Indian Phytopathology
https://doi.org/10.1007/s42360-024-00768-4

RESEARCH ARTICLE

Establishment of axenic culture from basidiospores of an ectomycorrhizal fungus *Astraeus asiaticus* and *A. odoratus*

Vineet Vishal[1,3] · Sweta Sushmita Tigga[2] · Pitchapa Thongsuwan[5] · Pisit Thamvithayakorn[5] · Nuttika Suwannasai[5] · Cherdchai Phosri[6,7] · Manoj Emanuel Hembrom[4] · Geetanjali Singh[1] · Shalini lal[1,2]

Received: 21 November 2023 / Revised: 15 May 2024 / Accepted: 31 May 2024

Abstract

Astraeus is a genus of edible ectomycorrhizal star-shaped fungus with commercial value. The inner tissue of basidiomata has previously been utilized to study the macro– and micro-cultural traits of *Astraeus*; though, a handful of studies have focused on basidiospores. Here we report the first pure cultural traits of Indian *Astraeus asiaticus* and *A. odoratus* obtained from basidiospores dropping/dusting method. Four distinct solid culture media namely potato dextrose agar (PDA), modified Melin–Norkrans (MMN), modified Norklan' s C (MNC), and Yeast media (YM) were used to culture the basidiospores. After about 42 days of culture in dark at 30 °C all the cultures exhibit diffused pale–yellow pigmentation followed by radial mycelial growth with fluffy and velvety surface. Monomitic, generative hyphae, and septate clamps with paariage branching were observed which are the typical traits of the cultured mycelium. *A. odoratus* were successfully grown on all the four media but attempts to grow *A. asiaticus* on MMN, MNC, and YM were unsuccessful. The fungal culture's identity was validated by phylogenetical approach using ITS and LSU nrDNA. The findings suggest PDA medium as the most suitable medium for culture of *Astraeus* and provide critical information on the culture traits that could be useful for wild mushroom cultivation thus increasing the country's mycoprotein richness.

Keywords Basidiospores · Cultivation · Earthstar · Mushroom · Phylogeny

Introduction

Basidiomycota spores are either ballistosporic or statismosporic when mature and are symbolized as the most fundamental component of their life cycle. These basidiospores after repeated mitotic divisions, give rise to genetically identical monokaryotic or primary mycelium which upon plasmogamy with two distinct hyphae, initiates the development of dikaryotic or secondary mycelium later on responsible for basidiocarp primordium formation (Webster and Weber 2007). Raudaskoski and Viitanen (1982) investigated the early stages of basidiocarp formation. Although the majority of the diagnostic traits used to identify basidiomycetes were provided by basidiocarps, establishing pure cultures derived from those structures makes it very simple to identify unknown fungi (Lodge et al. 2004). Since then, most cultural studies have relied heavily on inner tissue carefully extracted from basidiocarps (Phosri 2004; Fangfuk et al. 2010; Suwannasai et al. 2020). Scrase (1995) observed that the inner tissue of the gilled fungi *Hygrocybe* (Fr.) P. Kumm. can sometimes be contaminated with

✉ Shalini lal
shalini.kumari@gmail.com

1 Department of Botany, Dr Shyama Prasad Mukherjee University, Ranchi, Jharkhand, India

2 Department of Microbiology, Dr Shyama Prasad Mukherjee University, Ranchi, Jharkhand, India

3 Department of Botany, Bangabasi Evening College, Kolkata, West Bengal, India

4 Acharya Jagadish Chandra Bose Indian Botanic Garden, Botanical Survey of India, Howrah, India

5 Department of Microbiology, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand

6 Department of Biology, Faculty of Science, Nakhon Phanom University, Nakhon Phanom, Thailand

7 The Knowledge Hub for Integrated Economic Trees: Plantation Establishment, Management, Utilization and Industry, Kasetsart University, Bangkok, Thailand

Published online: 27 June 2024

bacteria and other fungi, making it challenging to establish a pure culture.

The basidiospore suspension technique is more effective, shorter, and less prone to contamination. Basidiospore isolation is direct, simple, rapid technique where the chances of recovery of a true product of meiosis are quite high (Pandey et al. 2016). The pure cultures of *Agaricus brunnescens* Peck (Kumar and Munjal 1981); *A. bisporus* (J.E. Lange) Imbach (Feofilova et al. 2004); oyster mushroom (Pandey et al. 2016) were all obtained from spore suspension culture. Similarly, the effects of liquid culture media, pH, temperature, on the basidiospore germination of *Lentinus swartzii* and *L. strigosus* using basidiospore suspension culture was studied (Dulay et al. 2020). However, it has been observed that in many cases in vitro basidiospore germination is a daunting task (Madelin 1962) as there are problem of poor germination and contamination by other microorganisms. To address these issues, several innovative approaches and refinements to existing methods have been suggested. Watling (1981) outlines many strategies for establishing pure cultures from basidiospores using spore suspension in sterile water. Scrase (1995) and Ainsworth (1995) later proposed similar methodologies for isolating the spores and even suggested using colonies of yeast (*Rhodotorula mucilaginosa* var. *sanguinea* (Schimon) Lodder, Verh. K. Akad) as a germination inducer, particularly for litter-decomposing gasteromycetes. To minimize the contamination by other fungi and bacteria in the culture medium, Lakkireddy and Kues (2017), used electrostatic attraction to isolate bulk basidiospores from wild mushrooms (*Coprinellus domesticus* (Bolton) Vilgalys, Hopple & Jacq. Johnson, *Schizophyllum* Fr. and *Psathyrella conopilea* (Fr.). However, the prerequisite nutritional and physiological conditions of most fungi remain unknown and may not be fulfilled in culture, which explains why several fungi cannot be cultured or are challenging to cultivate (Rama and Quandt 2021; Rahimlou et al. 2024).

Astraeus Morgan is a common gasteromycetes genus belonging to the Diplocystidiaceae family of the order Boletales. They are found in Dipterocarp forests in Southeast Asia and India, and even Pinaceae and Fagaceae forests in Europe and North America (Phosri et al. 2013). These star-shaped fungi grew as the monsoon season began, and they were always ripe, usually dehisced like a star, and with a whitish or brownish rhizomorph at the bottom of fruitification. The members such as *A. asiaticus* Phosri, M.P. Martin & Watling and *A. odoratus* Phosri, Watling, M.P. Martin & Whalley are edible fungi that have substantial markets in Northern Thailand, Laos, Vietnam, Cambodia, and India. However, there is no reliable evidence on the annual yield, preservation, or storage of these mushrooms (Phosri et al. 2014; Vishal et al. 2022).

Till date, eleven species have been identified, out of which *Astraeus pteridis* (Shear) Zeller, *A. hygrometricus* Pers., *A. odoratus* and *A. sirindhorniae* Watling, Phosri, Sihan., A.W. Wilson & M.P. Martín have had pure culture studies attempted to optimized mycorrhization capabilities of the fungus (Malajczuk et al. 1982; Fangfuk et al. 2010; Kaewgrajang et al. 2013; Suwannasai et al. 2020). In all these studies, inner tissue was extracted from the basidiocarp to establish a pure culture (Phosri 2004; Fangfuk et al. 2010; Suwannasai et al. 2020). Gaie and Heinemann (1980), on the other hand, reported successful pure culture establishment utilising an *Astraeus* glebal fragment. Their report appears to be the first trustworthy one in this field of research.

However, no such studies for *A. asiaticus* have been reported to date. The current gasteroid fungus project was part of an endeavor to document the distribution of various Indian *Astraeus* specimens associated with Indian Dipterocarp forests, particularly *Shorea robusta* Gaertn. The practices of artificially inoculating agaricomycetes under axenic circumstances have opened up new opportunities in fungi–based restoration, and other fungal biotechnological processes (Kumar et al. 2023).

However, here, we focused on enhancing *Astraeus* biomass production by cultivating pure culture strains of *A. asiaticus* and *A. odoratus*. Our major goal was to develop a reliable method of establishing pure fungal strains. In addition, we used macro– and micromorphology, as well as a combined phylogeny based on ITS and LSU genes, to assess the identity of pure mycelial strains.

Materials and methods

Study site and description

Young fruiting bodies of *A. asiaticus* (n = 10) and *A. odoratus* (n = 9) were collected from the dry deciduous tropical *Shorea* forest of Chainpur (23° 16’ 58.0” N, 84° 14’ 23.1” E) village in the Gumla district of Jharkhand, India (Fig. 1). The district’s landscape is characterized by gradual slopes with hills, valleys, and plateaus, and lush forests dominated by *Shorea* trees. The elevation ranges from 400 to 1,000 m above mean sea level. The average annual rainfall is 1200 to 1300 mm, and the temperature ranges from 10 to 30 °C. The soil in the forests was red, lateritic, sandy, and less humid. The forests are home to several IUCN-listed endangered wild animals and birds.

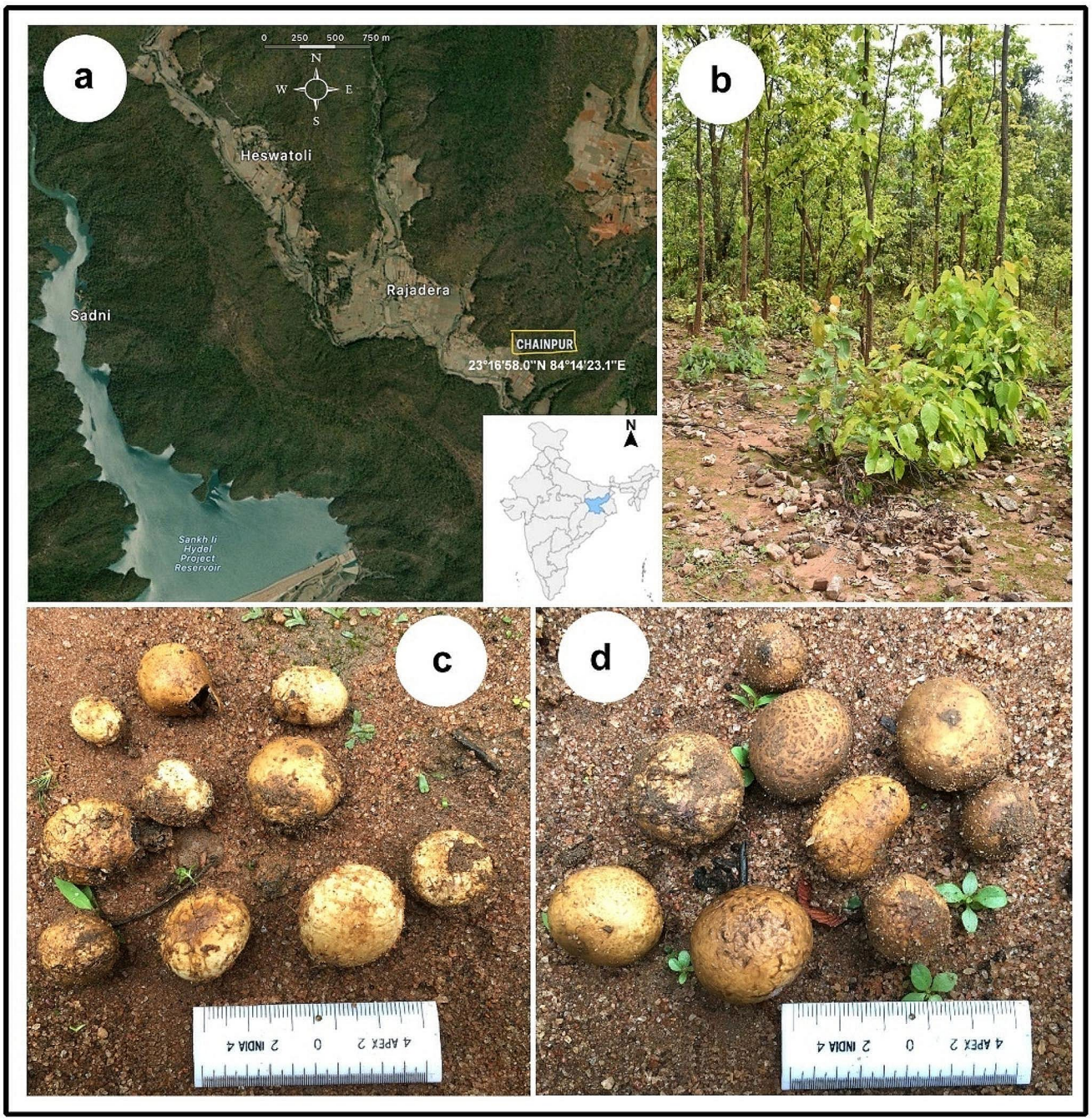

Fig. 1 Satellite image and protocol for axenic culture establishment of wild edible mushroom. **a** satellite view of the area of collections (India: Jharkhand: Gumla: Chainpur); **b** habitat and habit of *Astraeus*; **c** immature basidiomes of *A. asiaticus*; **d** immature basidiomes of *A. odoratus*. Bar, B–D = 10 mm

Isolation and culture preparations

Basidiospores from basidiomata were selected for *A. asiaticus* and *A. odoratus* spore cultivation and pure culture. Individual fruiting bodies were sterilized for 20 min with a 10% sodium hypochlorite solution before culture, according to the Watling (1981) approach. PDA, MNC, MMN, and YM have been used (Yamada and Katsuya 1995; Phosri 2004). Streptomycin (100 µg/ml) was added to the melting mediums after autoclaving to prevent contamination; however, it did not affect on the growth of *A. asiaticus* and *A. odoratus*. Cultures were first grown on PDA using the spore dropping/dusting method. Basidiospore dropping/dusting is a method in which portions of glebal fragments, containing spores, are sliced transversely and then dropped or dusted directly onto agar petri plates. Approximately 0.1 mg of

Table 1 Molecular markers, as well as their PCR primers and cycles, were applied in this study

Gene	Primers	Sequence (5'-3')	Position	Cycles	References
ITS	ITS1	TCCGTAGGTGAACC TGCGG	1773–1791 (18 S)	94 °C: 5 min; (94 °C 1 min, 50 °C 1 min, 72 °C 1 min, 72 °C 1 min) x 35 cycles	White et al. (1990)
	ITS4	TCCTCCGCTTATTGA TATGC	57 – 38 (25 S)		
LSU	LR0R	ACCCGCTGAACTTA AGC	26–42	94 °C: 5 min; (94 °C 1 min, 50 °C 90 s, 72 °C 2 min, 72 °C 10 min) x 35 cycles	Vilgalys and Hester (1990)
	LR5	TCCTGAGGGAAACT TCG	964 – 948		

spores were dropped/dusted in the agar plates. Incubation was done in a dark chamber at 30 °C for 5–6 days (Phosri 2004). The freshly developed primary mycelium was then transferred to MNC, MMN, and YM for pure culture establishment and stored in the incubators (BJPX–H30II, Bio-Base Pvt Ltd., China) for a month. The pH of the MNC and MMN mediums was merely adjusted to 5.4–5.8 using 1 N HCl or 1 N NaOH. (Phosri 2004; Fangfuk et al. 2010). All of the experiments were carried out in triplicate and with a completely arbitrary design. Uninoculated plates with each medium were utilized as a control. Mycelial growth was measured and recorded every two days during the incubation until mycelial growth reached the edge of 90 mm Petri plates. For subsequent experimentation, established pure mycelium cultures were subcultured monthly. Pure mycelia cultured on PDA, MNC, MMN, and YM agar media were kept at 30 °C for a few months and examined for macro– and micromorphological study. A triplicate of pure fungal stains was deposited at the Department of Botany, DSPMU, Ranchi, Jharkhand, India, under the voucher numbers Rug–10a–JH, Rug–10ab–JH for *A. asiaticus* and Rug–9a–JH for *A. odoratus*. A few fresh basidiomata from both specimens were preserved at 4 °C for future basidiospore inoculation experimentation.

Morphological identification of fungi

The main macro– and micromorphological traits of pure culture were studied using an Olympus CX 41 light compound microscope, including line drawing at 1000x magnification. Color and tint on colony, smell or fragrance of culture, surface structure, pattern, margin and growth pigment exuded; hyphal analysis includes hyphal system, wall thickness, septations, branching patterns, and inflations were all noted. The mycelium was mounted in water, 5% KOH, and stained with Melzer reagent. Olympus BX 53 camera was used to capture microscopic images. Colour codes were based on the Methuen Handbook of colour (Kornerup and Wanscher 1967). The images were edited and prepared using Adobe Photoshop 7.0.

DNA extraction, amplification, sequencing and sequence alignment

A plant genomic DNA Extraction kit was used to extract genomic DNA (Farvogen, Taiwan) from the mycelium of the outer part of the potato dextrose agar plates. The sequencing of internal transcribed spacer regions with the intervening 5.8 S nrDNA gene (nrITS) and the partial large subunit nrDNA gene (nrLSU) validated the authenticity of fungal specimens cultured on agar plates.

The nrITS (ITS1 + 5.8 S + ITS2) and nrLSU (28 S) regions (Table 1) were amplified using primers ITS1/ITS4 and LROR/LR5 (White et al. 1990; Vilgalys and Hester 1990). The PCR reaction and cycles were carried out by Phosri et al. (2014). The resulting amplicons were purified with a PCR/gel purification kit (Farvogen, Taiwan) and sequenced at Macrogen (South Korea). Chromas 2.4 (Technelysium Pty Ltd., South Brisbane, Australia) was used to analyze the chromatograms to determine the quality of each nucleotide base. Five of the consensus sequences were submitted to GenBank to procure accession numbers.

Phylogenetic analysis

Six newly generated sequences of nrITS and nrLSU from pure culture mycelium of *A asiaticus* and *A. odoratus* including their homologues were retrieved (Table 2) from nBLAST runs against the NCBI GenBank nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and other relevant phylogenetic publications in order to infer a functional and phylogenetic linkage between the sequences (Phosri et al. 2007, 2014; Wilson et al. 2011; Fangfuk et al. 2010; Crous et al. 2019). Two distinct datasets were generated, one including ITS sequences and the other comprising LSU nrDNA sequences. The MUSCLE tool was used to align both datasets individually. To represent missing data, missing genes for individual samples were recorded as "–" in the dataset. MEGA software version 10.1.1812 (Kumar et al. 2018) was used to check, trim, and concatenate these datasets. Two locus datasets were employed for maximum likelihood (ML) and Bayesian analysis in the phylogenetic study. To estimate the bootstrap support value, ML analysis was

Table 2 List of specimens used in this study

Specimens	Voucher no	ITS	LSU	Locations	References
Astraeus asiaticus	ASTRAE-54	AJ629397	HE681776	Thailand	Phosri et al. (2007)
A. asiaticus	ASTRAE-65	AJ629401	HE681778	Thailand	
A. asiaticus	**Rug-10a-JH**	**OQ253540**	**OQ253547**	**India**	**This study**
A. asiaticus	**Rug-10ab-JH**	**OQ253541**	**OQ253558**	**India**	
A. odoratus	**Rug-9a-JH**	**OR617082**	**OR625446**	**India**	**This study**
A. odoratus	ASTRAE-58	AJ629874	HE681779	Thailand	Phosri et al. (2007)
A. odoratus	ASTRAE-59	AJ629875	HE681780	Thailand	
A. odoratus	ASTRAE-62	AJ629877	HE681781	Thailand	
A. hygrometricus	MB05-029	EU718087	DQ682996	USA	Wilson et al. (2011)
A. hygrometricus	ASTRAE-43	AJ629406	-	France	Phosri et al. (2007)
A. hygrometricus	Aswan28	AB535112	-	Japan	Fangfuk et al. (2010)
A. hygrometricus	Aswan56	AB535114	-	Japan	
A. sirindhorniae	GAPK1	HE681772	HE681782	Thailand	Phosri et al. (2014)
A. sirindhorniae	GAPK2	HE681773	HE681783	Thailand	
A. sirindhorniae	GAPK3	HE681774	HE681784	Thailand	
A. sirindhorniae	GAPK4	HE681775	HE681785	Thailand	
A. pteridis	Ashy3	EU718088	AF336238	Switzerland	Wilson et al. (2011)
A. pteridis	PDD88503	FJ710188	EU718158	New Zealand	
A. macedonicus	05MCF5221	MK491320	MK496886	Macedonia	Crous et al. 2019
A. macedonicus	07MCF6706	MK491317	MK496884	Macedonia	
A. macedonicus	08MCF10410	MK491318	MK496885	Macedonia	
A. macedonicus	09MCF11183	MK491316	MK496883	Macedonia	
Pisolithus albus	MDBF151	KY689591	KY689569	outgroup	Phosri et al. (2007)

performed with raxmlGUI 2.0 (Edler et al. 2021) using the best substitution model and one thousand replicates. For the Bayesian analysis, the best–fit model, TIM3ef + G4, generated from jMoldelTest, was utilized. MrBayes 3.2.7a (Ronquist et al. 2012) was executed using the CIPRES XSEDE web-server *(*https://www.phylo.org/portal2/mrbayes xsede! input.action*)* with the default configuration (Nst = 6, 2 runs and 4 chains per run, each run searching for 10^6 generations and sampling every 1000th generations). The tree depicts maximum likelihood bootstrap values (MLBS) of ≥ 70% and Bayesian posterior probabilities (PP) of ≥ 0.7.

Results

Culture and growth characteristics of *Astraeus*

A total of seven strains of *Astraeus* were established using the basidiospore dropping or dusting method. These strains grow well in natural (PDA) and synthetic media (MMN, MNC and YM). All these strains were placed in a dark room at 30 °C for incubation period of 42 days. Initially after 2–3 days of incubation all the strains produced a pale yellowish (2A3) pigment that diffused through the media, resulting in a change of color. The colonies show radial outgrowth with regular edges. The surface was matted, smooth, fluffy, and velvety. The aerial brown hyphae displayed different growth rate on the medium (Fig. 2G, H). Both *A. asiaticus* and *A. odoratus* both have monomitic and generative type of hyphal system with numerous, septate clamp, present at both simple and multiple or in intercalary junctions (Figs. 3B-D and 4B-D). Mycelial cords show further branching and moderately encrusting in KOH.

Growth characteristics of *Astraeus asiaticus* and *A. odoratus*

Three strains of *Astraeus asiaticus* and four strains of *A. odoratus* were successfully established by this method. *Astraeus asiaticus* strains was grown primarily on PDA, however *A. odoratus* strains were cultured on PDA, MMN, MNC, and YM media. Strains of *A. asiaticus* and *A. odoratus* showed different pigmentation in PDA, with *A. asiaticus* (Fig. 2A) showing pale yellow (2A3) on the top, reverse center-light brown(6D8), and dark brownish pigments on the periphery (6FB) whereas *A. odoratus* (Fig. 2B) shows brownish–green (6E8–27A8) on the top. The *Astraeus asiaticus* and *A. odoratus* PDA strains have light brown aerial hypha, which grow slowly in case of *A. asiaticus* (up to 29 mm in diameter after 42 days) and rapidly in *A. odoratus* (up to 88 mm in diameter). The radial colony of *A. asiaticus* grew at 1.2 mm/day during the incubation phase, but decreased to 0.6 mm/day as the duration increased. *Astraeus odoratus* radial colony grew at 3.5 mm/day but decreased to 2.1 mm/day as the incubation period increased. The study found that *A. asiaticus* hyphae exude a light brown

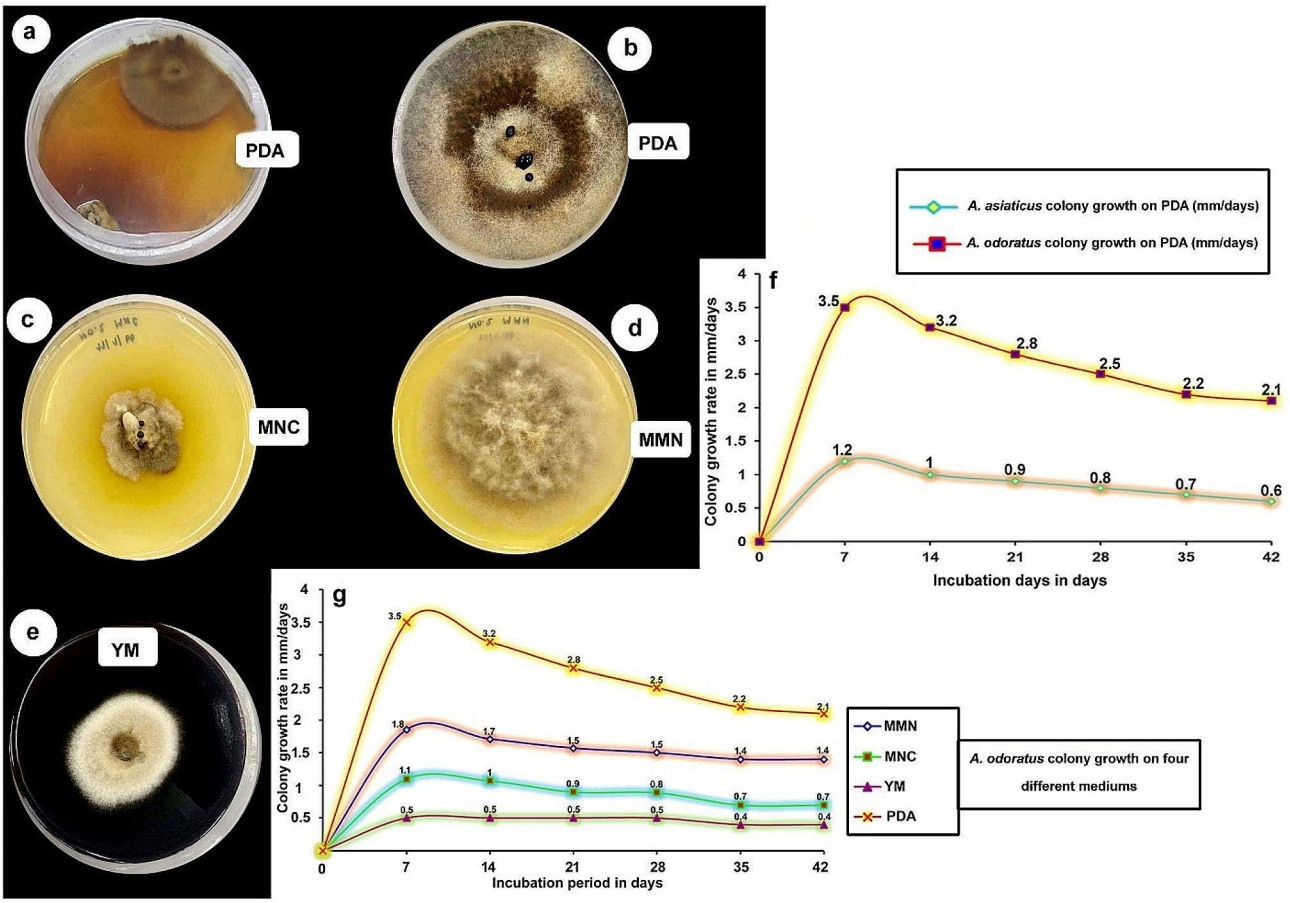

Fig. 2 Photoplate of pure culture plates of *Astraeus asiaticus* and *A. odoratus*. **a***A. asiaticus* obtained from PDA; **b***A. odoratus* obtained from PDA; **c***A. odoratus* obtained from MNC; **d***A. odoratus* obtained from MMN; **e***A. odoratus* obtained from Yeast Media (YM); **f** scatter with smooth line graph showing colony growth rate of *A. asiaticus* and *A. odoratus* after 42 days of incubation; **g** scatter with smooth line graph showing colony growth rate of *A. odoratus* obtained from four different media throughout the same incubation period

to slightly dark greenish-brown (27F8–6E8) colored pigment that diffused through the PDA, resulting in a change of color (Fig. 2A) but no watery fluid was observed. However, *A. odoratus* plates (Fig. 2B) exude watery brown pigments (6D8). Despite several attempts to obtain cultures of *A. asiaticus* on MMN, MNC, and YM were unsuccessful.

In contrast, *A. odoratus* grew well on MMN, MNC, and YM agar mediums. MMN plates showed pale brownish (6D8) to light greenish-brown (27A8–6E8) pigments on the top and reverse center light brownish (6D8) (Fig. 2D); MNC plates displayed pale yellow (2A8) to dark greenish-brown (6E8–27AB) pigments on the top and reverse center light brown (6D8) (Fig. 2C). The YM agar plate had a light-brown (6D8) pigment on the top and a dark brown (6D8) pigment on the reverse (Fig. 2E). The MMN, MNC, and YM agar plates had radial aerial hyphae with diameters of 62 mm (Fig. 2D), 33 mm (Fig. 2C), and 20 mm (Fig. 2E) during a 42-day incubation period. The colonies grew at 1.8 mm/day, 1.1 mm/day, and 0.5 mm/day, but decreased as the incubation period grew longer (Fig. 2G). PDA and MNC agar plates exudates watery brown (6D8) pigments (Fig. 2B, C).

Micro–morphological culture characterization of *Astraeus asiaticus and A. odoratus*

All strains developed monomitic and generative hyphal systems. *Astraeus asiaticus* and *A. odoratus* strains produced generative hyphae which were 2.1–5.3 µm and 2.5–6.0 µm wide, clamped, thin to thick-walled (wall up to 1.3 µm thick), long, straight, frequently branched (few branches either at an acute angle from the main branches or nearly at the right angle to them), with frequent paarige branching (is a branching in which a pair of branches is given off immediately below a transverse wall), pigmented, hyaline to greenish in KOH. Some olieferous hyphae (with dense cytoplasmic contents) and a few producing dense contents with irregular bulges turned into chlamydospores were found in *A. asiaticus*, whereas few dense-content hyphae without bulges had been observed in *A. odoratus*. Those

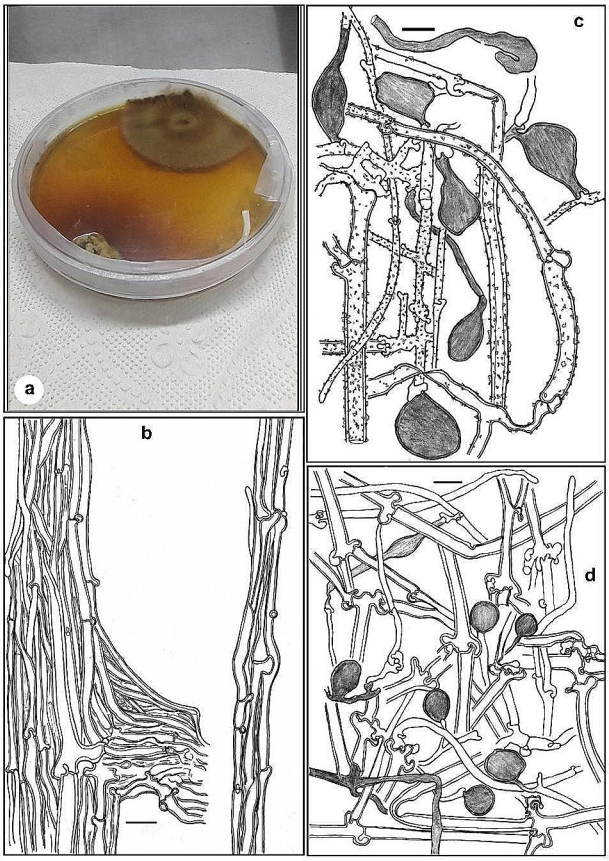

Fig. 3 Line diagram of pure culture hyphal system of *Astraeus asiaticus* (Rug-10a-JH) at 1000x magnification. **a** 42days old culture plate; **b** corded mycelium with multiple clamp-connection and thick-walled hyphae; **c** olieferous hyphae (with dense cytoplasmic), encrusted hyphae, and chlamydospores in KOH; **d** paarige branching with multiple clamp-connection and bulges. Bars = b-d 10 µm

asexual chlamydospore observed in *A. asiaticus* were 6.9–38.5 × 5.1–40.5 µm in diam., circular, oval to elliptical, terminal to intercalary of hyphae, moderate to thick-walled, wall smooth (in KOH and Melzer's) (Fig. 3C-D), bright yellowish (2A8) (Fig. 3A). However, no such asexual chlamydospore was observed in *A. odoratus*. Furthermore, mycelial cords of *A. asiaticus* were much wider (22.3–25.5 µm in diam.) than those of *A. odoratus* (13.5–18.6 µm in diam.). In *Astraeus asiaticus*, these mycelial cords are moderately encrusted in KOH, but incrustation dissolves in Melzer, orange-yellow (4A8). No such traits were observed in *A. odoratus*, but some hyphae of the strains have inflated inflations and incrusted hyphal walls.

Phylogenetic analysis

We constructed a two-locus (ITS + LSU nrDNA) combined phylogenetic tree to elucidate the placement of the newly generated six sequences from the pure mycelial culture of

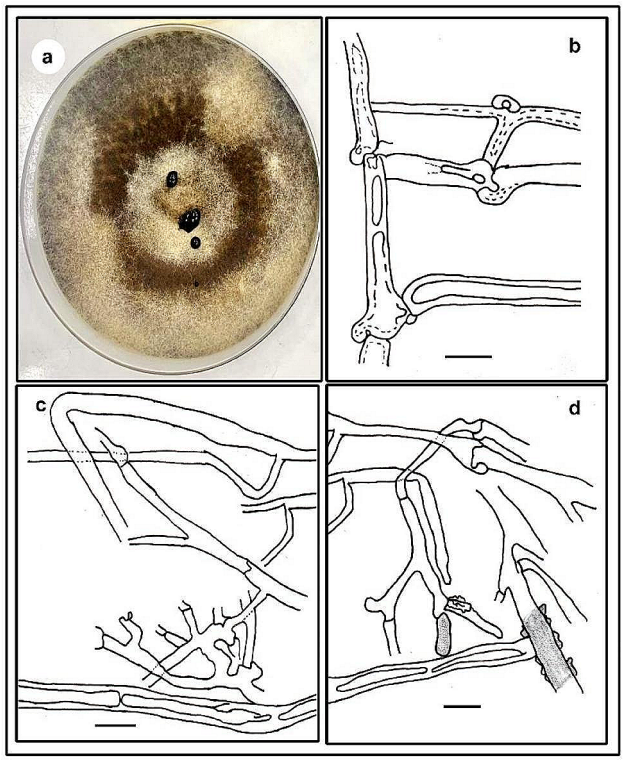

Fig. 4 Line diagram of pure culture hyphal system of *Astraeus odoratus* (Rug-9a-JH) at 1000x magnification. **a** 42 days old PDA-plate; **b-c** thick-walled hyphae with infrequent clamp-connection; **d** dense cytoplasmic with encrusted hyphae and paarige branching. Bars, b-d = 10 µm

Astraeus. The sixteen sequences were retrieved from published literature and the GenBank nucleotide database, and six newly generated (Rug–10a–JH, ITS = OQ253540 and LSU = OQ253547; Rug–10ab–JH, ITS = OQ253541; LSU = OQ253558 and; Rug–9a–JH, ITS = OR617082; LSU = OR625446) sequences were used to construct the phyllogram (TABAL 2). For rooting purposes, *Pisolithus albus* was employed as an outgroup (Fig. 4). The final alignment dataset contains 787 distinctive alignment patterns, with 39.44% of characters undetermined, including gaps. Both RAxML and Bayesian analysis produced trees with comparable topologies, Only the RAxML tree with the final ML optimization likelihood of − 8768.710351 (ln) is taken into account and shown in Fig. 5.

The length of the tree is 1.103530. The two–locus datasets demonstrated six major clades in the tree. The first three clades include all Southeast Asian specimens, such as *A. odoratus, A. asiaticus*, and *A. sirindhorniae*. The remaining three monophyletic clades are represented by European and North American specimens: *A. macedonicus, A. hygrometricus*, and *A. pteridis*. The newly discovered sequences from the pure culture of Indian *A. asiaticus* (Rug–10a–JH and Rug–10ab–JH) form a sister clade with Thai *A.*

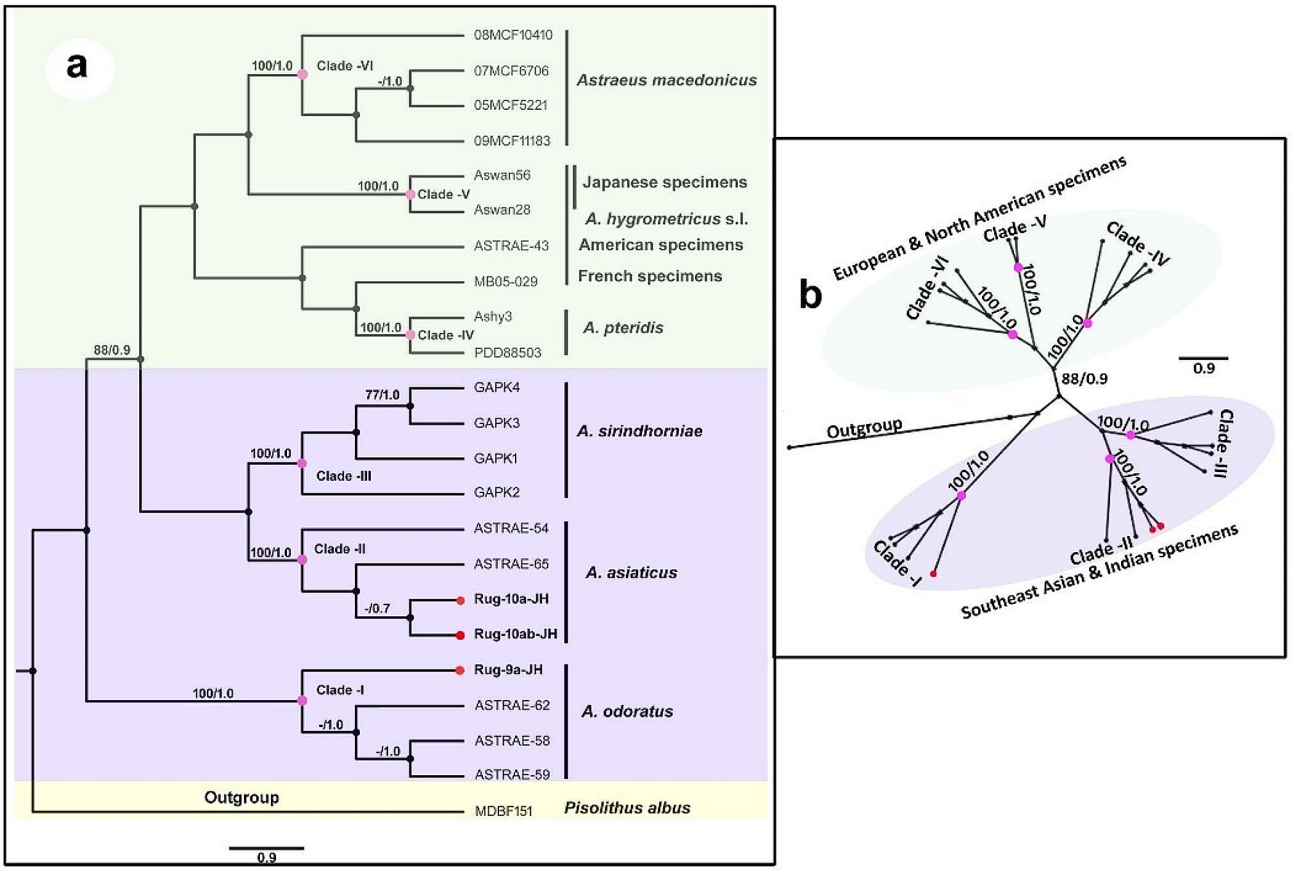

Fig. 5 Two gene (nrITS and nrLSU) RAxML cladogram of *Astraeus* and allied species. **a** cladogram showing the placements of Indian voucher specimens (Rug–10a–JH, Rug–10ab–JH and Rug-9a-JH); **b** radial tree showing delimitation of Indian specimens from North American and European specimens. Note- Terminal branches showing vouchers numbers. Number on the nodes represents the percentage of MLBS and PP values. *Pisolithus alba* was used as an outgroup for rooting purpose

asiaticus (ASTRAE–54 and ASTRAE–64) and have a high bootstrap support value (MLBS = 100% and PP = 1.00). Similarly, Indian *A. odoratus* (Rug–9a–JH) forms a sister clade with Thai *A. odoratus* (ASTRAE–58, ASTRAE–59, and ASTRAE–62) with a high bootstrap support value (MLBS = 100% and PP = 1.00). However, posterior probability support was often higher than bootstrap support, indicating a more acceptable phylogenetic structure, as demonstrated in the RAxML tree (Fig. 5).

Discussion

The inner cortex tissue of ectomycorrhizal fungi has received the most attention in pure culture studies, rather than basidiospores, rhizomorphs, sclerotia, or other vegetative organs. According to Watling (1963), most of the mushroom's parts may be dormant, except basidiospore-producing tissue. The basidiospores were the sexual spores of mushrooms, and they were notoriously difficult to germinate (Scrase 1995). Due to the limited survivability of hybrid crosses it was predicted that around 5–20% of basidiospores develop into mature colonies under axenic conditions (Forsythe et al. 2016). The most useful technique for culturing ectomycorrhizal fungi, however, is to germinate their basidiospores following a standard protocol. To obtain pure cultures, one of the simplest methods is to allow a fungus to drop or dust spores directly onto a nutrient-rich agar surface. Fuchs (1911), Kneebone (1950), Gaie and Heinemann (1980), and Nakano et al. (2017) have successfully germinated spores of the ectomycorrhizal fungi *Lactarius deliciosus* (L.) Gray, *L. luteolus* Peck, and *Astraeus hygrometricus* and *Rhizopogon roseolus*, respectively.

Culturing a particolous ectomycorrhizal fungus from basidiospore has undoubtedly proven challenging for several decades. *Astraeus asiaticus* and *A. odoratus* are common earthstar edible ectomycorrhizal fungi that were described and separated from *A. hygrometricus* by Phosri et al. (2004, 2007). They are well known for their nutritional, nutraceutical, and therapeutic characteristics; nevertheless, little is known about their mycorrhizal symbiosis, host preference,

Table 3 Cultural comparison of the *Astraeus* and allied species

Characteristics	A. odoratus Fangfuk et al. (2010)	A. sirindhorniae Suwannasai et al. (2020)	A. hygrometricus Gaie and Heinemann (1980)	A. asiaticus (This study)	A. odoratus (This study)
Inoculation	Inner tissue	Inner tissue	Basidiospores	Basidiospore	Basidiospore
Media	MNC	MNC	PCM*	PDA	PDA
Incubation(days)	4–7	4–5	–	6–7	6–7
pH	–	6.0	5.0	–	–
Colony (Growth observed)	within 4–7 days	within 4–5 days	-	within 2–3 days	within 2–3 days
Pigmentation	yellowish to reddish, dark brown pigment	reddish–brown pigments	Fairly light cinnamon brown	light brown to slightly dark greenish–brown color pigment	Light brown to pale yellow
Hyphal morphology	2.63–5.39 µm in diam; curved to straight; clamp connections on septa	hyphae 2.5–3. 0 µm in diam; clamp connections on septa;	Hyphae cylindrical; 2–5 µm in diam; smooth or often verrucose; thick–walled; encrustation	2.1–5.3 µm in diam; clamp connection on septa; Thick–walled (up to 1.3 µm);	2.5–6.0 µm in diam; long; clamped septate; thin– to thick–walled (wall up to 1.4 µm thick).
Hyphal pigments	laterally or intercalarily pigmented.	–	–	Intercellularly pigmented	Intercellularly pigmented

***note PCM is* Palmer culture medium

and fruiting ecology. There is a dearth of study on the traits and cultivations of pure cultures.

Therefore, we commence *A. asiaticus* and *A. odoratus* cultural studies in a axenic condition by modifying Choi et al. (1999) spore shooting approach to germinating spores. The spore-drop or dusting technique was chosen because the basidiospores, which are enclosed within the hymenium (gleba), are themselves contamination free zones and did not require any special chemical treatment. Obtaining a pure culture from inner tissue, on the other hand, can be contaminated with endobacteria or other microbes, making isolation difficult, as demonstrated in the gilled fungus *Hygrocybe* sp. (Scrase 1995). Another drawback of tissue-cultured stains is that they are not monokaryons and do not reflect the actual outcome of meiotic division, making them unsuitable for mass genetic breeding programs. The preliminary selection of isolates of interest, accompanied by the breeding of the selected isolates to boost productivity in terms of metabolite synthesis or production of biomass necessitates genetically pure cultures. According to Pandey et al. (2016) they are the genuine outcome of meiosis. Our approach of germinating involves dusting 0.1 mg of spores, which is easier, faster, and more efficient compared to isolating single spores using vaseline or the spore suspension serial dilution method (Watling 1981; Scrase 1995; Ainsworth 1995).

Gaie and Heinemann (1980) are the only mycologists to successfully germinate basidiospores of *A. hygrometricus* using glebal fragments with 50% success rate; Archer (1962) and Phosri (2004) failed to do so with other *Astraeus* specimens. Although few mycologists use germination inducers *Rhodotorula mucilaginosa* var. *sanguinea* or *Rhodotorula glutinis* (Fresen.) F.C. Harrison for germination to obtain monosporous mycelia in *Amanita* Dill. ex Boehm., *Tricholoma* (Fr.) Staude, *Clitopilus* (Fr. ex Rabenh.) P. Kumm., *Suillus* P. Micheli (Fries 1943), and *Rhizopogon roseolus* (Corda) Th. Fr. (Nakano et al. 2017) for other litter–decomposing gasteromycetes (Scrase 1995).

In this study, no germination inducers were used. On the nutrient medium, a pale yellowish (2A3) to light brownish (6D8) pigment was formed on PDA, MMN, MNC and YM after 2–3 days of incubation, indicating both developmental competency and survivability (Fangfuk et al. 2010; Forsythe et al. 2016). The pigmentation in PDA ranges from yellowish–light brown (2A8–6D8) to slightly dark greenish–brown (27F8–6E8) in *A. asiaticus* and brownish–green (6E8–27A8) in *A. odoratus* (Fig. 2A-B). In MMN, MNC, and YM, the pigments were pale brownish (6D8) to light greenish–brown (27A8–6E8), pale yellow (2A8) to dark greenish–brown (6E8–27AB), and light–brown (6D8), respectively, in a few days old agar plates. (Fig. 2C-E). A similar phenomenon was also reported by Phosri (2004); Fangfuk et al. (2010); and Suwannasai et al. (2020). However, Burgess et al. (1995) and Phosri (2004) mentioned that pH and temperature are very crucial parameters for Boletales pure culture, therefore, we maintained a pH of 5.4 to 5.8 for MNC and MMN and a temperature of 30 °C, which were found to be effective conditions for stimulating spore germination.

After 42 days of incubation, the vegetative hyphae of *A. asiaticus* develop more slowly (29 mm) than those of *A. odoratus* (88 mm). Cultures of *A. asiaticus* on MMN, MNC, and YM were unsuccessful. *A. odoratus*, on the other hand, grows well on all four mediums (Fig. 2B-E). According to our findings, PDA was the optimal media for cultivating *A. asiaticus* and; PDA and MMN for *A. odoratus*. All the PDA triplicates of *A. asiaticus* and *A. odoratus* have a fluffy, velvety, smooth, or matted texture, with distinct zones around the edges. The cultural strains were examined on 5% KOH, Melzer reagent, and water successively. On the water and 5% KOH, moderate encrustation was observed with olieferous hyphae (dense cytoplasmic) content (Fig. 3C) observed in *A. asiaticus*, the same phenomenon was also reported by Gaie and Heinemann (1980) in *A. hygrometricus*. The vegetative hyphae established many clamp connections at the septa, with occasional paarige branching. These hyphae are monomitic, generative, thick to thin-walled, pigmented and moderately encrusted.

However, *A. asiaticus* and *A. odoratus* vegetative hyphae can be distinguished by hyphal cord and generative hypha thickness, the presence or absence of olieferous hyphae, encrustation and asexual chlamydospores. The hyphal cord thickness of *A. asiaticus* is larger (22.3–25.3 m in diam) than that of *A. odoratus* (13.5–18.6 m in diam), but the generative hyphal thickness of the latter is larger (2.5–6.0 m in diam) than that of former (2.1–5.3 m in diam) (Fig. 2F-G). *A. odoratus* agar plates also show no olieferous hyphae or asexual chlamydospore elements (Fig. 3C). Some hyphal branches in *A. asiaticus* were heavily encrusted (Fig. 3B), but few hyphae were encrusted in *A. odoratus* (Fig. 4D). Some hyphal branches of *A. asiaticus* agar plates are either at an acute angle or nearly at a right angle to the major branches, but few hyphal branches of *A. odoratus* agar plates contain inflated inflations and an incrusted hyphal wall. All of these micromorphological cultural traits were comparable to those of European *A. hygrometricus* s.l. (Gaie and Heinemann 1980), *A. pteridis* (Malajczuk et al. 1982), Thai *A. odoratus*. (Phosri 2004) and *Astraeus* sp. (Saensuk and Suntararak 2018) and *A. sirindhorniae* (Suwannasai et al. 2020). Except for Gaie and Heinemann (1980), these mycologists used inner tissue from *Astraeus* basidiocarps to inoculate and germinate mycelium on artificial media: MMN, MNC, YM, and Malt Extract Agar (MEA), Table 3. Our PDA (Fig. 4A) and MNC (Fig. 2C) culture plates of *A. odoratus* exuded dark brownish watery droplets. These dark, watery droplets could be pulvinic acid derivatives, according to Watling (2008).

Our phylogeny (ITS and LSU nrDNA) demonstrates the phylogenetic placements of pure culture strains. The vouchers no: Rug–10a–JH and Rug–10ab–JH were placed in the Southeast Asian clade of *A. asiaticus*. Similarly, voucher no: Rug–9a–JH placed was in the Southeast Asian clade of *A. odoratus* based on genetic sequence and morphology. Thus, validates the pure culture strain's identity (Fig. 5A). The radial phylogenetic tree further provides evidence for the Southeast Asian origin of the species (Fig. 5B).

To the best of our knowledge, this is the first report of *Astraeus* pure culture and cultivation from basidiospores in axenic conditions using the dropping/dusting method. For in vitro culture of *A. asiaticus* and *A. odoratus*, potato dextrose agar (PDA) seems to be the most suitable medium. This is because PDA offers several advantages: it has the right nutrients, it's easy to prepare and maintain, and it doesn't require any special solutions to keep the fungus growing well. Cultivating ectomycorrhizal fungi provides a low-cost, nature-based strategy that aids in forest restoration, conserves biodiversity, and enhances the yield of forest mycoprotein, ultimately stimulating the local economy. This method not only benefits the environment in these ways, but it also plays a crucial role in green research by capturing greenhouse gases and promoting carbon sequestration (Thomas and Vazquez 2022).

Conclusion

Astraeus is a therapeutic and commercially valuable wild mushroom. Obtaining pure cultures would greatly aid in Mycoforestry, and Myco–economy of the country, although wild mushrooms are extremely difficult to culture. Establishing artificial nutritional media, temperature, and pH may be critical conditions for culture. This study not only describes the cultural traits of *Astraeus*, but it also suggests that PDA and MMN would be the best natural and synthetic media for the growth of the taxon. However, more study is required to investigate various unexplored areas, such as their mycorrhizal symbiosis, host choice, fruiting ecology, and factors influencing mushroom production longevity.

Acknowledgements We would like to express our gratitude to the Head of the Department of Botany, DSPMU, Ranchi, Jharkhand, the Principal of Bangabasi Evening College, Kolkata, West Bengal, India and Head of Department of Microbiology, Faculty of Science, Srinakharinwirot University, Bangkok, and Dean of Faculty of Science, Nakhon Phanom University, Nakhon Phanom, Thailand, for providing the necessary equipment. We are thankful to the Scientist of Central National Herbarium (CAL) and Dr. Kanad Das, of the Botanical Survey of India, Howrah, West Bengal, India, for their motivation and guidance.

Author contributions The ideas and project were developed by SL, NS, CP, and VV. VV, SST, PTO and PTA, were involved in the research and investigation process, particularly in experimentation. VV, SL, NS, and CP have equally contributed to data interpretation, analysis and writing of the manuscript. MEH and VV were doing line diagrams. All of the authors provided constructive feedback on how to implement the study, analysis, and development of the article. The

version to be submitted, as well as any revised versions, received final approval from SL, NS, and CP.

Funding Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

References

Ainsworth AM (1995) Technical information sheet 11: isolation techniques for basidiomycetes. World J Microbiol Biotechnol 11:364–366

Archer S (1962) Preliminary studies on the germination of the basidiospores of *Astreaus Hygrometricus*. National Science Foundation, Oklahoma, p 13

Burgess T, Malajczuk N, Dell B (1995) Variation in *Pisolithus* based on basidiome and basidiospore morphology, culture characteristics and analysis of polypeptides using 1D SDS–PAGE. Mycol Res 99(1):1–13. https://doi.org/1016/S0953-7562(09)80309-2

Choi YW, Hyde KD, Ho WH (1999) Single spore isolation of fungi. Fungal Divers 3:29–38

cosci.MYC51234

Crous PW, Carnegie AJ, Wingfield MJ, Sharma R, Mughini G, Noordeloos ME, Silva BDB (2019) Fungal planet description sheets: 868–950. Pers Mol Phylogeny Evol Fungi 42:291

Dulay RM, Cabrera EC, Kalaw SP, Reyes RG, Hou CT (2020) Cultural conditions for basidiospore germination of *Lentinus Swartzii* and *Lentinus strigosus* and their morphogenesis. Asian J Agric Biol 8:377–385. https://doi.org/10.35495/ajab.2020.04.240

Edler D, Klein J, Antonelli A, Silvestro D (2021) raxmlGUI 2.0: a graphical interface and toolkit for phylogenetic analyses using RAxML. Methods Ecol Evol 12(2):373?377. https://doi.org/10.1111/2041-210X.13512

Fangfuk W, Okada K, Fukuda M, Yamada A, Petchang R, To–anun C (2010) In vitro mycorrhization of edible *Astraeus mushrooms* and their morphological characterization. Mycoscience 51(3):234–241. https://doi.org/10.1007/S10267-009-0031-1

Feofilova EP, Tereshina VM, Garibova LV, Zav'yalova LA, Memorskaya AS, Maryshova NS (2004) Germination of basidiospores of *Agaricus Bisporus*. Appl Biochem Microbiol 40:186–191

Forsythe A, Vogan A, Xu J (2016) Genetic and environmental influences on the germination of basidiospores in the *Cryptococcus neoformans* species complex. Sci Rep 6(1):1–12. https://doi.org/10.1038/srep33828

Fries N (1943) Untersuchungen über Sporenkeimung Und Mycelentwicklung Odenbewohnender Hymenomyceten [Studies on spore germination and mycelium development of soil–dwelling Hymenomycetes in German]. Symb Bot Ups (6):1–81

Fuchs I (1911) Uber die Beziehungen Von Agaricineen und anderen humusbewohnenden Pilzen Zur Mycorrhizenbiidung Der Waldbaume [On the relationship of agaricines and their humus–dwelling fungi to mycorrhizal formation in forest trees in German]. Biliotheca Bot 18:1–32

Gaie W, Heinemann P (1980) Synthese et morphologie de l'ectomycorhize *Betula pendula*/*Astraeus hygrometricus* [Synthesis and morphology of ectomycorrhiza *Betula pendula*/*Astraeus hygrometricus*, in French]. Bull Garden natl Bot belg 161–165

Kaewgrajang T, Sangwanit U, Iwase K, Kodama M, Yamato M (2013) Effects of ectomycorrhizal fungus *Astraeus odoratus* on *Dipterocarpus alatus* seedlings. J Trop Sci 200–205

Kneebone LR (1950) An investigation of basidiospore germination in the Hymenomycetes, especially in the Agaricaceae. Doctoral dissertation, Pennsylvania State College, USA

Kornerup A, Wanscher JH (1967) Methuen handbook of colour. Methuen handbook of colour. Hasting House, New York, USA

Kumar S, Munjal RL (1981) Studies on evolving new strains of the cultivated mushroom (*Agaricus Brunnescens* Peck) by single spore isolation method. Indian J Mushroom 7:37–42

Kumar S, Stecher G, Li M, Knyaz, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35(6):1547. https://doi.org/10.1093/molbev/msy096

Kumar V, Kumar H, Vishal V, Lal S (2023) Studies on the morphology, phylogeny, and bioremediation potential of *Penicillium Citrinum* and *Paecilomyces Variotii* (Eurotiales) from oil–contaminated areas. Arch Microbiol 205(1):50. https://doi.org/10.1007/s00203-022-03383-x

Lakkireddy K, Kües U (2017) Bulk isolation of basidiospores from wild mushrooms by electrostatic attraction with low risk of microbial contaminations. AMB Express 7:1–22. https://doi.org/10.1186/s13568-017-0326-0

Lodge DJ, Ammirati JF, O'Dell TE, Mueller GM (2004) Collecting and describing macrofungi. Biodiversity of fungi: inventory and monitoring methods. Elsevier Academic, Oxford, UK, pp 128–158

Madelin MF (ed) (1962) The fungus spore. Butterworth: London

Malajczuk N, Molina R, Trappe JM (1982) Ectomycorrhiza formation in *Eucalyptus*: I. pure culture synthesis, host specificity and mycorrhizal compatibility with *Pinus radiata*. New Phytol 91(3):467–482

Nakano S, Gao Q, Aimi T, Shimomura N (2017) Improvement of basidiospore germination and mycelial growth of the ectomycorrhizal mushroom *Rhizopogon Roseolus* in axenic conditions. Mush Sci Biotech 25(3):94–99. https://doi.org/10.24465/msb.25.3_94

Pandey NK, Maravi KK, Kerketta A, Taram L (2016) A simple technique for isolation of single basidiospore from *Pleurotus Flabellatus*. Res J Agri Sci 7(3):703–704

Phosri C (2004) Characterization and development of tropical gasteromycete fungi in ectomycorrhizal associations. Doctoral dissertation, Liverpool John Moores University, UK

Phosri C, Martín MP, Sihanonth P, Whalley AJ, Watling R (2007) Molecular study of the genus *Astraeus*. Mycol Res 111(3):275–286. https://doi.org/10.1016/j.mycres.2007.01.004

Phosri C, Martín MP, Watling R (2013) *Astraeus*: hidden dimensions. IMA Fungus 4(2):347–356. https://doi.org/10.5598/imafungus.2013.04.02.13

Phosri C, Watling R, Suwannasai N, Wilson A, Martín MP (2014) A new representative of star–shaped fungi: *Astraeus sirindhorniae* sp. nov. from Thailand. PLoS ONE 9(5):e71160. https://doi.org/10.1371/journal.pone.0071160

Rahimlou S, Quandt CA, James TY (2024) Metabolic Constraints and Dependencies Between Uncultivable Fungi and Their Hosts. In: Hsueh, YP., Blackwell, M. (eds) Fungal Associations. The Mycota, vol 9, Springer Cham. https://doi.org/10.1007/978-3-031-41648-4_2

Rama T, Quandt CA (2021) Improving fungal cultivability for natural products discovery. Front Microbiol 12:2673. https://doi.org/10.3389/fmicb.2021.706044

Raudaskoski M, Viitanen H (1982) Effect of aeration and light on fruit body induction in *Schizophyllum commune*. Trans Br Mycol Soc 78(1):89–96

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Huelsenbeck JP (2012) MrBayes 3.2: efficient bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61(3):539–542. https://doi.org/10.1093/sysbio/sys029

Saensuk T, Suntararak S (2018) Mycelial cultivation of *Astraeus* Sp. From Khao Kra–Dong Volcano Forest Park on Agar Medium in Laboratory. Life Sci Environ J 19(2):234–242

Scrase R (1995) Cultivating mushrooms—from pure culture to spawn production. Mycologist 9(2):53–56

Suwannasai N, Dokmai P, Yamada A, Watling R, Phosri C (2020) First ectomycorrhizal syntheses between *Astraeus sirindhorniae* and *Dipterocarpus alatus* (Dipterocarpaceae), pure culture characteristics, and molecular detection. Biodiversitas 21(1):231–238. https://doi.org/10.13057/biodiv/d210130

Thomas PW, Vazquez I-B (2022) A novel approach to combine food production with carbon sequestration, biodiversity and conservation goals. Sci Total Environ 806:151301. https://doi.org/10.1016/j.scitotenv.2021.151301

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 172:4238–4246

Vishal V, Munda SS, Singh G, Lal S (2022) Hidden Earthstar Diversity in the Jharkhand State of India. In: Rajpal, V.R., Singh, I., Navi, S.S. (eds) Fungal diversity, ecology and control management. Fungal Biology Springer Singapore 135–164. https://doi.org/10.1007/978-981-16-8877-5_9

Watling R (1963) Germination of basidiospores and production of fructifications of members of the agaric family Bolbitiaceae using herbarium material. Nature 197(4868):717–718

Watling R (1981) How to identify mushrooms to genus. V. Cultural and development features. Mad River. pp, Eureka, CA

Watling R (2008) A manual and source book on the boletes and their allies (Vol. 24). Fungiflora. Oslo, Norway pp 13–14

Webster J, Weber R (2007) Introduction to fungi, 3rd edn. Cambridge University Press, UK, pp 487–588

White T, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications. Academic, San Diego, pp 315–322

Wilson AW, Binder M, Hibbett DS (2011) Effects of gasteroid fruiting body morphology on diversification rates in three independent clades of fungi estimated using binary state speciation and extinction analysis. Evol Int J Org Evol 65(5):1305–1322. https://doi.org/10.1111/j.1558-5646.2010.01214.x

Yamada A, Katsuya K (1995) Mycorrhizal association of isolates from sporocarps and ectomycorrhizas with *Pinus densiflora* seedlings. Mycoscience 36:315–323. https://doi.org/10.1007/BF02268607