**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 Ypt1. 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, Ypt1 gene.
Table 1. Collection IDs numbers, source and HRM genotypes of P. cambivora isolates.

<table>
<thead>
<tr>
<th>Collection ID number</th>
<th>Source</th>
<th>Collection Site</th>
<th>HRM genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>P95</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-1</td>
</tr>
<tr>
<td>P96</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-1</td>
</tr>
<tr>
<td>P105</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-1</td>
</tr>
<tr>
<td>P98</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-2</td>
</tr>
<tr>
<td>P142</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-2</td>
</tr>
<tr>
<td>P102</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-3</td>
</tr>
<tr>
<td>P104</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-3</td>
</tr>
<tr>
<td>P128</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-4</td>
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<tr>
<td>P143</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-4</td>
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<td>P90</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-5</td>
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<td>P92</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-6</td>
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<td>P109</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-7</td>
</tr>
<tr>
<td>P120</td>
<td>C. sattiva rhizosphere</td>
<td>Rieti (Italy)</td>
<td>Pc-8</td>
</tr>
</tbody>
</table>

isoyme 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 Ypt1 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 specialiae (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 Ypt1 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 (Vetraino 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). Phytophtora species-specific degenerated primers (YPH6F: GAGYTACATCCTGACCAYGG and YPh10R: TCTTGCCGTCCARCTCRAT) were designed in conserved regions of exons 3 and 4 of the Ypt1 gene locus, using the Primer 3 software (Rozen and Skalesky, 2000). The sizes of all PCR products were expected to be approximately 130 bp.

All HRM reactions, after optimization of the DNA and MgCl2 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 MgCl2, 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 ddH2O 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 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 Ypt1 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).
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 of several Phytophthoras (Quinn et al., 2013; Vannini et al., 2014). HRM 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., 2015).

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 Ypt1, revealed a high variability among these isolates. Thus, HRM analysis using the Ypt1 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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REFERENCES


