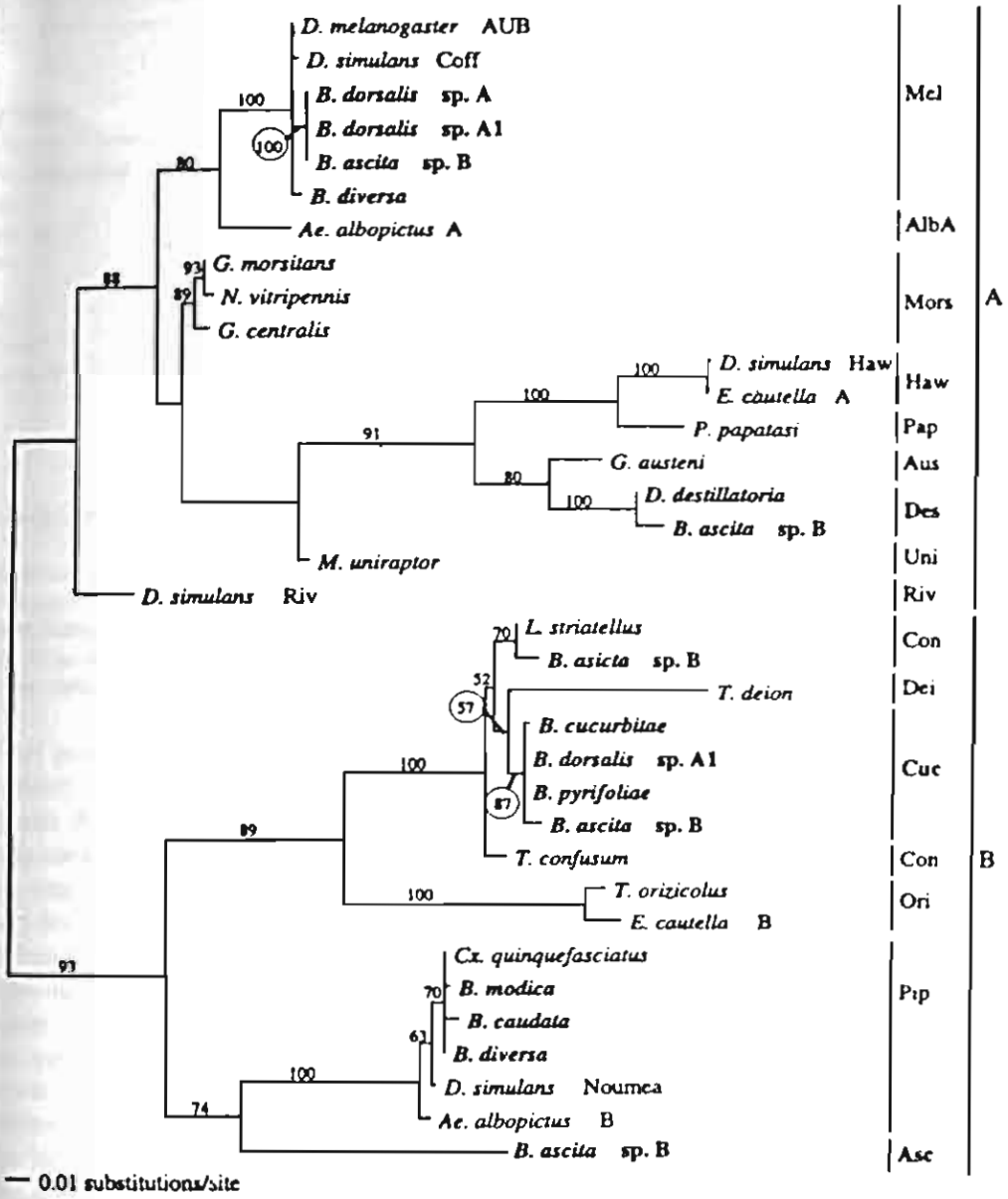

Fig 1. Maximum likelihood tree based on *wsp* gene sequences showing the relationships among arthropod hosts and *Wolbachia*. Tree generated by step-wise addition method using HKY model with consideration of gamma-distributed rate heterogeneity across sites and was midpoint rooted. Bootstrap values (500 replicates) are labeled above branches. The fruit fly hosts, *Wolbachia* strains, and new *Wolbachia* subgroups are represented in bold.

cloned *wsp* fragments indicated that nearly all of these inconsistencies could be explained by cross-reacting primers used in PCR typing. The only case that could not be explained this way was *B. pyrifoliae*, where PCR typing indicated the presence of a *Dei* group infection, but sequence analysis of cloned fragments could not detect a *Dei* group infection, and *Dei* group PCR primers did not cross-react with plasmids containing the only *wsp* fragment found in this species. It is possible in this case that another infection may exist in this species, but we could not detect it in the sequence analysis we did.

Discussion

In this study, we found 15 *Wolbachia* strains infecting nine fruit flies species. Five *Wolbachia* infections were found to simultaneously infect *B. ascita* sp. B, and two infected hosts (*B. diversa* and *B. dorsalis* sp. A1) each harbored two *Wolbachia* strains. Examination of the phylogenetic tree in Fig. 1 shows that the five infections of *B. ascita* sp. B are all divergent, implying that each strain was independently acquired. This is in contrast to a recent report suggesting seven *Wolbachia ftsZ* gene sequences in the beetle *Byturus tomentosus* [9]. In this case the different sequences constituted a monophyletic clade that would suggest a radiation from a single infection event. It is also not clear to what extent these latter sequences represent within-strain variation or actually represent distinct strains. Laboratory studies had indicated that *Drosophila* hosts could maintain three infections with no competitive effects between individual strains [11], suggesting that higher-order infections

ble 3. Group typing *Wolbachia* strains with specific *wsp* primers [20]

uit fly species	PCR typing of total DNA from insect hosts[a]	Strain typing by sequence analysis	No. of clones sequenced	PCR typing of plasmids carrying individual clones used from previous sequence analysis (no. of positive clones)
trocera cucurbitae	*Con*	*Cuc*	3	*Con* (3)
caudata	*Pip*	*Pip*	3	*Pip* (3)
dorsalis sp. A[b]	*Dei, Pip*	*Mel*	3	*Mel* (3), *Dei* (3), *Pip* (0)
diversa	No amplification	*Pip*	3	*Pip* (3), *Mel* (0)
		Mel	3	*Pip* (0), *Mel* (3)
modica	*Pip*	*Pip*	3	*Pip* (3)
pyrifoliae[b]	*Dei, Pip*	*Cuc*	3	*Con* (3), *Dei* (0), *Pip* (0)
ascita sp. B[c]	No amplification	*Mel*	3	*Mel* (3), *Con* (1), *Pip* (0), *Aus* (0)
		Des	3	*Mel* (0), *Con* (3), *Pip* (0), *Aus* (3)
		Con	3	*Mel* (0), *Con* (3), *Pip* (0), *Aus* (0)
		Cuc	3	*Mel* (0), *Con* (3), *Pip* (0), *Aus* (0)
		Asc	3	*Mel* (0), *Con* (2), *Pip* (0), *Aus* (0)
dorsalis sp. A1[b]	No amplification	*Mel*	5	*Mel* (5), *Con* (5)
		Cuc	3	*Mel* (0), *Con* (3)
cus destillatoria	*Aus, Dei*	*Des*	3	*Aus* (3), *Dei* (3)

ata from Kittayapong et al. [8].
lember of the *B. dorsalis* complex [1, 4].
Volbachia multiply infected tephritid fruit fly species.

ould be possible. In this case, the presence of five dependently acquired infections in field-caught tephrit- s suggests that at least five independent infections must e able to coexist without detrimental competitive effects tween strains. It is not clear if this represents an upper undary for *Wolbachia* superinfections or if other cases ill be found in which even higher order of superinfec- ons with distinct strains exist. Future studies are needed at assess the transmission efficiency of these strains tween generations as well as the relative densities of ch strain in different strain backgrounds so as to pro- de evidence for possible competitive effects.

The high level of multiple infections found in some phritid species could be explained by several hypoth- es. For example, it may be possible that certain te- hritid species are predisposed to invasion of new *Wol- achia* strains through a combination of their habitats id susceptibility to parasitoids or their ability to phys- logically support diverse *Wolbachia* strains. At the esent time, only limited data exist indicating how *Wol- achia* invade new species, but parasitic Hymenoptera e one incriminated vector [19]. Alternatively, insects ay cycle *Wolbachia* infections through time, as has en theoretically proposed [5], with new infections be- g gained and old infections lost. It may be possible that rtain insect hosts maintain higher levels of superinfec- on by a steady accumulation of strains over time with- t any subsequent loss of strains. Alternatively, some uit fly species may be predisposed to environmental uring of strains through exposure to naturally occurring tibiotics.

It has been proposed that *Wolbachia* might act as a speciation agent by generating reproductive isolation [18]. Our observations found different *Wolbachia* strains infecting *B. dorsalis* species complex fruit flies collected from the same host fruit. As such it may be possible that *Wolbachia* has contributed to the generation of reproductive isolation in this complex.

The use of group-specific *wsp* primers was proposed as a fast method for typing *Wolbachia* infections without the need to clone and sequence *wsp* genes from individual hosts [20]. Our data clearly show that the PCR-based typing system cannot adequately predict *Wolbachia* infections in new hosts. We found that some of these primers cross-reacted with other groups with our materials. In fruit fly hosts that had more than one strain of *Wolbachia*, group primers gave no amplification, whereas in single infected hosts, more than one primer set gave PCR amplification (Table 3). For preliminary identification of *Wolbachia*, *wsp* group primers might be used, but they cannot be reliably used without supporting sequence data of *wsp* gene fragments.

ACKNOWLEDGMENTS

We thank John R. Milne and Elizabeth A. McGraw for helpful suggestions and for reviewing the manuscript; Saen Tigvattananont for collecting part of the original samples; Samnieng Theinthong and 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 /01/2541, RGJ/PHD/0095/2541 and BGJ/16/2543).

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Molecular Ecology (2003) 12, 1049–1060

Wolbachia infection complexity among insects in the tropical rice-field community

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Abstract

Wolbachia are a group of intracellular bacteria that cause reproductive alterations in their arthropod hosts. Widely discordant host and *Wolbachia* phylogenies indicate that horizontal transmission of these bacteria among species sometimes occurs. A likely means of horizontal transfer is through the feeding relations of organisms within communities. Feeding interactions among insects within the rice-field insect community have been well documented in the past. Here, we present the results of a polymerase chain reaction-based survey and phylogenetic analysis of *Wolbachia* strains in the rice-field insect community of Thailand. Our field survey indicated that 49 of 209 (23.4%) rice-field insect species were infected with *Wolbachia*. Of the 49 infected species, 27 were members of two feeding complexes: (i) a group of 13 hoppers preyed on by 2 mirid species and parasitized by a fly species, and (ii) 2 lepidopteran pests parasitized by 9 wasp species. *Wolbachia* strains found in three hoppers, *Recilia dorsalis*, *Nephotettix malayanus* and *Nisia nervosa*, the two mirid predators, *Cyrtorhinus lividipennis* and *Tytthus chinensis*, and the fly parasitoid, *Tomosvaryella subvirescens*, were all in the same *Wolbachia* clade. In the second complex, the two lepidopteran pests, *Cnaphalocrocis medinalis* and *Scirpophaga incertulas*, were both infected with *Wolbachia* from the same clade, as was the parasitoid *Tropobracon schoenobii*. However, none of the other infected parasitoid species in this feeding complex was infected by *Wolbachia* from this clade. Mean (± SD) genetic distance of *Wolbachia wsp* sequences among interacting species pairs of the hopper feeding complex (0.118 ± 0.091 nucleotide sequence differences), but not for the other two complexes, was significantly smaller than that between noninteracting species pairs (0.162 ± 0.079 nucleotide sequence differences). Our results suggest that some feeding complexes, such as the hopper complex described here, could be an important means by which *Wolbachia* spreads among species within arthropod communities.

Keywords: endosymbiont, horizontal transfer, natural enemies, rice pests, *Wolbachia*

Received 2 October 2002; revision received 9 December 2002; accepted 6 January 2003

Introduction

Endosymbiotic bacteria of the genus *Wolbachia* are widespread in insect species. Recent polymerase chain reaction (PCR)-based surveys for *Wolbachia* using a standard PCR approach have detected these bacteria in 16.9% of neotropical insects (Werren *et al.* 1995a), 50% of Indo-Australian ants (Wenseleers *et al.* 1998), 53.6% of Cynipid gall wasps (Plantard *et al.* 1999), 28.1% of southeast Asian mosquitoes (Kittayapong *et al.* 2000a), 28.3% of tephritid fruit flies (Kittayapong *et al.* 2000b) and 19.3% of temperate North American insects (Werren & Windsor 2000), although *Wolbachia* has not been detected in some surveyed insect groups, for example, aphids and aphid parasitoids (West *et al.* 1998), and thelytokous social Hymenoptera (Wenseleers & Billen 2000). Using a long PCR approach, i.e. one in which two polymerase enzymes were used, 76% of tested insect species were found to be infected with *Wolbachia* (Jeyaprakash & Hoy 2000). Long PCR is more sensitive than standard PCR for amplifying *Wolbachia* sequences (Jeyaprakash & Hoy 2000), probably

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resulting in the high proportion of *Wolbachia*-infected species detected.

Sequencing of *Wolbachia* genes from different host species has revealed patterns of closely related *Wolbachia* strains distributed across widely divergent hosts (O'Neill *et al.* 1992; Rousset *et al.* 1992; Werren *et al.* 1995b; Schilthuizen & Stouthamer 1997). Horizontal transmission between insect species has been hypothesized to explain this lack of concordance between host and *Wolbachia* phylogenies.

For *Wolbachia* transmission between species, an ecological interaction between the two species is necessary to provide the means of transfer (Hurst *et al.* 1992). The types of interaction that have received the most research attention as a potential means of *Wolbachia* horizontal transmission are those between parasitoids and their hosts. *Wolbachia* has, indeed, been found to infect both hosts and parasitoids. Thus, West *et al.* (1998) detected *Wolbachia* in 38.1% of leaf-mining Gracillarid moths and 27.8% of their associated wasp parasitoids, whereas Schilthuizen & Stouthamer (1997) found *Wolbachia* in four of the seven wasp parasitoids associated with the galls of the Cynipid wasp, *Diplolepis rosae*. However, no close relationships were detected using phylogenetic analysis between *Wolbachia* strains in the hosts and parasitoids of either study. In contrast, Vavre *et al.* (1999) determined close relationships between several *Wolbachia* strains infecting *Drosophila* species and their wasp parasitoids. Werren *et al.* (1995b), and Van Meer *et al.* (1999) each reported a close phylogenetic relationship between a parasitoid and its host. In only one case, reported in Heath *et al.* (1999), has horizontal transmission between a host and its parasitoid been shown in the laboratory.

Feeding interactions other than that of parasitoid–host relationships may potentially result in *Wolbachia* transmission between species and remain to be explored. The insect community of a rice field is perhaps one of the best studied insect communities with numerous feeding interactions among insect species that have been well documented (e.g. Yasumatsu *et al.* 1982; Heong *et al.* 1991; Ooi & Shepard 1994). Here, we present the results of a PCR-based survey and phylogenetic analysis of *Wolbachia* among the rice-field insects of Thailand. In particular, we identify participant species in interactions within the rice insect community, which are infected with closely related *Wolbachia* strains. Such interactions may have been in the past, or may still be, important for the horizontal transmission of these bacteria within the rice-field insect community.

Materials and methods

Collection of rice insects

Insects were collected from rice fields in 29 provinces in different regions of Thailand between June 1997 and October 2000 using simple sweep nets and aspirators. All specimens were frozen in dry ice and transported to the laboratory in Bangkok where adults were identified using morphological keys (Barrion & Litsinger 1994; Roffey 1979; Reissig *et al.* 1985; Shepard *et al.* 1987; Wilson & Claridge 1991). Only adult specimens were tested for *Wolbachia*. Some specimens were stored at –70 °C before they were tested.

DNA extraction

Individual insect specimens were dissected for ovaries or testes in distilled water using sterile dissecting equipment. For very small insects, whole bodies were used for DNA extraction. The DNA extraction method followed O'Neill *et al.* (1992). Whole bodies or gonads of each insect were ground using a hand-held polypropylene homogenizer in a 1.5-mL microcentrifuge tube filled with 100 µL of STE buffer (100 mM NaCl, 1 mM EDTA, pH 8.0, 10 mM Tris–HCl, pH 8.0). The homogenate was heated at 95 °C for 10 min and then centrifuged at 1400 *g* for 1 min. One microlitre of the supernatant was used to PCR screen for *Wolbachia*.

Polymerase chain reaction

PCRs were performed in 20 µL reaction volumes: 50 mM Tris (pH 9.2), 16 mM ammonium sulfate, 1.75 mM $MgCl_2$, 350 µM dNTPs, 0.5 µL of 20 µM forward and reverse primers (Jeyaprakash & Hoy 2000), 1 unit of *Pwo* and 5 units of *Taq* DNA polymerase. The *ftsZ* primers for long PCR were used to screen for *Wolbachia* infection and primers based on the mitochondrial 12S rDNA gene of host insects were used to check for the quality of DNA extraction. Samples negative for 12S rRNA were discarded. The thermal profile for DNA amplification was according to Jeyaprakash & Hoy (2000). Ten microlitres of each PCR product were run on a 1% agarose gel stained with ethidium bromide and visualized under a UV transilluminator to determine the presence and size of amplified DNA. One specimen of each species that was *Wolbachia*-negative using the standard PCR method described above but positive for 12S rRNA when tested again for *Wolbachia* using long PCR (Jeyaprakash & Hoy 2000). DNA extracts of *Wolbachia*-infected *Aedes albopictus* were used as positive controls. Negative controls containing only double-distilled water were also included to check for contamination.

Cloning and sequencing

Insect samples that were positive using *ftsZ* primers were reamplified with *wsp* primers using long PCR with the PCR conditions described above (Jeyaprakash & Hoy 2000). PCR products were cloned into a plasmid (pGEM-T vector; Promega) and transformed into *Escherichia coli* cells.

asmids with inserts were then purified using QIAprep bin Miniprep Kit (Qiagen) and sequenced on an ABI utomated sequencer. At least three independent clones ere sequenced from each *Wolbachia* strain in order to lentify polymerase errors. Both strands of the plasmids ere sequenced using T7 and SP6 primers. Consensus equences from each *Wolbachia* strain were constructed nd used for subsequent analyses.

hylogenetic analysis

he *wsp* sequences from this study as well as sequences lescribed previously (Zhou *et al.* 1998) were used in this nalysis. All *wsp* sequences were aligned using the LUSTAL algorithm followed by manual modification ased on amino acid translation of the different genes. The hird hypervariable region of the gene (positions 519–559) Braig *et al.* 1998) was excluded from the analysis because t could not be aligned with confidence. The *wsp* sequences rom this study have been deposited in GenBank under ccession number AF481160–AF481202.

Phylogenies were generated using both maximum parsimony and distance (neighbour-joining; Saitou & Nei 1987) methods using PAUP* 4.0 (Swofford 2000). In the maximum parsimony analysis, heuristic searches were performed, gaps were treated as missing data and bootstrap analysis was carried out with 1000 replications. A neighbour-joining tree was generated using a HKY distance model with consideration of the proportion of invariable sites and of the gamma shape parameter which was chosen using the MODELTEST program (Posada & Crandall 1998). The neighbour-joining tree was assessed using 1000 bootstrap replicates.

Feeding relationships of Wolbachia*-infected insects*

The feeding relations of those insect species on rice plants infected with *Wolbachia* were determined from Yasumatsu *et al.* (1982) and Ooi & Shepard (1994). Although only adults were tested for *Wolbachia*, it is often the larval stage that is the important feeding stage on rice. Where appropriate, therefore, the larval stage was used to indicate the feeding relations of an insect species within the rice-field community. Insect species were classified as rice herbivores, generalist predators, specialist predators or specialist parasitoids. Specialist predators/parasitoids were linked with their prey/hosts.

Results

A total of 2585 insects from tropical rice fields comprising 209 species were tested for *Wolbachia* (Table 1). Forty-nine species (23.4%) were found to be infected. These infected species represented the seven orders, Coleoptera (7.1% of species infected), Diptera (30.8%), Hemiptera (11.6%), Homoptera (54.2%), Hymenoptera (31.0%), Lepidoptera (40.0%) and Orthoptera (18.2%). Most insects that were collected were from these orders. Few insects (< 4) were collected from each of the other five orders, Blattaria, Dermaptera, Ephemeroptera, Mantodea and Thysanoptera, and none of these insects was infected.

Twenty-five of the 49 insect species (51.0%) infected with *Wolbachia* were rice herbivores and 17 (34.7%) were predators or parasitoids (Fig. 2). The feeding relations of seven species were unknown. Of the predator species, four were generalists (Fig. 2B), whereas two (mirid bugs, Fig. 2A) were specialists that fed on planthopper and leafhopper eggs. Most (9/11) parasitoid species parasitized larvae of two pest Lepidoptera, i.e. the leaffolder and the rice yellow stem borer. One of these parasitoids, i.e. *Trichomalopsis apanteloctena*, is also a secondary parasitoid of the wasp, *Goniozus* sp., which in turn parasitizes leaffolder larvae. However, two parasitoids, the pipunculid fly, *Tomosvaryella subvirescens*, and the wasp, *Opius* sp., parasitize non-lepidopteran hosts, i.e. *Nephotettix* spp. and *Hydrellia* spp., respectively. In total, 29 of the 49 infected species (59.2%) are participants in known feeding interactions between species (Fig. 2A). The remaining infected species (Fig. 2B) may be involved in feeding interactions with other infected species, e.g. as predator or prey, but information concerning the feeding relations of these species was unavailable.

Partial sequences of the 43 *wsp* genes amplified from each of the insect host species together with 16 sequences described previously (Zhou *et al.* 1998) were analysed using both maximum parsimony and distance methods. Phylogenetic trees generated using both phylogenetic methods produced similar tree topologies. There were 200 most parsimonious trees with 944 steps. The tree calculated using the neighbour-joining method is shown in Fig. 1, which also shows neighbour-joining and maximum parsimony bootstrap support for the different clades.

Of 43 *Wolbachia* strains found in insects in the rice-field community, 29 were in the B supergroup and the remaining 14 were in the A supergroup (Fig. 1). The black bug, *Scotinophara coarctata* was found to be infected with three *Wolbachia* strains, each from a different clade.

Most *Wolbachia* strains found in planthoppers and leafhoppers as well as their mirid predators and the hopper parasitoid, *Tomosvaryella subvirescens* (Fig. 2A) were in the B supergroup. The leafhopper, *Recilia dorsalis*, however, was doubly infected with both A and B supergroups. Identical *wsp* sequences were found in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*.

A close relationship among *Wolbachia* strains was also found for the wasp parasitoid, *Tropobracon schoenobii* and its two lepidopteran rice pest hosts, *Cnaphalocrosis medinalis* and *Scirpophaga incertulus*. All three strains were in the same clade with sequence differences of 0.37 and 0.56%, respectively (Fig. 1). *Wolbachia* strains were found in both

Table 1 PCR-based distribution of *Wolbachia* in insects collected from rice fields in Thailand from January 1998 to November 2000

Order and species	Common name	Total no. tested	No. positive	*Wolbachia* strain	Accession no.
Blattaria					
Blattella germanica (Linnaeus)	German cockroach	3	0		
Coleoptera					
Adoretus sp.	Scarab beetle	4	0		
Aeoloderma brachmana (Candeze)	Click beetle	2	0		
Anomala humeralis (Burmeister)	Scarab beetle	3	0		
Apalochrus rufofasciatus Pic	Soft-winged flower beetle	2	0		
Berosus sp.	Water scavenger beetle	8	0		
Brumoides lineatus (Weise)	Lady beetle	2	0		
Brumoides suturalis (Fabricius)	Lady beetle	1	0		
Chlaenius circumdatus (Brulle)	Ground beetle	1	0		
Chlaenius quadricolor Fabricius	Ground beetle	6	0		
Chlaenius sp. 1	Ground beetle	3	0		
Chlaenius sp. 2	Ground beetle	3	B (1)*	–	–
Chlaenius sp. 3	Ground beetle	3	0		
Chlaenius sp. 4	Ground beetle	1	0		
Chlaenius sp. 5	Ground beetle	3	0		
Cicindela sp.	Tiger beetle	1	0		
Coccinella repanda Thunberg	Lady beetle	2	0		
Dicladispa armigera (Oliver)	Hispa beetle	12	B (1)†	wDic	AF481160
Drypta japonica (Bates)	Ground beetle	1	0		
Echinocnemus oryzae Marshall	Weevil	2	0		
Ectomocoris biguttulus Stal	Ground beetle	1	0		
Formicomus braminus La Ferte Senectere	Anthicid beetle	6	0		
Formicomus sp.	Anthicid beetle	1	0		
Harmonia octomaculata (Fabricius)	Lady beetle	5	0		
Hydronomidius molitor Faust	Paddy-root weevil	1	A (1)†	wMol	AF481161
Hydrophilus acuminatus Motschulsky	Water scavenger beetle	7	0		
Lissorhoptrus oryzophilus Kuschel	Weevil	2	0		
Micraspis discolor (Fabricius)	Lady beetle	131	0		
Micraspis tenera (Gorham)	Lady beetle	5	0		
Monolepta signata Olivier	Leaf beetle	33	0		
Ophionea indica (Thunberg)	Ground beetle	15	0		
Ophionea ishii hoshii Habu	Ground beetle	1	0		
Ophionea ishii ishii Habu	Ground beetle	2	0		
Ophionea nigrofasciata Schmidt-Goebel	Ground beetle	4	0		
Oulema sp.	Leaf beetle	1	0		
Paederus fuscipes Curtis	Rove beetle	17	0		
Paederus tamulus Erichson	Rove beetle	2	0		
Paederus sp.	Rove beetle	4	0		
Pentagonica sp.	Ground beetle	5	0		
Pheropsophus sp.	Ground beetle	1	0		
Propylaea japonica (Thunberg)	Lady beetle	1	0		
Scarites sp.	Ground beetle	3	0		
Sulcacis latifrons (Dejean)	Ground beetle	7	0		
Dermaptera					
Euborellia philippinensis Srivastava	Earwig	1	0		
Diptera					
Agromyza oryzae (Munakata)	Leaf-miner fly	2	B (2)†	wAory	AF481201
Argyrophylax nigrotibialis Baranov	Tachinid fly	8	0		
Argyrophylax plueda Townsend	Tachinid fly	6	0		
Argyrophylax sp.	Tachinid fly	7	0		
Carcelia sp.	Tachinid fly	1	0		
Ceracia sp.	Tachinid fly	1	0		
Ceratitis sp.	Fruit fly	3	0		
Chironomus dorsalis Meigen	Midge	1	0		
Chironomus kiiensis Tokunaga	Midge	7	0		
Chironomus tepperi Skuse	Midge	3	0		
Chironomus sp. 1	Midge	6	A (1)*	–	–

ble 1 *Continued*

der and species	Common name	Total no. tested	No. positive	*Wolbachia* strain	Accession no.
Chironomus sp. 2	Midge	12	0		
Chironomus sp. 3	Midge	10	0		
Chironomus sp. 4	Midge	4	0		
Chlorops oryzae Matsumura	Chloropid fly	15	A (3)*	–	–
Diopsis apicalis Dalman	Stalk-eyed borer	3	A (2)†	wApi	AF481162
Drapetis sp.	Humpbacked fly	3	0		
Hydrellia sp. 1	Whorl maggot	7	A (2)†	wHyd	AF481163
Hydrellia sp. 2	Whorl maggot	9	0		
Hydrellia sp. 3	Whorl maggot	12	0		
Hydrellia sp. 4	Whorl maggot	3	0		
Notiphila sp.	Ephydrid fly	2	0		
Ochthera brevitibialis de Meijere	Ephydrid fly	5	0		
Orseolia oryzae (Wood-Mason)	Gall midge	38	B (7)†	wOry	AF481164
Paleosarcophaga sp.	Tachinid fly	2	0		
Pipunculus javanensis de Meijere	Pipunculid fly	4	0		
Pipunculus orientalis (Koizumi)	Pipunculid fly	1	0		
Pipunculus sp.	Pipunculid fly	10	0		
Poecilotraphera taeniata (Macquart)	Picture-winged fly	7	0		
Psilopa sp.	Ephydrid fly	1	0		
Tipula aino Alexander	Crane fly	17	B (14)†	wAin	AF481165
Tomosvaryella subvirescens (Loew)	Pipunculid fly	1	B (1)*	wPyr	AF481166
phemeroptera					
Ephemera sp.	Mayfly	1	0		
emiptera					
Arthus indicus (Westwood)	Burrower bug	8	0		
Andrallus spinidens (Fabricius)	Stink bug	1	0		
Anisops sardea (Herr-Schaff)	Back swimmer	16	0		
Cletus trigonus (Thunberg)	Coreid bug	1	0		
Cyrtorhinus lividipennis Reuter	Mirid bug	221	B (51)†	wLiv	AF481167
Dysdercus sp.	Red bug	1	0		
Eysarcoris ventralis Distant	Stink bug	6	0		
Geotomus pygmaeus (Dallas)	Burrower bug	8	0		
Geotomus sp.	Burrower bug	1	0		
Hydrometra vittata Stal	Marsh treader	4	0		
Leptocorisa acuta (Thunberg)	Rice stink bug	1	0		
Leptocorisa oratorius (Fabricius)	Rice stink bug	49	0		
Limnogonus fossarum (Fabricius)	Water strider	3	0		
Limnogonus nitidus (Mayr)	Water strider	2	0		
Lygaeus hospes Fabricius	Lygaeid bug	2	0		
Menida formosa (Westwood)	Stink bug	2	0		
Mesovelia sp.	Water treader	2	0		
Micronecta quadristrigata Breddin	Water boatman	8	0		
Mononyx sp.	Toad bug	1	0		
Nabis sp. 1	Damsel bug	3	0		
Nabis sp. 2	Damsel bug	22	0		
Nezara viridula (Linnaeus)	Green stink bug	3	0		
Ninus insignis Stal	Lygaeid bug	1	? (1)‡	–	–
Pachybrachius pacificus Stal	Lygaeid bug	1	0		
Pachybrachius sobrinus (Distant)	Lygaeid bug	1	0		
Paraeucosmetus pallicornis (Dallas)	Lygaeid bug	8	A, B (1)*	–	–
Polytoxus sp. 1	Assassin bug	5	0		
Polytoxus sp. 2	Assassin bug	2	0		
Polytoxus sp. 3	Assassin bug	10	0		
Pygomenida sp. 1	Stink bug	1	0		
Pygomenida sp. 2	Stink bug	1	0		
Pygomenida sp. 3	Stink bug	4	0		
Rhinocoris sp.	Assassin bug	1	0		
Riptortus linearis (Fabricius)	Squash bug	2	0		

Table 1 *Continued*

Order and species	Common name	Total no. tested	No. positive	*Wolbachia* strain	Accession no.
Riptortus pedestris (Fabricius)	Squash bug	1	0		
Saldula sp.	Shore bug	6	0		
Scotinophara coarctata (Fabricius)	Black bug	18	A, B (5)†	wCoaR	AF481168
				wCoaA	AF481169
				wCoaC	AF481170
Scotinophara inermiceps (Breddin)	Black bug	1	0		0
Scotinophara scotti Horvath	Black bug	1	0		0
Scutellera nobilis Fabricius	Shield-backed bug	1	0		0
Tetroda histeroides Fabricius	Stink bug	1	0		0
Tetroda transversalis Westwood	Stink bug	8	0		0
Tytthus chinensis Stal	Mirid bug	15	B (1)†	wChi	AF481171
Homoptera					
Balclutha sp.	Leafhopper	25	B (7)†	wBal	AF481172
Cicadulina bipunctata (Melichar)	Leafhopper	10	0	–	
Cofana spectra (Distant)	Leafhopper	33	B (1)†	wSpe	AF481173
Cofana unimaculata (Signoret)	Leafhopper	7	0		
Empoascanara alami (Ahmed)	Leafhopper	37	B (1)†	wAla	AF481174
Exitianus sp.	Planthopper	8	0		
Hecalus sp.	Leafhopper	2	0		
Hysteroneura setariae (Thomas)	Aphid	9	0		
Laodelphax striatellus (Fallen)	Planthopper	6	B (1)†	wStri	AF481175
Macrosteles striifrons Anufriev	Leafhopper	4	0		
Nephotettix malayanus Ishihara & Kawase	Green leafhopper	6	B (2)†	wMal	AF481176
Nephotettix nigropictus (Stal)	Green leafhopper	46	B (3)†	wNig	AF481177
Nephotettix virescens (Distant)	Green leafthopper	292	B (35)†	wVirP	AF481178
				wVir	AF481179
Nilaparvata bakeri (Muir)	Brown planthopper	9	B (3)†	wBak	AF481180
Nilaparvata lugens (Stal)	Brown planthopper	237	B (39)†	wLug	AF481181
Nisia nervosa (Motschulsky)	Planthopper	15	B (7)†	wNer	AF481182
Olurus sp.	Planthopper	1	0		
Poophilus costalis Walker	Leafhopper	5	0		
Pyrilla perpusilla (Walker)	Planthopper	3	0		
Recilia dorsalis (Motschulsky)	Zig-zag leafhopper	116	A, B (6)†	wRecM	AF481183
				wRecC	AF481184
Sogatella furcifera (Horvath)	White-backed planthopper	275	B (90)†	wFur	AF481185
Stirellus sp.	Green leafhopper	6	? (2)‡	–	–
Tagosodes pusanus (Distant)	Leafhopper	1	0		
Thaia oryzivora Ghauri	Orange-headed leafhopper	45	0		
Hymenoptera					
Amauromorpha sp.	Parasitic wasp	1	0		
Brachymeria excarinata Gahan	Chalcidid wasp	4	A (2)†	wExc	AF481186
Brachymeria lasus (Walker)	Chalcidid wasp	3	0		
Brachymeria megaspila Cameron	Chalcidid wasp	1	0		
Camponotus sp.	Ant	5	0		
Campsomeris sp.	Parasitic wasp	1	0		
Charops brachypterum (Cameron)	Parasitic wasp	2	0		
Charops sp.	Parasitic wasp	1	0		
Copidosomopsis nacoleiae (Eady)	Parasitic wasp	1	0		
Cotesia angustibasis (Gahan)	Parasitic wasp	4	0		
Cotesia cypris Nixon	Parasitic wasp	1	? (1)‡	–	–
Cotesia flavipes Cameron	Parasitic wasp	3	A (1)†	wFla	AF481187
Diacamma sp.	Ant	3	0		
Elasmus philippinensis Ashmead	Parasitic wasp	1	0		
Gambrus sp.	Parasitic wasp	2	0		
Goniozus nr. *triangulifer* Kieffer	Parasitic wasp	2	0		
Goniozus sp.	Parasitic wasp	5	B (1)†	wGon	AF481188
Macrocentrus philippinensis Ashmead	Parasitic wasp	5	A (1)†	wMph	AF481189
Macrocentrus sp.	Parasitic wasp	4	0		

ble 1 *Continued*

der and species	Common name	Total no. tested	No. positive	*Wolbachia* strain	Accession no.
Neanastatus sp.	Eupelmid wasp	1	0		
Oecophylla smaragdina Fabricius	Ant	4	0		
Opius sp.	Parasitic wasp	13	B (1)†	wDveP	AF481190
Platygaster oryzae Cameron	Parasitic wasp	2	0		
Polyrachis sp. 1	Ant	1	0		
Polyrachis sp. 2	Ant	1	? (1)‡	—	—
Polyrachis sp. 3	Ant	1	0		
Polyrachis sp. 4	Ant	3	0		
Prosevania sp.	Ensign wasp	1	A (1)‡	wPro	AF481202
Ropalidia cyathiformis (Fabricius)	Yellow jacket	4	0		
Ropalidia sp.	Yellow jacket	1	0		
Sceliphron madraspatanum canapicillatum (Costa)	Sphecid wasp	1	0		
Solenopsis sp.	Ant	2	A (1)*	—	—
Telenomus rowani (Gahan)	Parasitic wasp	1	0		
Temelucha philippinensis (Ashmead)	Parasitic wasp	13	A (4)†	wPhi	AF481196
Temelucha stangli (Ashmead)	Parasitic wasp	1	0		
Tetrastichus schoenobii Ferriere	Parasitic wasp	2	0		
Trichogramma sp.	Parasitic wasp	3	0		
Trichogrammatoidea sp.	Parasitic wasp	1	0		
Trichomalopsis apanteloctena (Crawford)	Parasitic wasp	2	B (1)†	wApa	AF481191
Trichomma cnaphalocrosis Uchida	Parasitic wasp	15	B (2)†	wCnaM	AF481192
				wCnaC	AF481193
Tropobracon schoenobii (Viereck)	Parasitic wasp	4	B (1)†	wSch	AF481194
Xanthopimpla flavolineata Cameron	Parasitic wasp	7	0		
Lepidoptera					
Chilo suppressalis (Walker)	Rice stem borer	1	0		
Cnaphalocrocis medinalis (Guenee)	Rice leaffolder	86	B (42)†	wMed	AF481195
Marasmia sp.	Grass moth	1	A (1)*	—	—
Melanitis sp.	Green-horned caterpillar	2	0		
Mythimna separata (Walker)	Ear-cutting caterpillar	1	0		
Nymphula depunctalis (Guenee)	Grass moth	4	B (2)*	—	—
Pelopidas mathias (Fabricius)	Skipper	1	0		
Polytremis pellucida (Murray)	Skipper	1	0		
Scirpophaga incertulas (Walker)	Rice yellow stem borer	20	B (2)†	wInsO	AF481197
				wInsP	AF481198
Sesamia inferens (Walker)	Pink stem borer	4	0		
Mantodea					
Hierodula sp.	Praying mantid	1	0		
Orthoptera					
Acrida willemsei Dirsh	Grasshopper	5	0		
Anaxipha longipennis (Serville)	Cricket	6	A (4)†	wLon	AF481199
Ceracris fasciata (Brunner)	Grasshopper	1	0		
Conocephalus longipennis (de Haan)	Grasshopper	53	0		
Euscyrtus concinnus (Haan)	Cricket	27	0		
Gryllotalpa orientalis Burmeister	Mole cricket	1	0		
Hieroglyphus banian (Fabricius)	Grasshopper	1	0		
Metioche vittaticolis (Stal)	Cricket	5	0		
Oxya japonica japonica (Thunberg)	Grasshopper	44	0		
Teleogryllus sp.	Cricket	1	A (1)†	wTel	AF481200
Tetrigidea sp.	Pygmy grasshopper	2	0		
Thysanoptera					
Stenchaetothrips biformis (Bagnall)	Rice thrips	1	0		

Wolbachia group described by A-B group specific primers (Zhou *et al.* 1998).
†*Wolbachia* group described by sequence typing.
‡Unidentified *Wolbachia* group.

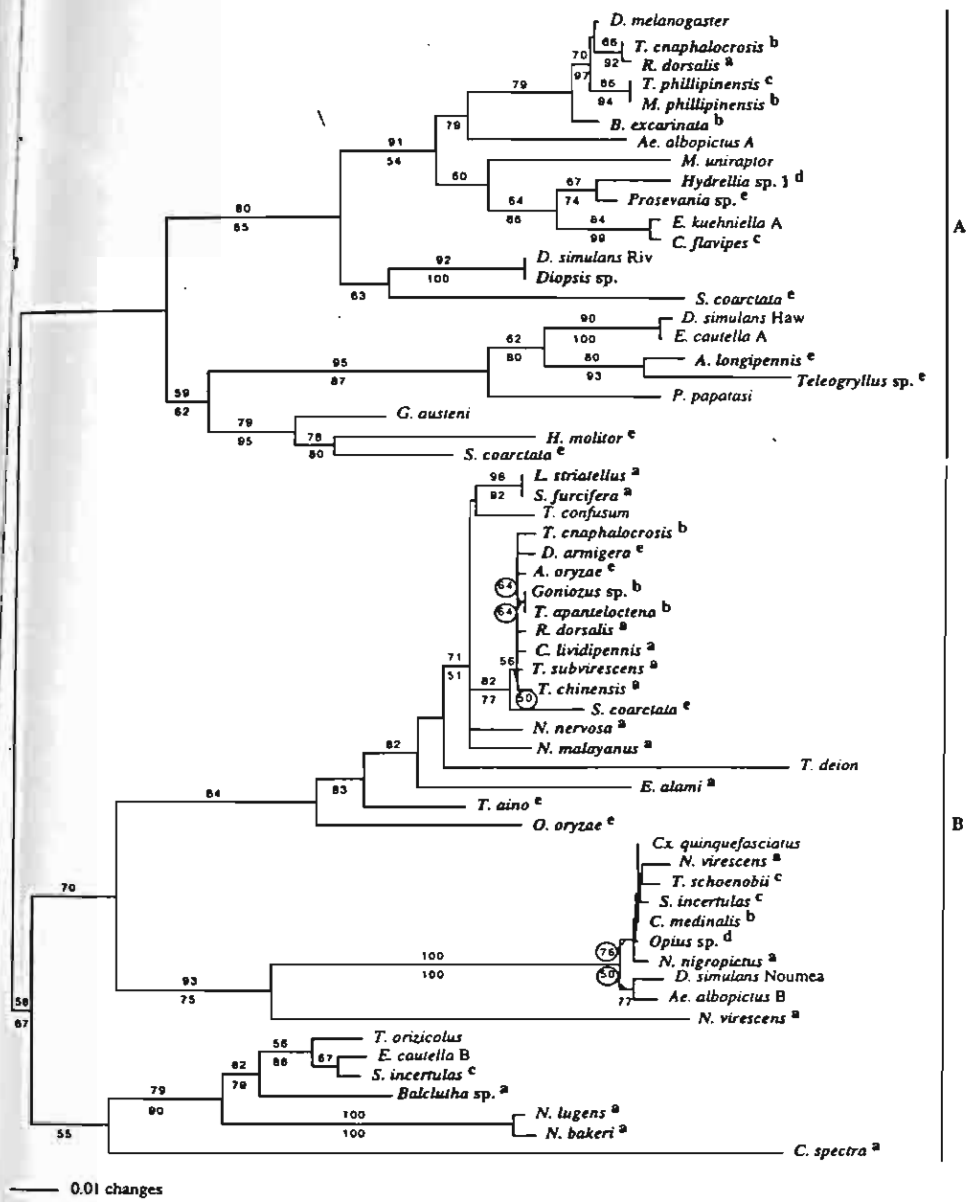

Fig. 1 Phylogenetic relationships of *Wolbachia* strains found in insects of the rice field community. The figure shows the neighbour-joining tree based on *wsp* gene sequences. Bootstrap values (1000 replicates) for nodes are shown for both neighbour-joining (above the branch) and maximum parsimony (below the branch) analyses. *Wolbachia* strains are named according to their insect host and are represented in bold. Superscript letters: a represents *Wolbachia* strains from the hopper feeding complex, b represents *Wolbachia* strains from the leaf-folder feeding complex, c represents *Wolbachia* strains from the yellow stem borer feeding complex, d represents *Wolbachia* strains from the whorl maggot feeding complex, and e represents *Wolbachia* strains from rice-field insects with uncertain feeding relationships.

A and B supergroups for both hosts and parasitoids. Identical *wsp* sequences were found in *Temelucha philippinensis* and *Macrocentrus philippinensis*, although they parasitize different hosts, i.e. *S. incertulas* and *C. medinalis*, respectively. No relationship was found between the *Wolbachia* strains in the whorl maggot, *Hydrellia* sp., and its parasitoid, *Opius* sp. (Fig. 1).

A total of 29 *Wolbachia*-infected insect species were grouped into three feeding complexes based on their known feeding relations (Fig. 2A): a hopper feeding complex, a lepidopteran feeding complex based on the rice leaffolder, *C. medinalis*, and the rice yellow stem borer, *S. incertulas*, and a whorl maggot feeding complex. The feeding relations of the remaining 20 infected species (Fig. 2B) were too poorly known for the species to be grouped. The mean *wsp* genetic distance among species pairs that interacted within the hopper feeding complex and within the lepidopteran complex was each statistically compared with the mean genetic distance among all pairs that did not interact within a complex. The whorl maggot complex had too few interactions ($n = 1$) for statistical analysis. For the hopper complex comparison, variances were not homogeneous, so a t-test assuming unequal variances (SPSS 1997) was used to analyse the data. Mean ($\pm$ SD) genetic distance among interacting pairs of the hopper complex (0.118 ± 0.091 nucleotide sequence differences) was found to be significantly smaller than that of all noninteracting pairs (0.162 ± 0.079 nucleotide sequence differences) ($t = 2.905$, d.f. = 38.5, $P = 0.006$). For the lepidopteran complex comparison, variances were homogeneous, so a t-test assuming equal variances (SPSS 1997) was used. Mean genetic distance among interacting pairs within this complex (0.159 ± 0.080 nucleotide sequence differences) was not significantly different to that among

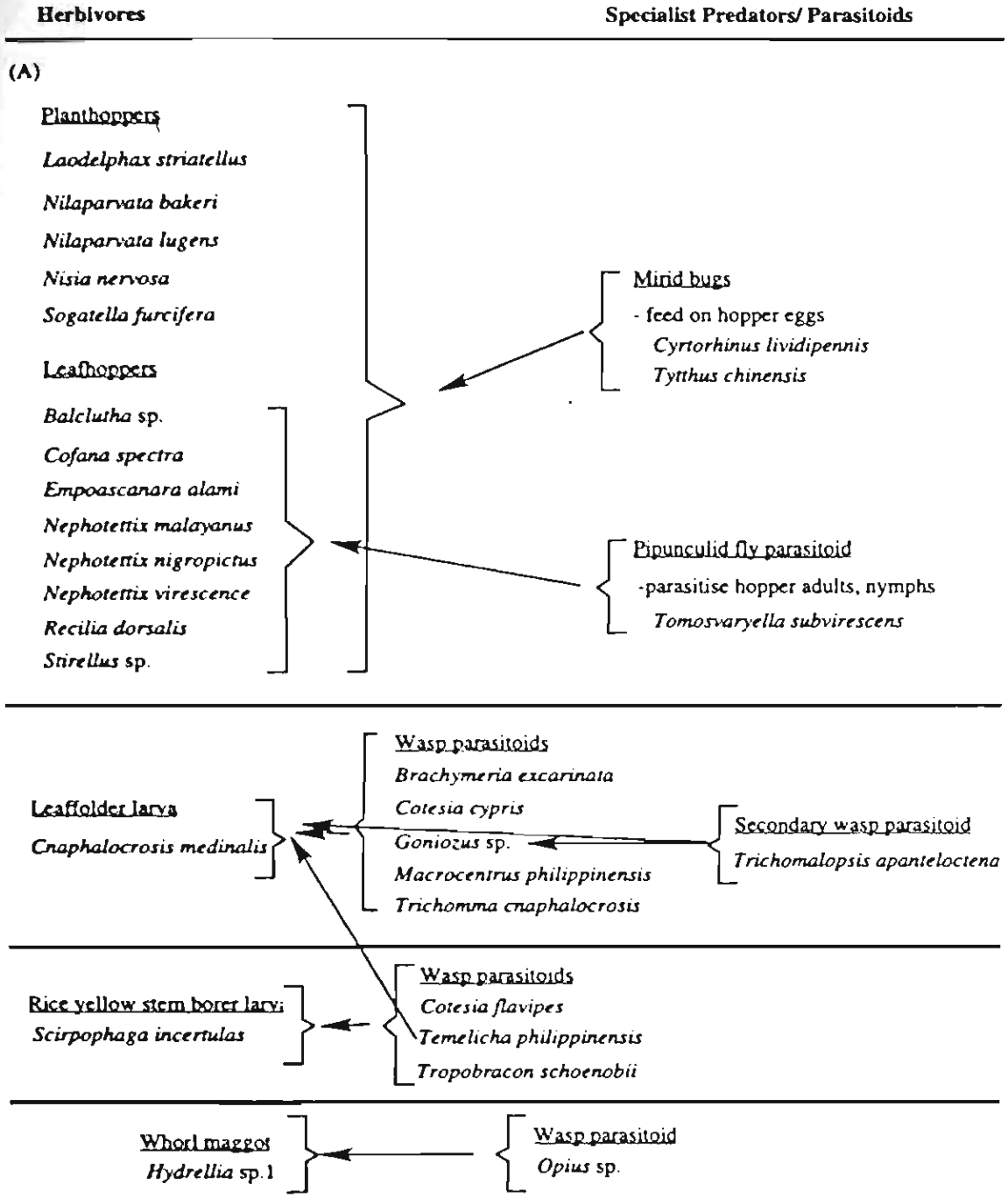

Fig. 2 *Wolbachia*-infected rice insects classified by their feeding relations. Insects were caught as adults. For many species, however, the larval stage is the important feeding stage on rice. For such species, the larval stage is indicated. (A) Interactions between herbivores and specialist predators and parasitoids. The arrow begins at the predator/parasitoid and ends at the prey/host. (B) *Wolbachia*-infected rice insects for which feeding interactions were uncertain or unknown.

noninteracting pairs (0.160 ± 0.070 nucleotide sequence differences) ($t = -0.12$, d.f. = 905, $P = 0.991$).

Discussion

Our field survey has indicated that 23.4% of tropical rice-field insect species are infected with *Wolbachia*, although this percentage would undoubtedly increase if more specimens were tested per species or different methods of DNA extraction or PCR were used. Previous field surveys (Werren *et al.* 1995a; Wenseleers *et al.* 1998; Plantard *et al.* 1999; Kittayapong *et al.* 2000a,b; Werren & Windsor 2000) detected *Wolbachia* in insects from 16.9%(Werren *et al.* 1995a) to 53.6% (Plantard *et al.* 1999) of species.

Among rice-field insects, the order Homoptera had the highest proportion of species (54.2%) infected with

Herbivores	Generalist Predators	Others-feeding relations on rice unknown
(B)		
Black bug *Scotinophora coarctata*		
Fly larvae *Agromyza oryzae* *Chlorops oryzae* *Chaphalocrocis medinalis* *Orseolia oryzae*	Ants *Solenopsis* sp. *Polyrachis* sp. 2 Ground beetle *Chlaenius* sp. 2	Flies *Chironomus* sp. 1 *Diopsis apicalis* *Tipula aino* Moths *Nymphula depunctalis*
Beetle larva *Dicladispa armigera*	Cricket *Anaxipha longipennis*	True bugs *Ninus insignis* *Paraeucosmetus pallicornis*
Moth larva *Marasmia* sp.		Wasp *Prosevania* sp.
Paddy-root weevil *Hydronomidius molitor*		
Cricket *Teleogryllus* sp.		

Fig. 2 *Continued*

Wolbachia. Although *Wolbachia* infection has been reported before for natural populations of the two homopteran species, *Sogatella furcifera* and *Laodelphax striatellus* (Noda *et al.* 2001), extensive surveys for these bacteria in populations of many homopteran species have not been reported previously for this insect order. Werren *et al.* (1995a), West *et al.* (1998) and Werren & Windsor (2000) tested two (F. Cicadidae), four (F. Aphidae) and three (F. Aphidae, F. Cicadellidae, F. Membracidae) homopteran species, respectively, and none of them were positive. Using a long PCR method, Jeyaprakash & Hoy (2000) detected *Wolbachia* in three of seven homopteran species during a survey of mainly laboratory colonies. Therefore, long PCR might improve *Wolbachia* DNA amplification.

The next three orders, in order of species infection rates, were Lepidoptera (40.0%), Hymenoptera (31.0%) and Diptera (30.8%). These same three orders had the highest species infection rates in the survey of Werren *et al.* (1995a), but only Hymenoptera and Orthoptera had species infection rates > 20% in the survey conducted by Werren & Windsor (2000). Hymenoptera are also reported to have high species infection rates in other published surveys. Thus *Wolbachia* has been found in 62.5% of wasp species inhabiting *Diplolepis rosae* galls (Schilthuizen & Stouthamer 1998), 50% of Indo-Australian ant species (Wenseleers *et al.* 1998), 27.8% of leaf-miner parasitoids (West *et al.* 1998) and 59.3% of Cynipid gallwasps (Plantard *et al.* 1999).

The widespread distribution of *Wolbachia* in some orders compared with others may be because species of some orders are more favoured for *Wolbachia* horizontal transmission and establishment of *Wolbachia* infections within new species. A lack of concordance is commonly observed between host and *Wolbachia* phylogenies, which is explained most parsimoniously by extensive horizontal transmission among species (e.g. O'Neill *et al.* 1992; Werren *et al.* 1995b; Zhou *et al.* 1998).

An ecological interaction between an infected and uninfected species is considered necessary for horizontal transmission to occur. A possible route for *Wolbachia* horizontal transmission is through one insect becoming infected when it feeds on another infected insect. In particular, the relatedness of *Wolbachia* between parasitoids and their hosts has been targeted in recent studies. Several phylogenies, in which closely related *Wolbachia* strains occur in both the host and parasitoid species, have been published (e.g. Werren *et al.* 1995b; Van Meer *et al.* 1999; Vavre *et al.*

1999). In addition, a laboratory study (Heath *et al.* 1999) has recently demonstrated that *Wolbachia* transfer from host to parasitoid is possible, although the infection rate eventually decreased to zero with subsequent generations. Based on phylogenetic relations among *Wolbachia* strains, our results suggest several such host–parasitoid interactions in the rice insect community through which horizontal transmission may have occurred in the past or still occur (Fig. 2A). In addition, we found a new possible route of *Wolbachia* transfer between specialist predators, i.e. mirid bugs, and their prey, i.e. leaf- and planthoppers, which may be important for horizontal transmission between predator and prey. This hypothesized route could be either way, i.e. from prey to predator or from predator to prey. Thus, a mirid bug could possibly acquire *Wolbachia* by feeding on infected hopper eggs. Alternatively, probing of hopper eggs by an infected mirid bug might lead to injection of *Wolbachia*-contaminated fluid into an egg.

Within the rice-field insect community it seems that infected species tended to group together such that feeding 'complexes' of *Wolbachia*-infected species were apparent. Planthopper and leafhopper species were all interconnected through the two mirid species, *Cyrtorhinus lividipennis* and *Tytthus chinensis*, which feed on hopper eggs (Fig. 2A). A highly specialized parasitoid, the pipunculid fly, *Tomosvaryella subvirescens*, is also part of this hopper feeding complex by means of parasitizing leafhoppers of the genus, *Nephotettix*, which are among the rice hopper assemblage infected with *Wolbachia*. From our phylogenetic analysis, *Wolbachia* strains found in the hoppers, *Recilia dorsalis*, *Nephotettix malayanus* and *Nisia nervosa*, their egg predators, *Cyrtorhinus lividipennis* and *Tytthus chinensis*, and the *Nephotettix* parasitoid *Tomosvaryella subvirescens*, are all in the same *Wolbachia* clade (Fig. 1). Furthermore, the average genetic distance between interacting pairs in this feeding complex was significantly smaller than for noninteracting pairs, which suggests possible *Wolbachia* horizontal transfer among members of this complex.

Another feeding 'complex' is centred on two lepidopteran larval pests of rice, i.e. larvae of the leaffolder, *Cnaphalocrocis medinalis*, and the rice yellow stem borer, *Scirpophaga incertulas* (Fig. 2A). Larvae of each species are parasitized by several *Wolbachia*-infected parasitoid species, thus indicating possible routes of horizontal *Wolbachia* transfer. The leaffolder and the stem borer were both infected with *Wolbachia* from the same clade, as was the parasitoid, *Tropobracon schoenobii*, which parasitizes larvae of the rice yellow stem borer. However, none of the other infected parasitoid species were infected by *Wolbachia* of this clade. Another parasitoid species, *Trichomalopsis apanteloctena*, parasitizes not only leaffolder larvae, but also larvae of another parasitoid species, *Gonozus* sp., which in turn parasitizes leaffolder larvae. The two parasitoid species have *Wolbachia* strains of the same clade. However, statistical support for this feeding complex being important for *Wolbachia* horizontal transfer was not found.

Although these results point to potential *Wolbachia* horizontal transmission routes, they can, however, only be regarded as speculative for at least three reasons. First, the insect samples were collected from not just one rice field but from 29 rice fields throughout Thailand and, so, infected species may not be directly linked in one area. Second, many insect species would not have been sampled using our methods and, so, key feeding interactions that may be important for horizontal transmission have probably been overlooked. For example, there is good evidence that the strepsipteran endoparasite, *Elenchus japonicus*, may mediate *Wolbachia* transfer between two of the rice planthoppers sampled in this study, *L. striatellus* and *S. furcifera* (Noda *et al.* 2001), but our sampling methods never picked up this parasite. Third, even though rice-field insect communities have been much studied, there are probably still many species whose exact feeding relations are unknown. For example, we recorded seven *Wolbachia*-infected species whose feeding relations in tropical rice fields have not been determined. An understanding of these species place in the rice food web may highlight other potential horizontal transmission routes.

We have classified interactions among *Wolbachia*-infected species from the rice-field insect community into two major feeding complexes. When relationships among *Wolbachia* strains were compared, we found that some strains were closely related within each feeding complex. The feeding interactions of the species having closely related *Wolbachia* strains may therefore be of potential importance to *Wolbachia* horizontal transmission. Of particular interest is the hopper feeding complex in which *Wolbachia* sequences in three hopper species occurred in the same clade as their mirid predators as well as a *Nephotettix* leafhopper parasitoid. However, many insect species with *Wolbachia* strains in the same clade were not ecologically connected by any known feeding interaction, thus highlighting the difficulty in unravelling the evolutionary history of *Wolbachia* relations with their hosts.

Acknowledgements

The authors would like to thank Scott L. O'Neill for helpful advice and suggestions, Monpop Tripop for his help in specimen collection, Sammieng Thonthong and Natchava Klinpikul for their technical assistance. The work was supported by the TRF/BIOTEC Special Program for Biodiversity Research and Training (BRT 139026) and the Thailand Research Fund (BGJ/16/2543).

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Pattamaporn Kittayapong and Wanwisa Jamnongluk research interests include host reproductive alterations caused by *Wolbachia* as well as evolutionary relationships among *Wolbachia* strains. John Milne's research focuses on interactions among insects, plants and associated microorganisms. Apisit Thipaksorn and Chalerm Sindhusake are interested in the taxonomy and ecology of agricultural insect pests.

Molecular phylogeny of tephritid fruit flies in the *Bactrocera tau* complex using the mitochondrial COI sequences

Wanwisa Jamnongluk, Visut Baimai, and Pattamaporn Kittayapong

Abstract: We compared sequences of the mitochondrial cytochrome oxidase I gene of eight species of the *Bactrocera tau* complex using *Bactrocera dorsalis*, *Bactrocera pyrifoliae*, *Ceratitis capitata*, *Anopheles gambiae*, and *Locusta migratoria* as outgroups. A 639-bp variable region was sequenced. The sequence divergence between species in the *B. tau* complex ranged from 0.06 to 28%, and up to 29% between the complex and its tephritid outgroups, *B. dorsalis* and *C. capitata*. According to the phylogenetic relationships, these members of the *B. tau* complex could be classified into four clades. Thus, species A and D form clades 1 and 3, respectively, while species C and I belong to clade 4. However, species B, E, and F form a distinct group, clade 2, and infested the fruits of non-cucurbit hosts. Host-plant shifts resulting in adaptive radiation and premating isolation among species might play an important role in species differentiation of the *B. tau* complex.

Key words: *Bactrocera*, cytochrome oxidase I, species complex, mitochondrial DNA, phylogeny.

Résumé : Les auteurs ont comparé les séquences du gène codant pour la cytochrome oxydase I mitochondriale chez huit espèces du complexe *Bactrocera tau*, en utilisant *Bactrocera dorsalis*, *Bactrocera pyrifoliae*, *Ceratitis capitata*, *Anopheles gambiae* et *Locusta migratoria* comme groupes externes. Une région variable de 639 bp a été séquencée. La divergence nucléotidique au sein des espèces du complexe *B. tau* variait entre 0,06 et 28 % ; elle atteignait jusqu'à 29 % entre le complexe et les tephrétidés *B. dorsalis* et *C. capitata*, utilisées comme groupe externe. Selon les relations phylogénétiques, les membres du complexe *B. tau* forment quatre clades. Les espèces A et C forment les clades 1 et 3, respectivement, tandis que les espèces C et I appartiennent au clade 4. Cependant, les espèces B, E et F forment un groupe distinct, le clade 2. Des changements dans les espèces hôtes auraient résulté en une radiation adaptative et un isolement précédant la reproduction pourraient jouer un rôle important au niveau de la différenciation des espèces au sein du complexe *B. tau*.

Mots clés :

[Traduit par la Rédaction]

Introduction

The fruit flies of the family Tephritidae are one of the most diverse families comprising some 4000 species distributed throughout the world. Some of these fruit flies are important agricultural pests (Foote et al. 1993). In Thailand and other Southeast Asian countries, fruit flies of the genus *Bactrocera* (Tribe Dacinae, Family Tephritidae), particularly the *Bactrocera dorsalis* species of the subgenus *Bactrocera*, are known to be one of the major pests of tropical fruits (Drew and Romig 1997). Hence, this genus of tephritid fruit flies is an excellent candidate for study of species diversity and evolutionary processes. Recently, it has been demonstrated that the taxon *Bactrocera dorsalis* s.l. is a species complex comprising at least 40 species (Drew 1989; Baimai et al. 1995, 1999*a*, 1999*b*, 2000*a*). Moreover, *Bactrocera tau* s.l. of the subgenus *Zeugodacus* is another taxon considered to consist of closely related species or sibling species (Drew and Romig 1997). These fruit flies have a strong preference for attacking plants in the Cucurbitacae family, e.g., cucumber, luffa, and young melon fruit, throughout Thailand (Tigvattananont 1986; Meksongsee et al. 1991) and other regions in the Far East (White and Elson-Harris 1992).

Although *B. tau* is a major cucurbit pest, little work has been done on the genetic relationships among members of this species complex. Morphologically, members of the *B. tau* complex show differences in the three yellow stripes on the thorax and the size and shape of dark bands on the dorsal abdomen (S. Tigvattananont, Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's University of Techonology, Bangkok, Thailand, personal communication). However, some species of the *B. tau* complex could not be easily distinguished morphologically. Recently, mitotic karyotype and electrophoresis analyses of the *B. tau* complex have been demonstrated

Received 15 January 2002. Accepted 23 October 2002. Published on the NRC Research Press Web site at http://genome.nrc.ca on xx xxxx 2003.

Corresponding Editor: B. Golding.

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 doi: 10.1139/G02-113

Table 1. List of eight species of the *Bactrocera tau* complex used in this study.

Species	Host plant species (Family)	Location*	Accession No.
A	*Cucurbita moschata* (Cucurbitaceae)	KB	AF400067
B	*Siphonodosa celastrineus* (Celastraceae)	KB	AF400068
C	*Momordica cochinchinensis* (Cucurbitaceae)	KB	AF400069
D	*Trichosanthes tricuspidata* (Cucurbitaceae)	RN	AF400070
E	*Strychnos thorelii* (Strychnaceae)	KB	AF400071
F	*Hydnocarpus anthelminthicus* (Flacourtiaceae)	RN	AF400072
G	*Hydnocarpus anthelminthicus* (Flacourtiaceae)	KB	AY151138
I	*Trichosanthes tricuspidata* (Cucurbitaceae)	SO	AF400073

*Abbreviated name of locations where fruit flies have been collected: KB, Kanchanaburi Province (western Thailand), RN, Ranong Province (southern Thailand), and SO, Songkhla Province (southern Thailand).

to be useful tools for separation of these closely related species, although the methods are some what tedious and time consuming (Saelee 1999; Baimai et al. 2000*b*).

In this study, we used the variable region of the mitochondrial cytochrome oxidase subunit I (COI) gene to obtain better estimates of divergence for each species among the eight members of the *B. tau* complex. The phylogenetic relationships among these species and between the outgroups were determined.

Materials and methods

Insect samples

Tephritid fruit fly larvae were collected from fruits and flowers in different parts of Thailand. Details of specimen collections and storage procedures are given in Kittayapong et al. (2000). The identification of collected tephritid fruit flies was done by S. Tigvattananont based on the band pattern of the adult's dorsal thorax and abdomen, the size and shape of their ovipositors, and the flies' preferred host plants. The voucher specimens of each species were kept by S. Tigvattananont at the Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's University of Techonology, Bangkok, Thailand. A single fruit fly of each species was randomly sampled. Species and sample localities for sequenced specimens are listed in Table 1.

DNA extraction

Total DNA was extracted from the ovaries or testes of individual fruit fly adults using the crude boiling methods (O'Neill et al. 1992). The ovaries or testes were homogenized with a sterilized pestle in a 1.5-mL microcentrifuge tube filled with 100 µL of STE buffer (100 mM NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM 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. Two microlitres of supernatant was used as the DNA template for the polymerase chain reaction (PCR).

PCR amplification and sequencing

A 639-bp-long COI fragment was polymerized by using the sense primer UEA 7 and the antisense primer UEA 10, both of which were developed by Lunt et al. (1996). PCR amplification was done in 20-µL reaction volumes: 12.5 µL ddH2O, 2 µL 10× PCR buffer (Promega, Madison, Wis.), 2 µL of 25 mM $MgCl_2$, 0.5 µL dNTP (10 mM each), 0.5 µL of 20 µM forward and reverse primers, and 1 U *Taq* DNA polymerase (Promega). PCR amplification was done with initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and final extension step at 72°C for 30 min. Ten microlitres of PCR product was run on a 1% w/v agarose gel to determine the presence and size of amplified DNA.

One microliter of the PCR product was directly ligated into a pGEM-T vector (Promega) in a 10-µL reaction by incubation at 4°C for two nights. Plasmids were isolated using the Qiaprep plasmid miniprep kit (Qiagen, Valencia, Calif.) and sequenced on an ABI Prism automated sequencer (Applied Biosystems, Foster City, Calif.). At least three independent clones were sequenced from each tephritid fruit fly specimen. Both strands of the plasmids were sequenced using T7 and SP6 primers. Consensus sequences of COI fragments from each fruit fly specimen were constructed and used for later analyses. Sequences of all eight tephritid fruit fly species have been deposited in GenBank under accession Nos. AF400067–AF400073 and AY151138.

Data analysis

The alignment was done manually; however, there were no gaps in any of the analyzed sequences. Using MEGA software version 2.0 (Kumar et al. 2001), nucleotide and amino acid sequence differences among the eight tephritid species of the *B. tau* complex were calculated. The Kimura two-parameter distance method (Kimura 1980) was used to calculate nucleotide sequence differences and the *p* distance method was used to calculate amino acid sequence differences. The overall transition–transversion ratio was calculated by the general time reversible method (Yang 1994) chosen by the Modeltest program (Posada and Crandall 1998). A synonymous substitution rate was determined by the MEGA program version 2.0 (Kumar et al. 2001), which employed Nei and Gojobori calculation methods (Nei and Gojobori 1986).

Maximum parsimony, maximum likelihood, and neighbor-joining methods were used in phylogeny reconstruction using PAUP version 4.0 b2 (Swofford 1999). *Bactrocera dorsalis* (Jamnongluk 2001), *B. pyrifoliae* (Jamnongluk 2001), *C. capitata* (Spanos et al. 2000), *An. gambiae* (Beard et al. 1993), and *L. migratoria* (Flook et al. 1995) were used as outgroups in all analyses. A maximum parsimony analysis was performed using a heuristic search procedure with 1000 bootstrap replications. Characters were weighted

Table 2. Proportion of substitutions represented by transition–transversion ratio (above diagonal) and nucleotide sequence differences (below diagonal) of the cytochrome oxidase I gene of the tephritid fruit flies *Bactrocera* spp. and *Ceratitis capitata* using the Kimura two-parameter distance method.

Species	1	2	3	4	5	6	7	8	9	10	11
(1) *B. tau* sp. A		4.704	5.119	5.033	6.335	4.772	1.685	4.208	1.790	2.353	1.142
(2) *B. tau* sp. B	0.136		4.68	5.989	4.168	3.053	2.727	6.414	2.003	2.871	1.223
(3) *B. tau* sp. C	0.204	0.167		4.410	4.656	4.776	2.825	9.483	2.558	3.659	1.625
(4) *B. tau* sp. D	0.122	0.166	0.180		5.212	5.448	1.631	4.313	2.085	2.282	1.169
(5) *B. tau* sp. E	0.135	0.054	0.178	0.137		4.167	2.540	5.585	1.796	2.401	1.571
(6) *B. tau* sp. F	0.148	0.006	0.181	0.166	0.068		2.847	6.526	2.002	2.861	1.222
(7) *B. tau* sp. G	0.164	0.162	0.225	0.161	0.162	0.176		2.408	1.154	1.425	0.885
(8) *B. tau* sp. I	0.205	0.262	0.193	0.209	0.246	0.281	0.258		1.794	2.174	1.479
(9) *B. dorsalis*	0.211	0.227	0.243	0.189	0.233	0.235	0.247	0.226		9.738	1.255
(10) *B. pyrifolia*	0.245	0.283	0.285	0.193	0.275	0.212	0.278	0.232	0.051		1.337
(11) *C. capitata*	0.205	0.231	0.286	0.223	0.239	0.239	0.290	0.311	0.220	0.240	

equally. For maximum likelihood analysis, we used stepwise addition using a general time reversible model chosen by the above calculation with consideration of gamma distributed rate across sites. Bootstrap analysis was done with 100 replications. In the neighbor-joining analysis, a phylogenetic tree was produced based on the above calculated Kimura two-parameter distance method with gamma correction. Bootstrap analysis was performed with 1000 replications.

Results and discussion

We observed 145 synonymous and 35 non-synonymous substitutions totaling 180 sites scattered across the entire 639-bp downstream region of COI among the 8 species of the *B. tau* complex. The average transition–transversion ratio was 4.490 (range from 1.631–9.483, Table 2). Except for *B. tau* sp. G, strong transition bias was also found. High transition bias was found among *B. tau* spp. C and I. These two tephritid species were morphologically identical, but were found in different host plant species and feeding habitats (Fig. 1). A strong transition bias for nucleotide substitutions of mtDNA sequences between closely related taxa has been repeatedly observed (Brown et al. 1982; DeSalle et al. 1987; Beckenbach et al. 1993). Beckenbach et al. (1993) proposed that transitions are involved in sequence divergence among closely related species because transitions at silent sites reached saturation more quickly than do silent transversions. The majority of transitions (72%) were thymine–cytosine substitutions. The bias in A+T content of the 639-bp downstream segment of COI in the *B. tau* complex was 65% (ranged from 63–68%); it is relatively similar to the *B. dorsalis* complex (66%). However, it is lower than that of other outgroup species along the same segment (69–71%). The biases were much stronger for third-base positions (80%) than for second- (61%) and first-base positions (57%). An A+T bias has been found in most insect mtDNA genes (Lunt et al. 1996; Han and McPheron 1997; Langor and Sperling 1997) and it has been suggested that regions with high A+T content might be useful for studying phylogenetic relationships among insect species in a species complex (Lunt et al. 1996).

Using the Kimura two-parameter method, the mean sequence divergence among the eight species of the *B. tau* complex ranged from 0.6 to 28% and between the

Fig. 1. Map of Thailand showing the collection localities for each of the eight forms of the *B. tau* complex. The symbols representing each locality are as follows: ●, *B. tau* sp. A; ■, *B. tau* sp. B; ◇, *B. tau* sp. C; □, *B. tau* sp. D; ★, *B. tau* sp. E; ▼, *B. tau* sp. F; ♠, *B. tau* sp. G; and ◆, *B. tau* sp. I.

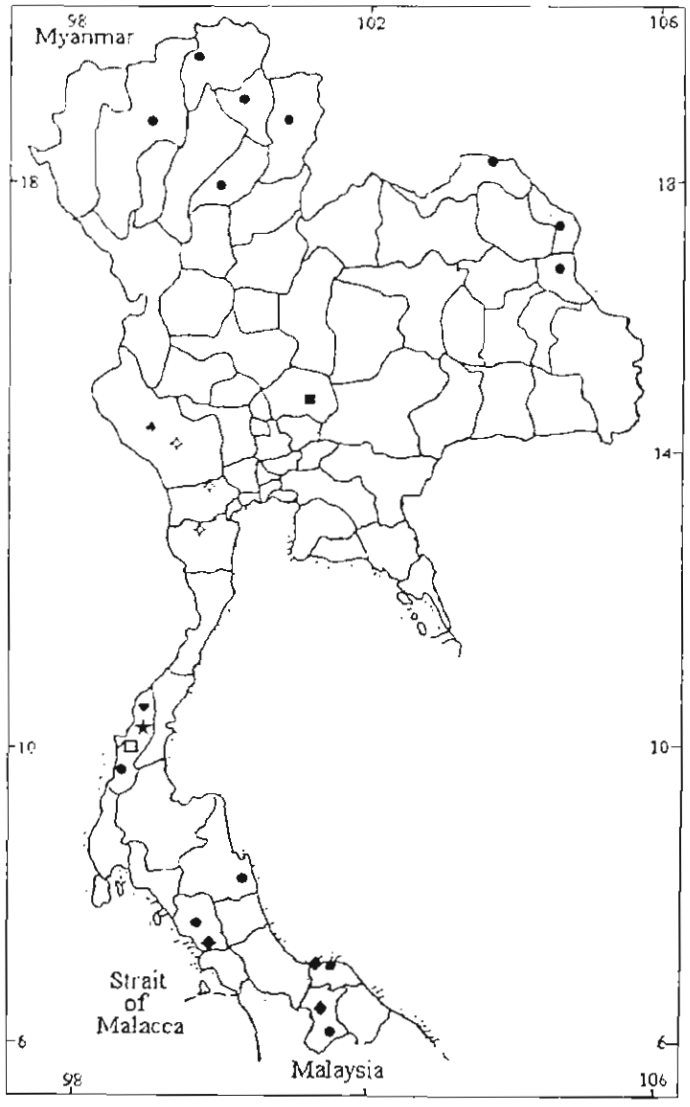
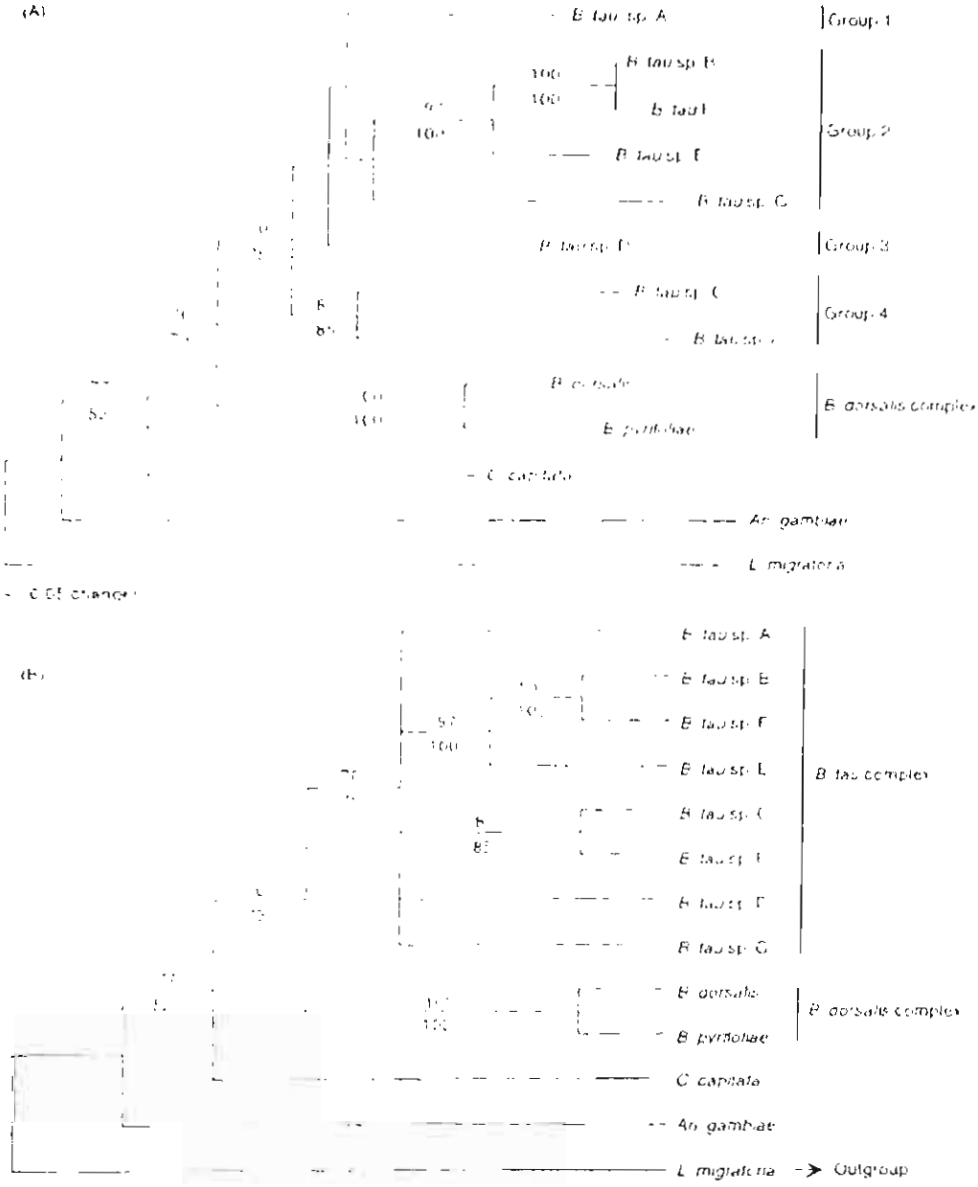


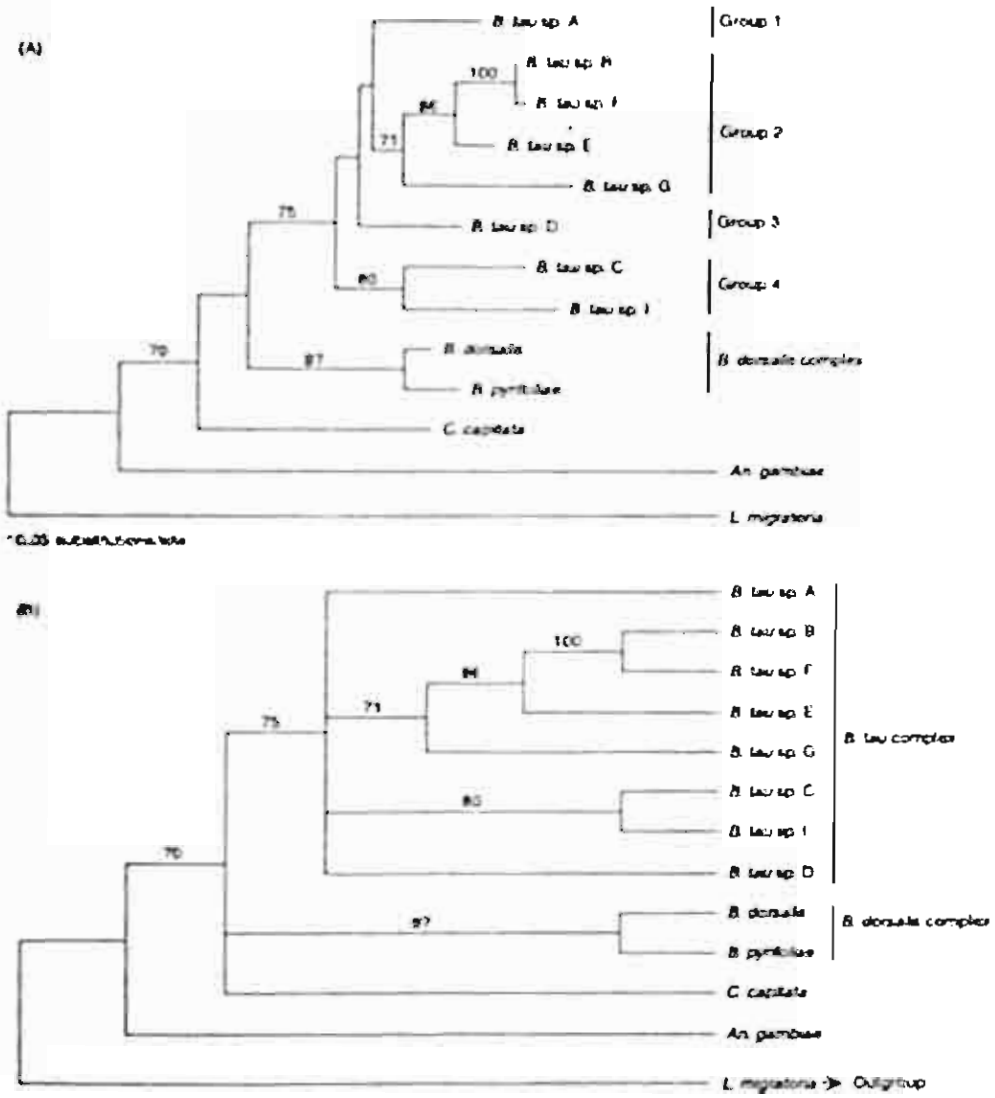
Fig. 2. (A) Maximum parsimony tree based on COI gene sequences showing the relationships between eight members of the *Bactrocera tau* complex. The tree was generated by a heuristic method and was rooted at *L. migratoria*. (B) Same tree topology after collapsing branches with bootstrap values <50. Numbers above and below the branches are bootstrap values calculated by neighbor joining (1000 replicates) and maximum parsimony (1000 replicates), respectively. Two *Bactrocera dorsalis* complex species *B. dorsalis*, AY053507; *B. pyrifoliae*, AY053514, Jamnongluk 2001), *Ceratitis capitata* (AJ242872; Spanos et al. 2000), *Anopheles gambiae* (L20934; Beard et al. 1993), and *Locusta migratoria* (X80245, Flook et al. 1995) were used as outgroups.



B. dorsalis and *B. tau* complexes it ranged from 19 to 29% (Table 2). Therefore, the saturation of nucleotide sequence might occur. Interspecific sequence divergences for the *B. tau* complex are much higher than for other insects studied thus far (reviewed in Langor and Sperling 1997). High sequence divergence in the *B. tau* complex is in accordance with the results of gel electrophoresis based on 12 loci (Saelee 1999). Moreover, the gel electrophoresis data suggested a low gene flow value ($N_m < 1$) among members of the *B. tau* complex.

Previous studies on the phylogenetic relationships among Tephritidae using the 16S rRNA gene showed no significant statistical support in the higher classification (Han and McPheron 1997). Based on studies of insect mtDNA, Brower (1994) suggested that the molecular clock could be calibrated to 2.3% pairwise sequence divergence per million years. Using this value, the *B. tau* complex could have arisen some 5 million years ago, which is apparently older than other species complexes that have been studied (Langor and Sperling 1997). Using the same ratio, the possible time that the *B. tau* complex diverged from the closely related tephritid species, *B. dorsalis* complex, and the divergence time between *Bactrocera* and their sister tephritid species, *C. capitata*, could also be estimated. The *B. tau* complex

Fig. 3. (A) Maximum-likelihood tree based on COI gene sequences showing the relationships among eight members of the *Bactrocera tau* complex. The tree was generated by the step-wise addition method using a general time reversible model with consideration of gamma-distributed rate heterogeneity across sites (−ln*L* = 3472.1213, gamma shape parameter = 0.3482). Trees were rooted with *L. migratoria*. (B) Same tree topology after collapsing branches with bootstrap values <50. Bootstrap values were calculated by maximum likelihood (100 replicates). Two *Bactrocera dorsalis* complex species (*B. dorsalis*, AY053507; *B. pyrifoliae*, AY053514; Armongluk 2001), *Ceratitis capitata* (AJ242872; Spanos et al. 2000), *Anopheles gambiae* (L20934; Beard et al. 1993), and *Locusta migratoria* (X80245; Flook et al. 1995) were used as outgroups.



might have diverged from the *B. dorsalis* complex approximately 10.6 million years ago. The phylogenetic tree showed that *B. tau* and *B. dorsalis* shared a common ancestor and might have diverged from *C. capitata* approximately 87 000 years ago. On the other hand, the mtDNA genome of the *B. tau* complex could have evolved at a much faster rate than for other insects. Rapid adaptive radiation of tephritid fruit flies has been proposed by Han and McPheron (1997). Moreover, selective forces like genetic drift might also be involved in the evolutionary divergence of the fruit fly species in the *B. tau* complex.

Phylogenetic trees of the *B. tau* complex generated by maximum likelihood, maximum parsimony, and neighbor-joining analyses showed similar tree topologies. Our data showed that all eight species of the *B. tau* complex formed a monophyletic grouping with support of at least 70% of 1000 bootstrap replicates, 75% of 1000 bootstrap replicates, and 75% of 100 bootstrap replicates when calculated using the distance and (or) neighbor-joining, maximum parsimony, and maximum likelihood methods, respectively, (Figs. 2 and 3). Phylogenetic relationships among the eight species of the *B. tau* complex clearly exhibited four clades (Figs. 2 and 3). Thus clades 1 and 3 containing species A and D, respectively, form somewhat remote groups among the eight species of the complex. These results are similar to the previously reported cytological data and are identical to the phylogenetic tree calculated from allozyme data of the *B. tau* species complex (Baimai et al. 2000*b*; SaeLee 1999).

A correlation between morphological and nucleotide sequence differences among *B. tau* complex species was found (S. Tigvattananont, personal communication), especially in clades 2 (*B. tau* spp. [illegible]) and [illegible] and D. From the SEM data it was found that the [illegible] of the apex of the aculeus in species [illegible] and [illegible] were similar in shape (S. Tigvattananont, unpublished data).

Clade 2 consists of species [illegible] with a bootstrap support of 71% for the maximum parsimony tree (Fig. 3). However, the bootstrap values calculated by maximum parsimony and neighbor-joining [illegible] enough to support the [illegible] (Fig. 2). In this clade, *B. tau* spp. [illegible] related and clustered in the same clade with a bootstrap value of [illegible]. This result [illegible] from mitotic karyotypes (Baimai et al. [illegible]). [illegible] species of the *B. tau* complex [illegible] host plants that are [illegible] Celastraceae, Strychnaceae, and [illegible] (Table 1). Moreover, *B. tau* spp. [illegible] host plant species [illegible] and I were genetically and [illegible] clustered in clade 4 with [illegible] joining and [illegible] for maximum likelihood [illegible] support morphological (S. Tigvattananont, unpublished data), cytological (Baimai et al. [illegible]) [illegible] evidence (Saelee 1996).

Adaptive radiation [illegible] considered to be an important factor [illegible] phytophagous insects. In a [illegible] fruit flies, rapid adaptive radiation [illegible] (Han and McPheron 1997). [illegible] between insects and plants [illegible] Farrell 1998). Genetic differentiation [illegible] patric host races of *Rhagoletis* [illegible] ported (Feder et al. 1988; McPheron et al. [illegible]). [illegible] effects of host plant [illegible] host preference [illegible] (Wood and Keese [illegible]).

Except for *B. tau* sp. A, which [illegible], all members of the *B. tau* complex are [illegible] and found only in certain areas (Saelee [illegible]; Baimai et al. 2000). Hence, host plant [illegible] the *B. tau* complex and [illegible] could play a significant role in [illegible] tephritid fruit flies in Thailand. [illegible] isolation might also promote speciation [illegible] *B. tau* complex. The interesting [illegible] tiation among members of the *B. tau* complex warrant further detailed investigation.

Acknowledgements

We would like to thank John E. [illegible] and Elizabeth McGraw for reviewing the manuscript and Saen Tigvattananont and Anchalee Saelee for providing specimens of the *Bactrocera tau* complex used in this study. This work was supported by the TRF/BIOTEC Special Program for Research and Training in Biodiversity (BRT 139026) and the Thailand Research Fund (RGJ PHD 0095/2541, RTA/01/2541, and BGJ/16/2544).

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[illegible] 10 [illegible].

[illegible] DNA sequence [illegible] 3 [illegible].

[illegible] 18: [illegible].

[illegible] DNA [illegible] 26: [illegible].

[illegible] The [illegible] fruit flies (Diptera: Tephritidae) [illegible] 26: [illegible].

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Genetica 1802: 1–7, 2002

Molecular evolution of tephritid fruit flies in the genus *Bactrocera* based on the cytochrome oxidase I gene

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Received 28 December 2001 Accepted 24 October 2002

Key words: Bactrocera spp., cytochrome oxidase I gene, mitochondrial DNA, molecular phylogeny

Abstract

Fruit flies of the genus *Bactrocera* (Diptera: Tephritidae) are one of the major economically important insects in Asia and Australia. Little attention has been given to analyses of molecular phylogenetic relationships among *Bactrocera* subgenera. By using mitochondrial cytochrome oxidase I gene (COI) sequences, the phylogenetic relationships among four subgenera, *Asiadacus*, *Bactrocera*, *Hemigymnodacus*, and *Zeugodacus*, were investigated. Nucleotide diversity within subgenera ranged from 11.7 to 12.4%, and the net divergence among subgenera ranged from 11.2 to 15.7%. Phylogenetic trees calculated from both maximum parsimony and neighbor-joining phylogenetic analysis methods were highly congruent in terms of tree topologies. Phylogenetic analysis of mitochondrial COI sequences suggests that tephritid fruit fly species, which attack cucurbit plants, that is, *Asiadacus*, *Hemigymnodacus* and *Zeugodacus*, were more closely related to each other than to fruit fly species of the subgenus *Bactrocera*, which attack plants of numerous families. Our data supports previous classification of *Bactrocera* based on morphological characters. However, the phylogenetic tree showed the polyphyletic of fruit flies in subgenus *Zeugodacus*. Possible causes of speciation among fruit flies species in this genus was also discussed.

Introduction

Tephritid fruit flies in the genus *Bactrocera* (Diptera: Tephritidae) are widespread in Asia and Australia. Several *Bactrocera* species are serious pests of fruits and vegetables (Allwood et al., 1999). The genus *Bactrocera* is highly diverse and is a large genus with many species still to be described (Drew & Hancock, 2000). At least 28 *Bactrocera* subgenera have been described and these are divided into four groups, namely *Bactrocera*, *Melanodacus*, *Queenslandacus*, and *Zeugodacus* (Drew, 1989). In Southeast Asia, tephritid fruit flies from the *Bactrocera* and *Zeugodacus* groups are dominant groups in terms of the number of species (Allwood et al., 1999) and cause serious reductions in yields and quality of fruits and vegetables in this region (Hardy, 1973). At least 50% of all known species in the *Zeugodacus* group are cucurbit feeders whereas fruit flies in the *Bactrocera* group infest more diverse host plants (Allwood et al., 1999). Therefore, host plants might be one of the factors that cause species diversity in *Bactrocera* fruit flies.

Four subgenera of *Bactrocera* fruit flies from two groups, namely the subgenus *Bactrocera* from the *Bactrocera* group and the subgenera, *Asiadacus*, *Hemigymnodacus* and *Zeugodacus*, from the *Zeugodacus* group were used in the study. These taxa have been defined on the basis of morphological characters and ecological data. However, genetic data in relation to phylogenetic relationships among these groups are poorly known. Preliminary work on mitotic karyotypes of some species of the genus *Bactrocera* was reported by Hunwattanakul and Baimai (1994). Recently, cytological and allozyme studies of the *Bactrocera dorsalis* and the *Bactrocera tau* complexes have been conducted (Baimai et al., 1995, 2000a,b;

Baimai, Phinchongsakuldit & Trinachartvanit, 1999; Baimai, Sumrandee & Tigvattananont, 1999; Saelee, 1999).

Mitochondrial DNA has become the common molecular marker in phylogenetic analysis and population genetic studies in animals (Boyce, Zwick & Aquadro, 1994; Langor & Sperling, 1997). The advantage of using mitochondrial genes in evolutionary study is that mutations that create new haplotypes are rare. Therefore, two individuals that share the same haplotype are likely to have a common ancestor (Li, 1997). Mitochondrial DNA has also been used in phylogenetic relationship among tephritid fruit fly species but the relationship among higher taxa could not be resolved (Han & McPheron, 1997; Han, 2000). Recently, by using 1.6 kb sequences of mitochondrial DNA, the more resolved phylogenetic relationship among higher taxa of genus *Bactrocera* has been reported (Muraji & Nakahara, 2001).

In this study, we used shorter nucleotide sequence, the 639 bp variable region of the mitochondrial cytochrome oxidase I gene (COI) (Lunt et al., 1996) for construction phylogenetic relationships among the four subgenera of *Bactrocera* fruit flies. Thus, it could reduce calculation time for phylogenetic relationship among higher taxa of the genus *Bactrocera*.

Materials and methods

Fruit fly collection and handling

Fruits and flowers infested with tephritid larvae were collected from nine host plant species in different parts of Thailand (Table 1). The larval specimens were reared to adult in the laboratory at Mahidol University, Bangkok. The adults were allowed to mature, identified and then stored at −70°C until required for molecular analysis.

Template preparation and DNA manipulation

Total DNA was extracted from the ovaries or testes of individual adult flies using the STE boiling method (O'Neill et al., 1992). The ovaries or testes were then homogenized with a sterilized polypropylene pestle in a 1.5 µl microcentrifuge tube filled with 100 µl of STE buffer (100 mM NaCl, 10 mM Tris–Cl pH 8.0, 1 mM EDTA). The homogenate was heated at 95°C for 10 min before being centrifuged at 14,000 rpm for 1 min at room temperature.

Partial sequences of the mitochondrial COI gene was amplified from all genomic DNA samples by polymerase chain reaction (PCR) using the forward primer (UEA7) and reverse primer (UEA 10) developed by Lunt et al. (1996).

PCR amplification was done in 20 µl reaction volumes: 12.5 µl dd H_2O, 2 µl 10× PCR buffer (Promega), 2 µl 25 mM $MgCl_2$, 0.5 µl dNTPs (10 mM each), 0.5 µl 20 µM forward and reverse primers and 1 unit of Taq DNA polymerase (Promega). Two microliters of supernatant were used as DNA template for the PCR. Thermal cycling consisted of an initial denaturation period of 3 min at 94°C, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min and a final extension step at 72°C for 30 min. Ten microliters of each PCR product was run on a 1% agarose gel to determine the presence and size of amplified DNA.

One microliter of the PCR product was directly ligated into a pGEM-T vector (Promega) in a 10 µl reaction and incubated at 4°C for two nights. Plasmids were isolated using a Qiaprep Plasmid miniprep kit (Qiagen) according to the manufacturer's recommendations. Three independent clones were sequenced from individual tephritid fruit fly specimens. The sequencing of inserts in both directions was carried out on an ABI 377 automated sequencer of a commercial laboratory (Bioservice Unit, NSTDA, Bangkok, Thailand) using T7 and SP6 primers.

Data analysis

A consensus sequence of COI fragments from one specimen of each fruit fly species was constructed by using the SeqMan program (DNAstar, Lasergene). The sequences of the 13 species of *Bactrocera* have been deposited in GenBank. Accession numbers are listed in Table 1.

In addition to mitochondrial COI gene sequences of the 13 *Bactrocera* species, COI sequences of the same region for *Ceratitis capitata*, *Anopheles gambiae*, and *Locusta migratoria* were used in the analysis as outgroups. The sequences were initially aligned using the Crustal algorithm of the MegAlign program (DNAstar, Lasergene) and then adjusted by eye.

Nucleotide and amino acid sequence differences among the 13 species of *Bactrocera* were calculated using MEGA software 2.1 (Kumar et al., 2001). The overall transition–transversion ratio was calculated by the general time reversible method (Yang, 1994) chosen using the modeltest program (Posada &

Table 1 List of *Bactrocera* species used in this study and their collection locations and host plants

Species	Location	Host plant species (Family)	Accession number
B. (B.) correcta sp. A[a] (Hendel)	Ranong	*Syzygium samarangense* (Myrtaceae)[b]	AF423102
B. (B.) correcta sp. B[a]	Ranong	*Syzygium samarangense* (Myrtaceae)[b]	AF423104
B. (B.) dorsalis sp. A[a] (Bezzi)	Ranong	*Sandoricum koetjape* (Meliaceae)[b]	AY053507
B. (B.) sp.	Ranong	*Siphonodon celastrineus* (Celastraceae)[b]	AF423112
B. (B.) latifrons (Hendel)	Chiangmai	*Lycopersicon esculentum* (Solanaceae)[b]	AF423103
B. (B.) tuberculata (Bezzi)	Ranong	*Careya sphaerica* (Barringtoniaceae)[b]	AF423105
B. (A.) modesta (Hardy)	Phetchaburi	*Diplocyclos palmatus* (Cucurbitaceae)[c]	AF423106
B. (H.) diversa (Coquillett)	Amnat Charoen	*Cucurbita moschata* (Cucurbitaceae)[c]	AF423107
B. (Z.) aesta sp. A[a] (Hardy)	Kanchanaburi	*Luffa aegyptiaca* (Cucurbitaceae)[c]	AF423108
B. (Z.) aesta sp. B[a]	Machonpsorn	*Trichosanthes tricuspidata* (Cucurbitaceae)[c]	AF423111
B. (Z.) caudata (Fabricius)	Ranong	*Cucurbita moschata* (Cucurbitaceae)[c]	AF423109
B. (Z.) cucurbitae (Coquillett)	Chiangmai	*Lycopersicon esculentum* (Solanaceae)[b]	AF423110
B. (Z.) tau (Walker)	Kanchanaburi	*Cucurbita moschata* (Cucurbitaceae)[c]	AF408067

[a] Members of sibling species

[b] *Bactrocera* fruit flies collected from fruits

[c] *Bactrocera* fruit flies collected from flowers

Crandall, 1998). The Jukes-Cantor distance method (Jukes & Cantor, 1969) was used to calculate nucleotide sequence differences. The p-distance method was used to calculate amino acid sequence differences. Synonymous substitution rates were determined by the MEGA program version 2.1 (Kumar et al., 2001), which employs the Nei and Gojobori method (Nei & Gojobori, 1986).

We conducted a neighbor-joining (NJ) analysis using heuristic searches in the Jukes-Cantor model (Jukes & Cantor, 1969) with gamma parameter of 0.3048 (from previous calculation). Bootstrap analyses were done with 1000 replicates using PAUP, version 4.0 b2 (Swofford, 1999).

The data set was also analyzed by maximum parsimony method using PAUP, version 4.0 b2 (Swofford, 1999). The maximum parsimony tree was reconstructed by heuristic search procedure with TBR swapping and 100 maxtree options. We first performed unweighted parsimony search, only one most parsimonious tree was obtained from the analysis. Then we performed character weighting using the REWEIGHT command with an index = RC option of PAUP*, treating the consensus tree of the unweighted parsimony analysis as the starting tree. Bootstrap support was performed with 1000 replications.

Results and discussion

A total of 639 bp nucleotide sequences of the COI gene among the genus *Bactrocera* were used in the analyses. The overall mean sequence divergence among the 13 species of *Bactrocera* was 13.6%. Between different subgenera, the highest nucleotide sequence divergence was found between *Bactrocera* and *Asiadacus* (15.7%) and the lowest between *Hemigymnodacus* and *Zeugodacus* (11.2%).

Among the 203 variable sites found in this study, only 19 were non-synonymous substitutions while 184 were synonymous substitutions. The overall transition to transversion ratio was 2.037. The nucleotide substitutions were biased toward thymine–cytosine transitions (62.5%). From the analyses, parsimony uninformative and informative characters were 104 and 190, respectively. Like other insects (Langor & Sperling, 1997), the COI gene in *Bactrocera* was most variable at the third position in a codon and least variable in the second position. In the subgenus *Zeugodacus*, only six non-synonymous substitutions out of a total of 122 variable sites were found, whereas 13 non-synonymous substitutions from a total of 149 variable sites were encountered in the subgenus *Bactrocera*.

The A + T content of the 639 bp downstream segment of COI in species of the genus *Bactrocera* was slightly lower (63–68%) than those found in other insects over the same segment, for example, 71% in *L. migratoria* (Flook, Rowell & Gellissen, 1995), 69% in *An. gambiae* (Beard, Hamm & Collins, 1993) and 70% in *C. capitata* (Spanos et al., 2000).

Amino acids varied at 18 locations across the 213 amino acid sequences of the downstream segment of COI among the 13 *Bactrocera* species (Table 2).

Table 2 Amino acid variation among 15 tephritid fly species of the genus *Bactrocera* within a 658 bp segment of COI gene. Vertical numbers at the tops of columns represent the variable nucleotide sites of the *Drosophila yakuba* mitochondrial COI gene (Lewis, Farr & Kaguni, 1995). The symbol indicates the same amino acid sequence as that in the first row.

Species	2	2	2	2	3	3	3	3	3	3	4	4	4	4	5	5	5
	3	4	4	4	4	5	5	6	7	7	0	1	8	8	9	0	0
	8	1	7	7	8	3	6	0	1	4	8	1	2	5	0	0	3
	7	4	4	7	7	4	4	0	8	1	0	4	1	0	7	8	1
B. dorsalis A	S	T	P	S	T	S	C	K	T	T	R	G	S	F	S	P	S	T
B. correcta A																		
B. correcta B	W		Q			C						G					T	
B. latifrons				T				T							T	T		
B. tuberculata											W		T					F
Bactrocera sp.																		F
B. musae		R			M								T					F
B. diversa									A					L				F
B. ascita A										S								F
B. ascita B									T				T	L	T			F
B. cucurbitae									T					L	A			F
B. tau							T											F

Within the subgenus *Bactrocera*, 12 amino acid variation sites were found, whereas fewer amino acid variation sites (six sites) were found within the *Zeugodacus* subgenus.

A phylogenetic tree of the 15 tephritid fruit fly species representing four subgenera of *Bactrocera* was reconstructed by both neighbor-joining method based on Jukes–Cantor distances and maximum parsimony method. Tree topologies calculated from both methods were identical and revealed the existence of two distinct mitochondrial DNA lineages. The first lineage clade consisted of the subgenera *Zeugodacus*, *Hemigymnodacus* and *Asiadacus*. This clade will be defined here as the *Zeugodacus* group. The second lineage clade consisted of the subgenus *Bactrocera*. Figure 1 shows weighted parsimonious tree with tree length: 244.9, CI: 0.842, RI: 0.736, RC: 0.620.

The divergences of both nucleotide and amino acid sequences within the *Bactrocera* group were higher than those of the *Zeugodacus* group. The nucleotide and amino acid sequence divergences within the *Bactrocera* clade were 12.4 and 2.0%, respectively. In *Zeugodacus*, the nucleotide and amino acid sequence divergences were 11.7 and 1.5%, respectively.

From the phylogenetic analyses, our data support the morphological group classification, *Bactrocera* group (*Bactrocera*) and *Zeugodacus* group (*Zeugodacus* + *Hemigymnodacus* + *Asiadacus*), (Drew & Hancock, 2000). However, our results disagree with the morphologically based classification in grouping tephritid fruit fly species of the subgenus *Zeugodacus*. The disagreement between morphological classification and molecular phylogeny was also reported previously (Muraji & Nakahara, 2001). With regard to the subgenus *Zeugodacus*, tree topologies calculated from both neighbor-joining and maximum parsimony showed that fruit flies in this subgenus are polyphyletic. *B. tau* and *B. cucurbitae* were clustered in the distinct clade from other fruit flies in the *Zeugodacus* subgenus. This was supported by bootstrap analyses with confidence levels equal to 100%. Unlike *Zeugodacus* subgenus, fruit flies of the *Bactrocera* subgenus were monophyletic with bootstrap support of 97%.

In this study, we found two sibling species of *B. ascita* and *B. correcta*. The sibling species of *B. ascita* s.s. was morphologically identical with the previously described species, *B. ascita* s.s. (sp. A) but was found in different host plants and locations. Therefore, it was designated as *B. ascita* sp. B. The *B. correcta* s.s. (sp. A) was morphologically similar to its sibling species, namely *B. correcta* sp. B and was found in the same host fruit species collected from the same location. An undescribed species, *Bactrocera* sp., was

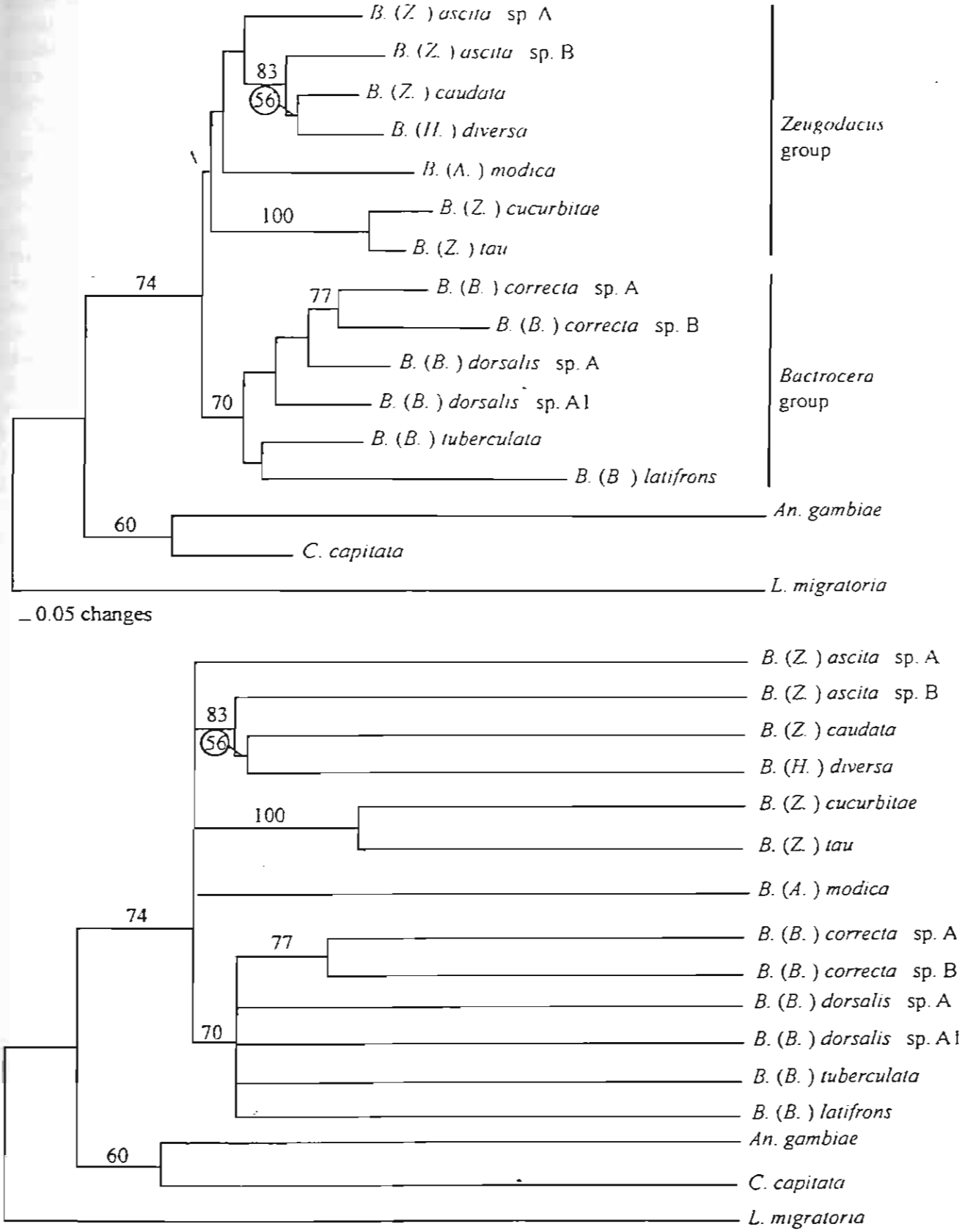

Figure 1. The unrooted most parsimonious tree (tree length: 244.9, CI: 0.842) was generated by reweighing of the result of equally weighted parsimonious analysis. Bootstrap support values of 1000 replications are given on the nodes. *Ceratitis capitata* (AJ242872; Spanos et al., 2000), *Anopheles gambiae* (L20934; Beard, Hamm & Collins, 1993) and *Locusta migratoria* (X80245; Flook, Rowell & Gellisen, 1995) were used as outgroups.

highly specific to both host plant and location. It was found only in *Siphonodon celastrineus* fruit in the southern part of Thailand. Although this insect has not been described as a new species, our data revealed that this species is a member of the subgenus *Bactrocera*.

We have observed associations between plant species and mitochondrial haplotypes of the tephritid fruit flies that infest their fruits and flowers. In the *Zeugodacus* clade, *B. cucurbitae* and *B. tau* were closely related with 100% bootstrap support and they often share the same host plant species. The findings of a close relationship between these two species are supported by both cytological and allozyme data (Baimai et al., 1995; Saelee, 1999). Likewise, close phylogenetic relationships of the two sibling species, *B. ascita* sp. A and *B. ascita* sp. B, seem to be closely associated with host plant species. *B. ascita* sp. A has been found infesting several species of the host plant family Cucurbitaceae, such as *Lagenaria siceraria* and *Luffa cylindrica*, while *B. ascita* sp. B. infests only in flowers of *Trichosanthes tricuspidata* which is also in the family Cucurbitaceae (Kittayapong et al., 2000). However, it is not known if these two fruit flies are reproductively isolated.

Adaptation to host plants might accelerate speciation in *Bactrocera*. The classic example of genetic change caused by host plant adaptation is that of another tephritid species, the apple maggot fly, *Rhagoletis* pomonella (Bush, 1969). It has been determined that sympatric host races of *Rhagoletis* are genetically differentiated (Feder, Chilcote & Bush, 1988; McPheron, Smith & Berlocher, 1988).

Reproductive isolation may sometimes be caused by inherited elements that are not conventional genes (Hurst & Pomiankowski, 1991). In this regard, it is interesting to note that one of these inherited elements, namely the bacteria *Wolbachia*, has been recently found in several *Bactrocera* species (Kittayapong et al., 2000). These bacteria are known to cause hybrid inviability in many insect species. *Wolbachia* bacteria occur at least in five orders of insects (O'Neill et al., 1992; Werren, Zhang & Guo, 1995). Infection of a single population produces inviability in only one direction of a cross, but different infections in geographically isolated populations can cause partial or complete reproductive isolation (Hoffmann & Turelli, 1997).

Wolbachia, by causing reproductive isolation between populations, might be a possible speciation agent or accelerate speciation rates among sibling species of the tephritid fruit flies. For example, *Wolbachia* infection has been reported in *B. ascita* sp. B whereas no infection has been found in *B. ascita* sp. A (Kittayapong et al., 2000). Similarly, *Wolbachia* might also be involved in the speciation process of the two sibling within the *B. correcta* complex. The *B. correcta* sp. A fruit flies have been found infected with *Wolbachia* whereas no *Wolbachia* infection have been found in its sibling species, *B. correcta* sp. B. These two species have been found infesting the same host plant in certain populations (V. Baimai, unpublished data).

In the present study, only 13 species of tephritid fruit flies genus *Bactrocera* were included in the phylogenetic analyses. However, our results can resolve the *Bactrocera* relationships at various taxonomic levels, which might be useful information for further taxonomic classification of genus *Bactrocera*. We also found associations between host plant species and the tephritid flies that infest them. Tephritid fruit flies that infested the same host plant species and lived sympatrically were more closely related than the ones that infested different host plants. Therefore, our data suggests that adaptation to the environmental conditions produced by the host plants might play a role in speciation of tephritid fruit flies in the genus *Bactrocera*. Moreover, infection of tephritid flies by *Wolbachia* bacteria, which can cause cytoplasmic incompatibility, might accelerate speciation rates among *Bactrocera* sibling species as evidenced from the present study. It has been demonstrated that studies on the population biology of tephritids have contributed to a better understanding of speciation and evolutionary processes (Bush, 1975; Feder, Chilcote & Bush, 1988). Thus, the existence of sibling species and closely related taxa of the *Bactrocera* in Thailand and Southeast Asia warrants further molecular study of this group of tephritid fruit flies in this region.

Acknowledgements

We would like to thank John Milne for critically editing the manuscript; and Saen Tigvattananont and Anchalee SaeLee for collecting and identifying insect samples. This work was supported by the TRF/BIOTEC Special Program for Biodiversity Research and Training (BRT 139026) and the Thailand Research Fund (RGJ/PHD/0095/2541, RTA/01/2541, and BGJ/16/2543).

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MOLECULAR EVIDENCE OF *WOLBACHIA* INFECTION IN NATURAL POPULATIONS OF AN ODONATE.

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Running Title: *Wolbachia* infections in an Odonate

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ABSTRACT

Wolbachia are endosymbiotic bacteria that cause reproductive alterations in numerous arthropod species. Using a PCR-based method, we found that, out of 33 odonate species, four species were infected with *Wolbachia*. This finding represents the first record of *Wolbachia* infection in tropical odonates. Identical *wsp* gene sequences were found in the *Wolbachia*-infected common odonate species, *Agriocnemis f. femina*, collected from different locations in Thailand. The infection frequencies in several natural populations suggest that replacement of uninfected populations by *Wolbachia*-infected ones has recently occurred in this damselfly species.

Introduction

Wolbachia comprise a group of α-proteobacteria that infect reproductive tissues of numerous arthropod species. *Wolbachia* are transmitted through the egg cytoplasm and are the cause of several types of reproductive alterations in their arthropod hosts. *Wolbachia* have been associated with post-zygotic cytoplasmic incompatibility (CI) in a wide range of insects [6, 20], parthenogenesis induction (PI) in wasps [23], and feminization in an isopod [22].

By using polymerase chain reaction (PCR) amplification and sequencing of the bacterial 16S rDNA genes [6, 20, 22, 23] and *fts*Z genes [28], it has been found that cytoplasmic incompatibility, parthenogenesis and feminizing bacteria form a closely related group in *Wolbachia* phylogeny. *Wolbachia* bacteria have been divided into two major groups (designated A and B groups) by *fts*Z gene sequence differences [28]. Both groups are widespread in insects, based upon synonymous substitution rates. Bacterial 16S rDNA sequence divergence is estimate to be 1-2 % per 50 million years [17, 18].

Wolbachia have been found in over 500 arthropod species [16, 20, 23, 28-31]. Even though most of these hosts are insects, *Wolbachia* have also been found in 88 crustaceans [3, 22, 30], 17 arachnids [5, 29, 30], 2 Chilopoda and 1 Diplopoda [30]. Among insects, *Wolbachia* have been found in all major orders, including Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Lepidoptera and Orthoptera [29].

Odonates are common insects of paddy fields in Thailand. They usually have two or three generations per year and their larval stage is found in stagnant water in and around rice fields [2]. They are important predators of rice pests such as leafhoppers, planthoppers [8], and rice stem borers [17]. In addition, [27] reported 7 species of Odonata larvae found in a paddy as predators of mosquito larvae. Using a normal PCR method, three neotropical dragonflies were tested for *Wolbachia* infection; however, none of them were found positive [29, 30]. Recently, the long PCR method has been used to improve the detection of *Wolbachia* infection [15]. Based on the long PCR method, a single odonate species, *Perithemis tenera*, has been found positive with *Wolbachia*. However, there has been no published report of a systematic survey for *Wolbachia* in the order Odonata collected from a tropical region.

The purpose of our study is to investigate the presence and distribution of *Wolbachia* among the tropical odonates collected from rice fields in Thailand using a polymerase chain reaction (PCR) amplification of the *Wolbachia wsp* protein-coding gene. Relationships of *Wolbachia* strains found in tropical odonates were also determined based on *wsp* nucleotide sequence data.

Materials and Methods

Specimen collection and handling

Adult odonates were collected using a simple sweep net from rice fields in 36 provinces representing the four major regions of Thailand. Collections were carried out during the month of October in 1998, 1999 and 2000. Live odonates were fixed at -78°C in a foam box filled with dry ice and transported to the laboratory at Mahidol University in Bangkok for further analysis. Odonate specimens were identified to species by using morphological keys [1, 7, 9, 10, 11]. After identification, the specimens were temporarily kept in a -20°C freezer until DNA was extracted.

DNA extraction and polymerase chain reaction

Individual insect specimens were dissected for ovaries or testes using a sterile technique. The DNA extraction method followed O'Neill et al. [20], insect gonads were ground in 100 µl of STE buffer (100 mM NaCl; 1mM EDTA, pH 8.0; 10mM Tris-HCl, pH 8.0). The homogenate was heated at 95°C for 10 minutes. One microliter of supernatant was used in polymerase chain reactions.

Polymerase chain reactions were performed in 20 µl reaction volumes containing 2 µl of 10X buffer (Promega), 2 µl of 25 mM $MgCl_2$, 0.5 µl of dNTPs (10mM each), 0.5 µl of forward and reverse *wsp* primers [32] and 1 unit of *Taq* DNA polymerase. The thermal profile for DNA amplification was: 95°C for 1 min., 55°C for 1 min., and 72°C for 1 min. per cycle for a total of 35 cycles. If the samples were negative with *wsp* primers, DNA was then amplified using a long PCR procedure with *wsp*L primers following the methods of Jeyaprakash and Hoy [15]. DNA samples that failed to amplify using both methods were then amplified again using 12S DNA primer [20] to check for the quality of DNA extraction. Ten microliters of each PCR product was run on a 1% agarose gel stained with ethidium bromide and visualized under a UV transilluminator. DNA extracts of *Wolbachia*-infected *Aedes albopictus* were used as positive controls.

Cloning and sequencing

For cloning, PCR products were incubated for an additional time of 90 minutes at 72°C after 35 cycles of amplification. One microliter of PCR product was then directly ligated into a pGEM-T vector (Promega) in a 10 µl reaction for 2 nights at 4°C. Three independent clones were sequenced for each of three *Wolbachia* strains in order to make sure that there was no polymerase error. Plasmids were purified using a High Pure Plasmid Isolation Kit (Boerhinger Mannheim). Sequencing was carried out on an ABI automated sequencer. Both strands of the plasmids were fully sequenced using T7 and SP6 primers.

Sequence alignment and phylogenetic analysis

Wolbachia DNA sequences from *A. f. femina* were aligned together with 22 previously published *Wolbachia* strains [32] using a clustal algorithm followed by manual modifications based on the amino acid translation of different genes. The third hypervariable region of the gene (position 519-559) [4] was excluded because no accurate alignment could be made. *Wolbachia wsp* sequences from tropical odonates are deposited under the accession numbers **XXXX-XXXX (please add)**. The data set was analyzed by maximum parsimony, maximum likelihood and neighbor-joining methods using PAUP 4.0 b1 [24] and Modeltest 2.0 [21]. For maximum parsimony analysis, heuristic searches were done, gaps were treated as missing data and bootstrap analysis was done with 1,000 replications. For maximum likelihood analysis, we performed step-wise addition using a general time reversible model with consideration of a gamma-distributed rate of heterogeneity across sites. Bootstrap analysis was conducted with 100 replications. A neighbor-joining tree was generated using a general time-reversible DNA/RNA distance approach and bootstrap analysis was done with 1,000 replications. Trees were unrooted. Nucleotide substitution rates were determined using Kimura two-parameter distances (K) in PAUP 4.0 b1 [24].

Results and Discussion

A total of 427 odonate individuals from 19 genera and 33 species (17 species of Zygoptera and 16 species of Anisoptera) were screened for *Wolbachia* by both regular and long PCR assay using *Wolbachia*-specific *wsp* gene primers. The infection status of each odonate species tested and the number of individuals screened are listed in Table 1. Four odonate species, *Agriocnemis f. femina*, *Pseudagrion pruinosum* (Zygoptera), *Brachythemis contaminata*, *Neurothemis t. tullia* (Anisoptera), were found to be infected with *Wolbachia*. The percentage of *Wolbachia* infection among species of the Order Odonata was 12.12 % (4/33) which is very low. Based on the data presented here, it appears that *Wolbachia* is not widespread in the Odonate species that we tested. Only four odonate species, i.e., two zygopteran and two anisopteran species, were found infected with *Wolbachia*. In the common odonate species, *Agriocnemis f. femina*, a total of 69 individuals of this infected species were tested and six samples (8.7%) from three provinces were found positive (Table 2). Infection rates were 100% (2/2) in Chaiyaphum, 25% (1/4) in Nakornratsima and 21.4% (3/14) in Suphanburi. In the common odonate species, *A. f. femina*, low infection rate was also found. Only 8.7% of tested individuals were infected. However, when only infected populations are considered, more than 20% of individuals were found infected. Of the other three infected species, only a single individual from each species was infected with *Wolbachia*.

Low *Wolbachia* densities in insect individuals may have caused false negatives and have led to low *Wolbachia* infection frequencies being recorded for Odonate species. A survey of *Wolbachia* using the long PCR method indicated that 76% of 62 tested arthropod species were infected [15], which is considerably higher than that previously reported using the standard PCR technique [28]. From our study, long PCR method was found to be more sensitive for amplifying *Wolbachia* DNA in odonates and could give positive amplification in insect samples originally found negative for *Wolbachia* using standard PCR.

A phylogenetic analysis of *Wolbachia* strains found in infected odonates with nine, including the strain from the odonate, *Perithemis tenera*, and two previously published A group *Wolbachia* strains [15, 32] was conducted. All procedures used for phylogenetic reconstruction (maximum parsimony, maximum likelihood and neighbor-joining methods) place all odonate *Wolbachia* strains in a monophyletic group with those of B group *Wolbachia* with bootstrap values of 100 (from maximum likelihood and neighbor-

joining analysis). Figure 1 shows the phylogenetic tree obtained using the neighbor-joining method with 1,000 bootstrap replicates. The *Wolbachia* strains found in the four rice field odonate species were in the Con and Pip subgroups. The *wsp* gene sequences of *A. f. femina* and *B. contaminata* were in the Pip subgroup with 100% of sequences similar to those of *Cx. quinquefasciatus* while the wsp gene sequences of *N. t. tullia* and *P. pruinosum* were identical and in the same subgroup as those of *Perithemis tenera*, i.e., Con, with bootstrap support of 97% for the maximum likelihood and 94% for the neighbor-joining analyses (Figure 1).

The low *Wolbachia* infection frequencies and identical *wsp* gene sequences in related odonate species that are not closely related suggest that *Wolbachia* might have recently invaded rice field odonate populations through some means of horizontal transmission. Several studies have shown that *Wolbachia* may have transferred from a single host species to unrelated uninfected host species [12, 26, 29]. Identical *wsp* gene sequences were found from all three positive populations of *A. f. femina* collected from different regions of Thailand. This finding supports the hypothesis that *Wolbachia*-infected damselflies spread into uninfected populations. Further study should be done to investigate the rates at which *Wolbachia*-infected damselflies could spread into uninfected populations. Because damselflies are one of the major predators of rice pests, an understanding of the spreading mechanism of *Wolbachia*-infected damselflies might be useful for future use of infected damselflies as a biological control agent of rice pests.

Acknowledgements

We would like to thank Mr. Monphob Triphob, Mr. Worawit Uamcharoen and Mr. Kitti Theinthong for their help in collecting specimens around Thailand. We also thank Mrs. Samnieng Thienthong and Miss Natchaya Klinpikul for their help in PCR and cloning methods. We are also indebted to Bro. Amnuay Pinratana for loaning odonate references and specimens, to Dr. John R. Milne for reading the manuscript and his comments and to Dr. Chalerm Sindhusake for guidance in field sampled. This work was supported by the TRF/BIOTEC Special Program for Biodiversity Research and Training (BRT 139026).

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Table 1. Results of PCR-screening for *Wolbachia* using specific *wsp* primers in Odonates sampled from different regions of Thailand.

Taxon	No. tested	Standard PCR	Long PCR	Infection status
Zygoptera				
Aciagrion occidentale Laidlaw	2	-	-	-
Agriocnemis clauseni Fraser	1	-	-	-
Agriocnemis f. femina (Brauer)	69	6	6 (B)	6 (B)
Agriocnemis minima Selys	5	-	-	-
Agriocnemis nana (Laidlaw)	1	-	-	-
Agriocnemis pygmaea (Rambur)	235	-	-	-
Ceriagrion a. auranticum Fraser	3	-	-	-
Ceriagrion azureum (Selys)	3	-	-	-
Ceriagrion cerinorubellum (Brauer)	6	-	-	-
Ceriagrion indochinense Asahina	9	-	-	-
Ceriagrion o. olivaceum Laidlaw	2	-	-	-
Enallagma cyathigerum (Charpentier)	3	-	-	-
Enallagma parvum Selys	4	-	-	-
Ischnura a. aurora (Brauer)	10	-	-	-
Ischnura senegalensis (Rambur)	2	-	-	-
Pseudagrion microcephalum (Rambur)	2	-	-	-
Pseudagrion pruinosum (Burmeister)	3	-	1 (B)	1 (B)
Anisoptera				
Acisoma p. panorpoides Rambur	2	-	-	-
Aethriamanta aethra Ris	4	-	-	-
Brachydiplax c. chalybea Brauer	2	-	-	-
Brachythemis contaminata (Fabricius)	4	-	1 (B)	1 (B)
Crocothemis s. servilia (Drury)	7	-	-	-
Diplacodes nebulosa (Fabricius)	2	-	-	-
Diplacodes trivialis (Rambur)	14	-	-	-
Neurothemis fluctuans (Fabricius)	1	-	-	-
Neurothemis intermedia atalanta Ris	1	-	-	-
Neurothemis t. tullia (Drury)	16	-	1 (B)	1 (B)
Orthetrum s. sabina (Drury)	2	-	-	-
Pantala flavescens (Fabricius)	1	-	-	-
Rhyothemis p. phyllis (Sulzer)	2	-	-	-
Tholymis tillarga (Fabricius)	1	-	-	-
Trithemis aurora (Burmeister)	3	-	-	-
Urothemis s. signata (Rambur)	5	-	-	-

Table 2. Infection rates of *Wolbachia* in different infected populations of *A. f. femina*.

Province (Region)	No. tested	No. PCR-positive	Infection rate
North			
Chiangmai	4	0	0
Phayao	2	0	0
Phrae	2	0	0
Northeast			
Chaiyaphum	2	2	100%
Nakhonratchasima	4	1	25%
Roi-et	6	0	0
Surin	7	0	0
Ubonratchathani	3	0	0
Yasothon	1	0	0
Central			
Chainat	2	0	0
Pathumthani	8	0	0
Suphanburi	14	3	21.4%
South			
Chumphon	2	0	0
Nakhonsrithammarat	5	0	0
Pattani	5	0	0
Suratthani	2	0	0
Total	69	6	8.7

Figure 1. A phylogenetic tree of *Wolbachia* based on *wsp* gene sequences that includes Wolbachia strains from odonates. The tree was generated using the neighbor-joining method. The tree was midpoint rooted. Bootstrap values of 1,000 replicates are presented above the branch (bootstrap scores less than 50 are not shown). Sequences a identified by the host species from which they were isolated.

Molecular phylogeny of tephritid fruit flies in the *Bactrocera dorsalis* complex using the mitochondrial COI sequences.

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Keywords: *Bactrocera dorsalis*, cytochrome oxidase I, complex species, mitochondrial DNA, phylogeny

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Abstract

The *Bactrocera* (*Bactrocera*) *dorsalis* complex consists of more than 50 species whose evolutionary relationships are poorly known. We analyzed 638 coding nucleotides downstream from the mitochondrial gene encoding subunit I of cytochrome oxidase (COI) in ten species of the *B. dorsalis* complex and five outgroups, *B.* (*Zeugodacus*) *tau*, *Ceratitis capitata*, *Anopheles gambiae*, *Compsomyiops callipes*, and *Locusta migratoria*. Based on Kimura-2 parameter method, sequence divergence between the fruit flies of this species complex ranged from 0.6 to 16.2%. As in other insects, A+T content and transition bias in the *B. dorsalis* complex were high. However, amino acid sequence divergence within the species complex was low. According to the phylogenetic relationships, these members of the *B. dorsalis* complex could be classified into three clades. Except for *B. dorsalis* sp. A, *B. dorsalis* sp. O and *B. carambolae*, the phylogenetic relationships among fruit fly species in the *B. dorsalis* complex are concordance with mitotic chromosome data. Possible causes of speciation among fruit flies species in this species complex was also discussed.

Introduction

The fruit flies of the genus *Bactrocera* belonging to the subfamily Dacinae (Diptera: Tephritidae) are widely distributed in subtropical and tropical forests of Southeast Asia, Australia, and the Pacific region. A total of 234 species have been recognized and formally described in this genus (Hardy and Adachi, 1954, Hardy, 1973, Drew 1989, Drew and Hancock, 1994). Among the fruit flies of Southeast Asia and Australia, the *B. dorsalis* group has received close attention because of its species diversity, cryptic species problems and the economic importance of several member species (White and Elson-Harris, 1992). Based on morphological and geographical distributions, the *B. dorsalis* complex consists of at least 40 fruit flies species (Drew, 1989). Cytologically, Baimai and coworkers have described mitotic karyotypes of 27 species of the *B. dorsalis* complex (Baimai *et al.*, 1995, 1999 a, b, 2000).

Mitochondrial DNA (mtDNA) has become the common molecular marker in phylogenetic and population genetic studies in animals. The advantage of using mitochondrial genes in evolutionary studies is that their DNA represents a lineage of historical events that can reflect species differences since mitochondria are vertically transmitted without recombination (Strickberger, 2000). In this regard, the region of COI gene was a good candidate for constructing the phylogenetic relationships in species complex or sibling species (Lunt *et al.*, 1996).

The appearance and radiation of the angiosperms has been hypothesized to accelerate the evolution of several herbivorous insect orders including Diptera (Strong *et al.*, 1984). From previous phylogenetic study of tephritid fruit flies, rapid evolution of this fruit fly group was suggested (Han and McPheron, 1997). In the present study, the mtDNA was used to determine the phylogenetic relationships of ten species of the *B. dorsalis* complex using the variable region (638 bp) of the mitochondrial gene

encoding subunit I of cytochrome oxidase (COI). The effect of host plant on the *B. dorsalis* fruit fly species diversity has also been investigated.

Materials and methods

The specimens

The ten species of the *B. dorsalis* complex were collected from naturally infested fruits from different parts of Thailand (Table 1). The larval specimens were reared to adult in the laboratory at Mahidol University, Bangkok. The adults were allowed to mature, identified and then stored at −70°C until required for molecular analysis.

Template preparation and DNA manipulations

Total DNA was extracted from the ovaries or testes of individual adult flies using the STE boiling method (O'Neill et al., 1992). The ovaries or testes were then homogenized with a sterilized polypropylene pestle in a 1.5 µl microcentrifuge tube filled with 100 µl of STE buffer (100 mM NaCl, 10 mM TrisCl pH 8.0, 1 mM EDTA). The homogenate was heated at 95°C for 10 min before being centrifuged at 14,000 rpm for 1 min at room temperature.

Partial sequences of mitochondrial COI gene were amplified from all genomic DNA samples by polymerase chain reaction using sense primer (UEA 7; 5'-TAC AGT TGG AAT AGA CGT TGA TAC-3') and antisense primer (UEA 10; 5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3') developed by Lunt et al. (1996). PCR amplification was done in 20 µl reaction volumes: 12.5 µl dd H_2O, 2 µl 10X PCR buffer (Promega), 2 µl 25 mM $MgCL_2$, 0.5 µl dNTPs (10 mM each), 0.5 µl 20 µM of both primers and 1 units of Taq DNA polymerase (Promega). Thermal cycling used an initial denaturation period of 3 min at 94 °C, followed by 35 cycles of 94 °C for 1 min., 50 °C for 1 min. and 72 °C for 1 min. and final extension step at 72 °C for 30

min. Ten microliters of PCR product was run on a 1% agarose gel to determine the presence and size of amplified DNA.

One microliter of the PCR product was directly ligated into pGEM-T vector (Promega) in a 10 µl reaction and incubated at 4 °C for 2 nights. Plasmids were isolated using Qiaprep Plasmid miniprep kit (Qiagen) according to the manufacturer's recommendation. At least three independent clones were sequenced from each tephritid fruit fly specimens. The sequencing of inserts in both directions was carried out using T7 and SP6 primers on an ABI 377 automated sequencer (Bioservice Unit, NSTDA). Concensus sequences of COI fragments from each fruit fly specimens were constructed and used for later analyses. The sequences for the ten species of the *B. dorsalis* complex were deposited in GenBank. The accession numbers are listed in Table 1.

Data analysis

The nucleotide sequence differences among ten species of the *B. dorsalis* complex were calculated using MEGA software version 2.0 (Kumar et al., 2001). Kimura-2-parameter distance methods (Kimura, 1980) were used to calculate nucleotide sequence differences. The overall transition-transversion ratio was calculated by general time reversible method (Yang, 1994) chosen by Modeltest program (Posada and Crandall, 1998). Synonymous substitution rate was determined by the MEGA program version 2.0 (Kumar et al., 2001), which employed Modified Nei and Gojobori (Nei and Gojobori, 1986).

In addition to the ten species of the *B. dorsalis* complex, mitochondrial COI gene sequence of the same region from *B. tau* s.s., *Ceratitis capitata*, *Anopheles gambiae*, *Compsomyiops callipes* and *Locusta migratoria* were used in the analysis as outgroups. The sequences were initially aligned using the clustal algorithm and then

adjusted by eye. The data set was analyzed by maximum parsimony (Fitch, 1971), neighbor-joining (Saitou and Nei, 1987) and maximum likelihood (Felsenstein, 1981) using PAUP 4.0 b1 (Swofford, 2000). The maximum parsimony trees were reconstructed by heuristic search and bootstrap test was performed with 1,000 replicates. The characters were weighted equally. The neighbor-joining tree was reconstructed by heuristic search using the Kimura-2-parameters model (Kimura, 1980) and bootstrap analyses were performed with 1,000 replicates. For maximum likelihood tree, the initial tree searching and model fitting were calculated using Modeltest 2.0 (Posada and Crandall, 1998). The estimated rates for six nucleotide substitution types, nucleotide frequencies and the proportion of invariable sites were used in the later analyses. Rates of substitution at variable sites were assumed to follow a gamma distribution of four rate categories, with the average rate for each category represented by the mean. The shape parameter of gamma distribution was set to 0.4396 from the previous calculation. The likelihood model is a general time-reversible (Yang, 1994) with rate heterogeneity. Heuristic search was conducted with step-wise addition method using the model parameters mentioned previously. Bootstrap test was performed with 500 replicates.

Results

From 638 bp, downstream region of COI gene, the average nucleotide divergence in the *B. dorsalis* complex ranged from 0.6% to 16.2%. Among 90 variable sites found in the *B. dorsalis* complex, 15 were non-synonymous substitutions and 75 were synonymous substitutions. Transition to transversion ratios ranged from 0 to 9.56. The majority (67.14 %) of transitions were thymine-cytosine substitutions, while most of transversions were adenine-thymine (41.94 %) and

adenine-cytosine (45.16 %). The third and second codon positions were biased towards adenine and thymine (81.3 % and 80.3 %, respectively).

There was variation in amino acids at 15 locations across the 213 COI amino acid sequences downstream (Table 2). From the total 15 locations of amino acid variation, *B. arecae* and *B. dorsalis* sp. L had the highest amino acid divergence complex with ten amino acid differences compared with other species of the *B. dorsalis* complex used in this study.

We reconstructed a phylogenetic tree for ten species of the *B. dorsalis* complex together with five outgroup species including *B. tau* s.s. Tree topologies calculated from three phylogenetic analysis methods, neighbor-joining, maximum likelihood and maximum parsimony were similar. Figure 1 shows the maximum likelihood tree based on General time reversible model with gamma shape parameter = 0.44. Its reliability was assessed with 500 replications of the bootstrap algorithm. The fruit flies of the *B. dorsalis* complex clustered together with 99% bootstrap support when calculated using maximum likelihood and neighbor-joining methods and 100% bootstrap support when calculated using maximum parsimony method. Within the *B. dorsalis* complex, the phylogenetic tree represents three distinct clades. First, the clade consisting of *B. pyrifoliae* and *B. dorsalis* sp. L showed bootstrap support of 100% when calculated using all three methods. The sequence divergence between these two species was 1.1% (7 bp difference). Second, the clade consists of seven species, *B. dorsalis* s.s. (sp. A), *B. dorsalis* sp. D, *B. dorsalis* sp. J, *B. dorsalis* sp. O, *B. kanchanaburi*, *B. carambolae*, and *B. raiensis*, with bootstrap support of at least 82%. In this clade, the sequence divergences were ranged from 0.6 % (4 bp difference) to 1.8% (11 bp difference). The third clade has only one species in this

study, i.e., *B. arecae*, which represents the oldest lineage within this species complex, with 12.7% - 16.2% divergence from the other species.

Using the same model, the neighbor-joining trees were also reconstructed based on either transition or transversion sites. Some branching orders in the neighbor-joining tree using only transition sites (Ti-NJ) were not compatible with those using only transversion sites (Tv-NJ). In the Ti-NJ tree, *B. dorsalis* sp. L and *B. pyrifoliae* formed a monophyletic clade, but it is not so in the Tv-NJ tree (tree not shown).

Discussion

Tephritid fruit flies of the genus *Bactrocera* have been considered a large group of species diversity with economical importance (Drew, 1989, Hardy, 1973, Hardy and Adachi, 1954). Of these, the *B. dorsalis* complex represents a good candidate for study of species complexity. Morphological identification of the fruit flies belonging to the *B. dorsalis* complex has been problematic for taxonomists (White and Elson-Harris, 1994). We used 638 bp of the downstream COI gene to infer the phylogenetic relationships among ten species of the *B. dorsalis* complex. According to our phylogenetic tree, the ten species of the *B. dorsalis* complex are monophyletic and it is related to *B. tau* s.s. more than other outgroups. High nucleotide sequence divergence was found among members of the *B. dorsalis* complex similar to those in the *B. tau* complex (Jamnongluk *et al*, 2002).

Like other closely related species groups, the nucleotide sequence substitution of COI gene in the *B. dorsalis* complex was transition bias. Beckenbach *et al.* (1993) suggested that transition substitutions might involve in sequence divergence among closely related species because transitions at silent sites reach saturation more quickly than do silent transversions. Phylogenetic tree reconstructed from only transitional

sites showed similar tree topology to phylogenetic tree reconstructed from all sites. However, the bootstrap supporting for all branches of Ti-NJ tree was less than the tree constructed from all sites.

Based on mitotic chromosome evidence, 27 species of the *B. dorsalis* group in Thailand were roughly classified into six cytological groups (Baimai *et al.*, 1995, 1999 a, b, 2000). Based on partial mitochondrial COI gene in this study, we could arrange the ten species of the *B. dorsalis* complex into three groups (Figure 1). The phylogenetic analyses of tephritid fruit fly species in the *B. dorsalis* complex revealed the concordance of mitotic chromosome and mitochondrial COI sequence data (Figure 1).

The simplest model of speciation process leading to geographical isolation and subsequently reproductive isolation between populations as a by-product of adaptation to alternative selection process could possibly apply to some members of the *B. dorsalis* complex. Several species of the *B. dorsalis* complex were host-plant specific and found only in certain areas. For example, the two closely related species, *B. dorsalis* sp. D and *B. dorsalis* sp. J, were found infested different host plants in different locations in Thailand (Baimai, unpublished data). A true specialist, *B. arecae* attacks only palm nuts of the Arecaceae family (Allwood *et al.*, 1999). Adaptation to specific host-plant species could have led to genetic differentiation and subsequently reproductive isolation between *B. arecae* and its ancestor.

The cytoplasmic incompatible-inducing bacteria, *Wolbachia* might also be one of the speciation agents involving in the *Bactrocera* species complex by means of cytoplasmic incompatibility mechanism. The cytoplasmic incompatibility phenomena has been found in several insect species (Hoffmann and Turelli, 1997) and suggested to be possible cause of reproductive isolation (Werren, 1997). The bidirectional

cytoplasmic incompatibility phenomenon has been found when insect hosts were infected with different *Wolbachia* strains (Hoffmann and Turelli, 1997). Therefore, *Wolbachia* have been suggested to be possible agent causing reproductive isolation and speciation in their host (Bordenstein *et al.*, 2001). The phylogenetic evidences showed a non-concordance of *Wolbachia* strains in two sympatric species of *B. dorsalis* complex, *B. dorsalis* sp. A and *B. pyrifoliae* (Jamnongluk *et al.*, 2001). Thus, through bidirectional incompatibility mechanism, *Wolbachia* might cause speciation between these two *B. dorsalis* complex species.

As in other phytophagous insects, i.e. the apple maggot fly, *Rhagoletis pomonella* (Bush, 1969) and the pea aphids, *Acyrthosiphon pisumon* (Via, 1999), speciation adaptation to different host plant species in tephritid fruit flies in the *B. dorsalis* complex is indicated in our results. Moreover, reproductive isolation caused by *Wolbachia* via the bidirectional cytoplasmic incompatibility phenomenon might lead to speciation between two sibling species, which infected with two different *Wolbachia* strains.

Acknowledgements:

We would like to thank Saen Tigvattananont, Anchalee SaeLee and Chalow Samrandee for collecting and identifying part of the original samples and Elizabeth McGraw for reviewing the manuscript. This work was supported by the TRF/BIOTEC Special Program for Research and Training in Biodiversity (BRT 139026) and the Thailand Research Fund (RGJ/PHD/0095/2541, RTA /01/2541, and BGJ/16/2543).

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Table 1. Taxa examined

Taxon name	Accession number	Plant Host (Family)	Reference
Diptera: Tephritidae			
Bactrocera arecae	AY053508	*Areca catechu* (Palmae)	This study
Bactrocera carambolae	AY053509	*Psidium guajava* (Myrtaceae)	This study
Bactrocera dorsalis s.s. (RN-SK)	AY053507	*Sandoricum koetjape* (Meliaceae)	Jamnongluk *et al.* 2002
Bactrocera dorsalis s.s. (RN-MA)		*Musa acuminata* (Musaceae)	This study
Bactrocera dorsalis s.s. (KB-PG)		*Psidium guajava* (Myrtaceae)	This study
Bactrocera dorsalis sp. D		*Solanum erianthum*	This study
Bactrocera dorsalis sp. J		*Syzygium claviflora*	This study
Bactrocera dorsalis sp. L	AY053511	*Platea* sp. (Icacinaceae)	This study
Bactrocera dorsalis sp. O	AY053512	*Willughbeia firma* (Apocynaceae)	This study
Bactrocera kanchanaburi		*Artabotrys siamensis*	This study
Bactrocera pyrifoliae	AY053514	*Psidium guajava* (Myrtaceae)	This study
Bactrocera raiensis	AY053515	*Sandoricum koetjape* (Meliaceae)	This study
Bactrocera tau s.s.	AF400067	*Cucurbita moschata* (Cucurbitaceae)	Jamnongluk *et al.* 2002 b
Ceratitis capitata	AJ242872	Many species (Mediterranean fruit fly)	Spanos *et al.*, 2000
Diptera: Culicidae			
Anopheles gambiae	L20934	-	Beard *et al.*, 1993
Diptera: Calliphoridae			
Compsomyiops callipes	AF295549	-	Wells and Sperling, 2001
Orthoptera: Acrididae			
Locusta migratoria	X80245	Sorghum	Flook *et al.*, 1995

Table 2. Amino acid variation among ten species of the *B. dorsalis* complex within a 638 bp segment of mtDNA. Vertical numbers at top of columns represent the nucleotide site that varies. The nucleotide locations were according to *Drosophila melanogaster* mitochondrial DNA (Lewis *et al.*, 1995)

Species	259 6	266 2 6	267 7 8	269 1	282 2	282 4	289 6	289 9	290 8	291 4	295 0	295 6	298 9	299 5	300 5
B. dorsalis sp. A 1	F	F	V	K	V	V	V	T	Q	I	L	N	L	L	N
B. dorsalis sp. A 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B. dorsalis sp. A 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B. dorsalis sp. D	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.
B. dorsalis sp. J	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B. dorsalis sp. O	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B. kanchanaburi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B. carambolae	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.
B. raiensis	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
B. pyrifoliae	.	.	.	.	.	.	.	.	.	V	.	.	.	.	T
B. dorsalis sp. L	.	.	.	.	G	.	.	P	.	V	.	D	M	.	T
B. arecae	.	.	T	N	.	.	I	.	.	V	F	.	.	I	.