



Amanita griseofusca: A new species of *Amanita* in section *Vaginatae* from Malam Jabba, Swat, Pakistan

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Abstract

A new species, *Amanita griseofusca* in section *Vaginatae* is described and illustrated here from Pakistan. Distinguishing characters of the new species include medium-sized basidiomata, greyish brown pileus surface with white to beige, membranous volval remnants present as one (large) to a few (small) warts, close lamellae which are cream colored with a pink tone, striations one third of the total pileus radius, broadly ellipsoidal to ellipsoidal basidiospores and white loose saccate volva turning beige at maturity. Molecular data inferred from partial nuc rDNA internal transcribed spacer region (ITS), partial nuc rDNA larger subunit region (LSU) and partial translation elongation factor 1-alpha (*tef1*) confirms the novelty of the present taxon.

Keywords: Amanitaceae, molecular phylogeny, new taxon, *Quercus dilatata*

Introduction

Amanita Pers. (1794: 145) is a well-known genus of Basidiomycota in the family Amanitaceae that exhibits worldwide distribution. It contains both deadly poisonous and valued gourmet species. It plays important roles in forest ecosystems, as a large majority of the species are known to be ectomycorrhizal fungi. *Amanita* is an easily recognizable taxon due to the presence of bilateral gill trama, presence of acrophysalides and schizophymenial development of fruit body (Bas 1969). Moreover, the genus is rich in striking macroscopic characters. Some species have warts on the pileus surface derived from the break-up of universal veil but others have smooth pileus with sulcate margins (Yang & Oberwinkler 1999). Most of the species have a partial veil (annulus) that protects lamellae in young specimens and a universal veil (volva) that covers the stipe base. Lamellae are mostly free or slightly attached. The spore print is usually white. However, species of section *Vaginatae* (Fries 1854:2, Quélet 1872:64) are recognized by the sulcate margin of the pileus, lack of an annulus and a bulbous base, non-amyloid basidiospores and the lack of clamp connections in the basidiomata (Yang 1997, 2005). Recently, Lieu *et al.* (2017) reported an annulate species of sect. *Vaginatae* from subtropical China other than those found in tropical Africa and Europe.

Genus *Amanita* comprises nearly 500 described taxa (Kirk *et al.* 2008). About 220 new taxa (new species, new varieties and new forms) in *Amanita* have been reported especially from East Asia, Central and South America, South Africa and Australia over the last two decades (Yang 2011). Zhang *et al.* (2010) recently reported three lethal amanitas in East Asia. Deng *et al.* (2014) and Li and Cai (2014) each described a new *Amanita* species from South China. There are 296 names listed under section *Vaginatae* on <http://www.amanitaceae.org/?section%20Vaginatae> with 97 accepted species from all over the world.

Two taxa in *Amanita*: *A. nana* Sing. and *A. vaginata* (Bull. : Fr.) Lamarck were reported from Pakistan by Ahmad (1956). Later, Ahmad *et al.* (1997) reported fifteen species from Pakistan. Tulloss *et al.* (2001) reported three previously described taxa: *A. flavipes* Imai, *A. orsonii* Kumar & Lakh, and *A. umbrinolutea* (Secr. ex Gillet) Bataille as new for Pakistan. They illustrated five additional taxa believed to be distinct and probably new to science. *Amanita cinnamomescens* Tulloss, S. H. Iqbal, A. N. Khalid & Bhandary, *A. battarae* (Bound.) Bon. and *A. ovoidea* (Bull.)

Link, were also reported from different parts of Pakistan (Tulloss *et al.* 2005, Sultana *et al.* 2011, Razaq & Shahzad 2015). Jabeen *et al.* (2017) added a novel species *A.glarea* S. Jabeen, M. Kiran & Sadiqullah to this list. Most of these reports from Pakistan are based on morphological data (Ahmad *et al.* 1997, Tulloss *et al.* 2001, Razaq & Shahzad 2015, Sultana *et al.* 2011). To date, there are only two published reports about ectomycorrhizal associations of *Amanita* species with host trees from Pakistan (Niazi *et al.* 2009, Kiran *et al.* 2017). Recent phylogenetic studies indicate that there are many species yet to be discovered.

The present study provides information about *Amanita griseofusca*, a new species in section *Vaginatae*. It is reported from moist temperate forests of the Malam Jabba valley, district Swat, Khyber Pakhtunkhwa province, Pakistan. Morpho-anatomical characterization together with analysis of three nuclear ribosomal gene regions contributed to the designation of this taxon as a new species.

Materials and Methods

Study area

District Swat is part of Malakand Division of Khyber Pakhtunkhwa province. It lies between 34° 34' to 35° 55' north latitudes and 72° 08' to 72° 50' east longitudes. The district is mountainous with the highest peak of Falak Sar towering more than 5918 m above sea level (Kiran *et al.* 2017). Based on varying elevation and exposure, the area can be divided into many vegetation and climatic zones that vary from sub-tropical chir pine forests to alpine pastures and meadows. Malam Jabba valley is one of the scenic spot of the Swat district, located in the north of Pakistan between the Himalayan and the Hindu Kush foothills. The valley is mountainous with elevation ranging from 990 m a.s.l. at the valley entrance to 2880 m at the highest peak of Shagar Sar. The valley is dominated by coniferous vegetation with some mixed coniferous vegetation in the foothills.

Morphological studies

Collection of basidiomata, photography and field annotations followed Tulloss & Yang (2011). Basidiomata were dug out using a sharp knife and photographed in their natural habitat. For a meaningful length of pileus striation, pileus diameter was measured along with the pileus surface (Tulloss & Yang 2011). Size of the basidiomata was annotated following Bas (1969).

Color codes followed Munsell Color System (1975) and are presented in parenthesis after common color names. The specimens were dried using a fan heater adjusted at temperature 50–60°C (Tulloss & Yang 2011). Properly dried specimens were deposited to Herbarium University of Swat (SWAT) and Lahore Herbarium, University of the Punjab (LAH).

Microscopic examinations were made under a compound microscope (Olympus, BX40) and visualized on the computer screen using a microscopic camera (Olympus, XC50). Measurements of the microscopic structures are based on calibrated Piximètre software (<http://www.piximetre.fr/>). Tissues were rehydrated using distilled water and 2% aqueous KOH (w/v). For contrast purpose, 1 % Congo red solution was used and Melzer's reagent was used for checking amyloidy of the basidiospores. The abbreviation [n/b/p] indicates 'n' basidiospores measured from 'b' basidiomata from 'p' collections. For basidiospores, L is the average length of the measured basidiospores, Similarly, W is the average width of the measured basidiospores, Q is the range of L/W ratio of all the measured basidiospores, Qe is the average L/W ratio of the all measured basidiospores. Shape of the basidiospores is quoted according to Bas (1969).

Molecular studies

DNA extraction and Polymerase chain reaction (PCR) amplification

DNA was extracted from small portions of lamellae from dried specimens using modified CTAB method (Gardes & Bruns 1993). Universal primer pair ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3', Gardes & Bruns 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3', White *et al.* 1990) was used to amplify the ITS (ITS1-5.8S-ITS2) rDNA region. For amplification of LSU region the primer pair LROR (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-TCCTGAGGGAACTTCG-3') (Vilgalys & Hester 1990) were used. PCR amplification of the translation elongation factor 1-alpha (*tef1*) gene was carried out with the primer pair 983F (5'-GCYCCYGGHCAYCGTGAYTTYAT-3') and 1567R (5'-ACHGTRCCRATACCACCSATCTT-3') using Q5 High fidelity DNA polymerase (New England Biolabs) under the PCR conditions given by Rehner & Buckley (2005). PCR procedure for ITS region had initial 4 min

denaturation at 94°C, 40 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension of 10 min at 72°C. The primer pair LR5 and LR0R (Vilgaly's lab <http://sites.biology.duke.edu/fungi/mycolab/primers.htm>) were used to amplify the rDNA large subunit (LSU). Thermo-cycling profile for LSU region comprised of initial denaturation at 94°C for 2 min, then 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, and final extension at 72°C for 7 min.

Visualization of PCR products were carried out using 1 % agarose gel stained with 3 µl ethidium bromide. Sequencing of the amplified products (ITS and LSU) was done by the Beijing Genomic Institute, Hong Kong. Additionally, some amplified LSU and *tef1* products were also sequenced at Bauer Core, Northwest Laboratory, Harvard University, USA.

Phylogenetic analysis

All the sequences generated in this study were BLAST searched at NCBI (<http://www.ncbi.nlm.nih.gov/>). The representative species of section *Vaginatae*, which are related to the proposed new species, were selected to reconstruct the phylogeny. Two separate data sets for ITS and LSU were made since very little data is available for the *tef1* region. For both ITS and LSU datasets, two species of section *Caesareae*, *A. loosii* Beeli (1936: 90) and *A. yuani* Yang (1994: 464) and three species of section *Amanita* (*A. altipes* Yang, M. Weiss & Oberw (2004: 636), *A. sinensis* Yang (1997: 23), and *A. subfrostiana* Yang (1997:12)) along with species of section *Vaginatae* were used as in-group taxa (Table 1). *Limacella pitereka* Grgur (1997: 417, KX443412) and *L. subillinita* Guzmán (1974: 89; KR919759) was used as outgroup taxa in the ITS dataset and *L. subillinita* (KP313599, KP313601) in the LSU dataset. Downloaded sequences were then aligned using online MUSCLE by EMBL-EBI, Wellcome Trust Genome Campus, UK (<http://www.ebi.ac.uk/Tools/msa/muscle/>) and edited manually with BioEdit v7.0.9 (Hall 1999). Maximum likelihood analyses were performed for individual gene regions via CIPRES Science Gateway (Miller *et al.* 2010) employing RAxML-HPC v.8. Rapid bootstrap analysis/search for best-scoring ML tree (-f a) was configured for each dataset. For the bootstrapping phase, the GTRCAT model was selected. One thousand rapid bootstrap replicates were run. A bootstrap proportion of $\geq 70\%$ was considered significant.

TABLE 1. List of taxa used in phylogenetic analysis with their vouchers and accession numbers.

Taxon	Voucher	ITS	LSU	<i>tef</i>
<i>Amanita</i> sp.H909	H909	GQ925401	GQ925378	
<i>Amanita</i> aff. <i>lignitincta</i>	LE295454	KM658297	KM658306	
<i>Amanita</i> sp. <i>albiceps</i>	RET 521-10	KP224316		
<i>Amanita altipes</i>	HKAS 36609	AY436445	AY436487	
<i>Amanita basiana</i>	RET 308-4	KP258986	KP258987	
<i>Amanita battarrae</i>	LE296458	KM658290	KM658305	
<i>Amanita battarrae</i>	LE296459	KM658289	KM658309	
<i>Amanita beckeri</i>	7826	JF907758		
<i>Amanita ceciliae</i>	HKAS26935	KU139512	KU139439	
<i>Amanita ceciliae</i>	ASIS26247	KU139510	KU139437	
<i>Amanita cistetorum</i>	MC261008	KX834236		
<i>Amanita cistetorum</i>	MC141208	KX834235		
<i>Amanita constricta</i>	BW_Mycoblitz		HQ539684	
<i>Amanita crocea</i>	HKAS 31489	AY436484		
<i>Amanita dryophila</i>	MC130902	KX834237		
<i>Amanita friabilis</i>	RET 395-10	KU248103		
<i>Amanita friabilis</i>	RET 396-1	KU248104	KU248120	
<i>Amanita fulva</i>	ASIS26388	KU139518	KU139448	
<i>Amanita fulva</i>	ASIS26398	KU139519	KU139446	
<i>Amanita glarea</i>	LAH35217	KY817312	KY781175	
<i>Amanita glarea</i>	LAH35044	NR151657	KY788653	
<i>Amanita</i> sp. <i>glenosomma</i>	RET 556-8	KY952145	KY952146	
<i>Amanita griseofusca</i>	SWAT000137	MH241057	MH241058	
<i>Amanita griseofusca</i>	LAH35366	MH241055	MH241056	MH282854
<i>Amanita griseofusca</i>	SWAT000138	MH241059		
<i>Amanita groenlandica</i>	IB20050079	KX834241		
<i>Amanita</i> sp. <i>insinuans</i>	RET 685-8	KY435394	KY435395	
<i>Amanita</i> sp. <i>justicei</i>	RET 444-8	KY614228	KY614224	
<i>Amanita</i> sp. <i>justicei</i>	RET 691-2	KY614229	KY614225	

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TABLE 1. (Continued)

Taxon	Voucher	ITS	LSU	tef
<i>Amanita lippiae</i>	RET 418-2	KP258991	KP258992	
<i>Amanita liquii</i>	HKAS 36611	AY436462	AY436493	
<i>Amanita lividopallescens</i>	MC121009	KX834245		
<i>Amanita lividopallescens</i>	GC93231	KX834242		
<i>Amanita lividopallescens</i> var. <i>globosispora</i>	M0200751	KX834247		
<i>Amanita loosii</i>	PC-0084409	JQ512092	JQ512081	
<i>Amanita madagascariensis</i>	PC-0084420	JQ512096		
<i>Amanita madagascariensis</i>	PC-0084421		JQ512087	
<i>Amanita malleata</i>	GC02017	KX834249		
<i>Amanita mairei</i>	MC271104	KX834248		
<i>Amanita mortenii</i>	RET 294-8	KT317713	KT317710	
<i>Amanita oblongospora</i>	15831	JF907762		
<i>Amanita olivaceogrisea</i>	RET 523-2	KU867878	KU867877	
<i>Amanita orientifulva</i>	HKAS 32522	NR137544	AY436496	
<i>Amanita orientifulva</i>	KA12-0642	KF017940	KF021679	
<i>Amanita populiphila</i>	RET 266-9	KP224323	KP224346	
<i>Amanita populiphila</i>	RET 423-2	KP224322	KP224345	
<i>Amanita populiphila</i>	RET 035-8		KP221314	
<i>Amanita populiphila</i>	RET 068-7		KP221315	
<i>Amanita protecta</i>	RET 277-5	KP224326		
<i>Amanita pseudovaginata</i>	HKAS38323	AY436470		
<i>Amanita pseudovaginata</i>	HKAS29524	KM658285	AF024472	
<i>Amanita</i> sp. <i>rhacopus</i>	RET 627-2	KY655762	KY655753	
<i>Amanita</i> sp. <i>rhacopus</i>	RET 627-10	KY655764	KY655755	
<i>Amanita</i> sp. <i>semiobruta</i>	RET 691-1	KY435402	KY435401	
<i>Amanita separata</i>	MC071002	KX834251		
<i>Amanita sinensis</i>	HKAS 25761	NR119389	AF024474	
<i>Amanita simulans</i>	PA151006	KX834261		
<i>Amanita simulans</i>	GC99066	KX834252		
<i>Amanita simulans</i>	GK200910	KX834257		
<i>Amanita simulans</i>	AM161112	KX834255		
<i>Amanita simulans</i>	JMM101012	KX834258		
<i>Amanita simulans</i>	FB031211	KX834256		
<i>Amanita simulans</i>	LIP_PAM13112104	KX834253		
<i>Amanita</i> sp. AW03	RET 573-1		KX270344	
<i>Amanita</i> sp. <i>kryorhodon</i>	RET 691-9		KY435398	
<i>Amanita</i> sp. <i>luzernensis</i>	RET 556-3		KY799113	
<i>Amanita</i> sp. N66	RET 704-6		KY799115	
<i>Amanita</i> sp. <i>pahasapaensi</i>	RET 566-7	KP313577		
<i>Amanita</i> sp. <i>penetratrix</i>	RET 492-9	KU186827	KU186831	
<i>Amanita</i> sp. <i>penetratrix</i>	RET 704-1	KU186828	KU186832	
<i>Amanita</i> sp.-Arora_01-555	RET 345-3		KU186808	
<i>Amanita subfrostiana</i>	HKAS 57042	JN943173	JN941162	
<i>Amanita submembranacea</i>	LE215542	KM658288		
<i>Amanita</i> sp. <i>subnigra</i>	RET 522-2	KP258996		
<i>Amanita supravolvata</i>	RET 103-7	KP258995		
<i>Amanita</i> sp. <i>texasorora</i>	RET 703-4	KY435408	KY435409	
<i>Amanita</i> sp. <i>texasorora</i>	RET 622-8	KY952157	KY952156	
<i>Amanita</i> sp. 'trygonion'	RET 622-5	KU186809	KU186810	
<i>Amanita vaginata</i>	KA12-0962	KF017950	KF021689	
<i>Amanita vaginata</i>	KA12-1190	KF017949	KF021688	
<i>Amanita velosa</i>	APNo.1	EU909453		
<i>Amanita yuaniana</i>	HKAS 29516	AY436479	AF024488	
<i>Limacella pitereka</i>	RET 496-5	KX443412		
<i>Limacella subbillinata</i>	RET 159-2		KP313601	
<i>Limacella subbillinata</i>	RET 158-6		KP313599	
<i>Limacella subbillinata</i>	NY 0027729	KR919759		

Results

Molecular characterization

The ITS sequences obtained from Pakistani collections (MJ1558, MJ1630 and MJ1664) were of 610, 590 and 580bp in length, respectively. The entire query ITS sequences were subjected to BLAST analysis. They showed 97% similarity matches with *Amanita beckeri* Huijsman (1962: 349–350, JF907758) and *A. oblongospora* Contu ex Tulloss & Contu (1994: 358, JF907762), followed by *Amanita crocea* (Quél.) Singer (1951: 386, JQ912665) with 95 % identity. Similarly, the LSU region of MJ1558 and MJ1630 upon amplification resulted in 955 and 960bp sequences, respectively. Both the query sequences showed 98% similarity with several species of section *Vaginatae* from USA including *Amanita* sp. 'sp-N66' from (KY799115), *A. populiphila* Tulloss & E. Moses (1995:455; KP221315), *Amanita* sp. 'kryorhodon' (KY435398), *Amanita* sp. 'luzernensis' (KY799113), and *Amanita* aff. *vaginata* (KM658313).

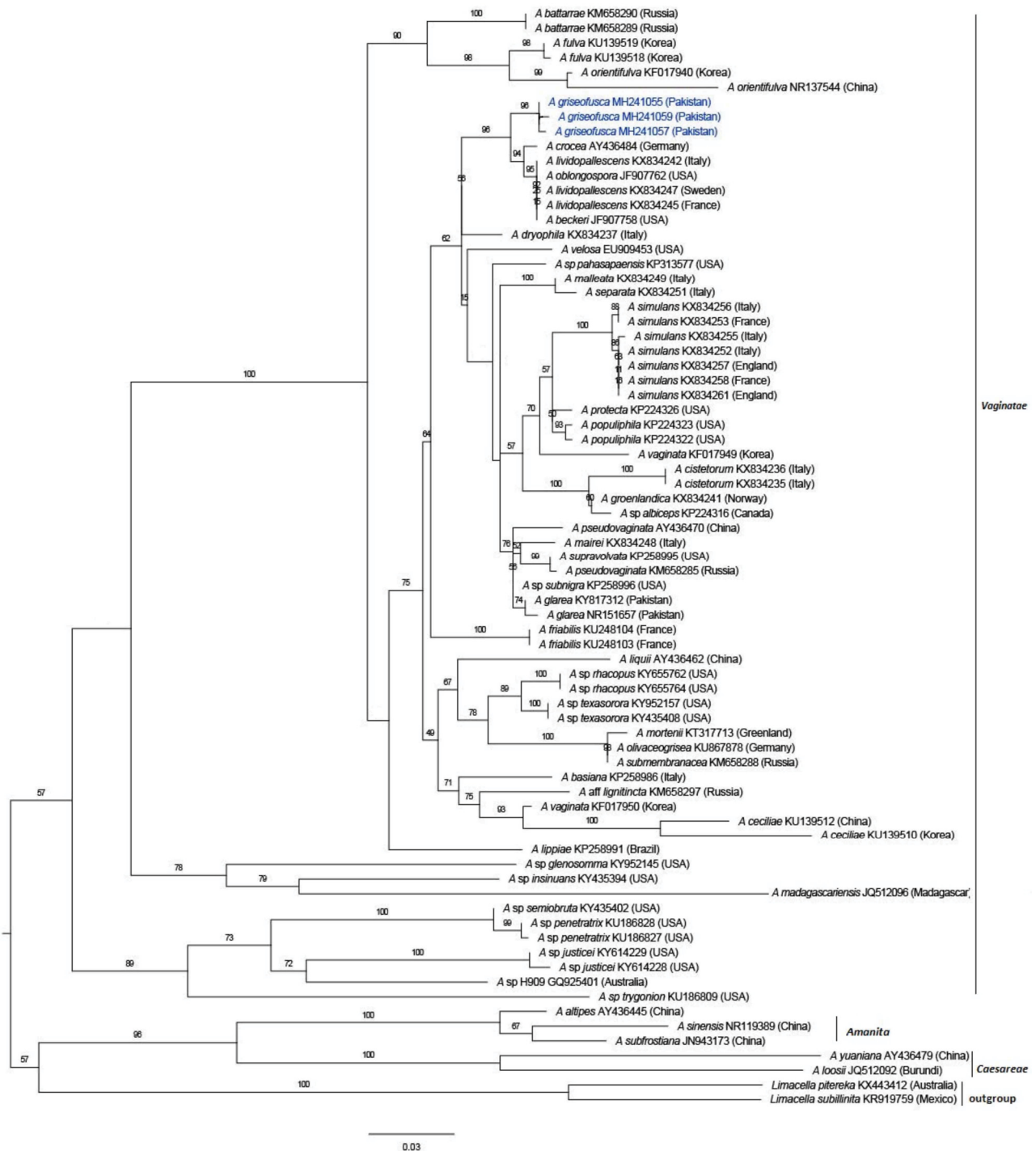


FIGURE 1. Maximum-Likelihood phylogenetic tree generated from the ITS dataset. ML bootstrap values are shown above the nodes. *Amanita griseofusca* (Pakistani collections) is represented in blue.

The *tefl* region yielded a 633bp consensus sequence which showed 88% similarity with species of *Amanita* section *Vaginatae* from Colombia including *A. colombiana* Tulloss, Ovrebo & Halling (1992: 8; KT008012) and *A. fuligineodisca* Tulloss, Ovrebo & Halling (1992:10, KT008011). Due to low number of matching sequences availability, the generated *tefl* sequence was not used in the phylogenetic re-construction, however, this was submitted to GenBank under accession number (MH282854) for future reference (Table 1).

The final ITS and LSU dataset consisted of 59 and 53 nucleotide sequences, respectively. An independent phylogenetic tree for each gene was constructed. In the ITS based phylogram (Fig. 1), three Pakistani sequences of putative new species (*A. griseofusca* sp.nov.) formed a sister clade with *A. beckeri*, *A. crocea*, and *A. oblongospora*. In the LSU based phylogram (Fig. 2), the two sequences of *A. griseofusca* sp.nov. formed a clade of its own further supporting its unique position.

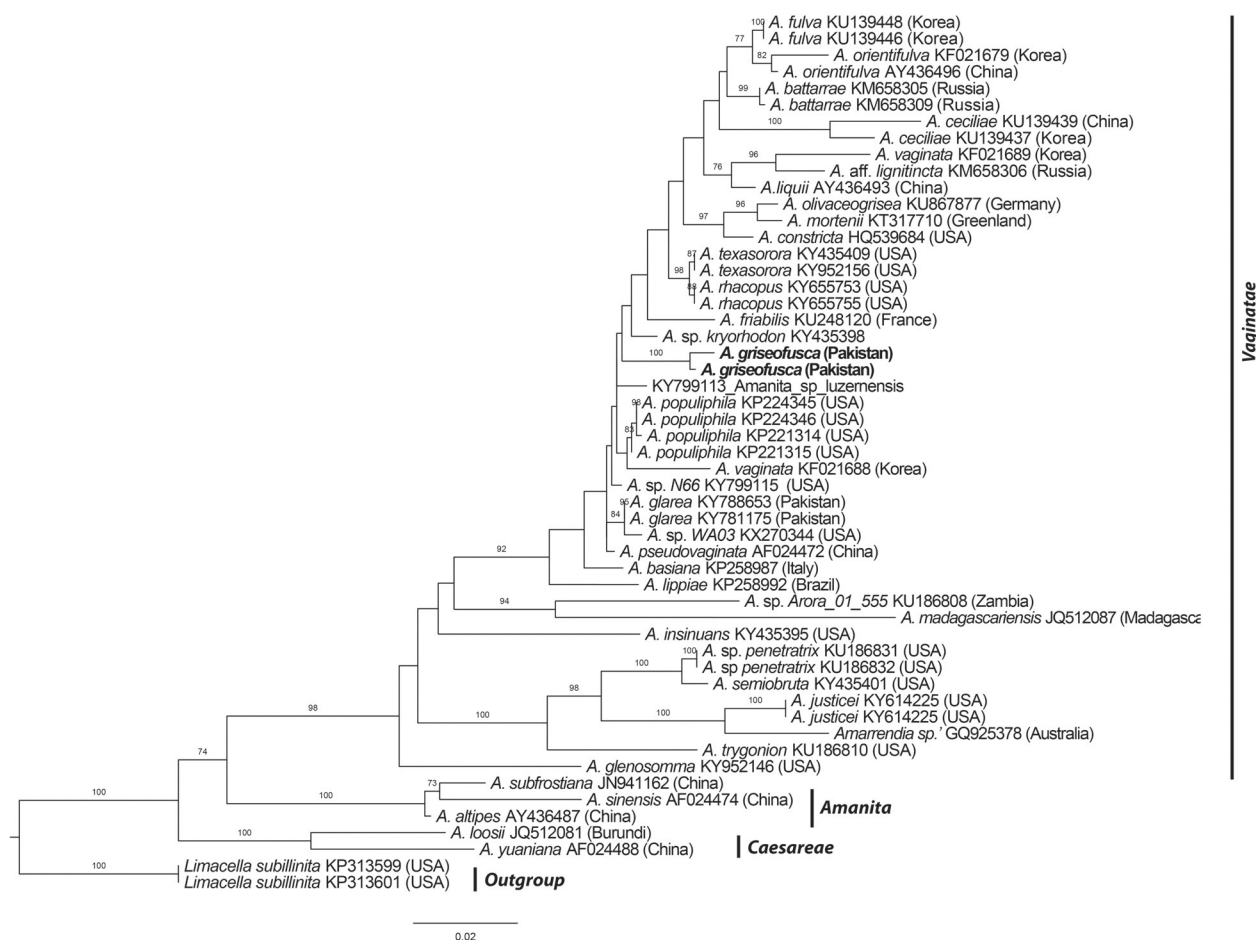


FIGURE 2. Maximum-Likelihood phylogenetic tree inferred from the LSU dataset. ML bootstrap values $\geq 70\%$ are shown above the nodes. *Amanita griseofusca* is represented in bold.

Taxonomy

Amanita griseofusca J. Khan & M. Kiran *sp.nov.* (FIGURES. 3 & 4)

Mycobank no: MB 825012

Etymology:—the epithet “*griseofusca*” Latin refers to greyish brown pileus color characterizing the present taxon

Holotype:—PAKISTAN. Khyber Pakhtunkhwa, Swat District, Malam Jabba valley, 1950 m a.s.l., under *Quercus dilatata* Royle in mixed coniferous forest, 16 August 2015, *Junaid Khan MJ1558* (Holotype *SWAT000137!*, Genbank no. MH241057 for ITS & MH241058 for LSU).

Diagnosis:—greyish brown pileus with white to beige, membranous volval remnants present as one (large) to a few (small) warts, striations one third of the total pileus radius, close, cream lamellae with a pink tone, fibrillose stipe, with a white loose and saccate volva turning beige at maturity and broadly ellipsoidal to ellipsoidal inamyloid basidiospores.



FIGURE 3: A–D: Morphology of *Amanita griseofusca*. A: young stage fruiting body of MJ1558 (SWAT000137!). B: Basidiomata of MJ1558 in natural habitat. C: View of the lamellae and stipe. D: Basidiomata of MJ1630 (LAH35366!). Scale bars: A = 8 cm; B = 16.5 cm; C = 10 cm; D = 20cm. Photographs by Junaid Khan.

Description:—*Basidiomata* (Fig. 3) medium-sized. *Pileus* 50–80 mm diam., hemispherical to elliptical when young, mostly flat or broadly convex with a depressed center at maturity, greyish brown (10YR 5/2) with a darker (10YR 3/2) disc, striated to almost one third from the margin, smooth at the center, volval remnants present as one (large) to a few (small) warts, warts membranous, white at first, turning greyish (2.5Y 7/4), context creamy white, fragile, thicker at the center (2–3 mm). *Lamellae* free, close, pruinose, especially in young specimens, broader (5–7 mm) at the center, lamellar edge even, creamy (7.5Y 9/4) at first, with a pinkish tone (2.5Y 9/4) at maturity, fragile, lamellulae rare, short, mostly in a single tier, rarely in three tiers, of varying lengths. *Stipe* 80–100 × 8–12 mm, slightly thickening downward, basal bulb absent, central, creamy white (10Y 9/4) to pale yellowish tan (2.5Y 9/4), fibrillose especially at the base, fibrils whitish to greyish white in young specimens, browning (10YR 5/6) with age, *Annulus* absent. *Volva* saccate, non-coherent in the upper part, white at first, turning beige (10Y 9/4) upon maturity, reduced in mature specimens. *Odour* not observed.

Lamellar trama bilateral, mediostatum 30–50 µm wide composed of ellipsoid to sub-fusiform inflated cells (25–50 × 10–15 µm) with abundant interwoven filamentous hyphae, 5–7 µm wide, vascular hyphae rare, 3–7 µm in diam. Lateral stratum composed of ellipsoid to sub-fusiform hyphae (25–40 × 10–15 µm) with abundant interwoven filamentous hyphae which are 3–7 µm wide and often branching, vascular hyphae rare, 3–7 µm. *Sub-hymenium* 2–3 layers, composed of subglobose to ovoid or rarely short ellipsoidal cells, 20–30 × 10–20 µm, inflated cells rare, 3–7 µm. *Basidia* 40–45 × 10–15 µm, club-shaped, 2–4 spored, basal clamp connections absent. *Basidiospores* [n/b/p] [60/3/3] (9.6–) 10–12 (–12.5) × (8–) 8.5–10 (–10.5) µm, L × W = 11 × 9 µm, Q = (1.1–) 1.2–1.3 (–1.5), Q_e = 1.22, broadly ellipsoid to ellipsoid, inamyloid. *Lamellar edge* sterile, 200–250 µm wide, composed of subglobose to ovoid cells (20–50 × 15–30 µm), filamentous hyphae abundant, 3–7 µm in diam., irregularly arranged, hyaline or rarely with a brownish pigment. *Pileipellis* 90–130 µm thick, upper layer 30–50 µm thick, gelatinized, composed of moderately

compact hyaline cells, 2–5 μm in diam., lower layer 60–80 μm thick, composed of radial, compact, filamentous hyphae 3–5 μm wide, hyaline or with a brownish pigment in KOH, vascular hyphae rare 2–5 μm wide. *Stipe trama* composed of longitudinally arranged filamentous hyphae, 5–10 μm wide, hyaline in KOH, terminal cells clavate to broadly clavate 100–300 \times 15–30 μm . *Volval remnants on the pileus* composed of filamentous hyphae 3–5 μm wide, frequently branching, interwoven, thin-walled, hyaline, scattered inflated cells, variable, subglobose to globose, rarely ellipsoid to ovoid, 25–30 \times 18–25 μm wide, mostly single, terminal, thin-walled, hyaline in KOH. *Volva at stipe base* two-layered, outer volval layer: filamentous hyphae (4–7 μm diameter) abundant to very abundant, hyaline, thin walled, compact, scattered, infrequent, globose to ellipsoidal inflated cells; inner volval layer: inflated cells abundant to fairly abundant, frequent, globose to subglobose to ovoid (45–50 \times 55–60 μm), usually single and terminal. *Clamp connections* absent in all tissues.

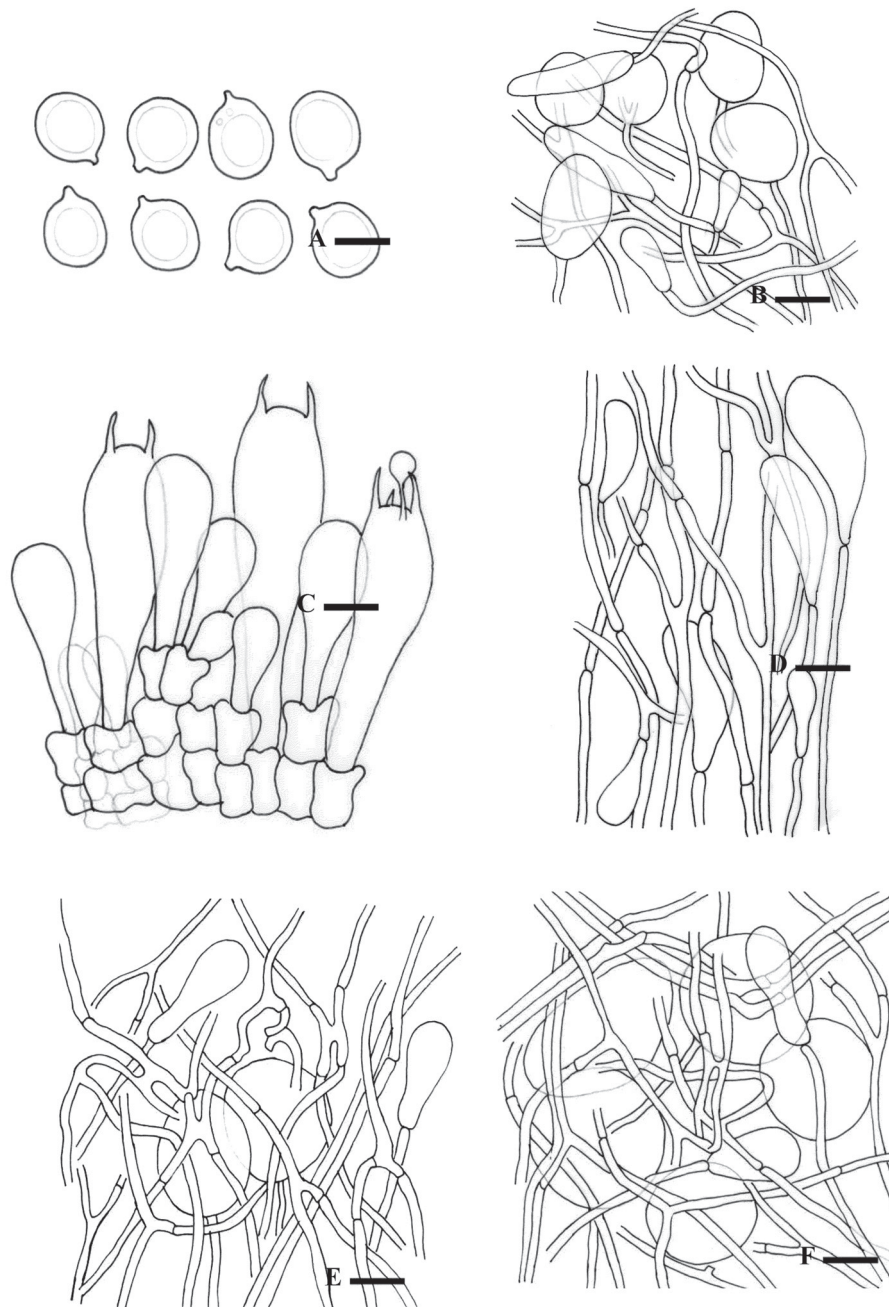


FIGURE 4. *Amanita griseofusca* (SWAT000137!). A. basidiospores; B. volval remnants from pileus surface; C. basidia and subhymenium; D. stipitipellis. E. outer layer of volval elements at stipe base F. inner layer of volval elements at stipe base. Scale bars: A = 8.7 μm ; B = 15 μm ; C = 8.5 μm ; D = 20 μm ; E = 20 μm . Drawings by Munazza Kiran.

Habit and Habitat:—solitary on soil under *Quercus dilatata* Royle.

Distribution:—currently only collected from Kishawra village, Malam Jabba Valley, Swat district, Khyber Pakhtunkhwa province, Pakistan.

Additional materials examined:—PAKISTAN. Khyber Pakhtunkhwa, Swat District, Malam Jabba valley, 1950 m a.s.l., under *Quercus dilatata* in mixed coniferous forest, 10 August 2016, *Junaid Khan* MJ1630 (*LAH35366!*, Genbank no. MH241055 for ITS & MH241056 for LSU) 31 August 2016, *Junaid Khan* MJ1664 (*SWAT000138!*, Genbank no. MH241059 for ITS).

Discussion

Taxa belonging to section *Vaginatae* are more or less difficult to distinguish on the basis of morphology. Moreover, there is a lack of ample reliable molecular data of this section in Genbank (Liu *et al.* 2017). *Amanita griseofusca* is characterized here by greyish brown pileus with a darker disc, one third striation on the cap margin, close lamellae which are cream colored with a pinkish tone, and loose saccate volva. Anatomically, it has broadly ellipsoidal to ellipsoidal basidiospores.

Phylogenetic analysis based on ITS data set, places the new species in a sister clade to *A. beckeri*, *A. crocea* and *A. oblongospora* (Fig. 1) besides *A. lividopallescens* puzzle (Vizzini *et al.* 2016). However, *A. griseofusca* is significantly different from all the three said species based on macro-and micromorphological characters other than molecular analysis. Greyish brown pileus of *A. griseofusca* is comparable to pearl grey pileus of *A. oblongospora*, a European species. However, striations on the pileus margin of *A. griseofusca* occupy one third of the pileus radius whereas in *A. oblongospora* one fifth of the pileus radius is striated. It also has a prominent and broad umbo which is lacking in *A. griseofusca*. *A. oblongospora* also has larger basidiospores ($10.5\text{--}13.8 \times 7.8\text{--}10.8 \mu\text{m}$). Moreover, clamp connections are commonly present at the bases of basidia in *A. oblongospora* (Tulloss 1994) but are absent in *A. griseofusca*.

A. griseofusca resembles *A. mairei* Foley (1949:117) morphologically as both have greyish tints on pileus surface, saccate volva and ellipsoid to broadly ellipsoid inamyloid basidiospores. However, *A. mairei* develops olivaceous or violaceous tint at maturity and is never a pure gray pileus surface whereas *A. griseofusca* has greyish brown pileus with one third striations. Further, clamps may occasionally be found at bases of *A. mairei* basidia which are absent from all tissues of *A. griseofusca*. The greyish brown cap of *A. pseudovaginata* Hongo (1983: 39) is slightly depressed at the centre like *A. griseofusca* but its gills are white or sometimes grey but never develop a pink tint like *A. griseofusca*. Moreover, the stipe of *A. griseofusca* has distinct fibrillose surface unlike *A. pseudovaginata* which has a nearly smooth stipe surface. The upper half of the volva becomes grey in *A. pseudovaginata* whereas it develops a beige color in *A. griseofusca* at maturity. *A. pseudovaginata* has subglobose to broadly ellipsoid basidiospores in contrast to ellipsoid to broadly ellipsoid spores of *A. griseofusca*. *Amanita beckeri* can be easily distinguished from *A. griseofusca* on the basis of its golden walnut color and smaller, subglobose to globose basidiospores measuring $9.8\text{--}11.8 \times 9.0\text{--}11.0 \mu\text{m}$ (Huijsman 1962a, b, Tulloss 1994) while in *A. griseofusca* pileus color is greyish brown and has ellipsoidal to broadly ellipsoidal basidiospores. *Amanita crocea* has a saffron orange cap with a central umbo as compared to greyish brown pileus with a darker disc characterizing *A. griseofusca*. Pileus of *A. crocea* is without any volval patches whereas *A. griseofusca* has numerous volval remnants on pileus. Shape of basidiospores is subglobose to globose (Singer 1949) in *A. crocea* in contrast to ellipsoidal to broadly ellipsoidal basidiospores of *A. griseofusca*.

Amanita malleata (Piane ex Bon) Contu (1986: 43) resembles morphologically with *A. griseofusca* in its greyish pileus color but the cap of *A. malleata* becomes white with age. Both of these have creamy white lamellae with a pinkish tone upon drying. However, it differs from *A. griseofusca* in shape of the pileus which is hemispherical to elliptical when young and flat at maturity, while in *A. malleata* it becomes more or less conic or campanulate. *A. griseofusca* has ellipsoidal to broadly ellipsoidal basidiospores compared with subglobose to broadly ellipsoidal basidiospores of *A. malleata* (Contu 1986, Tulloss 1994). *A. separata* Contu (1999: 162) has an umbonate bistre to dark brownish pileus in contrast to flat or broadly convex greyish brown pileus of *A. griseofusca* which has a depressed center at maturity. Further, sphaerocysts are abundant in the volva of *A. griseofusca* while they are very rare in the universal veil of *A. separata* (Venturella *et al.* 2006). White gills of *A. cistetorum* Contu & Pacioni (1998:438) sometimes develop pink ochraceous tint, often turning to ash grey with age unlike lamellae of *A. griseofusca* which develop a pink tint at maturity. Presence of fibrils on stipe and saccate volva also make *A. griseofusca* different from *A. cistetorum* where the stipe is densely covered by creamy powder and the volva is submembranous and fragile. *A. simulans* Contu (1999:11) is another species which resembles *A. griseofusca* morphologically but mainly differs from it by the presence of

large obtuse umbo, black spotted lamellae, fulvous orange spotted volva and globose to subglobose basidiospores (Vizzini *et al.* 2016). Our phylogenetic analyses based on LSU data set also indicated that *A. griseofusca* is a distinct species without any close phylogenetic relationships with already known taxa. Thus, morphological and molecular data evidently supports *Amanita griseofusca* as a new taxon.

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References

- Ahmad, S. (1956) *Fungi of West Pakistan*. Biol. Soc. Pak., Government College, Lahore, 126 pp.
- Ahmad, S., Iqbal, S.H. & Khalid, AN. (1997) *Fungi of Pakistan*. Sultan Ahmad Mycological Society of Pakistan.
- Bas, C. (1969) Morphology and subdivision of *Amanita* and a monograph of its section *Lepidella*. *Persoonia* 5: 285–579.
- Beeli, M. (1936) Contribution to the study of mycological flora of Congo XI. Fungi Goossensiani XII. *Bulletin du Jardin Botanique de l'État à Bruxelles* 14: 83–91.
<https://doi.org/10.2307/3666668>
- Contu, M. (1986) Notes on genus *Amanita*. *Bollettino dell'Associazione Micologica ed Ecologica Romana* 6 (7): 43–50.
- Contu, M. (1994) *Amanita oblongospora*. Contu ex Tullio & Contu. *Mycotaxon* 52 (1): 358.
- Contu, M. (1987) Notas sobre el género *Amanita*. IV. Una nueva especie Mediterránea de la sección *Ovigerae* Singer: *Amanita oblongospora* sp. nov. *Boletín de la Sociedad Micológica de Madrid* 12: 89–92.
- Contu, M. (1999) Appunti sul genere *Amanita*-IX. nuove specie e studi tassonomico-nomenclaturali nella sezione *Vaginatae*. *Bollettino dell'Associazione Micologica ed Ecologica Romana* 15 (46): 3–22.
- Contu, M. & Pacioni, G. (1998) *Amanita cistetorum* and *Psathyrella liciosae*, two new Mediterranean species. *Mycotaxon* 69: 437–446.
- Deng, W.Q., Li, T.H. Li, P. & Yang, Z.L. (2014) A new species of *Amanita* section *Lepidella* from South China. *Mycological Progress* 13: 211–217.
<https://doi.org/10.1007/s11557-013-0906-6>
- Foley, H. (1949) Une amanite nord-africaine nouvelle *Amanita mairei* Foley, n. sp. *Mémoires de la Société d'Histoire Naturelle d'Afrique du Nord*. 2: 117–120.
- Gardes, M. & Bruns, T.D. (1993) ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae rusts. *Molecular Ecology* 2: 113–118.
<https://doi.org/10.1111/j.1365-294X.1993.tb00005.x>
- Grgurinovic, C.A. (1997) *Larger Fungi of South Australia*. Botanic Gardens of Adelaide and State Herbarium, Australia, pp. 1–725.
- Guzmán, G. & Johnson, P. (1974) Registration and new species of fungi from Palenque, Chiapas. *Boletín de la Sociedad Mexicana de Micología* 8: 77–105.
- Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.
<https://doi.org/10.1073/pnas.1117018109>
- Hongo, T. (1983) *Notulae mycologicae* (18). *Memoirs of Shiga University*. 33: 37–41.
- Huijsman, H.S.C. (1962a) [*1961*] *Amanita beckeri* nov. sp. *Bulletin de la Société Mycologique de France* 77: 349–350.
- Huijsman, H.S.C. (1962b) *Amanita beckeri* nov. sp. (Diagnose latine). *Bulletin de la Société Mycologique de France* 78: 217.
- Jabeen, S., Kiran, M., Ullah, S., Wilson, A.W., Mueller, G.M., Fiaz, M. & Khalid, A.N. (2017) *Amanita glareae*, a new species in section *Vaginatae* from Pakistan. *Phytotaxa* 306 (2): 135–145.
<https://doi.org/10.11646/phytotaxa.306.2.3>
- Kiran, M., Khan, J., Naseer, A., Sher, H. & Khalid, A.N. (2017) *Amanita pallidorosea* in Pakistan and its ectomycorrhizal association with *Quercus oblongata*. *Mycotaxon* 132: 799–81.
<https://doi.org/10.5248/132.799>

- Kirk, P.M., Cannon, P.F., Minter, D.W. & Stalpers, J.A. (2008) *Ainsworth Bisby's Dictionary of Fungi*. 10th ed. CABI, Wallingford, UK.
- Li, F. & Cai, Q. (2014) *Amanita heishidingensis*, a new species of *Amanita* sect. *Lepidella* from China. *Mycological Progress* 13: 1191–1197.
<https://doi.org/10.1007/s11557-014-1008-9>
- Liu, J.W., Cai, Q., Cui, Y.Y. & Yang, Z.L. (2017) *Amanita cingulata*, a new annulate species of *Amanita* sect. *Vaginatae* from subtropical China. *Phytotaxa* 326 (1): 41–53.
<https://doi.org/10.11646/phytotaxa.326.1.3>
- Miller, M.A., Pfeiffer, W. & Schwartz, T. (2010) *Creating the CIPRES Science Gateway for inference of large phylogenetic trees*. In: *Proceedings of the Gateway computing environments workshop (GCE)*. New Orleans, Louisiana, pp. 1–8.
<https://doi.org/10.1109/GCE.2010.5676129>
- Munsell, A.H. (1975) Soil color charts. Munsell™. Baltimore.
- Niazi A.R., Iqbal, S.H. & Khalid, A.N. (2009) Ectomycorrhizae between *Amanita rubescens* Himalayan Spruce (*Picea smithiana*) from Pakistan. *Mycotaxon* 107: 73–80.
<https://doi.org/10.5248/107.73>
- Persoon, C.H. (1794) Neuer Versuch einer systematischen Eintheilung der Schwämme. *Neues Magazin für die Botanik* 1: 63–80.
- Razaq, A. & Shahzad, S. (2015) New record of Amanitaceae and Strophariaceae from Pakistan. *Pakistan Journal of Phytopathology* 27 (2): 127–129.
- Rehner, S.A. & Buckley, E.P. (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF1 sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* 97: 84–98.
<https://doi.org/10.3852/mycologia.97.1.84>
- Singer, R. (1951) [1949] The Agaricales in modern taxonomy. *Lilloa* 22: 1–832.
- Sultana, K., Rauf, C.A., Riaz, A., Naz, F., Irshad, G. & Haque, M.I. (2011) Check list of agarics of Kaghan valley-1. *Pakistan journal of Botany* 43: 1777–1787.
- Tulloss, R.E. (1994) Type studies in *Amanita* section *Vaginatae* I: some taxa described in this century (studied 1–23) with notes on description of basidiospores and refractive hyphae in *Amanita*. *Mycotaxon* 52: 305–396.
- Tulloss, R.E., Iqbal, S.H., Khalid, A.N., Bhatt, R.P. & Bhatt, V.K. (2001) Studies in *Amanita* (*Amanitaceae*) from southern Asia. I. Some species of Pakistan's Northwest Frontier Province. *Mycotaxon* 77: 455–490.
- Tulloss, R.E., Iqbal, S.H. & Khalid, A.N. (2005) Studies in *Amanita* (*Amanitaceae*) from southern Asia II. *Amanita cinnamomescens*-new species of Pakistan's North West Frontier Province. *Mycotaxon* 94: 1001–1005.
- Tulloss, R.E. & Moses, E. (1995) *Amanita populiphila*-a new species from the Central United States. *Mycotaxon* 53: 455–466.
- Tulloss, R.E., Ovrebø, C.L. & Halling, R.E. (1992) Studies on *Amanita* (*Amanitaceae*) from Andean Colombia. *Memoirs of the New York Botanical Garden* 66: 1–46.
- Tulloss, R.E. & Yang, Z.L. (2011) Morphological study of *Amanita* (Fungi: Agaricales)—notes on methodology. *Amanita Workshop*, 6th ed.-R. E. Tulloss and C. Rodríguez Caycedo.
- Venturella, G., Saitta, A. & Contu, M. (2006) A key for the identification of *Amanita* species, sect. *Vaginatae*, subsect. *Ovigerae* (*Basidiomycota*, *Amanitaceae*) from Sicily. *Naturalista siciliano, Serie IV* 30 (3–4): 419–430.
- Vilgalys, R. & Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* 172 (8): 4238–4246.
<https://doi.org/10.1128/jb.172.8.4238-4246.1990>
- Vizzini, A., Mircazotti, Traversso, M., Ercole, E., Moreau, P.A., Consiglio, G., Cullington, P., Ardron, P., Moingeon, J.M. & Peintner, U. (2016) Variability, host range, delimitation and neotypification of *Amanita simulans* (*Amanita* section *Vaginatae*): collections associated with *Helianthemum* grasslands, and etypification of *A. lividopallescens*. *Phytotaxa* 280 (1): 1–22.
<https://doi.org/10.11646/phytotaxa.280.1.1>
- White, T.J., Bruns, T.D. & Taylor, L.J. (1990) Amplification direct sequencing of fungal ribosomal RNA genes for Phylogenetics. In: Innis, M.A., Gelf, D.H., Sninsky, J.J. & White, T.J. (Eds.) *PCR protocols: A guide to methods applications*. Academic Press, New York, pp. 315–322.
<https://doi.org/10.1016/b978-0-12-372180-8.50042-1>
- Yang, Z.L. (1994) Studies of the genus *Amanita* from Southwestem China (I). *Mycotaxon* 51: 459–470.
- Yang, Z.L. (1997) Die *Amanita*-Arten von Südwestchina. *Bibliotheca Mycologica* 170: 1–240.
- Yang, Z.L. (2005) *Amanitaceae. Flora Fungorum Sinicorum* 27. Science Press, Beijing, China, pp. 258.
- Yang Z.L. (2011) Molecular techniques revolutionize knowledge of basidiomycete evolution. *Fungal Diversity* 50: 47–58.
<https://doi.org/10.1007/s13225-011-0121-1>

- Yang, Z.L. & Oberwinkler, F. (1999) The fruit body development of *Amanita muscaria* (Basidiomycetes). *Nova Hedwigia* 68: 441–468.
- Yang, Z.L., Weiß, M. & Oberwinkler, F. (2004) New species of *Amanita* from the eastern Himalaya and adjacent regions. *Mycologia* 96 (3): 636–646.
<https://doi.org/10.2307/3762180>
- Zhang, P., Chen, Z.H., Xiao, B., Tolgor, B., Bao, H.Y. & Yang, Z.L. (2010) Lethal amanitas of East Asia characterized by morphological and molecular data. *Fungal Diversity* 42 (1): 119–133.
<https://doi.org/10.1007/s13225-010-0018-4>