Neotrop Entomol (2013) 42:436–438
DOI 10.1007/s13744-013-0146-0

SCIENTIFIC NOTE

Genetic Diversity and Pathogenicity of *Metarhizium* spp. Associated with the White Grub *Phyllophaga capillata* (Blanchard) (Coleoptera: Melolonthidae) in a Soybean Field

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Keywords
Entomopathogenic fungi, *Metarhizium* diversity, *Metarhizium robertsii*, microbial control, molecular characterization

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Edited by Ítalo Delalibera Jr – ESALQ/USP

Received 19 October 2012 and accepted 27 May 2013
Published online 19 June 2013

Abstract
Phyllophaga capillata (Blanchard) was recently described as a soybean pest in Brazil. The occurrence of *Metarhizium* spp. associated with adults and their pathogenicity were investigated. Natural prevalence of *Metarhizium anisopliae* was 0.51% in adults collected by light trap. Most *Metarhizium* isolates were identified as *Metarhizium robertsii*, although *M. anisopliae* sensu stricto and a few unidentified isolates were also found. Bioassays with representative isolates selected from different genetic groups resulted in ≤5.6% confirmed mortality against larvae and adults, suggesting low potential of microbial control of this pest by use of indigenous *Metarhizium* isolates.

White grubs in the genus *Phyllophaga* have been reported to be among the most destructive pests in Central and South America (Morón 1997). *Phyllophaga capillata* (Blanchard) was recently described as a rhizophagous soybean pest in Middle–Western Brazil (Oliveira *et al* 2007). In the present study, we evaluated the natural prevalence of entomopathogenic fungi associated with *P. capillata* adults and their genetic diversity, as well as the pathogenicity of indigenous *Metarhizium* isolates against the adult and larval stages.

Phyllophaga capillata adults were collected in October 2010 from a soybean field in the state of Goiás following emergence using a light trap set up on a white cloth, and put inside plastic containers filled with soil sample (20–50 cm deep) collected near the trap. In the laboratory, adults remained in soil for a 20-day incubation period at room temperature and every 2 days, dead insects were removed and transferred to wet chambers. Insect-associated fungi were isolated and cultivated on PDA medium for a 10-day incubation period at 25±0.5°C. A total of 2,556 *P. capillata* adults were collected and 13 insects were infected by *Metarhizium anisopliae* (s.l.).

Phylogenetic analyses were used to assess the genetic variability of these isolates. Total genomic DNA was extracted from mycelia using the protocol described by Raeder & Broda (1985). Identification was performed by amplifying the TEF-1α gene fragment based on Bischoff *et al* (2009). Analyses of the consensus sequences were carried out under the Maximum Likelihood method and bootstrap support (BS) values were provided. Additionally, we used Bayesian phylogenetic inference and BS values were included in the Bayesian tree. One major group aggregating eight isolates was confirmed as *Metarhizium robertsii*. In the second group, two isolates were identified as *M. anisopliae* sensu stricto (s.s.), clearly clustering with sequences from the GenBank database (Fig 1). The three remaining isolates (CG1231, CG1233, and CMA12) were closest to *M. anisopliae* (s.s.) (98.98% of similarity with ARSEF7487), although additional studies are required before any definitive identification is made. Likewise, Rocha *et al* (2012) reported the isolation of *M. robertsii*, *M. anisopliae* (s.s.), and over 50 unidentified *Metarhizium* isolates from soil samples in Central Brazil. Interestingly, in a survey conducted by our team in a near municipality, another

Metarhizium species (*Metarhizium lepidiotae*, also included in Fig 1) was isolated from the melolonthid *Aegopsis bolboceridus* (Thomson) in a corn field. In contrast to *M. anisopliae* (s.s.) and *M. robertsii* (Faria *et al* 2010, Xavier-Santos *et al* 2011), this is the first record of *M. lepidiotae* infecting an insect in Brazil. Accession numbers in GenBank for *Metarhizium* isolates incorporated in the Invertebrate Fungal Collection at EMBRAPA Genetic Resources and Biotechnology are KC832295 (*M. anisopliae* CG1232), KC832294 (*M. anisopliae* s.l. CG1231), KC832296 (*M. anisopliae* s.l. CG1233), KC832297 (*M. robertsii* CG1234), and KC832298 (*M. lepidiotae* CG1238).

Third-instars larvae from the same soybean site, extracted from soil a few months later (February/2011), were immersed in conidial suspensions (1×10^7 viable conidia mL^{-1}) or water. Four representative isolates were selected from different genetic groups [one *M. robertsii*, one *M. anisopliae* (s.s.), and two *M. anisopliae* (s.l.)]. For each isolate, six groups of 10 insects were confined in 1,000-mL round plastic containers filled with soil and soybean seedlings as food source, and kept in a room at 25±2°C and 70% RH. Mortality was assessed at 20-day post-inoculation. For bioassays with adults, individuals collected in the field 1 year later (October–November 2011) were observed for 20 days

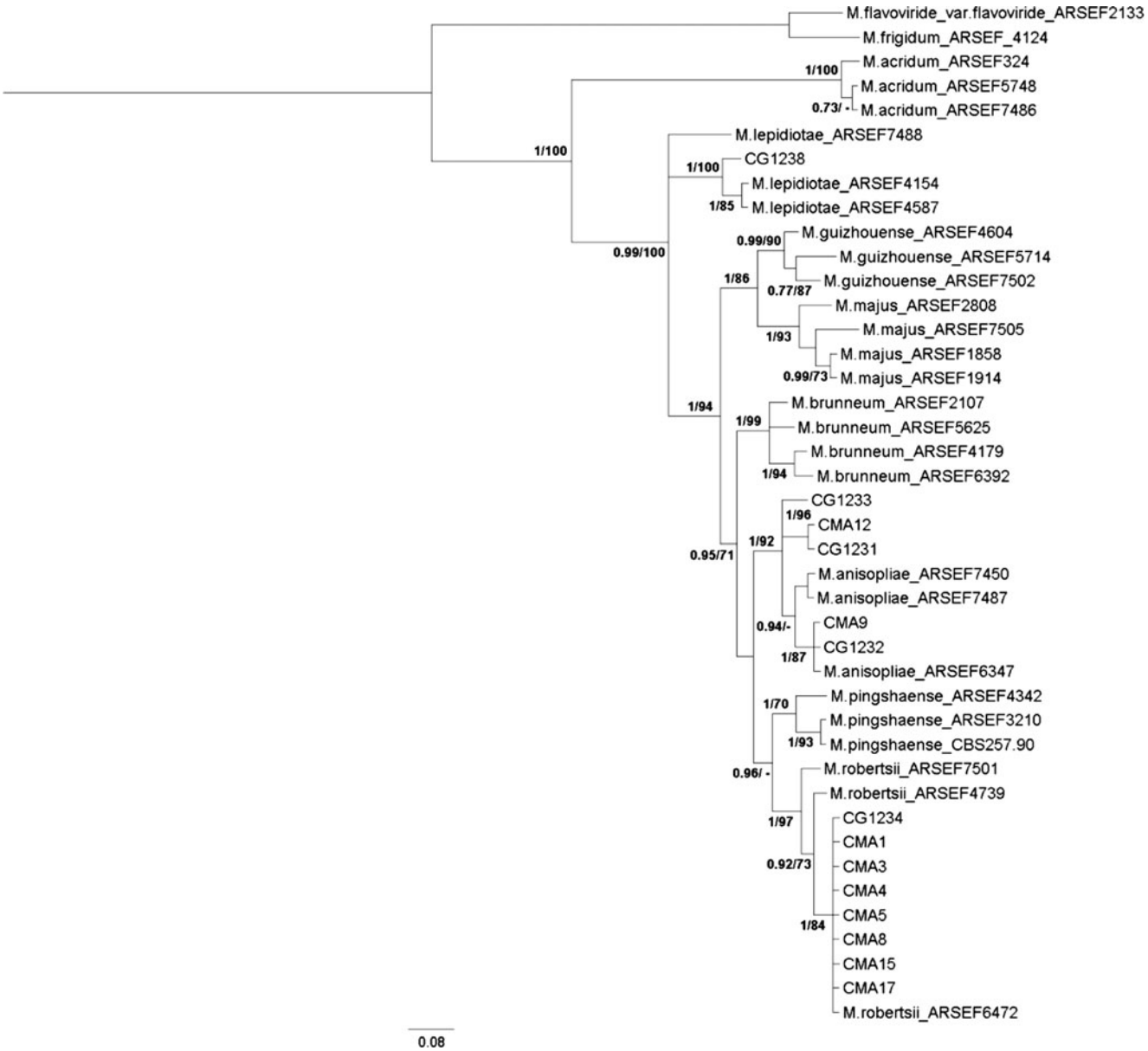

Fig 1 Bayesian majority rule consensus phylogram of aligned 5′ end of TEF-1α gene sequence data for *Metarhizium* isolates. Sequence data from ARSEF isolates are from the GenBank database, whereas non-ARSEF isolates (coded CG or CMA) correspond to those from *Phyllophaga capillata* adults collected in October 2010 in state of Goiás, Brazil (exception is CG1238, isolated from an adult of the melolonthid *Aegopsis bolboceridus*).

and then inoculated on the thorax with 5 µL of conidial suspensions (5×10^7 viable conidia mL^{-1}) or water (control), using a glass micro syringe coupled to a micro applicator. Following inoculation, six groups of 12 insects (4 males and 8 females) were transferred to 1,000-mL round plastic containers and incubated as previously described. A tubular plastic cage with a screened lid was used on top of each container to allow insect movement at night (mating behavior) and to avoid escape. Mortality was assessed at 12-day post-inoculation. In general, *Metarhizium* isolates tested against *P. capillata* were not pathogenic or caused very low levels of confirmed mortality (≤5.6 and ≤1.7% for adults and larvae, respectively), whereas fungal infections were not seen in non-treated insects (data not shown). Mortality of the larval stage of the melolonthid *Phyllophaga* sp. treated with different *Metarhizium* spp. isolates was less than 20% after a 36-day incubation period and using the same dipping methodology adopted in our work, although their conidial concentration was 10× higher (Guzmán-Franco *et al* 2012).

In summary, we identified at least two *Metarhizium* species associated with *P. capilatta* adults within the same population, although natural infection was low. Besides, *P. capilatta* adults and larvae showed low susceptibility to fungal infections under laboratory conditions.

Acknowledgments We appreciate the funding provided by the Brazilian National Research Council (CNPq).

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