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Metarhizium dendrolimatilis, a novel *Metarhizium* species parasitic on *Dendrolimus* sp. larvae

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Abstract

A novel species of the genus *Meatrhizium*, *Metarhizium dendrolimatilis*, parasitic on *Dendrolimus* sp. larvae, collected in Huaxi, Guiyang, Guizhou Province, China, is described based on morphological and phylogenetic evidences. This species differs morphologically from other species in the genus by its determinate synnemata, ellipsoidal conidia, and globose phialides. The phylogenetic analyses based on four loci (EF1a, RPB1, RPB2 and TUB), strongly support the novel species designation of this fungus within the *Metarhizium* genus, *Metarhizium dendrolimatilis* sp. nov.

Key words – entomopathogenic fungi – morphology – multi-gene – phylogeny

Introduction

The genus *Metarhizium* (Metschn.) Sorokin consists of a diverse group of asexual entomopathogenic fungi with a global distribution and a wide range of host insects. The organism has been used as bio pesticide (Roberts & St. Leger 2004) for mite and tick control (Maniania et al. 2008, Stafford et al. 2010, Wekesa et al. 2005, 2006). Since this genus was initially established, several new species and varieties have been described based on seemingly minor morphological differences from *M. anisopliae* (Metschn.) Sorokīn, the type species of the genus (Latch 1965, Petch 1931, 1935). Tulloch (1976) recognized only two species, *M. anisopliae* and *M. flavoviride* W. Gams & Rozsypal, along with a single variety *M. anisopliae* var. *majus* (J.R. Johnst.) M. C. Tulloch (as var. *major*) (Tulloh) in her revision of the genus. Rombach et al. (1986) segregated *M. flavoviride* W. Gams & Rozsypal into the varieties *M. flavoviride* var. *flavoviride*, and by virtue of its smaller conidia, *M. flavoviride* var. *minus* Rombach, Humber & D.W. Roberts. Liang et al. (1991) named *M. taii* Z.Q. Liang & A.Y. Liu isolated from the new species, *Cordyceps taii* Z.Q. Liang & A.Y. Liu, and postulated a connection between *C. taii* and *M. taii* based on microcyclic conidiation. Liu et al. (2001) collected a single specimen and described it as a new species,

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Cordyceps brittlebankisoides Zuo Y. Liu, Z.Q. Liang, Whalley, Y.J. Yao & A.Y. Liu, whose anamorph (confirmed by the identical ITS sequences from the teleomorphic stroma and in vitro culture) was identified as *M. anisopliae* var. *majus*.

The development of molecular techniques, in particular DNA sequencing, provides valuable tools for studying phylogenetic relationships and clarifying the taxonomic position of confusing species. Single and multi-locus were used in the phylogenetic classification of *Metarhizium* (Bischoff et al. 2006, 2009, Curran et al. 1994, Driver et al. 2000, Rakotonirainy et al. 1994). These studies highlight the superiority of the multigene phylogenetic approaches for determination of species boundaries and relationships in *Metarhizium*. Kepler et al. (2014) proposed application of the name *Metarhizium* in a manner that approximates the concept of *Metacordyceps* sensu Sung et al. (2007), minus *Pochonia*. The newly proposed definition of *Metarhizium* includes green-spored species in *Nomuraea*, the genus *Chamaeleomyces*, and several species formerly included in *Paecilomyces*. However, no *Metarhizium* species have previously been found with naturally determinate synnemata (fruit body).

Recently, we screened the synnematous entomopathogenic fungi of southwest China and isolated a *Metarhizium* with determinate synnemata infecting a *Dendrolimus* larvae. Based on a combined of the morphological characters and our phylogenetic analysis, we concluded that the strain GZAC IFR1006 represents a new species and describe it here as *Metarhizium dendrolimatilis*.

Materials & Methods

Field collections

A novel specimen HXDX.1006 of nomuraea-like structure was collected in the Tongmuling, Huaxi, Guiyang, China during a survey of the divergence of synnematous entomogenous fungi. The collected material was returned to the laboratory in plastic bags and stored in a refrigerator until used for microscopic examination and molecular phylogenic analysis. Strain GZAC IFR1006 was isolated from the specimen HXDX.1006 on improved PDA (1% w/v peptone). The specimen and the strain were deposited in the Institute of Fungus Resources, Guizhou University, formally Herbarium of Guizhou Agricultural College (code, GZAC), Guiyang City, Guizhou, China.

Morphological examinations

Specimen HXDX.1006 was prepared for microscopic observation using a small amount of synnemata mounted in lactic acid-phenol-glycerin solution. The strain GZAC IFR1006, was incubated on Sabouraud's dextrose and potato dextrose agars at 25 °C for 14 d. Observations of microscopic features were made using the Motic B series microscope fitted with a Motic Digital Moticam 1300 imaging system (Motic China Group Co. Ltd. Xiamen, China). All measurements were made with the ruler tool in Photoimpact 6.0 ESD extended (Ulead Systems, Inc., Taipei, Tw).

DNA extraction, PCR amplification and nucleotide sequencing

The extracted DNA was stored at −20 °C. Taq enzyme and dNTP were from Shanghai Tiangen. DNA extraction was according to Liang et al. (2011). Amplification of Beta tubulin (TUB) was performed with Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') (Glass & Donaldson 1995). RNA polymerase II largest subunit (RPB1) was with the primer CRPB1A (5'-CAYCCWGGYTTYATCAAGAA-3') and RPB1Cr (5'-CCNGCDATNTCRTTRTCCATRTA-3') (Castlebury et al. 2004). For the amplification of RNA polymerase II subunit two (RPB2) the forward primer 5'-GACGACCGTG ATCACTTTGG-3' and the reverse primer 5'-CCCATGGCCTGTTTGCCCAT-3' were used (Houbraken et al. 2007), and translation elongation factor 1 alpha (EF1a) was amplified with forward primer 5'-GCCCCCGGCCATCGTGACTTCAT-3' and reverse primer 5'-ATGACACCGACAGCGACGGT

CTG-3'(Houbraken et al. 2007). The PCR products were purified using the UNIQ-10 column PCR Products Purification kit (no. SK1141; Sangon Biotech (Shanghai) Co. Ltd', Shanghai, China) according to the manufacturer's protocol and sequenced with the above primers at Sangon Biotech (Shanghai) Co. Ltd.

Phylogenetic analysis

DNA sequences generated in this study were assembled and edited using Lasergene software (version 6.0, DNASTAR). Sequences of EF1a, RPB1, RPB2 and TUB from 33 taxa (with *Nomuraea atypicola* as an outgroup) based on the BLAST sequence similarity to our sample and the literature (Kepler et al. 2014) were downloaded from GenBank. Multiple sequence alignments for EF1a, RPB1, RPB2 and TUB were constructed with MAFFT v7.037b (Katoh et al. 2013). Sequences alignments were manually edited with MEGA v. 6 (Tamura et al. 2013). A concatenated dataset (EF1a+RPB1+RPB2+TUB) was assembled using SequenceMatrix v. 1.7.8 (Vaidya et al. 2011). Concordance between genes was assessed with the 'hompart' command of PAUP4.0b10 (Swofford 2002).

A phylogenetic tree was inferred from the combined four-locus dataset with MrBayes 3.2 (Ronquist et al. 2012). Two runs were executed simultaneously for 10 000 000 generations, saving trees every 500 generations. The SYM+G nucleotide substitution model and GTRGAMMA rate variation model were used for all partitions. All phylogenetic reconstructions were performed with services available from the CIPRES web portal (Miller et al. 2010). Our aligned EF1a+RPB1+RPB2+TUB dataset is 3,982 bp long, and is available in TreeBASE under submission ID 19899.

Results

Phylogenetic analyses

Newly generated EF1a, RPB1, RPB2 and TUB sequences of GZAC IFR1006 were deposited in GenBank (KT166031, KT961694, KT166032, and KT166033, respectively). *M. dendrolimatilis* assorts to a single clade by itself, supported by a high posterior probability in our maximum likelihood and Bayesian analysis (Fig. 1).

Metarhizium dendrolimatilis Z.Q. Liang, W.H. Chen, Y.F. Han & D.C. Jin, **sp. nov.** Fig. 2
MycoBank 812866
Facesoffungi number: FoF 02608

Etymology – named for its host *Dendrolimus* sp..

On *Dendrolimus* sp. caterpillar, 2.5 cm long, 0.9 cm wide. Asexual morph: Synnemata are scattered, white to earth yellow at the base, white to green at the upper edges. The spore mass, when present, is 0.6–1.2 cm long, 282–333 μm wide. Conidiophores arise from the lateral hyphae of the synnemata, bearing loosely verticillate, with each branch bearing 2–10 phialides; the verticillate space is 22.5–35 μm. Phialides are closely appressed, rarely solitary, and directly on the conidiophores, with base globes, and a short neck, 2.2–3.2 μm. Conidia are one-celled, smooth-walled, ellipsoid, 3.2–5.4 × 2.2–3.2 μm, or globose, 2.2–4.3 μm long.

Colonies grow moderately fast on PDA at 25°C; after 14 days, 20.5–24.5 mm in diameter, velutinous, light yellow green, with white floccose margin. The reverse is light yellow. Hyphae are septate, smooth-walled, hyaline, and 1.5–1.8 μm wide. Conidiophores are hyaline, smooth walled, cylindrical, arise from aerial hyphae, and 9.3–15.0 × 1.2–3.4 μm. Phialides are in a cluster of two to five, arising on the lateral and end of conidiophores, subglobose at the base, and 4.3–5.4 μm long. Conidia are single or formed in long chains, one celled, hyaline, smooth walled, ellipsoid, and 3.8–10 × 2–2.5 μm.

Colonies grow slowly on Czapek agar at 25°C; after 14 days, 18.5–20.0 mm in diameter, thinly lawned, with an off white margin. Conidiophores 13.5–14.0 × 1.1–1.6 μm. Phialides 3.9–6.5 × 1.9–2.5 μm. Conidia 3.0–3.9 × 1.3–2.2 μm. Other characteristics were similar to those on PDA growth.

Sexual morph – Unknown

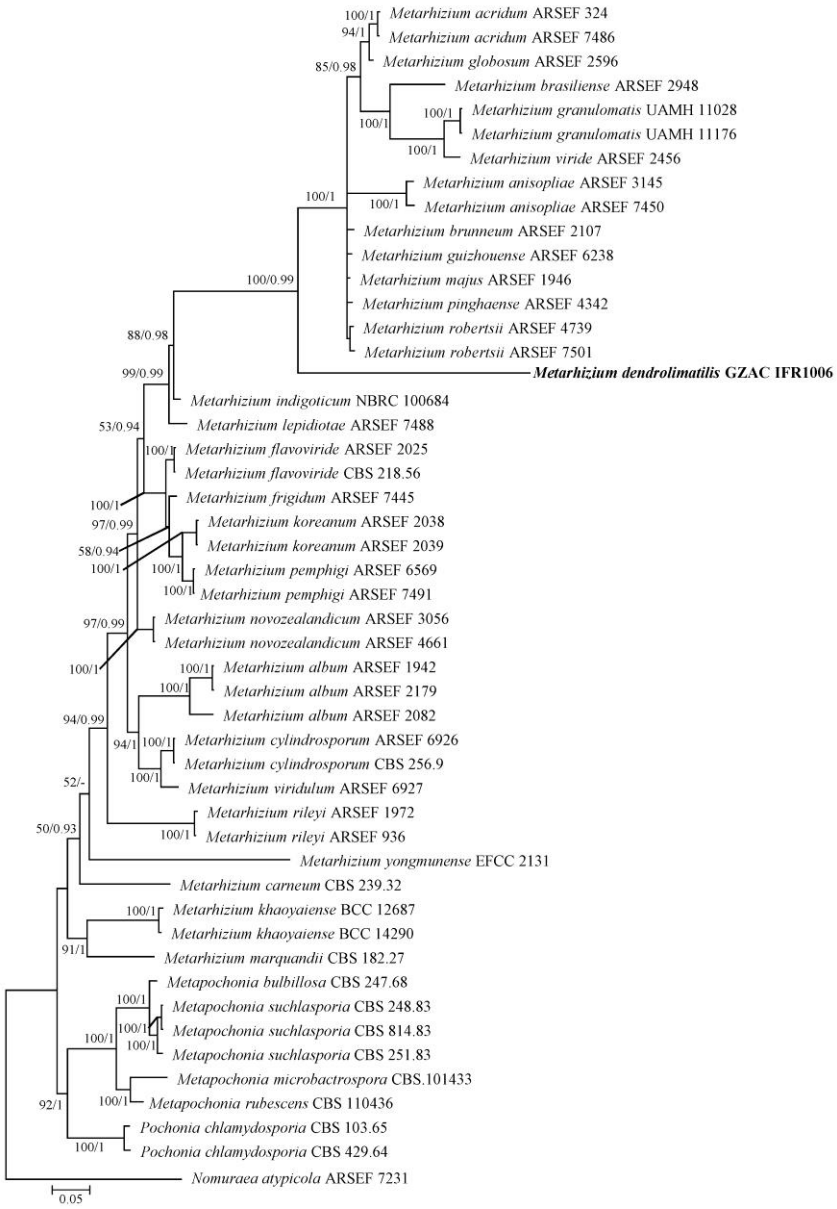

Fig. 1 – Phylogenetic tree generated from maximum parsimony and Bayesian method analysis based on EF1a+RPB1+RPB2+TUB sequences. Statistical support values of Maximum-parsimony bootstrap values (⩾50 %) and Bayesian posterior probabilities (⩾0.9) are shown at nodes.

Type – China, Guizhou Province, Guiyang, Tongmuling, N26°23′, E106°40', on the *Dendrolimus* sp. in the pinewood, 6 October 2013, W.H. Chen (HXDX.1006, holotype)

Known distribution – Guiyang and Qiandongnan Miao and Dong Autonomous Prefecture Guizhou Province, China

Material examined – China, Guizhou Province, Qiandongnan Miao and Dong Autonomous Prefecture, Cenggong County (27°12′N, 108°84′E), on the *Dendrolimus* sp. in the pinewood, 24 October 2015, L.Q. Yu, paratype CC1024, ex-paratype culture GZAC JS102401).

Notes – We compared *M. dendrolimatilis* with similar species of this genus. Only one species has ellipsoidal conidia and synnemata, *M. flavoviride* var. *minus* Rombach, Humber & D.W. Roberts, but *M. dendrolimatilis* is easily distinguishable from *M. flavoviride* var. *minus* by its globose phialides (2.2–3.2 μm).

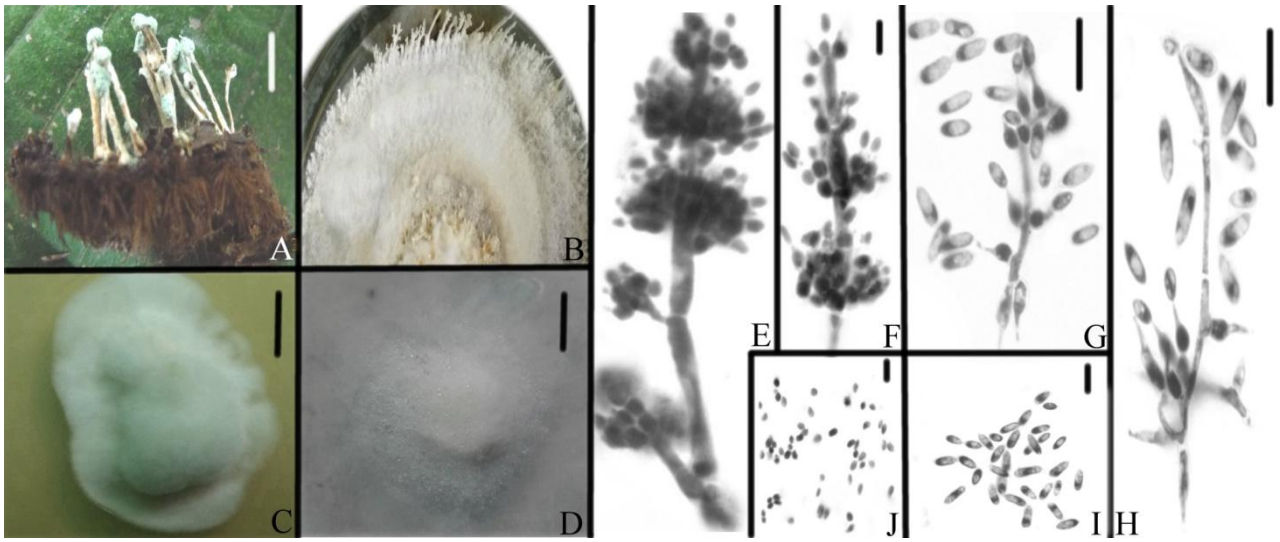

Fig. 2 – ***Metarhizium dendrolimatilis* (HXDX.1006, holotype)**. **A** Synnemata on the *Dendrolimus* sp. **B** Synnemata on the edge of the PDA plate after 20 d at 25 °C. **C, D.** The colony on PDA and Czapek after 14 d at 25 °C. **E, F, J.** Conidiophores, phialides and conidia arising on the natural substrate. **G, H, I.** Conidiophores, phialides and conidia formed on PDA. – Bar: A, C, D = 0.5 cm; E, F, G, H, I, J = 10 μm.

Discussion

As the originally described by Sorokīn and subsequently emended (Rombach et al. 1987), the main taxonomic criteria for the genus *Metarhizium* are the shape of its phialides and conidia, with or without a sporulation zone in a hymenium-like layer, as well as a clustering behavior forming aprismatic spore masses. Conidia size has great significance in *Metarhizium* classification; the color of hyphae and conidia is of secondary significance. We compared *M. dendrolimatilis* with similar species of this genus. Only one species has ellipsoidal conidia and synnemata, *M. flavoviride* var. *minus* Rombach, Humber & D.W. Roberts, but *M. dendrolimatilis* is easily distinguishable from *M. flavoviride* var. *minus* by its globose phialides (2.2–3.2 μm).

The most complete taxonomic treatments of *Metarhizium* to date were conducted using a four-locus phylogeny (Kepler et al. 2014, Montalva et al. 2016). We decided to use the same four-locus multigene phylogenetic approach in our present study. Most branches are strongly supported in the phylogenetic tree inferred from our Bayesian analysis. Strain GZAC IFR1006 assorts to a single clade alone, supporting its unique identification based on morphological characters. *Metarhizium dendrolimatilis* is a new species in the genus *Metarhizium*.

Despite our evidence, based on morphological characters and a phylogenetic analysis, which shows that *Metarhizium dendrolimatilis* is a new species, a crucial question remains: What is the molecular genetic background for the presence of synnemata in *M. dendrolimatilis*. Gao et al. (2011) presented a comparative analysis of *M. anisopliae* and *M. acridum* genome sequences, and speculates that the two species diverged from an ancestral plant endophytic fungi similar to *Epichloe fetucae*, or an ancestral wheat pathogenic fungi similar to *Fusarium graminearum*, ca. 33–43 million years ago. *Metarhizium*'s habitat changed from plant to soil during this divergence; a concurrent host shift occurred to soil inhabiting insects. *Metarhizium taii* Z.Q. Liang & A.Y. Liu and M. majus (J.R. Johnst.) J.F. Bisch. (the anamorph of *M. brittlebankisoides* (Zuo Y. Liu, Z.Q. Liang, Whalley, Y.J. Yao & A.Y. Liu) Kepler, S.A. Rehner & Humber) (Liang et al. 1991, Liu et al. 2001) are two species that continued to adaptively radiate into different forest ecological conditions, and evolved different kinds of synnemata for different means of spore dispersal (Kepler

et al. 2014). The formation of synnemata as a newly derived character in *Metarhizium* is a product of convergent evolution, and serves as an adaption for the dispersal of spores in very specific ecological conditions.

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