



Metarhizium baoshanense sp. nov., a New Entomopathogen Fungus from Southwestern China

Zihong Chen¹, Ling Xu², Xiaona Yang², Yaguan Zhang^{3*} and Yuming Yang⁴

¹Institute of Biological Resources of Gaoligong Mountains, Baoshan University, Baoshan 678000 Yunnan, China

²College of Resources and Environment, Baoshan University, Baoshan 678000, Yunnan, China

³College of Chemistry and Environmental Science, Qujing Normal University, Qujing 655011 Yunnan, China

⁴Yunnan Province Key Laboratory of Forest Plant Cultivation and Utilization, Yunnan Academy of Forestry, Kunming 650201 Yunnan, China

ABSTRACT

Metarhizium is an entomopathogenic fungal genus relevant in biological protection. A new species of the genus, collected from Taibao mountain, Baoshan City, Yunnan Province, southwestern China, was described here as *Metarhizium baoshanense*. It was proposed and determined based on morphological characters combined with a multigene phylogenetics analysis involving 5.8S-ITS, nrSSU, nrLSU, EF-1 α , RPB1 and RPB2. In multilocus phylogeny, *M. baoshanense* was grouped as a sister clade to *Metarhizium majus*, *Metarhizium guizhouense*, *Metarhizium lepidiotae* and *Metarhizium indigoticum*, but conclusively separated from these allied species with high support value. Morphological character differentiated *M. baoshanense* from its relatives on that its conidial size was obviously smaller than *M. majus* and *M. indigoticum*, a little shorter than *M. guizhouense* and thicker than *M. lepidiotae*. Still the new species was characterized by that its colony colour became slightly shallow with culture time on PDA plates at 25°C, being grayish green at 12 d and turning gray black at 30 d of cultivation.

Article Information

Received 23 May 2018

Revised 30 June 2018

Accepted 12 July 2018

Available online 03 August 2018

Authors' Contribution

ZC designed the study, performed experimental work and analyzed the data. LX performed the experiment, analyzed the data and wrote the manuscript. XY helped in DNA extraction and PCR. YZ helped in morphological evaluation. YY helped in paper writing.

Key words

Metarhizium, Morphology character, Multigene phylogenetics, Taxonomy.

INTRODUCTION

Metarhizium is one of the ubiquitous anamorphic genera of entomopathogenic fungi (Driver *et al.*, 2000). *Entomophthora anisopliae* Metsch was described from Russia as a pathogen of wheat cockchafer (*Anisolia austriaca* Hbs.) and turned to be a type species of a new genus *Metarhizium* (Metsch.) Sorokin. Teleomorph of *Metarhizium* was firstly confirmed as *Cordyceps* s. l. by Liang *et al.* (1991) and then assigned to a new genus *Metacordyceps* by Sung *et al.* (2007).

A high number of species and varieties of *Metarhizium* had been discovered in recent decades and their convergent morphologies hampered the taxonomic studies. Molecular evidence enabled great resolution of fungi taxonomy than allowed by morphology alone, and multigene phylogenetic approaches particularly urged/forced the clarification of the genetic relationship at species rank (Kepler *et al.*, 2012; Nonaka *et al.*, 2013). Based on 4-gene (EF-1 α , RPB1,

RPB2 and β -tubulin) dataset, Bischoff *et al.* (2009) revealed that the *Metarhizium anisopliae* complex included 9 species. Based on 5-gene (nrSSU, nrLSU, EF-1 α , RPB1 and RPB2) dataset, Kepler *et al.* (2012) identified that *Metacordyceps* contained 14 species and 1 varieties, and then Kepler *et al.* (2014) revised the boundaries of *Metarhizium* as including the majority of species recognized in *Metacordyceps*, green-spored species in *Nomuraea*, *Chamaeleomyces* and several species formerly in *Paecilomyces*.

During a survey of entomogenous fungi at Taibao mountain in Baoshan City, Yunnan Province, China in 2015, a new *Metarhizium* species, *M. baoshanense* was discovered. The objective of this study was to confirm its taxonomy position based on morphological characteristics and 6-locus phylogenetic analysis. The anamorph of the related species, *M. indigoticum* (Kobayasi & Shimizu) Kepler, S.A. Rehner & Humber, was firstly described.

MATERIALS AND METHODS

Fungal isolation and morphological evaluation

Soil specimens were collected from plant rhizosphere

* Corresponding author: zhangyaguan2013@163.com
0030-9923/2018/0005-1739 \$ 9.00/0

Copyright 2018 Zoological Society of Pakistan

at mid–montane of humid evergreen broad–leaved forest in Taibao Mountain, Baoshan city, Yunnan Province in China. Fungi were isolated from soil by insect baiting method according to [Keyser *et al.* \(2015\)](#). Soil samples (~100 mL) were moistened to slightly damp in sterile glass canned bottles (150 mL). Ten healthy 6–7th instar *Tenebrio molitor* L. larvae were cultured in each bottle and checked every two days. Dead insects were transferred to a sterile petri dish with moist filter paper and maintained at 25°C for about 10 d. *Metarhizium* conidia were transferred to PDA plates and cultured at 25°C. Pure (axenic) strains were indentified morphologically and evaluated microscopically under a motic BA410 microscope. Morphological characteristics of the new species were compared with its related species among *M. anisopliae* complex. Anamorphic strains of the related species, *M. indigoticum*, were isolated from Gaoligong mountains and morphologically described.

DNA extraction and PCR

Genomic DNA was extracted from mycelia and conidia with the Plant Genomic DNA Purification Kit (Qiagen) following the manufacturer instructions. The partial sequences of 6 nuclear loci, including 5.8S–ITS, nrSSU, nrLSU, EF-1 α , RPB1 and RPB2, were amplified. Primer pairs of the 6–locus were referred to [Chen *et al.* \(2013\)](#). PCR reactions were carried out in 20 μ L reaction mixture containing 10 μ L 2 \times EasyTaq PCR Supermix (TransGen Biotech, Beijing, China), 1 μ L of each primer (10 μ M), 1 μ L of template DNA (1–2 ng) and 7 μ L sterile water. PCR programs of ITS–5.8S, nrSSU and nrLSU were referred to [Chen *et al.* \(2013\)](#) and that of EF-1 α , RPB1 and

RPB2 were referred to [Bischoff *et al.* \(2006\)](#).

Cloning and sequencing

PCR products were purified with Gel Purification Kit (Bioteke, Beijing, China) and the fragments were cloned with TaKaRa PMDTM18–T vector system (TaKaRa Bio, Dalian, China). DNA sequencing was performed at SinoGenoMax Co. Ltd., and the resulting datasets were submitted to GenBank.

Phylogenetic analysis

DNA sequences of 6–locus included 34 species, *M. baoshanense*, all dataset (30 species) in *Metarhizium* used by [Kepler *et al.* \(2014\)](#), *Metarhizium martialis* (Speg.) Kepler, G.H. Sung & Spatafora used by [Kepler *et al.* \(2012\)](#), *Metarhizium shibinensis* T.C. Wen, J.C. Kang & K.D. Hyde used by [Wen *et al.* \(2015\)](#) and *Beauveria bassiana* (Bals.) Vuill. as the outgroup. All sequences were retrieved from the GenBank with accession number detailed in [Table I](#).

The combined dataset of 6–locus were aligned with Clustal X2.0 ([Larkin *et al.* \(2007\)](#)). Ambiguous regions in both sides were excluded and gaps were treated as missing data in the subsequent phylogenetic analyses. Phylogenic consensus tree was analyzed with the program MEGA6 ([Tamura *et al.* \(2013\)](#)). Maximum Likelihood (ML) estimation was performed with 1000 replicates. T92+G+I (Tamura 3–parameter + Gamma distribution (+G) with invariant sites (+I) nucleotide substitution model was taken as suggested by looking for a best–fit substitution model (ML). Clades supported with ML values of $\geq 70\%$ were considered significantly supported by the data.

Table I. Taxon information and GenBank accession numbers of the sequences used for phylogenetic analysis.

Species	Strain code	GenBank accession number					
		nrSSU	nrLSU	EF-1 α	RPB1	RPB2	5.8S-ITS
<i>Metarhizium acridum</i>	ARSEF 324	Data missing	Data missing	EU248844.1	EU248896.1	EU248924.1	HM055449.1
<i>M. acridum</i>	ARSEF 7486	Data missing	Data missing	EU248845.1	EU248897.1	EU248925.1	NR132019.1
<i>M. album</i>	ARSEF 2082	DQ518775.1	DQ522560.1	DQ522352.1	DQ522398.1	DQ522452.1	Data missing
<i>M. album</i>	ARSEF 2179	Data missing	Data missing	KJ398807.1	KJ398618.1	Data missing	HM055452.1
<i>M. anisopliae</i>	ARSEF 7450	Data missing	Data missing	EU248852.1	EU248904.1	EU248932.1	HQ331464.1
<i>M. anisopliae</i>	ARSEF 7487	Data missing	Data missing	DQ463996.2	DQ468355.1	DQ468370.1	NR132017.1
<i>M. baoshanense</i>	CCTCC M 2016589	KY264177	KY264174	KY264169	KY264180	KY264183	KY264172
<i>M. baoshanense</i>	BUM 63.4	KY264178	KY264175	KY264170	KY264181	KY264184	KY264173
<i>M. baoshanense</i>	BUM 1700	KY264179	KY264176	KY264171	KY264182	KY264185	Data missing
<i>M. brasiliense</i>	ARSEF 2948	Data missing	Data missing	KJ398809.1	KJ398620.1	Data missing	Data missing
<i>M. brunneum</i>	ARSEF 2107	Data missing	Data missing	EU248855.1	EU248907.1	EU248935.1	NR132023.1
<i>M. brunneum</i>	ARSEF 4179	Data missing	Data missing	EU248854.1	EU248906.1	EU248934.1	HQ331451.1
<i>M. carneum</i>	CBS 239.32	EF468843.1	EF468988.1	EF468789.1	EF468894.1	EF468938.1	NR131993.1
<i>M. cylindrosporium</i>	ARSEF 6926	Data missing	Data missing	KJ398814.1	KJ398625.1	Data missing	AF368270.1

Species	Strain code	GenBank accession number					
		nrSSU	nrLSU	EF-1 α	RPB1	RPB2	5.8S-ITS
<i>Metarhizium flavoviride</i>	ARSEF 2133	Data missing	Data missing	DQ463999.1	DQ468358.1	DQ468373.1	Data missing
<i>M. flavoviride</i>	ARSEF 2025	Data missing	AF138269.1	KJ398804.1	KJ398614.1	DQ468374.1	AF138269.1
<i>M. frigidum</i>	ARSEF 7445	Data missing	Data missing	KJ398818.1	KJ398628.1	Data missing	Data missing
<i>M. frigidum</i>	ARSEF 4124	Data missing	Data missing	DQ464002.1	DQ468361.1	DQ468376.1	NR132012.1
<i>M. globosum</i>	ARSEF 2596	Data missing	Data missing	EU248846.1	EU248898.1	EU248926.1	NR132020.1
<i>M. granulomatis</i>	UAMH 11176	HM635078.1	Data missing	KJ398782.1	KJ398593.1	Data missing	HM195306.1
<i>M. granulomatis</i>	UAMH 11028	HM195304.1	HM635076.1	KJ398781.1	Data missing	Data missing	NR132013.1
<i>M. guizhouense</i>	CBS 258.90	Data missing	Data missing	EU248862.1	EU248914.1	EU248942.1	HQ331448.1
<i>M. guizhouense</i>	ARSEF 6238	Data missing	Data missing	EU248857.1	EU248909.1	EU248937.1	HQ331447.1
<i>M. indigoticum</i>	TNS-F 18553	JF415968.1	JF415952.1	JF416010.1	JN049886.1	JF415992.1	JN049874.1
<i>M. indigoticum</i>	TNS-F 18554	JF415952.1	JF415969.1	JF416011.1	JN049887.1	JF415993.1	JN049875.1
<i>M. indigoticum</i>	BUM 1512.8	KY264192	KY264190	KY264186	KY264194	KY264196	KY264188
<i>M. indigoticum</i>	BUM 2600	KY264193	KY264191	KY264187	KY264195	KY264197	KY264189
<i>M. khaoyaiense</i>	BCC 12687	JF415971.1	Data missing	KJ398796.1	JN049889.1	Data missing	Data missing
<i>M. khaoyaiense</i>	BCC 14290	JF415970.1	Data missing	KJ398797.1	JN049888.1	Data missing	JN049869.1
<i>M. koreanum</i>	ARSEF 2039	Data missing	Data missing	KJ398806.1	KJ398616.1	Data missing	Data missing
<i>M. koreanum</i>	ARSEF 2038	Data missing	Data missing	KJ398805.1	KJ398615.1	Data missing	Data missing
<i>M. kusanagiensis</i>	TNS-F 18494	JF415972.1	JF415954.1	JF416014.1	JN049890.1	Data missing	JN049873.1
<i>M. lepidiotae</i>	ARSEF 7412	Data missing	Data missing	EU248864.1	EU248916.1	EU248944.1	HQ331455.1
<i>M. majus</i>	ARSEF 1946	Data missing	Data missing	EU248867.1	EU248919.1	EU248947.1	HM055450.1
<i>M. majus</i>	ARSEF 1914	Data missing	Data missing	KJ398801.1	KJ398610.1	Data missing	HQ331445.1
<i>M. marquandii</i>	CBS 182.27	EF468845.1	EF468990.1	EF468793.1	EF468899.1	EF468942.1	NR131994.1
<i>M. martialis</i>	HMAS 197472	JF415956.1	JF415975.1	JF416016.1	JN049892.1	JF415995.1	JN049881.1
<i>M. minus</i>	ARSEF 1764	AF280635.1	AF280632.1	DQ464006.1	KJ398609.1	DQ468380.1	HM055453.1
<i>M. minus</i>	ARSEF 2037	AF339531.1	AF339580.1	DQ464007.1	DQ468366.1	DQ468381.1	AF138271.1
<i>M. novozealandicum</i>	ARSEF 4661	Data missing	Data missing	KJ398811.1	KJ398622.1	Data missing	Data missing
<i>M. novozealandicum</i>	ARSEF 4674	Data missing	Data missing	KJ398812.1	KJ398623.1	Data missing	Data missing
<i>M. owariense</i>	NBRC 33258	HQ165730.1	HQ165669.1	HQ165689.1	HQ1665747.1	Data missing	HQ165712.1
<i>M. pemphigi</i>	ARSEF 7491	Data missing	Data missing	KJ398819.1	KJ398629.1	DQ468379.1	Data missing
<i>M. pemphigi</i>	ARSEF 6569	Data missing	Data missing	KJ398813.1	KJ398624.1	DQ468378.1	Data missing
<i>M. pinghaense</i>	CBS 257.90	Data missing	Data missing	EU248850.1	EU248902.1	EU248930.1	NR077205.1
<i>M. pinghaense</i>	ARSEF 4342	Data missing	Data missing	EU248851.1	EU248903.1	EU248931.1	HQ331454.1
<i>M. pseudoatrovirens</i>	TNSF 16380	JF415977.1	Data missing	Data missing	JN049893.1	JF415997.1	JN049870.1
<i>M. rileyi</i>	ARSEF 1972	Data missing	Data missing	KJ398803.1	KJ398613.1	Data missing	Data missing
<i>M. rileyi</i>	CBS 806.71	Data missing	AY526491.2	EF468787.1	EF468893.1	EF468937.1	NR119513.1
<i>M. robertsii</i>	ARSEF 727	Data missing	Data missing	DQ463994.1	DQ468353.1	DQ468368.1	HQ331453.1
<i>M. viride</i>	ARSEF 2456	Data missing	Data missing	KJ398808.1	KJ398619.1	Data missing	EU553291.1
<i>M. viride</i>	CBS 659.71	HQ165735.1	HQ165673.1	HQ165692.1	Data missing	HQ165652.1	HQ165714.1
<i>M. viridulum</i>	ARSEF 6927	Data missing	Data missing	KJ398815.1	KJ398626.1	Data missing	Data missing
<i>M. yongmunense</i>	EFCC 2135	Data missing	EF468979	EF468834	EF468769	EF468877	Data missing
<i>M. yongmunense</i>	EFCC 2131	EF468833.1	EF468977.1	EF468770.1	EF468876.1	Data missing	JN049856.1
<i>M. shibinensis</i>	GZUHSB 13050311	KR153588.1	Data missing	KR153589.1	KR153590.1	Data missing	KR153585.1
<i>Beauveria bassiana</i>	ARSEF 7518	HQ880975.1	HQ880975.1	HQ880975.1	HQ880834.1	HQ880834.1	HQ880762.1

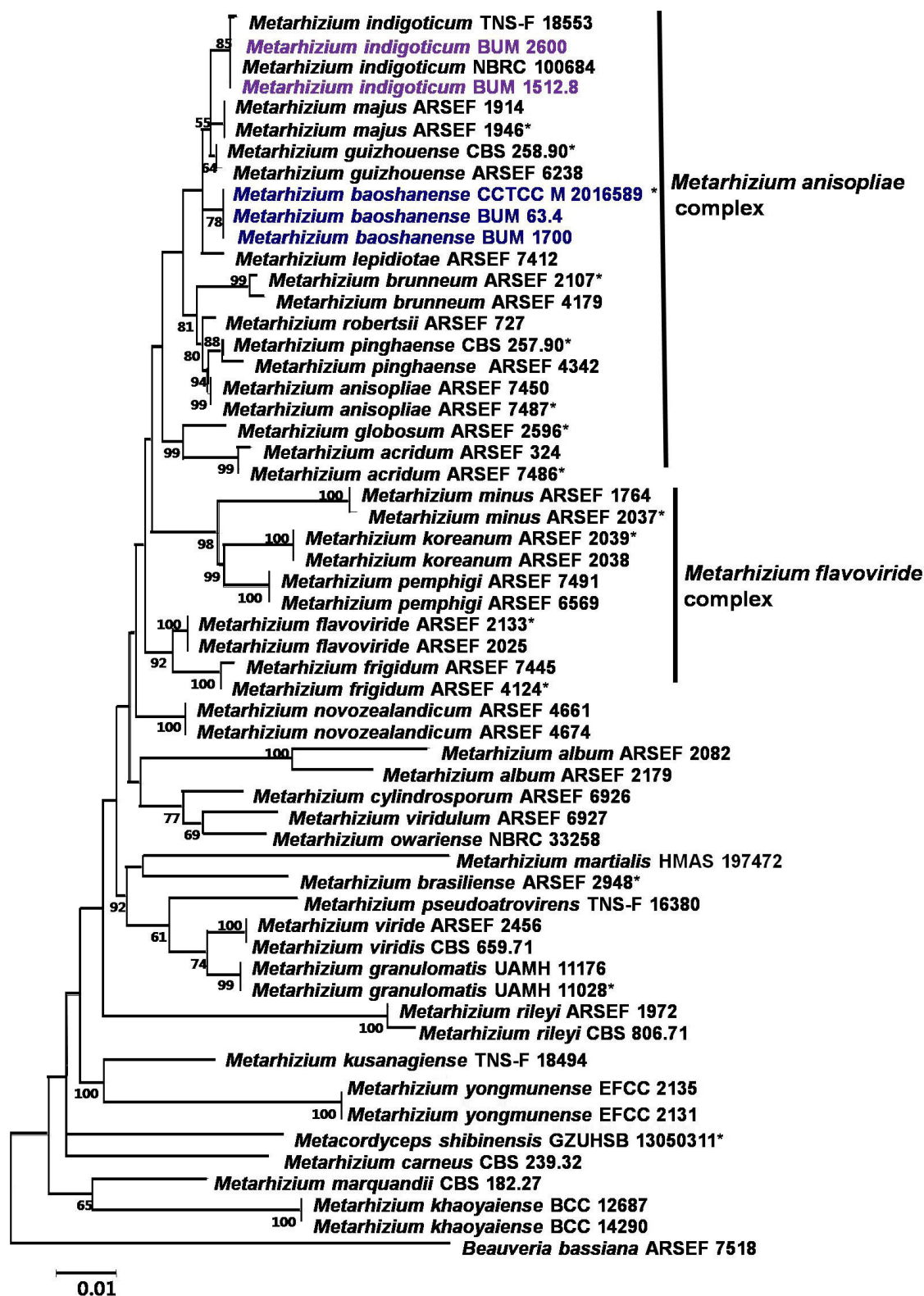


Fig. 1. Phylogenetic tree of *Metarhizium* based on ML analysis of 6-locus (5.8S-ITS, nrSSU, nrLSU, EF-1 α , RPB1 and RPB2) dataset. *Denotes ex-type strains.

RESULTS

Phylogenetic analyses

BLAST analysis revealed that 6 genes of *M. baoshanense* respectively shared sequences identities with different species of *Metarhizium* and *Metacordyceps*, ITS 98% with *M. anisopliae* M3419 (FJ609313.1), EF-1 α 99% with *M. majus* ARSEF 7505 (EU248870.1), RPB1 99% with *M. indigoticum* NBRC 100684 (KJ398595.1), RPB2 99% with *Metacordyceps brittlebankisoides* (KC429019.1), nrLSU and nrSSU 98% with *Metacordyceps indigotica* TNS-F 18554 (JF415953.1). It was hard to determine the species position with a single gene. Further multilocus analysis was employed to confirm the species taxonomic status.

The combined alignment of 6-locus dataset of *Metarhizium* comprised 5049 base pairs (5.8S-ITS: 717 bp; nrSSU: 1018 bp; nrLSU: 846 bp; EF-1 α : 917 bp; RPB1: 713 bp; RPB2: 838 bp). Phylogenetic tree of combined 6-locus for 34 species revealed similar affiliation inferred from 5 nuclear loci by [Kepler et al. \(2014\)](#) (Fig. 1). *M. baoshanense* was clustered in *M. anisopliae* complex which contained 11 individuals. The 3 strains (CCTCC M 2016589, BUM 63.4 and BUM 1700) of *M. baoshanense* were clustered in the same clade with *M. majus*, *M.*

indigoticum, *M. guizhouense* Q.T. Chen & H.L. Guo and *M. lepidiotae* (Driver & Milner) J.F. Bisch., Rehner & Humber, but formed a distinct clade and was clearly separated from the allied species with high support value.

The position of the strains BUM 2600 and BUM 1512.8 isolated from Gaoligong mountains was also confirmed. Their 6-locus dataset was clustered in the same clade with the reported sequences (TNS-F18553 and NBRC 100684) of *M. indigoticum*, illustrating that they were the anamorph stage of *M. indigoticum* and their morphological data in this study was credible.

Morphological comparison of M. Baoshanense and its related species

M. baoshanense was obviously distinguished from *M. majus* and *M. indigoticum* for the conidial feature, *M. majus* possessing the biggest conidia (8.5–13.0 \times 3.0–5.0 μ m) in *M. anisopliae* complex and *M. indigoticum* (8.2–12.3 \times 2.6–4.2 μ m) being next to it, much greater than *M. baoshanense* (6.7–8.5 \times 2.6–3.3 μ m) (Table II). The conidia of *M. baoshanense* were close to *M. lepidiotae* (6.0–7.5 \times 2.0–3.0 μ m) for the length and a little shorter than *M. guizhouense* (7.0–10.0 \times 2.0–3.0 μ m), while being a little thicker than *M. guizhouense* and *M. lepidiotae* (Table II; Fig. 2I–L).

Table II.- Morphological characters comparison among *Metarhizium anisopliae* complex.

Species	Strain	Geography	Conidia(μ m)	Phialides(μ m)	Reference
<i>Metarhizium majus</i>	ARSEF 1914	Philippines	10.5-13.0 \times 2.5-4.0	10.0-18.0 \times 2.5-3.5	Bischoof et al. (2009)
<i>M. majus</i>	ARSEF 7505	Australia	8.5-13.0 \times 3.0-5.0	9.0-18.5 \times 2.5-4.5	Bischoof et al. (2009)
<i>M. indigoticum</i>	BUM 1512.8	China	8.2-12.3 \times 2.6-4.2 (9.7 \pm 1.0 \times 3.1 \pm 0.6)	8.5-20.5 \times 1.7-3.5 (13.7 \pm 3.7 \times 2.5 \pm 0.6)	This study
<i>M. guizhouense</i>	ARSEF 4321	Australia	5.5-8.0 \times 2.5-3.5	8.0-12.0 \times 2.5-3.5	Bischoof et al. (2009)
<i>M. guizhouense</i>	CBS 258.90	China	7.0-10.0 \times 2.0-3.0	7.0-12.0 \times 2.0-3.0	Bischoof et al. (2009)
<i>M. guizhouense</i>	ARSEF 4321	Australia	5.5-8.0 \times 2.5-3.5	8.0-12.0 \times 2.5-3.5	Bischoof et al. (2009)
<i>M. baoshanense</i>	CCTCC M 2016589	China	6.7-8.5 \times 2.6-3.3 (7.4 \pm 0.5 \times 2.9 \pm 0.3)	8.2-19.2 \times 2.1-3.5 (11.9 \pm 3.3 \times 2.5 \pm 0.4)	This study
<i>M. lepidiotae</i>	ARSEF 4154	Australia	6.0-7.5 \times 2.0-3.0	6.5-12.0 \times 2.0-3.0	Bischoof et al. (2009)
<i>M. lepidiotae</i>	ARSEF 7488	Australia	5.0-7.0 \times 3.0-4.0	8.0-12.0 \times 2.5-3.5	Bischoof et al. (2009)
<i>M. pingshaense</i>	CBS 257.90	China	6.0-8.0 \times 2.5-3.5	7.0-17.0 \times 2.5-3.5	Bischoof et al. (2009)
<i>M. pingshaense</i>	ARSEF 4342	Solomon islands	4.5-7.0 \times 2.0-3.0	7.0-13.0 \times 2.0-3.0	Bischoof et al. (2009)
<i>M. robertsii</i>	ARSEF 7501	Australia	5.0-7.5 \times 2.0-3.0	6.0-12.0 \times 2.0-3.0	Bischoof et al. (2009)
<i>M. robertsii</i>	ARSEF 727	Brazil	5.0-7.0 \times 2.0-3.5	7.0-14.5 \times 2.5-3.5	Bischoof et al. (2009)
<i>M. anisopliae</i>	ARSEF 7487	Eritrea	5.0-7.0 \times 2.0-3.5	8.0-11.5 \times 2.0-3.0	Bischoof et al. (2009)
<i>M. brunneum</i>	ARSEF 4179	Australia	5.5-8.0 \times 2.0-3.0	6.0-13.0 \times 2.0-3.0	Bischoof et al. (2009)
<i>M. brunneum</i>	ARSEF 2107	USA	4.5-7.5 \times 2.0-3.0	9.5-18.0 \times 2.0-5.0	Bischoof et al. (2009)
<i>M. brunneum</i>	ARSEF 4152	Australia	5.0-7.0 \times 2.5-3.5	7.5-13.5 \times 2.5-4.0	Bischoof et al. (2009)
<i>M. acridum</i>	ARSEF 5748	Mexico	4.0-5.0 \times 2.5-3.5	6.0-13.0 \times 2.0-3.5	Bischoof et al. (2009)
<i>M. acridum</i>	ARSEF 7486	Niger	4.0-5.5 \times 3.0-4.0	4.5-12.5 \times 2.5-4.5	Bischoof et al. (2009)
<i>M. globosum</i>	ARSEF 2596	India	4.0-5.0 \times 4.0-5.0	5.0-12.0 \times 3.0-4.0	Bischoof et al. (2009)

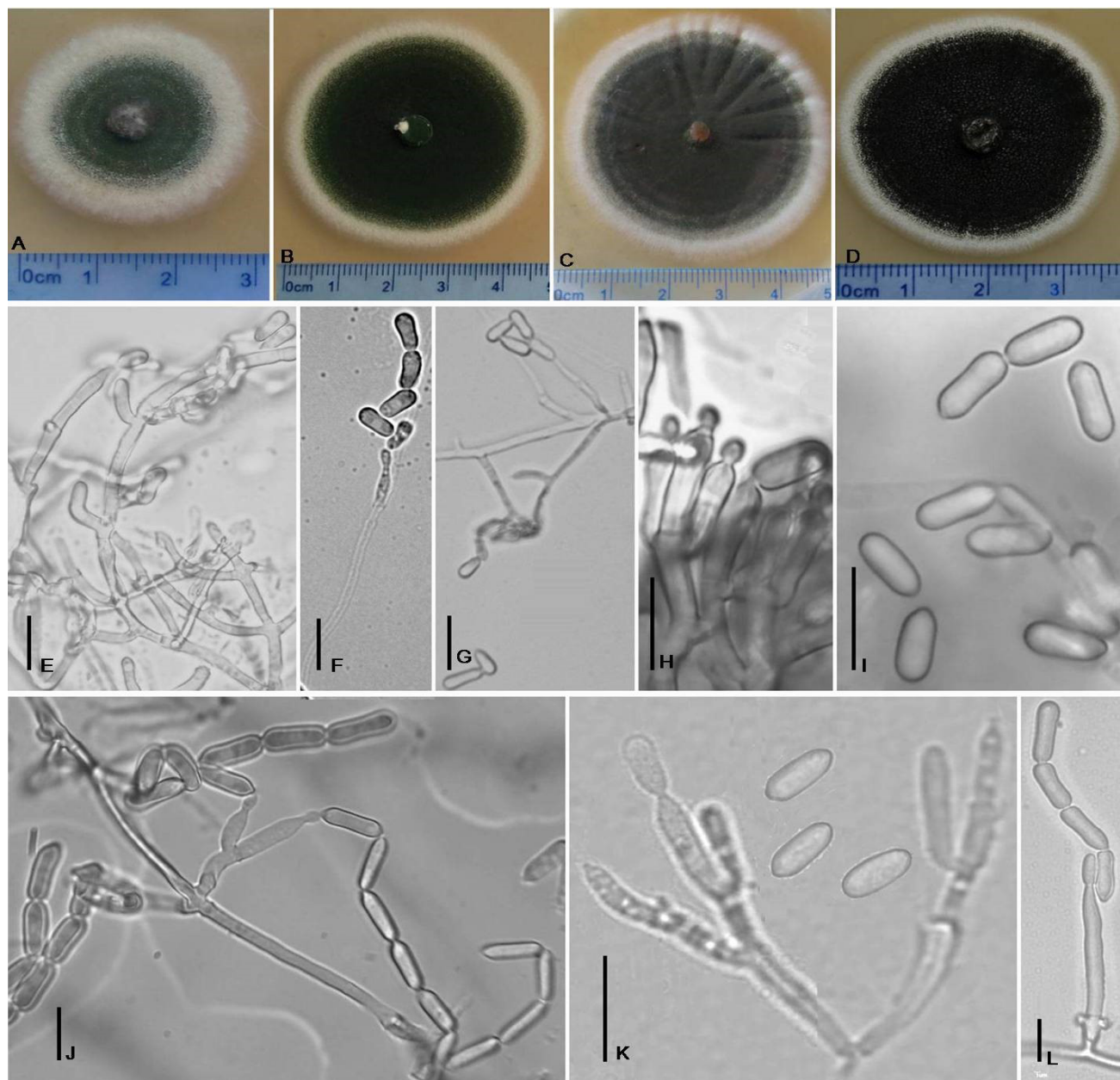


Fig. 2. Morphology of *Metarhizium baoshanense* and its close related species. A, colony of *Metarhizium baoshanense* cultured 12 d; B, colony of *Metarhizium lepidiotae* cultured 12 d; C, colony of *Metarhizium guizhouense* cultured 12 d; D, colony of *Metarhizium indigoticum* cultured 12 d; E, phialides formation of *Metarhizium baoshanense*; F and G, phialide with conidia in chain of *Metarhizium baoshanense*; H, phialide with budding conidium of *Metarhizium baoshanense*; I, conidia in chain and separated of *Metarhizium baoshanens*; J, phialides with conidia in chain of *Metarhizium guizhouense*; K, conidia and phialides of *Metarhizium lepidiotae*; L, conidia and phialides of *Metarhizium indigoticum*. Bars, E–L 10 μ m.

The culture character was further conducted among *M. baoshanense*, *M. lepidiotae*, *M. guizhouense* and *M. indigoticum* (Fig. 2 A–D). The conidia layers of all the four species were white in early day, while showed apparent discrepancy with culture time. After 12 d of cultivation on

PDA plates at 25°C, *M. baoshanense* was grayish green (Fig. 2A), *M. guizhouense* was blackish green (Fig. 2B), *M. lepidiotae* was light black (Fig. 2C) and *M. indigoticum* was dark black (Fig. 2D). After one month of cultivation, *M. baoshanense* and *M. lepidiotae* were gray black, *M.*

guizhouense was dark green, and *M. indigoticum* was black.

Taxonomy

MycoBank number
MB 819373.

Species diagnosis

The new species is clustered with *M. guizhouense*, *M. lepidiotae*, *M. majus* and *M. indigoticum* in the same clade in the multiple gene (5.8S-ITS, nrSSU, nrLSU, EF-1 α , RPB1 and RPB2) sequence analyses, but the conidial size of *M. baoshanense* was obviously smaller than *M. majus* and *M. indigoticum*, a little shorter than *M. guizhouense* and thicker than *M. lepidiotae*.

Species description

Colonies on PDA medium being 61 mm in diameter after 16 d at 25 °C, white at first, turning grayish green after 16 d of cultivation. Hyphae hyaline, septate, branched, smooth-walled, 2.0–3.2 (\bar{x} = 2.5 \pm 0.6) μ m wide. Conidiophores solitary or branched. Phialides cylindrical, 8.2–19.2 \times 2.1–3.5 (\bar{x} = 11.9 \pm 3.3 \times 2.5 \pm 0.4) μ m. Conidia forming columns in culture and hyaline, aseptate, smooth, long oval or cylindrical, grayish green en masse, 6.7–8.5 \times 2.6–3.3 (\bar{x} = 7.4 \pm 0.5 \times 2.9 \pm 0.3) μ m.

Type: China. Yunnan Province: Taibao mountain, alt. 1740 m, 3 May 2015, Zihong Chen (Holotype, CCTCC M 2016589; ex-type culture, BUM 63.4)

Sexual state: Unknown.

Host: Unknown.

Habitat and distribution: The soil at mid-montane of humid evergreen broad-leaved forest in Taibao mountain, Baoshan city, Yunnan Province, China.

DISCUSSION

Most species in *Metarhizium* are useful biological control agents and often act as regulators of insect populations (Kepler *et al.*, 2014; Pattemore *et al.*, 2014; Shoukat *et al.*, 2018). Further understanding their diversity can provide new genetic resource for development the entomopathogen as a biopesticide. In this study, *M. baoshanense* was determined to be a new species in *Metarhizium* based on morphological study and molecular evidence.

Muti-locus phylogeny is routinely used to delimit species of Ascomycota (Wang *et al.*, 2015). Six loci including 5.8S-ITS, nrSSU, nrLSU, EF-1 α , RPB1 and RPB2 were performed analysis in this study for their wide usage in phylogenetic reconstructions of fungi (Kepler *et al.*, 2012; Sanjuan *et al.*, 2014; Wen *et al.*, 2015). Multi-

genes analyses involved almost all currently recognized species of *Metarhizium* and the resulted affiliation was consistent with the works of Kepler *et al.* (2014), being able to provide strong evidence for the taxonomic status of *M. baoshanense*. The 2 strains BUM 2600 and BUM 1512.8 were placed as *M. indigoticum* and the 3 strains of *M. baoshanense* were resolved as a novel clade in *M. anisopliae* complex, clearly separated from its allied species, *M. majus*, *M. guizhouense*, *M. lepidiotae* and *M. indigoticum*.

Cordyceps indigotica Kobayasi & Shimiz was firstly reported by Kobayasi and Shimizu (1978) and determined to be a member of *Metacordyceps* by Kepler *et al.* (2012). However, so far the asexual morphological description of *M. indigoticum* had been still missing. To better clarify the relationship of *M. baoshanense* and its related species, 2 strains from Gaoligong Mountains were confirmed as *M. indigoticum* by molecular analysis and morphologically compared with *M. baoshanense* (Table II). The conidial size of *M. baoshanense* was obviously smaller than *M. majus* and *M. indigoticum*, a little shorter than *M. guizhouense*, and a little thicker than *Metarhizium lepidiotae*. The colony color could well clarify the interspecific relationship, that of *M. baoshanense* being grayish green, obviously shallow than *M. lepidiotae*, *M. guizhouense* and *M. indigoticum* after 12 d of cultivation on PDA plates at 25 °C.

CONCLUSION

In this study, a new species of *Metarhizium*, *M. baoshanense* was determined based on morphological characters and 6-locus (5.8S-ITS, nrSSU, nrLSU, EF-1 α , RPB1 and RPB2) molecular data. *M. baoshanense* was obviously separated from its sister species, *M. majus*, *M. guizhouense*, *M. lepidiotae* and *M. indigoticum* in phylogenetic tree and was also clearly morphologically distinguished from its relatives.

ACKNOWLEDGEMENT

The authors wish to thank Dr. Yong-Dong Dai (Institute of Herbal Biotic Resources, Yunnan University, Yunnan, China) for his kindly help to analyse the multigene sequences. This work was funded by the National Natural Science Area Fund Projects of China (31460153), Key Project of Universities Joint Foundation in Yunnan province (2017FH001-126), Surface Project of Universities Joint Foundation OF Yunnan province (2017FH001-029).

Statement of conflict of interest

The authors declare no conflicts of interest. All the

experiments undertaken in this study comply with the current laws of the country where they were performed.

REFERENCES

- Bischoff, J.F., Rehner, S.A. and Humber, R.A., 2006. *Metarhizium frigidum* spp. Nov.: a cryptic species of *M. anisopliae* and a member of the *M. flavoviride* complex. *Mycologia*, **98**: 737-745. <https://doi.org/10.3852/mycologia.98.5.737>
- Bischoff, J.F., Rehner, S.A. and Humber, R.A., 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia*, **101**: 512-530. <https://doi.org/10.3852/07-202>
- Chen, Z.H., Dai, Y.D., Yu, H., Yang, K., Yang, Z.L., Yuan, F. and Zeng, W.B., 2013. Systematic analyses of *Ophiocordyceps lanpingensis* sp. nov., a new species of *Ophiocordyceps* in China. *Microbiol. Res.*, **168**: 525-532. <https://doi.org/10.1016/j.micres.2013.02.010>
- Driver, F., Milner, R.J. and Trueman, J.W.H., 2000. A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. *Mycol. Res.*, **104**: 134-150. <https://doi.org/10.1017/S0953756299001756>
- Kepler, R.M., Sung, G.H., Ban, S., Nakagiri, A., Chen, M.J., Huang, B., Li, Z.Z. and Spatafora, J.W., 2012. New teleomorph combinations in the entomopathogenic genus *Metacordyceps*. *Mycologia*, **104**: 182-197. <https://doi.org/10.3852/11-070>
- Kepler, R.M., Humber, R.A., Bischoff, J.F. and Rehner, S.A., 2014. Clarification of generic and species boundaries for *Metarhizium* and related fungi through multigene phylogenetics. *Mycologia*, **106**: 811-829. <https://doi.org/10.3852/13-319>
- Keyser, C.A., Licht, H.H.D.F., Steinwender, B.M. and Meyling, N.V., 2015. Diversity within the entomopathogenic fungal species *Metarhizium flavoviride* associated with agricultural crops in Denmark. *BMC Microbiol.*, **15**: 249. <https://doi.org/10.1186/s12866-015-0589-z>
- Kobayasi, Y. and Shimizu, D., 1978. *Cordyceps* species from Japan. *Bull. natl. Sci. Mus., Tokyo Ser. B*, **4**: 43-63.
- Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. and Higgins, D.G., 2007. Clustal W and clustal X version 2.0. *Bioinformatics*, **23**: 2947-2948. <https://doi.org/10.1093/bioinformatics/btm404>
- Liang, Z.Q., Liu, A.Y. and Liu, J.L., 1991. A new species of the genus *Cordyceps* and its *Metarhizium* anamorph. *Acta Mycol. Sin.*, **10**: 257-262.
- Nonaka, K., ōmura, S., Masuma, R., Kaifuchi, S. and Masuma, R., 2013. Three new *Pochonia* taxa (Clavicipitaceae) from soils in Japan. *Mycologia*, **105**: 1202-1218. <https://doi.org/10.3852/12-132>
- Pattemore, J.A., Hane, J.K., Williams, A.H., Wilson, B.A., Stodart, B.J. and Ash, G.J., 2014. The genome sequence of the biocontrol fungus *Metarhizium anisopliae* and comparative genomics of *Metarhizium* species. *BMC Genom.*, **15**: 660. <https://doi.org/10.1186/1471-2164-15-660>
- Sanjuan, T., Tabima, J., Restrepo, S., Læssøe, T., Spatafora, J.W. and Franco-Molano, A.E., 2014. Entomopathogens of Amazonian stick insects and locusts are members of the *Beauveria* species complex (*Cordyceps sensu stricto*). *Mycologia*, **106**: 260-275. <https://doi.org/10.3852/13-020>
- Shoukat, R.F., Freed, S. and Ahmad, K.W., 2018. Assessment of binary mixtures of entomopathogenic fungi and chemical insecticides on biological parameters of *Culex pipiens* (Diptera: Culicidae) under laboratory and field conditions. *Pakistan J. Zool.*, **50**: 299-309. <https://doi.org/10.17582/journal.pjz/2018.50.1.299.309>
- Sung, G.H., Hywel-Jones, N.L., Sung, J.M., Luangsa-ard, J.J., Shrestha, B. and Spatafora, J.W., 2007. Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Stud. Mycol.*, **57**: 5-59. <https://doi.org/10.3114/sim.2007.57.01>
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., Nei, M. and Kumar, S., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evolut.*, **30**: 2725-2729. <https://doi.org/10.1093/molbev/mst197>
- Wang, Y.B., Yu, H., Dai, Y.D., Chen, Z.H., Zen, W.B., Yuan, F. and Liang, Z.Q., 2015. *Polycephalomyces yunnanensis* (Hypocreales), a new species of *Polycephalomyces* parasitizing *Ophiocordyceps nutans* and stink bugs (hemipteran adults). *Phytotaxa*, **208**: 34-44. <https://doi.org/10.11646/phytotaxa.208.1.3>
- Wen, T.C., Zha, L.S., Xiao, Y.P., Wang, Q., Kang, J.C. and Hyde, K.D., 2015. *Metacordyceps shibinensis* sp. nov. from larvae of Lepidoptera in Guizhou Province, southwest China. *Phytotaxa*, **226**: 51-62. <https://doi.org/10.11646/phytotaxa.226.1.5>