Distribution of the Reproduction-modifying Bacteria, *Wolbachia*, in Natural Populations of Tephritid Fruit Flies in Thailand

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Received 8 Nov 1999
Accepted 24 Mar 2000

ABSTRACT *Wolbachia* are bacteria that infect the reproductive tissues of numerous arthropod species, including tephritid fruit flies. These bacteria are potentially useful for management of tephritid fruit fly pest populations and may also influence tephritid speciation. The extent to which *Wolbachia* is distributed among tephritid fruit flies is currently unknown. We conducted a PCR-based survey from October 1995 to June 1998 to determine the prevalence and types of *Wolbachia* present in tephritid fruit flies in Thailand. Infected flies emerged from twenty of 126 fruit and flower collections and comprised 13 (of 46) species, twelve of which were in the genus *Bactrocera*. However, only 40% of individuals from infected populations, on average, were infected. Further, for infected species collected at numerous locations, less than one-half of locations had infected flies. Finally, 80% of infected collections were taken during just 3 months (October to December 1997) throughout the 33 month long survey. The use of PCR primers revealed that members of both A and B *Wolbachia* groups were present. They comprised the *Aus* subgroup (Group A), and the *Con*, *Dei* and *Pip* subgroups (Group B). *Wolbachia* from flies of nine collections could not be typed and may represent new subgroups, although there are other possibilities. Most flies carried a single subgroup of *Wolbachia*. Flies of two collections, however, were doubly infected. Our results indicate that *Wolbachia* prevalence and type varies substantially among and within tephritid fruit fly species. It is, therefore, crucial to understand the effects of these subgroups on tephritids as well as the factors that control W*olbachia* prevalence before a role in pest management or speciation can be assessed for these bacteria.

KEYWORDS: *Wolbachia*, PCR-based survey, fruit flies, Tephritidae, Thailand.

INTRODUCTION

Endosymbiotic bacteria of the genus *Wolbachia* infect the ovaries and testes of numerous arthropod species, especially insects.[1,2,3,4] For most species studied to date, *Wolbachia* is associated with the reproduction-modifying phenomenon called cytoplasmic incompatibility,[5,6] an incompatibility between egg and sperm that causes death of the zygote.[7] Two other reproductive effects of *Wolbachia* have been reported, namely, the induction of parthenogenesis in some parasitoid species and feminization of genetic males in isopods.[5,7]

Interest in *Wolbachia* has grown considerably in recent years primarily because of the potential importance of these bacteria to two research areas, those of pest management and sympatric speciation. The controlled introduction of *Wolbachia* strains into pest populations may result in significant reductions in fertility of pest populations, whereas natural *Wolbachia* movement into new populations may promote rapid speciation by causing reproductive incompatibility between populations.[7]

Molecular genetic studies have allowed the typing of *Wolbachia* from different host species, originally into two groups, A and B, based on the *ftsZ* gene[4] and, more recently, into 12 subgroups using the *wsp* gene[6]. Such typing now means that extensive surveys can be made to determine the subgroups of *Wolbachia* infecting particular invertebrate taxa. Knowledge of the subgroups and distribution of *Wolbachia* in diverse invertebrate taxa may help refine pest management programs that include these bacteria as well as lay a foundation for study of the importance of *Wolbachia* to speciation.

Tropical fruit flies (Diptera: Tephritidae) are widely distributed throughout Southeast Asia, Australia and the Pacific region where several species, mainly in the genus *Bactrocera* cause considerable economic losses.[8] Besides their

economic importance, tephritid fruit flies are of particular evolutionary interest because of the existence of many closely-related species, often with overlapping distributions. The presence of several closely-related species of one tephritid genus, *ie*, *Rhagoletis*, within the same area has been hypothesized to be the result of sympatric speciation,[9] one possible reason being that *Wolbachia*-induced cytoplasmic incompatibility between populations preceded speciation. Further, *Wolbachia* has been reported in three tephritid fruit fly species that occur in temperate regions, *Anastrepha suspensa*, *R. mendax* and *R. pomonella* (R. Giordano, unpublished data cited in [1]), but its effects on these flies are unknown. Cytoplasmic incompatibility is known to occur in another temperate species, *Rhagolettis cerasi*,[10] although infection by *Wolbachia* was not investigated. Neither parthenogenesis nor femininization have been reported for tephritid flies.

Because of the potential of *Wolbachia* for use in pest control programs and for their potential role in tephritid speciation, we conducted a survey for these bacteria in tropical tephritids throughout Thailand.

Materials and Methods

Fruit fly collection and handling

Fruits and flowers infested with tephritid larvae were collected from 65 locations within 33 provinces throughout Thailand from October 1995 to December 1998. Each collection of fruit or flowers was placed in a plastic container (21 cm high x 25 cm diameter) with a gauze-covered hole in the lid, for ventilation, and a 1 cm layer of sawdust on the bottom, as a pupation medium. The collections were brought back to the laboratory at Mahidol University and placed in an insectary. Approximately seven days later, each collection's pupae were sifted from the sawdust and placed in a plastic box (17 x 31 x 24 cm, with a gauze-covered hole in the lid) for adult emergence. Adult flies were left in boxes for seven days to allow color patterns to develop. They were then identified[11,12,13] before being stored in a freezer (-70°C) until required for template preparation.

Template preparation

Total DNA was extracted from the ovaries or testes of individual fruit fly adults using the crude boiling methods of O'Neill *et al*.[1] The ovaries or testes were removed from each adult fly using sterilized dissecting equipment and homogenized with a sterilized pestle in a 1.5 ml microcentrifuge tube filled with 100 µl of STE buffer (100 mM NaCl; 10mM Tris Cl, pH 8.0; 1mM EDTA, pH 8.0). The homogenate was heated at 95°C for 10 min before being centrifuged at 14,000 rpm for 1 min at room temperature. One microliter of supernatant was used as DNA template for the polymerase chain reaction (PCR).

Polymerase chain reaction

PCR reactions were conducted in 0.5 ml microcentrifuge tubes with each reaction volume consisting of 2 µl 10X buffer (Promega), 0.5 µl dNTP's (10 mM each), 0.5 µl of each primer (20 µM each), 2 µl 25 mM $MgCl_2$ and 1 unit of *Taq* polymerase (Promega) made up to 20 µl with distilled water. The mixture was overlain with one drop of mineral oil to prevent evaporation.

The efficiency of DNA extraction from flies was tested by using eukaryotic 12S rDNA primers (12SAI, 12SBI).[1] A negative result indicated that insect DNA was not extracted from the sample. *Wolbachia* DNA was assumed, correspondingly, not to have been extracted and the sample was not processed further.

Primers used to detect *Wolbachia* in infected fruit flies were based on the *ftsZ* cell cycle gene.[14,15] Amplification using these primers typically results in a 730 bp product. Typing of *Wolbachia* was carried out using primers for the *Wolbachia* outer surface protein (*wsp*) gene, which should yield products varying from 403 to 556 bp.[6] First, *wsp* primers specific to the A and B groups were used to type *Wolbachia*. Then, subgroup specific primers were used for further typing. For *Wolbachia* type A, primers specific to the subgroups, *Mel*, *AlbA*, *Mors*, *Uni*, *Riv*, *Haw*, *Pap* and *Aus*,[6] were employed. Primers specific to the subgroups, *Con*, *Dei*, *Ori* and *Pip*, were used for *Wolbachia* type B.[6] All DNA samples that were scored as positive for subgroups were tested at least twice to confirm subgroups. Amplifications were performed on a Hybaid OmniGene thermal cycler. The PCR temperature parameters were an initial denaturation at 95°C for 3 min in the first cycle and 1 min for subsequent cycles, primer annealing at 50°C for 1 min and primer extension at 72°C for 1 min. The total number of cycles was 30. *Wolbachia*-infected *Drosophila simulans* or *Aedes albopictus* were used as positive controls for PCR-screening. Negative controls, consisting of reaction volume without DNA template, were randomly included to check for contamination.

PCR products were run on a 1% agarose gel stained with ethidium bromide and visualized and photographed under 312 nm UV light. A 1 Kb DNA ladder (Gibco) was used to determine the size of amplified DNA.

RESULTS

One hundred and twenty-six fruit and flower collections of 54 host plant species encompassing 22 plant families (Tables 1 & 2) were made. Flies of 46 tephritid fruit fly species emerged from the collections. Most species were in the genus *Bactrocera* but one species from each of the genera, *Anomoia*, *Carpomya* and *Dacus*, were also present. Usually, only one fruit fly species was associated with each collection (Table 1), but adults from two species emerged from each of 13 collections and three species emerged from each of two collections (Table 2). Fly species that emerged from three or more host species (*ie*, *B. correcta*, *B. cucurbitae*, *B. diversa*, *B. dorsalis*, *B. tau*) are here termed as polyphagous. For those collections from which two or three fly species emerged, usually at least one of the species was polyphagous (13 out of 15 collections, Table 2). The two fruit fly species from two collections, KB(T)11 and RB(C)14, however, were not polyphagous.

DNA was extracted from each of 1,133 flies that emerged from these collections. Insect DNA extraction seemed efficient because only 11 (= 1%) of the fly extracts did not produce PCR bands when amplified with the 12S rDNA primers. These extracts were not processed further. *Wolbachia* DNA extraction from flies was assumed to be correspondingly efficient. *Wolbachia* infection rates of flies are analyzed by fruit fly taxon, by location, by collection period and by host plant taxon in what follows.

Thirteen (28.3%) of the 46 fruit fly species, including the five polyphagous species, had *Wolbachia* (Tables 1 & 2). The infected species occurred in two of the four fruit fly genera, *viz Bactrocera* and *Dacus*. Species infection rates were significantly higher among polyphagous species (100.0%) than other species (17.5%) (χ^2 =15.89, df = 1, P < 0.0001). A mean of 40.0% (sd = 19.7%, n = 22) of flies per infected population were infected (Table 3). Only for one population of *B. caudata* were 100% of flies infected (RB(C)14, n=10).

The effect of location was next considered. Data from collections from which the same fruit fly species emerged at the same location were pooled to represent that species at the location. For species collected from several locations, less than one-half of locations had infected flies. The five polyphagous species illustrate this. *Bactrocera cucurbitae* had the highest proportion of locations (44.4%, n = 9) with infected individuals, followed by *B. diversa* (28.6%, n = 7), *B. tau* (15%, n = 20), *B. dorsalis* (14.3%, n = 21) and, lastly, *B. correcta* (12.5%, n = 8). Statistically for all fruit fly species, no species was infected with *Wolbachia* at more locations than for any other species (χ^2 = 38.25, df = 45, P = 0.75).

Collections were then grouped according to the 3-month period during which they were gathered, for the next analysis. There were eleven 3-month periods throughout the course of the survey, from October 1995 through to June 1998. The majority, *viz* 80%, of *Wolbachia*-infected collections were taken during just one period, October to December in 1997 (Table 3). This result was supported statistically when the frequencies of infected and uninfected collections were found to be dependent on the collection period (χ^2 = 34.0, df = 9, P < 0.0001). Multiple comparisons using a series of Chi-squared tests were conducted between periods. A more conservative test is recommended for such comparisons and so the Dunn-Sidak calculation[16] was used to determine the level of significance as α'=0.0011. The frequency of infected collections was found to be significantly higher during the October to December period in 1997 than both those of the same period in 1996 (χ^2 = 10.7, df = 1, P < 0.0011) and the April to June period of 1998 (χ^2 = 15.1, df = 1, P = 0.0001). No other significant differences were detected, probably because of the small sample sizes for other periods.

Finally, the dependence of *Wolbachia*-infection of flies on host plant species and family was investigated. For the polyphagous species, not all host plant species yielded infected flies (*B. correcta*, 1 of 7 host species; *B. cucurbitae*, 3 of 7; *B. diversa*, 1 of 3; *B. dorsalis*, 3 of 20; *B. tau*, 4 of 9). Collections, however, were too few to statistically associate any polyphagous species with host plant taxa. Disregarding the taxa of fruit flies, then collections of twelve host plant species from five families contained infected flies (Table 3). Two plant species, pumpkin (*Cucurbita moschata*) and smooth loofah (*Luffa cylindrica*), each made up four (or 20%) and the family Cucurbitaceae made up 14 (or 70%) of the 20 collections with infected fruit flies. Frequencies of collections from which infected fruit flies emerged, however, were neither dependent on host species (χ^2 = 41.42, df = 53, P = 0.88) nor family (χ^2 = 13.80, df = 21, P = 0.88).

Both A and B *Wolbachia* groups as well as four of the 12 subgroups, *viz Aus*, *Con*, *Dei* and *Pip*, were found in Thai tephritid fruit flies (Table 3). Infected flies from the same collection always carried the same group. For example, four of ten *B. diversa* flies that emerged from pumpkin flowers were *Wolbachia*-infected. All four flies carried the same *Wolbachia*

Table 1. Distribution of *Wolbachia* infections among tephritid fruit fly species from fruit and flower collections and from which one fly species emerged per collection.

Fruit fly species	No collections	Total no larvae tested	No. collections with infected fruit flies	Host plant species[c]
Anomoia kraussi	1	2	0	*Gmelina philippensis* (VER)
Bactrocera albistrigata	1	2	0	*Terminalia catappa* (COM)
B. caudata	1	6	0	*Cucurbita moschata* (CUC) flowers
B. correcta	5	51	0	*Careya sphaerica* (BAR), *Terminalia catappa*, *Zizyphus mauritiana* (RHA)
B. cucurbitae	6	48	2	*Cucumis sativus* (CUC), *Cucurbita moschata*, *Luffa acutangula* (CUC), *Luffa cylindrica* (CUC), *Trichosanthes tricuspidata* (CUC) flowers
B. diaphoropsis	1	10	0	*Strychnos nux-blanda* (LOG)
B. diversa	7	46	2	*Cucurbita moschata* flowers, *Lagenaria siceraria* (CUC) flowers, *Luffa cylindrica* flowers
B. dorsalis complex[a]				
B. arecae	5	19	0	*Areca catechu* (PAL)
B. carambolae	2	20	0	*Syzygium grande* (MYR), *Syzigium jambos* (MYR)
B. dorsalis sp. A	28	222	2	*Polyalthia longifolia* (ANN), *Siphonodon celastrineus* (CEL), *Terminalia catappa*, *Luffa cylindrica* flowers, *Gnetum* sp. (GNE), *Artocarpus chaplasha* (MOR), *Artocarpus* sp. (MOR), *Musa acuminata* (MUS), *Musa balbisiana* (MUS), *Musa sapientum* (MUS), *Psidium guajava* (MYR), *Syzygium malaccensis* (MYR), *Syzygium samarangense* (MYR), *Zizyphus mauritiana*, *Citrus mitis* (RUT), *Chrysophyllum cainito* (SAP), *Mimusops elengi* (SAP), *Pouteria campechiana* (SAP), *Solanum verbascifolium* (SOL)
B. dorsalis sp. C	1	2	0	*Artocarpus lakoocha* (MOR)
B. dorsalis sp. E	2	18	0	*Nauclea orientalis* (RUB), *Anthocephalus chinensis* (RUB)
B. dorsalis sp. J	1	5	0	*Syzygium samarangense*
B. dorsalis sp. K	1	2	1	*Payena* sp. (SAP)
B. dorsalis sp. L	1	2	0	*Platea* sp. (ICA)
B. dorsalis sp. V	1	10	0	unidentified sp. (ANN)
B. kanchanaburi	1	2	0	*Artabotrys siamensis* (COM)
B. papayae	1	5	0	*Willughbeia firma* (APO)
B. propinqua	2	17	0	*Garcinia costata* (GUT)
B. verbascifoliae	1	10	0	*Solanum verbasifolium*
B. expandens	1	10	0	*Garcinia* sp. (GUT)
B. infesta	1	3	0	*Luffa cylindrica* flowers
B. maculata	1	2	0	*Cucurbita moschata* flowers
B. maculifacies	1	6	0	*Siphonodon celastrineus*
B. modica	2	12	1	*Bryonopsis laciniosa* (CUC) flowers
B. rubella	1	10	0	*Bryonopsis laciniosa*
B. tau complex[a]				
B. tau sp. A	18	205	2	*Benincasa hispida* (CUC), *Gymnopetalum cochinchinense* (CUC), *Luffa cylindrica*, *Momordica cochinchinensis* (CUC), *Trichosanthes cordata* (CUC) flowers and fruit, *Trichosanthes tricuspidata*
B. tau sp. C	1	10	1	*Momordica cochinchinensis*
B. tau sp. D	1	2	0	*Trichosanthes tricuspidata*
B. tau sp. E	1	2	0	*Strychnos ignatii* (LOG)
B. umbrosa	2	16	0	*Artocarpus heterophyllus* (MOR), *Artocarpus integer* (MOR)
B. sp. 1[b]	1	10	1	*Trichosanthes tricuspidata* flowers
B. sp. 2	2	18	0	*Spondias pinnata* (ANA)
B. sp. 3	1	12	0	*Zehneria indica* (CUC)
B. sp. 4	1	1	0	*Crataeva magna* (CAP)
B. sp. 5	1	2	0	*Crataeva magna*
B. sp. 6	1	20	0	*Siphonodon celastrineus*
B. sp. 7	1	2	0	*Syzygium* sp. (MYR)
B. sp. 8	1	5	1	*Siphonodon celastrineus*
Carpomya vesuviana	1	2	0	*Zizyphus mauritiana*
Dacus destillatoria	2	11	2	*Luffa cylindrica*

[a] Members of the *B. dorsalis* and *B. tau* species complexes distinguished by cytological (Baimai *et al*[17], V Baimai, unpublished) and morphological comparison (Drew & Hancock,[13] S Tigvattananont, unpublished).

[b] *Bactrocera* sp. 1 to 8, new species (S Tigvattananont, unpublished).

[c] Plant family names are abbreviated in parentheses at first appearance of plant species in table: ANA - Anacardiaceae, ANN - Annonaceae, APO - Apocynaceae, BAR - Barringtoniaceae, CAP - Capparidaceae, CEL - Celastraceae, COM - Combretaceae, CUC - Cucurbitaceae, GNE - Gnetaceae, GUT - Guttiferae, ICA - Icacinaceae, LOG - Loganiaceae, MOR - Moraceae, MUS - Musaceae, MYR - Myrtaceae, PAL - Palmae, RHA - Rhamnaceae, RUB - Rubiaceae, RUT - Rutaceae, SAP - Sapotaceae, SOL - Solanaceae, VER - Verbenaceae.

Most flies emerged from fruit collections. When flies emerged from flowers or from both flowers and fruit, this is indicated after the plant name.

Table 2. Distribution of *Wolbachia* infections among tephritid fruit flies from individual fruit and flower collections and from which more than one fly species emerged per collection.

Fruit fly species	Collection code[d]	Total no larvae tested	Infected	Host plant species[e]
Two species emerged				
Bactrocera caudata	RB(C)14	10	Yes	*Cucurbita moschata* flowers
B. diversa		12	No	
B. cilifer	RB(C)18	10	No	*Momordica cochinchinensis*
B. sp. 9[a]		10	No	
B. correcta	RN645	10	No	*Artocarpus chaplasha*
B. dorsalis sp. A[b]		10	No	
B. correcta	RN646	8	No	*Cleistocalyx operculatus*[f]
B. dorsalis sp. A[b]		10	No	
B. correcta	PU(A)11	2	No	*Polyalthia longifolia*
B. dorsalis sp. A[b]		6	No	
B. correcta	CP(K)2	2	No	*Syzygium samarangense*
B. dorsalis sp. A[b]		10	No	
B. correcta	PU(A)10	5	No	*Syzygium samarangense*
B. dorsalis sp. A[b]		10	No	
B. correcta	RN648	10	No	*Careya sphaerica*
B. tuberculata		6	No	
B. cucurbitae	KB(S)26	18	Yes	*Coccinia cordifolia*[g]
B. tau sp. A[c]		9	No	
B. cucurbitae	SK(D)5	4	Yes	*Cucurbita moschata* flowers
B. tau sp. A[c]		4	Yes	
B. cucurbitae	NT(I)26	5	No	*Trichosanthes tricuspidata*
B. tau sp. A[c]		7	No	flowers
B. dorsalis sp. A[b]	MS(E)17	10	Yes	*Psidium guajava*
B. pyrifoliae[b]		7	Yes	
B. sp. 1[a]	KB(T)11	9	No	*Trichosanthes tricuspidata*
B. sp. 10[a]		10	No	flowers
Three species emerged				
B. carambolae[b]	RN522	6	No	*Syzygium samarangense*
B. correcta		2	Yes	
B. dorsalis sp. A[b]		10	No	
B. cucurbitae	PH(M)9	5	No	*Bryonopsis laciniosa*
B. rubella		10	No	
B. tau sp. A[c]		10	No	

[a] New species (S. Tigvattananont, unpublished).

[b] Member of the *B. dorsalis* complex[13,17].

[c] Member of the *B. tau* complex (V. Baimai, unpublished, S. Tigvattananont unpublished).

[d] Province: CP - Chumphon; KB - Kanchanaburi; MS - Maehongsorn; NT - Nakorn Sithammarat; PH - Phetchaburi; PU - Phuket; RB - Ratchaburi; RN - Ranong; SK - Sakonnakhon.

[e] Most flies emerged from fruit collections. When flies emerged from flowers, this is indicated after the plant name.

[f] Family Myrtaceae.

[g] Family Cucurbitaceae.

Table 3. *Wolbachia* groups and subgroups present in infected tephritid fruit flies that emerged from fruit and flower collections.

Fruit fly species	Collection code[d]	Month / Year of collection	Host plant species[e]	*Wolbachia* group[f]	*Wolbachia* subgroup[f]	No. larvae tested	No. +ve larvae
One species emerged							
Bactrocera cucurbitae	KA(A)1	10 / 97	*Luffa cylindrica*	Unk	Unk	10	2
	NK(A)11	10 / 97	*Luffa cylindrica*	B	*Con*	10	2
B. diversa	AC(C)1	10 / 97	*Cucurbita moschata* flowers	Unk	Unk	10	4
	RN537	5 / 97	*Cucurbita moschata* flowers	B	*Pip*	4	2
B. dorsalis sp. A[a]	PB(F)1	10 / 97	*Terminalia catappa*	B	*Dei*	5	2
	TK(C)2	10 / 97	*Terminalia catappa*	B	*Pip*	8	1
B. dorsalis sp. K[a]	RN233	4 / 96	*Payena* sp.	Unk	Unk	2	1
B. modica	PH(M)10	12 / 97	*Bryonopsis laciniosa* flowers	B	*Pip*	10	6
B. tau sp. A[b]	KB(T)7	11 / 97	*Benincasa hispida*	Unk	Unk	10	3
	KB(U)8	11 / 97	*Gymnopetalum cochinchinense*	Unk	Unk	10	2
B. tau sp. C[b]	KB(S)50	11 / 97	*Momordica cochinchinensis*	A	*Aus*	10	4
B. sp. 1[c]	MS(E)16	10 / 97	*Trichosanthes tricuspidata* flowers	Unk	Unk	10	4
B. sp. 8[c]	RB(U)11	12 / 97	*Siphonodon celastrineus*	Unk	Unk	5	2
Dacus destillatoria	NM(A)1	10 / 97	*Luffa cylindrica*	A, B	*Aus, Dei*	8	3
	UB(I)2	10 / 97	*Luffa cylindrica*	Unk	Unk	3	1
Two species emerged							
B. caudata	RB(C)14	12 / 97	*Cucurbita moschata*	B	*Pip*	10	10
B. diversa			flowers	-	-	12	0
B. cucurbitae	KB(S)26	2 / 97	*Coccinia cordifolia*	Unk	Unk	18	2
B. tau sp. A[b]				-	-	9	0
B. cucurbitae	SK(D)5	10 / 97	*Cucurbita moschata*	B	*Con*	3	2
B. tau sp. A[b]			flowers	B	*Con*	4	2
B. dorsalis sp. A[a]	MS(E)17	10 / 97	*Psidium guajava*	B, B	*Dei, Pip*	10	4
B. pyrifoliae[a]				B, B	*Dei, Pip*	7	2
Three species emerged							
B. carambolae[a]	RN522	4 / 97	*Syzygium*	-	-	6	0
B. correcta			*samarangense*	B	*Con*	2	1
B. dorsalis sp. A[a]				-	-	10	0

[a] Member of the *B. dorsalis* complex[13,17].

[b] Member of the *B. tau* complex (V. Baimai, unpublished, S. Tigvattananont, unpublished).

[c] New species (S. Tigvattananont, unpublished).

[d] Province abbreviations as in Table 2. Additional provinces: AC - Amnat Charoen; KA - Kalasin; NK- Nong-Khai; NM - Nakornphanom; PB - Prachinburi; TK - Tak; UB - Ubon Ratchathani.

[e] Most flies emerged from fruit collections. When flies emerged from flowers, this is indicated after the plant name.

[f] Unk: *Wolbachia* group or subgroup not known; - not infected

subgroup, *ie*, *Con*. Flies from nine collections, although they tested positive for *Wolbachia*, did not produce bands using either the A or B *wsp* primers or the 12 *wsp* subgroup primers.

On four (of 13) occasions, infected flies that emerged from collections comprised two fly species (Table 3). On two of these occasions, both fly species in each collection were infected. Further, the two species from each collection carried the same *Wolbachia* subgroup(s). Thus, *B. cucurbitae* and *B. tau* flies that emerged from the same pumpkin flowers were both infected with the *Con* subgroup. Similarly, *B. dorsalis* and *B. pyrifoliae*, which emerged from the same guava (*Psidium guajava*) fruit, were both doubly infected with *Dei* and *Pip* subgroups. On the other hand, different *Wolbachia* subgroups were found in infected flies that emerged from collections taken at the same location but from different hosts. Thus, infected fruit flies were reared from both *Trichosanthes tricuspidata* flowers (*B. ascitoides*, MS(E)16) and guava fruit (*B. dorsalis* and *B. pyrifoliae*, MS(E)17) at the same location but were infected with an unknown *Wolbachia* subgroup and with *Dei* and *Pip* subgroups respectively. Flies infected with *Wolbachia* that emerged from *Coccinia cordifolia* (*B. cucurbitae*, KB(S)26) and *Momordica cochinchinensis* flowers (*B. samlanensis*, KB(S)50) collected at the same location were infected with an unknown subgroup and with the *Aus* subgroup respectively. Finally, infected flies from pumpkin flowers (*B. diversa*, RN 537) and *Syzigium samarangense* fruit (*B. correcta*, RN522) from the same location carried *Pip* and *Con* subgroups respectively.

The majority of infected flies were infected with only one *Wolbachia* subgroup but flies of two collections were doubly infected (Table 3). As mentioned previously, both *B. dorsalis* and *B. pyrifoliae* from the same collection were doubly infected. In addition, *D. destillatoria* that emerged from smooth loofah fruit was infected with both *Aus* and *Dei* subgroups on one occasion.

Discussion

Wolbachia appears to be widespread among tropical tephritid fruit flies, having been recorded in 13 (28.3%) of 46 sampled Thai species. All 13 represent new records of insect species infected with these bacteria. Among these species, we report *Wolbachia* infections in *B. correcta*, *B. cucurbitae*, *B. dorsalis*, *B. pyrifoliae* and *B. tau*, which are considered to be major pest species in Southeast Asia.[8]

Species infection rates in which less than 50% of species are infected with *Wolbachia* appear to be common in arthropod surveys at the family or higher taxonomic level. Similar PCR surveys have detected *Wolbachia* in neotropical insect species[4] (16.9% of species), oniscidean isopods[18] (46.3%) and temperate leaf-mining moths (Lepidoptera: Gracillariidae) and their parasitoids[19] (38.1% and 27.8% respectively). In contrast, surveys of amphipods[18], and of temperate aphids and their primary parasitoids[19] failed to detect *Wolbachia*.

At least three hypotheses can be formulated to explain differences in species infection rates among surveys. First, the number of individuals examined for *Wolbachia* per species may differ among surveys. In our tephritid fruit fly survey, we tested more than four flies for most species. Bouchon *et al.*[18] examined two or more individuals for most species in their isopod survey. In both our survey and that of Bouchon *et al,*[18] infection rates higher than 25% of species were found. In contrast, Werren *et al,*[4] who recorded the lowest species infection rate of all surveys, usually tested only one individual per species. West *et al,*[19] however, examined only one or two individuals per species but recorded infection rates similar to ours. So, factors in addition to sample size, discussed in the following two paragraphs, also seem important.

Second, *Wolbachia* infection may be more common in some taxa than in others. For example, the absence of *Wolbachia* in amphipods, despite numerous tested individuals (55 tested individuals from 12 species),[18] indicates a biased *Wolbachia* distribution across arthropod taxa. Diptera, in particular, were recorded by Werren *et al*[4] to have high species infection rates (35.7%) compared with most other sampled insect orders (e.g., Coleoptera 10.5%, Hemiptera/Homoptera 14.3%, Lepidoptera 16.3%, Orthoptera 12.5%) in their survey of neotropical insects. The results of our survey of tephritid dipterans also indicate a high species infection rate relative to most other orders surveyed by Werren *et al.*[4]

Third, species infection rates may be similar within but vary among geographic regions. Our results yielded an overall tephritid species infection rate only 0.2% higher than that for mosquito species collected from the same tropical region[20] (28.1%). West *et al,*[19] however, has also shown species infection within temperate species of gracillariid moths at rates very similar to ours. If the second hypotheses is correct, then such comparisons among taxa may confound any comparisons among regions.

Rather than compare different taxa, regional comparisons among species of the same taxon may reveal geographical differences. On a finer scale, location may be an important correlate of infection. For example, *B. dorsalis* was recorded from 21 locations within Thailand in our survey, but collections from only three locations yielded infected individuals. Although proportions varied (12.5 to 44.4% of locations), the absence of infected individuals from many collection locations was typical of all five infected polyphagous tephritid species. Bouchon *et al*[18] also recorded differences in species infection rates among geographic locations for several isopod species. *Wolbachia* infection of widely distributed species may have remained undetected if the survey had been restricted to one or two locations. Indeed, most fruit fly species were collected from only one or two locations. *Wolbachia* infection of those species determined in this survey as being uninfected, therefore, can not be discounted. Surveys that sample arthropod species across their distributions may more accurately track *Wolbachia* infection within a species.

Not all flies from infected populations were infected, *ie*, *Wolbachia* infection did not appear to be fixed (= 100% of flies infected). On average, *Wolbachia* was detected in 40% of flies from infected populations (n = 22). One hundred percent infection occurred for only one population of *B. caudata*. Bouchon *et al*[18] also recorded much less than 100% infection for most isopod populations. In a similar fashion, for many infected insect species in which two or three individuals were tested, both Werren *et al*.[4] and West *et al*[19] recorded some uninfected individuals. *Wolbachia* prevalence, therefore, seems commonly to be much less than 100% in natural populations of arthropods. In addition, *Wolbachia* prevalence has been shown to be unstable and vary markedly throughout the year for *Drosophila melanogaster* in some locations[21] and may do so for tephritid fly species as well. Thus, almost all *Wolbachia*-infected collections in this 3-year survey were taken during the last three months of 1997 and may indicate that conditions for *Wolbachia* spread were most favorable during this period. More intensive survey work than that presented here, however, would be needed to track seasonal changes in *Wolbachia* prevalence as well as to correlate environmental conditions to infection rates.

At least five factors could account for the incomplete infection of populations detected in this survey.

(i) Maternal transmission of *Wolbachia* may not be perfect,[7,22,23,24,25] that is, a proportion of an infected female fruit fly's progeny may be uninfected. Imperfect maternal transmission of *Wolbachia* (2.6% uninfected offspring) has been demonstrated for the drosophilid, *D. melanogaster*[21].

(ii) Natural curing of *Wolbachia* infections may occur.[7,24] Naturally occurring antibiotics in larval host plant tissues, for example, may cure *Wolbachia* infections in field populations of tephritid fruit flies. Cytoplasmic incompatibility among *Tribolium confusum* beetles, a condition associated with *Wolbachia* infection, was eliminated in most individuals reared on wheat molded with natural tetracycline-producing fungi.[26] Further, commercial tetracycline has been demonstrated to cure *Wolbachia* infections in the mosquito, *Culex pipiens,*[27] the drosophilids, *D. simulans and D. melanogaster*[28] and the hymenopteran parasitoid, *Nasonia vitripennis.*[28,29] Prolonged larval diapause has also cured *N. vitripennis* individuals of their *Wolbachia* infections[30]. High temperatures,[31,32] high larval densities,[15,21] low nutrition levels,[32] and crosses with older males[31,32,33,34] have all been associated with reduction of cytoplasmic incompatibility in non-tephritid hosts, possibly through reducing *Wolbachia* densities, and may also be occasionally responsible for complete elimination of *Wolbachia* infections from arthropod populations.

(iii) Reduced fitness of infected compared with uninfected individuals may result in less than 100% prevalence of *Wolbachia* infections in populations.[7,24,25] Infected *D. simulans* females in laboratory stocks had lower fecundity than their uninfected counterparts[35]. However, it was not clear if this was a laboratory artifact because field-collected infected females oviposited similar numbers of eggs as did uninfected females that were also collected from the field.[34] A particularly virulent form of *Wolbachia*, called the *popcorn* strain, that results in early death of *D. melanogaster* adults has also been described[36] but, thus far, only from a laboratory fly strain.

(iv) Migration of infected and uninfected individuals into and out of infected populations may influence *Wolbachia* prevalence rates.[37] Tephritid fruit flies, particularly *Bactrocera* species, are known to fly long distances.[38] Low rates of migration are expected to maintain stable *Wolbachia* prevalences, whereas higher migration rates may reduce or increase prevalences.[37]

(v) It is also possible that *Wolbachia* prevalence may be underestimated due to limitations in the PCR assays. The 12S rDNA assay for insect DNA is more sensitive than the *Wolbachia* assays and so it is

possible to get a proportion of false negative results.[20]

Numerous factors could, therefore, be responsible for low *Wolbachia* prevalence rates detected within fruit fly populations.

Only four of the *Wolbachia* subgroups typed by Zhou *et al* [6] were present in tephritid fruit flies (Table 3). This contrasts with Thai mosquitoes in which nine of the subgroups were found.[20] However, 43 of the 46 tephritid species were in one genus, *ie*, *Bactrocera*. When the tephritid and mosquito families are compared on a per genus basis, the results are similar. For example, the two mosquito genera with the greatest numbers of sampled species, *Aedes* and *Culex*, each had six subgroups[20] whereas *Bactrocera* had four. In addition, *Wolbachia* in *Bactrocera* species from nine collections could not be typed and these may represent new subgroups, although there may be other reasons for the lack of amplification. Thus, these untyped *Wolbachia* could belong to existing subgroups but substitutions have accumulated in the PCR priming regions resulting in no amplification. Alternatively, DNA stored after initial *Wolbachia* detection may have decomposed in the time lapse to *Wolbachia* typing. Future sequencing may establish the relations of the untyped *Wolbachia* with other subgroups.

Two of the four subgroups present in tephritid fruit flies, *viz Con* and *Pip*, were also present in mosquito species.[20] Of these, the *Pip* subgroup has been found, thus far, only in Tephritidae (this survey), Culicidae[6,20] and Drosophilidae[6] and may be restricted to dipteran species. Only further surveys will determine the full extent of the *Pip* subgroup's distribution among dipteran and other insect taxa. The *Con* subgroup, although found in mosquito[20] and tephritid species (this survey) was originally described from the beetle, *T. confusum*,[6] and may be more widespread among different insect orders than the *Pip* subgroup. The *Aus* and *Dei* subgroups were originally typed from *Wolbachia* isolated from the tsetse fly, *Glossina austeni*, and the egg parasitoid, *Trichogramma deion*, respectively.[6] The distribution of these subgroups in insects other than tephritid fruit flies and the two original insect hosts is currently unknown.

Infections of a single insect species with two *Wolbachia* groups have been documented frequently.[4,15,30,39,40,41] Similarly, we recorded three fly species with double infections, *B. dorsalis* and *B. pyrifoliae* both doubly infected with *Dei* and *Pip*, and *D. destillatoria* doubly infected with *Aus* and *Dei*.

Subgroup typing in this survey was conducted using primers based on the *wsp* gene. This gene's high variability that makes it so suitable for strain typing and diagnostics has also raised problems for phylogenetic analysis, in particular that subgroups in the A group are only weakly supported by bootstrap analysis.[6] It is possible, therefore, that the subgroup primers used in this survey may cross-react with novel sequences in newly discovered hosts and produce false positives. Records of subgroups within new species as was found in this survey should, therefore, be corroborated by sequencing before subgroup identifications can be confirmed.

Effects of *Wolbachia* infections, whether they be single or double, on tephritid fruit flies are currently unknown. Cytoplasmic incompatibility, of unknown cause, has been recorded in one tephritid fruit fly species, *R. cerasi*.[10] The *Pip* subgroup is known to cause cytoplasmic incompatibility in the mosquito species, *Cx. quinquefasciatus*, *Cx. pipiens* and *Ae. albopictus*.[6] It is also known to cause cytoplasmic incompatibility in a Noumean strain of the drosophilid, *D. simulans*, but has no known effects on another strain (DSW (Mau)) of the same species[6]. The *Aus* and *Con* subgroups cause cytoplasmic incompatibility in *G. austeni* and *T. confusum* respectively, whereas the *Dei* subgroup induces parthenogenesis in the egg parasitoid species, *T. deion*.[6] Our future efforts will be directed at elucidating some of these effects of *Wolbachia* infection on tephritid fruit flies.

Horizontal transmission of *Wolbachia* among taxa has been implied by a lack of congruence between *Wolbachia* and host phylogenies.[1,2,4,42] In addition, experimental transmission of *Wolbachia* to a parasitic wasp (*Leptopilina boulardi*) from its infected host (*D. simulans*) by a naturally-occurring mechanism has recently been demonstrated.[43] Interspecific behavioral interactions may, therefore, be the major means by which horizontal transmission occurs. We found that when two infected fly species emerged from the same host collection, they both were infected with the same *Wolbachia* subgroup(s). This occurred in only two collections and consequently is not statistically significant. In contrast, infected flies that emerged from different host species but at the same location always carried different subgroups. Interactions between fruit fly species on the same host may be conducive to horizontal transmission. A more detailed survey at the same collection sites is needed to determine if this pattern predominates for these combinations of *Wolbachia* subgroups, fly species and fruit hosts.

Just as host sharing by two fly species possibly facilitates horizontal transmission, so associations

of fly populations with different hosts may result in little or no transmission among populations. Thus, flies from fruit and flower collections of the family Cucurbitaceae appeared to be far more frequently infected (70% of collections) than collections from other plant families. Fly populations that associate with cucurbit hosts may, rarely if ever, transfer *Wolbachia* to populations on different plant hosts. Statistically, however, Cucurbitaceae did not have a higher frequency of infected collections than did other plant families. There are two reasons for this and both contribute to the lack of statistical differences that we observed. First, collections of Cucurbitaceae were over-represented among plant taxa and accounted for 45.5% of all collections made during the survey. The high percentage representation of this taxon among infected collections may simply be because they were collected more often than other plant taxa. Second, sample sizes of most other plant taxa were insufficient to determine statistical differences among taxa. Despite the lack of statistical significance, an influence of fruit fly host plant on *Wolbachia* infection remains an intriguing possibility and one that we are investigating further.

Through this survey, we have demonstrated the presence of *Wolbachia* in many tropical tephritid fruit fly species. In most species, *Wolbachia* infection was not fixed and, for those species sampled at many sites, varied markedly with location. Further, the *Wolbachia* subgroups infecting flies varied both among and within species and occurred both as single and double infections. Several potential applications of *Wolbachia* to pest management have been discussed in the literature[44]. Further, it has been suggested that *Wolbachia* may promote rapid speciation by causing reproductive incompatibility between populations.[7] Most discussion of *Wolbachia* for both pest management applications and as important influences on speciation rely on these bacteria becoming fixed or nearly so in populations. Our results indicate that *Wolbachia* fixation is rare in tephritid fruit fly populations. It is, therefore, crucial to understand the factors affecting prevalence of these infections before being able to assess *Wolbachia*'s potential for use in a tephritid pest management program or the role of these bacteria in tephritid speciation.

Acknowledgements

The authors would like to thank Dr Scott O'Neill for his encouragement and for his helpful comments on the paper, two anonymous referees for their useful suggestions, Mr Chalao Sumrandee for his assistance in collecting fruit flies and Ms Samnieng Theinthong and Ms Nutchaya Klinpikul for their technical assistance. This work was supported by the TRF/BIOTEC Special Program for Biodiversity Research and Training (BRT 139026) and the Thailand Research Fund (RTA/38/80008).

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