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Pathogenicity, Morpho-Species and Mating Types of *Alternaria* spp. causing Alternaria blight in *Pistacia* spp. in Turkey

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Abstract *Alternaria* genus includes many plant pathogens on numerous hosts, causing leaf spots, rots and blights. Alternaria blight has been observed as one of the important fungal diseases of pistachio (*Pistacia vera* L.) as well as its wild relatives (*P. terebinthus, P. lentiscus, P. khinjuk, P. atlantica, P. mutica*) in Turkey. *Alternaria* species were sampled from *Pistacia* spp. hosts from different geographic regions in Turkey during field trips in late spring to early fall of 2013. Alternaria blight symptoms were observed mainly on fruits and rarely on leaves. Four hundred and twenty two of the isolates were morphologically defined as *A. alternata*, *A. tenuissima*, *A. arborescens* and also intermediate morpho-species between *A. alternata/A. arborescens*. Pathogenicity of the isolates was confirmed with host inoculations on detached fruits. Mating types of 270 isolates of *Alternaria* spp. from the collection were identified using a PCR-based mating type assay that amplifies either a *MAT1-1* or a *MAT1-2* fragment from the mating locus. Although a strongly clonal population structure was expected due to the putative asexual reproduction of these fungi, both idiomorphs were detected at equal frequencies at several different spatial scales. The distribution of mating types within each geographic region, within host species as well as in overall collection was not significantly different from 1:1. Amplified fragments of partial idiomorph sequences were obtained for representative isolates. Parsimony trees were depicted based on sequence data of mating type genes for these representative isolates as well as some other *Alternaria* species obtained by Genebank. Several point mutations presented a few clusters which are supported by high bootsrapped values. The Alternaria blight disease agents both from cultivated and wild hosts were pathogenic on pistachio which may cause difficulties to control the disease because of extensity of pathogen sources. Besides, equal mating type distribution of the pathogen at both geographic and host species levels suggests a potential for sexual reproduction of *Alternaria* spp. in Turkey.

Keywords *Alternaria* · Mating type · Pistachio · Alternaria blight · Pathogenicity

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Introduction

Turkey is one of the centers of origin of pistachio (*Pistacia vera* L.) and many wild relatives (*Pistacia* spp.) grow sympatrically with cultivated pistachio in

Turkey (Zohary 1952; Whitehouse 1957). Alternaria blight of pistachio is one of the most economically significant diseases of this important crop. *Alternaria* spp. from pistachio has been reported from in Egypt (Wasfy et al. 1974), California/USA (Michailides et al. 1995), South Africa (Swart and Blodgett 1998), Australia (Ash and Lanoiselet 2001). The disease causes premature defoliation, blight symptoms on leaves and the staining of nutshells, thus reducing fruit quality (Michailides 2005). Under favourable climatic and environmental conditions, the disease is highly destructive. Turkey is the third largest producer of pistachio in the world and severe disease epidemics have been observed in recent years in Turkey. The disease has been observed on many wild relatives of pistachio in Turkey 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 (Pryor and Michailides 2002). There is no detailed information about the disease and its pathogens in Turkey and no report about Alternaria blight of wild *Pistacia* spp. worldwide.

It has been known that morphology based identifications for section *Alternaria* is 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 (Rotem 1994; Simmons 2007; Tymon et al. 2016; Ozkilinc et al. 2017). 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 recoginition as applied in some previous studies (Peever et al. 2004; Andrew et al. 2009; Stewart et al. 2014; Tymon et al. 2016; Ozkilinc et al. 2017).

The mating type locus (*MAT*) controls sexual reproduction in many fungi and the structure of *MAT* locus differs among ascomycete fungi (Turgeon 1998). A single mating type locus carries either of the two alternate alleles, *MAT1-1* or *MAT1-2*, termed idiomorphs rather than alleles due to their significant sequence dissimiliarity (Turgeon and Yoder 2000). Even though the *Alternaria* genus comprises mostly asexual species, mating type genes have been identified in several *Alternaria* spp. (Arie et al. 2000; Berbee et al. 2003; Stewart et al. 2011) as well as in other putatively asexual

ascomycete fungi (Sharon et al. 1996; Paoletti et al. 2005; Groenewald et al. 2006). The *MAT1* locus of asexual *Alternaria* species was shown to be functional when used to transform a *MAT1*-deficient mutant of a closely related sexual species (Arie et al. 2000; Berbee et al. 2003). The mating type locus of several *Alternaria* species has been characterized and primers designed to estimate mating type ratios in populations via PCR (Arie et al. 2000; Berbee et al. 2003; Stewart et al. 2011). Both mating types from *A. alternata* and only *MAT1-2* from *A. arborescens* were reported in small scale samples from several hosts (Arie et al. 2000; Berbee et al. 2003; Stewart et al. 2011). A 1:1 ratio of mating types was found among 90 isolates of *A. alternata* sampled from diverse hosts and geographic locations (Armitage et al. 2013). Sequence variation detected at the mating type locus can be used to detect recombination and to estimate phylogenies. Rate of evolution and sequence variation at the *MAT* locus can vary at the species or genus level. For example, *MAT* sequence variation of *Cochliobolus* samples was low within species but high between species (Turgeon 1998). Recently, Stewart et al. (2011) evaluated sequences of mating type genes in *Alternaria* species considering nucleotide diversity, substitution ratios and codon usage statistics. Purifying selection and biased codon usage were detected at the *MAT1* locus in *Alternaria* spp. and it was explained by a recent sexual past, cryptic sexual present or additional important cellular role of the mating type genes (Stewart et al. 2011).

Mating type ratios in fungal populations are widely used indicators of reproductive mode. For example, distribution of *MAT* idiomorphs in a global collection of *Aspergillus fumigatus*, 43% of isolates carried the *MAT1-1* idiomorph and 57% carried the *MAT1-2* idiomorphs (Paoletti et al. 2005). Detection of a near 1:1 distribution ratio of *MAT* idiomorphs for a worldwide collection of 2035 *Mycosphaerella graminicola* isolates was concluded as evidence of sexual reproduction (Zhan et al. 2002). Mating type distribution of Alternaria blight disease agents should be informative to learn about pathogen population biology. There is no information about mating type ratios and the distribution of mating types for *Alternaria* pathogens in Turkey. Besides, there is no information about mating types of *Alternaria* pathogens specifically from wild *Pistacia* hosts anywhere in the world. Determination of mating type distributions over different locations and hosts may reflect reproductive behaviour of the pathogen and

selection pressures on mating types. Moreover, diversity of mating types of these pathogens in cultivated and wild *Pistacia* ecosystems will give an insight to understand disease and pathogen evolution in a broad ecological range.

The objectives of this research were (i) to identify the pathogens causing Alternaria blight of *Pistacia* spp. in Turkey using morphological criteria; (ii) to confirm their pathogenicity on pistachio; (iii) to determine mating types of the *Alternaria* spp. isolates within and among populations sampled from wild and cultivated pistachio in different geographic locations; (iv) to compare sequence variation of each mating type idiomorph. This study will give first information about the pathogens causing Alternaria blight in wild and cultivated *Pistacia* spp. in Turkey and could be useful to improve disease management through increased knowledge of pathogen biology.

Materials and methods

Fungal Sampling

Collecting trips were carried out from late spring (May-June) to early fall (August-September) of 2013 across Turkey. Pistachio and its wild relatives occur mostly in Southeastern, Mediterrenean, Agean and South Marmara regions of Turkey with both cultivated species and wild relatives with sympatric distribution in these regions (Fig. 1). Sampling was carried out from both pistachio trees (*P. vera*) and wild relatives (*P. terebinthus, P. lentiscus, P. khinjuk, P. atlantica, P. mutica*). Sampling information showing host and region are shown in Supplementary Table. Thus, sampling was done from 332 trees at 287 different locations in 17 cities taking place in four geographic regions (Supplemantary Table). Dark brown to black blighted lesions were seen commonly on fruits and rarely on leaves. Each plant sample were treated seperately to discriminate pathogen sampling origin. Several fruits showing Alternaria bight symptoms were collected from a tree and kept in a paper bag and were taken to the laboratory within 24-48 h to isolate pathogens. Fruits were washed with tap water and then cut into small pieces (such as about 0.5-1 cm^3 dimensions) bearing disease symptoms. These pieces were surface sterilized with 3% NaOCl solution for three minutes, then, rinsed in sterile distilled water three times and plated on potato

dextrose agar (PDA) (Merck). Plates were incubated 3-5 days under cool white fluorescent light at 23 °C and 12 h photoperiod. *Alternaria* colonies were subcultured for single spore isolation. Cultures were maintained as single spore colonies and stored in filter papers at −20 °C. Four hundred-twenty two isolates were obtained. A specific code was given each isolate from one tree. Codes represented sampling province, orchard number and tree number. Additional isolation number if there is one more isolation per tree (Supplemantary Table). G or GY symbols were added if sampling was done in the late season (Supplemantary Table).

Morphological identification

Colony type and sporulation pattern was recorded for all isolates using the morphological criteria of Pryor and Michailides (2002) and Simmons (2007). Isolates were grown on PDA and weak-PDA for 7-10 days in incubators providing a 12 h dark/12 h light cycle at 20 ± 2 °C (Pryor and Michailides 2002). Moreover, conidiophore lengths were measured for some representative isolates following the criteria of Simmons (2007) and Tymon et al. (2016). Isolates were grown on potato carrot agar at 23 °C at 16 h dark and 8 h light cycle during 5-7 days prior to condiophore length measurements (Simmons 2007; Tymon et al. 2016). Morphologies were also observed and recorded for reference isolates *A. arborescens* (EGS 39-128), *A. alternata* (EGS 34-016 and EGS34-039) and *A. tenuissima* (EGS 34-015), kindly provided by Prof. Dr. B.M. Pryor (Arizona State University, Dept. of Plant Pathology, Tucson, Arizona/ U.S.A.) and compared with the pistachio isolates sampled from Turkey.

Pathogenicity

Pathogenicity of the isolates was tested on pistachio fruits (cultivar Ohadi) obtained from pistachio trees grown in orchards of Pistachio Research Institute (Gaziantep, Turkey). Healthy fruits were washed with tap water and sterilized with 3% NaOCl solution for three minutes, rinsed in sterile distilled water three times then stored between filter papers until completely dry. Fruits were placed on plastic mesh above moistened filter papers in the bottom of clean plastic boxes to maintain humidity. Each box was divided in three zones using aluminum foil strips. Fifteen fruits were placed per box with five fruits per zone. One box was prepared for one fungal isolate. The

Fig. 1 Map of Turkey showing cities where *Pistacia* spp. samples diseased with Alternaria blight were collected. Thin lines show city borders, thick lines show borders of geographic regions. Map

source: https://tr.wikipedia.org/wiki/Dosya:Turkey_region_map_with_province_borders.svg (creative commons)

fruits were sprayed with conidial suspensions of 1×10^6 conidia ml^{-1} produced from 7 to 10 days old cultures grown on PDA, and controls were sprayed with sterile dH_2O. Three hundred fifty-eight isolates were tested. Due to low sporulation of 54 of the isolates, mycelium was harvested directly from the culture plate and applied to the fruit surface using a sterile rod to test their pathogenicity. Following inoculation, boxes were sealed and put into growth chambers with 12 h dark/light cycle at 20 ± 2 °C. Disease symptoms were evaluated after 7 days. The isolates originating from pistachio leaves (coded as 33-34, 35-37/Y1, 35-45, 35-22, 45-64, 17-13-GY), leaf stalk (coded as 35-37/leaf stalk) and one randomly selected isolate from pistachio fruit (coded as 35-13) (Supplemantary Table) were also tested for pathogenicity to pistachio leaves. Tests were performed on detached leaves of cv. Ohadi. For each isolate, four fully expanded and healthy leaves were used. Two leaves (one with abaxial side up the other with adaxial side up) were placed in plastic boxes prepared as described above but not divided in zones. For the leaf inoculation, 20 µl of a conidial suspension (10^6 conidia ml^{-1}) were placed in two different spots on each leaf. Following inoculation, boxes were sealed and placed into growth chambers with a 12 h dark/light cycle at 20 ± 2 °C. As control, 20 µl of sterile distilled water were placed in two different spots on leaves and incubated in the same conditions. Leaves were monitored until the symptoms appeared and the experiment was ended at day 7th after inoculation.

Fungal DNA isolation

For DNA extraction, fungal mycelia were grown in potato dextrose broth (PDB) on a rotary shaker at 120 rpm at room temperature for 5-7 days. Growth medium was removed using a vacuum pump and mycelia were freeze-dried for 48 h. Dried mycelia were powdered using sterile metal rod before DNA isolation. Fungal DNA isolation was performed using the i-genomic Plant DNA isolation kit (Invitrogen). DNA concentrations were estimated using a Nanoquant spectrophotometer (NanoQuant Infinite M200, Tecan, Austria) and DNAs adjusted to 10-15 ng $µl^{-1}$ concentrations.

Mating type determination

Two hundred and seventy isolates were chosen from the collection considering to represent different geographic location and host origin. The PCR-based mating-type marker system developed for *A. alternata* by Stewart (2011) was used to identify mating types in *A. alternata*, *A. tenuissima* and *A. arborescens* populations sampled from *Pistacia* spp. Primers ALMAT-L (GCAAGATT CTAGGCCCAACG) /AA-MAT1-867 (TGCGGTGG GGAGTAGTGT) and AAMAT2-1691 (CAGCACCC CGACTAC AAGTAT)/AsM1-8 (GGTCGTGA GTCGTGATCG) were used to amplify *MAT1-1* and *MAT1-2* genes, respectively (Stewart 2011). These

primers were designed designed to the *MAT1-1* [GenBank: AB009451] and *MAT1-2* [GenBank: AB009452] idiomorphs of the Japanese pear pathotype of *A. alternata* (Arie et al. 2000) by Stewart (2011). Several isolates of *A. alternata* isolated from citrus with known mating type (SH-MIL-34 s, SH-MIL-11 s, SH-MIL-22 s for MAT1-1, and SH-MIL-14 s for MAT1-2), kindly provided by T.L. Peever (Washington State University, Plant Pathology Dept., Pullman/WA, U.S.A.), were used as positive controls. These isolates, plus *A. arborescens* isolate EGS39-128 (MAT1-2) and the other two reference isolates (EGS 34-016 and EGS 34-015 for MAT1-2) used in morphological identifications were included in the analysis. Twenty five μL PCR reaction mixtures contained 10-15 ng of template DNA, 1X PCR buffer (Applied Biological Materials Inc., Canada), 1.5 mM MgCl$_2$ (Applied Biological Materials Inc., Canada) 200 μM dNTPs, and 1 μM of each primer. PCR conditions were 95 °C for 5 min, 35 cycles at 95 °C for 20 s, 58 °C for 30 s, and 72 °C for 30 s followed by a final step at 72 °C for 10 min. PCR amplifications were conducted separately for each *MAT* primer pair. Reactions were carried out with a Bio-Rad T100 thermocycler (USA). PCR products were detected on 1.5% agarose gel and visualized under UV light using a Vilber Lourmat Quantum ST4 1100 gel documentation system (Vilber Lourmat, France). Each isolate gave product 640 bp for *MAT1-1* and 880 bp for *MAT1-2*. No isolate produced both amplicons. Negative control reactions without template DNA gave no amplicon. Mating-type distributions were tested for deviation from the expected ratios of 1:1 using chi-square goodness-of-fit tests.

Sequences of *MAT1-1* and *MAT1-2* idiomorphs

MAT1-1 and *MAT1-2* amplicons were sequenced for 16 mating type 1 and 22 mating type 2 isolates from the collection, respectively. Representative isolates of *A. alternata, A. tenuissima* and *A. arborescens* morphospecies carrying either the *MAT1-1* or *MAT1-2* idiomorph were chosen to represent *Alternaria* species sampled from *Pistacia* spp. Seven isolates were chosen as positive control isolates including the MAT1-1 isolates SH-ML-11 s, SH-ML-34 s, and SH-ML-22 s, and MAT1-2 isolates EGS 39-128, EGS 34-016, EGS 34-015, and SH-ML-14 s. PCR amplicons were sequenced using an ABI 3500xL Genetic Analyzer (Applied Biosystems, MedSanTek Lab, Turkey). Sequences of both strands were obtained for each

amplified fragment and edited in Bioedit v7.0.53 for Windows software (Hall 1999) and aligned using clustalW implemented in BioEdit software. Each consensus sequence was also used as a query to search similarities using BLASTn of the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990). Coding regions were identified using NCBI database Blastn-tools. Number of haplotypes, Tajima's neutrality test and substitution rates were estimated using DnaSP v.5 (Rozas et al. 2003). *MAT1-1* and *MAT1-2* sequences of different *Alternaria* species deposited in Genebank were obtained to compare phylogenies based on mating type sequences within the sampled isolates. *Alternaria* species Genebank were *A. brassicae* (AB444165.1 and AY042091), *A. brassicicola* (AB444163.1 and AB444162.1), *A. citriarbusti* (AB444181.1 and AB444168.1), *A. citrimacularis* (AB444184.1 and AB444185.1), *A. gaisen* (AB444187.1), *Lewia infectoria* (AB444188.1), *A. longipes* (AB444192.1), *A. perangusta* (AB444194.1), *A. solani* (AB444198.1 and AB444196.1), *A. colombiana* (AB444186.1), *A. limicola* (AB444189.1), *A. limoniasperae* (AB444190.1), *A. toxicogenica* (AB444201.1). *Stemphylum* sp. (AY339861.1 and AY340942) was selected as outgroups. ClustalW implemented in BioEdit v7.0.53 was used for multiple sequence alignment for each data set. Parsimony analysis was performed in PAUP* ver. 4.0b10 (Swofford 2003). Analyses were conducted by 20 replicate heuristic searches consisting of 1000 stepwise random addition replicates with branch swapping by the tree-bisection-reconnection algorithm. The trees were rooted with outgroup. Branch stability for each dataset was evaluated by 1000 bootstrap replications using a heuristic search with simple sequence addition to produce a strict-rule consensus tree with nodal support values. Nodes with greater than 70% bootstrap support values were considered significant. Trees were visualized using Acrhaeopteryx v.09901 beta (Han and Zmasek 2009).

Results

Morpho-species of *Alternaria* causing Alternaria blight of *Pistacia* spp.

Among total 422 isolates of *Alternaria* spp. sampled from *Pistacia* spp. throughout Turkey, 330 were identified morphologically as *A. alternata*, 88 as *A. tenuissima,* 1 isolate as *A. arborescens* and 3 isolates

as *A. alternata/A. arborescens* intermediates (Supplementary Table). Isolate 02-28 showed typical arborecens type branching pattern and had a conidiophore length of 201 μm. Three isolates (02-29-1, 07-17-2/GY, and 31-09) showed intermediate characters between *A. alternata* and *A. arborescens* in terms of sporulation pattern. Conidiophore lengths were 127 and 171 μm for isolate 02-29-1 and 31-09, respectively. Conidiophore lengths were measured for representative isolates randomly chosen from collection which were *A. alternata* or *A. tenuissima* and conidiophore lengths of these isolates were less than 100 μm.

Pathogenicity of *Alternaria* spp. isolates from *Pistacia* spp.

All isolates, inoculated as spore suspension or as mycelium, caused typical Alternaria blight symptoms. Brown to dark lesions were observed on fruit shell. Thus, all of the isolates tested were confirmed as pathogenic to pistachio fruits. Isolates tested on leaves were also pathogenic on pistachio leaves and caused necrotic lesions.

Distribution of mating types

Mating type of 270 isolates of *Alternaria* spp. were determined. On average, across all populations analyzed, mating type frequencies did not differ significantly from a 1:1 ratio (Table 1). Distribution of mating types according to the geographic region and host species did not differ from 1:1 in each of the category, as well (Table 1). Mating type distribution of isolates based on morphospecies were evaluated for *A. alternata/A.tenuissima*, *A. arborescens* and intermediate types. Mating type of isolate 02-28, the only *A. arborescens* morphospecies identified in the sample was MAT1-2. Isolates represented as intermediate morpho-species were evaluated seperately. Isolate 02-29-1 was MAT1-2, isolates 31-09 and 07-17-2_GY were MAT1-1. The mating types ratio in the overall sample was 126:138 for isolates *A. alternata/A. tenuissima* (Chi-square value = 0.545, *P* = 0.460).

Sequence analysis of mating type idiomorphs

Five hundred sixty-eight bp of sequence data was obtained for MAT1-1 isolates representing *A. alternata, A. tenuissima,* and *A. alternata/arborescens* morphospecies

Table 1 Mating type ratios of *Alternaria* spp. sampled from *Pistacia* spp.according to geographic regions, host ans well as a regional scale

Sampling Source	MAT1-1: MAT1-2	Chi-Square	*P* value
Geographic Region			
Southeastern Anatolia	57:74	2.20	0.14
Mediterranean	24:21	0.2	0.65
Aegean	37:33	0.262	0.63
Marmara	11:13	0.0	0.68
Host			
P. vera	85:100	1.21	0.27
P. terebinthus	30:28	0.06	0.79
P. khinjuk	1:1	0.0	1.00
P. atlantica	7:6	0.07	0.78
P. lentiscus	4:3	0.14	0.70
P. mutica	2:3	0.2	0.65
All collection	129:141	0.533	0.465

from our collection. Nineteen variable sites including 13 parsimony-informative sites were observed. The sequenced region included both intron (349-568 bp) and exon (89-568 bp). One singleton mutation and 1 parsimony informative mutation were observed in the exon. Nine haplotypes were observed among 19 isolates based on *MAT1-1* sequence data. 712 bp sequence data was obtained for *MAT1-2* for 26 *A. alternata/tenuissima/arborescens* samples from our collection and 9 haplotypes were detected. 50 variable sites including 28 parsimony informative sites were recorded. Sequenced *MAT1-2* region included both exon (1-356 bp) and intron (357-712 bp) regions. 10 singleton and 10 parsimony informative mutations were seen in exon part. The estimated

Fig. 2 Phylogenetic trees inferred from Maximum Parsimony (MP) analysis of sequences from mating type 1 (a) and mating type 2 (b) for *Alternaria* spp. Information about isolates from *Pistacia* spp. were provided by Supplementary Table. Morphospecies of the isolates were presented in parantheses. Reference isolates used for morphology are EGS39.128 (*A. arborescens*), EGS 34.16 (*A. alternata*) and EGS34.15 (*A. tenuissima*). SH-ML coded isolates were used as reference which their mating types were known. Sequences of *MAT1-1* and *MAT1-2* genes obtained in this study deposited in Genebank (accession numbers from KY559241 to KY559286). Sequence data of other *Alternaria* species were obtained from NCBI-genebank. Branch lengths are proprotional to number of substitutions. Bootstrap support values (1000 replicates) above 70% are shown

a

b
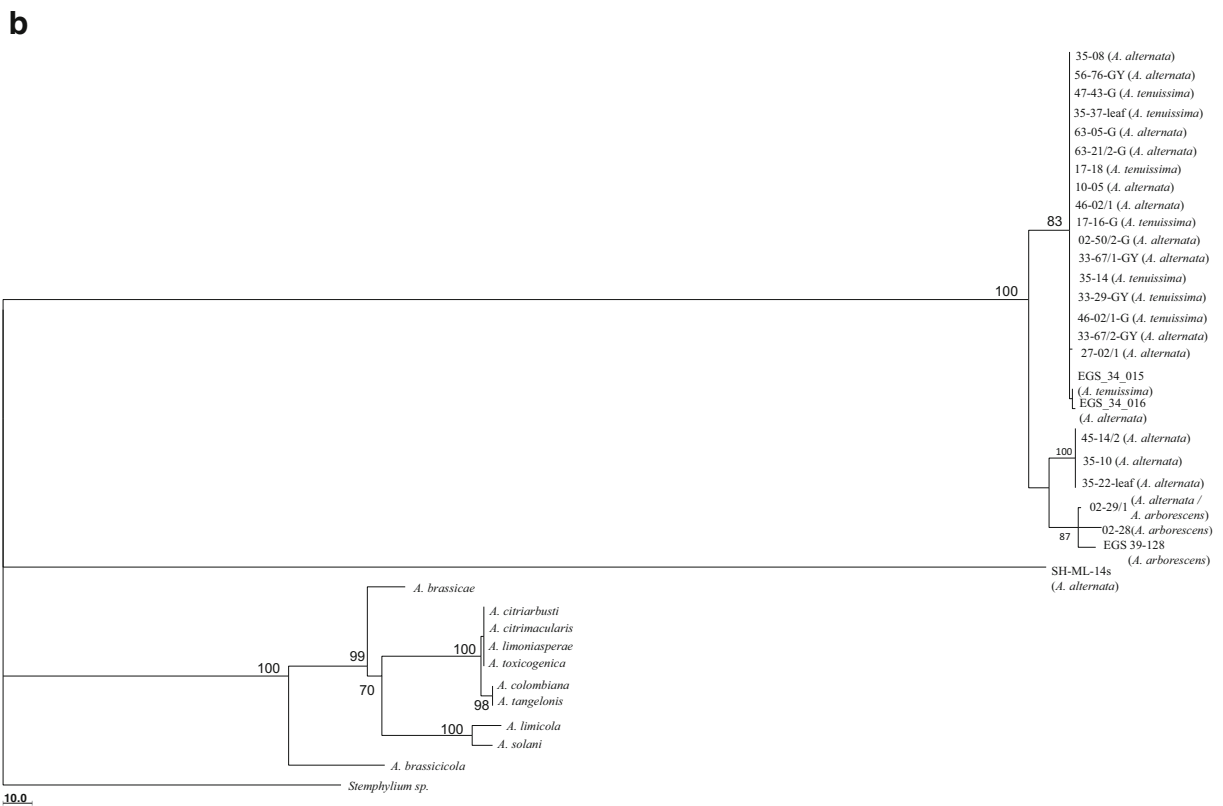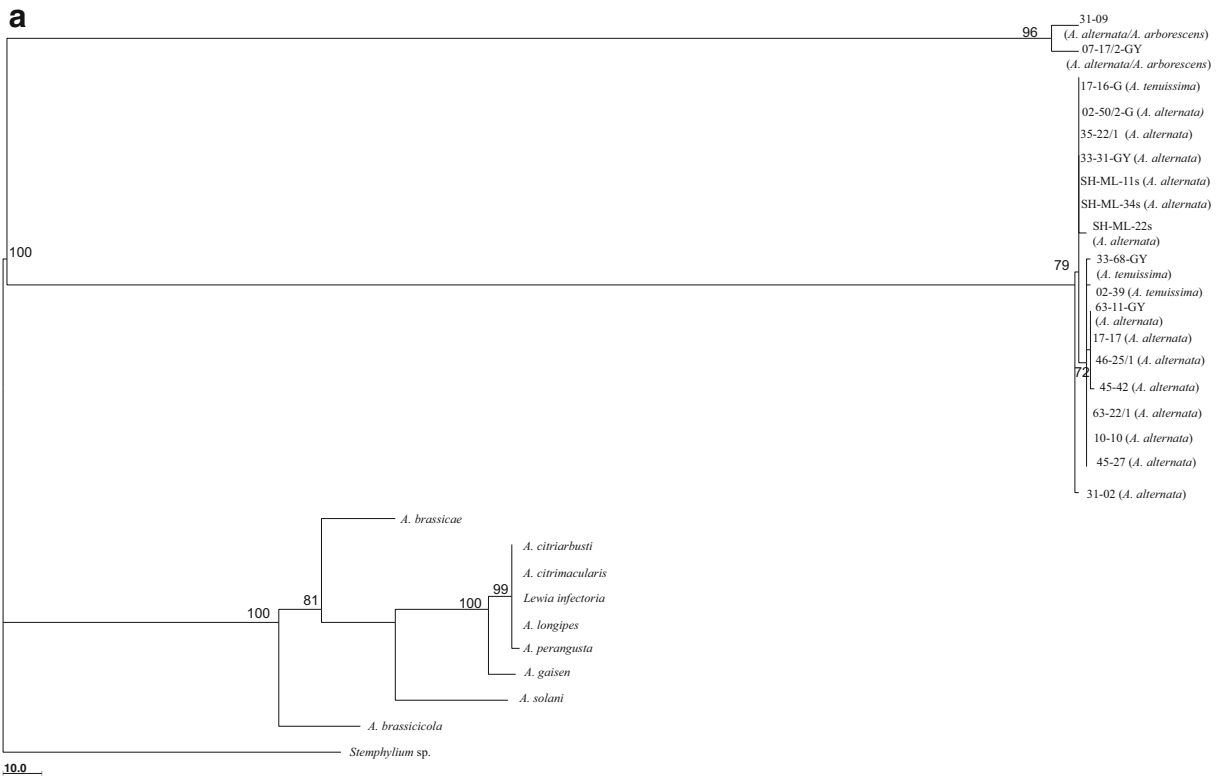
Transition/Transversion bias was $R = 5.73$ for *MAT1-1* region and $R = 3.59$ for *MAT1-2* region. Samples presented insignificant negative Tajima's values for both *MAT1-1* and *MAT1-2* regions which indicates possible population expansion. Sequences of *MAT1-1* and *MAT1-2* genes for our collection and reference isolates used in this study were deposited in Genebank (accession numbers from KY559241 to KY559286).

Sequence comparison were made within MAT1-1 and MAT1-2 isolates of *Alternaria* spp. and parsimony based trees were depicted (Fig. 2a-b). Moreover, several sequence data of *MAT1* region of *Alternaria* spp. deposited in NCBI used for comparison. One most parsimonious tree was obtained for *MAT1-2* and one of the tree was arbitrarily selected among two most parsimonious trees for *MAT1-1*. For both idiomorphs, sequences of these additional *Alternaria* species were highly distinct from our small spored *Alternaria* sequences (Fig. 2). Several number of mutations supported few clusters within the samples. Moreover, parsimony based analysis of *MAT1-1* and *MAT1-2* sequences indicated a distinction between morpho-species of *A. alternata/tenuissima* and *A. arborescens*.

Discussion

This is the first comprehensive work characterizing the casual agent of Alternaria blight of cultivated pistachio and its wild relatives in Turkey on a large geographic and host scale. Most of the fungi causing this disease on both cultivated and wild hosts were classified morphologically as *A. alternata*. Even though the same disease mainly effects leaves of pistachio in U.S.A. (Pryor and Michailides 2002), the disease mainly occurs on fruits rather than leaves in Turkey. Only seven isolates were isolated from leaves among four hundred-twenty two isolates in the collection. This could be due the fact that *Septoria* and *Septoria*-like pathogens are very common disease agents on leaves of pistachio in Turkey (Crous et al. 2013) and probably competition does not allow for *Alternaria* infection on leaves. Because *Alternaria* pathogens obtained both from leaves and fruits have a capability to infect pistachio leaves as shown in our experiments.

A. alternata was found as the most common pathogen of this disease. Only one typical *A. arborescens* was found in a large geographic and host scale sampling collection. These three morpho-species were reported

as Alternaria blight pathogen of pistachio in U.S.A. and frequency of these morpho-species did not present extreme differences in pathogen samples from both leaves and fruit parts (Pryor and Michailides 2002). Moreover there were three *A. alternata / A.arborescens* intermediate type isolates within the collection. Morphologically intermediate types were reported in small-spored *Alternaria* species collection from a wide host ranges (Andrew et al. 2009). Tymon et al. (2016) used morphology based keys to distinguish small-spored *Alternaria* species from potato. According to branching pattern, length of conida and conidiaphore, moprhospecies *A. alternata*, *A. tenuissima* and *A. arborescens* were determined, but, restriction digest assay using ApaI restriction site in the OPA1-3 regions was distinguished the isolates either *A. alternata/tenuissima* or *A. arborescens* (Tymon et al. 2016). Besides, inconsistent between morphology and molecular based assay was observed for few isolates of *A. alternata/tenuissima* and *A. arborescens* (Tymon et al. 2016). Defining *A. alternata* and *A. tenuissima* as a distinct morpho-species is difficult because morphological characters are highly overlapping and vulnerable to show plasticity or absence/inadequate of key morphological characters. Besides, these two morhopspecies can not be distinguished by molecular phylogenetics, as well. (Ozkilinc et al. 2017; Ozkilinc H., unpublished data). In a recent study, *A. alternata* and *A. tenuissima* obtained from a diverse host range were considered as one species group in phylogenetic analysis comparing two sections of *Alternaria* genus (Ozkilinc et al. 2017). While *A. alternata* and *A. tenuissima* can not be distinguished based on molecular and phylogenetic approaches, *A. arborescens* is recognized as a distinct species from *A. alternata/tenuissima* (Tymon et al. 2016; Ozkilinc et al. 2017; Ozkilinc H. unpublished data). Some phylogenetic and molecular approaches were applied on an isolate collection used in this study and will be discussed in a future publication (Ozkilinc H. unpublished data).

Equal ratios of mating types grouped based on geographic region, host as well as across all collection could be an evidence that balancing selection is acting on these idiomorphs. In some other haploid, heterothallic fungi, similarly, the ratio of mating type idiomorphs was used as evidence of sexuality (Zhan et al. 2002; Paoletti et al. 2005). Even though indirect indication of sexuality cannot be a proof of sexuality (Schurko et al. 2009), our data is also a contribution to a previous results of

indirect evidence for possible sexual reproduction in the *Alternaria* genus, specifically in small-spored *Alternaria* species (Linde et al. 2010; Stewart et al. 2011, 2014). Thus, these populations may not really asexual or some kind of selection on *MAT* locus may maintain both idiomorphs in equal frequency which requires detailed investigation of cellular roles of mating type gene products.

While there are report for mating types of *A. alternata/tenuissima* in literature, there has been only one report for *A. arborescens* which is for EGS39-128 isolate carrying mating type MAT1-2. We also detected MAT1-2 type for *A. arborescens* isolate coded as 02-28 within the collection. Among *A. alternata/arborescens* intermediate type isolates, two of these isolates (31-09 and 07-17-2-GY) were MAT1-1 type and the other one was MAT1-2 type. Moreover, it was detected a genetically distinct cluster among *A. alternata* isolates based on sequences of *MAT1-2* region (Fig. 2b) and the isolates were from wild *Pistacia* species in this cluster (Supplementary Table) which indicated diversity in populations from native hosts.

Mutations occuring in the mating type idiomorphic sequences revealed several different haplotypes within small-spored *Alternaria* species from *Pistacia* hosts. Partial sequences of *MAT1-2* idiomorph presented more mutational sites, especially in the exon, comparing to the *MAT1-1* region which indicates different evolutionary rates on these two idiomorphs. Additionally, mutations in *MAT1-2* presented transversional bias which may indicate a directional selection on mating type genes.

A. arborescens and intermediate isolates clustered as a distinct group. Close phylogenetic relationship between *A. arborescens* and intermediate isolates was also observed in another work (Ozkilinc H., unpublished data). Besides, *A alternata/A. tenuissima* isolates indicated at least two distinct clusters within populations according to the both mating type idiomorphs. These small-spored *Alternaria* species are distinctly grouped from the other *Alternaria* species based on mating type sequences. The clustering implied that these regions could be phylogenetically informative within *Alternaria* genus, but species level distinction is required more genetic information to resolve species boundaries within small-spored *Alternaria* species.

Our results showed that both pistachio and wild relatives bear both mating types of *Alternaria* species causing Alternaria blight at equal frequencies across Turkey. Even though absence of teleomorph evidence for these species, sexual reproduction still could be questioned due to some indirect evidences such as equal frequencies of mating type genes, recombination detection and genetically distinct clusters. On the other hand, different mating types and genetic variations in sequences of mating type genes indicate for a high diversity for these pathogens in Turkey as expected in the origin centers of hosts as well its pathogens. Moreover ongoing research about genetic variability in other gene region will give more insights about the pathogen genetic variability and phylogenetic lineages (Ozkilinc H., unpublished data).

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