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Hirsutella liboensis, a new entomopathogenic species affecting *Cossidae (Lepidoptera)* in China

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Abstract — *Hirsutella liboensis* was isolated from the larva of *Cossidae (Lepidoptera)* in Libo Natural Reserves, Guizhou Province. The fungus produces fasciculate synnemata and mono- and polyphialidic conidiogenous cells with necks twisted in two or three helical turns. Conidia are one-celled or (rarely) one-septate, fusiform or like orange segments that are enveloped in a mucous sheath. Morphological characters and phylogenetic analyses of ITS1-5.8S-ITS2 sequences support this fungus as a new species.

Key words — *Cordyceps*, taxonomy, entomopathogen

Introduction

The genus *Hirsutella* Pat. (Patouillard 1892) is important because of its capacity for serving as a natural control factor to insects, mites, and nematodes. Recent research has shown that some *Hirsutella* species produce various valuable bioactive compounds that could be used in anti-tumor (He et al. 2008), anti-tuberculosis (Isaka et al. 2008), and anti-malaria (Thongtan et al. 2006) capacities. Some authors consider the helical neck of conidiogenous cell is a crucial character in differentiating individual *Hirsutella* species (Mains 1951; Liang 1990a,b; Hodge 1998). Six species with conidiogenous cells possessing helical or wavy necks are currently known: *Hirsutella nodulosa* Petch (Petch 1926); *Hirsutella parasitica* (Henn.) Samson & H.C. Evans and *Hirsutella dendritica* Samson & H.C. Evans (Samson & Evans 1991); *Hirsutella brownorum* Minter & B.L. Brady (Minter & Brady 1980); *Hirsutella leizhouensis* H.M. Fang & S.M. Tan (Fang & Tan 1992); and *Hirsutella vermicola* M.C. Xiang & Xing Z. Liu (Xiang et al. 2006). In this paper we report a new species with conidiogenous cells that also have a helical neck from larvae of *Cossidae* (*Lepidoptera*).

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Table 1. List of fungi and GenBank accession numbers used in this paper.

Fungus	GenBank #	Fungus	GenBank #
Chaunopycnis pustulata	AF389189	*H. vermicola*	DQ345592
Elaphocordyceps capitata	AB027364	*Ophiocordyceps agriotis*	AY245626
E. inegoensis	AB027368	*O. cochlidiicola*	AB027377
E. ophioglossoides	AB027367	*O. emeiensis*	AJ309347
E. ophioglossoides	AJ309360	*O. multiaxialis*	AJ309359
E. paradoxa	AB027369	*O. nepalensis*	AJ309358
Hirsutella gregis	EF194155	*O. robertsii*	AJ309335
H. liboensis	FJ957852	*O. rubiginosoperitheciata*	AB294423
H. nodulosa	EF194146	*O. sinensis*	AB067715
H. rhossiliensis	AB109740	*O. sinensis*	AJ309357
H. rhossiliensis	DQ345587	*Tolypocladium cylindrosporum*	AB044645
H. sinensis	AJ309353	*T. inflatum*	AB208110
H. sinensis	AJ309355		

Materials and methods

Isolation and culture of collections

Infected hosts were surface-sterilized for 5 s with 75% ethanol, broken open to expose the body cavity, and then small tissue blocks and fungal hyphae were placed aseptically on potato dextrose agar (PDA) plates. The plates were incubated at 22°C and dark. After 14 d, the colonies can be observed. Select these pure colonies for identification and deposition of strains. The strain of GZUIFR-Libo1 was cultured on Czapek and Sabouraud media for identification. The cultures of this strain were maintained under 12 h:12 h /light:dark at 22°C or at 20–28 °C.

DNA extraction, amplification, and sequencing

Axenic mycelia (about 1 g) of *H. liboensis* strains were collected from PDA plate and used for DNA extraction according to Tigano-Milani et al. (1995). The extracted DNA was stored at -20 °C. The rDNA gene ITS-5.8S region was amplified using the primers ITS5 (5'-GGT GAGAGATTTCTGTGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). PCR amplification was performed as follows: at 94°C for 5 min and 35 cycles with at 94°C for 40 s, at 49°C for 40 s, at 72°C for 60 s; followed by a final elongation step at 72°C for 10 min. PCR products were purified and sequenced by Beijing Sunbiotech Co. Ltd. The sequence of ITS1-5.8S-ITS2 rDNA region of strain GZUIFR-Libo1 was submitted to GenBank.

Phylogenetic analysis

The ITS1-5.8S-ITS2 nucleotide sequence of strain GZUIFR-Libo1 was aligned using the Clustal X1.83 with related fungi sequences (Table. 1) from GenBank. The phylogenetic analysis was performed by neighbor-joining method (NJ) of MEGA version 4.0 (Kumar et al. 2004). Confidence values for individual branches were determined by bootstrap analysis (1000 replications).

Table 2. Morphological comparison between *Hirsutella liboensis* and similar *Hirsutella* species.

Species	Phialides	Conidia	Host	Synnemata
H. brownorum	not poly-phialidic	lemon-shaped 5–6 × 4–5 µm	Mite	none
H. dendritica	wavy neck	fusiform, non-mucoid, 6–8 × 2–3 µm	Pupae	single
H. leizhouensis	warted, in opposite pairs	oviform, non-mucoid, 3.5–4.5 × 2–3 µm	*Phragmatoecia castaneae*	clustered
H. liboensis	single, smooth, polyphialidic,	fusiform, in mucoid sheath, 6–10 × 1.5–4 µm	*Cossidae*	clustered larva
H. nodulosa	rough-surfaced	non-mucoid, 3–5 × 3 µm	Mite	none
H. parasitica	wavy neck	cylindric, non-mucoid 12–25 × 2.5–4 µm	Unknown	clustered
H. vermicola	singly or in opposite pairs	ellipsoid or like orange segments, 7–8 × 1.5–3 µm	Nematode	none

Results and discussion

Taxonomy

Hirsutella liboensis X. Zou, A.Y. Liu & Z.Q. Liang, **sp. nov.** Fig. 1

MycoBank MB 513283; GenBank FJ957852

Coloniae in Czapek agaro lente crescentes, albae, 2–3 mm diam post 21 dies. Mycelium hyalinum, septatum, laeve. Conidiophoris ad cellulas conidiogenas singulatim producentis ex hyphis vegetativis. Cellulis conidiogenis mono-phialidicis, rarius polyphialidicis, 28–30 µm longis, basi parte inflatis, 18–20 × 3–4.5 µm, apice 1–2 µm latis, 2–3 helicinis. Conidiis aseptatis vel septatis, levibus, singulatim vel 2 aggregate ad colli apicem facientibus, plus minusve ellipsoideis, 6–8 µm longis, 3–5 µm latis, in muco involutis.

***Holotypus**: GZUXIFR-Libo1, isolatus ex insectus, X. Zou, Libo, Provincia Guizhou, VI, 2007; in Guizhou Univ., conservatus.* The holotype and isolated strains are deposited in the Institute of Fungus Resources, Guizhou University.

Etymology: *liboensis*, referring to the collection location.

Colonies on Czapek agar, 25°C, growing very slowly, up to a diam of 2–3 mm after 3 weeks. Mycelium hyaline, septate, smooth. Conidiogenous cells single, almost at right angles from vegetative hyphae, monophialidic or polyphialidic, hyaline, smooth, 28–30 µm long, significantly swollen in basal portion, 3–4.5 µm width, tapering to 1–2 µm wide and 10–13 µm overall length, twisting in 2–3 helices at the apex. Conidia hyaline, aseptate or with a septum, smooth, fusiform or like orange segments, arising singly (or both-cell) from the apex of the neck, 6–8 µm long, 3–5 µm wide, enveloped in a hyaline mucous sheath. Colonies on PDA, 22–24°C, in light, producing synnemata after 60 d; synnemata up to 40–50 mm length after 90 d. No teleomorph was observed.

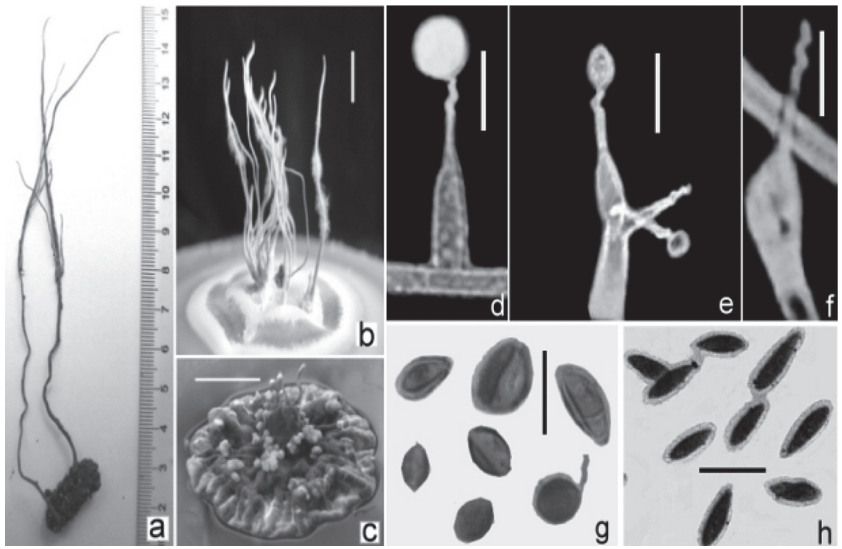

Fig. 1 Synnemata and conidiogenous structures of *Hirsutella liboensis*. a. Synnemata on insect; b. Synnemata on PDA; c. Culture on Sabouraud medium; d. Single erect phialides; e. Proliferating phialides; f. Phialides with 2–3 helices; g. Conidia with mucus sheath; h. Conidia from specimen synnemata. Bar = 10 mm (b–c) or 10 μm (d–h).

Host: Larva of a species of *Cossidae (Lepidoptera)* in a tree hole.

Additional specimen examined (Paratype): GZUIFR-Libo1, Libo, Provincia Guizhou, VII, 2007; Guizhou Univ.

The most typical character of *H. liboensis* is the helical twist of the necks of the phialides, a feature shared in common with six other *Hirsutella* species. The differences separating the seven species are compared in Table 2 below.

Molecular analyses

A BLAST search of GenBank was performed by using the *H. liboensis* ITS-5.8S sequence. Close matches showing maximal sequence identities of 94–97% included *Ophiocordyceps cochlidiicola* (Kobayasi & Shimizu) G.H. Sung et al., *H. nodulosa,* and *H. vermicola*. The ITS sequences of these species, related species of *Hirsutella,* and other entomogenous fungi were retrieved from GenBank for phylogenetic analysis, which clearly supported three clades (Fig. 2). Clade-B included an independent sub-clade comprising *O. cochlidiicola*, *H. nodulosa, H. vermicola,* and *H. liboensis.*

Many *Hirsutella* species are the anamorphs of *Ophiocordyceps* (Liang 1990a). Recent data showed also that *Hirsutella* is an anamorphic genus of *Ophiocordyceps* Petch (Sung et al. 2007). Liu et al. (2005) described a fungus

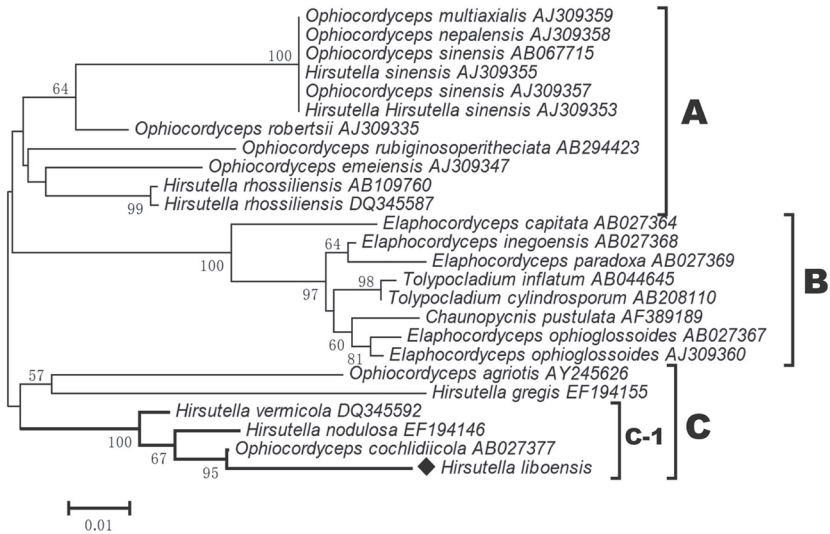

Fig. 2 Phylogenetic tree based on rDNA ITS1–5.8S–ITS2 sequences of *H. liboensis* and related species.

with pleoanamorphic states characteristic of both *Aschersonia* and *Hirsutella*. *Aschersonia* species linked exclusively to teleomorphs in the genus *Hypocrella*. It can be seen that *Hirsutella* has a mixed and paraphyletic phylogenetic background.

Hirsutella liboensis clustered in subclade C–1, which included *H. nodulosa* and *H. vermicola* with helical or curved necks atop the phialides. *Hirsutella vermicola* infects nematodes and has oppositely paired phialides while *H. nodulosa* has phialides with a warted surface and a greater length/width ratio. Genetic distances show *Hirsutella liboensis* and *Ophiocordyceps cochlidiicola* with the closest relationship. Kobayasi & Shimizu (1980) reported that *O. cochlidiicola* could infect larva of *Cochlidiidae (Lepidoptera)* and its stroma was single, thin, fibers, dust-color, 70–100 × 1 mm. Until now, the *O. cochlidiicola* anamorph has been unknown. As the *H. liboensis* teleomorph is also unknown, the relationship between the two forms will be further studied and analyzed. Two other related species, *O. agriotis* Sung GH et al. [as '*agriotidis*', nom. inval.] and *H. gregis* Minter et al. were also grouped in clade C (supported by 57%) but group together outside the *H. liboensis* sub-clade, C-1.

In a word, both morphological characters and phylogenetic analysis support *H. liboensis* as a new species of *Hirsutella*.

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