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Structural characterization of wild edible *Pleurotus* collected from Punjab, India

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Changes in global climate and over harvesting of mushroom diversity growing in wild are responsible for depletion of certain naturally grown mushroom species. Conservation of wild diversity of mushrooms before extinction is crucial to sustainably nurture the ecosystem. Genus *Pleurotus* recorded 70 spp. but few species are commercially cultivated (Gargano *et al.* 2013), such as *P. florida, P. sajor-caju* and *P. ostreatus*. Species from the genus *Pleurotus* exhibit a diverse range of morphological characters in varied environment and climates (Estrada *et al.* 2010, Maftoun *et al.* 2015). Hence, morphological classification is not a preferred method to identify *Pleurotus* spp. from wild as it may lead to misidentification of *Pleurotus* spp. (Maftoun *et al.* 2015). Different molecular tools based on PCR or non-PCR are mainly used for characterization of *Pleurotus*. Internal transcribed spacer (ITS) region of DNA is an easier and reliable method to amplify the highest degree of variation among closely related species (Schoch *et al.* 2012). Hence, the aim of the study was to identify the wild *Pleurotus* (consumed by locals) variability using ITS based sequencing collected from Punjab.

The field survey was conducted for collection of wild *Pleurotus* spp. i.e. *P. sapidus* and *P. floridanus*. A research trial to domesticate these wild mushrooms was conducted and these mushrooms were identified through ITS based sequencing and their protein profile was examined.

Sample collection and procurement of cultures: Wild mushroom *P. sapidus* was collected from Danour, Pathankot (32º19'50"N and 75º49'92"E) and *P. floridanus* was collected from Sirhind, District Fatehgarh Sahib (75°22'47"N and 31°22'47"E). They were described morphologically by their colour, shape, size and habitat. Spore print and pure mycelial cultures were obtained (Stamets 2000, Dhouib *et al.* 2005).

DNA extraction and purification: Pure mycelial cultures were grown on CYM (complete yeast medium) broth and total fungal DNA was extracted by using CTAB methodology.

Primer design: ITS-1, (5'-TCCGTAGGTGAACC TGCGG-3') and ITS-4(5'-TCCTCCGCTTATTGAT ATGC-3') were used as forward and reverse primers respectively (White *et al.* 1990) to amplify ITS region of 5.8S r RNA gene by PCR.

Amplification with PCR: Amplification of DNA through Eppendorf thermal cycler was performed with a final volume of 25 µl which includes 90 ng of extracted DNA approximately, 2.0 mM Mg Cl_2, 0.2 mM of each dNTPs, 5X green Taq buffer, 10v pmole of both the primers and 3.75 units of DNA Polymerase (Promega).

Phylogenetic analysis: BLAST of ITS region was carried out with NCBI Genbank database. Clustal W was used to generate distance matrix and MEGA 7 was used to construct the phylogenetic tree (Kumar *et al.* 2016). Maximum Likelihood method based on the Kimura 2-parameter model (1980) was used to infer evolutionary history.

Electrophoresis: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a vertical slab gel apparatus (Bio-Rad Protean II, Bio-Rad, USA) using 2-mercaptoethanol (Laemmli 1970).

Densitometry: Electrophoretic bands of polypeptide subunits was analyzed on Proteoscan II Computer System using transmittance mode. Photographs were taken to record the protein separation.

Scanning electron microscopy of mycelial samples of three Pluerotus samples: Imaging (high resolution) of the fungal hyphae using scanning electron microscope (Hitachi S2400N) was performed to compare ultra-structural of the hyphae of one week old *Pluerotus* cultures (Two cultures collected from wild and one commercially available culture of *Pleurotus florida* as control).

Two *Pleurotus* spp. i.e. *P. sapidus* (AMN 39) and *P. floridanus* (AMN 202) were collected from wild areas of Punjab, India. The morphological characters of these wild mushrooms were compared with the morphology of

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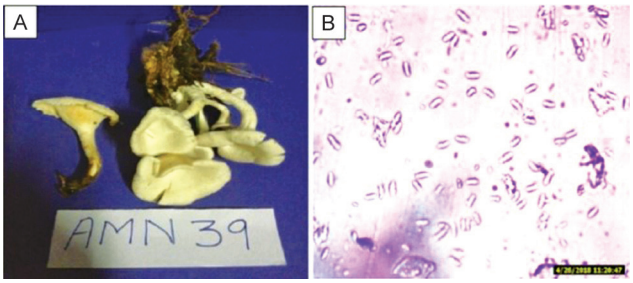

Fig. 1 (A) *Pleurotus sapidus* (AMN 39); (B) Spore prints of *Pleurotus sapidus* (AMN 39).

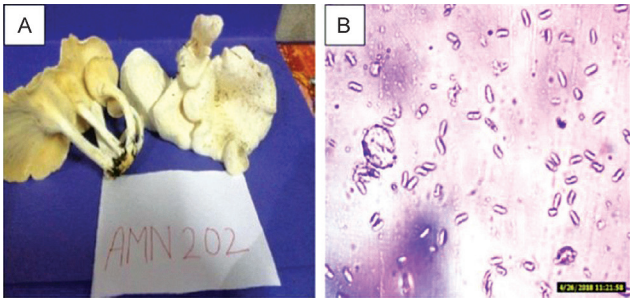

Fig. 2 (A) *Pleurotus floridanus* (AMN 202); (B) Spore prints of *Pleurotus floridanus* (AMN 202).

mushrooms given by Hunter (1975), Singer (1975) and www.mushroomexport.com.

Description of Wild Pleurotus: Morphology such as macroscopic characters pertaining to gross morphology is described as follows:

AMN 39 (Pleurotus sapidus): It was observed in clusters. The caps were irregular or not fan shaped, densely crowded, smooth and white in colour. The flesh was observed as thick and white (Fig. 1A). The gills were white, broad in size. The stipe was generally short, solid, either laterally or eccentrically attached with cap. Spores were oblong and white but after a short exposure to air, exhibit a pale lilac colour (Fig. 1B). Cap size varied from 3–5 cm. It was observed on wooden stumps in the month of July.

AMN 202 (Pleurotus floridanus): Pileus was soft, 4–8 cm broad, fleshy, fan shaped or slightly depressed behind, (Fig. 2A). Gills were broad, shortly decurrent, and white in colour, branched at the base, the stipe was very short. Spores were observed to be oblong, white, 0.010 mm long, 0.004 mm broad (Fig. 2B). It was found in the month of August.

Molecular characterization of AMN 39 and AMN 202: Fragment of the rDNA-ITS region was amplified using ITS1 and ITS4 primers and forward and reverse DNA sequencing of PCR amplicon was carried out. Single band of DNA was observed around 700 bps.

The analysis involved 11 nucleotide sequences. A total of 665 positions were observed for AMN39 and 670 positions for AMN202 in the final dataset. The aligned sequence was submitted to gene bank and accessioned number as AMN 39 (MK 281339) and AMN 202 (MK 281340). Based on the BLAST, first 10 sequences with maximum similarity were selected and phylogenetic tree was generated (Supplementary Fig. 1 and 2). AMN 39 showed high similarity with *Pleurotus sapidus* and AMN 202 was similar to *Pleurotus floridanus* based on nucleotide homology and phylogenetic analysis (Table 1). Morphological characters of AMN 39 and AMN 202 ensures the reliability of molecular techniques.

Genetic diversification studies of rDNA internal transcribed spacer (ITS) has become popular method for identification (Maftoun *et al.* 2015) which provides grounds to the present study to amplify ITS region of wild fungus. Similar method of amplification based upon ITS region was used by various authors i.e. four *Pleurotus* spp. viz. *P. sajor-caju, P. djamor*, *P. floridanus* and *P. ostreatus* were identified by Rajaratnam and Thiagarajan (2012), Roy and Chakraborty (2023) identified wild mushrooms from *Perenniporia* species. To ensure the viability of molecular based techniques it is important to compare its morphological characters with the specimen (Khaund and Joshi 2014). Morales-Flores *et al.* (2022) identified wild strain of *Pleurotus* spp., *Pleurotus djamor* by sequencing the region with ITS1-5.8S-ITS2 and studied the morphological characters of wild mushroom. Eleven wild Basidiomycota species from three different genera (*Pleurotus, Agaricus, and Pisolithus*), three different families (Pleurotaceae, Agaricaceae, and Sclerodermataceae) and two orders (Agaricales and Boletales) were diagnosed at molecular level and morphologically by Chechan *et al.* (2020). .

SDS-PAGE of extracellular enzymes: In both the species, AMN 39 (*Pleurotus sapidus*) and AMN 202 (*Pleurotus floridanus*), two proteins of molecular weight 24.81 KDa and 28.43 KDa were observed to be same. AMN 39 contains one more protein of MW of 16.16 KDa and AMN 202 contains two more proteins of MW 17.47 KDa and 42.8 KDa (Table 2).

Estimation of protein among wild edible mushrooms is limited, which is crucial to know the role of wild mushrooms in protein supply. Moreover, quality and quantity

Table 1 Summary of the BLAST results to show homology with the wild isolate AMN 39 (*P. sapidus*) and AMN 202 (*P. floridanus*)

Description	Query cover	E value	Identity	Accession number
Pleurotus floridanus (FJ810170.1)	99%	0	100%	MK 281340 (AMN 202)
Pleurotus sapidus (HM561980.1)	100%	0	100%	MK 281339 (AMN 39)

Table 2 Extracellular proteins in AMN 39 and AMN 202

Pleurotus spp.	No. of bands	Molecular weight
P. sapidus (AMN 39)	3	16.16, 24.81, 28.43
P. floridanus (AMN 202)	4	17.47, 24.81, 28.43,42.8

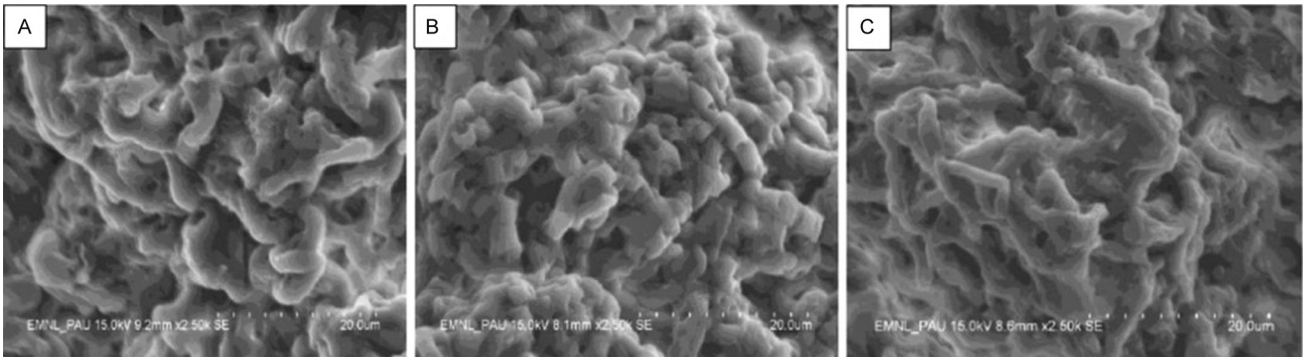

Fig. 3 Scanning electron micrograph of (A) *Pleurotus florida*; (B) AMN 39 (*Pleurotus sapidus*); and (C) AMN 202 (*Pleurotus floridanus*).

of mushroom protein can help to reveal the intra and inter species dissimilarity (Uzun *et al.* 2009). Variability in protein content depends upon many factors, namely, type of the mushroom, development stage, environmental and genetic factor. Thus, quantification of wild mushrooms becomes essential at every location (Kavhumbura 2022).

During the present study, molecular weight of proteins varies from 16.16 KDa–42.8 KDa which was in accordance to previous work, proteins, ostreolysin and aegerolysin with MW 16 KDa (Berne *et al.* 2002) were observed in fruiting bodies of the edible mushrooms *Pleurotus ostreatus* and *Agrocybe aegerita.* Another protein, pleurotolysin A consists of MW 17 KDa was purified by Sakurai *et al.* (2004). Similarly, Wu *et al.* (2023) detected low molecular weight (17 KDa) protein in *Pleurotus eryngii*. Zuchowski and Grzywnowicz (2006) detected three different proteins having molecular weight of 14.5, 16.6, and 20 kDa. Petrovska (2001) found proteins of molecular weight ranging from 30–90 kDa in wild mushrooms. .

Scanning electron microscopy of Pleurotus florida, Pleurotus sapidus *(AMN 39) and* Pleurotus floridanus *(AMN 202)*: Ultra-structure of three mushrooms was examined through scanning electron microscopy. It was observed that hyphae of commercial strain, *Pleurotus florida* (Fig. 3A) was seen to be more tightly packed and denser in comparison to wild mushrooms, *Pleurotus sapidus* (AMN 39) (Fig. 3B) and *Pleurotus floridanus* (AMN 202) (Fig. 3C). Hyphae of *Pleurotus sapidus* (AMN 39) was packed tightly in comparison to *Pleurotus floridanus* (AMN 202).

SEM is widely used technique in life science to compare the surface of biological material but few studies of hyphal examination were recorded i.e. the hyphae of *Agaricus bisporus* (Lang) Sing from casing and composting were compared by Masaphy *et al.* (1987) and difference was recorded amidst both the hyphae. Boekstein *et al.* (1993) also compared hyphae wild mushroom specimens.

This study was an attempt to identify *Pleurotus* mushroom species from wild areas of Punjab to keep a database of wild species before getting extinct. Two wild *Pleurotus* mushrooms collected, analyzed using molecular level techniques and submitted to NCBI gene bank under the accession no. MK 281339 (AMN 39) and MK 281340 (AMN 202). In conclusion, the examination of unidentified mushrooms presents an intriguing opportunity for further research to exploit wild species for human benefits and generate database before wild mushrooms get eloped. Further, GenBank database should be sufficient enough to identify different species of wild mushrooms otherwise it will be difficult to recognize the wild mushrooms at species level.

SUMMARY

Oyster mushroom (*Pleurotus* spp.) belongs to the class Basidiomycetes, family Agaricaceae, commonly known as ‘dhingri’ in India and proliferates naturally on dead, decay wooden logs or trunks of deciduous or coniferous woods in tropical and temperate forests. Although *Pleurotus* mushrooms are commercially important mushrooms but wild *Pleurotus* still require public recognition. Hence, the present study aimed to conduct a field survey for collection of *Pleurotus* mushrooms from wild regions of of Punjab Two wild *Pleurotus* were collected and domesticated at Mushroom Research Complex, Punjab Agricultural University, Ludhiana, Punjab which were further analyzed at molecular and biochemical levels. These mushrooms were identified at species level through ITS (Internal transcribed spacer region) based method (18S ribosomal RNA gene, internal transcribed spacer, 5.8S ribosomal RNA gene, complete sequence; and 28S ribosomal RNA gene) was carried out. The gene bank accessioned *Pleurotus sapidus* as MK 281339 and *Pleurotus floridanus* as MK 281340. Hyphae of *Pleurotus sapidus* was observed to be packed tightly to a greater extent as compared to *Pleurotus floridanus* according to the ultra-structure recorded by scanning electron microscope. Both species of Pleurotus share two different proteins of same molecular weight 24.81 KDa and 28.43 KDa. Collecting and identifying wild species is crucial to preserve the wild fruits and fathom out the benefits of wild edible *Pleurotus* spp.

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