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Detection of *Penicillium* species in complex food samples using the polymerase chain reaction

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Abstract

Rapid identification of filamentous fungi is becoming increasingly important in food mycology both for monitoring the production process and for the identification of food spoilers. This paper describes the development and trial of two specific PCR primer sets. A 336 bp fragment from species belonging to *Penicillium* subgenus *Penicillium* was amplified by the primers ITS 212d and ITS 549. The other primer set, ITS 183 and ITS 401 specifically identified two species, *Penicillium roqueforti* and *P. carneum*, both known as spoilers in the bread industry, by amplification of a 300 bp fragment. The future perspectives of PCR based identification of filamentous fungi in food are discussed. © 1997 Elsevier Science B.V.

Keywords: Polymerase chain reaction; Penicillium; Food spoilers

1. Introduction

Current practices in food mycology rely primarily on conventional cultivation and microscopic techniques (Deak, 1994). In particular, identification of *Penicillium* species is to a large extent based on the examination of macro- and micromorphological characteristics (such as colour, size and shape of colonies and conidia) and gross physiological criteria (Pitt, 1991). These techniques are time consuming and require skilled taxonomists, and standardisation has proved difficult as minor differences in medium composition can impair effective comparison of colony characters (Okuda, 1994). Recently, secondary metabolite profiles, isoenzyme analysis and molecular techniques have been introduced as powerful tools for detecting and identifying fungi such as *Aspergillus* and *Penicillium* (Frisvad and Filtenborg, 1983; Cruickshank and Pitt, 1987; Peterson, 1993; Spreadbury et al., 1993).

Detection techniques using nucleic acids are based on the use of unique oligonucleotide se-

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quences either as probes in hybridization assays, or as primers for enzymatic amplification of DNA fragments using the polymerase chain reaction (PCR).

In fungi, direct sequencing of PCR-amplified DNA using primers (White et al., 1990) that are complementary to conserved sequences within the nuclear and mitochondrial ribosomal genes (rDNA) as well as the internal transcribed spacer regions (ITS I and ITS II) have been used for phylogenetic studies and for identification of specific groups of fungi (Berbee and Taylor, 1992; Bowman et al., 1992; Gardes and Bruns, 1993; Berbee et al., 1995).

Eukaryotic fungal ribosomal genes are arranged in a tandem repeat and within the rDNA repeat, the two variable non-coding ITS regions are nested between the highly conserved nuclear small subunit rRNA (SrDNA), 5.8S and large subunit rRNA genes. The ribosomal region spanning ITS I, 5.8S and ITS II is often between 600–800 bp long (Fig. 1) and is found in multiple copies, which makes it feasible to amplify DNA fragments from samples containing target DNA (Gardes and Bruns, 1993). The fungal ribosomal genes are highly conserved at the genus level or even higher (Bruns et al., 1991). The internally transcribed spacers (ITS I and ITS II) and the intergenic spacer (IGS) have evolved faster than the ribosomal genes and may therefore be more useful for the development of specific oligonucleotide primers or probes, aimed at differentiating at the genus, species or subspecies level (White et al., 1990).

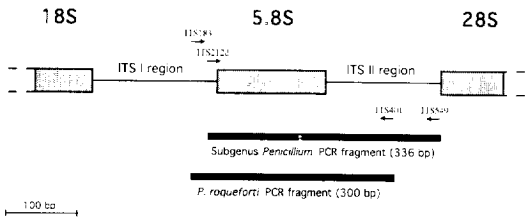

Fig. 1. Map of the ITS I, 5.8S and ITS II ribosomal regions. The location of the subgenus *Penicillium* specific primers (ITS 212d and ITS 549), and the *P. roqueforti P. carneum* primer set (ITS 183 and ITS 401), are indicated. The subgenus specific primer set amplifies a 336 bp fragment whereas the *P. roqueforti P. carneum* primer set amplifies a 300 bp PCR fragment as indicated by the black boxes.

Several studies have shown that the ITS regions are highly variable among and within different fungal species (Chen et al., 1992; O'Donnell, 1992; Muthumeenakshi et al., 1994). We have analysed the sequence variation within the ITS regions of 28 *Penicillium* subgenus *Penicillium* species and in general, the ITS regions were too conserved to be useful for differentiating between all the individual species (Skouboe, 1996, unpublished data). However, it was possible to identify specific oligonucleotides for some species, e.g. *P. roqueforti* and the closely related *P. carneum* (Boysen et al., 1996).

P. roqueforti is well known for its role in the production of blue-veined cheeses such as Gorgonzola, Stilton and Roquefort. However, this filamentous fungus and the closely related species *P. carneum* are also widely distributed food and feed spoilage fungi. They are particularly found on rye bread and other preserved or acid-treated foods and on products stored at low oxygen tension (Gravesen et al., 1994), as *P. roqueforti* and *P. carneum* are the only known Penicillia with tolerance to 0.5% acetic acid (Engel and Teuber, 1978; Frisvad, 1981).

In this report we have used ITS sequences from *Penicillium* species to identify and test two sets of primers, one which specifically identifies all members of *Penicillium* subgenus *Penicillium*, and one which specifically recognizes *P. roqueforti* and *P. carneum*.

2. Methods

2.1. Fungi

The *Penicillium* species and other isolates used in this study are listed in Table 1. The IBT strains are maintained at Department of Biotechnology, Technical University of Denmark, Lyngby, Denmark.

2.2. Extraction of mycelial DNA for specificity tests

Fungi were grown in 200 ml Czapek Yeast Autolysate (CYA) broth (Pitt, 1979) for 3 days at

Table 1
List of strains examined and results of tests with *Penicillium* subgenus *Penicillium* specific PCR primers (ITS 212d and ITS 549) and the *P. roqueforti P.carneum* specific primer set

Isolates used	IBT no.	Source	PCR products	
			ITS212d ITS549	ITS183 ITS401
Penicillium aurantiogriseum	6215	Barley	+	−
	10 047	Sunflower	+	ND
P. freii	3464	Wheat	+	−
P. tricolor	12 493	Wheat	+	ND
P. polonicum	11 388	Barley	+	ND
P. aurantiovirens	11 330	Barley	+	ND
P. melanoconidium	3442	Barley	+	−
	6794	Salami	+	ND
P. viridicatum	5273	Barley	+	ND
	10 057	Sesam seed	+	ND
P. cyclopium	5311	*Amaranthus* flower	+	ND
	10 085	Oats	+	ND
P. sp.1	12 708	Kangaroo rat	+	ND
P. sp. 2	12 396	Kangaroo rat	+	ND
P. neoechinulatum	3462	Seed cache of kangaroo rat	+	ND
P. verrucosum	5010	Barley	+	ND
	11 621	Wheat	+	−
P. camemberti	3505	Brie cheese	+	−
	11 568	Camembert cheese	+	...
P. commune	10 253	Cheese	+	
	6359	Cheese	+	−
P. roqueforti	12 845	Cheese	+	+
	14 429	Starter culture	ND	+
	14 431	Starter culture	ND	+
	914 430	Starter culture	ND	+
	914 433	Starter culture	ND	+
	914 432	Starter culture	ND	+
	14 408	Silage	ND	+ −
	14 412	Silage	ND	+ −
	14 420	Silage	ND	+ −
	14 425	Silage	ND	+ −
	6754[a]	Blue cheese	ND	+
P. carneum	6884[a]	Rye bread	ND	+
	3477	Rye bread	ND	+
	6885	Meat	ND	+
	6753	Cheddar cheese	ND	+
	14 042	Rye bread	ND	+
P. paneum	12 407	Rye bread	+	−
	11 839	Rye bread	ND	+
	12 392	Chocolate sauce	−	−
	13 321	Soft drink	ND	−
	13 929	Baker's yeast	ND	−
P. chrysogenum	3182	Volcano dust	+	ND
	5848	Sesame seed	+	−
P. nalgiovense	5746	Desert sand	+	ND
	12 051	Fermented sausage	+	ND
	12 108	Cheese	+	ND
P. dipodomyis	5324	Seed cache of kangaroo rat	+	ND

Table 1 (continued)

Isolates used	IBT no.	Source	PCR products	
			ITS212d ITS549	ITS183 ITS401
P. crustosum	13 049	Rye bread	ND	–
	13 769	Cheese	+	–
P. discolor	3086	Jerusalem artichoke	+	ND
P. hirsutum	10 628	Aphid	+	–
P. hirsutum var. *venetum*	10 594	Hot water tank	+	ND
P. hordei	3083	Onion	+	ND
P citrinum	11 819	Unknown	–	ND
P. janczewskii	13 105	Unknown	–	ND
P. steckii	14 691	Bermuda grass hay	–	ND
P. hispanicum	14 196	Spices	–	ND
P. selandiae	6828	Unknown	–	ND
P. scabrosum	14 401	Processed wood	–	ND
P. thomii	12 891	Cranberry	–	ND
Eupenicillium shearii	14 694	Unknown	–	ND
Aspergillus terreus	6207	Acid preserved grain	–	–
	6253	Soil	ND	–
Aspergillus flavus	5694	Peanut	–	–
	3593	Soil	ND	–
Aspergillus versicolor	13 719	Cheese	–	–
	12 388	Cheese	ND	–
Aspergillus candidus	5826	Rye bread	–	–
	13 302	Rye bread	ND	–
Paecilomyces variotii	6326	Wooden pallet	–	–
	7580	Unknown	ND	–
Eurotium repens	13 486	Nut	–	–
Scopulariopsis brevicaulis	13 784	Creamery	ND	–
	13 316	Cheese	–	–
Fusarium avenaceum	8089	Cereal mix	–	–
Fusarium poae	8062	Stored wheat	–	–
Geotrichum candidum	7644	Unknown	ND	–
	7646	Unknown	ND	–
Hyphopichia burtonii	–	Unknown	ND	–
	–	Unknown	ND	–
Lactococcus lactis	–	Unknown	ND	–
	–	Unknown	ND	–

ND, not determined: +, indicates that a specific band was seen after PCR with primers: –, no specific band was seen.
[a] Type culture.

25°C with agitation (200 rpm). Mycelium was harvested by filtration, washed with 0.9% NaCl and frozen in dry ice ethanol. For isolation of DNA, mycelium (500 mg wet weight) was pulverized by repeatedly grinding with a glass spatula in an Eppendorf tube and freeze-thawing. The disrupted cell suspension was added to 800 μl lysis buffer (50 mM EDTA pH 8.5, 0.2% SDS), incubated at 68°C (30 min), cooled to room temperature, and centrifuged (10 000 × g for 15 min). The supernatant was transferred to another tube and 5 M KAc (80 μl) carefully added. After incubation on ice (1 h) and centrifugation (20 000 × g, 15 min at 4°C), the nucleic acids in the supernatant were precipitated with an equal volume of isopropanol at room temperature and pelleted by centrifugation (10 000 × g for 15 min). The pellet was washed with 80% ethanol, dried and dissolved in

TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0; 50 μl).

2.3. PCR primers

Penicillium subgenus *Penicillium* specific primers used were: ITS 212d (5′-AAA TAT AAA TTA TTT AAA ACT TTC-3′) and ITS 549 (5′-CTG GAT AAA AAT TTG GGT TG-3′). The specific primer set for *P. roqueforti* and *P. carneum* were ITS 183 (5′-CTG TCT GAA GAA TGC AGT CTG AGA AC-3′) and ITS 401 (5′-CCA TAC GCT CGA GGA CCG GAC-3′).

2.4. PCR conditions

The PCR conditions were optimized by varying the concentrations of KCl, $MgCl_2$ and Tris–HCl pH 8.3 using purified *Penicillium* DNA as template (data not shown).

For the *P. roqueforti*/*P. carneum* specific primer set, 5 μl template DNA (10 ng) was amplified in a 50 μl reaction containing 10 mM Tris–HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatine, 200 μM dNTPs, 0.25% Tween 20, 10% DMSO, 0.3 μM of each of ITS 183 and ITS 401, and 2.5 U AmpliTaq polymerase (Perkin Elmer Cetus). Amplification conditions (Perkin Elmer 9600 Thermocycler) were 45 cycles for 30 s at 94°C, 1 min at 60°C and 1 min at 72°C. Final extension at 72°C was for 10 min.

For the *Penicillium* subgenus *Penicillium* primer set, 1–5 μl DNA (10 ng) was added to a reaction mixture containing 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 200 μM, dNTPs, 1 μM ITS 212d, 1 μM ITS 549 and 2.5 U AmpliTaq polymerase in a final volume of 100 μl. Amplification conditions were 45 cycles at 94°C for 30 s, 48°C for 30 s, 72°C for 30 s, and finally 72°C for 10 min on the Perkin Elmer 9600 Thermocycler. Alternatively, the PCR was run on the Perkin Elmer 480 thermocycler for 40 cycles at 94°C for 30 s, 48°C for 60 s and 72°C for 60 s followed by 72°C for 10 min.

PCR products (10 μl aliquots) were visualised by electrophoresis in a 2.5% agarose gel in TAE buffer followed by staining in 1 μg/ml ethidium bromide (Sambrook et al., 1989).

2.5. Preparation of food samples

To investigate the possibility of using the primers and PCR conditions on biological material, DNA was extracted from two types of soft cheese fermented either by *P. camemberti* or *P. roqueforti*. Two different extraction procedures were used, a proteinase treatment and an aqueous polymer two phase separation method.

To obtain cheese samples containing *P. roqueforti*, Roquefort cheese (25 g) was homogenised in 250 ml water using a Stomacher 400 (Struers, DK) for 120 s at medium speed. A cheese sample containing *P. camemberti* was obtained by scraping fungal tissue (2 g) from the surface of Camembert cheese and stomaching for 120 s at medium speed in 20 ml of water. These preparations were used for either proteinase treatment or two-phase separation.

2.6. Proteinase treatment

Camembert and Roquefort cheese homogenates (4 ml) were digested with proteinase K in a 10 ml volume consisting of 0.01 M Tris–HCl pH 8.0, 0.005 M EDTA, 0.5% SDS and 250 μg/ml Proteinase K. The mixture was incubated for 1 h at room temperature (Sambrook et al., 1989). Aliquots were removed for DNA extraction.

2.7. Two-phase separation

Cheese homogenate (4 ml) was added to a polymer system to separate fungal tissue from the cheese fraction. The system contained 10 mM PBS pH 7.0, 100 mM NaCl, 5.0% PEG 3350, 5.0% Dextran 500 in a 10 ml final volume. The tubes were inverted 30 times and then centrifuged at 20 000 × *g* for 5 min. Aliquots were removed from the PEG rich upper phase for DNA extraction (Lantz et al., 1994)

2.8. DNA extraction of cheese homogenates

Two volumes of 2.0% Hexadecyltrimethylammonium bromide (CTAB), 0.1 M Tris–HCl pH 8.0, 20 mM EDTA and 1.4 M NaCl were added to a 500 μl aliquot and the mixture was incubated

at 65°C for 10 min and centrifuged at 20 000 × *g* for 10 min (Murray and Thompson, 1980). The supernatant was extracted in an equal volume of chloroform by inversion and centrifuged at 20 000 × *g* for 10 min. An equal volume of 1.0% CTAB, 50 mM Tris–HCl pH 8.0, 10 mM EDTA was added to the supernatant, which was held at room temperature for 30 min, centrifuged for 10 min and the supernatant discarded. To the pellet 1 M NaCl (450 μl) and 96% EtOH (900 μl) were added; after 5 min, the mixture was centrifuged for 5 min at 20 000 × *g* and the supernatant was discarded. The DNA was washed with 80% EtOH, and dissolved in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA; 15 μl).

3. Results

3.1. Selection and test of subgenus Penicillium specific primers

Based on alignment of 70 DNA sequences spanning the ITS regions and the 5.8S gene from 28 species of *Penicillium* (Boysen et al., 1996; Skouboe, 1996, unpublished data) the primer set ITS 212d/ITS 549 was selected and tested for specificity against species belonging to *Penicillium* subgenus *Penicillium* (Fig. 1). Furthermore, the primer set was tested on chromosomal DNA extracted from a range of fungi covering both related and unrelated genera and subgenera (Table 1). The primer set ITS 212d/ITS 549 was specific for subgenus *Penicillium* amplifying a 336 bp DNA fragment from all *Penicillium* subgenus *Penicillium* isolates tested (Fig. 2, Table 1) but the primers did not amplify DNA from other *Penicillium* species or isolates belonging to other genera, e.g. *Aspergillus*, *Fusarium* and *Eurotium* (Table 1).

3.2. Selection and test of the P. roqueforti/P. carneum specific primer set

The primer set ITS 183/ITS 401 was tested for specificity to *P. roqueforti* and *P. carneum* and a number of fungi covering both species belonging to subgenus *Penicillium* and ecologically important species found in food samples (e.g. cheese) and the environment (Table 1). Only *P. roqueforti* and *P. carneum* gave positive PCR signals, producing the expected 300 bp fragment (Fig. 3, Table 1).

3.3. PCR tests on different cheeses

Two different techniques were used to separate fungal mycelia and spores from the complex cheese matrix. The proteinase digestion made the cheese homogenate less viscous, but it still contained aggregates. Aqueous polymer two-phase partitioning, which was used as an alternative to the proteinase treatment, separated most of the hyphae, spores and cheese homogenate to the Dextran-rich bottom phase (results not shown). The PEG-rich top phase contained less hyphae and spores, but only minor traces of cheese homogenate, and thereby some purification was obtained.

After proteinase digestion or phase separation, DNA was extracted by the CTAB method. The

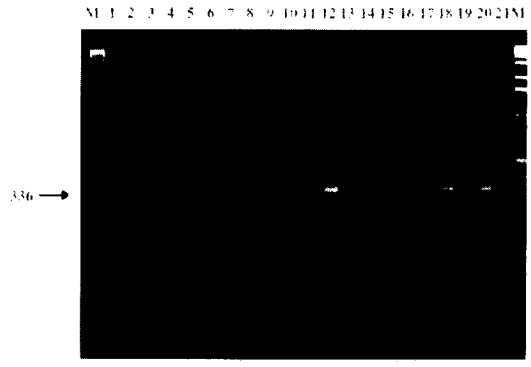

Fig. 2. Gel electrophoresis of PCR products amplified from DNA extracted from different *Penicillium* isolates. The PCR products (336 bp indicated by the arrow) were amplified by the primers ITS 212d ITS 549. Lanes: M, molecular weight marker, DNA ladder (GIBCO, BRL); 1, *P. freii* (IBT 3464); 2, *P. aurantiogriseum* (IBT 6215); 3, *P. tricolor* (IBT 12 493); 4, *P. sp. 1* (IBT 12 708); 5, *P. polonicum*(IBT 11 294); 6, *P. sp. 2* (IBT 12 396); 7, *P. viridicatum* (IBT 10 057); 8, *P. cyclopium* (10 085); 9, *P. neoechinulatum* (IBT 3462); 10, *P. verrucosum* (IBT 11 621); 11, *P. camemberti* (IBT 3505); 12, *P. commune* (IBT 10 253); 13, *P. roqueforti* (IBT 12 845); 14, *P. carneum* (IBT 12 392); 15, *P. chrysogenum* (IBT 5848); 16, *P. nalgiovense* (IBT 12 051); 17, *P. crustosum* (IBT 13 769); 18, *P. discolor* (IBT 3086); 19, *P. hirsutum* (IBT 10 628); 20, *P. hordei* (IBT 3083); 21, negative control (IBT-DNA).

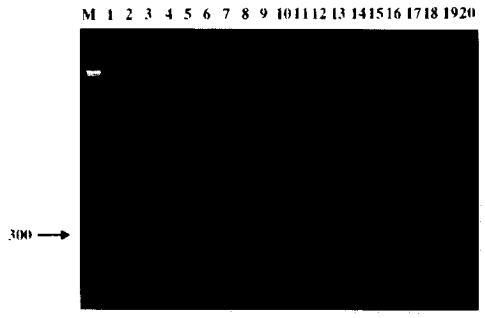

Fig. 3. Gel electrophoresis of PCR products amplified from DNA extracted from different *Penicillium* isolates. The PCR products (300 bp indicated by the arrow) were amplified by the species primers ITS 183/ITS 401. The IBT collection numbers are given in parenthesis. Lanes 1–10: *P. roqueforti* (12429, 14431, 914430, 914433, 914432, 14408, 14412, 14420, 14425, 6754). Lanes 11–15: *P. carneum* (3477, 6885, 6753, 14042, 6884). Lanes 16–20: negative controls, *P. paneum* (Boysen et al., 1996) (12407, 11839, 12392, 13321, 13929).

samples were analysed by PCR using both sets of primers (Fig. 4). The subgenus primers (ITS 212d and ITS 549) amplified the 336 bp fragment from DNA extracts prepared from both types of cheese whereas the *P. roqueforti*/*P. carneum* primer set (ITS 183 and 401) amplified the 300 bp fragment only from DNA samples derived from the Roquefort cheese. A number of bands were amplified when the *P. roqueforti*/*P. carneum* primer set was used on *P. camemberti* DNA (Fig. 4, lane 10), but these all differed in size from the specific 300 bp PCR fragment. These non-specific bands have also been observed in previous *P. camemberti* PCR amplifications (unpublished observations) with the *P. roqueforti*/*P. carneum* primers.

4. Discussion

We have shown that identification of *Penicillium* species can be performed as a PCR assay with two oligonucleotide primers, with at least one unique to the relevant taxon. Identification can also be performed by dot blot hybridisation experiments using a specific oligonucleotide primer as probe as shown by Boysen et al., 1996. When the target is present in multiple copies on the genome, as in the case of ribosomal DNA a fairly low detection limit is possible.

Based on a study of 52 isolates belonging to 28 species from *Penicillium* subgenus *Penicillium* (Skouboe, 1996, unpublished data) it was possible to identify regions general to all species as well as regions unique to individual species or groups of closely related species. In this paper we have presented one primer set unique to two closely related species, together with a subgenus specific primer set, as an illustration of the detection of *Penicillium* based on ribosomal ITS sequences. Alternatively, detection of closely related species within the genus *Penicillium* could be performed by using fingerprinting techniques (e.g. RAPD), or by identifying other regions with more sequence variation (e.g. IGS regions, specific functional genes, etc.) (Loudon et al., 1993; Spreadbury et al., 1993).

It is well established that a number of inhibitory substances can interfere both with the ability of the polymerase enzyme to amplify the specific sequences and with the hybridisation of primers to the template DNA (Wernars et al.,

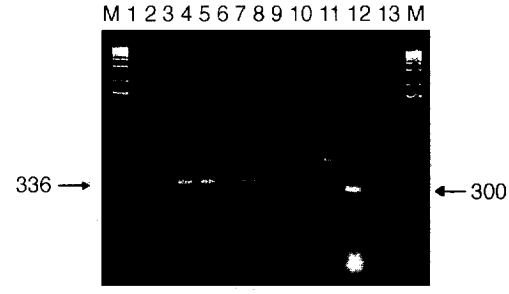

Fig. 4. Gel electrophoresis of PCR products amplified from DNA extracted from roquefort and camembert cheeses. PCR products in lanes 1–7 were amplified using the ITS 549/ITS 212d primers (arrow points to the 336 bp PCR fragment). PCR products in lanes 8–13 were amplified using ITS 183/ITS 401 primers (arrow points to the 300 bp PCR fragment). ITS 212d primers. Lanes: M, DNA ladder marker (Gibco BRL); 1, Camembert cheese proteinase extract; 2, Camembert cheese extract from two-phase system; 3, pure *P. camemberti* (11568) DNA; 4, pure *P. roqueforti* (14429) DNA; 5, negative control; 6, Roquefort cheese proteinase extract; 7, Roquefort cheese extract from two-phase system; 8, Camembert cheese proteinase extract; 9, Camembert cheese extract from two-phase system; 10, pure *P. camemberti* (11568) DNA; 11, pure *P. roqueforti* (14429) DNA; 12, Roquefort cheese proteinase extract; 13, Roquefort cheese extract from the two-phase system.

1991; Panaccio and Lew, 1991; Rossen et al., 1992). We used a proteinase digestion method, and an aqueous polymer two-phase extraction method, to separate fungal hyphae or spores from two complex cheese samples: a blue mould cheese and a white mould cheese, both with 60% fat (Sambrook et al., 1989; Lantz et al., 1994). Both procedures worked well although the recovery of fungal elements, estimated by microscopic observation, seemed to be lower when the latter method was used (unpublished observation). The concomitant PCR reactions showed that it was possible to amplify DNA from both cheeses with the subgenus specific primers. The *P. roqueforti*/*P. carneum* specific primer set amplified DNA from the Roquefort cheese only. The integrity of the PCR products derived from the cheese samples was confirmed by DNA sequencing (results not shown). Thus, it was possible to obtain detectable signals with DNA derived from complex food matrices, which shows that the PCR method is relatively robust. However, further experiments are needed in order to assess the detection limit.

The total detection time, i.e. the period of time from when the samples were taken until the PCR products were visualized, was approximately 7 h. This detection time can be reduced to 4 h if more rapid extraction methods and fluorescent detection systems are used (Holmstrøm et al., 1993). Therefore, PCR and related nucleic acid based methods may be useful for detecting contaminating fungi during process control.

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