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PHYLOGENETIC ANALYSIS OF *LENTINUS SQUARROSULUS*, MACROFUNGUS FROM CENTRAL PUNJAB, PAKISTAN BASED ON DNA MARKER

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Abstract

A lignicolous macrofungus, *Lentinus squarrosulus*, is collected from dead logs of bamboo vegetation at Rana resort and Safari park located in central Punjab region of Pakistan that has never been recorded from plain areas of Pakistan. The identification of this taxon is based on morphological and molecular characterizations. Morphological characteristics such as pileus size, form, colour, basidiomata and scales, stipe length, width, and texture, and spore anatomy have been documented and found in accordance with characteristics of European collection of the same species. Molecularly, ITS-rDNA, universal fungal marker of the internal transcribed spacers of ribosomal DNA sequences,, from local collections have been compared to GenBank database sequences by using BLAST analysis and further the evolutionary affinities of local collection determined by phylogenetic analysis. All of the data examined has been found to be consistent with the morpho-anatomical and molecular characteristics of *L. squarrosulus* collections from Europe and Asia.

Key words: Edible mushroom, polyporaceae, DNA barcoding, *Lentinus*

Introduction

Lentinus belongs to Agaricomycetes and Polyporaceae that are generally considered as lignicolous or saprophytic edible fungi comprising of about sixty three (63) species (Kirk et al., 2008). Several species of this genus are known for growing spontaneously on species specific substrates and even capable can grow upon pasteurised substrates (Corner, 1981; Philippoussis et al., 2001; Karunarathna et al., 2011; De Leon et al., 2017). They have strong, hard fruiting bodies, dimitic tissues in the basidicarps, decurrent to sub-decurrent lamellae with hyaline, cylindrical to ellipsoidal basidiospores. *Lentinus* species can be further characterized by having hyphal pegs (Pegler, 1983). Due to its basidiocarp and lamellate nature, *Lentinus* is a distinctive genus within the Polyporales family (Hermawan, 2021). *Neolentinus* is another sister genus of *Lentinus* but it can be separated because it causes brown rot in plants whereas the latter causes white rot (Redhead and Ginns, 1985). Mycologists have been interested in the white rot fungus genus *Lentinus* for a long time due to its numerous taxonomic issues (Manjunathan et al., 2011). *Lentinus* can further be distinguished by its xeromorphic hard carpophores with serrated gill borders. *Lentinus* has a wide range of fruiting bodies that are often lignicolous, especially in subtropical areas (Pegler, 1977). The genus is recognized by its solid and permanent texture, dimitic hyphal system, and generative hyphae that can be either skeletal or binding (Pegler 1977; Singer 1986).

In Pakistan, a wide range of macrofungi are found especially in hilly areas of northern Pakistan. During the rainy season, *Lentinus squarrosulus* is found growing on decomposing wood logs and has been collected from safari park present in central Punjab of

Pakistan. *Lentinus squarosulus* is famous for its edibility and herbal uses for having high amount of vitamin and mineral nutrients (Gulati *et al.,* 2011; Adenipekun et al., 2021). This fungus can grow on a broad range of substrates and habitats. There have been reports of numerous *Lentinus* species growing on unique substrates in the wild and on pasteurised substrates (Morais *et al.,* 2000; Philippousis *et al.,* 2001). Ishaq et al., (2022) reported *L. squarrosulus* is an edible macro-fungus from the moist temperate Himalayan forests of Pakistan. This species has been described using morphological and molecular details using Internal Transcribed Spacers (ITS) as molecular marker. Molecular markers and the Internal Transcribed Spacers (ITS) of rDNA are employed in Pakistan for species identification and fungal taxonomy (Jabeen et al., 2017).

According to phylogenetic analysis, the Pakistani collection recovered with related same species sequences from the rest of the world using a universal marker for fungi, ITS-nrDNA marker (Grades and Bruns 1993; Vellinga, 2003; Ilyas et al., 2013; Razaq et al., 2012, 2016, 2017). The goal of study is to report an addition of novel locality for described taxon from central part of Pakistan that will also be add to the local fungal diversity of central Punjab and plain areas of Pakistan.

Materials and Methods

1. Morpho-anatomical characterization

In the current study, fruiting body of mushroom were collected from Rana Resort and Safari Park, which is located near "Bamboo" in "Bhai Pheru, More Khunda Road Head Baloki District Nankana, Pakistan. Morphological characters were noted on the spot from collection site, the mushrooms were photographed and a field observations were recorded. Morpho-anatomically the collections were characterized following Vellinga (2001). Free hand sections of lamella were prepared for anatomical characters after dipping the material dissolved in 5% KOH solution and observed using light microscope.

2. **Molecular characterization**

For DNA extraction a fragment of dried basidiomata was extracted and dissolved in CTAB buffer by following protocol of Gardes and Bruns, (1993). Internal Transcribed Spacers (ITS) part of nrDNA was amplified following protocol described by (White *et al.,* 1990; Gardes & Bruns, 1993). For visualizing and sequencing of amplified product, Razaq *et al.,* 2016, 2017 was followed.

The sequences were created and edited with the current version of the BioEdit alignment software (Tom Hall, Ibis Biosciences, Carlsbad and California). Local species nucleotide sequence comparisons were carried out using online databases. Molecular Evolutionary Genetics Analysis (MEGA) software was used to perform sequence alignments and phylogenetic analysis (Tamura *et al.,* 2011). All of the sequences in the study are aligned with the default settings. After completing the BLAST (Basic Local Alignment Search Tool) closely related sequences were downloaded from GenBank for alignment and phylogenetic analysis. Sequences alignment and phylogenetic tree construction was carried by using MEGA ver. 6.0 software (Tamura *et al.,* 2013). In addition, the alignment was trimmed with conserved motifs to allow for the inclusion of full ITS parts of sequences (Dentinger et al., 2011). On the bases of Jukes-Cantor model of nrITS sequencing, the maximum likelihood (ML) technique used the nearest-neighbor-interchange (NNI) heuristic search technique. The bootstrap value of 1000 replicates was used to test the phylogeny. All recently produced

sequences were uploaded to GenBank, and in the phylogenetic tree their accession codes also mentioned.

Results

Morpho anatomical characterization.

Pileus 2-8 cm diam., 3.5cm height; white cream to yellowish, smooth surface, sometimes light brown scales scattered from center to margins, lighter towards margins, surface convex to straight, margins smooth to slightly dentate, context, hard, pale yellow to cream, somewhat thick. Gills decurrent, brownish, packed, with dentate edges; lamellulae, variable in length and distribution among gills, vary from small marginal to full length of lamellulae, mainly marginal. Stipe 2.5 × 0.6 cm wide, solid, cream to white rooty, woody texture Context is pale yellow brown, with no volva. There was no mention of taste or odour.

Basidiospores 40-7.50 × 3.2 -11.2 µm, ovoid to ellipsoidal, thin walled, sharpe apiculus. Basidia 15.0–21.0 × 4.5–6.05 µm, clavate narrowly clavate, 4-spored. Cheilocystidia are of two types, the longer ones (21.0-30.5 5.0-7.5 µm) and the shorter ones (18.0-23.5 5.0-7.5 µm), clamped connections present, cylindrical to nearly clavate, colorles to light green in KOH solution. Pleurocystidia are not present. There are Hymenium trama hyphal, regular, parallel Clamp connections. Dimitic hyphae present, generative hyphae and skeletal ones. Generative hyphae 2.0–6.0 µm wide, frequently branched, scarcely inflated, colorless, thin walled, clamp connections present. Skeletal hyphae 2.0–10.0 µm wide, thick walled, colourless, and not frequently branched.

Material Examined:

Pakistan, Punjab, Kasur, Nankana, Rana resort, rich soil of Bamboo vegetation, solitary to gregarious , collector, Razaq, NA1, AS1, 23/8/2018; submitted to herbarium, Department of Biological Sciences, (UVAS), Pattoki, Pakistan.

Molecular characterizations

The genetically informative and polymorphic part of ribosomal DNA (ITS1, 5.8S, ITS2) after amplification using PCR with universal primer pair (ITS1F & ITS4) produce amplicons of ranges from 700-800bp. Web based BLAST analysis of sequences showed maximum comparative nucleotide similarity with *L. squarrosulus* (accession# KP283482). Other closely related sequences are: *L. sajorcaju* KP283494, *L. badius* KP283481, *L. badius* KP283481. To determine the placement of samples collected from Pakistan with previously reported closely related genera, phylogenetic analysis was performed.

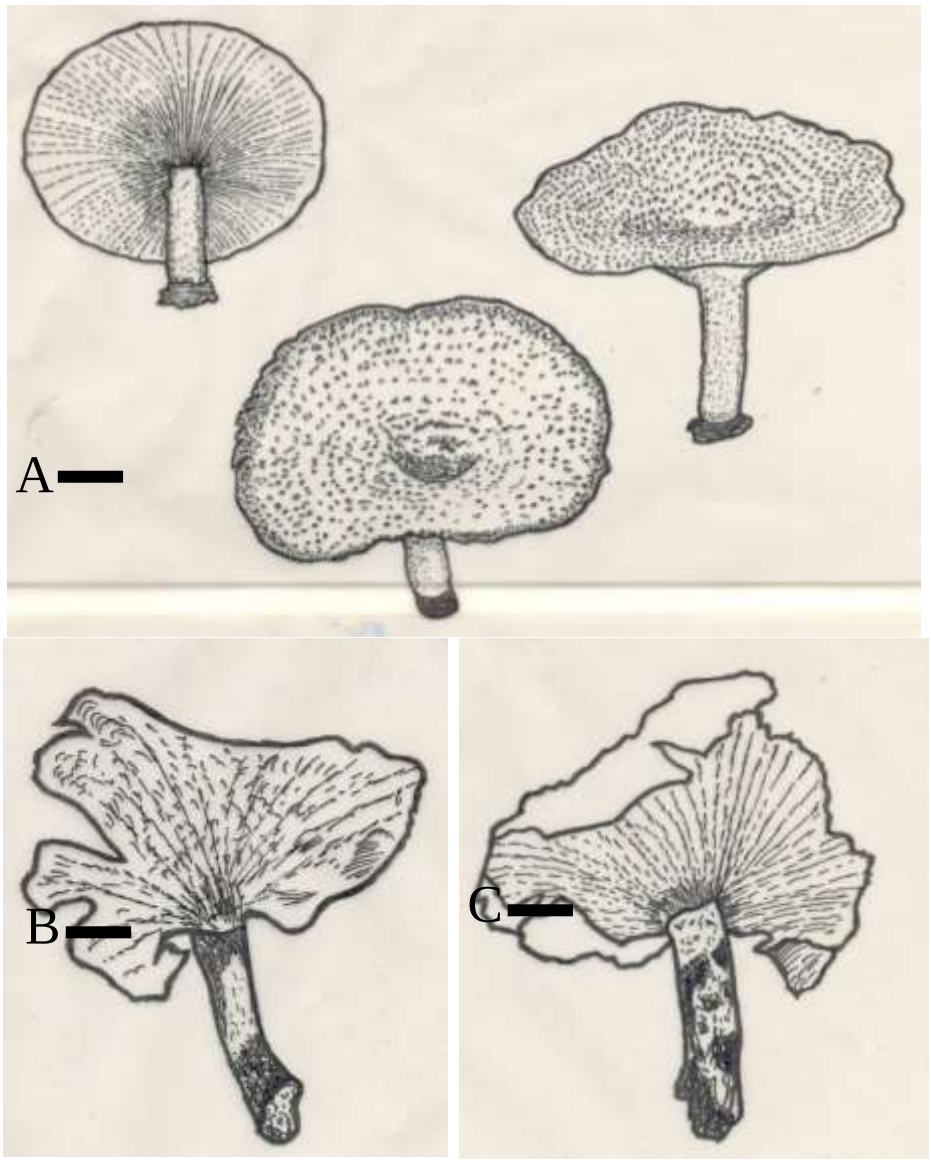

Fig. 1. Morphological features of *Lentinus squarrosulus* (a) Surface view with squarrose scales. (b, c) Lamellae.

Evolutionary relationships deduced from 29 taxa's ITS-rDNA sequences using maximum parsimony analysis. Utilizing the greatest likelihood method, *Lentinus squarrosulus* species obtained in Pakistan were analysed phylogenetically. This research is based on the 5.8 S rDNA marker and the fungal ITS1 and ITS2 sequences. Gaps are treated as data in phylogenetic analysis. In the phylogenetic tree clades were formed. KP283502, KP283503, KP283505, KP283504, KP283501, KP283498, KP283497, KP283499, KP283496, KP283481, KP283480, KP283479, KP283508, KP283494, KP283493, KP283492, KP283482, KP283484, KP283483 were included in six different clades. The maximum likelihood method used to reconstruct the phylogenetic tree from indigenous collections of

Lentinus squarrosulus. A sum of 29 closely similar *Lentinus* sequences were used in the evolutionary analysis. In this study, the local collections of *L. squarrosulus* recovered among the sequences of the same species (Clade VIII, Fig.3).

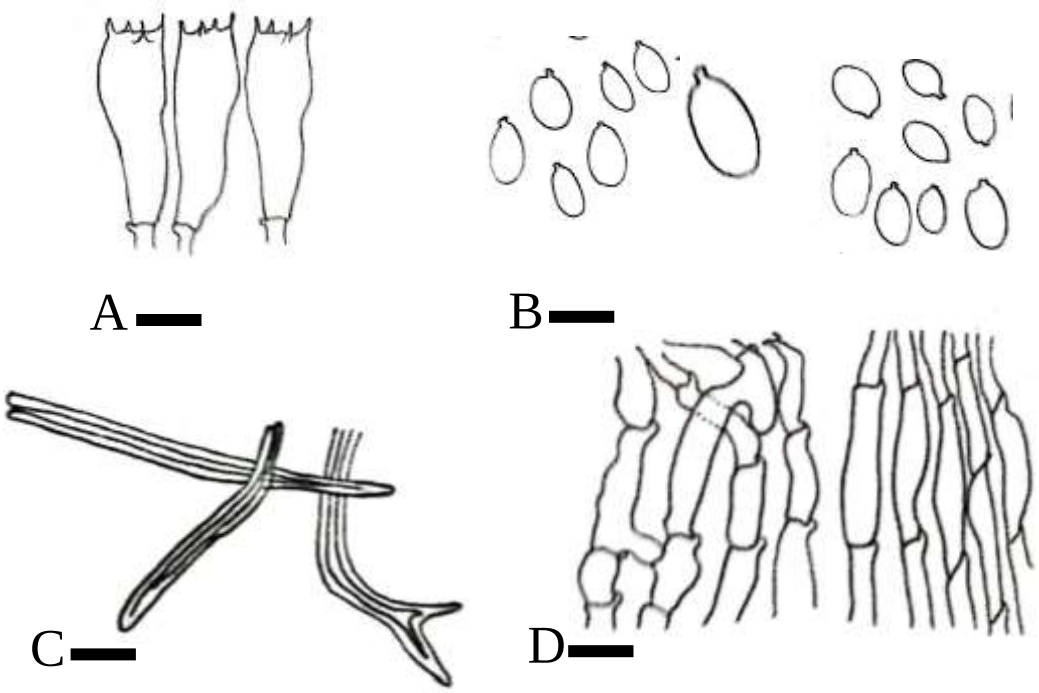

Fig. 2. Anatomical features of *Lentinus squarrosulus*. (a) Basidia. (b) Basidiospores. (c) Cheiliocystidia. (d) Pileipellis.

Discussion

Polypores of Polyporaceae have been actively investigated in the previous period because these fungus can breakdown hemicellulose, cellulose and lignin from cell walls of plants, and therefore these fungi play a important role in the recycling process of nutrients in the most of forest ecosystems of the world (Lindner and Banik 2008; Cui et al., 2019). With the recent advancement of molecular tools in recent years, DNA markers have been greatly used for the identifications and taxonomy of polypores and therefore this data have remarkably contributed to a realistic classification of the family. Nuclear small-subunit ribosomal DNA data has been used to establish evolutionary connections of the members of Polyporaceae (Hibbett and Donoghue, 1995). Binder et al., (2013) described an evolutionary details of the taxa of Polyporales suggesting that the core polyporoid clade of their work could represent Polyporaceae. From Pakistan, a small numbers of this gnus are reported so far, only four species of *Lentinus* have reported record (Ahmad et al., 1997). *Lentinus squarrosulus* is a tough and hard mushroom that belongs to the family Polyporaceae and is infamous for inedibility (Ugbogu et al., 2019).

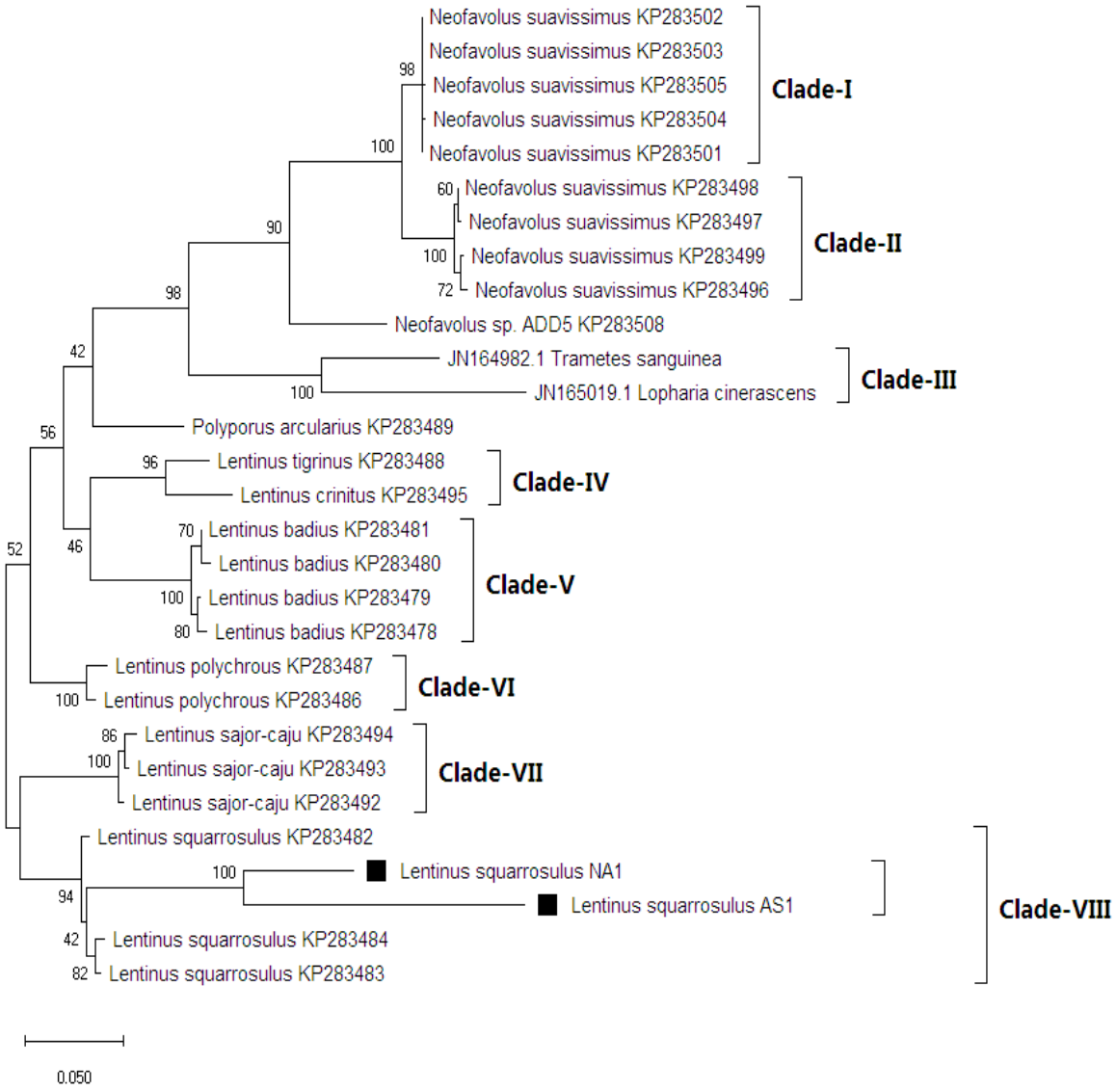

Fig. 3. Phylogenetic tree of *Lentinus squarrosulus* based on ITS-DNA marker using maximum likelihood method. The numerical numbers mentioned on branch node represent bootstrap values. Studied sequences in current research have been labeled with a box (■).

Morphologically, Pakistani *L. squarrosulus* collections include rough, leathery, and scaly pileus with spores, indicating a similar resemblance. *L. sajor-caju* is widely distributed. According to Pegler (1983), the fungus reported as *L. crinitus* by Natarajan and Raman (1981) from southern India represents *L. squarrosulus*. Spores of the Kerala collections measured 6-10 x 3-5 (7.68±1.08 x 4.35±0.45) µm. Jordan (1995) described same species with larged sized cap (16cm broad) and with long stipe (8cm long) while the Pakistani collection has smaller sized of cap (1.5-10 cm x 3-20 mm with cap) but the molecular data supported the Pakistani collection as *L. squarrosulus* (Fig.2). This difference in legths and size the collections can be justified as both these collection are collected from different geographical areas, the description of premature specimen can also be reason for this difference but the other features like the colour of lamellae, pale yellow, brown scales on surface of pileus especially their presence on central concave part, spore size (Pakistani 5.82–12.12 × 4.85–10.29 µm, Vs Kerala 6-10 x 3-5 (7.68±1.08 x 4.35±0.45) µm Vs India7.0 - 10.5 x 3.0 - 4.5 mm) indicate that both the Indian and Pakistani collections are members of the same species (Bas et al., 1990).

The spore size specified for the Kerala collection is enough for the Pakistani specimens of this species. The Pakistani collection's large stem sizes nevertheless exhibit some phenotypic variety which can be detected genetically in the form of intraspecific variation.

Lentinus squarrosulus is widely found in Pakistan's woods. This collection is found on the decaying wood logs of pine this species also very close relative of Pakistani collections of *Neolentinus* species in the forests of Pakistan (Ginns, 1986; Farr et al., 1989; Bas, 1990).

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