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## A NEW ACCURATE GENOTYPING HRM METHOD FOR *ALTERNARIA* SPECIES RELATED TO FRUIT ROT DISEASES OF APPLE AND POMEGRANATE

<sup>a</sup>Antonios Zambounis, <sup>b</sup>Aliki Xanthopoulou, <sup>c</sup>George Karaoglanidis, <sup>a,b</sup>Athanasios Tsafaris, <sup>b</sup>Panagiotis Madesis\*

<sup>a</sup> Laboratory of Genetics and Plant Breeding, School of Agriculture, Forestry and Natural Environment, Aristotle University of Thessaloniki, P.O. Box 261, Thessaloniki GR-54124, Greece.

<sup>b</sup> Institute of Applied Biosciences, CERTH, Themi, Thessaloniki, Greece.

<sup>c</sup> Laboratory of Plant Pathology, School of Agriculture, Forestry and Natural Environment, Aristotle University of Thessaloniki, P.O. Box 261, Thessaloniki GR-54124, Greece.

### ABSTRACT

*Alternaria* core rot and *Alternaria* black heart rot of apple and pomegranate fruit, respectively, are major pre- and postharvest diseases worldwide. However, it is very difficult to differentiate the rot related *Alternaria* species in the *Alternaria* complex as they are not always correlate to species-groups based upon morphological characteristics and due to the limited genetic variation these species exhibit among each other. Therefore, it is crucial to exploit novel assays towards the accurate identification and differentiation of these *Alternaria* species. We have developed, a real-time PCR assay [using species specific primers targeting the endopolygalacturonase (*EndoPG*) gene] combined with a high-resolution melting (HRM) curve analysis for discrimination of the 14 single nucleotide polymorphisms (SNPs)-based *Alternaria* haplotypes, which were assigned based on the aligned sequence profiles of 138 *Alternaria* spp. strains previously isolated from apple and pomegranate rotted fruit. This analysis specifically generated 14 unique HRM curve haplotype profiles among the *Alternaria* complex species tested. The results showed that HRM curve analysis allows the rapid and adequate identification and genotyping of the three *Alternaria* species (*A. alternata*, *A. tenuissima* and *A. arborescens*) responsible mostly for the apple and pomegranate fruit rot diseases.

**Keywords:** *Alternaria*-species complex; apple fruit core rot; endopolygalacturonase gene; HRM genotyping; pomegranate fruit black heart rot.

### INTRODUCTION

*Alternaria* core rot of apple fruit (*Malus domestica*) is considered as the most severe postharvest disease of apples occurring in several places of the world, including Greece (Gao *et al.*, 2013; Ntasiou *et al.*, 2015). The disease initiates during bloom from the loculus and spreads into the fruit mesoderm leading finally to either a dry or wet core rot (Shtienberg, 2012). External symptoms are only evident after fruit harvest, while during storage the rot still progresses and may be observed on the fruit epidermis after several months (Shtienberg, 2012). Additionally, pomegranate (*Punica granatum* L.) is a rapidly expanding crop throughout the world because of the fruit great nutritional value and human diet benefits (Gil *et al.*, 2000). As apple fruits, pomegranate fruits suffer

also from the so called *Alternaria* black heart disease that occurs both as a pre- or postharvest disease, and that has been found in several countries worldwide (Ezra *et al.*, 2015; Michailides *et al.*, 2008). Pathogen may penetrate either through injuries of fruit exocarp or during the blooming period, and remain latent until the onset of fruit maturation, becoming evident during storage (Palou *et al.*, 2013). *Alternaria* decays not only reduce the yield, but may also deteriorate the quality of the fresh or processed fruit because of the production of mycotoxins. Mycotoxins could be quite dangerous if contaminated apples or pomegranates are designated to processing, especially for juices and baby foods production, since they have mutagenic and carcinogenic effects (Ostry, 2008).

Despite preliminary studies, based mainly on morphological evidences, suggested *A. alternata* as the causal agent of the disease both in apples and pomegranates, more recent molecular surveys indicated

\* Corresponding Author:

Email: [pmadesis@certh.gr](mailto:pmadesis@certh.gr)

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that several other small-spored *Alternaria* species such as *A. tenuissima*, *A. arborescens* and *A. infectoria* might be the causal agents of the disease (Gao *et al.*, 2013; Kou *et al.*, 2014). Recently, it was shown that *Alternaria* species obtained from in stored apple fruits which depicted core rot disease symptoms in Greece, were recognized as *A. tenuissima* or *A. arborescens* based on the sequencing of regions of endopolygalacturonase (*EndoPG*) gene (Ntasiou *et al.*, 2015). Similarly, in another study aiming to identify the agents of pre- and postharvest rots of pomegranate fruit in Greece and Cyprus, it was shown that black heart rots of pomegranate fruit were caused by *A. alternata*, *A. tenuissima* and *A. arborescens* (Kanetis *et al.*, 2015).

Recently, molecular surveys revealed a wealth of non-monophyletic genera both within the *Alternaria* complex and *Alternaria* species lineages, which do not always being associated to species-groups based upon morphological features (Woudenberg *et al.*, 2013). This diversity in *Alternaria* complex suggests that both in apples and pomegranate a number of distinct *Alternaria* species lineages, being associated with small-spored *Alternaria*, encompasses this rot disease. Thus, in order to understand and solve the challenging issue of differentiating *Alternaria* rot species, the development of precise and efficient molecular fungal diagnostics is of paramount importance (Woudenberg *et al.*, 2013).

A novel molecular method, termed high-resolution melting (HRM), has been extensively used for DNA genotyping applications (Reed and Wittwer, 2004). According to this technique, the rate of double-stranded DNA dissociation is measured under gradually increasing temperatures (Reed and Wittwer, 2004). The advantage of this technique is actually based on a suitable fluorescent dye homogeneously intercalated into the DNA. Upon the melting of the PCR products, the rate of dissociation is relevant with various thermodynamic properties of these products (Ganopoulos *et al.*, 2012). Consequently, these thermodynamic properties of the PCR products trigger specific changes in fluorescence measurements and alter the shape of melting curve profiles during this dissociation. HRM has already been employed in the successful identification and differentiation of other fungal species, while at the same time helps eliminating contamination risks during the whole process (for review see Zambounis *et al.*, 2015).

The *EndoPG* gene has been previously employed in the delineation of the closely related species within the *Alternaria* complex and was used for pathogen identification and phylogenetics (Hong *et al.*, 2005;

Andrew *et al.*, 2009). Therefore, based on the sequencing data obtained from two previous surveys (Kanetis *et al.*, 2015; Ntasiou *et al.*, 2015), the scope of this survey was to establish HRM technique as method of choice for the accurate and rapid discrimination of *Alternaria* complex species associated with apple and pomegranate fruit rots.

## MATERIALS AND METHODS

**Fungal isolates and sequences:** In the present study, we used the genomic DNA of 138 fungal isolates, obtained from apples and pomegranate fruit showing decay symptoms and accurately identified at their *Alternaria* species level (*A. alternata*, *A. tenuissima* and *A. arborescens*), by a combination of molecular (direct sequencing approaches) and morphological evidences (Kanetis *et al.*, 2015; Ntasiou *et al.*, 2015). We also retrieved from NCBI the corresponding 138 sequences including: i) 67 *A. tenuissima* and 8 *A. arborescens* sequences from apples (GenBank Accession numbers: LK054418-LK054484 and LK054410-LK054417, respectively), and ii) 30 *A. alternata*, 19 *A. tenuissima* and 14 *A. arborescens* strains from pomegranate (GenBank Accession numbers: KP789517-789546, KP789498-KP789516 and KP789484-KP78947, respectively) (Table 1). These related amplicons have been previously obtained (Kanetis *et al.*, 2015; Ntasiou *et al.*, 2015) using the universal primers PG3 and PG2b (Andrew *et al.*, 2009) targeting a region of the *EndoPG* gene.

**Sequences alignment, haplotypes assignment and HRM analysis:** The MUSCLE software (Edgar, 2004) inferred in the Geneious package (Drummond *et al.*, 2009) was used for the alignment of the 138 *Alternaria* species sequences. We identified a fragment in the aligned homologous region, where adequate single nucleotide polymorphisms (SNPs) have been detected, permitting their clustering in unique SNPs-based *Alternaria* haplotypes (Table 1). Based on this alignment profile specific primers for the *EndoPG* gene, *Alternaria\_For* (5'- CAACTTTGGACCTCTCTAGTCTGG -3') and *Alternaria\_Rev* (5'- GTCTTTCACCATTTCACCCTT -3') were manually designed; these primers were capable for amplifying a 199 bp amplicon for all the 138 *Alternaria* species isolates. Two independent representative isolates of each *Alternaria* haplotype were analysed in HRM experiments.

HRM analysis, were performed on *EndoPG* PCR products obtained by the specific primers, in a final volume of 15 µl using the Rotor-Gene 6000 real-time 5P HRM PCR Thermocycler (Corbett Research, Sydney, Australia).

We used an initial denaturing step of 95°C for 10 min followed by 35 cycles of 95°C for 5 s, 52°C for 15 s and 72°C for 10 s, following by a final extension step of 72°C for 2 min. Before HRM, the products were denatured (95°C for 5 s), and then annealed at 50°C for 30 s to randomly form DNA duplexes. HRM was performed as follows: pre-melting at the first appropriate temperature for 90 s, and melting at a ramp of 10°C in an appropriate temperature range setting at 0.1°C increments every 2 s. End point fluorescence measurements were retrieved following the melting process by holding at 60°C for 5 min and for 5 cycles at 60°C for 20 s, while fluorescence data being obtained at the end of each cycle step. The reaction mixture contained 20 ng fungal DNA, 1X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 300 nM forward and reverse primers, 1.5 mM Syto9® green fluorescent nucleic acid stain, and 0.5 U Kapa Taq DNA polymerase (Kapa Nippon Genetics). Finally, each *Alternaria* haplotype profile was assigned as a genotype, and the average HRM genotype confidence percentages (GCPs) for each haplotype were applied according to Hewson *et al.*, (2009). A GCP value of 100 implied an accurate genotype match. The correct sizes of all PCR amplicons were confirmed by agarose gel visualization.

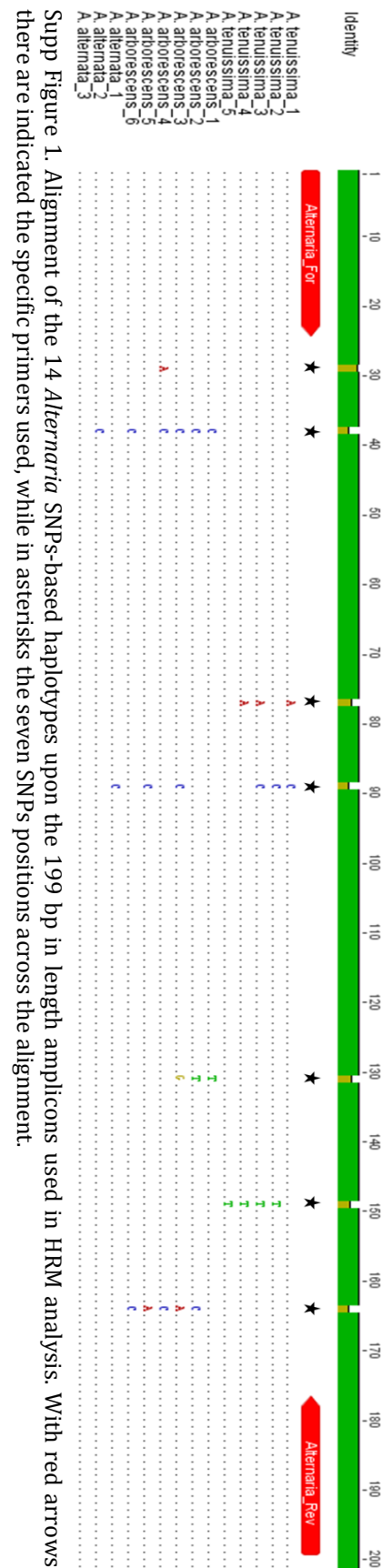
## RESULTS AND DISCUSSION

Taking into account that *Alternaria* core rot and black heart rot diseases represent major threats for apple and pomegranate fruit production, respectively, the application of innovative molecular diagnostic approaches is undoubtedly nowadays a crucial challenge

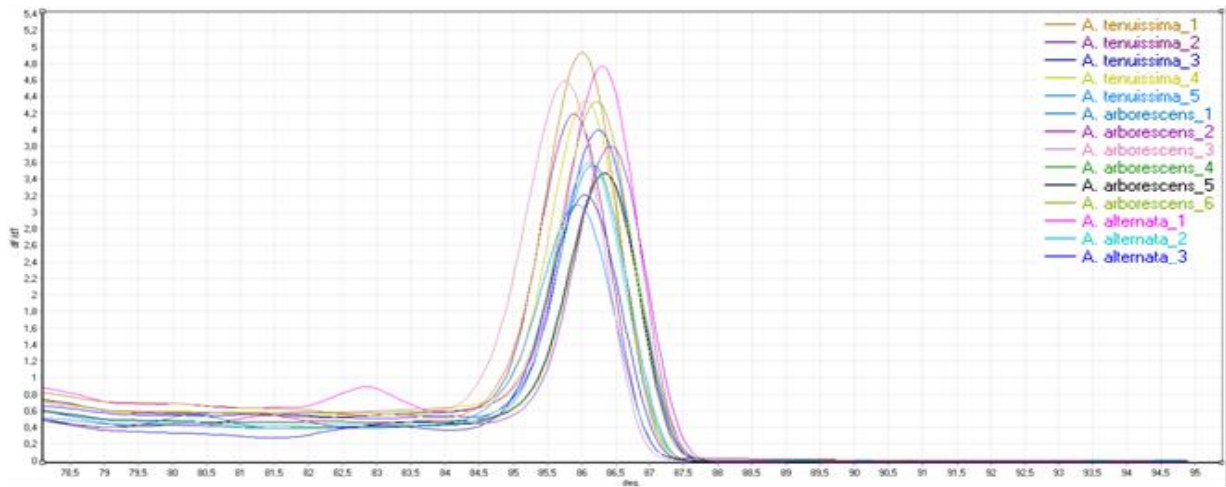
(Ntasiou *et al.*, 2015). Moreover, considering the well documented taxonomic problems and the complexity of this collective group at species level with associated small-spored *Alternaria*, rapid and accurate molecular techniques should contribute towards the precise identification of *Alternaria* species being involved in this disease (Hartevelde *et al.*, 2013).

**SNPs-based *Alternaria* complex haplotypes assignment:** Alignment of the 138 sequences of *Alternaria* species *EndoPG* gene was performed using the MUSCLE software (Edgar, 2004); the overall identity was 99.4% among these sequences. We identified various SNPs, even among the same species sequences, which were distributed in seven different positions along the 199 bp in length amplicons (Supp Figure 1). Except point mutations in the above ungapped alignment, there were not observed any insertions or deletions (Supp Figure 1). Taking into account the alignment profile, 14 SNPs-based *Alternaria* complex haplotypes were assigned, from now on called simply as “haplotypes” (six for *A. arborescens*, five for *A. tenuissima* and three for *A. alternata*). The distribution of *Alternaria* spp. sequences among these 14 haplotypes and their hosts of origin are shown in Table 1.

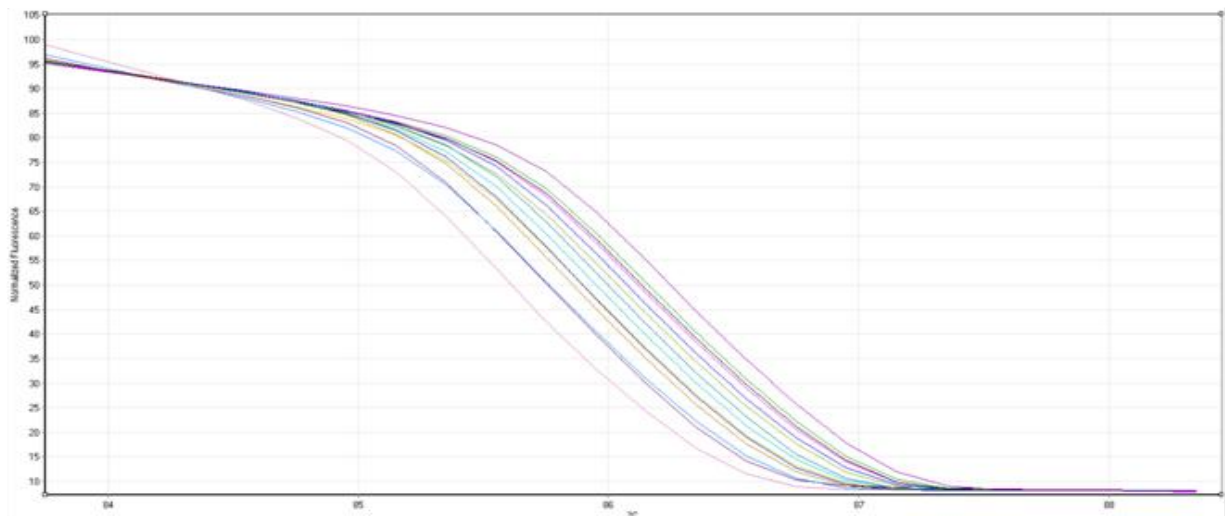
**The HRM analysis allow an accurate genotyping and differentiation of the *Alternaria* complex haplotypes:** Specific primers were designed for the HRM analysis of the above *Alternaria* haplotypes; these primers amplified constantly an amplicon of 199 bp, which was harbouring the identified SNPs observed in the sequences alignment.



(a)



(a)



(a)

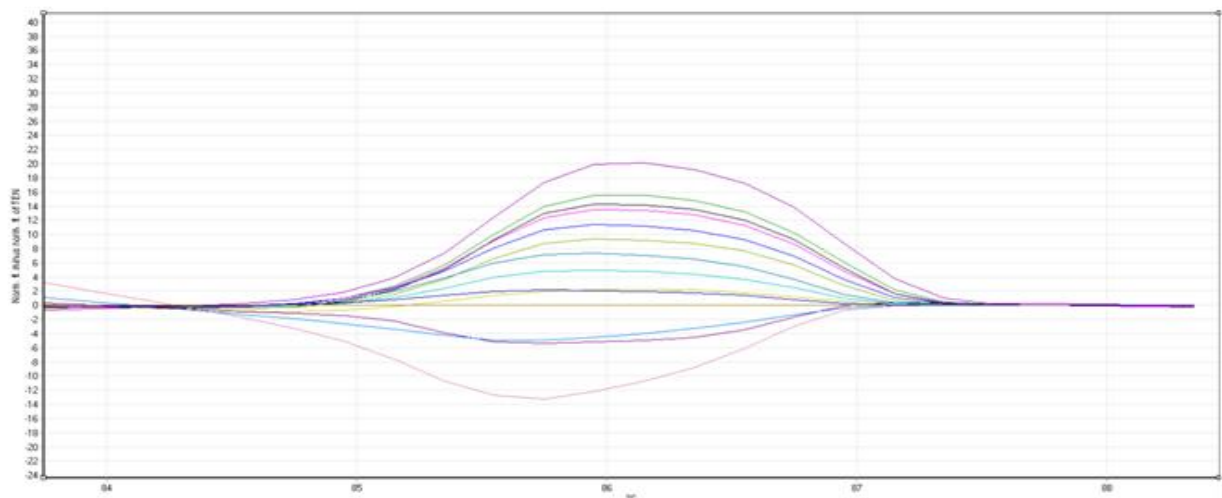


Figure 1. (a) *EndoPG* gene conventional HRM curves, (b) HRM profiles produced by the 14 *Alternaria* complex species haplotypes, (c) *Alternaria* haplotypes difference normalized graphs assigning '*A. tenuissima\_1*' haplotype as a normalized reference genotype. Identical genotypes were assigned using a cutoff GCP value of 95%.

Figure 1a depicts the results of *Alternaria* haplotypes conventional genotyping plots. Each genotype presented a unique peak, in a range from 81 to 85°C (data not shown). The different *Alternaria* strains tested, corresponding to each haplotype, generated distinctive normalized HRM profiles, facilitating the accurate identification of each haplotype. In general, the resolving efficiency of the data obtained is much higher than other similar conventional melting curve analysis, because the HRM curves efficiently differentiated amplicons even when they shared exactly the same T<sub>m</sub>, because of curve shape as the result of the heterozygosis related effects (Liew *et al.*, 2004).

In this study, we based the discrimination of 14

*Alternaria* haplotypes upon their informative HRM curve profiles (Figure 1b). Analysis of the *EndoPG* gene marker produced normalized HRM curves (Figure 1c), indicating that all of the haplotypes were efficiently distinguished from each other, despite the similarity of certain HRM profiles of some haplotypes (Figure 1b). We assigned the 'A. *tenuissima*\_1' haplotype as a reference genotype, as this haplotype comprised the highest number of *Alternaria* spp. sequences tested in the present study (Table 1). The GCPs similarity values between this reference haplotype and the remaining ones (Table 2) indicated that *EndoPG* gene was a promising locus for identification and differentiation of the haplotypes sequences under investigation (Figure 1c).

Table 1. *Alternaria* species, SNPs-based haplotypes, number of isolates sequences assigned to each haplotype and their hosts of origin.

<i>Alternaria</i> species identified according to previous studies <sup>1</sup>	SNPs-based haplotypes	Number of isolates sequences assigned to haplotypes	Hosts of origin
<i>A. tenuissima</i>	A. <i>tenuissima</i> _1	67	apple
	A. <i>tenuissima</i> _2	2	pomegranate
	A. <i>tenuissima</i> _3	11	pomegranate
	A. <i>tenuissima</i> _4	2	pomegranate
	A. <i>tenuissima</i> _5	4	pomegranate
<i>A. arborescens</i>	A. <i>arborescens</i> _1	6	apple
	A. <i>arborescens</i> _2	2	apple
	A. <i>arborescens</i> _3	2	pomegranate
	A. <i>arborescens</i> _4	2	pomegranate
	A. <i>arborescens</i> _5	4	pomegranate
	A. <i>arborescens</i> _6	6	pomegranate
<i>A. alternata</i>	A. <i>alternata</i> _1	22	pomegranate
	A. <i>alternata</i> _2	2	pomegranate
	A. <i>alternata</i> _3	6	pomegranate

The GCPs values were employed as described previously (Hewson *et al.*, 2009) using a cutoff value of 95% to assign an identical genotype. The highest GCP value (88.84) was observed between the 'A. *tenuissima*\_3' and 'A. *tenuissima*\_4' haplotypes both comprising isolates from pomegranate, while the lowest (0.02) was between 'A. *arborescens*\_2' and 'A. *arborescens*\_3' haplotypes which were considered distinctly different profiles, assigning isolates from apples and pomegranate, respectively. Furthermore, the 'A. *tenuissima*\_1' haplotype, to which 67 *A. tenuissima* sequences from apples were assigned, depicted quite high GCPs among the other four *A. tenuissima* haplotypes (Table 2), implying that in principle differentiation of this haplotype was quite challenging against the other *A. tenuissima* haplotypes in the HRM analysis.

Overall, the 138 *Alternaria* sequences, which were previously classified in the three *Alternaria* species, were efficiently clustered in the 14 different haplotypes according to their SNPs homology profiles, possessing point mutations capable of revealing shape fluctuations in the respective HRM haplotype curves, even at their species level (Figure 1a). By this way, variations of haplotype sequences at a specific region of the *EndoPG* gene was translated into an informative and reproducible HRM curve genotyping analysis for the examined haplotypes (Figure 1b). In the present study we transferred direct DNA sequencing results previously acquired to discriminate *Alternaria* species to a more sensitive and precise HRM assay; this was exemplified by the successful genotyping of the 14 *Alternaria* haplotypes tested.

Table 2. The average genotype confidence percentage values ( $\pm 2.7$ ) for the 14 *Alternaria* haplotypes, derived from the HRM analysis, using a ramp of 0.1 °C.

	A. tenuissima_1	A. tenuissima_2	A. tenuissima_3	A. tenuissima_4	A. tenuissima_5	A. arborescens_1	A. arborescens_2	A. arborescens_3	A. arborescens_4	A. arborescens_5	A. arborescens_6	A. alternata_1	A. alternata_2	A. alternata_3
A. tenuissima_1	100	-	-	-	-	-	-	-	-	-	-	-	-	-
A. tenuissima_2	71.39	100	-	-	-	-	-	-	-	-	-	-	-	-
A. tenuissima_3	85.83	54.3	100	-	-	-	-	-	-	-	-	-	-	-
A. tenuissima_4	84.14	53.36	88.84	100	-	-	-	-	-	-	-	-	-	-
A. tenuissima_5	82.01	83.43	66.41	66.55	100	-	-	-	-	-	-	-	-	-
A. arborescens_1	58.98	19.02	75.73	77.22	27.53	100	-	-	-	-	-	-	-	-
A. arborescens_2	2.59	0.32	4.81	5.38	0.59	19.8	100	-	-	-	-	-	-	-
A. arborescens_3	24.25	61.09	14.62	14.77	55.79	3.16	0.02	100	-	-	-	-	-	-
A. arborescens_4	10.05	1.7	16.57	18.16	2.94	47.8	83.33	0.17	100	-	-	-	-	-
A. arborescens_5	13.3	2.42	21.28	23.26	4.16	56.29	74.17	0.38	88.47	100	-	-	-	-
A. arborescens_6	41.5	11.44	56.71	59.63	17.53	83.12	34.72	1.77	69.08	77.75	100	-	-	-
A. alternata_1	17.26	3.37	26.87	28.95	5.61	64.89	67.48	0.38	85.91	88.8	84.75	100	-	-
A. alternata_2	78.56	32.96	59.95	82.87	44.31	84.44	11.38	6.91	32.18	39.42	80.35	47.18	100	-
A. alternata_3	29.07	6.77	42.34	44.75	10.71	83.05	48.39	0.88	82.87	89.62	86.62	84.81	66.1	100

Conclusively, this is the first survey according to our knowledge using the HRM technique aiming at differentiating the *Alternaria* species complex at species level. We demonstrate that this approach is both an accurate and a rapid molecular assay for the genotyping of the small-spored *Alternaria* species (*A. alternata*, *A. tenuissima* and *A. arborescens*), associated with apple and pomegranate fruit decays in Greece. Finally, this genotyping method could be used potentially to

allocate even unknown *Alternaria* isolates to the HRM melting profiles of the tested species haplotypes.

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