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Ribosomal DNA analysis of three *Phytophthora* species occurring in India

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ABSTRACT: Three Indian *Phytophthora* isolates from black pepper, coconut and cardamom were characterized based on internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). The sequence analysis and the predicted secondary structure of RNA of these species of *Phytophthora* revealed that black pepper isolate of *Phytophthora* shares characteristics of both *P. capsici* and *P. tropicalis* whereas coconut and cardamom isolates matched that of *P. palmivora and P. meadii* respectively.

Key words: *Phytophthora capsici*, *P. palmivora*, *P. meadii,* ribosomal DNA, internal transcribed spacer

The genus *Phytophthora* comprises of a devastating group of plant pathogens existing in a wide range of ecological niches. It causes severe loss in many plant species which can have a serious impact on native vegetation (Erwin and Ribeiro, 1996). The morphological and physiological features of *Phytophthora* are unique to each species; however these characters are highly variable and identification thus becomes difficult. Waterhouse (1963) categorized *Phytophthora* species into six groups based on a series of morphological and physiological parameters. There are however difficulties in using phenotypic taxonomic characters, in that some characters overlap between species, and significant variation can occur among isolates of same species (Appiah, 2001; Appiah *et al.*, 2003; Park *et al.,* 2008). Molecular tools used in phylogenetic studies of Oomycetes (Forster and Coffey, 1990) have included analysis of large and small subunit ribosomal RNA genes and sequence analysis of the internal transcribed spacer (ITS) regions of the r RNA genes (Crawford *et al.*, 1996). Cooke *et al.* (2000) reported an ITS based molecular phylogeny, which included 50 *Phytophthora* species, and is the most comprehensive to date. ITS sequence data have also been used to study the phylogenetic relationships of many *Phytophthora* species (Lee and Taylor, 1992; Brasier *et al.,* 1999). Lee and Taylor (1992) reported that the ITS sequence differences have been used to distinguish between some *Phytophthora* species. The objective of the present study was to identify and characterize three isolates of *Phytophthora* from black pepper (*Piper nigrum* L), coconut (*Cocos nucifera* L) and cardamom (*Elettaria cardamom* Maton) by analyzing ITS regions of ribosomal DNA.

MATERIALS AND METHODS

Phytophthora cultures and Culture conditions

The isolates used in the present study were obtained from National Repository of *Phytophthora* (NRPH), Indian Institute of Spices Research (IISR), Calicut. The cultures included black pepper isolate of *Phytophthora* (99-166) collected from Peruvannamuzhi, Kerala, coconut isolate (96-30) and a cardamom isolate (99-180) collected from Adimali in Kerala. The isolates were grown in 500 ml conical flasks containing 50ml of Ribeiro's liquid medium. Each flask was inoculated with five mycelial disks, each of five mm diameter, cut from the advancing margin of three-day-old cultures grown on carrot agar (CA) in dark. The inoculated flasks were incubated in dark at 24±1°C for five days. Then, the mycelial mat was harvested by filtering through Whatman No. 1 filter paper and damp dried.

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**Contribution number 443

DNA extraction

DNA of pure culture was extracted by the method of Cooke and Duncan (1997). Mycelium from the 5-day old liquid culture was harvested and excess medium was removed by filtration. Approximately 500mg of fungal mycelium was ground in a sterile 1.5 ml micro centrifuge tube containing 750µl of STE buffer (1M Tris, 5M NaCl, 0.5M EDTA, 10% SDS) using minigrinder (Sigma) by adding 25 mg of PVPP and 20 mg of sterile glass powder. After centrifugation at 13000 rpm for 5 minutes, the aqueous phase was collected and extracted with equal volume of Phenol: chloroform: isoamyl alcohol (25:24:1). Further extraction was repeated using chloroform: isoamyl alcohol (24:1) and pelletted using 0.6 volumes of isopropanol. The pellet was washed with 70% ethanol and the dried pellet was resuspended in 50µl of TE (Tris EDTA buffer; Tris HCl 10mM, EDTA 1mM, pH.8.0). DNA concentrations were estimated visually in ethidium bromide stained agarose gels (0.8%) by comparing band density with known quantity of pBR322 DNA (Genie, Bangalore, India) and also through UV spectrophotometer. Template DNA was diluted to a concentration of 50ng/µl and it was used for PCR amplification.

PCR amplification of ITS regions

The ITS region of the three isolates were amplified by using the primers ITS 6 (5'-GAAGGTGAAGTCGTAACAAGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATG-3') (White *et al.*, 1990). PCR was performed in 25µl reaction volumes with each reaction mixture containing 10X PCR buffer, 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 20pmole each of primers, 10mM each of the dNTPs (Genei, Bangalore, India), and 1.5 units of *Taq* polymerase (Genei, Bangalore, India). Amplification was performed in an automated thermal cycler (Eppendorf master cycler) gradient and the programme consisted of initial denaturation at 94 °C for 3 min followed by 34 cycle repeats involving denaturation at 94°C for 30, annealing 55°C for 30s and synthesis at 72°C for 1min and a final extension at 72°C for 10 min. The amplified products were analysed on Ethidium bromide stained agarose gels (2%) along with size standard 500bp ladder (Genei, Bangalore, India), visualized under UV and photographed using alpha imager (Alpha Innotech corporation).

Cloning and sequencing of PCR products

PCR fragments were excised from agarose gels and purified using Genie Spin gel extraction kit (Genei, Bangalore, India). Purified fragments were ligated into T-vector (INSTANT cloning kit, Bangalore Genei, India) according to the manufacturer's instructions and transformed into *Escherichia coli* strain DH5-α using standard molecular biology procedures (Sambrook and Russel, 2001). Plasmids were extracted using alkaline lysis method and the recombinant clones were identified by restriction endonuclease digestion using the enzyme *Nco1* and by PCR using specific primers. Selected clones were sequenced at the automated DNA sequencing facility at Bangalore Genei, Bangalore, India.

Sequence Analysis and Secondary structure prediction

Vector sequences were identified and trimmed by using NCBI VecScreen. (URL:http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). The vector-trimmed sequences were used for similarity search in the database by using BLAST (Altschul *et al.*, 1990). Multiple sequence alignment was performed using CLUSTALW version 1.83 (Thompson *et al.,* 1994). The RNA secondary structures for ITS were predicted using RNADRAW (Matzura and Wennborg 1996). RNADRAW predicts RNA structures by identifying suboptimal structures using the free energy optimization methodology at a default temperature of 37°C. In the current study, ITS region was used for RNA structure prediction. The minimum energy structure prediction algorithm in RNADRAW was ported from the RNAFOLD program included in the Vienna RNA package (Hofacker *et al.*, 1994). The dynamic programming algorithm employed in RNADRAW was based on the effort of Zuker and Stiegler (1981) and uses energy parameters taken from Turner *et al.* (1988).

Phylogenetic analysis

The phylogenetic relationships among sequences were established using Neighbour-Joining (NJ) (Saitou and Nei, 1987). Trees and genetic distances were based on 10,000 replicates in order to assess the degree of confidence for each branch on the trees.

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Phylogenetic analysis

The phylogenetic relationships among sequences were established using Neighbour-Joining (NJ) (Saitou and Nei, 1987). Trees and genetic distances were based on 10,000 replicates in order to assess the degree of confidence for each branch on the trees.

RESULTS AND DISCUSSION

ITS-PCR Amplification and Sequence analysis

The polymerase chain reaction (PCR) amplified a product of ~900bp, from the isolate used in the study as routinely observed in the amplification of ITS region using ITS 4 and ITS 6 universal primers (Fig.1).

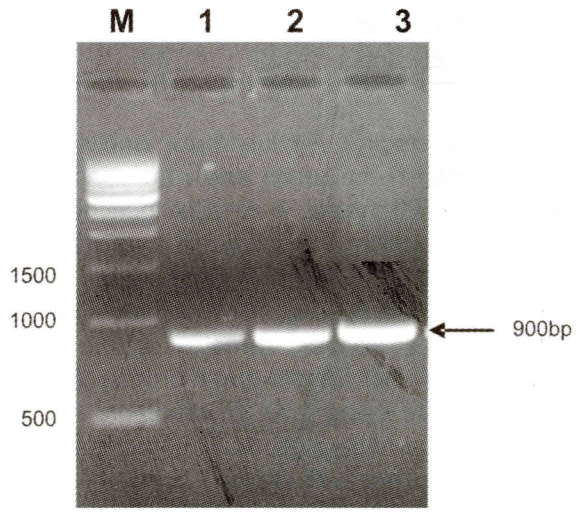

Fig. 1. ITS –PCR amplicons of *Phytophthora* isolates. **M**-500bp ladder; Lane 1. Black pepper isolate of *Phytophthora;* Lane 2. Coconut isolate of *Phytophthora*; Lane 3. Cardamom isolate of *Phytophthora*

The sequenced product of black pepper isolate had a length of 864 bps, which comprised of the complete ITS1 (174bps), 5.8S ribosomal subunit (159bps), ITS 2 region (419 bps) along with partial sequences of 28S-large (59bps) and 18S-small subunit (53bps) genes of r DNA. BLAST searches of ITS rDNA resulted in match with Cp-2 tropical isolate maintained in USA (DQ464038) as well as *P. tropicalis* SCRP730 isolate of Italy (DQ118649) by limiting our search to Oomycetes taxa. All the hits had a reliable E value 0.0 showed that the product is 99% similar to *P. capsici* as well as *P. tropicalis.* The sequence alignment with *P. capsici* (DQ464038) showed a transition at position 93 (G to A), whereas *P. tropicalis* (DQ118649) showed a transition at position 369 (C to T). The sequence was submitted in GenBank (Benson *et al.,* 1998) (AM422703).

The same BLAST search methodology was extended to the sequenced coconut isolate (96-30) which had a length of 898 bps, which comprised of the complete ITS1 (213 bps), 5.8S ribosomal subunit (159bps), ITS 2 region (414 bps) along with partial sequences of 28S-large (59bps) and 18S-small subunit (53bps) genes of r DNA. BLAST searches resulted in match with *P. palmivora* by limiting our search to Oomycetes taxa. BLAST results showed 100 hits and our query matched 100% against *P. palmivora* TARS8 isolate (DQ987922) which had a reliable E value 0.0 confirms the product is *P. palmivora.* The identified *P. palmivora* rDNA sequence was also submitted in GenBank (AM422704). Cardamom isolate (99-180) had a length of 809bps, which comprised of the complete ITS1 (191bps), 5.8S ribosomal subunit (162bps), ITS 2 region (106bps) along with partial sequences 18S-small subunit (32bps) genes of r DNA. BLAST searches resulted in match with *P. meadii* by limiting our search to Oomycetes taxa. BLAST results showed 109 hits, out of 109 hits our query matched 100% against *P. meadii* isolate (AY251649) which had a reliable E-value 0.0 confirms the product is *P. meadii.* The identified *P. meadii* rDNA sequence was also submitted to GenBank (AM422705). These findings are in agreement with earlier report of Anandaraj (2000) that destruction of black pepper by *P. capsici*, immature nut fall of coconut caused by *P. palmivora* and fruit rot of cardamom by *P. meadii* are some of the destructive diseases of plantation crops.

Predicted RNA secondary structures of ITS

The inter-species specific sequence variation is contemplated in the predicted RNA secondary structures of *P. tropicalis* (AJ299733) and *P. capsici* (DQ464038), which have lowest free energy -197.94 Kcal and -196.47 Kcal, respectively (Table 1).

The black pepper isolate showed similarity in the secondary structures by having free energy (-196.12 Kcal) that tend towards *P. capsici* (Table 1). Whereas, when stem formation was considered, it matched with that of *P. tropicalis* (Fig.2a,b,c&d) *P. palmivora* isolates from coconut studied here showed similarity in secondary structure and free energy (-229.02 Kcal) with *P. arecae* (AF266781) but showed significant difference in free energy with *P. palmivora* (AY742734) from GenBank (Table.2).

Table 1. ITS secondary structure profiles of *Phytophthora* isolates

GenBank Accession number	Isolates Used	ITS-Length	GC %	Number of Stems	Energy	Number of G-C	Number of A-U	Number of G-U
AM422704	*P. palmivora* IISR	786	49	45	-229.02 Kcal	115	75	68
AY742734	*P. palmivora*	786	49	45	-231.12 Kcal	116	75	67
AM422703	*P. capsici* IISR	752	50	50	-196.12 Kcal	111	82	62
AJ299733	*P. tropicalis*	752	50	50	-197.94 Kcal	112	82	61
DQ464038	*P. capsici*	752	50	46	-196.47 Kcal	107	78	64
AY251649	*P.meadii*	780	50	49	-216.74 Kcal	120	81	64
AM422705	*P.meadii* IISR	781	50	51	-219.88 Kcal	123	87	65

However, it did not show any difference in the phylogram and formed a homogeneous cluster with other *P. palmivora* isolates (Fig.3). The IISR isolate of *P. meadii* from cardamom showed slight variation in the free energy (-219.88 Kcal) with *P. meadii*, AY251649 from GenBank (-216.74 Kcal) (Table 1) but showed similar secondary structure (Fig. 2 g, h).

Phylogenetic analysis

The phylogenetic tree construction methods (as detailed in Materials and Methods) yielded trees with similar topologies. The characterized three isolates grouped in three major clusters (Fig.3). The first major cluster comprises two different sub clusters, the first one is a homogenous group of *P. capsici* isolates (Clade IA) and the second a heterogenous group of *P. capsici* and *P. tropicalis* isolates (Clade IB). The black pepper isolate fall in the Clade I B. The second major cluster (Clade II) includes *P. meadii* isolate of cardamom with an available *P. meadii* sequence. The third major cluster comprises of the isolates of *P. palmivora* (Clade III) (showed more divergence with group I & II) with some deviations within the populations. These difference may be explained by a higher mutational saturation in the ITS region. In order to assess the confidence of the cladistic status of black pepper isolate with other isolates of *P. capsici* and *P. tropicalis* from GenBank, *Pythium aphanidermatum* was used as an out-group and added to the alignment profile.

P. capsici was first described on pepper by Leonian (1922) and is classified in Group II. Tucker (1931) classified *P. capsici* as a host-specific fungus, pathogenic on pepper. Subsequently, taxonomists studied *Phytophthora* isolates from various hosts in the world and re-described the taxonomy of *P. capsici*. A detailed description of *P. capsici* taxonomy is provided by Erwin and Ribeiro (1996). In 2001, Aragaki and Uchida described a new species *Phytophthora tropicalis* from diseased pepper, tomato, eggplant and cucurbit. From their comparative morphological and cultural studies using 100 *Phytophthora* isolates with deciduous, long-pedicellate sporangia which have been referred to *P. capsici,* revealed that they were separable into two taxa: *P. capsici* with broad sporangia, sporangial length to diameter ratio less than 1.8, predominantly round sporangial bases, absence of chlamydospores, good growth at 35º C, and pathogenicity to *capsicum*; and *P. tropicalis* sp. nov. distinguished from *P. capsici* by narrow sporangia less than 26 µm in diameter, sporangial length to diameter ratio of more than 1.8, predominately tapered sporangial bases, production of chlamydospores by most isolates, poor or no growth at 35º C, and weak or no virulence to *capsicum*. Although *P. tropicalis* and *P. capsici* have similar morphological and physiological attributes, the molecular distinctions between them indicated *P. tropicalis* is a separate species (Zhang *et al.*, 2004).

The analysis of black pepper isolate revealed that the isolate shares characteristics of both the species, *P. capsici* and *P. tropicalis*. Pathogenicity on capsicums and some other characters like production of chlamydospores and pedicel length support that black pepper isolate is *P. capsici*, whereas some morphological characters like sporangial length to diameter ratio of more than 1.8 and narrow sporangia similar to that of *P. tropicalis*

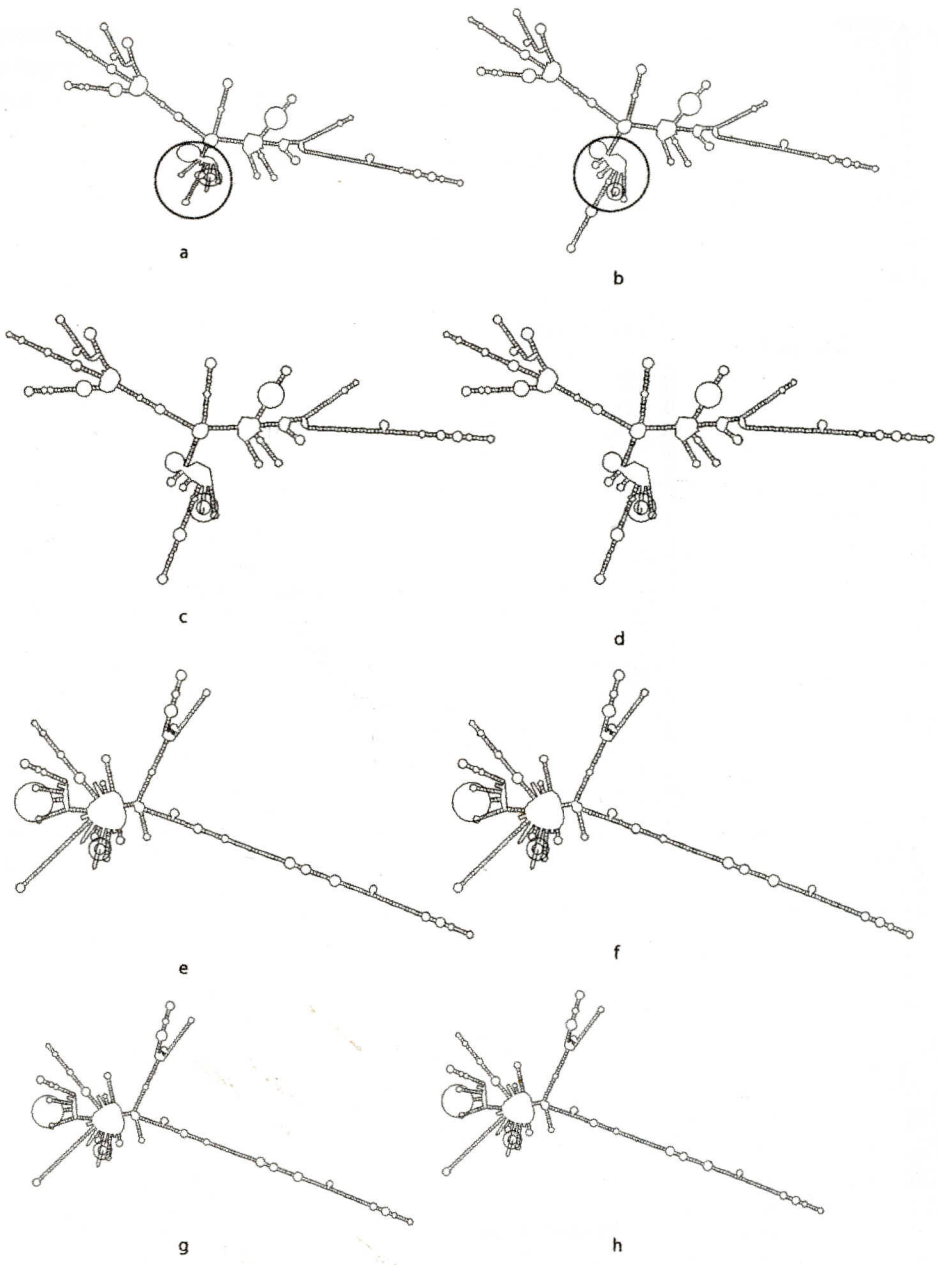

Fig. 2. Predicted secondary structure models for ITS sequences of *Phytophthora* species
a. *P. capsici* from database, b. Black pepper isolate of *P. capsici* (Note the differences indicated in circles). c. *P. tropicalis* from database, d. Black pepper isolate of *P. capsici*, e. *P. meadii* from database, f. Cardmom isolate of *P. meadii*, g. *P. palmivora* from database, h. Coconut isolate *P. palmivora*

(unpublished data). Molecular data of ITS rDNA matched 99% with *P. capsici* and *P. tropicalis*. The predicted ITS secondary structure profile matches with *P. tropicalis.* It is further evident from the cladogram IB, that it is a heterogenous group. For solving this confusion, we undertook a separate sub-grafting and pruning (SPR) analysis supported by 10,000 bootstrap replications which also favoured that black pepper isolate grouped in a separate cluster that includes *P. capsici* and *P. tropicalis.* Hence, we conclude that the black pepper isolate of *Phytophthora* shares characteristics of both *P. capsici* and *P. tropicalis* suggesting that black pepper isolate possess distinct features. Further studies are required for the confirmation of origin of a new *Phytophthora* species showing characters of

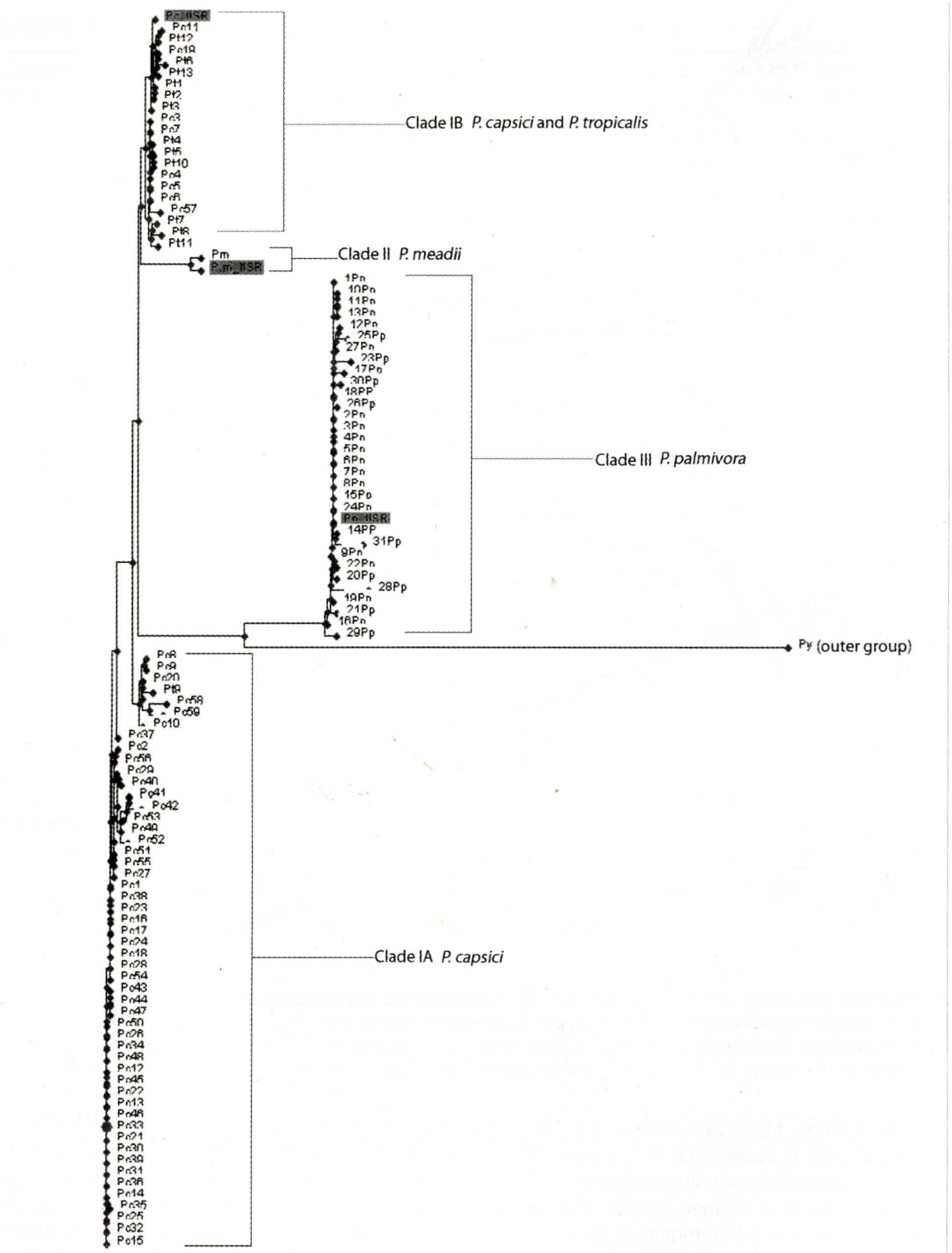

Fig. 3. Phylogram of *Phytophthora* species Black pepper isolate of *P. capsici* grouped with clade IB where as *P. meadii* and *P. palmivora* grouped with respective clades

both *P. capsici* and *P. tropicalis*. The present study will be highly useful for disease diagnosis of the destructive diseases of plantation crops such as black pepper, coconut and cardamom.

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