Tropical Plant Research
An International Journal
© Society for Tropical Plant Research

ISSN (E): 2349 – 1183
ISSN (P): 2349 – 9265
4(1): 62–70, 2017
DOI: 10.22271/tpr.2017.v4.i1.009

Research article

Morphology and phylogeny reveal two new records of boletoid mushrooms for the Indian mycobiota

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Abstract: A detailed macro- and micromorphological studies coupled with the LSU-based phylogenetic inference of *Aureoboletus nephrosporus*, a tubulose member of the family Boletaceae is presented. Similarly, another tubulose bolete, *Strobilomyces mirandus* which was collected both from Eastern and Western Himalayas of India is also reported here with morphological details and ITS-based phylogeny. Both are the new records for this country.
Keywords: Boletales - India - Macrofungi - New records - Phylogeny - Sikkim.

[Cite as: Chakraborty D, Semwal KC, Adhikari S, Mukherjee SK & Das K (2017) Morphology and phylogeny reveal two new records of boletoid mushrooms for the Indian mycobiota. *Tropical Plant Research* 4(1): 62–70]

INTRODUCTION

The members of the family Boletaceae are mostly ectomycorrhizal in tropical to subalpine regions and thus are well represented in Sikkim of the Eastern Himalaya (Lakhanpal 1996, Das 2009, 2012, 2013, Chakraborty & Das 2015, Das & Chakraborty 2014, Das & Dentinger 2015, Das *et al.* 2012, 2013, 2014, 2015) because of the abundance of suitable host trees like *Abies* Mill., *Picea* Mill., *Tsuga* Carrière, *Lithocarpus* Blume, *Castanopsis* (D. Don) Spach, *Quercus* L., etc. and favourable microclimates. A hitherto poorly recorded (from India) ectomycorrhizal and tubuloid bolete genus *Aureoboletus* Pouzar is separated from the rest of the tubulose members of Boletaceae by its glutinous pileus, brightly yellow colored hymenophore which is unchanging on bruising, ixotrichodemis pileipellis (rarely trichodermis) and smooth basidiospores. In a recent macrofungal foray in 2016 to South and East districts of Sikkim, a number of boletoid mushrooms were collected by authors (DC & KD) along with other mushroom members and after thorough morphological examination and molecular phylogenetic studies of those mushrooms, one appeared as a recently established species: *Aureoboletus nephrosporus* G. Wu & Zhu L. Yang which is reported so far from China (Wu *et al.* 2016). It is described here for the first time from India with a detailed macro- and micromorphology along with an LSU-based phylogeny.

The representatives of another genus *Strobilomyces* Berk. (Boletaceae) are characterised mainly by greyish or blackish pileus, the presence of squamules or scales on pilear surface, ornamented basidiospores (Smith & Thiers 1971, Singer 1986). They are fairly common in India and are distributed from subtropical to subalpine zones (Bilgrami *et al.* 1991, Lakhanpal 1996, Kour 2013, Das *et al.* 2014). During last couple of forays to Eastern and Western Himalayas of India from 2007 to 2016 authors (DC, KCS & KD) came across repeatedly an interesting member of this boletoid genus. Thorough studies (morphology and ITS-based phylogeny) of the collected materials revealed a species of this genus with unusual morphology *i.e. Strobilomyses mirandus* Corner. A detailed macro- and micromorphology along with the ITS-based phylogeny of this species are also presented here for the first time from the country.

MATERIALS AND METHODS

Morphology

Macrofungal forays were undertaken by three of us (DC, KCS & KD) to different parts of Eastern (South and East districts of Sikkim) and Western Himalaya during the rainy season (July–August) in 2016.

Received: 08 November 2016 Published online: 28 February 2017
https://doi.org/10.22271/tpr.2017.v4.i1.009

Macromorphological characters were observed in the field and or basecamp from the fresh and dissected young to mature basidiomata. Samples were duly dried with respective field-drier. Images of these basidiomata were captured with the help of Canon Power Shot SX 220 HS and Nikon Coolpix P510. Micromorphological characters were observed with the help of a compound microscope (Nikon Eclipse N*i*-U) from the dry samples mounted in a mixture of 5% KOH, 1% Phloxin and 1% Congo red or in distilled water. Color codes and terms mentioned here are mostly after Methuen Handbook of Color (Kornerup & Wanscher 1978). Micromorphological drawings were prepared with a drawing tube (attached to the Nikon Eclipse N*i* or Olympus CX 41) at 1000×. Basidium length excludes sterigmata. Basidiospore measurements were recorded in profile view from 20 basidiospores mounted from a spore print. Spore measurements and length/width ratios (Q) are given here as minimum–mean–maximum. Methods for SEM follow Das *et al.* (2015). Herbarium codes follow Thiers (continuously updated).

DNA extraction, polymerase chain reaction (PCR) and sequencing

Genomic DNA (for a molecular phylogeny) was extracted from 100 mg of dried basidiome of each of the species with the help of InstaGeneTM Matrix Genomic DNA isolation kit (Biorad, USA) following the manufacturer's instructions. The nrITS gene region was amplified with primer pairs ITS5 and ITS4 (White *et al.* 1990). Similarly, the nrLSU gene region was amplified with primer pairs LR0R and LR7 (http://www.biology.duke.edu/fungi/mycolab/primers.htm). PCR was performed in volumes of 25 µL containing 2.5 µL of 10x assay buffer (100 mM Tris–Cl; pH 8.3, 500 mM KCl, 15 mM MgCl2), 200 μM dNTP mix (Bangalore Genei, Bangalore, India), 10 picomoles of primer, 1.0 unit of Taq DNA polymerase (Bangalore Genei), and 30 ng of template DNA. Then PCR-amplification was done with a thermal cycler (Eppendorf, Germany) programmed for 2 min at 94°C, followed by 35 cycles of 45 sec at 94°C, 1 min at 55°C, 1 min at 72°C and a final stage of 10 min at 72°C for ITS region. The PCR condition for nLSU was as follows: 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 30s at 52°C (for D1D2 region), 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 DNA sequence of the reverse strand was edited with Sequence Navigator version 1.0.1 (Applied Biosystems). The final consensus sequences were deposited at GenBank to procure the accession numbers (KY412776 for LSU of *Aureoboletus nephrosporus* and KY412777 for ITS of *Strobilomyces mirandus*).

Phylogenetic analysis

Phylogenetic analyses based on ITS and LSU sequences data were carried out to establish the phylogenetic placement of our isolated taxa. Reference sequences and outgroups were selected from the relevant literature and GenBank. Alignment were performed using CLUSTAL W (http://www.ebi.ac.uk/clustalw/) and phylogenetic analyses were conducted in MEGA 6.0 (Tamura *et al.* 2013). No manual editing was done within the alignment. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). One thousand bootstrap replicates were analysed to obtain nodal support values. The European material of *Boletus edulis* was chosen as out group taxon in our ITS-based analysis whereas, *Phylloporus rhodoxanthus* (North American sample) and *Xerocomus subtomentosus* (European sample) were chosen as outgroups in our LSU-based analysis.

RESULTS

Phylogeny

Our LSU-based phylogenetic analysis (Fig. 1) with 22 LSU sequences (including the present species) resolved genus *Aureoboletus* with full support. The sequence (GenBank accession no. KY412776) derived from Indian collection of *Aureoboletus nephrosporus* G. Wu & Zhu L. Yang is clustered with the sequences derived from its Chinese counterpart (represented by GenBank accession numbers KT990516 and KT990517) showing the conspecificity (100% identity in BLAST search) with strong support (BS value). Similarly, our ITS based phylogeny (Fig. 2) clearly supports the existence of two distinct clades (Clade A and Clade B) in the genus *Strobilomyces* which are also reported by the earlier workers like, Gelardi *et al.* (2012) and Antonin *et al.* (2015). Clade A includes *Strobilomyces confusus* Singer, *Strobilomyces seminudus* Hongo, *Strobilomyces verruculosus* Hirot. Sato where basidiospores are never with reticulation. Distinguishingly, Clade B represented by *Strobilomyces strobilaceous* (Scop.: Fr) Berk., *Strobilomyces* sp. (from India), *S. echinocephalus* Gelardi &

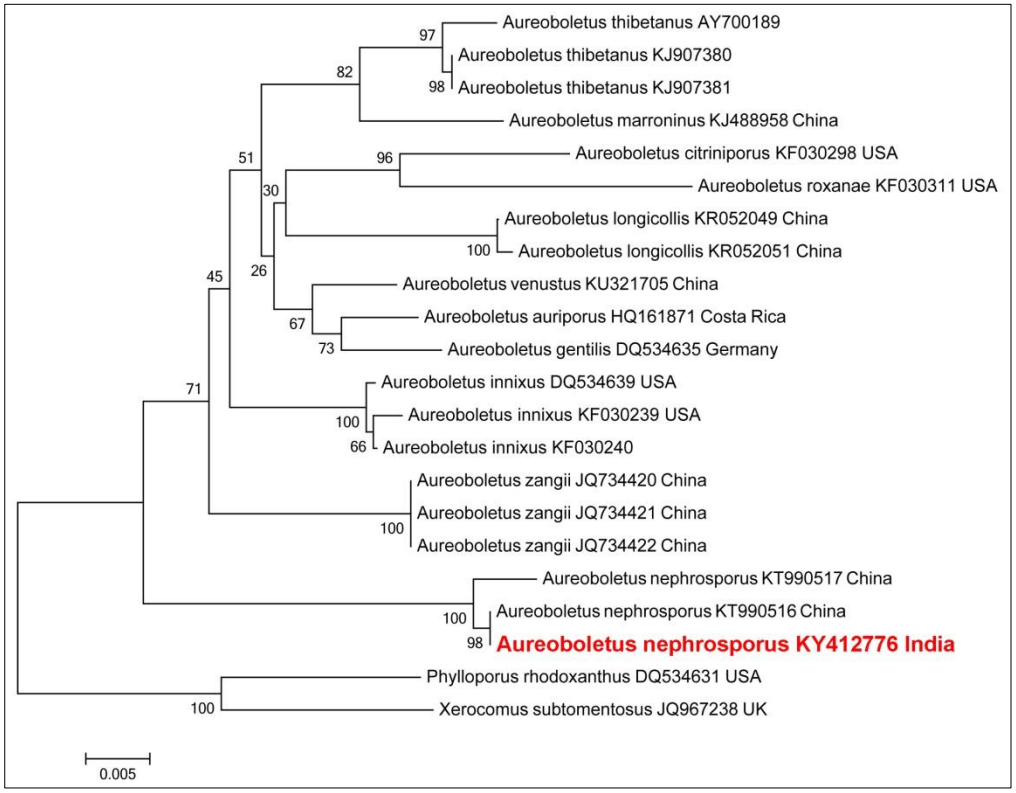

Figure 1. Phylogram of DC 16-29 (*Aureoboletus nephrosporus* G. Wu & Zhu L. Yang, in bold and red font) inferred from Maximum Likelihood analysis of nrLSU sequences using MEGA 6.0.

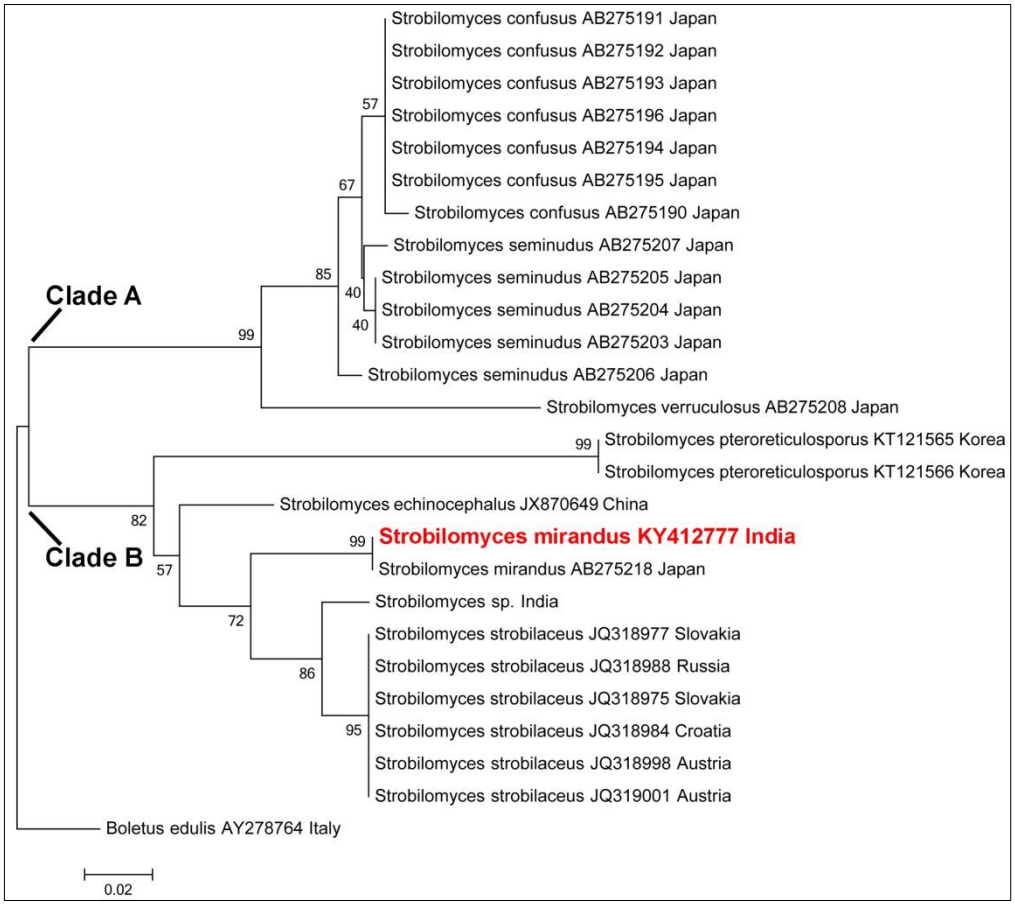

Figure 2. Phylogram of DC 16-27 (*Strobilomyces mirandus* Corner, in bold and red font) inferred from Maximum Likelihood analysis of nrITS sequences using MEGA 6.0.

Vizzini., *S. pteroreticulosporus* Antonín & Vizzini, *S. mirandus* where basidiospores are typically reticulate (Gelardi *et al.* 2012, Antonin *et al.* 2015). The sequence (GenBank accession no. KY412777) from our present Indian material of *Strobilomyces* (DC 16-27) showing conspecificity (99% identity in BLAST search) is clustered with the sequence derived from *S. mirandus* from Japan with strong support (bootstrap value 99).

Taxonomy

Aureoboletus nephrosporus G. Wu & Zhu L. Yang, Fungal Diversity, DOI 10.1007/s13225-016-0375-8

(Figs. 3 & 4)

Pileus 45–70 mm diam, subhemispherical to convex, sometimes with a broad umbo when young; surface mat, subvelvety, reddish brown (8D6) or brownish red to greyish red (10D6–5) when dry, turning dark brown (8F8) with KOH and greenish grey (26E2) with $FeSO_4$; margin with sterile flap of tissue. Pore surface yellow (2A8), becoming dingy with time, unchanging when bruised; pores 0.7 per mm in mature basidiomata, rounded to angular, compound. Tubes up to 50 mm long, subdecurrent to decurrent, light yellow (2A5). Stipe 90–170 × 7–15 mm, central, cylindrical, tapering at base; surface covered with striations; light yellow (5A6) to yellow ochre (5C7), olive (3E3) or greenish black when bruised. Context pale yellow (3A3) at pileus, turning golden yellow (5B7) with KOH; pastel yellow (3A4) or darker at stipe, turning reddish brown (8D8) with KOH.

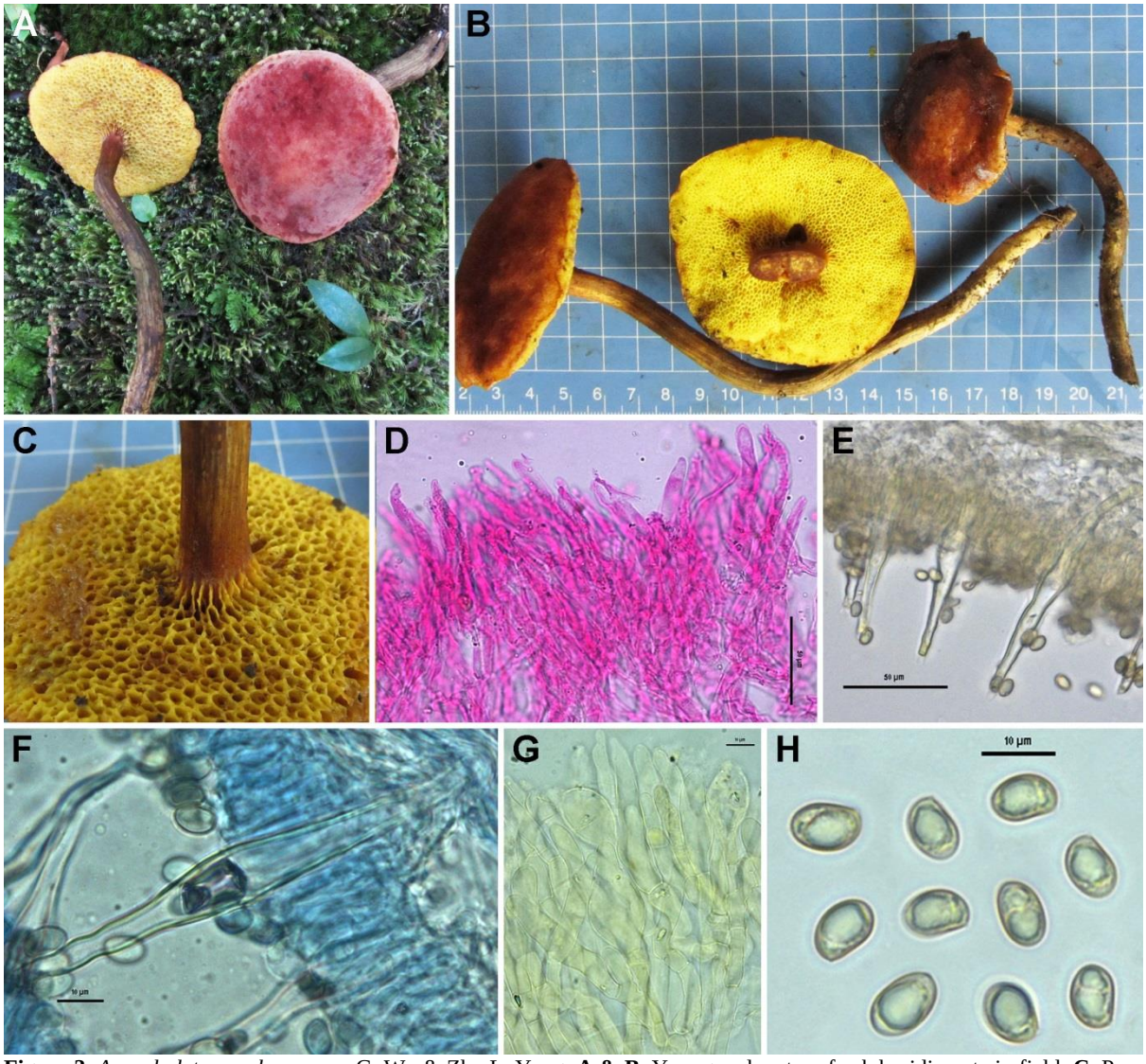

Figure 3. *Aureoboletus nephrosporus* G. Wu & Zhu L. Yang: **A & B,** Young and mature fresh basidiomata in field; **C,** Pore surface; **D,** Pileipellis; **E & F,** Pleurocystidia; **G,** Caulocystidia; **H,** Basidiospores. Bars. [Scale: **D–E,** 50 µm; **F–H,** 10 µm]

Basidiospores 8.5–9.5(–10.8) × 5.6–6.2(–6.7) µm, (Q = 1.41–1.53(–1.71)), ovoid to ellipsoid or nephroid, inequilateral, smooth under a light microscope. Basidia 37–55 × 10–13 µm, 4-spored, clavate. Pleurocystidia 50–70 × 9–13 µm, emergent up to 60 µm, fusoid to ventricose, mostly with thick covering, rarely thin walled.

Subhymenial layer up to 25 µm thick, hyphal. Tube edge fertile with basidia and cystidia. Hymenophoral trama mostly parallel to subparallel or sometimes interwoven. Pileipellis a trichodermis, up to 200 µm thick, composed of erect hyphae of slightly inflated cells; terminal cells 23–44 × 7.2–10 µm, cylindrical to subcylindrical, sometimes subfusoid. Stipitipellis up to 160 µm thick, composed of hyphae and cystidia in several clusters; basidia not observed; caulocystidia 40–55 × 8–20 µm, broadly ventricose to lanceolate, clavate to subclavate or cylindrical with fusoid apex.

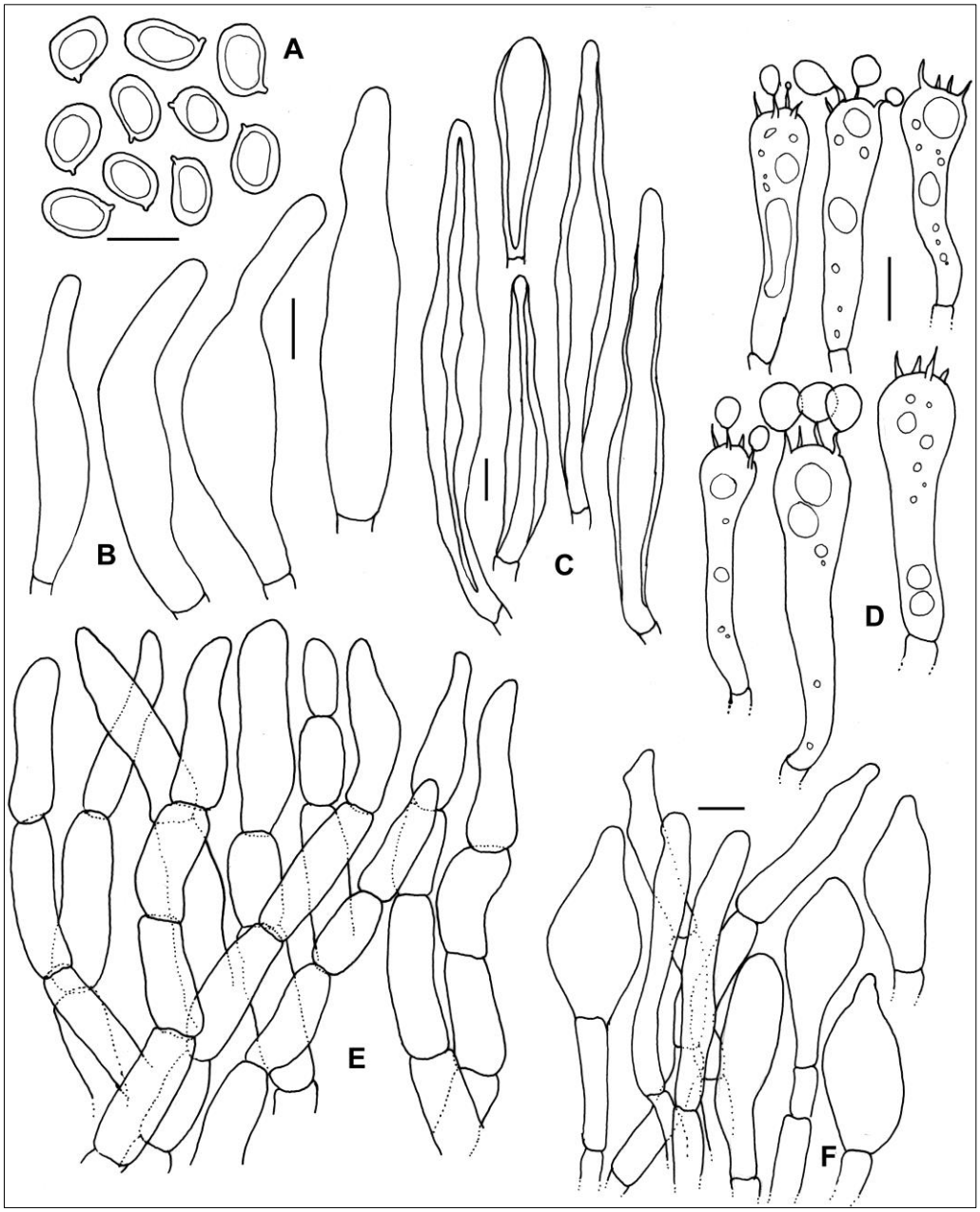

Figure 4. *Aureoboletus nephrosporus* G. Wu & Zhu L. Yang: **A,** Basidiospore; **B,** Pleurocystidia without covering of refractive substance; **C,** Pleurocystidia with covering of refractive substance; **D,** Basidia; **E,** Pileipellis; **F,** Caulocystidia. [Scale: **A–F,** 10 µm]

Distribution: China and India (Sikkim).

Specimen examined: India, Sikkim, South district, Maenum Wild life Sanctuary (Maenum top 2), 2315 m, N27°19’18.7" E88°22’07.9", under *Quercus* sp., 21st August 2016, *D. Chakraborty & K. Das*, DC 16-29 (CAL).

Note: The combination of macro- and micromorphological characters like fleshy tubulose basidiomata with reddish, dry or mat pilear surface, bright yellow pore surface, unchanging (on bruising) context color and smooth nephroid basidiospores place the Indian collection under the genus *Aureoboletus*. Further, *A. nephrosporus* is micromorphologically distinct from rest of the species of this genus by the presence of nephroid

basidiospores and surface of pleurocystidia being covered with a thick layer of a strongly refractive substance which is often dissolved in KOH (Wu *et al.* 2016). Morphology of the Indian material [except the stipe (which is longer) and pleurocystidia (which is smaller)] is mostly in conformity with that of its counterpart from China. Moreover, our LSU-based phylogeny strongly shows the conspecificity of our collection to *Aureoboletus nephrosporus* (represented by KT990516 in Fig. 1), the Chinese counterpart both in morphology and phylogeny. But, the Chinese material shows somewhat different altitudinal variation (collected from subtropical belt: 1700 m). Another Chinese species, *Aureoboletus zangii* X.F. Shi & P.G. Liu (represented by JQ734420 to JQ734422 in Fig. 1) is quite similar to *A. nephrosporus*, however, the former differs by its viscid pileus and stipe, fox red or English red colored stipe-surface and narrower basidiospores (3–4 µm) (Wu *et al.* 2016) from the latter. *Aureoboletus thibetanus* (Pat.) Hongo & Nagas. (the only other species from this genus reported from India) can easily separated from *A. nephrosporus* by presence of strongly reticulate and highly glutinous pileus surface (Sharma *et al.* 2005).

Strobilomyces mirandus Corner, *Boletus* in Malaysia: 61 (1972). **(Figs. 5, 6)**

Pileus 37–75 mm. diam.; convex, vivid yellow to sunflower yellow (3A8–4A7); surface densely squamulose with flat to bluntly pyramidal or wart-like squamules, which are more dense towards centre, brown (7E–F4) to blackish brown, surface brownish red (8–9C8) with KOH; margin wavy with cottony veiler remnant, vivid yellow (3A8). Pore surface covered by partial veil when young, smoky white; depressed near stipe, brownish initially then blackish on bruising; pore 2/mm, simple, angular. Tube 12–16 mm long, adnate–sinuate, chalky to smoky white, then greyish brown (7D3). Stipe 55–90 × 17–28 mm, central, concolorous with pileus, with the cotton-like annular region; surface with reticulations on the upper portion of the annular region, striations with pit like openings throughout the rest; basal mycelium greyish magenta (13B3). Context solid in pileus and stipe; context in pileus chalky white but immediately turning greyish orange (7C4) and then brownish grey (7E2) or brownish black on exposure, turning reddish orange to brownish orange (7B–C8) with KOH, greenish grey to dull green (26B2–26D3) with $FeSO_4$ in pileus, stipe context chalky white turning dark brown to brownish black with an intermediate orange brown to red brown coloration when exposed. Spore print blackish brown. Taste indistinct. Odour indistinct.

Basidiospores 7.2–8.8(–10.5) × 6–6.9(–7.7) µm, (Q = 1.09–1.26(–1.45)), mostly subglobose or broadly ellipsoid, ornamented, forming complete reticulation. Basidia 38–48 × 10–16 µm, four-spored, clavate. Pleurocystidia 60–90 × 15–20 µm, fusoid to ventricose or ventricose rostrate, brown pigmented. Hymenophoral trama divergent. Pileipellis a trichodermis, 200–400 µm thick, composed of erect to suberect hyphae of slightly inflated to elongated cells, with minute incrustations; terminal cells 22–75 × 8–112 µm, cylindrical to subcylindrical. Stipitipellis hyphal, same as pileal hyphae; cystidia in several clusters; basidia not observed; caulocystidia 32–53 × 01–13 µm, broadly ventricose to lanceolate, clavate to fusoid.

Distribution: Malaysia, Japan, China and India (Sikkim).

Specimens examined: India, Sikkim, South district, Rabangla, 1985 m, N27°15’14.8’’ E88°23’03.7’’, under *Lithocarpus* sp., 20th August 2016, *D. Chakraborty & K. Das*, DC 16-27; ibid., South district, Kewzing, 1888 m, N27°17’46.5’’ E88°21’26.6’’, under *Lithocarpus* sp., 21st August 2016, *D. Chakraborty & K. Das* DC 16-028 (CAL); Uttarakhand, Rudraprayag, Kund, 1160 m, under *Cinnamomum tamala* and *Quercus glauca*, 13th August 2007, *K.C. Semwal*, KCS 1102; 12th August 2016, *K.C. Semwal*, KCS 2556.

Note: Unlike other (Asian or extralimital) species of *Strobilomyces*, present one i.e. *S. mirandus* has entirely distinct combination of macromorphological features like golden yellow to yellowish orange or yellow colored pileus (distinct from any known species of this genus) with blackish brown squamules, pileus margin with yellow veilar remnants, smoky white pore surface (turning brownish after bruising), yellow stipe with cottony annular region. Morphological features of Indian collection is on conformity to its counterparts reported from other Asian countries like Malaysia, Japan or China (Corner 1972, Sato *et al.* 2005, Ge &Yang 2005) and the combination of our ITS-based phylogeny and morphological studies further warrants its wider range of distribution in different countries of Asia. Moreover, the occurrence of *S. mirandus* strengthens the representation of this genus in India with eight species (*Strobilomyces strobilaceus* (Scop.) Berk., *S. nigricans* Berk., *S. polypyramis* Hook. f., *S. velutipes* Cooke & Massee, *S. annulatus* Corner, *S. mollis* Corner, *S. mirandus* Corner and *Strobilomyces* sp. (unpublished and represented by “Strobilomyces sp.” in our ITS-based tree: Fig. 2).

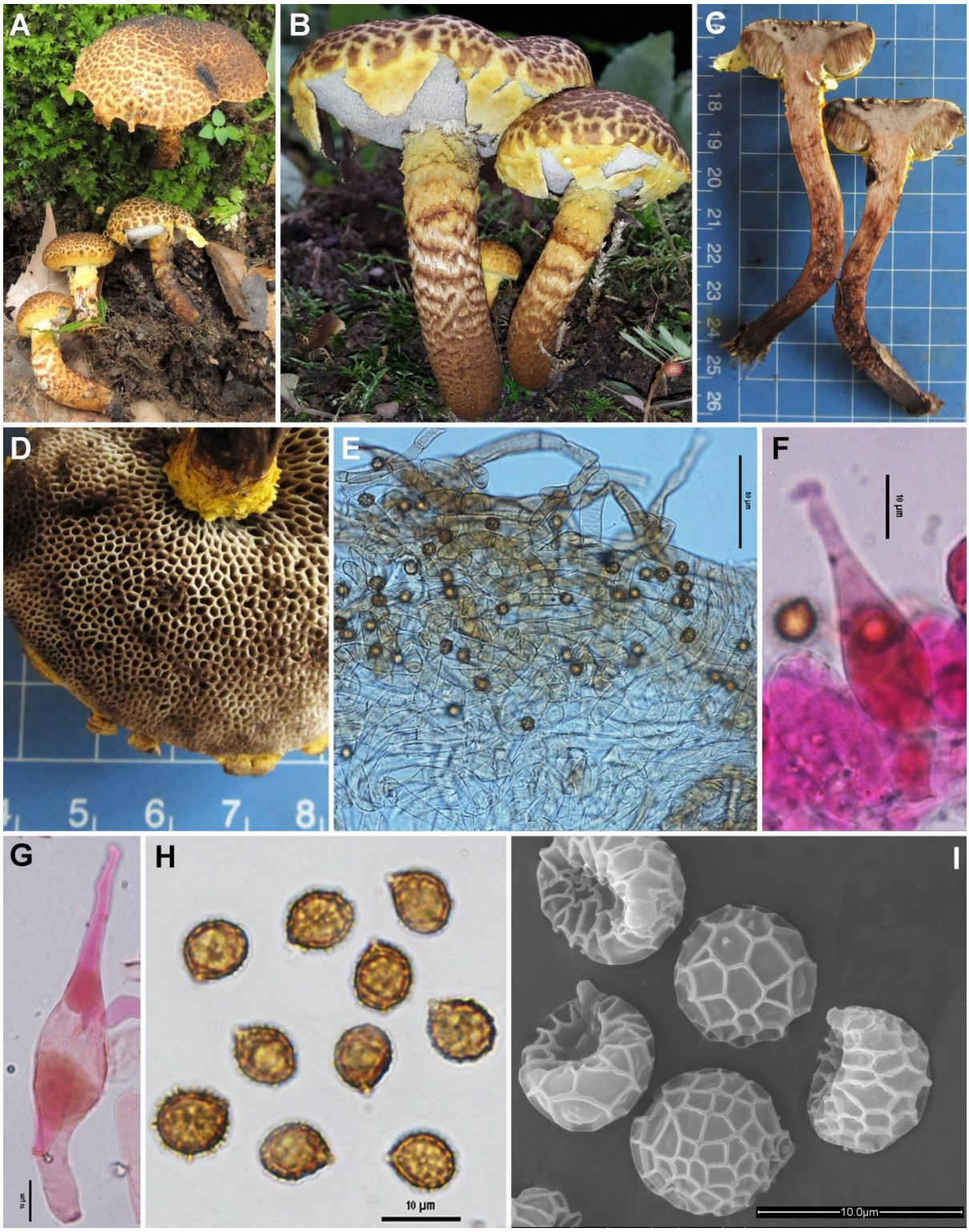

Figure 5. *Strobilomyces mirandus* Corner: **A–C,** Young and mature fresh basidiomata in field and in basecamp; **D,** Pore surface; **E,** Pileipellis; **F–G,** Pleurocystidia; **H,** Basidiospores; **I,** SEM of basidiospores. [Scale: **E,** 50 µm; **F–I,** 10 µm]

In the western Himalaya, *S. mirandus* was collected from the present locality (Kund Forest) very first time in 2007. That time the forest was so fruitful with abundant suitable hosts, leaf litter and humus and about 25 mushroom species had been encountered in a small area in a day on August 13, 2007, but in the year of 2016 when the forest area has been visited again on August, 12, it was observed that the forest trees were so less with very less leaf litter and humus. Only 3 wild mushrooms species has been encountered. So from August 2007 to August 2016 several factors dramatically altered the scenario of the concerned forest. It has been assumed that the several anthropogenic activities (construction of hydropower dam and subsequent shifting of the respective

national highway) which developed in recent years are the cause of the declination of the mushroom diversity in this area. Moreover, the host trees are likely to be cut shortly in order to shift the national highway. Therefore, we are afraid that there will be the declination of *S. mirandus* from Western Himalaya of India once all these on-going construction activities will be over.

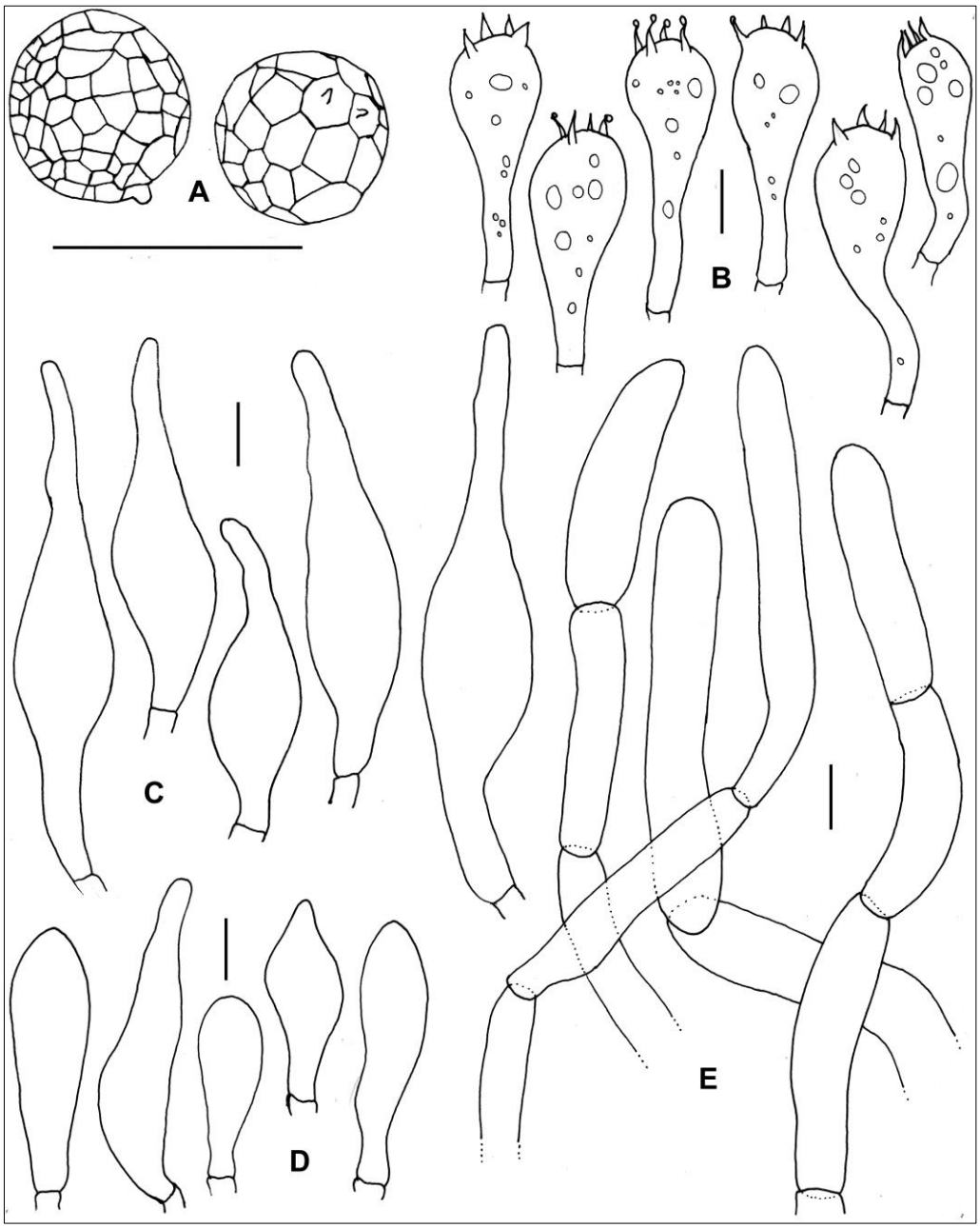

Figure 6. *Strobilomyces mirandus* Corner: **A,** Basidiospore; **B,** Basidia; **C,** Pleurocystidia; **D,** Caulocystidia; **E,** Pileipellis. [Scale: **A–E,** 10 µm]

ACKNOWLEDGEMENTS

The authors are grateful to the Director, Botanical Survey of India (Kolkata) and the Scientist-in-Charge, Botanical Survey of India (Gangtok) for providing facilities. DC & KD are thankful to the entire forest department of Sikkim for allowing us (KD & DC) to undertake the macrofungal surveys to the restricted areas of South district of Sikkim. Assistance (to KD & DC) rendered by Subhash Pradhan (BSI, Gangtok) in the field is duly acknowledged.

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