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

Marianne Boysen,¹ Pernille Skouboe,¹ Jens Frisvad² and Lone Rossen¹

Author for correspondence: Lone Rossen. Tel: +45 45 87 66 99. Fax: +45 45 93 28 88. e-mail: lro@bio.atv.dk

*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, marcraftines 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 botryodiplodioidin. 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).

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 conidiphore 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, isofumigaiacalvina 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 nontoxic 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 mycotoxicoses observed (Häggblom, 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. gorgonae), 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 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**

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

*Type culture.
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'-TCCCTCGCTATGGATATGC) and ITS 5 (5'-GGAGATCTAAAGTCTAGAACAAGG), 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 60 s. 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 (x-32P)ATP (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 x 40 x 0.04 cm) using wedged spacers. The gels were judged and exposed to Kodak X-Oмат 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'-CTGAGATGCGTCTGAGA-3') was selected by examination of the P. roqueforti DNA sequence, and labelled by kination of [y-32P]ATP (Amersham) according to the method of Sambrook et al. (1989). Hybridization was performed in a Hybridization oven. The filter was pre-hybridized for 3–5 h at 60 °C in 6X SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0), 1% (w/v) SDS, 10 x Denhardt's solution (2 g Ficoll 1%, 2 g polyvinylpyrrolidone 1%, 2 g BSA 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 6X SSC, 1% (w/v) SDS (0.1 ml cm-2 filter) and 1 µl 32P-labelled oligonucleotide (ml hybridization buffer)-1. After hybridization, the filters were washed three times in 6X 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 1X 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. Tag polymerase (AmpliTaq) from Perkin Elmer Cetus was used. The fingerprint was visualized by UV after running 10–20% 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] and Mycological Institute (IMI) no. 293204] bore a strong sequence resemblance to the P. roqueforti isolates, and the other five isolates (P. panum 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. panum 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 was identical to P. crustosum, and P. carneum was identical and different from P. panum. In 9 of these 11 positions, P. carneum was identical to P. crustosum. In one position (no. 147), P. carneum and P. panum 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. panum uniquely exhibited a C compared to other sequenced isolates from Penicillium subgenus Penicillum, 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. 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. panum (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), Paeclomyces 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.
P. roqueforti comprises three different species

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. carneum 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 terrestri acid, roquefortine C, viridicatin and penitrem A (Frisvad & Filtenborg, 1989; Frisvad & Thrane, 1995). In a similar

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

<table>
<thead>
<tr>
<th>Metabolite</th>
<th><em>P. roqueforti</em></th>
<th><em>P. carneum</em></th>
<th><em>P. paneum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>PR toxin</td>
<td>X X X X X X X X</td>
<td>X X X X X X</td>
<td></td>
</tr>
<tr>
<td>Roquefortine C</td>
<td>X X X X X X X X</td>
<td>X X X X X X</td>
<td></td>
</tr>
<tr>
<td>Marcornitine</td>
<td>X X X X X X X X</td>
<td>X X X X X X</td>
<td></td>
</tr>
<tr>
<td>Metabolite A</td>
<td>X X X X X X X X</td>
<td>X X X X X X</td>
<td></td>
</tr>
<tr>
<td>Fumiglactin A</td>
<td>X X X X X X X X</td>
<td>X X X X X X</td>
<td></td>
</tr>
<tr>
<td>Mycophenolic acid</td>
<td>X X X X X X</td>
<td>X X X X X X</td>
<td></td>
</tr>
<tr>
<td>Patulin</td>
<td>X X X X X X X X</td>
<td>X X X X X X</td>
<td></td>
</tr>
<tr>
<td>Cyclopaldic acid</td>
<td>X X X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolite W</td>
<td>X X X X X X X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penitrem A</td>
<td>X X X X X X X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillic acid</td>
<td>X X X X X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolite CAN 1-4</td>
<td>X X X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolite NB</td>
<td>X X X X X X X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolite AR</td>
<td>X X X X X X X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Botryodiploidin</td>
<td>X X X X X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolite QQ</td>
<td>X X X X X X X X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

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. gorgonzeae* 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.**


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.

**P. roqueforti** comprises three different species

**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, metulæ 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, botryodiplodin 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-characterised 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 1 region within a stretch of 85 bp. The two differences that separate *P. roqueforti* from *P. carneum* are both found within the ITS 1 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 lists few ex-type cultures of other species regarded as synonyms of *P. roqueforti* such as *P. stilton* Biourge, *P. atroviride* Sopp, etc., as they are not available in any culture collection. The only available ex-types were those of *P. gorgonolae* 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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