*J. Hortl. Sci.*
*Vol. 17(2) : 00-00, 2022*

***Original Research Paper***

# Morphological and molecular diversity of *Ganoderma* spp. causal agent of basal stem rot of coconut in Southern dry tracts of Karnataka

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## ABSTRACT

**Morphological and molecular diversity of *Ganoderma* species causing basal stem rot of coconut in Southern dry tracts of Karnataka, India was carried out during 2016-17. A total of 20 isolates were isolated from Chitradurga, Chikamagalore, Hassan and Tumkur districts of Karnataka and were identified based on morphological and molecular characteristics. Sporocarps and diseased root bits were found as good source for isolation of *Ganoderma*. In all the isolates there were high variability in cultural, morphological and molecular characteristics. The dendrogram generated from the cultural and morphological characteristics showed clear variations among *Ganoderma* isolates and formed two main clusters, one cluster consisted of 13 isolates and another cluster consisted of 7 isolates. Several isolates showed 100 per cent similarity in the morphological characters regardless of their geographical origin. All the *Ganoderma* isolates amplified a fragment of 650 bp with fungal universal primers (ITS1 and ITS4). The ITS gene sequences of five isolates *viz*., $CG_1$ (MK 681870), $CG_7$ (MK681871), $CG_{11}$ (MK681872), $CG_{14}$ (MK681873) and $CG_{20}$ (MK681874) were deposited in NCBI gene bank. Taxonomic comparison of the isolates with NCBI database proved that the isolates were genetically related to *Ganoderma* spp. with 80-100 per cent identity. However, all the tested isolates could not amplify *G. lucidum* species specific markers which indicate its absence in the region. The phylogenetic analysis of the *Ganoderma* isolates (ITS1 and ITS4) of coconut with other known species of *Ganoderma* from GenBank emphasized the close relationship with India, China and Sri Lanka isolates. The isolate $CG_1$ grouped with *Ganoderma carnosum* (KR 733545.1) with 98.97 per cent identity which is isolated from Sri Lanka and $CG_{14}$ and $CG_{20}$ grouped with *G. applanatum* (MF 072395.1) and *G. gibbosum* (OM 350473.1) with 98 to 99 per cent identity and $CG_7$ and $CG_{11}$ isolates of coconut grouped into distinct sub cluster and clearly indicated the species diversity in *Ganoderma* infecting coconut in Southern Karnataka.**

**Keywords**: Coconut, DNA sequence, *ganoderma* wilt, ITS, phylogeny and variability

## INTRODUCTION

Coconut (*Cocos nucifera* L.) belonging to family Arecaceae is an important plantation crops of India providing livelihood to a substantial number of farm families. The versatile palm popularly known as 'King of Palms', 'Tree of Heaven', 'Tree of life', 'Tree of Abundance', as well as 'God's gift to mankind', is grown in more than 93 countries within an area of 12.8 million hectares and production of 10.9 m MT (copra equivalent) in 2001. The total area and the production in Asian Pacific Coconut Committee (APCC) countries are estimated at 11.4 mha and 9.2 m MT respectively, which is 90 and 84 per cent of world area and production (Rethinam and Taufikkurahman 2002). In India, coconut palms are grown in an area of 2.17 million hectares with a production of 20,308.70 million nuts and a productivity of 9345 nuts/ha annually (CDB, 2019-20). Kerala ranks first in terms of area and production followed by Tamil Nadu, Karnataka and Andhra Pradesh, while, Tamil Nadu ranks first in the productivity followed by Andhra Pradesh and Kerala.

Coconut palms are normally affected by various biotic and abiotic stresses resulting in drastic reduction in yields. Among the various biotic stresses that affect coconut production in India, Basal Stem Rot (BSR) or *Ganoderma* wilt caused by *Ganoderma applanatum* Pers and *G. lucidum* (*Leys*). Karst. is a major constraint in coconut production, especially in dry tracts of Southern Karnataka. The disease is reported from various places all over the tropical world *viz*., India, Sri Lanka, West Indies, Seycheles, Guam etc., Though the disease was first recorded by Dr. Butler in the beginning of 20$^{th}$ century and later by Venkatanarayan (1936) from Karnataka, a severe outbreak occurred in 1652 in Thanjavur district of Tamil Nadu and hence named as Thanjavur wilt. The disease is also reported from Andhra Pradesh, Kerala, Maharashtra, Gujarat and Orissa (Bhaskaran, 1994; Wilson *et al,* 1987). *Ganoderma* species are important wood decaying fungi occurring throughout the world. They are diverse in the tropics affecting plantation crops such as coconut, arecanut and oil palm by causing basal stem rot (Flood *et al.,* 2000 and Pilotti, 2005) and they also affect ornamental and forest trees in tropical and temperate areas causing disease and wood rots of timber (Lee, 2000).

The taxonomy of basidiomycetes has traditionally been based on the morphological features of the basidiocarps. Identification based on the basidiocarp features, however, is prone to problems such as absence of basidiocarps during certain times of the year, their morphological plasticity and presence of cryptic species (Moncalvo and Ryvarden, 1997; Gottlieb and Wright, 1999). However, studies had shown that *Ganoderma* species were genetically heterogeneous since wide range of genetic variation were 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 combinations of these for identification of the pathogen. Recently, molecular approach has been adapted to identify *Ganoderma* species such as 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. Accurate identification of the pathogen is pre requisite for designing management strategies. Hence, the present study was undertaken to investigate the diversity of *Ganoderma* species isolated from BSR infected coconut palms in terms of their molecular and morphological characteristics.

## MATERIALS AND METHODS

### Collection of diseased root samples/stem bit and sporocarps of coconut from different places of Southern Karnataka

Different parts of the coconut palms such as diseased root bits/stem bits affected by *Ganoderma* wilt showing typical symptoms and sporocarps were collected from infected palms from various places of Southern Karnataka (Table 1). The samples were labeled and packed in polythene bags for the purpose of isolation of the causal organism.

### Isolation and designation of the causal organism isolates

Infected roots/ stem bits collected from infected palms were washed thoroughly with sterile water and cut into small bits/pieces and were surface sterilized in 1 per cent sodium hypochlorite solution for 30 seconds and rinsed with sterile distilled water thrice serially to remove the traces of sodium hypochlorite. 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 was observed and that was transferred into potato dextrose agar (PDA) medium. The inoculated plates were incubated at room temperature (28 °C ± 2 °C) for 3-5 days to facilitate growth of the fungus. Later, the bit of fungal growth was transferred to PDA slants. The pure culture of the fungus was obtained by following hyphal tip culture technique under aseptic conditions. The isolated *Ganoderma* isolates of coconut were designated as $CG_1$, $CG_2$, $CG_3$, $CG_4$, $CG_5$, $CG_6$, $CG_7$, $CG_8$, $CG_9$, $CG_{10}$, $CG_{11}$, $CG_{12}$, $CG_{13}$, $CG_{14}$, $CG_{15}$, $CG_{16}$, $CG_{17}$, $CG_{18}$, $CG_{19}$ and $CG_{20}$.

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

| Sl. No. | Source forisolation | Place of collection | Designation of *Ganoderma* Isolates |
|---|---|---|---|
| 1 | Sporocarp | Karekodihally, Arsikere Tq. Hassan Dist. | $CG_1$ |
| 2 | Root sample | Haranahally, ArsikereTq. Hassan Dist. | $CG_2$ |
| 3 | Sporocarp | Vittalapura, ArsikereTq. Hassan Dist. | $CG_3$ |
| 4 | Sporocarp | Nagenakoppalu, CR Pattana Tq. Hassan Dist. | $CG_4$ |
| 5 | Root sample | Badarahally, Channarayapattana Tq. Hassan Dist. | $CG_5$ |
| 6 | Root sample | Belagralli, Tiptur Tq. Tumkur Dist. | $CG_6$ |
| 7 | Sporocarp | Hindiskere, Tiptur Tq. Tumkur Dist | $CG_7$ |
| 8 | Sporocarp | Thimmanahali, C.N.Halli Tq. Tumkur Dist. | $CG_8$ |
| 9 | Sporocarp | Anesidri, Hiriyur Tq. Tumkur Dist. | $CG_9$ |
| 10 | Root sample | Dharmapura(H), Hiriyur Tq. Chitradurga Dist. | $CG_{10}$ |
| 11 | Root sample | Venglapura, Hosdurga Tq. Chitradurga Dist. | $CG_{11}$ |
| 12 | Sporocarp | Shettihalli, Hosdurga Tq. Chitradurga Dist. | $CG_{12}$ |
| 13 | Root sample | Thirumalapura Holalkere Tq. Chitradurga Dist. | $CG_{13}$ |
| 14 | Sporocarp | Thalakatta, HosdurgaTq. Chitradurga Dist. | $CG_{14}$ |
| 15 | Sporocarp | Vaderahalli, Holalkere Tq. Chitradurga Dist. | $CG_{15}$ |
| 16 | Root sample | Doddanaramangala, Tumkur Tq. Tumkur Dist. | $CG_{16}$ |
| 17 | Root sample | Kodipalya, Tumkur Tq. Tumkur Dist | $CG_{17}$ |
| 18 | Sporocarp | Shettikere, C.N.Halli Tq. Tumkur Dist. | $CG_{18}$ |
| 19 | Sporocarp | Hullekere, Turvekere Tq. Tumkur Dist. | $CG_{19}$ |
| 20 | Sporocarp | Thyagaturu, Gubbi Tq. Tumkur Dist. | $CG_{20}$ |

**Note**: CG-Coconut *Ganoderma*

**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 in 2 to 3 months.

**Study on variability of *Ganoderma* isolates of coconut**

Twenty *Ganoderma* isolates of coconut isolated during course of investigation were used in variability study.

**Cultural morphological variability of *Ganoderma* isolates**

**Growth on potato dextrose agar**

Twenty *Ganoderma* isolates [$CG_1$, $CG_2$, $CG_3$, $CG_4$, $CG_5$, $CG_6$, $CG_7$, $CG_8$, $CG_9$, $CG_{10}$, $CG_{11}$, $CG_{12}$, $CG_{13}$, $CG_{14}$, $CG_{15}$, $CG_{16}$, $CG_{17}$, $CG_{18}$, $CG_{19}$ and $CG_{20}$] of coconut collected from different geographic locations were cultured on PDA. The morphological characters like colony diameter/growth, biomass production, colony colour, colony margin, mycelial density, appearance of zones, reverse pigmentation etc were studied.

Mycelia plug (6 mm) from seven days 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 with three replications was run simultaneously to avoid bias due to external factors. The diameter was measured daily and the number of days required for maximum growth of mycelium was also recorded. The colony texture, appearance of zone, reverse pigmentation colour, type of colony margin and mycelial density were recorded after seventh day of incubation.

**Growth on liquid media**

The flasks containing 100 ml of sterilized potato dextrose broth (PDB) were inoculated with the 0.6 cm mycelial discs of *Ganoderma* isolates of coconut. 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. The 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 bio mass were transformed into code and a numerical data matrix was generated (Table 2). The data was subjected to cluster analysis using multivariate statistical package (MVSP version 3.13). Similarity matrices were calculated using the simple matching coefficient and a dendrogram was generated using the unweighted pair group method of arithmetic averages (UPGMA) (Pilotti *et al*., 2004).

**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 & dense at corner | 22 |
| | Dense at center | 23 |
| Margin | Filamentous | 24 |
| | Even | 25 |
| | Undulate | 26 |
| | Erose | 27 |
| | Lobate | 28 |

**Molecular characterization of *Ganoderma***

The 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 (M/s Sigma Ltd. and M/s Merck Ltd.). 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) were ground to fine powder using liquid nitrogen. Pre-warmed extraction buffer (1 ml) was added to the samples and it was ground once more in the buffer. All the samples were transferred to 2.0 ml Eppendorf tubes and 5 µL proteinase K (10 mg $ml^{-1}$) 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 RT 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 in version 30-40 times. The samples were centrifuged at 10,000 x g for 10 min at RT. 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 until the traces of ethanol was removed and dissolved in an appropriate amount of TE buffer (50-70 µL). The nucleic acid dissolved in TE buffer were treated with ribonuclease (RNase 10 $mgml^{-1}$), incubated at 37 °C for 30 min and stored at -20°C until further use. The experiment was repeated thrice and the results described as the mean of three independent experiments (Sambrook and Russel, 2001).

**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. The DNA from all the isolates produced clear sharp bands in one per cent agarose gel indicating good quality of the DNA. The DNA has been quantified by comparing with the 1 kb size marker (Genei, Bangalore) and by spectrophotometer (Nanodrop ND 1000).

**PCR amplification of internal transcribed spacer (ITS) region**

The ribosomal DNA (rDNA) unit contains genetic and non-genetic or spacer region. Each repeat unit consisted of a copy of 18S, 5.8S and 28S like rDNA and its spacer like internal transcribed spacers (ITS) and Inter-Genic Spacers (IGS). The rDNA has been employed to analyze evolutionary events because it is highly conserved whereas ITS rDNA is more variable. Hence, it has been 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. 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' -CCCTAAACCTCTCAAA GTCA-3') and GSR (5' -TATCGTACAGGTTCT CGTG -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 1min and 72° C for 1min and a final elongation step of 72° C for 5min.

**Separation of amplified products by Agarose gel electrophoresis**

Agarose gel electrophoresis was performed to resolve the amplified product using 1.4 per cent 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). Four µL of the loading dye was added to 5 µL of PCR product and loaded to the agarose gel. Electrophoresis was carried at 65 V for 1.5 hrs. The gel was observed under UV light and documented using gel documentation unit.

**Sequencing of ITS region:** The ITS region was sequenced from isolates of *Ganoderma* species to confirm the organism and to know the variability present in them. Homology search was done using BLAST algorithm (Basic Local Alignment Search Tool).

## RESULTS

### Cultural and morphological variability/ characteristics of *Ganoderma* isolates of coconut

The results revealed that there were cultural morphological variations between isolates of *Ganoderma* isolated from infected palms of coconut in Southern dry tracts of Karnataka. The colony diameter on 5$^{th}$, 7$^{th}$ and 9$^{th}$ day after inoculation was significantly varied, where radial growth ranged from 1.87 to 8.53 cm on 5$^{th}$ day after inoculation. Similarly on 7$^{th}$ and 9$^{th}$ day after inoculation it ranged from 2.63 to 9.00 cm and 4.75 to 9.00 cm respectively. The number of days taken to cover full plate ranged from 7 to 18 days and most of the isolates covered entire plate in 7 days as noted in $CG_{4,}$ $CG_{7,}$ $CG_{10}$, $CG_{11}$, $CG_{12}$, $CG_{13}$, $CG_{14}$ and $CG_{20}$. However, some of isolates taken <10 days to cover entire plate. The bio mass production also varied significantly between different isolates and it ranged from 0.56 to 1.46 g/ 100ml. There were lot of variations observed with respect to colony/ mycelial characteristics *viz*., concentric rings. reverse pigmentation, density of mycelium and colony margin. However, there was not much variations were observed with respect to colour and texture of the colony (Fig.1 & 2 and Table 3)

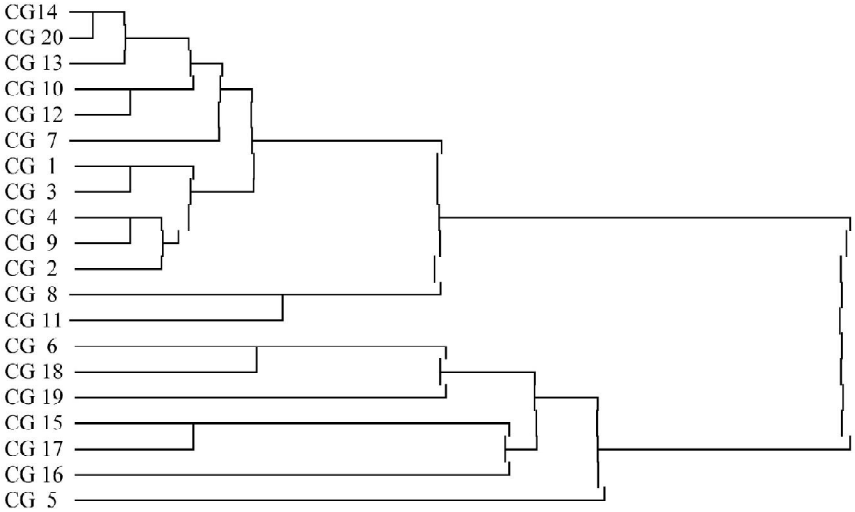



Fig. 1 : Dendrogram showing relationships of *Ganoderma* isolates of coconut based on similarity matrix of cultural/morphological characteristics

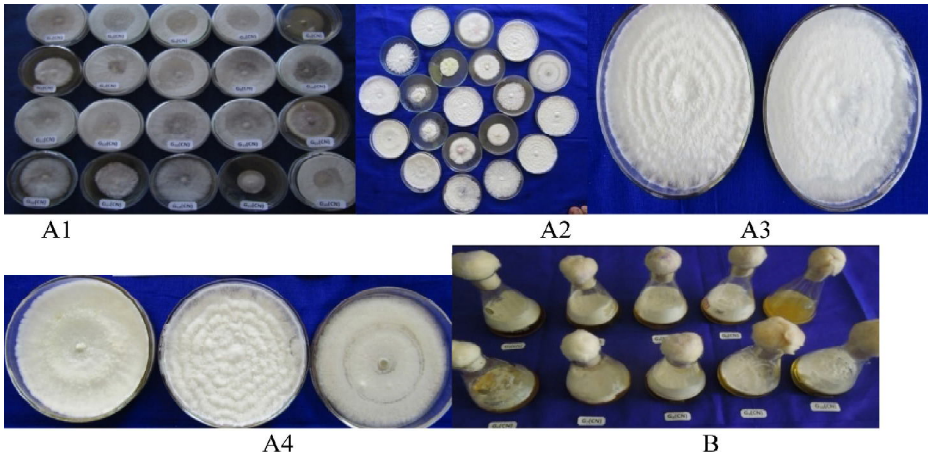



Fig. 2 : Cultural morphological variability *Ganoderma* isolates. A1-A4, ) *Ganoderma* isolates on PDA and B) *Ganoderma* isolates on PDB

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

| Isolates | Radial growth (cm) | | | | | Colony/ mycelial characters | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 5 DAI | 7 DAI | 9 DAI | Days taken | Biomass g/100ml | Colour/reverse pigmentation | Texture/ Density | Concentric Rings | Margin |
| $CG_1$ | 6.16 | 7.75 | 9.00 | 9 | 1.27 (6.48) | White /white | Fluffy/dense | - | Filamentous |
| $CG_2$ | 7.08 | 8.68 | 9.00 | 8 | 1.17 (6.14) | White/white | Fluffy/dense | - | Even |
| $CG_3$ | 7.63 | 8.95 | 9.00 | 8 | 1.36 (6.69) | White/pale yellow | Fluffy/dense | - | Filamentous |
| $CG_4$ | 8.36 | 9.00 | 9.00 | 7 | 1.09 (5.98) | White/white | Fluffy/dense | - | Filamentous |
| $CG_5$ | 2.50 | 4.75 | 6.25 | 14 | 1.01 (5.75) | Creamy white/pale yellow | Smooth/dense | - | Even |
| $CG_6$ | 2.67 | 4.33 | 6.00 | 11 | 0.77 (5.05) | White/ pale yellow | Fluffy/thin | - | Even |
| $CG_7$ | 8.53 | 9.00 | 9.00 | 7 | 1.46 (6.94) | White/yellowish | Fluffy/dense | - | Filamentous |
| $CG_8$ | 7.89 | 8.64 | 9.00 | 8 | 0.87 (5.33) | White/yellowish | Fluffy/dense | - | Filamentous |
| $CG_9$ | 6.58 | 8.22 | 9.00 | 9 | 1.16 (6.18) | White/White | Fluffy/dense | - | Filamentous |
| $CG_{10}$ | 8.39 | 9.00 | 9.00 | 7 | 1.14 (6.12) | White/ pale yellow | Fluffy/thin | - | Filamentous |
| $CG_{11}$ | 8.34 | 9.00 | 9.00 | 7 | 1.20 (6.28) | White/yellow | Fluffy/thin | - | Filamentous |
| $CG_{12}$ | 8.22 | 9.00 | 9.00 | 7 | 1.07 (5.95) | White/ pale yellow | Fluffy/dense | - | Filamentous |
| $CG_{13}$ | 8.34 | 9.00 | 9.00 | 7 | 1.05 (5.86) | White/ pale yellow | Fluffy/dense | - | Filamentous |
| $CG_{14}$ | 8.26 | 9.00 | 9.00 | 7 | 1.32 (6.58) | White/ pale yellow | Fluffy/dense | + | Filamentous |
| $CG_{15}$ | 2.64 | 5.58 | 7.00 | 14 | 0.95 (5.53) | White/yellow | Leathery/dense | + | Even |
| $CG_{16}$ | 2.47 | 5.50 | 7.50 | 11 | 1.30 (6.54) | White/ yellowish | Fluffy/thin | - | Undulate |
| $CG_{17}$ | 2.08 | 4.08 | 6.25 | 17 | 0.71 (4.81) | White/ yellow | Fluffy/dense | - | Undulate |
| $CG_{18}$ | 3.08 | 6.08 | 8.00 | 15 | 0.87 (5.36) | White/ pale yellow | Fluffy/dense | + | Even |
| $CG_{19}$ | 1.87 | 2.63 | 4.75 | 18 | 0.56 (4.26) | White/white | Fluffy/thin | - | Erose |
| $CG_{20}$ | 8.08 | 9.00 | 9.00 | 7 | 1.27 (6.45) | White / pale yellow | Fluffy/dense | + | Filamentous |
| **SEm ±** | 0.086 | 0.155 | 0.014 | - | 0.226 | - | - | - | - |
| **CD (p=0.01)** | 0.850 | 1.12 | 0.395 | - | 1.348 | - | - | - | - |
| **CV (%)** | 4.998 | 5.273 | 1.719 | - | 7.978 | - | - | - | - |

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

The dendrogram generated from the cultural morphological characteristics showed clearly the variations among *Ganoderma* isolates and dendrogram formed two main groups (Fig.1). The isolate $CG_{14}$ and $CG_5$ are distinct. The complete similarity (100%) was found in several isolates of *Ganoderma* regardless of their geographical origin. All the isolates used under study showed high variability in cultural and morphological characteristics. Rakib *et al*. (2014) who had studied the genetic morphological variability of forty six isolates of *Ganoderma* causing basal stem rot and upper stem rot in oil palm stated that, there were significant variations within and between *Ganoderma* species in terms of their cultural morphology and basidiospore characteristics and they also reported that, cluster analysis of the cultural morphology and scattered plot of basidiospore features indicated that there was no distinct relationship within and between species, disease types or geographical origins of *Ganoderma* species.

The wide range of variation in morphological characteristic can be related to the heterogeneity of *Ganoderma* species. The cultural characteristic that appeared to distinguish *G. zonatum* from *G. boninense* and *G. miniatocinctum* was the strongly wavy characteristic of the colony in *G. zonatum*. However, this characteristic also varied and was not present in all of the *G. zonatum* isolates. Furthermore, the cultural appearances of fungi are 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 same species of *Ganoderma* may be separated by up to 40 per cent dissimilarity, while different species of *Ganoderma* may have up to 92 per cent similarity. This indicates that *Ganoderma* species in an oil palm plantation could not be separated according to their species, disease type or geographical origins based on their cultural morphological features. More precise tool such molecular techniques/tools should be used to identify the *Ganoderma* species accurately.

**Molecular characterization of *Ganoderma* isolates**

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 concentration of DNA was determined using nanodrop equipment which was 75µg/µl.

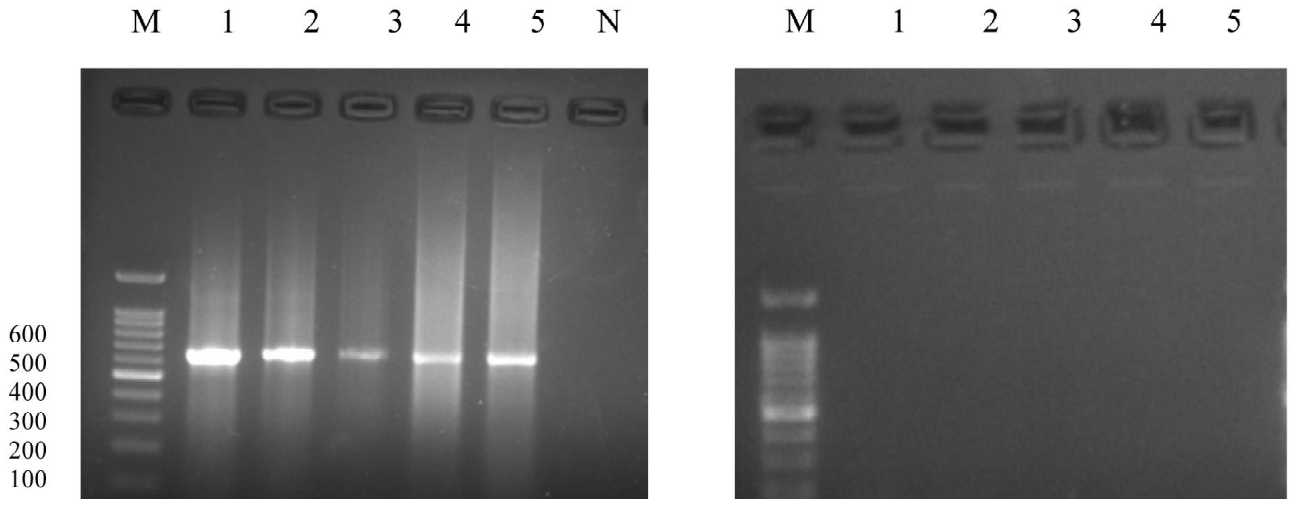
**Amplification of ITS1 and ITS4 region of rDNA**

The full length ITS rDNA region was amplified with ITS region with fungal universal primers (ITS1 and ITS4) and *G. lucidum* specific primers from the total genomic DNA of all the 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 and results revealed that, the *G. lucidum* species was absent in coconut isolates tested. Further, the species identity was confirmed with DNA sequencing.

**DNA sequencing and specific amplification of *Ganoderma* isolates**

The ITS rDNA fragments of *Ganoderma* isolates sequences 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 coconut was compared using bioinformatics tool like NCBI (National Centre for Bioinformatics) BLAST programme. Based on the sequence comparison, the identification of *Ganoderma* isolates was confirmed and all the ITS rDNA sequences of the isolates were confirmed as *Ganoderma* sp. with 80-100 per cent identity. The GenBank accession number for the ITS sequences for the isolates $CG_1$, $CG_7$, $CG_{11}$, $CG_{14}$ and $CG_{20}$ were MK681870, MK681871, MK681872, MK681873, MK681874. and Phylogenetic tree of *Ganoderma* constructed with ITS region sequences is shown in Fig. 4.

The phylogeny of the *Ganoderma* isolates of coconut revealed that, the isolate $CG_1$ grouped with *Ganoderma carnosum* (KR 733545.1) which is originated from Sri Lanka and $CG_{14}$ and $CG_{20}$ grouped with *Ganoderma* sp. (KR154930) and *Ganoderma* sp. (KM229652). These species were originated from India and $CG_7$ and $CG_{11}$ isolates of coconut grouped into distinct sub cluster and indicated the species




**Legend**: Lane M = 100bp Ladder; Lane 1-5 = *Ganoderma* isolates of coconut; Lane N = Negative control

Fig. 3 : Gel picture showing PCR amplification of rDNA of *Ganoderma* isolates of coconut with ITS1, ITS4 and *G lucidum* primers

diversity and dissimilarity of *Ganoderma* in Southern Karnataka.

Abundance and uniform distribution of genetic markers in any pathogen is necessary for applications like diversity analysis at various levels. Almost unlimited in number, they are widely and evenly distributed in the genome. Unaffected by other genes and environment, the 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 with *G. lucidum* specific markers and ITS sequence was used in the present investigation.

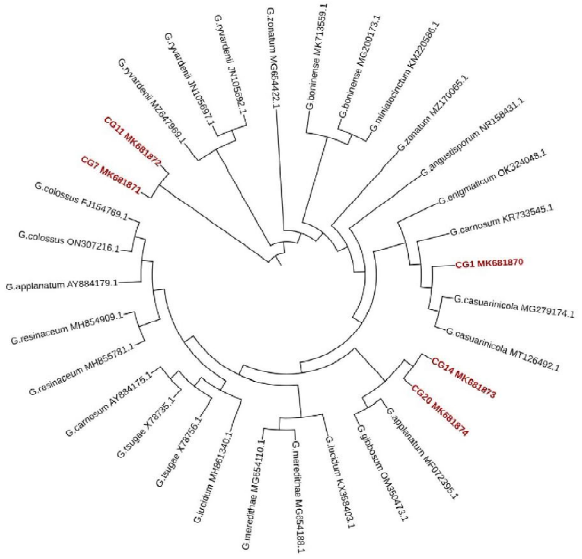



Fig. 4 : Phylogenetic relationships of *Ganoderma* isolates of coconut inferred from the sequences of the ITS region

Variations in morphological characteristics of *Ganoderma* have led many taxonomists to introduce biochemical and molecular methods to differentiate *Ganoderma* species. 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. The sequencing and phylogenetic analysis of selected isolates *Ganoderma* infecting coconut revealed the *Ganoderma* species diversity in dry tracts of Southern Karnataka.

Nuclear rDNA, including the small and large subunits, 5.8S, and the Internal Transcribed Spacer (ITS) region, proved an ideal target for specific PCR primers, as each sequence is variable at the family,

genus, or species level. Internal Transcribed Spacer (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 a potentially more sensitive and accurate approach than conventional microscopic techniques. Nucleotide sequences from certain regions of the DNA reflect phylogeny at various taxonomic levels. Such regions need to be evolving 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 comparisons of different copies in different species (paralogous comparisons) if the region exists as multicopy. Also, the region should have the same function in all organisms (Mitchell *et al*., 1995). The ribosomal RNA (rRNA) genes, certain ribosomal elongation factors, and genes from the nuclear and the 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 the ITS (Moncalvo *et al*., 1995a, b; Smith and Sivasithamparam, 2000a), the 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. This is because the variability of these regions, which is harboured mainly in the introns, provides sufficient resolution at various taxonomic levels.

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 the sub generic level, however this relationship was difficult to visualize at the species level. Singh *et al*. (2003) characterized 61 accessions using DNA finger printing technique and RAPD/ AFLP analysis which revealed highly significant genetic variability among *G. lucidum* isolates collected from coconut gardens in Coimbatore. Phylogenetic analysis of the ITS sequence data was used to resolve Australian *Ganoderma* isolates into five terminal clades, and showed that a number of isolates had been misnamed (Smith and Sivasithamparam, 2000a). Based on the phylogenetic analysis of the ITS and 5.8S sequence, Latiffah *et al.* (2002) showed that *Ganoderma* isolates from infected oil palm and coconut stumps belong to the same group as classified by PCR-RFLP. Gottlieb *et al*. (2000) also used ITS-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 the subgeneric level, but that it was difficult to determine relationships at the species level.

Earlier studies based on morphological identification asserted that North American *G. lucidum* and European *G. resinaceum* belong to the same biological species (Adaskaveg and Gilbertson, 1986). Based on phylogenetic relationships and nucleotide sequence variations of the ITS (Moncalvo *et al*., 1995a, b) as well as the mtSSU (Hong and Jung, 2004), these two species were shown to be different. The gene phylogeny by Moncalvo *et al*. (1995b) has indicated that isolates that were morphologically identified as *G. lucidum* did not cluster together, neither did 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 that were identified based on morphological characters. Species that were identified as *G. lucidum* were scattered over three of the groups, the Asian *G. lucidum* group, the *G. resinaceum* group and the *G. tsugae* group. Also, isolates that were identified as *G. oregonense* 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 thing do not form a monophyletic group.

From the preceding discussion it is clear that DNA sequence analysis of the ribosomal DNA region has provided an alternative approach to elucidate 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 that have been used for the same purpose. Misidentification and species synonyms based on morphological identification have been reduced using the molecular techniques. Among 5 isolates sequenced, isolate $CG_{14}$ and $CG_{20}$ are grouped

in same cluster both in morphological and molecular phylogeny. However, other isolates *viz*., $CG_7$ and $CG_{11}$ which are genetically 100 per cent similar and grouped in same cluster are morphologically different as evidenced by grouping in different clusters in morphological phylogeny. In this study, a combination of cultural/morphological characteristics and molecular techniques allowed identification of groups within *Ganoderma* isolates of coconut and results indicated existence of morphological and molecular variability of *Ganoderma* isolates of coconut causing BSR in dry tracts of Southern Karnataka. Further, molecular characterization with *G. lucidum* species specific markers and fungal universal primers also indicated species diversity in *Ganoderma* causing basal stem rot/ *Ganoderma* wilt in coconut. In the present study based on phylogenetic analysis isolate $CG_1$ was identified as *G. carnosum* with 98.97 per cent identity and isolates $CG_{14}$ and $CG_{20}$ showed maximum (98.96 to 99.46 %) identity with *G. gibbosum* and *G. applanatum* species and indicating the different species associated with *Ganoderma* wilt of coconut in dry tracts of Southern Karnataka. However, the species identity has to be confirmed by systematic investigation with polyphasic taxonomic approach to unravel the species diversity of *Ganoderma* causing basal stem rot in coconut in Karnataka.

## ACKNOWLEDGEMENT

The authors gratefully acknowledge ICAR-AICRP on palms, Agriculture Research Station, Arsikere and Department of Horticulture, Government of Karnataka for providing base line information on coconut cultivation and Department of Plant Pathology, University of Agricultural Sciences, GKVK, Bengaluru for providing facilities to carry out this work.

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