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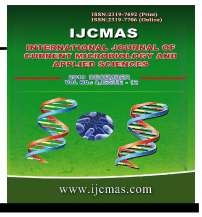

Original Research Article

Ganoderma adspersum and *Ganoderma cupreum* from South India, First report based on molecular phylogeny

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Keywords

Ganoderma; molecular phylogenitic study; ribosomal RNA coding gene; ITS1 & ITS2

A B S T R A C T

A large number of *Ganoderma* were collected from different states of South India and characterized though conventional methods of identification. The isolates which were identified through conventional method were eliminated and those which were not identified were included for molecular phylogenitic study. In such as way, ten numbers of unidentified isolates ware taken to this study and subjected to ribosomal RNA coding gene (ITS1 & ITS2) sequence study. The sequence results showed that the nucleotide sequence size between 600 to 650 base pairs and alignment showed 618 aligned nucleotide positions. ITS1 region ranged upto 220 positions and ITS2 region ranging from 380 to 618 positions. The maximum parsimony tree was constructed with the comparative sequence of different species of Ganoderma derived from GenBank/NCBI. Based on the cladistics, 5 of isolates were identified as *G.adspersum* and remaining 5 of isolates were identified as *G.cupreum*. In the cladistic, the native isolates of *G.adspersum* clustered with the isolates of Germany, Finland and Italy collection with 88% boot strap support and *G.cupreum* isolates associated with Australian collection with 98% of strong bootstrap support. Since there are not previous reports from India, These could be a first report on identification of *G.adspersum* and *G.cupreum* in South India.

Introduction

The genus *Ganoderma* was established by Karsten (1881) for the laccate and stipitate white rot fungus *Polyporus lucidus*. The genus *Elfvingia* was erected later by Karsten (1889) based on *Polyporus applanatus* for the non-laccate species. Since then, over 290 taxonomic names in the genus of *Ganoderma* (Ryvarden, 2000) have been published, indicating that this genus is morphologically complex. The taxonomy of *Ganoderma* species is not clear and it has been noted that the genus is in a state of taxonomic crisis (Ryvarden, 1991). Traditional identification of

Ganoderma species based on the morphological and physiological features and its results are neither well established nor universally accepted (Gottlieb *et al.*, 2000). Besides, it has been shown that the nature of the fruiting bodies and cultural characteristics of species from the same genus can be greatly affected by environment. This signifies that a large number of synonyms may exist due to the number of species that have been identified based on morphology (Chen, 1993 and Moncalvo, 2000). Identification of *Ganoderma* based on these characteristics have contributed greatly to the confusion of naming species within this genus and have resulted in traditional taxonomic methods being inconclusive for establishing a stable classification system for *Ganoderma* species (Hseu et al. 1996; Hong et al. 2002).

Traditional identification parameters are rapidly becoming outdated and new identification methods are being investigated. Alternative approaches that have been used to identify *Ganoderma* species includes RFLP method (Miller et al. 1999), DNA sequence analysis (Smith and Sivasithamparam, 2000a; Hong et al. 2002) and isoenzyme electrophoresis (Smith and Sivasithamparam, 2000b). The phylogenetic analysis of amino acid or DNA sequences is also known to have the highest resolving power (Bruns *et al.*, 1991). The different sequences used are internal transcribed spacers (ITS-rDNA) (Moncalvo *et al.*, 1995a) , RNA polymerase subunit genes (RPB) (Nawrath *et al.*, 1990), ATP synthase gene (atp6) (Morelli and Macino, 1984), elongation factor gene (EF-1α) (Roger *et al.*, 1999), β- tubulin gene (Bruns *et al.*, 1991) and manganese superoxide dismutase gene (Mn-SOD) (Wang, 1996). These modern techniques have helped to clarify the distribution of the different species complexes in the genus *Ganoderma.* In the present study, identification of *G. adspersum* and *G. cupreum* from south India has been made based on the ITS - rDNA gene sequences.

Materials and Methods

Collection of Basidiomata and raising of pure culture

The basidiocarps of *Ganoderma* were collected from various locations of Southern India during southwest monsoon from July to September and northwest monsoon, October to December. The details of collected isolates have been shown in table.1.

The pure culture was raised using malt extract agar medium. The actively growing margin region of the basidiomata was surface sterilized with 95% ethanol. A small portion which measured around 2 mm was sliced and placed in sterile malt extract agar medium containing 0.1% Amphistin antibiotic. The plates were incubated for two days at room temperature. The pure culture was raised by subsequent sub-culturing of mycelium into sterile malt extract agar.

Macro and Micromorphological characterization

The isolates of *Ganoderma* were preliminarily identified based on their macro and microscopic characterization. The shape, size and nature of attachment with the host tree were measured as macroscopic characters. The nature of basidiospores and chlamydospores were analysed as microscopic characters thorough wet mount preparations using Melzer's reagent as reactive stain.

Table.1 Details of Host and GenBank accession number of native collections

S.No	Name of the isolate	Host	*GenBank Accession Number for ITS sequences*
1.	*G. adspersum* KL10	*Terminalia bellerica*	FJ655447
2.	*G. adspersum* KL3	*Tamarindus indica*	FJ655449
3.	*G. adspersum* KR24	*Prunus armeniaca*	FJ655451
4.	*G. adspersum* TN20	*Melia azadirachta*	FJ655452
5.	*G. adspersum* TN22	*Grevillea parallela*	FJ655453
6.	*G. cupreum* KL16	*Terminalia bellerica*	FJ655466
7.	*G. cupreum* KR11	*Prunus armeniaca*	FJ655468
8.	*G. cupreum* KR15	*Terminalia bellerica*	FJ655469
9.	*G. cupreum* KR4	*Prunus armeniaca*	FJ655467
10.	*G. cupreum* KR6	*Prunus armeniaca*	FJ655470

The investigation of basidiospores were made in the porous surface of the basidiomata. The clamydospore investigation was carried out from the pure cultures. The isolated pure strains were grown in sterile malt extract agar medium and incubated at room temperature for 10-12 days. The wet mount preparation was made using Melzer's reagent and observed under light microscope. The characters of chlamydospores like occurrence, abundance, shape, size and reaction to the Melzer's reagent were recorded. If the reaction with Melzer's reagent turns the chlamydospore colour into blue or black that was considered as amyloid, brown or reddish brown was considered as pseudoamyloid and no colour change was considered as inamyloid (Wang and Hau, 1991).

Molecular Phylogeny

The molecular phylogeny was carried out through ITS1 & ITS2 gene sequence of ribosomal RNA coding gene. Among the huge number of isolates, the isolates that were identified as other species of *Ganoderma* other than *G. adspersum* and *G. cupreum* through micro and macroscopic characterization was not included for molecular phylogenic study.

DNA extraction

The genomic DNA extraction and PCR amplification were done with few modifications that described elsewhere (Moncalvo *et al*., 1995). The DNA was extracted from freshly raised mycelium. The mycelial sample (ca. 50 mg) was homogenized with 500µl of SDS extraction buffer (3% SDS, 50 mM Tris, 150 mM NaCl and 80 mM Na_2EDTA) and was ground well mechanically. After incubation at 60 °C for 40 min, The protein contents were extracted with an addition of equal volume of chloroform:isoamyl alcohol (24:1, v/v) and centrifuged for 13 000 g for 10 min. The upper aqueous phase was transferred to a new tube and equal volume of ice cold isopropanol was added to precipitate the DNA. The DNA was collected by centrifugation and gently washed with 80% ethanol thrice. Finally, the pellet was resuspended in ca. 50 µl of ddH2O.

PCR amplification and purification of ITS Rdna

The 5.8S nuclear ribosomal RNA gene and the flanking internal transcribed spacers (ITS1 and ITS2) were amplified using primers ITS1 and ITS4 (White *et al*., 1990). The PCR cocktail with a total reaction volume of 50µl contains the following: 1.25 mM each dNTP, PCR buffer with 1.5mM MgCl2, 10 mM of each primer, 0.1U of Taq polymerase and 10 ng genomic DNA. Thermal cycler (PTC-100, MJ Research Inc. USA) was programmed as follows: Single cycle of 95°C for 2 min for initial denaturation; 36 repeated cycles of 94°C for 45 sec for denaturation, 50°C for 45 sec for annealing, and 72°C for 90 sec for extension and a final single cycle of 72°C for 5 min for final extension. The amplified products were determined in 1.2% agarose gel electrophoresis.

Gene sequencing

The purification of amplified product was carried out using QIAQuick (Qiagen) spin column. Twenty microlitres of amplified sample was mixed with 20 µl of eluting buffer (provided in the kit) and the mixture was loaded in the spin column. The spin column was fitted with collecting vial and centrifuged at 2000 rpm for 5 minutes at 4 °C. The purified sample was transferred to fresh vial and stored at 4 °C for further study.

The direct gene sequencing was carried out by the method of Sanger *et al.,* (1977) using DTCS quick start Dye terminator kit (Beckman Coulter) and Beckman Coulter CEQ 8000 auto analyzer. The cycle sequencing was carried out in the following conditions. The reaction mixture contain 5 µl of sterile water, 2 µl amplified product DNA (10 ng), 5 µl of sequencing primer (1 µM) and 8 µl DTCS master mix. The cycling conditions were as follows: 30 repeated cycles of 90°C for 20 seconds for denaturation, 48°C for 20 sec for primer annealing and 60°C for 60 seconds for polymerization. After cycle sequencing, the removal of unbound dye and nucleotides from cycle sequenced product was carried out using DyeEx spin columns (Qiagen). The purified samples were sequenced in CEQ8000 auto analyzer, Beckman Coulter Inc.USA.

Phylogenetic analysis

DNA sequences obtained from both strands were edited and contigs were assembled using Sequencher TM ver. 4.2.2. The sequences were subjected to BLAST in NCBI website for sequence confirmation and deposited in GenBank to obtained accession numbers. The sequences were aligned manually in multiple alignment mode using Clustal-X Ver.2.0. alignment tool and Maximum Parsimony tree was constructed using MEGA (Molecular Evolutionary Genetic Analysis) Ver.4.0 (Nei and Kumar, 2000). Gaps were introduced into sequences to increase their alignment similarity and the sequence of conserved region 5.8 S located in between ITS 1 and ITS 2 region was excluded from the analysis. The gaps were considered as missing data.

Results and Discussion

The preliminary Microscopic and macroscopic characterization was useful to eliminate the other *Ganoderma* sp. other than the strain of interest. There are four different species like *G. lucidum, G. weberianum, G. tropicum* and *G. resinaceum* were characterized through macro and microscopic characterization was eliminated from phylogenic study. The isolates which could not be identified

were subjected to molecular phylogeny. The size of basidiomata of unidentified isolates showed circular, semicircular in shape and mostly brown or white pileal surface (Fig.1). Most of them are stipitate with short stipes. The basidiospores were semicircular in nature semicircular in nature (fig.2), the culture pattern showed development of white colour mycelium which turned brown in some cultures. There was no presence of chlamydospore was observed.

The DNA extraction was made from native collection of 10 isolates namely *Ganoderma* KL3, KL10, KL16, KR4, KR6, KR11, KR15, KR24, TN20 and TN22 (Table.1). The amplified PCR product of ITS rDNA size was around 600 to 650 bps in 1.2% agarose gel which included the sequences of ITS1, 5.8S subunit and ITS2. The sequence results of the native isolates showed that the total alignment of 618 aligned nucleotide positions. ITS1 region ranged upto 220 positions were found as variable and ITS2 region ranging from 380 to 618 were found as highly variable. Variations of nucleotides and deletions occurred in initial, central and terminal positions. The transitional and transversional ratio was 1.6 and the average nucleotide composition was Adenine - 25.1, Thiamine-29.8, Guanine-25.0 and Cytosine-23.6.

A Maximum parsimony (MP) tree for ITS sequence data set was constructed for 10 sequences of native collection along with 25 ITS rDNA sequences of various species of *Ganoderma* downloaded from GenBank, NCBI (Table.2). A single sequence of *Amauroderma rude* was included as out group taxa. The expanded parsimony tree was constructed and the major clades were resolved with bootstrap value with cutoff of 50%. The cladistics results showed that the native isolates has clusters as two major clades (Fig.3). The 5 native isolates KL3, TN22, TN20, KR24, KL10 had grouped as a major clade along with *G.adspersum* isolates of Germany, Finland and Italy collection with 88% boot strap support. The isolates of TN20, KR24 and KL10 had formed sub group resolving 73% bootstrap with previous clade. Another five native isolates KL16, KR6, KR11, KR15 and KR4 grouped together and was associated with 98% of strong bootstrap support with Australian isolate of *G.cupreum*. Among these 5 isolates, KL16 and KR6 formed a separate group and associated with remaining three isolates with 88% boot strap support.

Ryvarden (1991) stated that the high phenotypic plasticity observed in *Ganoderma* indicates that the group is young and that strong speciation has not yet been achieved. This hypothesis was supported from molecular evidence (Moncalvo *et al.,* 1995).

This study provides novel evidence of the recent origin of laccate *Ganoderma* species. Besides showing low divergence time between species of the *G.lucidum* aggregate, results indicate that temperate taxa believed to be widespread *G. lucidum and G. tsugae* are in fact geographically restricted, and therefore might be too young to have spread worldwide. Using a nucleotide sequence database of type specimens, virtually any *Ganoderma* collection could be identified correctly and synonymies could be established with better confidence. ITS sequences may serve this purpose, but inter and intraspecies nucleotide variation in the ITS region has yet to be investigated (Moncalvo, 1995).

Table.2 Details of comparative ITS rDNA gene sequences of *Ganoderma* spp. obtained from GenBank, NCBI.

S.No	Name of the Isolate	Geographical origin	Accession Number
1.	*Amauroderma rude*	Taiwan	X78753
2.	*G. adspersum* 12	United Kingdom	AJ006685
3.	*G. adspersum* CBS 351.74	Germany	EU162053
4.	*G. adspersum* GaGE99	Italy	AM906056
5.	*G. adspersum* ITA 42	Finland	EF060010
6.	*G. applanatum* E3795	Australia	AJ608709
7.	*G. applanatum* voucher K(M)120829	England	AY884179
8.	*G. australe* UWA 92	Australia	AJ627592
9.	*G. australe* K(M)120828	England	AY884183
10.	*G. cupreum* SUT H1	Australia	AY569450
11.	*G. fornicatum* AS5.539 type 2	Taiwan	AY593860
12.	*G. gibbosum* isolate XSD-63	China	EU326219
13.	*G. japonicum* AS5.69 type 1	China	AY593864
14.	*G. lipsiense* 131R610	Finland	EF060004
15.	*G. lucidum* G1T099	Italy	AM269773
16.	*G. lucidum* HMAS86597	England	AY884176
17.	*G. neojaponicum* AS5.541 type 1	Taiwan	AY593866
18.	*G. oerstedii*	China	DQ425011
19.	*G. pfeifferi* 874	Italy	AM906059
20.	*G. philippii* E7425	Malaysia	AJ608713
21.	*G. resinaceum* G4/13	Italy	AM269778
22.	*G. resinaceum* HMAS86599	England	AY884177
23.	*G. sinense*	China	DQ425014
24.	*G. tsugae* AFTOL-ID 771	USA	DQ206985
25.	*G. weberianum* SUT H2	Australia	AY569451

Fig.1 Basidiomata of *Ganoderma* isolate KL3

Fig.2 Basidiospores of *Ganoderma* isolate KL3

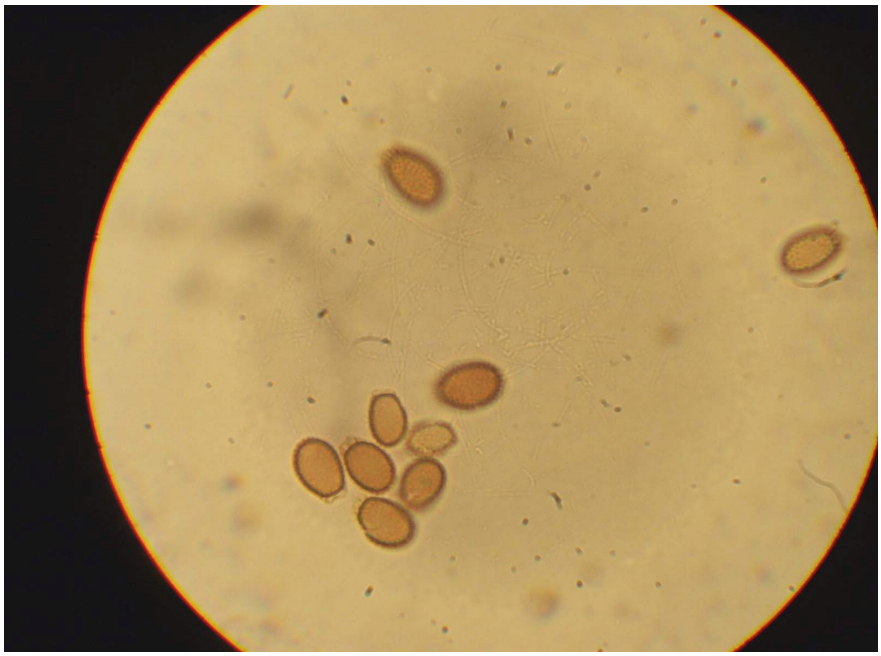
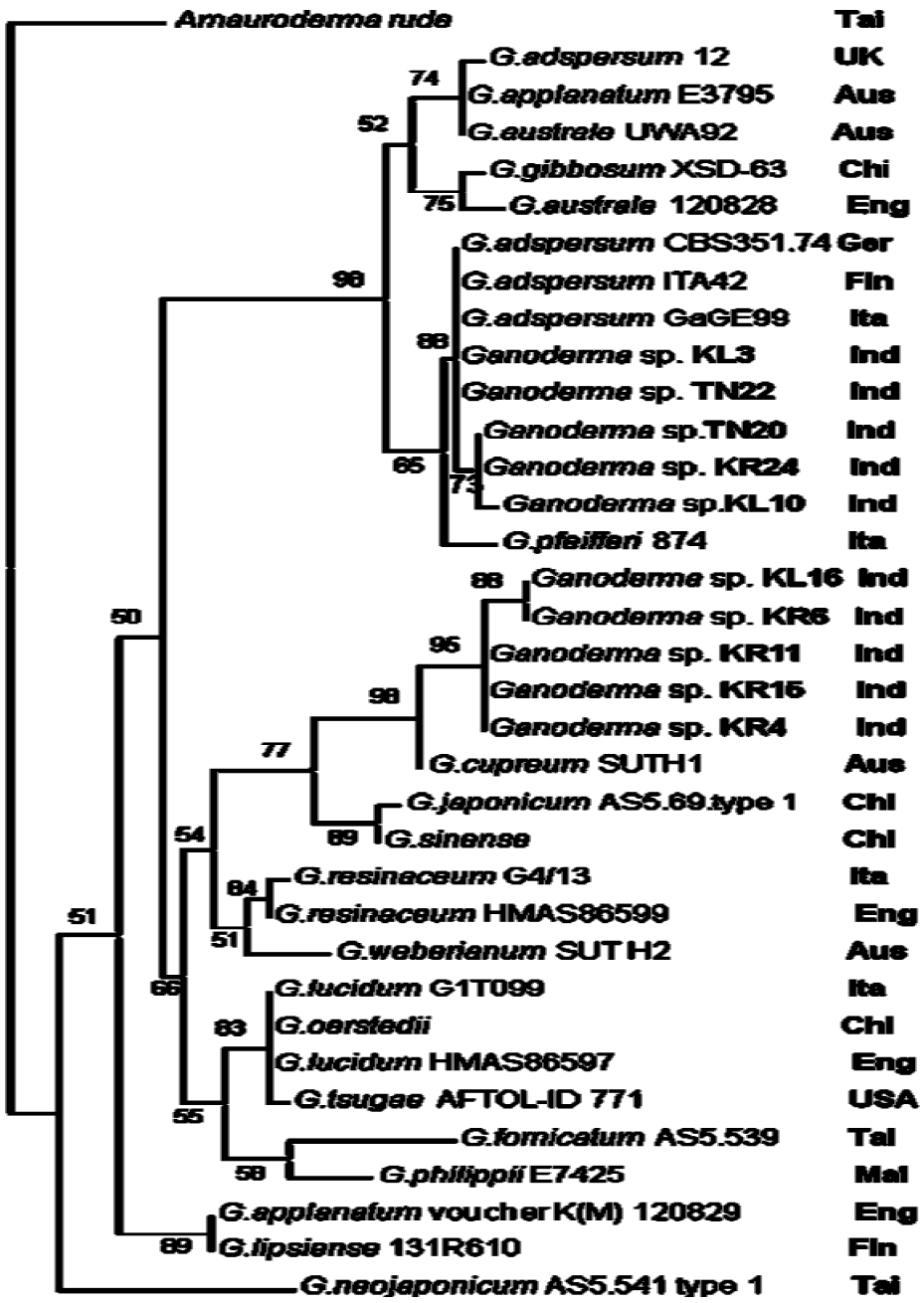


Figure.3 Phylogenetic tree obtained by Maximum Parsimony analysis of the ITS-rDNA showing phylogenetic relationships of *Ganoderma* spp.



The tree was rooted with *Amauroderma rude* as a out group. The native isolates sequenced in this study have been shown in bold. The bold abbreviated codes after the isolate indicates the geographical location; Aus – Australia, Chi – China, Eng – England, Fin – Finland, Ger – Germany, Ita – Italy, Mal – Malaysia, Tai – Taiwan, UK – United Kingdom, USA – United States of America

Our data provided a first significant report on genetic diversity of two biological species of *G. adspersum* and *G. cupreum* from South India. This paper presents the first molecular evidence of *G.adspersum* and *G. cupreum* in South India, which was substantially supported by bootstrapping and nucleotide differences in ITS sequences. The present study indicates DNA sequence divergence rates may serve as a measurement which is indicative of the possibility of gene flow among the *Ganoderma* species. Such gene flow can have a major impact on isolation of biological species and further disjunction into a new species. *G. adspersum* is a wood decaying pathogen which begins its colonization as a parasite, later develops saprophytically (Petersen, 1983). The *G. adspersum* certainly occurs in Europe where the specimens were collected. The morphological, distribution and initial ribosomal sequence analysis could not separate the *G. adspersum* from *G. australe* (Moncalvo *et al.,* 1995a). The correct name of the *G. adspersum* was considered as synonym of *G.australe* by Ryvarden (1976) and Ryevarden and Gilbertson (1993). But comparision of ITS rDNA dataset clearly separated the *G. adspersum* from Australian *G. australe* which was inferred as single species. Molecular characterization or sexual compatibility studies are needed to clarify the status and distribution of the European species in relation to *G.applanatum* (Smith and Sivasithamparam, 2000).

G. cupreum has been referred as *G.chalceum* in previous literatures of Corner (1983) and Hood *et al.,* (1996). According to Smith and Sivasithamparam (2000b), *G.cupreum* has nomenclature priority. Basidiome features were similar to that of Australian collections reported by Smith and Sivasithamparam (2003). The distribution of *G. cupreum* only in Australia and Asia was disagreed with concepts of Steyaert (1967, 1972) but still his interpretations were followed for *G. cupreum* as a priority over *G. chalceum***.** This species seems to be distributed in Australia, Pacific, Northern Africa (Smith and Sivasithamparam, 2003) and our present study has supported its presence in India. Additional molecular evidences from Eastern Asia and province of South Africa are needed for more studies on taxonomy and distribution pattern of *G. cupreum*. Further, di-monokaryotic studies within the biological species are still needed to resolve the gene flow boundaries. There were several attempts made to raise the monokaryotic culture from basidiospores for the compatibility studies but unfortunately it was unsuccessful. The sampling exploration through other parts of India and other continents are needed to elucidate the genetic diversity of *Ganoderma* species complex. In future, the phylogenetic structure will be increased through the additional gene sequences.

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