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Comprehensive morphological and phylogenetic inferences of star-shaped fungus *Astraeus* (*Diplocystidiaceae*) from sal-dominant tropical and subtropical *Pinus-Shorea* forests in India: an integrative taxonomic analysis

Vineet Vishal[1,2], Pitchapa Thongsuwan[3], Pisit Thamvithayakorn[3], Nuttika Suwannasai[3], Cherdchai Phosri[4,5], María P. Martín[6], Geetanjali Singh[1] & Shalini Lal[1*]

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Abstract. Classifying fungus based on morphological traits is an effective strategy to distinguish between puffballs, earthballs, earthstars, and other gasteroid fungi, as well as for identifying sub-groups of closely related taxon. However, to delimitate taxa it should be addressed with caution, since cryptic species have been described recently in different genera of gasteroid fungi. *Astraeus* is a star-shaped fungus that has piqued the interest of mycologists worldwide, including India. These endearing fungi have tremendous nutritional and therapeutic benefits, but their molecular and phylogenetic placement in India is uncertain. An integrative taxonomic approach was used to identify and resolve ambiguities within the genus. This study, the first to elucidate the distribution, taxonomy, and phylogeny of the genus *Astraeus* in the mixed subtropical *Pinus-Shorea* forest and tropical dry deciduous *Shorea* dominated forest in India, aims to shed light on these indispensable ectomycorrhizal fungi. The phylogenetic analysis assigned all thirteen ITS DNA barcoding Indian sequences of *Astraeus* to the Southeast Asian clade, reinforcing the genus Southeast Asian origin. Detailed species descriptions, line diagrams, SEM images of basidiospores, ITS nrDNA based phylogeny, and a dichotomous key are provided. Mycoobservations of the taxon from *Pinus-Shorea* subtropical and *Shorea*-dominated forests were additionally reviewed.

Key words: boletoid, diversity, earthstar, India, ITS nrDNA, mushroom, phylogeny

Introduction

India's biogeographic, physiographic, and agroclimatic conditions have led to the presence of a rich diversity of macrofungi, including *Astraeus*. This epigeous wild earthstar mushroom is usually found in *Dipterocarpaceae*-dominated forests. The young gasterocarp has a globose, sub-globose, depressed, or dome-shaped body that lacks a stalk and cap (Phosri 2004). These gasterocarps are mostly hypogeous to sub-hypogeous and occasionally epigeous in nature and are united by the fact that their spores are enclosed in hymenium and dehisce by a passive bellowing mechanism. The taxonomic history of this earthstar fungus, however, is exceptionally cryptic, perplexing, and complex in comparison to other earthstar fungi like *Geastrum* and *Myriostoma*. The name *Geastrum hygrometricum* was initially given by Persoon (1801). Morgan (1889) established the new genus *Astraeus* for these rare fungi and included them to the *Lycoperdaceae* family. The barometer earthstar *Astraeus hygrometricus* (syn. *Geastrum hygrometricum*) serves as the type species (Weber & Webster 2007). Martin (1936) then gave *Astraeus* the new family name *Astraeaceae*. Kreisel (1974) later placed these earthstar mushrooms as members of the *Diplocystidiaceae* family, which includes the genera *Diplocystis*, *Endogonopsis* and *Tremellogaster*. There are currently eleven valid *Astraeus*

[1] Department of Botany, Dr Shyama Prasad Mukherjee University, Ranchi, Jharkhand, India
(V. Vishal, ORCID: 0000-0003-0641-4979; G. Singh, ORCID: 0000-0002-5690-379X; S. Lal, ORCID: 0000-0002-4974-7709)
[2] Department of Botany, Bangabasi Evening College, Kolkata, West Bengal, India
[3] Department of Microbiology, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand
(P. Thongsuwan, ORCID: 0009-0007-5717-5003; P. Thamvithayakorn, ORCID: 0000-0002-7773-4016; N. Suwannasai, ORCID: 0000-0003-0599-5732)
[4] Department of Biology, Faculty of Science, Nakhon Phanom University, Nakhon Phanom, Thailand
ORCID: 0000-0003-3963-752X
[5] The Knowledge Hub for Integrated Economic Trees: Plantation Establishment, Management, Utilization and Industry, Kasetsart University, Bangkok, Thailand
[6] Department of Mycology, Royal Botanical Garden, RJB-CSIC, Madrid, Spain
ORCID: 0000-0002-1235-4418
[*] Corresponding author e-mail: shalini.kumari@gmail.com

species listed in the Index Fungorum database (https://www.indexfungorum.org/) out of 20 records, the majority of which have been documented in North America, Europe, and Southeast Asia. However, *Astraeus* species have rarely been discovered in South America, including Neotropical regions, the boreal forest regions, and the Himalayan low to montane region (Phosri et al. 2013).

Ahmed (1950) was the first Indian to identify *Astraeus hygrometricus* s.l. from the Himalayan regions while re-examining *Geastrum lilacinum* Massee (as *Geaster lilacinus*; Massee 1889). *A. hygrometricus* s.l. is the most studied and documented species from India (Biswas et al. 2011; Semwal et al. 2014; Pavithra et al. 2015; Mallick et al. 2015; Singha et al. 2017; Verma et al. 2018; Singh 2020; Khan & Chandra 2022; Roy et al. 2022). It should be noted that in 2007 and 2013, Phosri et al. separated one Thai, one European and two North American species from specimens previously identified as *A. hygrometricus* to *A. asiaticus*, *A. telleriae*, *A. smithii*, and *A. morganii*; and, in Crous et al. (2019), *A. macedonicus* is described as a new species by Rusevska, Karadelev, Telleria, and Martín from southeastern Europe. This type of research highlighted the taxon's cryptic nature, which should be addressed with caution. Hence, it is suggested that the presence and naming of *Astraeus* species as *A. hygrometricus* throughout the India should be reconsidered. Unfortunately, none of the Indian mycologists sought to investigate the fungus's phylogenetic position or any molecular analysis. For identification, they mostly rely on checklists or documents from various locations in India (Semwal et al. 2014; Singha et al. 2017; Roy et al. 2022).

Mycologists in the past have attempted to classify fungi into categories with similar traits, and this traditional method was successful in separating out the puffballs, earthballs, earthstars, stinkhorns, and other agaricomycetes, as well as identifying sub-groups of closely related taxa. As science and technology advanced, factors such as morphological, molecular, genetical, ecological, and chorological data constituted the integrative approach to delimitate species. Mycologists can infer the link between taxa based on a variety of parameters (Zamora et al. 2015). Current molecular researchers have shown that the taxonomic diversity of fungi is grossly underestimated (Wilson et al. 2011; Hawksworth 2012). Here, we have carried out a comprehensive study that is required to reconcile taxonomic uncertainties among *Astraeus* and allied species.

The province of Jharkhand lies on the Chota-Nagpur plateau and is densely covered with forest. The province's total forest area is 29.61% of its overall geographical area, covering 23,605 square kilometers. Tropical dry deciduous forest occupies 93.25% of the province's forest area. The dry deciduous forest ecological systems are interspersed with a variety of plants, including *Terminalia tomentosa*, *Madhuca longifolia*, *Butea monosperma*, *Buchanania lanzan*, *Diospyros melanoxylon*, bamboo, and others. *Shorea robusta* is the dominant species, forming mono-specific canopies in dry deciduous forests (Mishra et al. 2021).

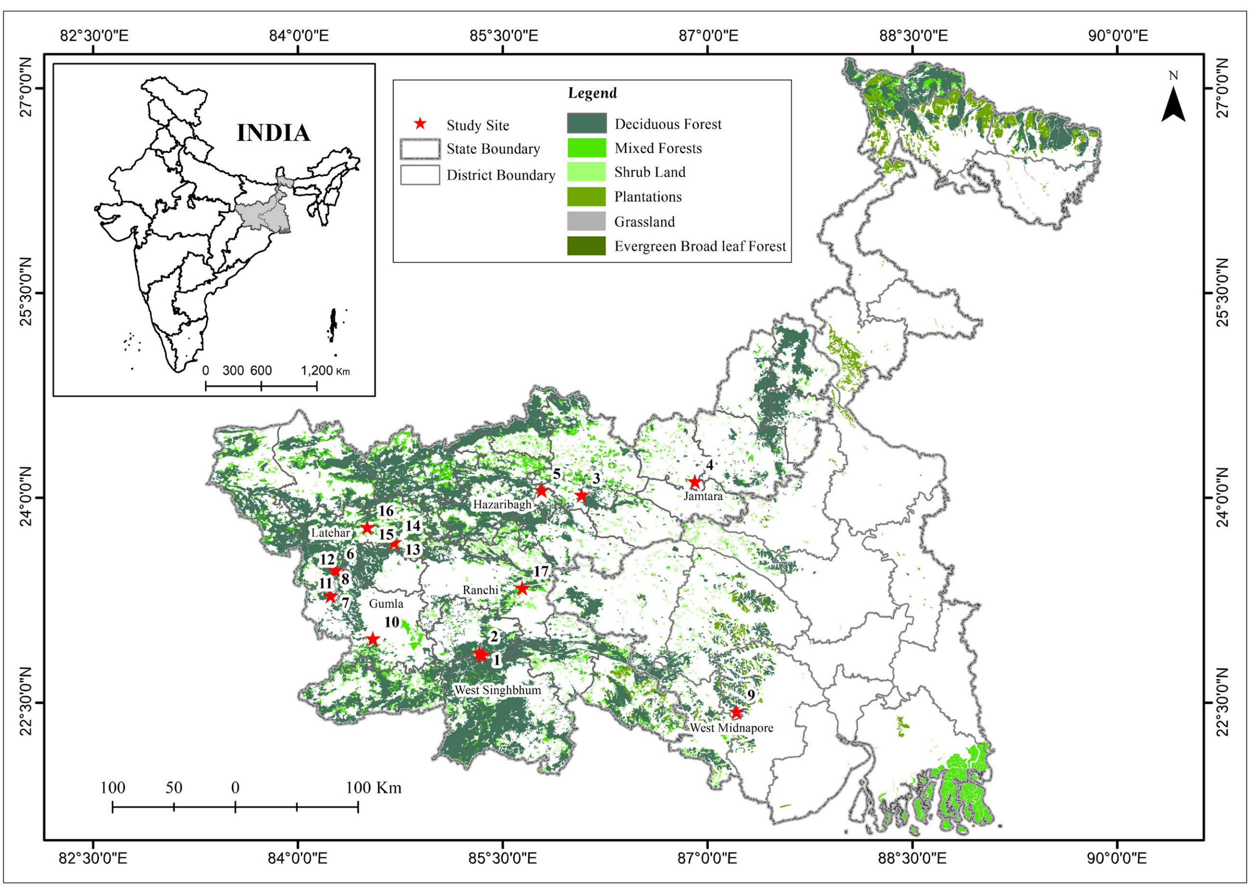

Figure 1. Map of study area and collections of *Astraeus* spp. sites highlighted in numbers and stars (red) in the provinces of Jharkhand and West Bengal, India. The detailed information of legend presented in Table 1.

Furthermore, *Shorea* forest acts as a green corridor for elephant movement. On the other side, the province has moist deciduous mixed forest-covered subtropical hills on the south Chota-Nagpur plateau. *Shorea robusta*, *Pinus roxburghii*, *Madhuca longifolia*, *Diospyros melanoxylon*, *Eucalyptus*, and *Pyrus* grow in the thick forests of the hills. However, *Shorea-Pinus* species dominate the subtropical moist deciduous forest hills. These two varied habitats are a rich source of flora, fauna, and fungal biodiversity, but the area has only been partially addressed by expeditions or mycoobservations to identify macrofungi associated with dry deciduous *Shorea* forest and moist deciduous *Pinus-Shorea* mixed forest.

The aim of this study is to gain insight into the diversity and geographical distribution *Astraeus* mushrooms in Jharkhand and West Bengal provinces using an integrative characterization and taxonomy including detailed morpho-anatomical, ecological, molecular, and phylogenetical studies to shed more light on these fungi. Extensive macrofungal expeditions and mycoobservations were conducted in moist deciduous subtropical *Pinus-Shorea* mixed forest and dry deciduous tropical *Shorea* forest and *Astraeus* specimens were collected from 2019 to 2022 (Fig. 1). The study includes detailed macro- and micromorphological descriptions coupled with the illustrations, along with a phylogenetic tree based on ITS nrDNA sequences for eleven *Astraeus* collections. A dichotomous key is also provided.

Materials and methods

Site description

Fresh and dried specimens were collected from July 2019 to August 2022 from a moist deciduous mixed forest of *Pinus-Shorea* in subtropical hills in Netarhat, Jharkhand and 11 tropical deciduous scrub forest areas in Jharkhand and the Southwestern districts of West Bengal (Figs 1–2; Table 1). Red lateritic or sandy soils are found in the forest. In terms of geography, the *Shorea* forest is made up of valleys, hills, and waterfalls that are distributed 300–1,300 meters above sea level. This forest is home to an amazing diversity of IUCN-listed birds and animals.

Sample collection and morphological analysis

Macromorphological characteristics such as peridial and glebal color or texture were recorded from the fresh basidiomes in the field according to Phosri et al. (2007). The Kornerup & Wanscher (1978) were followed for color code descriptions. Micromorphological characters were observed in a free-hand section of preserved dried tissue. Line drawings of micromorphological characters were made using an Olympus CX41 light microscope with a dedicated drawing attachment at 1000× magnification. Dried basidiospores were mounted in a mixture of 5% KOH in lactophenol and cotton blue, and stained with Congo red and Melzer reagent. The spores (n = 30) were examined, and their sizes were assessed based on Vishal et al. (2021). For SEM observation, specimens were air–dried and observed in Joel JSM–7600F Field Emission scanning electron microscope (Jeol Ltd., Japan). Adobe Photoshop 7.0 was used to edit and prepare the photographs. The GIS-based map was prepared with ArcGIS version 3 and is licensed by Dr. Shyama Prasad Mukherjee University. All collected specimens were archived at the Department of Botany, Dr Shyama Prasad Mukherjee University, Ranchi, Jharkhand, India.

DNA extraction, amplification and sequencing

DNA extraction of mushroom samples was carried out from 10 mg of dried basidiomes (for both species). Tissues were retrieved from the inner part of the basidiomes to prevent contamination by The ITS1 + 5.8S + ITS2 nrDNA region was amplified utilizing primer pairs ITS1F/ITS4B and ITS1/ITS4 as mentioned by White et al. (1990) and Gardes & Bruns (1993). In each run of amplifications, a negative control was prepared in order

Table 1. India study site area and GPS coordination of macrofungi expeditions between 2019 to 2022. Locality numbers correspond to those indicates in Fig. 1.

Locality number	Location	Latitude	Longitude	Altitude
1	Jharkhand: Khunti: Bandhgaon	22°50′56″N	85°21′1″E	287 m
2	Jharkhand: Khunti: Bandhgaon	22°51′36″N	85°19′50″E	240 m
3	Jharkhand: Giridih: Parasnath: Chainpur	24°01′51.3″N	86°04′46.9″E	332 m
4	Jharkhand: Jamtara: Baliapur	24°07′11.5″N	86°54′31.0″E	146 m
5	Jharkhand: Hazaribag: Chontha	24°03′14.7″N	85°47′13.6″E	308 m
6	Jharkhand: Latehar: Netarhat	23°28′06.8″N	84°16′33.6″E	1071 m
7	Jharkhand: Gumla: Rajadera	23°16′58.0″N	84°14′23.1″E	643 m
8	Jharkhand: Gumla: Rajadera	23°16′58.0″N	84°14′23.1″E	643 m
9	West Bengal: West Midnapore: Khasjangal Trailokyapur	22°25′57.3″N	87°12′45.1″E	53 m
10	Jharkhand: Gumla: Silam	22º58′07″N	84°33′02″E	452 m
11	Jharkhand: Gumla: Banari	23°25′39.5″N	84°20′15.5″E	643 m
12	Jharkhand: Gumla: Banari	23°25′39.1″N	84°20′15.7″E	643 m
13	Jharkhand: Latehar: Chandwa: Chiro	23°40′28.3″N	84°42′19.9″E	515 m
14	Jharkhand: Latehar: Chandwa: Chiro	23°40′28.5″N	84°42′19.3″E	515 m
15	Jharkhand: Latehar: Lawagara: Kura	23°47′02.4″N	84°30′32.1″E	381 m
16	Jharkhand: Latehar: Lawagara: Kura	23°47′01.8″N	84°30′32.0″E	381 m
17	Jharkhand: Ranchi: Angara: Dumargarhi	23°20′23.7″N	85°38′37.9″E	444 m

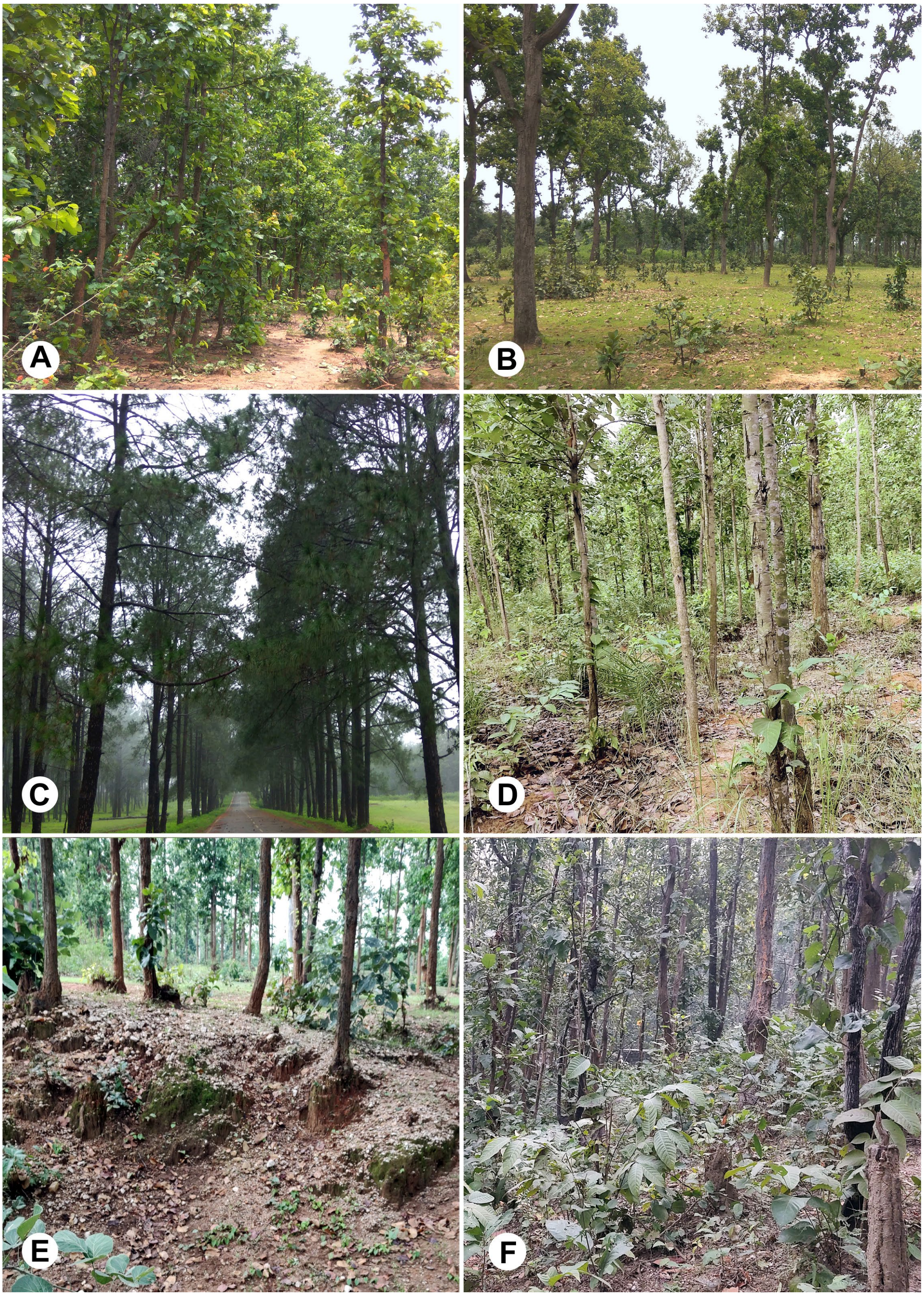

Figure 2. Photoplate of habitat and habit of *Astraeus* spp. (A–B); D–F – collected from scrub fire affected tropical deciduous forest of *Shorea*; C – sub-tropical mixed *Pinus-Shorea* forest.

to detect contamination in the reaction mixture. The PCR master mix contained 250–400 ng μL^{-1} of DNA template, 3 μM of each primer, 200 μM dNTP, 1.5 mM MgC_{l2}, 1 × buffer, and 1 U μL^{-1} of Taq DNA polymerase (Qiagen, Germany). The PCR amplification was carried out on an Eppendorf thermal cycler (Eppendorf, Germany) configured for 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C and a final stage of 10 min at 72°C. The GEL/PCR purification Mini Kit (Favorgen Biotech Corp., Taiwan) was used to purify the amplicons, which were subsequently sequenced at ATGC Co. Ltd. (Thailand). Chromas 2.4 (Technelysium Pty Ltd., South Brisbane, Australia) was used to assess the quality of each nucleotide base based on the chromatograms. The final consensus sequences were deposited at GenBank to get accession numbers.

Phylogenetic analyses

The new ITS nrDNA sequences, as well as other homologues, were retrieved using a MEGABLAST (McGinnis & Madden 2004) of NCBI, and relevant literature (Phosri et al. 2007, 2013, 2014; Wilson et al. 2011; Hembrom et al. 2014; Ryoo et al. 2017; Crous et al. 2019) (Table 2). The final ITS nrDNA dataset was aligned with the default parameters of the web–based multiple sequence alignment program MAFFT version (https://mafft.cbrc.jp/alignment/server/). The online tool Gblocks 0.91b (Talavera & Castresana 2007) was used to delete ambiguous aligned regions in the alignment as much objectively as possible. Maximum likelihood (ML) analysis was performed with raxmlGUI 2.0 (Edler et al. 2021) using the best nucleotide substitution model and 1,000 replicates to determine nodal support values. The best fit–model GTR derived from jModelTest was used for the Bayesian

Table 2. List of species, voucher collection numbers, origin and GenBank accession numbers used in this study. The described specimens used in this study are in bold font.

Scientific name	Voucher collection no.	Origin	ITS accession no.	References
Astraeus sirindhorniae	GAPK1	Thailand	HE681772	Phosri et al. 2014
Astraeus sirindhorniae	GAPK2	Thailand	HE681773	Phosri et al. 2014
Astraeus sirindhorniae	GAPK3	Thailand	HE681774	Phosri et al. 2014
Astraeus asiaticus	ASTRAE-64	Thailand	AJ629400	Phosri et al. 2007
Astraeus asiaticus	Arora02-121	Thailand	EU718089	Wilson et al. 2011
Astraeus asiaticus	ASTRAE-15	Laos	AJ629384	Phosri et al. 2007
Astraeus asiaticus	ASTRAE-44	Sri Lanka	AJ629395	Phosri et al. 2007
Astraeus asiaticus	ASTRAE-8	Thailand	AJ629382	Phosri et al. 2007
Astraeus asiaticus	ASTRAE-17	Laos	AJ629385	Phosri et al. 2007
Astraeus asiaticus	CAL 1869	JH, India	**MN257431**	This study
Astraeus asiaticus	Rug-5a-JH	JH, India	**OP198447**	This study
Astraeus asiaticus	Rug-11a-WB	WB, India	**OP964740**	This study
Astraeus asiaticus	Rug-11b-WB	WB, India	**OP964795**	This study
Astraeus asiaticus	Rug-14a-JH	JH, India	**OP965021**	This study
Astraeus asiaticus	Rug-14a1-JH	JH, India	**OP965022**	This study
Astraeus asiaticus	Rug-12a-JH	JH, India	**OP964930**	This study
Astraeus asiaticus	Rug-12b-JH	JH, India	**OP964931**	This study
Astraeus odoratus	Rug-3a-Jh	JH, India	**MN262679**	This study
Astraeus odoratus	Rug-7-JH	JH, India	**OP965383**	This study
Astraeus odoratus	Rug-10b-JH	JH, India	**OP965392**	This study
Astraeus odoratus	Rug-13b-JH	JH, India	**OP965538**	This study
Astraeus odoratus	Rug-16b-Jh	JH, India	**OP965540**	This study
Astraeus odoratus	ASTRAE-61	Thailand	AJ629876	Phosri et al. 2007
Astraeus odoratus	ASTRAE-62	Thailand	AJ629877	Phosri et al. 2008
Astraeus odoratus	MEH-13-036	India	KJ847767	Hembrom et al. 2014
Astraeus ryoocheoninii	KFRI-DMZ002	South Korea	KC985146	Ryoo et al. 2017
Astraeus macedonicus	11MCF12901	Macedonia	MK491321	in Crous et al. 2019
Astraeus macedonicus	07MCF8434	Macedonia	MK491322	in Crous et al. 2019
Astraeus macedonicus	14MCF11641	Macedonia	MK491323	in Crous et al. 2019
Astraeus koreanus	ASTRAE-97	China	AJ629900	Phosri et al. 2007
Astraeus koreanus	ASTRAE-98	China	AJ629893	Phosri et al. 2007
Astraeus koreanus	OTU526	Japan	MT596222	unpublished
Astraeus koreanus	TNS: F-70414	Japan	KY629425	unpublished
Astraeus terlleriae	88MCF9574	Macedonia	MK491295	unpublished
Astraeus terlleriae	ASTRAE-72	Spain	AJ629408	Phosri et al. 2013
Astraeus terlleriae	ASTRAE-87	Greece	AJ629404	Phosri et al. 2013
Astraeus smithii	ASTRAE-74	USA	AJ629399	Phosri et al. 2013
Astraeus smithii	ASTRAE-85	USA	AJ629402	Phosri et al. 2013
Astraeus smithii	MB05-029	USA	EU718087	Wilson et al. 2011
Astraeus smithii	AWW220	USA	FJ710187	Wilson et al. 2011
Astraeus smithii	ASTRAE-86	USA	AJ629403	Phosri et al. 2013
Astraeus hygrometricus	ASTRAE-43	France	AJ629406	Phosri et al. 2007
Astraeus hygrometricus	ASTRAE-42	France	AJ629394	Phosri et al. 2007
Astraeus pteridis	ASTRAE-48	USA	AJ629407	Phosri et al. 2007
Astraeus pteridis	Ashy3	Switzerland	EU718088	Wilson et al. 2011
Astraeus pteridis	ASTRAE-37	Spain	AJ629393	Phosri et al. 2007
Pisolithus arhizus	PISOLI-12	Thailand	AJ629887	Phosri et al. 2009
Scleroderma verrucosum	VERSCLE-4	Spain	AJ629886	Phosri et al. 2008

analysis (BI). MrBayes v. 3.2.6 (Ronquist et al. 2012) was used for Bayesian tree construction with the default parameters (Nst = 6 with 2 runs, 4 chains per run, each run searching for the 100,000th generations and sampling every 1,000th generations). Maximum likelihood bootstrap values (MLBS) ≥70% and Bayesian posterior probabilities (PP) ≥0.5% are shown in the phylogenetic tree.

Results

Phylogenetic analysis

The current dataset of ITS nrDNA contains 47 homologous sequences, including our 11-consensus sequence from India. *Scleroderma verrucosum* and *Pisolithus arhizus* were used as an outgroup for rooting purpose, as indicated in previous papers (Phosri et al. 2007). The final alignment dataset contains 1,201 characters, including gaps. Because both the ML and Bayesian analyses displayed the same topology, only the Bayesian tree with both MLBS and PP values is shown (Fig. 4). The Bayesian phylogram yielded ten major clades within the genus *Astraeus*. Clades I–III and IX were Asian clades with strong support (MLBS = 100% and PP = 1.00), represented by *A. asiaticus*, *A. sirindhorniae*, *A. odoratus*, and *A. koreanus*, respectively. In particular, the sequences from Indian specimens further grouped in clades I and III; the clade I consists of Indian *A. asiaticus* (OP198447, OP964740, OP964795, OP964930, OP964931, OP965021, and OP965022) and the clade III consists of Indian *A. odoratus* (OP965383, OP965538, OP965392, and OP965540); both clades have strong bootstrap and posterior probability support (MLBS = 100% and PP = 1.00). Furthermore, the clades V–VIII and IV and X were European and American clades. Therefore, our molecular results clearly distinguish Asian *Astraeus* from European and American specimens. Wilson et al. (2011) and Phosri et al. (2013) speculated the delimitation of Asian specimens based on 18S nrDNA barcoding and phylogenetic analysis.

Taxonomy

Astraeus asiaticus Phosri, M.P. Martín & Watling, Mycological Research 111(3): 279. 2007.
(Figs 3A–C, 5A–J, 7A–C)

Holotype: Thailand. Ban Dutung, Muang District, Yasothon province, 4 Jun. 2002, C. Phosri ASTO_07 (E-Wat28787) (ITS nrDNA AJ293396).

Description. Basidiomata sessile, subepigeous, globose to depressed globose, 10–25 × 12–25 mm, whitish (A1), covered with debris, slight odor, rhizomorph thread or cordlike, whitish (A1), persistent, 2–3 mm long, partly buried in soil. Expanded basidiomata saccate, 15–40 × 15–30 mm. Exoperidium, three-layered, hygroscopic, split into 9–12 rays, recurved when moist, umber brown (6F4), incurves when dry, granulate surface, opening by longitudinal cracking, 8–18 mm long, 2–6 mm wide, ovate to elliptical. Exoperidial suprapellis layer thick, encrusted, whitish when young, ages becoming smoke grey (E1) to brownish (6E8). Exoperidial mediopellis layer, fleshy, coriaceous, cigar brown (6D5), glabrous. Exoperidial subpellis pseudoparenchymatous layer sepia brown (6E4), thin, fleshy, glabrous, collar absent. Endoperidium globose to depressed, sessile, 12–30 mm diam., whitish (A1) to ash grey (E1), opening by irregular apical pore, peristome, columella & apophysis absent. Gleba powdery dark brown (6E8) at maturity. Basidiospore globose, thick–walled, acyanophilous, 9.3–12.5 × 9.1–12.3 µm (excluding ornamentation) [x = 10.7 ± 0.9 × 10.6 ± 0.9 µm, Q_m = 1.01, n = 20], ornamentation includes coalescent to spinoid, spine 0.9 – 1.4 µm long and tapered, fused, very dense, narrow, apiculus conspicuous. Capillitial hyphae thin to thick-walled, clamped, hyaline to greenish (27AB), branched, with or without encrustation, 3.5–5.5 µm diam., sinuous to straight hyphae, lumen present. Endoperidial hyphae hyaline to greenish (27AB), thin to very thick-walled, 3–5 µm diam., encrusted, hyphae straight to sinuous with lumen, clamped, branched. Exoperidial suprapellis layer hyphae composed of sinuous to straight, thin to thick-walled hyphae, 1.5–3.5 µm diam., heavily encrusted, lumen present, hyaline to greenish (27AB) in KOH. Exoperidial mediopellis layer hyphae composed of periclinal (3–6 µm diam.) and diagonal hyphae (6–9 µm diam.), sinuous to straight, thick-walled, diagonal hyphae, 1.5–3 µm diam., not encrusted, lumen present, hyaline to greenish (27AB) in KOH. Exoperidial subpellis pseudoparenchymatous layer hyphae hyaline in KOH, globose, few elongated and cylindrical cell; cell 12–25 × 14–20 µm in size, irregular, thick-walled. Rhizomorph hyphae hyaline, branched, heavily encrusted, thin to thick-walled, up to 5 µm diam., clamped, interwoven, lumen present.

Myco-chemical test. Non-reactive with 10% KOH and water, but reactive (yellowish) with 10% $FeSO_4$.

Edibility. Edible.

Odor. Mild odor**.**

Substrate. Found growing on sandy lateritic (Jharkhand province); clay, alluvial and lateritic soil (West Bengal province), covered with litter. Fruiting frequently from June to September as solitary or scattered in small groups in the deciduous *Dipterocarpaceae Shorea* dominated forest.

Materials examined. INDIA: Jharkhand, Gumla-district, Banari, alt. 643 m, 23°25′39.5″N, 84°20′15.5″E, 20.07.2022, on ground near *Shorea robusta*, V. Vishal (Rug-13a-JH); Gumla-district, Rajadera village, alt. 643 m, 23°16′58.0″N, 84°14′23.1″E, 06.07.2020, on ground near *Shorea robusta*, V. Vishal and S. Bara (Rug-10a-JH); Gumla-district, Silam, alt. 452 m, 22º58′07″N, 84º33′02″E, 13.07.2022, on ground near *Shorea robusta*, V. Vishal and S. Kumari (Rug-12a-JH); Jamtara-district, Baliapur Village, alt. 146 m, 24°07′11.5″N, 86°54′31.0″E, 27.07.2019, on ground near *Shorea robusta*, V. Vishal (Rug-4a-JH); Hazaribag-district, Chontha village, alt. 308 m 24°03′14.7″N, 85°47′13.6″E, 30.07.2019, on ground near *Shorea robusta*, V. Vishal and S.S. Munda (Rug-5a-JH); Khunti-district, Bandhgaon village, alt. 287 m, 22°50′56″N, 85°21′1″E, 18.07.2019, on ground near *Shorea robusta*, V. Vishal and S.S. Munda (CAL 1869); Latehar district, Chiro village, alt. 515 m, 23°40′28.3″N, 84°42′19.9″E, 26.07.2022, on

Figure 3. Photoplate showing *Astraeus* specimens in the natural habitat. A–C – expanded and unexpanded basidiomes of *A. asiaticus*; D–F – expanded and unexpanded basidiomes of *A. odoratus*; G – macrofungi expedition and collection of basidiomes; H–I – *Astraeus* fresh basidiome sold in the local market. Scales: D–E = 5 mm.

ground near *Shorea robusta*, V. Vishal (Rug-14a-JH); Latehar, Lawagara, Kura village, alt. 381m, 23°47′02.4″N, 84°30′32.1″E, 02.08.2022, on ground near *Shorea robusta* and *Butea monosperma*, V. Vishal (Rug-16a-JH); Ranchi, Angara, Dumargarhi, alt. 444 m, 23°20′23.7″N, 85°38′37.9″E, 20.09.2022, on ground near *Shorea robusta* V. Vishal, G Singh, A. Ghosh and S. Singh (Rug-17a-JH). West Bengal, West Midnapore-district, Khasjangal Trailokyapur village, alt. 53 m, 22°25′57.3″N, 87°12′45.1″E, 08.07.2021, on ground near *Shorea robusta*, V. Vishal (Rug-11a-WB).

GenBank accession numbers. MN257431 (CAL 1869); OP198447 (Rug-5a-JH); OP964740, OP964795 (Rug-11a-WB); OP965021, OP965022 (Rug-14a-JH); OP964930, OP964931 (Rug-12a-JH).

Notes. *Astraeus asiaticus* was discovered in many consecutive mycoobservations and surveys conducted between 2019–2022 monsoon in the Dipterocarp tropical dry deciduous *Shorea* forests of Jharkhand and West Bengal province of India. Temperatures varied from 26 to 35°C, relative humidity from 78 to 94%, and average rainfall from 500 to 800 mm over this time.

Astraeus odoratus Phosri, Watling, M.P. Martín & Whalley, Mycotaxon 89(2): 458. 2004.

(Figs 3D–F, 6A–H, 7D–F)

Holotype: Thailand. Pranomprai district, Roi Et province, 6 June 2002, C. Phosri ASTO_14 (E159386) (ITS nrDNA AJ629874).

Description. Basidiomata globose to sub-globose, epigeous, smooth, mosaic surface, brownish (6E8), coriaceous, not encrusted, rounded apex, 20–45 × 16–42 mm, rhizomorph brownish (6E8) persistent. Expanded Basidiomata saccate 48–62 × 18–52 mm. Exoperidium multilayered (≤ 1 mm), split into 5–7 rays, hygroscopic, surface smooth, recurved when wet, incurved at dry, rays ovate to elliptical in shape. Exoperidial suprapellis layer thick, not encrusted, grey brown (6E8) to yellowish brown (5E8), coriaceous exposing to suprapellis layer.

Exoperidial mediopellis layer fleshy, thick, coriaceous, sepia brown (6E4) to smoke grey (E1), glabrous. Exoperidial subpellis Pseudoparenchymatous layer, fleshy, thin, pale straw or olivaceous, glabrous. Endoperidium subglobose, 15–35 mm diam., brownish (6E8) to violaceous black (17H8), sessile, thin, papery, sub-globose, opening by specialized apical pore, peristome, columella & apophysis absent. Gleba powdery, dark brown (6E8) to coffee color (6E3) at maturity. Basidiospore globose, thick-walled, acyanophilous, 6.5–10.2 × 6.4–10.1 µm (excluding ornament) [x = 8.6 ± 1.38 × 8.5 ± 1.37 µm, Q_m = 1.02, n = 20], ornamentation includes long, narrow, less dense, coalescent to spinoid, spine 0.9–1.55 µm long, apiculus conspicuous. Capillitial hyphae thick walled, thin, hyaline to greenish (27AB), branched, encrusted, 2–5 µm, sinuous to straight hyphae with lumen. Endoperidial hyphae hyaline to greenish (27AB), thick-to thin walled, 1.5–84.5 µm, heavy encrustation, hyphae straight to sinuous with lumen, clamped, branched. Exoperidial suprapellis layer hyphae composed of sinuous to straight, clamped, thin to thick -walled hyphae, 2–5 µm diam., surface smooth, encrusted, few heavily encrusted, lumen present, hyaline to greenish (27AB) in KOH. Exoperidial mediopellis layer hyphae composed of periclinal hyphae (1–2 µm) and diagonal hyphae (2.5–7.5 µm) sinuous to straight, thin to thick-walled, heavily encrusted, lumen present, hyaline to greenish (27AB) in KOH. Exoperidial subpellis pseudoparenchymatous layer hyphae hyaline in KOH, irregular, spherical; cell 10–40 × 8–38 µm in size, thick-walled. Rhizomorph hyphae thick-walled, up to 6 µm diam., branched, encrusted, clamped, lumen prominent.

Myco-chemical test. Non-reactive with 10% KOH and water, but reactive (yellowish) with 10% $FeSO_4$.

Edibility. Edible.

Odor. Odor like sandal (*Santalum*) or Chandana (in local dialect).

Substrate. Growing on sandy, lateritic, sloppy and with small stones pebbles. Fruiting frequently from May to August as solitary or scattered in small groups in the deciduous *Dipterocarpaceae Shorea* dominated forest.

Materials examined. INDIA, Jharkhand, Giridih-district, Parasnath, Chainpur, alt. 332 m, 24°01′51.3″N, 86°04′46.9″E, 18.06.2021, on ground near *Shorea robusta*, V. Vishal (Rug-3-JH); Gumla-district, Banari, alt. 643 m, 23°25′39.1″N, 84°20′15.7″E, 20.07.2022, on ground near *Shorea robusta*, V. Vishal (Rug-13b-JH); Gumla-district, Rajadera village, alt. 643 m, 23°16′58.0″N, 84°14′23.1″E, 06.07.2020, on ground near *Shorea robusta*, V. Vishal and S. Bara (Rug-10b-JH); Khunti district, Bandhgaon village, alt. 240 m, 22°51′36″N, 85°19′50″E, 24.07.2019, on ground near *Shorea robusta*, V. Vishal and S. Munda (Rug-2a-JH); Latehar-district, Chiro village, alt. 515 m, 23°40′28.5″N, 84°42′19.3″E, 26. 07. 2022, on ground near *Shorea robusta*, V. Vishal (Rug-14b-JH); Latehar-district, Lawagara, Kura village alt. 343 m, 23°47′01.8″N, 84°30′32.0″E, 02.08.2022, on ground near *Shorea robusta*, V. Vishal (Rug-16b-JH); Latehar-district, Netarhat, alt. 1071 m, 22°51′36″N, 85°19′50″E, 31.07.2021, on ground near *Pinus* and *Shorea robusta*, V. Vishal and S.S. Munda (Rug-7-JH).

GenBank accession numbers. MN262679 (Rug-3a-Jh); OP965383 (Rug-7-JH); OP965392 (Rug-10b-JH); OP965538 (Rug-13b-JH); OP965540 (Rug-16b-Jh).

Notes. *Astraeus odoratus* was discovered in many consecutive mycoobservations and surveys conducted between 2019–2022 monsoon in the Dipterocarp tropical dry deciduous *Shorea* forests and mixed forest of *Pinus-Shorea* subtropical hills (1,071 m) of Jharkhand province. The mixed forest of *Pinus-Shorea* subtropical hills has temperatures ranging from 10−24°C, humidity varying from 88−94%, and an average rainfall of over 1,300 mm over this time period.

Key to the Asian species of *Astraeus*

1 Basidiome hygroscopic; columella, apophysis and peristome absent . . . . 2 (***Astraeus***)
– Basidiome not hygroscopic; columella, apophysis and peristome present . . . . ***Geastrum***

2(1) Basidiome globose or depressed globose; endoperidium sessile 3
– Basidiome subglobose to ellipsoid; endoperidium stipitate 4

3(2) Expanded basidiome (≤ 37 mm in diam.); 7–10 broad stellate rays; spore 9–10.5 µm in diameter . . . . ***A. koreanus***
– Expanded basidiome (≤ 65 mm in diam.); 5–7 broad stellate rays; spore 7.5–15.2 µm in diameter . . . . ***A. odoratus***

4(2) Exoperidium layer 3–5 mm thick when fresh; clamp-connection absent in capillitium . . . . ***A. sirindhorniae***
– Exoperidium layer 1–2 mm thick when fresh; clamp-connection present in capillitium 5

5(4) Very dense ornamentation; spine 0.9–1.4 µm long; capillitium hyphae 3.5–5.5 µm in diameter . . . . ***A. asiaticus***
– Moderately dense ornamentation; spine 0.6–1.3 µm long; capillitium hyphae 4.0–6.5 µm in diameter . . . . ***A. ryoocheoninii***

Discussion

Shorea tropical deciduous forests are widespread in Indo-Malayan regions and have a diverse mycoflora. In India, *Astraeus* (false earthstar) diversity is vaguely described morphologically, with inadequate molecular and phylogenetic data. They are frequently misidentified as puffball, earthballs or other gasteroid fungi (Topno & Srivastava 2017). Zamora et al. (2015) and Sousa et al. (2017) used integrative approach to reveal hidden species within *Geastrum* and *Myriostoma*. These approaches have been especially helpful for addressing cryptic or semi-cryptic taxa within the species. In this study, an integrative taxonomic approach has been used to characterize, distinguish, and identify Indian *Astraeus* and allied species, which were previously identified as *A. hygrometricus.* As shown in the bayesian phylogenetical analysis of ITS nrDNA, the Indian specimens were distributed in clade I and III (Fig. 4), that correspond to *A. asiaticus* and *A. odoratus*, respectively.

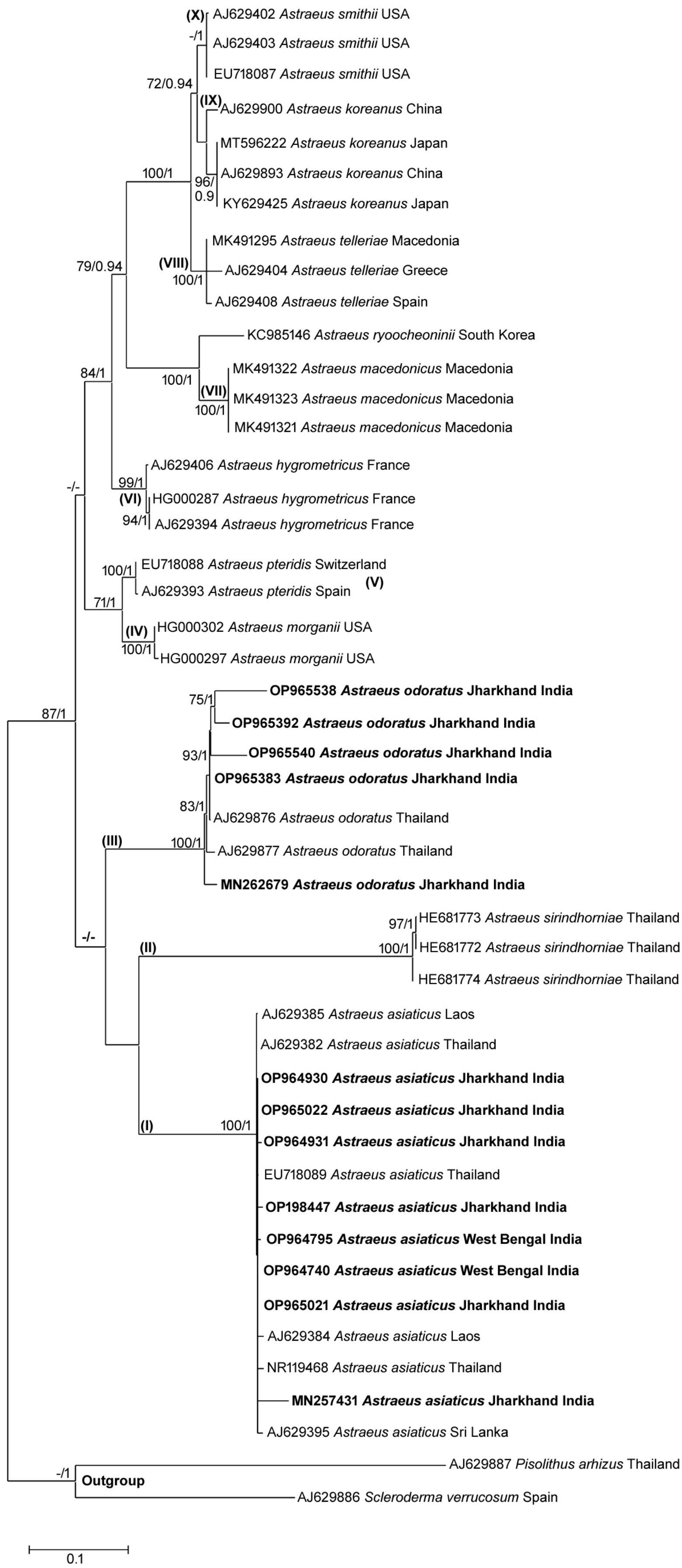

Figure 4. Bayesian tree inferred from a heuristic search of ITS nrDNA sequence of *Astraeus* spp. resulted into Clade I–X. The sequence of *Pisolithus* and *Scleroderma* sp. is used as an outgroup. Terminal branches are labeled with the appropriate GenBank accession number and country of origin. Numbers at the nodes indicate the percentage of bootstrap values obtained from maximum likelihood analysis and the posterior probabilities from Bayesian analysis (MLbs/PP).

Similarly in 2004/2007, Thai *A. hygrometricus* was evaluated and placed in different clades when compared with European collections; and Thai collections were described as a new species, *A. odoratus* (Phosri et al. 2004) and *A. asiaticus* (Phosri et al. 2007). Fangfuk et al. (2010b) also found that the Japanese *Astraeus* species identified as *A. hygrometricus*, could be a new species, but later Ryoo et al. (2017) reestablished it as *A. ryoocheoninii* Ryoo Similarly, P.-A. Moreau (in Paz et al. 2017) redescribed *A. sapidus* from European deer truffles (*Elaphomyces*). Thus, in order to distinguish possible cryptic or semi-cryptic species in the genus *Astraeus*, it is necessary to obtain at least the barcoding sequence (ITS nrDNA) from different phytogeographical zones, such as in Phosri et al. (2014). Balasundaram et al. (2015) highlighted the significance of collecting more specimens from a broader phytogeographical zones in order to investigate intra- and interspecific species differences.

This study pertains to three years of extensive macrofungal expeditions conducted in eleven different forest areas (Figs 1–2). *A. odoratus* basidiomes were collected from tropical deciduous *Shorea* forests and subtropical mixed *Pinus-Shorea* forests of Jharkhand province (Figs 2C, 3D) and *A. asiaticus* was only collected from tropical deciduous *Shorea* forests of Jharkhand and West Bengal province (Fig. 2A–C, E–F). However, after the three years of extensive macrofungal expeditions between the monsoon seasons of 2019 to 2022 we could not find any of the *A. hygrometricus* basidiomes as reported by Khan & Chandra (2022) and Singh (2020) from the province of Jharkhand, and by

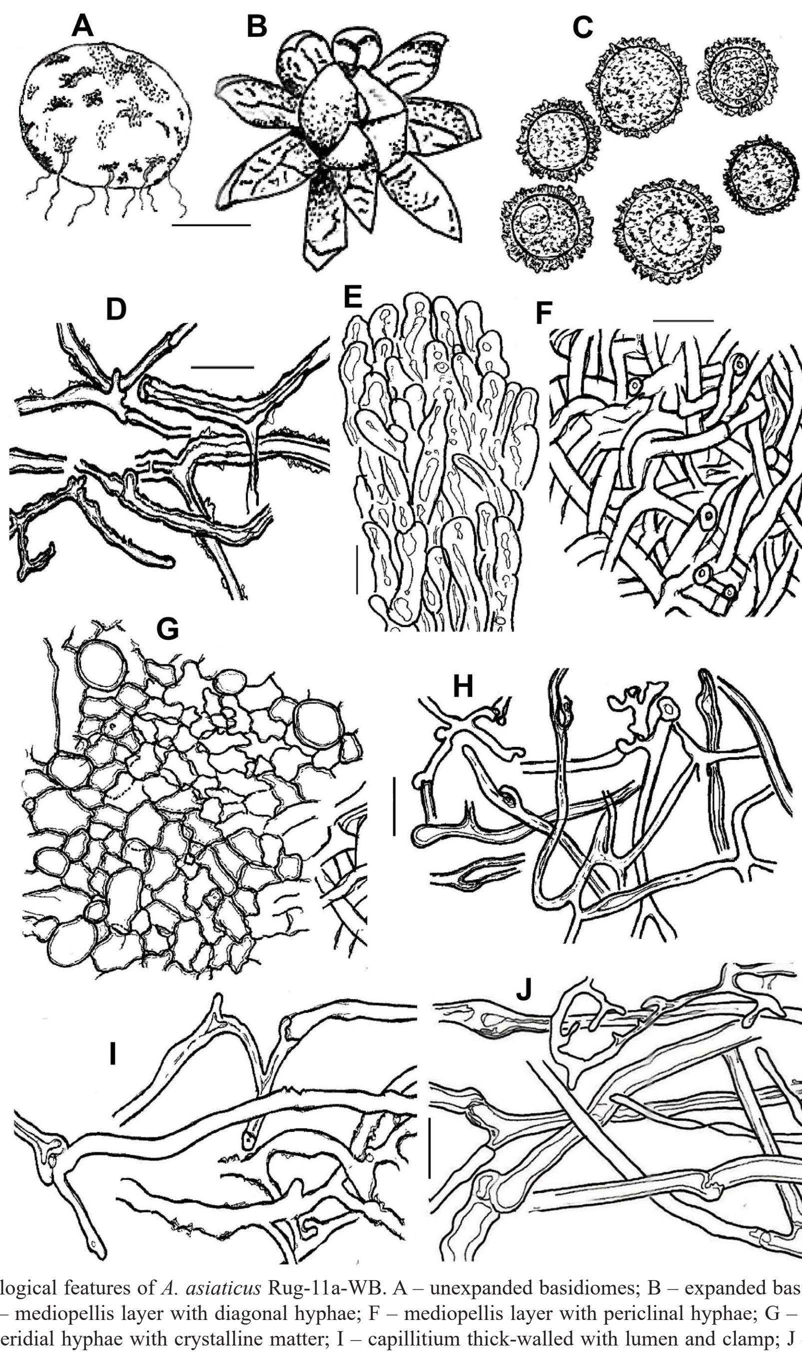

Figure 5. Micro-morphological features of *A. asiaticus* Rug-11a-WB. A – unexpanded basidiomes; B – expanded basidiomes; C – basidiospore; D – suprapellis layer; E – mediopellis layer with diagonal hyphae; F – mediopellis layer with periclinal hyphae; G – subpellis pseudoparenchymatous layer; H – endoperidial hyphae with crystalline matter; I – capillitium thick-walled with lumen and clamp; J – rhizomorph hyphae with encrustation and clamp connection. Scales: A–B = 10 mm; C = 10 µm; D–J = 10 µm.

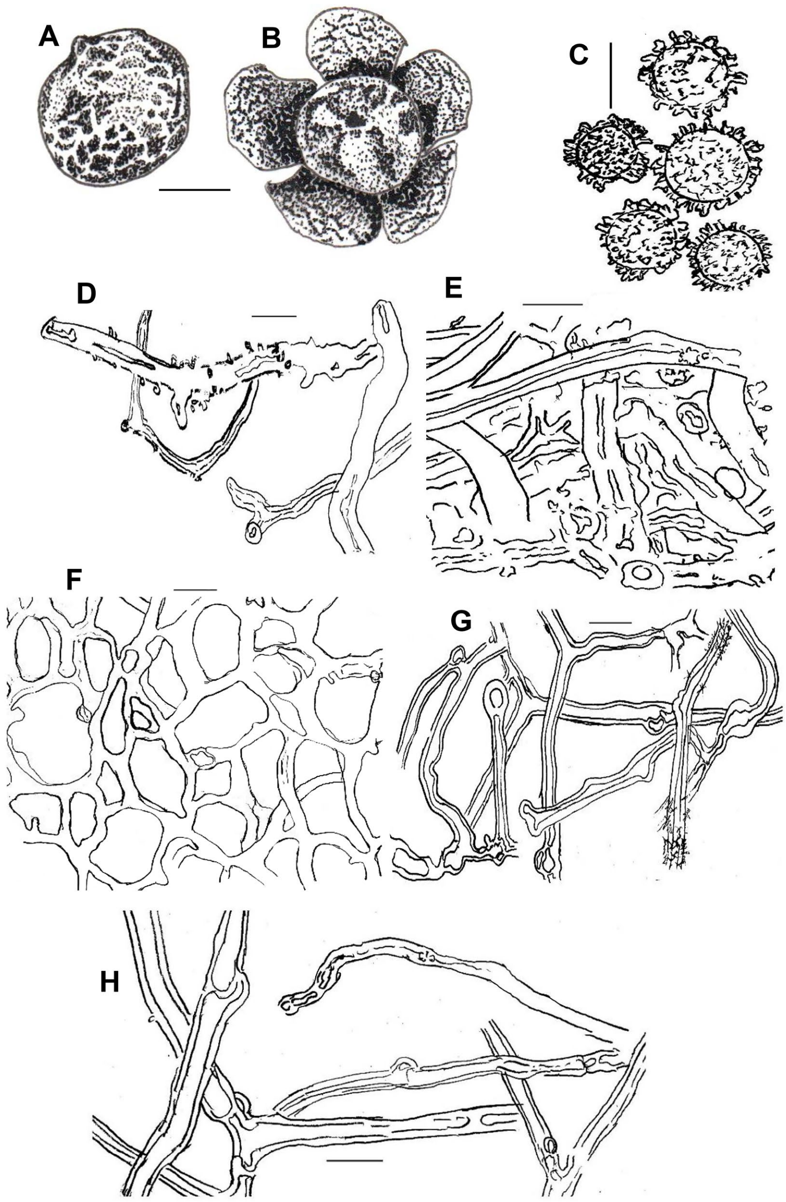

Figure 6. Micro-morphological features of *A. odoratus* (Rug-7-JH). A – unexpanded basidiomes; B – expanded basidiomes; C – basidiospore; D – suprapellis layer; E – mediopellis layer hyphae; F – subpellis pseudoparenchymatous layer; G – capillitium hyphae with encrustation and clamp connection; H – rhizomorph hyphae with clamp connection. Scales: A–B = 10 mm; C–H = 10 µm.

Mallick et al. (2015) from West Bengal where the specimens have been identified as *A. hygrometricus* solely based on conventional morphology and literature review.

Astraeus hygrometricus was considered for many years to be the only species of the genus. However, molecular studies by Phosri et al. (2004, 2007, 2013) have revealed that there are more species and they can be distinguished by their ITS nrDNA sequences, and also by morphological and ecological characters. A second morphological revision is always necessary to confirm if they are cryptic species or not. According to the data obtained from previous authors (Nouhra & Toledo 1998; Pegler et al. 1995; Baseia & Galvao 2002; Phosri et al. 2004, 2007, 2013) and our own study, morphological analyses based on the peridial surface, number of the rays, basidiospore size and ornamentations, as well as the ecology, distinguish *A. hygrometricus* (neotype from France) from *A. odoratus* and *A. asiaticus* (both with Thai holotypes) among the India specimens (Table S1). Lack of granulate exoperidial surface or mosaic-like pattern over basidiomes, thicker capillitial hyphae, smaller basidiospores, and less dense ornamentation (herbarium specimen CAL 5353: 0.5–1.2 µm, Fig. 7G–H) are adequate morphological features to distinguish *A. hygrometricus* s.l. from the collected Indian *A. asiaticus* and *A. odoratus.* The Indian specimens are similar to the Thai specimen (holotype), but the Thai specimens had larger basidiospores (*A. asiaticus* 8.7–15.2 µm; *A. odoratus* 7.5–15.2 µm) than their Indian counterparts (*A. asiaticus* 7.5–12.3 × 7.3–12.5 µm; *A. odoratus* 6.5–10.2 × 6.4–10.1 µm) (Figs 5–7).

The Boletoid genus *Astraeus* benefits the forest ecology by establishing symbiotic relationships with tropical or subtropical forest trees from the *Pinaceae*, *Fagaceae*, *Dipterocarpaceae*, *Betulaceae*, *Myrtaceae*, and *Ericaceae* families. In the Indian and Southeast Asian continents, the earthstar fungi (*A. odoratus*, *A. asiaticus*,

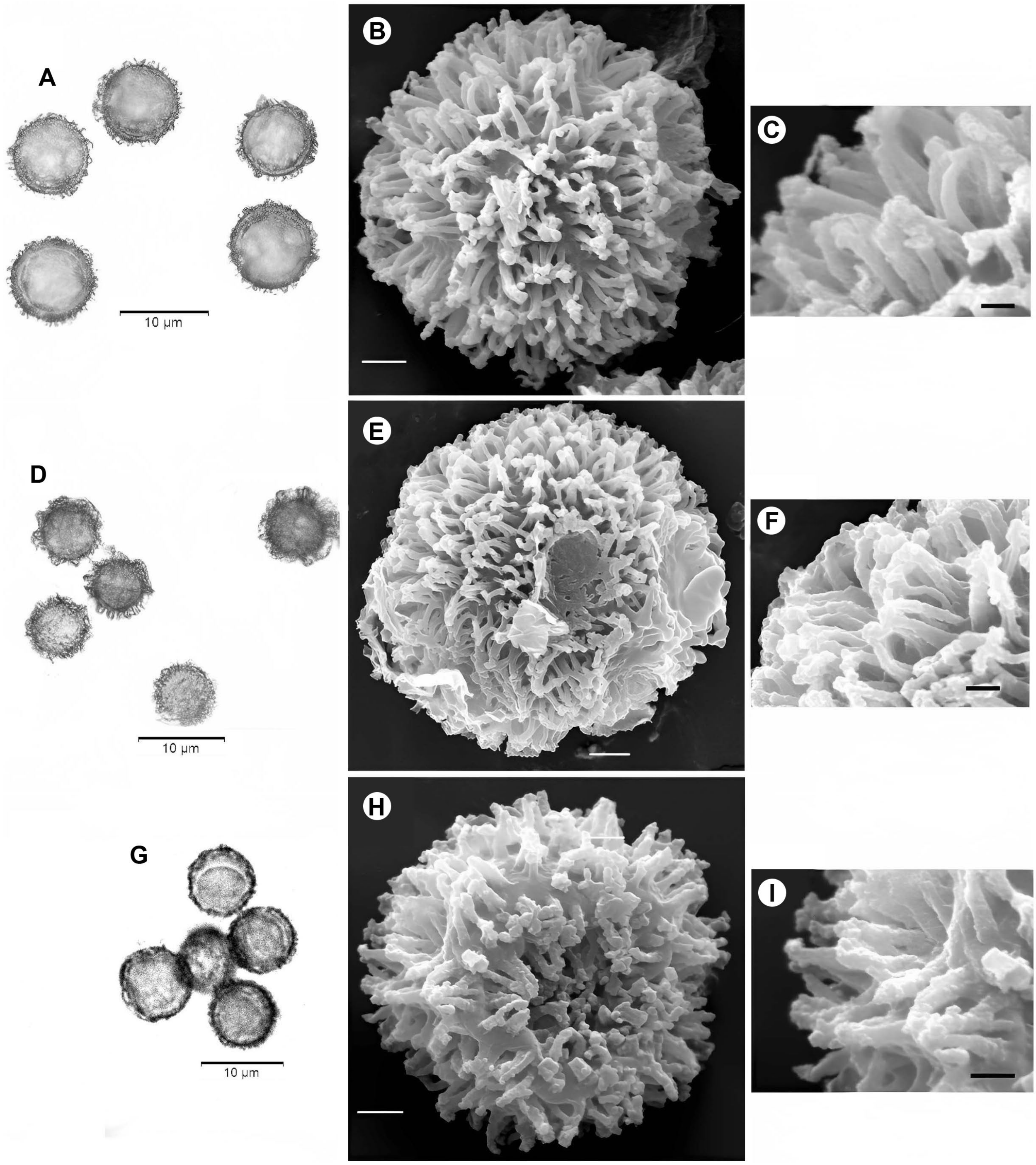

Figure 7. Light microscope and scanning electron microscope of mature spores of *Astraeus* species showing different spine morphologies. A–C – *A. asiaticus* showing a thick-walled globose basidiospore and ornamentation, including coalescent spines that are narrow, long, tapered, fused, and very dense (Rug-11a-WB); D–F – *A. odoratus* with long, thick-walled globose basidiospores and ornamentation, including narrow, long, and less dense coalescent spines (Rug-7-JH); G–I – herbarium specimen of *A. hygrometricus* (CAL 5353**) with thick-walled basidiospore and ornamentation including coalescent spines that are short, narrow, rounded, less dense, and sparsely arranged. Scales: A, D, G = 10 µm; B, E, H = 400 nm; C, F, I = 200 nm. ** CAL 5353 was a herbarium specimen archived at Central National Herbarium, AJC Bose Indian Botanic Garden (CAL), Howrah, W.B, India.

and *A. sirindhorniae*) primarily formed ectomycorrhizal associations with trees such as *Shorea roxburghii*, *Dipterocarpus obtusifolius*, *Dipterocarpus alatus*, *Anisoptera costata* and *Hopea odorata* (*Dipterocarpaceae*) (Phosri et al. 2004, 2007, 2014; Kaewgrajang et al. 2013; Suwannasai et al. 2020). However, in the Northern hemisphere, *Astraeus* (*A. hygrometricus* s.l., *A. pteridis*, *A. macedonicus*, *A. telleriae*, *A. morganii*, and *A. smithii*) is associated with a large number of trees such as *Pinus banksiana*, *P. resinosa*, *P. densiflora*, *P. kesiya*, *Larix occidentalis* and *Pseudotsuga menziesii* (Mirbel) Franco (*Pinaceae*), *Fagus*, *Castanea*, *Quercus* and *Castanopsis* (*Fagaceae*), *Alnus incana* (*Betulaceae*), *Eucalyptus camaldulensis* (*Myrtaceae*), and *Arbutus menziesii* (*Ericaceae*) (Trappe 1967; Malajczuk et al. 1982; Phosri et al. 2013; Crous et al. 2019). Thus, it was surprisingly noted that the northern hemisphere has a vast range of hosts for *Astraeus*, with *Pinus* being the dominating host; conversely, *Astraeus* has a small range of hosts on the Indian and Southeast Asian continents, with the dipterocarp tree being the dominant host. Tedersoo et al. (2014) advocated that *Pinaceae* should be the dominant ECM partner because it is the longest-surviving plant family and while geographic, climatic, and edaphic variables (pH, calcium, and phosphorus) might be responsible for the richness of ectomycorrhiza (ECM) in the Northern Hemisphere.

Trappe (1967), Malajczuk et al. (1982), Phosri (2004), Fangfuk et al. (2010a), Kaewgrajang et al. (2013), and Suwannasai et al. (2020) have all explored ectomycorrhizal syntheses under *in vitro* condition using *Astraeus* cultures. Species of *Astraeus* also serve as a lucrative fungus, because they are collected for sales as an edible mushroom in the local markets (Fig. 3H–I).

Abiotic variables, such as global boiling (previously global warming) due to climate change, deforestation and a lack of rainfall, all impose an additional load on the development and germination of these enticing mushrooms. Hence, the government, stakeholders, and non-governmental organizations (NGOs) must take strong actions to ensure conservation and protection of the exquisite mushroom, *Astraeus*, which is a vital source of nutrition for tribals, villagers, and the rest of the world.

Conclusion

Gasteroid fungi are one of the most noteworthy groups of mushrooms due to their edibility, nutrient and medicinal benefits, and ability to support forest ecosystems via ectomycorrhizal association. Although reported by other mycologists, we could not find any basidiome of *A. hygrometricus* in the forest areas, despite the fact that it resembles *A. asiaticus* morphologically. As a result, additional collections should be made in the neighboring state for appropriate species identification, based on integrative taxonomic approach in tandem with morphological, molecular, ecological, and multigenetic phylogenies which would help resolve the taxonomic issues in the genus *Astraeus* in India.

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Supplementary electronic materials

Table S1. Structural difference between French, Thai and Indian Astraeus specimens. IM: Immature basidiome; M: Mature basidiome. Download file

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