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Original article

Multigene phylogeny and haplotype analysis reveals predominance of oomycetous fungus, *Phytophthora meadii* (McRae) associated with fruit rot disease of arecanut in India

Balanagouda Patil [a,b], Vinayaka Hegde [b], Shankarappa Sridhara [c], R. Thava Prakasa Pandian [d], Shivaji H. Thube [d], Gangaraj Karyath Palliath [b], Sunil S. Gangurde [e,f], Prakash Kumar Jha [g,h,*]

[a] Department of Plant Pathology, University of Agricultural and Horticultural Sciences, Shivamogga, Karnataka 577255, India
[b] Division of Crop Protection, ICAR-Central Plantation Crops Research Institute, Kasaragod, Kerala 671124, India
[c] Center for Climate Resilient Agriculture, University of Agricultural and Horticultural Sciences, Shivamogga, Karnataka 577255, India
[d] Division of Crop Protection, ICAR-Central Plantation Crops Research Institute, Regional Station, Vittal, Karnataka 574243, India
[e] International Crops Research Institute for Semi-Arid Tropics, Patancheru, Hyderabad, Telangana 502324, India
[f] Crop Genetics and Breeding Research Unit, University of Georgia, Tifton, GA 31793, USA
[g] Sustainable Intensification Innovation Lab, Kansas State University, Manhattan, KS 66506, USA
[h] Department of Agronomy, Kansas State University, Manhattan, KS 66506, USA

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ABSTRACT

An oomycetous fungus *Phytophthora* causing fruit rot is the most devastating disease of arecanut in different agro-climatic zones of Karnataka with varied climatic profiles. The main aim of this investigation was to characterize the geo-distant *Phytophthora* populations infecting arecanut using robust morphological, multi-gene phylogeny and haplotype analysis. A total of 48 geo-distant fruit rot infected samples were collected during the South-West monsoon of 2017–19. Pure culture of the suspected pathogen was isolated from the infected nuts and pathogenic ability was confirmed and characterized. Colony morphology revealed typical whitish mycelium with stellate or petalloid pattern and appearance with torulose hyphae. Sporangia were caducous, semipapillate or papillate, globose, ellipsoid or ovoid-obpyriform in shape and sporangiophores were irregularly branched or simple sympodial in nature. Subsequent multi-gene phylogeny (ITS, β-tub, TEF-1α and Cox-II) and sequence analysis confirmed the identity of oomycete as *Phytophthora meadii* which is predominant across the regions studied. We identified 49 haplotypes representing the higher haplotype diversity with varying relative haplotype frequency. Comprehensive study confirmed the existence of substantial variability among geo-distant populations (n = 48) of *P. meadii*. The knowledge on population dynamics of the pathogen causing fruit rot of arecanut generated from this investigation would aid in developing appropriate disease management strategies to curtail its further occurrence and spread in arecanut ecosystem.
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1. Introduction

Arecanut (*Areca catechu* L.) is a tropical palm that plays a significant role in providing livelihood, and nutritional security to millions of marginal farmers in India (Balanagouda et al., 2021). India stands first in the world with respect to area (5,12,266 ha) and production (8,27,639 t) of arecanut contributing 49% and 50%, respectively. Karnataka, Kerala, West Bengal, and Assam are the major arecanut producing states in India which share more than 70% of production (Mitra and Devi, 2018). Among the various production constraints, fruit rot disease (FRD) incited by *Phytophthora meadii* (McRae) is the most devastating disease resulting in economic losses ranging from 10 to 90% (Bavappa, 1963; Jose

* Corresponding author.
E-mail addresses: balupat007@gmail.com (B. Patil), vinayaka.hegde@icar.gov.in (V. Hegde), sridharas1968@gmail.com (S. Sridhara), r.pandian@icar.gov.in (R. Thava Prakasa Pandian), shivaji.thube@icar.gov.in (S.H. Thube), gangarajkp@gmail.com (G. Karyath Palliath), sgangurde40@gmail.com (S.S. Gangurde), pjha@ksu.edu (P.K. Jha).

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et al., 2008; Chowdappa et al., 2014; Sarma et al., 2002; Prathibha et al., 2019).

In India, FRD is more predominant in the South (Western-Ghats and foothills) which is a major area of arecanut cultivation. The devastating FRD epidemics are common in traditional arecanut-growing ecosystems of Karnataka during South-West monsoon inflicting huge economic losses (Coleman, 1910; Chowdappa et al., 2003; Jose et al., 2008). The cultivation of arecanut in Karnataka has expanded over the last few decades to non-traditional areas (DASD, 2019) in different agro-climatic zones and concurrent occurrence of FRD with varying intensity has been noticed in these areas. Hence, the arecanut FRD prevailing areas in Karnataka can be grouped into three major tracts *viz.,* Malnad, Coastal and Maidan regions. The Malnad (gateway of Western-Ghats) and Coastal (seashore) regions are traditional arecanut cultivating tracts receiving annual rainfall ranging from 2500 mm to 5000 mm. Whereas, the Maidan (transitional) region where arecanut cultivation expanded in the last few decades receives < 1000 mm rainfall annually. Though different species of *Phytophthora* such as *P. palmivora* (Das and Cheeran, 1986), *P. meadii* (Sastry and Hedge, 1985), *P. heveae* (Chowdappa et al., 2002) and *P. arecae* (Pethybridge, 1913) were reported earlier as the causal agents of FRD of arecanut, but *P. meadii* has been identified as the most prevalent species causing fruit rot of arecanut based on restriction fragment length polymorphism (RFLP) studies (Chowdappa et al., 2003). No species dominance studies were conducted in traditional and non-traditional areas and are considered as most identified research gap.

Previously, the characterization, variability and diversity analysis of *P. meadii* has been studied by employing ITS-RAPD (Internal Transcribed Spacer-Randomly Amplified Polymorphic DNA) and RFLP markers to determine genetic variation among *Phytophthora* isolates collected from South Canara, Shivamogga infecting arecanut, rubber (*Hevea brasiliensis* L.) and cardamom (*Elettaria cardamomum* L.) with identical patterns among isolates which predicted the presence of *P. meadii* on arecanut (Chowdappa et al., 2003). To overcome the taxonomic conflicts, numerous *Phytophthora* species have been identified through morphological, molecular and diversity analysis employing microsatellite profiles which evidenced that molecular phylogenies can provide better identification of the pathogen (Prathibha et al., 2020). In order to understand the complexity and differentiate *Phytophthora* on arecanut sampled from North and South Canara districts of Karnataka state, a high-resolution melting (HRM) analysis was performed which revealed *P. meadii* on arecanut with some intra-specific variations (Prathibha et al., 2019). Molecular approaches integrated with phenotypic studies have demonstrate best strategy for identification of *P. capsici* on black pepper (*Piper nigrum* L.; Truong et al., 2010), *P. palmivora* on cocoa (*Theobroma cacao* L.; Chowdappa, 1995), *P. palmivora* on coconut (*Cocos nucifera* L. Sharadraj, 2010) and *P. colocasiae* in taro (*Colocasia esculenta* L.; Nath et al., 2015). However, morphological characterization has been mostly utilized for the identification of *P. meadii* in Karnataka (Saraswathy, 1994; Chowdappa, 2000). However, most of the prior research lacked a combinatorial approach to analyze phenotypic and molecular diversity which can provide most precise and useful information on the pathogen biology/life cycle.

Understanding the variability and dynamics of the pathogenic populations is of utmost importance to develop an eco-friendly and effective disease management strategies. Previous reports have established the taxonomy and variability of *P. meadii* to a limited extent and needs attention. To date, there are no reports on population dynamics and species complexity of fruit rot pathogen in different agro-climatic zones of Karnataka where the arecanut cultivation has expanded. A detailed study of the pathogenic variability or population dynamics of arecanut fruit rot pathogen across the varied agro-climatic zones of Karnataka will help in understanding the changes in epidemiology, host-pathogen interaction, and pathogen management.

Therefore, the objective of this study was to determine the extent of morphological, molecular variability and dynamics among the populations of *P. meadii* collected from varied agro-climatic zones over different growing seasons in Karnataka. To the best of our knowledge, this is the first intensive study where morphological and molecular approaches are integrated to characterize and analyze to understand the diversity including regional occurrence of the pathogen across studied regions. The generated information would provide a better understanding of the pathogen and thereby, aid in developing effective management approaches for varied agro-climatic zones.

2. Materials and methods

2.1. Sampling area and isolation of the pathogen

The fruit rot infected immature arecanut samples (n = 48) were collected from major growing agro-climatic zones of Karnataka, India *viz.*, Malnad (Shivamogga, Chickmagalur and parts of North Canara), Coastal (South Canara and Udupi) and Maidan (Davanagere, parts of Shivamogga and Chickmagalur) regions during 2018 to 2019 (Fig. 1). Isolation from naturally obtained symptomatic arecanut samples (n = 48) was attempted. Sampled nuts were cut into tiny pieces, thoroughly washed with tap water, surface-sterilized with 2 percent NaOCl for 60 s followed by thrice rinsed in distilled water, and air-dried. Sterilized portion of infected tissue was kept on 20% carrot agar (CA,) plates and incubated at 24 ± 2 °C for 4–6 days (Ribeiro, 1978) and used for further analysis.

2.2. Morphological identification

The phenotypic and colony characteristics of one-week-old cultures of isolates (n = 48) were recorded under stereomicroscopy (Nikon E100, Japan) at 40x magnification. Colony characters of the collected fungal isolates were observed on CA (Carrot Agar) medium and the patterns of growth with other features was documented. The size and shape of sporangia (n = 25) were recorded with other characters *viz.,* ramification of sporangiophores, caducity, papillae and more. All isolates were phenotypically identified on the basis of colony and sporangial characters (Erwin and Ribeiro, 1996) by comparing with the lineage standard isolate (CPCRI Pm1) of *P. meadii* maintained at Culture Repository, Central Plantation Crops Research Institute (CPCRI), Kasaragod, Kerala, India.

2.3. Cultural characterization

The morphology of colonies was analyzed on three media: potato dextrose agar (PDA; 250 g l^{-1} potato, 20 g l^{-1} dextrose and 20 g l^{-1} agar), Carrot Agar (CA; 250 g l^{-1} carrot juice and 20 g l^{-1} agar), and V8 (V8 juice 200 ml l^{-1}, $CaCO_3$ 2 g l^{-1}, 15 g l^{-1} agar). A 5 mm disc was taken from the active colony edge was kept at the center of Petri dishes with the above-mentioned media and then incubated at 24 ± 2 °C with 95% humidity for 5–7 days. The morphology of isolates was determined on the basis of mycelial texture and three replicates were maintained for each isolate. However, CA medium supports good mycelial growth and sporangial production, further comprehensive studies were performed in CA medium, and the colony growth rate of each isolate was recorded (mm per day). To study the effect of temperature

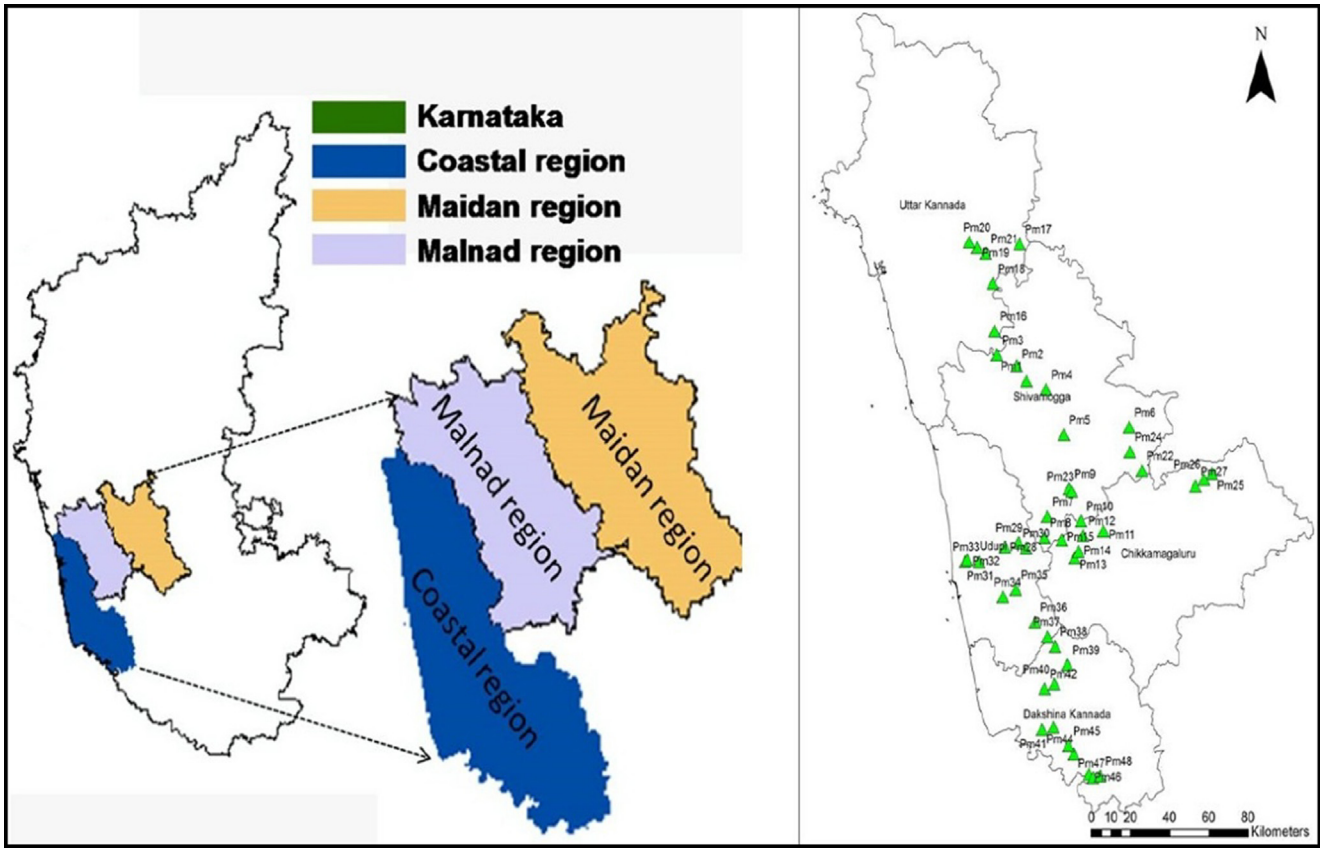

Fig. 1. Location map showing selected regions for investigation (left) and *Phytophthora* geo-distant isolates (n = 48) sampled from arecanut growing varied agro-climatic zones of Karnataka, India (right).

on various growth characteristics, isolates were incubated at 15, 20, 25, 28, 30, 35, 40 °C in triplicates.

2.4. Mating type determination

The mating type of each isolate was determined according to the method described by Chowdappa and Chandramohanan (1997). The mating test of isolates were analyzed by pairing each obtained isolate with a known isolate (Isolate IISR, related species *P. capsici* A2 mating type) on carrot agar (CA) medium at 5 cm apart. After incubation at 28 °C in the dark for 4 weeks, agar discs were examined microscopically. The absence of oospores at the interface between colonies was indicated the same mating type, whereas the presence of oospores was indicated opposite mating type. The solo culture of each isolate was examined for oospore formation as a control. Three replicates were used for each isolate.

2.5. Moleular confirmation through multigene analysis

17 representative isolates were selected from three varied agro-climatic zones of Karnataka, India based on morpho-metrical types and colony groups for molecular characterization and phylogenetic analysis. Eight Malnad (P3, P4, P9, P10, P12, P15, P18 and P21), one Maidan (P43) and eight Coastal (P25, P28, P31, P32, P37, P39, P40, and P41) isolates were molecularly characterized.

2.5.1. DNA extraction, amplification, and sequencing

Following manufacturer's instructions, the total genomic DNA was isolated by using DNeasy plant Mini DNA purification kit (Product code- 69204, QIAGen, USA). The relative quantity and quality of genomic DNA was measured spectrophotometrically (Nanodrop ND-100, Thermo Fisher Scientific Inc., Waltham, MA, USA) and electrophoretically on agarose gel (0.8%), respectively. The descriptions of the genetic regions with primer sequences and PCR setup are listed in Table 1. PCR was performed in T100™ thermal cycler (Bio-Rad, USA) for 20 µL reaction volumes consist of 80 ng genomic DNA and ready to use Red Dye PCR Master mix (Genei, Bangalore, India).

The amplified products were diluted with 1.5% agarose gel in Tris-Borate-EDTA (TBE) buffer containing 0.5 µg ml^{-1} ethidium bromide and amplification was detected by Gel Doc System (Alpha Imager, Alpha Innotech, USA). The amplified products obtained were cleaned using a Nucleospine® PCR clean up and purification kit (product code- 740606.50, Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions and sequenced using a service provider (AgriGenome Labs Pvt Ltd, Kochi, Kerala, India).

2.5.2. Sequence alignment and phylogenetic analysis

The nucleotide sequences obtained in this study were analyzed to remove amalgamated primer sequences and low-quality readings, converted to consistent sequences with Geneious Pro software version 5.6. The resultant consensus sequences were aligned with additional accessions obtained from GenBank using BLASTn program (https://blast.ncbi.nlm.nih.gov) to confirm the authenticity of the isolates. In the event, all sequences of *P. palmivora, P. capsici, P. nicotianae, P. infestans*, *P. colocasiae,* and *P. citrophthora* available in GenBank were retrieved for confounding of species complexity as out grouping. The multiple sequence alignments were performed using the Clustal W module (Thompson et al., 1994) and aligned sequences were trimmed using TrimAl. The trimmed alignment sequences of four genes were concatenated using Molecular Evolutionary Genetic Analysis (MEGA) soft-

Table 1
Details of various genes, primer sequences, polymerase chain reaction (PCR) conditions, amplicon size and references used in the study.

Locus	Primer Sequence (5′-3′)	PCR Cycle	Size of Amplicon	Reference
Beta-tubulin (β-tub)	Btub-F1 5′-GCCAAGTTCTGGGAGGTCATC −3′ Btub-R1 5′-CCTGGTACTGCTGGTACTCAG −3′	(94 °C: 30 s, 60 °C: 30 s, 72 °C: 1 min) × 35 cycles	1250 bp	Martin and Tooley (2003)
Translation elongation factor-1-alpha (TEF-1α)	EF1A-for 5′-TCACGATCGACATTGCCCTG −3′ EF1A-rev 5′-ACGGCTCGAGGATGACCATG −3′	(94 °C: 30 s, 60 °C: 30 s, 72 °C: 1 min) × 35 cycles	970 bp	Kroon et al. (2004)
Internal Transcribed Spacer (ITS)	ITS45′- TCCTCCGCTTATTGATATGC-3 ITS65′-GAAGGTGAAGTCGTAACAAGG −3′	(95 °C: 30 s, 55 °C: 30 s, 72 °C: 1 min) × 35 cycles	900 bp	White et al. (1990);Cooke et al., (2000)
Mitochondrial cytochrome *c* oxidase subunit II (*Cox-II*)	FMPhy-8b 5′-AAAAGAGAAGGTGTTTTTTATGGA-3′ FMPhy-10b 5′-GCAAAAGCACTAAAAATTAAATATAA-3′	(95 °C: 2 min, 56 °C: 1 min, 72 °C: 2 min) × 35 cycles	825 bp	Kroon et al. (2004)

ware 7.0 × version (Tamura et al., 2013) and phylogenetic analyses was done by the Neighbour-Joining (NJ) method (Saitou and Nei, 1987) with Jukes Cantor substitution model in MAFFT (Multiple Alignment using Fast Fourier Transform). The robustness of the tree topology was assessed by bootstrap analysis using 1000 repetitions (Tamuraet al., 2011).

2.6. SNP calling and haplotype analysis

Haplotype analysis for each gene was carried out by aligning the nucleotide sequences of representative isolates (n = 17) of *P. meadii*. MUSCLE (Multiple Sequence Comparison by Log-Expectation) tool was used for multiple sequence alignment (Edgar, 2004). MUSCLE has high accuracy and faster than ClustalW2.The alignment file generated in FASTA format in MUSCLE was used as an input for calling polymorphic sites (SNPs/InDels) for each gene. SNPs called for each gene was used as input for the haplotype analysis in Haploview (Barrett et al., 2005). The Haplophyle program based on the "Median-Joining Network" algorithm linked to the Graph *viz.*, a graph display tool was used to create a synthetic neural network. The sizes of the circles to be represented are equal to haplotype frequency, and the lengths of the connection lines are equal to the number of mutational steps between haplotypes. Linkage disequilibrium (a genetic linking structure) plot for each gene was constructed at Haploview.

2.7. Virulence assay

Virulence assay was preceded with *Phytophthora* isolates (n = 48) to understand the pathogenic fitness/aggressiveness on tender green arecanut (Four months old) as described by Prathibha et al. (2020). Virulence assay was accomplished by inoculating the pathogen zoospore suspension (1×10^6 zoospores ml^{-1}) on surface-sterilized (70% ethanol) healthy, green, tender detached arecanut and sterile distilled water (SDW) inoculated nuts were considered as control. Inoculated nuts were kept in a plastic container to retain humidity and stored in an incubator at 22 ± 2 °C with relative humidity of 95% for 5 days. Five replicates were kept in a completely randomized design, and the trial was tripled. The identity of the pathogen re-isolated from the inoculated nuts showing typical symptoms like appearance of water-soaked lesions near perianth region, presence of whitish mycelial growth, mummifying of fruits during advance stage of infection due to pathogen was confirmed rigorously through molecular characterization.

2.8. Statistical analysis

The data on culture characteristics and virulence assay of *P. meadii* isolates were analyzed by one way analysis of variance (ANOVA) and the mean data were compared by Duncan's Multiple Range Test (DMRT) using SPSS (version17. 0; SPSS, Inc., Chicago, IL, USA). Probability (*P*) level of 0.05 was set for analyzing the critical difference among the isolates in each treatment, and values $P \leq 0.05$ were considered significantly different. Phenotypic correlation and frequency distribution was analyzed for all morphological characters of 48 isolates and analysis was performed in R software (Version, Ri3864.0.5, R core team, USA). Grouping of isolates (n = 48) was performed based on morphological components by analyzing Principal Component Analysis (PCA) through GenStat software (version, 19.1, VSN international, UK).

3. Results

3.1. Symptomatology and fungal isolation

The fruit rot disease (FRD) of arecanut is characterized by rotting and extensive falling of the immature nuts scattered near the base of the palm. The first symptom appeared as dark green/yellowish watery lesions in the nut area near the perianth (calyx). Later, fruits sores gradually spread throughout the area before or after dropping which consequently rot. A pile of white mycelial mass covered the entire surface of the fallen nuts and as the disease progressed, the fruit the fruit stalks and the axis of the inflorescence were rotten and dried. A total of 48 *Phytophthora* isolates (denoted as P1 to P48 and standard isolate CPCRI Pm1) were obtained from the samples collected from varied agro-climatic zones (n = 3) of Karnataka. Out of 48 isolates, 21 isolates each were sampled from Malnad (P1 to P21) and Coastal (P22 to P42) regions which characterized as fruit rot prevalent, traditional, and regular occurring areas, whereas six isolates were sampled from Maidan region (P43 to P48) that characterized the non-traditional, and sporadic nature of the disease (Table 2).

Table 2
Passport data of *P. meadii* geo-distant isolates (n = 48) obtained from the FRD affected arecanut samples in Karnataka during the study.

Isolate ID	Cultivars	Place of Collection	Longitude	Latitude
P1	Sagara local	Varadamula, Sagara	75.03°N	14.11°E
P2	Sagara local	Galipura, Sagara	74.99°N	14.16°E
P3	Sagara local	Talaguppa, Sagara	74.90°N	14.21°E
P4	Thirthahalli local	Hebbailu, Hosanagara	75.12°N	14.08°E
P5	Sagara local	Hugudi, Hosanagara	75.20°N	13.90°E
P6	Thirthahalli local	Nagara, Hosanagara	75.51°N	13.93°E
P7	Thirthahalli local	Nalooru, Thirthahalli	75.13°N	13.59°E
P8	Thirthahalli local	Agumbe, Thirthahalli	75.12°N	13.51°E
P9	Thirthahalli local	AHRS, Thirthahalli	75.23°N	13.69°E
P10	Thirthahalli local	Bandigadi, Koppa	75.28°N	13.57°E
P11	Thirthahalli local	Balagaru, Koppa	75.39°N	13.53°E
P12	Thirthahalli local	Sooraly, Koppa,	75.29°N	13.51°E
P13	Thirthahalli local	AHRS, Sringeri	75.25°N	13.43°E
P14	Thirthahalli local	Torehadlu, Sringeri	75.27°N	13.45°E
P15	Thirthahalli local	Bolur, Sringeri	75.20°N	13.50°E
P16	Sagara local	Akkunji, Siddapura	74.89°N	14.30°E
P17	SAS-1	Hosalli, Siddapura	75.00°N	14.63°E
P18	Sagara local	Kalgadde, Siddapura	74.88°N	14.48°E
P19	Sirsi local	Halalla, Sirsi	74.81°N	14.62°E
P20	SAS-1	Balegadde, Sirsi	74.77°N	14.64°E
P21	Sirsi local	Vanalli, Sirsi	75.81°N	13.70°E
P22	Mangala	Santhekatte, Hebri	74.93°N	13.47°E
P23	Mangala	Kucchuru, Hebri	75.03°N	13.49°E
P24	South Kanara local	Seethanadi, Hebri	75.13°N	13.47°E
P25	Sumangala	AHRS, Brahmavara	74.75°N	13.42°E
P26	Mohitnagar	Chantaru, Brahmavara	74.76°N	13.42°E
P27	South Kanara local	Pethri, Brahmavara	74.82°N	13.41°E
P28	Mohitnagar	Bailur, Karkala	74.92°N	13.28°E
P29	Sreemangala	Ajekar, Karkala	74.98°N	13.31°E
P30	Mangala	Nallur, Karkala	75.07°N	13.18°E
P31	Mohitnagar	Naaravi, Belthangady	75.13°N	13.13°E
P32	South Kanara local	Kutluru, Belthangady	75.16°N	13.09°E
P33	Sumangala	Kapinadka, Belthangady	75.22°N	13.02°E
P34	South Kanara local	Punjalakatte, Bantwal	75.16°N	12.94°E
P35	Mangala	Vittal, Bantwal	75.10°N	12.77°E
P36	Sreemangala	Madva, Bantwal	75.12°N	12.93°E
P37	Mohitnagar	Kabaka, Puttur	75.16°N	12.78°E
P38	South Kanara local	Ariyadaka, Puttur	75.25°N	12.68°E
P39	Mangala	Aryapura, Puttur	75.23°N	12.71°E
P40	South Kanara local	Kanakamajjalu, Sullia	75.32°N	12.60°E
P41	South Kanara local	Jalsoor, Sullia	75.34°N	12.58°E
P42	Mangala	Davsuru, Sullia	75.37°N	12.45°E
P43	Maidan local	Umblebailu, Shivamogga	74.85°N	14.60°E
P44	Maidan local	Mandagadde, Shivamogga	75.57°N	13.76°E
P45	Maidan local	Gajanur, Shivamogga	75.24°N	13.68°E
P46	Tarikere tall	Vittlapura, Tarikere	75.52°N	13.82°E
P47	Tarikere tall	Nagenahalli, Tarikere	75.89°N	13.75°E
P48	Tarikere tall	Amruthapura, Tarikere	75.85°N	13.73°E

3.2. Morphological and cultural characterization

After a comprehensive morphological analysis, isolates (n = 48) were classified into six morphological groups based on the morphological, cultural and virulence nature (Tables 3 and 4; Figs. 2 and 3). The significant variation was observed among the quantitative characters such as sporangium length, sporangium breadth, L/B ratio, pedicel length and colony diameter (Fig. 3). Morphological characteristics of *P. meadii* were obviously different and are provided below in detail.

Morpho group A: Colonies on CA were stellate pattern with well-diffused margins; papillate, caducous sporangia, ovoid and

Table 3
Features of various isolates (n = 48) of *P. meadii* morpho groups identified in this study.

Morpho Group[a]	Sporangial Shape	No. of isolates	Sporangial Dimensions (µm)[b]		Pedicel Length (µm)	Ontogeny
			Mean Length (L)	Mean Breadth (B)		
Group A	Ovoid	15	37.80 ± 0.11[a*]	30.83 ± 0.16[d]	1.70–5.90	Simple Sympodial
Group B	Ellipsoid	8	41.76 ± 0.14[b]	32.26 ± 0.17[e]	1.25–4.73	Irregular
Group C	Globose to ovoid	7	41.80 ± 0.11[b]	32.20 ± 0.11[e]	2.33–7.86	Irregular
Group D	Ovoid-Obpyriform	10	52.60 ± 0.23[e]	27.70 ± 0.15[c]	3.70–5.90	Simple Sympodial
Group E	Lemoniform	3	47.26 ± 0.14[c]	32.26 ± 0.14[b]	Absent	Irregular
Group F	Obpyriform	5	49.66 ± 0.17[d]	19.70 ± 0.17[a]	3.54–4.10	Irregular

[*] Values represent the mean ± SE of three replicates. Duncan's multiple range tests at $P \leq 0.05$ showed that the mean values in the same column followed by the same letter are not substantially different.
[a] Based on colony appearance on Carrot Agar (CA) medium; [b] HAI = Hours after inoculation.

Table 4
Grouping of *P. meadii* isolates (n = 48) causing fruit rot of arecanut based on cultural characteristics.

Colony Group[a]	Colony Pattern	No. of isolates	Chlamydo-Spores	Hyphal swelling	Growth rate (mm day $^{-1}$)	Lesion diameter 72 HAI (cm)[b]
Group A	Stellate	13	Abundant	Absent	5.66 ± 0.03^{e}	1.66 ± 0.05^{d}
Group B	Stellate striated	10	Abundant	Absent	5.73 ± 0.03^{d}	0.68 ± 0.00^{a}
Group C	Petaloid	8	Moderate	Absent	5.43 ± 0.03^{c}	1.16 ± 0.05^{c}
Group D	Plain with irregular concentric rings	11	Absent	Absent	5.23 ± 0.03^{b}	0.98 ± 0.05^{c}
Group E	Chrysanthemum (Floral)	3	Moderate	Absent	5.03 ± 0.03^{a}	1.33 ± 0.05^{b}
Group F	No definite pattern	4	Moderate	Absent	5.10 ± 0.00^{a}	1.80 ± 0.10^{e}

*Values are the mean ± SE of three replicates. Mean values in the same column followed by the same letter are not significantly different according to Duncan's multiple range tests at $P \leq 0.05$.
[a] Based on colony appearance on Carrot Agar (CA) medium; [b] HAI = Hours after inoculation.

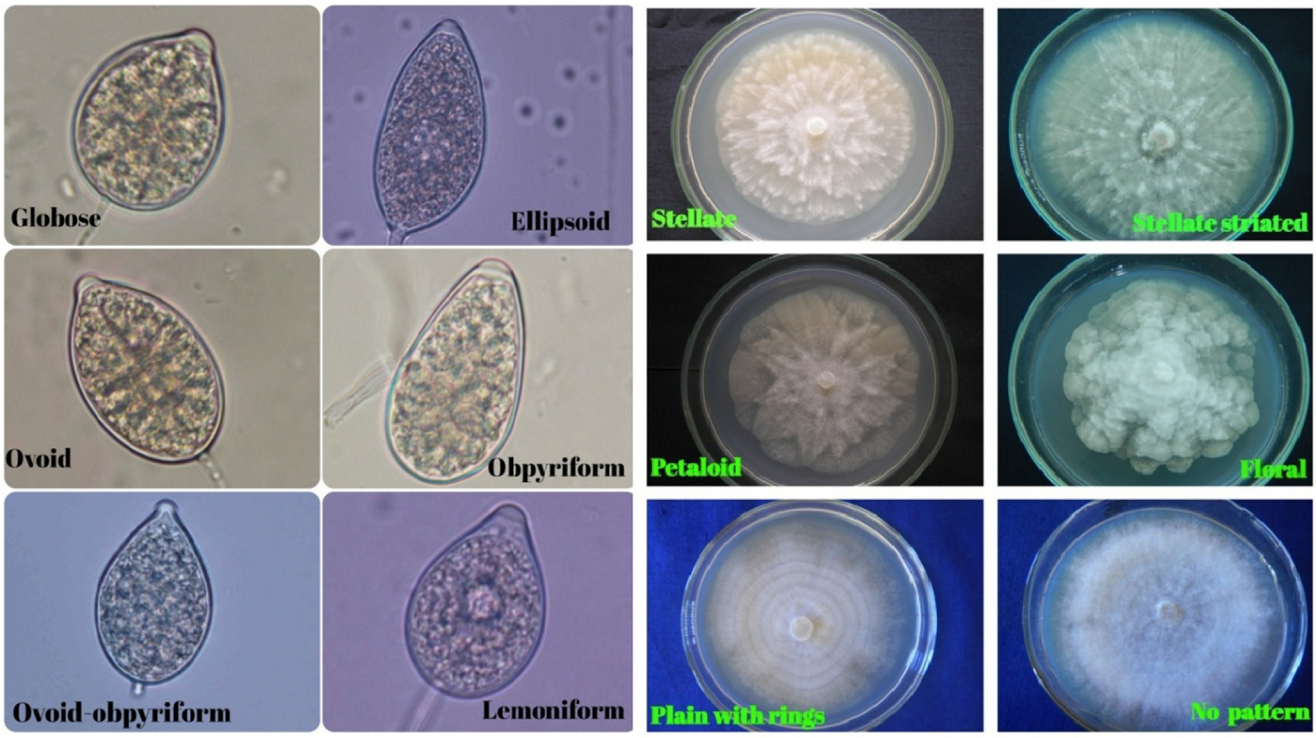

Fig. 2. Variations in sporangial and colony morphology among *P. meadii* geo-distant isolates (n = 48) infecting arecanut.

occasionally globose or ellipsoid (n = 25) with narrow to broad pedicel length and grew at 5.66 mm/day at 25 ℃. Sporangia were 36.19–39.84 × 25.45–36.83 µm (n = 25) sized, L/B ratio 1.20 × 1.38 (n = 25) and pedicel length 1.70–5.90 µm (n = 25). Sporangiophores were irregular or simple sympodial ontogeny, abundant chlamydospore production without hyphal swelling and lesion diameter of 1.66 ± 0.05 cm.

Morpho group B: Colonies on CA medium were woolly and loose towards center and sparse in the periphery with more or less stellate striated pattern and grew at 5.73 mm/day at 25 ℃. Sporangia were caducous, ellipsoidal and occasionally ovoid or ellipsoidal (n = 25) with distinct and prominent papillae, 40.80–46.40 × 31.50–35.40 µm (n = 25) sized, L/B ratio varied from 1.33 to 2.03 (n = 25), irregular or simple sympodial ontogeny, abundant chlamydospore production without hyphal swelling and lesion diameter of 0.68 ± 0.00 cm.

Morpho group C: Abundant aerial mycelium production with dense growth along the edge of petalloid pattern on CA medium and grew at 5.43 mm/day at 25℃. Sporangia were caducous with broad pedicel (average 4.80 µm), globose to ovoid (n = 25), semi-papillate, size of 47.62–49.96 × 31.38–33.48 µm (n = 25) sized, L/B ratio 1.82 (1.77–1.87) (n = 25); moderate chlamydospore production with lesion diameter of 1.16 ± 0.05 cm.

Morpho group D: Colonies on CA appeared plain with irregular concentric rings; uniform, well defined and sharp margin, moderate and adpressed as a thin layer and grew 5.23 mm/day at 25 ℃. Sporangia were papillate, Ovoid-Obpyriform (n = 25), caducous with broad pedicel length (3.7–5.9 µm), size of 46.19–59.84 × 25.45–36.83 µm (n = 25) sized, L/B ratio was 1.40 (n = 25); non chlamydospore producers without hyphal swelling and with lesion diameter of 0.98 ± 0.05 cm.

Morpho group E: The colony appeared as floral or chrysanthemum pattern, uniformly abundant, raised, flocculose, woolly and loosely interwoven and grew 5.03 mm/day at 25 ℃. Sporangia were semi-papillate, caducous, lemoniform but occasionally ovoid (n = 25) without pedicel; size about were 45.80–50.40 × 31.50–35.40 µm (n = 25) sized, L/B ratio was 1.21–1.83 (n = 25); moderate chlamydospore producers without hyphal swelling and with lesion diameter of 1.33 cm and only three isolates were categorized in this group.

Morpho group F: Isolates which did not show any definite pattern of colony growth on CA medium, abundant and flocculose and

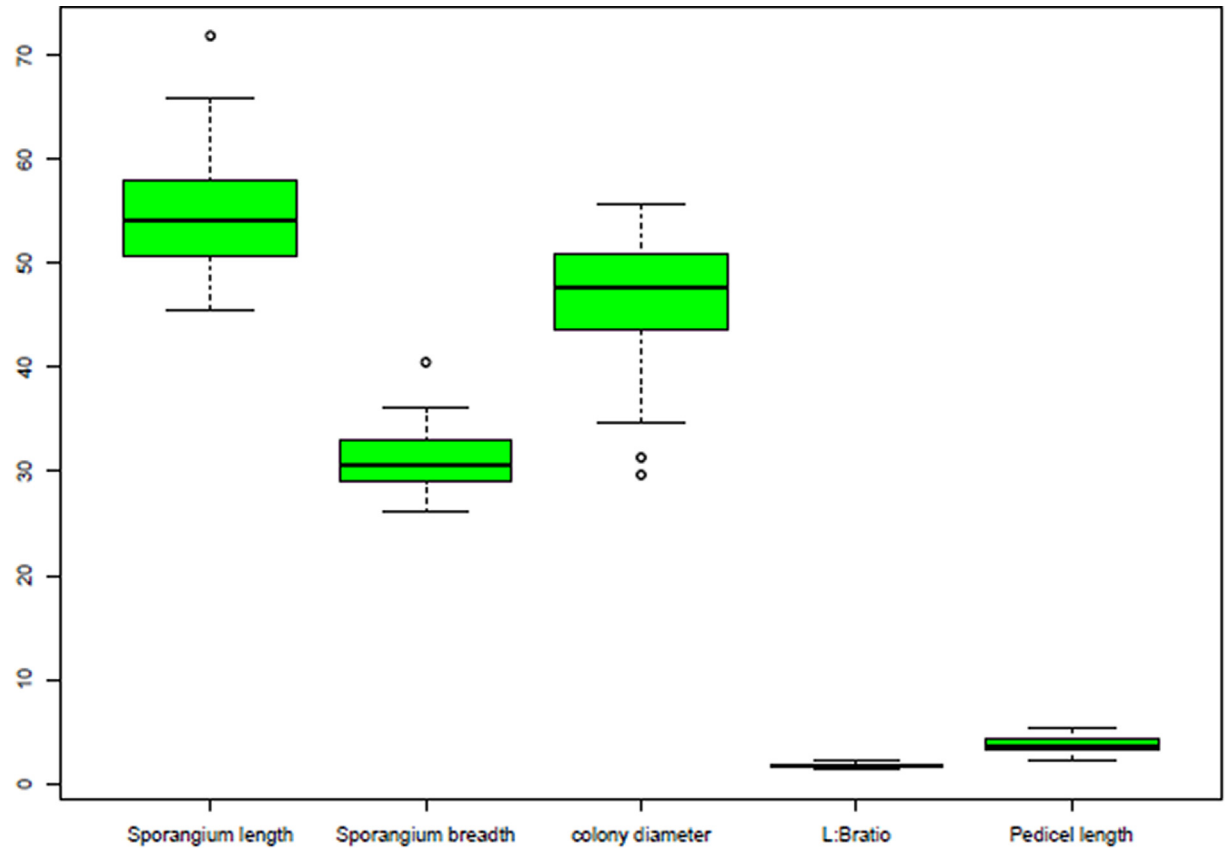

Fig. 3. Box plots representing significant variation among quantitative features of *P. meadii* isolates (n = 48). The bottom limit of the box indicating median (black line in the box is the means) denotes standard deviation, while the upper boundary shows the 95% of occureences.

grew 5.10 mm/day at 25 ℃. Sporangia were caducous, obpyriform (n = 25), semi-papillate with medium pedicel 3.54–4.10 μm (n = 25); 47.62–49.96 × 17.38–23.48 μm (n = 25) sized, L/B ratio 1.62 (n = 25) with lesion diameter of 1.80 ± 0.10 cm and four isolates exhibited similar morphology.

Cultural characterization indicated considerable differences between the *P. meadii* isolates (n = 48), and colony morphology varied on different media used. Based on the variation of the colony pattern shown in CA medium, *P. meadii* isolates (n = 48) were identified into six colony groups (Table 4) and as a common trend, isolates from the same field/region have exhibited same colony patterns. There was a significant difference in the growth rate of isolates from different morphotypes ($P \leq 0.05$). Isolates showing stellate and stellate striated pattern had faster growth rates (5.73 ± 0.03 and 5.66 ± 0.03, respectively), while isolates with floral or chrysanthemum pattern were slow-growers (5.03 ± 0.03) and the remaining isolates had an intermediate growth rate (Table 4). The cardinal temperature for growth of *P. meadii* isolates was 25 to 30 °C but the growth of the isolates was inhibited below temperature of 15 °C and higher temperature of 35 °C, demonstrating their temperature sensitivity.

A phenotypic correlation was computed between morphological characters (n = 7) among *P. meadii* isolates (n = 48) sampled from three varied topography and climatic profiles (Fig. 4). A significant positive association was observed between sporangium length and sporangium breadth (r = 0.57, $P \leq 0.01$) with L/B ratio (r = 0.51, $P \leq 0.01$) exhibiting identical magnitude towards variability. Similarly, sporangium length showed considerable positive relations with L/B ratio (r = 0.25, $P \leq 0.01$) and pedicel length (r = 0.36, $P \leq 0.01$). Sporangium shape showed a positive association (not significantly differed) with papillation (r = 0.099, $P \leq 0.01$), but negatively associated with sporangium length (r = –0.16, $P \leq 0.01$) and sporangium breadth (r = – 0.21, $P \leq 0.01$). Phenotypic correlation analysis evidenced the existence of maximum correlation among sporangium length, sporangium breadth, L/B ratio and sporangium shape features with identical tendency of variability.

The morphometric variation was analyzed with seven principal components (PCs) and the Eigen values for PC1 (sporangium shape), PC2 (L: B ratio) and PC3 (sporangium length) are 1.93, 1.67 and 1.24, respectively. Initial three PCs together contributed 69.37 per cent to the total variability among the components (Table 5). The results of the Biplot principal component analysis (PCA), revealed that contributions of sporangium length, sporangium width, L / B ratio and pedicel length were in the right direction, looking at PC1 forming a single group, as angles between for them it was less than 90° (Fig. 5). Similarly, the pedicel type has contributed significantly to the better understanding of PC2 and together they form another group. Sporangium formation and papillation contribute significantly to the negative path towards PC3, forming a third group. PCA analysis illustrated that, isolates (n = 48) were scattered along both axes representing large extent of morphometric variations by considering PCs (n = 7). This might represent the evolution of highly variable isolates in major arecanut growing tracts of Karnataka irrespective of their geographical origin.

The phenotypic diversity analysis among morphometrical characters (n = 7) of *P. meadii* isolates (n = 48) with heat map is depicted in Fig. 6. Based on the hierarchical dendrogram, all the collected isolates were clustered into two sub-groups depending upon the similarities among the isolates. The heat map indicated that, sporangium length and sporangium breadth are found to be

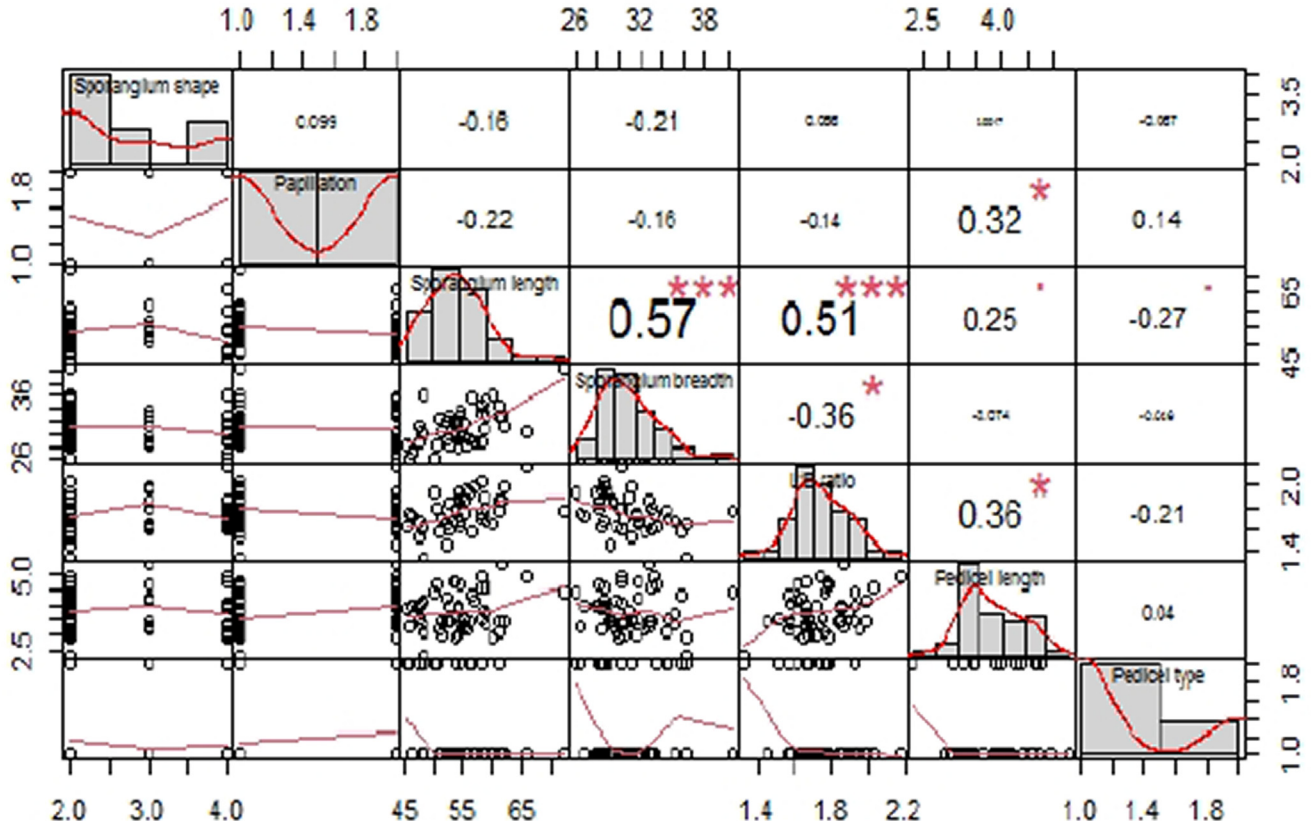

Fig. 4. Phenotypic corre lation and frequency distribution of morphological features of *P. meadii* isolates(n = 48) from varied agro-climatic zones of Karnataka.

Table 5
Eigen value and percent variability contribution of each component based on Principal Component Analysis (PCA).

PCA	Eigen value	Variability (%)	Cumulative Variability (%)
PC1	1.93	27.64	27.64
PC2	1.67	23.88	51.52
PC3	1.24	17.85	69.37
PC4	0.94	13.47	82.84
PC5	0.74	10.68	93.52
PC6	0.45	6.45	99.97
PC7	0.02	0.03	100

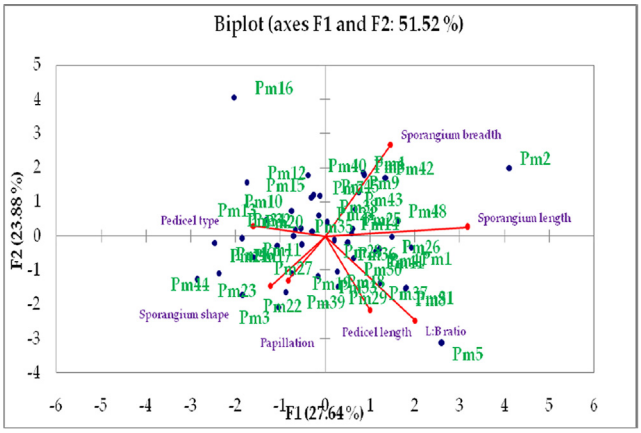

Fig. 5. Principal Component Analysis (PCA) from components of morphological variables of *P. meadii* isolates on variability.

closely related compared to other characteristics analyzed. However, the second sub-group consisted of remaining features and within that again two clusters were formed based on similarities between morphotypes and isolates over a geographical region. This comprehending the existence of substantial differences observed among the morphological characters (n = 7) and between isolates (n = 48) analyzed.

3.3. Mating type test

Mating-type of isolates (n = 48) revealed predominance of A_2 mating-type across the studied regions in Karnataka, India. The populations sampled from diverse agro-climatic zones of Karnataka consisting of A_2 mating-type when tested with known isolates of related species.

3.4. Molecular confirmation and phylogenetic analysis

To confirm the identity of *P. meadii*, multigene phylogenetic analysis of the representative isolates (n = 17) was comprehensively studied using four genetic regions, ITS, β-tub, TEF-1α and Cox-II. The sequences of the 17 isolates were deposited in NCBI GenBank and assigned accession numbers are furnished in Table 6 and the phylogenetic tree is shown in Fig. 7.

Successful amplification of expected amplicon size of 900 bp (ITS), 1250 bp (β-tub), 970 bp (TEF-1α) and 825 bp (Cox-II) were detected. The phylogenetic tree resulting from the concatenated data set had some significant differences among the isolates. Concatenated phylogenetic analysis of four genetic regions of representative isolates (n = 17) were clustered in clade-II (Cooke et al., 2000) which consisting of other closely related *Phytophthora* species, *P. capsici, P. colocasiae, P. citrophthora* and all the isolates gen-

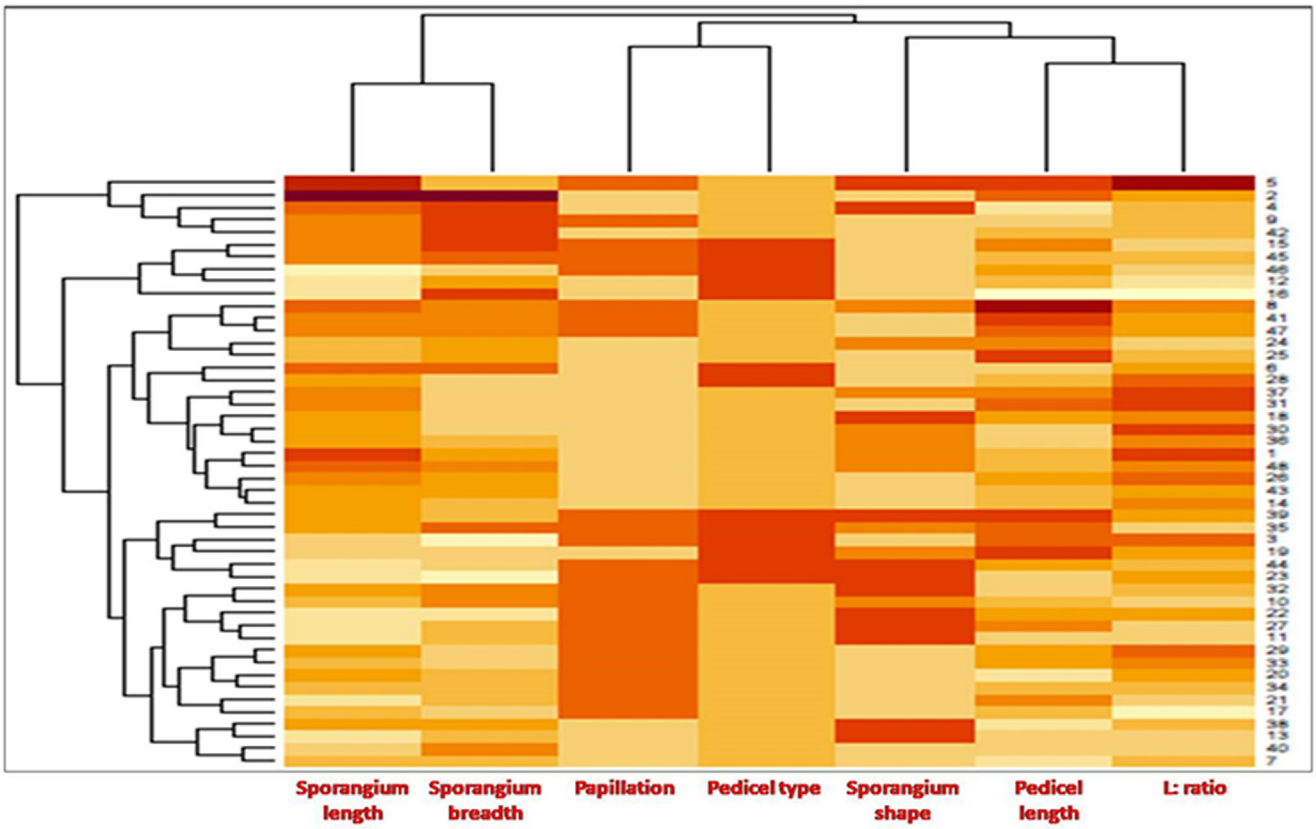

Fig. 6. Heat map depicting morphological variability among *P. meadii* isolates (n = 48) infecting arecanut in Karnataka.

Table 6
Representative isolates of *P. meadii* characterized during the study with passport data on host, origin and GenBank accession number of sequences.

Species	Isolate	Agro-Climatic Zone	Origin	Host	GenBank accession number			
					ITS	*β-Tub*	*TEF-1α*	*Cox-II*
P. meadii	P3	Malnad		*Areca*	MT680639	MT826734	MT826751	MT826768
	P4	Malnad		*Catechu*	MT680640	MT826735	MT826752	MT826769
	P9	Malnad			MT680641	MT826736	MT826753	MT826770
	P10	Malnad			MT680642	MT826737	MT826754	MT826771
	P12	Malnad			MT680643	MT826738	MT826755	MT826772
	P15	Malnad			MT680644	MT826739	MT826756	MT826773
	P18	Malnad			MT680645	MT826740	MT826757	MT826774
	P21	Malnad			MT680646	MT826741	MT826758	MT826775
	P25	Coastal			MT680647	MT826742	MT826759	MT826776
	P28	Coastal	Karnataka		MT680648	MT826743	MT826760	MT826777
	P31	Coastal			MT680649	MT826744	MT826761	MT826778
	P32	Coastal			MT680650	MT826745	MT826762	MT826779
	P37	Coastal			MT680651	MT826746	MT826763	MT826780
	P39	Coastal			MT680652	MT826747	MT826764	MT826781
	P40	Coastal			MT680653	MT826748	MT826765	MT826782
	P41	Coastal			MT680654	MT826749	MT826766	MT826783
	P43	Maidan			MT680655	MT826750	MT826767	MT826784
P. citrophthora*	CH90-19		India	*Citrus* sp.	AB366374	KX250552	EU080539	GU221973
P. capsici	LEV6717		Mexico	*Capsicum annum*	MH025884	EU080852	KU518466	DQ469734
P. colocasiae	PC-112–12		India	*Colocasia esculenta*	JX134654	EU080125	EU080126	LC596018
P. palmivora	P80		India	*Cocos nucifera*	MT052675	AY729822	MH760176	EU427470
P. nicotianae	Dos-4	Data	Turkey	*Punica granatum*	MH219859	EU080716	EU080717	MH221078
P. infestans	P6166	not available	USA	*Solanum tuberosum*	FJ801899	JN654387	KR046670	GU318302

*The partial sequences of different *Phytophthora* species retrieved from GenBank were highlighted with blue colour.

erated in study were exhibited homology of > 99% with *P. meadii* (Fig. 7). Isolates sampled from varied regions were shower similarity and clustered with standard lineage isolate (CPCRI Pm1) with high support sister position to closely related species (Fig. 7). This indicating that, *P. meadii* is a distinct species with moderate to stronger support pertaining close evolutionary relationship with other clade-II species. Therefore, the representative isolates (n = 17) sampled from varied agro-climatic zones were identical at all loci and we confirmed that *P. meadii* predominantly associated with fruit rot of arecanut.

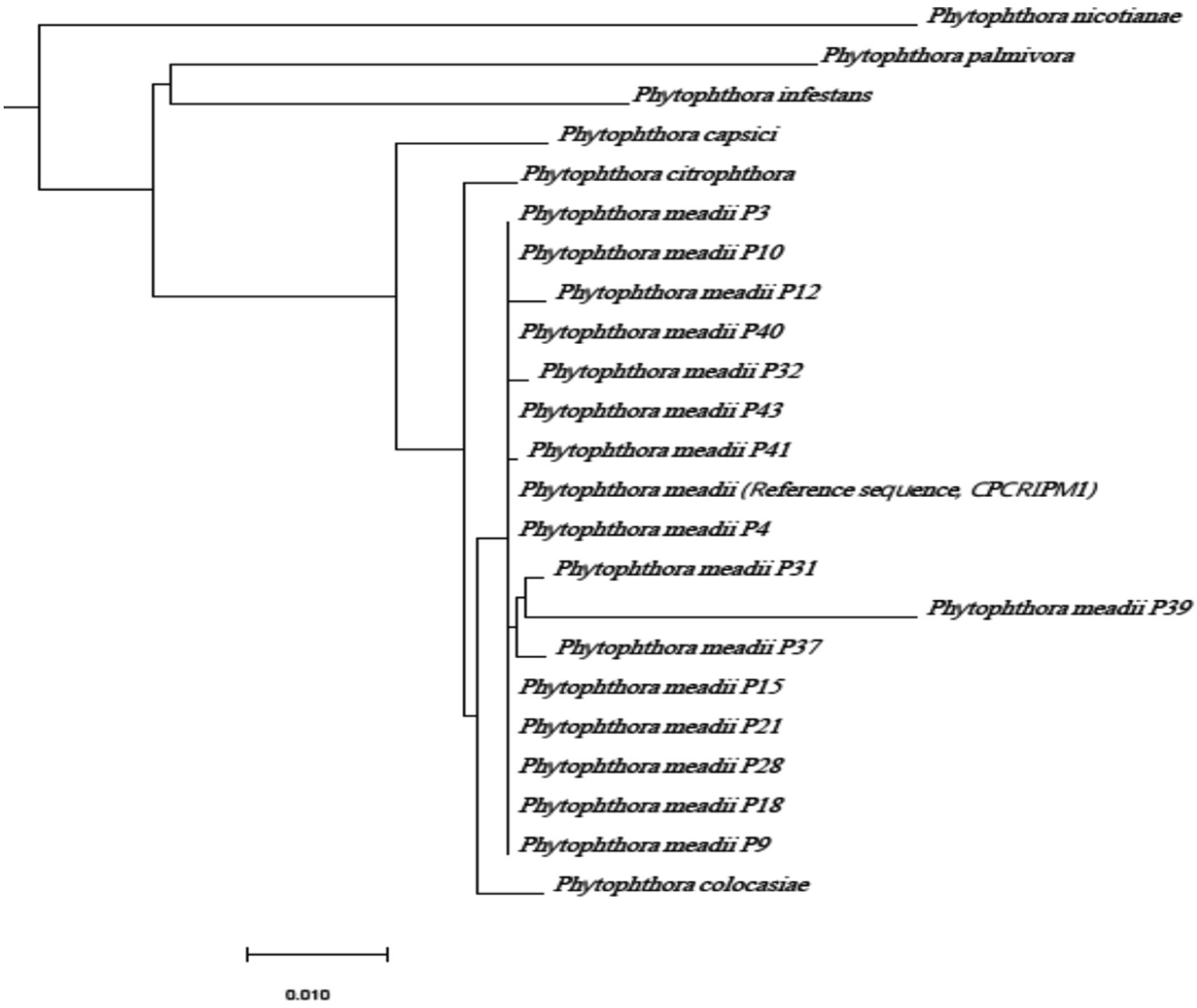

Fig. 7. Consensus phylogenetic tree derived from Jukes Cantor substitution model by Neighbour –Joining method by four locus ITS, TEF-1α, β-tub and Cox-II dataset of the 17 represntative *P. meadii* isolates. The model computes the probabilities of substitution from nucleotides of isolates. *P. nicotianae*, *P. infestans*, *P. palmivora*, *P. citrophthora*, *P. colocasiae, P. capsici* and standard reference isolate CPCRI Pm1 were used as outgroup taxa. Scale bar = 0.010 expected changes per site per branch.

3.5. Haplotype diversity and frequency analysis in four candidate genes of P. Meadii

Based on morphotypes and colony groups of *P. meadii*, about 17 sequences at four loci (Cox-II, ITS, TEF1α, and β-tub) were examined for haplotype analysis (Table 7). Multiple haplotypes were identified at nuclear and mitochondrial genes such as Cox-II, ITS, TEF1α, and β-tub regions of *P. meadii*. A total of 49 haplotypes (h) were identified for all four genes across 17 isolates with different haplotype diversities (hd) and haplotype frequencies (hf). Highest number of haplotypes were identified for TEF1α (h = 16) followed by Cox-II (h = 12), ITS (h = 11) and β-tub (h = 10). Interestingly, the 46 haplotypes covered all the 17 representative isolates of *P. meadii*. Similarly, the haplotype diversity was found to be highest for TEF1α (hd = 0.9112), followed by Cox-II (hd = 0.7845), ITS (hd = 0.6987), and β-tub (hd = 0.6652).

Among the 12 haplotypes in Cox-II region, haplo_10 represents the highest number of isolates (n = 3) with frequency of 18.2% followed by haplo_1 and halpo_8 which represented two isolates each with frequency (12.1%) and remaining haplotypes were comprised of one isolate each with frequency (6.1%). While in case of ITS region, a total of 11 haplotypes were identified and each consists of one isolate with frequency of 8.7% which indicated higher haplotype diversity exists among isolates sampled from three varied agro-climatic zones of Karnataka (Table 7). Similarly, a total of 16 haplotypes were determined in TEF1α region and each haplotype was comprised one isolate with frequency (6.1%) representing large variation among haplotypes. Whereas, in the region of β-tub, about 10 haplotypes were identified and haplo_1 was present in maximum isolates (n = 3) with frequency of 22.2% followed by haplo_6 which represented two isolates with relative frequency (14.8%); while remaining haplotypes were comprised of one isolate each with frequency of 7.4%. Surprisingly, haplotype groups identified in our study had larger variation and diversity among 17 isolates of *P. meadii* infecting arecanut in Karnataka. The haplotype groups obtained in our study have been represented in haplotype network constructed using median-joining method (Fig. 8).

3.6. Linkage disequilibrium blocks

A random set of SNP markers identified in genomic region of each gene was used for calculating important measures of linkage

Table 7
Haplotype groups identified in genomic regions of Cox-II, ITS, TEF1α, and β-tub in 17 isolates of *P. meadii* infecting arecanut.

Gene	Haplotype	Size	Frequency (%)	Haplotype Representing Isolates
Cox-II	haplo1	2	12.1	Pm21, Pm 32
	haplo2	1	6.1	Pm 18
	haplo3	1	6.1	Pm 37
	haplo4	1	6.1	Pm 03
	haplo5	1	6.1	Pm 40
	haplo6	1	6.1	Pm 43
	haplo7	1	6.1	Pm 41
	haplo8	2	12.1	Pm 10, Pm 09
	haplo9	1	6.1	Pm 12
	haplo10	3	18.2	Pm 15, Pm 04, Pm 39
	haplo11	1	6.1	Pm 31
	haplo12	1	6.1	Pm 28
ITS	haplo1	1	8.7	Pm 40
	haplo2	1	8.7	Pm 15
	haplo3	1	8.7	Pm 28
	haplo4	1	8.7	Pm 10
	haplo5	1	8.7	Pm 43
	haplo6	1	8.7	Pm 37
	haplo7	1	8.7	Pm 12
	haplo8	1	8.7	Pm 39
	haplo9	1	8.7	Pm 32
	haplo10	1	8.7	Pm 41
	haplo11	1	8.7	Pm 31
TEF1α	haplo1	1	6.1	Pm 18
	haplo2	1	6.1	Pm 32
	haplo3	1	6.1	Pm 43
	haplo4	1	6.1	Pm 10
	haplo5	1	6.1	Pm 9
	haplo6	1	6.1	Pm 31
	haplo7	1	6.1	Pm 39
	haplo8	1	6.1	Pm 40
	haplo9	1	6.1	Pm 21
	haplo10	1	6.1	Pm 37
	haplo11	1	6.1	Pm 41
	haplo12	1	6.1	Pm 15
	haplo13	1	6.1	Pm 4
	haplo14	1	6.1	Pm 8
	haplo15	1	6.1	Pm 12
	haplo16	1	6.1	Pm 03
β-tub	haplo1	3	22.2	Pm 41, Pm 18, Pm 31
	haplo2	1	7.4	Pm 04
	haplo3	1	7.4	Pm 39
	haplo4	1	7.4	Pm 43
	haplo5	1	7.4	Pm 40
	haplo6	2	14.8	Pm 21, Pm 12
	haplo7	1	7.4	Pm 28
	haplo8	1	7.4	Pm 09
	haplo9	1	7.4	Pm 32
	haplo10	1	7.4	Pm 10

disequilibrium such as interallelic r^2 values (association between any two random pairs alleles of from different loci), and their significance (D'). Linkage disequilibrium blocks were identified in the genomic region of each gene. In the genomic region of Cox-II gene, a total of 13 polymorphic SNPs were housed four LD blocks. Block 3 and block 4 were highly informative than block 1 and block 2 with higher r^2 values represented by darker red squares. In the genomic region of TEF1α, a total of 28 SNPs were identified in one block. 13 SNPs were identified in the region of β-tub region with 3 LD blocks. Highest number of polymorphic SNPs (99 SNPs) with 14 LD blocks were identified in the region of ITS genomic region (Fig. 9).

3.7. Virulence assay

Pathogenic variability among the isolates was analyzed based on the lesion area (cm^2) and area of infection (%), further isolates were categorized based on nature of virulence (Padmanaban et al., 1997). Among the three agro-climatic populations of *P. meadii* infecting arecanut, a substantial and statistically significant variation in virulence components were observed and isolates showed wide variation in their virulence capacity. Out of 48 isolates tested, 17 isolates were found highly virulent with higher lesion development, 25 isolates were exhibited moderately virulent with medium lesion area of infection and 6 isolates showed less virulent or non-virulent reaction with least or no lesion area development (Fig. 10).

Comparing the virulence capacity with the geographic origin of isolates revealed interesting results. The maximum number of isolates (n = 11) which exhibited maximum area of infection (>50%) and lesion development (>10 cm^2) on tender green arecanut were obtained from Malnad regional populations followed by Coastal isolates (n = 6). While none of the isolates sampled from Maidan region exhibited higher virulence components. The highest number of isolates with moderate level of virulence were recorded from Coastal isolates (n = 12) followed by few Malnad isolates (n = 10) and Maidan populations (n = 3) with an area of infection (25–50%) and lesion development (7–10 cm^2). The remaining isolates (n = 6) from all the studied regions had shown lesser virulence capacity indicating the lowest area of infection (<25%) and lesion development (<7cm^2). Virulence assay clearly evidenced that, iso-

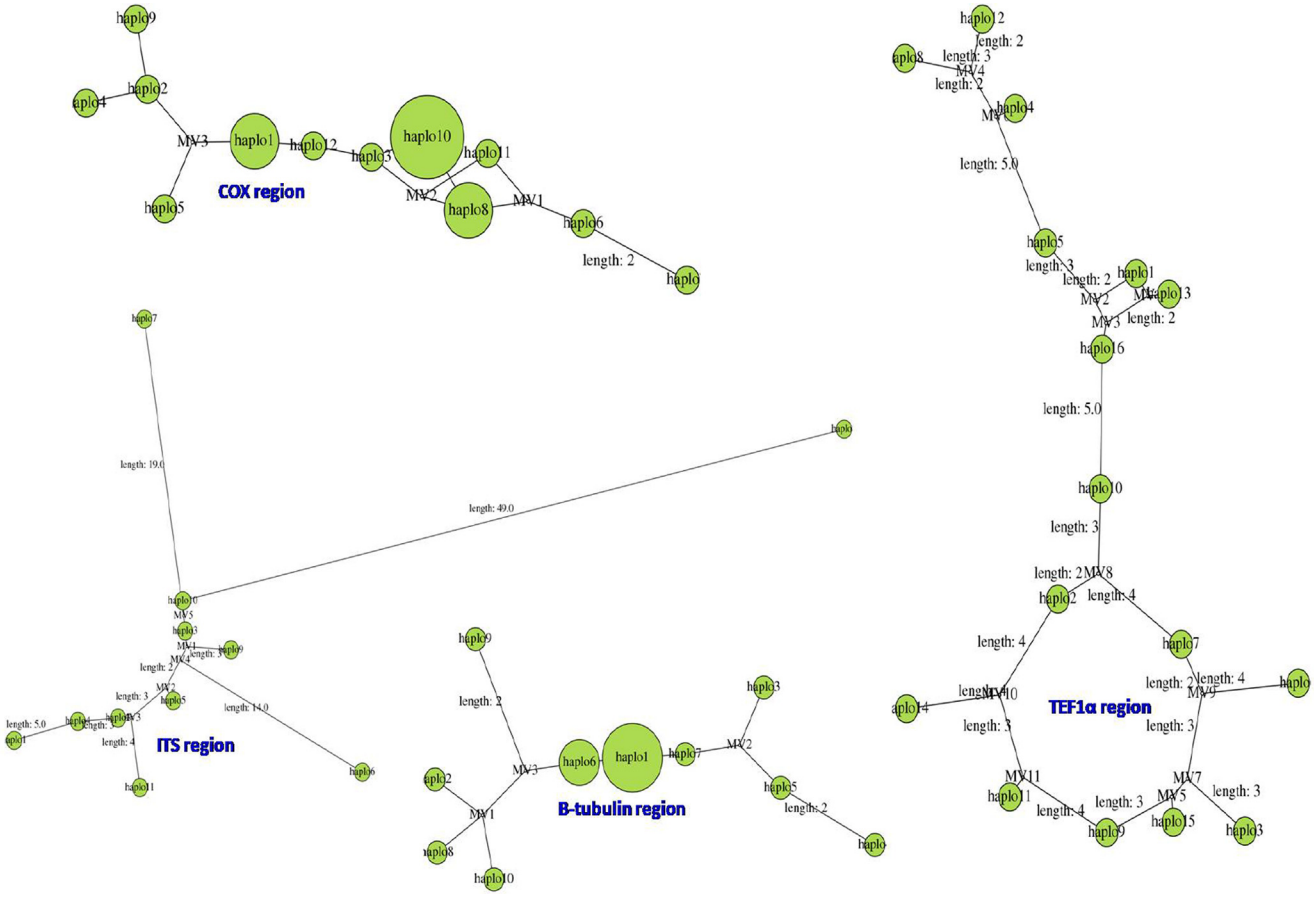

Fig. 8. Median-joining haplotype network of Cox, ITS, TEF1α, and β-tub regions of *P. meadii* haplotypes constructed using haplophile. The network analysis included multiple haplotypes of *P. meadii* infecting arecanut. Each circle indicates a unique haplotype; the size of the circle is proportional to the number of *P. meadii* isolates included. MV represents ''median vector" hypothesized (often ancestral) sequences which are required to connect existing sequences within the network.

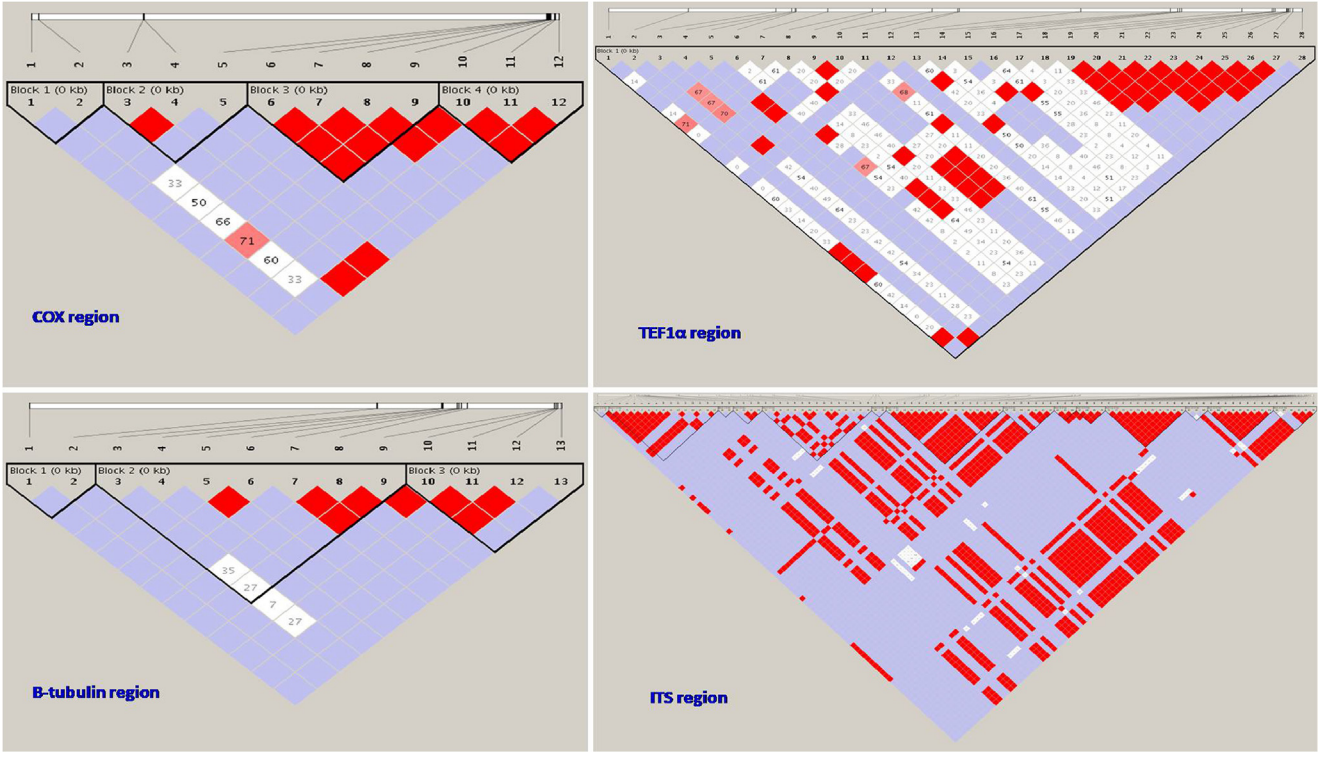

Fig. 9. Linkage disequilibrium plots of different single nucleotide polymorphisms (SNPs) of Cox, TEF1α, β-tub and ITS regions generated in Haploview represents the haplotype block structure using the solid spine definition. 100* [D'] values are given, an empty cell indicated D'=1 and the darker the red shading, the larger the [D'].

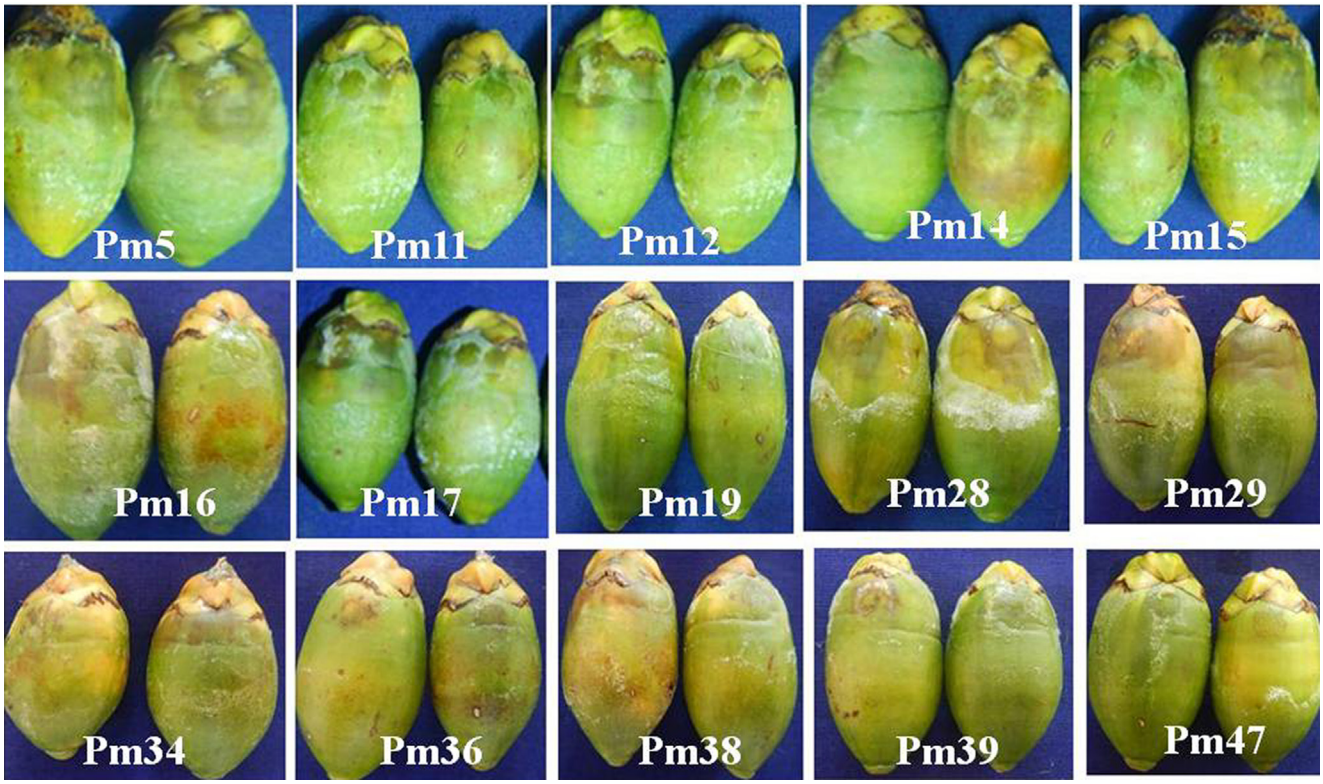

Fig. 10. Comparative virulence capacity of *P. meadii* isolates originated from diverse agro-climatic zones of Karnataka, India.

lates originated from Malnad parts of Karnataka including few isolates from Coastal belts found to be highly virulent and posed a higher risk of infection on arecanut plantations which is distributed evenly in both regions.

4. Discussion

Phytophthora causes destructive diseases in many agricultural and horticultural plants, natural vegetation, and forests around the world (Chowdappa, 2017). *Phytophthora* species has emerged as a biosecurity threat due to an increase in international plant trade (Brasier, 2008). Accurate diagnosis and appropriate risk management of *Phytophthora* species are required in order to formulate suitable control strategies. The evolution of new populations by sexual recombination, genetic modifications and global migration has necessitated the use of sensitive and reliable diagnostic tools for rapid identification and characterization of *Phytophthora* species infecting arecanut (Chowdappa, 2017).

Only 59 species of *Phytophthora* were recorded worldwide until 1996 according to Erwin and Rebeiro (1996). Later, many species of *Phytophthora* have been identified from various parts of the world and so far, 120 species have been described molecularly (Kroon et al., 2012). Different *Phytophthora* species namely, *P. palmivora* (Das and Cheeran, 1986), *P. meadii* (Sastry and Hedge, 1985), *P. heveae* (Chowdappa et al., 2002) and *P. arecae* (Pethybridge, 1913) were previously recorded as causative agent of arecanut fruit rot. However, the studies carried out later identified the *Phytophthora* species associated with fruit rot of arecanut as *P. meadii* (Chowdappa et al., 2003) and further, association of *P. meadii* as incitant of fruit rot was confirmed (Prathibha et al., 2019, 2020). Though the characterization of *P. meadii* has been confirmed in the previous studies, the isolates collected were limited extent in Coastal and Malnad areas and did not cover entire arecanut growing areas of Karnataka. Therefore, our results provide a fully comprehensive analysis for the entire region. In addition, a virulence assay was performed to understand aggressiveness of isolates, virulence profile and to fulfill Koch's postulate. We demonstrated that *P. meadii* have been responsible for causal agent of fruit rot epidemic across varied agro-climatic zones in Karnataka during South-West monsoon season of 2018 to 2019.

Morphological characteristics revealed the existence of substantial variation in many parameters tested. *P. meadii* isolates depicted diverse sporangial and colony morphology in various media used. All the isolates of *P. meadii* were classified into six morphotypes and colony groups based on distinct morphological characteristics. Our study revealed more morphological diverse groups as compared to the earlier studies (Sastry and Hedge, 1987; Saraswathy, 1994; Misra et al., 2011; Nath et al., 2015; Prathibha et al., 2019, 2020) and it could be due to the sampling of more number of populations from different climatic features which provides a better coverage of varied agro-climatic zones of Karnataka. Studies with respect to temperature response have shown that the distinct variation of morphotypes is tolerance to high temperatures (30–35 °C). Significant differences were noted in sporangial and colony morphology among *P. meadii* isolates. These results are in consistent with previous studies (Sastry and Hedge, 1987; Saraswathy, 1994; Chowdappa, 1995; Rasmi, 2003; Misra et al., 2011; Prathibha et al., 2020), except that in our study no growth was noticed for any of the isolates at 40 °C.

Virulence assay showed that the isolates collected from Malnad (n = 21) and Coastal region (n = 21) were found to be more virulent with significant differences in mean lesion diameter (>10 cm^2) compared to Maidan region isolates (n = 6), which exhibited relatively least infection rate (<25%) and lesion diameter (<7cm^2). Variations in lesion area as observed in this study were reported in *P. meadii* (Prathibha et al., 2016) and other *Phytophthora* species

(Pereira et al., 1987; Pinto et al., 1989; Liyanage and wheeler, 1989; Chowdappa, 1995; Misra et al., 2011; Granke et al., 2011; Costamilan et al., 2012; Nath et al., 2015). Virulence assay showed that, *P. meadii* isolates studied under the present investigations consist of high degree of genetic diversity. Our research confirmed that, the isolates from Malnad regions are highly virulent as similar to the isolates of Maidan and Coastal in contrasting to the previous report by Prathibha et al. (2016). The difference in virulence of isolates may be credited to the large number of sampling sites used in the current study that provides broader area coverage compared to previous findings (Prathibha et al., 2016). Increased virulent populations should be handled with caution as this indirectly raises the potential for evolutionary of the isolates. The isolates sampled from maidan region proved to be less virulent, this might be attributed to the lesser amount of initial inoculum under field condition coupled with higher temperature and lesser humidity as compared to other studied regions. No clear correlations were observed between colony morphology, pathogenicity tests, and geographical origin of the isolates examined.

Alignment of ITS, β-tub, TEF-1α and Cox-II sequences revealed considerable variation/polymorphism/mutation in the target regions of genes in the isolates (n = 48) studied. Similar observations were reported (Prathibha et al., 2020), who confirmed more polymorphism in rDNA region of *P. meadii*. Similarly, (Anandaraj et al., 2020) characterized *Phytophthora* species from nutmeg using ITS and MLST (multi-locus sequence typing) reveled diversification in species that clustered in clade-2 with three different groups. The phylogenetic tree based on genetic distance clearly indicated the higher degree of genetic diversity among isolates of *P. meadii* collected from Karnataka. The clustering of the isolates could not be associated with geographical origin, morphotypes and colony groups. This can be attributed to the fact that fruit rot was reported in India in early 1906 (Butler, 1906; Coleman, 1910) and population of the pathogen tend to have higher variability after many years evolution (McDonald and Linde, 2002). Existence of high rate of variability in all genes indicates that the population is continued to evolve naturally. Alternatively, migration events are more common in *Phytophthora*, which could be credited to witness diversity of in *P. meadii* isolates of Karnataka. However, further studies on sequencing of a large number of isolates from diverse geographical boundaries are required to confirm the general population composition of the existing pathogen in India.

In the present study, a total of 49 haplotypes were identified at four loci (Cox, ITS, TEF1α, and β-tub) for 17 representative isolates of *P. meadii* from Karnataka with the higher haplotype diversity (hd) and relative haplotype frequency (hf) of isolates. The higher haplotype variation between the isolates showed that the haplotypes were closely related, and they differed in smaller nucleotide variations. These results were confirmed by a high haplotype diversity of 0.9163 and a low nucleotide variation among 45 haplotypes identified in 71 isolates of *Ustilaginoidea virens* infecting rice (Sharanabasav et al., 2021). A high degree of sequence divergence between isolates of *Phytophthora cinnamomi* has been reported in a total of 45 haplotypes (Martin and Coffey, 2012). 63 isolates *Phytophthora infestans* from potato and 94 isolates from tomato were identified haplotype Ia group (Chowdappa et al., 2015).

Prevalence and distribution of pathogen associated with fruit rot disease of arecanut is mainly related to various factors such as sampling area, geographical origin, aggressiveness and arecanut variety. In the present investigation, *P. meadii* is the most predominant species represented 100 per cent of the total isolates (n = 48) collected in all the sampled sites. Populations of *P. meadii* from Karnataka exhibited wider geographical distribution in major arecanut growing regions (Sastry and Hedge, 1985; Chowdappa et al., 1993; Jose et al., 2008) which could be attributed to the prevalence of this species in Karnataka.

In summary, the present investigation deployed the conventional techniques like morphological, cultural and microscopic characterization with multi-gene (*ITS*, *β-tub*, *TEF-1α* and *Cox-II*) phylogenetic analysis for the identification of the pathogen. Most of the isolates from varied agro-climatic zones of Karnataka witnessed similar morphological characteristics *viz.*, colony growth rate, pathogenic ability, shape and size of sporangia, production of chlamydospores, ontogeny, colony pattern and other *in vitro* cultural characteristics are related to confirm *Phytophthora* species associated with fruit rot disease (Sastry and Hedge, 1985; Saraswathy, 1994; Prathibha et al., 2019, 2020). *P. meadii* isolates identified during the study, exhibited varied colony patterns representing six colony groups, six type of sporangial morphology, varied colony growth rate, differed virulence capacity is confirmed by suitable approaches (morpho-cultural characterization). However, comprehending morphology, multigene phylogeny, and haplotype analysis revealed the dominance of *P. meadii* associated with fruit rot disease of arecanut in Karnataka which is corroborated with earlier findings on limited area (Erwin and Ribeiro, 1996; Chowdappa, 1995; Sharadraj and Chandramohanan, 2010).

5. Conclusion

We characterized the *P. meadii* populations from various agro-climatic zones of Karnataka and confirmed the existence of extensive phenotypic and genotypic variability in *P. meadii* isolates. These data will be extremely valuable in understanding and breeding for fruit rot disease resistance. Additionally, a significant degree of variability has been shown in the *P. meadii* populations which can respond quickly to selection exerted by introduced host resistance or fungicides, emphasizing the importance of relying on integrated disease management. Haplotype and SNP analysis substantially provides the variation in the descent nucleotides of different genetic regions sequenced and clearly demonstrated the existing of similar species of *Phytophthora* on arecanut. The results represented here would provide benchmark information for designing appropriate management approaches in combating the fruit rot disease of arecanut.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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