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SHORT COMMUNICATION

RAPD Technique is a Useful Tool to Distinguish *Penicillium* Species

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Abstract

Random amplified polymorphic DNA (RAPD) analysis was used to evaluate genetic diversity among 13 soil *Penicillium* strains originating from widely dispersed areas. Twenty one of the 34 synthetic random primers were found to identify polymorphism in amplification products. The results show a high level of diversity of RAPD markers among the strains. All the strains could be identified by their characteristic amplification profile, using selected random primers. This suggests that RAPD analysis is a useful and reliable assay for characterizing the species of *Penicillium* genus.

Key words: *Penicillium* spp., Genetic diversity determination, RAPD, xylanase

Penicillium is one of the most heterogeneous and difficult to classify fungal genera. Because of the inherent variability in the genus, only 70 to 80% of isolates are readily identifiable (Pitt, 1988).

The taxonomy of the genus *Penicillium* is confusing and controversial. The need of a system for the identification and classification of *Penicillium* sp. is justified by the ecological and industrial importance of these microorganisms. Many of the taxonomic keys to identify *Penicillium* sp. are based primarily on morphological criteria. However, some of these characteristics (*i.e*. colony texture) are found to be highly variable and the morphological attributes do not allow unambiguous classification (Frisvad and Filtenborg, 1983). Several methods have also been used to identify *Penicillium* sp., such as physiological criteria (Pitt, 1988), secondary metabolite profiles (Jimenez *et al.*, 1990) and isoenzyme electrophoretic patterns (Frisvad, 1981). However, these methods are not generally accepted as standard systems for the evaluation of *Penicillium* sp., since a standard method should be simple, rapid, inexpensive, reliable, and applicable in any kind of routine laboratory.

One of the tools for the study of genetic divergence between different isolates is the random amplified polymorphic DNA (RAPD) technique, which allows the detection of polymorphisms in rapid, direct, consistent and low-cost manner (Jawhar *et al.*, 2000).

Xylanase is a hydrolytic enzyme, which has been used in many processing industries, such as pulp and paper, food and textile. Filamentous fungi such as *Penicillium* have been widely used to produce this enzyme (Bailey *et al.*, 1992).

The objective of this study was to evaluate RAPD methodology as a tool for the identification and classification of strains within and between some different *Penicillium* species. The relationship between xylanase production of strains and RAPD profiles is also discussed.

The strains utilized in this study were obtained from the CWBI (Centre Wallon de Biologie Industrielle, Belgium). The mutant strain *P. canescens* 10-10c producing a high level of xylanase (Bakri *et al.*, 2003) was used as a reference for xylanase assays (originating from the Institute of Plant Biotechnology, Academy of Science, Tbilisi, Georgia). The stock cultures were maintained in tubes with compost-agar and malt-agar media at 20°C. The strains were labeled as CWBI followed by one digit referring to the number given to the isolate. Three local soil *Penicillium* sp. strains Sy1, Sy8 and Sy14 were included in the experiment. The 13 strains are listed in Table I. The fungi were cultured in malt-agar medium; monosporic cultures of each *Penicillium* isolate were obtained using the same medium.

Xylanase was assayed by the optimized method described by Bailey *et al.* (1992), using 1% birchwood

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Table I
Relationship among primers utilized and results obtained by RAPD analysis of *Penicillium* sp.

Primer OP	Sequence	Polymorphism
A6	5 'GAGTCTCAGG 3'	X
A11	CAATCGCCGT	+
A12	TCGGCGATAG	+
A13	CAGCACCCAC	+
A14	TCTGTGCTGG	+
B6	GTGACATGCC	X
B7	AGATGCAGCC	X
B11	GTAGACCCGT	+
B12	CCTTGACGCA	+
B15	GCAGGGTGTT	+
B17	AGGGAACGAG	+
B18	AGGTGACCGT	+
C14	TGCGTGCTTG	+
C20	ACTTCGCCAC	+
E5	TCAGGGAGGTC	-
F11	TTGGTACCCC	X
F16	GGAGTACTGG	-
I-18	TGCCCAGCCT	-
J1	CCCGGCATAA	+
J7	CCTCTCGACA	+
J5	CTCCATGGGG	+
J4	CCGAACACGG	+
J15	TGTAGCAGGG	-
K8	GAACACTGGG	-
K12	TGGCCCTCAC	+
K13	GGTTGTACCC	+
K17	CCCAGCTGTG	+
L8	AGCAGGTGGAC	-
L15	AAGAGAGGGGC	X
N2	ACCAGGGGCAC	X
W17	GTCCTGGGTT	+
Y10	CAAACGTGGG	X
Z19	GTGCGAGCAA	+
Z20	CCTACGGGGA	+

Symbols: + with polymorphism; – without polymorphism and X without amplification

xylan as substrate; solutions of xylan and the enzyme at appropriate dilution were incubated at 55°C for 2 min and reducing sugars were determined by the dinitrosalicylic acid procedure (Miller, 1959), with xylose as standard. The released xylose was measured spectrophotometrically at 540 nm. One unit (U) of enzyme activity is defined as the amount of enzyme releasing 1 µmol xylanase/ml per minute under the described assay conditions. All experiments were repeated twice. Statistical analyses were performed using the Statview program (Abacus concepts, 1996) to test for differences in xylanase production among strains.

Thirteen isolates were grown on malt-agar medium for 3 weeks at 21±1°C and stored at 4°C for further study. Mycelium was harvested and DNA was extracted according to standard protocols (Leach *et al.*, 1986), resuspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1mM EDTA) and stored at –20°C.

DNA samples from each isolate were amplified with 34 random oligonucleotide (10-mer) primers from Operon Technologies, Inc. (Alameda, CA, USA). Reactions were carried out in a final volume of 25 µl containing 1×PCR buffer, 1 U Taq polymerase (Eppendorf AG, Hamburg, Germany), 0.2 mM dNTPs (Promega), 2.0 mM $MgCl_2$, 0.35 µM of primer and 1 ng of genomic DNA per 1 µl of reaction mixture. PCR was performed in a Thermocycler (BIO-RAD system, USA). Initial denaturation of 94°C for 2 min was followed by 45 cycles (1 cycle consists of denaturation for 1 min at 94°C, annealing for 1 min at 35°C and extension for 2 min at 72°C). A final extension of 72°C for 5 min was incorporated into the program, followed by cooling to 4°C until recovery of the samples. Amplicons were electrophoresed in 1.5% agarose gels that were photographed under UV light (302 nm) after staining with ethidium bromide, and the presence (1) or absence (0) of bands was recorded. The experiments were repeated twice for each isolate to confirm the repeatability and the monomorphic bands were removed from the analysis. The data were converted to a distance matrix using Nei and Li's (1979) coefficient, which was used to construct a dendrogram by the UPGMA (unweighted pair-group method with arithmetic averages) provided on the computer package (STATSOFT 2003).

Significant differences ($P<0.05$) in the mean yield values were detected among isolates, with high values being consistently higher in the reference strain F58 with mean value 54.01 U/ml (Fig. 2). From the 34 primers utilized, only 21 showed polymorphic bands, generating a total of 204 polymorphic bands, utilized for the analysis of the results, while the other primers either did not yield consistent results, or produced indistinguishable bands under the conditions used (Fig. 1; Table I).

Dendogram analysis of the RAPD profiles (Fig. 2) showed that the strains of *Penicillium* clustered into three groups. The *P. chrysogenum* strain (F271) clustered in one group. The other isolates were clustered in two different groups. No correlation exists between the differentiation of the isolates according to the RAPD analysis and their capacity to produce xylanase enzyme. However, despite the high variation observed, RAPD profiles exhibited some bands that were common to strains yielded high xylanase, and absent to that produced less xylanase. However, the banding pattern produced by primer OPA-11 (CAATCGCCGT) was polymorphic between low and high xylanase producing strains. This primer might be used to identify fragments for the synthesis of RAPD-derived probes for specific detection of newly studied *Penicillium* strains.

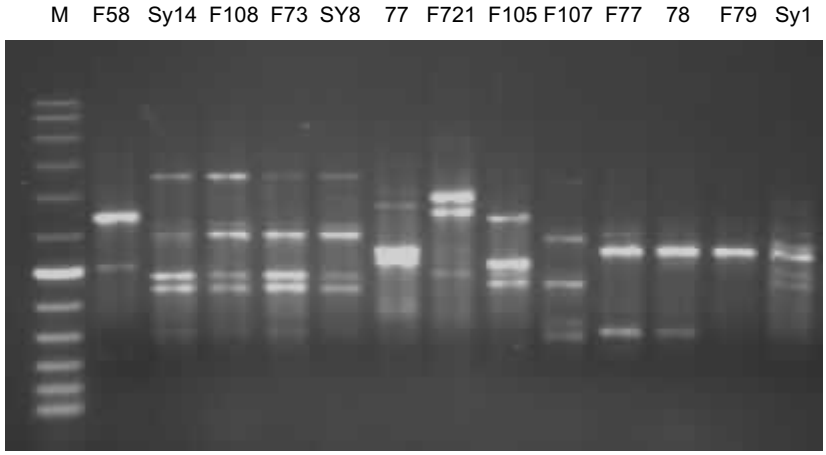

Fig. 1. PCR amplification using primers OPA-11 with template DNA from the 13 strains of *Penicillium* species.
M: Molecular weight markers (100 bp-Q.BIOgene).

This approach has been previously used to design PCR primers for the identification of *Aeromonas hydrophila* (Oakey *et al.*, 1999).

In this study the *Penicillium* strains from different species could be distinguished by RAPD markers. However, differentiation among strains could be due to selection occurring in these strains. Random changes in allele frequencies coupled with restricted migration may have resulted in genetic differentiation in *Penicillium* populations (Pitt, 1988).

Our results are supported by the fact that the RAPD data detect genetic diversity between related species and also within species (Lee and Taylor, 1990). However, RAPD markers evolve more rapidly than other genomic markers such as isozymes or rDNA sequences, and they tend to produce genetic

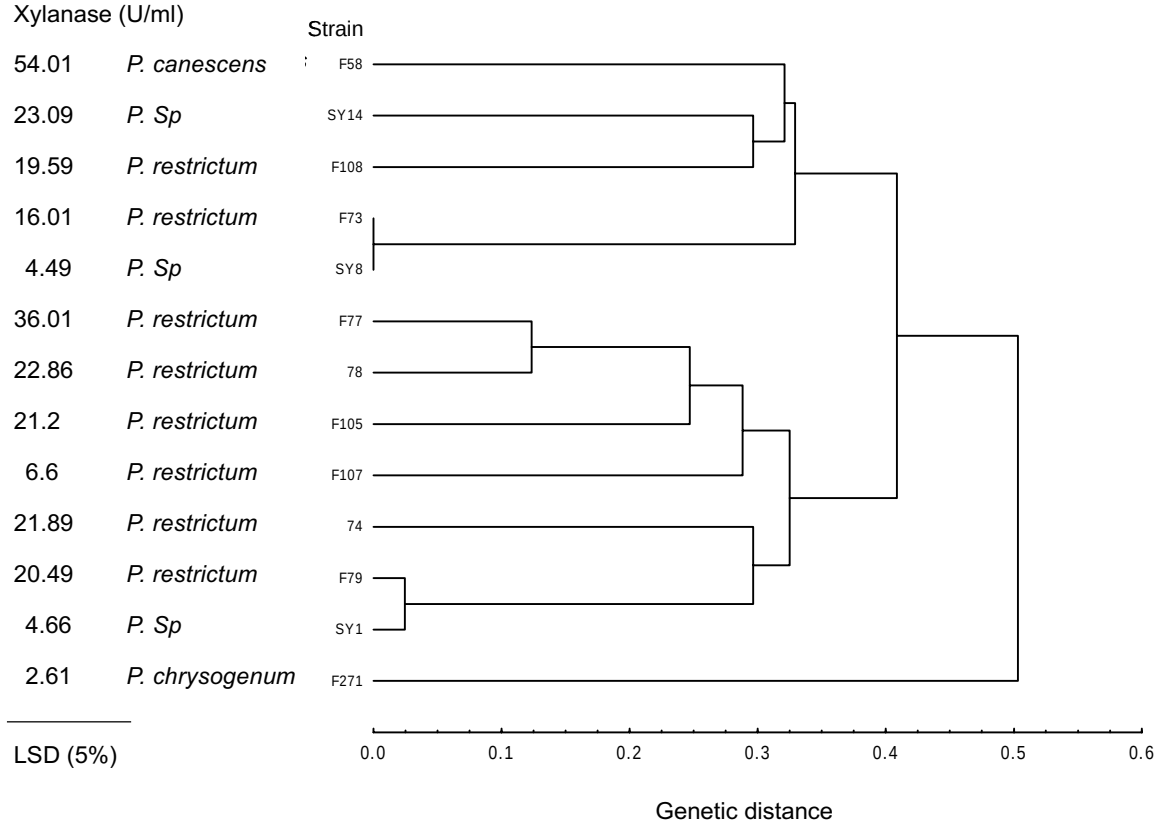

Fig. 2. Dendrogram showing relative genetic distances between 13 *Penicilium* strains.
The dendrogram was produced using similarity coefficients obtained from 204 polymorphic bands. LSD (5%): Least Significant Differences at $P < 0.05$.

distance values much greater than other methods (van de Zande and Bijlsma, 1995).

On the other hand, the results show that among all soil fungi tested for xylanase production, F58 and F77 strains were the highest xylanase producers with a small genetic distance value (Fig. 2), which might be attributed to the genetic differentiation (DNA fragments) between species. However, these two strains could be a good candidate for biotechnological applications.

Numerical analysis of profiles obtained with the selected RAPD primers showed genetic diversity among the strains and allowed clear differentiation of *Penicillium* species. We suggest that the RAPD technique is a rapid and reliable assay to distinguish the *Penicillium* species. It would be useful to perform this characterization for some other *Penicillium* species in order to determine the general application of the method. However, RAPD analysis potentially provides information across an entire genome, and further genetic analysis using internal transcribed spacer (ITS) region approach could be made to understand the relationship between xylanase production and DNA profiles.

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