Molecular characterization and pathogenicity of isolates of *Beauveria* spp. to fall armyworm

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Abstract – The objective of this work was to evaluate the pathogenicity of 24 *Beauveria* isolates to *Spodoptera frugiperda* larvae, and characterize them molecularly through rDNA-ITS sequencing and RAPD markers. Sequencing of rDNA-ITS fragments of 570 bp allowed the identification of isolates as *B. bassiana* or *B. brongniarti* by sequence comparison to GenBank. Sixty seven polymorphic RAPD fragments were capable to differentiate 20 among 24 *Beauveria* isolates, grouping them according to the derived host insect and to pathogenicity against maize fall armyworm larvae. Three RAPD markers were highly associated to the pathogenicity against *S. frugiperda*, explaining up to 67% of the phenotypic variation. Besides identification and molecular characterization of *Beauveria* isolates, ITS sequence and RAPD markers proved to be very useful in selecting the isolates potentially effective against *S. frugiperda* larvae and in monitoring field release of these microorganisms in biocontrol programs.

Index terms: *Spodoptera frugiperda*, entomopathogenic fungi, RAPD, rDNA-ITS.

Caracterização molecular e virulência de isolados de *Beauveria* spp. contra a lagarta-do-cartucho

Resumo – O objetivo deste trabalho foi avaliar a patogenicidade de 24 isolados de *Beauveria* contra larvas de *Spodoptera frugiperda* e caracterizá-los molecularmente, por meio do seqüenciamento da região ITS do rDNA e de marcadores RAPD. O seqüenciamento de fragmentos de 570 pares de bases, da região ITS do rDNA, possibilitou a identificação dos isolados como *B. bassiana* e *B. brongniarti*, pela comparação com seqüências depositadas no GenBank. Sessenta e sete fragmentos polimórficos de RAPD foram capazes de diferenciar 20 entre 24 isolados de *Beauveria*, e agrupá-los de acordo com o inseto hospedeiro e com a patogenicidade contra a lagarta-do-cartucho do milho. Os marcadores RAPD foram altamente associados à patogenicidade contra *S. frugiperda* e explicaram até 67% da variação fenotípica. Além da identificação e caracterização molecular de isolados de *Beauveria*, o seqüenciamento da região ITS, aliado aos marcadores RAPD, é útil na seleção de isolados potencialmente eficazes contra larvas de *S. frugiperda* e no monitoramento de liberações desses microrganismos em programas de biocontrole.

Termos para indexação: *Spodoptera frugiperda*, fungo entomopatogênico, RAPD, rDNA-ITS.

Introduction

Fall armyworm [*Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae)] is one of the main pests in maize fields in Brazil, and its severity has increased in various agricultural areas (Cruz et al., 1999). *S. frugiperda* is able to damage maize plants in all physiological stages, and drastically reduces the crop production, if not controlled.

Control of *S. frugiperda* is made mainly by chemical treatment, as soon as the first symptoms appear. However, the constant use of these products has caused problems such as the development of resistance, reduction of natural enemy populations and environmental contamination (Yu et al., 2003). To avoid such problems, the search for alternative methods of pests control has become mandatory (Cruz et al., 1999).

One of the alternatives to control *S. frugiperda* infestations is based on microbial agents with low environmental impact and high specificity and efficiency in reducing this insect ability to cause injury to the host plant. Therefore, entomopathogenous fungi are interesting biocontrol agents, due to their epizootics and pathogenicity (Devi et al., 2001). Among them, *Beauveria* is one of the most studied fungi which infects many insect species in different parts of the world (Hajek & St. Leger, 1994), being frequently isolated from mycosed insect corpses (Devi et al., 2001), and used as entomopathogenic agent in biological control programs (Wang et al., 2005; Cruz et al., 2006; Dolci et al., 2006).

A number of *Beauveria* isolates have been studied due to their potential use as biopesticides (Smith et al., 1999; Devi et al., 2001). However, once phenotypic characteristics are neither sufficient to distinguish different *Beauveria* isolates nor enough to monitor field releases of biocontrol agents (Gaitan et al., 2002; Castrillo et al., 2003), molecular analysis is demanded. Most of the methods used to characterize genotypic variations in fungi are based on PCR, DNA/RNA probes, and protein assays. Among them, RAPD markers (Maurer et al., 1997; Berreta et al., 1998; Castrillo & Brooks, 1998; Glare & Inwood, 1998; Devi et al., 2001; Gaitan et al., 2002; Dolci et al., 2006), and the internal transcribed spacers of the ribosomal DNA (rDNA-ITS) sequencing (Coates et al., 2002; Gaitan et al., 2002; Muro et al., 2003, 2005; Wada et al., 2003) have been successfully employed to assess the genetic variability of *Beauveria* spp. Nevertheless, the identification of molecular markers highly associated to *Beauveria* spp. pathogenicity against *S. frugiperda* has not been made yet.

The objective of this work was to evaluate the pathogenicity of 24 *Beauveria* isolates against *S. frugiperda* larvae, and to characterize them molecularly, through rDNA-ITS sequencing and RAPD markers.

Materials and Methods

Twenty four *Beauveria* spp. isolates were recovered from infected dead corpses of *S. frugiperda* and *Dalbulus maidis* collected in different maize fields of Central Brazil (Table 1). Dead insects were incubated under 80–90% humidity at 25ºC for 1–2 weeks to induce mycelia growth of infecting fungi. *Beauveria* isolates obtained were plated onto potato-dextrose agar (PDA) and stored in glycerol 20% at -20ºC. Single spore cultures were established for each isolate, and cultured on PDA for 10 days at 25ºC.

S. frugiperda used in the bioassays were obtained from the Laboratório de Controle Biológico (Biological Control Laboratory) of Embrapa Milho e Sorgo, where larvae and adult colonies are maintained in artificial diet at 26ºC. Pathogenicity against *S. frugiperda* was determined using six replicates of eight second instar larvae, which were soaked in aqueous conidial suspensions (10^9 spores mL^{-1}) of each isolate, with 0.01% Triton XL. After treatment, larvae were transferred to a 50 mL plastic cup containing pieces of fresh maize leaves. As control, eight larvae were treated with water and 0.01% Triton XL. Recipients containing larvae were maintained at 26ºC and 80–90% relative humidity. Larvae mortality was recorded as the percentage of dead larvae, 10 days after infection.

Taxonomic identification of *Beauveria* spp. was performed according to Alves et al. (1999). Monosporic cultures of each isolate were observed using a phase contrast microscopy and scanning microscopy. Phenotypic traits were observed, such as mycelium septation and coloration, sexual and asexual reproduction, presence or absence of phyalide with zigzag ends, form and size of conidias.

For DNA extraction, fungi isolates were grown on 25 mL of liquid PDA medium for 96 hours at 26ºC, after which mycelia formed were collected by filtration, washed three times with distilled water, freeze-dried and grounded in liquid nitrogen. Five volumes of extraction buffer (50 mM Tris-HCl pH 7.2; 50 mM EDTA pH 8; 3% SDS; 1% beta-mercaptoetanol) were added to 0.5 g of powdered fungal mycelia (Lee & Taylor, 1990), and kept at 65ºC for 20 min. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the mixture, stirred gently and then centrifuged at room temperature for 20 min at 12,000 *g*. The aqueous phase was re-extracted with an equal volume of chloroform:isoamyl alcohol (24:1), and then DNA was precipitated by adding 0.6 volume of isopropanol and 100 mM NaCl. DNA was spooled out with a glass hook, washed twice with 70% (v/v) ethanol and eluted in distilled water. DNA concentration was determined using a spectrophotometer LAMBDA Bio.

To perform the rDNA analysis, primers ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3') and ITS4 (5' - TCC TCC GCT TAT TGA TAT GC - 3') were

used to amplify a rDNA-ITS region (White et al., 1990). Amplification reactions were achieved in a total volume of 50 µL containing 0.2 µM of each primer; 20 mM Tris-HCl; 50 mM KCl; 2.5 mM $MgCl_2$; 0.1 mM each deoxinucleotide (dATP, dCTP, dGTP and dTTP); 1 U Taq DNA polymerase; and 50 ng genomic DNA. Samples were amplified using an initial step of 15 s at 94°C, followed by 40 cycles (94°C at 15 s, 50°C at 30 s, 72°C at 30 s), and one final extension of 7 min at 72°C.

For the RAPD analysis, eight random decamer primers (OPA02, OPA03, OPA09, OPA13, OPQ01, OPQ04, OPZ13 and OPZ19) were selected out of 15 RAPD primers tested. Amplification reactions were carried out in a total volume of 25 µL containing 0.4 µM each primer; 20 mM Tris-HCl pH 8.4; 50 mM KCl; 2.5 mM $MgCl_2$; 0.1 mM each deoxinucleotide; 25 ng genomic DNA; and 0.5 U Taq DNA polymerase. Amplifications were executed using an initial step of 15 s at 94°C, followed by 40 cycles (94°C at 15 s, 36°C at 30 s, 72°C at 1 min), and a 7 min final extension at 72°C. RAPD amplifications were repeated two times for each primer. Negative controls containing water instead of DNA were included in each experiment. All amplifications were performed in a PTC-200 termocycler. RAPD products were analyzed by electrophoresis in 1.5% agarose gels with TAE buffer 1 X (40 mM Tris-Acetate, 1 mM EDTA pH 8), stained with ethidium bromide (0.5 µg mL^{-1}) and 1 kb ladder was used as a standard molecular weight. Amplified bands were visualized under UV lights, and images were captured and stored using a photo-documentation system Eagle Eye II.

PCR-amplified ITS fragments of the 24 *Beauveria* isolates were purified using the QIAquick gel extraction kit, according to the manufacturer's instructions. Each ITS fragment was sequenced in both directions with the primers ITS1 and ITS4, using Big Dye Terminator v. 3.1 in an ABI Prism 3100 sequencer. Nucleotide sequences were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases (accession numbers DQ 153016 to DQ 153039).

Amplified ITS sequences were edited by the module SeqMan of DNAStar, and were compared with the GenBank Nucleotide Database (http://www.ncbi.nlm.nih.gov) using the algorithm Blast N (Altschul et al., 1997). The entire sequences were aligned

Table 1. Host, ITS sequences analyzed, geographical origin, and pathogenicity against *Spodoptera frugiperda* larvae of isolates of *Beauveria* spp.

Isolate	Species	Host	ITS sequences[1]	Origin	Pathogenicity (%)[2]
CNPMS07	*B. bassiana*	*S. frugiperda*	DQ153016	Sete Lagoas, MG - 19.47S/44.25W	0.0
CNPMS08	*B. bassiana*	*S. frugiperda*	DQ153017	Sete Lagoas, MG - 19.47S/44.25W	0.0
CNPMS09	*B. bassiana*	*S. frugiperda*	DQ153018	Sete Lagoas, MG - 19.47S/44.25W	0.0
CNPMS10	*B. bassiana*	*S. frugiperda*	DQ153019	Sete Lagoas, MG - 19.47S/44.25W	0.0
CNPMS11	*B. bassiana*	*S. frugiperda*	DQ153020	Sete Lagoas, MG - 19.47S/44.25W	0.0
CNPMS12	*B. bassiana*	*S. frugiperda*	DQ153021	Sete Lagoas, MG - 19.47S/44.25W	0.0
CNPMS15	*B. bassiana*	*S. frugiperda*	DQ153022	Goiânia, GO - 16.67S/49.25W	0.0
CNPMS21	*B. bassiana*	*S. frugiperda*	DQ153023	Sete Lagoas, MG - 19.47S/44.25W	0.0
CNPMS22	*B. bassiana*	*S. frugiperda*	DQ153024	Londrina, PR - 23.37S/51.17W	11.7
CNPMS48	*B. bassiana*	*S. frugiperda*	DQ153025	Sete Lagoas, MG - 19.47S/44.25W	0.0
CNPMS49	*B. bassiana*	*S. frugiperda*	DQ153026	Sete Lagoas, MG - 19.47S/44.25W	0.0
CNPMS50	*B. brongniartii*	*S. frugiperda*	DQ153027	Sete Lagoas, MG - 19.47S/44.25W	0.0
CNPMS63	*B. brongniartii*	*D. maidis*	DQ153028	Sete Lagoas, MG - 19.47S/44.25W	77.2
CNPMS67	*B. brongniartii*	*S. frugiperda*	DQ153029	Fortuna de Minas, MG - 19.56S/44.45W	49.3
CNPMS70	*B. brongniartii*	*S. frugiperda*	DQ153030	Sete Lagoas, MG - 19.47S/44.25W	50.0
CNPMS71	*B. bassiana*	*D. maidis*	DQ153031	Sete Lagoas, MG - 19.47S/44.25W	100.0
CNPMS72	*B. brongniartii*	*D. maidis*	DQ153032	Jardinópolis, SP - 21.02S/47.76W	100.0
CNPMS73	*B. brongniartii*	*D. maidis*	DQ153033	Sete Lagoas, MG - 19.47S/44.25W	100.0
CNPMS74	*B. brongniartii*	*S. frugiperda*	DQ153034	Sete Lagoas, MG - 19.47S/44.25W	44.7
CNPMS75	*B. brongniartii*	*D. maidis*	DQ153035	Sete Lagoas, MG - 19.47S/44.25W	37.5
CNPMS76	*B. brongniartii*	*D. maidis*	DQ153036	Dourados, MS - 22.23S/54.98W	0.0
CNPMS78	*B. bassiana*	*D. maidis*	DQ153037	Barreiras, BA - 12.15S/45.00W	63.0
CNPMS79	*B. brongniartii*	*D. maidis*	DQ153038	Capão Bonito, SP - 24.03S/48.37W	36.2
CNPMS91	*B. brongniartii*	*S. frugiperda*	DQ153039	Mineiros, GO - 17.57S/52.55W	100.0

[1]Accession number. [2]Larvae mortality in bioassays.

using the Clustal W 1.8 software (Thompson et al., 1994), and were grouped with other *Beauveria* sequences deposited in the GenBank. A consensus tree was constructed, after 1,000 bootstraps resampling steps, by maximum parsimony 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, respectively. Genetic distance measurements were estimated according to Nei & Li (1979) index. Cluster analysis among the isolates was carried out using the UPGMA method (unweighted pair group method algorithm), and performed using the Statistica software version 4.2 (StatSoft Inc.). The support for clustering was estimated by bootstrap analysis using 10,000 sampling.

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 of the SAS.

Results and Discussion

Attempts to taxonomic classification of the white colonies grown on PDA medium were not conclusive. Under light and scanning microscopy those colonies had the appearance of dense clusters of globose spherical conidiogenous cells, with apical denticulate rachis, which gave them a zigzag appearance (Samson et al., 1988). Also, the length (L) and width (W) of *Beauveria* conidia, measured through a scanning microscope, were not different among the isolates, ranging from 1.8 to 3.1 µm x 1.1 to 2.1 µm, and L/W ratios from 1 to 1.5 µm. Even though Mugnai et al. (1989), Glare & Inwood (1998) and Alves et al. (1999) could distinguish *Beauveria* species using spore shape and size, these authors suggested that additional traits should be used to confirm the classification. Besides, spore shape of *Beauveria* can change, when it is cultured in vitro (Wada et al., 2003) or when it is isolated from different hosts (Mugnai et al., 1989).

Although isolates cultured in PDA showed morphological homogeneity, bioassays revealed a wide variation in their pathogenicity against the *S. frugiperda*, where the larvae mortality ranged from 0 to 100% (Table 1). Only 12 out of the 24 isolates were infectious to the fall armyworm and, among them, four – CNPMS71, CNPMS72, CNPMS73 and CNPMS91 – were able to kill 100%. Out of these four, the isolate CNPMS91 was the only one recovered from *S. frugiperda*, while the others were isolated from *D. maidis*. This difference in pathogenicity was probably a consequence of the fact that *Beauveria* were isolated as a saprophytic microorganism, instead of the main cause of insect death.

In this work, no polymorphism in length of the amplified rDNA region was observed among the *Beauveria* isolates, since a fragment of 570 bp was amplified for all the 24 isolates. Sequences of these fragments were compared with data from the GenBank, and the isolates were characterized as *B. bassiana* or *B. brongniartii* with high degree of identity. A low level of sequence variation was detected within the ITS region, which corroborates other authors (Glare & Inwood, 1998; Muro et al., 2003, 2005; Wada et al., 2003; Rehner & Buckley, 2005; Cruz et al., 2006). Only four out of 571 nucleotides were different between *B. bassiana* and *B. brongniartii* species. A consensus tree based on the ITS sequences clustered the isolates in three groups (Figure 1). All the isolates identified as *B. brongniartii*, recovered either from *S. frugiperda* or from *D. maidis* (Group A), were separated from the ones identified as *B. bassiana*. However, the isolates identified as *B. bassiana* were clustered based on the host of origin, from *D. maidis* (Group B) and from *S. frugiperda* (Group C).

Eight RAPD primers generated 72 scorable bands. Out of those, only 5 (7%) were monomorphic among all *Beauveria* isolates (Table 2). There were no fragments amplified in the negative control. The number of bands amplified by each primer varied from 4 (OPA09) to 13 (OPA03 and OPQ01), with an average of 5.3 bands per primer. The sizes of the bands ranged from 300 (OPZ19) to 3,800 (OPA13) base pairs.

A total of 67 polymorphic RAPD fragments were able to discriminate 20 among 24 *Beauveria* isolates, and detected a higher amount of genetic variation compared with the ITS sequences. Among all pair wise comparisons, the smallest genetic distance was between the isolates CNPMS09 and CNPMS10 and the isolates CNPMS48 and CNPMS49 (0%), while the greatest divergence was between CNPMS78 and CNPMS79 (75%). However, most of these variations were below the genetic distance of 0.30, showing a close relatedness among all the isolates. This can be justified by the fact that *Beauveria* spp. is a haploid fungus with a predominant asexual reproduction, so most of its genetic variation is due to

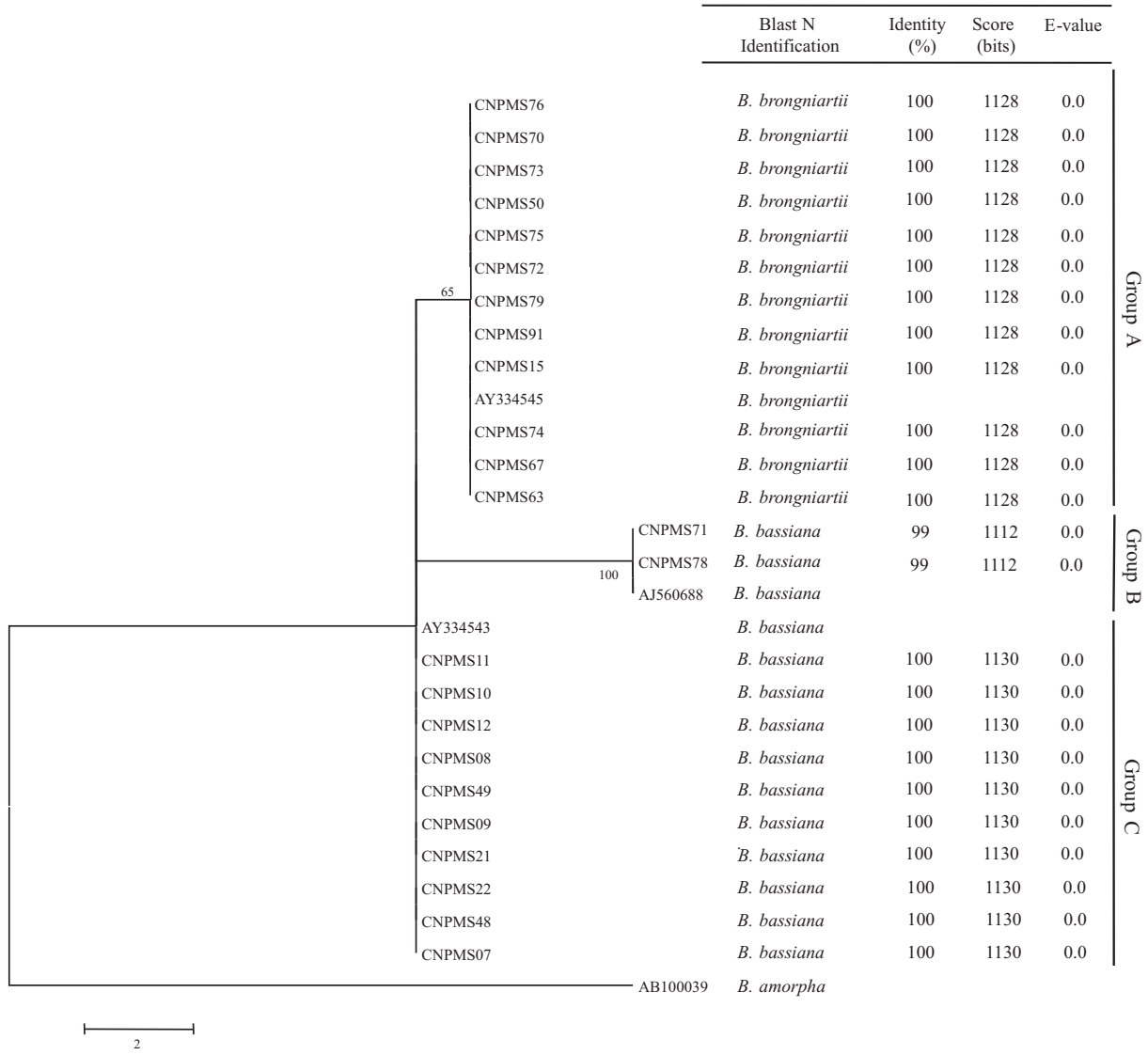

Figure 1. Dendrogram of the 24 ITS sequences from *Beauveria* isolates, generated by Clustal W using the maximum parsimony method, and 1,000 bootstrap resampling steps. The tree was rooted with the outgroup *B. amorpha.* Bar: nucleotide substitutions (x100).

Table 2. Primers used in RAPD amplification, sequences, number of analyzed bands, number of polymorphic bands and percentage of polymorphism generated.

Primer	Sequence (5' 3')	Analyzed fragments	Polymorphic fragments	Polymorphism (%)
OPA02	TGCCGAGCTG	11	11	100.0
OPA03	AGTCAGCCAG	13	13	100.0
OPA09	GGGTAACGCC	4	4	100.0
OPA13	CAGCACCCAC	8	8	100.0
OPQ01	GGGACGATGG	13	11	84.6
OPQ04	AGTGCGCTGA	8	7	87.5
OPZ13	GACTAAGCCC	7	7	100.0
OPZ19	GTGCGAGCAA	8	6	75.0
Total		72	67	93.0

mutation or parasexual recombination (Castrillo & Brooks, 1998). In addition, most of the isolates were collected from the same geographic area. A high genetic similarity level among the isolates from different climatic zones and hosts was observed by Devi et al. (2001) and Gaitan et al. (2002). In contrast, Berretta et al. (1998) and Castrillo & Brooks (1998) detected high levels of genetic variability among *B. bassiana* isolates using RAPD markers.

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 (Figure 2). Cluster A comprised most of *B. brongniartii* isolated from *D. maidis*, except for isolates CNPMS67, CNPMS70 and CNPMS91, which derived from *S. frugiperda*, while cluster B grouped all *B. bassiana* isolated from *S. frugiperda* . Most of the isolates in cluster B were not able to infected *S. frugiperda* under laboratory conditions. In contrast, almost all *B. brongniartii* isolates grouped in cluster A, except for CNPMS76, caused *S. frugiperda* larvae mortality ranging from 36 to 100%, in laboratory bioassays. Group B clustered two *B. bassiana* isolated from *D. maidis*, showing high levels of pathogenicity against fall armyworm larvae. Correlations between insect host, geographical origin and pathogenicity in *Beauveria* spp. have been controversial; while some reports demonstrated significant correlations among these factors, others found no relationship between them (Maurer et al., 1997; Beretta et al., 1998; Castrillo & Brooks, 1998; Castrillo et al., 1999; Devi et al., 2001). The data emphasize that the grouping factors, in this set of entomopathogenic fungi, were: species, the host insect and the pathogenicity to the *S. frugiperda* in bioassays.

Single linear regression analysis indicated that three RAPD polymorphic bands among *Beauveria* isolates were significantly ($p<0.001$) associated with the levels of pathogenicity against *S. frugiperda*, and explained from 32 to 67% of the phenotypic variation (Table 3). This result suggests a potential use of these markers, as

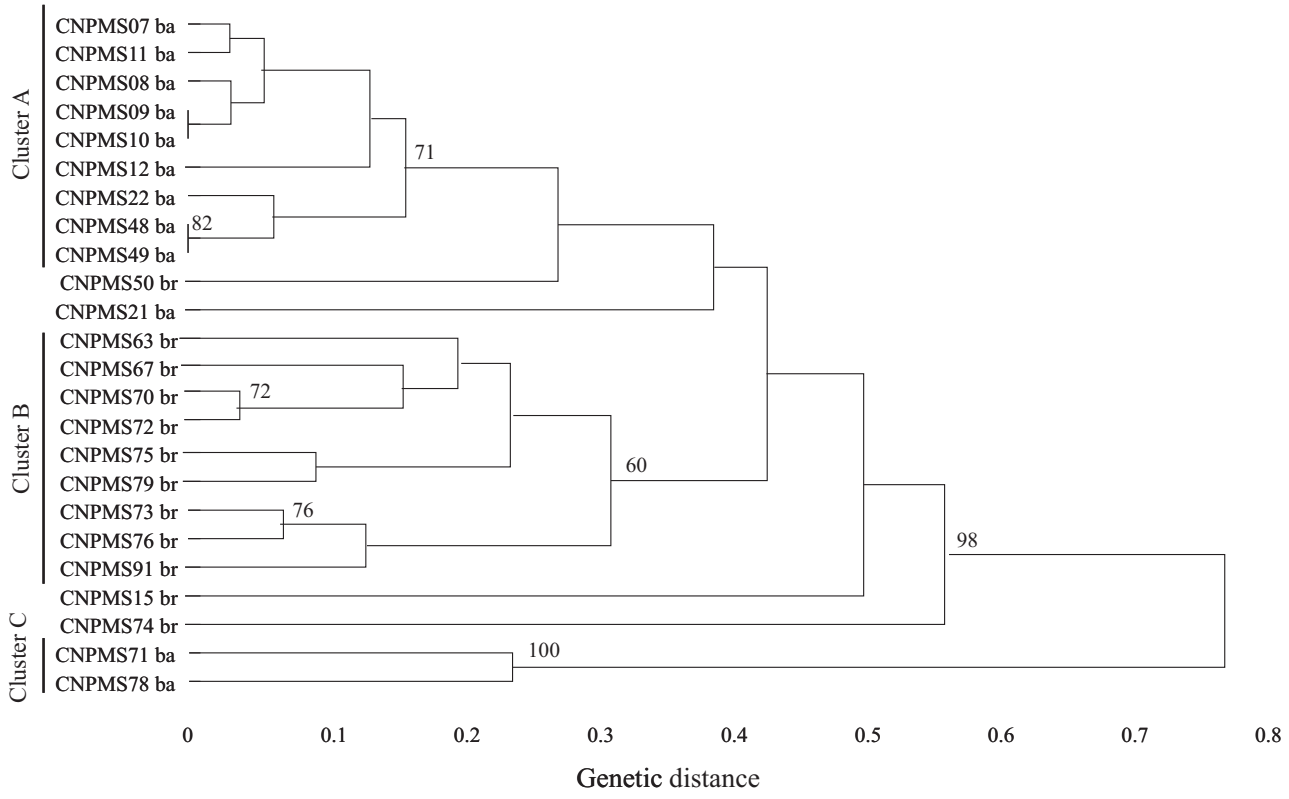

Figure 2. Cluster analysis of 24 *Beauveria* spp. isolates by using RAPD markers. The dendrogram was generated by UPGMA method using 67 RAPD polymorphics. Bootstrap values that exceeded 60% are shown on the respective branches in percentage. Group identification was the same as that designated by ITS sequence on Figure 1. ba: *B. bassiana*; br: *B. brongniartii.*

Table 3. RAPD loci significantly associated with *Beauveria* spp. pathogenicity against *Spodoptera frugiperda* larvae, by using linear regression analysis.

RAPD markers	Effect±SD	R^2 adjusted (%)	F ratio	Probability
OPA03-2	-58.71±12.78	50.13	21.11	0.0002
OPA13-4	51.55±15.72	32.79	10.76	0.0039
OPA02-8	-67.24±10.33	67.41	42.37	<0.0001

a first screening strategy of *Beauveria* isolates for biological control against *S. frugiperda* . However, to validate the association of these markers with insect mortality, a higher number of *Beauveria* isolates should be screened, and their mortality against *S. frugiperda* should be quantitatively evaluated by Probit methodology.

Conclusions

1. rDNA-ITS sequence and RAPD markers used simultaneously are able to identify and to characterize *Beauveria* isolates, and are useful to detect inter and intraspecific variability within this genus.

2. Three RAPD markers are associated with the pathogenicity against *Spodoptera frugiperda*, and can be used to screen *Beauveria* isolates for biological pests' control.

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