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NOTES ON GEOGRAPHIC DISTRIBUTION

Check List 18 (3): 553–562
https://doi.org/10.15560/18.3.553

PENSOFT

First records of *Tylopilus glutinosus* Iqbal Hosen (Boletaceae) from *Shorea robusta*-dominated forests in tropical India: morphological description and phylogenetic estimation

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Abstract

During the routine survey for exploring the hidden macrofungal wealth of tropical Sal (*Shorea robusta* Roth) forests in West Bengal and Jharkhand (India), we found a specimen similar to *Tylopilus plumbeoviolaceous* (Snell & E.A.Dick) Snell & E.A.Dick. After careful morphological observations and phylogenetic analysis, the species was found to be conspecific with *Tylopilus glutinosus* Iqbal Hosen, a recently established taxon from Bangladesh. We report *T. glutinosus* for the first time from India and provide a detailed description, figures, a multigene phylogenetic analysis, and comprehensive comparisons with similar species. A distributional map is also provided.

Keywords

Dipterocarpaceae, macrofungi, 2-loci phylogram, taxonomy

Academic editor: Ajay Kumar Gautam | Received 10 March 2022 | Accepted 20 May 2022 | Published 2 June 2022

Citation: Chakraborty D, Gelardi M, Hembrom ME, Ghosh A (2022) First records of *Tylopilus glutinosus* Iqbal Hosen (Boletaceae) from *Shorea robusta*-dominated forests in tropical India: morphological description and phylogenetic estimation. Check List 18 (3): 553–562. https://doi.org/10.15560/18.3.553

Introduction

One of the most diverse groups of mushrooms is the family Boletaceae, and a genus in this family, *Tylopilus* P. Karst., also exhibits a remarkable morphological diversity. Molecular phylogeny has shown that *Tylopilus* is polyphyletic; a number of species previously assigned to it have been reassigned to newly described genera like *Zangia* Yan C. Li & Zhu L. Yang, *Harrya* Halling, Nuhn & Osmundson, *Sutorius* Halling, Nuhn & Fechner, and *Australopilus* Halling & Fechner and species complexes were detected (Binder and Hibbet 2006; Li et al. 2011; Halling et al. 2012a, 2012b; Nuhn et al. 2013; Wu et al. 2014). Molecular study of some representatives of *Tylopilus* s.s. reveals species complexes such as *Tylopilus balloui* (Peck) Singer or T. *plumbeoviolaceous* (Snell and E.A. Dick) Singer (Halling et al. 2008; Gelardi et al. 2015, 2019; Chakraborty et al. 2018).

Tropical Sal forests (*Shorea robusta* Roth; Dipterocarpaceae) in India harbor a many mushroom species

(Dutta et al. 2015; Khatua et al. 2015; Kumar and Atri 2016; Verma and Pandro 2018), but the diversity of boletes are, unfortunately, little explored. Few species of boletes have been reported from India (Parihar et al. 2014, 2018; Verma and Pandro 2018). During macrofungal surveys to several *Shorea robusta*-dominated forests in West Bengal and Jharkhand, India, we collected several basidiomata allied to *Tylopilus plumbeoviolaceus* (Snell & E.A.Dick) Snell & E.A.Dick. Critical macro- and micromorphological characterization, coupled with combined nrITS and nrLSU-based phylogenetic studies, revealed our specimens to be *T. glutinosus* Iqbal Hosen, a recently established taxon from Bangladesh (Iqbal Hosen 2021), which had not been recorded from India until now. Here, we report *T. glutinosus* in India for the first time, based on a detailed morphological description and a multigene (nrITS + nrLSU) phylogenetic inference.

Methods

We collected fresh young to mature basidiomata during surveys to various areas of Jharkhand and West Bengal during the monsoon season, July and August, in 2019–2021. Macromorphological characters were recorded in the field or at basecamp from fresh and dissected fruitbodies. We photographed basidiomata in the field with a Sony DSC-RX100 camera. Colour codes and terms mostly follow Kornerup and Wanscher (1978). Samples were dried in a field drier. We observed micromorphological characters of freehand sections of dried materials mounted in a solution of 5% KOH, 1% Phloxin, and 1% ammoniacal Congo red with an Olympus CX 41 compound microscope. Drawings of the anatomical features were made with a drawing tube at 1000× magnification. Microscopic photographs were taken with an Olympus BX 53 camera. The basidiospores were measured in lateral view. Basidiospore measurements and length/width ratios (Q) are recorded as: minimum–mean–maximum. Basidium length excludes the length of sterigmata. Herbarium codes follow Thiers (2021). The distributional map (Fig. 1) was produced in ArcGIS v. 10.5.

DNA extraction, PCR amplification and sequencing. Genomic DNA was extracted from 100 mg of dried basidiomes with the InstaGeneTM Matrix Genomic DNA isolation kit (Biorad, USA) following the manufacturer's instructions. The nrITS and nrLSU genes regions were amplified with primer pairs ITS-1F and ITS-4 (White et al. 1990; Gardes and Bruns 1993) and LR0R and LR7 (Vilgalys and Hester 1990), respectively. PCR amplification was performed on a thermal cycler (Eppendorf, Germany) programmed for 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 30 s at 52 °C, 2 min at 72 °C, and a final 7 min extension step at 72 °C. The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany). Both strands of the PCR fragment were sequenced on the 3730xl DNA Analyzer (Applied Biosystems, USA) using the amplifying primers. The sequence quality was checked using Sequence Scanner Software v. 1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious v. 5.1 (Drummond et al. 2010). The newly generated sequences in this study were submitted to GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). Accession numbers of species used in the phylogenetic analysis are listed in Table 1.

Sequence alignment and phylogenetic analysis. The newly generated nrITS and nrLSU sequences of *T. glutinosus* from India and its close relatives were retrieved from the nBLAST search against GenBank (https://www.ncbi.nlm.nih.gov/genbank, accessed on 2022-2-22), UNITE database (https://unite.ut.ee, accessed on 2022-2-22), and relevant published phylogenies (Gelardi et al. 2015, 2019; Chakraborty et al. 2018; Hosen 2021). Two datasets (ITS and LSU) were created separately. Both the datasets were aligned separately using the online version of MAFFT v. 7 with the L-INS-i strategy (Katoh et al. 2019) with default settings and then trailing ends of the alignment trimmed manually with MEGA v. 7 (Kumar et al. 2016). To eliminate ambiguously aligned positions in the alignment as objectively as possible, Gblocks 0.91b (Talavera and Castresana 2007) was used. The program was run with settings allowing for smaller blocks, gaps within these blocks and less strict flanking positions. Species delimitation was first examined using single locus phylogenies. When significant conflict was not observed among the single locus phylogenies, we concatenated all single locus alignments into one multilocus dataset using BioEdit v. 7.2 (Hall 1999). The concatenated dataset was then phylogenetically analyzed using the maximum-likelihood (ML) and Bayesian-inference (BI) methods. For the ML analysis, the concatenated alignment was carried out using raxmlGUI v. 2.0 (Edler et al. 2021) with the GTRGAMMA substitution model. The ML analysis was executed applying the rapid bootstrap algorithm with 1000 replicates to obtain nodal support values. For BI, the 2-loci dataset was divided into four partitions: ITS1, 5.8S, ITS2, and LSU. PartitionFinder2 was used to find the appropriate partitioning scheme and substitution models for each partition (SYM+I+G for ITS1, SYM for 5.8S, GTR+G for ITS2 and GTR+I+G for 28S) using the Akaike information criterion (AICc) with a greedy search over all models (Lanfear et al. 2016). BI was computed in MrBayes v. 3.2.6 (Ronquist et al. 2012) with four Markov chain Monte Carlo (MCMC) chains for 1,000,000 iterations until the standard deviation of split frequencies reached below the 0.01 threshold. Trees were sampled every 100th generation. The first 25% of trees were discarded as burn-in. The convergence of runs was visually assessed using the Trace function in Tracer v. 1.6 (Rambaut et al. 2014). Maximum-likelihood bootstrap (MLB) values ≥70% and Bayesian posterior-probabilities (BPP) values ≥0.95 are shown in the phylogenetic tree.

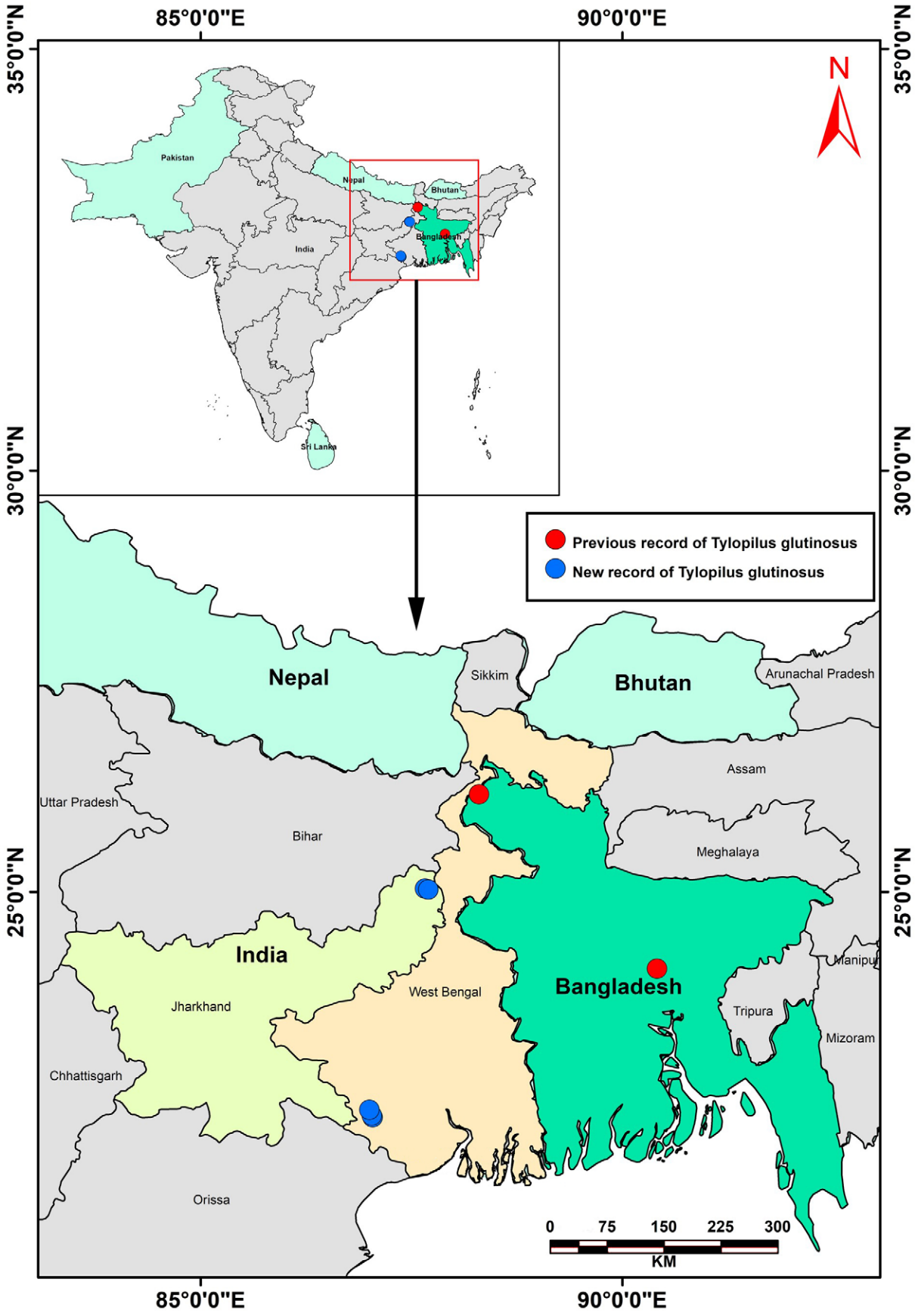

Figure 1. Geographic distribution of *Tylopilus glutinosus*: first collections records (red circle) from Bangladesh and new records in India (blue circle).

Table 1. A list of species, voucher no., GenBank accession no. and reference of species used in this study.

Species	Voucher no.	GenBank accession no.		Reference
		nrITS	nrLSU	
Porphyrellus porphyrosporus	MB97-023	DQ534643	DQ534563	Gelardi et al. 2015
Tylopilus aff. *balloui*	HKAS 59700	—	KF112458	Chakraborty et al. 2018
T. argillaceus	HKAS90186	—	KT990589	Wu et al. 2016
T. argillaceus	HKAS90201	—	KT990588	Wu et al. 2016
T. atroviolaceobrunneus	HKAS84351	—	KT990625	Wu et al. 2016
T. badiceps	MB03-052	—	KF030336	Gelardi et al. 2015
T. badiceps	78206	—	KF030335	Gelardi et al. 2015
T. balloui	FMNH 1073250 (F)	—	EU430733	Chakraborty et al. 2018
T. balloui	T.W. Osmundson 1198 (NY)	—	EU430740	Chakraborty et al. 2018
T. balloui	R.E. Halling 8292 (NY)	—	EU430734	Halling et al. 2008
T. balloui	R.E. Halling 8521 (NY)	—	EU430735	Chakraborty et al. 2018
T. balloui	REH9467	—	JX889676	Chakraborty et al. 2018
T. balloui	TH8593	—	HQ161872	Chakraborty et al. 2018
T. balloui	T. W. Osmundson 1132 (NY)	—	EU430739	Chakraborty et al. 2018
T. balloui	118-121	AB509625	—	Gelardi et al. 2015
T. balloui	118-393	AB509735	—	Gelardi et al. 2015
T. dunensis	FLOR 51718	MF113419	MF113428	Gelardi et al. 2019
T. felleus	F PRL5803MAN	GQ166878	—	Chakraborty et al. 2018
T. felleus	F PRL5803	GQ166904	—	Chakraborty et al. 2018
T. felleus	18	JN182869	—	Chakraborty et al. 2018
T. felleus	Q38	KC414274	—	Gelardi et al. 2015
T. felleus	HKAS54926	—	HQ326933	Chakraborty et al. 2018
T. felleus	TM03 453	—	EU522827	Chakraborty et al. 2018
T. ferrugineus	MB06-053	—	JQ326994	Gelardi et al. 2015
T. formosus	PDD 72637	HM060320	HM060319	Gelardi et al. 2015
T. glutinosus	HKAS 75116	MZ351437	—	Hosen 2021
T. glutinosus	HKAS 81369	MZ351438	MZ351442	Hosen 2021
T. glutinosus	**AGDC_21-14**	**OM903877**	**OM899731**	**This study**
T. glutinosus	**NPDF917-45**	—	**MW675784**	**This study**
T. glutinosus	**NPDF917-60**	—	**MW675802**	**This study**
T. griseipurpureus	MG521a	KM975484	KM975493	Gelardi et al. 2015
T. griseipurpureus	HKAS90200	—	KT990624	Wu et al. 2016
T. griseipurpureus	USMBo3	KF442407	—	Gelardi et al. 2015
T. griseipurpureus	Songkhla	JQ726597	—	Gelardi et al. 2015
T. himalayanus	DC 17-25	MG799322	MG799328	Chakraborty et al. 2018
T. himalayanus	DC 17-31	MG799323	MG799326	Chakraborty et al. 2018
T. microsporus	HMAS:84730	KM975485	KM975494	Gelardi et al. 2015
T. neofelleus	DC 16-63	MG777523	MG777525	Chakraborty et al. 2018
T. neofelleus	DC 16-64	MG777524	MG777529	Chakraborty et al. 2018
T. neofelleus	YT20121007	KM975488	KM975496	Gelardi et al. 2015
T. neofelleus	YT20120811	KM975487	KM975495	Gelardi et al. 2015
T. neofelleus	HKAS50319	—	HQ326936	Gelardi et al. 2015
T. oradivensis	R.E. Halling 8087	—	EU430731	Chakraborty et al. 2018
T. otsuensis	HKAS:53401	—	KF112449	Gelardi et al. 2015
T. plumbeoviolaceoides	GDGM:42624	—	KM975498	Chakraborty et al. 2018
T. plumbeoviolaceoides	GDGM21040-1	—	KT990640	Wu et al. 2016
T. plumbeoviolaceoides	GDGM21040-2	—	KT990641	Wu et al. 2016
T. plumbeoviolaceus	MB06-056	—	KF030350	Gelardi et al. 2015
T. plumbeoviolaceus	NYBG:0009	KY432830	KY432825	GenBank
T. pseudoballoui	DC 17-30	MG799329	MG799327	Chakraborty et al. 2018
T. pseudoballoui	DC 17-35	MG799324	MG799325	Chakraborty et al. 2018
T. rubrobrunneus	BD329	—	HQ161876	Chakraborty et al. 2018
T. rubrobrunneus	152/98	—	DQ534629	Chakraborty et al. 2018
T. sp.	MG509a	KM975492	KM975499	Gelardi et al. 2015
T. vinaceipallidus	HKAS90184	—	KT990703	Wu et al. 2016
T. violaceobrunneus	HKAS89443	—	KT990702	Gelardi et al. 2019
T. violatinctus	HKAS:50208	—	KF112472	Gelardi et al. 2015
T. violatinctus	HKAS50279	—	HQ326935	Gelardi et al. 2015

Results

Phylogenetic inferences. Both ML and BI analyses produced the same topology; therefore, only the Bayesian trees with both MLBS and BPP values are shown (Fig. 2). The ITS data matrix comprised a total of 26 sequences; the alignment comprised 446 characters. The LSU matrix consisted of 50 sequences; the alignment comprised 831 characters. The 2-loci (ITS + LSU) final dataset consisted of 58 sequences including our consensus sequence for each species. The final alignment comprised 1277 characters including gaps.

Our phylogenetic analysis using nrITS and nrLSU genes shows that our Indian *Tylopilus glutinosus* (vouchers AGDC_21-14, NPDF917-45, and NPDF917-60) are nested (with strong support; MLBS = 100%, BPP = 1)

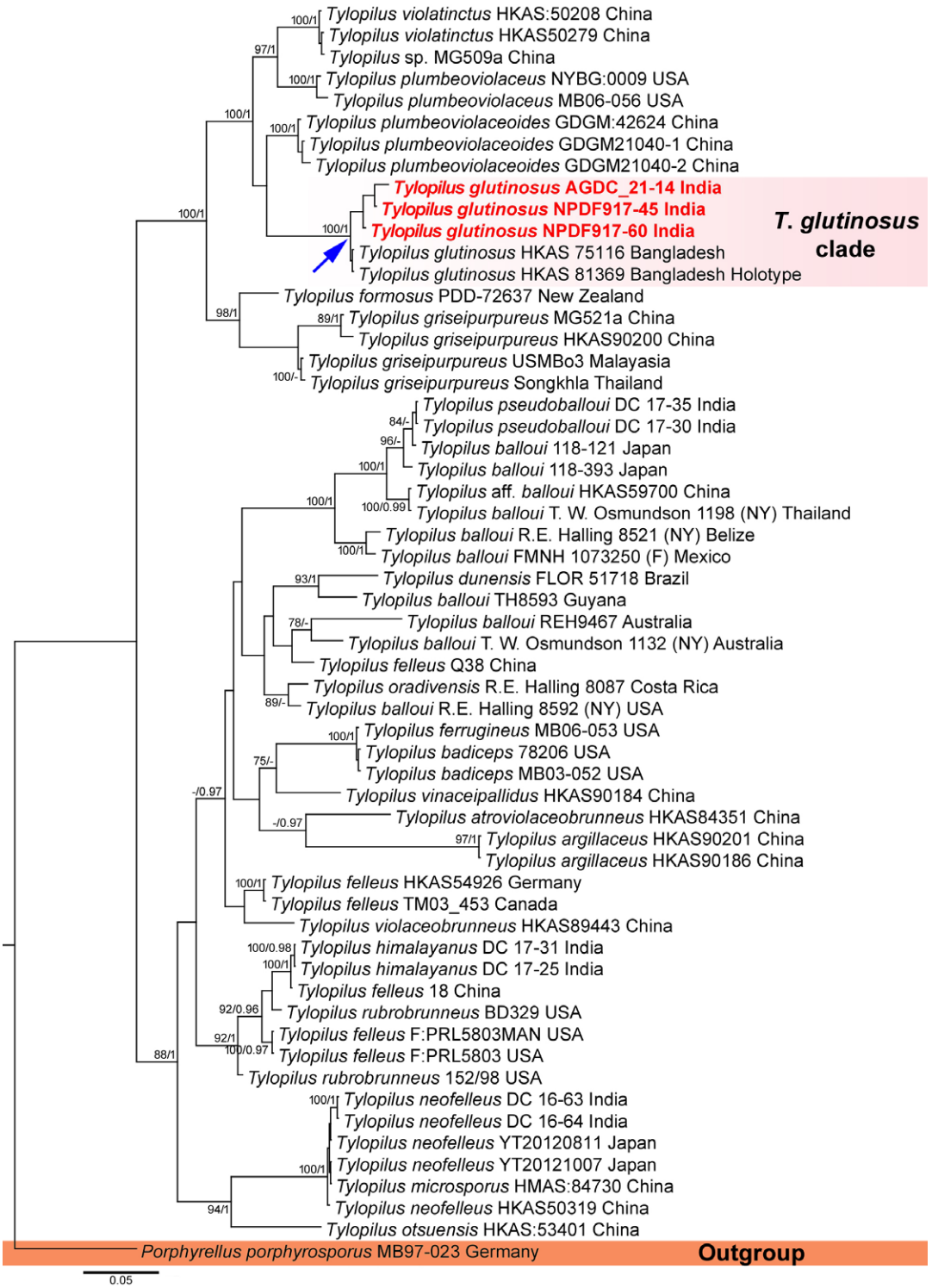

Figure 2. Maximum-likelihood (ML) and Bayesian (BI) phylogram inferred from raxmlGUI 2.0 on a concatenated dataset of nrITS and nrLSU sequence data of *Tylopilus* s.s. species. *Porphyrellus porphyrosporus* is used as outgroup taxon. Support values in either the ML Bootstrap percentage or BI posterior probabilities values are indicated. MLB ≥70% are shown on the left of "/" and BPP ≥0.95 are shown on the right above or below the branches at nodes. Three Indian collections of *Tylopilus glutinosus* are highlighted in red and bold font in the phylogram.

within the *T. glutinosus* clade consisting of sample vouchers HKAS 75116 and HKAS 81369) collected from Bangladesh.

***Tylopilus glutinosus* Iqbal Hosen**
Figures 3, 4

Material examined. INDIA – **West Bengal** • Jhargram district, Tuluha; 22°19′44″N, 087°02′39″E; alt. 80 m; on soil in a forest dominated by *Shorea robusta*; 13.VIII.2020; D. Chakraborty leg.; (NPDF917-45) • Jhargram district, Chandra; 22°21′01″N, 087°02′00″E; alt. 90 m; on soil in a forest dominated by *S. robusta*; 13.VIII.2020; D. Chakraborty leg.; (NPDF917-60) • Jhargram district, Jhargram city; 22°25′01″N, 087°00′14″E; alt. 103 m; on soil in a forest dominated by *S. robusta*; 12.VIII.2021; A. Ghosh, D. Chakraborty leg.; AGDC_21-14 – **Jharkhand** • Rajmahal hills, Sahibganj district, Borio block, Dhogoda paharia cemetery, north of Teenpahar-Borio road, Dhogada-paharia burial ground forest; 25°02′24″N, 087°39′36″E; alt. 110 m; on soil in a forest dominated by *S. robusta*; 12.VII.2019, M.E. Hembrom leg.; (MEH-19-22) • Taljhari block, Brindaban Joshkuti reserve forest; 25°01′52″N, 087°42′17″E; alt. 63 m; on soil in a forest dominated by *S. robusta*; 12.VII.2019; M.E. Hembrom leg.; (MEH-19-28).

Identification. Pileus 6–110 mm in diameter, initially subglobose then convex; surface dry but viscid when wet, matte to subvelvety, greyish brown (7D3), brown to purple brown (14D3–D2), paler towards margin, fading to ash grey (1B–C2) with age; margin entire, decurved with a narrow flap of tissue. Pore surface greyish orange (6B3–B2) when young, greyish brown (6C3) with age, unchanging on bruising; pores angular, stuffed when young. Tubes adnate, 3–4 mm long, grey whitish (4C1), unchanging on bruising. Stipe 14–140 × 8–37 mm, mostly subclavate with wider base or broadly cylindrical, solid, greyish magenta to dark purple (14E3–F4), white towards base; surface with faint longitudinal striations, without reticulum. Veil absent. Basal mycelium white. Context up to 16 mm thick in the pileus, milky white (1A2), unchanging when exposed; no color change with 5% KOH, $FeSO_4$, and 10% NH_4OH. Odour mushroomoid. Taste bitter. Spore print not obtained.

Basidiospores 7–8.9–10 × 3.7–4.1–4.5 µm, (n = 30; Q = 1.8–2.17–2.47), elongated to fusiform, inequilateral, thin-walled, smooth under light microscope. Basidia 27–33 × 7–9 µm, 4-spored, clavate. Pleurocystidia 35–60 × 8–12 µm, emergent up to 15 µm from the hymenial palisade, fusoid to ventricose with granular refractive content, some are deeply rooted into hymenial trama. Tube edge fertile. Cheilocystidia 40–48 × 8–11 µm, common, mostly subfusoid to ventricose. Hymenophoral trama divergent, hyphae septate, gelatinous, up to 5 µm wide. Pileipellis an ixotrichoderm, up to 280 µm thick, composed of erect, somewhat interwoven, brown pigmented hyphae; terminal elements 20–60 × 5–18 µm, cylindrical to subcylindrical, sometimes subfusoid, content brown pigmented. Stipitipellis a cutis, up to 80 µm thick, made up of subparallel repent hyphae. Caulocystidia 26–45 × 8–11 µm, completely pigmented or only partly pigmented; caulobasidia present. Clamp connections absent.

Habit and habitat. Mostly gregarious to subcaespitose, growing in association with *Shorea robusta* in tropical deciduous forests.

Discussion

Our collections of *Tylopilus glutinosus* from India morphologically alike to the species, as recently described from Bandladesh (Iqbal Hosen 2021). However, our material differs from the type collection in having longer pleurocystidia, 35–60 versus 30–45 µm according to Hosen (2021). Our 2-loci phylogenetic results confirm sequences from our Indian samples are nested within the *T. glutinosus* clade (Fig. 2, indicated with an arrow) from Bangladesh with strong support (MLBS= 100%, BPP= 1). Morphologically, *T. plumbeoviolaceoides* T.H. Li, B. Song & Y.H. Shen, *T. plumbeoviolaceous*, *T. violaceobrunneus* Y.C. Li & Zhu L. Yang, *T. vinosobrunneus* Hongo, *T. atroviolaceobrunneus* Y.C. Li & Zhu L. Yang, *T. atripurpureus* (Corner) E. Horak., and *T. alboater* (Schwein.) Murrill resemble *T. glutinosus*, but all of them differ by their mycorrhizal association with members of the Fagaceae. Although these species may be close relatives of *T. glutinosus*, they are not contaxic, as *T. glutinosus* associates with *Shorea* in tropical regions of Bangladesh and India. Our phylogenetic reconstruction places *T. glutinosus* sister to the Chinese *T. plumbeoviolaceoides*, which morphologically differs by its pale pink to flesh-pink or pale vinaceous tan-colored tubes and pore surfaces and the subpruinose stipe (Li et al. 2002). Another morphologically similar taxon, the American *T. plumbeoviolaceus*, can also be separated from this Asian lookalike by its larger basidiomata (pileus 3–15 cm in diameter), the amber-orange and pale brownish amber reactions with KOH and NH_4OH on the pileus surface, respectively, and longer basidiospores (10–13 µm long) (Bessette et al. 2010). *Tylopilus violaceobrunneus* can also be distinguished from this species by its reddish-brown to brownish-violet pileus and distinctly reticulate stipe, especially at apex (Wu et al. 2016). *Tylopilus vinosobrunneus* is separated by its reticulate stipe at apex and pallid tubes that turns wood-brown when bruised (Chen et al. 2004). Similarly, *T. atroviolaceobrunneus* is another morphologically related species which differs from *T. glutinosus* in turning reddish in the context and tubes on injury and in having longer basidiospores (10–13 µm long) (Wu et al. 2016). *Tylopilus alboater* can easily be distinguished in the field by its robust pileus (3–15 cm in diameter), purple-black to dark purple pileus, and mild taste (Bessette et al. 2010; Wu et al. 2016). *Tylopilus atripurpureus* differs from *T. glutinosus* by its purple-black to dark purple pileus, longer pleurocystidia (75–100 µm long), and palisadoderm pileipellis (Horak 2011; Gelardi et al. 2015). *Tylopilus neofelleus* Hongo, which

Figure 3. *Tylopilus glutinosus*. **A–D.** Fresh basidiomata in the field and basecamp. **E.** Cross section through pileipellis. **F.** Terminal cells of the pileipellis. **G.** Caulocystidia. **H.** Pleurocystidia and basidia. **I.** Tube edge showing cheilocystidia. **J.** Basidiospores. Scale bars: E = 100 µm; F–J = 10 µm.

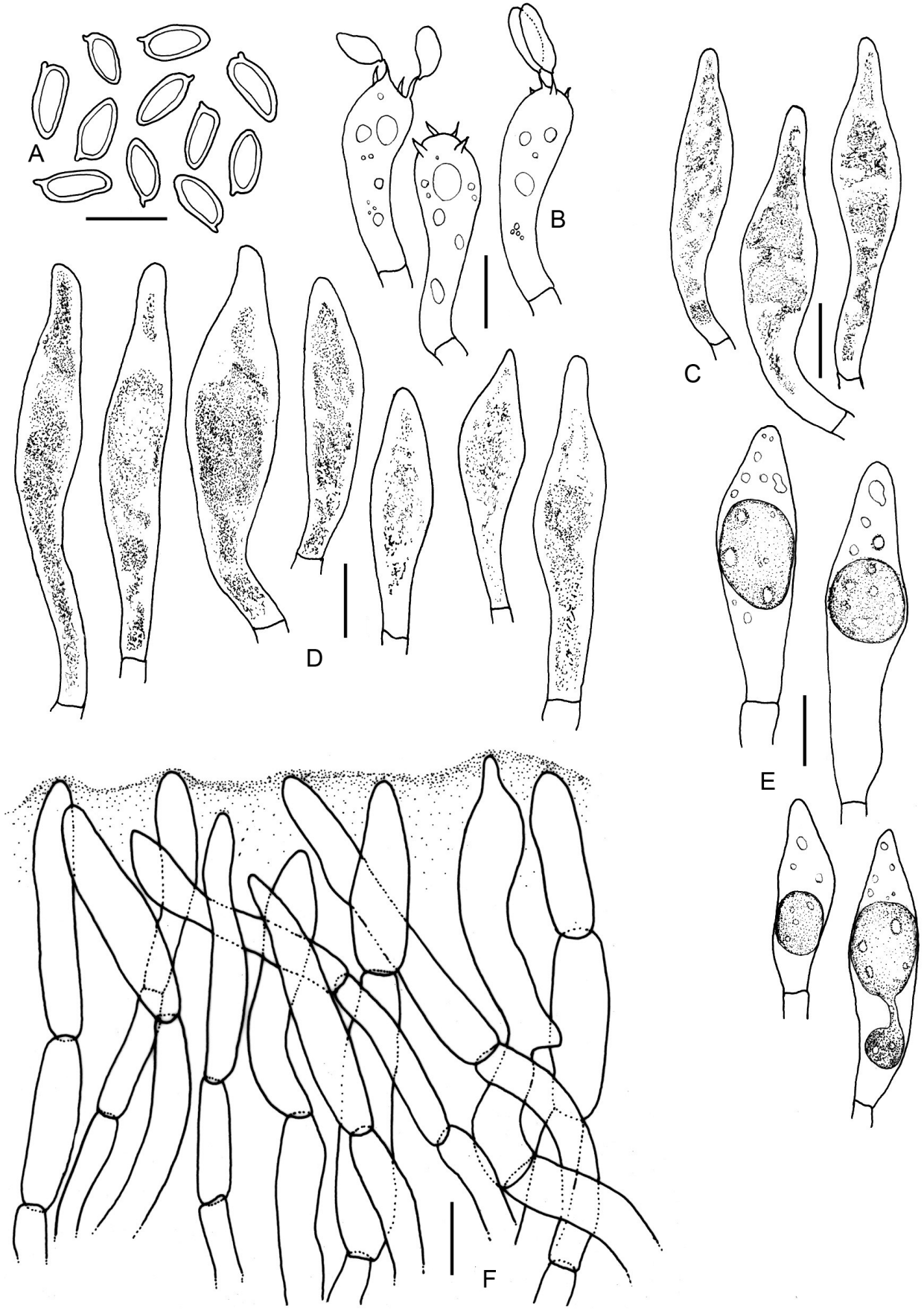

Figure 4. *Tylopilus glutinosus*. **A.** Basidiospores. **B.** Basidia. **C.** Cheilocystidia. **D.** Pleurocystidia. E. Caulocystidia. **F.** Cross section through pileipellis. Scale bars = 10 µm.

has also been reported from India, is distinguished from *T. glutinosus* by the pale violet reticulum on the upper part of the stipe (Chakraborty et al. 2018; Gelardi et al. 2019).

Acknowledgements

We are grateful to the Botanical Survey of India, Kolkata, and the Acharya Jagadish Chandra Bose Indian Botanic Garden, Howrah, for providing facilities. Dyutiparna Chakraborty and Aniket Ghosh are thankful to the Science and Engineering Research Board (SERB) for providing the National Post-Doctoral Research Fellowship (NPDF/2019/000917 and PDF/2021/000183, respectively). Ishika Bera is thanked for assisting AG, DC, and MEH in the field.

Authors' Contributions

Conceptualization: DC. Data curation: DC, AG. Formal analysis: MG, AG. Funding acquisition: DC. Investigation: DC. Methodology: DC, MG, AG. Software: AG. Supervision: DC. Validation: MG. Writing – original draft: DC. Writing – review and editing: MEH, DC, AG.

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