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A new species of the genus *Hymenagaricus* (Basidiomycota) from Taiwan and its phylogenetic position inferred from ITS and nLSU sequences

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Abstract – A new species of the genus *Hymenagaricus* (*Agaricaceae*) collected from Taiwan, *H. taiwanensis*, is described and illustrated with line drawings. It is characterized by the yellow-brown pileus covered with fuscous black squamules, the white membranous annulus, ellipsoid basidiospores, and the pseudoparenchymatous elements in the pileal squamules which are often encrusted with brownish pigments. The analyses of internal transcribed spacer and large subunit of nuclear ribosomal DNA sequences suggest that *Hymenagaricus* has a close relationship with *Micropsalliota* and *Allopsalliota* (Agaricus clade) in the tribe *Agariceae*, and represents an independent line of evolution.

Agaricales / new taxon / phylogeny / rDNA / taxonomy

INTRODUCTION

The genus *Hymenagaricus* Heinem. harbours two subgenera in which at least 28 species are accommodated, and these are mainly equatorial-palaeotropical in distribution (Heinemann 1981; Heinemann & Little Flower 1984; Pegler 1986; Reid & Eicker 1995, 1998, 1999). It is distinguished from other genera in the tribe *Agariceae* (*Agaricaceae*) mainly by the hymenidermal to pseudoparenchymatous structure of the squamules on the pileus (pileal epicutis) confined to the central region of the pileal surface. Although this genus has become relatively well known, its systematic position based on molecular data has not yet been investigated. In this study, a new species of the genus *Hymenagaricus* collected from Taiwan is described. Its phylogenetic position is also investigated using the internal transcribed spacer (ITS) and large subunit of nuclear ribosomal DNA (nLSU-rDNA) sequences from the holotype.

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MATERIALS AND METHODS

Materials and morphological study

Material was collected in Taiwan by the second author. The holotype specimen is deposited in the Herbarium of Cryptogams, Kunming Institute of Botany of the Chinese Academy of Sciences (HKAS). The macro-morphological description is based on the field notes and colour slides of the material; micro-morphology is based on observation of the material under microscope. Colour names with first letters capitalized are from Ridgway (1912). Colour designations (*e.g.*, 8E5) are from Kornerup & Wanscher (1981). In the descriptions of basidiospores, the abbreviation [*n*/*m*/*p*] means *n* basidiospores measured from *m* basidiomata of *p* collections in 5% KOH solution; *Q* is used to mean "length/width ratio" of a spore in side view; *avQ* means average *Q* of all basidiospores ± sample standard deviation; *x* = means range of basidiospore length × width.

DNA extraction, PCR and sequencing

For molecular investigation, DNA was extracted from herbarium material of the holotype. Approximately 25 mg of tissue was ground in liquid nitrogen and extracted in 600 ml of extraction buffer (1% SDS, 0.15 M NaCl, 50 mM EDTA) at 75 °C for 1 h and purified with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with 95% ethanol and 3M NaCl overnight. Crude DNA extracts were diluted with distilled water up to 100-fold for use as PCR templates. The ITS and part of the nLSU gene of the nuclear ribosomal repeat were amplified by polymerase chain reaction (PCR). ITS regions were amplified and sequenced with fungal specific primers ITS1F and ITS4 (Gardes & Bruns, 1993). For nLSU region, LR0R and LR7 were used as PCR primers, and LR0R, LR5, LR16, and LR3R as sequencing primers (http://www.biology.duke.edu/fungi/mycolab). Sequencing reactions were purified using Pellet Paint (Novagen, Madison, Wisconsin, USA) and were run on an Applied Biosystems 377 XL automated DNA sequencer. Sequence chromatograms were compiled with Sequencher 4.1 software (GeneCodes Corporation, Ann Arbor, Michigan, USA). The ITS and nLSU sequences of the holotype generated in this study were deposited in GenBank with accession number DQ006271 and DQ006270 respectively.

Phylogenetic analyses

When investigating the phylogenetic relationships of *Hymenagaricus taiwanensis*, sequences of *H. taiwanensis* were compared with ITS/nLSU sequences of genera in the family *Agaricaceae*, and then three most homologous genera (*Agaricus*, *Micropsalliota* and *Allopsalliota*) in the tribe *Agariceae* and related taxa of *Chlorophyllum* were chosen for phylogenetic reconstruction. *Leucoagaricus americanus* and *Leucocoprinus cepaestipes* were used as outgroup. Sequences retrieved from GenBank and used in the analyses are as following: U85307, U85273, AF161014, U11911, AF482836, U85274, AF482849, AY176345, AF482835, AF482879, AF482857, AF482888, AY176407, AF482891, U85338 and U85306. ITS and nLSU sequences were combined since both ribosomal units are linked within a single array (Hillis & Dixon 1991), then aligned using Partial

Order Alignment (Lee *et al.* 2002), and minutely adjusted manually where necessary. The ITS/nLSU combined data matrix is available in NEXUS format on request. Phylogenetic calculations were run in PAUP* version 4.0b10 (Swofford, 2003). Likelihood and parsimony trees were generated using heuristic searches (with 1000 random taxon addition replicates, TBR swapping and collapse of zero-length branches), and character state changes were equally weighted in the analyses. Gaps were treated as missing data. Bootstrap analyses were estimated based on 1000 replicates with the heuristic search options using parsimony.

RESULTS

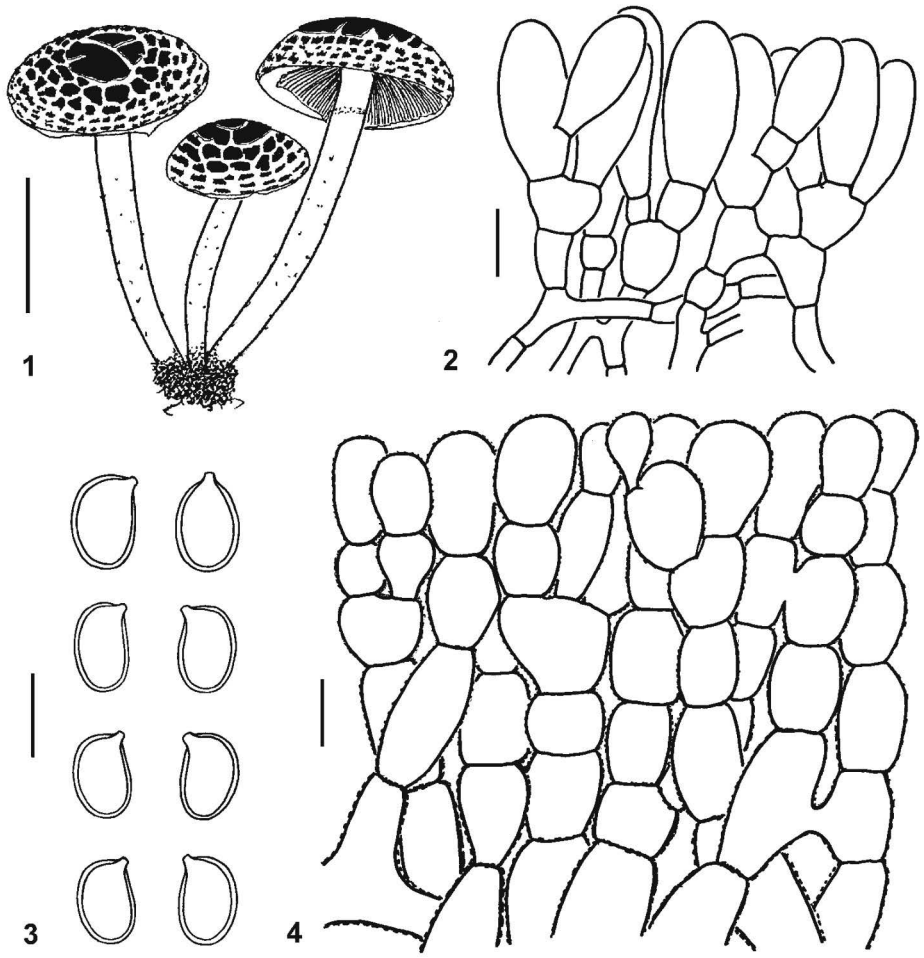
Taxonomy

Hymenagaricus taiwanensis Zhu L. Yang, Z. W. Ge & C. M. Chen, **sp. nov.**

Etymology: The specific epithet refers to the holotype locality of this species.
Pileus 1.5-3.5 cm latus, conico-campanulatus vel convexus, non viscidus, luteo-brunneus, squamulis atrobrunneis. Lamellae liberae, brunneae vel atrobrunneae. Stipes 4.0-7.0 × 0.6-0.9 (1.2) cm, subcylindricus, albus vel albidus, flavescens, glaber vel fibrillosus, annulatus. Annulus superus, albidus, membranaceus. Basidia clavata, 4-sporigera. Basidiosporae (4.5) 5.0-5.5 (6.0) × 3.0- 4.0 (4.5) µm, ellipsoideae, brunneoluteae. Pleurocystidia absentia. Cheilocystidia ellipsoidea vel obovoidea, (11) 20-28 (32) × 7.5-10 (14) µm. Squamulae pilei ex cellulis subglobsis, oblongo-ellipsoideis vel subclavatis compositae. Fibulae absentes. Habitatio: terrestris. Holotypus: C. M. Chen 3636 (HKAS 42545), 20 March 2003, Nantou, Taiwan, China.

Basidiomata (Fig.1) small to moderate, in groups, terrestrial. **Pileus** 1.5-3.5 cm in diam., conico-campanulate to hemispherical to convex, not umbonate; surface dry, yellow-brown, without any greenish tinge in the stages of development of basidioma, at first covered by a smooth, dark brown to fuscous black (Seal Brown to Fuscous Black; 8E5-8E6-8E7) pellicle soon disrupting except at the disc where it is retained as one or more large squamules, with small, scattered, dark brown squamules toward the margin; margin at first incurved, often appendiculate with white membranous remnants of annulus. **Lamellae** free, pinkish to pink when young, then becoming grayish pink (Livid Pink to Pale Brownish Vinaceous; 13A2 to 14A2), and finally brown to fuscous brown, up to 3 mm wide, densely crowded, with lamellulae of 2-3 lengths. **Stipe** central, 4.0-7.0 × 0.6-0.9 (1.2) cm, subcylindrical or somewhat attenuate toward the base, fistulous to hollow; surface white to whitish, becoming yellowish when bruised, smooth, sometimes covered with white fine squamules or fibrils. **Annulus** membranous, whitish, superior, fugacious or persistent. **Smell** indistinct.

Basidia 15-22 × 7-8 µm, clavate, hyaline, 4-spored; sterigmata up to 3 µm long. **Basidiospores** (Fig. 3) [50/3/1] (4.5) 5.0-5.5 (6.0) × 3.0-4.0 (4.5) µm (x = 5.18 ± 0.30 × 3.58 ± 0.33 µm), Q = (1.33) 1.38-1.67 (avQ = 1.46 ± 0.09), ellipsoid, brownish yellow, smooth, slightly thick-walled (< 0.5 µm thick), without germ pore, not or very weakly metachromatic in cresyl blue, congophilous. **Pleurocystidia** absent. **Cheilocystidia** (Fig. 2) (11) 20-28 (32) × 7.5-10 (14) µm, ellipsoid to subclavate, sometimes obovoid, colourless, hyaline, thin-walled. **Subhymenium layer** composed of thin-walled inflated subglobose to irregular cells 9-15 µm in diam. **Lamellar trama**



Figs 1-4. *Hymenagaricus taiwanensis* (from holotype). **1.** Basidiomata. **2.** Cheilocystidia. **3.** Basidiospores. **4.** Squamules on pileus. Bars: **1** = 2 cm; **2** = 10 μm; **3** = 5 μm; **4** = 10 μm.

regular, composed of sub-cylindrical hyphae (3) 6-8.5 (13) μm in width. **Squamules** on pileus (Fig. 4) 60-80 μm thick, composed of pseudoparenchymatous elements of agglutinated subglobose to oblong ellipsoid, sometimes subclavate, slightly thick-walled (about 0.5 μm thick) cells measuring 10-22 × 8-12 μm and often encrusted with brownish pigments. **Clamp connections** absent.

Habitat and known distribution: On soil, so far only known from Taiwan.

Material examined: CHINA: Taiwan, Nantou County, Jiujiufeng, 20 March 2003, C. M. Chen 3636 (HKAS 42545, holotype).

Commentary: *Hymenagaricus taiwanensis* is characterized by its ellipsoid basidiospores, and the fuscous black squamules on the pileus made up of a pseudoparenchymatous epithelium of agglutinated subglobose to subclavate, slightly thick-walled cells. Based on the form and structure of the pileus squamules, as well as the basidiospore colour, Heinemann & Little Flower (1984) divided the

genus *Hymenagaricus* into two subgenera, i.e., subgen. *Hymenagaricus*, with pileus squamules of a hymeniform structure and brown basidiospores, and subgen. *Xanthagaricus* with squamules of hymeniform to pseudoparenchymatous structure and often yellow basidiospores. *Hymenagaricus taiwanensis* may belong to the subgen. *Xanthagaricus* due to the brownish yellow basidiospores and the squamules on the pileus made up of pseudoparenchymatous elements.

H. taiwanensis is very similar to *H. subaeruginosus* (Berk. & Broome) Heinem. & Little Flower which was originally described from Sri Lanka, and belongs to subgenus *Xanthagaricus* Heinemann & Little Flower (1984). However, *H. subaeruginosus* has a distinctly umbonate pileus, a pileal surface with some dull greenish tints in the early stage of basidioma development, and "cymbiform" or ovoid basidiospores with a broad, almost truncated base (Heinemann & Little Flower 1984; Pegler 1986).

Because of the ellipsoid basidiospores and the absence of a greenish tinge in the pileus, *Hymenagaricus taiwanensis* is also similar to *H. myriostictus* (Berk. & Broome) Heinem. & Little Flower and *H. flavidorufus* (Berk. & Broome) Heinem. & Little Flower, both originally described from Sri Lanka. However, both taxa differ from *H. taiwanensis* in having an umbonate pileus with furfuraceous squamules made up of short chains of detersile sphaerocysts. Furthermore, *H. myriostictus* has relatively smaller basidiospores measuring (3.6) 3.7-4.4 × 2.6-3.0 μm, while *H. flavidorufus* has a white stipe context discolouring reddish on exposure (Heinemann & Little Flower 1984). The latter two species are so close that they were regarded as conspecific by Pegler (1986) under the name *Agaricus flavidorufus* Berk. & Broome.

Phylogenetic Results

The aligned ITS/nLSU dataset was 1760 base pairs in length (including gaps). After excluding regions (36-49, 61-62, 129, 143-277, 284-288, 294, 308, 326-332, 340, 347, 361-362, 545-546, 555-615, 659, 707-709, 729-741, 748-755, 791-822, 1298, 1313, 1749-1760) that were not unambiguously alignable (304 characters in total), 1456 characters were included in the analyses. Among these sites, 1217 were constant, 122 were variable but parsimony-uninformative, and 117 were parsimony-informative. Maximum likelihood (ML) and Maximum Parsimony (MP) analytic methods resulted in almost the same topology trees. Fig. 5 illustrates the ML tree (-ln = 4077.23492) calculated with HKY85 model (number of substitution types = 2; number of distinct data patterns under this model = 241) with bootstrap values (above branches, distance; below branches, parsimony). MP analysis of the combined ITS/nLSU sequences resulted in 2 most parsimonious trees, with a tree length (L) of 362, a consistency index (CI) of 0.749, a retention index (RI) of 0.573, and a rescaled consistency index (RC) of 0.429. The only difference between the two MP trees is that one of the two trees shows that *Chlorophyllum* is not a monophyletic group, but this result has not got uphold from the bootstrap analysis. Compared with the ML tree, one of the two MP trees shows the same topology with the ML tree as shown in Fig. 5, but with a slightly different bootstrap value. According to the ML analysis (Fig. 5), *Hymenagaricus taiwanensis* is nested in a clade of its own within *Agariceae*, and forms a sister clade of *Micropsalliota* and *Allopsalliota* with 70% bootstrap support. The Agaricus clade (including the genera *Agaricus*, *Micropsalliota*, *Allopsalliota* and *Hymenagaricus* of tribe *Agariceae*) is a monophyletic group with a strong (95%) bootstrap uphold.

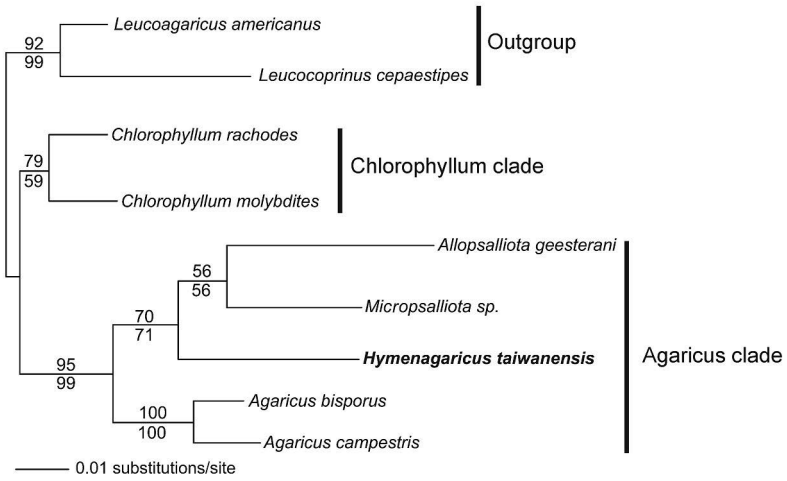


Fig. 5. The Maximum likelihood (ML) tree of *Hymenagaricus* based on ITS/nLSU-rDNA nucleotide sequences (-ln = 4077.23492). *Hymenagaricus taiwanensis* is the sister group of *Micropsalliota* and *Allopsalliota*. ML and Maximum Parsimony (MP) bootstraps greater than 50% are indicated above and below branches, respectively. *Leucoagaricus americanus* and *Leucocoprinus cepaestipes* were used to root the tree. Branch lengths reflect the number of substitutions per site.

DISCUSSION

Generic-level relationships within *Agaricaceae* based on molecular data have been investigated by previous studies (e.g. Moncalvo *et al.,* 2002; Vellinga, 2004). However, sequences of the genus *Hymenagaricus* were not included, thus made the phylogenetic position of *Hymenagaricus* an unresolved question. According to the phylogram inferred from ITS/nLSU sequences (Fig. 5) in this study, *Hymenagaricus* has an independent position in the Agaricus clade in *Agaricaceae*, and is related with *Micropsalliota* and *Allopsalliota*.

Since Heinemann (1981) elevated the section *Hymenopilei* in *Agaricus* subgenus *Conioagaricus* Heinem. to the genus rank, at least 28 species were reported in the genus, and two subgenera, *Hymenagaricus* and *Xanthagaricus* were proposed (Heinemann & Little Flower, 1984; Reid & Eicker, 1995, 1998, 1999). However, considering lack of sound characters to separate *Hymenagaricus* from *Agaricus*, Singer (1986) treated *Hymenagaricus* (as "*Hymenoagaricus*") as a synonym of *Agaricus.* More recently, *Hymenagaricus* Heinem. subgenus *Xanthagaricus* Heinem. was elevated to genus level (Little Flower *et al.* 1997). According to our morphological observation on *H. taiwanensis*, as well as the descriptions of *Hymenagaricus* species made by Heinemann (1981), Heinemann & Little Flower (1984), Pegler (1986), and Reid & Eicker (1995, 1998, 1999), the hymenidermal to pseudoparenchymatous structure of squamules confined to the central region of the pileal surface is typical of the genus. Thus, we prefer to recognize *Hymenagaricus* as a separate genus, and this is consistent with our molecular phylogenetic result.

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