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Morphological and phylogenetic characterisation of two species of family *Russulaceae* from Jammu and Kashmir, India

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ABSTRACT

In the present paper two species of family *Russulaceae* i.e., *Lactarius abieticola* and *Russula lakhanpalii* are reported for the first time from Jammu and Kashmir, India. *Russula lakhanpalii* belongs to subgenus *Heterophyllidia* of genus *Russula* and *Lactarius abieticola* belongs to subg. *Lactarius* of genus *Lactarius*. A detailed macro- and micromorphological descriptions coupled with the illustrations and nrITS-based molecular analyses are presented here.

Keywords: Macrofungi, nrITS, Phylogeny, Regional records

INTRODUCTION

The family *Russulaceae* Losty is one of the ectomycorrhizal families recognized under the order *Russulales*. It is well known for its ectomycorrhizal association with a wide range of host plants in both temperate and tropical forested biomes (Verbeken and Nuytinck, 2013; Looney *et al.,* 2018). The family exhibits global distribution and is represented by more than 3800 species (Wijayawardene *et al.,* 2022). Molecular phylogenetic analyses consistently recovered a monophyletic *Russulaceae* clade which now comprises 4 genera: *Russula* Pers., *Lactarius* Pers., *Lactifluus* (Pers.) Roussel and *Multifurca* Buyck & Hofstetter. Amongst all the genera of the family *Russulaceae*, *Russula* is the most species rich ectomycorrhizal genus with about 2,000 species known worldwide (Adamčík *et al.,* 2019), followed by *Lactarius* with more than 450 species (Verma *et al.,* 2022) and *Lactifluus* with more than 226 species (Lee *et al.,* 2021). Till date, more than 180 taxa of *Russula*, 95 taxa of *Lactarius* and around 33 taxa of *Lactifluus* are known from India (Das *et al.,* 2020; Verma *et al.,* 2022; Ghosh *et al.,* 2022).

Russula and *Lactarius* constitute about 80% of the family *Russulaceae*. Members of the genus *Russula* are distinguished by their brightly coloured, fragile sporocarps, amyloid spore ornamentation, brittle contexts with many sphaerocytes, and the presence of gloeoplerous components in various areas of their fruiting bodies (Das *et al.,* 2020; Ghosh *et al.,* 2020). On the other hand, species of *Lactarius* are characterized by the presence of latex; azonate to strongly zonate, glutinous to dry pileus surface with bearded to glabrous margins; absence of thick-walled elements in pileipellis and stipitipellis; basidiospores with amyloid ornamentation; sphaerocytes in the context; hymenial pseudocystidia and presence of lactiferous hyphae (Heilmann-Clausen *et al.,* 1998; Verma *et al.,* 2022).

During macrofungal surveys to different parts of the Union Territory of Jammu and Kashmir, India, the first author collected several specimens of macro fungi from coniferous and broadleaf forests. Detailed morphological examination along with molecular evidences indicated that these species are characterized for the first time from Jammu and Kashmir. Detailed macro- and micromorphological descriptions together with illustrations and nrITS-based phylogeny are presented in this paper.

MATERIALS AND METHODS

Site description

The Union Territory of Jammu & Kashmir is stretched between 32º 17' N to 37º 05' N latitude and 72º 31' E to 80º 20' E longitude and covers a total area of 42, 241km^2, with an average rainfall of 103 cm. The vast geographical area abundant in forests, varied topography and various climatic regimes provides a conducive environment for the growth of a large variety of mushrooms, and thus, there is an equal opportunity to explore the mushroom flora of various climatic and ecological regimes within this region of the country.

Macro- and micromorphology

Fresh basidiomes were collected and photographed in the field using a Nikon D5300 camera. Macromorphological descriptions were recorded from fresh specimens in the field along with habitat and associated hosts. Terminology of macromorphology is in accordance with Vellinga (1988). Colour codes were designated according to Kornerup and Wanscher (1978). Chemical spot tests with 10% KOH, $FeSO_4$, phenol, and guaiacol were performed on the pileus surface, stipe surface, and context of fresh basidiomes. All anatomical details were observed from dried samples by making freehand sections which were mounted in either 5% KOH, 1% Phloxine, or 1% Congo Red and examined under an Olympus CH20i compound. Micromorphological elements were drawn with a Camera lucida at 2000× magnification. Photomicrographs of the various elements were captured with a digital camera attached to an Olympus CX33 compound microscope. 40 basidiospores were measured from each of the specimens in Melzer's Reagent. Basidiospore measurements are represented as minimum-mean-maximum length × minimum-mean-maximum width and Q = length/ width ratio with Qm the average Q of all basidiospores.

DNA extraction, PCR amplification, and sequencing

A Plant II Kit (Macherey-Nagel) was used to isolate nuclear genomic DNA from 100 mg of dried basidiocarps. The ITS regions of the nuclear ribosomal DNA were amplified using primers ITS1 and ITS4 (White *et al.,* 1990). PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2 mM each dNTPs, 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA, 3% DMSO, 0.5M Betaine, and 5pM of forward and reverse primers. PCR amplification was carried out in 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 sec at 50 °C, and a final stage of 4 min at 60 °C. The PCR products were purified with QIAquick Gel Extraction Kit (QIAGEN, Germany) and then subjected to Sanger sequencing in an automated DNA sequencer (ABI3730xl DNA Analyzer, Applied Biosystems, USA) using the same primers used for amplification. All generated ITS sequences obtained were deposited in GenBank and accession numbers procured.

Phylogenetic analysis

In this study, two separate datasets including our taxa were created for phylogenetic analysis. The dataset for *Lactarius abieticola* contained 32 nrITS sequences, including ours, and the dataset for *Russula lakhanpalii* contained 30 nrITS sequences. The datasets consist of nrITS sequences of collections and reference sequences acquired from nBLAST search against GenBank (https://www.ncbi.nlm.nih.gov/genbank; (Altschul *et al.,* 1997; Clark *et al.,* 2016) and relevant published phylogenies (Bera *et al.,* 2020; Wang 2016; Ghosh *et al.,* 2020). Then these datasets were aligned with Mafft ver. 7 (Katoh and Standley, 2013). Maximum likelihood (ML) phylogenetic analysis of the nrITS sequences was performed using MEGA7 (Kumar *et al.,* 2016). One-thousand bootstrap (BS) replicates were analysed to obtain nodal support values. *Lactarius rufus* (KF241543, EF685089) were used as outgroups for *Lactarius abieticola* while *Russula albonigra* (DQ422029) and *R. nigricans* (DQ422010) was used as an outgroup for *Russula lakhanpalii*.

RESULTS

Phylogenetic inferences

The sequences generated for this study were deposited in the GenBank to obtain accession numbers. Initial BLAST search result of the nrITS sequence of the *Lactarius abieticola* collection (LAC-02) against the NCBI database exhibited 100% identity with *Lactarius abieticola* from China whereas BLAST search for *Russula lakhanpalii* exhibited 99.8% identity with *Russula lakhanpalii* from India. In the ITS phylogenetic tree our nrITS sequences obtained from our collections clustered within a clade formed by Chinese sequences of *Lactarius abieticola* with strong bootstrap support 95% (**Fig.1**) and *Russula lakhanpalii* sequences from India with bootstrap support 99.6% (**Fig.2**)

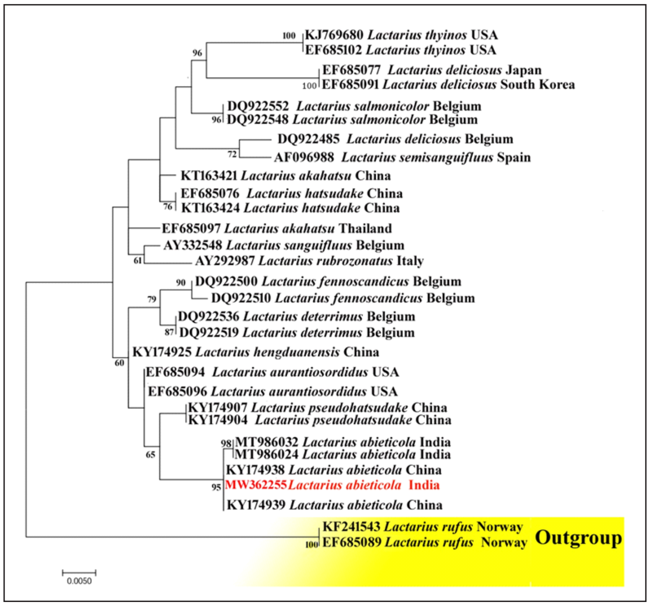

Fig. 1: Phylogram resulting from ITS-rDNA sequences: The evolutionary history was inferred by applying maximum likelihood in Mega 7.0. Bootstrap support values (>50%) obtained from the ML analysis are shown above or below the branches at nodes. *Lactarius abieticola* is highlighted in bold red.

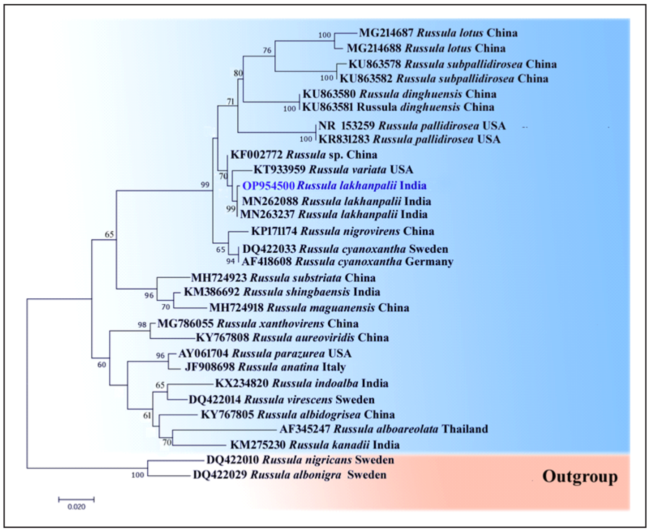

Fig. 2: Phylogram resulting from ITS-rDNA sequences: The evolutionary history was inferred by applying maximum likelihood in Mega 7.0. Bootstrap support values (>50%) obtained from the ML analysis are shown above or below the branches at nodes. *Russula lakhanpalii* is highlighted in bold blue

Taxonomy

Lactarius abieticola X.H. Wang *Cryptogamie Mycologie*, 2016, **37(4):** 493-508. **(Fig. 3 & 4)**

Genbank: MW362255

Basidiomata 70-90 mm long, Pileus 45-55 mm diam., convex to planoconvex with broadly depressed centre when young, gradually becoming infundibuliform when mature; surface smooth, moist, sticky, greasy, light orange (5A5), brownish orange (5C5), deep orange (6A8), faintly zonate; white tomentum when young, often transparently striate in the margin, never peeling; having greenish tinge on pileus when mature, brittle in consistency; margin involute when mature. Gills 1.5-3 mm broad, decurrent, rather crowded (16/cm at pilear margin), smooth, dark orange (5A8); lamellulae present in 2-3 length; bifurcation, edge entire. Stipe 60-75 × 10-12 mm, central, cylindrical sometime slightly swollen at the base, deep orange (5A8), with no bruising having green colouration on it. Context thick at pileus, stuffed in stipe when young and becomes hallow when mature, light orange (5A4), and deep red (10C8) with guaiac. Latex scanty, orange colour, unchanging. Taste hardly to weakly acrid. Odour pleasant.

Fig. 3: ***Lactarius abieticola*****. a-c.** Basidiomata in the field; **d&e.** Basidiomata in the base camp; **f.** Cheilomacrocystidia; **g.** Basidia; **h&i.** Pleuromacrocystidia; **j.** Pleuropseudocystidia; **k&l.** Transverse section through pileipellis. **m.** Basidiospores under light microscope. Scale bars: a-b =20 mm, f m =10 µm

Basidiospores 7.5-8.7 - 9.5 × 6.2-7.1 - 7.8 µm (n= 40, Q = 1.0-8-1.32-1.38), subglobose to ellipsoid; amyloid, ornamented, composed of irregular ridges and warts forming incomplete recticulum; suprahilar spot inamyloid. Basidia 40-62 × 12.3-16.8 µm, clavate to subclavate, hyaline, thin-walled, four-spored; sterigmata 5-10.5 × 0.4-1 µm. Pleuromacrocystidia abundant, 37-75 × 4.5-8.5 µm, emergent up to 35 µm, sublanceolate, subfusiform, acuminate to subobtuse apices, originating from sub-hymenial region, dense, granular to crystalline content. Gill edge sterile; Cheilomacrocystidia scarce, 25-55.5 × 4.0-7.0 µm, emergent up to 25 µm, cylindric to subcylindric with fusoid, thin-walled; dense, granular to crystalline content. Pseudocystidia present, 6-7.9 µm wide, emergent up to 20 µm, cylindrical obtuse at apex. Subhymenium up to 30 µm thick, cellular. Lamellar trama composed of golden lactifers and abundant sphaerocytes with connecting hyphae. Pileipellis up to 85-130 µm thick, ixocutis, composed of compact interwoven thin-walled, septate, branched repent hyphae (2-4 µm wide) under a very thin layer of gluten. Stipitipellis up to 50-80 µm thick, with an ixocutis composed of interwoven, septate, thin-walled hyphae under a very thin layer of gluten; hyphae 3-5 µm wide. Clamp connections absent in all tissues.

Habit and habitat: Solitary to scattered in association with *Abies* sp. in coniferous forests of Sarthal in Kathua district of Jammu and Kashmir.

Distribution: Originally described from China and recently

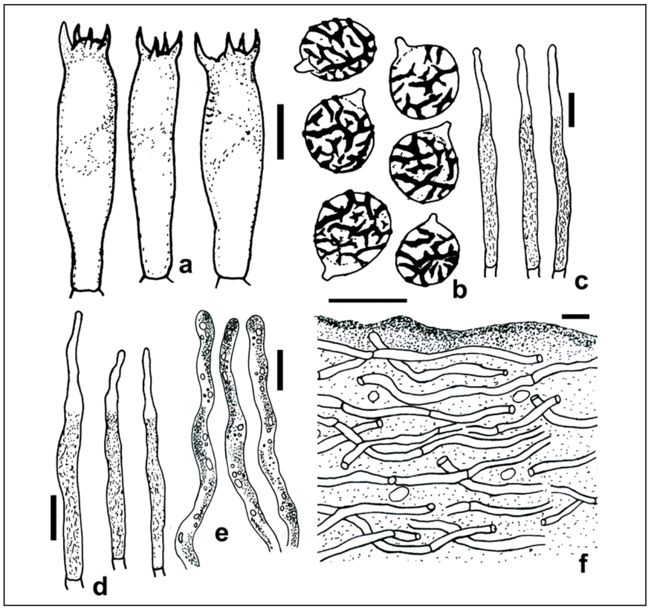

Fig. 4: ***Lactarius abieticola.*** **a.** Basidia; **b.** Basidiospores; **c.** Cheilomacrocystidia; **d.** Pleuromacrocystidia; **e.** Pleuropseudocystidia; **f.** Transverse section through Pileipellis. Scale bars: a-e =10 µm

reported from Sikkim and Arunachal Pradesh, and is now know from Jammu and Kashmir, India

Specimens examined: India, Jammu and Kashmir, Sarthal, Kathua district, 32°50′247″N, 074°46′370 ″E 3146 m a.s.l., forest dominated by *Abies* sp. 24 August 2019, Komal Verma, LAC-02, JUH-745.

Notes

Lactarius abieticola was originally reported from China (Wang 2016), but recent reports of this species have come from the Indian states of Sikkim and Arunachal Pradesh. (Bera *et al.,* 2020). It belongs to *L.* sect. *Deliciosi* of *L.* subg. *Lactarius*. This species is characterized by the combination of macro- and micromorphological characters such as medium sized orange colored basidiomata (pileus 30-65 mm diam, stipe 55-60 × 10-15 mm) with faint greenish coloration on bruising, the white aspect on pileus, a faintly zonate cap, orange unchanging latex, ixocutis nature of pileipellis and stipitipellis and its occurrence under *Abies* sp. (Wang 2016, Bera *et al.,* 2020). The present collection is morphologically and microscopically similar with the holotype reported from China and the specimen reported from the Indian states of Sikkim and Arunachal Pradesh. Moreover, the nrITS sequence also shows the present collection's placement within the clade formed by the Indian and Chinese collection.

Russula lakhanpalii A. Ghosh, K. Das & R.P. Bhatt *Nova Hedwigia* 2020, **111 (2):** 115-130. **(Fig. 5 & 6)**

Genbank: OP954500

Basidiomata medium sized, Pileus 35-89 mm in diam., hemispherical when young, becoming convex, planoconvex, applanate to uplifted, with broadly depressed center, margin decurved to plane, sometimes uplifted with maturity, entire; surface dull when dry, shiny, viscid when moist, smooth, becoming areolate when mature, yellowish white (2A2), pale yellow to pastel yellow (3A3-4) and centrally light yellow to greyish yellow (4B4-5) or brownish orange (5C4-6); cuticle peeling 1/3rd of the radius. Pileus context 12-20 mm thick, compact, brittle, firm, chalky white (1-2A1), unchanging on bruising or cutting. Lamellae adnexed or subdecurrent, crowded (22-25/cm at pileus margin), chalky white (1-2A1), 3-5 mm broad, unequal, irregularly forked, soft and flexible, edges entire, concolorous. Lamellue present, up to 3-4 series. Stipe 45-70 × 15-20 mm, cylindrical, dry, smooth, tapered at base, central, solid, firm, chalky white (1-2A1) with pale yellow to pastel yellow (3A3-4) flush present at base or up to middle, surface longitudinally venose, turning light yellow to yellow with KOH and reddish brown with guaiacol respectively, $FeSO_4$ negative. Stipe context solid, chalky white (1-2A1), unchanging after bruising or on exposure, solid; turning reddish brown (8E6-8) after application of guaiacol, $FeSO_4$ negative. Odour not distinctive. Taste mild. Spore print white.

Fig. 5: ***Russula lakhanpalii***. **a&b.** Basidiomata in the field; **c&d.** Basidiomata in the base camp; **e.** Cheilocystidia; **f-h.** Basidia; **h&i.** Transverse section through lamellae showing basidia and Pleuromacrocystidia; **j.** Pleuropseudocystidia; **i&j.** Transverse section through pileipellis **k.** Basidiospores under light microscope. Scale bars: a-b =20 mm; e h =10 μm; i= 5 μm; j-k 10 μm

Basidiospores 7.5-8.5-9.5 × 6.5-7.0-7.5 μm, Q= 1.15-1.20-1.26) μm, subglobose to broadly ellipsoid; ornamentation amyloid, consisting of numerous blunt warts that are connected by ridges forming incomplete reticulation and intermixed with isolated warts; suprahilar plage inamyloid; apiculi up to 1 μm high. Basidia 32-34.5-40 × 8-10.5-12 μm, 4-spored, subclavate to clavate; sterigmata up to 4 μm long. Pleurocystidia 48-59.0-70 × 8.0-9.5-10.5 μm subclavate to fusiform with capitate apex, emergent up to 20 μm beyond the basidiole tips; filled with dense, fibrillose to thin crystalline content, turning grey-black with sulphovanillin. Lamellae edges fertile with basidia and cystidia. Cheilocystidia 38-42.5-47.5 × 7-7.5-9.5 μm, cylindrical to subclavate with capitate apex, completely or partly filled with dense, heteromorphous fibrillose to thin crysrtalline content, turning grey-black with sulphovanillin. Subhymenium layer up to 12 μm thick, pseudo-parenchymatous. Hymenophoral trama mainly composed of large nests of sphaerocytes and hyphal elements. Pileipellis metachromatic in cresyl blue, sharply delimited from the underlying sphaerocytes of the context, up to 170 μm thick, two-layered, divided up to 60 μm deep suprapellis composed of suberect to erect branched, septate hyphal terminations, arranged in a densely turf of trichodermal structure and dispersed pileocystidia; subpellis up to 110 µm deep, composed of horizontally, irregularly moderately dense, pilear hyphae. Hyphal terminations near the pileus margin usually branched at the subterminal cells or the cells just below, occasionally slightly flexuous, thin-walled; terminal cells (10-15.0-30) × (3.5-4.5-5.5) µm, mainly cylindrical to subcylindrical, apically obtuse and wider near base; subterminal cells usually equal in size. Hyphal terminations near the pileus centre with slightly shorter terminal cells measuring (8-13.5-20) × (1.5-2.5-3.5) µm, mainly cylindrical to subcylindrical, apically obtuse wider near base; subterminal cells equally wide. Pileocystidia near the pileus margin 1-celled, numerous, cylindrical to subclavate, small and scattered in the tufts of the suprapellis, subpellis and underlying context becoming gradually longer for becoming embedded in the tissue; terminal cells (25-45.5-68.5) × (3-5.4-8) µm, apically capitate, mucronate, obtuse or appendiculate, filled with dense, heteromorphous, fibrillose to thin crystalline content, negative in sulphovanillin. Pileocystidia near the pileus centre 1-celled; terminal cells (20-40-58) × (4-5.5-6.5) µm, cylindrical to subclavate or slightly tapered towards tips, rounded-obtuse apex. Clamp connections absent from all tissues.

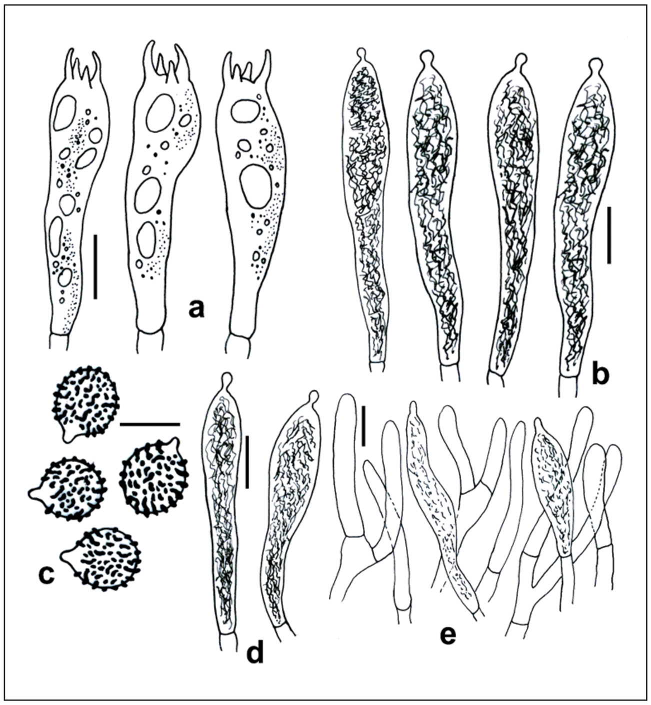

Fig. 6: ***Russula lakhanpalii*** **a.** Basidia; **b.** Pleurocystidia; **c.** Basidiospores; **d.** Cheilomacrocystidia; **e**. Transverse section through Pileipellis showing Pileocystidia. Scale bars: a-e =10 µm

Habit and habitat: Solitary, scattered to gregarious, growing in association with species of *Quercus* sp. in temperate mixed forests.

Distribution: Previously reported from Uttarakhand, and now reported from Jammu & Kashmir, India.

Specimen examined: India, Jammu and Kashmir, Rajouri, Darhal, Dodaj, N 33°27.651' E 074°25.029', 1602 m a.s.l., 09 September, 2021, Komal Verma, KV-42/21, JUH-895.

Notes

Russula lakhanpalii is a member of sect. *Cyanoxanthinae* of subg. *Heterophyllidia* of genus *Russula*. This species was recently discovered in the Indian state of Uttarakhand and characterized by the combination of a yellowish white to pale yellow areolate pileus with orange-brown centre, presence of lamellulae, frequently forked lamellae, white spore print, cystidia content that is unchanging in sulphovanillin and pileipellis metachromatic in cresyl blue (Ghosh *et al.,* 2020). In terms of macro and micromorphology, the current species from Jammu & Kashmir is quite similar to the holotype. In addition to this, phylogenetically the nrITS sequence of present collection exactly clustered in the clade formed by holotype of *Russula lakhanpalii*.

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