Molecular identification of *Trichogramma* species from South and South-East Asia and natural *Wolbachia* infection

Yu-Di Liu, Mao-Lin Hou & Kai Song

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Trichogramma wasps were collected from the parasitized eggs of lepidopteran pests from 21 sampling sites in East Asia and South-East Asia. Six *Trichogramma* species were identified based on the molecular identification method using the internal transcribed spacer 2 (ITS2) region of the rDNA of *Trichogramma chilonis*, *T. evanescens*, *T. ostriniae*, *T. embryophagum*, *T. dendrolimi* and *T. japonicum*. The results of molecular identification were confirmed by morphological identification. Additionally, natural populations were screened for the prevalence of *Wolbachia*. Five out of 21 populations were infected by the same *Wolbachia* strain, which was identified by using *Wolbachia wsp* gene and multi–locus sequencing approach. The phylogenetic analysis of *Wolbachia wsp* sequences revealed that the *Wolbachia* strain was classified in the strain wEvaA in the group of EvA of the supergroup A.

Y.-D. Liu & M.-L. Hou, State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, No. 2, West Yuan Ming Yuan Road, Beijing 100193, China; *Corresponding author's e-mail: ydliu@ippcaas.cn*
K. Song, Dryland Farming Institute, Hebei Academy of Agricultural and Forestry Sciences, Hengshui, China

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1. Introduction

There are about 650 species in the family Trichogrammatidae (Grissel & Schauff 1990), and they are the most widely used parasitoids in biological control programs (Kumar *et al.* 2009). For successful biological control purpose, the identification of *Trichogramma* species is the important first step (Hassan 1994). Unfortunately, species identification in this group is difficult due to their small size, the large number of species, and the lack of clear morphological characteristics. Identification is time consuming and requires specialized skills (Pinto *et al.* 1989, Pinto & Stouthamer 1994, Poorjavad *et al.* 2012). In order to be of economic importance in biological control projects, it is essential to be able to identify the Trichogrammatid species quickly, and the methods have to be simple and widely applicable.

Molecular approaches based on DNA sequences of the internal transcribed spacer 2 (ITS2) have helped to solve the above difficulty (Stouthamer *et al.* 1999). Several studies have used the ITS region to identify the *Trichogramma* species occurring in different regions (Silva *et al.* 1999, Kumar *et al.* 2009, Sumer *et al.* 2009,

Poorjavad *et al.* 2012, Nasir *et al.* 2013, Pino *et al.* 2013). The ITS2 region has been used to distinguish *Trichogramma* species collected from tomato fields in Portugal by sequencing and restriction analysis (Silva *et al.* 1999). Pino *et al.* (2013) rapidly identified five *Trichogramma* species occurring in the Canary Islands by using multiplex PCR method based on amplification of ITS2 region. Poorjavad *et al.* (2012) used PCR amplification of ITS2 region to identify seven Iranian *Trichogramma* species, which were identical in external morphology. Nasir *et al.* (2013) used the ITS2 region of rDNA to distinguish six *Trichogramma* species collected from different ecological zones of Pakistan.

In East Asia and South-East Asia, rice (*Oryza sativa* L.) is a staple food source for more than half of the world's population (Gross & Zhao 2014). To reduce the application of pesticides in control of the rice pests, integrated pest management (IPM) based on biological control by *Trichogramma* releases was launched (Ko *et al.* 2014). Therefore, in this study, comprehensive field surveys were conducted in these regions to collect *Trichogramma* species. Finally, *Trichogramma* were collected from 21 sampling sites in East Asia and South-East Asia. The molecular method based on ITS2 region was used to identify these *Trichogramma* species.

On the other hand, *Wolbachia*, as a symbiotic bacterium, plays important roles in evolution, ecology, and reproduction of their hosts (Werren 1997). They are extremely common, with 20–76% of insect species being infected (Harris *et al.* 2003). More than 20 *Trichogramma* species have been partly or completely infected by *Wolbachia* (Poorjavad *et al.* 2012). Because *Wolbachia* infection can affect the wasp's fitness, it is necessary to investigate the infection status of a population, which may give important aids for *Trichogramma* application in biological control programs (Stouthamer & Kazmer 1994, Horjus & Stouthamer 1995, Poorjavad *et al.* 2012). Therefore, in this study, the collected *Trichogramma* wasps were screened for the infection with *Wolbachia*, and the respective *Wolbachia* strain was identified by sequences of *Wolbachia* surface protein (*wsp*), Cytochrome *c* oxidase, subunit I (*cox*A) and Fructose-bisphosphate aldolase (*fbp*A).

2. Materials and methods

2.1. *Trichogramma* collection

Parasitized *Trichogramma* eggs were collected from 21 paddy fields in China and Korea in 2011 and 2012 (Table 1), and kept individually in glass tubes until adult emergence. In some fields, fresh sentinel eggs of *Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae) were used to trap *Trichogramma*. Therefore, the originating host of *Trichogramma* for some sites is the sentinel host *C. cephalonica*. After collection, the adults were reared on eggs of the grain moth, *Sitotroga cerealella* Olivier (Lepidoptera: Gelenchiidae), in climate chambers at 25 ± 1 °C, 70 ± 5% RH with the photoperiod of 14:10 (L:D) h. Populations are defined as the progeny from one egg batch, which are collected from the same species at the same location on the same day.

2.2. DNA isolation

DNA was extracted using chelating agent Chelex–100 (5%) (Biorad) method according to Stouthamer *et al.* (1999). One to three wasps from the single parasitized egg were ground in 100 µl 5% Chelex–100 (Biorad) and 3 µl proteinase K (20 mg/ml) and incubated for 2 h at 56 °C, followed by 10 min at 95 °C. The supernatant was stored at –20 °C for subsequent molecular analysis.

2.3. PCR amplification

The ITS2 region was amplified using the following primers: forward, 5 –TGTGAACTGCAGGACACATG–3, located in the 5.8S rDNA; and reverse, 5 –GTCTTGCCTGCTCTGAG–3, located in the 28S rDNA closer to the 3 end of the ITS2 (Stouthamer *et al.* 1999). Touchdown thermal cycling programs encompassing a 5 °C span of annealing temperatures at 55–50 °C were performed for the amplification using a S1000™ Thermal Cycler (Bio–Rad). After an initial denaturation at 94 °C for 4 min, cycling parameters were 10 cycles of 95 °C for 20 s, highest annealing temperature (decreased 0.5 °C per cycle) for 30 s, and 72 °C for 30 s; and 30 cycles of 95 °C for 20 s, lowest annealing temperature for 30 s, and

Table 1. Origins of *Trichogramma* species and strains with originating hosts, no. of base pairs in ITS2 (internal transcribed spacer 2 region of rDNA) sequences and GenBank accession numbers.

Sample no. and Code	Species	Population	Host	ITS2 and Acc. no.
1. Tc-CJ	*T. chilonis*	Cuijia, Xing'an, China 34°55'46"N, 109°37'51"E	*Corcyra cephalonica*	426 KR148947
2. Tc-WLP	*T. chilonis*	Wulipai, Xing'an, China 24°24'0"N, 120°37'58"E	*C. cephalonica*	426 KR148947
3. Tc-Lo	*T. chilonis*	Vientiane, Laos 17°58'0"N, 102°36'0"E	*Chilo suppressalis*	426 KR148948
4. Tc-NB	*T. chilonis*	Ningbo, China 29°52'48"N, 121°33'0"E	*C. suppressalis*	426 KR148948
5. Tc-MM	*T. chilonis*	Hmawbi, Myanmar 17°5'0"N, 95°57'0"E	*Scirpophaga incertulas*	426 KR148948
6. Tc-TW	*T. chilonis*	Taiwan, China 25°3'0"N, 121°31'0"E	*C. suppressalis*	426 KR148948
7. Tc-HS	*T. chilonis*	Hengshui, China 37°32'14"N, 115°28'59"E	*C. suppressalis*	426 KR148949
8. Te-HS	*T. evanescens*	Hengshui, China 37°32'14"N, 115°28'59"E	*Ostrinia furnacalis*	546 KR148950
9. Te-BJ	*T. evanescens*	Beijing, China 39°54'27"N, 116°23'17"E	*C. cephalonica*	546 KR148950
10. Te-CQ	*T. evanescens*	Chongqing, China 29°10'47"N, 106°9'36"E	*C. cephalonica*	546 KR148950
11. Te- K1	*T. evanescens*	Korea 37°28'N, 126°37'E	*O. furnacalis*	546 KR148950
12. Te- K2	*T. evanescens*	Korea 37°28'N, 126°37'E	*O. furnacalis*	546 KR148950
13. Te-Tconf	*T. evanescens*	Guangdong, China 23°4'48"N, 113°8'24"E	*O. furnacalis*	546 KR148951
14. Te-GD	*T. evanescens*	Guangdong, China 23°4'48"N, 113°8'24"E	*O. furnacalis*	544 KR148952
15. To-HBZ	*T. ostriniae*	Hebianzai, Dehong, China 24°16'57"N, 104°25'21"E	*C. cephalonica*	566 KR148945
16. To-MZ	*T. ostriniae*	Mangzai, Dehong, China 24°0'16"N, 101°6'26"E	*C. cephalonica*	566 KR148945
17. To-HS	*T. ostriniae*	Hengsui, China 37°32'14"N, 115°28'59"E	*C. cephalonica*	566 KR148946
18. Tem-Tcac	*T. embryophagum*	Husa, Dehong, China 24°27'48"N, 97°53'24"E	*C. cephalonica*	587 KR148953
19. Td-NJ	*T. dendrolimi*	Nanjing, China 32°3'0"N, 118°46'60"E	*Cnaphalocrocis medinalis*	520 KR148954
20. TJ-GD	*T. japonicum*	Guangdong, China 23°4'48"N, 113°8'24"E	*C. suppressalis*	432 KR148955
21. Tj-HS	*T. japonicum*	Husa, Dehong, China 24°27'48"N, 97°53'24"E	*S. incertulas*	432 KR148955

72 °C for 30 s. This was followed by a final extension step at 72 °C for 7 min. Each reaction was run in a volume of 30 µL, containing 3 µL (10×) Taq assay buffer, 300 µM dNTP, 0.4 µM of each primer, 1 U of Taq DNA polymerase (TaKaRa Biotechnology, Dalian, China), and 50–100 ng genomic DNA.

The primer used to amplify the *wsp* fragment from *Trichogramma* samples was that described by Braig *et al.* (1998). We also used two conserved *Wolbachia* genes, *fbp*A and *cox*A, to characterize *Wolbachia* strain for *Trichogramma* samples, which were amplified based on the methods of Baldo *et al.* (2006). The PCR primers were designed also according to Baldo *et al.* (2006). The PCR reaction conditions and the

Table 2. Information of *wsp* gene for constructing the phylogenic tree of *Wolbachia* strains in Fig 1.

Group	*Wolbachia* host species	*Wolbachia* strain	GenBank Acc. no.
Supergroup A			
Ha	*Drosophila sechella*	*w*Ha	AF020073
Ha	*Cadra cautella*	*w*CauA	AF020075
Aus	*Glossina austeni*	*w*Aus	AF020077
Kue	*Trichogramma ourarachae*	*w*Bou	AF071913
Kue	*Trichogramma evanescens*	*w*EvaB	AY390280
Kue	*Trichogramma kaykai*	*w*KayA	AF071912
Mel	*Drosophila simulans*	*w*Cof	AF020067
Mel	*Amitus fuscipennis*	*w*Fus	AF071909
Mel	*Drosophila melanogaster*	*w*Mel	AF020063
Dro	*Trichogramma drosophilae*	*w*Dro	AF071910
Eva	*Trichogramma evanescens*	*w*EvaA	AY390279
Pap	*Phlebotomus papatasi*	*w*Pap	AF020082
Uni	*Muscidifurax uniraptor*	*w*Uni	AF020071
Mors	*Nasonia vitripennis*	*w*VitA	AF020081
Supergroup B			
Ori	*Leptopilina australis*	*w*Aus	AF071920
Ori	*Cadra cautella*	*w*CauB	AF020076
Ori	*Spalangia fuscipes*	*w*Fu	AF071921
Con	*Trichogramma bedeguaris*	*w*Bed	AF071915
Dei	*Trichogramma deion*	*w*Dei	AF020084
Vul	*Armadillidium vulgare*	*w*Vul	AF071917
Pip	*Trichogramma chilonis*	*w*Chi	AY311486
Pip	*Drosophila simulans*	*w*Ma	AF020069
Pip	*Culex pipiens*	*w*Pip	AF020061
	Spodoptera exigua	*w*ExiB	EU332344
Pip	*Ostrinia furnacalis*	*w*FurB	EU294312
Kay	*Trichogramma kaykai*	*w*KayB	AF071924
Kay	*Trichogramma nubilale*	*w*Nub	AF071926
Supergroup D			
	Brugia malayi		JX506736
Supergroup F			
	Cimex lectularius		DQ842459

thermal cycling protocol were identical to those described above.

After purified with PCR Cleanup Kit (Axygen, USA), the PCR products were directly sequenced on an Automated DNA Sequencer (ABIPRISMTM 3730XL, APPLIEDBIOSYSTEMS, INC. Foster City, CA). All sequences were aligned using CLUSTAL_X (Thompson *et al.* 1997) and rechecked by eye to verify for accuracy.

2.4. Phylogenetic analysis

The *wsp*, *cox*A, and *fbp*A sequences of *Wolbachia* from the different *Trichogramma* populations were first blasted in NCBI, then analyzed and aligned with Clustal X1.83 (www.clustal.org). Some reference sequences of *Wolbachia wsp*, *cox*A, and *fbp*A sequences of other species were downloaded from GenBank for the phylogenetic analysis of *Wolbachia* (Tables 2 and 3). In order to verify the consistency of the tree, phylogenetic trees were constructed using two methods, neighbor joining (NJ) and maximum parsimony (MP) (Mega 4.0 software, MEGA, www.megasoftware.net). Bootstrap analysis was done with 1,000 replications, and bootstrap values were calculated using a 50% majority rule. Two reference sequences belonging to the *Wolbachia* D and F supergroups were used as the outgroups in the phylogenetic trees of *wsp*, *cox*A, and *fbp*A; *Brugia malayi* (Brug, 1927) (*wsp* JX506736,

Table 3. Host species, supergroups and GenBank accession numbers of *cox*A and *fbp*A genes for constructing the phylogenic tree (Fig. 2) of *Wolbachia* strains.

Host species or subspecies	Supergroup	*cox*A Accession no.	*fbp*A Accession no.
Brugia malayi	D	DQ842273	DQ842347
Cimex lectularius	F	DQ842275	DQ842349
Acromis sparsa	A	DQ842271	DQ842345
Aedes albopictus	A	DQ842268	DQ842342
Camponotus pennsylvanicus	A	DQ842276	DQ842350
Drosophila bifasciata	A	DQ842279	DQ842353
Drosophila innubila	A	DQ842280	DQ842354
Drosophila melanogaster	A	DQ842304	DQ842378
Drosophila neotestacea	A	DQ842281	EU126408
Drosophila orientacea	A	DQ842282	EU126398
Drosophila recens	A	DQ842283	DQ842357
Ephestia kuehniella	A	DQ842289	DQ842363
Incisitermes snyderi	A	DQ842292	DQ842366
Muscidifurax uniraptor	A	DQ842293	DQ842367
Nasonia giraulti	A	DQ842294	DQ842368
Nasonia longicornis	A	DQ842295	DQ842369
Nasonia vitripennis	A	FJ390240	DQ842370
Solenopsis invicta	A	DQ842300	DQ842374
Acraea encedon	B	DQ842269	DQ842343
Acraea eponina	B	DQ842270	DQ842344
Armadillidium vulgare	B	FJ390241	EF451552
Chelymorpha alternans	B	DQ842274	DQ842348
Culex pipiens pipiens	B	DQ842277	DQ842351
Culex pipiens quinquefasciatus	B	DQ842278	DQ842352
Drosophila simulans	B	KF987018	KF987033
Encarsia formosa	B	DQ842288	DQ842362
Gryllus firmus	B	DQ842291	DQ842365
Nasonia vitripennis	B	DQ842297	DQ842371
Ostrinia scapulalis	B	DQ842298	DQ842372
Protocalliphora sialia	B	DQ842299	DQ842373
Teleogryllus taiwanemma	B	DQ842303	DQ842377
Tribolium confusum	B	DQ842301	DQ842375
Trichogramma deion	B	DQ842302	DQ842376

*cox*A DQ842273, *fbp*A DQ842347) and *Cimex lectularius* (Latreille, 1802) (*wsp* DQ842459, *cox*A DQ842275, *fbp*A DQ842349).

3. Results

3.1. *Trichogramma* identification and distribution

The ITS2 sequences that were obtained from each population were compared with the identified ITS2 sequences of rDNA from GenBank to confirm the identification of *Trichogramma* species (Table 4). Six *Trichogramma* species were identified: *T. chilonis* (Ishii, 1941) (seven populations), *T. evanescens* (Westwood, 1833) (seven populations), *T. ostriniae* (Pang & Chen, 1974) (three populations), *T. embryophagum* (Hartig, 1838) (one population), *T. dendrolimi* (Matsumura, 1926) (one population) and *T. japonicum* (Ashmead, 1904) (two populations). The ITS2 sequences obtained from each species were (97–100% Max Ident score in BLAST) similar to those present in GenBank. In this study, a total of eleven ITS2 sequences were deposited in GenBank (accession numbers KR148945–KR148955) (Table 1), and these sequences were complete ITS2 sequences plus flanking sequences of 5.8S and 28S. Although these sequences display

Table 4. Sequences used from GenBank for comparison of the *Trichogramma* species in this study.

Sample no. and spp.	Sequence	Base pairs	Accession no. similarity (%)	Sequence
1. *T. chilonis*	partial ITS-2	1,155	AY167415	99
2. *T. chilonis*	partial ITS-2	1,158	AY167418	99
3. *T. chilonis*	Complete ITS-2	560	DQ088055	99
4. *T. chilonis*	Complete ITS-2	538	GU562445	99
5. *T. evanescens*	Complete ITS-2	559	DQ088059	99
6. *T. evanescens*	Complete ITS-2	546	FJ436332	99
7. *T. evanescens*	Complete ITS-2	546	JN315373	99
8. *T. evanescens*	Complete ITS-2	546	JN315380	99
9. *T. ostriniae*	Complete ITS-2	446	AY244463	98
10. *T. ostriniae*	Complete ITS-2	447	AY518695	99
11. *T. ostriniae*	Complete ITS-2	491	GQ324625	98
12. *T. embryophagum*	Complete ITS-2	479	AF453562	100
13. *T. embryophagum*	Complete ITS-2	474	AY244465	100
14. *T. embryophagum*	Complete ITS-2	593	DQ344044	97
15. *T. embryophagum*	Complete ITS-2	530	JF920430	97
16. *T. dendrolimi*	partial ITS-2	510	AB094398	98
17. *T. dendrolimi*	Complete ITS-2	412	AF453555	99
18. *T. dendrolimi*	Complete ITS-2	540	AF517576	98
19. *T. dendrolimi*	Complete ITS-2	465	DQ344045	97
20. *T. japonicum*	Complete ITS-2	578	DQ471294	98
21. *T. japonicum*	partial ITS-2	436	FN822756	98
22. *T. japonicum*	partial ITS-2	438	FN822758	98
23. *T. japonicum*	partial ITS-2	436	FN822759	98

significant interspecies differences, they showed low intraspecific variability in length (2–10 bases).

The comparison showed that *T. chilonis* was found from seven populations with three different sequences, Tc–CJ and Tc–WLP (KR148947); Tc–Lo, Tc–NB, Tc–MM, Tc–TW (KR148948); Tc–HS (KR148949) (Table 1). The three sequences had only three mutation sites, and the sequences were closely similar (99%) to *T. chilonis* in GenBank accession numbers AY167415, AY167418, DQ088055 and GU562445 (Table 4).

The *T. evanescens* specimens from seven populations had three different sequences: Te–HS, Te–BJ, Te–CQ, Te–K1 and Te–K2 (KR148950); Te–Tconf (KR148951); Te–GD (KR148952) (Table 1). The three sequences had 10 mutation sites, and the sequences were closely similar (99%) to *T. evanescens* in GenBank accession numbers DQ088059, FJ436332, JN315373 and JN315380 (Table 4).

The *T. ostriniae* specimens from three populations had two different sequences: To–HBZ and To–MZ (KR148945); To–HS (KR148946), which had two mutation sites (Table 1). The two sequences were closely similar (98–99%) to *T. ostriniae* in GenBank accession numbers AY244463, AY518695 and GQ324625 (Table 4).

The sequence of *T. embryophagum*, Tem–Tcac (KR148953), was closely similar (97–100%) to GenBank accession numbers AF-453562, AY244465, DQ344044 and JF920430 (Table 4).

The sequence of *T. dendrolimi* (Td–NJ, KR148954) was closely similar (97–99%) to that of *T. dendrolimi* available in GenBank (AB-094398, AF453555, AF517576 and DQ344045) (Table 4).

The sequence of *T. japonicum* (Tj–GD and Tj–HS KR148955) was closely similar (98%) to that of *T. japonicum* available in GenBank (DQ-471294, FN822756, FN822758 and FN822759) (Table 4).

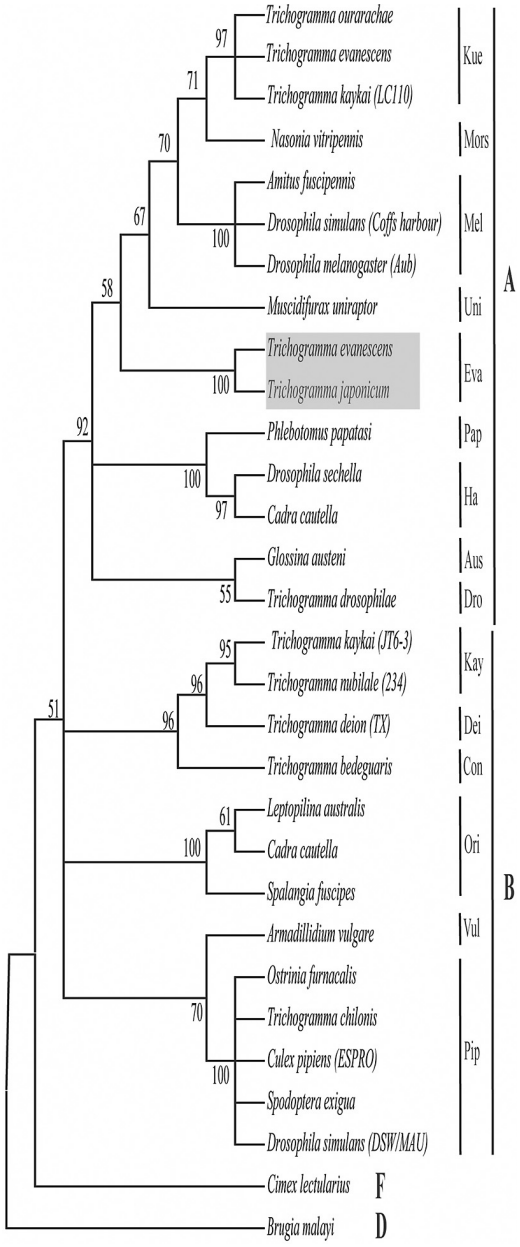

Fig. 1. Phylogenetic neighbor joining tree of *Wolbachia* based on *wsp* sequences. For information of *wsp* gene, see Table 2. Sequences of *Brugia malayi* and *Cimex lectularius* were used as outgroups. Names of host species and their related groups are listed on the right side of the figure. Supergroups of *Wolbachia* are shown as uppercase letters. The infected strain of *Trichogramma japonicum* clustered together with that of *Trichogramma evanescens*, which were the strain Eva. They are highlighted by shaded area. Bootstrap values >50% are shown above branches.

3.2. Identification of the associated *Wolbachia* strain

The detection of *Wolbachia* was performed for *Trichogramma* species from 21 sites. The infection existed in only five populations, which were Tc-TW, Te-BJ, Te-K1, Tem-Tcac and Tj-GD, and all tested individuals of these five populations were infected. Furthermore, all of the five populations were found to have identical sequences of the *wsp*, *cox*A and *fbp*A genes. As these five populations were infected with the same *Wolbachia* strain, only one of them (*Trichogramma japonicum*) was used for the phylogenetic analysis based on *wsp*, *cox*A and *fbp*A genes.

The phylogenetic analysis was performed for the *Trichogramma Wolbachia wsp* sequences and 29 reference sequences. The tree has two major branches, corresponding to supergroups A and B (Fig. 1). The *wsp* sequence in *Trichogramma* samples shared 100% identity with the sequences from *T. evanescens* (AY390279). This *Wolbachia* strain was defined in the strain *wEva*A in the group of Eva of the supergroup A (Fig. 1). The phylogenetic tree for the concatenated sequences of *Wolbachia cox*A and *fbp*A is shown in Fig. 2. Similar to the *wsp* tree, the concatenated sequences were first clustered into supergroup A branch. The concatenated sequences were clustered into a subclade with *Aedes albopictus* (Skuse, 1894) (*cox*A DQ842268 and *fbp*A DQ842342), sharing 99% identity (Fig. 2). As the topologies of the trees inferred from neighbor joining and maximum parsimony were similar based on *wsp* and concatenated *cox*A and *fbp*A sequences, we only displayed the trees constructed by neighbor joining (Figs 1 and 2). The sequences for the *wsp*, *fbp*A and *cox*A genes were deposited in GenBank with the accession numbers KR906068, KR906069 and KR906070.

4. Discussion

ITS2 provides an excellent method for separating closely related species of *Trichogramma*. The main advantage of the DNA identification system over the morphological system is that it is fast and requires few specialized skills, and can work well on the dried or 100% alcohol stored specimen(s).

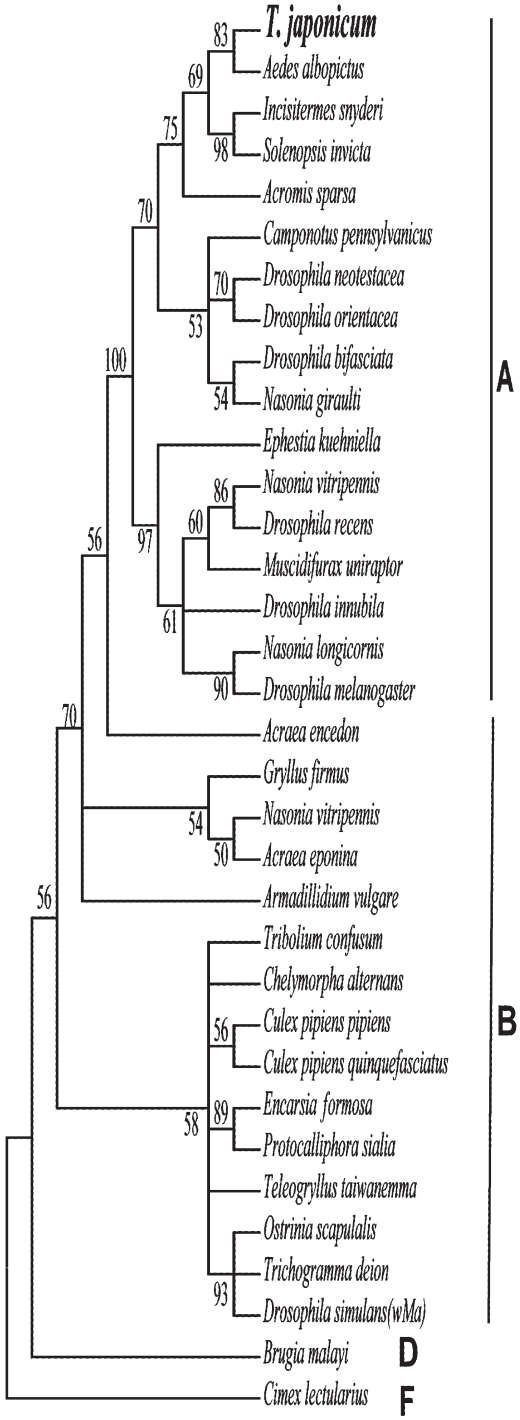

Fig. 2. Phylogenetic neighbor joining tree of *Wolbachia* based on concatenated sequences of *cox*A and *fbp*A. For information of *cox*A and *fbp*A genes, see Table 3. Sequences of *Brugia malayi* and *Cimex lectularius* were used as outgroups. Names of host species and their related groups are listed on the right side of the figure. Supergroups of *Wolbachia* are shown as uppercase letters. Population of *Trichogramma japonicum* is highlighted by boldface in the figure. Bootstrap values >50% are shown above branches.

More and more resources of *Trichogramma* ITS2 genes are becoming publicly available from NCBI database, which provides a rich source of information for the identification of *Trichogramma* species.

In this study, the sequences of the tested *Trichogramma* samples were blasted in NCBI and the species with 98–100% identity were identified as the same species. Finally, the identification results of molecular methods and morphological characters were identical. Therefore, it can be deduced that the results of the molecular identification based on ITS2 genes are reliable.

The phylogeny of *Wolbachia* has been studied extensively based on different gene sequences (Rousset *et al.* 1992, Braig *et al.* 1998). As the extensive recombination and strong diversifying selection affect the *wsp* gene, it is an unreliable tool for the characterization of *Wolbachia* (Werren & Bartos 2001, Baldo *et al.* 2002, Jiggins *et al.* 2002, Baldo *et al.* 2005). Therefore, another two *Wolbachia* genes, *cox*A and *fbp*A, were also applied.

In this study, *Wolbachia* infection was found only in five populations of *Trichogramma* from 21 sites. The phylogenetic analysis of the *Wolbachia wsp* sequences revealed that the *Wolbachia* strain belonged to the strain *wEva*A in the group of Eva of the supergroup A. The phylogenetic tree of also the concatenated *cox*A and *fbp*A revealed that the *Wolbachia* strain of *Trichogramma* was defined as supergroup A, and it was closely, with 83% identity, related to the *Wolbachia* of *Aedes albopictus*.

In a recent study using the *wsp* gene and five genes in multilocus sequence typing (MLST) (Poorjavad *et al.* 2012), only two populations of *Trichogramma brassicae* (Bezdenko, 1968) from 34 tested *Trichogramma* populations were infected by *Wolbachia*. The two populations were

infected with the same *Wolbachia* strain, which was defined in supergroup B in that study.

Although the *Wolbachia* infection rates were low in both our current study and in that of Poorjavad *et al.* (2012), the *Wolbachia* strains were different. The two *T. brassicae* populations were from Iran, i.e. Western Asia, whereas the five infected populations in our study were from East Asia and South-East Asia. It is thus possible that the *Trichogramma* populations in Western Asia are infected with different *Wolbachia* strains than those in East Asia and South-East Asia.

The molecular identification and *Wolbachia* infection test for the *Trichogramma* species from 21 populations can provide an important aid for biological control programs using *Trichogramma* spp.

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