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ORIGINAL ARTICLE

Characterization, identification and virulence of *Metarhizium* species from Cuba to control the sweet potato weevil, *Cylas formicarius* Fabricius (Coleoptera: Brentidae)

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Abstract

Aims: Entomopathogenic *Metarhizium* fungi are widely recognized for their biological control potential. In Cuba, several fungus-based bio-insecticides have been developed and are produced as part of integrated pest management (IPM) programmes for economically relevant agricultural pests. Screening of fungal isolates from the INISAV strain collection was used for the development of bio-insecticides against important pest insects as, for example the sweet potato weevil, *Cylas formicarius*.

Methods and results: Six fungal isolates from Cuba were microscopically, morphologically and molecular-taxonomically characterized using marker sequences *ef1a, rpb1* and *rpb2*, and the 5TEF region of the *ef1a* gene. Five isolates were assigned to the species *Metarhizium anisopliae* sensu stricto and one isolate to *Metarhizium robertsii*. The pathogenic potential was evaluated against adults of *C. formicarius,* and growth and conidial production on different nutritional media were determined. *Metarhizium anisopliae* strain LBM-267 displayed pronounced virulence against the sweet potato weevil and abundant conidia production on several culture media.

Conclusions: Entomopathogenic fungal isolates from Cuba were assigned to the taxonomic species *M. anisopliae* sensu stricto and *M. robertsii*. Virulence assessment with respect to *C. formicarius* led to the identification of two *M. anisopliae* isolates holding biocontrol potential. Isolate LBM-11 has previously been developed into the bio-insecticide METASAVE-11 that is widely used to control several species of plant pathogenic weevils, Lepidoptera and thrips in Cuba. Isolate LBM-267 has not been employed previously but is as virulent against *C. formicarius* as LBM-11; its growth and conidial production capacities on different nutritional media will likely facilitate economically feasible bio-insecticide development.

Significance and Impact of the Study: *Metarhizium anisopliae* isolate LBM-267 has been selected as a promising candidate for biocontrol of the sweet potato weevil, an economically important agricultural pest in Cuba, and for further R&D activities within the framework of the Biological Control Program of Cuba.

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KEYWORDS

biological control, entomopathogenic fungi, integrated pest management (IPM), *Metarhizium anisopliae*, *Metarhizium robertsii*, METASAVE-11, PARB clade, species delineation

INTRODUCTION

Entomopathogenic fungi of the genus *Metarhizium* Sorokin (Hypocreales; Clavicipitaceae) are used globally for biological insect control and act as regulators of insect populations in nature (Li et al. 2010). They are pathogenic to a wide range of arthropod pests and can occupy a great diversity of ecological niches (Li et al. 2010; Pattemore et al. 2014). The entomopathogenic and biocontrol potential of *Metarhizium* fungi has been used in the development of successful biopesticides that combine high effectiveness with a low environmental impact on the beneficial invertebrate fauna (Zimmermann 2007; Souza et al. 2014; Tóthné Bogdányi et al., 2019).

Traditionally, the identification of *Metarhizium* is based on phenotypic traits such as conidial morphology and dimensions, the structure of conidiophores or phyalides and biochemical methods as, for example extracellular protein profiles. However, cultural characteristics can be variable, depending on cultivation conditions and the importance of genotypic traits for the determination of genus boundaries and species delineation has been stressed (Driver et al. 2000). In particular, a multilocus sequence analysis (MLSA) scheme comprising as taxonomic markers partial sequences of the genes encoding translation elongation factor 1-alpha (*ef1a*), DNA-dependent RNA polymerase II subunit 1 (*rpb1*), and DNA-dependent RNA polymerase II subunit 2 (*rpb2*) together with an intron rich partial sequence of the *ef1a* gene, termed 5TEF, has been introduced (Bischoff et al. 2006, 2009) and employed extensively to corroborate or redefine species delineations (Kepler et al. 2014; Montalva et al. 2016; Rehner & Kepler 2017; Lopes et al. 2018; Gutierrez et al. 2019; Mongkolsamrit et al. 2020). One main result of these molecular studies is the existence of a species complex, termed *Metarhizium anisopliae* sensu *lato,* within the genus *Metarhizium*. This *M. anisopliae* complex is organized around a presumably monophyletic clade, informally termed "PARB" clade with respect to the four distinct *Metarhizium* species—*M. pinghaense*. *Metarhizium anisopliae (*sensu stricto), *M. robertsii* and *M. brunneum*—classically comprising therein (Rehner & Kepler 2017). However, a further new PARB species named *Metarhizium humberi* has been described recently (Luz et al. 2019).

Moreover, in order to provide a diagnostic tool for sequencing-independent species assignment within the PARB clade, a species-specific multiplex-PCR protocol based on a set of previously established intergenic spacer (IGS) sequence markers (Rehner & Kepler 2017) has been introduced (Mayerhofer et al. 2019).

In Cuba, the studies of *Metarhizium* as biological control agents date back to the 1980s. These studies were focused on fungal virulence and the selection of endemic isolates, including strain LBM-11, for the control of economically important pests such as rice water weevils, *Lissorhoptrus brevirostris* Suffrian (Coleoptera: Curculionidae) and *Lissorhoptrus oryzoplhilus* Kuschel (Coleoptera: Curculionidae), the banana weevil, *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae), and spittlebugs, *Monecphora bicinta fraterna* Uhler (Hemiptera: Cercopidae), in different cropping systems (Castiñeiras et al. 1990; Luján et al. 1990). Currently, several fungus-based bio-insecticides are produced in the nation-wide network of plant-laboratory units referred to as CREE (Centros de Reproducción de Entomófagos y Entomopatógenos = Entomophage and Entomopathogen Reproduction Centers) as part of integrated pest management (IPM) programmes for relevant agricultural pests, with isolate LBM-11 being the active ingredient of the bio-insecticide product METASAVE-11. However, few advances have been made in the molecular characterization of biocontrol isolates and the selection and evaluation of fungal isolates for the control of a more diversified pest spectrum to satisfy the demand for biopesticides in Cuban agriculture.

The sweet potato weevil, *Cylas formicarius* Fabricius (Coleoptera: Brentidae), is the economically most important insect pest in sweet potato cultivation in Cuba and numerous further countries worldwide (Gurr et al. 2016). The sweet potato constitutes an important food in the diet of the Cuban population; every year approximately 6300 ha are cultivated. Currently, apart from chemical insecticides, *C. formicarius* is controlled with a Cuban commercial product based on the entomopathogenic fungus *Beauveria bassiana*, with pheromones, with the ant *Pheidole megacephala* and with entomopathogenic nematodes; however, these control methods together are not sufficient to satisfy growers' demand. This situation has contributed to foster screenings for new fungal isolates with activity against a wide spectrum of arthropod pests that hold potential for the development of innovative bio-insecticides.

It has been the objective of the present study to characterize at a molecular-taxonomic level fungal isolates from Cuba belonging to the micro-organism culture collection of the Plant Health Research Institute (INISAV) and to evaluate the fungal biocontrol potential with respect to the sweet potato weevil.

MATERIALS AND METHODS

Fungal isolates

Six fungal isolates from the INISAV culture collection of micro-organism were used in this study that stem from insect and soil samples from different environments in Cuba (Table 1). Isolates were recovered from sterile mineral oil preservation and grown on Sabouraud dextrose agar (SDA) at 26°C for approximately 15 days. Single spore cultures were prepared using the method described by Inglis et al. (2012).

Morphological and cultural characterization of fungal isolates

For morphological and cultural characterization, a conidial suspension at 10^7 conidia per ml was prepared for each fungal isolate. Fungal suspension (0.1 ml) was spread on a Petri dish with SDA culture media. Petri dishes were incubated in the dark at 26°C for 72 h. A mycelium disc of 5 mm diameter from 3 days old cultures were transferred to the centre of the 9 cm Petri dish containing potato dextrose agar (PDA). Five replicates were prepared for each isolate and incubated under the conditions described above. The growth of the isolates was observed from 72 h to 14 days. Cultural characteristics of fungal growth as colony diameter, colour, border and texture were noted.

Conidial morphology was described, and size was measured (length × width) in a phase-contrast microscope. Fifty conidia were measured for each isolate and the minimum and maximum values were determined according to Fernandes et al. (2010).

Metarhizium was identified according to the taxonomic descriptions by Driver et al. (2000) and Bischoff et al. (2009).

Fungal mycelial growth and conidial production on different culture media

The radial growth of colonies in different culture media was determined according to the method of Onofre et al. (2001). The culture media used were: SDA, PDA, malt extract agar (MEA), Czapek-Dox Agar (CDA) and complete medium (CM) (0.4 g l^{-1} KH_2PO_4, 1.4 g l^{-1} Na_2HPO_4, 0.6 g l^{-1} $MgSO_4$, 1.0 g l^{-1} KCl, 0.7 g l^{-1} NH_4NO_3, 10 g l^{-1} glucose, 15 g l^{-1} bacteriologic agar, 5 g l^{-1} yeast extract). Colony diameters were measured for up to 14 days. The cultural characteristics of the isolates were described, and the production of conidia was calculated according to the procedure of Paccola-Meirelles and Azevedo (1990). In brief, conidia were removed from plates with 5 ml of distilled water and 0.01% of Tween 80. The number of conidia per ml was determined by serial dilution and Neubauer chamber counting.

For both experiments, five replicates per treatment were performed. Data of the growth and conidial production of the isolates in different culture media were processed by means of the simple classification variance analysis (ANOVA) using the Stat Soft program (6.0). In case of significant differences, the Tukey comparison test was applied ($p \leq 0.05$).

DNA extraction, PCR amplification and sequencing

For DNA extraction, fungal isolates were grown on YPG agar (*2* g l^{-1} yeast extract, 10 g l^{-1} peptone, 20 g l^{-1} glucose) containing 34 μg ml^{-1} chloramphenicol at 25°C. Approximately 100 mg of mycelium was transferred to a screw-capped 2 ml microcentrifuge tube containing Lysing Matrix C (MP Biomedicals). Samples were frozen in liquid nitrogen and processed for up to 1 min at 6 m s^{-1} in a Fastprep 24 homogenizer (MP Biomedicals). DNA from homogenized samples was further extracted using the DNeasy Plant kit (Qiagen) according to the standard protocol as provided by the manufacturer. Purified DNA was finally eluted in 100 μl elution buffer (10 mM Tris–Cl, pH 8.5).

For DNA sequencing purposes, the following markers were amplified from fungal DNA samples using standard Taq DNA polymerase (New England Biolabs) with the PCR primers and conditions indicated in Table 2: internal partial sequences of the *ef1a* gene (primer pair EF1A-983F/EF1A-2218R), the *rpb1* gene (RPB1Af/

TABLE 1 Fungal isolates from the INISAV culture collection used in this study

Isolates	Source	Geographic origin
LBM-5	Hemipteran insect. Unknown host	Cuba
LBM-10	Hemipteran insect. Unknown host	Cuba
LBM-11	*Mocis latipes* Guenée (Lepidoptera: Erebidae)	Cuba, Havana
LBM-12	*Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae)	Cuba
LBM-146	Leaf hopper (Hemiptera: Cicadellidae)	Cuba
LBM-267	Leaf hopper (Hemiptera: Cicadellidae)	Cuba

TABLE 2 Oligonucleotide primers and reaction-specific PCR parameters used in this study

Primer designation	Primer sequence	Annealing temperature (°C)	Elongation time (s)	Reference
EF1T	5′-ATGGGTAAGGARGACAAGAC	64	60	Bischoff et al. (2006)
EF2T	5′-GGAAGTACCAGTGATCATGTT			
EF1A-983F	5′-GCYCCYGGHCAYCGTGAYTTYAT	52	120	Rehner and Buckley (2005)
EF1A-2218R	5′-ATGACACCRACRGCRACRGTYTG			
EF1A-1567R	5′-ACHGTRCCRATACCACCSATCTT	Sequencing primer		
RPB1Af	5′-GARTGYCCDGGDCAYTTYGG	52	120	Stiller and Hall (1997)
RPB1Cr	5′-CCNGCDATNTCRTTRTCCATRTA			
RPB2-5f	5′-GAYGAYMGWGATCAYTTYGG	58	120	Liu et al. (1999)
RPB2-7r	5′-CCCATRGCTTGYTTRCCCAT			
RPB2-6f	5′-TGGGGKWTGGTYTGYCCTGC	Sequencing primers		Goetsch et al. (2005)
RPB2-6r	5′-GCAGGRCARACCAWMCCCCA			

RPB1Cr) and the *rpb2* gene (RPB2-5f/RPB2-7r) as well as the 5TEF region of gene *ef1a* (EF1T/EF2T). The generalized PCR protocol employed for marker amplification consisted of one initial denaturation step of 95°C for 2 min, 35 cycles of 45 s at 95°C, 45 s at the primer specific annealing temperature, and a 68°C elongation step of amplicon specific time; followed by a 5 min final elongation step (Table 2). PCR product size was checked by agarose gel electrophoresis, and PCR products were purified using the Qiaquick PCR purification kit (Qiagen). Sanger sequencing of PCR products was performed by StarSEQ using respective PCR primers and the additional internal sequencing primers indicated in Table 2. Raw sequence data were combined into a single consensus sequence for each fungal isolate and marker using version 6 of the MEGA software package (Tamura et al. 2013).

Phylogenetic reconstruction

For molecular taxonomic identification of the isolates under study, the MLSA scheme introduced by Bischoff et al. (2009) has been used. Reference sequences employed for phylogenetic reconstruction are identified in Table S1. Nucleotide sequences of the four markers were aligned using the CLUSTAL W function (Thompson et al. 1994) as implemented in the MEGA 6 software package. For comprehensive analysis of protein-encoding sequences, a concatenation of the *ef1a, rpb1* and *rpb2* marker sequences was aligned as well. Pairwise sequence similarity percentages were assessed from p-distance matrices calculated in MEGA 6 from unfiltered nucleotide sequence data under pairwise deletion of alignment gaps and missing data. Phylogenies were reconstructed using a p-distance matrix-based Neighbour-joining (NJ) method as implemented in MEGA 6. Tree topology confidence limits were explored in non-parametric bootstrap analyses over 1000 pseudo-replicates.

Virulence bioassays against *C. formicarius*

For in vivo assays, adults of the sweet potato weevil were reared in the Arthropod Laboratory at INISAV. Insects were fed with sweet potato and kept in glass containers at 25 °C.

To evaluate the pathogenic effect of fungal isolates on adults of *C. formicarius*, a suspension of each isolate to be tested was prepared at concentrations of 1×10^6, 5×10^6, 1×10^7, 5×10^7 and 1×10^8 conidia per ml. The concentration was corroborated using microscope and Neubauer chamber counting. Each treatment consisted of 50 adult insects that were immersed in a conidial suspension of each fungus for 30 s. For control treatments, an equal number of insects were dipped in sterile water. All treatment solutions contained 0.01% Tween 80. The insects were placed in a Petri dish and were fed with sweet potato. Mortality was recorded daily for a period of up to 21 days and mortality percentage was calculated using the formula given by Abbott (1925). For each concentration, lethal times (TL_{50} and TL_{90}) were calculated from the regressions between accumulated mortality and time. Lethal concentrations (CL_{50} and CL_{90}) were derived from regressions between the probit of mortality and the logarithm of the concentration. All analyses were done using the statistics program StatSoft (6.0). Dead insects were removed from the container and transferred to a wet chamber to check for fungal presence. Insects showing signs of mycosis were observed

under a microscope and the fungus was re-isolated from the dead insects.

RESULTS

Morphological and cultural characterization of fungal isolates

The fungal isolates studied had the following cultural and morphological features: cottony colonies with a white mycelial margin, with clumps of conidiophores which become coloured with the development of conidia varying from olive green to yellow green or dark green. Some of the isolates produced layers of conidia breaking off as crust, others showed overall only poor conidial production or formed halos or concentric rings of conidia. The colony reverse was colourless or honey-coloured, and several isolates produced yellow pigment diffusing into the medium (Figure 1).

The conidial shape was cylindrical to ellipsoidal and conidia sizes were similar and mostly overlapping between different isolates (Table 3). However, it is possible to subdivide fungal isolates into three groups according to conidial dimensions: (i) conidia of isolates LBM-5, LBM-10, LBM-12 and LBM-267 had dimensions (4.88–9.76 × 2.77 μm) corresponding to those measured by Bischoff et al. (2009) for *Metarhizium* strains belonging to PARB clade species whereas (ii) conidia of isolate LBM-11 were more oblong in shape (10–14 × 3.10 μm) approaching conidial dimensions typically measured for the species *Metarhizium majus* and (iii) those of isolate LBM-146 appeared shorter in average (2.44–7.32 × 2.68 μm).

Phenotypic differences in colony appearance were observed for all isolates in different culture media (Figure 2). In CDA the fungi produced more mycelial growth than conidial production and the colony diameters were smaller, while in other cultures more abundant conidia in variable green colours were produced. Individual isolates showed visible cultural variability in colony colours and conidial production capacity when cultivated on different nutritional sources.

Fungal mycelial growth and conidial production on different culture media

Culture media composition influenced fungal growth, colony characteristics and the production of conidia. All isolates studied were able to grow in the culture media evaluated.

Fungal growth was more favourable in MEA, CM and SDA. Independent of the culture medium, isolate LBM-11 grew faster than the other isolates and had the highest value of fungal growth in CM (5.4 cm) and SDA (5.38 cm). Isolate LBM-267, in contrast, displayed slow to intermediate growth on all culture media tested. The culture medium less favourable for fungal growth was CDA

TABLE 3 Conidial size and shape for Cuban fungal isolates

Isolate	Conidia size[a]	Conidia shape[b]
LBM-5	4.88–7.32 (5.94) × 2.44–4.88 (2.77)	C to E
LBM-10	4.88–9.76 (7.07) × 2.44–4.88 (2.77)	C to E
LBM-11	10.0–14.0 (12.0) × 1.80–4.50 (3.10)	C
LBM-12	4.88–7.32 (5.53) × 2.44–4.88 (2.77)	C
LBM-146	2.44–7.32 (4.11) × 2.44–3.66 (2.68)	C to E
LBM-267	4.88–7.32 (5.77) × 2.44–4.88 (2.77)	C

Note: Average values are indicated in brackets.

[a]Cylindrical (C) or ellipsoidal (E).

[b]All measurements in μm, length × width.

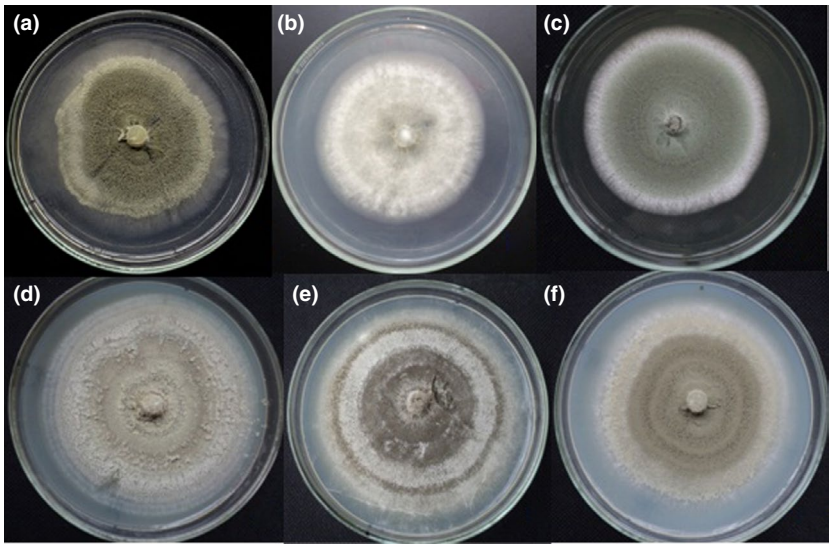

FIGURE 1 Cultural characteristics of fungal isolates on PDA plates after 14 days of growth at 26 ± 1°C. (a) LBM-5, (b) LBM-10, (c) LBM-11, (d) LBM-12, (e) LBM-146, (f) LBM-267

where colony diameters ranged from 2.43 cm to 3.52 cm (Table 4).

In general, the conidial production was higher in CM and SDA as opposed to PDA and CDA medium with lower values for all fungi evaluated (Table 5). Isolate LBM-267 produced the highest number of conidia in SDA medium and produced abundant conidia in CM and MEA. The lowest number of conidia was produced by isolate LBM-11.

Molecular taxonomy

For all fungal isolates investigated, consistent consensus sequences were obtained for the four molecular taxonomic markers used. Consensus sequences have been submitted to the Genbank database under accession numbers MZ292434–MZ292451 (*ef1a, rpb1* and *rpb2*)

TABLE 4 Colony diameters of fungal isolates in different culture media

	Colony diameters of isolates in different culture media				
Isolates	**SDA**	**PDA**	**CM**	**MEA**	**CDA**
LBM-5	5.17^{bc}	4.80^{a}	4.97^{c}	4.73^{bc}	2.67^{a}
LBM-10	5.22^{bc}	4.98^{b}	5.12^{cd}	4.82^{c}	2.76^{a}
LBM-11	5.38^{c}	5.10^{b}	5.4^{d}	4.86^{c}	3.52^{c}
LBM-12	3.30^{a}	4.90^{ab}	3.93^{ab}	4.47^{abc}	2.83^{a}
LBM-146	4.13^{b}	4.57^{a}	3.53^{a}	3.90^{a}	3.03^{bc}
LBM-267	4.27^{b}	4.60^{a}	4.07^{bc}	4.10^{ab}	2.43^{a}

Note: Same letters in columns mean insignificant differences in these variants.

Tukey test $p < 0.05$.

Abbreviations: CDA, Czapek-Dox agar; CM, Complete Medium; PDA, Potato dextrose agar; SDA, sabouraud dextrose agar, MEA: malt extract agar.

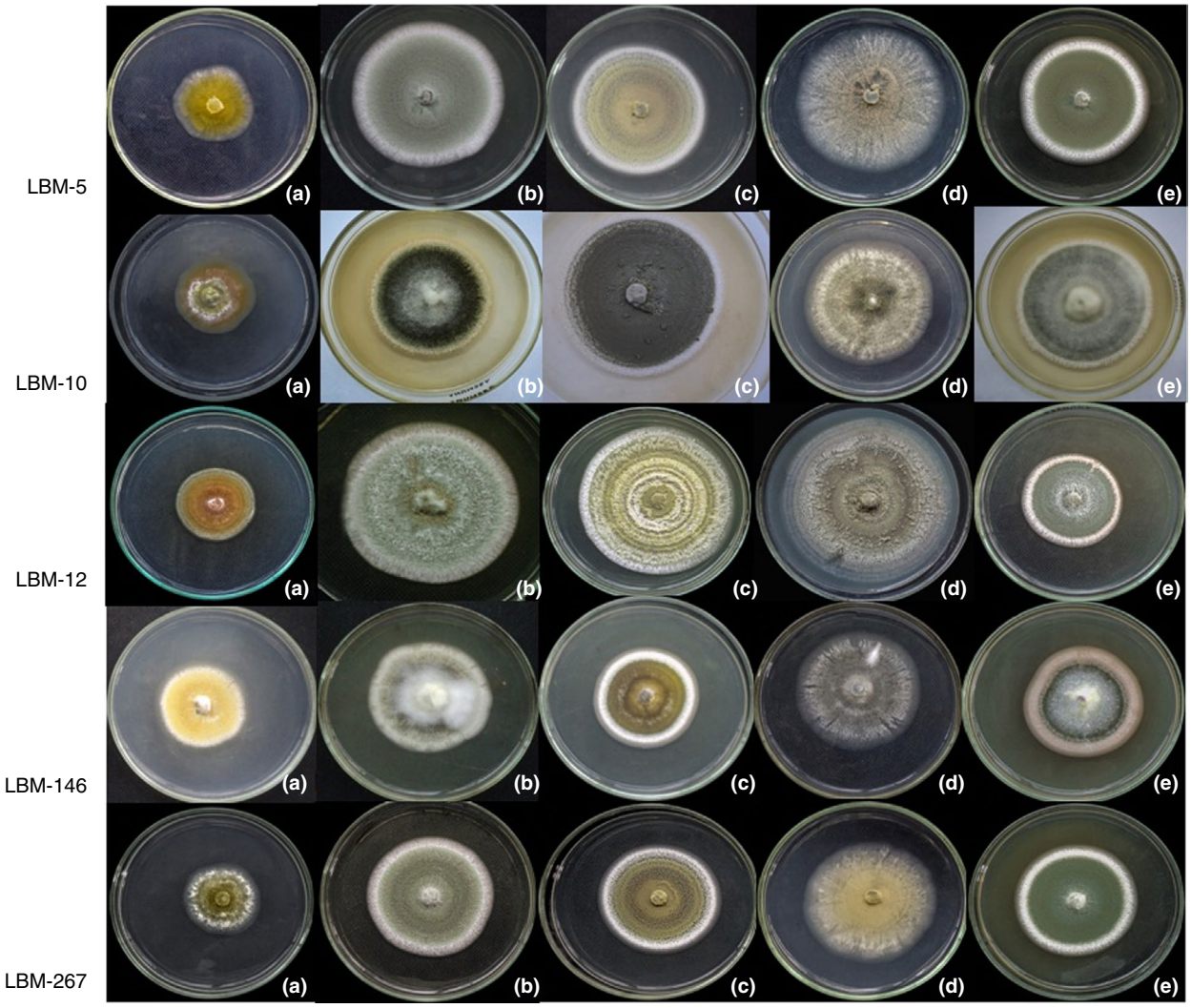

FIGURE 2 Cultural variability of fungal isolates LBM-5, LBM-10, LBM-12, LBM-146 and LBM-267 grown for 14 days at 26 ± 1°C on different culture media. (a) Czapek-dox agar; (b) malt extract agar; (c) complete medium; (d) potato dextrose agar; (e) Sabouraud dextrose agar

and MZ329397–MZ329402 (5TEF). Comparisons with reference sequences gave rise to alignments comprising in length 882 bp (*ef1a*), 522 bp (*rpb1*), 978 bp (*rpb2*), and 675 bp (5TEF), respectively. Pairwise sequence similarities for concatenated *ef1a, rpb1* and *rpb2* sequences ranged from 99.3% to 99.9% for the six Cuban isolates, that is the isolates were all different with a moderate level of diversity.

In a phylogenetic tree reconstructed from an alignment of concatenated *ef1a, rpb1* and *rpb2* marker sequences comprising all *Metarhizium* species distinguished in the most recent systematic review of the genus (Mongkolsamrit et al. 2020) the six Cuban isolates were located in the *M. anisopliae* sensu *lato* complex and, within the latter, in the PARB clade (Figure S1). Whereas strain LBM-146 clustered with several reference sequences representing the species *M. robertsii*, the remaining five isolates appeared all together most closely related to a single isolate assigned to the recently introduced species *M. humberi* (Figure 3). However, reference sequences representing the species *M. anisopliae (*sensu stricto*)* and *M. humberi* were not delineated to different clades in this phylogeny, and bootstrap support values for both the PARB clade itself (76%) and the presumed PARB species within were far from sufficient to support conclusive species assignments.

In the phylogenetic tree of the PARB species reconstructed from an alignment of 5TEF sequences (Figure 4), Cuban isolate LBM-146 clustered as before with the *M. robertsii* reference strains, now with 96% bootstrap support, whereas isolates LBM-5, LBM-10, LBM-11, LBM-12 and LBM-267 were located within an 85% bootstrap supported clade comprising uniquely *M. anisopliae (*sensu stricto) reference strains. Importantly, all *M. humberi* reference strains formed a separate clade in a sister position to *M. anisopliae*. Within the *M. anisopliae* clade, Cuban isolates formed two different sub-clades comprising only LBM-10 and LBM-11, on the one hand, and LBM-5, LBM-12 and LBM-267 together with several reference strains, on the other hand, 5TEF sequences were identical for all strains comprised in one of these sub-clades.

TABLE 5 Numbers of conidia (10^5 conidia per ml) of isolates in the culture media evaluated

	Conidia concentration (10^5 conidia per ml) of isolates in the culture media				
Isolates	**SDA**	**PDA**	**CM**	**MEA**	**CDA**
LBM-5	466.2^{c}	36.7^{a}	251.2^{b}	350.4^{bc}	15.4^{a}
LBM-10	126.3^{b}	13.8^{a}	300.6^{c}	166.7^{b}	31.7^{a}
LBM-11	141.3^{b}	3.75^{a}	302.7^{c}	202.2^{b}	29.6^{a}
LBM-12	76.9^{ab}	4.58^{a}	298.3^{c}	151.3^{b}	23.8^{ab}
LBM-146	134.3^{a}	61.7^{c}	187^{d}	107.5^{a}	157.5^{b}
LBM-267	568.8^{c}	37.9^{a}	326.3^{b}	379.6^{b}	5.4^{a}

Note: Same letters in rows mean no significant differences in these variants. Tukey test $p < 0.05$.

Virulence bioassays against *C. formicarius*

The virulence of Cuban *Metarhizium* isolates against adults of the sweet potato weevil, *C. formicarius,* was determined in laboratory assays. All isolates tested were pathogenic for the test insect with mortalities ranging from 10% to 100% at 14 days postinoculation. At a concentration of

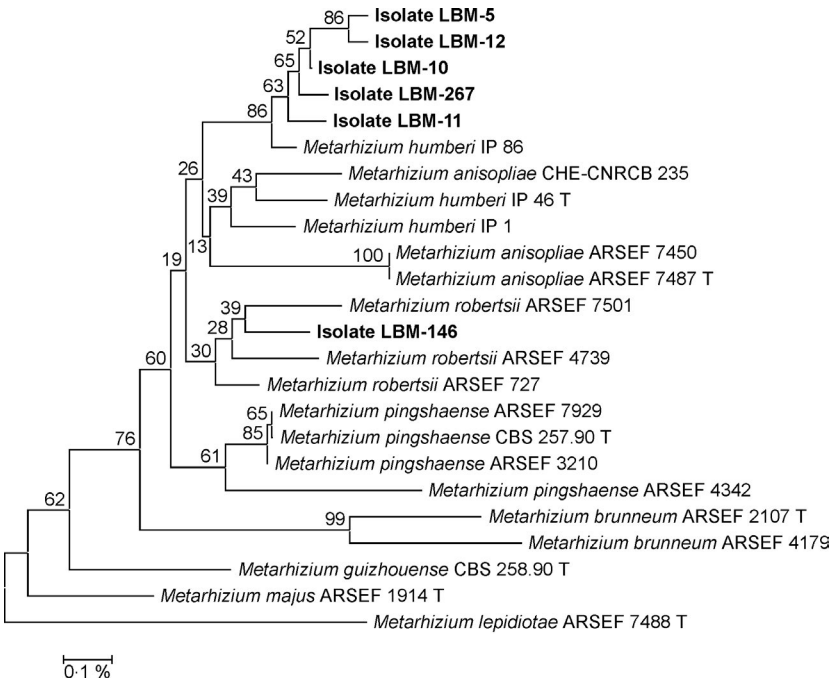

FIGURE 3 Neighbour-joining (NJ) phylogeny of *Metarhizium* fungi comprised by the PARB clade as reconstructed from concatenated *ef1a, rpb1 and rpb2* nucleotide sequences. Terminal branches are labelled by genus, species and strain designations; "T" following a strain designation denotes a specific type of strain. Fungal isolates from Cuba are displayed in bold type. Numbers on branches indicate bootstrap support values. The size bar indicates a branch length corresponding to 0.1% sequence divergence. A concatenation of orthologous sequences from the non-PARB species *Metarhizium lepidiotae* has been used as outgroup

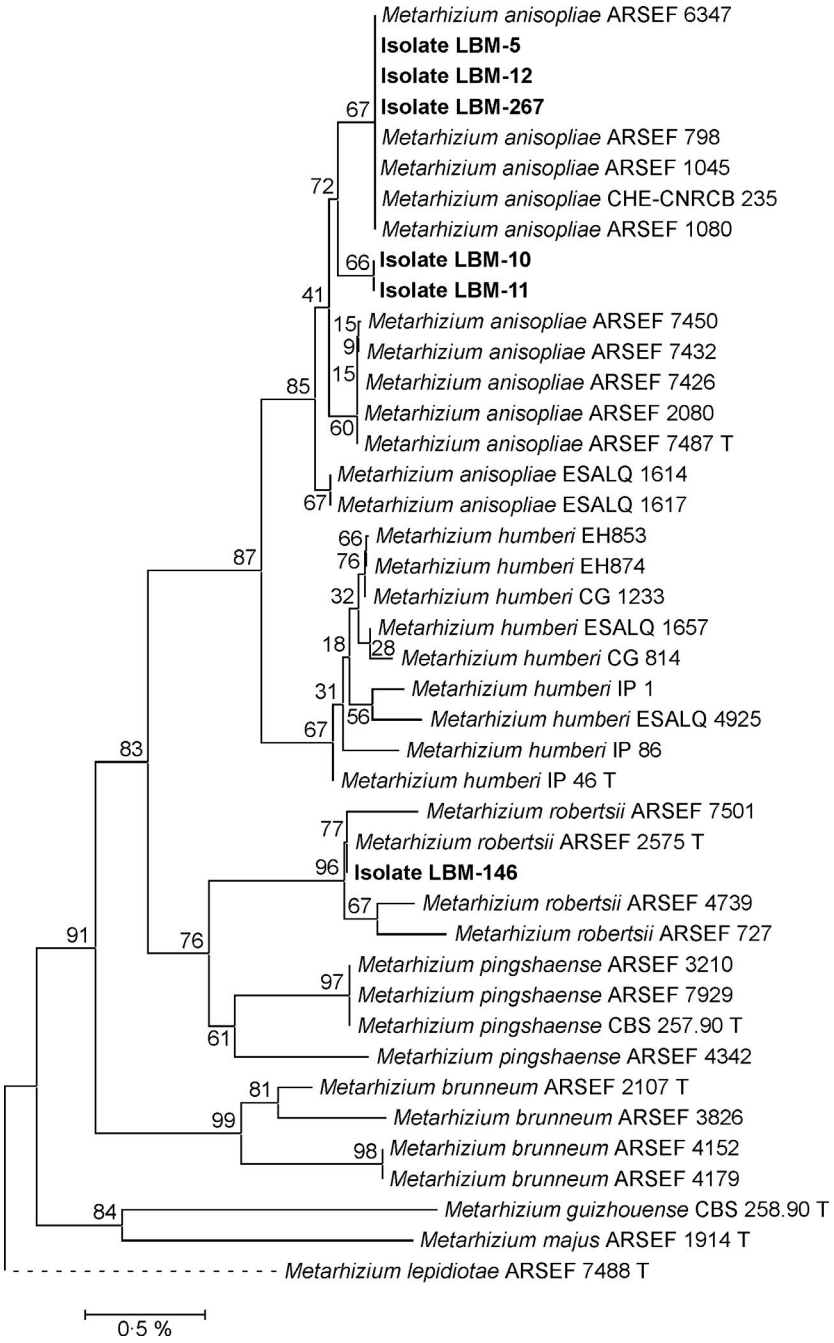

FIGURE 4 Neighbour-joining (NJ) phylogeny of *Metarhizium* fungi comprised by the PARB clade as reconstructed from 5TEF nucleotide sequences. Terminal branches are labelled by genus, species and strain designations; "T" following a strain designation denotes a specific type of strain. Fungal isolates from Cuba are displayed in bold type. Numbers on branches indicate bootstrap support values. The size bar indicates a branch length corresponding to 0.5% sequence divergence. A concatenation of orthologous sequences from the non-PARB species *Metarhizium lepidiotae* has been used as outgroup

1×10^6 conidia per ml the mortalities were low, ranging from 10% to 40%. The most virulent isolate was LBM-267 with 100% mortality at a concentration of 5×10^7 conidia per ml at 7 days postinoculation, with LT_{50} values ranging from 3.83 to 4.58 days, LT_{90} values between 6.18 and 7.34 days (Table 6) and a LC_{50} value of 2.7×10^6 conidia per ml (Table 7). Both LBM-267 and LBM-11 had the lowest LC_{90} value (1.1×10^7 conidia per ml), whereas the lowest LC_{50} value (1.8×10^6 conidia per ml) was determined for isolate LBM-5 (Table 7).

Rich fungal colonization of the insect body was visible after approximately 7 days of wet chamber incubation of dead test insects (Figure 5).

DISCUSSION

Morphological characteristics observed with Cuban isolates broadly corresponded to descriptions by Bischoff et al. (2009) and Fernandes et al. (2010) for fungi of the PARB clade within the *M. anisopliae* species complex, with the notable exception of isolate LBM-11 that produced large oblong conidia (with average measures 12.0 μm × 3.10 μm) more similar in dimensions to conidia of *M. majus* fungi (typical range: 8.5–14.5 μm × 2.5–5.0 μm) within the MGT clade of *M. anisopliae* sensu *lato*. Although these descriptions are useful tools for approximate isolate

TABLE 6 Susceptibility of *Cylas formicarius* to fungal isolates

Isolates	Concentration (conidia ml^{-1})	% Mortality (7 days)	% Mortality (14 days)	LT_{50} (days)	LT_{90} (days)	Regression equation
LBM-5	1×10^7	90	100	7.94	13.0	$y = 7.9x–12.7$
	5×10^7	100	—	5.09	5.09	$y = 13.3x–24.3$
	1×10^8	100	—	1.56	4.84	$y = 12.2x + 31.0$
LBM-10	1×10^7	64	82	7.88	15.0	$y = 5.63x + 5.64$
	5×10^7	66	100	6.91	12.4	$y = 7.35x–0.80$
	1×10^8	98	100	4.41	6.91	$y = 16.0x–20.6$
LBM-11	1×10^7	50	100	7.2	11.0	$y = 0.55x + 0.26$
	5×10^7	96	100	6.2	9.2	$y = 0.50x + 0.02$
	1×10^8	98	100	3.8	6.8	$y = 0.37x–1.27$
LBM-12	1×10^7	70	80	7.68	12.6	$y = 8.06x–11.9$
	5×10^7	80	100	7.38	12.8	$y = 7.41x–4.65$
	1×10^8	100	—	2.08	5.75	$y = 10.9x + 27.3$
LBM-146	1×10^7	76.7	96	5.75	12.0	$y = 6.37x + 13.4$
	5×10^7	100	—	3.34	5.06	$y = 23.3x–28.0$
	1×10^8	100	—	1.75	4.01	$y = 17.7x + 19.0$
LBM-267	1×10^7	90	93,3	3.83	7.34	$y = 11.4x + 6.3$
	5×10^7	100	—	4.58	8.75	$y = 9.59x + 6.1$
	1×10^8	100	—	3.46	6.18	$y = 14.7x + 0.9$

TABLE 7 Lethal concentrations of fungal isolates against *Cylas formicarius*

Isolates	LC_{50} (conidia ml^{-1})	LC_{90} (conidia ml^{-1})	Regression equation	Determination coefficient (R^2)
LBM-5	1.8×10^6	4.4×10^7	$y = 3.4x–19.7$	0.58 ns
LBM-10	4.2×10^6	3.5×10^7	$y = 1.41x–4.34$	0.987**
LBM-11	2.7×10^6	1.1×10^7	$y = 2.1x–8.5$	0.98**
LBM-12	3.5×10^6	1.3×10^7	$y = 2.2x–9.4$	0.63 ns
LBM-146	4.4×10^6	1.2×10^7	$y = 2.8x–13,6$	0.95**
LBM-267	2.7×10^6	1.1×10^7	$y = 2.1x–8.5$	0.83*

Note: "ns" indicates insignificant differences.

"*" and "**" denote significance at the 5 % and 1 % significance level, respectively.

identification, sound species-level assignment within the *M. anisopliae* complex, and especially within the PARB clade, is generally not possible on a morphological basis, with the notable exception of the species *Metarhizium globosum* that is recognized by its globose conidial shape (Bischoff et al. 2009).

Cultural characteristics of all isolates studied were in broad agreement with those expected for colonies belonging to the *M. anisopliae* complex. These are white during the early conidial development (typically 4–7 days), later turn yellow and dark green with conidial maturation (10–14 days). In this sense, Bischoff et al. (2009) suggested that the morphology of the *M. anisopliae* species complex provides information for a limited diagnosis.

Molecular taxonomic characterization demonstrated that the fungal isolates from Cuba were all genetically different from each other and belonged to a group of comparatively closely related fungal species informally referred to as the PARB clade within the *M. anisopliae* species complex. The degree of similarity of the sets of selected marker genes — originally ribosomal RNA gene sequences Stackebrandt & Goebel (1994) and subsequently sets of protein-encoding markers (Maiden 2006; Sung et al. 2007) — has become the de facto criterion for species delineation and identification in the microbiology of asexual organisms as bacteria or mitosporic fungi to which the classical species concept developed for higher organisms does not apply. The set of MLSA

FIGURE 5 Sweet potato weevils, *C. formicarius,* with conidia of isolates LBM-5 (left and mid) and LBM-267 (right) growing out from the cadaver 7 days postmortality under laboratory conditions

marker genes *ef1a, rpb1* and *rpb2* used here is currently accepted for phylogenetic and molecular taxonomic studies of the genus *Metarhizium* (Bischoff et al. 2009; Kepler et al. 2014; Rehner & Kepler 2017; Mongkolsamrit et al. 2020). However, species delineation within the PARB clade and, in particular, differentiation of the species *M. anisopliae* sensu stricto and *M. humberi* was insufficient using the combined MLSA sequence data. In a previous study comprising the same isolates, the use of ribosomal RNA operon internal transcribed spacer (ITS) sequences gave rise to similarly insufficient species delineation (Gato et al., 2017b). Moreover, a diagnostic multiplex PCR approach developed to facilitate species-level identification within the PARB clade (Mayerhofer et al. 2019) was not helpful in the present context as it has to date not been extended to the recently introduced species *M. humberi*. However, better species delineation, especially with respect to *M. anisopliae* and *M. humberi*, has been obtained using the 5TEF marker sequence that had previously been shown to provide a better resolution for the species in question (Luz et al. 2019). Based on the results obtained with the 5TEF marker, Cuban isolates LBM-5, LBM-10, LBM-11, LBM-12 and LBM-267 were assigned to the taxonomic species *M. anisopliae* sensu stricto, whereas isolate LBM-146 was identified as a *M. robertsii* strain. These assignments coincided only partly with the conidial dimension-based characterization: in particular, close clustering of LBM-10 and LBM-11 in the 5TEF marker-based phylogeny did not appear to reflect the extreme differences in shape between conidia of these two isolates.

The nutrient composition of culture media, the carbon and nitrogen sources, C/N proportion and mineral salts are important factors for fungal growth-regulating germ tube germination, hyphal growth and conidial production, that is significant parameters to be considered for isolate selection for mass production (Zimmerman 2007; Obando et al. 2013). The cultivation on different media showed differences among isolates, with the best results for fungal conidial production being obtained in SDA, MEA and complete media. Similar results were found by Onofre et al. (2001), while Ruiz-Sanchez et al. (2011) reported high growth rates in SDA for five *Metarhizium* isolates. However, the results underline the importance to evaluate different substrates for fungal commercial production.

The pathogenic activity of Cuban *Metarhizium* isolates against the target insect *C. formicarius* demonstrated good potential as biological control agent. *Metarhizium anisopliae* isolate LBM-267 has been retained as a promising candidate for biocontrol agent development because of its high virulence against *C. formicarius* that, remarkably, is slightly higher than that of the established biocontrol isolate *M. anisopliae* LBM-11. Isolate LBM-267 has previously been demonstrated to secrete hydrolytic enzymes as quitinases, proteases, amylases and caseinases (Gato et al., 2017a) that are supposed to contribute to high virulence of entomopathogenic fungi.

Interestingly, both highly virulent isolates *M. anisopliae* LBM-267 and LBM-11 showed very different growth and conidial production: whereas hyphal growth was fast in LBM-11 and comparatively few conidia were formed, LBM-267 grew slower but produced a much higher quantity of equally virulent conidia. These differences might express a different evolutionary adaptation at the level of host relationships and the switch between saprophytic and pathogenic lifestyles. Both fungal strains have been isolated from different hosts, LBM-11 from a lepidopteran insect and LBM-267 from a hemipteran insect (Table 1), and clustered to different sub-clades within the *M. anisopliae* clade in the 5TEF phylogeny (Figure 4).

Under an application-oriented perspective, *M. anisopliae* LBM-267 displayed appropriate characteristics of conidial production and growth in different culture media, which are considered important parameters for isolate selection for used in biological pest control. This offers the possibility to evaluate and choose diverse substrates considering economic alternatives for mass production.

Entomopathogenic fungal isolates from Cuba have been microscopically, morphologically and molecularly characterized and assigned to the taxonomic species

M. anisopliae sensu stricto and *Metarhizium robertsii*. Virulence assessment with respect to the sweet potato weevil, *C. formicarius*, that is an economically important agricultural pest in Cuba, have led to the identification of two *M. anisopliae* isolates holding biocontrol potential. One of both, isolate LBM-11, has previously been developed into the bio-insecticide METASAVE-11 that is widely used to control rice water weevils, the banana weevil, the fall armyworm and several species of thrips in Cuba. The second isolate, LBM-267, has not previously been employed in biocontrol but has been found at least as virulent against *C. formicarius* as LBM-11. Its growth and conidial production capacities on different nutritional media will facilitate economically feasible bio-insecticide development.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the Genbank database under accession numbers MZ292434 - MZ292451 and MZ329397 - MZ329402. Genbank accession numbers of reference sequences are available in the supplementary material of this article, Suppl. Table S1. Fungal isolates described are available upon reasonable request from the INISAV culture collection (http://www.inisav.cu) or the first author.

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REFERENCES

Abbott, W.S. (1925) A method for computing the effectiveness of an insecticide. *Journal of Economic Entomology*, 18, 265–267.

Bischoff, J.F., Rehner, S.A. & Humber, R.A. (2009) A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia*, 10, 1512–1530.

Bischoff, J.F., Rehner, S.A. & Humber, R.A. (2006) *Metarhizium frigidum* sp. nov.: a cryptic species of *M. anisopliae* and a member of the *M. flavoviride* complex. *Mycologia*, 98, 737–745.

Castiñeiras, A., López, M., Calderón, A., Cabrera, T. & Luján, M. (1990) Virulencia de 17 aislamientos de *Beauveria bassiana* y 11 de *Metarhizium anisopliae* sobre adultos de *Cosmopolites sordidus*. *Ciencia y Técnica en la Agricultura, Protección de Plantas*, 13, 45–51.

Driver, F., Milner, R.J. & Trueman, J.W. (2000) A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. *Mycological Research*, 104, 134–150.

Fernandes, E.K., Keyser, C.A., Chong, J.P., Rangel, D.E., Miller, M.P. & Roberts, D.W. (2010) Characterization of *Metarhizium* species and varieties based on molecular analysis, heat tolerance and cold activity. *Journal of Applied Microbiology*, 108, 115–128.

Gato, Y., Márquez, M.E., Baró, Y. & Calle, J. (2017a) Detección de enzimas extracelulares en cepas cubanas del complejo *Metarhizium anisopliae* con acción entomopatogénica contra *Cylas formicarius* Fabricius (Coleoptera: Brentidae). *Actualités Biologiques*, 39, 71–78.

Gato, Y., Schuster, C., Leclerque, A., Márquez, M.E. & Baró, Y. (2017b) Group-I intron based strain-specific diagnosis of entomopathogenic *Metarhizium* fungi from Cuba. *IOBC/Wprs Bulletin*, 129, 47–51.

Goetsch, L., Eckert, A.J. & Hall, B.D. (2005) The molecular systematics of *Rhododendron* (Ericaceae): a phylogeny based upon *RPB2* gene sequences. *Systematic Botany*, 30, 616–626.

Gurr, G.M., Liu, J., Johnson, A.C., Woruba, D.N., Kirchhof, G., Fujinuma, R. et al. (2016) Pests, diseases and crop protection practices in the smallholder sweet potato production system of the highlands of Papua New Guinea. *PeerJ*, 4(12), 1–20.

Gutierrez, A.C., Leclerque, A., Manfrino, R.G., Luz, C., Ferrari, W.A.O., Barneche, J. et al. (2019) Natural occurrence in Argentina of a new fungal pathogen of cockroaches, *Metarhizium argentinense* sp. nov. *Fungal Biology*, 123, 364–372.

Inglis, D., Enkerli, J. & Goettel, M. (2012) Laboratory tecniques used for entomopathogenic fungi: hypocreales. In: Lacey, L.A. (Ed.) *Manual of techniques in invertebrate pathology*, 2nd edition. London: Academic Press, pp. 189–243.

Kepler, R.M., Humber, R.A., Bischoff, J.F. & Rehner, S.A. (2014) Clarification of generic and species boundaries for *Metarhizium* and related fungi through multigene phylogenetics. *Mycologia*, 106(4), 811–829.

Li, Z.Z., Alves, S.B., Roberts, D.W., Fan, M.Z., Delalibera, I. & Tan, J. (2010) Biological control of insects in Brazil and China: history, current programs and reasons for their successesusing entomopathogenic fungi. *Biocontrol Science and Technology*, 20, 117–136.

Liu, Y.J., Whelen, S. & Hall, B.D. (1999) Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. *Molecular Biology and Evolution*, 16, 1799–1808.

Lopes, R.B., Souza, D.A., Rocha, L.F.N., Montalva, C., Luz, C., Humber, R.A. et al. (2018) *Metarhizium alvesii* sp. nov.: a new member of *the Metarhizium anisopliae* species complex. *Journal of Invertebrate Pathology*, 151, 165–168.

Luján, M., Cabrera, T., Vázquez, T. & Sánchez, E. (1990) Metodología para la reproducción de *Metarhizium anisopliae*, virulencia y conservación. Ciencia y Técnica en la Agricultura. *Protección de Plantas*, 13, 43–49.

Luz, C., Rocha, L.F.N., Montalva, C., Souza, D.A., Botelho, A.B.R.Z., Lopes, R.B. et al. (2019) *Metarhizium humberi* sp. nov. (Hypocreales: Clavicipitaceae), a new member of the PARB clade in the *Metarhizium anisopliae* complex from Latin America. *Journal of Invertebrate Pathology*, 166, 107–216.

Maiden, M.C. (2006) Multilocus sequence typing of bacteria. *Annual Review of Microbiology*, 60, 561–588.

Mayerhofer, J., Lutz, A., Dennert, F., Rehner, S.A., Kepler, R.M., Widmer, F. et al. (2019) A species-specific multiplexed PCR amplicon assay for distinguishing between *Metarhizium anisopliae*, *M. brunneum*, *M. pingshaense* and *M. robertsii*. *Journal of Invertebrate Pathology*, 161, 23–28.

Mongkolsamrit, S., Khonsanit, A., Thanakitpipattana, D., Tasanathai, K., Noisripoom, W., Lamlertthon, S. et al. (2020) Revisiting *Metarhizium* and the description of new species from Thailand. *Studies in Mycology*, 95, 171–251.

Montalva, C., Collier, K., Rocha, L.F., Inglis, P.W., Lopes, R.B., Luz, C. et al. (2016) A natural fungal infection of a sylvatic cockroach with *Metarhizium blattodeae* sp. nov., a member of the *M. flavoviride* species complex. *Fungal Biology*, 120, 655–665.

Obando, J., Bustillo, A., Castro, U. & Mesa, N. (2013) Selección de cepas de *Metarhizium anisopliae* para el control de *Aeneolamia varia* (Hemiptera: Cercopidae). *Revista Colombiana de Entomología*, 39, 26–33.

Onofre, S., Miniuk, C., Monteiro, N. & Azevedo, J. (2001) Growth and sporulation of *Metarhizium flavoviride var. flavoviride* on culture media and lighting regimens. *Science in Agriculture*, 58, 613–616.

Paccola-Meirelles, L.D. & Azevedo, J.L. (1990) Variabilidae natural no fungo entomopatogénico *Beauveria bassiana*. *Arquivos de Biologia e Tecnologia*, 33, 657–672.

Pattemore, J.A., Hane, J.K., Williams, A.H., Wilson, B.A., Stodart, B.J. & Ash, G.J. (2014) The genome sequence of the biocontrol fungus *Metarhizium anisopliae* and comparative genomics of *Metarhizium* species. *BMC Genomics*, 15, 660.

Rehner, S.A. & Buckley, E. (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF1-alpha sequences: evidence for cryptic diversification and links to cordyceps teleomorphs. *Mycologia*, 97, 84–98.

Rehner, S.A. & Kepler, R.M. (2017) Species limits, phylogeography and reproductive mode in the *Metarhizium anisopliae* complex. *Journal of Invertebrate Pathology*, 148, 60–66.

Ruiz-Sanchez, E., Chan, C.W., Pérez, G.A., Cristóbal, A.J., Uch, V.B., Tun, S.J. et al. (2011) Crecimiento, esporulación y germinación in vitro de cinco cepas de *Metarhizium* y su virulencia en huevos y ninfas de *Bemisia tabaci*. *Revista mexicana de micología*, 33, 9–15.

Souza, R., Azevedo, R., Lobo, A.O. & Rangel, D.E.N. (2014) Conidial water affinity is an important characteristic for thermotolerance in entomopathogenic fungi. *Biocontrol Science and Technology*, 24, 448–461.

Stackebrandt, E. & Goebel, B.M. (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology*, 44, 846–849.

Stiller, J.W. & Hall, B.D. (1997) The origin of red algae: implications for plastid evolution. *Proceedings of the National Academy of Sciences of the USA*, 94, 4520–4525.

Sung, G.H., Hywel-Jones, N.L., Sung, J.M., Luangsa-Ard, J.J., Shrestha, B. & Spatafora, J.W. (2007) Phylogenetic classification of *cordyceps* and the clavicipitaceous fungi. *Studies in Mycology*, 57, 5–59.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30, 2725–2729.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673–4680.

Tóthné Bogdányi, F., Petrikovszki, R., Balog, A., Putnoky-Csicsó, B., Gódor, A., Bálint, J. et al. (2019) Current knowledge of the entomopathogenic fungal species *Metarhizium flavoviride* Sensu Lato and its potential in sustainable pest control. *Insects*, 10, 385.

Zimmermann, G. (2007) Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology*, 17, 879–920.

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