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Natural occurrence in Argentina of a new fungal pathogen of cockroaches, *Metarhizium argentinense* sp. nov.

Alejandra C. Gutierrez [a, *], Andreas Leclerque [b], Romina G. Manfrino [a], Christian Luz [c], Walter A.O. Ferrari [a], Jorge Barneche [a], Juan J. García [a, d], Claudia C. López Lastra [a]

[a] Centro de Estudios Parasitológicos y de Vectores – CEPAVE (CONICET, Consejo Nacional de Investigaciones Científicas; UNLP, Universidad Nacional de La Plata), La Plata, 1900, Buenos Aires, Argentina
[b] Institute for Microbiology and Biochemistry, Geisenheim University, Geisenheim, D-65366, Germany
[c] Instituto de Patologia Tropical e Saúde Pública (IPTSP), Universidade Federal de Goiás (UFG), Goiânia, 74605-050, Goiás, Brazil
[d] Comisión de Investigaciones Científicas (CIC), La Plata, 1900, Buenos Aires, Argentina

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ABSTRACT

The aim of this study was to search for entomopathogenic fungi that infect wild cockroaches in forest ecosystems in two protected natural areas of Argentina. Two isolates of *Metarhizium argentinense* were obtained and identified from wild cockroaches (*Blaberidae*: *Epilamprinae*) through the use of morphological characteristics and molecular phylogenetic analyses. This novel species was found in Argentina and is a member of the *Metarhizium flavoviride* species complex. Phylogenetic analyses, based on sequence similarity analysis using internal transcribed spacer (ITS) and a set of four protein-coding marker sequences (*EF1A*, *RPB1*, *RPB2* and *BTUB*), supported the status of this fungus as a new species. In addition, we tested the biological activity of the new species through assays against *Blattella germanica* nymphs and found that the two evaluated isolates were pathogenic. However, isolate CEP424 was more virulent and caused a confirmed mortality of 76 % with a median lethal time of 7.2 d. This study reports the southernmost worldwide location of a *Metarhizium* species that infects cockroaches and will help expand the knowledge of the biodiversity of pathogenic fungi of Argentine cockroaches.

1. Introduction

Approximately 4000 cockroach species have been described worldwide, and about 1 % of them are considered pests (Cochran, 2003). Urban cockroaches act as mechanical vectors of pathogenic microorganisms for humans and contribute to allergic processes and exacerbation of asthma (Pai et al., 2004; Fu et al., 2009; Pomés et al., 2017). There are no significant natural enemies of synanthropic cockroaches in human environments, and this factor contributes to the high levels of home infestations (Schal and Hamilton, 1990). The control of these urban pests in large cities is difficult due to prevalent insecticide (pyrethroids, carbamates and others) resistance (Zhu et al., 2016; Wu and Appel, 2017). In addition, several studies have shown that the exclusive use of insecticides does not promote satisfactory long-term control when compared to an integrated pest management approach (Wang and Bennett, 2010).

Natural infection records of Blattodea pathogens and parasites are scare and fragmented (Roth and Willis, 1960; Suiter, 1997). The few recorded fungal pathogens of cockroaches are *Hymenostilbe ventricosa*, described on nymphs that attached to the underside of leaves of forest plants in Thailand (Hywel-Jones, 1995); *Ophiocordyceps blattae* (Petch), found on cockroaches in Sri Lankan gardens (Petch, 1931); *Ophiocordyceps blattarioides*, described on wild adult cockroaches from Colombia (Sanjuan et al., 2015). Recently, Montalva et al., (2016) described the novel species *Metarhizium blattodeae* as a natural fungal pathogen of a sylvatic cockroach in Brazil and evaluated its pathogenicity against *Periplaneta americana* nymphs.

* Corresponding author. Centro de Estudios Parasitológicos y de Vectores – CEPAVE (CONICET-UNLP), La Plata, 1900, Buenos Aires, Argentina. Fax: +54 0221 4232140.
E-mail addresses: gutierrez@cepave.edu.ar, gutialeja@gmail.com (A.C. Gutierrez), andreas.leclerque@hs-gm.de (A. Leclerque), manfrino@cepave.edu.ar (R.G. Manfrino), wchrisluz@hotmail.com (C. Luz), ferrariwalter@gmail.com (W.A.O. Ferrari), jorgebarneche@cepave.edu.ar (J. Barneche), juan@cepave.edu.ar (J.J. García), claudia@cepave.edu.ar (C.C. López Lastra).

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Genus *Metarhizium* (Metschn.) Sorokin was originally described based on its anamorphic stage. Liang et al. (1991) were the first to confirm the relationship between *Metarhizium* and its sexual form, which has been demonstrated by culturing. This genus level relationship between anamorph-teleomorph was supported by phylogenetic studies conducted by Liu et al. (2002) and Huang et al. (2005). The teleomorphic stages through sexual forms were included in the Genera *Cordyceps* or *Metacordyceps* (Sung et al., 2007; Kepler et al., 2012). Phylogenetic analysis of nuclear ribosomal RNA operon internal transcriber spacer (ITS) sequences demonstrated that the *Metarhizium anisopliae* species complex is monophyletic (Driver et al., 2000). Subsequently, molecular taxonomic studies of the fungal genus *Metarhizium* Sorokin (Hypocreales; Clavicipitaceae) have increased considerably in recent years. However, species delineation within this genus, as in many other entomopathogenic fungal genera, has remained a difficult task on the basis of morphological characteristics and ITS sequence data alone (Crous et al., 2005; Rehner and Buckley, 2005; Tsui et al., 2006). A more sensitive and robust basis for molecular taxonomic studies of *Metarhizium* fungi was achieved by the introduction (Bischoff et al., 2006, 2009) and successful application (Kepler et al., 2014; Montalva et al., 2016; Rehner and Kepler, 2017) of an additional Multilocus Sequence Analysis (MLSA) scheme. This analysis utilises partial sequences of four gene markers: *translation elongation factor 1-alpha* (*EF1A*), *RNA polymerase II subunit 1* (*RPB1*), *RNA polymerase II subunit 2* (*RPB2*) and *beta tubulin* (*BTUB*).

Since previous records of natural infections of cockroaches by entomopathogenic fungi are scarce, the aim of this study was to search for entomopathogenic fungi that infect wild cockroaches in forest protected natural areas of Argentina. We describe a novel species of *Metarhizium* and characterise its pathogenicity against the urban pest *Blatella germanica*.

2. Material and methods

2.1. Collection of infected cockroaches

Cockroaches with signs of mycoses were collected between Jun 2013 and Mar 2015 in two natural protected areas of Argentina: El Palmar National Park (Entre Ríos province; 31° 51′ 11″ S, 58° 19′ 21″ W) the predominant vegetation types are the deciduous xerophilous forest, palm groves, savannahs and graminoid steppes and shrub steppes. Reserve “El Destino” (Magdalena, Buenos Aires province; 35° 08′ S, 57° 25′ W). The environments were principally forests of *Celtis tala* Gillet ex Planchon (Ulmaceae), associated with *Jodina rombifolia* Hook et Arn. (Santalaceae) and *Acacia caven* (Mol.) Mol. (Leguminosae). These areas have soil with sedimentary shells, and they are subject to seasonal flooding. Cockroaches with signs of fungal infection were individually hand collected, mainly from under trees (leaf litter and under stones close to vegetation) and from dead trees in the soil of native forest, and deposited with fine forceps into small, clean capped plastic tubes that were identified with the site, date and collector's name. In addition, alive wild cockroaches were collected, for later taxonomic identification by the experts (see Acknowledgments).

2.2. Fungal isolation

Infected insects with evidence of external fungal growth were examined under a stereomicroscope (Stemi DV4, Zeiss). Fungi were isolated directly from insect cadavers that supported fungal sporulation by using Sabouraud dextrose agar with 1 % yeast extract (SDAY 1 %) medium that contained antibiotics (80,000 units/mL gentamycin and 40,000 units/mL chloramphenicol; Parafarm, Argentina). Cultures were incubated at 25 ± 1 °C in darkness for 10–15 d. Emergence of hyphae was monitored daily, and the fungus was reisolated in fresh culture medium, SDAY 1 % and potato dextrose agar (PDA), without antibiotics to obtain the pure fungal culture.

2.2.1. Morphological characterisation

Fungal species were identified based on macroscopic and microscopic features. Macroscopic features included the aspect, colour and mycelium texture of the fungal colonies. Microscopic characteristics were obtained from material mounted in lactophenol/cotton blue (0.01 % w/v) and observed under brightfield optics microscopy (Olympus CH3). The observed features were the shape and size of the conidia, conidiogenous cells and mycelium. These characteristics were photographed using a digital camera (Sony DSCP73). Measurements were based on 50 objects per microstructure and were used to calculate the mean, standard error of the mean (SEM) and range (minimum and maximum values), all in micrometer (μm). Semi-permanent slides were mounted according to Humber (2012a). Fungi were initially identified according to taxonomic keys of Humber (2012a) and for molecular taxonomic studies were used Bischoff et al. (2006, 2009), Kepler et al. (2014) and Rehner and Kepler (2017). Conidial germination percentage was calculated according to Lane et al. (1988). Isolates were preserved by cryopreservation at −20 °C and subsequent lyophilisation (Humber, 2012b); preserved samples were deposited at the Entomopathogenic Fungal Culture Collection of CEPAVE (La Plata, Argentina) and the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF, Ithaca, New York, USA).

2.2.2. DNA extraction, PCR amplification and sequencing

The fungal strains were cultured on potato dextrose agar (PDA) medium and incubated at 25 ± 1 °C with a 12 h light and 12 h dark cycle. A small portion of mycelium was harvested with a sterile loop from five-day-old cultures and placed in a 1.5 mL microcentrifuge tube. DNA was extracted using the ionic exchange resin in the Insta-Gene Matrix kit (Bio-Rad) according to the manufacturer's instructions, and the supernatants that contained the genetic material were transferred to a 1.5 mL microcentrifuge tube. DNA samples were stored at −20 °C until use. Partial sequences of four nuclear protein coding genes were amplified as follows: *TUBB*, using PCR primers T1 and T22 (O'Donnell and Cigelnik, 1997); a large exon region of *EF1A*, using primers EF1-983F and EF1-2218R (Rehner and Buckley, 2005); *RPB1* and *RPB2*, using primer pairs RPB1Af and RPB1Cr (Stiller and Hall, 1997; Matheny et al., 2002) and fRPB2-5F and RPB2-7cR (Liu et al., 1999), respectively. PCR primers ITS4 and ITS5 (White et al., 1990) were used to amplify ribosomal RNA operon internal transcribed spacer (*ITS1-5.8S-ITS2*) sequences. Reaction conditions were the same as those used by Bischoff et al. (2009). PCR product size was examined and confirmed by agarose gel electrophoresis, and DNA was purified using Puriprep-GP Kit (Inbio Highway, Argentina). Additional primers used in combination with PCR primers for sequencing by Macrogen Co. (Republic of Korea) were: EF1-1567R and EF1-2212R (Rehner and Buckley, 2005), RPB1-int2-2f and RPB1-int2-1r (Frøslev et al., 2005; Binder et al., 2010) and RPB2-6F and RPB2-6R (Goetsch et al., 2005).

2.2.3. Molecular taxonomic characterisation

Raw sequence data were combined into a single consensus sequence for each fungal isolate and marker and, where applicable, translated into peptide sequences using MEGA 6 (Tamura et al., 2013). Orthologous GenBank database entries were searched using BLASTN (Altschul et al., 1997; Zhang et al., 2000). GenBank entries with the highest similarity to query sequences were retained for phylogenetic reconstruction along with the sets of reference sequences used by Kepler et al. (2014) and Montalva et al. (2016); other sequences were obtained from GenBank and are listed in Figs. 2 and 3.

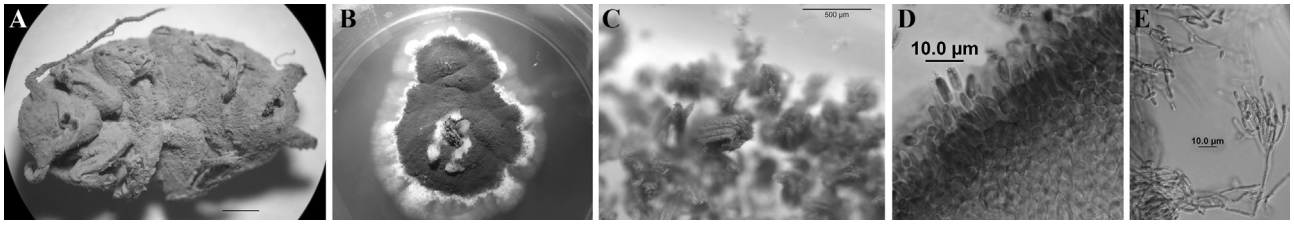

Fig. 1. (A) Nymphs of *Epilampra* sp. (Blaberidae: Epilamprinae) infected with *Metarhizium argentinense*, scale 1 mm (Holotype specimen). (B) Culture of CEP424 from Argentina on PDA, after 7 d. (C) Masses of parallel conidial chains forming prismatic columns. (D) Conidiogenous cells surface showing production of conidial. (E) Details of conidiophores, conidiogenous cells and conidia.

ITS and protein-coding marker sequences were either aligned or codon-aligned using the ClustalW function (Thompson et al., 1994) as implemented in MEGA 6 using an IUB DNA weight matrix. For comprehensive analysis of the 4 protein coding genes, a codon-aligned concatenation of the MLSA marker sequences was created. TREE-PUZZLE 5.2 software (Schmidt et al., 2002) was used to estimate data-set-specific parameters, including nucleotide frequencies, the percentage of invariable sites, the transition to transversion ratio and the alpha parameter for gamma-distribution-based correction of rate heterogeneity among sites.

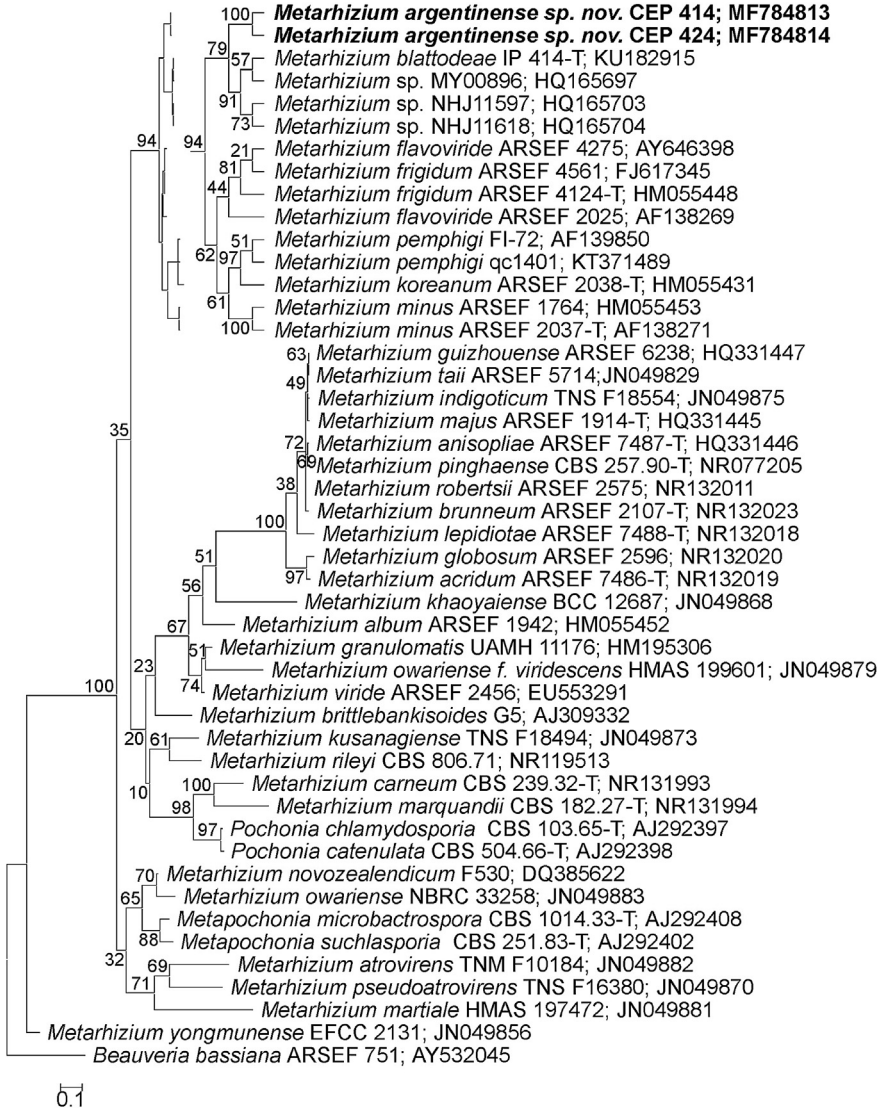

Fig. 2. Maximum Likelihood (ML) phylogeny of *Metarhizium* fungi as reconstructed from ribosomal RNA operon internal transcribed spacer (ITS) sequences. Terminal branches are labelled by genus, species and strain designations together with GenBank accession numbers; "-T" following a strain designation denotes a specific type strain. Numbers on internal branches indicate bootstrap support values. The clade that represents the *Metarhizium flavoviride* species complex comprising *M. argentinense* isolates CEP 414 and CEP 424 (shown in bold type) has been expanded into a cladogram for better resolution. The size bar corresponds to 10 % sequence divergence with respect to phylogram branch lengths. An ITS sequence from the distantly related fungal species *Beauveria bassiana* has been used as outgroup to root the phylogenetic tree.

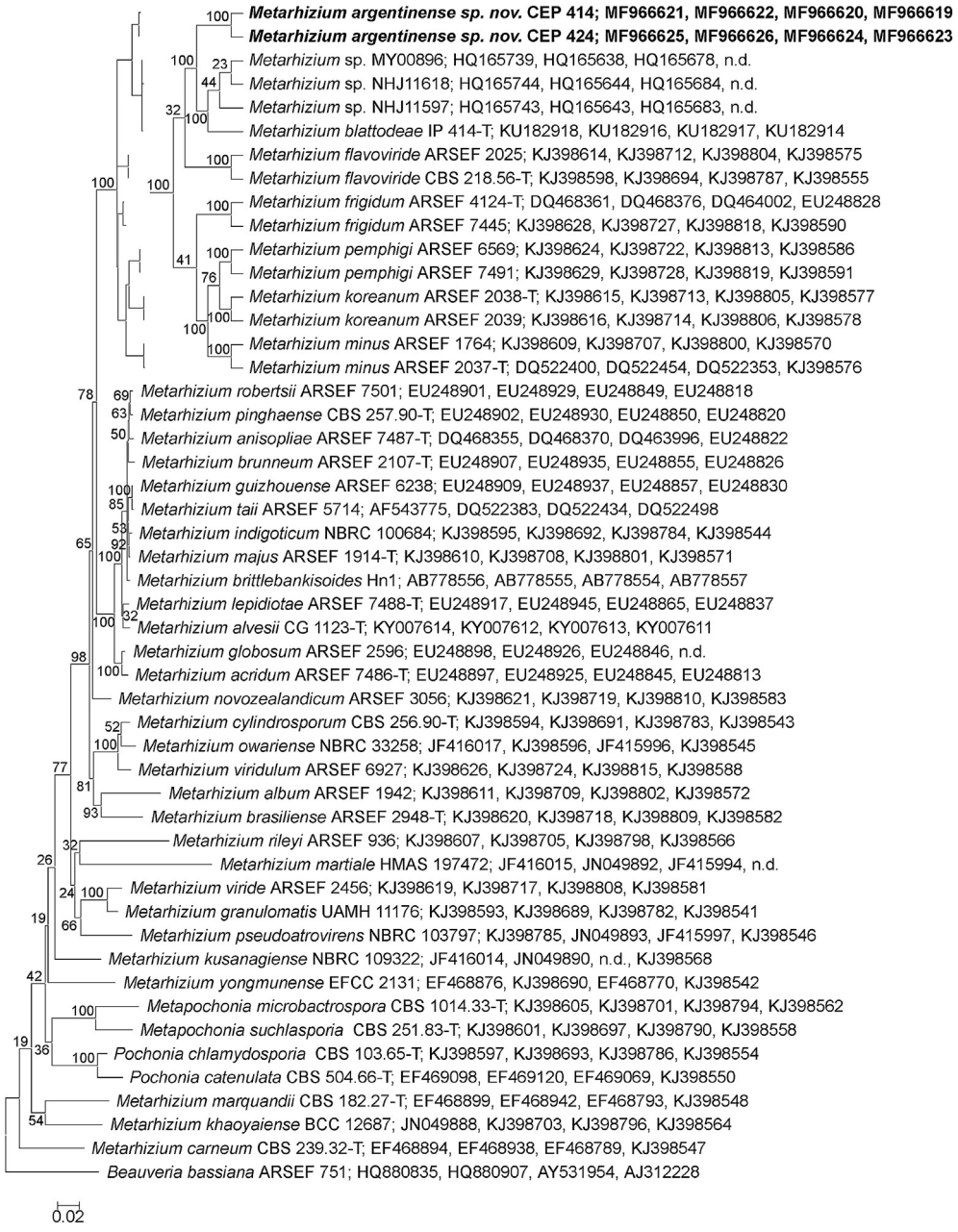

Fig. 3. Maximum Likelihood (ML) phylogeny of *Metarhizium* fungi as reconstructed from concatenated MLSA marker nucleotide sequences. Terminal branches are labelled by genus, species and strain designations together with GenBank accession numbers of the four marker sequences in the order RPB1, RPB2, EF1A, and BTUB; "-T" following a strain designation denotes a specific type strain; "n.d." indicates that the respective marker sequence is not available. Numbers on internal branches indicate bootstrap support values. The clade that represents the *Metarhizium flavoviride* species complex comprising *M. argentinense* isolates CEP 414 and CEP 424 (shown in bold type) has been expanded into a cladogram for better resolution. The size bar corresponds to 2 % sequence divergence with respect to phylogram branch lengths. A concatenation of orthologous sequences from the distantly related fungal species *Beauveria bassiana* has been used as outgroup to root the phylogenetic tree.

For phylogenetic reconstruction from nucleotide sequence alignments, the most appropriate models of DNA sequence evolution were chosen according to the rationale outlined by Posada and Crandall (1998). Phylogenies were reconstructed with the maximum likelihood (ML) method in PhyML software (Guindon and Gascuel, 2003) using the Hasegawa-Kishino-Yano model of nucleotide substitution (Hasegawa et al., 1985) under the assumption of a gamma-distribution-based model of rate heterogeneity (Yang, 1993). This method allowed for eight rate categories with and without systematic suppression of third codon positions in the case of protein coding sequences. Pairwise sequence similarity percentages were assessed from p-distance matrices calculated from unfiltered nucleotide sequence data under pairwise deletion of alignment gaps and missing data, and p-distance matrix-based neighbour joining (NJ) phylogenies were reconstructed in MEGA 6. Tree topology confidence limits were explored in non-parametric bootstrap analyses over 1,000 pseudo-replicates.

2.3. Pathogenicity assays

The virulence of two *Metarhizium* isolates (CEP414 and CEP424) toward third instar *B. germanica* nymphs reared under laboratory conditions was evaluated. Nymphs were sedated by a 15 s exposure to CO_2, or until complete sedation. Inoculation was performed by

topical application of conidia with a semiautomatic micropipette on the ventral abdominal region (Gutierrez et al., 2015). Based on the results of a preliminary experiment, we applied a dose of 6×10^5 conidia per cockroach. Cockroaches were treated with 6 μL of conidial suspension in 0.01 % Tween 80 (v/v). Controls were treated with 6 μL of 0.01 % Tween 80. For each replicate, a total of 20 *B. germanica* nymphs were used. The test was repeated three times, each on a different date. Afterwards, cockroaches were transferred to plastic cups (250 cm^3) at 25 ± 1 °C and 70 ± 5 % relative humidity (RH). Five cockroach nymphs were placed in each container. Dry dog food (Purina Dog Chow, Nestlé Argentina S.A., Buenos Aires) and water were placed inside the containers and changed every 2 d. Mortality was monitored daily up to 15 d post treatment. Dead cockroaches were removed daily. Cadavers were placed on wet filter paper disks into sterile 100 mm Petri dishes sealed with Parafilm and maintained in an incubator chamber at 25 ± 1 °C and 70 ± 5 % RH. Emergence of mycelium was monitored for eight d. Infected insects with evidence of external fungal growth were examined under a stereomicroscope. Fungal structures were stained with 0.01 % (w/v) lactophenol-cotton blue 1 % (w/v), observed under an Olympus microscope and compared with the previously inoculated fungus.

2.4. Statistical analysis

After log transformation of the data, cockroach mortality among isolates was compared using parametric analysis of variance (ANOVA) and Tukey's HSD post-hoc test with $P < 0.05$ considered statistically significant. Median lethal time (LT_{50}) values were calculated by using statistical software for correlated data developed by Throne et al. (1995).

3. Results

3.1. Collection of infected cockroaches

Two infected nymphs were collected from El Palmar National Park, and 17 infected nymphs were recorded in El Destino Natural Reserve (Table 1). Only wild cockroaches collected in El Destino were identified by specialists as *Epilampra* sp. (Blaberidae: Epilamprinae) (Table 1), and nymphs collected in El Palmar were identified up to Order level as Blattodea. The infected specimens were completely covered with mycelium and conidia in parallel chains that formed columns or plate-like masses; this pattern is typical for the entomopathogenic genus *Metarhizium* (Fig. 1A). Only 2 fungi were successfully isolated from conidia and maintained in pure cultures on PDA: CEP 414 and CEP 424 (Table 1).

3.2. Taxonomy

Metarhizium argentinense Gutierrez, Leclerque & López Lastra sp. nov.

Mycobank MB 822918.

Colonies on PDA presented irregular borders and creeping mycelium. Sporulation began as cream-coloured, transitioned to yellow-green and then became olivaceus green to dull green in colour in mass (Plate 25-4-d; Kornerup and Wanscher, 1967) at maturity (Fig. 1B); they produced uncoloured exudates in culture, whereas the reverse colony showed yellow to brownish colouration. From each conidiophore emerged 1 to 5 conidiogenous cell formed a dense hymenium. Conidiogenous cells were 8 ± 1 × 2 ± 0.3 μm (overall range: 5.9–9.9 × 1.4–2.7 μm), and conidiogenous cell were cylindrical with a defined neck. Conidia in parallel chains formed columns or plate-like masses (Fig. 1C). Conidia were cylindrical, 6.9 ± 0.6 × 2.3 ± 0.3 μm (overall range: 5.1–7.7 × 1.7–2.9 μm), and formed in heads at the apex of the conidiogenous cell (Fig. 1D and E). Conidia from the holotype were cylindrical, 4.2 ± 0.5 × 1.6 ± 0.2 μm (overall range: 3.4–5.6 × 1.3–2.4 μm) (Table 2). We observed significant differences in the size of the conidia that originated from the host and three different culture media (length $F = 215.8$; $df = 3$; $p < 0.05$; width $F = 56.4$; $df = 3$; $p < 0.05$) (Table 2). However, the shape of the conidia did not vary when found on the host or in the culture media (Table 2). These variations in size were perhaps due to different nutritional conditions present in the host and culture media.

Holotype: LPS49098, a single infected cockroach, in good physical condition, was deposited at the Herbarium of the Spegazzini Institute, La Plata, Buenos Aires, Argentina (LPS).

Ex-type culture: CEP424, Entomopathogenic Fungal Culture Collection of CEPAVE (La Plata, Buenos Aires, Argentina), Determined by Gutierrez, A.G. Leg. J. Barneche, Aug 14, 2013. In addition, this culture was deposited for preservation in the USDA-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, New York) as ARSEF 13510.

Host type: Cockroaches that belonged to *Epilampra* sp. (Blaberidae: Epilamprinae).

Locality: Argentina, Buenos Aires province, Magdalena, Reserve natural El Destino (35°8′33.07″ S, 57°23′11.51″ W).

Etymology: the species epithet refers to the Argentine origin of the fungus.

Notes: Infections were detected in cockroach nymphs; the colour of the fungus on the infected wild host was malachite green, whereas when cultured *in vitro* it was olive green to dull green in mass at maturity (Kornerup and Wanscher, 1967). The size and shape of the conidia and conidiogenous cell of *M. argentinense* were similar to those of *Metarhizium frigidum*, but *M. argentinense* was distinguishable from it because the conidiogenous cell were cylindrical with a defined neck and the conidia were cylindrical (Table 3).

3.2.1. Molecular taxonomic characterisation

We obtained consistent consensus sequences for the five molecular taxonomic markers (ITS, *EF1A*, *RPB1*, *RPB2* and *BTUB*)

Table 1
Collected sites of cockroaches with signs of fungal infection from El Palmar National park, Entre Rios province and Reserve El Destino, Buenos Aires province, Argentina.

Sites	Latitude	Longitude	Number of insects collected with fungus	Insects host	Date	Strain[b]
El Palmar National Park	31° 51′46.40″S	58° 13′44.63″O	1	Blattodea	10/6/2013	CEP 414/ARSEF13509
El Palmar National Park	31° 52′31.12″S	58° 12′48.45″O	1	Blattodea	11/6/2013	–
Reserve "El Destino"	35° 8′33.07″S	57° 23′11.51″O	2	*Epilampra* sp.[a]	14/8/2013	CEP 424/ARSEF13510
Reserve "El Destino"	35° 8′33.07″S	57° 23′11.51″O	6	*Epilampra* sp.[a]	16/5/2014	–
Reserve "El Destino"	35° 8′17.64″S	57° 23′44.43″O	8	*Epilampra* sp.[a]	29/5/2014	–
Reserve "El Destino"	35° 8′17.64″S	57° 23′44.43″O	1	*Epilampra* sp.[a]	6/3/2015	–

[a] Blattodea: Blaberidae: Epilamprinae.
[b] Abbreviations for collections: CEP, CEPAVE, Entomopathogenic Fungal Collections of Argentina; ARSEF, USDA-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, New York).

Table 2
Variation in size of conidia of *Metarhizium argentinense* (CEP 424) on host and different media.

Host/culture media	Conidia	
	Length	Width
Holotype: *Epilampra* sp. (Blattodea)	4.2 ± 0.5 (3.4–5.6) a	1.6 ± 0.2 (1.3–2.4) a
YPSS	5.5 ± 0.6 (4.2–6.6) b	2.1 ± 0.3 (1.4–2.7) b
SDYA/4	6.7 ± 0.6 (5.3–8.5) c	2.2 ± 0.3 (1.6–2.9) bc
PDA	6.9 ± 0.6 (5.1–7.7) c	2.3 ± 0.3 (1.7–2.9) c

Different letters in the column indicate significant differences (Tukey's test, $p < 0.05$). Emerson's YPSS (YPSS); Sabouraud dextrose agar + yeast extract (SDYA/4); potato dextrose agar (PDA).

investigated for both fungal isolates. When these sequences were queried with BLASTN to find the most similar GenBank database entries, with results sorted by decreasing sequence similarity percentage, the orthologs from *M. blattodeae* IP414 and three closely related *Metarhizium* strains, MY00896, NHJ11597 and NHJ11618 (Montalva et al., 2016), were identified as the best hits due to high similarity percentages for ITS (96 %), *EF1A* (98 %), *RPB1* (98 %) and *RPB2* (99 %). For the *BTUB* marker, *M. blattodeae* IP414 alone was identified as the best hit with a 98 % sequence similarity; no orthologs from the latter three strains (MY00896, NHJ11597 and NHJ11618) were available. In all cases, best hits were followed by a plethora of sequence entries from several other *Metarhizium* species, mainly *Metarhizium flavoviride* and *M. frigidum*, at slightly lower similarity values.

For both ITS- and concatenated MLSA-marker-sequence-based ML phylogenies (Figs. 2 and 3, respectively), CEP414 and CEP424 formed a clade that was located in a well-supported sister clade to one that was comprised of *M. blattodeae* IP414 and strains MY00896, NHJ11597 and NHJ11618. Both clades had at the basal branch 100 % bootstrap support from the MLSA and 91 % support from the ITS tree. The superclade that comprised both above clades had 94 % (ITS) and 100 % (MLSA) bootstrap support and was identified as part of the *M. flavoviride* species complex. These topological features were essentially reproduced in the NJ phylogenies reconstructed from the same ITS and concatenated MLSA marker sequence data as well as the four single gene trees reconstructed one by one from MLSA markers. Pairwise p-distance matrix-derived ITS and concatenated MLSA marker sequence similarities across the *M. flavoviride* species complex (Table 4) supported the view that both isolated Argentine *Metarhizium* strains were genetically different but very closely related: the concatenated MLSA marker and ITS sequences of strains CEP414 and CEP424 were, respectively, 99.9 % and 99.4 % similar to each other. These values were well in line with intra-specific similarity values across the species complex, which range, respectively, from 99.6 % to 98.1 %, and thus CEP414 and CEP424 can be consistently described as two different strains of the same *Metarhizium* species (see Table 5).

In line with results from GenBank database searches and phylogenetic reconstruction, with regards to p-distance derived pairwise sequence similarities, both Argentine strains appeared particularly closely related to the species *M. blattodeae*. Pairwise similarities between CEP414 or CEP424, on the one hand, and one of the four strains that comprised the *M. blattodeae* clade, on the other hand, ranged from 98.1 % to 98.5 % for the concatenated MLSA marker sequences. Importantly, these values were considerably lower than the corresponding intra-specific similarity percentages across the *M. flavoviride* complex. Moreover, similarity percentages matched perfectly with the range of inter-specific sequence similarities between, for instance, members of the core species *M. flavoviride* and *M. frigidum* (98.1–98.3 %). When comparing the corresponding values obtained from the ITS sequence data, CEP414 and CEP424 appeared considerably more distantly related to *M. blattodeae* (95.9–96.4 %) than *M. flavoviride* is to *M. frigidum* (98.7 %–99.4 %). The sequence similarity analysis therefore supported that CEP414 and CEP424 were the first members of a new fungal species different from *M. blattodeae*.

3.3. Pathogenicity assays

In laboratory bioassays, both isolates caused *B. germanica* nymph mortality. The first dead cockroaches were observed 4 d post-treatment with CEP424. Cadavers showed external mycelium growth first in the intersegmental regions of the abdomen, thorax, mouth and legs that produced green-coloured conidia when subjected to high humidity. The mortality of cockroaches treated with *M. argentinense* showed significant differences between each isolate and between the isolates and controls ($F = 42.9$, $df = 2$, $P < 0.05$). Likewise, there were significant differences between isolates CEP414 and CEP424 ($P < 0.05$). CEP424 caused 76.7 ± 2.3 % mortality, while mortality caused by CEP414 was 36.7 ± 1.8 %. LT_{50}

Table 3
Morphological characteristics of species of the *Metarhizium flavoviride* complex.

	Strains[a]	Host/ substrate	Location	Conidia (μm)	Phialides (μm)	Colour description	Colour code	
Metarhizium argentinense	CEP424	Blattodea	Argentina	7.3–5.1 × 2.8–1.8	9.6–5.9 × 2.7–1.5	dull green	25-4-d (Kornerup and Wanscher, 1967)	Present paper
M. blattodea	IP414	Blattodea	Brazil	4.9–7.4 × 1.7–3	5.8–8.4 × 1.8–2.6	greyish-green	28-6-d (Kornerup and Wanscher, 1967)	Montalva et al. (2016)
M. flavoviride	CBS 218.56	Coleoptera	Czech Republic	7–11 × 4.5–5.5	9–14 × 3–4	pale olivaceous	Buff (Rayner, 1970)	Gams and Roszypal (1973)
M. frigidium	ARSEF 4124	Coleoptera	Australia	4.5–7.5 × 2.5–3.5	–	bright green	28E7	Bischoff et al. (2009)
M. minus	ARSEF 2037	Hemiptera	Philippines	4.6–7 × 2–3	8.4 ± 1.2 × 2.8 ± 0.3	dull grey-green	–	Rombach et al., 1986
M. koreanum	ARSEF 2038	Hemiptera	Korea	7–10.5 × 2.5–4.5	8–16.5 × 3–4.5	olive to olive brown	S90, C40, Y50	Kepler et al. (2014)
M. pemphigi	ARSEF 6569	Hemiptera	UK	5.4 < 9 ± 0.47 × 2.4 ± 0.43	–	light green	(Munsell greenish yellow 7,5Y 4/4).	Driver et al. (2000)

[a] Abbreviations for collections: ARSEF, USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CEP, Entomopathogenic Fungal Culture Collection of Argentina; IP, Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia, Goiás, Brazil.

Table 4
Pairwise nucleotide sequence similarities (in %) as calculated from a p-distance matrix for ITS and MLSA markers from strains representing species within the *Metarhizium flavoviride* species complex; the corresponding sequences of *M. anisopliae* strain ARSEF 7487 have been used as external standard. Values calculated from internal transcribed spacer (ITS) sequences are displayed in the right upper, those from concatenated MLSA marker sequences in the lower left part of the table. Intra- and comparison relevant inter-species sequence similarities have been marked green and yellow, respectively, under the hypothesis that Argentine isolates CEP 414 and CEP 424 form a new species. The abbreviation "ARS" has been used to refer to the ARSEF strain collection.

Pairwise sequence similarities (%)		*M.argentinense (Marg)*		*M.blattodeae (Mbla)*				*M.flavoviride (Mfla)*		*M.frigidum (Mfri)*		*M.koreanum (Mkor)*		*M.pemphigi (Mpem)*		*M.minus (Mmin)*		*M.anisopliae (Mani)*	ITS	
		CEP 414	CEP 424	IP 414	MY 00896	NHJ 11597	NHJ 11618	ARS 2025	ARS 4275	ARS 4561	ARS 4124	ARSEF 2038		Qc 1401	FI-72	ARS 1764	ARS 2037	ARSEF 7487		
Marg	CEP 414		99.4	96.3	96.1	96.4	96.4	96.1	95.8	96.5	96.5	93.1		93.6	94.4	93.6	93.6	87.4	CEP 414	Marg
	CEP 424	99.9		96.1	95.9	96.2	96.2	95.9	95.6	96.3	96.3	92.9		93.3	94.2	93.4	93.4	87.2	CEP 424	
Mbla	IP 414	98.1	98.3		99.8	99.6	99.6	95.4	95.2	95.8	95.8	93.6		93.6	94.2	93.6	93.6	86.5	IP 414	Mbla
	MY00896	98.3	98.5	100		99.4	99.4	95.2	95.0	95.6	95.6	93.3		93.3	94.0	93.4	93.4	86.5	MY00896	
	NHJ11597	98.3	98.5	100	100		100	95.4	95.1	95.8	95.8	93.7		93.7	94.2	93.5	93.5	86.2	NHJ11597	
	NHJ11618	98.3	98.4	99.8	99.9	99.9		95.4	95.1	95.8	95.8	93.7		93.7	94.2	93.5	93.5	86.2	NHJ11618	
Mfla	ARS 2025	96.8	96.9	96.7	96.9	96.9	96.7		98.1	98.7	98.7	94.8		95.8	95.9	95.6	95.7	87.2	ARS 2025	Mfla
	CBS21856	96.8	96.9	96.7	96.8	96.8	96.7	100		99.4	99.4	94.8		95.4	95.9	94.8	94.8	86.7	ARS 4275	
Mfri	ARS 7445	97.0	97.1	96.8	96.9	96.9	96.7	98.1	98.1		100	95.4		96.0	96.6	95.4	95.4	87.4	ARS 4561	Mfri
	ARS 4124	97.2	97.2	96.9	97.0	97.0	96.8	98.3	98.3	99.6		95.4		96.0	96.6	95.4	95.4	87.4	ARS 4124	
Mkor	ARS 2038	95.8	95.9	95.6	95.4	95.4	95.3	96.6	96.5	96.9	97.0			98.3	98.5	95.2	95.2	84.6	ARS 2038	Mkor
	ARS 2039	95.8	95.9	95.6	95.4	95.4	95.3	96.6	96.5	96.9	97.0	100								
Mpem	ARS 6569	96.0	96.0	95.8	95.9	95.9	95.8	96.9	96.9	97.2	97.3	97.5	97.5		99.4	94.8	94.8	84.8	qc1401	Mpem
	ARS 7491	96.0	96.0	95.8	95.9	95.9	95.8	96.9	96.9	97.2	97.3	97.5	97.5	100		95.1	95.1	85.6	FI-72	
Mmin	ARS 1764	96.7	95.8	95.6	95.6	95.6	95.5	96.5	96.5	96.8	96.9	96.9	96.9	97.1	97.1		99.6	86.5	ARS 1764	Mmin
	ARS 2037	95.6	96.7	95.6	95.6	95.6	95.5	96.4	96.4	96.7	96.8	97.0	97.0	97.0	97.0	99.9		86.0	ARS 2037	
Mani	ARS 7487	94.1	94.2	94.0	94.6	94.6	94.5	94.7	94.7	94.7	94.9	93.8	93.8	94.0	94.0	93.8	93.8		ARS 7487	Mani
MLSA concatenation		CEP 414	CEP 424	IP 414	MY 00896	NHJ 11597	NHJ 11618	ARS 2025	CBS 21856	ARS 7445	ARS 4124	ARS 2038	ARS 2039	ARS 6569	ARS 7491	ARS 1764	ARS 2037	ARSEF 7487		
		M.argentinense		*M.blattodeae*				*M.flavoviride*		*M.frigidum*		*M.koreanum*		*M.pemphigi*		*M.minus*		*M.anisopliae*		

for CEP424 was 7.2 d (6.1–8.6 d). Overall, CEP424 was significantly more virulent to *B. germanica* nymphs.

4. Discussion

The *M. flavoviride* species complex can be difficult to distinguish using only morphological characteristics, and several species of the genus are morphologically cryptic species (Bischoff et al., 2009). Kepler et al. (2014) considered that conidial and phialidic morphologies in *Metarhizium koreanum* were apparently variable between isolates. Bischoff et al. (2006) described *M. anisopliae* conidia and conidiogenous cell as more consistently cylindrical than those of *M. frigidum*. Conidia and conidiogenous cells of the Argentine *Metarhizium* isolates in this study were similar in size and shape to those of both *M. blattodeae* and *M. frigidum*. In addition, mature cultures of *M. anisopliae* are more darkly pigmented. *M. frigidum* appears to be restricted to Australia, and its host range has been associated only with coleopterans. However, both species can be isolated from the soil (Bischoff et al., 2006). Bischoff et al. (2006) pointed that many species now recognized in this genus based on their genomic content rather than their morphologies, for example *M. frigidum* is a somewhat of a cryptic species with respect to *M. anisopliae*. Isolates of the new Argentine *Metarhizium* species were very similar to *M. frigidum* with respect to conidial colour in culture. However, Rombach et al. (1987) and Latch (1965) reported that changes in conidial colour could be artificially induced in isolates of *Metarhizium* species. In the present study, we observed different colours between isolates growing *in vivo* from *Epilampra* sp. (malachite green) and those growing in PDA (dull green).

Molecular characteristics are the most definitive way to distinguish the *M. flavoviride* species complex (Bischoff et al., 2006). Molecular taxonomy, using ITS and a set of four protein coding MLSA marker sequences, demonstrated that cockroach pathogens CEP414 and CEP424 belonged to a group of fungi currently recognised as the *M. flavoviride* species complex. Within this complex, CEP414 and CEP424 are most closely related to each other; they belong to the same fungal species and form a tight clade that is a sister to one that contains the cockroach pathogenic species *M. blattodeae*. Therefore, these species are presumably part of a monophyletic clade of cockroach pathogens comprised of *M. blattodeae*-type strains and the Argentine isolates. It is tempting to speculate that evolution of these fungi has been driven by host group adaptation.

The topological features of the phylogenies of the fungal genus *Metarhizium* are consistent with the perception that strains CEP414 and CEP424 are the first members of a new *Metarhizium* species or a subclade within *M. blattodeae*. The degree of similarity among the sets of selected marker genes—originally ribosomal RNA gene sequences (Stackebrandt and Goebel, 1994) and subsequently sets of protein-coding markers (Maiden 2006; Sung et al., 2007)—has become the *de facto* criterion for species delineation in the microbiology of asexual organisms (e.g., bacteria or mitosporic fungi) to which the classical species concept developed for higher organisms does not apply. The set of MLSA marker genes, namely *EF1A*, *RPB1*, *RPB2* and *BTUB*, which is currently accepted for phylogenetic and molecular taxonomic studies of the genus *Metarhizium*, was introduced by Bischoff et al. (2009). The above analysis of pairwise p-distances for ITS and MLSA marker sequences across the *M. flavoviride* species complex assessed the taxonomic position of the new Argentine cockroach pathogen on the basis of the current practice of species delineation.

There are few records of fungal infections that occur in wild cockroaches. The pathogenicity of fungal pathogens has not been deeply studied and is most of the time unknown (Milner and Pereira, 2007; Suiter, 1997). Montalva et al. (2016) studied the pathogenicity of *M. blattodeae* on the urban pest *P. americana* and found that this fungus caused 96 % mortality after 10 d. In the present study, isolate CEP424 caused 76 % mortality, with a LT_{50} of 7 d. CEP424 caused much higher mortality than other *Metarhizium* spp. isolates against *B. germanica* nymphs, where mortality rates were not higher than 50 % (Quesada Moraga et al., 2004; Lopes and Alves, 2011; Gutierrez et al., 2014, 2015). This study described the second set of *Metarhizium* isolates from wild cockroaches in South America; this species currently represents the southernmost location record for the world. We propose the introduction of a new taxonomic species of the *M. flavoviride* species complex to be designated "*M. argentinense*" which references the common

Table 5
Summary of data for sequences used for the phylogenetic analysis.

Species	Strain[a]	Host/Substrate	Location	GenBank accession number				
				ITS	EF1A	BTUB	RPB1	RPB2
Beauveria bassiana	ARSEF 751	Coleoptera	Vietnam	AY532045	AY531954			
Metarhizium sp.	NHJ11597	[b]	[c]	HQ165703/AY646375	HQ165683		HQ165743	HQ165643
Metarhizium sp.	NHJ11618	[b]	[c]	HQ165704/AY646376	HQ165684		HQ165744	HQ165644
Metarhizium sp.	MY00896	[b]	[c]	HQ165697	HQ165678		HQ165739	HQ165638
Metarhizium argentinense	**CEP414/ARSEF13509**	**Blattodea**	**Argentina**	MF784813	MF966620	MF966619	MF966621	MF966622
Metarhizium argentinense	**CEP424/ARSEF13510**	**Blattodea**	**Argentina**	MF784814	MF966624	MF966623	MF966625	MF966626
Metarhizium blattodeae	IP414	Blattodea	Brazil	KU182915	KU182917	KU182914	KU182918	KU182916
Metarhizium flavoviride	ARSEF2025	soil	Germany	AF138269	KJ398804	KJ398575	KJ398614	KJ398712
Metarhizium flavoviride	CBS 218.56	Coleoptera	Czech Republic		KJ398787	KJ398555	KJ398598	KJ398694
Metarhizium frigidum	ARSEF 4124	Coleoptera	Australia	HM055448	DQ464002	EU248828	DQ468361	DQ468376
Metarhizium frigidum	ARSEF 7445	Isoptera	Australia		KJ398818	KJ398590	KJ398628	KJ398727
Metarhizium minus	ARSEF 1764	Hemiptera	Solomon Isl.	HM055453	KJ398800	KJ398570	KJ398609	KJ398707
Metarhizium koreanum	ARSEF 2038	Hemiptera	Korea	HM055431	KJ398805	KJ398577	KJ398615	KJ398713
Metarhizium koreanum	ARSEF 2039	Hemiptera	Korea		KJ398806	KJ398578	KJ398616	KJ398714
Metarhizium pemphigi	ARSEF 6569	Hemiptera	UK		KJ398813	KJ398586	KJ398624	KJ398722
Metarhizium pemphigi	ARSEF 7491	Hemiptera	UK		KJ398819	KJ398591	KJ398629	KJ398728
Metarhizium acridum	ARSEF 7486	Orthoptera	Niger		EU248845	EU248813	EU248897	EU248925
Metarhizium indigoticum	NBRC 100684	Lepidoptera	Japan		KJ398784	KJ398544	KJ398595	KJ398692
Metarhizium majus	ARSEF 1914	Coleoptera	Philippines		KJ398801	KJ398571	KJ398610	KJ398708
Metarhizium guizhouense	ARSEF 6238	Lepidoptera	China		EU248857	EU248830	EU248909	EU248937
Metarhizium pinghaense	CBS 257.90	Coleoptera	China		EU248850	EU248820	EU248902	EU248930
Metarhizium robertsii	ARSEF 7501	Coleoptera	Australia		EU248849	EU248818	EU248901	EU248929
Metarhizium novozealandicum	ARSEF 3056	Coleoptera	New Zealand		KJ398810	KJ398583	KJ398621	KJ398719
Metarhizium cylindrosporum	CBS 256.90	Hemiptera	China		KJ398783	KJ398543	KJ398594	KJ398691
Metarhizium viridulum	ARSEF 6927	Hemiptera	Taiwan		KJ398815	KJ398588	KJ398626	KJ398724
Metarhizium album	ARSEF 1942	Hemiptera	Philippines	HM055452	KJ398802	KJ398572	KJ398611	KJ398709
Metarhizium brasiliense	ARSEF 2948	Hemiptera	Brazil		KJ398809	KJ398582	KJ398620	KJ398718
Metarhizium granulomatis	UAMH 11176	Squamata	Denmark		KJ398782	KJ398541	KJ398593	KJ398689
Metarhizium viride	ARSEF 2456	Squamata	[c]		KJ398808	KJ398581	KJ398619	KJ398717
Metarhizium rileyi	ARSEF 936	Lepidoptera	Brazil		KJ398798	KJ398566	KJ398607	KJ398705
Metarhizium yongmunense	EFCC 2131	Lepidoptera	Korea		EF468770	KJ398542	EF468876	KJ398690
Metapochonia suchlasporia var. *suchlasporia*	CBS 251.83	nematode eggs	Sweden		KJ398790	KJ398558	KJ398601	KJ398697
Metapochonia microbactrospora	CBS 1014.33	Bdelloida	Japan		KJ398794	KJ398562	KJ398605	KJ398701
Pochonia chlamydosporia var. *chlamydosporia*	CBS 103.65	soil	Germany		KJ398786	KJ398554	KJ398597	KJ398693
Pochonia chlamydosporia var. *chlamydosporia*	CBS 594.66	soil	Guinea		KJ398792	KJ398560	KJ398603	KJ398699
Metarhizium carneum	CBS 239.32	dune sand	France		EF468789	KJ398547	EF468894	EF468938
Metarhizium khaoyaiense	BCC 12687	Lepidoptera	Thailand		KJ398796	KJ398564	JN049888	KJ398703
Metarhizium marquandii	CBS 182.27	soil	USA		EF468793	KJ398548	EF468899	EF468942
Metarhizium alvesii	ARSEF 13308	soil	Brazil		KY007614	KY007611	KY007612	KY007613
Metarhizium taii	ARSEF 5714	Lepidoptera	China	JN049829	AF543775	DQ522498	DQ522383	DQ522434
Metarhizium owariense f. viridescens	HMAS199601	[c]	[c]	JN049879				
Metarhizium owariense	NBRC33258	[c]	[c]	JN049883	JF416017	KJ398545	KJ398596	JF415996
Metarhizium brittlebankisoides	[b]	[c]	China	AJ309332				
Metarhizium brittlebankisoides	MAFF243306	Coleoptera	Japan		AB778556	AB778557	AB778555	AB778554
Metarhizium kusanagiense	TNS-F18494	[c]	[c]	JN049873	JF416014	KJ398568	JN049890	
Metarhizium atrovirens	TNM F10184	[c]	[c]	JN049882				
Metarhizium pseudoatrovirens	TNS-F 16380	[c]	[c]	JN049870	KJ398785	KJ398546	JN049893	JF415997
Metarhizium martiale	HMAS 197472	[c]	[c]	JN049881	JF416015		JN049892	JF415994

Bold entries correspond to fungus specie described in this document.

[a] Abbreviations for collections: ARSEF, USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA; BCC, BIOTEC culture collection, Pathum Thani, Thailand; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CEP, Entomopathogenic Fungal Culture Collection of Argentina; EFCC, Entomopathogenic Fungal Culture Collection, Chuncheon, Korea; IP, Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia, Goiás, Brazil; MAFF, NARO Genebank, Microorganism Section; NBRC, Biological Resource Centre, National Institute of Technology and Evaluation, Tokyo, Japan; UAMH Centre for Global Microfungal Biodiversity, Toronto, Canada.

[b] Collection information not available.

[c] Location not specified.

geographic origin of the strains. We also tested the strains pathogenicity through assays against *B. germanica* nymphs and found promising results for it to be used as biological control for cockroaches.

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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.2019.02.005.

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