PHYLOGENETIC RELATIONSHIPS OF *GANODERMA* SPECIES BASED ON MITOCHONDRIAL AND NUCLEAR DNA SEQUENCES FROM TAMIL NADU, INDIA

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ABSTRACT

Phylogenetic relationship of *Ganoderma* species confining to Tamil Nadu was assessed by three molecular markers viz., internal transcribed spacer region (ITS) 1 and 2, β-tubulin and ATP synthase subunit 6 (*atp*6). Forty-three isolates representing 11 species of *Ganoderma* were identified from ITS rDNA sequence data. The nucleotide variations found in the ITS region were suitable for discriminating the eleven species viz., *G. australe*, *G. cupreum*, *G. lucidum*, *G. resinaceum*, *G. tropicum*, *G. weberianum*, *Ganoderma* sp. 1, *Ganoderma* sp. 2, *Ganoderma* sp. 3, *Ganoderma* sp. 4 and *Ganoderma* sp. 5. The terminal clade represent that species and/or species complex were consistent with geographical origin of the isolates and cultural characters. The β-tubulin gene phylogeny provided more robust phylogenetic information for separating ten *Ganoderma* spp. than the ITS region and found to be suitable in identifying the *Ganoderma* species and/or species complex. The *atp*6 gene sequence data acquired highest phylogenetic informative character than nuclear DNA genes; however, due to the very low levels of nucleotide divergence, the gene is suitable at genus or lower level. *Ganoderma* spp. inferred from these three un-linked loci resulted in a robust phylogeny. The terminal clades of nuclear DNA genes represented the species or species complex and consistent with cultural characteristic, host relationship and biogeographical distribution rather than *atp*6. This study implies that the higher variability in the nucleotide sequences of the nuclear DNA genes have the potential to be the robust markers for molecular identification than *atp*6. Chlamydospores in culture provided valuable information in identifying *Ganoderma* spp.

Keywords: *Ganoderma*, ITS rDNA, β-tubulin gene, *atp*6, chlamydospores, multigene phylogeny, biogeography

INTRODUCTION

The genus *Ganoderma* is a member of the Ganodermataceae, a family distinguished by unique double-walled basidiospores. *Ganoderma* has a world wide distribution growing on numerous perennial, coniferous and palmaceous hosts. Species of *Ganoderma* are the causal agents of root and stem rots [1] however, they are considered as the 'herb of longevity' [2]. The genus *Ganoderma* for the laccate and stipitate *Polyporus lucidus* W.Curt was established in 1881 [3] and later erected to the family Ganodermataceae based on the unique double-walled basidiospores [4]. Mycologists from different parts of the world used various criteria for the identification of *Ganoderma* sp. Approximately, 219 species have been described so far and 30% of name in *Ganoderma* have been proposed as synonyms [5]. The macroscopic (pileus, stipe, shape, size, color, context, tube) and microscopic (hyphal system, basidiospores and pilocystidia) characters have been used to distinguish the species [6]. However, the macro-morphological characters are influenced by environmental factors [7], the species identification and circumscription are often difficult and controversial [8] and there are many synonyms and several species complexes have been recognized [6, 7, 9]. Besides basidiomata characters, cultural studies were also investigated [6, 10-13] to discriminate the *Ganoderma* sp. The cultural characters are less polymorphic than the morphological characters [14]. The presences of chlamydospores in the cultures are considered to be useful character in distinguishing between the taxa [15]. Besides, isozyme analysis was carried out to investigate the species [16]. The molecular data have great potential in elucidation of the relationships between taxa and together with recent methodological advances, have instigated a resurgence of interest in phylogeny reconstruction. DNA taxonomy system provides a new scaffold for the accumulated taxonomic knowledge and convenient tool for species identification and description where traditional micro-morphological approaches have failed. Fungal systematists have begun to explore various protein-coding genes, including β-tubulin [17] and mitochondrial *ATP*6 [18]. The number of loci that are used in fungal molecular systematics will continue to grow, especially as more complete fungal genome sequences are produced. For the past one decade, *Ganoderma*

have been explored in different parts of the world using molecular techniques. Phylogenetic studies in *Ganoderma* using rDNA region and confirmed that variation found in the ITS rDNA could be appropriate for the species level identification [14]. Their study implied that many names reported earlier were synonyms. Similarly, ITS marker was used to distinguish the *Ganoderma* species in Australia and South America respectively [16, 19]. In India, all studies for identification was confined to macro-morphological, microscopical characters and cultural studies of the basidiomata alone. Therefore, many names used could be synonym, especially *G. lucidum*, *G. australe* and *G. applanatum* are more frequently described, which led to contradictory view of *Ganoderma* spp. in India and this also emphasized the importance of molecular tool in taxonomic studies. The aim of the present investigation is to identify the *Ganoderma* isolates collected from Tamil Nadu, India through ITS rDNA locus and to infer the phylogenetic relationship of *Ganoderma* spp. Furthermore it is to investigate the utility of partial β-tubulin (nuclear) and *atp*6 (mitochondrial) genes in molecular systematics of *Ganoderma*. Systematics of *Ganoderma* isolates are also ascertained from the combined dataset of nuclear (ITS and partial β-tubulin) and a mitochondrial gene (*atp*6).

MATERIALS AND METHODS

Organisms

Table 1 lists the isolates from Tamil Nadu examined in this study, which includes both basidiocarps and culture collections. Cultures were raised from context tissue of the fresh basidiomata and grown on PDA medium (Difco) at 25 °C.

Cultural characters

The chlamydospore characters were studied only in twelve isolates that could be cultured *in vitro*. The occurrence, abundance, shape, size, and reaction to Melzer's reagent were recorded. The results were later compared with molecular data.

Molecular techniques

DNA was isolated using 3% SDS extraction buffer according to the method described elsewhere [14].The ITS-rDNA region was amplified using primers ITS1 and ITS4 [20] in a reaction volume of 25 ml, as follows: 1.25 mM of each dNTP, 3.5 ml of ddH_2O, 2.5 ml of 10x PCR buffer containing 1.5 mM $MgCl_2$, 2.5 ml of BSA (10 mg / ml), 10 mM of each primer, 0.5 U of *Taq* polymerase and 10 ml of genomic DNA (5-10 ng). Thermal cycling was as follows: one cycle of 95 °C for 2 min; 36 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 1.30 min; and one final cycle of 72 °C for 5 min. Partial β-tubulin gene was amplified by a modified method [21]. The PCR amplification of β-tubulin gene was performed with a primer pair B36/B12 (5'-CACCCACTCCCTCGGTGGTG-3') and (5'-CATGAAGAAGTGAAGACGCGGGAA-3') targeting exon 5 and 6 respectively. Mitochondrial gene, *atp*6was amplified by the modified method [18]. The ligonucleotide primers, ATP6-3 (5'-TCTCCTTTAGAACAATTTGA-3') and ATP6-2 (5'-AATTCTANWGCATCTTTAATRTA-3') were used to amplify the partial *atp*6 gene region. PCR products were purified with QIA quick PCR purification Kit (QIAGEN) and sequenced by the Big Dye v. 3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according the manufacturer's instruction, except that 2 ml of Big Dye was used and the reaction volume was 10 ml. PCR templates were sequenced in both directions using the respective forward and reverse primers. Sequencing reactions were run in an ABI PRISM® 3100 Genetic Analyzer (AppliedBiosystems). Bidirectional reads were assembled and edited in Sequencher™ version 4.2.2. (Gene Codes Corporation, Ann Arbor, MI, USA).

Phylogenetic analyses

First analysis was conducted using only the Tamil Nadu sequences listed in Table 1 with the addition of a sequence of *Amauroderma rude* from Taiwan as outgroup taxon [14]. Sequence alignment was done manually and exported to PAUP* [22] for maximum-parsimony (MP) analyses. MP analyses in PAUP* was conducted as follows: alignment gaps treated as missing data, characters weighted equally, 100 heuristic searches with random addition sequence keeping no more than 100 trees in each replicates, TBR branch swapping, and branches collapsed when minimum length was zero. Bootstrap analyses used 1,000 replicates of random addition sequence with TBR branch-swapping, keeping no more than

10 trees per replicate, and retaining groups with frequency > 70%. The method of assessing the combinability of data sets, and the one adopted in this study, is by simply comparing highly supported clades among trees generated from different data sets to detect conflict [23]. If no conflict exists between the highly supported clades in trees generated from individual data sets, it suggests that the genes share similar phylogenetic histories and combining the data sets could ultimately increase phylogenetic resolution and support. BLAST searches in the NCBI database (Genbank) were used to retrieved highly similar sequences to the Tamil Nadu sequences produced in this study.

RESULTS AND DISCUSSION

Seventeen isolates of *Ganoderma* were collected from different parts of Tamil Nadu were listed in Table 1.

Cultural studies

Ganoderma sp. 1, 3, 4, 5 and *G. australe* do not produce chlamydospores in culture. *Ganoderma* sp. 2 produced inamyloid and amyloid chlamydospores were observed in cultures. The size ranges from 8 µm × 9-11 µm; globose or elliptical; intercalary or terminal in position. In *G. resinaceum*, they were ellipsoidal, hyaline, smooth, dexrinoid, 8.5-14 × 11.5-14 µm; intercalary in position and rarely terminal. *G. tropicum* produce inamyloid cylindrical chlamydospores with the size ranges from 4.5-6.0 µm × 12-6 µm. *G. weberianum* produced abundant chlamydospore with the size ranging from 4.5-7 µm × 8-12 µm; globose, subglobose and elliptical shape but they were not striated; intercalary in position; negative in Melzer's reagent. In addition, gastrospores were observed after 5 weeks.

Phylogenetic analysis of ITS rDNA region

PCR amplification of ITS region of *Ganoderma* was ca. 560 bp. All the positions were generally able to be aligned within the collections of the same species; whereas alignment across the species required gaps. The ITS region varies in length from 546 to 562; the length of ITS1 ranges from 196 to 208 and sequences were aligned in 227 positions; ITS2 ranges from 184 to 199 and aligned in 212 positions. There is negligible amount of difference in AT% (50.3) and GC% (49.7) in ITS region. Seventeen different sequences were identified among the native collections examined (Table 1). Of the 429 characters included in the maximum parsimony (MP) analysis, 240 were constant; 63 variable characters were parsimony uninformative; 126 were parsimony informative. Tree statistics were; tree length=403, consistency index (CI)=0.6402, retension index (RI)=0.8294, rescaled consistency index (RC)=0.5310, homoplasy index (HI)=0.3598. Parsimony analysis based on an alignment of these sequences indicated that the isolates were grouped into 10 well supported clades as measured by bootstrapping (Fig 1). Phylogenetic analysis of ITS rDNA was useful in the identification and classification of 17 isolates of *Ganoderma* into 11 species viz., *G. australe* (5), *G. cupreum* (1), *G. lucidum* (11), *G. resinaceum* (2), *G. tropicum* (2),*G. weberianum* (1), *Ganoderma* sp. 1 (1), *Ganoderm*sp. 2 (1), *Ganoderma* sp. 3 (7), *Ganoderma* sp. 4 (2) and *Ganoderma* sp. 5 (10) (Table 1). The five *Ganoderma* spp. 1, 2, 3, 4 and 5 remained unidentified since they occupied isolated position in ITS phylogenetic tree (Fig. 1).

Non-laccate species (*Ganoderma* subg. *Elfvingia*)

Two strains viz., YER03 and K39 clustered with intersterile groups 1 and 2 of Taiwan isolates TAI-01 (94% Bootstrapping (BS)) and TAI-05 (100% BS), respectively. Although *G. australe* Group 1 (Yercaud) and Group 2 (Kodaikanal) were collected from different localities but they fall on different altitude i.e. below 1500 mts and above 2133 mts respectively. The result obtained in the present study is consistent with the earlier reports [24, 25]. *Ganoderma* sp. 5 was placed in isolated position with 100% BS, and hence considered as a distinct species. More samples were required from other parts of world to study the phylogenetic relationship.

Laccate species (*Ganoderma* subg. *Ganoderma*)

G. cupreum MYC1 tended to group together with collections from Australia with 100% BS. *Ganoderma cupreum* has been referred to as *G. chalceum* in previous literatures [7] however, *G. cupreum* has nomenclatural priority [5, 16]. *G. cupreum* seems to be distributed in Australia, Pacific, Northern Africa [26] and Asia (present study). Additional molecular

Table 1. *Ganoderma* isolates sequenced in this study and sequenced by Dr. Moncalvo

Determination	Source & Collection Number[a]	Geographical origin, Host	Gene Bank Accession
G. adspersum	CBS351.74	Europe, *Salix* sp.	X78742/X78763
G. ahmadii	FWP 14329	Pakistan, *Dalbergiasissoo*	Z37047/Z37098
G. australe	**YER03**	**Yercaud, India, *Bougainvillaea* sp.**	**AY993914/AY993915**
G. australe	**K39**	**Kodaikanal, India, Hardwood,**	**AY993916/AY993917**
. australe	RSH.0705	Taiwan, N.d.	X78750/X78771
G. boninense	LKM254	Sabah, Malaysia, Palm	
G. cupreum	DFP3896	Australia, *Casuarina* sp.	AJ27586/AJ627587
G. cupreum	QFRI4336	Australia, Dead wood	AJ627588/ AJ627589
G. cuperum	**MYC1**	**Coutrallum, India, Hard wood**	**DQ051906/DQ015907**
G. capense	ACCC5.71	China, N.d.	Z37070/Z37104
G. curtisii	JM96/80	North Carolina, USA,	Check
G. lobatum	BAFC651	Chile, Dead stump	AF169983/AF169984
G. lucidum	**I5**	**Chennai, India, Hard wood**	**DQ015904/DQ015905**
G. lucidum	**G14**	**Chennai, India, *Borassusflabillifer***	**DQ011079/DQ011080**
"*G. lucidum*"	ATCC32472	India, N.d.	X87351/ X87361
"*G. lucidum*"	RSH.G001	Taiwan,	X87345/ X87355
G. lucidum	CCRC37053	Taiwan	-
G. lucidum	RYV33217	Norway, Hardwood	Z37096/Z37073
G. microsporum	RSH.0821	Taiwan, *Salix babylonica*	X78751/X78772
G. praelongum	ATCC52410	Argentina, *Platanusacerifolia* (?)	-
G. resinaceum	CBS152.27	United Kingdom, N.d.	Z37062/Z37085
G. resinaceum	**PTK3**	**Chennai, India, *Tamarindusindica***	**DQ011095/DQ011096**
G. subamboinense	ATCC52419	Argentina, *P. acerifolia*	X78736/X78757
G. trophicum	**MYC6**	**Coutrallum, India, *Albizzialebbeck***	**DQ011093/DQ011094**
G. cf. tropicum	RSH.1111	Taiwan, N.d.	Z37068/Z37088
G. valesiacum	CBS 282.33	United Kingdom, *Larix*sp.	Z37056/Z37081
G. weberianum	CBS219.36	Philippines, *Mangifera* sp.	X78734/X78755
G. weberianum	**19**	**Vandalur, India, *Polyalthia* sp.**	**DQ001761/DQ001762**
G. weberianum	DFP8405	NSW, Australia, *Mangifera*sp.	-
G. zonatum	ME-GAN-33	Florida, Oil palm	-
***Ganoderma* sp.**	**CNB1 TN**	**Coonoor, Hard wood**	**DQ011635/DQ011636**
Ganoderma sp.	MW1	Ontario, Canada, Chestnut.	-
***Ganoderma* sp.**	**CN2**	**Coonoor, India, hardwood**	**DQ011633/DQ011634**
Ganoderma sp.	TNAU-CRS-1	Veppankulam, India, *Cocosnucifera*	AY508882
Ganoderma sp.	QFRI8647.1	QLD, Australia, Dead wood	-
Ganoderma sp.	JMHK93.9	Honk Kong, Hardwood	Z37069/Z37089?
Ganoderma sp.	JMCR.132	Costo Rica, N.d.	AF255138
Ganoderma sp.	JM97/3	North Carolina, N.d.	AF255094
Ganoderma sp	TAI-01	Taiwan, N.d.	AF255110/AF255111
Ganoderma sp.	ME-GAN-14	Florida, N.d.	AF255130
Ganoderma sp	TAI-05	Taiwan, N.d.	AF255193/AF255194
***Ganoderma* sp.**	**MC3**	**Tambaram, India, Hard wood**	**DQ011623/DQ011624**
***Ganoderma* sp.**	**G09**	**Chennai, India, *Tamarindusindica***	**DQ015908/DQ015909**
***Ganoderma* sp.**	**MC5**	**Tambaram, India, *Pterolobium* sp.**	**DQ011625/DQ011626**
***Ganoderma* sp.**	**G05**	**Chennai, India, *B. flabellifer***	**DQ056318 /DQ056319**
***Ganoderma* sp.**	**ANN3**	**Chennai, India, *Cassia roxburgii***	**DQ011097/DQ011098**
***Ganoderma* sp.**	**CPT**	**Chengalpet, India, *Mangiferaindica***	**DQ011103/DQ011104**
***Ganoderma* sp.**	**IFE**	**Chennai, India, *Feroniaelephantum***	**DQ011109/DQ011110**
Ganoderma sp.	BJ-1	Indonesia, *Elaeisguineensis*	AY220539
Ganoderma sp.	MUCL27886	India, N.d.	AF255190
Ganoderma sp.	MUCL38595	Zimbabwe, N.d.	-
Amaruoderma rude	JM/ASP.1	Taiwan, on buried roots	X78753/X78744

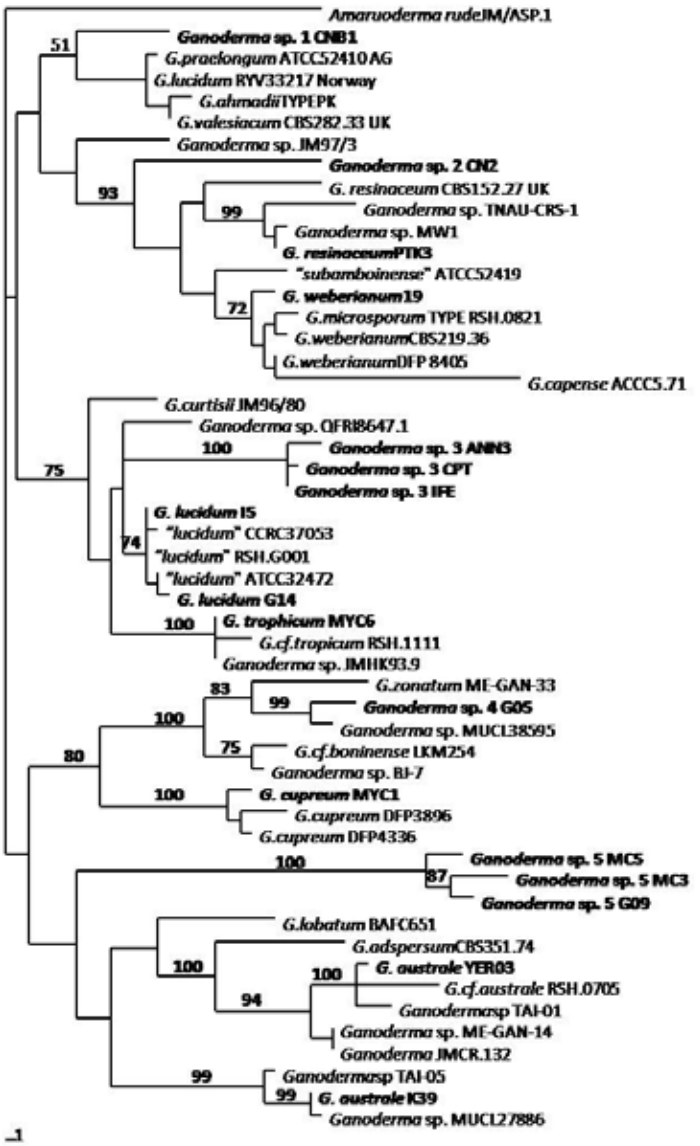

Figure 1. One of the most parsimonious trees inferred from ITS rDNA gene rooted with *Amauroderma rude* JM/ASP.1 sequence. The bold names indicate the eleven *Ganoderma* species from present study. The numbers on branches represent the bootstrap values

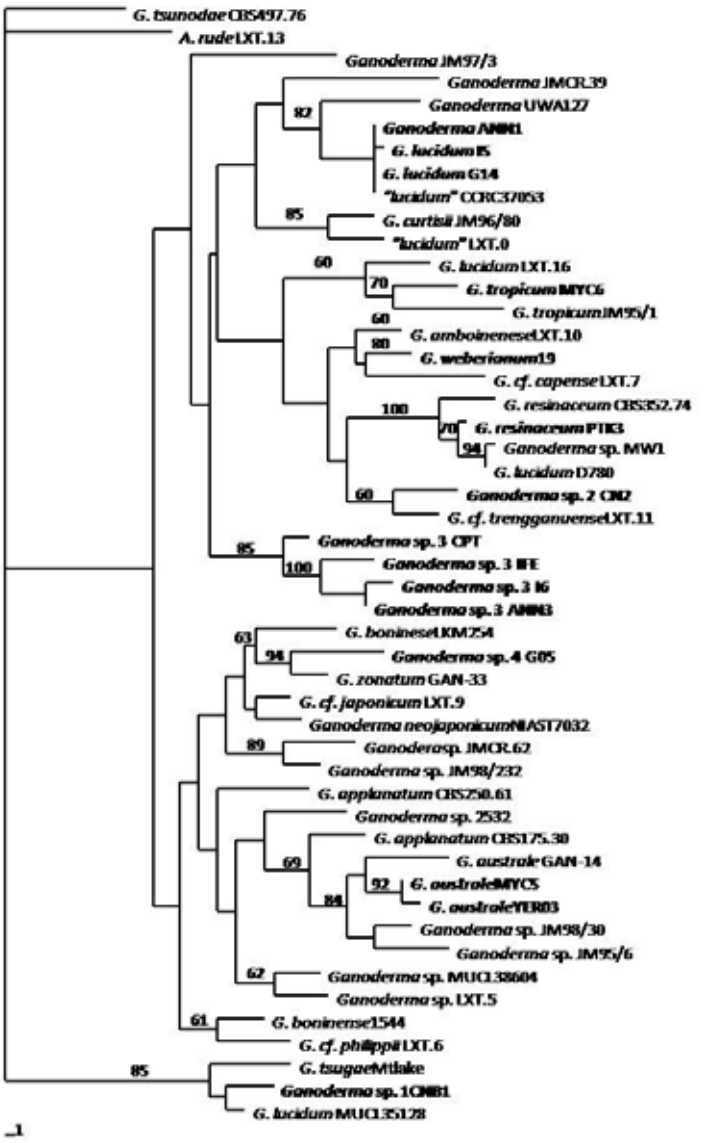

Figure 2. Maximium parsimonious trees inferred from partial β-tubulin gene sequences of *Ganoderma* spp. rooted with *Amauroderma rude* JM/ASP.1. The bold names indicate the *Ganoderma* species from present study. The numbers on branches represent the bootstrap values

evidences from Eastern Asia or South Africa are needed for more studies on taxonomy and distribution pattern of *G. cupreum*, *G. lucidum* (G14 and I5) closely clustered with Asian isolates with strong BS (74%). This clade comprises of *G. lucidum* from Asian counties. The Asian *G. lucidum* were similar with a lower level of nucleotide divergence [14]; they were intercompatible in di-monokaryotic mating studies [12]. But reports are not available whether this species are conspecific or not. Asian *G. lucidum* collected from hardwoods produced chlamydospores, which agrees with earlier reports [14]. *G. tropicum* (MYC6) was closely clustered with Asian collections with 100% BS. This group produces abundant, inamyloid, long, cylindrical chlamydospores which was consistent with earlier reports [27]. These isolates can be distinguished by morphological and cultural evidences [12]. *G. resinaceum* (PTK3) was closely clustered with Indian (TNAU-CRS-1) and North American collection (MW1) with 99 % BS and there is no nucleotide variation. They were completely conspecific irrespective of their geographical distribution or macro-morphological differences [6, 14]. The ITS sequence analysis clearly demarcated the populations of Europe, North America and South America and they were genetically isolated from each other and hence these populations needs more justification at the species level [27]. However, additional sampling

from South East Asia and Australia is required to confirm the taxonomic conclusion. This group produced abundant chlamydospores which were smooth, ellipsoid or subglobose; dextrinoid or inamyloid; intercalary or terminal in position [6, 13]. This species was more or less cosmopolitan in its distribution. *G. tropicum* is distributed throughout the tropical and subtropical regions. Only few collections were reported from Asian countries and additional molecular evidence from Africa would provide the firm taxonomic conclusion of this species. The monophyletic lineage of *Ganoderma* sp. 3, *G. lucidum* and *G. tropicum* are was well resolved (75 % BS). *G. weberianum* was considered to be the counter part of *G. resinaceum* in tropical countries and can be distinguished by cultural and molecular data. The former species closely clustered with Asia-Australian isolates with 72% BS. The robustness of the *G. resinaceum* and *G. weberianum* clade was highly supported (93% BS) and comparable to literature [14]. There is strong support for separating Asian and Australian collections as reported earlier [16]. The chlamydospores of *G. weberianum* were of two types viz., ellipsoid, striated and ellipsoid, non-striated [13]. The latter type was found in *G. weberainum* and was also reported earlier in a Taiwanian collection CCRC37081. In addition, globose, elliptical with echinulate and fissured gastrospores were also produced in less numbers as reported earlier in Australian [16] and Indonesian collections [7]. This result shows that chlamydospore and gastrospore producing groups were clearly demarcated in ITS phylogeny tree of the present study. This species seems to be distributed in temperate and tropical Asia, Australia and Pacific regions [16]. *Ganoderma* sp. 1 (CNB1) was isolated in its position but remain clustered with collections from temperate regions representing *G. lucidum* sensustricto and stands alone in phylogenetic analysis with no clearly supported relationships. *Ganoderma* sp. 1 is phylogenetically distinct from other members by >2.75% nucleotide substitution in ITS region without bootstrapping, therefore, we consider this taxa to be a distinct species. Members of this group lack chlamydospores and seem to be distributed in temperate region of both northern and southern hemisphere [14]. *Ganoderma* sp. 2 differed with *G. resinaceum* by >3.5% nucleotide variation in the ITS region. The distinction in cultural and molecular data was consider, *Ganoderma* sp. 2 could be a distinct species which might be closely related to *G. resinaceum* rather than *G. weberainum*. It produced abundant chlamydospores in culture which is comparatively smaller than that of *G. resinaceum* group but larger than *G. weberianum*. It produces both amyloid and inamyloid chlamydospore. ITS phylogeny shows that *Ganoderma* sp. 2 might be distributed in tropical and subtropical regions; still additional sampling from southern hemisphere is needed to confirm the distribution pattern. Three strains of *Ganoderma* sp. 3 represents only Indian isolates and stands alone in the ITS phylogenetic tree with high BS (100 %) and named as distinct ITS species. Additional data are required to confirm its phylogenetic relationship. *Ganoderma* sp. 4 closely clustered with pathogenic strains isolated from palm with 99% BS. ITS phylogeny clearly shows that the *Ganoderma* sp. 4 might be restricted to southern hemisphere (Zimbabwe and India) (unpublished data). However additional molecular evidence would provide more information concerning the distribution. The *Ganoderma* species 1, 2, 3, 4, and 5 are represented by only a few collections from Asia and South America thus the correct naming of species remains problematic and additional sampling would provide more information.

Phylogenetic analysis of β-tubulin gene

Partial β-tubulin, nuclear DNA gene was amplified with B36 / B12 primer pair which yielded ca. 450 bps. The present investigation revealed that the partial β-tubulin sequences proved to be an excellent tool for the identification of *Ganoderma* species/species complex and also resolved the relationship of *Ganoderma* species. The nucleotide composition was varied among the nuclear DNA genes; in β-tubulin, GC content was 55.5% and AT was 44.6% whereas in ITS rDNA, the GC (49.73%) content comparatively lesser than AT (50.3%). The transition and transversion ratio was comparatively higher (2.84) than ITS rDNA (2.299) gene. 56.1% of GC content of β-tubulin gene was reported in "*G. lucidum*" WC721 [21]. Heuristic searches performed under parsimony criterion (as described in Materials and Methods) produced 20 most parsimonious trees with tree length = 547, consistency index = 0.459, retention index = 0.631. Phylogenetic analysis of β-tubulin sequences was concurrent with ITS dataset and provided greater resolution as assessed by bootstrapping. However *Ganoderma* sp. 5 (MC3, MC5 and G09) and *G. australe* (K39) has been eliminated due to template or PCR product contamination. The remaining nine species remain clustered with respective clades and were consistent with ITS rDNA gene (Fig. 2). The GC content was higher (55.5 %) than that of AT content (44.6 %) and the transition and transversion ratio was comparatively higher (2.84) than ITS rDNA (2.299) gene.

Phylogenetic analysis of *atp*6 gene

The mitochondrial protein coding gene *atp*6 was amplified with ATP3/ATP2 primers which yielded a single PCR of approximately 664 bps. Phylogenetic analysis of *atp*6 gene showed that there was less nucleotide divergence when compared to the nuclear DNA genes (ITS rDNA and β-tubulin). The percentage of the AT (73.5 %) content was comparatively higher than GC (26.5 %) content. The abundance of A/T transversion caused lowest transition / transversion ratio (0.730) than the other two nuclear DNA genes. *G. australe*, *G. lucidum*, *G. resinaceum*, *G. tropicum*, *G. weberianum*, *Ganoderma* sp. 2, *Ganoderma* sp. 3, *Ganoderma* sp. 4 and *Ganoderma* sp. 5 were well resolved as assessed by bootstrapping (Fig. 3). One of the strains of *Ganoderma* sp. 3 (CPT) conflicts with nuclear DNA gene and remain clustered with *G. lucidum*. When compared to ITS rDNA and β-tubulin, *atp*6 provided very little informative character towards the phylogenetics study. However it contributed important information for the resolution of the *Ganoderma* spp.

Multigene phylogenetic analysis

Ganoderma phylogeny inferred from three unlinked loci resulted in a robust phylogeny that is largely in congruence with individual sequence dataset. The terminal and deep branches were well resolved when comparing the ITS, β-tubulin and *atp*6. Combined dataset clearly separated all the 10 species viz., *G. resinaceum, G. weberianum, G. tropicum, G. australe*, *G. lucidum* and unidentified species *Ganoderma* sp. 1, *Ganoderma* sp. 2, *Ganoderma* sp. 3, *Ganoderma* sp. 4 and *Ganoderma* sp. 5. However, *Ganoderma* sp. 3 (CPT) alone conflicted and closely clustered with *G. lucidum* clade with moderate support (70 %) (Fig. 4).

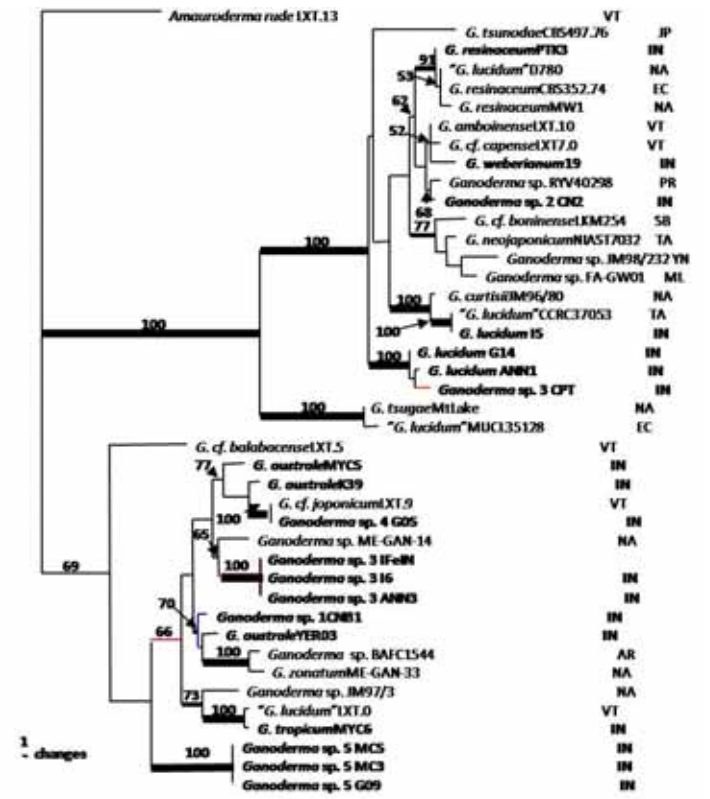

Figure 3. Maximium parsimonious trees inferred from partial *atp6*gene sequences of *Ganoderma* spp. rooted with *Amauroderma rude* JM/ASP.1. The bold names indicate the *Ganoderma* species from present study. The numbers on branches represent the bootstrap values

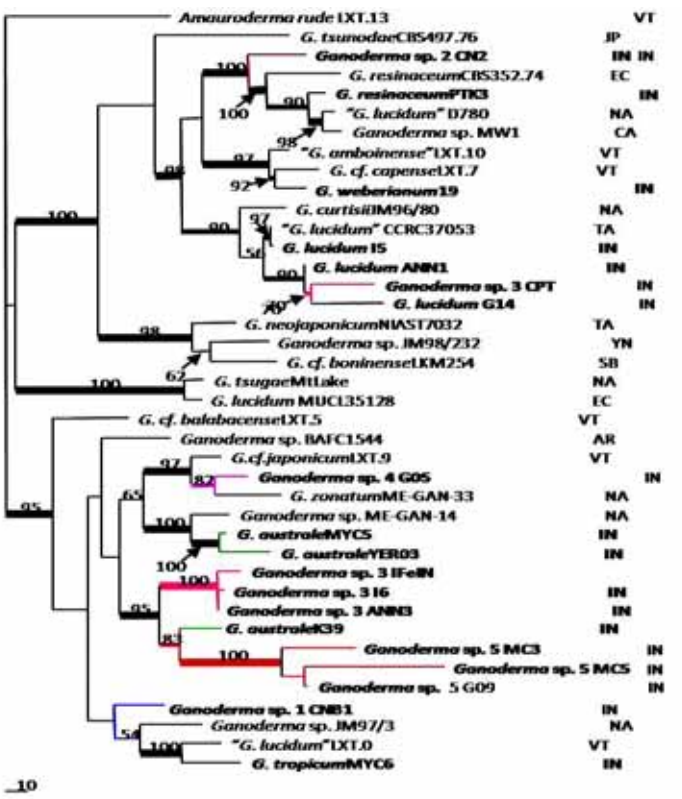

Figure 4. Maximium parsimonious trees inferred from ITS r DNA region, β-tubulin and partial *atp6* gene sequences of *Ganoderma* spp. rooted with *Amauroderma rude* JM/ASP.1. The bold names indicate the *Ganoderma* species from present study. The numbers on branches represent the bootstrap values

CONCLUSION

This study has contributed important details to the understanding of the natural relationships within the *Ganoderma* species. Now it is clear that the nuclear ITS rDNA and β-tubulin genes do contain useful information to resolve the relationship of species, species complex and closely related species of *Ganoderma*. On the other hand, though *atp*6 contained low nucleotide divergence it provided significant information towards the *Ganoderma* phylogeny. However, *atp*6 based tree topology was in line with the multiple gene phylogeny. The results were consistent with cultural and geographical distribution of species. Since this is the first report of protein coding gene in *Ganoderma* phylogenetic study, no literature is available for the comparative analyses.

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