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Molecular Identification of *Lyophyllum connatum* and *Paneolus sphinctrinus* (Basidiomycota, Agaricales) from Himalyan Moist Temperate Forests of Pakistan

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ABSTRACT

Two gilled fungi: *Lyophyllum connatum* and *Paneolus sphinctrinus* (Basidiomycota, Agaricales) have been identified from Pakistan using the fungal molecular marker (ITS-rDNA). The target ITS-rDNA of both species was amplified using polymerase chain reaction (PCR) with universal fungal primers (ITS1F & ITS4), which generated 700-750bp fragments. After sequencing of amplified product, the initial blast analysis revealed and confirmed the identification of both species by comparing the sequences of these respective species present in GenBank. The nucleotide base parentage similarity of both sequences is more than 99% with sequences of *L. connatum* (HM119488, EF421104) and *P. sphinctrinus* (FJ755221, DQ182503.1) respectively. Further, in phylogenetic analysis both species distinctly clustered with their respective groups. Morphological and molecular descriptions of both these species are provided. Shape, size and color of basidiomata, basidiospore size, basidial lengths, shape and size of cheilocystidia of both collections have also been measured and compared with data given in literature. © 2012 Friends Science Publishers

Key Words: Biodiversity; Gilled fungi; Molecular techniques; rDNA

INTRODUCTION

The fungal order Agaricales (Basidiomycota) possessing gilled fungi (mushrooms), contains some of the most familiar types of fungi. Among the forest mycological communities these fungi are either saprobs on decaying wood and other organic material like cow dung, or are symbiotic with the living cells of plant roots, forming mycorrhizal associations with trees or parasitic on living plants (Bruns *et al.*, 1991; O'Brien *et al.*, 2005). These gilled fungi are very essential component of forest ecosystem.

Pakistan is fertile and ecologically rich country. Its forests have not only a lot of biodiversity of fauna and flora but also mushroom flora (Tulloss *et al*., 2001, 2007; Burni *et al.*, 2006; Niazi *et al.*, 2006; Niazi, 2008; Razaq *et al.*, 2012). During this study two gilled fungi collected from Himalayan moist temperate forests of Pakistan from cow dung and decaying wood separately were identified using molecular techniques in which ITS-rDNA was used as molecular marker. Phylogenetic analysis of both species also confirmed their identification. Molecular results further supported by morpho-anatomical description and illustrations. The objective this publication is to update mycoflora of Pakistan using molecular techniques.

MATERIALS AND METHODS

Basidiocarps were carefully dugout with the help of a knife and photographed in the field. Collected material was characterized morphologically and microscopically. Sections of lamellae of basidiocarp were prepared and observed under the light microscope equipped with camera lucida. For microscopic observation, free hand sections were made and stained with Congo Red and Melzer's reagent. Size dimensions were determined for 25 basidiospores, 20 basidia, 20 cystidia, from each basidioma.

A sample from a dried specimen of each species was ground in liquid nitrogen and placed in 2% CTAB buffer and DNA was extracted using Porebski *et al.* (1997). ITS regions of rDNA were amplified using universal primer pair ITS1F and ITS4 (White *et al*., 1990). PCR was performed in 25-µL reaction volume following the protocol given by Gardes and Bruns (1993). The PCR product of the ITS-amplified region containing ITS-1, 5.8 and ITS-2 was directly sequenced in both directions using the same pair of amplification primers (Macrogen, Korea). For initial comparison and alignment of the sequence, BLAST (Basic Local Alignment Search Tool) analysis was performed using the National Center for Biotechnology Information (NCBI), USA database. For further phylogenetic analysis and alignment of sequence, closely related sequences were

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retrieved from GenBank. The sequence alignments and phylogenetic analysis were performed using Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura *et al.*, 2011). Maximum Likelihood (ML) method was based on the Jukes-Cantor model of nrITS sequences using Nearest-Neighbor-Interchange (NNI) as ML heuristic search method. Phylogeny was tested by bootsrap value of 1000 replicates. Consensus nucleotide sequences of *P. sphinctrinus* and *L. connatum* were submitted to European Molecular Biology Laboratory (EMBL) database under accession numbers HE819397 and HE819396 respectively.

RESULTS

Molecular characterization and phylogenetic analysis of *Lyophyllum connatum*: For internal transcribed spacers (ITS) regions and 5.8S region of rDNA, fungal genomic DNA was amplified with ITS1 and ITS4 primers pair generating a fragment approximately 700bp on 1% gel electrophoresis. Nucleotide sequence of Pakistani collection on Blast revealed that it matches 99% with *L. connatum* (GenBank accession # HM119488, EF421104, JF908332) with sequences in National Center for Biotechnology Information (NCBI). After removing and editing of ambiguous letters from alignment, the percentage similarity was calculated by Megalign software (DNA Star. Inc.), which remained same as revealed by Blast analysis. A phylogentic analysis was inferred in which sequences of closely related genera were included for further confirmation of its identity. Three clades were formed: clade I *Hypsizygus* Singer, Clade II *Clitocybe* (Fr.) Staude, Clade III *Lyophyllum* P. Karst. Pakistani collection (accession # HE819396) clearly lies in Clade III among *Lyophyllum* species especially with *L. connatum* under a significant bootsrap value and branch length. Further these molecular results were determined by morpho-anatomical emuration of Pakistani collection.

Taxonomic Description

***L. connatum*:** (Schumach.) Singer, *Schweiz. Z. Pilzk.* 17: 55 (1939) (Fig. 2. A-D).

Pileus 3-5 cm diameter, hemispherical when young, becoming convex with an incurved margin; eventually plane and often irregularly undulating; surface moist when fresh, smooth, off-white to gray-white, flesh moderately thick, white, cartilaginous. Lamellae white when young to pale white, distant, decurrent to subdecurrent, some forked, edges smooth; 3-tiers of lamellulae present. Stipe 3.5–8×0.7–2 cm thick; smooth; equal to tapered at the base, dry, whitish, sometimes becoming brownish toward the base, without partial veil, Context: white, firm, not changing on exposure.

Basidiospores 4–7 × 5–6 µm, broadly clavate to ovoid, circular in side view, hyaline, transparant to whitish, smooth, inamyloid, colourless in Melzer's reagent, apiculate. Basidia 26.0–30.0 × 4.5–6.0 µm, 4-spored, clavate to cylindrical, hyaline to pale yellow in 5% KOH, oil contents present, sterigmata sharp, clamp connections at basidial base. Cystidia absent.

Material examined: Pakistan, Gilgit-Baltistan, Fairy Meadows (34.400570°S 150.891738°E), Pine forest of Himalayan moist temperate region, distributed along road side in sandy soil among the coniferous trees during late summer, at 3306 m a.s.l., on decaying logs of coniferous tree, 21st July, 2010, A Razaq, AN Khalid & S Ilyas, SR-32 (Herbarium # LAH 21071032), GenBank accession # HE819396.

Comments: Genus *Lyophyllum* P. Karst. consists of 40 species found across the world in temperate north hemisphere (Kirk *et al*., 2008). *L. connatum* is a good representative of this genus found in large groups on decaying woods, litter etc. It is commonly collected from Pine forest of Fairy Meadows, Gillgit-Pakistan we noted this forest region was very rich in coniferous logs and decaying fallen trees in summer 2010. This part of Himalayan moist temperate forests is present at 3300 m and the whole winter it experiences high snow fall. Molecular characterization of Pakistani collections showed its similarity of sequences with European collections and from other part of the world. To confirm molecular results, a complete morphological description is also provided. All the morphological characters were compared with the description available in literature (Breitenbach & Kränzlin, 1991). The genetic analysis data and morpho-anatomical characterization and comparison proved Pakistani collection as *L. connatum*.

Molecular characterization and phylogenetic analysis of *Panaeolus sphinctrinus*: From fungal genomic DNA fragments approximately 700bp were amplified with ITS1f and ITS4 primers pair, which were observed on 1% gel electrophoresis. In blast our sequence shows similarity with only *Paneolus* (Fr.) Quél. species but the maximum similarity was with *P. sphinctrinus* (GenBank accession # FJ755227.1). Nucleotide sequence of Pakistani collection on Blast revealed that it matches 99% with *P. sphinctrinus.* Other some sequences of *P. sphinctrinus* also showed significant similarity with our sequence (accession # FJ755221, DQ182503.1, JF908513.1). After removing and editing of ambiguous letters from alignment, the percentage similarity was calculated by Megalign software (DNA star. Inc.,), which remained same as revealed by Blast analysis. Base similarity of observed sequence was further determined by a phylogenetic analysis. A phylogenetic analysis was inferred in which sequences of closely related sequences included for confirmation of its identity. Pakistani collection (accession # HE819397) clearly separates with *P. sphinctrinus* under a significant bootstrapping value and branch length. All the sequences retrieved from GenBank do not fall in a single clade (Fig. 3) because of intra-specific dissimilarity. Further these molecular results were determined by morpho-anatomical description of Pakistani collection.

Fig. 1: Phylogenetic relationship of *Lyophyllm connatum* (■) with other related members based on Maximum Likelihood method inferred from nrITS sequences. Bootstrap values based on 1000 replicates are shown. The analysis involved 22 sequences. There were a total of 553 positions in the final dataset

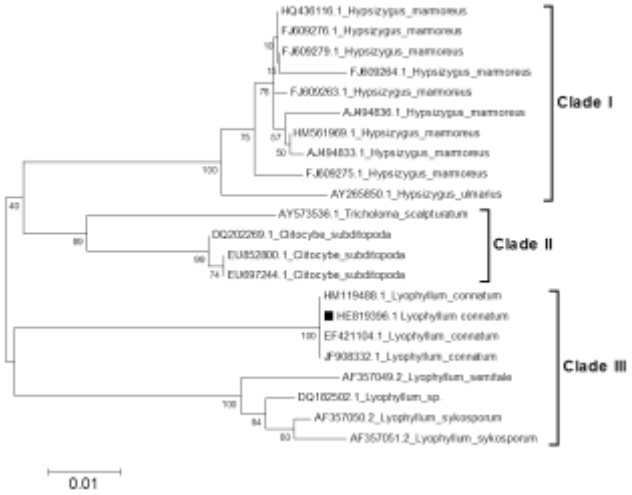
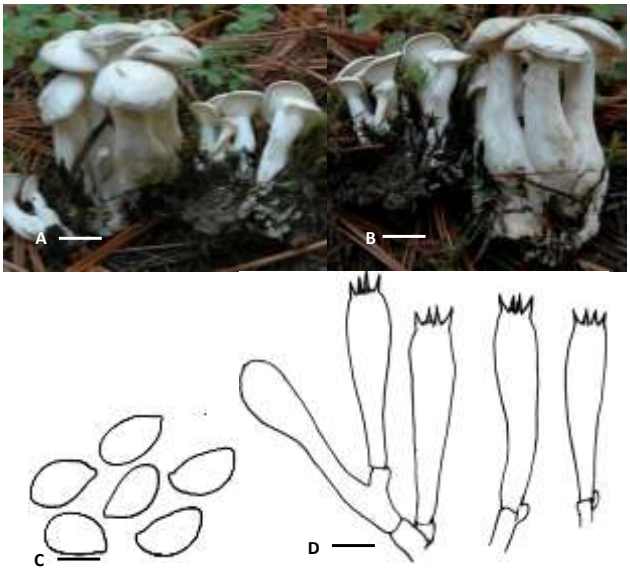


Fig. 2A–D: *Lyophyllum connatum*. A–B Photographs of basidiomata showing pileus shape and surface and lamellae C-Basidiospores D- Basidia with clamped bases. Scale Bar A–B= 2 cm C= 3 µm D= 6 µm



Taxonomic Description

***Panaeolus sphinctrinus*:** (Fr.) Quél., *Mém. Soc. Émul. Montbéliard*, Sér. 2 5: 151 (1872) (Fig. 4 A-E)

Pileus 1.6 cm wide, bell-shaped to conical, brownish gray to blackish gray or olivaceaus grey, glabrous, moist or sometimes more or less areolate when dry, central disc more darkened brown towards margins grayish, the margins smooth, brittle, slightly incurved and appendiculate from

Fig. 3: Phylogenetic relationship of *Paneolus sphinctrinus* (■) with its allies based on Maximum Likelihood method inferred from nrITS sequences. Bootstrap values based on 1000 replicates are shown above the branches. The analysis involved 20 sequences. All positions containing gaps and missing data were eliminated. There were a total of 384 positions in the final dataset

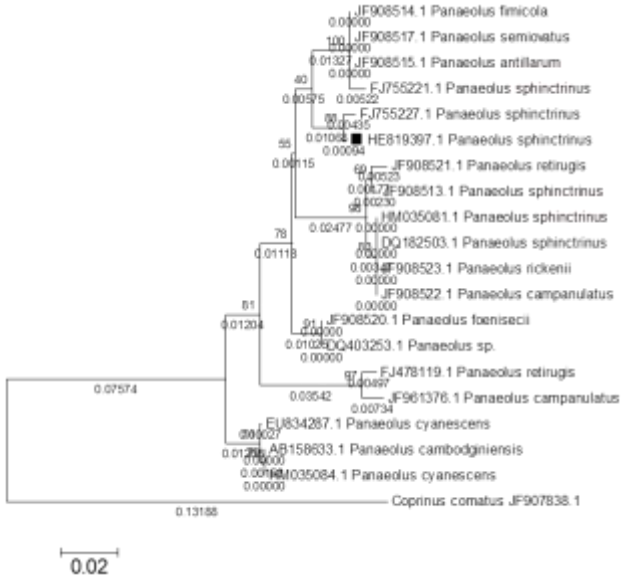
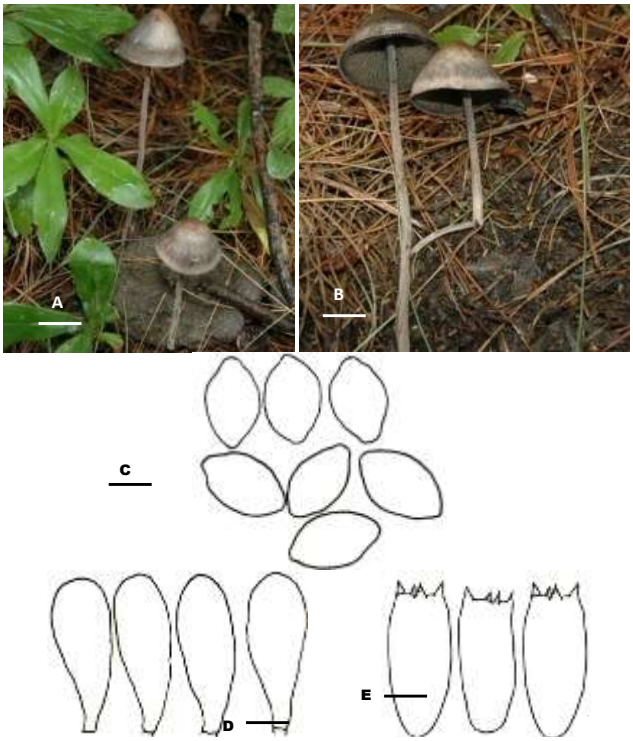


Fig. 4A–E: *Panaeolus sphinctrinus*. A–B. Photographs of basidiomata C. Basidiospores. D. Cheilocystidia E. Basidia Scale Bar = A and B= 3 cm, C– E= 7 µm



fragments of the veil. Context thin to moderately thick, very soft and fleshy, dark gray, unchanging when bruised or cut. Lamellae ascending to adnate, seceding, subdistant, broad, at first grayish, becoming mottled blackish, edges white to flocculose, shorter lamellae present. Stipe 6.3×0.18 cm, grayish, cylindrical, firm, smooth; thick, equal, reddish brown with a grayish to pruinose covering, hollow, striate at the apex, Context grayish, dark brown under stipipellis, without volva and annulus ring, partial veil absent.

Basidiospores 15.0–18.0 × 11.0–12.5 µm, avl × avw = 16.8–11.6 µm, Q= 1.36–1.44, avQ= 1.41; apiculate, moderately thick walled, smooth, broadly ellipsoid to lemon shaped, proximal and distal poles taper, middle broad, black 5% KOH, inamyloid, yellow in Melzer's reagent. Basidia 23.5–33.0 × 11.5–15.5 µm, 4-sterigmata 3.0–4.3 µm in length, septate bases, hyaline to pale yellow in 5% KOH, thin walled, clavate to sac like transparant. Cystidia 19.5–28.0 × 10.0–13.5 µm, hyaline, thin walled, clavate.

Material examined: Pakistan, Khyber Pakhtunkhwa, Abbottabad, Himalayan Moist Temperate Forests, Nathia Gali (34° 4' 0" N, 73° 24' 0" E), at 2450 m a.s.l., gregarious, decaying dried cow dung, 23 August 2010, Abdul Razaq (K-41) LAH.No.230841.' GenBank Accsession # HE819397.

Comments: *Paneolus sphinctrinus* grows usually in groups on cow or horse dung in pastures during rainy season from May to September. This species is fairly common and known to be poisonous. It produces symptoms of intoxication. *P. retirugis* Fr. is similar to *P. sphinctrinus* but has a more wrinkled or reticulate pileus; it is also believed to be poisonous (Groves, 1962). Morphological and anatomical characterizations of the specimen were compared in the literature and there are very careful differences among different species of *Paneolus* for example their spore size, shape, colour and habitat are very close (Groves, 1962; Ola'h 1969; Smith *et al*., 1979).

Molecular characterization of *P. sphinctrinus* in the form of a phylogenetic tree is provided in which seven published/unpublished sequences of the species from different areas of the world were included and compared (FJ755221, FJ755227, DQ182503.1, JF908513.1, HM035081.1, AY129348, AY152728.1). Some of the sequences are very short therefore they have been excluded from alignment. These sequences are intraspecifically different (Fig. 3) even two sequences (FJ755221, FJ755227) deposited from China by the same author behave in a totally different way. Other possible reason may be misidentification of different species because there are close similarities of species of *Paneolus* genus and the same habitat usually on dung (Smith *et al*., 1979). This may easily lead to misidentification of *Paneolus* species. A significant molecular data about *Paneolus* genus is still to be developed.

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