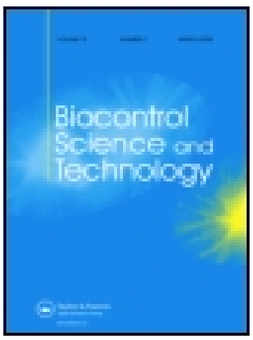

Biocontrol Science and Technology

ISSN: 0958-3157 (Print) 1360-0478 (Online) Journal homepage: https://www.tandfonline.com/loi/cbst20

Identification and molecular characterisation of fifteen tropical isolates of the entomopathogenic fungus *Metarhizium*

R. M. Castro-Vásquez, S. Hernández-Villalobos, M. Montero-Astúa, A. Vargas-Martínez, A. González-Herrera & R. Molina-Bravo

To cite this article: R. M. Castro-Vásquez, S. Hernández-Villalobos, M. Montero-Astúa, A. Vargas-Martínez, A. González-Herrera & R. Molina-Bravo (2020): Identification and molecular characterisation of fifteen tropical isolates of the entomopathogenic fungus *Metarhizium*, Biocontrol Science and Technology, DOI: 10.1080/09583157.2020.1732296

To link to this article: https://doi.org/10.1080/09583157.2020.1732296

Published online: 26 Feb 2020.

Submit your article to this journal

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=cbst20

BIOCONTROL SCIENCE AND TECHNOLOGY
https://doi.org/10.1080/09583157.2020.1732296

Taylor & Francis
Taylor & Francis Group

SHORT COMMUNICATION

Check for updates

Identification and molecular characterisation of fifteen tropical isolates of the entomopathogenic fungus *Metarhizium*

R. M. Castro-Vásquez[a,b], S. Hernández-Villalobos[b], M. Montero-Astúa[a,c], A. Vargas-Martínez[b], A. González-Herrera[b] and R. Molina-Bravo[b]

[a]Centro de Investigación en Biología Celular y Molecular, Universidad de Costa Rica, San José, Costa Rica; [b]Escuela de Ciencias Agrarias, Universidad Nacional, Heredia, Costa Rica; [c]Escuela de Agronomía, Universidad de Costa Rica, San José, Costa Rica

ABSTRACT

Twelve *Metarhizium* isolates from Costa Rica, two from Honduras and one from Nicaragua were characterised using molecular and morphological information. Two gene sequences, TEF1-α and β-TUB, identified isolates as *M. anisopliae*, *M. brunneum* and *M. robertsii*, and has provided new sequence information. Eleven SSRs revealed fourteen different genotypes. Morphological variability coincided with allelic diversity within species. This work provides a starting point for the application of integral biocontrol programmes, but more comprehensive and detailed surveys are warranted to understand the species composition and genetic diversity of the *Metarhizium* in Central America.

ARTICLE HISTORY
Received 27 March 2019
Accepted 15 February 2020

KEYWORDS
Metarhizium; tropical fungi; internal transcribed spacer; translation elongation factor 1-α; β-tubulin; SSR

Metarhizium is comprised of haploid, entomopathogenic fungi found throughout the globe and is associated with more than 200 host species (Hajek & Leger, 1994). In nature, *Metarhizium* species act as regulators of insect populations and have been used in biocontrol (Lacey, Frutos, Kaya, & Vail, 2001). *Metarhizium* phylogeny has been examined through a multigene approach and more than 20 species have been recognised (Kepler, Humber, Bischoff, & Rehner, 2014). Within the *M. anisopliae* complex, there is considerable cryptic diversity and some species such as *M. anisopliae*, *M. brunneum*, *M. robertsii* and *M. pingshaense* are morphologically indistinguishable (Bischoff, Rehner, & Humber, 2009; Kepler et al., 2014).

In North and South American countries many diversity and characterisation studies have been conducted and have reported numerous previously recognised species (Brunner-Mendoza, Moonjely, del Rocío Reyes-Montes, Toriello, & Bidochka, 2017; Hernández-Domínguez et al., 2016; Lopes et al., 2013; Pérez-González et al., 2014; Rezende, Zanardo, da Silva Lopes, Delalibera, & Rehner, 2015; Rocha, Inglis, Humber, Kipnis, & Luz, 2013), new species (Gutierrez et al., 2019; Kepler et al., 2014; Lopes et al., 2018) and unassigned lineages (Botelho, Alves-Pereira, Prado, Zucchi, & Júnior, 2019; Iwanicki et al., 2019; Lopes et al., 2014). Conversely, only one of these studies has been conducted in

CONTACT R. Molina-Bravo ramon.molina.bravo@una.cr Escuela de Ciencias Agrarias, Universidad Nacional, Apartado Postal 86-3000, Heredia 40101, Costa Rica
Supplemental data for this article can be accessed at https://doi.org/10.1080/09583157.2020.1732296.
© 2020 Informa UK Limited, trading as Taylor & Francis Group

Central America (Hughes, Thomsen, Eilenberg, & Boomsma, 2004). The aim of this study was to identify and characterise a set of 15 *Metarhizium* isolates, which are part of a collection of entomopathogenic fungi housed in *Universidad Nacional de Costa Rica* and intended for insect biocontrol.

Fifteen isolates were collected, 12 from diverse agrosystems in Costa Rica, two from Honduras and one from Nicaragua, either directly from soil or from infected insects (Table 1). A baiting method with *Galleria mellonella* larvae was used to isolate the fungi from soil samples (Zimmermann, 1986). DNA was extracted using 100 mg of 10–15 d-old mycelial mats grown in PDA following a modified protocol outlined by Hoyos-Carvajal, Duque, and Orduz P (2008). PCR amplification of the internal transcribed spacer (ITS) region was performed using the ITS4 and ITS5 primers (White, Bruns, Lee, & Taylor, 1990). Two nuclear genes were amplified: the translation elongation factor 1 alpha (TEF1-α) using the 983F and 2218R primers (Rehner & Buckley, 2005), and the beta-tubulin gene (β-TUB) using the T1, T22, T1 and T222 primers (O'Donnell & Cigelnik, 1997). PCR products were visualised on 1% agarose gels, stained with GelRed™ (Biotium, USA) and sent to Macrogen Inc. (Seoul, Korea) for Sanger sequencing. ITS sequences from the 15 isolates were deposited at the National Center of Biotechnology Information (NCBI) GenBank database (accession numbers MG786728 to MG786742). TEF1-α and β-TUB accession numbers, ex-type *Metarhizium* species and other reference sequences are detailed in Table S1. Sequences from the TEF1-α and β-TUB were edited and aligned using BioEdit 7.2.5 (Hall, 1999) and MUSCLE (Edgar, 2004). Single and concatenated sequences of both genes were used to infer phylogenetic relationships. Maximum likelihood (ML) and Bayesian inference (BI) phylogenetics were analysed using MEGA7 (Kumar, Stecher, & Tamura, 2016) and MrBayes 3.1.2 (Ronquist et al., 2012), respectively. Maximum likelihood trees were run with Tamura-Nei as the appropriate model and group bootstrap values were estimated with 1000 iterations. Bayesian inference analysis was performed using a mixed model and two partitions were defined for the concatenated sequences of the TEF1-α and β-TUB genes. The final tree was generated by running a total of 10,000,000 generations. Sampling was performed every 500 generations. The first 25% of trees were omitted from each run to obtain stationarity values in the log-likelihood scores. The value 0.95 was defined as the optimal posterior probability (PP) and the nodes with bootstrap values ≥ 70% were considered supported. Short Sequence Repeat (SSR) analysis was performed using 12 markers develop by Oulevey, Widmer, Kölliker, and Enkerli (2009): Ma2049, Ma2054, Ma2055, Ma2063, Ma2064, Ma2065, Ma2069, Ma2077, Ma2097, Ma2098, Ma2099, Ma2274. Polymerase chain reactions were performed using a modified M13 universal primer protocol (Schuelke, 2000) to generate fluorescently-labeled fragments. PCR reactions were mixed in a total volume of 10 µL that contained 1X DreamTaq Polymerase Buffer (Thermo Scientific, USA), 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.02 µM forward primer containing the M13 tail, 0.1 µM reverse primer, 0.08 µM IRDye 800®-labeled M13 primer and DreamTaq Polymerase at 0.01 U μL^{-1}. Thermal profiles were performed as described by Oulevey et al. (2009). Allelic fragments were resolved in 8% acrylamide gels on a LI-COR 4300 DNA Analyser (LI-COR, Biosciences, Lincoln NE, USA). Allele sizes were estimated using Cross Checker (Buntjer & Otsen, 1999). SSR size variation, number of alleles and genetic diversity indices were determined using the *poppr* software package for R (Kamvar, Tabima, & Grünwald, 2014).

Table 1. *Metarhizium* isolates from Central America extracted from soil or insect in agrosystems.

Isolate	Species	Country	Latitute	Longitute	Locality	Isolation source	Conidia (µm)*
MTR-ECA0	*M. anisopliae*	Honduras	14°00′36″N	87°00′40″W	San Antonio de Oriente, Fco. Morazán	Soil	$4.7–9.5^{a} \times 1.8–2.9^{a}$
MTR-ECA1	*M. brunneum*	Costa Rica	10°00′36″N	84°06′53″W	Barva, Heredia	Unknown insect	$4.3–6.9^{e} \times 2.1–3.7^{a}$
MTR-ECA3	*M. brunneum*	Nicaragua	–	–	–	Soil	$4.7–6.7^{c} \times 1.8–3.4^{c}$
MTR-ECA4	*M. brunneum*	Costa Rica	9°13′01″N	83°20′10″W	Puntarenas, Puntarenas	*Phylophaga vicina*	$4.7–7.3^{cd} \times 2.0–3.3^{c}$
MTR-ECA5	*M. anisopliae*	Costa Rica	10°03′36″N	84°26′16″W	Palmares, Alajuela	Soil	$3.9–6.7^{b} \times 2.0–3.6^{bc}$
MTR-ECA6	*M. anisopliae*	Costa Rica	9°59′26″N	83°02′09″W	Limón, Limón	Soil	$5.0–7.0^{b} \times 2.1–5.6^{c}$
MTR-ECA7	*M. anisopliae*	Costa Rica	8°32′10″N	82°56′25″W	Ciudad Neily, Puntarenas	Soil	$5.0–7.0^{b} \times 2.1–3.0^{c}$
MTR-ECA8	*M. brunneum*	Honduras	14°23′04′′N	88°10′48″W	La Esperanza, Intibucá	Soil	$3.2–6.4^{cde} \times 2.0–3.4^{c}$
MTR-ECA9	*M. anisopliae*	Costa Rica	8°49′15″N	82°58′15″W	Coto Brus, San José	Soil	$4.6–6.7^{de} \times 2.0–3.1^{c}$
MTR-ECA11	*M. robertsii*	Costa Rica	9°13′01″N	83°20′10″W	Puntarenas, Puntarenas	Soil	$4.6–6.2^{de} \times 2.0–3.1^{c}$
MTR-ECA16	*M. anisopliae*	Costa Rica	10°13′01″N	83°47′05″W	Guápiles, Limón	Soil	$5.0–6.1^{c} \times 2.1–5.4^{c}$
MTR-ECA28	*M. brunneum*	Costa Rica	10°00′59″N	84°12′42″W	Alajuela, Alajuela	Soil	$4.4–6.1^{cde} \times 2.0–3.0^{c}$
MTR-ECA29	*M. anisopliae*	Costa Rica	8°36′12″N	83°6′48″W	Golfito, Puntarenas	Soil	$5.0–6.6^{b} \times 2.0–3.8^{a}$
MTR-ECA34	*M. anisopliae*	Costa Rica	8°32′10″N	82°56′25″W	Ciudad Neily, Puntarenas	Soil	$4.6–6.1^{cde} \times 2.1–3.3^{c}$
MTR-ECA38	*M. anisopliae*	Costa Rica	9°13′01″N	83°20′10″W	Puntarenas, Puntarenas	Soil	$4.6–7.0^{b} \times 2.2–3.3^{ab}$

Note: Collection data and morphological measurements.
*Conidia mean length and width compared using a Kruskal-Wallis and t-student post hoc tests, $\alpha = 0.01$. Size ranges with the same letter have means that are not significantly different.

For morphological analysis, monosporic isolate cultures were grown on potato dextrose agar (PDA) at 26°C. Conidial slides were prepared using 8–10 d-old cultures and mounted on glass slides with lactophenol. Twenty conidia per isolate were measured in triplicate using a Nikon Eclipse 80i microscope. Images were captured on a Nikon DS-fi1 camera. Conidia shape and colour, arrangement of phialides and colonial growth were recorded. Radial growth was measured on four replicate plates in PDA media. Plates were inoculated with 5 mm-long agar discs from 8 to 10 d old mycelia grown on PDA. Plates were kept at 26°C and a 24 h photoperiod. Radial growth was recorded at 3, 7, 10, 17 and 21 d after inoculation. Conidial size (length and width) differences were analysed by Kruskal Wallis (KW) and Student *t* tests ($\alpha = 0.01$). Growth was analysed by repeated measures ANOVA.

Taxonomic identification using the ITS sequences determined isolates at the genus level but were not included in the phylogenetic analyses due to low resolution (Driver, Milner, & Trueman, 2000; Huang et al., 2005b; Huang, Humber, Li, Li, & Hodge, 2005a). Phylogenetic analyses of TEF1-α, β-TUB and combined TEF1-α/β-TUB partial sequences yielded alignments of 851, 856 and 1707 bp, respectively, excluding gaps. A total of 155 parsimonious informative sites were included; 55 were from TEF1-α and 100 from β-TUB. Compatible trees from BI and ML analyses were obtained; only the BI phylogram for concatenated sequences is shown (Figure 1). The clustering observed was consistent with the multilocus phylogeny reported by Kepler et al. (2014). Based on the concatenated gene sequences, the isolates from Costa Rica, Honduras and Nicaragua were assigned as *M. anisopliae, M. robertsii and M. brunneum*, corresponding to the PARB clade together with *M. pingshaense* (PP support of 95%). Isolates ARSEF 1914 and ARSEF 1015 (*M. majus*), and CBS 258.9 and ARSEF 7507 (*M. guizhouense*) clustered together to form the MGT clade (composed of *M. majus* and *M. guizhouense* = *M. taii*; PP 98%) as defined by Bischoff et al. (2009). *Metarhizium acridum* (ARSEF 7486) was distantly related to the PARB and MGT clades, as established by previous studies (Bischoff et al., 2009). Two subgroups were observed in *M. anisopliae* isolates: a cluster of five isolates (MTR-ECA6, 9, 29, 34, 38) related (PP 98%) to the ex-type reference sequence from Eritrea (ARSEF 7487); and four isolates (MTR-ECA0, 5, 7, 16) clustering (PP 99%) with sequences from Brazil (Genbank AY445082) and Mexico (GenBank KF981907). *Metarhizium brunneum* isolates were more closely related (PP 85%) to an isolate from Mexico (ARSEF 3297) than the ex-type reference from the USA (ARSEF 2107). Moreover, the single *M. robertsii* (MTR-ECA11) isolate, corresponding references ARSEF 4739, 727, 7501 and isolate A103 (GenBank KC461031, from Mexico) formed a closely related group (PP 100%). SSR analysis revealed genetic diversity within *M. anisopliae* and *M. brunneum*. Locus Ma2274 and isolate MTR-ECA16 represented 33.3% and 75% of the null alleles, respectively, and were excluded from further analysis. All eleven SSR markers incorporated in the analysis were polymorphic (3–6 alleles per locus). Allele size range per locus was similar to that of previously reported research (Oulevey et al., 2009). *Metarhizium anisopliae* isolates had the most unique alleles across all loci (21). Only one allele (120 pb) of one locus (Ma2065) was common among all isolates. MTR-ECA11, an *M. robertsii* isolate, showed unique alleles throughout five loci. Locus Ma2054 yielded different alleles for all three species and can potentially be used to distinguish among species, while loci Ma2063 and Ma2097 had the greatest number of alleles (Table S3). SSR

Figure 1. Consensus tree based on Bayesian inferences (BI) of the TEF1-α and β-TUB concatenated partial gene sequences (1707 bp). Posterior probabilities ≥70% from Bayesian analysis are shown at branching points. *Metarhizium anisopliae* (ARSEF 7487), *M. brunneum* (ARSEF 2107), *M. guizhouense* (CBS 258.90), *M. humberi* (IP 46), *M. majus* (ARSEF 1914) and *M. pingshaense* (CBS 257.90) are shown in blue as ex-type sequences. *Metarhizium acridum* (ARSEF 7486) was used as an outgroup.

analysis revealed a different genotype for each *M. anisopliae* isolate, while the five *M. brunneum* isolates corresponded to four genotypes. The high genetic diversity and polymorphisms observed were unexpected due to the number of isolates. In Denmark, few genotypes have been found within two of the species identified herein; six genotypes out of 126 isolates of *M. brunneum* and *M. robertsii* (Steinwender et al., 2015). In three *M. anisopliae* fungal collections from Switzerland comprised of 104 isolates, 66 genotypes were detected using 41 SSR markers (Oulevey et al., 2009). However, in Mexico and Chile there was greater genotype diversity (Becerra Velásquez, Paredes Cárcamo, Rojo Meriño, France Iglesias, & Franco Durán, 2007; Serna-Domínguez et al., 2019). We attribute this phenomenon to two factors: (i) the previously reported high polymorphisms in the PARB clade species complex (Mayerhofer et al., 2015) and (ii) the diversity of ecosystems found in the tropics. Morphological descriptions determined that colony pigmentation was white or yellow before sporulation and turned olive green upon reaching maturity. There were no observed differences in

appearance of mature conidiogenous cells or mycelial colour among species (Figure S1). Conidia sizes were within the species range as identified by sequence analyses (4.5 to 7.5 µm by 2.0 to 3.5 µm; Bischoff et al., 2009). Differences in length ($KW = 271.1$, $df = 14$, $P < 0.01$) and width ($KW = 120.1$, $df = 14$, $P < 0.01$) were significant among isolates (Table 1). Although conidial size ranges overlapped among species, MTR-ECA0, an *M. anisopliae* isolate collected in Honduras, produced significantly longer conidia (a mean of 6.7 µm) compared with all other isolates ($KW = 271.1$, $df = 14$, $P < 0.01$; Figure S2). *Metarhizium anisopliae* isolates MTR-ECA5 and 9 showed the highest and the lowest radial growth after 21 d after transfer, respectively (Table S2). MTR-ECA6 grew significantly faster compared to all other isolates with a mean of 56.1 mm at 17 d ($P < 0.01$; Table S2), while other isolates grew ≤56.9 mm at 21 d within the same statistical group.

The present study demonstrated that three *Metarhizium* species are currently recognised to occur in Central American agrosystems and suggested a high degree of genetic diversity (11 SSR markers indicated high variability within 15 isolates).

To our knowledge, this is the first study to report a detailed morphological and molecular characterisation of *Metarhizium* isolates from Costa Rica, Honduras and Nicaragua. Most studies in this geographical region have focused mainly on the performance of *Metarhizium* isolates in controlling insect pests or on the identification of *Metarhizium* associated with other biological systems, such as rain forest litter and insect microbiota (Badilla Fernández, 2002; Bills & Polishook, 1994; Rivera-Méndez 2016; Vargas-Asensio et al., 2014). None of the fifteen isolates analysed in this study were identified as *M. humberi*, the recently described species for the PARB clade from Brazil and Mexico (Luz et al., 2019). However, based on a recent report of *M. humberi* in Mexico (Brunner-Mendoza et al., 2017), Luz et al. (2019) suggest that the species is likely to be found throughout the tropical Americas with distinct rainy and dry seasons, which includes Costa Rica. More inclusive surveys and characterisation work is needed to describe the diversity of the genus in the region.

Metarhizium and other entomopathogenic fungi have been used in many crops. In Costa Rica and in other Central American countries, several products, including *Metarhizium*, have been used as biopesticides for over thirty years (Rivera-Méndez, 2016). However, insect control using these products has been practiced with a limited understanding of the genetic and species composition of isolates. In spite of Central America's rich biological diversity, the NCBI database housed a mere 13 *Metarhizium* nucleotide sequence entries (11 ITS and two 18S) from Costa Rica and none from Honduras or Nicaragua (https://www.ncbi.nlm.nih.gov, accessed 24 October 2019). A better knowledge of *Metarhizium* species identification and genetic diversity will provide a platform for a more strategic and knowledge-based approach for integraded biological control in the region.

Acknowledgements

We would like to thank Walter Peraza-Padilla for the acquisition of digital images. We would also like to thank Eduardo Mora from the Tropical Agricultural Research and Higher Education Center (CATIE), Rommel Vega from *Instituto Nacional de Aprendizaje* (INA) and Ruth León from *Instituto Nacional de Innovación y Transferencia en Tecnología Agropecuaria* (INTA) for helping us recover missing biogeographical data.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by Fondos Especiales para la Educación Superior (FEES) of the Consejo Nacional de Rectores (CONARE) [grant agreement ACUERDO-VI-171-2013]. Additional support provided by [grant number 801-B6-131] Vicerrectoría de Investigación, Universidad de Costa Rica. This work has been submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Sciences of Ruth Castro-Vásquez.

ORCID

M. Montero-Astúa http://orcid.org/0000-0003-1458-242X
R. Molina-Bravo http://orcid.org/0000-0001-5564-4426

References

Badilla Fernández, F. (2002). Un programa exitoso de control biológico de insectos plaga de la caña de azúcar en Costa Rica. *Manejo Integrado de Plagas y Agroecología (Costa Rica)*, *64*, 77–87.

Becerra Velásquez, V., Paredes Cárcamo, M., Rojo Meriño, C., France Iglesias, A., & Franco Durán, J. (2007). Intraspecific differentiation of Chilean isolates of the entomopathogenic fungi *Metarhizium anisopliae* var. *anisopliae* as revealed by RAPD, SSR and ITS markers. *Genetics and Molecular Biology*, *30*, 89–99.

Bills, G. F., & Polishook, J. D. (1994). Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica. *Mycologia*, *86*, 187–198.

Bischoff, J. F., Rehner, S. A., & Humber, R. A. (2009). A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia*, *101*, 512–530.

Botelho, A. B. R. Z., Alves-Pereira, A., Prado, R. C., Zucchi, M. I., & Júnior, I. D. (2019). *Metarhizium* species in soil from Brazilian biomes: A study of diversity, distribution, and association with natural and agricultural environments. *Fungal Ecology*, *41*, 289–300.

Brunner-Mendoza, C., Moonjely, S., del Rocío Reyes-Montes, M., Toriello, C., & Bidochka, M. (2017). Physiological and phylogenetic variability of Mexican *Metarhizium* strains. *BioControl*, *80*, 1–13.

Buntjer, J. B., & Otsen, M. (1999). Cross Checker provides computer-assisted marker interpretation. *Journal of Agricultural Genomics*, *4*, 1–6.

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

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*, 1792–1797.

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

Hajek, A. E., & Leger, R. J. S. (1994). Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology*, *39*, 293–322.

Hall, T. A. (1999). Bioedit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Hernández-Domínguez, C., Guzmán-Franco, A. W., Carrillo-Benítez, M. G., Alatorre-Rosas, R., Rodríguez-Leyva, E., & Villanueva-Jiménez, J. A. (2016). Specific diversity of *Metarhizium* isolates infecting *Aeneolamia* spp. (Hemiptera: Cercopidae) in sugarcane plantations. *Neotropical Entomology*, *45*, 80–87.

Hoyos-Carvajal, L., Duque, G., & Orduz P, S. (2008). Antagonismo in vitro de *Trichoderma* spp. sobre aislamientos de *Sclerotinia* spp. y *Rhizoctonia* spp. *Revista Colombiana de Ciencias Hortícolas*, *2*, 76–86.

Huang, B., Humber, R. A., Li, S., Li, Z., & Hodge, K. T. (2005a). Further notes on the molecular taxonomy of *Metarhizium*. *Mycotaxon*, *94*, 181–187.

Huang, B., Li, C., Humber, R. A., Hodge, K. T., Fan, M., & Li, Z. (2005b). Molecular evidence for the taxonomic status of *Metarhizium taii* and its teleomorph, *Cordyceps taii* (Hypocreales, Clavicipitaceae). *Mycotaxon*, *94*, 137–147.

Hughes, W. O. H., Thomsen, L., Eilenberg, J., & Boomsma, J. J. (2004). Diversity of entomopathogenic fungi near leaf-cutting ant nests in a neotropical forest, with particular reference to *Metarhizium anisopliae* var. *anisopliae*. *Journal of Invertebrate Pathology*, *85*, 46–53.

Iwanicki, N. S., Pereira, A. A., Botelho, A. B. R. Z., Rezende, J. M., Moral, R. d. A., Zucchi, M. I., & Delalibera Júnior, I. (2019). Monitoring of the field application of *Metarhizium anisopliae* in Brazil revealed high molecular diversity of *Metarhizium* spp. in insects, soil and sugarcane roots. *Scientific Reports*, *9*, 4443.

Kamvar, Z. N., Tabima, J. F., & Grünwald, N. J. (2014). Poppr: An R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ*, *2*, e281–e214.

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*, 811–829.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*, 1870–1874.

Lacey, L. A., Frutos, R., Kaya, H. K., & Vail, P. (2001). Insect pathogens as biological control agents: Do they have a future? *Biological Control*, *21*, 230–248.

Lopes, R. B., Faria, M., Souza, D. A., BlochJrC., Silva, L. P., & Humber, R. A. (2014). MALDI-TOF mass spectrometry applied to identifying species of insect-pathogenic fungi from the *Metarhizium anisopliae* complex. *Mycologia*, *106*, 865–878.

Lopes, R. B., Mesquita, A. L. M., Tigano, M. S., Souza, D. A., Martins, I., & Faria, M. (2013). Diversity of indigenous *Beauveria* and *Metarhizium* spp. in a commercial banana field and their virulence toward *Cosmopolites sordidus* (Coleoptera: Curculionidae). *Fungal Ecology*, *6*, 356–364.

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

Luz, C., Rocha, L. F. N., Montalva, C., Souza, D. A., Botelho, A. B. R. Z., Lopes, R. B., … Júnior, I. D. (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*, 107216.

Mayerhofer, J., Lutz, A., Widmer, F., Rehner, S. A., Leuchtmann, A., & Enkerli, J. (2015). Multiplexed microsatellite markers for seven *Metarhizium* species. *Journal of Invertebrate Pathology*, *132*, 132–134.

O'Donnell, K., & Cigelnik, E. (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution*, *7*, 103–116.

Oulevey, C., Widmer, F., Kölliker, R., & Enkerli, J. (2009). An optimized microsatellite marker set for detection of *Metarhizium anisopliae* genotype diversity on field and regional scales. *Mycological Research*, *113*, 1016–1024.

Pérez-González, V. H., Guzmán-Franco, A. W., Alatorre-Rosas, R., Hernández-López, J., Hernández-López, A., Carrillo-Benítez, M. G., & Baverstock, J. (2014). Specific diversity of the entomopathogenic fungi *Beauveria* and *Metarhizium* in Mexican agricultural soils. *Journal of Invertebrate Pathology*, *119*, 54–61.

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

Rezende, J. M., Zanardo, A. B. R., da Silva Lopes, M., Delalibera, I., & Rehner, S. A. (2015). Phylogenetic diversity of Brazilian *Metarhizium* associated with sugarcane agriculture. *BioControl*, *60*, 495–505.

Rivera-Méndez, W. (2016). Control microbiológico como experiencia de sostenibilidad local en la agricultura centroamericana. *Tecnología en Marcha*, *29*, 31–40.

Rocha, L. F., Inglis, P. W., Humber, R. A., Kipnis, A., & Luz, C. (2013). Occurrence of *Metarhizium* spp. in Central Brazilian soils. *Journal of Basic Microbiology*, *53*, 251–259.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., … Huelsenbeck, J. P. (2012). Mrbayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*, 539–542.

Schuelke, M. (2000). An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology*, *18*, 233–234.

Serna-Domínguez, M. G., Andrade-Michel, G. Y., Rosas-Valdez, R., Castro-Félix, P., Arredondo-Bernal, H. C., & Gallou, A. (2019). Genetic diversity of the *Metarhizium anisopliae* complex in Colima, Mexico, using microsatellites. *Fungal Biology*, *123*, 855–863. In Press.

Steinwender, B. M., Enkerli, J., Widmer, F., Eilenberg, J., Kristensen, H. L., Bidochka, M. J., & Meyling, N. V. (2015). Root isolations of *Metarhizium* spp. from crops reflect diversity in the soil and indicate no plant specificity. *Journal of Invertebrate Pathology*, *132*, 142–148.

Vargas-Asensio, G., Pinto-Tomas, A., Rivera, B., Hernandez, M., Hernandez, C., Soto-Montero, S., … Tamayo-Castillo, G. (2014). Uncovering the cultivable microbial diversity of Costa Rican beetles and its ability to break down plant cell wall components. *PLoS ONE*, *9*, e113303–11.

White, T. J., Bruns, T., Lee, S., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR protocols* (pp. 315–322). New York: Academic Press.

Zimmermann, G. (1986). The '*Galleria* bait method' for detection of entomopathogenic fungi in soil. *Journal of Applied Entomology*, *102*, 213–215.