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Research article

Determination of the Genetic Variation within the Egg Parasitoid, *Trichogramma brassicae*, Bezdenko (Hymenoptera: Trichogrammatidae) Populations in Düzce Province, Turkey

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Abstract. Members of *Trichogramma* genus are the most widely used as natural enemies in biological control programs. Choice of natural enemies is known to have a critical important for the succession of biological control. Their morphologic identification has some bottlenecks as laborious, time-consuming and mostly based on very small structures of the bodies. Molecular techniques have been widely used for detection, discrimination, phylogenetic analysis, identification and characterization in biological control agents belong to *Trichogramma spp.* In this study, the efficacy of the direct sequencing analysis of internal transcribed spacer locus of rDNA was investigated for analyzing the genetic variation within nine *Trichogramma brassicae* population from corn growing areas in Düzce province. The comparison of the sequences was performed with other ITS2 locus of *Trichogramma* species available in GenBank database. The result of analyses indicated that no genetic variation was determined in the Düzce population.

Yumurta Parazitoiti, *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae)'nin Düzce İlindeki Popülasyonları İçerisindeki Genetik Varyasyonların Belirlenmesi

Anahtar kelimeler:
Trichogramma brassicae, yumurta parazitoiti, genetik varyasyon, Düzce

Özet. *Trichogramma* türleri, biyolojik mücadele programlarında en çok kullanılan doğal düşmanlardandır. Doğal düşmanın seçiminin, biyolojik kontrolün başarısı açısından kritik bir öneme sahip olduğu bilinmektedir. Bu cinse bağlı türlerin morfolojik teşhisleri zahmetli olmaları, zaman kaybı oluşturmaları ve vücut yapılarının oldukça küçük boyutlarda olmasına bağlı olarak problem oluşturmaktadır. Moleküler teknikler, *Trichogramma* cinsine ait biyolojik kontrol ajanlarında tespit etme, ayırt etme, filogenetik analiz, tanımlama ve karakterizasyon için yaygın olarak kullanılmaktadır. Bu çalışmada, Düzce ilinde mısır yetiştirilen alanlardan elde edilen dokuz *Trichogramma brassicae* popülasyonunun genetik varyasyon analizi için rDNA'nın ITS bölgesinin doğrudan sekanslama analizinin etkinliği araştırılmıştır. Sekansların karşılaştırılması, GenBank veri tabanında mevcut olan *Trichogramma* türlerinin ITS2 bölgeleri ile gerçekleştirilmiştir. Analiz sonuçları, Düzce popülasyonlarında genetik varyasyonun tespit edilmediğini göstermiştir.

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INTRODUCTION

The genus *Trichogramma* belongs to the *Trichogrammatidae* family, which comprises of 80 genera and about 620 species. (Pinto and Stouthamer 1994). *Trichogramma* Westwood (Hymenoptera: *Trichogrammatidae*) species which are egg parasitoids have been used as biological control agents for more than 100 years and these species are polyphagous egg parasitoids on ten insect orders, containing Diptera, Lepidoptera, Hemiptera, Coleoptera, Neuroptera and Hymenoptera (Smith 1996).

Among the 180 *Trichogramma* species identified, the 70 of them are used in control of insect pests in agro-ecosystem (Li 1994). This genus has been very effective in biological control especially Lepidopteran pests. It is thought by researchers that this genus contains a large number of cryptic species because the general morphology within the group is similar (Knutson 1998). *Trichogramma* species are difficult to determine because of their small size and low interspecific morphological character leading to many diagnosis problems (Nagarkatti and Nagaraja 1977; Smith and Hubbes 1986). Previously, diagnosis of these species has been based chiefly on the male genitalia and the male antennae (Nagarkatti and Nagaraja 1977; Pinto 1999). According to recent studies, they have been focused on the use of mtDNA-COI and rDNA-ITS2 regions for species diagnosis. The rDNA occurs of three preserved regions that encode ribosomal RNA and is separated by two conserved regions within the species, but vary fairly amongst species. Because of these regions are so preserved, specific primers work in a wide variety of taxa (Sumer *et al*., 2009). The rDNA-ITS2 sequences are useful as a general diagnosis technique for *Trichogramma* species. Before any character source can be proposed as appropriate for separating species, potential intraspecific variation must be taken into consideration (Stouthamer *et al*., 1999). rDNA-ITS2 and COI ensure a powerful tool for phylogenetics at low taxonomic levels (Cruickshank 2002).

Meksem and Kahl (2005) reported that polymorphism occurs naturally and depends on the variation in the DNA sequence, it also creates the basis of intra-species and inter-species variation in plant and animal genomes. The aim of this research was to determine whether current DNA techniques could differentiate intra-population genetic variation of these nine *T. brassicae* populations and other *Trichogramma* species present in GenBank database of National Center for Biotechnology Information (NCBI).

MATERIALS AND METHODS

Trichogramma Cultures

Corn fields which are infested with European corn borer, *Ostrinia nubilalis* (Hübner) in Düzce province have been identified and the populations of pests and egg parasitoids have been followed up by the weekly intervals since the first egg was determined. The collected *O. nubilalis* egg clusters were placed in glass tubes individually. The eggs were maintained at 25 ºC until European corn borer larvae and parasitoids emerged. After the parasitoids and larvae emergence are complete, the eggs are counted as parasitized and non-parasitized. *Trichogramma* species, which are emerged from parasitized eggs have continued to produce under laboratory conditions. Eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were used as hosts for this purpose. The obtained *E. kuehniella* eggs were glued onto cardboards as a daily. The cardboards containing the *E. kuehniella* eggs were placed in tubes with the *Trichogramma* adults and maintained in climatic chambers regulated at 25±1 °C, 70±5% RH, and 16:8 h photoperiod. About 9-10 days later, the *Trichogramma* adults began to appear. Insect culture was continued until the identification of the *Trichogramma* species was completed.

DNA Extraction and PCR Amplification

DNA was isolated as identified by Waeyenberge *et al*. (2000) with some revisions described follow; one to ten frozen parasitoids were crushed with a crusher into 45 µl of distilled water (ddH2O) in 1.5 ml eppendorf tube and the crushed parasitoid contents were centrifuged. After that 40 µl of the sample mix were transferred to PCR tube comprising 50 µl of WLB (Worm Lysis Buffer) and 10 µl of Proteinase K (20 mg ml-1). The PCR tubes were frozen at -80 ºC for at least 10 minute, then incubated at 65 for 1 hour and 95 ºC for 10 minute in a thermocycler. After, the tubes were

centrifuged for 1 minute at 14000 rpm and these extracted DNA samples were stored at -20 ºC until PCR amplification (Waeyenberge *et al*., 2000).

The amplification of the rDNA-ITS region was carried out in a total 50 µl reaction volume in PCR thermocycler. The reaction volume including that 30 µl ddH2O, 15 µl of Dream Taq PCR Master Mix (2X) (Fermentas Life Sciences, Germany), 1 µM of each forward and reverse primer (Table 1), and 3 µl of DNA. The PCR condition were initial denaturation for 3 min at 94 °C; 35 cycles of 94 °C denaturation (45 s), 53 °C annealing (45 s) and 72 °C extension (45 s); a final extension for 3 min at 72 °C. After PCR amplification, 10 µl of each PCR product was mixed with 1 µl of 6X loading buffer (Fermentas Life Sciences, Germany) and then loaded on a 1.5 % standard TAE buffered agarose gel. After electrophoresis (100 V for 60 min), the gel was stained with ethidium bromide (0.1 µg ml-1) for 20 min, and visualized under UV-light and photographed. The remaining PCR products were kept at -20 °C (Subbotin *et al*., 2003; Tanha Maafi *et al*., 2003).

Sequencing

The remainder of the PCR products were loaded on a 1 % agarose gel for electrophoresis (100 V, 60 min). The purification method was done as defined in the manufacturer's instructions (Wizard® SV Gel and PCR Clean - Up System Kit, Promega). DNA from nine samples, representing nine populations, were sequenced in a sequencing facility (Refgen, Ankara, Turkey) in both directions to get matching sequences of both DNA strands. The sequences were formatted and evaluated. Eventually, all sequences were added to GenBank (Sequin v. 9.00, http://www.ncbi.nlm.nih.gov) and accession numbers were obtained for each population. The sequences of nine DNA samples from this research were placed in GenBank with accession numbers (Table 2).

Phylogenetic Analysis

The sequences of nine DNA samples were edited and alignments were made by CLUSTALW as implemented in BIOEDIT (Hall 1999). All aligned characters were implemented in the phylogenetic analysis. Phylogenetic trees were derived using maximum likelihood and this was constructed with using the MEGA v 7.0. (Kumar *et al*., 2016). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980).

Table 1. Primers used in the amplification of DNA.
Çizelge 1. DNA amplifikasyonunda kullanılan primerler.

Primer	5'_3'	References
Forward	TGTGAACTGCAGGACACATG	Staouthammer *et al*., 1999
Reverse	GTCTTGCCTGCTCTGAG	Staouthammer *et al*., 1999

Table 2. Identified *Trichogramma brassicae* and accession numbers.
Çizelge 2. Tanımlanan Trichogramma brassicae ve erişim numaraları.

Sample No	Species	Original Host	Accession Number
1	*Trichogramma brassicae*	*Ostrinia nubilalis*	KX010935
2	*Trichogramma brassicae*	*Ostrinia nubilalis*	KX010936
3	*Trichogramma brassicae*	*Ostrinia nubilalis*	KX010937
4	*Trichogramma brassicae*	*Ostrinia nubilalis*	KX010938
5	*Trichogramma brassicae*	*Ostrinia nubilalis*	KX010939
6	*Trichogramma brassicae*	*Ostrinia nubilalis*	KX010940
7	*Trichogramma brassicae*	*Ostrinia nubilalis*	KX010941
8	*Trichogramma brassicae*	*Ostrinia nubilalis*	KX010942
9	*Trichogramma brassicae*	*Ostrinia nubilalis*	KX010943

RESULTS AND DISCUSSION

Trichogramma specimens were collected from corn fields in Düzce province of Turkey. Nine sequences were obtained from the Düzce samples and amplified partial sequences of the rDNA-ITS2 of *Trichogramma* samples by use the polymerase chain reaction (PCR). All tested populations were defined as *T. brassicae*. A general consensus sequence was derived from the obtained sequences and used for phylogenetic analysis. *T. brassicae* sequences were placed in GenBank with the numbers from KX010935 to KX010943 (Table 2). Dendograms were formed together with sequences of different *Trichogramma* species obtained from the GenBank.

The rDNA-ITS2 sequences of nine *T. brassicae* samples obtained from Düzce province were 100% similarity to each other, in other words, there is no genetic variation among the populations (Figure 1.). However, when the samples belonging to *T. brassicae* in different countries (AY182766, JF920441, JF920451) available in GenBank database were compared with our samples, the similarity rate was found to be 62% (Figure 2.). In addition, these samples were most similar to *T. evanescens*, and were found to be quite different from other *Trichogramma* species.

Our results show that there is no genetic variation in *T. brassicae* populations. Parasitization rate obtained in different corn field in Düzce province is very high (98%) and very close to each other (Kutuk 2016). This supports homogenity of identified *T. brassicae* population in Düzce province.

The rDNA-ITS2 is a significant molecular marker that can be used for comparing closely related subspecies, species and populations. The molecular method provides specific and precision results for the diagnosis of single and multiple species of egg parasitoids in agricultural systems (Sumer Ercan 2011).

Chang *et al.* (2001) sequenced the ITS1 region of *Trichogramma chilonis* and *T. ostriniae* species which are egg parasitic wasps of Asian corn borer (*Ostrinia furnacalis* Guenée). It was found that the two parasitic wasps were 86,1% similar. Alvarez and Hoy (2002) used the sequences of rDNA-ITS2 for separating populations of the parasitoid *Ageniaspis citricola* (Hymenoptera: Encyrtidae) from different geographical zones. Moreover, Thomson *et al.* (2003) studied the identification of *Trichogramma* species in southeastern Australia and used for ITS2 sequence analysis. It was found that the length of ITS2 is different for each species. Kumar *et al.* (2009) used the ITS2-RFLP method to distinguish 12 indigenous and exotic *Trichogramma* species in India. Sumer Ercan *et al.* (2011) reported that the differences between *T. brassicae* and *T. euproctidis* in their ITS2 sequence. Similarly, Sumer Ercan *et al.* (2013) analyzed that sequence variation of ITS2 examined differentiation of *T. euproctidis* and *T. brassicae* populations. Joeng *et al.* (2010) ITS2 gene sequences have been used to identify two *Trichogramma* species, *T. ostriniae* and *T. brassicae*, in Korea. Thiruvengadam *et al.* (2016) have studied the evolutionary relationships based on ITS2 gene of these species in order to identify *Trichogramma* species and to determine their differences.

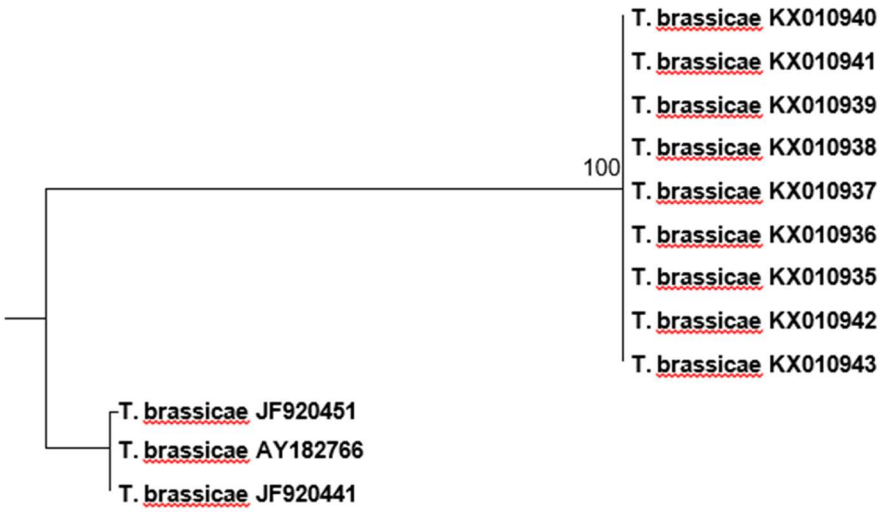

Figure 1. Phylogenetic tree (Maximum Likelihood) of *T. brassicae* sub-tree separately.
Şekil 2. T. brassicae'nin alt dallarına ayrılmış filogenetik ağacı.

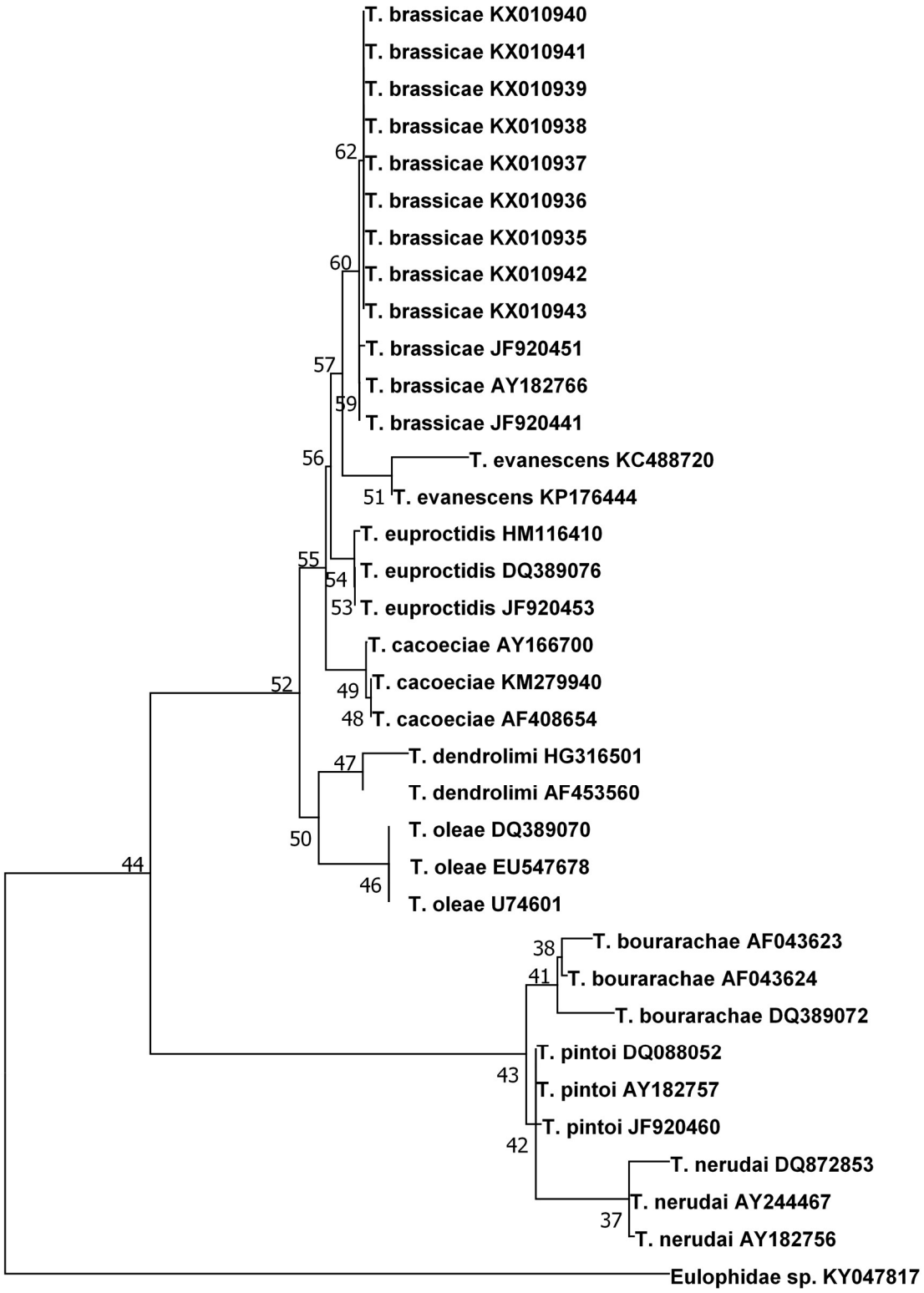

Figure 1. Phylogenetic tree created through the rDNA-ITS sequence alignment from nine *T. brassicae* populations. *Eulophidae* sp. is used as an outgroup.

Şekil 1. Dokuz T. brassicae popülasyonundan rDNA-ITS sekans grupları ile oluşturulan filogenetik ağaç. Eulophidae sp. grup dışı olarak kullanılmıştır.

CONCLUSION

Trichogramma species are important biological agents in biological control programs. The choice of the appropriate natural enemy in the control of the target pest is very essential for reaching the successful biological control. Techniques based upon sequence analysis of the ITS2 locus of rDNA are used effectively in the identification of these species. Sequence analysis of the rDNA-ITS region of *Trichogramma brassicae* samples showed that the samples were genetically similar, but were quite different from other *Trichogramma* species. Genetic variations among different *Trichogramma* species are thought to be useful in the development of rapid diagnosis and detection methods based on PCR.

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