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Combined Study of Morphology and Molecular Phylogeny Unveils the First Report of the Mushroom Genus *Singerocybe* (*Tricholomataceae*) from India

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ABSTRACT

The genus *Singerocybe* is characterized by the presence of vesicles in the pileus and stipe cuticle. *Singerocybe alboinfundibuliformis*, collected from two Himalayan states (Sikkim and Uttarakhand), is described as a new generic and species record for the Indian mycobiota based on morphological features coupled with the phylogenetic evaluation based on ITS sequence data.

Keywords: Basidiomycota, Conspecificity, Molecular phylogeny, Sikkim, Taxonomy

INTRODUCTION

The genus *Singerocybe* Harmaja was erected by a Finnish mycologist Harmaja in 1988, with *Singerocybe viscida* Harmaja as the type (Harmaja, 1988). Describing *Singerocybe*, Harmaja replaced his older illegitimate name *Singerella* Harmaja (Harmaja, 1974). Later authors (especially in the "pre-molecular time") have usually followed the broader conception of the genus *Clitocybe* and included *Singerocybe* among synonyms (e.g. Singer 1986 as *Singerella*, Corner 1994; Kuyper 1995; Bon 1997; Horak 2005 as *Singerocybe*). However, Qin *et al*. (2014) raised it to the level of an independent genus based mostly on phylogenetic studies. The genus *Singerocybe* is characterized by the presence of vesicles in the pileipellis (Harmaja, 1988). This genus is distributed worldwide in temperate and tropical regions (Qin *et al*., 2014; Kumla *et al*., 2016) and only six species are known across the world (www.indexfungorum.org). Species of this genus are saprotrophic and usually grow on soils rich in humus, dead wood, or rotting leaves (Peck 1873; Harmaja 1988; Takahashi 2000; Seok *et al*., 2009, Qin *et al*., 2014).

During the macrofugal forays to Sikkim (Eastern Himalaya) and Uttarakhand (Western Himalaya) the authors collected some translucent specimens of agaricoid macrofungi. The thorough morphological examination followed by ITS-based phylogenetic analysis revealed their identity as *Singerocybe alboinfundibuliformis* **(**Seok, Yang S. Kim, K.M. Park, W.G. Kim, K.H. Yoo & I.C. Park) Zhu L. Yang, J. Qin & Har. Takah. Here we report this species for the first time from India on the basis of macro- and micromorphological characters supported by ITS sequence based phylogenetic inferences.

MATERIAL & METHODS

Morphological studies

Macromorphological features, including macro chemical colour reactions (with KOH, $FeSO_4$ and Guaiacol) and habitat details, were recorded from the fresh young and mature basidiomata in the field or in base camp, before they were dried with a portable dryer. Photographs of fresh basidiomata and photomicrographs were taken with Cannon SX 220 HS and Olympus Pen Lite EPL-6 belonging to the Olympus CX-41 microscope cameras. Colour codes and terms have been followed as per Kornerup & Wanscher (1978). Micromorphological characters were recorded with the help of compound microscope (Olympus CX-41) from free hand sections of dried basidiomata samples mounted in 5% KOH or stained in a mixture of 5% KOH and phloxin and mounted in 30% glycerol. The outline of the microscopic

structures was drawn with a drawing tube attached to Olympus CX-41 microscope at 1000×. The basidium length excludes length of sterigmata. Spore measurements were recorded in profile view from forty basidiospores and measurements are presented as the form a–b–c, where the a and c contain minimum and maximum values respectively, while underlined b represents an average value of all measurement. "Q" stands for length/width quotient and is constructed by the same way as basidiospores measurements. Herbarium codes follow Thiers (continuously updated; assessed on 15 March, 2020).

DNA Extraction, Polymerase Chain Reaction (PCR) and Sequencing

Genomic DNA was extracted from 100 mg of dried basidioma using the InstaGeneTM Matrix Genomic DNA isolation kit (Biorad, USA) following the manufacturer's instructions. The nuclear ITS region was amplified with ITS1 and ITS4 primer pairs (Vilgalys and Hester 1990; http://www.biology .duke.edu/fungi/mycolab/primers.htm). PCR amplification was performed on a thermal cycler (Eppendorf, Germany) programmed for 2 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 30 secs at 50°C, 45 sec at 72°C and a final stage of 5 min at 72°C. The PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN, Germany) and directly sequenced on the 3730xl DNA Analyzer (Applied Biosystems, USA) using the amplifying primers. The newly generated sequence was deposited at GenBank vide accession numbers MH032768 and MH031702.

Phylogenetic analysis

The newly generated ITS sequences of *Singerocybe alboinfundibuliformis* and their close relatives were retrieved from nBLAST search against GenBank (https://www.ncbi.nlm.nih.gov/genbank) and relevant published phylogenies (Qin *et al*., 2014; Kumla *et al*., 2016). The ITS dataset was aligned using the online version of the multiple sequence alignment program MAFFTv.7 (https://mafft.cbrc.jp/alignment/software/) with L-INS-i strategy. The alignment was checked and trimmed manually with MEGA v. 7 (Kumar *et al*. 2016). Phylogenetic analysis was undertaken with the help of raxmlGUI 2.0 (Edler et al. 2021) based on the maximum likelihood (ML) criterion. Maximum Likelihood (ML) analysis was conducted using the IQ-tree tool version 2.2.2.6 (Nguyen *et al*. 2015). Additionally, ultrafast bootstrap with 1,000 replicates was applied to obtain nodal support values. *Clitocybe nebularis* (Batsch) P. Kumm. and *Leucocybe candicans* (Pers.) Vizzini, P. Alvarado, G. Moreno & Consiglio were chosen as the outgroup.

RESULTS

Phylogenetic inferences

Present ITS-based phylogenetic analysis (Fig. 1) with 27 ITS sequences (including two sequences from the Indian collections) resolved the genus *Singerocybe* with full support. Six species of *Singerocybe* (*S. adirondakensis*, *S. phaeophthalma*, *S. umbilicata*, *S. clitocyboides*, *S. humilis* and *S. alboinfundibuliformis*) are separated in this analysis as ingroup. Sequences derived from two Indian collections (KD 17-17 and KD 17-40 represented by GenBank numbers MH032768 and MH031702 respectively) have appeared as monophyletic and nested in the *S. alboinfundibuliformis* clade (highlighted in Fig. 1) including all the known Asian sequences of derived from Chinese and Japanese collections indicating the conspecificity or similarity between the collections from three Asian countries.

Taxonomy

Singerocybe alboinfundibuliformis (Seok, Yang S. Kim, K.M. Park, W.G. Kim, K.H. Yoo & I.C. Park) Zhu L. Yang, J. Qin & Har. Takah., Mycologia 106(5): 1022 (2014). *Clitocybe alboinfundibu liformis* Seok, Yang S. Kim, K.M. Park, W.G. Kim, K.H. Yoo & I.C. Park [as 'alboinfundibuliforme'], Mycobiology 37(4): 295 (2009) **(Figures 2 and 3)**

Pileus 20–60 mm in diam., caespitose, infundibuliform, hollow till the base of stipe, smooth, white (4A1) to smoky-white, paler than brownish orange (5C3) when fresh, yellowish grey (3B2) to greyish yellow (4B3) on drying, margin involute when young becoming incurved in older specimens. Lamellae decurrent, narrow (≤1.5 mm wide), strongly anastomosing, interveined, reticulate, transparent to white (4A1) when young, becoming greyish yellow (4B3–4) on drying. Stipe central, 20–60 × 4–10 mm, cylindrical, tubular, tapering towards the base, smooth, concolorous to the pileus. Odour mild, pleasant. Context thin, unchanging with 5% KOH, 10% $FeSO_4$ and Guaiacol. Spore print white (1A1).

Pileipellis 200–250 µm thick, composed of parallel to subparallel dense, 3–10 µm wide hyphae, hyphae septate, clamped and interspersed with vesicles (19–35 × 12–25 µm). Basidia 22–30 × 5–8 µm, clavate, usually 4-, occasionally 2-sterigmate, with basal clamp connections and long sterigmata (up to 5 µm).

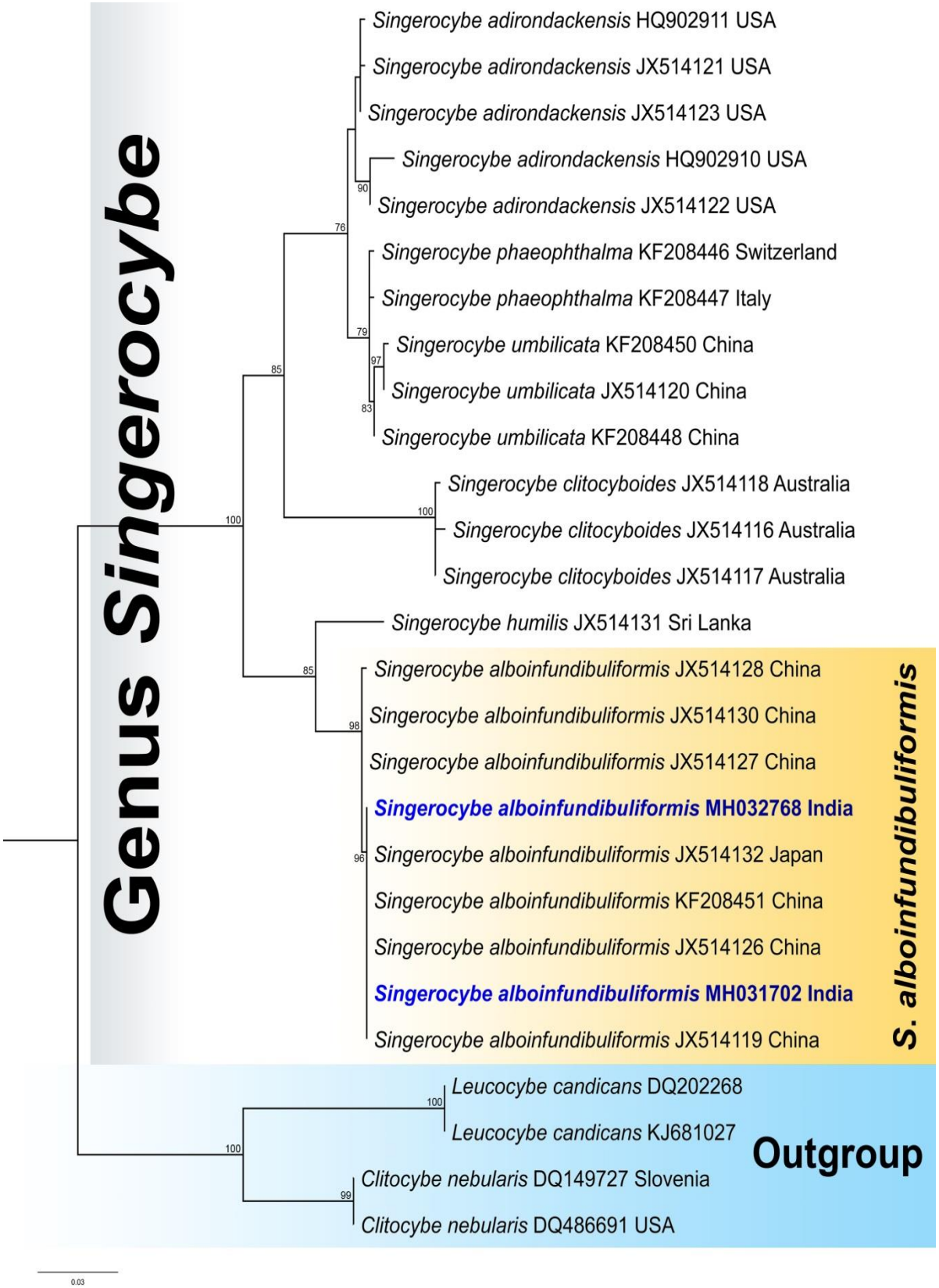

Figure 1: Phylogram generated from ITS-rDNA sequences. The evolutionary history was inferred by using the Maximum Likelihood method in raxmlGUI 2.0. Bootstrap support values (>70%) obtained from the ML analysis are shown above or below the branches at nodes. Two sequences derived from two Indian collections of *Singerocybe alboinfundibuliformis* (KD 17-17 & KD 17-40) are shown in bold and blue font.

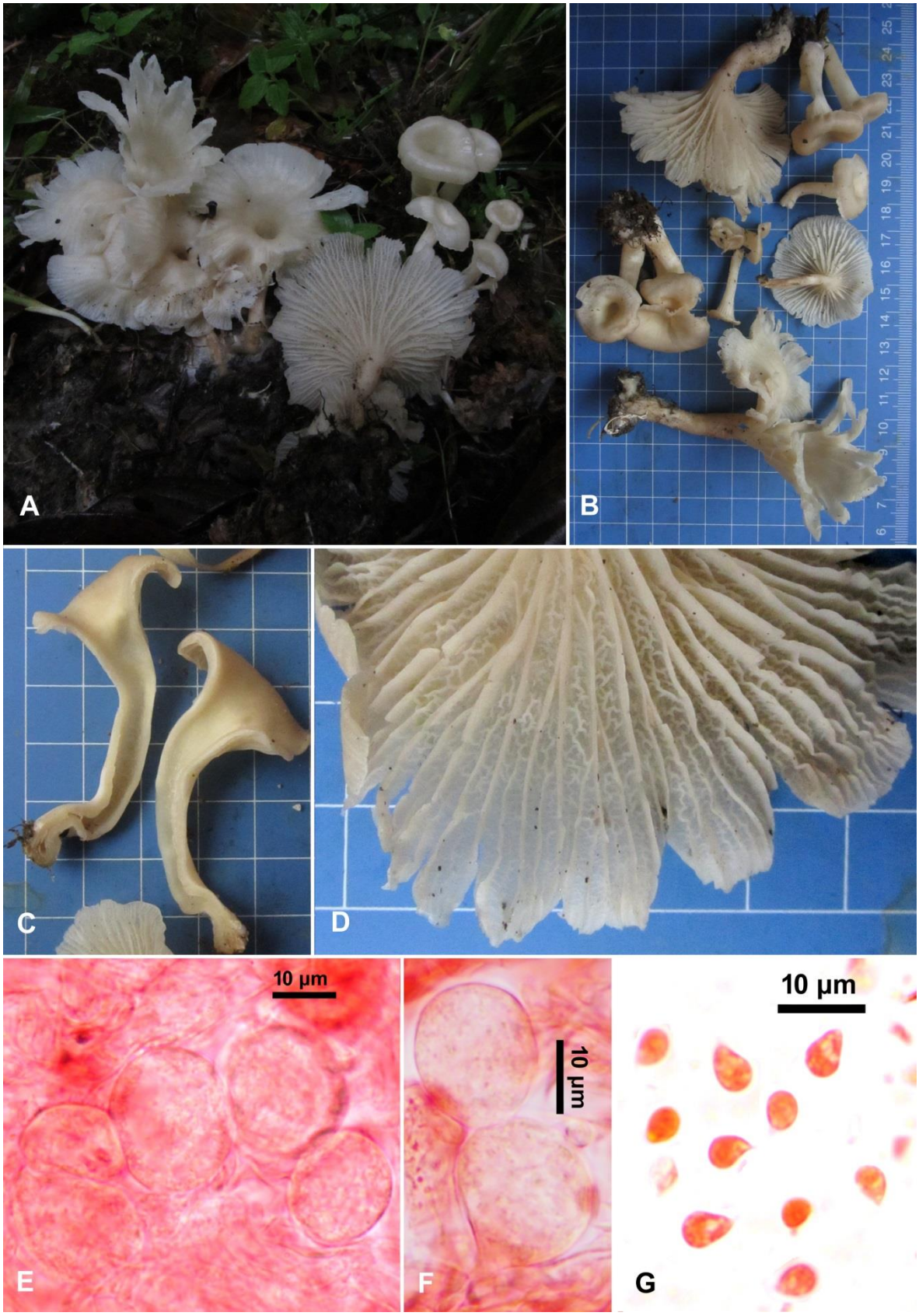

Figure 2: *Singerocybe alboinfundibuliformis* (KD 17-40). A–B. Basidiomata; C. Longitudinal section of basidiomata showing the hollow stipe; D. Narrow anastomosed gills; E. Photomicrograph of the cross-section of pileus showing pileipellis interspersed with vesicles; F. Photomicrograph showing vesicles; G. Photomicrograph showing the basidiospores.

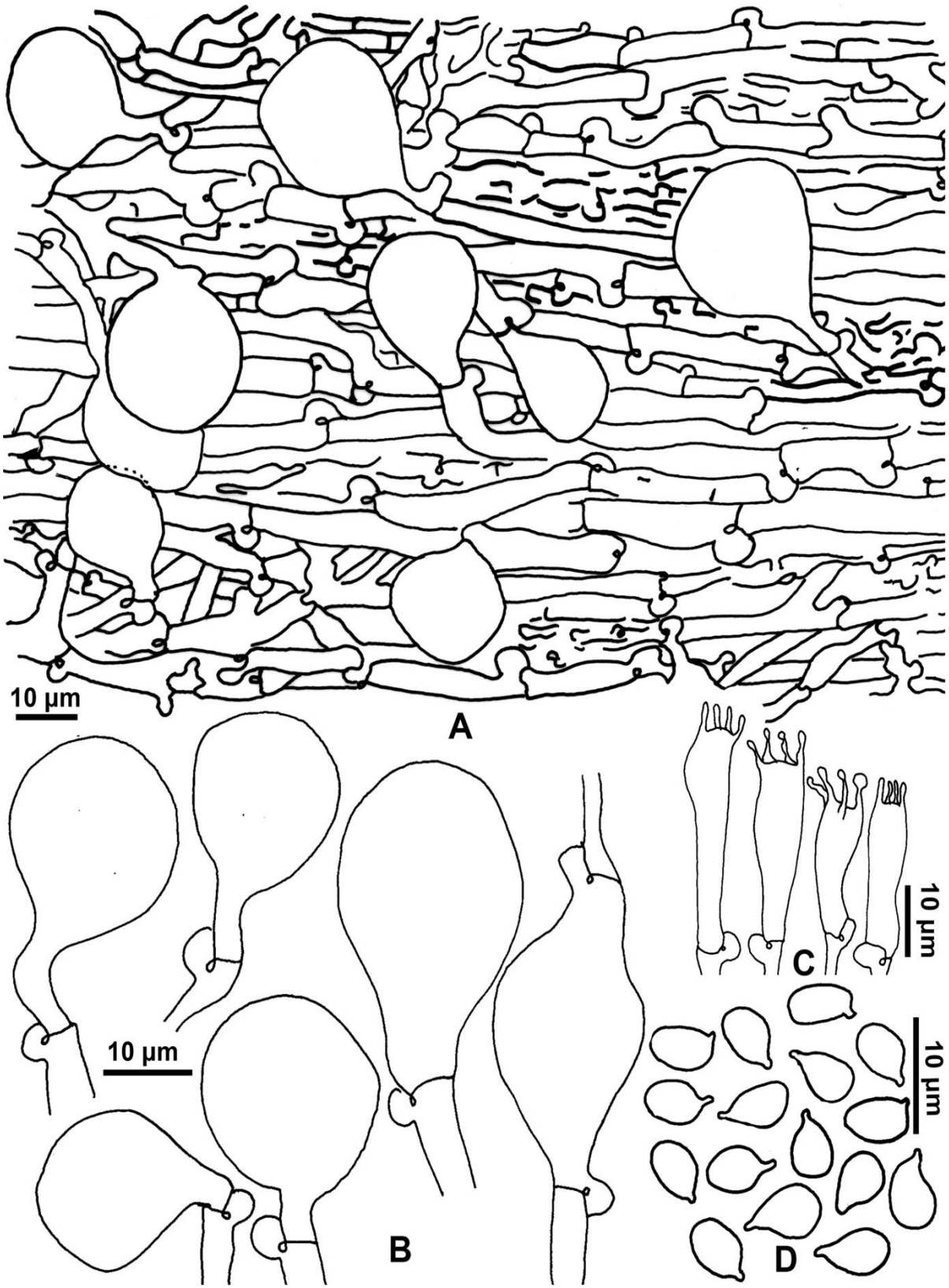

Figure 3: *Singerocybe alboinfundibuliformis* (KD 17-40). A, Cross-section through pileus showing pileipellis interspersed with vesicles; B, Vesicles; C, Basidia; D, Basidiospores.

Basidiospores 4.4–6.1–7.1 × 2.7–3.9–4.5 [n = 40; Q = 1.2–1.5–1.7], ellipsoid, smooth, thin-walled, hyaline, inamyloid. Stipitipellis same as pileipellis, interspersed with vesicles.

Habitat

Caespitose to gregarious, rarely scattered, under *Castanopsis* sp. and *Quercus* sp., in temperate broadleaf forests.

Specimens examined

India, Sikkim, East district, Fambonglho wildlife sanctuary, 27°21.631″N 88°33.922″E, alt. 2098 m, 26.08.2017, *K. Das*, KD 17-40 (CAL); Uttarakhand, Pauri district, Buakhal, 30°07.325″N 78°47.594″E, alt. 1708 m, 31.07.2017, *K. Das and M. E. Hembrom*, KD 17-17 (CAL).

DISCUSSION

The combination of morphological features like translucent nature of basidiomata and the presence of vesicles in pileipellis and stipitipellis placed the Indian collections as a member of the genus *Singerocybe* Harmaja. Further, with the help of existing key for the currently known species of *Singerocybe* (Qin *et al*., 2014), the present Indian specimens were identified as *S. alboinfundibuliformis.* The macro- and micromorphological details of the Indian collections also agreed with description of *S. alboinfundibuliformis* published by Seok *et al*., 2009, (as *Clitocybe alboinfundibuliformis*) and other available descriptions provided by Qin *et al*. (2014) and Kumla *et al.* (2016). The presence of a deeply infundibuliform white pileus, narrow and intervenose gills, a transluscently striate pileus margin and ellipsoid smooth basidiospores easily distinguish it from other known species of *Singerocybe*. Among these *S. phaeophthalma* (Pers.) Harmaja and *S. viscida* Harmaja (both known only from Europe; Kuyper, 1981; Harmaja, 1988), *S. adirondackensis* (Peck) Zhu L. Yang & J. Qin (known from North America; Peck 1873; Qin *et al*., 2014) and *S. clitocyboides* (Cooke & Massee) Zhu L.Yang, J. Qin & G.M. Gates (known from Australia and New Zealand; Pegler 1965; Qin *et al*. 2014) are not yet known from Asia. Macromorphological features like the shape of the pileus, stipe and lamellae are mainly used to differentiate various species in *Singerocybe*. However, the differences in micromorphological features among the species are not quite distinct (Qin *et al*., 2014) and are considered insufficient species differentiation in the genus *Singerocybe*.

In the field, *Singerocybe alboinfundibuliformis* resembles *S. humilis* (Berk. & Broome) Zhu L. Yang & J. Qin which was originally reported from Peradeniya, Sri Lanka), but the presence of narrow intervenose gills in the former distinguishes it from the latter. Moreover, the habitat of *S. humilis* is tropical, whereas the present species grows in temperate Himalayan forests. *Singerocybe clitocyboides*, *S. adirondackensis* and *S. phaeophthalma* can be separated by their shallowly depressed pileus and partially stuffed stipe (deeply infundibuliform pileus and tubular stipe in S. *alboinfundibuliformis*). Another Asian species *S. umbilicata* Zhu L. Yang & J. Qin can easily be differentiated by wavy to undulate (never striate) margin, one- or two-spored basidia and slightly larger basidiospores (Qin *et al*., 2014). Finally, *S. viscida* has slightly viscid pileal surface (Harmaja, 1988).

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