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SEQUENCE ANALYSIS OF THE RIBOSOMAL DNA ITS2 REGION IN TWO *TRICHOGRAMMA* SPECIES (HYMENOPTERA: TRICHOGRAMMATIDAE)

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Abstract - Two egg parasitoid wasps, *Trichogramma euproctidis* (Girault) and *Trichogramma brassicae* (Bezdenko) (Hymenoptera: Trichogrammatidae) were identified in the study. The taxonomy of these wasps is problematic because of their small size and lack of distinguishable morphological characters. The DNA sequence variation from the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA (rDNA) was analyzed from these two *Trichogramma* species. This technique provides quick, simple and reliable molecular identification of *Trichogramma* species.

Key words: Trichogramma euproctidis, *Trichogramma brassicae*, molecular identification, rDNA ITS2 region

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INTRODUCTION

Trichogramma wasps are one of the most important biological control agents of insects, especially eggs of moths and butterflies in different crops. *Trichogramma* are extremely tiny wasps in the family Trichogrammatidae. The family contains 80 genera and approximately 620 species (Pinto and Stouthamer, 1994). The identification of these wasps is difficult due to the small size (< 1mm long) and has typically uniform morphological characters. The identification and using of correct species of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) before releasing in the field are very important step in biological control programs. Classical morphological methods are sometimes not able to differentiate microhymenopteran species at the strain level (Landry et al., 1993). Nagarkatti and Nagaraja (1971) discovered the usefulness of male genitalia as a taxonomic character for their identification. The most important limitation of this technique is that female *Trichogramma* remain unidentifiable (Stouthamer et al., 1999). There are some populations within the species of *Trichogramma* that consist of only females. These are examples of thelytokous reproduction. Thelytoky is a type of parthenogenesis which is often induced by bacteria of the genus *Wolbachia*; however in some cases thelytoky has a purely genetic basis (Vavre et al., 2004). The sequence analysis of the ITS2 rDNA has been described in recent studies as a tool for *Trichogramma* identification as well (Kan et al., 1996, Pinto et al., 1997, Silva et al., 1999, Stouthamer et al., 1999). rDNA consists of three highly conserved regions that code for the ribosomal RNA, separated by two spacer regions that are conserved within species but vary substantially between species. These ITS spacers can be amplified using universal primers that bind in the highly conserved regions. Pinto et al. (2002) found a new *Trichogramma* species, *T. itsybitsi,* by using ITS2 sequence. Sumer et al., (2009)

have created a molecular key for the identification of *Trichogramma* species found around the Mediterranean by using ITS2 of the ribosomal cistron. Using the technique it was possible to identify the two *Trichogramma* species: *T. euproctidis* and *T. brassicae,* and thus to provide a reliable identification method for these species recorded from Turkey.

MATERIALS AND METHODS

Trichogramma samples

T. euproctidis and *T. brassicae* originated from the eggs of the European corn borer *Ostrinia nubilalis* (Lepidoptera: Noctuidae) collected in a corn field in the south of Turkey. The origins of *Trichogramma* used in the study are shown in Table 1. Females that emerged from parasitized corn borer eggs were used to initiate isofemale lines. Cultures are reared in Biology Department of Erciyes University, Turkey, on eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae).

DNA Extraction

DNA was extracted from one wasp for each species. They were ground in 60µl 5% Chelex-100 and 2µl Proteinase K (20mg/ml). Then they incubated for 1 h at 55ºC, followed by 10min at 96ºC. The PCR was done in a total volume of 25µl. It contains 2µl DNA template, 2.5µl PCR buffer, 5µl dNTPs, 0.5µl forward and reverse primers (ITS2 forward: 5'-TGTGAACTGCAGGACACATG-3', ITS2 reverse: 5'-GTCTTGCCTGCTCTGAG-3'), 0.2µl Taq Polymerase and 14.3µl of sterile distilled water. Primers were the same as those used by Stouthamer et al. (1999) for distinguishing sibling species of *Trichogramma.* The cycling program was also the same as used by Stouthamer et al. (1999). The size of the PCR product was determined by 1% agarose gel electrophoresis with a size standard.

Cloning and sequencing

After electrophoresis, the PCR products were purified with Wizard® PCR Preps DNA Purification System.

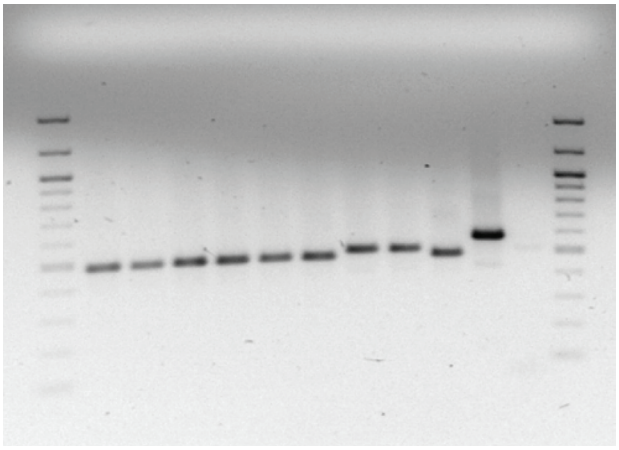

Fig. 1. Gel showing the PCR products of the ITS2 for the species of *T. euproctidis* and *T. brassicae* from different cultures. L: low ladder size standard, 1: *T. euproctidis*, 2: *T. euproctidis*, 3: *T. euproctidis*, 4: *T. euproctidis*, 5: *T. euproctidis*, 6: *T. euproctidis*, 7: *T. brassicae*, 8: *T. brassicae*, 9: *T. euproctidis*, P: Positive Control (*T. deion*), N: Negative Control.

Following the purification the PCR products were ligated into a Pgem-T® Vector (Promega). 2µl of the ligation mix was transformed in the heat shock cells of DH5-α *Escherichia coli* and plated in a LB agar medium containing aAmpicillin, X-GAL and IPTG. The plates were stored overnight at 37ºC. The next day white colonies of each plate were picked using sterile toothpicks; the bacteria attached to the toothpicks were dispersed in Ependorf tubes containing 50µl sterile distilled water. 2 µl of this solution was used for PCR reaction using the ITS2 primers in a PCR reaction as described above to determine the correct size of insert in the Pgem plasmid in each sample. The size of the PCR product was determined using agarose gel electrophoresis as described above. Following electrophoresis, the PCR products were purified using the Wizard purification system. Tthe PCR products were then sent to an automatic sequencer for direct sequencing (Institute for Integrative Genome Biology UCR).

RESULTS AND DISCUSSION

The size of ITS2 sequences of *T. euproctidis* and *T. brassicae* differed from each other (Fig. 1). The length of ITS2 region was found to be 428bp for *T.*

Table1. Origins of *Trichogramma*

Population	Locality/Origin	Original Host	Date collected
1: *T. euproctidis*	**N** 37° 15' 24.0" **E** 35° 3' 32.0" **Elevation:** 251m	*Ostrinia nubilalis*	September 2002
2: *T. euproctidis*	**N** 37° 15' 24.0" **E** 35° 3' 32.0" **Elevation:** 251m	*Ostrinia nubilalis*	September 2004
3: *T. euproctidis*	**N** 37° 15' 24.0" **E** 35° 3' 32.0" **Elevation:** 251m	*Ostrinia nubilalis*	September 2004
4: *T. euproctidis*	**N** 37° 15' 24.0" **E** 35° 3' 32.0" **Elevation:** 251m	*Ostrinia nubilalis*	September 1998
5: *T. euproctidis*	**N** 37° 15' 24.0" **E** 35° 3' 32.0" **Elevation:** 251m	*Ostrinia nubilalis*	September 2002
6: *T. euproctidis*	**N** 37° 2' 28.0" **E** 35° 24' 46.0" **Elevation:** 220m	*Ostrinia nubilalis*	September 2006
7: *T. brassicae*	**N** 37° 13' 34.0" **E** 35° 5' 37.0" **Elevation:** 173m	*Ostrinia nubilalis*	September 2006
8: *T. brassicae*	**N** 37° 6' 17.0" **E** 35° 6' 39.8" **Elevation:** 86m	*Ostrinia nubilalis*	September 2006
9: *T. euproctidis*	**N** 37° 6' 41.2" **E** 35° 6' 43.6" **Elevation**: 95m	*Ostrinia nubilalis*	September 2006

euproctidis and 456bp for *T. brassicae*. We sequenced the ITS2 region of *Trichogramma* cultures and compared them to homologous sequences of other *Trichogramma* species available in GenBank database of National Center for Biotechnology Information. Following comparison, two different species, *T. euproctidis* and *T. brassicae,* were found from the cultures of the study. Table 2 shows the differences between *T. euproctidis* and *T. brassicae* in their ITS2 sequence.

If a molecular marker is rapidly evolving and located within an otherwise highly conserved gene region, it can be used successfully for distinguishing closely related taxa. ITS2 is an important molecular marker that can be used for comparing closely related species, subspecies and populations. Alvarez and Hoy (2002) used ITS2 DNA sequences for separating closely related populations of the parasitoid *Ageniaspis*. Amplification of ITS2 by PCR is very easy, because there are many copies of this gene per nucleus. Zhu and Williams (2002) cloned and sequenced the ITS2 region of the Mymarid parasitoids and their host by using the primers of rDNA sequences. This molecular technique provided specific and sensitive results for the identification of single and multiple species of egg parasitoids in agricultural and natural systems.

Different biochemical and molecular methods have been developed to determine the differences

Table 2 Aligned sequences of ITS2 region of rDNA from *T*. euproctidis (Te) and *T*. brassicae (Tb).

Lines	ITS2 sequence of rDNA	
Te	GTTTATAAAAACGAACCCGACTGCTCTCTCGCAAGAGAGAGCGTTGATCT	50
Tb	.....	
Te	GGGCGCTCGTGTCTCTATCTCGCTCTCTACTCTTCTTCGAAGTGTATAGA	100
Tb	.....--.....--....-.....C.C.G-..	
Te	GCAGTGTGTGATACGTCGCCTCAAACGATTAGCAAGAAAAAAGACGAATT	150
Tb	.....--.....ACG.....T.....	
Te	CGTTCGTCTAGCTGGCGCGCGCGCTTACCGCTTGGAGAGTACTCGCTCGC	200
Tb	.....	
Te	GCGAGTACTTCCGATCGTTCTGCGTCGAGTCCCGGAGCTTTCTCGACTCG	250
Tb	.....	
Te	TCGAGCAGCGGACCGACGTCTAGCACACGATCAGGCTCGTCCATGCATCG	300
Tb	.....	
Te	GTCATTGAACGCGCGCGCGCG----------	350
Tb	.....CACTTTTTTTAAACGCACACACACACCGT	
Te	-------TGCACTTTTACACATGGCTAGCTCGAATTTTTGCTGAACGAGT	400
Tb	GTGTGCG.TT.T.AAG.A.A.....	
Te	CTTTTTTCTTCGAT	414
Tb	.....TC.....	

- Dash means insertion or deletion; dot indicates where the sequence of Tb is identical to that of Te.

L 1 2 3 4 5 6 7 8 9 P N L

between closely related taxa: allozyme electrophoresis (Miura et al., 1990, Pintureau, 1993, Ram et al., 1995, Basso et al., 1999, Pinto et al., 2003), random amplified polymorphic DNA variability (Landry et al., 1993, Chang et al., 2001, Al-Barrak et al., 2004), restriction fragment length polymorphism (Vanlerberghe-Masutti, 1994, Sappal et al. 1995), mitochondrial cytochrome oxidase subunit I (COI) (Monti et al., 2005), microsatellite markers (Pizzol et al., 2005). An allozymic analysis, especially esterase electrophoresis, is another useful technique that easily distinguishes the variation in the genus *Trichogramma*. However, the allozymic method requires fresh material or material stored in liquid nitrogen. An important advantage of the ITS2-DNA method over the allozymic methods is that specimens can be dried or stored in 100% alcohol without affecting our ability to identify them.

In addition, the ITS2 sequence contains more potentially different sites for species-specific characters than a single allozyme with at best 2-5 distinguishable alleles (Stouthamer et al. 1999). Thomson et al., (2003) used ITS2 sequence analyses for the identification of *Trichogramma* species from southeastern Australia. They found that the length of ITS2 is different for each species. Chang et al., (2001) found that the ITS1 regions of two egg parasitoids of *Ostrinia furnacalis* Guenée (Lepidoptera: Pyralidae) present in Taiwan, *T. ostriniae* and *T. chilonis* were 86.1% identical. The length of ITS1 was different in these two species, 458bp and 322bp respectively. In our study, the ITS2 sequences of *T. euproctidis* and *T. brassicae* were different in length, 428bp for *T. euproctidis* and 456bp for *T. brassicae* (Fig. 1). Chang et al., (2001) also used RAPD PCR for identification: they tested 30 RAPD primers but only one of them gave species specific DNA products. The application

of RAPD markers is quick and simple: nevertheless the RAPD PCR system has some problems; repeatability, results are sensitive to laboratory conditions and it requires many more samples of the wasp to obtain results. Ciociola et al. (2001) used five individuals for DNA extraction: the system that we used required only one specimen and it gave enough DNA extract for successful PCR and subsequent sequencing of the ITS2 region of rDNA.

Choosing the best molecular marker for identification of closely related species is very important for success. Our study showed that the ITS2 region of rDNA can be used for the differentiation of the two *Trichogramma* species, *T. brassicae* and *T. euproctidis*, commonly found in agricultural areas in Turkey. Molecular identification of additional *Trichogramma* species from Turkey will help to clarify the *Trichogramma* fauna native to Turkey and it will help in a rational choice for testing and identifying the best species to be used for biological control.

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