Genotyping Isolates of *Beauveria* spp. by rDNA-ITS Sequencing and RAPD Markers

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INTRODUCTION

Maize armyworm, *Spodoptera frugiperda*, is one of the main pests in maize fields in Brazil, whose severity has been increasing in various agricultural areas (Cruz et al., 1999). Control of *S. frugiperda* has been made mainly by chemical treatment as soon as the first symptoms appear. One of the alternatives to control of *S. frugiperda* infestations is based on microbial agents with low environmental impact, high specificity and efficiency in reducing its ability to cause injury to the host plant. In this way, the entomopathogenous fungi *Beauveria* are interesting biocontrol agents due to their epizootics and aggressiveness (Devi et al., 2001). Many *Beauveria* isolates have been studied due to their potential use as biopesticides (Devi et al., 2001). However, once phenotypic characteristics are not sufficient to distinguish different *Beauveria* strains or to monitor field releases of biocontrol agents, molecular analysis are demanded. The most used methods to characterize genotypic variation in fungi are based on PCR, DNA / RNA probes, and protein technologies. Among then, RAPD technology Gaitan et al., 2002) and rDNA-ITS sequencing studies (Wada et al., 2003; Muro et al., 2005) have already been employed successfully for assessing the degree of genetic variation existent among strains of *Beauveria* spp. As an effort to develop an integrated pest management control program for *S. frugiperda*, mycosed dead insects from different regions of central Brazil were collected and different fungi pathogens were isolated, among them 24 *Beauveria* spp. strains. These isolates were tested against *S. frugiperda* and analyzed by RAPD and rDNA-ITS sequencing in order to evaluate their genetic variability.

MATERIAL AND METHODS

Strains and growth conditions: Twenty four *Beauveria* spp. strains isolated from *S. frugiperda* and *Dalbulus maidis* at different maize fields of central Brazil were utilized in this study. Mycelia and conidia were germinated in potato-dextrose agar (PDA) and stored in glycerol 20% at –20 ^{o}C at the Embrapa Maize and Sorghum Insect Propagation Laboratory, Brazil.
Pathogenicity assays: *Beauveria* virulence towards *S. frugiperda* was determined using six replicates of eight second instar larvae. Mortality was recorded as the percentage of dead larvae 10 days after infection.
Morphology: *Beauveria* spp. taxonomic identification was performed according to Alves et al., (1999). Monosporic cultures of each isolate were observed using a Phase Contrast Microscopy (Axioplan – Zeiss, Germany) and Scanning Microscopy (DMS940 A – Zeiss, Germany. *DNA extraction*: Fungi strains were grown on 25 ml liquid PDA medium for 96 hours at 26 ^{o}C for DNA extraction, according Lee and Taylor (1990).

DNA Amplification e Sequencing: Primers ITS1 and ITS4 were used to amplify a rDNA ITS region (White et al., 1990). PCR-amplified ITS fragments were purified using the QIAquick

Gel Extraction kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). Each ITS purified fragment was sequenced in both directions with the primers ITS1 and ITS4 using Big Dye Terminator v. 3.1 (Applied Biosystems, Foster City, CA) in an ABI Prism 3100 sequencer (Applied Biosystems). For the RAPD analysis, eight random decamer primers from Operon Technologies Inc. (Alameda, CA, USA) were used.

Data analysis: Amplified ITS sequences were compared with the GenBank Nucleotide Database (http://www.ncbi.nlm.nih.gov) using the algorithm Blast N (Altschul et al., 1997). A consensus tree was constructed, after 1,000 bootstraps resampling steps, by UPGMA method with p-distance using the software Mega 3 (Kumar et al., 2004). RAPD data were recorded as a binary matrix of 0 and 1 corresponding to the absence or presence of reproducible bands. Cluster analysis among the isolates was carried out using the UPGMA method using the Statistica software version 4.2 (StatSoft Inc., 1995). Linear regression models were applied to detect associations between the RAPD data and larvae mortality, considering the molecular markers as independent variables using Jump version 3.1.6.2 software (SAS Institute, Inc.).

RESULTS AND DISCUSSION

Under light and scanning microscopy, *Beauveria* spp. cultures had the appearance of dense clusters of globose spherical conidiogenous cells with apical denticulate rachis, which give it a zigzag appearance (Samson et al., 1988) (Fig 1). Even though, strains cultured in PDA showed morphological homogeneity, bioassays results showed considerable variability among the *Beauveria* isolates aggressiveness to the maize armyworm larvae, in which the insect mortality ranged from 0 to 100%. Once phenotypical characterization of *Beauveria* species was controversial and not sufficient to differentiate among strains (Gaitan et al., 2002), a further molecular study using rDNA-ITS sequencing and RAPD markers was carried out among the Brazilian isolates. rDNA sequences of approximately 570 bp revealed almost 100% identity with sequences deposited in the GenBank Nucleotide Database, which were able to distinguish both species of *B. bassiana* and *B. brongniartii* (Fig 2). As the similarity among the sequences was high, the isolates could not be distinguished from each other. A consensus tree based on the ITS sequences clustered the isolates in three groups (Fig 2). Eight RAPD primers generated 72 scorable bands, out of those only 5 (7%) were monomorphic among all *Beauveria* strains. Among all pair wise comparisons, the smallest genetic distances was between the isolates CNPMS09 and CNPMS10, and the isolates CNPMS48 and CNPMS49 (0%), while the greatest divergence was between CNPMS78 and CNPMS79 (75%). The dendrogram generated by RAPD markers revealed three major phenetic groups supported by bootstrap values higher than 60%, at genetic distances of 0.32, 0.16 and 0.25, for the groups A, B and C, respectively. Single linear regression analysis indicated that three RAPD polymorphic bands among *Beauveria* isolates were significantly ($P<0.001$) associated with the levels of virulence against *S. frugiperda*, explaining from 32 to 67% of the phenotypic variation. Among then, two loci OPAO3-2 and OPAO2-8 were negatively associated with the isolates virulence. A total of 67 polymorphic RAPD fragments were able to discriminate the *Beauveria* strains evaluated in this study, detecting a higher amount of genetic variation compared to the ITS sequence. However, most of these variations were below the genetic distance of 0.30, showing a close relatedness among these strains. This can be justified by the fact that *Beauveria* spp. is an haploid fungi and reproduces predominantly via the asexual mode, so most of its genetic variation is due to mutations or parasexual recombination (Castrillo and Brooks, 1998). In addition of genetic diversity analysis, three RAPD markers were highly associated with the maize armyworm larvae mortality, suggesting a potential use of these markers as a first screening strategy of *Beauveria* strains for biological

control against *S. frugiperda*. Indeed, to validate the association of these markers, a higher number of *Beauveria* strains should be screened.

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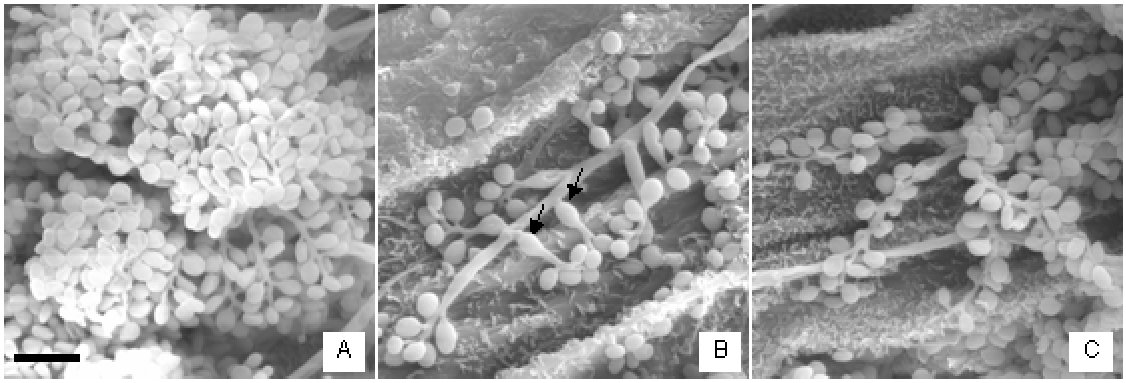

Fig 1 - *Beauveria* spp. morphology under scaning microscopy. (A) Cluster of conidiogenous cells; (B) Arrows indicate phyalides; (C) Rachis in a zigzag appearance. Bar: 5.0 µm.

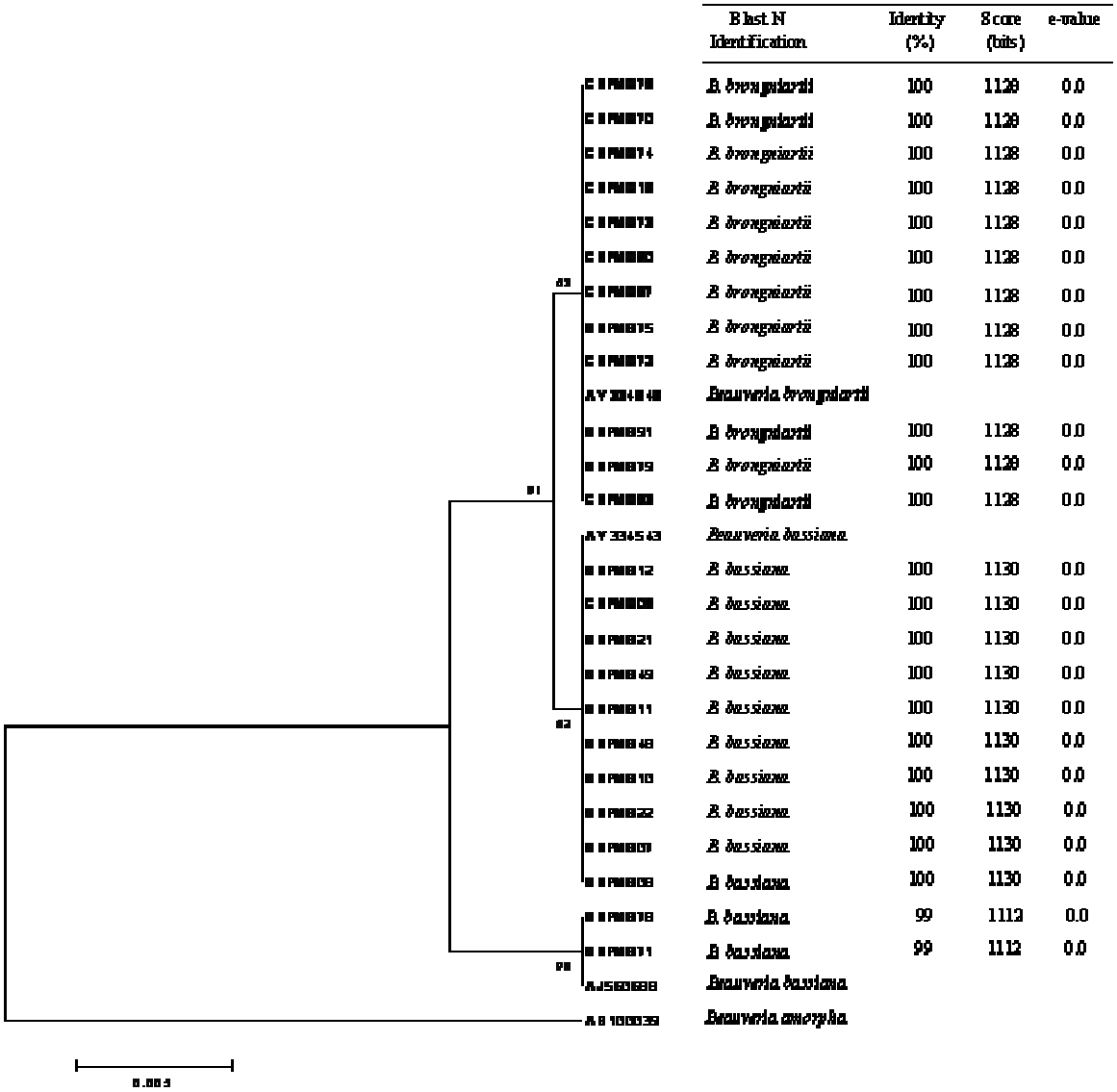

Fig 2 – Dendrogram of the 24 ITS sequences from the *Beauveria* isolates generated by Clustal W using UPGMA method and 1,000 bootstrap resampling steps. The tree was rooted with the outgroup *Beauveria amorpha. Bar*: Nucleotide substitutions (x100).