

MORPHOLOGICAL AND MOLECULAR CONFIRMATION OF THE FIRST FINDING OF *LECCINUM DURIUSCULUM* (BOLETACEAE, BOLETALES) IN GLACIAL ROCKY MOUNTAINS OF HUNZA VALLEY (CENTRAL KARAKORUM), GILGIT, NORTHERN PAKISTAN

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Abstract

The of slate bolete *Leccinum duriusculum* is reported for the first time from Pakistan based on morphological, scanning electron microscopical, ecological and phylogenetic evidences. Sampling was done from glacial rocky mountains of Hunza valley (Central Karakorum), Gilgit, Northern Pakistan, dominated by *Populus alba* trees at their foothills. *Populus* trees are the ectomycorrhizal partners of *Leccinum duriusculum*. A comprehensive description of the macro- and micro-morphological characteristics is included, supplemented with color photographs of fresh basidiomata in their natural habitat and microscopic images with main anatomical features. Phylogenetic analysis of the studied sample was conducted using internal transcribed spacer (ITS) and larger subunit (LSU) sequences of nuclear ribosomal DNA. This analysis supports the taxonomic identification of *Leccinum duriusculum* and confirms its phylogenetic position. Additionally, comparisons are made with closely related taxa that exhibit phenotypic and molecular genetic similarities. To our knowledge, this represents the first record of this taxon occurring in Pakistan.

Key words: LSU, ITS, Morphology, SEM, Taxonomy

Introduction

Leccinum is a diverse genus within the boletes. Many species in this genus form ectomycorrhizal relationships with different plants. Some of these species are popular edible mushrooms, highlighting their ecological and economic significance (Wang *et al.*, 2023, Meng *et al.*, 2021). It features a whitish or yellowish hymenophore and has a white to cream-colored flesh that remains the same or changes to greenish blue or pale reddish when damaged. The stem has brown to blackish tiny squamules, and the basidiospores are relatively long and smooth (Den Bakker & Noordeloos, 2005). Typically, species from this genus are found across subarctic, boreal, temperate, and Mediterranean zones, with some extending into the neotropics (Den Bakker *et al.*, 2004a, 2004b). This genus comprises roughly 150 species (Meng *et al.*, 2021). North America hosts the greatest species diversity of this genus, with 118 species documented in this region (Thiers, 1971). In Europe, Singer subdivided the genus in four sections (Singer, 1986). In the Southern Hemisphere, four species are known: one in New Zealand and three in Australia (Watling, 2001; Segedin, 1987; McNabb, 1968).

Leccinum species have been identified in Asia, specifically in Malaysia, Japan, and China, by examining their morphological and molecular genetic characteristics (Mao *et*

al., 2000; Meng *et al.*, 2021). Despite that Northern Pakistan has biodiversity-rich hotspots regarding fungi, limited research has been done on *Leccinum* species and the species diversity of this genus in Pakistan has not been well elucidated yet. Until now only three *Leccinum* species, *Leccinum scabrum* (Bull.) Gray, *Leccinum ustale* (Berk.) E. Horak, and *Leccinum versipelle* (Fr. and Hök) Snell, have been reported based on morphological analysis (Aman *et al.*, 2022; Razaq & Shahzad, 2017). In this research, we combined morphological data with molecular genetic analysis, utilizing sequences from the nuclear ribosomal internal transcribed spacer (ITS) and the large subunit (LSU) of the nuclear ribosomal RNA. We aimed to assess the phylogenetic relationships of *Leccinum duriusculum*, marking its first confirmed occurrence in the Hunza Valley, Gilgit.

Material and Methods

Site description and sampling

Sampling sites: During summer 2021, a survey of the study area and macromycete collecting were conducted at multiple locations within Hunza Valley. Situated 112 kilometers north of Gilgit, the study area lies on the western bank of the Hunza River and is surrounded by numerous steep peaks exceeding 7000 m s.m. This region has a

temperate climate, with summer temperatures ranging from a high of 27°C to a low of 14°C. During winter, temperatures fluctuate between 10°C and –10°C. The territory of Hunza spans about 7900 square kilometers at an altitude of 3000 meters above sea level. In this valley, the forested region spans approximately 17,028 hectares, characterized mainly by montane dry temperate sub-alpine vegetation. During the monsoon season, these forests receive substantial rainfall. The average humidity in these regions is around 57%, and the mean annual rainfall is 59.3 cm (Hussain, 1995). Macromorphological characters were meticulously recorded from fresh basidiomata, alongside with photographs capturing them in their natural settings. The basidiomata were then dried using a fan heater and stored in envelopes for further laboratory analysis.

Macro- and micromorphological studies: The sample was examined both macro- and microscopically, based on Zang (2006). The macroscopic features were documented from fresh basidiomata, including: 1) the shape, diameter, texture, ornamentation, and surface color of the pileus, along with any bruising reactions in the context, and the color and shape of the margin; and 2) the width, length, color, shape, texture, ornamentation, and attachment of the stipe to the pileus, the presence or absence of an annulus on the stipe, and any bruising reactions in the context; 3) hymenium: color of lamellae, bruising reactions of the hymenial surface. Microscopic examination was conducted for examining the size, shape, cytoplasmic contents, and color reactions of basidia, hymenial cystidia, stipitipellis, pileipellis, basidiospores and terminal cells following Den Bakker & Noordeloos (2005), and Meng *et al.*, (2021). Spore dimensions including length and width of 30 spores were taken.

Molecular phylogenetic studies: Genomic DNA was extracted using the GeneJET Plant Genomic DNA Purification Kit from Thermo Scientific. Bands were observed with the Bio-Rad Gel Doc™ 2000 system. PCR was performed utilizing both fungus-specific and universal primers (ITS1F, ITS4, and ITS4B) to amplify the internal transcribed spacer (ITS) region of nuclear ribosomal DNA, along with LR0R and LR5 primers for the large subunit (LSU) region (White *et al.*, 1990). The PCR products were checked using the nanodrop technique and subsequently cleaned with an enzymatic PCR cleanup method (Werle *et al.*, 1994) following the protocol detailed in Voglmayr & Jaklitsch (2008). DNA sequencing was conducted utilizing the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems, Warrington, UK). This sequencing was carried out on an automated DNA sequencer (ABI 3730xl Genetic Analyzer, Applied Biosystems). The obtained sequences were analyzed using BioEdit sequence alignment editor version 7.2.5 (Hall, 1999). The consensus sequence was then searched using BLAST at NCBI (<http://www.ncbi.nlm.nih.gov/>). For phylogenetic reconstruction, sequences from GenBank with the closest match, as well as additional sequences from *Leccinum* species, were selected (Table 1). The sequences were aligned with the online MUSCLE tool at EMBL-EBI (<http://www.ebi.ac.uk/>) and manually edited as needed using BioEdit (Hall, 1999). MEGA11 software (Tamura *et al.*,

2021) was utilized to construct the phylogenetic tree. Initial trees for the heuristic search were generated automatically by employing the Neighbor-Joining and BioNJ algorithms on a pairwise distance matrix estimated with the Jukes-Cantor model, followed by the selection of the topology with the highest log-likelihood value.

Results

Molecular phylogenetic characterization: The BLAST results of ITS-LSU region revealed a concordance of more than 98 % with *Leccinum duriusculum* (ITS sequences with MT965822, OQ550146 and LSU sequences with MZ675541). The final ITS dataset comprised 23 sequences and 1956 characters. Similarly, the final aligned data set of nrLSU sequences included 25 sequences and 908 characters. Maximum Likelihood method and Jukes-Cantor model (Jukes & Cantor, 1969) was used for inferring the evolutionary history. The tree with the highest log likelihood -5789.38 for ITS phylogeny and -2217.47 for LSU phylogeny is shown (Figs. 1, 2). Bootstrap consensus tree was determined from 1000 replicates, and the corresponding bootstrap values (> 50%) were mentioned in the tree. *Leccinellum albellum* was used as outgroup.

Description of the Pakistani specimen

Leccinum duriusculum (S. Schulz.) Singer - Figs. 3-4

Basidiomata medium-sized. **Pileus** about 5 cm in diameter, sub-hemispherical to convex, surface dry, yellowish brown, brown squamulose all over, small patches towards margins, context white to pale. **Hymenophore** poroid, adnexed to narrowly adnate, creamy; pores rounded, small, extremely dense. **Stipe** measuring 5-6 × 1.4-3 cm, noticeably swollen (ventricose) and tapering towards the base, with slight rooting; covered by fine greyish to blackish squamules on a pale background, forming a net-like structure in the upper part. Whitish at both the top and base, solid, and centrally located. The squamules becoming larger towards the base, often arranged in longitudinal rows or ridges. In some cases, especially in the upper part of the stipe, merging into a reticulate pattern and rupturing at maturity. Context and mycelium white. **Basidiospores** 12-16 × 4-6 µm, fusiform to subfusiform, slightly thick-walled, smooth, with conical apex. **Basidia** 19-33 × 5.0-9.0 µm, clavate, and 4-spored. **Cystidia** 35-55 × 8-12 µm, with a subfusiform to fusiform shape. **Hymenophoral trama** boletoid, with hyphae subcylindrical and 4-10 µm wide. **Hymenial cystidia** abundant, 23-81 × 6-17 µm, lageniform, often with a long, tapering neck. **Pileipellis** a cutis-like structure made up of septate hyphae, with terminal elements measuring 10-85 × 2.7-8 µm. These elements irregularly cylindrical and containing intracellular granular pigment. **Stipitipellis** also displaying a cutis arrangement, characterized by narrow, cylindrical hyphae ranging from 3.4-9.0 µm in width. **Caulocystidia** 23-105 × 4.5-12 µm, lageniform or fusiform, with either thin or thick walls. **Clamp-connections** absent.

Table 1. Accession numbers from GenBank and the geographic origins of the taxa analyzed for the phylogenetic study.

Taxa	Voucher/isolate/ strain	Origin	GenBank accession number		Reference
			ITS	nrLSU	
<i>Leccinum duriusculum</i>	taxon:121049	China	KJ476662		Unpublished
<i>Leccinum duriusculum</i>	SWFU-XJ	China	MT965822		Unpublished
<i>Leccinum duriusculum</i>		France	AF484445		Unpublished
<i>Leccinum duriusculum</i>	Yang5971	Austria	MZ485402	MZ675541	Meng <i>et al.</i> , 2021
<i>Leccinum aurantiacum</i>	WA0000071058	Poland	MK028914		Unpublished
<i>Leccinum versipelle</i>	WA0000071010	Poland	MK028873		Unpublished
<i>Leccinum manzanitae</i>	MF06316 iNat #35018251	USA	MT636961		Unpublished
<i>Leccinum piceinum</i>	men2048	Austria	AF454579		den Bakker <i>et al.</i> , 2004a
<i>Leccinum vulpinum</i>	KPM-NC-0017834	United Kingdom	KC552013		Orihara <i>et al.</i> , 2016
<i>Leccinum rotundifoliae</i>	O73610	Netherlands	GU234155		Geml <i>et al.</i> , 2012
<i>Leccinum scabrum</i>	MQ18R057-QFB30140	Canada	MN992213		Unpublished
<i>Leccinum affvulpinum</i>	Mushroom Observer 207392 (Mycoflora 54417)	USA	MW882080		Unpublished
<i>Leccinum scabrum</i>	hdb022	Sweden	AF454583		Unpublished
<i>Leccinum alaskanum</i>	SAT-16-237-15	USA	MW597143		Unpublished
<i>Leccinum snellii</i>	2835	Canada	KM248968		Unpublished
<i>Leccinum roseotinctum</i>	hdb74	Sweden	AF454575		Unpublished
<i>Leccinum melaneum</i>	IK-00555	Poland	KX610697		Kalucka <i>et al.</i> , 2016
<i>Leccinum schistophilum</i>	F33286UBC	Canada	MH752446		Unpublished
<i>Leccinum albostipitatum</i>	Mushroom Observer #379895	Lithuania	ON692953		Unpublished
<i>Leccinum insolens</i>	iNat66933796	Canada	ON943316		Unpublished
<i>Leccinum insignis</i>	OMDL K. Canan iNaturalist # 185182902	USA	PP156164		Unpublished
<i>Leccinellum albellum</i>	S.D. Russell Mushroom Observer # 207876	USA	ON209218		Unpublished
<i>Leccinellum albellum</i>	FLAS-F-61742	USA		MH620338	Unpublished
<i>Leccinum affsnellii</i>	Mushroom Observer #290898	USA		OQ754971	Unpublished
<i>Leccinum duriusculum</i>	GL4676	Germany		AF139699	Binder and Besl, 2000
<i>Leccinum duriusculum</i>	GR92103	Germany		AF139690	Binder and Besl, 2000
<i>Leccinum duriusculum</i>	KPM-NC 24518	Japan		MT812736	Unpublished
<i>Leccinum subradicatum</i>	NCJ26	USA		AY612814	Unpublished
<i>Leccinum scabrum</i>	JAC12883	New Zealand		OP141527	Unpublished
<i>Leccinum schistophilum</i>	HN1573	USA		AY612810	Unpublished
<i>Leccinum aurantiacum</i>	CFMR DLC2002-122	USA		MK601778	Unpublished
<i>Leccinum versipelle</i>	KUN-HKAS3417	China		MW413907	Kuo and Ortiz Santana, 2000
<i>Leccinum album</i>	HKAS51277	China		HQ326929	Meng <i>et al.</i> , 2021
<i>Leccinum manzanitae</i>	MICH KUO-09150707	USA		MK601763	Li & Yang, 2011
<i>Leccinum holopus</i>	NY-00815448 REH-8591	Costa Rica		MK601767	Kuo & Ortiz-Santana, 2000
<i>Leccinum monticola</i>	Lp1	Austria		DQ534614	Kuo & Ortiz-Santana, 2020
<i>Leccinum piceinum</i>	24/98	Austria		AF139696	Binder & Hibbett, 2006
<i>Leccinum flavostipitatum</i>	9210040	Germany		AF139702	Binder & Besl, 2000
<i>Leccinum percardidum</i>	Lvar1	Germany		AF139706	Binder & Besl, 2000
<i>Leccinum varicolor</i>	MK11800	Germany		AF139692	Binder & Besl, 2000
<i>Leccinum cerinum</i>	MB-06026	USA		MH620341	Unpublished
<i>Leccinum scabrum</i>	24/98	Germany		AF139696	Binder & Besl, 2000
<i>Leccinum flavostipitatum</i>	Yang5972	Austria		MW413906	Meng <i>et al.</i> , 2021
<i>Leccinum holopus</i>	TM0280	Canada		EU522793	Porter <i>et al.</i> , 2008
<i>Leccinum crocipodium</i>	MK11107	Germany		AF139701	Binder & Besl, 2000
<i>Leccinum palustre</i>	SS-12-PakFM04082021	Pakistan	pp660354 ITS	pp660355 LSU	Sequences from Pakistan (from this study)

Note: Sequences generated for this study are shown in bold

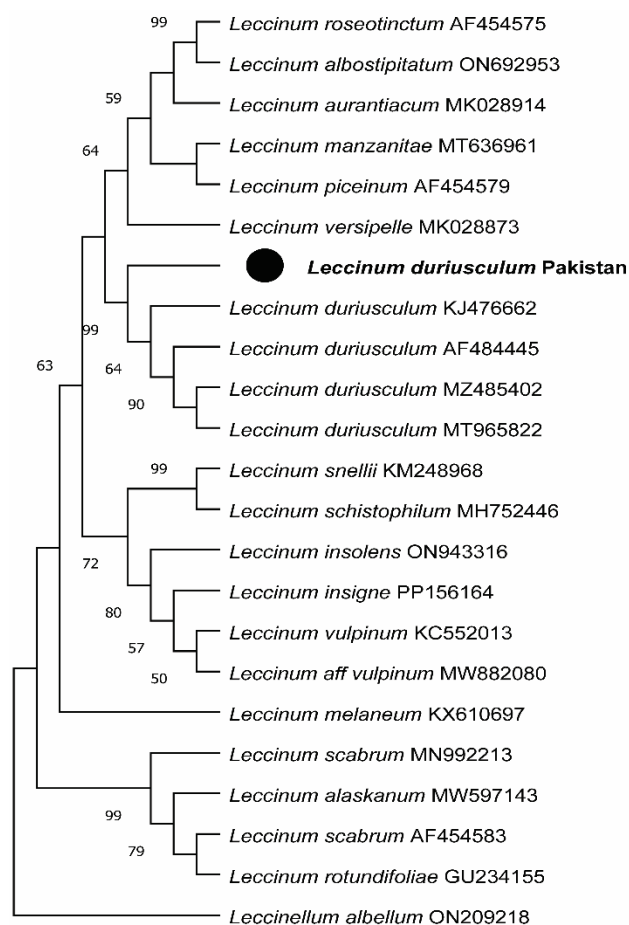


Fig. 1. Phylogenetic tree of *Leccinum duriusculum* represented by (●) based on Maximum Likelihood (ML) analysis of nrITS sequences.

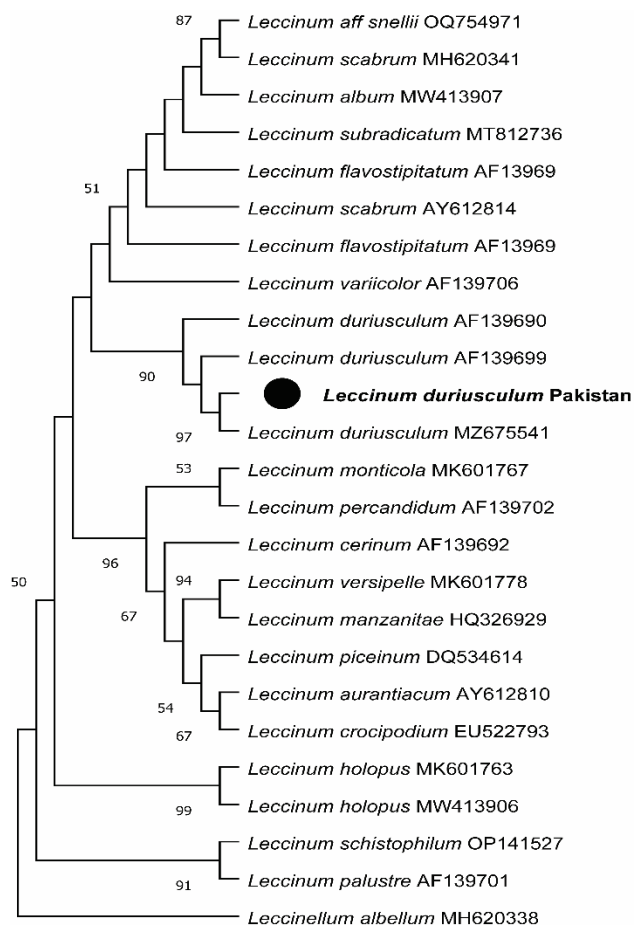


Fig. 2. Phylogenetic tree of *Leccinum duriusculum* represented by (●) based on Maximum Likelihood (ML) analysis of nrLSU sequences.



Fig. 3. Habit of *Leccinum duriusculum*. Bar = 1 cm.

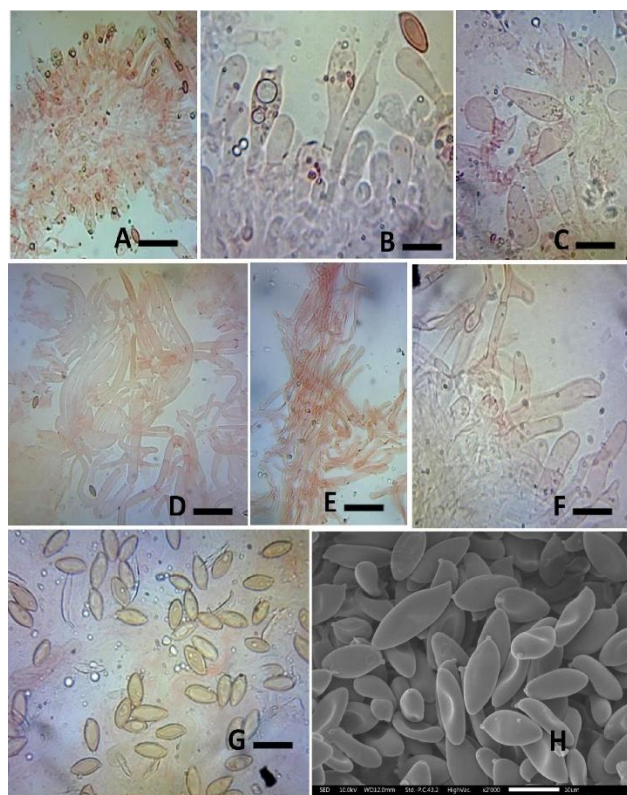


Fig. 4. Microscopic features of *Leccinum duriusculum*. (A-C) Basidia and cystidia (D) Pileipellis (E-F) Stiptipellis and terminal hyphae (G) Basidiospores. (Bar = 10 µm), (H) SEM photograph of basidiospores.

Material examined: Pakistan, Hunza Valley, Gilgit-Baltistan, from rocky mountains, 12-08-2021, leg. S. Sawar, LCWUBOT. SS-12-PakFM04082021 (Herbarium, Dept. of Botany, LCWU, Lahore). (Genbank no. PP660354 for nrITS and PP660355 for nrLSU).

Discussion

One of our specimens found during the mycological survey of Hunza Valley was revealed as *Leccinum duriusculum* and is the first record for Pakistan. Previously, only three species of this genus were known from Pakistan but without molecular and phylogenetic analysis (Aman *et al.*, 2022). *Leccinum duriusculum* was analyzed macro- and microscopically, as well as by sequencing ITS and LSU regions (Figs. 1-2). In both phylogenetic trees, our sequences form a clade with *Leccinum duriusculum* reported from other countries with high bootstrap value.

Leccinum duriusculum can be identified by its yellowish-brown pileus, distinguishing it from the closely related *L. nigellum*, which has a dark, almost blackish pileus (Den Bakker *et al.*, 2004b). While *L. parascabrum* also has similar pileus and hymenophore colors and a slender stipe, *L. duriusculum* stands out with its yellowish-brown pileus, white flesh, relatively small spores, and genetic distinctions (Horak, 2011). *Leccinum cyaneobasileucum* has a context turning blue near the stipe base, and *L. versipelle* features a more orange pileus and bruises blue-green at the stipe base.

Leccinum duriusculum forms an ectomycorrhizal association with *Poulus alba* and *P. tremula* (Selosse, 2003; Jarzyńska and Falandysz, 2012). Different species of *Leccinum* are associated with specific trees i.e., *L. cyaneobasileucum*, *L. scabrum* and *L. albobostipitatum* found in association with *Betula*. *Leccinum cyaneobasileucum* has a bluing stipe base, and its white flesh turns pink when cut, then slowly blue. The mycorrhizal partner and the smaller size reveal its difference from Slate Bolet. The context of *Leccinum scabrum* remains white after being cut, and its stipe base does not stain blue. *Leccinum albobostipitatum* has a bright orange to brick colored pileus which makes it easy to separate.

In conclusion, after morphological and molecular genetic analysis, the Slate Bolet *Leccinum duriusculum* is reported here for the first time for Pakistan from Hunza Valley's glacial rocky mountains.

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