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Morphological and phylogenetic characterization of novel *Metarhizium* species in Guizhou, China

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Abstract

Two new species, *Metarhizium lepidopterorum* and *Metarhizium rongjiangense*, which are both parasitic on lepidopteran pupae, are reported. Both the morphological characteristics and DNA-based phylogenies from a multigene dataset-based analysis supported the identification of two new species. Both species can be distinguished from other species of *Metarhizium* by their lecanicillium-like conidiophores, longer phialides and the presence of only one type of conidium, which is smaller and fusiform in *M. lepidopterorum* and smaller and of subwedge shape in *M. rongjiangense*. The combined dataset (*ITS*, *RPB1*, *RPB2* and *TEF*) was analysed phylogenetically in *Metarhizium* spp. The new species described herein are clearly distinct from other *Metarhizium* species.

Key words: convergent evolution, lecanicillium-like, *Metarhizium*, morphology, phylogeny

Introduction

The genus *Metarhizium* was established based on the species *Metarhizium anisopliae* (Metschn.) Sorokîn, which was initially described as *Entomophthora anisopliae* Metsch. by Metschnikoff (Sorokin 1883). Petch (1931, 1935) reported two species, *M. album* Petch and *M. brunneum* Petch, which were isolated from a leafhopper and hemipteran larva, respectively. Tulloch (1976) re-evaluated the genus *Metarhizium* and delimited it into three species, *M. anisopliae*, *M. flavoviride* W. Gams & Rozsypal and *M. anisopliae* var. *majus* (J.R. Johnst.) M.C. Tulloch. The connection between *Metarhizium* and *Cordyceps* was confirmed based on the microcycle conidiation and ITS sequences analyses by Liang *et al*. (1991) and Liu *et al*. (2001), respectively.

Driver *et al*. (2000) redefined the taxonomy of *Metarhizium* using ITS sequences. *Metarhizium album*, *M. flavoviride* var. *flavoviride* and *M. flavoviride* var. *minus* were recognized, and four new varieties, *M. anisopliae* var. *lepidiotae* Driver & Milner, *M. anisopliae* var. *acridum* Driver & Milner, *M. flavoviride* var. *novozealandicum* Driver & Milner and *M. flavoviride* var. *pemphigi* Driver & Milner were described. *Metarhizium anisopliae* var. *frigidum* A.C. Rath, C.J. Carr & B.R. Graham was treated as a synonym of *M. flavoviride* based on ITS sequence analysis. Bischoff *et al*. (2006) investigated whether *M. anisopliae* var. *frigidum* was a synonym of *M. flavoviride* by analyzing three protein-coding genes (*TEF*, *RPB1* and *RPB2*), and defined the new species, *Metarhizium frigidum* J.F. Bisch. & S.A. Rehner.

Four protein-coding genes (*TUB*, *RPB1*, *RPB2* and *TEF*) were assessed in *Metarhizium* and its allies by Kepler *et al*. (2014). As a result, they transferred many/several species of *Metacordyceps* G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, *Nomuraea* Maubl., *Chamaeleomyces* Sigler and *Pochonia* Bat. & O.M. Fonseca to *Metarhizium*. New *Metarhizium* species were described later (Montalva *et al*. 2016, Chu *et al*. 2016, Chen *et al*. 2017, Luangsa-ard *et al*. 2017, Lopes *et al*. 2018, Chen *et al*. 2018, Nishi *et al*. 2018, Gutierrez *et al*. 2019, Luz *et al*. 2019). Recently, two infected insect specimens were collected during a survey of araneogenous fungi and their allies in southwestern China. Morphological and molecular phylogenetic analyses suggested that these specimens represented two new species, which are described here as *Metarhizium lepidopterorum* and *M. rongjiangense*.

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Materials & methods

Specimen collection and identification

Two infected lepidopteran pupae (DL1022 and DL1030) were collected from Dali, Rongjiang County (26°01′58.70″ N, 108°24′48.06″ E), Guizhou Province, on October 1, 2018. Fungal strains DL10221, DL10222, DL10301 and DL10302 were isolated and cultured on agar plates containing an improved potato dextrose agar (PDA, 1% w/v peptone) medium. The specimens and the isolated strains were deposited in the Institute of Fungus Resources, Guizhou University (formally Herbarium of Guizhou Agricultural College; code, GZAC), Guiyang City, Guizhou, China.

The strains were incubated on PDA at 25 °C for 14 d. Macroscopic and microscopic morphological characteristics of the fungi were examined using classical mycological techniques, and the growth rates were determined. Fresh hyphae were observed with an optical microscope (OM, BX35, Olympus, Japan) following pretreatment with lactophenol cotton blue solution or normal saline.

DNA extraction, *PCR amplification and nucleotide sequencing*

DNA extraction was carried out in accordance with Liang *et al*. (2011). The extracted DNA was stored at −20 °C. Translation elongation factor 1 alpha (*TEF*) and RNA polymerase II largest subunit 2 (*RPB2*) were amplified using 983F/2218R and RPB2-5F/RPB2-7Cr primers according to van den Brink *et al*. (2012). RNA polymerase II largest subunit 1 (*RPB1*) was amplified with the primer pair CRPB1 and RPB1-Cr (Castlebury *et al*. 2004). The internal transcribed spacer (*ITS*) region was amplified using ITS4/ITS5 primers by PCR as described by White *et al*. (1990). PCR products were purified using the UNIQ-10 column PCR products purification kit [no. SK1141; Sangon Biotech (Shanghai) Co., Shanghai, China] in accordance with the manufacturer's protocol and sequenced at Sangon Biotech (Shanghai) Co. The resulting sequences were submitted to GenBank.

Sequence alignment and phylogenetic analyses

The DNA sequences generated in this study were assembled and edited using DNASTAR Lasergene software (version 6.0). Sequences of *ITS*, *RPB1*, *RPB2* and *TEF* were selected based on previously published data by Gutierrez *et al*. (2019) and the results of a BLAST algorithm-based search of the GenBank databases. Multiple sequence alignments of *ITS*, *RPB1*, *RPB2* and *TEF* were carried out using MAFFT v7.037b (Katoh & Standley 2013). Sequence editing was performed with MEGA6 (Tamura *et al*. 2013), and the resulting output was in the Fasta file format. The concatenated *ITS+RPB1+RPB2+TEF* sequences were assembled by SequenceMatrix v.1.7.8 (Vaidya *et al*. 2011). Gene concordance was assessed using the 'hompart' command in PAUP4.0b10 (Swofford 2002).

The combined dataset of the four genes (*ITS+RPB1+RPB2+TEF*) were analyzed phylogenetically using Bayesian inference (BI) and maximum likelihood (ML) methods. For the BI analysis, two runs were executed simultaneously for 10,000,000 generations, saving trees every 500 generations, with the GTR+G nucleotide substitution model across all the partitions, in MrBayes 3.2 (Ronquist *et al*. 2012). After the analysis was finished, each run was examined using the program Tracer v1.5 (Drummond & Rambaut 2007) to determine the burn-in and confirm that both runs had converged. For the ML analysis in RAxML (Stamatakis 2014), the GTRGAMMA model was used for all the partitions in accordance with recommendations in the RAxML manual against the use of invariant sites. The final alignment is available from TreeBASE under submission ID: 24831 (http://www.treebase.org).

Sequencing and phylogenetic analysis

The dataset of the combined four locus sequences contained 2,573 characters with gaps (*ITS*: 557, *RPB1*: 524, *RPB2*: 691 and *TEF*: 801). No significant differences in topology were observed in either the BI or ML phylogeny.

The RAxML analysis of the combined dataset (*ITS+RPB1+RPB2+TEF*) yielded a best scoring tree (Fig. 1) with a final ML optimization likelihood value of –18261.029390. Parameters for the GTR model of the concatenated data set was: estimated base frequencies; A = 0.240916, C = 0.277545, G = 0.269022, T = 0.212517; substitution rates AC = 0.907379, AG = 3.311030, AT = 0.837415, CG = 0.878474, CT = 6.972383, GT = 1.000000; gamma distribution shape parameter $\alpha = 0.270549$. The Bayesian analysis resulted in 20,001 trees after 10,000,000 generations. The first 4,000 trees, representing the burn-in phase of the analyses, were discarded while the remaining 16,001 trees were used for calculating posterior probabilities in the majority rule consensus tree.

The new species *Metarhizium lepidopterorum* and *M. rongjiangense* formed a separate clade from other *Metarhizium* species, which was statistically well supported by the ML and BI analyses (Fig. 1).

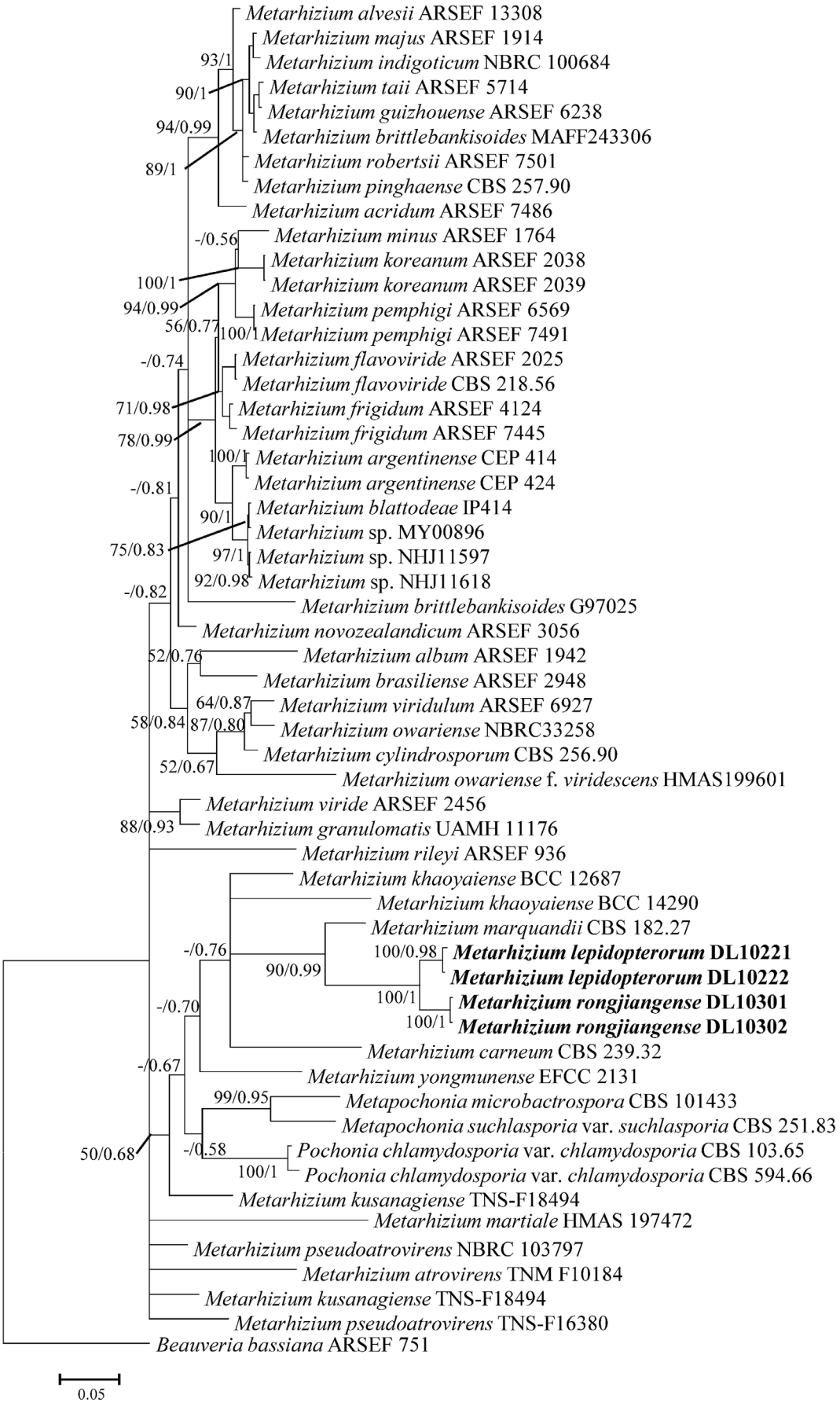

FIGURE 1. Phylogenetic analysis of *Metarhizium lepidopterorum*, *M. rongjiangense* and other *Metarhizium* species based on combined partial *ITS+RPB1+RPB2+TEF* sequences. Statistical support values (≥50 %) are shown at nodes, and presented as bootstrap values/ Bayesian posterior probabilities.

Taxonomy

Metarhizium lepidopterorum W.H. Chen, Y.F. Han & Z.Q. Liang, ***sp. nov.*** (Fig. 2)

Mycobank No.: MB 831960

Type:—CHINA. Guizhou Province: Qiandongnan Miao and Dong Autonomous Prefecture, Rongjiang County (26°01′58.70″ N, 108°24′48.06″ E), on a lepidopteran pupa, 1 October 2018, W.H. Chen, holotype GZAC DL1022, ex-type culture GZAC DL10221. Sequences from this strain have been deposited in GenBank with accession numbers: *ITS*=MN165990, *RPB1*=MN172267, *TEF*=MN172269.

Colonies on PDA at 25 °C after 14 d reaching 18–20 mm in diameter, velutinous, light yellow, with white floccose margin, powdery when sporulating, and yellow synnemata arising in the middle over time; reverse light brown. *Hyphae* septate, smooth-walled, hyaline, 1.2–1.8 µm wide. *Conidiophores* lecanicillium-like, hyaline, smooth-walled, cylindrical, arising from aerial hyphae. *Phialides* in a cluster of two to three, arising on the lateral sides and ends of conidiophores, cylindrical at the base, 12.6–18.1 × 1.4–1.7 µm. *Conidia* single or in long chains, one-celled, hyaline, smooth-walled, fusiform, 2.7–4.1 × 1.2–1.4 µm.

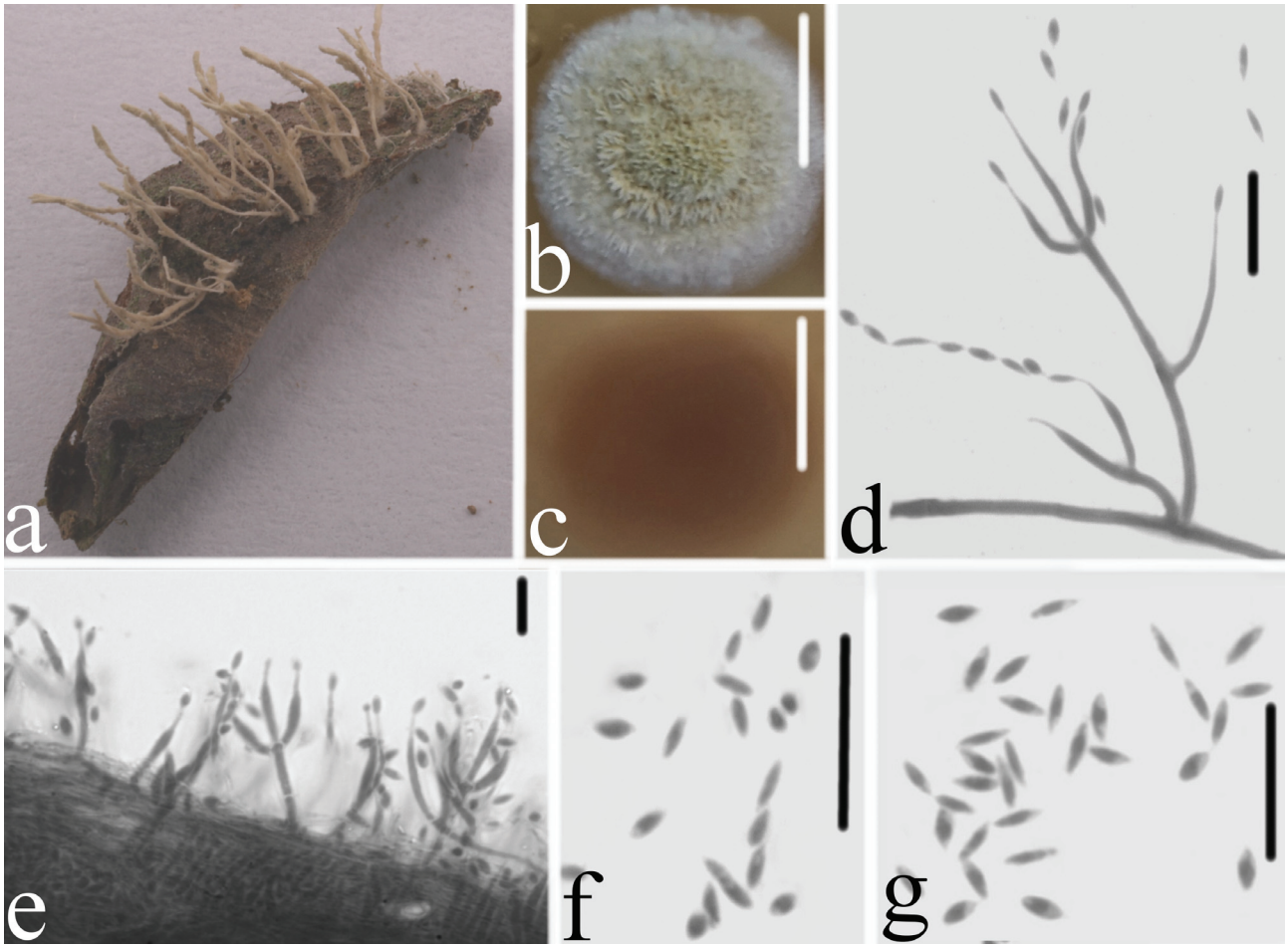

FIGURE 2. *Metarhizium lepidopterorum sp. nov*. **a.** Infected insect. **b, c.** Colony on PDA after 14 d at 25 °C (upper surface and lower surface). **d, g**. Condiogenous structures and conidia in chains on PDA. **e, f**. Condiogenous structures and conidia on the lateral of synnemata. Bars: b, c = 10 mm; d, e, f, d = 10 µm.

On lepidopteran pupae, synnemata scattered, white to earth yellow, powdery at the top when sporulating. *Conidiophores* arise from the lateral hyphae of the synnemata, bearing loosely verticillate, with each branch bearing 2–3 phialides; *Phialides* in a cluster of two to three, or solitary, and directly on the conidiophores, cylindrical at the base, 7.3–14.4 × 1.0–1.7 µm. *Conidia* single or in long chains, one-celled, hyaline, smooth-walled, fusiform, 2.3–3.1 × 1.0–1.4 µm.

Etymology:—referring to its insect host in the order Lepidoptera.

Additional material and strain examined:—CHINA. Guizhou Province: Qiandongnan Miao and Dong Autonomous Prefecture, Rongjiang County (26°01′58.70″ N, 108°24′48.06″ E), on a lepidopteran pupa, 1 October 2018, W.H. Chen, paratype DL1018, ex-paratype culture GZAC DL10181; *ibid*. DL10222. Sequences from DL10222 have been deposited in GenBank with accession numbers: *ITS*=MN165993.

Known distribution:—Qiandongnan Miao and Dong Autonomous Prefecture Guizhou Province, China.

Notes:—*M. lepidopterorum* is distinguished from other species of *Metarhizium* by its lecanicillium-like conidiophores, long phialides (12.6–18.1 × 1.4–1.7 μm) and the presence of only one type and smaller fusiform conidium (2.7–4.1 × 1.2–1.4 μm).

Metarhizium rongjiangense W.H. Chen, Y.F. Han & Z.Q. Liang, ***sp. nov.*** (Fig. 3)

Mycobank No.: MB 831961

Type:—CHINA. Guizhou Province: Qiandongnan Miao and Dong Autonomous Prefecture, Rongjiang County (26°01′58.70″ N, 108°24′48.06″ E), on a lepidopteran pupa, 1 October 2018, W.H. Chen, holotype GZAC DL1030, ex-type culture GZAC DL10301. Sequences from this strain have been deposited in GenBank with accession numbers: *ITS*=MN165995, *RPB1*=MN172268, *RPB2*=MN172272.

Colonies on PDA at 25°C after 14 d reaching 13–14 mm diameter, velutinous, white, with white floccose margin; reverse light yellow to red. Synnemata not present over time. *Hyphae* septate, smooth-walled, hyaline, 1.2–1.5 μm wide. *Conidiophores* mononematous, lecanicillium-like, hyaline, smooth-walled, cylindrical, arising from aerial hyphae. *Phialides* in a cluster of two to three or solitary, arising on the lateral sides and ends of conidiophores, cylindrical at the base, 19.7–34.3 × 0.7–1.1 μm. *Conidia* single or forming long chains, one-celled, hyaline, smooth-walled, subwedge, 2.5–3.5 × 1.0–1.1 μm.

Etymology:—referring to the location, Rongjiang County, where the type specimen was collected.

Additional strain examined:—CHINA. Guizhou Province: Qiandongnan Miao and Dong Autonomous Prefecture, Rongjiang County (26°01′58.70″ N, 108°24′48.06″ E), on a lepidopteran pupa, 1 October 2018, W.H. Chen (DL10302). Sequences from this strain have been deposited in GenBank with accession numbers: *RPB2*=MN172271, *TEF*=MN172270.

Known distribution:—Qiandongnan Miao and Dong Autonomous Prefecture Guizhou Province, China.

Notes:—*M. rongjiangense* is distinguished from other species by its lecanicillium-like conidiophores, longer phialides (19.7–34.3 × 0.7–1.1 μm) and the presence of only one type and smaller subwedge conidium (2.5–3.5 × 1.0–1.1 μm).

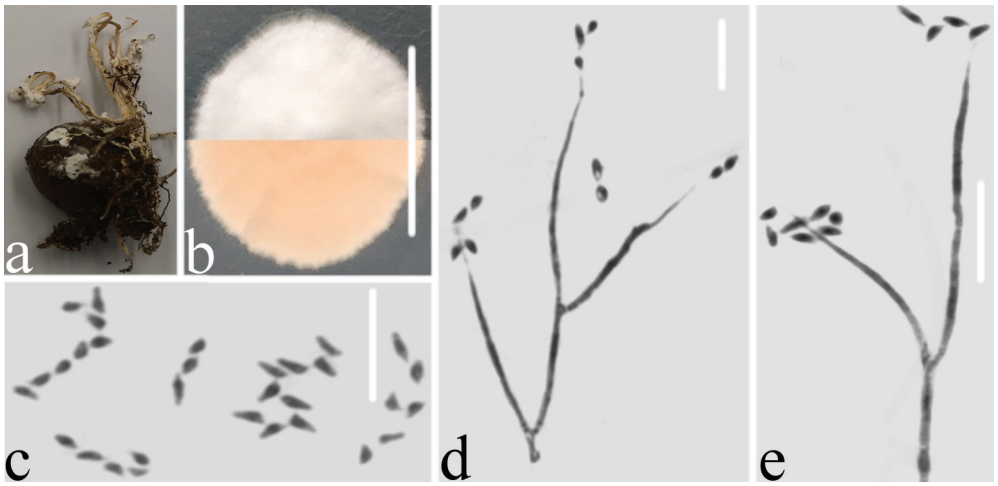

FIGURE 3. *Metarhizium rongjiangense sp. nov*. **a.** Infected insect. **b**. Colony on PDA after 14 d at 25 °C (upper surface and lower surface). **c**. Conidia. **d, e**. Condiogenous structures and conidia in chains. Bars: b = 10 mm; c–e = 10 μm.

Discussion

The typical characteristics of *Metarhizium* are conidiophores variously branched, occasionally simple, with apices of branches bearing one to several phialides (Rombach *et al*. 1987). Conidial size is an important identifying characteristic in the taxonomy of *Metarhizium*, followed by the hyphal characteristics and conidial colour. Kepler *et al*. (2014) mentioned that the accepted characteristics of *Metahizium* were as originally described by Sorokîn and subsequently emended by Rombach *et al*. (1987), but included anamorphic species not producing synnemata. *Metarhizium* contains species with both sexual and/or asexual spore states, and some species have synnemata (Kepler *et al*. 2014).

The synnematous entomopathogenic fungi, such as *Akanthomyces* Lebert, *Hymenostilbe* Petch, *Hirsutella* Pat. and *Polycephalomyces* Kobayasi, appear in the shrubbery of the original forest, litter layer or shallow soil (Hywel-Jones 1996). Because air flows under the forest canopy is slow and humidity is high, dispersal of conidia through airflow diffusion is difficult. Consequently, these entomopathogenic fungi have developed a strategy in which they produce different synnematal types and sticky conidia to accommodate various arthropod activities for conidial spread (Abbott 2002). The present synnemata and lecanicillium-like conidiophores of *M. lepidopterorum* and *M. rongjiangense* may be the result of convergent evolution, which could help them increase their fitness under different environmental conditions.

The *ITS*, *TUB*, *RPB1*, *RPB2* and *TEF* sequences have been widely applied in the identification of *Metarhizium* (Driver *et al*. 2000, Bischoff *et al*. 2006, Kepler *et al*. 2014, Montalva *et al*. 2016, Chu *et al*. 2016, Chen *et al*. 2017, Luangsa-ard *et al*. 2017, Lopes *et al*. 2018, Chen *et al*. 2018, Gutierrez *et al*. 2019). In the present study, *ITS*, *RPB1*, *RPB2* and *TEF* sequences of the new *Metarhizium* isolates clustered into two subclades, which were distinctly different from other *Metarhizium* species. Therefore, the new strains represent two new species, *M. lepidopterorum* and *M. rongjiangense*, and this identification was supported by combining the morphological characteristic and phylogenetic analyses.

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