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Morphological and molecular intervention in identifying *Phytophthora* spp. causing leaf and nut fall in nutmeg (*Myristica fragrans Houtt.*)

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Abstract Nutmeg, a major crop of India provides two important spices. During the monsoon season of 2011, a serious leaf and nut fall occurred in nutmeg plantations and *Phytophthora ramorum* was reported as the causal organism. *P. ramorum* being an alien species, the aetiology warranted a detailed investigation. *Phytophthora* isolated from different endemic locations were studied for morphological and molecular characterization using ITS, ITS-RFLP, MLST, SSCP and hybrid analysis. The isolates showed papillate, ovoid-obovoid sporangia with pedicel length > 20 μm which is distinct from the semi-papillate, caducous sporangia with short pedicel of <5 μm characteristic of *P ramorum*. The isolates failed to grow at 10 °C or at 37 °C and chlamydopsores were not produced in culture, a characteristic distinct to *P. ramorun*. MLST analysis placed the isolates in Clade 2 while *P. ramorum* belongs to Clade 8. *P. ramorum* has a unique set of morphological characteristics and a unique molecular sequence at the ITS region which delineate it from other *Phytopthora* species. Among the eight isolates studied, ITS region of six isolates (13–01–13-06) showed similarity to *P. meadii* and two isolates (13–55 and 98–68) to *P. tropicalis*. In SSCP, the isolates formed a separate group distinct from *P.meadii* of cocoa and cardamom. The double peaks in the ITS chromatograms indicated hybridity and further hybrid analysis placed them under *P. citrophthora* and *P. meadii* (Cit-Med) lineage. Thus due to the distinct differences from *P. ramorum* and evidences of shared lineage with *P. citrophthora* and *P. meadii,* we propose to call these isolates as *P. citromeadii* (13–01- 13-06) and *P. citrocaptalis* (13–55 and 98–68).

Keywords Fruit rot · Hybrid analysis · MLST analysis · Nutmeg · *Phytophthora ramorum* · *Phytophthora meadii* · Spices

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Introduction

Nutmeg (*Myristica fragrans* Houtt.) is one of the major spice crops in south India. It is cultivated as an intercrop in coconut and arecanut gardens. It produces two major spices, viz. nutmeg and mace which are commercially valued for medicinal and culinary purposes. Nutmeg is the dried kernel of the seed and mace is the dried aril surrounding the

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seed. The plant is susceptible to a number of fungal diseases like fruit rot induced by species of *Phytophthora*, white thread blight and horse hair blight induced by *Marasmius* spp., shot hole disease incited by *Colletotrichum gloeosporioides* etc. During the peak monsoon period of 2011, occurrence of a serious leaf and fruit fall was reported from major nutmeg growing areas of Thrissur, Emakulam, and Kottayam districts of Kerala especially in places like Kalady, Mambara, Desham, Parakkadavu and Poovathuserry. The disease appeared as severe defoliation and fruit fall and on close observation, symptoms were manifested as dark brown water-soaked lesions on the midrib of the leaves which enlarged and spread along the lateral veins to lamina resulting in blighting of the leaves (Fig. 1) Petioles of the infected leaves become black and on young shoots, black lesions appeared, which enlarged in size resulting in rotting and drying up of shoots from the tip downwards. Leaf and stem infections resulted in extensive defoliation. A group of researchers reported the disease as caused by *P. ramorum* (Mathew and Beena 2012). But *P. ramorum* being an alien species hither to unreported in the country, there arose a controversy in the causal organism which warranted a detailed investigation (Anandaraj et al. 2015). So the present study was focussed on understanding the actual aetiology of the disease by characterization of the *Phytophthora* isolates from nutmeg collected from different locations reported to be hot spots. The study comprises morphological, physiological and molecular characterisation of the isolates which include analysis of ITS rDNA, ITS-RFLP, MLST SSCP and Hybrid analysis.

Fig 1 Leaf and nut fall in nutmeg

Materials and methods

Morphological characterization

Source of Phytophthora isolates

Eight *Phytophthora* isolates, of which six isolates from Kerala Agricultural University, Vellanikkara, Thrissur, collected from different nutmeg growing areas of Kalady, Mambara, Desham, Parakakdavu, Poovathuserry (13–01 to 13–06) (Ernakulam, Thrissur, and Kottayam districts of Kerala) and two isolates (13–55 and 98–68) from the National Repository of *Phytophthora* at ICAR-Indian Institute of Spices Research, Kozhikode, were used for characterization. The details of isolates are given in Table 1.

Colony morphology, sporangial morphology and growth rate

The *Phytophthora* isolates obtained were purified using PVPH medium (Tsao and Guy 1977) and sub cultured onto carrot agar (CA) for further studies.

For colony morphology, the cultures were grown in CA for 72–96 h and observed for phenotypic characters as well as growth rate by measuring the colony diameter. For sporulation, 5 mm culture plugs cut from the periphery of 72 h old CA grown culture, were immersed in sterile water in Petri dishes and exposed under continuous white light for 24–48 h. The sporangia produced in 48 h were documented for details such as sporangial morphology, ontogeny and pedicel length. The sporangial length and breadth were also measured to calculate the l: b ratio.

Table 1 Source of *Phytophthora* isolates from nutmeg leaf fall

Isolate code	Location of collection	Plant part
13–01	Mambara (Ernakulam dt)	Leaf
13–02	Desham (Ernakulam dt.)	Leaf
13–03	Kalady (Ernakulam dt.)	Leaf
13–04	Parakkadavu (Ernakulam dt.)	Leaf
13–05	Poovathussery (Ernakulam dt.)	Leaf
13–06	Kodissery (Ernakulam dt.)	Leaf
13–55	Anakkampoyil (Kozhikode)	Fruit
98–68	Kaladi (Ernakulam)	Leaf

Determination of reproductive type

The mating type was determined by dual culturing of the isolates with known A1 (05–06 isolate *P. capsici* of Black Pepper) and A2 (12–19 *P. meadii* isolate of cocao). The dual culture was done on Ribeiro's medium supplemented with beta- sitosterol and incubated in dark at 24-25 °C for 7–21 days. Oospore formation was monitored from 7 days onwards using a Leica (DM500) Research Microscope and documented using a DFC 310FX camera.

Effect of temperature

To study the cardinal temperature for growth, 5 mm mycelial plugs cut from the periphery of 72 h old CA grown culture was placed in CA and incubated at temperatures ranging from 10 to 35 °C for 72–96 h and observations were recorded on radial growth.

Pathogenicity

Pathogenicity of the isolates was tested on probable host plants such as cardamom (*Elettaria cardamomum)* cacao (*Theobroma cacao*), arecanut (*Areca catechu)* and rubber (*Hevea brasieliensis)* as well as on nutmeg by detached inoculation method. The nutmeg isolates were inoculated to nutmeg fruits, leaves as well as twigs and on crops such as cardamom, cocoa, arecanut and rubber. Similarly *P. meadii* isolates (since ITS sequencing showed sequence similarity to *P.meadii,*) from these crops were also inoculated to nutmeg. Briefly, inoculum plugs of 5 mm size, cut from the periphery of 72 h actively growing culture was inoculated to detached plant parts of the respective plants in a mist chamber maintaining a temperature of 24 °C ± 2 °C and incubated for 4–7 days. The progress of infection was recorded as water soaked lesions and necrosis on the inoculated portions.

Molecular characterization

ITS sequencing and phylogeny

DNA extraction

Phytophthora isolates were grown as stationary cultures in Ribeiro's liquid medium (Ribeiro's 1978) for 4 days at room temperature (28–30 °C) for DNA extraction. Mycelia were harvested by filtration through sterile Whatman No.1 filter paper. Genomic DNA was extracted according to Cooke et al. (2000) protocol. Mycelia were ground in a mortar with extraction buffer and extracted with phenol: chloroform: isoamyl alcohol (25:24:1 *v*/v). Precipitation of DNA was carried out with ice-cold isopropanol, and the pellet was washed with 70% ethanol and re-suspended in sterile double-distilled water. The purified DNA was quantified using Eppendorf biophotometer (Eppendorf India Limited, Chennai) and subjected to PCR amplification. The PCR conditions are given below.

Amplification of ITS region of rDNA was carried out using primers ITS 4 (5' TCCTCCGCT TATTGATA TGC3') and ITS 6 (5' GAAGGTGAAGTCGT AACAAGG 3′). The PCR products were purified using GenElute PCR Clean Up kit (Sigma-Aldrich) and sequenced. The sequence data of these amplified fragments were obtained using ABI DNA sequencer (Xcelris Labs Limited). The forward and reverse sequences were assembled into contigs using DNA Baser software. Sequence similarity was analysed with NCBI-Blast algorithm.

PCR component	Volume	Step.	Temperature	Temperature	Cycle
Nuclease free water	15.4 µl	Denaturation	94 °C	5 min	1X
10 X reaction buffer	2.5 µl	Denaturation	94 °C	30 s	
$MgCl_2$ (25 mM)	2.0 µl	Annealing	55 °C	45 s	35 X
dNTP mix (2.5 mM each)	1.0 µl	72 °C	1 min		
Forward primer (10 pm)	1.0 µl	Extension	72 °C	20 min	1X
Reverse primer (10 pm)	1.0 µl	Final extension			
Template DNA (50 ng)	2.0 µl				
Taq DNA polymerase (5 U/µl)	0.1 µl	Hold	4 °C	∝	

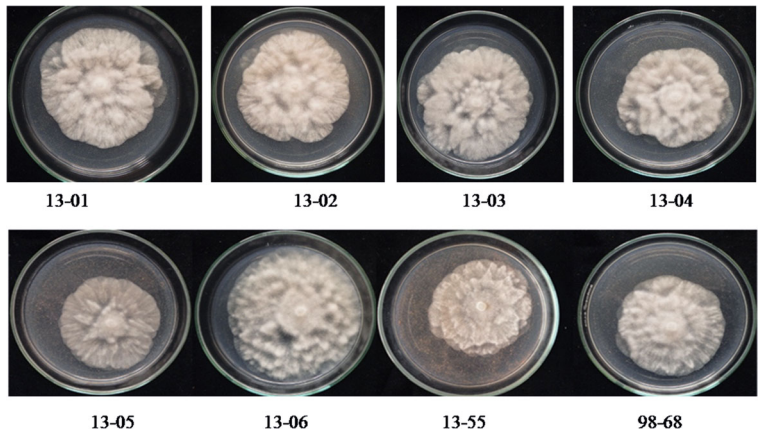


Fig. 2 Colony morphology of nutmeg P*hytophthora* isolates

A final alignment of 835 nucleotides of the ITS region was used in the analyses with ClustalW2 programme (Larkin et al. 2007). The analysis included species such as *P. nicotianae* from Clade 1 and *P. ramorum* and *P. lateralis* from Clade 8 and all other *Phytophthora* species from Clade 2. Phylogenetic analysis was performed using different methods such as Bayesian analysis, maximum likelihood and neighbor-joining method. The consensus tree was identified using consense package of Phylip 3.69. Bayesian analysis was performed in MrBayes version 3.1 (Ronquist and Huelsenbeck 2003) with two searches run simultaneously for at least two million generations. Flat Dirichlet parameters were used for the gamma shape parameter and the proportion of invariable sites. Three heated chains (temperature 0.2) and one cold chain were used in each search. The parameter was then fixed for a bootstrap analysis with 10,000 replicates. Tracer version 1.5 (Rambaut and Drummond 2009) was used to evaluate mixing and convergence, and to estimate appropriate burn-in period. Maximum likelihood analysis was performed using GARLI version 2.0 (Zwickl 2006) with two replicates were used to estimate model parameters. These were then fixed for a bootstrap analysis with 10,000 replicates and NJ method (Saitou and Nei 1987) was calculated using MEGA5 (Tamura et al. 2007). The majority-rule consensus of the bootstrap replicate trees was calculated using consense and

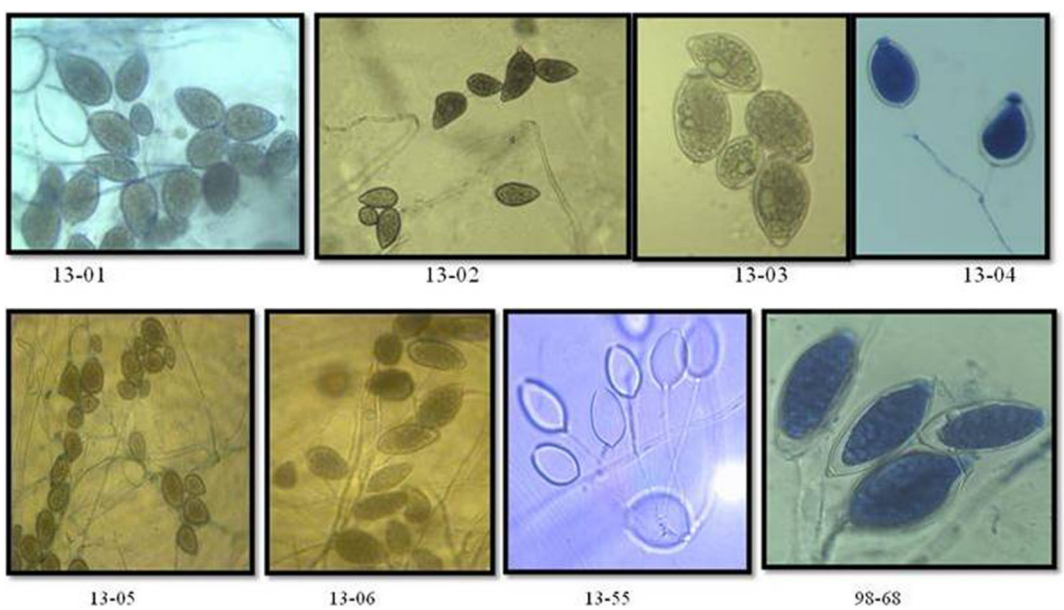


Fig. 3 Sporangial morphology of nutmeg *Phytophthora* isolates

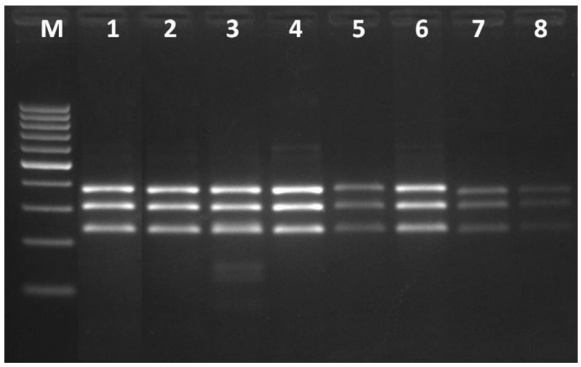


Fig. 4 ITS-RFLP (*Msp*I) banding pattern of *Phytophthora* isolates from nutmeg M: 100 bp ladder; 1: 13–01; 2. 13–02; 3. 13–03; 4. 13–04; 5. 13–05; 6. 13–06; 7. 98–68; 8.13–55.

seqboot package in the PHYLIP (Felsenstein Felsenstein 1985, Felsenstein 1993). When consense is run the majority rule consensus tree will result that retains the relationships found in majority of the trees and allows bootstrapping on different methods. *Phytopthora nicotianae* (AF266776) of clade1 species was designated as out-group for the selected sequences.

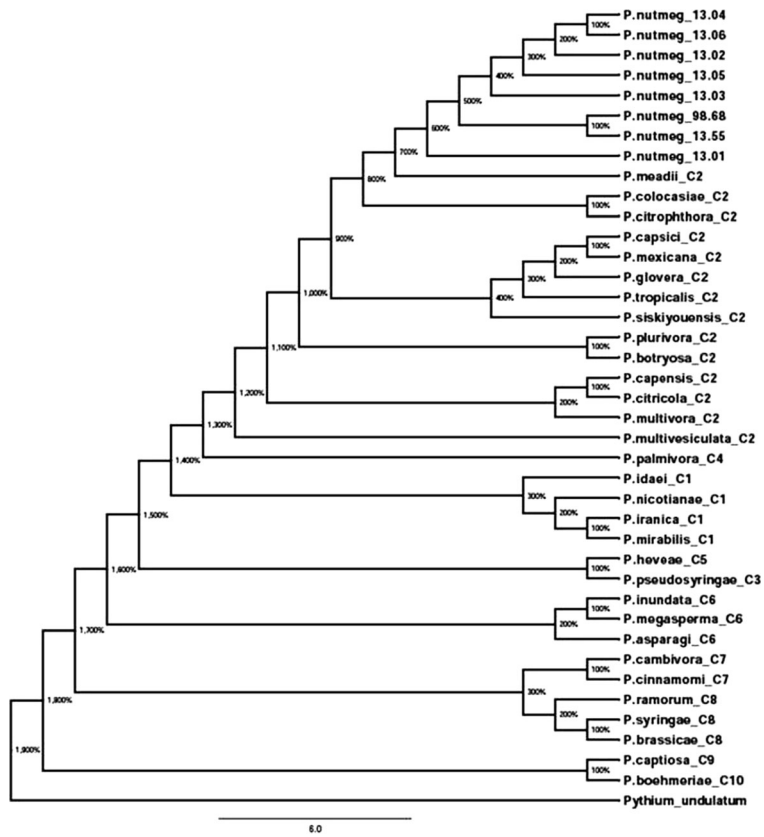
Fig. 5 ITS Phylogeny of Nutmeg *Phytophthora* isolates: consensus tree from MP, ML, NJ & Bayesian methods

Its-RFLP

The amplified PCR product was digested with the restriction enzyme *Msp*I. Restriction digestions were performed in 20 µl reaction containing 10 µl PCR product, 1X restriction buffer with bovine serum albumin, 6 µl PCR-grade water, and restriction enzyme (2 U/reaction) and briefly centrifuged and incubated at 37 °C for 2 h.

MLST analysis

The sequence diversity was analyzed among the six *Phytophthora* isolates from nutmeg as per the procedure of Kroon et al. (2004) and Schena et al. (2006). Nuclear genes such as *28sRNA*, *60s* Ribosomal Protein *L10*, *B-tubulin*, *Elongation factor 1*$^{\alpha}$, *Enolase*, *Hsp 90*, *Tig A* fusion protein, *Ypt Ras related Protein* and *Ig Cox* and Mitochondrial gens such as *Nad 9*, *rsp10* and *SecY* were used for MLST analysis.



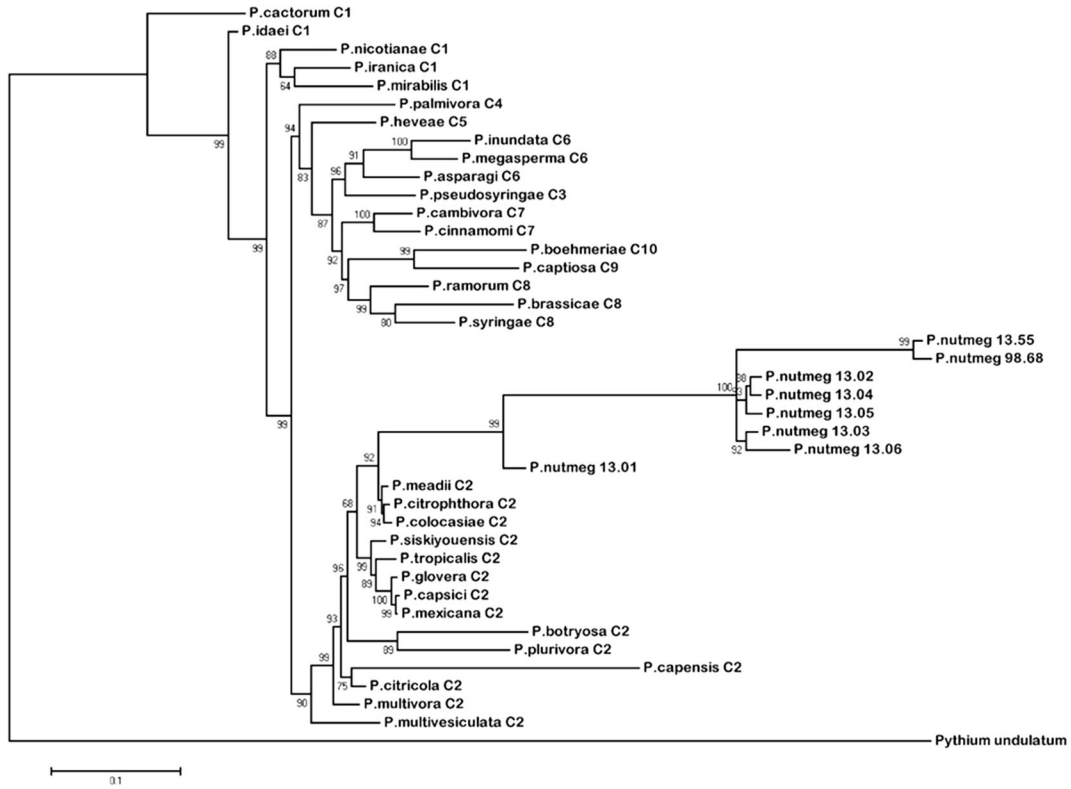


Fig. 6 MLST analysis of nutmeg *Phytophthora* isolates using 7 nuclear genes viz. ITS, *Beta-tubulin*, *Elongation factor*, *HSP*90, *60SL10*, *Enolase* and *TigA*

Single Strand conformation polymorphism analysis

DNA amplification and SSCP analysis of the PCR products were performed as described previously (Kong et al. 2004). Amplification utilized a pair of primers that favour oomycetes (Cooke et al. 2000) – forward primer ITS6: 5′-GAA GGT GAA GTC GTA ACA AGG-3′, located in the 18S rDNA and reverse primer ITS7: 5'-AGCGTT CTT CAT CGA TGT GC-3′, located in the 5.8S rDNA. PCR was performed in a total volume of 25 μl containing 100 ng of DNA. The PCR conditions were as mentioned elsewhere. 1 μL of individual PCR products was mixed with 9 mL of the denaturing buffer (95% formamide, 20 mM EDTA and 0.5% bromophenol blue). After a brief spin, mixtures were heated at 96 °C for 10 min then chilled on ice. 5 μL of each mixture was loaded on an 8% acrylamide: bis-acrylamide (29: 1) non-denaturing minigel cast using a

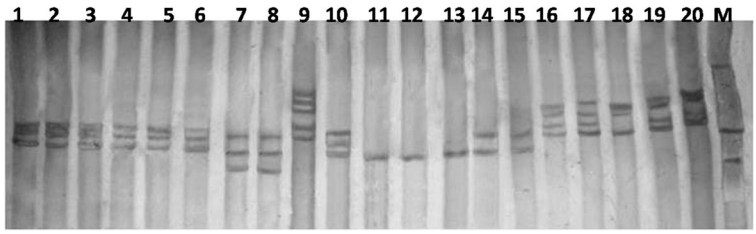


Fig. 7 Single strand confirmation polymorphism (SSCP) among nutmeg *Phytophthora* isolates in comparison with other *Phytophthora* isolates of the same clade - Lane M: ss DNA ladder; 1–8 *Phytophthora* isolates from nutmeg.1–13-01; 2–13-02; 3–13-03; 4–13-04; 5–13-05; 6–13-06; 7–98-68; 8–13-55; 9–12-19 – *P. meadii* (cocoa) 10–12-30 – *P. meadi*i (coconut) 11–13 *P. meadii* (cardamom); (11: 10–12 12. 13–16;13. 13–32) 14–99-188 *P. citrophthora (*black pepper); 15–12-15 *P. citrophthora* (cocoa);16–98-01 *P. palmivora* (black pepper)*;17*–12-13 *P. palmivora (*cocoa); 18–12-18 *P. palmivora* (coco); 19–12-29 *P. palmivora* (coconut); 20–14-04 *P. palmivora* (citrus)

Table 2 Phenotypic characteristics of nutmeg *Phytophthora* isolates

Isolate No.	Colony morphology in CA	Colony dia (72 h) cm	Ontogeny	Caducity	Sporangial shape	Chlamydospores
13–01	Petalloid	7.2	Sympodial	caducous	Obpyriform	Absent
13–02	Floral	6.9	Sympodial	caducous	Obpyriform	Absent
13–03	Floral	7.6	Sympodial	caducous	Ovoid	Absent
13–04	Floral	6.7	Sympodial	caducous	Ovoid	Absent
13–05	Floral	6.4	Sympodial	caducous	Ovoid	Absent
13–06	Rosaceous	8.4	Sympodial	caducous	Obpyriform	Absent
13–55	stellate	6.3	sympodial	caducous	ovoid	Absent
98–68	Floral	6.3	Umbellate	caducous	Elliptical	Present

Mini-PROTEAN 3 Cell (BioRad Laboratories, Hercules, CA, USA). An aliquot of 25 ng of a single-stranded DNA (ssDNA) ladder was also included to facilitate comparison of SSCP patterns (Kong et al. 2004). Denatured PCR products were eletrophoresed in prechilled 1X TBE buffer at 200 V for 2 h at room temperature. After electrophoresis, the gel was subjected to silver staining. SSCP banding patterns of individual isolates were analysed with the aid of the ssDNA ladder.

Hybrid analysis

Since MLST analysis of eight *Phytophthora* isolates from nutmeg showed similarity to two or more species in Clade2, it was assumed that these isolates are putative hybrid isolates. So, it warrants an understanding of the ancestral relationship. For hybridity analysis, seven species from clade 2, viz. *P. capsici, P. citrophthora, P. colocasiae, P. glovera, P.meadii*, *P. siskiyouensis* and *P. tropicalis,* were selected as parental species for hybrid analysis. Nuclear genes and mitochondrial genes were used for studying hybrid analysis. Eight Nuclear genes viz. *28S, 60S, Btub, EF, ENO, HSP90, Tig A* and *ITS* were used for paternal identification and four mitochondrial genes viz. SecY, rps10, Nad9 and Cox1 were used for maternal identification. All the available sequences of selected nuclear genes and mitochondrial genes were collected from different databases like Phytophthora.org, Q-bank and NCBI. From the selected parental species, 21 parental (hybrid) combinations were made viz. Cap **X** Cit, Cap **X** Col, Cap **X** Glo, Cap **X** Med, Cap **X** Sis, Cap **X** Tro, Cit **X** Col, Cit **X** Glo, Cit **X** Med, Cis **X** Sis, Cit **X** Tro, Col **X** Glo, Col **X** Med, Col **X** Sis, Col **X** Tro, Glo **X** Med, Glo **X** Sis, Glo **X** Tro, Med **X** Sis, Med **X** Tro, Sis **X** Tro [where Cap - *P. capsici*, Cit-*P. citrophthora*,Col-*P.colocasiae*,Glo-*P. glovera*; *P. meadii*, Trop-*P.tropicalis*, Sis- *P.siskiyouensis*). A total of 168 combinations were made for identifying the paternal parent. Consensus of the parental combinations was made from the collected sequences. The consensus sequences of the hybrid isolates were then aligned together with the parental species and the identity of the base at each of the identified positions were recorded. Alignments of the consensus of parental combination and all the nutmeg isolates were predicted through Geneious R9. From the obtained alignments, variable parsimony sites were identified and colours were given to the sites. According to the IUPAC codes, SNPs were also identified. Percentage of identity was calculated

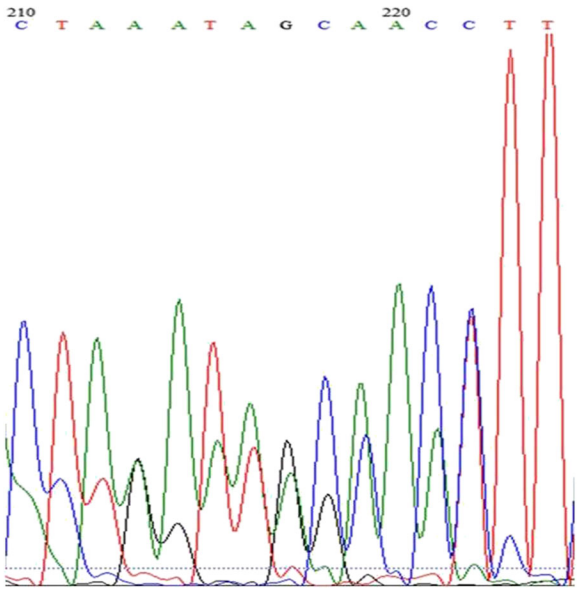


Fig. 8 Double peaks in ITS chromatograms

Table 3 Sporangial morphology and mating type

Isolate No.	Sporangial characters (μm)			Pedicel length(μm)	Papilla (μm)	Mating type
	length	breadth	l/b			
13–01	36.70 (29.76–2.16)	28.9 (24.8–32.24)	1.73 (1.58–1.96)	14.39 (6.32–18.77)	5–6	A1
13–02	27.94 (19.84–32.24)	21.99 (12.4–27.28)	1.78 (1.48–2.36)	21.21 (17.95–25.81)	5–7	A1
13–03	29.76 (22.32–34.72)	22.24 (14.88–29.76)	1.71 (1. 28–1.92)	18.34 (13.29–23.15)	5–7	A1
13–04	29.93 (24.8–37.2)	21.91 (17.36–28.52)	1.65 (1.47–2.05)	20.22 (13.95–25.83)	7–11	A1
13–05	20.72 (12.40–33.48)	14.36 (8.68–23.56)	1.42 (1.17–1.60)	21.83 (17.63–80.71)	8–16	A1
13–06	27.45 (19.84–34.72)	18.10(12.4–24.8)	1.79 (1.58–2.13)	30.01 (12.61–41-09)	5–6	A2
13–55	35.46 (27.28–44.64)	25. 54 (19.84–34.72)	1.39 (1.2–1.66)	35.67 (11–57-54.67)	5–8	A1
98–68	32.41(22.32–39.68)	19.18 (14.88–22.32)	2.07(1.84–2.37)	130.9 (82.53–200.97)	6–10	A1

through Geneious R9. Isolates with more than 95 percentage of identity were taken for the identification of hybrids.

Results and discussion

Cultural and morphological characterization

The eight *Phytophthora* isolates collected from endemic locations (13–01, 13–02, 13–03, 13–04, 13–05, 13–06, 13–55 and 98–68) (Table 1) were characterized morphologically. All the isolates were fast growing in carrot agar. The average diametrical growth of the isolates varied from 63 mm to 84 mm in 72 h at 25 ± 2 °C (Table 2). The colony morphology in carrot agar showed varying colony patterns like petalloid, floral, stellate and chrysanthemum (Fig. 2). Sporangia were rarely produced in solid culture. However abundant sporangial formation was observed when the mycelial plugs were suspended in sterile distilled water at optimum temperature.

The sporangial type of the isolates viz.13–01,13–02, 13–03, 13–04, 13–05, 13–06 were papillate, caducous with medium pedicel length of >20 μm and formed sympodially and ranged in shape from ovoid to obpyriform showing similarity to *P. meadii*. The two isolates 98–68 and 13–55 showed umbellate ontogeny with long pedicel and elongated sporangia with tapered base, characteristic of *P. tropicalis* and *P.capsici* (Fig. 3, Table 2). The average sporangial length of the nutmeg isolates varied from 20.72–36.70 μm and width varied from 14.36–28.9 μm with an average length/breadth ratio (l/b) of 1.85–1.91(Table 3. Chlamydospores were not produced in carrot agar culture except 98–68 where chlamydospores were produced in culture.

Determination of mating type

The isolates were heterothallic and produced oogonia when paired with known *P. meadii* isolate of A2 mating type except one isolate 13–06 which produced oogonia when paired with *P. capsici* (A1). So all the isolates

Table 4 Radial Growth (mm) at different temperatures at 72 h

Isolate No.	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C	40 °C
13–02	0*	16.84	40.95	56.33	44.5	0	0
13–03	0	16.17	44.17	63.83	66.5	0	0
13–04	0	16.83	43.00	58.66	50.5	0	0
13–05	0	20.17	43.17	58.17	52.00	0	0
13–06	0	19.84	60.26	79.5	65.5	0	0
13–55	0	24.83	53.00	60.00	54.5	0	0
98–68	0	24.00	50.66	59.83	64.17	0	0

Table 5 Average Comparative identity of mitochondrial genes of nutmeg isolates (*Cox Nad9 rps10 SecY*) For maternal parent analysis

Nutmeg *Phytophthora* isolates	*P.colocasiae_C2*	*P.glovera_C2*	*P.meadii_C2*	*P.citrophthora_C2*	*P.capsici_C2*	*P.siskiyouensis*_C2	*P.tropicalis*_C2
13–01	85.24033	83.59833	85.389	85.216	83.677333	84.13567	84.09467
13.02	85.24033	83.59833	85.389	85.216	83.677333	84.13567	84.09467
13.03	85.80133	84.15933	85.95	85.777	84.191667	84.69667	84.65567
13.04	85.80133	84.15933	85.95	85.777	84.191667	84.69667	84.65567
13.05	80.521	79.53	81.368	76.67575	80.712	76.15	79.39475
13.06	81.56875	80.57525	82.606	77.60375	81.727	77.06525	80.44475
13.55	81.1765	82.567	82.47925	75.6385	83.6455	76.69725	82.786
98.68	80.52	81.75375	82.0065	76.036	82.8315	76.68	82.29625

except 13–06 were of A1 mating type (Table 3**,** Suppl. Fig. 1**)**.

Effect of temperature

The optimum temperature required for growth was found to be 25–28 °C. The minimum growth occurred at >10 °C and maximum growth at <35 °C. None of the isolates grew at 10 °C or at 35 °C (Table 4).

Pathogenicity

All the isolates showed pathogenicity on fruit, leaves and twigs of nutmeg thereby proved Koch's postulates (Suppl. Fig. 2-5) within 3–5 days of inoculation. They were also able to infect Cardamom (*Elettaria cardamom)*, Cocoa (*Theobroma cacao*), Arecanut (*Areca catechu*) and Rubber (*Hevea brasielinsis*) with few exceptions (Table 5). However none of the isolates were found infective on black pepper. Though *P. capsici* and *P. tropicalis* are predominant pathogens of black pepper with occasional occurrence of *P. meadii*, the *Phytophthora* isolates from nutmeg (showing close similarity to *P.meadii*, and certain isolates to *P. capsici* and *P. tropicalis,* failed to infect black pepper showing their deviation from original *P. capsici, P. tropicalis* or *P.meadii* which warranted detailed species analysis. So also, *P. meadii* isolates from cardamom, cocoa and coconut were not infective on nutmeg and hence these

Table 6 Comparison of nut meg isolates with *P. ramorum* and other closely related species

Phytophthora isolates	Pedicel length	Papilla	Caducity	Chlamydospore	Reproduction	Antheridia	Plerotic/ Aplerotic	Optimum temp.	Max.Temp.
13–01	M	P	C	_	HT	A	A	25	<35
13–02	M	P	C	_	HT	A	A	25	<35
13–03	M	P	C	_	HT	A	A	25–30	<35
13–04	M	P	C	_	HT	A	A	25	<35
13–05	M	P	C	_	HT	A	A	25	<35
13–06	M	P	C	_	HT	A	A	25	<35
13–55	L	P	C	_	HT	A	A	25	<35
98–68	L	P	C	+	HT	A	A	30	<35
P. meadii	M	P/sp.	C	+/_	HO/HT	A	A	25–30	<33
P. citrophthora									
P. capsici	L	P	C	_	HT	A	A	27–33	>35
P. tropicalis	L	P	C	+	HT	A	A	27–30	<33
P. ramorum	S	SP	C	+	HT	A	P	15–21	<27

isolates were subjected to detailed genetic analysis Figs. 4, 5, 6, 7 and 8.

Molecular characterization

ITS sequencing and phylogeny

The isolates showed 900 bp amplification product and on BLAST analysis, the sequences of 13–01 to 13–06 showed similarity to *P. meadii,* while the sequences of 98–68 and 13–55 showed similarity to *P tropicalis* (http://www.q-bank.eu/Fungi) (Supplementary Table 1).

Its-RFLP

The amplified PCR product was digested with the restriction enzyme *Msp*I. Restriction digestion (RFLP) of the PCR products generated three bands in all the eight isolates with a single band exhibiting polymorphism (Fig. 9). The Neighbour Joining tree based on ITS sequences with eight *Phytophthora* isolates from nutmeg along with selected *Phytophthora* spp. showed a separate grouping for nutmeg isolates within the clade 2 along with *P. colocasiae, P.citrophthora* and *P. meadii* (Fig. 10).

MLST analysis

Using MLST analysis of seven nuclear genes, viz. *ITS, Beta-tubulin, Elongation factor, HSP90, 60SL10, Enolase and Tig A*, it was found that all the nutmeg *Phytophthora* isolates form a separate group very close to clade 2 isolates of *Phytophthora* and very close to *P.meadii.* However, analysis of 11 mitochondrial genes revealed that *Phytophthora* isolates from nutmeg form three separate groups in clade 2 indicated species diversification (Fig. 11).

SSCP analysis

Single-strand conformation polymorphism (SSCP) analysis is a powerful tool, which can detect single base mutations or variations (Orita et al. 1989; Rubio et al. 1996; Kong et al. 2000; Sambrook and Russell 2001). Single Strand Confirmation Polymorphism (SSCP) among nutmeg *Phytophthora* isolates in comparison with other *Phytophthora* isolates of the same Clade showed a characteristic banding pattern for nutmeg isolates showing the deviation of nutmeg isolates from closely related species viz. *P. meadii*, *P. citrophthora* and *P. plamivora* (Fig. 12).

Hybrid analysis

The double peaks in ITS chromatograms (Fig. 7) suggest the hybrid nature of nutmeg *Phytophthora* isolates. Furthermore, the hybrid analysis of nuclear and mitochondrial genes revealed its probable divergence from *P. citrophthora* and *P. meadii* (Cit-Med) lineage. For instance, six of the isolates (13–01–13-06) fall under P. *citrophthora* or *P. meadii lineage*. The identity analysis of ITS genes revealed the similarity of these isolates (13–01(99.32), 13–02(99.471), 13–03(99.321), 13–04(99.471), 13–05(98.284), 13–06 (99.471) to *P. meadii* lineage and other two of the isolates 13–55 (99.47) and 98–68 (99.32) to *P. tropicalis* lineage (Supplementary Table 2). Analysis of mitochondrial genes *NaD9, rps10, and SecY* also gave evidence for grouping the isolates under *P. meadii - P. citrophthora* lineage and two other isolates, viz.13–55 and 98–68, under *P.capsici/ P. tropicalis -P. citrophthora* lineage as well. Collectively the data indicated that these isolates had *P. citrophthora* as their paternal parent and *P. meadii* and *P. capsici /P. tropicalis* as the maternal parent (Table 5).

As stated by Cooke et al. (2000) and Martin and Tooley (2003), only molecular analysis can reveal the phylogenetic relationships between *Phytophthora* isolates. But in many cases, these analysis were based on sequence information of ITS or genes like cytochrome oxidase II (*Cox* II or *Cox* I). Here we employed multiple gene analysis for deciphering species boundaries among morphologically cryptic *Phytophthora* species as done by Kroon et al. (2004). So the present study was focused on characterizing the nutmeg isolates based on detailed morphological, physiological and biological approaches as well as molecular tools like ITS Phylogeny and MLST (Multi locus sequence typing) analysis. We also adopted another approach called Single-Strand conformation polymorphism (SSCP), or single-strand chain polymorphism where double peaks in the chromatogram indicate the heteozygosity of the organism (Felsenstein 1993, Oto et al. 1993). Here sequences were prominent by means of gel electrophoresis which separates fragments according to their different conformations. In the present study we analysed nutmeg isolates for double peaks in the chromatogram for finding the hybridity among the isolates. To understand ancestral relationship, it was assumed that

they are putative hybrid isolates. Based on MLST analysis, seven species from Clade2 were selected as parental species for hybrid analysis and confirmed that the nutmeg isolates were hybrids of two species from Clade 2 (Supplementary Table 4–7).

Thus both morphological and molecular characterization clearly indicated that *Phytophthora* isolates from nutmeg were very distinct from *P. ramorum*, as previously reported by Mathew and Beena (2012). The delimiting factors were:

1) The colony morphology of nutmeg isolates in carrot agar was petalloid, floral and stellate with sympodial, papillate sporangia and caducous with intermediate pedicel length of >20 μm, while *P. ramorum* is semi-papillate, caducous short pedicellate (< 5 μm) sporangia produced singly or in clusters of 2–12 on sympodially branched sporangiophores (Werres et al. 2001).
2) The average sporangial length of nutmeg isolates ranged from 20.72–36.70 μm and breadth ranged from 14.36–28.9 μm with an average l/b ratio ranging from 1.85–1.91. The shape varied from ovoid-obpyriform or elongated in contrast to *P. ramorum* where the average sporangial length ranged from 46 to 65 μm and breadth ranged from 21 to 28 μm. Sporangia formed both on agar and in water, and the shape of the sporangia varied from ellipsoid, spindle-shaped or elongate-ovoid (Werres et al. 2001).
3) The nutmeg isolates failed to grow at 10 °C or at 37 °C and additionally chlamydopsores were not ordinarily produced in culture in contrast to P. *ramorun,* where the most distinguishing characteristics are the presence of large (22 to 72 μm) terminal chlamydospores. Also, the mature chlamydospores from *P. ramorum* tend to change from hyaline to cinnamon brown which was absent altogether in nutmeg isolates. However, in both cases, the isolates were heterothallic and produced oogonia when paired with known mating type.
4) *P. ramorum* belongs to an entirely different clade (Clade 8) highly distinct from nutmeg isolates further confirming the lack of similarities between the two.
5) As stated by Werres et al. (2001) the distinctive morphological features make *P. ramorum* a fairly easy organism to identify in culture with characteristics similar to *P. palmivora* where the pedicel length is short (<5 μm). Moreover, *P. ramorum* has a unique molecular sequence at the ITS region which distinguishes it from other *Phytopthora* species (Schena et al. 2006), especially isolates from nutmeg.
6) Although morphologically similar to *P.meadii,* the nutmeg isolates were different from known *P.meadii* isolates of cocoa, cardamom and rubber. This was also supported by cross infectivity studies where the *P. meadii* isolates from cocoa, cardamom, coconut and black pepper failed to infect leaves or fruits of nutmeg showing the host specificity.

A comparative analysis performed between *Phytophthora* isolates of nutmeg and other long and medium pedicellate isolates (Table 6) with *P. ramorum* (short pedicellate) revealed distinguishing features, further asserting the fact that nutmeg isolates were distinct from *P. ramorum*. Several *Phytophthora* sp. isolated from nutmeg were reported from the Island of Grenada. This includes species such as *P. capsici, P. heveae, P. macrochlamydospora, P. nicotianae, P. palmivora, P. tropicalis*, and nine hitherto unknown species (Balci 2014). According to this report *P. palmivora* and an unknown species were closely related to *P. katsurae* but not *P. meadii*. Here we reported the presence of yet another species of *Phytophthora* for the first time from India that is related to *P. meadii*' causing defoliation and fruit fall. It is to be noted that these newly identified isolates although related to *P. meadii,* multigene analysis clearly indicate their divergence from *P. meadii*, forming a separate clade.

There are several reports of novel *Phytophthora* species isolated from irrigation reservoirs at several ornamental plant production facilities in eastern Virginia (Yang et al. 2014a). The Identification of these species was based on initial sequencing of the ITS rDNA (Yang et al. 2014b), cloning and sequencing of the ITS region and sequencing of the mitochondrially encoded cytochrome *c* oxidase 1 and beta-tubulin genes revealed its hybridity between *P.* taxon *PgChlamydo* as its paternal parent and an unknown species genetically close to *P. mississippiae* as its maternal parent. This new hybrid was named *Phytophthora×stagnum*. Similarly Abad et al. 2008 described a new species called *P. bisheria* isolated from strawberry, rose and raspberry roots in USA and Australia. *P. bisheria* is a self-fertile (homothallic), slow growing species that produces semipapillate, persistent sporangia and unique

paragynous antheridia that broadly attach to the oogonial walls. Analysis was based on sequences of the internally transcribed spacer rDNA region (ITS1–5.8S-ITS2) of this taxon and those from other *Phytophthora* species from GenBank. In the phylogenetic tree with other reported *Phytophthora* species at the GenBank, this new species was more closely related to others in ITS Clade 2 comprising semipapillate taxa. Similarly, *P. borealis* and *P. riparia*, identified from *Phytophthora* surveys of forest streams in Oregon, California and Alaska, were described as new species in *Phytophthora* ITS Clade 6 (Everett et al. 2012). They were similar in growth form and morphology to *P. gonapodyides* and were predominantly sterile. They presented unique DNA sequences but differed in temperature/growth relations and geographic distribution. *P. pluvialis* was described from Oregon, USA in 2013 where it was not associated with any disease (Dick et al. 2014). Similarly, two new species, *P. nagaii* sp. nov. and P. *fragariaefolia* sp. nov., causing serious diseases in rose and strawberry plants were reported from Japan. They were also identified in the multilocus phylogenetic tree constructed using sequences from the rDNA ITS regions, rDNA LSU, and the translation elongation factor 1-a, beta-tubulin and cox I genes. They formed a distinct monophyletic group in Clade7 with strong bootstrap support. *P. sansomeana* was detected on soybean for the first time in Wisconsin in 2012 by Wisconsin Department of Agriculture, Trade, and Consumer Protection (DATCP). *P. sansomeana* has also been reported on corn in Ohio, soybeans in Indiana, Douglas fir seedlings in Oregon, and weeds such as white clover, wild carrot, and white cockle in alfalfa fields in New York. Container-grown *Ceanothus sanguineus, C. velutinus,* and *C.integerrimus* showed leaf wilt associated with stem lesions developing above diseased roots and isolated and described the species as *P. taxon Ceanothus* (Reeser et al. 2012). The ceanothus Phytophthora produced a flame-like radiate colony reminiscent of *P. citrophthora* which produces sporangia that are papillate and caducous, with shapes ranging from ovoid to bizarre, including many bifurcate, trifurcate, and compound shapes similar to those depicted for *P. himalsilva* (Vettraino et al. 2011). Another new *Phytophthora* species was detected in the USA, infecting foliage of *Kalmia latifolia* called *P. obscura* sp. nov. It was formally named based on phylogenetic analysis, host range, Koch's postulates and morphology. It is genetically closely related to *P. syringae* and *P. austrocedrae* and together these three species define a new *Phytophthora* sub Clade 8d, (Grünwalda et al. 2012). During surveys of dying vegetation in Australia many new taxa were identified falling under *Phytophthora* ITS Clade 6. Based on phylogenetic analysis and morphological and physiological comparison, four species and one informally designated taxon were described viz. *P. gibbosa, P. gregata, P. litoralis, P. thermophila* and *P. taxon paludosa* respectively (Jung et al. 2011; Burgess 2015). They formed a new cluster and shared a common ancestry. Among them, *P. thermophila* and *P. litoralis* was sister species to each other and more distantly related to *P. gonapodyides.* Yet another species *P. alni* isolated from riparian alder's stands in Europe was responsible for the devastating losses. An emergent hybrid pathogen with multiple variants has been placed in subspecies. *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*. In April 2011, a survey along the Deza River in Galicia was carried out to clarify the *Phytophthora* sp. associated with the alder decline. This was the first time that *P. alni* subsp. *uniformis* has been reported from Spain.

Such identification of new sub-species in a region could result in hybridization between individuals of different species or sub species which may progressively allow the rapid evolution and adaptation of such species to new hosts or environmental conditions (Varela et al. 2012). In view of this, the new *Phytophthora* sub-species that we reported in this study raises serious concern with respect to adaptability and reach in infecting new crops other than nutmeg and requires posible quarantine.

Collectively in MLST analysis nutmeg isolates (13–01 to 13–06) showed close genetic similarity to *P.meadii* and *P.citrophthora* but also formed a separate clade showing its deviation from both, indicating that these isolates were species hybrids of *P. meadii* and *P.citrophthora*. (Sansome et al. 1991) This is supported by mmorphological dissimilarity from *P. meadii* due to the absence of hyphal swellings and junction thickenings at the point of origin of the sporangiospores as observed in the *P. meadii* isolates of cardamom. Hence, we proposed the names of these isolates having *P. citrophthora X P. meadii* hybridity as *Phytophthora citromeadii* sp.de novo. Similarly isolates 13–55 and 98–68 with *P. citrophthora* and *P. tropicalis* as their paternal parent and *P. capsici* as their maternal parent (Supplementary Table 3–6) and named as *P. citrocaptalis* sp.de novo.

Taxonomic description of nutmeg *Phytophthora* isolates

P. Citromeadii

The colony morphology of nutmeg *Phytophthora* isolates (13–01,13–02, 13–03, 13–04, 13–05, 13–06) in carrot agar is petalloid, floral and stellate; sporangia sympodial, papillate, and caducous with intermediate pedicel length (>20 μm). The average sporangial length varied from 20.72–36.70 μm and breadth from 14.36–28.9 μm with average l/b ratio ranging from 1.85–1.91. The shape varied from ovoid-obpyriform or elongated. Chlamydopsores were not ordinarily produced in culture and isolates were heterothallic and produced oogonia when paired with known mating type. The optimum temperature required for growth was 25–30 °C. and minimum growth occurred at 15 °C and maximum growth at 30 °C. None of the isolates grew at 10 °C or at 35 °C.

P. Citrocaptalis

The colony morphology of nutmeg isolates (13–55 and 98–68) in carrot agar is floral and stellate. The sporangial ontogeny is umbellate to sympodial, long pedicellate with tapered base. The pedicel length vary from 11.57–200.97 to 3.67–130.9 μm. The average sporangial length varied from 22.32–44.64 μm and width varied from 14.88–34.72 μm. The average length/width ratio is 1.2–2.37. Chlamydospores are occasionally produced in culture. The isolates are heterothallic and produced oogonia when paired with known A2 mating type. The optimum temperature required for growth is 27–33 °C and the minimum growth occurred at 15 °C and maximum at 30 °C. None of the isolates grew at 10 °C or at 35 °C.

Conclusion

Based on the detailed investigations on morphological, biological and molecular analysis, it became clear that nutmeg *Phytophthora* isolates are very distinct and have no similarity with *P. ramorum*. The isolates showed different morphological and molecular identity and could be given the status of a new species(s). All the morphological and molecular data clearly delineated nutmeg *Phytophthora* isolates from *P. ramorum* as described above. Hence *Phytophthora* isolates from nutmeg formed a separate group and are species hybrids of *P. citrophthora* X *P. meadii* (13–01 to 13–06) and called as *P. citromeadii* and of *P. citrophthora* X *P. tropicalis* (98–68 and 13–55) called as *P. citrocaptalis*.

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Conflict of interest The authors declare that they have no conflict of interest.

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