



## Research Article

# *Asterophora lycoperdoides* (Bull.) Ditmar, a New Generic Record from Sub-Himalayan Forests of Pakistan

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**Abstract** | The genus *Asterophora* belongs to the mycoparasitic class of fungi and mostly infects fruiting bodies of *Russula* and *Lactarius*. During a survey for the collection of macrofungi from forests of sub-himalayas, Bhurban, Punjab, Pakistan, a unique fungus *Asterophora lycoperdoides* found growing on a decaying *Russula* species. Previously, no species of *Asterophora* has been described so far from Pakistan. Descriptions and illustrations are made using morpho-anatomical details. Moreover, molecular techniques have been used to amplify its ITS region of nrDNA and sequenced for phylogenetic analyses. This genus is a new record for Pakistan.

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## Introduction

The genus *Asterophora* Ditmar (Lyophyllaceae) is commonly known as fungicolous fungi or mycoparasitic fungi. These fungicolous fungi are diversified, trophic and ecological groups of organisms associated with other fungi such as symbionts, saprotrophs and even neutrals (Sun *et al.*, 2019). The species of *Asterophora* mostly grow on basidiomata of other fungi (common hosts include the species of family Russulaceae, notably genus *Lactarius* Pers. and *Russula* Pers.). Besides its unique habitat, the genus can easily be distinguished by the production of chlamydo-spores (Redhead and Seifert, 2001). According to an estimation, approximately 18 species

of *Asterophora* have been described from all over the world so far (<http://www.indexfungorum.org/>).

*Asterophora lycoperdoides* (Bull.) Ditmar is one of the well-documented species of this genus and is mainly recognized by its habitat (on mushrooms); small, whitish, globose to pulvinate pileus that become brown and powdery by the formation of stellate chlamydo-spores and poorly formed gills. These chlamydo-spores are very large (often greater than 20 µm long), pigmented and profusely produced early, hence they are conspicuous and dominant (Redhead and Seifert, 2001). It is found in gregarious clusters on rotting *Russula* (especially the *R. dissimulans*-*R. nigricans* complex) and *Lactarius* spp.

The moist temperate forests of the Himalayan region of Pakistan are recognized as one of the twenty-five biodiversity hotspots (Myers *et al.*, 2000). These forests are characterized by their lush vegetation and evergreen cover, host a diverse array of plant species, thus indicating an anticipated high diversity of fungal species. During a macrofungal survey conducted in various parts of the Himalayan forests in Bhurban, Punjab, Pakistan, a rare species *Asterophora lycoperdoides* was identified as thriving on decaying *Russula* species. This study provides a concise diagnosis, a detailed description and employs phylogenetic analyses based on ITS sequence data.

## Materials and Methods

### *Sampling and morpho-anatomical characterization*

A mycological investigation was carried out between August 2020 and September 2021 in the Himalayan temperate forests of Bhurban. These forests have high humidity and a wide range of elevations from 1800 to 2420 meters above sea level. Precipitation occurs throughout the entire year in the area, with an average rainfall of approximately 1800 mm (30 years period: World Weather Information Service, 2018). The vegetation in this region primarily consists of *Pinus wallichiana* B. Jacks, *Quercus dilata* Royle, and *Cedrus deodara* (Roxb. ex D. Don) G. Don. These conditions create an ideal environment for the proliferation of mycorrhizal fungi (Hameed *et al.*, 2012).

Fresh basidiomata of the specimen were collected from two distinct locations (Golf club and Kashmiri Bazar) of Bhurban, (Coordinates: 33°56'26.24N to 73°26'59.48E; 1828 m a.s.l) Punjab, Pakistan. All the environmental factors like habitat, location and type of soil were noted and images were taken. The standardized color system given by Munsell's (1975) was used to give color codes and Vellinga (2001) terminology was followed. A fan heater at 40–50°C was used to dry the samples which were later deposited in the LAH Herbarium, Institute of Botany, University of the Punjab, Lahore, Pakistan.

For the anatomical research, 5% KOH (w/v) was used for slide preparation. 1% aqueous solution of Congo Red and Melzer's reagent was using the microscopic procedures of Liang *et al.* (2018). The anatomical characters were observed using a light microscope through an HDCE-X5 camera under 400X and oil immersion 1000X magnification. The measurements

are described in description as (a)b–c(d), where 'a' is the lowest reading, 'b–c' are as a minimum 90% of readings, and 'd' is the highest reading, whereas 'Q' is the length to width ratio of basidiospore (Bas, 1969; Yu *et al.*, 2020); and av. L × av. W describes the average length and average width.

### *DNA extraction, PCR and sequencing*

DNA was extracted from dried herbarium specimens using a 2% CTAB procedure (Bruns, 1995), with minor changes given by Zhao *et al.* (2011). For the qualitative investigation of extracted DNA, gel electrophoresis was carried out by means of 1% agarose gel (Voytas, 2000). A thermocycler was used to amplify the extracted DNA using polymerase chain reaction. For molecular analysis, ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer pairs were used to amplify the Internal Transcribed Spacer (ITS) region (White *et al.*, 1990; Gardes and Bruns, 1993). PCR was carried out in a thermocycler (Perkin-Elmer, Applied Biosystems) using a 25 µl reaction mixture following the conditions of Yan and Bau (2018). Bidirectional sequencing was done using the same pairs of primers. All the new sequences were deposited in GenBank (ITS OP160530; OP160531; OP537167; MG822871).

### *Phylogenetic analysis*

Consensus sequences consisting of 610–650 bps were generated using BioEdit software version 7.0.4.1 (Hall, 1999). These consensus sequences were then subjected to BLAST searches on the NCBI (National Center for Biotechnology Information) platform (<https://www.ncbi.nlm.nih.gov/>). The closely related *Asterophora* sequences were retrieved from GenBank and were aligned using the online MUSCLE tool at EMBL-EBI (<http://www.ebi.ac.uk/>). For phylogenetic analysis of ITS dataset, Maximum likelihood (ML) bootstrap analyses were performed in MEGA11 that involved the utilization of the ultrafast bootstrap approximation, employing 1000 replicates.

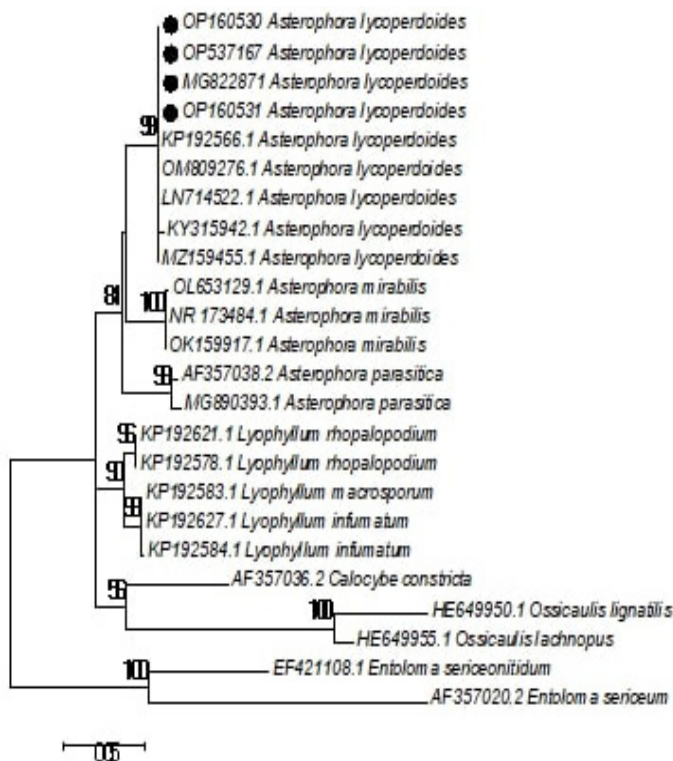
## Results and Discussion

### *Phylogenetic analysis*

The aligned ITS dataset comprises 22 nucleotide sequences, which were subjected to BLAST searching against GenBank. The dataset includes sequences from *Entoloma sericeum* Qué. and *E. sericeonitidum*

P.D. Orton Arnolds, serving as outgroup references (Bellanger *et al.* 2015). The ITS sequences of our collection showed 99.67 % similarity with sequences of *Asterophora lycoperdoides* (KP192566 from France, OM809276 and MZ159455 from the USA, LN714522 from Czech Republic). The newly generated sequences clustered in a clade with *Asterophora lycoperdoides* specimens that is in sister relationship with *Asterophora mirabilis* but with no support. The branch representing the *Asterophora lycoperdoides* specimens show the strong bootstrap value of 100%. Newly generated sequences are highlighted with a bullet (Figure 1).

completely lacking; 0.3–2mm deep; when present normal, moderately crowded to distant, adnate to decurrent; white to pale or cream, concolorous with pileus at young basidiomata becoming brown or cinnamon colored by the formation of chlamydospores; edge even or entire. Stipe long and thin, 5–60 × 4–18 mm; central; cylindrical, often curved; whitish at young stage becoming pale brown at maturity; surface dry, dull, concolorous with pileus, with longitudinally appressed fibrils. Context white when young becoming cream in the pileus, stipe, and over the lamellae. Veil absent. Odor insignificant. Taste not recorded. Basidiospores very sparse and hard to obtain; chlamydospores cover the lamellae at mature stages. Chlamydospores or secondary spores abundant in the lamellar and pileal trama and even numerous on the host tissue, (12.3–) 12.9–15.6 (–15.9) × (11.3–) 11.9–14.2 (–14.4) μm, avL × avW = 14.3 × 13.2 μm; globose, thick-walled, starburst shape, hyaline to brown; containing granular contents, thick, conical and large projections 3–6 μm; sometime obtuse or truncate apex (Figure 2). Stiptipellis a cutis of parallel cylindrical hyphae, 4–18 μm, hyaline to pale yellow.



**Figure 1:** Molecular phylogenetic analysis of *Asterophora lycoperdoides* (Bull.) Ditmar. based on maximum likelihood (ML) method of nrITS sequences.

**Taxonomy**

*Asterophora lycoperdoides* (Bull.) Ditmar. 1809: Pileus 3–25 mm, pin head size at initial stage, hemispherical convex at young stage, becoming broadly convex to plano convex at maturity; incurved margins; at first surface smooth, dry, non-striate, non-fibrillose rapidly becoming rough, powdery and fibrillose; whitish at young stage forming a prominent and bright appearance over decaying brown host, brown patches appeared on the surface of pileus and stipe with time and ultimately covered with brown or cinnamon colored mass by asexual spores (chlamydospores) formation. Hymenophore usually less developed or



**Figure 2:** (A) *Asterophora lycoperdoides* (Bull.) Ditmar growing on its host *Russula* sp, (B–D) basidiocarps of *A. lycoperdoides*. (E–F) chlamydospores of *A. lycoperdoides*.

*Material examined*

Pakistan, Punjab province, Bhurban town, 1828 m asl; September 7, 2020, Annum Razzaq GB108 (LAH38040), GenBank (ITS; OP160530); September 10, 2020, Annum Razzaq GB111 (LAH38041), Golf club, 1828m asl; August 10, 2019, Annum Razzaq GC36 (LAH38042), GenBank (ITS; OP160530); clustered on pileus of a decaying *Russula* sp. under coniferous tree of sub-himalayan temperate forests.

The *Asterophora* is an uncommon genus and has received limited attention in available reports. *Russula* and *Lactarius* are the most common the host of this genus and produce edible fruiting bodies. These are frequently gathered from natural forests without a comprehensive understanding of the potential long-term consequences of such harvesting. Both *Russula* and *Lactarius* are ectomycorrhizal and serve as a food source for the local tribal communities.

In this study, a mycoparasitic mushroom, *Asterophora lycoperdoides* is described as a new generic record for Pakistan on morpho-anatomical and phylogenetic bases. The proper identification of *Asterophora* species is often difficult to perform as the taxon has rudimentary lamellae and does not produce basidiospores. However, the reproduction takes place through the formation of enormous stellate chlamydospores in the upper pileal trama, hence, giving the brown powdery appearance to the basidiomata (May and Fuhrer, 1995; Redhead and Seifert, 2001). The Pakistani collections of *A. lycoperdoides* show similar morphological features; like the shape and size of pileus and chlamydospores to the *A. lycoperdoides* described by Sharma *et al.* (2007) from India. The nrITS sequences of the present collections (GB111, GB108, GC36) phylogenetically cluster with *A. lycoperdoides* KP192566 (France), OM809276, MZ159455 (USA), LN714522 (Czech Republic) indicating the cosmopolitan distribution of this species. Previously, the genus has already been described from North America, Europe, Asia, North Africa and Papua New Guinea (Corner, 1966; Horak, 1980; Singer, 1986). However, no species of this genus has ever been described from Pakistan.

**Conclusions and Recommendations**

The forests of Pakistan hold substantial and yet unexplored mushroom biodiversity. Continued efforts in characterizing mycorrhizal genera are

crucial for comprehending their significance as fungal symbionts. This understanding is essential for future management strategies aimed at ensuring ecosystem sustainability and productivity. In this context, this report marks the initial generic documentation of the species *A. lycoperdoides* in the temperate forests of Pakistan, contributing to the expanding mycoflora knowledge of the region.

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**Novelty Statement**

By exploring the diversity of macrofungi, their phylogenetic relationship can be identified and will provide a baseline data to use macrofungi in applied sciences research.

**Author's Contribution**

**Annum Razzaq:** Data curation, conceptualization, methodology, writing original draft, editing.

**Zia Ullah:** Collection, DNA extraction, PCR, sequencing.

**Arooj Naseer:** Supervision of formal analysis, supervision.

**Abdul Nasir Khalid:** Supervision, review and editing.

*Conflict of interest*

The authors have declared no conflict of interest.

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