WOLBACHIA SUPERGROUPS A AND B IN NATURAL POPULATIONS OF MEDICALLY IMPORTANT FILTH FLIES (DIPTERA: MUSCIDAE, CALLIPHORIDAE, AND SARCOPHAGIDAE) IN THAILAND

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Abstract. Filth flies, belonging to suborder Brachycera (Family; Muscidae, Calliphoridae and Sarcophagidae), are a major cause of nuisance and able to transmit pathogens to humans and animals. These insects are distributed worldwide and their populations are increasing especially in sub-tropical and tropical areas. One strategy for controlling insects employs *Wolbachia*, which is a group of maternally inherited intracellular bacteria, found in many insect species. The bacteria can cause reproductive abnormalities in their hosts, such as cytoplasmic incompatibility, feminization, parthenogenesis, and male lethality. In this study we determined *Wolbachia* endosymbionts in natural population of medically important flies (42 females and 9 males) from several geographic regions of Thailand. *Wolbachia* supergroups A or B were detected in 7 of female flies using PCR specific for *wsp*. Sequence analysis of *wsp* showed variations between and within the *Wolbachia* supergroup. Phylogenetics demonstrated that *wsp* is able to diverge between *Wolbachia* supergroups A and B. These data should be useful in future *Wolbachia*-based programs of fly control.

Keywords: *Wolbachia*, fly control, medically important fly, *wsp*, Thailand

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INTRODUCTION

Filth flies are classified in suborder Brachycera (Family; Muscidae, Calliphoridae and Sarcophagidae). These files are commonly known as house fly, blow fly and flesh fly (Monzon *et al*, 1991). Filth

flies are found worldwide especially in tropical and sub-tropical countries. It can cause a nuisance and transmit pathogens to humans and animals (Greenberg, 1971). Several strategies have been employed for controlling fly populations, including environmental management, biological and insecticidal control, but the fly population continues to increase, especially in sub-tropical and tropical areas.

Insecticides have been the most effective strategy used for fly control, but continuous applications of these chemicals have led to increase in resistance of the insects; moreover it contaminated the environment (Sirisuda *et al*, 2008). An alternative strategy for controlling fly population is biological control. However, traditional biological control using parasitoids or insect predators requires continuous release of these predators into the environment. Recently, *Wolbachia* bacteria from various insect species have investigated for their potential application for biological control of agricultural and medical importance arthropods (Moreira *et al*, 2009). Previous reports have shown that *Wolbachia* strain *w*MelPop isolated from *Drosophila* is able to invade and sustain themselves in the mosquito population and can reduce mosquito lifespan (Kambris *et al*, 2009).

Wolbachia are intracellular maternally inherited bacteria and are classified to Class Alphaproteobacteria, Order Rickettsiales. They are found mainly in arthropods including spiders, terrestrial crustaceans and insects, as well as in filarial nematodes (O'Neill *et al*, 1992; Werren, 1997). *Wolbachia* is classified into 12 supergroups, A-G and H-L, based on the *Wolbachia* surface protein (*wsp*) gene (Ros *et al*, 2009; Ravikumar *et al*, 2010), but the supergroup G-*wsp* is very similar to the sequences of supergroups A and B (Rowley *et al*, 2004; Baldo and Werren, 2007). Supergroups A and B are the most widely distributed in insects (Zhou *et al*, 1998; Ravikumar *et al*, 2010). Recently, *Wolbachia* classified based on 16S rRNA gene sequencing, showed two new *Wolbachia* supergroups M and N in aphids (Augustinos *et al*, 2011). Many studies reported that *Wolbachia* are found at least 20% of all insect species (Werren *et al*, 1995; Werren and Windsor, 2000; Hilgenboecker *et al*, 2008). Relationships between *Wolbachia* and their hosts have many forms ranging from reproductive parasitism to mutualistic symbiosis, which can induce reproductive alterations such as cytoplasmic incompatibility, male lethality, parthenogenesis, and feminization (Rousset *et al*, 1992). Some insect species are unable to produce offspring without *Wolbachia*, viz. bed bug (Hosokawa *et al*, 2010).

In Thailand, information regarding *Wolbachia* endosymbionts in medically important filth flies (Diptera: Muscidae, Calliphoridae, and Sarcophagidae) has never been investigated. In this study, we present preliminary data of *Wolbachia* infection in these medically important flies from different geographical populations in Thailand using PCR-based detection of *wsp*.

MATERIALS AND METHODS

Fly collection and identification

Fifty-one adult fly samples were collected from various regions of Thailand: 9 from Chiang Mai (northern Thailand), 4 from Bangkok (central), 3 from Prachuap Khiri Khan (central), 1 from Nakhon Ratchasima (north eastern), 4 from Nong Khai (north eastern), 20 from Phuket (southern), 6 from Ranong (southern), and 4 from Satun (southern) (Table 1).

Collected samples were identified based on morphological characteristics and molecular techniques as described by Preativatanyou *et al* (2010) and Bhakdeenuan *et al* (2012).

PCR amplification of *wsp*

Genomic DNA was isolated using Invisorb® Spin Tissue Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the manufacturer's instructions. Each fly sample was ground in 200 µl of lysis buffer using sterile plastic pestle. Extracted DNA was stored in 50 µl of elution buffer, concentration determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Singapore) and kept at -20ºC until used.

Primers used were 136AF (5'-TGAAATTTTACCTCTTTTC- 3') and 691AR (5-'AAAAATTAAACGCTACTCCA-3') for amplification of *Wolbachia* supergroup A (550 bp) and primers 81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and 522 R (5'-ACCAGCTTTTGCTTGATA-3') for supergroup B (450 bp) (Zhou *et al*, 1998). PCR, in a final volume of 25 µl, contained 100 ng of DNA, 10 µM each primer, 2.5 mM $MgCl_2$, 2 mM dNTPs and 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Thermocycling (Veriti; Applied Biosystems, Carlsbad, CA) conditions were as follows: 95ºC for 3 minutes; 40 cycles of 95ºC for 30 seconds, 50ºC for 30 seconds, and 72ºC for 30 seconds; and a final step at 72ºC for 7 minutes. Double-distilled water was used as negative control and DNA from female Asian tiger mosquito (*Aedes albopictus*) was positive control. Amplicons were analyzed by 1.5% agarose gel-electrophoresis, stained with ethidium bromide and recorded using Gel Doc EQ Quantity One quantification analysis software version 4.5.2 system (Bio-Rad, Hercules, CA).

Cloning and sequencing of *wsp* amplicons

PCR amplicons were ligated into pGEM-T Easy Vector (Promega, Madison, WI) and the recombinant plasmids were used to transform competent *Escherichia coli* DH5± α strain. Transformed cells were cultured and recombinant plasmids were extracted using Invisorb® Spin Plasmid Mini kit (STRATEC Molecular, Berlin, Germany) following the manufacturer's instructions. Plasmids were sequenced by 1st Base Laboratories, Malaysia.

Sequence analysis and phylogenetic tree construction

Nucleotide sequences were analyzed by comparison with available sequence data in GenBank using BLAST search (http://www.ncbi.nlm.gov/BLAST). The nucleotide sequences generated in this study have been deposited in GenBank (Table 2). Sequences were aligned and the percentage of sequence similarity was calculated using BioEdit Sequence Alignment Editor Version 7.1.9 (Hall, 1999). Phylogenetic tree was constructed by Neighbor-joining method using Kimura's 2-parameter model implemented in MEGA© version 5.1 (Tamura *et al*, 2011). The reliability of an inferred tree was tested by 1000 bootstraps using *Wolbachia* endosymbiont of *Aprostocetus* spp accession no. HQ121415 as an outgroup.

RESULTS

Filth flies used in this study belonged to 3 families, namely, Muscidae, Calliphoridae and Sarcophagidae, and 15 fly species were identified using morphology and molecular techniques (Table 1). PCR-based detection of *wsp* showed *Wolbachia* in 7/51 (14%) of the collected samples (Table 2). No co-infection of *Wolbachia* supergroups A and B was detected (Fig 1).

Table 1
Flies collected from various regions of Thailand.

Family	Species	Province	Region of Thailand	Sex		Total (no.)
				Female (no.)	Male (no.)	
Muscidae	*Hydrotaea spinigera*	Chiang Mai	Northern	1	1	2
	Musca domestica	Ranong	Southern	2	1	3
		Phuket	Southern	1	0	1
		Satun	Southern	2	0	2
		Nong Khai	Northeasthern	1	0	1
	M. sorbens	Nong Khai	Northeasthern	2	0	2
		Bangkok	Central	1	0	1
		Phuket	Southern	3	0	3
Calliphoridae	*Chrysomya chani*	Chiang Mai	Northern	1	1	2
		Phuket	Southern	2	0	2
	C. megacephala	Prachuab Khiri Khan	Central	2	0	2
		Phuket	Southern	1	0	1
		Ranong	Southern	1	1	2
		Satun	Southern	0	2	2
	C. pinguis	Chiang Mai	Northern	1	0	1
	Hemipyrellia ligurriens	Phuket	Southern	1	0	1
	H. pulchra	Chiang Mai	Northern	2	0	2
		Phuket	Southern	1	0	1
	Lucilia cuprina	Phuket	Southern	3	2	5
	L. porphyrina	Chiang Mai	Northern	1	0	1
Sarcophagidae	*Sarcophaga dux*	Bangkok	Central	2	0	2
		Prachuab Khiri Khan	Southern	1	0	1
		Phuket	Southern	4	0	4
	S. indica	Nakhon Ratchasima	Northeasthern	1	0	1
	S. peregrina	Chiang Mai	Northern	1	0	1
	S. ruficornis	Nong Khai	Northeasthern	0	1	1
		Bangkok	Central	1	0	1
		Phuket	Southern	2	0	2
	S. scopariiformis	Ranong	Southern	1	0	1
Total				42	9	51

There were 4 samples with *Wolbachia* supergroup A, consisting of a female *M. sorbens* and *S. dux* from Bangkok, a female *C. megacephala* from Phuket, and a female *H. pulchra* from Chiang Mai (Fig 1A), whereas *Wolbachia* supergroup B were detected in 3 samples, consisting of a female *M. domestica* and *S. scopariiformis* from Ranong and a female *C. megacephala* from Prachuab Khiri Khan (Fig 1B).

Analysis of the mean G+C content of the partial *wsp* sequences revealed 37.90% of supergroup A and 35.50% of supergroup B. Both *Wolbachia* supergroups A and B partial *wsp* sequences did not show intra-variation of nucleotide sequences, but inter-variations among *Wolbachia* supergroup A showed the inter-species

Table 2
Wolbachia supergroups A and B in flies.

Species	Location	Sex	Supergroup	Clone no.	Code	GenBank accession no.
H. pulchra	Chiang Mai	Female	A	1	CM62_1	KC668275
H. pulchra	Chiang Mai	Female	A	3	CM62_3	KC668276
H. pulchra	Chiang Mai	Female	A	5	CM62_5	KC668277
M. sorbens	Bangkok	Female	A	1	BK7_1	KC668284
M. sorbens	Bangkok	Female	A	2	BK7_2	KC668285
M. sorbens	Bangkok	Female	A	3	BK7_3	KC668286
S. dux	Bangkok	Female	A	1	BK8_1	KC668278
S. dux	Bangkok	Female	A	2	BK8_2	KC668279
S. dux	Bangkok	Female	A	3	BK8_3	KC668280
C. megacephala	Phuket	Female	A	1	PK26_1	KC668281
C. megacephala	Phuket	Female	A	2	PK26_2	KC668282
C. megacephala	Phuket	Female	A	3	PK26_3	KC668283
S. scopariiformis	Ranong	Female	B	6	RN4_6	KC668287
S. scopariiformis	Ranong	Female	B	7	RN4_7	KC668288
S. scopariiformis	Ranong	Female	B	9	RN4_9	KC668289
M. domestica	Ranong	Female	B	1	RN8_1	KC668290
M. domestica	Ranong	Female	B	2	RN8_2	KC668291
M. domestica	Ranong	Female	B	3	RN8_3	KC668292
C. megacephala	Prachuab Khiri Khan	Female	B	1	PJ9_1	KC668293
C. megacephala	Prachuab Khiri Khan	Female	B	3	PJ9_3	KC668294
C. megacephala	Prachuab Khiri Khan	Female	B	5	PJ9_5	KC668295

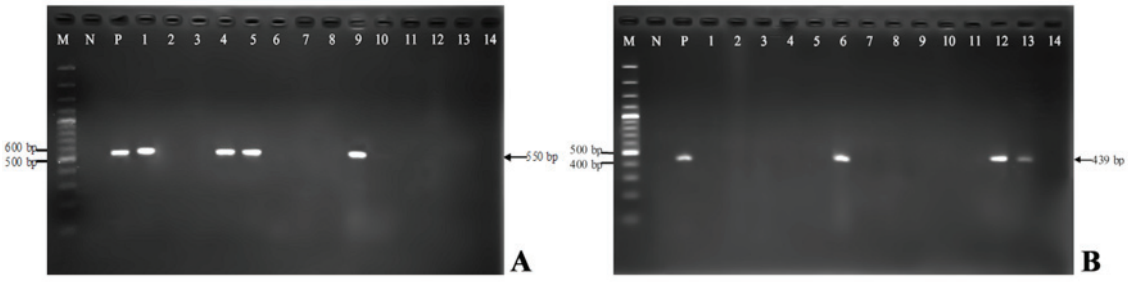

Fig 1–PCR amplicons of *wsp* specific to *Wolbachia* supergroup A (A) and supergroup B (B). Primers used and PCR condition are described in Materials and Methods. Lane M: 100 bp molecular weight markers, lane N: negative control, lane P: positive control *Ae. albopictus*, lane 1: *H. pulchra* from Chiang Mai (CM62), lane 2: *L. porphyrina* from Chiang Mai (CM95), lane 3: *S. ruficornis* from Nong Khai (NK 11), lane 4: *M. sorbens* from Bangkok (BK7), lane 5: *S. dux* from Bangkok (BK8), lane 6: *C. megacephala* from Prajuab Kiri Khan (PJ9), lane 7: *S. dux* from Prachuab Khiri Khan (PJ 15), lane 8: *M. domestica* from Phuket (PK5), lane 9: *C. megacephala* from Phuket (PK26), lane 10: *S. ruficornis* from Phuket (PK42), lane 11: *L. cuprina* from Phuket (PK61), lane 12: *S. scopariiformis* from Ranong (RN4), lane 13: *M. domestica* from Ranong (RN8), lane 14: *C. megacephala* from Ranong (RN15).

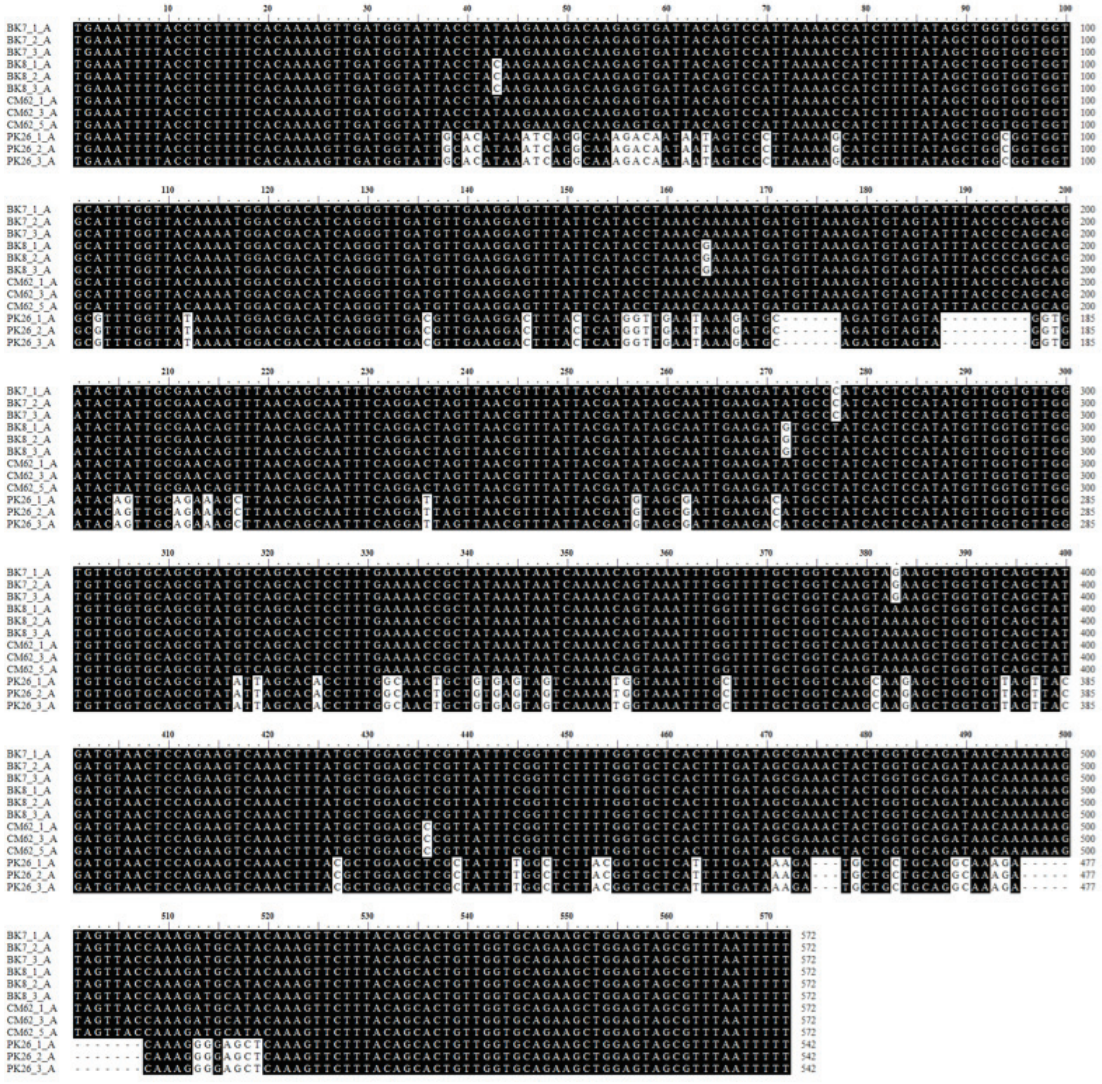

Fig 2–Comparison of partial *wsp* sequences of *Wolbachia* supergroup A from various sources of flies. *M. sorbens* from Bangkok (BK7)_1,_2, and _3 clones; *S. dux* from Bangkok (BK8)_1, _2, and _3 clones; *H. pulchra* from Chiang Mai (CM62)_1, _3, and _5 clones; *C. megacephala* from Phuket (PK26)_ 1, _2, and _3 clones.

variation between *H. pulchra* from Chiang Mai (CM62) and *C. megacephala* from Phuket (PK26) (20%), *S. dux* from Bangkok (BK8) (0.9%), and *M. sorbens* from Bangkok (BK7) (0.6%) (Fig 2). However, *Wolbachia* supergroup B did not show inter-variation in all positive isolates (Fig 3). Phylogenetic tree, constructed basing on the sequences obtained and that of *Wolbachia* sequence from *Aprostocetus* spp as outgroup clearly indicated that *Wolbachia* endosymbionts of flies in Thailand were divided into two major clades, with significant differences between supergroup A and B. *Wolbachia* supergroup A of *C. megacephala* from Phuket (PK26) was iso-

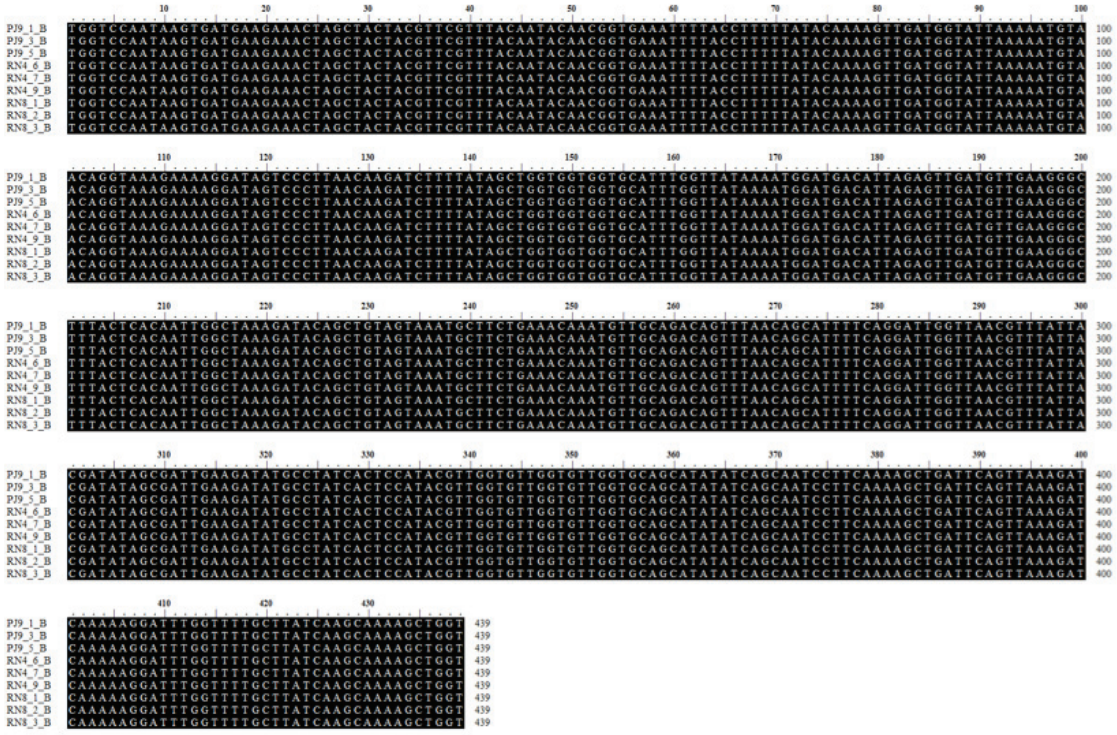

Fig 3–Comparison of partial *wsp* sequences of *Wolbachia* supergroup B from various sources of flies. *C. megacephala* from Prachuab Khiri Khan (PJ9)_1, _3, and _5 clones; *S. scopariiformis* from Ranong (RN4)_6, _7, and _9 clones; *M. domestica* from Ranong (RN8)_1, _2, and _3 clones.

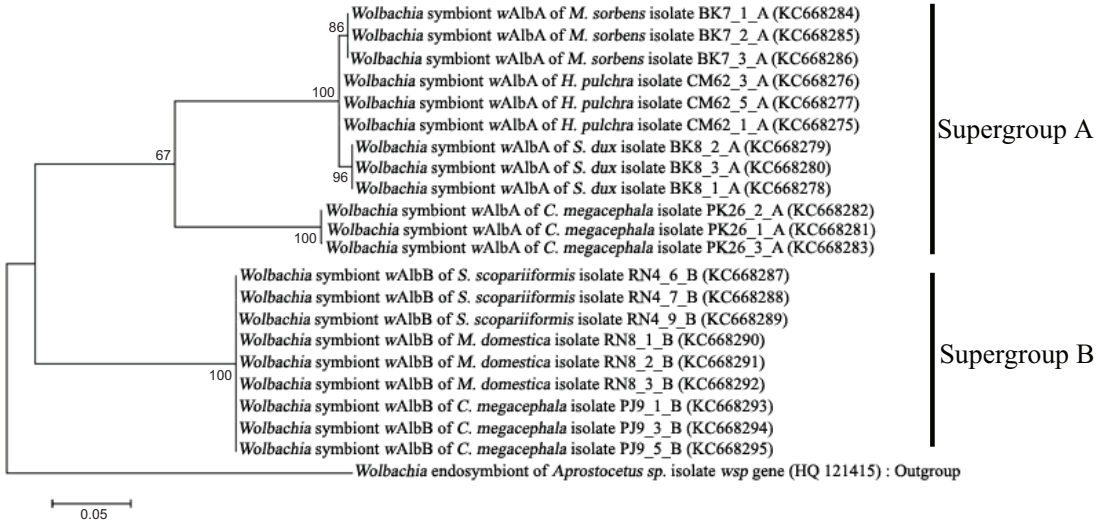

Fig 4–Phylogenetic tree of *Wolbachia* constructed from partial *wsp* sequences. Three isolates of each sample and *Wolbachia* endosymbiont of *Aprotocetus* spp as an outgroup were employed.

lated from other supergroup A samples, but *Wolbachia* supergroup B showed no differences among samples from various geographic areas and fly species (Fig 4).

DISCUSSION

This is the first survey study of *Wolbachia* endosymbionts in natural populations of medically important filth flies (Diptera: Muscidae, Calliphoridae, and Sarcophagidae) in several geographic regions of Thailand. The most common *Wolbachia* endosymbionts found in arthropods are classified into supergroups A and B (Zhou *et al*, 1998; Ravikumar *et al*, 2010). *Wolbachia* endosymbionts were detected in 14% of the samples, all being females with no specific host species or geographical regions. *Wolbachia* supergroup A was not specifically associated with any fly species or geographical regions, but supergroup B was found mostly in southern region of Thailand and there are no co-infections of *Wolbachia* supergroups A and B in any single fly sample. Baudry *et al* (2003) reported in the bird nest blow fly, *Protocalliphora sialia*, that *Wolbachia* endosymbionts in western North America are composed of two types of A supergroup, with some flies being singly and others doubly infected, but in eastern North America mostly of the flies are infected with only the B supergroup with both supergroups present in the Midwest. However it is not yet known whether the bacteria induce cytoplasmic incompatibility or other phenotypes in *Protocalliphora* (Baudry *et al*, 2003; Whitworth *et al*, 2007).

Marker genes used for detecting *Wolbachia* include ribosomal gene (16S rDNA) and a regulatory gene of the bacterial cell cycle (*ftsZ*) (Holden *et al*, 1993; Braig *et al*, 1998). PCR amplification was performed using *wsp*, which is capable of classifying *Wolbachia* into supergroups A and B (Zhou *et al*, 1998; Ravikumar *et al*, 2010).

Sequence analysis showed only slightly difference of mean G+C content between *Wolbachia* supergroups A and B. The low G+C content results are similar to those of *wsp* genes from other insects belonging to order Diptera, such as *Drosophila paulistorum* (mean G+C content of 38.03%) (Miller *et al*, 2010). There was 100% intra-specific similarity of the partial *wsp* sequences but 80-100% of inter-specific similarity of each supergroup. Phylogenetic analysis showed significant difference between *Wolbachia* supergroups. The NJ tree based on *wsp* was able to separate *Wolbachia* supergroups A and B in filth fly samples of this study. Moreover, *Wolbachia* supergroup A of the *C. megacephala* from Phuket (PK26) was clearly separated from other A supergroup. *Wolbachia* supergroup A of *S. dux* from Bangkok (BK8), *M. sorbens* from Bangkok (BK7), and *H. pulchra* from Chiang Mai (CM62) showed monophyletic clade and minor nucleotide variations in different regions and host. All *Wolbachia* supergroup B isolates clustered together and did not show differentiation between geographical regions and hosts.

This study is a preliminary survey of *Wolbachia* in medically important flies in Thailand. Extensive survey of *Wolbachia* infection in flies covering more areas of the country would provide valuable data for developing an effective *Wolbachia*-based fly control strategy.

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REFERENCES

Augustinos AA, Santos-Garcia D, Dionyssopoulou E, *et al*. Detection and characterization of *Wolbachia* infections in natural populations of aphids: is the hidden diversity fully unraveled? *PLoS One* 2011; 6: e28695.

Baldo L, Werren JH. Revisiting *Wolbachia* supergroup typing based on WSP: spurious lineages and discordance with MLST. *Curr Microbiol* 2007; 55: 81-7.

Baudry E, Bartos J, Emerson K, Whitworth T, Werren JH.*Wolbachia* and genetic variability in the birdnest blowfly *Protocalliphora sialia*. *Mol Ecol* 2003; 12: 1843-54.

Bhakdeenuan P, Siriyasatien P, Payungporn S, *et al*. Molecular analysis of medically and veterinary important Muscid flies (Diptera: Muscidae) in Thailand. *Thai J Vet Med* 2012; 42: 333-42.

Braig HR, Zhou W, Dobson S, O'Neil SL. Cloning and characterization of the gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia*. *J Bacteriol* 1998; 180: 2373-8.

Greenberg B. Flies and disease: ecology, classification and biotic associations. Vol I. Princeton: Princeton University Press, 1971.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* 1999; 41: 95-8.

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. How many species are infected with *Wolbachia*? statistical analysis of current data. *FEMS Microbiol Lett* 2008; 281: 215-20.

Holden PR, Brookfield JFY, Jones P. Cloning and characterization of an *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster*. *Mol Gen Genet* 1993; 240: 213-20.

Hosokawa T, Koga R, Kikuchi Y, Meng XY, Fukatsu T.*Wolbachia* as a bacteriocyte-associated nutritional mutualist. *PNAS* 2010; 107: 769-74.

Kambris Z, Cook PE, Phuc HK, Sinkins SP. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science* 2009; 326: 134-6.

Miller WJ, Ehrman L, Schneider D. Infectious speciation revisited: impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. *PLoS Pathog* 2010; 6(12): e1001214.

Monzon RB, Sanchez AR, Tadiaman BM, *et al*. A comparison of the role of *Musca domestica* (Linnaeus) and *Chrysomya megacephala* (Fabricius) as mechanical vectors of helminthic parasites in a typical slum area of Metropolitan Manila. *Southeast Asian J Trop Med Public Health* 1991; 22: 222-8.

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, *et al*. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and Plasmodium. *Cell* 2009; 139: 1268-78.

O'Neill SL, Giordano R, Colbert AM, Karr TL, Robertson HM. 16S rRNA phylogenetic analysis of the bacterial endosymbionts symbionts associated with cytoplasmic incompatibility in insects. *Proc Natl Acad Sci USA* 1992; 89: 2699-702.

Preativatanyou K, Sirisup N, Payungporn S, *et al*. Mitochondrial DNA-based identification of some forensically important blowflies in Thailand. *Forensic Sci Int* 2010; 202(1-3): 97-101.

Ravikumar H, Ramachandraswamy N, Sampathkumar S, *et al*. A preliminary survey for *Wolbachia* and bacteriophage WO infections in Indian mosquitoes (Diptera: Culicidae). *Trop Biomed* 2010; 27: 384-93.

Ros VI, Fleming VM, Feil EJ, Breeuwer JA. How

diverse is the genus *Wolbachia*? Multiple-gene sequencing reveals a putatively new *Wolbachia* supergroup recovered from spider mites (Acari: Tetranychidae). *Appl Environ Microbiol* 2009; 75: 1036-43.

Rousset F, Bouchon D, Pintureau B, Juchault P, Solignac M. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc R Soc London Ser B* 1992; 250: 91-8.

Rowley SM, Raven RJ, McGraw EA. *Wolbachia pipientis* in Australian spiders. *Curr Microbiol* 2004; 49: 208-14.

Sirisuda S, Kabkaew LS, Budsabong K, Kom S. Ultrastructural alteration of larvae and puparia of blow fly *Chrysomya megacephala* (F.) (Diptera: Calliphoridae) and house fly *Musca domestica* L. (Diptera: Muscidae) exposed to neem extract. *Trop Biomed* 2008; 25: 107-16.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; 28: 2731-9.

Werren JH. Biology of *Wolbachia*. *Annu Rev Entomol* 1997; 42: 587-609.

Werren JH, Windsor DM. *Wolbachia* infection frequency in insects: evidence of a global equilibrium?. *Proc R Soc Lond B Biol Sci* 2000; 267: 1277-85.

Werren JH, Windsor D, Guo L. Distribution of *Wolbachia* among neothropical arthropods. *Proc R Soc Lond B Biol Sci* 1995; 262: 197-204.

Whitworth TL, Dawson RD, Magalon H, Baudry E. DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). *Proc Biol Sci* 2007; 274(1619): 1731-9.

Zhou W, Rousset F, O'Neill SL. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc R Soc London Ser B* 1998; 265: 509-15.