



## *Inocybe griseorubida*, a new species of Pseudosperma clade from tropical India

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### Abstract

*Inocybe griseorubida* sp. nov. is described from Kerala State, India. A comprehensive description, photographs, and comparisons with phenetically similar species are provided. The nuclear ribosomal internal transcribed spacer region (ITS), a portion of the nuclear ribosomal large subunit (nLSU) and a portion of the nuclear second-largest subunit of RNA polymerase II (*rpb2*) gene of this species were sequenced and analyzed. Phylogenetic analysis of *rpb2* sequences confirmed both the novelty of the species and its placement within the Pseudosperma clade.

**Key words:** Agaricales, Basidiomycota, Inocybaceae, phylogeny, taxonomy

### Introduction

*Inocybe* (Fries 1821: 254) Fries (1863: 346) (Inocybaceae, Agaricales, Basidiomycota, Fungi) is a large genus represented by about 500 species world-wide (Kirk *et al.* 2008; Ryberg *et al.* 2010). The genus is noted for both its ectomycorrhizal ecology and the toxicity of several of its species (Kosentka *et al.* 2013). The genus is macroscopically characterized by mostly brownish or rarely whitish basidiomata occasionally with a purplish or lilac hue and sometimes with characteristic odors, a fibrillose-rimose or squamulose pileus, brownish lamellae, a brown spore-print, and a fibrillose-pruinose stipe at times with a distinct marginate-bulbous base. Microscopically, this genus is characterized by smooth, warty, nodulose or spinulose basidiospores lacking a true germ pore. The lamellae of several species exhibit metuloidal cystidia often with a crystalloid deposit at the apex. Several species are devoid of metuloidal cystidia and they are characterized by copious, thin-walled cheilocystidia (Matheny 2005; Larsson *et al.* 2009). Molecular phylogenetic analyses of *Inocybe* using ribosomal and protein coding genes have confirmed that *Inocybe* is monophyletic with several divergent lineages existing within it (Matheny *et al.* 2002; Matheny 2005; Matheny *et al.* 2009).

Kerala State is located in the south-west corner of Peninsular India and has a tropical, maritime and monsoonal climate. Most of the forested areas of Kerala are parts of the Western Ghats, a mountain range, which is one of the biodiversity hotspots of the world that has been included in the World Heritage list. To date, nineteen species of *Inocybe* have been reported from this region (Farook *et al.* 2013). In the course of our studies on the genus *Inocybe* of Kerala, we came across a remarkable species of *Inocybe* that has a grayish red pileus with a well-developed velipellis and thin- to slightly thick-walled, versiform cheilocystidia often covered with a resinous substance towards the apex. It is described here as a new species belonging to the Pseudosperma clade.

### Materials and Methods

#### *Morphological studies*

Conventional morphology-based taxonomic methods as well as molecular methods were employed. Light microscopic studies were made on thin sections of dried material stained with 1% aqueous solutions of Congo red and mounted in 3% aqueous KOH. For evaluation of the range of spore size, 20 basidiospores each from one specimen of each collection cited were measured. The hilar appendix is included in the basidiospore length. Basidiospore measurements include both the mean and the standard deviation for both the length and the width, together with the range of spore quotient (Q, the length/width ratio) and its mean value (Q<sub>m</sub>). Alphanumeric color codes from both Kornerup & Wanscher

(1978) (e.g., 12E4) and the Online Auction Color Chart (Anonymous 2004) (e.g., OAC448) accompany color names in the description. The examined collections are deposited at Central National Herbarium (CAL), Kolkotha, India and the CAL accession numbers (e.g., CAL 1253) are provided.

#### DNA extraction, PCR and sequencing

The ITS, nLSU and *rpb2* gene regions were analyzed in this study. Genomic DNA was extracted from dried specimens (holotype: CAL 1253) of the new *Inocybe* species employing the procedure described by Izumitsu *et al.* (2012). PCR reactions were performed with the primer pairs ITS1 & ITS4 for ITS (White *et al.* 1990), LROR & LR7 for nLSU (Vilgalys & Hester 1990) and b6f & b7.1R for *rpb2* (Matheny 2005). The amplification reaction mixture (final volume 30 µL) contained the following: 15 µL of EmeraldAmp GT PCR Master Mix, 6 µL of ddH<sub>2</sub>O, 3 µL of 10 µM primers (ITS1-ITS4; LROR-LR7; b6f-b7.1R) and 3 µL of template DNA. PCR reactions were performed in a GeneAtlas™ Thermal cycler (Astec, Fukuoka, Japan). Thermal profile of PCR for ITS and nLSU were 2' 95°C, 1' 50°C, 1' 72°C; 34 cycles of 30" 94°C, 1' 50°C, 1' 72°C and a final extension step of 10' 72°C. PCR thermal profile followed for *rpb2* was: 5' 95°C; 30 cycles of 1' 95°C, 1.30' 53°C, 1' 72°C and a final extension step of 10' 72°C. The PCR products were examined on 1.0% agarose gel, stained with ethidium bromide and visualized under a UV transilluminator. Amplified PCR products were purified using column purification (GeneJet™ PCR Purification Kit, Thermo Fisher Scientific, Mumbai, India) as per manufacturer's guidelines and were subjected to automated DNA sequencing on ABI3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using the same primers used for PCR. The generated sequences were edited manually using BioEdit sequence alignment editor v.7.0.9.0 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA). The edited sequences were then used for BLAST search in the GenBank database (www.ncbi.nlm.nih.gov). The newly generated sequences were deposited in GenBank (ITS: KT180326; nLSU: KT180327; *rpb2*: KT180328).

#### Alignment assembly and phylogenetic analysis

The molecular phylogenetic analysis was performed using *rpb2* sequences. Initial BLAST searches of the GenBank database were performed on 10 June 2015 to identify related taxa, for which sequences were available. The newly generated *rpb2* (761 bp) sequence of the newly discovered *Inocybe* species along with those retrieved from GenBank (61 sequences) were aligned using MUSCLE v.3.8.31 (Edgar 2004). A final dataset of *rpb2* sequences of 62 taxa including one outgroup (Table 1) was manually edited in AliView v.1.15 (Larsson 2014). The sequences of *Inocybe* species were selected based on BLASTn search similarity indices with an E value of zero; query coverage of >99% and >90% sequence identity. Available sequences of the species of Pseudosperma clade (Kropp *et al.* 2013) and those of representative species of the remaining six clades of Inocybaceae (Alvarado *et al.* 2010; Matheny *et al.* 2012) were also added to the dataset. *Tubaria vinicolor* (Peck 1909: 334) Ammirati, Matheny & Vellinga (2007: 580) was selected as an outgroup taxon for rooting purpose following Matheny *et al.* (2012). Maximum likelihood analysis was conducted using RAxML v.8.0.26 (Stamatakis 2014) employed in raxmlGUI v.1.3.1 (Silvestro & Michalak 2012), implementing GTR+G model with 1000 rapid ML bootstrap replicates. GTR+G model of molecular evolution was selected using an online model selection tool of TOPALi v2.5 (Milne *et al.* 2009). Bootstrap values ≥70% were considered significant. The aligned sequence dataset has been deposited in TreeBase (<http://purl.org/phylo/treebase/phyloids/study/TB2:S17814>). The phylogeny from ML analysis was displayed with FigTree 1.4.2 (Rambaut 2014).

**TABLE 1.** List of species, geographic origin and GenBank accession numbers of *rpb2* sequences used in the molecular analysis.

Species	Geographic origin	GenBank no.
<i>Auritella arenicolens</i>	Australia	KJ729920
<i>A. dolichocystis</i>	Australia	AY337371
<i>A. geoaustralis</i>	Australia	AY333774
<i>A. serpentinocystis</i>	Australia	AY333773
<i>Inocybe adaequata</i>	Finland	AY333771
<i>I. aestivalis</i>	Utah	EU600846
<i>Inocybe</i> aff. <i>renispora</i> E7054	Australia	JQ421069
<i>Inocybe</i> aff. <i>renispora</i> E7042	Australia	EU555465
<i>Inocybe</i> aff. <i>rimosa</i>	Papua New Guinea	JQ421070
<i>Inocybe</i> aff. <i>sororia</i>	Costa Rica	JQ421076
<i>I. breviterincarnata</i> BK28080407	USA	EU555450

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**TABLE 1.** (Continued)

Species	Geographic origin	GenBank no.
<i>I. breviterincarnata</i> SAT0712518	USA	JQ421066
<i>I. calamistrata</i>	USA	AY333764
<i>I. calospora</i>	Sweden	AY337365
<i>I. candipes</i>	USA	AY337366
<i>I. chelanensis</i>	USA	AY337368
<i>I. cercocarp</i>	USA	EU600889
<i>I. corydalina</i>	Belgium	AY337370
<i>Inocybe</i> cf. <i>rimosa</i>	USA	JQ421071
<i>I. dulcamara</i>	Mexico	AY388644
<i>I. flocculosa</i>	Norway	AY337375
<i>I. geophylla</i>	Finland	AY333777
<b><i>I. griseorubida</i></b>	<b>India</b>	<b>KT180328</b>
<i>I. godeyi</i>	Italy	AY337378
<i>I. heimii</i>	Italy	AY337380
<i>I. hystrix</i>	Finland	AY337381
<i>I. lacera</i>	USA	KM245991
<i>I. lanatodisca</i>	USA	JQ846480
<i>I. lanuginosa</i>	USA	KM245992
<i>I. leptocystis</i>	Finland	AY337386
<i>I. leucoblema</i>	—	AY333310
<i>I. maculata</i>	USA	EU569863
<i>I. malenconii</i>	Finland	EU569869
<i>I. misakaensis</i>	Zambia	AY333767
<i>I. napipes</i>	Norway	AY337390
<i>I. niveivelata</i>	USA	AY333776
<i>I. occidentalis</i> PBM525	USA	AY333775
<i>I. occidentalis</i> BK27089703	USA	EU600892
<i>I. obsoleta</i>	USA	EU600849
<i>I. praetervisa</i>	USA	AY337392
<i>Inocybe</i> sp. IND02	India	KM656098
<i>Inocybe</i> sp. MCA562	Japan	JQ421077
<i>Inocybe</i> sp. PC96073	Zambia	EU600869
<i>Inocybe</i> sp. PBM2863	USA	JQ846495
<i>Inocybe</i> sp. PBM2983	USA	JQ421078
<i>Inocybe</i> sp. TR49_05	Papua New Guinea	JQ421079
<i>Inocybe</i> sp. ZT8944	India	EU600902
<i>Inocybe</i> sp. ZT9250	India	EU600904
<i>I. spuria</i>	USA	EU600867
<i>I. sororia</i> ADW0057	USA	JQ421072
<i>I. sororia</i> ADW0063	USA	JQ421073
<i>I. sororia</i> PBM3042	USA	JQ421074
<i>I. sororia</i> PBM3055	USA	JQ421075
<i>I. sororia</i> SAT0427801	USA	JQ846492
<i>I. stellatospora</i>	USA	AY337403
<i>I. terrigena</i>	Finland	AY333309
<i>I. umbrinella</i>	USA	JQ846497
<i>I. unicolor</i>	USA	AY337409
<i>Tubaria vinicolor</i>	—	DQ536418
<i>Tubariomyces hygrophoroides</i>	France	GU907090
<i>T. inexpectatus</i>	Spain	GU907088
<i>Tubariomyces</i> sp. 2 BB6018	Zambia	EU600886

## Results

### Taxonomy

***Inocybe griseorubida*** K. P. D. Latha & Manim., *sp. nov.* Figs. 1A–F; Figs. 2A–F

*Etymology*:—The specific epithet (Latin) refers to the grayish red pileus of this species.

*Diagnosis*:—This species is characterized by a grayish red pileus with a well-developed, grayish, sericeous velipellis; ellipsoid, oblong or subphaseoliform basidiospores ( $7\text{--}13 \times 5\text{--}7 \mu\text{m}$ ); versiform cheilocystidia often with a resinous substance towards the apex; an ecological association with Dipterocarpaceae; and is phylogenetically distinguished from other species of *Inocybe*.

*Holotype*:—INDIA. Kerala State: Ernakulam District, Perumbavoor, Iringolkavu Sacred Grove, 31 July 2012, *K. P. Deepna Latha*, DKP8 (CAL 1253).

*Description*:—Basidiomata small. Pileus 5–23 mm diam., almost parabolic when very young, becoming convex, finally plano-convex with a shallow central depression or occasionally with a small umbo surrounded by a slight depression; surface grayish red (12E4–5/OAC448) when very young, becoming grayish (12E4/OAC504) at the center, grayish red (12D3, 12D4/OAC505) elsewhere, appressed-fibrillose, radially rimose towards the margin; grayish, sericeous, well-developed velipellis observed in all collections, which covers the pileus surface and often dense at the center; margin initially incurved, becoming decurved to slightly reflexed with age, appendiculate when very young, becoming crenate or somewhat wavy, slightly fissile. Lamellae emarginate with a small decurrent tooth, moderately crowded, initially orange-gray (6B2/OAC669), becoming reddish gray (7B2/OAC599), up to 3 mm deep, with lamellulae of 3 lengths; edges crisped, whitish. Stipe 13–37  $\times$  2.2–5 mm, central, terete, equal or slightly tapering towards the apex, cartilaginous, solid; surface reddish gray (12C2/OAC591) when very young, becoming grayish rose (12B3/OAC562), fading reddish white (12A2) towards the apex with age, with appressed as well as slightly recurved fibrils all over, more so towards the apex; base slightly enlarged, not bulbous, whitish. Context soft, up to 3 mm wide, off-white. Odor and taste not distinctive.

Basidiospores  $(7)8\text{--}12(13) \times 5\text{--}6.5(7) (9.6\pm 0.1.2 \times 5.4\pm 0.5) \mu\text{m}$ ,  $Q = 1.2\text{--}2.2$ ,  $Q_m = 1.8$ , smooth, ellipsoid to slightly oblong or subphaseoliform or occasionally with a slight median constriction, slightly thick-walled, pale brown or pale yellowish brown. Basidia 20–47  $\times$  6–17  $\mu\text{m}$ , clavate, thin-walled, hyaline or pale yellow, 4-spored; sterigmata up to 6  $\mu\text{m}$  long. Pleurocystidia absent. Lamella edge sterile or occasionally heterogeneous. Cheilocystidia 25–66  $\times$  8–19  $\mu\text{m}$ , abundant, versiform: clavate, broadly clavate, cylindrical, cylindro-clavate, ovoid, oblong, narrowly fusiform, narrowly utriform or with a capitate or subcapitate apex, rarely septate, often coated with a resinous substance especially towards the apex, thin- to slightly thick-walled, hyaline, occasionally with tiny amorphous contents. Lamellar trama subregular; hyphae 3.5–10  $\mu\text{m}$  wide, inflated up to 27  $\mu\text{m}$ , thin-walled, hyaline or very pale yellow. Subhymenium inconspicuous. Pileus trama subregular; hyphae 4–20, thin-walled, hyaline or pale yellow. Pileipellis a disrupted cutis with supra- and subpellis regions; suprapellis composed of compact, closely septate, non-encrusted hyphae (elements 22–40  $\times$  8–10  $\mu\text{m}$ ), with a pale violet plasmatic pigment; subpellis made up of both narrow and inflated, parallel hyphae (6–32  $\mu\text{m}$  wide) with dense, brown or yellowish brown, external spiral encrusting pigment. Stipitipellis a cutis often disrupted with loose, entangled, superficial covering of hyphae especially towards the apex; hyphae 4–15  $\mu\text{m}$  wide, thin- to slightly thick-walled, with a pale violet plasmatic pigment and faint hyaline encrustations, sometimes with yellowish brown amorphous contents. Caulocystidia absent. Oleiferous hyphae observed in all trama. Clamp connections present on all hyphae.

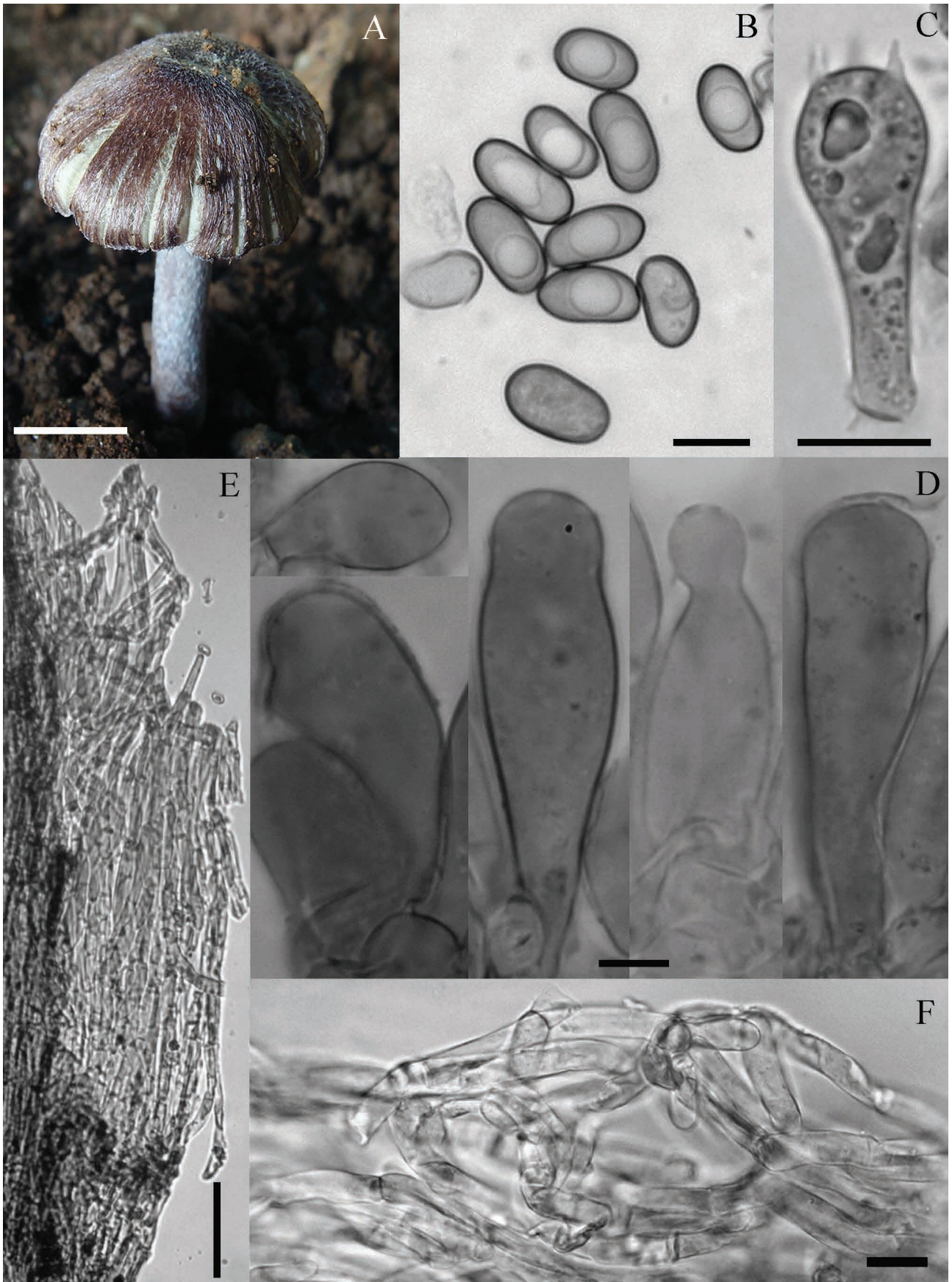
*Habitat*:—in small groups, on soil, near *Vateria indica* L. (Dipterocarpaceae, broad-leaved) trees.

*Geographical distribution range*:—known only from the type locality in Kerala State, India.

*Additional specimens examined*:—INDIA. Kerala State: Ernakulam District, Perumbavoor, Iringolkavu Sacred Grove, 30 July 2012, *K. P. Deepna Latha*, DKP7 (CAL 1254); 26 August 2014, *K. P. Deepna Latha*, DKP269 (CAL 1255).

*Comments*:—The authors could not find a species description in the literature that matches exactly with *Inocybe griseorubida*. Comparison of the ITS (678 bp), nLSU (870 bp) and *rpb2* (761 bp) sequences derived from *I. griseorubida* with the nucleotide sequences of taxa available in GenBank indicate that the derived ITS, nLSU and *rpb2* sequences of *I. griseorubida* are distinct. Based on BLASTn search using the ITS sequence, no close hit was obtained with an e-value of zero. An unnamed species, *Inocybe* sp. IND02 was the closest hit in BLASTn search for both nLSU (GenBank KP171060; Identities = 833/873 (95 %), Gaps = 10/873 (1 %)) and *rpb2* (GenBank KM656098; Identities = 701/750 (93 %), Gaps = 2/750 (0 %)) sequences. *Inocybe* sp. IND02 is an undescribed species from Kerala, the details of which are not available for comparison.





**FIGURE 1.** A–F: *Inocybe griseorubida* (CAL 1253, holotype). A. Basidioma; B. Basidiospores; C. Basidium; D. Cheilocystidia; E. Pileipellis; F. Stiptipellis. Scale bars: A=10 mm; B–F=10  $\mu$ m. Photos: By K.P.D. Latha.

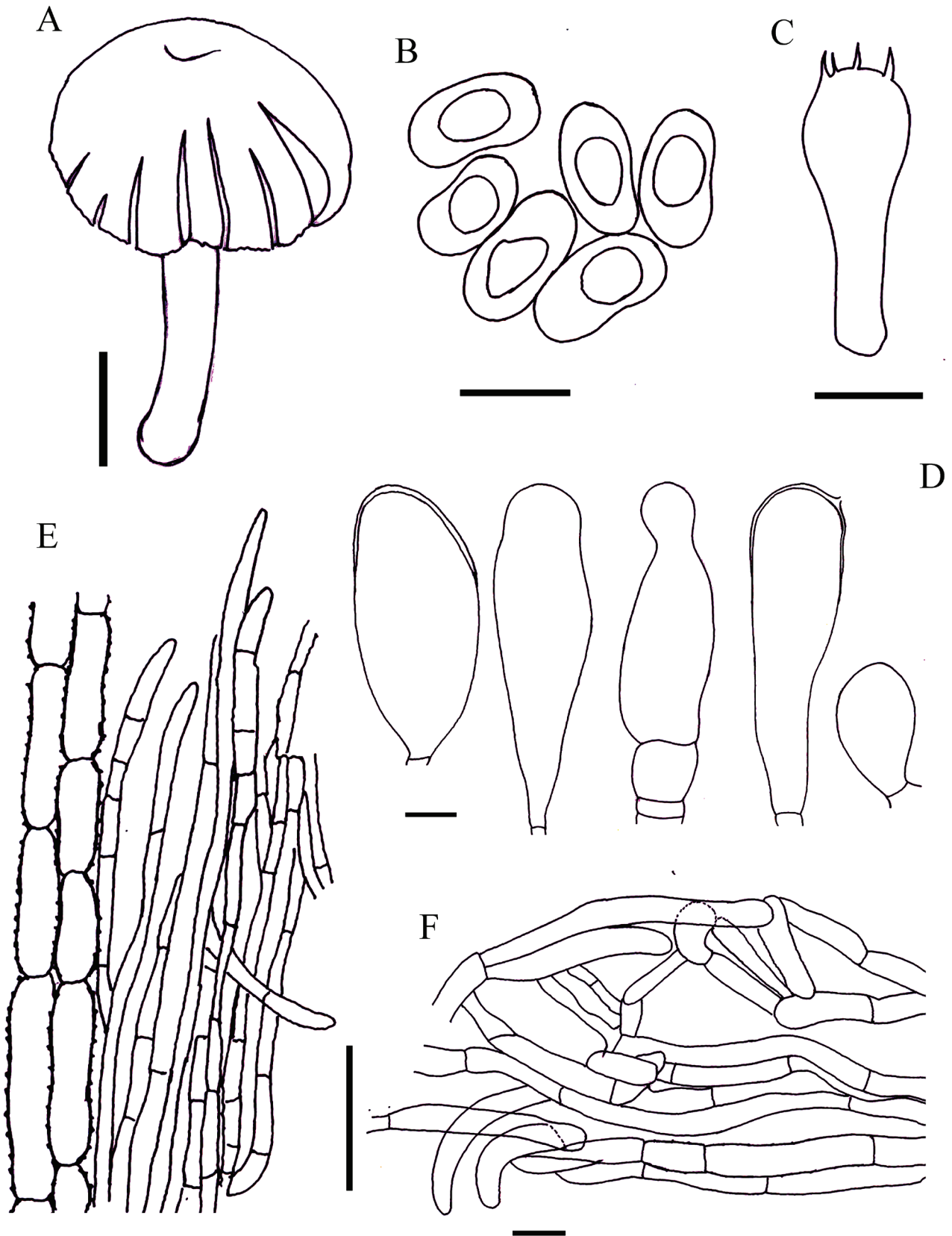
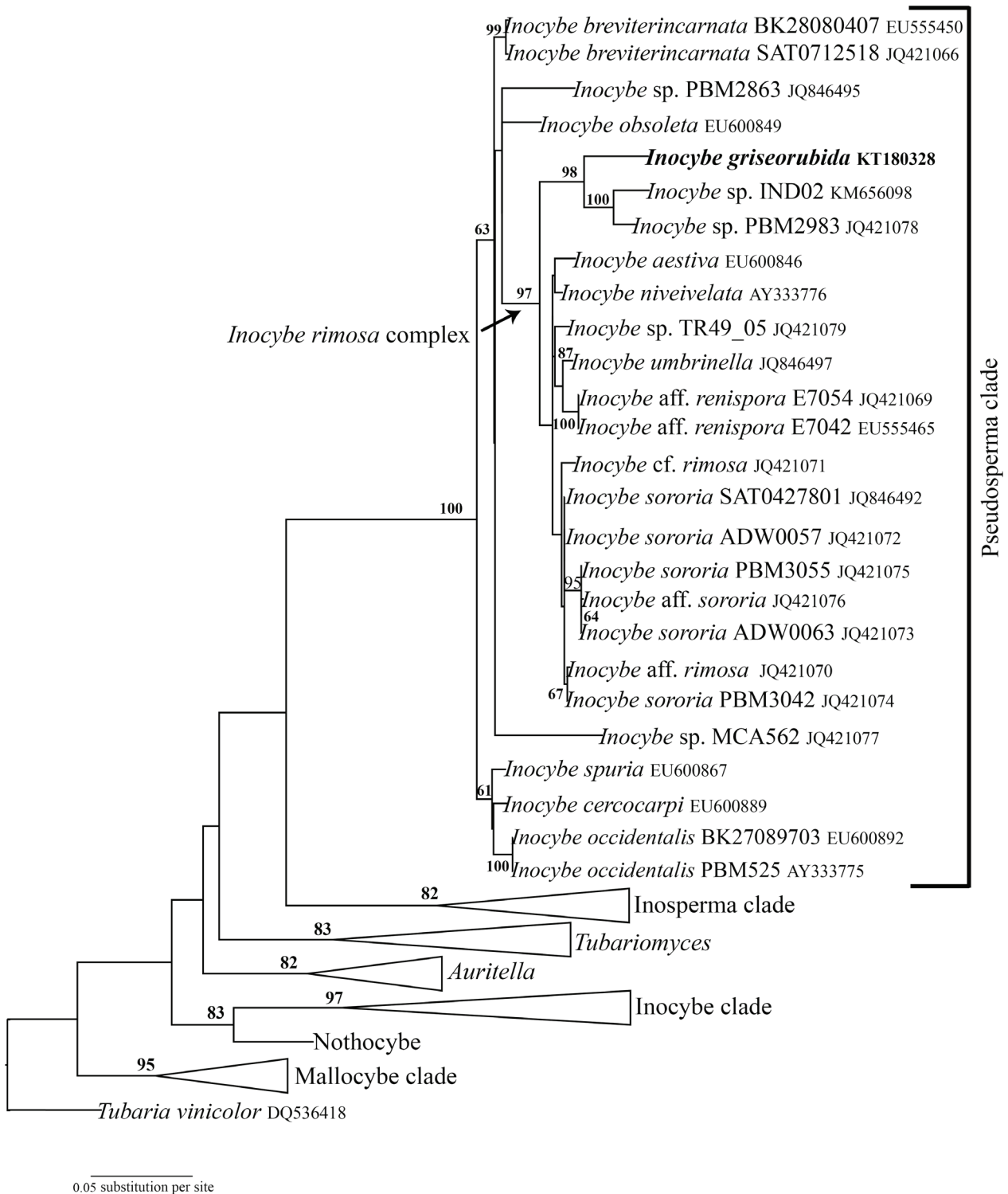


FIGURE 2. A–F: *Inocybe griseorubida* (CAL 1253, holotype). A. Basidioma; B. Basidiospores; C. Basidium; D. Cheilocystidia; E. Pileipellis; F. Stipitipellis. Scale bars: A=10 mm; B–F=10 µm. Drawings: By K.P.D. Latha.

Phylogenetic analysis generated a tree that showed the relative placement of *I. griseorubida*. The phylogeny was inferred from maximum likelihood (ML) analysis which is shown in Fig. 3. The ML analysis of the *rpb2* dataset placed

*I. griseorubida* in the Pseudosperma clade with full ML bootstrap support (100% BS). In the Pseudosperma clade, *I. griseorubida* nested in the *I. rimosa* complex with significant ML bootstrap support (97% BS). Within this complex, *I. griseorubida*, *Inocybe* sp. IND02 and *Inocybe* sp. PBM2983 clustered together as a discrete clade. Within this clade, the present species, *I. griseorubida* formed a lineage distinct from other two taxa of that clade with high ML bootstrap support (98% BS).



**FIGURE 3.** ML tree of *rpb2* sequences showing the placement of *I. griseorubida* within the Pseudosperma clade. Values above or below branches indicate bootstrap proportions. BS values  $\geq 50\%$  are shown. GenBank accession numbers are given after the name of each taxon.



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