Phylogenetic analysis of nucleotide sequences from the ITS region of terverticillate *Penicillium* species

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The genetic variability within 600 bp of DNA sequence from the ribosomal internal transcribed spacers (ITS 1 and ITS 2) and the 5·8 S rRNA gene was examined in 52 strains belonging to 29 terverticillate taxa in *Penicillium* subg. *Penicillium* from diverse sources and locations. The sequenced region is extremely conserved within the terverticillate penicillia, with only 29 positions differing in one or more taxa. The highest degree of ITS variability was seen among the species close to *Penicillium roqueforti* (*P. roqueforti*, *P. carneum* and *P. paneum*) whereas the ITS variability was very low between closely related taxa, e.g. among the taxa near *P. aurantiogriseum*. The relationships among the terverticillate penicillia, and related teleomorphs in *Eupenicillium*, were analysed from bootstrapped ITS sequence data sets using the neighbour-joining method. The terverticillate penicillia form a well supported clade with *Eupenicillium crustaceum*. Sequence analysis generally confirmed the overall taxonomy in *Penicillium* subg. *Penicillium* but relationships between all the terverticillate taxa could not be clearly established due to the low degree of ITS variability. Nevertheless, clades of *Penicillium* species sharing environmental characteristics did emerge, e.g. species growing on protein and lipid rich substrates (e.g. *P. crustosum* and *P. commune*), species prevalent in dry habitats (e.g. *P. chrysogenum* and *P. nalgiovense*), and those prevalent on carbohydrate rich substrates (e.g. *P. aurantiogriseum* and *P. freii*).

The terverticillate taxa in *Penicillium* subg. *Penicillium* (Pitt, 1979) (= section *Asymmetrica*, subsection *Fasciculata*, Raper & Thom, 1949) [hereafter 'terverticillate penicillia'] are characterized by producing terverticillate (and in some cases quaterverticillate) penicilli. The subgenus is very widespread and economically important, and includes common food and feed spoilage fungi as well as industrial starter cultures for fermented meat production (*P. chrysogenum* and *P. nalgiovense*) and for fermentation of cheese (*P. roqueforti* and *P. camemberti*). The taxonomy of *Penicillium* species has traditionally been based on morphology (Samson, Stolk & Hadlok, 1976; Pitt, 1979), but it is very difficult to classify and identify the food-borne terverticillate penicillia referable to *Eupenicillium* because of their morphological similarity. In addition to morphological comparison, several other approaches have been used. Cruickshank & Pitt (1987) developed a technique based on isoenzyme electrophoresis for classification of subg. *Penicillium* species and profiles of secondary metabolites have been used as consistent taxonomic characters for separating the terverticillate penicillia (Frisvad & Filtenborg, 1983, 1989; Svendsen & Frisvad, 1994; Larsen & Frisvad, 1995; Smedsgaard & Frisvad, 1997).

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In recent years, molecular techniques exploiting variations in nuclear and mitochondrial rDNA have been used extensively for phylogenetic and systematic studies in fungi, including *Penicillium*. Different regions of the rDNA diverge at different rates, allowing their sequences to be exploited at different taxonomic levels (Bruns, White & Taylor, 1991; Seifert, Wingfield & Wingfield, 1995). The non-coding rDNA regions, especially the ITS 1 and ITS 2 region but also to a lesser extent the IGS region, are generally more variable than the rRNA genes and have been most widely used for taxonomic studies near the species level (Bruns *et al*., 1991; Bainbridge, 1994). For example, species-specific variation within ITS regions have been determined across a broad range of *Phytophthora* species (Lee & Taylor, 1992; Cooke & Duncan, 1997) and between several phytopathogenic and saprotrophic *Colletotrichum* species (Sreenivasaprasad *et al*., 1996). Furthermore, nucleotide sequence data from the ITS 1–5·8S–ITS 2 region have been used for development of specific oligonucleotide primers for PCR identification of the human pathogenic *Penicillium marneffei* (LoBuglio & Taylor, 1995) and for detection of basidiomycetes (Gardes & Bruns, 1993). Some species complexes have, however, not yet been resolved using ITS sequences. Carbone & Kohn (1993) revealed very little variation in the ITS 1 region between closely related species of the Sclerotiniaceae, whereas the ITS 1 region exhibited sufficient variability for studying intra-

specific variation within plant pathogens such as *Fusarium* (O'Donnell, 1992), *Leptosphaeria* (Xue, Goodwin & Annis, 1992) and *Seiridium* (Viljoen, Wingfield & Wingfield, 1993).

Molecular methods have been used to improve the understanding of the natural taxonomy of *Penicillium* and its teleomorphs *Eupenicillium* and *Talaromyces*. Comparison of nucleotide sequence data from the ITS spacers and D1–D2 region of the 28S rRNA gene has suggested a close phylogenetic relationship between the terverticillate penicillia and related teleomorphs in *Eupenicillium* (Peterson, 1993), similar to the close phylogenetic relationship existing between the strictly anamorphic species in *Penicillium* subg. *Biverticillium* and *Talaromyces* species (LoBuglio, Pitt & Taylor, 1993).

In the present study, we have used sequence data from the rDNA ITS regions to characterize 29 terverticillate penicillia in order to identify species-specific differences and to examine the phylogenetic relationship (i) among these taxa, and (ii) between terverticillate penicillia and related teleomorphs in *Eupenicillium*.

MATERIALS AND METHODS

Fungal strains

The isolates of terverticillate penicillia, *Penicillium* subg. *Furcatum*, *Eupenicillium*, and *Aspergillus* teleomorphs, used in this study are listed in Table 1. The isolates are maintained at IBT Culture Collection, Department of Biotechnology, Technical University of Denmark, Lyngby, Denmark. Representative species of the terverticillate penicillia were selected based on comparison with cultures ex type. Whenever possible at least two isolates of each taxon were used for rDNA sequencing (Table 1).

Growth conditions

Fungi were grown in Czapek yeast autolysate (Pitt, 1979) (CYA) broth (Difco Laboratories, Detroit, MI) supplemented with trace elements (Frisvad & Filtenborg, 1983). The cultures were incubated in the dark at 25 °C for 3 d in a rotary shaker (200 rpm) using 500 ml Erlenmeyer baffled flasks containing 200 ml of medium.

Isolation of genomic DNA

Total fungal DNA was isolated by a procedure modified from Yelton, Hamer & Timberlake (1984). The mycelium was harvested by filtration, washed several times with 0·9% NaCl or distilled sterile water, aliquoted (500 mg, wet weight) to Eppendorf tubes, and stored at −80°. For extraction of high molecular weight DNA, an aliquot of frozen mycelium was manually crushed in the Eppendorf tube using a glass spatula. This step was repeated twice while the mycelium was frozen in dry ice/ethanol in between. The pulverized mycelium was mixed with 800 µl extraction buffer (50 mM EDTA, pH 8·5; 0·2% SDS) and incubated at 68° for 30 min. After cooling to room temperature and centrifuging (10000 ***g*** for 15 min), the supernatant was transferred to another tube and 1/10 vol. of 5 M K acetate was added by mixing gently. The samples were placed on ice for 1 h and then centrifuged for 15 min (20000 ***g***, 4°C). The supernatant was transferred to a fresh tube and the DNA precipitated with an equal volume of isopropanol at room temperature. The precipitated DNA was pelleted by centrifugation (10000 ***g*** for 15 min) and washed with 80% ethanol. The pellet was dried and resuspended in 50 µl of TE buffer (10 mM Tris/HCl; pH 8, 1 mM EDTA). An aliquot (2 µl) was run on a 1% (w/v) agarose gel stained with ethidium bromide for estimation of DNA concentration.

PCR amplification of ribosomal DNA regions

The ITS 1–5·8S-ITS 2 region was amplified by PCR using the primers ITS4 and ITS5 essentially as described by White *et al*. (1990). Each PCR reaction mixture contained 5 µl of genomic DNA (5–10 ng), 1 µM each of primers ITS4 and ITS5, reaction buffer (50 mM KCl, 50 mM Tris/HCl; pH 8·3, 0·1 mg/ml bovine serum albumin), 3 mM $MgCl_2$, 200 µM each of dNTP, 10% dimethylsulphoxide (DMSO), 0·25% Tween 20 and 2·5 U of *AmpliTaq* DNA polymerase (Perkin-Elmer Corp., Norwalk, CT, U.S.A) in a total volume of 50 µl. Alternatively, asymmetric PCR reactions were performed by using the asymmetric primer ratio method (Gyllensten & Erlich, 1988), i.e. using primer ratios of 50:1 generating single-stranded templates of both strands in separate reactions. Amplification was performed in a Perkin Elmer DNA thermal cycler 480 or a GeneAmp PCR system 2400 model (Perkin-Elmer Corp., Norwalk, CT, U.S.A.). The temperature cycling parameters were denaturation at 93° for 30 s followed by primer annealing at 53° for 30 s and primer extension at 72° for 1 min for a total of 40 cycles plus a final 10 min elongation step at 72°. Following amplification, excess primers and deoxynucleotide triphosphates were removed from symmetric PCR reactions using MicroSpin S-400 HR Columns (Pharmacia Biotech, Uppsala, Sweden). The asymmetric PCR products were precipitated in 2·5 M ammonium acetate plus 2 vol of 100% ethanol for 10 min at room temperature and pelleted in a microfuge (10000 ***g***, 10 min at room temperature). The asymmetric PCR products were resuspended in 17 µl of TE buffer.

DNA sequencing

The primers ITS1, ITS2, ITS3, ITS4 (for primer sequences see White *et al*., 1990) and ITS6 (5′-GGATCATTACCGAGTGAG) were used for direct sequencing of the amplified fragments in both directions. Using asymmetric PCR products as templates, the ITS 1–5·8S–ITS 2 region was sequenced on both strands according to the Sanger dideoxy method (Sanger, Nicklen & Coulsen, 1977) and the Sequenase kit Version 2.0 (U.S. Biochemicals, Cleveland, Ohio) using ^{35}S-labelled dATP (Amersham Int., Little Chalfont, U.K.) according to the manufacturer's instructions with the following modifications of the initial step: approximately 1 µg of template and 50 ng of primer was mixed with 0·7 µl of 1 M NaOH and dH_2O to a total vol. of 10 µl. After denaturing at 68° for 10 min, 2·8 µl of TDMN containing in final concentration: 0·28 M TES (Sigma Chemical Co. No. T-4152), 0·12 M HCl,

Table 1. List of isolates of terverticillate penicillia, and outgroup species representing *Penicillium* subg. *Furcatum*, *Eupenicillium*, and *Aspergillus* teleomorphs, included in this study

	IBT number[1]	Source	Geographical origin	EMBL accession number[2]
P. albocoremium (Frisvad) Frisvad & Samson	10682*	Salami	Denmark	AJ004819
P. allii Vincent & Pitt	3056	Food item	Great Britain	AJ005484
	3058	Garlic	Denmark	AJ005484
P. aurantiogriseum Dierckx	6215	Barley	Denmark	AJ005488
	10047	Sunflower seed	Denmark	AJ005488
P. aurantiovirens Biourge	11330	Barley	Denmark	AJ005490
P. camemberti Thom	3505	Cheese	France	AJ004814
	11568	Camembert cheese	Germany	AJ004814
P. carneum (Frisvad) Frisvad	6884*	Rye bread	Denmark	X82359
P. chrysogenum Thom	3182	Volcano dust	Italy	AJ004812
	5848	Sesame seed	Korea	AJ004812
P. commune Thom	6327	Turnip	Denmark	AJ004813
	10763	Cheese	France	AJ004813
	6359	Cheese	Denmark	AJ004813
P. crustosum Thom	13049	Rye bread	Denmark	X82361
	13769	Cheese	Denmark	X82361
P. cyclopium Westling	5311	*Amaranthus* flower	U.S.A.	AJ005491
	10085	Oats	Denmark	AJ005491
P. dipodomyis (Frisvad, Filt. & Wicklow) Banke, Frisvad & S. Rosend.	5324	Seed cache of kangaroo rat	U.S.A.	AJ004896
P. discolor Frisvad & Samson	3086	Jerusalem artichoke	Denmark	AJ004816
	3087*	Radish	Israel	AJ004816
P. echinulatum Raper & Thom ex Fassat.	3171	Pomelo	Israel	AJ004815
	3238	Mouldy gravy	Denmark	AJ004815
P. freii Frisvad & Samson	3464	Wheat	Great Britain	AJ005479
	11306	Barley	Denmark	AJ005479
P. hirsutum Dierckx	10623	Rotten apple	Czech Republic	AJ004818
	10628*	Aphid	Netherlands	AJ004818
P. hordei Stolk	3083	Wheat	Denmark	AJ004817
	4900	Greenhouse fern	Netherlands	AJ004817
P. melanoconidium (Frisvad) Frisvad & Samson	3442	Barley	Denmark	AJ005483
	6794	Salami	Germany	AJ005483
P. nalgiovense Laxa	5746	Desert sand	U.S.A.	AJ004894
	12108	Cheese	Denmark	AJ004895
P. neoechinulatum (Frisvad, Filt. & Wicklow) Frisvad & Samson	3493	Seed cache of Kangaroo rat	U.S.A.	AJ005481
	3462	Seed cache of Kangaroo rat	U.S.A.	AJ005481
P. paneum Frisvad	12392*	Chocolate sauce	Norway	X82360
P. polonicum K. M. Zalessky	11388	Barley	Denmark	AJ005492
	11239	Mixed feed	Norway	AJ005492
P. roqueforti Thom	12845	Cheese	Denmark	AJ005677
	12846	Pickled acid-preserved pumpkins	Denmark	AJ005677
P. sp. 1	12708	Kangaroo rat	New Mexico	AJ005480
P. sp. 2	12396	Kangaroo rat	New Mexico	AJ005493
P. sp. 3	10697	Soil	Denmark	AJ004820
P. tricolor Frisvad, Seifert, Samson & J. T. Mills	12471	Wheat	Canada	AJ005489
	12493	Wheat	Canada	AJ005489
	12494	Wheat	Canada	AJ005489
P. venetum (Frisvad) Frisvad & Samson	5464	Flower bulb	Denmark	AJ005485
	10594	Water (hot water tank)	Denmark	AJ005485
P. verrucosum Dierckx	5010	Barley	Denmark	AJ005486
	11621	Wheat	Canada	AJ005487
P. viridicatum Westling	5273	Barley	Denmark	AJ005482
	10057	Sesame seed	U.S.A.	AJ005482
P. coralligerum Nicot & Pionnat	17894 (= ATCC48993)	Soil	Canada	AJ010484
Eupenicillium crustaceum F. Ludw.	14680 (= CBS 183·72)	Soil	Netherlands	AJ004892
	14683 (= CBS 653·82)	Soil	India	AJ004892
Eupenicillium shearii Stolk & D. B. Scott	14694 (= CBS 488·66)	Soil		AJ004893
	14695 (= CBS 343·54)	Soil	Zaire	AJ004893
Petromyces muricatus Udagawa, Uchiy. & Kamiya	19374 (= IMI 368521)	Soil	Philippines	AJ005674
Petromyces albertensis J. P. Tewari	14317 (= UAHM 2476)	Ear swab	Canada	AJ005673

* ex. Type culture.

[1] IBT (Dept of Biotechnology) Culture Collection.

[2] ITS1–5·8S–ITS2 sequences are available through EMBL.

0·05 M DTT (dithiothreitol), 0·08 M $MgCl_2$, and 0·2 M NaCl was added (Del Sal, Manfioletti & Schneider, 1989). After 10 min of annealing at room temperature, the labelling and termination reactions were performed as described for the Sequenase kit Version 2.0. The reaction were run on a denaturing 7 M urea/6% polyacrylamide standard premixed 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 d. Using symmetric PCR products as templates, cycle sequencing were performed using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Int., Little Chalfont, U.K.) according to the manufacturers protocol, and analysed on an automated DNA sequencer (A.L.F. express, Pharmacia Biotech, Uppsala, Sweden).

Sequence analysis

Computer-aided alignment of the ITS sequences were initially performed using the CLUSTAL option in the IntelliGenetics software package, PC Gene Screen Device, Version 5.03 (IntelliGenetics, Inc., Mountain View, CA). We then used the program MacClade 3.05 (Maddison & Maddison, 1992) to improve the alignment by eye.

The data set had approximately 660 aligned nucleotide positions, some of which were scored as deletions or unknowns in one or more taxa. Phylogenetic analysis was performed with the software PAUP* (a prerelease version generously provided by D. Swofford, Smithsonian Institute of Natural History, U.S.A.) using neighbour joining and maximum likelihood distances (two substitution types with the ratio of transversions to transitions estimated from the data, empirical base frequencies, and starting branch lengths estimated by the Rogers–Swofford method). Bootstrapping was used to resample the data (1000 resamplings) and the proportion of neighbour-joining trees possessing internal branches was used to assess their support.

The data were also subjected to parsimony analysis. Heuristic analysis with 1000 repetitions using random taxon order was performed to increase the chance of finding the shortest tree. Bootstrapping was used to resample the data (1000 resamplings) and the proportion of consensus trees for each bootstrapped data set possessing internal branches was used to assess their support.

The ITS 1–5·8S–ITS 2 rDNA sequences of the terverticillate penicillia have been deposited at the EMBL Nucleotide Sequence Database and are identified by the accession numbers listed in Table 1. Strains belonging to the same taxa with identical ITS sequences are registered under the same accession number. The EMBL accession numbers for the outgroup species reported in this paper are *Penicillium coralligerum* (AJ010484), *Eupenicillium crustaceum* (AJ004892), *Eupenicillium shearii* (AJ004893), *Petromyces muricatus* (AJ005674), and *Petromyces albertensis* (AJ005673). Furthermore, the outgroup species (with corresponding GenBank accession numbers) *Neosartorya fischeri* (U18355), *Eupenicillium javanicum* (U18358), *Emericella nidulans* (L76746), *P. duclauxii* L14534), and *P. variabile* (L14507) were included in the study.

RESULTS AND DISCUSSION

Amplification of the ITS regions

Using the primers ITS4 and ITS5 (White *et al*., 1990) the ribosomal DNA region containing the ITS 1, ITS 2 regions and the 5·8S rRNA gene was amplified from 52 terverticillate *Penicillium* isolates belonging to 29 taxa (Table 1). Using asymmetric PCR amplification with molar excess of ITS4, or symmetric PCR amplification, the PCR product was in all cases a unique fragment of approximately 600 bp. Using molar excess of ITS5, however, some additional unspecific bands were amplified (data not shown).

Sequence analysis of the ITS regions

Alignment of the ITS 1–5·8S–ITS 2 sequences from the 52 *Penicillium* strains showed no nucleotide differences within the 5·8S rRNA gene. The number of sequence differences within the ITS regions was low, considering the amount of variation found in *Penicillium* subg. *Biverticillium* (LoBuglio *et al*., 1993) and other genera (O'Donnell, 1992). Comparison of the ITS sequences revealed only 29 nucleotide positions differing in one or more taxa, four of these were insertions or deletions (Fig. 1). Eighteen of these positions were found in the ITS 1 region, and the rest (11) in the ITS 2 region.

Several closely related taxa shared identical or nearly identical ITS sequences, e.g. *P. echinulatum* and *P. discolor* had identical ITS sequences as well as *P. commune* and *P. camemberti* (*P. camemberti* is a domesticated form of *P. commune*, a creatine positive species that has adapted to growth on cheese and other substrates high in protein). The 11 closely related taxa belonging to the *P. aurantiogriseum* group; *P. aurantiogriseum*, *P. freii*, *P. aurantiovirens*, *P. tricolor*, *P. polonicum*, *P. melanoconidium*, *P. viridicatum*, *P. cyclopium*, *P. neoechinulatum* and two unnamed species (sp. 1 and sp. 2) (Lund & Frisvad, 1994) could be differentiated from the other 18 terverticillate taxa by a T in position 70, while the three species from the *P. chrysogenum* group could be identified by a T in position 202 and a C in position 430 (Fig. 1). The largest number of sequence differences were seen between the species related to *P. roqueforti* (*P. roqueforti*, *P. carneum* and *P. paneum*) and the other terverticillate *Penicillium* species. Peterson (1993) found no differences in the D2 region between *P. roqueforti* and *P. crustosum*, but according to our analysis sequence differences are more pronounced in the ITS regions between selected taxa. On the contrary *P. crustosum* can be regarded as one of the closest relatives to *P. roqueforti* and *P. carneum* based on morphological, physiological and secondary metabolite data (Frisvad & Filtenborg, 1989).

No intraspecific ITS variation was detected among the taxa with the exception of the two species *P. verrucosum* and *P. nalgiovense* (Fig. 1), where a single nucleotide sequence difference was observed. No explanation could be found for the differences in these two taxa, however, intraspecific ITS variation has been reported in other fungi (Burt *et al*., 1996; Sreenivasaprasad *et al*., 1996).

Sequence analysis generally confirmed the results from morphology within the terverticillate penicillia, except that in some cases additional variation could be detected using other

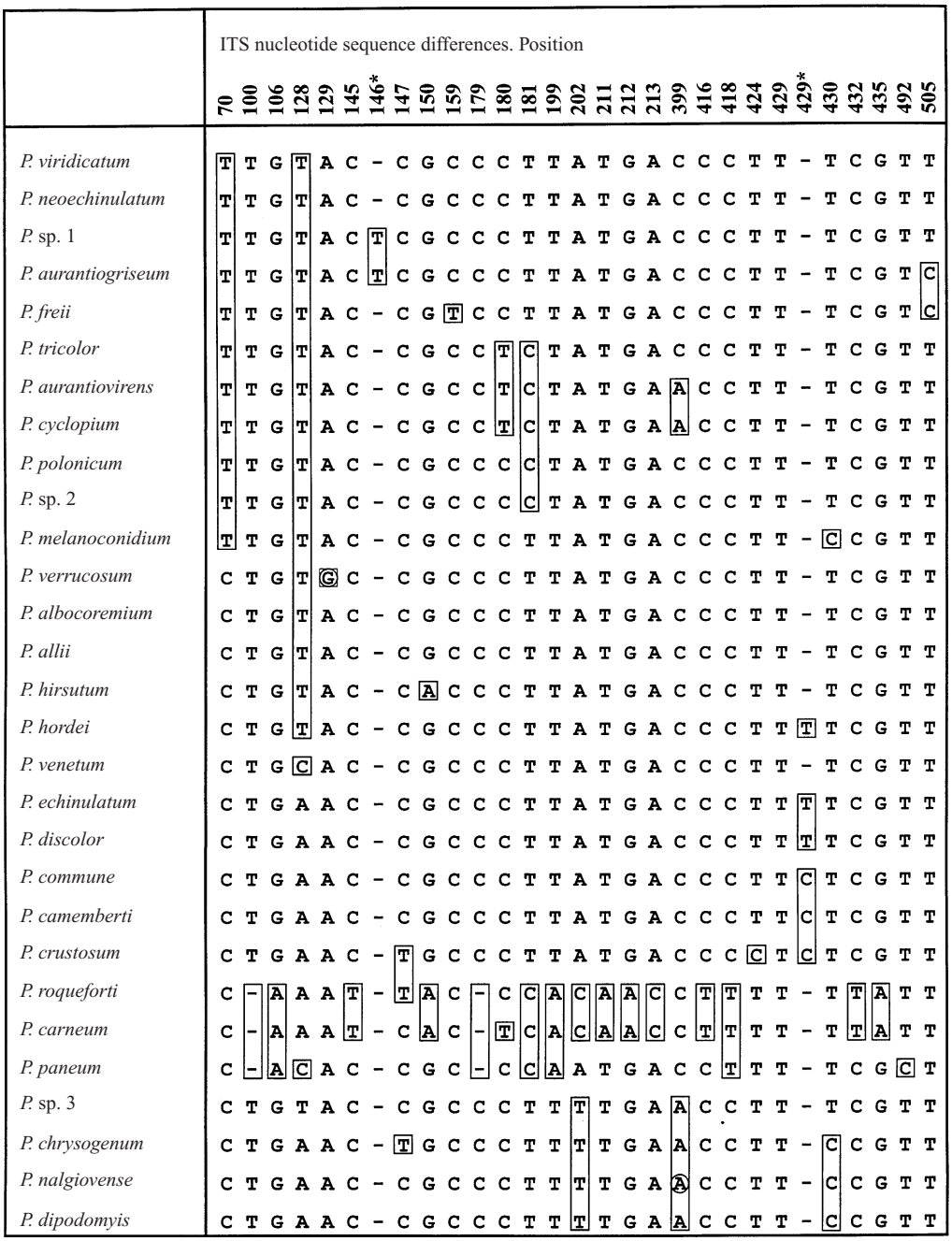

Fig. 1. Comparative sequence analysis of the ITS1–5·8S–ITS2 rDNA sequences from 29 terverticillate *Penicillium* taxa. The sequences were aligned and nucleotide differences noted. The matrix illustrates the total number of positions at which nucleotides differ between one or more taxa. Unique nucleotide sequence differences are boxed. Circles indicate the two nucleotides that vary between isolates belonging to the same taxa; for *P. verrucosum*, the sequence of IBT 5010 is shown, whereas IBT 11621 has an A at position 129; for *P. nalgiovense* the sequence of IBT 5746 is shown, whereas IBT 12108 has a C at position 399. A dash (—) indicates introduced gaps. Position no. 1 refers to the 5′ end of the conserved primer ITS 5 (White *et al.*, 1990). The 5·8 S rRNA gene is located between position no. 230 and 385. *: 146* and 429* indicates the insertion of an extra nucleotide immediately after position 146 and 429, respectively. Position 429 is included in the table as three of the insertions at position 429* are the same base (T) and it is, therefore, not possible to say which is the inserted one.

characters, i.e. taxa with identical ITS sequences are known to be different on the basis of secondary metabolite profiles and micromorphological characters.

Phylogenetic relationships

Phylogenetic relationships among penicillia were inferred from neighbour-joining and heuristic parsimony analysis of the aligned ITS sequences. The analysis showed that the topology of both types of trees was nearly identical and was identical for all branches with even 50% bootstrap support. The neighbour-joining trees are shown in Figs 2 and 3, because the branch lengths should be better estimates than those calculated for parsimony analysis. In the analysis of all taxa (Fig. 2), *P. duclauxii* and *P. variabile* were designated as outgroups based on comparisons of morphology and DNA

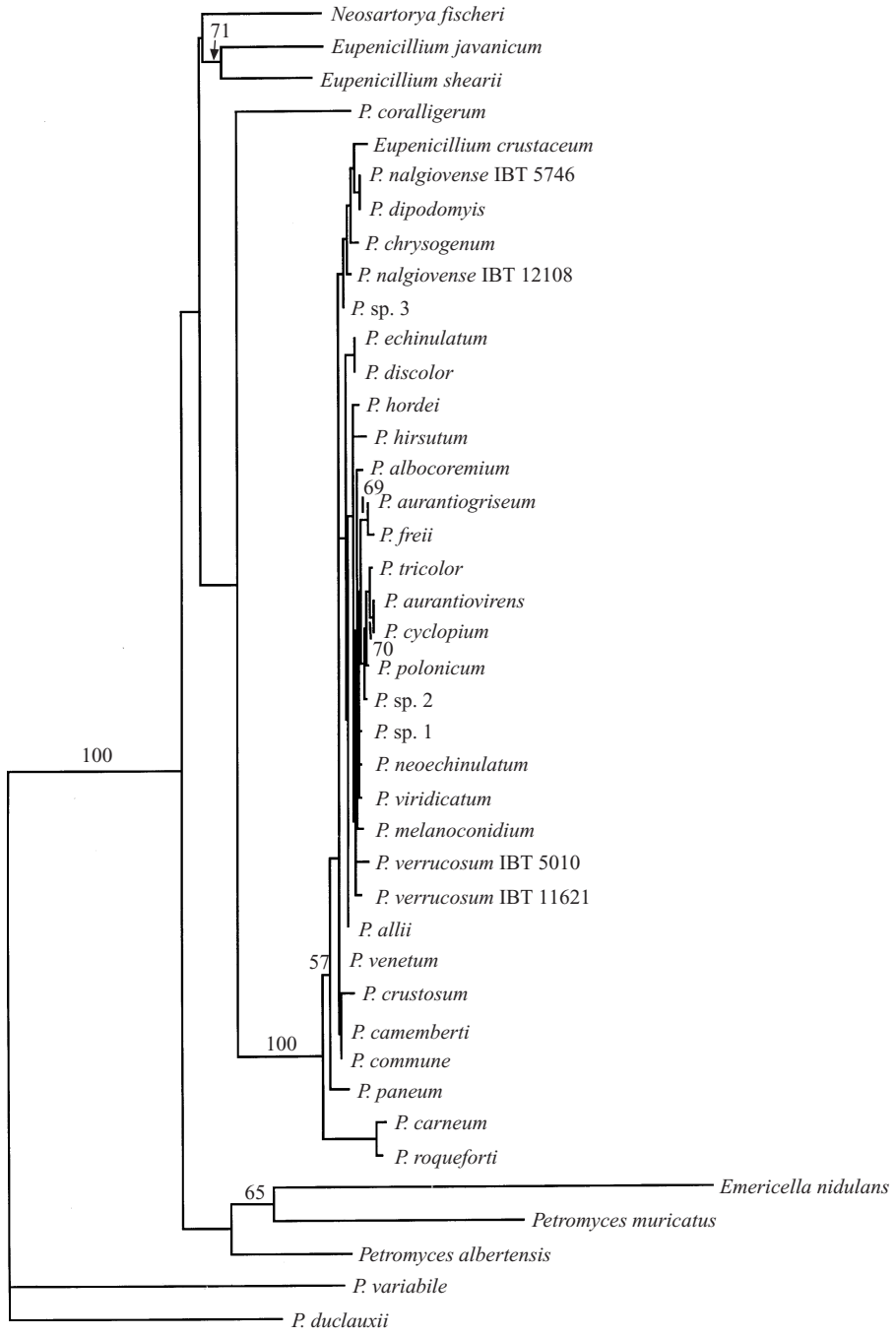

Fig. 2. Neighbour-joining tree based on phylogenetic analysis of the ITS1–5·8S–ITS2 rDNA sequences showing the relationships of terverticillate penicillia with all outgroup taxa. Numbers at branching points are percentages of 1000 bootstrapped data sets supporting the specific internal branches. Bootstrap values below 50% are not shown. *Penicillium duclauxii* and *P. variabile* were chosen to root the tree based on morphological data and 18 S rDNA sequence information (Berbee *et al.*, 1995).

sequence from 18S rRNA genes (Berbee *et al.*, 1995). Support for specific branches in the trees was assessed by the percentage of neighbour-joining trees of 1000 bootstrapped data sets possessing the branch. In Fig. 2, fungi with *Aspergillus* mitosporic anamorphs states are basal, including *Neosartorya fischeri* and a clade with *Emericella nidulans* and two *Petromyces* species. *Neosartroya fischeri* is the sister taxon to the *Penicillium* clade, with *Eupenicillium* species and *P. coralligerum* basal to the terverticillate penicillia. Adequate bootstrap support was, however, only found on the branches leading to the clade comprising the two *Petromyces* species. Removing the outgroup taxa representing *Penicillium* subg. *Biverticillium* (*P. duclauxii* and *P. variabile*) did not change the phylogenetic analysis significantly, except that high bootstrap support (91) was obtained on the branch leading to *P. coralligerum* (data not shown). The outgroups are now *Emericella nidulans* and the two *Petromyces* species. *Penicillium* subg. *Furcatum*, as represented by *P. coralligerum* (Frisvad & Filtenborg, 1990), is the sister group to a well supported clade of terverticillate penicillia plus *Eupenicillium crustaceum*, consistent with

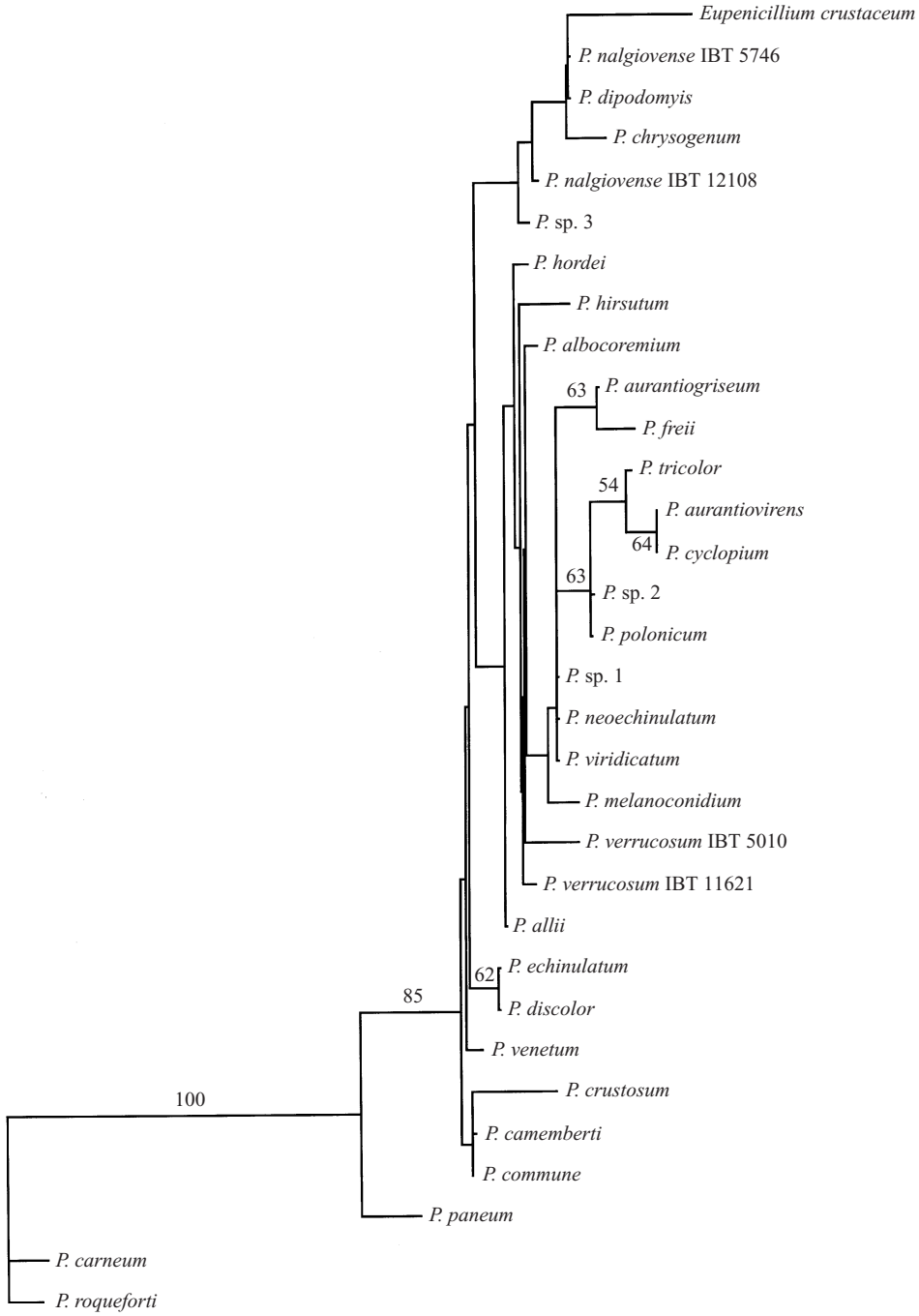

Fig. 3. Neighbour-joining tree based on phylogenetic analysis of the ITS1–5·8S–ITS2 rDNA sequences showing the relationships among 29 taxa of terverticillate penicillia and *Eupenicillium crustaceum* after removing all outgroup taxa included in Fig. 2. Numbers at branching points are percentages of 1000 bootstrapped data sets supporting the specific internal branches. Bootstrap values below 50% are not shown. A long branch leads to *P. carneum*, *P. roqueforti*, and *P. paneum*. This is the same branch that had the root in Fig. 2. In the main group of subg. *Penicillium* plus *E. crustaceum*, few clades are well supported.

Peterson (1993). Within the terverticillate species, there are two basal clades, one with *P. carneum*, *P. roqueforti*, and *P. paneum*, and the other with the remaining taxa.

To make available as much variation as possible within the terverticillate penicillia, all outgroup taxa were removed. Neighbour-joining analysis of the penicillia and *E. crustaceum* (Fig. 3) still left many ambiguities due to the few nucleotide substitutions distinguishing the terverticillate penicillia. A long branch leads to *P. carneum*, *P. roqueforti*, and *P. paneum*, a species related to *P. carneum* (Boysen *et al.*, 1996). This is the same branch that had the root in Fig. 2. In the main group of the subg. *Penicillium* plus *Eupenicillium crustaceum*, few clades are well supported, the best support being found for *P. echinulatum* plus *P. discolor* (62), *P. aurantiogriseum* plus *P. freii* (63), and *P. cyclopium* plus *P. aurantiovirens* (64). These are all closely related species (Lund & Frisvad, 1994; Frisvad *et al.*, 1997).

A number of species appeared less well supported in the bootstrap analysis; of these three groups of fungi reflecting the habitat of the species were apparent (Fig. 3). One clade,

comprising *P. crustosum*, *P. commune* and its domesticated form *P. camemberti* appears to be a similarity group found on substrates with high contents of lipids and protein (cheese, meat, nuts etc.) (Frisvad & Filtenborg, 1993). Another clade comprised *P. chrysogenum*, *P. nalgiovense* (both isolates) and *P. dipodomyis*, species that grow at low water activities and are often found in deserts (Frisvad & Filtenborg, 1989, 1993; Banke, Frisvad & Rosendahl, 1997). This clade also includes *E. crustaceum*. *Eupenicillium egyptiacum*, a species closely related to *E. crustaceum*, is reported to produce xanthocillin in common with *P. chrysogenum* (Turner & Aldridge, 1983) thus indicating a similarity between these eupenicillia and *P. chrysogenum*.

The last clade consisted of species in the *P. aurantiogriseum* group (Lund & Frisvad, 1994), which are species that grow in carbohydrate rich substrates such as cereals (Frisvad & Filtenborg, 1989, 1993). The remaining species were poorly resolved in the phylogenetic analysis and more sequence data from other regions may help elucidate their proper phylogenetic status. They include other cereal-borne taxa such as *P. verrucosum* and taxa associated to onions and bulbs such as *P. allii*. Within most of the clades, the species in the same clade produced some secondary metabolites in common, i.e. different combinations of several secondary metabolites such as xanthomegnin, penicillic acid, verrucofortine, and verrucosidin produced by the species in the *P. aurantiogriseum* group, or roquefortine C produced by the species in another clade, *P. crustosum* and *P. roqueforti*. Some metabolites are, however, produced by species from more than one clade. Roquefortine C for example is found also in the *P. chrysogenum* clade and in *P. melanoconidium* (Frisvad & Filtenborg, 1989).

Using profiles of secondary metabolites and micromorphological characters, it has been shown that the species examined in this work are distinct (Frisvad & Filtenborg, 1989; Frisvad, 1992, 1994*a*, *b*; Svendsen & Frisvad, 1994; Larsen & Frisvad, 1995; Smedsgaard & Frisvad, 1997). Despite this, the DNA sequences were in some cases identical. One example is *P. viridicatum* and *P. neoechinulatum* which had identical ITS sequences (Fig. 1). *Penicillium viridicatum* is a widely distributed species while *P. neoechinulatum* has been found only in kangaroo rat environments, albeit co-occurring with the former species (Frisvad, Filtenborg & Wicklow, 1987). *Penicillium viridicatum* has finely roughened green conidia and produce viridic acid, viridamine, brevianamide A and the xathomegnin biosynthetic family as its principal secondary metabolites, while *P. neoechinulatum* has echinulate blue-green conidia and produce aurantiamine, penicillic acid and the 3-methoxyviridicatin biosynthetic family, so they appear to be some of the most distantly related species in the *P. aurantiogriseum* group (Lund & Frisvad, 1994). The only obvious synapomorphic phenotypic character is the diketopiperazine basic chemical structure common to both aurantiamine and viridamine, which differs only in the location and stereochemistry of a terpene unit (Larsen, Frisvad & Jensen, 1992). Although it would be an insurmountable task to characterize all the genes coding for the enzymes which produce these very specific secondary metabolites, it would be of great interest to find other DNA sequences that reflect these differences in phenotype.

Among the more than twenty terverticillate *Penicillium* species examined, only one is known to reproduce sexually, *Eupenicillium crustaceum*. Its presence shows that sexual reproduction is a feature of the terverticillate penicillia, and suggests that loss of sex in this group is a recent and short-lived phenomenon as with biverticillate penicillia (LoBuglio *et al*., 1993). The position of *E. crustaceum* in the *P. chrysogenum* clade, however, leaves open the possibility that other clades of terverticillate penicillia may have evolved without sex. This speculation assumes that mitosporic taxa are truly asexual and do not recombine in nature. Until this assumption is challenged, the possibility remains that terverticillate penicillia are not clonal, but are recombining in nature, as has been shown for other ascomycota (Burt *et al*., 1996; Geiser, Pitt & Taylor, 1998; Gräser *et al*., 1996).

In conclusion, the ITS 1 and ITS 2 regions do contain information that can be used to support taxonomical, ecological and physiological data for common food-borne *Penicillium* species, but the degree of ITS variability is too low to facilitate separation of all these closely related taxa. Additional sequence data from other genes may provide sufficient characters to establish the phylogenetic relationships between the terverticillate penicillia.

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