

## MORPHOLOGICAL AND MOLECULAR DIFFERENTIATION OF THE PECTINASE PRODUCING FUNGI *PENICILLIUM EXPANSUM* AND *PENICILLIUM GRISEOROSEUM*

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Submitted: May 15, 2006; Returned to authors for corrections: August 28, 2006; Approved: January 18, 2007.

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### ABSTRACT

Two species from the genus *Penicillium*, *Penicillium expansum* and *P. griseoroseum* (Brazilian isolates) were characterized morphologic and molecularly. Morphological variability was detected among isolates in regard to colony morphology and to conidia coloration. The molecular characterization was based on the RAPD markers, telomeric fingerprinting and ITS sequencing. A total of 78 RAPD primers were used and 8 presented differences in band patterns with 54% of the amplified polymorphic fragments. The monomorphic fragments of 600 bp (*P. expansum*) and 594 bp (*P. griseoroseum*) were amplified. The only internal transcribed spacer region variation detected between the two species was the additional six initial nucleotides. The analysis by telomeric fingerprinting showed polymorphism between both species and the chromosome minimal numbers estimated were three. The polymorphism observed in the organization of the subtelomeric region in the genome of two *Penicillium* species within the high homogeneous *Penicillium* subgenus is for the first time reported and perhaps can be employed in future phylogenetic studies.

**Key words:** Ribosomal DNA, Internal Transcribed Spacer, Subtelomeric Region, *Penicillium* spp.

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### INTRODUCTION

The genus *Penicillium* is worldwide known for production of secondary metabolites and extracellular enzymes of commercial value, including the pectinases, utilized in fruit juice industry during the stage of pulp maceration, juice liquefaction or depectinization (19). Most *Penicillium* species are considered ubiquitous, opportunistic saprophytes. Nutritionally, they are supremely undemanding being able to grow in almost any environment with a sprinkling of mineral salts, any but the most complex forms of organic carbon, and a wide range of physical-chemical environments,  $a_w$ , temperature, pH and redox potential. The taxonomy of this genus is hard as its classification is based mainly on conidiophore and conidia structure, although the colony diameter after incubation under standardized conditions has greater importance for classification (31). The genus *Penicillium* is subdivided in four subgenera (*Aspergilloides*, *Penicillium*, *Biverticillium* and

*Furcatum*) determined by the number of branch points between phialide and stipe, down the main axis of the penicillus and others characters, like ratio of metula length to phialide, length and colony diameter on G25N, when the number of branch points is the same (31).

Two species from the genus *Penicillium*, *P. expansum* and *P. griseoroseum* have been selected in our laboratory and they were shown to be excellent pectinase producers (4). These species have been extensively studied physiologically, showing clear differences in their answers to the influence of inoculum age and concentration, as the effect of some cultural conditions (growth period, substrate concentration and different carbon sources) on the production of pectin lyase (PL) and polygalacturonase (PG). The *P. griseoroseum* PL activity was highest with 9-day-old spore,  $5 \times 10^4$  spores/mL after 48 h of growth and the *P. expansum* PL activity with 5-day-old spore,  $10^6$  spores/mL after 72 h of growth (1-3,10,11,26,27). Several genetic studies have been carried out in our laboratory in order to isolate strains with enhanced

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pectinolytic enzyme activities. Different methodologies which have been used to achieve these goals include protoplast production and regeneration, mutant isolation and characterization, protoplast transformation and isolation and characterization of genes coding pectinolytic enzyme in *P. griseoroseum* and *P. expansum*. (5,9,12,13,16,21,33,34-36,38,41). Incompatibility was observed between these two species for the formation of heterokaryons and diploids when conidia were mixed (41). When the parasexual cycle of *P. expansum* was analyzed, it was noted that diploids were rather unstable and that recombinant sectors could easily be isolated (21). Nevertheless, was observed different results with *P. griseoroseum*, in which the parasexual cycle was not obtained spontaneously, and the diploids isolated by protoplast fusion were stable and produced few discrete haploid sectors even when placed in complete medium supplemented with benomil (38). High identity in amino acid sequence and similar global organization in PL and PG genes from *P. expansum* and *P. griseoroseum* was showed (5,12,34-36). *Penicillium expansum* PLE1 shares 100% amino acids identity with PLG1 from *P. griseoroseum* and the organization of PL genes showed same hybridization pattern what suggests that PL genes have a similar global organization in the genome segment in which they are found. Some doubts emerged from the genetics studies with respect to the basic differences that are required for strains to be considered different species.

The objective of this study was to analyze the differentiation of the pectinase-producing fungi *Penicillium expansum* and *Penicillium griseoroseum* employing both morphological and molecular methods. The use of molecular techniques based on the random amplified polymorphic DNA (RAPD) and sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) have been useful for the identification and classification taxonomy of filamentous fungi.

## MATERIALS AND METHODS

### Strains and growth conditions

*Penicillium expansum* and *P. griseoroseum* isolates were obtained from forest tree seeds collected at Universidade Federal de Viçosa, Minas Gerais, Brazil. The isolate of *Penicillium griseoroseum* was deposited at Fundação Tropical de Tecnologia e Pesquisas André Toselo (Campinas, São Paulo, Brazil), under the code register CCT 6241, and the *P. expansum* isolate was deposited at VIC Herbarium (Viçosa, Minas Gerais, Brazil), under the code register VIC 28728. Cultures were also kept in incubation on agar/H<sub>2</sub>O at 4°C for long-term storage. The inoculum was prepared from 7-day-old cultures grown at 25°C. Erlenmeyer flasks (125 mL) containing 50 mL of minimal medium (MM) (32) were inoculated for a final spore concentration of 10<sup>6</sup> spores mL<sup>-1</sup> and shaken (150 rpm; 25°C). Bacterial transformation was carried out using method proposed by Inoue *et al.* (20).

### Morphological characterization of *P. expansum* and *P. griseoroseum*

Fungi were grown in specific medium to differentiate species of *Penicillium*, Czapek Yeast Extract Agar (CYA) and Neutral Creatine Sucrose Agar (CSN), as described by Pitt and Hocking (31). Colors of colony were recorded under bright tungsten illumination and designated according to Munsell Color Charts (23).

### DNA extraction and Southern blot

*Penicillium expansum* and *P. griseoroseum* total DNA was extracted according to Specht *et al.* (40) and cleaved with restriction enzymes *AluI*, *BamHI*, *EcoRI*, *HindIII* and *SmaI*. The reactions were analyzed on 0.8% agarose gel, and then transferred to Duralon membranes (Stratagene) according to Sambrook *et al.* (37). The membrane was probed with the plasmid pTel13 (22) containing 225 bp DNA fragment labeled with [ $\alpha$ -<sup>32</sup>P]d-ATP, using "Random Primer-It II Labeling Kit" (Stratagene). Hybridization was carried out overnight at 60°C in standard hybridization buffer, washed twice with 2 X SSC, 0.1% SDS for 20 min, and 1 X SSC, 0.1% SDS for 10 min. Autoradiographs were taken by three-day exposure of XOMAT K film (Kodak), with an intensifying screen.

### PCR amplification of ribosomal DNA regions

The ribosomal ITS1-5.8S rRNA gene-ITS2 region was amplified with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') constructed for molecular phylogenetic studies (43). PCR amplification was performed in 25 mL reaction mixtures containing 10 ng of genomic DNA template, 1.0 U of Taq DNA polymerase (Promega), 40 pmol of each primer, 10 mM Tris-HCl (pH 8,3), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.1 mM of each dNTP (dATP, dCTP, dGTP e dTTP). Amplification was performed in a thermocycler PCT-100 (MJ Research, Inc.) for 40 cycles, each cycle consisting of a denaturation step (15s; 94°C), an annealing step (30s; 35°C), and a extension step (60 s; 72°C). After the 40th cycle, a final extension step was performed (60s; 72°C). ITS PCR products were cloned into pGEM-T Easy Vector System (Promega). This vector was used to transform *E. coli* DH5a and the plasmidial DNA was extracted by alkaline lysis method (37). The primers M13 forward and reverse (Promega) and "BigDye™" kit Terminator Cycle Sequencing Ready Reaction" (Applied Biosystems) were used to sequence both strands. The ITS sequences were analysed and aligned using BLAST and CLUSTAL W software. Sequence data were deposited in the GenBank Nucleotide Database.

### RAPD analysis

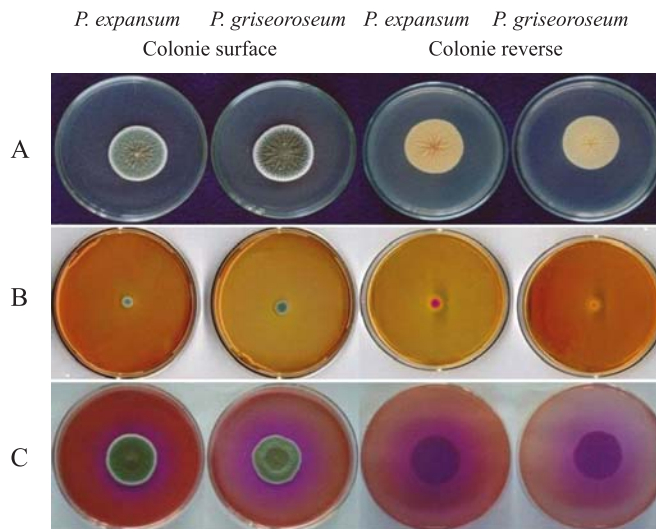
PCR amplification of DNA from *P. expansum* and *P. griseoroseum* was performed with 78 random primers (Operon Technologies Inc., Alameda, CA) as described by Williams *et*

al. (44). Amplification products were electrophoresed in 1.5% (w/v) agarose gels immersed in TEB running buffer (90 mM Tris-borate, 2 mM.L<sup>-1</sup> EDTA, pH 8.0) and run for 4h at 100 V. The gels were stained with ethidium bromide (0.5 µg mL<sup>-1</sup>). The images were captured and stored using a photo documentation system (Eagle Eye II, Stratagene, La Jolla, California).

## RESULTS AND DISCUSSION

The taxonomy of *Penicillium* has always been complex due to its great number of species (nearly 250), which have very few differences. This fact complicates researchers understanding of their ecology, diversity and consequently its exploration by industry. Classification systems of organisms are historically based on observable characteristics. The growth of isolates in appropriate culture media, enabling their most characteristic features to be recognized, is still the most common procedure used in practice. Many species classified in sub-genus *Penicillium* are morphologically similar, and identification using traditional morphological techniques remains difficult. Pitt (28,30) and Frisvad (17,18) media introduced for identification of *Penicillium* species provide a very useful tool to distinguishing between these difficult and closely related species. The *P. expansum* and *P. griseoroseum* strains were grown on specific medium CYA and CSN at 5, 25, and 37°C. No growth was observed at 5 and 37°C for 7 days on medium CYA. At 25°C, vigorous growth with distinctive features between *P. expansum* and *P. griseoroseum* colonies (Fig. 1) was recorded. Colonies on CYA produce 30-37 diameter green conidia, with mycelium white at the margins. *P. expansum* conidia presented light green color with radially sulcate, velutinous texture, reverse yellow. Dull green conidia, surface texture from granular to floccose, and presence of an orange brown soluble pigment, reverse pale, was observed in *P. griseoroseum* colonies. On CSN (2 days; 25°C), it was observed differences in the diameter and coloration of colony reverses. *P. expansum* colonies produce green conidia and violet reverse. Smaller colonies with green conidia and pale yellow reverse were observed in *P. griseoroseum*. After incubation for 7 days on CSN, *P. expansum* colonies showed irregular growth, presented pale green conidia, velutinous texture, violet reverse. Dull green conidia, regular surface texture, reverse reddish-violet were observed in *P. griseoroseum* colonies. These isolates give a very strong alkaline reaction on Neutral Creatine Sucrose agar, although Pitt and Hocking (31) described an acid reaction for *P. expansum*.

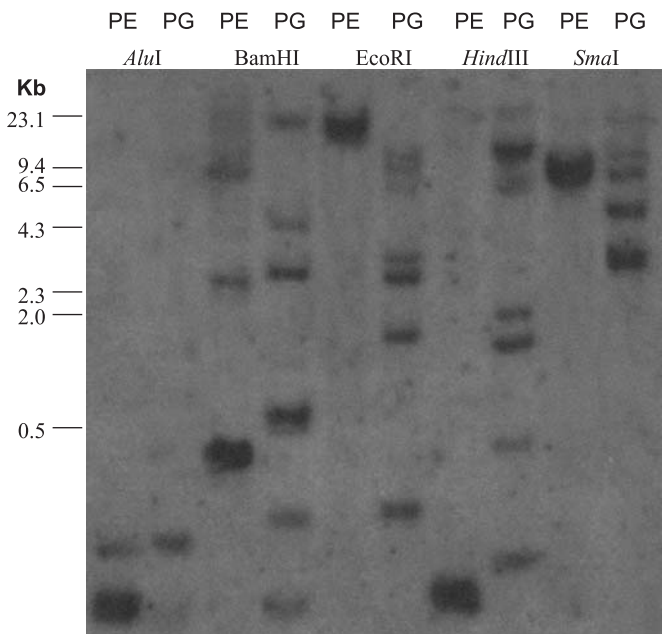
Although identification by the traditional morphological criteria is very effective in large and complex *Penicillium* genera, the large number of existing species has created a need for alternative approaches. Molecular methods have been used to improve the understanding of the natural taxonomy of *Penicillium*. As telomere-associated sequences generally vary



**Figure 1.** Morphological characterization of *P. expansum* and *P. griseoroseum*. Colonies have been grown in medium CYA for 7 days (A), CSN for 2 (B) and 7 days (C), at 25°C.

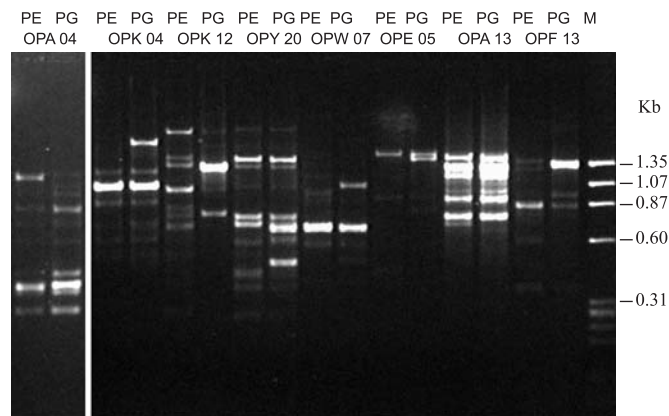
considerably in length, complexity and species-specific sequences, the telomeric molecular marker could be an excellent tool for fingerprint and further epidemiological studies (24). Telomeres are essential for genome stability in all eukaryotes. These terminal structures serve as functional complexes that protect chromosomal ends by preventing terminal fusions and degradation (6,7). Telomere DNA has been isolated from diverse filamentous fungi and all have the same TTAGGG repeat (22). The plasmid pTel13 contain 8 repeats at one end and 3 at the other end and only 159 bp of the 225 bp correspond to a subtelomeric region specific from the phytopathogenic fungus *Botrytis cinerea*. This probe is therefore non-specific to *B. cinerea*, and it is likely that the observed signal with these other fungi is not due to the specific *B. cinerea* sequence, but to the TTAGGG repeats present in pTel13 making it a convenient tool for probing telomeric sequences (22). In the case of *Beauveria bassiana*, an entomopathogenic fungus, the use of pTel13 for fingerprint allowed to suggest that the observed polymorphism corresponds to the insect-host and not to the geographic origins of isolates (42). The presence of TTAGGG repeats and organization of the subtelomeric region of cromossomes of *P. expansum* and *P. griseoroseum* was checked using pTel13 as probe (Fig. 2). This probe was hybridized to genomic DNA from *P. expansum* and *P. griseoroseum*, and cut with five different restriction enzymes. The Southern blot results show the presence of TTAGGG repeats of the subtelomeric region of cromossomes of these fungi and the polymorphism in the organization of the region, indicating that the genomes of these two species are not identical. The analysis showed that the chromosome minimal numbers estimated were three.





**Figure 2.** Southern blot analysis of digest DNA from *P. expansum* (PE) and *P. griseoroseum* (PG). The blot was probe with radiolabelled pTel13. The sizes of DNA fragments are indicated in kilobases on the left.

The genetic diversity of some *Penicillium* species was also studied by random amplified polymorphic DNA (15, 39). RAPD analyses were performed for *P. expansum* and *P. griseoroseum* using 78 single primers of arbitrary sequences. These species presented differences in band patterns using only 8 primers, since 54% of the amplified fragments were polymorphic (Fig. 3). In spite of the small number of amplified polymorphic fragments between *P. expansum* and *P. griseoroseum*, those results indicate differences in the genome of these two species. It was therefore expected that very distinct patterns would be observed when used to study different species, but identical patterns from RAPD analysis of different *Penicillium* spp. with 21 primers was reported by Dupond *et al.* (14). Molecular characterization based on the RAPD markers among 10 *Penicillium* species was reported by Pereira *et al.* (25). High polymorphism among the species *P. roqueforti*, *P. oxalicum*, *P. griseofulvum*, *P. brevicompactum*, *P. camembertii* and *P. charlesii* was demonstrated by the amplifications (25). The species *P. purpurogenum* and *P. crustossum* did not present polymorphism nor did *P. expansum* and *P. griseoroseum*. In our study, a higher polymorphism was detected among *P. expansum* and *P. griseoroseum* by RAPD analysis than reported by Pereira *et al.* (25), probably due to the different number of primers utilized in both studies. RAPD analyses were performed for *P. expansum* and *P. griseoroseum* using 78 single primers of arbitrary sequences, despite the fact that in Pereira *et al.* (25) PCR

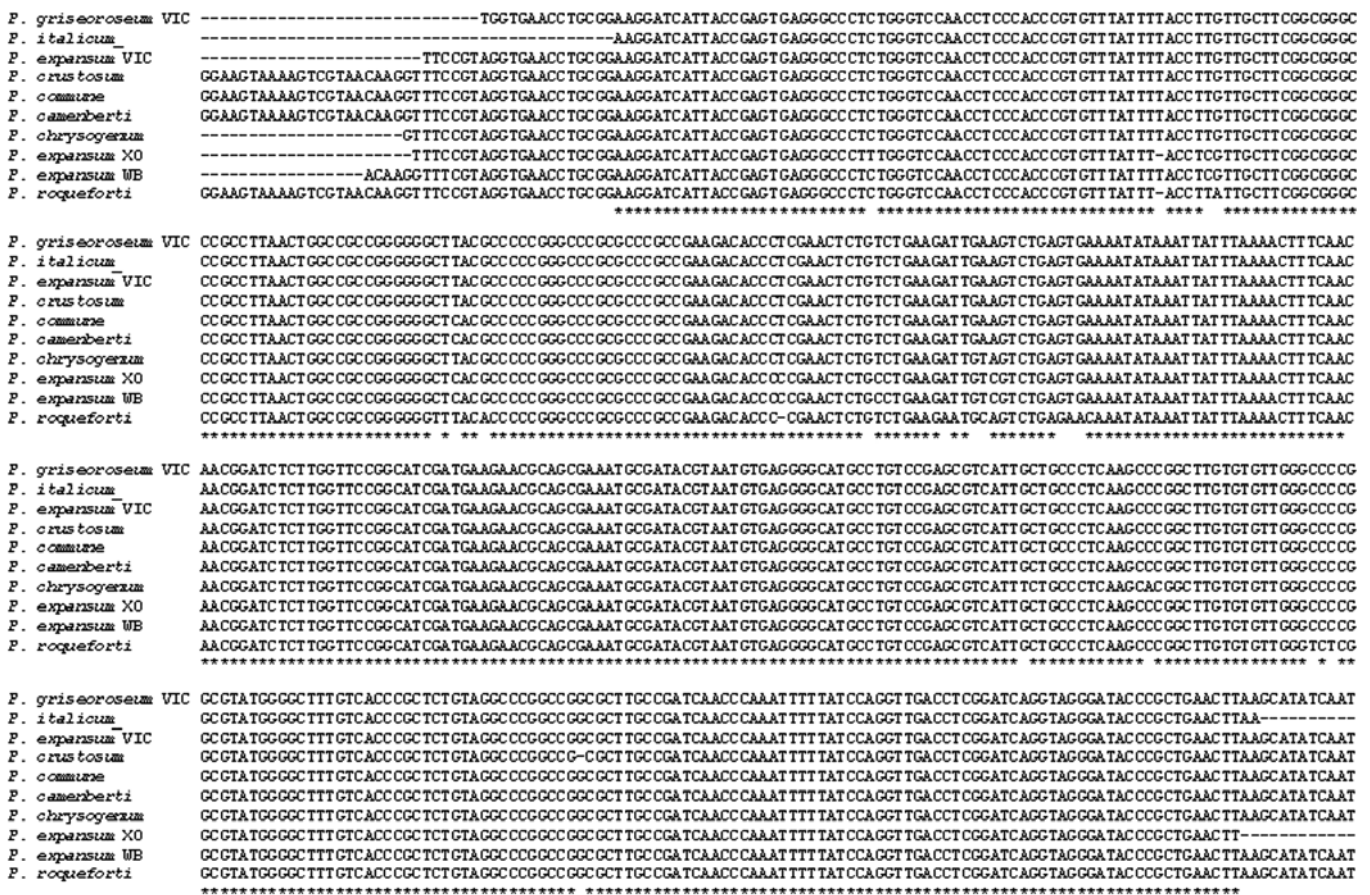


**Figure 3.** Gel electrophoresis of the RAPD amplified products from *P. expansum* (PE) and *P. griseoroseum* (PG) obtained with different primers. Column M shows the band pattern for the fragment size marker ( $\phi$ X174 digested with *Hae*III).

amplifications was performed with 18 random primers only, so the polymorphism between species in the subgenus *Penicillium* was probably underestimated. Our results emphasize the need to use the largest possible number of random primers to generate greater polymorphism in the high homogeneous subgenera *Penicillium*.

The rDNA ITS region was obtained for *P. expansum* and *P. griseoroseum* using the specific primers ITS1 and ITS4. The monomorphic fragment of 600 bp was amplified for *P. expansum* and nucleotide sequence deposited into GenBank with accession number AY425984. For *P. griseoroseum*, the size of the amplified ITS region was of 594 bp and nucleotide sequence deposited into GenBank with accession number AY425983. The size of the amplified ITS region is similar to that reported for other *Penicillium* species (8,14,39). ITS sequence of *P. expansum* and *P. griseoroseum* were aligned with ITS sequences of others *Penicillium* species (Fig. 4). The multiple sequence alignment showed high homology of nucleotide among all species. No ITS sequence variation was detected between *P. expansum* and *P. griseoroseum* excepting the additional six initial nucleotides.

Our results show that the comparison of nucleotide sequence ITS region between species of the genus *Penicillium* does not reveal a satisfactory discrimination because of the very low degree of ITS variability. On the other hand, the organization of the subtelomeric region and RAPD allows the analysis of the entire genome and gives a discriminatory level even between species indicating that the genomes of these two species are not identical. Despite the fact that Pitt (30) introduced an approach based on gross physiology under standardized conditions, these procedures are not yet effective for all species of the genus creating a need for alternative approaches (29).



**Figure 4.** Multiple alignment of the ITS region of *P. expansum* (this study), *P. griseoroseum* (this study), *P. italicum* (AJ250548), *P. crustosum* (X82361), *P. commune* (AJ004813), *P. camemberti* (AJ004814), *P. chrysogenum* (AF455490), *P. expansum* (XO) (AJ005676), *P. expansum* isolate wb342 (WB) (AF455466) and *P. roqueforti* (AJ005677). GenBank accession numbers in parentheses. Asterisks indicate identical nucleotides.

The polymorphism observed in the organization of the subtelomeric region in the genome of two *Penicillium* species within the high homogeneous *Penicillium* subgenera is for the first time reported and perhaps can be employed in future phylogenetics studies for the species belonging to this fungi subgenus. The integration of different methods and techniques should lead to the identification of useful markers for the standardization of global taxonomical studies of *Penicillium* species.

**ACKNOWLEDGMENTS**

The authors thank the Brazilian agencies CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), MCT/CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) for financial support.

**RESUMO**

**Diferenciação morfológica e molecular de fungos produtores de pectinases *Penicillium expansum* e *Penicillium griseoroseum***

*Penicillium expansum* e *P. griseoroseum* foram caracterizados morfológica e molecularmente. Variações na morfologia das colônias e coloração dos conídios foram observadas entre os isolados. A caracterização molecular foi baseada em marcadores RAPD, sequenciamento da região do espaçador interno transcrito do DNA ribossomal e “fingerprinting” telomérico. Foi usado um total de 78 primers RAPD, sendo que 8 apresentaram 54% de fragmentos de DNA polimórficos. Os produtos da amplificação da região ITS de *P. expansum* e *P. griseoroseum* foram de 600 e 594 pb, respectivamente. Não foi detectada nenhuma variação na seqüência de nucleotídeos dessa região, comparando-se *P.*

*expansum* e *P. griseoroseum*, exceto em relação aos seis nucleotídeos iniciais adicionais. Observou-se a ocorrência de polimorfismo na organização da região subtelomérica no genoma destes fungos e estimou-se um número mínimo de três cromossomos para estas espécies. Este é o primeiro trabalho que descreve a existência de polimorfismo na organização da região subtelomérica do genoma de espécies de fungos pertencentes ao gênero *Penicillium*, altamente homogêneo, indicando uma possível utilização da abordagem empregada neste estudo para pesquisas filogenéticas futuras.

**Palavras-chave:** DNA ribossômico, Espaçador Transcrito Interno, Região Subtelomérica, *Penicillium* spp.

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