



# Morphology and Phylogeny of *Lactarius Wallichianae* sp. nov and *Xerula magnispora* sp. nov. from India

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## Abstract

The present paper discusses two new species of gilled fungi on the basis of polyphasic approach, the species are described as new to the science on the account of detailed morphological and anatomical details and further, confirmed through molecular characterization. These species were found growing in the pristine coniferous forest of the Kashmir Himalaya of India. They are described as incipient species to science and denominated as *Lactarius wallichianae* sp. nov. and *Xerula magnispora* sp. nov. These do not resemble with any of the known species of genus *Lactarius* and *Xerula*.

**Keywords:** Taxonomy; Molecular Phylogeny; Transcribed; Identification; *Xerula*

## Introduction

India being the 6<sup>th</sup> mega spot of biodiversity is known for its rich flora and fauna thus, harboring diversity of agaric species as the favourable climatic conditions favours the agarics to flourish. In the present study taxonomic and phylogenetic studies are based mainly on morphological and anatomical characters. The identification with a classical taxonomic approach has been further strengthened by using molecular genetic markers internal transcribed spacer region (ITS) was followed extensively sequenced DNA region with a higher degree of variation than other genetic regions of rDNA [1]. The internal transcribed spacer (ITS) region Schoch CL, et al. [2] has been widely used in the identification of new species [3,4].

In the present study, the morphoanatomical description and molecular nucleotide analysis of *Lactarius wallichianae* sp. nov. and *Xerula magnispora* sp. nov. were used as a tool for their characterization and identification using integrated study of classical as well as molecular taxonomy. DNA

sequence analysis of the ITS region using ITS region (ITS1, 5.8S, and ITS2) were helpful in identifying these taxa.

Genus *Lactarius* Pers. was described by Persoon CH, et al. [5] with *Lactarius piperatus* (L.) Pers. as type species. At present its type species is *L. torminosus* (Schaeff.) Pers. [6,7]. It is an ectomycorrhizal genus that plays a critical ecological role in terrestrial ecosystems with various plants [8].

Genus *Xerula* Maire and *Oudemansiella* Speg. belongs to family *Physalacriaceae* Corner. *Xerula* Maire is cosmopolitan in distribution but mainly found in North temperate regions. It is saprotrophic, associated by pseudorhiza with roots or subterranean parts of wood, mainly of *Fagus*. Genus *Xerula* has an exannulate, central stipe with or without thick-walled setae both on pileus and stipe. This genus is close to the *Oudemansiella* which is without rooting base of the stipe and grow directly on wood, without persistent annulus. They are collybioid and some authors have accommodated collybioid fruit body with rooting, pseudorhizal stipes (*Xerula*), together with similar species growing on dead wood in the

genus *Oudemansiella* [9,10] while others kept these groups separate [11-13].

## Methodology

The mushrooms were collected from the Kashmir Himalayas. The fungal forays were aimed at picking fresh and healthy fruit bodies. The morphological characters were noted down on the 'Field key' provided by Atri NS, et al. [14,15]. The colour terminology used for describing the colour variations in the basidiomata parts is after Kornerup A, et al. [16]. A small portion of the material was preserved in the liquid preservative for conducting the microscopic studies. The major portion of the same collection was hot air dried and were deposited in the Herbarium of Botany Department, Punjabi University, Patiala, India under PUN. The basidiospores were stained and observed under microscope and Camera Lucida drawings at different magnification were made.

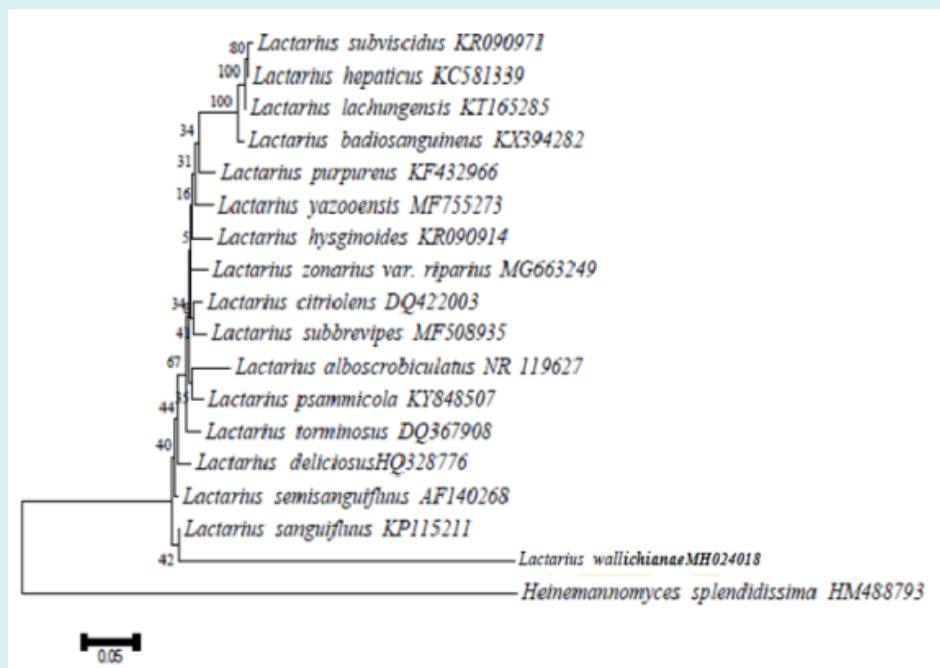
For molecular characterization and identification, genomic DNA was isolated, amplified, purified and sequenced. The DNeasy Plant Mini Kit (Qiagen, Stanford, CA) was used for DNA extraction as per the manufacturer's procedures. PCR amplification of ITS was done using primers ITS1 and ITS4

[17]. PCR conditions were as follows: The thermal cycling conditions were initial denaturation for 5 min at 95°C, 34 cycles of 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72 °C followed by a final extension of 7 min at 72°C. The reaction mixture was cooled at 4°C to stop the reaction and stored at -20°C. 5µl of amplified product of each sample were loaded on to a 1.5% agarose gel and electrophoresed for 1hr to 2 hr. DNA was analyzed by electrophoresis in 1.5% agarose gel. The amplified product were immediately sent to the 'Ist Base' (Singapore) through its distributor 'Agile Lifescience Technologies India Pvt. Ltd.' for nucleotide sequencing. The ITS sequences obtained in the present study were compared to the NCBI GenBank database using a nucleotide BLAST search (<http://www.ncbi.nlm.nih.gov>). Sequences were downloaded from GenBank. The evolutionary distances among isolates and related taxa were determined with the Molecular Evolutionary Genetics Analysis 5.0 software Tamura K, et al. [18], using Kimura's two-parameter model [19].

## Taxonomic Studies

*Lactarius wallichianae* Malik, N. A & Saini, M. K, sp. nov. – (Figure 1-3).

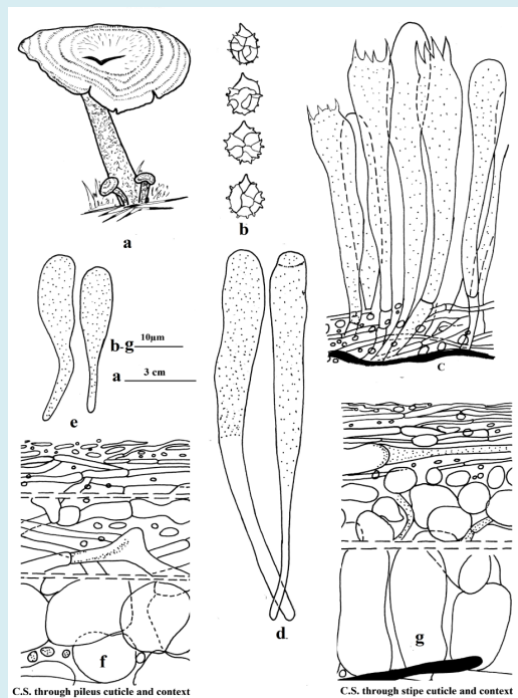
MycoBank no.: MB 830256



**Figure 1:** The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site.



**Figure 2:** (A-C) - *Lactarius wallichianae* sp. nov. a. Carpophores growing in groups in putative ECM association with *Pinus wallichiana*. b. Young developing carpophores in groups along with mature carpophores. c. Young developing carpophores with bluish latex and bands and also the furcate lamellae near the stipe of the mature carpophore.



**Figure 3:** *Lactarius wallichianae* sp. nov. a. Carpophore. b. Basidiospores. c. C.S. through hymenophore. d. Pleurocystidia. e. Cheilocystidia. f. C.S. through pileus cuticle and context. g. C.S. through stipe cuticle and context.

Basidiomata 4.5 - 6.5 cm in height. Pileus 5.0 - 7.0 cm broad, convex when young, infundibuliform at maturity; surface, dirty white to grey (8E1) when young, reddish or pinkish white (9A2), pinkish silvery tinge at maturity; zonate, 3 - 4 concentric rings, 0.1 - 0.3 cm broad zones; scaly; scales, appressed fibrillose, high red (9A8), present in bluish bands at places in developing young dirty cream caps; margin regular, involute, not splitting at maturity; moist; apex depressed; cuticle half peeling; flesh up to 0.5 cm thick, white, spongy, changing to reddish on exposure; odour good; taste delicious. Latex, bluish in young, red in mature carpophores. Spore print creamy white. Lamellae up to 0.1 cm broad, decurrent, close, unequal, furcate near the stipe, pale red (9A3), changing to brownish orange or reddish golden (6C4) on bruising; lamellulae present; gill edges smooth. Stipe excentric, 4.0 - 5.0 cm long, up to 1.0 cm broad throughout, equal in diameter; bluish grey (2B4) in young, pastel red (9A4) at maturity; tomentose base; white mycelium form ring near the apex of mature stipe; bluish latex oozes out from the young stipe, red latex in mature stipe; surface smooth, glabrous; solid; exannulate.

Basidiospores 8.8 - 11.2 × 8.0 - 9.6 μm (excluding ornamentation), Q = 1.1, subglobose to ellipsoidal, warty, warts up to 0.83 μm high, thin to thick, warts connected forming a complete reticulum; ornamentation type VI, VIII; amyloid; apiculate. Basidia 72.0 - 88.0 × 8.0 - 14.5 μm, clavate, tetrastragimate; sterigmata 4.8 - 7.2 μm long with pointed apices. Pleurocystidia 96.0 - 120.0 × 11.2 - 14.0 μm, clavate, sparsely granular, protruding beyond the hymenium and deeply seated, not abundant; cheilocystidia 32.0 - 48.0 × 6.4 - 10.4 μm, clavate, granular, rare. Gill edge heteromorphous. Hymenophoral trama regular, formed of closely septate, gelatinized, hyaline to granular hyphae. Pileus cuticle hyphal, differentiated into epicutis and subcutis; epicutis ixocutis, made up of 1.6 - 4.8 μm broad, horizontally tangled, loosely packed, hyaline, septate hyphae; subcutis an ixocutis is made of 2.5 - 5.0 μm broad, gelatinized, hyaline, branched, septate hyphae; context made up of 3.2 - 4.8 μm broad, septate, granular hyphae intermingled with hyaline rosettes of sphaerocysts. Stipe cuticle hyphal, ixocutis, made up of 1.66 - 5.0 μm broad, longitudinally running, loosely packed, hyaline hyphae; context made up of rosettes of spherical to elongated sphaerocysts intermixed with 3.2 - 5.6 μm broad, septate, granular hyphae. Laticifers hyphae present throughout. Clamp connection absent.

**Etymology** – The name of this species is based on its putative ECM association with *Pinus wallichiana*.

**Habitat or host plant** – *Pinus wallichiana*

**Material examined.** – Kashmir Himalayas, India (2500m), 33034.221'N - 075031.331E, growing groups in putative ectomycorrhizal association with *Pinus wallichiana* in pure coniferous forest, 9 Sep 2015, Nazir Ahmad Malik, PUN 9057 (Holotype).

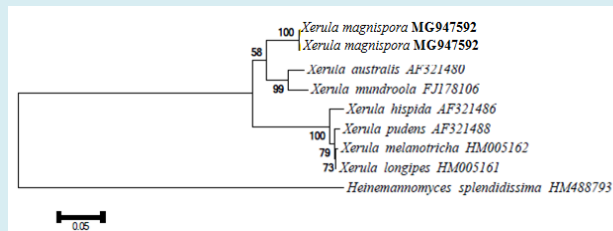
**Edibility**– The taste of flesh and latex is delicious and can be recommended for eating purpose.

**Notes** – Genus *Lactarius* Pers. was described by Persoon CH, et al. [5] with *Lactarius piperatus* (L.) Pers. as type species. At present its type species is *L. torminosus* (Schaeff.) Pers. [6,7]. It is an ectomycorrhizal genus that plays a critical ecological role in terrestrial ecosystems with various plants [8].

In the morphology and anatomical characters of the present collection does not fit in to any of the known species of *Lactarius*, but it does fall under the sub section *Lactarius* described by Hesler LH, et al. [20]. It is characterized by the young convex carpophores having dirty cream cap with few bluish scaly zones, blue latex oozing out from the cap as well as the stipe while the mature fruit bodies are infundibuliform with light silvery pinkish cream cap with rusty red patches forming 3 - 4 concentric zonations, the stipe is concolours with the pileus, the stipe base covered by white cottony mass, somewhat decurrent lamellae, not bluing or greening on the exposure or with age, basidioles forming a uniform row, basidia and pleurocystidia protruding beyond the basidioles, deeply seated pleurocystidia, gill edge heteromorphous and gelatinized pileus and stipe cuticle. For confirmation of the morphoanatomical observation and proposing a new species, DNA barcoding of this collection was done. Its ITS1 and ITS4 regions were sequenced and sequences were analyzed using the gapped BLASTn <http://www.ncbi.nlm.nih.gov> search algorithm and aligned to the nearest neighbours It has 74% identification and 94% Query cover with its allied species *Lactarius sanguifluus* which shows it has less identification percentage and good query cover, hence proposed as new to science. Evolutionary analyses were conducted in MEGA6 by taking *Heinemannomyces splendidissima* as an out group. Sequence was submitted in NCBI with accession number MH024018 [21-25].

*Xerula magnispora* Malik, N. A & Saini, M. K sp. nov. – (Figures 4-6).

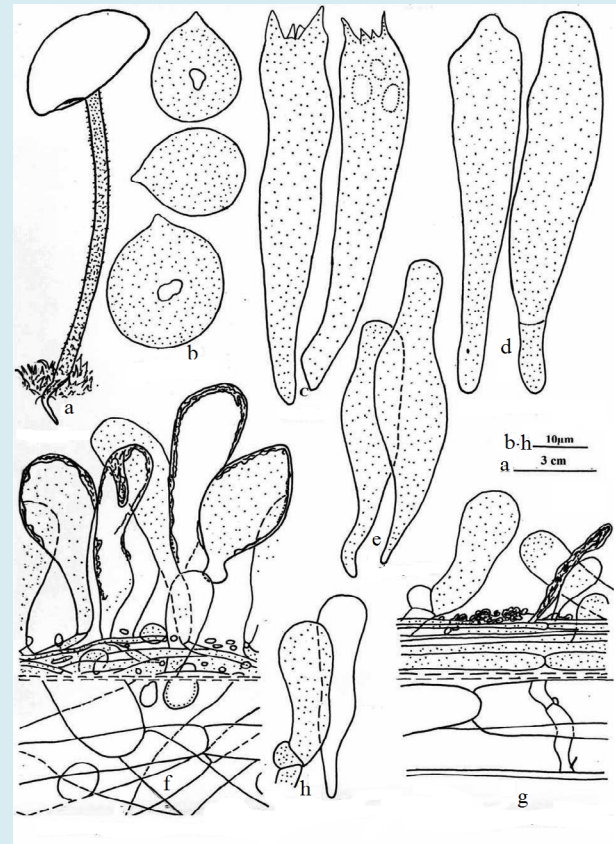
MycoBank no.: MB 833873



**Figure 4:** The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site.



**Figure 5:** *Xerula magnispora* sp. nov. a. Carpophore solitary, convex with subumbonate cap. b. Underview of cap bearing distant lamellae, sinuous to incurved tips and the violet brown furfuraceous stipe with white apex. c. C.S through hymenophore with basidia and pleurocystidia. d. C.S. through gelatinized pileus cuticle consisting clavate to sphaeropedunculate, thick walled, often pedicellate pilocystidia.



**Figure 6:** *Xerula magnispora* sp. nov. a. Carpophore. b. Basidiospores. c. Basidia. d. Pleurocystidia. e. Cheilocystidia. f. C.S. through pileus cuticle and context. g. C.S. through stipe cuticle and context. h. Caulocystidia.

Carpophore up to 12.0 cm in height. Pileus up to 4.5 cm broad, convex; subumbonate; surface white (2A1) to dull red (8B3); moist; margin regular, creanulate, involute; cuticle fully peeling; flesh up to 0.1 cm thick, white, changing slowly; taste and odor mild. Spore print creamy white (2A1). Pileal veil warty. Lamellae up to 1.0 cm broad, adnate to subdecurrent, distant, unequal, present in 3 to 4 tiers, white (2A1) with white floccose edges, unchanging; lamellulae present with incurved tips, sinuous; gill edges serrate to dentate. Stipe central, up to 12.0 cm long (excluding rooting base), up to 0.4 cm broad, equal in diameter, curved, not straight; expanded rooting base present; surface violet brown (10F5), off white near apex; white mycelium present at base; surface furfuraceous; scaly, scales fibrillose, violet brown (10F5), white floccose near apex; hairy; solid; exannulate [25-30].

Basidiospores 15.2 - 24.0 × 13.41 - 21.6 μm, Q = 1.1, globose to ellipsoidal, thickly granular, looks warty, single thick walled, monoguttulate; inamyloid; apiculate, apiculus 0.83 - 1.66 μm long. Basidia 66.4 - 91.3 × 9.96 - 14.94 μm, clavate, clamp connections absent at base, granular, thick walled, tetrasterigmate; sterigmata 3.2 - 8.0 μm long, granular. Pleurocystidia 59.76 - 73.04 × 9.13 - 16.6 μm, clavate, granular, not deeply seated, broad as compared to basidia; cheilocystidia 41.5 - 58.1 × 9.13 - 16.6 μm, clavate, ventricose to lageniform with pedicellate base, partially double walled, granular, crowded. Hymenophoral trama regular. Gill edge sterile. Pileus cuticle hyphal, ixocutis, made up of 1.6 - 6.4 μm broad, septate, granular hyphae giving rise to regular turf of pilocystidia without pileal hairs; pilocystidia 24.0 - 64.0 × 8.0 - 14.4 μm broad, clavate to sphaeropedunculate, thick walled, often pedicellate; context hyphal, gelatinized, made up of 6.4 - 24.0 μm broad, septate, hyaline, longitudinally placed, septate, inflated hyphae. Pileal hairs (setae) absent. Stipe cuticle hyphal, ixocutis, made up of 1.66 - 8.3 μm broad, longitudinally placed, septate, granular hyphae, giving rise to caulocystidia; caulocystidia 24.09 - 48.14 × 8.3 - 14.94 μm broad, clavate, hyaline, rarely granular; context hyphal, made up of 8.3 - 13.28 μm broad, longitudinally placed, hyaline, hyphae. Clamp connections present [31-37].

Etymology – The name of the species is based on large size of basidiospores.

Habitat or host plant – humicolous soil.

Material examined – South Kashmir, Tsimer (2136m), 330 34.219'N-075000.540, growing solitary on humicolous soil in coniferous forest, 15 July 2013, Nazir Ahmad Malik, PUN 9041 (Holotype).

Notes – Genus *Xerula* Maire and *Oudemansiella* Speg. belongs to family *Physalacriaceae* Corner. *Xerula* Maire is cosmopolitan in distribution but mainly found in North temperate regions. It is saprotropic, associated by pseudorhiza with roots or subterranean parts of wood, mainly of *Fagus*. Genus *Xerula* has an exannulate, central stipe with or without thick-walled setae both on pileus and stipe.

Present collection is characterized by adnate to sub decurrent gills present in 3 to 4 series with incurved tips near margin, basidiospores are much larger than any of the known species of genus *Xerula* Maire. The presently examined collection has been compared with other allied species viz. *X. asparata* which is golden yellow to more orange with smaller basidiospores i.e. 7.5 - 10 × 5.0 - 7.5 (9 × 5.8) μm (Pegler 1977, as a *Xerulina asparata*). For further confirmation of the result based on morphoanatomical data, the molecular identification with ITS1 and ITS4 regions have been sequenced and analyzed using the gapped BLASTn <http://www.ncbi.nlm.nih.gov> search algorithm and aligned to the

nearest species. The evolutionary analysis was conducted in MEGA6 by taking *Heinemannomyces splendidissima* as an out group. The sequence was submitted in NCBI with accession number MG947592.

## Summary

The fungal furays results two new species of gilled fungi from Kashmir Himalayas based upon the detailed morphological and anatomical studies and molecular approach. These species are described as new to science viz. *Lactarius wallichianae* sp. nov. and *Xerula magnispora* sp. nov. All the morphology and anatomical details of *Lactarius wallichianae* sp. nov. vary from any of the known *Lactarius* species. It is having blue latex. The stipe is not bluing or greening on the exposure or with age. *Xerula magnispora* sp. nov. with much larger basidiospores than any species of *Xerula* Maire. For morphoanatomical observation, DNA barcoding confirms both species as new to science.

## Conclusion

The present study has a valuable cause for the exploration of unknown agaric species. The Kashmir Himalaya is still unexplored. Its exploration of fungal diversity is important. We made some fungal forays and identified two new species that are not discovered before this study.

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