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Original article (Orijinal araştırma)

New records of the parasitoids of *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae) in newly invaded areas in Turkey: molecular identification

Türkiye'de yeni istila edilen alanlarda *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae)'nin yeni parazitoit kayıtları: moleküler tanımlamaları

Gülay KAÇAR[1*]

Abstract

Drosophila suzukii (Matsumura, 1931) (Diptera: Drosophilidae) is an invasive pest species of various fruit crops in the USA and Europe. Although *D. suzukii* has been recently reported in strawberry in Erzurum and other newly invaded areas in Turkey (e.g., Ankara, Bolu, Çanakkale and Düzce), there is only limited information on its indigenous parasitoids. In this study, four hymenopteran parasitoids, the larval parasitoids of *Leptopilina boulardi* (Barbotin, Carton & Kelner-Pillault, 1979), *Leptopilina heterotoma* (Thomson, 1862) (Figitidae) and the pupal parasitoids of *Pachycrepoideus vindemmiae* (Rondani, 1875) (Pteromalidae) and *Trichopria drosophilae* (Perkins, 1910) (Diapriidae), were collected from frugivorous drosophilid species. *Leptopilina boulardi* and *T. drosophilae* were found for the first time in Turkey. *Leptopilina heterotoma* and *P. vindemmiae* were the most common parasitoid species, reared from field-collected fruit samples in this study. The laboratory assays revealed that both pupal parasitoids developed from *D. suzukii* pupae, but the association of *L. heterotoma* and *L. boulardi* with *D. suzukii* is yet to be confirmed. The PCR amplification of the cytochrome c oxidase subunit I loci of mtDNA of the representative four parasitoid samples produced different lengths of DNA fragments, ranging from 633 bp to 658 bp. BLASTn queries based on the COI of the parasitoid samples showed that the sequences were 99-100% identical to those of the corresponding species in the GenBank database.

Keywords: Molecular diagnostic, new parasitoids, spotted wing drosophila, Turkey

Öz

Drosophila suzukii (Matsumura, 1931) (Diptera: Drosophilidae), Avrupa ve Amerika'da çeşitli meyvelerde zarara neden olan istilacı bir türdür. *Drosophila suzukii*, son zamanlarda Türkiye'de Erzurum'da çilekte rapor edilmesine ve o zamandan beri yeni alanları (Ankara, Bolu, Çanakkale ve Düzce vs.) istila etmesine rağmen, onun yerli parazitoitleri çok az bilinmektedir. Bu çalışmada, dört hymenopter parazitoit, larval parazitoitler *Leptopilina boulardi* (Barbotin, Carton & Kelner-Pillault, 1979), *Leptopilina heterotoma* (Thomson, 1862) (Figitidae) ve pupal parazitoitler *Pachycrepoideus vindemmiae* (Rondani, 1875) (Pteromalidae) ve *Trichopria drosophilae* (Perkins, 1910) (Diapriidae) türleri, meyvelerde bulunan drosophilid türlerinden elde edilmiştir. *Leptopilina boulardi* ve *T. drosophilae* türlerinin Türkiye'de ilk defa varlığı belirlenmiştir. *Leptopilina heterotoma* ve *P. vindemmiae,* toplanan örneklerden en fazla bulunan türler olmuştur. Laboratuvar denemeleri iki pupa parazitoitinin *D. suzukii* pupalarından geliştiğini belirlenmesine rağmen, *L. heterotoma* ve *L. boulardi*'nin *D. suzukii*'den geliştiklerini hala doğrulanamamıştır. Dört parazitoit türü temsil eden örneklerin mitokondrial DNA'sının cytochrome c oxidase subunit I lokusunun PCR ile çoğaltılması sonucu 633 bp ile 658 bp arasında DNA segmentlerini ürettiği belirlenmiştir. Bu ürünlerin BLASTn analizi sonucu GenBank'daki referans bireylerin sekanslarıyla %99-100 benzerliğe sahip olduğu tespit edilmiştir.

Anahtar sözcükler: Moleküler tanımlama, yeni parazitoitler, noktalı kanatlı drosophila, Türkiye

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Introduction

Drosophila suzukii (Matsumura, 1931) (Diptera: Drosophilidae), which is primarily known as spotted wing drosophila in the USA and indigenous to southeast Asia (Kanzawa, 1939), has rapidly spread to Europe, South and North America (Bolda et al., 2010; Hauser, 2011; Walsh et al., 2011; Berry, 2012; Depra et al., 2014; Emiljanowicz et al., 2014). After the fly was first found in Spain and Italy in 2008 (Walsh et al., 2011), *D. suzukii* has increasingly spread into many other countries of Europe, such as Croatia, France, Portugal and Slovenia (Milek et al., 2011; Withers & Allemand, 2012). In Turkey, it was first recorded in Erzurum in 2014 (Orhan et al., 2016; Tozlu et al., 2016), and further found Ankara in 2015 (Önder et al., 2016), Bolu and Düzce in 2016 (Kaçar & Koca, 2017), Karaman in 2017 (Ögür et al., 2018) and Çanakkale in 2018 (Efil, 2018; Kasap & Özdamar, 2019). *Drosophila suzukii* appears to be spreading rapidly in Turkey. The most probable reason for this rapid spread is the international trade of infested fruits (EPPO, 2002; Westphal et al., 2008). *D. suzukii* is an extremely polyphagous invasive pest which principally damages soft-skinned fruits (Lee et al., 2011). The female of *D. suzukii* lays eggs in undamaged fruit owing to the serrated female ovipositor (Sasaki & Sato, 1995). Thus, it usually lays eggs on ripe fruit (Mitsui et al., 2007). *Drosophila suzukii* causes physical damage to fresh fruit by means of secondary pathogen infections, which can access the damaged skin of the fruit and cause faster deterioration, resulting in the yield losses of 30-100% in fruit production (Bolda et al., 2010).

Drosophila species are attacked by about 50 hymenopteran parasitoids which have an important function in controlling the drosophilids (Carton et al., 1986). Hymenopteran parasitoids mostly from the genera *Asobara* (Braconidae), *Leptopilina* and *Ganaspis* (Figitidae), *Trichopria* (Diapriidae), and *Pachycrepoideus* (Pteromalidae) commonly parasitize drosophilids (Carton et al., 1986; Hertlein, 1986). Several larval parasitoids, including *Leptopilina boulardi* (Barbotin, Carton & Kelner-Pillault, 1979), *Leptopilina heterotoma* (Thomson, 1862), *Leptopilina japonica* Novković & Kimura, 2011*, Leptopilina japonica formosana* Novković & Kimura, 2011*, Asobara japonica* Belokobylskij, 1998*, Asobara brevicauda* Guerrieri, Giorgini, Cascone, Carpenito & Achterberg, 2016*, Asobara leveri* (Nixon, 1939)*, Asobara tabida* (Nees, 1834)*, Ganaspis xanthopoda* (Ashmead, 1896) and *Ganaspis brasiliensis* (Ihering, 1905)*,* and pupal parasitoids*;* e.g., *Pachycrepoideus vindemmiae* (Rondani, 1875) (Pteromalidae) and *Trichopria drosophilae* (Perkins, 1910) (Diapriidae), are commonly associated with *Drosophila* species (Fleury et al., 2004; Mitsui et al., 2007; Chabert et al., 2012; Novkovic et al., 2011; Poyet et al., 2013; Kimura & Novkovic, 2015; Daane et al., 2016; Girod et al., 2018a, b, c; Giorgini et al., 2019). Recently, several studies conducted in the USA and European countries have shown that most of the larval drosophila parasitoids are unable to successfully develop in *D. suzukii* (Chabert et al., 2012; Poyet et al., 2013; Rossi Stacconi et al., 2013; Girod et al., 2018a, b, c; Giorgini et al., 2019*). Asobara japonica* successfully developed in *D. suzukii* compared with other widespread larval parasitoids (*A. tabida* and *G. xanthopoda*) although *L. heterotoma* may be country-specific (Mitsui et al., 2007; Kimura & Novkovic, 2015; Rossi Stacconi et al., 2015; Girold et al., 2018). Only two pupal parasitoids, *P. vindemmiae* and *T. drosophilae*, readily attack *D. suzukii* (Rossi Stacconi et al., 2015, 2017; Kaçar et al., 2017). Although *T. drosophilae* is polyphagous parasitoid, it may be one candidate for controlling populations of *D. suzukii* (Girod et al., 2018c; Rossi Stacconi et al., 2018).

The parasitoids of the order Hymenoptera are generally identified according to their morphological characteristics, which leads to challenges in diagnosis due to their body size and undefined characteristics (Tomanovic et al., 2003). The molecular analysis of DNA sequences is considered as a complementary tool for the morphological identification of insects (Farrokhzadeh et al., 2014). Molecular identification has allowed quick recognition, distinction and identification of diverse species based on the DNA sequencing of single samples. However, since researchers perform the identification of species using only key morphological characteristics, the results may not be definitive and they are mostly at genus level. The molecular techniques recently developed allow identification of insects at the species level. For example, the cytochrome c oxidase subunit I (COI) locus of mitochondrial DNA has been employed as a reliable tool to accurately identify parasitoid species (Fre´zal & Leblois, 2008; Linares et al., 2009).

The current study aimed to explore and identify drosophilid parasitoids in different geographical regions of Turkey and present an alternative identification technique for these parasitoids based on phenotypic characteristics to support diagnoses.

Materials and Methods

Collection areas and methods

The survey for the parasitoids of frugivorous drosophila was conducted in two provinces in Turkey, Bolu and Düzce, from May 2016 to December 2018. The samples of drosophilids and parasitoids were collected from infested fresh and fallen decaying fruit (including apples, blackberries, cherries, figs, grapes, pears, persimmons, plums, raspberries and strawberries) in the field. Samples were collected during or after the harvesting seasons for each fruit. The fruit samples were placed in a cooler box to be transferred to a climate room. The location, date and collector were recorded, and each fruit sample was labeled accordingly. The fruit samples collected in the field were separately put in transparent containers with ventilated lids on a layer of moist filter paper until pupation.

All fly pupa reared from the field-collected larvae were inspected and separately placed in 5-cm Petri dishes until the appearance of drosophilid adults or parasitoids which were preserved in alcohol (95%) for later identification. The samples were kept in a climate cabin at 40-70% RH, 22±3°C and 12:12-h L:D photoperiod with natural light in the laboratory. Four parasitoid species were paired and maintained on *D. suzukii* and *Drosophila melanogaster* (Meigen, 1830) supplied with artificial food of 10% honey-water provided for the parasitoids with wet paper towel in plastic container as additional water source. The flies were collected from the field fruits in Düzce and provided an artificial corn meal (Dalton et al., 2011). Petri dishes (9 cm) were filled with 35 g of artificial diet were placed in cages as an oviposition media and changed in every 2 d. These were used for rearing drosophilids and their parasitoids. The parasitoid species were tested in the laboratory to confirm their suitability as a host for *D. suzukii*. Emerged parasitoids were separated in *L. heterotoma* and *L. boulardi* males and females. Each parasitoid pair were individually released, as soon as they were collected, in rearing tubes with drosophila medium and 10 *D. suzukii* 1-2-d old larvae or pupae and honey-water droplets for confirming tests. After all parasitoids had emerged, all dead unemerged pupae were dissected. The majority of emerged flies (notably those from the same dishes where were parasitoids have emerged) were preliminary identified.

Molecular identification of the parasitoids

The fly species were identified using the identification key according to Markow & O'Grady (2006). *D. suzukii* pupae were distinguished considering the existence of a couple of distinct breathing tubes on the anterior end (Kanzawa, 1939). The parasitoids species were identified using the identification key described by Legner et al. (1976), Boucek & Rasplus (1991) and Carton et al. (1986). The identification of the four parasitoids were confirmed by all experts. The figitids were identified by Dr. Mattias Forshage and deposited with the Entomology Collection of the Swedish Museum of Natural History (Stockholm, Sweden). *Trichopria drosophilae* was confirmed or identified by Dr. Ovidiu Gavrilovici (Department of Psychology and Education Sciences, Universitatea Alexandru Ioan Cuza, Iaşi, Romania). *Pachycrepoideus vindemmiae* were confirmed or identified by Dr. Habil Mircea-Dan Mitroiu (Biology Faculty, Alexandru Ioan Cuza University, Romania). All parasitoid samples were also deposited at Bolu Abant Izzet Baysal University, Agriculture and Science Faculty, Bolu, Turkey.

The extraction of the nucleic acids of all specimens either dried or preserved in 95% ethyl alcohol was conducted using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The DNA extracts were quantified spectrophotometrically by a DS-11 FX series spectrophotometer (DeNovix Inc., Wilmington, DE, USA) and ultimately diluted to 10 ng/µl using sterile ddH_2O. The PCR amplification based on part on the mitochondrial protein-coding gene and COI

gene was performed using the universal primer pair LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al., 1994). Amplification reactions were conducted in a 50 µl reaction mixture containing 5 µl 10× PCR reaction buffer, 0.4 µM of each primer, 50 ng DNA template, 0.2 mM each dNTPs, and 1.25 unit Taq DNA Polymerase (New England BioLabs, MA, USA; Neb #M0320S). The amplification program for the COI locus was as follows: an initial denaturation step at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 1 min, and a 5-min final extension at 72°C. All the PCR products were confirmed electrophoretically using agarose gel (1.2% w/v) and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) following the manufacturer's instructions. The DNA fragments were sequenced by the Sanger nucleotide sequencing method provided by a commercial company (Macrogen Inc., Seoul, South Korea).

The resulting sequences were aligned with ClustalW, which is a multiple sequence alignment method (Thompson et al., 1994). The sequences were analyzed and BLAST-searched against GenBank for the identification of the closest presented reference sequences in the NCBI nucleotide collection (http://blast.ncbi.nlm.nih.gov/Blast). The phylogenetic analyses of the sequences and reference sequences available in GenBank were performed using the MEGA 7 software (Kumar et al., 2016). A neighbor-joining tree was constructed using Tamura & Nei's (1993) model with 1000 bootstrap replicates. The sequence of *Trichogramma evanescens* Westwood, 1833 (Hymenoptera: Trichogrammatidae) was included as the out group to root the phylogenetic tree.

Results and Discussion

The adult parasitoid species were determined in field-collected drosophilid specimens from various fruit samples. In the present study, four drosophilid parasitoid species were collected from Bolu and Düzce and identified as *L. boulardi* and *L. heterotoma*, and *P. vindemmiae* and *T. drosophilae*, which are new to the fauna of Turkey (Figure 1). Both pupal parasitoids are usually solitary; *P. vindamiae* is ectophagous but *T. drosophilae* is endophagous (Legner et al., 1976). Four parasitoid species were determined from the field-collected fruit samples from Düzce: *L. boulardi* from strawberry, *P. vindemmiae* from wild blackberry in 2016, and *L. heterotoma* and *T. drosophilae* from pear in 2017 (Figure 1). *Leptopilina heterotoma* from a yellow plum in 2017 and *P. vindemmiae* from apple in 2016 in Bolu. While the laboratory assays confirmed that *P. vindemmiae* and *T. drosophilae* successfully parasitized *D. suzukii* pupae, there was no evidence that the growth of the larval parasitoid, *L. heterotoma*, was associated with *D. suzukii* in the laboratory or field.

Leptopilina species are common parasitoids of Drosophilidae throughout the world (Allemand et al., 2002). *Leptopilina* individuals are solitary koinobiont endoparasitoid that attacks a single host that continues feeding and growing during parasitism (van Noort et al., 2015; Harvey et al., 2016). The typical *Leptopilina* are often superficially similar to *Ganaspis*. *Leptopilina* males have the third and fourth antennomeres slightly curved and have a distinct hair tuft on the metapleural corner (van Noort et al., 2015). *Leptopilina heterotoma* and *L. boulardi* are the most known parasitoids in Africa, Asia, Europe and North America, attack drosophilid hosts, especially *D. melanogaster*, but neither developed from the immune resistance of SWDs under laboratory conditions (Chabert et al., 2012; Poyet et al., 2013; van Noort et al., 2015). However, *T. drosophilae* and *P. vindemmiae* attack the pupae of *D. suzukii* and can effectively parasitize in the laboratory (Kaçar et al., 2017; Rossi Stacconi et al., 2017). *Pachycrepoideus vindemmiae* was reported that the generalist solitary ectoparasitoid which attacked more than 60 fly species (Carton et al., 1986; Hanson & Gauld, 1995; Wang & Messing, 2004). The parasitism rate of *P. vindemmiae* was found over 80% in raspberries in laboratory tests (Chabert et al., 2012; Gabarra et al., 2015). *Pachycrepoideus vindemmiae* was also highly reproduced in *D. suzukii* in the laboratory (Rossi Stacconi et al., 2015). *Trichopria drosophilae* is an idiobiont endoparasitoid specialized in fruit drosophilids (Carton et al., 1986; Chabert et al., 2012; Wang et al., 2016). Of the parasitoids, *T. drosophilae* has been reported several times in the USA and Europe and proven to attack either *D. melanogaster* or *D. suzukii* (Gabarra et al., 2015;

Rossi Stacconi et al., 2015; Mazzetto et al., 2016). Currently, this species is considered to be one of the best candidate parasitoids for the biological control of *D. suzukii.* Field trials in Italy showed that *T. drosophilae* is widely spread all over the country and presents as the promising candidate for the augmentative control of *D. suzukii* in fields (Mazzetto et al., 2016; Rossi Stacconi et al., 2018).

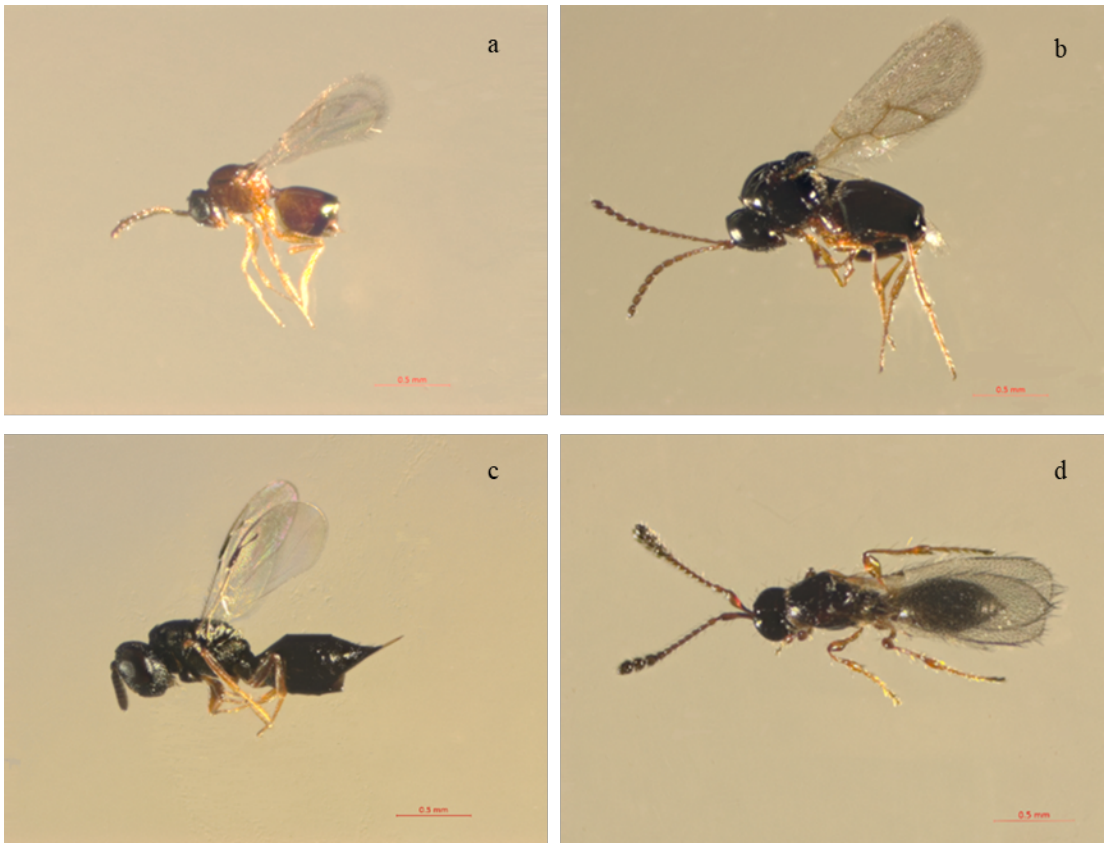

Figure 1. a) *Leptopilina boulardi* Barbotin, Carton & Keiner-Pillault ♀; b) *Leptopilina heterotoma* Thomson ♂; c) *Pachycrepoideus vindemmiae* (Rondani) ♀; d) *Trichopria drosophilae* Perkins ♀.

The PCR amplification of the COI loci of the mtDNA of the representative parasitoid samples produced different lengths of DNA fragments, ranging from 633 to 658 bp. The BLASTn queries based on the COI of the samples showed that the sequences were 99-100% identical to those of the corresponding species in the database of GenBank. There were two sequences of *Trichopria* sp. matching the current sequence at 641/642 sequence identity. The sequences data were deposited in the GenBank with the accession numbers of MK798163, MK813907, MK798164 and MK798165 for the *T. drosophilae, P. vindemmiae, L. boulardi*, and *L. heterotoma* samples, respectively. Morphologically cryptic species are known in *L. heterotoma* (Novkovic et al., 2011). The phylogenetic tree was included these cryptic species. Dzc06 was on a branch with a clone from Sapporo, Japan (AB583568). Phylogenetic analyses based on the COI sequences of the samples obtained from this study and the reference sequences of the corresponding species found in the GenBank indicated that the samples belonging to the same species were clearly separated from each other and *T. evanescens* with a bootstrap support of 100% (Figure 2).

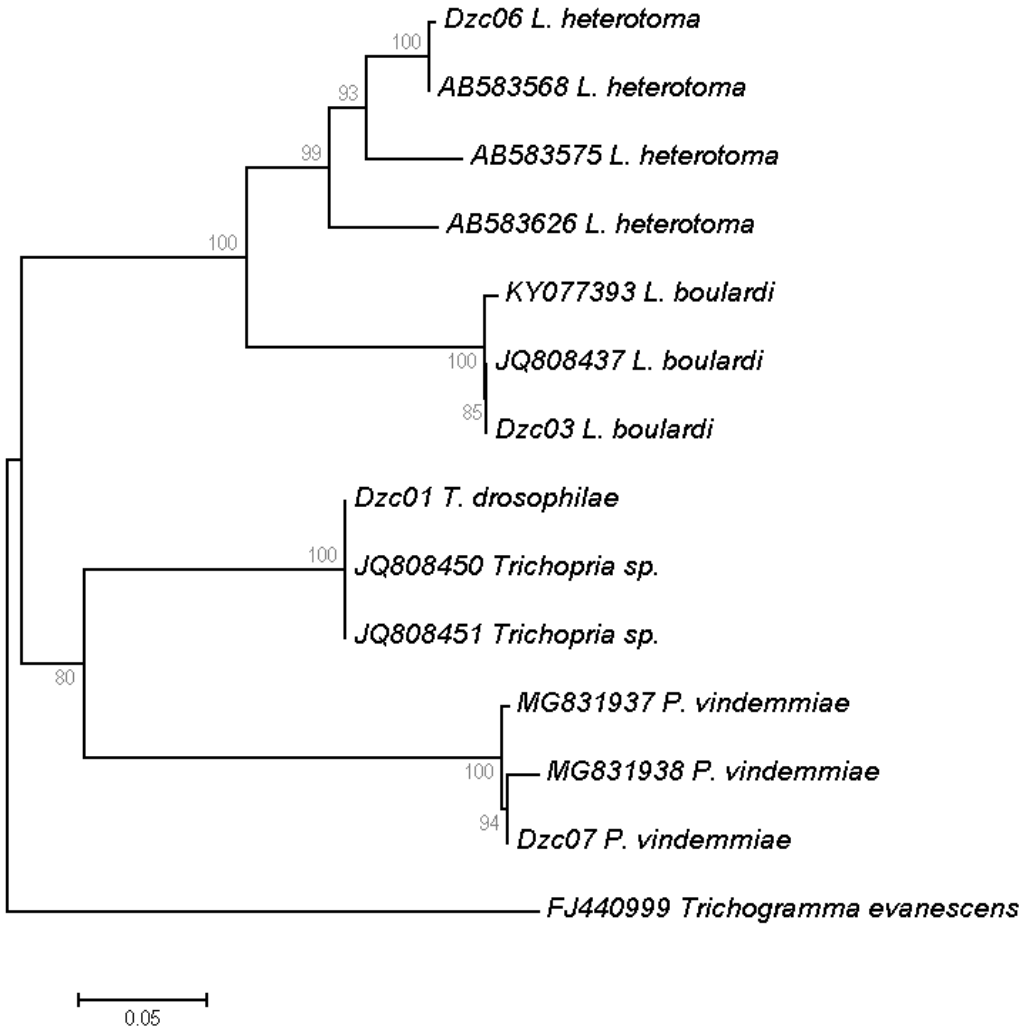

Figure 2. Phylogeny of the parasitoids associated with *Trichogramma evanescens* Westwood based on the COI locus (neighbor-joining). The bootstrap values (percentage, based on 1000 replicates) are shown on the branches.

Drosophila suzukii is a new quarantine fly pest attacking all types of fruit throughout the world. This fly is one of the serious economic pests that especially damage soft fruit. The native parasitoids of *D. suzukii* significant contribute to its management worldwide, but they are not fully known in Turkey. In the current study, two drosophilid parasitoids were determined in geographical areas that had not been previously surveyed. In this study, rather than morphological identification, the molecular diagnostic method was used for the drosophilid parasitoid species of *D. suzukii* since it is a faster and easier alternative identification technique. The pupae of *D. suzukii* were successfully parasitized by *P. vindemmiae* and *T. drosophilae* according to the laboratory experiments. However, further investigations are needed to offer more definite results related to larval parasitoids.

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