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PCR-RFLP Characterization of *Alternaria* spp. from Pistachio in Türkiye and Evidence for a Putative Clonal Lineage

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ABSTRACT

Purpose: *Pistacia vera* is an important crop species in Türkiye with considerable economic income. *Alternaria* spp. cause yield loss in *P. vera* and many other agriculturally important plants. Information on population structure is critical in breeding for resistance to Alternaria blight in pistachio. This study was carried out to characterize *Alternaria* isolates collected from local cultivars of different pistachio growing districts of Türkiye through PCR-RFLP.

Materials and Methods: The PCR method was utilized for the genetic characterization for identification of *Alternaria* spp. using specific primers designed from nuclear ribosomal *ITS* (Internal Transcribed Spacer), *Actin* and *Tef1-α* (Translation Elongation Factor 1 alpha) regions.

Results: Approximately 600 bp, 250 bp and 280 bp amplicons were obtained for ITS, Actin and *Tef1-α*, respectively. The PCR products were cut with *Hind*III, *Eco*RI, *Taq*I, *Hinf*, *Hha*I and uncut with *Pst*I restriction endonucleases. Identical restriction band profiles were obtained in all isolates as a result of RFLP.

Conclusion: Restriction analysis revealed identical banding profiles across all isolates for each enzyme tested. There was no polymorphism among *Alternaria* spp. isolates at ITS, Actin and *Tef1-α* regions, and it is suggested that the isolates may belong to a single clonal species.

Keywords: Pistachio, *Alternaria*, PCR-RFLP, Genetic diversity

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Introduction

Cultivated pistachio (*Pistacia vera* L.), a member of the Anacardiaceae, is native to the eastern Mediterranean and eastern to central Asia (1). Iran has a significant share of the worldwide pistachio production, followed by the USA, Türkiye, China and Syria (2). Pistachio is produced mostly in the Southeastern Anatolia region of Türkiye, including Adıyaman, Gaziantep, Şanliurfa and Siirt provinces where production in this region is not only an economic but also a social element of sustainability (3). Many fungal diseases affect pistachio trees in the East-Mediterranean and Southeast Anatolian regions of Türkiye causing damage to hulls and kernels reducing economic income (3-5, 49). Alternaria blight is considered as one of the most important diseases of pistachio (6-9). Morphologically three species (*Alternaria alternata*, *A. tenuissima* and *A. arborescens*) and phylogenetically two species (*A. alternata* and *A. arborescens*) are responsible for the disease. Section 'Alternaria' is one of the most current debates in the literature since the species boundaries are not well-resolved that leads to serious disruptions in disease management and quarantine processes (6, 10). The pathogen infects both leaves and fruit, causes severe premature defoliation and staining of nutshells, resulting in reduced fruit quality (11, 12). The disease is characterized by the development of large necrotic lesions that eventually coalesce and consume the entire leaf. The lesions generally are black in the center due to the abundant production of fungal spores and typically are surrounded by a chlorotic halo (6, 13). Infection causes brownish-darkening lesions on fruits (9). The major impacts for growers are reduction in yield due to defoliation and rejection of nuts by processors. Irrigation management and the use of fungicides can reduce the damage (11). Also, the most cost-effective and long-term disease management is to develop resistant cultivars. It was reported that one highly virulent isolate of *Alternaria* fungi was used to test reactions of 'Uzun', 'Ohadi' and 'Siirt' pistachio cultivars under field conditions, and 'Uzun' was found to be the most tolerant cultivar followed by 'Siirt' and 'Ohadi' (14). Different varieties elicit different responses, making knowledge of the genetic diversity or clonal structure of the pathogen population in the region essential.

Alternaria blight disease has been observed both in pistachio and its wild relatives in Türkiye and the disease agents were characterized based on morphology, pathology and molecular/ phylogenetic aspects. The disease is common wherever pistachio trees are grown in Türkiye (8, 9).

It has been known that morphology-based identifications for section *Alternaria* are highly difficult due to plasticity in their morphological characters which are also affected by environmental conditions and species boundaries are not well-resolved within this section (9, 15, 16). Still, traditional identification based on morphology is used as a first attempt to characterize pathogen isolates. But molecular and/or phylogenetic tools should be used to confirm species recognition as applied in some previous studies (9, 17-19).

Polymerase Chain Reaction (PCR)-based tests have increasingly been used to identify numerous fungal species (20). Several methods have been reported for DNA-based identification of *Alternaria* spp. including PCR-sequencing of the non-coding Internal Transcribed Spacer regions (ITS) due to their high degree of sequence variation (21), calmodulin, actin, β-tubulin (β-tub), rodlet A (rodA) (22, 23) and PCR-restriction fragment length polymorphism (PCR-RFLP) for identification of *Alternaria* at the genetic level (24-27). Although Whole Genome Sequencing (WGS) is popular today, it should be emphasized that PCR-RFLP is still a cost-effective, fast, and applicable method for single-nucleotide change detection. It is widely used in differentiation between mycotoxigenic species. RFLP 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 (28).

This study aimed at determining the polymorphism and genetic diversity among *Alternaria* spp. isolates that were isolated from Pistachio in different provinces in Türkiye. Restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified specific primers designed from nuclear ribosomal ITS (Internal Transcribed Spacer), Actin and *Tef1-α* (Translation Elongation Factor) regions were used.

Materials and Methods

Alternaria spp. Isolates

Twenty-nine *Alternaria* spp. isolates, previously obtained from pistachio trees with *Alternaria* blight symptoms and shown to be pathogenic in a previous study, were used in the present study. The samples were collected from different areas of the Southeastern Anatolia region in Türkiye between 2009-2013 (5, 29) (Table 1).

Table 1. Provinces, collection sites and number/code of *Alternaria* spp. isolates from pistachio

Province	Collection site	Isolate Number	Isolate Code
Adana	Central	2	01-AP/14
			01-AT/14
Adıyaman	Pazarcık-Gölbaşı	1	02-PG/12
	Pazarcık	1	02-P/12
	Besni-Köseceli	2	02-BK1/12
			02-BK2/12
	Nemrut	1	02-Nt1/12
Batman	Central	2	72-Bt1/12
			27-Bt2/12
Gaziantep	Karakuyu	3	27-Kr1/12
			27-Kr2/12
			27-Kr3/12
	Gaziantep Univ. Campus	4	27-K1/12
			27-K2/12
			27-K3/12
			27-K4/12
	Pisrachio Res. Inst.	1	27-FA1/12
	Central	1	27-GK1/12
İzmir	Alaçatı	1	35-At1/12
Kilis	Central	1	79-KM1/12
Mersin	Aydıncık	1	33-İD1/12
Şanlıurfa	Surtepe	1	63-BS1/12
	Hilvan	1	63-HG1/12
	Gelenek-Hilvan	1	63-HG2/12
	Central	4	63-SF1/12
			63-SF2/12
			63-SF3/12
			63-SF4/12
Tunceli	Ovacık	1	62-OV1/12
Total	**18**	**29**	**29**

These isolates, collected during the early epidemic outbreaks in the region, were used as reference isolates because they represent the historical genetic composition of the pathogen population and provide a baseline for evaluating temporal changes in population structure and diversity. The isolates were preserved at -80 ºC in tubes containing 100ml glycerol in the laboratory of Biology Department, Faculty of Science and Letter, Gaziantep University, Türkiye.

The sensitivity of the molecular studies requires the use of the single spore colonies belonging to *Alternaria* spp. For this purpose, the spores taken from the surface of the spores-formed fungal culture which were transferred to a new Potato Dextrose Agar (PDA) medium (39g PDA/ 1 liter distilled water). Petri dishes were supplemented with antibiotics (Streptomycin) to prevent bacterial contamination. Then it was incubated for 4-6 days at 25-30ºC. Developing samples were examined under a light microscope and an inverted microscope, and hypha from a single

spore were marked. Then a small piece was taken from the marked area with the help of a sterile scalpel and transferred to PDA medium for development. Samples taken into PDA medium were incubated for 4-6 days at room temperature to be used in the next study (14). The pure cultures (formed through the previous isolation process) were developed on Whatman paper placed on the PDA medium. For this purpose, a small piece of stylet scalpel was taken from the developing samples, planted on sterile Whatman paper and left for incubation for 6-10 days at 24 ± 2 °C to grow. Whatman paper, on which fungi developed, was removed from the medium with the help of sterile forceps after incubation and dried in sterile Petri dishes. The dried isolates were placed in sterile paper envelopes and stored at -20 ° C in the laboratory.

DNA Isolation

Modified method of DNA extraction reported by (30) was used. The mycelium was cultured in liquid PDB medium. Later, this medium was incubated on shaker at 22-28 ºC for 35 days, and the mycelium developed after this period were used, then the prepared samples were kept at -80 ° C in the laboratory. Sufficiently developed mycelium was crushed by adding 10 - 15 ml liquid nitrogen by using mortar and pestle. The mycelium powder obtained was placed in a 1.5 ml tube, then preserved in the liquid nitrogen at -196 °C. Then CTAB (18) and extraction buffer (2% w/v Hexadecyltrimethyl-ammoniumbromide (CTAB), 8.2% w / v NaCl (1.4M)), 1.2 % w / v EDTA (20 mM) 1% w / v Tris-Base (pH 8, 100 mM) (31) was prepared. 600 µl of CTAB buffer solution was added to 2 ml tubes with powder in the form of mycelium. It was gently mixed in a water bath following a 30 minutes' incubation at 65 ° C. The tubes were then cooled to room temperature and 600 µl of chloroform-isoamylalcohol (24:1) was added and the tubes were shaken at room temperature for 10 minutes. After this process, it was centrifuged at 5000 rpm for 20 minutes at 15 °C. The top layer (about 300 µl) was taken into new tubes by centrifugation. Then cold isopropanol (400 µl) was added, and the tubes were gently mixed. Following this procedure, the tubes were incubated in ice for 15 minutes to obtain DNA precipitate. The samples were centrifuged at 5000 rpm for 20 minutes at 15 °C, the pellet was washed with 70% 400 µl of 70% ethanol and the tubes were allowed to dry to remove ethanol. The completely dried DNA pellet was dissolved in 0.15ml TE [0.122% w / v Tris-Base (pH = 8, 10 mM), 0.02% w / v EDTA (1 mM)] and the samples were stored at -20 °C. DNA concentration and purity were assessed using a spectrophotometer before PCR amplification. A no-template control (NTC) was included in each PCR reaction to monitor potential contamination, and no amplification was detected in the negative controls.

Polymerase Chain Reaction (PCR)

Alternaria spp. isolates were analyzed by PCR using primers ITS4, ITS5, ACT512, ACT783, TEF2 and TEF4 (21). Of the mentioned primers, ITS amplifies approximately 600 bp, Actin approximately 250 bp and *Tef1-α* 280 bp DNA fragment (Table 2).

Table 2. PCR primers and sequences (20, 21)

Regions	Primer Name	Primer Sequence (5'-3')
ITS	ITS4	TCCTCCGCTTATTGATATGC
	ITS5	GGAAGTAAAAGTCGTAACAAGG
Tef1-α	TEF2 F	CATCGAGAAGTTCGAGAAGG
	TEF4 R	TACTTGAAGGAACCCTTACC
Actin	ACT-512 F	ATGTGCAAGGCCGGTTTCGC
	ACT-783 R	TACGAGTCCTTCTGGCCCAT

The PCR reactions were 2 minutes (1 cycle) at 94 °C, 30 seconds at 93 °C, 2 minutes at 53 °C, 2 minutes at 72 °C (30 cycles) and final extension for 10 minutes (1 cycle) at 72 °C (20, 21).

Agarose Gel Electrophoresis

The agarose gel prepared in 1-1.5% was dissolved by heating in 1X TAE (40 mM Tris, 20 mM glacial acetic acid, 1mM EDTA) solution. 60 µl (1 mM stock) ethidium bromide was added in agarose gel to see DNA under UV light. It was then allowed to cool for 30 minutes. The solution was poured into the gel tank after cooling, and after gel polymerization, the combs were removed and the gel was taken into the electrophoresis tank containing 1xTAE buffer; after loading DNA extracts and samples, they were electrophoresed at 80-100 volts for 50-60 minutes. After this process, the agarose gel electrophoresis process was completed, the gel obtained was placed on the imaging device and the dimensions of ITS, ACT and *Tef1-α* were determined (20, 21).

PCR-RFLP

A reaction mixture containing 10 µl of PCR product, 1 unit of restriction enzyme, enzyme buffer and ddH2O, containing DNA regions replicated to cut with restriction enzymes. *Pst*1, *Hind*III, *Taq*I, *Eco*RI, *Hinf*I and *Hha*I enzymes were used in this study. The reaction mixtures were incubated at optimal temperatures of restriction enzymes. Following incubation, 6X loading dye was added to the samples and agarose gel electrophoresis was performed. The bands obtained after electrophoresis procedures were placed on the gel imaging device and examined.

Results

PCR-RFLP of ITS Region

A total of 29 *Alternaria* spp. isolates were tested in this study, and when ITS4 and ITS5 primes were used, the results showed that a single band of approximately 600 bp was obtained (Figure 1).

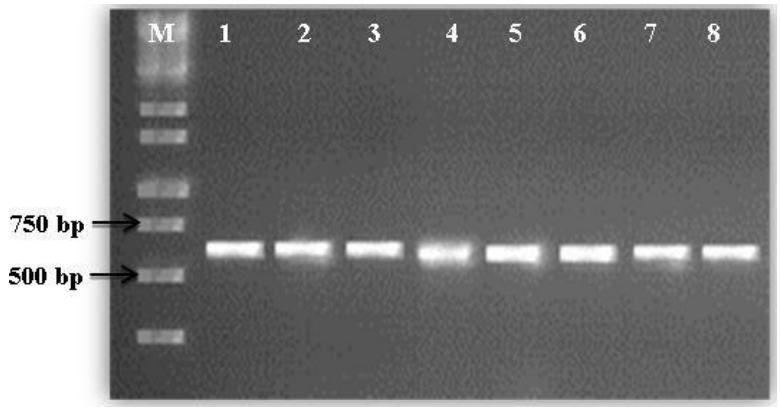

Figure 1. *Alternaria* spp. PCR of rDNA 5.8S ITS in isolates amplification. M: 1 kb DNA marker, 1-8 *Alternaria* spp. isolates

Within the scope of the study, 6 restriction enzymes (*Pst* I, *Hinf* I, *Hind* III, *Eco*R I, *Taq* I and *Hha* I) were used with RFLP technique in the ITS region (Figure 2). The results showed that no fragment formation was observed because of cutting with the *Pst*I enzyme in the ITS region and showed 600 bp band (Figure 2a). Whilst 3 fragments (350, 150, 100 bp) were formed because of the *Hinf*I restriction enzyme cut in the ITS region (Figure 2b). The *Hind* III enzyme recognizes the 5'-A ↓ AGCTT-3 '/ 3'-TTCGA ↑ A-5' point, and because of cutting the ITS region with *Hind* III enzyme in the *Alternaria* spp isolates, 2 fragments (200, 400 bp) were observed (Figure 2c). The *Eco*R I restriction enzyme recognizes the G/ AATTC region. In agarose gel electrophoresis, two DNA fragments of the same size (e.g., 300 bp and 300 bp) will run in line and appear as a single thick/bright fargment on the gel (Figure 2d). The *Taq* I restriction enzyme recognizes the T / CGA region and provides optimum cutting at 65 °C. with this enzyme, and because of the ITS region cut of the *Alternaria* spp. isolates, 4 fragments (250x2, 50x2 bp) were obtained (Figure 2e). For *Hha* I restriction enzyme, 3 fragments (350, 125x2 bp) were formed in the ITS region of the *Alternaria* spp. isolates (Figure 2f).

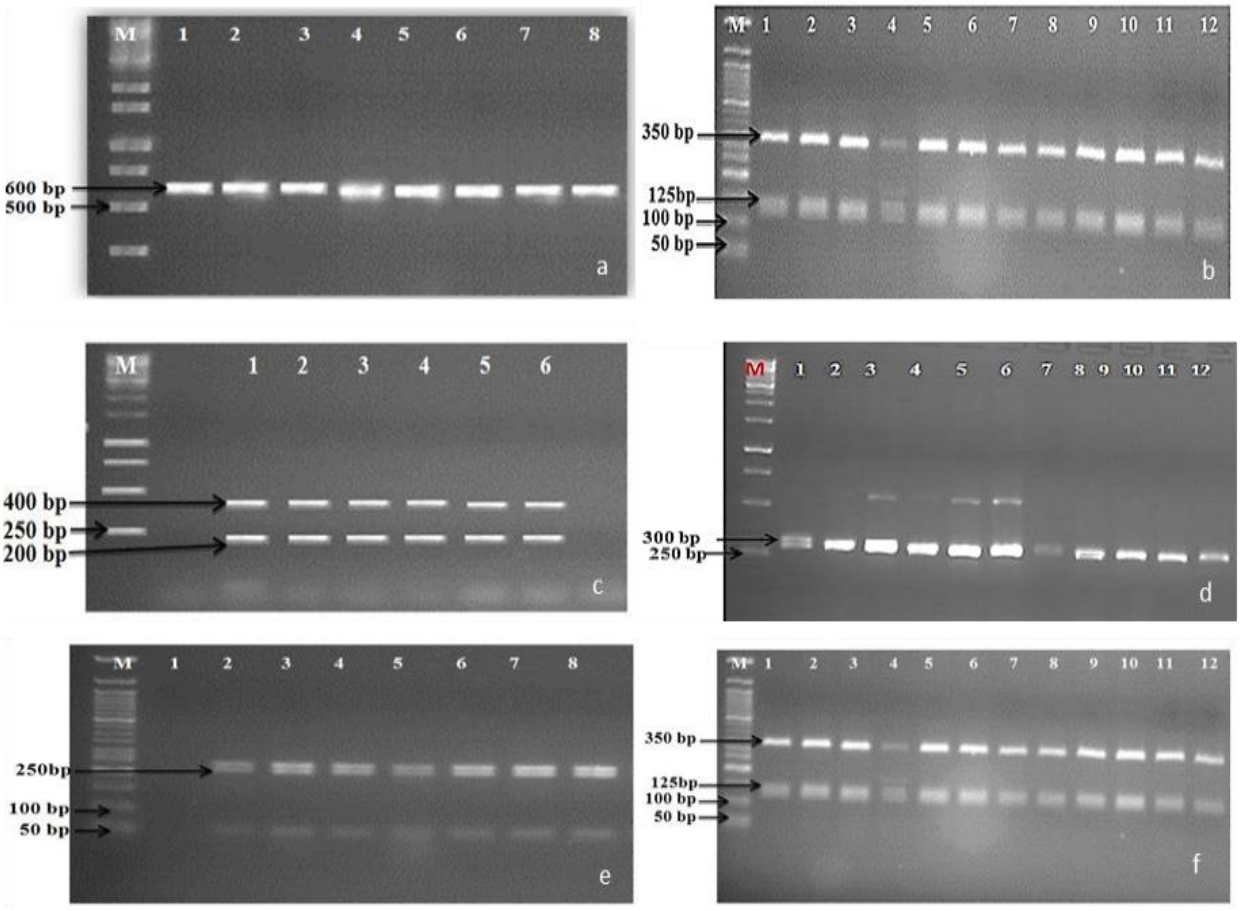

Figure 2. PCR-RFLP band profiles of the *Alternaria* spp ITS region**. a**. 5.8S rDNA ITS PCR-RFLP products of *Pst*I enzyme. M: 1kb DNA marker; 1-8 *Alternaria* spp. isolates; **b**. 5.8S rDNA ITS PCR-RFLP products of *Hinf*I enzyme. M: 50 bp DNA mark; 1-12 *Alternaria* spp. isolates; **c**. 5.8S rDNA ITS PCR-RFLP products of *Hind* III enzyme. M: 1kb DNA mark; 1-6 *Alternaria* spp. isolates; **d.** 5.8S rDNA ITS PCR-RFLP products of *Eco*RI enzyme. M: 1 kb DNA marker; 1-12 *Alternaria* spp. isolates; **e.** 5.8S rDNA ITS PCR-RFLP products of *Taq*I enzyme. M: 50 bp DNA marker; 1-8 *Alternaria* spp. isolates; **f.** 5.8S rDNA ITS PCR-RFLP products of *Hha*I enzyme. M: 50 bp DNA marker; 1-12 *Alternaria* spp. isolates.

PCR-RFLP of Actin region

A single band of approximately 250 bp was obtained from the reactions of 29 *Alternaria* spp. isolates by using ACT-512 F and ACT-783 R primers (Figure 3).

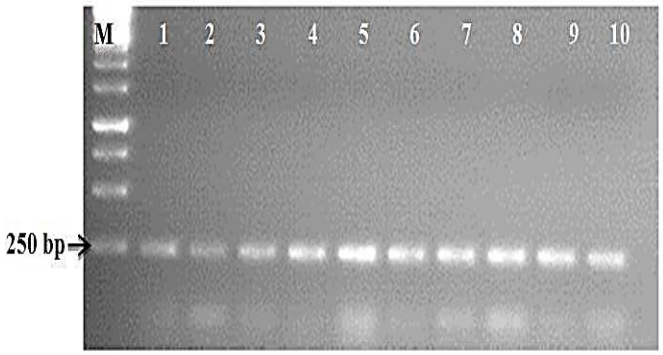

Figure 3. Actin region PCR amplification in *Alternaria* spp. M: 1 kb DNA marker, 1-10 *Alternaria* spp. isolates

PCR-RFLP analysis was performed on the 29 isolates by using ACT-512F and ACT-783R primers and *Pst* I, *Hinf* I, *Hind* III, *Eco*R I, *Taq* I and *Hha* I enzymes. The result of using the *Pst* I, *Eco*R I and *Hind*III enzymes showed that the cut was not obtained (Figure 4), whilst two bands (150, 100 bp) were observed with *Hinf* I and *Taq* I enzymes (Figure 5).

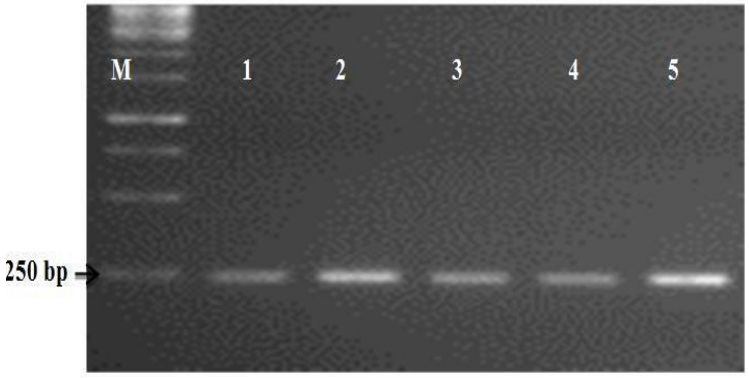

Figure 4. PCR-RFLP image of the Actin region with *Pst* I, *Hind* III and *Hind* III enzymes in *Alternaria* spp. isolates. M: 1 Kb DNA marker; 1-5 *Alternaria* spp. isolates

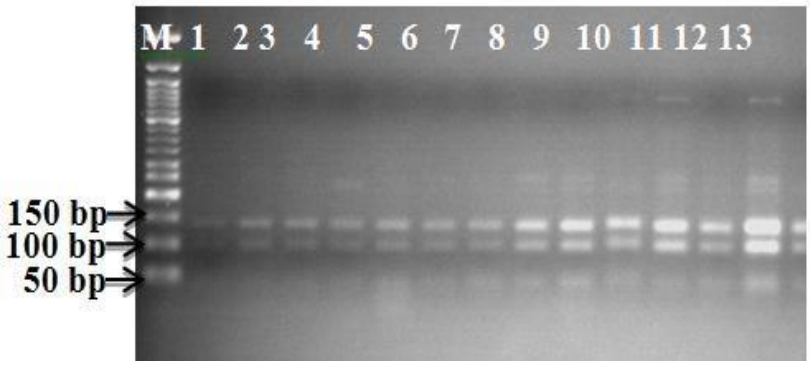

Figure 5. *Alternaria* spp. PCR-RFLP image with *Hinf* I and *Taq* I enzymes of Actin region. M: 50 bp DNA marker; 1-13 *Alternaria* spp. isolates

Moreover, *Hha* I enzyme results in agarose gel were divided into two bands and showed that all samples had the same band size (approximately 180 + 70 bp) by using 50 and 100 bp marker ladder (Figure 6).

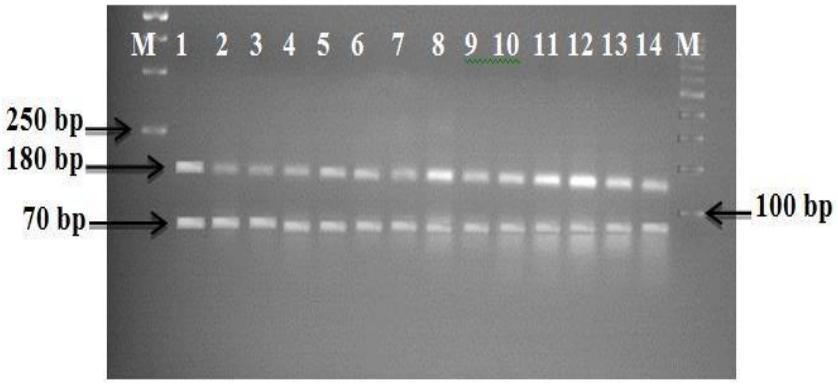

Figure 6. *Alternaria* spp. PCR-RFLP image with *Hha* I enzyme of the Actin region. M: 1 kb and 100 bp DNA are marked; 1-14 *Alternaria* spp. isolates

PCR-RFLP of *Tef1-α* region

A single band of 280 bp was obtained in all isolates of *Alternaria* spp. (Figure 7).

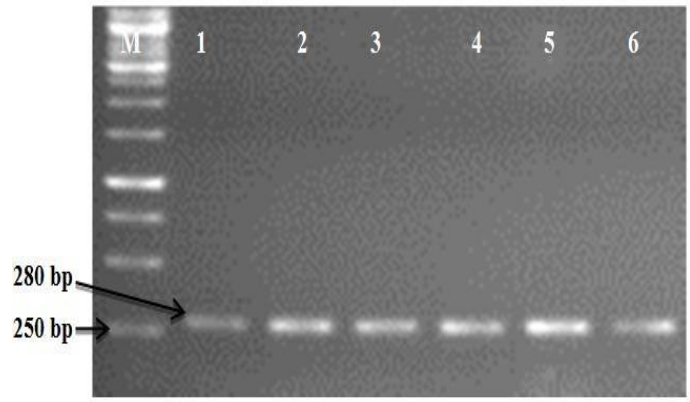

Figure 7. PCR amplification in *Alternaria* isolates using TEF2 and TEF4 primers. M: 1 kb DNA marker, 1-6 *Alternaria* spp. isolates

RFLP analysis was performed using *Pst* I , *Hinf* I, *Hind* III, *Eco*R I and *Taq* I enzymes. The same band size (280 bp) was obtained in all isolates (Figure 8).

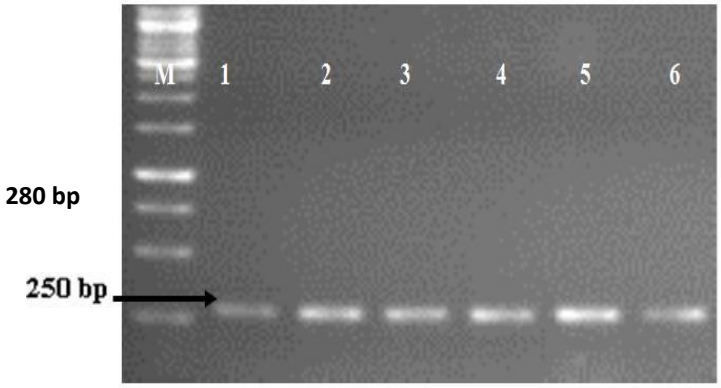

Figure 8. *Alternaria* spp. PCR-RFLP image of the isolates of *Tef1-α* DNA region with *Pst* I, *Hinf* I, *Hind* III, *Eco*R I and *Taq* I enzymes (lines 1-6, respectively). M: 1 kb DNA marker; 1-6 *Alternaria* spp. isolates

As a result of *Hha* I enzyme cutting for the 29 *Alternaria* spp., two bands (180 and 100 bp) were detected(Figure 9).

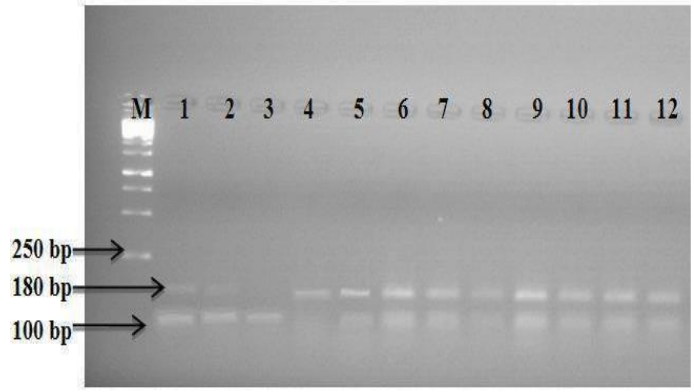

Figure 9. *Alternaria* spp. PCR-RFLP image of the isolates of the *Tef1-α* DNA region with *Hha* I enzyme. M: 1 kb DNA marker; 1-12 *Alternaria* spp. isolates

PCR-RFLP profiles were obtained from using *Pst* I, *Hinf* I, *Hind* III, *Eco*R I, *Taq* I and *Hha* I restriction enzymes of ITS, Actin and *Tef 1-α* regions. it was found that all *Alternaria* spp. isolates did not have polymorphism in terms of ITS, Actin and *Tef1-α* regions (Table 3).

Table 3. PCR-RFLP results of the ITS, ACT and *Tef1-α* regions of *Alternaria* spp.

Regions	**PCR-RFLP**					
	Pst*1	***Hind*III****	***Hinf*I**	***Eco*RI**	***Taq*I**	***Hha*I**
ITS	-	+	+	+	+	+
		NP	NP	NP	NP	NP
Actin	-	-	+	-	+	+
			NP		NP	NP
Tef1-α	-	-	-	-	-	+
						NP

* -: uncut, +:cut
** NP: no polymorphism

Discussion

The current study focused on the Alternaria late blight disease of pistachio in Türkiye since Türkiye is the third largest producer of pistachio in the world and severe disease epidemics have been observed in recent years in Türkiye (9). The disease has been observed on many wild relatives of pistachio in Türkiye and this may contribute to pathogen variability and provides an inoculum source for epidemics on cultivated pistachio. *A. alternata, A. tenuissima* and *A. arborescens* are all small-spored *Alternaria* species that are primarily responsible for the disease worldwide and these pathogens have been isolated from both fruits and leaves (6, 9). The aim of this study was to determine the PCR-RFLP characterization of *Alternaria* isolates collected from pistachios in various cities of Southeast Anatolia region using ITS, *Tef1-α* and Actin gene regions. As a result of the work done, all *Alternaria* spp. species are similar and have conserved sequences in ITS, Actin and *Tef1-α* gene regions.

Since pistachio is of high economic and medical importance, it is naturally important to identify pathogenic species correctly and accurately (12, 13, 18). There are considerable morphological differences between the species in the *Alternaria* genus and this may cause confusion in the definition (32, 33). Inaccurate diagnoses for this genus, genes such as ITS (house-keeping genes) and the mitochondrial small subunit (mtSSU) has become more complex due to the inability of ribosomal DNA to identify small spored *Alternaria* species (34). In Basidiomycetes, the DNA sequence of the ITS region has been reported to be insufficient to distinguish all species (35, 36). In these studies, it has been reported that the sequences of the CT and histone regions should be used to distinguish *Cylindrocladium spathiphylli* and *C. floridanum* species complexes, otherwise no distinction can be made when adhering to the ITS data set (37). Mitochondrial large subunit (mtLSU) gave positive results in ribosomal DNA, CT, actin, calmodulin, chitin synthase, *Tef1-α* or 1.3.8-trihydroxynaphthalene (THN) reductase gene-type discrimination (17, 30).

In the current study, 29 *Alternaria* spp. isolates were analyzed using PCR-RFLP. ITS, Actin and *Tef1-α* regions were amplified with PCR and 600 bp, 250 bp and 280 bp band profiles were obtained, respectively. The absence of polymorphisms in the ITS, Actin, and *Tef1-*α gene regions used does not constitute absolute proof of clonality, but rather indicates that these genes are highly conserved among small spore-forming Alternaria species. This agrees with another studies (6) who could not make a definite distinction because *A. alternata* and *A. tenuissima* groups are very similar to *A. infectoria*.

Restriction fragment length polymorphisms (RFLPs) technique was used to determine the polymorphism of *Alternaria* spp. isolates isolated from pistachio in Türkiye, and this in line with many studies have been conducted using RFLP which has been used extensively in analyzing genetic variability and structure in natural

populations of plant pathogenic fungi (24-26, 35, 38)

Small-spored *Alternaria* species, also known as the *Alternaria* species group or section *Alternaria*, are a well-supported phylogenetic lineage within the genus *Alternaria* (39). *Alternaria alternata, A. tenuissima*, and *A. arborescens* are the most common morpho-species within this group (6, 33, 40).

These taxa are important plant pathogens around the world. Thus, accurate identification is important for pathogen reports, quarantine procedures, as well as exploring biodiversity. Delimitation and identification of species boundaries within the section *Alternaria* are challenging due to similar characteristics with high variability, intermediate characters, and plasticity in their morphological features (15, 16, 38).

In addition to using morphological characters to identify *Alternaria* species, molecular phylogenetic approaches have been utilized in recent years. Mitochondrial large-subunit (mtLSU), ribosomal DNA, beta-tubulin, actin, chitin synthase, translation elongation factor 1-alpha, and 1,3,8-trihydroxynaphthalene reductase regions were insufficient to resolve phylogenies within section *Alternaria* (17, 41). *Endo_PG, ATPase, Alt_a1*, some anonymous regions, and SCAR markers were informative in estimating phylogenies within section *Alternaria* (10, 17-19). However, phylogenetically, the number of species or lineages is involved with this disease is unknown. Moreover, distinguishing species within section *Alternaria* with molecular approaches is required and the previous studies reported some techniques (42). Because of some incongruences between OPA1.3 and morpho-species (43) as well as identification problems of some intermediate types, some additional markers are required. Thus, we aimed to resolve phylogenetic lineages/species within section *Alternaria* causing Alternaria blight of pistachio and its wild relatives and to identify species based on molecular phylogenetic approaches.

Supporting these results, restriction fragment length polymorphism (RFLP) analyses using the λ phage clone *Alt1* as a hybridization probe were not able to differentiate small-spore host-specific *Alternaria* spp. from *A. alternata* (44). However, RFLP analyses using a fragment of the *Alt1* clone were able to segregate small-spored *Alternaria* isolates from pistachio into three distinct groups, although critical morphological characterization of these isolates was not performed (39, 45). Sequence analysis of the nuclear rDNA internal transcribed spacer (ITS) region has revealed variation (1 to 4 nucleotides) among small-spored *Alternaria* spp., but failed to resolve these taxa as phylogenetically distinct from *A. alternata* (46). However, random amplified polymorphism DNA (RAPD) analyses of *A. alternata*, *A. tenuissima*, *A. infectoria*, and small-spored hostspecific species revealed distinctive RAPD fragment patterns for all species, and cluster analysis did resolve these species into distinct clades, which suggests that these taxa constitute well-defined species (6, 47, 48).

Conclusion

The purpose of this work was to evaluate the phylogenetic relationships among *Alternaria* isolates from pistachio using a RFLP molecular techniques to further our understanding of the taxonomic status of these *Alternaria* spp.. Previous molecular studies have not produced a consensus on the genetic relationships among small spored catenulate *Alternaria* spp., which underscores the controversial taxonomic status of this group of fungi. In future studies, the use of more distinctive markers (or Next-Generation Sequencing - WGS techniques) such as Alt_a1 and endoPG will reveal population dynamics more clearly.

Declarations

Ethical Approval Statement

Not applicable to this article.

Author Contribution Statement

All the authors read, approved the final manuscript and all the authors contributed to the study. S.J.T.A, A.A and S.B. performed molecular characterization. O.A., C.C supervised the manuscript.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

Declaration on the Use of AI

During the preparation of this study, artificial intelligence tools were used exclusively for language assistance and for correcting spelling and grammar. The author retains full responsibility for all scientific content, as well as for the interpretations and conclusions presented in this work.

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