Evaluation of High-Resolution Melting for Rapid Differentiation of *Phytophthora* Hybrids and Their Parental Species

Maria F. Ratti,^{1,2,†} Rhys A. Farrer,³ Liliana M. Cano,³ Roberto Faedda,⁴ and Erica M. Goss^{1,†}

¹ Department of Plant Pathology and Emerging Pathogens Institute, University of Florida, Gainesville, FL 32611 U.S.A.

² Facultad de Ciencias de la Vida, Escuela Superior Politécnica del Litoral, Campus Gustavo Galindo, Km 30.5 Vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador

³ The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, United Kingdom

⁴ Department of Agriculture, Food and Environment, University of Catania, Catania, Italy

Abstract

Phytophthora species hybrids have been repeatedly reported as causing damaging diseases to cultivated and wild plants. Two known hybrids, *P. andina* and *P. × pelgrandis*, are pathogens of Solanaceae and ornamentals, respectively, although the extent of their host ranges are unknown. *P. andina* emerged from hybridization of *P. infestans* and an unidentified related species, whereas *P. × pelgrandis* emerged from *P. nicotianae* and *P. cactorum*. Considering that hybrids and parental species can coexist in the same regions and to distinguish them usually requires cloning or whole genome sequencing, we aimed to develop a rapid tool to distinguish them. Specifically, we used high-resolution melting (HRM) assays to differentiate genotypes based on their amplicon melting profiles. We

designed primers for *P. × pelgrandis* and parental species based on available sequences of *P. nicotianae* and *P. cactorum* nuclear genes containing polymorphisms between species. For *P. andina*, heterozygous sites from Illumina short reads were used for the same purpose. We identified multiple amplicons exhibiting differences in melting curves between parental species and hybrids. We propose HRM as a rapid method for differentiation of *P. andina* and *P. × pelgrandis* hybrids from parental species that could be employed to advance research on these pathogens.

Keywords: HRM analysis, heterozygosity, *Phytophthora andina*, *P. × pelgrandis*

Human transportation and other forms of migration have moved plant pathogens out of their evolutionary origin and into habitats containing other pathogens. When previously allopatric pathogens lack reproductive isolation, interspecific hybridization may occur (Beckerman et al. 2014; Brasier 2008; Harrison and Larson 2014). Pathogenic hybrids can pose different economic and ecological risks than parental species (Brasier 2000). Indeed, interspecific hybridization introduces genetic variation to a newly introduced pathogen and may allow rapid adaptation to a new host or environment, resulting in the emergence of new diseases (Aguayo et al. 2016). Hybrid offspring may persist as independent species, particularly when they are reproductively isolated from the parental species or exhibit advantageous phenotypes or increased fitness. The prevalence of interspecific hybrids may be under-reported because molecular markers are generally required to confirm hybrid status (Burgess 2015).

Current address of R. A. Farrer: Medical Research Council Centre for Medical Mycology at The University of Aberdeen, Aberdeen, AB243FX, United Kingdom.

Current address of L. M. Cano: Department of Plant Pathology, Indian River Research and Education Center, University of Florida UF/IFAS, Fort Pierce, FL 34945, U.S.A.

†Corresponding authors: M. F. Ratti; mratti@espol.edu.ec and E. M. Goss; emgoss@ufl.edu

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Evolutionary adaptability and effective dispersal have enabled *Phytophthora* species to colonize new niches and become invasive pathogens (Érsek and Nagy 2008; Schardl and Craven 2003). There are several instances of hybridization between *Phytophthora* species that are associated with host shifts and aggressive new taxa (Brasier et al. 1999; Goss et al. 2011; Husson et al. 2015; Man in 't Veld et al. 2012; Oliva et al. 2010; Yang et al. 2014). The most well-explored case is *P. × alni*, which is hypothesized to have emerged through several hybridization events, possibly in nurseries, before establishing as an aggressive alder pathogen in natural ecosystems in Europe (Brasier et al. 2004). Host range change via hybridization has been explored through laboratory crosses between *P. mirabilis* and *P. infestans*, whose progeny were not pathogenic on *Mirabilis jalapa* (host of *P. mirabilis*) or potato (host of *P. infestans*). However, some progeny could infect tomato (Goodwin and Fry 1994), which demonstrates the ability of hybrids to have altered host range.

In the Andean region of Ecuador, *P. infestans* was thought to have a simple population genetic structure, until blight symptoms were found on wild *Solanum* species. Atypical *P. infestans* isolates were isolated from wild hosts, as well as *S. quitoense* (lulo) and *S. betaceum* (tree tomato), which are commercialized in the Andes and cultivated elsewhere (Oliva et al. 2010; Ordoñez et al. 2000). The atypical isolates were described as a new species, *P. andina*, based on amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism, and allozyme analyses (Oliva et al. 2010). *P. andina* was subsequently identified as an interspecific hybrid of *P. infestans* and an unknown closely related species based on the two phylogenetically diverged alleles of nuclear housekeeping genes found in *P. andina* (Blair et al. 2012; Goss et al. 2011). The second parental species may reside cryptically in the Andean region and nearby Amazon basin of Ecuador, Colombia, and Peru (Adler et al. 2002; Érsek and Man in 't Veld 2013; Goss et al. 2011). Both *P. infestans* and *P. andina* are able to infect *S. muricatum*, which could have served as a bridge between the parental species (Oliva et al. 2010). *S. betaceum* is attacked by *P. andina* genotype EC-3, recently redescribed as *P. betacei* (Mideros et al. 2018). Presently, isolates causing late blight on *Solanum* hosts in the Andes are genotyped at simple sequence repeat (SSR) loci to assign them to species (Forbes et al. 2016).

P. × pelgrandis was reported for the first time in Dutch hydroponic greenhouses, where it was recovered from the ornamentals *Spathiphyllum* and *Primula* with root and crown rot (Man in 't Veld et al. 1998). *P. × pelgrandis* produces abortive oospores, suggesting that its sexual reproductive system is nonfunctional (Man in 't Veld et al. 2012). Random amplification of polymorphic DNA (RAPD) and isozyme analysis determined its relationship with *P. nicotianae*, followed by AFLP analysis and ITS sequencing to recognize *P. cactorum* as the second parent of this interspecific hybrid (Érsek and Man in 't Veld 2013; Man in 't Veld et al. 2012). *P. cactorum* and *P. nicotianae* are closely related species in *Phytophthora* clade 1 (Blair et al. 2008; Cooke et al. 2000). Mitochondrial DNA from Dutch *P. × pelgrandis* isolates was of *P. nicotianae* origin (Man in 't Veld et al. 1998). In contrast, German isolates had a mitochondrial *coxI* locus of *P. nicotianae* origin, and RAPD analysis of nuclear DNA indicated a greater resemblance to *P. cactorum* than *P. nicotianae*, showing the difficulty of identification of *P. × pelgrandis* with traditional methods (Nirenberg et al. 2009). *P. × pelgrandis* represents a more widespread risk to agriculture than *P. andina*, because it has been reported from the Netherlands, Taiwan, Peru, Germany, the United States, Hungary, and Italy and has a wide host range including ornamentals of commercial importance as well as conifers (Faedda et al. 2013; Hurtado-Gonzales et al. 2009; Leonberger et al. 2013; Man in 't Veld et al. 1998; Nirenberg et al. 2009; Szigethy et al. 2013).

A method for routine and rapid detection of *Phytophthora* hybrid species would facilitate study of their host ranges and distributions and would accelerate management decisions. A characteristic of *Phytophthora* hybrids is high heterozygosity in the nuclear genome, a result of the polymorphism between homologous chromosomes from parent species (Harrison and Larson 2014; Man in 't Veld et al. 2012; Ordoñez et al. 2000). Loci that differentiate parental species may therefore be heterozygous in the hybrid. High-resolution melting (HRM) is a quantitative PCR (qPCR)-dependent technique that measures the rate dissociation of double-stranded to single-stranded DNA as temperature increases (Martini et al. 2015; Taylor 2009). This technique allows the identification of different single nucleotide polymorphism (SNP) and heterozygous genotypes by taking advantage of their distinct melting temperatures (T_m) (Hansen et al. 2016). During the HRM, gradual increases in temperature cause double-stranded DNA to dissociate, releasing a dye bound to the double-stranded DNA and decreasing the fluorescence level. The rate of dissociation depends on the thermodynamics of the DNA sequence being analyzed. Polymorphisms within and among samples cause small but detectable differences in T_m , thus forming unique fluorescence profiles (Ganopoulos et al. 2012; Hansen et al. 2016; Reed and Wittwer 2004). Importantly for *Phytophthora* hybrids, HRM can be used to differentiate heterozygous from homozygous genotypes. HRM has been used in a variety of plant pathology-related

studies in which accurate differentiation of one or a few polymorphisms is required. These methods have been used to distinguish seven *Fusarium oxysporum* formae speciales (Ganopoulos et al. 2012), closely related *Monilinia* spp. associated with brown rot of pome fruits (Papavasileiou et al. 2016), multiple *Phytophthora* species (Veerappa Hanumanthappa et al. 2018; Zambounis et al. 2016), and *P. infestans* lineages in the United States (Hansen et al. 2016), among others.

We hypothesized that HRM analysis can differentiate hybrid *Phytophthora* from their parent species using loci that are heterozygous in the hybrid. To test this hypothesis, we used the hybrids *P. andina* and *P. × pelgrandis*. Thus, our objectives were to (i) identify loci that distinguish *P. andina* and *P. × pelgrandis* from parental species; (ii) design primers for HRM assays based on these sites; and (iii) test primer pairs for differentiation of *P. andina* and *P. × pelgrandis* from parental species by HRM. Here we show proof of concept for using HRM to differentiate *P. andina* and *P. × pelgrandis* from parental species, which could aid study of the distribution and host range of *Phytophthora* hybrids.

Materials and Methods

Isolates used in this study. Isolates or DNA of *P. infestans*, *P. andina*, *P. nicotianae*, *P. cactorum*, and *P. × pelgrandis* were kindly provided by collaborators. Host and country of origin are described in Table 1. For genomic DNA extraction, we used the FastDNA isolation kit (MP Biomedicals, U.S.A.) for *P. infestans* and *P. andina* and used the DNeasy Plant mini kit (Qiagen, Valencia, U.S.A.) for *P. nicotianae*, *P. cactorum*, and *P. × pelgrandis*. We adjusted extracted genomic DNA to a concentration of 20 ng/μl.

Identification of polymorphic sites and primer design. To identify polymorphisms between *P. infestans* and *P. andina* we used heterozygous sites identified from Illumina short reads of *P. andina* isolate EC3425 (SRA accession PRJNA52431) mapped against *P. infestans* isolate T-30-4 contigs. Methods for generation of sequence data and mapping are as described in Raffaele et al. (2010). A Samtools pileup file summarizing polymorphisms in 50 genes conserved between *P. andina* and closely related species was explored using Artemis 16.0.0 (Rutherford et al. 2000). Sections of 80 to 100 bp with sites that appeared to be polymorphic in the sequencing data were selected to design primers suitable for amplifying these short regions. Primer3 version 0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012) generated several sets of primers that would amplify 50 to 90 bp with optimum melting temperatures of 60°C. These amplicons were tested in silico using uMelt software (Dwight et al. 2011), which produces a virtual melting profile curve for each sample, with values of fluorescence versus temperature and fluorescence derivative ($-dF/dT$) versus temperature at 0.5°C intervals. Derivative values were transferred to a spreadsheet, where they were plotted. The temperature at which the peak reached a maximum was recorded

Table 1. Species, name, host, and country of collection of *Phytophthora* isolates used in this study

Species	Isolate name	Host genus	Origin	Provided by	Reference
<i>P. infestans</i>	J107	<i>Solanum</i>	Mexico	S. Fernandez-Pavia	Wang et al. (2017)
	Mich7043	<i>Solanum</i>	Mexico	S. Fernandez-Pavia	Wang et al. (2017)
	Mich7039	<i>Solanum</i>	Mexico	S. Fernandez-Pavia	Wang et al. (2017)
	Mich7061	<i>Solanum</i>	Mexico	S. Fernandez-Pavia	Wang et al. (2017)
<i>P. andina</i>	EC 3821	<i>Anarrichomenum</i>	Ecuador	R. Oliva/G. Forbes	Goss et al. (2011)
	EC 3865	<i>Solanum</i>	Ecuador	R. Oliva/G. Forbes	Oliva (2009)
	EC 3818	<i>Anarrichomenum</i>	Ecuador	R. Oliva/G. Forbes	Goss et al. (2011)
<i>P. nicotianae</i>	F11	<i>Ananas</i>	Ecuador	M. Ratti	Ratti et al. (2018)
	M231	<i>Ananas</i>	Ecuador	M. Ratti	Ratti et al. (2018)
	G194	<i>Ananas</i>	Ecuador	M. Ratti	Ratti et al. (2018)
	Correa2	<i>Correa</i>	Italy	F. Martin/L. Schena	Biasi et al. (2016)
	C301	<i>Myrtus</i>	Italy	F. Martin/L. Schena	Biasi et al. (2016)
	FerraraR1	<i>Citrus</i>	Italy	F. Martin/L. Schena	Biasi et al. (2016)
<i>P. cactorum</i>	11-1	<i>Fragaria</i>	U.S.A.	N. Peres	Souza et al. (2012)
	12-12	<i>Fragaria</i>	U.S.A.	N. Peres	Souza et al. (2012)
<i>P. × pelgrandis</i>	956/07	<i>Lavandula</i>	Italy	R. Faedda/S. O. Cacciola	Faedda et al. (2013)

and compared between *P. infestans* and *P. andina*. If this difference was $\geq 0.5^{\circ}\text{C}$, we considered the amplicon a candidate for HRM. Resulting primers were tested on hybrid and parental isolates by visualizing PCR products in 2% agarose gels after end-point PCR before qPCR and HRM. Primers were also examined for potential specificity to *P. infestans* and *P. andina* by searching their sequences against closely related *Phytophthora* species using the BLASTn tool.

To identify heterozygous SNPs in *P. × pelgrandis*, available sequences of nuclear genes from *P. nicotianae* and *P. cactorum* were aligned and compared visually in MEGA version 6.0 (Tamura et al. 2013). For most genes, one sequence of *P. × pelgrandis* was included in the alignment, whereas three to nine *P. cactorum* and four to 42 *P. nicotianae* sequences were available. Regions of 80 to 100 bp containing one or more SNPs between *P. nicotianae* and *P. cactorum* were identified. These regions were then tested in uMelt and used to generate primers, and virtual amplicons were evaluated as above.

PCR and HRM. PCR and HRM were performed in the Applied Biosystems 7500Fast system. MeltDoctor reagents or master mix were used in 20- μl reactions. MeltDoctor HRM reagent kit was added in the following manner: 1 $\times$ buffer, 3 mM MgCl_2 , 0.2 mM dNTP mix, 0.4 μM each primer, 1 $\times$ HRM dye, 0.1 U of AmpliTaq Gold DNA polymerase, and 5 ng of DNA. PCR cycling was done as follows: 95 $^{\circ}\text{C}$ for 10 min, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 15 s, annealing temperature for 30 s, and 60 $^{\circ}\text{C}$ for 45 s. The HRM protocol followed the manufacturer's instructions. Fluorescence was recorded every 0.1 $^{\circ}\text{C}$. Each isolate was repeated three times for each primer set.

Melting curves were visualized and analyzed with Applied Biosystems HRM software version 3.0.1 (Life Technologies). Melting profiles were generated by the software, which involved calculating pre- and postmelting regions, derivative melting curve, and aligned and difference melting curves and then plotting these curves. To visualize melting profiles among samples amplified with the same primer set, we used change in fluorescence at each temperature step. Most primer sets used default determination of pre- and postmelting temperature to define the region of analysis, but if complex curves were generated, we considered only one peak, which was either the first peak or the peak present for all isolates. Variants were called according to the software analysis parameters, which are proprietary (Applied Biosystems 2009). The software groups curves based on their dissociation differences, which are owing to mismatches, deletions, or insertions. If variant calls were consistent with species

tested, we considered the primer set to be successful in hybrid/parental discrimination. For visualization of melting curve variation within and between species, curves were aligned with the fluorescence values at the pre- and postmelting temperatures set to 100 and 0, respectively.

Initially, one or two isolates of each parental and hybrid species were run for each primer set. When the software was able to identify different variants, these primer sets were retested with additional isolates, if available. Information on gene targets, reference sequences, annealing temperature, and amplicon sizes of candidate primer sets are detailed in Table 2.

To visualize performance of the test, we performed receiver operating characteristics (ROC) curve analysis and calculated the area under the curve (AUC) for each primer set. ROC analysis has been used in medicine to evaluate the accuracy of diagnostic tests with dichotomous responses, by describing the relationship of test sensitivity to specificity (Hajian-Tilaki 2013). We used the fluorescence derivative ($-dF/dT$) and produced ROC curves in blocks of 0.5 $^{\circ}\text{C}$, for which there were observable differences in derivative values between variants. ROC curves and AUC were calculated and visualized in R using the pROC package (Robin et al. 2011). The temperature range with the highest AUC values indicates the threshold temperatures that distinguish between hybrid and parental species.

Results

***P. andina* and *P. infestans*.** For *P. andina*, heterozygous sites were found at several loci within protein coding genes that were annotated as hypothetical proteins or were putative known proteins (Table 2). We tested 24 primer sets (Supplementary Table S1) based on amplicon simulations in uMelt. End-point PCR produced 14 primer sets that amplified in both *P. infestans* and *P. andina*.

Candidate primer sets PaM2, PaM18, PaM19, PaM27, and PaM57 amplify regions that contained one or two SNPs in the sequence data for EC3425 (Table 2). All SNPs caused transitions, except for amplicon PaM2, which contained one transversion. Five primer sets successfully distinguished *P. infestans* and *P. andina* as two different variants by HRM, whereas PaM33 misassigned *P. infestans* isolates Mich7039 and Mich7043. Primers PaM2 and PaM18 were tested with two isolates per species, PaM19 with four *P. infestans* and two *P. andina* isolates, and the others with four *P. infestans* and three *P. andina* isolates. Supplementary Figure S1 shows the derivative melting graphic for all six primer sets, and pre- and postmelting temperatures that defined the region of analysis are provided in Table 3.

Table 2. Amplicons that were suitable for high-resolution melting analysis^a

Amplicon designation	Location/accession number of reference sequence ^b	Polymorphic sites	Annealing temperature ($^{\circ}\text{C}$)	Amplicon size (bp)
PaM2	PITG_18049 Chromosome segregation protein / XM_002997638.1	2	50	87
PaM18	PITG_03974 Conserved hypothetical protein / XM_002906958.1	1	50	63
PaM19	PITG_04010 APS kinase/ATP sulfurylase/pyrophosphatase fusion protein / XM_002906988.1	1	51	51
PaM27	PITG_08406 Conserved hypothetical protein / XM_002903744.1	1	52	56
PaM33	PITG_09705 SUMO-conjugating enzyme (SCE), putative	1	52	63
PaM57	PITG_20799 / XM_002903264.1 Conserved hypothetical protein / XM_002895373.1	1	51	57
Ph9	Beta (β) tubulin / MH101875.1, MH493911.1	3	51	66
Ph11	Beta (β) tubulin / MH101875.1, MH493911.1	4	54	67
Ph14	Tryptophan biosynthesis protein (TRP1) / JN664185.1, JN664180.1	2	53	59
Ph25	Triosephosphate isomerase/glyceraldehyde-3-phosphate dehydrogenase (TigA) / EU080604.1, EU080283.1	5	51	70
Ph29	Triosephosphate isomerase/glyceraldehyde-3-phosphate dehydrogenase (TigA) / EU080604.1, EU080283.1	12	52	90

^a Primer sequences for each amplicon are provided in the supplementary tables.

^b Ph primer sets have two reference sequence accession numbers that correspond to *Phytophthora cactorum* and *P. nicotianae*, respectively.

Figures 1 and 2 show aligned melting curves for each primer set, by isolate and by variant. We observed variation among replicates and isolates within a species, but variants could be differentiated using pre- and postmelting temperature settings that were modified to include a single peak. PaM2, the only amplicon that contained two SNPs (one transition and one transversion), provided wider separation between curves for *P. infestans* and *P. andina*. The type of SNPs present in amplicons can affect the shift in curves, with A/T and C/G SNPs causing smaller shifts than other types of SNPs (Applied Biosystems 2009).

We also visualized difference data, where one melting curve was designated as the “standard” and was set to 0 for each temperature, and other reactions using the same primer set were compared with this standard (Supplementary Fig. S2). These graphics show thermodynamic differences among melting curves and variation among replicate reactions. Melting curves for *P. andina* had negative differences when compared with *P. infestans* for all primer sets except for PaM2. The negative values indicate that *P. andina* released a given level of fluorescence at a lower temperature than *P. infestans*, which is related to the nucleotide present at the polymorphic site or sites. All primer sets produced ROC curves with AUC values greater than 0.98, except for PaM33, which misassigned two isolates and produced an AUC of 0.86 (Supplementary Fig. S6A).

***P. nicotianae*, *P. cactorum*, and *P. × pelgrandis*.** A total of seven nuclear genes were examined for polymorphic sites between *P. cactorum* and *P. nicotianae*: *Btub*, *TRP1*, *Ypt1*, *TEF1*, *TigA*, *Hsp90*, and *ENL*. Based on the assumption that *P. × pelgrandis* is heterozygous in these loci, 22 primer sets were designed (Supplementary Table S2), and 10 amplified both parental species and the hybrid. Five sets successfully distinguished *P. × pelgrandis* as a variant distinct from one or both parental species based on melting profiles (Table 2). For distinguishing *P. × pelgrandis*, we designed primers for regions with two to 12 SNPs that were mostly transitions or a mix of transitions and transversions.

To distinguish *P. × pelgrandis* from its parental species, we used settings described in Table 3, and derivative graphics are shown in Supplementary Figure S3. Primer set Ph29 produced complex melting profiles, and thus we examined two different pre- and postmelting temperature settings. Aligned melting curves for each primer set are shown in Figures 3 and 4.

Ph14 successfully differentiated all three species. Ph9 was able to distinguish *P. nicotianae* but not *P. cactorum* from *P. × pelgrandis*, producing two variants. Ph11 and Ph25 distinguished *P. cactorum* but not *P. nicotianae* from *P. × pelgrandis*, also producing two variants. Ph29 was able to differentiate all three species as variants, although the curve separation was only strong for *P. cactorum* versus *P. × pelgrandis*. Note that we used only one isolate of *P. × pelgrandis* for assays; thus, we were not able to assess variation among isolates of this hybrid. Details on the curves that were generated for primer sets Ph9, Ph11, and Ph25, which were not called as three variants when analyzed together, are shown in Supplementary Figure S5.

We noticed that a higher number of SNPs (three or more) did not reliably produce greater separation of curves among species. This was found using primer set Ph9 (four SNPs) compared with Ph11 and Ph14 (three and two SNPs, respectively). Likewise, Ph25 and Ph29 (five and 12 SNPs, respectively) showed clear separation according to the species tested but no greater divergence among curves. The SNPs in reference amplicon sequences are shown in Supplementary Table S3.

Difference plots showed variation within species and among replicates. For Ph29 (Supplementary Fig. S4F), there was variation among *P. nicotianae* isolates (shades of brown) but still clear differentiation from *P. × pelgrandis* (blue). Ph9 showed less separation than the other primer sets in the aligned curves graphic (Fig. 3A), but species were called correctly. All primer sets had AUC values of 1.

Discussion

Interspecific hybrids of *Phytophthora* species are being reported with increasing frequency and include emerging pathogens (Brasier et al. 2004; Goss et al. 2011; Man in 't Veld et al. 2012). Two hybrids that pose economic risks and are the subject of ongoing research are *P. andina* and *P. × pelgrandis*. We showed that HRM can differentiate isolates of the hybrid pathogens *P. andina* and *P. × pelgrandis* from isolates of their parental species. For researchers interested in identifying these hybrids and their parental species from diseased samples, the technique we describe in this study can distinguish hybrid from parental isolates faster than traditional genotyping or sequencing.

Survival in a changing environment is a driving force in evolution, and hybridization is a remarkable way to obtain significant genetic changes in relatively short time. Interspecific hybridization impacts genome evolution enormously and can result in increased pathogenicity of filamentous microbes (Depotter et al. 2016). Interspecific hybridization is limited by reproductive barriers and genome divergence, but the movement of live plants and their pathogens into new environments by commercial trade increases the possibility of hybridization among closely related species that have not encountered each other for thousands or millions of years (Beckerman et al. 2014; Burgess 2015; Depotter et al. 2016; Faedda et al. 2013; Man in 't Veld et al. 2012; Safaiefarahani et al. 2016; Stukenbrock 2016). Multiple hybrids have been found in nurseries where plant genera such as *Rhododendron* can act as melting pots for multiple *Phytophthora* pathogens (Beckerman et al. 2014). Hybrids may then shift or spread to new hosts or escape from the nursery to wild hosts, causing economic and environmental problems. Both *P. × pelgrandis* and *P. andina* were initially investigated because they were causing disease on new hosts, relative to parental species. Hybrids may outcompete parental species on their primary host or may shift from the parental host when they are less fit or competitive on that host than the parental species (Depotter et al. 2016). Detection of these host range shifts and host jumps is a critical first step in understanding the risk of hybrid pathogens as they arise and spread.

Table 3. Recommended pre- and postmelting temperature settings for analysis of melting curves for each primer set^a

Primer set	Premelting start / stop	Postmelting start / stop	Isolates successfully assigned to species-specific variants ^b
PaM2	79.2 / 79.6	84.2 / 84.6	PiJ107, PiMich7061, PaEC 3821, PaEC 3865
PaM18	79.8 / 80.1	84.8 / 85.2	PiJ107, PiMich7061, PaEC 3821, PaEC 3865
PaM19	76.9 / 77.3	83.5 / 83.9	PiJ107, PiMich7043, PiMich7061, PaEC 3818, PaEC 3821, PaEC 3865
PaM27	75.9 / 76.3	83.1 / 83.5	PiJ107, PiMich7043, PiMich7061, PaEC 3818, PaEC 3821, PaEC 3865
PaM33	77.9 / 78.2	82.8 / 83.1	PiJ107, PiMich7061, PaEC 3818, PaEC 3821, PaEC 3865
PaM57	73.1 / 73.5	83.7 / 84.0	PiJ107, PiMich7043, PiMich7061, PaEC 3818, PaEC 3821, PaEC 3865
Ph9	75.4 / 75.7	82.6 / 82.9	PnG194, PnFerraraR1, PnC301, Pp956/07
Ph11	77.5 / 77.9	83.6 / 84.0	Pc11-1, Pc12-12, Pp956/07
Ph14	77.7 / 78.0	84.4 / 84.7	PnC301, PnCorrea2, PnM231, Pc11-1, Pc12-12, Pp956/08
Ph25	81.3 / 81.7	88.2 / 88.6	Pc11-1, Pc12-12, Pp956/07
Ph29	76.1 / 76.4	83.6 / 83.9	Pc11-1, Pc12-12, Pp956/07
Ph29	76.1 / 76.4	88.0 / 88.3	PnC301, PnCorrea2, PnM231, Pc11-1, Pc12-12, Pp956/08

^a See the supplementary figures.

^b Pi = *Phytophthora infestans*; Pa = *P. andina*; Pn = *P. nicotianae*; Pp = *P. × pelgrandis*; and Pc = *P. cactorum*.

To distinguish *P. andina* and *P. × pelgrandis* from their parents, multiple primer sets should be used in parallel to increase the accuracy of the assays. Specifically, the targeted heterozygous SNPs may be present in some loci but not in others owing to loss of heterozygosity that varies among hybrid isolates (Lamour et al. 2012). In addition, amplicons may contain additional variation that was not present in the reference sequences used to design HRM primers. When amplicons contained more than one SNP, melting profiles

were complex and more difficult to interpret. However, the presence of multiple SNPs could increase the robustness of the analysis to within-species variation. For *P. andina*, we developed five primer sets that produced results consistent with heterozygosity in the five conserved genes that the primers targeted. Together, these primers should provide robust detection of hybrid status. Primer set PaM33 incorrectly grouped *P. infestans* isolates from Michoacán, Mexico, with *P. andina* isolates, which can be explained by the

Fig. 1. Aligned curves, generated for primer sets PaM2 (A), PaM18 (B), and PaM19 (C). Left figures show the curves color coded by isolate and right figures by the variant calls, which correspond to the different species. Pi = *Phytophthora infestans*; and Pa = *P. andina*.

high diversity and fast evolution of *P. infestans*, particularly in Mexico (Wang et al. 2017). For *P. × pelgrandis*, differentiation from both parental species can be obtained with only Ph14 or Ph29. Despite separation between curves among *P. nicotianae* isolates, the software correctly assigned the isolates to the same variant. Small curve shifts within species can be owing to point variations among isolates or differences in DNA concentration. Isolates not tested in this study could introduce additional SNPs that

may hamper interpretation using these primers. We suggest that these primers could be combined with others that distinguish between *P. × pelgrandis* and one or the other parent for confirmation. We were initially limited to multilocus sequencing data for *P. cactorum*, but draft genomes of *P. cactorum* (Grenville-Briggs et al. 2017) and *P. nicotianae* (Liu et al. 2016) have since become available, which could be used to identify additional loci for HRM. In addition, future analyses of genome sequences for *P. × pelgrandis*

A PaM27

B PaM33

C PaM57

Fig. 2. Aligned curves, generated for primer sets PaM27 (A), PaM33 (B), and PaM57 (C). Left figures show the curves color coded by isolate and right figures by variant calls, which correspond to the different species except for PaM33, which misassigned Mich7039 and Mich7043 (*Phytophthora infestans*) to *P. andina*. Pi = *P. infestans*; and Pa = *P. andina*.

could identify additional heterozygous sites in conserved genes to increase the robustness of the HRM assay.

We are proposing HRM analysis as a faster technique compared with SSR genotyping or sequencing to differentiate hybrids from parental species. The protocol for a 96-well plate can be completed in ~3 h, and analysis is fast (~1 h) when appropriate software is available. We recommend using three to five primer sets, which could increase the processing time if primers do not anneal at the same

temperature. The laboratory time for SSR analysis or Sanger sequencing may be similar, but time to results depends on availability of a capillary sequencer and the level of automation of analysis of resulting chromatograms. There are also considerations of cost and convenience in HRM analysis. “New generation” saturation dyes increase the resolution and accuracy of HRM assays without inhibiting PCR reactions (Reed et al. 2007; Wittwer 2009; Wittwer et al. 2003) but are more expensive than nonsaturating dyes. Because oligo-specific

Fig. 3. Aligned curves for primer sets Ph9 (A), Ph11 (B), and Ph14 (C). Left figures show the curves color coded by isolate and right figures according to the variant calls, which correspond to the species tested. Pn = *Phytophthora nicotianae*; Pc = *P. cactorum*; and Pp = *P. × pelgrandis*.

fluorescent probes are not required, the same dye can be used across loci, which lowers the start-up cost of HRM analysis. The method requires DNA content uniformity and DNA of high quality, which can be obtained by most commercial kits. If there are large differences in DNA concentration among isolates, the behavior of melting curves could be affected and cause false variants. This can be overcome by standardizing extraction protocols among isolates being tested and using aliquots with similar DNA concentrations.

Finally, access to HRM software is essential for rapid analysis, and costs depend on the platform used to generate data. However, open-source programs are available for processing HRM data (Cousins et al. 2012).

In conclusion, we showed that HRM can be used for rapid discrimination of hybrid *Phytophthora* from parental species. Globally, many *Phytophthora* hybrids have been identified, from economically important crops and natural ecosystems. HRM could be deployed

A Ph25

B Ph29

C Ph29 (pre and post-melting temperature modified)

Fig. 4. Aligned curves generated for primer sets Ph25 (A), Ph29 (B), and Ph29 with modified pre- and postmelting temperature (C). Left figures show the curves color coded by isolate and right figures according to the variant calls, which correspond to the isolates tested. Pn = *Phytophthora nicotianae*; Pc = *P. cactorum*; and Pp = *P. × pelgrandis*.

locally for discrimination of hybrid species of particular concern. Multilocus assays are expected to be relatively stable through time owing to the asexual nature of *P. andina*, *P. × pelgrandis*, and other hybrid *Phytophthora* of concern. As long as routine genome sequencing of *Phytophthora* isolates for identification remains cost prohibitive, HRM has the potential to improve understanding of the distribution and host range of *Phytophthora* hybrids.

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