

Morphology and molecular characterization of three species of *Amanita* from north-western Himalaya of Jammu and Kashmir

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(Submitted on November 21, 2021; Accepted on January 09, 2022)

ABSTRACT

Three species of genus *Amanita* namely; *Amanita pseudorufobrunnescens*, *A. fritillaria* and *A. spissacea*, are identified from Jammu and Kashmir, India. Morphological details, illustrations and phylogenetic observations based on nrLSU data are given here.

Keywords: Morpho-taxonomy, Molecular phylogeny, Jammu and Kashmir, India

INTRODUCTION

Mushrooms in the family *Amanitaceae* are characterized by agaricoid or secotioid fleshy basidiomes with longitudinally acrophysalidic stipe tissue and bilateral, divergent lamellar trama (Bas 1969; Tulloss *et al.*, 2016). To date, a total of 614 species of *Amanita* are validly published all over the world and about 60 taxa are reported from India (Kumar *et al.*, 2021a). The genus is divided into three subgenera and eleven sections based on multi-locus analysis and morphological characters (Yang *et al.*, 2018). The subgenus *Amanita* Pers., includes *A. sect. Amanita*, *sect. Amarrendiae*, *sect. Caesareae* and *sect. Vaginatae* and the subgenus *Amanitina* (E.J. Gilbert) E.J. Gilbert includes *A. sect. Amidella*, *sect. Arenariae*, *sect. Phalloideae*, *sect. Roanokenses*, *sect. Strobiliformes*, and *sect. Validae*, while the subgenus *Lepidella* (E.J. Gilbert) Vesely includes *A. sect. Lepidella*. Presently, the main focus is to collect additional data on genus *Amanita* from this region of the country as many misidentified and doubtful taxa that may exist in the literature were identified and excluded (Kumar *et al.*, 2021).

Union Territory of Jammu and Kashmir (J&K) is stretched between 32° 17' N to 37° 05' N latitude and 72° 31' E to 80° 20' E longitude and cover a total area of 42, 241km², with an average rainfall of 103 cm. The vast geographical area abundant in forests, varied topography and various climatic regimes are conducive to the growth of a large variety of mushrooms, and there is an equal opportunity to explore the mushroom flora of various climatic and ecological regimes within this region of the country. During the course of macro-fungal forays in different parts of J&K, India, the first author collected several specimens of *Amanita* from coniferous and broadleaf forests. Morphological examination and molecular evidences indicated that these species are characterized for the first time from J&K.

MATERIALS AND METHODS

Morphological observations

Macro-morphological characteristics were documented in the forest or base camp from fresh and dissected young to mature basidiomata. Specimens were annotated and photographed in the field. Colour codes follow Kornerup and Wanscher (1978). The collected material was dried with a field drier. Herbarium codes follow Index Herbariorum Thiers (2018). Micromorphological features were carried out on dried samples. Microscopic analysis of the tissues were made from

free hand-cut sections of dried material. Sections were mounted in a mixture of 5% KOH, 1% Phloxine, and 1% Congo red and then observed under a microscope. Amyloidity was checked in Melzer's reagent. Biometric variables follow (Yang 1997) and Cui *et al.* (2018). Drawings of microscopic elements were made with a Camera lucida attached to a compound microscope (Olympus CH20i) at 1000× magnification. Microphotographs of the various elements were taken using a dedicated digital camera attached to an Olympus Ch20i compound microscope.

DNA isolation, amplification and sequencing

Genomic DNA was isolated from dry 100 mg of the material using NucleoSpin Plant II Kit (Macherey-Nagel) (RGCB, RFDF, and Thiruvananthapuram). For LSU amplification, partial sequence of the ribosomal large subunit of RNA (nrLSU) using universal primer pairs LR0R and LR5 primers were used (Vilgalys and Hester, 1990). PCR amplification was conducted on a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) programmed for 2 min at 96°C, followed by 30 cycles of 30 sec at 96°C, 40 seconds at 50°C and a final stage of 4 min at 60°C. The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany). Both strands of the PCR fragments were sequenced in ABI 3500 DNA Analyzer (Applied Biosystems) using the amplifying primers.

Phylogenetic analysis

Phylogenetic analyses based on nrLSU sequences data were carried out to establish the phylogenetic placement of our species. Sequences of *Amanita* were selected based on BLAST search results (Altschul *et al.*, 1997). Multiple sequence alignment was performed using MAFFT v.7 (Kato *et al.*, 2013) with minimal editing in BioEdit v.7.2.5 (Hall 1999). Phylogenetic analysis was undertaken based on maximum likelihood (ML) in MEGA 6.0. (Tamura *et al.*, 2013). One Thousand bootstrap replicates were analyzed to obtain nodal support values. Three nrLSU sequences were generated for this study and deposited to the GenBank to procure the accession numbers (OM562275, OM728179 and OM722135).

RESULTS

Phylogeny

Most of the identified *Amanita* species formed a group with

strong support in the nrLSU trees (Figs. 1, 4, 7). The isolated sequence (GenBank OM728179), *Amanita pseudorufobrunnescens* was closely clustered with reference sequence of *A. pseudorufobrunnescens* (GenBank KM116903) from Uttarakhand in nrLSU tree. *Amanita fritillaria*, (GenBank OM728179) was closely grouped with two sequences of *A. fritillaria* (GenBank MH486548, MH486549) from China in nrLSU phylogenetic trees and the isolated sequence of *Amanita spissacea* (GenBank OM722135) were closely clustered with reference sequences of *A. spissacea* GenBank (KR865979, KP866163) from Korea in nrLSU phylogenetic tree.

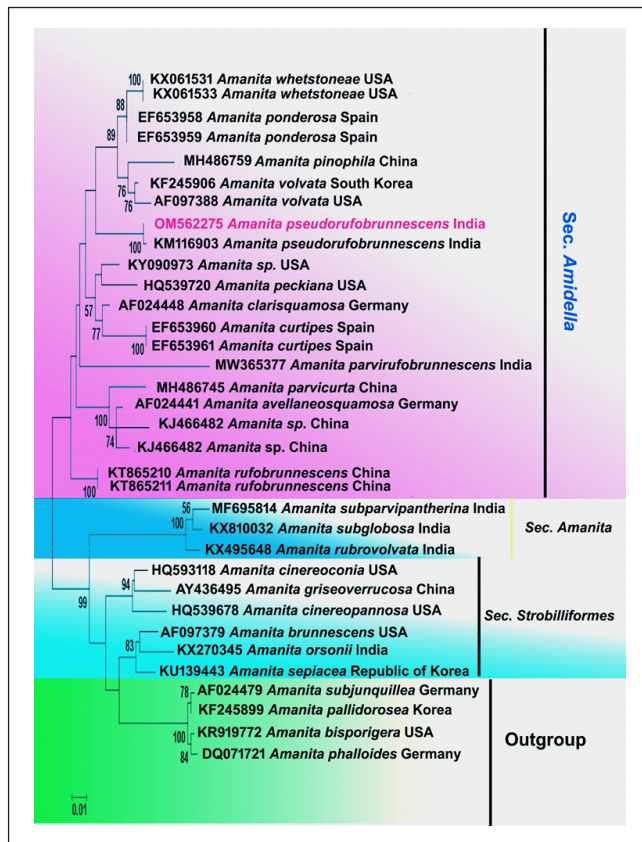


Fig. 1: Phylogenetic relationships *Amanita pseudorufobrunnescens* inferred from nrLSU sequences using Maximum Likelihood (ML) method. Bootstrap support values (>50%) obtained from maximum likelihood (ML) analysis are shown above or below the branches at nodes.

Taxonomic description

Amanita pseudorufobrunnescens K.C. Semwal, K. Das, R.P. Bhatt, Mehmood & V.K. Bhatt, *Kew Bull.* **75**: 22. (Figs. 2, 3)

Basidiomata medium-sized. Pileus 45 -70 mm diam., initially hemispherical, then convex to planoconvex, finally plane, initially white to milky-white (1A1), turning light orange (5A5) to greyish orange (6B3), radially striate; universal veil remnants scaly to radially fibrillose, white to whitish (1A1), light brown (6D5) to greyish orange (6B3-6C4), margin

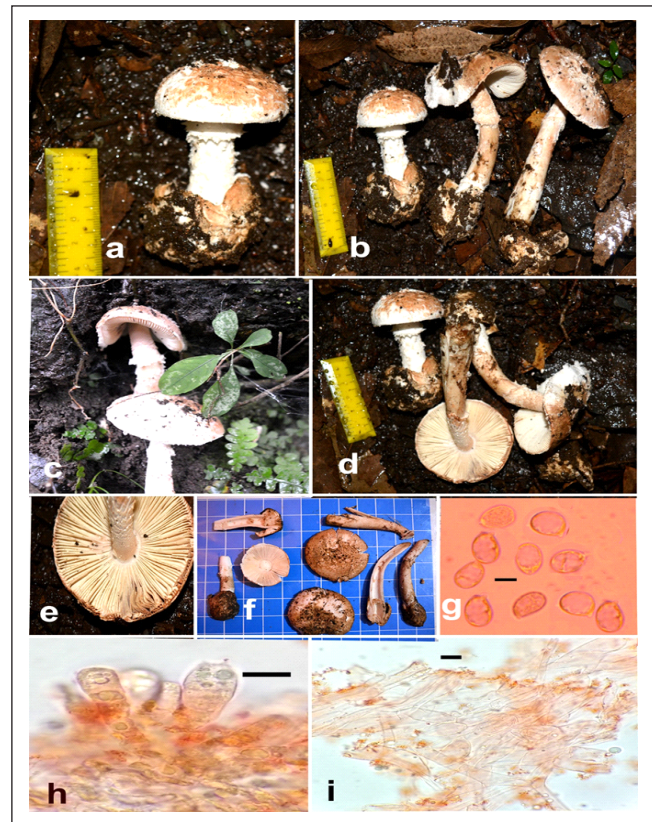


Fig. 2: *Amanita pseudorufobrunnescens*. a-f. Fresh basidiomata in the forest; g. Light micrograph (LM) of Basidiospores; h. Basidia (LM); i. Volval remnants at stipe base. Scale bars: g-i = 10 μm.

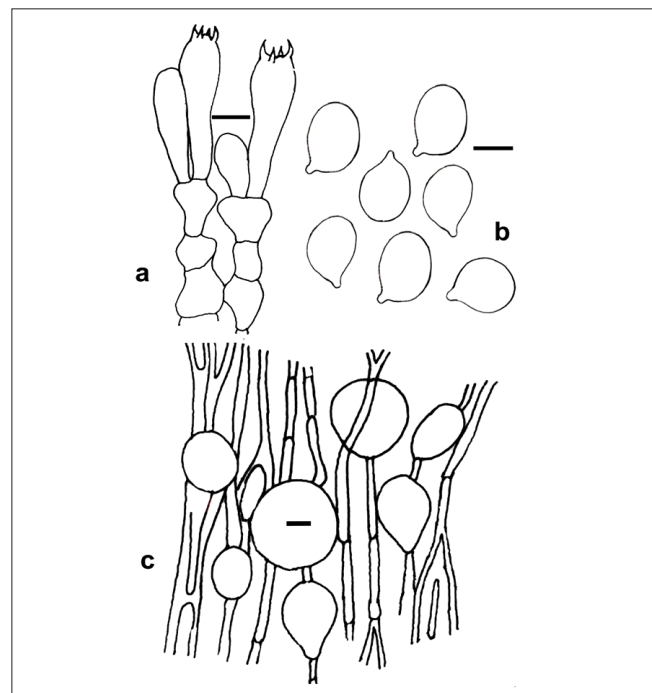


Fig. 3: Line drawings of *Amanita pseudorufobrunnescens* a. Basidia and elements of hymenium and subhymenium b. Basidiospores; c. Volval remnants at stipe base. Scale bars: a-c = 10 μm.

appendiculate with thick fibrils, context 25 mm thick, white, changing light brown (6D6) when bruised. Lamellae free, close, whitish to cream (4A2), changing light brown (6D5) with age. Lamellulae sub truncate to truncate. Stipe 170-110 × 12-15 mm, tapering upwards, white to whitish (1A1), covered with fibrillose to squamules, universal veil remnants at stipe base as light brown (6D5) to greyish orange (6B3). Stipe context white changing light orange (5A5) on exposure. Partial veil subapical, fragile, white. Volva saccate, thick, 30-40 × 20-30 mm, with 2-3 limbs, whitish to greyish orange (6B5). Odour indistinct. Taste not recorded. Spore print white. Basidiospores (9-) 11-12 (-13) × (6-) 7-8 (-9) μm [Q = (1.22-) 1.37-1.57 (-1.71), Qav = 1.53], ellipsoid, amyloid, colourless, hyaline, thin-walled, smooth. Basidia 35-46 × 8-11 μm, clavate, 4 spored; sterigmata 3-5 μm long. Hymenophoral trama bilateral, divergent. Mediostratum 50-100 μm wide, composed of abundant broadly ellipsoid to elongated inflated cells (25-90 × 13-28 μm), filamentous, undifferentiated hyphae 4-12 μm wide. Subhymenium 25-50 μm thick, basidia arising from subglobose (10-18 × 8-14 μm). Pileipellis 100-160 μm thick, two-layered; upper layer 40-60 μm thick, filamentous, undifferentiated hyphae 3-8 μm wide, thin-walled, hyaline. Universal veil remnants on pileus; filamentous undifferentiated hyphae up to 5-12 μm wide, thin-walled, colorless, hyaline; inflated cells subglobose to ellipsoid 35-115 × 10-28 μm, hyaline, thin-walled. Universal veil on stipe base with elements irregularly arranged; filamentous undifferentiated hyphae up to 3-11 μm wide, yellowish brown, slightly thick-walled; inflated cells scattered, clavate to subglobose 15-25 × 10-20 μm, Stipe context longitudinally acrophysalidic; acrophysalides 110-250 × 20-35 μm; filamentous undifferentiated hyphae 5-9 μm wide. Clamp connections are absent in all tissues.

Habit and habitat: Solitary to scattered on the ground in a mixed forest of *Quercus* sp.

Distribution: This species was originally described from Uttarakhand, (Semwal *et al.*, 2020), and is now known from Jammu and Kashmir, India.

Specimens examined: India, Jammu & Kashmir, district Rajouri, Dharal, 33°29'0.59"N, 74°26'31.49"E, alt. 1718 m a.s.l., 08 September 2021, Anil Kumar (AKA11); Distt. Kathua, Machedi, 08 September 2020, Anil Kumar (AKS05).

Discussion: *Amanita pseudorufobrunnescens* is a member of [subgenus *Amanitina*] section *Amidella*. In the field, can be recognized by its whitish basidiomata that turn brownish orange with age, its whitish to light brown, floccose to radially fibrillose universal veil remnants on the pileus, its close lamellae becoming light brown on exposure, its ellipsoidal basidiospores (9-) 11.0-12 (-13) × (6-) 7-8 (-9) μm. The size of basidiomata, as well as the size and shape of basidiospores of our collections, is very similar to the original description from Uttarakhand (Semwal *et al.*, 2020).

Amanita fritillaria (Berk.) Sacc., *Sylogae Fungorum* 9: 2 (1891). (**Figs. 5, 6**)

Basidiomata small to medium. Pileus 30-100 mm diam., hemispherical at first, then convex to planoconvex, greyish brown (6D3) to brownish grey (5D2), chocolate brown (6F5) to brownish (6D7), slate grey (3F2) darker over centre, surface shiny, viscid when moist; context 6-8 mm thick thinning slowly toward margin, white (2A1) unchanging on cutting or bruising; margin non-striate and non-appendiculate. Universal veil on pileus as verrucose to felted olive-grey (1F2) to brownish grey (4D2). Lamellae free, crowded (9-12 lamellae/10 mm at the margin), white. Lamellulae attenuate of several lengths, plentiful. Stipe 50-120 × 20-30 mm, narrowing upward, subbulbous, stuffed, covered with greyish brown (5D3) fibrils, turning slightly darker when bruised. Partial veil apical, membranous, skirt-like, dark grey (1F1), covered with very small greyish warts on edges. Bulb ovate 14-20 × 20-32 mm, greyish brown (6D3). Universal veil as dark grey (1F1) warts. Odour indistinct. Taste not recorded. Spore print white. Basidiospores (7-) 8-9.5 (-10.5) × (6-) 7-8 (-9.5) μm, Q = 1.21-1.26, Qav = 1.25) hyaline, colorless, thin-walled, smooth, amyloid, subglobose to broadly ellipsoid with sublateral apiculus, about 1 × 1.5 μm, with monogutulate contents. Basidia (35-) 38-45 (-50) × (8-) 9-11 (-12) μm, thin-walled, 2-4 spored, sterigmata up to 4-2 μm long, basal clamps absent. Lamellar edge cells sterile, filamentous, undifferentiated hyphae 2-4 μm wide, hyaline, thin-walled; inflated cells dominating, mostly globose to subglobose 10-20 × 10-16 μm, colorless, thin-walled. Subhymenium, 27-40 μm, basidia arising from subglobose to ovoid cells 8-15 × 9-12 μm. Hymenophoral trama bilateral, divergent, filamentous, undifferentiated hyphae 2-6 μm wide. Pileipellis up to 145 μm thick, not differentiated into suprapellis and subpellis; filamentous, undifferentiated hyphae 4-8 μm wide, horizontally arranged, thin to slightly thick-walled, with yellowish brown vacuolar pigments. Lamellar edge sterile inflated cells mostly subglobose to pyriform 16-20 × 9-15 μm, colorless, thin-walled. Universal veil on the pileus with elements irregularly arranged, filamentous, undifferentiated hyphae 3-6 μm wide, dominant, branched; inflated cells dominant; globose to subglobose 30-80 × 25-75 μm, sometimes broadly ellipsoid to ellipsoid 35-60 × 12-20 μm, thin-walled, hyaline, yellowish brown vacuolar pigments. Partial veil filamentous, undifferentiated hyphae 3-7 μm wide; inflated cells broadly ellipsoid 37-45 × 75-162 μm. Stipe trama longitudinally acrophysalidic; acrophysalides 122-315 × 20-45 μm; filamentous. Clamp connections are absent in all tissues.

Habit and habitat: Solitary to sub gregarious in temperate mixed coniferous/broad-leaved forest, under *Quercus* sp.

Specimens examined: India, Jammu & Kashmir, district Udhampur, 32°54'.43"N, 75°27'.51"E, alt. 1940 m. a.s.l., Latti (Dhuna), 2 August 2021, Anil Kumar (AKA36).

Distribution: This species was originally described from Assam, China, (Zhang *et al.*, 2004) and is now known from Jammu and Kashmir, India.

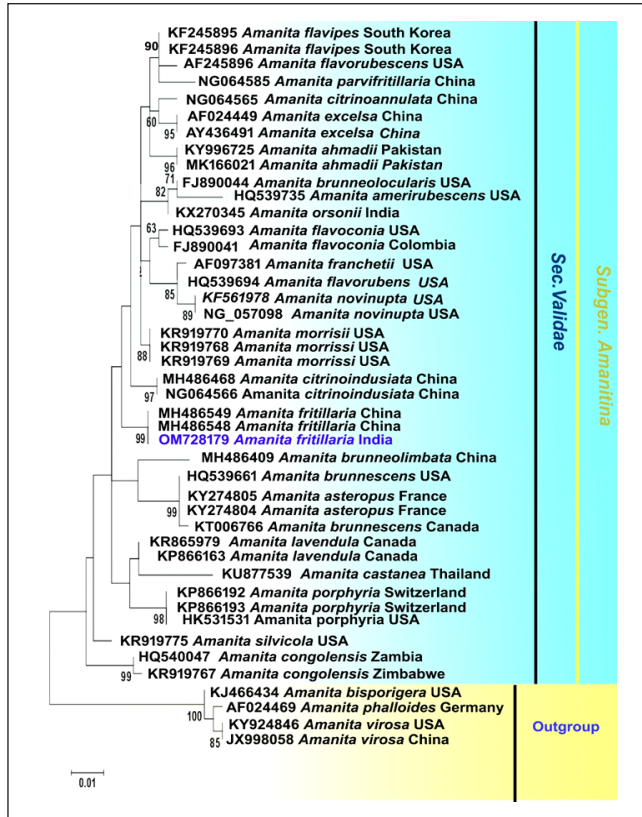


Fig. 4: Phylogenetic relationships *Amanita fritillaria* inferred from nrLSU sequences using Maximum Likelihood (ML) method. Bootstrap support values (>50%) obtained from maximum likelihood (ML) analysis are shown above or below the branches at nodes. *Amanita fritillaria* is highlighted in bold blue in the tree.

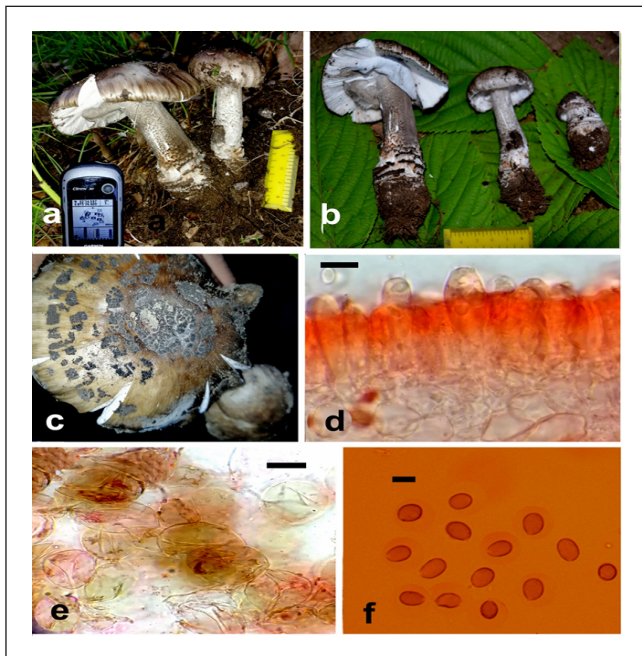


Fig. 5: *Amanita fritillaria*. a-c. Fresh basidiomata in the field; d. Basidia (LM); e. Elements of universal veil from pileus surface. f. Basidiospores. Scale bars: d-f = 10 μ m.

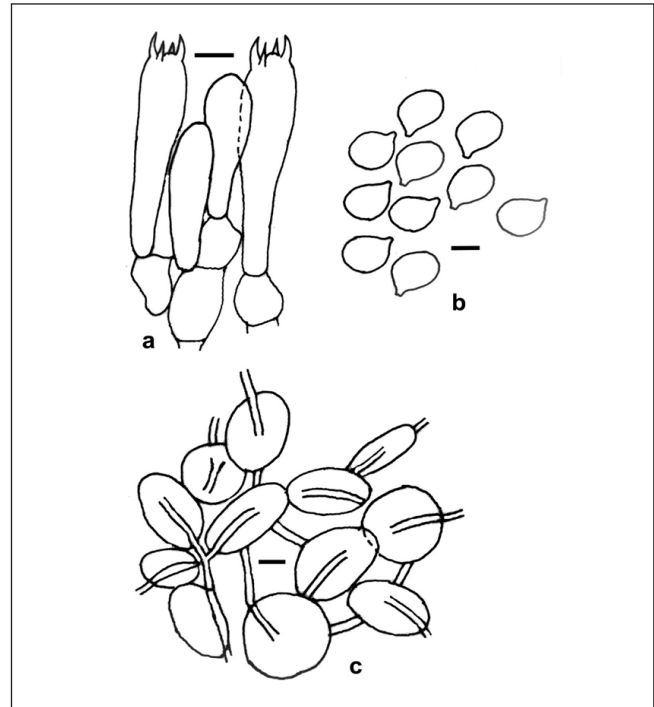


Fig. 6: Line drawings of *Amanita fritillaria*. a. Basidia and elements of hymenium and subhymenium. b. Basidiospores. c. Elements of universal veil pileus. Scale bars: a-c = 10 μ m.

Discussion: *Amanita fritillaria*, belongs to [subgenus *Amanitina*] section *Validae*, which is characterized by amyloid spores, non-appendiculate pileus margin and non-membranous universal veil (Corner and Bas 1962; Bas 1969). In the field, *Amanita fritillaria* is characterized by its greyish brown to brownish grey pileus covered by dark brown to chocolate brown universal veil remnants and subglobose to broadly ellipsoid basidiospores (8.5-9.5 \times 7-8).

Amanita spissacea S. Imai, *Bot. Mag. (Tokyo)* **47**: 427 (1933). (Figs. 8, 9)

Basidiomata small to medium-sized. Pileus 20-60 mm in diam., convex to plano-convex, grayish-green (1D4) to gray-brown (1E3-5) to gray (1D1-4); surface shiny, dry; context 4-6 mm thick white (2A1) unchanging on cutting or bruising; margin non-striate and non-appendiculate. Universal veil on pileus as felted to patchy, dark gray (1E1-4) to gray-brown (1E3-5). Lamellae free, white (1A1); lamellulae attenuate. Stipe 80-105 \times 5-15 mm wide, tapering upward, stuffed, white (1A1) to grayish-brown (5D3) covered with grayish (1C1) to brownish (3C2) squamules; context white (1A1), unchanging. Partial veil membranous, superior, dirty white (2B1), grayish (2C1) to brownish (3C2). Bulb subglobose 14 \times 11 μ m thick covered with floccose, gray brown (5E3) universal veil remnants. Basidiospores (8-) 9-10 (-12) \times (5-) 6-7 (-8) μ m, Q = (1.28) 1.42-1.57 (1.69), Qm = 1.45) hyaline, colourless, thin-walled, smooth, amyloid, ellipsoid. Subhymenium = 20-46 μ m, Hymenophoral trama bilateral, divergent, filamentous, undifferentiated hyphae 2-5 μ m wide.

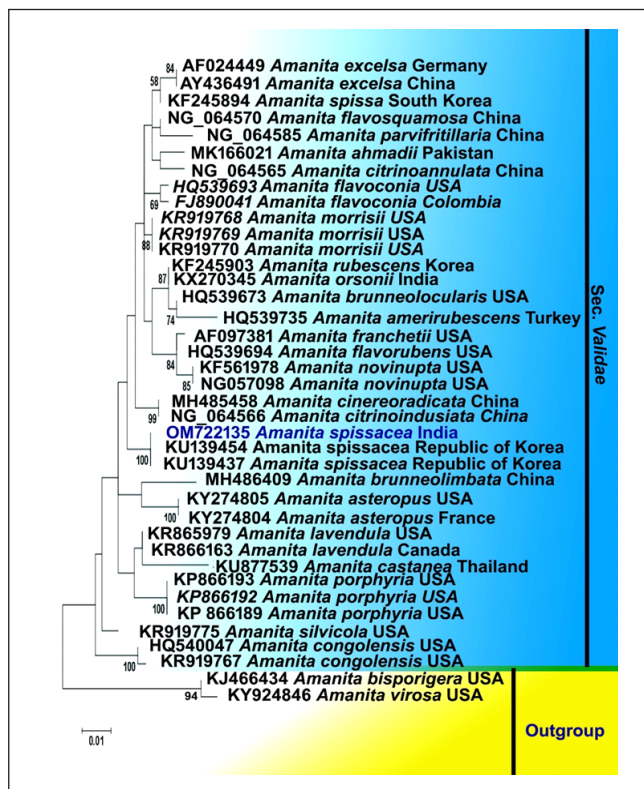


Fig. 7: Phylogenetic relationships *Amanita spissacea* inferred from nrLSU sequences using Maximum Likelihood (ML) method. Bootstrap support values (>50%) obtained from maximum likelihood (ML) analysis are shown above or below the branches at nodes. *Amanita spissacea* is highlighted in bold blue in the tree.

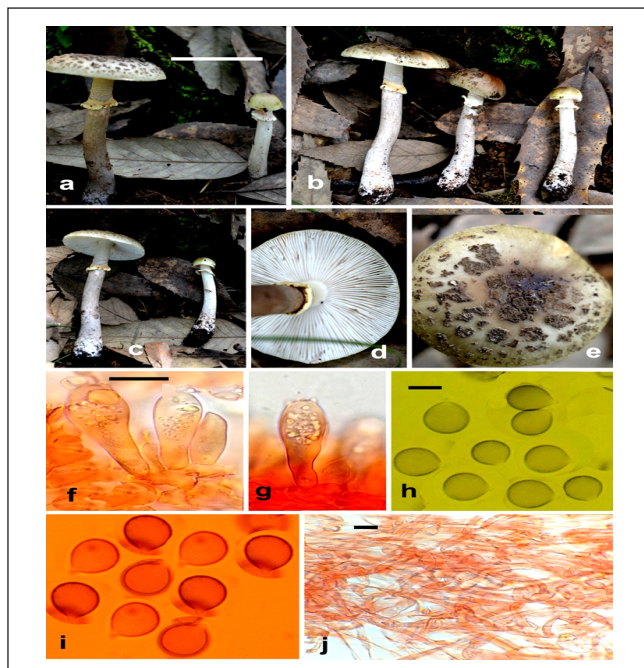


Fig. 8: *Amanita spissacea*. a-e. Fresh basidiomata in the field; f-g. Basidia (LM); h-i. Light micrograph (LM) of Basidiospores; h. Volval remnants at stipe base. Scale bars: a-e = 50 mm; f-j = 10 µm.

Pileipellis upto 150 µm thick, not differentiated into suprapellis and subpellis; filamentous, undifferentiated hyphae 3-6 µm wide. Pileus context filamentous, undifferentiated hyphae 4-12 µm wide, thin-walled, hyaline; inflated cells broadly ellipsoid to cylindrical up to 100 × 30 µm, thin-walled, colorless, hyaline. Lamellar edge sterile inflated cells mostly subglobose to pyriform 12-15 × 7-14 µm, colorless, thin-walled, hyaline. Universal veil on the pileus with elements irregularly arranged, filamentous, undifferentiated hyphae 3-6 µm wide.

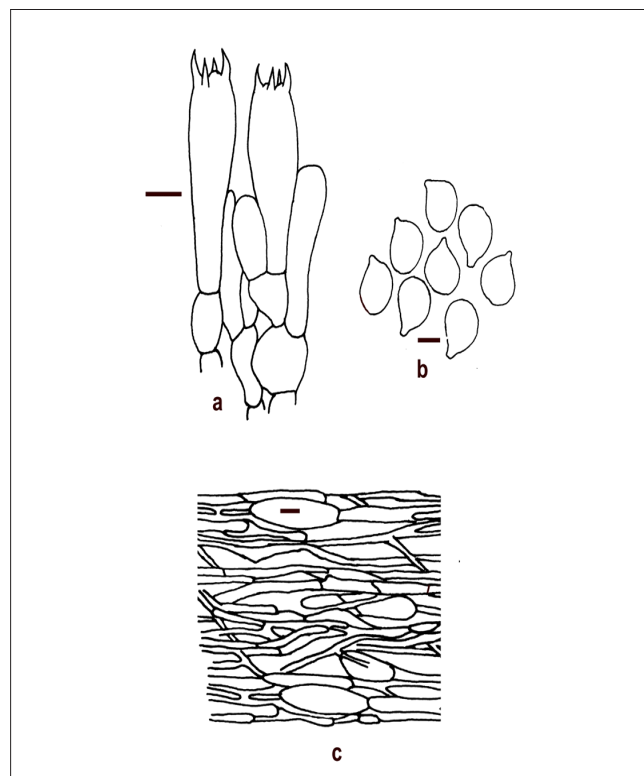


Fig. 9: Line drawings of *Amanita spissacea*. a. Basidia and elements of hymenium and subhymenium b. Basidiospores; c. Elements of universal veil pileus. Scale bars: a-c = 10 µm.

Habitat: Solitary to scattered in temperate mixed forest dominated by *Quercus* sp. alt. 1618m a.s.l.

Specimens examined India, Jammu & Kashmir, district Rajouri, 33°29'0.59"N, 74°26'31.49"E, alt. 1618m a.s.l., August 2021, Anil Kumar (AKA10).

Distribution: This species was earlier described from Mizoram state of India by Lalrinawmi *et al.* (2018) and is now known from Jammu and Kashmir, India.

Discussion: *Amanita spissacea* belongs to [subgenus *Amanitina*] section *Validae*, which is characterized by amyloid spores, non-appendiculate pileus margin, and non-membranous universal veil (Corner and Bas 1962; Bas 1969). *Amanita spissacea* is characterized by its grayish-green to gray-brown pileus, ellipsoidal basidiospores 9-10 × 6-7.

ACKNOWLEDGMENTS

The authors are grateful to the Head, Department of Botany (UGC-SAP DRS II) University of Jammu, Jammu for providing laboratory facilities during the present study. The first author also acknowledges the financial assistance received from UGC in the form of Junior Research Fellowship.

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