

## CHARACTERIZATION AND fAFLP GENOTYPING OF *PENICILLIUM* STRAINS FROM POSTHARVEST SAMPLES AND PACKINGHOUSE ENVIRONMENTS

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### SUMMARY

We examined 41 isolates of *Penicillium* spp. recovered from rotten fruits (including oranges, grapefruits, pears, lemons, strawberries, apples, loquats, prickly pears) and from air and surfaces of markets and packinghouses. *Penicillium* isolates were identified as *P. expansum*, *P. italicum*, *P. digitatum*, *P. olsonii*, *P. chrysogenum* or *P. citrinum*. Isolates were tested for sensitivity to commonly used postharvest fungicides, and pathogenicity on different potentially susceptible fruit hosts. Genetic characterization was performed with ITS4 and ITS5 primers that specifically identified *Penicillium* isolates by amplification of a 600-bp fragment, with PEF and PER primers used to identify *P. expansum* isolates by amplification of a 404-bp fragment, and with fluorescent amplified fragment length polymorphism analysis (fAFLP). Cluster analysis of fAFLP data divided the isolates into five well-separated *P. italicum*, *P. digitatum*, *P. citrinum*, *P. chrysogenum* and *P. olsonii* clusters, whereas *P. expansum* isolates were divided in three distinct clusters. Within all the eight clusters, isolates were well differentiated. Results obtained with fAFLP analysis confirmed the reliability of the method to characterize and identify strains at intraspecific level.

*Key words:* *Penicillium*, characterization, DNA fingerprinting, fAFLP, postharvest.

### INTRODUCTION

Postharvest stored foodstuffs are subject to fungal alteration caused by a broad range of *Penicillium* spp. While fungicides are still the primary means of control of post harvest diseases, the public is demanding reduced application of chemicals to agricultural products, stimulated by the growing concern for the environment and human health and the spread of pathogen strains tolerant to pesticides.

The genus *Penicillium* includes about 150 species but only a minor fraction of these cause infection to important plant and processed foodstuffs (Pitt and Hocking, 1997). Species identification relies on evaluation of macro-morphological characters, microscopic observation of reproductive structures, easily recognisable secondary metabolite production (such as diffusible pigments) or isoenzyme profiling (Frisvad, 1981; Frisvad and Filtenborg, 1983). However, many authors have shown the high variability of some of these characteristics and that morphological criteria do not always allow unambiguous classification (Pitt, 2000), especially for *P. expansum* (Frisvad and Filtenborg, 1983). More recently, genotypic characterization has proven very useful to identify *Penicillium* spp. and several methods have been used to assess intraspecific and interspecific variation in *Penicillium* (Dupont *et al.*, 1999; Lund *et al.*, 2003). These methods include amplification of the internal transcribed spacers (ITS1 and ITS2), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). Amplification of ITS regions is effective in identifying the genus *Penicillium* (White *et al.*, 1990; Peterson, 2000), and some primer combinations allow selective amplification at the sub-genus level (Pedersen *et al.*, 1997). Restriction of amplicons generated by PCR (RFLP) can be used to differentiate *Penicillium* isolates at the species level (Pianzola *et al.*, 2004).

The use of RAPD markers in studying intra- and interspecific variation in *Penicillium* has been also reported (Geisen *et al.*, 2001; Pianzola *et al.*, 2004).

AFLP analysis is a broadly applicable genotyping method with high degrees of reproducibility and discriminatory power. The method can type microorganisms at the isolate level (Vos *et al.*, 1995; Olive and Bean, 1999). Several applications in taxonomy, diagnostics and epidemiology have been realized (Savelkoul *et al.*, 1999). AFLP has been used for differentiation of bacteria (Vos *et al.*, 1995; Lin *et al.*, 1996; Ripabelli *et al.*, 2000; Zewdu *et al.*, 2001), fungi (Majer *et al.*, 1996; Rosendhal *et al.*, 1997; Abd-Elsalam *et al.*, 2003; Kure *et al.*, 2003; Schmidt *et al.*, 2003) and plants (Vos *et al.*, 1995; Diaz *et al.*, 2003). AFLP fingerprinting has also

been used in several studies regarding diversity of *Penicillium commune*, *P. palitans* and *P. verrucosum* isolates (Lund *et al.*, 2003; Kure *et al.*, 2003; Frisvad *et al.*, 2005). Unlike other PCR-based fingerprinting techniques, AFLP reactions are less dependent on minor changes in PCR conditions, due to the use of selective primers with more stringent annealing temperatures that limit the number of bands (Terefework *et al.*, 2001). Different polymerase enzymes, slight deviations of the optimal MgCl<sub>2</sub> concentration and template concentration that are attributed to problems with reproducibility of other fingerprinting techniques, for instance, RAPD and rep-PCR, did not show any effect on the fingerprint patterns in AFLP reactions (Bakkeren *et al.*, 2000; Tuthill, 2004).

Fluorescence labelling and semi-automated detection of fragments have increased the speed and accuracy of the AFLP technique (Wenz *et al.*, 1998; Huang and Sun, 1999).

Capillary electrophoresis coupled with fluorescent fragment analysis has improved the power of AFLP by adding the sharp precision of digital analysis to the resolving power of traditional polyacrylamide gels. The modified AFLP technique avoids the use of isotopes or silver staining, and gives a much higher resolution than other AFLP detection systems.

Fluorescence based AFLP (fAFLP) has been shown to be an excellent alternative to other characterization and detection methods (Arnold *et al.*, 1999; Dresler-Nurmi *et al.*, 2000; De Curtis *et al.*, 2004; Scuderi *et al.*, 2005).

The aim of our study was to characterize *Penicillium* isolates obtained from rotten fruits and environmental samples by morphological, physiological, biological and molecular criteria and to evaluate fAFLP as a tool for identification of *Penicillium* isolates. This appears to be the first report of using fAFLP for this purpose.

## MATERIALS AND METHODS

**Strains.** *Penicillium* strains were previously isolated (Oliveri *et al.*, 2004) from oranges, grapefruits, pears, lemons, strawberries, apples, loquats and prickly pears showing mould symptoms, and from air samples and surfaces at five commercial packinghouses and eleven local markets in Eastern Sicily (Table 1). Single-spore cultures of each *Penicillium* strain were routinely grown at 25°C in the dark on potato dextrose agar (PDA, Oxoid, Basingstoke, UK) supplemented with chloramphenicol (250 mg l<sup>-1</sup>; Sigma, St. Louis, MO, USA) to inhibit bacterial contamination. All strains were stored on PDA under mineral oil (Sigma) at 4°C.

Conidial suspensions (10<sup>6</sup> CFU ml<sup>-1</sup>) were stored over longer periods in 15% glycerol at -80°C. *P. digitatum* MUCL 14256, *P. expansum* MUCL 273 and *P. itali-*

*cum* MUCL 958, purchased from BCCM™ / MUCL Micothèque de l'Université Catholique de Louvain-Belgium, were used as reference strains in all experiments.

### Morphological characterization of fungal isolates.

Twenty-two *Penicillium* strains isolated from fruits and sixteen *Penicillium* strains isolated from air and surface samples were identified at the species level by the key system of Pitt (2000) using standard parameters such as morphology and growth on three standard media: Czapek-yeast agar (CYA), malt extract agar (MEA) and 25% glycerol nitrate agar (G25N). To observe morphological characteristics, a suspension was prepared from spores removed with a sterile loop from sporulating edges of a 1 week-old culture and suspending them in 1ml sterile distilled water (SDW). Petri dishes (Ø 90 mm) containing MEA and CYA were inoculated with a single-spore culture (1 ml of 10<sup>6</sup> cfu ml<sup>-1</sup>) at three points, equidistant from the centre and the edge of the plate, and from each other. Plates of G25N were inoculated at two points per plate. MEA and G25N cultures were incubated at 25°C, and CYA cultures at 5°, 25° and 37°C. All plates were incubated for 7 days. Diameter, colour and texture of the colonies and microscopic features (stipes, branches, metulae, phialides, conidium shape and length) were observed on optical microscope slides with a drop of 0.1% lactofuchsin stain and photographed (Carmichael, 1955). To study fruiting structure morphology a 40X objective was used. Observation by SEM of the *penicillus* structure and conidia allowed clarification of ambiguous results (data not shown).

Isolates were routinely grown on plates of Sabouraud (Oxoid, Basingstoke, UK) or PDA (Oxoid, Basingstoke, UK) medium.

**Pathogenicity assay.** Ripe fruits (apricots, pears, apples, lemons, oranges, prickly pears, grapes) from organic agriculture were surface disinfected with 70% ethanol, then rinsed twice with sterile water. Each fruit was wounded with a steel sterile needle (2 mm diameter x 2 mm deep) at four locations in the equatorial region. Each wound was inoculated with 10 µl of conidial suspension (10<sup>6</sup> conidia ml<sup>-1</sup>) from a 7 day-old plate. Control fruits were treated with SDW. Inoculated fruits were placed in separate polyethylene bags and incubated in a moist chamber at room temperature (21-26°C). Three repetitions per treatment were carried out and the experiment was repeated twice. The number of wounds showing symptoms of sporulation was counted seven days after inoculation and converted to percentage values.

**Assessment of fungicide sensitivity.** Minimal inhibitory concentrations (MIC) of benomyl and imazalil were determined for all isolates. Fungicides were tested in plate radial growth assays by inoculating each fungal isolate (20 µl of 10<sup>4</sup> conidia ml<sup>-1</sup> from 7 day old PDA

culture) on PDA plates amended with 10, 50 and 100 ppm of benomyl (Benlate® - DuPont) or 0.1, 0.5 and 1 ppm of imazalil (DeccoZil® 50 - Cerexagri). Fungal growth was measured after 7 days of incubation at 25°C in darkness. MIC was defined as the lowest concentration that inhibited fungal growth. Two repetitions per treatment were performed. Experiments were repeated twice.

**Extraction of genomic DNA.** Total genomic DNA from *Penicillium* strains was extracted using Puregene DNA Purification Kit (Puregene, Gentra, Minneapolis, MN, USA) according to the manufacturer's instructions with some modifications. A loopful of conidia from single-spore cultures were inoculated in 10 ml Sabouraud broth (Oxoid, Basingstoke, UK) in canted neck flasks for 4-5 days at 25°C with orbital shaking at 50 rpm. The mycelium was harvested by filtration through a double layer of sterile cheese cloth and washed three times with SDW, aliquoted (10-20 mg, wet weight) to 1.5 ml tubes and stored at -20°C. The fungal tissue was ground with a sterile micropestle (Eppendorf, Hamburg, Germany) in the same tube and resuspended in 600 µl of Cell Lysis Solution with Proteinase K (100 mg/ml). The cell lysate was incubated at 55°C overnight to inactivate DNases. RNase A Solution (3 µl) was added to the sample and incubated at 37°C for 15 minutes. The supernatant containing the DNA was collected after selective precipitation of proteins by the addition of Protein Precipitation Solution to the cell lysate and mixing. DNA was precipitated by adding an equal volume of isopropanol. The precipitated DNA was pelleted by centrifugation (13,000 - 16,000 g for 1 minute) and the pellet washed with 70% ethanol, dried and resuspended in 50 µl of TE buffer (10 mM Tris/HCl, pH 8; 1 mM EDTA). A 2 µl aliquot was run on a 1% (w/v) agarose gel stained with ethidium bromide for estimation of DNA concentration.

**PCR amplification of the ribosomal DNA region for RFLP studies.** The ITS1 - 5.8S - ITS2 region of the rDNA was amplified by PCR using the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAG-3') essentially as described by White *et al.*, (1990). For each 50 µl reaction, a mixture was prepared containing 10 ng of genomic DNA, 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 0.2 mM of deoxynucleoside triphosphates, 0.25% Tween 20, 10% dimethyl sulfoxide (DMSO), 1 µM each of primers ITS4 and ITS5 (MWG Biotech Ltd., Milton Keynes, UK), and 2.5 U of *Taq* DNA polymerase Recombinant (Invitrogen Life Technologies, Carlsbad, CA, USA). Amplification was performed in a GeneAmp 9700 PCR system (Perkin Elmer Corp., Norwalk, CT, USA) with an initial denaturation of 30 sec at 93°C, followed by 40 cycles of 94°C for 15 sec,

53°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 10 min.

PCR products (10 µl) were digested with the restriction enzymes *Hinf*I (Invitrogen Life Technologies, Carlsbad, CA, USA) and *Taq* I (Invitrogen Life Technologies, Carlsbad, CA, USA) and the reactions incubated overnight at 37° and 65°C respectively. The DNA fragments were resolved by electrophoresis in 3% agarose. Ethidium bromide (1 µg/ml) was added to the gels prior to electrophoresis and digitized by ChemiDoc™ EQ (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A 50-bp DNA Ladder (Invitrogen Life Technologies, Carlsbad, CA, USA) was used as a size standard.

**Polymerase chain reaction with PEF and PER primers for the polygalacturonase gene of *P. expansum*.**

PCR was carried out for specific identification of *P. expansum* as described by Marek *et al.* (2003). Primers based on the polygalacturonase gene of *P. expansum* PEF (5'-ATC GGC TGC GGA TTG AAA G-3') and PER (5'-AGT CAC GGG TTT GGA GGG A-3') were purchased from MWG Biotech. Amplification was carried out in 50 µl reaction mixtures containing 2.5 mM MgCl<sub>2</sub>, 200 mM of each nucleotide, 1 µM of each primer, 1.25 U of *Taq* DNA polymerase Recombinant (Invitrogen Life Technologies, Carlsbad, CA, USA) and 10 ng of genomic DNA from each *P. expansum* isolate. All PCR Reactions were performed in a GeneAmp 9700 PCR system (Perkin-Elmer Corp., Norwalk, CT, USA) and the temperature cycling conditions were as described by Marek *et al.* (2003). A 20 µl volume of each reaction product was analyzed by electrophoresis on 1.2% agarose gel in 1x TAE buffer and stained with ethidium bromide.

**Fluorescent AFLP analysis.** The fAFLP analysis was performed according to the manufacturer's protocol (AFLP® Core Reagent Kit - Invitrogen Life Technologies, Carlsbad, CA, USA). Fungal genomic DNA (250 ng) was digested with *Eco*RI/*Mse*I restriction enzymes mix (1.25 U each) in 1x Reaction Buffer for 2 hours at 37°C in a final volume of 25 µl. The mixture was incubated for 15 minutes at 70°C to inactivate the restriction endonucleases. In the same reaction mixture, 24 µl Adapter/Ligation Solution (*Eco*RI forward: 5'-CTCG-TAGACTGCGTACC-3', *Eco*RI reverse: 5'-AATTGG-TACGCAGTCTAC-3', *Mse*I forward: 5'-GACGATG-AGTCCTGAG-3', *Mse*I reverse: 5'-TACTCAGGACT-CAT-3') and 1 µl of T4 DNA ligase (AFLP® Core Reagent Kit - Invitrogen Life Technologies, Carlsbad, CA, USA) were added at final concentrations of 2 and 20 µM, respectively. The final ligation mixture volume was 25 µl. The ligation mixture was incubated at 20°C for 2 hours.

A two-step procedure was adopted for DNA amplification. Pre-amp Primer Mix I (Invitrogen Life Tecnolo-

**Table 1.** Original source, pathogenicity tests and fungicide minimal inhibitory concentration (MIC) for *Penicillium* strains used in this study.

Strains <sup>a</sup>	Area of origin	Source <sup>b</sup>	Symptoms on inoculated fruits <sup>c</sup>							MIC (ppm)	
			Apricot	Pear	Apple	Lemon	Orange	Prickly pears	Grape	IMZ	Benomyl
<i>P. citrinum</i>											
MPVCT 200	Lentini-SR	Surface (P1)	-	-	-	-	++	++	+	>1	<50
MPVCT 202	Lentini-SR	Surface (P1)	-	-	-	-	-	-	+	>1	>100
MPVCT 214	S.M di Licodia-CT	Surface (P3)	-	-	-	-	-	-	+	>1	>100
MPVCT 204	Lentini-SR	Air (P1)	-	-	-	-	-	-	-	>1	>100
MPVCT 210	S.M di Licodia-CT	Air (P3)	-	-	-	-	-	-	-	>1	>100
<i>P. chrysogenum</i>											
MPVCT 201	Lentini-SR	Surface (P1)	-	-	-	-	-	-	+	>1	>100
MPVCT 203	Lentini-SR	Surface (P1)	-	-	-	-	-	-	-	>1	>100
MPVCT 206	Lentini-SR	Surface (P2)	-	-	-	-	-	-	++	>1	>100
MPVCT 208	Lentini-SR	Surface (P2)	++	+	-	-	-	-	-	>1	>100
MPVCT 211	S.M di Licodia-CT	Air (P3)	+	++	-	-	-	-	-	>1	>100
<i>P. olsonii</i>											
MPVCT 205	Lentini-SR	Surface (P2)	++	-	-	-	-	-	-	>1	>100
MPVCT 212	S.M di Licodia-CT	Surface (P3)	+	-	-	-	-	-	-	>1	>100
MPVCT 213	S.M di Licodia-CT	Surface (P3)	+	-	-	-	-	-	+	>1	>100
MPVCT 215	S.M di Licodia-CT	Air (P3)	-	-	-	-	-	-	-	>1	>100
<i>P. italicum</i>											
MUCL 958	Louvain-Belgium	Grapefruit	+	+	-	-	-	-	++	>1	>100
MPVCT 207	Lentini-SR	Surface (P2)	+	+	-	++	++	-	++	<0.1	>100
MPVCT 209	Lentini-SR	Air (P2)	++	+	-	-	-	-	-	<0.1	<10
MPVCT 236	Motta S.Anastasia-CT	Orange (P4)	++	++	-	++	++	-	++	<0.1	<50
MPVCT 237	Catania-CT	Orange (M1)	++	++	-	++	++	-	++	<0.1	<10
MPVCT 239	Catania-CT	Orange (M2)	++	++	-	++	++	-	++	<0.1	<10
<i>P. expansum</i>											
MUCL 273	Louvain-Belgium	Apple	++	++	++	-	-	+	++	>1	<50
MPVCT 221	Catania-CT	Apple (M5)	++	++	++	-	-	+	++	>1	>100
MPVCT 222	Catania-CT	Apple (M5)	++	++	++	-	-	-	++	>1	>100
MPVCT 231	Catania-CT	Apple (M7)	++	++	++	-	-	+	++	>1	>100
MPVCT 216	Motta S.Anastasia-CT	Pear (P4)	-	+	+	-	-	-	-	>1	<50
MPVCT 223	Catania-CT	Pear (M5)	++	+	+	-	-	-	++	>1	<50
MPVCT 228	Sant'Agata Li Battiati-CT	Pear (M6)	++	++	++	-	-	-	++	>1	<10
MPVCT 229	Sant'Agata Li Battiati-CT	Pear (M6)	++	++	++	-	-	+	++	>1	>100
MPVCT 217	Catania-CT	Pear (M3)	++	++	++	-	-	++	++	>1	>100
MPVCT 218	Assoro ValDittaino-EN	Bread (P5)	++	++	++	-	-	+	++	>1	>100
MPVCT 219	Acireale-CT	Strawberry (M4)	++	++	++	-	-	++	++	>1	<50
MPVCT 225	Sant'Agata Li Battiati-CT	Loquat (M6)	++	++	++	-	-	+	++	>1	>100
MPVCT 226	Sant'Agata Li Battiati-CT	Loquat (M6)	++	++	++	-	-	-	++	>1	>100
MPVCT 227	Sant'Agata Li Battiati-CT	Loquat (M6)	++	+	++	-	-	-	++	<0.1	<50
MPVCT 232	Catania-CT	Prickly pears (M8)	++	-	+	-	-	++	++	<0.1	<10
<i>P. digitatum</i>											
MUCL 14256	Louvain-Belgium	Orange	++	++	-	++	-	+	++	1	<10
MPVCT 233	Motta S.Anastasia-CT	Orange (P4)	++	++	-	++	++	-	++	1	<50
MPVCT 234	Giarre-CT	Orange (M9)	++	++	-	++	++	-	++	>1	<10
MPVCT 235	Motta S.Anastasia-CT	Orange (P4)	++	++	-	++	++	-	++	1	<50
MPVCT 238	Mascali-CT	Orange (M10)	++	+	-	++	++	-	++	>1	>100
MPVCT 230	Catania-CT	Lemon (M11)	++	++	-	+	+	-	++	>1	<10

<sup>a</sup> MPVCT: fungi collection of the Plant Pathology laboratory, University of Catania, Italy; MUCL: Micothèque de l'Université Catholique de Louvain- Belgium

<sup>b</sup> Letters in parentheses designate packinghouses (P) and markets (M). Numbers designate the 5 packinghouses and 11 markets.

<sup>c</sup> - = no visible symptoms on more than of inoculated sites

+= sporulation on more than 40% of inoculated sites

++= sporulation on more than 80% of inoculated sites

gies, Carlsbad, CA, USA) containing adapter-complementary AFLP primers, each with one selective nucleotide (M-C; E-A), was used in the pre-amplification reaction to amplify genomic DNA following restriction-digestion. Five microliters of each restriction-ligation reaction were processed in a preselective PCR in 50 µl mixture containing 2 µl of each 5 mM preselective primer *EcoRI* (5'-GACTGCGTACCAATTCA-3') and *MseI* (5'-GATGAGTCCTGAGTAAC-3'), 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 1x PCR reaction buffer and 0.5 µl of *Taq* polymerase Recombinant (Invitrogen Life Technologies). The 1:5 diluted pre-amplification PCR product was used as template for a subsequent selective amplification reaction. In the selective amplification step, *EcoRI* (5'-GACTGCGTACCAATTCA-3') labelled at 5' ends with Cy5 fluorophore and unlabeled selective *MseI* (5'-GATGAGTCCTGAGTAACAG-3') primers had two and three selective nucleotides respectively.

All PCR reactions were performed in a GeneAmp 9700 PCR system (Perkin-Elmer Corp., Norwalk, CT, USA) and the temperature cycling conditions were as described by Vos *et al.* (1995). Primers were purchased from MWG Biotech. The AFLP products were separated with a CEQ 8000 Genetic Analysis System automated DNA sequencer (Beckman Coulter Inc., Fullerton, CA, USA). A 1:5 dilution of amplified DNA with sample loading solution (SLS; Beckman Coulter, Inc., Fullerton, CA, USA) containing formamide, was required before loading samples on the sequencer. CEQ DNA Size Standard kit 600 (Beckman Coulter, Inc., Fullerton, CA, USA) was used to normalize the profiles. The sequencer was set up according to the manufacturer's instructions. Reproducibility of the results was checked by repeating AFLP reactions on the fungal strains examined. A binary data matrix was generated reflecting the presence or absence (1-0) of fragments obtained by AFLP from different isolates.

Dendrograms were constructed based on the unweighted pair-group method using arithmetic average (UPGMA) with the PHYLIP<sup>®</sup> software package (Sneath and Sokal, 1973; Felsenstein, 2004). Results in the form of Nei's genetic diversity statistics (Nei, 1978) were calculated using Popgene v1.31 software (Population Genetic Analysis © 1997).

## RESULTS

### Morphological characterization of fungal strains.

Using identification keys (Pitt, 2000), five isolates were identified as *P. citrinum*, five as *P. chrysogenum* and four as *P. olsonii*. All strains belonging to these three species were isolated from air and surfaces of five different packinghouses (Table 1). Five isolates were identified as *P. italicum*, five as *P. digitatum* and fourteen as *P. expansum*. All these strains were isolated from rotten fruits

collected from eleven local markets and packinghouses (Table 1).

By lactofuchsin staining, young actively growing structures were preferentially stained bright pink, so penicilli were clearly distinguished by light microscopy against a background of older mycelium. *P. citrinum* was readily recognisable by its penicilli, which consisted of 3-5 divergent and vesiculate metulae, bearing long well-defined columns of conidia. Penicilli were typically biverticillate and asymmetrical. Conidia were spherical, 2.2-3.0 µm in diameter. *P. chrysogenum* isolates showed typically terverticillate penicilli, with 1-2 rami. *P. olsonii* stipes were characterized by terminal multiramulate and terverticillate penicilli.

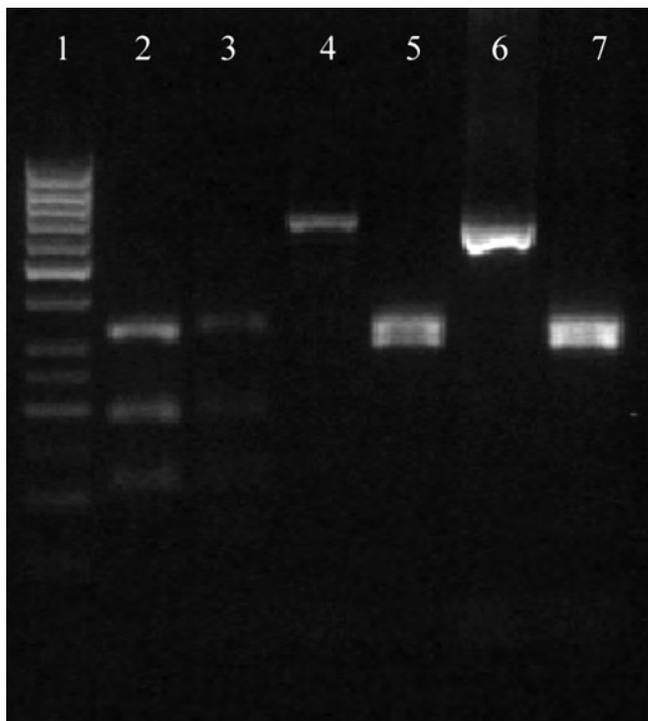
Conidia were ellipsoidal, 3.0-4.0 µm long. All isolates belonging to *P. expansum* showed typically terverticillate penicilli and ellipsoidal conidia (3.0-3.5 µm long) borne in long irregular chains.

The macromorphology of *P. expansum* cultures on CYA was variable. Examination under the stereomicroscope revealed some differences in colony texture, extent of sporulation and reverse colour of the strains. In culture, *P. italicum* formed broad grey-green colonies with deep brown reverse colours and terminal terverticillate penicilli. Production of yellow-green to olive conidia on all substrates tested is a distinctive characteristic of *P. digitatum* species. *P. digitatum* conidia were ellipsoidal, 2.5-5.0 µm long. Stipes with terminal terverticillate or biverticillate penicilli were observed frequently.

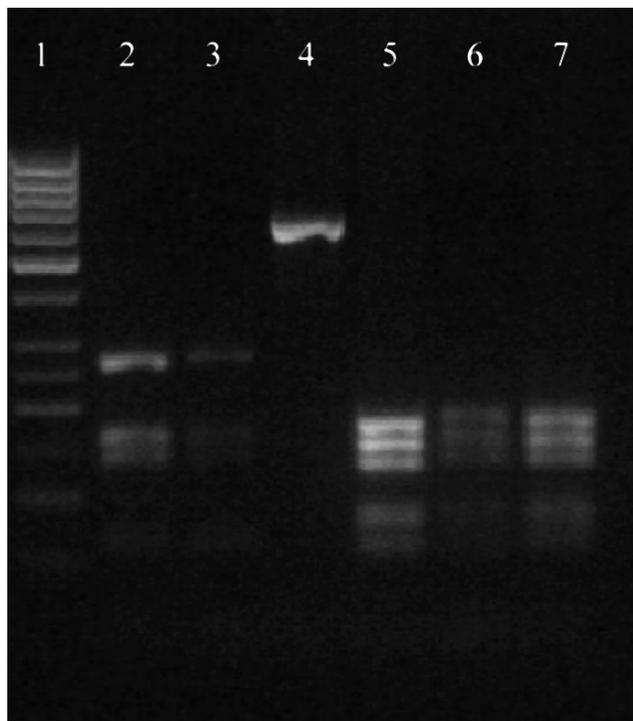
**Pathogenicity assay.** *Penicillium* spp. isolated from different sources behaved differently when assayed for pathogenicity (Table 1). *P. citrinum*, *P. chrysogenum* and *P. olsonii* strains isolated from air and surfaces of different packinghouses generally did not cause mould on the fruits assayed and only four of them were pathogens on apricots, pears and grapes. *P. expansum* strains isolated from decayed fruits were highly pathogenic towards apricots, pears, apples and grapes, whereas *P. digitatum* and *P. italicum* strains were highly pathogenic also towards lemons, oranges and prickly-pears (Table 1). The more aggressive strains of *P. digitatum* and *P. italicum* did not show strict host specificity, usually attacking five kinds of fruit.

**Assessment of fungicide sensitivity.** Fungicide resistance was detected in strains belonging to all species. All *P. citrinum*, *P. chrysogenum* and *P. olsonii* strains showed high resistance to imazalil and benomyl. About 50% of the *P. expansum* strains were resistant to benomyl, whereas all of them were resistant to imazalil. Two *P. italicum* strains and three *P. digitatum* strains were resistant to imazalil and benomyl (Table 1).

**PCR amplification of ribosomal DNA region for RFLP studies.** The PCR products of the 41 strains te-



**Fig. 1.** Restriction patterns of PCR-amplified internal transcribed regions digested with *Hinf*I, after 3% agarose gel electrophoresis. Lanes: 1, 50-bp molecular weight marker (Invitrogen Life Technologies); 2, *P. italicum* MUCL 958; 3, *P. expansum* MUCL 273; 4, *P. digitatum* MUCL 14256; 5, *P. citrinum* MPVCT 204; 6, *P. chrysogenum* MPVCT 201; 7, *P. olsonii* MPVCT 213.



**Fig. 2.** Restriction patterns of PCR-amplified internal transcribed regions digested with *Taq*I after 3% agarose gel electrophoresis. Lanes: 1, 50-bp molecular weight marker (Invitrogen Life Technologies); 2, *P. italicum* MUCL 958; 3, *P. expansum* MUCL 273; 4, *P. digitatum* MUCL 14256; 5, *P. citrinum* MPVCT 204; 6, *P. chrysogenum* MPVCT 201; 7, *P. olsonii* MPVCT 213.

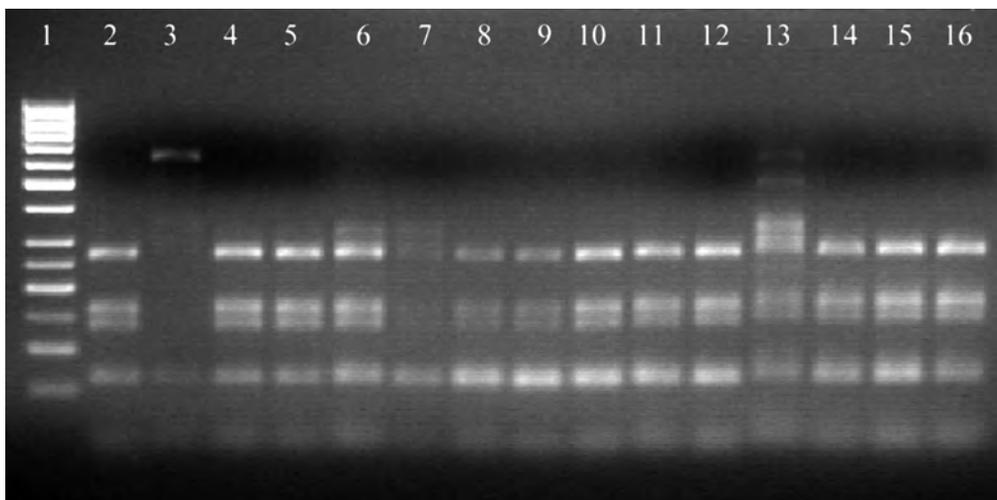
sted, amplified with primers ITS4 and ITS5, had a similar size of about 600 bp. RFLP of the ITS1 - ITS2 regions with *Hinf*I and *Taq*I revealed enough variation to distinguish among four species. With the use of *Hinf*I, *P. italicum* and *P. expansum* could be distinguished from *P. digitatum* / *P. chrysogenum* and *P. citrinum* / *P. olsonii* (Fig. 1). With the use of *Taq*I, *P. italicum* and *P. expansum* could be distinguished from *P. digitatum* and *P. citrinum* and from *P. chrysogenum* / *P. olsonii* (Fig. 2). Homogeneous patterns were revealed for strains of *P. italicum*, *P. digitatum*, *P. olsonii*, *P. chrysogenum* and *P. citrinum* species (data not shown). Two of the 15 *P. expansum* isolates (MPVCT 218 and MPVCT 219) revealed polymorphisms with *Taq*I (Fig. 3), whereas *Hinf*I digestion gave the same pattern for all *P. expansum* strains (Fig. 4).

**Polymerase chain reaction with PEF and PER primers for the polygalacturonase gene of *P. expansum*.** The primers PEF and PER amplified a 404 bp DNA product from all 15 *P. expansum* isolates used (Fig. 5, lanes 2-16). No amplification was observed for other *Penicillium* species (*P. digitatum*, *P. italicum*, *P. citrinum*, *P. chrysogenum*) (Fig. 5, lanes 17-20).

**Fluorescent AFLP analysis.** Analysis allowed identification of 229 fAFLP polymorphic markers in the size range from 60 to 640 bp. Each amplification with the primer pair used produced from 6 to 33 detectable peaks. A “core profile” was recognised, allowing discrimination of species-specific peaks (Fig. 6). Species-specific common fragments of 15 *P. expansum* strains were detected (107, 221 and 491 bp). The six *P. italicum* strains shared common fragments of 112, 145, 222 and 299 bp. The *P. chrysogenum* strains showed 75, 119, 278 and 132 bp common fragments.

The six *P. digitatum* strains showed three specific fragments (146, 264, 343 bp). *P. citrinum* and *P. olsonii* isolates did not show common fragments.

The resulting dendrogram (Fig. 7) shows clustering of isolates belonging to the same species under the same clade, with the exception of *P. expansum* isolates, which clustered in three separated clades. Strains MPVCT 218 and MPVCT 219, showing polymorphism in RFLP with *Taq*I, were included in two different clades (*P. expansum* I and *P. expansum* III). Statistical analysis of genetic diversity within the six species identified was performed using POPGENE v 1.31 software. In Table 2 we report the statistics calculated using data relative to 229 fAFLP polymorphic markers. The primer combinations *Eco*-



**Fig. 3.** Restriction patterns of PCR-amplified internal transcribed regions digested with *TaqI* after 3% agarose gel electrophoresis. Lanes: 1, 50-bp molecular weight marker (Invitrogen Life Technologies); 2-16, *P. expansum* strains: MPVCT 226; MPVCT 218; MUCL 273; MPVCT 221; MPVCT 231; MPVCT 228; MPVCT 232; MPVCT 223; MPVCT 229; MPVCT 225; MPVCT 216; MPVCT 219; MPVCT 227; MPVCT 217; MPVCT 222.

**Table 2.** Descriptive statistics on number ( $N_{Ip}$ ) and proportion ( $\%_{Ip}$ ) of polymorphic loci, observed ( $n_o$ ) and effective ( $n_e$ ) number of alleles per locus, Nei's (1978) genetic diversity (H), Shannon's Information index (I).

Species	$N_{Ip}$	$\%_{Ip}$	$n_o$	H	$n_e$	I
<i>P. italicum</i>	37	16.16	1.161	0.047	1.079	0.073
<i>P. citrinum</i>	66	28.82	1.288	0.084	1.130	0.133
<i>P. chrysogenum</i>	42	18.34	1.183	0.050	1.077	0.081
<i>P. olsonii</i>	50	21.83	1.218	0.058	1.084	0.095
<i>P. expansum</i>	95	41.48	1.414	0.051	1.064	0.097
<i>P. digitatum</i>	39	17.03	1.170	0.040	1.059	0.067
Total	54.8	23.94	1.882	0.069	1.082	0.141
St. Dev.			0.323	0.071	0.099	0.116

Legend:  $n_i$ = number of isolates;  $n_o$ =observed number of alleles per locus ;  $n_e$ = effective number of alleles; H= genetic diversity; I= Shannon's Information index;  $N_{Ip}$ = number of polymorphic loci;  $\%_{Ip}$ = proportion of polymorphic loci.

AT / *Mse*-CAG allowed testing of an average number of polymorphic loci ( $N_{Ip}$ ) of 54.8 with an average proportion ( $\%_{Ip}$ ) of 23.9%. The number of alleles observed per locus ( $n_o$ ) varied from 1.414 (*P. expansum*) to 1.161 (*P. italicum*). Total genetic diversity (H) was 0.069 within the genus *Penicillium*, while it was 0.047 in *P. italicum*, 0.084 in *P. citrinum*, 0.050 in *P. chrysogenum*, 0.058 in *P. olsonii*, 0.051 in *P. expansum* and 0.040 in *P. digitatum* (Table 2). The most heterogeneous species was *P. expansum*, who showed the highest number of polymorphic loci ( $N_{Ip}$ ) (95).

## DISCUSSION

Only a minor fraction of *Penicillium* species cause food spoilage (Pitt, 2000). The six *Penicillium* species

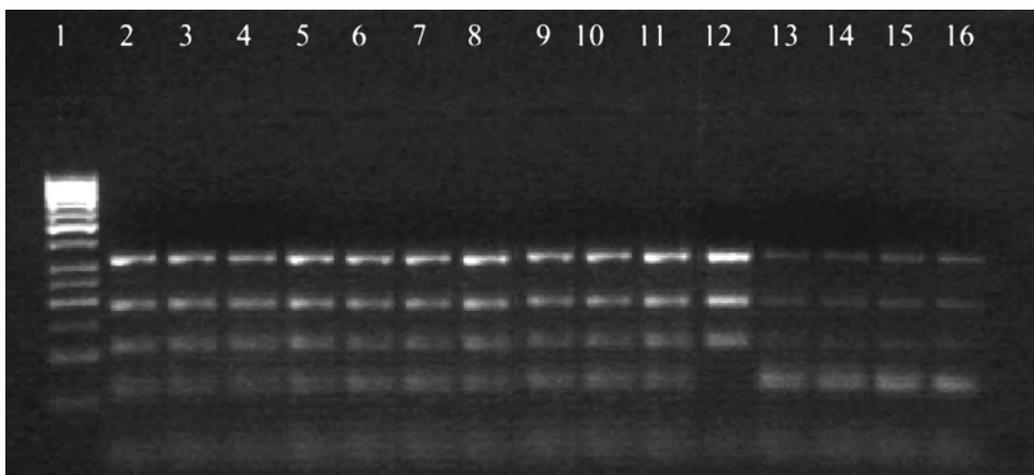
identified and examined in the present study showed different behaviours when assayed for pathogenicity and for tolerance to fungicides, correlated with the source of isolation. *P. italicum*, *P. digitatum* and *P. expansum* strains from decaying fruits were the most aggressive species against all fruits tested. Moreover, most strains of these species were sensitive to all benzimidazole and imidazole concentrations tested. All *P. chrysogenum*, *P. olsonii*, *P. citrinum* strains isolated from air and surfaces, sampled in a strongly selective environment such as packinghouses, showed pathogenicity toward a narrow host range and the highest benzimidazole and imidazole tolerance. As previously reported, *P. citrinum*, *P. chrysogenum*, *P. olsonii* are not frequently associated with mould losses during fruit storage, whereas they have been reported as air and surface contaminants in packinghouses (Scott *et al.*, 2004).

ITS amplification with primers ITS4 and ITS5 allowed molecular identification of the 41 *Penicillium* strains studied. Moreover, PCR using the species-specific primers PER and PEF for *P. expansum* amplified a 404-bp product from all the *P. expansum* isolates tested, thus confirming their identity (Marek *et al.*, 2003). RFLP of the ITS1 - ITS2 region distinguished among four species and revealed a very limited diversity only among *P. expansum* isolates, but not among isolates of other species.

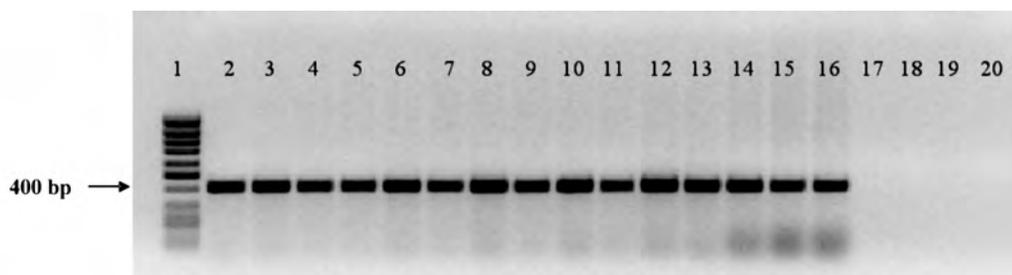
The DNA sequence of the ITS region may vary among species within a genus or even among populations, thus rDNA ITS analysis is frequently used in phylogenetic studies of closely related taxa (Bridge and Arora, 1998). These sequence differences can be exploited to rapidly identify specific fungal taxa. Once amplified, species can be identified through either RFLP or sequencing. The ITS1 and ITS2 regions do contain information that can be used to support taxonomic,

ecological and physiological data for common food-borne *Penicillium* species, but the degree of ITS variability is too low to facilitate separation of all these closely related taxa (Skouboe *et al.*, 1999) and it is not advisable to use these sequences as the only criterion for identification (Boysen *et al.*, 2000).

In this paper, fAFLP analysis was used to assess inter- and intraspecific variability of postharvest pathogens belonging to the genus *Penicillium* isolated from different hosts and environments. Polymorphisms obtained by AFLP fingerprinting are multilocus markers, which allow the individuals to be genotyped and differentiated on the basis of respective alleles (Mueller and Wolfenbarger, 1999; Olive and Bean, 1999). The 229 fAFLP polymorphic markers obtained just with one primer pair allowed genetic characterization of all *Penicillium* strains and showed a high degree of variability among isolates that were correlated with the origin and pathogenicity of strains. Strains belonging to the same species showed si-



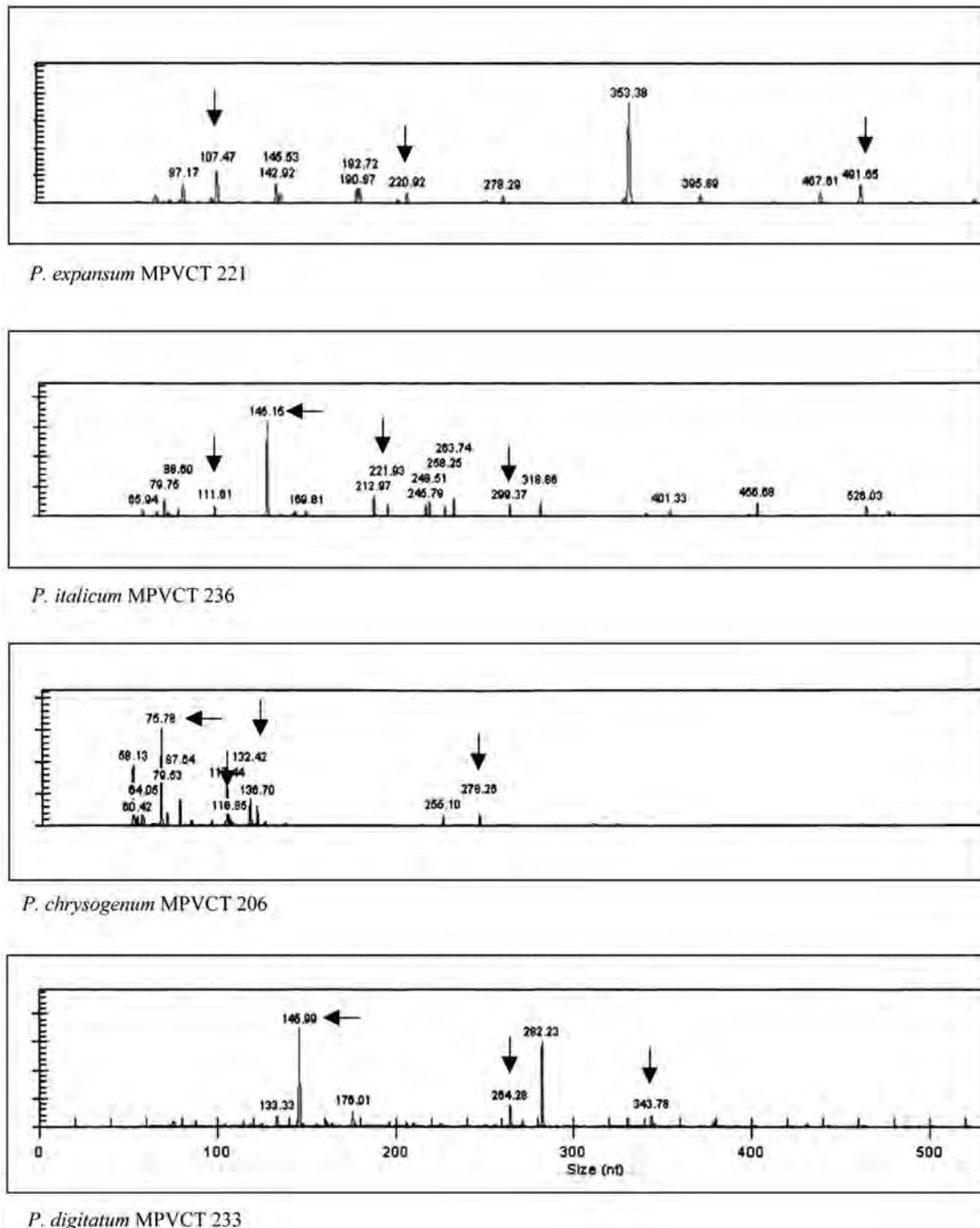
**Fig. 4.** Restriction patterns of PCR-amplified internal transcribed regions digested with *Hinf*I after 3% agarose gel electrophoresis. Lanes: 1, 50-bp molecular weight marker (Invitrogen Life Technologies); 2-16, *P. expansum* strains: MPVCT 226; MPVCT 218; MUCL 273; MPVCT 221; MPVCT 231; MPVCT 228; MPVCT 232; MPVCT 223; MPVCT 229; MPVCT 225; MPVCT 216; MPVCT 219; MPVCT 227; MPVCT 217; MPVCT 222.



**Fig. 5.** Agarose gel electrophoresis of PCR products from *Penicillium* spp. with primers PER and PEF. Lane 1, 50 bp molecular weight marker (Invitrogen Life Technologies); lanes 2- 16, *P. expansum* strains: MPVCT 216; MPVCT 217; MPVCT 218; MPVCT 219; MPVCT 221; MPVCT 222; MPVCT 223; MPVCT 225; MPVCT 226; MPVCT 227; MPVCT 228; MPVCT 229; MPVCT 231; MPVCT 232; MUCL 273; lane 17, *P. digitatum* MUCL 14256; lane 18, *P. italicum* MUCL 958; lane 19, *P. citrinum* MPVCT 200; lane 20, *P. chrysogenum* MPVCT 201.

milar band patterns and common species-specific fragments, and clustered together when numerical analysis was performed. The *P. italicum*, *P. digitatum*, *P. citrinum*, *P. chrysogenum* and *P. olsonii* strains studied clustered in homogeneous groups (Fig. 7). Conversely, the *P. expansum* strains appeared less homogeneous. The percentage of polymorphic loci was minimal (16.16%) in *P. italicum* and 41.48% in *P. expansum*, which showed the highest polymorphism (Table 2).

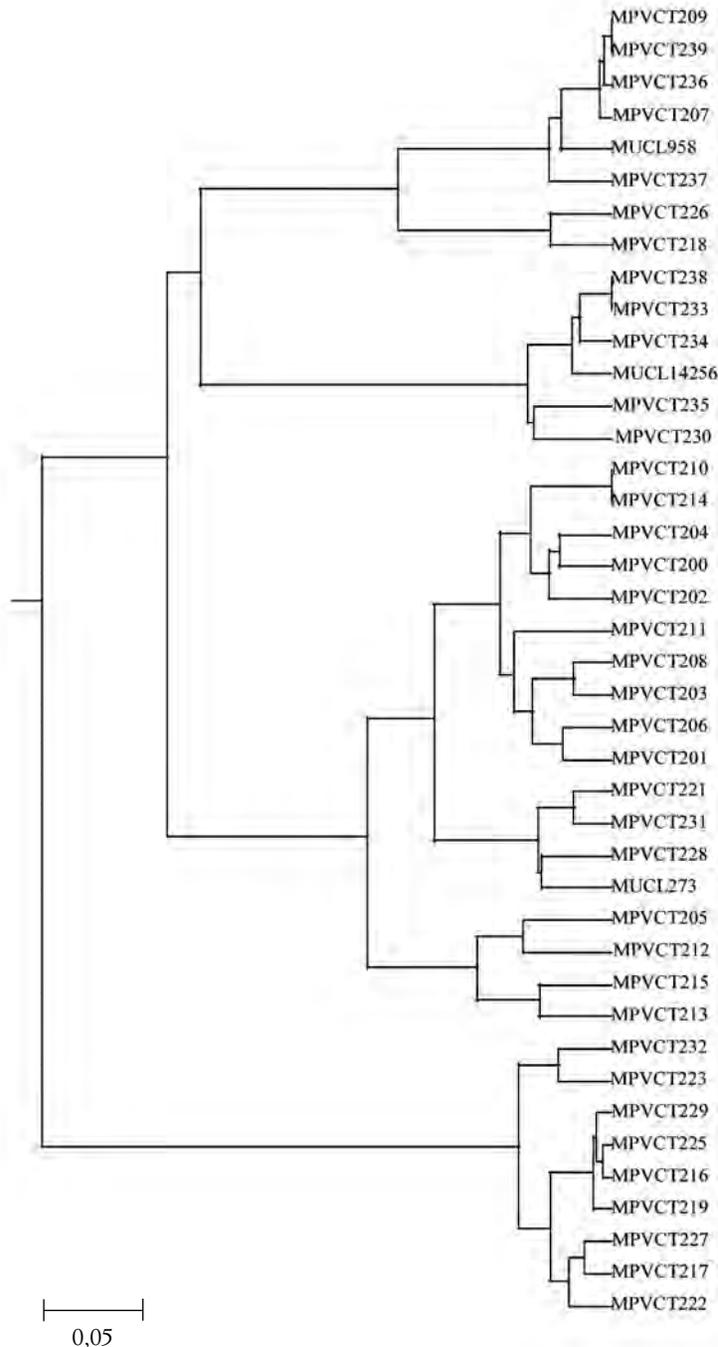
Strains here identified as *P. expansum* by means of traditional and molecular criteria formed three distinct clusters, suggesting molecular heterogeneity in this species. This is consistent with previous studies indicating that the species is morphologically highly variable (Frivad and Filtenborg, 1983). The UPGMA algorithm used in this study to analyse the results is an unweighted method, by which bands strongly discriminative of distinguishing the species are as important as markers oc-



**Fig. 6.** Species-specific fragments in the range of 60-640 bp of electropherograms of *P. italicum*, *P. digitatum*, *P. expansum* and *P. chrysogenum* strains obtained with AFLP analysis. Fragments that appear to be specific are indicated by arrows.

curing occasionally. We point out that these common bands do not necessarily correspond to the same locus and may represent different fragments with identical sizes in different species, thus confusing the UPGMA analysis. The complex AFLP patterns allow a distinction of very closely related strains, but incidental similarities among the fingerprints of distantly related organisms may occur.

High genetic diversity has been detected by AFLP analysis in *P. verrucosum* (Frisvad *et al.*, 2005) and *P. miczynskii* (Tuthill, 2004), revealing a mechanism of genetic recombination still not described. Use of additional primers will increase the power of the technique and the amount of data available. Isolation of DNA fragments linked to interesting characters will be followed by cloning and sequence analysis.



**Fig. 7.** Dendrogram obtained from fAFLP analyses of 41 *Penicillium* isolates. Clusters were constructed using the unweighted pair-group method with average linkages (UPGMA) based on 229 fAFLP polymorphic markers. Similarity between fingerprints was calculated with the Dice coefficient.

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## REFERENCES

- Abd-Elsalam K.A., Schnieder F., Khalil M.S., Asran-Amal A., Verrete A., 2003. Use of AFLP fingerprinting to analyze genetic variation within and between populations of *Fusarium* spp. derived from Egyptian cotton cultivars. *Journal of Plant Pathology* **85**: 99-103.
- Arnold C., Metherell L., Clewley J.P., Stanley J., 1999. Predictive modeling of fluorescent AFLP: a new approach to the molecular epidemiology of *E. coli*. *Research in Microbiology* **150**: 33-44.
- Bakkeren G., Kronstad J.W., Levesque A.C., 2000. Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in Ustilaginomycetes. *Mycologia* **92**: 510-521.
- Boysen M.E., Jacobsson K.G., Schnürer J., 2000. Molecular identification of species from the *Penicillium roqueforti* group associated with spoiled animal feed. *Applied and Environmental Microbiology* **66**: 1523-1526.
- Bridge P.D., Arora D.K., 1998. Interpretation of PCR methods for species definition. In: Bridge P.D., Arora D.K., Reddy C.A., and Elander R.P. (eds). *Application of PCR in Mycology*, pp. 62-84. CAB International, N.Y., USA.
- Carmichael J.W., 1955. Lacto-fuchsin: a new medium for mounting fungi. *Mycologia* **47**: 611.
- De Curtis F., Caputo L., Castoria R., Lima G., Stea G., De Cicco V., 2004. Use of fluorescent amplified fragment length polymorphism (fAFLP) to identify specific molecular markers for the biocontrol agent *Aureobasidium pullulans* strain LS30. *Postharvest Biology and Biotechnology* **34**: 179-186.
- Diaz S., Pire C., Ferrer J., Bonete M.J., 2003. Identification of *Phoenix dactylifera* L. varieties based on amplified fragment length polymorphism. *Cellular and Molecular Biology Letters* **8**: 891-899.
- Dresler-Nurmi A., Terefework Z., Kajjalainen S., Lindstro M.K., Hatakka A., 2000. Silver stained polyacrylamide gels and fluorescence-based automated capillary electrophoresis for detection of amplified fragment polymorphism patterns obtained from white-rot fungi in the genus *Trametes*. *Journal of Microbiological Methods* **41**: 161-172.
- Dupont J., Magnin S., Marti A., Brousse M., 1999. Molecular tools for identification of *Penicillium* starter cultures used in food industry. *International Journal of Food Microbiology* **49**: 109-118.
- Felsenstein J., 2004. PHYLIP (Phylogeny Inference Package) version 3.6. Department of Genome Sciences, University of Washington, Seattle, USA.
- Frisvad J.C., 1981. Physiological criteria and mycotoxin production as aids in identification of common asymmetric penicillia. *Applied of Environmental Microbiology* **41**: 568-579.
- Frisvad J.C., Filtenborg O., 1983. Classification of terverticillate *Penicillia* based on profiles of mycotoxins and other secondary metabolites. *Applied and Environmental Microbiology* **46**: 1301-1310.
- Frisvad J.C., Lund F., Elmholt S., 2005. Ochratoxin A producing *Penicillium verrucosum* isolates from cereals reveal large AFLP fingerprinting variability. *Journal and Applied Microbiology* **98**: 684-692.
- Geisen R., Cantor M.D., Hansen T.K., Holzapfel W.H., Jakobsen M., 2001. Characterization of *Penicillium roqueforti* strains used as cheese starter cultures by RAPD typing. *International Journal of Food Microbiology* **65**: 183-191.
- Huang J., Sun M., 1999. A modified AFLP with fluorescence-labelled primers and automated DNA. *Biotechnology Techniques* **13**: 277-278.
- Kure C.F., Skaar I., Holst-Jensen A., Abeln E.C.A., 2003. The use of AFLP to relate cheese-contaminating *Penicillium* strains to specific points in the production plants. *International Journal of Food Microbiology* **83**: 195-204.
- Lin J.J., Kuo J., Ma J., 1996. A PCR-based DNA fingerprinting technique AFLP for molecular typing of bacteria. *Nucleic Acids Research* **24**: 3649-3650.
- Lund F., Nielsen A.B., Skouboe P., 2003. Distribution of *Penicillium commune* isolates in cheese dairies mapped using secondary metabolite profiles, morphotypes, RAPD and AFLP fingerprinting. *Food Microbiology* **20**: 725-734.
- Majer D., Mithen R., Lewis B.G., Vos P., Oliver R.P., 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research* **100**: 1107-1111.
- Marek P., Annamalai T., Venkitanarayanan K., 2003. Detection of *Penicillium expansum* by polymerase chain reaction. *International Journal of Food Microbiology* **89**: 139-144.
- Mueller U.G., Wolfenbarger L., 1999. AFLP genotyping and fingerprinting. *Tree* **14**: 389-394.
- Nei M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**: 583-590.
- Olive D.M., Bean P., 1999. Principles and applications of methods for DNA-based typing of microbial organisms. *Journal of Clinical Microbiology* **37**: 1661-1669.
- Oliveri C., Campisano A., Cirvilleri G., Catara A., 2004. Fingerprinting analysis of pathogenic and non pathogenic postharvest *Penicillium* strains. *Journal of Plant Pathology* **86**: 300.
- Pedersen L.H., Skouboe P., Boysen M., Soule J., Rossen L., 1997. Detection of *Penicillium* in complex food samples using the polymerase chain reaction. *International Journal of Food Microbiology* **35**: 169-177.
- Peterson S.W., 2000. Phylogenetic analysis of *Penicillium* species based on ITS and LSU-rDNA nucleotide sequences. In: *Integration of Modern Taxonomic Methods for Penicillium and Aspergillus classification*. Samson R.A. and Pitt J.I. pp. 163-178. Harwood Academic Publishers, U.K.

- Pianzola M. J., Moscatelli M., Vero S., 2004. Characterization of *Penicillium* isolates associated with blue mold on apple in Uruguay. *Plant Disease* **88**: 23-28.
- Pitt J.I., 2000. A laboratory guide to common *Penicillium* species. CSIRO Division of Food Processing, North Ryde, New South Wales, Australia.
- Pitt J.I., Hocking, A.D., 1997. Fungi and food spoilage. 2nd Ed. Blackie Academic and Professional, London, UK.
- Ripabelli G., McLauchlin J., Mithani V., Threfall E.J., 2000. Epidemiological typing of *Bacillus cereus* by amplified fragment length polymorphism. *Letters in Applied Microbiology* **30**: 358-363.
- Rosendhal S., Taylor J.W., 1997. Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP. *Molecular Ecology* **6**: 821-829.
- Savelkoul P.H.M., Aarts H.J.M., Haas J., Dijkshoorn L., Duim B., Otsen M., Rademaker J.L.W., Schouls L., Lestra J.A., 1999. Amplified fragment length polymorphism analysis: the state of art. *Journal of Clinical Microbiology* **37**: 3083-3091.
- Schmidt H., Ehmann M., Vogel R.R., Aniwaki M.H., Niessen I., 2003. Molecular typing of *Aspergillus ochraceus* and construction of species specific SCAR primers based on AFLP. *Systematic and Applied Microbiology* **26**: 138-146.
- Scott J., Untereiner W.A., Wong B., Straus A., Malloch D., 2004. Genotypic variation in *Penicillium chrysogenum* from indoor environments. *Mycologia* **96**: 1095-1105.
- Scuderi G., Bonaccorsi A., Scortichini M., Cirvilleri G., 2005. Preliminary assessment of *Pseudomonas syringae* isolates using fluorescent amplified fragment length polymorphism. *Journal of Plant Pathology* **87**: 283.
- Skouboe P., Frisvad J.C., Taylor J.W., Lauritsen D., Boysen M., Rossen L., 1999. Phylogenetic analysis of nucleotide sequences from the ITS region of terverticillate *Penicillium* species. *Mycological Research* **103**: 873-881.
- Sneath P.H.A., Sokal R.R., 1973. Numerical Taxonomy. Freeman W.H. (ed.), San Francisco, USA.
- Terefework Z., Kaijalainen S., Lindstro M.K., 2001. AFLP fingerprinting as a tool to study the genetic diversity of *Rhizobium galegae* isolated from *Galega orientalis* and *Galega officinalis*. *Journal of Biotechnology* **91**: 169-180.
- Tuthill D.E., 2004. Genetic variation and recombination in *Penicillium miczynskii* and *Eupenicillium* species. *Mycological Research* **103**: 1593-1603.
- Vos P., Hogers R., Bleeker M., Reijans M., Vander Lee T., Miranda H., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M., 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407-4414.
- Wenz H.M., Robertson J.M., Menchen S., Oaks F., Demorest D.M., Scheibler D., Rosenblum B.B., Wike C., Gilbert D.A., Efcavith J.W., 1998. High-precision genotyping by denaturing capillary electrophoresis. *Genomic Research* **8**: 69-80.
- White T.J., Bruns T., Lee S., Taylor J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Sninsky J.J. and White T.J. (eds.). PCR Protocols: A Guide to Methods and Applications, pp. 315-322. Academic Press, London, U.K.
- Zewdu T., Seppo K., Kristina L., 2001. AFLP fingerprinting as a tool to study the genetic diversity of *Rhizobium galegae* isolated from *Galega orientalis* and *Galega officinalis*. *Journal of Biotechnology* **91**: 169-180.

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