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A N I N T E R N A T I O N A L J O U R N A L

Pulsed Field Gel Electrophoresis Reveals Chromosome Length and Number Differences in Brazilian Strains of *Metarhizium Anisopliae*

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ABSTRACT

Electrophoretic karyotypes of eight wild-type strains of Metarhizium anisopliae *var.* anisopliae *were obtained by pulsed-field gel electrophoresis. These strains were isolated from insects of six different Brazilian states. The chromosomal DNA molecules of three strains were separated into seven bands and of five strains into eight bands. Chromosome length polymorphisms were also observed. The size of the chromosomal DNA of all strains varied between 7.7 and 0.9 Mb using the* Aspergillus nidulans *chromosomes as size standards. The total genome size of these strains was estimated in at least 29.7 Mb. Some correlations between differences in karyotype and occurrence of parasexual cycle likewise the host specificity were discussed.*

Key words: *Metarhizium anisopliae*, pulsed-field gel electrophoresis, chromosome polymorphisms, parasexual cycle, entomopathogenic fungus

INTRODUCTION

Metarhizium anisopliae is an entomopathogenic hyphomycete fungus, which has been recognized as a powerful tool in the control of the pests in agriculture. In Brazil, *M. anisopliae* var. *anisopliae* has been mainly employed to control Cercopidae: Homoptera, such as *Mahanarva posticata* in sugarcane and *Deois flavopicta* and *Zulia entreriana* in pasture grasses. The nature and extent of genetic variation in this species have been assessed in several ways: isoenzyme analysis (De Conti, et al., 1980; St. Leger et al., 1992); pyrolisis-gas chromatography (Messias et al., 1983); growth rates (Huxam et al., 1989); virulence (Daoust and Roberts, 1982); production of extracellular enzymes (Rosato et al., 1981); RFLP, restriction fragment length polymorphisms (Pipe et al., 1995) and RAPD, random amplified polymorphic DNA (Bidochka et al., 1994; Fegan et al., 1993 and Fungaro et al., 1996). All data showed great genetic diversity among the isolates analyzed. The results derived from RAPD analysis (Fungaro et al., 1996) showed for the first time that the genetic variability among insect isolates was much lower than the genetic variability observed

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among soil isolates, suggesting that this fungus was developed a certain degree of host specificity. An effective technique designated pulsed-field gel electrophoresis (PFGE), was used to separate chromosome-sized DNA molecules of *M. anisopliae* (Shimizu et al., 1992 and Valadares-Inglis and Peberdy, 1998). However, so far only a few isolates have been tested by this technique and most of them derived from a single species host.

The present work was carried out in an attempt to improve the conditions used in the chromosome separation for this fungus; to study the possible chromosome-sized DNA molecules differences in number and size of eight isolates of *M. anisopliae* var. *anisopliae* from different insect-hosts and regions and find some correlation between the occurrence of parasexual cycle described by other authors and the karyotype found in the present work using the same strains.

MATERIALS AND METHODS

Strains

Strains of *M. anisopliae* used in this work were obtained from the "Laboratório de Genética de Microrganismos" (ESALQ/University of São Paulo, Brazil). The eight wild-type strains were isolated from insects of six different states of Brazil and were designated as follows: E6 and E9 (from the state of Espirito Santo), M5 (Pernambuco), MT (Mato Grosso), RJ (Rio de Janeiro) and AL (Alagoas), all from *Deois flavopicta*. Strains A4 and A19 were isolated from Bahia state, from *Mahanarva posticata* and *Deois schach*, respectively. The MSE strain from *Aspergillus nidulans* was used as source of chromosomal DNA size standard.

Preparation of intact chromosomal DNA

Agarose plugs containing *A. nidulans* intact chromosome DNAs were prepared as described by Brody and Carbon (1989). The protoplasts from the *M. anisopliae* strains were obtained as described by Silveira and Azevedo (1987) in a 0.7M KCl phosphate buffer as osmotic stabilizer. Chromosome-sized DNA was prepared based on procedures currently used for *A. nidulans*, with modifications. Protoplast suspensions were centrifuged for 10 minutes at 4000 rpm and protoplasts were resuspended in GMB buffer (0.125M EDTA pH 7.5, 0.9M sorbitol) and centrifuged again for five minutes under the same conditions. This procedure was repeated twice to remove lytic enzymes used in protoplast production. The protoplasts were then resuspended in GMB buffer, to obtain a final concentration of 10^9 cells per mL. The suspension was then placed at 42^o C and an equal volume of 1.4% agarose (LGT-Low Gelling Temperature) at the same temperature was added. The mixture was gently homogenized, placed into a plug mould (Bio-Rad) and kept on an ice-bath for ten minutes. The plugs were then removed and incubated in NDS buffer (0.5M EDTA, pH 8.0; 10mM Tris-HCl, pH 9.5; 1% (w/v) sodium N-lauroylsarcosinate) containing proteinase K (1mg/mL) at 50^oC overnight. The NDS plus proteinase K was replaced by 50mM EDTA (pH 8.0) plus 1% (w/v) sodium N-lauroylsarcosinate and incubated at 50^o C for 30 minutes. Finally, the plugs were washed three times in 50mM EDTA (pH 8.0) at 50^o C with one hour interval between each wash with occasional swirling. The plugs were stored in 50mM EDTA (pH 8.0) at 4^oC.

Pulsed Field Gel Electrophoresis conditions

Electrophoresis was performed in a CHEF-DRII (Bio-Rad) system. Gels of 0.6% and 0.8% (w/v) chromosomal grade agarose (Sigma) were prepared and run in 0.5x TBE buffer (44.5mM Tris, 44.5mM boric acid, 1mM EDTA). The plugs were inserted into the gel wells and sealed with the same agarose used to prepare the gel. The electrophoresis conditions (pulse intervals and durations) were: A) 50 min, 45min and 37 min, during 73h, 18h and 73h, respectively, with a voltage of 46V; B) 90 and 60 min during 72h each one with a voltage of 40V (Shimizu et al., 1992). During the run, the temperature was kept at 12^o C. Gels were stained in ethidium bromide (0.5 mg/mL) for 20 min, destained in distilled water for 20 min and photographed under ultraviolet transillumination using Ilford 50 film.

RESULTS AND DISCUSSION

The method used in this work to prepare chromosome-sized DNA molecules of eight strains

of *M. anisopliae* var. *anisopliae* proved to be efficient considering that good resolution bands were obtained for all the analyzed strains. Using parameters described by Shimuzi et al.(1992) we were not able to resolve the chromosomal DNAs in seven bands as described by these authors. Three strains of *M. anisopliae* isolated in Japan were from soil and two from insects (*Bombyx mori* and *Anomala cuprea*). Each of these strains showed a unique banding profile. Valadares-Inglis and Peberdy (1998) using four strains of *M. anisopliae*, isolated from *Deois* sp. in Brazil, were able to resolve chromosomal DNA into eight bands for one strain and seven bands for others. They used linearly ramped initial and final pulse lengths of 3000 s and 1300 s, respectively, for 167 hours.

Among three groups of *M. anisopliae* chromosomal DNA, Group I showed two to three bands corresponding to molecules between 0.9 and 2.8Mb; Group II had three bands corresponding to molecules between 3.6 and 4.9 Mb and, Group III had two to three bands corresponding to molecules between 6.0 and 7.7 Mb (Table 1 and Fig. 1). Three bands in Group III were only resolved using B electrophoresis conditions (Shimizu et al., 1992) (data not shown). These parameters were useful to show that some single bands showed in Group III with condition A were doublets. In Group I and Group III polymorphisms were observed in the length and in the number of chromosomal DNA while in Group II only length polymorphisms were detected. The total genome size of these strains was estimated in at least 29.4 Mb. Molecular sizes of the chromosomal DNAs were estimated with reference to the size standards, using Kodak 1D Imaging Analysis Software.

Table 1 - Size estimated (Mb) and number of *M. anisopliae* chromosomal DNAs as determined by CHEF analysis.

Group and	Strain							
Band nº	E6	E9	RJ	A4	A19	M5	MT	AL
III 3	7.6	7.6	7.6	7.6	7.2	7.7	7.7	7.6
III 2	6.7	-	6.8	6.9	6.6	6.7	6.9	7.3
III 1	6.1	6.2	-	-	6.1	-	6.0	7.0
II 3	4.7	4.5	4.6	4.7	4.5	4.7	4.5	4.9
II 2	4.3	3.8	4.2	4.3	3.8	4.4	3.8	4.3
II 1	3.8	3.6	3.6	3.6	3.7	3.6	3.7	3.6
I 3	2.5	2.3	2.5	2.0	2.4	1.5	2.4	2.8
I 2	1.5	1.4	1.4	1.5	1.4	1.4	1.4	1.4
I 1	-	-	0.9	-	-	-	-	-
Total	37.2 (8)	29.4 (7)	31.6 (8)	30.6 (7)	35.7 (8)	30.0 (7)	36.4 (8)	38.9 (8)

Differences in chromosome number and length are common not only in *Metarhizium anisopliae* (Shimizu et al., 1992, Valadares-Inglis and Peberdy, 1998) but also in other fungi such as *Cladosporium fulvum* (Talbot et al. 1991), *Neurospora crassa* (Orbach et al., 1988), *Colletotrichum gloeosporioides* (Masel et al., 1990), *Ustilago hordei* (McCluskey and Mills, 1990), *Fusarium solani* (Bruschi and Nazareth, 1994; Suga et al. 2002) and *Fusarium oxysporum* (Davière et al., 2001). These differences were attributed mainly to translocations (Orbach et al., 1988 and Talbot et al. 1991) and other chromosomal rearrangements (Masel et al., 1990). Davière et al. (2001) observed a correlation between the high level of chromosomal polymorphism in *F. oxysporum* and concentrations of transposables elements (TEs) but these elements were not detected in *M. anisopliae*.

In *M. anisopliae*, recombinants were obtained by parasexuality (Bagagli et al., 1991; Messias and Azevedo, 1980; Valadares-Inglis and Azevedo, 1997) but, in some cases it was extremely difficult to produce heterokaryons and stable recombinants between some strains. Silveira and Azevedo (1987) obtained recombinants between E6 and RJ only by protoplast fusion and even then, the fusion products were very unstable but produced more stable sectors which were proved to be recombinants strains. These results were in agreement with the differences found among electrophoretic karyotypes of E6 and RJ strains in this study.

Apart from translocations, other factors may account for degree of incompatibility between RJ and E6 strains. Pamphile (1992) was not able to produce recombinants between different strains like E6 and MT, which showed a very similar karyotype.

The similarities and differences found in the molecular karyotypes of the strains studied in the present work, partialy agreed with results from other authors. De Conti et al. (1980), using esterase profile analysis, distinguished three groups of strains, the first one including E6 and E9 strains, a second one comprising A19 strain and a third one including A4 strain. In the present work, it was not possible to compare E9 with others, but E6, A4 and A19 showed different karyotypes.

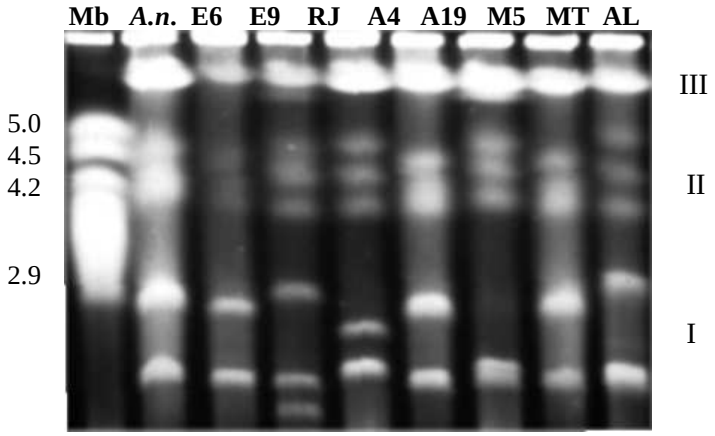

Figure 1 - Separation of *Metarhizium anisopliae* intact chromosomal DNAs on a CHEF gel. Gel (0.6% w/v) was electrophoresed in A condition. Group I) two to three bands corresponding to molecules between 0.9 and 2.8Mb; Group II) three bands corresponding to molecules between 3.6 and 4.9 Mb and, Group III) with these parameters presents two bands corresponding to molecules between 6.0 and 7.7 Mb. *A.n.* shows the *Aspergillus nidulans* chromosome size standards.

The RAPD analysis between E6, E9 M5, AL and RJ strains showed distinct profile for each strain, except for E6 and E9 that showed the same profile. UPGMA dendrogram data showed that E6/E9 and M5 were the most similar to AL in the same group and RJ strain was the least similar (Fungaro et al., 1996). In the present work, distinct electrophoretic karyotypes were found from these strains, derived from the same host, *D. flavopicta*, and RJ was the only strain that showed a small chromosomal band (0.9 Mb).

These results were in part in accordance wit h the work of Fungaro et al. (1996). The strain A19 isolated from another species of *Deois* (*D. schach*) showed almost the same electrophoretic karyotype from MT and E6. These strains were isolated from different regions far apart from each other. Based on the present data, it is not possible to do some correlation between the electrophoretic karyotype of each strain and their hosts or the geographic localization. Using data derived from AFLP, amplified fragment length polymorphism, Muro et al. (2003) were not able to do some correlation between 50 isolates of the entomopathogenic fungus *Beauveria bassiana* and hosts or geographical origins.

The data shown here indicated that this species presented a wide genetic diversity. The genus *Metarhizium* could be basically divided in three species: *M. anisopliae*, *M. flavoviride* and *M. albus*, with some described varieties inside these species. A more detailed study including electrophoretic karyotype should be carried out, and may prove to be valuable for a better definition of the *Metarhizium* genus taxonomy.

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RESUMO

Cariótipos de oito linhagens selvagens do fungo entomopatogênico *Metarhizium anisopliae* var. *anisopliae* foram obtidos em gel, por eletroforese em campo pulsado. As linhagens foram isoladas de insetos provenientes de seis estados brasileiros. As moléculas de DNA cromossômico de três linhagens foram separadas em sete bandas e, de cinco linhagens, em oito bandas. Polimorfismo de tamanho cromossômico também foi observado. O tamanho do DNA cromossômico de todas as linhagens variou de 7,7 a 0,9 Mb, utilizando-se DNA cromossômico de *Aspergillus nidulans* como padrão. O tamanho do genoma total foi estimado em pelo menos 29,7 Mb. Algumas correlações entre semelhanças e diferenças no cariótipo eletroforético e a ocorrência do ciclo parassexual como também a especificidade com insetos hospedeiros foram discutidas.

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