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Finding a needle in a haystack: morphology and multigene molecular analysis unveil a novel species, *Neoboletus angiocarpus*, the first sequestrate Indian bolete (Boletaceae)

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ABSTRACT: Featured with unconventional pattern, sequestrate mushrooms in Basidiomycota always elicit never-ending curiosity among mycologists and mushroom hunters across the continents. Our quest for the hidden treasure of Boletoid mushrooms in Indian Himalaya led to the discovery of the first sequestrate form of mushroom in the immensely diverse and one of the fast-growing mushroom families i.e. Boletaceae. *Neoboletus angiocarpus* sp. nov. is proposed and described here with morphological details and multigene molecular phylogenetic estimation. Allied species are also compared morphologically with this new species.

KEY WORDS: Boletales, Himachal Pradesh, macromorphology, micromorphology, new species, phylogenetic inferences.

INTRODUCTION

Sequestrate (truffle-like) mushrooms within Basidiomycota attract the mycologists since long back. They include morphology-based artificial assemblage of those angiocarpic taxa with basidiospores that are either not forcibly discharged (statismospores) or with mass of basidia that become mature inside an enclosed, hypogeous to epigeous basidioma. It is interpreted that they are derived through convergent evolution from epigeous habit with exposed hymenium (gymnocarpic) (Kendrick, 1992; Bougher and Lebel, 2001). Latest concept proposes bidirectional evolutionary process behind their morphological aberration 1) neoteny: retention of juvenile features in adult basidiomata showing mature reproductive structures or 2) progenesis: the onset of sexual maturity in morphologically young (immature) basidiomata that never reach at maturity (Kuhar *et al*., 2023). Spore dispersal of these mushrooms is mainly regulated by animal interference (Bougher and Lebel, 2001). The major problems with the sequestrate mushrooms are something else. Their tissues are often compressed, distorted and morphologically so different from their closest epigeous/conventional relatives that it is quite impossible to identify them solely with their morphology. Morphology-based few polyphyletic genera of sequestrate mushrooms are now fall apart into several genera with the combined study of morphology and molecular phylogeny. Except some sporadic reports, these sequestrate mushrooms (Basidiomycota) are remained unexplored or seriously underexplored from the vast country like India (Ahmad, 1941; Natarajan *et al*., 1988; Beig *et al*., 2011; Castellano *et al*., 2012; Buyck *et al*., 2017; Malik *et al*., 2017; Talie *et al*., 2020). Recently, during a macrofungal foray undertaken in search of boletoid mushrooms to Shimla district of Himachal Pradesh two of us (KD and SD) came across two different collections of sequestrate mushrooms which after thorough morphological examination followed by multigene molecular phylogeny were revealed as a novel species of *Neoboletus* Gelardi, Simonini & Vizzini, the genus where most of the members are conventional boletoid mushroom-former and are proposed herein as *Neoboletus angiocarpus* sp. nov. This noteworthy finding is the first record of sequestrate mushroom in Boletaceae Chevall. from India as well. In this present paper, this hidden treasure is described with its detailed morphology along with multigene molecular phylogeny.

MATERIAL AND METHODS

Morphological studies

In search of boletoid mushrooms, a routine macrofungal survey to different forests of the state of Himachal Pradesh was carried out during the rainy season of 2024 (August) and several fresh conventional basidiomata belonging to different species of boletoid mushrooms were collected. Along with these mushrooms, two specimens of nonconventional sequestrate boletoid mushrooms were also collected. Macromorphological characters were noted in the field (forest) or in the basecamp. Images of the fresh and dissected basidiomata (in their habitat and basecamp, respectively) were duly captured with digital cameras: Canon Power Shot SX50 HS and Canon Power Shot SX220 HS. Colour codes and their corresponding terminologies primarily followed Kornerup and Wanscher (1978). After recording all the macromorphological characters, dissected samples were kept in an electric fruit-drier overnight for drying. Macrochemical colour tests were also performed by applying $FeSO_4$ and 5–10% KOH on surface and context of basidiomata. Micromorphological characters were

Table 1. ***Neoboletus*** and allied sequences used in Maximum Likelihood analyses of this study. Newly barcoded collections are in bold.

Name of the species	Voucher no.	Country	28S	tef 1-α	rpb2
Caloboletu spanniformis	HKAS 56164	China	KJ605674	KJ619466	
Caloboletus xiangtoushanensis	GDGM 44833	China	KY800415	KY800418	
Neoboletus angiocarpus	KD 24HP-134 (Type)	India	PQ578768	PQ613840	PQ613843
Neoboletus angiocarpus	KD 24HP-142	India	PQ578864	PQ613841	PQ613842
Neoboletus antillanus	JBSD 127417	Dominican Republic	MK388302		MK488082
Neoboletus brunneissimus	HKAS 50538	China	KM605138	KM605150	KM605173
Neoboletus brunneissimus	HKAS 57451	China	KM605137	KM605149	KM605172
Neoboletus erythropus	VDKO 0690	Belgium		KT824048	KT824015
Neoboletus ferrugineus	HKAS 77617	China	KT990595	KT990788	KT990430
Neoboletus ferrugineus	HKAS 77718	China	KT990596	KT990789	KT990431
Neoboletus flavidus	HKAS 58724	China	KU974140	KU974137	KF739724
Neoboletus flavidus	HKAS 59443	China	KU974139	KU974136	KU974144
Neoboletus hainanensis	HKAS 74880	China	KT990597	KT990790	KT990432
Neoboletus hainanensis	HKAS 90209	China	KT990615	KT990809	KT990450
Neoboletus hainanensis	HKAS 59469	China	KF112359	KF112175	KF112669
Neoboletus infuscatus	N.K. Zeng 3352 (FHMU3370)	China	MW293785	MW307255	
Neoboletus infuscatus	N.K. Zeng 4030 (FHMU3371)	China	MW293786	MW307256	
Neoboletus infuscatus	N.K. Zeng 4031 (FHMU3372)	China	MW293787	MW307257	
Neoboletus junquilleus	AF2922	France		MG212596	MG212638
Neoboletus luridiformis	AT2001087	England	JQ326995	JQ327023	
Neoboletus magnificus	HKAS 54096	China	KF112324	KF112149	KF112654
Neoboletus magnificus	HKAS 74939	China	KF112320	KF112148	KF112653
Neoboletus magnificus	N.K. Zeng 4038 (FHMU3373)	China	MW293788	MW307258	
Neoboletus multipunctatus	HKAS 76851	China	KF112321	KF112144	KF112651
Neoboletus multipunctatus	N.K. Zeng 2498 (FHMU1620)	China	MH879693	MH879722	
Neoboletus multipunctatus	OR0 128	Thailand		MH614734	MH614781
Neoboletus obscureumbrinus	CMU 58-ST-0237	Thailand	KX017292		
Neoboletus obscureumbrinus	HKAS 89014	China	KT990599	KT990793	
Neoboletus obscureumbrinus	N.K. Zeng3091 (FHMU2052)	China	MH879694	MH879723	MH879742
Neoboletus rubriporus	HKAS 57512	China	KF112327	KF112151	KF112656
Neoboletus rubriporus	HKAS 90210	China	KT990604	KT990798	KT990439
Neoboletus rubriporus	L.P. Tang 1958	China		MH879726	
Neoboletus sanguineoides	HKAS 57766	China	KT990605	KT990799	KT990440
Neoboletus sanguineoides	HKAS 74733	China	KT990606	KT990800	KT990441
Neoboletus sanguineus	HKAS 80823	China	KT990608	KT990802	KT990442
Neoboletus sanguineus	HKAS 80849	China	KT990609	KT990803	KT990443
Neoboletus sp.	HKAS 50351	China	KF112318		KF112658
Neoboletus sp.	HKAS 76660	China	KF112328	KF112180	KF112731
Neoboletus subvelutipes	Mushroom Observer #285181	USA	MH244204	MH347318	
Neoboletus subvelutipes	Mushroom Observer #242312	USA	MH230086	MH337277	
Neoboletus thibetanus	HKAS 57093	China	KF112326		KF112655
Neoboletus tomentulosus	HKAS 53369	China	KF112323	KF112154	KF112659
Neoboletus tomentulosus	HKAS 77656	China	KT990611	KT990806	KT990446
Neoboletus tomentulosus	N.K. Zeng 1285 (FHMU841)	China	MH879691	MH879720	
Neoboletus venenatus	HKAS 57489	China	KF112325	KF112158	
Neoboletus venenatus	HKAS 63535	China	KT990613	KT990807	KT990448

observed and duly recorded in the laboratory after mounting freehand sections from dry samples in a solution of 5% KOH, 1% Phloxin, and 1% ammoniacal Congo red under an Olympus CX 41 compound microscope. Drawings of these micromorphological features were prepared with the help of a dedicated drawing tube attached to Olympus CX 41 compound microscope at 1000× magnification. Micro-photographs were captured with a dedicated camera (Olympus DP22) attached to an Olympus BX 53 microscope. The basidiospores were observed and measured in lateral view. Basidiospore measurements along with length/width ratios (Q) were noted as: minimum–mean–maximum. Basidium length excludes that of sterigmata. Herbarium codes followed Thiers (continuously updated).

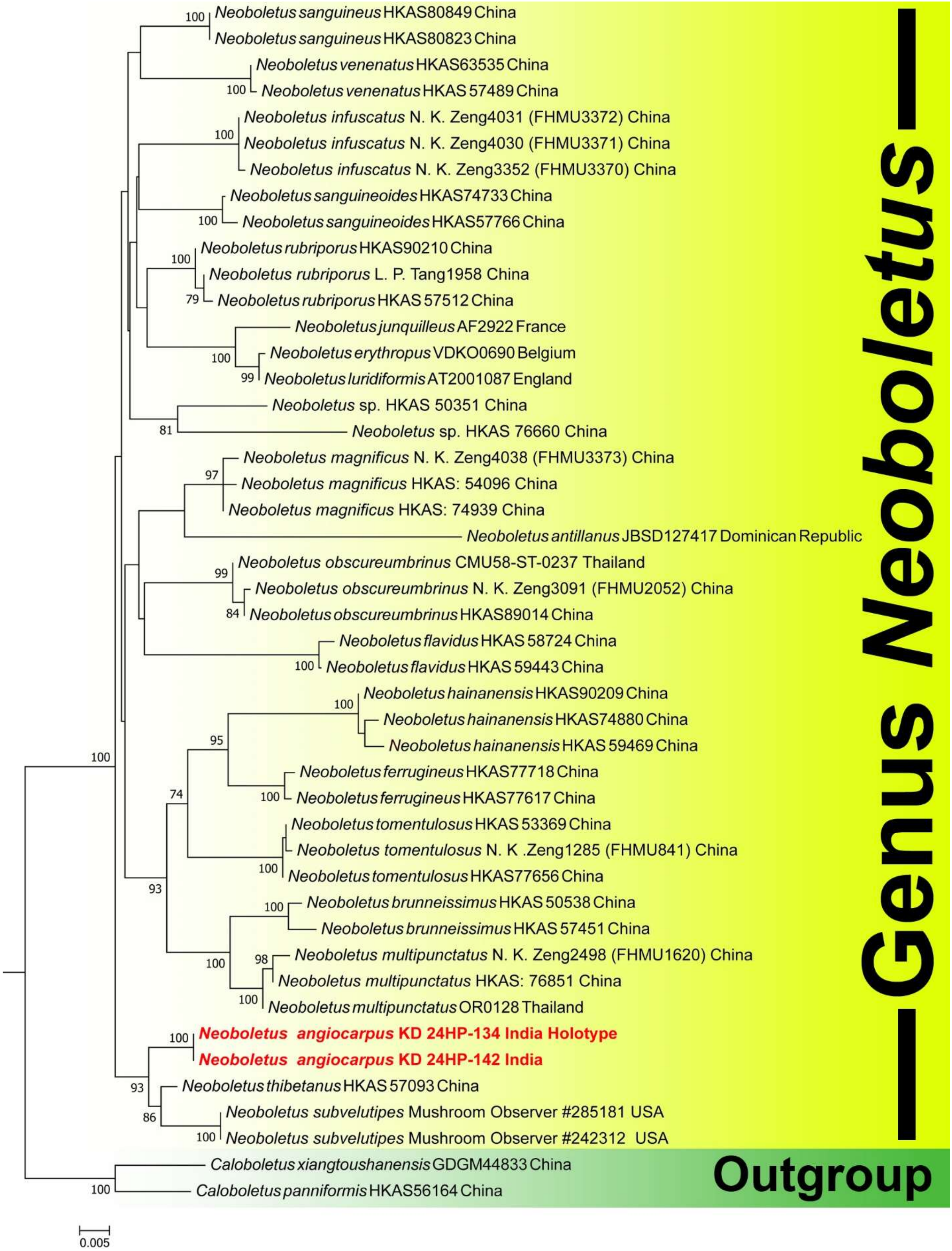

Fig. 1. Phylogram generated by Maximum Likelihood analysis based on 3-locus (28S, tef 1-α and rpb2) sequence data for ***Neoboletus angiocarpus*** and allied species. Maximum Likelihood bootstrap support values (MLbs) ≥70% are shown above or below the branches at nodes. ***Neoboletus angiocarpus*** is placed in bold red font to highlight its phylogenetic position in the tree.

DNA extraction, polymerase chain reaction (PCR) 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 three nuclear loci, partial nuc 28S rDNA D1-D2 regions (28S), region between conserved domains 6 and 7 of second largest subunit of RNA polymerase II (rpb2), and translation elongation factor 1-α (tef 1-α) were done using the primer pairs LR0R and LR5, b*rpb2*-6F and f*rpb2*-7cR, 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 placed at 72 °C for 7 min. The amplified PCR products were then 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, six sequences (Two each for 28S, rpb2 and tef 1-α) were generated from two separate collections of *Neoboletus angiocarpus* (voucher nos. KD 24HP-0134 and KD 24HP-0142). All newly generated sequences for this study were submitted to GenBank. Accession numbers used in phylogenetic analysis (Fig. 1) are listed in the Table 1.

Phylogenetic analysis

28S, rpb2 and tef 1-α sequences of the newly described *Neoboletus* species plus close relatives were retrieved from BLASTn search against GenBank (https://www.ncbi.nlm.nih.gov/genbank) and relevant published phylogenies (Jiang *et al*., 2021; Chai *et al*., 2019) Three raw datasets (28S, rpb2 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, three alignments (28S, rpb2 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 three-locus dataset (28S+rpb2+tef 1-α) of *Neoboletus*, sequence lengths were determined to be 840 bp for 28S, 668 bp for rpb2 and 506 bp for tef 1-α. The combined dataset was phylogenetically analysed using maximum likelihood (ML). The ML analysis was performed using raxmlGUI 2.0 [42] with the GTRGAMMA substitution model. This ML analysis was executed using the rapid bootstrap algorithm with 1000 replicates to obtain nodal support values. Maximum likelihood bootstrap (MLbs) values ≥ 70% are shown in the phylogenetic tree (Fig. 1).

RESULTS

Phylogenetic inferences

The three-gene combined phylogenetic (ML) tree is consistent and the phylogenetic analysis (Fig. 1) including the present novel species resolved the genus *Neoboletus* as monophyletic with full support. This analysis revealed that the sequences isolated from our species, *N. angiocarpus* (voucher nos. KD 24HP-134 and KD 24HP-142) clustered in a clade consisting of *N. thibetanus* (voucher no. HKAS 57093) and *N. subvelutipes* (voucher nos. Mushroom observer 242312 and Mushroom observer 285181) with a strong support (MLbs = 93%), forming a distinct clade within the *Neoboletus* lineage. However, our specimens were recovered as distinct species within the phylogenetic tree (Fig. 1).

TAXONOMIC TREATMENTS

Neoboletus angiocarpus Su. Datta, K. Das & Vizzini, ***sp. nov.*** **Figs. 2 & 3**

***MycoBank*: MB856564**

Type: INDIA, HIMACHAL PRADESH: Shimla District, Hattu hill, in subalpine forest under *Quercus* sp., 28 Aug 2024, alt. 3105 m, N 31°14.925′ E 77°29.889′, *Kanad Das & Sudeshna Datta*, *KD 24HP-134* (holotype! CAL 2119).

GenBank: PQ578768 (28S, CAL 2119); PQ613843 (rpb2, CAL 2119); PQ613840 (tef 1-α, CAL 2119).

Diagnosis: Distinguished from the closely allied species *Neoboletus thibetanus* by scrobiculate to pitted peridium surface of basidiomata, non-dendroid stipe-columella, presence of abundant cystidia, occurrence under broadleaf trees and 28S, rpb2 and tef 1-α sequence data.

Description: Basidiomata sequestrate (gastrocarps), 28–40 × 25–30 mm, partially epigeous, rounded, pear-shaped or ellipsoid, mostly irregular in outline with a tapering, rudimentary base. Peridium surface moist but never viscid, intricately ridged to somewhat scrobiculated or pitted, mainly pastel yellow to light yellow (3A4–5) with reddish madder red to jasper red (9A–B7) to lobster red (9B8) areas at several places including the tapered base, often turning slate grey to olive (3F2–4) or blackish on bruising, slowly turning oxblood red to cuba or reddish brown (9E7–8) with the application of KOH and greenish with $FeSO_4$. Context of reduced pileus 1 – 3 mm thick, light yellow (3A5–6), instantly changing to dark turquoise

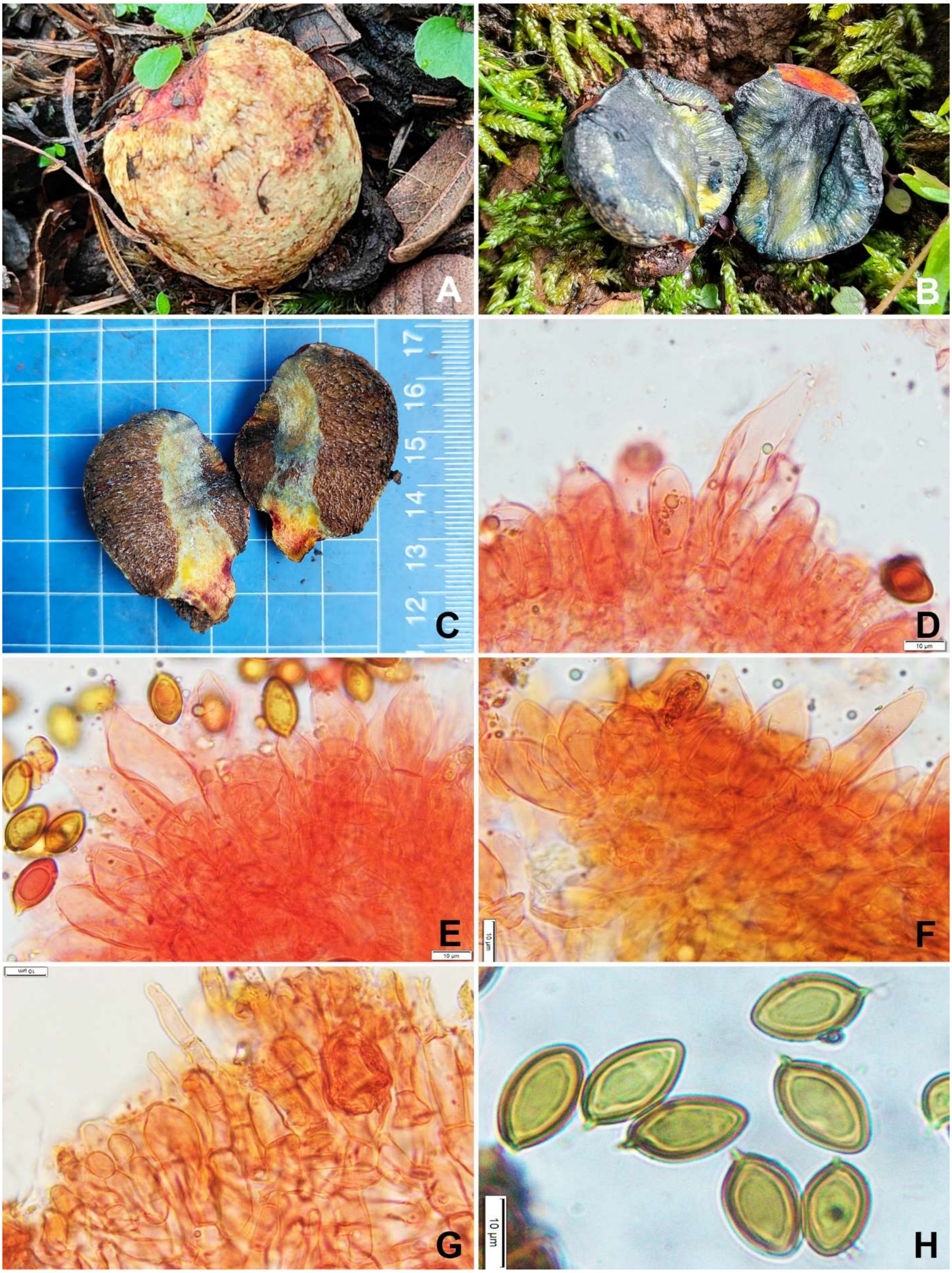

Fig. 2. Photoplate of ***Neoboletus angiocarpus*** (CAL 2119). **A–C.** Fresh and dissected basidiomata in the field and basecamp. **D.** Hymenial basidia and cystidia. **E.** Clusters of hymenial cystidia. **F.** Transverse section through epicutis of peridium showing its elements. **G.** Transverse section through epicutis of stipe-columella showing its elements. **H.** Basidiospores. Scale bars: **D−H** = 10 µm.

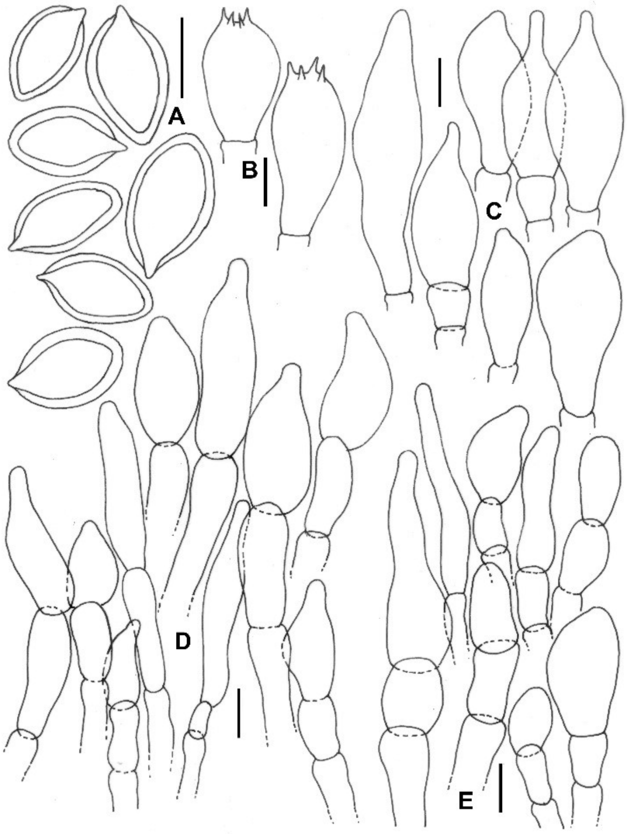

Fig. 3. Drawings of ***Neoboletus angiocarpus*** (from CAL 2119). **A.** Basidiospores. **B.** Basidia. **C.** Hymenial cystidia. **D.** Elements of the epicutis of peridium. **E.** Elements of the epicutis of stipe-columella. Scale bars: **A**–**E** = 10 μm.

(24F5–6). Stipe-columella 35–45 × 8–12 mm, prominent, truncate (but never dendroid) with indistinctively radiating branches, mostly yellow (2A6–7) or light yellow to yellow (3–4A5–7) but base in combination with reddish orange to orange red (7–8A–B8), instantly changing to greenish blue or dark turquoise (24F4–5) on exposure, turning reddish brown to dark brown (9F7–8) with the application of KOH and greenish with $FeSO_4$. Gleba tubulose, attached to the stipe-columella, the tubes 6–18 mm long, but tapering in length towards apex and base. In very young (immature) basidiomata, concolorous to columella or context, light yellow to yellow (2–3A5–7) changing instantly to dark turquoise (24F5–6) on exposure; in mature basidiomata never concolorous to columella, olive brown, mustard brown, yellowish brown or rust brown (5–6E6–8), bluing or becoming darker instantly on exposure. Pores small, up to 2 mm diam. Odour mushroomy to fruity.

Basidiospores 13.9–16.33–18.9 × 9–9.93–11.5 μm (Q = 1.45–1.63–1.84), ellipsoid to oblong, ochraceous to brown in KOH, thick-walled (wall 1.2–1.7 μm thick), statismosporic. Basidia 23.5–40.3 × 12–18 μm, subclavate to broadly clavate, 4-spored, thin-walled, hyaline; sterigmata 3–4 μm long. Cystidia 27–57.5 × 9.5–15.8 μm, ventricose rostrate to fusiform or appendiculate, frequent in tubes, scattered or in clusters along with basidia, thin-walled. Hymenophoral trama, parallel, hyphae 3–7 μm wide, septate, thin-walled. Epicutis of peridium a closely packed trichoderm to palisadoderm in nature, terminal elements 17–38.7 × 5.5–13.6 μm, mostly inflated, fusiform, cystidioid, bulbous or rarely cylindric with papillate, appendiculate to rounded apex. Epicutis of stipe-columella trichoderm to palisadoderm in nature, sterile, terminal elements 13.4–44.5 × 6–44.4 μm, mostly inflated, fusiform, cystidioid, bulbous or rarely cylindric mostly with rounded apex. Trama of stipe columella composed of compactly arranged slightly interwoven thin-walled hyphae (5–7 μm wide). Clamp connections absent in all tissues.

Etymology: species epithet “angiocarpus” refers to the hymenium produced in an enclosed basidiomata.

Additional specimen examined: INDIA, HIMACHAL PRADESH: Shimla District, Hattu hill, in subalpine forest under *Quercus* sp., 28 Aug 2024, alt. 2974 m, N 31°15.014′ E 77°29.642′, *Kanad Das* and *Sudeshna Datta*, KD 24HP-142 (CAL 2120).

GenBank: PQ578864 (28S, CAL 2120); PQ613842 (rpb2, CAL 2120); PQ613841 (tef 1-α, CAL 2120).

DISCUSSION

The present species is a wonder representing the first sequestrate member in the family Boletaceae from India. It belongs to the genus *Neoboletus*, typified with *Boletus luridiformis* Rostk. (Vizzini, 2014). Species included in *Neoboletus* are characterized by boletoid to rarely sequestrate habit, tomentose to velvety pileus, brownish, red to orange or more rarely yellow pores, stipe surface usually minutely dotted-punctate, context quickly and intensely bluing on handling or exposure, ellipsoid-fusiform, smooth basidiospores, trichodermic pileipellis, hymenophoral trama of the “Boletus-type”, fertile caulohymenium, inamyloid hyphae in the stipe trama, and ectomycorrhizal association with members of the Pinaceae and Fagaceae (Vizzini, 2014; Simonini and Vizzini, 2015; Urban and Klofac, 2015; Bessette *et al.*, 2016; Wu *et al.*, 2016a, 2023a; Chai *et al.*, 2019; Gelardi *et al.*, 2019; Jiang *et al.*, 2021; Mao *et al.*, 2023). Based on a small taxon sampling, Wu *et al.*, (2016b), after having firstly accepted *Neoboletus* as an independent genus (Wu *et al.*, 2016a), have reduced it as a later synonym of *Sutorius* Halling, Nuhn & Fechner. Subsequent phylogenetic and phylogenomic analyses indicated the independence of *Neoboletus* from *Sutorius* (Chai *et al.*, 2019; Gelardi *et al.*, 2019; Mao *et al.*, 2023; Shumskaya *et al.*, 2023; Wu *et al.*, 2023a,b; Tremble *et al.*, 2024).

Morphologically, another sequestrate Asian species, *N. thibetanus* (Shu R. Wang & Yu Li) Zhu L. Yang, B. Feng & G. Wu (morphologically and genetically closest), resembles our novel species *N. angiocarpus* in the field, however the former (originally reported from China) has a smooth peridium surface (scrobiculate to pitted in *N. angiocarpus*), distinctively dendroid stipe-columella, absence of cystidia and occurrence under coniferous trees (*Abies*) (Wang *et al.*, 2014). *Neoboletus subvelutipes* (Peck) Yang Wang, B. Zhang & Yu Li, a species originally reported from North America, is also phylogenetically quite close to *N. angiocarpus*, however the former is clearly distinct from our present species by exhibiting pileate-stipitate basidiomata with tubular hymenophore (like most of the boletoid mushrooms) (Bessette *et al.*, 2010).

Another sequestrate bolete, *Gymnogaster boletoides* J.W. Cribb (originally reported from Australia), resembles our species but the former can easily be segregated by possessing completely and permanently exposed hymenophore, restricted (as a small apical, appressed disc) pileus or absence of pileus, a defined stipe (0.5–1.5 × 0.1– 0.8 cm) and occurrence (gregariously or scattered) in forest dominated by Myrtaceae (*Eucalyptus* L'Hér., *Lophostemon* Schott. and *Corymbia* K.D. Hill & L.A.S. Johnson). Micromorphologically, the smaller basidiospores (11.3–13.1× 7.1–8.1 µm) and moderately thick-walled cheilocystidia of *G. boletoides* are very distinctive (Gelardi *et al.*, 2017).

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