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Research Article

MORPHOLOGY AND PHYLOGENY OF *RETIBOLETUS KAUFFMANII* (BOLETACEAE): A NEW RECORD OF WILD MUSHROOM FROM INDIAN HIMALAYA

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ABSTRACT
Retiboletus kauffmanii is reported for the first time from India. Details of macro- and micro-morphology along with nrLSU-based phylogenetical analysis are presented for the Indian collection. Similar looking Asian or extra limital species are compared.

Keywords: *Boletales, Macrofungi, Phylogenetic Study, Sikkim, Taxonomy*

INTRODUCTION
Continuous development in molecular phylogeny corresponds with the revolutionary changes on the systematic studies of boletes in recent years (Nuhn *et al.*, 2013; Wu *et al.*, 2014, 2016). The family Boletaceae (one of the 17 families in Boletales) alone has a worldwide representation of 66 generic clades including nearly 1050 species (Li *et al.*, 2016; Wu *et al.*, 2016). The genus *Retiboletus* Binder & Bresinsky (2002) represented by one of these 66 generic clades is mainly characterised morphologically by: dry to subtomentose, black, dark gray, mustard yellow to olive brown colored pileus; pallid, grayish to yellow tubes and pores; centrally placed stipe with reticulation; pallid, yellow or vividly yellow context that changes slightly or to orange brown on bruising; smooth basidiospores; presence of cystidia and absence of clamp connections. Recent molecular phylogeny of the family Boletaceae placed them under subfamily *Leccinoideae* i.e. clade 15 (Wu *et al.*, 2014). Combination of molecular phylogenetic and morphological studies also clearly shows the existence of 13 distinct species from all over the world (Zeng *et al.*, 2016).
Sikkim (one of the Himalayan states in India) which comes under one of the 18 biodiversity hot spots of the world was seriously under explored in terms of boletes until recent past (since 2008) when Botanical Survey of India undertook the extensive and intensive macrofungal explorations of fleshy wild mushrooms. During a routine foray to South and East district of Sikkim in 2016 two (KD & DC) of us collected a number of Boletes. Thorough morphological examination followed by LSU-based phylogenetic analysis of these mushrooms revealed one of them as *Retiboletus kauffmanii*, a recently established species from China. We report this species for the first time from India.

MATERIALS AND METHODS
Morphology
Macromorphological features were recorded from the fresh basidiomata in the field, including macro chemical (with KOH, $FeSO_4$ and Guiacol) colour-reactions, prior to drying with a portable dryer. Photographs of fresh basidiomata and microphotographs were taken with Cannon SX 220 HS and Nikon-DS-R*i*1 (dedicated to Nikon Eclipse N*i* compound microscope) cameras. Colour codes and terms mostly follow Kornerup & Wanscher (1978). Micro morphological characters were recorded with the help of compound microscope (Nikon Eclipse N*i*) from free hand sections of dry samples mounted in 5 % KOH, or stained in a mixture of 5 % KOH and phloxin and mounted in 30 % glycerol. Drawings were made with a drawing tube (attached to Nikon Eclipse N*i* microscope) at 1000×. Basidium length excludes

length of sterigmata. Spore-measurements were recorded in profile view from twenty basidiospores. Spore-size measurements and length/width ratios (Q) are given as: minimum–*mean*–maximum. Herbarium codes follow Thiers (continuously updated).

DNA Extraction, Polymerase Chain Reaction (PCR) and Sequencing

Genomic DNA was extracted from 100 mg of dried basidiome using the InstaGeneTM Matrix Genomic DNA isolation kit (Biorad, USA) following the manufacturer's instructions. The Nuclear LSU region was amplified with primer pairs LR0R and LR5 (Vilgalys & 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 sec 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 to procure the accession number (KY290586).

Phylogenetic Analysis

Phylogenetic analyses based on nrLSU sequence data were carried out to establish the phylogenetic placement of our isolated taxon. The reference sequences and outgroup taxa were selected from relevant literature (Zeng *et al.,* 2016; Desjardin *et al.,* 2009) and information retrieved from GenBank. All sequences were aligned with MAFFT v. 7 (Katoh *et al.,* 2002). No manual editing was done within the alignment. Phylogenetic analysis was conducted with Maximum Likelihood (ML) method implemented in MEGA 6.0 (Tamura *et al.,* 2013). One-thousand bootstrap replicates were analysed to obtain nodal support values. *Spongiforma thailandica* and *S. squarepantsii* were chosen as out group taxa.

RESULTS AND DISCUSSION

Phylogeny

Our LSU-based phylogenetic analysis (Figure 1) with 34 LSU sequences (including the present species) resolved genus *Retiboletus* with full support. This analysis clearly supports the studies undertaken by Zeng *et al.,* (2016) indicating the existence of 13 species level subclades within the genus *Retiboletus*. Out of existing 13 subclades six (*R. sinensis*, *R. kauffmanii*, *R. zhangfeii*, *R. pseudogriseus*, *R. fuscus*, *Retiboletus* sp. HKAS 52680) are represented by China, five (*R. ornatipes*, *R. retipes* 1 & 2, *R. griseus*, *Retiboletus* sp. AF456813) are represented by USA, one (*R. nigerrimus*) from Japan and one (*R. flavoniger*) from Costa Rica.

Like the analysis of Zeng *et al.,* (2016) our analysis also shows polyphyletic existence of *R. retipes* (American species showing as "R. retipes 1" and "R. retipes 2" respectively in Figure 1). Present Indian collection (DC 16-58) is recovered here as sister to the Chinese *R. kauffmanii* with very high support (100% BS). The 100% bootstrap value supports the monophyly of R. kauffmanii + DC 16-58. In spite of our repeated efforts we failed to get good ITS sequence which further could give addition support to this interesting species.

Taxonomy

Retiboletus kauffmanii (Lohwag) N.K. Zeng & Zhu L. Yang; Mycologia 108, 2016 Figures 2 & 3.

Pileus 45–62 mm diam, subhemispherical to convex, sometimes helmet-shaped; surface dry, with blunt warts (verrucae) or blister-like protuberances, greyish brown (5D3) or brown (5E4), olive yellow (3D7) with NH_4OH and KOH and unchanging with $FeSO_4$ on pileus surface; margin with sterile flap of tissue. Pore surface light yellow to yellow (2A5–2A6), slightly darker when bruised; pores 0.8 per mm, rounded to somewhat angular, simple to compound. Tubes 5–7 mm long, subdecurrent to decurrent, concolorous to pore surface. Stipe 95–120 × 20–30 mm, central, cylindrical, gradually broader towards base; surface distinctly covered with reticulation (covering 1/3 to 1/2 of the stipe length from apex) which gradually becoming indistinct towards base; light yellow (2A5), slightly paler towards base, brownish orange (5C6) with NH_4OH, reddish brown (8F8) with KOH and unchanging with $FeSO_4$. Context pastel yellow (1A4) at pileus, greenish yellow (1A7 and 1A8) with NH_4OH and KOH; pastel yellow (2A4) or darker at stipe but yellowish orange (4A6) at base. Odor indistinct. Spore print olive-brown (4D4).

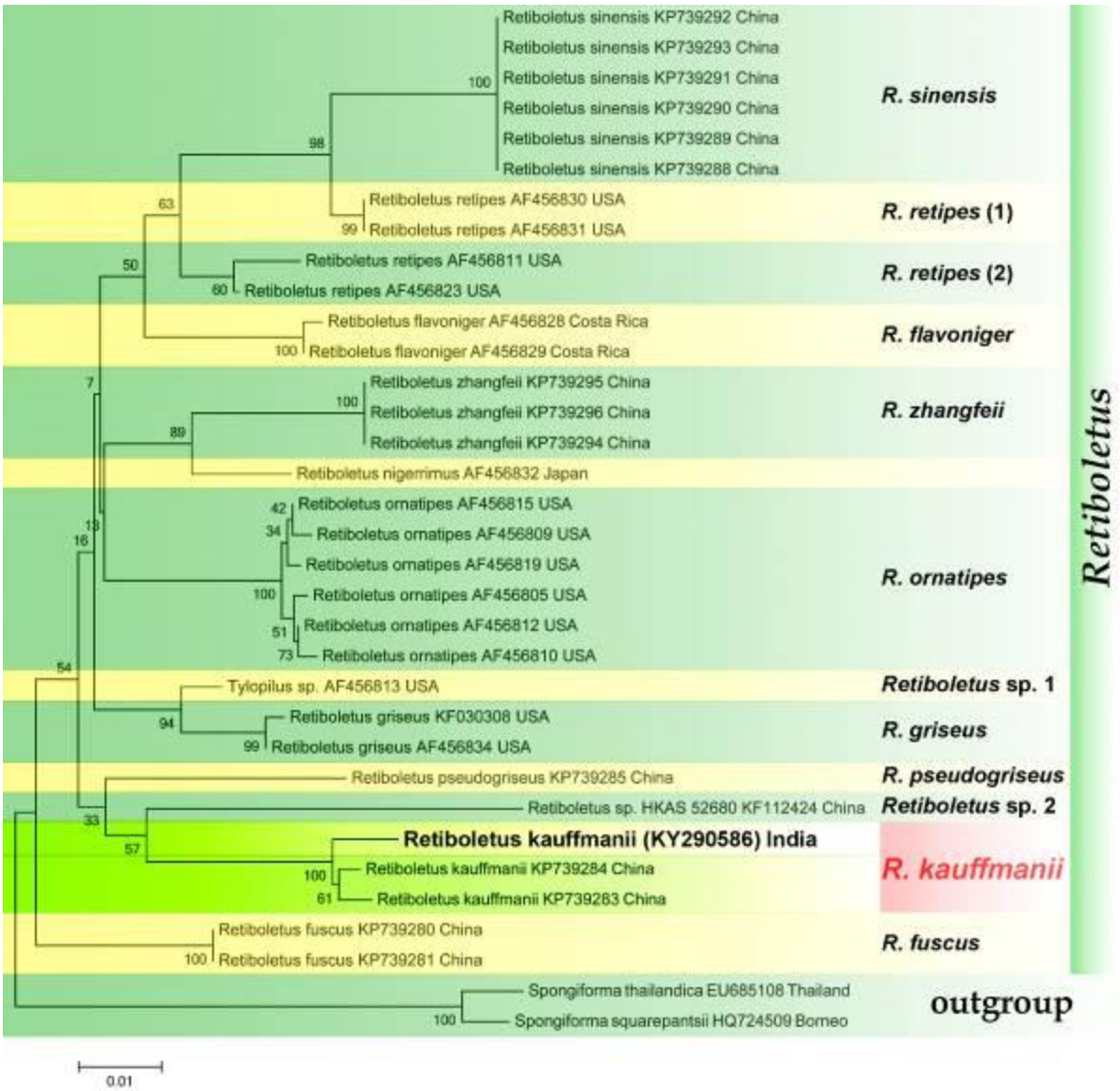

Figure 1: Phylogeny of DC 16-58 (Indian Collection of *Retiboletus Kauffmanii*, is in Bold and Black Font) Inferred from Maximum Likelihood Analysis of nrLSU Sequences Using MEGA 6.0

Basidiospores 9.9–11.8–13 × 4.3–4.6–5.1 µm (n = 20, Q = 2.25–2.53–2.79), oblong, inequilateral, smooth under light microscope, olivacious brown. Basidia 28–44 ×11–15 µm, 4-spored, clavate to subclavate. Pleurocystidia 45–60 × 8–12 µm, common, subfusiform to ventricose; content dense, granular, yellowish brown in KOH. Tube edge fertile, composed of basidia and cheilocystidia. Cheilocystidia shorter than pleurocystidia, 34–50 × 7–11 µm, cylindrical to subventricose. Hymenophoral trama divergent. Pileipellis 180–250 µm thick, a trichodermium, composed of more or less vertically arranged, sometimes interwoven, olive brown pigmented, highly encrusted hyphae; terminal cells 30–100 × 10–16 µm, cylindrical to subfusoid, with rounded apex, no color change in Melzer's reagent; incrustations in the form of rings or belts placed in parallel to subparallel tiers. Stipitipellis 40–60 µm thick, fertile, composed of caulobasidia, caulocystidia and marginal cells. Caulobasidia 30–46 × 9–12 µm, clavate, 2- to 4-spored. Caulocystidia 20–63 × 8–13 µm, subfusiform to fusiform, ventricose or appendiculate, mostly brown pigmented, content granular. Clamp connections absent in all tissues.

Habitat/Distribution:—Gregarious, under *Lithocarpus* sp., in temperate broadleaf forest (dominated by *Lithocarpus*, *Castanopsis* and *Quercus*) of Sikkim.
Specimen Examined:—India: Sikkim, East Distr., Maenam Top, alt. 2315 m, N27°19'18.7'' E88°22'07.9'', 21 Aug. 2016, *K. Das & D. Chakraborty*, DC 16-058.

Figure 2: *Retiboletus kauffmanii* (DC 16-58); A & B: Fresh and Dissected Basidiomata; C: Pore Surface; D: Transverse Section through Pileipellis; E & F: Heavily Encrusted Hyphae of Pileipellis; G: Caulocystidia; H: Pleurocystidia; I & J: Transverse Section through Stipitipellis; K & L: Basidiospores in Phloxin and KOH Respectively; Scale bars: D = 100 µm; E–L = 10 µm

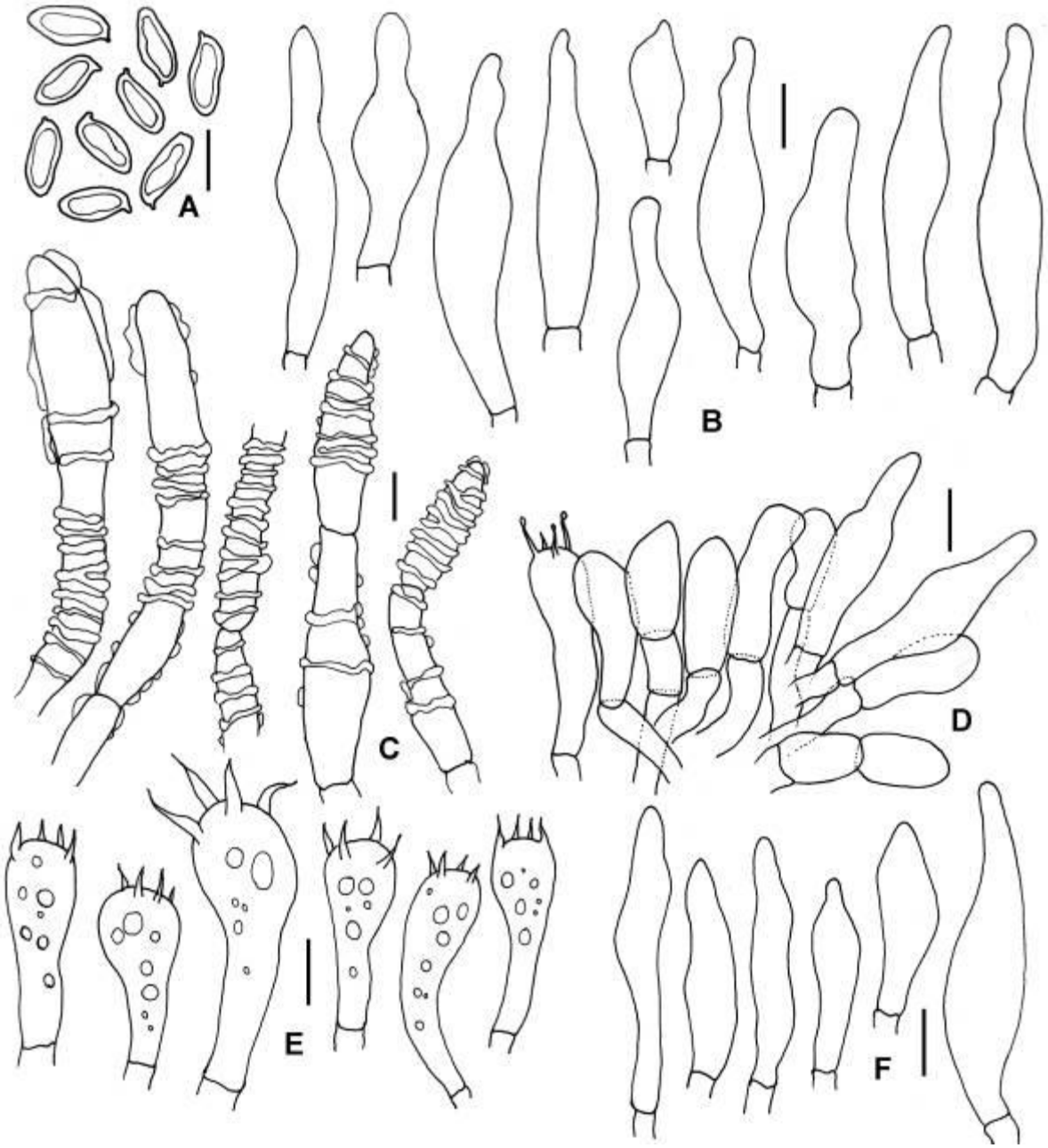

Figure 3: *Retiboletus kauffmanii* (Drawings from DC 16-58); A: Basidiospores; B: Caulocystidia; C: Terminal and Subterminal Cells of Heavily Encrusted Hyphae in Pileipellis; D: Transverse Section through Stipitipellis; E: Tube Basidia; F: Cheilocystidia; Scale Bars: A–F = 10 µm

Notes: Macro- and micro-morphology of the present Indian collection mostly agree with that of Chinese specimens of *R. kauffmanii* except the prominent verrucoid pilear surface in our specimen. But, these verrucae might be either anomalous growth or well be of teratological origin. Present description is based on two basidiomata from a single collection site. So, additional collections are required to confirm the stability of this macro-morphological feature. Micro-morphologically, unlike the Chinese material Indian collection possesses encrusted hyphae (in pileipellis) which could be considered as a diagnostic feature for an undescribed species if the phylogeny is allowed us to do so. Our nrLSU-based analysis is not adequate to discriminate it up to a species rank, rather it indicate the conspecificity of Indian and Chinese

collections. Until more field numbers are examined, those features can be considered here as morphological variations within *R. kauffmanii* only.

Retiboletus sinensis N.K. Zeng & Zhu L. Yang (represented by KP739288 to KP739293 in Figure 1) is morphologically close to *R. kauffmanii* but, differs by absence of wart or blister-like numerous projections on pileus surface, presence of yellow pores which turns brownish when injured, reticulatios on stipe surface that extended nearly up to base and hyphal elements of pileipellis without any ring like incrustations and smaller basidiospores (8–10 × 3.5–4 µm) as mentioned in Zeng *et al.*, (2016).

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