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Cover: A bag worm with its beautiful heap of junk. Acrylics on 300 GSM paper by Dupati Poojitha based on a picture by Sanjay Molur.

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SHORT COMMUNICATION

Calvatia craniiformis (Schwein.) Fr. ex De Toni (Agaricomycetes: Lycoperdaceae): a new puffball mushroom record from eastern India

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Abstract: The present study reports the occurrence of *Calvatia craniiformis*, collected during early monsoon, from the forest floor of Ajodhya Hills, near Teliabhasa village in the Baghmundi Block of Purulia District, West Bengal. The identity of the taxon was revealed through macro- and micro-morphological characterization, followed by nrITS based phylogenetic analyses, representing a first report from eastern India. A detailed description, scanning electron microscopy micrographs and molecular phylogeny are provided.

Keywords: Agaricales, brain puffball, micro-morphology, nrITS phylogeny, SEM, taxonomy.

Calvatia Fr. is a gasteroid fungus belonging to the family Lycoperdaceae F.Bercht. & J.Presl., commonly known as puffball mushrooms. The genus was established in 1859 by Fries and is typified by *Calvatia craniiformis* (originally described as *Bovista craniiformis* Schwein.). It represents a group that includes some of the largest-sized puffballs. The name *Calvatia* is derived from the Latin word 'calvus' meaning 'bald' (Hedavoo 2020). The specific epithet *craniiformis* is derived from two Latin words: *cranion* meaning 'skull' and *forma* meaning 'form', referring to the resemblance of the puffball to a human brain. Consequently, it is also commonly called Skull Puffball or Brain Puffball (Marshall 2003; Hard 2009; Hawkeswood 2019; Gogoi & Kumar 2020). The genus *Calvatia* is characterized by a soft, pyriform to globose, turbinate, and epigeous basidiome; cottony to pulverulent gleba; simple and well-developed subgleba, dehiscence by irregular rupturing of peridium rather than by an apical pore; *Calvatia*-type capillitium, and basidiospores smooth to ornamented (verrucose and echinate) (Krüger et al. 2001; Coetzee & Wyk 2009).

At present, 47 species of *Calvatia* are accepted. *C. craniiformis* has been reported from various countries, including Australia, China, Indonesia, Japan, Malayasia, South Korea, Thailand, and the United States (Jung 1995; Bates et al. 2009; Hosaka & Uno 2012; Hawkeswood 2019; Gogoi & Kumar 2020; Yuwa-Amornpitak & Yeunyaw 2020; Patel & Rajput 2024). In India, approximately 25 species of *Calvatia* have been reported from different regions. Based on their current taxonomic status, only 15 species are accepted, while several have been synonymised with other genera or species (Patel & Rajput 2024). Few reports on *C. craniiformis* from India have been achieved from southern India (Abrar et al. 2008), northeastern India (Gogoi & Kumar 2020), and northern part of Western Ghats (Kshirsagar et al. 2020). Earlier,

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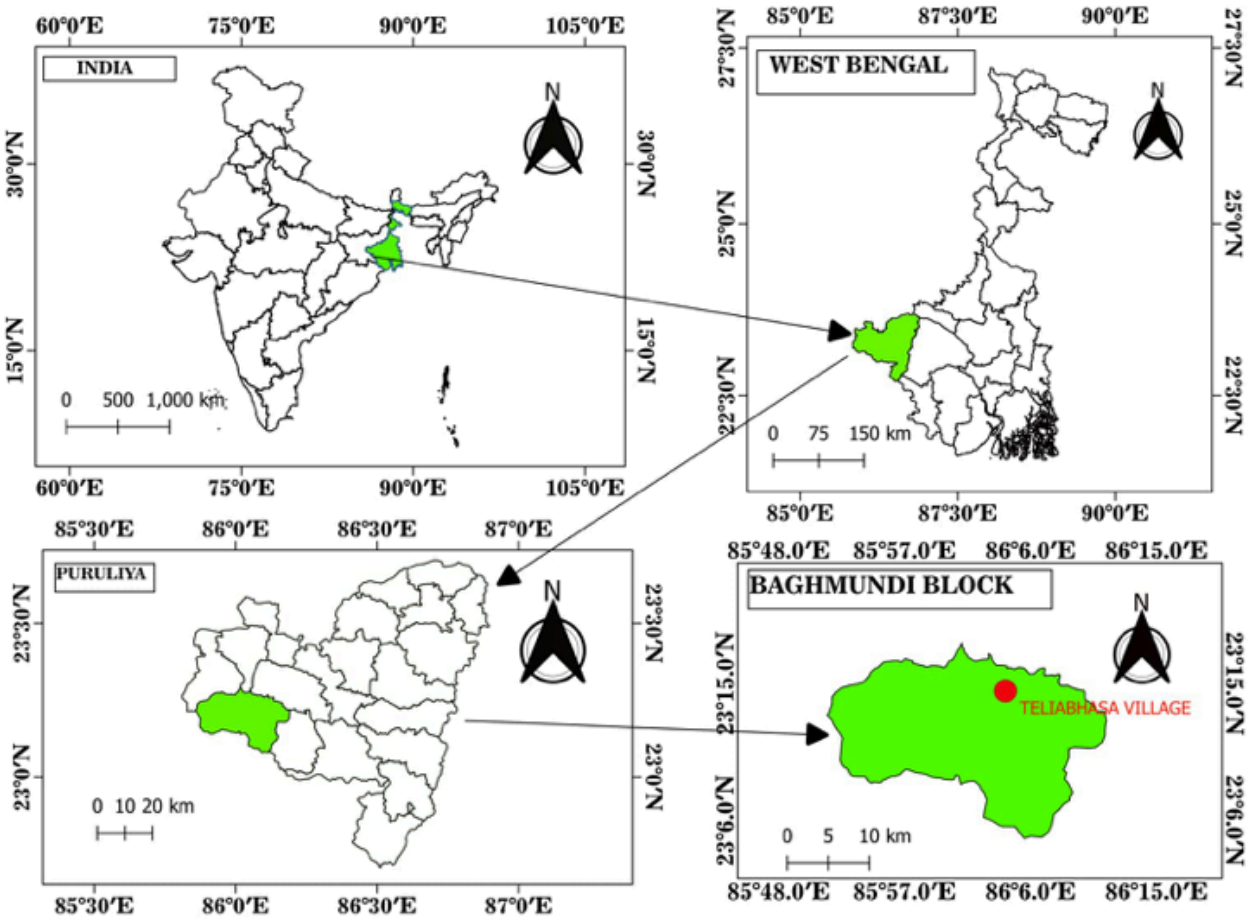

Figure 1. Location of study site on map.

the species has been found growing in humus-rich soil with leaf litter (Hawkeswood 2019), as well as in open ground and meadows (Abrar et al. 2008). Additionally, *C. craniiformis* is recognized as an important source of food and traditional medicine, with reported antifungal properties (Gogoi & Kumar 2020).

In the present investigation, the isolated fungal strain was identified as *Calvatia craniiformis* through combined approach of morpho-taxonomy and molecular phylogenetic characterization and revealed as first report from eastern India.

Materials And Methods

Collection site

The specimen was collected from the dry deciduous, humus-rich forest floor of a sacred groove, 'Jaherthan', near Teliabhasa Village in the Ajodhya Hills, Purulia, West Bengal at an altitude of 647 m (Figure 1). The specimens were found growing either in clusters or scattered during June 2023. Fresh fruiting bodies were sampled, dried and preserved for further studies.

Morpho-anatomical analysis

Macro-morphological and substrate details of fresh, young to mature basidiomata were recorded in the field or at the respective basecamps, including colour, odour, texture, substratum, and size of the basidiomata. Images of the basidiomata were captured by Realme 8, 64MP AI quad-camera. The collected specimens were dried overnight in a hot air oven at 60°C and preserved in sealed plastic bags with silica gel. An herbarium record of the collected specimens was deposited in the Department of Botany, Sidho-Kanho-Birsha University, Purulia. Colour code followed the Methuen Handbook of Colour (Kornerup & Wanscher 1967). Micromorphological characters were observed by preparing free hand sections of dried samples, mounted in a mixture of 3% KOH, 2% Congo Red and observed under the microscope (Leica DM 3000 LED). Images were captured using a digital camera (Leica MC 190 HD). SEM analyses were done to study the ornamentation of basidiospores using the model JEOL JCM-6000 Plus Benchtop. Basidiospores were collected from dried gleba, placed in a water

droplet, and mixed gently. The mixture was immediately pipetted onto a cover glass, dried, placed on a stub and coated with the gold (Hansen et al. 1999).

Molecular characterization and phylogenetic tree analysis

Genomic DNA of *C. craniiformis* was extracted from a dried powder sample of basidiomata following Aamir et al. (2015) and amplified using ITS1 and ITS4 as forward and reverse primers, following White et al. (1990). PCR products were purified using the QIAGEN QIA quick PCR Purification Kit and sequenced using the Sanger sequencing method (Kshirsagar et al. 2020). The size of PCR product was estimated by comparing the migration distance of SRAM-220626 to the loaded DNA ladder and confirmed using NEBcutter V1.0 (Vincze et al. 2003). The nBlast program of NCBI (National Centre for Biotechnology Information) database was used to analyze the obtained raw sequences and compare them with available fungal sequences in the database. The dataset was prepared using partial 18S rRNA gene sequence of SRAM-220626 obtained in this study, along with other retrieved sequences from the GenBank database, with *Termitomyces heimii* Natarajan as an outgroup. Sequences were aligned using the ClustalW program in MEGA11 (Tamura et al. 2021). Molecular phylogeny was determined using the RAxML-HPC2 Workflow on XSEDE programme of RAxML v.8.2.10 with a bootstrap value of 1000 (Kantharaja & Krishnappa 2022) and visualized with FigTree software v 1.4.4 (Rambaut 2018). The newly generated sequence has been submitted to GenBank.

Results

Taxonomy

Calvatia craniiformis (Schwein.)
Fr. ex De Toni, Syll. Fung. 7: 106 (1888)
(Image 1 A–D)

GenBank accession number OR185460

Basidiomata gasteroid, 50–80 mm high and 40–70

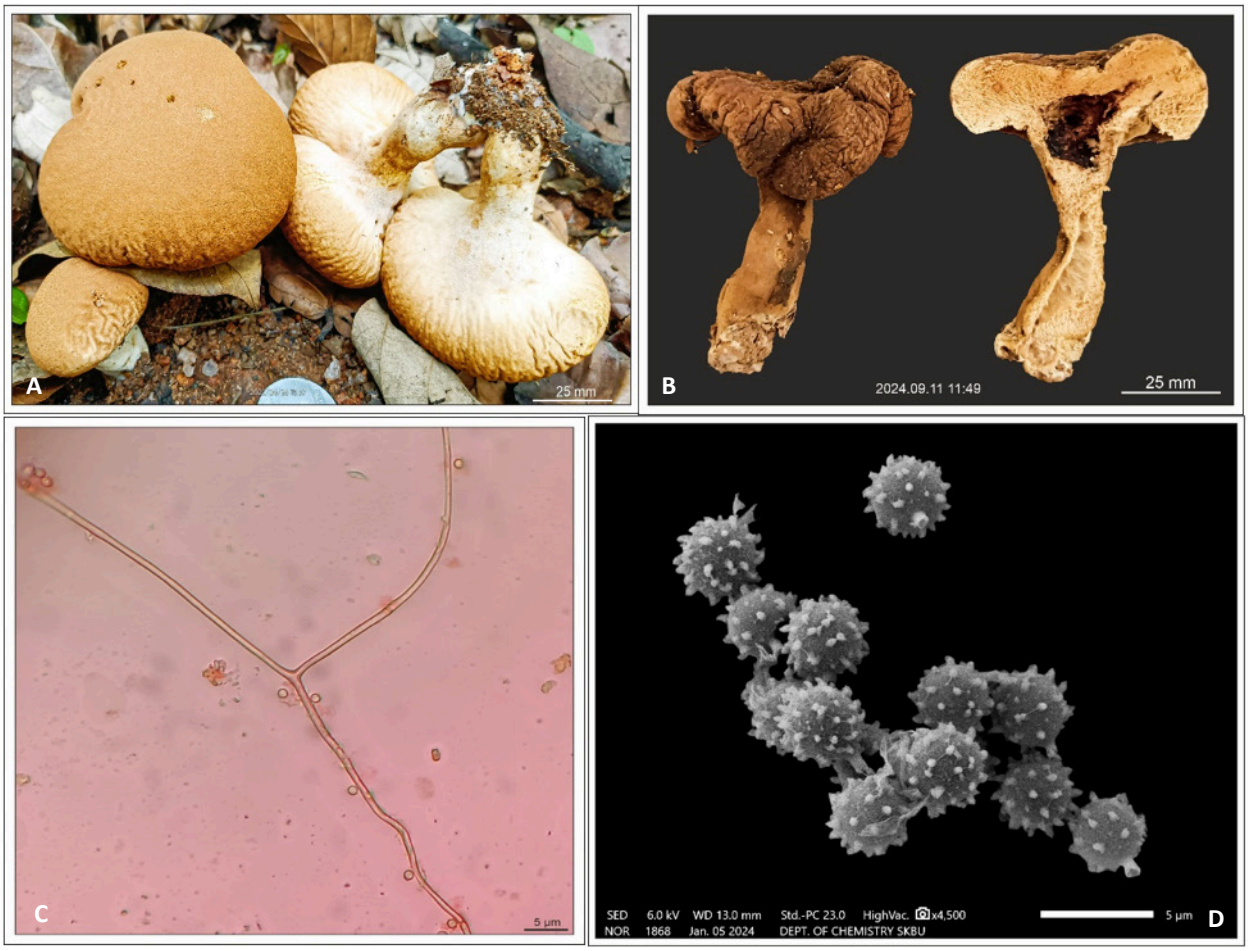

Image 1. Basidiomata, capillitial hyphae and spores of *Calvatia craniiformis*: A—Basidiomata of *Calvatia craniiformis* | B—Gleba of dry fruit body | C—Capillitial thread | D—SEM of spores. © Asit Mahato.

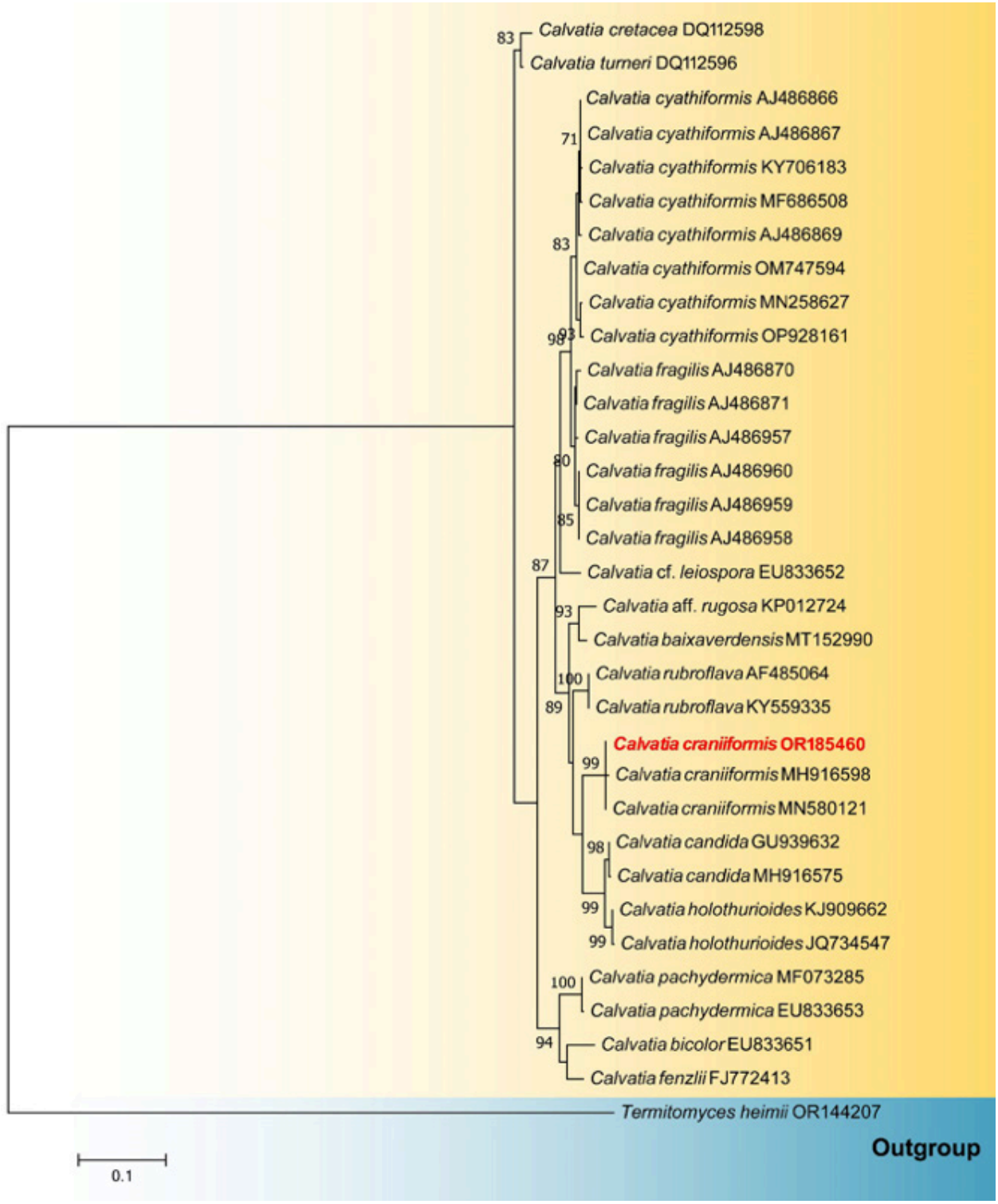

Figure 2. Phylogenetic tree based on 18S rRNA sequence analysis showing genetic relationship among different *Calvatia* species, including isolate *Calvatia craniiformis* SRAM-220626 (OR185460) and *Termitomyces heimii* used as outgroup. The tree was inferred by RAxml programme with 1,000 bootstrap value.

mm broad, epigeous, medium to large-sized, globose to turbinate, dry, low hygrophanous, wrinkled, splitted, lacinate, rivulose, brownish-yellow (5C7) to yellowish-brown (5D8), with an anise-like to unpleasant odor, and a mild taste. Two or more separate fruit bodies arise from a single basal position; ostiole absent. Stipe central, subclavate, glandular-dotted, arid, with moderate basal tomentum, white to brownish, unbranched or branched.

Peridium smooth to wrinkled, folded, and pulverulent. Exoperidium thin, granulose, yellowish-brown, and darker than the endoperidium. Endoperidium papery, white to brownish-white.

Gleba yellowish-white (4A2) to light brown (5D7), solid when young, becoming spongy and cottony at maturity. Capillitia *Calvatia*-type, occasionally branched, light brown, septate 2–4 µm, straight to undulate. Basidia not observed.

Basidiospores (2.3) 2.5–3.5 (3.8) × (2) 2.2–3.1 (3.3), 3.1 ± 0.43 × 2.6 ± 0.73 µm, globose to subglobose, echinulate with spinulose to spinose ornamented under SEM, spines measuring 0.65–0.75 µm in length; pedicellate, with hyaline pedicels.

ITS Sequences and Phylogeny analysis

NEBcutter V1.0 indicated that the length and GC content of the generated sequence are approximately 600 bp and 44.2%, respectively. The BLAST program of NCBI inferred that the strain, SRAM-220626, is closely clustered with *C. craniiformis* CMIS (MN580121) from Thailand and *C. craniiformis* C2 (MH916598) from India. Phylogenetic tree analysis based on nrITS sequences of 52 different *Calvatia* species, along with the Indian isolate of *C. craniiformis*, was conducted using *Termitomyces heimii* as an outgroup (Figure 2). The analysis revealed that *C. craniiformis* CMIS, *C. craniiformis* SRAM-220626 and *C. craniiformis* C2 cluster together in the same clade with a strong ML bootstrap support (MLbs = 99%).

Discussion

Based on a combined approach of macro- and micro-morphological characterization, along with molecular phylogenetic analyses, the Indian collection was confirmed as *Calvatia craniiformis*. The present collection also shows similarities with other Asian collections of *C. craniiformis* from different regions of India, as reported by Abrar et al. (2008), Gogoi & Kumar (2020), Kshirsagar et al. (2020), and mentioned the size of the mature basidiomata as: 50–150 × 60–120 mm, 70–200 × 70–180 mm, and 30–35 × 20–30 mm, respectively. Notably, the size of the mature basidiomata in the present study is relatively smaller than the specimens examined by Gogoi & Kumar (2020) and Abrar et al. (2008) but significantly larger than the collections described by Kshirsagar et al. (2020), while the basidiospores are comparatively smaller than those described by Kshirsagar et al. (2020). This investigation represents the first report of *Calvatia craniiformis* from West Bengal and eastern India, contributing to regional mycological knowledge and expanding the distributional range of the species in the Indian subcontinent.

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