Molecular phylogenetic species in *Alternaria* pathogens infecting pistachio and wild relatives

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Abstract

Many important pathogens of crops worldwide are members of section *Alternaria* within the genus *Alternaria*. Representative species in this section such as *Alternaria alternata*, *Alternaria tenuissima*, and *Alternaria arborescens* show high variability, intermediate characters and plasticity in morphological features, which makes species identification difficult. The aim of this study was to characterize *Alternaria* species associated with pistachio and wild relatives in Turkey using molecular phylogenetics. One hundred isolates of *Alternaria* spp. from pistachio and wild relatives from Turkey were investigated. In addition, standard morphological reference isolates and *Alternaria* blight pathogens of pistachio from USA were included. Sequence data from *major allergen a1*, *ATPase*, *endopolygalacturanase*, and anonymous regions *OPA1.3* and *SCAR2* were obtained. Gene trees were estimated based on maximum parsimony, maximum-likelihood, and bayesian inference methods. Species tree estimation was performed based on Yule speciation and strict molecular clock assumption. Among the collection of *Alternaria* spp. from Turkey, only one *A. arborescens* isolate and three isolates which were morphologically *A. alternata*/*A. arborescens* intermediate types, but, phylogenetically close to *A. arborescens* were discovered. While *A. alternata* and *A. tenuissima* formed one phylogenetic species, *A. alternataltenuissima* were phylogenetically distinct from *A. arborescens*. Furthermore, a *TaqI* restriction site in the *endopolygalacturanase* gene was explored as a novel diagnosis for identification of *A. alternataltenuissima* and *A. arborescens*. All these molecular phylogenetic approaches allow to distinguish morphologically similar *Alternaria* pathogens and molecular phylogenies of *Alternaria* pathogens from pistachio and wild relatives in Turkey are described for the first time.

Keywords *Alternaria* · *Alternaria* blight · Phylogenetics

Introduction

Small-spored *Alternaria* species, also known as the *Alternaria* species group or section *Alternaria*, are a well-supported phylogenetic lineage within the genus *Alternaria* (Lawrence et al. 2013; Woudenberg et al. 2013). *Alternaria*

alternata, *A. tenuissima*, and *A. arborescens* are the most common morpho-species within this group (Pryor and Michailides 2002; Lawrence et al. 2013; Woudenberg et al. 2015). 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 (Rotem 1994; Simmons 2007; Tymon et al. 2016). Besides, providing perfectly standardized environmental conditions, finding experienced mycologists make species identification of these species challenging. Personal qualifications are other difficulties for accurate identification of these species. In addition to considerable and overlapping morphological similarities of *A. alternata* and *A. tenuissima*, intermediate morphological characters have been observed between *A.*

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alternata and *A. tenuissima* and between *A. alternata* and *A. arborescens* (Andrew et al. 2009; Rotondo et al. 2017). Besides, morphology-based characterization cannot be applied for cultures showing low sporulation. Thus, solely morphology-based identification may lead to misidentification. Even though morphology-based species classification agrees with phylogenetic species classification in most of the times, incongruence between these two classifications has been observed in some studies (Andrew et al. 2009; Tymon et al. 2016).

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* (Pryor and Gilbertson 2000; Chou and Wu 2002; Rang et al. 2002; Pryor and Bigelow 2003; Peever et al. 2004). *Endo_PG*, *ATPase*, *Alt_al*, some anonymous regions, and SCAR markers were informative in estimating phylogenies within section *Alternaria* (Peever et al. 2004; Andrew et al. 2009; Stewart et al. 2014; Zhu and Xiao 2015; Ozkilinc et al. 2018). Incongruence among individual gene trees due to incomplete lineage sorting, horizontal gene transfer, or recombination may result in gene trees that do not represent the species tree (Maddison 1997). When multilocus phylogenies were estimated among isolates of the small-spored *Alternaria* species causing citrus brown spot, three well-supported lineages were identified (Stewart et al. 2014). However, species tree estimation revealed that these three lineages should be considered a single species, *A. alternata* (Stewart et al. 2014).

Alternaria blight of pistachio is one of the important diseases caused by small-spored *Alternaria* pathogens. *Alternaria alternata*, *A. tenuissima*, and *A. arborescens* within section *Alternaria* are characterized as primary causal agents of *Alternaria* blight disease of pistachio in California/USA. Pryor and Michailides (2002) separated *A. arborescens* from *A. alternata*/*tenuissima* but could not resolve *A. alternata* from *A. tenuissima* based on molecular phylogenies. This disease has been observed in Turkey in recent years (Ozkilinc et al. 2017). Infection causes brownish-darkening lesions on fruits (Ozkilinc et al. 2017) which decrease fruit quality. Morphology-based identification showed *A. alternata*, *A. tenuissima*, and *A. arborescens* are causal agents of this disease in Turkey (Ozkilinc et al. 2017) as reported in USA, as well (Pryor and Michailides 2002). 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 (Tymon et al. 2016). Because of some incongruences between OPA1.3 and morpho-species (Tymon et al. 2016) 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.

Materials and methods

Fungal samples, culturing, and DNA extraction

One hundred and fourteen isolates of *Alternaria* spp. from *Pistacia* spp. (100 isolates from Turkey and 14 isolates from USA) and 4 reference isolates were used in this study (Supplementary Table 1). Isolates from Turkey were chosen from a single-spore collection which was collected in 2013, morphologically identified, and stored in filter papers at -20°C (Ozkilinc et al. 2017). Reference isolates and *Alternaria* isolates from pistachio from USA were kindly provided by Dr. B.M. Pryor, University of Arizona. Fungal isolates were grown on potato dextrose agar and then transferred to potato dextrose broth for DNA extraction. Mycelia incubated 5–7 days on a rotary shaker at room temperature were harvested, lyophilized, and extracted DNA using i-Genomic Plant DNA Extraction Kit (Intron Biotechnology).

PCR and sequencing

Sequences from three genes (*Alt_al*, *ATPase*, *Endopolygalacturonase*) and two anonymous regions (*OPA1.3* and *SCAR2*) were used in the analysis. There were some missing data due to problems with amplification or sequencing of some samples. Primers and annealing temperatures for each locus were listed in Supplementary Table 2. PCR was performed in 25 μl reaction volumes containing 15–20 ng template DNA, 1 $\times$ PCR buffer (Invitrogen, Life Technologies, USA), 0.2 mM dNTPs (Invitrogen, Life Technologies, USA), 1.5 mM MgCl_2 , 0.4 μM of each forward and reverse primer, and 1 unit of Taq polymerase (Invitrogen, Life Technologies, USA). Thermal cycling was performed on a MyCycler thermocycler (Bio-Rad Laboratories) with 5 min denaturation at 95°C followed by 35 cycles of 20 s at 95°C , 20 s at $55\text{--}61^{\circ}\text{C}$ and 30 s at 72°C , and a final extension step of 10 min at 72°C . PCR products were detected on 1.5% agarose gels, stained with 5 $\mu\text{l}/100\text{ ml}$ of SafeView (Applied Biological Materials Inc., Canada) dye, and visualized under UV light on a gel documentation system Vilber Lourmat Quantum ST4 1100 (Vilber Lourmat, France). After successful amplification, PCR products were sequenced in ABI 3500 $\times$ L Genetic Analyzer (Applied Biosystems, MedSanTek Lab., Turkey). Sequence data were aligned and edited using clustalW implemented in BioEdit v7.0.53 for Windows (Hall 1999).

Phylogenetic analysis

Unique haplotypes and segregating sites were identified by using DNASP v. 5 (Librado and Rozas 2009). Phylogenetic trees were estimated using maximum parsimony (MP), maximum-likelihood (ML), and Bayesian methods. DT_ModSel (Minin et al. 2003) was used to estimate the best-fit model of nucleotide evolution for the datasets. TrNef+G, TrNef, TrNef, JC, and HKY models were applied for *Alt_a1*, *ATPase*, *Endo_PG*, *OPA1.3*, and *SCAR2* regions, respectively. MP and ML analyses were performed in PAUP* ver. 4.0b10 (Swofford 2003). Gaps were treated as missing data. Heuristic searches consisting of 1000 stepwise random addition replicates with branch swapping by the tree-bisection-reconnection algorithm were used in the analyses. Branch stabilities were evaluated by 1000 bootstrap replications and produced a majority-rule consensus tree with nodal support values for each dataset. Bayesian analyses were performed in MrBayes ver. 3.1.1 (Huelsenbeck and Ronquist 2001) and the first 2.5 million generations were discarded as burn-in. Trees were visualized using Archaeopteryx v.09901 beta (Han and Zmasek 2009). *Alternaria solani* was used as outgroup in all phylogenetic analyses.

Phylogenetic species tree estimations were done using Bayesian approaches using *BEAST software (Drummond and Rambaut 2007), which estimates species tree by avoiding incomplete lineage sorting (Heled and Drummond 2010). BEAUTi was used to create XML-formatted input files for *BEAST v1.6.1. (Heled and Drummond 2010). Unlinked models were treated for each data set. Yule process was chosen for species tree prior. Species tree estimation was performed based on strict molecular clock assumption. Data set was run for 100 million generations in *BEAST, sampling every 10,000 generations. Log file was evaluated in TRACER version 1.5. (Heled and Drummond 2010). Summarized maximum clade credibility tree from estimated species trees was calculated using TreeAnnotator version 1.7.2. (Heled and Drummond 2010) with a burn-in of 10% of trees. FigTree version 1.4.2. (Rambaut 2008) was used to visualize the consensus tree, node ages, branch lengths, and posterior probabilities. Densitree software (Bouckaert 2010) was used to illustrate resolution of species tree estimation.

Screening for restriction sites to distinguish species

All genomic regions sequenced and aligned to identify possible restriction sites discriminating morphologically and phylogenetically distinct *Alternaria* species using Geneious software (Biomatters, Auckland, NZ). There were no distinguishable sites within *Alt_a1*, *ATPase*, and *SCAR2*. The *Apa I* site in region *OPA1.3* identified by Tymon et al. (2016) was evaluated through the sequence data in this study and restriction digestion was confirmed for several isolates which

ones as *A. arborescens* and *A. alternataltenuissima*. Moreover, a novel restriction site, *Taq I*, which distinguishes *A. alternataltenuissima* and *A. arborescens* within *Endo_PG* region amplified was found in this study. Sequence data were evaluated for all isolates used in *Endo_PG* amplifications. Restriction digest assay was applied to some representative isolates for confirmation. *Taq I* enzyme with its buffer (Fermentas) applied to DNA samples following the manufacturer's instructions. Band profiles were observed on 2% agarose gels, stained with 5 µl/100 ml of SafeView (Applied Biological Materials Inc., Canada) dye, and visualized under UV light on a gel documentation system Vilber Lourmat Quantum ST4 1100 (Vilber Lourmat, France).

Results

Haplotype diversity and phylogenetics of *Alternaria* spp.

Sequences of *Alt_a1*, *ATPase*, *Endo_PG*, *OPA1.3*, and *SCAR2* resulted in 368, 722, 119, 821, and 440 bp length data, respectively. All the sequences obtained in this study deposited in Genbank (accession numbers KY561821—KY561852 for *Alt_a1*, KY561853—KY561894 for *ATPase*, KY561895—KY561923 for *Endo_PG*, KY561924—KY561950 for *SCAR2*, KY561951—KY561989 for *OPA1.3* from *Alternaria* spp. from *Pistacia* spp., and KY561990—KY561994 for five sequences from outgroup *A. solani* isolate). A number of segregating sites of *Alt_a1*, *ATPase*, *Endo_PG*, *OPA1.3*, and *SCAR2* were 21, 24, 11, 23, and 9 for all isolates analyzed, respectively. Sequences of *Alt_a1*, *ATPase*, *Endo_PG*, *OPA1.3*, and *SCAR2* presented different number of haplotypes and variations within *Alternaria* spp. from *Pistacia* spp. from Turkey (Fig. 1 and Supplementary Fig. 1). Mostly, many of the isolates were placed in a one haplotype for each region as seen in Fig. 1 and Supplementary Fig. 1. Individual gene trees based on MP, ML, and Bayesian estimations were obtained and all tree topologies were similar (Fig. 1 and Supplementary Fig. 1). In all trees, one isolate within collection from Turkey (isolate 02–28) clustered with *A. arborescens*-EGS39-128. *Alternaria arborescens* isolates from USA were also clustered in the same or sister branches of isolates EGS39-128 and 02–28. Moreover, three isolates (02-29-1, 31-09, and 07-17-2/GY coded) which morphologically exhibited intermediate characters of *A. alternata* and *A. arborescens* were clustered closely with *A. arborescens* isolates on *Alt_a1*, *ATPase*, *OPA1.3*, and *SCAR2* gene trees and clustered together with *A. arborescens* isolates on *Endo_PG* tree. *Endo_PG* was the most powerful marker to distinguish the *A. arborescens* branch, but was not powerful enough to identify further genetic diversities within species. *OPA1.3*, *SCAR2*,

Fig. 1 Phylogenetic trees inferred from maximum-likelihood (ML), maximum parsimony (MP), and Bayesian analysis of *Endo_PG* sequences for *Alternaria* spp. from *Pistacia* spp. and reference isolates of *Alternaria* spp. beginning with EGS codes. *A. solani* was selected as outgroup. Isolates beginning with BMP code were from pistachio in California. Isolates from *Pistacia* spp. from Turkey were represented by unique haplotypes per genomic region. Isolates repre-

sented with a haplotype number are listed in Supplementary Tables 1 and the number of isolates per representative haplotype was shown in parentheses. Bootstrap support values (1000 replicates) above 70% for ML and MP, and above 0.95 for Bayesian analysis are shown. Phylogenetic trees of the other four regions (*Alt_a1*, *ATPase*, *OPA1.3* and *SCAR2*) are represented in Supplementary Fig. 1

and *ATPase* regions indicated also species diversification as well as revealed genetically diverse groups. *OPA1.3* was the most variable region and exhibited the highest number of haplotypes within the collection. *Alt_a1* indicated species divergences but not with highly supported bootstrap values, and did not exhibit diversities within species. Considering all of the trees, one isolate within the collection from Turkey belonged to *A. arborescens* and three isolates were closer to *A. arborescens* than to the *A. alternata/tenuissima* group. *Alternaria alternata* and *A. tenuissima* were not resolved at all and there was no indication for a consistent and highly supported genetic/phylogenetic lineage within *A. alternata/tenuissima*.

Phylogenetic species tree estimation

Assigning of *A. alternata* and *A. tenuissima* as a separate species did not result any good convergence and ESS (Effective Sample Size) values were lower than 100. Another attempt was to assignment of three intermediate isolates as a separate species. This attempt did not provide a resolved species tree due to insufficient divergence from *A. arborescens*. Thus, *A. alternata* and *A. tenuissima* isolates were assigned as one species and *A. arborescens* isolates together with three intermediate isolates were assigned as another species. *BEAST analysis showed that the majority of the parameters had ESS values > 200.

The tree topologies revealed strong statistically supported clades for *A. alternata/tenuissima* and *A. arborescens* species groups (Fig. 2 and Supplementary Fig. 2).

A restriction site on *Endo_PG* to distinguish *A. alternata/tenuissima* and *A. arborescens*

Sequence data of *Endo_PG* region had a *TaqI* restriction site (recognizes 5'TCGA3') distinguishing *A. alternata/tenuissima* and *A. arborescens*. *Alternaria alternata/tenuissima* has two restriction sites and *A. arborescens* has three restriction sites within this region. Moreover, restriction digestion reaction for PCR product of *Endo_PG* with *Taq I* enzyme resulted in detectable species diagnosis by banding pattern. Based on sequence data 31, 333, and 55 bp products for *A. alternata/tenuissima* and 31, 282, 51, and 55 bp products for *A. arborescens* are expected. Since agarose gel cannot show small band product successfully, the biggest band fragment which is easily visible on 2% agarose gel provides species detection. Thus, about 330 and 280 bp fragments indicate for *A. alternata/tenuissima* and *A. arborescens*, respectively. Moreover, this region distinguished *A. alternata/arborescens* intermediate isolates as *A. arborescens* as supported by phylogenetic species tree estimation.

Fig. 2 *BEAST species tree estimation based on the five genomic regions. This tree represents consensus species tree estimation by Figtree visualization. Species assignments were performed considering divergences in gene trees as well as morpho-species. **a** *A.*

alternata/tenuissima, **b** *A. arborescens*, outgroup: *A. solani*. Branch lengths and posterior probabilities are shown on branches and nodes, respectively

Discussion

This research represents the first attempt to characterize the genetic diversity of *Alternaria* species causing Alternaria blight of pistachio and its wild relatives. Based on gene trees and species tree estimations, two species were distinguished: *A. alternataltenuissima* and *A. arborescens*. *Alternaria alternataltenuissima* is the most dominant and prevalent pathogen of this disease in Turkey. Even *A. alternata* and *A. tenuissima* were mostly introduced as two different species based on morphological classification, these two morpho-species are not diverged as two distinct species according to the phylogenies in this study as well as in the previous studies (Pryor and Michailides 2002; Andrew et al. 2009; Rotondo et al. 2012; Woudenberg et al. 2015). Recently, *A. alternata* and *A. tenuissima* were treated as one species (*A. alternataltenuissima*) in phylogenetic analyses of sections Alternaria and Porri (Ozkilinc et al. 2018). In addition to that, their morphological specifications overlap and are difficult to differentiate due to the considerable variations in culture morphology, low sporulation, and/or intermediate characteristics. Still, morphological species concept is widely applied to these groups worldwide, *A. alternata* and *A. tenuissima* are presented two different morpho-species. In view of phylogenetic species concept, these pathogens represent one species according to this study including a population-level sampling and applying some other markers by supporting the previous studies. Moreover, 35 morpho-species within section Alternaria were considered as one phylogenetic species, *A. alternata* based on the multi-gene phylogenies (Woudenberg et al. 2015). Species boundaries may change according to the species concept accepted. Eventually, two morpho-species: *A. alternata* and *A. tenuissima*, are revealed as one phylogenetic species: *A. alternataltenuissima*. These pathogens could be a species complex, though, which cannot be resolved, yet.

Alternaria alternataltenuissima populations showed polymorphisms in each genomic region studied and represented different haplotypes based on data sets, but none of the clusters were supported as a distinct phylogenetic lineage. All these haplotypes indicate an important diversity within a presumably asexual species. None of the clusters or haplotypes were related to origin of geographic location or host which may cause free spread of pathogen among locations and between cultivated and wild hosts. Similarly, genetic groups within small-spored Alternaria pathogens from different hosts were not related with origin of host in the previous studies, as well (Andrew et al. 2009; Ozkilinc et al. 2018). *Alternaria arborescens* isolates from USA showed variability within species, but we do not know about variability for *A. arborescens* pathogens

from Turkey due to just one precise *A. arborescens* isolate found within a large collection. Molecular diversities and phylogenies of these pathogens from Turkey are described in detailed for the first time in this research, and thus, these results will be highly informative for comparative population studies among the *Alternaria* pathogens worldwide.

Describing some representative isolates from phylogenetic trees, morphology and phylogeny were consistent. On the other hand, there were three isolates which could not be distinguished as either *A. alternataltenuissima* or *A. arborescens* based on their morphologies. These isolates were resolved by phylogenies. Even though this species divergence was well visualized, short branch lengths may indicate recent divergence or still diverging species.

DNA-based approaches are very successful to resolve phylogenies among major species groups within *Alternaria* genus (Pryor and Michailides 2002; Lawrence et al. 2013; Woudenberg et al. 2015; Ozkilinc et al. 2018); however, section Alternaria was needed more effort to resolve phylogenies (Andrew et al. 2009; Stewart et al. 2014; Ozkilinc et al. 2018). Recently, Tymon et al. (2016) showed that *Apa I* restriction digestion of *OPA.1.3* amplified region can distinguish *A. alternataltenuissima* and *A. arborescens*, but the molecular data and the morphology did not always support each other. In our study, we found that *TaqI* restriction sites within amplified *Endo_PG* region can distinguish *A. alternataltenuissima* and *A. arborescens*. Moreover, intermediate-type isolates were also identified as *A. arborescens* by this restriction digestion as supported by species tree estimation. Therefore, we highly recommend using this region for fast and accurate species identification for these two closely related species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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