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Molecular tools for identification of *Penicillium* starter cultures used in the food industry

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Abstract

The main goal of this work was to develop rapid and accurate molecular tools to discriminate species of white industrial *Penicillia*. We applied three different polymerase chain reaction (PCR) based techniques. Sequences of the ITS region of the rRNA gene unit and of the 5′ end of the β tubulin gene yielded 1.2% and 5.8% nucleotide variability respectively, between *Penicillium camembertii* and *Penicillium nalgiovense*. Polymorphic restriction sites were found in both sequences. These may be used in diagnostic PCR–RFLP analysis to rapidly distinguish between the two *Penicillium* species. Random amplified polymorphic DNA (RAPD) markers were also useful to differentiate these two species, but no polymorphism was found at the subspecific level, which evidenced a high level of homogeneity of the isolates studied. By means of these three techniques, the real identity of industrial strains of *Penicillium chrysogenum* and *P. nalgiovense* could be demonstrated. The comparison of these isolates with type strains of the two species suggested that the former corresponds to *P. nalgiovense*. The genetic relatedness between *P. naglovense* and *Penicillium dipodomyis* was also confirmed. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Penicillium* starters; Species identification; ITS; β Tubulin; PCR–RFLP; RAPD

1. Introduction

Penicillium camembertii Thom, *Penicillium nalgiovense* Laxa and white sporulating mutants of *Penicillium chrysogenum* Thom, are currently used as starter cultures in the food industry. The domestic habitats of these fungi, which are cheeses and fermented meats, have imposed selection pressure towards reduced pigmentation and sporulation (Pitt et al., 1986). This loss of the original distinctive colour of the conidia, coupled with occasionally altered conidiogenous structures, means these strains are difficult to distinguish by nonspecialists. The additional growth data regularly used for identification (Pitt, 1979) also cannot be used in these cases.

Molecular tools based on the polymerase chain

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reaction (PCR) method are now frequently applied in food mycology for diagnostic purposes and to accurately characterise the fungi employed (White et al., 1990). In the genus *Penicillium* the noncoding ITS region of the rDNA unit has been particularly investigated to clarify the subdivisions within the genus (Lobuglio et al., 1993; Peterson, 1993; Lobuglio et al., 1994; Lobuglio and Taylor, 1995), and to investigate the genetic structure of some species. For example, the blue-veined cheese fungus *Penicillium roquefortii* was reclassified into three species on the basis of ITS sequence polymorphisms (Boysen et al., 1996) and specific primers were developed to identify *P. roquefortii* and *Penicillium carneum* (Pedersen et al., 1997). Genes coding for metabolic and structural functions have also been explored as tools for rapid differentiation of some fungal species. Among these, the β tubulin gene was found to be highly polymorphic (Glass and Donaldson, 1995), and was more discriminating than ITS when examining varieties of the thermoresistant food spoilage mold *Neosartorya fischeri* (Chrzavzez, 1995).

The RAPD technique (Williams et al., 1990), is very convenient for subspecific analysis (Bruns et al., 1991). The high degree of polymorphism of RAPD markers has been exploited to differentiate numerous fungal plant pathogens at the population level [see for example Huff et al. (1994), Peever and Milgroom (1994), Fabre et al. (1995), Lees et al. (1995), Nelson et al. (1997), Wang (1997)]. Within the genus *Penicillium*, RAPD has been applied to the nodule forming *Penicillium nodositatum* (Sequerra et al., 1997), and among industrial food starter cultures, RAPD markers have demonstrated the homogeneity of the species *P. nalgiovense*, and have clearly distinguished *P. nalgiovense* from *P. chrysogenum* (Geisen, 1995), suggested to be its ancestral form (Stolk et al., 1990).

We therefore initiated the current study to find molecular markers to differentiate industrial white isolates of *Penicillium* and to clarify their relationships with type isolates of ancestral and domesticated species. ITS and partial β tubulin gene sequences have been used to establish the molecular identification of the white industrial *Penicillia*, *P. camembertii*, *P. nalgiovense* and *P. chrysogenum*. Sequencing and restriction analysis were performed using an isolate of each species. In addition, RAPD amplifications were done on eight isolates of *P. camembertii*, four of *P. chrysogenum* and one of *P. nalgiovense* which were compared with type strains of the three species. *Penicillium dipodomyis*, recently considered as the more closely related species of *P. nalgiovense* (Banke et al., 1997) was also included.

2. Materials and methods

2.1. Fungal isolates

Fungal isolates of *P. camembertii*, *P. chrysogenum*, *P. dipodomyis* and *P. nalgiovense* used in this study are listed in Table 1. The starter cultures were obtained from industry and are now included in the LCP culture collection (MNHN, Laboratoire de Cryptogamie, Paris, France).

2.2. Isolation of fungal DNA

Fresh mycelia were harvested from the surface of Czapek yeast extract agar plates, which had been grown for 2 days at 25°C. Fungal DNA was extracted following the method of Lee and Taylor (1990). It was necessary to dilute the DNA extracts 100-fold in double-distilled water to achieve positive PCR amplifications.

2.3. DNA amplification prior to sequencing

Primers used in PCR amplifications were ITS4 and ITS5, which amplify across the ITS1-5.8S-ITS2 rDNA region (White et al., 1990), and Bt2a and Bt2b which amplify the 5′ end of the β tubulin gene (Glass and Donaldson, 1995). The PCR reactions were done in a final volume of 25 μl containing: 12.5 μl of diluted genomic DNA, 0.625 unit of Taq DNA polymerase (Appligène Oncor, Illkirch, France), 2.5 μl of 10 X Taq DNA Polymerase buffer, 1.5 μl of glycerol, 1.0 μl of 5 mM dNTPs (Eurogentec, Seraing, Belgium) and 1 μl of each 10 μM primer. The amplifications were performed on a Perkin Elmer Cetus thermal cycler model 2400 using an initial denaturation of 94°C for 2 min followed by 30 cycles of 94°C for 10 s, 50°C (ITS4/ITS5) or 58°C (Bt2a/Bt2b) for 10 s and 72°C for 20 s. A final extension step of 5 min at 72°C was included.

Table 1
Penicillium isolates used in this study

Species	Strain no	Habitat and origin
P. camembertii	CBS 299.48[a]	Camembert cheese, France
	Ca1	Starter culture, France
	Ca2	Starter culture, France
	Ca3	Starter culture, France
	Ca4	Starter culture, France
	Ca5	Starter culture, France
	Ca6	Starter culture, France
	Ca7	Starter culture, France
	Ca8	Starter culture, France
P. chrysogenum	MUCL 29145[a]	Cheese, USA
	MUCL 31327[a]	Rotting branch, Norway
	Ch1	Starter culture, France
	Ch2	Starter culture, France
	Ch3	Starter culture, France
	Ch4	Starter culture, France
P. dipodomyis	IBT 12701	Cheek pouch of living *Dipodomys*
	IBT 3353	Cheek pouch of living *Dipodomys*
P. nalgiovense	MUCL 31194[a]	Cheese, Czechoslovakia
	Na1	Starter culture, France

[a] Type strains.

2.4. DNA sequencing

Sequences were obtained on both DNA strands using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) with the amplification primers ITS4, ITS5, Bt2b, and a specially designed sequencing primer, Bt2abis (5′CGTTGGGTATCAATTGAC3′). Sequencing assays were analysed on an ABI PRISM 377 automated DNA Sequencer (Applied BioSystems), and sequences were aligned manually using the MUST computer package (Philippe, 1993).

2.5. Restriction enzyme digestions

Full restriction maps of the ITS and β tubulin sequences were defined using the DNA Strider program. Three of the most common enzymes generating discriminant profiles between the species were selected to design partial restriction maps and single digests of 15 μl of amplified products were performed with the following enzymes: 2 units of *Ava*I (Eurogentec) for ITS, 1.5 units of *Mbo*I (Appligène Oncor) and 1.2 units of *Msp*I (Eurogentec) for β tubulin. The restriction fragments were separated on 2% agarose gel, stained with ethidium bromide (10 μg/μl) and photographed. The molecular size marker was the Superladder-low 1000 bp Ladder (Eurogentec).

2.6. RAPD protocol

The RAPD protocol described by Williams et al. (1990) was modified as follows: PCR reactions included 1.5 unit of Taq polymerase (Appligène Oncor); 2.5 μl of 10 X Taq DNA Polymerase buffer [10 mM Tris HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_{2,}$ 0.1% (w/v) Triton X 100, 0.2 mg/ml bovine serum albumin, 2.0 μl of 12.5 mM $MgCl_2$ (added to give a final concentration of 2.5 mM $MgCl_2$)]; 2.0 μl of 1.25 mM dNTPs (Eurogentec); 1 μl of 10 μM primer and 2 μl of diluted genomic DNAs (approximatively 20 ng DNA). Random primers F01, F02, F03, F04, F05, F11, F19, G01, G02, G03, G04, G18, I01, I02, I06, I08, J01, J05, J06, J09 and J20 from Operon (Alameda, CA, USA) were used.

PCR was done in a Perkin Elmer Cetus 2400 thermal cycler using an initial denaturation of 94°C

for 4 min, followed by 40 cycles of 10 s at 94°C, 10 s at 35°C, 20 s at 72°C and a final extension of 72°C for 4 min. Eight μl of PCR product was electrophoresed in a 2 or 2.5% (w/v) agarose gel for 30 min at 100 V. Gels were stained with ethidium bromide (10 μg/μl) and RAPD profiles were visualized by UV transillumination. RAPD analysis were repeated three times per isolate.

2.7. Southern hybridization

The RAPD products generated from DNA of the type strain of *P. nalgiovense* with primer G02 were used to probe Southern blots of RAPD gels. Ten μl of the RAPD products were purified using the Wizard PCR Preps DNA Purification System (Promega) and labelled with the DIG DNA Labelling and

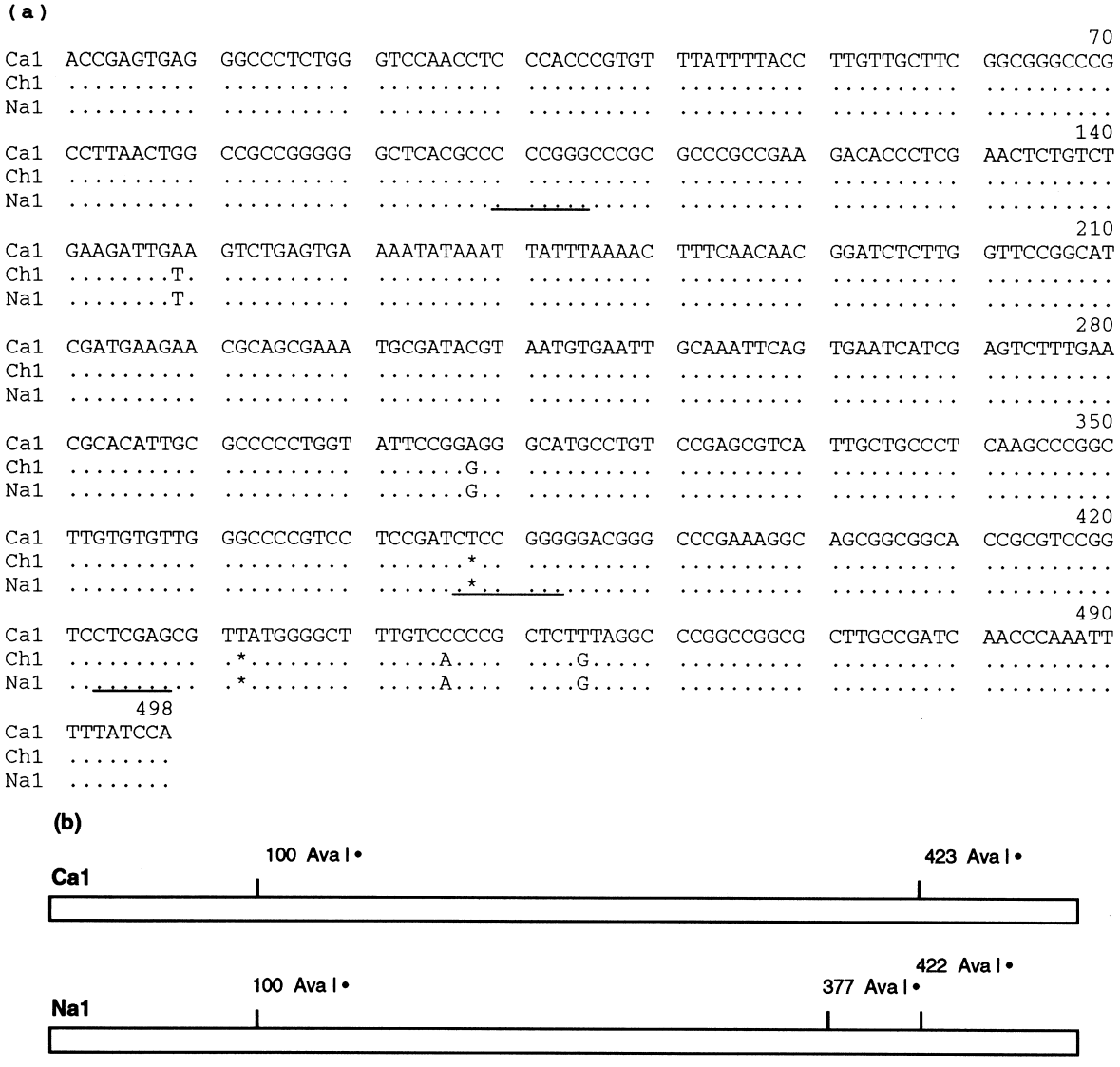

Fig. 1. (a) DNA sequence alignment of the ITS1-5.8S-ITS2 regions from *P. camembertii* (Ca1), *P. chrysogenum* (Ch1) and *P. nalgiovense* (Na1). Dots represent nucleotides identical to those in the top sequence and asterisks indicate gaps. The 5.8S region extends from position 178 to position 332. The underlined positions represent restriction endonuclease sites of *Ava*I (c/ycgrg). (b) Physical restriction maps for Ca1 and Na1, using *Ava*I.

Detection Kit (Boehringer Mannheim, Meylan, France) according to the manufacturer's protocol. After hybridization of the probe and binding of the antibody against digoxigenin, the blot was washed (2 × SSC, 0.1% sodium dodecyl sulfate at 68°C) and the signals were visualized by colourimetric alkaline phosphatase reaction.

3. Results

The analysis of the nucleotide variability within the ITS regions of the rRNA gene and the 5′ end of the β tubulin gene was performed on *P. camembertii* Ca1, *P. chrysogenum* Ch1 and *P. nalgiovense* Na1.

3.1. Analysis of the nucleotide variability within ITS sequences and restriction data analysis

PCR amplifications using the primers ITS4 and ITS5 resulted in products of approximately 600 base pairs (bp) for each of the three DNAs. The sequences (498 bp) were aligned unambiguously and showed a high level of homology (Fig. 1a).

The sequences from *P. chrysogenum* Ch1 and *P. nalgiovense* Na1 were completely identical, and differed from *P. camembertii* Ca1 by six base changes (1.2%), with five of them located within ITS2. The similarity of *P. chrysogenum* and *P. nalgiovense* was surprising and we questioned whether the two species were really so closely related phylogenetically, or whether there had been a strain misidentification. Comparison of the ITS sequence of *P. chrysogenum* strain NRRL 807 from GenBank (AF033465) with Ch1 revealed two differences (T at position 94 and A at position 346). Type strains of these two species were included in the RAPD analysis to determine the real identity of Ch1 and Na1.

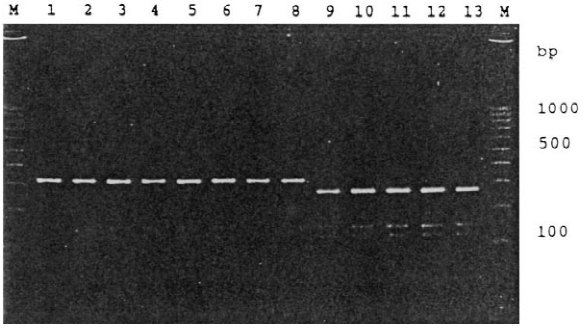

Fig. 2. RFLP patterns of ITS1-5.8S-ITS2 PCR-amplified region of industrial strains of *P. camembertii*, *P. chrysogenum* and *P. nalgiovense* using restriction endonuclease *Ava*I. Lane 1 to 8: *P. camembertii* Ca1, Ca2, Ca3, Ca4, Ca5, Ca6, Ca7, Ca8; lane 9 to 12: *P. chrysogenum* Ch1, Ch2, Ch3, Ch4; lane 13: *P. nalgiovense* Na1; M: size marker.

Physical restriction maps of *P. camembertii* Ca1 and P. *nalgiovense* Na1 were determined for *Ava*I (Fig. 1b). Na1 differed from Ca1 by gaining one restriction site on the 3′ end of the sequence (at position 377). However, the 45 bp extra band generated was too small to be visualised in the electrophoresis conditions used, and the RFLP patterns differed only by a longer fragment for *P. camembertii* (323 bp) than for the other strains (277 bp) (Fig. 2).

3.2. Analysis of the nucleotide variability in the 5′ end of the β tubulin gene and restriction data analysis

The results for the β tubulin sequences were concordant with those obtained for the ITS region, but a higher variability was seen. Ch1 and Na1 sequences were again identical, and differed from Ca1 at 24 positions among the 410 bp sequenced (5.8%) (Fig. 3a). The variability was located within introns (determined with reference to the structure of *Neurospora crassa* gene, Orbach et al., 1986), as was also noted by Glass and Donaldson (1995). Bt2abis was designed for reverse sequencing, as good sequence could not be obtained using primer Bt2a due to an upstream stretch of polyT.

Physical restriction maps of Ca1 and Na1 were determined for *Mbo*I and *Msp*I (Fig. 3b). Digestion of the PCR fragments (around 480 bp) produced discriminant patterns between *P. camembertii* and the others strains (Fig. 4), but showed fewer bands than expected for the reason explained above (small size bands not visualised) (Fig. 4b).

3.3. Comparison of RAPD patterns from industrial strains with those from type strains

Of the 21 primers tested, G02 (5′GGCACTGAGG3′), G03 (5′GAGCCCTCCA3′), G18 (5′GGCTCATGTG3′), I02 (5′GGAGGAGA-

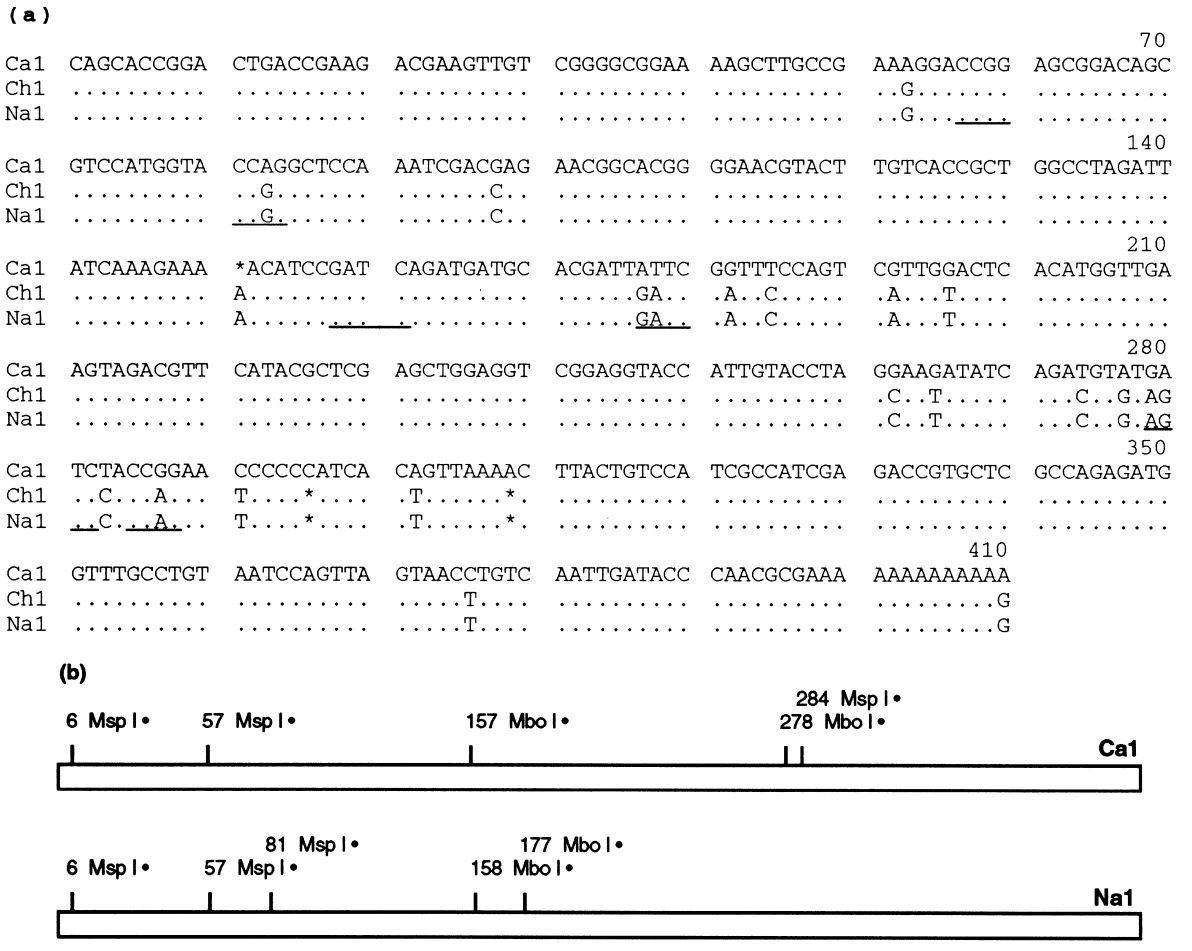

Fig. 3. (a) DNA sequences alignment of the 5′ end of the β tubulin gene from *P. camembertii* (Ca1), *P. chrysogenum* (Ch1) and *P. nalgiovense* (Na1). Dots represent nucleotides identical to those in the top sequence and asterisks indicate gaps. The underlined positions represent restriction endonuclease sites of *Mbo*I (/gatc) and *Msp*I (c/cgg). (b) Physical restriction maps for Ca1 and Na1, using *Mbo*I and *Msp*I.

GG3′) and I06 (5′AAGGCGGCAG3′) generated reproducible patterns containing four to six major prominent bands, allowing a straightforward comparison. Examples of the RAPD patterns generated by primers I06 and G02 are presented in Fig. 5(a and b).

Two different and very homogeneous patterns were revealed among the 13 industrial isolates with all of the primers used. One corresponded to *P. camembertii* (Ca1 to Ca8), authenticated with reference to the type strain CBS 299.48 (lane 9). The other was common to the four *P. chrysogenum* isolates (Ch1 to Ch4) and to *P. nalgiovense* Na1. To check the real identity of these five latter strains, we compared them with type strains of *P. chrysogenum* MUCL 29145, *Penicillium notatum* Westling MUCL 31327 (considered as a synonym of *P. chrysogenum*) and *P. nalgiovense* MUCL 31194, using the primer G02. The RAPD patterns of the five industrial strains were always identical to the pattern of the type strain of *P. nalgiovense* (Fig. 6a).

In order to check the homology of the sequences of the fragments observed by RAPD, the products generated with primer G02 from DNA of the type

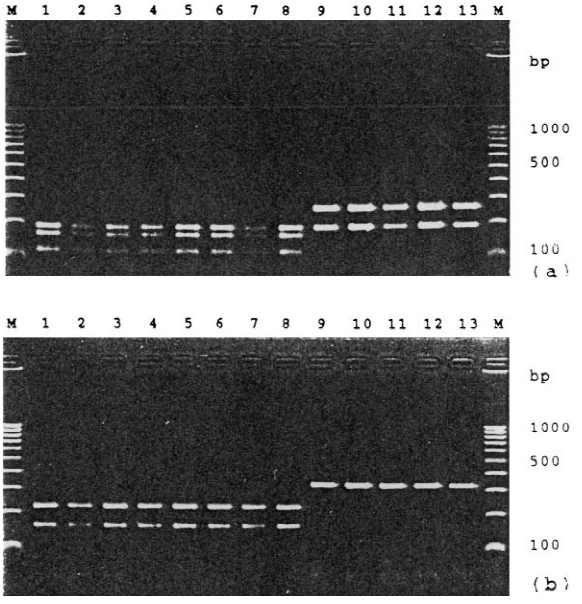

Fig. 4. RFLP patterns of the 5′ end of the β tubulin PCR-amplified region of industrial strains of *P. camembertii*, *P. chrysogenum* and *P. nalgiovense* using restriction endonucleases *Mbo*I (a) and *Msp*I (b). Lane 1 to 8: *P. camembertii* Ca1, Ca2, Ca3, Ca4, Ca5, Ca6, Ca7, Ca8; lane 9 to 12: *P. chrysogenum* Ch1, Ch2, Ch3, Ch4; lane 13: *P. nalgiovense* Na1; M: size marker.

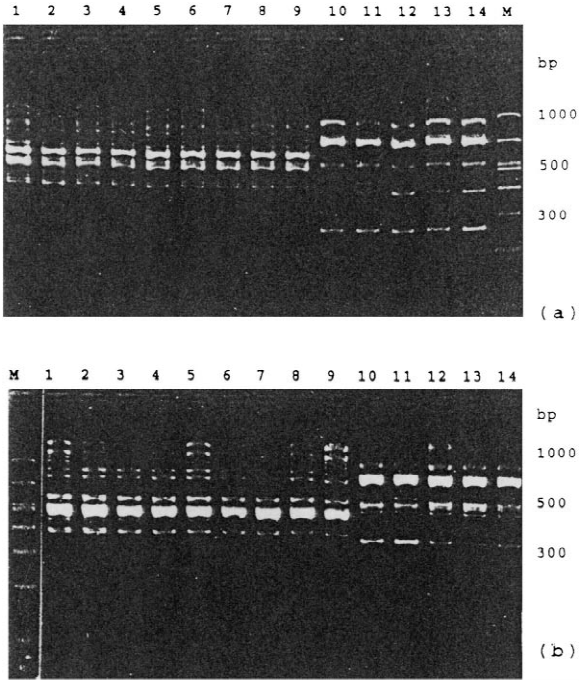

Fig. 5. RAPD patterns of industrial strains of *P. camembertii*, *P. chrysogenum* and *P. nalgiovense* generated with primer I06 (a) and G02 (b). Lane 1 to 9: *P. camembertii* Ca1, Ca2, Ca3, Ca4, Ca5, Ca6, Ca7, Ca8, CBS 299.48; lane 10 to 13: *P. chrysogenum* Ch1, Ch2, Ch3, Ch4; lane 14: *P. nalgiovense* Na1; M: size marker 1 kb.

strain of *P. nalgiovense* were labelled with digoxigenin and used as a hybridization probe. The RAPD products from the industrial and type strains of *P. chrysogenum* and *P. nalgiovense*, and from two strains of *P. dipodomyis* [thought to be the ancestral species for *P. nalgiovense* by Banke et al. (1997)], were included on the Southern blot. The result is shown in Fig. 6b. A strong positive signal was observed between the *P. nalgiovense* probe and the industrial strains of *P. chrysogenum* (lanes 1 to 4) and *P. nalgiovense* (lane 9). Each major band hybridized with the probe demonstrating their sequence homology. In contrast, only one band (0.5 kilobase [kb]) in the RAPD patterns from the type strains of *P. chrysogenum* (lanes 5 and 6) hybridized with the *P. nalgiovense* probe, showing a very weak homology between these two species. Stronger hybridization was obtained with the patterns of the two strains of *P. dipodomyis* (IBT 12701 and IBT 3353, lanes 7 and 8). Although some differences were observed between the patterns from the two isolates of *P. dipodomyis*, several bands hybridized strongly with the probe, showing high levels of homology between some regions of the genomes of both species.

4. Discussion

These results indicated that white industrial *Penicillia* can be differentiated by PCR–RFLP and RAPD techniques, and identified with reference to type strain material of these species.

Distinct genotypes have been evidenced for the species *P. camembertii* and *P. nalgiovense*. However, the relationships between industrial *P. chrysogenum* and *P. nalgiovense* could not be clarified, as the *P. chrysogenum* isolates analysed in this study were found to belong to the species *P. nalgiovense*. This result explained the great phenotypical resemblance observed between these five isolates, all showing

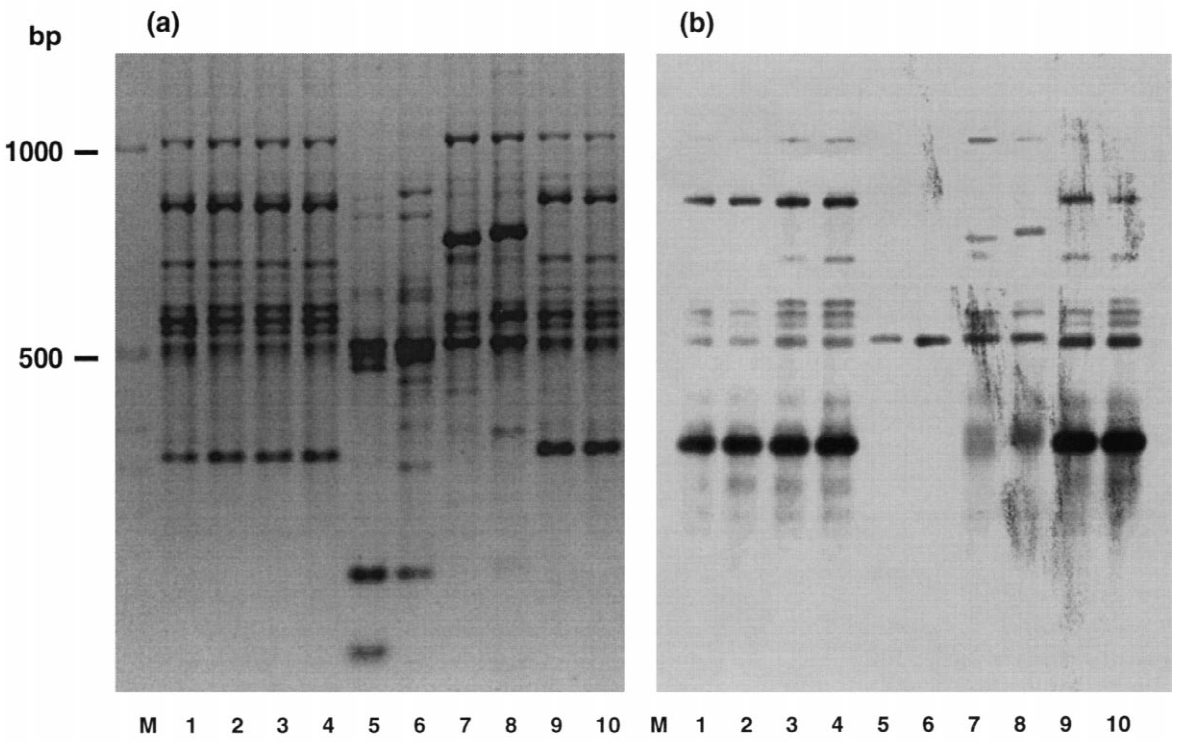

Fig. 6. (a) Comparison of RAPD patterns of industrial strains of *P. chrysogenum* and *P. nalgiovense* with type strains of the same species and with *P. dipodomyis*, suggested as a possible ancestor for *P. nalgiovense*, generated with primer G02. (b) Southern blot of RAPD–PCR products of *P. chrysogenum*, *P. dipodomyis* and *P. nalgiovense* generated with primer G02, probed with the RAPD–PCR products of the type strain of *P. nalgiovense* (MUCL 31194) generated with the same primer (G02). Lanes 1 to 4: *P. chrysogenum* industrial strains: lanes 5 and 6: *P. chrysogenum* type strains (MUCL 29145 and MUCL 31327); lanes 7 and 8: *P. dipodomyis* (IBT 12701 and IBT 3353); lanes 9: *P. nalgiovense* industrial strain; lane 10: *P. nalgiovense* type strain (MUCL 31194), used as hybridization probe; M: size marker.

particularly the typical orange–brown reverse of the colony of *P. nalgiovense* grown on Czapek yeast agar, the distinctive features between the two species, i.e., the penicillus type and the colour of the conidia (Pitt, 1979), being altered by the domesticated conditions.

No subspecies polymorphism was observed in any of the species analysed, indicating a very high level of relatedness between the industrial strains of each species, as was previously shown for *P. nalgiovense* (Geisen, 1995). The genealogy of these natural *P. camembertii* and *P. nalgiovense* variants could explain their genomic uniformity with regards to RAPD and PCR–RFLP results. The different morphological or physiological characteristics for which each strain has been selected could result from point mutations that are too minor to affect the patterns generated by random amplification. Inversely, an experiment on corn demonstrated that apparent variations in RAPD profiles may not be caused by point mutations in the recognition sites but rather by competition between PCR products and primers (Heun and Helentjaris, 1993). Moreover, *P. camembertii* and *P. nalgiovense* are strictly asexual species and it has been shown that genetic diversity is low in asexual populations compared with sexual ones (Wang, 1997).

A high level of DNA homology was found between *P. nalgiovense* and *P. dipodomyis*. Conversely, *P. chrysogenum* which was originally thought to be the ancestral form of *P. nalgiovense* (Stolk et al., 1990; Andersen, 1995; Geisen, 1995; Pitt, 1995) presented a lower homology. This result confirms the hypothesis that *P. nalgiovense* could be a subspecific variant, or perhaps a clonal lineage, of *P. dipodomyis* (Banke et al., 1997). *P. dipodomyis*, previously considered as a variety of *P. chrysogenum* (Frisvad et al., 1987) was raised to the species level,

based on isozyme analysis (Banke et al., 1997), in agreement with chemotypes demonstrated by Svendsen and Frisvad (1994).

In summary, PCR–RFLP and RAPD techniques provide an effective means to differentiate phenotypically near-identical species of industrial white *Penicillia*. The relatively simple procedures outlined in this paper could easily be transfered to industrial laboratories in need of rapid and accurate diagnostic tools for fungal identification.

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