Two new species of *Hirsutella* (Ophiocordycipitaceae, Sordariomycetes) that are parasitic on lepidopteran insects from China

Jiaojiao Qu¹, Xiao Zou², Wei Cao², Zhongshun Xu², Zongqi Liang²

¹ College of Tea Sciences, Guizhou University, Guiyang, 550025, China ² Institute of Fungal Resources, College of Life Sciences, Guizhou University, Guiyang, 550025, China

Corresponding author: Xiao Zou (xzou@gzu.edu.cn)

Academic editor: Kevin D. Hyde | Received 5 April 2021 | Accepted 13 June 2021 | Published 9 August 2021

Citation: Qu J, Zou X, Cao W, Xu Z, Liang Z (2021) Two new species of *Hirsutella* (Ophiocordycipitaceae, Sordariomycetes) that are parasitic on lepidopteran insects from China. MycoKeys 82: 81–96. https://doi.org/10.3897/mycokeys.82.66927

Abstract

*Hirsutella* are globally distributed entomopathogenic fungi that offer important economic applications in biological control and biomedicine. *Hirsutella* was suppressed in favour of *Ophiocordyceps* affected by the ending of dual nomenclature for pleomorphic fungi in 2011. Currently, *Hirsutella* has been resurrected as a genus under Ophiocordycipitaceae. In this study, we introduce two new species of *Hirsutella*, based on morphological and phylogenetic analyses. *Hirsutella flava* and *H. kuankuoshuiensis* are pathogenic on different species of larval Lepidoptera in China. *Hirsutella flava* primarily differs from related species by its awl-shaped base; long and narrow neck, 24–40.8 × 2.2–2.5 μm; long and narrow cymbiform or fusoid conidia, 6.5–10 × 2.1–4.3 μm. *Hirsutella kuankuoshuiensis* has two types of phialides and distinctive 9.9–12.6 × 2.7–4.5 μm, clavate or botuliform conidia. The distinctions amongst the new species and phylogenetic relationships with other *Hirsutella* species are discussed.

Keywords

entomopathogenic fungi, *Hirsutella*, *Ophiocordyceps*, two new taxa

Introduction

The entomopathogenic fungal genus *Hirsutella* Pat. was erected by Patouillard (1892) based on the type species *H. entomophila*. The genus was introduced to the family Ophiocordycipitaceae and its sexual morph was linked to *Ophiocordyceps* (Sung et al. 2011).
In Hirsutella sensu stricto, conidiation is synnematous and phialides typically have a swollen base that tapers abruptly into a long neck producing either a single conidium or 2–3 conidia coated with mucus. The colour of the synnemata ranges from ash-grey or brown to dark brown. The size and shape of the hyaline conidia vary from citriform to oblong, subcylindric, globose, rhombic, or reniform (Luangsa-ard et al. 2017; Quandt et al. 2014). These taxa are important pathogens of agricultural pests and are used as popular traditional medicine and a nutritious food in many Asian countries (Evans 1974; Quandt et al. 2014; Hyde et al. 2019). Several common species of Hirsutella, such as H. thompsonii and H. rhossiliensis, are potentially important biological control agents for nematodes and mites (Jaffee 1992; Van der Geest 2010; Hyde et al. 2019). Further uses involve the development and application of several effective bioactive secondary metabolites (Mazet and Vey 1995; Lang et al. 2005; Qu et al. 2017).

Research on Hirsutella originated in the 1920s. Through the 1950s, Speare (1920), Petch (1924) and subsequent researchers reported 25 new species of the genus. However, many of these species were not described in detail and lacked adequate drawings, as well as holotypes. In addition, many specimens were damaged or lost during wartime (Zou et al. 2016a). In the 1970s and 1980s, Miner, Samson and Evans re-examined the status of Hirsutella and established the modern scientific definition for the genus (Minter and Brady 1980; Evans and Samson 1982, 1984). Since the beginning of the 21st century, the taxonomy, molecular evolution and phylogeny of Hirsutella have been addressed by a small number of Chinese and international studies, with sporadic reports of new species (Seifert 2004; Xiang et al. 2006; Zou et al. 2010). However, it is likely that further new species remain to be discovered, and specific information on insect hosts, pathogenicity and habitats are lacking (Sung et al. 2007a; Hoyos-Carvajal et al. 2009).

Quandt et al. (2014) proposed that Hirsutella should be suppressed in favour of Ophiocordyceps affected by the ending of dual nomenclature for pleomorphic fungi in 2011 (McNeill et al. 2012). Ophiocordyceps is the type genus in the family Ophiocordycipitaceae (Hypocreales, Sordariomycetes) (Sung et al. 2007a). The main characteristics of the sexual morphs of Ophiocordyceps are fibrous, hard, pliant-to-wiry, dark stromata with superficial to immersed perithecia (Sung et al. 2007a; Xiao et al. 2019). Most of the sexual species of Ophiocordyceps were transferred from the genus Cordyceps (Cordycipitaceae) by Sung et al. (2007a). Since many species of Hirsutella are closely related to Cordyceps, the asexual morphs in most of the species in Ophiocordyceps have hirsutella-like features (Kepler et al. 2013; Quandt et al. 2014; Maharachchikumbura et al. 2015, 2016). Therefore, Hirsutella was treated as a separate genus from Ophiocordyceps before the taxonomic revision (Sung et al. 2007a; McNeill et al. 2012; Quandt et al. 2014). For example, some new species only known from a Hirsutella morph have been accepted into Ophiocordyceps (Simmons et al. 2015a; Qu et al. 2018b).

In recent years, the taxonomic transitions of Ophiocordycipitaceae changed rapidly under the new rules. Quandt et al. (2014) included Ophiocordyceps, Tolypocladium, Polycephalomyces, Purpureocillium, Drechmeria and Harposporium in Ophiocordycipi-
Two new species of Ophiocordyceps from China

Based on morphological and phylogenetic analyses. In the paper “Outline of Ascomycota: 2017”, the genus Hymenostilbe was added into the Ophiocordycipitaceae families (Wijayawardene et al. 2018). According to the latest taxonomic report, the number of genera included in Ophiocordycipitaceae has increased to ten, and among them, Hirsutella, Paraisaria and Perennicordyceps are new additions (Hyde et al. 2020). The taxonomic revision of Ascomycota is continuing. Further research into the phylogeny of these organisms is needed. Examples include investigating the new resources to supplement the available taxonomic information and perform phylogenetic research.

During an investigation of the genetic resources of entomopathogenic fungi in southwest China, we collected two specimens of Lepidoptera insects that were infected by fungi. Two hirsutella-like species were isolated and their gene sequences and morphological traits were shown to be related to Hirsutella sensu stricto. In this study, two new species of Hirsutella are introduced.

Materials and methods

Specimens

The specimens HKAS112884 and HKAS112885 were deposited at the Kunming Institute of Botany, Chinese Academy of Sciences (KIB), Kunming, China. The isolated strains of their asexual stage were deposited at the Institute of Fungal Resources of Guizhou University (GZAC), Guiyang, China. More information about these specimens is shown in Suppl. material 1: Table S1.

Fungal isolation and culture

The fungi were isolated as described by Qu et al. (2018). The surface of specimens was rinsed with sterile water, followed by surface sterilisation with 75% ethanol for 3-5 s. Parts of the insect body were cut off and a piece of tissue was inoculated in haemocoel on a PDA plate for 20 days at 16 °C.

LM and SEM observation

For light microscopy (LM) observations and imaging, the morphological characteristics of mycelia were observed using an optical microscope (OM, BK5000, OPTEC, Chicago, IL, USA) after staining with a lactic acid/phenol cotton blue solution. The captured images of new species were edited and digitally contrasted using Paint Shop Pro v. 5.0.1 (Corel, Ottawa, Canada).

Electron microscopy was performed as described by Qu et al. (2018). Briefly, 1 cubic cm of hyphae with conidia were cut from the fungus on PDA cultures, fixed with 4% glutaraldehyde at 4 °C overnight, and then washed three times with phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄ and 1.5 mM NaH₂PO₄)
KH₂PO₄, pH 7.4) for 10 min each time. Fixed hyphae and conidia were dehydrated using 50%, 70%, 90% and 100% ethanol, 10 min for each concentration, and were finally dehydrated with super-critical carbon dioxide. After being sprayed with gold, the conidia and mucilage were examined by scanning electron microscopy (SEM) (S-3400N, Hitachi, Tokyo, Japan) and photographed.

**DNA extraction, PCR amplification and sequencing**

Axenic and fresh mycelia (0.05–0.1 g) of the new species were transferred to 1.5 ml Eppendorf tubes for genomic DNA extraction using a Fungal DNA MiniKit (Omega Bio-Tek, Norcross, GA, USA). The universal known primers were used for PCR amplification: (1) NS1/NS4 for the partial small subunit ribosomal RNA gene region (SSU) (White et al. 1990), (2) LROR/LR5 for the partial large subunit rDNA gene region (LSU) (Vilgalys and Hester 1990; Rehner and Samuels 1994), (3) ITS4/ITS5 for the internal transcribed spacer gene region (ITS) (White et al. 1990), (4) 983F/2218R for the partial translation elongation factor 1-alpha gene region (TEF1α) (Sung et al. 2007b) and (5) CRPB1A/CRPB1Cr for the partial RNA polymerase II largest subunit gene region (RPB1) (Castlebury et al. 2004).

**Molecular phylogeny**

To construct a phylogeny of major lineages, 71 representative species were chosen to represent the ecological diversity of *Hirsutella* and *Ophiocordyceps* based on previous phylogenetic studies (Simmons et al. 2015b; Xiao et al. 2017; Qu et al. 2018; Xiao et al. 2019). *Tolyphocladium inflatum* and *T. ophioglossoides* were selected as the outgroup taxa and are classified within Ophiocordycipitaceae (Xiao et al. 2019). The sequences used in this study were combined with published data on hirsutella-like species and Ophiocordycipitaceae. All the other sequences were collected from GenBank and the accession numbers are shown in Table 1.

All the sequences were edited for multi-alignment using the BioEdit Sequence Alignment Editor v.7.0.5.3 (Hall 1999) with the Clustal X v.1.83 software package (Thompson et al. 1999). Gaps were excluded from the phylogenetic analysis based on previous research (Qu et al. 2018). The ITS, SSU, LSU, TEF1α and RPB1 regions were aligned in combined datasets using MAFFT v.7 (Katoh and Standley 2013, http://mafft.cbrc.jp/alignment/server/). The Akaike Information Criterion (AIC) in jModeltest 0.1.1 (Guindon and Gascuel 2003; Posada 2008) was used to select the nucleotide substitution model for each region. The combined data included a 4778 bp character set of the five regions and were analysed. Maximum likelihood phylogenetic analyses were conducted in RAxML (Stamatakis et al. 2008) with the recommended partition parameters to determine the best tree topology. The bootstrap support values were achieved after 500 search replicates and summarised in TreeGraph. Bayesian Posterior Probabilities (BPP) were estimated in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with the same partition parameters. In this analysis, two runs of
Two new species of Ophiocordyceps from China

| Species                          | Insecta                   | Voucher          | GenBank accession no.               |
|---------------------------------|---------------------------|------------------|-------------------------------------|
| Hirastella cf. haptorpora       | Diptera: Ironididae       | ARSEF 2228       | KM652166 KM652118 KM652075 KM652041 KM652001 |
| H. changbeiianensis             | Homoptera: leafhopper     | GZUFR-hir106527  | KY415578 KY415586 KY415592          |
| H. citrifirmai                  | Hemiptera: Delphacidae    | ARSEF 490        | KM652151 KM652103 KM651987         |
| H. citrifirmai (Coxidae)        | Hemiptera: Coxidae        | ARSEF 1035       | KM652153 KM652105 KM652064 KM652030 KM651989 |
| H. citrifirmai (Pyllidae)       | Hemiptera: Pyllidae       | ARSEF 2598       | KM652155 KM652107 KM651991         |
| H. cryptococetium               | Hemiptera: Delphacidae    | ARSEF 4517       | KM652157 KM652109 KM652066 KM652032 KM651992 |
| H. flavus                       | Lepidoptera: Delphacidae  | GZUFR-hir100627-1| KY415598 KY415599 KY945366 KY415601 |
| H. fulviflavus                  | Coleoptera: Curculionidae | ARSEF 5474       | KM652110 KM652067 KM652033 KM651993 |
| H. goyana                       | Hemiptera: Cicadellidae   | ARSEF 878        | KM652158 KM652111 KM652068 KM652035 KM651994 |
| H. haptorpora                   | Acari: Uropodina           | ARSEF 1026       | KM652159 KM652036 KM651995         |
| H. ilustris                     | Hemiptera: Aphiidiidae    | ARSEF 5539       | KM652160 KM652112 KM652069 KM652037 KM651996 |
| H. kirchneri                   | Acari: Etrophiiidae       | ARSEF 5551       | KM652161 KM652113 KM652070 KM651997 |
| H. kuanloushiuimensis           | Lepidoptera: Delphacidae  | ARSEF 5473       | KM652167 KM652120 KM652077 KM652003 |
| H. leizhouensis                 | Lepidoptera: Pyralidae    | GZUFR-hir140506  | KY415573 KY415580 KY945360 KY415590 |
| H. lecanicola                   | Hemiptera: Coccidae       | ARSEF 8888       | KM652162 KM652114 KM652071 KM652038 KM651998 |
| H. liobonis                     | Lepidoptera: Cossiidae    | ARSEF 9603       | KM652163 KM652115 KM652072         |
| H. necatrix                     | Acari: Uropodina           | ARSEF 5549       | KM652164 KM652116 KM652073 KM652039 KM651999 |
| H. nodulata                     | Lepidoptera: Pyralidae    | ARSEF 5473       | KM652165 KM652117 KM652074 KM652040 KM652000 |
| H. radissa                      | Diptera: Delphacidae      | ARSEF 1369       | KM652167 KM652119 KM652076 KM652042 KM652002 |
| H. repes nom. inval.            | Hemiptera: Delphacidae    | ARSEF 2348       | KM652167 KM652120 KM652077 KM652003 |
| H. rhousienisi (Heteroderidae)  | Tylennycha: Tylennycha     | ARSEF 2931       | KM652168 KM652121 KM652078 KM652043 KM652004 |
| H. rhousienisi                  | Tylennycha: Tylennycha     | ARSEF 3747       | KM652170 KM652123 KM652080 KM652045 KM652006 |
| H. iattamatiensis               | Lepidoptera: Pyralidae    | ARSEF 996        | KM652172 KM652125 KM652082 KM652047 KM652008 |
| H. sinensis                     | Lepidoptera: Heliidae     | ARSEF 6282       | KM652173 KM652126 KM652083 KM652048 KM652009 |
| H. tristia(Cicadellidae)        | Hemiptera: Cicadellidae   | ARSEF 2197       | KM652175 KM652129 KM652085 KM652050 KM652012 |
| H. tristia(Delphacidae)         | Hemiptera: Delphacidae    | ARSEF 2044       | KM652174 KM652128 KM652043 KM652011 |
| H. tubulata                     | Lepidoptera: Microlepoidoptera | ARSEF 2227 | KM652176 KM652130 KM652086 KM652051 KM652013 |
| H. thompsonii (Eriophyidae)     | Acari: Eriophyidae        | ARSEF 253        | KM652179 KM652133 KM652088 KM652016 |
| H. thompsonii (Tetranychidae)   | Acari: Tenuipalpidae      | ARSEF 3233       | KM652188 KM652143 KM652096 KM652059 KM652024 |
| H. thompsonii vari. synennatina | Acari: Tetranychidae      | ARSEF 5412       | KM652193 KM652148 KM652100         |
| H. thompsonii vari. thomsonii   | Acari: Eriophyidae        | ARSEF 137        | KM652177 KM652131 KM652087 KM652052 KM652014 |
| H. versicolor                   | Hemiptera: Membracidae    | ARSEF 1037       | KM652190 KM652102 KM652063 KM652029 |
| Ophiocordyceps acindaris        | Coleoptera: Coleoptera    | OSC 110988       | EF468804 EF468951 EF468853 EF468745 |
| O. agristidis                   | Coleoptera: Coleoptera    | ARSEF 5602       | JN049819 DQ518754 DQ522540 DQ522368 DQ522322 |
| O. apodoli                      | Coleoptera: Coleoptera    | ARSEF 5498       | DQ518757 DQ522541 DQ522325         |
| O. appendicicata                | Coleoptera: Coleoptera    | NBC 106960       | JN943326 JN941413 JN941728 JN922462 AB980577 |
| O. breunneopustulata            | Coleoptera: Blattidae     | OSC 128576       | DQ518756 DQ522542 DQ522369 DQ522324 |
| O. clavata                      | Coleoptera: Coleoptera    | NBC 106962       | JN943328 JN941415 JN941726 JN922460 AB980587 |
| O. coccidicola                  | Insect: Coleoptera        | HMAS19612       | AB027377 KJ878884 KJ878917 KJ878998 KJ878965 |
| O. communis                     | Coleoptera: Coleoptera    | NH 12581         | EF468831 EF468973 EF468775         |
| O. dipertigena                  | Diptera: Coleoptera       | OSC 151912       | KJ878887 KJ878920 KJ879001 KJ878967 |
| O. elongata                     | Lepidoptera: Coleoptera   | OSC 110989       | EF468808 EF468856 EF468748         |

Table 1. GenBank accession numbers for sequences used in the phylogenetic analysis.
| Species            | Insecta                       | Voucher               | GenBank accession no.          |
|-------------------|-------------------------------|-----------------------|-------------------------------|
| *O. entomorrhiza* | Lepidoptera                  | KEW 53484             | ITS: JN049850                 |
|                   |                               |                       | LSU: EF468809                 |
|                   |                               |                       | RPB1: EF468954                |
|                   |                               |                       | TEF1α: EF468875               |
|                   |                               |                       |                               |
| *O. evansi*       | Hymenoptera (Pachycondylaharpax) | Ophup 858             |                               |
|                   |                               |                       |                               |
| *O. forquignonii* | Diptera (adult fly)           | OSC 151908            |                               |
|                   |                               |                       |                               |
| *O. geometridicola* | Lepidoptera (Geometridae)   | TRBC 8095             |                               |
|                   |                               |                       |                               |
| *O. gracilis*     | Lepidoptera (larva)           | EFCC 8572             |                               |
| *O. heteropoda*   | Hemiptera (ciada nymph)      | OSC 106404            |                               |
|                   |                               |                       |                               |
| *O. irangensis*   | Hymenoptera (adult ant)      | OSC 128579            |                               |
|                   |                               |                       |                               |
| *O. konnoana*     | Coleoptera (larva)            | EFCC 7315             |                               |
| *O. lanpingensis* | Hymenoptera                  | OSC 151913            |                               |
| *O. lloydii*      | Coleoptera (larva)            | BCC 105888            |                               |
|                   |                               |                       |                               |
| *O. macroacicularis* | Coleoptera (larva)           | OSC 110993            |                               |
|                   |                               |                       |                               |
| *O. multiporitibicata* | Lepidoptera (larva)    | BCC 69008             |                               |
| *O. myrmicarum*   | Formicidae (adult ant)       | ARSEF 11864           |                               |
| *O. nigrella*     | Lepidoptera (larva)           | EFCC 9247             |                               |
|                   |                               |                       |                               |
| *O. paucivoritibicata* | Lepidoptera (larva)    | TRBC 8106             |                               |
| *O. pseudocarciculis* | Lepidoptera (larva)    | TRBC 8102             |                               |
| *O. ramossimum*   | Lepidoptera (larva)           | GZUH8HN8              |                               |
| *O. robertii*     | Lepidoptera (Hepialidae larva) | KEW 27083             |                               |
| *O. inensis*      | Lepidopteran pupa             | EFCC7287              |                               |
| *O. sporonigeria* | Lepidoptera (Cossidae)       | MFLUC 18-0492         |                               |
| *O. styphlon*     | Coleoptera (Elateridae larva) | OSC 111000            |                               |
| *O. xuefengensis* | Lepidoptera (Hepialidae larva) | GZUH2012HN11         |                               |
| *Tolypocladium inflatum* | Coleoptera (larva)    | OBC 71255             |                               |
| *T. ophioglossoides* | Fungi (Elaphomyces sp.) | NBRC 106332           |                               |

Results

Phylogenetic analyses

The tree was constructed with maximum likelihood and Bayesian posterior probabilities with *Tolypocladium inflatum* and *T. ophioglossoides* as the outgroup taxa based on RPB1, *tef1*, ITS, 18S rDNA and 28S rDNA gene datasets (SSU: 1391 bp, LSU: 903 bp, ITS: 721 bp, TEF1α: 946 bp and RPB2: 817 bp) (Fig. 1). In this phylogenetic tree, *Hirsutella flavia* and *H. kuankuoshuiensis* formed a separate clade from the other species with credible bootstrap values (85% ML and 0.90 PP), suggesting that these two species are truly related. Within a separate branch, *H. flavia* and *H. kuanku-
Two new species of Ophiocordyceps from China

Figure 1. Phylogenetic tree of Hirsutella species combined with RPB1, rpb1, ITS, 18S rDNA and 28S rDNA datasets, using the maximum likelihood method. Numbers below the branches are bootstrap percentage values, based on 10,000 replicates, ML/BPP.
oshuiensis were allied with the *H. sinensis* and *H. strigosa* clade, distant from the other hirsutella-like species, particularly the *H. thompsonii* clade. A molecular phylogenetic analysis further confirmed the differences among the two new species and other related species. Based on the morphological characteristics and molecular phylogenetic analysis, these two new species are introduced as new members of *Hirsutella* species in the Ophiocordycipitaceae family.

**Taxonomy**

*Hirsutella flava* X. Zou, J.J. Qu, Z.A. Chen & Z.Q. Liang, sp. nov.

MycoBank No: 819552

**Fig. 2**

**Diagnosis.** Characterised by phialides slender awl-shaped and tapered; a width of base 24–40.8 × 2.2–2.5 μm; tapering to narrow neck, 7.2–9 μm long × 0.5 μm wide. Conidia narrow cymbiform, long fusoid or limoniform, 6.5–10 × 2.1–4.3 μm.

**Type.** China, Zhejiang Province, Tianmu Mountain National Nature Reserve (30°18'N, 119°28'E, approximately 600–1200 m a.s.l.), 27 June 2010, presented by Prof. Zhuan Chen. The holotype has been deposited at KIB (HKAS112884). Sequences from isolated strains (GZUIFR-hir100627-1, GZUIFR-hir100627-2 and GZUIFR-hir100627-3) have been deposited in GenBank.

**Description.** *Synnemata* extending from the head of insect, 3–10 cm × 0.5–1 mm, simple or irregularly branched, dark brown and changing to faint yellow toward the apex; no conidiation was observed (Fig. 2A). The fungus grows slowly at 22 ± 1 °C on Czapek-Dox agar medium to a diam. of 8–12 mm; the colony surface was flat and flocculent with white aerial hyphae. On PDA agar, fungal colonies grew quickly to a diam. of 15–23 mm after 20 d at 22 ± 1 °C, when the colonies were blanket-like with rough mycelia, radiating beam-like from the centre; centre lunate concave, pale yellow; colony surface with yellowish liquid exudation (Fig. 2B, C). *Mycelium* hyaline, smooth, septate, 3.6–4.5 μm wide. *Conidiogenous cells* form directly from the mycelial end, monophialidic or polyphialidic, and borne perpendicular or at acute angles (80°–85°) to the subtending hyphae. *Phialides* slender awl-shaped and tapered, width of the base 24–40.8 × 2.2–2.5 μm, tapering to a narrow neck, 7.2–9 μm long × 0.5 μm wide. *Conidia* narrow cymbiform, long fusoid or limoniform, 6.5–10 × 2.1–4.3 μm; single- or double-enveloped in a hyaline mucus, thickness 2.0–3.0 μm (Fig. 2D–K).

**Host.** Larva of a species of Lepidoptera.

**Habitat and distribution.** On decaying leaves in broadleaved forests, Zhejiang Province, China.

**Etymology.** Refers to the yellow colour (Lat. ‘flava’) of the holotype and colony.

**Teleomorph.** Unknown.

**Remarks.** This species is allied with the *H. sinensis* and *H. strigosa* clade. The phialides of *H. flava* are subulate, and the necks are slenderer. In particular, the colony
Two new species of Ophiocordyceps from China

Figure 2. Morphological characteristics of *Hirsutella flava* A the infected insect specimens with a long and single synnemata (HKAS112884) B, C, colonial morphology on PDA agar media for 20 d B shows the front of the colony and C shows the back of the colony D–G LM images of the general morphology of conidiogenous cells and conidia H–K SEM images showing conidiogenous cells and conidial structure; Scale bars: 1 cm (A); 5 cm (B, C), 10 μm (D–G); the rest of the bars are shown in the figure. LM, light microscopy; PDA, potato dextrose agar; SEM, scanning electron microscopy.

Morphology of this fungus is unique among the *Hirsutella* species. The colony surface appears very rough, and the hyphae are gathered into outwardly radiating filamentous bundles of varying sizes.

*Hirsutella kuankuoshuiensis* X. Zou, J.J. Qu & Z.Q. Liang, sp. nov.

MycoBank No: 819591

Fig. 3

**Diagnosis.** *Hirsutella kuankuoshuiensis* differs from other species in this genus primarily by its clavate, narrow fusiform or botuliform conidia and subulate or slender columnar phialide.

**Type.** China, Guizhou Province, Suiyang County, Kuankuoshui Nature Reserve (28°08’N, 107°02’E, approximately 1400 m a.s.l.), July 2012, collected by X. Zou. The holotype has been deposited at KIB (HKAS112885). Sequences from isolated strains (GZUIFR-2012KKS3-1, GZUIFR-2012KKS3-2 and GZUIFR-2012KKS3-3) have been deposited in GenBank.
Figure 3. Morphological characteristics of *Hirsutella kuankuoshuiensis*. A, the insect specimens with single and thin synnemata (HKAS112885). B, C, colonial morphology on PDA agar media for 20 d. B shows the front of the colony and C shows the back of the colony. D–G, LM images showing conidiogenous cells and conidia. D, E, the structure of conidiogenous cells on mycelia. F, the images of conidiogenous cells on synnemata (optical microscope). H–J, conidial morphology (LM). G, conidia with mucilage (SEM). Scale bars: 10 mm (A–C); bar of G was shown in the figure; the rest of the bars were 10 μm. LM, light microscopy; PDA, potato dextrose agar; SEM, scanning electron microscopy.

**Description.** *Synnemata* are single, extending from the head of insect; 8.6 cm long, dark brown and changing to brown towards the apex; no conidiation was observed (Fig. 3A). The fungus spreads slowly on PDA agar at 20–22 °C and grows to a diam. of 22–30 mm after 14 d; the colony is round, centre of surface with brown dense bulges and grey-white sparse flocculent aerial hyphae. Colony margin is flat with radial groove; a large amount of brown pigment secreted into the medium causes the back of colony to appear dark brown; thickness 10–12 mm (Fig. 3B, C). *Mycelium* hyaline, smooth, septate, 1.5–3.0 μm wide. *Conidiogenous cells* monophialidic, hyaline, borne perpendicular or at an acute angle to the subtending hyphae. *Phialides* subulate or slender columnar, tapering gradually to a long and narrow neck, 30–45 × 1–3 μm long. *Conidia* clavate, narrow fusiform or botuliform without a diaphragm, 9.9–12.6 × 2.7–4.5 μm, single- or double-enveloped in a hyaline mucus, thickness 2.0–3.0 μm (Fig. 3D–J).

**Etymology.** Referring to the locality of the specimen, kuankuoshui (Lat. ‘kuankuoshuiensis’).

**Host.** Lepidoptera larva.

**Habitat and distribution.** On the decaying leaves of broadleaved forests, Guizhou Province, China.

**Teleomorph.** Unknown.
Remarks. This species possesses two types of conidiogenous cells and long fusiform or clavate without diaphragm conidia (9.9–12.6 × 2.7–4.5 μm), which is extremely rare in *Hirsutella* species. In addition, *H. kuankuoshuiensis* could produce long thin synnemata on the culture media that contain few or no conidia.

Discussion

Previous taxonomic studies have shown that the *Hirsutella* species are reconstructed in five main groups, and clustering taxa shared the same phialide structures (Simmons et al. 2015b; Qu et al. 2017; Qu et al. 2018). In general, the *H. nodulosa* lineage possesses phialides with apical helical twists. The *H. citriformis* clade is primarily represented by a squat ovoid base and a single slender neck. The *H. thompsonii* clade, the most widely studied hirsutella-like species and a potential biocontrol agent for mite pests, has a small cylindrical or round phialide, usually less than 25 μm, while the *H. sinensis* clade includes isolates that originate from a variety of taxa, including nematodes, mites and both hemi (Hemiptera) and holometabolous (Coleoptera, Lepidoptera) insect hosts (Simmons et al. 2015b). The majority of these species share a cylindrical base and an average phialide length greater than 40 μm. In our phylogenetic tree, these five typical branches of *Hirsutella* were more dispersed owing to the addition of more *Ophiocordyceps* species. *Hirsutella flava* and *H. kuankuoshuiensis* formed a separate clade that is represented by the subulate phialides and narrow fusiform conidia and have a close relationship with the *H. sinensis* and *H. strigosa* clades. In addition, this separate clade is distant from the *H. thompsonii* and *H. citriformis* clades. Species in these clades primarily share similarly large phialides and long fusiform conidia (Qu et al. 2018).

The phylogenetic tree confirmed the distinction between two new species and extant species. Among the species with an awl-shaped base and a long narrow neck, *H. flava* differs in its subulate phialides (e.g. *H. danubiensis* Balazy et al., 2008; *H. tunicate* Ciancio et al., 2013), cylindrical phialides (e.g. *H. changbeisanensis* Liang, 1991; *H. strigosa* Petch, 1939) and two types of conidiogenous cells (e.g. *H. stilbelliformis* Evans & Samson, 1982; *H. shennongjiaensis* Zou et al., 2016b) (Suppl. material 1: Table S2). In addition, *H. flava* is unique in the colony morphology of isolated strains. The fungus spreads more quickly than other hirsutella-like species on PDA media, and the colony surface appears very rough, owing to the hyphae being gathered into outwardly radiating filamentous bundles of varying sizes. *H. flava* could be distinguished from similar species by the shape and size of the conidiogenous cells. Morphological comparisons of relevant taxa are shown in Suppl. material 1: Table S2.

*Hirsutella kuankuoshuiensis* possesses two types of conidiogenous cells and long fusiform or clavate conidia, which are unique to *Hirsutella*. Furthermore, this species can readily produce long thin synnemata on culture media, but it produces few or no conidia. There are five other species similar to this species: *H. shennongjiaensis* (Zou et al. 2016), *H. stilbelliformis* var. *stilbelliformis* (Evans and Samson 1982), *H. sporodochialis*
(Evans and Samson 1984), *H. subramanianii* (Samson and Evans 1985), and *H. zhangjiajiensis* (Liang et al. 2005). Among them, the conidia of *H. shennongjiaensis* are primarily rod-like and slender; *H. stilbelliformis* var. *stilbelliformis* has a larger base with thorny phialides, greater than 50 μm long; *H. sporodochialis* has longer conidia; *H. subramanianii* has hymenopteran hosts and thinner stick-shaped conidia, 10–13.5 × 1.8–2.5 μm; and *H. zhangjiajiensis* conidia are lanceolate or resemble an orange segment (Suppl. material 1: Table S3). Within the framework of the available data for the genus, the phylogenetic tree and the morphological analysis confirmed the status of *Hirsutella flava* and *H. kuankuoshuiensis* as new species.

**Acknowledgements**

We would like to thank Professor Zhuan Chen for presenting us with the specimen HKAS112884. This work was supported by the National Natural Science Foundation of China (No. 32060038, 31860037), the Science and Technology Project of Guizhou Province ([2021]080, [2020] 1Z009), and the Talent Fund of Guizhou University (2019)10.

**References**

Balazy S, Miętkiewski R, Tkaczuk C, Wegensteiner R, Wrzosek M (2008) Diversity of acaropathogenic fungi in Poland and other European countries. Experimental and Applied Acarology 46: 53–70. https://doi.org/10.1007/978-1-4020-9695-2_7

Castlebury LA, Rossman AY, Sung GH, Hyten AS, Spatafora JW (2004) Multigene phylogeny reveals new lineage for *Stachybotrys chartarum*, the indoor air fungus. Mycological Research 108(8): 864–872. https://doi.org/10.1017/S0953756204000607

Ciancio A, Colagiero M, Rosso LC, Murga Gutierrez SN, Grasso G (2013) Phylogeny and morphology of *Hirsutella tunicata* sp. nov. (Ophiocordycipitaceae), a novel mite parasite from Peru. Mycoscience 54: 378–386. https://doi.org/10.1016/j.myc.2013.01.002

Evans HC (1974) Natural control of arthropods, with special reference to ants (Formicidae), by fungi in the tropical high forest of Ghana. Journal of Applied Ecology 37–49. https://doi.org/10.2307/2402003

Evans HC, Samson RA (1982) *Cordyceps* species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems I. The Cephalotes (Myrmicinae) complex. Transactions of the British Mycological Society 79: 431–453. https://doi.org/10.1016/S0007-1536(82)80037-5

Evans HC, Samson RA (1984) *Cordyceps* species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems II. The Camponotus (Formicinae) complex. Transactions of the British Mycological Society 82: 127–150. https://doi.org/10.1016/S0007-1536(84)80219-3

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696–704. https://doi.org/10.1080/10635150390235520
Two new species of Ophiocordyceps from China

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series 41: 95–98. https://doi.org/10.1021/bk-1999-0734.ch008

Hoyos-Carvajal L, Orduz S, Bisett J (2009) Genetic and metabolic biodiversity of Trichoderma from Colombia and adjacent neotropic regions. Fungal Genetics and Biology 46: 615–631. https://doi.org/10.1016/j.fgb.2009.04.006

Hyde KD, Xu J, Rapior S, Jeewon R, Stadler M (2019) The amazing potential of fungi: 50 ways we can exploit fungi industrially. Fungal Diversity: 1–136. https://doi.org/10.1007/s13225-019-00430-9

Hyde KD, Norphanphoun C, Maharachchikumbura SSN, Bhat DJ, Jones EBG, Bundhun D, Chen YJ, Bao DF, Boonmee S, Calabon MS, Chaiwan N, Chethana KWT, Dai DQ, Dayarathne MC, Devadatha B, Dissanayake AJ, Dissanayake LS, Doilom M, Dong W, Fan XL, Goonasekara ID, Hongsanan S, Huang SK, Jayawardena RS, Jeewon R, Karunarathna A, Konta S, Kumar V, Lin CG, Liu JK, Liu NG, Luangsaraard J, Lumyong S, Luo ZL, Marasinghe DS, McKenzie EHC, Niego AGT, Niranjan M, Perera RH, Phukhamsakda C, Rathnavayaka AR, Samarakoon MC, Samarakoon SMBC, Sarma VV, Senanayake IC, Shang QJ, Stadler M, Tibpromma S, Wanasinghe DN, Wei DP, Wijayawardene NN, Xiao YP, Yang J, Zeng XY, Zhang SN, Xiang MM (2020) Refined families of Sordariomycetes. Mycosphere 11(1): 305–1059. https://doi.org/10.5943/mycosphere/11/1/7

Jaffee BA (1992) Population biology and biological control of nematodes. Canadian Journal of Microbiology 38: 359–364. https://doi.org/10.1139/m92-061

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30(4): 772–780. https://doi.org/10.1093/molbev/mst010

Kepler RM, Rehner SA (2013) Genome-assisted development of nuclear intergenic sequence markers for entomopathogenic fungi of the Metarhizium anisopliae species complex. Molecular ecology resources 13: 210–217. https://doi.org/10.1111/1755-0998.12058

Kumar PS, Singh L (2008) Enabling mycelial application of Hirsutella thompsonii for managing the coconut mite. In: Bruin J, van der Geest LPS (Eds) Diseases of Mites and Ticks, Springer, Dordrecht, 169–182. https://doi.org/10.1007/978-1-4020-9695-2_14

Lang G, Blunt JW, Cummings NJ, Cole ALJ, Munro MHG (2005) Hirsutidae, a cyclic tetrapeptide from a spider-derived entomopathogenic fungus, Hirsutella sp. Journal of Natural Products 68: 1303–1305. https://doi.org/10.1021/np0501536

Liang ZQ (1991) Two new entomopathogenic fungi of the genus Hirsutella. Southwest China Journal of Agricultural Sciences 1: e008. https://doi.org/10.16213/j.cnki.scjas.1991.01.009

Liang ZQ, Han YF, Liu AY, Huang JZ (2005) Some entomogenous fungi from Wuyishan and Zhangiajie Nature Reserves. Mycotaxon 94: 349–355. https://doi.org/10.1007/s11769-002-0037-5

Luangsaraard JJ, Mongkolsamrit S, Noisripoom W, Thanakitpipattana D, Khonsanit A, Wuthikun T (2017) Helicocollum, a new clavicipitalean genus pathogenic to scale insects (he-miptera) in thailand. Mycological Progress 16(4): 1–13. https://doi.org/10.1007/s11769-017-1283-3

Maharachchikumbura SSN, Hyde KD, Jones EBG, McKenzie EHC, Huang SK, Abdel-Wahab MA, Daranagama DA, Dayarathe M, D’souza MJ, Goonasekara ID, Hongsanan S, Ruvi-
shika SJ, Kirk PM, Konta S, Liu JK, Liu ZY, Norphanphoun C, Pang KL, Perera RH, Senanayake IC, Shang QJ, Shenoy BD, Xiao YP, BahkaliJichuan AH, Kang JC, Somrothipol S, Suetreong S, Wen TC, Xu JC (2015) Towards a natural classification and backbone tree for Sordariomycetes. Fungal Diversity 72(1): 199–301. https://doi.org/10.1007/s13225-015-0331-z

Maharachchikumbura SSN, Hyde KD, Jones EBG, McKenzie EHC, Bhat DJ, Dayarathne MC, Huang SK, Norphanphoun C, Senanayake IC, Perera RH, Shang QJ, Xiao YP, D’souza MJ, Hongsonan S, Jayawardena RS, Daranagama DA, Konta S, Goonasekara ID, Zhuang WY, Jeewon R, Phillips AJL, Abdel-Wahab MA, Al-Sadi AM, Bahkali AH, Boonmee S, Boonyuen N, Cheewangkoon R, Dissanayake AJ, Kang JC, Li QR, Liu JK, Liu XZ, Liu ZY, Luangsa-ard JJ, Pang KL, Phookamsak R, Promputtha I, Suetreong S, Stadler M, Wen TC, Wijayawardene NN (2016) Families of Sordariomycetes. Fungal Diversity 79 (1): 1–317. https://doi.org/10.1007/s13225-016-0369-6

Mazet I, Vey A (1995) Hirsutellin A, a toxic protein produced in vitro by Hirsutella thompsonii. Microbiology 141: 1343–1348. https://doi.org/10.1099/13500872-141-6-1343

McNeill J, Barrie FR, Buck WR, Demoulin V, Greuter W, Hawksworth D, Herendeen P, Knapp S, Marhold K, Prado J, Prud’homme Van Reine W, Smith G, Wiersema J, Turland N (2012) International Code of Nomenclature for algae, fungi and plants. Regnum Vegetabile 154: 62–74.

Minter DW, Brady BL (1980) Mononematous species of Hirsutella. Transactions of the British Mycological Society 74: 271–282. https://doi.org/10.1016/S0007-1536(80)80157-4

Patouillard N (1892) Une Clavariée entomogène. Revue de Mycologie 3: 67–70.

Petch T (1924) Studies in entomogenous fungi: IV. Some Ceylon Cordyceps. Transactions of the British Mycological Society 10: 28–36. https://doi.org/10.1016/S0007-1536(24)80005-0

Petch T (1936) Isaria exoleta Fr. The Naturalist 1936: 250–251. https://doi.org/10.25291/VR/1936-VLR-250

Petch T (1937) Notes on entomogenous fungi. Transactions of the British Mycological Society 21: 34–67. https://doi.org/10.1016/S0007-1536(31)80006-3

Petch T (1939) Notes on entomogenous fungi. Transactions of the British Mycological Society 23: 127–148. https://doi.org/10.1016/S0007-1536(33)80026-X

Posada D (2008) jModeltest: phylogenetic model averaging. Molecular Biology and Evolution 25: 1253–1256. https://doi.org/10.1093/molbev/msn083

Quandt CA, Kepler RM, Gams W, Araújo JPM, Ban S, Evans HC, Hghes D, Humber R, Hywel-Jones N, Li ZZ, Luangsa-ard J, Rehner SA, Sanjuan T, Sato H, Shrestha B, Sung GH, Yao YJ, Zare R, Spatafora JW (2014) Phylogenetic-based nomenclatural proposals for Ophiocordycipitaceae (Hypocreales) with new combinations in Tolypocladium. IMA Fungus 5: 121–134. https://doi.org/10.5598/imagfungus.2014.05.01.12

Qu JJ, Zou X, Yu JP, Zhou YM (2017) The conidial mucilage, natural film coatings, is involved in environmental adaptability and pathogenicity of Hirsutella satumaensis Aoki. Scientific Reports 7: e1301. https://doi.org/10.1038/s41598-017-01368-1

Qu JJ, Yu LQ, Zhang J, Han YF, Zou X (2018a) A new entomopathogenic fungus, Ophiocordyceps ponerus sp. nov., from China. Phytotaxa 343: 116–126. https://doi.org/10.11646/phytotaxa.343.2.2
Two new species of Ophiocordyceps from China

Qu JJ, Zhou YM, Yu JP, Zhang J, Han YF, Zou X (2018b) Estimated divergence times of Hirsutella (asexual morphs) in Ophiocordyceps provides insight into evolution of phialide structure. BMC Evolutionary Biology 18: e111. https://doi.org/10.1186/s12862-018-1223-0

Rehner SA, Samuels GJ (1994) Taxonomy and phylogeny of Gliocladium analysed from nuclear large subunit ribosomal DNA sequences. Mycological Research 98: 625–634. https://doi.org/10.1016/S0953-7562(09)80409-7

Rehner SA, Buckley EA (2005) Beauveria phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to Cordyceps teleomorphs. Mycologia 97: 84–98. https://doi.org/10.3852/mycologia.97.1.84

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Samson RA, Evans HC (1985) New and rare entomogenous fungi from Amazonia. Proceedings: Plant Sciences 94: 309–317. https://doi.org/10.1134/S1062359006060082

Simmons DR, Lund J, Levitsky T, Groden E (2015a) Ophiocordyces myrmicarum, a new species infecting invasive Myrmica rubra in Maine. Journal of Invertebrate Pathology 125: 23–30. https://doi.org/10.1016/j.jip.2014.12.010

Simmons DR, Kepler RM, Rehner SA, Groden E (2015b) Phylogeny of Hirsutella species (Ophiocordycipitaceae) from the USA: remedying the paucity of Hirsutella sequence data. IMA fungus 6: 345–356. https://doi.org/10.5598/Imafungus.2015.06.02.06

Spatafora JW, Quandt CA, Kepler RM, Sung GH, Shrestha B, Hywel-Jones NL, Luangsa-ard JJ (2015) New 1F1N species combinations in Ophiocordycipitaceae (Hypocreales). IMA fungus 6: 357–362. https://doi.org/10.5598/Imafungus.2015.06.02.07

Speare AT (1920) On certain entomogenous fungi. Mycologia 12: 62–76. https://doi.org/10.1080/00275514.1920.12016820

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML Web-Servers. Systematic Biology 75: 758–771. https://doi.org/10.1080/10635150802429642

Sung GH, Hywel-Jones NL, Sung JM, Luangsa-ard JJ, Shrestha B, Spatafora JW (2007a) Phylogenetic classification of Cordyceps and the clavicipitaceous fungi. Studies in Mycology 57: 50–59. https://doi.org/10.3114/sim.2007.57.01

Sung GH, Sung JM, Hywel-Jones NL, Spatafora JW (2007b) A multi-gene phylogeny of Clavicipitaceae (Ascomycota, Fungi): identification of localized incongruence using a combinatorial bootstrap approach. Molecular Phylogenetics and Evolution 44(3): 1204–1223. https://doi.org/10.1016/j.ympev.2007.03.011

Thompson J, Plewniak F, Poch O (1999) A comprehensive comparison of multiple sequence alignment programs. Nucleic acids research 27: 2682–2690. https://doi.org/10.1093/nar/27.13.2682

Van der Geest LPS (2010) IPM potentials of microbial pathogens and diseases of mites. In: Ciancio A, Mukerji KG (Eds) Integrated Management of Arthropod Pests and Insect Borne Diseases. Springer, Dordrecht, 249–309. https://doi.org/10.1007/978-90-481-8606-8_11

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. Journal of Bacteriology 172(8): 4238–4246. https://doi.org/10.1128/jb.172.8.4238-4246.1990

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ
Supplementary material I

Tables S1–S3. A total of 71 taxa were selected to represent the morphological and ecological diversity of *Hirsutella* asexual morphs and *Ophiocordyceps*

Authors: Jiaojiao Qu, Xiao Zou, Wei Cao, Zhongshun Xu, Zongqi Liang

Data type: phylogenetic data

Explanation note: The GenBank accession numbers of all species are shown in Table 1.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mycokeys.82.66927.suppl1