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Metarhizium humberi sp. nov. (Hypocreales: Clavicipitaceae), a new member of the PARB clade in the *Metarhizium anisopliae* complex from Latin America

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ABSTRACT

A new species, *Metarhizium humberi*, from the *M. anisopliae* complex and sister lineage of the *M. anisopliae* s.str. in the PARB clade, including *M. pingshaense*, *M. anisopliae*, *M. robertsii* and *M. brunneum*, is described based on phylogenetic analyses [translation elongation factor 1-alpha (5'TEF and 3'TEF), RNA polymerase II largest subunit (RPB1a), RNA polymerase II second largest subunit (RPB2a) and β-tubulin (BTUB)]. *Metarhizium humberi* was first collected in 2001 in the Central Brazilian state of Goiás, later found to be a common fungus in soils in Brazil, and since then has also been isolated from coleopteran, hemipteran and lepidopteran insects in Brazil and Mexico. This new species, named in honor of Richard A. Humber, a well-known insect pathologist and taxonomist of entomopathogenic fungi, is characterized by a high insecticidal activity against different developmental stages of arthropod pests with importance in agriculture and vectors of diseases to human and animals.

1. Introduction

The genus *Metarhizium* contains well-known entomopathogenic fungi that occur mainly in soils (Lenteren et al., 2018) but that are also common as endophytes and in the rhizosphere associates of plants (Bamisile et al., 2018). There is high interest in these fungi for the control of agricultural or vector pests and as plant growth promoters and nutrient recyclers (Vitorino and Bessa, 2017; Karabörklü et al., 2018; Mascarin et al., in press; Thomas, 2018). Studies on phylogenetics, distribution and ecology of species in the genus *Metarhizium* increased in the last years (Rocha et al., 2013; Rezende et al., 2015; Brunner-Mendoza et al., 2017; Hernández-Domínguez and Guzmán-Franco, 2017; Kryukov et al., 2017; Rehner and Kepler, 2017; Masoudi et al., 2018; Iwanicki et al., 2019), and more new species have been described from China (Chen et al., 2017, 2018a, 2018b; Chu et al., 2016; Yang et al., 2009), Thailand (Luangsa-ard et al., 2017), Japan (Nishi et al., 2017), Brazil (Montalva et al., 2016; Lopes et al., 2018) and Argentina (Gutierrez et al., 2019). The last major revision of the genus *Metarhizium* was published by Kepler et al. (2014) and highlighted the superiority of the multigene phylogenetic approach for determination of species boundaries and relationships in *Metarhizium*.

The *Metarhizium anisopliae* species complex currently consists of 13 known species with *M. pingshaense*, *M. anisopliae* s.str., *M. robertsii* and *M. brunneum* (collectively referred to as the PARB clade, Bischoff et al., 2009), *M. acridum*, *M. globosum*, *M. guizhouense*, *M. indigoticum*, *M. majus, M. lepidiotae,* and the recently described species *M. kalasinense*, isolated in 2012 from an elaterid larva (Coleoptera) collected in a tropical forest in Thailand (Luangsa-ard et al., 2017), *M. alvesii* isolated in 2009 from a soil sample collected in a banana plantation in northeastern Brazil (Lopes et al., 2018), and *M. baoshanense* from soil of native forest in southwestern China (Chen et al., 2018a).

Several *M. anisopliae* s.l. isolates that originated from soil samples collected in the Brazilian Cerrado biome and other locations in Brazil and from a wide range of insects have been demonstrated to represent a novel lineage within the *M. anisopliae* species complex based on molecular analyses. The genomic evidence that separates isolates of this clade from *M. anisopliae* s.str. includes sequences of the 5'intron-rich region of the translation elongation factor 1-alpha (5'TEF), internal

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transcribed spacer (ITS) or the nuclear intergenic region (Rocha et al., 2013; Rezende et al., 2015) and mass spectrometric (MALDI-TOF) studies (Lopes et al., 2014). Recently, two more isolates of this new clade were collected from lepidopteran specimens in Mexico (Brunner-Mendoza et al., 2017). An isolate from this clade, IP 46, originally identified as *M. anisopliae* has been investigated extensively for its promising activity against such vector insects of diseases in humans (Mascarin et al., in press), as the mosquitoes *Aedes aegypti* and *Anopheles gambiae* (Silva et al., 2004; Albernaz et al., 2009; Santos et al., 2009; Leles et al., 2010, 2012; Mnyone et al., 2009, 2010; Sousa et al., 2013; Lobo et al., 2016; Falvo et al., 2016, 2018; Rodrigues et al., 2019), triatomine vectors of Chagas disease (Rocha and Luz, 2011; Luz et al., 2012; Rodrigues et al., 2015), cockroaches (Hubner-Campos et al., 2013; Gutierrez et al., 2016), as well as tick vectors (Luz et al., 2016), and even against *Biomphalaria glabrata*, the molluscan intermediate host of schistosomiasis (Duarte et al., 2015).

This study presents a multi-locus phylogenetic analysis to determine the placement of this important clade in the *M. anisopliae* complex, and to describe it as a new species, *M. humberi,* that we name in honor of Richard A. Humber, a well-known insect pathologist and taxonomist of invertebrate-associated fungi and for his support of the progress of insect mycology and training of scientists in Brazil.

2. Material and methods

2.1. Origin and culture of IP isolates

All nine Brazilian IP strains studied (IP 1; IP 16; IP 41; IP 46; IP 59; IP 86; IP 101; IP 118; and IP 151) were isolated from soil samples collected in Central Brazil (Rocha et al., 2013) and grown routinely on SDAY/4 medium (SDAY/4: 2.5 g L^{-1} peptone, 10 g L^{-1} dextrose, 2.5 g L^{-1} yeast extract, 20 g L^{-1} agar) in Petri dishes (100 × 20 mm) for 15 days at 25 ± 1 °C and 12 h photophase. The fungi were stored in the IPTSP and co-deposited in Embrapa Genetic Resources and Biotechnology, Brasília, Brazil, and in the USDA Collection of Entomopathogenic Fungi (Ithaca, NY).

2.2. Morphological evaluations

IP 46 was investigated based on morphological characteristics using semi-permanent slide mounts prepared in lactophenol-cotton blue according to Humber (2012). The isolate was grown on SDAY/4 medium for 5–7 days at 25 ± 1 °C and 12 photophase. Fungal microstructures (conidiophores, conidiogenous cells, and conidia) were examined by brightfield or phase contrast microscopy (Nikon Eclipse E600), documented with a Nikon DS-Fi1 digital camera, and measured with Motic Images Plus 2.0 software. Measurements were based on 50 objects per microstructure from which we calculated mean values and their respective standard errors of the mean (± SEM). The color of the conidial mass was determined using the Pantone color system (Eiseman and Herbert, 1990).

2.3. Molecular characterization

The nine IP isolates (Table 1) were grown in 150 mL in SDY/4 broth for 7 days in a shaker at 125 rpm and 25 ± 1 °C. DNA was extracted from mycelium using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Partial sequences of the following four genes were amplified by PCR: β-tubulin (BTUB) using the primers BT1F and BT1R (Bischoff et al., 2009); RNA polymerase II largest subunit (RPB1a) with RPB1C and RPB1Af (Stiller and Hall, 1997); RNA polymerase II second largest subunit (RPB2a) with fRPB2-5F and RPB2-7cR (Liu et al., 1999); and translation elongation factor 1 alpha [3' end of the TEF-1α (3'TEF)] with primers 983F and 2218R (Rehner and Buckley, 2005). The 5' end of TEF-1α (5'TEF) was previously sequenced by Rocha et al. (2013). The PCR products were checked using agarose gel electrophoresis and sent for purification and sequencing by Helixxa Genomic Services (Paulínia, SP, Brazil). Sequencing of both strands of the PCR products was accomplished with the Applied Biosystems Big Dye v.3.1 kit, using the same primers described above and an ABI 3500 automatic sequencer. Contigs of the isolates sequence data were assembled using Chromas Pro (v. 1.5, Technelysium Pty Ltd).

Sequences from ex-type cultures or taxonomically authenticated reference isolates used in two studies about taxonomic re-evaluations of *Metarhizium* (Bischoff et al., 2009, Kepler et al., 2014) and other sequences were obtained from GenBank database with information about host or substrate and geographical origin presented in Table 1. Multiple sequence alignments of each gene were made with Mega 5.0.3 by ClustalW and adjusted. The program MrModeltest (Nylander, 2004) obtained by PAUP (Phylogenetic Analysis Using Parsimony; v.4.0 b10) was used to identify the best-fit models of nucleotide substitutions using the corrected Akaike Information Criteria for each gene. A concatenated alignment [(3'TEF (GTR + I + G), 5'TEF (HKY + I), RPB1a (K80 + G), RPB2a (*SYM* + I + G) and BTUB (GTR + I)] was generated with Mesquite 3.04 software (Maddison and Maddison, 2015). Analyses of the consensus sequences of 5'TEF and the concatenated alignment were carried out under the Maximum Parsimony (MP) method, and bootstrap support (BS) values were provided. Additionally, we used Bayesian phylogenetic inference by MrBayes v.3.2.1 (Ronquist et al., 2012), and posterior probability values were included in the Bayesian trees. Bayesian analysis was run over ten million generations, with tree sampling every 100 generations, and the first 25% of trees were discarded prior to consensus tree calculation.

3. Results

3.1. Morphological identification of the Metarhizium strain

Taxonomy— *Metarhizium humberi* Luz, Rocha & Delalibera sp. nov. (Fig. 1a – c)

MycoBank registration: MB 828706

The colonies on SDAY/4 were initially colourless, and became increasingly yellow immediately below developing conidial hymenia (typically after 5–8 days) and then producing plate-like masses of laterally appressed conidial chains with a grey-green color after 5 days (Pantone 15-6414, “Reseda”; similar to CMYK 54:70:70:10 at 83% opacity) and with the conidial mass becoming slightly darker in color in the next days (Fig. 1c). Conidiogenous cells ovoid to broadly ellipsoid, 10.08 ± 0.59 × 2.09 ± 0.06 µm (overall range: 6.60–12.85 × 1.77–2.45 µm) (Fig. 1a). Conidia cylindrical, 5.17 ± 0.05 × 2.22 ± 0.03 µm (overall range: 4.14–6.05 × 1.69–2.59 µm) (Fig. 1 b).

Holotype: UFG 50751, is a dried culture of IP 46 deposited in the Herbarium of the Federal University of Goiás, Goiânia, GO, Brazil.

Ex-Type culture: IP 46, Collection of Entomopathogenic Fungi, at the Institute of Tropical Pathology and Public Health (Goiânia, Goiás, Brazil), collected by Christian Luz, Luiz Fernando Nunes Rocha, Regiane Oliveira Silva and Martin Unterseher, 14 September 2001, and co-deposited as CG620 in the Invertebrate-Associated Fungal Collection (CFI) at Embrapa Genetic Resources and Biotechnology (Brasília, Federal District, Brazil), and also co-deposited as ARSEF 12874 in the USDA-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, New York).

Type locality: Parque Nacional das Emas, Goiás State, Brazil; S 18°10′56.1″, W 52°44′34.5″.
Type substrate: soil sample from a tropical gallery forest in a Cerrado ecosystem.
Sexual state: unknown.

Etymology: *M. humberi* is named in honor of Richard Alan Humber,

Table 1
Reference of *Metarhizium* spp. strains used in phylogenetic analyses and their isolation source, country of origin and Genbank accession numbers.

Species	Strain code[a]	Substrate	Origin	Accession number				
				3'TEF	5'TEF	RPB1a	RPB2a	BTUB
M. acridum	ARSEF 324	Orthoptera	Australia	EU248844	EU248844	EU248896	EU248924	EU248812
M. acridum	ARSEF 7486[b]	Orthoptera	Niger	EU248845	EU248845	EU248897	EU248925	EU248813
M. alvesii	CG1123[b]	soil	Brazil	KY007614	KC520541	KY007612	KY007613	KY007611
M. anisopliae	ARSEF 6347	Homoptera	Colombia	—	EU248881	—	—	—
M. anisopliae	ARSEF 7450	Coleoptera	Australia	EU248852	EU248852	EU248904	EU248932	EU248823
M. anisopliae	ARSEF 7487[b]	Orthoptera	Ethiopia	DQ463996	DQ463996	DQ468355	DQ468370	EU248822
M. anisopliae	CHE CNRCB 235	Hemiptera	Mexico	KU725694	KU725694	KU725697	KU725702	KU725705
M. anisopliae	ESALQ 1614	soil	Brazil	—	KP027962	—	—	—
M. anisopliae	ESALQ 1617	soil	Brazil	—	KP027957	—	—	—
M. baoshanense	CCTCC M 2016589	soil	China	KY264169	—	KY264180	KY264183	—
M. baoshanense	BUM 63.4[b]	soil	China	KY264170	—	KY264181	KY264184	—
M. brunneum	ARSEF 2107[b]	Coleoptera	USA	EU248855	EU248855	EU248907	EU248935	EU248826
M. brunneum	ARSEF 4179	soil	Australia	EU248854	EU248854	EU248906	EU248934	EU248825
M. frigidum	ARSEF 4124[b]	Coleoptera	Australia	DQ464002	DQ463978	DQ468361	DQ468376	EU248828
M. globosum	ARSEF 2596[b]	Lepidoptera	India	EU248846	—	EU248898	EU248926	—
M. guizhouense	ARSEF 6238	Lepidoptera	China	EU248857	EU248857	EU248909	EU248937	EU248830
M. guizhouense	CBS 258.90[b]	Lepidoptera	China	EU248862	EU248862	EU248914	EU248942	EU248834
M. humberi	CG814	Coleoptera	Brazil, PR[c]	—	KF357928	—	—	—
M. humberi	CG835	Hemiptera	Brazil, MT[d]	—	KF357929	—	—	—
M. humberi	CG1233	Coleoptera	Brazil, GO[e]	—	KC832296	—	—	—
M. humberi	EH853	Lepidoptera	Mexico, TAM[f]	—	KY616806	—	—	—
M. humberi	EH874	Lepidoptera	Mexico, OAX[g]	—	KY616808	—	—	—
M. humberi	ESALQ 1638	soil	Brazil, GO	—	KP027955	—	—	—
M. humberi	ESALQ 1657	soil	Brazil, SP[h]	—	MH596726	—	—	—
M. humberi	ESALQ 4614	soil	Brazil, MT	—	MH719718	—	—	—
M. humberi	ESALQ 4829	soil	Brazil, GO	—	MH719700	—	—	—
M. humberi	ESALQ 4925	soil	Brazil, MT	—	MH719696	—	—	—
M. humberi	IP 1	soil	Brazil, GO	MH837571	JQ061188	MH837553	MH837562	MH837544
M. humberi	IP 16	soil	Brazil, GO	MH837572	JQ061196	MH837554	MH837563	MH837545
M. humberi	IP 41	soil	Brazil, GO	MH837573	JQ061199	MH837555	MH837564	MH837546
M. humberi	IP 46[b]	soil	Brazil, GO	MH837574	JQ061205	MH837556	MH837565	MH837547
M. humberi	IP 59	soil	Brazil, GO	MH837575	JQ061187	MH837557	MH837566	MH837548
M. humberi	IP 86	soil	Brazil, GO	MH837576	JQ061186	MH837558	MH837567	MH837549
M. humberi	IP 101	soil	Brazil, GO	MH837577	JQ061195	MH837559	MH837568	MH837550
M. humberi	IP 118	soil	Brazil, GO	MH837578	JQ061185	MH837560	MH837569	MH837551
M. humberi	IP 151	soil	Brazil, GO	MH837579	JQ061208	MH837561	MH837570	MH837552
M. indigoticum	NBRC 100684	Lepidoptera	Japan	KJ398784	—	KJ398544	KJ398692	KJ398544
M. indigoticum	TNS-F 18553	Lepidoptera	Japan	JF416010	—	JN049886	JF415992	KJ398569
M. kalasinense	BCC53581	Coleoptera	Thailand	—	KX823944	—	—	—
M. kalasinense	BCC53582[b]	Coleoptera	Thailand	—	KX823945	—	—	—
M. lepidiotae	ARSEF 7412	Coleoptera	Australia	EU248864	EU248864	EU248916	EU248944	EU248836
M. lepidiotae	ARSEF 7488[b]	Coleoptera	Australia	EU248865	EU248865	EU248917	EU248945	EU248837
M. majus	ARSEF 1914[b]	Coleoptera	Philippines	KJ398801	KJ398801	KJ398610	KJ398708	KJ398571
M. majus	ARSEF 1946	Coleoptera	Philippines	EU248867	EU248867	EU248919	EU248947	EU248839
M. pingshaense	ARSEF 4342	Coleoptera	Solomon Islands	EU248851	EU248851	EU248903	EU248931	EU248821
M. pingshaense	CBS 257.90[b]	Coleoptera	China	EU248850	EU248850	EU248902	EU248930	EU248820
M. robertsii	ARSEF 23	Coleoptera	USA	—	KX342726	—	—	—
M. robertsii	ARSEF 727	Orthoptera	Brazil	DQ463994	DQ463994	DQ468353	DQ468368	EU248816
M. robertsii	ARSEF 4739	soil	Australia	—	EU248848	—	—	—
M. robertsii	ARSEF 7501	Coleoptera	Australia	EU248849	EU248849	EU248901	EU248929	EU248818
M. robertsii	ESALQ 1621	soil	Brazil	—	KP027980	—	—	—
M. robertsii	ESALQ 1625	soil	Brazil	—	KP027974	—	—	—
M. robertsii	ESALQ 1634	soil	Brazil	—	KP027971	—	—	—
M. robertsii	ESALQ 1635	soil	Brazil	—	KP027977	—	—	—

[a] Abbreviations for collections: ARSEF, USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA; BCC, BIOTEC Culture Collection, Microbe Interaction and Ecology Laboratory, BIOTEC, National Science and Technology Development Agency, KhlongLuang, PathumThani, Thailand; BUM, Baoshan University, Baoshan, China; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CG, Invertebrate-Associated Fungal Collection at Embrapa Genetic Resources and Biotechnology, Brasília, Brazil; CCTCC, China Center fot Type Culture Collection, Wuhan University, Wuhan, China; CHE CNRCB, Centro Nacional de Referencia de Control Biológico, Tecomán, Colima, Mexico; EH, Laboratorio de Micología Básica, Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad de México, Mexico; ESALQ, Collection of Entomopathogenic Microorganisms "Prof. Sérgio Batista Alves" at Department of Entomology and Acarology, Luiz de Queiroz College of Agriculture, University of São Paulo (ESALQ/USP), Piracicaba, São Paulo, Brazil; IP, Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia, Goiás, Brazil; NBRC, National Institute of Technology and Evaluation, Biological Resource Center, Chiba, Japan; TNS, National Museum of Science and Nature, Tsukuba, Japan

[b] Ex-type isolates.

[c] Paraná State.

[d] Mato Grosso State.

[e] Goiás State.

[f] Tamaulipas State.

[g] Oaxaca State.

[h] São Paulo State.

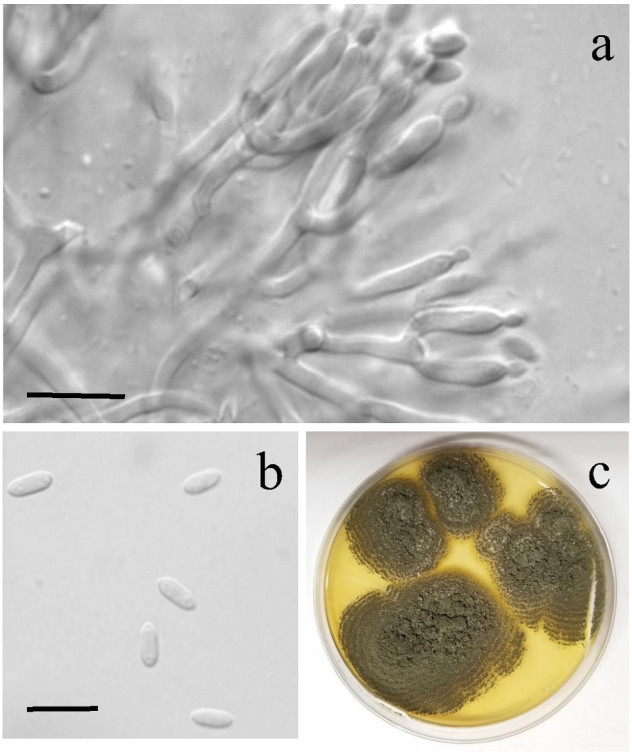

Fig. 1. *Metarhizium humberi*: mature phialides with conidiogenous cells and conidia (a); conidia (b) and 15-day old culture on SDAY/4 medium at 25 °C and 12 h photoperiod (c). Bar (Fig. 1 a, b) = 10 µm.

internationally recognized insect mycologist and formerly curator of the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) at the USDA-ARS Robert W Holley Center for Agriculture & Health in Ithaca, NY, USA. The co-authors of this publication agreed to apply this species epithet prior to informing Dr. Humber. We recognize his long experience and merit in insect pathology and taxonomy of entomopathogenic fungi, especially in Entomophthoromycota, and his long and much appreciated services in support of the progress of insect mycology and training of scientists in Brazil.

3.2. Molecular characterization

The alignment of the characters obtained from partial sequencing of five loci comprised 3998 base pairs (5'TEF: 708 bp, 3'TEF: 922 bp, BTUB: 652 bp, RPB1a: 675 bp and RPB2a: 1041 bp). The Bayesian and MP phylogenetic analysis of these combined loci produced a strongly supported and distinct specific branch named *M. humberi*, consisting of all tested Brazilian IP strains (IP 1, IP 16, IP 41, IP 46, IP 59, IP 86, IP 101, IP 118 and IP 151). *M. humberi* clustered as a distinctly separate group nested within the PARB clade and as a sister group to *M. anisopliae* s.str. (Fig. 2). High bootstrap values of 89% (MP) and 1 (Bayesian posterior probability) were obtained for the clade in which the strains described above are placed. The MP and Bayesian analyses produced slightly divergent topologies in relation to the clustering among Brazilian strains with six haplotypes in the Bayesian inference (Fig. 2) while the MP analysis indicated only four Brazilian haplotypes (Mhum 1 = IP 86; Mhum 2 = IP 46; Mhum 3 = IP 151; Mhum 4 = IP 1, IP 16, IP 41, IP 59, IP 101 and IP 118) in the MP analysis.

The phylogenetic analysis using the 5'TEF sequences of the IP strains and other strains from widely dispersed sites in Brazil and Mexico (Rocha et. al., 2013; Lopes et al., 2014; Rezende et al., 2015; Brunner-Mendoza et al., 2017), showed all 19 strains to cluster within the new species, with 88% bootstrap support and 1 Bayesian posterior probability (Fig. 3). This analysis also completely confirmed the currently recognized species limits in the *M. anisopliae* complex and that *M. humberi* is, indeed, a genomically distinct new species within the PARB complex. Not surprisingly for so relatively common and widely distributed a species, *M. humberi* currently comprises nine haplotypes based on 5'TEF sequences that are designated as H1 through H9. Six of these haplotypes (H1–H6) originated from isolates recovered only in soil samples from distantly separated regions in Brazil: H1 (IP 118), H2 (IP 101), H3 (IP 86 and IP 59) and H4 (ESALQ 4829) collected in the State of Goiás. H5 (ESALQ 4614) and H6 (ESALQ 4925) originated from the State of Mato Grosso. H7 was found both in soil samples in Goiás (IP 41 and IP 16) and from a coleopteran insect (CG814) from the State of Paraná; H8 was recovered from soil in Goiás (IP 1) and from a hemipteran insect in the State of Mato Grosso (CG835). H9, the last and most frequently encountered haplotype (CG1233, IP 46, IP 151, ESALQ 1657, ESALQ 1638, EH 853 and EH 874), was detected either in soils or from cadavers of two insect orders from several different States in Brazil as well as from Mexico (Table 1). Other phylogenetic trees obtained by Bayesian analysis of 3'TEF, RPB1a, RPB2a, and BTUB between *M. humberi* IP 46 and the members of the PARB clade are available as supplementary material (Suppl. 1–4).

4. Discussion

Results of the multilocus analysis clearly support the recognition of *M. humberi* as a new species closely allied with M. *anisopliae* s.str. in the PARB clade of the *M. anisopliae* complex. In fact, 3'TEF, 5'TEF, RPB1a, RPB2a and BTUB are considered the principal genes for distinguishing individual species in the genus *Metarhizium* (Bischoff et al., 2009; Kepler et al., 2014). The description of *M. humberi* as a new species is also supported by sequencing the ITS, MzIGS3 and MZFG543igs regions as well as by mass spectrometric data (Rocha et al., 2013; Lopes et al., 2014; Rezende et al., 2015). Although both molecular and mass spectrometric findings provide independent evidence of a new species, *M. humberi* cannot be distinguished by its characteristic asexual reproductive morphologies from other fungi of the PARB clade examined by Bischoff et al. (2009).

This new species, together with *M. robertsii* (named after Donald W. Roberts, Emeritus Research Professor of the Utah State University, USA; Bischoff et al., 2009) and *M. alvesii* (named after Sérgio B. Alves from the University of São Paulo, Brazil; Lopes et al., 2018) increases the group of species described in the genus *Metarhizium* dedicated to honorable and influential researchers who contributed decisively to the development of insect mycology in Brazil.

Extensive biological survey data now suggest that the most common species of *Metarhizium* in Brazilian soils is *M. robertsii* followed by *M. humberi* and *M. anisopliae* s.str. (Rocha et al., 2013; Lopes et al., 2013, 2014; Rezende et al., 2015; Zanardo, 2015; Castro, 2016; Moreira, 2016; Iwanicki et al., 2019). Recent reports from Mexico (Brunner-Mendoza et al., 2017) proved that the occurence of *M. humberi* is not restricted only to Brazil, but suggest that this new species probably occurs in other regions in the Americas with tropical climate and distinct rainy and dry seasons (Kottek et al., 2006). This new species was referred to by Rezende et al. (2015) as *Metarhizium* sp. indet. 1 and forms a strongly supported group sharing the same haplotype with other ESALQ strains based on its 5'TEF gene which Bischoff et al. (2009) regarded at that time to be the most informative for distinguishing individual species in the genus. In a study, using 303 MzIG3 sequences originated from five Brazilian biomes (Zanardo, 2015) *Metarhizium* sp. indet. 1 (now identified as *M. humberi*) has shown higher haplotype and nucleotide diversities than *M. robertsii*, *M. anisopliae*, *M. pingshaense* and two other lineages–*Metarhizium* sp. indet. 2 and *Metarhizium* sp. indet. 3– whose taxonomies remain incompletely characterized.

Of the 126 isolates currently identified as *M. humberi*, the vast majority (98.4%) were reported from the Brazilian states of Goiás (67.5%), Minas Gerais (19%), Mato Grosso (8.7%), São Paulo (2.4%), and Paraná (0.8%). Two other isolates were found in Mexico where

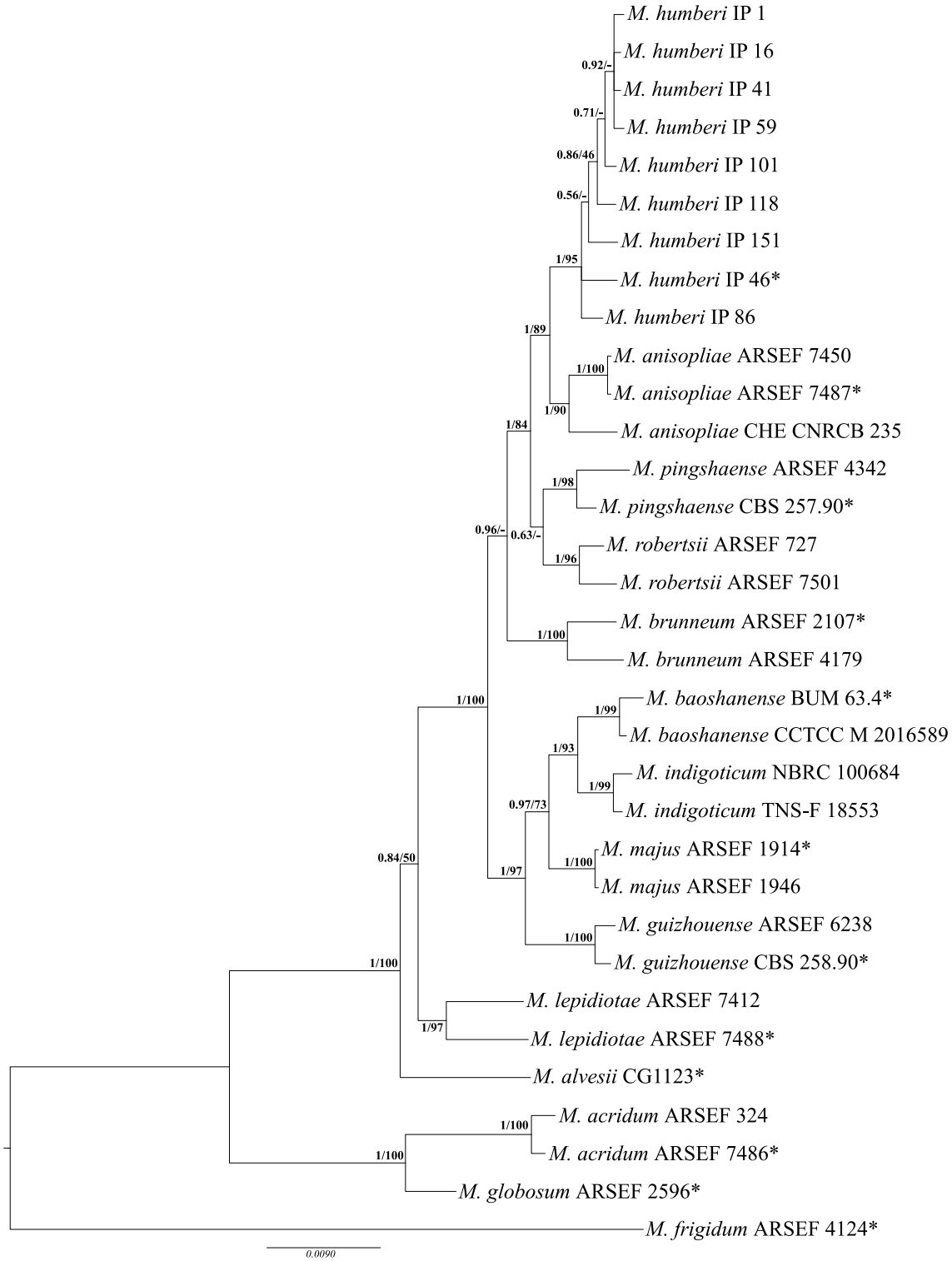

Fig. 2. Majority rule consensus phylogram from the Bayesian analysis of a concatenated dataset comprising partial 5'TEF, 3'TEF, RPB1a, RPB2a and BTUB gene sequences. Trees were rooted using the sequence from *Metarhizium frigidum* ARSEF 4124 as outgroup. Support for branches were given as the Bayesian posterior probability (first number) and percentage of bootstrap support derived from a MP analysis (second number). "−" indicates the inexistence of support value since trees from MP and Bayesian analyses do not have similar topology in the specified branch; * represents the ex-type strains and the scale bar the number of expected substitutions per site.

single isolates were collected from each of the states of Tamaulipas and Oaxaca (Fig. 4) (Rocha et al., 2013; Lopes et al., 2014; Rezende et al., 2015; Zanardo, 2015; Castro, 2016; Moreira, 2016; Brunner-Mendoza et al., 2017; Iwanicki et al., 2019).

Some 95.2% of known *M. humberi* strains were isolated from soil samples, with 60.8% of those is from predominantly conserved areas of native savanna in Central Brazil's Cerrado biome. Nonetheless, this species is not restricted to natural soils in the Cerrado biome but occurs also in other ecosystems and from cultivated agricultural soils. In the Cerrado, only 10.8% of the strains were collected from agricultural

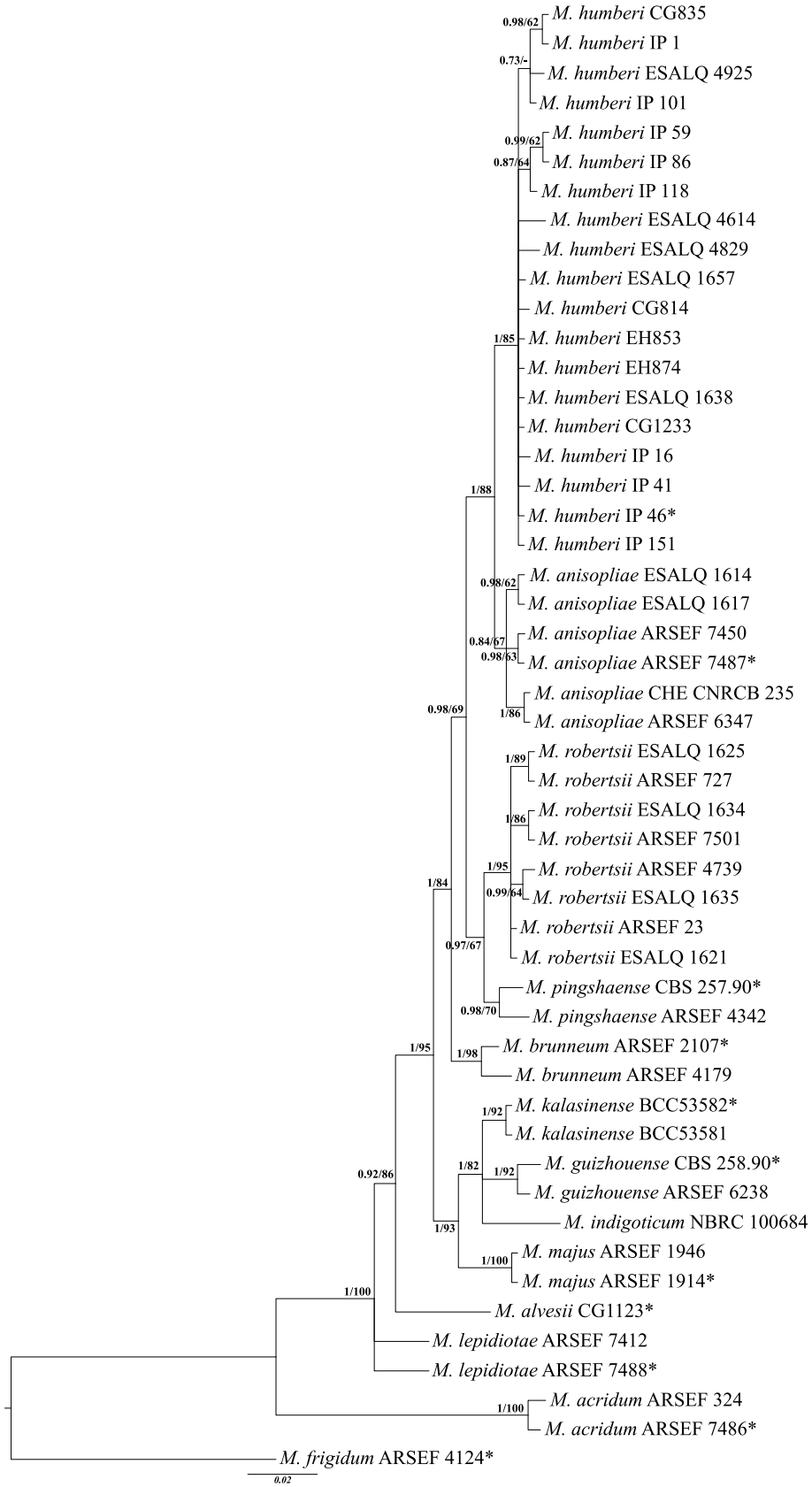

Fig. 3. Phylogenetic hypothesis based on Bayesian analysis of 5'intron-rich region of the translation elongation factor 1-alpha (5'TEF) gene sequences. Trees were rooted using the sequence from *Metarhizium frigidum* ARSEF 4124 as outgroup. Support values were given as the Bayesian posterior probability (first number) and percentage of bootstrap support derived from a MP analysis (second number). "−" indicates the inexistence of support value since trees from MP and Bayesian analyses do not have similar topology in the specified branch; * represents the ex-type strains and the scale bar the number of expected substitutions per site.

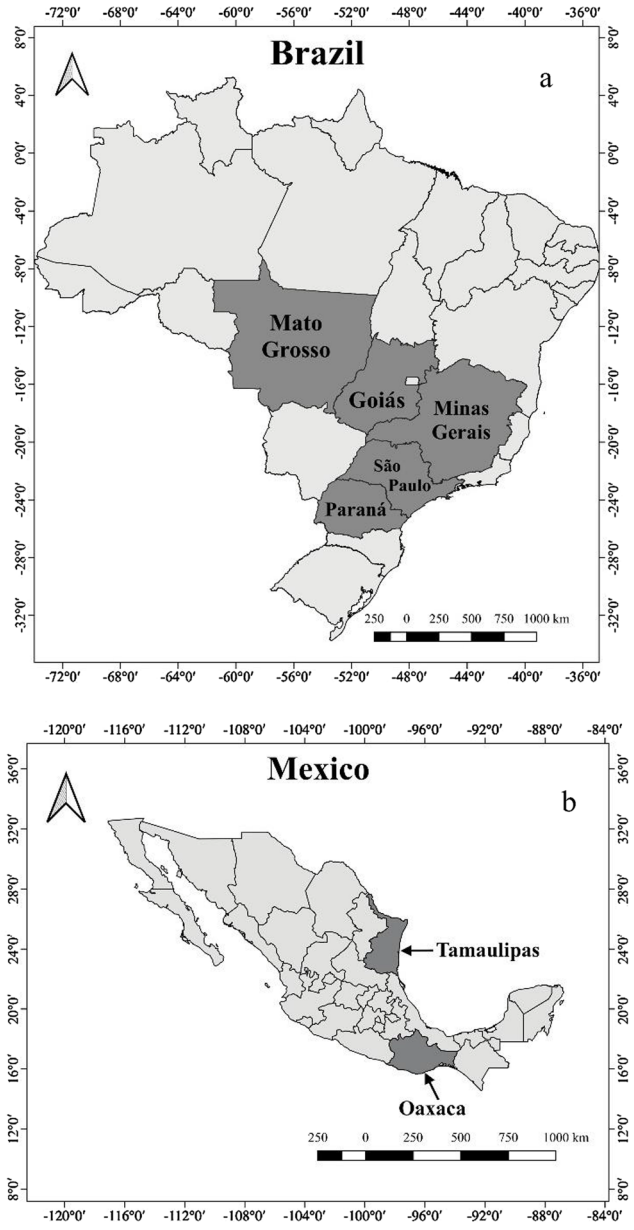

Fig. 4. Maps indicating the locations of the collecting regions in the states in Brazil (a) and Mexico (b) from which *Metarhizium humberi* has been recovered.

plantations, mostly growing sugarcane or soybean. Another 8.4% of isolates originated from tropical forest soils in Brazil with 3.4% in native vegetation and 5% in agricultural areas. These results seem to be highly influenced by the focus of surveys on soils from conserved areas. Other isolates were detected in soils sampled in agricultural areas from mountainous or wet lands in Minas Gerais, Brazil (Rocha et al., 2013; Lopes et al., 2014; Rezende et al., 2015; Zanardo 2015; Castro, 2016; Moreira, 2016; Brunner-Mendoza et al., 2017; Iwanicki et al., 2019).

Isolates of *M. humberi* from soil samples collected in Brazil and Mexico were isolated using semi-selective media or baited with *Triatoma infestans* (Reduviidae, Hemiptera), *Galleria mellonella* (Pyralidae, Lepidoptera) and *Tenebrio molitor* (Tenebrionidae, Coleoptera). For these studies, bait insects were either exposed directly to soils or to fungal colonies growing on semi-selective Chase medium after inoculation with filtered aqueous suspensions of soil. Fungal cultures were then isolated from dead individual bait hosts that were incubated in a humid chamber (Luz et al., 2004; Rocha et al., 2013; Rezende et al., 2015; Zanardo, 2015; Castro, 2016; Moreira, 2016; Iwanicki et al., 2019).

Apart from soils, a total of 4.8% of all *M. humberi* isolates were isolated from a diverse spectrum of mycotized insect from the orders Coleoptera (33.3%), Hemiptera (33.3%) and Lepidoptera (33.3%) in field crops in Brazil and Mexico (Lopes et al., 2014; Rezende et al., 2015; Brunner-Mendoza et al., 2017; Table 1). These insect-derived isolates were not genomically different from the soil-derived isolates.

That *M. humberi* is, in fact, distributed across Brazil and elsewhere in the Americas and recoverable from soil samples as well as from diverse diseased insects both underscores and amplifies the interest about its possible roles in the natural environment. The large body of research involving IP 46 confirms *M. humberi* to be a highly effective pathogen affecting such a wide a range of invertebrate hosts. These facts also indisputably place this new taxon among an elite group of fungal taxa with broad suitability for use as biological control agents against dipteran, hemipteran, lepidopteran and coleopteran pests of medical, veterinary and agricultural importance. These potential target pests include mosquitoes, triatomines, cockroaches, ticks and even snails.

In addition to IP 46, another strain of *M. humberi*–ESALQ 1638, *Metarhizium* sp. indet. 1–has been extensively studied and proved to be highly virulent against the two-spotted spider mite, *Tetranychus urticae* (Castro et al., 2018). This ESALQ isolate of *M. humberi* is under consideration for registration as a mycoinsecticide in Brazil (Italo Delalibera Jr., personal communication), and its rhizosphere competence has been demonstrated in sugarcane, strawberry and soybean plants. The potential for ESALQ 1638 to enhance plant growth while simultaneously providing protection against insect pests and plant pathogens has been demonstrated (Italo Delalibera Jr., personal communication). The approvals for registration in Brazil of biological control agents require the explicit and unambiguous identification to the species level of any microbe being used. The results of the studies reported here to describe and to characterize *M. humberi* specifically facilitate a current effort in Brazil to register a new biopesticide product based on *M. humberi* for use against pests.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2019.107216.

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