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ORIGINAL ARTICLE

Differentiation of *Phytophthora* species associated with plantation crops using PCR and high-resolution melting curve analysis

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Abstract

Diseases caused by closely related species of *Phytophthora* such as *P. palmivora*, *P. nicotianae*, *P. capsici*, *P. meadii*, *P. citrophthora* and *P. colocasiae* are major production constraints of plantation crops in India. A reliable method for the rapid identification of these *Phytophthora* species is required for production of disease-free planting material and promote exports. A specific PCR assay with primer pairs designed from the ITS region effectively differentiated *Phytophthora* species belonging to different clades viz., *P. palmivora, P. nicotianae* and *P. capsici*. In order to differentiate closely related species (*P. meadii, P. citrophthora* and *P. colocasiae*), a high-resolution melting **(**HRM) curve analysis, using primers designed based on single nucleotide differences in A/T and C/A alleles was developed and validated. HRM curve analysis proved to be a fast and accurate technique for differentiation of closely related species of *Phytophthora*.

Keywords Coconut · Arecanut palm · *Phytophthora* · HRM analysis · Rapid detection · PCR

Introduction

Plantation crops play an important role in employment generation as well as poverty alleviation in rural India and provide livelihood security to millions of small and marginal farmers besides earning huge foreign exchange. They blend effectively with the environment contributing to sustainability, conservation of bio-diversity and stable ecosystem. In India *Phytophthora* diseases pose a serious challenge to production of plantation crops like coconut (bud rot), arecanut palm (fruit rot) and cocoa (black pod) and their inter crops (Sarma et al. 2002). Various species of *Phytophthora* viz., *P. palmivora* (on coconut and cocoa), *P. nicotianae* (cardamom), *P. capsici* (black pepper and cocoa), *P. meadii* (arecanut palm, rubber and cardamom), *P. citrophthora* (cocoa) and *P. colocasiae* (taro) have been reported to cause crop losses of up to 100% (Chowdappa and Chandra Mohanan 1993, 1996; Chowdappa et al. 1993).

Accurate identification of the *Phytophthora* species affecting plantation crops is a key factor for adopting eco-friendly and effective control strategies (Chowdappa et al. 2016). Identification of *Phytophthora* species based on morphology is time consuming, labour intensive and difficult due to overlapping and limited number of morphological criteria and the need for mycological expertise (Chowdappa et al. 2003a). In addition to morphological approaches, PCR amplification and sequencing (Bowers et al. 2007) and restriction enzyme digestion patterns (Chowdappa et al. 2003b; Cooke et al. 2000; Cooke and Duncan 1997) have been used to identify, characterize and separate closely related *Phytophthora* species. Whereas conventional PCR with species-specific primers was used for the identification of *Phytophthora* species (Drenth et al. 2006), *P. capsici* in capsicum (Silvar et al. 2005; Ristaino et al. 1998) and *P. citrophthora* in citrus (Ersek et al. 1994), the more sensitive real time PCR with a TaqMan probes was extensively employed for detection and differentiation of many *Phytophthora* species (Bilodeau et al. 2007). However, the molecular detection of *Phytophthora* species affecting plantation crops such as coconut, arecanut palm and cocoa in a single round PCR assay are limited, except for two reports on restriction digestion patterns of the ITS region of rDNA (Chowdappa et al. 2003a, b). Also, there are no reports on the use of single round PCR for differentiation of two or more *Phytophthora* spp.

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High resolution melting (HRM) analysis, is a relatively new technique successfully applied for the characterization of human (Chen et al. 2011; Goldschmidt et al. 2012; Van der Stoep et al. 2009) and poultry pathogens (Ghorashi et al. 2010; Steer et al. 2009), and authentication of plant and food products (Ganopoulos et al. 2011). In recent years, HRM curve analysis has also been utilized to discriminate plant pathogenic viruses (Bester et al. 2012), bacteria (Ricchi et al. 2011) and also plant pathogenic *Fusarium oxysporum* f. sp. *vasinfectum* on cotton (Ganopoulos et al. 2012), *Phyllosticta* species on banana (Wong et al. 2013) and *Pseudoperonospora cubensis* and *P. humuli* on cucurbit (Summers et al. 2015). Thus, the objective of present study was to develop a robust detection tool that can differentiate closely related species of *Phytophthora* affecting plantation crops which would immensely contribute to enhanced disease management strategies.

Materials and methods

Sampling and *Phytophthora* isolation Isolates of *Phytophthora* were obtained from naturally infected meristematic region of coconut and fruits of arecanut palm and cocoa from different localities in five Indian states viz., Kerala, Tamil Nadu, Karnataka, Andhra Pradesh and Goa, during 2012 to 2014. For isolation of the pathogen, arecanut palm fruits, cocoa pod and coconut meristematic tissues with characteristic symptoms were thoroughly washed with sterile water. The tissues were excised from the edge of advancing lesions, inoculated on to V8 agar plates amended with PARP (100 ml V8 juice, 2.5 g $CaCO_3$ and 18 g agar per liter amended with 100 ppm pimaricin, 250 ppm ampicillin, 100 ppm rifampicin and 100 ppm pentachloronitrobenzene) and incubated for 3 days at 25 °C. Hyphal tips from advancing mycelium were then sub-cultured on plates containing carrot agar (CA) medium. The isolation from tissues with advanced infection was achieved by the baiting method as described by Sharadraj and Chandramohanan (2016).

Morphological characterization Colony characters and sporangial morphology was studied according to Erwin and Ribeiro (1996). Colony characters of the collected isolates of *Phytophthora* spp. were recorded on CA medium. The pattern of growth and production of aerial mycelium were recorded against a black background. Three replications were maintained for each fungal isolate.

The sporangial width and length of each isolate were determined using a light microscope (at 40×). The width and length of 10 sporangia per isolate were obtained by flooding 8-days-old cultures on CA medium with water. Other morphological characteristics, including the shape of sporangia, ramification of sporangiophores and presence of papillae, were recorded. All isolates were identified based on colony and sporangial morphology (Erwin and Ribeiro 1996). Mating or compatibility type of each isolate was determined by according to Chowdappa and Chandramohanan (1997). Compatibility types of the *Phytophthora* causing disease of coconut, arecanut palm and cocoa were evaluated by pairing them with known A1 (*P. capsici*, isolate ID. 98–75) and A2 (*P. palmivora*, isolate ID. 98–01) isolates obtained from ICAR-Indian Institute of Spices Research (IISR), Kozhikode, Kerala, India.

DNA extraction All *Phytophthora* isolates were cultured on CA medium at 27 °C for 3 days. Two discs of 0.5 mm were then cut from the mycelial margin, transferred to 100 ml of broth and incubated for 5 days at 27 °C. The mycelia were recovered, washed twice in sterile distilled water and dried on sterile filter paper for 5–10 min under a laminar air flow hood. Dry mycelia were frozen with liquid nitrogen and crushed to fine powder with mortar and pestle. DNA was extracted with DNeasy Plant Mini Kit (Qiagen, USA) following manufacturer's instructions and the DNA was eluted in 50 μl of TE buffer. Quality and quantity of the extracted DNA was verified using agarose gel electrophoresis and spectrophotometer.

DNA sequencing and analysis Using 20 ng of template DNA, the ITS region of the isolates was amplified using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5'-TCCTCCGC TTATTGATATGC-3′) as described by White et al. (1990). Amplification was carried out with PCR System (Biorad, USA) with a cycling profile of 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s with an initial denaturation of 2 min at 94 °C before cycling and final extension of 10 min at 72 °C after cycling. Amplified PCR products were separated in 1% agarose gel in Tris-Borate-EDTA (TBE) buffer and visualized under UV after staining with ethidium bromide (0.5 μg/ml). The bands were eluted using an elution kit (Qiagen, USA) and sent to M/S Scigenom (Kochi, India) for sequencing.

Phylogenetic analysis The ITS region sequences were compared to study the diversity among the *Phytophthora* isolates. Sequences were aligned with ClustalW followed by construction of a neighbor-joining phylogenetic tree using MEGA 5.01. The sequence of *Phytophthora colocasiae* (CTCRI-P24), obtained from ICAR-CTCRI, Thiruvananthapuram, India was also included for comparison. The bootstrap consensus tree was inferred from 1000 replicates using the software MEGA 5.01 (Tamura et al. 2011).

Primer design for species specific identification To detect *Phytophthora* species belonging to different clades infecting coconut, arecanut palm and cocoa, the ITS region sequences

of *Phytophthora* species were aligned using ClustalW and regions with differences were identified. Primers were then designed to regions with dissimilarities using the Primer3 software (http://primer3.ut.ee/). Candidate primers were analyzed for dimer and hairpin loop structures using the tool Oligoanalyzer (http://eu.idtdna.com/calc/analyzer). A ready to use Red Dye PCR Master mix (Genei, India) was used for PCR amplification which contains premixed dNTPs, *Taq* polymerase, $MgCl_2$ and buffer at optimum concentrations. The gel loading dye was also incorporated to the master mix. The PCR product was run on 1.0% agarose gel to determine the band size.

Table 1 *Phytophthora* isolates recovered from disease infected coconut, arecanut palm and cocoa ecosystem

Sl. No.	Isolate number	Disease	Cropping system	Host	District	State	Species	Mating type	Accession number
1	KL-CO/1	Bud rot	Coconut monocrop	Coconut	Kasaragod	Kerala	*P. palmivora*	A2	JX155789
2	KL-CO/6	Bud rot	Coconut monocrop	Coconut	Kasaragod	Kerala	*P. palmivora*	A2	JX155790
3	KL-CO/8	Bud rot	Coconut monocrop	Coconut	Kasaragod	Kerala	*P. palmivora*	A2	JX155791
4	KL-CO/9	Bud rot	Coconut monocrop	Coconut	Kasaragod	Kerala	*P. palmivora*	A2	JX155792
5	KL-CO/61	Bud rot	Coconut monocrop	Coconut	Waynad	Kerala	*P. palmivora*	A2	JX155794
6	KL-CO/74	Bud rot	Coconut + Pepper	Coconut	Waynad	Kerala	*P. palmivora*	A2	JX155795
7	KL-CO/77	Bud rot	Coconut + Coffee + Pepper	Coconut	Waynad	Kerala	*P. nicotianae*	A2	KC771052
8	KL-CO/78	Bud rot	Coconut + Pepper	Coconut	Waynad	Kerala	*P. palmivora*	A2	JX155796
9	KL-CO/16	Bud rot	Coconut monocrop	Coconut	Palakkad	Kerala	*P. meadii*	A2	JX155793
10	KA -CO/83	Bud rot	Coconut monocrop	Coconut	Kundapura	Karnataka	*P. nicotianae*	A2	JX155788
11	KA-CO/110	Bud rot	Coconut monocrop	Coconut	Chikkamaga-luru	Karnataka	*P. capsici*	A2	KC771053
12	TN-CO/127	Bud rot	Coconut monocrop	Coconut	Coimbatore	Tamil Nadu	*P. nicotianae*	A2	JX155797
13	KA/CA-258	BPD	Cocoa + Arecanut	Cocoa	Dakshina Kannada	Karnataka	*P. capsici*	A1	JX198554
14	KA/CA-260	BPD	Cocoa + Arecanut	Cocoa	Dakshina Kannada	Karnataka	*P. palmivora*	A2	JX198555
15	KA/CA-267	BPD	HDMS	Cocoa	Dakshina Kannada	Karnataka	*P. meadii*	A2	JX198556
16	KL/CA-246	SD	Cocoa + Coconut	Cocoa	Kannur	Kerala	*P. citrophth-ora*	A1	JX198561
17	KL/CA-221	SD	Cocoa + Coconut + Arecanut	Cocoa	Calicut	Kerala	*P. palmivora*	A2	JX198560
18	KL/CA-196	BPD	Cocoa + Coconut + Pepper	Cocoa	Thrissur	Kerala	*P. palmivora*	A2	JX198558
19	KL/CA-216	BPD	Cocoa + Coconut	Cocoa	Kozhikkode	Kerala	*P. palmivora*	A2	JX198559
20	TN/CA-301	BPD	Cocoa monocrop	Cocoa	Coimbatore	Tamil Nadu	*P. palmivora*	A2	JX198562
21	AP/CA-334	SC	Cocoa + Coconut + Banana	Cocoa	East Godavari	Andhra Pradesh	*P. palmivora*	A2	JX198553
22	P1	Fruit rot	Arecanut	Arec-anut	Dakshina Kannada	Karnataka	*P. meadii*	A2	LC076467
23	P45	Crown rot	Arecanut	Arec-anut	Uttara Kannada	Karnataka	*P. meadii*	A2	LC076468
24	P28	Fruit rot	Arecanut	Arec-anut	Kasaragod	Kerala	*P. meadii*	A2	LC076469
25	P41	Fruit rot	Arecanut	Arec-anut	Thrissur	Kerala	*P. meadii*	A2	LC076470
26	P36	Fruit rot	Arecanut	Arec-anut	North Goa	Goa	*P. meadii*	A2	LC076471

**BP* Black Pod Disease, *SC* Stem Canker of cocoa, *SD* Seedling Dieback

High-resolution melting (HRM) analysis For the HRM analysis, the sequences of closely related species, which could not be differentiated using single round PCR, were aligned using ClustalW and the regions with single nucleotide polymorphism (SNP) were identified. Primers were then designed to those regions with dissimilarities using Primer3 software considering each SNP separately with a T_m between 55 and 65 °C. Candidate primers were analyzed for dimer and hairpin loop structures using the tool Oligoanalyzer (http://eu.idtdna.com/calc/analyzer). All samples were tested in triplicate along with distilled water as blank control. PCR was performed from ca. 10 ng of DNA, using 0.3 mM each of the applicable forward and reverse primers in a total volume of 10 μl containing 5 μl MeltDoctor™ HRM Master Mix (Applied Biosystem, USA), 3 μl primers and 2 μl DNA template. The PCR and high-resolution melting analysis for species specificity was carried out with amplification conditions: 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min using 7900HT Fast Real-Time PCR System thermal cycler (Life Technologies, USA). Melting analysis was carried with the following steps: (i) denaturation at 95 °C for 10 s, (ii) annealing at 60 °C for 1 min and (iii) HRM curves generation at 95 °C for 15 s and annealing at 60 °C for 15 s. The ramp rate of high resolution melting step was 1% of the rate of the annealing step.

The amplification and dissociation curves were analysed and the post PCR analysis of melt curves was carried out using Step One Realtime HRM software (Applied Biosystem, USA). The melting curves were normalized and temperature shifted to allow samples to be directly compared. The melt curve analysis was repeated thrice to check reproducibility. By selecting any one as the baseline, the difference plots were generated. The fluorescence of all other samples was plotted relative to this sample.

Results

***Phytophthora* isolates and morphological identification** A total of 26 isolates of *Phytophthora* spp. comprising five isolates from arecanut palm, 12 from coconut and nine from cocoa were purified from infected samples collected from five Indian states viz., Kerala, Tamil Nadu, Karnataka, Andhra Pradesh and Goa (Table 1).

Colony morphology of *P. palmivora* isolates varied from stellate pattern without aerial mycelium to stellate pattern with cottony aerial mycelium. Ontogeny of sporangiophore was simple sympodial, sporangia were papillate, caducous, elliptical to ovoid with a short occluded pedicel. In *P. meadii* isolates, colony morphology varied from stellate to slightly petaloid with less aerial mycelium. Ontogeny of sporangiophores was simple sympodia to irregular and sporangia were papillate, caducous, ovoid to ellipsoidal with medium length pedicel (Fig. 1).

In *P. nicotianae,* colony morphology was cottony with fluffy aerial mycelium. Ontogeny of sporangiophore was simple sympodial and sporangia were non-caducous, papillate, ovoid to globose with hyphal swellings. Colonies of *P. capsici* isolates were slightly rosaceous and the aerial mycelium was dense cottony. Ontogeny of sporangiophore was simple sympodia and sporangia were papillate, caducous, fusiform shaped with long pedicel. *P. citrophthora* isolate expressed stellate pattern of the colony with dense cottony aerial mycelium. Sporangia were papillate, non-caducous, ovoid to ellipsoidal in shape with offset pedicel attachment (Fig. 1).

Out of 26 *Phytophthora* isolates, one isolate each of *P. capsici* (KA/CA-258) and *P. citrophthora* (KL/CA-246)

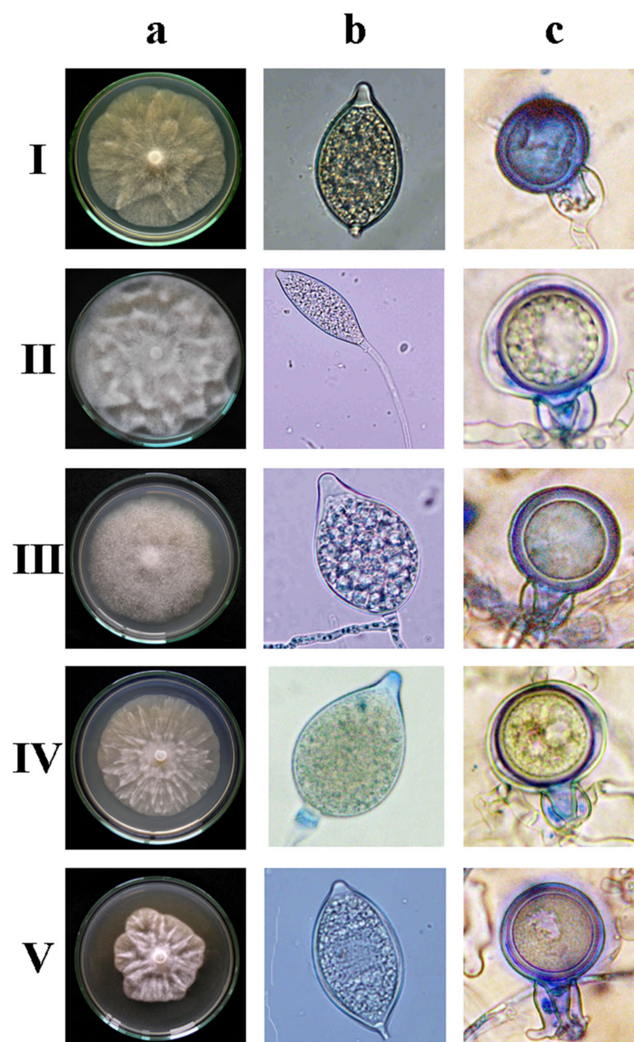

Fig. 1 Cultural and morphometric characterization of *Phytophthora* spp. (**a**) Cultural morphology; (**b**) sporangial morphology; (**c**) oogonial morphology. (I) *P. palmivora*; (II) *P. capsici*; (III) *P. nicotianae*; (IV) *P. citrophthora*; (V) *P. meadii*

from cocoa was A1 mating type, whereas all the remaining isolates belonged to the A2 mating type (Table 1, Fig. 1).

Molecular identification Amplification with primers ITS1 and ITS4 of DNA from 26 isolates belonging to *P. palmivora, P. nicotianae, P. capsici, P. meadii, P. citrophthora* and *P. colocasiae* produced amplicons of ca. 900 bp that were sequenced. BLAST similarity searches were carried out and the results revealed sequence identities with their respective species (Table 1). A neighbour joining analysis of ITS sequences resulted in three main groups (Fig. 2). Group I contained all the *P. palmivora* isolates. However, 2 *P. palmivora* isolates viz., TN/CA-301 and AP/CA-334 from Tamil Nadu and Andhra Pradesh, respectively, stood out separately in the first group, which indicates existence of geographic differences within the *P. palmivora* population (Fig. 2). Group II consisted of *P. capsici, P. meadii, P. citrophthora* and *P. colocasiae*. Group II could be further subdivided into two sub clusters, one containing *P. capsici* alone and the other consisting of *P. meadii, P. citrophthora* and *P. colocasiae*. Group III consisted exclusively of *P. nicotianae* isolates.

Conventional PCR In order to differentiate and detect *Phytophthora* species belonging to different clades infecting coconut, arecanut palm and cocoa, specific primers were designed based on the alignment of the ITS region sequences using ClustalW. One forward (PF1) and three reverse primers (PR1, PR2, PR3) were designed for the identification of *P. nicotaiane, P. palmivora* and *P. capsici* respectively (Table 2). When these primers were used for the single round PCR, *P. nicotianae*, *P. palmivora* and *P. capsici* could easily be distinguished based on the band sizes of ca. 300, 325 and 400 bp, respectively. However, these ITS primers could not distinguish three species (*P. meadii, P. citrophthora* and *P. colocasiae*) coming under sub-cluster 2 of Group II.

HRM analysis Analysis of multiple sequence alignment of ITS sequences of *P. meadii*, *P. citrophthora* and *P. colocasiae* revealed a difference of A/T and C/A alleles that could consistently separate the three species. Based on these differences, primers were designed for HRM analysis (Table 2). HRM assay could successfully distinguish all the three *Phytophthora* spp. viz., *P. meadii*, *P. citrophthora* and

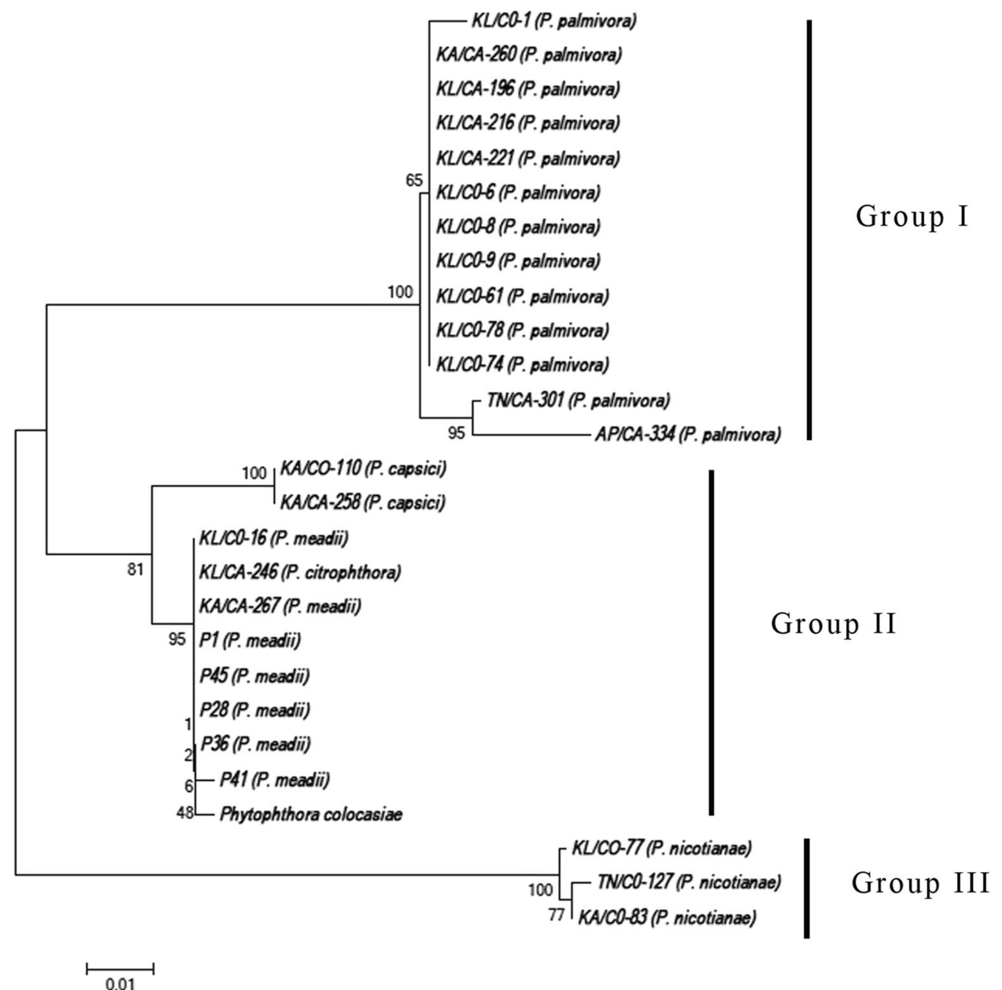

Fig. 2 Evolutionary relationships of rDNA ITS1-ITS4 sequences of *Phytophthora* spp. infecting plantation crops. The numbers at the branch points indicate the percentages of bootstrap values (based on 1000 bootstraps)

Table 2 Primers designed based on ITS region, used for single round PCR and High-Resolution Melt Curve analysis to identify different *Phytophthora* species

Primer	Forward primer	Reverse primer	Band size
P. nicotianae	GATGAAGAACGCTGCGAAC (PF1)	GACACTTCACATCTGACATC (PR1)	300 bp
P. palmivora		CAAAAGCGTGGCGTTGC (PR2)	325 bp
P. capsici		GCGCTTATTGAATGCTTTTC (PR3)	400 bp
Primers for differentiating *P. meadii, P. citrophthora* and *P. colocasia* using HRM analysis	CGGTTTGTGTGCCTTCGG	GCATACAATAAGCGTCTGTTC	206 bp

P. colocasiae. The GC content of the three amplicons ranged from 46.21 to 47.93%, displaying different melting points based on sequence differences. The initial melt curves differentiated each species by small shift in peaks and Tm for each species could be determined clearly (Fig. 3a). The melting profiles of three strains resulted in a single peak with differentiable melting temperature for *P. meadii*, *P. colocasiae* and *P. citrophthora* respectively (Table 3; Fig. 3b). The Tm values were highly reproducible across three repeated melt curve runs. The dissociation curve plot could facilitate accurate identification of the three species by revealing difference in peaks when any one species was considered as base (Fig. 3c).

Discussion

Rapid and accurate identification of *Phytophthora* species associated with diseases of plantation crops is crucial for developing effective disease management strategies, for the selection of the appropriate pathogen isolates to use in resistance screening programs and to address phytosanitary issues to boost the agricultural trade. Many approaches have been employed in diagnostics of *Phytophthora* species - these include sporangium morphology including pedicel length (Brasier and Griffin 1979), species-specific PCR (Bilodeau et al. 2014), PCR amplification and sequencing (White et al.

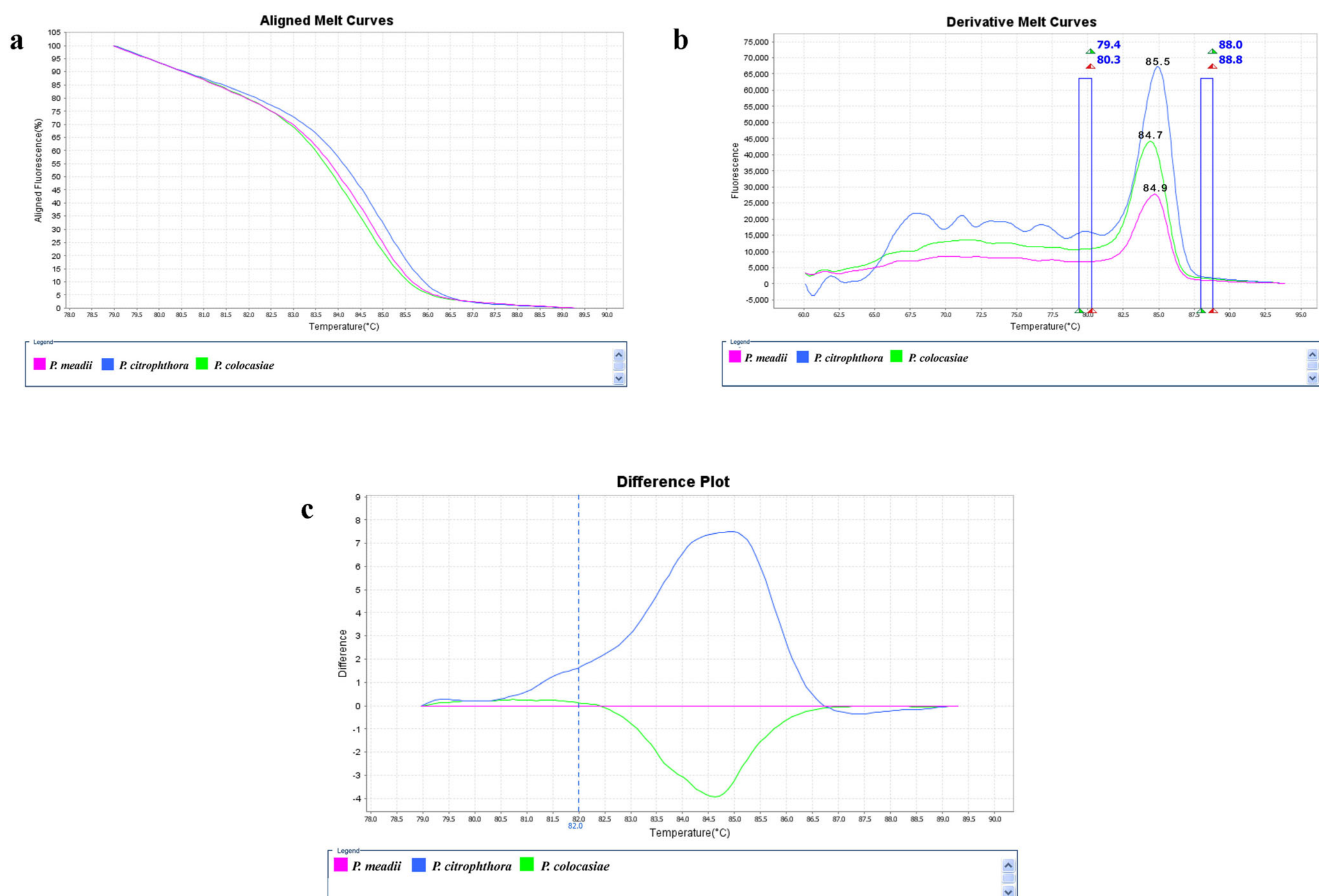

Fig. 3 Representative high-resolution melt curve analysis (HRM) curves: (**a**) aligned melt curves, (**b**) derivative melt curves and (**c**) difference plot

Table 3 Melt peak temperature of *Phytophthora* species in HRM analysis

Phytophthora species	Melting temperature		
	Tm-1	Tm-2	Tm-3
P. meadii	83.7 ± 0.2^{0}c	84.2 ± 0.1^{0}c	84.9 ± 0.4^{0}c
P. citrophthora	85.5 ± 0.1^{0}c	85.8 ± 0.2^{0}c	86.1 ± 0.1^{0}c
P. colocasia	84.3 ± 0.1^{0}c	84.1 ± 0.1^{0}c	85.1 ± 0.2^{0}c

1990) and restriction enzyme digestion patterns (Chowdappa et al. 2003b; Cooke et al. 2000) which have been used to identify, characterize, and separate species of *Phytophthora*. In the present study, 26 *Phytophthora* isolates were identified based on morphological characters described by Erwin and Ribeiro (1996).

The genetic relationships among *Phytophthora* isolates have also been resolved using a repertoire of molecular techniques, which include RFLP analysis of PCR-amplified ITS regions (Chowdappa et al. 2003b; Cooke et al. 2000), amplicons obtained using the genus-specific primers A2 and I2 (Drenth et al. 2006), multi-locus gene phylogeny (Blair et al. 2008) and real time PCR assay (Tomlinson et al. 2005). The choice of the DNA region to be amplified plays a substantial role in design and selection of molecular markers to differentiate fungi (Khadempour et al. 2010). The ITS region of the nuclear ribosomal DNA has been predominantly utilized for the molecular markers studies (Cooke et al. 2000).

In this study, phylogeny tree constructed using ITS rDNA sequences of 26 isolates grouped the isolates into three major clusters and the clustering was in accordance with the clade specific classification of *Phytophthora* (Blair et al. 2008). The first group corresponded to Clade 4 and comprised of all *P. palmivora* isolates. The second group corresponding to Clade 2 consisted of *P. capsici*, *P. meadii*, *P. citrophthora* and *P. colocasiae*. The last major group had *P. nicotianae,* which corresponded to Clade 1.

Based on the ITS region sequences, one forward and three reverse primers were designed and used for single round PCR. Three of the species, viz., *P. nicotianae*, *P. palmivora* and *P. capsici* could easily be distinguished based on the differential band sizes. However, since the ITS regions of the other three species (namely *P. meadii*, *P. citrophthora* and *P. colocasiae*) were highly conserved, they could not be differentiated by normal PCR and agarose gel electrophoresis. Closer analyses of multiple sequence alignment of ITS sequences of these three species revealed SNPs; these differences were exploited in order to differentiate three species using HRM approach. With a single pair of primers, three *Phytophthora* spp. viz., *P. meadii*, *P. citrophthora* and *P. colocasiae* could successfully be discriminated based on peaks developed with respect to differentiable melting temperatures. Ganopoulos et al. (2012) reported a real-time PCR assay, using universal ITS primers coupled with HRM analysis to differentiate closely related *F. oxysporum formae speciales* based on differences in melting curve characteristics. In another study, HRM analysis assay targeting the ITS region was utilized to differentiate three closely related species of *Phyllosticta* (*P. musarum*, *P. maculate* and *P. cavendishii*) (Wong et al. 2013).

The data presented in this study therefore supports the utility of HRM technique in differentiating closely related *Phytophthor*a species. The reliability, discriminatory capability and sensitivity of HRM analysis in molecular diagnostics of plant fungi has been extensively reviewed recently by Zambounis et al. (2015). Previously, HRM technique has been used for identification of *Phytophthora* spp. having wide host range (Zambounis et al. 2016a) and also for the assessment of genetic diversity in *Phytophthora cambivora* isolates causing root rot on several woody species (Zambounis et al. 2016b).

In conclusion, single round PCR was developed for identification of *P. palmivora, P. nicotianae* and *P. capsici.* Also, HRM analysis assay was developed and evaluated for its robustness for identification and differentiation of the three closely related species of *Phytophthora* namely *P. meadii, P. citrophthora* and *P. colocasiae* infecting plantation crops. Thus, the assay can be used not only for rapid separation of the three species but also as diagnostic research tool that enhance quarantine and management strategies for Phytophthora diseases in plantation crops.

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