ELSEVIER

Food Control 14 (2003) 137–140

FOOD CONTROL

www.elsevier.com/locate/foodcont

Identification of *Penicillium aurantiogriseum* species with a method of polymerase chain reaction-restriction fragment length polymorphism

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Received 22 March 2002; received in revised form 10 June 2002; accepted 17 June 2002

Abstract

A polymerase chain reaction method, added of restriction fragment length polymorphism analysis, was utilised to identify moulds, isolated from foods, belonging to *Penicillium aurantiogriseum* species. Suitable restriction enzymes were found. This simple method could be useful to face mycotoxin problem, discriminating toxic from non-toxic strains.

Keywords: PCR; *Penicillium aurantiogriseum*; RFLP

1. Introduction

Traditional identification methods of *Penicillium* genus' moulds, mainly based on cultural and morphological parameters, are sometimes unsuited when an objective analysis is required (Dragoni, Cantoni, Papa, & Vallone, 1997; Pitt, 1979; Pitt & Hocking, 1997; Samson & Van Reenen-Hoekstra, 1998). Some identification methods based on polymerase chain reaction (PCR) technique were set-up. PCR was in many cases followed by other technologies (amplification product denaturation or restriction enzymes digestion, etc.) (Dupont, Magnin, Marti, & Brousse, 1999; Karen, Hogberg, Dahlberg, Jonsson, & Nylund, 1997; Lo Buglio & Taylor, 1995; Nielsen, Frisvald, & Nielsen, 1998; Sequerra et al., 1997).

Molecular identification methods allow to securely face the mycotoxin problem (Sweeney & Dobson, 1998).

In this work, an easy-to-execute method for *Penicillium aurantiogriseum*'s identification was set-up.

2. Materials and methods

2.1. Sampling and DNA extraction

Lyophilized mycelia belonging to five *Penicillium* strains from a collection (Emisab, West Britain University) and fresh mycelia coming from five strains isolated from foods and identified with traditional methods were utilised (Table 1).

DNA extraction was performed with "Genomic Prep Cells and Tissue DNA Isolation Kit" (Amersham Pharmacia Biotech), according to the protocol for plant tissues.

2.2. Amplification (PCR)

As target sequences for amplification, nearly 350 and 600 bp DNA tracts, corresponding to 5.8 S and ITS I–II zones of fungal r-RNA genes, were selected (Boysen, Skouboe, Frisvad, & Rossen, 1996); primer pairs ITS5-ITS4 and ITS3-ITS4 were utilised (White, Bruns, Lee, & Taylor, 1990) (Table 2). Amplification protocol: 5 min at 95 °C, 35 cycles as below: 1 min at 95 °C, 1 min at 59 °C (primers ITS4-ITS5) or 61 °C (primers ITS3-ITS4), 2 min at 72 °C.

2.3. RFLP analysis

Restriction fragment length polymorphism (RFLP) analysis was performed on PCR amplification product with *AvaI*, *BgII* and *HaeIII* enzymes (Sigma).

Enzymes selection, based on *P. aurantiogriseum* and *P. chrysogenum* gene sequences found on the "Japan Genome Database" (http://www.ddbj.nig.ac.jp/searchese.html), was performed with public domain software "Digest" (author R. Nakisa).

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0956-7135/02/$ - see front matter
PII: S0956-7135(02)00056-7

Table 1
Strains utilised for the trial

Collection strains
P. aurantiogriseum n. 225–290 (AU1 or AU1X)
P. aurantiogriseum n. 792–795 (AU2 or AU2X)
P. chrysogenum n. 478–484 (CR2 or CRX)
P. commune n. 269–297 (PC2 or PCX)
P. solitum n. 141–186 (PS2 or PSX)
Strains isolated from foods
P. aurantiogriseum (AUA)
P. aurantiogriseum (AUB)
P. chrysogenum (CHA)
P. commune (COB)
P. solitum (SOA)

Table 2
Primer sequences

ITS3 GCATCGATGAAGAACGCAGC
ITS4 TCCTCCGCTTATTGATATGC
ITS5 GGAAGTAAAAGTCGTAACAAGG

For each specimen, 10 units of each enzyme and 10 μl of amplification product were used, in a total volume of 50 μl of buffer supplied with restriction enzymes. Digestion was performed at 37 °C for 3 h (Kumeda & Asao, 1996).

2.4. Electrophoresis and staining

An horizontal gel electrophoresis was performed (Gene Gel Excel 12.5/24 kit, Pharmacia Biotech) on a Multiphor II apparatus (Pharmacia Biotech), setting up electric parameters as follows: $V = 600$, mA = 25, $W = 15$, for nearly 80 min.

In addiction to *Penicillium* specimens, negative controls (without DNA), amplification standards (Pharmacia Biotech) and a molecular marker (100 Base Pair Ladder—Pharmacia Biotech) were seeded on the gel. This marker was seeded in several lanes.

Electrophoretic bands revelation was performed with silver staining (DNA Plus One Silver Staining Kit, Pharmacia Biotech) (Colombo, Viacava, & Bonfanti, 1998).

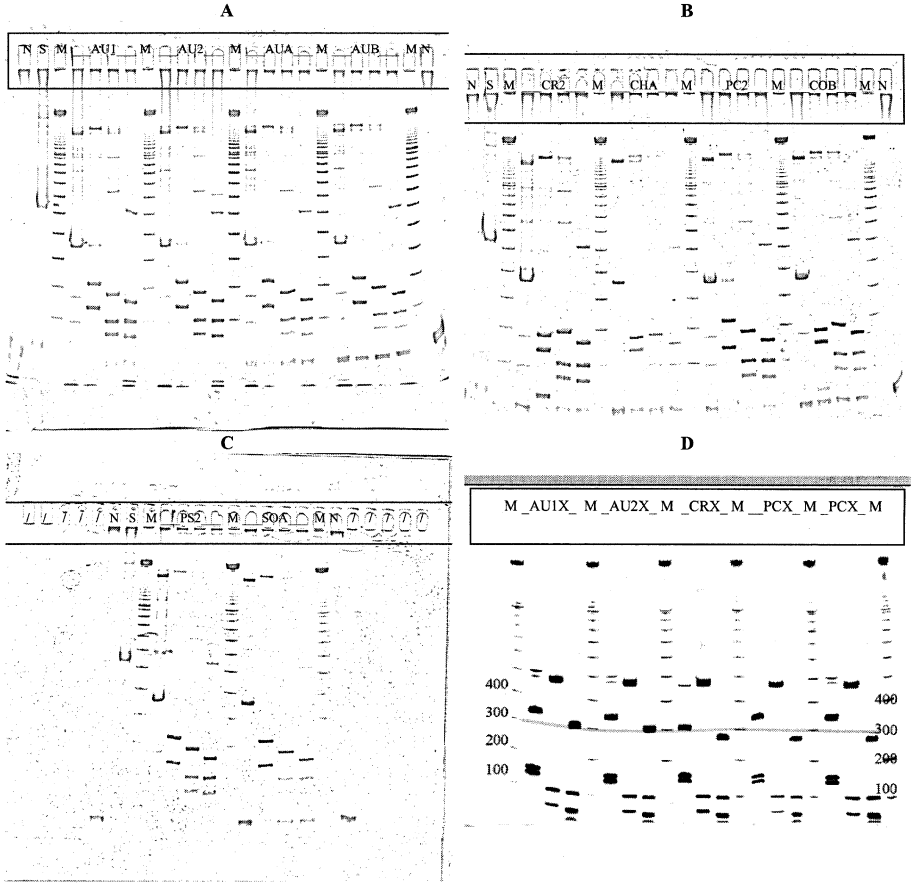

Fig. 1. Electrophoretic gels S—standard (500 bp); N—negative sample (no DNA in PCR reaction). M—molecular marker. /—Empty lane. Other labels correspond to those of mould samples listed in Table 1. A–C: Electrophoretic gel of RFLP fragments from PCR amplification with ITS3-ITS4 primers. Every mould sample is replicated in 4 lanes: the first contains undigested PCR product. In the other three PCR product is respectively digested with *AvaI*, *BgII* and *HaeIII* restriction enzymes. D: Electrophoretic gel of RFLP fragments from PCR amplification with ITS4-ITS5 primers. Every mould sample is replicated in 3 lanes the first being the PCR product digested with *AvaI*, the other two are respectively digested with *BgII* and *HaeIII* restriction enzymes. The line drawn on the gel image shows the 300 bp threshold discriminating *P. aurantiogriseum* 's from other species (see text).

2.5. Gel analysis

Electrophoretical gels were acquired with a scanner (AGFA Snapscan 600).

Gel analysis was performed calculating the bands' ratio frontis with "Sigma Gel" software (SPSS, Chicago). A cluster analysis was done on resulting data utilising "SPSS" software (SPSS, Chicago) yielding

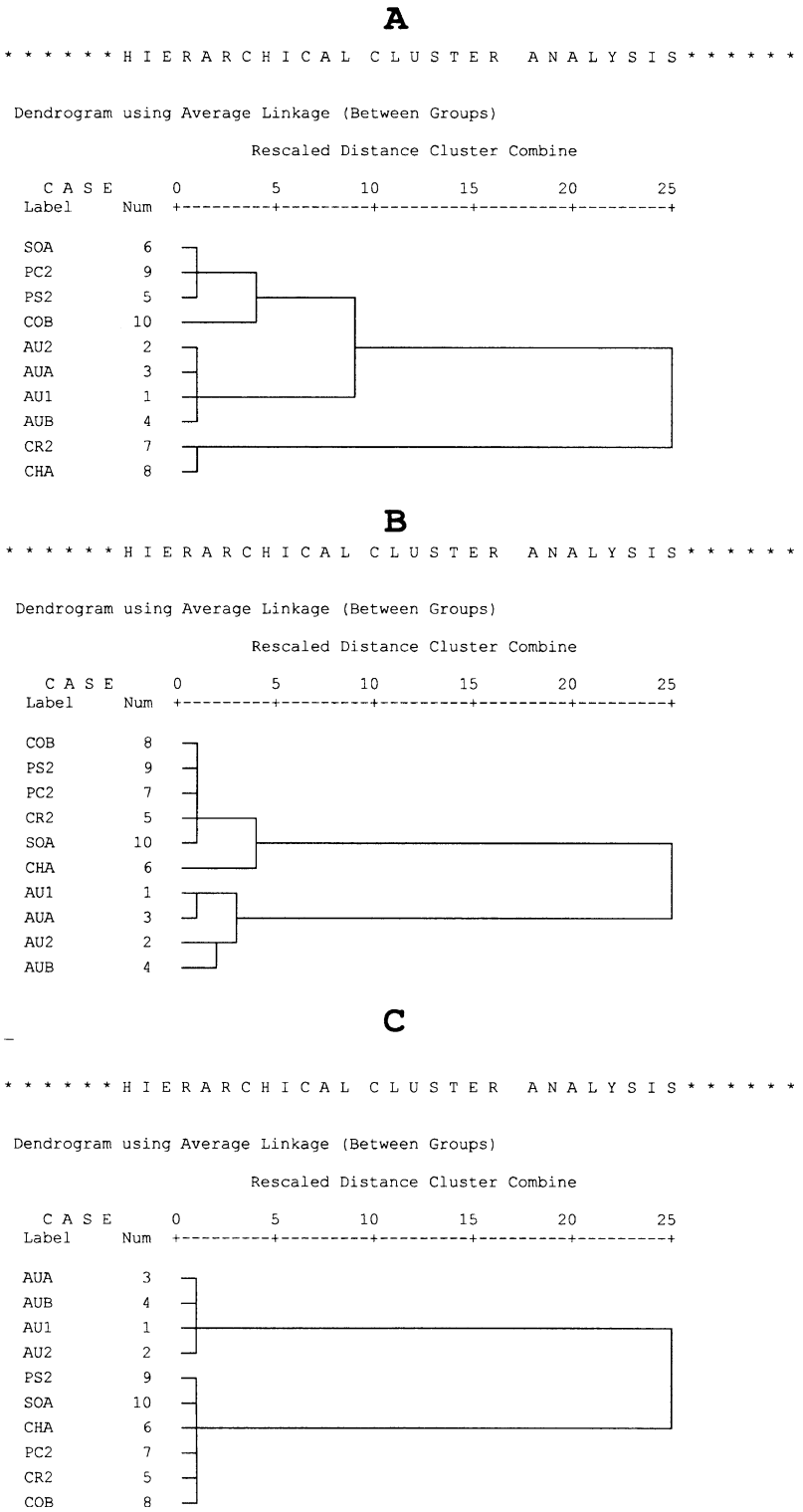

Fig. 2. Dendrogram from pattern of digestion fragments produced by *AvaI* (A), *BgII* (B) and *HaeIII* (C) enzymes on ITS3-ITS4 amplicon. See Table 1 for label explanation.

tree-diagrams (dendrograms). When detection of characteristic bands was clear and unambiguous, the gel was not analysed with a computer-assisted method but only by visual observation.

3. Results and discussion

Electrophoretic gels resulting from digestion of PCR products of ITS3-ITS4 tract are shown in Fig. 1A–C.

Related dendrograms shown in Fig. 2A–C clearly reveal that the three enzymes used isolate *P. aurantiogriseum* from other species; *BgII* enzyme, furthermore, allows an intra-specific level distinction.

This difference does not clearly appear to a gel's visual analysis: it is probable that, to give this distinctive effect, little but statistically significant differences in length of the digestion fragment, are determinant.

Referring to the experiment made on the ITS4-ITS5 zone (Fig. 1D), where only collection strains were analysed, *HaeIII* enzyme has proven to be effective to identify *P. aurantiogriseum*, because it gave a big characteristic band in the 300 bp zone which is almost exactly aligned with 300 bp band of marker: in other species that band is clearly shifted below the 300 bp front.

It seems important, in the right instances, to distinguish this species from others, as well as analysing it at intra-specific or at strain level: *P. aurantiogriseum*'s collection strains (225.90 and 792.95) were previously biochemically analysed and secondary toxic metabolites, penicillic and cyclopiazoic acid, were found (data not shown). Aurantiamine, a non-toxic secondary metabolite was only found in strain 225.90. These data suggest that RFLP profiles could correlate with corresponding metabolic ones, permitting to evaluate toxicity from DNA patterns.

Mould toxic secondary metabolites could be present in foods as some cheese and salami where spontaneous mould growth is requested for product typicalness: quick and reliable analytic techniques as PCR could be very useful to control these food industry products.

Regarding the adopted DNA extraction method, it is a non-toxic, easy-to-perform one: this allows using it for routine work.

This method, originally aimed to DNA extraction from plant tissues, in this work, as far we know, has been successfully applied for the first time to fungal mycelia.

High-resolution electrophoresis adopted in this trial is useful to detect small size (<100 bp) digestion fragments differing from one another in few base pairs: this can be important for differential examinations adopting electrophoretic gels' statistical analysis.

Acknowledgement

The authors thank Rossana Capoferri for collaboration.

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