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MATERNAL TRANSMISSION EFFICIENCY OF *WOLBACHIA* SUPERINFECTIONS IN *AEDES ALBOPICTUS* POPULATIONS IN THAILAND

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Abstract. We examined the transmission efficiency of 2 strains of *Wolbachia* bacteria that cause cytoplasmic incompatibility in field populations of *Aedes albopictus* by polymerase chain reaction assay. We found mainland and island populations throughout Thailand to be superinfected with group A and B bacteria. Of 320 *Wolbachia*-positive adult mosquitoes, 97.5% were infected with both groups. Single infected individuals of each *Wolbachia* group were encountered in nearly equal numbers. We screened 550 offspring from 80 field-collected mothers and found the transmission efficiency of group A *Wolbachia* to be 96.7% and that of group B *Wolbachia* to be 99.6%. Mothers that did not transmit both *Wolbachia* infections to all of their offspring were significantly larger in size than those with perfect transmission fidelity. We discuss our findings in relation to the prospects of the use of *Wolbachia* as a gene-driving mechanism.

INTRODUCTION

Cytoplasmic incompatibility is a common phenomenon in insects, caused by maternally inherited bacteria of the genus *Wolbachia.*[1] When a *Wolbachia*-infected male mates with an uninfected female, the eggs or embryos most commonly die. The net effect is a decrease in the fitness of uninfected females, which over time results in the spread of *Wolbachia* infection through the population. Individuals may possess more than one strain of *Wolbachia,*[2,3] in which case cytoplasmic incompatibility occurs between superinfected individuals if the female is infected with fewer *Wolbachia* strains than the male with which she mates. The net effect is a decrease in the fitness of such females, and thus the higher-level superinfection spreads.[4,5] Phylogenetic analyses of 16S ribosomal RNA and *ftsZ* gene sequences have established that *Wolbachia* associated with host reproductive alterations form a monophyletic clade in the α-proteobacteria.[3,6–8] Within this clade, the 2 major groups of *Wolbachia,* designated A and B, are estimated to have diverged 58–67 million years ago.[3]

It has been suggested that the capacity of *Wolbachia* for population invasion may be harnessed to push desirable genes, such as those that block parasite transmission, into natural populations of arthropod disease vectors.[9–11] These bacteria have a number of properties that make them particularly attractive for this goal, including a wide host range,[12] wide tissue distribution,[13] and an ability to sweep into insect populations repeatedly. Such repeated invasions are made possible by the independent cytoplasmic incompatibility properties of each *Wolbachia* strain, as demonstrated by the dynamics of superinfections in several insect hosts.[4,5,14]

The success of *Wolbachia* as a gene-driving mechanism is critically dependent on the efficiency of its maternal transmission under field conditions.[11] This parameter has only been measured in *Drosophila,* where it averages 96.4% in field-collected *Drosophila melanogaster*[15] and 95–97% in field-collected *Drosophila simulans,* with transmission efficiency of individual females varying 60–100%.[16,17] In contrast, perfect maternal transmission has been recorded in several hundred lines of laboratory-bred *Drosophila simulans.*[18] Although good data exist for transmission efficiencies of *Wolbachia* in *Drosophila* populations, almost no data are available for other species.[11] In addition, no data have been gathered on the transmission efficiencies of *Wolbachia* superinfections under field conditions. Laboratory studies suggest that they may be lower than single infections.[4] We have measured the vertical transmission efficiency of *Wolbachia* superinfection in field populations of the Asian tiger mosquito *Aedes albopictus* (Skuse), a vector of dengue fever.[19]

MATERIALS AND METHODS

Mosquito specimens. Adult mosquitoes were collected from 21 provinces in mainland Thailand and 7 islands located 20–70 km offshore between August 1995 and December 1997 (Figure 1). Collections were made with Centers for Disease Control and Prevention light traps (BioQuip, Gardena, CA) and mosquito landing catches by standard techniques.[20] Individuals were identified to species level with the morphological keys of Buei[21] and of Rattanarithikul and Panthusiri.[22] Specimens that could not be processed immediately were stored at −70°C for later use.

Polymerase chain reaction (PCR) amplification. Mosquitoes were screened for the presence of *Wolbachia* by PCR with general *ftsZ* bacterial cell cycle gene primers.[4,23] Individual mosquitoes were dissected and the ovaries or testes removed in distilled water by use of sterile dissecting equipment. Crude DNA extractions were performed by homogenizing gonadal tissue in 100 μl of STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), by use of the methods of O'Neill and others.[6] One microliter of supernatant was used as the DNA template in the PCR reaction. Specimens yielding a product of the expected size (730 base pairs [bp]) were scored as positive for *Wolbachia. Wolbachia*-infected *D. simulans* or *Ae. albopictus* were used as a positive control. Negative controls were randomly included to check for contamination.

The PCR amplifications were carried out on a Hybaid Omnigene thermal cycler (Hybaid Limited, Middlesex, United Kingdom) with 20 μL reaction volumes: 2 μL 10× buffer (Promega, Madison, WI), 2 μL 25 mM $MgCl_2$, 0.5 μL dNTPs (10 mM each), 0.5 μL 20 μM forward and reverse primers, and 1 U of DNA polymerase (Promega). The following temperature profiles were used: an initial denaturation at 95°C for 3 min, then 95°C for 1 min, 50°C for 1

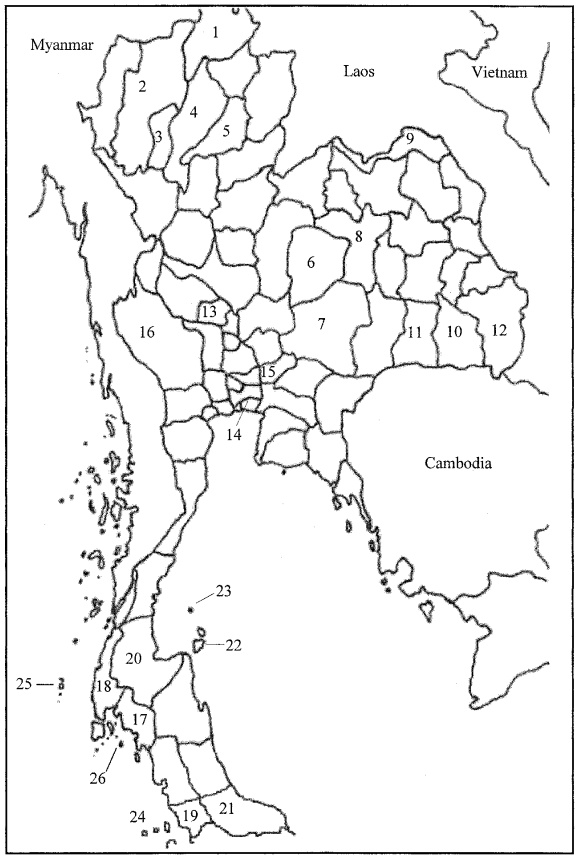

FIGURE 1. Map of Thailand showing the locations of provinces and islands where field collections were made. 1 = Chiangrai; 2 = Chiangmai; 3 = Lamphun; 4 = Lampang; 5 = Phrae; 6 = Chaiyaphum; 7 = Nakornratchasima; 8 = Khonkaen; 9 = Nongkai; 10 = Sisaket;11 = Surin; 12 = Ubonratchathani; 13 = Chainat; 14 = Chachoengsao;15 = Nakornnayok; 16 = Kanchanaburi; 17 = Krabi; 18 = Phang Nga; 19 = Satul; 20 = Suratthani; 21 = Songkhla; 22 = Samui Island; 23 = Tao Island; 24 = Adang, Lipe, and Tarutao Islands; 25 = Miang Island; 26 = Phi-phi Island.

min, and 72°C for 1 min per cycle for 30 cycles. The PCR products were run on a 1% agarose gel with a 1-kb ladder (Gibco BRL, Gaithersburg, MD) to determine the presence and size of amplified DNA.

We did not attempt to measure the number of uninfected individuals because the assay we used is susceptible to false-negative findings in this mosquito species, presumably due to PCR inhibitory compounds present in some DNA extractions. Because we could not confirm the infection status of uninfected individuals, this study is restricted to an analysis of the production of single infected offspring from superinfected mothers and an estimate of the frequency of single infection in the field. Among field-caught females, we were able to confirm the infection status of all single infected individuals by typing 2 of their offspring.

Infections were typed into *Wolbachia* groups A and B with 1 of 2 methods: 1) restriction fragment length polymorphism (RFLP) analysis with *Eco*RV (Promega) to digest the *ftsZ*-amplified product,[4] or 2) PCR screening with group-specific primers designed from the bacterial outer surface protein gene *wsp*.[24] For the RFLP analysis, specimens without a restriction site were classified as group A bacteria; those with a single *Eco*RV restriction site were classified as group B. Group A and B *wsp* primers yield fragments of 556 bp and 442 bp, respectively.

Group A and B *wsp* PCR products were sequenced to confirm the correct amplification and to identify the specific strain of *Wolbachia* infection in wild-caught *Ae. albopictus.* Products were cleaned by means of spin columns (Wizard PCR Preps, Promega) and cloned into a pGEM-T vector (Promega). Plasmids were extracted (Wizard Minipreps, Promega) and sequenced in both directions with primers T7 and SP6 (AmpliTaq DNA polymerase, FS, Applied Biosystems on an automated sequencer, ABI 377, Perkin-Elmer, Norwalk, CT).

Measure of maternal-transmission efficiency. Field-collected adult female mosquitoes from every region of Thailand were selected at random, fed blood, then transferred to an individual vial to lay eggs. After oviposition, parent females were PCR tested to confirm their *Wolbachia* infection status. Random samples of adult F1 offspring from each female were also PCR screened. In addition, for the Samui Island population, wing-length measurements were obtained for all parent females. F1 larvae from each female were reared under insectary conditions in 1 L of distilled water in separate autoclaved trays. Larvae were fed daily on autoclaved fish-food pellets until pupation.

Statistical analysis. The significance of differences in *Wolbachia* infection and transmission frequency between mosquito groups was evaluated with the chi-square test by Statistix version 4.1 (Analytical Software, Tallahassee, FL). Mean wing lengths between groups were compared with Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Field single and double infection frequencies. Amplified PCR products were confirmed as belonging to either group A or B *Wolbachia* clades by sequencing. The group A infection was from the *AlbA* subgroup and the group B infection was from the *Pip* subgroup, as described by Zhou and others.[24]

A total of 320 individuals tested positive for *Wolbachia* by PCR screening (Table 1). Of these, 312 (97.5%) were double-infected with *Wolbachia* groups A and B bacteria. Only 8 single-infected individuals were detected, 6 from central Thailand and 2 from southern Thailand. Single infections of both *Wolbachia* groups were encountered, with 5 individuals positive for group B bacteria only and 3 positive for group A only. The frequency of single infections did not differ significantly between geographical regions (chi-square = 4.26, degrees of freedom [df] = 2, $P = 0.13$).

Of the 320 *Wolbachia*-positive individuals, 110 were collected from island populations off the east and west coasts of southern Thailand (Table 1). A total of 108 were superinfected with group A and B *Wolbachia* (98.2%). In addition, all individuals tested from the 3 islands located furthest from the mainland, Miang, Lipe, and Adang, were found to be double infected. Miang is located >60 km off the Thai mainland, whereas Lipe and Adang are ~70 km offshore. Only 2 single-infected individuals were detected in collections made from island populations; one from Samui Island was positive for group B *Wolbachia* only, and one from Tao

TABLE 1

Frequency of *Wolbachia* superinfection in field-collected *Aedes albopictus* from Thailand (includes mainland and island populations)*

Region and total infected (% AB)	Province or island	AB	A only	B only	Total (% AB)
North	Chiangrai	9	0	0	9 (100.0)
30 (100.0)	Chiangmai	17	0	0	17 (100.0)
	Lamphun	2	0	0	2 (100.0)
	Lampang	1	0	0	1 (100.0)
	Phrae	1	0	0	1 (100.0)
Central	Chaiyaphum	5	0	0	5 (100.0)
128 (95.3)	Nakornratchasima	3	0	0	3 (100.0)
	Khonkaen	17	0	0	17 (100.0)
	Nongkai	1	0	0	1 (100.0)
	Sisaket	3	0	0	3 (100.0)
	Surin	3	0	0	3 (100.0)
	Ubonratchathani	18	0	0	18 (100.0)
	Chainat	38	0	2	40 (95.0)
	Chachoengsao	11	0	2	13 (84.6)
	Nakornnayok	3	0	0	3 (100.0)
	Kanchanaburi	20	2	0	22 (90.9)
South	Krabi	10	0	0	10 (100.0)
162 (99.4)	Phang Nga	12	0	0	12 (100.0)
	Satul	5	0	0	5 (100.0)
	Suratthani	3	0	0	3 (100.0)
	Songkhla	17	0	0	17 (100.0)
	Samui Island†	57	0	1	58 (98.3)
	Tao Island‡	19	1	0	20 (95.0)
	Lipe Island‡	5	0	0	5 (100.0)
	Adang Island‡	5	0	0	5 (100.0)
	Tarutao Island‡	20	0	0	20 (100.0)
	Miang Island‡	2	0	0	2 (100.0)
	Phi-phi Island‡	5	0	0	5 (100.0)
Total		312	3	5	320 (97.5)

* Infections were typed into *Wolbachia* groups A and B by restriction fragment length polymorphism analysis of *ftsZ*-amplified products, except as indicated.
† Some individuals were typed with group-specific *wsp* primers.
‡ All individuals were typed with group-specific *wsp* primers.

Island was positive for group A *Wolbachia* only. The frequency of single infections did not differ significantly between the island and mainland populations (chi-square = 0.36, df = 1, $P = 0.55$).

Transmission efficiencies. The F1 offspring were obtained from 80 field-collected adult females from 12 provinces throughout Thailand, representing 1 island and 11 mainland locations (Table 2). All parent females were positive for both A and B group *Wolbachia* by PCR assay. A total of 550 offspring were PCR screened for *Wolbachia,* of which 530 (96.4%) were superinfected with both *Wolbachia* groups. Transmission efficiency (the fraction of infected offspring produced by infected females) was 96.7% (532 of 550) for group A bacteria and 99.6% (548 of 550) for group B bacteria.

TABLE 3

Infection status of F1 offspring from females with imperfect transmission of *Wolbachia*

Female	Province	No. offspring tested	No. offspring of each infection status			
1	Satul	10	7	0	3	30.0
2	Kanchanaburi	14	11	1	2	21.4
3	Kanchanaburi	18	14	0	4	22.2
4	Samui Island	6	4	0	2	33.3
5	Samui Island	15	14	1	0	6.7
6	Samui Island	8	6	0	2	25.0
7	Samui Island	18	17	0	1	5.6
8	Samui Island	4	2	0	2	50.0
9	Samui Island	9	8	0	1	11.1
10	Samui Island	6	0	0	1	16.7
Total		103	83	2	18	17.5

The 20 single-infected offspring were produced by 10 mothers, or 12.5% of all mothers sampled. Eight mothers produced offspring that were single-infected with group B bacteria, one mother produced offspring that were single-infected with group A bacteria, and one mother produced single-infected offspring of each *Wolbachia* group (Table 3). The number of mothers producing offspring that were single-infected with group A bacteria was not significantly different from those producing offspring that were single-infected with group B bacteria (chi-square = 3.08, df = 1, $P = 0.08$). Among those females that did not transmit both *Wolbachia* infections to all their offspring, the proportion of single-infected progeny ranged from 5.6% (1 of 18) to 50% (2 of 4), with an overall mean of 17.5%. The number of females producing at least one single-infected offspring did not differ significantly between regions (chi-square = 1.17, df = 2, $P = 0.56$).

Wing-length measurements were obtained for 41 super-

TABLE 2

Segregation of double *Wolbachia* infections in F1 progeny of field-collected *Aedes albopictus* from mainland and island locations*

Region	Province or island	Total no. females	No. females with imperfect transmission	No. F1 progeny of each infection status			
				AB	A	B	Total (% AB)
North	Chiangrai	3	0	21	0	0	21 (100.0)
	Chiangmai	2	0	3	0	0	3 (100.0)
Central	Ubonratchathani	2	0	21	0	0	21 (100.0)
	Khonkaen	5	0	36	0	0	36 (100.0)
	Chainat	5	0	29	0	0	29 (100.0)
	Nakornnayok	2	0	12	0	0	12 (100.0)
	Kanchanaburi	6	2	48	1	6	55 (87.3)
South	Satul	2	1	17	0	3	20 (85.0)
	Krabi	3	0	16	0	0	16 (100.0)
	Songkhla	3	0	10	0	0	10 (100.0)
	Phang Nga	4	0	33	0	0	33 (100.0)
	Samui Island†	43	7	284	1	9	294 (96.6)
Total		80	10	530	2	18	550 (96.4)

* Infections were typed into *Wolbachia* groups A and B by restriction fragment length polymorphism analysis of *ftsZ*-amplified products, except as indicated.
† Infection status of F1 from some females typed with group-specific *wsp* primers.

infected parent females collected from Samui Island, 7 of which did not transmit both *Wolbachia* infections to all their F1 offspring. Mean wing length was significantly greater in those females that had produced at least one single-infected offspring (2.53 mm versus 2.37 mm; $P = 0.04$).

DISCUSSION

Our data show that *Wolbachia*-infected *Ae. albopictus* field populations in Thailand are predominantly superinfected with 2 different *Wolbachia* strains (Table 1). The frequency of superinfection in field-collected populations was not significantly different between regions of the country. Among those single-infected individuals sampled, group A and B *Wolbachia* were encountered in nearly equal numbers ($n = 3$ and 5, respectively).

The high frequency of superinfections observed in all regions of the country, including remote islands, suggests that both group A and B *Wolbachia* infections have been present in *Ae. albopictus* populations in Thailand for a considerable length of time and that both infections are near fixation. Sinkins and others,[4] in their work with *Ae. albopictus,* reported a single-strain *Wolbachia* infection in a colony that originated from Samui Island. In contrast, their colony, founded from females collected on mainland Thailand, was noted to be superinfected. The infection in the Samui colony has since been typed as group A *Wolbachia* and those in the mainland colony as groups A and B (O'Neill SL, unpublished data). The authors hypothesized that the group A infection might be more ancient and that the group B infection had subsequently swept through *Ae. albopictus* populations on the Thai mainland but had not yet reached isolated oceanic islands.

However, our data do not support this hypothesis. We sampled remote islands on both coasts of Thailand, including those that were located furthest from the mainland; superinfection was present in all *Ae. albopictus* populations that we tested. The Samui colony that Sinkins and others[4] used in their work was founded before 1970; their colony from the mainland was founded in 1992. It is possible that the group B infection spread to Samui, and to other remote islands, in the last 20–30 years. However, given the prevalence of superinfection on remote islands, a more likely explanation is that both infections have been near fixation for much longer, and that the Samui colony material lost the B group infection while in the laboratory.

Our data from field-collected mothers show a high rate of maternal transmission of the *Wolbachia* superinfection with, on average, 96% of F1 offspring from superinfected females receiving both infections (Table 2). Most females passed both infections to all their offspring, with only 12.5% (10 of 80) of females producing some single-infected progeny. Although not significantly different, more females produced single B infections (9 of 80) than single A infections (2 of 80), suggesting that group A infections may be lost more frequently than group B. This is consistent with earlier studies that suggest that group A *Wolbachia* are at lower densities than group B *Wolbachia* in *Ae. albopictus* and thus would be expected to be transmitted less efficiently.[25]

Of those females with imperfect transmission, 6 produced more than one single-infected offspring. Similar variation in the rates at which females transmit *Wolbachia* infection has been reported in *D. simulans.* Turelli and Hoffmann,[17] in their work with single-infected *D. simulans,* noted that although some females failed to show any segregation in their progeny, others produced nearly 50% uninfected offspring. Our study did not attempt to measure the proportion of uninfected individuals produced by superinfected mothers. This was due to limitations in the assay that produced a proportion of false-negative results. As a result, our results on transmission efficiency are likely to be an overestimate because it would be expected that superinfected mothers would produce some uninfected offspring, as well as single infected offspring. However, it is reasonable to suppose that the proportion of uninfected offspring would be less than that of single infected offspring from superinfected mothers.[4]

Studies of *D. simulans* have shown that maternal transmission rates of *Wolbachia* may vary in response to environmental conditions that affect larval development.[1] When reared under crowded conditions, 21% of offspring from superinfected *D. simulans* females were found to be single infected.[4] Merçot and Poinsot[26] demonstrated the effect of crowding on *wNo* strain *Wolbachia* infection in *D. simulans*; after 10 generations, 67% of *wNo* lines lost their infection. Recent phylogenetic analysis has demonstrated the *wNo* infection to be the same *Wolbachia* subgroup as the group B infection in *Ae. albopictus.*[24] Clancy and Hoffmann[27] showed that female *D. simulans* exposed to low doses of tetracycline produced 10–60% uninfected eggs; the authors suggested that partial curing due to naturally occurring antibiotics may contribute to imperfect maternal transmission in the field. Our data for the Samui Island population showed that field-collected females producing at least one single-infected offspring had significantly greater wing lengths than females that transmitted both infections to all offspring. This observation is not consistent with findings in *D. simulans,*[4] in which larval stressing resulted in smaller adults and lower *Wolbachia* transmission rates.

Two explanations may account for our findings in *Ae. albopictus.* Larger females may have proportionately lower bacterial densities, and therefore they may transmit their infections with lower fidelity. Indeed, infected hosts have been shown to vary in their levels of *Wolbachia* density.[16,25,28,29] Alternatively, the larger females may represent a cohort that experienced conditions during larval development that were less favorable for *Wolbachia* transmission, such as high temperatures or environmental antibiotics.

Because of their capacity for population invasion and cytoplasmic replacement, *Wolbachia* have been suggested as a driving mechanism for spreading useful genes, such as those that may confer resistance to disease transmission. One essential property of any gene-driving system is that it can be used to achieve repeated population replacements. The high fidelity with which the *Ae. albopictus* superinfection is passed to offspring in the field further supports the possible use of *Wolbachia* superinfections for this purpose.

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