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Metacordyceps guniujiangensis and its *Metarhizium* anamorph: a new pathogen on cicada nymphs

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Abstract — A new species, *Metacordyceps guniujiangensis*, collected from Guniujiang Nature Preserve in Anhui Province, southeastern China, is described and illustrated. The anamorph isolated from the ascospores is determined as *Metarhizium* aff. *cylindrosporum*. Sequence comparisons of 5.8S ribosomal RNA and complete internal transcribed spacer (ITS) regions from *Metacordyceps guniujiangensis* and *M.* aff. *cylindrosporum* show that the two taxa share the same ITS1-5.8S-ITS2 nucleotide sequences, strongly supporting *M. guniujiangensis* as the teleomorph of *M.* aff. *cylindrosporum*. Molecular phylogenetic analysis agrees with the morphological results and demonstrates that the *M. guniujiangensis* conidial isolate belongs to the genus *Metarhizium*. The phylogenetic tree and ITS sequence show the anamorphic species closely related to *M. cylindrosporum* with a variance at 9.3% (35 bp). The type specimen is deposited at Research Center for Entomogenous Fungi (RCEF), Anhui Agricultural University, China.

Keywords — anamorph-teleomorph connection, *Clavicipitaceae*, entomopathogenic fungi

Introduction

The genus *Cordyceps* Fr. is a group of entomogenous fungi. The earliest description of a *Cordyceps* (*Clavicipitales: Clavicipitaceae*) can be traced to 800 A.D. (Wang 1995). About 400 species of *Cordyceps* and related genera have been reported around the world (Kobayasi & Shimizu 1983, Shimizu 1994), of which approximately 120 have been recorded in China (Liang 2007). Sung et al. (2007) recently segregated the polyphyletic genus into several genera. Among them is the genus *Metacordyceps* G.H. Sung et al., established for several species of *Cordyceps* sensu lato, based on the phylogenetic placement of *C. taii* (Sung et al. 2007).

It is important to identify the anamorphic states of *Cordyceps* sensu lato species correctly for both academic and practical reasons because some species

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have potential value as pharmaceuticals and biological pest controls. However, progress in isolating and verifying *Cordyceps* anamorphs has been very slow. So far, over around seventy *Cordyceps* anamorphs representing twenty genera have been reported. *Hirsutella* Pat., *Hymenostilbe* Petch, and *Paecilomyces* Bainier represent the three principal genera. Other genera such as *Metarhizium* Sorokin and *Nomuraea* Maubl. contain a few anamorphs of *Cordyceps*.

The difficulty of inducing sexual reproduction in artificial culture has limited our knowledge of anamorph-teleomorph connections in these fungi. Kobayasi (1941) presented five criteria for determining *Cordyceps* anamorphs. Liang (1991) proposed an approach based upon microcycle conidiation of secondary ascospores. Currently, ribosomal RNA sequence analysis has provided a powerful molecular tool to clarify the relationships between ascogenous and conidial states. The internal transcribed spacers (ITS), which are more variable than the large and small subunit sequences, have been used to analyze intrageneric or intraspecific relationships by many researchers (Huang et al. 2002; Liu et al. 2001, 2002; Chen et al. 2001; Obornik et al. 2001; Sung et al. 1999; Zhao et al. 1999).

In *Metarhizium,* species relationships and delimitation have been systematically studied using RAPD-PCR (Leal et al. 1994) and ITS analyses (Curran et al. 1994, Driver et al. 2000). Liu et al. (2001) demonstrated the connection of *M. guizhouense* Q.T. Chen & H.L. Guo to its teleomorph, *Cordyceps taii* Z.Q. Liang & A.Y. Liu, through ITS analysis. *M. anisopliae* var. *majus* (J.R. Johnst.) M.C. Tulloch was confirmed as the anamorph of *C. brittlebankisoides* Zuo Y. Liu et al. when the ITS sequence from field–collected stroma proved to be identical to that of the culture (Liu et al. 2001).

An entomogenous fungus collected from southern Anhui, China, during the present study is described here as a new species, *Metacordyceps guniujiangensis*. An isolate derived from its ascospores was determined to be its anamorph, *M.* aff. *cylindrosporum.* This conclusion was supported by comparison of 5.8S rRNA and ITS sequences.

Materials and methods

Specimen and fungal isolates

A specimen (GNJ020527–04) was collected from the National Nature Conservation of Guniujiang, Shitai County, southern Anhui, China, N30°02′05′′ E117°26′26′′, altitude about 500 m. Its host was the soil-dwelling nymph of an unidentified cicada. A hyphomycete was isolated from ascospores discharged onto glass slides according to the method of Li et al. (1999) and was identified as *M.* aff. *cylindrosporum* (RCEF2001). The specimen was freeze-dried and stored at 4°C after isolation and teleomorphic description. Morphological characteristics of the cultures incubated on malt extract agar (MEA) and Czapek-Dox agar at 25°C for 14 days were recorded (Tzean et al. 1993).

Sequence data and analysis

DNA preparation, PCR amplification, purification of amplification products, DNA cloning and sequencing of the amplified ITS region followed the protocol of Huang et al. (2002) using primers ITS1 and ITS4.

Sequence data of *M. guniujiangensis* and *M.* aff. *cylindrosporum* were submitted to GenBank (Accession numbers AY913757 and AY913758, respectively) and were compared with available ITS sequences of *M. cylindrosporum* (ACCC30114, ex type strain), *M. viridulum* (AF368500), *Nomuraea rileyi* (AF368501), *M. anisopliae* var. *anisopliae* (AF135210) and *M. flavoviride* var. *flavoviride* (AF138270) retrieved from GenBank.

Alignments and analysis

Sequences were aligned with Clustal X (Thompson et al. 1994) and positions with gaps (coded as a 5th character) are included. A phylogenetic tree was constructed using neighbor-joining methods using TreeconW in the Treecon software package (Van de Peer & De Wachter 1994). *Beauveria bassiana* (AF347162) was used as an outgroup. 1000 replicates of bootstrap analysis were completed.

Results

Metacordyceps guniujiangensis C.R. Li, B. Huang, M.Z. Fan & Z.Z. Li, **sp. nov.**

MycoBank MB 511341 Fig. 1

Stromata 2, stipite basi confluentia, ex capite hospitis, atrovirentia, curvata, 40.3–42.5 mm longa, stipite cylindrica, 2.5–2.7 mm crassa. Pars fertilis 8.8–11.1 × 2.7–3.2 mm, sterili apice attenuato, luteo, glabello, 5.6–11.1 mm longo, 2.5–3.0 mm crasso basi. Perithecia curvato–ampullacea, penitus oblique immersa, 640–770 × 240–320 μm. Asci cylindrici, 310–380 × 4.0–4.8 μm, cum pileo asci 2.8–3.0 μm diam. Ascosporae filiformes, 240–330 × 0.8–1.0 μm, laeves, multiseptatae, nonsecedentes, cellulis 8–17 μm longis. In larvis cicada.

Holotype—National Natural Reserve of Guniujiang, Shitai County, southern Anhui, P.R. China, 27 V 2002. On nymph of a cicada (*Homoptera: Cicadidae*). Coll. C. R. Li. GNJ020527-04; deposited at Research Center on Entomogenous Fungi, Anhui Agricultural University, China.

Anamorph—*Metarhizium* aff. *cylindrosporum*

Stromata two, stipes 2.5–2.7 mm thick, confluent at basal part, arising from the head of the larval cicada host, dark green, curving, 40.3–42.5 mm long; Fertile part 8.8–11.1 × 2.7–3.2 mm, not clearly defined from the stipes, apically subulate, with acute, yellow and glabrous sterile tip, 5.6–11.1 mm long, 2.5–3.0 mm wide at the base of sterile tip; Perithecia ampullaceous, obliquely immersed, with curved neck, 640–770 × 240–320 μm. Asci cylindrical, 8-spored, 310–380 × 4.0–4.8 μm, ascus cap 2.8–3.0 μm in diameter. Ascospores hyaline, filiform, 240–330 × 0.8–1.0 μm when discharged, smooth, multiseptate with cells 8–17 μm long, not breaking into secondary ascospores.

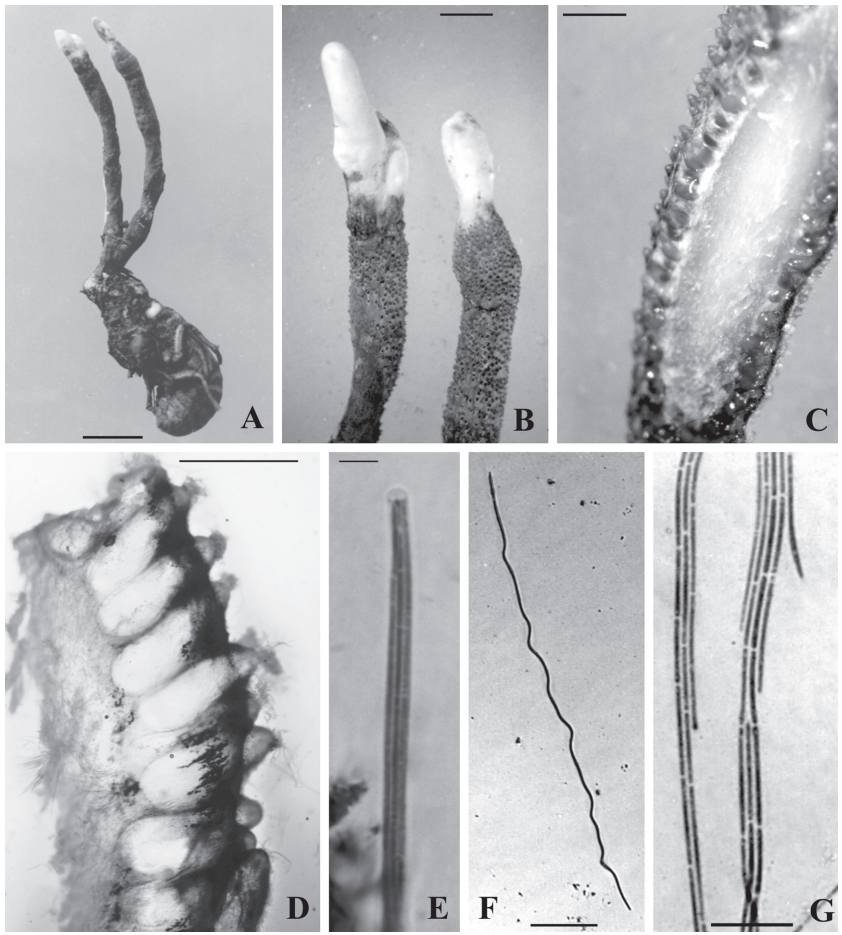

Fig. 1. *Metacordyceps guniujiangensis* A. Stromata of *Metacordyceps guniujiangensis*, bar = 1 cm; B. Fertile stromata showing fertile part and sterile tip, bar = 2.5 mm; C. Section showing oblique orientation of perithecia, bar = 1 mm; D. Perithecia, bar = 500 μm; E. Upper part of an ascus, bar = 5 μm; F. A discharged ascospore, bar = 50 μm; G. Multiseptate nonfragmenting ascospores, bar = 10 μm.

Comments—*Cordyceps owariensis* f. *viridescens* Uchiy. & Udagawa (Uchiyama & Udagawa 2002) and *C. brittlebankisoides* (Liu et al. 2001) are close to this species. The former is more similar to *M. guniujiangensis* in that both attack cicada nymphs and have similar stromata. However, they are quite different in other features.

The two stromata of *M. guniujiangensis* are confluent at their bases; the fertile parts are apically subulate with an acute, yellow, glabrous sterile tip. In comparison, the stromata of *C. owariensis* f. *viridescens* are solitary, simple or 3–4 branched, subulate apically with an acute, white, glabrous sterile tip. Furthermore, the perithecia and asci of *M. guniujiangensis* are longer (440–640 μm) than those of *C. owariensis* f. *viridescens* (180–300 μm), and the ascus cap of the new species is 2.8–3.0 μm in diameter and narrower (4.0–5.0 μm) than that of the latter.

There are obvious differences between *C. brittlebankisoides,* found on larvae of *Coleoptera,* and *M. guniujiangensis*, found on nymphs of *Homoptera*. The arrangement of their perithecia, the sizes of their perithecia and asci, and ascospores are all different. Additionally, the anamorphs of the three species are also different (Table 1).

Table 1. Morphological comparison of *Metacordyceps guniujiangensis* with allied species.

Character	Species		
	C. owariensis f. *viridescens* (Uchiyama & Udagawa 2002)	*C. brittlebankisoides* (Liu et al. 2001)	*M. guniujiangensis* (this publication)
Location	Amami–oshima Island, sw Japan.	Wawu Mountains, Sichuan, sw China	Guniujiang nature preserve, Anhui, se China
Stroma color	greyish–green or dark herbage green	pale green	dark green
Host	cicada nymph	scarabaeid larva	cicada nymph
Fertile part	acute, white, glabrous sterile tip	terminal tapering sterile tip	yellow, acute, glabrous sterile tip
Perithecium (μm)	obliquely inserted, 440–640 × 180–320	vertically immersed, 406–531 × 170–220	obliquely inserted, 640–770 × 240–320
Ascus (μm)	180–300 × 5–6.5	188–313 × 3	310–380 × 4.0–4.8
Ascus cap (μm diam)	4–5	3	2.8–3.0
Ascospore shape. size	multiseptate, nonfragmenting	5.7–8.1 × 0.9 μm	multiseptate, nonfragmenting, 8–17μm long
Anamorph	*Nomuraea owariensis*	*Metarhizium anisopliae* var. *majus*	*Metarhizium* aff. *cylindrosporum*

The culture (RCEF2001) of *M. guniujiangensis* (GNJ020527–04) is kept at the Research Center on Entomogenous Fungi, Anhui Agricultural University, China.

Metarhizium aff. ***cylindrosporum*** (Anamorph) Fig. 2

Colonies on MEA grow moderately at 25°C after 14 days, 28.5–36.5 mm in diam., zonate with radiate veins, velutinous to farinose, light yellow green to light blue, with white floccose, margin. Reverse center light blue, with light crocus margin and radiating veins.

Hyphae septate, smooth–walled, hyaline, 1.5–2.5 µm wide. Conidiophores hyaline, smooth walled, cylindrical, arising from aerial hyphae, mostly branched,

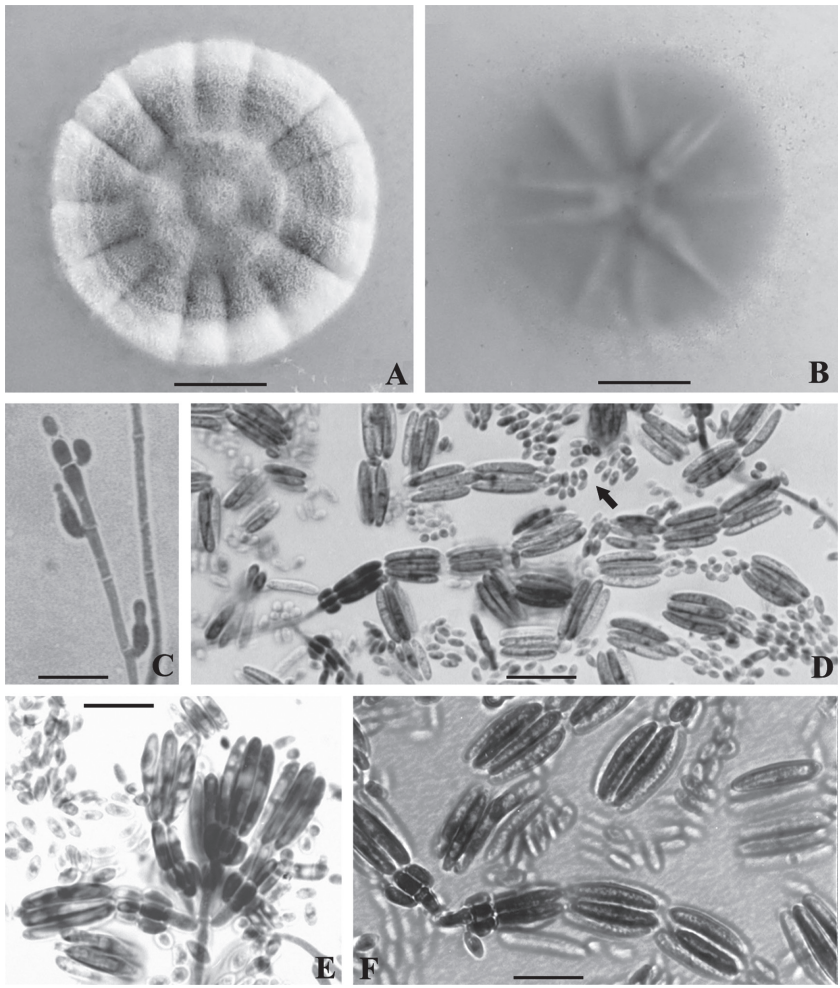

Fig. 2. *Metarhizium* aff. *cylindrosporum*, the anamorph of *Metacordyceps guniujiangensis*. A. A colony of *Metarhizium* aff. *cylindrosporum* on MEA (25°C, 14 d), bar = 10 mm; B. Reverse side, bar = 10 mm; C–F. Conidiophores and conidia: C, E, F, bar = 10 µm; D, bar = 20 µm. Note the small conidia in D (see arrow).

6.0–7.0 × 2.9–3.9 μm. Phialides solitary, or in a cluster of two to five, arising from short branches at the ends of conidiophores, clavate, 4.0–5.5 × 2.8–3.0 μm. Two types of conidia formed in long chains and united in a column structure, one celled, hyaline, smooth walled: small conidia are ovoid to ellipsoid, 5.0–6.5 × 3.0–4.0 μm (Fig. 2 D), and large conidia cylindric to banana–shaped, with both ends narrower, 14.5–24.0 × 3.0–4.0 μm (Fig. 2 D–F).

Colonies growing moderately, reaching 35.7–36.4 mm in diameter after 14 days on Czapek agar at 25°C; lawn thin, with ivory white margin; conidial masses light yellow green to light blue; reverse pale yellow. Conidiophores 5.0–6.3 × 2.0–3.75 μm. Phialides 4.0–6.3 × 2.2–3.0 μm. Two types of conidia: small conidia 5.0–10 × 2.0–3.3 μm, and large conidia 14.0–18.5 × 2.5–3.5 μm. Other characteristics similar to those on MEA.

A culture (RCEF2001) derived from ascospores of *M. guniujiangensis* (GNJ020527-04) is accessioned in the Research Center on Entomogenous Fungi, Anhui Agricultural University, China.

Comments—Both *Metarhizium cylindrosporum* Q.T. Chen & H.L. Guo and *Metarhizium viridulum* (Tzean et al.) B. Huang & Z.Z. Li (Huang et al. 2004) grow on cicada nymphs and adults and have two types of conidia, close to *M.* aff. *cylindrosporum*, but they are quite different in their conidial size, the colour of conidial masses, and teleomorphic state (Table 2).

Table 2. Morphological and ITS sequence comparison of *Metarhizium* aff. *cylindrosporum* to allied *Metarhizium* species

Character	Species			
	M. cylindrosporum (Guo et al. 1986; Shimazu 1989)	*M. viridulum* (Tzean et al. 1992)	*M. viridulum* (Huang et al. 2004)	*M.* aff. *cylindrosporum*
Location	Guiyang, Guizhou, China; Ibaraki, Japan	Taipei, Taiwan	Anhui, China	Guniujiang nature preserve, Anhui, se China
Conidial mass (colour)	On PDA: light to dark green; pale grayish green to olive green	On MEA: greyish green to dull green	On cadaver: white to greyish green	On MEA: light yellow green to light blue
# of conidial shapes	2	1	2	2
Conidial size (μm)	5.4 × 2.7; 17.2–19.7 × 2.5–3.7	14.4–19.4 × 3.8–4.4	4.2–9.0 × 4.0–5.5; 10.8–16.5 × 4.0–5.2	5.0–6.5 × 3.0–4.0; 14.5–24.0 × 3.0—4.0
% ITS sequence divergence* (# bps)	9.3% (35) (type strain)	Unknown	8.2% (31)	0
Teleomorph	Unknown	Unknown	Unknown	*Metacordyceps guniujiangensis*

*note: ITS divergence from *Metarhizium* aff. *cylindrosporum*

Taxonomic placement of the conidial isolate within *Metarhizium*– To determine the phylogenetic position of the anamorph of *M. guniujiangensis* in the *Metarhizium* genus, the sequence of strain RCEF2001 (GenBank No. AY913758) was compared with all available ITS sequences of *M. cylindrosporum* (ACCC30114), *M. viridulum* (AF368500), *Nomuraea rileyi* (AF368501), *M. anisopliae* var. *anisopliae* (AF135210), and *M. flavoviride* var. *flavoviride* (AF138270) retrieved from the GenBank Nucleotide database and analyzed by neighbour-joining, with *Beauveria bassiana* (AF347162) as outgroup (Fig. 3). The ITS sequence of the *M. guniujiangensis* anamorph differs from those of all the other *Metarhizium* spp., although *M. cylindrosporum* and *M. viridulum* are the closest with ITS sequence divergences of 9.3% (35 bp) and 8.2% (31 bp), respectively. The divergence between *M. cylindrosporum* and *M.* aff. *cylindrosporum* is even greater than that between *M. viridulum* and *M.* aff. *cylindrosporum*, although morphological differences between *M. cylindrosporum* and *M.* aff. *cylindrosporum* are less noticeable. The molecular evidence suggests that *M.* aff. *cylindrosporum* may be a new species.

Anamorphic–teleomorphic relationships—The size of amplified fragments spanning the ITS1-5.8S-ITS2 region of *Metacordyceps guniujiangensis* and *Metarhizium* aff. *cylindrosporum* (GenBank no. AY913757 and AY913758) are identical (534 bp). The sizes of ITS1, 5.8S rRNA and ITS2 in the ITS1-5.8S-ITS2 region are also identical at 187 bp, 158 bp and 189 bp, respectively. This molecular evidence confirms that the anamorph of *M. guniujiangensis* is *M.* aff. *cylindrosporum* identified based on the strain RCEF2001.

Discussion

Based on Sung's system (2007), the present specimen should be assigned to genus *Metacordyceps* in the clavicipitaceaen clade that includes the *Cordyceps* sensu lato species with *Metarhizium* anamorphs. A closely related species is *C. owariensis* f. *viridescens*, which also pathogenic on cicada nymphs. The hosts of more than 30 *Cordyceps* and related genera (about 8.5% of all known *Cordyceps* spp.) are reported as homopteran. In addition, among all the species of *Metacordyceps* with *Metarhizium* anamorphs, *C. brittlebankisoides* is closely related to the present species but its host is a scarabaeid.

In 1991, Liang et al. first established the relationship between *Metarhizium* and *Cordyceps*. Microcyclic conidiation confirmed *Metarhizium taii* as the anamorph of *Cordyceps taii* (≡ *Metacordyceps taii*). *Metacordyceps taii* and *M. guizhouense* are the same fungus based on their morphology and esterase isozyme profile (Liu et al., 1995). The name *M. guizhouense* has priority because it was published earlier than *M. taii*. However, Huang et al. (2005) suggested that *M. taii* should be treated as a synonym of *Metarhizium anisopliae* var. *anisopliae*

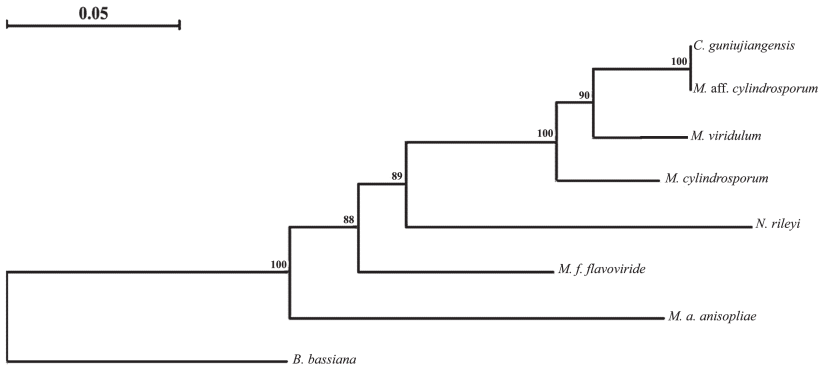

Fig. 3. Neighbour-joining tree based on ITS_1-5.8S-ITS_2 region sequence data from some *Metarhizium* spp. Values above the branches indicate bootstrap support.

based on sequence data of the ITS1-5.8S-ITS2 region, while its teleomorph was *C. taii*. Liu et al. (2001) confirmed *M. anisopliae* var. *majus* as the anamorph of *Cordyceps brittlebankisoides* (≡ *Metacordyceps brittlebankisoides*) on the basis of shared morphological features between an isolate from stromal tissue and one from ascospores. Furthermore, ITS sequences of ribosomal DNA from the field–collected stroma were identical to those from the culture. In addition, Zhang et al. (2004) reported *Cordyceps campsosterni* (≡ *Metacordyceps campsosterni*), a new pathogen of a wireworm, *Campsosternus auratus,* and the third *Cordyceps* species with a *Metarhizium* anamorph. Therefore, including the present species there are four reported species of *Metarhizium* anamorphs connected to *Metacordyceps* after phylogenetic revision of *Cordyceps* and the clavicipitaceous fungi.

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