Lactarius subg. Lactarius (Russulaceae) in Indian Himalaya: two new species with morphology and phylogenetic inferences

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Abstract – The present paper deals with two novel species in Lactarius subg. Lactarius collected from western Himalaya (India): Lactarius indozonarius sp. nov. and Lactarius thindii sp. nov. Morphological descriptions, illustrations and nrITS-based phylogenetic analyses of these species are presented.

INTRODUCTION

Lactarius Pers. is a large ectomycorrhizal genus playing a significant role as a mycobiont in different ecosystems, from tropical to temperate and arctic-alpine regions (Verbeken & Nuytinck 2013, Leonardi et al. 2016). Species traditionally placed in L. subg. Lactarius (one of the three subgenera) are characterized in the field by a slimy to viscid or shiny, rarely dry, zonate to azonate pileus, with or without a hairy margin; zonate to azonate pileus context; a dry or sticky stipe which is often scrobiculate and variable latex colour. Microscopically, they typically have an ixocutis to ixotrichoderm (more rarely a cutis to a trichoderm) type of pileipellis (Le et al. 2007, Verbeken & Nuytinck 2013). This subgenus is very well represented in all Northern hemisphere continents (Le et al. 2007).

In Indian Himalaya, macrofungal explorations by various workers, especially in the past 25 years, reported 29 species so far. These are Lactarius abbotanus K. Das & J. R. Sharma, L. alnicola A. H. Sm., L. byssaceus K. Das & Verbeken, L. controversus Pers., L. dařianus K. Das et al., L. deliciosus (L.) Gray, L. deterrimus Gröger, L. dhakurianus K. Das et al., L. elaioviscidus K Das & Verbeken, L. fennoscandicus Verbeken & Vesterh., L. indigo (Schwein.) Fr.,

Fig 1. Map of Indian Himalayan region showing sites for the occurrences (only small patches in three states) of Lactarius subg. Lactarius members with green dots.
in Uttarakhand, Sikkim and Himachal Pradesh (Fig. 1) while the rest of Indian Himalaya remains unexplored.

While undertaking routine macrofungal surveys in temperate forests of Uttarakhand (Figs. 3-6), two interesting taxa were encountered by one (PU) of us. Thorough morphological examination and nrITS-based phylogenetic data of these taxa revealed two novel species within *Lactarius* subg. *Lactarius* and we describe them here as *Lactarius indozonarius* (*L*. sect. *Zonarii*) and *Lactarius thindii* (*L*. sect. *Colorati*).

**MATERIAL AND METHODS**

Collecting and morphology

Macromorphological characters and field data were recorded in the forest or basecamp from the fresh and dissected young to mature basidiomata. Macrochemical colour reactions on pileus, stipe surface, context and latex were also recorded. Images of the fresh basidiocarps were captured with the help of Canon Power Shot SX 50 HS and Sony Cyber Shot W730 cameras. Color codes and terms follow Methuen Handbook of Color (Kornerup & Wanscher 1978). Micromorphological characters were observed with the help of a light microscope (Olympus CH20i) from dry material mounted in a mixture of 5% KOH, 1% Phtloxin and 1% Congo red. Drawings of micromorphological elements were made with a camera lucida at 2000 × magnification. Microphotographs were captured with a digital camera attached to the Olympus CH20i and Olympus-CX2iLED light microscopes. Basidiospores were mounted in Melzer’s reagent and measured in lateral view excluding the height of ornamentation and apiculus. Basidiospore measurements are presented as minimum-mean-maximum length × minimum-mean-maximum width and Q = length/width ratio.

Scanning Electron Microscope (SEM) images of basidiospores were obtained from dry basidiospores that were directly mounted on a double-sided adhesive tape pasted on a metallic specimen-stub and then scanned with silver coating at different magnifications in high vacuum mode (20 KV). SEM-studies were carried out with a ZEISS EVO 18 SPECIAL EDITION model installed at USIC Dept., HNBGU Srinagar (Garhwal), India. Herbarium codes follow Thiers (continuously updated).

DNA isolation, amplification and sequencing

Genomic DNA was isolated from 100 mg of (dry) tissue of the basidiomata using the XcelGen Plant Fungal gDNA kit (XG2416-01) following the manufacturer’s instructions. The nrITS region was amplified with primer pairs ITS1-F and ITS4-B (White *et al*. 1990). PCR amplification was performed using a thermocycler (ABI, Veriti) programmed for 3 min at 95°C, followed by 1 min at 94°C, 30 cycles of 45 sec at 48°C, 45 seconds at 72°C and a final stage of 10 min at 72°C. The PCR amplicon was purified with exosap enzymatic purification as per the manufacturer instruction (ABI). After the purification the products were subjected to Sanger sequencing using an ABI 3730XL DNA analyzer. Forward and reverse strands were
Fig 2. Phylogram generated from ITS-rDNA sequences: The evolutionary history was inferred by using the Maximum Likelihood method in RAxML v. 8.0.24. Bootstrap support values (>50%) obtained from the ML analysis are shown above or below the branches at nodes.
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Two novel species of Lactarius from India sequenced separately with the ITS1 and ITS4 primers using BDT v3.1 Cycle sequencing kit. Sequences generated in this study are deposited in GenBank.

Phylogenetic analysis

A dataset with reference nrITS sequences was assembled by considering the sequences from studies on L. subg. Lactarius (Le et al. 2007, Nuytinck et al. 2007, Das et al. 2015, Uniyal et al. 2017, Wang 2017, Shi et al. 2018), and a BLAST (Altschul et al. 1997) search in GenBank (Clark et al. 2016) and UNITE (Kõljalg et al. 2013). We aimed for the inclusion of as many Asian representatives as possible. Phylogenetic analyses were carried out to establish the relationships of our new taxa. Species of L. subg Plinhogalus were selected as outgroup. Multiple sequence alignment was performed using MAFFT v.7 (Katoh & Standley 2013), applying the E-INS-I strategy. Alignments were manually edited in Bioedit v 7.2.5 (Hall et al. 1999). Maximum Likelihood (ML) analyses were conducted in RAxML v. 8.0.24 (Stamatakis 2014) on the CIPRES Science Gateway (Miller et al. 2010).

RESULTS

Phylogenetic inference

Our ITS-based phylogenetic analysis being conducted with 109 nucleotide sequences (Fig. 2) supports the placement of L. indozonarius sp. nov. and L. thindii sp. nov. in L. subg. Lactarius. Although this subgenus is paraphyletic in our analysis with L. subg. Russularia nested inside, all close relatives of the newly described species are traditionally considered to be members of L. subg. Lactarius.

Delimiting sections and subsections in Lactarius is impossible based on ITS sequences only, but the two new species described here fall in well-supported species groups that also make sense morphologically. This is discussed in more detail in the taxonomy section of this manuscript.

Taxonomy

Lactarius indozonarius Uniyal, K. Das & Nuytinck, sp. nov. Figs 7-20

MycoBank: MB823734
Genbank: MG028638 (holotype), MG456761 (paratype)
Diagnosis: Differs from L. zonarius by its zoned context and smaller basidiospores with higher (up to 1.5 µm) ornamentations and by its nrITS data.
Holotype: INDIA: Uttarakhand, Pauri Garhwal, Chaubatta, N30º 09.697’ E078º 51.195’, 1913 m asl., growing in a mixed temperate forest dominated by Quercus, Rhododendron, Myrica, Cedrus and Pinus sp., 16th July 2016, P. Uniyal, PU-1087 (CAL:).
Etymology: Referring to morphological resemblance and close affinity with L. zonarius, a European species.
Pileus 60-122 mm diam., convex to plano-convex, shallowly depressed, finally infundibuliform; surface viscid to glutinous, zonate, increasingly matted-
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fibrillose towards margin, straw yellow to wax yellow (3B4-5) when young, light yellow (4A4-5) to greyish yellow (4B3) or corn yellow (4B5) with age, finally brownish yellow (5C6-8), sometimes paler near margin, hairs up to 2 mm long, turning brownish yellow (5C7); zonations greyish orange (5B3-4); margin inrolled to decurved, undulate to entire, radially ridged when young, hairy. Lamellae subdecurrent, very crowded (15-17 L+1/cm), pale yellow to pastel yellow (3A3-4) when young, greyish yellow (4B4-5) with age, brownish orange (5C5) where damaged, some forked near attachment; lamellulae numerous. Stipe 45-72 × 13-29 mm, cylindrical, equal, scrobiculated, pale yellow (3A3) to greyish yellow orange (4B5) on handling or with age. Context 7–10 mm thick, white to yellowish white (2A2), zonate, zonations watery, continuations in zones of pileus surface, gradually disappearing toward centre; unchanging with FeSO4 and KOH, turning pastel red (8A4–5) at first with guaia, then brownish red (8C8); taste mild to slightly acrid; stuffed to hollow in stipe. Latex scarce, white, unchanging; taste acrid on tissue and in isolation. Odor pleasant, fruity. Spore deposit yellowish white (1A2).

Basidiospores 7-7.5-8.5 × 6.5-6.9-7.5 µm, subglobose to broadly ellipsoid, (n = 20, Q = 1.07-1.13-1.23), ornamentations ≤1.5 µm high, rounded to triangular warts and connecting ridges never forming a complete reticulum; plage inamyloid. Basidia 40-51 × 9-12 µm, subclavate, 2-, 4-spored; sterigmata 4-5 µm long. Pleuromacrocytisma abundant, 30-63 × 4-7.5 µm, cylindric to subfusiform, apices capitate to mucronate and constricted, mostly moniliform; contents dense, small crystal-like. Pleuropseudocystidium up to 5.5 µm thick, nonemergent to slightly emergent, cylindric, with a round apex and often forked near apex. Lamellae edge sterile. Cheilomacrocytisma 36-42 × 4-5.5 µm, subcylindric to subfusiform, apex moniliform to constricted; contents dense. Lactifiers in hymenophoral trama up to 10 µm thick. Pileipellis an ixocutis, 80-137 µm thick, composed of densely packed parallel, often shrivelled hyphae; hyphae 1-2.5 µm wide. Lactifiers in pilear trama up to 9 µm thick.

Ecology: Growing in association with Quercus leucotrichophora in mixed temperate forest dominated by Quercus, Rhododendron, Myrica, Cedrus and Pinus sp.


Comments: The viscid to glutinous, zonate, yellowish pileus with hairy margin, and the white, unchanging latex, scrobiculate stipe and fruity odour undoubtedly place the present taxon in Lactarius sect. Zonarii. This is also confirmed in the molecular phylogeny by the placement of L. indozonarius as the sister species of L. zonarius, although the monophyly of L. sect. Zonarii in its traditional morphological sense cannot be confirmed. A Nepalese sample (LC150021) is clustered with L. indozonarius but lacks any morphological description. Lactarius indozonarius is quite distinct due to the zonations in the pileus context. This character has only been reported in Thai species of L. sect. Zonarii: L. alboscrobiculatus var. roseopurpureus H.T. Le & A. Verbeken, L. austrotorminosus H.T. Le & A. Verbeken and L. austrozonarius H.T. Le & A. Verbeken (Le et al. 2007), but is a generic character for its sister genus Multifurca (Wang et al. 2018). Microscopically, L. indozonarius is characterized by subglobose to broadly ellipsoid basidiospores, abundant macrocystidia and an ixocutis pattern of the pileipellis.
Lactarius indozonarius is morphologically similar to *L. zonarius* by the presence of a strongly zonate, yellowish pileus, brownish discoloration in lamellae on damage, burning acrid taste, pleasant odour and abundant pleurocystidia. Yet, our
new species is clearly distinct from it due to its hairy pileus and zoned context. Microscopically, *L. zonarius* differs because of its smaller (6.9-7.7 × 5.8-6.2 μm) basidiospores with lower (0.7 μm) ornamentation (Basso 1999; Heilmann-Clausen et al. 1998).

*L. alboscrobiculatus* var. *roseopurpureus* (95% similarity for 100% query coverage in BLAST search) is distinct by its white to cream-coloured pileus, distant lamellae and very low (0.5-0.8 μm) ornamentations on the basidiospores. *L. austrozonarius* differs by its subdistant lamellae, very abundant, white to watery cream-coloured latex and distinctively larger (7.2-12.2 × 7-10.2 μm) basidiospores with very high ridges (≤ 2.5 μm). *Lactarius indozonarius* shares with *L. austrotorminosus* a hairy cap margin, dense lamellae with brownish orange discoloration and ≤ 1.5 μm high ornamentations on the basidiospores, but the latter species is still recognizable by its pinkish orange basidiomes and smaller (30-80 mm diam.), papillate pileus. Micromorphologically, *L. austrotorminosus* is quite distinct from *L. indozonarius* due to the structure of the pileipellis being a cutis or trichoderm, and the zebroid ornamentations of the basidiospores (Le et al. 2007).

*Lactarius thindii* Uniyal, K. Das & Nuytinck, **sp. nov.**

**Figs 21-33**

*Mycobank*: MB823735

*Genbank*: MG028639 (holotype), MG456762 (paratype)

**Diagnosis**: Differs from *Lactarius pallidizonatus* by the presence of an azonate to faintly zonate pileus, non striate pileus margin, unchanging latex, smaller (6.0-7.0 × 5.0-6.0 μm) basidiospores and nrITS sequence data.


**Etymology**: in commemoration of Prof. K. S. Thind for his contributions to the study of Indian mycobiota.

**Pileus** 24-37 mm diam., convex to plano-convex with depressed center, surface smooth, sticky to slightly glutinous, azonate to faintly zonate near margin, light orange (5A4-5) to greyish orange (5B3-4), brownish yellow (5C7-8) at centre, pale yellow (4A3) near margin; margin undulate, decurved. **Lamellae** subdecurrent, rather close (6-7 L/cm), white to yellowish white (2A2), unchanging when damaged; lamellulae 1-2 between two lamellae. **Stipe** 25-36 × 6-11 mm, brownish orange (5C4-5), whitish toward apex. **Context** white to orange white (5A2), greyish orange (5B4) in stipe when mature, stuffed to hollow, unchanging on exposure and with FeSO4 and KOH; taste faintly acrid; odour insignificant. **Latex** scarce, white, unchanging; taste acrid. **Spore deposit** yellowish white (2A2).

**Basidiospores** 6-6.3-7 × 5-5.2-6 μm, (n = 20, Q = (1) 1.10–1.20–1.27); broadly ellipsoid to ellipsoid, ornamentations composed of some small round to conical warts and ridges (≤ 1.2 μm high) that form a close incomplete reticulum; plage inamyloid. **Basidia** 33-45 × 9-11 μm, subclavate, 4-spored; sterigmata 3-5 μm long. **Pleuromacrocystidia** 54-82 × 6.5-12 μm, fusiform to subfusiform, often forked near apex; apices acute, capitate and mucronate to moniliform, contents dense, needle shaped. **Pleuroseudocystidia** up to 6.5 μm thick, cylindrical, sometimes forked. **Cheilomacrocystidia** 45-58 × 6.5-8 μm; apex acute to mucronate. **Lactifers in hymenophoral trama** up to 9 μm thick. **Pileipellis** an ixocutis, 85-165 μm thick; hyphae densely packed, septate, repent to slightly ascending, interwoven in the form of a reticulum, 1-4 μm thick.
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Ecology: Growing in association with Quercus semecarpifolia in a mixed subalpine forest dominated by Quercus, Rhododendron, Lyonia, Myrica and Abies spp.

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Comments: Lactarius thindii is morphologically characterized by its small sized basidiomes, its sticky to slightly glutinous, greyish orange to brownish yellow pileus that is almost white towards the margin; unchanging, white latex; subglobose to broadly ellipsoid basidiospores with a dense, incomplete reticulum; often forked pleurocystidia and ixocutis type of pileipellis. Molecular data suggest its close relation with L. purpureus R. Heim ex R. Heim, L. pallidizotonatus X.H. Wang and L. pallido-ochraceus X.H. Wang. The Asian species L. purpureus is unmistakably different from L. thindii by its wine red to pink, scaly pileus surface; larger (5.2-8.6 × 4.5-7.9 μm) basidiospores and a cutis to trichoderm type of pileipellis. Lactarius pallidizotonatus and L. pallido-ochraceus share similarities with L. thindii in having a faintly zonate to zonate, smooth pileus surface, acrid tasting white latex, ixocutis type of pileipellis and occurrence under fagaceous trees. However, both species are different from L. thindii due to presence of hygrophanous pileus surface with sub-transparently striate margin. In addition, L. pallidizotonatus is distinct by latex changing to watery, larger (7.5-9.0 × 6.0-7.5 μm) basidiospores with lower (0.4-0.6 μm) ornamentations and very thick (200-500 μm) pileipellis. Lactarius pallido-ochraceus is different from L. thindii due to presence of brownish to greyish ochraceous discoloration in lamellae on bruising and unchanging to glaucous green discourting latex (Wang 2017).

Lactarius fraxineus Romagn. is remarkably similar to L. thindii in having crowded, cream-coloured lamellae, white latex, basidiospores (6-7 × 5-6 µm) with incomplete reticulation and an ixocutis type of pileipellis. However, this European species differs from the present taxon by its larger (≤ 85 mm) pileus with scaly surface, scrobiculate stipe and greyish-olive latex discoloration (Basso 1999).

In India, Lactarius dafianus is the sole representative of L. sect. Colorati and can be confused with L. thindii due to its reddish orange pileus with darker centre, dense lamellae and unchanging latex. The former is different on the basis of its watery latex, white spore deposit, abundant isolated warts on the basidiospores and trichodermal pileipellis (Das & Sharma 2005).

A provisional key to the known species of Lactarius subg. Lactarius from Indian Himalaya

1a. Latex strikingly coloured, presence of greenish discoloration in tissue .......... 2
1b. Latex white to watery, absence of greenish discoloration in tissue .......... 10
2a. Pileus greyish blue to blue with silvery lusture ............................................. 3
2b. Pileus pinkish, brownish or orange ................................................................. 5
3a. Latex vinaceous red, occurrence under Pinus ............................................. L. paradoxus
3b. Latex blue ........................................................................................................ 4
4a. Latex turning green on exposure, basidiospores 7-9.5 × 6-7.5 μm, occurrence under Pinus and Quercus ................................................................. L. indigo
4b. Latex unchanging on exposure, basidiospores 6.6-8.3 × 5.4-6.5 μm, occurrence under Castanopsis and Lithocarpus ........................................... L. subindigo
5a. Lamellae subdistant to distant (3-4/cm) .......................................................... 6
5b. Lamellae crowded (12-18/cm) ........................................................................ 7
6a. Pileus brownish orange to brown, pleuromacrocystidia scarce, occurrence under Pinus................................................................. L. rubrifluus
6b. Pileus dirty pinkish, pleuromacrocystidia abundant, occurrence under Tsuga ................................................................. L. subpurpureus
7a. Pileus distinctly or faintly zonate .................................................................... 8
7b. Pileus azonate, occurrence under Pinus ............................................... L. sanguifluus
8a. Stipe nonscrobiculate, basidiospore ornamentation consisting of isolated warts and short ridges, never forming reticulum
8b. Stipe scrobiculate, basidiospores ornamentation forming an incomplete reticulum, occurrence under Pinus
9a. Pileus typically azonate or with a few zones near the margin, with mainly bright orange colours and green tinges, occurrence under Picea
9b. Pileus clearly zonate and brown-orange with sometimes lilac-grey tinges, occurrence under Picea
10a. Pileus distinctly to faintly zonate
10b. Pileus azonate
11a. Pileus pubescent to fibrillose
11b. Pileus neither pubescent nor fibrillose
12a. Pileus large (≥ 120 mm diam.)
12b. Pileus smaller (≤ 60 mm)
13a. Latex changing to sulphur yellow on exposure, occurrence under Picea
13b. Latex unchanging on exposure, occurrence under Quercus
14a. Latex unchanging on exposure, stipe scrobiculate, occurrence under Castanopsis
14b. Latex changing to lemon yellow on exposure, stipe non scrobiculate, occurrence under Rhododendron
15a. Pileus dark olivaceous to vinaceous
15b. Pileus light orange to pale yellow or pinkish
16a. Latex turning greenish on exposure
16b. Latex unchanging on isolation
17a. Context turning lilac with KOH, basidiospores with broken to partial reticulum, occurrence under Abies
17b. Context not turning lilac with KOH, basidiospores with zebroid ornamentation
18a. Occurrence in subtropical to temperate forest under Quercus, pleuromacrocytisdia with needle-like contents
18b. Occurrence in subalpine forest under Betula, pleuromacrocytisdia without needle-like contents
19a. Latex staining lamellae and context lilac, under Abies and Tsuga
19b. Latex not staining lamellae and context, under Quercus
20a. Pileipellis a trichoderm, occurrence under Rhododendron
20b. Pileipellis an ixocutis
21a. Stipe scrobiculate, latex changing to bright grey to pale brownish grey on lamellae edges, occurrence under Quercus
21b. Stipe not scrobiculate, latex unchanging
22a. Lamellae white to yellowish white, latex scarce, basidiospores 6-7 × 5-6 µm, occurrence under Quercus
22b. Lamellae pale vinaceous-cinnamon, latex copious, basidiospores 7-9 × 6-7.5 µm, occurrence under Quercus
23a. Pileus hairy or fibrillose
23b. Pileus smooth
24a. Latex immediately changing to yellow on exposure
24b. Latex unchanging on exposure
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25a. Pileus surface viscid to glutinous, stipe scrobiculate, larger basidiospores (7.5-10 × 6-7.5 µm), occurrence under Abies ........................................ L. alnicola
25b. Pileus surface dry, stipe non scrobiculate, smaller basidiospores (6.6-7.4 × 4.9-5.2 µm), occurrence under Betula ......................... L. pubescens
26a. Lamellae subdistant to distant (2-5/cm) .......................................................... 27
26b. Lamellae crowded (17-21/cm) ................................................................. 28
27a. Lamellae orange yellow, pileus margin regular, basidiospore ornamentations >0.5 µm high, occurrence under Rhododendron and Quercus .... L. dhakurianus
27b. Lamellae yellowish white, pileus margin crenate, basidiospore ornamentations 2-2.4 µm high, occurrence under Quercus ..................................... L. kumaonensis
28a. Pileus surface viscid, pileipellis an ixocutis ................................................... 29
28b. Pileus surface very glutinous to slimy, pileipellis an ixotrichoderm ............ 30
29a. Lamellae white to yellowish buff, latex copious, changing to greyish, staining lamellae violaceous to brownish lilac............................ L. pseudoaspideus
29b. Lamellae pinkish, latex scarce, unchanging and not staining the lamellae, occurrence under Salix and Populus ..................................... L. controversus
30a. Latex unchanging, smaller basidiospores (7-8.7 × 6-7.1 µm), occurrence under Tsuga ..................................................... L. elaiovascidus
30b. Latex changing to pinkish on cut lamellae, larger basidiospores (8-10.1 × 6.7-7.8 µm), occurrence under Abies ......................... L. olivaceoglutinus

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