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Restriction endonuclease analysis of mitochondrial DNA of *Metarhizium anisopliae* strains

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ABSTRACT

Mitochondrial DNAs from strains A4, A19, AL, E6, E9 and MT of *Metarhizium anisopliae* var. *anisopliae* were isolated and characterized by restriction-endonucleases. The banding pattern obtained was the same for all strains investigated, indicating that their mtDNAs have the same molecular sizes (about 36 Kb) and the same restriction sites for the enzymes used. Thus, the restriction pattern of the mtDNA of these strains may serve as a specific marker for the identification of the variety *anisopliae* or of subpopulations of this variety.

INTRODUCTION

The species *Metarhizium anisopliae* contains two recognized varieties, *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus*, which mainly differ in spore size (Tulloch, 1976; Fegan *et al.*, 1993). *Metarhizium anisopliae* var. *anisopliae* isolated from various geographical locations is currently being used for the biological control of insects (Riba *et al.*, 1986). The variability of some strains of this species isolated from several Brazilian States has been determinated by isoenzyme polymorphism (Conti *et al.*, 1980; Riba *et al.*, 1986), genetic differences in allozymes (St. Leger *et al.*, 1992) and production of extracellular enzymes (Rosato *et al.*, 1981). However, enzyme production may be dependent on culture medium conditions affecting catabolite-repression events (St. Leger *et al.*, 1992; Bidochka *et al.*, 1994). Furthermore, the genetics of *M. anisopliae* has not been well characterized and its genetic markers are rare, a fact limiting the development of more adequate strains to be used in the biocontrol of insects. In addition, the phylogenetic relationships between groups of the genus *Metarhizium* are still to be determined.

The genomic variability of the genus *Metarhizium* has been recently analyzed by molecular biology approaches using random amplified polymorphic DNA (RAPD) markers (Cobb and Clarkson, 1993; Fegan *et al.*, 1993; Biodochka *et al.*, 1994). On the other hand, molecular analysis of mitochondrial DNA of several organisms has been extremely useful for the understanding of the genetic roles and functions of this molecule in the cell. In fungi, this analysis has also permitted mitochondrial DNA mapping (Pfeifer *et al.*, 1992) and has been of help in distinguishing among species (Kozlowski and Stepien, 1982; Camougrand *et al.*, 1988) and among isolates of the same species (Camougrand *et al.*, 1988), at times even leading to a questioning of classic taxonomy (Okamoto *et al.*, 1991).

Since studies of *M. anisopliae* at the molecular level are still quite limited, we undertook a study of its mtDNA in order to obtain a pattern that might be useful in the characterization of its isolates and in comparison

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with other species in a study of phylogenetic relationships.

MATERIAL AND METHODS

Organisms and growth conditions

Strains of *M. anisopliae* var. *anisopliae* were kindly supplied by Prof. J.L. Azevedo and Dr. A. Pizzirani-Kleiner, Department of Genetics, ESALQ-USP, Piracicaba, Brazil. These strains were isolated from several Brazilian states: A4 and A19 from Bahia; AL from Alagoas; E6 and E9 from Espírito Santo and MT from Mato Grosso do Sul. They were grown in complete liquid medium (Pontecorvo *et al.*, 1953) in a orbital incubator (160 rpm) for 18 hours at 28°C.

Isolation of mtDNA

MtDNA was isolated by the method described for yeast (Defontaine *et al.*, 1991) adapted to filamentous fungi, with minor modifications. MtDNA to be used as probe was also purified by centrifugation in CsCl-bisbenzimide gradients (Hauswirth *et al.*, 1987).

Restriction analysis and Southern blot

MtDNA samples were digested with restriction endonucleases, heated at 75°C for 30 min., subjected to electrophoresis in 0.8 to 1.2% TAE agarose gels, stained with ethidium bromide and photographed with transillumination at 302 nm. Electrophoretically separated DNAs were blotted to nitrocellulose according to supplier recommendations (Gibco-BRL). All hybridizations and washes were carried out at high stringency. Probes were the mix of mtDNA isolated by CsCl from all strains (A4, A19, AL, E6, E9, and MT) and labelled by the nick translation method using [α-32P]dCTP (Sambrook *et al.*, 1989).

RESULTS AND DISCUSSION

The mtDNAs isolated from strains A4, A19, AL, E6, E9 and MT of *M. anisopliae* were digested with various restriction endonucleases. The electrophoretic patterns of restriction enzyme fragments of each strain treated with PvuII and HindII are shown in Figure 1. Figure 2 shows the electrophoretic pattern obtained after HaeIII treatment. In both Figures, each restriction enzyme produced the same band pattern in all strains investigated. A single pattern was obtained for the mtDNA of these same strains, also after treatment with BglII, EcoRI, PstI, SalI, XbaI and XhoI (data not shown), indicating the absence of marked structural differences between the mtDNA of these isolates of the variety *anisopliae*. In contrast, random amplified polymorphic total DNA of *M. anisopliae* var. *anisopliae* isolates from Australia has shown broad genetic diversity (Fegan *et al.*, 1993). The isolates of *M. anisopliae* used in the present study also presented variability in morphology, resistance to UV light and to fungicides (data not shown) and in the electrophoretic pattern of the esterase system (Conti *et al.*, 1980).

The size of each restriction fragment was determined after electrophoresis, by reference to restriction fragments of lambda-HindIII DNA or to the 1 Kb-ladder DNA mixture. The number and the size of mtDNA fragments generated with the different

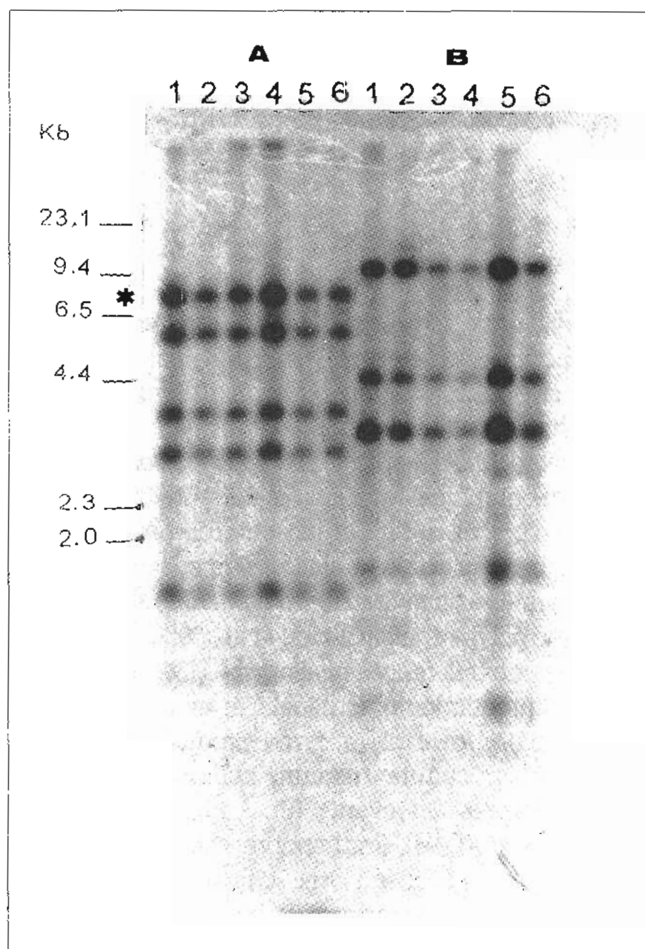

Figure 1 - Southern blotting of mtDNA of strains MT (Line 1), A4 (Line 2), A19 (Line 3), AL (Line 4), E6 (Line 5) and E9 (Line 6) of *Metarhizium anisopliae* digested with PvuII (A) and HindII (B). The samples were hybridized with a mix of mtDNA from the same strains, labelled with [α 32P]dCTP.
*Double band.

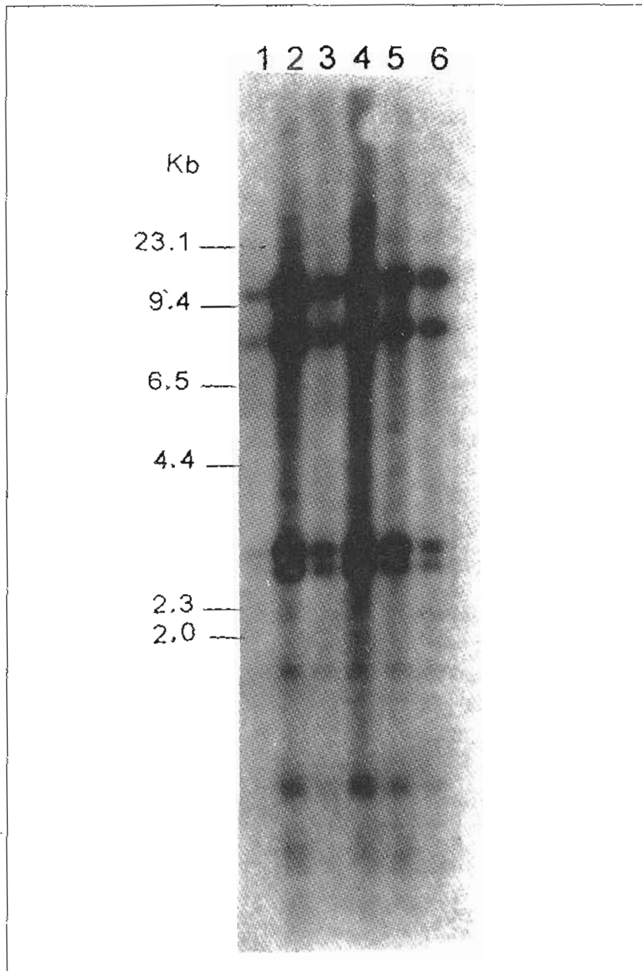

Figure 2 - Southern blotting of mtDNA of strains MT (Line 1), A4 (Line 2), A19 (Line 3), AL (Line 4), E6 (Line 5), and E9 (Line 6) of *Metarhizium anisopliae* digested with HaeIII. The samples were hybridized with a mix of mtDNA from the same strains, labelled with [α 32P]dCTP.

enzymes are presented in Table I. The sum of the fragments produced by each restriction enzyme showed that the molecular size of the mtDNA of these strains is approximately 36 Kb.

The mitochondrial genomes of fungi range in size from 17.6 Kb for *Schizosaccharomyces pombe* (Zimmer *et al.*, 1987) to 176 Kb for *Agaricus bitorquis* (Hintz *et al.*, 1985). The mitochondrial genomes of several filamentous fungi are around 35 Kb, as observed in *Aspergillus nidulans* (Brown *et al.*, 1985), *Aspergillus flavus* (Moody and Tyler, 1990) and *Aspergillus niger* (Kirimura *et al.*, 1992). Although little is known about the genetics or molecular biology of other Deuteromycetes, *Beauveria bassiana* has been reported to have mtDNA of about 28.5 Kb (Pfeifer *et al.*, 1992).

The restriction-banding pattern may vary among species and among isolates of the same species, with the possibility of grouping banding patterns according to degree of relatedness (Kirumura *et al.*,

Table I - Restriction enzyme fragments produced from mtDNA of strains MT, A4, A19, AL, E6 and E9 of *M. anisopliae* var. *anisopliae*.

BglII	EcoRI	HaeIII	HindII	PstI	PvuII	SalI	XbaI	XhoI
13.0	17.5	12.2	14.5	33.0	9.2	34.0	16.5	26.5
11.0	9.0	8.9	4.5	1.9	8.3	1.2	6.2	9.0
9.0	5.4	2.9	3.3		5.9		5.4	
2.4	3.3	2.6	2.8		3.8		3.6	
1.0	1.3	1.9	2.1		3.0		2.8	
0.9		1.4	1.9		1.8		2.0	
		1.2	1.7		1.3			
		1.1	1.1		1.0			
		1.0	1.0		0.95			
		0.95						
37.3	36.5	34.15	32.9	34.9	35.25	35.2	36.5	35.5

The fragment size were calibrated for length in kb by using a standard marker lambda/HindIII digest or 1 kb-ladder DNA mix. The last row indicates the sums of sizes of fragments generated by each endonuclease.

1992). In the present study we demonstrated the highly conserved nature of *M. anisopliae* var. *anisopliae* mtDNA in strains from different locations. A strain of *M. anisopliae* var. *majus* analyzed with some of the restriction enzymes used here showed an electrophoretic pattern that differed markedly from that of var. *anisopliae* (data not shown). Thus, the restriction pattern of mtDNA may serve as a specific marker in the identification of var. *anisopliae* or of some sub-population of this variety. MtDNA analysis in other isolates of this variety will be needed to test this hypothesis.

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RESUMO

Os DNAs mitocondriais das linhagens A4, A19, AL, E6, E9 e MT de *Metarhizium anisopliae* var. *anisopliae* foram isolados e caracterizados por enzimas de restrição. O padrão de bandas obtido para todas as linhagens investigadas foi o mesmo indicando que seus mtDNA tem o mesmo tamanho molecular (cerca de 36 Kb) e os mesmos sítios de restrição para as enzimas utilizadas. Portanto o padrão de restrição do mtDNA destas linhagens pode se constituir

num marcador específico na identificação da variedade *anisopliae*, ou de alguma sub-população desta variedade.

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