
First report of *Ganoderma resinaceum* and *G. weberianum* from north India based on ITS sequence analysis and micromorphology

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The genus *Ganoderma* is a notorious pathogen causing root rot and butt rot in more than 144 tree species. There is high variability in the basidiome morphology, and complicated speciation, which often leads to inconclusive identification by traditional taxonomic methods. We analyzed the specimen collected from *Acacia nilotica* and *Tectona grandis* using morphological characters, sequencing of the ribosomal 5.8S RNA gene and the flanking internal transcribed spacers (ITS). The species status was confirmed as *Ganoderma resinaceum* and *Ganoderma weberianum* both from sequence and micromorphological study.

Key words – Internal transcribed spacer – *Ganoderma* – Cutis

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Introduction

Ganoderma Karst. is a cosmopolitan basidiomycete, which causes white rot of hardwoods by decomposing lignin as well as cellulose and related polysaccharides (Hepting 1971, Adaskaveg et al. 1991). Root rot and stem rot caused by *Ganoderma* species results in loss in crops and trees worldwide (Martinez et al. 1994). However, the genus also has medicinal value and is a traditional Chinese medicine. The genus *Ganoderma* was introduced by Finnish mycologist Peter Adolf Karsten in 1881 for the lactate species, *Polyporus lucidus* W. Curt. Various species were added to the genus by several other early mycologists (e.g., Patouillard 1889, Boudier & Fischer 1894, Boudier 1895, Murrill 1902, 1908). The important characteristics of family Ganodermataceae, which distinguish it from other families are the shape and size of basidiospores and the texture of the pileus surface. More than 250 species have been described in the Ganodermataceae (Ryvarden 1991). Variability in morphological characteristics often imposes difficulties

in the identification of *Ganoderma* species, and species are often identified as *Ganoderma lucidum* or *G. applanatum*. Due to this confusion it was proposed that new species of *Ganoderma* should only be established when molecular evidence is presented (Buchanan & Wilke 1995). In India, the identification of fungal species is mainly done on the basis of morphological and microscopical characters, which is not suitable for differentiating closely related species of *Ganoderma*.

In the present study *G. resinaceum* and *G. weberianum* are described for the first time from North India based on internal transcribed spacer analysis as well as morphological (both microscopical and macroscopical) characteristics.

Materials and methods

Isolates

Fruiting bodies of *Ganoderma* species were collected from plantations in north India. Fruiting bodies were cleaned and dried in

sunlight, stored at room temperature in paper bags and deposited in Forest Pathology Division herbarium, Forest Research Institute, Dehradun, India. Isolations were done onto potato dextrose agar (PDA) medium and pure cultures were maintained on PDA slants.

For confirmation of identification, taxonomic keys and descriptions were consulted (Ryvarden 1991). Descriptions of basidiomes were made according to their macro- and microscopic features. Dermic elements were carefully examined and measured in thin sections perpendicular to the pileus surface, and stained with a drop of 5% KOH aqueous solution. Three types of dermis were studied: 1) hymenodermis *vera* type composed of cylindrical-claviform, smooth elements; 2) hymenodermis diverticulated type with hymenodermis formed by diverticulated hyphae, with similar endings, though wider through the apex and with lateral branches; 3) hymenodermis spheroid-pedunculate type composed of claviform filaments, with or without lateral diverticules, but always with emerging spheroid-pedunculate or capitate knobs at the apex. Morphological characters such as size, shape, colour of upper and lower surface, pileus and stipe were noted as described by Gottlieb & Wright (1999).

Culture study

Isolates were grown on malt extract agar (MEA) medium at 25±1°C for one month for cultural study. They were examined at 3-day intervals for chlamydospore formation. Growth rate of isolates was measured on days 6, 9, 12 and 15.

DNA extraction, amplification and sequencing:

Isolates were grown in potato dextrose broth (PDB) for 10 days, filtered and lyophilized for DNA isolation. Lyophilized mycelium was ground in liquid nitrogen and DNA extraction done by the method described by Zolan & Pukkila (1986). DNA was quantified and diluted to 30 ng DNA/μl. Internal transcribed spacers, ITS-I, ITS-II along with 5.8S rDNA were amplified by using primer ITS5 and ITS4. PCR reactions were carried out in 25 μl of reaction mixture containing 10 mM Tris-HCl (pH=8.3), 50 mM KCl, 1.5 mM MgCl₂,

0.0001% gelatin, 400 μM dNTPs, 1 μM of each forward and reverse primer, 1 unit of Taq DNA polymerase and 30 ng of template DNA. The reactions were carried out on a gradient thermal cycler (Eppendorf) with an initial denaturation temperature 96°C for 3 min, followed by 30 cycles of 92°C for 30 sec, 55°C for 45 sec and 72°C for 1 minute. The reaction was completed at a final extension temperature of 72°C for 10 minutes.

All the PCR products were sequenced at Genomics Laboratory of Axygen India Private Ltd, New Delhi. The ITS region including ITS-I, ITS-II along with 5.8s rDNA was sequenced directly from the PCR products with primer ITS5. DNA sequences obtained were submitted to GenBank (NCBI, USA) (Accession numbers: GU451246, GU451247, GU726934, GU726935, HM053459, HM053460 and HM053461). Additional sequences were retrieved from GenBank.

Phylogenetic analysis:

The evolutionary history was inferred using the Maximum Parsimony (MP) method (Eck & Dayhoff 1966). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei & Kumar, 2000) with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree was drawn to scale, with branch lengths calculated using the average pathway method and were in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

Results

Morphology

Ganoderma resinaceum Boud. in Pat., Bull. Soc. Mycol. France 5: 72, 1889.

Basidiomata annual, stipitate, laccate, brazil brown, thick, 8.0–9.0 × 6.0–7.5 × 2.0–2.5 cm; pileus surface concave, smooth, irregularly rugose; margin rounded, light ochraceous

buff, 0.7–1.2 cm; stipe liver brown, lateral, 5–6 × 2–3 cm; hymenial surface oyster white to brown, pores circular, 2–6 per mm, 97–306 µm diam; context light brown, melanoid, 10.6–16.2 mm thick, cutis formed by claviform hyphae, arranged in hymenodermis vera type, cylindrical, smooth, lumen narrow with rings, 85–100 × 7.5–10 µm (Fig. 1a); hyphal system trimitic, generative hyphae thin-walled, with clamp connections, septa restricted to clamps, 7–15 µm thick, branched; skeletal hyphae arboriform, clampless, aseptate, thick-walled, branched, 3–8 µm thick; binding hyphae bovista type, clampless, aseptate, thick-walled, much branched, 1–4 µm thick; basidiospores light yellowish, ovate, fuscous, semirugose with thick episporium, 9–13 × 5–8 µm.

Collected from Kotdwar, Uttarakhand, on *Acacia nilotica*, (L.) Willd. ex Del., 2009.

Ganoderma weberianum (Bres. & Henn. ex Sacc.) Steyaert, Persoonia 7: 79, 1972.

Basidiomata annual, sessile or pseudostipitate, snuff brown, thick, 16.5–18.3 × 9.5–11.5 × 2.0–2.8 cm; pileus surface flabelliform, subundulate, smooth; margin subacute, yellow buff, 1.1–1.4 cm; hymenial surface light yellowish, pores circular to angular, 4–5 per mm, 110–200 µm dia; context light brown, nonmelanoid, 8.0–11.3 mm thick, cutis of hymenodermis divericulated hypae, wider to narrow lumen not reaching to the apex, 65–100 × 6–13 µm (Fig. 1b); hyphal system trimitic; generative hyphae thin-walled, aseptate, 2–5 µm thick, rarely branched; skeletal hyphae clampless, septate, thick-walled, branched, 3–5.5 µm thick; binding hyphae bovista type, clampless, aseptate, thick-walled, much branched, 1–5 µm thick; basidiospores light yellowish, broadly ellipsoid, 3.0–3.5 × 2.0–3.0 µm.

Collected from Dehradun, Uttarakhand, *Tectona grandis*, Linn., 2009.

Ganoderma lucium (Curtis .) P. Karst. Rev. Mycol. 3(9): 17, 1881.

Basidiomata annual, stipitate, brilliantly laccate, liver brown, thick, 5.0–6.5 × 4.0–4.5 × 1.0–2.0 cm; pileus surface concave, woody; margin rounded, light ochraceous buff, 0.5–1.5 cm; stipe liver brown, central, 5.0–10.0 × 3.0–5.0 cm; hymenial surface ochraceous,

pores circular, 4.0–7.0 per mm, 120–360 µm diam; context light brown, melanoid, 10.6–16.2 mm thick, cutis formed by claviform hyphae, arranged in hymenodermis vera type, cylindrical, smooth, narrow lumen, 60–85 × 7–10 µm (Fig. 1c); hyphal system trimitic, generative hyphae thin-walled with clamp connections 5.0–15.0 µm thick, branched; skeletal hyphae arboriform, clampless, aseptate, thick-walled, branched, 4–9 µm thick; binding hyphae bovista type, clampless, aseptate, thick-walled, profusely branched, 1–3 µm thick; basidiospores light yellowish, ovate, double walled, 9.0–13.0 × 5–8 µm.

Collected from Dehradun, Uttarakhand, *Cassia javanica* L., 2009

Cultural characteristics

Ganoderma resinaceum

Growth rate slow to rapid, 1.5–3.5 cm/week, covering Petri dish in 12–15 days. Advancing zone white, appressed. Mycelial mat white at first, latter cream coloured with a few light yellowish zones. Hyphae in the advancing zone hyaline, thin-walled, branched. Texture of mycelia mat farinaceous during first week, later whole surface becoming farinaceous and much denser. Aerial mycelium thin to slightly thick-walled, hyaline, branched, aseptate with narrow lumen, 2.5–2.5 µm. Chlamydospores both intercalary and terminal, thick-walled, golden color, ellipsoid or ovoid, 9.0–22.0 × 7.0–18.0 µm.

Ganoderma weberianum

Growth rate moderate, 1.0–3.0 cm/week, covering Petri dish in 15–18 days. Advancing zone white, submerged and appressed. Mycelial mat white, scattered, more or less extensive. Hyphae in the advancing zone hyaline, thin-walled, branched, aseptate. Texture of mycelial mat farinaceous with scattered dots. Aerial mycelium thin, hyaline, branched, aseptate, 1.5–2.0 µm. Chlamydospores both intercalary and terminal, thick-walled, light yellowish, ellipsoid 4.0–5.0 × 2.5–3.0 µm.

Ganoderma lucidum

Growth rate moderate to rapid, 1.5–3.5 cm/week, covering Petri dish in 12–15 days. Advancing zone white to hyaline, even,

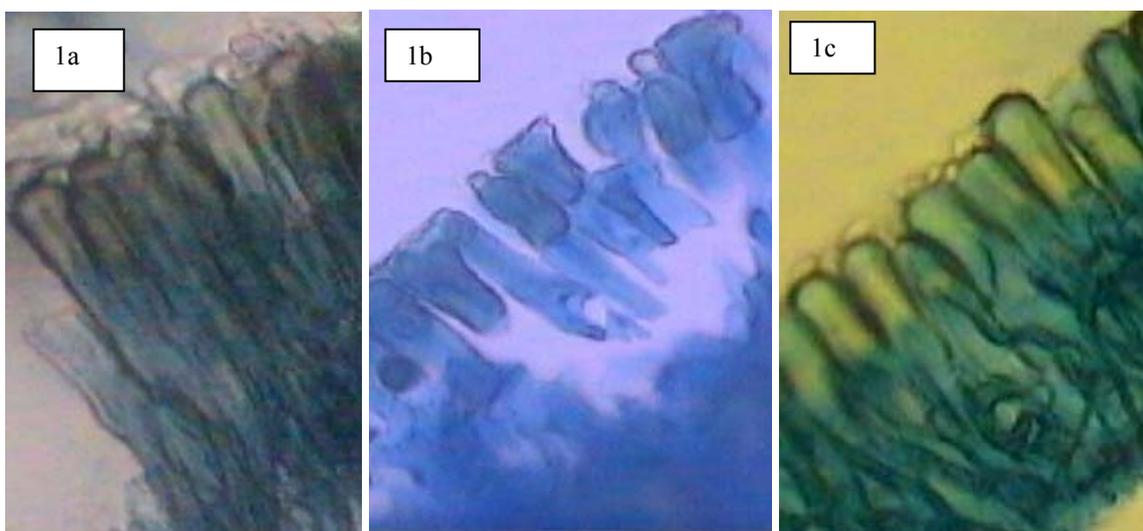


Fig. 1 – Cutis structure in *Ganoderma*. **1a)** Cutis of *G. resinaceum*, **1b)** Cutis of *G. weberianum*, **1c)** Cutis of *G. lucidum*.

appressed. Mycelial mat white to light cream, scattered, more or less extensive. Hyphae in the advancing zone hyaline, thin-walled, branched. Texture of mycelial mat appressed farinaceous-felty. Aerial mycelium thick-walled, hyaline, branched, aseptate, 1.5–3.0 μm . Chlamydospores both intercalary and terminal, thick-walled, pale in colour, ellipsoid to ovoid, 8.5–12.5 \times 5.5–7.5 μm .

Phylogenetic analysis

The length of the ITS region varies from 545 to 561 bp. There were a total of 561 positions included in the final dataset, of which 88 were parsimony informative (15.68%). A strict consensus tree was generated having length 122 (Fig. 2). The consistency index is 0.7735850, the retention index is 0.9175260, and the composite index is 0.729315. The tree is divided into two main clusters (A and B). In cluster (A) *G. applanatum*, *G. adpersum* and *G. australe* were grouped together at a bootstrap value of 99%. Cluster B was further divided into five subclusters (B1 to B5). In cluster B5 isolates of *G. resinaceum* were grouped together at a bootstrap value of 74%. *G. weberianum* grouped together in cluster B4 at a bootstrap value of 78%. In cluster B1 isolates of *G. lucidum* were grouped at a bootstrap value of 99%. The over all transition/transversion bias was 3.5.

Discussion

This study provides molecular evidence for occurrence of *G. lucidum*, *G. resinaceum* and *G. weberianum* in north India. Little work had been done for molecular typification of the two latter species. Gottlieb & Wright (1999) attempted to classify *G. lucidum* complex on the basis of isozyme analysis but, unfortunately, the typification of *G. resinaceum* remained unresolved. Hong & Jung (2004) found that *G. resinaceum* could not be distinguished phylogenetically from *G. lucidum*. Moncalvo (2000) differentiated *G. resinaceum* and *G. weberianum* from related species of *Ganoderma*. In this study *G. resinaceum* and *G. weberianum* were differentiated from *G. lucidum* on the basis of variation in internal transcribed spacers ITS1 and ITS2. The result was substantially supported by bootstrap values of three clusters (B1, B4 and B5) in which the three species were grouped. The tree also confined that cluster B1, in which the isolates of *G. lucidum* were placed, was distant to clusters B4 and B5 in which *G. weberianum* and *G. resinaceum* were grouped, respectively. Morphological and anatomical data clearly differentiated these three species from each other. Although these three species have the cutis structure of hymenodermis vera type, there is clear difference found in the lumen structure. In *G. resinaceum* concentric rings were confined to the lumen

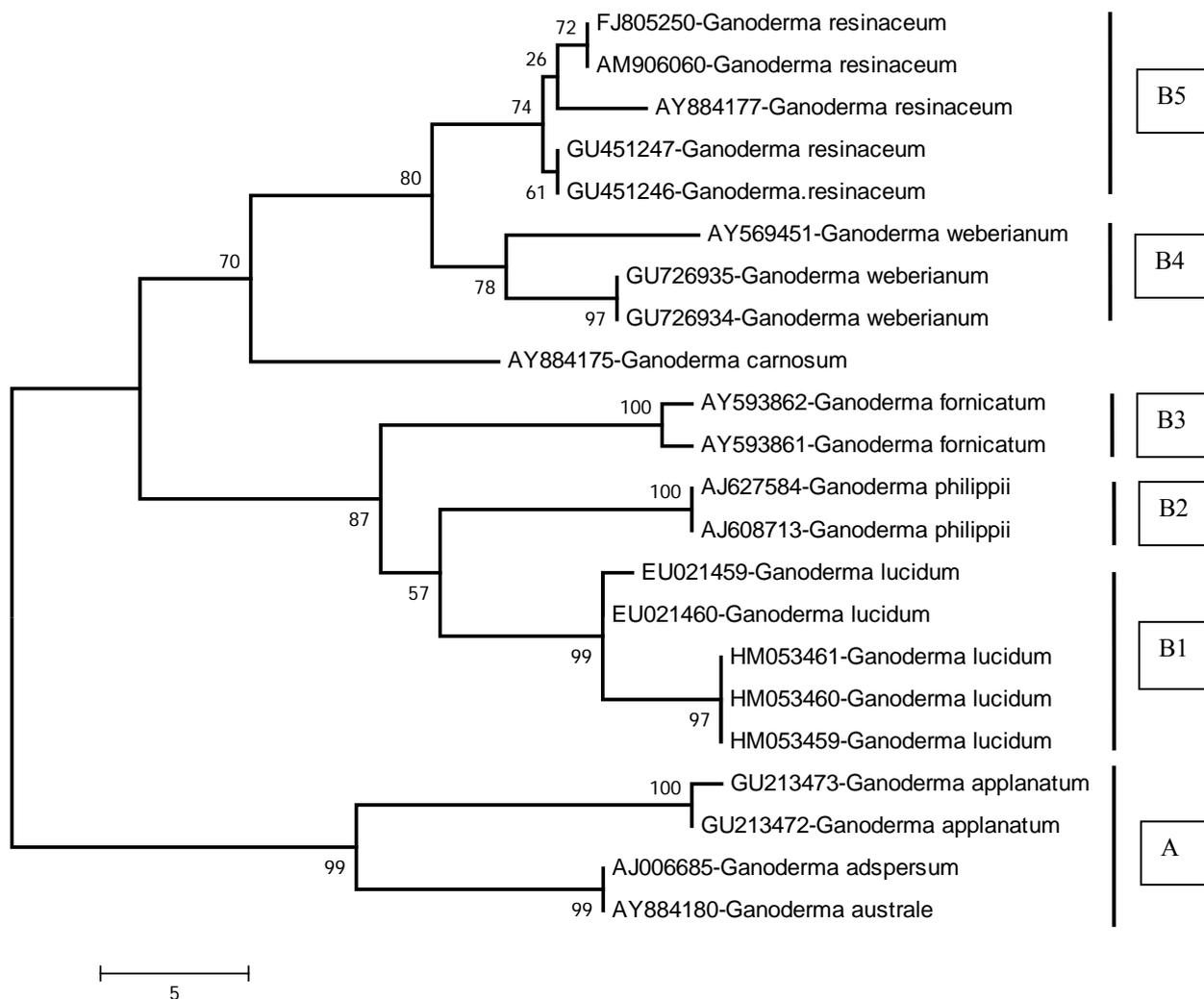


Fig. 2 – Phylogenetic relationship between *Ganoderma* species, inferred from ITS nucleotide sequence data. The tree depicted represents the strict consensus tree by use of close neighbor interchange algorithm from 88 parsimony informative characters and the consistency index = 0.7735850; retention index = 0.9175260 and composite index = 0.729315. The values above and below branches are confidence levels estimated by 1000 bootstrap replicates.

whereas this type of ring was absent in *G. lucidum* and in *G. weberianum*. The lumen structure of *G. lucidum* and *G. weberianum* were somewhat similar but in *G. weberianum* it did not reach to the apex. Furthermore, *G. weberianum* is sessile or pseudostipitate whereas *G. lucidum* is stipitate. From the cultural study it was found that the chlamydospores of *G. resinaceum* were larger than those of the two other species, but other cultural characteristics did not differ significantly.

This is the first report of the occurrence of *G. resinaceum* and *G. weberianum* from north India. The present study indicated that DNA sequence variability and cutis structure may serve as a measurement for species identi-

fication for *G. lucidum* complex. Tiwari et al. (2009) reported *G. resinaceum* from central India and described it on the basis of morphological and microscopic characters. Increased taxon sampling from other parts of India and other continents are needed to elucidate the genetic diversity of *G. lucidum* complex. In future, the phylogenetic structure will be increased through additional gene sequences.

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