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Molecular identification of *Trichogramma* Westwood, 1833 species as egg parasitoids of *Ostrinia nubilalis* (Hübner, 1796) in corn production areas of Sakarya province in Türkiye (Insecta: Lepidoptera, Hymenoptera)

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Abstract

The study focuses on using ITS2 sequences from rDNA for systematic studies of *Trichogramma* Westwood, 1833 species. ITS2 sequences have shown to be reliable in distinguishing *Trichogramma* species. Accurate identification of natural enemies is essential for biological control programs. The aim of the study is to compare rDNA-ITS2 sequences from *Trichogramma* samples with sequences in GenBank. By utilizing ITS2 as a barcode, the study aims to achieve dependable species identification and evaluate genetic diversity within *Trichogramma* species. ITS2 sequence was used to differentiate two *Trichogramma* species, *Trichogramma euproctidis* (Girault, 1911) and *Trichogramma brassicae* Bezdenko, 1968 collected from Sakarya province of Türkiye. The ITS2 sequences of the two *Trichogramma* species were aligned using Clustal W, and genetic distances as well as a phylogenetic tree were calculated using MEGA V7.0. In the study, rDNA-ITS2 sequences of fifty four *Trichogramma* specimens were confirmed in GenBank. Additionally Mfold web server was used to predict secondary structures of ITS2 sequences. The construction of all secondary structures was carried out at 37ºC using RNA version 2.3 default parameters. The study identified a new species that expands the Trichogramma fauna of Sakarya Province in northwestern Türkiye, where only one species had previously been detected through rDNA-ITS2 sequence analysis. This shows the importance of molecular markers in species identification and biological control strategies.
Keywords: Insecta, Lepidoptera, Hymenoptera, ITS2, *Trichogramma*, *Ostrinia*, biological control, molecular systematics, Türkiye.

Identificación molecular de especies de *Trichogramma* Westwood, 1833 como parasitoides de huevos de *Ostrinia nubilalis* (Hübner, 1796) en zonas de producción de maíz de la provincia de Sakarya en Turquía (Insecta: Lepidoptera, Hymenoptera)

Resumen

El estudio se centra en la utilización de las secuencias ITS2 del ADNr para estudios sistemáticos de las especies de *Trichogramma* Westwood, 1833. Las secuencias ITS2 han demostrado ser fiables para distinguir las especies de Trichogramma. La identificación precisa de los enemigos naturales es esencial para los programas de control biológico. El objetivo de este estudio es comparar secuencias de ADNr-ITS2 de muestras de Trichogramma con secuencias del GenBank. Utilizando ITS2 como código de barras, el estudio pretende lograr una identificación fiable de las especies y evaluar la diversidad genética dentro de las especies de *Trichogramma*. Se utilizó la secuencia ITS2 para diferenciar dos especies de *Trichogramma*, *Trichogramma euproctidis* (Girault, 1911) y *Trichogramma brassicae* Bezdenko, 1968, recogidas en la provincia turca de Sakarya. Las secuencias ITS2 de las dos especies de *Trichogramma* se alinearon utilizando Clustal W, y las distancias genéticas, así

como un árbol filogenético, se calcularon utilizando MEGA V7.0. En el estudio se confirmaron en GenBank las secuencias de ADNr-ITS2 de cincuenta y cuatro especímenes de *Trichogramma*. Además, se utilizó el servidor web Mfold para predecir las estructuras secundarias de las secuencias ITS2. La construcción de todas las estructuras secundarias se llevó a cabo a 37ºC utilizando los parámetros por defecto de la versión 2.3 de RNA. El estudio identificó una nueva especie que amplía la fauna de *Trichogramma* de la provincia de Sakarya, en el noroeste de Turquía, donde anteriormente sólo se había detectado una especie mediante el análisis de secuencias de ADNr-ITS2, lo que demuestra la importancia de los marcadores moleculares en la identificación de especies y las estrategias de control biológico.
Palabras clave: Insecta, Lepidoptera, Hymenoptera, ITS2, *Trichogramma*, *Ostrinia*, control biológico, sistemática molecular, Turquía.

Introduction

Biological control is based on the reduction of harmful insect populations by using their natural enemies. Natural enemies of harmful insects are also defined as biological control agents, and one of the most important among them is parasitoids. The selection of the natural enemy is the most critical step for the biological control to reach the target. Accordingly, the natural enemy in the application area can be protected, increased or brought from a different region to the application area (Wiedenmann, 2000).

Among the egg parasitoids, the species belonging to the genus *Trichogramma* Westwood, 1833 are the most used group. These microscopic wasps parasitize over 400 pest species and especially economically important pest species belonging to the order Lepidoptera. Although it can successfully parasitize the eggs of pests belonging to the order Lepidoptera, it can also parasitize the eggs of species belonging to the orders Coleoptera, Diptera, Hemiptera, Hymenoptera and Neuroptera (Li, 1994; Smith, 1996; Pinto, 1999).

The key to the success of biological control programs lies in the accurate selection of the appropriate species or strains of natural enemies. In biological control programs in which *Trichogramma* species are used, it is important to select the most suitable and effective *Trichogramma* species for successful mass production and control of the target pest. The identification of species belonging to this genus is problematic due to their small size. For the identification of these parasitoids, which are approximately 0.5 mm long, the morphological characters of the male genitalia were used as a basis (Nagarkatti & Nagaraja, 1971). However, many important species have very similar genitalia (Pinto & Stouthamer, 1994). Their very small size makes it difficult to identify visible and reliable features that enable them to be distinguished among themselves and from other insects. However, the most important limitation is that female individuals cannot be identified based on morphological characters (Stouthamer et al. 1990).

The reasons such as the unreliable morphological features and the absence of male individuals have directed researchers to different methods that are more reliable and stable for diagnosis. As a result, various biochemical and molecular techniques have been created to differentiate closely related taxa. A technique based on the sequence analysis of the ITS2 locus of rDNA was developed and started to be used effectively in the diagnosis of *Trichogramma* (Stouthamer et al. 1999). Thus, the problem of species identification, which is limited to morphological diagnosis depending on the characteristics of male individuals, has been solved, and at the same time, an alternative and more useful method to biochemical analysis has been developed.

It can be said that most of the *Trichogramma* species names used in the literature of previous years are misidentified or suspicious. Because species included in published information are rarely preserved. Therefore, it has become impossible to review the original diagnoses or to make a decision by comparing new and correct nomenclature based on current information. As a result, a small amount of literature can be used by researchers. For this reason, it is absolutely necessary for researchers to protect the species they have determined as a result of their studies (Rosen, 1986). There are many *Trichogramma* species and they vary greatly in their adaptability to different environmental conditions and the different insects that controlled by them (Silva 1999). Molecular identification of *Trichogramma* species, which are distributed in different regions of Türkiye, has been carried out with our previous studies (Sümer et al. 2009; Sümer et al. 2010; Ercan et al. 2011; Ercan et al. 2013; Ercan et al. 2022).

This study aims to identify the *Trichogramma* species collected from the Sakarya region of Türkiye using molecular techniques. Although there are many studies on the cultivation of *Trichogramma* and its use in biological control in our country, studies on the use of molecular methods in the identification of this important

biological control agent have gained importance in recent years. With this study, *Trichogramma* species found in corn fields in Sakarya province were detected using molecular methods and contributed to future biological control studies.

Materials and methods

Collection of *Trichogramma* Specimens

Trichogramma samples were collected from corn fields in Sakarya province between July and September 2022. During the field study, sampling was made on 100 plants from each field from randomly selected corn fields in the Sakarya region in May-September. All the leaves and leaf undersides of the selected maize were carefully examined, and the maize leaves with parasitized (blackened) and non-parasitized egg packs of *Ostrinia nubilalis* (Hübner, 1796). Were cut without damaging the egg pack, taken into plastic culture containers and brought to the laboratory in an ice container. Each of the egg packages was placed in separate glass test tubes and followed in a long-day illuminated air-conditioning cabinet adjusted to 25±1ºC temperature and 70±5% proportional humidity. As a result of the field study, parasitoid hatching was detected from pest eggs obtained from 3 different localities (N 40º 68'78.86"'/E 30º 39'69.33"', N 40º 69'56.53"'/E 30º 39'07.25" and N 40º 75'58.75"'/E 30º 41'53.56"'). *Trichogramma* adults emerging from egg packages collected from the field were transferred into 70% alcohol to be used in DNA isolation and labeled. (Ercan et al. 2011)

Molecular Studies

DNA extraction from *Trichogramma* samples was performed from individual wasp of each sample. Firstly, 60 µl 5% Chelex-100 and 2 µl Proteinase K (20 mg/ml) were used to grind the samples. Then ground samples incubated at 1 h at 55º C, followed by 10 min at 96º C. The rDNA-ITS2 amplification was performed to reveal the phylogenetic relationship between the samples, ITS2 froward and reverse primers were used (5′-TGT GAA CTG CAG GAC ACATG-3′ and 5′-GTC TTG CCT GCT CTGAG-3′, respectively) in PCR reaction (Stouthamer et al. 1999). After electrophoresis cloning was veri as described in Ercan et al. (2011). PCR products were visualized by electrophoresis after cloning and sent for automatic sequencing in a sequencing facility (BM Laboratory, Türkiye).

Structural characterization of ITS2

The mFOLD web server is used for predicting RNA secondary structures and calculating the Gibbs free energy (ΔG) of these structures. This prediction was veri at 37 °C using RNA version 2.3 with the default parameters provided by the program (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3) (Santa Lucia, 1998; Zuker, 2003).

Phylogenetic Studies

Mega 7 yazılım was used to determine inter- and intra-species nucleotide differences. Kimura 2-Parameter (K2P) distance model is a widely used method to examine the evolutionary similarities of DNA sequences (Kimura, 1980). Kimura's model calculates the genetic distance between species by taking into account the nucleotide change rates (Kumar et al. 2016). In the study, the probability of the best phylogenetic tree was determined according to the polymorphism in the nucleotide sequences. For this purpose, Maximum Likelihood (ML) analysis was used. In ML analyses, jModelTest v.0.1.1 program was used to determine the most appropriate model for sequence variation and the model with the lowest AIC (Akaike Information Criteria) value was selected to determine the phylogenetic tree. In addition, the reliability of the created model phylogenetic trees was checked with 1000 replication Bootstrap test.

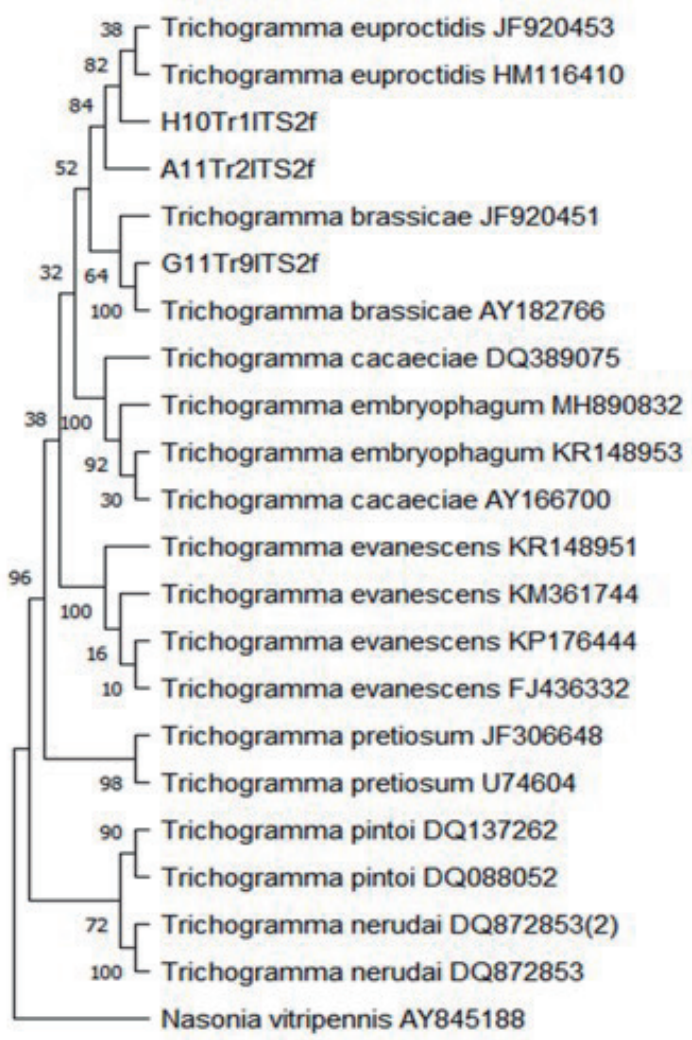
Results and Discussion

Trichogramma samples were collected from *Ostrinia nubilalis* eggs in Sakarya province of Türkiye. Fifty

four individuals were emerged from the collected host eggs.

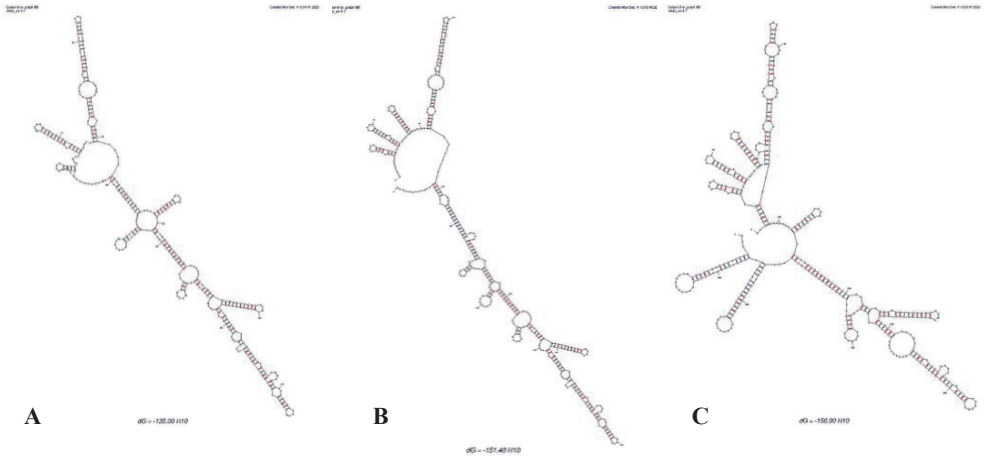
RDNA-ITS2 sequences specimens were searched in GenBank database of National Center for Biotechnology Information. We compared them with all obtained homologous sequences of other *Trichogramma* species in GenBank. From the samples collected 41 out of 54 specimens were identified as *Trichogramma euproctidis* (Girault, 1911) and other 13 individuals as *Trichogramma brassicae* Bezdenko, 1968. The ITS2 sequences of *Trichogramma euproctidis* samples varied in length between 415 and 424 bp while the ITS2 sequence length of all of the samples from *T. brassicae* was determined as 435 bp. Sequence results were used to construct a phylogenetic tree. GenBank provides a large database of genetic sequences, allowing researchers to study the genetic material of a variety of organisms. Genetic analysis of parasitoid insects such as *Trichogramma* species is a common method of constructing dendrograms using the sequences obtained. (Figure 1). *T. euproctidis* A11 coded, *T. euproctidis* H10 coded and *T. brassicae* G11 coded samples were selected randomly.

Figure 1. Phylogenetic tree based on the ITS2 gene region of *Trichogramma* samples (except for the samples coded A11, H10 and G11, the samples in the tree were taken from NCBI. *Nasonia vitripennis* is included in the tree as an outgroup.



ITS2 secondary structures of *T. euproctidis* and *T. brassicae* were generated using the Mfold web server (Figure 2). Analysis of predicted secondary structures using the Mfold web server can reveal genetic and morphological differences among species. Branching structures were found to be remarkable in *T. brassicae*, which led to the emphasis of structural differences between species. In the constructed phylogenetic tree, similarities and differences are reflected by helices and angles over the ITS2 sequence. This provides an important element in understanding the evolutionary relationships of species. The calculated ΔG values as- 135.00 kcal/mol for *T. euproctidis* and-156.90 kcal/mol for *T. brassicae* reveal the thermodynamic differences between the species. Since the lower ΔG value means a more stable structure, it provides additional information about the secondary structure of these species. The secondary structure of the ITS2 region can be interpreted as a clear observation of the nucleotide sequences and can be evaluated as morphological characteristics of the species.

Figure 2. Secondary structure predictions and ΔG (Gibbs) free energy values (bottom) of T. euproctidis (A: A11, B: H10) and T. brassicae (C: G11) http://www.unafold. Calculated and analyzed on the mFOLD web server org/mfold/applications. /rna-fold-form-v2.php).



Trichogramma are a priority group for molecular identificaiton due to their importance in biological control against especially lepidopteran pests. Their relatively small size and morphological similarity lead to inability to accurately identify species and to understand the effects of these parasitoids on pest populations. PCR of intraspecies conserved regions is very important for molecular identification of *Trichogramma* and reliable results were obtained by selecting the rDNA-ITS2 region as the target region for analysis. *Trichogramma* is a genus of parasitic insects that are important in agricultural pest control. DNA sequence analysis plays a vital role in correctly identifying these species and distinguishing similar species. Identifying species with the genetic data and analysis results obtained allows the development of more effective and sustainable methods in agricultural practices.

Although there are many studies on the cultivation of *Trichogramma* and its use in biological control in our country, studies on the use of molecular techniques in the diagnosis of this important biological control agent have gained importance in recent years. Sakarya region, which was sampled in our study, ranks first in its region in corn cultivation and is economically important. Therefore, it is important to detect and identify the natural biological control agents of the region for the development of alternative control methods against European cornborer, *Ostrinia nubilalis*, which is one of the main pests that cause product loss in corn production. In this study, the local *Trichogramma* species(s) of the region were identified molecularly for the first time.

The secondary structure of the ITS2 sequence reveals notable branched formations in *T. brassicae* when compared to *T. euproctidis*. Furthermore, the similarities and differences evident in the phylogenetic tree are also reflected in the helices and secondary structural composition of ITS2 among the species. Consequently, thermodynamic calculations for T. euproctidis and T. brassicae, along with the computation of ΔG (Gibbs) free energy values using mFold program parameters based on helices and angles in the secondary structure, varied by

species. The calculated values were -135.00 kcal/mol for the *T. euproctidis* A11 coded sample, -151.40 kcal/mol for the *T. euproctidis* H10 coded sample, and -156.90 kcal/mol for the *T. brassicae* G11 coded sample. Therefore, the secondary structural formation of the ITS2 region has also been utilized as a morphological characteristic of the species due to the clear visualization of the nucleotide sequences (Figure 2).

Molecular markers that are rapidly evolving and located in highly conserved gene regions can be effectively utilized to differentiate closely related taxa. The ITS2 region is a significant molecular marker that enables comparison of closely related species, sub-species, and populations. Zhu & Williams (2002) cloned and sequenced the ITS2 region of Mymarid parasitoids and their hosts using rDNA sequence primers. This molecular technique demonstrates potential for the accurate and sensitive identification of both single and multiple species of egg parasitoids in agricultural and natural environments.Various biochemical and molecular methods have been developed to discern differences between closely related taxa, such as allozyme electrophoresis, random amplified polymorphic DNA variability (RAPD), Restriction Enzyme Fragment Length Diversity (RFLP), mitochondrial cytochrome oxidase subunit I (COI), and microsatellite markers (Miura et al. 1990; Pintureau, 1993; Vanlerberghe-Masutti, 1994; Sappal et al. 1995; Chang et al. 2001; Al-Barrak et al. 2004; Monti et al. 2005; Pizzol et al. 2005).

Thomson et al. (2003) used ITS2 sequence analyzes to identify *Trichogramma* species from southeastern Australia. They found that ITS2 length differs for each species. Chang et al. (2001) found that the ITS1 regions of *T. ostriniae* Pang & Chen, 1974 and *T. chilonis* Ishaii, 1941, two egg parasitoids of *Ostrinia furnacalis* (Guenée, 1854) (Lepidoptera: Pyralidae) found in Taiwan, were 86.1% identical. They determined that the length of ITS1 is different in these two species, 458 bp and 322 bp, respectively. In our study, *T. euproctidis* and *T. brassicae* species were identified according to the ITS2 sequence, and the sequence lengths of the ITS2 region were determined as 415 and 424 bp for *T. euproctidis* (A11 and H10, respectively) and 435 bp for *T. brassicae*. Ciociola et al. (2001) used five adult individuals for DNA extraction. With the system we used in our study, sufficient DNA extract could be obtained from only one adult parasitoid for successful PCR and subsequent sequencing of the ITS2 region of the rDNA.

Today, it can be said that most of the *Trichogramma* species names used in the literature of the past years are incorrect or at least doubtful. Because species included in published information are rarely protected. Therefore, it has become impossible to review the original diagnoses or to make decisions by comparing new and correct nomenclature based on current information. As a result, a small number of literature can be used by researchers. It is estimated that only 10-30% of parasitic Hymenoptera have been identified so far. The species identified as a result of our study will be protected. This is especially important for entomophagous insects, due to their small body size, morphological homogeneity, and taxonomic status, which is much less known than many other groups. Thus, it will be possible to molecularly compare the *Trichogramma* obtained in subsequent studies. By identifying natural populations collected from the field, misdiagnoses will be corrected and a contribution to the literature will be made.

With this study, a new species was added to the *Trichogramma* fauna of Sakarya Province, North western of Türkiye where a single species was previously detected. The importance and necessity of molecular diagnostic studies for biological control studies to be carried out in the corn fields of Sakarya province in the future have been revealed with this study. The possibilities of using this species in biological control should be studied in corn fields of Sakarya province where corn planting is high. Economical production can be achieved through biological control applications, and ecological benefits can be achieved by preserving the natural balance and biodiversity. Producers will use less chemical input and social awareness will be raised. It is very important to have an accurate molecular diagnosis of biological control agents that can be used commercially in the future. This provided basic data for new research projects.

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Conflict of Interest

The authors declare that there is no known financial interest or personal relationship that could have influenced the work presented in this article.

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