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***Orijinal araştırma* (*Original article*)**

Genetic diversity in some *Lysiphlebus confusus* (Hymenoptera: Braconidae) populations

Gül SATAR[1]*, Petek TOKLU[1]

Bazı *Lysiphlebus confusus* (Hymenoptera: Braconidae) popülasyonlarında genetik çeşitlilik

Öz: *Lysiphlebus confusus* Tremblay & Eady (Hymenoptera: Braconidae), geniş bir konukçu dizisine sahip önemli bir yaprakbiti parazitoitidir. Bu çalışmada *L. confusus*'taki genetik çeşitlilik, Adana ve Hatay illerinde yaprakbiti türü, konukçu bitki ve yükseklik açısından PCR-RFLP yöntemi kullanılarak incelenmiştir, böylece *L. confusus*'un biyolojik mücadelede daha etkin kullanılabilmesi için yeni bulgular elde edilmiştir. PCR-RFLP yöntemi ile SacI, AccI, HpyCH4III ve EcoP151 enzimlerinin 709 bp mitokondriyal COI gen bölgesinde enzim kesim noktaları saptanmıştır. Gen dizileri farklı ülkelerden (Sırbistan, Çin, Fransa ve İran) örnekler ile karşılaştırılmış ve net sapmaları belirlenmiştir. Balcalı (11) ve Ceyhan (4) örnekleri İran örneğine yakın bulunurken, Erzin (5) örneği Sırp ve diğer bir İran örneğine yakın bulunmuştur. Seyhan'dan *Cucumis melo* L, Gülek (Pozantı)'den *Solanum nigrum* L., Saimbeyli'den *Citrullus lanatus* (Tunb.), Ceyhan'dan *S. nigrum* ve Balcalı'dan *Vicia sativa* L. örnekleri, PCR-RFLP ile sırasıyla SacI, HpyCH4III, EcoP151 ve EcoP151 ile AccI enzimleri tarafından sırayla kesildiği belirlenmiştir. Yükseklik, konukçu yaprakbiti ve bitki türlerine bağlı spesifik genetik çeşitlilik saptanmamıştır.

Anahtar sözcükler: Genetik çeşitlilik, Braconidae, PCR-RFLP, biyolojik kontrol, filogenetik.

Abstract: *Lysiphlebus confusus* Tremblay & Eady (Hymenoptera: Braconidae: Aphidiinae) is an important aphid parasitoid that has a wide host range. Genetic diversity in *L. confusus* was studied by the PCR-RFLP method in terms of host aphid, host plant, and altitude in Adana and Hatay Provinces, Turkey in this research. Thus, new data was obtained to use more efficient of *L. confusus* which is common parasitoid in the Mediterranean Region at biological control. Restriction sites of SacI, AccI, HpyCH4III, and EcoP151 enzymes on the 709 bp mitochondrial COI gene region was detected by PCR-RFLP method. The sequences were compared with specimens from other countries (Serbia, China, France and Iran) and their net divergence were determined. While the Balcalı (11) and Ceyhan (4) populations were close to the Iran specimen, Erzin (5) was close to the Serbian and other Iran specimens. The specimens of *Cucumis melo* L. from Seyhan, *Solanum nigrum* L. from Gülek (Pozantı), *Citrullus lanatus* (Tunb.) from Saimbeyli, *S. nigrum* from Ceyhan, and *Vicia sativa* L. from Balcalı were digested with SacI, HpyCH4III, EcoP151, and both EcoP151 and AccI by PCR-RFLP, respectively. Specific genetic diversity related to altitude, host aphid and plant species was not determined.

Key words: Genetic diversity, Braconidae, PCR-RFLP, biological control, phylogenetic.

[1]Çukurova University Biotechnology Research and Application Center, Adana, TURKEY
*Sorumlu yazar (Corresponding author) e-mail: satarg@cu.edu.tr
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Introduction

Intraspecific identification of a parasitoid on a host from different habitats and locations is necessary to reveal ecological end evolutionary processes between host and parasitoid (Tilmon et al. 2000). Genetic variation is important for the parasitoid to successfully parasitize its hosts (Henter & Via 1995; Carius et al. 2001). Furthermore, genetic diversity within a natural enemy species is essential for climatic tolerance, exploitation of prey, habitat variation and synchrony with hosts (Crowder & Jabbour 2014). In order to increase the chances of success in biological control, controlling the pest species with the right suite of natural enemies is essential. However, an imported biological control agent in a new area sometimes cannot control its target species (Hopper & Powell 1993). Sometimes, both aphids and their parasitoids are not correctly described, and that is a barrier to be resolved for successful biological control (Vanlerberghe-Masutti & Chavigny 1998; Hufbauer et al. 2004; Satar et al. 2013). For instance, *Lysiphlebia japonica* (Ashmead) (Hymenoptera: Aphididae), exported to control *Aphis spiraecola* Patch (Hemiptera: Aphididae) from Japan, could not able to settle into Eastern Mediterranean Region, because of probably wrong strain (Satar & Uygun 2011). However, it successfully parasitized this aphid species in North America (Deng & Tsai 1998). While the walnut aphid parasitoid *Trioxys pallidus* (Haliday) (Hymenoptera:Aphidiidae) strain from Persian region managed to control the aphid in California, France strain could not be able to control (Van den Bosch et al. 1970).

Lysiphlebus confusus belongs to the Aphidinae subfamily and is present in all Mediterranean regions (Satar et al. 2009; 2013) and has been reported from Diyarbakir (Ölmez & Ulusoy 2003), Ankara (Güz & Kılınçer 2005), Kahramanmaraş (Aslan et al. 2004) and many other cities in Turkey. It attacks more than 30 aphid species, including *Aphis gossypii* Glover, *A. fabae* (Scopoli) and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), which are important pests on cultured and uncultured plants; it has been reported on more than 20 host plants that included citrus, ornamental plants, different weeds, and cotton in the Mediterranean and Aegean Regions of Turkey (Satar et al. 2009; 2013). The distribution, host aphids and plant range of the parasitoid may increase its genetic variation.

In that context, the importance of the molecular methods is increasing. Molecular markers provide new characters for the study of phylogenetic relatedness, identification of cryptic species and biotypes, and assessment of heritable variation for population genetics and ecological investigations. Thus, it can be valuable insights and organizing principles for selecting natural enemies (Unruh & Woolley 1999). Two of the most targeted regions in insect systematic and population genetic studies are mitochondrial and ribosomal DNA genes (Mandal et al. 2014). Although specific PCR is very powerful, often enabling detection of just a single base-pair difference, it is not always possible to adjust PCR conditions to enable discrimination of all species of interest. For this reason, the RFLP method refers digestion of the PCR product with a restriction enzyme can be a good alternative (Greenstone 2006). It is applied for the detection of intraspecies as well as interspecies variation. On the

other hand, the method is inexpensive, easy to design, applicable to analysis of single nucleotide polymorphisms, no requirement for expensive instruments, and for extensive training of laboratory staff (Rasmussen 2012).

This study aimed to better understand the genetic variation of *L. confusus* in order to help develop better biological control programs via the selection of the best strain for a particular host/plant environment.

Materials and methods

Collection the *Lysiphlebus confusus* specimens and their identification

The *L. confusus* specimens from Adana and Hatay provinces were selected to survey the genetic diversity on the basis of the different plant, aphid species, and altitude. The plants that had parasitized aphids were collected and cultured in paper bags in 2015. The collecting date, location, and host plants were recorded. In the laboratory, a glass tube was attached to each bag to monitor parasitoid emergence with daily observation. A total of 42 specimens representing 21 specimens emerged from parasitized aphids belong to different species in the laboratory collected on various hosts plant was analyzed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (Table 1). The specimens identified as *L. confusus* were stored at -80 °C until DNA extraction.

DNA extraction

Genomic DNA was extracted from the one parasitoid specimen with a DNeasy Tissue Kit (QIAGEN, Germany), according to the manufacturer's instructions. Both DNA quality and quantity were measured with a microplate reader (Multiscan GO, Thermo, USA) and stored at -20 ºC.

PCR amplification

PCR reactions of specimens 4, 5, and 11 were set with primers from the COI gene region. These three specimens collected on different plants and aphid species were chosen for sequencing to increase the potential for the detection of genetic diversity and for select restriction enzymes (Table 1). The bare coding primer pairs, LCO1490: GGTCAACAAATCATAAAGATATTGG and HC02198: TAAACTTC AGGGTGACCAAAAAATCA, were used for the applications (Folmer et al. 1994). The reaction mixture was prepared to achieve a final volume of 25 µl with the inclusion of Taq buffer (10X), 2,5 mM $MgCI_2$, 250 µM dNTPs, 1 µM primer, 0,5 U Taq and 5 µl DNA template. The thermocycler were set as 5 min at 94°C for pre-denaturation, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final period of 72°C for 7 min. The PCR products were run on 2% agarose gel, stained with ethidium bromide, and viewed with gel imaging system (Versa Doc 4000MP-Biorad, USA).

PCR product belong to specimen 4 was cloned to NEB 10-beta Competent *Esceria coli* to be sequenced. For this purpose, DNA extraction from the gel was

done according to the Qiagen gel purification instructions. The PCR product was then inserted into the vector E1202S (NEB, USA) and cloned into *E. coli*. For plasmid purification, a Wizard Plus SV Minipreps DNA purification kit (Promega, USA) was used. The plasmids were digested with Eco*RI* to confirm insertion of the amplified DNA.

The PCR reactions for the other collected parasitoid specimens were performed as the methodology described above (Table 1).

Table 1. The collection date, location, host plant and aphid species of *Lysiphlebus confusus* specimens in Adana, Hatay Provinces, Turkey.

No	Date	District	Altitude (m)	Host Plant	Host aphid species
1	10.05.07	Yüreğir	28	*Malva grandifolia* C. Moren	*Aphis gossypii*
2	23.05.07	Yüreğir	28	*Citrus reticulata* Blanco	*A.gossypii*
3	23.05.07	Yüreğir	28	*Solanum nigrum* L.	*Aphis fabae solanella*
4	29.11.07	Ceyhan	50	*S. nigrum*	*A. fabae subsp. sollanella* Theobald
5*	17.01.08	Erzin	165	*Capsella bursa pastoris* L.	*Rhopalosiphum padi* L.
6	21.03.08	Seyhan	23	*C. bursa pastoris*	*Myzus persicae* (Sulzer)
7	13.05.08	Yüreğir	25	*Citrus limon* L.	*A. gossypii*
8	07.05.08	Kadirli		*Citrus sinensis* L.	*A. gossypii*
9	13.05.08	Karataş		*C. sinensis*	*A. gossypii*
10	10.05.09	Yüreğir	25	*Vicia sativa* L.	*Aphis craccivora* Koch
11	10.05.09	Balcalı	127	*V. sativa*	*A. craccivora*
12	6.05.15	Karataş	14	*Punica granatum* L.	**
13	6.05.15	Yumurtalık	18	*Malva sylvestris* Zebrina	*A. gossypii*
14	6.05.15	Ceyhan	29	*Sinapis arvensis* L.	*Aphis nasturtii* (Kaltenbach)
15	12.05.15	Kozan		*V. sativa*	*Aphis fabae* Scopoli
16	21.05.15	Karaisalı		*Chenopodium album* L.	**
17	17.06.15	Seyhan	23	*Cucumis melo* L.	*A. gossypii*
18	21.05.15	Karaisalı	105	*S. arvensis*	*Brevicoryne brassicae* (L.)
19	03.09.15	Gülek	1020	*S. nigrum*	*A. fabae subsp. sollanella*
20	06.10.15	Saimbeyli	1050	*Phaseolus vulgaris* L.	*A. craccivora*
21	06.10.15	Saimbeyli	1050	*Citrullus lanatus* (Thunb.)	*A. gossypii*

*Hatay population

**The specimens had inadequate morphological character for identification

Sequencing

The specimen 4 with the primer which bind to plasmid from the E1202S (NEB) kit, and specimens 5 and 11 with LCO1490 and HC02198 primers, respectively, were sequenced using an ABI 3131xl machine. A QIA quick PCR Purification Kit was used to clean up the PCR products. Cycle-Sequencing PCR reactions were set after purification with a BigDye® Terminator v3.1 Cycle Sequencing Kit. The PCR product was cleaned up with the BigDye XTerminator® Purification Kit and then sequenced.

Phylogenetic Analysis

The evolutionary history was inferred using the Maximum Parsimony (MP) method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 60% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). The MP tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm (Nei & Kumar 2000). The analysis involved 26 nucleotide sequences. All positions with less than 5% site coverage were eliminated. That is, fewer than 95% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 365 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura et al. 2013). *Lysiphlebus confusus* specimens from France, China, Iran, Serbia, Switzerland, and *L. fabarum* and *L. testaceipes* specimens were compared with 4, 5, and 11. *Bemisia tabaci* (Gennadius) (Hemiptera:Aleyrodidae) and *A. gossypii* were selected as outgroup.

Selection of relevant restriction enzymes

The sequences (4, 5, and 11) were compared with homologous sequences retrieved from Genebank. The selection of the relevant restriction enzymes was based on 709 bp mtCOI sequences deriving from 4, 5, and 11 specimens collected on following insect hosts *A. fabae subsp. sollanella*, *R. padi*, and *A. craccivora* taken from following host plants *S. nigrum*, *C. bursa pastoris*, and *V. sativa*, respectively. Firstly, the sequences were translated to the Fasta format and then transferred to the Mega 6 program to make the comparisons. Editing of sequences was then done on the Finch TV program. The data was further processed with the GeneDoc program. The NEB website (http://nc2.neb.com/NEBcutter2/) was used to determine the enzyme restriction sites of three sequenced specimens. These restriction sites for each enzyme were compared manually. Based on the size of bands is going to produce at agarose gel electrophoresis, three enzymes, namely AccI, HpyCH4III,

EcoP15I, were selected to distinguish the specimens of interest; SacI digests the PCR product of three specimens at the same size was used as positive control.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

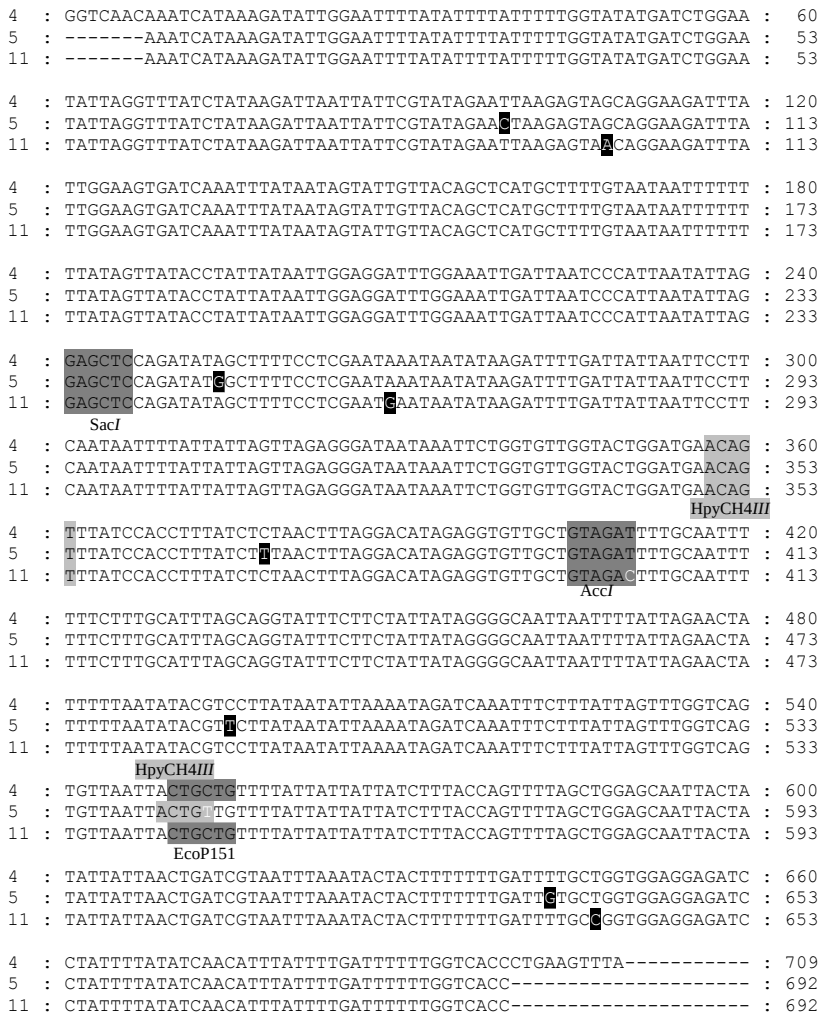
The restriction enzymes (NEB) identified after sequencing were used to determine the extent of intra-species genetic diversity shown by the PCR-RFLP studies. Enzyme digestion with AccI, HpyCH4III, EcoP15I, and SacI were done with 12.5 µl of the PCR product, contain 0.25 µl enzyme, 1.25 µl Buffer, 3 µl DNA, in a thermocycler at one hour at 37ºC and 20 minute at 65 ºC, according to the manufacturer's instructions. Digested specimens were checked on agarose gel (1%) and scanned on the gel imaging system.

Results and discussion

The genetic diversity was examined in the *L. confusus* specimens collected from different plants, aphid species and altitudes in Adana and Hatay Provinces, Turkey by using PCR- RFLP and sequencing methods. Specimens 4, 5, and 11 (Fig. 1, Table 1) yielded 709 bp of DNA fragments after sequencing and were compared with other sequences on the NCBI web site. The specimens showed99 % similarity with other *L. confusus* populations in the database. The phylogenetic analyses showed that specimens 4 and 11 were branched and had a higher bootstrap value than specimen 5 and specimens from other countries. This difference may lead to *L. confusus* to evolving into new species or subspecies in the future. The six genetically divergent populations in the present study, even with narrow population ranges, have this inherent potential. However, the specimen from Iran, a neighbor of Turkey, grouped with specimens 4 and 11, possibly because of their close location to each other. Specimen 5 is close to Serbia, and other Iran specimens, and *L. fabarum* reference genes, because of generally being found *L. confusus* and *L. fabarum* as group in nature (Fig. 2). *L. confusus* can be defined as the *L. confusus* group (Laamari et al. 2012; Rahimi et al. 2012) and separation is not easy by marker system (Tomanovic et al. 2018). The results show that our COI marker is also inadequate to distinguish *L. confusus* and *L. fabarum* from each other.

The AccI, HpyCH4III, EcoP15I and SacI enzymes were chosen for PCR-RFLP studies after comparison of the enzyme restriction site of the specimens (Fig. 1). The specimens collected on the different plants and aphid species showed some basic variations, especially specimen 5 (Fig. 1). All specimens, except 4, 5, and 11, were cut with AccI, HpyCH4III, EcoP15I and SacI by using the PCR-RFLP method (Table 1). Sac*I* was chosen as the positive control because it produced 250 and 459 bp fragments for all PCR specimens (Fig.1), but this enzyme did not digest specimen 17 (Fig. 3). Therefore, this result showed that the degree of genetic diversity was higher than might be expected.

Figure 1. Compression of enzyme cut site of 4, 5 and 11 numbered specimens of *Lysiphlebus confusus* (black color (▉) shows base differences, gray color (▒) shows enzyme cut sites)



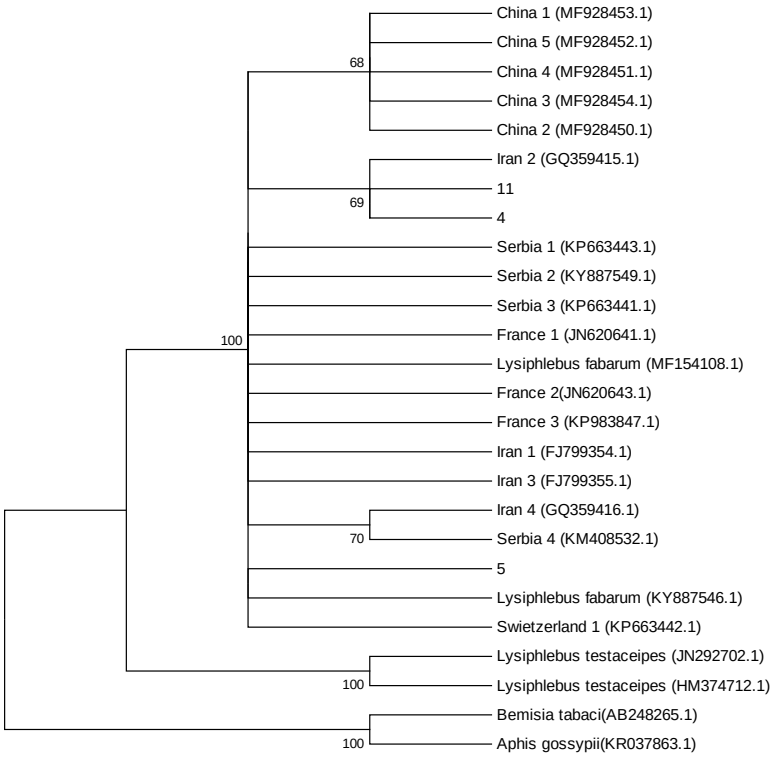


Figure 2. Phylogenetic relationships of *Lysiphlebus confusus* from Adana, Hatay Provinces (specimens 4, 5, 11) and reference genes from different countries from the NCBI by using the Neighbors joining method (Bootstrap 2000).

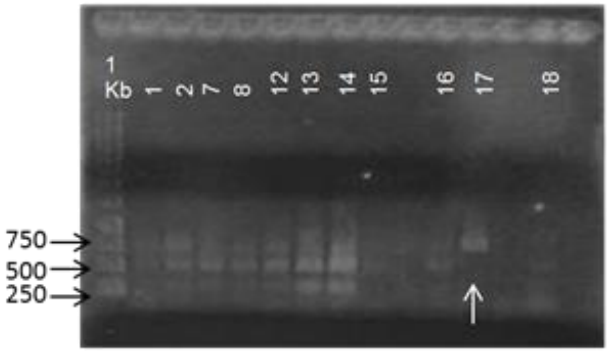


Figure 3. Sac I enzyme cutting site on the COI gene of *Lysiphlebus confusus*.

The second enzyme, HpyCh4III, had two different variations among the populations. Specimens 5 and 11 gave two different fragments (342 and 367 bp) and specimen 4 gave three different fragments (150, 192 and 367 bp), based on the sequencing results (Fig. 1). The bands on the gel were around 400 and 150 bp for all specimens (150 and 192 bp bands were not separated) like specimen 4 (Fig. 4). The gel showed that this enzyme did not digest specimen 19 differently from the other.

When the enzyme EcoPI5I was evaluated, it gave two bands of 176 and 533 bp (5, 11) (Fig. 1), only specimen 21 had a weak band around 500 bp and the other specimens were undigested by this enzyme (Figs. 1, 5). The enzyme AccI digested specimen 11 into 292 and 417 bp sizes but it failed to differentiate the other specimens.

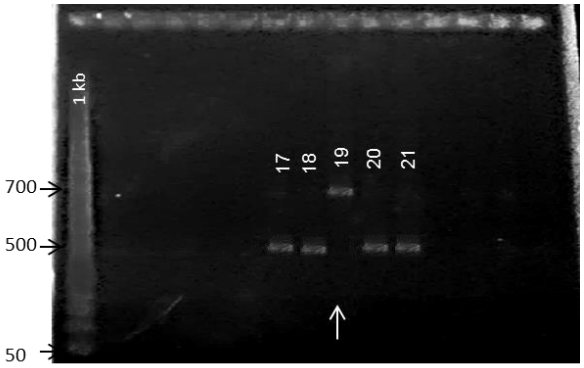


Figure 4 HpyCH4III c (a, b) enzyme cutting site on the COI gene of *Lysiphlebus confusus.*

The specimens that were collected on *C. melo* from Seyhan (17), *S. nigrum* from Gülek (Pozantı) (19), and Ceyhan (4), *C. lanatus* from Saimbeyli (21), and *V. sativa* from Balcalı (11), were genetically divergent with AccI, SacI, HpyCH4III, and EcoP151, respectively. Two of the variations (19, 21) were identified from the plateau region (Saimbeyli (1050 m), Gülek (1020 m)) and others (4, 11, and 17) from

the plains region (Seyhan 23 m, Ceyhan 50 m, Balcalı 127 m). When the specimens were evaluated in terms of host aphids, the genetically divergent specimens were detected on *A. gossypii* (17, 21), *A. fabae subsp. sollanella* Theobald (4, 19), and *A. craccivora* Koch (11). These aphid species were also collected in other specimens and no species specificity was detected (Table 1).

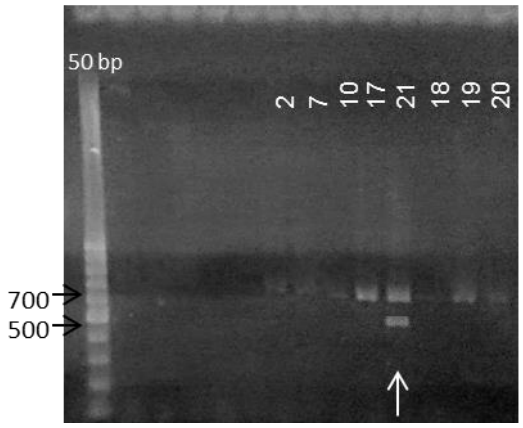


Figure 5. EcoP15I enzyme cutting site on the COI gene of *Lysiphlebus confusus*.

Molecular tools are an important and easy way to define inter and intraspecific variation of the species. In this study, we investigated genetic variation of *L. confusus* to keep the light for better biological control of aphids. Differences across the parasitoid populations could be attributable to their being from different locations, host plants and aphid species (Starý et al. 2014; Derocles et al. 2011). The variations were detected for different aphid, altitude, and host plant among *L. confusus* populations, but they were not correlated with these parameters. However, this study is the first record revealing genetic diversity among aphid parasitoids by using the PCR-RFLP method. Vaughn & Antolin (1998) detected genetic diversity on a small spatial scale in *Diaeretiella rapae* (Hymenoptera: Braconidae) through the use of the RAPD-PCR technique. *Lysiphlebus testaceipes* was collected on oleander and citrus and *L. fabarum* was shown to be heterozygous by using microsatellite markers (Fauvergue et al. 2005; Sandrock et al. 2007).

In conclusion, to generate more data, it would be useful to carry out this study with more enzymes. For more precise results, complete sequencing of all specimens may produce clearer data for the differentiation of genetic diversity in the COI gene region and population genetic analysis of *L. confusus*. Furthermore, the fitness cost for the aphid and the encapsulation rate of the parasitoid should be investigated to better understand the relationship.

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