



## Research Article

# Taxonomic Investigation and Addition of *Marasmiellus agrianum* N. Jabeen and M. Lodhi sp. nov. (Agaricales, Omphalotaceae) from Hazara Division Mansehra, Pakistan

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**Abstract** | *Marasmiellus agrianum* N. Jabeen and M. Lodhi was added as a new science species in the genus *Marasmiellus* (*Omphalotaceae*), collected from the lawn and grassy soil of Hazara University, Mansehra. The comparative studies revealed that *Marasmiellus agrianum* N. Jabeen and M. Lodhi was grown on lawn soil and is a flattened convex whereas *Marasmiellus scandens* being pathogenic was found mostly on the trunk of trees and is a semicircle convex-shaped basidiocarp. The species were studied for morphological characteristics. For molecular characterization, DNA extraction was performed from the samples using a kit, and PCR amplification was done using ITS4 and ITS1F primers designed from the conserved regions as described in the previous studies. The amplified PCR products were sequenced from Macrogen Korea, via worldwide scientific Pakistan. Phylogenetic analysis of the obtained sequences was done based on the maximum similarity method using Mega 6.0. The spore was smaller as compared to *Marasmiellus scandens* (6.2-8.7 μm). The newly described species i.e., *Marasmiellus agrianum* N. Jabeen and M. Lodhi differs from other species based on the molecular study as well with few morphological characteristics; size, color, shape, and texture of pileus, and stipe, and the molecular characterization reveals that the species was placed at a new place in the phylogenetic tree, compared with the NCBI Blast sequences. The species were preserved by sun and oven drying methods. The area of District Mansehra is enriched with natural vegetation and can be explored for new species.

**Received** | February 11, 2023; **Accepted** | June 05, 2023; **Published** | July 20, 2023

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**Citation** | Jabeen, N., A.M. Lodhi, R.N. Syed, M.A. Khanzada and A. Gul. 2023. Taxonomic investigation and addition of *Marasmiellus agrianum* N. Jabeen and M. Lodhi sp. nov. (Agaricales, Omphalotaceae) from Hazara Division Mansehra, Pakistan. *Sarhad Journal of Agriculture*, 39(3): 609-615.

**DOI** | <https://dx.doi.org/10.17582/journal.sja/2023/39.3.609.615>

**Keywords** | *Agaricales*, Morphology, Phylogenetic analysis, nov. species identification



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## Introduction

The genus *Marasmiellus* was identified and placed in *Omphalotaceae* with more than 400 different species on a molecular basis worldwide (Wilson

and Desjardin, 2005). The genus was introduced for the first time by Murril in 1915 which later was altered and modified by Singer (1973a, 1973b, 1986). Some of the species belonging to this genus are host specific, parasitic in nature, and exclusively habitat the

decaying matter (Singer, 1973). These are very small-sized mushrooms to observe and overlook with keen eyes on the grassy lands and grounds. The accepted species are approximately 250 but some published names exceed 402 (Singer, 1973; Pegler, 1977, 1983, 1986; Antonín and Noordeloos, 2010; Corner, 1996; Kirk *et al.*, 2008).

Recently many species were reported by mycologists all around the world on molecular and morphological basis *Marasmiellus istanbulensis* was reported from Belgrade Forest (Istanbul-Turkey), (Seseli *et al.*, 2018). *M. rhizomorph* genus and *M. Koreans* were found in excessive distribution in Korea (Vladimir *et al.*, 2010). *M. palmivorus* was collected from the tree trunk and identified added to the genus by different mycologists all around the world (Schneider *et al.*, 2018). From Pakistan work was done on this Genus and new to science and a new Pakistan species was reported identified and described. *Marasmius margallensis*, *Marasmius oreades*, reported by Razaq and Khalid (2023) and Razaq *et al.* (2013) reported the novel species from the genus. Aman *et al.* (2022) published “A compendium of macrofungi from Pakistan” described species from the Genus. A check list of macrofungi of Pakistan was published by Abdul Nasir Khalid from 1998–2020 describing more than 200 articles from Pakistan. *Marasmiellus longistipes*, *Marasmiellus luxurians*, *Marasmiellus Menebune*, *Marasmiellus omphaloides*, and *Marasmiellus subnudus* reported by Khalid (2022) from Pakistan. *Marasmiellus palmivorus* is also reported by Hussain (2016) from Malakand Pakistan in his dissertation from Hazara University Mansehra Pakistan.

District Mansehra is enriched with natural vegetation and can be explored for new species as well as for Mushrooms hunting. Many national and international mycologists worked on the wild species of macro-fungi and compiled data related to the wild mushroom species of Pakistan. The specie collected can be further studied for their importance as it is newly identified from the region.

## Materials and Methods

Current investigations aimed to explore different mushroom species. This research work was conducted during 2017–2020 in the Mansehra District of Pakistan. Our specimen species were collected from the lawn area of the Agriculture Department Hazara

University, Mansehra (*Marasmiellus agrianum* Jabeen and Lodhi, 201113) and the Pheasant area of Hazara University (*Marasmiellus agrianum* N. Jabeen 201112) and Mansehra Channai (*Marasmiellus agrianum* N. Jabeen and M. Lodhi 201111) and submitted to NCBI (National Center for Biotechnology Information) for Accession number.

*Marasmiellus agrianum* N. Jabeen and M. Lodhi was added as a new science species in the genus *Marasmiellus* (*Omphalotaceae*), collected from the lawn and grassy soil of Hazara University, Mansehra. The comparative studies revealed that *Marasmiellus agrianum* N. Jabeen and M. Lodhi was grown on lawn soil and is a flattened convex whereas *Marasmiellus scandens* being pathogenic was found mostly on the trunk of trees and is a semicircle convex-shaped basidiocarp. The species were studied for morphological characteristics (Table 1). For molecular characterization, DNA extraction was performed from the samples using a kit, and PCR amplification was done using ITS4 and ITS1F primers designed from the conserved regions as described in the previous studies. The amplified PCR products were sequenced from Macrogen Korea, via worldwide scientific Pakistan. Phylogenetic analysis of the obtained sequences was done based on the maximum similarity method using Mega 6.0. The spore was smaller as compared to *Marasmiellus scandens* (6.2–8.7  $\mu\text{m}$ ). The newly described species i.e., *Marasmiellus agrianum* N. Jabeen and M. Lodhi differs from other species based on the molecular study as well with few morphological characteristics; size, color, shape, and texture of pileus, and stipe, and the molecular characterization reveals that the species was placed at a new place in the phylogenetic tree, compared with the NCBI (National Center for Biotechnology Information's) Blast sequences. The species were preserved by sun and oven drying methods.

### Morphological observations

The species were studied for their macro and micro morphological characteristics, ecological data was recorded at the time of collection. The species were photographed by Canon Power Shot A460 5.0 MP Digital Japan Camera on the substrate and observed for morphological data like size, shape, and color of the sporocarp as well as the stipe (Figure 1). Altitude and Latitude were taken by GPS Garmin e Trex 10–GPS China and temperature, humidity, and month of the collection were noted. Samples were collected in a

paper bag, brought to the Department of Agriculture Laboratory, and washed with distilled water to remove the soil particles (Lodge *et al.*, 2004). Sun drying method was used for preservation, keeping the specie for 5-6 hours in the sun for two days. Light Microscopy (Compound Microscope with digital Camera CB, 10) was done to observe the spores of the fungus under a binocular microscope using a lens scale. For identification the Protocol followed from "Collecting and Describing Macrofungi" (Halling, 1996).

#### Molecular studies for identification

**DNA extraction:** All the collected samples having sporocarp and stripe were sun-dried and later crushed to powder form for DNA extraction using a DNA extraction kit (EZ- 10Spin column Genomic DNA Minipreps) in accordance with the manufacturer's protocol.

1. PCL (Plant Cell Lysis) solution was used by adding RNase A (100 µg/ ml). The entire content of RNase A was added to the PCL solution and stored at 4°C. The precipitate was formed after storing the PCL solution and 2mg was dissolved at room temperature. The proteinase K solution was kept at 20°C as the storage time is kept long for this.
2. The solution was prepared and added 48ml of 100% ethanol 212ml wash solution for BS424, 120ml of 100% ethanol 230ml wash solution for BS626 (volume of added ethanol: volume of wash solution = 4:1).
3. Elution buffer is 2.0 mM tris-HCl pH 8.0~8.5 (TE buffer pH 8.0).

#### Procedures for isolation of genomic DNA from macrofungi

**Sample preparation:** The dried samples were sent to the Karachi University Molecular lab for DNA, Extraction by Kit- method (Kit-Protocol was used).

For amplification of the rDNA ITS region (Ribosomal Internal Transcribed Spacer region of the DNA), fungal specific forward primer, ITS1F 5'-CTTGGTCATTTAGAAGTAA-3' in combination with the reverse primer, ITS4 5'-TCCTCCGCTTATTGATATGC-3' were used (White, 1990; Hanif and Khalid, 2015). PCR was performed in 20µl PCR tubes containing 10µl 2X Ready mix (Sigma-Aldrich), 8.3µl water, and 0.1µl

of each primer with 1.5µl of DNA extract. PCR reactions were performed with 3.00 minutes of initial denaturation and 30s of final denaturation at 95°C followed by 35 cycles at 53°C for 35s, initial extension for 1.35+5s at 72°C and final extension for 2 minutes at 72°C.

#### Phylogenetic analysis

The PCR products were sequenced from Macrogen, Korea. The DNA sequences were edited using Sequence Scanner (v1.0) and Bioedit (v 7.0) and BLAST-searched for sequence comparison and identification by submitting the query in the GenBank database using default settings.

Sequences were aligned using Mega 6.0 (Tamura *et al.*, 2013). The divergence in rDNA-ITS was measured by comparing sequence pairs reconstructed by using Meg Align (DNA STAR). For Phylogenetic analysis, MEGA 6.0 was used. Employing the Tamura-Nai model, maximum likelihood (ML) trees were constructed and bootstrap consensus trees were generated. The bootstrap consensus trees were inferred from 1000 replicates and corresponding bootstrap values were cited in the tree.

All the samples were sent for sequencing to Macrogen Korea via Worldwide Scientific Pakistan and the results were collected within a month and a half.

## Results and Discussion

*Marasmiellus scandens* (Masse) Dennis & D.A.Reid  
Synonym=*Marasmius byssicola* Petch

#### Morphological characteristics

The species were grown like small white flower textures with whitish to creamy white color, gilled, and measured as 1.5-2 cm on average. The stem of the fungus appeared smooth and measured as 2-3.6cm long and 1.5-6mm thick. The spores of the specie were ellipsoid and 5-6.5µm on average and the spore print was whitish (Figure 2). The specie belongs to *Basidiomycota*, *Agaricales*, and *Marasmiellus*.

The comparative studies revealed that *Marasmiellus agrianum* N. Jabeen was grown in the lawn on soils as flattened convex shaped and was Pathogenic mostly found on a tree trunk and appeared as semicircle convex shaped basidiocarp. The spores appear to be smaller as compared to *Marasmiellus scandens* (6.2-8.7µm). According to the molecular basis, the newly described

specie is different as well with few morphological characteristics. The size, color, shape, and texture of pileus, stipe, and gills were studied and identified using. Sharples (1928), Turner (1981), and Hemmes and Desjardin (2002) studied and identified different species of this Genera and described them by using a light microscope and scanning electron microscope.

**Specimen region:** The specimen was collected from the enriched soil of the Department of Agriculture Hazara University lawn grass, Mansehra. *Marasmiellus agrianum* N. Jabeen and M. Lodhi 201113 (NCBI Accession No. MZ044841), was collected during the month of March 2018 from Hazara University Mansehra.

Location coordinates: (N= 34° 26,0" E=73° 15,0" Alt = 1060m)

**Fungal isolates:** The same species *Marasmiellus agrianum* N. Jabeen and M. Lodhi 201111D (NCBI Accession No. MZ044839) (N= 34° 20'0" E=73° 12'0" Alt= 977m/3205feet) from Mansehra Channai and *Marasmiellus agrianum* N. Jabeen 201112 (NCBI Accession No. MZ044840) (N= 34° 26'0" E=73° 15'0" Alt= 1060) from Pheasant area of Hazara University, Mansehra during March-April 2018.

**Properties:** Most of the species belonging to the genera are parasitic to economically important plants such as banana, maize, sugarcane, etc. and a few are saprophytic in nature. i.e., bananas, sugar cane, maize, and coconut palms (Singer, 1973). Many other properties of the species are unknown.

**Remarks:** *Marasmiellus agrianum* N. Jabeen is different from the previously reported species, no previous record is related to this specie. The species was proved new in morphological and molecular characterization.

*Taxonomy*

*Marasmiellus agrianum* N. Jabeen and M. Lodhi sp. Nov

**Type:** Pakistan Mansehra District; HU, grown on garden soil along *Cynodon dactylon* on soil (enriched with organic matter) elv. 1060m 2018, (holotype).

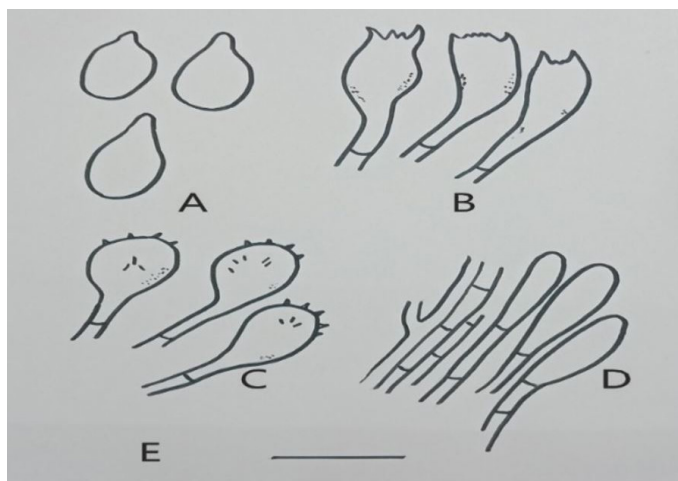
*Phylogenetic analysis*

Twenty-eight sequences amplified using ITS1F and ITS4 primers pair were identified. BLAST analysis to find out the closest match to the sequences found

in GenBank. BLAST result showed the maximum identity of amplified products to *Marasmiellus species*. Three sequences *Marasmiellus agrianum* N. Jabeen and M. Lodhi 201113, *Marasmiellus agrianum* N. Jabeen and M. Lodhi 201112, and *Marasmiellus agrianum* N. Jabeen and M. Lodhi 201111 have maximum similarities to each other and observed the same and new specie as found at a different place in the phylogenetic tree. The phylogenetic tree was constructed with these sequences the results showed that all the collected species are in one clade. Besides three amplified and sequenced rDNA-ITS sequences, 28 similar sequences from NCBI GenBank data were used to construct a phylogenetic tree with maximum likelihood as an optimality criterion. After sequence alignment using Mega 6.0, unaligned regions were trimmed from the alignment data sheet's 5' and 3' ends prior to analysis. *Coprinopsis cinereal* was used as an out-group.



**Figure 1:** Habitat and substrate of *Marasmiellus agrianum* N. Jabeen and M. Lodhi. (A) Basidiocarp; (B) Morphological features of mushroom (Hymenium).

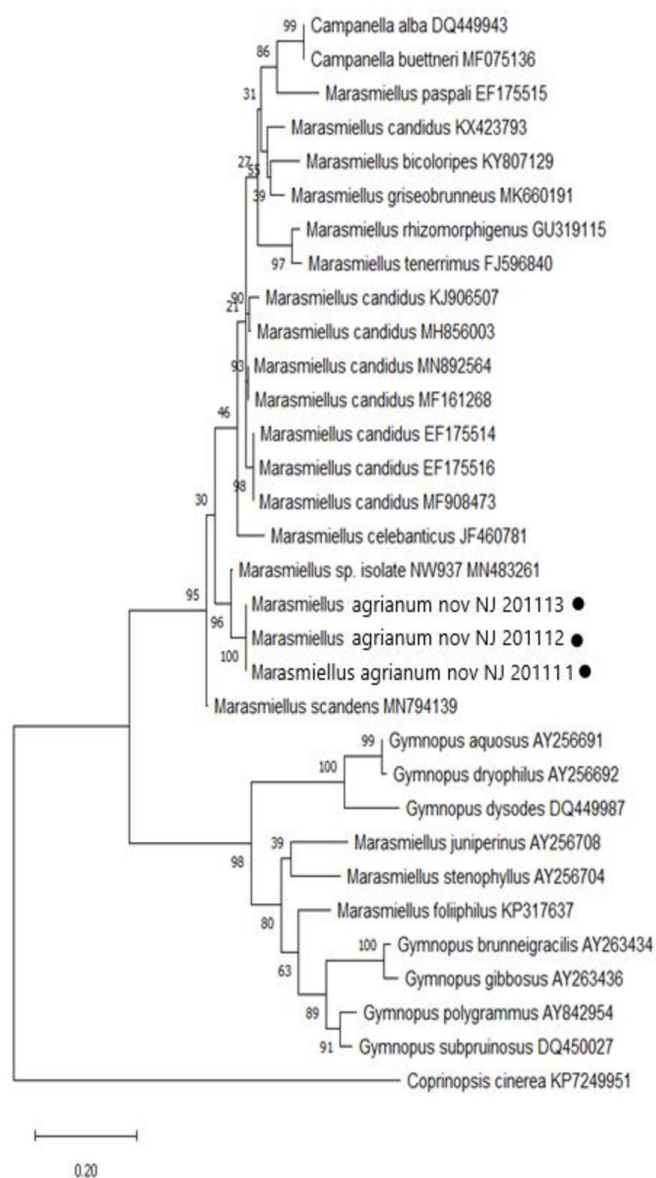


**Figure 2:** Anatomical features of *Marasmiellus agrianum* N. Jabeen and M. Lodhi. (A) Basidiospores, (B) Basidia, (C) Cheilocystidia, (D) Pleurocystidia. Scale bar = 5µm, (E) Scale bar = 20 µm.

Phylogenetically, the 3 *Marasmiellus* sp. isolated from the Mansehra region clustered in the same clade. Among the finding species, 3 species were showing a 100% bootstrap value (Figure 3).

**Table 1:** Morphological comparison of *Marasmiellus agrianum* and *Marasmiellus scandens*.

Character	<i>Marasmiellus agrianum</i>	<i>Marasmiellus scandens</i>
Habitat/ Substrate	Grown on grassland, enriched with organic matter as soil improved with the incorporation of FYM(Farm Yard Manure), found as solitary or single	Found on wood logs as well as on leaves and twigs as plant pathogenic fungus, grown in aggregation
Stipe	Equal cylindrical, Smooth, hallow, creamy white, size 1.5-2cm on average	Cylindrical, brown, short, size 0.5-1mm in length
Basidiocarp	Creamy white texture, semi-circle convex, Gilled, Delicate and whitish, size 1.5-2cm on average	Powdery-cream texture, gilled, dark on the top surface
Basidiospore	Hyaline, oval to elliptical, apiculate, size 7-8 x 3-5.2 µm, spore print was whitish/ creamy white color of sporocarp	Hyaline, oval, ellipsoid, size 1.5-2 on average, apiculate
Spore print	Whitish in observation	Light brown in color
Pileus	Central, convex, umbonate, irregular obtuse, serrate	Smooth, cream pruinose, orbicular to slightly rein form, size 8mm
Species characterized and reported Nomenclature	<i>Marasmiellus agrianum</i> N. Jabeen and M. Lodhi	<i>Marasmiellus scandens</i> (Masse) Dennis and D.A. Reid



**Figure 3:** Phylogenetic tree of *Marasmiellus agrianum* N. Jabeen and M. Lodhi. Generated with 1000 bootstrap values based on sequences of different species and *Coprinopsis cinerea* as an out-group. Novel species in the tree shown by •.

### Conclusions and Recommendations

Morphological and molecular characteristics of the *Marasmiellus agrianum* N. Jabeen and M. Lodhi proved that the District Mansehra is enriched with unexplored flora and can be identified morphological and molecular basis.

The region district Mansehra is not fully explored for different types of Myco-flora to add valuable species of many genera as well as species of the area, needed to conduct research for further investigations. New to science species can be introduced and characterized for their beneficial and other aspects. this is a newly described species in the Genus. The results were further confirmed by constructing the phylogenetic tree that revealed the specie is at a new position that needs further exploration.

### Acknowledgments

Authors greatly admire the efforts of Khurram Shahzad and all the facilities provided during the research work, visits to the difficult areas, and financial and moral support for the collection of the specimens. The Allelopathy, Plant Systematics, and Biodiversity lab of Dr. Alia Gul were used for Microscopy. Plant Pathology Diagnostic Lab of Sindh Agriculture University Tandojam is appreciated for their support for sample preservations.

### Novelty Statement

The current work was conducted to explore the

species of Basidiomycota in the Mansehra Region and identified different reported and unreported species from the area. The described species was founded novel due to its morphological as well as molecular characterization. Compared with the species in NCBI and Gene Bank.

## Author's Contribution

**Nadia Jabeen:** Exploration and collection of specimens, conducted the research, and drafted the manuscript.

**Abdul Mubeen Lodhi:** Contributed to molecular work, and conducted the conceptualization and validation of the study.

**Rehana Naz Syed:** Helped in the formation of the figures.

**Muhammad Ali Khanzada:** Helped in figures setting.

**Alia Gul:** Nicely participated in writing, critically reviewed, helped to describe new species, and edited the manuscript.

## Abbreviations

DNA, deoxyribose nucleic acid; PCR, polymerase chain reaction; ITS, internal transcribed spacer; NCBI, national center for biotechnology information; BLAST, basic local alignment search; MEGA6, molecular evolutionary genetics analysis.

## Conflict of interest

The authors have declared no conflict of interest.

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