Morphological and phylogenetic characterization of genus *Amanita* from Uttarakhand, India: I

Mehmood T¹, Bhatt RP¹, Uniyal P¹, Singh U¹* and Chowdhary AK²

¹ Department of Botany & Microbiology, H.N.B. Garhwal University, Srinagar, Garhwal – 246174, Uttarakhand, India
² Department of Zoology & Biotechnology, H.N.B. Garhwal University, Srinagar, Garhwal – 246174, Uttarakhand, India


Abstract

Four species of genus *Amanita* namely; *A. orsonii*, *A. rubrovolvata*, *A. subglobosa* and *A. hemibapha* are identified from Uttarakhand, India. Morphological details, illustrations and phylogenetic observations based on ITS and nrLSU data are given here.

Key words – *Amanitaceae* – molecular phylogeny – taxonomy – Uttarakhand Himalaya

Introduction

The genus *Amanita* Pers. is widely distributed from tropical to temperate regions of the world and consists of both edible as well as poisonous species. To date, a total of 534 species of *Amanita* are validly published all over the world and about 60 taxa are reported from India (Yang 2000, Bhatt et al. 2003, Semwal et al. 2007, Tulloss & Yang 2016, Bhatt et al. 2017, Das et al. 2017, Tibpromma et al. 2017). Moreover, most species of this genus form ectomycorrhizal associations with vascular plants and play an important role in the sustenance of forest ecosystems (Yang & Doi 1999). The genus is divided into two subgenera: subg. *Amanita* (with inamyloid spores) consisting of three sections namely sect. *Amanita*, sect. *Caesareae* Singer, sect. *Vaginatae* (Fr.) Quélet. and subg. *Lepidella* (E.-J. Gilbert) Veselý (with amyloid spores) includes four sections namely, sect. *Amidella* (E. J. Gilbert) Konrad & Maubl., sect. *Lepidella* sensu Bas, sect. *Phalloideae* (Fr.) Quél., and sect. *Validae* (Fr.) Quél.

*Amanita orsonii*, *A. rubrovolvata*, *A. subglobosa* and *A. hemibapha* are reported earlier (Bhatt et al. 2003, Semwal et al. 2007, Bhatt et al. 2016) from Uttarakhand Himalaya. However, all of these lack a molecular data for identification and phylogenetic relations. These species are described here with macro and microscopic details along with nrITS and nrLSU based phylogenetic data.

Materials and Methods

Morphological observations

Macromorphological characters like shape, size, colour, texture, smell, spore print, habit and habitat were documented in the forest or base camp from the fresh and dissected young to mature
basidiomata. The photography was accomplished using digital camera (Sony cyber-shot W730 and Canon Power Shot SX 50). Color codes and terms mostly follow Methuen Handbook of Color (Kornerup & Wanscher 1978). Samples were dried with a field drier. Micromorphological characters were observed with the help of a compound microscope (Olympus CH20i) from the dry materials mounted in a mixture of 5% KOH, 1% Phloxin and 1% Congo red. Biometric variables for spores follow Tulloss 2008), i.e. ‘L’ = the average spore length computed for one specimen examined and the range of such averages, L’ = the average spore length computed for all spores measured, W = the average spore width computed for one specimen examined and the range of such averages, W’ = the average spore length computed for all spores measured, Q = the ratio of length/breadth for a single spore and the range of the ratio of length/breadth for all spores measured; Q’ = average value of Q computed for one specimen examined and the range of such averages; Q’ = average value of Q computed for all spores measured’. Drawings of microscopic elements were made with the Camera lucida at 2000 × magnifications. Microphotography was made with the respective dedicated cameras attached to the compound microscopes: Olympus CH20i and Olympus CX21i LED.

DNA isolation, amplification and sequencing
Genomic DNA was isolated from dried basidiome following the modified CTAB method Doyle & Doyle (1987). PCR was performed to amplify partial sequence of the ribosomal large subunit of RNA (nrLSU) using universal primer pairs LROR and LR5 (Vilgalys & Hester 1990). ITS region was amplified using primers ITS 1 and ITS 4 (White et al. 1990). PCR amplification was conducted on a thermal cycler (Eppendorf, Germany) programmed for 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 1 min at 55°C, 1 min at 72°C and a final stage of 8 min at 72°C. The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany). Both strands of the PCR fragment were sequenced on the 3730xl DNA Analyzer (Applied Bionsystems, USA) using the same primer pairs.

Phylogenetic analysis
Phylogenetic analyses based on internal transcribed spacer (nrITS) and (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). Limacella spp. were chosen as outgroup. Multiple sequence alignment was performed using MAFFT v.7 (Katoh et al. 2005) 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). Five hundred bootstrap replicates were analyzed to obtain nodal support values. Four nrLSU and four ITS sequences were generated for this study and deposited to the GenBank to procure the accession numbers (KX270327, KX270345, KX810031, KX810032, KX495648, KX539266, KY214404 and KY214405).

Results and discussion

Phylogeny
Most of the identified Amanita species formed a group with strong support in the ITS and nrLSU trees (Figs 1, 2). Two isolated sequences (GenBank KX270327, KX270345) of Amanita orsonii were closely grouped with reference sequence of A. orsonii (GenBank KU 248131) from Pakistan both in nrLSU and ITS phylogenetic trees. A. rubrovolvata (GenBank KX495648) was closely grouped with sequence of A. rubrovolvata (GenBank AF024474) from China in nrLSU tree. The isolated sequences of A. subglobosa from India (GenBank KX810031, KX810032) were closely grouped with reference sequence of A. subglobosa GenBank (KU248106) from China both in ITS and nrLSU phylogenetic trees. Also, two ITS sequences of A. hemibapha (GenBank KY349225, KY214405) were closely grouped with reference sequence of Amanita hemibapha (GenBank LC056764) from Japan in ITS phylogenetic tree.
Taxonomy


Figs 3 & 4

Basidiomata medium-sized to large. Pileus 89–125 mm wide, light brownish (5D5) turning pale orange (5A3) to golden yellow (5B7) with exposure or handling, initially hemispherical then convex to plano-convex and finally plane, viscid when moist, shining; context 5–7 mm thick, thinning slowly toward margin, whitish, turning dull pinkish or reddish white (7A2) when aged or bruised; margin non–striate, non–appendiculate, slightly uplifted with age. Universal veil on pileus as whitish (3A1) floccose, felled to sub-pyramidal patches, diminishing in size toward margin.

**Fig. 1** – Maximum likelihood phylogenetic tree of *Amanita*, showing the position of *A. orsonii*, *A. rubrovolvata*, *A. subglobosa* and *A. hemibapha*. Our isolates are highlighted in blue font on the tree. Bootstrap support values > 50% are mentioned above branches. *Limacella glioderma* and *L. illinita* are rooted as outgroup.
Fig. 2 – Maximum likelihood phylogenetic tree of *Amanita*, showing the position of *A. orsonii*, *A. subglobosa* and *A. hemibapha*. Our isolates are highlighted in blue font on the tree. Bootstrap support values > 50% are mentioned above branches. *Limacella glioderma* is rooted as outgroup.

Lamellae free, crowded, (7–9 lamellae/10 mm at margin) white, staining reddish brown or deep orange (6A8) when cut or bruised. Lamellulce attenuate, of several lengths, plentiful, forked. Stipe 105–135 × 12–25 mm, tapering upward, reddish white (7A2), turning pale red (7A3) when aged or bruised, covered by pale yellow (2A3) squamules above partial veil, with dusty yellow or light yellow (4A5) fibrils below, stuffed to solid. Partial veil superior, membranous, whitish or dusty yellow or yellowish white (4A2), striate above. Bulb 21–23 × 24–26 mm, covered by yellowish to grayish white (1B1) patches. Odour indistinct. Taste not recorded. Spore print white.

Basidiospore (7.5–) 8–9 (–10) × (5.5–) 6–7 (–8) μm, L= 8–8.5 μm; L’ = 8.35 μm, W = 5.5–6.5 μm; W’ = 6.1 μm; Q = (1.25–) 1.33–1.45 (–1.5); Q = 1.35–1.42; Q’ = 1.38, amyloid, broadly ellipsoid to ellipsoid, thin–walled, with monoguttulate content; apiculus 1.5 μm long, sublateral, hyaline. Basidia (30–) 32–35(–41) × (9–) 9–9.5(–10) μm, 2–4-spored, thin-walled; sterigmata up to
2.5–3 μm long. Clamp connection absent at the base of basidia. Lamellar edge cells sterile, with inflated cells clavate or pyriform, 22–35 × 14–20 μm, colourless, frequent to abundant. Subhymenium \( w_{st-near} = 32–47 \) μm thick, \( w_{st-far} = 35–54 \) μm, inflated cells, with two to three layers of subgloboso to ovoid cells up to 12 × 15 μm wide. Hymenophoral trama bilateral, divergent, \( w_{st} = 43–78 \) μm, filamentous, undifferentiated hyphae 4–6 μm wide. Pileipellis up to 210 μm thick, slightly gelatinising, filamentous, undifferentiated hyphae 2–5 μm wide, thin-walled, hyaline, orientation mainly radial with some loosely interwoven; vascular hyphae 7–14 μm wide. 

Subhymenium \( w_{st-near} = 32–47 \) μm thick, \( w_{st-far} = 35–54 \) μm, inflated cells, with two to three layers of subglobose to ovoid cells up to 12 × 15 μm wide. Hymenophoral trama bilateral, divergent, \( w_{st} = 43–78 \) μm, filamentous, undifferentiated hyphae 4–6 μm wide. Pileipellis up to 210 μm thick, slightly gelatinising, filamentous, undifferentiated hyphae 2–5 μm wide, thin-walled, hyaline, orientation mainly radial with some loosely interwoven; vascular hyphae 7–14 μm wide. 

Pileus context filamentous, undifferentiated hyphae 4–10 μm wide, thin-walled, hyaline; ellipsoid cells 23–56 × 13–27 μm. Universal veil on pileus filamentous, undifferentiated hyphae 2–5 μm wide, globose cells up to 30–50 × 32–53 μm, subglobose to ovoid 22–27 × 32–38 μm. Partial veil filamentous, undifferentiated hyphae 2.5–8 μm wide, inflated cells, narrow ellipsoid to elongate up to 36 × 14 μm, subclavate up to 43 × 16 μm, pyriform up to 28 × 16 μm. Stipe context longitudinally acrophysalidic; acrophysalides 124–214 × 23–38 μm; filamentous, undifferentiated hyphae 3–7 μm wide. Clamp connection absent in all tissue.

Habit & habitat – Solitary to scattered in temperate mixed forest under Quercus floribunda and Q. semicarpifolia and Abies pindrow.


Notes – Amanita orsonii belongs to [subgenus Lepidella] section Validae. In the field, Amanita orsonii is distinct from all other species of sect. Validae by its light brownish pileus turning pale orange to golden yellow with exposure, broadly ellipsoid to ellipsoid basidiospore. Two rubescent taxa of this section Validae; Amanita rubescens var. rubescens (originally reported from the Netherlands) and Amanita brunneolocularis (reported from Colombia) are somewhat close to Amanita orsonii but Amanita rubescens var. rubescens differs from Amanita orsonii by its brown pileus and ellipsoid to elongate basidiospores 8–10.6 × 5.5–7) μm whereas Amanita brunneolocularis is separated from Amanita orsonii by its reddish brown pileus discolouring reddish where bruised (Tulloss et al. 1992). The closest blast hit for the LSU sequence of our specimen from India (RET 717-8) is KU248131 (A. orsonii voucher RET 390-4), with 100% identity and 100% query cover. The closest blast hit for the ITS is the sequence KU248133 (A. orsonii voucher RET 390-4), with 100% identity and 88% query cover. In the nrLSU phylogenetic tree our Indian specimen A. orsonii sequence clustered with Amanita orsonii sequences from Pakistan (Figs 1, 2).


Basidiomata small to medium sized. Pileus 20–55 mm wide, initially campanulate and finally plano-convex, reddish orange (7A6–7) over centre, orange-red to yellowish orange (4A7) toward margin, viscid, shiny; context 2–4 mm thick, thinning slowly toward margin, white to light yellow (2A5), orangish beneath disc, unchanging when cut or bruised; margin short striated 5–9 mm, non-appendiculate, uplifted with age. Universal veil on pileus as yellow to orange-red (8A7), floccose to crust like patches, randomly distributed, diminishing in size toward margin. Lamellae free, crowded (11–13 lamellae/10 mm at margin) 3–4 mm broad, white to cream. Lamellulae truncate, of various lengths. Stipe 90–115 × 5–8 mm, tapering upward, yellow to pale yellow (3A3), sometimes deep yellow (4A8) entire, covered by orange-yellow (4B8) floccose above annulus and light yellow (2A5) fibrillos below; context white (3A1) to light yellowish
Fig. 3 – *Amanita orsonii* a–b Fresh basidiomata in the field. c Basidium and basidioles. d Lamellae edges cell. e Basidiospores. f Elements of universal veil from pileus surface. g Elements of partial veil.
Fig. 4 – *Amanita orsonii*. a Basidiomata. b Basidia and element of subhymenium. c Basidiospores. d Lamellae edge cells. e Elements of universal veil on pileus surface. f Elements of partial veil. Scale bars: a = 10 mm; b–f = 10 μm.

(3A4), stuffed with white cottony material, unchanging when bruised or exposed. Partial veil superior to median, membranous, thin, creamy or yellowish orange (4A7), striate above covered by reddish orange (7A7) warts. Bulb 13–17 × 11–15 mm, subglobose, white, covered by floccose to felted reddish warts in an incomplete ring around the bulb. Odour indistinct. Taste not recorded. Spore print white.

Basidiospores (8.5–) 9–10(–10.5) × (7.5–)8.1–9.1(–9.8) μm, L= 9–10 μm; L’ = 9.45 μm, W =7.9–9.0 μm; W’ = 8.7 μm; Q = (1.07–) 1.09–1.12 (–1.4); Q = 1.08–1.13; Q’ = 1.10, colourless,

Habit & Habitat – Solitary to gregarious in temperate mixed forest dominated by Abies pindrow, Quercus semicarpifolia and Q. leucotricophora.


Notes – Amanita rubrovolvata is characterized by its reddish orange pileus over centre, orange-red to yellowish orange toward margin and subglobose basidiospores. Amanita rubrovolvata might be confused with Amanita subfrostiana but it differs from A. rubrovolvata by its red colour pileus over centre, pale orange towards the margin and globose to subglobose basidiospores (Yang 1997). The closest blast hit for the LSU sequence of our specimen from India (TM-0126) is KY747477 (A. rubrovolvata voucher voucher BZ2015_68) with 99% identity and 100% query cover). In the nrLSU phylogenetic tree our Indian specimen Amanita rubrovolvata sequence clustered with Amanita rubrovolvata sequences (AF024473) from China (Fig. 1)


Figs 7 & 8

Basidiomata small to medium sized. Pileus 40–90 mm wide, light brown to tea brownish (6D5–6) at centre, pale yellow to light yellow (1A3-5) toward margin, initially hemispherical then convex to plane, slightly depressed in centre, surface slightly viscid when moist, shiny; context 4–6 mm thick, thinning slowly toward margin, off-white, unchanging when bruised or exposed; margin short striated, non-appendiculate, slightly uplifted. Universal veil on pileus as granular subfelted warts, 1–1.5 mm wide, up to 1.3 mm high, dirty white to cream. Lamellae free, crowded, white to cream colour, unchanging, 3–8 mm broad; lamellulae truncate, plentiful, in several lengths. Stipe 60–130 × 5–10 mm, narrowing upwards, white, stuffed. Partial veil subapical, membranous, white, pendent. Bulb 10–19 × 8–15 mm, subglobose, remnants of universal veil on top of the bulb are white granular warts. Odour indistinct. Taste not recorded. Spore print white.

Basidiospores (8–) 9.5–11(–11.5) × (6.5–) 7–7.5 (–8) µm, L =9.5–10.5 µm; L’ = 9.7 µm, W = 6.8–7.5 µm; W’ = 7.1 µm; Q = (1.25–)1.33–1.37(–1.5); Q = 1.36–1.4; Q’ = 1.33, broadly ellipsoid
Fig. 5 – *Amanita rurovolvata*. a Fresh basidiomata in the field. b–c Basidiomata in the base camp. d Elements of universal veil from pileus surface. e Basidiospores. f Hymenium and subhymenium.
Fig. 6 – *Amanita rurovolvata*. a Basidiomata. b Basidiospores c Hymenium and subhymenium. d Elements of universal veil from pileus surface. e Elements of partial veil. Scale bars: a = 10 mm; b–e = 10 µm.

to ellipsoid, hyaline, thin-walled, smooth, inamyloid, apiculus up to 1.5 µm long. Basidia (42–)48–51(–58) × (10–)11–12(–12.5) µm, thin-walled, colourless, 2-4-spored, sterigmata up to 2–4 µm long; basal clamps sometimes present. Subhymenium wst-near = 35–55 µm; wst-far = 56–72 µm thick; Hymenophoral trama bilateral, divergent; wcs = 45–78 µm, inflated cells up to 82 × 30 µm, filamentous, undifferentiated hyphae 5–6 µm wide, thin-walled, hyaline; clamps absent. Lamellar edge cells sterile; with inflated cells clavate to pyriform up to 22–25 × 9–10 µm, colourless.
**Amanita subglobosa** (Amanita subglobosa voucher Yang 2488) with 99% identity and 92% query cover. In the both nrLSU phylogenetic tree our Indian specimen *Amanita subglobosa* sequence clustered with *A. subglobosa* sequences from China (Figs 1, 2).
Fig. 7 – *Amanita subgloboasa*. a–b Fresh basidiomata in the field. c Basidiospores. d Elements of universal veil from pileus surface. e Basidium and basidioles.
Fig. 8 – *Amanita subglobose*. a Basidiomata. b Basidia and element of subhymenium. c Elements of partial veil. d Basidiospores. e Elements of universal veil on pileus surface. Scale bars: a = 10 mm; b–e = 10 µm.

Ellipsoid to elongated 20–40 × 10–16 µm; clamps present. Partial veil filamentous, undifferentiated hyphae 4–8 µm wide, inflated cells dominant, globose to clavate up to 105–75 µm wide, colourless, thin-walled, hyaline. Stipe context longitudinally acrophysalidic; acrophysalides 130–235 × 28–39 µm; filamentous, undifferentiated hyphae 5–7 µm wide, hyaline. Clamp connections present in all tissue.
Fig. 9 – *Amanita hemibapha*. a–c Fresh basidiomata in the field. d Basidiomata in the base camp. e Basidia and basidiole. g–f Basidiospores. h Elements of universal veil from stipe.
Fig. 10 – *Amanita hemibapha*. a Basidiomata. b Hymenium and Subhymenium. c Basidiospores. d Lamellar edge cells. e Elements of universal veil pileus surface. Scale bars: a = 10 mm, b–e= 10 µm.
Habit & habitat – Solitary to gregarious, in the temperate coniferous forests dominated by Pinus roxburghii.


Notes – Amanita hemibapha is characterized by its yellowish orange to reddish yellow pileus slightly depressed over centre, white lamellae, broadly ellipsoid to ellipsoid basidiospores and occurrence under Pinus roxburghii.

Morphologically, several species are similar to Amanita hemibapha such as A. caesareoides Lj. N. Vassiljeva, A. javanica (Corner & Bas 1962) T.oda, C.Tanaka & Tsuda, A. caesarea (Scop: Fr.) Pers. and Amanita similis Boedijn. Amanita caesareoides differs from A. hemibapha by its completely bright orange-red pileus (Sanmee et al. 2008, Bhatt et al. 2017). Whereas, Amanita javanica originally described from Java differs from A. hemibapha by its orange-yellow to ochre yellow pileus (Oda et al. 1999). Amanita caesarea known from Europe differs from Amanita hemibapha by its shorter stipe, shorter marginal striations on the pileus (Breitenbach & Kranzlin 1995). Amanita similis is easily distinguished from A. hemibapha by its dark brown olivaceous pileus (Boedijn 1951). Our nrLSU & ITS phylogenetic tree clearly indicate the genetic dissimilarities of A. hemibapha from these taxa (Fig. 2)

Acknowledgements

The authors are grateful to R.E Tulloss (Herbarium Amanitarum Rooseveltensis, USA) and Linas V. Kudzma (USA) for providing sequence data. The Head, Department of Botany & Microbiology, HNB Garhwal University, Srinagar Garhwal for providing all kinds of facilities during the present study. Tahir Mehmood is thankful to UGC for providing fellowship. Mr. Aniket Ghosh is highly acknowledged for assistance in the field. Financial assistance received from Govind Ballabh Pant Institute of Himalayan Environment and Development (GBPPIHED) is gratefully acknowledged.

References


Oda T, Tanaka C, Tsuda M. 1999 – Molecular phylogeny of Japanese Amanita species based on nucleotide sequences of the internal transcribed spacer region of nuclear ribosomal DNA. Mycoscience 40, 57–64.