A natural fungal infection of a sylvatic cockroach with *Metarhizium blattodeae* sp. nov., a member of the *M. flavoviride* species complex

CrossMark

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ABSTRACT

A wild, forest-dwelling cockroach from the subfamily *Ectobiidae* (order *Blattodea*) in a nature reserve in Cavalcante, in the state of Goiás, Brazil, was found to be infected by a new, genetically distinct species in the *Metarhizium flavoviride* species complex that we describe here as *Metarhizium blattodeae*. The status of this fungus as a new species is supported by both multigenic sequence comparisons and protein profiles generated by MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry. This is one of the first reports of a naturally occurring fungal pathogen affecting any sylvatic (forest-dwelling) cockroach from any part of the world. *M. blattodeae* caused up to 96 % mortality of *Periplaneta americana* nymphs (a serious peridomestic cockroach species) after 10 d.

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Introduction

Hypocrealean entomopathogenic fungi have long been studied as candidates for the microbial control of synanthropic cockroach pests. The cosmopolitan German and American cockroaches, *Blattella germanica* and *Periplaneta americana* (*Blattodea*), respectively, are among the most troublesome of household insect pests and are well known for both their

hardiness and their ability to evade diverse control measures. Fungal activity against these insects has been shown with the important and widely used fungal biocontrol agents *Beauveria bassiana* and *Metarhizium anisopliae* (Mohan *et al.* 1999; Quesada-Moraga *et al.* 2004; Abedi & Dayer 2005; Lopes & Alves 2011; Hubner-Campos *et al.* 2013; Gutierrez *et al.* 2015). Knowledge about the potential of these fungi for cockroach control is based mainly on assays done under laboratory conditions with fungal strains originating from other host insects or substrates. Studies on cockroaches as target hosts have often emphasized the combined effects of fungus and synthetic insecticides (Kaakeh *et al.* 1996, 1997; Zurek *et al.* 2002). One commercialized mycoinsecticide based on *M. anisopliae* was registered with the US Environmental Protection Agency (Registration #70644-8) for cockroach control (Faria & Wraight 2007), but this registration has since been cancelled, and the manufacturer, EcoScience Corporation, is no longer in business. In Guatemala, however, a *Metarhizium*-based product (Zero QK-S 0,4 DP) has been sold by Agricola El Sol for use against domestic cockroach pests for many years.

More than 4000 cockroach species are known worldwide, but most have no importance as pests and mostly live far from human habitations (Bell *et al.* 2007; Beccaloni 2014). There are surprisingly few reports of natural fatal infections of any cockroach species, synanthropic or not, caused by any fungus that is clearly pathogenic for these insects, and these reports generally suggest that only very few cockroach individuals were negatively affected by these fungi. Although Roth & Willis (1960) compiled a surprisingly long list of fungi and yeasts from cockroaches, most of these fungi are primarily saprobic or facultative pathogens, and many were reported only from gut contents or feces. The cockroach fungi noted by Roth & Willis (1960) that cause diseases of insects include ectoparasitic ascomycetes (*Herpomyces* species: *Laboulbeniales*) and the cordycipitoid fungi now classified as *Ophiocordyceps amazonica* and *Ophiocordyceps blattae* (*Hypocreales*: *Ophiocordycipitaceae*), as well as a few poorly supported reports of *Metarhizium*.

Archbold *et al.* (1987) and Appel *et al.* (1987) studied an entomopathogenic yeast affecting the German cockroach, *B. germanica*. One of the authors (RAH) is aware of an unpublished finding in the early 1980's of an entomophthoralean fungus, probably *Batkoa* species based on the globose shape of the conidia, affecting cockroaches in a grain field near the Rothamsted Experimental Station in the United Kingdom. *Hymenostilbe ventricosa* was described from forest-dwelling cockroach nymphs in Thailand (Hywel-Jones 1995), and Cummings (2009) reported finding *B. bassiana* affecting single cockroaches at two different locations within New Zealand.

The cockroach from which this new fungus was isolated was collected as part of routine survey of vegetation and soil surfaces adjacent to aquatic sites where the primary intention of the field work was to find fungal pathogens of mosquitoes and other dipteran vectors of serious human and animal diseases. These vegetation surveys were undertaken to help to expand the comparatively poor understanding of the biota of Brazilian fungal entomopathogens (Sosa-Gómez *et al.* 2010). The scarcity of information about such fungi, especially in nonagricultural sites, is primarily a result of the lack of collecting efforts to find such fungi.

The present study reports a natural infection by *Metarhizium blattodeae* sp. nov. affecting a sylvatic cockroach species collected in Central Brazil. The status of this new species is supported by both gene sequence and mass spectrometric data about the proteins on its conidial surfaces, and we characterize the morphology and confirm the pathogenicity of this new fungus against *P. americana* nymphs under laboratory conditions.

Material and methods

Field location, collecting and initial processing of material

The survey for entomopathogenic fungi in which this new fungus was found was done during the rainy season (February 2015) in a tropical secondary gallery forest in the privately owned and operated Bacupari Reserve, close to the city of Cavalcante in northern Goiás state, Central Brazil. Leaves in the vegetation up to 2 m high, as well as plant remains on the soil, were checked for mycotized arthropod cadavers. Material with dead specimens was carefully removed, transferred to paper bags and placed in a polystyrene cooler at 20 °C (Benjamin *et al.* 2004) for later examination and processing in a field laboratory.

Dead arthropods and their fungal pathogens were examined microscopically for their taxonomically significant morphological characteristics, and conidia were subsequently transferred to quarter-strength Sabouraud dextrose agar + yeast extract (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) amended with chloramphenicol (0.05 %) in 60 × 15 mm Petri dishes. Dishes were sealed with parafilm and incubated at 25 ± 3 °C and natural photophase. The development of fungi and of any contaminants was checked daily, and contaminants were removed or clean transfers of uncontaminated fungal growth from the presumptive pathogen were made to fresh medium. Once a pure culture was established, all further transfers were onto SDAY/4 without additional antibiotics.

The fungus reported here was prepared for long-term preservation in the IPTSP Laboratory of Invertebrate Pathology as IP 414 following protocols from Humber (2012b), and was also co-deposited in the USDA Collection of Entomopathogenic Fungi (Ithaca, NY) as ARSEF 12850.

Morphological evaluations

An infected cockroach was identified morphologically based on keys in Costa Lima (1938). Conidial inoculum from this fungus was grown at 25 ± 1 °C, 75 ± 10 % relative humidity (RH) and a 12 h photophase on Sabouraud dextrose agar for 14 d (Bischoff *et al.* 2009). The fungus was investigated based on morphological characteristics, and semi-permanent slide mounts were prepared in lactophenol-cotton blue according to Humber (2012a). Fungal microstructures were examined by brightfield or phase contrast microscopy (Leica DMLS 020-518.500), measured microscopically (Nova 180i-T; Toupview) and documented with a digital camera (UCMOS01300KPA). Photomontages of multiple focal planes for the conidia and conidiophores were prepared using Helicon Focus Pro software (www.heliconsoft.com). Measurements were based on

50 objects per microstructure from which we calculated mean values, standard error of the mean (±SEM), and a range with the maximum and minimum values.

In vivo characterization

The pathogenicity of the fungus was tested in laboratory-reared first up to fourth instar *Periplaneta americana* nymphs (Hubner-Campos *et al.* 2013). Ten nymphs for each repetition, and the same number for all controls, were sedated with carbon dioxide and then treated with an undefined quantity of conidia on the thorax and abdomen of each nymph by a very brief direct contact with the sporulated culture. Viability of conidia was checked as reported by Sousa *et al.* (2013). Nymphs were then incubated in a Petri dish (90 × 18 mm) at 25 ± 1 °C, RH close to saturation (>98 %) and a 12 h photoperiod. Every three days, the infected nymphs were provided with new ground, dry cat food (0.5 g) (Whiskas®, Mars Brazil, Guararema, SP, Brazil) and water through moistened cotton wool (0.1 ml) arranged in separate small aluminium containers. Control nymphs were not treated with conidia and were held as mentioned. Mortality was monitored daily, and dead nymphs were transferred to sterile filter paper in new Petri dishes and incubated in a humid chamber at 25 ± 1 °C. Development of mycelium and conidia on cadavers was checked daily for up to 15 d, and the fungus was examined morphologically with a stereomicroscope and compared with the fungus inoculated previously.

Molecular characterization

The fungal isolate was grown in liquid SDY/4 for 7 d in a shaker at 150 rpm and 25 ± 1 °C. DNA was extracted from mycelium using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Partial sequences of four genes were amplified by PCR including: beta tubulin (BTUB) using the primers BT1F and BT1R (Bischoff *et al.* 2009); RNA polymerase II largest subunit (RPB1a) with RPB1C and RPB1Af (Stiller & 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 (TEF) with primers 983F and 2218R (Rehner & Buckley 2005). The internal transcribed spacer (ITS) regions ITS1 and ITS2 as well as the central 5.8S rDNA were also amplified using the primers ITS1 and ITS4 (White *et al.* 1990). The PCR products were checked using agarose gel electrophoresis, and bands were then purified. 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 IP 414 sequence data were assembled using Chromas Pro (V. 1.5, Technelysium Pty Ltd). Reference sequences used in two recent taxonomic re-evaluations of *Metarhizium* (Bischoff *et al.* 2009; Kepler *et al.* 2014) and others sequences were obtained from GenBank and are listed in Table 1. Individual gene regions were aligned using MAFFT v. 7 with the G-INS-i option (Katoh & Standley 2013), and the multiple alignments were trimmed to the length of the IP 414 sequences. A concatenated matrix comprising partial BTUB, RPB1a, RPB2a and TEF sequences was assembled in Sequence Matrix v. 1.7.8 (Meier *et al.* 2006), totalling 3205 aligned characters.

A phylogenetic hypothesis based on the concatenated matrix was obtained using the Bayesian Markov Chain Monte Carlo method as implemented in MrBayes 3.2.5 (Ronquist *et al.* 2012). Default program settings were used, except that reversible-jump MCMC (Huelsenbeck *et al.* 2004) was implemented to optimize the analysis within the GTR model family (nst = mixed rates = invgamma). Model parameters varied freely over the four component data partitions, and the analysis run for five million generations, with the first 25 % of trees discarded as burn-in. This run-time was sufficient to allow the convergence diagnostic, the standard deviation of split frequencies, to fall to 0.0002365. The analysis was repeated to ensure reproducibility of the resultant majority rule phylogram.

To supplement the results of the combined gene analysis, rRNA-ITS sequences from *Metarhizium* species close to IP 414, including as many of the same isolates as in the previous analysis as currently available, were also obtained from GenBank. The ITS sequences were aligned as above and trimmed, producing a matrix of 621 aligned characters. A phylogenetic hypothesis was again calculated in MrBayes 3.2.5 as above, but with two million generations being sufficient to produce a standard deviation of split frequencies of 0.006008.

Protein analysis by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS)

Conidia were collected from 10 to 12-d-old cultures on potato dextrose agar (PDA) and transferred to 2-mL plastic tubes containing 1 mL of ethanol (75 %). The suspension was agitated manually for 30 s and then centrifuged at $16\,100\times$ g for 2 min. The supernatant was discarded, and the pellets were air-dried at room temperature for 15 min. Protein extraction and spectral analysis were performed as described by Lopes *et al.* (2014a). Briefly, conidial pellets were acid digested in 50 μL formic acid (70 %) and 50 μL of acetonitrile (100 %) before centrifugation ($16\,100\times$ g for 2 min). The supernatant was diluted in deionized water (1:10 v/v), directly spotted (1 μL droplet) onto a steel target plate, and allowed to air-dry for 15 min. A 1-μL droplet of α-cyano-4-hydroxycinnamic acid matrix solution was applied to the dried sample and air-dried for 15 min. Analyses were performed on a MicroFlex Speed™ MALDI-TOF mass spectrometer (Bruker Daltonick GmbH, Bremen, Germany). Each spectrum was obtained after an average of 240 laser shots, and the signals were automatically collected at a mass range between 2000 and 20 000 *m/z*. Six independent samples were prepared for the IP 414 isolate. The collected spectra were analysed with the Biotyper software algorithm (version 3.1) to match the number and intensity of peaks in the raw spectra with those in the *Metarhizium* reference main spectrum profile (MSP) database. Spectra matching results were expressed as log score values in a 0−3 scale. The IP 414 consensus spectrum was incorporated into the MSP database and submitted to cluster analyses (MSP Dendrogram Creation Standard Method, version 1.4), comparing with spectrum profiles from different species within the genus *Metarhizium*.

Species	Strain[a]	Host/substrate	Location	RPB1a	RPB2a	TEF	BTUB	ITS
Metapochonia bulbillosa	CBS 247.68	wheat field soil	Germany	KJ398599	KJ398695	KJ398788	KJ398556	
Metapochonia microbactrospora	CBS 1014.33[b]	bdelloid rotifers	Japan	KJ398605	KJ398701	KJ398794	KJ398562	
Metapochonia rubescens	CBS 1104.36	soil	Netherlands	KJ398606	KJ398702	KJ398795	KJ398563	
Metapochonia suchlasporia var. *catenata*	CBS 248.83[b]	nematode eggs	Sweden	KJ398600	KJ398696	KJ398789	KJ398557	
Metapochonia suchlasporia var. *catenata*	CBS 814.83	nematode eggs	Sweden	KJ398604	KJ398700	KJ398793	KJ398561	
Metapochonia suchlasporia var. *suchlasporia*	CBS 251.83[b]	nematode eggs	Sweden	KJ398601	KJ398697	KJ398790	KJ398558	
Metarhizium acridum	ARSEF 324	Orthoptera	Australia	EU248896	EU248924	EU248844	EU248812	HM055449
Metarhizium acridum	ARSEF 7486[b]	Orthoptera	Niger	EU248897	EU248925	EU248845	EU248813	
Metarhizium album	ARSEF 1942	Hemiptera	Philippines	KJ398611	KJ398709	KJ398802	KJ398572	HM055452
Metarhizium album	ARSEF 2082	Hemiptera	Indonesia	KJ398617	KJ398715	DQ522352	KJ398579	
Metarhizium album	ARSEF 2179	Hemiptera	Philippines	KJ398618	KJ398716	KJ398807	KJ398580	
Metarhizium anisopliae	ARSEF 7450	Coleoptera	Australia					HQ331464
Metarhizium anisopliae	ARSEF 7487[b]	Orthoptera	Ethiopia	DQ468355	DQ468370	DQ463996	EU248822	HQ331446
Metarhizium blattodeae	**IP 414[b]**	**Ectobiidae**	**Brazil**	**KU182918**	**KU182916**	**KU182917**	**KU182914**	**KU182915**
Metarhizium brasiliense	ARSEF 2948[b]	Hemiptera	Brazil	KJ398620	KJ398718	KJ398809	KJ398582	
Metarhizium brunneum	ARSEF 2107[b]	Coleoptera	USA	EU248907	EU248935	EU248855	EU248826	
Metarhizium brunneum	ARSEF 4179	soil	Australia	EU248906	EU248934	EU248854	EU248825	
Metarhizium carneum	CBS 239.32[b]	dune sand	France	EF468894	EF468938	EF468789	KJ398547	
Metarhizium cylindrosporum	ARSEF 6926	Hemiptera	Taiwan	KJ398625	KJ398723	KJ398814	KJ398587	
Metarhizium cylindrosporum	CBS 256.90[b]	Hemiptera	China	KJ398594	KJ398691	KJ398783	KJ398543	
Metarhizium flavoviride	ARSEF 1184	*Otiorhynchus sulcatus*	France					AY646383
Metarhizium flavoviride	ARSEF 2025	soil	Germany	KJ398614	KJ398712	KJ398804	KJ398575	AF138269
Metarhizium flavoviride	ARSEF 4275	soil	Australia					AY646398
Metarhizium flavoviride	CBS 218.56[b]	Coleoptera	Czech Republic	KJ398598	KJ398694	KJ398787	KJ398555	
Metarhizium frigidum	ARSEF 4124[b]	Coleoptera	Australia	DQ468361	DQ468376	DQ464002	EU248828	HM055448
Metarhizium frigidum	ARSEF 4343	soil	Australia					FJ617324
Metarhizium frigidum	ARSEF 4561	soil	Australia					FJ617345
Metarhizium frigidum	ARSEF 7445	Isoptera	Australia	KJ398628	KJ398727	KJ398818	KJ398590	
Metarhizium globosum	ARSEF 2596	Lepidoptera	India	EU248898	EU248926	EU248846	EU248814	
Metarhizium granulomatis	UAMH 11028[b]	*Chamaeleo calyptratus*	Denmark		KJ398688	KJ398781	KJ398540	
Metarhizium granulomatis	UAMH 11176	*Chamaeleo calyptratus*	Denmark	KJ398593	KJ398689	KJ398782	KJ398541	
Metarhizium guizhouense	ARSEF 6238	Lepidoptera	China	EU248909	EU248937	EU248857	EU248830	
Metarhizium guizhouense	CBS 258.90[b]	Lepidoptera	China	EU248914	EU248942	EU248862	EU248834	
Metarhizium indigoticum	NBRC 100684	Lepidoptera	Japan	KJ398595	KJ398692	KJ398784	KJ398544	
Metarhizium khaoyaiense	BCC 12687	Lepidoptera	Thailand	JN049888	KJ398703	KJ398796	KJ398564	
Metarhizium khaoyaiense	BCC 14290	Lepidoptera	Thailand	JN049889	KJ398704	KJ398797	KJ398565	
Metarhizium koreanum	ARSEF 2038[b]	Hemiptera	Korea	KJ398615	KJ398713	KJ398805	KJ398577	HM055431
Metarhizium koreanum	ARSEF 2039	Hemiptera	Korea	KJ398616	KJ398714	KJ398806	KJ398578	
Metarhizium lepidiotae	ARSEF 7412	Coleoptera	Australia	EU248916	EU248944	EU248864	EU248836	
Metarhizium lepidiotae	ARSEF 7488[b]	Coleoptera	Australia	EU248917	EU248945	EU248865	EU248837	
Metarhizium majus	ARSEF 1914[b]	Coleoptera	Philippines	KJ398610	KJ398708	KJ398801	KJ398571	
Metarhizium majus	ARSEF 1946	Coleoptera	Philippines	EU248919	EU248947	EU248867	EU248839	
Metarhizium marquandii	CBS 182.27[b]	soil	USA	EF468899	EF468942	EF468793	KJ398548	
Metarhizium minus	ARSEF 1764	Hemiptera	Solomon Isl.	KJ398609	KJ398707	KJ398800	KJ398570	HM055453

Table 1 – Reference sequences used for phylogenetic analysis; genes and locus with no deposited sequences are left blank.

Species	Isolate	Host	Location					
Metarhizium minus	ARSEF 2037[b]	Hemiptera	Philippines	DQ522400	DQ522454	DQ522353	KJ398576	AF138271
Metarhizium novozealandicum	ARSEF 3056	Coleoptera	New Zealand	KJ398621	KJ398719	KJ398810	KJ398583	
Metarhizium novozealandicum	ARSEF 4661	soil	Australia	KJ398622	KJ398720	KJ398811	KJ398584	
Metarhizium novozealandicum	F530	*Platypus* sp.	New Zealand					DQ385622
Metarhizium novozealandicum	FI-1125	soil	Australia					AF139853
Metarhizium pemphigi	ARSEF 6569	Hemiptera	UK	KJ398624	KJ398722	KJ398813	KJ398586	
Metarhizium pemphigi	ARSEF 7491	Hemiptera	UK	KJ398629	KJ398728	KJ398819	KJ398591	
Metarhizium pemphigi	CABI 177416	Hemiptera	UK	KJ398630	KJ398729	KJ398820	KJ398592	
Metarhizium pemphigi	FI-72	Homoptera	UK					AF139850
Metarhizium pemphigi	qc1401	*Melanotus cribricollis*	China					KT371489
Metarhizium pinghaense	ARSEF 4342	Coleoptera	Solomon Isl.	EU248903	EU248931	EU248851	EU248821	
Metarhizium pinghaense	ARSEF 7929	Isoptera	Australia	EU248899	EU248927	EU248847	EU248815	
Metarhizium pinghaense	CBS 257.90[b]	Coleoptera	China	EU248902	EU248930	EU248850	EU248820	
Metarhizium rileyi	ARSEF 936	Lepidoptera	Brazil	KJ398607	KJ398705	KJ398798	KJ398566	
Metarhizium rileyi	ARSEF 1972	Lepidoptera	Brazil	KJ398613	KJ398711	KJ398803	KJ398574	
Metarhizium robertsii	ARSEF 4739	soil	Australia	EU248900	EU248928	EU248848	EU248817	
Metarhizium robertsii	ARSEF 7501	Coleoptera	Australia	EU248901	EU248929	EU248849	EU248818	
Metarhizium sp.	NHJ11597	[c]	[c]	HQ165743	HQ165643	HQ165683		HQ165703/AY646375
Metarhizium sp.	NHJ11618	[c]	[c]	HQ165744	HQ165644	HQ165684		HQ165704/AY646376
Metarhizium sp.	MY00896	[c]	[c]	HQ165739	HQ165638	HQ165678		HQ165697
Metarhizium viride	ARSEF 2456	*Chamaeleo lateralis*	[c]	KJ398619	KJ398717	KJ398808	KJ398581	
Metarhizium viridulum	ARSEF 6927	Hemiptera	Taiwan	KJ398626	KJ398724	KJ398815	KJ398588	
Metarhizium yongmunense	EFCC 2131	Lepidoptera	Korea	EF468876	KJ398690	EF468770	KJ398542	
Pochonia chlamydosporia var. *chlamydosporia*	CBS 103.65[b]	soil under *Brassica napus*	Germany	KJ398597	KJ398693	KJ398786	KJ398554	
Pochonia chlamydosporia var. *chlamydosporia*	CBS 429.64[b]	soil	Brazil	KJ398602	KJ398698	KJ398791	KJ398559	
Pochonia chlamydosporia var. *chlamydosporia*	CBS 594.66[b]	soil	Guinea	KJ398603	KJ398699	KJ398792	KJ398560	

a Abbreviations for collections: ARSEF, USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY, USA; CABI, CAB International Bioscience, Egham, UK; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; EFCC, Entomopathogenic Fungal Culture Collection, Chuncheon, Korea; F, AgResearch Insect Pathogen Culture Collection, Lincoln, New Zealand; FI, CSIRO Collection, Canberra, Australia; IMI, International Mycological Institute, CAB Bioscience, Egham, UK; IP, Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia, Goiás, Brazil; NBRC, Biological Resource Center, National Institute of Technology and Evaluation, Tokyo, Japan; UAMH Centre for Global Microfungal Biodiversity, Toronto, Canada.

b Denotes ex-type isolate.

c Collection source information not available.

Results

Observations on the field-collection and initial processing of material

A brownish adult dead mycotized cockroach (13 mm length), was detected fixed to the underside of a leaf at a height of ca. 50 cm in the Bacupari Reserve (S 13°48′16.85″ and W 47°25′38.43″, WGS 84) on 4 February 2015. The surface of the cadaver was extensively covered by mycelium, and a large amount of light grey-green conidia forming columnar to plate-like masses, typical for the entomopathogenic genus *Metarhizium* (Figs 1—4) was observed. This cockroach and one other similarly infected individual collected at this site showed significant growth of a *Metarhizium*-like fungus on its surface and were collected from the field. Both insects were returned to the field laboratory in the evening for microscopic determination of the identity of the fungus and to attempt to isolate cultures. The specimen reported on here proved to be sufficiently fresh to enable the isolation of the fungus. The second specimen was too old and in a somewhat degraded condition, and produced no subsequent fungal growth.

Both cockroach specimens were identified morphologically as belonging to the family *Ectobiidae* (*Blattodea*). Ectobiid cockroaches are wild non-synanthropic, mostly diurnal feeders on decaying organic material in forests and are common in Brazil and other South American countries (Costa Lima 1938; Lopes *et al.* 2014b; Beccaloni 2014).

Pathogenicity against Periplaneta americana

The first infected nymph died within 24 h after exposure to conidia. Cumulative mortality of nymphs increased markedly in the following days, and reached 96 % (±1.9 %) after 10 d (Fig 2). All control nymphs had survived at this time. Mycelium and conidia, respectively, of the *Metarhizium* isolate developed on dead nymphs after 5 and 8 d of incubation of cadavers in a humid chamber. A total of 20 ± 20 % dead nymphs were found over the following 10 d with mycelium and conidia of the previously inoculated fungus.

Taxonomy

Metarhizium blattodeae Montalva, Humber, Collier & Luz, sp. nov. (Figs. 1—4)
Mycobank MB 815131
Colonies on SDAY/4 initially colourless, becoming increasingly yellow below developing conidial hymenia. Conidiophores showing obtuse, candalabrum-like open branching, becoming intertwined in dense hymenia with closely crowded conidiogenous cells at apices of 1—5 branches per conidiophore. Conidiogenous cells ovoid to broadly ellipsoid, 7.1 ± 0.3 × 2.1 ± 0.1 (overall range: 5.8—8.4 × 1.8—2.6 μm). Conidia cylindrical, 5.9 ± 0.1 × 2.3 ± 0.0 μm (overall range: 4.9—7.4 × 1.7—3 μm), greyish-green in mass (plate 28-6-d; Kornerup & Wanscher 1967), formed in long, laterally adherent chains forming columns or plates.
Holotype: UFG 49886, deposited in the Herbarium of the Federal University of Goiás, Goiânia, Brazil; a single infected cockroach, now detached from the subtending leaf.
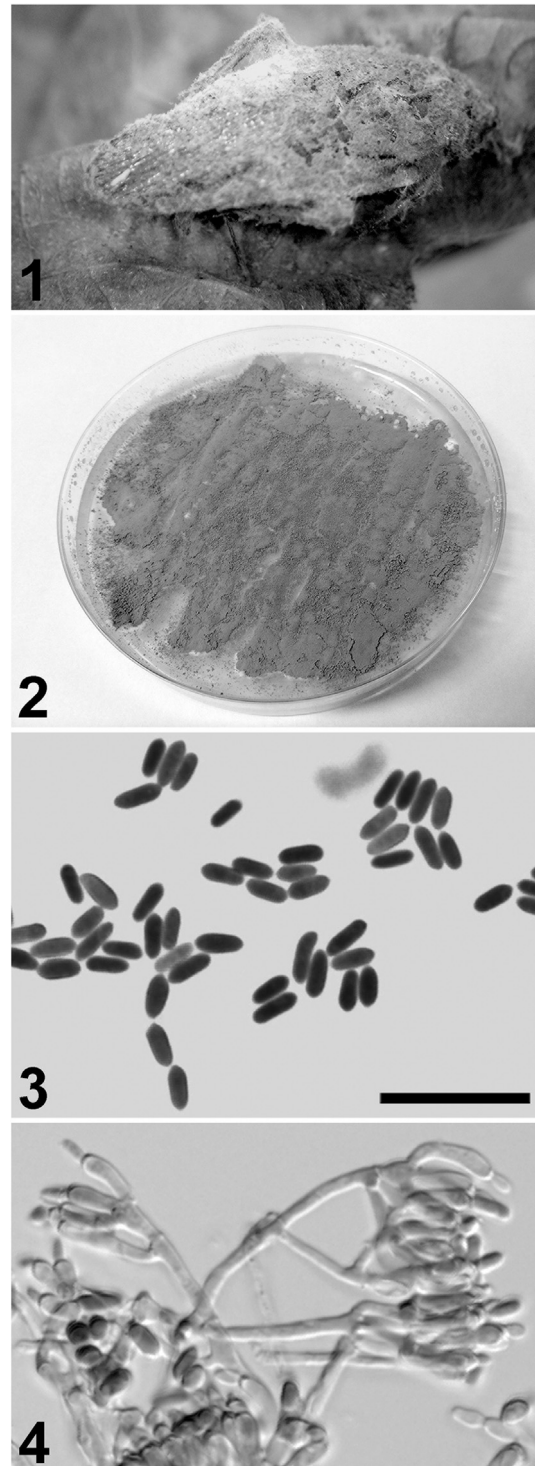
Figs 1 — 4 *Metarhizium blattodeae*. 1. Holotype specimen on leaf surface. 2. Culture of IP 414 on SDAY/4. 3. Conidia. 4. Conidiogenous cells. The micrographs of conidia and conidiogenous cells were prepared as photomontages combining several focal planes. Bar (Figs. 3 and 4) = 20 μm).

Ex-Type culture: ARSEF 12850 (= IP 414), USDA-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, New York), collected by Cristian Montalva, 4 February 2015.
Type host: An undetermined species of *Dictyoptera*: *Blattodea*: *Ectobiidae*.
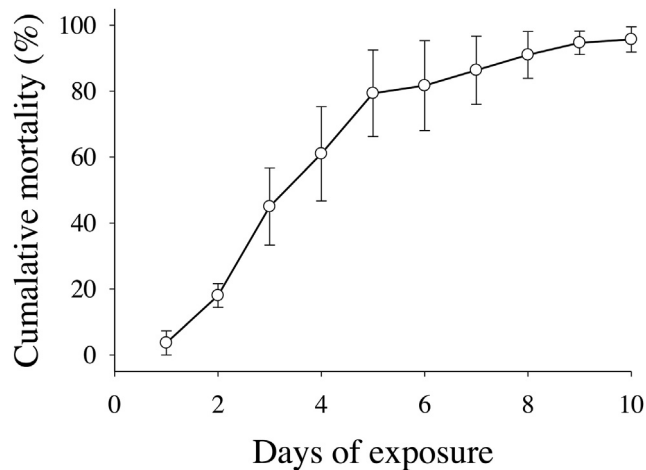
Fig 2 — Cumulative mortality of *Periplaneta americana* nymphs infected with *Metarhizium blattodeae* conidia under laboratory conditions. The scale bars represent the standard error of the means for three replicates.

Type locality: Bacupari Reserve, Cavalcante, Goiás, Brazil; S 13°48′16.85″, W 47°25′38.43″.
Etymology: The specific epithet refers to the name of the order of the host cockroach from which this species was recovered.

Phylogenetic and other molecular placements of Metarhizium blattodeae *in its genus*

The phylogenetic analysis of the combined partial BTUB, RPB1a, RPB2a and TEF gene dataset clearly place isolate IP 414 within the strongly supported clade comprising the *Metarhizium flavoviride* species complex (Fig 3). The short branch uniting the IP 414-*Metarhizium minus*-*Metarhizium koreanum*-*Metarhizium pemphigi* clade with the remainder of the *M. flavoviride* species complex is unsupported (Fig 3), however, as is the short branch uniting *Metarhizium frigidum* and *M. flavoviride*, which agrees with the most recently published phylogenetic and nomenclatural treatment of *Metarhizium* (Kepler *et al*. 2014). IP 414 should therefore be considered as part of a basal polytomy in the *M. flavoviride* species complex, along with *M. frigidum* and *M. flavoviride* itself. With the exception of *M. pemphigi*, all the species comprising this complex are represented in the analysis by ex-type isolates (Kepler *et al*. 2014). The relationships revealed in the ITS analysis (Fig 4) agree with and support the multi-gene analysis, where the IP 414 isolate was distinct from both the *M. minus-M. koreanum-M. pemphigi* clade and from the unsupported *M. frigidum-M. flavoviride* clade.

IP 414 could not be identified by MS from within the previously established MSP database since the average log score value was only 0.97 (0.87−1.16), lower than the cut-off value of 1.70 suggested by Lopes *et al*. (2014a) for this genus. The dendrogram of protein-based MALDI-TOF MS profiles (Fig 5) clearly distinguished IP 414 from any other identified *Metarhizium* species in the MSP. Strains of the same species

(spectra matching with log scores ≥ 1.70) show more than 90 % protein profile similarity according to the software algorithm.

Discussion

The IP 414 isolate was immediately and unambiguously recognizable from its general appearance, conidial shape and aggregation of conidial chains in dense columns and plates as a species of *Metarhizium* (Tulloch 1976; Rombach *et al*. 1986). In the current systematics of hypocrealean entomopathogens, it is recognized that the species can be and often are defined only by their genomic characters, and that morphological characters can no longer be regarded as necessarily adequate to identify species within this extremely large and taxonomically complex group of fungi.

In addition to the now virtually mandatory use of key gene sequences to distinguish phylogenetically justified species of *Metarhizium* (Bischoff *et al*. 2009; Kepler *et al*. 2014), it was recently shown that a mass spectrometric method of examining and comparing *Metarhizium* species can be useful (Lopes *et al*. 2014a). MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry produced a protein profile for IP 414 unambiguously differentiating this fungus from all species of *Metarhizium* currently included in the MSP library. This protein-based analysis underscored the great variability within this genus and reinforced the multigenic sequence analysis supporting the description of this new species, even though the MALDI-TOF dendrogram is not congruent with the DNA-based phylogenetic trees (Figs 3 and 4) in every aspect. That differences between gene- and MS-based dendrogram topologies can occur was reported for both *Alternaria* (Brun *et al*. 2013) and *Metarhizium* (Lopes *et al*. 2014a). These differences may be due to metabolites produced by one species that resemble or are the same as those produced by other species.

BLAST searches of GenBank records, using the IP 414 Brazilian cockroach pathogen TEF, RPB1, RPB2 and ITS sequences, all returned strong matches to three unnamed *Metarhizium* sp. strains (MY00896, NHJ11597, and NHJ11618), for which no description of a new species seems to have been published. The GenBank records, submitted in 2010, do not indicate the geographical origin or host insect (if any) of these isolates. Duplicate ITS sequences from the NHJ11597 and NHJ11618 strains, submitted earlier to Genbank in 2004, are recorded as *Metarhizium flavoviride*. These three strains were included in our phylogenetic analyses, where they clustered closely together with IP 414 in both the ITS and combined four-locus trees. Although the MY00896, NHJ11597 and NHJ11618 strains were found to differ from IP 414 by between one to three base substitutions or indels in the ITS, TEF and RPB1a sequences and by zero to one base substitution in RPB2a (see Appendix A), they are most appropriately identified as *Metarhizium blattodeae*.

A diverse range of sylvatic cockroaches is now moving into increasingly large-scale production as pets or as alternative food sources for animals and humans (Anankware *et al*. 2015). Whether or not *M. blattodeae* is well adapted to cockroach hosts, this species and, potentially, other entomopathogenic fungi could represent a distinct threat to this new
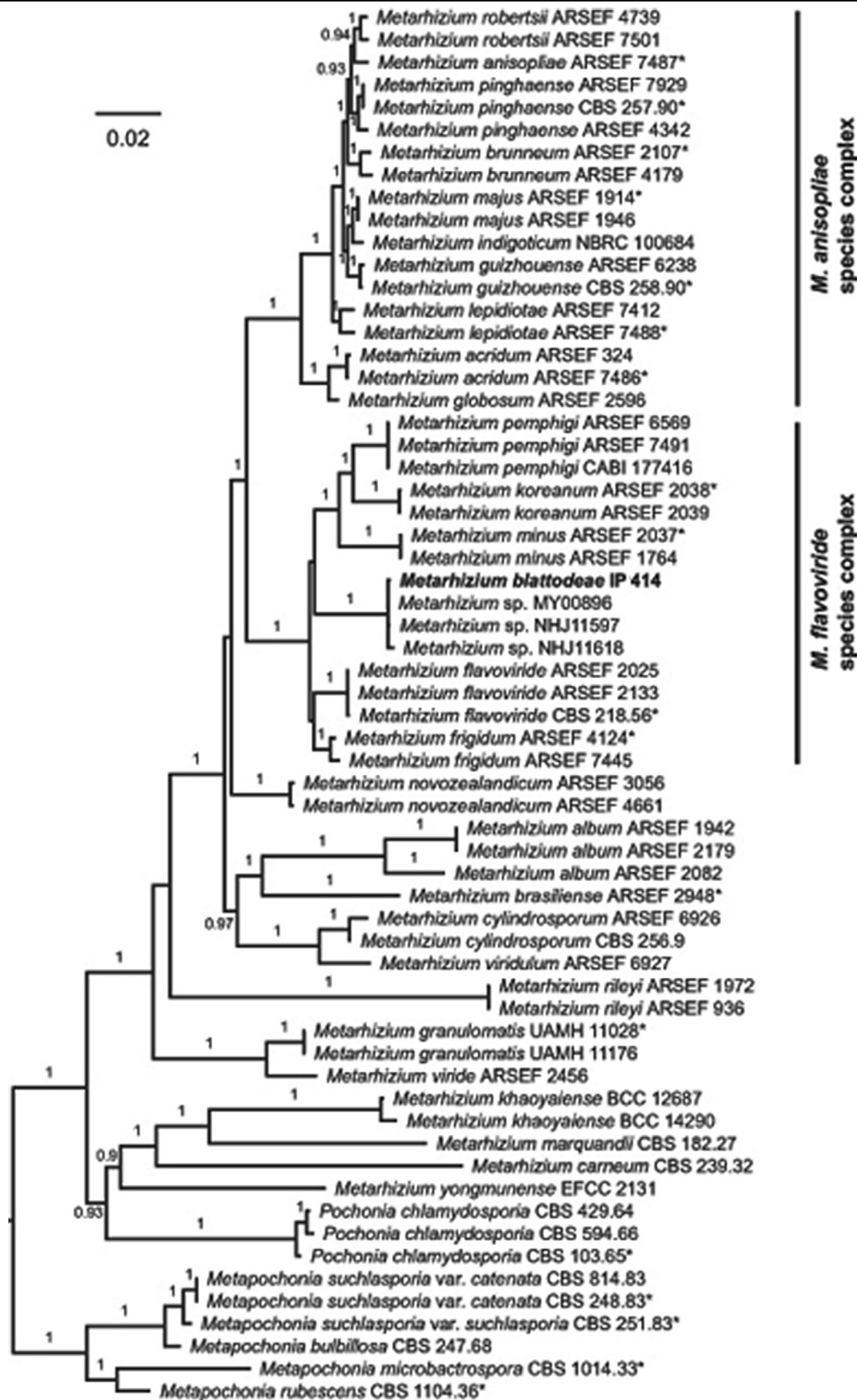
Fig 3 — Phylogenetic hypothesis based on Bayesian analysis of a concatenated dataset comprising partial BTUB, RPB1a, RPB2a and TEF gene sequences. Trees were rooted using *Metapochonia* spp. as outgroup, and posterior probabilities >0.9 are indicated. *ex-type isolate. The scale bar represents the number of expected substitutions per site.
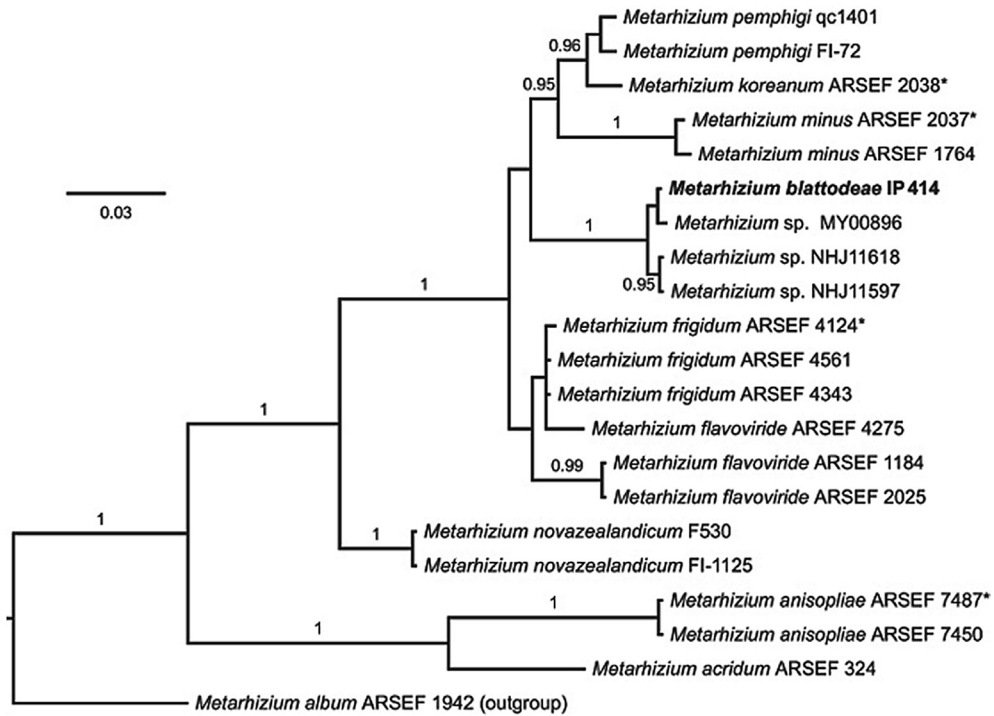
Fig 4 — Phylogenetic hypothesis based on Bayesian analysis of rRNA-ITS gene sequences. Trees were rooted using *Metarhizium album* as outgroup, and posterior probabilities >0.9 are indicated. *ex-type isolate. The scale bar represents the number of expected substitutions per site.

alternative foods industry, and increased vigilance against fungal and other diseases of these insects would be prudent.

Naturally occurring populations of cockroaches in forests or fields are not regarded to be pests needing to be managed by the use of pesticides or other control strategies. It is significant, however, that *M. blattodeae* was shown here to be able to cause high mortality to nymphs of the serious domestic pest species, *Periplaneta americana* (American cockroach). No tests have yet been conducted to determine whether this new fungal species also shows activity against any other important cockroach
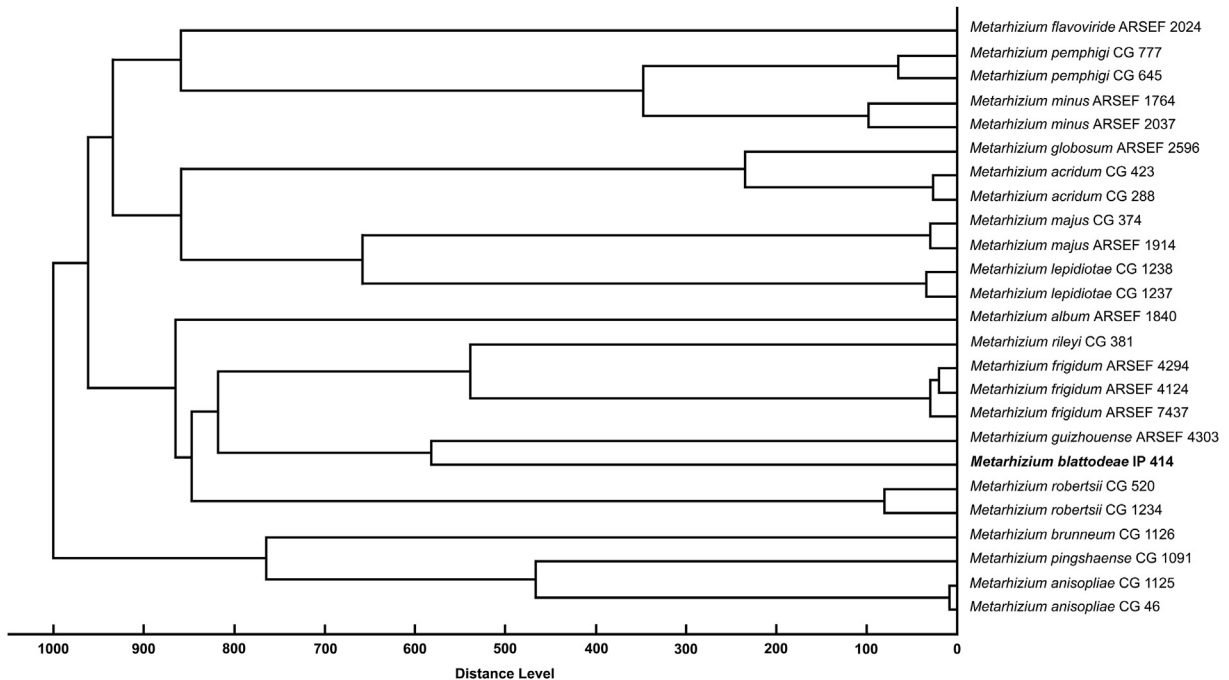

Fig 5 — Dendrogram showing cluster analysis of MALDI-TOF mass spectra.

species. Despite being significantly pathogenic for *P. americana*, this new fungus did not sporulate well on those individuals that it had killed; the degree of sporulation on these American cockroach cadavers was notably less than is usually seen on insects that are highly susceptible to various *Metarhizium* species. If *M. blattodeae* were to be developed for the applied biological control of domestic cockroach pests, its apparent reluctance to sporulate on those hosts might be seen as a significant benefit for the formulated product. The impaired ability of the fungus to establish itself at its application sites or to start any sort of outbreak in its host populations could result in increased product sales while also limiting any threats to non-target hosts.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2016.03.004.

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