

A new species of *Russula* (Russulaceae) from India based on morphological and molecular (ITS sequence) data

Arun Kumar DUTTA, Soumitra PALOI, Prakash PRADHAN, Krishnendu ACHARYA*

Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University College of Science and Agriculture, University of Calcutta, Kolkata, West Bengal, India

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Abstract: *Russula kanadii* (Russulaceae, Basidiomycota) is described as a new species from West Bengal, India, and is assigned to the subgenus *Heterophyllidia*, section *Heterophyllae*, and subsection *Virescentinae*. A comprehensive description, photographs, and comparisons with morphologically similar and phylogenetically related species are provided. Inferences of its phylogenetic relationships within the genus are provided based on the sequence of the nuclear internal transcribed spacer (ITS) region.

Key words: Macrofungi, taxonomy, West Bengal

1. Introduction

West Bengal covers an area of approximately 88,752 km² (21°20'–27°32'N and 85°50'–89°52'E) and lies in the eastern part of India, bordering Bangladesh, Nepal, and Bhutan. This state is typical in its geographical position, having high Himalayan peaks in the north, a combination of Gangetic delta and coastal regions in the south, and large plains in between. Plant communities range from littoral and swamp forests (Sundarbans) and subtropical broadleaved forests (North Bengal hills 300 m–1650 m alt.) to subalpine forests (Sandakpu, Sabarkum, Phalut), while the tropical regions (Bankura, Purulia, Midnapur, Birbhum, Burdwan) are mostly covered by dry deciduous forests. The predominant trees present in the dry deciduous forest are *Anogeissus latifolia* (Roxb.) Bedd. (Combretaceae), *Terminalia bellirica* (Gaertn.) Roxb. (Combretaceae), *Shorea robusta* C.F.Gaertn. (Dipterocarpaceae), *Diospyros melanoxylon* Willd. (Ebenaceae), *Pterocarpus marsupium* Roxb. (Fabaceae), *Schleichera trijuga* Willd. (Sapindaceae), *Madhuca longifolia* J.F.Macbr. (Sapotaceae) etc. During the rainy season, the forest ecosystem dominated by *Shorea robusta* facilitates the fruiting of most ectomycorrhizal basidiomycetes (Pradhan et al., 2012, 2013), among which the members of *Russula* Pers. are very common.

The genus *Russula* is considered one of the most abundant and widely distributed ectomycorrhizal agaric genera (Buyck and Horak, 1999; Buyck et al., 2008), and the Indian subcontinent is no exception to it. Berkeley (1851, 1852, 1854, 1856, 1876) was the pioneer who made a

significant contribution by reporting *Russula cinnabarina* Berk., *Russula emetica* (Schaeff.) Pers., *Russula grossa* Berk., *Russula lepida* Fr., *Russula heterophylla* (Fr.) Fr. (as *R. furcata*), and *Russula sanguinea* Fr. (as *R. roseacea*) from Sikkim Himalaya (nestled in the Himalayan Mountains of the eastern side of India), and *Russula alutacea* (Fr.) Fr. and *Russula brevipes* Peck from Jammu and Kashmir (located in the northernmost part of the Indian subcontinent in the vicinity of the Karakoram and western Himalayan mountain ranges). A thorough literature survey reveals that, at present, *Russula* is represented by ca. 132 species from India, most of which have been reported from the subtropical to subalpine Himalayan region and a few from tropical regions (Das et al., 2014). Among the total number of reported species, ca. 24 taxa belong to the subgenus *Heterophyllidia* (Manjula, 1983; Atri et al., 1993, 1997; Das and Sharma, 2005; Buyck and Atri, 2011; Das et al., 2013, 2014; Kumar et al., 2014), most of which were originally described from either Europe or North America (viz. *Russula aeruginea* Lindblad, *R. anatina* Romagn., *R. cyanoxantha* (Schaeff.) Fr., *R. grisea* Fr., *R. heterophylla*, *Russula ionochlora* Romagn., *R. mustelina* Fr., *R. parazurea* Jul. Schäff., *Russula pseudoaeruginea* (Romagn.) Kuyper & Vuure, *Russula vesca* Fr., *R. virescens* (Schaeff.) Fr. etc.). Some newly described species from India under *Russula* subgenus *Heterophyllidia* include *Russula appendiculata* K. Das, S.L. Mill. & J.R. Sharma (Das et al., 2006), *Russula shingbaensis* K. Das & S.L. Mill. (Das et al., 2014), and *Russula sikkimensis* K. Das, Atri & Buyck (Das et al., 2013).

* Correspondence: krish_paper@yahoo.com

During a survey on the family Russulaceae in the tropical region of the state of West Bengal, one interesting *Russula* was found possessing characters distinct from those found in any previously described species, and is described here as a new species.

2. Materials and methods

2.1. Morphological protocols

Basidiocarps of the specimen were collected from West Bengal, India, during several field trips from 2011 to 2013. The morphological and ecological features of the collected specimen were noted in the field. Microscopic features were obtained from dried material by mounting free-hand sections of basidiocarps in 5% KOH, Melzer's reagent, and congo red. All tissues were also examined in cresyl blue to verify the presence of ortho- or metachromatic reactions. Specimens were then examined with a Carl Zeiss AX10 Imager A1 phase contrast microscope. Color codes and terms (mostly) follow *Methuen Handbook of Colour* (Kornerup and Wanscher, 1978). The terms used to describe lamellae spacing refer to the number of lamellae that run from the stipe to the pileus margin and do not include the lamellulae, whose spacing is indicated by the number of series present. Q value denotes the length/width ratio of the spores excluding ornamentation. Statistics for measurements of spores are based on 25 measurements from each of the collected four basidiocarps and given as a mean value (underlined); values in parentheses indicate minimum or maximum measured values. Scanning electron microscope (SEM) illustrations of basidiospores were obtained from dry spores (spore print) with platinum coating at different magnifications in high vacuum mode to observe patterns of spore ornamentation. This work was carried out with a Zeiss EVO-MA10 electron microscope at the Centre for Research in Nanoscience & Nanotechnology, University of Calcutta, Kolkata, India. The holotype specimen has been deposited in the Botanical Survey of India (CAL) and the isotype along with other specimens in the Calcutta University Herbarium (CUH).

2.2. Phylogenetic protocols

2.2.1. Taxon sampling

Twenty-one internal transcribed spacer (ITS) nrDNA sequences representing twelve species were used in the analyses, of which three sequences of the newly described species of *Russula* were generated as part of this study. The sequences represent ten species of *Russula* distributed over two well accepted subgenera: subgenus *Russula* emend. Sarnari (2 sequences) and *Heterophyllidia* Romagn. (17 sequences). *Albatrellus flettii* Morse ex Pouzar and *Boidinia aculeata* (Sheng H. Wu) E. Larss. & K.H. Larss. (as *Gloeocystidiellum aculeatum*) were selected as out-group taxa for rooting purposes following Miller and Buyck

(2002). The accession numbers of the newly generated ITS sequence as well as those pulled from GenBank for the purpose of conducting phylogenetic analysis for this study are cited in Figure 1.

2.2.2. DNA extraction, polymerase chain reaction, and sequencing

Genomic DNA was extracted from dried herbarium specimens (10–50 mg) using the Fungal gDNA Mini Kit (Xcelris Genomics, Ahmedabad, India). ITS region 1 and 2, and the 5.8S rDNA were amplified using primer pair ITS1 and ITS4 (White et al., 1990). The DNA fragments were amplified on an Applied Biosystems 2720 automated thermal cycler. A hot start of 4 min at 94 °C was followed by 35 cycles consisting of 1 min at 94 °C, 1 min at 56 °C, 1 min at 72 °C, and a final elongation step of 7 min at 72 °C. PCR products were checked on 2% agarose gel stained with ethidium bromide. PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN, Germany) and were subjected to automated DNA sequencing on an ABI3730xl DNA Analyzer (Applied Biosystems, USA) using primers identical with amplification for the ITS rDNA region. The newly generated sequences were then deposited in GenBank (www.ncbi.nlm.nih.gov).

2.2.3. Phylogenetic analysis

Sequences were edited with the CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA). The newly generated three ITS1-5.8S-ITS2 sequences of *R. kanadii* and those retrieved from GenBank were aligned with the help of ClustalX (Thomson et al., 1997) using the default setting. A final set of 24 sequences were aligned.

Phylogenetic analyses of the sequence data were performed in MEGA6 (Tamura et al., 2013) with the help of the maximum parsimony (MP) method, using the tree-bisection-regrafting (TBR) algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The branch lengths were calculated using the average pathway method (Nei and Kumar, 2000) and are in the units of the number of changes over the whole sequence. Branches corresponding to partitions reproduced in less than 50% trees are collapsed.

Beside MP analyses in MEGA6, Bayesian phylogenetic analyses were also carried out using MrBayes v3.2.2 (Ronquist et al., 2012) to determine whether different methods (MP vs. Bayesian) alter the resulting phylogenetic tree. For a given data set, the general time reversible (GTR) model was employed with gamma-distributed substitution rates. Markov chains were run for 100,000 generations, saving a tree every 100th generation. Default settings in MrBayes were used for the incremental heating scheme for the chains (3 heated and 1 cold chain), unconstrained branch length (unconstrained: exponential (10.0)), and uninformative topology (uniform) priors. The first 25%

of the sampling trees were discarded as the burn-in phase of each analysis, and the remaining trees were used to compute a 50% majority rule consensus to obtain Bayesian posterior probabilities (PPs).

3. Results

3.1. Molecular phylogeny

Phylogenetic analyses were performed on an ITS dataset of 24 sequences of which 22 sequences were *Russula* species, and the remaining two, viz. *A. flettii* and *B. aculeata* (as *Gloeocystidiellum aculeatum*), were used as an out-group for rooting purposes. Sequencing products of the newly described species collected from different places in subsequent years were approximately 675 nucleotides each. All sequences were aligned and the ends trimmed to create a dataset of 560 nucleotides that included 249 positions in the final dataset. The resulting consensus phylogram inferred from the four most parsimonious trees is shown in Figure 1, where the consistency index is 0.637584, the retention index is 0.767241, and the composite index is 0.562137 (0.489181) for all sites and

parsimony-informative sites. The phylogram obtained using Bayesian (MCMC) analyses showing the Bayesian posterior probability displayed overall similar topology with the phylogram obtained using MP analyses in MEGA6. Data obtained from MP analyses (bootstrap percentage) and Bayesian (MCMC) analyses (PPs) are indicated in Figure 1.

The analysis of nrDNA ITS sequence data distributed over twenty-two in-group species of *Russula* resulted in two well-supported clades (Clade-I and Clade-II) with high bootstrap support (100% BS) and posterior probability value (1.0 PP) (Figure 1). Selected members of *Russula* under subgen. *Heterophyllidia* Romagn. form a monophyletic clade with relatively strong support (100% BS and 0.8 PP; clade-I). Members belonging to Clade-I comprised species with a combination of characters like mild taste; a nonamyloid suprahilar spot on the spores; typically dense, septate, ramifying, and more or less inflated hyphal extremities on the pileus surface; one-celled pileocystidia when present; absence of deep colored spore-print; and presence of granular, extracellular

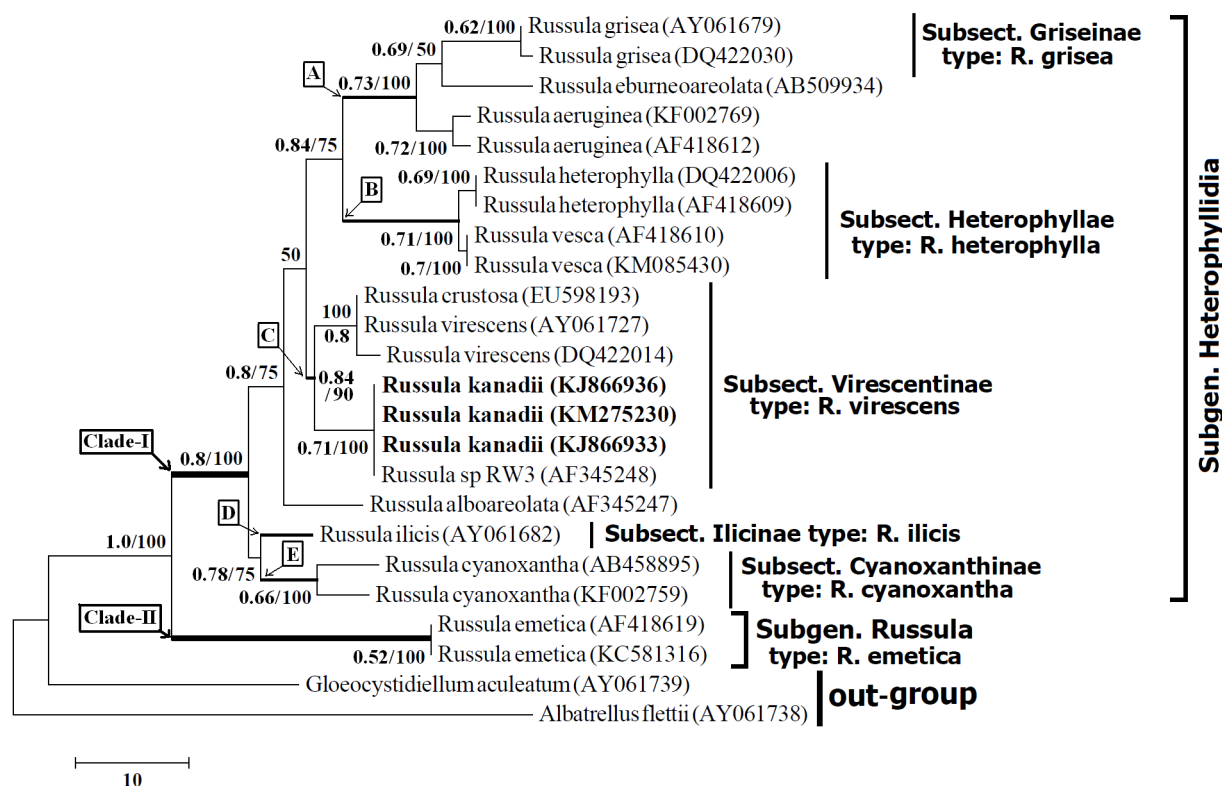


Figure 1. Maximum parsimony tree inferred from internal transcribed spacer nuclear ribosomal DNA sequences showing the position of *Russula kanadii*. Values to the left of the “/” are Bayesian posterior probabilities (PP), and those to the right indicate the maximum parsimony bootstrap (BS) support of that clade. PP values > 0.5 and BS values \geq 50% are shown. *Russula kanadii* is placed in bold font to highlight its phylogenetic position in the tree. Categorizations of *Russula* species within the tree follow the classification of Sarnari (1998).

pigments in the pileipellis when fresh (Sarnari, 1998; Buyck and Adamick, 2011). Clade B, with 100% BS and 0.52 PP support, contains two sequences of *Russula emetica* of subgen. *Russula* emend. Sarnari and are known to possess characters like a brightly colored pileus ranging from red, purple, or yellowish with smooth to pruinose or rarely areolate surface, smooth sulcate or tuberculately striate margin, white to reddish purple stipe, amyloid suprahilar spot, presence of hymenial and pileocystidia (Sarnari, 1998).

Altogether eleven species within Clade-I fall into five major subclades (A to E) with relatively high to significant bootstrap support and significant to moderate posterior probability values (PP). Subclade-A consists of five sequences, representing three species, viz. *Russula aeruginea* Lindblad, *Russula eburneoareolata* Hongo, and the type species *Russula grisea* of the subsection *Griseinae* Jul. Schäff., with 100% BS support and 0.73 PP. Clustering of *R. eburneoareolata* (GenBank accession no. AB509934) within the same subclade together with *R. grisea* is in contrast with the result obtained by Shimono et al. (2004) using nucLSU sequences. Subsection *Heterophyllinae* (Fr.) Jul. Schäff. (characterized by mild taste, bright-orange coloration of the context with FeSO_4 , poorly developed pileocystidia that never turn blue in Sulfovanillin; Sarnari, 1998) is represented here by both *R. heterophylla* and *R. vesca* Fr., clustered together with 100% BS support and 0.71 PP (subclade-B). *Russula virescens*, the type species of subsect. *Virescentinae* Singer, forms subclade-C together with *R. crustosa* Peck and the newly described *Russula kanadii* with 90% BS support and 0.84 PP. Subclade-D represents only a single species (*R. ilicis* Romagn.) of the subsect. *Ilicinae* (Romagn.) Buyck. Two sequences representing subsect. *Cyanoxanthinae* Singer (type species: *R. cyanoxantha*) cluster together forming subclade-E (MP-BS: 100%, PP: 0.66). Based on morphological characters and nucLSU sequence data although *R. alboareolata* belongs to the subsect. *Virescentinae* (Hongo, 1979; Shimono et al., 2004), in the present study, the position of *R. alboareolata* within clade-I remains unresolved as that species does not cluster with any of the five major subclades representing five subsections within the section *Heterophyllae* Fr. of the subgenus *Heterophyllidia* (Sarnari, 1998).

3.2. Taxonomy

Russula kanadii A.K. Dutta & K. Acharya, sp. nov. (Figures 2 and 3)

Mycobank: MB809301

Holotype: INDIA, West Bengal, West Midnapur District, Gurguripal forest, under *Shorea robusta* tree, 22°26'48"N, 87°12'25"E, alt. 56 m, 30 July 2013, Soumitra Paloi, SOUMITRA-03, (holotype: CAL 1162).

Diagnosis

Medium-sized (39–45 mm) broadly convex, white, cracked pileus, creamy lamellae with no lamellulae, entirely white stipe, white spore print, mild taste, presence of 3–5-layered pseudoparenchyma in pileipellis, narrow ($25\text{--}35.8 \times 3.5\text{--}6.1 \mu\text{m}$), one-celled, sulfovanillin-negative distinct pileocystidia towards the cap center, subglobose smaller ($4.5\text{--}7.2 \times 4.5\text{--}6.1 \mu\text{m}$; $Q = 1.08$) basidiospores with a nonamyloid suprahilar spot, virescens-type structure of pileipellis, finely disrupted-areolate suprapellis, presence of lanceolate to fusiform with appendiculate to moniliform apex hymenial cystidia on gill sides ($63\text{--}72 \times 8\text{--}11 \mu\text{m}$) and edges ($34\text{--}53 \times 9.5\text{--}11 \mu\text{m}$) filled with heteromorphous contents, and presence of abundant fusiform caulocystidia ($39\text{--}43 \times 7\text{--}14 \mu\text{m}$) with moniliform to appendiculate apex.

Description: Pileus 39–45 mm broad, broadly convex, plane to depressed at center; margin incurved, white (1A1), smooth when young, becoming cracked when mature, sulcate; surface creamy cinnamon, sometimes rusty spotted (Figures 2A and 3A), unchanging with KOH and NH_4OH but, saffron yellow (4A8) with FeSO_4 ; context 2 mm thick in half radius, thinning towards margin, creamy, unchanging when exposed but, turning faded lemon yellow or pale yellow to pastel yellow (1A3–1A4) with KOH. **Lamellae** adnexed, 4 mm wide, regular, cream colored throughout, saffron with FeSO_4 , lemon yellow with KOH, red with sulfovanillin; edges even, concolorous; lamellulae absent. **Stipe** central, 29 mm long, 8 mm broad, tapering towards the base, cavernate, fleshy, straight to slightly curved, moist, surface smooth, white; context white, turning saffron yellow (4A8) with FeSO_4 , translucent with KOH but, unchanging with NH_4OH . **Spore print** white (1A1). **Odor and taste** mild.

Basidiospores ($4.5\text{--}5.5\text{--}5.7\text{--}6.5\text{--}(7.2) \times 4.5\text{--}5.3\text{--}5.5\text{--}(6.1) \mu\text{m}$, $Q = (1.00\text{--}1.02\text{--}1.08\text{--}1.14\text{--}1.17)$, subglobose, hyaline, ornamentation amyloid, composed of small conical warts, measuring $0.25\text{--}0.32\text{--}(0.4) \mu\text{m}$ high and ridges forming incomplete reticulum and with few isolated warts, measuring $0.45\text{--}0.52\text{--}(0.6) \mu\text{m}$ high; suprahilar spot not amyloid (Figures 2B and 3B). **Basidia** ($32\text{--}34\text{--}47\text{--}(48) \times 6\text{--}9\text{--}(10) \mu\text{m}$, 2–4 spored, clavate, with so many oil droplets when viewed in KOH; sterigmata $3.6\text{--}3.9\text{--}(4.3) \times 0.7\text{--}0.9\text{--}(1.1) \mu\text{m}$ (Figure 2C). **Subhymenium** pseudoparenchymatous. **Lamellar trama** mainly composed of sphaerocytes measuring $25\text{--}26 \times 21\text{--}22 \mu\text{m}$. **Hymenial cystidia** ca. ($63\text{--}65\text{--}70\text{--}(72) \times 8\text{--}10\text{--}(11) \mu\text{m}$ on gill sides (Figure 2D), near gill edge ca. ($34\text{--}39\text{--}41\text{--}(53) \times 9.5\text{--}10\text{--}(11) \mu\text{m}$ (Figure 2E), lanceolate to fusiform with appendiculate to moniliform apex, thin-walled, mostly with heteromorphous contents that are restricted to the central or upper part of the cystidium. **Pileipellis** orthochromatic in cresyl blue, sharply delimited from

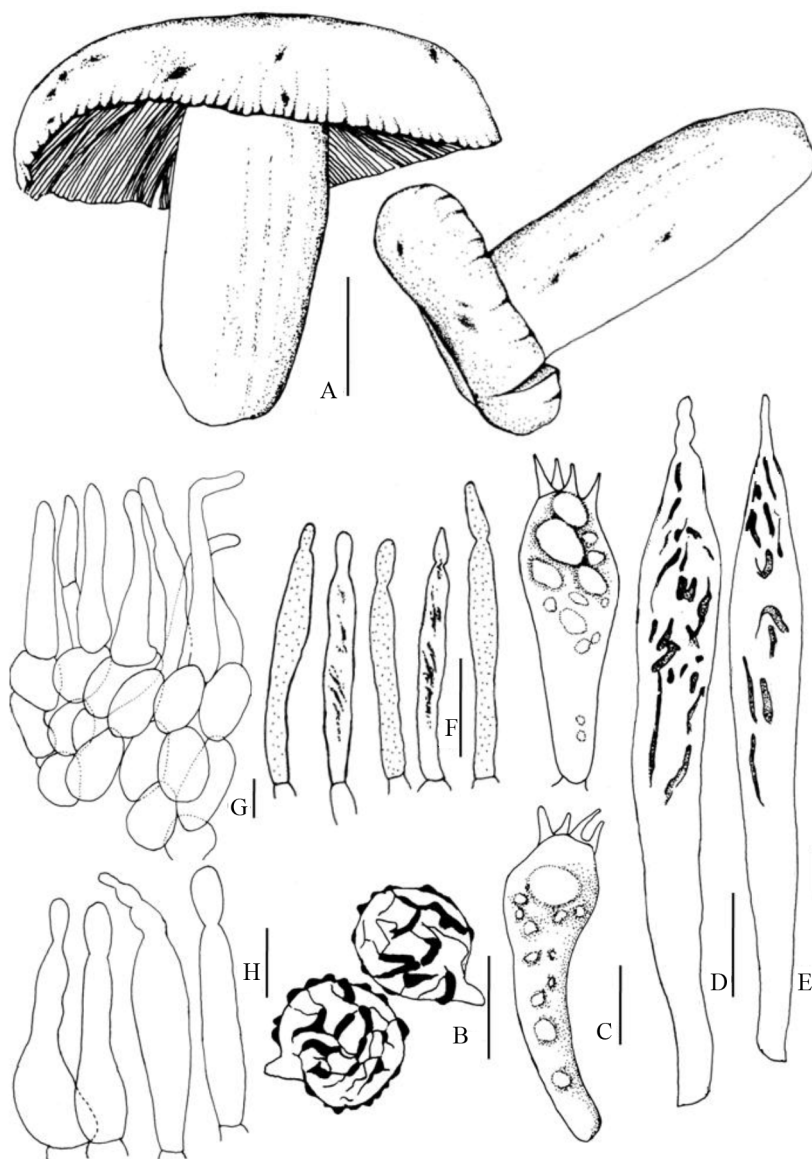


Figure 2. *Russula kanadii* (from the holotype). (A) Basidiomes (B) Basidiospores as seen in Melzer's reagent (C) Basidia (D) Hymenial cystidia on gill sides with contents indicated in some elements as seen in congo red (E) Hymenial cystidia near gill edge with contents indicated in some elements as seen in congo red (F) Pileocystidia with contents indicated in some elements as seen in congo red (G) Suprapellis near margin of pileus showing terminal and subterminal cells (H) Caulocystidia. Scale bars = 10 mm (A); 5 μ m (B) and 10 μ m (C-H).

underlying sphaerocytes of the context, distinctly divided into a dense, strongly gelatinized, ca. 47–65 μ m deep subpellis composed of horizontally oriented hyphae (2.5–3.5 μ m wide), some scattered with oleiferous fragments (3.5–5 μ m wide) often branched and less dense and less gelatinized, 53–75 μ m deep suprapellis of erect or repent hyphal ends. Incrustations absent. Pileocystidia (25–)28–32(–35.8) \times 3.5–5.7(–6.1) μ m, distinct towards the center

of the pileus but scattered to absent towards the margin, one-celled, very slender, subulate, mostly with mucronate-appendiculate to subterminally constricted, thin-walled, recognizable by their distinct, heteromorphous, sulfovanillin-negative contents (Figure 2F). Suprapellis near margin of pileus disrupted-areolate, composed of inflated hyphal terminations, terminal cells 21.5–40(–71) \times 3.5–5.7 μ m, subulate; subterminal cells 5.7 \times 4.3 μ m,



Figure 3. *Russula kanadii* (A) Habit (B) Scanning electron micrograph of basidiospores.

ellipsoid or subglobose (Figure 2G). **Stipitipellis** up to 36–57 μm thick, composed of subulate hyphal end, up to 3.5–6.5 μm broad and abundant caulocystidia measuring 39–43 \times 7–10.5(–14) μm , fusiform with moniliform to appendiculate apex (Figure 2H). **Stipe trama** composed of nested subglobose sphaerocytes, measuring 28.5–39 \times 25–28 μm .

Distribution and habitat: Growing solitary, common, under *Shorea robusta* tree, tropical deciduous forest, West Bengal, INDIA.

Etymology: named after Kanad Das for his contribution to the genus *Russula*.

Additional specimens examined: INDIA, West Bengal, Birbhum District, Shantiniketan, under *Shorea robusta*, 23°40'57"N, 87°40'24"E, alt. 67 m, 28 July 2013, Payel Mitra, CUH AM086 (isotype); East Midnapur District, Salbani forest, under *Shorea robusta*, 22°37'14"N, 87°14'58"E, alt. 77 m, 16 July 2012, Soumitra Paloi, CUH AM087; West Midnapur District, Gophghar forest, under *Shorea robusta*, 03 August 2011, Soumitra Paloi, CUH AM088.

4. Discussion

Russula kanadii is a not uncommon edible species in this part of West Bengal. Both the macro- and

micromorphological characters (see diagnosis) as well as the significant support for its phylogenetic placement (MLBS = 90%, PP = 0.84) confirm *Russula kanadii* within subclade-C of clade-I (Figure 1) or subsect. *Virescentinae* as it is defined in modern revisions (Sarnari, 1998; Buyck and Adamcik, 2011).

Russula sp. RW-3 (GenBank accession no. AF345248), collected from the northeastern part of Thailand, is suggested to be the same species as our Indian collections (99% identity for a 96% query coverage). Unfortunately, there is no other information available for this collection other than the white cap color. We doubt the correctness of the identification of *Russula* sp. RW-3 as *R. alboareolata* (Manassila et al., 2005) as it is genetically quite different (a BLAST result of 91% identity for a 92% coverage) from the voucher for *R. alboareolata* (GenBank accession no. AF345247) collected from the same part of Thailand (Figure 1).

Although *R. kanadii* is morphologically somewhat similar to *R. alboareolata* in terms of pileal shape, absence of lamellulae, hyphal pattern of the pileipellis, basidiospores shape and ornamentation, the combination of features like presence of pale dull yellowish coloured pileus at the center, minutely pruinose pileal surface, almost free and often forked lamellae, and larger sized spore (6.5–8.5 \times 5.5–7.0 μm) (Hongo, 1979) together with blasting through the NCBI databank (77% query coverage; 90% identity) distinctly separate *R. alboareolata* from *R. kanadii*.

Considering the white cap color, the present species resembles some of the previously reported taxa from India. However, earlier reported *Russula delica* Fr. and *Russula brevipes* have abundant lamellulae, larger spore size, capitate hymenial cystidia on gill sides and near gill edge, and amyloid suprahilar spot, signifying these species as completely unrelated to the newly described *R. kanadii* (Sarnari, 1998; Das and Sharma, 2005).

Russula eburneoareolata Hongo, originally described from Papua New Guinea and subsequently reported to occur in Japan (Shimono, 2004) and Korea (Shin, 2010), differs from the new species by its pale yellow to grayish yellow (ivory) pileus with a cuticle that is soon broken up into patches, developing a tuberculately striate pileus margin when old, and possesses almost free lamellae that may often be with a decurrent line, and broadly oval to subspherical (7–8 \times 5–6.5 μm) basidiospores (Hongo, 1973; Shin, 2010). Another possible close species within *Virescentinae* is *Russula heterosporoides* Murrill described from Florida (USA) (Buyck, 2010). The original description of the type specimen of *R. heterosporoides* by Murrill (1944) and later type studies by Buyck and Adamcik (2011) reveal a dull white pileus with slight yellowish center, striate-tuberculate pileus margin, but with distinctly ellipsoid basidiospores with a mean Q value of 1.55 and therefore quite different from the newly described *R. kanadii*.

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