

The use of RAPD-PCR analysis in characterization of *Liriomyza trifolii* (Burgess, 1880), *Liriomyza congesta* (Becker 1903), *Agromyza apfelbecki* Strobl, 1902 and *Chromatomyia horticola* (Goureau, 1851) species collected from Turkey*

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Summary

The *Liriomyza trifolii* (Burgess, 1880), *Liriomyza congesta* (Becker, 1903), *Agromyza apfelbecki* Strobl, 1902 and *Chromatomyia horticola* (Goureau, 1851) species have become important vegetable pests in the agricultural areas in Turkey. These species are not easily differentiated by their morphological characteristics. The aim of the present study was to obtain genetic markers to unambiguously distinguish these species and gain insight into genetic variation between the individuals of *L. trifolii* strains collected from various geographic locations in Turkey. Thus, four random primers were employed to generate RAPD markers. Different RAPD profiles were observed for the different species indicating that the RAPD-PCR analysis can be applied as a useful tool in quickly screening the strains to aid in discriminating these species that was routinely done via classical methods. Identification of biotypes of the species is crucial in order to designing control strategies to avoid the spread of the pests because of economic losses caused by the damages to vegetable crops. Our results show that RAPD is promisingly an effective, fast and economic way, hence proposed as a valuable alternative to traditional identification of the insect species and strains.

Key words: Agromyzidae, leafminer, molecular identification, RAPD

Anahtar sözcükler: Agromyzidae, galerisineği, moleküler teşhis, RAPD

* This study was supported by TUBITAK, 106T122 project.

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Alınış (Received): 20.02.2007

Introduction

Agromyzidae (leaf mining flies) is one of the largest fly families, with more than 2742 valid species belonging to 27 genera worldwide (Spencer, 1990). From this family, about 1165 species were identified in the Palearctic region (Scheirs et al., 1999). Adults can be minute, with wing length of little more than 1 mm. The maximum size known is 6.5 mm. The majority of species are in the range of 2 to 3 mm. There is a high degree of host specificity (Spencer, 1989). Agromyzids are typically phytophagous as their larvae live in tissues of living plants. Larvae of most leaf miners feed with the leaf parenchyma. Most species are miners in leaves where they produce a characteristic form of mine, in most of the cases a substantial aid in identifying the agromyzid. Some species are stem-borers or develop in roots, seeds or galls. One genus develops exclusively in the cambium of young and old trees. Most species are monophagous, a considerable number are oligophagous, and while very few are truly polyphagous (Spencer, 1972). Common characteristic of 150 species are known as feeding regularly on cultivated plants. Normally, most of these species do not reach high population levels, but occasional outbreaks can occur. Some species are serious pests of cultivated plants such as *Liriomyza* spp. (Spencer, 1973; Cerny et al., 2001). *Liriomyza* is a cosmopolitan group of pests that consists of more than 300 species. Larvae of this genus are polyphagous, attacking ornamental and vegetable crops in the families of Asteraceae, Brassicaceae, Cucurbitaceae, Fabaceae, Solanaceae, and many other families of plants. Infestation by *Liriomyza* spp. can cause both direct and indirect damages (Musgrave et al., 1975; Minkenberg & Van Lenteren, 1986). Direct damage given by larval feeding on palisade parenchyma tissue can reduce the photosynthetic capacity of the plant up to 62 % (Johnson et al., 1983) and several infested leaves may fall. Indirect injury occurs when both adult males and adult females feed. Also when females lay eggs, they may act as vectors for the disease (Zitter & Tsai, 1977; Matteoni & Broadbent, 1988).

Agromyzidae is one of the most important fly families in Turkey, because of their pest status especially on vegetable and ornamental plants in the greenhouses. The Turkish agromyzid fauna is poorly known. Until now, only 113 species have been identified in Turkey (Giray, 1980; Uygun et al., 1995; Deeming & Civelek, 1997; Civelek & Demirkan, 1998; Campobasso et al., 1999; Civelek et al., 2000; Civelek 2002, 2003, 2004; Cikman & Civelek, 2005; Mart et al., 2005; Cerny & Merz, 2006; Civelek et al., 2007).

The studies performed during the last 10 years have yielded new information regarding the detrimental effects of the insects especially on the agricultural economy. It has been reported that *Liriomyza huidobrensis* (Blanchard, 1926), *Liriomyza sativae* (Blanchard, 1938), *Liriomyza strigata* (Meigen, 1830), *Liriomyza trifolii* (Burgess, 1880), *Agromyza apfelbecki* Strobl, 1902, *Agromyza frontella*

(Rondani, 1875), ***Agromyza rondensis*** (Strobl, 1900) and ***Chromatomyia horticola*** (Goureaux, 1851) cause damages on numerous crops. Therefore, identification of these strains is crucial in order to design strategies to prevent the harmful effects on the economy.

Species identification for the Agromyzidae family is routinely done by inspecting male genital organs. However, it may possible that molecular methods such as RAPD can be potentially applied. The advantage of using molecular methods is that the genotypic characters are the basis for classification and any form of the organism during the life cycle can be used as a source of genetic material. A study using PCR-restriction fragment length polymorphism (PCR-RFLP) method was performed by Scheffer et al. (2001) in order to identify the organism at the species level. Other molecular studies involving the Agromyzidae family have also been reported (Scheffer, 2000; Scheffer & Wiegmann, 2000; Scheffer & Lewis, 2001; Scheffer et al., 2001; Kox et al., 2005).

To date, there are 113 species of the Agromyzidae family reported in Turkey. To our knowledge, no molecular studies have been carried out on the species of ***L. trifolii***, ***Liriomyza congesta*** (Becker, 1903), ***A. apfelbecki*** and ***C. horticola*** collected from Turkey. Therefore, this study is the first in characterizing these species in that regard and could be a stepping stone for assessing the biodiversity and determining the insecticide resistance of the insect species at hand. Specifically, in this study, the random amplified polymorphic DNA (RAPD) approach has been applied in order to assess the possibility in distinguishing some Agromyzidae species more rapidly.

Materials and Methods

Agromyzidae specimens and identifications

This study was carried out during 2006 in some provinces of Anatolian part (Mugla, Aydın, Erzurum and Antalya) of Turkey. Mugla, Aydın and Antalya are all located in the west with relatively close proximity to each other. Erzurum is located in the east region of Anatolia. ***L. trifolii***, ***L. congesta***, ***A. apfelbecki*** ve ***C. horticola*** specimens were collected from both cultured and non-cultured plants during 6 months in 2006 (Table 1). The adults were obtained by sweeping. Since the male genitalia are important characters for identification of leaf miners, slide preparations were made. The following general procedures were applied: The abdomen of each male was boiled in 10 % KOH, transferred into 5 % glacial acetic acid for 5 minutes and subsequently transferred to 96 % alcohol for 5 minutes. Then, the abdomen was further dissected under a stereoscopic microscope. The male genitalia were transferred into euparal on a micro mount pinned under the individual specimen in order to preserve the material perpetually. Identifications of the species were made as described by Spencer (1972, 1973, 1976, 1989, 1990).

Table 1. Agromyzidae species used in this study

Locality Number	Species	Date of collection	Province	Host plant
1	<i>L. trifolii</i>	24.01.2007	Antalya/Calkaya	<i>Lycopersicon esculentum/ Phaseolus vulgaris</i>
2	<i>L. trifolii</i>	23.01.2007	Antalya/Kumluca	<i>L. esculentum/ Phaseolus vulgaris</i>
3	<i>L. trifolii</i>	30.09.2006	Mugla/Yemisendere village	Vegetable and wild plant
4	<i>L. trifolii</i>	01.10.2006	Mugla/Dokuzcam village	Vegetable and wild plant
5	<i>C. horticola</i>	18.01.2007	Mugla/Ortaca	<i>Brassica oleracea/Eruca sativa</i>
6	<i>C. horticola</i>	23.01.2007	Antalya/Finike	<i>L. esculentum/ Pisum sativum</i>
7	<i>L. sp</i>	19.01.2007	Mugla/Fethiye	<i>L. esculentum</i>
8	<i>A. apfelbecki</i>	14.12.2006	Aydın / Centre	<i>Cynara scolymus</i>
9	<i>L. congesta</i>	09.09.2006	Erzurum	<i>Medicago sativa/ Vicia sativa</i>
10	<i>A. apfelbecki</i>	14.12.2006	Aydın / Yenipazar	<i>Cynara scolymus</i>
11	<i>L. sp.</i>	30.09.2006	Mugla/Yemisendere village	<i>Cucumis melo/ Solanum nigrum</i>

Molecular Methods

DNA extraction

Specimens of *L. trifolii*, *L. congesta*, *A. apfelbecki* and *C. horticola* were stored in 70 % ethanol in dry at room temperature before DNA extraction. Genomic DNAs were isolated from pupae or adults by using Lifton method (Bender et al., 1983). This method briefly includes the following steps: Individual flies were homogenized in 500 µl Lifton solution (0.1 M Tris-HCl, 0.05 M EDTA (pH=9.1)) with 0.5 % sodium dodecyl sulfate (SDS) and incubated at 65 °C for 35 minutes. Then, 250 µl 0.6 M potassium acetate was added and inverted to mix and left on ice for 60 minutes. The homogenate was centrifuged at 14.000 rpm for 10 minutes at room temperature and then supernatant was removed into a new microfuge tube. 500 µl phenol was added to supernatant, inverted to mix and centrifuged at 14.000 rpm for 5 minutes at room temperature. The aqueous phase was taken and 250 µl phenol, 250 µl chloroform: isoamylalcohol (24:1) was added and centrifuged at 14.000 rpm at room temperature for 5 minutes. In the next step,

500 µl chloroform: isoamylalcohol (24:1) was added into tubes and previous step was repeated. The supernatant was taken to a new tube and 1 µl RNAase (10 mg/ml) added and incubated at 37 °C for 30 min. After this step, 500 µl of 70 % ethanol was added and centrifuged for 15 min at 14.000 rpm. The pellet was washed with 80 % ethanol. After briefly drying, DNA was resuspended in 50 µl of MQH₂O and stored at 4 °C for overnight and visualized on 1 % agarose gel. Genomic DNAs samples were diluted to 25 ng of DNA / µl.

RAPD analysis

DNA was amplified by the RAPD-PCR technique. The 20 µl reaction mixture in each tube consisted of 1,5 µl 10x reaction buffer, 1,2 µl dNTP mix, 1 Unit Taq DNA polymerase of primer, 5 µl of template DNA, 4,1 µl of sterile distilled water. All polymerase chain reaction amplification reactions were performed in an PTC-100 Programmable Thermal Controller programmed with the following program: initial denaturing step at 94 °C for 30 s, 94 °C for 25 s, annealing 35 °C at 45 s, 72 °C for 1 min, next 35 cycles until from 2 to 5 step, 72 °C for 5 m and a final extension step of 4 °C until endless. Four 10mer random primers were used for amplification (primer F04 (5'-GGTGATCAGG-3'), primer I16 (5'-TCTCCGCCT-3'), primer P06 (5'-GTGGGCTGAC-3') and primer N07 (5'-CAGCCCAGAG-3')). Amplified DNA fragments were separated in a 1% agarose TBE gel at 60 Watts, stained with ethidium bromide and were photographed under Kodak EDAS 290 High Performance UV Transilluminator.

Dendogram analysis and estimation of genetic distances

RAPD bands produced by all primers were scored for the 11 individuals. A matrix has been created by taking into account the presence (1) or absence (0) of the bands. Using this matrix, Genedist application in the PHYLIP program was used to calculate the genetic distance between every insect and all data was shown as a table. Also, JMP program was used to create the dendogram tree from the matrix.

Results and Discussion

PCR amplifications were done using as template the genomic DNA isolated from the Agromyzidae specimens. The four primers used showed some same and different profiles. Illustrative examples of the RAPD results obtained with the primers F04 (5'-GGTGATCAGG-3'), I16 (5'-TCTCCGCCT-3'), P06 (5'-GTGGGCTGAC-3') and N07 (5'-CAGCCCAGAG-3') are shown in figures 1 and 2.

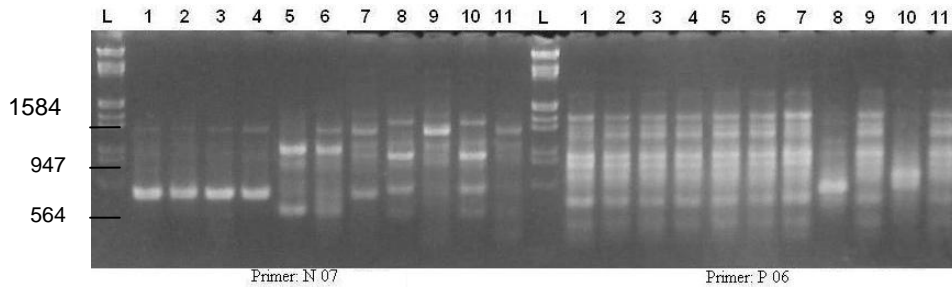


Figure 1. The RAPD profiles of the strains collected from several geographic locations in Turkey using primer N07 and P06. 1. *Liriomyza trifolii* (Burgess) 2. *L. trifolii* 3. *L. trifolii* 4. *L. trifolii* 5. *Chromatomyia horticola* (Goureau) 6. *C. horticola* 7. *L. sp.* 8. *Agromyza apfelbecki* Strobl 9. *Liriomyza congesta* (Becker) 10. *A. apfelbecki* 11. *L. sp.* “L” on the figure indicates ladder (molecular size markers) and numbers indicate the sizes in base pairs (bp). Each lane was numbered from 1 to 11, which corresponds to the name of the species given on Table 1. Primer names (N07 and P06) are shown below the gel picture.

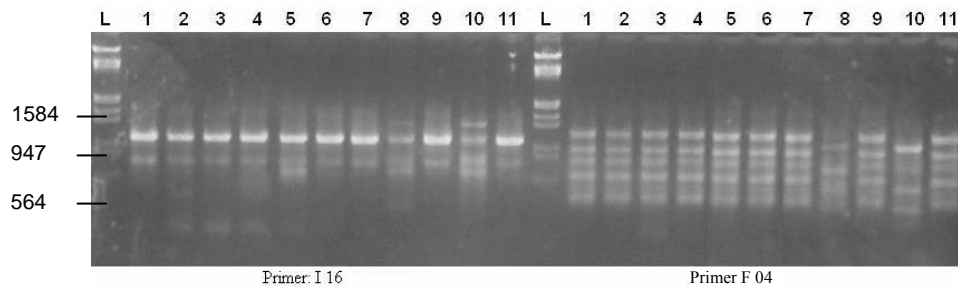


Figure 2. The RAPD profiles of the strains using primer I16 and F04. 1. *Liriomyza trifoliii*, (Burgess) 2. *L. trifolii* 3. *L. trifolii*, 4. *L. trifolii*, 5. *Chromatomyia horticola* (Goureau), 6. *C. horticola*, 7. *L. sp.* 8. *Agromyza apfelbecki* Strobl, 9. *Liriomyza congesta*, (Becker) 10. *A. apfelbecki*, 11. *L. sp.* Numbers and letters are as explained in the legend of figure 1. Primers used were I16 and F04.

The most striking result was produced when the primer N07 was used. The RAPD bands created by the primer N07 indicate that this primer could discriminate *L. trifolii* species from others. As seen in Figure 1, the RAPD band for the first four lanes (*L. trifolii*) created a PCR band around 500 kb, whereas the same band was not detected in the other lanes in which the PCR products obtained from the genomic DNA of *L. congesta*, *C. horticola* and *A. apfelbecki* were loaded. This primer, therefore, is a good candidate in quickly screening the strains explained here and can be used as a molecular tool in helping identify these insects.

In terms of comparing the individuals of the same species, *L. trifolii* collected from Mugla and Antalya regions showed the same RAPD profiles suggesting the low degree of polymorphism. This was expected because these regions are geographically and ecologically similar. More individuals collected from

different locations and more primers are needed to study the polymorphism within a species.

The primer F04 produced the fragments that share the same pattern in all of the species except for *A. apfelbecki*. The DNA band for *A. apfelbecki* is single or double around 600 kb (Figure 1, lanes 8 and 10 for primer F04). This primer has given rise to numerous RAPD bands in other lanes. So, this primer can possibly be used in experiments involving *A. apfelbecki*.

In the case of primer P06, a bright band was detected in all of the strains. However, a polymorphism seems to be evident when considering other weak bands (Figure 2). On the other hand, primer F 04 produced 5 DNA bands in all except for lanes 8 and 10, which are *A. apfelbecki* (Figure 2).

During the classical identification steps involving the morphological approaches, specimens 7 and 11 could only be named as *L. sp.* due to limitations in the classical method. The molecular method has suggested that *L. sp.* in lane 7 is *L. trifoli*, while the sample 11 is likely to be *L. congesta* (Figure 3).

Following the RAPD assessment, the genetic distances between the samples were estimated (Table 2). The values 0.1000 on the table indicate the least genetic distance or in other words the most genetically close individuals. For example, individuals 2 and 3 are very close to each other (0.1000). The most genetically distant individuals are found to be 7 and 8 (0.9538), which are *L. sp.* and *A. apfelbecki*, respectively (Table 2, A7 and A10).

Table 2. Estimation of genetic distance between the Agromyzidae species after RAPD assessment

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
A1	0.1000										
A2	0.9375	0.1000									
A3	0.9375	0.1000	0.1000								
A4	0.9375	0.1000	0.1000	0.1000							
A5	0.7647	0.7222	0.7222	0.7222	0.1000						
A6	0.8235	0.7777	0.7777	0.7777	0.8235	0.1000					
A7	0.1000	0.9375	0.9375	0.9375	0.7647	0.8235	0.1000				
A8	0.9524	0.9091	0.9091	0.9091	0.1500	0.9091	0.9538	0.1000			
A9	0.8750	0.8235	0.8235	0.8235	0.7647	0.8235	0.8750	0.9524	0.1000		
A10	0.2500	0.2381	0.2381	0.2381	0.3158	0.3000	0.2500	0.3846	0.1905	0.1000	
A11	0.7777	0.7368	0.7368	0.7368	0.7778	0.7368	0.7778	0.1905	0.8824	0.2273	0.1000

A dendrogram was constructed based on the RAPD data generated in this study (Figure 3). The purpose in doing so was only to form clusters of the insect

specimens for identification purposes, not for determining biological diversity. Certainly, more samples and primers are needed for a meaningful dendrogram tree in concluding the biological diversity of these organisms. However, our results have shown that the RAPD-PCR analysis can be used in species identification. We propose that RAPD should be integrated into classical identification methods since it is faster, more economical and reliable. This technique will likely offer more solid and certain results when identifying these morphologically related organisms. One could screen more than 50 samples at one time as opposed to one using the classical method where the characteristics of the genital organ of males is the decisive point. Also, the RAPD technique can identify the female individuals, which is not easily possible by the classical method.

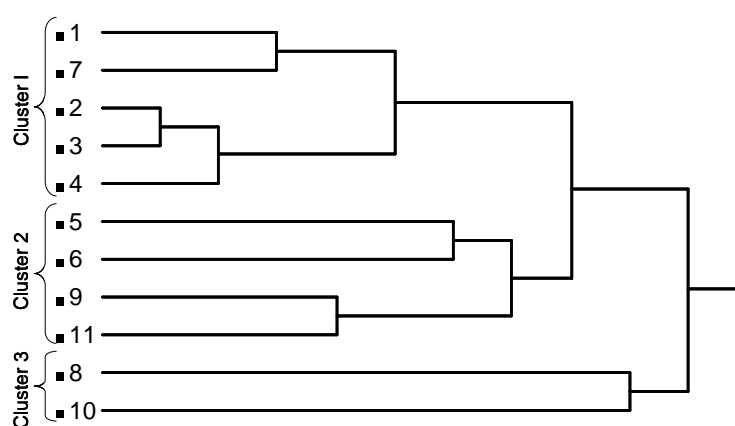


Figure 3. Dendrogram based on the RAPD assessment for the Agromyzidae species. Numbers indicate the species whose names and collection sites are given on table 1. Three clusters are shown. Cluster 1 (1, 7, 2, 3 and 4) is composed of the species *Liriomyza trifolii* (Burgess) and *L. sp.* Cluster 2 species are *Chromatomyia horticola* (Goureau) (5 and 6) and *Liriomyza congesta* (Becker) along with *L. sp.* (9 and 11). The species in the Cluster 3 (8 and 10) are *Agromyza apfelbecki* Strobl.

There are no RAPD-PCR studies found in the literature using these species. Most of the studies have taken advantage of the RFLP-PCR technique. Scheffer et al (2001) used the RFLP technique and showed the polymorphism between *L. huidobrensis* and *L. langei*. Similarly, Kox et al (2005) used the RFLP technique in analyzing the molecular differences between the species of *L. bryoniae*, *L. huidobrensis*, *L. sativae* and *L. trifolii*. However, as important as these studies are, the RFLP technique has certain limitations as it takes account only one or several genes or genome sites. The RAPD technique as shown by our results in this study will be more instrumental as it scans numerous DNA regions in the genome of the insect, randomly. Further studies will be performed using the RAPD-PCR analysis to achieve the goal of documenting the biological diversity, insecticide resistance status and evolutionary relationship of these economically important Agromyzidae species.

Özet

Türkiye’den toplanan *Liriomyza trifolii* (Burgess, 1880), *L. congesta* (Becker, 1903), *Agromyza apfelbecki* Strobl, 1902 ve *Chromatomyia horticola* (Goureau, 1851) türlerinin karakterize edilmesinde RAPD-PCR analizinin kullanımı

Liriomyza trifolii (Burgess, 1880), *Liriomyza congesta*, (Becker, 1903) *Agromyza apfelbecki* Strobl, 1902 ve *Chromatomyia horticola* (Goureau, 1851) türleri Türkiye’nin tarımsal alanlarında önemli sebze zararlılarındandır. Bu türler morfolojik karakterleri ile kolaylıkla ayırt edilemezler. Bu çalışmanın amacı, bu türleri kesin bir şekilde ayırt edebilmek için genetik işaretleyicileri oluşturmak ve Türkiye’deki değişik coğrafik alanlardan toplanan *L. trifolii* bireyleri arasındaki genetik varyasyonları kavramaktır. Böylece 4 rastgele primer kullanılarak RAPD işaretleyicileri çalıştırılmıştır. Farklı RAPD profilleri farklı türlerin belirlenmesinde kullanılmış ve böylece RAPD-PCR analizlerinin, klasik metodlar yoluyla rutin bir şekilde ele alınarak bu türlerin ayırt edilmesine yardımcı olma doğrultusunda hızlı bir tarama için kullanışlı bir araç olarak başvurulabileceğini göstermiştir. Sebze ürünlerine zarar vererek önemli ekonomik kayıplara neden olduğundan dolayı bu zararlının yayılımını önlemeye yönelik kontrol stratejilerinin geliştirilmesi için bu türlerin biyotiplerinin tanımlanması önemlidir. Bu çalışmadan elde edilen sonuçlar RAPD’in, türlerin ve ırklarının geleneksel teşhislerine önemli bir alternatif olarak umut verici bir şekilde etkili, hızlı ve ekonomik bir yöntem olduğunu, göstermiştir.

Acknowledgements

We would like to thank TUBITAK (The Scientific and Technical Research Council of Turkey) for funding this project (Research Project No: 106T122).

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