ARTICLE

Sex in *Penicillium* series *Roqueforti*

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Abstract: Various fungi were isolated during the course of a survey in a cold-store of apples in the Netherlands. One of these fungi belongs to the genus *Penicillium* and produces cleistothecia at 9 and 15 °C. A detailed study using a combination of phenotypic characters, sequences and extrolite patterns showed that these isolates belong to a new species within the series *Roqueforti*. The formation of cleistothecia at low temperatures and the inability to produce roquefortine C, together with a unique phylogenetic placement, make these isolates a novel entity in the *Roqueforti* series. The name *Penicillium psychrosexualis* sp. nov. (CBS 128137^T) is proposed here for these isolates.

Key words:
Penicillium
roqueforti
P. carneum
P. paneum
taxonomy
phylogeny

Article info: Submitted: 28 October 2010; Accepted: 19 November 2010; Published: 23 November 2010.

INTRODUCTION

Penicillium species are commonly occurring worldwide, and have been isolated from various substrates including air, soil, various food and feed products and indoor environments (Pitt 1979, Samson *et al.* 2010, Houbraken *et al.* 2010). *Penicillium roqueforti* is a member of this genus is and this species has both adverse and beneficial properties. The main beneficial property of this species is its role in the production of blue-veined cheeses, such as Roquefort, Danish blue, and Gorgonzola (Nichol 2000). However, this species is also frequently encountered as a spoilage organism, and is able to damage a vast array of food and feed products, due to its ability to grow under harsh conditions. These conditions include growth at low oxygen and high carbon dioxide levels, in the presence of preservatives and/or at low temperatures (Samson *et al*. 2010).

The taxonomy of series *Roqueforti* was studied by Samson & Frisvad (2004) using a polyphasic approach, combining partial β-tubulin sequences, extrolite patterns, phenotypic and physiological data. They showed that *P. paneum* and *P. carneum* are closely related to *P. roqueforti*, together forming the series *Roqueforti*. This series shares certain characters, such as a fast growth rate on agar media, the ability to grow on malt extract agar supplemented with acetic acid and the production of the extrolite roquefortine C. Despite the various shared characters, also various features are known to differentiate between these species (Frisvad & Samson 2004, Karlshøj & Larsen 2005, O'Brien *et al*. 2008). These include the growth rate at 30 °C, reverse colours on Czapek yeast agar and yeast extract agar, extrolite patterns and Ehrlich reaction (Samson & Frisvad 2004, Samson *et al.* 2010).

Various fungi were isolated during the course of a survey in a cold-store of apples in The Netherlands. The apples were stored in wooden crates, which were covered by a white fungal growth of *Fubulorhizoctonia psychrophila*. The latter species only grows at temperatures below 20 °C, and during the isolation of this species growth of an ascospore-forming *Penicillium* species was detected. This species appeared to be related to the series *Roqueforti* and a detailed study was performed on these isolates using a polyphasic approach. For the phylogenetic analysis, ITS, partial β-tubulin and calmodulin sequences were used, and these data were combined with extrolite analysis and macro- and microscopical characteristics. The combination of these datasets show that this species is new and is here described as *Penicillium psychrosexualis*.

MATERIAL AND METHODS

Strains and morphological examination

All examined strains belong to the *Penicillium* series *Roqueforti*. The strains (Table 1) were grown for 7 d as three point inoculations on Czapek yeast agar (CYA), malt extract agar, yeast extract sucrose agar (YES), creatine sucrose agar (CREA) and oatmeal agar (OA). The effect of various incubation temperatures (9–36 °C with intervals of 3 °C) on the growth was studied on CYA and OA.

Molecular analysis

Genomic DNA was isolated using the Ultraclean™ Microbial DNA Isolation Kit (MoBio, Solana Beach, CA, USA) according

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Table 1. Overview of *Penicillium* strains used in this study.

CBS no.	Other no.	Name	Substrate, locality
449.78	IBT 21509=IBT 3473=IBT 6753	*P. carneum*	Cheddar cheese
466.95	ATCC 46837=IBT 6885	*P. carneum*	Cured meat, Germany
467.95	IBT 3466	*P. carneum*	Hotwater tank, North Sealand, Denmark
112297^{T}	IBT 6884	*P. carneum*	Type, mouldy rye bread, Denmark
463.95	IBT 12392	*P. paneum*	Chocolate sauce, Norway
464.95	IBT 11839	*P. paneum*	Rye bread (non preserved), Odense, Denmark
465.95	IBT 13929	*P. paneum*	Mouldy baker's yeast, Vangede, Denmark
101032^{T}	IBT 21541=IBT 12407	*P. paneum*	Type, mouldy rye bread, Denmark
112296	IBT 21729	*P. paneum*	Cassava chips, Africa
112294	IBT 16402=NRRL 1168	*P. paneum*	Unknown substrate, Ottawa , Canada
128137^{T}	DTO 70G9 = IBT 29551	*P. psychrosexualis*	Type, wooden crate in cold-store of apples, the Netherlands
128136	DTO 70H7	*P. psychrosexualis*	Wooden crate in cold-store of apples, the Netherlands
128035	DTO 70H4	*P. psychrosexualis*	Wooden crate in cold-store of apples, the Netherlands
128036	DTO 70H9	*P. psychrosexualis*	Wooden crate in cold-store of apples, the Netherlands
135.67	IBT 19475=MUCL 8491	*P. roqueforti*	Blue veined cheese, Germany
221.30^{NT}	ATCC 10110=ATCC 1129=CECT 2905=IBT 6754=IFO 5459=IMI 024313=NRRL 849	*P. roqueforti*	Neotype, French Roquefort cheese, USA
234.38	IBT 19781=IMI 291202	*P. roqueforti*	Blue Cheshire cheese
479.84	IBT 21543	*P. roqueforti*	Mouldy baker's yeast, Denmark
498.73	ATCC 24720=FRR 1480=IBT 19476=IMI 174718=IMI 291199=VKM F-1748	*P. roqueforti*	Fruit of *Malus sylvestris* (apple), Russia

the manufacturer's instructions. The ITS regions (ITS), a part of the β-tubulin (BenA) or calmodulin (Cmd) gene were amplified and sequenced according the method described in Houbraken *et al.* (2007). Each dataset was aligned using the Clustal W program in MEGA5 (Tamura *et al.* 2007), and subsequently manually optimised. The evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura & Nei 1993). The bootstrap consensus tree inferred from 1 000 replicates is taken to represent the evolutionary history of the taxa analysed (Tamura *et al.* 2007). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites is < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Felsenstein 1985, Tamura *et al.* 2007). All phylograms were rooted with *Penicillium egyptiacum* CBS 244.32^{NT}. The newly obtained sequences were deposited in GenBank under accession numbers HQ442319–HQ442359.

Extrolite analysis

Plugs with mycelium and agar were extracted from 7 d old cultures grown on CYA and YES. Extracts were prepared using the method described by Smedsgaard (1997). Each extract was filtrated through a 0.45 PTFE filter and subsequently analysed using HPLC with diode array detection (DAD) detection. The UV spectrum and the RI value, and comparison with authentic chemical standards, were used to characterise the extrolites produced (Frisvad & Thrane 1987).

RESULTS

Phylogeny

The ITS regions and parts of the β-tubulin (BenA) and calmodulin (Cmd) gene were sequenced and analysed. The BenA alignment included 432 positions, and 35 positions were parsimony informative. The bootstrap consensus tree based on the results of the maximum likelihood analysis of this alignment is shown in Fig. 1. The total length of the calmodulin alignment was 500 positions long, and 27 sites were parsimony informative. The bootstrap consensus tree derived from the maximum likelihood analysis is shown in Fig. 2. The phylogram in Fig. 3 is based on the ITS regions (incl 5.8S rDNA), and 585 bases were used in the maximum likelihood analysis. Of these 585 characters, 16 were parsimony informative (including alignment gaps).

The result of the analysis of the three datasets shows that *P. psychrosexualis* belongs to the series *Roqueforti*. The species is related to *P. carneum* and *P. roqueforti* in all three analysed loci, and *P. paneum* is basal to these three species. *Penicillium carneum* is the closest relative of *P. psychrosexualis* in the tubulin phylogram (99 %, Fig. 1), and *P. roqueforti* is basal to these two species. However, this relationship is not supported in the phylograms based on the calmodulin and ITS sequences. In these datasets, *P. carneum* and *P. roqueforti* are

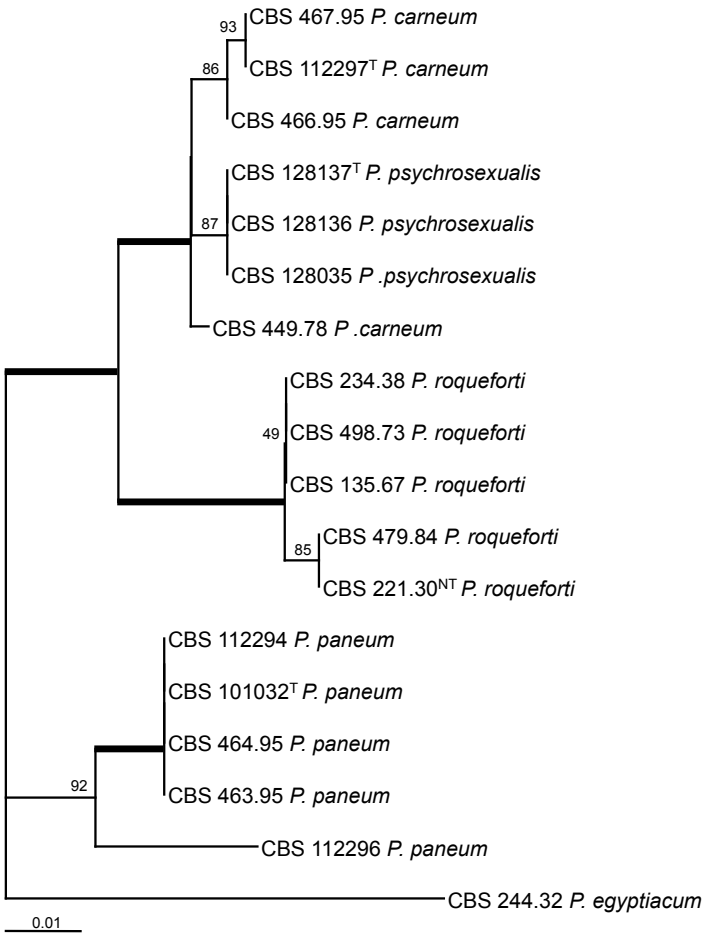

Fig. 1. Bootstrap consensus tree from a maximum likelihood analysis of partial β-tubulin sequences. The bootstrap values from 1 000 replicates are shown at the nodes, the branches in bold have a bootstrap support higher than 95 %. The tree was rooted with *Penicillium egyptiacum* CBS 244.32^{NT}.

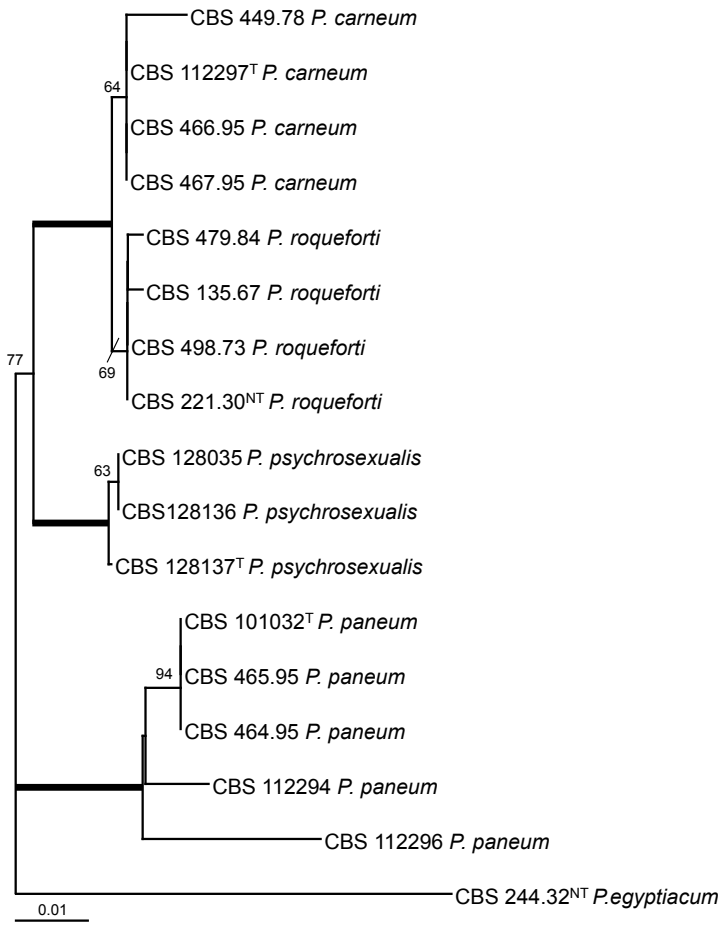

Fig. 2. Bootstrap consensus tree from a maximum likelihood analysis of partial calmodulin sequences. The bootstrap values from 1 000 replicates are shown at the nodes, the branches in bold have a bootstrap support higher than 95 %. The tree was rooted with *Penicillium egyptiacum* CBS 244.32^{NT}.

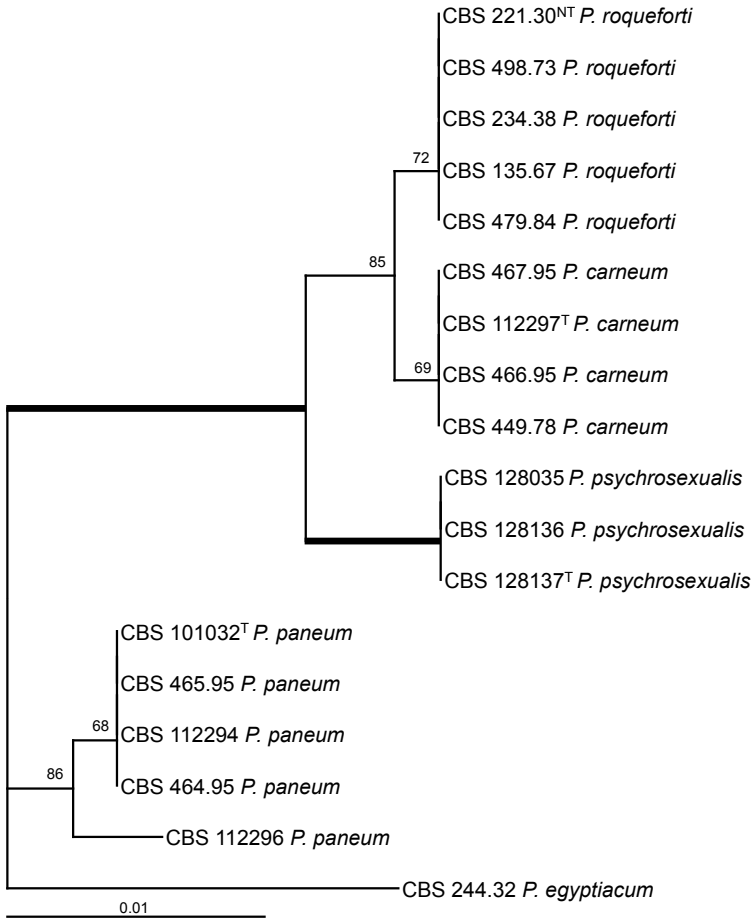

Fig. 3. Bootstrap consensus tree from a maximum likelihood analysis of ITS sequences. The bootstrap values from 1 000 replicates are shown at the nodes, the branches in bold have a bootstrap support higher than 95 %. The tree was rooted with *Penicillium egyptiacum* CBS 244.32NT.

sister species and in both cases *P. psychrosexualis* is basal to these two species. Two isolates (CBS 449.78 and CBS 112296) warrant further attention. *Penicillium carneum* CBS 449.78, an isolate from cheddar cheese, has a unique position in the tubulin and calmodulin phylograms (Figs 1, 2). In addition, this strain is morphologically slightly deviating from the majority of examined *P. carneum* isolates. Isolate CBS 449.78 is cream-brown in reverse on CYA, more restricted colonies on creatine agar and slightly slower growth rate at 30 °C. The other isolate which warrants attention is *P. paneum* CBS 112296. This strain has a unique β-tubulin, calmodulin and ITS sequence. However, extrolite analysis shows that this strain produces a typical array of *P. paneum* extrolites. More strains of these two types should be collected and examined to determine whether these strains should be raised to species level.

Taxonomy

Penicillium psychrosexualis Houbraken & Samson, **sp. nov.**
MycoBank MB519086
(Fig. 4)
In *Penicillium* subgenus *Penicillium* sect. *Roqueforti* ser. *Roqueforti*

Coloniis in MEA cum 0.5 % acore acetica crescentibus et item in agaro MEA, CYS et YES celeriter crescentibus, et formatione cleistotheciorum ad temperationem exiguam. Roquefortino C haud producenti.

Typus: The Netherlands: wooden crate in cold-store of apples covered by growth of *Fubulorhizoctonia psychrophila*, 3 Apr. 2008, *J. Houbraken & F. van der Geijn* (CBS H-20501 holotype; cultures ex type – CBS 128137 = IBT 29551 = DTO 70G9).

Colony diameter at 7 d (in mm): CYA, 25 °C, 47–55; CYA, 15 °C, 35–46; CYA, 30 °C, 14–27; no growth on CYA at 37 °C; MEA >60; YES >60; DG18, 40–50; ratio CYAS : CYA 1.2–1.4; creatine agar 15–25, good growth and no or weak acid production (under colony), delayed base production.

Strong sporulation on CYA, velvety, slightly floccose in centre, dull green or dark dull green conidia, mycelium inconspicuous, exudates absent, soluble pigment absent, radial sulcate, reverse warm brown. Good sporulation on YES, conidia dull-green, soluble pigments absent, reverse mustard-yellow, none sporulating edge 6–10 mm. Good sporulation on DG18, conidia dull-green, reverse pale. Colonies on MEA dull-green towards pure-green, velvety, soluble pigments absent. No reaction with an Ehrlich test.

Cleistothecia on OA at 25 °C sparsely produced and not visible due to the presence of a layer of conidia, formation of cleistothecia induced and sporulation reduced at low temperatures (9–15 °C, Fig. 5), cleistothecia white, soft and sterile when young, maturing slowly and becoming pale orange-brown after 3–4 mo of incubation, (50–)100–175 µm diam. *Ascospores* ellipsoidal, 4–5 × 3–4 µm, with two distinct equatorial ridges, often with additional secondary ridges, one

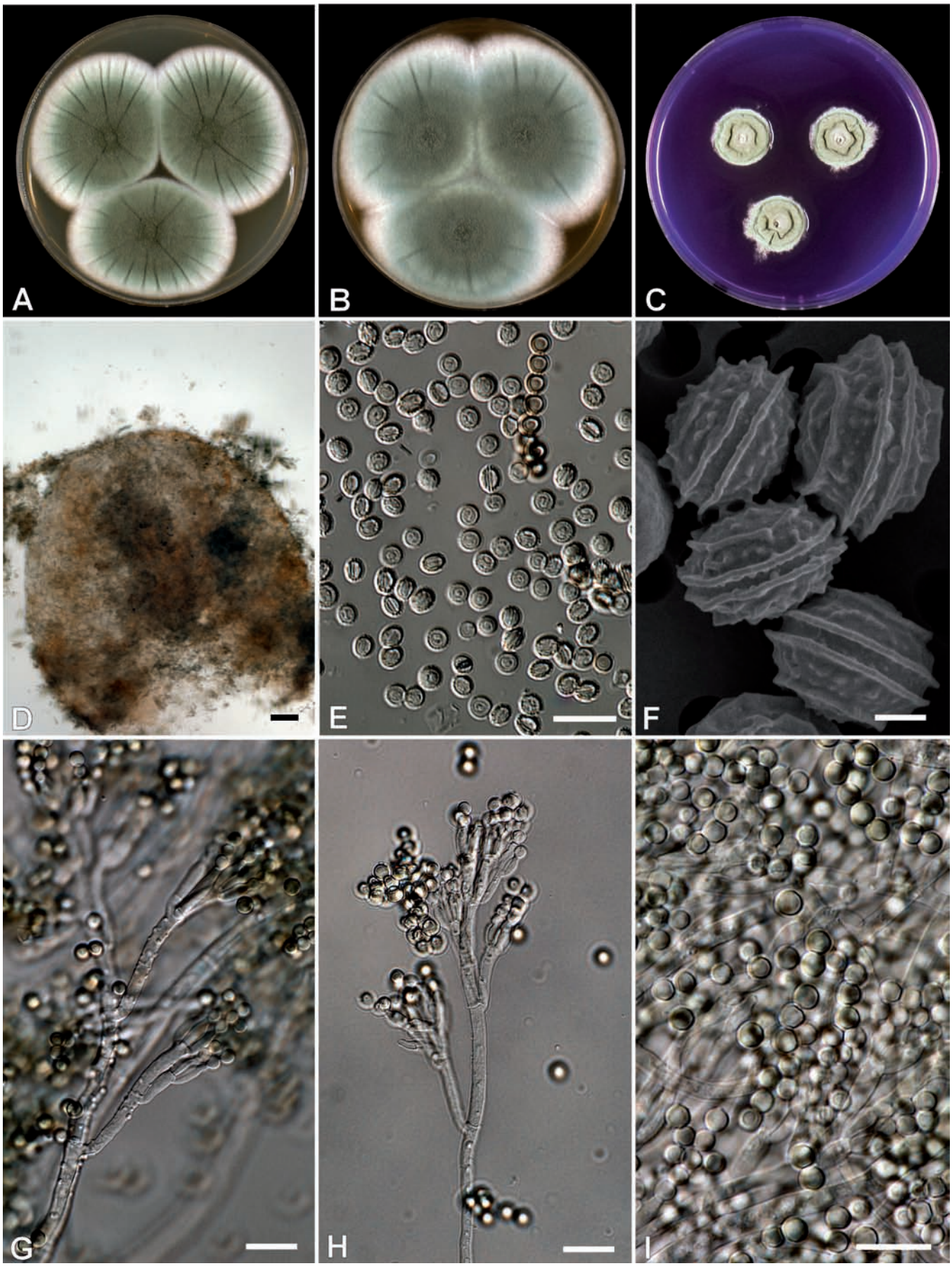

Fig. 4. *Penicillium psychrosexualis* (CBS 128036 , ex wooden crate in cold-store of apples, the Netherlands). (A–C) Colonies grown at 25 °C for 7 d on (A) CYA, (B) MEA, and (C) YES; (D) cleistothecium; (E–F) ascospores; (G) conidiophores on DG18 with warted stipes; (H) conidiophore with smooth stipe; (I) conidia. Bar = 10 µm, except (F) = 1 µm.

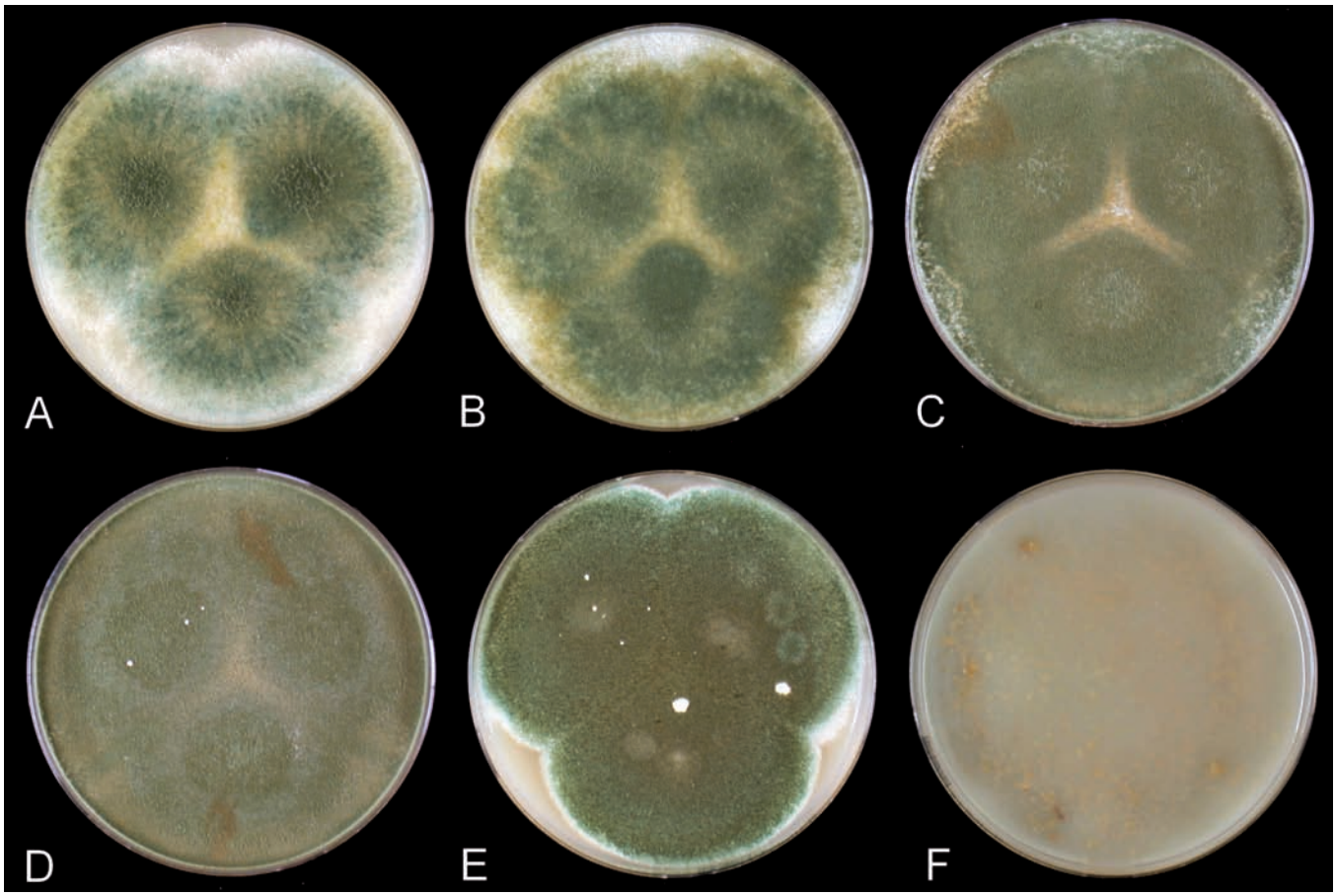

Fig. 5. Growth of *Penicillium psychrosexualis* CBS 128036 on oatmeal agar at various incubation temperatures. A–F: 9, 12, 18, 24, 27 and 33 °C.

on either side of the main ones, suggesting the presence of four ridges when observed with light microscopy, valves slightly roughened when viewed with SEM. *Conidiophores* terverticillate, slightly reduced conidiophores with smooth walled stipes on MEA and other agar media (PDA, PCA), on DG18 robust conidiophores with warted stipes, 3–4 µm. *Metulae* 10–15 × 3–4 µm. *Conidiogenous cells* (phialides) ampulliform, 8–10 × 3–4 µm. *Conidia* globose, smooth, 3.5–4 µm.

Extrolites: *Penicillium psychrosexualis* produces the extrolites andrastin A, mycophenolic, patulin, roquefortine C and the uncharacterized extrolite tentatively named “fumu”. Furthermore, *P. psychrosexualis* produces the same odour as *P. roqueforti*.

Diagnostic features: The growth on MEA containing 0.5 % acetic acid, the formation of cleistothecia at relatively low temperatures for the genus (9 °C) and fast growth rate on MEA, CYA and YES are diagnostic features of *P. psychrosexualis*. An overview of characteristics of *P. psychrosexualis* in comparison with other members of the series *Roqueforti* is shown in Table 2.

Similar species and taxonomy: Phylogenetically *P. psychrosexualis* belongs to series *Roqueforti*. This species shares a fast growth rate on agar media, the ability to grow on MEA supplemented with 0.5 % acetic acid and forms conidiophores with warted stipes on DG18. This species produces the extrolites andrastin A, mycophenolic, patulin and roquefortine C and is chemically close to *P. carneum*. However, *P. carneum* also produces penitrem A, isofumigaclavine A and cyclopaldic aicd, while *P. psychrosexualis* produces the uncharacterised extrolite “fumu”. *Penicillium psychrosexualis* produces the same odour as *P. roqueforti*, and is thus very different from the strong odour of *P. carneum*. Another difference between *P. psychrosexualis* and the other members of the *Roqueforti* series is the production of cleistothecia by the former species. The growth rate on CYA at 30 °C is a diagnostic tool to differentiate between *P. roqueforti* and *P. carneum* on one hand and *P. paneum* on the other. *Penicillium psychrosexualis* has similar growth rates at 30 °C as *P. roqueforti* and *P. carneum*. This observation is concordant with the phylogeny, which also shows that these three species are closely related and that *P. paneum* is basal to these species. An overview of growth rates on CYA at various temperatures is shown in Fig. 6.

Nomenclature: Although the new species produces cleistothecia, we decided to describe the taxon in *Penicillium* rather than *Eupenicillium* in accordance with the recommendations of Hawksworth (2010) on best-practice in such instances in a period when the rules of nomenclature that permit the dual naming of pleomorphic fungi are under revision.

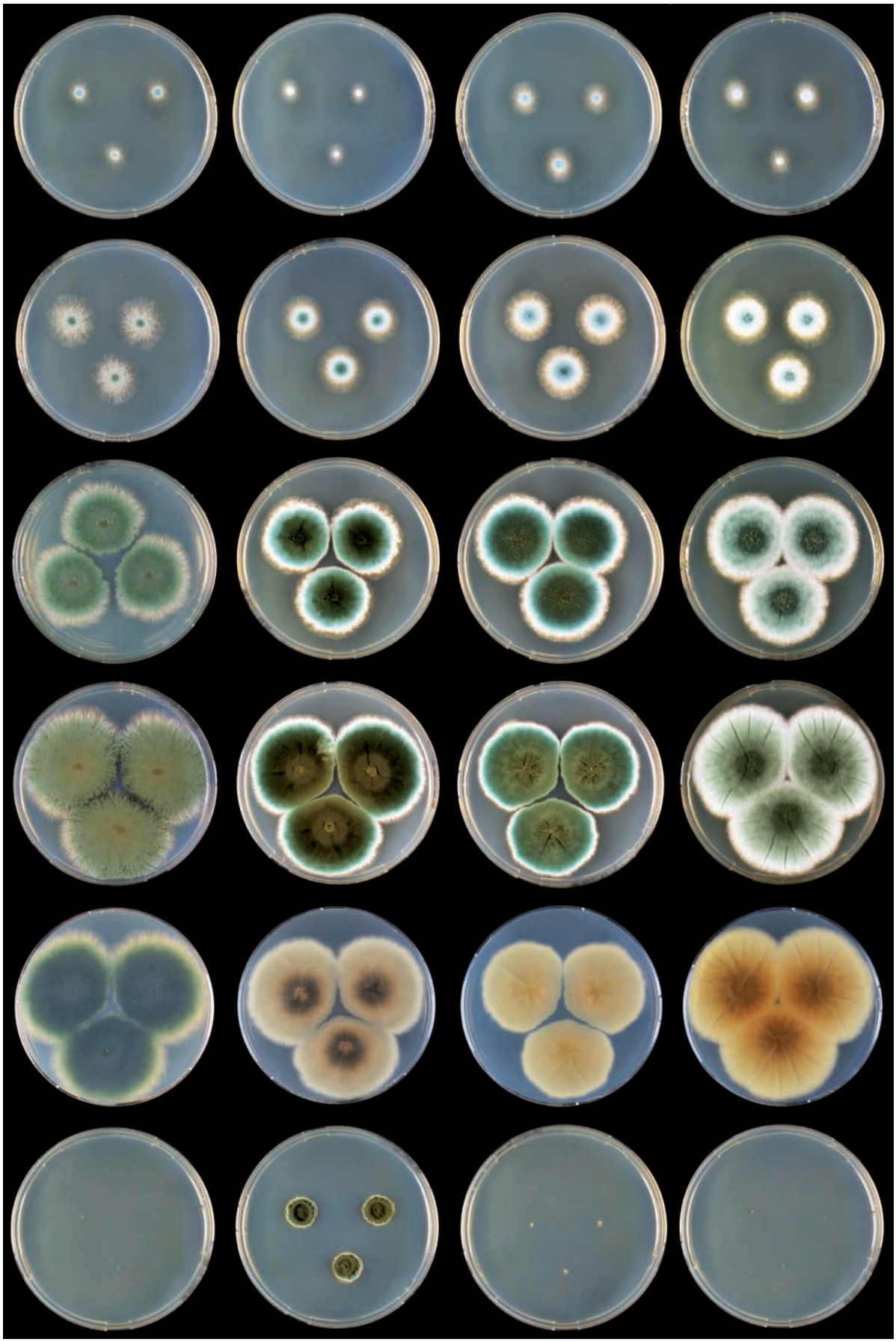

Fig. 6. Overview of growth rates of the members of *Penicillium* series *Roqueforti* on CYA at various temperatures. Row, top to bottom: 9, 12, 18, 24, 24 (reverse), 30 °C; columns, left to right: *P. roqueforti* DTO 81D6, *P. paneum* DTO 28G8, *P. carneum* DTO 128A9 and *P. psychrosexualis* CBS 128036.

Table 2. Overview of selected characters of members of *Penicillium* series *Roqueforti* (after Frisvad & Samson 2004, Sumarah *et al*. 2005, Nielsen *et al*. 2006, O'Brien *et al*. 2006, Månsson *et al*. 2010). See Karlshøj & Larsen (2005) for further differences in volatiles.

Species	Ehrlich reaction	Reverse on YES	Cleistothecia/ sclerotia	Growth rate on CYA30 °C (mm)	Extrolites**
P. carneum	Violet	Cream-beige	-	15–30	Roquefortine C, isofumigaclavine A&B, mycophenolic acid, patulin, cyclopaldic acid, penitrem A, andrastin A, (penicillic acid in CBS 449.78)
P. paneum	Negative	Cream yellow/ beige*	-	30–45	Roquefortine C, marcfortin A, patulin, andrastin A, citreoisocoumarin, (botryodiploidin)
P. psychrosexualis	Negative	Mustard-yellow	+	15–25	Andrastin A, mycophenolic, patulin and roquefortine C and the uncharacterized extrolite tentatively named "fumu"
P. roqueforti	Violet	Blackish green	-/(+)	(0–)5–15	Roquefortine C, isofumigaclavine A&B, PR-toxin, andrastin A, citreoisocoumarin, (mycophenolic acid)

*Often turning strawberry-red with age; with colour diffusing into the medium.

**The extrolites mentioned between brackets are not produced by the majority of isolates.

Distribution and ecology: This species has been isolated from wood and apples (Elstar) stored in a cold-store in the Netherlands. The conditions in the cold-store were 1.5–2.0 °C in combination with an oxygen level of 1.0–1.5 %, a carbon dioxide level of 2.0 % and a relative humidity of 92–95 %. These conditions strongly inhibit the growth of most fungi; however, a low temperature and microaerophilic conditions do not prevent growth of members of the *Roqueforti* series (Samson *et al*. 2010).

DISCUSSION

The taxonomy of *Penicillium* series *Roqueforti* has been studied extensively in the past, mainly due to its role in cheese manufacture. These studies were based on phenotypic characters (Thom 1906, 1910, Raper & Thom 1949, Pitt 1980, Samson *et al.* 1977), extrolite patterns (Frisvad & Filtenborg 1989, Boysen *et al.* 1996, Samson & Frisvad 2004, Smedsgaard *et al.* 2004) and/or molecules (Boysen *et al.* 1996, Skouboe *et al.* 1999, Samson *et al.* 2004). This is the first study using a multigene approach to determine the relationship of species belonging to the *Roqueforti* series. All three studied loci are suitable for species recognition. Even the ITS regions, normally not recommended for species identification in *Penicillium*, have enough variation in this series (Skouboe *et al*. 1999, Houbraken *et al.* 2010, Samson *et al.* 2010). Incongruence was detected during the phylogenetic analysis of the calmodulin, β-tubulin and ITS loci. *Penicillium psychrosexualis* was, with high bootstrap support, basal to *P. carneum* and *P. roqueforti* in the ITS and calmodulin dataset, while *P. roqueforti* was basal to *P. carneum* and *P. psychrosexualis* in the β-tubulin dataset. The use of β-tubulin in taxonomy was debated by Peterson (2008) and he excluded this locus in his study due to his doubt about the homology of this locus between members of sections in *Aspergillus*. Furthermore, Hubka & Kolařík (2010) showed that the commonly used primers Bt2a and Bt2b could amplify the β-tubulin paralog *tubC* in Aspergilli. The interpretation of paralogous genes with non-homologous function in the same phylogenetic analysis posses a great risk and might create incongruence within and between datasets (Hubka & Kolařík 2010).

A limited number of penicillia are able to produce cleistothecia and ascospores, and these species were referred to the genus *Eupenicillium* in a number of studies. Only a limited number of penicillia known to reproduce sexually belong in subgenus *Penicillium*. Samson & Frisvad (2004) omitted these species in their monograph of this subgenus, and they recommended that a multigene study needs to be conducted to resolve the placement of these teleomorphic penicillia within the subgenus *Penicillium*. Peterson (2000) included various *Eupenicillium* species in his phylogenetic study of *Penicillium,* and showed that *E. crustaceum*, *E. egyptiacum*, *E. baarnense*, *E. tularense,* and *Hemicarpenteles paradoxus* belonged to Group 6. This group largely corresponds with the subgenus *Penicillium* as circumscribed by Samson & Frisvad (2004). Until now, only homothallic species are described in this subgenus; however, recent studies indicated that various species belonging to this subgenus are heterothallic. Hoff *et al.* (2008)

showed that *P. chrysogenum* is heterothallic, and analysis of 12 *P. chrysogenum* isolates showed an equal mating type distribution, indicating the potential of this species to reproduce sexually. In addition, Eagle (2009) detected either MAT1-1-1 or MAT1-2-1 gene fragments in isolates of *P. camemberti*, *P. roqueforti* and *P. verrucosum*, also indicating heterothallism. Although various trials were undertaken to inducing mating in *P. chrysogenum* (Hoff *et al.* 2008, Eagle 2009, Houbraken unpubl. data) none of them have been successful. In addition, mating trials with *P. roqueforti* under conditions known to induce sex in *Aspergillus fumigatus* were unsuccessful and no cleistothecia were detected after 6 mo of incubation (Eagle 2009). Various growth factors induce formation of cleistothecia, such as temperature, light, nutrients and oxygen levels (Han *et al.* 2003). In this study, we show that *P. psychrosexualis*, a species related to *P. roqueforti*, produces cleistothecia abundantly at 9 °C. The production of a sexual stage at low temperatures might be more widespread in *Penicillium,* and mating experiments with *P. roqueforti* at this temperature might result in a sexual stage. Furthermore, *P. psychrosexualis* might be a good model species for comparison purposes in sex induction experiments or expression studies of genes required for sex in *P. roqueforti*. There are also indications of a sexual stage in *P. roqueforti*. Sclerotia were observed in cultures in *P. roqueforti* (Samson *et al.* 1977, Shimada & Ichinoe 1998) and it was postulated that similar structures have a dual function in the life-cycle in *Aspergillus* sect. *Flavi*. Survival of adverse conditions is one of them; the other is providing genetic variation in populations through sexual reproduction as a cleistothecium (McAlpin & Wicklow 2005, Horn *et al.* 2009). The possible discovery of the sexual stage in *P. roqueforti* could have consequences for the stability of starter cultures and might have advantages in strain improvement programs using conventional genetical approaches.

The effect of temperature on sexual reproduction in species belonging to the subgenus *Penicillium* is poorly studied. Many of these species are capable to grow at low temperatures and are therefore common spoilage organisms in refrigerators. McCulloch & Cain (1928) found an effect of the temperature on the formation of sclerotia of *Penicillium gladioli*. This species produces blue-green conidial structures abundantly when incubated at 14–15 °C, but produced comparatively a high number of sclerotia and only a few conidial structures, when incubated at 22 °C or higher. This observation is opposite to the results reported here, if the assumption is followed that sclerotia are immature cleistothecia. On the other hand, large white sclerotia are occasionally seen in *P. italicum*, a species related to *P. psychrosexualis* and also belonging to the subgenus *Penicillium*. These structures have been observed in cultures incubated in darkness at 0 °C for 3 mo (Raper & Thom 1949, Samson & Frisvad 2004), also suggesting the induction of a sexual cycle at low temperatures.

Members of series *Roqueforti* have a worldwide distribution, mainly related to human environments, and occur on various substrates. *Penicillium roqueforti*, *P. paneum,* and *P. carneum* occur on (preserved) food and silage, and only *P. roqueforti* has been frequently isolated as a saprobe in nature. Reports of the occurrence of *P. carneum* and *P. paneum* in nature are rare, and recently *P. paneum* has been found in stone tombs in Japan (An *et al.* 2009). *Penicillium psychrosexualis* is the second saprobic species in this series and has also been isolated from wood. Several reports are made on the occurrence of *P. roqueforti* on woods such as sawn wood (logs), wood stakes in soil, wood in sea, cut lumber, *Quercus robur*, and very wet wood in indoor environments (Picci 1966, Pitt 1980, Land *et al.* 1985, Kubátová 2000, Seifert & Frisvad 2000, Sumarah *et al.* 2005).

ACKNOWLEDGEMENTS

We thank Frank van de Geijn (Agrotechnology & Food Innovations BV, Wageningen, the Netherlands) for collecting the wood and apples samples. Dae-Hoo Kim is greatly acknowledged for the preparations of the SEM images of the ascospores, and we thank Uwe Braun for providing the Latin diagnosis.

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