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Genetic diversity of the *Metarhizium anisopliae* complex in Colima, Mexico, using microsatellites

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ABSTRACT

Metarhizium anisopliae is a complex of cryptic species with wide geographical distribution and versatile lifestyles. In this study, 45 isolates of the *Metarhizium* genus harbored in the "Colección de Hongos Entomopatógenos" of the "Centro Nacional de Referencia de Control Biológico" from different substrates, insect-host, and localities from Colima, Mexico, were phylogenetically identified using the 5′end of translation elongation factor 1-α (5′TEF) and intergenic nuclear region MzFG543igs. Seven species were recognized, *M. acridum* (n = 26), *M. pemphigi* (n = 1), and within the PARB and MGT clades: *M. anisopliae* (N = 7; *sensu stricto*: n = 2; *sensu lato*: n = 5), *M. brunneum* (n = 2), *M. guizhouense* (n = 2), *M. pingshaense* (n = 2), and *M. robertsii* (n = 5). Twenty-nine SSR markers were developed for *M. acridum*; according to the analysis of 12 polymorphic SSR loci, *M. acridum* showed low genetic diversity, revealing five genotypes with a dominant one (n = 21). Based on the analysis of 13 specific SSR loci, 14 genotypes were identified within the PARB and MGT clades. This study contributes to generating valuable information about the community structure and genotypic diversity of *Metharhizum* species in the state of Colima, Mexico.

1. Introduction

Metarhizium Sorokin (Hypocreales: Clavicipitceae) is a cosmopolitan genus with a versatile lifestyle as soil-inhabitants (i.e., saprobes or rhizosphere entomopathogens), endophytes, and antagonists of fungal plant pathogens (Brunner-Mendoza et al., 2018; Vega et al., 2012; Vega, 2018). Species of *Metarhizium* genus (e.g., *Metarhizium anisopliae* and *M. acridum*) have been recognized as biological control agents (BCA) and attained considerable importance in commercial formulations as an alternative to chemical insecticides (de Faria and Wraight, 2007; Lacey et al., 2015).

Morphological diagnosis has often been used for *Metarhizium* strain identification; unfortunately, these features are insufficient to adequately differentiate the cryptic species of this genus (Bischoff et al., 2009). Recently, a multigene analysis of the *Metarhizium* genus has distinguished 30 species, including sexual and asexual forms (Kepler et al., 2014). In this respect, Bischoff et al. (2009) recognized nine species within the species complex of *M. anisopliae*, which includes the PARB clade (i.e., *Metarhizium pinsghaense*, *M. anisopliae*, *Metarhizium robertsii*, and *M. brunneum*), the MGT clade (i.e., *Metarhizium majus* and *Metarhizium guizhouense*), and three additional species, *M. acridum*, *Metarhizium lepidiotae*, and *Metarhizium globosum*. Notably, the 5′end of the translation elongation factor 1-α (i.e., 5′TEF) region displayed a high power of discrimination within the *M. anisopliae* complex (Rehner and Kepler, 2017). Additionally, several studies have combined the use of 5′TEF with different nuclear intergenic loci (Kepler and Rehner, 2013) for a deep phylogenetic understanding of the intraspecific variation of the *M. anisopliae* complex (Brunner-Mendoza et al., 2017; Kepler et al., 2016; Rezende et al., 2015; Rehner and Kepler, 2017). Particularly, the 5′TEF and MzFG543igs loci have been suggested for a major resolution of cryptic genetic structure, mainly for the PARB and MGT clades (Rehner and Kepler, 2017).

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Microsatellite (SSR) markers have been widely used in *Metarhizium* for a deep understanding of the genotypic diversity, genetic structure, and in the assessment of insect-host association (Enkerli and Widmer, 2010; Hernández-Domínguez and Guzmán-Franco, 2017; Mayerhofer et al., 2015). Furthermore, SSRs have been suggested as a diagnostic tool for tracking fungal strains in the environment (Lacey et al., 2015; Mayerhofer et al., 2015) and in the evaluation of possible effects over indigenous *Metarhizium* populations after strain application (Enkerli and Widmer, 2010; Hernández-Domínguez and Guzmán-Franco, 2017; Kepler et al., 2015; Steinwender et al., 2015). Sets of polymorphic SSR have been assayed to acquire a robust and useful genotyping tool for several different *Metarhizium* species (i.e., PARB and MGT clades and *M. lepidiotae*) (Mayerhofer et al., 2015). Currently, 41 SSR markers had been characterized from three species of *Metharhizium*: *M. anisopliae*, *M. brunneum*, and *M. robertsii*, originally identified as *M. anisopliae* (Enkerli et al., 2005; Oulevey et al., 2009). However, to date, non-specific SSR markers for *M. acridum* have been developed, and only the SSR marker Ma325 from *M. robertsii* (Enkerli et al., 2005) has been revealed to be transferable and informative for *M. acridum* (Mayerhofer et al., 2015).

Cryptic species of the *M. anisopliae* complex are frequently associated with specific types of habitat with high exposure to UV radiation (i.e., cultivate soils and disturbed environments); however, certain *Metarhizium* species (i.e., *M. brunneum* and *M. robertsii*) are also detected in both rhizospheres of natural landscapes and agricultural fields (Bidochka et al., 2001; Meyling and Eilenberg, 2007; Nishi et al., 2017; Wyrebeck et al., 2011). Also, it has been suggested that the genetic diversity of some species is related to specific plants and microhabitats (Hernández-Domínguez et al., 2016; Meyling and Eilenberg, 2007; Rezende et al., 2015; Wyrebeck et al., 2011) with a potential of movement between ground environments (Hernández-Domínguez and Guzmán-Franco, 2017). In Mexico, several studies have exposed a high species diversity of *Metarhizium* genus infecting different insect hosts and associated with specific agricultural environments (Brunner-Mendoza et al., 2017; Carrillo-Benítez et al., 2013; Pérez-González et al., 2014; Hernández-Domínguez et al., 2016) and have even detected the presence of specific haplotypes over time (Hernández-Domínguez and Guzmán-Franco, 2017).

Even though biological control in Mexico based on *Metarhizium* species (e.g., *M. anisopliae* and *M.* acridum) has great success within different insect control campaigns (Brunner-Mendoza et al., 2018), the assessment of the occurrence and diversity of *Metarhizium* species in a greater number of specific environments is highly required. Therefore, the aim of this study was to characterize the genetic diversity of 45 isolates of *Metarhizium* genus from the state of Colima, Mexico, harbored in the "Colección de Hongos Entomopatógenos (CHE)" of the "Centro Nacional de Referencia de Control Biológico (CNRCB)", to contribute in the selection of isolates with potential as BCA. Species identification was carried out with the phylogenetic analysis of 5′TEF and MzFG543igs regions, while the assessment of genetic diversity of the *M. anisopliae* complex was accomplished by SSR markers.

2. Materials and methods

2.1. Fungal isolates

In this study, forty-five *Metarhizium* isolates from the state of Colima, Mexico were used. The isolates were morphologically identified and provided by the "Colección de Hongos Entomopatógenos" of the "Centro Nacional de Referencia de Control Biológico" (Tecoman, Colima, Mexico). Information for each isolate is detailed in Table 1.

2.2. DNA extraction

Initially, all isolates were grown on Sabouraud dextrose agar medium with yeast extract (i.e., SDAY/4) for 15 d at 25 ± 2 °C and were then cultivated in 75 mL of liquid medium (i.e., 40 g L^{-1} glucose, 10 g L^{-1} polypeptone, and 10 g L^{-1} yeast extract) with a constant agitation (150 rpm) at 27 ± 2 °C for 3 d. The biomass of each isolate was collected and immediately lyophilized with FreeZone 4.5 L Cascade Benchtop Freeze Dry System (Labconco Corp., Kansas City, USA) (Gallou et al., 2016). Cell lysis procedure was achieved with 10 mg of lyophilized mycelia and ground with liquid nitrogen on a sterile mortar and pestle. The extraction of genomic DNA was performed using DNeasy® Plant Mini Kit (Qiagen, Valencia, USA) in agreement with the manufacturer's instructions. The DNA concentration and quality were estimated with Fragment Analyzer™ Automated CE system with the High Sensitivity Genomic DNA Analysis kit according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). On the other hand, the purity of extracted DNA in the absorbance ratio A260/A280 was achieved using a BioTek Epoch spectrophotometer (BioTek, Winooski, USA).

2.3. Molecular identification

2.3.1. PCR and sequencing

To identify the *Metarhizium* isolates at the species level, we used the partial sequences of intron-rich region of the 5′end of translation elongation factor 1-α (i.e., 5′TEF) (Rehner and Buckley, 2005) and intergenic region MzFG543igs (Kepler and Rehner, 2013). These loci were selected due to their high degree of differentiation among species and congruency of the topology with the *Metarhizium* genus speciation (Bischoff et al., 2009; Kepler and Rehner, 2013; Rezende et al., 2015). Information about amplification and sequencing primers for both loci are shown in supplementary Table S1. Amplification reaction mixtures for both loci were prepared in a final volume of 20 μL with Phire™ reaction buffer 1X (Thermo Fisher Scientific, Waltham, USA), 0.2 mM dNTP mix (Promega, Madison, WI, USA), 0.4 μM of each primer, 0.4 μL of Phire™ Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, USA), and 1 or 20 ng of genomic DNA for 5′TEF and MzFG543igs, respectively. 5′TEF locus was amplified with the touchdown PCR protocol previously established by Don et al. (1991) and in accordance with the Phire™ Hot Start II DNA Polymerase cycling conditions. 5′TEF thermal cycling was performed as follows: initial denaturalization at 98 °C for 30 s, continuing with 10 cycles of denaturation at 98 °C for 5 s, annealing at 66 °C for 5 s with a decrease of 1 °C in the next 9 cycles, and extension at 72 °C for 12 s. The next 36 cycles were accomplished with denaturation at 98 °C for 5 s, annealing at 56 °C for 12 s, and extension at 72 °C for 12 s, concluding with a final extension step at 72 °C for 1 min. Meanwhile, the MzFG543igs locus thermal cycling was achieved according to Kepler and Rehner (2013) and the Phire™ Hot Start II DNA Polymerase cycling conditions as presented below: initial denaturalization at 98 °C for 30 s, continuing with 35 cycles of denaturing at 98 °C for 5 s, annealing of 56 °C for 5 s, and extension of 72 °C for 12 s, with a cycle of final extension at 72 °C for 1 min. Visualization of PCR products was accomplished on 1.3 % agarose gel stained with 3X GelRed™ (Biotium, USA). PCR product purification procedure was completed with Wizard® SV Gel and PCR Clean-Up System Kit (Promega, Madison, USA). The sequencing process was performed by Macrogen (Seoul, Korea).

2.3.2. Sequence alignment and phylogenetic analyses

Edition and assembling of sequences were carried out with BioEdit v 7.2.5 (Hall, 1999). Sequences were deposited at GenBank

Table 1
Information (i.e., acronym of the collection, identification at species level, year, locality, substrate, host, and host order and family) and GenBank accession numbers from 5′ end of translation elongation factor 1-α (5′TEF) and MzFG543igs intergenic region of *Metarhizium* spp. isolates from Colima, Mexico used in this study.

Collection accession number	Phylogenetic identification	Year of collection	Locality	Substrate	Host	Host Order: Family	GenBank accession number	
							5′TEF	MzFG543igs
CHE-CNRCB 181	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188596	MK188641
CHE-CNRCB 183	*Metarhizium anisopliae s.l.*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188626	MK188671
CHE-CNRCB 184	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188597	MK188642
CHE-CNRCB 185	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188598	MK188643
CHE-CNRCB 186	*Metarhizium anisopliae s.l.*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188627	MK188672
CHE-CNRCB 187	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188599	MK188644
CHE-CNRCB 189	*Metarhizium acridum*	1995	Tecoman	Eggplant	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188600	MK188645
CHE-CNRCB 190	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188601	MK188646
CHE-CNRCB 192	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188602	MK188647
CHE-CNRCB 193	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188603	MK188648
CHE-CNRCB 194	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188603	MK188649
CHE-CNRCB 195	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188605	MK188650
CHE-CNRCB 196	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188606	MK188651
CHE-CNRCB 197	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188607	MK188652
CHE-CNRCB 198	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188608	MK188653
CHE-CNRCB 199	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188609	MK188654
CHE-CNRCB 200	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188609	MK188655
CHE-CNRCB 202	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188611	MK188656
CHE-CNRCB 204	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188612	MK188657
CHE-CNRCB 205	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188613	MK188658
CHE-CNRCB 206	*Metarhizium acridum*	1992	Tecoman	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188614	MK188659
CHE-CNRCB 207	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188615	MK188660
CHE-CNRCB 208	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188616	MK188661
CHE-CNRCB 209	*Metarhizium robertsii*	1992	Tecoman	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188631	MK188676
CHE-CNRCB 210	*Metarhizium acridum*	1992	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188617	MK188662
CHE-CNRCB 211	*Metarhizium acridum*	1995	Isla Socorro	INR	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188618	MK188663
CHE-CNRCB 212	*Metarhizium acridum*	1995	Isla Socorro	INR	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188619	MK188664
CHE-CNRCB 213	*Metarhizium acridum*	1996	Isla Socorro	INR	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188620	MK188665
CHE-CNRCB 215	*Metarhizium anisopliae s.l.*	1988	Cuauhtemoc	Sugarcane	*Diatraea saccharalis*	Lepidoptera: Crambidae	MK188628	MK188673
CHE-CNRCB 216	*Metarhizium anisopliae s.s.*	1987	Cuauhtemoc	Sugarcane	*Diatraea saccharalis*	Lepidoptera: Crambidae	MK188624	MK188669
CHE-CNRCB 217	*Metarhizium guizhouense*	1987	INR	Corn	*Spodoptera frugiperda*	Lepidoptera: Noctuidae	MK188638	MK188683
CHE-CNRCB 218	*Metarhizium anisopliae s.s.*	1989	INR	Corn	*Spodoptera frugiperda*	Lepidoptera: Noctuidae	MK188625	MK188670
CHE-CNRCB 236	*Metarhizium anisopliae s.l.*	2002	Cuauhtemoc	Sugarcane	*Aeneolamia* sp.	Hemiptera: INR	MK188629	MK188674
CHE-CNRCB 253	*Metarhizium robertsii*	1995	INR	Soil	*Phyllophaga* sp.	Coleoptera: INR	MK188632	MK188677
CHE-CNRCB 397	*Metarhizium acridum*	1997	INR	Grass	*Schistocerca piceifrons*	Orthoptera: Acrididae	MK188621	MK188666
CHE-CNRCB 399	*Metarhizium robertsii*	1997	INR	Soil	*Phyllophaga* sp.	Coleoptera: INR	MK188633	MK188678
CHE-CNRCB 413	*Metarhizium anisopliae s.l.*	2014	Minatitlan	Soil/Forest	*Galleria mellonella*	Lepidoptera: Pyralidae	MK188630	MK188675
CHE-CNRCB 474	*Metarhizium pemphigi*	2015	Comala	Coffee	INR	INR	MK188640	MK188685
CHE-CNRCB 475	*Metarhizium brunneum*	2015	Comala	Soil/Avocado	*Galleria mellonella*	Lepidoptera: Pyralidae	MK188636	MK188681
CHE-CNRCB 476	*Metarhizium brunneum*	2015	Comala	Soil/Avocado	*Galleria mellonella*	Lepidoptera: Pyralidae	MK188637	MK188682
CHE-CNRCB 477	*Metarhizium guizhouense*	2015	Comala	Soil/Avocado	*Galleria mellonella*	Lepidoptera: Pyralidae	MK188639	MK188684
CHE-CNRCB 480	*Metarhizium pingshaense*	2015	Comala	Soil/Avocado	*Galleria mellonella*	Lepidoptera: Pyralidae	MK188622	MK188667
CHE-CNRCB 481	*Metarhizium robertsii*	2015	Comala	Soil/Avocado	*Galleria mellonella*	Lepidoptera: Pyralidae	MK188634	MK188679
CHE-CNRCB 482	*Metarhizium pingshaense*	2015	Comala	Soil/Avocado	*Galleria mellonella*	Lepidoptera: Pyralidae	MK188623	MK188668
CHE-CNRCB 488	*Metarhizium robertsii*	2015	Cuauhtemoc	Soil/Blackberry	*Galleria mellonella*	Lepidoptera: Pyralidae	MK188635	MK188680

CHE-CNRCB, "Colección de Hongos Entomopatógenos" of the "Centro Nacional de Referencia de Control Biológico", Tecoman, Colima, Mexico.
s.s., sensu stricto.
s.l., sensu lato.
INR, information not registered.

public collection of the National Center for Biotechnology Information (NCBI) (Table 1). The multiple sequence alignments (MSA) of individual 5′TEF and MzFG543igs regions and concatenated dataset (i.e., 5′TEF-MzFG543igs) were conducted using MAFFT (Katoh et al., 2017) (http://mafft.cbrc.jp/aligment/server/), and final MSA were deposited in the TreeBASE database with the ID 24246. The reference sequences from Bischoff et al. (2006, 2009), Fisher et al. (2011), Kepler and Rehner (2013), Kepler et al. (2015), Rezende et al. (2015), and Rocha et al. (2013) were included (Suppl. Table S2). Evolutionary model selection for each MSA was done with jModelTest 2.10 software (Darriba et al., 2012) using the Bayesian information criteria (BIC). The K80+Γ and K80 + I models were selected for 5′TEF and MzFG543igs dataset, respectively, while for the phylogenetic reconstruction of concatenated 5′TEF-MzFG543igs both individual models were used. Phylogenetic hypotheses of individual and concatenated datasets were developed under Bayesian inference (BI) using MrBayes 3.2.5 software (Ronquist and Huelsenbeck, 2003). The BI analyses were assayed with two runs of eight Markov chains run simultaneously for 1 050 000 and 1 050 000 generations for 5′TEF, MzFG543igs, and 5′TEF-MzFG543igs datasets, respectively. Sampling was developed every 100 generations, discarding the first 25 % trees (i.e., burn-in). The 50 % majority-rule consensus tree was visualized with FigTree 1.4.2 (Rambaut, 2014).

2.4. SSR genotyping

2.4.1. SSR analysis of M. acridum

To characterize specific SSR marker for *M. acridum*, the *M. acridum* strain CQMa 102 genome (Gao et al., 2011; project accession number PRJNA245139) was used as an input for the Tandem repeats finder program (Benson, 1999) to locate and display the microsatellite regions with the parameter 'Maximum Period Size' set on five. Specific forward and reverse primers of selected SSR loci were designed with the Primer-BLAST program (Ye et al., 2012). Forty SSR markers of *M. acridum* were tested for PCR amplification with two isolates (i.e., CHE-CNRCB 189 and 212) in a Veriti™ Thermal Cycler (Thermo Fisher Scientific, Waltham, USA). PCR amplification reactions were performed in a final volume of 20 μL, consisting of 1X Taq Buffer (BioTecMol, D.F., Mexico), 2.5 mM of $MgCl_2$ (BioTecMol, D.F., Mexico), 0.2 mM of each dNTP (Promega, Madison, WI, USA), 0.2 μM of forward and reverse primer, 0.5 U Amplificasa® (BioTecMol, D.F., Mexico), and 1 ng of genomic DNA with the next thermal cycling parameters: 94 °C for 3 min followed by 36 cycles at 94 °C for 30 s, T_a (see Suppl. Table S3) for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 5 min. Twenty-nine SSR markers produced single amplicons of the expected size and were used for further analyses (Suppl. Table S3). A total of 30 SSR loci (i.e., 29 SSR selected in this study and the Ma325 [Enkerli et al., 2005] revealed to be polymorphic for *M. acridum* [Mayerhofer et al., 2015]) were analyzed through the 26 isolates identified as *M. acridum* (Table 1). *M. acridum* strains ARSEF 5736 (Madagascar) and CHE-CNRCB 214 (Chiapas, Mexico) were included as outgroup to assess the polymorphism exposed by the SSR loci. The SSR products were resolved by capillary electrophoresis on Fragment Analyzer™ Automated CE system (Agilent Technologies, Santa Clara, CA, USA) with the DNA-900 Reagent Kit (i.e., DNA size range: 35–500 bp). Determination of amplicon size and fragment analyses were carried out with the PROSize™ 2.0 software (Agilent Technologies, Santa Clara, CA, USA). A matrix containing alleles per locus of SSR was generated. Measures of genetic diversity (i.e., number of alleles (Na), number of effective alleles (Ne), the percentage of polymorphic loci, diversity index (h), and unbiased diversity index (uh) per locus) were estimated with GenAlex 6.5 package (Peakall and Smouse, 2012). Finally, a minimum spanning network based on Bruvo's genetic distances (Bruvo et al., 2004) was constructed using the package Poppr (Kamvar et al., 2014) in R (R Core Team, 2013) to know the SSR multilocus genotypes of *M. acridum*.

2.4.2. SSR analyses of PARB and MGT clades

Eighteen SSR loci (i.e, Ma164, Ma195, Ma307, Ma327, Ma375, Ma416, Ma2049, Ma2054, Ma2055, Ma2060, Ma2063, Ma2064, Ma2089, Ma2097, Ma2098, Ma2108, Ma2287, and Ma2296) previously characterized (Enkerli et al., 2005; Oulevey et al., 2009) were assayed within the 18 isolates identified as belonging to the PARB and MGT clades (Table 1). PCR reaction mixtures were accomplished as described above to *M. acridum* SSR analysis. The thermocycling conditions were performed as mentioned by Enkerli et al. (2005) and Oulevey et al. (2009). Visualization of SSR products, determination of size, fragment analyses, and descriptive genetic measures were determined as previously stated for *M. acridum*.

3. Results

3.1. Phylogenetic identification of Metarhizium isolates

The MSA of 5′TEF, MzFG543igs, and 5′TEF-MzFG543igs (i.e., concatenated dataset) comprised 674, 876, and 1550 positions including gaps treated as missing data. Bayesian inference of 5′TEF-MzFG543igs (Fig. 1) showed congruent topologies with multilocus phylogeny of Bischoff et al. (2009). Seven species of *Metarhizium* genus were identified: *M. acridum* (n = 26), *M. anisopliae* (N = 7; *sensu stricto*: n = 2; *sensu lato*: n = 5), *M. brunneum* (n = 2), *M. guizhouense* (n = 2), *Metarhizium pemphigi* (n = 1), *Metarhizium pingshaense* (n = 2), and *M. robertsii* (n = 5) (Table 1). Sequences of *M. acridum* isolates were placed at the monophyletic clade of *M. acridum* with 100 BI posterior probabilities (i.e., BI-PP) in each 5′TEF BI (Suppl. Fig. S1), MzFG543igs BI (Suppl. Fig. S2), and 5′TEF-MzFG543igs BI (Fig. 1). Only two genotypes were identified in the monophyletic clade of *M. acridum*. The cluster of the isolates CHE-CNRCB 198 and 205, was detected only in the 5′TEF-MzFG543igs BI and 5′TEF BI with 99 and 97 BI-PP, respectively (Fig. 1; Suppl. Fig. S1). On the other hand, 18 isolates were distributed in the six terminal clades that formed the PARB and MGT clades. Moreover, these groupings were congruent in the three phylogenetic reconstructions (Fig. 1; Suppl. Figs. S1 and S2), except for the *M. pingshaense* terminal clade which showed topological conflicts in 5′TEF-MZFG543igs BI and MzFG543igs BI (Fig. 1; Suppl. Fig. S2). Notably, the *M. anisopliae* clade contained three subclades, previously determined by Rezende et al. (2015), named Mani1, Mani2, and Mani3 perfectly defined and well supported (>96 of BI-PP) in the 5′TEF-MzFG543igs BI and 5′TEF BI (Fig. 1; Suppl. Fig. S1). The isolates CHE-CNRCB 216 and 218 were grouped in the subclade Mani1, which includes the ex-type strain *M. anisopliae* ARSEF 7487 and is referred with the circumscription *sensu stricto*. Meanwhile, the subclade Mani2 contained most of the *M. anisopliae sensu lato* isolates (i.e., CHE-CNRCB 183, 186, 215, 236, and 413). Mani3 was composed only of two isolates proceeding from Brazil. Topology congruencies were shown at the monophyletic clades of *M. anisopliae*, *M. robertsii*, and *M. brunneum* highly supported in the concatenated dataset and individuals 5′TEF and MzFG543igs BI (Fig. 1; Suppl. Figs. S1 and S2), but not for *M. pingshaense* and *M. guizhouense* in the individual MzFG543igs BI (Suppl. Fig. S2). The isolates identified as *M. pingshaense* (CHE-CNRCB 480 and 482) and

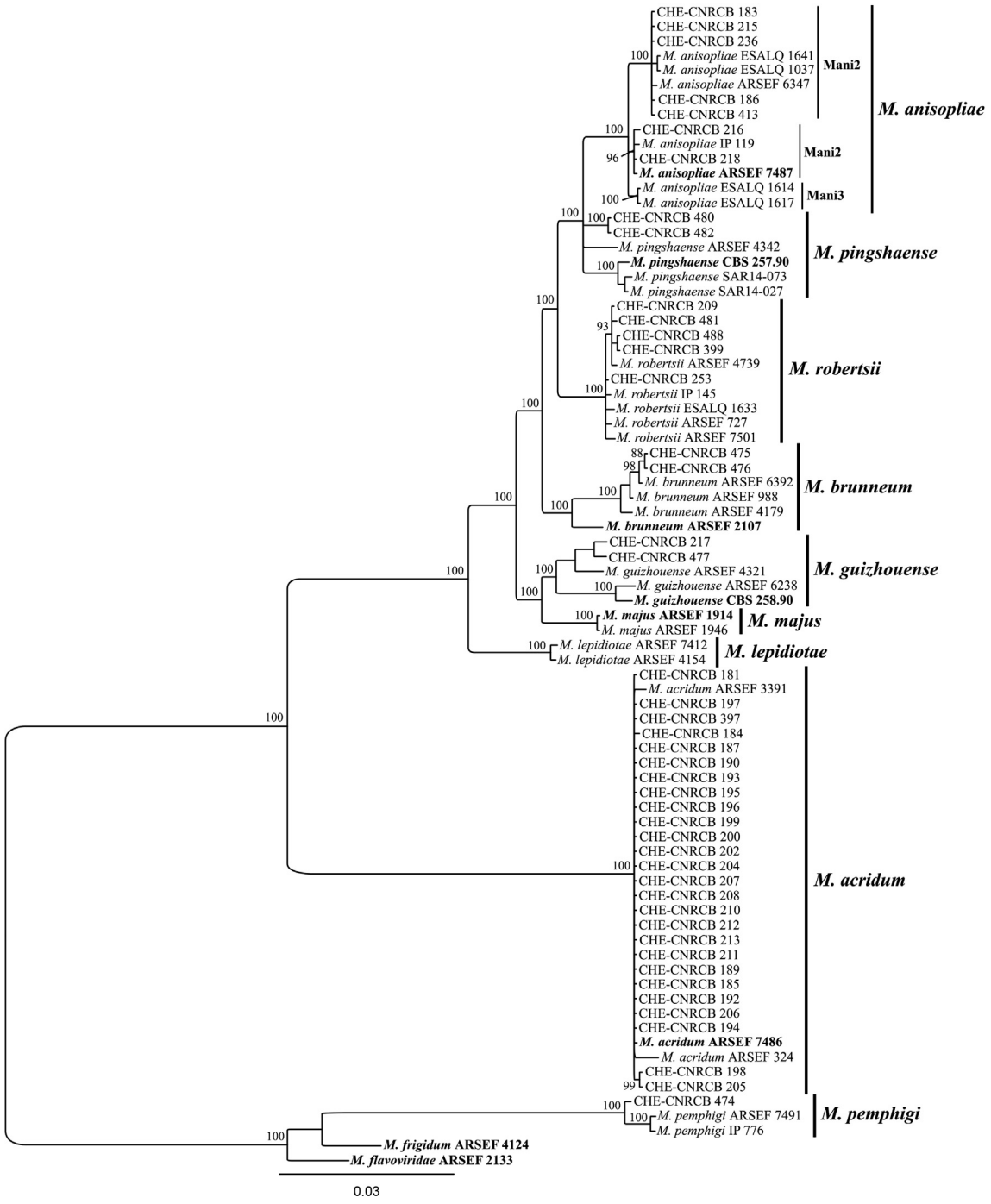

Fig. 1. Bayesian inference (BI) based on combined data set of intron-rich region of the 5′end of translation elongation factor 1-α (i.e., 5′TEF) and the intergenic nuclear region MzFG543igs from *Metarhizium* genus. Reference sequences were obtained from Bischoff et al. (2006, 2009), Fisher et al. (2011), Kepler and Rehner (2013), Kepler et al. (2015), Rocha et al. (2013), and Rezende et al. (2015). The GenBank accession numbers are shown on Supplementary Table 2. *Metarhizium flavoviride* ARSEF 2133 and *M. frigidum* ARSEF 4124 were used as outgroup. Sequences from reference ex-type material are marked in bold. Bayesian posterior probabilities (BI-PP) are exhibited as numerical value expressed in percentage over the node of each branch and only values >80 are exposed. Subclades Mani1, Mani2, and Mani3 are referenced from Rezende et al. (2015).

M. guizhouense (CHE-CNRCB 217 and 477) were phylogenetically placed separately of monophyletic *M. pingshaense* and *M. guizhouense* clades in MzFG543igs IB (Suppl. Fig. S2). Particularly, phylogenetic group topologies of MzFG543igs BI (i.e., *M. anisopliae*, *M. guizhouense*, and *M. pingshaense*) were mostly incongruent with respect to 5′TEF-MzFG543igs BI and 5′TEF BI, however, no identification problems were achieved. By last, sequences of *M. pemphigi* (CHE-CNRCB 474) were shown as a monophyletic group for the three phylogenetic analyses.

3.2. SSR analysis of *M. acridum*

Amplification of the 30 SSR loci (i.e., 29 designed in this study and Ma325) were consistent through the 28 isolates of *M. acridum* (i.e., 26 isolates from Colima and the two reference strains ARSEF 5736 and CHE-CNRCB 214). To assess the polymorphism of the SSR developed in this study, a first SSR analysis showed twenty-seven polymorphic SSR loci while two resulted monomorphic (i.e., Mac004 and Mac021). On the other hand, the genetic diversity

analysis of *M. acridum* was conducted with the 12 SSR polymorphic loci for the isolates from the state of Colima, Mexico. Measures of genetic diversity are given in Table 2. A total of 30 alleles with a range of size of 138–372 bp were obtained. As shown in the minimum spanning network, five genotypes were identified among the isolates (Fig. 2). One genotype resulted dominant with 21 isolates, and another genotype detected only in two isolates (i.e., CHE-CNRCB 198 and 205) was also revealed in the 5′TEF BI (Suppl. Fig. S1). Low levels of genetic diversity were observed ($h = 0.198 \pm 0.025$; $uh = 0.206 \pm 0.026$) in *M. acridum* (Table 3). Seven SSR loci (i.e., Mac002, Mac005, Mac012, Mac016, Mac019, Mac034, and Ma325) showed uh values greater than 0.271, whereas the minimum uh value was 0.077 for Mac031, Mac035, and Mac037 (Table 2).

3.3. SSR analysis of PARB and MGT clades

The amplification of 18 SSR loci was accomplished through the eighteen isolates identified inside the PARB and MGT clades. Five SSR loci (i.e., Ma327, Ma2049, Ma2060, Ma2098, and Ma2296) were eliminated from the SSR analysis due to the inconsistency of amplification and missing data (data not shown). Owing to the limited number of isolates per species for both PARB and MGT clades, only descriptive genetic measures were performed as shown in Table 3. Most of the SSR markers resulted monomorphic for *M. anisopliae s.s*, *M. brunneum*, *M. pingshaense*, and *M. guizhouense*. As expected, the number of genotypes obtained was greater for the species with the highest number of isolates, i.e., *M. anisopliae* (3) *s.l.* and *M. robertsii* (5). On the other hand, the two isolates of *M. anisopliae s.s.* and *M. guizhouense* corresponded to different genotypes, while the isolates of *M. brunneum* and *M. pingshaense* conformed to a single genotype.

4. Discussion

In Mexico, some species of *M. anisopliae* complex (i.e., *M. anisopliae* and *M. acridum*) have been successfully used to control insect pest of agricultural importance (Brunner-Mendoza et al., 2018). Nevertheless, knowledge about biodiversity, phylogenetic status, and genetic diversity becomes overriding in seeking potential candidates for the biocontrol of insect pests. Here, we identified and assessed the genetic diversity of the species of the *M. anisopliae* complex in the state of Colima, Mexico using the reference isolates harbored in the "Colección de Hongos Entomopatógenos" (CHE) of the "Centro Nacional de Referencia de Control Biológico" (CNRCB) obtained from different substrates, insect-host, and localities.

Table 2
Measures of genetic diversity through SSR analysis (i.e., number of alleles (Na), number of effective alleles (Ne), diversity (h), and unbiased diversity (uh)) from the 26 isolates identified as *Metarhizium acridum* in Colima, Mexico.

Locus	Na [Allele size (bp)]	Ne	h	Uh
Mac002	4 [225–295]	1.380	0.275	0.286
Mac005	3 [354–372]	1.368	0.269	0.280
Mac012	3 [333–366]	1.374	0.272	0.283
Mac016	2 [336–342]	1.352	0.260	0.271
Mac019	3 [260–296]	1.368	0.269	0.280
Mac022	2 [325–327]	1.166	0.142	0.148
Mac028	3 [313–341]	1.170	0.145	0.151
Mac031	2 [294–296]	1.080	0.074	0.077
Mac034	2 [360–363]	1.352	0.260	0.271
Mac035	2 [268–271]	1.080	0.074	0.077
Mac037	2 [294–297]	1.080	0.074	0.077
Ma325	2 [138–140]	1.352	0.260	0.271
Mean	3.083 [138–372]	1.308	0.226	0.235

Phylogenetic placement based on two highly discriminatory molecular markers (i.e., 5′TEF and MzFG543igs) defined seven species of the *Metarhizium* genus (i.e., *M. acridum*, *M. anisopliae*, *M. brunneum*, *M. guizhouense*, *M. pingshaense*, *M. pemphigi*, and *M. robertsii*) for the 45 isolates from the state of Colima, Mexico. Most of the isolates identified in this study belonged to the species *M. acridum*, this lineage exhibited a low intra-specific variation, in this respect only the isolates CHE-CNRCB 198 and 205 were grouped separately in 5′TEF-MzGF543igs BI and 5′TEF BI (Fig. 1; Suppl. Fig. S1). In fact, these isolates differed in a single nucleotide on the sequence of the 5′TEF region (data not shown). Even though *M. acridum* has been successfully applied for the control of a complex of grasshoppers in Mexico (Barrientos-Lozano et al., 2002; Brunner-Mendoza et al., 2018), knowledge about this fungus in natural and agricultural fields remains very limited (Guerrero-Guerra et al., 2012; Hernández-Velázquez et al., 2003), therefore, this study is a first approach about the presence of *M. acridum* in Mexico. *M. anisopliae s.l.* and *M. robertsii* were predominantly identified within the PARB clade, similarly to what was previously reported for South America (Carrillo-Benítez et al., 2013; Rezende et al., 2015; Rocha et al., 2013). In fact, both *Metarhizium* species are the most abundant and geographically widespread globally (Rezende et al., 2015); likewise, intra-specific variation was also observed for both lineages, particularly for *M. robertsii* (Fig. 1) (Kepler et al., 2015). Finally, one isolate was identified as *M. pemphigi* representing the first report for this species in Mexico (Brunner-Mendoza et al., 2017, 2018; Hernández-Domínguez et al., 2016; Hernández-Domínguez and Guzmán-Franco, 2017).

SSR analysis showed a low genetic diversity of *M. acridum* (Table 2). We obtained five genotypes, and one genotype was prevalent ($n = 21$) within the isolates (Fig. 2). Notably, a genotype composed of two isolates (i.e., CHE-CNRCB 198 and 205) was also identified with 5′TEF region (Suppl. Fig. S1), revealing a certain intra-specific variability for the monophyletic clade of *M. acridum*. This confirms the 5′TEF as a marker with a high phylogenetic resolution for the species of *Metarhizium* genus (Bischoff et al., 2009; Rehner and Kepler, 2017). On the other hand, we suggested that the low genetic diversity observed in *M. acridum* was mainly due to its geographical origin even when this information is missing in most of the accessions (Table 1). In fact, even in the analysis to assay the polymorphism of the developed SSR markers to *M. acridum*, the strain CHE-CNRCB 214 from Chiapas, Mexico located in Mexico's Southern Pacific Coast was clustered in the dominant genotype, whereas the strain ARSEF 5736 from Madagascar resulted in a distinct genotype (data not shown). Additionally, the low intra-specific variation could also be explained by the significant influence of biotic factors (i.e., UV-light resistance, elevated temperatures), climatic conditions and the narrow of host range (i.e., Orthoptera order) and fungal-host coevolution of *M. acridum* (Brunner-Mendoza et al., 2018; Gao et al., 2011; Hernández-Velázquez et al., 2003; Meyling and Eilenberg, 2007; Wang et al., 2016). These results confirmed that *M. acridum* is genetically uniform (Driver et al., 2000; Milner et al., 2003), although, a comprehensive population study with a wide range of *M. acridum* strains and different habitats is mandatory.

As we mentioned previously, only *M. anisopliae s.l.*, *M. anisopliae s.s.*, *M. guizhouense*, *M. pingshaense*, and *M. robertsii* have been reported for Mexico (Brunner-Mendoza et al., 2018), nevertheless, we identified two isolates as *M. brunneum*. According to Brunner-Mendoza et al. (2017), until date, *M. brunneum* has not been detected in Mexico, suggesting a low occurrence of this species in the country, indeed, the two isolates identified as *M. brunneum* conformed to a single multilocus SSR genotype (Table 3). On the other hand, *M. robertsii* showed great genetic heterogeneity, i.e., each isolate ($n = 5$) identified corresponded to a single genotype

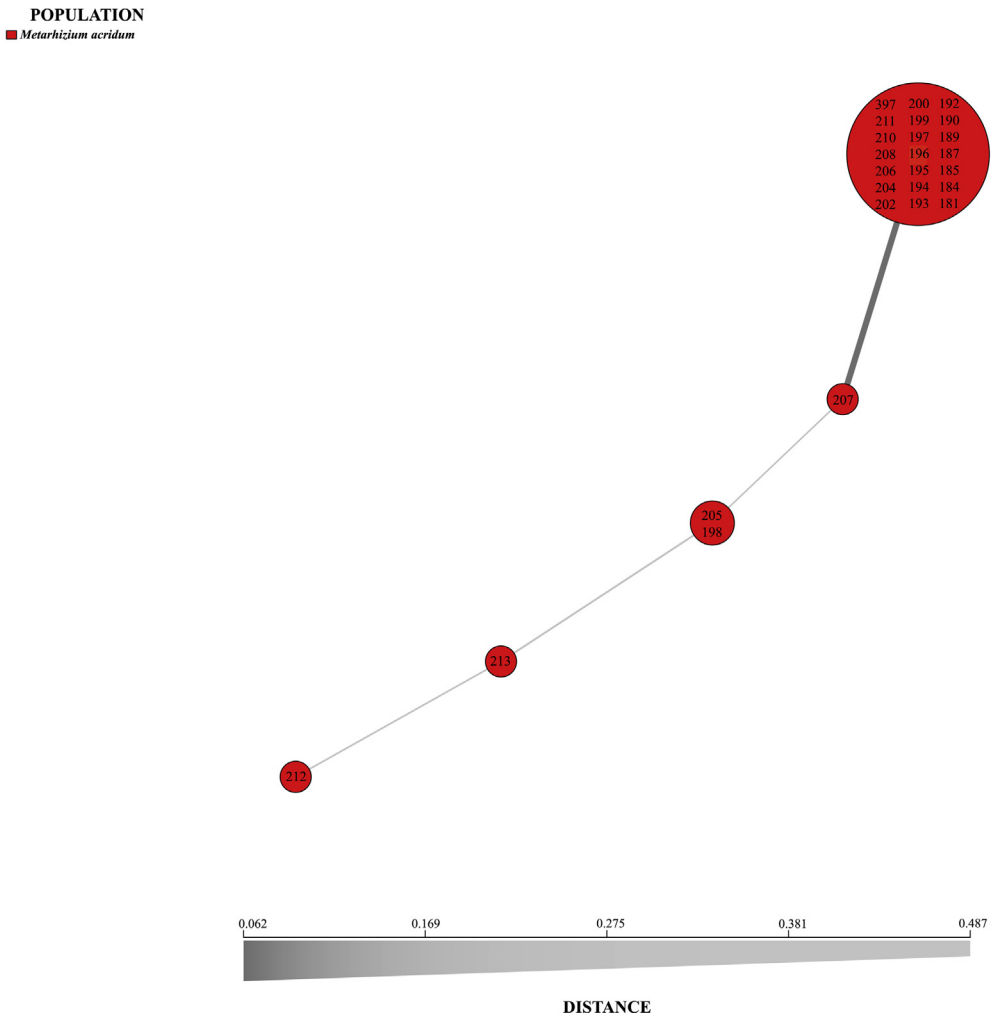

Fig. 2. Minimum spanning network for SSR multilocus genotypes of *Metarhizium acridum* based on Bruvo's genetic distances. The vertex size is proportional to the number of clones recovered for each genotype (one or two isolates and 21 isolates in the dominant genotype). Edge thickness is proportional to Bruvo's genetic distance between two nodes.

Table 3
Descriptive genetic measures through the analysis of 13 SSR loci (i.e., Ma164, Ma195, Ma307, Ma375, Ma416, Ma2054, Ma2055, Ma2063, Ma2064, Ma2089, Ma2097, Ma2108, and Ma2287; Enkerli et al., 2005 and Oulevey et al., 2009) from the *Metarhizium* species identified on PARB and MGT clades in Colima, Mexico.

Clade	Species	No. of isolates	Polymorphic SSR	Monomorphic SSR	Na [Allele size (bp)]	Ne	No. of genotypes
PARB	*M. anisopliae s.l.*	5	6	7	19 [106–300]	1.217	3
	M. anisopliae s.s.	2	1	12	14 [120–294]	1.077	2
	M. brunneum	2	–	13	13 [106–284]	1.000	1
	M. pingshaense	2	–	13	13 [102–290]	1.000	1
	M. robertsii	5	6	7	22 [98–298]	1.588	5
MGT	*M. guizhouense*	2	1	12	14 [104–280]	1.077	2

Na, number of alleles.
Ne, number of effective alleles.

(Table 3). Similar results were also exposed by Kepler et al. (2015) in corn and soybean soils, in fact, the five *M. robertsii* isolates identified in this study were collected from a distinct source of substrate (Table 1) hence this result could provide evidence of an association of a certain genotype with a particular host-plant species. Nevertheless, no specific genotype association with host-plant was observed for *M. brunneum* and *M. robertsii* (Steinwender et al., 2014, 2015).

In summary, the present study identified seven species belonging to *Metarhizium* genus from 45 isolates harbored in CHE of CNRCB from the state of Colima, Mexico. For the first time, we recorded the presence of *M. brunneum* and *M. pemphigi* in Mexico. On the other hand, *M. acridum* was the most predominant species identified on the *M. anisopliae* complex and according to SSR analysis showed low genetic diversity. Likewise, we developed and reported 29 informative SSR loci for *M. acridum*, this is a first approach towards the determination of genetic diversity for this *Metarhizium* species. We suggest a comprehensive population study that includes a large number of *M. acridum* strains along the country in other regions with different ecological conditions. Such knowledge could contribute to the development of molecular tools for monitoring specific strains or isolates released as BCA against

specific pests in agroecosystems or in enhancing the understanding about specific diversity of *Metarhizium* genus.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.09.005.

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