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Insight into the "*Sutorius eximius* species complex" with concordant genealogies and morphology to unveil the novel species *Sutorius apleurocystidiatus* (Boletaceae) from India

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ABSTRACT: Although India belongs to one of the 17 megadiverse countries of the world, little is known about its ectomycorrhizal fungi in general, and Boletaceae in particular. In the present communication, with the combined approach of multigene molecular phylogeny and morphological study, the diversity within *Sutorius eximius* sensu lato has been revealed and has supported the recognition of at least eight species in this complex. Moreover, a new species in this complex namely, *Sutorius apleurocystidiatus*, is also proposed and described from India with morphological details and molecular phylogeny.

KEY WORDS: Boletales, macrofungi, molecular phylogeny, new species, *Sutorius alpinus*, *Sutorius apleurocystidiatus* taxonomy.

INTRODUCTION

Immense anthropogenic pressure followed by environmental catastrophe has dramatically impacted on biodiversity patterns causing extinctions of many species before they are explored and discovered. Fungi, representing the second largest Kingdom (after animals) of eukaryotes (Hawksworth and Lücking, 2017; Antonelli *et al*., 2023), are no exception of this. Boletaceae, one of the dominating mushroom former ectomycorrhizal families of fungi in Indian Himalaya, is expanding rapidly with combination of multigene molecular phylogeny and morphology (Das *et al*., 2024). Our attempts to understand, uncover, assess, conserve, and restore their biodiversity, however, are obstructed due to our limited manpower and taxonomic knowledge. *Sutorius eximius* (Peck) Halling, Nuhn & Osmundson [synonyms: *Boletus eximius* Peck, *Boletus robustus* Frost, *Ceriomyces eximius* (Peck) Murrill, *Leccinum eximium* (Peck) Singer, *Tylopilus eximius* (Peck) Singer] is well-known with morphology and molecular phylogenetic research and is characterized by purplish-brown velvety pileus, densely scabrous-dotted purplish-grey stipe, tobacco-brown hymenophore and reddish-brown spore print (Both, 1993; Halling *et al*., 2012).

The genus *Sutorius* Halling, Nuhn & Fechner was erected to accommodate *Boletus eximius* Peck and accordingly, the new combination *Sutorius eximius* (Peck) Halling, Nuhn & Osmundson had been proposed (Halling *et al*., 2012). Earlier *Boletus eximius* Peck was also proposed as a *nomen novum* (new name) by Peck (1887) for North American collection of *Boletus robustus* Frost (1874) non Fries (1851) (Halling *et al*., 2012). Later, Snell and Dick (1970), Smith and Thiers (1971) and Grund and Harrison (1976) also described this taxon based on North American collections. As the holotype (original collection from North America) was not cited, Halling (1983) designated a lectotype to an original specimen from Frost's collection (from North America). Description was then upgraded by Bessette *et al*., (2000) and Roody (2003) as "*Tylopilus eximius*", Halling and Mueller (2005) as "*Leccinum eximium*", and finally Bessette *et al*., (2016) as *Sutorius eximius*. Gradually, the name (*Sutorius eximius*/*Boletus eximius*) had been applied for morphological look-alikes from Costa Rica, China, Papua New Guinea (*B. nigroviolaceus* Heim), Japan and China (Hongo, 1973, 1975, 1979, 1980; Imazeki and Hongo, 1989; Teng, 1996; Halling *et al*., 2012; Wu *et al*., 2016). These patterns of distributions were justified by calling them as morphological disjuncts (Halling *et al*., 2012). However, based on our multigene molecular phylogenetic analysis with the available sequence data (ITS, 28S, *rpb*2 and *tef* 1-α) it is well evident that *S. eximius*, as previously highlighted by Halling *et al*., (2012) and Vadthanarat *et al*., (2021), is not a single widespread species or disjunct, but a complex of multiple closely related species (*S. eximius* sensu lato/*S. eximius* species complex) where *S. eximius* sensu stricto has more limited geographical distribution i.e. North America. Other lookalikes in this complex represent different species and are from different continents or countries. In this context, a thorough morphological examination of two recent collections from Uttarakhand (India) followed by their molecular phylogenetic estimation enabled us to uncover a novel species in *Sutorius eximius* species complex. Accordingly, *S. apleurocystidiatus* is proposed herein as a novel species with morphological details and multigene molecular phylogenetic inferences.

Table 1. ***Sutorius*** and allied sequences used in the phylogenetic analysis of this study. Newly sequenced collections are in bold.

Sl. No.	Species name (as in GenBank)	Voucher No.	Country	GenBank accession numbers			
				ITS	28S	*rpb2*	*tef 1-α*
1.	*Sutorius alpinus* Type	HKAS 52672	China		KF112399	KF112802	KF112207
2.	*Sutorius microsporus*	S.D.Yang010	China	MH885359	MH879697		MH879727
3.	*Sutorius microsporus*	HKAS 56291	China		KF112400	KF112803	KF112208
4.	*Sutorius microsporus* Type	HKAS 68720	China		NG_088137		
5.	*Sutorius australiensis*	REH9280	Australia		JQ327005		JQ327031
6.	*Sutorius eximius*	TWO986	Costa Rica		JQ327009		JQ327028
7.	*Sutorius australiensis*	REH9441	Australia		JQ327006	MG212652	JQ327032
8.	*Sutorius eximius*	JLF2547	Unites States	KC812313	KC812314		
9.	*Sutorius eximius*	NY:02449711	Unites States		MK601813	MK766369	MK721167
10.	*Sutorius eximius*	REH9400	Unites States		JQ327004	MG212653	JQ327029
11.	*Sutorius alpinus*	HKAS50420	China		KT990549	KT990387	KT990750
12.	*Sutorius alpinus*	HKAS59657	China		KT990707	KT990505	KT990887
13.	*Sutorius pseudotylopilus*	Wu499	China		MT154774		
14.	*Sutorius pseudotylopilus*	Wu938	China		MT154775		
15.	*Sutorius pseudotylopilus*	SV0401	Thailand			MN067502	MN067486
16.	*Sutorius pseudotylopilus*	SV0415	Thailand			MN067503	MN067487
17.	*Sutorius subrufus*	N.K.Zeng3043	China	MH885360	MH879698	MH879745	MH879728
18.	*Sutorius subrufus*	N.K.Zeng3045	China	MH885361	MH879699	MH879746	MH879729
19.	*Sutorius obscuripellis* Type	OR0949	Thailand			MN067510	MN067494
20.	*Sutorius obscuripellis*	Wu2070	China		MT154772		MW165273
21.	*Sutorius ubonensis* Type	SV0032	Thailand			MN067507	MN067491
22.	*Sutorius ubonensis*	SV0203	Thailand			MN067508	MN067492
23.	*Sutorius rubinus*	OR0403	Thailand			MN067504	MN067488
24.	*Sutorius rubinus*	OR0409	Thailand			MN067505	MN067489
25.	*Sutorius pachypus*	SV0098	Thailand			MN067501	MN067485
26.	*Sutorius pachypus* Type	OR0411	Thailand			MN067500	MN067484
27.	*Sutorius mucosus* Type	OR0851	Thailand			MN067499	MN067483
28.	*Sutorius maculatoides*	OR0758	Thailand			MN067498	MN067481
29.	*Sutorius maculatoides*	OR0626	Thailand				MN067480
30.	*Sutorius* sp.	ADK4369	Togo			MN067512	MN067496
31.	*Sutorius* sp.	ADK2396	Zimbabwe			MN067511	MN067495
32.	*Sutorius vellingae* Type	ECV3603	Thailand		JQ327000		JQ327033
33.	*Pulveroboletus ravenelii*	REH2565	United States			KU665637	KU665636
34.	*Pulveroboletus* sp.	HKAS 57665	China			KF112715	KF112264
35.	*Neoboletus venenatus*	HKAS63535	China			KT990448	KT990807
36.	*Neoboletus tomentulosus*	HKAS77614	China			KT990445	KT990805
37.	*Suillellus subamygdalinus*	HKAS:53641	China			KT990478	KT990841
38.	*Crocinoboletus rufoaureus*	HKAS53424	China			KF112710	KF112206
39.	***Sutorius apleurocystidiatus* Type**	**KD 23-015**	**India**	**PP838747**	**PP838811**	**PP855517**	**PP855518**
40.	***Sutorius apleurocystidiatus***	**KD 23-017**	**India**	**PP840340**	**PP838750**	**PP855519**	**PP855520**
41.	*Sutorius eximius*	REH8594	Costa Rica		JQ327008		JQ327027
42.	*Sutorius eximius*	TWO995	Costa Rica		JQ327010		JQ327030
43.	*Sutorius eximius*	NY:1393562	Costa Rica			MK766383	MK721180
44.	*Sutorius eximius*	MyCoPortal_03817572	United States	MW899061			
45.	*Sutorius eximius*	HKAS91261	China			MT110444	
46.	*Sutorius pseudotylopilus* Type	OR0378B	Thailand			MH614787	MH614740
47.	*Sutorius* sp.	OR0379	Thailand			MH614788	MH614741
48.	*Sutorius* sp.	N.K.Zeng3297	China		MH87970		MH879731
49.	*Sutorius* sp.	JD669	Burundi				MN067497
50.	*Sutorius* sp.	TS-2021-8-5	China		OL998816		
51.	*Sutorius* sp.	JLF11199	United States	OP580495	OP578227		

MATERIAL AND METHODS

Morphological studies - A macrofungal survey to different forests of Chamoli district was undertaken during rainy season of 2023 (July to August) and several fresh basidiomata belonging to different species of

boletoid mushrooms were collected. Field observations and macromorphological characters were recorded in the field or in the basecamp. Images of the fresh and dissected basidiomata were duly captured with digital cameras: Canon Power Shot SX50 HS and Canon Power Shot SX220 HS. Colour codes and terminology used are primarily after Kornerup and Wanscher (1978). After noting all the macromorphological characters, dissected samples were placed for drying in an aluminium field drier. Macrochemical colour tests were also noted by applying $FeSO_4$, 5% KOH and/or NH_4OH on surface and context of pileus and stipe from fresh basidiomata. Micromorphological characters were observed in the laboratory after mounting freehand sections of dried samples in a solution of 5% KOH, 1% Phloxin, and 1% ammoniacal Congo red under an Olympus CX 41 compound microscope. Drawings of the micromorphological features were made with the help of drawing tube attached to Olympus CX 41 compound microscope at 1000× magnification. Micro-photographs were captured with a dedicated camera attached to an Olympus BX 53 microscope. The basidiospores were observed and measured in lateral view. Basidiospore measurements along with length/width ratios (Q) are recorded as: minimum–mean–maximum. Basidium length excludes that of sterigmata. Herbarium codes follow Thiers (continuously updated).

DNA extraction, PCR amplification and sequencing - Genomic DNA was isolated from 100 mg of dried basidiome with the HiPurA Fungal DNA Purification Kit (HIMEDIA) following the manufacturer's instructions mentioned on the Kit. The amplification (PCR) of four nuclear loci, the internal transcribed spacer (ITS1-5.8S-ITS2 = ITS), partial nuc 28S rDNA D1-D2 regions (28S), region between conserved domains 6 and 7 of second largest subunit of RNA polymerase II (*rpb*2), and translation elongation factor 1-α (*tef* 1) were done using the primer pairs ITS1-F and ITS4, LR0R and LR5, ef1-983F and ef1-1567R respectively (White *et al*., 1990; Liu *et al*., 1999; Gardes and Bruns, 1993; Matheny, 2005; Rehner and Buckley, 2005). Amplification for these loci were conducted in a ProFlex PCR system (Applied Biosystems) programmed for an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 30 sec, and extension at 72 °C for 1 min. The final extension was kept at 72 °C for 7 min. The amplified PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Germany). Both strands of the PCR fragment were sequenced on the ABI 3500 DNA Analyzer (Applied Biosystems, USA) using the amplifying primers. The sequence quality was checked and confirmed using Sequence Scanner Software ver. 1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro ver. 5.1 (Drummond *et al*., 2010). In this study, eight sequences (Two each for ITS, 28S, *rpb*2 and *tef* 1-α) were generated from two separate collections of *Sutorius apleurocystidiatus* (voucher nos. KD 23-015 and KD 23-017).

Phylogenetic analysis - ITS, 28S, *rpb*2 and *tef* 1-α sequences of the newly described *Sutorius* species plus close relatives were retrieved from BLASTn search against GenBank (https://www.ncbi.nlm.nih.gov/genbank) and relevant published phylogenies (Halling *et al*., 2012; Wu *et al*., 2016; Li *et al*., 2021; Li and Yang, 2021; Vadthanarat *et al*., 2021). Four raw datasets (ITS, 28S, *rpb*2 and *tef* 1-α) were prepared separately. All the datasets were aligned separately using the online version of the multiple sequence alignment program MAFFT v. 7 (https://mafft.cbrc.jp/alignment/software/) with L-INS-i strategy and normal alignment mode, respectively. The alignment was checked and trimmed with the conserved motifs manually with MEGA v. 7 (Kumar *et al*., 2016). Furthermore, four alignments (ITS, 28S, *rpb*2 and *tef* 1-α) were concatenated into multi-locus dataset using BioEdit v. 7.0.9 (Hall, 1999) and processed for the phylogenetic analyses. In the four-locus dataset (ITS+28S+*rpb*2+*tef* 1-α) of *Sutorius*, sequence lengths were determined to be 640 bp for ITS, 848 bp for 28S, 754 bp for *rpb*2 and 1196 bp for *tef* 1-α. The combined dataset was phylogenetically analysed using maximum likelihood (ML) method. The ML analysis was conducted using the IQ-tree tool version 2.2.2.6 (Nguyen *et al*., 2015), employing the best model for each locus chosen by ModelFinder (Kalyaanamoorthy *et al*., 2017). Additionally, ultrafast bootstrap with 1,000 replicates was applied to obtain nodal support values. Gaps in the alignment were treated as missing data in phylogenetic analyses. Our novel species is highlighted in the combined phylogenetic tree using bold red font. Maximum likelihood bootstrap (MLbs) values ≥70% are shown in the phylogenetic tree (Fig. 1).

RESULTS

Phylogenetic inferences

The four-gene phylogenetic (ML) tree is consistent and the phylogenetic analysis (Fig. 1) including the present novel species resolved the genus *Sutorius* as monophyletic with full support. It also reveals eight distinct species of *Sutorius* in "*Sutorius eximius* sensu lato" (*Sutorius eximius* species complex) out of which seven species namely, *S. pseudotylopilus* (from Thailand), *S. apleurocystidiatus* (from India and proposed here as new species), *Sutorius alpinus* (from China), *Sutorius microsporus* (from China), *Sutorius* sp. 1 (from Costa Rica), *Sutorius* sp. 2 (from China), *Sutorius* sp. 3 (from China) are different from the putative authentic *S. eximius* (*S. eximius* sensu stricto) from USA. The sequences derived from our Indian collections of *S. apleurocystidiatus* (KD 23-0015 and KD 23-017) clustered together with full support and appear to be sister

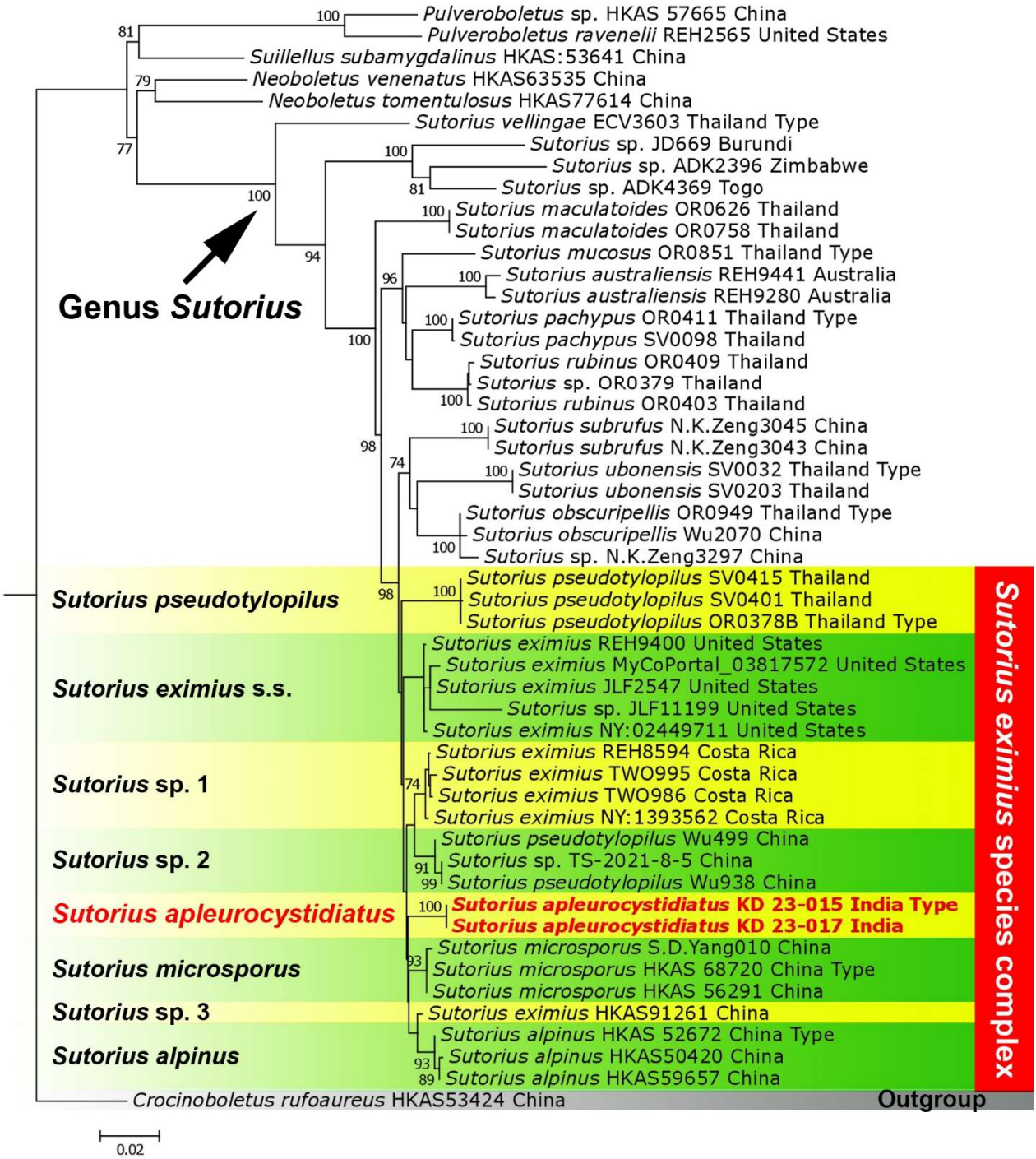

Fig. 1. Phylogram generated by maximum likelihood analysis based on combined sequence data of ITS, 28S, *rpb*2 and *tef* 1-α for *Sutorius apleurocystidiatus* and allied species. Maximum likelihood bootstrap support values (MLbs) ≥ 70% are shown above or below the branches at nodes. *Sutorius apleurocystidiatus* is placed in bold red font to highlight its phylogenetic positions in the tree. *Sutorius eximius* species complex is indicated with red shade on the right. The entire clade bearing the taxa belonging to the genus *Sutorius* is indicated with a black arrow.

to a clade containing *Sutorius alpinus* (represented by voucher numbers HKAS52672, HKAS50420 and HKAS59657) and *Sutorius* sp. 3 (HKAS91261) however, our species is recovered as a distinct species supporting its novelty.

Morphology

Sutorius apleurocystidiatus K. Das, Su.Datta, A. Ghosh & Vizzini, ***sp. nov.*** **Figs. 2 & 3**

MycoBank: MB 854149

***Typification*:** INDIA, UTTARAKHAND: Chamoli

District, Didna, in temperate forest under *Quercus* sp., 7 Aug 2023, alt. 2600 m, N 30°9′59″ E 79°38′19″, *Kanad Das*, *KD 23-015* (**holotype**! CAL 2005).

***GenBank*:** PP838747 (ITS, CAL 2005), PP840340 (ITS); PP838811 (28S, CAL 2005), PP838750 (28S); PP855517 (*rpb*2, CAL 2005), PP855519 (*rpb*2); PP855518 (*tef* 1-α, CAL 2005), PP855520 (*tef* 1-α).

***Diagnosis*:** Distinguished from the closely allied species *Sutorius alpinus* by viscid pileus and stipe, light brown to reddish brown unchanging pore surface, rounded pores, absence of pleurocystidia, smaller basidiospores and ITS, 28S, *rpb*2 and *tef* 1-α sequence data.

***Etymology*:** the specific epithet refers to the lack of pleurocystidia.

Description: Basidiomata medium to large. Pileus 105–120 mm in diam., hemispherical to applanate; reddish brown (9E5) at centre, more reddish towards mid (9E6–8) and paler towards margin, sometimes in combination with pink orange to light orange (5A3–4) and dull red (8B4) tinges along margin; surface even, subtomentose with matted fine squamules, sticky when wet, with a narrow sterile flap of tissue 1–1.5 mm wide. Hymenophore adnate, light brown to agate (7D–E6–8), unchanging when bruised; pores rounded, 2/mm; tubes 6.5–10 mm long, burnt red (7D8); unchanging when bruised. Stipe 70–75 × 16–20 mm, cylindrical to subclavate, greyish brown (8D–E3) to greyish red (10–11D4) with a whitish tinge, with dark brown dot-like squamules. Context in pileus yellowish white to orange white (4–5A2), up to 10 mm thick, becoming dark yellow (4C8) with KOH, greenish with $FeSO_4$; in stipe pale yellow (4A3) gradually light yellow (4A4), much later light orange, to greyish orange along margins (5A–B5), fleshy, becoming beige (4C3) to olive brown (4D4) with KOH, greenish grey to greyish green (27C2–3) with $FeSO_4$. Basal mycelium white forming a white band. Odour indistinct. Taste not recorded. Spore print not obtained.

Basidiospores 11.1–12.6–13.9 × 4.4–5.1–6.7 µm [Q = 1.92–2.52–3.13, n = 30], subfusoid, inequilateral in side view, smooth. Basidia 19–33 × 7–9 µm, clavate, 4-spored; sterigmata 2.5–4 × 1–1.5 µm. Pleurocystidia absent. Tube edge fertile, composed of cystidia and basidia. Cheilocystidia 25–33.5 × 4–8.5 µm, subcylindric, fusiform, ventricose to appendiculate. Subhymenium layer 5–10 µm wide; trama divergent, hyphae thin-walled, septate, gelatinous. Pileipellis 130–160 µm thick, submerged under a thin layer of gluten, a trichodermium, composed of thin-walled, septate, branched, erect to suberect hyphae forming a compactly arranged entangled mass, often in erect clusters at regular intervals forming the squamules of pileus; terminal cells of hyphae 19.4–25 × 4–10 µm, cylindric to subcylindric, subventricose to subfusiform, rarely inflated to clavate. Stipitipellis 50–70 µm thick, with a thin layer of gluten, hymeniform, composed of thin-walled, erect hyphae; terminal cells of hyphae 25–33 × 7–8 µm, cystidioid, bulbous, broadly clavate or ventricose. Caulobasidia, clavate to subclavate, 4-spored, similar to the hymenial basidia, infrequent. Clamp connections absent in all tissues.

Additional specimen examined: INDIA, UTTARAKHAND: Chamoli District, Didna, in temperate forest under *Quercus* sp., 7 Aug 2023, alt. 2600 m, N 30°9′59″ E 79°38′19″, *Kanad Das*, KD 23-017 (CAL 2006).

DISCUSSION

All the species in the "*S. eximius* species complex" including our novel species are morphologically quite close irrespective of their origin and distribution. However, *S. apleurocystidiatus* is distinct with the help of combination of morphological features: reddish brown slightly viscid pileus, brown unchanging pore surface and tubes, greyish stipe surface with brown dot-like squamules, yellowish white to orange-white pileus context that changes to greenish with $FeSO_4$, comparatively large basidiospores (11.1–12.6–13.9 × 4.4–5.1–6.7 µm), absence of pleurocystidia and occurrence under *Quercus* in temperate Himalayan forest.

Three recently established Asian species, viz. *S. alpinus* Yan C. Li & Zhu L. Yang, *S. microsporus* Yan C. Li & Zhu L. Yang, *S. pseudotylopilus* Vadthanarat, Raspé and Lumyong may easily be confused in the field with *S. apleurocystidiatus*. However, *S. alpinus* (originally reported from China and morphologically the closest relatives of *S. thindii*) is distinguished by a dry pileus surface, greyish red to dark reddish purple or reddish purple pore surface that stains reddish brown when bruised, purplish grey to dark purplish grey tubes, angular pores, presence of pleurocystidia, larger basidiospores (14–15.5 × 4.5–5.5 µm) and the occurrence in subtropical to alpine forests in China under coniferous trees belonging to Pinaceae (Li and Yang, 2021). Similarly, *S. microsporus* (also originally reported from China) appears very close to the Indian species by possessing similar size of basidiomata, reddish brown to orange brown pileus, pale brown to greyish brown stipe with dark brown to blackish brown dotted squamules, but the former differs in a dry pileus (non-viscid), pale reddish brown to violet-brown pore surface which changes to reddish brown when bruised, presence of pleurocystidia and occurrence in subalpine to alpine forests under coniferous trees of the Pinaceae (Li and Yang, 2021). *Sutorius pseudotylopilus* (originally reported from Thailand) has a purple (11F3–4) pore surface that becomes dark brown to brown (8–9F5–6 to 7F5–6) with age, greenish white stipe context at base, fusiform to broadly fusiform caulocystidia and occurrence in tropical (700–750 m) forests dominated by plants of the families Fagaceae and Pinaceae (Vadthanarat *et al*., 2021). Our 4-gene molecular phylogeny also clearly separates all these closely allied species from *S. apleurocystidiatus*. Moreover, our phylogenetic estimation strongly shows

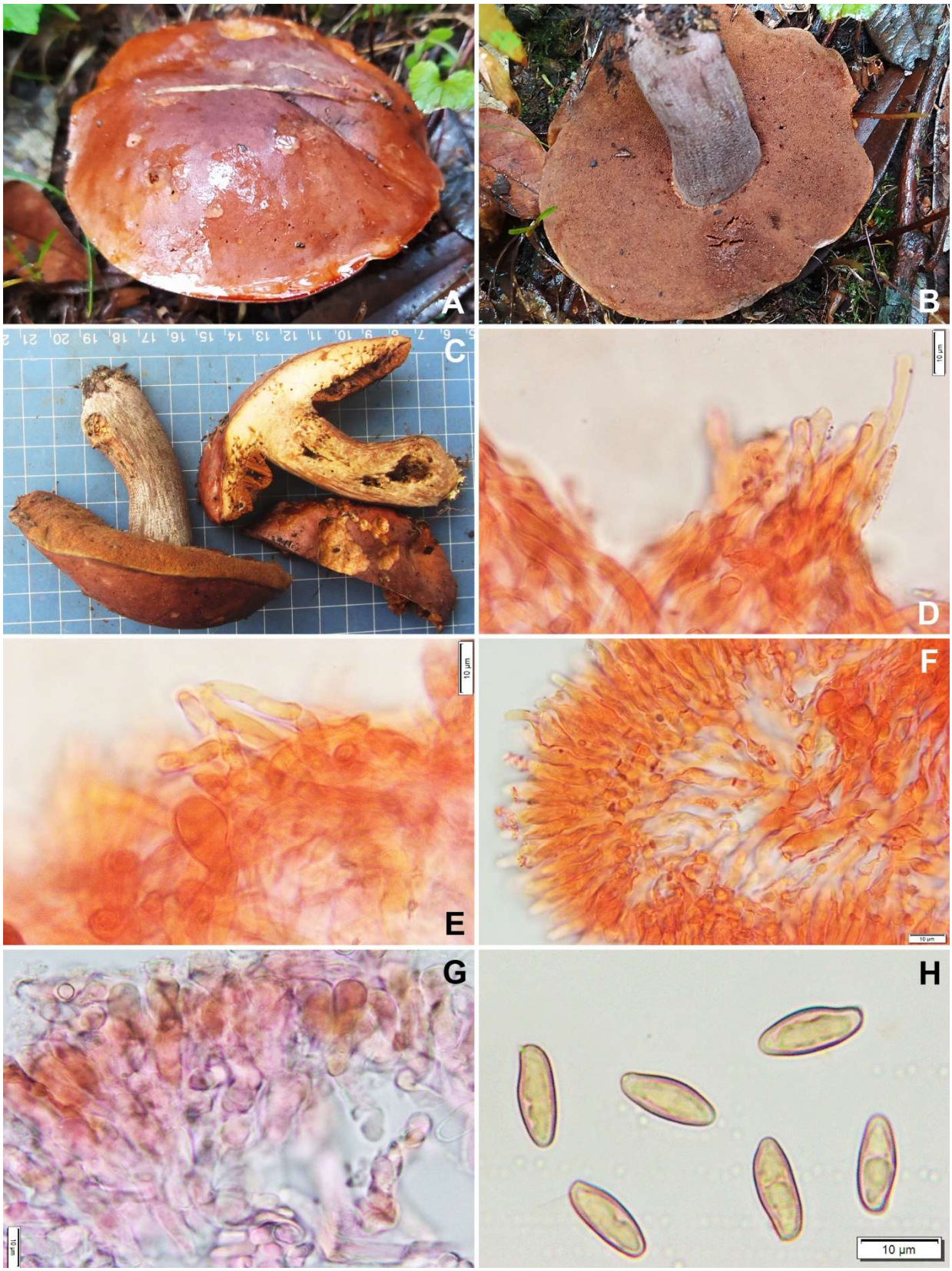

Fig. 2. Photoplate of ***Sutorius apleurocystidiatus*** (CAL 2005) **A–C.** Fresh and dissected basidiomata in the field and basecamp. **D–E.** Transverse section through the pileipellis. **F.** Lamellae edge showing cheilocystidia. **G.** Transverse section through the stipitipellis. **H.** Basidiospores. Scale bars: D−H = 10 µm.

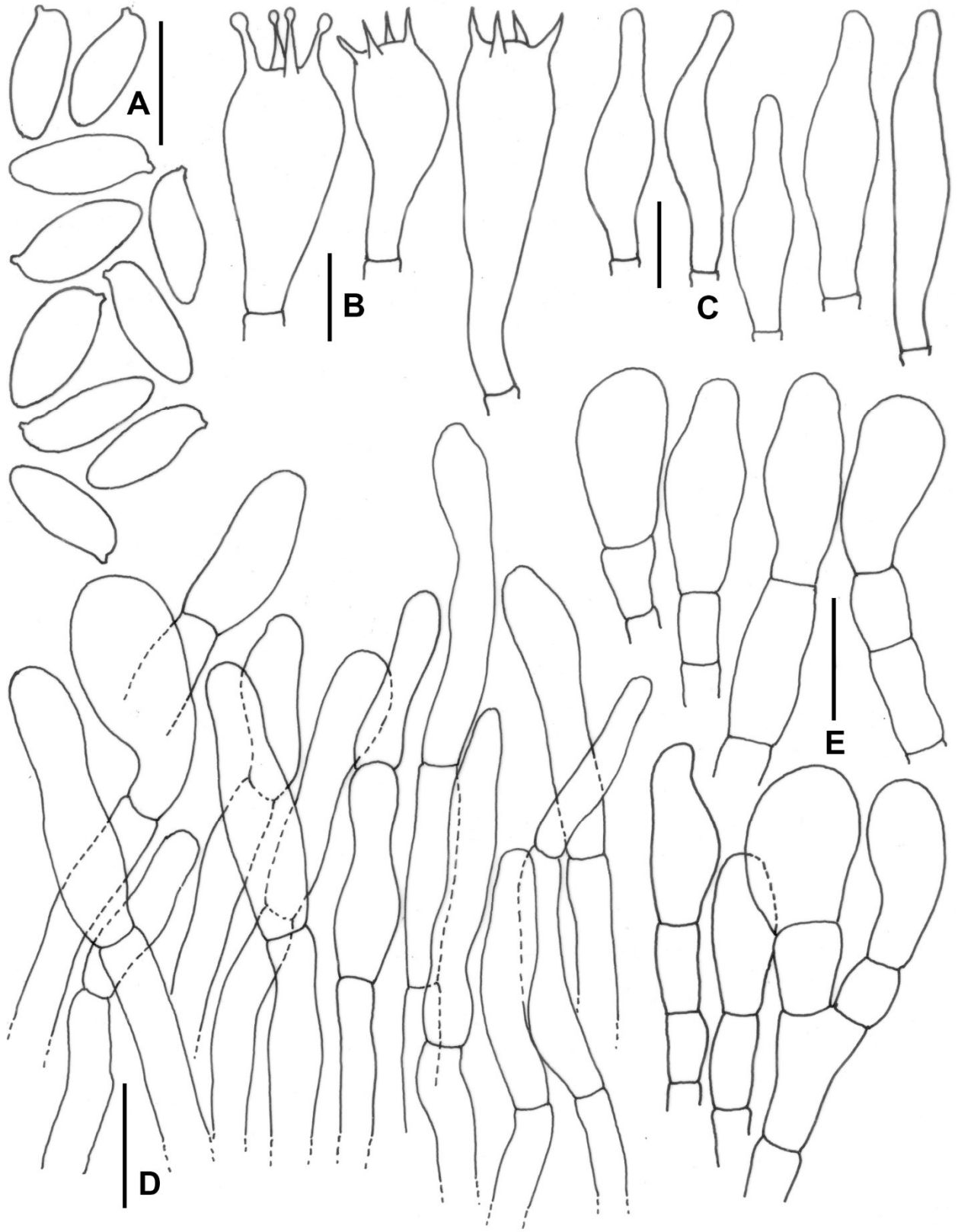

Fig. 3. Drawings of ***Sutorius apleurocystidiatus*** (from CAL 2005) **A.** Basidiospores. **B.** Basidia. **C.** Cheilocystidia. **D.** Elements of the pileipellis. **E.** Elements of the stipitipellis. Scale bars: A–E = 10 μm.

that true *S. eximius* (labelled as "*S. eximius* s.s." in Fig. 1) forms a distinct clade apart from Asian species and only consists of North American collections. *Sutorius eximius* s.s., distributed in northeastern North America (Peck, 1887; Smith and Thiers, 1971; Both, 1993; Bessette *et al*., 2000, 2016; Halling *et al*., 2012), differs from *S. apleurocystidiatus* by a dry pileus without red or oranges tinges, brown chocolate pores, presence of pleurocystidia, and longer spores (11–17 µm long) (Smith and Thiers, 1971; Bessette *et al*., 2000, 2016).

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