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Identification of *Phytophthora* Species by a High Resolution Melting Analysis: an Innovative Tool for Rapid Differentiation

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Abstract

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A new molecular method via the high resolution melting (HRM) analysis of the *Ypt1* gene non-coding regions was validated for ten *Phytophthora* species with a broad host range from forest trees to crop species. The melting curve analysis of the amplicons specifically grouped all species into 10 respective unique and distinct HRM curve profiles. The analysis of the normalised HRM melting curves, assigning *P. nicotianae* as a normalised reference genotype, revealed that the genotype similarities among all the species were adequately low, indicating that *Ypt1* marker was sufficient to identify and differentiate the tested species. This HRM method is rapid and reproducible allowing the identification of *Phytophthora* species and the screening of eventual variants eliminating the separate steps and reducing the risk of contamination.

Keywords: fungal differentiation; fungi of vegetable and tree plants; HRM; plant breeding; *Ypt1* gene

Oomycete pathogens of the genus *Phytophthora* comprise destructive plant pathogens on a global scale, threatening crops, forests, and other natural ecosystems (Cooke *et al.* 2007). There are over 140 species in the genus with a wide host range (Kroon *et al.* 2012; Martin *et al.* 2012). Except the most famous *P. infestans,* which is the causative oomycete of the Irish potato famine, other species such as *P. ramorum* and *P. cinnamomi* rapidly emerging during the last decade are of major importance (Balci *et al.* 2007; Cahill *et al.* 2008). Therefore, reliable and accurate detection of *Phytophthora* species is undoubtedly fundamental in disease management (Li *et al.* 2013). This aspect is particularly important in consideration of the many outbreaks that have recently emerged, often associated with previously uncharacterised species, both in agricultural and natural ecosystems (Prigigallo *et al.* 2015). Additionally, the easy movement of infected plants in the last years promotes the formation of hybrid species with new pathogenic characteristics (Brasier *et al.* 2008). Thus, preventing the spread of *Phytophthoras* requires the development of accurate detection techniques of high specificity and sensitivity (Sanzani *et al.* 2014).

Over the last years, there has been intensive research into the development of molecular tools, mainly based on conventional and real-time quantitative PCR, which were used for both the identification

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and detection of various *Phytophthoras* in different plant species, tissues, and environmental samples (Schena *et al.* 2013). However, these methods are inappropriate for the identification of *Phytophthoras* at culture level due to difficulties in differentiating species variants, interspecific hybrids or even previously uncharacterised species due to the increasing number of *Phytophthora* species that have still been found and described worldwide (Kroon *et al.* 2012).

A robust and sensitive molecular analytical technique named high resolution melting (HRM) analysis has been employed, apart from other applications, also for fungal genotyping and differentiation (for review see Zambounis *et al.* 2015). Briefly, this technique exploits the dissociation of double to single stranded DNA by elevating temperatures and the release of a homogeneously intercalated fluorescent dye (Reed & Wittwer 2004). The thermodynamic properties of the amplicons, such as sequence lengths and GC contents, motivate specific fluctuations in fluorescence and the revealing melting curve profiles (Zambounis *et al.* 2015). The possibility of contamination is astonishingly low as the entire process is fulfilled in a single step (Madesis *et al.* 2014). Finally, the significant elimination of post-handling steps, plus the exclusion of coming in touch with hazardous chemicals promotes this method as an excellent alternative molecular fungal diagnostic approach.

HMR methods for phytopathogenic fungi identification and differentiation have recently been developed targeting mostly on internal transcribed spacer (ITS) regions (Ganopoulos *et al.* 2012; Wong *et al.* 2013). Although ITS regions have many advantages, they frequently fail in differentiating closely related species because of the insufficient genetic variability; this is particularly relevant for *Phytophthoras* in consideration of the many species frequently characterized by very similar or even identical ITS sequences (Robideau *et al.* 2011). Another disadvantage is that these regions are too long to enable a perfect optimisation of HMR analyses. However, the ras-related *Ypt1* gene is a promising candidate locus by means of phylogenetic studies and for the establishment of accurate molecular diagnostics in order to differentiate *Phytophthora* species (Ioos *et al.* 2006; Schena & Cooke 2006; Meng & Wang 2010). The high differentiation power of the *Ypt1* gene upon *Phytophthora* species is a consequence of the high variation in the non-coding regions of this gene (Schena & Cooke 2006). These regions have already been utilised to develop specific identification assays based both on conventional and on quantitative PCR approaches in the cases of 15 different forest *Phytophthora* species (Schena & Cook 2006; Schena *et al.* 2008).

Therefore, the objective of the study was to develop and validate the HRM technique for the identification of *Phytophthora* species using a representative panel of 10 species with broad host ranges. The results based on differentiations of the melting curve profiles using degenerated primers for the *Ypt1* gene confirmed that HRM analysis might be a rapid and reliable diagnostic method for *Phytophthora* species differentiation.

MATERIAL AND METHODS

Fungal isolates, DNA extractions, and primer design. Pure cultures of 10 *Phytophthora* species were provided by the oomycetes collection of Benaki Phytopathological Institute (http://www.bpi.gr) (Table 1). Fungal genomic DNA was extracted according to Ganopoulos *et al.* (2012) and DNA concentrations were determined spectrophotometrically. Degenerated primers for *Phytophthora* species (YPh6F "GAGYTACATCTCGACCATYGG" and YPh10R "TCTTGCCGTCCARCTCRAT") were designed in conserved regions of exons 3 and 4 of the Ras-like GTP-binding protein YPT1 in order to efficiently amplify the flanked intron from all species used in this study. Therefore, all currently available sequences of this gene including those from genome sequencing projects were aligned and accurately compared all together. Primer 3 software was used to design the primer pairs (Rozen & Skaletsky 2000) while primers degenerations were added manually. Primers were designed to amplify amplicons of approximately 130 bp.

HRM analysis. To evaluate these primers, three representative isolates of each *Phytophthora* species (Table 1) were analysed in HRM. PCR reactions and HRM melting curve analyses were performed in a final volume of 15 µl using a Rotor-Gene 6000 real-time 5P HRM PCR Thermocycler (Corbett Research, Sydney, Australia) as described before (Ganopoulos *et al.* 2012). The HRM analyses were performed three times with independent isolates. For the current analysis each *Phytophthora* profile was considered as a genotype, and the average genotype confidence percentages (GCPs) attributed to each P*hytophthora* melting curve profile were estimated according to Hewson *et al.* (2009). A GCP value of 100 indicated an exact genotype match. All PCR products were visualised on a 1% agarose gel in order to confirm the correct amplicon sizes.

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Table 1. *Phytophthora* species, number of isolates, their hosts, and collection sites

Phytophthora species	No of isolates	Hosts	Collection sites
P. cactorum	3	*Malus sylvestris*	Tripoli
P. cambivora	3	*Castanea sativa*	Kilkis
P. capsici	3	*Capsicum annuum*	Drama
P. cinnamomi	3	*Castanea sativa*	Chania
P. citrophthora	3	*Prunus dulcis*	Preveza
P. cryptogea	3	*Solanum melongena*	Attiki
P. erythroseptica	3	*Solanum tuberosum*	Lefkada
P. megasperma	3	*Prunus dulcis*	Attiki
P. nicotianae	3	*Nicotiana tabacum*	Drama
P. palmivora	3	*Cocos nucifera*	Chania

RESULTS

In the present survey, the HRM data were accurately interpreted by means of conventional derivative genotype plots with each of the 10 *Phytophthora* species (Table 1) being assigned in a respective unique genotype profile (Figures 1B and C). These *Phytophthoras* genotypes were represented by three different melting curves (Tm) peaks profiles (Table 2 and Figure 1A). Despite that, each of these genotypes showing similar melting peak values was efficiently differentiated from the others. The ranging of the first peak was from 77.84 ± 0.2°C (*P. cryptogea*) up to 79.0 ± 0.1°C (*P. capsici*), of the second peak from 77.8 ± 0.3°C (*P. nicotianae*) up to 84.44 ± 0.4°C (*P. cactorum*), and finally of the third peak from 80.3 ± 0.2°C (*P. cambivora*) up to 88.94 ± 0.3°C (*P. erythroseptica*) (Table 2).

As mentioned above, although *Phytophthora* species melting profiles were very similar to each other, they could also be clearly discerned by the plotting of the temperature-shifted fluorescence differences (Figure 1B) and the normalised melting curves (Figure 1C). Thus, analysis of the revealed normalised HRM melting curves inferring the *Ypt1* marker allowed us to differentiate the *Phytophthora* species quite easily, even though the melting curve profiles of some species were quite similar (Figure 1C). Furthermore, a closer examination of the *Phytophthora* species HRM difference curves with the means of *P. cactorum, P. cambivora, P. capsici, P. cinnamomi, P. citrophthora, P. cryptogea, P. erythroseptica, P. megasperma, P. palmivora,* assigning *P. nicotianae* curve as the baseline, revealed part of the curve sitting outside the 90% confidence interval (CI) curve, suggesting that all the *Phytophthora* species HRM curves are indeed different (Figure 1C). Therefore, we conclude that assigning the *P. nicotianae* as a normalised reference genotype, it was really feasible to estimate the confidence similarity values among all the 10 tested species and to conclude that *Ypt1* marker was sufficient to identify and differentiate the tested species (Figure 1C).

The average GCPs values resulting from the HRM analysis are given in Table 3. A cut-off value of 90% was set in order to assign an identical genotype. The highest GCP value (63.58) was found between *P. cambivora* and *P. cinnamomi,* both infecting the forest species *Cas-*

Table 2. Melting analysis of *Phytophthora* species with their three melting peaks distribution

Phytophthora species	Peak 1 (°C)	Peak 2 (°C)	Peak 3 (°C)
P. cactorum	77.45 ± 0.2	84.44 ± 0.4	87.2 ± 0.3
P. cambivora	76.56 ± 0.2	77.9 ± 0.1	80.3 ± 0.2
P. capsici	79.00 ± 0.1	83.64 ± 0.2	86.7 ± 0.2
P. cinnamomi	77.70 ± 0.2	79.05 ± 0.2	80.74 ± 0.2
P. citrophthora	77.36 ± 0.2	78.34 ± 0.2	83.45 ± 0.1
P. cryptogea	77.84 ± 0.2	79.56 ± 0.3	83.46 ± 0.1
P. erythroseptica	77.80 ± 0.3	82.6 ± 0.2	88.94 ± 0.3
P. megasperma	77.44 ± 0.2	78.2 ± 0.2	81.7 ± 0.1
P. nicotianae	77.06 ± 0.4	77.8 ± 0.3	81.6 ± 0.1
P. palmivora	77.46 ± 0.2	79.06 ± 0.2	80.94 ± 0.2

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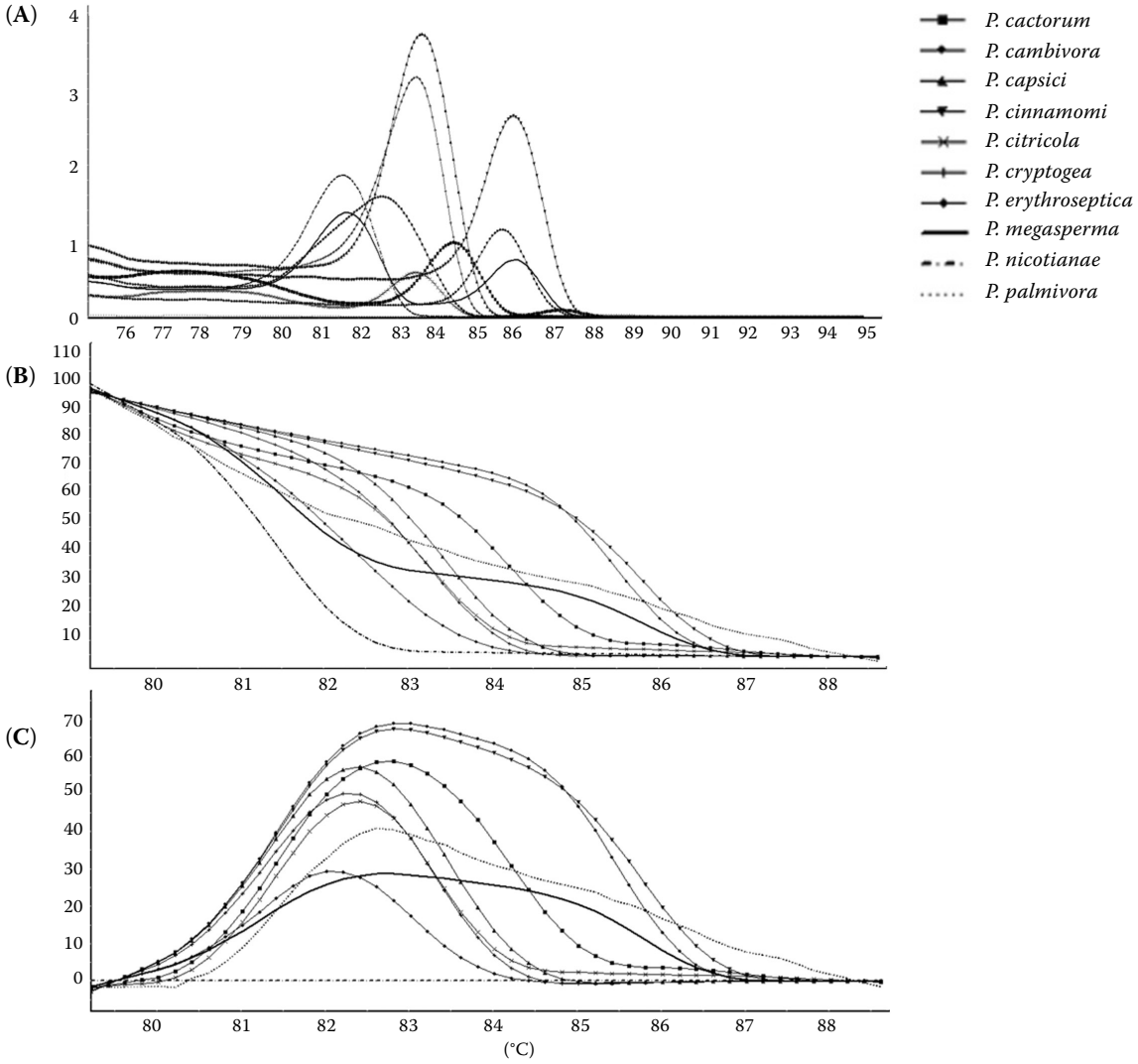

Figure 1. (**A**) Conventional melting curve profiles of the *Ypt*1 gene marker, (**B**) derived genotyping profiles of ten *Phytophthora* species developed by the HRM analysis, and (**C**) difference graphs of representative samples of *Phytophthora* species assigning *P. nicotianae* as a normalised reference genotype. Identical *Phytophthora* genotypes were assigned using a cut-off genotype confidence percentage (GCP) value of 90%. The HRM profiles of all the other *Phytophthora* species were compared to this *P. nicotianae* normalised reference genotype and were assigned as *P. nicotianae* genotypes if respective GCP values were higher than 90% or as variations if lower than 90%

tanea sativa (Table 1), indicating that the identification and differentiation of these two *Phytophthora* species among all the others species was the most challenging task, but nevertheless still quite feasible following the HRM approach. On the contrary, many species (e.g. *P. megasperma* and *P. erythroseptica*) showed constantly very low GCPs values, in some cases GCPs values at zero, indicative of quite distinct HRM profiles and phylogenies. For example, both *P. cambivora* and *P. cinnamomi* isolates revealed GCPs values at zero across all the pairwise comparisons among the other eight *Phytophthora* species (Table 3) tested in the present study.

DISCUSSION

Phytophthoras diseases represent a major threat for crops, forestry, and native ecosystems; therefore, the reliable detection and differentiation of *Phytophthora* species is a prerequisite in the diseases management (Li *et al*. 2013). This aspect is particularly important in consideration of the many *Phytophthoras* outbreaks that have recently emerged, often associated with previously undescribed species in agricultural ecosystems (Sanzani *et al*. 2014).

In the present survey, we develop and validate an innovative HRM method based on the differences in

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Table 3. Average genotype confidence percentages (GCPs) values (± 2.9) as resulting from the HRM analysis inferring the *YPT1* gene

	P. cactorum	*P. cambivora*	*P. capsici*	*P. cinnamomi*	*P. citricola*	*P. cryptogea*	*P. erythroseptica*	*P. megasperma*	*P. nicotianae*	*P. palmivora*
P. cactorum	100									
P. cambivora	0.00	100								
P. capsici	0.69	0.00	100							
P. cinnamomi	0.00	63.58	0.00	100						
P. citricola	0.06	0.00	16.60	0.00	100					
P. cryptogea	0.02	0.00	40.58	0.00	52.32	100				
P. erythroseptica	0.00	0.00	0.01	0.00	1.55	0.78	100			
P. megasperma	0.00	0.00	0.00	0.00	0.09	0.01	0.15	100		
P. nicotianae	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	100	
P. palmivora	0.10	0.00	0.00	0.00	0.03	0.00	0.00	7.41	0.00	100

the melting curve of specific amplicons of the *Ypt1* gene for the rapid differentiation of 10 *Phytophthora* species with broad host range. The *Ypt1* gene was previously shown as efficient to delineate closely related *Phytophthora* species (Ioos *et al*. 2006; Meng & Wang 2010). The different species tested generated distinctive HRM profiles, thus permitting an accurate identification and discrimination of each *Phytophthora* species. The potential discriminative efficiency of this technique is much higher than that of conventional melting curve approaches because in HRM analysis, melting curves can be efficiently differentiated even when they show the same Tm, because of the heterozygosity-derived composite melting curve profiles (Ganopoulos *et al*. 2012).

Herein, the HRM melting curve profiles formed the bases of our analysis. These HRM curves were highly informative even if compared with simple melting curves and gave enhanced information, which allowed the efficient differentiation of the tested *Phytophthora* species. A stunning aspect of our HRM analysis is that we successfully adapted degenerated primers for the *Ypt1* marker which was previously employed successfully under standard PCR conditions to discriminate *Phytophthora* species by sequencing approaches (Ioos *et al*. 2006; Meng & Wang 2010) with a more accurate and reliable molecular analysis. This was entirely confirmed by the successful genotyping of all the 10 *Phytophthora* species tested.

In conclusion, to our knowledge this is the first study employing the HRM analysis for the accurate identification and differentiation of *Phytophthora* species with broad host ranges. The current study demonstrates that the HRM method could potentially be a low cost, accurate, and rapid closed-tubed assay for the differentiation and genotyping of many *Phytophthora* species in a large scale. The target region utilised in this study offered a high variability to differentiate closely related *Phytophthora* species; in parallel, the short length of the amplicons enabled good optimisation of the reactions with positive results on the resolution of the analyses. Although our genotyping method is a promising approach to assign new unclassified and rapidly evolved *Phytophthora* strains to a specific species, this potential has not been tested so far, mainly because of the limited data available, regarding the undoubtedly encrypted genetic variation among *Phtytophthora* species. However, this is the first survey regarding identification and discrimination of ten different *Phytophthora* species using the HRM analysis.

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