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Amanita mansehraensis, a new species in section *Vaginatae* from Pakistan

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Abstract

A new species of *Amanita* subgenus *Amanita* sect. *Vaginatae* is described and illustrated based on material collected in pine forests in district Mansehra, Khyber Pakhtoonkhaw, Pakistan. *Amanita mansehraensis* is recognized by the presence of a light brown or light greyish olive pileus with strong brown or deep brown pileus center; non-appendiculate, rimose, sulcate or plicate striate pileus margin; subglobose to ellipsoid basidiospores; and a saccate volva. 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 *A. mansehraensis* with other species of *Amanita* were inferred by means of Maximum Likelihood and Bayesian inferences of the nrLSU dataset and concatenated ITS+nrLSU dataset. *Amanita mansehraensis* is most closely related to *A. brunneofuliginea*, *A. pseudovaginata*, and the recently described *A. glarea*.

Keywords: Amanitaceae, Ectomycorrhizae, Phylogeny, Pine Forests, Taxonomy

Introduction

Amanita Pers. (1794: 145) (Basidiomycota, Agaricomycetes, Agaricales) is a large genus that includes ca. 868 described taxa (Tulloss 2017). It contains both poisonous and edible mushrooms (Bas 1969). Most species of *Amanita* are ectomycorrhizal (ECM) with several vascular plants, and therefore play an important role in forest ecosystems (Yang 1997, 2005). Since the 1990s, several reports of *Amanita* spp. from Pakistan were made, and several species have been described based on Pakistani material. However, no list is available of all taxa known for this country. Only 21 species of this genus are known from Pakistan; we summarized these in Table 1.

The genus is divided into two subgenera, subgenus *Amanita* and subg. *Lepidella* (E.-J. Gilbert) Veselý, based on the reaction of basidiospore walls with Melzer's reagent and some other characters (Cui *et al*. 2018). Members of subg. *Amanita* include species that are characterized by a non-bulbous stipe base (Corner & Bas 1962, Bas 1969). This subgenus is divided further into three sections: sect. *Amanita*, sect. *Caesareae* Singer ex Singer and sect. *Vaginatae* (Fr.) Qúel. *sensu stricto* (Yang 1997). Sect. *Vaginatae* includes those species that lack an annulus and clamp connections at the base of basidia (Yang 1997, 2005). However, certain African and South Asian (Bangladesh) species in this section possess an annulus (Tang *et al*. 2015). These are *Amanita annulatovaginata* var. *citrina* Beeli, *A. infusca sensu* Pegler & Shah-Sm., *A. loosii* Beeli, *A. madagascariensis* L.P. Tang, Zhu L. Yang & B. Buyck, *A. mafingensis* Härk. & Saarim., *A. masasiensis* Härk. & Saarim., *A. strobilaceovolvata* Beeli, and a number of undescribed species. Tulloss & Yang (2019) list 335 species under sect. *Vaginatae*, including 64 as *nom. prov.* and 133 unnamed ones.

During the exploration of Pakistani ectomycorrhizal fungi associated with *Pinus* species during 2014–2017, three collections from different localities in the Mansehra district were examined and identified morphologically in combination with molecular sequence data. Based on these analyses, a new species, *A. mansehraensis*, is described and placed in *Amanita* sect. *Vaginatae*. The morphological characters of this new species are compared with those of the closely related taxa, and its phylogenetic placement is assessed using large nuclear ribosomal RNA gene (nrLSU) sequences and a combined dataset of internal transcribed spacer (ITS) and nrLSU sequences. Due to high success rates

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of PCR amplification and subsequent sequencing, even for older specimens, the nrLSU marker has been proposed as a candidate barcode for the genus *Amanita* (Cai *et al*. 2012). The nrLSU region has also proved very useful in resolving phylogenetic relationships and species delimitation within the genus *Amanita* (Weiß *et al*. 1998; Drehmel *et al*. 1999; Zhang *et al*. 2004; Justo *et al*. 2010; Zhang *et al*. 2010; Deng *et al*. 2014; Cai *et al*. 2014; Li & Cai 2014; Hosen *et al*. 2015, 2018).

TABLE 1. All species of *Amanita* known in Pakistan to date, with reference of first mentioning in Pakistan.

Species	Authority	Reference
Amanita caesarea	(Scop.) Pers.	Iqbal & Khalid (1996)
A. ceciliae	(Berk. & Broome) Bas	Murakami (1993)
A. flavipes	S. Imai	Murakami (1993)
A. glarea	Jabeen, M. Kiran & Sadiqullah	Jabeen *et al*. (2017)
A. hemibapha	(Berk & Broome) Sacc.	Murakami (1993)
A. longistriata	S. Imai	Iqbal & Khalid (1996)
A. mansehraensis	Saba & Khalid	This study
A. muscaria	(L.) Lam.	Iqbal & Khalid (1996)
A. nana	Singer	Ahmad (1956)
A. orsonii	Ash. Kumar & T.N. Lakh.	Tulloss *et al*. (2001)
A. pakistanica	Tulloss, S.H. Iqbal & Khalid	Tulloss *et al*. (2001)
A. pallidorosea	P. Zhang & Zhu L. Yang	Kiran *et al*. (2017)
A. pantherina	(DC.) Krombh.	Shibata (1992)
A. phalloides	(Vaill. ex Fr.) Link	Iqbal & Khalid (1996)
A. porphyria	Alb & Schwein.	Iqbal & Khalid (1996)
A. pseudovaginata	Hongo	Razaq (2013)
A. rubescens	Pers.	Iqbal & Khalid (1996)
A. umbrinolutea	(Secr. ex Gillet) Bataille	Tulloss *et al*. (2001)
A. vaginata	(Bull.) Lam.	Ahmad (1956)
A. verna	(Bull.) Lam.	Iqbal & Khalid (1996)
A. virosa	Bertill.	Iqbal & Khalid (1996)
A. watlingii	Ash. Kumar & T.N. Lakh.	Iqbal & Khalid (1996)

Materials and Methods

Field study:—Collections were done at selected sampling sites in Mansehra District, Pakistan: Batrasi (33°56’37.8”N, 72°54’53.0”E) and Chattar Plain (34°36’53.2”N, 73°07’04.8”E) (Figure 1). Specimens were photographed in the field and macro-morphological features were described on site. Color designations were derived from the Munsell color system (1975). Collections were dried using a food dehydrator (at 39 ˚C for 7–9 hours) and deposited at the University of the Punjab Herbarium (LAH) in Pakistan and Farlow Herbarium (FH) at Harvard University, USA.

Morphological studies:—Sections of specimens were mounted in H_2O and 5% aqueous 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 an Olympus BX40 light microscope (Waltham, Massachusetts) with Olympus XC50 digital camera and Microsuite special edition software 3.1 (Soft Imaging Solutions GmbH). Dimensions of basidiospores 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 between parentheses. Line drawings were made with a Leitz Camera Lucida (Wetzlar, Germany). The following abbreviations are used: KOH = 5% potassium hydroxide, L = spore length (arithmetic average of all spores), W = spore width (arithmetic average of all spores), Q = length/width ratio of a basidiospore in side view, [n/m/p] = n basidiospores from m basidiomata of p collection with minimum of 20 basidiospores from each basidioma.

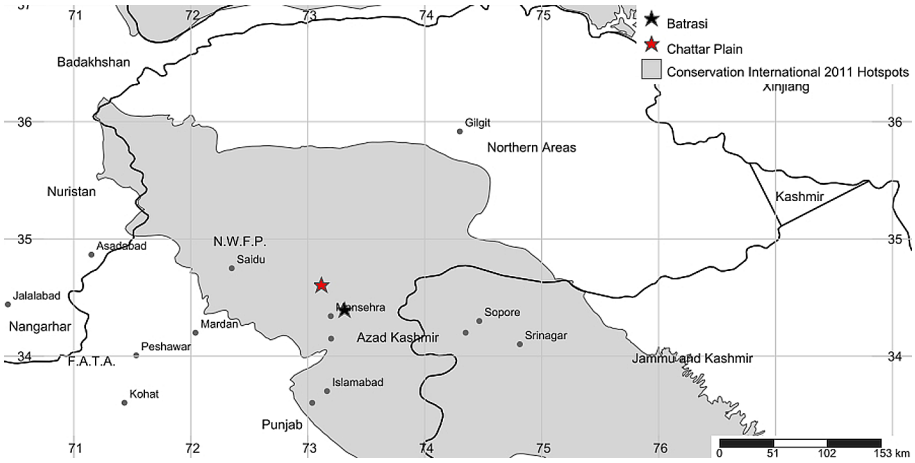

FIGURE 1. Map showing sampling sites. The Himalayan region is highlighted in grey (Shorthouse 2010).

DNA extraction, PCR amplification, DNA sequencing:—Genomic DNA was extracted from wheat grain-sized pieces of lamellae of basidiomata using a CTAB method (Lee *et al.* 1988). We amplified the complete ITS region (ITS1–5.8S–ITS2) of the nuclear RNA gene, the nrLSU region and the translation elongation factor 1-alpha (*tef1-α*) gene.

Primer pairs used were ITS1f/ITS4 (White *et al*. 1990, Gardes & Bruns 1993) for ITS, LR0R/LR5 (Vilgalys & Hester 1990) for nrLSU, and 983F/1567R (Rehner & Buckley 2005) for *tef1-α*. The PCR mixture contained the following components: 2.5 µL Econo buffer, 0.5 µL dNTPs, 1.25 µL of each primer, 0.125 µL EconoTaq, 14.375 µL ddH_2O, and 5 µL of DNA extract. 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. To amplify *tef1-α* we used a touchdown protocol: initial denaturation at 95 °C for 5 min; followed by 10 cycles of 95 °C for 1 min, 66 °C (decreasing 1 °C every three cycles) for 1 min and 72 °C for 90 s; then 30 to 36 cycles of 95 °C for 30 s, 56 °C for 90 s, 72 °C for 1 min; and final extension at 72 °C for 7 min (after Don *et al*. 1991, Haelewaters *et al*. 2018).

PCR products were run on 1% agarose gel, stained with ethidium bromide and bands were visualized under a UV trans-illuminator. Amplified PCR products of the ITS region were sent for purification and bidirectional sequencing to Macrogen (South Korea). Amplified PCR products of nrLSU and *tef1-α* were purified using the QIAquick PCR purification kit (Qiagen, Stanford, California) as per manufacturer's guidelines and sequencing reactions were performed using the Big Dye® Terminator v3.1 Cycle Kit (Life Technologies, Carlsbad, California). The same primers were used for sequencing as those used for PCR amplification.

Sequence alignment and phylogenetic analyses:—Sequences were assembled and manually edited in Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan). ITS sequences were trimmed to the conserved motifs 5'–(…GAT) CATTA– and –GACCT (CAAA…)–3' for complete ITS sequences (Dentinger *et al*. 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). Closely related nrLSU sequences were retrieved from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/), following Tang *et al*. (2015) who studied taxa in *Amanita* sect. *Vaginatae*. ITS sequences of the same taxa were obtained from GenBank to construct the phylogeny using the combined ITS+nrLSU dataset. We also added sequences of the recently described species *A. glarea* Jabeen, M. Kiran & Sadiqullah (2017: 140) from Pakistan to the final dataset (Jabeen *et al*. 2017). Finally, we included *Limacella glischra* (Morgan) Murrill and *L. glioderma* (Fr.) Maire as outgroup taxa; *Limacella sensu lato* is a polyphyletic genus in Amanitaceae (Yang *et al.* 2018). Sequences were aligned using Muscle v3.7 with default parameters in MEGA7 (Kumar *et al*. 2016).

To estimate the placement and phylogenetic relationships of the new species, Maximum Likelihood (ML) and Bayesian analyses of the nrLSU and concatenated ITS+nrLSU datasets were conducted. Phylogenetic analyses were done with RAxML 8.2.X (Stamatakis 2014), which is available on the Cipres Science Gateway (Miller *et al*. 2010). ML was inferred under GTRCAT model with 1000 bootstrap (BS) replicates. We used jModelTest 2 (Darriba *et al*. 2012) to select the nucleotide substitution model, by considering the Akaike Information Criterion. For both datasets, the GTR+I+G model was selected out of 88 candidate models (-lnL = 5373.2209 for the nrLSU dataset; -lnL = 9337.7183

for the ITS+nrLSU dataset). Bayesian inference was done with an MCMC coalescent approach implemented in BEAST v1.8.4 (Drummond *et al*. 2012), with an uncorrelated lognormal relaxed clock. A GMRF Bayesian skyride coalescent tree prior was used in all simulations with the GTR+I+ G model of substitution with randomly generated starting tree. Four independent runs of 40 million generations were undertaken, with sampling every 4000 generations. Tracer v1.6 (Rambaut *et al*. 2014) was used to check the effective sample size (ESS), and burnin values were adjusted to achieve a net ESS of at least 200. Consensus trees with 0% burnin were generated and a Maximum Clade Credibility tree was inferred using TreeAnnotator 1.8.4. Obtained trees were visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

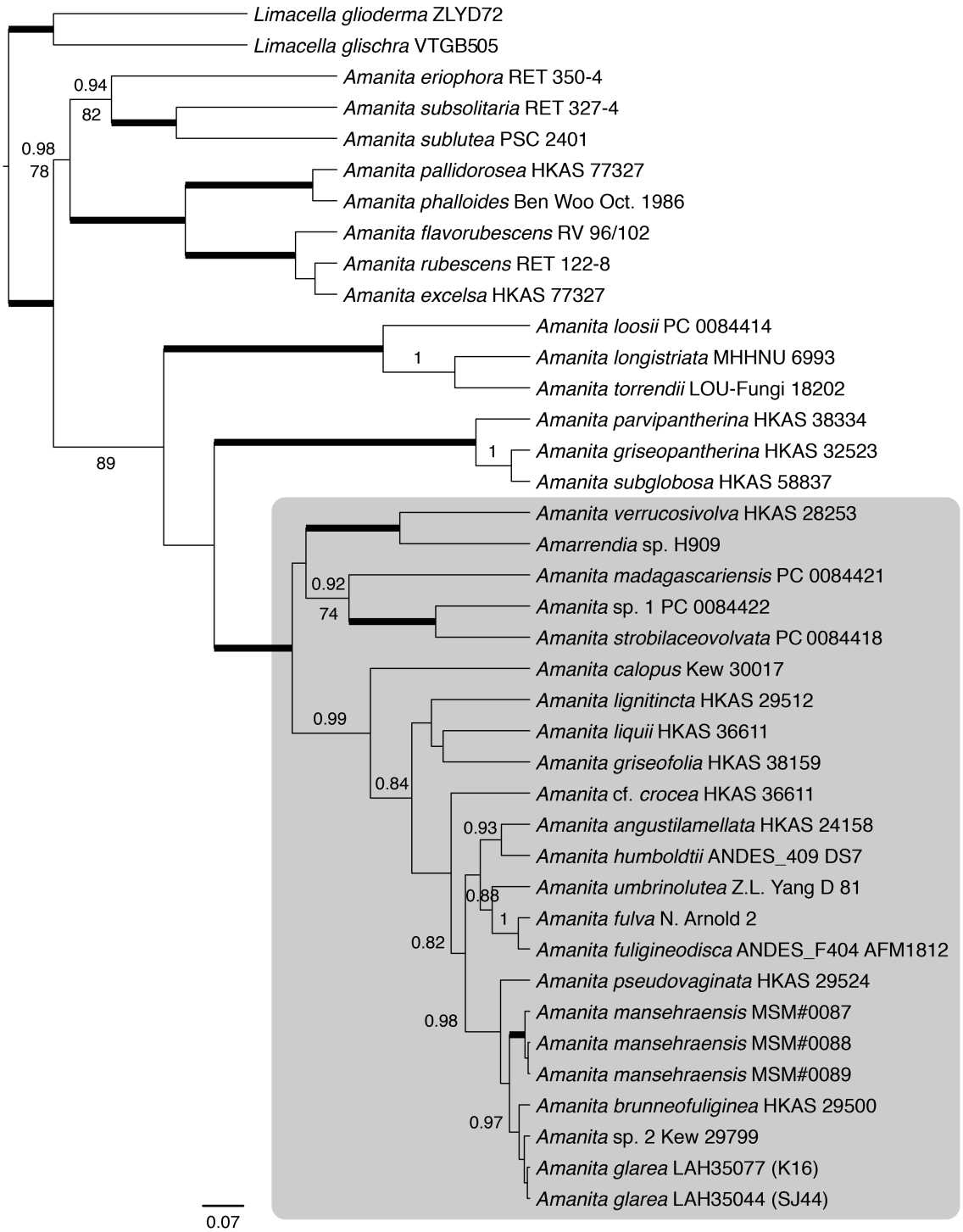

FIGURE 2. Phylogeny of *Amanita* species produced from Bayesian inference using nrLSU sequences. Numbers above branches are ML bootstrap values (only ≥ 70); numbers below branches are posterior probabilities (only ≥ 0.7). Branches in bold have ML bootstrap of ≥ 97 and posterior probability of ≥ 0.97. *Amanita* sect. *Vaginatae* is highlighted in grey.

TABLE 2. Taxa of *Amanita* included in our molecular phylogenetic analyses.

Species	Isolate	Location	nrLSU	ITS	Reference
Amanita angustilamellata	HKAS 24158	China	AF024440	N/A	Weiss *et al*. 1998
A. brunneofuliginea	HKAS 29500	China	AF024442	N/A	Weiss *et al*. 1998
A. calopus	Kew 30017	Zambia	JF710812	N/A	Tang *et al*. 2015
A. cf. *crocea*	HKAS 38461	China: Yunnan	AY436490	AY436483	Zhang *et al*. 2004
A. eriophora	RET 350-4	Cambodia: Angkor	HQ539672	N/A	B.E. Wolfe & A. Pringle unpubl.
A. excelsa	Ge 816	China: Sichuan	HQ539691	N/A	B.E. Wolfe & A. Pringle unpubl.
A. excelsa	HKAS 31510	Germany: Baden-Württemberg	AY436491	AY436453	Zhang *et al*. 2004
A. flavorubescens	RV 96/102	USA: Virginia	AF097380	-	Drehmel *et al*. 1999
A. flavorubescens	TENN61660	USA: Tennessee	-	JF313650	K.W. Hughes *et al*. unpubl.
A. fuligineodisca	ANDES_F404 AFM1812	Colombia: Antioquia	FJ890039	FJ890027	Vargas *et al*. 2011
A. fulva	N. Arnold 2	China	AF024455	-	Weiss *et al*. 1998
A. fulva	KA12-1406	South Korea	-	KF017933	Kim *et al*. 2013
A. glarea	LAH35077 (K16)	Pakistan: Khyber Pakhtunkhwa	KY788653	KY788649	Jabeen *et al*. 2017
A. glarea	LAH35044 (SJ44)	Pakistan: Khyber Pakhtunkhwa	KY781175	KY781174	Jabeen *et al*. 2017
A. griseofolia	HKAS 38159	China: Yunnan	AY436488	AY436448	Zhang *et al*. 2004
A. griseopantherina	HKAS 32523	China: Sichuan	AY436494	AY436459	Zhang *et al*. 2004
A. humboldtii	ANDES_409 DS7	Colombia: Antioquia	FJ890045	N/A	Vargas *et al*. 2011
A. lignitincta	HKAS 29512	China	AF024461	N/A*	Weiss *et al*. 1998
A. liquii	HKAS 36611	China: Yunnan	AY436493	AY436462	Zhang *et al*. 2004
A. longistriata	MHHNU 6993	China: Hunan	KP276142	-	Tang *et al*. 2015
A. longistriata	KA12-1372	South Korea	-	KR673582	Kim *et al*. 2015
A. loosii	PC 0084414	Burundi	JQ512084	JQ512095	Tang *et al*. 2015
A.madagascariensis	PC 0084421	Madagascar	JQ512087	N/A	Tang *et al*. 2015
A. mansehraensis	MSM#0087	Pakistan: Khyber Pakhtunkhwa	**MG195979**	**MG195984**	This study
A. mansehraensis	MSM#0088	Pakistan: Khyber Pakhtunkhwa	**MG195980**	**MG195982**	This study
A. mansehraensis	MSM#0089	Pakistan: Khyber Pakhtunkhwa	**MG195981**	**MG195983**	This study
A. pallidorosea	HKAS 77327	China	KJ466446	-	Cai *et al*. 2014
A. pallidorosea	RET 406-5	India: Uttarakhand	-	KX270316	R.E. Tulloss *et al*. unpubl.
A. parvipantherina	HKAS 38334	China: Yunnan	AY436498	AY436469	Zhang *et al*. 2004
A. phalloides	Ben Woo Oct. 1986	USA: Washington	AY380359	N/A	Matheny 2005
A. phalloides	RET 053-2	USA: New Jersey	KF561979	KF561975	R.E. Tulloss *et al*. unpubl.
A. pseudovaginata	HKAS 29524	China	AF024472	-	Weiss *et al*. 1998
A. pseudovaginata	HKAS 38323	China: Yunnan	-	AY436470	Zhang *et al*. 2004
A. rubescens	RET 122-8	Turkey: Trabzon	HQ539735	-	B.E. Wolfe & A. Pringle unpubl.
A. rubescens	TENN62894	Sweden	-	JF313654	K.W. Hughes *et al*. unpubl.
A. sp. 1	PC 0084422	Madagascar	JQ512088	JQ512097	Tang *et al*. 2015
A. sp. 2	Kew 29799	Malawi	JF710817	JF710841	Tang *et al*. 2015
A. strobilaceovolvata	PC 0084418	Zambia	JQ512085	N/A	Tang *et al*. 2015

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TABLE 2 (Continued**)**

Species	Isolate	Location	nrLSU	ITS	Reference
A.subglobosa	HKAS 58837	China	JN941152	JN943177	Schoch *et al*. 2012
A. sublutea	PSC 2401	Australia: South Australia	HQ539749	N/A	B.E. Wolfe & A. Pringle unpubl.
A. subsolitaria	RET 327-4	USA: New Jersey	HQ539750	N/A	B.E. Wolfe & A. Pringle unpubl.
A. torrendii	LOU-Fungi 18202	Spain: Ourense	GQ925368	GQ925387	Justo *et al*. 2010
A. umbrinolutea	Z.L. Yang D 81	China	AF024481	-	Weiss *et al*. 1998
A. umbrinolutea	HKAS 31451	Germany: Baden-Württemberg	-	AY436478	Zhang *et al*. 2004
A. vaginata	KA12-1190	South Korea	KF021688	KF017949	Kim *et al*. 2013
A. vaginata	H. A. v. d. Aa s. n.	N/A	AF024482	N/A	Weiss *et al*. 1998
A. verrucosivolva	HKAS 28253	China	AF024483	N/A	Weiss *et al*. 1998
Amarrendia sp.	H909	Australia	GQ925378	GQ925403	Justo *et al*. 2010
Limacella glioderma	ZLYD72	N/A	DQ071728	-	T.Y. James *et al*. unpubl.
L. glioderma	xsd08047	China	-	FJ478086	J. Jiang *et al.* unpubl.
L. glischra	VTGB505	N/A	AY612843	N/A	T.Y. James *et al*. unpubl.
L. glischra	RET 502-8	USA: Missouri	KT168222	KT168221	R.E. Tulloss *et al*. unpubl.

For each isolate is provided: location, GenBank accession numbers for nrLSU and ITS sequences, and reference. Newly generated sequences from Pakistan are highlighted in bold. N/A = no information/sequences available.

Results

Molecular analyses:—Sequences of *A. mansehraensis* generated during this study were submitted to GenBank (details in Table 2). The nrLSU sequences generated for the three collections were identical. An initial BLAST search of the nrLSU nucleotide sequences from the new species resulted in *Amanita* sp. 'sp-WA03', *Amanita* sp. 'sp-N66', and *A.* aff. *vaginata* as closest hits, with maximum similarities of 99% with 98% query coverage. The ITS sequence from MSM#0087 (Chattar Plain) differed from the other two sequences in two nucleotides (on a total of 516 nucleotides: C at position 387 instead of T and G at position 482 instead of A). An initial BLAST search of the ITS nucleotide sequences from the new species resulted in *A. pseudovaginata* Hongo (1983: 39) and *A. supravolvata* Lanne (1979: 24) as closest hits, with maximum similarities of 99% with 100% query coverage.

The nrLSU dataset consisted of 39 taxa (Table 2) and 968 characters, of which 633 were constant and 227 were parsimony-informative. Our ITS+nrLSU dataset consisted of 44 taxa (Table 2) and 1271 characters, of which 686 were constant and 388 were parsimony-informative. Tree topologies obtained from ML and Bayesian analyses were almost identical except for the closest relatives of the new species. Both ML bootstraps (BS) and Bayesian posterior probabilities (pp) strongly support the placement of the new species within sect. *Vaginatae*. Based on the ML topology of the nrLSU dataset, we can only conclude that it is positioned in *A*. sect. *Vaginatae* (Figure 2). Bayesian inference of the nrLSU provides high support (pp = 0.98) for the placement of the new species in a group with *A. brunneofuliginea* Zhu L. Yang (1997: 96), *A. glarea*, *A. pseudovaginata*, and *Amanita* sp.2 (Figure 2). Phylogenetic relationships were best inferred using the combined ITS+nrLSU dataset (Figure 3). Both ML and Bayesian inference provide support (BS = 79, pp = 1.0) for again clustering of *A. mansehraensis* with *A. brunneofuliginea*, *A. glarea*, *A. pseudovaginata*, and *Amanita* sp. 2.

Amanita glarea, recently described from Pakistan by Jabeen *et al*. (2017), is not supported as a separate species in our phylogenetic analyses based on nrLSU sequences (BS = 50, pp = 0.33). In the ITS+nrLSU dataset, *A. glarea* is supported and placed as sister taxon of *A. mansehraensis* with very poor support (BS = 44, pp = 0.41). *Amanita glarea* may represent a case of incipient speciation. *Amanita mansehraensis* is well supported as separate taxon in both analyses; it has maximum support in the phylogenetic reconstruction based on nrLSU (= the proposed barcode for *Amanita*, *fide* Cai *et al*. 2012).

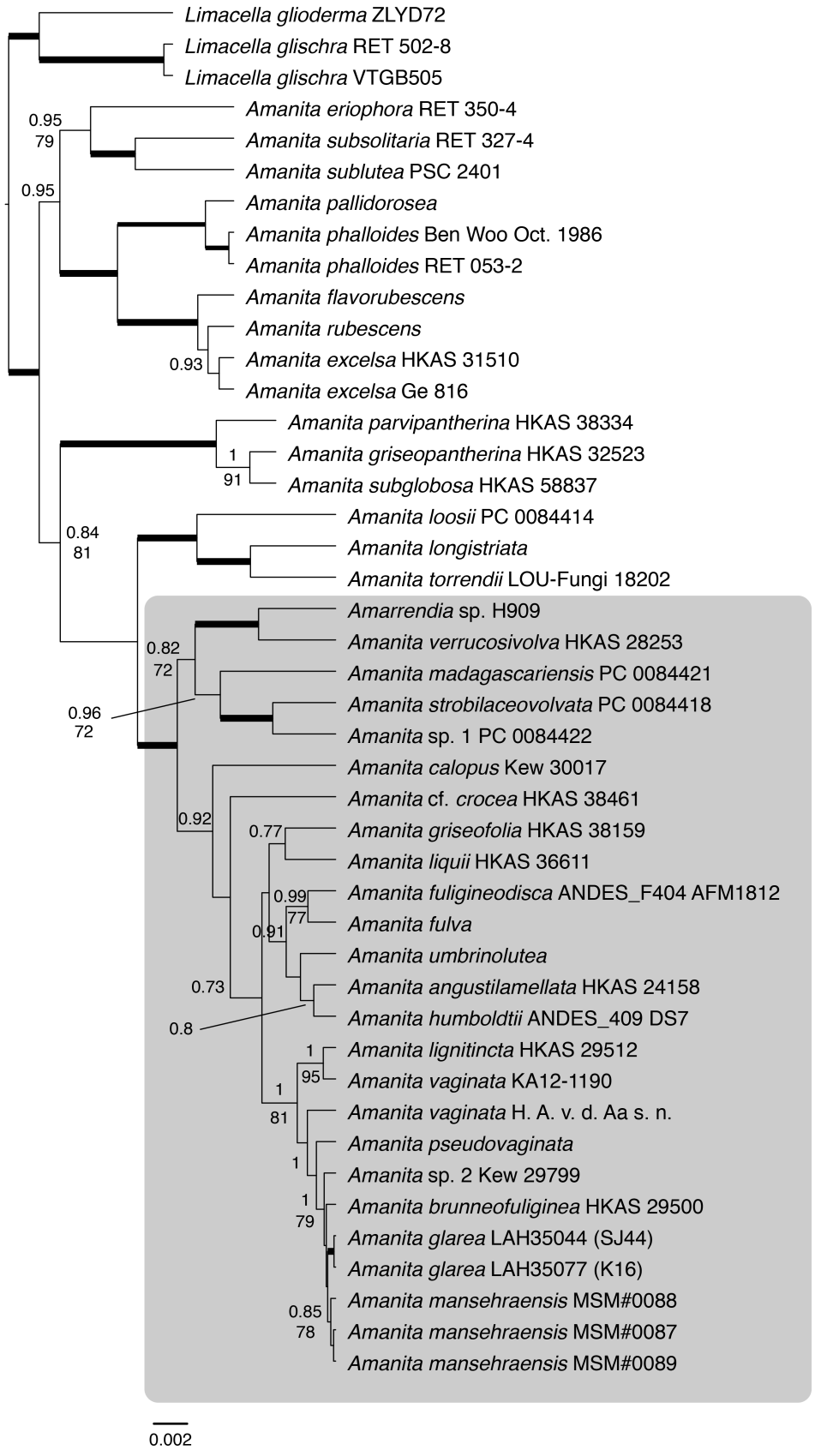

FIGURE 3. Phylogeny of *Amanita* species produced from Bayesian inference using combined dataset of ITS and nrLSU sequences. Numbers above branches are ML bootstrap values (only ≥ 70); numbers below branches are posterior probabilities (only ≥ 0.7). Branches in bold have ML bootstrap of ≥ 97 and posterior probability of ≥ 0.97. *Amanita* sect. *Vaginatae* is highlighted in grey.

Taxonomy

Amanita mansehraensis Saba, Haelew. & Khalid, *sp. nov*. **Figures 4 & 5**
MycoBank MB823035

FIGURE 4. *Amanita mansehraensis* basidiomata. A: MSM#0087. B, E: MSM#0088 (holotype). C, D, F: MSM#0089. Scale bars = 20 mm.

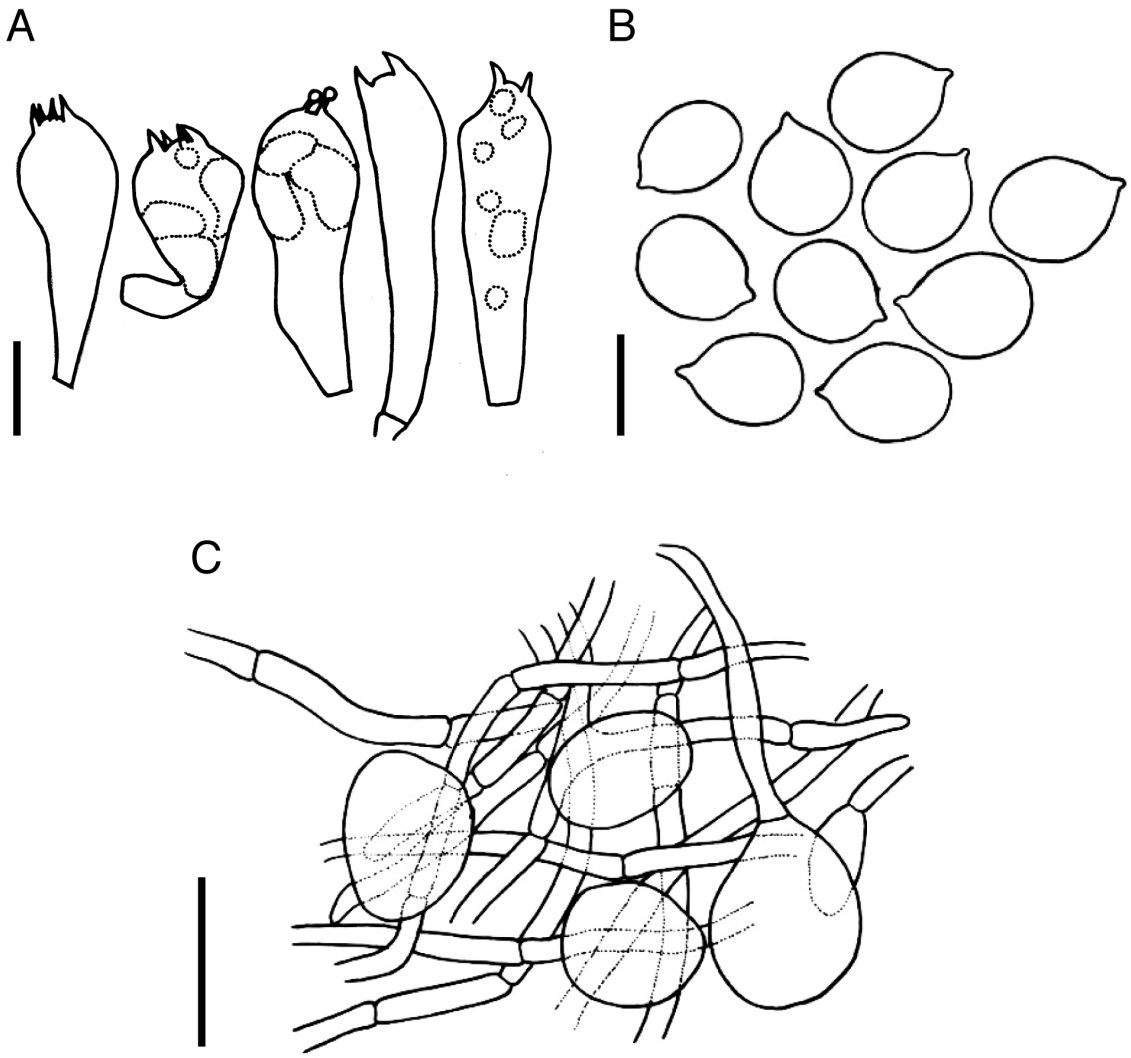

FIGURE 5. *Amanita mansehraensis* (MSM#0088). A. Basidia; B. Basidiospores; C. Volval elements. Bars = 10 µm.

Diagnosis:—Characterized by light brown or light greyish olive pileus becoming strong brown or deep brown in the center; non-appendiculate, rimose, sulcate or plicate striate pileus margin with non-persistent universal veil remnants; a long ringless stipe; subglobose to ellipsoid basidiospores and saccate volva. Associated with *Pinus* spp. Phylogenetically distinguished from other species within *A.* sect. *Vaginatae*.

Types:—PAKISTAN, Prov. Khyber Pakhtunkhwa, Mansehra District, Batrasi, 33°56'37.8"N 72°54'53.0"E, under *Pinus roxburghii* Sarg., 3 August 2014, *leg.* M. Saba & A.N. Khalid, MSM#0088 (LAH31005, **holotype**); GenBank accession nos. MG195982 (ITS), MG195980 (nrLSU), MH495970 (*tef1-α*). *Ibid.*, 12 September 2017, MSM#0089 (LAH31006, **paratype**); GenBank accession nos. MG195983 (ITS), MG195981 (nrLSU). PAKISTAN, Prov. Khyber Pakhtunkhwa, Mansehra District, Chattar Plain, 34°36'53.2"N 73°07'04.8"E, under *Pinus wallichiana* A.B. Jacks., 15 September 2012, *leg.* M. Saba & A.N. Khalid, MSM#0087 (LAH31004, **paratype**); GenBank accession nos. MG195984 (ITS), MG195979 (nrLSU).

Etymology:—Referring to the locality (Mansehra) where the holotype was collected.

Description:—Basidiomata medium to large, solitary. Pileus 53.7–85 mm in diameter, subglobose when young to convex at maturity, center plane or umbonate or slightly depressed, brown (5YR4/6) or deep brown (2.5YR2/8) from center and becoming light brown (5YR5/4) or light greyish olive (10Y6/2) towards margin, dry, sometimes rimose, splitting towards center, sometimes covered with off-white with brownish tinge or dirty white volval remnants; margin deflexed, straight or uplifted, sulcate striate or plicate striate, up to 7–13 mm, non-appendiculate; cuticle thin. Lamellae regular, free or sinuate, close to crowded, off-white (7.5Y9/2) or cream; margin entire: lamellulae truncate. Stipe 84–92 mm, 5–6 mm wide at upper end, 10–11 mm wide at base, central, clavate or subcylindrical, attenuate

upwards, with apex sometime expanded, light brown (5YR6/4), pale yellowish pink (7.5R9/2) or cream with brownish tinge, flocculose; annulus absent; volva saccate, 27–40 mm high, membranous, with round base, often exhibiting longitudinal splitting. Context white, unchanging when injured. Odor and taste unknown.

Basidiospores [60/3/3] **(**8.26) 9.9–11.8 × (8.26) 8.7–9.4 (10.7) µm [x = 10.46 × 9.1 µm, Q = 1.1–1.35], subglobose or ellipsoid, guttulate, smooth, thin-walled, hyaline in KOH, inamyloid, apiculus 0.8 µm long. Basidia 41–51 × 11.8–14.8 µm, narrowly clavate to clavate, usually four-spored, sometimes two-spored, thin-walled, hyaline in KOH; sterigmata 2.4–5 µm, acute. Subhymenium of irregular subglobose cells, 10–14 × 8–12 µm. Lamellar trama of filamentous hyphae, 6–11 µm wide. Lamellar edge tissue sterile, mainly composed of inflated globose to subglobose cells. Pileipellis a cutis of cylindrical hyphae, 3–8 µm wide, hyaline in KOH. Stipe hyphae cylindrical, hyaline in KOH, 6.39–12.40 µm, hyphal endings 25–49 × 10–20.5 µm, clavate, hyaline. Volval elements consist of hyphae and clavate or globose cells, hyphae hyaline in KOH, 4–10 µm wide, globular cells hyaline in KOH, 23.5–57 × 13–42 µm. Clamp connections absent.

Habit and habitat:—Found fruiting in August to September, solitary or scattered on the forest floor in pure stands of *Pinus roxburghii* and *P. wallichiana* (Pinaceae).

Known distribution:—Only known from Western Himalayas, Pakistan (Figure 1).

Discussion

Based on nrLSU sequence analysis, we recovered *A*. sect. *Vaginatae* as a monophyletic group, sister to *A*. sect. *Amanita* (Figure 2). The monophyly of this section has also been retrieved in previous molecular phylogenetic studies (Tang *et al*. 2015, Jabeen *et al*. 2017). *Amanita mansehraensis* falls in *A*. sect. *Vaginatae*. In addition, the different isolates of *A. mansehraensis* form a strongly supported monophyletic clade, clearly separated from other taxa. It clusters most closely with three South Asian species: *A. brunneofuliginea*, *A. glarea*, and *A. pseudovaginata*. In the ITS+nrLSU phylogenetic reconstruction, *A. glarea* and *A. mansehraensis* are sister species, which is interesting from a geographical point of view.

Amanita glarea is a recently described species within *A*. sect. *Vaginatae* from Pakistan (Jabeen *et al*. 2017) and resembles *A*. *mansehraensis* morphologically. However, *A*. *glarea* is differentiated by its larger and broader basidiospores (10–11.5 × 10.5–11 µm) that are globose to subglobose in shape. *Amanita brunneofuliginea*, a species from China (Yang 1997), can be differentiated by the pileus color (dark brown to brown black) and the basidiospores, which are larger and broader: (10–)10.5–13(–14) × (9–)9.5–12(–12.5) µm. *Amanita pseudovaginata*, a species originally described from Japan (Hongo 1983), is similar to *A. mansehraensis* in having a similar pileus color. However, *A. pseudovaginata* differs in having smaller basidiomata and subdistant lamellae, and its restricted volva, covering up to 15–20 mm of the stipe. In addition, *A*. *pseudovaginata* is clearly phylogenetically separated from the new species (Figures 2 & 3).

The BLAST search of ITS sequences of *A. mansehraensis* resulted in *A. supravolvata* as a close match (98–99% similarity). This is a European species (described from France), which can be morphologically distinguished by the following characteristics: pale grey color of pileus; ellipsoid to broadly ellipsoid, somewhat larger basidiospores [(8.8–)9.8–13.5(–16.5) × (6.4–)7.3–10.5(–14) µm]; and its volva covering a large portion of the stem (up to 35–67 mm) (Lann 1978).

Amanita mansehraensis is reported from subtropical pine forests in Batrasi, Mansehra dominated by *Pinus roxburghii* and from Himalayan moist temperate forests in Chattar Plain, Mansehra dominated by *P. wallichiana*. Our three collections were associated with *P. roxburghii* and *P*. *wallichiana*, but whether *A. mansehraensis* is strictly associated with these two *Pinus* species or with more species of *Pinus* needs to be verified in subsequent studies. As a comparison, *A*. *glarea* has a broad host range. It has been reported in association with *Abies pindrow* Royle, *Cedrus deodara* (Roxb. ex D. Don) G. Don, and *Pinus wallichiana* (Jabeen *et al*. 2017). *Amanita pseudovaginata* is known to occur in forests dominated by *Pinus* and *Quercus* L. (Hongo 1983) whereas *A*. *brunneofuliginea* is reported from mixed forests of *Abies* D. Don and *Quercus* (Yang 1997). *Amanita supravolvata* has been found in association with *Pinus* on sandy soils (Lanne 1978).

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