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Russula shanglaensis sp. nov. (Basidiomycota: Russulales), a new species from the mixed coniferous forests in District Shangla, Pakistan

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Abstract: A new species in the genus *Russula* from the mixed coniferous forest of District Shangla, Khyber Pakhtunkhwa, Pakistan is described and illustrated. Phylogenetic data derived from DNA sequences of nuclear ribosomal internal transcribed spacer (ITS), along with morphological characterizations, indicate the species is novel. *Russula shanglaensis* sp. nov. is distinct from other known species in subsect. *Virescentinae.* It is characterized by the convex to slightly depressed grayish pilei that often reflect rusty brown to light purplish spots, with very lightly striated to tuberculate striated margins. The occurrence of subglobose to ellipsoidal basidiospores (6.5–8 × 6–7 µm) with irregular incomplete reticulation is another distinguishing feature of the species. The species description includes photographs and line drawings illustrating key morphological features and a discussion comparing this species to morphologically and phylogenetically related species.

Key words: Himalaya, Hindu Kush, mycorrhizae, Russulaceae, taxonomy

1. Introduction

The genus *Russula* Pers. is one of the most abundant and widely distributed ectomycorrhizal fungal genera (Buyck et al., 2008) and is represented by 780 species worldwide (Kirk et al., 2008). Compared to several other genera, *Russula* fruiting bodies exhibit a high diversity of macro- and micromorphological as well as chemical features (Buyck et al., 2018). In Pakistan, the genus *Russula* is represented by only 28 species (Ahmad et al., 1997; Jabeen et al., 2017; Crous et al., 2018).

Species of *Russula* subsect. *Virescentinae* Singer are characterized by the presence of dermatocystidia, highly septate and more or less inflated hyphal extremities on the pileus surface, pileic epicutis consisting of an underlying stratum of rounded cells from which arise erect terminal binding hyphae or inflated pseudoparenchymatous hyphae, a nonamyloid suprahilar spot on the basidiospores, mild taste, pale colored spore prints, and granular, extracellular pigments in the pileipellis (Bi et al., 1993; Buyck and Adamcik, 2011).

During an exploration of the basidiomycetous fungi of Shangla, Pakistan in the years 2013 to 2015, several collections of *Russula* were encountered. This has resulted in the publication of one new species, *Russula mansehraensis* Saba, Caboň & Adamčík (Crous et al., 2018), while three collections, presumably belonging to *Russula* subsect. *Virescentinae* Singer, were selected for use in this study. Phylogenetic analyses reveal that these samples belong to a new species, *R. shanglaensis*, which is described in detail in this paper.

2. Materials and methods

2.1. Study area

District Shangla is an administrative unit of the Khyber Pakhtunkhwa Province of Pakistan (33.08° to 34.31° N, 72.33° to 73.01° E). This area is a part of the Hindu Kush mountain range and extends west to the great Himalayan range with an average elevation of 2000 to 3500 m above sea level (a.s.l.) (Ullah et al., 2019b). Climatically, District Shangla belongs to a moist temperate area, and the vegetation resembles Himalayan Mountain vegetation (Ullah et al., 2019a). Almost 90% of the area consists of mountains covered with coniferous and broad-leaved

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mixed forests, which provide an ideal habitat for a large number of mycorrhizal fungi. Due to the unique geographic position and rich floristic composition, the area is considered a hot spot of fungal diversity (Ullah et al., 2019c).

2.2. Sampling

Mushrooms were collected in the mixed coniferous forests of Shangla, Khyber Pakhtunkhwa, Pakistan between 2013 and 2015. Specimens were photographed using a digital camera (Nikon DS3300) and tagged. Field notes were made, and specimens were dried with a fan heater or oven at 50–60 °C, or with silica gel, until their water content was <15% and then stored in labeled packets or boxes. The specimens were deposited in the herbarium of Hazara University, Mansehra, Pakistan (HUP).

2.3. Morphological observations

Macromorphological features including color of the basidiomata; size and shape of pileus; lamellae color, lamellar attachment, and edge; stipe size, shape, color, and attachment to cap; and site data such as vegetation type, associated plants, and soil type were noted. For color descriptions, the Munsell soil color chart was followed (Munsell, 1975).

Micromorphological characteristics were observed using a compound light microscope (MX4300H Techno Co., Ltd., Japan) with an oil-immersion lens at a magnification of 1000×. Sections of lamellae, pileipellis, and stipitipellis were made and illustrated as observed in 3% aqueous KOH and 1% Congo red (w/v). Basidiospores were observed on the lamellae with Melzer's reagent. Measurements were recorded using a Carl Zeiss Jena ocular micrometer, and line drawings were made using a camera lucida. A total of 100 basidiospores were measured following Jabeen et al. (2017).

2.4. Molecular and phylogenetic analyses

DNA was extracted from 5–15 mg of dried specimen using a DNeasy Plant Mini Kit (QIAGEN Valencia, CA, USA). Polymerase chain reaction (PCR) and cycle sequencing were performed to obtain sequences of nuc-rDNA internal transcribed spacer region (ITS) using primer pairs ITS1F/ITS4 (White et al., 1990; Gardes and Bruns, 1993). For PCR conditions, we followed Ullah et al. (2019a, 2019c). DNA extraction, PCR, and cycle sequencing reactions were performed at the Chicago Botanic Garden Negaunee Institute for Plant Conservation Science and Action, Glencoe, Illinois. Sequencing was performed using an ABI-3730-XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) in the Pritzker Laboratory at the Field Museum of Natural History, Chicago, Illinois. Sequences produced for this study have been deposited in GenBank.

Sequences generated for this study were processed, edited, and assembled using Codon Code Aligner v.3.5.7 (CodonCode Corporation, Dedham, MA) and BioEdit (v 7.0). Sequences were screened for percentage of sequence identity using a BLAST search of GenBank (www.ncbi.nlm.nih.gov/genbank/). Nearest matches from the BLAST search and sequences from the study of Song et al. (2018) and Das et al. (2017) were retrieved in phylogenetic analyses. The ITS dataset was aligned automatically using MUSCLE v.3.8 (Edgar, 2004), followed by manual alignment with MESQUITE v.2.75 (Maddison and Maddison, 2005).

Phylogenetic analyses were conducted using Bayesian and maximum likelihood (ML) methods. Bayesian analysis was undertaken using BEAST 1.8.2 (Drummond and Rambaut, 2007) following Ullah et al. (2019c). Maximum likelihood analyses were run in RAxML-II-HPC (Stamatakis, 2006). One thousand rapid bootstrap replicates were run. Nodes were considered strongly supported when maximum likelihood bootstrap (MLB) results were ≥70% and Bayesian posterior probability (BPP) results were ≥0.95.

3. Results

3.1. Phylogenetic analyses

An initial BLAST search using sequences of the new species *Russula shanglaensis* sp. nov. and GenBank sequences of the ITS region returned matches of up to 94% sequence identity to *Russula mustelina* Fries (GenBank accessions: KT934005, AY061693) from Germany and Europe, respectively, with a third match of up to 93% identity to *Russula crustosa* Peck (EU598194) from the USA. These sequences are included in the dataset represented in Figure 1.

The ITS dataset (Table) consisted of 45 sequences and 731 characters, after it was manually trimmed in Mesquite. *Russula archaea* R. Heim and *R. camarophylla* Romagn., in subg. *Archaea*, plus *R. densifolia* Gillet, *R. nigricans* Fr., and *R. albonigra* (Krombh.) Fr., in subg. *Compactae*, were chosen as outgroups, following the studies of Song et al. (2018) and Das et al. (2017). *Russula shanglaensis* is resolved in subg. *Heterophyllidia* subsect. *Virescentinae* and is represented by three specimens (MK579185 = type, MK579183, and MK579184) with strong statistical support in both analyses, bootstrap and Bayesian posterior probability, MBS 100%, and BPP 1, respectively (Figure 1). The three specimens are separated and resolved as sister to a clade consisting of *R. xanthovirens* Y. Song & L. H. Qiu (MG786055, China) and *R. aureoviridis* J.W. Li & L.H. Qiu (KY767809, China); these clades are resolved as sister to the clade including *R. mustelina* Fr. (AY061693, KT934005), *R. crustosa* Peck (EU598194), and *R. cadaverolens* (KT935739, USA).

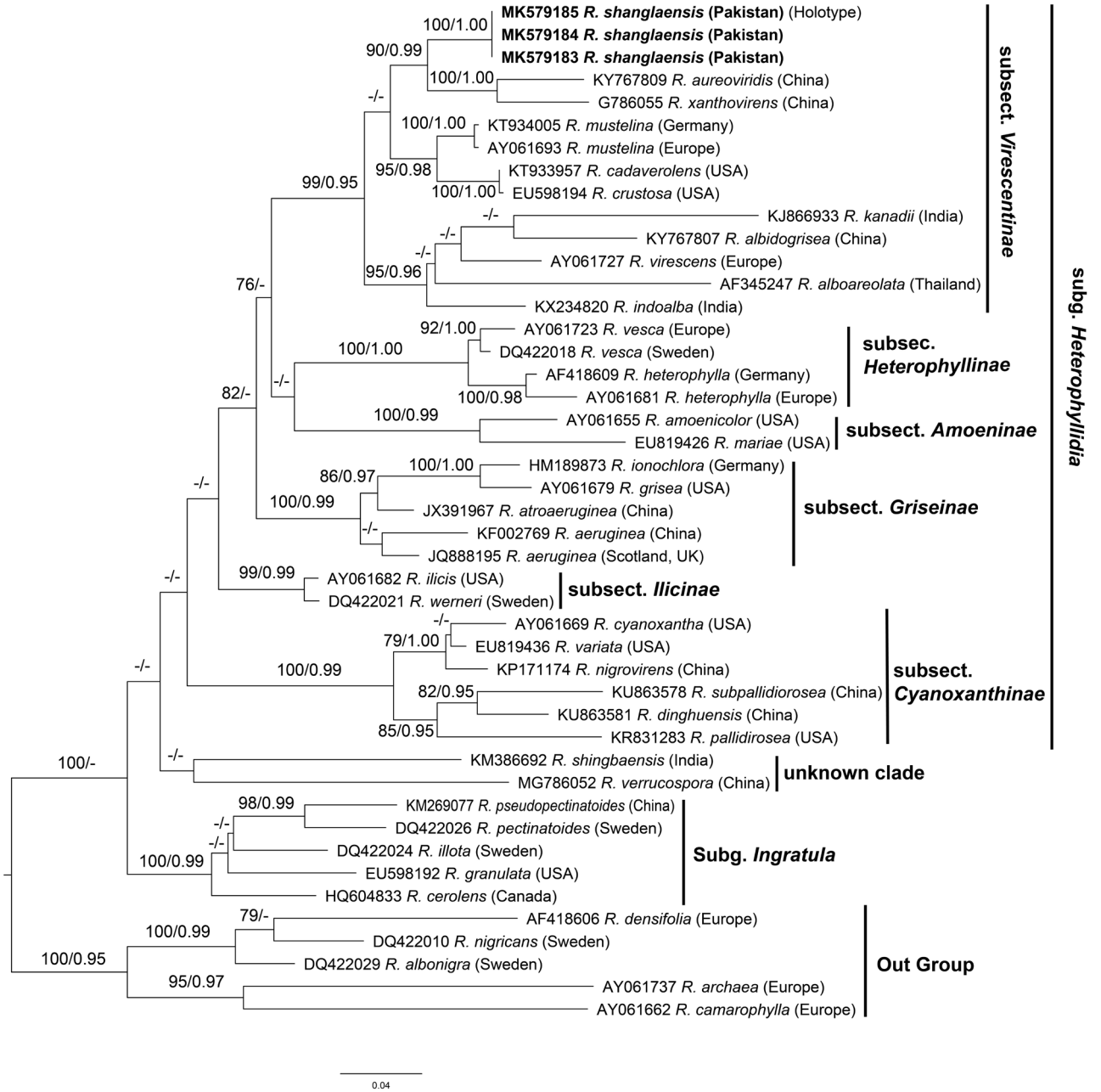

Figure 1. Phylogram generated from maximum likelihood (ML) analysis based on 45 rDNA ITS sequence data performed by RAxML. Sequences generated for this study are indicated in bold. Numbers above or below the branches indicate ML bootstrap percentages followed by Bayesian posterior probabilities.

3.2. Taxonomy

Russula shanglaensis S. Ullah, Khalid & Fiaz sp. nov., **Figures 2–3**

MycoBank: MB 830082

Etymology: The species epithet "*shanglaensis*" refers to its type locality, District Shangla.

Diagnosis: Distinguishing features of *Russula shanglaensis* include: pileus convex, slightly depressed, grayish, often with rusty brown to light purplish pink spots on the pileus, margins tuberculate striated, stipe ventricose when mature, often with yellowish tints at the base, basidiospores 6.5–8.5 × 5.8–7.8 µm, subglobose to ellipsoidal, ornamented with 0.2–0.4 µm high lines and ridges connected by a partial network or an incomplete irregular reticulation.

Holotype: Pakistan, Khyber Pakhtunkhwa, Shangla District, Ajmir, Sham Burj, solitary on humus soil at 2400

Table. Taxa of *Russula* included in molecular analyses, with voucher specimen numbers, country of origin, GenBank accession numbers, and references.

Taxon	Voucher	Location	ITS	References
R. mustelina	GENT:FH-12-226	Germany	KT934005	Looney et al., 2016
R. mustelina	503IS88	USA	AY061693	Miller and Buyck, 2002
R. shanglaensis	HUP-SUR433 (Holotype)	Pakistan	MK579185	This study
R. shanglaensis	HUP-SUR24	Pakistan	MK579183	This study
R. shanglaensis	HUP-SUR833	Pakistan	MK579184	This study
R. cadaverolens	TENN:067226	USA	KT933957	Looney et al., 2016
R. crustosa	PC BB2004-208	USA	EU598194	Data from GeneBank
R. xanthovirens	GDGM71147	China	MG786055	Das et al., 2017
R. aureoviridis	GDGM48786	China	KY767809	Das et al., 2017
R. indoalba	AG15–628	India	KX234820	Unpublished
R. virescens	1-211RUF24	Europe	AY061727	Miller and Buyck, 2002
Russula alboareolata	SUT-1	Thailand	AF345247	Manassila et al., 2005
R. kanadii	CAL1162	India	KJ866933	Dutta et al., 2015
R. vesca	210RUS24	Europe	AY061723	Miller and Buyck, 2002
R. heterophylla	209RUF	Europe	AY061681	Miller and Buyck 2002
R. albidogrisea	K15091234	China	Ky767807	Das et al. 2017
R. aeruginea	DG88	UK	JQ888195	Pickles et al. 2012
R. atroaeruginea	53626	China	JX391967	Li et al., 2013
R. grisea	2-1129IS75	USA	AY061679	Miller and Buyck 2002
R. ionochlora	BB28_302_Bv_Fa_070507	Germany	HM189873	Data from GeneBank
R. aeruginea	HKAS 78379	China	KF002769	Data from GeneBank
R. heterophylla	hue103 (TUB)	Germany	AF418609	Eberhardt, 2002
R. shingbaensis	1578736	India	KM386692	Das et al., 2014
R. pseudopectinatoides	HMAS251523	china	KM269077	Li et al., 2015
R. subpallidiorosea	K15052627	China	KU863578	Data from GeneBank
R. dinguensis	K15052704-3	China	KU863581	Zhang et al., 2017
R. pallidorosea	UTC00274382	USA	KR831283	Kropp, 2016
R. nigrovirens	HKAS 55042	China	KP171174	Zhao et al., 2015
R. cyanoxantha	207RUS24	USA	AY061669	Miller and Buyck, 2002
R. ilicis	563IC52	USA	AY061682	Miller and Buyck, 2002
R. werneri	IB1997/0786	Sweden	DQ422021	Data from GeneBank
R. mariae	JMP0063	USA	EU819426	Palmer et al., 2008
R. amoenicolor	311IX76	USA	AY061655	Miller and Buyck, 2002
R. vesca	AT2002091	Sweden	DQ422018	Data from GeneBank
R. verrucospora	K17092512	China	MG786052	Song et al., 2018
R. granulata	BB2004-226, PC	USA	EU598192	Looney et al., 2016
R. archaea	IS79	Europe	AY061737	Miller and Buyck, 2002
R. camarophylla	IS68	Europe	AY061662	Miller and Buyck, 2002
R. pectinatoides	AT2001049, UPS	Sweden	DQ422026	Data from GeneBank
R. illota	UE26.07.2002-3, UPS	Sweden	DQ422024	Data from GeneBank
Russula cerolens	UBC:F18895	Canada	HQ604833	Data from GeneBank
R. densifolia	ue116 (TUB)	Germany	AF418606	Eberhardt, 2002
R. nigricans	UE20.09.2004-07, UPS	Sweden	DQ422010	Data from GeneBank
R. albonigra	AT2002064, UPS	Sweden	DQ422029	Data from GeneBank

Figure 2. A–I. Basidiomata of *R. Shanglaensis* illustrating different features (A–D = HUP-SUR433 HOLOTYPE; E and G = HUP-SUR24; F, H, and I= HUP-SUR833). Scale bar: A–D = 25 mm; E and G = 15 mm, F, H, and I = 26 mm.

m a.s.l., in mixed forest under *Abies pindrow* (Royle ex D. Don) Royle, September 01, 2013, Sadiq Ullah HUP-SUR433.

Descriptions: Pileus 30–65 mm diam., first hemispheric convex with incurved margins then broadly convex with decurved margins, slightly depressed at center, light grayish olive (10Y 6/2) to grayish yellow-green (5GY 5/2) at disc, light grayish toward margin, often with rusty brown to pale yellow to light purplish pink spots on the pileus; surface smooth, slightly viscid when young, cuticle peeled easily; margins slightly striated to tuberculate striated, context white. Lamellae adnexed to adnate, 4–8 mm wide, whitish or pale yellow, unchanging after bruising, rarely unequal, rarely forking at midpoint, edges concolorous; lamellulae rare. Stipe 50–80 × 3.5–7.5 mm, central, clavate when young then ventricose at maturity, often tapering at both ends at maturity, irregularly hollow; surface whitish, dry, longitudinally rugulose. Context 1–3 mm thick, whitish, unchanging after bruising, often with yellowish tints at the base. Taste and odor not observed.

Basidiospores subglobose to broadly ellipsoidal to ellipsoidal, small, [100/3/3] (6) 6.5–8 (9) × (5.5) 6–7 (8) µm, avL = 7.5 ± 0.9, avW = 6.3 ± 0.9, Q = (1.02) 1.1–1.2 (1.28), avQ = 1.16 ± 0.12, hyaline in 3% KOH; ornamented with amyloid lines and ridges, 0.2–0.4 µm high, connected by a partial network or incomplete irregular reticulation; suprahilar spot not amyloid, apiculus relatively small. Basidia (30–) 37.5–50 (–53) × 7.5–10 µm, 2-4–spored, clavate, sterigmata 2.8–7.5 × 1.2–2.6 µm. Cheilocystidia 55–67 × 7.5–10 µm, emergent, fusiform to subcylindrical, often with mucronate or moniliform apices, thin-walled, with dispersed heteromorphous contents. Pleurocystidia

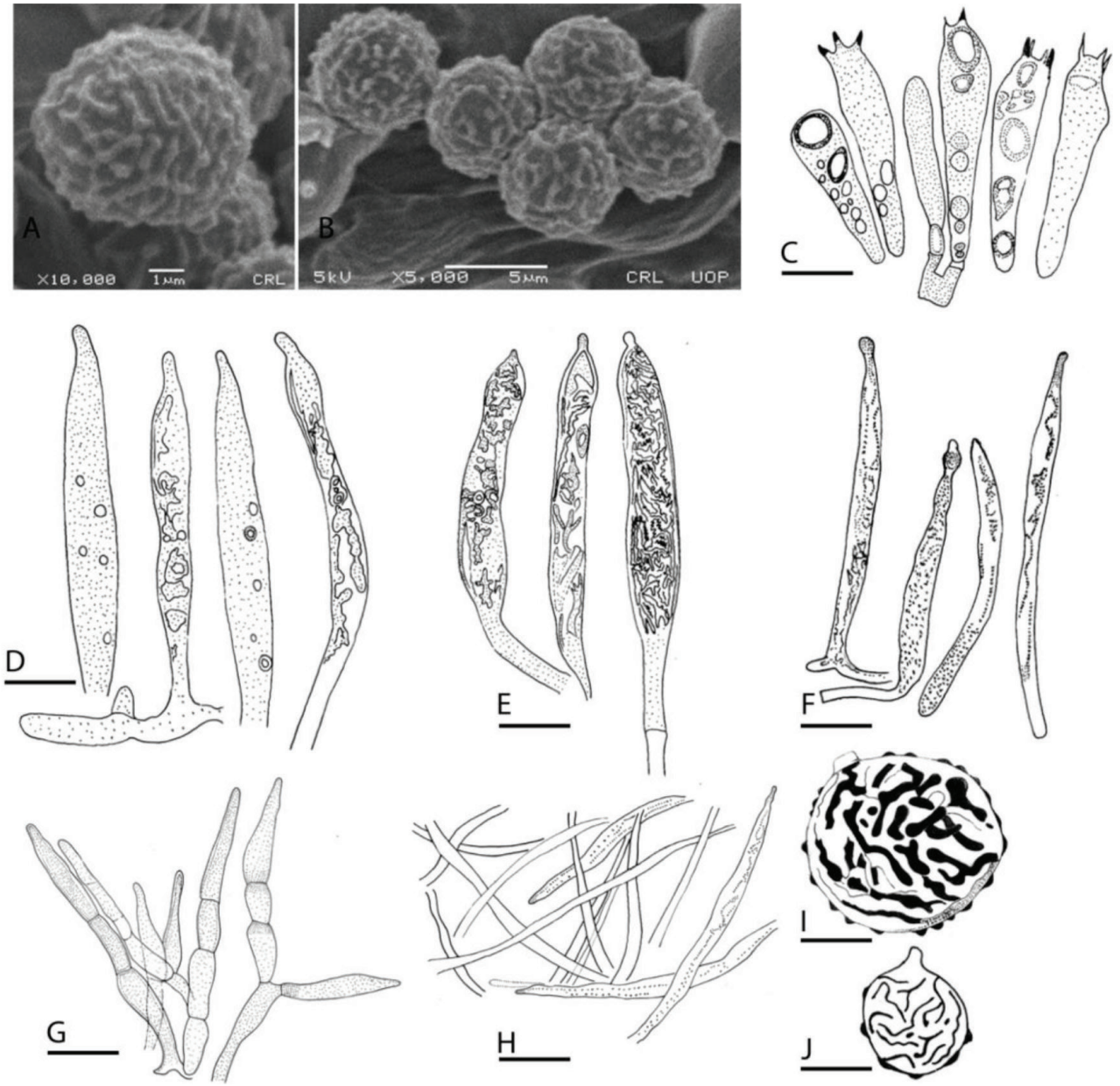

Figure 3. Micromorphological features of *Russula shanglaensis* (SUR433 HOLOTYPE). A–B. Basidiospores; C. basidia; D. cheilocystidia with contents in Congo red; E. pleurocystidia with contents in Congo red; F. pileocystidia with contents in Congo red; G. hyphal extremities in pileipellis (similar throughout cuticle); H. stipitipellis. Scale bars: A = 1 µm, B = 5 µm, C = 13.6 µm, D = 13 µm, E = 14.5 µm, F = 14 µm, G = 20 µm, H = 30 µm, I = 2.5 µm, J = 4.8 µm.

67.5–82.5 × 7–10.5 µm, clavate to subcylindrical, with mucronate or appendiculate apices, abundant, thin-walled, mostly with dispersed refractive heteromorphous content. Pileipellis composed of subparallel to erect hyphae (up to 5 µm broad) and cystidia; hyphal extremities having terminal cells measuring 17.5–37.5 × 5–7.5 (–15) µm, cylindrical to subconical with rounded apices. Pileocystidia abundant, one-celled, 45–70 × 2–7 µm, subulate to cylindric, with mucronate or capitate apices, with dispersed heteromorphous content. Hyphal extremities in pileipellis similar throughout cuticle. Stipitipellis composed of irregularly arranged to parallel hyphae (up to 5 µm broad); caulocystidia 40–130 × 3–7 µm, fusoid to lanceolate, with mucronate to moniliform apices.

Known distribution: In mixed coniferous forests of the Hindu Kush and Himalayan regions of Pakistan between 2300 m to 3000 m a.s.l.

Additional materials examined: Pakistan, Khyber Pakhtunkhwa, Shangla District, Ajmer forests, 2500 m a.s.l., on moist humus rich soil among/with mosses under *Juglans regia* and *Abies pindrow*, September 01, 2014, Sadiq Ullah HUP-SU24; Chakesar banda 2600 m a.s.l., on moist dark soil under *Abies pindrow* among/with mosses, August 27, 2015, Sadiq Ullah HUP-SUR833.

4. Discussion

Russula shanglaensis sp. nov. differs from other known species in subg. *Heterophyllidia* subsec. *Virescentinae* by its grayish pileus often with rusty brown to light purplish spots on the pileus, with lightly striated to tuberculate striated margins, rarely forked lamellae at midpoint mixed with rare lamellulae and basidiospores measuring 5.5–8 × 6–9 µm, subglobose to broadly ellipsoidal to ellipsoidal, weakly ornamented with 0.2–0.4 µm high lines and ridges, connected by irregular, incomplete reticulation.

The generated phylogram is mostly congruent with the studies of Das et al. (2017) and Song et al. (2018), with the exception of the clade labeled unknown, comprised of *R. verrucospora* Y. Song & L. H. Qiu and *R. shingbaensis* K. Das & S.L. Mill. that was resolved with nonsignificant bootstrap support and nonsignificant Bayesian posterior probability (Figure 1). The ITS phylogram and morphological characterizations resolve that *R. shanglaensis* is related to *R. xanthovirens*, *R. aureoviridis*, *R. mustelina*, and *R. crustosa*. However, *R. xanthovirens* has a yellowish green (#AAC381) to deep green (#182720), dry glabrous pileus surface, sulcate and cracked margin, solid stipe, short cheilocystidia (38–58.5 × 6.5–13.5 µm) and basidiospores with conical to subcylindrical warts measuring 0.4–0.8 µm high plus ridges up to 0.5 µm high (Song et al., 2018). Furthermore, in *R. xanthovirens* lamellulae are absent, while they are present in *R. shanglaensis*.

Russula shanglaensis is similar to *R. aureoviridis* as both have basidiospore ornamentation composed of ridges forming an incomplete reticulum; however, the latter has yellowish green to golden green pilei, frequently forked lamellae near the stipe, no lamellulae, short cheilocystidia (27–40 × 6–10 µm), short pleurocystidia (38–50 × 7–12 µm), and small basidiospores 5.3–6.1 × 4.8–5.5 µm (Das et al., 2017). Pilei of *R. mustelina* are very fleshy, thick, firm, and hard, and vary from yellow to pale yellow-brown, sometimes with green tones, large basidiospores (7–11 × 6–9 µm) ornamented with low isolated warts, heavy ridges, and fine lines, large basidia (50–70 × 6–10 µm) and long cystidia (70–100 × 8–12 µm) (Thiers, 1997). *Russula crustosa* differs by having a cap surface which breaks up into greenish patches around the margin, a pileus margin with radial grooves that match the gills on the underside, closely spaced adnate gills, and basidiospores that are elliptical and somewhat warted with a few fine interconnecting lines (Buyck, 2010; Buyck and Adamcik, 2011).

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