Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles

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Penicillium roqueforti is currently divided into two varieties, one used for cheese starter cultures, *P. roqueforti* var. *roqueforti*, and one ubiquitous patulin-producing variety, *P. roqueforti* var. *carneum*. The ribosomal regions comprising the 5.8S gene and the internal transcribed spacers, ITS I and ITS II, have been analysed from 10 isolates belonging to each variety. The 10 *P. roqueforti* var. *carneum* isolates were separated into two groups of five on the basis of 12 base-pair differences in the ITS regions. One of the groups of *P. roqueforti* var. *carneum*, in the following designated *P. carneum*, differed from *P. roqueforti* var. *roqueforti*, here designated *P. roqueforti*, in just two positions, while the other group, here called *P. paneum*, differed from *P. roqueforti* in 12 positions. Random Amplified Polymorphic DNA (RAPD) analysis substantiated these findings, and a comparison of secondary metabolites produced by the three groups showed that the *P. roqueforti* isolates all produce Penicillium Roqueforti (PR) toxin, marcfortines and fumigaclavine A, while the *P. carneum* isolates produce patulin, penitrem A and mycophenolic acid, as well as unidentified metabolites. *P. paneum* produces secondary metabolites in five chromophore families including the known mycotoxins patulin and botryodiploidin. On the basis of these findings it is proposed that *P. roqueforti* is reclassified into three species named *P. roqueforti*, *P. carneum* and *P. paneum*.

Keywords: *Penicillium roqueforti*, *Penicillium carneum* comb. nov., *Penicillium paneum* sp. nov., ITS sequences, taxonomy

INTRODUCTION

Penicillium roqueforti Thom is used extensively as a starter culture for the production of blue-veined cheese (e.g. Engel & Teuber, 1989). It was included in the *P. roqueforti* series together with *P. casei* Staub in the monographic treatment by Raper & Thom (1949), and in the same series together with *P. farinosum* Novobranova, *P. fagi* Martínez et Ramírez, *P. mali* Novobranova and *P. cordubense* Ramírez et Martínez in the monograph of Ramírez (1982).

Abbreviations: IBT, Institute of BioTechnology; IMI, International Mycological Institute; ITS, internal transcribed spacer; PR toxin, Penicillium Roqueforti toxin; RAPD, Random Amplified Polymorphic DNA.

The EMBL accession numbers for the sequences of the ITS I, 5.8S and ITS II regions of *P. roqueforti* (IBT no. 914432), *P. carneum* (IBT no. 6753), *P. paneum* (IBT no. 12392) and *P. crustosum* (IBT no. 13049) are X82358, X82359, X82360 and X82361, respectively.

As noted by Pitt (1979) it is only *P. crustosum* (= *P. farinosum*) that is obviously related to *P. roqueforti* and this has been confirmed subsequently using other characters, such as growth on creatine as sole nitrogen source and production of roquefortine C (Frisvad & Filtenborg, 1989; Frisvad *et al.*, 1990; Lund & Frisvad, 1994). *P. roqueforti* is the only species of *Penicillium* that can grow on media containing 0.5% acetic acid, at high ethanol concentrations and at very low oxygen concentrations (Engel & Teuber, 1978; Frisvad & Samson, 1991). Furthermore, it is one of the easiest of the penicillia to recognize because of its tuberculate conidiophore stipes, large globose smooth conidia and good growth on creatine sucrose agar (CREA) (Samson *et al.*, 1977; Pitt, 1979; Frisvad, 1981; Frisvad & Filtenborg, 1989). Another typical character is the blackish-green colony reverse of *P. roqueforti* on most laboratory media (Ramírez, 1982). However, the discovery that all strains with pale

brown reverse produced patulin in place of the *Penicillium Roqueforti* (PR) toxin typical of *P. roqueforti* strains, led Frisvad & Filtenborg (1989) to describe such isolates as a separate variety, *P. roqueforti* var. *carneum*. Strains of the latter taxon have never been used for, or found in, fermented blue cheeses. Other secondary metabolites produced by one or both varieties of *P. roqueforti* include mycophenolic acid (Lafont *et al.*, 1979), roquefortine C, isofumigaclavine A, festuclavine (Scott *et al.*, 1976; Ohmomo *et al.*, 1994), botryodiploidin (Moreau *et al.*, 1982), penicillic acid (Olivigni & Bullerman, 1976), marcfortines (Polonsky *et al.*, 1980; Prange *et al.*, 1981), and roquecin (Brückner & Reinecke, 1988).

Several of the secondary metabolites isolated from *P. roqueforti* are toxic, but it appears that they represent a low public health risk, even when blue-veined cheeses are consumed daily in large quantities (Teuber & Engel, 1983). Some of the known mycotoxins, such as PR toxin, are unstable in cheese and will be transformed into non-toxic PR imine and PR amide (Chang *et al.*, 1993). Roquefortine C has, however, been isolated from a dairy cow feed sample naturally contaminated with *P. roqueforti* and was possibly implicated in the mycotoxicosis observed (Häggbloom, 1990). PR toxin is believed to have caused mycotoxicoses in livestock that had consumed silage in which *P. roqueforti* had grown (Kanota, 1970; Wei *et al.*, 1973).

Since a large number of toxic secondary metabolites are produced by different strains of *P. roqueforti*, better methods for classification and identification are required at both the species and the strain level. This will ensure that the most appropriate strains are used for producing blue-veined cheeses and that starter cultures are not contaminated. Engel & Teuber (1983) subdivided *P. roqueforti* into 15 groups based on different TLC patterns of secondary metabolites, but further attempts to classify *P. roqueforti* at the strain level have not been attempted.

Profiles of secondary metabolites (Frisvad, 1994) and the use of molecular methods (Reynolds & Taylor, 1993; Avise, 1994; Hawksworth, 1994) have been exploited both as tools for clarifying the phylogenetic relationships among species, as well as for classifying species. Several methods have been used such as the DNA sequence determination of various regions including the ribosomal genes, and fingerprinting methods such as ribotyping or Random Amplified Polymorphic DNA (RAPD) (Reynolds & Taylor, 1993). The ribosomal DNA region has previously been used for taxonomic analysis (Logrieco *et al.*, 1990; Chen *et al.*, 1992; Peterson, 1993). In particular, the internal transcribed spacer (ITS) regions have been shown to be useful for identification at the genus and species level (Gardes & Bruns, 1993; Muthumeenakshi *et al.*, 1994). In this study, we examine the relationship between two previously described *Penicillium* varieties of the same *Penicillium* species, using both secondary metabolite profile and DNA analysis and demonstrate that these represent three species, described here as *P. roqueforti*, *P. carneum* and *P. paneum*.

METHODS

Fungal strains. The strains used in this study are listed in Table 1. Ex-type cultures for *P. roqueforti* and *P. carneum* were included as well as several industrial mutants which can be difficult to identify as *P. roqueforti* as some of them have grey or white conidia and/or a pale reverse colour. In addition, the available cultures of synonyms and older cultures discussed by Raper & Thom (1949) were examined to determine whether priority names exist for the new taxa proposed here. They included NRRL 851, NRRL 852, NRRL 853, NRRL 857 (ex-type of *P. gorgonzolae*), NRRL 858, NRRL 1165 and U35498.73 (ex-type of *P. conservandi*).

Growth media and conditions. All fungi were inoculated on Czapek Yeast Autolysate (CYA) agar, malt extract agar (MEA), yeast extract sucrose (YES) agar and CREA for 1 week at 25 °C prior to analysis for secondary metabolites. For formulations of media see Samson *et al.* (1995).

DNA preparation. Fungal mycelium was cultivated at 25 °C for 3–5 d with shaking in liquid CYA medium, harvested by filtration and stored at –80 °C. Aliquots of approximately 0.5 g fresh wt mycelium were pulverized in a 1.5 ml Eppendorf tube using a glass spatula, refrozen on dry ice and crushed once more, and suspended in 800 µl extraction buffer (50 mM EDTA, pH 8.5, 0.2% SDS) prior to incubation at 65 °C for 30 min. The mycelium was pelleted and the supernatant mixed with 80 µl 5 M potassium acetate and incubated on ice for at least 1 h. After pelleting, the DNA was precipitated from the tube at room temperature for 10 min by adding an equal volume of 2-propanol. The pellet was washed in 500 µl 80% (v/v) ice-cold ethanol, dried at 37 °C, and suspended in 50 µl TE buffer, pH 7.5 (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, pH 8.0).

ssDNA preparation. ssDNA was prepared from 10 ng DNA

Table 1. *Penicillium* isolates used in this study showing origin and IBT culture collection number

Strain	IBT no.	Origin
<i>P. roqueforti</i>	14429	Starter culture
<i>P. roqueforti</i>	14431	Starter culture
<i>P. roqueforti</i>	914430	Starter culture
<i>P. roqueforti</i>	914433	Starter culture
<i>P. roqueforti</i>	914432	Starter culture
<i>P. roqueforti</i>	14408	Silage
<i>P. roqueforti</i>	14412	Silage
<i>P. roqueforti</i>	14420	Silage
<i>P. roqueforti</i>	14425	Silage
<i>P. roqueforti</i>	6754*	Blue cheese
<i>P. carneum</i>	3477	Rye bread
<i>P. carneum</i>	6885	Meat
<i>P. carneum</i>	6753	Cheddar cheese
<i>P. carneum</i>	14042	Rye bread
<i>P. carneum</i>	6884*	Rye bread
<i>P. paneum</i>	12407	Rye bread
<i>P. paneum</i>	11839	Rye bread
<i>P. paneum</i>	12392	Chocolate sauce
<i>P. paneum</i>	13321	Soda water
<i>P. paneum</i>	13929	Baking yeast
<i>P. crustosum</i>	13049	Rye bread

* Type culture.

60							
<i>P. roq.</i>	GGAAGTAAAA	GTCGTAACAA	GGTTTCCGTA	GGTGAACCTG	CGGAAGGATC	ATTACCGAGT	
<i>P. car.</i>							
<i>P. pan.</i>							
<i>P. cru.</i>							
120							
<i>P. roq.</i>	GAGGGCCCTC	TGGGTCCAAC	CTCCACCCG	TGTTTATTT-	ACCTTATTGC	TTCGGCGGGC	
<i>P. car.</i>					A.....		
<i>P. pan.</i>					A.....		
<i>P. cru.</i>				T.....	G.....		
180							
<i>P. roq.</i>	CCGCCTTAAC	TGGCCGCCGG	GGGGTTTACA	CCCCCGGGCC	CGCGCCCGCC	GAAGACAC-C	
<i>P. car.</i>	A.....		T.C..A.....			-T	
<i>P. pan.</i>	C.....		C.C..G.....			-C	
<i>P. cru.</i>	A.....		C.T..G.....			CC	
240							
<i>P. roq.</i>	CCGAATCTG	TCTGAAGAAT	GCAGTCTGAG	AACAAATATA	AATTATTTAA	AACCTTCAAC	
<i>P. car.</i>	C.....	A.....	.C.....	AAC.....			
<i>P. pan.</i>	C.....	A.....	.A.....	TGA.....			
<i>P. cru.</i>	T.....	T.....	.A.....	TGA.....			
300							
<i>P. roq.</i>	AACGGATCTC	TTGGTTCCGG	CATCGATGAA	GAACGCAGCG	AAATGCGATA	CGTAATGTGA	
<i>P. car.</i>							
<i>P. pan.</i>							
<i>P. cru.</i>							
360							
<i>P. roq.</i>	ATTGCAAAAT	CAGTGAATCA	TCGAGTCTTT	GAACGCACAT	TGCGCCCCCT	GGTATTCGGG	
<i>P. car.</i>							
<i>P. pan.</i>							
<i>P. cru.</i>							
420							
<i>P. roq.</i>	GGGGCATGCC	TGTCCGAGCG	TCATTGCTGC	CCTCAAGCCC	GGCTTGCTGTG	TTGGGTCTCG	
<i>P. car.</i>						T.T..	
<i>P. pan.</i>						C.T..	
<i>P. cru.</i>						C.C..	
480							
<i>P. roq.</i>	TCCTCCGAT-	TCTGGAGGAC	GGGCCCCGAA	GGCAGCGGCG	GCACCGCGTC	CGGTCTCTGA	
<i>P. car.</i>	...T.....-	..T..A....					
<i>P. pan.</i>	...T.....-	..C..G....					
<i>P. cru.</i>	...C.....C	..C..G....					
540							
<i>P. roq.</i>	GCGTATGGGG	CTTTGTCACC	CGCTCTGTAG	GCCCGGCCGC	GCTTGCCGAT	CAACCCAAAT	
<i>P. car.</i>		..T.....					
<i>P. pan.</i>		..C.....					
<i>P. cru.</i>		..T.....					
600							
<i>P. roq.</i>	TTTATCCAG	GTTGACCTCG	GATCAGGTAG	GGATACCCGC	TGAACTTAAG	CATATCAATA	
<i>P. car.</i>							
<i>P. pan.</i>							
<i>P. cru.</i>							
609							
<i>P. roq.</i>	AGCGGAGGA						
<i>P. car.</i>							
<i>P. pan.</i>							
<i>P. cru.</i>							

Fig. 1. DNA sequence alignments of the ITS I, 5-8S and ITS II regions from *P. roqueforti* (*P. roq.*), *P. carneum* (*P. car.*), *P. paneum* (*P. pan.*) and *P. crustosum* (*P. cru.*). Identical sequences are indicated with a dot, and dashes indicate a deletion. Position 1 is the 5' end of the universal primer ITS 5. Position 609 is the 5' end of the primer ITS 4. The 5-8S region is located between position 230 and 385.

using an asymmetric PCR essentially as described by White *et al.* (1990) with the addition of 0.25% Tween 20 and 10% (v/v) DMSO using the primers ITS 4 (5'-TCCTCCGCTTAT-TGATATGC) and ITS 5 (5'-GGAAGTAAAAGTCGT-AACAAGG), and the enzyme *Taq* polymerase (Perkin Elmer Cetus). Amplification was performed on a Perkin Elmer Cetus thermal cycler model 9600 using an initial denaturation at 94 °C for 1 min followed by 40 cycles of 94 °C for 5 s, 53 °C for 30 s, 72 °C for 60s. A final extension step of 10 min at 72 °C was

included. The PCR product was precipitated for 10 min in an equal volume of 5 M ammonium acetate and 2.5 vols 96% (v/v) ethanol and suspended in TE buffer, pH 7.5, to a concentration of approximately 1 mg ml⁻¹.

DNA sequencing. DNA sequencing reactions were performed in both directions according to Sanger *et al.* (1977) using Sequenase version 2.0 from USB according to the manufacturer's instructions and the modification described by Li & Schweizer (1993). PCR fragments were used directly as

templates to avoid mistakes due to random PCR-induced errors. The universal primers described by White *et al.* (1990) were used as sequencing primers and [α - 35 S]dATP (Amersham) was used as label. The fragments were separated on a denaturing 7 M urea/6% (w/v) polyacrylamide standard pre-mixed gel cast in a Gibco BRL sequence gel model S2 (30 × 40 × 0.04 cm) using wedged spacers. The gels were dried and exposed to Kodak X-OMAT AR films for 1–3 d. The sequences were processed, aligned and compared by IntelliGenetics PC-GENE analysis software.

Nucleic acid hybridization. Genomic DNA was blotted to a Schleicher & Schuell Nytran filter using a dot blot manifold essentially as described by Datta *et al.* (1987). A primer (ITS 187, 5'-CTGAAGAATGCAGTCTGAGAAC-3') was selected by examination of the *P. roqueforti* DNA sequence, and labelled by kination of [γ - 32 P]ATP (Amersham) according to the method of Sambrook *et al.* (1989). Hybridization was performed in a Hybaid hybridization oven. The filter was pre-hybridized for 3–5 h at 60 °C in 6× SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0), 1% (w/v) SDS, 10× Denhardt's solution (2 g Ficoll l⁻¹, 2 g polyvinylpyrrolidone l⁻¹, 2 g BSA l⁻¹) (0.5 ml cm⁻²) and 50 µg heterologous salmon sperm DNA (ml pre-hybridization buffer)⁻¹. Hybridization was carried out overnight at 50 °C in 6× SSC, 1% (w/v) SDS (0.1 ml cm⁻² filter) and 1 µl 32 P-labelled oligonucleotide (ml hybridization buffer)⁻¹. After hybridization, the filter was washed three times in 6× SSC, 1% (w/v) SDS, at room temperature for 1, 2 and 3 min, respectively, followed by washing at 55 °C for 5 min in 1× SSC, 1% (w/v) SDS. A Kodak X-OMAT AR film was exposed for 1–7 d at –80 °C with an intensifying screen.

DNA fingerprinting. Fingerprinting using RAPD was performed by a modification of the PCR procedure of Saiki (1990) using universal primers NS2, 3, 5 and 7 (White *et al.*, 1990) and the following modifications: 200 ng primer in 50 µl reaction mixture, 0.25% Tween 20, 10% (v/v) DMSO, 10 ng genomic DNA. Initial denaturation at 94 °C for 1 min was followed by 40 cycles of 94 °C for 10 s, 40 °C for 60 s, 72 °C for 60 s, on a Perkin Elmer Cetus thermal cycler model 9600. Finally, the temperature was kept at 72 °C for 10 min. *Taq* polymerase (Ampli Taq) from Perkin Elmer Cetus was used. The fingerprint was visualized by UV after running 10 µl PCR product on a 15 cm 2.0% (w/v) agarose gel for 4 h at 90 mA and staining with ethidium bromide. The fingerprinting technique was performed at least three times for each isolate.

Secondary metabolite profiles. Extracts of strains grown on four different media were analysed by HPLC with diode array detection for secondary metabolites using the methods described by Frisvad & Thrane (1987, 1993) and Svendsen & Frisvad (1994).

RESULTS

Sequence analysis of *Penicillium* isolates

The 600 bp region between the 18S and 28S genes including the 5.8S gene were amplified and sequenced from 10 strains each of *P. roqueforti* var. *roqueforti* and *P. roqueforti* var. *carneum* as previously defined. The sequences were compared to the sequence of *P. crustosum* (see Fig. 1). The 10 *P. roqueforti* var. *carneum* isolates were clearly separated into two distinct groups, each of five isolates. Five isolates (*P. carneum* as described here) including the culture ex-type [Institute of BioTechnology (IBT) no. 6886/International Mycological Institute (IMI) no. 293204] bore a strong sequence resemblance to the *P.*

Fig. 2. Dot blot of 45 fungi using labelled ITS 187 as probe hybridizing to 10 isolates of *P. roqueforti* (nos 4, 7, 9, 19, 22, 33, 36, 37, 45 and 47), and to five *P. carneum* isolates (nos 1, 11, 16, 29 and 41). No signal was obtained from five isolates of *P. paneum* (nos 13, 25, 32, 35 and 43), *Aspergillus terreus* (nos 8 and 28), *A. flavus* (nos 14 and 34), *A. versicolor* (nos 3 and 39), *A. candidus* (nos 20 and 44), *Paecilomyces variotii* (nos 6 and 31), *Scopulariopsis brevicaulis* (nos 5 and 17), *Geotrichum candidum* (nos 27 and 46), *P. commune* (nos 2 and 40), *P. crustosum* (nos 15 and 30), *P. camemberti* (nos 18 and 42), *Hyphopichia burtonii* (nos 12 and 23), *Eurotium repens* (no. 10), *Fusarium avenaceum* (no. 24) and *F. poae* (no. 26) are included. *Lactococcus lactis* was used as a bacterial control (nos 21 and 38). Hybridization in the area around dot number 38 is classified as background as the dots have been applied using a symmetrical dot blotting manifold.

roqueforti isolates, and the other five isolates (*P. paneum* as described here) were very similar to *P. crustosum* (Fig. 1). All the isolates within each of the three groups had identical sequences, except for a single position (no. 493) in a *P. paneum* isolate (IBT 13321) where a C was replaced with a T. As can be seen in Fig. 1, sequence differences among the three species were found in a total of 13 positions. In 11 of these, *P. roqueforti* and *P. carneum* were identical and different from *P. paneum*. In 9 of these 11 positions, *P. paneum* was identical to *P. crustosum*. In one position (no. 147), *P. carneum* and *P. paneum* were identical (C) and different from *P. roqueforti* (T). A parallel study covering 28 species of *Penicillium* (unpublished data) showed variation between a C and a T in that position. In one position (no. 493), *P. paneum* uniquely exhibited a C compared to other sequenced isolates from *Penicillium* subgenus *Penicillium*, however, this was the position where one of the isolates (IBT 13321) differed from the others in having a T rather than a C. In eight positions, the three sequenced penicillia were identical but differed from *P. crustosum*.

Comparison of the *P. roqueforti* group with other fungi

To evaluate the specificity of the differences observed between *P. roqueforti* and *P. carneum* when compared to other fungi, a specific primer spanning the region from

Fig. 3. RAPD analysis comparing 10 isolates of *P. roqueforti* (lanes 1–10), five of *P. carneum* (lanes 11–15) and five of *P. paneum* (lanes 16–20) using either NS2 (a) or NS7 (b) as primer. The order of the isolates are as listed in Table 1. Numbers indicate approximate sizes in kb of corresponding DNA fragments.

pos. 192 to 213 was prepared and used in hybridization against a series of fungi. The probe hybridized only to the *P. roqueforti* and *P. carneum* isolates, indicating that these nucleotide substitutions are probably unique to that group of fungi (Fig. 2).

Characterization of the three groups using RAPD

To evaluate the extent of sequence variation between the 20 isolates, RAPD fingerprinting analysis was performed using the universal primers NS2, NS7, NS3 and NS5 (White *et al.*, 1990). The 10 *P. roqueforti* isolates showed extensive similarity within the group, i.e. a 0.9 kb band using NS2 or a 2.3 kb band using NS7 (see Fig. 3). Some similarity was found between *P. roqueforti* and *P. carneum*, e.g. the 1.1 kb band using NS2 (Fig. 3a), but the degree of similarity is larger between *P. carneum* and *P. paneum* (i.e. the 1.6 kb and the 0.7 kb bands using NS7) than it is to *P. roqueforti*. Unique bands were also observed within *P. roqueforti* and *P. paneum* using NS5 (data not shown). *P. roqueforti* appears to be separable into two subgroups, one comprising isolates found as starter cultures all producing

a 0.9 kb band with NS7 (Fig. 3b), and one comprising four natural isolates and the type culture isolated in 1904 (Pitt, 1979).

Biochemical characterization

As *P. carneum* seemed closely related to *P. roqueforti* based on ITS sequences, it was decided to investigate the production of secondary metabolites from the three groups of fungi. The results of the HPLC analysis of the 20 isolates showed a clear separation into three groups. Only the *P. roqueforti* isolates produced marcfortines and fumigaclavine A, only the *P. carneum* isolates produced metabolite W and penitrem A and only the *P. paneum* isolates produced metabolite NB, metabolite AR, botryodiploidin and metabolite QQ. In addition, metabolite A was produced by all isolates, patulin only by *P. carneum* and *P. paneum* and roquefortine C and mycophenolic acid by *P. carneum* isolates plus some *P. roqueforti* isolates (see Table 2). In comparison, *P. crustosum* produced terrestrial acid, roquefortine C, viridicatin and penitrem A (Frisvad & Filtenborg, 1989; Frisvad & Thrane, 1995). In a similar

Table 2. Secondary metabolites produced by *P. roqueforti* and the two related species

X indicates that a given metabolite was detected by HPLC. Metabolites A, W, NB, AR, QQ and CAN 1-4 are all unknown secondary metabolites with characteristic UV spectra.

Metabolite	<i>P. roqueforti</i>										<i>P. carneum</i>					<i>P. paneum</i>				
	14429	14431	914430	914433	914432	14408	14412	14420	14425	6754	3477	6885	6753	14042	6884	12407	11839	12392	13321	13929
PR toxin		X	X	X	X	X	X	X	X	X										
Roquefortine C		X	X	X	X	X	X	X	X	X	X	X	X	X	X					
Marcfortines	X	X	X	X	X	X	X	X	X	X										
Metabolite A	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Fumigaclavine A	X	X	X	X	X	X	X	X	X	X										
Mycophenolic acid						X	X	X	X	X	X	X	X	X	X					
Patulin											X	X	X	X	X	X	X	X	X	X
Cyclopaldic acid											X	X		X	X					
Metabolite W											X	X	X	X	X					
Penitrem A											X	X	X	X	X					
Penicillic acid													X							
Metabolite CAN 1-4																X	X	X	X	X
Metabolite NB																X	X	X	X	X
Metabolite AR																X	X	X	X	X
Botryodiploidin																X	X	X	X	X
Metabolite QQ																X	X	X	X	X

study where volatile secondary metabolites were investigated, Larsen & Frisvad (1995a, b) found clear differences between these three groups of fungi.

The older strains of *P. roqueforti*, including ex-type cultures of *P. gorgonzolae* and *P. conservandi*, produced the profile of secondary metabolites typical of *P. roqueforti*: PR toxin and associated metabolites, roquefortine C, mycophenolic acid (only NRRL 852 and NRRL 853) and metabolite A (data not shown).

Taxonomic description of new species

Based on the results reported above, we consider *P. roqueforti* var. *roqueforti* and *P. roqueforti* var. *carneum* to be so different that the latter taxon should be raised to species status.

Description of *Penicillium carneum* Frisvad comb. nov.

P. carneum Frisvad comb. nov., Basionym *P. roqueforti* Thom var. *carneum* Frisvad (Frisvad & Filtenborg, 1989, p. 858). Type strain: Herb. IMI 293204.

Conidia globose to slightly subglobose, smooth-walled (3.5–5.5 µm), phialides ampulliform, 8–11.5 × 2.8–3.3 µm, metulae 11–15 × 3–4.5 µm, rami 15–35 × 3–4.5 µm, 1 per stipe, stipes 100–200 × 4–5.5 µm. Sclerotia not present. Odour very characteristic, caused by consistent production of large amounts of 3-methyl-1-butanol and geosmin (Larsen & Frisvad, 1995a, b). Roquefortine C, mycophenolic acid, metabolite A, cyclopaldic acid, patulin

and penitrem A produced, the former three in common with isolates of *P. roqueforti*. Rather fast-growing colonies on all media: CYA, 34–52 mm, MEA, 35–56 mm, YES, 47–74 mm. Good growth on CREA, with nil or weak acid production. Very good growth on CYA with 0.5% acetic acid. Colony reverse on CYA cream to beige to pale brown and on YES creamish-yellow to pale brown. Colony margin entire and not arachnoid as in *P. roqueforti*. Conidium colour dark bluish-green on CYA and bluish-green on YES (as opposed to the pure green conidium colour of *P. roqueforti* on YES agar).

Latin description of *Penicillium paneum* Frisvad sp. nov.

Diagnosis. *A Penicillio roqueforti* Thom differt conidiorum massa atroviridi, reverso pallido et patulino et botryodiploidino generans.

Coloniae in agar CYA dicto post 7 dies 25 °C 39–55 mm diametro, velutinae, conidia atrovirenta, reversum eburneum vel spadiceum. Coloniae in agar malti post 7 dies 25 °C 45–67 mm diametro. Coloniae in agar YES dicto post 7 dies 25 °C 55–74 mm diametro, conidia veneta. 37 °C haud crescit. In CREA composito crescit. Conidiophora paecipue terverticillate, stipites 100–200 µm longi, 4–5.5 µm lati, tuberculati. Rami singuli, laterales, 15–35 × 3–4.5 µm, appressi, tuberculati. Penicilli appressi, 30–60 × 15–40 µm. Metulae binae ad quaternae, verticillatae, 11–15 × 3–4.5 µm, cylindricae. Phialides 4–8 verticillatae, 8–11.5 × 2.8–3.3 µm, ampulliformes, ad apicem distincte constrictae, collulo inconspicuo vel ad 1.5 µm longo. Conidia globosa vel subglobosa, 3.5–5.5 µm diametro. Producta secundaria accumulata: patulina et botryodiploidinum.

Typus: C 25000 (*Museum Botanicum Hauniense*) (IBT 12407).

Description of *Penicillium paneum* Frisvad sp. nov.

Conidia globose to slightly subglobose, smooth walled (3.5–5.5 µm), phialides ampulliform, 8–11.5 × 2.8–3.3 µm; metulae 11–15 × 3–4.5 µm, rami 15–35 × 3–4.5 µm, 1 per stipe, stipes 100–200 × 4–5.5 µm. Sclerotia not present. Metabolite A, patulin, botryodiploidin and a series of unknown secondary metabolites produced with characteristic UV spectra not seen in either of the other two species are produced. Metabolite A is also produced by isolates of *P. roqueforti*. However, rather fast growing colonies on all media: CYA, 39–55 mm, MEA, 45–67 mm, YES, 55–74 mm. Good growth on CREA, with nil acid production. Very good growth on CYA with 0.5% acetic acid. Colony reverse on CYA cream to beige, on YES creamish-yellow often with a pink centre. Colony margin entire as opposed to the arachnoid margin of *P. roqueforti*. Conidium colour dark bluish-green with a turquoise margin on CYA and bluish-grey-green on YES (as opposed to the pure green conidium colour of *P. roqueforti* on YES agar).

Type strain: C 25000 (fungal herbarium, Botanical Museum, Copenhagen) (living culture IBT 12407).

DISCUSSION

By complete sequencing of the ITS region of 10 isolates belonging to *P. roqueforti*, it was confirmed that this region was conserved within the group. An initial comparison between *P. roqueforti* and two *P. roqueforti* var. *carneum* isolates revealed a number of differences. However, only five of the 10 *P. roqueforti* var. *carneum* isolates subsequently sequenced showed these sequence differences and these isolates are now named *P. paneum*. The other five isolates, now named *P. carneum*, bear a much stronger resemblance to the *P. roqueforti* isolates.

It is noteworthy that *P. carneum* and *P. paneum* are indistinguishable using traditional morphological and physiological characters, but are nonetheless separable by a total of 12 nucleotide differences using the ITS region, whereas the difference between two well-characterized species such as *P. camemberti* and *P. echinulatum* in DNA terms amounts to no more than one character (unpublished data). Of the 12 differences observed in the 600 bp region, eight were located in the ITS I region within a stretch of 85 bp. The two differences that separate *P. roqueforti* from *P. carneum* are both found within the ITS I region.

It has previously been argued that a single base-pair difference within the ribosomal DNA region is sufficient to distinguish between two species (Logrieco *et al.*, 1990). The data presented here clearly demonstrate the potential risks associated with comparing the ribosomal DNA sequences of just one or two isolates of each species or variety. However, when DNA sequence data are used in combination with other characters (in this case secondary

metabolite profile and DNA fingerprinting), it is undoubtedly a very powerful tool.

Little or no variation was detected using either ribosomal DNA sequencing or HPLC analysis of metabolites within a species, as almost all the isolates within any species appeared to produce the same profile of secondary metabolites and to have the same ribosomal DNA sequence. Also, the RAPD fingerprinting profile showed qualitative as well as quantitative similarities among individual isolates within each species. The molecular structure of the characteristic metabolites produced by *P. paneum* has not been elucidated in this study. However, the UV spectrum of the secondary metabolites indicates that these molecules are very different from those produced by the other two species.

Two monographs (Raper & Thom, 1949; Pitt, 1979) and one paper (Samson *et al.*, 1977) discuss the taxonomy of *P. roqueforti*. These monographs list few ex-type cultures of other species regarded as synonyms of *P. roqueforti* such as *P. stilton* Biourge, *P. atroviride* Sopp *et al.*, as they are not available in any culture collection. The only available ex-types were those of *P. gorgonzolae* Weidemann and *P. conservandi* Novobranova, but these were typical of *P. roqueforti*. Most other strains examined by Raper & Thom (1949), Samson *et al.* (1977) and Pitt (1979) were typical of *P. roqueforti* with a black-green colony reverse.

The problem of varieties versus species has been discussed by many biologists, but authors of *Penicillium* monographs have generally raised varieties to species level (Pitt, 1979) or first used them (Samson *et al.*, 1976) and later discontinued their use (Samson *et al.*, 1995). Rogers (1989) suggested that varieties were indicated if there was one extra biosynthetic pathway in a group as compared with the species, but species level was indicated if more than one biosynthetic pathway or morphological character differed between the isolates. According to Rogers' (1989) definition, the three groups treated here must be species, as the number of correlated differences are even larger than required by Rogers (1989). Brasier & Rayner (1987) even proposed the abolition of the term variety in mycology.

This study has shown that a unique species (*P. roqueforti*) used for food production can be divided into three distinct species. We propose a name, *P. carneum*, for the species including the type culture for that previously known as *P. roqueforti* var. *carneum*, and the name *P. paneum* for the third species in the group.

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