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INOCYBE SINEOFIBULAE SP. NOV. (*SECT. MARGINATAE*), A NEW CLAMP-LESS ECTOMYCORRHIZAL SPECIES FROM KUMRAT VALLEY, KP, PAKISTAN

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Abstract

A new clamp-less ectomycorrhizal species, *Inocybe sineofibulae* sp. nov., collected from the Lamleek Forest of Kumrat Valley, KP, Pakistan. Morpho-anatomical and molecular characterization classified it in *Inocybe* sect. *Marginatae*. This newly described species is characterized by small sized basidiomata (pileus 1.8–2.3 cm) having pale brown fibrillose pileus with cracked margins, orange adnexed lamellae and a longitudinally striate stipe (3.1–5.6 × 0.4 cm). Large amyloid basidiospores (11.2–11.5 × 7.2–7.6 µm), exceptionally large metuloid cystidia and absence of clamp connections. It resembles *I. cryptica* in its small basidiomata and smooth, ellipsoidal spores but differs by having larger amyloid spores, significantly larger cystidia and the absence of clamp connections, which are abundant in *I. cryptica*. Phylogenetic analysis (ITS) declares its genetic distinctness, showing ~90% ITS similarity to *I. cryptica*. This finding highlights the unexplored fungal diversity of the Hindukush regions of Pakistan and emphasizes the value of integrative taxonomy in resolving cryptic lineages.

Key words: Amyloid spores; Ectomycorrhizal; *Inocybe*, Kumrat; *Marginatae*; Pakistan; Phylogeny

Introduction

The family *Inocybaceae* Jülich includes ectomycorrhizal fungi grouped within the order Agaricales and establish a monophyletic, cosmopolitan lineage. Its members develop symbiotic mycorrhizal associations to an extensive range of angiosperms and gymnosperms crossway temperate to tropical ecosystems, having sturdy preference with up to 23 families such as Fagaceae, Pinaceae and Salicaceae (Ahmed *et al*., 2025).

Phylogenetic studies have categorized the family into several well-distinct genera. *Inocybe* is amongst the most important and diverse genus in Agaricales, with more than 1,050 species presently documented worldwide (Kirk *et al*., 2008; Matheny *et al*., 2019; Bandini *et al*., 2022; Ahmed *et al*., 2025). Members are generally distinguished by a non-glutinous pileus, having brownish lamellae at maturity with pigmented large amyloid basidiospores without germ-pore and the occurrence of pleurocystidia and cheilocystidia (Khan *et al*., 2022; Ashraf *et al*., 2025).

Extensive studies have revealed significant *Inocybe* diversities world-widely across Europe, North America, Africa, Asia, China, and Japan (Fan & Bau, 2013, 2014; Latha & Manimohan, 2015; Buyck *et al*., 2022; Aïgnon *et al*., 2022). On the other hand, the global biodiversity hotspots of Hindukush moist temperate forests of Pakistan remain comparatively under-explored (Myers *et al*., 2000). To date, approximately 40 species from genus *Inocybe* have been reported from the different regions of Pakistan (Ahmed *et al*., 2025; Ashraf *et al*., 2025).

In the course of fungal surveys in the coniferous Lamleek forest of Kumrat Valley, Khyber Pakhtunkhwa, Pakistan, an undescribed *Inocybe* species was colletced. Here describes it as *Inocybe sineofibulae* sp. nov., based on integrated morphoanatomical and molecular confirmation, and assign it to sect. *Marginatae*. Exceptionally large amyloid spores, unusually long metuloid cystidia, and absence of clamp connections in hyphal system are distinctive characters which clearly distinguish it from morpho-anatomically related species, such as *I. cryptica*, *I. muricellata*, and *I. flocculosa*. This is the leading explosion of a clampless member of sect. *Marginatae* from Pakistan, escalating the known diversity of *Inocybe* in the region. This discovery accentuates the ecological significance of ectomycorrhizal fungi in Hindukush coniferous forests and emphasizes the need for continued integrative taxonomic exploration in this underexplored biodiversity hotspot. This research directly supports SDG 15 (Life on Land) by documenting and protecting terrestrial myco-diversity, a critical but often overlooked component of sustainable ecosystems.

Material and Methods

Sampling location: The fresh specimens of mushrooms were collected from Lamleek Forest, Kumrat Valley, Upper Dir, Khyber Pakhtunkhwa (35.3201°N, 72.4501°E; 2439–3048 m a.s.l.), during the monsoon month of July and August, 2023. Described as having granite, diorite, norite, and schist bedrocks; sandy loam soils with pH 6–6.5;

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annual precipitation 600–1200 mm (Ahmad & Nizami, 2014); seasonal temperatures of 25–31°C and high humidity during rains. The valley hosts mixed deciduous and coniferous forests. These offer perfect substrates (decaying wood, leaf litter, dung) to support various mushroom communities (Hameed *et al*., 2012).

Sampling and morphological- anatomical characterization: The basidiomata was photographed and tagged, together with field notes (e.g., morphological characters—, color, shape and texture), were done *in-situ*. Basidiomata colours were being noted using standard Munsell Color System (1975). Specimens were dried in a hot-air oven at 55°C. Anatomical characterization was made in 5% KOH solution by mounting thin section of the pileus, lamellae, and stipe and examining them under a compound microscope (OPTIKA Microscope, Optika SRL., Italia). Measurements of anatomical structures were taken using calibrated optikca vision pro plus; at least 20 basidiospores, basidia cystidia, pileipellis and stipitipellis elements measured. Line drawings were prepared using camera lucida. Voucher specimens were deposited to the Department of Botany in the Research Laboratory of Fungal Biology and Systematics, GC University Lahore, Pakistan.

Extraction of DNA, PCR and sequencing: Genomic DNA was extracted from the dried specimen with the 2% CTAB method (Bruns, 1995). The ITS region of nuclear rDNA was amplified with fungal-specific primer pairs: ITS1F & ITS4 for ITS (Grades & Bruns, 1993). The PCR product was observed by agarose gel electrophoresis with a gel documentation system (UVtec, Cambridge, UK). Purification of amplified products was done prior to the sequencing by Macrogen, Inc., Korea (Voytas, 2001).

Analysis of sequence data: Consensus sequence was obtained with software BioEdit-v.7.0.4.1 (Hall, 1999). It was subjected to BLAST search at NCBI and closely matched *Inocybe* sequences were downloaded from GenBank. All the sequences were aligned with MUSCLE. Maximum likelihood (ML) bootstrap analysis was conducted in MEGA11 with a General Time Reversible model (Tamura *et al*., 2021).

Results

Taxonomy

Inocybe sineofibulae Amanat & Hanif, sp. nov.
(Figs. 1-4)

MycoBank: MB860483

Etymology: “sineofibulae” refers to the clampless character of this species.

Diagnosis: Differs from related species in sect. *Marginatae* by its small basidiomata (pileus 1.8–2.3 cm), pale brown fibrillose pileus with cracked margins, adnexed dull orange lamellae, longitudinally striate stipe, exceptionally large amyloid spores (11.2–11.5 × 7.2–7.6 μm), very large metuloid cystidia (up to 93 μm long), and absence of clamp connections.

Description

Basidiomata small. **Pileus** 1.8–2.3 cm diam., pale brown at disc, lighter toward margins (10YR 7/4); conical to subglobose when young, later convex; surface uneven, silky-sericeous, centrally bumpy, becoming furfuraceous and appressed fibrillose; margin often cracking. **Lamellae** dull orange (5YR 5/14), adnexed, close to crowded, 2–3 mm wide; edges even. **Stipe** 3.1–5.6 × 0.4 cm, cylindrical, terete, equal, with basal tomentum, longitudinally striate, slightly clavate at base; apex whitish (5.1GY 8.1/1.6), becoming pale brown (5.7Y 6.6/3) below; fibrillose; stuffed; annulus and volva absent. Odor not distinctive.

Microanatomical features

Basidiospores 11.2–11.5(11.3) × 7.2–7.6(7.4) μm; av. 11.3 × 7.4 μm; smooth, oval to amygdaliform; light brown in KOH; prominent apiculus; strongly amyloid. **Basidia** 42.9–45.8 × 9.9–11.3 μm; av. 44.3 × 10.9 μm; thin-walled clavate, hyaline in KOH; tetra-spored. **Cheilocystidia** 83.7–93.2 × 23.1–25.9 μm; av. 86.7 × 23.4 μm; polymorphic, thick-walled, metuloid, often crystalliferous at apex; utriform to fusiform, pedicellate or spheropedunculate. **Pleurocystidia** 81.3–92.2 × 19.7–21.4 μm; av. 85.2 × 20.4 μm; hyaline, thick-walled, crystalliferous at apex. **Pileipellis** hyaline, thin-walled, filamentous, septate, branched hyphae without clamps with 7.5–13.6μm width. **Stipitipellis** hyaline, thin-walled, filamentous, septate, branched and clampless hyphae with 4.1–10.1 μm width.

Holotype

Pakistan Province Khyber Pakhtunkhwa (KP): District Upper Dir, Kumrat Valley, Lamleek Forest, under *Cedrus deodara* (Roxb. ex D.Don) G.Don woods, humus-rich soil, 28 July 2023, coll. R. Amanat, A. U. M. Zain & M. Hanif (Holotype: **MH-PFK 102**, Department of Botany, GC University Lahore, Pakistan). GenBank: ITS = PX113226.

MycoBank: MB860483

Phylogenetic analysis: A total of 54 ITS rDNA sequences were evaluated, including 53 from NCBI GenBank (Tables 1 and 2). The data matrix consisted of 1141 unambiguously aligned nucleotide positions (301 conserved, 667 variable, 552 parsimony-informative, 111 singletons). A BLAST search of the proposed *Inocybe sineofibulae* sequences showed 89.93% similarity with *Inocybe cryptica* (PQ279576). The specimen analyzed formed a distinct branch in the phylogenetic tree.

Fig. 1. A-D: Basidiomata of *Inocybe sineofibulae* sp. nov*;* A. Basidiomatas, B. Pileus Surface C. gills, D. Stipe.

Table 1. Morpho-anatomical comparison of *Inocybe sineofibulae* sp. nov. with closely related species (*I. cryptica*, *I. muricellata*, *I. flocculosa*, and *I. Rufuloides*.

Taxon	Habitat	Morphological characters	Anatomical characters	References
Inocybe sineofibulae sp. nov.	Kumrat valley, KPK, in leaf litter under Deodar forest	Pileus: 1.8–2.3 cm, pale brown, furfuraceous appressed fibrillose. Lamellae: Dull orange, regular, adnexed, close to crowded (2–3 mm wide), edges even. Stipe: 3.14–5.6 cm × 0.4 cm, terete, equal, longitudinally striate, fibrillose, slightly clavate at base with central basal tomentum.	Basidiospores: 11.2–11.5(11.3) × 7.2–7.6(7.4) µm, avL × avW = 11.3 × 7.4 µm; smooth; moderately elliptical to amygdaliform. Basidia: 42.9–45.8(44.3) × 9.9–11.3(10.9) µm, 44.3 × 10.9 µm; hyaline; thin-walled; clavate; tetraspored. Cheilocystidia: 83.7–93.2(86.7) × 23.1–25.9(23.4) µm, avL × avW = 86.7 × 23.4 µm; hyaline; metuloid with crystalliferous apex; utriform; thick-walled. Pleurocystidia: 81.3–92.2(85.2) × 19.7–21.4(20.4) µm, avL × avW = 85.2 × 20.4 µm; polymorphic; crystalliferous apex.	In Present study
I. cryptica	Spain, sandy soil, under Quercus ilex	Pileus: 13–30 mm, conical, brown to greyish brown, fibrillose-lanose surface. Lamellae: Moderately crowded, narrowly adnate, pale brownish; edges irregular, fimbriate, concolorous. Stipe: (20–40 × 2–7 mm), cylindrically curved, widen at base with whitish tomentum; no darkening on drying.	Basidiospores: 7.7–11.3 × 4.5–6.4 µm; smooth; (sub) amygdaloid to ellipsoid. Basidia: (27–36 × 7–11µm); predominantly tetraspored. Pleurocystidia: 58–86 × 9–18 µm; narrow (sub) fusiform. Caulocystidia: (61–95 × 9–16 µm); near stipe apex; (sub) cylindrical to (sub)utriform. Pileipellis: Parallelled hyphae (4–7 µm wide). Clamp connections: Abundant.	Bandini *et al*., 2022
I. muricellata	Burnham Beeches, Bucks, UK Under broadleaved trees, calcareous soil.	Pileus: 10–20(30) mm, applanate; disc smooth then roughened; orange-ochre brown. Gills: Ventricose at maturity, pale ochre with orange tint. Stipe: 30 × 3–4 mm, cylindrical; base sometimes swollen; fine white pruina throughout.	Basidiospores: 10–12 × 5–6 µm; smooth; amygdaliform with small apiculus. Basidia: Predominantly 4-spored. Hymenial cystidia: 60–68 × 12.5 µm; clavate; thin-walled. Caulocystidia: Abundant; similar to hymenial cystidia.	Cullington, 2010
I. flocculosa	Chuwarta, District Suliamanyia North Iraq Gregariously growing among leaf litter in mixed forest of Quercus spp.	Pileus: 20–25 mm, small, fibrous; bell-shaped/convex, slightly umbonate; pale brown. Gills: Adnexed, pale brown, crowded. Stipe: 40–60 × 3.0–3.8 mm, cylindrical; cream-colored, pruinose; bent at bulbose base.	Basidiospores: 8.0–11.25 × 5.5–6.5 µm; amygdaliform, smooth, light brown. Basidia: 20–25 × 6.25–7.5 µm; 4-spored. Cheilocystidia & Pleurocystidia: Similar, (90.6–112.0 × 20–25µm); hyaline, fusiform, apical crystals.	Sara *et al*., 2017
I. rufuloides	Western Australia, Jandakot Regional Park, Thomas Rd, Oakford, Perth, roadside edge near Pinus spp.	Pileus: 5–35 mm, convex to conico-campanulate; margin plane with whitish appendiculate remnants; dry, radially fibrillose. Gills: Broadly adnexed, crowded, abundant lamellulae. Stipe: 50 × 8 mm, cylindrical; base slightly swollen; longitudinally silky-fibrillose.	Basidiospores: 11–12.5(13) × 5.5–6.5 µm; smooth; ellipsoid to subamygdaliform; thick-walled. Basidia: 25–37 × 8.5–11.5 µm; quadrisporic; clavate. Pleurocystidia: 53–75 × 14–17 µm; lageniform, crystalliferous; thick-walled. Cheilocystidia: 20–56 × 11–15.5 µm; thin-walled. Clamp connections: Present.	Bougher and Matheny 2011

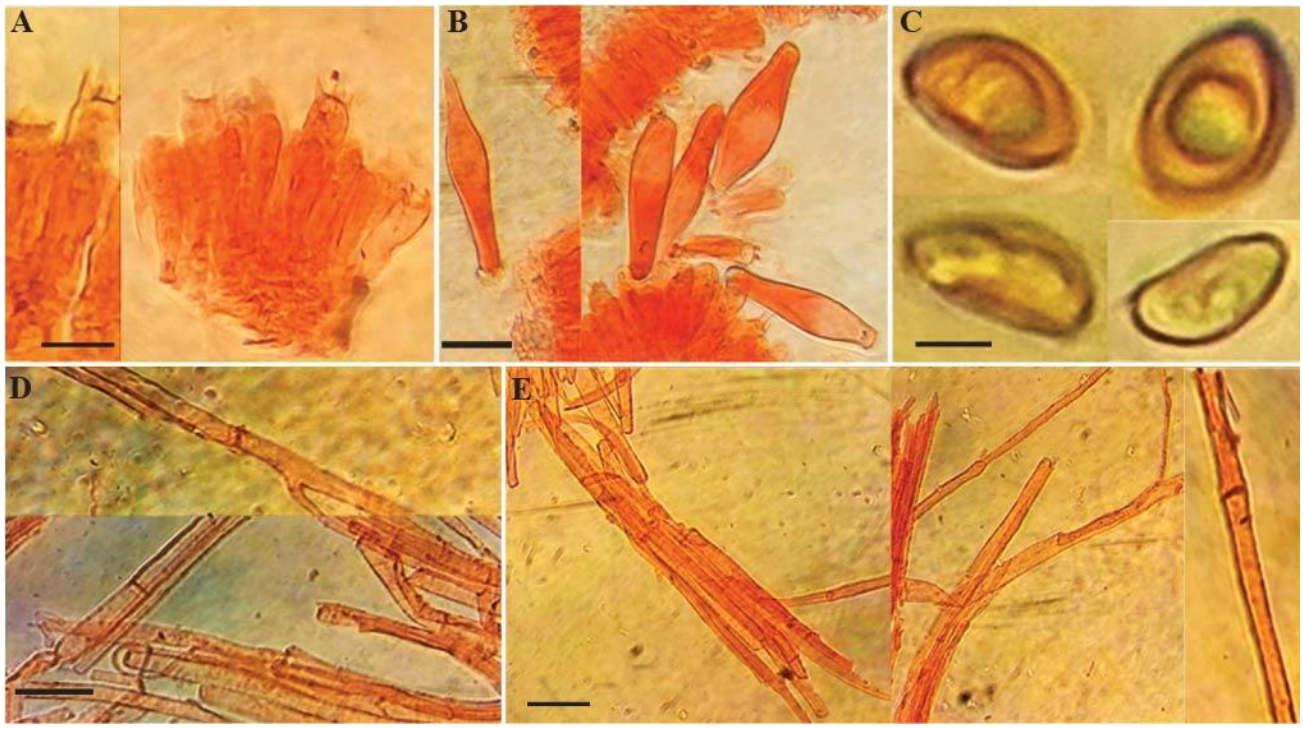

Fig. 2. Micrographs of *Inocybe sineofibulae* sp. nov. Anatomic features. A. Basidia, B. Cystidia, C. Basidiospores, D. Pelleipellis, E. Stiptipellis.

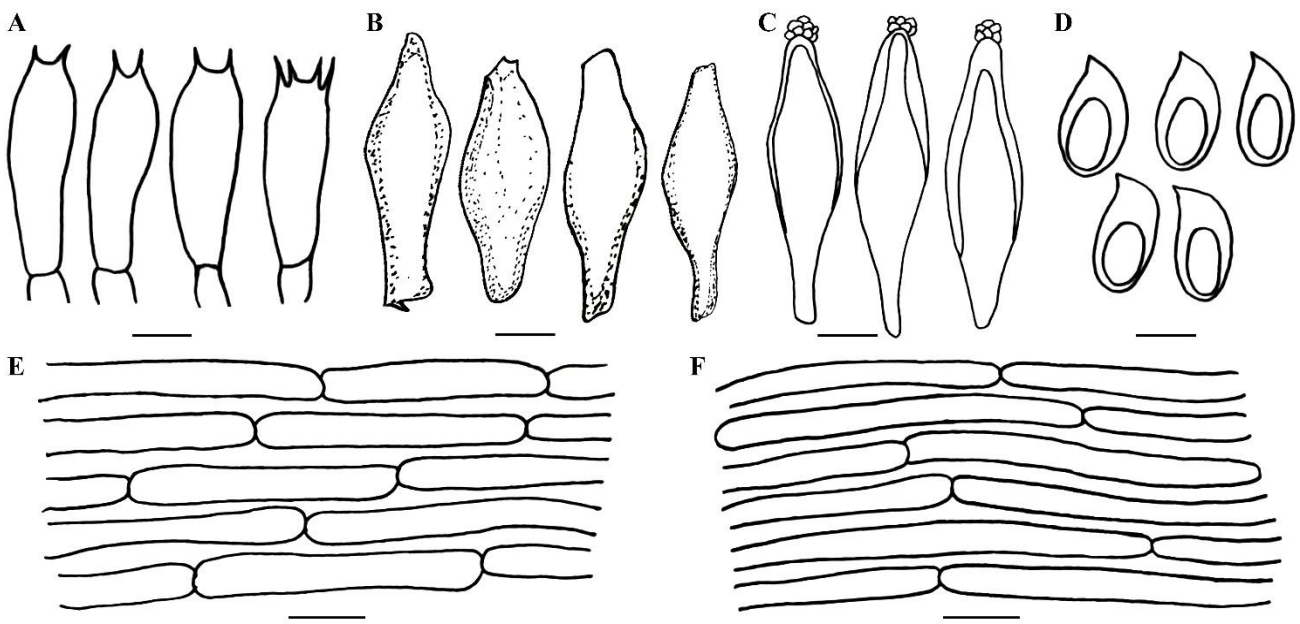

Fig. 3. Line Drawings of Microscopic features of *Inocybe sineofibulae* sp. nov. A. Basidia, B. Cheilocystedia, C. Pleurocyctedia, D. Basidiospores, E. Pelleipellis, E. Stiptipellis
Scale Bars: A=1.15cm, B=2.54µm, C=7.32µm, D=5.58µm, E=2.0µm, F=2.04µm,

Discussion

Inocybe sineofibulae is morphologically comparable to several species in sect. *Marginatae*, particularly *I. cryptica*, *I. muricellata*, *I. flocculosa*, and *I. rufuloides*. It resembles *I. cryptica* in its small basidiomata and smooth, ellipsoidal spores (Bandini *et al*., 2022), but differs by having larger amyloid spores (11.2–11.5 × 7.2–7.6 µm vs. 7.7–11.3 × 4.5–6.4 µm), significantly larger cystidia (up to 93 µm vs. 68 µm), and the absence of clamp connections, which are abundant in *I. cryptica*. Compared with *I. muricellata*, the new species has larger spores (up to 11.5 µm vs. 10–12 × 5–6 µm) and much larger cystidia (83–93 × 23–26 µm vs. 60–68 × 12.5 µm). From *I. flocculosa*, which has smaller spores (8.0–11.25 × 5.5–6.5 µm) and a bent bulbous stipe base (Sara *et al*., 2017), *I. sineofibulae* is readily separated by its straight striate stipe and strongly amyloid, larger spores. Finally, *I. rufuloides* possesses smaller cystidia (53–75 × 14–17 µm) and clamp connections (Bougher and Matheny 2011), both absent in *I. sineofibulae*. This unique combination of larger amyloid spores, exceptionally large metuloid cystidia, and lack of clamp connections provides strong evidence for the recognition of *I. sineofibulae* as a distinct species.

Beyond morphology, the discovery of *I. sineofibulae* highlights the underexplored mycological richness of Hinduush moist temperate forests. These forests, dominated by conifers such as *Cedrus deodara* and *Pinus wallichiana* **(A.B. Jacks.)** provide ideal substrates for ectomycorrhizal fungi, which in turn play critical roles in nutrient cycling, forest regeneration, and ecosystem stability (Ali *et al.,* 2021). As *Inocybe* species are well-known ectomycorrhizal partners of Fagaceae, Pinaceae, and Salicaceae, the presence of *I. sineofibulae* in association with Himalayan conifers emphasizes its potential ecological importance in sustaining forest health under changing climatic conditions.

This documentation of novel ectomycorrhizal species highlights significant contributions to SDG 15, which aims to cessation of biodiversity destruction by protecting ecosystems and encouraging the conservation of endemic species. This finding also aligns with recent explorations of new *Inocybe* taxa from different regions of Pakistan, including *I. crenata* (Ashraf *et al*., 2025) and *I. quercicola* (Khan *et al*., 2022). Together, these studies indicate that the Himalayan and sub-Himalayan regions harbor a cryptic diversity of ectomycorrhizal fungi that remain poorly characterized. Integrative taxonomic approaches, combining morphology with multilocus phylogenetics, are therefore essential to unravel the true diversity and ecological significance of *Inocybe* in Pakistan.

Table 2. ***Inocybe*** **sequences used for phylogenetic analyses; species name, geographical origin, voucher information & GenBank accession numbers (ITS region) are included.**

Taxon	Geographical area	Voucher	Genbank Accession number of ITS	References
Inocybe cryptica	Europe	MA-Fungi 98674	ON010593	Bandini *et al*., 2022
*I._flocculosa*_var._*flocculosa*	Canada	UBC:F19292	HQ604516	Berbee *et al*., 2010
I. cryptica	Europe	MA-Fungi 98672	ON010592	Bandini *et al*., 2022
I. muricellata	Europe	DB16-10-11-2	MW856432	Bandini *et al*., 2022
I. muricellata	Europe	STU:SMNS-STU-F-0900985	MW845931	Bandini *et al*., 2022
I. cf. microspora	Europe & North America	TAA185187	AM882808	Ryberg *et al*., 2008
I. rufuloides	Australia	PERTH7700598	JN035292	Bougher and matheny 2011
I. rufuloides	Australia	NLB00618	JN035291	Bougher and matheny 2011
I. nitidiuscula	Italy	voucher 80	JF908088	Osmundson *et al*., 2013
I. tarda	France	EL23506	FN550920	Ryberg *et al*., 2009
I. pseudodestricta	Italy	voucher 16041	JF908157	Osmundson *et al*., 2013
I. rufuloides	Italy		DQ067579	Bougher and matheny 2011
I. flocculosa var. *flocculosa*	Canada	UBC:F19374	HQ604506	Berbee *et al*., 2010
I. oblectabilis	Swedan	BJ920908	AM882831	Ryberg *et al*., 2008
I. flocculosa var. *flocculosa*	Canada	UBC:F19087	HQ604464	Berbee *et al*., 2010
I. pseudodestricta	Canada	UBC:F19094	HQ604460	Berbee *et al*., 2010
I. pseudodestricta	Canada	UBC:F19009	HQ604530	Berbee *et al*., 2010
I. squamata	Swedan	J85048	AM882778	Ryberg *et al*., 2008
I. squamata	Swedan	TK96109	AM882780	Ryberg *et al*., 2008
I. squamata	Italy	16576	JF908162	Osmundson *et al*., 2013
I. umbrinella	North Europe	JV17954	FJ904166	Larsson *et al*., 2009
I. umbrinella	Swedan	F14488	HM209796	Larsson *et al*., 2009
I. umbrinella	North Europe	JV13699	FJ904165	Larsson *et al*., 2009
I. nitidiuscula	japan	TAKK 08.8.18.22-2	AB594843	Kobayashi & Courtecuisse, 2000
I. nitidiuscula	Italy	3665	JF908112	Osmundson *et al*., 2013
I. oblectabilis	Italy	8458	JF908129	Osmundson *et al*., 2013
I. muricellata	Swedan	KGN980725	AM882916	Ryberg *et al*., 2008
I. muricellata	Swedan	BJKGN980720	AM882917	Ryberg *et al*., 2008
I. cf. hirtella	USA	PBM 2655	EU523582	Hughes *et al*., 2010
I. cf. hirtella	USA	PBM 2624	EU523572	Hughes *et al*., 2010
I. cf. hirtella	USA	PBM 2655	EU523583	Hughes *et al*., 2010
I. hirtella	USA	PBM 2650	EU523581	Hughes *et al*., 2010
I. hirtella	Norway	KH53	GU234131	Geml *et al*., 2011
I. hirtella	Norway	KH52	GU234122	Geml *et al*., 2011
I. cf. glabripes	Swedan	EL10805	AM882794	Ryberg *et al*., 2008
I. margaritispora	Italy	5050	JF908120	Osmundson *et al*., 2013
I. praetervisa	France	PAM01043008	HQ586860	Geml *et al*., 2011
I. praetervisa	Canada	UBC:F19231	HQ604598	Berbee *et al*., 2010
I. praetervisa	Canada	UBC:F19317	HQ604596	Berbee *et al*., 2010
I. cryptocystis	Swedan	BJ900914	AM882906	Ryberg *et al*., 2008
I. praetervisa	Italy	5052	JF908121	Osmundson *et al*., 2013
I. glabripes	Swedan	BJ940922	AM882807	Ryberg *et al*., 2008
I. cf. glabripes	Denmark	MC01-510	AJ889952	Ryberg *et al*., 2008
I. glabripes	Finland	TAA145067	AM882902	Ryberg *et al*., 2008
I. kohistanensis	Pakistan	LAH35024	KT897911	Jabeen *et al*., 2016
I. kohistanensis	Pakistan	LAH35002	KP316244	Jabeen *et al*., 2016
I. kohistanensis	Pakistan	LAH35003	KP316245	Jabeen *et al*., 2016
I. kohistanensis	Pakistan	LAH35001	KP316243	Jabeen *et al*., 2016
I. quercicola	Pakistan	LAH	MW412768	Khan *et al*., 2022
I. quercicola	Pakistan	HUP32966	MK368637	Khan *et al*., 2022
I. quercicola	Pakistan	FA167	MN812171	Khan *et al*., 2022

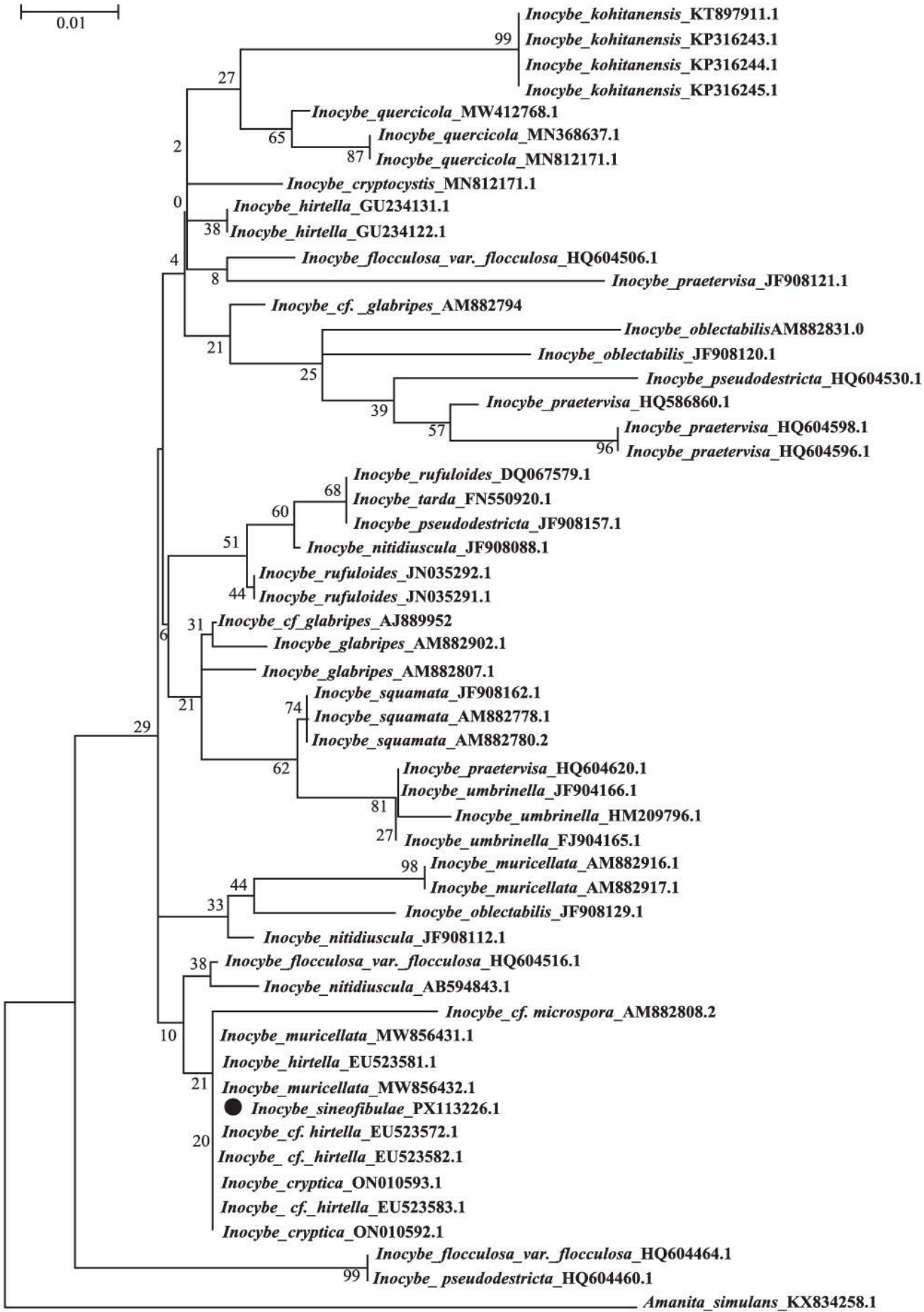

Fig. 4. Maximum Likelihood Phylogeny based on ITS sequences showing the placement of *Inocybe sineofibulae* sp. nov. within sect. *Marginatae*. Bootstrap support values (≥70%) are indicated at nodes. *Amanita simulans* was used as the outgroup.

Conclusion

Inocybe sineofibulae sp. nov. is distinguished by its unique morphological features and phylogenetic distinctness within sect. *Marginatae*. Its discovery from Himalayan coniferous forests underscores the ecological importance of ectomycorrhizal fungi in these ecosystems and highlights Pakistan as a reservoir of cryptic fungal diversity. Continued integrative taxonomic work is essential for documenting and conserving this underexplored mycobiota. Ultimately, this research work supports the overarching mission of SDG 17 (Partnership for the Goals) by providing a foundational taxonomic resource the enables future international collaborative research on fungal conservation, ecology and biogeography. It is recommended that monitoring of Lamleek Forest ecosystem be implemented to conserve this taxon and its unique habitat.

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Conflict of Interest: The authors declare no conflict of interest.

Authors' Contribution: Rizwan Amanat planned field work, collected specimens, conducted morpho-anatomical analysis and drafted the majority of the manuscript. Served as the primary point of contact for journal communication. Dr. Muhammad Hanif overall supervised the lab work and secured funding for the research. Atta Ull Mustafa Zain remained a key participant in fieldwork. Zubaria Ashraf provided the detailed description of ecology and geography of the sampling site. Sammar Bashir did phylogenetic analysis. Dr Nousheen Yousaf contributed to manuscript preparation. Dr Samina Sarwar reviewed the data interpretation.

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