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Metarhizium dianzhongense sp. nov. and New Record of *M. bibionidarum* (Clavicipitaceae, Hyocreales) Attacking Insects from China

Cui-Yuan Wei [1,2,3], Mei Tang [1,2,3], Liu-Yi Xie [2,3], Qi Fan [2,3,4], Shi-Kang Shen [5], Zhu-Liang Yang [2,3], Gang Deng [1,*] and Yuan-Bing Wang [2,3,*]

1 Institute of Agriculture, Yunnan University, Kunming 650091, China; weicuiyuan@mail.ynu.edu.cn (C.-Y.W.); Tangmei27@outlook.com (M.T.)
2 CAS Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China; xly_mic@outlook.com (L.-Y.X.); fanqi_666@outlook.com (Q.F.); fungi@mail.kib.ac.cn (Z.-L.Y.)
3 Yunnan Key Laboratory for Fungal Diversity and Green Development, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China
4 College of Life Science and Technology, Guangxi University, Nanning 530004, China
5 College of Ecology and Environment, Yunnan University, Kunming 650091, China; ssk168@ynu.edu.cn
* Correspondence: denggang1986@ynu.edu.cn (G.D.); wangyuanbing@mail.kib.ac.cn (Y.-B.W.)

Abstract: The genus *Metarhizium* is one of the most significant entomopathogenic fungi with diverse morphological characteristics and host species. Species of *Metarhizium* have been widely used for pest control as an environmentally safe alternative to chemical pesticides. This study reports a new species of *Metarhizium* and a new record of *M. bibionidarum* from China. The taxonomic positions of the two species within *Metarhizium* were assessed by morphological and multi-gene phylogenetic data. This assessment confirmed that the new species *M. dianzhongense* on white grubs (Coleoptera) is a sister to *M. ellipsoideum* on adult leafhoppers (Hemiptera) and represents a distinctive fungus according to the morphological and phylogenetic evidence. The two species, *M. dianzhongense* and *M. bibionidarum*, were described and illustrated. Pathogenicity tests by *M. bibionidarum* and *M. dianzhongense* were performed on early instar larvae of the significant agricultural pest *Spodoptera frugipera* (Lepidoptera). The results demonstrated that both *M. bibionidarum* and *M. dianzhongense* exhibit significant insecticidal activity against larvae of *S. frugipera*, providing new fungal resources for the development of an eco-friendly biocontrol agent against this pest.

Keywords: entomopathogenic fungi; phylogeny; taxonomy; biocontrol; *Spodoptera frugiperda*

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Citation: Wei, C.-Y.; Tang, M.; Xie, L.-Y.; Fan, Q.; Shen, S.-K.; Yang, Z.-L.; Deng, G.; Wang, Y.-B. *Metarhizium dianzhongense* sp. nov. and New Record of *M. bibionidarum* (Clavicipitaceae, Hyocreales) Attacking Insects from China. *Diversity* **2024**, *16*, 201. https://doi.org/10.3390/d16040201

Academic Editor: Ipek Kurtboke

Received: 29 February 2024
Revised: 20 March 2024
Accepted: 21 March 2024
Published: 27 March 2024

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1. Introduction

Metarhizium is one of the most interesting genera of entomopathogenic fungi (EPF); it is known as "green muscardine fungus" because it mostly produces green conidia on its arthropod hosts. The genus was established by Sorokīn (1879) based on the type species *M. anisopliae* (Metschn.) Sorokīn attacking the wheat cockchafer *Anisoplia austriaca* (Coleoptera) from Russia. Tulloch [1] revised the genus and recognized only two species, *M. anisopliae* and *M. flavoviride* W. Gams & Rozsypal, along with two varieties, *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus* (J.R. Johnst.) M.C. Tulloch. Species of *Metarhizium* were historically described by morphological characteristics such as colony color, conidial structure, and sporulation patterns [2], which led to considerable debate regarding their taxonomic statuses.

The advancement of molecular systematics, especially multi-gene phylogenetic analyses that include rDNA gene fragments and functional protein genes, has revolutionized the taxonomy of *Metarhizium*. The genus *Metacordyceps* G.H. Sung et al. was erected to include *Cordyceps* spp. linked to anamorphic *Metarhizium* and *Pochonia* Bat. & O.M. Fonsa within the

family Clavicipitaceae [3]. The recombination to *Metacordyceps* was made by adding several teleomorphic species, which accommodated 15 species and 1 variety [4]. Following the adoption of the "One Fungus One Name" standard, Kepler et al. [5] delimited the boundary of *Metarhizium* by combining the majority of species in *Metacordyceps*, *Nomuraea* Maubl., and *Chamaeleomyces* Sigler, recognizing 34 species. In recent years, numerous new taxa have been added to the genus *Metarhizium* and enriched its species diversity [6–14]. In the most complete taxonomic treatment of *Metarhizium* and its related genera, Mongkolsamrit et al. [11] reconstructed the phylogenetic framework of Clavicipitaceae focusing on the core *Metarhizium* clade and established six genera (*Keithomyces* Samson et al., *Marquandomyces* Samson et al., *Papiliomyces* Luangsa-ard et al., *Purpureomyces* Luangsa-ard et al., *Sungia* Luangsa-ard et al., and *Yosiokobayasia* Samson et al.) belonging to the core *Metarhizium* clade. To date, the genus *Metarhizium* accommodates 74 species, including 23 species in the *M. anisopliae* species complex and 13 species in the *M. flavoviride* species complex [11,14–23].

As an important and widespread group of EPF, *Metarhizium* has been used for pest control worldwide for approximately 140 years [24,25]. Various *Metarhizium*-based biocontrol products are available on the market and have been effectively implemented in managing a broad spectrum of pests worldwide [11,26]. Consequently, precise identifications for *Metarhizium* species and their host ranges are critical to the high-efficiency biocontrol strategy.

During surveys of EPF in southwestern China, we found a new species and a new record of *Metarhizium* attacking white grubs and March fly larvae, respectively. Collected specimens of the two species were identified by combining morphological and seven-gene (ITS, nrSSU, nrLSU, tef-1α, rpb1, rpb2, and 5′tef) phylogenetic analyses. The two species were described and illustrated. In addition, pathogenicity tests of the new species *M. bibionidarum* Nishi & Hiroki Sato and new record *M. dianzhongense* were performed on larvae of *Spodoptera frugipera* J. E. Smith.

2. Materials and Methods

2.1. Sample Collection and Isolation

Inst pathogenic specimens were meticulously collected in Yunnan, China, in 2022, with a keen examination of the forest understory, leaf litter, and the undersides of leaves for instances of EPF infection. Specimens were noted and photographed in the field, carefully placed in plastic boxes, and then transported to field stations or laboratories for detailed examination. The material was examined under an Olympus SZ60 microscope. Isolation from the material was conducted as described by Wang et al. [27] with modifications: the conidia from sporulating structures were touched by using a flame-sterilized inoculation needle and streaked on potato–dextrose agar (PDA: potato 200 g/L, dextrose 20 g/L, agar 20 g/L, in 1 L distilled water) plates. Purified strains were maintained in a culture room at 25 °C. Subculturing was performed on new PDA plates to ensure the development of pure colonies. Pure cultures were transplanted onto PDA slants and stored at 4 °C. Dried specimens were deposited in the Cryptogamic Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN-HKAS). Pure cultures were deposited in the Culture Collection of Kunming Institute of Botany, Chinese Academy of Sciences (KUNCC).

2.2. Test Material

The initial bioassay experiments were conducted using the selected strains of *M. bibionidarum* (KUNCC 10806) and *M. dianzhongense* (KUNCC 10811), which were collected, isolated, and purified in this study. Concurrently, field surveys were conducted to collect insect samples of *S. frugiperda* in 2022. The 4th-instar larvae of *S. frugiperda* were bred using lab colonies of the pest noctuids. The test strains were cultured on PDA plates for 14 d at 25 °C under a light/dark condition (L/D = 14:10) and the humidity of 70%. To harvest fungal spores, a solution containing 0.1% Tween 80 was poured onto a culture plate, and the surface of the fungal colony was gently scraped to release the spores. The spore

concentration was estimated using a hemocytometer and light microscope. For the bioassay, the spore suspensions were diluted to achieve a concentration of 1×10^8 spores/mL.

2.3. Morphological Observation

Specimens were photographed and examined in the laboratory using a Canon 750D camera and an Olympus SZ60 microscope. For descriptions of colony appearance, the isolated strains were cultured on PDA plates for 14 d at 25 °C under a light/dark condition (L/D = 14:10). The characteristics of colonies (size, texture, and color) were photographed with a Canon 750D camera. For morphological evaluation, microscope slide cultures were prepared by placing small amounts of mycelia on 5 mm diameter PDA medium blocks overlaid by a cover slip. The cultures were then incubated in a Petri dish at 25 °C with a small volume of sterile water. The asexual micro-morphological characteristics (hypha, conidiogenous structure, and conidia) were observed and examined under a light Olympus BX53 microscope.

2.4. DNA Extraction, PCR Amplification, and Sequencing

The genomic DNA of the two species was extracted using the modified CTAB method [27]. The extracted DNA used for PCR amplification was stored at −20 °C. Primer details used for amplifying seven genes (ITS, nrSSU, nrLSU, tef-1α, rpb1, rpb2, and 5′tef) in this study are provided in Table 1. Each 25 µL PCR reaction contained 12.5 µL of 2 × Taq PCR Master Mix (Tiangen Biotech Co., Ltd., Beijing, China), 9.5 µL of RNase-free water (Sangon Bio Co., Ltd., Shanghai, China), 1 µL of each forward and reverse primer (10 µmol/L), 1 µL of DNA template (500 ng/µL). PCR reactions were placed in a LongGene T20 multi-block thermal cycler (Hangzhou LongGene Scientific Instruments Co., Ltd., Hangzhou, China) under the following conditions: for ITS, (1) 3 min at 95 °C, (2) 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, (3) extension at 72 °C for 10 min and 14 °C soak; for tef-1α, (1) 3 min at 95 °C, (2) 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, (3) extension at 72 °C for 10 min and 14 °C soak; for rpb1, (1) 4 min at 95 °C, (2) 30 cycles of denaturation at 94 °C for 50 s, annealing at 52 °C for 50 s, and extension at 72 °C for 1 min, followed by (3) 8 cycles of denaturation at 94 °C for 50 s, annealing at 51 °C for 50 s, and extension at 72 °C for 1 min, (4) extension at 72 °C for 10 min and 14 °C soak; for rpb2, (1) 4 min at 95 °C, (2) 37 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, (3) extension at 72 °C for 10 min and 14 °C soak; for nrLSU, (1) 4 min at 95 °C, (2) 30 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min, (3) extension at 72 °C for 8 min and 14 °C soak; for nrSSU, (1) 2 min at 95 °C, (2) 35 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for 30 s, and extension at 72 °C for 1 min, (3) extension at 72 °C for 10 min and 14 °C soak; for 5′tef, (1) 3 min at 95 °C, (2) 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, (3) extension at 72 °C for 10 min and 14 °C soak. Standard DNA markers (Sangon Bio Co., Ltd., Shanghai, China) of known size and weight were used to quantify the PCR products. The PCR products were separated by electrophoresis in 1% agarose gels, purified using the Gel Band Purification Kit (Bio Teke Co., Ltd., Beijing, China), and then sent to Tsing ke Biotechnology Co., Ltd. for sequencing after the selection of impurity-free and distinct bands. The seven genes of two *Metarhizium* species generated in this study were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank, accessed on 26 February 2024).

Table 1. Sequences of primers used in this study.

Molecular Marker	Primer Name	Primer Sequence (5′–3′)	Reference
ITS	ITS4	TCCTCCGCTTATTGATATGC	[28]
	ITS5	GGAAGTAAAAGTCGTAACAAGG	[28]

Table 1. *Cont.*

Molecular Marker	Primer Name	Primer Sequence (5′–3′)	Reference
tef-1α	EF1α-EF	GCTCCYGGHCAYCGTGAYTTYAT	[17,18]
	EF1α-ER	ATGACACCRACRGCRACRGTYTG	[3]
rpb1	RPB1-5′F	CAYCCWGGYTTYATCAAGAA	[17,18]
	RPB1-5′R	CCNGCDATNTCRTTRTCCATRTA	[3]
rpb2	RPB2-5′F	CCCATRGCTTGTYYRCCCAT	[17]
	RPB2-5′R	GAYGAYMGWGATCAYTTYGG	[3]
nrLSU	LR5	ATCCTGAGGGAAACTTC	[29]
	LROR	GTACCCGCTGAACTTAAGC	[30]
nrSSU	nrSSU-CoF	TCTCAAAGATTAAGCCATGC	[31]
	nrSSU-CoR	TCACCAACGGAGACCTTG	[31]
5′tef	EF1α-IF	ATGGGTAAGGASGAMAAGAC	This study
	EF1α-IR	GGARGTACCAGTRATCATGTT	This study

2.5. Phylogenetic Analyses

Based on molecular data from recent research in the genus *Metarhizium*, seven gene sequences (ITS, nrSSU, nrLSU, tef-1α, rpb1, rpb2, and 5′tef) were retrieved and downloaded from the GenBank database. Detailed information is provided in Table 2. Along with the sequencing results from this study, gene sequences for 76 taxonomic units were ultimately obtained. Sequences of seven genes were aligned using MEGA v. 7.0 [32,33]. After sequence alignments, the aligned sequences of seven genes were concatenated. Ambiguously aligned sites were manually eliminated, and gaps were treated as missing data. The sequences of *Pochonia* Bat. & O.M. Fonsa (*P. boninensis* Nonaka et al. JCM 18597 and *P. chlamydosporia* (Goddard) Zare & W. Gams CBS 101244) were selected as the outgroup. Under the Akaike Information Criterion (AIC), ModelFinder is used to select the most appropriate nucleotide substitution model for Bayesian inference (BI) and maximum-likelihood (ML) analyses. The BI analysis was conducted using MrBayes v. 3.2 [34], employing the GTR+G+I model. Four simultaneous Markov chains were run for 2,000,000 generations with a sub-sampling frequency every 100 generations. A burn-in of the first 25% of the total run was discarded. The ML analysis was conducted using IQ-TREE v. 2.1.3 [35] under the TNe+I+R3 model with 1000 ultrafast bootstrap [36]. Trees were visualized with Bayesian posterior probability (BI-PP) and maximum-likelihood bootstrap proportions (ML-BS) in FigTree v. 1.4.4 and edited with Adobe Illustrator CS6.0.

Table 2. Voucher information and GenBank accession numbers of sequences used in this study.

Species	Voucher Information	GenBank Accession Number							Reference
		SSU	LSU	tef	rpb1	rpb2	ITS	5′tef	
Metarhizium acridum	ARSEF 7486^{T}	–	–	EU248845	EU248897	EU248925	HQ331458	EU248845	[18]
M. acridum	ARSEF 324	–	–	EU248844	EU248896	EU248924	HM055449	EU248844	[18]
M. album	ARSEF 2082	DQ522560	DQ518775	DQ522352	DQ522398	DQ522452	AY375446	–	[3]
M. album	ARSEF 2179	–	–	KJ398807	KJ398618	–	HM055452	–	[4]
M. alvesii	CG 1123^{T}	–	–	KY007614	KY007612	KY007613	–	KC520541	[9]
M. anisopliae	ARSEF 7487	–	–	DQ463996	DQ468355	DQ468370	MH604974	DQ463996	[18]
M. anisopliae	CBS 130.71^{T}	MT078868	MT078853	MT078845	MT078861	MT078918	MT078884	MT078928	[11]
M. argentinense	CEP 424^{T}	–	–	MF966624	MF966625	MF966626	–	–	[37]
M. argentinense	CEP 414	–	–	–	–	–	MF784813	–	[37]
M. atrovirens	TNM-F 10184	JF415950	JF415966	–	JN049884	–	JN049882	–	[4]
M. baoshanense	BUM 63.4	KY264178	KY264175	KY264170	KY264181	KY264184	KY264173	–	[21]
M. baoshanense	CCTCC M 2016588	KY087812	KY087816	KY087820	KY087824	KY087826	KY087808	–	[22]

Table 2. *Cont.*

Species	Voucher Information	GenBank Accession Number							Reference
		SSU	LSU	tef	rpb1	rpb2	ITS	5′tef	
M. baoshanense	CCTCC M 2016589^{T}	KY264177	KY264174	KY264169	KY264180	KY264183	KY264172	–	[21]
M. bibionidarum	CBS 64867	–	–	LC126075	LC125907	LC125923	–	AB807450	[20]
M. bibionidarum	NBRC 112661^{T}	–	–	LC126076	LC125908	LC125924	–	LC187676	[20]
M. bibionidarum	Hkd35-2	–	–	LC126077	LC125912	LC125925	–	AB807804	[20]
M. bibionidarum	KUNCC 10806	**PP256146**	**PP256148**	**PP328481**	**PP294690**	**PP314008**	**PP256140**	**PP413396**	This study
M. bibionidarum	KUNCC 10807	–	**PP256149**	**PP328482**	**PP294691**	**PP314009**	**PP256141**	**PP413397**	This study
M. bibionidarum	KUNCC 10808	**PP256145**	**PP256147**	**PP328480**	**PP294689**	**PP314007**	**PP256139**	**PP413395**	This study
M. biotecense	BCC 51812^{T}	MN781937	MN781838	MN781693	MN781745	MN781792	MN781878	–	[11]
M. biotecense	BCC 51813	MN781938	MN781839	MN781694	MN781746	MN781793	MN781879	–	[11]
M. blattodeae	ARSEF 12850^{T}	–	–	KU182917	KU182918	KU182916	KU182915	–	[38]
M. blattodeae	MY 00896	HQ165657	HQ165719	HQ165678	HQ165739	HQ165638	HQ165697	–	[8]
M. brachyspermum	CM 1^{T}	–	LC469749	LC469751	–	–	LC469747	LC469752	[13]
M. brasiliense	ARSEF 2948^{T}	–	–	KJ398809	KJ398620	–	–	–	[5]
M. brittlebankisoides	Hn 1	–	–	AB778556	AB778555	AB778554	–	–	[19]
M. brittlebankisoides	G 97025	–	–	–	–	–	AJ309332	–	[39]
M. brunneum	ARSEF 2107^{T}	–	–	EU248855	EU248907	EU248935	KC178691	EU248855	[18]
M. brunneum	CBS 316.51	MT078875	MT078860	MT078852	–	–	MT078888	MT078927	[11]
M. campsosterni	BUM 10	MH143832	MH143815	MH143849	MH143864	MH143879	MH143798	–	[2]
M. candelabrum	BCC 29224^{T}	MN781952	MN781853	MN781708	MN781755	MN781804	MN781881	–	[11]
M. cercopidarum	BCC 31660^{T}	MN781953	–	MN781709	MN781756	MN781805	MN781880	–	[11]
M. chaiyaphumense	BCC 28241	MN781932	MN781831	MN781684	MN781740	MN781784	MN781884	–	[11]
M. chaiyaphumense	BCC 78198^{T}	KX369596	KX369593	KX369592	KX369594	KX369595	MT078881	–	[8]
M. cicadae	BCC 48696	MN781948	MN781848	MN781703	–	MN781800	MN781885	–	[11]
M. cicadae	BCC 48881^{T}	MN781949	MN781849	MN781704	MN781752	–	–	–	[11]
M. clavatum	BCC 84543^{T}	–	MN781834	MN781689	MN781741	MN781789	MN781886	MT078929	[11]
M. clavatum	BCC 84558	–	MN781835	MN781690	MN781742	–	–	–	[11]
M. culicidarum	BCC 2673	MN781950	MN781851	MN781706	MN781753	MN781802	MN781887	–	[11]
M. culicidarum	BCC 7600^{T}	MN781951	MN781852	MN781707	MN781754	MN781803	MN781889	–	[11]
M. culicidarum	BCC 7625	–	MN781850	MN781705	–	MN781801	MN781888	–	[11]
M. cylindrosporum	CBS 256.90^{T}	–	MH873892	KJ398783	KJ398594	KJ398691	MH862209	–	[5]
M. cylindrosporum	RCEF 3632	JF415964	JF415987	JF416022	–	–	JN049872	–	[4]
M. cylindrosporum	TNS-F 16371	JF415963	JF415986	JF416027	JN049902	–	–	–	[4]
M. dianzhongense	KUNCC 10809	–	**PP256151**	**PP328484**	**PP294693**	**PP314011**	**PP256143**	-	This study
M. dianzhongense	KUNCC 10810	–	**PP256152**	**PP328485**	**PP294694**	**PP314012**	**PP256144**	-	This study
M. dianzhongense	KUNCC 10811	–	**PP256150**	**PP328483**	**PP294692**	**PP314010**	**PP256142**	-	This study
M. eburneum	BCC 79252^{T}	–	MN781829	MN781682	MN781736	–	MN781914	–	[11]
M. eburneum	BCC 79267	–	MN781826	–	MN781735	–	MN781915	–	[11]
M. ellipsoideum	BCC 12847	MN781959	MN781860	MN781715	MN781761	MN781810	MN781925	–	[11]
M. ellipsoideum	BCC 49285^{T}	MN781957	MN781858	MN781713	MN781759	MN781808	MT078876	–	[11]
M. ellipsoideum	BCC 53509	MN781958	MN781859	MN781714	MN781760	MN781809	MT078877	–	[11]
M. flavoviride	CBS 700.74	MT078870	MT078855	MT078847	MT078863	MT078920	–	MT078925	[11]

Table 2. *Cont.*

Species	Voucher Information	GenBank Accession Number							Reference
		SSU	LSU	tef	rpb1	rpb2	ITS	5′tef	
M. flavoviride	ARSEF 2133	–	–	–	–	–	NR131992	DQ463988	[17]
M. flavoviride	CBS 125.65	MT078869	MT078854	MT078846	MT078862	MT078919	MT078885	MT078926	[11]
M. flavoviride	CBS 218.56^{T}	–	–	KJ398787	KJ398598	–	–	–	[5]
M. flavum	BCC 90870^{T}	MN781965	MN781874	MN781731	MN781776	MN781822	–	–	[11]
M. flavum	BCC 90874	MN781966	MN781875	MN781732	MN781777	MN781823	–	–	[11]
M. frigidum	ARSEF 4124^{T}	–	–	DQ464002	DQ468361	DQ468376	NR132012	–	[17]
M. fusoideum	BCC 28246^{T}	MN781944	MN781844	MN781699	MN781749	MN781796	MN781893	–	[11]
M. fusoideum	BCC 41242	MN781942	MN781825	MN781679	–	MN781780	–	–	[11]
M. fusoideum	BCC 53130	MN781943	MN781843	MN781698	–	MN781795	MN781894	–	[11]
M. gaoligongense	BUM 3.5	KY087810	KY087814	KY087818	KY087822	–	KY087806	–	[22]
M. gaoligongense	CCTCC M 2016588^{T}	KY087812	KY087816	KY087820	KY087824	KY087826	KY087808	–	[22]
M. globosum	ARSEF 2596^{T}	–	–	EU248846	EU248898	EU248926	NR132020	–	[18]
M. granulomatis	UAMH 11028^{T}	HM635076	HM195304	KJ398781	–	–	NR132013	–	[40]
M. granulomatis	UAMH 11176	–	HM635078	KJ398782	KJ398593	–	HM195306	–	[40]
M. gryllidicola	BCC 37915	–	–	–	–	–	MN781896	–	[12]
M. gryllidicola	BCC 37918	MN781935	MN781836	MN781691	MN781743	MN781790	MN781897	–	[12]
M. gryllidicola	BCC 82988	MK632117	MK632091	MK632062	MK632166	MK632143	–	MT078891	[12]
M. guizhouense	ARSEF 6238	–	–	EU248857	EU248909	EU248937	–	EU248857	[18]
M. guizhouense	CBS 258.90^{T}	–	–	EU248862	EU248914	EU248942	HQ331448	EU248862	[18]
M. guniujiangense	RCEF 6740	MW718230	MW718256	MW723108	MW723140	MW723161	–	–	[14]
M. guniujiangense	RCEF 880^{T}	MW718229	MW718255	MW723106	MW723139	MW723160	–	–	[14]
M. huainamdangense	BCC 32190	MN781954	MN781855	MN781710	MN781757	–	MN781899	–	[11]
M. huainamdangense	BCC 44270^{T}	MN781956	MN781857	MN781712	–	MN781807	MN781898	–	[11]
M. huainamdangense	BCC 7672	MN781955	MN781856	MN781711	MN781758	MN781806	MN781901	–	[11]
M. humberi	IP 46^{T}	–	–	MH837574	MH837556	MH837565	–	JQ061205	[10]
M. indigoticum	TNS-F 18553	JF415953	JF415968	JF416010	JN049886	JF415992	JN049874	–	[4]
M. indigoticum	TNS-F 18554	JF415952	JF415969	JF416011	JN049887	JF415993	JN049875	–	[4]
M. kalasinense	BCC 53582^{T}	KC011175	KC011183	KC011189	–	–	KC011179	KX823945	[8]
M. koreanum	ARSEF 2038^{T}	–	–	KJ398805	KJ398615	KJ398713	HM055431	–	[5]
M. koreanum	BCC 27998	MN781945	MN781845	MN781700	–	MN781797	MN781903	–	[11]
M. koreanum	BCC 30455	MN781946	MN781846	MN781701	MN781750	MN781798	MN781904	–	[11]
M. lepidiotae	ARSEF 7488^{T}	–	–	EU248865	EU248917	EU248945	HQ331456	EU248865	[18]
M. macrosemiae	RCEF 6696^{T}	MW718233	MW718259	MW723113	MW723144	MW723164	MW718317	–	[14]
M. majus	ARSEF 1015	–	–	EU248866	EU248918	EU248946	HQ331444	EU248866	[18]
M. majus	ARSEF 1914^{T}	–	–	EU248868	EU248920	EU248948	HQ331445	EU248868	[18]
M. megapomponiae	BCC 25100^{T}	MN781947	MN781847	MN781702	MN781751	MN781799	MN781906	–	[11]
M. minus	ARSEF 1099	–	–	KJ398799	KJ398608	KJ398706	–	–	[5]
M. minus	ARSEF 2037^{T}	AF339580	AF339531	DQ522353	DQ522400	DQ522454	AF138271	–	[3]
M. niveum	BCC 52400^{T}	MN781933	MN781832	MN781685	–	MN781785	MN781907	–	[11]
M. nornnoi	BCC 19364	MN781940	MN781841	MN781696	MN781747	–	MN781891	–	[11]
M. nornnoi	BCC 25948^{T}	MN781941	MN781842	MN781697	MN781748	–	MN781892	–	[11]
M. novozealandicum	ARSEF 4661	–	–	KJ398811	KJ398622	–	–	–	[5]
M. novozealandicum	ARSEF 4674	–	–	KJ398812	KJ398623	–	–	–	[5]
M. ovoidosporum	BCC 29223	MN781960	MN781861	MN781716	MN781762	–	MN781909	–	[11]

Table 2. *Cont.*

Species	Voucher Information	GenBank Accession Number							Reference
		SSU	LSU	tef	rpb1	rpb2	ITS	5′tef	
M. ovoidosporum	BCC 32600^{T}	MN781961	MN781862	MN781717	MN781763	–	MN781910	–	[11]
M. ovoidosporum	BCC 7634	MN781962	MN781863	MN781718	MN781764	MN781811	MN781908	–	[11]
M. owariense	NBRC 33258	HQ165669	HQ165730	JF416017	KJ398596	JF415996	JN049883	–	[4]
M. pemphigi	ARSEF 6569	–	–	KJ398813	KJ398624	DQ468378	–	–	[5]
M. pemphigi	ARSEF 7491	–	–	KJ398819	KJ398629	DQ468379	–	–	[5]
M. pemphigi	BUM 1	–	–	–	–	–	MH143795	–	[22]
M. pemphigi	BUM 39.4	–	–	–	–	–	KY087809	–	[22]
M. phasmatodeae	BCC 2841	MN781931	MN781828	MN781681	MN781738	MN781782	MN781911	–	[12]
M. phasmatodeae	BCC 49272	MK632119	MK632093	MK632064	–	MK632145	MK632035	MT078893	[12]
M. phuwiangense	BCC 78206	–	–	MN781719	MN781765	MN781812	MT078879	–	[11]
M. phuwiangense	BCC 85068	–	MN781864	MN781720	MN781766	MN781813	MN781912	–	[11]
M. phuwiangense	BCC 85069^{T}	–	MN781865	MN781721	MN781767	MN781814	MN781913	–	[11]
M. pingshaense	CBS 257.90^{T}	–	–	EU248850	EU248902	EU248930	HQ331450	EU248850	[18]
M. prachinense	BCC 47950	KC011172	KC011180	KC011186	KC011184	–	KC011176	–	[8]
M. prachinense	BCC 47979^{T}	KC011173	KC011181	KC011187	KC011185	–	KC011177	–	[8]
M. pseudoatrovirens	TNS-F 16380	–	JF415977	KJ398785	JN049893	JF415997	JN049870	–	[4]
M. purpureogenum	ARSEF 12570	–	–	LC126079	LC125911	LC125922	–	–	[8]
M. purpureogenum	ARSEF 12571^{T}	–	AB700552	LC126078	LC125913	LC125920	–	–	[8]
M. purpureonigrum	BCC 89247^{T}	–	–	MN781725	MN781771	MN781817	–	–	[11]
M. purpureonigrum	BCC 89248	MN781964	MN781870	MN781728	–	MN781819	–	–	[11]
M. purpureonigrum	BCC 89249	MN781963	MN781869	MN781726	MN781772	MN781818	–	–	[11]
M. purpureum	BCC 82173	–	MN781866	MN781722	MN781768	MN781815	MN781919	–	[11]
M. purpureum	BCC 82642^{T}	–	MN781867	MN781723	MN781769	MN781816	MN781918	–	[11]
M. purpureum	BCC 83548	–	MN781868	MN781724	MN781770	–	MN781920	–	[11]
M. reniforme	ARSEF 429	HQ165671	HQ165733	HQ165690	–	HQ165650	DQ069284	–	[8]
M. reniforme	ARSEF 577	HQ165672	HQ165734	HQ165691	–	HQ165651	DQ069283	–	[8]
M. reniforme	IndGH 96	HQ165670	HQ165732	–	–	HQ165649	–	–	[8]
M. rileyi	AF 368501	–	–	–	–	–	AF368501	–	[16]
M. rileyi	CBS 806.71	AY526491	MH872111	EF468787	EF468893	EF468937	AY624205	–	[3]
M. rileyi	NBRC 8560	HQ165667	HQ165729	HQ165688	–	–	–	–	[8]
M. robertsii	ARSEF 4739	–	–	EU248848	EU248900	EU248928	–	EU248848	[18]
M. samlanense	BCC 17091^{T}	HQ165665	HQ165727	HQ165686	–	HQ165646	HQ165707	–	[8]
M. samlanense	BCC 39752	MN781939	MN781840	MN781695	–	MN781794	MT078880	–	[8]
M. sulphureum	BCC 36585	–	–	MN781686	–	MN781786	–	MT078931	[11]
M. sulphureum	BCC 36592^{T}	–	–	MN781687	–	MN781787	–	–	[11]
M. sulphureum	BCC 39045	MK632120	MK632095	MK632066	–	MK632147	MK632037	MT078930	[11]
M. takense	BCC 30934	HQ165658	HQ165720	HQ165679	HQ165740	HQ165639	HQ165698	–	[8]
M. takense	BCC 30939^{T}	HQ165659	HQ165721	–	HQ165741	HQ165640	HQ165699	–	[8]
M. viride	CBS 659.71	HQ165673	HQ165735	HQ165692	–	HQ165652	HQ165714	–	[14]
M. viridulum	ARSEF 6927	–	–	KJ398815	KJ398681	–	–	–	[5]
M. viridulum	BCC 36261	MN781930	MN781827	MN781680	MN781737	MN781781	MT078878	–	[11]
Pochonia boninensis	JCM 18597^{T}	AB758255	AB709831	AB758463	AB758666	AB758693	AB709858	–	[41]
P. chlamydosporia	CBS 101244	DQ522544	DQ518758	DQ522327	DQ522372	DQ522424	JN049821	–	[41]

Note. The accession numbers in bold font refer to newly generated sequences in this study.

2.6. Pathogenicity Test

The test strain's spore suspension with a concentration of 1×10^8 spores/mL was used to immerse the selected and consistent-sized healthy 4th-instar larvae of *S. frugiperda* for approximately 10 s. Subsequently, the larvae were removed and allowed to air dry. They were then placed in a controlled artificial climate chamber at 25 °C and a humidity level of 65% and were individually reared in plastic rearing containers measuring 3 cm in diameter and height, with corn seeds and leaves provided as their food source. The experiment was conducted with three repetitions, each consisting of 12 *S. frugiperda* larvae.

A 0.1% Tween-80 sterile water treatment was used as the blank control group. Infected and control samples were then reared under standard insect-rearing conditions (as mentioned above) for a duration of nine days. Deceased insects were promptly removed and placed in the moist culture room to confirm their infection. Mortality rates and adjusted mortality rates were calculated as follows. The mortality rate (%) was determined by the following formula: (number of deceased insects/total number of test insects) × 100%. The adjusted mortality rate (%) was calculated using the following formula: (mortality rate in treatment group—mortality rate in control group)/(1—mortality rate in control group) × 100%.

3. Results

3.1. Phylogenetic Analyses

The combined seven-gene dataset comprised 5581 base pairs (bp) after alignments (ITS 581 bp, nrSSU 968 bp, nrLSU 764 bp, tef-1α 871 bp, rpb1 738 bp, rpb2 919 bp, and 5′tef 740 bp). Phylogenetic trees from ML and BI analyses based on the combined seven-gene dataset of 78 taxa showed nearly congruent topologies (Figure 1). It was revealed that all *Metarhizium* species formed a monophyletic group with high support. The samples of *M. bibionidarum* collected in this study and described by Nishi et al. [20] both have the same hosts, the March fly larvae (Diptera: Bibionidae). Based on the seven-gene phylogeny, they clustered into a clade with credible bootstrap support (BI-PP = 1 and ML-BP = 100%). The new species *M. dianzhongense* was sister to *M. ellipsoideum* Luangsa-ard et al. in the *M. cercopidarum* complex. However, the three strains of *M. dianzhongense* (KUNCC 10809, KUNCC 10810, and KUNCC 10811) formed a single derived clade (BI-PP = 1 and ML-BP = 100%).

3.2. Taxonomy

Metarhizium bibionidarum Nishi & Hiroki Sato, Mycological Progress 16(10): 993 (2017) [20], Index Fungorum number: IF819797, Figure 2.

Description: Specimens were found on March fly larvae (Diptera: Bibionidae). The head and thorax of March fly larvae were covered with white mycelia and powdery conidia. The conidia were smooth-walled, yellowish-green to green, cylindrical to ellipsoid with semi-papillate apices, 2.5–3.5 × 5.5–7 µm. Colonies on PDA grew well, attaining a diameter of 27–29 mm in 7 d, and were white, fluffy, and cottony in appearance. Sporulation with peacock-green conidia produced on mycelia started at 7 d after inoculation, reverse white. Hyphae were smooth-walled, hyaline, branched, septate. Conidiophores arising from aerial mycelia were erect and smooth-walled. Phialides were smooth-walled, cylindrical or ellipsoidal, with papillate apices, 7.6–27.3 × 2–3.5 µm. Conidia were smooth-walled, cylindrical to ellipsoidal with semi-papillate apices, arranged in chains, 7.8–10.4 × 2–3 µm.

Host: March fly larvae (Bibionidae, Diptera).

Habitat: On the March fly larvae buried in soil or attached to fallen leaves.

Distribution: Known from France, Japan, and China.

Material examined: China. Yunnan Province: Kunming City, Heilongtan Park, alt. 2150 m, on a March fly larva, 5 September 2022, C.Y. Wei (HKAS 126221, KUNCC 10806); *Ibid*. 5 September 2022, C.Y. Wei (HKAS 126222, KUNCC 10807); *Ibid*. 5 September 2022, C.Y. Wei (HKAS 126223, KUNCC 10808).

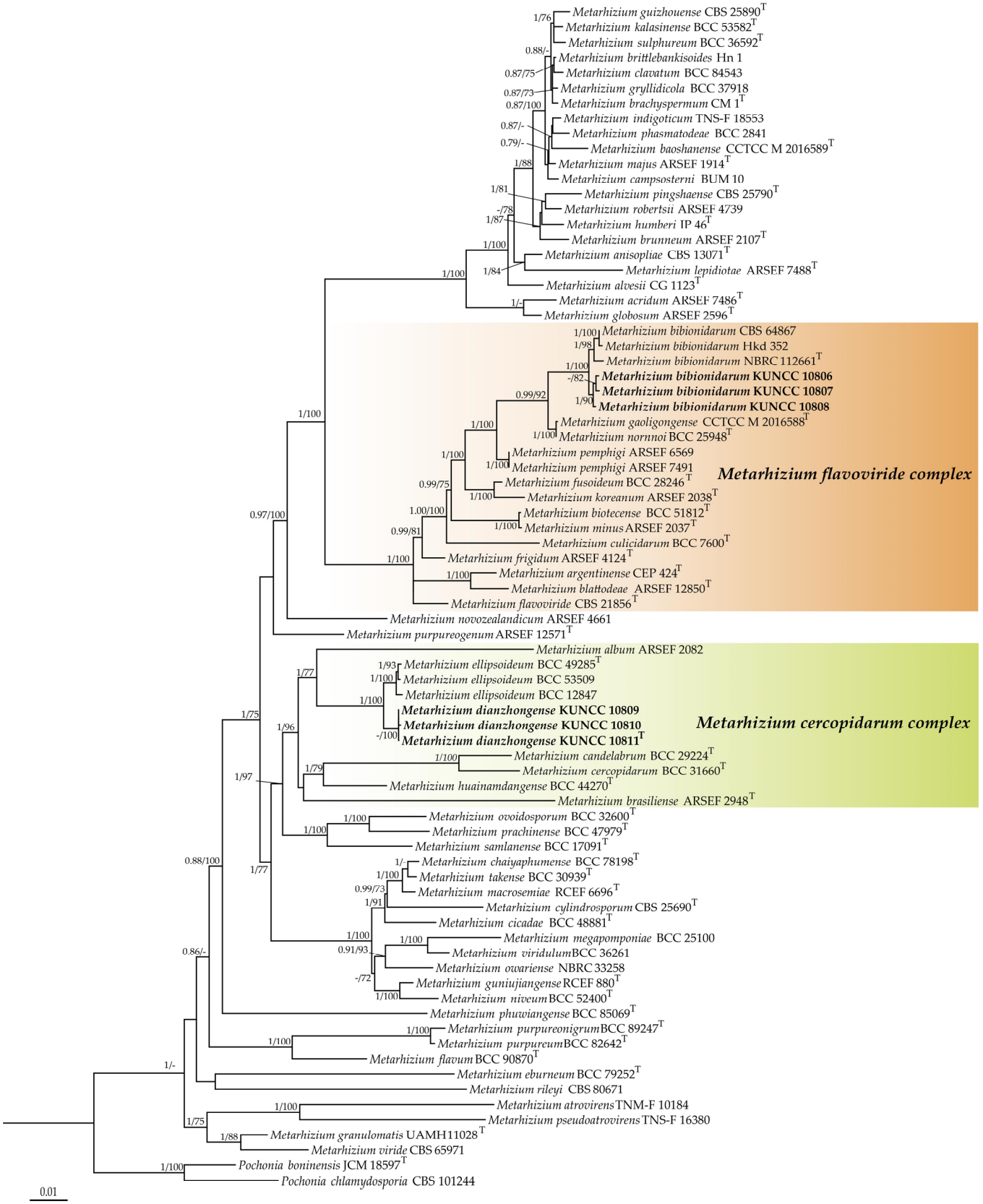

Figure 1. Phylogenetic placements of *Metarhizium dianzhongense* and *Metarhizium bibionidarum* in the genus *Metarhizium* inferred from BI and ML analyses based on multi-gene dataset (ITS, nrSSU, nrLSU, tef-1α, rpb1, rpb2, and 5′tef). Values at the nodes before and after the backslash are BI posterior probabilities (BI-PP > 0.70) and ML bootstrap proportions (ML-BP > 70%), respectively.

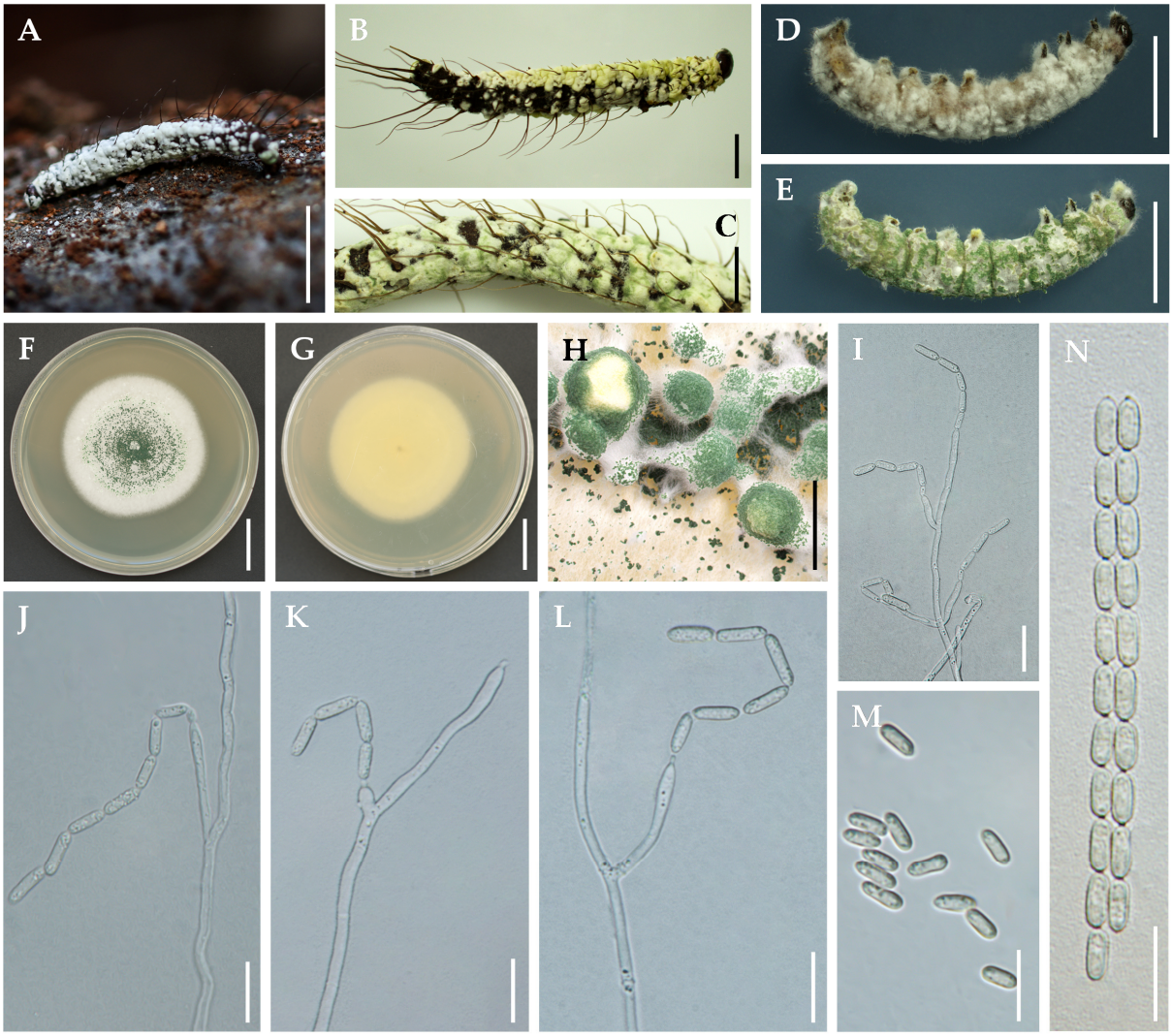

Figure 2. *Metarhizium bibionidarum*. (**A**–**C**) Larvae of March fly infected by *M. bibionidarum*; (**D**,**E**) 4th-instar larvae of *S. frugiperda* killed by *M. bibionidarum*; (**F**–**H**) colonies on PDA; (**I**–**L**) conidiophores and phialides on PDA; (**M**) conidia on PDA; (**N**) conidia on insect host. Scale bars: (**A**) = 5 mm, (**B**,**C**) = 2 mm, (**D**,**E**) =5 mm, (**F**,**G**) = 2 cm, (**H**) = 5 mm, (**I**) = 20 μm, (**J**–**N**) = 10 μm.

Notes: Our newly collected three samples are phylogenetically clustered with *Metarhizium bibionidarum* including strain NBRC 112661 (ex-type, Japan) and CBS 648.67 (France) within the *M. flavoviride* complex with strong statistical support. *M. bibionidarum* is closely related to *M. gaoligongense* Z.H. Chen & L. Xu, *M. nornnoi* Luangsa-ard et al., and *M. pemphigi* (Driver & Milner) Kepler et al. The three samples parasitize March fly larvae and produce similar-sized cylindrical to ellipsoidal conidia on PDA. The morphological characteristics on other media lack a clear distinction. *M. bibionidarum* is known to parasitize Diptera and Coleoptera, in addition to being commonly found in soil [20].

Metarhizium dianzhongense C.Y. Wei, Zhu L. Yang & Y.B. Wang, sp. nov. Fungal Names number: FN571888; Figure 3.

Etymology: The epithet '*dianzhong*' refers to central Yunnan Province in Chinese, where the holotype was collected.

Holotype: China. Yunnan Province, Chuxiong City, Sanjie Town, 100°69′233 E, 24°52′322 N, alt. 1880 m, on a white grub buried in soil, 5 September 2022, C.Y. Wei (HKAS 126224, holotype; KUNCC 10811, ex-holotype culture). GenBank accession Nos.: ITS = PP256142, nrLSU = PP256150, tef-1α = PP328483, rpb1 = PP294692, rpb2 = PP314010.

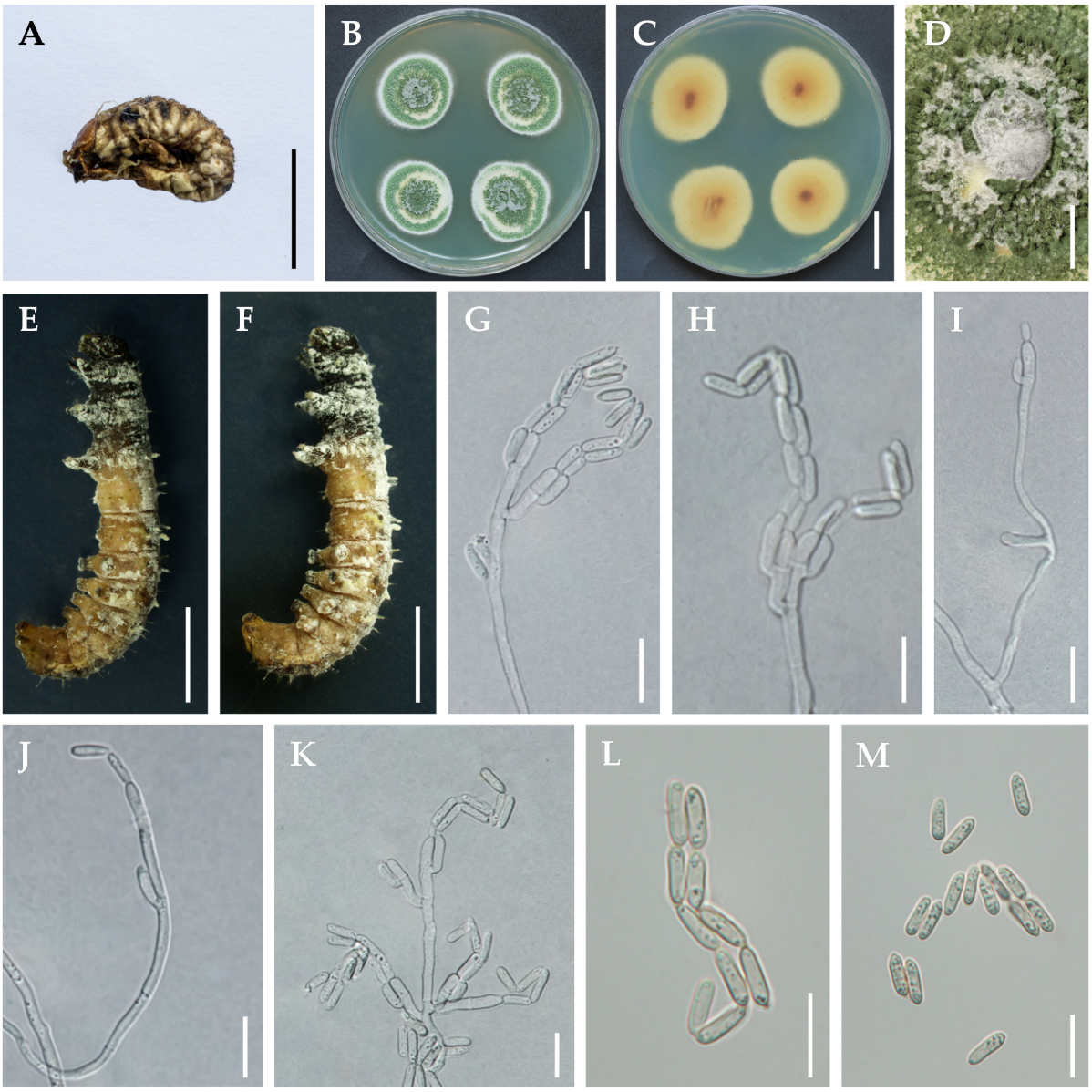

Figure 3. *Metarhizium dianzhongense*. (**A**) White grub infected by *M. dianzhongense*; (**B**–**D**) colonies on PDA; (**E**,**F**) 4th-instar larvae of *S. frugiperda* killed by *M. dianzhongense*; (**G**–**K**) conidiophores and phialides on PDA; (**L**,**M**) conidia on PDA. Scale bars: (**A**) = 1 cm, (**B**,**C**) = 2 cm, (**D**–**F**) = 5 mm, (**G**–**M**) = 10 μm.

Description: Specimens were found on larvae of Scarabaeidae (Coleoptera). The insect body curled up, stiffened, and formed sclerotia. Colonies on PDA attained a diameter of 23–25 mm in 14 d, with mycelia closely appressed to the agar surface, dense, flat. Margins were initially pale yellow to white, white turning to parrot-green with powdery conidia, becoming raised at the center of colonies. Sporulation started 3 d after inoculation, reverse pale yellow. Hyphae were smooth-walled. Conidiophores arising from aerial mycelia were erect, smooth-walled, cylindrical, 7.8–10.4 × 2–3 μm. Phialides were smooth-walled, cylindrical or ellipsoidal with semi-papillate apices, 4–7.5 × 1–3 μm. Conidia were smooth-walled, parrot-green, ellipsoidal or cylindrical with rounded apices, arranged in chains, 7–9 × 2–3 μm.

Host: White grubs, larvae of Scarabaeidae (Coleoptera).

Habitat: On the larvae of Scarabaeidae buried in soil from farmland.

Distribution: Known from Chuxiong City, Yunnan Province, China.

Other material examined: China. Yunnan Province, Chuxiong City, Sanjie Town, 100°53′346 E, 23°58′465 N, alt. 1873 m, on a white grub buried in soil, 13 November 2021, C.Y. Wei (HKAS 126224; KUNCC 10809); *Ibid*. 13 November 2021, C.Y. Wei (HKAS 126225, KUNCC 10810).

Notes: Phylogenetically, *M. dianzhongense* is sister to *M. ellipsoideum* with strong statistical support, forming a separate clade from other *Metarhizium* species in the *M. cercopidarum* complex. It is similar to *M. ellipsoideum*, *M. candelabrum*, *M. cercopidarum,* and *M. huainamdangense* due to the cylindrical and parrot-green conidia with rounded apices [11]. However, *M. dianzhongense* (14 d, 24 mm) grows faster than *M. ellipsoideum* (14 d, 14–15 mm), *M. candelabrum* (14 d, 15 mm), *M. cercopidarum* (14 d, 15 mm), and *M. huainamdangense* (14 d, 15 mm) on PDA. The distinctiveness of *M. dianzhongense* is indicated by its larger conidial size (7–9 × 2–3 µm) compared with *M. ellipsoideum* (5–7 × 1.5–2 µm) and *M. cercopidarum* (6–8 × 2 µm). In their natural habitats, *M. ellipsoideum*, *M. candelabrum*, *M. cercopidarum*, and *M. huainamdangense* parasitize insects of leafhoppers (Hemiptera). However, *M. dianzhongense* specifically targets larvae of Scarabaeidae (Coleoptera) in farmland and has been observed to infect larvae of *S. frugiperda* (Lepidoptera) during controlled experiments.

Additionally, there exist significant distinctions between *M. dianzhongense* and other unmentioned *Metarhizium* species in this study, namely *M. cicadinum* (Höhn.) Petch, *M. dendrolimatilis* Z.Q. Liang et al., *M. lepidopterorum* W.H. Chen et al., *M. lymantriidae* Z.H. Chen & L. Xu, *M. putuoense* Yi Li et al., and *M. rongjiangense* W.H. Chen et al. [11,15,42–45]. The six species are mainly parasitic on Hemiptera (*M. cicadinum*) and Lepidoptera (*M. dendrolimatilis*, *M. lepidopterorum*, *M. lymantriidae*, *M. putuoense,* and *M. rongjiangense*) hosts, which distinguishes them from *M. dianzhongense* (Coleoptera). The morphological characteristics of *M. dianzhongense* significantly differ from those of the six species in terms of colonies, conidiogenous structures, and conidia. Phylogenetically, *M. dianzhongense*, belonging to the *M. cercopidarum* complex clade, is distinct from *M. cicadinum* (the *M. anisopliae* complex), *M. putuoense* (the *M. purpureum* complex), and *M. dendrolimatilis* and *M. lymantriidae* (the *M. rileyi* complex) [43]. Particularly, *M. lepidopterorum* and *M. rongjiangense* now belong to the genus *Marquandomyces*, which was previously described as *Metarhizium* [44].

3.3. Pathogenicity to Spodoptera frugipera

After nine days of infection, the *M. bibionidarum* strain KUNCC 10806 exhibited the highest overall mortality in instar larvae of *S. frugiperda*, followed by the *M. dianzhongense* strain KUNCC 108011. The findings showed that mortality in *S. frugiperda* larvae caterpillars treated with KUNCC 10806 and KUNCC 10811 predominantly occurred within the initial three days after infection. The mortality rates of 94.44% and 86.11% were observed in *S. frugiperda* larvae caterpillars nine days after infection with KUNCC 10806 and KUNCC 10811, respectively, resulting in induced corrected mortality rates of 93.97% and 84.85%.

4. Discussion

The current species delimitation of fungal complex groups emphasizes the necessity of employing multi-gene phylogeny, as relying solely on morphological characteristics is insufficient for accurate species identification [3,5,11,46]. In this study, two *Metarhizium* species were identified by combining morphological and seven-gene phylogenetic evidence. The new record of *M. bibionidarum* infecting March fly larvae and belonging to the *M. flavoviride* complex is closely related to *M. gaoligongense*, *M. nornnoi*, and *M. pemphigi*. The taxonomic position of the new species *M. dianzhongense* infecting larvae of Scarabaeidae was determined based on phylogenetic analyses. *Metarhizium dianzhongense* is clustered in the *M. cercopidarum* complex and is clearly distinguished from its related species *M. ellipsoideum*.

In the *M. flavoviride* complex, thirteen species have been recognized, namely *M. argentinense* A.C. Gutierrez et al., *M. bibionidarum*, *M. biotecense* Luangsa-ard et al., *M. blattodeae* C. Montalva et al., *M. culicidarum* Luangsa-ard et al., *M. flavoviride*, *M. frigidum* J.F. Bisch. & S.A. Rehner, *M. fusoideum* Luangsa-ard et al., *M. gaoligongense*, *M. koreanum* Kepler

et al., *M. minus* (Rombach, Humber & D.W. Roberts) Kepler et al., *M. nornnoi* Luangsa-ard et al., and *M. pemphigi*. The asexual states of all species are naturally observed, while no instances of sexual states have been documented. Some species (*M. bibionidarum*, *M. flavoviride*, and *M. pemphigi*) appear to exhibit a worldwide distribution, while others (*M. minus*, *M. biotecense*, *M. culicidarum*, *M. fusoideum*, and *M. nornnoi*) have only been reported in tropical regions [2,3,5,8,11,17,20,22,37]. The *M. cercopidarum* complex comprises six recognized species, including *M. album* Petch, *M. brasiliense* Kepler et al., *M. candelabrum* Luangsa-ard et al., *M. cercopidarum*, *M. dianzhongense*, *M. ellipsoideum*, and *M. huainamdangense* Luangsa-ard et al. All species belonging to this group have been reported in tropical and subtropical regions, especially Thailand and China [5,11]. Some species (*M. album*, *M. brasiliense*, *M. candelabrum*, *M. cercopidarum*, *M. ellipsoideum*, and *M. huainamdangense*) parasitize insects of Hemiptera, while the new species *M. dianzhongense* parasitizes the larvae of Coleoptera [3,5,11,16]. These species, particularly, exhibit a candelabrum-like arrangement of cylindrical phialides, forming a densely packed hymenium [11].

Previous studies have demonstrated that *M. anisopliae*, *M. favoviride*, *M. majus* (J.R. Johnst.) J.F. Bisch. et al., *M. rileyi* (Farl.) Kepler et al., and *Beauveria bassiana* (Bals. -Criv.) Vuill. exhibit significant insecticidal activity against various insect pests [23,47–49]. *M. favoviride* is an effective microbial insecticidal against *S. litura*, with the fungus significantly impairing the immune response of the insect. The larval mortality rate reached 80% when exposed to treatment concentrations of 1×10^9 conidia/mL [50]. Reports have revealed the detection of a significant number of *S. frugiperda* larvae infected by *M. rileyi* in various maize-growing regions, particularly in countries such as Mexico and Brazi [49,51]. In laboratory conditions, Perumal et al. [23] observed that *M. majus* exhibited significant insecticidal activity against *S. frugiperda* larvae within nine days. The mortality rate of Colorado potato beetle adults caused by discovered native strains reached up to 100% when beetles were treated with conidia containing a concentration of 1×10^7 spores/mL [52]. Our findings demonstrate that the strains of *M. bibionidarum* and *M. dianzhongense*, which were isolated from naturally infested larvae of Bibionidae and Scarabaeidae, exhibited significant virulence against *S. frugiperda* larvae. These results suggest that these two species could serve as viable alternatives to broad-spectrum chemical insecticidal treatment.

Author Contributions: Conceptualization, S.-K.S. and Z.-L.Y.; supervision and validation, Q.F.; software and investigation, L.-Y.X.; methodology, formal analysis, and resources, C.-Y.W.; data management, M.T.; writing—original draft preparation, C.-Y.W. and M.T.; writing—review and editing, Y.-B.W. and C.-Y.W.; project administration and funding acquisition, Y.-B.W. and G.D. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the Science and Technology Planning Project of Yunnan Province (202207AB110016, 202401AS070030); the High Level Talent Introduction Plan, Kunming Institute of Botany, CAS (E16N61); and the Innovation Project of Guangxi Graduate Education (YCBZ2022028).

Institutional Review Board Statement: This article does not contain any studies with human participants or animals performed by any of the authors.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding authors on request. Moreover, sequences were deposited in GenBank http://www.ncbi.nlm.nih.gov/ (accessed on 20 August 2023) under the accession numbers mentioned in the text.

Acknowledgments: The authors gratefully acknowledge Baozheng Chen and Xinling Lai. of the Yunnan Agricultural University for their invaluable assistance and support during the sample collection process; and thanks Hui Xu. of the Yunnan University for technical support.

Conflicts of Interest: The authors declare no conflicts of interest.

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