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Species diversity of *Ganoderma* causing foot rot of arecanut in southern dry tracts of Karnataka

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Abstract

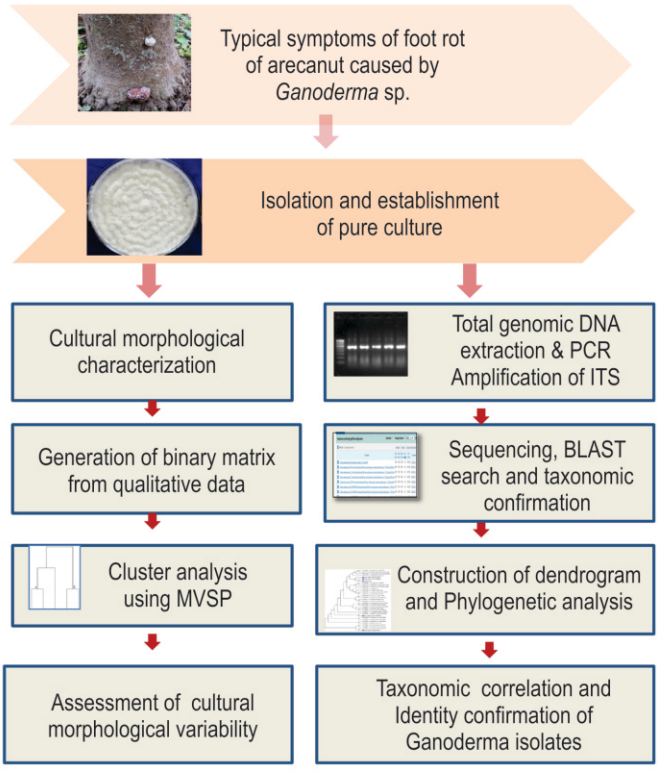
Aim: The present study was undertaken to study the morphological and molecular diversity of *Ganoderma* spp. causing foot rot of arecanut in dry tracts of Southern Karnataka.

Methodology: A total of 20 samples isolated from diseased areca palms in three districts of Karnataka were identified based on morphological and molecular characteristics. Qualitative data of cultural characteristics were transformed into code and a binary matrix was generated. Total genomic DNA was isolated and ITS region was amplified using universal primers ITS1 and ITS4. PCR amplicon was directly sequenced and phylogenetic analysis was carried out.

Results: The dendrogram generated from the cultural morphological characteristics showed clear variations among *Ganoderma* isolates causing foot rot. DNA amplification of *Ganoderma* isolates with fungal universal primers (ITS1 and ITS4) was observed at 650 bp in all isolates tested. Taxonomic correlation of isolates upon NCBI web proved that the isolates were genetically related to *Ganoderma* spp. with 89-99.49 per cent identity and confirmed the taxonomic identity of isolates used in this study.

Interpretation: Phylogenetic analysis of arecanut isolates of southern Karnataka are distinct as evidenced by forming separate cluster. Based on gene homology, *G. ryvardenni* and *G. casuarinicola* are new species reported as causal agent of foot rot in arecanut from Karnataka. The ITS gene sequences of four isolates *viz.*, AG_3 (MN 784436), AG_4 (MN 784437), AG_{11} (MN 784438), and AG_{20} (MN 78449) were deposited in NCBI gene bank.

Key words: Arecanut, Foot rot, *Ganoderma*, Phylogeny, Species diversity



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Introduction

Arecanut (*Areca catechu* L.) is a tropical plant found all over South-East Asia. The fruit (nut) of this tree is popularly known as betel nut or supari in India. This is an important commercial crop of the region and also forms a part of ritual offerings in Hindu religion. Areca is taken up from the Malayan language, which means 'cluster of nuts'. The current production of arecanut in the world is about 127 thousand tonnes from an area of 925 thousand ha. India ranks first in both area (55 %) and production (45 %) of arecanut. Arecanut is the major plantation crop of coastal and southern districts of the country under assured irrigation facility. Production of arecanut in India is mainly concentrated in six states, namely Karnataka, Kerala, Assam, Meghalaya, Tamil Nadu and West Bengal. The Karnataka and Kerala are predominant states in the production of arecanut. Karnataka stands in first place in terms both area (48 %) and production (56 %) and Kerala stands in second place in terms of area (21 %) and production (18 %). In Karnataka, six districts produce arecanut crop, out of which, Shivamogga stands first both in area and production (Bangarappa and Shiralashetti, 2018).

Areacanut palms are normally affected by various biotic and abiotic stresses resulting in drastic reduction in yield. Among various biotic stress that affect arecanut production in India, Foot rot (*Anabe roga* in Kannada) caused by *Ganoderma* is a major constraint in arecanut production, especially in dry tracts of Southern Karnataka. *Ganoderma* wilt in coconut and foot rot in arecanut caused by *Ganoderma* spp. is one of the most devastating disease affecting production and productivity of palms (Palanna, *et al.,* 2020). The disease is reported from various places all over the tropical world. Foot rot was reported by Coleman from Karnataka in 1911 (Coleman, 1911). This disease is also reported from Kerala, Assam, West Bengal (Sharples, 1928) and Nicobar Islands (Sangal *et al.,* 1961) and also from parts of Tamil Nadu. Naik *et al.* (2000) and Palanna *et al.* (2009, 2016) reported *Ganoderma* wilt incidence from Southern Karnataka on coconut.

The taxonomy of basidiomycetes is traditionally based on the morphological features of basidiocarps. Identification based on the basidiocarp features, however, is prone to problems such as absence of basidiocarps during certain time of the year, their morphological plasticity and presence of cryptic species (Moncalvo and Ryvarden, 1997; Gottlieb and Wright, 1999). However, studies have shown that *Ganoderma* species are genetically heterogeneous since wide range of genetic variation are reported and caused by out crossing over generations and different geographical origins (Miller *et al.,* 1999; Pilotti *et al.,* 2003). This leads to variation in their morphological characteristics even within same species (Hong *et al.,* 2001). For these reasons, contemporary taxonomists employ morphological studies, mating tests, analyses of biochemical and DNA sequence information or combination of these methods for the identification of the pathogen. Recently, molecular approach has been adopted to identify *Ganoderma* species through multiplex polymerase chain reaction (PCR) which is a more rapid and precise approach (Idris *et al.,* 2010; Wong *et al.,* 2012). Disease management is an important aspect to sustain the palm industry. Lack of knowledge of the pathogen may lead to inaccurate disease control strategies. Hence, the present study was undertaken to investigate the diversity of *Ganoderma* species isolated from foot rot infected arecanut palms in terms of their morphological and molecular characteristics.

Materials and Methods

Collection of diseased root samples/stem bit and sporocarps of arecanut: Different parts of arecanut palms such as diseased root bits/stem bits affected by *Ganoderma* foot rot showing typical symptoms and sporocarps were collected from infected palms from different places of Southern Karnataka (Table 1). Total twenty samples were collected, labeled and packed in polythene bags and brought to the laboratory for the isolation of causal organism.

Isolation and designation of causal organism isolates: Infected roots/ stem bits collected from infected arecanut palms were washed thoroughly with sterile water, cut into small bits/ pieces and surface sterilized in 0.1 % mercuric chloride for 30 sec and washed three times serially in sterile distilled water to remove the traces of mercuric chloride. After surface sterilization, diseased specimens were kept in sterilized bags along with wet cotton under room temperature for about 8 to 10 days. After 8 to 10 days of incubation period, slight mycelial growth observed was transferred onto Potato Dextrose Agar medium. The inoculated plates were incubated at room temperature (28 °C ± 2 °C) for 3-5 days to facilitate fungal growth. Later, a small portion of fungal growth was transferred to PDA slants. Pure culture of fungus was obtained by following hyphal tip culture technique under aseptic conditions. These isolates of *Ganoderma* isolated from arecanut were designated as AG_1, AG_2, AG_3, AG_4, AG_5, AG_6, AG_7, AG_8, AG_9, AG_{10}, AG_{11}, AG_{12}, AG_{13}, AG_{14}, AG_{15}, AG_{18}, AG_{19}, AG_{20}, AG_{21} and AG_{22}, respectively.

Maintenance of pure cultures: The isolated fungus was sub-cultured on PDA slants and allowed to grow at 28 °C ± 2°C temperature for 8-10 days. The cultures so obtained were stored in refrigerator at 4°C for further studies and they were cultured periodically once a month.

Study on variability of *Ganoderma* isolate isolated from arecanut: Twenty *Ganoderma* isolates of arecanut isolated during course of investigation were used for variability study.

Cultural morphological variability of *Ganoderma* isolates

Growth on potato dextrose agar: Twenty *Ganoderma* isolates [AG_1, AG_2, AG_3, AG_4, AG_5, AG_6, AG_7, AG_8, AG_9, AG_{10}, AG_{11}, AG_{12}, AG_{13}, AG_{14}, AG_{15}, AG_{18}, AG_{19}, AG_{20}, AG_{21} and AG_{22}] of arecanut collected from different geographic locations were cultured on PDA. Morphological characters like colony diameter/ growth, biomass production, colony colour, colony margin, mycelial density, appearance of zones, reverse pigmentation etc. were

studied. Mycelial plug (6 mm) from 7 day old active culture was transferred onto the centre of a standard 9 cm PDA plate and incubated for 7 days at an ambient temperature (Idris *et al.,* 2000). The test for all isolates was run simultaneously to avoid bias due to external factors. The experiment was conducted in three replications. The diameter was measured daily and the number of days required for maximum growth of mycelium was also recorded. The texture of colony, appearance of zone, reverse pigmentation colour, type of colony margin and mycelial density were recorded after 7th day of incubation.

Growth in liquid media: Flasks containing 100 ml of sterilized potato dextrose broth were inoculated with mycelial discs of *Ganoderma* isolates of arecanut. Three replications were maintained for each treatment. The inoculated flasks were incubated at room temperature (28±2 °C) for 10 days, and then mycelial mat was harvested on a previously weighed Whatman No. 4 filter paper and dried at 60 °C in a hot air oven till constant weight was obtained. Dry mycelial weight was recorded and expressed in mg 100 ml^{-1} broth and results were analysed statistically. Qualitative data of cultural characteristics on solid media and biomass were transformed into code and a binary matrix was generated (Table 2). Binary data was subjected to cluster analysis using multivariate statistical package (MVSP version 3.13). Similarity matrices were calculated using simple matching coefficient and a dendrogram was generated using unweighted pair group method of arithmetic averages (UPGMA) (Pilotti *et al.,* 2004).

Molecular characterization of *Ganoderma:* Isolates of *Ganoderma* species were identified through ITS (Internal Transcribed Spacer) region using universal primers ITS1 and ITS4 amplification.

Reagents and Chemicals: All the chemicals were of analytical grade and were from Sigma and Merck. The following buffers and solutions were prepared : Extraction buffer (100 mmM Tris-HCl (pH 8); 20 mM EDTA (pH 8); 2 M Nacl; 3 % CTAB (w/v); 1 % PVP; 2 % β-mercaptoethanol (v/v); phenol: chloroform (24:1); potassium acetate 7.5 M; proteinase K, 0.05 mg ml^{-1} ; wash solution [15 mM ammonium acetate in 75 % (v/v)ethanol]; TE buffer [10 mM Tris-HCl (pH 8), 1mM EDTA (pH 8)].

Fungal genomic DNA extraction: Fungal mycelia (100 mg) was ground in to fine powder using liquid nitrogen. Pre-warmed extraction buffer (1 ml) was added to the samples and grounded once more in buffer. All the samples were transferred to Eppendorf tubes and 5 µl proteinase K was added. The tube was incubated in 37 °C for 30 min and then at 65 °C for another 30 min with frequent swirling. Samples were centrifuged at 10,000 x g for 10 min at room temperature and the supernatant was transferred to fresh Eppendorf tube. To the supernatant, 100 µl of 7.5 M potassium acetate was added and incubated at 4 °C for 30 min. The samples were centrifuged at 13,000 x g for 10 min at RT; the supernatant was transferred to fresh tube, an equal volume of chloroform: isoamyl alcohol was added and mixed by gentle inversion 30-40 times. The samples were centrifuged at 10,000 x g for 10 min at room temperature. The supernatant was transferred to a fresh tube and precipitated with 2/3 volume of isopropanol. The precipitated nucleic acids were collected and washed twice with the wash solution. The nucleic acid pellet so obtained was air dried untill the traces of ethanol was removed and dissolved in an appropriate amount of TE buffer (50-70 µl). Nucleic acid dissolved in TE buffer were treated with ribonuclease, incubated at 37 °C for 30 min and stored at -20 °C until further use.

Qualitative and quantitative analysis of DNA: The quality and quantity of DNA was analyzed by running 2 µl of each sample mixed with 2 µl of 10x loading dye in one per cent agarose gel. DNA from all the isolates produced clear sharp bands in one per cent agarose gel indicating good quality of DNA. DNA was also quantified by spectrophotometer (Nanodrop ND 1000).

PCR amplification of Internal Transcribed Spacer (ITS) region: Ribosomal DNA (rDNA) unit contains genetic and non-genetic or spacer region. Each repeat unit consists of a copy of 18S, 5.8S and 28S like rDNA and its spacer like Internal Transcribed Spacers (ITS) and Inter-Genic Spacers (IGS). rDNA is used to analyze evolutionary events because it is highly conserved whereas ITS rDNA is more variable. Hence, it is used for investigating the species level relationships. The primers for amplification were custom synthesized at Bangalore Genie Pvt. Ltd., Bangalore and supplied as lyophilized products of desalted oligos. Primer sequences for amplification of ITS region used were as follows:

PCR was carried out in poly propylene tubes using universal primers ITS 1 (5' -AACGTTACCAAACTGTTA-3') and ITS 4 (5' -AAGTTCAGCGGGTATTCCT-3') and *G. lucidum* specific primers GSF (5' -CCCTAAACCTCTCAAAGTCA-3') and GSR (5' -TATCGTACAGGTTCTCGTG -3). PCR amplification was performed in 25 µl reaction mixture containing 10× reaction buffer supplied by the manufacturer, 100 ng of fungal DNA, each dNTP at a concentration of 0.5 mM, 20 pico moles of each primer and 1 U of Taq DNA polymerase (NEB, USA). Thermo cycling conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min and a final elongation step of 72°C for 5 min.

Separation of amplified products by agarose gel electrophoresis: Agarose gel electrophoresis was performed to resolve the amplified product using 1.4 % agarose in 1X TBE (Tris Borate EDTA) buffer, 0.5 mg ml^{-1} of ethidium bromide and loading buffer (0.25 % bromophenol blue in 40 % sucrose). One microliter of loading dye was added to 5 µl of PCR product and loaded into agarose gel. Electrophoresis was carried at 65 V for 1.5 hr. The gel was observed under UV light and documented using gel documentation unit.

Sequencing of ITS region: The ITS region was sequenced from the isolates of *Ganoderma* species to confirm the organism and to the variability present in them. Homology search was done using

BLAST algorithm (Basic Local Alignment Search Tool). www. ncbi. nlm. nih, gov/BLAST/available at the http://www.ncbi. nlm.nih.gov and phylogenetic trees constructed using the neighbor joining method (gopher:// megasum. bch, umntreal. ca: 70/11/GDE).

Results and Discussion

The perusal of data revealed cultural morphological variations between isolates of *Ganoderma* isolated from infected palms of arecanut in southern dry tracts of Karnataka. The colony diameters on 5th, 7th and 9th day after inoculation varied significantly among arecanut isolates, where radial growth ranged from 2.08 to 7.83 cm on 5th day after inoculation. Similarly on 7th and 9th day also it varied significantly. Many variations were observed with respect to colony/ mycelial characteristics in different isolates of arecanut (Table 3, Fig. 1 and 2). The number of days taken to cover full plate ranged from 7 to 18 days and few isolates covered entire plate in 7 days, as observed in AG_1, AG_7, AG_8, AG_9, AG_{21} and AG_{22}. However, most isolates took more than 10 days to cover the entire plate. The biomass production also varied significantly between different isolates of *Ganoderma*. Many of variation were observed with respect to colony/ mycelial characteristics of different isolates of *Ganoderma* (Table 3, Fig. 1 and 2). Dendrogram generated from cultural morphological characteristics of *Ganoderma* isolates showed clear variations among them. The isolate AG_{15} and AG_2 were distinct. Complete similarity (100 %) was found in several isolates of *Ganoderma* regardless of their geographical origin (Fig. 2). The present study is in agreement with the findings of Rakib *et al.* (2014) who had studied genetic morphological variability in forty six isolates of *Ganoderma* causing basal stem rot and upper stem rot in oil palm and reported significant variations within and between *Ganoderma* species in terms of their cultural morphology and basidiospore characteristics, and also reported that cluster analysis of cultural morphology and scattered plot of basidiospore features indicated in distinct relationship within and between species, disease types or geographical origins of *Ganoderma* species. Wide range of variation in morphological characteristic can be related to heterogeneity of *Ganoderma* species.

Cultural characteristic that distinguished *G. zonatum* from *G. boninense* and *G. miniatocinctum* was wavy characteristic of the colony in *G. zonatum*. However, this characteristic also varied and was not present in all *G. zonatum* isolates. Furthermore, the cultural appearances of fungi were also highly dependent on several factors such as type of media, pH and temperature (Adaskaveg and Gilbertson, 1989). Although similar (100 % similarity) cultural morphological features were observed between G_3 and G_4, G_{15} and G_{33}, G_{19} and G_{27}, and G_{30} and G_{31} based on the dendrogram generated, they were still genetically different based on the somatic incompatibility between the isolates. This showed that different genotype in *Ganoderma* species may express similar morphological features (phenotype). The dendrogram also showed similar species of *Ganoderma* may be separated by up to 40 % dissimilarity, while

Table 1: Identity and designation of *Ganoderma* isolates of arecanut and their source of collection

Source of isolation	Collection site	Identity and designation of *Ganoderma* isolates
Root sample	Mallenahally, Channarayapattana Tq. Hassan Dist.	Ag_1
Root sample	Jodigatte, Channarayapattana Tq. Hassan Dist.	Ag_2
Sporocarp	Shettikere, C.N.Halli Tq. Tumkur Dist.	AG_3
Sporocarp	Thimmanahalli, C.N.Halli Tq. Tumkur Dist.	AG_4
Root sample	Hosuru, BeluruTq. Hassan Dist.	Ag_5
Sporocarp	Bidarahally, HassanTq. Hassan Dist.	Ag_6
Root sample	Adivala Hiriyur Tq. Chitradurga Dist.	Ag_7
Root sample	Hosahally, TipturTq. Tumkur Dist.	Ag_8
Sporocarp	Kodipalya, Tumkur Tq. Tumkur Dist.	Ag_9
Root sample	Vadavalughatta, TipturTq. Tumkur Dist.	Ag_{10}
Sporocarp	Thyagaturu, Gubbi Tq. Tumkur Dist.	Ag_{11}
Sporocarp	Nagalapura, Gubbi Tq. Tumkur Dist.	Ag_{12}
Root sample	Kalkodi, Gubbi Tq. Tumkur Dist.	Ag_{13}
Root sample	Ammanagatta, Gubbi Tq. Tumkur Dist.	Ag_{14}
Root sample	Doddenahalli, Turvekere Tq. Tumkur Dist.	Ag_{15}
Root sample	Doddanaramangala Tumkur Tq Tumkur dist	Ag_{18}
Sporocarp	Chiknaramangla Tumkur Tq. Chitradurga Dist.	Ag_{19}
Root sample	Doddakittadahalli, HosdurgaTq. Chitradurga Dist.	Ag_{20}
Root sample	Krishnagiri, Hiriyur Tq. Chitradurga Dist.	Ag_{21}
Root sample	Malsandra Tumkur Tq. Chitradurga Dist.	Ag_{22}

Note: AG-Arecanut *Ganoderma*

Table 2: Cultural morphological characters and their corresponding codes used to describe *Ganoderma* isolates for assessment of cultural morphological characteristics

Characters	Description	Code
Days for full plate	< 8	1
	8-9	2
	10-11	3
	> 11	4
Biomass (g 100 ml^{-1})	< 1	5
	1-1.25	6
	> 1.25	7
Colony colour	White	8
	Creamy white	9
Mycelia texture	Smooth	10
	Leathery	11
	Fluffy	12
Concentric rings	Present	13
	Absent	14
Reverse pigmentation	No pigmentation (White)	15
	Pale yellow	16
	Yellowish	17
	Yellow	18
	Pinkish	19
Mycelia density	Thin	20
	Dense	21
	Thin at center and dense at corner	22
	Dense at center	23
Margin	Filamentous	24
	Even	25
	Undulate	26
	Erose	27
	Lobate	28

different species of *Ganoderma* may have up to 92 % similarity. This indicates that *Ganoderma* species in an oil palm plantation can not be separated according to their species, disease type or geographical origins based on their cultural morphological features. Hence, cultural characteristics observed in this study may not be useful as an identification tool. More precise tool such molecular techniques/tools should be used to identify the *Ganoderma* species accurately (Rakib *et al.,* 2014).

Genomic DNA of different isolates of *Ganoderma* was isolated by CTAB method, and the size was determined by resolving on one per cent agarose gel. The DNA obtained was about 600-650bp compared to the whole genomic DNA of *Ganoderma* spp. The concentration of DNA was determined using nanodrop equipment and the concentration was approximate by 75 µg $µl^{-1}$

Full length ITS rDNA region was amplified with ITS region with fungal universal primers (ITS1 and ITS4) and *G. lucidum* specific primers from total genomic DNA of all five isolates of *Ganoderma*. DNA amplicon was 600-650 bp in length in universal primers (Fig.3) and DNA was not amplified with *G. lucidum* specific primers. The results revealed that *G. lucidum* species was absent in *Ganoderma* isolates tested. Further, the species identity was confirmed with DNA sequencing.

The ITS rDNA fragments of *Ganoderma* isolates were sequenced and DNA amplification from *Ganoderma* was observed at good specificity for the genus *Ganoderma* and approximately 600-650 bp product was exclusively amplified in all the isolates tested with fungal universal primers. DNA sequences of selected isolates of arecanut were compared using bioinformatics tool like NCBI (National Centre for Bioinformatics) BLAST programme. Based on the sequence comparison, identification of *Ganoderma* isolates was confirmed and all the ITS rDNA sequences of the isolates were confirmed as of *Ganoderma* sp. with 89-99.49 % identity. The list of isolates, source of isolation and accession number are given in Table 4 and Phylogenetic tree of *Ganoderma* constructed with ITS region sequences is shown in Fig. 4 a and 4b.

Abundance and uniform distribution of genetic markers in any pathogen is necessary for diversity analysis at various levels. Presently, DNA markers are a class by themselves. Almost unlimited in number, they are widely and evenly distributed in the genome. Unaffected by other genes and environment, genotype of any individual of a population with respect to DNA based markers can be determined unequivocally at any stage of the development non-destructively. In addition, it is possible to generate markers to suite specific applications without altering the genotype of the individuals. It is difficult to distinguish these species using traditional morphological and physiological differences. To understand existence of variation among the isolates of pathogens, PCR based technique, *i.e.,* ITS (Internal Transcribed Sequence) was used in the present investigation. Variations in morphological characteristics of *Ganoderma* have led taxonomists to introduce biochemical and molecular methods to differentiate *Ganoderma* species (Muthelo, 2009).

DNA amplification from *Ganoderma* was observed at good specificity for the genus *Ganoderma,* and approximately 600-650 bp product was exclusively amplified in all the isolates tested with fungal universal primers. However, DNA amplification was not amplified with *G. lucidum* specific primers in the isolates tested. Ribosomal genes and their ITS and IGS spacer regions have been widely used for the identification and differentiation of species (Fouly *et al.,* 1997) as well as in taxonomic (Driver *et al.,* 2000), phylogenetic (Rakotonirainy *et al.,* 1994) and genetic diversity (Anderson *et al.,* 2001; Uetake *et al.,* 2002) studies with ITS sequences having been reported as useful for discriminating between different species of fungi (Neuveglise *et al.,* 1994; Fouly *et al.,* 1997; Jensen *et al.,* 2001; Anderson *et al.,* 2001; Thomsen and Jensen, 2002). ITS regions have been successfully used to generate specific primers capable of differentiating closely related fungal species.

Amplification of target DNA through PCR with taxon-specific primers is potentially more sensitive and accurate approach than conventional microscopic techniques. Nucleotide sequences from certain regions of DNA reflect phylogeny at

Table 3: Cultural and morphological characteristics/variability of *Ganoderma* isolates of arecanut*

Isolates	Radial growth (cm)			Days taken to cover plate	Colony/ Mycelial characters				
	5 DAI	7 DAI	9 DAI		Biomass g/100ml	Colour/reverse pigmentation	Texture/ density	Concentric Rings	Margin
AG_1	7.22	9.00	9.00	7	1.23	White/white	Fluffy/dense	-	Even
AG_2	3.75	4.87	6.21	11	0.80	White/white	Fluffy/dense	+	Lobate
AG_3	3.00	3.60	5.08	15	0.87	White/pinkish	Leathery	-	Erose
AG_4	2.08	2.90	4.25	17	0.89	White/yellowish	Leathery	-	Undulate
AG_5	2.84	3.98	6.00	12	1.00	White/pinkish	Fluffy/dense	-	Undulate
AG_6	2.22	3.46	5.75	13	0.76	White/white	Fluffy/dense	+	Even
AG_7	7.58	9.00	9.00	7	1.26	White/yellowish	Fluffy/dense	-	Filamentous
AG_8	6.87	8.57	9.00	7	1.30	White/yellowish	Fluffy/dense	-	Even
AG_9	7.31	9.00	9.00	7	1.36	White/yellowish	Fluffy/dense	-	Even
AG_{10}	4.18	6.08	7.87	11	0.84	White/yellowish	Fluffy/dense	+	Filamentous
AG_{11}	3.26	5.77	8.07	12	0.78	Creamy/ yellowish	Fluffy/thin	+	Even
AG_{12}	3.08	5.21	6.89	13	0.95	White/pinkish	Fluffy/dense	-	Undulate
AG_{13}	2.95	4.13	5.50	14	0.88	White/white	Fluffy/thin	-	Filamentous
AG_{14}	6.97	8.00	9.00	8	1.05	Creamy white/yellowish	Leathery/dense	-	Filamentous
AG_{15}	7.83	8.57	9.00	8	1.13	White/ yellowish	Fluffy/dense	+	Filamentous
AG_{18}	2.08	2.65	4.75	18	0.56	White/white	Fluffy/thin	-	Even
AG_{19}	2.14	3.00	6.45	16	0.74	Creamy White/white	Leathery/dense	+	Filamentous
AG_{20}	4.95	6.14	8.20	11	1.03	White/ yellowish	Fluffy/dense	-	Filamentous
AG_{21}	7.67	9.00	9.00	7	1.22	White/ yellowish	Leathery/dense	-	Filamentous
AG_{22}	5.26	9.00	9.00	7	1.20	White/ yellowish	Fluffy/dense	+	Filamentous
SEm ±	0.43	0.087	0.042		0.273	-	-	-	-
CD (p=0.01)	1.81	0.822	0.622		1.101	-	-	-	-
CV (%)	13.93	4.77	2.98		9.30	-	-	-	-

Note: + Present; - Absent; DAI-Days After Inoculation *Mean of three replications

Table 4: Details of *Ganoderma* spp. nucleotide sequence data submitted to GenBank

Crop	Source of isolation	Name of isolate	Gen Bank accession number	Site
Arecanut	Sporocarps	AG_3	MN784436	Shettikere, C.N.Halli Tq. Tumkur Dist.
Arecanut	Sporocarps	AG_4	MN784437	Thimmanahalli, C.N.Halli Tq. Tumkur Dist.
Arecanut	Sporocarps	AG_{11}	MN784438	Thyagaturu, Gubbi Tq. Tumkur Dist.
Arecanut	Root Samples	AG_{20}	MN78449	Doddakittadahalli, HosdurgaTq. Chitradurga Dist

various taxonomic levels. Such regions evolve at an appropriate rate in order to supply enough consistent differences to separate the taxa into statistically supported monophyletic groups.

These regions must be present as a single copy in the genome or evolve as a single copy region in order to avoid comparison of different copies in different species (paralogous comparisons) if the region exists as multicopy. Also, the region should have similar function in all organisms (Mitchell *et al.,* 1995). Ribosomal RNA (rRNA) genes, certain ribosomal elongation factors, and genes from nuclear and mitochondrial genomes have been useful for DNA sequence analysis in fungi (Tan and Niessen, 2003; Moreau *et al.,* 2006). Consequently, nucleotide sequence information from relatively conserved genes/DNA segments such as ITS (Moncalvo *et al.,* 1995a, b; Smith and Sivasithamparam, 2000), mitochondrial small subunit (mtSSU) (Hong and Jung, 2004), and nuclear large subunit (LSU) (Lee *et al.,* 2006) rDNA have been widely used in the taxonomy and phylogeny of *Ganoderma* species because the variability of these regions, which is harboured mainly in the introns, provide sufficient resolution at various taxonomic levels. Phylogenetic analysis of ITS sequence data was used to resolve Australian *Ganoderma* isolates into five terminal clades, and showed that number of isolates had been misnamed (Smith and Sivasithamparam, 2000). Based on the phylogenetic analysis of ITS and 5.8S sequence, Latiffah *et al.* (2002) showed that *Ganoderma* isolates from infected oil palm and coconut stumps belonged to same group as classified by PCR-RFLP. Gottlieb *et al.* (2000) also used ITS-

Fig. 1: Cultural and morphological characteristics/ variability of *Ganoderma* isolates of arecanut.

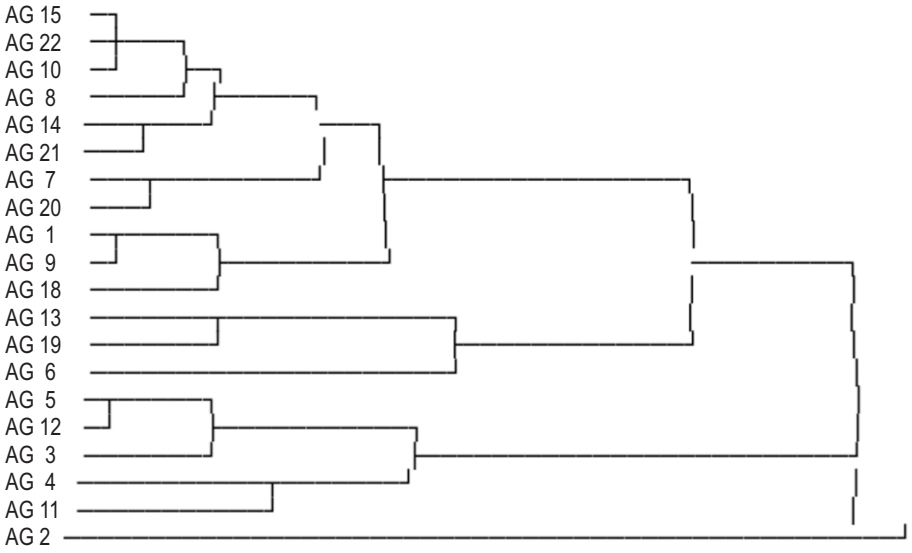

Fig. 2: Dendrogram showing relationships of *Ganoderma* isolates from arecanut based on similarity matrix of cultural/ morphological characteristics.

based phylogenetic analysis together with PCR-RFLPs to elucidate the taxonomy of *Ganoderma* species in South America. They reported that molecular and morphological data agree at subgeneric level, however, it was difficult to determine relationship at species level. Gottlieb *et al.* (2000) adopted rDNA analysis (ITS I and II of 5.8S rDNA) to identify South American isolates of *Ganoderma* and *Elfvingia* and found molecular and morphological agreement at subgeneric level, however, this relationship was difficult to visualize at species level. Singh *et al.* (2003) characterized 61 accessions using DNA finger printing technique and RAPD/ AFLP analysis revealed highly significant genetic variability among *G. lucidum* isolates collected from coconut gardens in Coimbatore. Earlier studies based on morphological identification asserted that North American *G. lucidum* and European *G. resinaceum* belong to same biological species (Adaskaveg and Gilbertson, 1986).

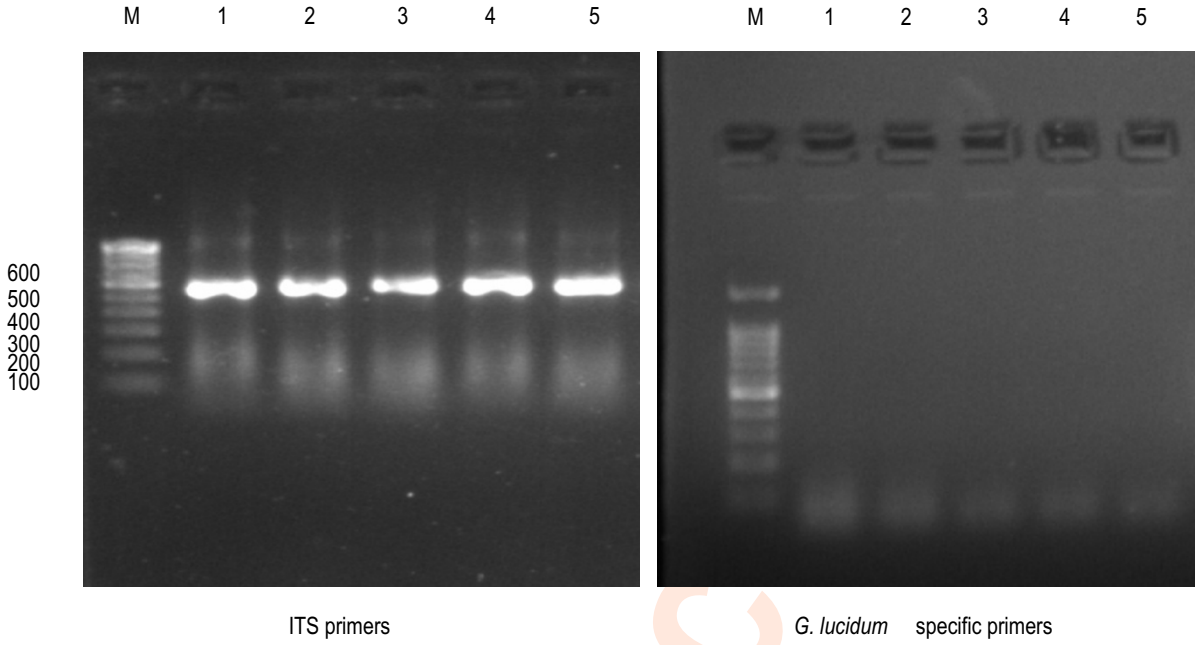

Fig. 3: Gel picture showing PCR amplification of rDNA of *Ganoderma* isolates of arecanut with ITS1, ITS4 and *G lucidum* specific primers. Lane M= 100bp Ladder; Lane 1-5= Ganoderma isolates of arecanut.

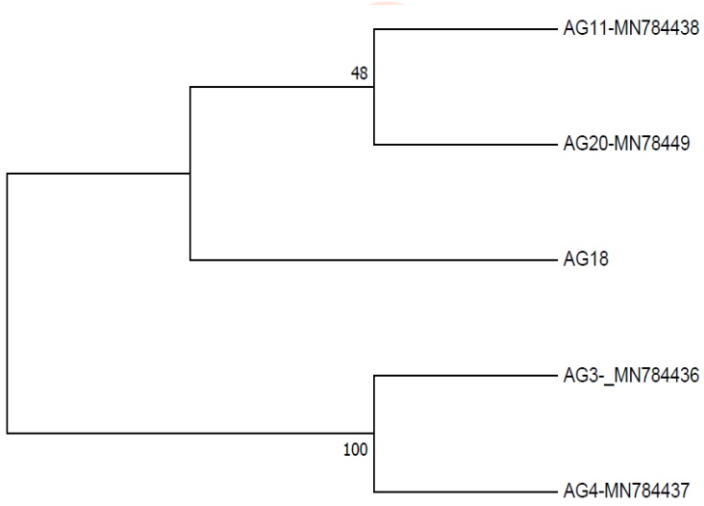

Fig. 4a: Phylogenetic relationships among *Ganoderma* isolates from arecanut inferred from the sequences of ITS region.

Based on phylogenetic relationships and nucleotide sequence variations of ITS (Moncalvo *et al.,* 1995a, b) as well as mtSSU (Hong and Jung, 2004), these two species were found to be different. Gene phylogeny by Moncalvo *et al.* (1995b) indicated that isolates that were morphologically identified as *G. lucidum* did not cluster together, neither those identified as *G. tsugae* or *G. resinaceum*. In the phylogenetic analysis of *Ganoderma* species using mtSSU sequence data by Hong and Jung (2004), *Ganoderma* species were divided into six monophyletic groups (*G. colossus* group, *G. applanatum* group, *G. tsugae* group, Asian *G. lucidum* group, *G. meredithiae* group and *G. resinaceum* group) that included different species identified on the basis of morphological characters. Species identified as *G. lucidum* were scattered over three groups, the Asian *G. lucidum* group, the *G. resinaceum* group and the *G. tsugae* group. Also, isolates that were identified as *G. oregonense*

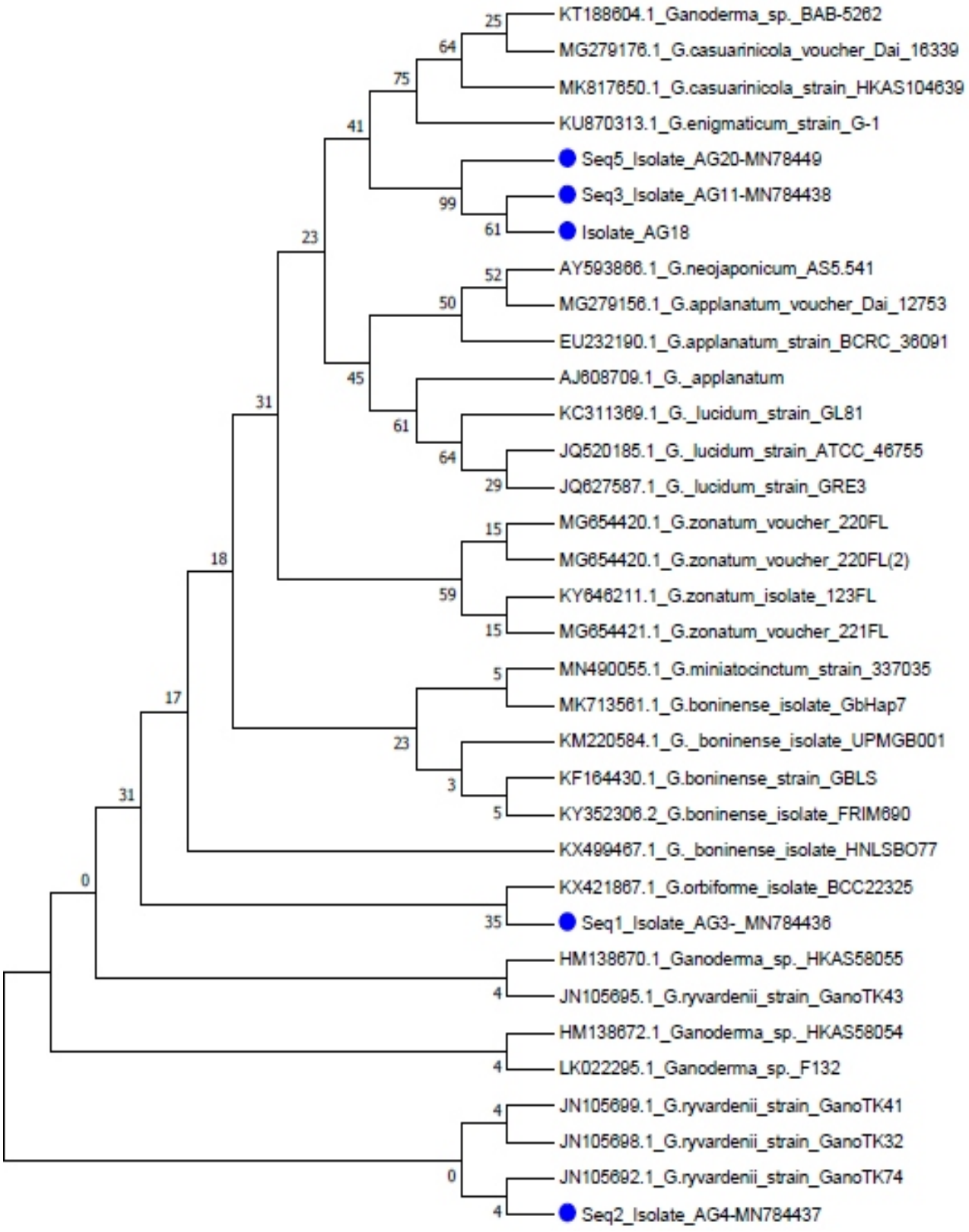

Fig. 4b: Phylogenetic relationships of *Ganoderma* isolates from arecanut inferred from the sequences of ITS region with known species of *Ganoderma.*

and *G. oerstedii* did not group together. These two studies indicate that some isolates were misidentified based on morphological characters since isolates that were identified as one did not form a monophyletic group. From the previous discussions, it is apparent that DNA sequence analysis of ribosomal DNA region has provided an alternative approach to illuminate the taxonomy of *Ganoderma*. These techniques have played an important role in the taxonomy of *Ganoderma*, and have proved to be more reliable than other techniques used for same purpose. Misidentification and species synonyms based on morphological identification have been reduced using molecular techniques (Muthelo, 2009).

Taxonomic correlation of isolates upon NCBI web proved that the isolate were genetically related to *Ganoderma* spp. with 94-100 % identity. Phylogenetic analysis with other known species of *Ganoderma* from Gene Bank, AG_2 and AG_3 emphasize close relationship with *G. ryvardenii* and *G. orbiforme* isolated from Cameroon and Thailand. Isolates AG_{11}, AG_{18} and Ag_{20} were found distinct by forming separate cluster and the results clearly indicated species diversity of *Ganoderma* in Southern Karnataka. Based on gene homology, *G. ryvardenii* and *G. orbiforme* are new species causing foot rot of arecanut in dry tracts Southern Karnataka. In this study, a combination of cultural/ morphological characteristics and molecular techniques allowed identification of groups within *Ganoderma* isolates of arecanut, and the results indicated existence of *Ganoderma* species diversity causing foot rot in dry tracts of Southern Karnataka.

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Add-on Information

Authors' contribution: K.B. Palanna: Conceptualization, Data curation, Investigation, Methodology, Resources, Software, Supervision, Validation, Writing-Original draft, Writing-review and editing; **S. Basavaraj, K.R. Shreenivasa, T. Narendrappa:** Invistigation, Data curation, Methodology, Resources, Supervision, Writing-review and editing.

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