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Development of a molecular detection and differentiation system for ochratoxin A producing *Penicillium* species and its application to analyse the occurrence of *Penicillium nordicum* in cured meats

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Abstract

A PCR method for differentiation and detection of the two known ochratoxin A producing *Penicillium* species, *Penicillium verrucosum* and *Penicillium nordicum* has been developed. It is based upon two genes of the ochratoxin A biosynthetic pathway, namely the ochratoxin A polyketide synthase gene (*otapks*PN) and a non-ribosomal peptide syntethase gene (*otanps*PN) from *P. nordicum*. Both ochratoxin A producing Penicillia differ characteristically in the PCR result, making a taxonomic differentiation possible. *P. verrucosum* gives consistently only a positive reaction with the primers for the *otanps*PN gene, whereas *P. nordicum* is positive for both genes. The PCR reaction is negative with all of other food related fungal species tested. This PCR system has been used to analyse 62 *Penicillium* strains isolated from cured meat products or ripening rooms, the natural habitat of *P. nordicum*. Among the 62 analysed strains 11 (18%) were positive with all specific PCR reactions. All 11 strains were able to produce ochratoxin A. In a RAPD analysis performed in parallel all 11 strains showed a pattern characteristic of *P. nordicum*, indicating the congruence of all data. None of the other strains isolated from cured meat produced ochratoxin A; most of them (30 out of 62) had a RAPD pattern characteristic for *Penicillium nalgiovense*. Interestingly some of the *P. nalgiovense* strains showed weak PCR product bands with varying length after electrophoresis. This was true for both primer pairs. None of these *P. nalgiovense* strains however produced detectable amounts of ochratoxin A. A more detailed analysis revealed that *P. nalgiovense* carries similar but non-transcribed sequences to the ochratoxin A biosynthetic genes of *P. nordicum*.

Keywords: *Penicillium nordicum*; Ochratoxin A; Cured meats; *Penicillium nalgiovense*; PCR; Biosynthetic genes

1. Introduction

Penicillium verrucosum and *Penicillium nordicum* are the two known ochratoxin A producing species of the genus *Penicillium* (Larsen et al., 2001; Castella et al., 2002). Both are related and morphologically very similar which hampers their correct taxonomic identification. Both ochratoxin A producing *Penicillium* species occupy different ecological niches. *P. verrucosum* is adapted to the cereal environment and is responsible for the occurrence of ochratoxin A in cereals and cereal products whereas *P. nordicum* can mainly be isolated from proteinaceous foods like cheeses and fermented meats (Lund and Frisvad, 2003). Generally *P. nordicum* is able to produce much more ochratoxin A than *P. verrucosum* in vitro, but until now nothing is known about the ability of *P. nordicum* to produce ochratoxin A in its natural environment. Ochratoxin A is a nephrotoxic mycotoxin which also has hepatotoxic, immunogenic and teratogenic properties (Höhler, 1998). Ochratoxin A is classified as a class II carcinogen by the WHO (Petzinger and Ziegler, 2000). It is a polyketide mycotoxin coupled to the amino acid phenylalanine. Recently a part of the gene cluster of the ochratoxin A biosynthetic pathway has been cloned (Karolewiez and Geisen, 2005). On this DNA fragment the ochratoxin A polyketide synthase (*otapks*PN, GeneBank accesion number AY196315) responsible for the biosynthesis of the polyketide part and the non-ribosomal peptide synthetase (*otanps*PN, GeneBank accession number AY534879), obviously responsible for the linkage of the phenylalanine moiety to the polyketide are localized. It

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could be demonstrated during this analysis that the *otanps*PN gene is obviously homologous in both ochratoxin A producing *Penicillium* species, whereas the polyketide synthase of both species must differ considerably. Based on these facts a PCR method for detection and differentiation of *P. nordicum* and *P. verrucosum* has been developed and is described. This method was applied to analyse the natural occurrence of *P. nordicum* in cured meat and ripening rooms.

2. Materials and methods

2.1. Strains and culture conditions

All strains with a BFE number were taken from the culture collection of the Federal Research Centre for Nutrition and Food. All strains with a MPVP number (University of Piacenza collection) were isolated from cured meat and from different production plants of this product. Strains were routinely grown on malt extract agar plates at 25 °C (malt extract agar (Merck, Darmstadt, Germany) 17 g/l, glucose 5 g/l). For the isolation of DNA, strains were grown in malt extract broth (Merck, Darmstadt, Germany) at 25 °C under shaking conditions for 5 days.

2.2. Isolation of the fungal strains from cured meat and from production plants

Four pieces of cured meats were sampled during ripening in 7 production plants for 1 year with a 3-month interval (112 samples). A tassel was cut from the surface; each tassel was incubated in a Petri dish with CYA (sucrose 30.0 g/l, yeast extract 5.0 g/l, $NaNO_3$ 3.0 g/l, K_2HPO_4 1.0 g/l, KCl 0.5 g/l, $MgSO_4 \times 7H_2O$ 0.5 g/l, $FeSO_4 \times 7H_2O$ 0.01 g/l, agar 15 g/l) for 7 days. Moulds developed were purified and identified at genus level.

In each production plant, pre-ripening and ripening rooms were monitored for fungal presence. Five Petri dishes with Potato Dextrose Agar (Merck, Darmstadt, Germany) were exposed for 6 h, with a 15-day interval for the whole year. After 7-day incubation at 25 °C, colonies were counted and representative isolates were purified and identified at the genus level. Most of the isolated strains, both on cured meat and from the air in ripening rooms, belong to Penicillia (Battilani et al., unpublished data). Eighteen and 44 strains, respectively isolated from meat and air, were selected among those forming colonies comparable to *P. verrucosum/P. nordicum* on CYA medium for molecular characterisation.

2.3. Isolation of fungal DNA

DNA was isolated using a method modified from that of Yelton et al. (1984). Seventy-two to ninety-six hour old mycelia were harvested from a submerged culture by filtration. The mycelia were transferred to a mortar and frozen in liquid nitrogen. The frozen mycelia were ground to a powder and resuspended in lysis buffer (50 mM EDTA; 0.2% SDS; pH 8.5). This suspension was heated to 68 °C for 15 min and centrifuged for 15 min at 15,000×*g*. After centrifugation 7 ml of the supernatant was transferred to a new centrifuge tube and 1 ml of 4 M sodium acetate was added. This solution was placed on ice for 1 h and centrifuged for 15 min at 15,000×*g*. After centrifugation 6 ml of the supernatant were transferred to a fresh tube. The solution was phenol extracted and the isolated DNA was precipitated by the addition of 2.5 volumes of ethanol. The isolated DNA was checked on an agarose gel and the concentrations were determined spectrophotometrically (Sambrook and Russel, 2001).

2.4. Diagnostic PCR for identification and differentiation of P. nordicum and P. verrucosum

For identification and differentiation of *P. nordicum* and *P. verrucosum* isolated DNA of fungal strains was subjected to PCR with specific primers targeted against the *otapks*PN gene (primer pair *otapks_for/otapks_rev*) and the *otanps*PN gene (primer pair *otanps_for/otanps_rev*). The primers had the following sequences:

otapks_for 5′ tac ggc cat ctt gag caa cgg cac tgc c 3′
otapks_rev 5′ atg cct ttc tgg gtc cag ta 3′
otanps_for 5′agt ctt cgc tgg gtg ctt cc 3′
otanps_rev 5′cag cac ttt tcc ctc cat cta tcc 3′.

The PCR was conducted in a Biorad iCycler (Hercules, USA) according to the following conditions: template DNA 5.0 µl (0.1 µg/ml); 10 × PCR buffer (Amersham-Pharmacia, Uppsala, Sweden) 5.0 µl; dNTP mix 8.0 µl (2.5 mM each nucleotide), each primer 1.25 µl (5 pM); Taq polymerase 0.5 µl (5 u/µl, Amersham-Pharmacia, Uppsala, Sweden), H_2O bidest 29.0 µl; PCR cycle scheme: 33 × (95 °C, 30 s; 60 °C, 40 s; 72 °C, 60 s). This PCR resulted in fragments of about 750 bp for the *otanps*PN primer and 500 bp for the *otapks*PN primer.

2.5. Quantitative determination of ochratoxin A by HPLC

Detection and quantitative determination of ochratoxin A from fungal colonies was performed according to the method described for cereals in the ISO 15141 standard adapted (1998, www.iso.ch). For this purpose 100 mg of the fungal colony was extracted under shaking conditions in 800 µl chloroform at room temperature for 20 min. The mycelial residue was discarded and the chloroform was evaporated to dryness in a Speed Vac concentrator (Savant Instruments, Farmingdale, USA). The residue was redissolved in 500 µl methanol and subjected to HPLC analysis (20 µl) in a Pharmacia HPLC apparatus LKB 2150 (Pharmacia, Uppsala, Sweden). A nucleosil 100-5 C18 CCV 250/4 column have been used for separation (Machery and Nagel, Düren, Germany) at a flow rate of 1 ml/min with acetonitril/water/acetic acid (40:60:1). The peak was determined with a fluorescence detector (Shimazu RF551, Düsseldorf, Germany).

2.6. RAPD analysis

The isolated chromosomal DNA was diluted to 2 µg ml^{-1} and used as template DNA for RAPD–PCR reactions. The PCR

reaction mixture contained: 5.0 µl Taq DNA polymerase buffer (10×, Pharmacia, Uppsala, Sweden), 8 µl nucleotide mixture (dATP, dCTP, dTTP, dGTP; 2.5 mM each, (Boehringer, Mannheim Germany)) 5.0 µl $MgCl_2$ (25 mM), 1.25 µl primer (120 pmol μl^{-1}), 0.1 µl Taq polymerase (5 U μl^{-1} (Pharmacia, Uppsala, Sweden)), 5.0 µl template DNA (2 µg ml^{-1}) and 25.0 µl H_2O. Polymerase chain reactions were performed in 44 cycles (Eppendorf Mastercycler 5330, Eppendorf, Hamburg, Germany): 1 min at 95 °C, 1 min at 36 °C, 4 min at 72 °C. The sequence of the random primer *ari1* was 5′TGC TTG GCA CAG TTG GCT TC3′. This primer is 21 nucleotides in length resulting in higher reproducibility of the PCR results than with a shorter primer. The RAPD–PCR products were separated on an 0.8% agarose gel and the band patterns were analysed with the BioNumerics software package (Ghent, Belgium).

2.7. Isolation of total RNA

An amount of 0.5 g of the mycelium was frozen in liquid nitrogen and ground to powder in a mortar. This powder (200 mg) was used for isolation of total RNA. For that purpose the E.Z.N.A. Fungal RNA kit (Peqlab, Erlangen, Germany) has been used according to the recommendations of the manufacturer. A volume of 80 µl of the RNA preparation was treated with 2 µl DNase I (2.5 Kunitz units/µl, Quiagen, Hilden, Germany) for degradation of traces of genomic DNA. The solution was incubated for 60 min at 37 °C and subsequently for 10 min at 65 °C to inactivate the DNase. An aliquote of the RNA was separated on an agarose gel, to check the integrity of the RNA. The RNA gel was prepared as described by Sambrook and Russel [12]. Before further experiments the RNA concentration for each sample was determined spectrophotometrically and brought to an identical concentration.

2.8. cDNA synthesis

For cDNA synthesis 8 µl of the DNase I treated total RNA were used along with the Omniscript Reverse Transcription kit (QIAGEN, Hilden, Germany). The reaction mixture was composed essentially as described by the manufacturer and incubated at 37 °C for 1 h. The cDNA was either directly used for Real Time PCR or stored at −20 °C.

2.9. Real Time PCR

The Real Time PCR reactions were performed in a GeneAmp 5700® Sequence Detection System (PE Applied Biosystems, Foster City, USA). The TaqMan® system with two primers and an internal fluorescence labelled probe was used. The optimal primers and the internal probe used in the reaction were identified within the *otapks*PN gene by the Primer Express 1.0 software (PE Applied Biosystems, Foster City, USA). The primer/probe set had the following nucleotide sequences: *otapkstaq*1, 5′-CACGGTTTGGAACACCACAAT-3′; *otapkstaq*2, 5′-TGAAGATCTCCCCCGCCT-3′; *otapksprobe* 5′-CGTACCAATCCCCATCCAGGGC-TC-3′ (labelled with the fluorescence marker FAM at the 5′end and with TAMRA at the 3′end). For the PCR reaction the TaqMan reagent kit (PE Applied Biosystems, Foster City, USA) was used according to the recommendations of the manufacturer. For each reaction 1 µl of the DNA sample solution (2 µg ml^{-1}) was mixed with 50 µl of the PCR stock solution containing 5 µl of 10× TaqMan™ buffer A, 7 µl of 25 mM $MgCl_2$, 1 µl of each dNTP mixture (10 mM dATP, dCTP, dGTP and 20 mM dUTP), 0.5 µl of the primers and probe (each 0.5 µM), 0.5 µl uracil-*N*-glycosylase (1 u/µl), 0.2 µl AmpliTaq Gold (5 u/µl) and 29.8 µl steril deionized water. After an incubation of 2 min at 50 °C to allow for uracil-*N*-glycosylase cleavage, AmpliTaq Gold polymerase was activated by an incubation step for 10 min at 95 °C. All 35 PCR cycles were performed according to the following temperature regime: 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s.

To generate the standard curve a larger PCR fragment of the *otapks*PN gene with the primer *otapks_for* and *otapks_rev* as template was used. The concentration of this standard PCR product was determined in a fluorometer (DyNa Quant 200, Pharmacia, Uppsala, Sweden) and the number of copies were calculated. These stock solutions were diluted serially by a factor of 10 and an aliquot of the dilutions was used as a copy number standard during each setup of the Real Time PCR reaction. The concentration of unknown samples was calculated by the GeneAmp 5700® system according to the generated standard curve.

2.10. PCR fragment cloning and sequencing

For PCR fragment cloning the TA cloning vector pGEM-T easy have been used according the suggestions of the manufacturer (Promega, Madison, USA). The PCR fragment was generated by using the primer pair *otapks_for/otapks_rev* with *P. nordicum* BFE487, *Penicillium nalgiovense* BFE335 and *P. nalgiovense* BFE852 chromosomal DNA as template. After successful cloning the sequencing of the insert have been carried out by a sequencing service (GATC Konstanz, Germany).

3. Results

3.1. Differentiation of both ochratoxin A producing Penicillia by PCR

Based on the previously described partial gene cluster of ochratoxin A biosynthetic genes in *P. nordicum* (Karolewiez and Geisen, 2005), primer pairs against the *otapks*PN gene and the adjacent *otanps*PN gene have been generated. It has been shown previously that the *otanps*PN gene is present in both ochratoxigenic Penicillia, whereas the *otapks*PN gene is present only in *P. nordicum*. As mentioned above *P. nordicum* and *P. verrucosum* are morphologically closely related and hardly to distinguish. The genetic situation however offers the possibility to use PCR as a diagnostic tool to differentiate *P. verrucosum* and *P. nordicum*. Strains of each species were subjected to PCR using the two primer pairs. The result is shown in Fig. 1. As expected there is a clear difference in the PCR results between *P. verrucosum* and *P. nordicum*. *P.*

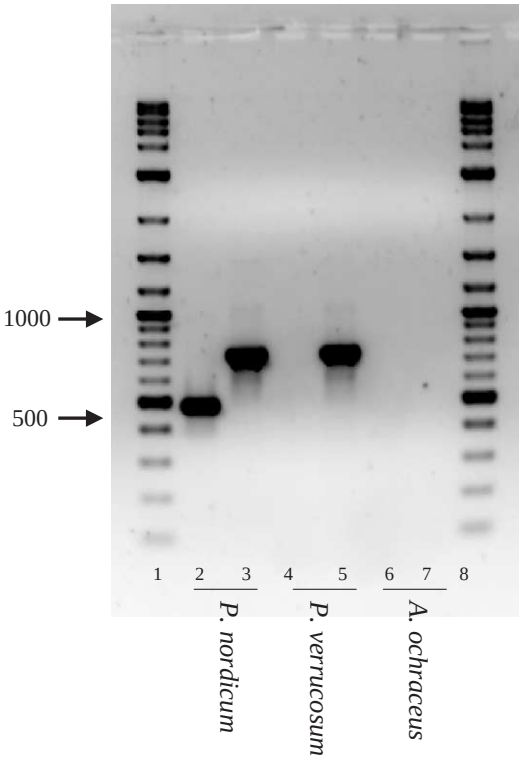

Fig. 1. Agarose gel with the PCR results of one *P. nordicum* (*P. nordicum* BFE487, lanes 2, 3), one *P. verrucosum* strain (*P. verrucosum* BFE500, lanes 3 and 5) and one *A. ochraceus* strain (*A. ochraceus* BFE635, lanes 6, 7) each with the primer pair *otapks_for/otapks_rev* (lanes 2, 4, 6) and the primer pair *otanps_for/otanps_rev* (lanes 3, 5, 7) Lanes 1 and 8 standards, important fragment lengths are indicated.

nordicum gave positive reactions with both primer pairs, whereas *P. verrucosum* only showed the expected PCR fragment with the primer pair for the *otanps*PN gene. This result was observed with all *P. nordicum* and *P. verrucosum* strains tested (about 20 for each species, data not shown). These results indicate a clear genetic difference of the ochratoxin A biosynthetic genes between the two ochratoxin A producing Penicillia which can be used for molecular differentiation.

3.2. Specificity of the PCR reaction

To demonstrate the specificity of the PCR reaction it was performed with various food related filamentous fungi including ochratoxin A producing *Aspergillus* species. The results are shown in Table 1. As already been shown, the tested strains of *P. nordicum* and *P. verrucosum* gave the expected results. None of the other species gave a positive signal with either PCR primer set used. Interestingly also the ochratoxin A producing

Table 1
Results of PCR reactions with the specific primer pairs with DNA of different food relevant fungi

BFE no	Species	*otapks*PN[a]	*otanps*PN[a]	OTA[b]
BFE487	*P. nordicum*	+	+	+
BFE500	*P. verrucosum*	–	+	+
BFE635	*A. ochraceus*	–	–	+
BFE631	*A. niger*	–	–	+
BFE640	*A. carbonarius*	–	–	+
BFE45	*P. italicum*	–	–	–
BFE574	*P. commune*	–	–	–
BFE66	*P. nalgiovense*	–	–	–
BFE141	*P. chrysogenum*	–	–	–
BFE221	*G. candidum*	–	–	–
BFE573	*P. crustosum*	–	–	–
BFE227	*F. solani*	–	–	–
BFE347	*F. proliferatum*	–	–	–
BFE312	*F. verticillioides*	–	–	–
BFE370	*C. cladosporoides*	–	–	–
BFE366	*P. variotii*	–	–	–

[a] Indicates the two different primer pairs: *otapks_for/otapks_rev* and *otanps_for/otanps_rev*.
[b] Capability of ochratoxin A production by the various species determined by HPLC.

Aspergillus species gave no positive signal with the primer pairs used (see also Fig. 1). The result implies that the genetic background of ochratoxin A biosynthesis in *Penicillium* and *Aspergillus* differs completely. Obviously the genes for ochratoxin A biosynthesis are heterologous in both geneva (Karolewiez and Geisen, 2005). These results indicate that the described PCR system is group specific for ochratoxin A producing Penicillia.

3.3. Application of the diagnostic PCR system to analyse the presence of P. nordicum on cured ham

The developed PCR system was used to analyse the occurrence of ochratoxin A producing *P. nordicum* strains on cured meat and in the ripening rooms. Sixty-two selected strains isolated as Penicillia were subjected to diagnostic PCR with the system described above. They also were analysed by HPLC for their capacity to produce ochratoxin A. The results are shown in Table 2. Most strains did not show the typical PCR pattern of *P. nordicum*, indicating that they did not belong to this species. All of these strains did not produce any detectable ochratoxin A when analysed by HPLC. Eleven strains, e.g. 18% of the strains were clearly positive in both PCR reactions, indicating that these strains belong to *P. nordicum*. All of these positive strains were capable of producing ochratoxin A at various amounts, some of them at very high rates. These results clearly showed the congruence of the positive PCR reactions and the ability to produce

Notes to Table 2:
[a] BFE no. = strain number of the strain collection of the Federal Research Centre for Nutrition; MPVP no. = strain number of the University of Piancenza.
[b] Indicates the two different primer pairs: *otapks_for*/*otapks_rev* and *otanps_for*/*otanps_rev*.
[c] Capability of ochratoxin A production by the various species determined by HPLC.
[d] The crosses in brackets (+) means that there were very faint bands at the same or at other positions.
All strains with the genotype and phenotype of *P. nordicum* are shaded.

Table 2
Results of the PCR reaction with the specific primer pairs and ochratoxin A production determined by HPLC from the various strains isolated from cured meat

BFE[a] no.	MPVP[a] no.	Origin	*Otapks*[b]	*Otanps*[b]	Ochratoxin A [∝g][c]
786	1350	Air	(+)[d]	–	n.d.
787	1412	Meat	–	–	n.d.
788	1413	Meat	(+)	–	n.d.
789	1414	Meat	–	–	n.d.
790	1416	Meat	(+)	–	n.d.
791	1417	Air			n.d.
792	1421	Air	–	–	n.d.
793	1437	Meat	–	–	n.d.
794	1466	Air	+	+	28.4
797	1524	Air	–	–	n.d.
798	1556	Meat	(+)	–	n.d.
799	1583	Air	–	–	n.d.
800	1605	Air	(+)	–	n.d.
806	1315	Air	–	–	n.d.
807	1319	Air	–	–	n.d.
808	1322	Air			n.d.
809	1328	Air	(+)	–	n.d.
810	1329	Air	–	–	n.d.
811	1335	Air	(+)	(+)	n.d.
812	1365	Meat	(+)	–	n.d.
813	1366	Meat	–	–	n.d.
814	1370	Meat	(+)	–	n.d.
815	1371	Meat	(+)	–	n.d.
816	1377	Meat	(+)	–	n.d.
817	1379	Meat	(+)	(+)	n.d.
818	1380	Meat	(+)	–	n.d.
819	1381	Meat	(+)	(+)	n.d.
820	1410	Meat	+	–	n.d.
821	1418	Air	(+)	(+)	n.d.
822	1423	Air	(+)	–	n.d.
823	1431	Air	+	(+)	n.d.
824	1439	Meat	(+)	–	n.d.
825	1447	Air	+	+	9.2
826	1449	Air	(+)	–	n.d.
827	1450	Air	(+)	–	n.d.
828	1454	Air	–	–	n.d.
829	1455	Air	(+)	–	n.d.
831	1468	Air	(+)	–	n.d.
832	1469	Air	(+)	–	n.d.
834	1499	Air			n.d.
835	1615	Air	+	+	5875.3
836	1647	Air	(+)	–	n.d.
837	1648	Air	(+)	–	n.d.
838	1669	Air	+	+	4355.5
839	1696	Air	(+)	–	n.d.
840	1757	Air	+	+	47.8
841	1772	Meat	+	+	6848.5
842	1789	Air	+	+	4217.0
843	1318	Air	–	–	n.d.
844	1320	Air	–	–	n.d.
845	1326	Air	–	–	n.d.
846	1334	Air	–	–	n.d.
847	1351	Air	+	+	4144.5
848	1415	Meat	–	–	n.d.
849	1426	Air	–	–	n.d.
850	1429	Air	+	+	152.5
851	1446	Air	+	+	n.d.
852	1448	Air	(+)	(+)	n.d.
853	1456	Air	(+)	(+)	n.d.
854	1463	Air	+	+	2974.8
855	1679	Air	–	–	n.d.
856	1692	Air	+	+	3024.8

ochratoxin A. However some of the ochratoxin A negative strains did show very faint bands sometimes at the same, sometimes at a different or additional positions as the expected bands of the *otapks*PN and *otanps*PN primer pairs (Table 2, Fig. 2). None of these strains however produced ochratoxin A. This results show that in the same habitat typical for *P. nordicum* another species exists which has some sequences in common with the ochratoxin A biosynthetic genes of *P. nordicum*.

3.4. RAPD analyses of Penicillium strains isolated from fermented ham and its environment

To get more detailed information and taxonomical confirmation about the species isolated from ham and its environment, an RAPD analysis with selected strains has been carried out. As control strains and for the sake of identification, characterized strains of *P. nordicum* (BFE487), *P. nalgiovense* (BFE55, BFE66) and *P. verrucosum* (BFE500) have been included in the analysis. The results are very clear and are shown in Fig. 3. All of the strains (11 from 62, however Fig. 3 shows only a subset) which produce ochratoxin A and which were positive in the two specific PCR reactions grouped with the control strain of *P. nordicum* BFE487 (group a). Most of the other strains (30 of 60), including them with the very faint PCR bands in some of the genes had the same pattern than the control strains of *P. nalgiovense* BFE55 and BFE66 (group b).

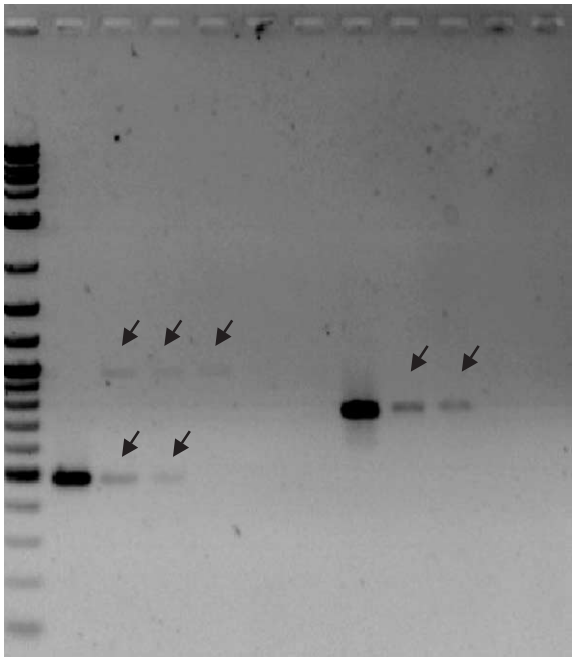

Fig. 2. Agarose gel of PCR reactions with *P. nordicum* 487 (lanes 2, 8) and 4 different *P. nalgiovense* strains (*P. nalgiovense* BFE67, lanes 3, 9; *P. nalgiovense* BFE133, lanes 4, 10; *P. nalgiovense* BFE68, lanes 5, 11; *P. nalgiovense* BFE500, lanes 6, 12) with different primer pairs (*otapks_for/otapks_rev*, lanes 2–6; *otanps_for/otanps_rev*, lanes 8–12). Lane 1 fragment length standards. Lane 7 empty. Weak bands of *P. nalgiovense* are indicated by an arrow.

This group separated into subgroups with the primer *ari1* used for RAPD analysis as was shown earlier (Geisen, 1995). All other species which were negative in the specific PCR showed different RAPD profiles. An ochratoxin A producing *P. verrucosum* strain (BFE500) has been included in the analysis for comparison. Taken together, the results indicate that the main mycobiota of this habitat consists of *P. nalgiovense* and *P. nordicum*. Both are clearly different in the RAPD patterns, but a weak and inconsistent cross hybridization occurs with some strains of *P. nalgiovense* with the specific primer pairs of the ochratoxin A biosynthetic genes.

3.5. Expression analysis and sequence comparison of the putative homologous of the ochratoxin A biosynthetic genes in P. nalgiovense

In order to get more information on the presence of putative sequences homologous to the ochratoxin A biosynthesis genes from *P. nordicum* PCR fragments obtained from *P. nalgiovense* genomic DNA (two strains) by the *otapks*PN primers have been cloned, sequenced and compared to the respective fragment of *P. nordicum* (Fig. 4). As the figure shows the sequences of this DNA region is very similar between *P. nordicum* and *P. nalgiovense*, indicating indeed the presence of sequences homologous to the ochratoxin A biosynthetic genes in *P. nordicum*. Only several single sequence variabilities, which are not identical in both analysed *P. nalgiovense* strains differ to *P. nordicum*.

Because *P. nalgiovense* is not able to produce ochratoxin A it could be assumed that these putative genes are silent and non-functional. To demonstrate this possibility an expression analysis have been performed by Real Time PCR. The primer for the Real Time PCR system have been chosen in a way that they are not located in DNA regions with variabilities (Fig. 4) to ensure the functionality of the system. The results, which are shown in Table 3 clearly indicate that the homologous gene sequences in *P. nalgiovense* are almost not expressed and obviously are non-functional. This fact explains the inability of *P. nalgiovense* to synthesise ochratoxin A, despite the presence of DNA sequences homologous to the ochratoxin A biosynthesis genes of *P. nordicum*.

4. Discussion

In the present work a PCR method for differentiation and detection of the two ochratoxin A producing *Penicillium* species has been described. Both species are morphologically very similar and taxonomical grouping simply by morphological means is not easy. In fact according to our experience most of the *P. nordicum* strains from from the BFE collection were formerly classified as *P. verrucosum* by morphological methods. This PCR system which is targeted against two genes of the ochratoxin A biosynthetic pathway is a very reliable and objective method to distinguish both species. Larsen et al. (2001) described another single phenotypic difference between both species based on the production of different secondary metabolites. *P. verrucosum* produces a

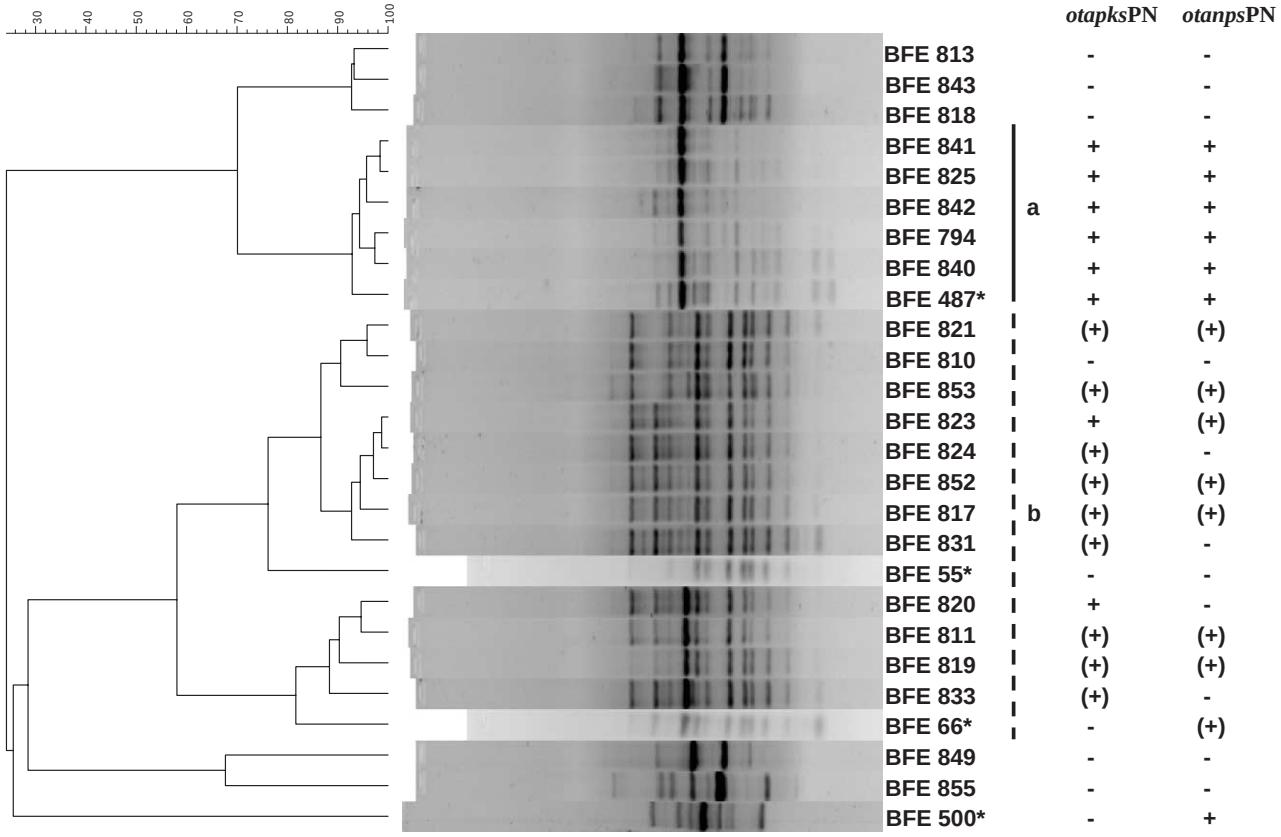

Fig. 3. Dendrogram of some selected *Penicillium* strains isolated from ham and its environment. The strain number is indicated according to Table 2. The type strains used for comparison (*P. nordicum* BFE487, *P. nalgiovense* BFE55 and *P. nalgiovense* BFE66) are indicated by an asterisk. *P. verrucosum* BFE500 was included to demonstrate the RAPD difference between both ochratoxin A producing *Penicillium* species. The two main groups a and b are indicated by different vertical bars. The results of the specific PCR reactions with the *otapks*PN and *otanps*PN primer pairs are indicated by crosses. Crosses in brackets means very faint bands.

brown colour after growth on YES medium, whereas *P. nordicum* does not. However this phenotypic difference is variable. The colour varies from brown, light brown, violet-brown to pale cream (Larsen et al., 2001).

The developed PCR system has been used to analyse the mycobiota of cured fermented ham. *P. nordicum* is specialized in the proteinacious food habitat, like cheeses and fermented meats (Lund and Frisvad, 2003). On the other hand *P. nalgiovense* is also adapted to these environments. In fact this species is used as a starter culture for the production of cured meat products and it is reported as the predominant fungal species from fermented meat products (Andersen, 1995a). This could be confirmed with the described results. After RAPD most of the strains had the same pattern than the *P. nalgiovense* type strains. The second most predominant species in the analysis was *P. nordicum*. Eighteen percent of all analysed *Penicillium* strains showed the phenotypic and genotypic features of *P. nordicum*. All of them were able to produce ochratoxin A. In another study done by Andersen (1995b) also a certain amount (5%) of the analysed strains from fermented meats has been classified as ochratoxin A producing *P. verrucosum* strains. According to the current knowledge it is straightforward to assume that these strains belong to *P. nordicum*. Because of the fact that most of the *P. nordicum* strains isolated from that environment are strong ochratoxin A producers, the question about importance of this fact in relation to food safety raises. To our knowledge nothing is known about the capability of *P. nordicum* to produce ochratoxin A in its natural environment. According to our own experience *P. nordicum* is able to produce ochratoxin A on model cheese or meat medium (data not shown). In a recent study the regulation of the *otapks*PN gene of *P. nordicum* has been analysed in relation to the environmental conditions relevant to the production of mould fermented meats (Geisen, 2004). According to this analysis it became clear, that the conditions during production of fermented meats allow induction of the ochratoxin A biosynthetic genes. This is at least an indication that ochratoxin A biosynthesis might be possible under these conditions. The results reported by Chiavaro et al. (2002) who could identify ochratoxin A in low amounts in 64% of the analysed samples of cured ham supports this possibility.

During the current study it became evident that most of the *P. nordicum* strains were isolated from the air of production plants, not from the ham itself. It might be possible that *P. nalgiovense* is more competitive on ham than *P. nordicum*, which would be an advantage for food safety. However no

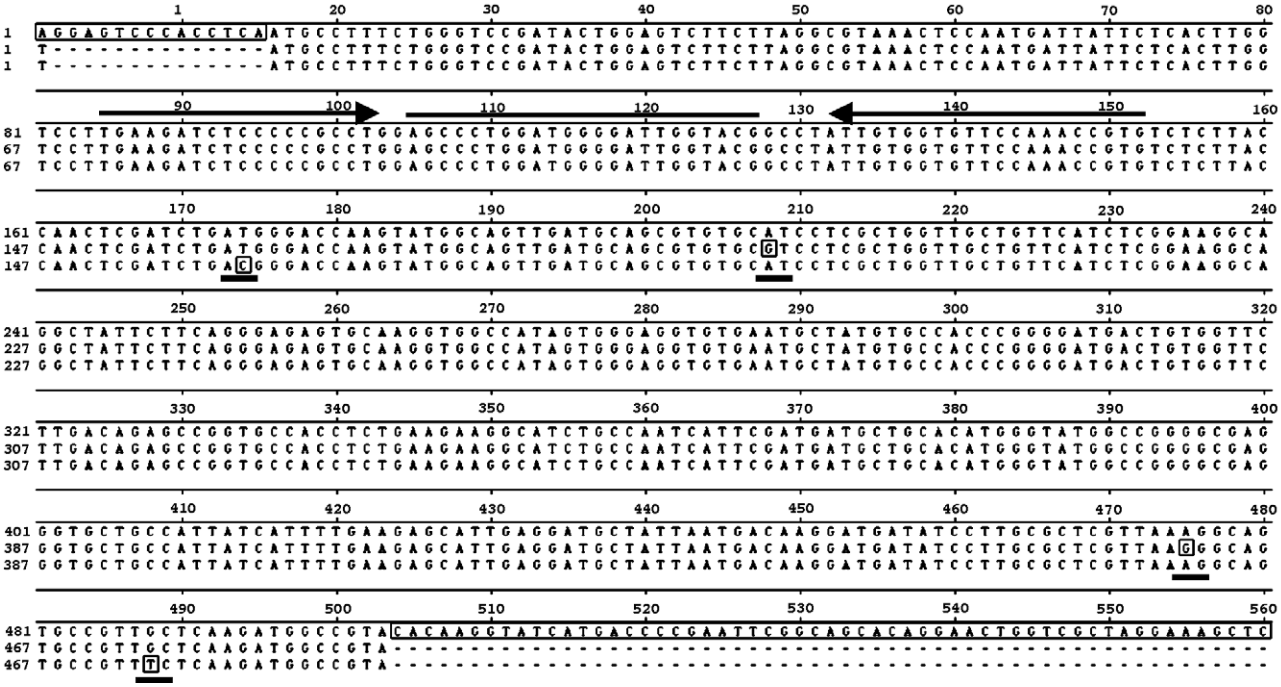

Fig. 4. Sequence comparisons of a part (PCR product generated with the primers *otapks_for/otapks_rev*) of the *otapks*PN gene of *P. nordicum* (upper row, *P. nordicum* BFE487) and its homologue in two strains of *P. nalgiovense* (middle row, *P. nalgiovense* BFE335, lower row *P. nalgiovense* BFE852). Positions with sequence differences are underlined. The location of the primer/probe system of the Real Time PCR used for expression studies are indicated by arrows and a bar above the sequences.

systematic analysis to clarify this point has been done yet and has still to be performed.

P. nalgiovense obviously carries sequences highly homologous to the ochratoxin A biosynthetic genes of *P. nordicum*. In the analysed region only a few bases differ between both species. However, according to the performed expression analysis, the putative genes in *P. nalgiovense* seems to be silent and non-functional. This is in agreement with the inability of *P. nalgiovense* to produce ochratoxin A. This situation resembles the relation between *A. flavus/A. oryzae* or *A. parasiticus/A. sojae*. The first two species are aflatoxigenic species, whereas both latter species are used in food production and do not produce aflatoxins, but carry defective aflatoxin biosynthetic genes (Klich et al., 1995).

With the developed PCR system cross hybridization with any other analysed fungal species have never been found. Even ochratoxin A producing Aspergilli proved to be negative, indicating that the ochratoxin A biosynthetic pathways in Penicillia and Aspergilli differ completely.

Table 3
Comparison of reverse transcriptase Real Time PCR results targeted against the *otapks*PN gene of different *P. nalgiovense* strains to *P. nordicum*

Strain		Quantitative expression
P. nordicum	BFE 487	2768[a]
P. nalgiovense	BFE 355	91
P. nalgiovense	BFE 852	34

[a] The numbers give the quantitative copy numbers per reaction starting with the same amount of mRNA for each strain.

The fact that *P. nalgiovense* shows some cross reactivity with the primer pairs used in the developed diagnostic PCR might lead to some ambiguities in the interpretation of the results, however the intensity of the bands usually differs considerably in most cases (Fig. 2). In addition the described RAPD system clearly differentiates between *P. nordicum* and *P. nalgiovense*. For future improvement of the system primers can be developed at positions where the sequences between *P. nordicum* and *P. nalgiovense* differ (Fig. 4).

We have described a system for the differentiation and detection of the two ochratoxingenic *Penicillium* species. This system has been used to analyse the mycobiota of cured meat products. It could be shown that a remarkable amount of strains of the environment of ham belongs to the ochratoxin A producing species *P. nordicum*. The impact of this fact on food safety has to be addressed in a further study. The exact relations of *P. nalgiovense* and *P. nordicum*, two species which obviously cover a limited habitat, have to be analysed in more detail, especially with respect to the presence of sequences which are related to ochratoxin A biosynthetic genes of *P. nordicum.*

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