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Short communication

Mallocybe velutina (Agaricales, Inocybaceae), a new species from Pakistan

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ABSTRACT

A new species of *Mallocybe* is described and illustrated based on material collected near the vicinity of *Pinus wallichiana* in mixed conifer forests in district Abbottabad, Khyber Pakhtoonkhaw, Pakistan. *Mallocybe velutina* is recognized by the presence of moderate yellow to light yellow pileal margin with deep yellow or fulvous pileus center; strikingly velvety pileus surface; subdistant moderate yellow lamellae; subphaseoliform to ellipsoid basidiospores, clavate to cylindrical cheilocystidia and an ecological association with Pinaceae. The internal transcribed spacer region (ITS) and large subunit of the nuclear ribosomal RNA gene (nrLSU) were used for the delimitation of this species based on sequence data. The evolutionary relationships of *M. velutina* with other closely related species of *Mallocybe* were inferred by means of maximum likelihood and maximum parsimony of concatenated ITS + nrLSU dataset. *Mallocybe velutina* is most closely related to *M. arenaria*, *M. heimii* and *M. tomentosula*.

The Inocybaceae Jülich is a monophyletic family of ectomycorrhizal fungi with worldwide distribution (Matheny et al., 2006) and is estimated to contain 1050 species (Matheny & Kudzma, 2019). *Mallocybe* is fairly widespread across the globe but has yet to be recorded from southern South America and the neotropics. About 55 species of *Mallocybe* have been recorded from different regions of Africa, Asia, Australia, Europe, New Zealand, and North America (Matheny, Hobbs, & Esteve-Raventós, 2019). Macroscopically, *Mallocybe* species are recognized by a fibrous or scaly often flattened pileus, a short stipe, ocher, brown, or red brown coloration, a cortina, adnate lamellae and absence of a spermatic odor. Microscopically, smooth spores, absence of pleurocystidia, thin-walled without crystals cheilocystidia and necropigment can be observed in basidia of fresh and dried specimens (Jacobsson, 2008, pp. 868–906; Kuyper, 1986; Matheny et al., 2019; Stangl, 1989). Though some studies have been conducted to explore the diversity of Inocybaceae, only one species of this genus is known from Pakistan (Ahmad, Iqbal, & Khalid, 1997).

During an investigation of ectomycorrhizal fungi associated with pine species in Pakistan, a novel species *M. velutina* was collected in Thandiani (34°14′0"N, 73°22′0"E; 2700 m above sea level), Abbottabad District, Pakistan. Specimens were photographed in the field and macro-morphological features were described on site. Color designations were derived from the Munsell Soil Color Charts (Munsell Color Company, 1975). Collections were dried using a food dehydrator (at 39 °C for 7–9 h) and deposited at the University of the Punjab Herbarium (LAH) in Pakistan.

Sections of specimens were mounted in H_2O and 5% aqueous potassium hydroxide (hereafter referred as KOH). Melzer's reagent and congo red were used to check the amyloid reaction of basidiospores and to increase the contrast of structures, respectively. Micro-morphological analyses, photographs, and measurements were made using a light microscope (BX40, Olympus, Waltham, Massachusetts, USA) with Olympus XC50 a digital camera (XC50, Olympus) and Microsuite special edition software 3.1 (Soft Imaging Solutions GmbH, Münster, Germany). Dimensions of anatomical features are presented in the following form: (a–)b–c(–d) in which 'b–c' contains at least 90% of the measured values and extreme values 'a' and 'd' are presented in parentheses. Line drawings were made with a Leitz Camera Lucida (Leitz, Wetzlar, Germany). Abbreviation Q means length/width ratio of a basidiospore in side view and x = mean values (average basidiospore length and width). About 20 basidiospores and cystidia from each basidiomata were measured.

Genomic DNA was extracted from wheat grain-sized pieces of

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lamellae of basidiomata using a CTAB method (Lee, Milgroom, & Taylor, 1988). We amplified the complete internal transcribed spacer (ITS) region including 5.8S and large subunit of the nuclear RNA gene (nrLSU). Primer pairs used were ITS1f/ITS4 (Gardes & Bruns, 1993; White, Bruns, Lee, & Taylor, 1990, pp. 315–322) for ITS and LR0R/LR5 (Vilgalys & Hester, 1990) for nrLSU. Thermal profile of PCR for ITS was as follows: initial denaturation at 94 °C for 1 min; then 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min and extension at 72 °C for 1 min; and final extension at 72 °C for 8 min. For nrLSU: initial denaturation at 94 °C for 2 min; then 40 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 90 s; and final extension at 72 °C for 5 min. Amplified PCR products of the ITS region were sent for purification and bidirectional sequencing to Macrogen (Seoul, South Korea). Amplified PCR products of nrLSU were purified using the QIAquick PCR purification kit (Qiagen, Stanford, California, USA) as per manufacturer's guidelines and sequencing reactions were performed using the Big Dye® Terminator v3.1 Cycle Kit (Life Technologies, Carlsbad, California, USA). The same primers were used for sequencing as those used for PCR amplification.

Sequences were assembled and manually edited in Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). ITS sequences were trimmed to the conserved motifs for complete ITS sequences (Dentinger, Didukh, & Moncalvo, 2011). The trimmed sequences were used for BLAST searches on GenBank (https://blast.ncbi.nlm.nih.gov/blast.cgi) using Nucleotide BLAST optimized for highly similar sequences (megablast). The ITS and nrLSU sequences generated for the three collections were identical. Sequences of *M. velutina* generated during this study were submitted to GenBank (Table 1).

An initial BLAST search of the nrLSU nucleotide sequences from the new species resulted in *M. heimii* (Bon) Matheny & Esteve-Rav. 'JV 14932F (WTU)' and *M. agardhii* var. *arenaria* 'EL25008' as closest hits, with maximum similarities of 99% with 100% query coverage. An initial BLAST search of the ITS nucleotide sequences from the new species resulted in *M. agardhii* var. *arenaria* 'EL25008' as closest hit, with maximum similarity of 95% with 87% query coverage.

(*M. agardhii* var. *arenaria* is not officially accepted species, so official scientific name of the species which is "*M. arenaria* (Bon) Matheny & Esteve-Rav." is adopted hereafter).

Closely related sequences were retrieved from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/), following Vauras & Larsson, 2011. We also added sequences from GenBank of the close hit after initial BLAST to construct the phylogeny using the combined ITS + nrLSU dataset. Finally, we included *M. arthrocystis* (Kühner) Matheny & Esteve-Rav. as outgroup following Vauras & Larsson, 2011. Sequences were aligned using Muscle v3.7 with default parameters in MEGA7 (Kumar, Stecher, & Tamura, 2016).

To estimate the placement and phylogenetic relationships of the new species, maximum likelihood (ML) and maximum parsimony (MP) analyses of the concatenated ITS + nrLSU datasets were conducted. MP analysis was performed in PAUP* version 4.0b10 (Swofford, 2002). All characters were equally weighted and gaps were treated as missing data. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Max-trees were set to 5,000, branches of zero length were collapsed and all parsimonious trees were saved. Clade robustness was assessed using a bootstrap (BS) analysis with 1000 replicates (Felsenstein, 1985). Descriptive tree statistics, tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated for each maximum parsimonious tree generated. Sequences were also analyzed using maximum likelihood (ML) with RAxML-HPC2 with

Table 1
Taxa of *Mallocybe* included in the molecular phylogenetic analyses.

Species	Specimen voucher/Isolate	Country	Accession numbers		Reference
			ITS	nrLSU	
Inocybe dulcamara (Pers.) P. Kumm.	EL59-05	Norway	GU980643	GU980643	Cripps, Larsson, & Horak (2010)
I. dulcamara	CLC 1333	USA	GU980635	GU980635	Cripps, Larsson, & Horak (2010)
M. agardhii (N. Lund) Matheny & Esteve-Rav.	AB980912	Denmark	HM209790	HM209790	Vauras & Larsson (2011)
M. arenaria (Bon) Matheny & Esteve-Rav.	EL25008	France	FN550937	FN550937	Ryberg, Larsson, & Jacobsson (2010)
M. arthrocystis (Kühner) Matheny & Esteve-Rav.	EL9207	Sweden	FN550941	FN550941	Ryberg, Larsson, & Jacobsson (2010)
M. fulvipes (Kühner) Matheny & Esteve-Rav.	EL99-07	Sweden	GU980600	GU980600	Cripps, Larsson, & Horak (2010)
M. fuscomarginata (Kühner) Matheny & Esteve-Rav.	EL10906	Sweden	FN550940	FN550940	Ryberg, Larsson, & Jacobsson (2010)
M. fuscomarginata	BJ890718	Sweden	GU980656	GU980656	Cripps, Larsson, & Horak (2010)
M. granulosa (Jacobsson & E. Larss.) Matheny & Esteve-Rav.	EL138-09	Sweden	KR029727	KR029727	Ariyawansa et al. (2015)
M. granulosa	SJ84030	Sweden	KR029725	KR029725	Ariyawansa et al. (2015)
M. gymnocarpa (Kühner) Matheny & Esteve-Rav.	SJ980707	Sweden	AM882866	AM882866	Ryberg et al. (2008)
M. heimii (Bon) Matheny & Esteve-Rav.	JV 14932F (WTU)	USA		AY380379	Matheny (2005)
M. latispora (Bon) Matheny & Esteve-Rav.	EL190-08	Not given	KR029724	KR029724	Ariyawansa et al. (2015)
M. leucoblema (Kühner) Matheny & Esteve-Rav.	SM2324	Sweden	GU980630	GU980630	Cripps, Larsson, & Horak (2010)
M. leucoblema	JV2898	Finland	HM209789	HM209789	Vauras & Larsson (2011)
M. leucoloma Kühner) Matheny & Esteve-Rav.	EL41-07	Sweden	GU980622	GU980622	Cripps, Larsson, & Horak (2010)
M. leucoloma	Ohenoja 880810	Svalbard	HM209786	HM209786	Vauras & Larsson (2011)
M. malenconii (R. Heim) Matheny & Esteve-Rav.	JV23101	Finland	HM209787	HM209787	Vauras & Larsson (2011)
M. malenconii	PAM98941302	France	HM209788	HM209788	Vauras & Larsson (2011)
M. myriadophylla (Vauras & E. Larss.) Matheny & Esteve-Rav.	EL121-08	Sweden	HM209792	HM209792	Vauras & Larsson (2011)
M. myriadophylla	JV19678	Finland	HM209793	HM209793	Vauras & Larsson (2011)
M. myriadophylla	JV5968	Finland	HM209794	HM209794	Vauras & Larsson (2011)
M. myriadophylla	JV19652	Finland	HM209791	HM209791	Vauras & Larsson (2011)
M. substraminipes (Kühner) Matheny & Esteve-Rav.	K70-148	USA	GU980601	GU980601	Cripps, Larsson, & Horak (2010)
M. substraminipes	EL12-08	USA	GU980607	GU980607	Cripps, Larsson, & Horak (2010)
M. terrigena (Fr.) Matheny, Vizzini & Esteve-Rav.	EL24-08	USA	GU980648	GU980648	Cripps, Larsson, & Horak (2010)
M. terrigena	EL11704	Sweden	AM882864	AM882864	Ryberg et al. (2008)
M. tomentosula Matheny & Esteve-Rav.	TENN:071837	USA	MG773814	MG773814	Unpublished
M. velutina	**MSM # 0048**	**Pakistan**	**MK990129**	**MK999927**	**This paper**
M. velutina	**MSM # 0049**	**Pakistan**	**MK990130**	**MK999928**	**This paper**
M. velutina	**MSM # 00050**	**Pakistan**	**MK990131**	**MK999929**	**This paper**

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general time reversible (GTR) model of site substitution including estimation of Gamma-distributed rate heterogeneity (+G) and a proportion of invariant sites (+I) on Abe through the Cipres Science Gateway (www.phylo.org; Miller, Pfeiffer, & Schwartz, 2010, pp. 1–8). Branch support for ML analysis was determined by 1000 bootstrap replicates (Hillis & Bull, 1993). Obtained trees were visualized in FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Our ITS + nrLSU dataset consisted of 31 specimens representing 18 taxa (Table 1) and 1752 characters, of which 1439 characters are constant, 113 are variable and parsimony-uninformative and 200 parsimony-informative. All characters are of unord type and have equal weight. One equally parsimonious tree (TL = 485, CI = 0.773, RI = 0.846, RC = 0.654) was derived from the MP analysis. Tree topologies obtained from MP and ML analyses were almost identical. Both ML and MP bootstraps strongly support the placement of the new species within *Mallocybe*. Both ML and MP inference provide support (BS = 100%) for clustering of *M. velutina* with *M. arenaria*, *M. heimii*, and *M. tomentosula* Matheny & Esteve-Rav. The ITS + nrLSU alignment was deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S26552).

Taxonomy

Mallocybe velutina Saba & Khalid, sp. nov. Fig. 1.

MycoBank no.: MB 823035.

Diagnosis: Most similar to *M. arenaria* and *M. heimii* but differs by the combination of smaller basidiomata, pileal color, strikingly velvety pileal appearance, smaller basidiospores, clavate to cylindrical cheilocystidia and an ecological association with Pinaceae. Phylogenetically separated from other species of *Mallocybe*.

Type: PAKISTAN, Prov. Khyber Pakhtunkhwa, Abbottabad, Thandiani, under *Pinus wallichiana* A.B.Jacks., 15 Sep 2012, leg. M. Saba & A.N. Khalid (holotype, MSM # 0048 (LAH310057); Gene sequences ex-holotype: MK990129 (ITS), MK999927 (nrLSU).

Etymology: Referring to the velvety appearance of pileus.

Pileus 17–22 mm diam, convex, hemispherical to plano-convex; margin turned down, without umbo; surface dull, uniformly scaly, velutinous, squamulose, moderate yellow (2.5Y7/6, 5Y7/6), light yellow (5Y9/6) margin, deep yellow (2.5YR6/10), fulvous center. Lamellae regular, adnate, subdistant, 4–6 mm broad, moderate yellow (2.5Y7/6, 5Y7/6) when young, deep yellow (2.5YR6/10) upon maturity, concolorous with pileus; margin even, 4 mm wide; two tiers of lamelullae. Cortina cobwebby, leaving tissue on cap margin

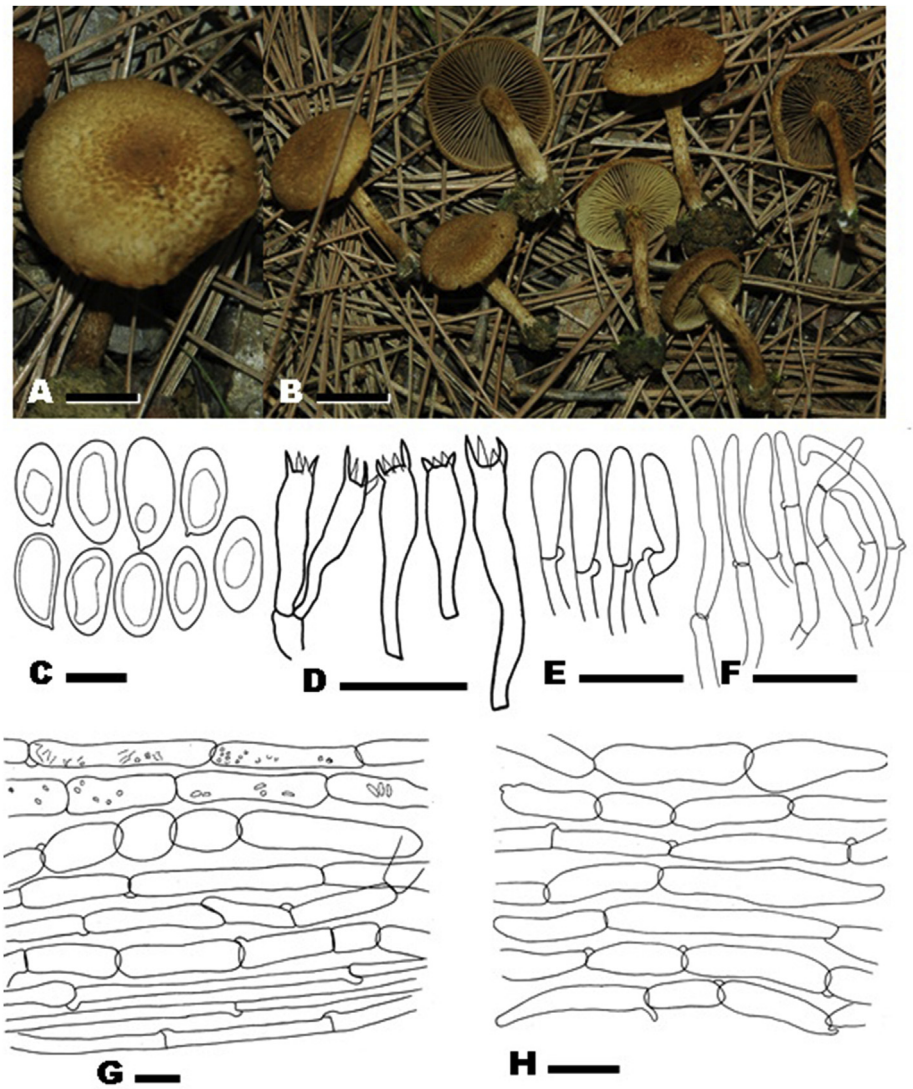

Fig. 1. *Mallocybe velutina*. A: MSM # 0049. B: MSM # 0048 (holotype). C: Basidiospores. D: Basidia. E: Cheilocystidia. F: Caulocystidia. G: Stipitipellis. H: Pileipellis. *Bars*: A, B 10 mm; C 10 μm; D–H 15 μm.

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and superior zone of stipe, strong brown (5YR4/8). Stipe 19–33 × 4–7 mm, central, equal, covered with strong brown fibrillose appressed scales that flare upward, brownish orange (5YR5/8), light orange yellow (7.5YR9/8); annulus absent; volva absent. Context pale yellow brown, up to 4 mm thick. Odor not distinctive to somewhat fungoid.

Basidiospores (6.6–)8.0–9.4 × 4.7–7.0 μm, 9.0 × 5.4 μm on average, Q = 1.2–2.1, subphaseoliform, ovoid or ellipsoid in profile, smooth, thin-walled, brown or reddish brown in KOH. Basidia with yellowish necropigment, (23.6–)26.6–37.2 × 6.3–8.0 μm, clavate, four spored, thin-walled, pale brown in KOH; sterigmata acute, 4.9–7.0 μm. Cheilocystidia 12.0–27.0 × 5.0–8.0 μm, clavate or cylindrical, hyaline, thin-walled. Pleurocystidia absent. Caulocystidia occurring mostly at stipe apex, clavate, similar to cheilocystidia but on average larger. Stipe hyphae cylindrical, 4.9–12.5 μm, hyaline to pale brown in KOH. Pileipellis a cutis of repent hyphae; hyphae cylindrical, 5.4–20.7 μm, thin-walled, light brown or pale brown in KOH, frequently septate. All structures inamyloid. Clamp connections present.

Habitat: Occurring in Sep, in groups, scattered on the forest floor in stands of *P. wallichiana* (Pinaceae).

Known distribution: Currently known from Western Himalayas, Pakistan.

Additional material studied: Paratype: Pakistan, Prov. Khyber Pakhtunkhwa, Abbottabad, Thandiani, under *P. wallichiana*, 22 Sep 2013, MSM # 0049 (LAH310058); GenBank accession nos. MK990130 (ITS), MK999928 (nrLSU). 13 Sep 2017, MSM # 00050, (LAH310060); GenBank accession nos. MK990131 (ITS), MK999929 (nrLSU).

Mallocybe velutina seems to be an ectomycorrhizal species of *P. wallichiana* inhabiting moist temperate forests where it grows as abundant groups. Other mycorrhizal trees growing in the vicinity are *Abies pindrow* Royle and *Cedrus deodara* (Roxb. ex D.Don) Loudon. Morphologically, the presence of yellowish or brown necropigment in basidia and stipe usually shorter than pileus diameter are the characters used to delimit taxon in *Mallocybe* (Kuyper, 1986). However, *M. velutina* has stipe which is somewhat larger than the pileus diameter. The phylogeny (Fig. 2) inferred from ITS + nrLSU sequences demonstrated that the three specimens from Pakistan fell into a well-supported lineage (99% BS) and grouped with *M. arenaria*, *M. heimii* and *M. tomentosula*. *Mallocybe arenaria* can be differentiated from the newly described taxon by the presence of larger pileus diameter (20–85 mm) and stipe size (40–60 mm) and septate utriform cheilocystidia (Ludwig, 2017). *Mallocybe heimii* is similar to *M. velutina* in inhabiting the pine forests, however, can be differentiated by larger size of pileus (20–70 mm), light grayish citrine, light oliveaceous brown to olivaceous reddish pileus which turns yellowish brown or fulvous (10 YR 5/6–5/8) to strong brown or nearly rusty brown (7.5 YR 4/4) with age, somewhat larger and narrower basidiospores (8.5–10.5(–11.0) × 4.5–5.5 μm) and its occurrence in Europe (Matheny, 2003). *Mallocybe tomentosula* is similar to *M. velutina* in having nearly similar shape, size and coloration of basidiomata, but can be distinguished by tomentose pileus, somewhat larger, septate cheilocystidia with pyriform or broadly clavate end cells, slightly larger and narrower basidiospores (9–10.5 × 5–6 μm) and its occurrence in North America (Matheny, unpublished data).

Mallocybe velutina is reported from Himalayan moist temperate forests in Thandiani, Abbottabad dominated by conifers i.e., *P. wallichiana*, *A. pindrow* and *C. deodara*. Our three collections were

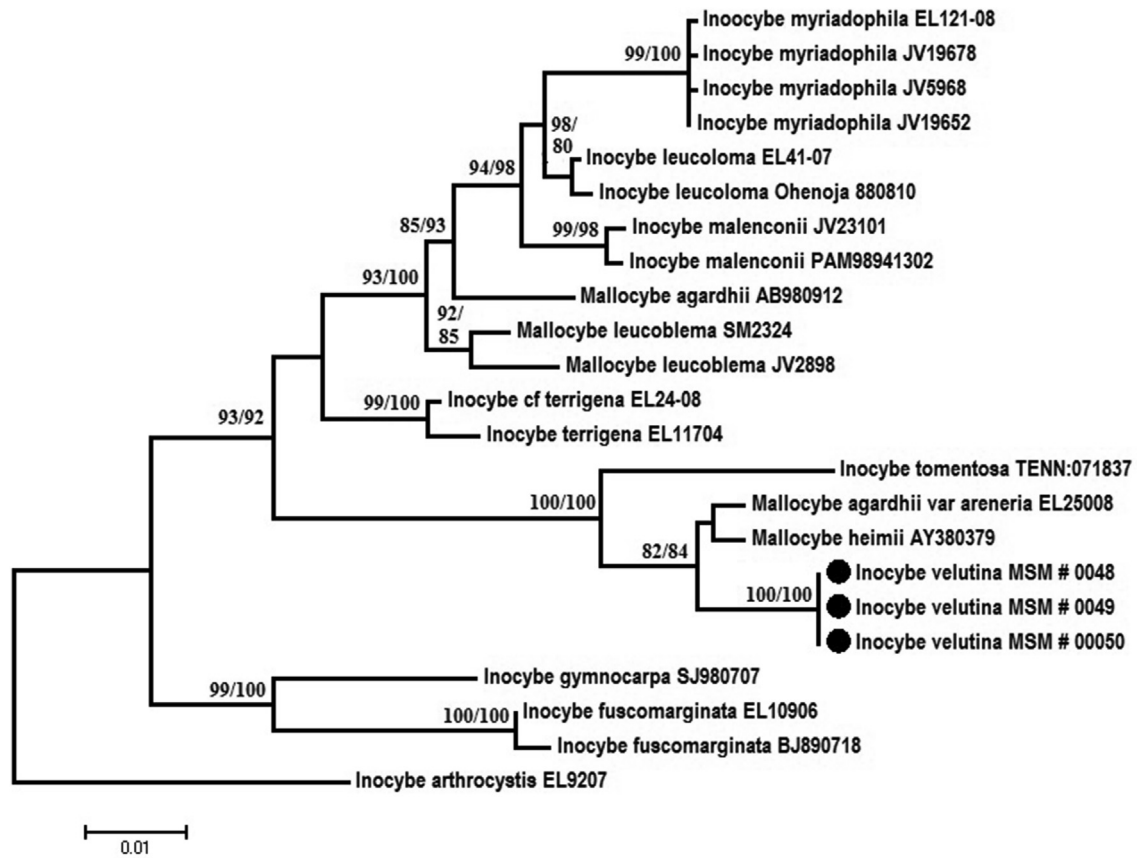

Fig. 2. Phylogeny of *Mallocybe velutina* and related taxa produced from maximum likelihood (ML) inference using combined dataset of ITS and nrLSU sequences. Numbers on branches are ML/MP bootstrap values (only ≥ 70). New sequences reported in this study are indicated with filled circles.

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collected in the vicinity of *P. wallichiana*, but whether *M. velutina* is strictly associated with the *Pinus* species or with more species of conifers needs to be verified in subsequent studies.

Species of the genus *Mallocybe* are poorly known in Pakistan. Currently, only one species is known from Pakistan: *M. leucoblema* (Kühner) Matheny & EsteveRav. (Ahmad et al., 1997). However, a considerable number of taxa have yet to be formally described, and the number of the species will likely increase as more collections are studied from under-explored localities.

Declaration of competing interset

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the countries where they were performed.

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