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Ganoderma Species of Mizoram, India

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ABSTRACT

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In the present study investigated the taxonomic identities and phylogenetic relationships of *Ganoderma* species isolated from different locations within Mizoram, India using a combination of morphological and molecular approaches. Six *Ganoderma* isolates were identified through morphology and further confirmed by molecular phylogenetic analysis using nuclear ribosomal DNA sequences, including both the internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene region. The 6 species of *Ganoderma* identified to the species level based on morphology and fungal DNA sequences were *G. lingzhi*, *G. mastoporum*, *G. mizoramense*, *G. multipileum*, *G. subresinosum*, and *G. williamsianum*.

Introduction

Ganoderma species belong to the kingdom of Fungi, the division of Basidiomycota, the class of Homobasidiomycetes, the order of Aphyllophorales, the family of Polyporaceae, (*Ganoderma* taceae) and the genus of *Ganoderma* (Wasser and Weis, 1999). They have a woody or leathery feel and the presence of pores are obvious characteristics that distinguish polypores from other common type of mushrooms. They degrade the wood and produce a fruiting body (or conk) on the surface of the wood. *Ganoderma* species are among those fungi that can thrive under hot and humid conditions and are commonly found in sub tropical and tropical regions (Moncalvo and Ryvardeen, 1998).

Although they are not classified as edible, several types of *Ganoderma* products are used for medical purposes including ground fruiting bodies or mycelium processed into capsule or tablet form; extracts from fruiting bodies or mycelium dried and processed into capsule or tablet form, *Ganoderma* beer and *Ganoderma* hair tonics (Jong and Birmingham, 1992) as well as many other products are made from various *Ganoderma* species. Within the genus *Ganoderma*, over 250 taxonomic names have been reported worldwide including *G. adspersum*, *G. applanatum*, *G. australe*, *G. boninense*, *G. cupreum*, *G. incrassatum*, *G. lipsience*, *G. lobatum*, *G. lucidum*, *G. oerstedii*, *G. oregonense*, *G. pfeifferi*, *G. platense*, *G. resinaceum*, *G. sessile*, *G. sinense*, *G.*

tornatum, *G. tsugae* and *G. webrianum*. (Moncalvo *et al.*, 1995).

Ganoderma species identification and circumscriptions have often been unclear and taxonomic segregation of the genus has been controversial (Moncalvo *et al.*, 1995). A number of *Ganoderma* collections and species have been misnamed. This is because the presence of heterogenic forms, taxonomic obstacles and inconsistencies in the way the genus has been subdivided (Mueller *et al.*, 2007). Since *Ganoderma* species are genetically heterogeneous, a wide range of genetic variation has been reported and caused by out crossing over generations and different geographical origins (Miller *et al.*, 1999, Pilotti *et al.*, 2003).

This has led to variation in their listed morphological characteristics, even within same species (Hong *et al.*, 2001). Environmental factors, variability, inter hybridization and individual morphological bias, mean identification of *Ganoderma* species is difficult (Zheng *et al.*, 2009). Naming a species within this genus is confused and traditional taxonomic methods based on morphology are inconclusive for establishing a stable classification system for *Ganoderma* species (Hseu *et al.*, 1996, Hong *et al.*, 2004). Hence an uncertain nomenclature has resulted.

This confusing situation is mainly the result of different authors using various criteria during identification. Some authors strictly only focus on host-specificity, geographical distribution and macro morphology of basidiomes, while other authors only focus on spore characters as the primarily taxonomic characters (Sun *et al.*, 2006).

Ganoderma lucidum (Bisht, 2011) and *Ganoderma mizoramense* (Zothanzama *et al.*, 2017) have been reported in Mizoram. The

present investigation is a first detailed report on the morphology and molecular phylogeny of *Ganoderma* in Mizoram (Fig. 2).

Materials and Methods

Collection and preservation of specimens

Samples of fruiting bodies or host substrate (wood, stumps or branches) were collected and kept in air-tight containers or plastics bags and kept cool until brought into the laboratory. Photograph of each sample collected were taken in the field and in the laboratory (Prasher, 2015). The specimens are preserved by air drying and deep freezing. Voucher numbers are given to the specimens and stored in the Department of Environmental Science, Mizoram University.

Identification of specimens

The collected specimens were identified according to standard macroscopic and microscopic characteristics through consultation with appropriate literature (Gilbertson and Ryvarden, 1986; Núñez and Ryvarden, 2000).

For microscopic study, thin sections of dried specimens were taken with a sharp razor blade and were mounted in 3% KOH solution and stained in 2% aqueous phloxine. Sections were mounted in Lactophenol or 60% lactic acid + cotton blue. Spore prints of the collected specimens were taken by cutting a section of the pore layer, placing it on a piece of white paper, and covering it.

Molecular analysis

DNA was extracted from tissue removed from the inside of fruiting bodies using a CTAB extraction. A small amount of tissue from the inside of the fruiting body was added to a sterile 1.7 ml microcentrifuge tube with glass

beads and 500 µL of CTAB lysis buffer. The centrifuge tube was then vortexed for a minute homogenize lyse fungal cells. For drier and harder fruiting bodies, the tissue was vortexed for 2 minutes to allow for a better homogenization of the tissue. The tubes were then briefly centrifuged to move the larger tissue segments to the bottom of the tube and then the supernatant was transferred to another tube. The new microcentrifuge tube was then placed into a 65°C hot water bath to further lyse the cells. After 20 minutes, the tubes were removed and 500 µL of chloroform were added to the tube, mixed and then centrifuged at 13,000 rpms for 5 minutes. The top layer of the supernatant was then transferred to a new microcentrifuge tube. The amount of liquid transferred was then measured and two thirds of that amount was added of isopropanol stored in a minus 20C freezer. The tube was then incubated for 5 minutes at room temperature before being centrifuged for 7 minutes at 15,000 rpm. The supernatant was removed and 500 µL of 70% ethanol was added. The tubes were centrifuged again at 15,000 rpm for 3 minutes and the supernatant was removed. The tubes were left open in a hood to allow the last of the ethanol to evaporate before the DNA pellet was re-suspended in 100 µL of sterile water.

PCR reactions were setup in 0.2 ml centrifuge tubes that contained 12.5 µl GoTaq Green Mastermix (Promega, Madison, WI), 9.5µl nuclease free water, 1µl forward primer (5M), 1µl reverse primer (5M) and 1µl of fungal DNA template for a total reaction volume of 25.5µl. PCR was performed using primers ITS1-F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') ITS4(5'-TCCTCCGCTTATTG ATATGC-3') (White *et al.*, 1990) with the following parameters; 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 52°C for 1 minute and 72°C for 1 minute with a final extension step of 72°C. PCR amplicons were verified by electrophoresis on a 1%

agarose gel with SYBR green and visualized on a Gel Documentation System. Sequencing was performed using both primers by using Sanger sequencing using a DNA sequencer. Consensus sequences for contigs were trimmed and aligned using Bioedit sequence alignment editor (Hall, 1997). Sequences were then compared to those in GenBank database using the BLASTn (Altschul *et al.*, 1990) search tool for similarities and were submitted to Genbank. The sequences were then aligned with Clustal W (Larkin *et al.*, 2007) and the phylogenetic tree was established using Maximum Likelihood in Mega 7 (Kumar *et al.*, 2015) software with the available sequences representing all the species of *Ganoderma* collected within Mizoram.

Phylogenetic analysis

Phylogenetic analysis was conducted based on the ITS gene data using both Maximum Likelihood (ML) and Neighbor Joining (NJ) approaches. ML and NJ searches were carried out using MEGA7. Alignment gaps were treated as missing data. NJ trees were constructed based on the total character differences and bootstrap values were calculated from 1,000 replications.

Results and Discussion

Morphological identification

Morphological identification of the 6 fungi from Mizoram was first carried out according to characteristics of the spores, and reproductive structures if discernible using previously described methods (Barnett and Hunter 1998, Zothanzama *et al.*, 2017).

Morphological details

Ganoderma subresinosum

Basidiocarp annual, stipitate laterally, applanate, rigid, becoming hard corky and

woody hard when dry. Pileus 6 – 14cm broad and upto 0.6 cm thick at the lateral, surface irregular/wrinkled, blackish blue, pore surface white to creamy in colour, sometimes extends upto the edge. Stalk stout upto 3 - 5 cm long, essentric (off-centric). Basidia club-shaped, broad at the base and narrower at top, tetrasterigmatoid, 2 - 4 x 6.8 - 8µm. Spore-print creamy to off-white, difficult to obtain, mostly obtained through section cuttings. Basidiospores 2 x 3.6 - 5.4 x 6.2 µm. Ellipsoidal, doubled wall.

Specimen Voucher No- MZU JZ014, MZU JZ067

Site of collection- Mizoram University Campus.

Ganoderma williamsianum

Basidiocarp sessile, annual or perennial, applanate and slightly ascending then more or less unguulate, sulcato-zoned, becoming stepped at the margin, at first pale warm brown, and reddish brown with age. Pileus 1.5 - 7(-9) cm in radius, upto 15 cm broad, or with a subdiscooid base, semicircular in outline. Tubes 35 mm long, 120-200 µm wide, dark brown, pores 60-70 µm wide, minute. Flesh 3-15 mm thick at the base, dense, firm, heavy, uniformly dark brown, dark. Spores 11-14(-15) x 8-10.5 µm, ellipsoid and double walled.

Specimen Voucher No- MZU JZ060.

Site of collection- Dampa

Ganoderma lingzhi

Basidiocarp sessile, rigid, corky to woody, crustose, dark brownish to blackish brown, irregular, rugose 4 - 8 cm wide, sometimes shows erect slender finger like projection. Stipe absent. Pore surface smooth, creamy when fresh and becomes pale brown when aged or bruised, pores circular to somewhat

ovoidal, 1.5 - 2.5 mm. Basidiopores 1.53-2.2 x 2.56-2.94 µm, more or less ellipsoidal, sometimes with truncate end, double walled. Basidia 2.24 - 2.91 x 3.41-4.6 µm, elongated, broadly clavated, tetrasterigmatic.

Specimen Voucher No- MZU JZ049.

Site of collection- Hmuifang.

Ganoderma mastoporium

Basidiocarp sessile, rigid, corky to woody, crustose, dark brownish to blackish brown, irregular, rugose 4 - 8 cm wide, sometimes shows erect slender finger like projection, Stipe absent. Pore surface smooth, creamy when fresh and becomes pale brown when aged or bruised, pores circular to somewhat ovoidal, 1.5 - 2.5 mm. Basidiopores 1.43-2.2 x 2.56-2.94 µm, more or less ellipsoidal, sometimes with truncate end, double walled. Basidia 2.24 - 2.91 x 3.41-4.6 µm, elongated, broadly clavated, tetrasterigmatic.

Specimen Voucher No- MZU JZ020.

Site of collection- Dampa.

Ganoderma mizoramense

Mature basidiocarp annual, pileate, stipitate, applanate, soft and leathery when fresh and woody to corky when dried, semi-circular, irregular surface, dark brownish to dark reddish brown, homogenous context structure 2–20 mm, upto 29 cm. Pileus upper surface reddish brown when fresh, liver brown when dry, surface hard, margin white, lower surface white when fresh, pale brown when dry. Stipe sometimes present and often prominent; twisted and irregular. Pore surface smooth, creamy to snuff brown when dry, pores 4–5 per mm, round to somewhat slightly oval, 187–278 × 134–228 µm. Basidia tetrasterigmatic. Basidiospores ellipsoid with a truncate base, 10–12.5 × 6–9 µm.

Specimen Voucher No- MZU-G, UMN-MZ5
Site of collection- Mizoram University
Campus.

Ganoderma multipileum

Basidiocarps annual, centrally to laterally stipitate, applanate, upto 15 cm or more broad, upper surface with thin varnished crust at maturity, yellow at the margin in actively growing specimens; pore surface creamy white at first, becoming light buff, staining dark purple-brown on bruising or when aged, pore circular to angular, 4 - 1 mm. Basidia 12-23 x 10-11 µm, broadly ellipsoid to barrel-shaped, tetrasterigmatic, with a basal clamp. Basidiopores 7-11 x 6-8 µm, often variable in length, ellipsoid, truncate at apex, wall two – layered.

Specimen Voucher No- MZU JZ021, MZU JZ078.

Site of collection- Hmuifang, Reiek.

Molecular analysis

In addition to the morphological characterization, molecular analyses were carried out to confirm the identification of 6 representative fungal isolates from the Mizoram. The ITS1-5.8S-ITS2 sequences of these isolates were compared to 14 corresponding sequences of reference fungal

taxa in the database. (Table-1) and the List of Species, VoucherNos, GenBank Accession Nos. and Locality used for the analysis is also given (Table-2).

The analysis involved 20 nucleotide sequences (Figure 1). In the maximum Likelihood tree generated, the specimens of the different species of *Ganoderma* clustered with their related species with high support value.

Blast search showed that isolate JZ14 is 99% identical to *G. subresinosum* (KJ654374) with query coverage of 99%. Isolate JZ20 was likely to be *G. mastoporum* with 99% identity with AJ627585 (98% coverage) and JQ409361 (99% coverage). Blast search showed that isolate JZ60 is 97% identical to *G. williamsianum* (KU219995) with query coverage of 98%. *Schizophyllum commune* (KX958030) was used as an out-group.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7.

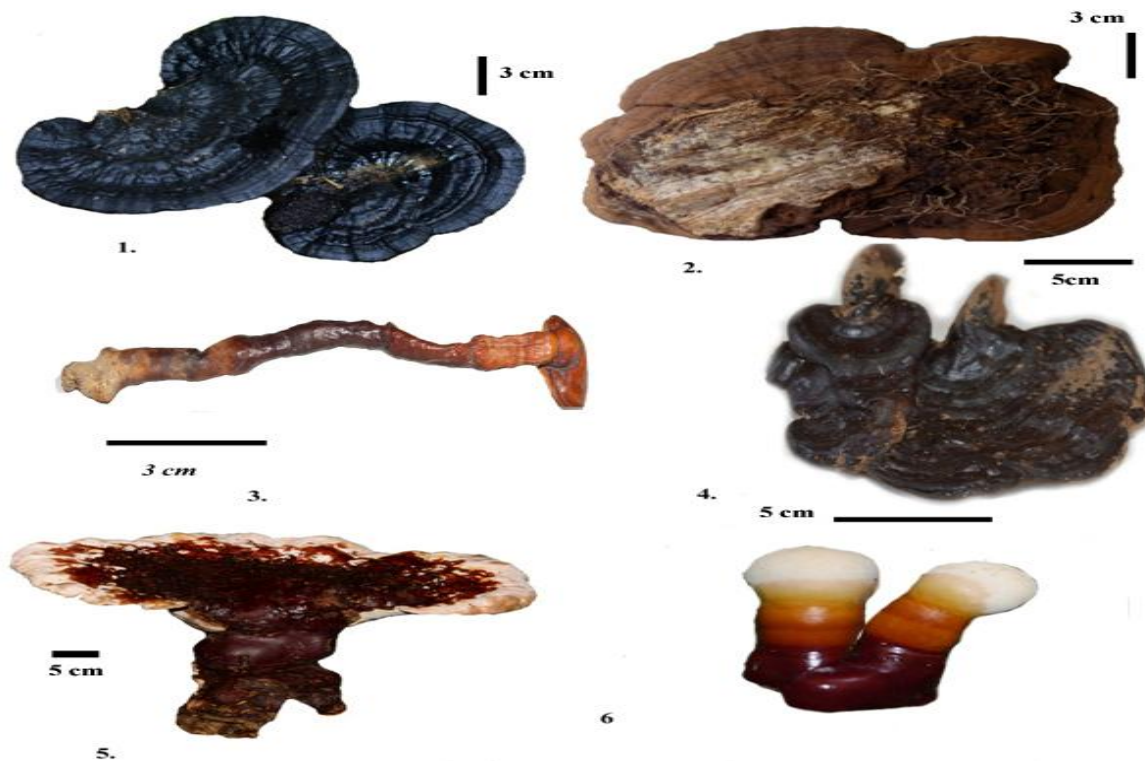
Table.1 List of *Ganoderma* species collected within Mizoram

Sl.No	Voucher No.	NCBI Accession No.	Morphological identification	Blast match	Max identity
1	JZ14	MG437401	<i>Ganoderma subresinosum</i>	KJ654374	99%
2	MZU-G/ UMN-MZ5	KY643751	<i>Ganoderma mizoramense</i>	KY643751	99%
3	JZ21	MG450346	<i>Ganoderma multipileum</i>	KX055556	97%
4	JZ20	MG448604	<i>Ganoderma mastoporum</i>	JQ409361	98%
5	JZ60	MH071336	<i>Ganoderma williamsianum</i>	KU219994	99%
6	JZ49	MH479057	<i>Ganoderma lingzhi</i>	AB811848	98%

Table.2 List of species, voucher, genbank accession nos. and locality used for the analysis

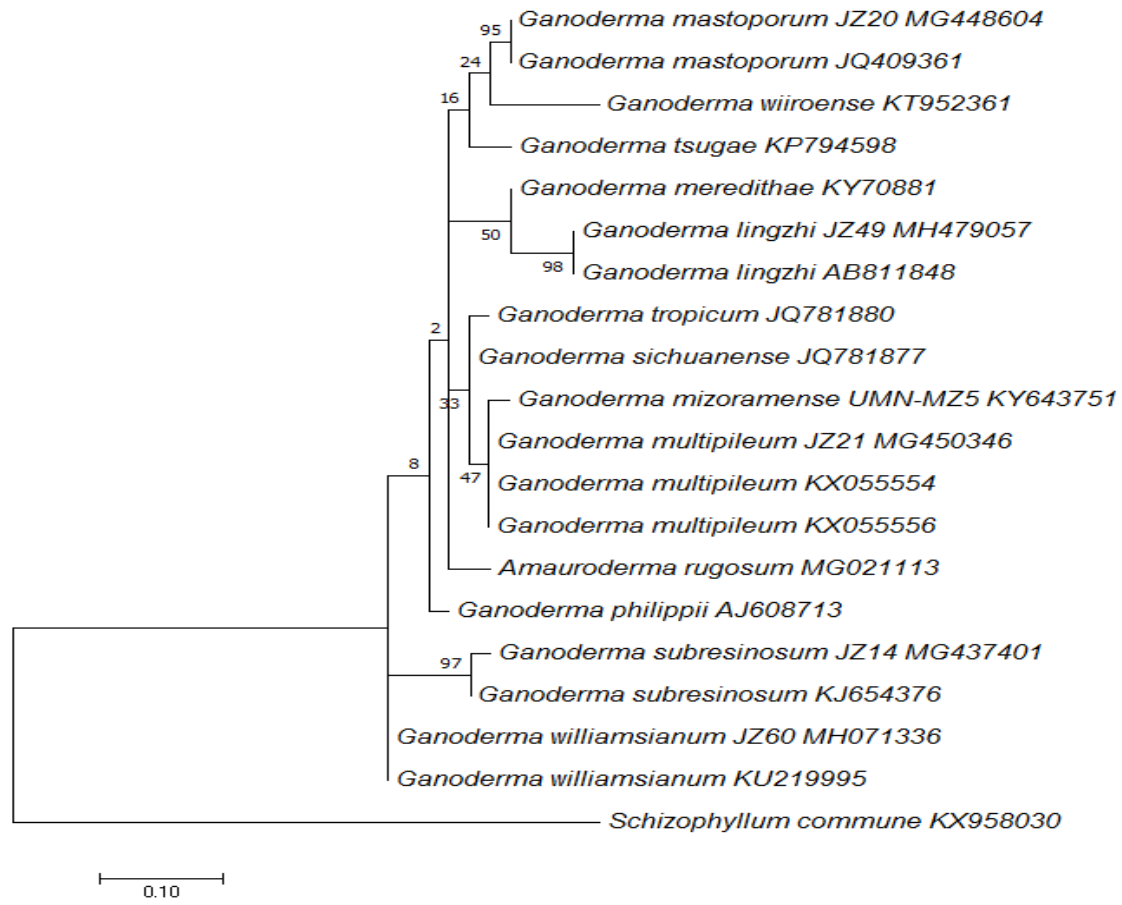
Species	Voucher	GenBank	Accession Nos.	Locality
<i>Ganoderma subresinosum</i>	T162	KJ654376		Indonesia
<i>Ganoderma mastoporum</i>	PM2	JQ409361		Malaysia
<i>Ganoderma philippii</i>	E7425	AJ608713		Indonesia
<i>Ganoderma wiiroense</i>	UMN-20-GHAKT	952361		Ghana
<i>Ganoderma merredithae</i>	124FL(ALL)	KY708881		United States
<i>Ganoderma tsugae</i>	UOC KAUNP MK26	KP794598		Sri Lanka
<i>Ganoderma sichuanense</i>	HMAS42798	JQ781877		China
<i>Amaurodermarugosum</i>	CH8-1	MG021113		China
<i>Schizophyllum commune</i>	15R-5-F01	KX958030		China
<i>Ganoderma multipileum</i>	G82	KX055556		China
<i>Ganoderma multipileum</i>	G74	KX055554		China
<i>Ganoderma williamsianum</i>	Yuan 5417	KU219995		China
<i>Ganoderma tropicum</i>	Yuan 3490	JQ781880		China
<i>Ganoderma lingzhi</i>	MQN002	AB811848		Japan

Fig.1 *Ganoderma* species collected in Mizoram



1. *Ganoderma subresinosum*, 2. *Ganoderma williamsianum*, 3. *Ganoderma lingzhi*, 4. *Ganoderma mastoporum* 5. *Ganoderma mizoramense*, 6. *Ganoderma multipileum*

Fig.2 Phylogenetic tree



Most of the major polygenetic relationships were congruent based on M.P (Maximum Parsimony), N.J (Neighbour Joining). Six species of *Ganoderma* have been collected and identified from different locations within Mizoram. *Ganoderma mizoramense* is a new species which was recently identified and described (Zothanzama *et al.*, 2017).JZ14 (MG437401) has slight variation in the nucleotide sequence (12 neucleotides differences) compared to *G. subresinosum* KJ654376.JZ60 (MH071336) collected from Dampa Tiger Reserved forest is quite similar to *G. australe*, but the phylogenic analysis indicates that it lies close to *G. williamsianum*. Only two sequences of *G. williamsianum* were found in the NCBI Genbank for comparison.

Molecular techniques have been successfully used for identifying fungi in recent studies. Culture-independent DNA methods, such as denaturing gradient gel electrophoresis (DGGE, Muyzer Gerard *et al.*, 1998), terminal restriction fragment length polymorphism (T-RFLP), and ITS sequencing, have been developed for the investigation of complex microbial communities. Our study also shows that molecular identification based on ITS sequences can be used to complement or verify morphological identification of unknown fungi.

Among the many wood rotting fungi in Mizoram, *Ganoderma* species are selected because they are the most common studied

species of wood rotting fungi. Some are known to cause disease while some has been used for centuries for promotion of good health longevity. Some *Ganoderma* species have been reported to have various pharmacological activities such as hepatoprotective, anti-diabetic, anti-hypertensive, cardioprotective, anticancer, antioxidant etc. More analysis and study should be done for species such as *G.mizoramense* which is the new species which may possess pharmacological properties.

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