



***Thelephora sikkimensis* sp. nov. (Thelephoraceae) from the Eastern Himalayas (India)**

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With 3 figures

Abstract: Thelephoroid fungi are abundant ectotrophic symbionts in temperate forests worldwide. The state of Sikkim, in India, constitutes a hot spot of fungal diversity due to the occurrence of many ectomycorrhizal tree species. Collections of a stipitate thelephoroid species were found under *Castanopsis hystrix* in the district South of this state. Molecular and morphological analyses support the placement of these collections as a monophyletic unit that is proposed here as *Thelephora sikkimensis* sp. nov. This species is characterized by its pileate-stipitate basidiomes; zonate-hairy pileus surface, papillate-odontoid hymenophore and large basidia [35–65 (infrequently up to 125) × 7–9 µm]. Morphological details of this new species are presented along with its phylogenetic affinities estimated on nrITS-based analyses.

Key words: Basidiomycota, macrofungi, Thelephorales, Sikkim, systematics.

Introduction

Species of the genera *Thelephora* Ehrh. ex Willd. and *Tomentella* Pers. ex Pat. form a monophyletic group of worldwide distributed ectomycorrhizal fungi (Tedersoo et al. 2014). The limits between both genera are not yet clear and molecular phylogenies

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(e.g. Yorou et al. 2012, Kuhar et al. 2016) actually show them as a complex entity (Tedesoo et al. 2014) referred to as the *Thelephora-Tomentella* complex (e.g. Taylor & McCormick 2008) or *Thelephora-Tomentella* clade (e.g. Vizzini et al. 2016). Despite the probably non-monophyletic nature of both taxa as distinct genera, *Tomentella* comprises fully resupinate species. Under *Thelephora*, however, more complex basidiomes occur, ranging from clavarioid, flabelliform to clearly stipitate (Ramírez-López et al. 2013). Indeed, high variation is reported even at intraspecific levels. Kõljalg et al. (1998) and Yorou & Agerer (2007) have proposed the conspecificity of the pileate *Thelephora terrestris* and the resupinate *Tomentella radiosa*. Since the confirmation of the ectotrophic nutritional form of species within this group, many works report high abundance of ectomorphotypes of both genera in temperate broadleaf and conifer forests and other climatic zones worldwide (e.g. Gao & Yang 2010, Nouhra et al. 2013, Álvarez-Manjarrez et al. 2016). Modern environmental sequencing methods have also revealed unexpected diversity within this group in this type of forests, but a deeper knowledge of the species from the morphoecological point of view is needed both to better interpret the information provided by metagenomic studies (Peay 2014) and to find the connection between ectomycorrhizal root tips and the species name (Jakucs & Erős-Honti 2008). Moreover, the recent segregation of species formerly treated as *Tomentella* to the saprotrophic genus *Odontia* (Tedesoo et al. 2014) implies that the correct phylogenetic placement of species within closely related groups provides invaluable information on the trophic mode of the studied taxa.

The state of Sikkim in the eastern Himalayas (India) displays a high diversity of landscapes and climatic zones due to the great altitudinal gradient and a great variety of geomorphological features. The biological richness of this state has attracted the attention of many naturalists like Joseph Dalton Hooker, who made innumerable notes and plant collections in the mid-nineteenth century. However, places like the Maenam Wildlife Sanctuary, with 2295 mm of annual rainfall mostly concentrated between June and September, are still a hot spot of hidden biological diversity (Das & Verbeke 2011). One of the most interesting communities is the ectotrophic mycobiota associated with *Castanopsis tribuloides* A. DC., *C. hystrix* A. DC., *Quercus lamellosa* Sm., *Schima wallichii* Choisy, *Lithocarpus pachyphyllus* Rehder, *Alnus nepalensis* D. Don, *Abies densa* Griff., *A. spectabilis* Spach, and *Pinus wallichiana* A.B. Jacks. among other ECM trees in the temperate forests of this natural reserve.

Probably due to their infrequent and sometimes inconspicuous fructifications, species within the Thelephorales have received less attention than other ectomycorrhizal taxa. However, species have been recorded from other regions of India (Kaur et al. 2010) and a list of 56 resupinate species has been proposed by Dhingra (2016) based on morphological identifications. A high richness of species in the *Thelephora/Tomentella* lineage has been reported from molecular studies in *Kobresia* communities in Eastern Himalaya (Gao & Yang 2010). Only a few reports acknowledge the presence of non-resupinate forms (Ghate & Sridhar 2016). During two recent macrofungi expedition in 2016 and 2017, basidiomes of a stipitate species were found in the aforementioned sanctuary of this state under *Castanopsis hystrix*. Morphological and molecular evidences from these collections revealed a novel species, which is described here as *Thelephora sikkimensis* sp. nov.

Materials and methods

MORPHOLOGICAL STUDIES: Ecological notes and macromorphological features were recorded from young to mature fresh basidiomes in the field and/or basecamp. Samples were dried with hot air using a portable field dryer. Photographs of fresh and dissected basidiomes were captured with a Canon SX 120 camera, whereas microphotography were made with Nikon-DS-Ni1 (attached to a Nikon Eclipse Ni-U compound microscope) camera. Color codes and terms are implemented mostly according to Methuen Handbook of Color (Kornerup & Wanscher 1978). Micromorphological characterization were made with the aid of two compound microscopes (Nikon Eclipse Ni-U and Olympus CX 41) from free-hand sections of dry basidiomes mounted in 5% KOH, or stained in a mixture of 5% KOH and phloxin and mounted in 30% glycerol or distilled water. Micromorphological drawings were prepared with the help of a drawing tube (attached and dedicated to Olympus CX 41) at 1000× magnification. Basidium length excludes length of sterigmata. Spore-measurements were recorded in profile view from twenty basidiospores (from spore print) excluding the ornamentations. Spore-size measurements and length/width ratios (Q) are given as: minimum–(mean)–maximum. SEM studies of basidiospores were performed following Ghosh et al. (2016). Herbarium codes follow Thiers (continuously updated).

DNA EXTRACTION, PCR AND SEQUENCING: Genomic DNA was extracted from 100 mg of a dry basidiome from each of the two collections (KD 16-003 and KD 16-042) using the InstaGene™ Matrix Genomic DNA isolation kit (Biorad, USA) following the manufacturer's instructions. For PCR amplification of ITS region, ITS1 (forward) and ITS4 (reverse) primers were used (White et al. 1990). PCR amplification on "ABI Veriti" thermal cycler protocol was (for this ITS region) 2 min at 94°C, 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. Amplified PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany). Both strands of the PCR fragments of every collection were sequenced on the 3730xl DNA Analyzer (Applied Biosystems, USA) using the PCR primers and then assembled using Sequencher (Gene Codes Corporation, USA). ITS sequence data (accession numbers: MF684017 and MF684018) was deposited in GenBank (<http://www.ncbi.nlm.gov>).

PHYLOGENETIC ANALYSIS: Similar sequences were retrieved from the public database UNITE (<https://unite.ut.ee/>) (Köljalg et al. 2013) using "Blastn" and Genbank (<http://www.ncbi.nlm.gov>) using Megablast. Only sequences showing high similarity were used in the analyses, as well as every available sequence of stipitate – theleporoid materials and geographically related species within the *Thelephora/Tomentella* clade. Low quality sequences were excluded from the matrix. Sequences of the sister genus (Tedersoo et al. 2014) *Odontia* Pers. (accession numbers: UDB028039 and UDB028043) were chosen as outgroup.

The resulting dataset was aligned using L-INS-i strategy as implemented in MAFFT v 7.0 (Katoh & Standley 2013). Maximum Likelihood analyses were performed in PhyML using SMS: (Smart Model Selection) (Lefort et al. 2017). The best model estimated was K80+G+I with ML optimized equilibrium frequencies, proportion of invariable sites 0.240, number of substitution rate categories: 4 and gamma shape parameter estimated: 0.677 and the Bootstrap branch support was calculated after 1000 replicates.

The output parameters of SMS PhyML were used in for the estimation of the Bayesian posterior probabilities in MrBayes using the same MAFFT aligned matrix (Huelsenbeck & Ronquist 2001). The number of generations was set in 8 000 000 starting with a random tree and using four simultaneous chains. The first 80 000 generations (i.e., 8000 trees) were discarded as the burn-in. TRACER1 (<http://evolve.zoo.ox.ac.uk/software.html/tracer/>) was used to ensure that stationarity was reached after the first 100 000 generations (Rambaut et al. 2013).

Results

Phylogeny

The nrITS-based phylogenetic analyses (Fig. 1) could not clearly resolve deeper nodes within the *Tomentella/Thelephora* clade. However the collections of the described

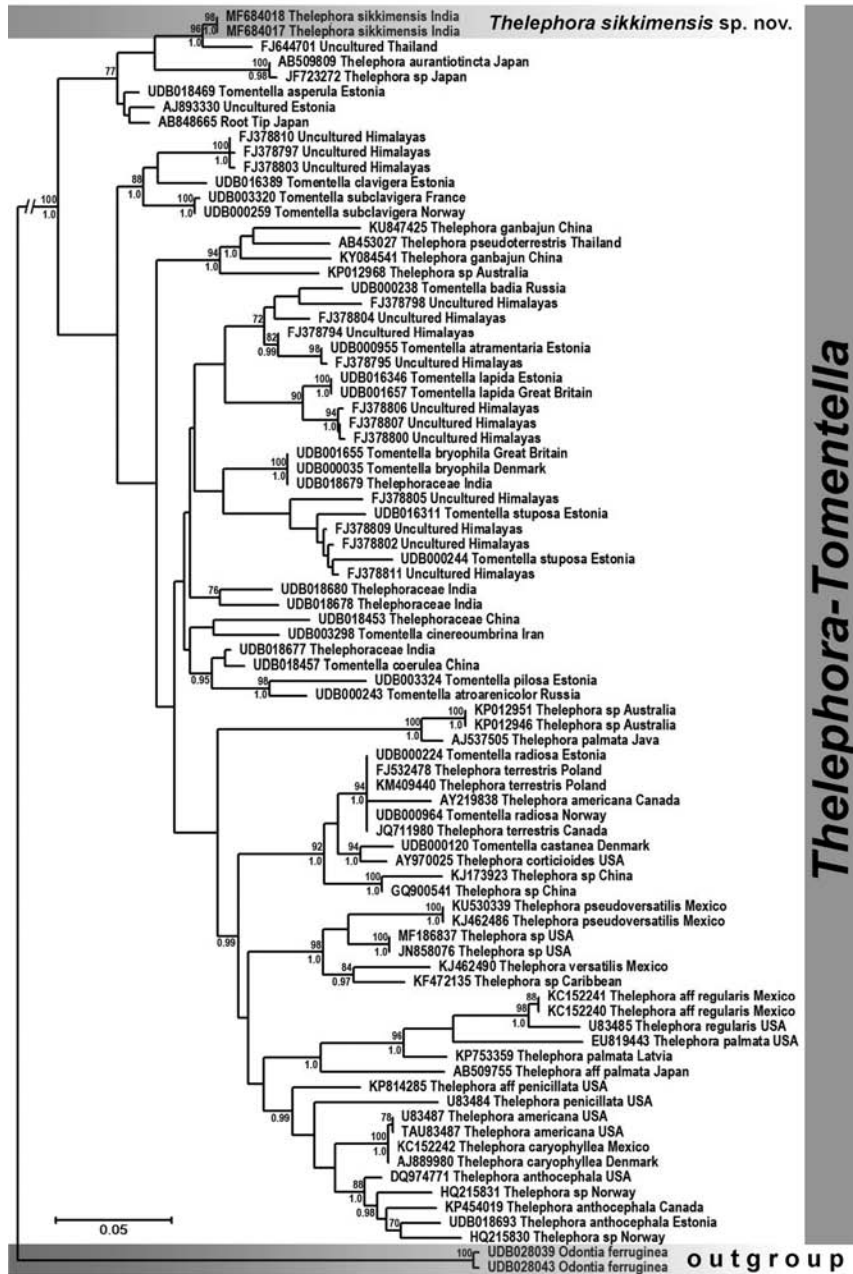


Fig. 1. Maximum Likelihood (ML) analysis of ITS sequences showing the affinities of *Thelephora sikkimensis*. Significant Bootstrap values from 1000 replicates and Bayesian posterior probability are indicated as support above and below the branches respectively. Scale bar indicates number of substitutions per site in the ML analysis.

species (KD 16-003 and KD 16-042) represented by its GenBank accession numbers (MF684017 and MF684018 respectively) are clearly nested amongst other *Thelephora-Tomentella* species as a distinct species in a strongly supported clade (98% ML bootstrap and 1.0 Bayesian posterior probability) close to sequences of an undescribed Thai species (GenBank accession number FJ644701).

Taxonomy

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Figs 2, 3

MycoBank No.: MB 822639.

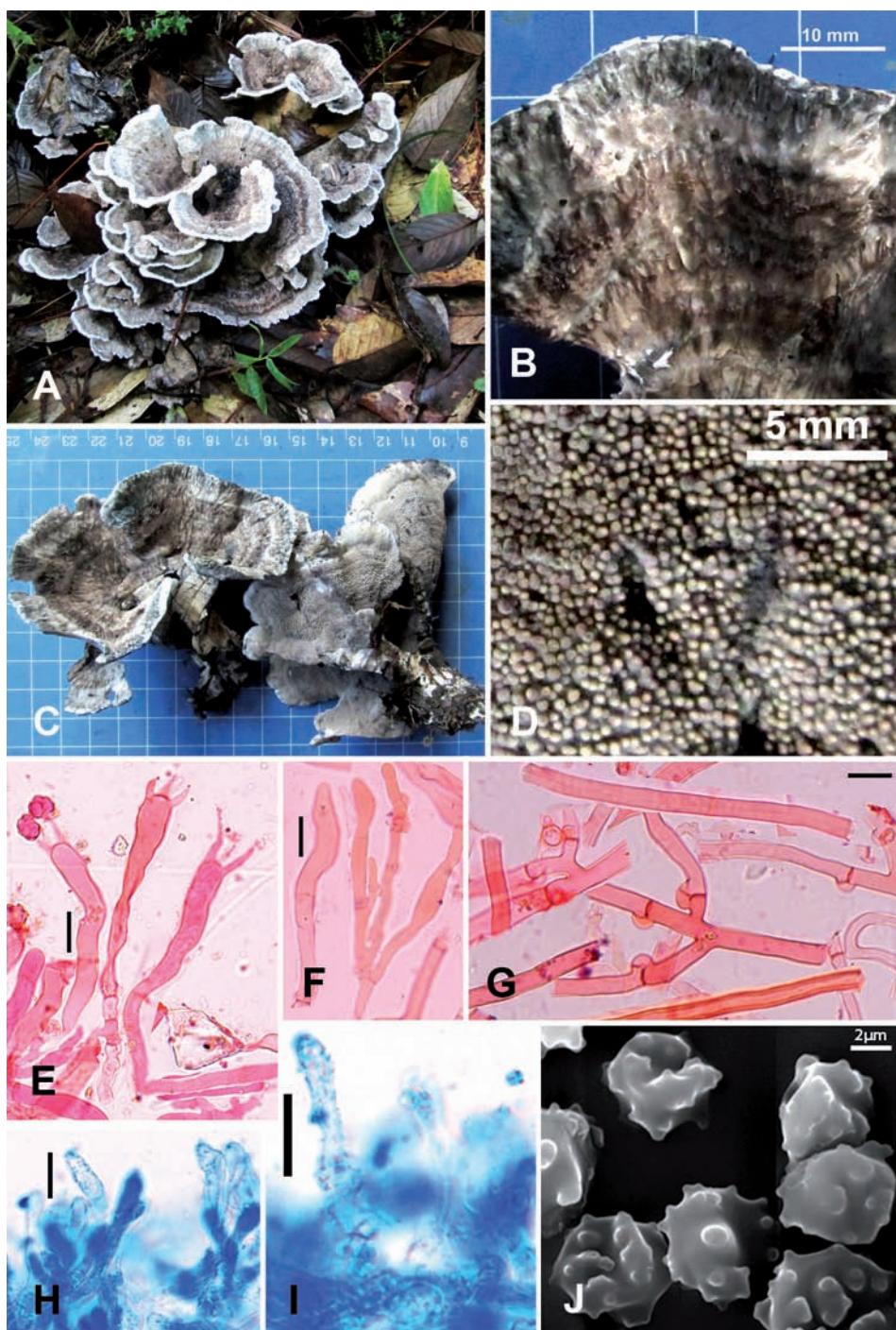
ETYMOLOGY: Referring to the Indian-Himalayan state of Sikkim, the type locality.

HOLOTYPE: CAL 1600

DIAGNOSIS: Pileate basidiomes, smooth, soft and brittle in consistency, with papillate to odontoid pale orange to turquoise hymenium.

BASIDIOMES 50–100 mm high, annual, gregarious to caespitose, leathery and moist when fresh, soft and brittle on drying, effused-reflexed, pileate to stipitate. PILEUS 30–70 × 27–75 × 0.5–2 mm, appanate to ligulate, imbricate, with multiple pilei arising from a common stipe or base and wrapping around to form rosette shape, with smaller pilei frequently arising from an old basidiome; pilear surface sulcate, zonate, hairy, charcoal black near the center then purplish grey to greyish magenta (13E2–13E3) to gradually grey–reddish grey (12D1–12D2) towards margin and becoming chalky white at the margin when fresh, orange white (5A2) with patches of brownish grey (5F2) on drying. MARGIN sterile, obtuse and entire to slightly undulate when fresh while acute and crenate dentate to almost lobed in older basidiomes, chalky white when young while gradually changes to grey to reddish grey (12D1–12D2) in older one. HYMENOPHORE papillate to odontoid, decurrent, papillae 0.5–2 mm high, 1–3 per mm, from gradually smooth to irregularly plicate near the margin, pale orange to orange white (5A3–6A2); overall hymenophore turquoise white to turquoise grey (24D1–24D2) to greyish turquoise (24C1), orange white (5A2) to almost white near the margin. CONTEXT up to 2 mm thick towards the base, homogenous, smooth, soft and brittle in consistency, chalky white. STIPE 30–50 × 3–10 mm, irregularly cylindrical, surface papillate to warted, glabrous, orange white (5A2) to pale orange (5A3).

HYPHAL SYSTEM monomitic, septate, irregularly clamped, thin- to thick-walled to almost solid, branched, hyaline. PILEIPELLIS and STIPETIPELLIS a trichoderm, hyphae 2.5–5 µm wide, rarely crystalline. CONTEXT hyphae 2.5–7 µm wide, interwoven, thin- to distinctly thick-walled (1–3 µm thick) to almost solid, branched at long intervals, smooth, hyaline. SUBHYMENIUM hyphae 3–5 µm wide, loosely interwoven, thin- to moderately thick-walled, frequently branched, densely crystalline. HYMENOPHORE composed of basidioles, basidia bearing abundant papillose basidiospores and infrequent, cylindrical cystidioles (smooth in 10% KOH), densely crystalline in Cotton blue, hyaline; basidia 35–65 (rarely up to 125) × 7–9 µm, elongated cylindrical to subclavate, (smooth in 10% KOH), densely crystalline in Cotton blue, 4-sterigmate, sterigmata 2–8 × 1–3 µm, hyaline; basidioles 30–50 × 3–10 µm, elongated cylindrical, smooth, hyaline. Teeth apex fertile, bearing abundant basidia and basidioles. Hymenophoral trama regular to



slightly divergent, with septate branched, clamped thin- to thick-walled hyphae (1.8–5 μm wide). BASIDIOSPORES 6–(7.3)–8.8 \times 5–(6.26)–7 μm , Q = 1–(1.17)–1.35, subglobose to irregularly lobed, with verruculose to verrucose and papillate ornamentations in groups from one to three, hyaline, acyanophilic, inamyloid, pale brown in KOH.

SPECIMENS EXAMINED: India, Sikkim, South district, Maenam Wild Life Sanctuary, 27°18'44.1"N, 88°21'53.6"E, 2096 m asl., 16th August 2016, K.Das, KD 16-003, (holotype: CAL 1600; isotype: BSHC 51744); *ibid.*, South district, Maenam Wild Life Sanctuary, 27°19'11.0"N, 88°22'02.4"E, 2185 m asl., 21st August 2016, K.Das, KD 16-42; *ibid.*, South district, Kewzing, 27°17'46.5"N, 88°21'26.6"E, 1888 m asl., 23rd August 2017, K.Das, KD 17-010 (CAL 1632).

HABITAT: Common, on soil, sometimes associated to buried woody matter under *Castanopsis hystrix* in temperate broadleaf forest.

NOTE: *Thelephora sikkimensis* is distinctively characterized by the combination of morphological features: papillate to odontoid hymenophore with presence of crystalline elements (in water and cotton blue) in correlation with the pale hymenial surface with turquoise tones and its occurrence under *Castanopsis*. The nrITS-based phylogenetic analyses (Fig. 1) show the placement of this novel species in a strongly supported clade that includes materials identified as *Thelephora aurantiotincta* (Corner). This species shares with *T. sikkimensis* the papillate hymenial surface and spore size and shape, but it can be easily distinguished from the new species by its azonate pale cream pileus and the pale to orange hymenial surface without turquoise tones (Corner 1968; Welden & Ovebro 1989). Another species nested in the same clade is *Tomentella asperula* (P. Karst.) Höhn. & Litsch., suggesting a close relation with our species despite its fully resupinate basidiome. *Thelephora sikkimensis* may also macro-morphologically resemble mature basidiomes of another Asian species; *T. ganbajun* Zang (originally described from China) in the field due to its pilaeate stipitate basidiomes. However, good diagnostic features to distinguish these species are the larger cystidia (52–80 \times 6–8 μm) and distinguishingly smaller basidia (25–35 \times 9–12 μm) of this species (Zang 1987) and its occurrence under *Pinus yunnanensis* as well as the clavarioid-palmate growth pattern and the fleshy and thicker consistency of *T. ganbajun* in young stages.

Discussion

The close phylogenetic affinity of *T. sikkimensis* with *T. aurantiotincta* is also well supported by morphological characters. Although being completely resupinate and bearing longer basidiospores (8–10 μm) (Köljalg 1996), *Tomentella asperula* shares with *T. sikkimensis* and *T. aurantiotincta* the strongly papillate to odontoid hymenophore structure and large basidia (50–62 \times 7–9 μm) (Köljalg 1996, Martini 2016). These

Fig. 2. Macro- and micromorphological features of *Thelephora sikkimensis* (CAL 1600, holotype). A & C. Fresh basidiomes in field or at basecamp: B. Pilear surface showing hairy zones and sulcus: D. Papillate hymenophore: E. Basidia and basidioles: F. Variations in generative hyphae: H. Encrusted pileipellis in cotton blue: I. Encrusted basidia in cotton blue: J. Basidiospores under SEM showing ornamentations. Scale bars: B 10 mm, D 5 mm, E–I 10 μm , J 2 μm .

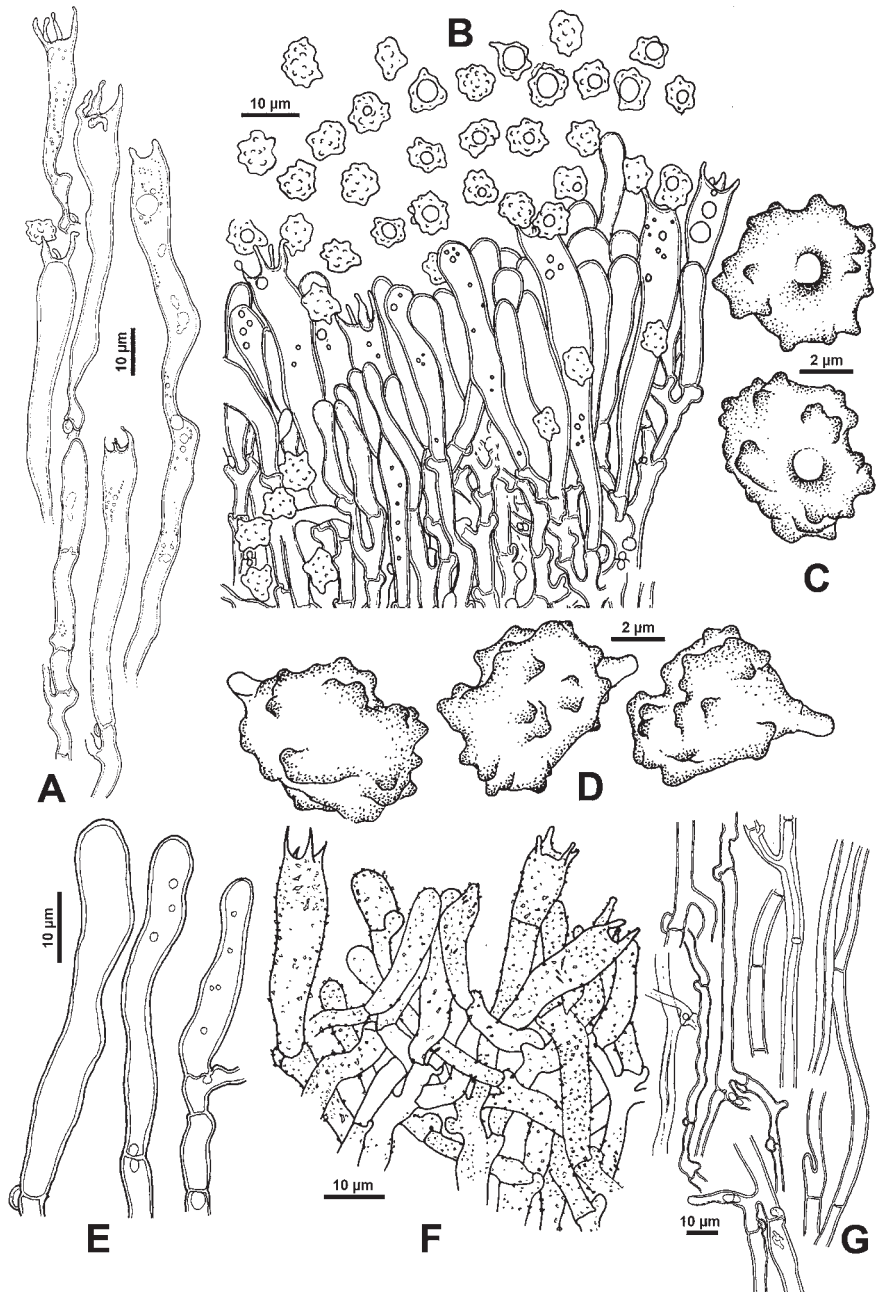


Fig. 3. Micromorphological line drawing of *Thelephora sikkimensis* (from CAL 1600, holotype). A. Basidia & cystidioles; B. Hymenophore showing basidia, basidioles and basidiospores; C. Frontal view of basidiospores; D. Lateral view of basidiospores; E. Basidioles; F. Encrusted basidia, basidioles and hyphae in cotton blue; G. Different types of generative hyphae.

morphological features also support the placement of this species of our molecular analysis and are also aligned with growing evidence that the pileate/resupinate constitution of the hymenophore do not show a clear phylogenetic signal separating the genera *Thelephora* and *Tomentella* (Yorou & Agerer 2007; Ramírez-López et al. 2015). The phylogenetic consistency of other features such as the hymenophore surface needs to be tested to determine whether it can act as a more informative character than the highly variable stipitate/resupinate configuration of the basidiome.

Many reports acknowledge the edibility (Wei 1986) and biomedical value of the closely related *T. aurantiotincta*. Anticancer activities (Norikura et al. 2011) as well as antioxidant effects (Liu et al. 2004) have been reported for this species. The fleshy consistency of *T. sikkimensis* may account for its edibility, but this remains to be verified. Biomedical activities also need to be tested in the new species but more collections are needed in order to verify these biochemical properties as well as the distribution and the host affinities.

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