Eur. J. Entomol. **104**: 363–367, 2007
http://www.eje.cz/scripts/viewabstract.php?abstract=1243
ISSN 1210-5759

Molecular differentiation of the four most commonly occurring *Trichogramma* (Hymenoptera: Trichogrammatidae) species in China

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Key words. Trichogrammatidae, *Trichogramma*, molecular identification, species-specific primers, restriction fragment length polymorphism, random amplified polymorphic DNA

Abstract. *Trichogramma dendrolimi*, *T. ostriniae*, *T. confusum* and *T. evanescens* are the four most commonly occurring *Trichogramma* species with overlapping distribution in China. They are the most frequently used egg parasitoids for biological control of lepidopterous crop pests in China. It is difficult to differentiate *Trichogramma* species because of their small size and lack of differences in morphological characters. Different molecular markers were employed to molecularly characterize and differentiate these species, including direct amplification of the internally transcribed spacer 2 (ITS2) of ribosomal DNA by polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and species-specific primers. The results showed that direct amplification of ITS2 could not clearly discriminate these species, but they could be differentiated using RFLP pattern obtained with endonucleases *Eco*RI and *Hin*dIII. The banding pattern produced by RAPD is irreproducible so it is not a suitable way to identify *Trichogramma* species. Finally, the species-specific primers designed based on ITS2 sequences could unequivocally distinguish the four species. The species-specific primer-based protocol proved to be the most convenient and time saving method for the identification of *Trichogramma* species by creating a unique PCR product, which can be used in surveying natural populations of *Trichogramma* species. This is the first report of the prompt identification of the four most commonly occurring *Trichogramma* species in China.

INTRODUCTION

T. dendrolimi Matsumura, *T. ostriniae* Pang et Chen, *T. confusum* Viggiani and *T. evanescens* Westwood are the most commonly occurring *Trichogramma* species in China. *T. ostriniae* is numerically dominant in cornfields, while *T. confusum* is the predominant *Trichogramma* species in cotton fields. *T. dendrolimi* mainly occurs in forests, whereas *T. evanscens* is often collected in vegetable fields. Their distribution ranges frequently overlap across the country. For example, over 80% of the samples collected from cornfields are of *T. ostriniae*, but *T. confusum*, *T. evanescens*, and *T. dendrolimi* can also occur simultaneously in cornfields. Therefore, it is always necessary to identify the samples collected from fields before further studies are undertaken.

The four *Trichogramma* species are also the most frequently used egg parasitoids in China. They are extensively used in the biological control of over 20 kinds of lepidopterous pests. It is reported that the annual acreage over which *Trichogramma* are released in China has reached about 660,000 ha (statistics of the Ministry of Agriculture, P.R.China). Use of species that are best adapted to local environment undoubtedly brings great economic benefits. Obviously, screening for the suitable wasps attaches great importance to species identification. However, protocols for rapid and precise identification of local *Trichogramma* species in China are still lacking. Therefore, the development of feasible methods for *Trichogramma* identification is important for making full use of these biocontrol agents.

Trichogramma species are very small (<1 mm) (Nagarkatti & Nagaraja, 1971) and morphological identification of them is usually time-consuming, sometimes uncertain. For instance, *T. confusum* has long been considered as the same species as *T. chilonis* (Lin, 1994), but careful re-examination of their taxonomic status based on ITS2 sequence and other biological characteristics revealed that they might be different species (Li et al., 2004). It was estimated that a total of 142 species belonging to the family Trichogrammatidae occur in China, and it is extremely difficult to distinguish many *Trichogramma* species based on morphology since these parasitoids are very small and lack of clear morphological differences. What is worse, it is almost impossible to identify the immature stages (eggs and larvae) based on morphological traits.

Various methods have been used to identify *Trichogramma* species, including morphological comparisons (Stouthamer et al., 1996; Pinto et al., 1997), allozymic analyses (Pinto et al., 1992; Pintureau, 1993) and reproductive compatibility tests (Pinto et al., 1991, 2003; Stouthamer et al., 2000). Recent advances in DNA-based techniques provide useful tools for the rapid identification of these minute wasps (Silva et al., 1999; Hoy et al., 2000). Different DNA- and polymerase chain reaction (PCR)-based molecular markers have been developed for diagnostic purpose, including random amplified polymorphic DNA (RAPD) (Landry et al., 1993; Laurent et al., 1998), restriction fragment length polymorphism (RFLP) (Sappal, 1995), satellite DNA (Landais et al., 2000), and

Inter-Simple Sequence Repeat (ISSR) (de León & Jones, 2005). Ribosomal DNA (rDNA) is present in all eukaryotic organisms and the internal non-coding transcribed spacer (ITS) region of rDNA has been extensively used in the examination of the taxonomic status of *Trichogramma* species (Orrego & Silva 1993; Van Kan et al., 1996; Pinto et al., 2002). Amornsak et al. (1998) designed a pair of unique primers based on the ITS2 sequences of *T. australicum* and its host for specific PCR amplification of *T. australicum* DNA from individual parasitized eggs, but the primers were unable to distinguish DNAs of different *Trichogramma* species. Thomson et al. (2003) also used the ITS2 region to identify *Trichogramma* species from southeastern Australia. Stouthamer et al. (1999) used the size and the difference in restriction fragment length polymorphism (RFLP) of rDNA-ITS2 to construct a simple and precise identification key to the sibling species of the *Trichogramma deion* Pinto & Oatman / *Trichogramma pretiosum* Riley complexes. Chang et al. (2001) used RAPD of genomic DNA and species-specific primers that were designed based on rDNA-ITS1 to differentiate two egg parasitic wasps of the Asian corn borer, i.e. *T. ostriniae* and *T. chilonis*. In this study, different molecular markers were compared and finally, the most suitable protocol was selected. This is the first report of a prompt identification of the four most commonly occurring *Trichogramma* species in China.

MATERIAL AND METHODS

Insects

T. dendrolimi, *T. confusum*, *T. evanescens* and *T. ostriniae* were collected in corn and cotton fields with their host eggs (Table 1). Lab-reared populations were established as isofemale lines by individual rearing in the eggs of rice moth, *Corcyra cephalonica* (25–27°C, RH 75%–80%, and 16L : 8D). The populations used in this study and the corresponding vouchers were all maintained in the Department of Entomology, China Agricultural University. *T. dendrolimi* were selected for intraspecies study because the largest number of collections is available for this species in our laboratory. Individual neonate female wasps from different cultures were directly used for analysis or preserved in 95% ethanol in sterilized 1.5 ml tubes at –20°C. Fresh wasps of the second generation from each line were soaked in acetic acid, and the male genitalia were examined microscopically for species identification using the procedures of Lin (1994).

TABLE 1. *Trichogramma* populations used in this study.

Populations*	Collection site and time
*T. dendrolimi*_JL	Changchun, Jilin, China, 1993
*T. confusum*_JL	Changchun, Jilin, China, 1999
*T. evanescens*_YQ	Yanqing, Beijing, China, 1999
*T. ostriniae*_JL	Changchun, Jilin, China, 1999
*T. dendrolimi*_YBL	Yabuli, Heilongjiang, China, 1994
*T. dendrolimi*_RH	Renhe, Jilin, China, 1994
*T. dendrolimi*_GZ	Guangzhou, Guangdong, China, 1996
*T. dendrolimi*_YQ	Yanqing, Beijing, China, 1999
*T. dendrolimi*_TTG	Tutougou, Heilongjiang, China, 1998
*T. dendrolimi*_XZ	Xuzhou, Jiangsu, China, 1994

* Populations were coded by combining species names with acronyms of collection sites.

Extraction of genomic DNA

Total genomic DNA was extracted from individual wasps. Neonate wasps were directly used for homogenization. If ethanol-preserved samples were used, the samples were firstly transferred onto sterilized filter paper and the ethanol evaporated prior to homogenization. Individuals were homogenized in 20 µl lytic buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.45% Tween, 20, 0.45% NP40, and 100 µg/ml proteinase K). The homogenate was incubated at 65°C for 30 min and then boiled for 10 min to inactivate the proteinase K.

PCR amplification and restriction profiles

Primers used for PCR amplification were: TrichF 5'-TTCTCGCATCGATGAAGAACG-3' (located in 5.8 S) and TrichR 5'-TCCTCCGCTTATTGATATGC-3' (located in 28 S) (Amornsak et al., 1998). PCR was performed by initially denaturing DNA at 95ºC for 3 min, followed by 35 cycles of amplification (94ºC 1 min, 53ºC 1 min, 72ºC 1 min 30 s) and extension at 72°C for 10 min. The 25 µl reactions contain 2 µl DNA lysate, 2.5 µl $MgCl_2$ (25 mM), 2 µl dNTPs (2.5 mM each), 1 µl primer mixture (25 µM each), and 0.4 µl *Taq* DNA polymerase (2.5 U/µl, TianGen, Beijing, China). Three microlitres of PCR product were subjected to electrophoresis on a 1.5% (w/v) agarose gel. Three microlitres of PCR product were digested with *Eco*RI and *Hin*dIII (Fermentas, Beijing, China) according to the manufacturer's instructions. DNA digestions were separated by electrophoresis on a 1.7% (w/v) agarose gel.

RAPD banding

Two microlitres of genomic DNA were used for RAPD PCR in 25 µl reactions, containing 1 µl random primer (25 µM), 2.5 µl $MgCl_2$ (25 mM), 2 µl dNTPs (2.5 mM each), and 0.5 µl *Taq* DNA polymerase (2.5 U/µl, TianGen, Beijing, China). Twenty random primers (Operon 10-mer A01-10, C01-10; Operon Technologies Inc., Alameda, CA) were tested. PCR was performed using the following cycling program: 94ºC for 3 min followed by 45 cycles of amplification (94ºC 30 s, 40ºC 25 s, 72ºC 1 min 30 s, and finally 72ºC 7 min). PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel. At least ten individuals of each culture were analyzed.

Species-specific primers

Species-specific primers were designed based on approximately 200 ITS2 sequences of the genus *Trichogramma*. These sequences were retrieved and screened from GenBank (June/2006). Primers were selected based on their specificity and general quality. PCR was carried out by initially denaturing DNA at 95ºC for 3 min, followed by 35 cycles of amplification (94ºC 50 s, 52ºC 30 s, 72ºC 1 min 50 s, and extension at 72°C for 10 min). The 15 µl reactions contain 1.5 µl DNA lysate, 0.6 µl primer mixture (25 µM each), 1.5 µl $MgCl_2$ (25 mM), 1 µl dNTPs (2.5 mM each), and 0.25 µl *Taq* DNA polymerase (2.5 U/µl, TianGen, Beijing, China).

RESULTS

PCR amplification

ITS2 fragments were successfully PCR amplified from each of the four *Trichogramma* species (not shown). The PCR amplicons of ITS2 from *T. dendrolimi* and *T. confusum* were nearly of the same size (~600 bp), and those from *T. ostriniae* and *T. evanescens* were similarly sized (~630 bp). These results demonstrate that ITS2 itself

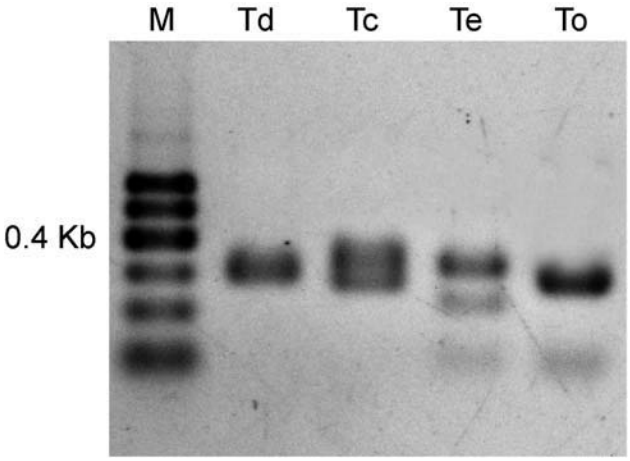

Fig. 1. RFLP profile of the ITS2s of different *Trichogramma* species using *Eco*RI and *Hin*dIII. Td – *T. dendrolimi*; Tc – *T. confusum*; Te – *T. evanescens*; To – *T. ostriniae*. M – molecular weight marker (100 bp ladder).

cannot be used to differentiate the four species. Intra-species analyses using 6 populations from the same species, *T. dendrolimi*, revealed that ITS2 size was consistent within a species (not shown).

RFLP

RFLP using *Eco*RI and *Hin*dIII produced species-specific banding patterns (Fig. 1). ITS2 of *T. dendrolimi* was cut into two overlapping bands of similar size around 300 bp (only one band visible); ITS2 of *T. confusum* was excised into two visible bands (300 ± 40 bp). Three visible bands (~100 bp, 200 bp, and 300 bp) and two clear bands (~300 bp and 100 bp) were seen, respectively, in the ITS2 of *T. evanescens* and *T. ostriniae*. RFLP patterns were reproducible within a species (not shown).

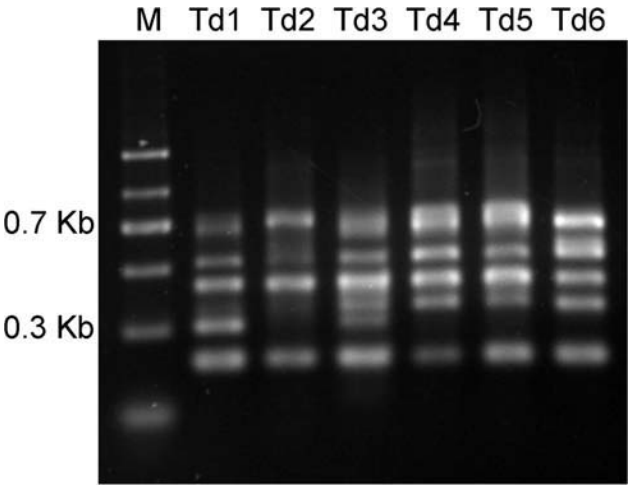

Fig. 2. RAPD banding pattern within a species. Td1 – *T. dendrolimi*_YBL; Td2 – *T. dendrolimi*_RH; Td3 – *T. dendrolimi*_GZ; Td4 – *T. dendrolimi*_YQ; Td5 – *T. dendrolimi*_TTG; Td6 – *T. dendrolimi*_XZ. M – molecular weight marker (200 bp ladder).

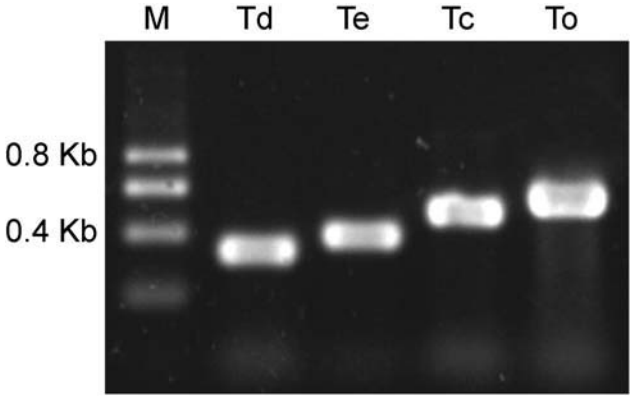

Fig. 3. Identification of *Trichogramma* species using species-specific primers. Td – *T. dendrolimi* (340 bp); Te – *T. evanscens* (390 bp); Tc – *T. confusum* (486 bp); To – *T. ostriniae* (545 bp). M – markers (200 bp ladder).

RAPD

Operon 10-mer C02 (5'-GTGAGGCGTC-3') produced polymorphic bands at species level, but it is not reproducible within a species (Fig. 2). As Fig. 2 shows, 3 major bands (~700 bp, 400 bp and 200 bp) are reproducible within *T. dendrolim*, but at least one not reproducible band is present, which makes RAPD markers unsuitable for species identification in this case.

Species-specific primer-based identification

Four pairs of primers were selected for identification of each species. Td340F / TrichR created a PCR product of 340 bp specific for *T. dendrolimi* DNA; Te390F / TrichR created a PCR product of 390 bp specific for *T. evanescens*; TrichF / Tc486R created a PCR product of 486 bp specific for *T. confusum*; TrichF / To545R created a PCR product of 545 bp specific for *T. ostriniae* (Table 2, Fig. 3). This diagnosis protocol is characterized by high species-specificity and detection sensitivity. This method is reproducible within a species as confirmed by six geographical populations of *T. dendrolimi*, which was confirmed by cloning and sequencing (GenBank accession numbers AF453554–AF453561).

DISCUSSION

Based on the data given in this study, it can be concluded that: (i) these species could not be discriminated only by the size of the amplified ITS2 fragments; (ii) these *Trichogramma* species could be differentiated using RFLP pattern and species-specific primers, but the latter are the most suitable as they can be used to identify each species quickly and with certainty; (iii) RAPD is not reproducible at intra-species level so it is not suitable for species identification. The species-specific primer-based protocol proved to be the most convenient and time saving method for identification of *Trichogramma* species and can be used for surveying natural populations of *Trichogramma* species in the field. This is the first report of rapid identification of the four most commonly occurring *Trichogramma* species in China. The method might be suitable for identifying other insects.

TABLE 2. Diagnostic primers used in this study.

Primer	Sequence (5'→ 3')	Primer pair	Product (bp)
TrichF	TTCTCGCATCGATGAAGAACG	TrichF/TrichR	Variable
TrichR	TCCTCCGCTTATTGATATGC		
Td340F	GCAGCAGTCAAGACGACA	Td340F/TrichR	340
Te390F	CGTAGAGAGAGAGTGCGC	Te390F/TrichR	390
Tc486R	GCTGCTGTTGTTGATACAACC	TrichF/Tc486R	486
To545R	GCCACTTTGACTCTGATAC	TrichF/To545R	545

Morphological identification of *Trichogramma* species is difficult and when the distribution ranges of different species overlap, several species may be confused during laboratory rearing and sample treatment. This is often the case if a lot of *Trichogramma* isolines are reared at the same time. Meanwhile, a protocol for rapid identification of *Trichogramma* species is particularly helpful for classifying specimens collected in a large-scale field survey, since traditional taxonomic methods are both labour intensive and slow, and therefore not suitable for handling large samples. Instead, the molecular method has been used to detect natural field populations (Prinsloo et al., 2002; Ratcliffe et al., 2002; Loxdale & MacDonald, 2004). Another reason why this method is advisable is that traditional methods require intact and well-preserved specimens, specialized expertise and taxonomic knowledge to fulfill a taxonomic task, but the species-specific primer-based method presented here can be used to identify incomplete specimens and researchers other than *Trichogramma* experts can identify them.

Detecting early instars and eggs of larval parasitoids, even with the aid of the best light microscopes and appropriate stains, is not an easy task. In contrast, PCR approaches for studying parasitism rates are sensitive and accurate (Greenstone & Edwards, 1998; Zhu & Williams, 2002; Erlandson et al., 2003; MacDonald & Loxdale, 2004). Amornsak et al. (1998) detected the DNA of the parasitoid *T. australicum* Girault in its host *Helicoverpa armigera* (Hübner) 12 h after parasitization. Similarly, Tilmon et al. (2000) found the PCR-based technique to be capable of detecting even a newly laid parasitoid egg within parasitized nymphs of *Lygus* spp., in which the parasitoid constituted only 0.01% of the host tissue. Agustí et al. (2005) demonstrated that the percentage of parasitism in *Ostrinia nubilalis* (Hübner) by *Lydella thompsoni* (Herting) and *Pseudoperichaeta nigrolineata* (Walker) given by a traditional method was three times lower than that revealed by the molecular method. Molecular detection and identification of immature stages is especially useful for quality control of *Trichogramma* species that are commercially reared and released as biocontrol agents. Laboratories that specialize in mass rearing of *Trichogramma*, where species identification, quality control and population monitoring are very important, usually have PCR thermocyclers that allow the use of this technique.

ACKNOWLEDGEMENTS. This study was supported by National Basic Research Program of China (Grant No. 2006CB102006) and National Science Foundation of China (Grant No. 30571218).

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Received December 21, 2006; revised and accepted February 26, 2007