Short Communication

HRM: A TOOL TO ASSESS GENETIC DIVERSITY OF *PHYTOPHTHORA CAMBIVORA* ISOLATES

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SUMMARY

Phytophthora cambivora (Petri) Buisman is a widespread soil born oomycete causing root rot on several woody species. Together with *P. cinnamomi*, it is considered the most pathogenic species associated with chestnut ink disease all over Europe. Currently, no efficient control strategies are available for ink disease. Information about the population genetic variability of *P. cambivora* is quite limited, despite that it might be useful for the development of integrated control programmes. To our knowledge no such studies have been conducted in chestnut-growing regions in Europe. Therefore, we set up a reliable and accurate genotyping approach named HRM (High Resolution Melting) analysis in order to assign efficiently and accurately the signatures of genetic variation among *P. cambivora* isolates collected in a chestnut growing area in Central Italy. Degenerated primers were designed in conserved regions of exons 3 and 4 of the gene locus *Ypt*1. The resulting melting curves profiles specifically assigned all *P. cambivora* isolates in eight unique HRM genotypes according to their normalized curve profiles implying high intraspecific genetic variation of these isolates. Data confirmed that the HRM approach might be on a wide scale a rapid and reproducible technique for efficient intraspecific genotyping of *P. cambivora* populations.

Keywords: *Castanea sativa*, fungal population genotyping, *Ypt*1 gene.

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Phytophthora cambivora (Petri) Buisman has a widespread distribution and is the cause of extensive root rot of several woody plants (Jönsson *et al*., 2003; Jung *et al*., 2000; Jung, 2009; Vettraino *et al*., 2002, 2005). All over Europe, it is well-known, along with *P. cinnamomi*, as the causal agent of ink disease, one of the most destructive diseases affecting chestnut (*Castanea sativa* Mill) (Vettraino *et al*., 2005). Sweet chestnut is an important wide-spread Mediterranean tree species in Europe, cultivated for both fruit and timber production, covering about 2,53 million hectares (Serrazina *et al*., 2015). *Phytophthora cambivora* causes severe root and collar rot of adult chestnut trees and seedlings in nurseries, plantations and forests (Vannini *et al*., 2013). Pathogen development in the host is usually very fast and the death of the tree follows within few years of infection, with evident economic repercussions on the timber and fruit trade (Vannini and Vettraino, 2011).

Isolates of *P. cambivora*, like those of other *Phytophthora* heterothallic species, form oospores when mated with isolates of the opposite mating type or, for isolates of A2 mating type, as a result of selfing due to stimulations by ubiquitous soil fungi, such as *Trichoderma*, or colony ageing and secondary plants products (Brasier, 1972; Zentmeyer, 1979). In Europe and Oregon, the A2 mating type is more frequently isolated from chestnut and chinquapin areas (Saavedra *et al*., 2007; Vettraino *et al*., 2005).

Clonal reproduction is predominant in populations of many *Phytophthora* heterothallic species (Pérez *et al*., 2001; Tian *et al*., 2015). The introduction of strains of different mating type in area where they were absent can promote sexual recombination with increased genetic diversity (Drenth *et al*., 1994). However, genetic variation can arise within *Phytophthora* clonal populations, most likely as result of mutation and mitotic recombination (Dobrowolski *et al*., 2003; Goodwin *et al*., 1995). No attention has been drawn to genetic diversity analysis within *P. cambivora* isolates from different parts of the world. However, in an

Table 1. Collection IDs numbers, source and HRM genotypes of *P. cambivora* isolates.

Collection ID number	Source	Collection Site	HRM genotypes
P95	*C. sativa* rhizosphere	Rieti (Italy)	Pc-1
P96	*C. sativa* rhizosphere	Rieti (Italy)	Pc-1
P105	*C. sativa* rhizosphere	Rieti (Italy)	Pc-1
P98	*C. sativa* rhizosphere	Rieti (Italy)	Pc-2
P142	*C. sativa* rhizosphere	Rieti (Italy)	Pc-2
P102	*C. sativa* rhizosphere	Rieti (Italy)	Pc-3
P104	*C. sativa* rhizosphere	Rieti (Italy)	Pc-3
P128	*C. sativa* rhizosphere	Rieti (Italy)	Pc-4
P143	*C. sativa* rhizosphere	Rieti (Italy)	Pc-4
P90	*C. sativa* rhizosphere	Rieti (Italy)	Pc-5
P92	*C. sativa* rhizosphere	Rieti (Italy)	Pc-6
P109	*C. sativa* rhizosphere	Rieti (Italy)	Pc-7
P120	*C. sativa* rhizosphere	Rieti (Italy)	Pc-8

isozyme study variation among isolates of *P. cambivora* has been reported by Oudemans and Coffey (1991). The knowledge of the genetic variability present in *P. cambivora* populations and how it affects the pathogen's capacity to cause disease, can ensure that chestnut breeding programmes use appropriate pathogen isolates for screening germplasms, as suggested for other Phytophthoras (Cooke *et al*., 2012; Li *et al*., 2012).

The aim of the current investigation was to evaluate the use of the High Resolution Melting (HRM) approach in order to screen for *Ypt*1 gene mutations in *P. cambivora* isolates. This quantitative technique exploits the dissociation of double to single stranded DNA by escalating temperatures resulting in a homogenously intercalated fluorescent dye release (Reed and Wittwer, 2004). HRM has already been employed for fungal genotyping and differentiation (Zambounis *et al*., 2015a) such as among *Fusarium oxysporum* formae speciales (Ganopoulos *et al*., 2012) or within and among rot-related species of the *Alternaria* complex (Zambounis *et al*., 2015b). In addition, HRM has been used for population genetics analyses of the nut rot agent of chestnut *Gnomoniopsis castaneae* in Europe (Sillo *et al*., 2016). The ras-related protein *Ypt*1 gene is a promising locus for phylogenetics and species differentiation because of the highly polymorphic nature of its non-coding regions (König *et al*., 2015; Meng and Wang, 2010; Schena and Cooke, 2006; Schena *et al*., 2008).

In this primary study, a total of 13 isolates of *P. cambivora* (Collection of the Laboratory of Mycology and Forest Pathology, DIBAF, University of Tuscia, Italy), from chestnut growing areas in Central Italy, were analysed (Table 1). These isolates were grown on carrot broth 20% (w/v) for one week at 20°C (Vettraino *et al*., 2012). Genomic DNA was extracted from mycelia using the Qiagen DNeasy Plant Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's protocols. DNA extracts were quantified using the Qubit Quantitation Kit (Life technologies, Monza, Italy). *Phytophthora* species-specific degenerated primers (YPh6F: GAGYTACATCTCGACCATYGG and YPh10R: TCTTGCCGTCCARCTCRAT) were designed in conserved regions of exons 3 and 4 of the *Ypt*1 gene locus, using the Primer 3 software (Rozen and Skaletsky, 2000). The sizes of all PCR products were expected to be approximately 130 bp.

All HRM reactions, after optimization of the DNA and $MgCl_2$ concentrations, were performed in a final volume of 15 µl using a Rotor-Gene 6000 real-time 5P HRM PCR Thermocycler (Corbett Research, Sydney, Australia) and contained 20 ng of fungal genomic DNA, 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 300 nM of each degenerated primer, 1.5 mM Syto®green fluorescent nucleic acid stain, and 1 U Kapa Taq DNA polymerase (Kapa Biosystems). Reactions were conducted at an initial denaturing step of 95°C for 3 min, followed by 35 cycles of 95°C for 20 s, 55°C for 45 s and 72°C for 50 s, with a final extension step of 72°C for 2 min. The fluorescent HRM data were acquired at the end of each extension step. Before HRM reactions, all products were denatured at 95°C for 5 s and then annealed at 50°C for 30 s in order to create the respective DNA duplexes. The HRM analysis was performed three times, using ddH_20 as a negative control; all PCR products were visualized in agarose gel to confirm their expected amplicon sizes.

The HRM data were accurately interpreted by means of conventional derivative genotype plots for all 13 *P. cambivora* isolates, according to Hewson *et al.* (2009). Results were analyzed as fluorescence versus temperature graphs by Rotor-Gene Q software version 2.3.1 (QIAGEN, Bio-Analytica S.A, Greece) with normalized, temperature-shifted melting curves displayed as difference plot. The Pc-1 genotype normalization curve was assigned as a baseline. An average cut-off genotype confidence percentage (GCP) value of 90% among the three HRM runs was set for assigning isolates in an identical genotype. A GCP value of 100 indicates an exact genotyping match. In contrast, GCP values of zero are indicative of entirely distinct HRM profiles, implying existence of high genetic diversity among isolates, and consequently different HRM genotypes (Zambounis *et al*., 2015a).

All the samples showed amplification with the HRM primer set and produced specific melting profiles, consisting of one main defined peak (Fig. 1A). After normalization and temperature shifting, a clear difference was evident among the samples (Fig. 1B). This difference was emphasized by using the fluorescence difference plots, where the curves for the samples were clustered around the baseline (Fig. 1C).

Analysis of the normalized HRM curves produced with the *Ypt*1 marker revealed that all isolates could easily be distinguished. Furthermore, closer examination of the *P. cambivora* curves, with the curve of strain P95 (Pc-1 genotype) as the baseline, revealed part of the curve sitting outside the 90% CI (confidence interval) curve, suggesting that a significant number of examined *P. cambivora* isolates via the HRM curves are indeed different (Fig. 1C).

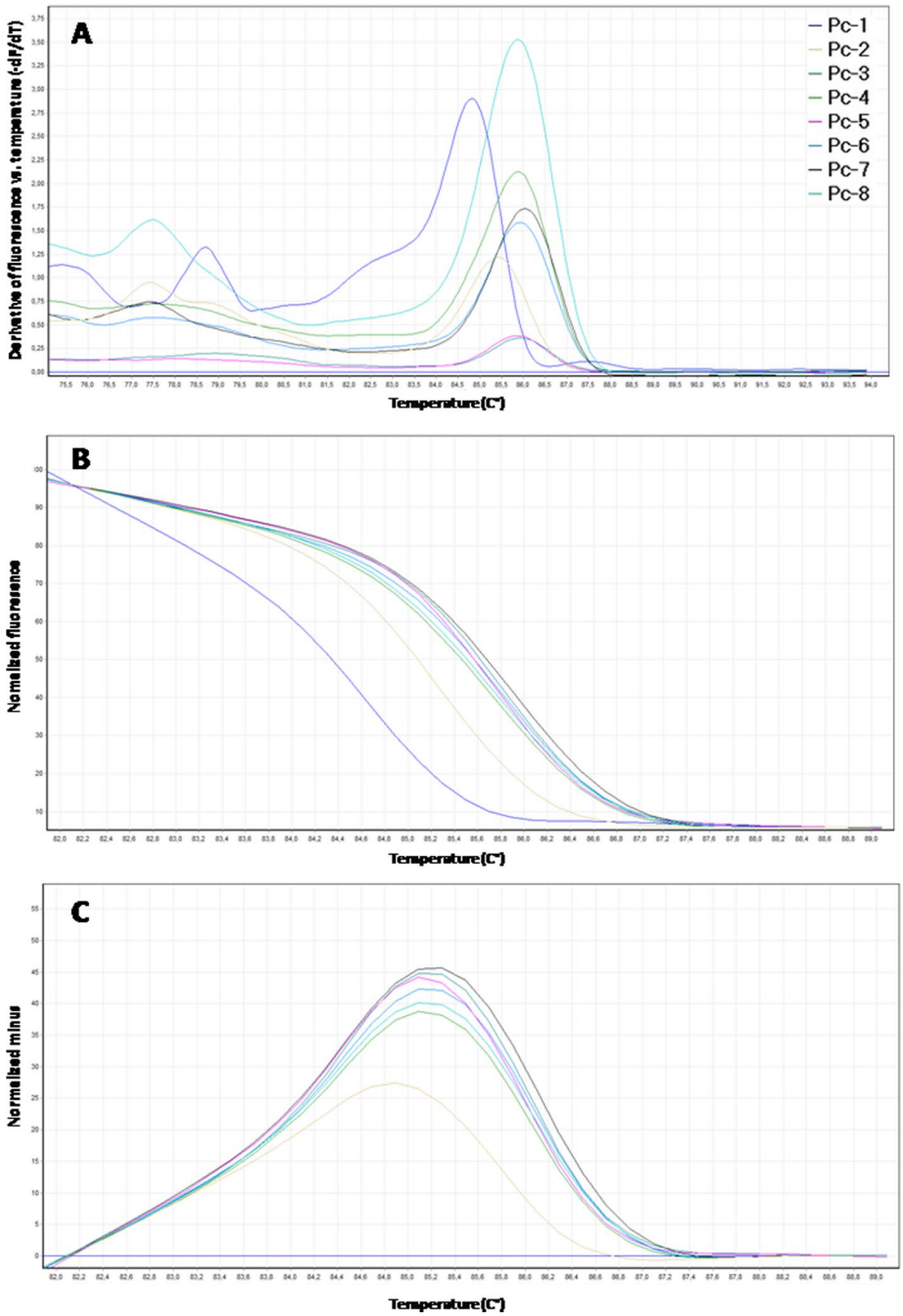

Fig. 1. A. Conventional melting curve profiles of the *Ypt*1 gene locus. B. Genotypes normalized profiles of the eight assigned *P. cambivora* genotypes developed by the HRM analysis. C. Difference graphs of representative samples of these *P. cambivora* genotypes assigning Pc-1 as a normalized reference genotype. The HRM profiles of all the other seven genotypes were compared to this normalized reference Pc-1 genotype. *P. cambivora* genotypes were assigned using a cut-off GCP value of 90%.

Phytophthora cambivora isolates were assigned to eight different genotypes. Each of these eight genotypes profiles was represented by different melting curve peak (Tm) values. The Tm values among these genotypes ranged from 78.70 ± 0.1°C (Pc-1) up to 91.50 ± 0.1°C (Pc-6). The Pc-1 genotype was the most popular containing the isolates P95, P96, P105. The genotypes Pc-2, Pc-3, and Pc-4 consisted of each two isolates. Finally, isolates P90, P92, P109 and P120 were allocated to separate genotypes Pc-5, Pc-6, Pc-7, Pc-8, respectively (Table 1).

Despite the importance of *P. cambivora* and the fact that much survey work on distribution and resistance has been carried out, studies of its molecular diversity have been limited (Oudemans and Coffey, 1991). A better understanding of pathogen population dynamics will contribute to more durable disease management strategies. However, genotyping pathogen populations pose several challenges, including the choice of the proper markers. In fact, their resolution should be sufficiently high to discriminate multilocus genotypes (MLGs) and also identify

sub-clonal variation. The level of genetic variability in Phytophthoras has been evaluated by a number of studies using various molecular markers. In the past, AFLP markers, simple sequence repeats (SSRs), RAPD analysis have been used to evaluate the genotypic variation of several Phytophthoras (Cooke *et al*., 2005; Mahuku *et al*., 2000; Mascheretti *et al*., 2008). The availability of entire genome sequences for an increasing number of species has provided novel opportunities to identify and evaluate potential SSR markers identified by computational tools (Biasi *et al*., 2015; Garnica *et al*., 2006; Lees *et al*., 2006; Li *et al*., 2013). Recent advance of genotyping-by-sequencing (GBS) methods are assigning them as less expensive and thus, are increasingly used for population genetic studies (Davey *et al*., 2011; Elshire *et al*., 2011; Lu *et al*., 2013). Additionally, advents of genome-wide approaches such as next generation sequencing enable the study of genetic variation in Phytophthoras (Quinn *et al*., 2013; Vannini *et al*., 2013; Vettraino *et al*., 2012). However, due to the complexity of analyzing large data sets, such applications may require substantial time, expertise, and computational resources. HRM-based genotyping techniques are promising alternatives in order to investigate the genetic diversity among fungi species and populations. HRM techniques are rapid, simple, and of low-cost compared with the others genotyping approaches (Athamanolap *et al*., 2014; Cousins *et al*., 2012; Sillo *et al*., 2016; Zambounis *et al*., 2015a).

In conclusion, in this primary study, we optimized the HRM method for the accurate genotyping of *P. cambivora* isolates collected from a chestnut growing area in Central Italy. The analysis, focused on only 13 isolates and the ras-related protein gene *Ypt*1, revealed a high variability among these isolates. Thus, HRM analysis using the *Ypt*1 locus is capable to detect intraspecific genetic variation. Using a larger population size and a multigene approach, this method has the potential to be employed to evaluate the intraspecific genetic variation of *P. cambivora* populations from different populations.

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