0.35	0.68	0.11	0.12	0.24
Anopheles stephensi	0	0	0.02	0	0.01
Anopheles sundaicus	0	0.22	0.35	0.03	0.22
Anopheles tessellates	0	0.15	0.01	0.04	0.05
Armigeres subalbatus	1.46	0.46	0.01	0.01	0.18
Culex bitaeniorhynchus	0	0	0.01	0	0.01
Culex fuscocephala	0.29	0	0.01	0	0.02
Culex gelidus	13.94	53.51	59.41	49.15	53.03
Culex quinquefasciatus	0.41	0	0.01	0	0.03
Culex tritaeniorhynchus	70.30	41.39	32.87	47.54	40.46
Mansonia annulifera	0.12	0.02	0	0	0.01
Mansonia indiana	1.00	0.14	0.39	0.12	0.31
Mansonia uniformis	11.36	1.78	3.49	1.55	3.10

uniformis (0.76–3.30% and 1.55–11.36%, respectively). *Cx. tritaeniorhynchus* were the most collected species in August (70.30%) and *Cx. gelidus* were the most collected species in October, November, and December (53.51, 59.41, and 49.15%, respectively).

Detection of *Wolbachia* by PCR

PCRs were performed for testing *Wolbachia* infection in *Cx. gelidus* and *Cx. tritaeniorhynchus* only since they were most collected species. The 16s rDNA *Wolbachia* PCR product had 438 base pairs (bps) and the 28s rDNA universal arthropod PCR product had 443 bps. Twenty eight pools of *Cx. gelidus* and 20 pools *Cx. tritaeniorhynchus* with 10 mosquitoes each were tested and there were 15 pools (54%) of *Cx. gelidus* were positive for *Wolbachia* however there was no *Wolbachia* infection in all *Cx. tritaeniorhynchus* tested pools.

Discussion

This study is the preliminary study on mosquito distribution at the white, open-billed stork-nested area, Phailom temple, Pathumthani province, central Thailand during the bird immigration season in 2006. Mosquitoes were collected before (August) and during (October–December) the bird immigration season. No mosquito was collected in September because of the flooding in this area in 2006. This place is located on the side of Chao Phraya River which is 46 km far from Bangkok. There are small ponds with water plants and high organic matters which are suitable bleeding places for *Culex* and *Mansonia* (Phan-Urai et al. 1976; Apiwathnasorn et al. 2006). This area is also surrounded by rice fields which are the bleeding places for *Anopheles* (Matthys et al. 2006). *Armigeres* is another mosquito genus found in this area but with a small number. However, no *Aedes* was collected in CDC light traps which may be because of their indoor and day time feeding habit (Scott et al. 2000). Normally, mosquito abundance in most areas in Thailand is high in monsoon (May–October), moderate in transition (March–April and November–December), and low in dry (January–February) seasons. This study, however, showed the number of collected mosquitoes increased significantly from August to October and November and eventually decreased in December.

Cx. gelidus and *Cx. tritaeniorhynchus* might play important roles in this area since they were most collected mosquitoes from this area. These mosquitoes play important roles in many arbovirus transmission cycles in nature particularly Japanese Encephalitis virus (Dirk Van Peenen et al. 1975). Blood meals from blood-fed mosquitoes in this area need to be studied and identified to confirm the role and relationship between species of mosquitoes and white, open-billed storks in this area. The study by Gingrich et al. (1987) showed 71–96% of all mosquitoes collected by CO_2-baited CDC light traps at three suburban sites of Bangkok from 1986 to 1987 were *Cx. tritaeniorhynchus* and *Cx. gelidus*, and showed Minimum Infection Rate (MIR per 1,000 mosquitoes) for Japanese Encephalitis virus in *Cx. gelidus* and *Cx. tritaeniorhynchus*, which were 0.47 and 0.17, respectively. The study by Tsuda et al. (1998) in northern Thailand with different rice fields also showed that Cx. *tritaeniorhynchus* and *Cx. gelidus* were dominant in all collected samples.

Wolbachia infection in mosquitoes caused reproductive alterations including cytoplasmic incompatibility (Werren 1997; Stouthamer et al. 1999; Jamnongluk et al. 2000). There were fifty-four percent (15/28 pools) of *Cx. gelidus* and none (0/20 pools) of *Cx. tritaeniorhynchus* were positive for *Wolbachia* infection in this study which similar to the study by Kittayapong et al. (2000), which indicated the *Wolbachia* infection in *Cx. gelidus* and no *Wolbachia* infection in *Cx. tritaeniorhynchus*. This present study, however, also showed percent infection in *Cx. gelidus* which never indicated in other previous studies.

Since *Wolbachia* infection causes few or no offspring in mosquitoes (Werren 1997), there is the considerable interest in using *Wolbachia* in biological control of mosquitoes to eradicate mosquito population or reduce the reproductive potential of mosquito population. However, the population of *Cx. gelidus* in this study area, which 54% of them were infected with *Wolbachia*, was still high when compared to the population of *Cx. tritaeniorhynchus*, which were free from *Wolbachia* infection. Cytoplasmic incompatibility in filed mosquito population may be not the main factor that plays a significant role in mosquito population in nature. Other factors, particularly ecology of mosquito habitat, might be the major influent in the mosquito population. *Wolbachia* infection in other mosquito species collected in this and other areas need to be investigated to understand species and geographic variation of *Wolbachia* infection in mosquitoes in nature. Arbovirus isolation from mosquitoes collected in this area and white, open-billed storks also need to be performed to point out the role of birds and mosquitoes in arbovirus transmission cycle in this area.

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Miscellaneous This study is the preliminary study on mosquito distribution at the white, open-billed stork-nested area, Phailom temple, Pathumthani province, central Thailand during the bird immigration season in 2006. No previous study on mosquito distribution at the immigration bird-nested area in Thailand was conducted. This study showed the distribution of mosquito species and

Wolbachia infection rates in *Cx. gelidus* and *Cx. tritaeniorhynchus*, the most collected species in this area. This information would be useful for the future works on epidemiological study, prevention, and control of emerging diseases from immigration birds in Thailand.

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