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FIRST RECORD OF *SPHAEROBOLUS CUPROPHILUS* FROM PAKISTAN BASED ON MORPHOLOGICAL AND MOLECULAR STUDIES

MUHAMMAD BILAL SHARIF AND SANA JABEEN*

Department of Botany, Division of Science and Technology, University of Education, Lahore, Pakistan

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Abstract

Sphaerobolus cuprophilus was collected from Haroonabad, Bahawalnagar District, Punjab, Pakistan. It was identified based on its cottony white basal mycelium, spherical basidiocarps with a yellow inner layer, and a centrally positioned greenish brown peridiole. Microscopic examination revealed elliptical, thick-walled spores (12.8 × 7.6 μm) and the presence of elongated gemmae. Molecular analysis of ITS region of the nrDNA further confirmed its identity, representing the first documented record of the genus *Sphaerobolus* from Pakistan.

Introduction

Sphaerobolus Tode commonly known as the cannonball mushroom, was first described as *Carpobolus* is a distinctive group of gasteroid basidiomycetes commonly known as "artillery fungi" due to their unique spore dispersal mechanism, in which the gleba is forcibly ejected and adheres to nearby substrates (Micheli, 1729). Species of *Sphaerobolus* are typically saprobic and occur on dung, decaying wood, leaf litter, and other organic substrates, playing an important role in nutrient cycling in terrestrial ecosystems. These fungi produce globose, pigmented basidiocarps containing a single peridiole bearing gleba (spore mass), which houses two spore types: thick-walled, uninucleate basidiospores and thin walled, dikaryotic gemmae which germinate by producing a germ tube that develops into septate hyphae with clamp connections. Basidiospores germinate optimally under proteolytic conditions (e.g., pepsin exposure) and high temperatures characteristic of herbivore gastrointestinal systems, accounting for their frequent occurrence on herbivore dung (Dykstra, 1982; Geml, 2004).

Sphaerobolus has been reported from several regions, including Asia, Europe, Africa, North and South America, and Oceania, indicating a nearly worldwide distribution (Walker, 1927; Butler & Bisby, 1931; Vasava *et al*., 2021; Oliveira *et al*., 2022; Kalayanamitra *et al*., 2023). To date, as per Index Fungorum, only nine species of the genus *Sphaerobolus* have been documented (Index Fungorum 2025, retrieved on 4 March 2026), whereas MycoBank recorded 19 legitimate taxa within the genus (MycoBank 2025, retrieved on 4 March 2026). In Pakistan, only a single species, *S. stellatus* Tode, has previously been documented, and this record was based solely on morphological evidence (Ahmad *et al*., 1997).

Basidiomata resembling members of the genus Sphaerobolus were collected from Haroonabad, Bahawalnagar District, Punjab, Pakistan, during the spring of 2024. Detailed morphological and anatomical examinations, along with molecular phylogenetic analysis based on the ITS (Internal Transcribed Spacer) region of nrDNA, were conducted to determine its taxonomic identity at the species level.

*Corresponding author: sanajabeen@ue.edu.pk

Materials and Methods

Sampling site

The district of Bahawalnagar, located next to the Cholistan desert in Punjab, Pakistan, was selected as sampling site. The area has a hot desert climate according to the Köppen climate classification (Peel *et al*., 2007). Summers are typically hot and dry, while winters are cold and dry, with temperatures ranging from 11–52°C throughout the year. The region receives an average annual rainfall of 99 mm (Ahmed *et al*., 2014).

Morphoanatomical studies

Basidiomata were collected and photographed in the field. The fresh specimens were studied for their morphological characteristics, such as size, shape, and color. Color codes were given by using Munsell color chart (Munsell, 1975). The specimens were carefully dried in food dehydrator at 45°C for eight hours.

For micro-morphological analysis, structures like spores, basidia, cystidia, and hyphae arrangements in different tissues were examined under a microscope (Coroscope, S/N-EU 2230362) using 5% KOH and Congo red, at 1000 × magnification. Measurements were recorded using Motic Images Plus 2.0 software. For basidiospores, the [s/b/c] format was used, where ‘s’ is the number of basidiospores measured, b’ refers to the number of basidiomata, and ‘c’ is the number of collections. The measurement range is expressed as (a)b–c(d), where 'a' is the minimum, 'b–c' covers 90% of the values, and 'd' is the maximum. The ‘Q’ ratio represents the length/width ratio of the spores (Bas, 1969), and avl × avw stands for the average length and width. The specimen was deposited in the herbarium of the Department of Botany, University of Education, Lahore, Pakistan (UEH).

Molecular studies

DNA extraction was carried out from small portions of basidiomata using the CTAB method of Bruns (1995). The ITS region of nrDNA was amplified and sequenced at the Beijing Genomic Institute, China. The obtained sequences were analyzed using BioEdit sequence alignment editor version 7.2.5 (Hall, 1999) and BLAST searched at NCBI (http://www.ncbi.nlm.nih.gov/). The sequence generated during this study, closest matching sequences from GenBank and published literature (Kalayanamitra *et al*., 2023) were selected to reconstruct a phylogenetic tree. For the alignment of these sequences in the ITS dataset, online MUSCLE tool was used (Edgar, 2004). Sequences were trimmed from conserved motifs in BioEdit version 7.2.5 (Hall, 1999). To assess phylogeny, Maximum Likelihood (ML) analysis was conducted with MEGA 12 software using K2+G model as best fit model for the alignment using 1000 bootstrap replicates (Tamura, 1992; Kumar *et al*., 2024).

Results and Discussion

Molecular phylogenetic analysis

The ITS region of nrDNA obtained from the Pakistani specimen *Sphaerobolus cuprophilus* UEH-F40054 yielded a sequence of 650 bp, which was compared with available sequences in GenBank using the BLAST algorithm. It showed 98% similarity with sequence of *S. cuprophilus* (OM980552–OM980554) from Thailand and 95% similarity with *S. jaysukhianus* A.M. Vasava, R.S. Patel & K.S. Rajput (MK208479) from India. These sequences along with other closely related sequences were used to reconstruct the phylogeny. Taxa from closely related genera; *Dacrymyces roseotinctus* Lloyd (OM980556) and *Dacryopinax spathularia* (Schwein.) G.W. Martin (MH230102) were used as outgroup to root the phylogenetic tree (Kalayanamitra *et al*.,

2023). This phylogenetic analysis involved 40 nucleotide sequences and 1060 positions. From which 459 were conserved, 580 were variable, 464 were parsimony informative and 116 were represented as singletons while the remaining 21 positions contained gaps and/or ambiguous characters. In phylogenetic tree, the sequence generated during this research was clustered with sequences of *S. cuprophilus* (OM980552–OM980554) from Thailand and separated from all other sequences with 100% bootstrap value (Fig. 1).

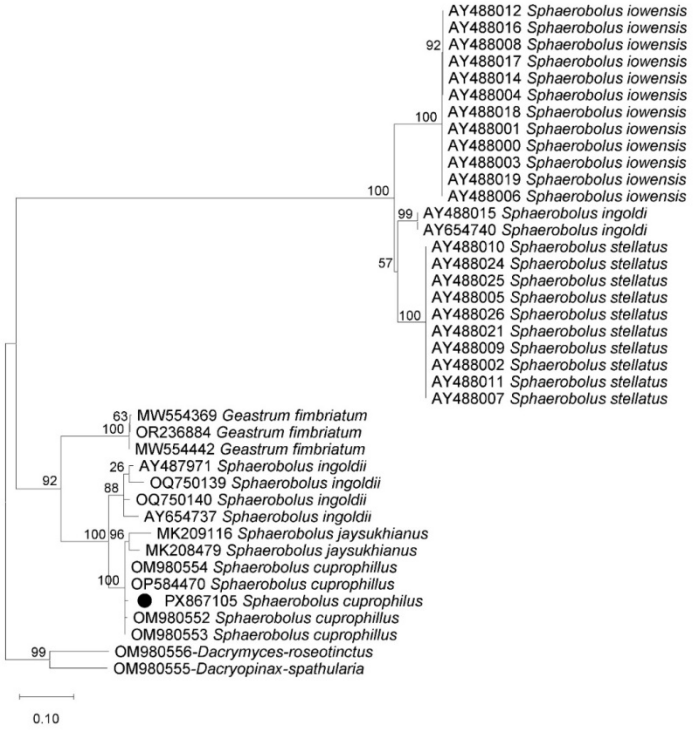

Fig. 1. Molecular phylogenetic analysis of *Sphaerobolus* spp. based on ITS sequences inferred by the Maximum Likelihood method. Values above nodes are the ML bootstrap percentages, the sequence generated during this study is marked with bullet.

Taxonomy

Sphaerobolus cuprophilus Kalayanamitra & Bussaban, Plants **12**(3): 480 (2023) (Figs 2 & 3)

Mycelium cottony white (N9.5) to off-white (10YR 9/1). Basidiomata is mostly in cluster, rarely solitary. Basidiocarps spherical (2)3–5(6) mm, spilt radiantly at maturity, exposing yellow layer (7.5Y 9/12); Out surface of exoperidium ramose (cracked) in reticulate manner. Peridiole single, spherical, light greenish brown (2.5GY 5/4) to dark greenish brown (2.5GY 3/4) and become dark brown (5YR 3/2) when dried.

Basidiospores [50/10/1] (10.6)11.1–14.6(15.6) µm × (6.3)7.1–8.7(8.9) µm, Q = 1.7; ellipsoid to broadly ellipsoid, hyaline, thick-walled, smooth surface. Basidia not found. Cystidia (9.5)10.1–12.1(13.2) µm in diameter, round, scattered in gleba, hyaline. Exoperidium layer 1 with hyphae (2.1)2.7–3(3.1) µm, septate, clamp connections present, branched hyaline. Exoperidium layer 2 with hyphae (0.9)1–2.5(2.8) µm with inflated cells, hyaline; layer 3 with hyphae (0.9)1.6–2.5(3.3)

µm, hyaline, unbranched and aseptate; layer 4 with hyphae (5)5.9–12.7(14.4) µm branched, hyaline, bulbous to various shapes. Gemmae septate with clamp connections, hyaline, 3.1(3.5)–9.1(10.5) µm wide.

Habitat and distribution: Found gregarious growing on cow dung, warm desert climate of Haroonabad, District Bahawalnagar, Punjab, Pakistan.

Material examined: Pakistan, Punjab, Bahawalnagar District, Tehsil Haroonbad, Village Chak No. 1/1R, clusters cow dung, 8 April 2024, Muhammad Bilal Sharif, (UEH-F40054); GenBank accession: PX867105.

Fig. 2. *Sphaerobolus cuprophilus* UEH-F40054. (A–D) Basidiomata on cow dung. Scale bars = 1 cm. Photographs captured and figure plate made by Muhammad Bilal Sharif.

The present study documents the first record of *Sphaerobolus cuprophilus* from Pakistan and constitutes only the second global report of the species since its original description from Thailand by Kalayanamitra *et al*. (2023). Despite the remarkable dispersal strategy and apparent ecological tolerance of *Sphaerobolus* species, their taxonomy and global distribution remain poorly documented, with many records relying solely on morphology and limited molecular data. By integrating detailed morphological observations with ITS-based molecular phylogenetic analysis, this study contributes robust evidence for the occurrence of *S. cuprophilus* in a new geographical region and climatic zone.

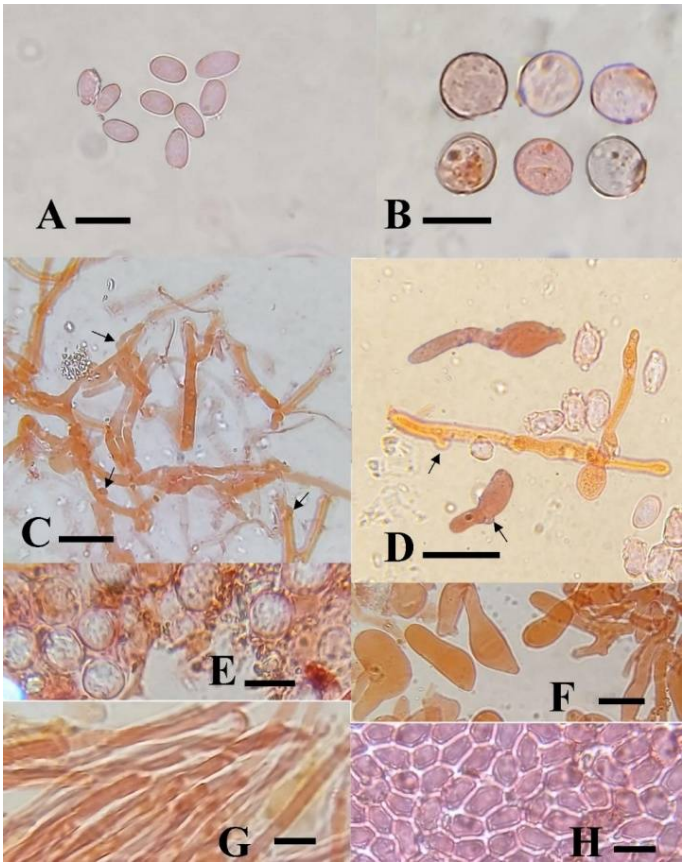

Fig. 3. *Sphaerobolus cuprophilus* UEH-F40054. A. Basidiospores; B. Cystidia; C. Exoperidium 1; D. Gemmae; E. Exoperidium 2; F. Exoperidium 3; G. Exoperidium 4; H. Hyphae of the peridiole wall. Scale bars: A–C, D, F = 16 µm; E, G = 3 µm; H = 6 µm. Arrows indicate clamp connections. Micrographs and figure plate made by Muhammad Bilal Sharif.

Vegetative growth in *Sphaerobolus* is reported to occur optimally at temperatures between 20°C and 25°C, whereas the development of reproductive structures is favored at lower temperatures (10–20°C), leading to their characterization as "cool-season fungi" (Alasoadura, 1963; Geml, 2004). In contrast to these typical growth preferences, the Pakistani collections were obtained from a warm desert climate, suggesting that *S. cuprophilus* can tolerate a broader thermal range than previously recognized.

Although, *Sphaerobolus* species play a role in nutrient cycling as saprobes, their forcible ejection of glebal masses can negatively affect agricultural and horticultural systems. They have been reported as the causal agent of black spot disease in Chinese kale (*Brassica alboglabra* L. H. Bailey) (Kalayanamitra *et al*., 2023) and have also been associated with turfgrass thatch collapse on golf courses (Baetsen-Young *et al*., 2015). These reports highlight the potential economic and aesthetic impact of the genus beyond its ecological role as a decomposer.

Sphaerobolus cuprophilus, a copper-tolerant species, was originally described from Thailand, where it produced abundant basidiocarps in Chinese kale plantations treated with copper-based

fungicides. Glebal masses strongly adhered to leaf and stem surfaces, reducing market quality without causing tissue infection, thereby constituting an important postharvest problem (Kalayanamitra *et al*., 2023). In the present study, basidiomata were recovered from cow dung in the warm desert region of Haroonabad, District Bahawalnagar, Punjab, Pakistan, representing a marked shift in both substrate and climatic conditions.

The Pakistani specimens were characterized by cottony white mycelium, spherical basidiocarps with a yellow inner peridial layer, and a centrally positioned dark greenish brown peridiole. Microscopic examination revealed elongated, thick-walled basidiospores and the presence of gemmae. Molecular phylogenetic analyses based on ITS sequences placed the Pakistani collections within the *S. cuprophilus* clade, confirming their taxonomic identity and excluding the possibility of cryptic speciation.

The occurrence of *S. cuprophilus* in cold agricultural systems of Thailand and in the hot desert environment of Pakistan reflects wide ecological amplitude within the species. Such divergence in habitat and substrate preference indicates strong adaptive capacity to contrasting thermal regimes and nutrient sources. This ecological plasticity may explain the apparent rarity of records and suggests that the species may be underreported rather than truly rare.

Previously, *S. cuprophilus* was known only from Thailand. Its discovery in Pakistan therefore represents the first national record and the second global report of this species, substantially extending its known geographical range.

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