Reseacrh Article
The Journal of Turkish Phytopathology
fitopatoloji.org.tr

PCR-RFLP Analyses of *Aspergillus* spp. Isolated from Pistachio in Turkey

Ali AL-AAYEDI[1] Safa ALI[1] Selçuk BAŞBUĞA[1] Omar ATIK[2] Canan CAN[1]

[1]Department of Biology, Gaziantep University, Gaziantep, Turkey
[2]Department of Plant Protection, Agricultural Scientific Research Center, Aleppo, Syria

ABSTRACT
Pistachio (*Pistacia vera* L.) is a strategic crop plant and Turkey is one of the major pistachio producing county together with Iran, Syria and the United States of America. Pistachio trees are affected by many fungal diseases. The *Aspergillus* spp. are one of the most important agent that produce mycotoxins and also known as human and animal pathogens. This study was conducted to determine the genomic DNA polymorphisms from *Aspergillus* spp. which were isolated from pistachio fruits collected from different climatic zones in Turkey. Total of 27 *Aspergillus* spp. isolates were used and specific primers for ITS, Actin and β-tubulin genes were subjected to RFLP analysis by using *Hind* III, *Pst* I, *Taq* I and *Eco* RI restriction enzymes. Three different groups were defined with the enzyme *Taq* I in the β-tubulin region. The results revealed low polymorphism and genetic diversity in *Aspergillus* spp. with PCR-RFLP of the ITS and actin regions.
Keywords: Pistachio*, Aspergillus* spp.*,* PCR-RFLP

ÖZ
Türkiye'de Antep Fıstığı'ndan İzole Edilen *Aspergillus* spp.'nin PCR-RFLP Analizleri
Antep fıstığı (*Pistacia vera* L.) stratejik öneme sahip bir ürün ve Türkiye, İran, Suriye ve Amerika Birleşik Devletleri ile birlikte önemli bir üretici ülke konumundadır. Antep fıstığı ağaçlarında hastalık yapan birçok fungus türü vardır. Bunlardan *Aspergillus* spp. aflatoksin üretimi yanında insan ve hayvan patojeni olması dolayısı ile Antep fıstığı için en önemli tehditlerden birisi olarak kabul edilmektedir. Bu çalışma ülkemizin farklı bölgelerinden toplanmış meyve örneklerinden izole edilmiş *Aspergillus* spp.'de genomik DNA polimorfizmini belirlemek amacı ile yapılmıştır. Toplam 27 *Aspergillus* spp. izolatında ITS, Aktin ve β-tubulin genlerinde *Hind* III, *Pst* I, *Taq* I ve *Eco* RI kesim enzimleri kullanılarak RFLP analizi yapılmıştır. β -tubulin bölgesinde *Taq* I enzimi ile üç farklı grup saptanmıştır. Sonuçlar, ITS ve Aktin bölgelerinde *Aspergillus* spp.'nin PCR-RFP ile düşük polimorfizm ve genetik çeşitlilik sergilediğini ortaya koymuştur.
Anahtar kelimeler: Antep fıstığı, *Aspergillus* spp., PCR-RFLP

INTRODUCTION

Cultivated pistachio (*Pistacia vera* L.), a member of the Anacardiaceae, is native to the eastern Mediterranean and eastern to central Asia. Iran has a significant share of the worldwide pistachio production, followed by the USA, Turkey, Syria, China, Greece and Afghanistan (FAOSTAT, 2018; Zheng et al., 2012). Pistachio is produced mostly in the Southeastern Anatolia Region of Turkey, including Adiyaman, Gaziantep, Sanliurfa and Siirt provinces (Sarpkaya, 2014). Pistachio contains many nutrient elements necessary for human health such as vitamins, potassium, phosphorus, calcium, minerals, protein and amino acids (Tsantili et al., 2010).

Article Info / Makale Bilgileri
Corresponding author e-mail: ayselcan1938@gmail.com
Received: August 13, 2020 *Accepted:* October 26, 2020
ORCID ID's of Authors in order:
0000-0003-0547-9295, 0000-0002-5014-1368
0000-0002-9558-8307, 0000-0002-7439-4343
0000-0002-0473-1914
Bu çalışma ilk yazarın yüksek lisans tezi ürünüdür.
ISSN 0378 - 8024 Turkish Phytopathology Society ©2020

Many fungal diseases affect pistachio trees in East-Mediterranean and Southeast Anatolian regions and causing damage to hulls and kernels (Eskalen et al., 2001; Denizel et al., 2006; Ozkilinc et al., 2017). The greatest postharvest disease threats are from *Aspergillus flavus* and *A. parasiticus* wherein. *flavus* is one of the most prevalent storage fungus colonizing pistachio kernels. Previous studies reported that 30.97 million tons of products, especially peanuts in Asian and African countries, are contaminated with *A. flavus* and *A. parasiticus* species before and after harvest (Dekoe et al., 2000; Bruce et al., 2003).

The danger is particularly serious because *Aspergillus* spp. fungi can produce aflatoxins which are secondary metabolites produced by some species of *Aspergillus* and toxic for animals and humans (Jalali and Avagyan, 2016), and significantly reduces the quality and value of pistachios as well as it directly affects farmers and consumers. (Doster and Michailides, 1994; Ferdaws et al., 2016). Aflatoxins are particularly produced by *A. flavus* and *A. parasiticus* where Aflatoxin B1 (AFB1) is

the most toxic aflatoxin with the highest carcinogenic effects (Afsah-Hejri et al., 2013; Ferdaws et al., 2016). Polymerase Chain Reaction (PCR)-based tests have increasingly been used in laboratories to identify numerous fungal species (Luchi, 2020). Several methods have been reported for DNA-based identification of *Aspergillus* spp. including sequencing of the non-coding Internal Transcribed Spacer regions (ITS) (Moody and Tyler, 1990; Schoch et al., 2012), calmodulin, actin, β-tubulin (β-tub), rodlet A (rodA) (Samson et al., 2007; Nasri et al., 2015) and PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) for identification of *Aspergillus* at the generic level (Mirhendi et al., 2007; Zarrin et al., 2017). PCR-RFLP is the simplest method for single-nucleotide change detection. It is widely used in differentiation between mycotoxigenic species. The technique is based on PCR amplification of a target region containing the variant site of the studied species followed by restriction endonuclease digestion and gel electrophoresis to visualize the RFLP patterns (Atoui and El Khoury, 2017)

This study aimed at determining the polymorphism and genetic diversity among *Aspergillus* spp. isolates that were isolated from pistachio fruits in different governorates in Turkey. Restriction Fragment Length Polymorphism (RFLP) analysis of the PCR-amplified ITS, Actin and βtubulin regions of ribosomal DNA, was used.

MATERIALS and METHODS

Aspergillus spp. isolates

This study was conducted in 2015 in Gaziantep University in Turkey. Total of 27 *Aspergillus* spp. isolates were used in this study and they were originally isolated from *Pistacia vera* in 2012-2013 from 13 different sites related to 8 provinces through previous studies conducted in Turkey and were determined to be pathogenic and produce mycotoxins (Konukoğlu et al., 2011; Özgün, 2013). The isolates were stored in the Mycology Laboratory in Biology Department, Faculty of Arts and Sciences in Gaziantep University as per the codes demonstrated in Table 1. In addition, two isolates of *A. flavus* and *A. parasiticus* were included in this study as positive controls (Özgün, 2013).

In order to reproduce the preserved isolates of *Aspergillus* spp., the isolates were inoculated to Petri dishes including Potato Dextrose Agar (PDA) medium, then the Petri dishes were incubated for 4-6 days at 26-28 °C. After incubation, macroscopic and microscopic examinations of *Aspergillus* species (Figure 1), which were conducted according to fungal colonies features on the PDA medium and microscope observation, including colony diameter, color of conidia, secretions, mycelium tissue, soluble pigment, shape and size of conidiophores, vesicles, fialides, conidia were recorded periodically (Klich, 2002).

Preservation of re-cultured *Aspergillus* spp. isolates

Two different methods have been used for long-term storage of samples to be used in other studies:

Enclosure on filter paper

Sterilized pieces of filter papers (Whatman), with a diameter of 2-3 cm, were placed aseptically on the PDA medium. Then discs from the *Aspergillus* spp. colonies (3 mm in diameter) were inoculated to the paper and incubated for 5-7 days until they completely covered the petri dishes including the filter paper. After incubation, sterile filter papers, on which fungi developed, were pulled out and left to dry in sterile Petri dishes for 2-3 days. The dried filter papers were placed in sterile paper envelopes and stored at -20 °C.

Freezing

Glycerol stock solution (glycerol, NaCL) was prepared and sterilized. After *Aspergillus* spp. isolates were reproduced at 26-28 °C for 7-10 days, small pieces (3 mm in diameter) of isolates were placed in tubes containing 750 ml of glycerol stock. The collection site, name, isolate number and storage date were written on each tube and then preserved at -80 °C.

Genomic DNA extraction from *Aspergillus* spp. isolates.

For mycelium production of *Aspergillus* spp. isolates, small pieces from each isolate were transferred to standard petri dishes containing Potato Dextrose Broth (PDB). Then the dishes were put on an orbital shaker and incubated for 4-5 days at 26-28 °C and 65 rpm. At the end of this period, the developed mycelium was washed with sterile distilled water, the medium was removed and the liquid was frozen in nitrogen. Each sample was covered with aluminum foil and stored at -80°C until using them (Peever et al., 1999).

The Genomic DNA was extracted from the mycelium of the *Aspergillus* spp. isolates through the CTAB method (Somashekar et al., 2004). Briefly, fungal mycelia were crushed in the presence of liquid nitrogen. Then 600 µL CTAB extraction buffer was added and incubated at 60 °C for 30 minutes. After incubation, 600 µL of chloroform: isoamyl alcohol (24:1) was purified, centrifuged at 15 °C for at 5000 rpm for 20 minutes. Following centrifugation, genomic DNA was precipitated with isopropanol and the DNA obtained was dissolved in 100 µL TE buffer and preserved at 20 °C.

Polymerase chain reaction (PCR)

In our study, three DNA regions were used; 1) Part of the nuclear Ribosomal RNA gene repeat region (5.8S-ITS), 2) Actin (ACT) gene region and 3) β--tubulin (BT) gene region.

Table 1. Collection sites, codes and number of isolates of *Aspergillus* species used in the study

Province	Collection site	Isolate Number	Isolate Code
Adana	Central	2	01-AF/14
			01-AP/14
Aydın	Germencik	1	09-AG1/12
Batman	Gercus	1	72-BG1
Gaziantep	Pistachio Research Institute	1	27-FA1/12
	Karakuyu	1	27-KK1/12
Icel	Aydıncik	4	33-AD1/12
			33-AD2/12
			33-AD3/12
	Bozyazi		33-MB1/12
Kahramanmaras	Pazarcik Golbasi	1	46-BGB1/12
	Pazarcik-Ganidagi	1	46-PG1/12
Mardin	Midyat	1	47-MM1/12
Sanliurfa	Surtepe	2	63-BS1/12
			63-BS2/12
	Bozova	3	63-SB1/12
			63-SB2/12
			63-SB3/12
	Merkez	9	63-SM1/12
			63-SF1/12
			63-SM3/12
			63-SM4 /12
			63-SM5/12
			63-SM6 /12
			63-SM7/12
			63-SM8 /12
			63-SM9/12
Total 8	**13**	**27**	**27**

The dimensions of each mentioned region (ITS, Actin and β-tubulin) were determined by PCR. The primers used for the 5.8S-rDNA region; ITS5(5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3') (White et al., 1990), for Actin region; ACT-512F (5'-ATGTGCAAGGCCGGTTTCC-3') ACT-783R (5'-TACGAGTCCTTCTGGCCCAT-3') (Carbone and Kohn 1999), and finally for β-tubulin region; Bt1a (5'-TTCCCCCGTCTCCACTTCTTCATG-3') and Bt1b (5 'GACGAGATCGTTCATGTTGAACTC-3') (Glass and Donaldson, 1995).

The PCR amplification of ITS, Actin and β-tubulin regions was carried out in a 30 µl reaction mixture containing 30 mM $MgCl_2$, 2mM dNTP, 0.250 µM forward primer, 1U *Taq* DNA polymerase, 10x *Taq* DNA polymerase buffer and 25-100 ng genomic DNA. The PCR amplification of 45 ITS zones, 1 cycle for 2 minutes at 94 °C was followed by 30 cycles of each amplification cycle denaturation (93 °C, 30 seconds), primer binding (53 °C, 2 minutes) and elongation (72 °C, 2 minutes). It was carried out in stages. Finally, final amplification was given for 10 minutes at 72 °C. The PCR amplification of Actin and β-tubulin zone was followed by 1 cycle for 5 minutes at 94 °C, 35 cycles of each amplification cycle denaturation (94 °C, 30 seconds), annealing (coupling), (59 °C actin, 55 °C β-tubulin, 30 minutes) and elongation (72 °C, 1 minute). Finally, 1 cycle was given for 10 minutes at 72 °C (White et al., 1990; Crous et al., 2013).

Agarose gel electrophoresis

For agarose gel electrophoresis, 1.5% agarose was dissolved in 1X TAE (Tris, Acetic acid, EDTA) solution in presence of heating. The agarose solution was then left to cool for 15 minutes and ethidium bromide was added. The agarose was poured into a gel tank, following the solidification of the gel, electrophoresis was performed in 1xTAE buffer for 80-100 volts at 60-100 minutes. After the electrophoresis process was completed, the gel was displayed under UV light.

Restriction fragment length polymorphism (RFLP)

RFLP treatment was performed using restriction enzymes (*Eco* RI, *Hind* III, *Pst* I, *Taq* I) of each PCR product (ITS, Actin and β-tubulin). *Eco* RI, *Hind* III and *Pst* I enzyme cuts were performed for 16 hours at 37 °C and *Taq* I enzyme cuts for 8 hours at 65 °C. Following the cutting process, the products were

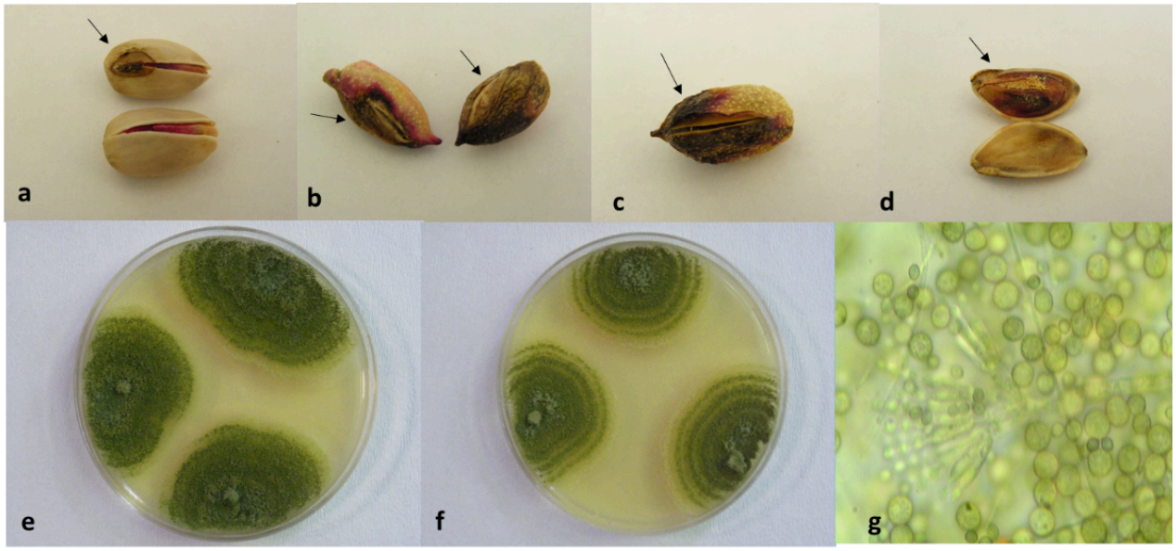

Figure 1. Pistachio nuts exhibiting *Aspergillus* spp. infections (a-d); colony morphology on PDA plates (e, f); conidia (g; 100 x magnification).

electrophoresed in 2-2.5% agarose gel and visualized in the UV system.

RESULTS and DISCUSSION

This study focused on Pistachios since it is an economically strategic crop plant in Turkey, where wild pistachio trees such as *P. terebinthus*, *P. atlantica* and *P. lentiscus* were also recorded (Kaska, 2005). In addition, it has high nutritional value and is consumed intensely as raw, salty and roasted products that is considered as an income-generating product for farmers, wholesalers, retailers and food industry owners (Shokraii, 1977; Maskan and Karatas, 1998). *Aspergillus* spp. isolates were studied to be classified since these fungi are prevalent on pistachio fruits in Turkey, and in the other hand produce mycotoxins (such as AFB and CPA, AFB and AFG) which is a big threat for healthy and high quality production of pistachios in Turkey and in the world (Denizel et al., 2006). This importance is supported by some studies which showed that detection of *A. niger* and *A. fumigatus* species are extremely important in preventing mycotoxins contaminations, since they are dangerous, allergy causing and associated with Aspergillosis (Abraca et al., 1994; Schuster et al., 2002; Noonimabc et al., 2009; Edwin et al., 2010).

PCR-RFLP results for the ITS region

Since the genus *Aspergillus* has many species (about 250), its taxonomy has always been quite complicated (Samson and Pitt, 2000; Samson et al., 2011). In order to identify the *Aspergillus* isolates used in this research, macroscopic (morphology), microscopic and molecular characterization were all conducted because the distinction based on morphological characters is one of the oldest and the most accepted methods. But since some species in the genus *Aspergillus* are morphologically similar, so it is difficult to distinguish these species. This shows that morphological characters are not sufficient alone in the identification of fungal species, making it necessary to use microscopic features as have been reported in many previous studies in the world (Mirhendi et al., 2007). Although, fungal identification using macroscopic and microscopic features has become one of the classic methods and is currently the most widely used method in the field of fungal taxonomy (Mirhendi et al., 2007; Afsah et al., 2013), the possibility of making mistakes in these methods also increases (Klich and Pitt, 1988; Samson et al., 2004), and disadvantages appeared such as posing a risk to the health of the laboratory staff.

The core objective of this study was to distinguish *Aspergillus* spp. isolates at the DNA level through PCR-RFLP. Many previous studies reported that the molecular markers, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and inter simple sequence repeats (ISSR), have been used broadly to prevent such situations and to assess the genetic variation and polymorphic fragments of a wide range of species. (Kesawat and Kumar, 2009; Muranty et al., 2014) The analysis of genomic DNA using PCR-based methods has proven to be a fast, sensitive and reliable method for determining genetic relationships among pathogenic microorganisms (Somashekar et al., 2004). Some studies reported that nuclear rDNA, and particularly the internal transcribed spacer (ITS) regions, are good targets for the phylogenetic analysis in fungi because the ITS regions are often highly variable between isolates of the same species (Schoch et al., 2012).

ITS PCR amplification was performed using ITS4 and ITS5 primers and the 5.8S rDNA gene was amplified. A band of approximately 600 bp was obtained for all the *Aspergillus* spp. (Figure 2).

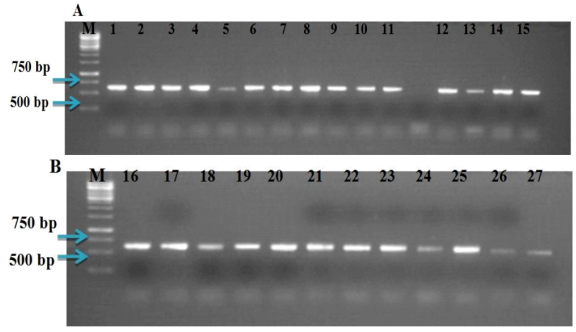

Figure 2. PCR amplification of 5.8S rDNA ITS region in *Aspergillus* spp. 1-25: *Aspergillus* spp.: 26: *A. flavus*: 27: *A. parasiticus*: M: 250 bp DNA marker.

RFLP analysis was performed using *EcoR* I, *Hind* III, *Pst* I and *Taq* I restriction endonucleases to demonstrate the difference between species. And the results showed that no polymorphism was observed in the ITS region when digested by *Pst* I and *Hind* III (Figure 3).

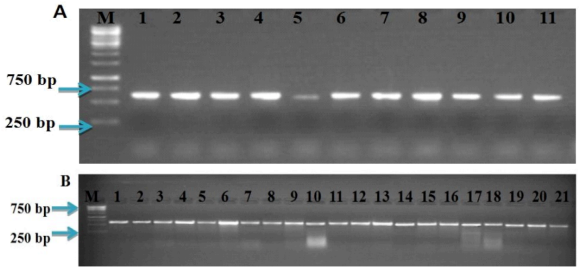

Figure 3. PCR-RFLP results of *Aspergillus* spp.; A) *Pst* I B) *Hind* III, M: 250 bp DNA ladder.

As a result of the *Taq* I cut of the 5.8S rDNA region of the *Aspergillus* spp. isolates, 3 bands, 280, 170 and 150 bp were observed in all samples (Figure 4). Similarly, no polymorphism was detected by *Eco* RI cut (Figure 5).

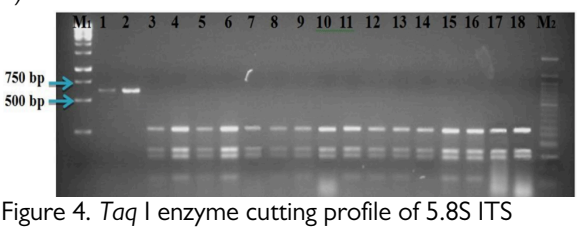

Figure 4. *Taq* I enzyme cutting profile of 5.8S ITS region in *Aspergillus* spp.; 1-18: *Aspergillus* spp.; M1: 250 bp DNA marker, M: 50 bp DNA ladder

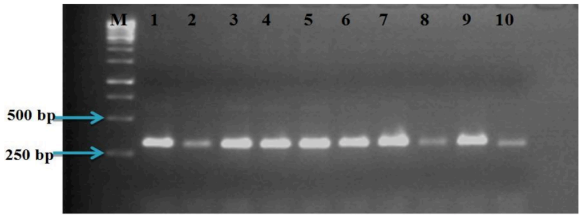

Figure 5. *Aspergillus* spp. 5.8S rDNA ITS region *Eco* RI cut profile; 1-8*: Aspergillus* spp.; 9: *A. flavus*; 10: *A. parasiticus*; M: 250 bp DNA ladder

As a result of this study, *Taq* I, *Eco* RI, *Hind* III and *Pst* I enzymes of 5.8S rDNA ITS region, polymorphism was not detected among *Aspergillus* spp. Considering this result, it can be stated that the restriction enzymes we used in PCR-RFLP of the ITS region were insufficient for detection of the difference among *Aspergillus* species. The obtained result is in line with the study that has been conducted by Nikkuni et al. (1998), who also reported that ITS sequence of species belonging to *Flavi* section is closely related. Similar results were stated by other researchers when analysis conducted on 5.8S ITS region comparison of *A. flavus* and *A. oryzae* species due to high genetic similarity (Kurtzman et al., 1986; Chang et al., 1991; Nikkuni et al., 1996).

PCR-RFLP results for the Actin region

A single band of approximately 370 bp was amplified for the Actin region in *Aspergillus* spp. (Figure 6).

No polymorphism was observed as a result of RFLP analysis of the Actin region of *Aspergillus* spp. using *Eco* RI, *Hind* III, *Pst* I and *Taq* I (Figure 7).

All *Aspergillus* spp. as a result of the *Eco* RI cut in the Actin region of the isolates, non-polymorphic two bands, approximately 150 and 200, were obtained (Figure 8).

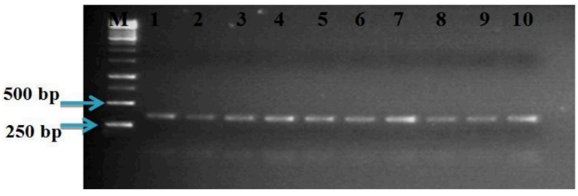

Figure 6. PCR amplification results of Actin region from *Aspergillus* spp.;1-8: *Aspergillus* spp.; 9: *A. flavus*; 10: *A. parasiticus*; M: 250 bp DNA ladder.

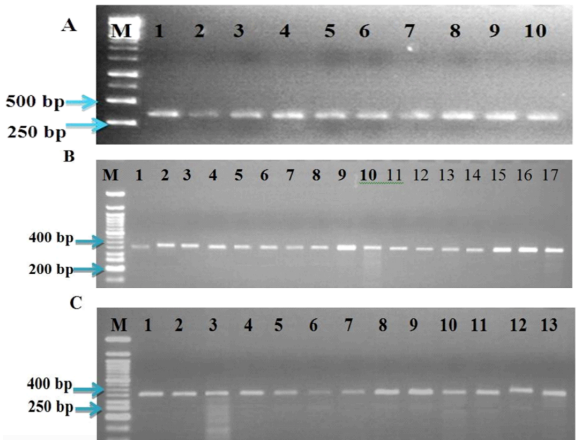

Figure 7. *Aspergillus* spp. Actin region PCR-RFLP results. *A)*-Profile obtained after cutting with *Pst* I; 1-8 *Aspergillus* spp.; 9: *A. flavus*; 10: *A. parasiticus;* M: 250 bp DNA ladder. B)-Profile obtained after cutting with *Hind* III; 1-15: *Aspergillus* spp.; 16: *A. flavus;* 17: *A. parasiticus;* M: 50 bp DNA ladder. C)-Profile obtained after cutting with *Taq* I;1-2: Actin region profiles; 3-11: *Aspergillus* spp.; 12: *A. flavus*; 13: *A. parasiticus*; M: 50 bp DNA ladder.

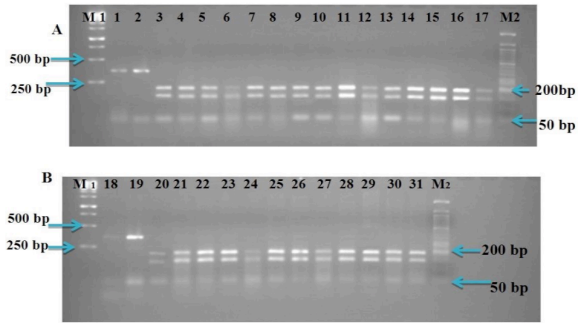

Figure 8. The band profile obtained as a result of *Eco* RI restriction of *Aspergillus* spp. Actin region, A). 1, 2: Actin PCR profile; 3-17: *Aspergillus* spp. B). 18-19: Actin PCR profile, 20-29: *Aspergillus* spp.; 30: *A. flavus*: 31: *A. parasiticus*: M: 250 bp DNA marker.

In our study, there was no difference between *Aspergillus* species as a result of cutting the Actin region with *Taq* I, *Eco* RI, *Hind* III, and *Pst* I enzymes. This result disagreed with Daniela and Meyera (2003), when they used the Actin gene in Ascomycetes yeasts and reported large number of sequence diversity.

PCR-RFLP results for the β-tubulin region

As a result of standard PCR reactions, a band of about 450 bp was obtained for all *Aspergillus* spp. isolates (Figure 9: A, B).

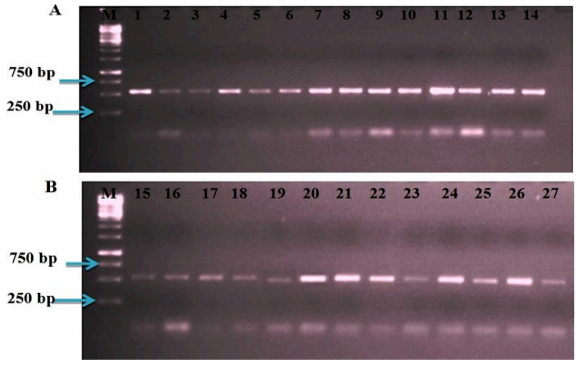

Figure 9. PCR amplification of genomic DNA using Bt1a and Bt1b primers. *A)*.1-14: *Aspergillus* spp. B).15-25: *Aspergillus* spp., 26: *A. flavus*, 27: *A. parasiticus*, M: 250 bp DNA marker.

Polymorphism was not detected in all samples as a result of analysis of the β-tubulin gene with PCR-RFLP using *Pst* I, *Hind* III and *Eco* RI enzymes (Figure 10).

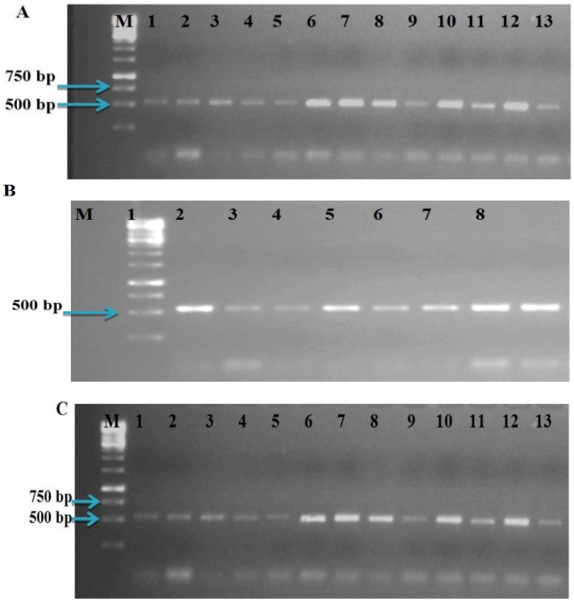

Figure 10. β -tubulin region PCR-RFLP results in *Aspergillus* spp. A) *Pst* I profile; 1-11: *Aspergillus* spp.: 12: *A. flavus*: 13: *A. parasiticus*; B) *Eco* RI profile. 1-6: *Aspergillus* spp., 7: *A. flavus*, 8: *A. parasiticus*; C) *Hind* III profile. 1-11: *Aspergillus* spp., 12: *A. flavus*, 13: *A. parasiticus*. M: 250 bp DNA marker.

According to the results obtained for the PCR-RFLP of the β -tubulin gene using *Taq* I enzyme, 3 different groups were identified in terms of size and polymorphism was detected. Of these, the first group included band patterns at approximately 250, 140, 60, 90 bp, the second group included band patterns at 250, 100, 90, 55 bp, while the third group included band pattern at approximately 90 bp (Figure 11).

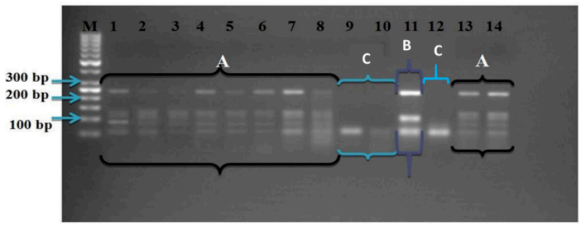

Figure 11. β -tubulin region *Taq* I enzyme PCR RFLP results in *Aspergillus* spp.; 1-14: *Aspergillus* spp. M: 50 bp DNA marker.

We claim that, it is necessary to conduct detailed studies on this region. These results are compatible with studies showing that RFLP technique is effective in identifying *Aspergillus* spp. at species level (Geiser et al., 1998; Balajee et al., 2005; Balajee et al., 2006; Peterson, 2008). Another study reported that, comparative sequence analysis of β-tubulin can be used for molecular identification of *Aspergillus* spp. (Balajee et al., 2009). Also Davidson et al. (2006) stated that the β-tubulin gene is the most conserved genes in fungi and can be used to control DNA quality for PCR reactions. Studies have reported that introns in the β-tubulin genes in fungi may provide some evidence for evolutionary relationships between species (Crous et al., 2013). β- tubulin gene has also been used to maintain the quality of fungal gDNA and PCR reaction conditions (Dao et al., 2005). The β-tubulin gene, isolated from the DNA library of *A. flvaus*, was also used as a selectable marker for transformation (Seip et al., 1990). All eukaryotic cells produce tubulin protein. Tubulins are essential components of eukaryotic cell microtubules. Tubulin gene family is divided into 3 classes as a, b and c depending on the protected sequence region and function. These genes provide information in terms of microtubule function and understanding of cell division (Hiraoka et al., 1984). The β -tubulin protein is the target sites of benzimidazole fungicides used in the control of many phytopathogenic fungi (Jones et al., 1987; Koenraadt et al., 1992, Cooley and Caten, 1993, Goldman et al., 1993). Therefore, the β -tubulin gene may be the subject to rapid mutations resulting polymorphism at the target regions (Nasri et al., 2015).

As a conclusion, when each of the ITS and Actin regions were used, it was found that they failed to identify different types of *Aspergillus* spp used in this study. The difference between *Aspergillus* species was determined based on the PCR-RFLP results of the β-tubulin region of *Aspergillus* spp. isolated from pistachio using the *Taq* I restriction enzyme. This difference may have occurred as a result of point mutations, deletions and additions. Further studies are needed on β-tubulin region sequence comparisons among the *Aspergillus* spp. isolates.

LITERATURE CITED

Abraca, M.L., Bragulat, G. and Cabanes, F.J. 1994. Ochratoxin A Production by Strains of *Aspergillus flavus* var. *niger*. App. Environ. Microbiol. 60: 2650-2652.

Afsah-Hejri, L., Jinap, S., Hajeb, P., Radu, S. and Shakibazadeh, S. 2013. A Review on Mycotoxins in Food and Feed: Malaysia Case Study. Comp. Rev. Food Sci. Food Saf. 12:629-651.

Atoui, A. and El Khoury, A. 2017. PCR-RFLP for *Aspergillus* Species. Pages 313-320 in Mycotoxigenic Fungi. Methods in Molecular Biology. Moretti, A. and Susca, A. eds. Humana Press, New York.

Balajee, S.A., Borman, A.M., Brandt, M.E., Cano, J., Cuenca-Estrella, M., Dannaoui, E., Guarro, J., Haase, G., Kibbler, C.C., Meyer, W., O'Donnell, K., Petti, C.A., Rodriguez-Tudela, J.L., Sutton, D., Velegraki, A. and Wickes, B.L. 2009. Sequence-based Identification of *Aspergillus, Fusarium*, and Mucorales Species in the Clinical Mycology Laboratory: Where are We and Where Should We Go from Here? J. Clin. Microbiol. 47:877–884.

Balajee, S.A., Gribskov, J.L., Hanley, E., Nickle, D. and Marr, K.A. 2005. *Aspergillus lentulus* sp. nov., A New Sibling Species of *A. fumigatus*. Eukaryotic Cell. 4: 625–632.

Balajee, S.A., Nickle, D., Varga, J. and Marr, K.A. 2006. Molecular Studies Reveal Frequent Misidentification of *Aspergillus fumigatus* by Morphotyping. Eukaryotic Cell. 5: 1705-1712.

Bruce, C., Campbell, H., Schatzk, F., Russell, J. and Monlyneux, T. 2003. Current Research On Reducing Pre and Post-Harvest Aflatoxin Contamination of USA Almond, Pistachio and Walnut. Toxin Rev. 22:225-260.

Carbone, I. and Kohn, L.M. 1999. A Method for Designing Primer Sets for Speciation Studies in Filamentous Ascomycetes. Mycologia 91: 553-556.

Chang, J. M., Oyaizu, H. and Sugiyama, J. 1991. Phylogenetic-Relationships Among Eleven Select Species of *Aspergillus* and Associated Teleomorphic Genera Estimated from 18S Ribosomal-RNA Partial Sequences. J. Gen. Appl. Microbiol. 37: 289-308.

Cooley, R.N. and Caten, C.E. 1993. Molecular Analysis of the *Septoria nodorum* Beta Tubulin Gene and Characterization of a Benomyl-Resistance Mutation. Mol. Gen. Genet. 237: 58-64.

Crous, P.W., Quaedvlieg, W., Sarpkaya, K., Can, C. and Erkılıç, A. 2013. Septoria-Like Pathogens Causing Leaf and Fruit Spot of Pistachio, IMA Fungus, 4(2): 187–199.

Daniela, H.M. and Meyera, W.C. 2003. Evaluation of Ribosomal RNA and Actin Gene Sequences for the Identification of Ascomycetous Yeasts. Int. J. Food Microbiol. 86: 61–78.

Dao, H.P., Mathieu F. and Lebrihi, A. 2005. Two Primer Pairs to Detect OTA Producers by PCR Method. Int. J. Food. Microbiol., 104: 61–67.

Davidson, R. M., Hanson, L. E., Franc, G. D. and Panella, L. 2006. Analysis of β-tubulin Gene Fragments from Benzimidazole-Sensitive and Tolerant *Cercospora beticola*. J. Phytopathol., 154: 321–328.

Denizel, T., Jarvis, B. and Rolf, E.J. 2006. A field Survey of Pistachio (*Pistacia vera*) Nut Production and Storage in Turkey with Particular Reference to Aflatoxin Contamination. J. Sci. Food Agric. 27 (11):1021-1026.

Dekoe, W.J., Sanson, R.A., Egmond, P.V., Gilbert, J. and Sabino, M. 2000. Proceedings of The International IUPAC Symposium on Mycotoxins and Phcotoxins, Guarja Brazil. pp. 21-25.

Doster, M.A. and Michailides, T.J. 1994. *Aspergillus* Molds and Aflatoxins in Pistachio Nuts in California. Phytopathology. 84 583-590.

Edwin, R., Palencia, I., Hinton, D.M. and Bacon, C.W. 2010. The Black *Aspergillus* Species of Maize and Peanuts and Their Potential for Mycotoxin Production. Toxins. 2: 399-416.

Eskalen, A., Küsek, M., Danisti, L. and Karadag, S. 2001. Fungal Diseases in Pistachio Trees in East Mediterranean and Southeast Anatolian Regions. In: Ak B.E. (ed.). XI GREMPA Seminar on Pistachios and Almonds, Zaragoza: CIHEAM, 2001, 261-264

FAOSTAT 2018. Food and Agriculture Organization Production Resource Statistics. (Web page: http://faostat.org.)

Ferdaws, B., Sobita, S. and Abhilasha, A. 2016. Effect of Selected Spices Against *Aspergillus flavus* of Pistachio (*Pistachia vera* L.) *in vitro* Condition. Int. J. Multidiscip. Res. Dev. 7: 185-187

Geiser, D.M., Frisvad, J.C. and Taylor, J.W. 1998. Evolutionary Relationships in *Aspergillus* Section Fumigati Inferred from Partial Beta-Tubulin and Hydrophobin DNA Sequences. Mycologia. 90: 831–845.

Glass, N.L. and Donaldson, G.C. 1995. Development of Primer Sets Designed for Use with The PCR to Amplify Conserved Genes from Filamentous Ascomycetes. App. Environ. Microbiol. 61: 1323–1330.

Goldman, G.H., Temmerman, W., Jacobs, D., Contreras, R., van Montagu, M. and Herrera-Estrella, A. 1993. A Nucleotide Substitution in One of the Beta-Tubulin Genes of *Trichoderma Viride* Confers Resistance to the Antimitotic Drug Methyl Benzimidazole-2-Yl-Carbamate. Mol. Gen. Genet. 240: 73–80.

Hiraoka, Y., Toda, T. and Yanagida, M. 1984. The NDA3 Gene of Fission Yeast Encodes Beta-Tubulin: A Cold Sensitive Nda3 Mutation Reversibly Blocks Spindle Formation and Chromosome Movement in Mitosis. Cell. 39: 349–358.

Jalali, L. and Avagyan, G. 2016. Evaluation of Contamination of *Aspergillus flavus* and Aflatoxin Production in Pistachio Cultivars and Investigation of a Chemical Controlling Method. J. Nuts. 7: 51–58.

Jones, A.L., Shabi, E. and Ehret, G.R. 1987. Genetics of Negatively Correlated Cross Resistance to a N-Phenylcarbamate in Benomyl-Resistant *Venturia inaqualis*. Can. J. Plant Pathol. 9: 195-199.

Kaska, N. 2005. Pistachio nut growing in Turkey. Acta Hortic. 419: 161-164.

Kesawat, M.S. and Kumar, B.D. 2009. Molecular Markers: Its Application in Crop Improvement. J. Crop. Sci. Biotechnol. 4: 169–181.

Klich, M.A. 2002. Identification of Common *Aspergillus* Species. Centraalbureau voor Schimmelcultures, Utrecht. The Netherlands, 128 pp.

Klich, M.A. and Pitt, J.I. 1988. Differentiation of *Aspergillus flavus* from *Aspergillus parasiticus* and Other Closely Related Species. T. Brit. Mycol. Soc. 91:99-108.

Koenraadt, H., Somerville, S.C. and Jones, A.L. 1992. Characterization of Mutations in the Beta-Tubulin Gene of Benomyl-Resistant Yield Strains Of *Venturia inaequalis* and Other Plant Pathogenic Fungi. Phytopathology. 82: 1348-1354.

Konukoğlu, F., Kusek, M., Sarpkaya, K., Karadag, S. and Can, C. 2011. Discoloration of Pistachio Nuts Grown in the Gaziantep Province of Turkey. Acta Horticulturae, 912:761-763.

Kurtzman, C.P., S, Smiley, M.J., Robnett, C.J. and Wicklow, D.T. 1986. DNA Relatedness Among Wild and Domesticated Species in *Aspergillus flavus* Group. Mycologia. 78: 955-959.

Maskan, M. and Karatas, S. 1998. Fatty Acid Oxidation of Pistachio Nuts Stored Under Various Atmospheric Conditions and Different Temperatures. J. Sci. Food Agric. 77: 334-340.

Mirhendi, S.H., Diba, K. and Kordbacheh, P. 2007. Identification of Pathogenis *Aspergillus* Species by a PCR-Restriction Enzyme Method. J. Med. Microbiol. 56:1568-1570.

Moody, S.F. and Tyler, B.M. 1990. Use of Nuclear DNA Restriction Fragment Length Polymorphism to Analyze the Diversity of the *Aspergillus flavus* group; *A. flavus*, *A. parasiticus* and *A. nomius*. J. App. Environ. Microbiol. 56: 2453-2461.

Muranty, H., Jorge, V., Bastien, C., Lepoittevin, C., Bouffier, L. and Sanchez, L. 2014. Potential for Marker-Assisted Selection for Forest Tree Breeding: Lessons from 20 Years of MAS in Crops. Tree Genet. Genom. 6: 1491–1510.

Nasri, T., Hedayati, M., Abastabar, M., Pasqualotto, A., Armaki, M., Hosseinnejad, A. and Nabili, M. 2015. PCR-RFLP on β-Tubulin Gene for Rapid Identification of the Most Clinically Important Species of *Aspergillus*. J. Microbiol. Meth. 117. 10.1016/j.mimet.2015.08.007.

Nikkuni, S., Kosaka, N., Suzuki, C. and Mori, K. 1996. Comparative Sequence Analysis on the 18S rRNA Gene of *Aspergillus oryzae*, *A. sojae*, *A. flavus*, *A. parasiticus*, *A. niger*, *A. awamori* and *A. tamarii*. J. Gen. App. Microbiol. 42:181-187.

Nikkuni, S., Nakajima, H., Hoshina, S., Ohno, M., Suzuki, C., Kashiwagi, Y. and Mori, K. 1998. Evolutionary Relationships Among *Aspergillus Oryzae* and Related Species Based on the Sequences of 18S rRNA Genes and Internal Transcribed Spacers. J. Gen. App. Microbiol. 44: 225-230.

Noonimabc, P., Mahakarnchanakulb, W., Nielsend, K.F., Frisvadd, J.C. and Samsona, R.A. 2009. Fumonisin B2 Production by *Aspergillus niger* in Thai Coffee Beans. Food Addit. Cont. 26: 94-100.

Ozkilinc, H., Sarpkaya, K., Kur,t S., Can, C., Polatbilek, H., Yasar, A., Sevinc, U., Uysal, A. and Konukoglu, F. 2017. Pathogenicity, Morpho-Species and Mating Types of *Alternaria* spp. Causing Alternaria Blight in *Pistacia* spp. in Turkey, Phytoparasitica. Doi: 10.1007/s12600-017-0624-8.

Özgün, E. 2013. Antep fıstığında (*Pistacia vera* L.) Aflatoksin Oluşturan *Aspergillus* Türlerinin Belirlenmesi. Gaziantep Üniversitesi, Yüksek Lisans Tezi, Türkiye, 52 pp.

Peever, T. L., Canihos, Y., Olsen, L., Ibanez, A., Liu, Y.C. and Timmer, L.W. 1999. Population Genetic Structure and Host Specificity of *Alternaria* spp. Causing Brown Spot of Minneola tangelo and Rough Lemon in Florida. Phytopathology. 89: 851-860.

Peterson, S.W. 2008. Phylogenetic Analysis of *Aspergillus* Species Using DNA Sequences from Four Loci. Mycologia. 100: 205-26.

Samson, R., Hong, S., Peterson, S.W., Frisvad, J.C. and Varga, J. 2007. Polyphasic Taxonomy Of *Aspergillus* Section Fumigati and Its Teleomorph Neosartorya. Stud. Mycol. 59: 147–203.

Samson, R.A. and Pitt, J. I. 2000. Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* Classification. Harwood Academic Publishers, Singapore, 510 pp.

Samson, R.A., Peterson, S.W., Frisvad, J.C. and Varga, J. 2011. New Species in *Aspergillus* Section Terrei. Stud. Mycol. 69: 39-55.

Samson, R.A., Seifert, K.A., Kuijpers, A.F.A. and Frisvad, J.C. 2004. Phylogenetic Analysis of *Penicillium* Subgenus *Penicillium* Using Partial β-Tubulin Sequences. Stud. Mycol. 49:175–200.

Sarpkaya, K., 2014. Antep Fıstığında Karazenk Hastalığına Neden Olan *Pseudocercospora Pistacina*'nın Biyolojisi, Epidemiyolojisi ve Mücadelesine Yönelik Çalışmalar, Ç.Ü. Doktora Tezi, 120 sayfa.

Schoch, CL, Seifert, KA, Huhndorf, S., Robert, V., Spouge J.L., Levesque, C.A. and Chen, W. 2012. Nuclear Ribosomal Internal Transcribed Spacer (ITS) Region as a Universal DNA Barcode Marker for Fungi. Proc Natl Acad Sci U S A. 2012; 109(16):6241-6246. doi:10.1073/pnas.1117018109.

Schuster, E., Dunn-Coleman, N., Frisvad, J.C. and Van Dijck, P.W.M. 2002. On the Safety of *Aspergillus niger* - A Review. App. Microbiol. and Biotechnol. 59: 426-435.

Seip, E.R., Woloshuk, C.P., Payne, G.A. and Curti, S.E. 1990. Isolation and Sequence Analysis of a β-Tubulin Gene from *Aspergillus flavus* and Its use as a Selectable Marker. App. Environ. Microbiol. 56: 3686–3692.

Shokraii, E.H. 1977. Chemical Composition of the Pistachio Nuts (*Pistacia vera* L.) of Kerman, Iran. J. Food Sci. 42: 244-245.

Somashekar, D., Ratl, E.R. and Chandrashekar, A. 2004. PCR-Restriction Fragment Length Analysis of aflR Gene for Differentiation and Detection of *Aspergillus flavus* and *Aspergillus parasiticus* in Maize. Int. J. Food Microbiol. 93:101-107.

Tsantili, E., Takidelli, C., Christopoulos, M.V., Lambrinea, E., Rouskas, D. and Roussos, P.A. 2010. Physical, Compositional and Sensory Differences in Nuts Among Pistachio (*Pistachia vera* L.) varieties. Sci. Hortic. 125: 562- 568.

White, T.J., Bruns, T., Lee, S. and Taylor. J. 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. Pages 315-322 in PCR protocols: a guide to methods and applications PCR protocols: a guide to methods and applications. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., eds. Academic Press, New York.

Zarrin, M., Rashidnia, Z., Faramarzi, S. and Harooni, L. 2017. Rapid Identification of *Aspergillus fumigatus* Using Beta-tubulin and RodletA Genes. Mac. J. Med. Sci. 5. 10.3889/oamjms.2017.203.

Zheng, Z., Saghaian, S. and Reedc, M. 2012. Factors Affecting the Export Demand for U.S. Pistachios. Int. Food Agribus. Man. 15: 139-154.