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Orijinal araştırma (Original article)

Mitochondrial and ribosomal DNA sequence analysis for discrimination of *Trichogramma euproctidis* Girault and *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae)

Trichogramma euproctidis Girault ve *Trichogramma brassicae* Bezdenko (Hymenoptera:Trichogrammatidae)'nin ayırımında mitokondriyal ve ribozomal DNA dizi analizi

Fahriye SÜMER ERCAN[1*] **Sevcan ÖZTEMİZ**[2] **Aydın Suzu TUNÇBİLEK**[3]

Summary

Sequence variation of ribosomal regions, namely the second internal transcribed spacer region of the nuclear ribosomal gene cluster (ITS2), the D2 domain of 28S ribosomal subunit (28SD2), and the mitochondrial cytochrome oxidase I (COI) were examined for differentiation of *Trichogramma euproctidis* Girault and *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) populations. The length of ITS2 region ranged from 378 bp to 406 bp. The sequences of 28SD2 and COI were 522 bp and 669 bp for *T. euproctidis* and 530 bp and 672 bp for *T. brassicae*, respectively. A phylogenetic relationship was constructed by using the maximum likelihood method for each marker. The ITS2 gene region more clearly differentiated between *T. euproctidis* and *T. brassicae* than the 28SD2 and COI genes.

Key words: *Trichogramma euproctidis*, *Trichogramma brassicae*, ITS2, 28SD2, COI

Özet

Ribosomal bölgeler dizi varyasyonu: nuclear ribosomal gen dizisinin ikinci genler arası bölgesi (ITS2), 28S ribosomal altünitenin D2 bölgesi (28SD2) ve mitokondrial sitokrom oksidaz I (COI) *Trichogramma euproctidis* Girault ve *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) popülasyonlarının ayırımı için incelenmiştir. ITS2 bölgesinin uzunluğu 378 bç ile 406 bç arasında değişmiştir. 28SD2 ve COI dizilerinin boyutu sırasıyla *T. euproctidis* için 522 bç ile 669 bç ve *T. brassicae* için 530 bç ile 672 bç olarak farklı bulunmuştur. Filogenetik ilişki her bir marker için maximum likelihood yöntemi kullanılarak oluşturulmuştur. ITS2 gen bölgesi, *T. euproctidis* ve *T. brassicae*'yi 28SD2 ve COI genlerinden daha net ayırt etmiştir.

Anahtar sözcükler: *Trichogramma euproctidis*, *Trichogramma brassicae*, ITS2, 28SD2, COI

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[1] Department of Biology, Faculty of Science and Art, Bozok University, Yozgat.
[2] Biological Control Research Station, Adana
[3] Department of Biology, Faculty of Science, Erciyes University, Kayseri
Sorumlu yazar e-mail: fahriyesumer@gmail.com
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Introduction

Egg parasitoids of genus *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) have been used as biological control agents for more than 100 years (Smith, 1996). Taxonomy of these parasitoid wasps has been difficult because of their small size and lack of distinguishable morphological characters (Stouthamer et al., 2000). Formerly, the identification of *Trichogramma* was based on male genitalia (Nagarkatti & Nagaraja, 1971), but the main problem was that the thelytokous *Trichogramma* population consists of only females. Stouthamer et al., (1990), used antibiotics to return thelytokous population to arrhenotoky. Thus, only males can occur but this is an unpractical procedure for routine identification. In recent years, with the development of molecular techniques, DNA-based identification methods have been used for characterization of closely related or cryptic species for biological control studies (Landry et al., 1993). Stouthamer et al. (1999), showed the usefulness of the internally transcribed spacer 2 regions of the ribosomal DNA (rDNA-ITS2) sequences for identification of *Trichogramma* species. They determined consistent differences between species and a little variation within *Trichogramma* species in the ITS2 sequence. The ITS regions evolve more quickly than most other gene regions and may vary substantially between closely related species. Consequently, they are most useful for detecting and distinguishing species. ITS2 and COI provide an effective tool for phylogenetics at low taxonomic levels (Cruickshank, 2002). Molecular information from the COI gene has been used for the understanding of phylogenetic relationships at different hierarchical levels of different hymenopteran groups (Mardulyn & Whitfield 1999). 28SD2 is one of the expansion segments of 28S rRNA that can vary greatly within Hymenoptera. The variation in expansion segments is widely used for phylogenetic studies among higher taxonomic levels (Gillespie et al., 2005). The advent of molecular taxonomy makes more sensitive techniques available for identification of cryptic species. Assessing morphological and ecological differences in cryptic species is quite difficult. DNA-based techniques provide an informative tool for detecting cryptic species.

In the study, both ribosomal and mitochondrial genes were used. The results of phylogenetic trees and genetic distances of *Trichogramma euproctidis* and *T. brassicae* are compared.

Materials and Methods

***Trichogramma* samples**

The *Trichogramma euproctidis* and *T. brassicae* populations originated from the eggs of the European corn borer *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae) collected from corn fields of the southeast of Turkey (Table 1). Females that emerged from parasitized corn borer eggs were used to initiate isofemale lines. Cultures were reared on eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) as a host (Ercan et al., 2011).

Table1. Population codes of samples used in the study

Population Code	Province/County	Host	Year of collection
A	Adana/Turkey	*O. nubilalis*	2007
B	Adana/Turkey	*O. nubilalis*	2007
C	Adana/Turkey	*O. nubilalis*	2007
D	Adana/Turkey	*O. nubilalis*	2007
E	Tarsus/ Turkey	*O. nubilalis*	2007
F	Tarsus/Turkey	*O. nubilalis*	2007
G	Adana/Turkey	*O. nubilalis*	2007

DNA extraction

DNA was extracted from one wasp of each species. They were ground in 60µl 5% Chelex-100 and 2µl Proteinase K (20mg/ml) and incubated at 1h at 55 °C, followed by 10min at 96 °C.

Internal transcribed spacer 2 amplification

For ITS2 amplification, the following primers were used: ITS2 forward, 5’-TGTGAACTGCAGGACACATG-3', and ITS2 reverse, 5’- GTCTTGCCTGCTCTGAG-3’ (Stouthamer et al., 1999). The PCR was done in a total volume of 25µl. It contained 2µl DNA template, 2.5µl PCR buffer (10X buffer with $(NH_4)_2SO_4$, Fermentas), 5µl dNTPs (10mM stock solution), 0.5µl forward and reverse primers, 0.2µl Taq Polymerase (5 u/µl, Fermentas) and 14.3µl of sterile distilled water. The cycling program was also the same as used by Stouthamer et al. (1999). The size of PCR product was determined with 1% agarose gel electrophoresis with a size standard.

Cloning and Sequencing

After electrophoresis, PCR products were purified with the Wizard® PCR Preps DNA Prufication System. Following the purification, the PCR products were ligated into a Pgem-T® Vector (Promega). 2µl of the ligation mix was transformed in the heatshock cells of DH5-α *Escherichia coli* and plated in a LB agar medium containing Ampicilin, X-GAL and IPTG. The plates were stored overnight at 37°C. The next day, white colonies on each plate were removed with sterile toothpicks, and the bacteria attached to the toothpicks were dispersed in eppendorf tubes containing 50µl sterile distilled water. Two µl of this solution was used for PCR reaction using the ITS2 primers in the PCR reaction described above to determine the correct size of insert in the Pgem plasmid in each sample. The size of PCR product was determined using agarose gel electrophoresis as described above. Following electrophoresis, PCR products were purified using the Wizard purification system. PCR products were then sent for automatic sequencing (Institute for Integrative Genome Biology, University of California – Riverside).

Second expansion segment of the 28S ribosomal subunit amplification

For 28SD2 amplification, the following primers were used: 28SD2 forward, 5’TACCGTGAGGGAAAGTTGAAA3’ and 28SD2 reverse, 5’AGACTCCTTGGTCCGTGTTT3’ (Rugman-Jones et al., 2010). The PCR was done in a total volume of 25µl. It contained 2µl DNA template, 2.5µl PCR buffer, 5µl dNTPs, 0.5µl forward and reverse primers, 0.2µl Taq Polymerase and 14.3µl of sterile distilled water. The cycling conditions were initial denaturation at 94 °C for 2 min followed by 38 cycles each of denaturation at 94 °C for 30 sn, annealing at 53 °C for 50 sn and extension at 72 °C for 1.5 min followed by a final extension step at 72 °C for 10 min. The size of PCR product was determined with 1% agarose gel electrophoresis with a size standard. The PCR products were then sent for automatic sequencing (Institute for Integrative Genome Biology, UCR).

Mitochondrial cytochrome oxidase I amplification

For COI amplification, the following primers were used: COI forward, 5’GGTCAACAAATCATAAAGATATTGG3’ and COI reverse, 5’TAAACTTCAGGGTGACCAAAAAATCA3’ (Rugman-Jones et al., 2009). The PCR was done in a total volume of 25µl. It contained 2µl DNA template, 2.5µl PCR buffer, 5µl dNTPs, 1µl forward and reverse primers, 2µl 25 mM $MgCl_2$, 1.2 µl BSA (10mg/ml), 0.2µl Taq Polymerase and 11.1µl of sterile distilled water. The cycling conditions were initial denaturation at 95 °C for 5 min followed by 34 cycles each of denaturation at 94 °C for 30 sn, annealing at 53 °C for 30 sn and extension at 72 °C for 1.5 min followed by a final extension step at 72 °C for 10 min.

The size of PCR product was determined with 1% agarose gel electrophoresis with a size standard. Then the PCR products were sent for automatic sequencing (Institute for Integrative Genome Biology, UCR).

Phylogenetic analysis

DNA sequences were aligned using the BioEdit program (Hall, 1999). Phylogenetic trees were inferred by using maximum likelihood. Dendograms were performed using MEGA 4 (Molecular Evolutionary Genetic Analysis) program (Tamura et al., 2007).

Results and Discussion

Using the polymerase chain reaction (PCR), we amplified partial sequences of the mitochondrial gene, COI and ribosomal regions, ITS2 and 28SD2 of *Trichogramma* specimens. Seven specimens of *Trichogramma* were collected from corn fields in southeastern Turkey (Table 1) and their ITS2, 28SD2 and COI regions were sequenced. After sequencing, a BLAST search analysis (National Center for Biotechnology) was performed to find regions of sequence similarity between database sequences. The ITS2 nucleotide sequences werefound in GenBank under Accession Nos. HQ332598 for *T. brassicae* and HM116410 for *T. euproctidis*.

Alignment of ITS2 sequences from seven *Trichogramma* samples, obtained through GenBank, revealed variation between *T. euproctidis* and *T. brassicae*. Length of ITS2 was 378 bp in *T. euproctidis*. The same region in *T. brassicae* was 406 nucleotides in length for all specimens. The sequences of 28SD2 and COI were distinctly different in size, namely 522 bp and 669 bp for *T. euproctidis* and 530 bp and 672 bp for *T. brassicae*. COI and 28SD2 sequences of *T. euproctidis* differed from those of *T. brassicae* by 0,8% and 3,5%, respectively. Substantially, the three gene regions, ITS2, 28SD2 and COI, divided the seven specimens into the two main groups (Figures 1, 2 and 3, respectively). However, all seven specimens complied with same Accession Nos. (FM210198 for COI and Accession Nos. AY599408) for the sequences of D2 domain of 28S. The data of these two sequences can be used to distinguish two species but only by the length of the region. The nuclear DNA sequence represented phylogenetically more valuable data. Phylogenetic analysis was performed with maximum likelihood in the MEGA 4 program for all gene regions.

Figure 1. Phylogenetic tree based on ITS2 gene region of *Trichogramma* population (Cluster I: *Trichogramma euproctidis*, Cluster II: *Trichogramma brassicae*).

Figure 2. Phylogenetic tree based on 28SD2 gene region of *Trichogramma* population (Cluster I: *Trichogramma euproctidis*, Cluster II: *Trichogramma brassicae*).

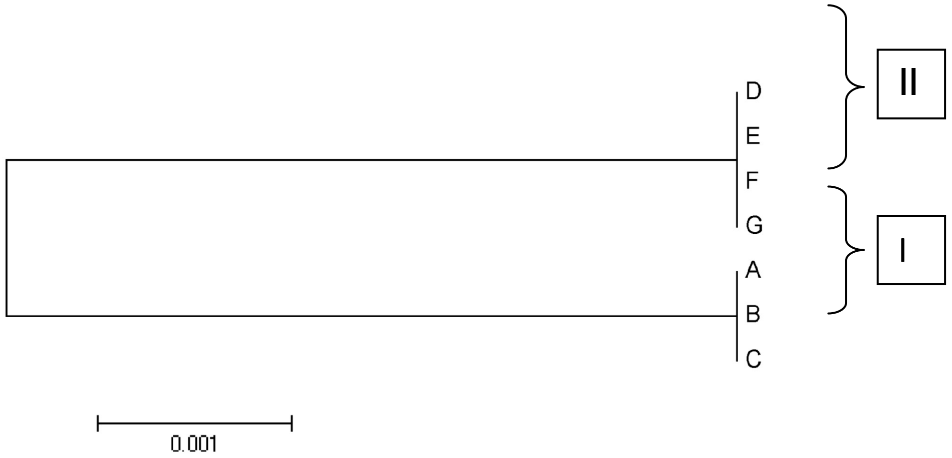

Figure 3. Phylogenetic tree based on COI gene region of *Trichogramma* population (Cluster I: *Trichogramma euproctidis*, Cluster II: *Trichogramma brassicae*).

In the Eastern Mediterranean Region of Turkey, six *Trichogramma* species (*T. evanescens* Westwood (=*Trichogramma turkestanica* Meyer), *T. brassicae* Bezdenko, *T. cacoeciae* Marchall, *T. pintoi* Voegelé, *T. embryophagum* Hartig and *T. dendrolimi* Matsumura) were collected from eleven host plants and nine lepidopteran pests (Oztemiz, 2007). According to our results from the southeast of Turkey, the surveyed natural communities of *Trichogramma* parasitoids in corn fields are composed of only two species from those six *Trichogramma* species; which are synonyms of *T. turkestanica*, *T. euproctidis* and *T. brassicae,* by using molecular techniques. In similar studies, new strains and species of *Trichogramma* and the most common *Trichogramma* species were identified by using the ITS2 region of ribosomal RNA (Sayed et.al., 2011; Karimi et al., 2012; Polaszek et al., 2012)

Many methods, such as allozyme electrophoresis, random amplified polymorphic DNA variability, restriction fragment length polymorphism, mitochondrial cytochrome oxidase subunit I (COI) and microsatellite markers, have been used for *Trichogramma* identification but it is important to choose the best one for distinguishing closely related taxa. A molecular marker which evolved rapidly and is located within a highly conserved gene region, like ITS2, can be used reliably for identification (Ercan et al., 2011). Vickerman et al. (2004) did not determine adequate variations in the COI, COII, ITS1, and ITS2 regions to differentiate the populations of *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae) from different localities. In our study, we differentiated between *Trichogramma* specimens by using the ITS2, 28SD2 and COI gene regions; the ITS2 region provided more reliable sequence divergence at the species level. Wagener et al. (2006) showed that sequence analyses of ITS2 and COI were phylogenetically informative for separating nine *Diadegma* (Hymenoptera: Ichneumonidae) species but the ITS2 gene region provided more valuable data than COI. Doganlar et al. (2010) determined three new species of *Ceranisus* (Chalcidoidea: Eulophidae) in Turkey by using the D2 domain of the 28S ribosomal DNA.

The most important part of a successful biological control is choosing the correct species of natural enemy. Therefore, correct identification of the natural enemy and the techniques used for identification are critical steps. The present study provides an understanding of genetic relationships between two species of egg parasitoid, *T. euproctidis* and *T. brassicae*, from south-eastern Turkey. Results of the study indicated that the ITS2 gene region can be used to effectively differentiate between these two species and that it is phylogenetically more valuable than COI and 28SD2 gene regions.

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