

MOLECULAR IDENTIFICATION OF NOTEWORTHY LIGNICOLOUS FUNGUS, *NEOLENTINUS LEPEDIUS* (FR.) REDHEAD & GINNS: A NEW GENUS FOR PAKISTAN USING PHENOTYPICAL AND PHYLOGENETIC APPROACHES

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Abstract

Neolentinus lepedius (Fr.) Redhead & Ginns is a lignicolous/saprophytic macrofungus found on the dead logs of pine vegetation of Himalayan forests, not previously reported from Pakistan. The purpose of this work is to report the occurrence of a European genus from pine forests of Pakistan, using morphological and molecular characterization. For species recognition, the phenotypic features like size, shape, colour of basidiocarps and scales on pileus, length, width and texture of stipe and spores have been compared with available literature. Internal Transcribed Spacers (ITS) region of ribosomal DNA of all local collections has been amplified using universal fungal primers. Local species sequences were confirmed by using BLAST analysis and construction of Phylogenetic tree. All the data analysed was found consistent with morpho-anatomical and molecular characters of *N. lepedius*. Morphological description, photographs, line drawings and phylogenetic tree of Pakistani collections have also been provided.

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

Introduction

Neolentinus Redhead & Ginns is small saprophytic genus with eleven species belonging to Gloeophyllaceae, characterized morphologically by tough, leathery hard fruiting bodies with serrate lamellae and anatomically it can be distinguished by binucleate, inamyloid, fusoid spores, unbranched sclerohyphae, pseudocystidia and basidia more than 20µm in lengths (Redhead & Ginns, 1985; Zmitrovich & Kovalenko, 2016). It was segregated from closely related genus, *Lentinus* as it produced brown rot in plant while latter produced white rot (Redhead & Ginns, 1985). Phylogenetic analysis based on DNA barcoding also proved that *Neolentinus* is a monophyletic clade distinctly related to *Lentinus* while closely related to other brown rot producing genera like *Gloeophyllum* and *Heliocybe* etc., (Thorn *et al.*, 2000; Hibbett & Donoghue, 2001; Binder *et al.*, 2005; Zmitrovich & Kovalenko, 2016). So, *Neolentinus* is now been treated in Gloeophyllaceae family of order Gloeophyllales while *Lentinus* belong to Polyporaceae of Polyporales (Zmitrovich & Kovalenko, 2016).

A lot of diversity of macrofungi can be observed in Pakistani forests, *Neolentinus* species have not been yet reported from Pakistan so far. Perhaps the close phenotypical resemblance of both genera was a reason for Pakistani mycologists, so they only reported *Lentinus* from local forests (Ahmad *et al.*, 1997). The use of molecular marker especially Internal Transcribed Spacers (ITS) of rDNA for species recognition has revolutionized the fungal taxonomy in Pakistan and now the mycologists are on way to filling the gaps (Ilyas *et al.*, 2013; Jabeen *et al.*, 2017; Razaq *et al.*, 2017). Molecular barcoding made possible the identification of first species of *Neolentinus* pine dominated Himalayan forests of northern areas of Pakistan.

In this study, a newly recorded species of *Neolentinus* is being described from Pakistan using morphological data and phylogenetic details. *Neolentinus lepedius* is hard and tough macrofungus whose pileus has brown scales on white background with long woody stipe always found associated with decaying log of wood giving brown rot. Phylogenetically, Pakistani collection clustered with its respective sequences based on ITS-rDNA marker, a universal DNA marker for fungi having interspecific variability (Gades & Bruns, 1993; Vellinga, 2003; Razaq & Shahzad, 2016; Razaq *et al.*, 2012, 2016). The objective of this work is to report *Neolentinus lepedius* a new generic and species record from Pakistan.

Materials and Methods

Morpho-anatomical characterization: Basidiocarps of this species were observed commonly in Himalayan moist temperate forests of northern Pakistan especially Muree hills of Galyat, near dead pine logs. The fruiting bodies were photographed and field note prepared on the spot. Morphological description and anatomical observation were noted following Vellinga (2001). For anatomical characterization, 20 basidia, 25 basidiospores, and 20 cystidia were measured from each basidiocarp and for line drawings light microscope with camera lucida was used.

Molecular characterization: A small piece of dried basidiocarp was macerated with ice-cold pestle mortar in liquid nitrogen. This fine powdered material was used to extract genomic DNA following protocol given by Gardes & Bruns (1993). For amplification of target region, Internal Transcribed Spacers (ITS) part of rDNA with ITS1f and universal ITS4 primers, a standard protocol was followed (Gardes & Bruns, 1993; White *et al.*, 1990). For visualization and sequencing of PCR product, the method used by Razaq *et al.*, (2016, 2017) was followed.

The consensus sequences were generated and edited manually using Bio Edit alignment software latest version (Tom Hall, Ibis Biosciences, Carlsbad, California). Closely related sequences were retrieved from GenBank after performing BLAST (Basic Local Alignment Search Tool) search for alignment Pakistani sequences and construction of phylogenetic tree, we followed Razaq *et al.*, 2017 using MEGA6 ver. 6.0 (Molecular Evolutionary Genetics Analysis) software (Tamura *et al.*, 2013). All extracted sequences were aligned using clustal W program of MEGA6 using default settings and alignment was trimmed with the conserved motifs 5'-(...GAT) CATTG-and-GACCT (CAAA...)-3' (Dentinger *et al.*, 2011) for inclusion of complete ITS portions of sequences. Consensus nucleotide sequences of all Pakistan collections have been submitted to DNA database, European Molecular Biology Laboratory (EMBL).

Results

Morpho anatomical characterization

Neolentinus lepideus: (Fr.) Redhead & Ginns, *Trans. Mycol. Soc. Japan.*, 26:357, 1985. Fig. 1 (i-v) Pileus 6.0 cm diam., white cream to pinkish with light brown umbonate scales, distributed from centre to periphery, less dense towards the margins, non-viscid when wet, surface straight, smooth margins, plane; context, moderately thick, firm, cream to little pale yellow, unchanging when

bruised; margin entire, undulating, thick. Lamellae/gills decurrent, light pinkish to cream, crowded, narrow, fleshy, edges dentate; lamellulae, diverse in length and distribution, marginal to half-length of lamellae, mostly marginal, alternate with lamellae. Stipe 17.0 × 0.76 cm, thick, firm, central, cream-white covered with orangish brown scales, none cylindrical, rooty baset apering towards the base, without cracking pattern, texture woody; Context pale yellow brown, volva absent. Taste and odor not noted.

Basidiospores 7.0–12.5 × 4.5–6.5 μm; rod to cylindrical to oblong, thin walled, apiculate, hyaline or colourless to yellowish pale in KOH, inamyloid. Basidia 18.0–38.5 × 6.0–9.5 μm, hyaline to yellowish pale in KOH, thin walled, 2–4-sterigmate, four sterigmata uncommon, sub-clavate to clavate with oil contents. Pleurocystidia absent. Cheilocystidia longer as well as shorter, 21.0–30.5 × 5.0–7.5 and shorter, 18.0–23.5 × 5.0–7.5 μm, clamped, cylindrical to narrowly clavate, hyaline to light green in 5% KOH. Hymenium trama hyphal, regular, parallel Clamp connections present.

Material examined: Pakistan, Khyber Pakhtankhaw (KPK), Abbotabad, Ayubia-Khanspur (wild vegetation), nearly 2400-2580m a.s.l, under rich soil of *Pinus wallichiana* A. B. Jacks. Plants, solitary, collector, Abdul Razaq, A08, KP-36, 24/08/2010, 18/08/2011; submitted to Department of Botany herbarium, University of the Punjab, Lahore. LAH. 18081108, 24081036.

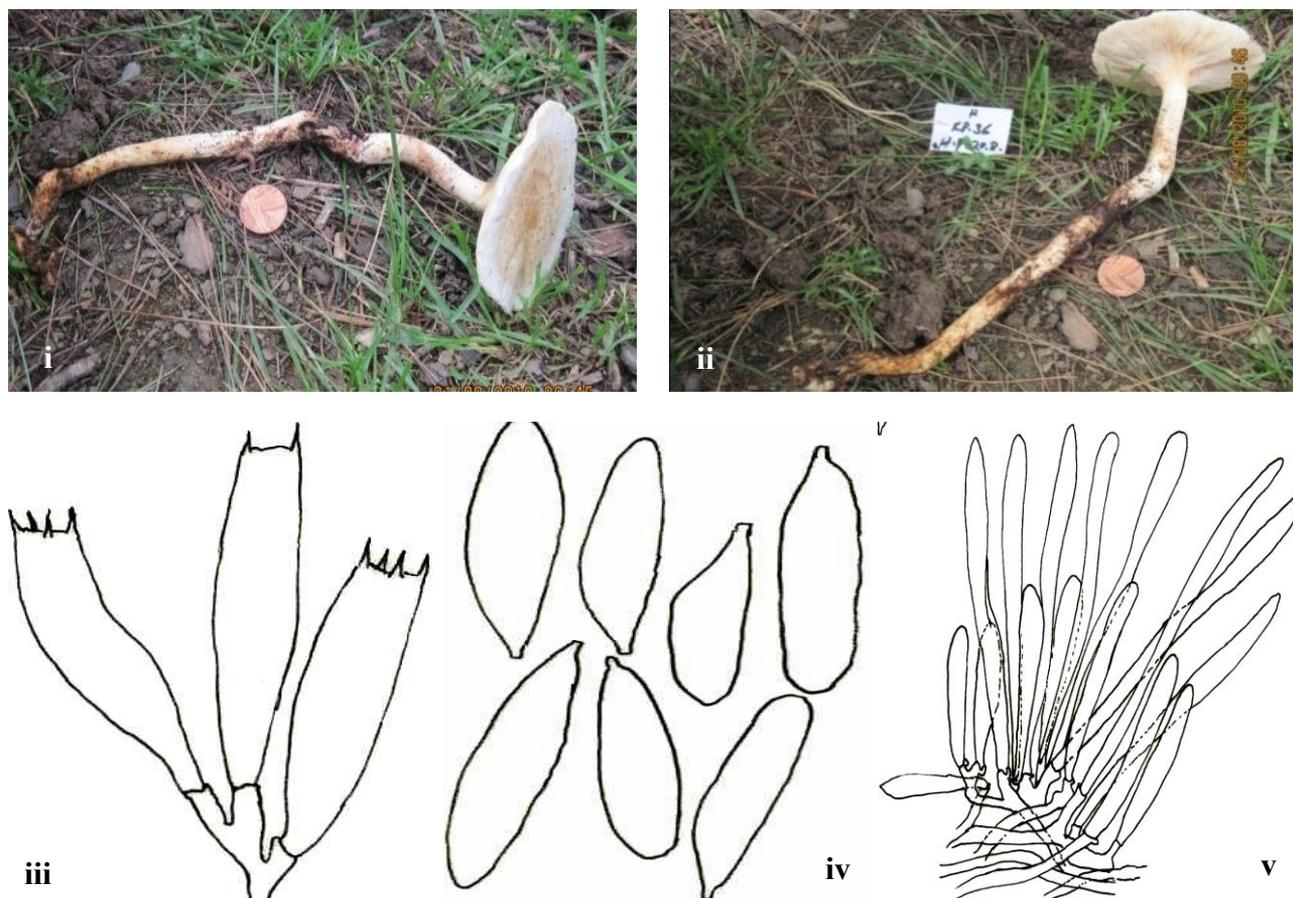


Fig. 1. i-v. *NeolentinusLepideus* (I) Basidioma (II) Lamellar side of basidioma (III) Basidia (IV) Basidiospore (V) Cheilocystidia. Bars = i & ii: 2.7cm, iii: 12μm, iv: 4 μm, v: 5 μm.

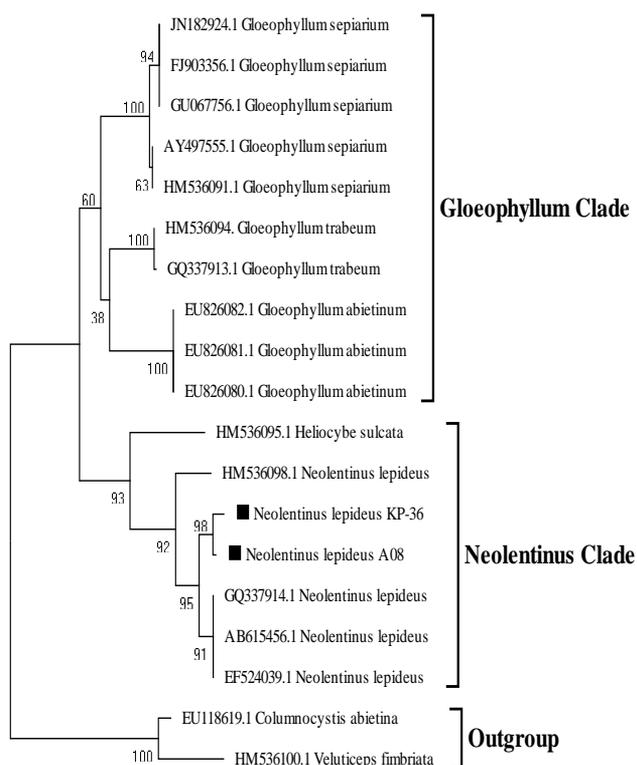


Fig. 2. Phylogenetic analysis of *Neolentinus lepideus* collections from Pakistan based on Jukes-Cantor model, maximum likelihood method using ITS-rDNA. The statistical values present on each branch node represent bootstraps. Pakistani collections have been labeled with a box (■).

Molecular characterization: The ribosomal DNA (ITS1, 5.8S, ITS2) produced fragments of 700-750 bp on gel after amplification with polymerase chain reaction using universal fungal primers (ITS1f & ITS4). In BLAST (Basic Local Alignment Search Tool) analysis, Pakistani sequences showed maximum nucleotide base similarity with *Neolentinus lepideus* (accession # AB615456) showing 96% same bases. This percentage base similarity is noted maximum with its Japanese, Asian collection. Other closely related sequences matches in BLAST window showed similarities with *N. ponderosus* (accession # FJ235147, USA), *Aureobasidium mansonii* (accession # AF121280, Korea), *Gloeophyllum sepiarium* (accession # JN182924, China) and *G. sepiarium* (accession # FJ903356, Latvia). To determine the position of Pakistani collection with rest of the world belonging to closely related genera, phylogenetic analysis has been conducted (Fig. 2).

Phylogenetic analysis of *Neolentinus lepideus*: The phylogeny of local collections of *Neolentinus lepideus* collected from Pakistan was performed using maximum likelihood method. Initially, sequences dataset contained total 2065 characters out of which 599 characters were found informative and included in alignment. A total 20 closely related sequences of *Neolentinus* and *Gloeophyllum* (closely related genus) were included in phylogenetic analysis to clarify the position of local collection. Two specimens of *N. lepideus* were collected, sequenced and phylogenetically analyzed from Pakistan in this work (Fig. 2).

Sequences included in this analysis mostly belong to *Neolentinus* and *Gloeophyllum* P. Karst., which recovered into two separate clades as *Gloeophyllum* clade, *Neolentinus* clade, (Fig. 2). Clade I (*Gloeophyllum* clade) contains only *Gloeophyllum* species; *G. trabeum* (Pers.) Murrill (GQ337913.1, HM536094.1) *G. abietinum* (Bull.) P. Karst. (EU826081.1, EU826080.1, EU826082.1), *G. sepiarium* (Wulfen) P. Karst. (FJ903356.1, JN182924.1, GU067756.1, HM536091.1, AY497555.1) while Clade II (*Neolentinus* clade) has *Neolentinus* species, especially *N. lepideus* Redhead & Ginns (AB615456.1, HM536098.1, GQ337914.1, EF524039.1). Pakistani collection recovered in clade II among *N. lepideus* sequences with significant bootstrap value. Pakistani sequences formed sister clade to three isolates (AB615456.1, EF524039.1, GQ337914.1) but at the same time the fourth one) isolate (HM536089.1 behaving in the same ways as Pakistani sequences but it is yet identified as *N. lepideus*.

Discussion

Morphologically, Pakistani collections of *Neolentinus lepideus*, have tough, leathery and scaly pileus with fusoid spores indicating that it has close resemblance to European one. Jordan (1995) described the European collection with pileus 12cm broad and stipe with 8cm long while the Pakistani collection was 17cm long with cap almost half of the European description. This difference can be related with geographical isolation of two collections and description of premature specimen from Himalayan part but brown colored scales on pileus surface especially concentrated to central depressed part, the colour of lamellae (white), their decurrent nature, spore size (Pakistani $7.0-12.0 \times 4.5-6.0 \mu\text{m}$ Vs European $8-12.5 \times 3.5-5 \mu\text{m}$ Vs North American $7-15 \times 3-6.5 \mu\text{m}$) showed that both European and Pakistani collections were conspecific (Bas *et al.*, 1990). North American mycologists, (Ginns, 1986; Farr *et al.*, 1989) have given pileus size from 6-12 cm which is according to range given for Pakistani collection. The long stem size of Pakistani collection still shows some morphological variation which can also be seen at genetic level as intraspecific variation.

Molecularly, it has been noted that Pakistani collection in BLAST analysis showed more than 97% similarity with Asian sequences which was considered threshold level for species identification (Niskanen *et al.*, 2009; Gao & Yang, 2010) but with European sequences similarity level rarely reached to 97% indicating intraspecific variations in internal transcribed spacers (ITS) of rDNA which could be linked with geographical boundaries. In the same way, in phylogenetic analysis (Fig. 2) the Pakistani collection clustered with those species which formed brown rot of plants in *Neolentinus* clade. Our phylogenetic tree showed two monophyletic clades (Fig. 2, *Gloeophyllum* Clade and *Neolentinus* Clade) and Pakistani collection recovered among *Neolentinus* species especially with European sequences of *N. lepideus* showing its conspecific DNA nature. It was noted that two local collections clustered together monophyletically while the minor nucleotide base difference with European collections was evident as these

collections formed sister clade pattern with European ones (Fig. 2, *Neolentinus* Clade). In our discussion it can be concluded that even a minor genotypic difference among the individuals of same species appear in morphology if we could be able to identify. Morphologically, the closely related genera, *Lentinus* and *Neolentinus* can be differentiated by the disease which they cause (Redhead & Ginns, 1985). This phenotypic difference was also proved by Zmitrovich & Kovalenko (2016), in their phylogenetic tree showing the monophyletic nature of *Neolentinus* species different from *Lentinus* ones on genetic basis. Former genus especially *N. lepedius* is well known from different parts of globe and famous for its non-poisonous nature.

Neolentinus lepedius is well distributed in the forests of Pakistan especially the Himalayan moist forests in Northern parts of Pakistan. This species is found linked with decaying logs of pine vegetation and considered edible but the edibility of Pakistani collection is still to be determined. The European and North American collections are considered edible especially when these are young (Ginns, 1986; Farr *et al.*, 1989; Bas, 1990). As our morphological and molecular phylogenetic data showed that Pakistani collection is conspecific with rest of *N. lepedius* collection, so it can be concluded that *N. lepedius* from Pakistan might be also edible.

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