MOLECULAR CHARACTERIZATION OF *Paecilomyces fumosoroseus (Deuteromycotina: Hyphomycetes)* ISOLATES

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ABSTRACT: ITS and RAPD analyses were used to investigate molecular variations within samples of *Paecilomyces* isolates and to resolve five morphologically atypical isolates resembling *P. fumosorosus*, obtained from whitefly in Northern Paraná State. The ITS4-ITS5 amplicon was 700 base pairs (bp) long in all isolates. The five isolates of *Paecilomyces* not assigned to species produced restriction profiles identical to all the reference strains of *P. fumosoroseus*. The extent of fingerprint variability observed by RAPD was sufficient to discriminate all the isolates. The genetic similarity among unidentified isolates and strains of *P. fumosoroseus* was even higher than that observed among reference strains of this species, allowing us to conclude that isolates CNPso-P77, CNPso-P78, CNPso-P80, CNPso-P85 and CNPso-P91 are *P. fumosoroseus*.
Key words: *Paecilomyces fumosoroseus*, *Bemisia*, RAPD, biological control, entomopathogenic fungus

CARACTERIZAÇÃO MOLECULAR DE ISOLADOS DE *Paecilomyces fumosoroseus (Deuteromycotina: Hyphomycetes)*

RESUMO: As análises de RAPD (Polimorfismo de DNA Amplificado ao Acaso) e ITS (Sequências Internas Transcritas) foram utilizadas para investigar a variabilidade molecular entre isolados de *Paecilomyces* e para identificar cinco isolados morfologicamente atípicos, obtidos a partir de mosca branca no Norte do Estado do Paraná, que possuíam alguma semelhança com *P. fumosoroseus*. O produto da amplificação com os primers ITS4 e ITS5 apresentou 700 pares de bases para todos os isolados investigados. Os cinco isolados não identificados deram origem a padrões de restrição idênticos a todas as linhagens de *P. fumosoroseus* utilizadas como referência. A variabilidade observada nos perfis de RAPD foi suficiente para discriminar todos os isolados. A similaridade genética entre os isolados não identificados e as linhagens de *P. fumosoroseus* foi maior do que aquela observada entre as linhagens referência desta espécie. Este fato permitiu concluir que os isolados CNPso-P77, CNPso-P78, CNPso-P80, CNPso-P85 and CNPso-P91 pertencem à espécie *P. fumosoroseus*.
Palavras-chave: *Paecilomyces fumosoroseus*, *Bemisia*, RAPD, controle biológico, fungo entomopatogênico

INTRODUCTION

The whitefly *Bemisia argentifolli* is one of the most severe agricultural pests of the 20th century. It is widespread throughout the world, and causes considerable damage to a variety of high value crops, including in Brazil where *B. argentifolli* was recently introduced. As chemical controls were shown to be ineffective, efforts have been made to develop natural remediation in applying biological controls of this pest. Over the world, one of the most common sources of whitefly population mortality is a mycosis caused by the entomopathogenic fungus *Paecilomyces fumosoroseus* (Wise) Brawn & Smith (Lacey et al., 1996; Humber, 1992). This fungus is associated with whitefly in different regions of the globe (Lacey et al., 1996), including observations performed in Brazil on soybean and other vegetables. The pathogenicity of *P. fumosoroseus* against whitefly has recently been studied (Vidal et al., 1997; Wraight, et al., 1998).

Despite common outbreaks of *B. argentifolli*, little is known about the genetic variability of its potential nemesis, *P. fumosoroseus*. The detection of variability and knowledge of various strain identities are basic prerequisities for either the development of an effective biocontrol strategy or to evaluate the impact of artificial epizootics. The morphological characteristics used for classification of *Paecilomyces* species frequently do not resolve new isolates into clearly defined species and provide no isolate-level characterisation. Currently, more sensitive genetic methods such as random amplification of polymorphic DNA (RAPD) (Williams et al., 1990) and nuclear rDNA analysis have been extensively employed for genetic variability analyses (Fungaro & Vieira, 1998) and for the identification of species and isolates including entomopathogenic fungi (Fegan et al., 1993; Leal et al., 1994, Bidochka et al, 1994; Tigano-Milani et al., 1995a; Tigano-Milani et al., 1995b; Fungaro et al., 1996; Cravanzola et al., 1997; Berretta et al., 1998, Cantone & Vandenberg, 1998; Piatti et al. 1998).

In this study polymorphisms of internal transcribed spacers (ITS) of ribosomal DNA amplified by polymerase chain reaction (PCR) and RAPD were used to investigate

molecular variations of *Paecilomyces* isolates and to resolve five morphologically atypical isolates resembling *P. fumosoroseus*, obtained from whitefly in Northern Paraná State (Sosa-Gómez, 1998).

MATERIAL AND METHODS

Fungal Isolates

ITS-rDNA and RAPD analyses were performed on five unidentified *Paecilomyces* isolates (CNPso-P77, CNPso-P78, CNPso-P80, CNPso-P81, and CNPso-P85) obtained from infected *B. argentofolli* in northern regions of Paraná State, Brazil, and seven strains of *P. fumosoroseus* from different hosts and geographic regions (CNPso-P92, CNPso-P93, ARSEF2658, ARSEF 3083, ARSEF3303, ARSEF3660, ARSEF3699) (TABLE 1). In addition, five isolates of *P. tenuipes* were used as an outgroup.

DNA isolation

Conidial suspensions of 17 isolates in complete medium (Pontecorvo et al., 1953), were incubated for 2 days under agitation at 28°C. The resulting mycelium was harvested by filtration. To extract the DNA, 1g of mycelium was ground to a fine powder in liquid nitrogen and incubated in 800 µL DNA extraction buffer (100mM Tris-HCl (pH8.0), 25mM EDTA, 1% SDS, 25mM NaCl) at 65°C for 20 min. The suspension was deproteinized by extracting once with an equal volume of phenol and once with an equal volume of chloroform: isoamylcohol (24:1). DNA was precipitated by adding two volumes of ice-cold ethanol and 10% 3M sodium acetate. The precipitate was collected by centrifugation, washed with 70% ethanol and dried. The pellet was resuspended in TE (10mM Tris, 1mM EDTA pH 8.0).

TABLE 1 - Origin of *Paecilomyces* isolates.

Isolates		Host	Location
CNPSo-P77 (ARSEF 5154)	*Paecilomyces* sp.	*B. argentifolii*	PR/Brazil
CNPSo-P78 (ARSEF 5155)	*Paecilomyces* sp.	*B. argentifolii*	PR/Brazil
CNPSo-P80 (ARSEF 5157)	*Paecilomyces* sp.	*B. argentifolii*	PR/Brazil
CNPSo-P81 (ARSEF 5158)	*Paecilomyces* sp.	*B. argentifolii*	PR/Brazil
CNPSo-P85	*Paecilomyces* sp.	*B. argentifolii*	PR/Brazil
CNPSo-P92 (ARSEF 3638)	*P. fumosoroseus*	Soil	PR/Brazil
CNPSo-P93 (ARSEF 2956)	*P. fumosoroseus*	*Spaethiella* sp.	AM/Brazil
ARSEF-2658	*P. fumosoroseus*	*Trialeurodes vaporariorum*	USA
ARSEF-3083	*P. fumosoroseus*	*B. tabaci*	USA
ARSEF-3303	*P. fumosoroseus*	*Bemisia* sp.	Mexico
ARSEF-3660	*P. fumosoroseus*	*B. tabaci*	USA
ARSEF- 3699	*P. fumosoroseus*	*B. tabaci*	India
CNPso-P95	*P. tenuipes*	*Noctuidae*	PR/ Brazil
CNPso-P96	*P. tenuipes*	*Noctuidae*	PR/Brazil
CNPso-P41	*P. tenuipes*	pupa of lepidopteran	PR/Brazil
CNPso-42	*P. tenuipes*	pupa of lepidopteran	PR/Brazil
ARSEF-2488	*P. tenuipes*	*Spodoptera frugiperda*	Mexico

rDNA ITS Regions Analyses

Primers ITS4 (5'TCCTCCGCTTATTGATATGC-3') and ITS5 (5'GGAAGTAAAAGTCGTAACAAGG-3') were used to amplify the ITS region of rDNA. The 50 µL of reaction mixture contained 10 ng template DNA, 1 µM of each primer, 100 µM of each dNTP, 5 µL 10x PCR buffer, 1.5 mM $MgCl_2$, and 2.5 Units of *Taq* Polymerase. The mixture was subjected to amplification in a MJ Research thermal cycler. An initial denaturation step of 2 min at 95°C was followed by 35 cycles of annealing for 1 min at 55°C, extension for 1 min at 72°C and denaturation for 2 min at 95°C before a final extension step of 5 min at 72°C. The resultant reaction mixture (10 µL) was separated on a 1.4% horizontal agarose gel, stained with ethidium bromide and visualised under UV illumination. For restriction analysis, 10 µL of each PCR product was digested in a 20 µL volume, using *AluI*, *Xho* I, *Dde* I, *Bam* H1, *Pst* I, *Pvu* I, *Hind* III, *Ras* II, *Sau* 3A1, *Eco* RI, *Hae* III under conditions supplied by the manufacturer (Gibco-BRL). The restriction reactions were separated by electrophoresis in 1.4% agarose gels. After staining, the gels were photographed over a UV transilluminator.

RAPD Analyses

The 10-mers used as the random primer in the RAPD procedure was purchased from Operon Technologies, Alameda, CA. Taq DNA polymerase and DNA polymerisation mix (dNTP) were supplied by Pharmacia. The 10x concentrated buffer, supplied with the enzyme, contained 100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$. The amplification reactions were conducted in a total volume of 25 µL. The mixture contained 8.8 µl of fresh ultra-pure sterile water, 2.5 µl buffer, 4.0 µL of dNTP (200 µM each), 2.5 µL of primer (2,5 mM), 0.3 µL of enzyme (1.5 units), 3.0 µL of $MgCl_2$ (12.5 mM) and 4.0 µL of DNA (20-25 ng). The final concentration of magnesium in the reaction was 3.0 mM. Control reactions were run containing all components except genomic DNA and none of the primers employed yielded detectable amplified products. The mixture was subjected to the following temperature conditions: 92°C for 5 min, followed by 40 cycles of 92°C for 45 sec, 39°C for 1 min 30 sec, 72°C for 2 min and ending with 3 min at 72°C. Samples of 22 µL of the RAPD products were assayed by electrophoresis in 1.4% agarose gels run with TBE buffer. Electrophoresis

was conducted at 3V/cm. Gels were stained with ethidium bromide and photographed under UV using a Pan F Ilford film. For data analysis, each isolate was scored for the presence or absence of amplification products. The data were entered into a binary matrix and a pairwise similarity matrix was constructed using the Sorensen coefficient (Sneath & Sokal, 1973). An UPGMA cluster based on Sorensen values was generated using the NTSYS (Numerical Taxonomy System, Applied Biostatistics, Setauket, New York) computer programme (Rohlf, 1994).

RESULTS AND DISCUSSION

The development of PCR and the design of primers for the amplification of the various rDNA regions have considerably facilitated taxonomic studies of fungi. ITS sequences are generally constant, or show little variation within species but vary between species in a particular genus. As such, these sequences have been widely used to develop rapid procedures for the identification of fungal species by PCR-RFLP analysis (Edel, 1998). In this study ITS analyses were conducted by restriction enzyme digestion of the amplified region using the primers ITS 4 and ITS 5. The ITS4-ITS5 amplicon was 700 base pairs (bp) long in all isolates of *Paecilomyces* sp., *P. fumosoroseus* and *P. tenuipes*. Of the 12 restriction enzymes used, differences between isolates were detected with *Hae* III only. No restriction sites were found when DNA was treated with *AluI*, *Xho* I, *Dde* I, *Bam* H1, *Pst* I, *Pvu* I and *Hind* III. The enzymes *Ras* II, *Sau* 3A1, *Eco* RI digested the amplicon, but showed no polymorphisms among isolates. Interspecific polymorphisms detected with *Hae* III, discriminated *P. fumosoroseus* from *P. tenuipes* (Figure 1).

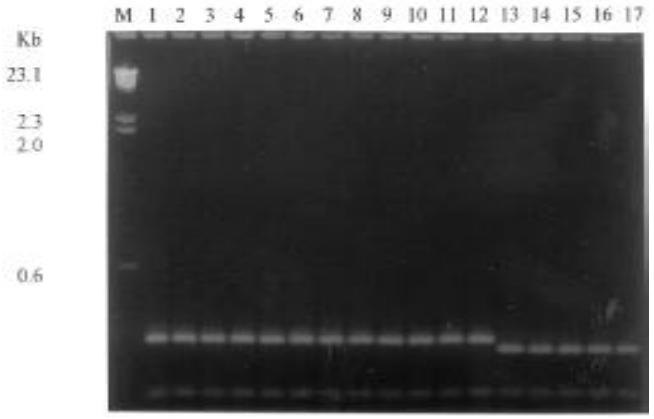

Figure 1 - ITS-RFLP patterns of *Paecilomyces* isolates using *Hae*III restriction enzyme. Lane 1, *P. fumosoroseus* ARSEF 3303; lane 2-6, *Paecilomyces* sp. CNPso-81, CNPso-77, CNPso-78, CNPso-P80, CNPso-P85, respectively; lane 7-12, *Paecilomyces fumosoroseus* ARSEF 3699, ARSEF 3083, CNPso-92, CNPso-P93, ARSEF 3660, ARSEF 2658, respectively; lane 13-17, *Paecilomyces tenuipes* CNPso-95, CNPso-96, ARSEF 2488, CNPso-41, CNPso-42, respectively. The lane marked with the letter M is l*Hind* III molecular mass standards. Molecular sizes in Kilobases are indicated on the right.

The five isolates of *Paecilomyces* not assigned to species produced restriction profiles identical to all the reference strains of *P. fumosoroseus*. In addition, ITS intraspecific polymorphisms were observed for neither *P. tenuipes* nor *P. fumosoroseus*.

RAPD analyses using 31 primers produced 536 scored bands within all of the 17 isolates. An example of a typical RAPD pattern is given in Figure 2. The extent of fingerprint variability was sufficient to discriminate all isolates. This feature is very important because the identification of isolates of *Paecilomyces*, has often turned out to be difficult or even impossible when based solely on morphological traits.

A dendrogram based on the amplification fragments is shown in Figure 3. The isolates were separated into 2 large phenetic groups. The first group is formed by all isolates of *P. fumosoroseus* and the five unidentified isolates. The second group includes all isolates of *P. tenuipes*. Despite the high genetic divergence between these two groups (0.75), a common amplification product obtained using the primer OPAK-16 (Figure 2) was observed for all isolates. The five unidentified isolates CNPso-P77, CNPso-P78, CNPso-P80, CNPso-P85 and CNPso-P91 were very similar to each other, with an average pairwise similarity of 92.5%. This high genetic similarity was expected since they were isolated from the same epizootic phase in northern regions of Paraná State. The genetic similarity among the unidentified isolates and strains of *P. fumosoroseus* was even higher than that observed among reference strains of this species. These data allow us to conclude that isolates CNPso-P77, CNPso-P78, CNPso-P80, CNPso-P85 and CNPso-P91 are *P. fumosoroseus*. A similar strategy was employed by Tigano-Milano et al. (1995b) to study 27 *P. fumosoroseus* isolates, of which 15 were obtained from *B. tabaci*, in comparison with one strain of *P. lilacinus* and nine strains of *Paecilomyces* not assigned to species.

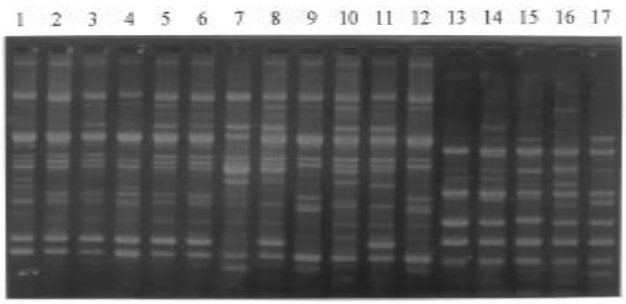

Figure 2 - RAPD profile obtained with primer OPAK-16. Lane 1, *P. fumosoroseus* ARSEF 3303; lane 2-6, *Paecilomyces* sp. CNPso-81, CNPso-77, CNPso-78, CNPso-P80, CNPso-P85, respectively; lane 7-12, *Paecilomyces fumosoroseus* ARSEF 3699, ARSEF 3083, CNPso-92, CNPso-P93, ARSEF 3660, ARSEF 2658, respectively; lane 13-17, *Paecilomyces tenuipes* CNPso-95, CNPso-96, ARSEF 2488, CNPso-41, CNPso-42, respectively.

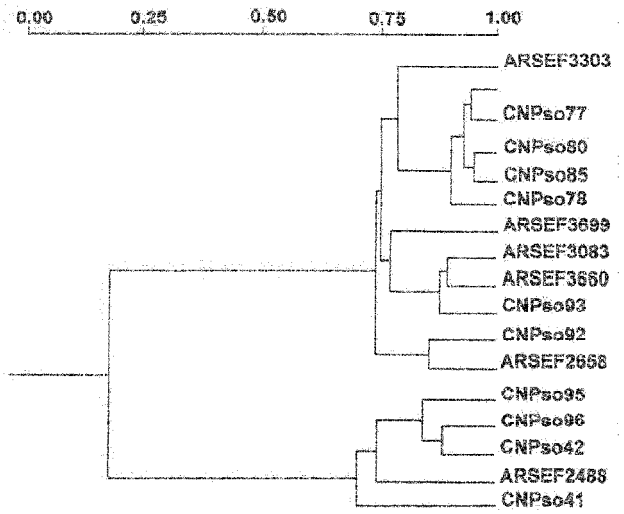

Figure 3 - Dendrogram constructed from analysis of DNA fragments of 7 *Paecilomyces fumosoroseus*, 5 *Paecilomyces* sp. and 5 *Paecilomyces tenuipes* isolates amplified by RAPD. The matrix was created with the Sorensen similarity coefficient, and clustering was performed with UPGMA. The scale on top indicates % similarity.

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REFERENCES

BERRETTA, M.F.; LECUONA, R. E.; ZANDOMENI, R. O.; GRAU, O. Genotyping isolates of the entomopathogenic fungus *Beauveria bassiana* by RAPD with fluorescent labels. **Journal of Invertebrate Pathology,** v.71, p.145-150, 1998.

BIDOCHKA, M.J.; MCDONALD, M.A.; ST. LEGER, R.J.; ROBERTS, D.W. Differentiation of species and strains of entomopathogenic fungi by random amplified polymorphic DNA (RAPD). **Current Genetics,** v.25, p.107-113, 1994.

CANTONE, F.A.; VANDENBERG, J.D. Intraspecific diversity in *Paecilomyces fumosoroseus*. **Mycological Research**, v.102, 801-806, 1998.

CRAVANZOLA, F.; PIATTI, P.; BRIDGE, P.D.; OZINO, O.I. Detection of genetic polymorphism by RAPD-PCR in strains of the entomopathogenic fungus *Beauveria brongniartii* isolated from European cockchafer (*Melolontha* spp.). **Letters in Applied Microbiology,** v.25, p.289-294, 1997.

EDEL, V. Polymerase Chain Reaction in Mycology: an Overview. In: BRIDGE,. P.D., ARORA, D.K., REDDY, C.A.; ELANDER, R.P. (Ed.) **Applications of PCR in Mycology**. Cambridge: University Press, 1998. p.1-20.

FEGAN, M.; MANNERS, J.M.; MACLEAN, D.J.; IRWIM, J.A.G.; SAMUELS, K.D.Z.; HOLDOM, D.G.; LI, D.P. Random amplified polymorphic DNA markers several a high degree of genetic diversity in the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae*. **Journal of General Microbiology**, v.139, p.2075-2081, 1993.

FUNGARO, M.H.P.; VIEIRA, M.L.C.; PIZZIRANI-KLEINER, A.A.; AZEVEDO, J.L. Diversity among soil and insect isolates of *Metarhizium anisopliae* var. *anisopliae* detected by RAPD. **Letters in Applied Microbioogy,** v.22**,** p.389-392, 1996.

FUNGARO, M. H. P.; VIEIRA, M. L. C. Aplicações da PCR em Ecologia Molecular. In: MELO, I.S., de AZEVEDO, J. L. (Ed.) **Ecologia Microbiana**. Jaguariúna: Embrapa, CNPMA, 1998. p.205-227.

HUMBER, R.A. **Collection of Entomopathogenic cultures:** ARSEF catalog of strains. Ithaca, 1992. (USDA, ARS Publication, 110). Update in http://www.ppru.cornell.edu/mycology/Insect_mycology.html

LACEY, L.A.; FRANSEN, J.J.; CARRUTHERS, R. Global distribution of naturally occurring fungi of *Bemisia*, their biologies and use as biological control agents. In: GERLING, D.; MAYER, R. (Ed.) ***Bemisia* 1995**: taxonomy, biology, damage, and management. Andover: Intercept, 1996.

LEAL, S.C.M.; BERTIOLI, D.J.; BUTT, T.M.; PEBERDY, J.F. Characterization of isolates of the entomopathogenic fungus *Metarhizium anisopliae* by RAPD-PCR**. Mycological Research**, v.98, p.1077-1081, 1994.

PIATTI, F.; CRAVANZOLA, F.; BRIDGE, P.D.; OZINO, O.I. Molecular characterization of *Beauveria brongniartii* isolates obtained from *Melolontha melolontha* in Valle d'Aosta (Italy) by RAPD-PCR. **Letters in Applied Microbiology,** v.26, p.317-324, 1998.

PONTECORVO, G.; ROPER, J.A.; HEMMONS, L.M.; MacDONALDS, K.D.; BUFTON, A. W.J. The genetics of *Aspergillus nidulans*. **Advances in Genetics**, v.5, p.141-148, 1953.

ROHLF, F.J. **NTSYS-pc:** numerical taxonomy and multivariant analysis system; version 1.80. Setauket: Exeter Publishing, 1994.

SOSA-GÓMEZ, D.R. **Coleção de fungos entomopatogênicos**: catálogo de isolados. Londrina: EMBRAPA, CNPso, 1998. 38p.

SNEATH, P.H.A; SOKAL, R.R. **Numerical taxonomy.** San Francisco: Freeman, 1973.

TIGANO-MILANI, M.S.; GOMES, A.C.M.M.; SOBRAL, B.W.S. Genetic variability among Brazilian isolates of the entomopathogenic fungus *Metarhizium anisopliae*. **Journal of Invertebrate Pathology,** v.65, p.206-210, 1995a.

TIGANO-MILANI, M.S.; HONEUCUTT, R.J.; LACEY, L.A.; ASSIS, R.; MCCLELLAND, M.; SOBRAL, B.W.S. Genetic variability of *Paecilomyces fumosoroseus* isolates revealed by molecular markers. **Journal of Invertebrate Pathology,** v.65, p.274-282, 1995b.

VIDAL, C.; LACEY, L.A.; FARGUES, J. Pathogenicity of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) against *Bemisia argentifolii* (Homoptera: Aleyrodidae) with a description of a biossay method. **Journal of Economic Entomology**, v.90, p.765-772, 1997.

WILLIAMS, J.G.K.; KUBELIK, A.R.; LIVAK, K.J.; RAFALSKI, J.A.; TINGEY, S.V. DNA polymorphisms amplified by arbitrary *primers.* **Nucleics Acids Research**, v.18, p.6531-6535, 1990.

WRAIGHT, S.P.; CARRUTHERS, R.I.; BRADLEY, C.A.; JARONSKI, S.T.; LACEY, L. A.; WOOD, P.; GALAINI-WRAIGHT, S. Pathogenicity of the entomopathogenic fungi *Paecilomyces* spp.. and *Beauveria bassiana* against the silverleaf whitefly, *Bemisia argentifolii*. **Journal of Invertebrate Pathology,** v.71, p.217-226, 1998.

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