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Delimitation of a novel member of genus *Metarhizium* (Clavicipitaceae) by phylogenetic and network analysis

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Abstract

An entomopathogenic taxon from a wetland area in Guiyang City, China was found to be a novel species of *Metarhizium* based on both morphology and phylogeny. The new species, which has unique, slender and cylindrical, *Akanthomyces*-like synnemata is described, illustrated and named *Metarhizium synnematis*. Phylogenetic analysis of ITS sequence data confirmed that *M. synnematis* belongs in *Metarhizium*. It differs from other species of *Metarhizium* in having relatively small, ellipsoidal to cylindrical or sub-clavate conidia, coated with a thin mucilaginous sheath which aggregates spores into sticky masses.

Key words: synnematous fungi, entomopathogenic fungi, pleoanamorph, convergent evolution, neighbor net

Introduction

The genus *Metarhizium* (Metschn.) Sorokin belong in the family Claviciptaceae, Hypocreales, Sordariomycetes (Maharachchikumbura *et al.* 2015, 2016) and comprises a diverse group of asexual entomopathogenic fungi, with a global distribution, occurring on a wide range of host insects (Roberts & St. Leger 2004). Rombach *et al.* (1987) and Samson *et al.* (1988) reported that *Metarhizium* has at least three distinct morphological species, *Metarhizium anisopliae* (Metschn.) Sorokīn, *M. flavoviride* W. Gams & Rozsypal and *M. album* Petch. Index Fungorum (2016) however, lists 65 epithets for *Metarhizium*. Driver *et al.* (2000) and Bischoff *et al.* (2006) introduced new species of *Metarhizium* using single and multigene analysis. Kepler *et al.* (2014) revised the genus based on a molecular dataset of four protein-coding genes. Twenty-six species are now presently recognized in *Metarhizium* by (Kepler *et al.* 2014).

Currently, about 17200 full-length fungal ITS sequences are deposited in GenBank, and 56% are associated with a Latin binominal, representing approximately 15,500 species and 2,500 genera (Schoch *et al.* 2011). ITS combines the highest resolving power for discriminating closely related species, with a high PCR and sequencing success rate across a broad range of fungi and ITS was proposed as the primary DNA barcording region for fungi (Schoch *et al.* 2011). Schoch *et al.* (2011, 2014) suggested that authors should include ITS sequences in the description of any new species. Nilsson *et al.* (2014) provided edited ITS sequence data to aid the identification of plant pathogenic fungi.

Several studies have shown that the information from a single gene such as ITS, LSU, ans SSU, was limited and could not effectively distinguish species that have close genetic relations (Kiss *et al*. 2012, Avin *et al.* 2014). However, on the other hand, conflicting phenomena from multi-gene trees has frequently been found (Zou & Ge 2008, Kepler *et al.* 2013, Short *et al.* 2014, Simmons *et al.* 2015). There has been increased interest in using networks in order to avoid the conflicting phenomenon with multi-gene trees (Morrison 2014, Liang *et al.* 2016). Phylogenetic networks could not only reveal multi-gene data conflict and ambiguous information, but also differentiate between allied species which were uncertain and concealed and could not be differentiated by bifurcating trees (Huson & Bryant 2006, Morrison 2014). In the present study, the split network and Min Spanning Network were applied to show the relationship among a new species and its allies.

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Material and methods

Specimens

During a survey of entomogenous stilbellaceous taxa conducted in June 2013, a novel specimen with *Akanthomyces*-like synnemata was collected from the Xiaochehe Forest Wetland Park, Guiyang, China. The material was returned to the laboratory in plastic bags and stored in a refrigerator for microscopic examination. The specimen is deposited in the Herbarium of Guizhou University (GZUH), Guiyang City, Guizhou, China. The Facesoffungi number is provided as detailed in Jayasiri *et al.* (2015).

Morphological examination

The specimen was prepared for microscopic observation by mounting synnemata in lactic acid-phenol-glycerin solution. Microscopic characteristics were observed using a Motic B series microscope fitted with a Motic Digital Moticam 1300 imaging system (Motic China Group Co., Xiamen, China). All measurements were made using the ruler tool in Photoimpact 6.0 ESD extended (Ulead Systems, Taipei, Taiwan).

DNA extraction, PCR amplification, and nucleotide sequencing

Taq enzyme and dNTP were obtained from Shanghai Sangon. The specimen GZUHXCHL12 was used for DNA extraction as described by Tigano-Milani *et al.* (1995). The extracted DNA was stored at –20°C. An internal transcribed spacer (ITS) region, including the 5.8SrDNA, was amplified by polymerase chain reaction (PCR) using the primers ITS5 (5′-GGTGAGAGATTTCTGTGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). After a first denaturation step at 94°C for 5 min, the amplification reaction was performed for 35 cycles with denaturation at 94°C for 40 S, annealing at 49°C for 40 S, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. The PCR products were purified using the UNIQ-10 column PCR product purification kit (no. SK1141; Sangon Biotech (Shanghai) Co., Shanghai, China) according to the manufacturer's protocol and sequenced by Sangon Biotech Co., Shanghai. The sequence of ITS1-5.8S-ITS2 rDNA region of GZUHXCHL12 was submitted to GenBank.

Sequence alignment and phylogenic analyses

The ITS1-5.8S-ITS2 rDNA sequences of GZUHXCHL12 and the closest related fungal species were compared using the similarity search by the NCBI's BLAST search to determine the closet relatives of the novel species and the representative sequences of similarity >50% were downloaded. ITS1-5.8S-ITS2 rDNA sequences of some known species of *Akanthomyces* were also downloaded from GenBank following Benson *et al.* (2000). These sequences were aligned using the CLUSTAL W program (Thompson *et al.* 1997) with the default settings followed by manual refinements. Sequence alignments were deposited at TreeBase (http://purl.org/phylo/treebase/; submission ID 18058). A maximum likelihood tree was constructed using MEGA 5 with the GTR+G+I model (six general time reversible substitution rates, assuming gamma distributed with invariant sites). Bootstrap analysis was conducted to test the reliability of the clades in the phylogeny for 1000 replications (Tamura *et al.* 2011). *Sordaria fimicola* (Roberge ex Desm.) Ces. & De Not. was used as the outgroup taxon.

Construction of the minimum spanning network and neighbor net

The minimum spanning network and neighbor net were generated by Splitstree version 4.10 (Huson & Bryant 2006) using sequence data, including GZUHXCHL12 and *Neotyphodium* spp., *Akanthomyces* spp. and *Metarhizium* spp.

Results

Phylogenetic analyses

The representative ITS-5.8S rDNA sequences of similarity >50% by BLAST and the genera *Metarhizium* and *Akanthomyces* were obtained from GenBank. Maximum likelihood analyses were constructed using MEGA5 software (Tamura *et al.* 2011) and this showed that species of *Akanthomyces* and *Metarhizium* clustered into three distinct clades (Fig. 1). Clade 1 comprised strains of *Metarhizium anisopliae*, *M. flavoviride*, and the plant endophytes, *Neotyphodium* spp. and *Epichloe festucae*, which clustered together with 62% bootstrap support, *M. synnematis* was in a separate subclade and apart from another clade containing *Akanthomyces* spp.

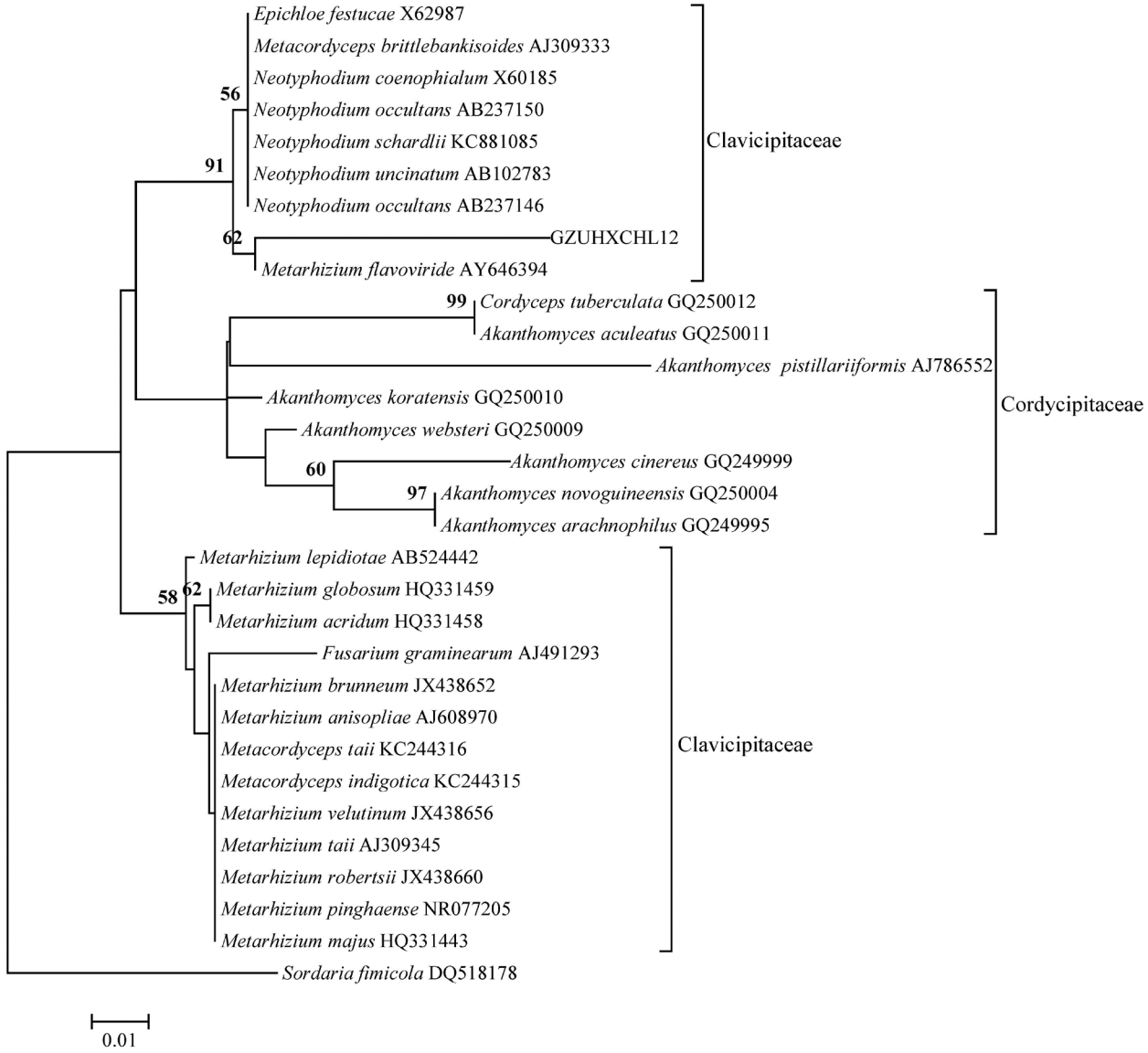

FIGURE 1. Phylogenetic relationship between *Metarhizium synnematis* GZUHXCHL12 and its allies based on ITS-5.8S rDNA sequence data. Bootstrap values (1,000 replicates) are indicated above the nodes.

The mini-spanning network and neighbor net analysis

To confirm the authenticity of *Metarhizium synnematis* as a new species, we reconstructed min-spanning network using sequence data from phylogenetic tree data set (Fig. 1). In the minimum spanning network graph, *M. synnematis* was closely related to *M. flavoviride*, *Cordyceps brittlebankisoides*, and *Neotyphodium* sp. by minimum distance. On the contrary, *Akanthomyces* spp. were distant relatives of *M. synnematis*, having no direct correlation (Fig. 2). The mini-spanning network analysis supported the results from the phylogenetic analysis. The neighbor net showed *Metarhizium* divided into two distinct groups, the *M. flavoviride* complex and *M. anisopliae* complex. *Metarhizium synnematis* closely clustered with the *M. flavoviride* complex, and the strain *M. synnematis* was located on the top of the quadrangle structure (Fig. 4).

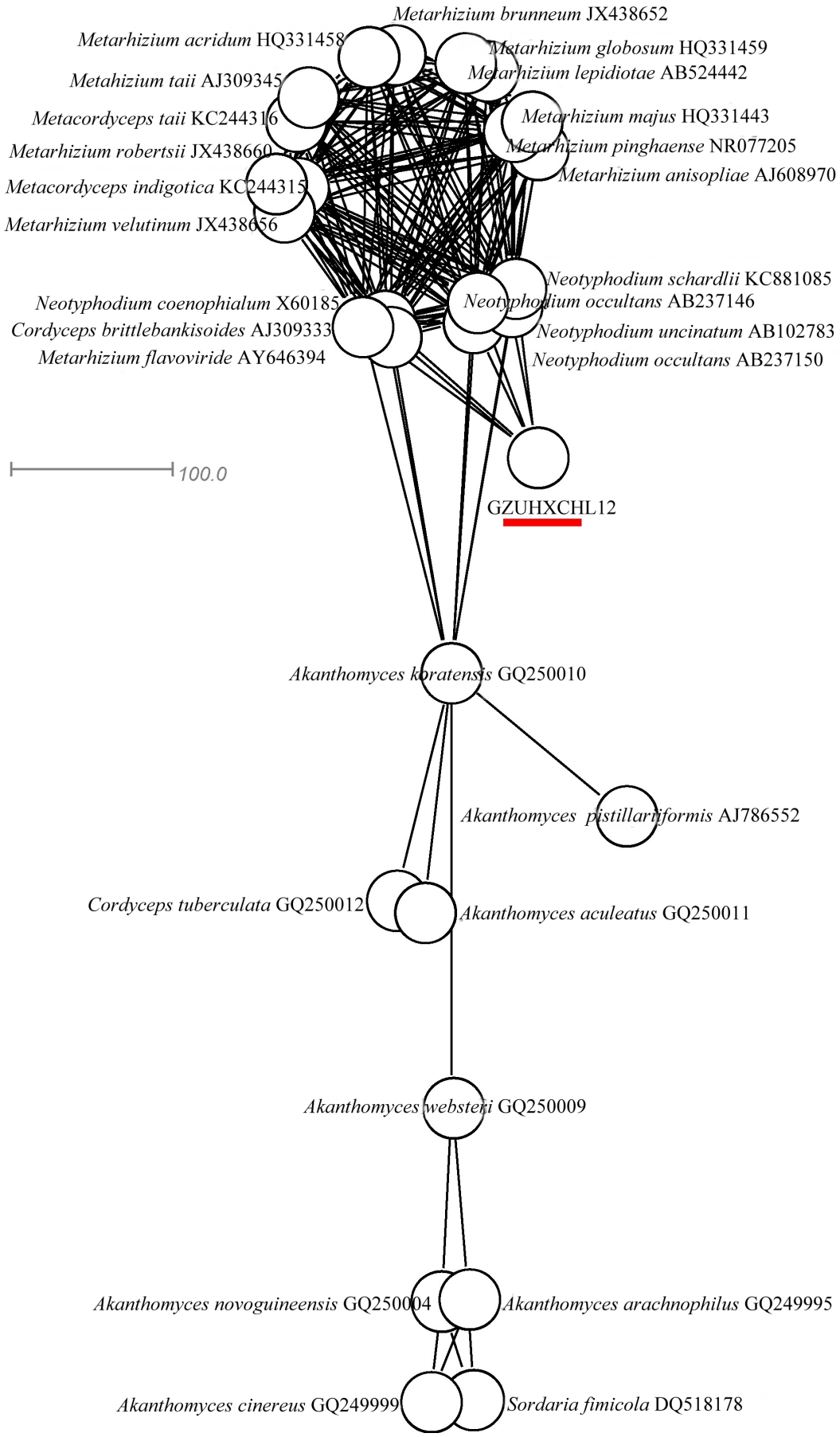

FIGURE 2. Minimum spanning network displaying the relationship among *Metarhizium synnematis*, *Neotyphodium* sp., *Akanthomyces* sp. and *Metarhizium* sp.

Taxonomy

Metarhizium synnematis Z.Q. Liang, H.L. Chu, & T.C. Wen, *sp. nov.* (Fig. 3)
MycoBank: MB808755, Facesoffungi number: FoF 02252.

Type:—CHINA. Guizhou Province: Guiyang City, Xiaochehe Forest Wetland Park, on a cocoon of an unidentified species of Lepidoptera, 12 July 2013, *Zong-Qi Laing* XCH.L12 (GZUHXCHL12 Holotype!).

Host a 30 × 20 mm Lepidopteran cocoon. Asexual morph: Synnemata slender, cylindrical, light brown, upper part pale green, 5–10 mm long and 0.05–0.1 mm wide. Phialides cylindrical to sub-clavate, 5.4–8.6 μm long and 1.1 μm wide with a short neck, most phialides produced either as lateral cells or as terminal cells of short, lateral branches of outer hyphae along the upper part of the synnemata, eventually covering the surface of the synnemata in a hymenium-like manner. Conidia ellipsoidal to cylindrical or sub-clavate, slightly acute at each end, 2.2–4.3 × 1.1 μm, coated with a thin sticky substance, and aggregating into sticky masses. Sexual morph: Undetermined.

Etymology:—Refers to the *Akanthomyces*-like synnemata.

Distribution:—Known only from China.

Host:—On a Lepidopteran cocoon.

Notes: *Metarhizium synnematis* is morphologically similar to *M. flavoviride* in both phialides and conidia. However, *M. flavoviride* does not produce synnemata, and forms larger phialides (7.2–9.6 × 2.5–3.1 μm), as well as larger conidia (4.5–7 × 2–3 μm), as compared both phialides (5.4–8.6 × 1.1 μm) and conidia (2.2–4.3 × 1.1 μm) of *M. synnematis* (Table 1). It differs from related *Metarhizium* species mainly by its small, ellipsoidal to cylindrical or sub-clavate conidia, which are coated with a thin sticky substance and are aggregate into sticky masses. The phylogenetic tree and network based on the maximum likelihood, mini-spanning and neighbor net method also revealed *M. synnematis* to be a new taxon of the genus *Metarhizium* (Fig. 3).

Metarhizium synnematis can form determinate *Akanthomyces*-like synnemata (Seifert 1985). This characteristic is indicative of an *Akanthomyces* sp. (Mains 1950, Samson & Evans 1974). However, the phialides of GZUHXCH12 were clearly cylindrical to subclavate with a short neck (Fig. 1, 4–6), and conidia were coated with mucus and aggregated into sticky masses. These characteristics clearly showed that the specimen belongs to the genus *Metarhizium* (Tulloch 1976). It could be distinguished from other species of *Metarhizium* by its slender synnemata (length: width = 50–100:1) and smaller conidia (2.2–4.3 × 1.1 μm; Table 1). Based on the morphological criterion, the specimen could be regarded as an intermediate between *Metarhizium* and *Akanthomyces*.

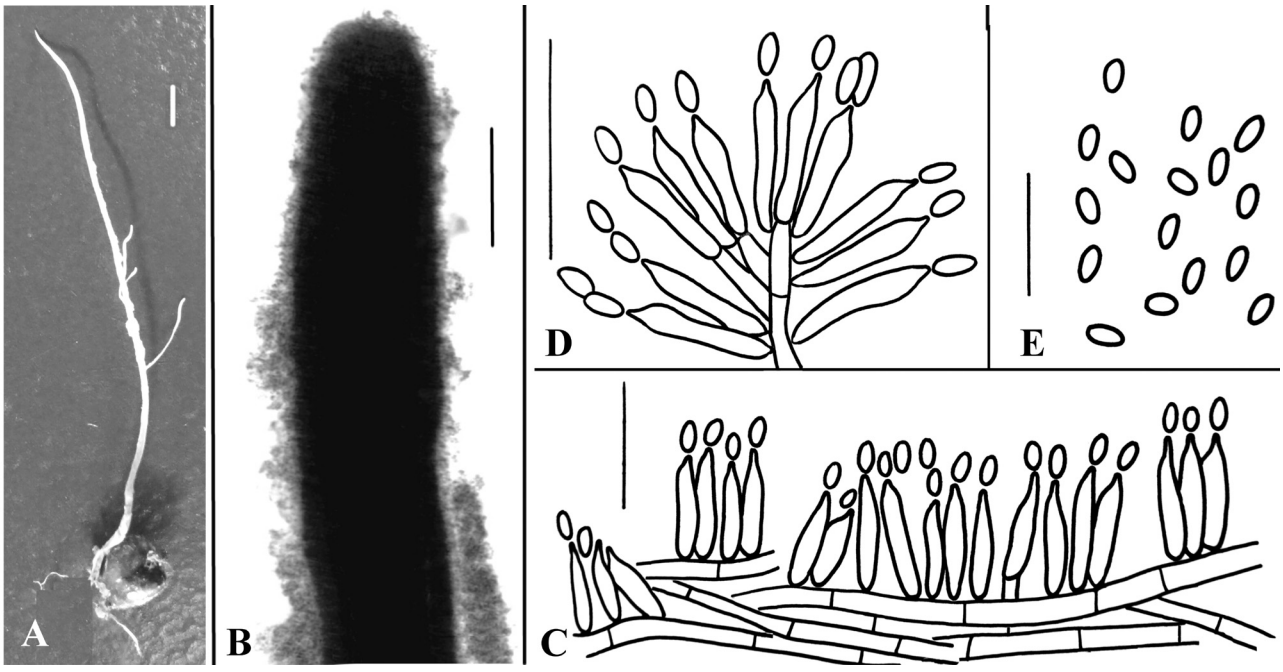

FIGURE 3. ***Metarhizium synnematis*** **(holotype) A.** Synnema on a lepidopteran cocoon. **B.** Upper part of a synnema. **C.** Phialides covering the surface of the synnema in a hymenium with conidia aggregating in sticky masses. **D.** Phialides. **E.** Conidia. Scale bars: A = 10 mm, B = 100 μm, C–E = 10 μm.

TABLE 1. Comparison between *Metarhizium synnematis* and its allies.

Taxa	Phialides (μm)	Conidia (μm)	Synnemata (mm)	Reference
Metarhizium anisopliae var. *lepidiotum*	Cylindric	7.3–10.6 × 3–4.1	Absent	Driver *et al.* 2000
M. anisopliae var. *minus*	Clavate, 9–14 long	Cylindric, 5–8 × 2.5–3.5	Absent	Rombach *et al.* 1986
M. flavoviride var. *flavoviride*	Clavate, 7.2–9.6 × 2.5–3.1	Ovoid to ellipsoidal, 4.5–7 × 2–3	Present 10 × 2	Gams & Roszypal 1973
M. flavoviride var. *novozealandicum*	Clavate to cylindric, 5.5–8.5 × 1.5–2.1	Elongate, 5.5–6.5 × 2.2–2.6	Absent	Driver *et al.* 2000
M. flavoviride var. *pemphigi*	Cylindric, less than 9 long	Ovoid to elongate, 4.9–5.8 × 2–2.8	Absent	Driver *et al.* 2000
M. frigidum	Ovoid to cylindric, 3.5–13.5 × 2.0–3.5	Sub-spherical to cylindric, 4.5–9.0 × 2.0–4.0	Absent	Bischoff *et al.* 2006
M. synnematis	Cylindric to clavate, 5.4–8.6 × 1.1	Ellipsoidal to cylindric, 2.2–4.3 × 1.1	Present 5–10 × 0.1	This work

Discussion

Phylogenetic reconstruction among various organisms not only helps understand their evolutionary history, but also answers several fundamental evolutionary questions. However, almost all of the widely used phylogenetic methods have limitations, which fail to eliminate systematic errors effectively, preventing the reconstruction of true organismal relationships. "Long-branch Attraction" (LBA) artifact is one of the most problematic factors in phylogenetic reconstruction (Bergsten *et al.* 2005). The LBA illusion can be caused by many reasons, such as the convergence and parallel evolution in the dataset (Li *et al*. 2007).

In our present analyses, *Metarhizium synnematis* clustered with *M. flavoviride*. The mini-spanning network revealed a close relationship between *M. synnematis* and the endophytic taxa *Neotyphodium* sp., *Neotyphodium* sp. and *Epichloe* sp., although the latter taxa have a more obligatory relationship to their host plants (Saikkonen *et al*. 2004, Schulz & Boyle 2005), and their conidiogenous structures are simple. *Metarhizium synnematis* is parasitic to insects, rather than being endophytic and it produces synnemata and forms hymenium-like phialides on the synnemata outer layer hyphae. Therefore, *M. synnematis* belongs in the genus *Metarhizium*, rather than the morphologically similar genus *Akanthomyces,* based on the combined data of maximum likelihood tree and the mini-spanning network.

Evidence indicates that *M. synnematis* belongs to the genus *Metarhizium*, however a critical question concerns the origin to long-branch of *M. synnematis*. In the neighbor-net, *Metarhizuim* species clustered in two groups, the *M. flavoviride* complex and *M. anisopliae* complex. *Metarhizium synnematis* grouped with the *M. flavoviride* complex and is consistent with the maximum likehood tree and the mini-spanning network.

Long-branches have often been found in the molecular phylogenetic studies. There was a long-branch effect in our phylogenetic tree and Neighbor-Net network. In general, the reasons for the long-branch effect are (1) the unit with a long-branch in the topological structure is due to significant acceleraion of evolution rate in the dataset, (2) the change of internal topological structure by the long-branch attraction in the in group because of the inappropriate or a too-distant outgroup, (3) a synapomorphies by the evolutionary characteristics of convergence or analogous evolution, the unit clustered in a wrong relationship with the sister group, (4) the inappropriate nucleotide substitution model in the dataset (Bergsten 2005, Li *et al.* 2007, Morrison 2011). In our study, the long-branch of *M. synnematis* occurred on the quadrangle vertex in the neighbor-net network. The main reason may be because of recombination evidence (Gandolfi *et al.* 2003, Bergsten 2005, Morrison 2011).

Synnematous entomopathogenic fungi, such as *Akanthomyces*, *Hymenostilbe*, *Hirsutella*, *Polycephalomyces*, *Stilbella*, and *Tilachlidiopsis* species are often found in the shrubbery of virgin forests, litter layers, or shallow soils (Hywel-Jones 1996). As air flow under the forest canopy is slow and humid, the dispersal of conidia through airflow diffusion should be difficult. Therefore, these entomopathogenic fungi should employ a particular strategy, by producing

different synnemata types and sticky conidia, to accommodate various arthropods activities and conidium spread (Abbott 2002). The pleoanamorphy in entomogenous fungi occurs. For example, most *Polycephalomyces* species produce *Hirsutella*-like and *Acremonium*-like asexual morphs in culture (Kepler *et al.* 2013). *Aschersonia insperata* also possesses characteristics of *Aschersonia* and *Hirsutella* morphs (Liu *et al.* 2005). *Metarhizium synnematis* with *Akanthomyces*-like synnemata, is also pleoanamorphic. The states of pleoanamorphs associated with sexual morphs may be a result of convergent evolution. Similar to other pleoanamorphic fungi, *M. synnematis* with *Akanthomyces*-like synnemata performed the functions of different types of phialides and conidia possibly because conidia were dispersed differently and utilize different infection strategies. These pleoanamorphic fungi increased their fitness in different environmental conditions. *M. synnematis* produces two types of conidia aggregation, one in masses of hydrophilic slime and another in a single, dry, short chain. The former can be dispersed through rainwater, and the latter can be spread by various moving arthropods.

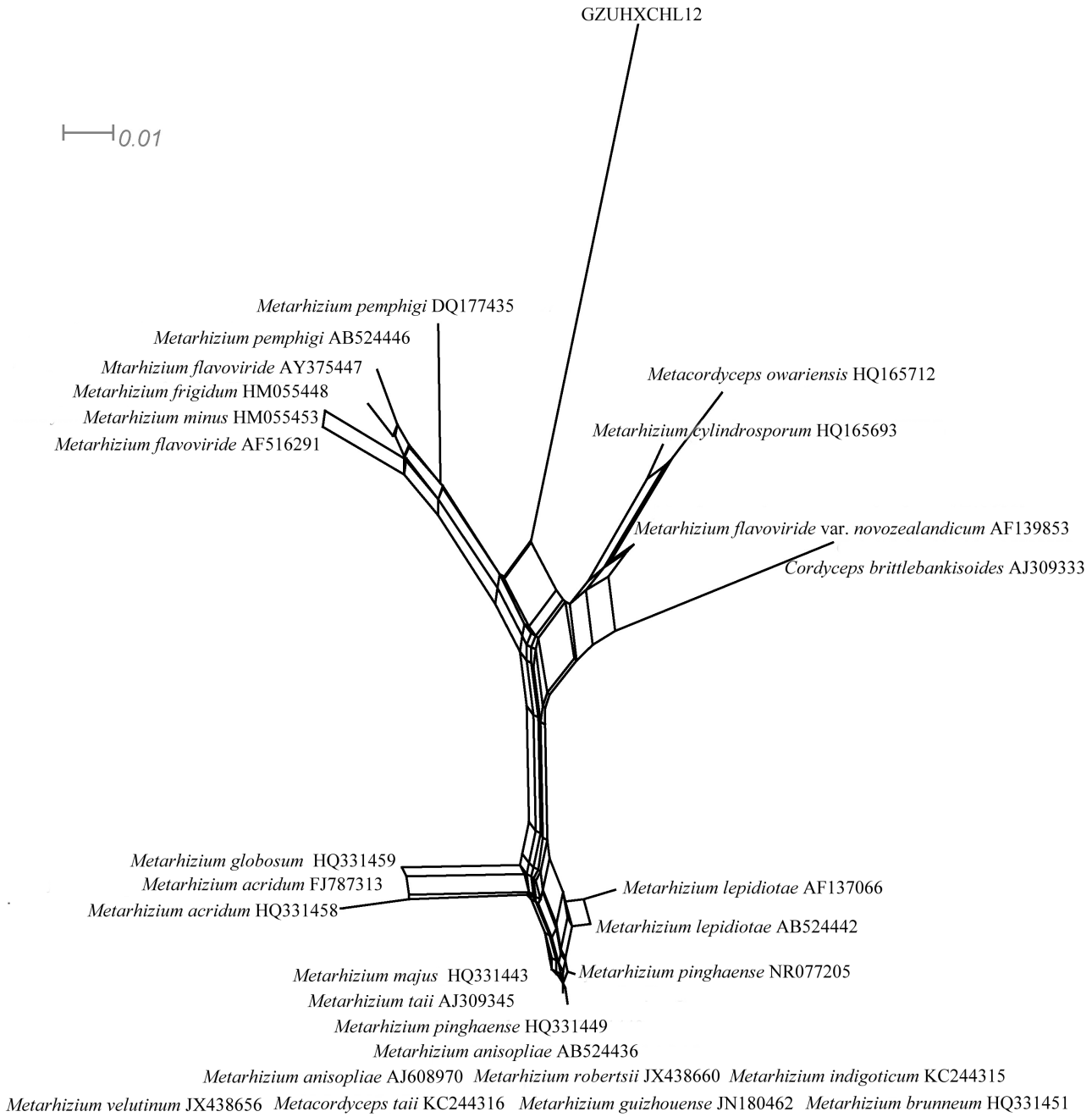

FIGURE 4. Reconstruction of Neighbor-Net network of *Metarhizium synnematis* and *Metarhizium* sp.

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