

Four psychrotolerant species with high chemical diversity consistently producing cycloaspeptide A, *Penicillium jamesonlandense* sp. nov., *Penicillium ribium* sp. nov., *Penicillium soppii* and *Penicillium lanosum*

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Penicillium jamesonlandense is a novel species from Greenland that grows exceptionally slowly at 25 °C and has an optimum temperature for growth of 17–18 °C. The novel species is more psychrotolerant than any other *Penicillium* species described to date. Isolates of this novel species produce a range of secondary metabolites with a high chemical diversity, represented by kojic acid, penicillic acid, griseofulvin, pseurotin, chrysogine, tryptoquivalins and cycloaspeptide. *Penicillium ribium*, another novel psychrotolerant species from the Rocky Mountains, Wyoming, USA, produces asperfuran, kojic acid and cycloaspeptide. Originally reported from an unidentified *Aspergillus* species isolated from Nepal, cycloaspeptide A is reported here for the first time from the two novel *Penicillium* species and two known psychrotolerant species with high chemical diversity, *Penicillium soppii* and *Penicillium lanosum*. All species, except *P. ribium*, produce a combination of cycloaspeptide and griseofulvin. However, *P. ribium* (3/5 strains) produced the precursor to griseofulvin, norlichexanthone. The type strain of *Penicillium jamesonlandense* sp. nov. is DAOM 234087^T (=IBT 21984^T=IBT 24411^T=CBS 102888^T) and the type strain of *Penicillium ribium* sp. nov. is DAOM 234091^T (=IBT 16537^T=IBT 24431^T).

It has often been claimed that there is a higher degree of biodiversity in the tropics compared with the cold regions of the world (Pointing & Hyde, 2001). Recently, it was shown that a high degree of fungal diversity exists in tundra soils, even those which are covered by snow for most of the year

(Schadt *et al.*, 2003). However, despite these findings, few novel species are currently being described from cold regions. In the fungal genus *Penicillium* (with the ascomycetous state *Eupenicillium*), only one novel species has been described from very cold regions. This species, *Penicillium antarcticum*, is not particularly psychrotolerant (McRae *et al.*, 1999). Many species of *Penicillium* grow rather well at 5 °C, especially most of the food-borne terverticillate penicillia, but most of these species have an optimum temperature for growth at around 25 °C (Pitt, 1979). Those *Penicillium* species that have been reported from cold regions have been found to be common ubiquitous fungi (McRae *et al.*, 1999) and no penicillia have been reported to be geographically confined to the Arctic or Antarctic;

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the ITS and β -tubulin gene sequences for the strains of the four *Penicillium* species examined in this study are DQ285608–DQ285627 and DQ267904–DQ257924, respectively.

A phylogenetic tree based on ITS gene sequences and a description of the methods used for X-ray analysis are available as supplementary material in IJSEM Online.

even *P. antarcticum* occurs worldwide (J. C. Frisvad and others, unpublished observations). Despite this, a recent paper (Gunde-Cimerman *et al.*, 2003) indicates that there may be several novel cold-tolerant species of *Penicillium* in Arctic areas. Currently there are no data concerning the chemical diversity of cold-tolerant fungi; however, the chemical diversity within the genus *Penicillium* is considered to be generally high (Frisvad & Filtenborg, 1989, 1990a, b). It is our hypothesis that not only tropical species, but also psychrotolerant species have high chemical diversity.

Many novel interesting metabolites have been described from unidentified species of common genera such as *Aspergillus* and *Penicillium*. One such example is the biosynthetic family of cyclic peptides, cycloaspeptide A, B and C, from an *Aspergillus* species (Kobayashi *et al.*, 1987). The original *Aspergillus* strain was not available for study. No producers were revealed after we performed a screen of the whole genus *Aspergillus* for cycloaspeptide producers by HPLC with diode array detection. In contrast, by screening the genus *Penicillium*, we found four species producing cycloaspeptides: two known species and two novel species (Table 1). We isolated this compound from the novel species described below in order to confirm the structure of cycloaspeptide A. Interestingly, these four species were all isolated from cold regions (alpine, northern temperate and Arctic regions). Species nova characterizations were derived from extrolite, morphological and colony data and supported by phylogenetic analyses of internal transcribed spacer (ITS) and β -tubulin gene sequences.

Soil samples taken from Greenland, tundra and alpine areas in Wyoming and Colorado (USA) and alpine areas in Slovenia were examined for *Penicillium* and *Aspergillus* species using a serial dilution technique and the selective low-water-activity medium DG18 incubated at 15 and 25 °C (Hocking & Pitt, 1980). The filamentous fungi isolated from the soil samples from cold areas showed a high diversity of *Penicillium* species, but a low diversity of *Aspergillus* species. Only *Aspergillus versicolor* was detected in a sample from Nuuk airport, Greenland. The remaining fungi consisted of *Geomyces pannorum*, *Cladosporium* spp., *Mucor* spp. and *Mortierella* spp. Among the species most often recovered in the samples were *Penicillium soppii* and *Penicillium lanosum* and the two novel species, described here as *Penicillium ribium* sp. nov. and *Penicillium jamesonlandense* sp. nov. *P. ribium* was found only in samples taken at different locations in Wyoming, USA, while *P. jamesonlandense* was recovered in samples from Greenland (both east and west) at low elevations and from Wyoming in tundra soil at a high elevation (2500–2800 m). *P. jamesonlandense* could not be recovered from DG18 plates incubated at 25 °C (see Table 1 for a list of representative isolates of the four species).

According to one of the definitions of psychrophilic fungi, the most cold-tolerant species described here, *P. jamesonlandense*, is not a true psychrophilic species as it can grow at 25 °C, albeit very weakly. It is, however, close to being a

true psychrophile (Weinstein *et al.*, 1997), as its optimum temperature for growth is 18 °C. We propose to call such species quasipsychrophilic, as they are clearly different in their temperature profile from the psychrotolerant species *P. ribium*, *P. lanosum* and *P. soppii* and several psychrotolerant species from foods that actually grow and sporulate well at 25 °C (Pitt, 1979). *P. jamesonlandense* is the first species described in the genus *Penicillium* that grows slowly, or not at all, at 25 °C and it can be distinguished solely on that basis from any other *Penicillium* species. Some species of *Eupenicillium*, e.g. *Eupenicillium fractum*, also grow quite slowly at 25 °C (Pitt, 1979), albeit not as slowly as *P. jamesonlandense*, but these fungi are xerotolerant, not psychrotolerant, and they grow and sporulate well on media with a lowered water activity, such as the medium G25N, at 25 °C (Pitt, 1979). Moreover, *P. jamesonlandense* does not sporulate at 25 °C, another indication that this species is psychrophilic. Pringle & Taylor (2002) have suggested that the fitness of filamentous fungi can be measured by their ability to sporulate and, if their suggestion is accepted, *P. jamesonlandense* is not fit at 25 °C. The three other species, also found in cold alpine or Arctic areas, grow well and sporulate well at 25 °C, except *P. soppii*, which produced a large number of sclerotia and relatively few conidia on the media used. *P. jamesonlandense* is thus the first quasipsychrotolerant species found in the genera *Eupenicillium* and *Penicillium*.

New sequences for the ITS gene and partial sequences of the β -tubulin gene were prepared for strains of *P. ribium*, *P. jamesonlandense*, *P. lanosum* and *P. soppii* using DNA isolated from conidia and mycelia produced on malt extract agar (MEA) using the FastPrep FP120 (BIO 101) or UltraClean microbial DNA isolation (Mo Bio Laboratories) kits. PCRs were performed in 25 μ l volumes using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech) and 2 μ l template, using a Techne Genius thermocycler (Techne). PCR cycling parameters included 30 cycles of denaturation at 95 °C for 1.5 min, annealing at 56 °C for 1 min and extension at 72 °C for 2 min, with an initial denaturation of 4 min and a final extension step of 10 min. Amplified products were purified using the UltraClean microbial PCR purification kit (Mo Bio Laboratories) and DNA concentrations were estimated from fragments stained by ethidium bromide and separated by agarose gel electrophoresis. Sequencing reactions were performed using the BigDye Terminator cycle sequencing system (Applied Biosystems) with the recommended cycling parameters. Reactions were purified by ethanol/sodium acetate precipitation. The sequences were determined using an ABI PRISM 3100 DNA automated sequencer (Applied Biosystems). The complete ITS and 5.8S rRNA genes were amplified using ITS1 and ITS4 primers, with the addition of ITS2 and ITS3 for cycle sequencing when necessary (White *et al.*, 1990). Exons 3–6 of the β -tubulin gene were amplified using T1, T10 and T224 or T222 primers (O'Donnell & Cigelnik, 1997) and sequenced using Bt2a and Bt2b primers (Glass & Donaldson, 1995). Consensus sequences were determined

Table 1. Isolates examined for cycloaspeptides

Only species containing at least one producer are listed.

| Strain | Origin |
|--|---|
| <i>P. jamesonlandense</i> sp. nov. | |
| IBT 21984 ^T (=IBT 24411 ^T =CBS 102888 ^T =DAOM 234087 ^T) | Soil under heather near Hugin Lake, Jamesonland, Greenland |
| IBT 22005 (=CBS 102887) | Soil under heather near Hugin Lake, Jamesonland, Greenland |
| IBT 22004 (=IBT 24439) | Soil under heather near Hugin Lake, Jamesonland, Greenland |
| IBT 22021 | Beach sand near Hugin Lake, Jamesonland, Greenland |
| IBT 22647 | Sandy soil, Fortune Bay, Disco Island, Greenland |
| IBT 22931 | Greenland |
| IBT 23955 (=RMF T72A) | Tundra soil, Middle Mountain, Wind River Range, WY, USA |
| IBT 23957 (=RMF T72B) | Tundra soil, Middle Mountain, Wind River Range, WY, USA |
| <i>P. ribium</i> sp. nov. | |
| IBT 16537 ^T (=IBT 24431 ^T =DAOM 234091 ^T) | Soil under redcurrant, Eagle Rock, Medicine Bow National Forest, WY, USA |
| IBT 21215 | Soil under pine tree, Eagle Rock, Medicine Bow National Forest, WY, USA |
| IBT 18067 | Soil under <i>Artemisia tridentata</i> , Table Rock Road, Highway 80, WY, USA |
| IBT 18294 | Soil under <i>Artemisia tridentata</i> and pine tree, Eagle Rock, Medicine Bow National Forest, WY, USA |
| IBT 18295 | Soil under <i>Artemisia tridentata</i> and pine tree, Eagle Rock, Medicine Bow National Forest, WY, USA |
| <i>P. lanosum</i> | |
| IBT 6619 ^T (=CBS 106.11 ^T =NRRL 2009 ^T) | Ex <i>Lecanora</i> sp., Norway |
| IBT 3265 | Unknown |
| IBT 4093 (=IBT 15554) | Salami factory, Hillerød, Denmark |
| IBT 4172 | Air, Denmark |
| IBT 4173 | Unknown, Denmark |
| IBT 4174 | Wrapping paper of bakers yeast, Århus, Denmark |
| IBT 4216 | Air filter, factory, Denmark |
| IBT 12213 | Soil near Lake Tesse, Norway |
| IBT 13448 | Wheat kernel, Denmark |
| IBT 13537 | Elder swamp, Denmark |
| IBT 13588 | Forest soil, Canada |
| IBT 15512 | Heath soil, Hjelm, Jutland, Denmark |
| IBT 15719 | Heath soil, Hjelm, Jutland, Denmark |
| IBT 15766 | Forest soil near Umeå, Sweden |
| IBT 17102 | Endophyte of <i>Sorbus</i> sp., Germany |
| IBT 17364 | Air, cake factory, Denmark |
| IBT 17537 | Unknown, Papua New Guinea |
| IBT 17741 | Soil under <i>Juniperus communis</i> , Wind River Canyon, 10 km south of Thermopolis, WY, USA |
| IBT 18326 | Pine soil, Eagle Rock, Laramie, WY, USA |
| IBT 18579 | Air, cake factory, Give, Denmark |
| IBT 18582 | Air, cake factory, Give, Denmark |
| IBT 18774 | Mouldy cake, Denmark |
| IBT 18952 (=WT 238) | Deciduous/conifer forest, India |
| IBT 19190 | Air, cake factory, Stege, Denmark |
| IBT 19714 | Air, cake factory, Stege, Denmark |
| IBT 19718 | Air, cake factory, Stege, Denmark |
| IBT 19933 | Air, cake factory, Give, Denmark |
| IBT 19992 | Air, cake factory, Stege, Denmark |
| IBT 20686 | Soil under spruce tree, Brandywine Falls, British Columbia, Canada |
| IBT 20812 | Soil under moss, Grouse Mountain, 1100 m elevation, British Columbia, Canada |
| IBT 22473 | Soil under <i>Nothofagus</i> sp., Chile |

Table 1. cont.

| Strain | Origin |
|---|--|
| IBT 22675 | Soil under spruce, 4 km east of Zelenogorsk, Russia |
| IBT 22684 | Soil under spruce, 4 km east of Zelenogorsk, Russia |
| IBT 23368 | Soil, Svalbard, Norway |
| IBT 23837 | Soil, Svalbard, Norway |
| IBT 23838 | Soil, Svalbard, Norway |
| IBT 24652 | Soil, Faroe Islands |
| <i>P. soppii</i> | |
| IBT 18220 ^T (=NRRL 2023 ^T) | Pine forest soil, Puszcza Bialowieska, Poland |
| IBT 3130 (=NRRL A-23325) | Green barley, Denmark |
| IBT 3331 (=CBS 869.70) | Root of <i>Picea abies</i> , Denmark |
| IBT 3377 (=CBS 144.83) | Sandy soil under pine, Spain |
| IBT 5397 (=CBS 271.73) | Soil, Canada |
| IBT 5400 (=IJFM 19000) | Soil, Russia |
| IBT 14908 | Soil, Greenland |
| IBT 15641 | Heath soil, Hjelm, Jutland, Denmark |
| IBT 16551 | Soil under grass, near Red Mountain, Sierra Madre, Houston Wilderness, WY, USA, 3430 m elevation |
| IBT 16841 | Soil under grass, near Red Mountain, Sierra Madre, Houston Wilderness, WY, USA, 3430 m elevation |
| IBT 17716 (=NRRL 701) | Mount Desert Island, ME, USA |
| IBT 17839 (=RMF 9021) | <i>Quercus savanna</i> soil, Cedar Creek, MN, USA |
| IBT 18041 (=RMF 203) | Lodgepole forest, WY, USA |
| IBT 18955 (=RMF 8829) | Deciduous forest, Coweeta, NC, USA |
| IBT 18956 (=RMF 205) | <i>Pinus contorta</i> forest soil, WY, USA |
| IBT 18957 (=WSF 2397) | Floodplain deciduous forest, WI, USA |
| IBT 19343 | Soil under <i>Juniper communis</i> , Near Red Mountain, Sierra Madre, Houston Wilderness, WY, USA, 3400 m elevation |
| IBT 19231 (=UAMH 1083) | Soil under spruce, Edmonton, Canada |
| IBT 21211 | Soil |
| IBT 22161 | Soil in spruce forest, NJ, USA |
| IBT 22628 | Soil under moss, Engelskmandens Havn, Disco Island, Greenland |
| IBT SL18-4 | Soil under grass, meadow, Ratitovec, Slovenia, 1620 m elevation |

from overlapping sequence data for both DNA strands, except where noted, using SEQUENCHER software (Gene Codes).

Datasets were compiled of sequences of the novel species and selected ITS gene sequences of species of *Penicillium*, subgenus *Furcatum*, mostly from the study of Peterson (2000), with individual sequences from the studies of Haugland *et al.* (2004), Rakeman *et al.* (2005) and H. A. Sabev, P. S. Handley & G. D. Robson (unpublished; GenBank accession number GI 53125189). Additional ITS and β -tubulin gene sequences from ongoing studies in the Seifert/Louis-Seize lab were included as relevant. The two datasets were not completely congruent due to the inclusion of ITS gene sequences from GenBank and the unavailability of a few strains for reciprocal sequencing. Both analyses were rooted with sequences for *Penicillium chrysogenum*, but using different strains. GenBank accession numbers for all sequences used are included in Fig. 1 and in Supplementary Fig. S1, available in IJSEM Online. Initial alignments were calculated using CLUSTAL W and adjusted using

SE-AL (version 1.d1; <http://evolve.zoo.ox.ac.uk/software/Se-AL/main.html>) to maximize alignment. Both data matrices were subjected to parsimony analysis using the heuristic search option of PAUP version 4.0b10 (Swofford, 1999) with simple stepwise addition of taxa, tree bisection-reconnection branch swapping, gaps treated as missing data and uninformative characters removed. The maximum number of trees to be saved to memory was set to 5000 to prevent saturation of the computer's memory, most relevant for the ITS dataset. The robustness of the phylogenies was tested using bootstrap analysis (1000 replications, 'fast' stepwise searches).

The ITS gene sequence alignment included 561 bp, of which 22 (4%) were parsimony-informative. The heuristic analysis yielded more than 5000 equally parsimonious trees 31 steps long (Supplementary Fig. S1 in IJSEM Online). The large number of trees reflects the presence of many identical sequences in the dataset, and most of the trees resulted from the rearrangement of 0-length branches, as is indicated by the support of the main structure of

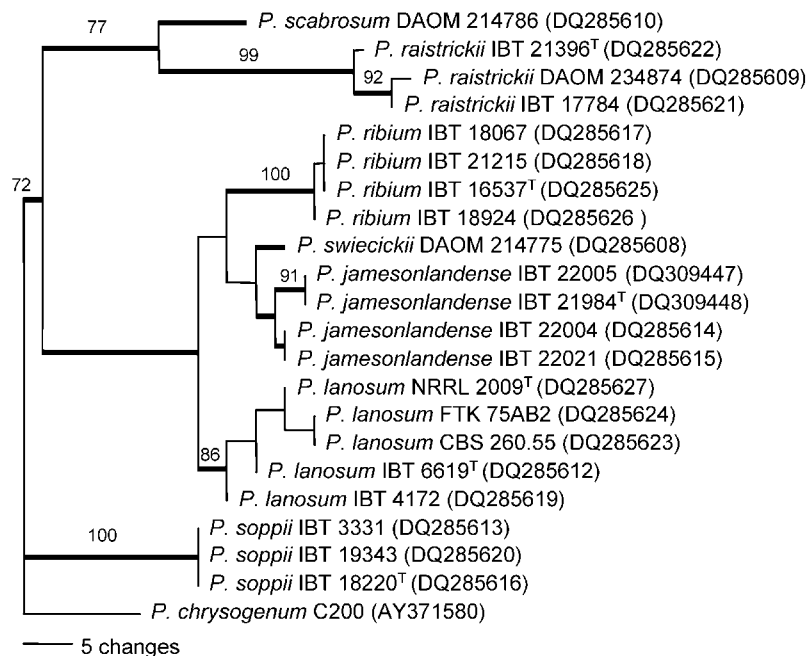


Fig. 1. Gene tree based on heuristic analysis of partial β -tubulin gene sequences of cycloaspeptide- and griseofulvin-producing species of *Penicillium* and their closest phylogenetic relatives. One of 20 equally parsimonious trees, 113 steps long (CI 0.726, RI 0.876, RC 0.635, HI 0.274). Branches in bold occur in the 100% consensus trees. Numbers above branches are 'fast' bootstrap support values above 70%. The cladogram is rooted with *Penicillium chrysogenum* C200. GenBank accession numbers (given in parentheses) beginning with DQ were generated in this study. Bar, 5 changes.

the tree by the strict consensus. Phylogenetic analyses of ITS gene sequences showed that *P. jamesonlandense*, *P. ribium*, *P. lanosum* and *P. soppii* formed a monophyletic group with 98% bootstrap support, together with the species *Penicillium kojigenum*, *Penicillium swiecickii* and *Penicillium scabrosum* (Supplementary Fig. S1). This clade is sister to *Eupenicillium baarnense* and *Penicillium turbatum* and would be inserted at the top of 'group 4' in the gene tree of Peterson (2000). Both of the novel species, *P. jamesonlandense* and *P. ribium*, had invariant and unique ITS gene sequences that distinguished them from their closest neighbours, although *P. jamesonlandense* differed from *P. swiecickii* by only a single base pair change.

The β -tubulin gene sequence dataset was more variable (Fig. 1), with 68 parsimony-informative characters present in the 513 bp alignment (13%). A poly (T) run of about 13 characters was omitted from the 5' end of the alignment because of uncertainties in reading the particular sequences. The heuristic analysis yielded 20 equally parsimonious trees of 113 steps. This confirmed the close phylogenetic relationship between *P. ribium*, *P. swiecickii*, *P. jamesonlandense* and *P. lanosum* suggested by the ITS gene sequence analysis. However, *P. soppii* and *Penicillium raistrickii* were not clearly allied with this clade in the β -tubulin gene sequence analysis and *P. scabrosum* was sister to *P. raistrickii*, rather than to *P. soppii*. The three strains of *P. soppii* were invariant in their β -tubulin gene sequences, whereas there was a single base pair substitution among the four sequenced strains of *P. ribium*. There was a 15 bp insertion in two of the strains of *P. jamesonlandense* (strains IBT 22005 and IBT 21984^T), which accounted for

the dichotomy within this species in Fig. 1. There was a fair amount of divergence among the strains of *P. lanosum* sequenced, including three base pair differences between two different versions of the type strain, IBT 4172^T and NRRL 2009^T (obtained from D. Malloch, University of Toronto, in 1995). These polymorphisms were confirmed by direct comparison of the sequence files. Evidently, there are two different strains in circulation as the type culture, an issue that requires investigation.

Culture extracts of at least two strains of each named species of *Aspergillus* and *Penicillium* from the IBT collection, as well as the novel strains isolated from soil samples, were screened for secondary metabolite production using HPLC with diode array detection according to Frisvad & Thrane (1987, 1993) as modified by Smedsgaard (1997). The screening of all *Penicillium* and *Aspergillus* species in our collection revealed only four *Penicillium* species that produced cycloaspeptide: *P. jamesonlandense*, *P. ribium*, *P. lanosum* and *P. soppii*. All four species produced a large number of both known and unknown secondary metabolites. *P. jamesonlandense* produced the glucose-derived kojic acid, the polyketides griseofulvin and penicillic acid, the amino acid-derived compounds cycloaspeptide A, tryptoquivalins and chrysogine and the phenylalanine- and hexaketide-derived pseurotin and some of the strains produced the terpene fumagillin. *P. ribium* produced kojic acid, the polyketides asperfuran, norlichexanthone, viridicatumtoxin, and an unknown anthraquinone in addition to cycloaspeptide A and D, psychrophilin A (Dalsgaard *et al.*, 2004) and 2-(4-hydroxyphenyl)-2-oxoacetaldehyde oxime (Amade *et al.*, 1994). *P. lanosum* produced kojic acid, the polyketide compactins, griseofulvins and pyripropens,

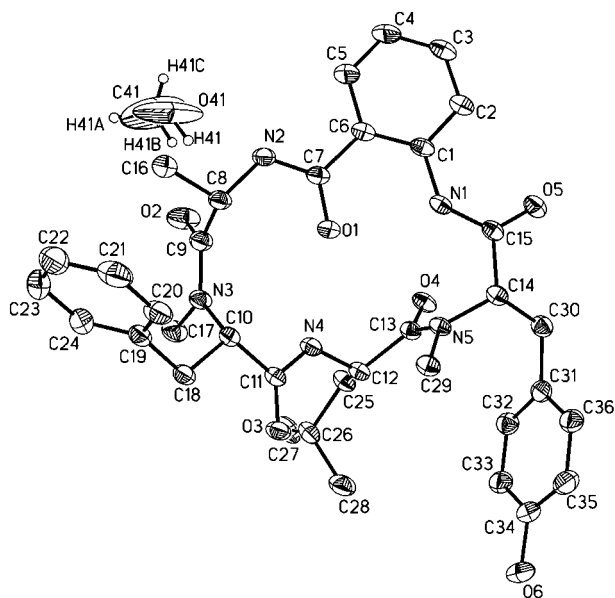


Fig. 2. X-ray-generated structure of cycloaspeptide isolated from *P. jamesonlandense* DAOM 234087^T.

and the amino acid-derived cycloaspeptide A and sclerotigenin. *P. soppii* produced the polyketides asperentins, terrein and griseofulvin, in addition to the amino acid-derived cycloaspeptide A, benzomalvins, asperphenamate and pseurotins, and the terpene fumagillin.

The consistency in cycloaspeptide production by the strains examined here is high. This consistent production of cycloaspeptide in soil-borne psychrotolerant species could indicate an ecophysiological function of this metabolite. Cycloaspeptides have never been found in the psychrotolerant food-borne penicillia (Frisvad & Filtenborg, 1989; Frisvad *et al.*, 2004; and this study). The other cyclic peptide, psychrophilin A, was only produced by *P. ribium* in this set of species and, like cycloaspeptide, psychrophilin A has not been found in any food-borne species of *Penicillium*.

For structural confirmation of the production of cycloaspeptide A, *P. jamesonlandense* strain IBT 21984^T was cultured on 200 Czapek yeast autolysate (CYA) agar plates in the dark at 12 °C for 3 weeks. Cultures were then macerated and extracted with 2 l ethylacetate (16 h, 22 °C), evaporated and partitioned between dichloromethane and water (CH₂Cl₂/H₂O; 55:45; v/v). The cycloaspeptide-enriched CH₂Cl₂ fraction was further separated on a Merck Lichroprep Si (310 × 25 mm i.d., 40–63 μm) column (30:69:1 to 0:99:1 heptane/ethylacetate/methanol gradient in 40 min at 14 ml min⁻¹). The third Lichroprep fraction was subsequently fractionated on a Sephadex LH20 column (25 × 300 mm) using CH₂Cl₂/methanol (50:50) flowing at 1 ml min⁻¹. Additionally, Sephadex fraction three was then further purified by HPLC on a Waters Prep Nova-Pak Si cartridge (100 × 25 mm i.d., 6 μm) using a gradient of CH₂Cl₂/methanol (99:1 to 95:5

gradient in 15 min at 14 ml min⁻¹) to give 32 mg pure cycloaspeptide A. Since a crystal of cycloaspeptide A could easily be obtained from methanol, X-ray analyses were performed, confirming the original structure proposed by Kobayashi *et al.* (1987) (Fig. 2; for X-ray methodology, see supplementary material in IJSEM Online).

Phylogenetic analyses of the ITS and partial β-tubulin gene sequences both showed that the two novel species, *P. ribium* and *P. jamesonlandense*, formed a monophyletic clade with

Table 2. Production of secondary metabolites by the four psychrotolerant species

Numbers in parentheses indicate the number of strains that produced the metabolite from the total number of strains.

| Secondary metabolite | Retention index |
|---|-----------------|
| <i>P. jamesonlandense</i> | |
| A tryptoquivalin (5/8) | 898 |
| Chrysogine (8/8)* | 681 |
| Cycloaspeptide A (8/8) | 1081 |
| Griseofulvin (5/8) | 941 |
| Kojic acid (7/8) | 630 |
| Penicillic acid (8/8) | 702 |
| Pseurotin A (6/8) | 811 |
| <i>P. ribium</i> | |
| 2-(4-hydroxyphenyl)-2-oxoacetaldehyde oxime (4/5) | 679 |
| Asperfuran (5/5) | 892 |
| Cycloaspeptide A (4/5) | 1081 |
| Cycloaspeptide D (4/5) | 1046 |
| Kojic acid (3/5) | 630 |
| Lichexanthone (3/5) | 863 |
| Psychrophilin A (5/5) | 1109 |
| Viridicatumtoxin (4/5) | 1181 |
| <i>P. lanosum</i> | |
| Compactin (31/37) | 1107 |
| Cycloaspeptide A (37/37) | 1081 |
| Dechlorogriseofulvin (36/37) | 885 |
| Griseofulvin (36/37) | 941 |
| Kojic acid (31/37) | 630 |
| Pyripyropen A (12/37) | 1034 |
| Sclerotigenin (32/37) | 754 |
| <i>P. soppii</i> | |
| Asperentin (21/22) | 910 |
| Asperphenamate (18/22) | 1197 |
| Benzomalvin A (22/22) | 1018 |
| Cycloaspeptide A (21/22) | 1081 |
| Fumagillin (6/22) | 1142 |
| Griseofulvin (12/22) | 941 |
| Pseurotin A (20/22) | 811 |
| Terrein (21/22) | 645 |

*All isolates produced chrysogine, except strain IBT 22021, which produced the chrysogine precursor, 2-acetyl-3H-quinazolin-4-one.

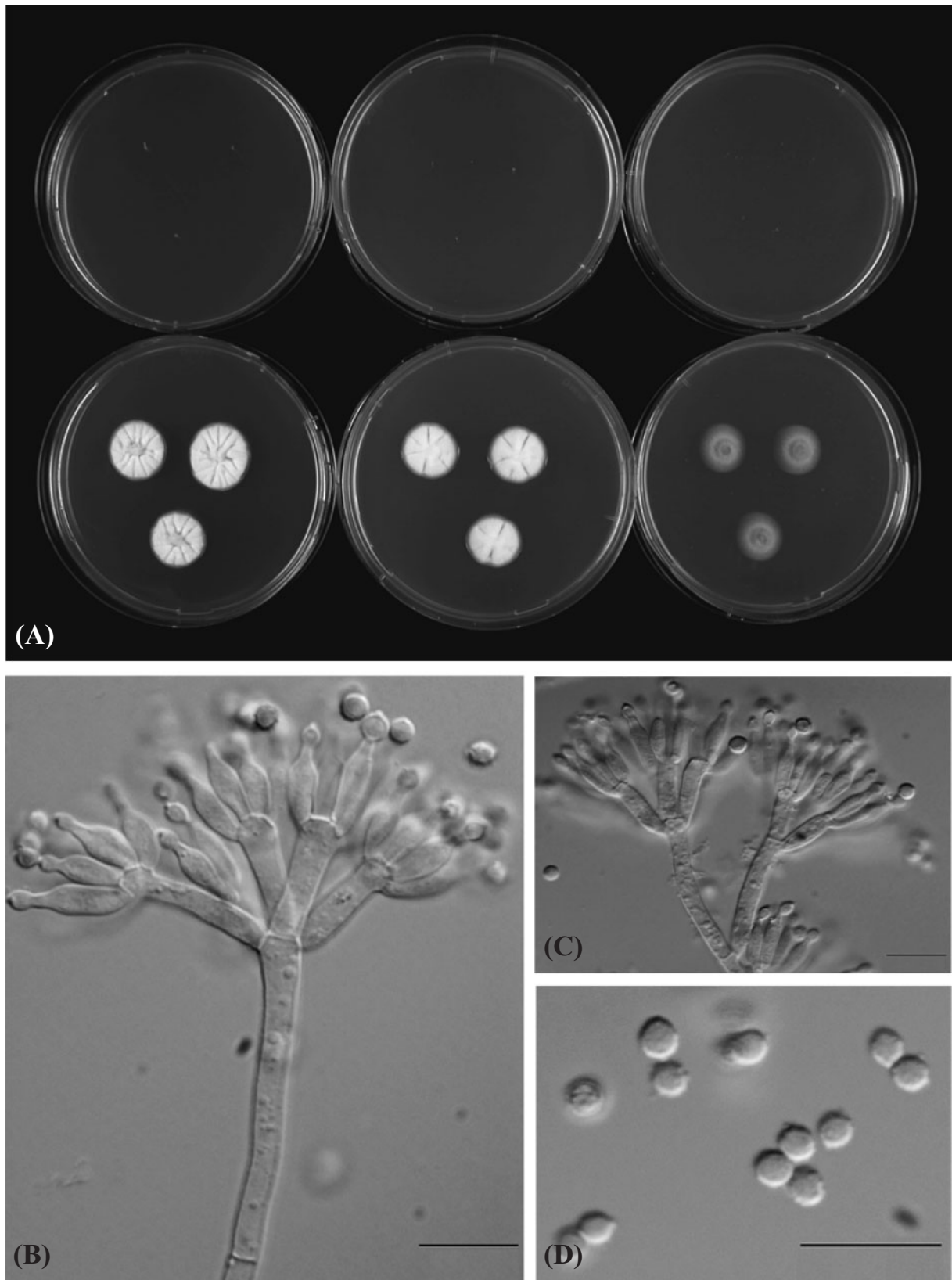


Fig. 3. Colony appearance and micromorphology of *P. jamesonlandense* DAOM 234087^T. (A) Colony appearance (front) after 7 days at 25 and 20 °C (top left, YES 25 °C; top centre, CYA 25 °C; top right, MEA 25 °C; bottom left, YES 20 °C; bottom centre, CYA 20 °C; bottom right, MEA 20 °C). (B, C) Conidiophores. (D) Conidia. Bars, 10 µm.

two other species producing cycloaspeptide, *P. swiecickii* and *P. lanosum*. The inclusion of the fifth cycloaspeptide producer *P. soppii* in this clade is equivocal in the β -tubulin gene sequence analysis. Some of the species belonging to this clade, *P. ribium*, *P. scabrosum*, *P. soppii* and *P. raistrickii*, do not produce kojic acid, but several species in the major

clade produce griseofulvin (Table 2). Both analyses supported the species concepts for the cycloaspeptide-producing species, including the two novel species. As expected, the less variable ITS region revealed invariant gene sequences for the four species producing cycloaspeptide, whereas the β -tubulin gene sequences had some infraspecific

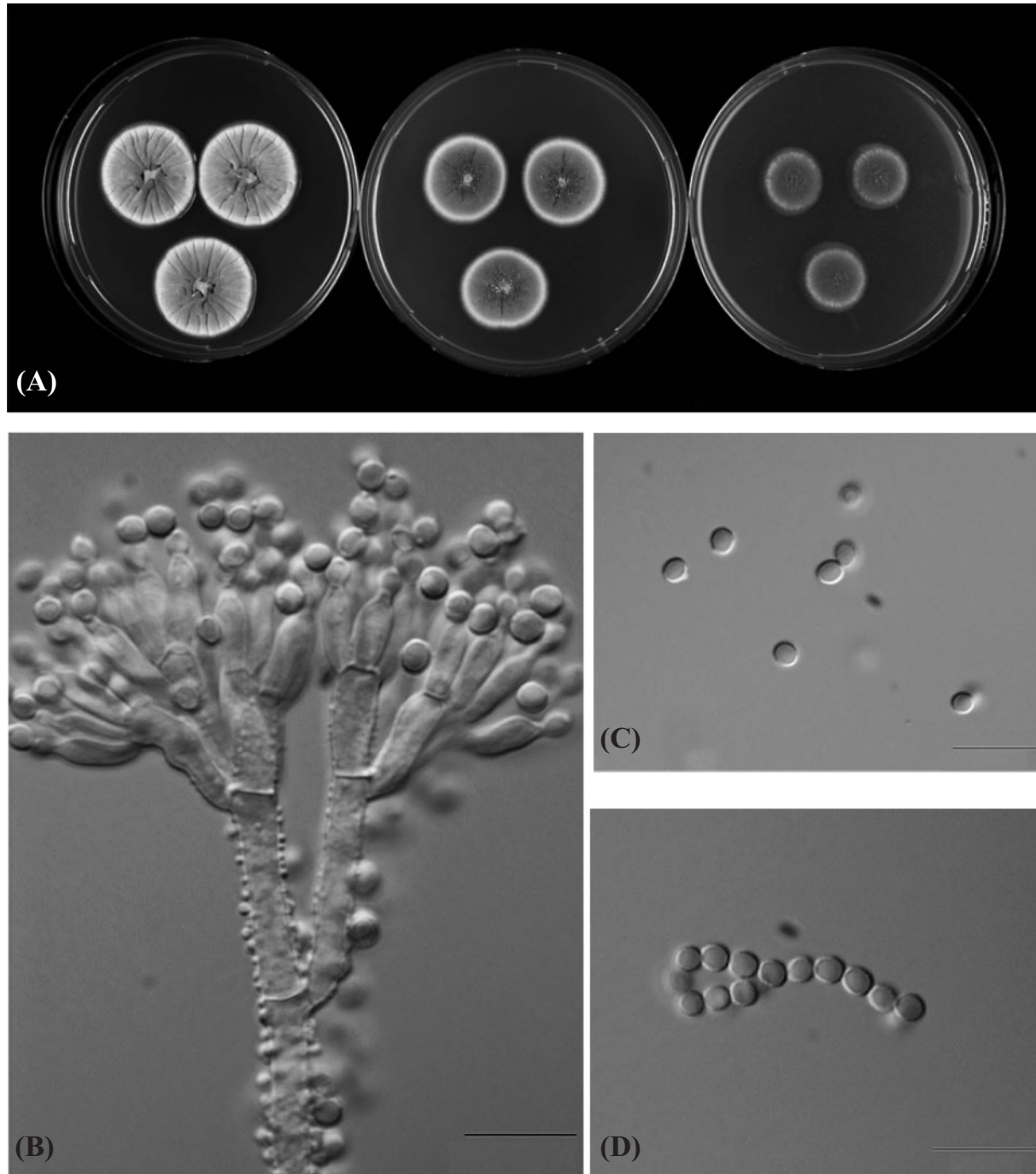


Fig. 4. Colony appearance and micromorphology of *P. ribium* DAOM 234091^T. (A) Colony appearance (front) after 7 days at 25 °C (left, MEA; centre, CYA; right, YES). (B) Conidiophore. (C, D) Conidia. Bars, 10 μ m.

variation, most notably a 15 bp insert in two of the four strains of *P. jamesonlandense*. However, the two datasets provide support that the phenotypically delimited species also meet the criteria of the phylogenetic species concept (Taylor *et al.*, 2000). In contrast with some other phylogenetic studies on complexes in *Penicillium* (Skouboe *et al.*, 1999), but in agreement with others (Boysen *et al.*, 1996), the ITS gene sequences provided species-level resolution for the species studied here. As in previous studies, partial β -tubulin gene sequences were more variable and provided more robust support for species concepts, despite some infraspecific variation in the sequences (Samson *et al.*, 2004b).

The chemical diversity and diversity of pharmaceutically active compounds is exceptionally high in these species from alpine areas and Arctic regions (Table 2). It is often claimed that most bioactive molecules are to be found in the tropics (Pointing & Hyde, 2001) but, among the secondary metabolites tested, griseofulvin, asperfuran, compactin, pyripyropens, benzomalvins, pseurotin and fumagillins have been suggested or used as drugs. Several compounds have antibiotic activity and some have been regarded as mycotoxins, including tryptoquivalins, viridicatumtoxin and penicillic acid (Cole & Cox, 1981). It is remarkable that bioactive secondary metabolites such as griseofulvin and viridicatumtoxin are found in tropical species such as *Penicillium aethiopicum* (Frisvad & Filtenborg, 1989) and also in the psychrotolerant species examined in this study. Apparently, climatic habitat is not necessarily a reliable indicator of production of particular bioactive secondary metabolites. On the other hand, metabolites such as cycloaspeptide A and D and psychrophilin A have only been found in psychrotolerant species. We conclude that polar regions are an untapped resource of biodiversity and chemical diversity.

For the purpose of species descriptions, isolated strains of *P. ribium* and *P. jamesonlandense* were cultured individually on multiple media [creatine-sucrose (CREA), CYA, MEA, oatmeal (OAT) and yeast extract-sucrose (YES) agar; for formulae see Samson *et al.*, 2004a] by incubating in the dark at 15, 20, 25 and 37 °C. After 7 days growth, colony appearance, exudate production, pigmentation and reverse colouration were assessed and colony diameters were measured. A set of 25 micromorphological dimensions was obtained for each characteristic at 40 × 10 and 100 × 10 magnification using an Olympus microscope, DP20 digital camera and DP-Soft Image Analysis software.

The results obtained in this study show that strain IBT 21984^T represents a novel species, *Penicillium jamesonlandense* sp. nov., and that strain IBT 16537^T represents a second novel species, *Penicillium ribium* sp. nov. The two species were unique morphologically, physiologically and in their extrolite profiles. Furthermore, they were clearly different from other *Penicillium* species in ITS and partial β -tubulin gene sequences. A list of the strains used in this study is provided in Table 1.

Latin diagnosis of *Penicillium jamesonlandense* Frisvad *et* Overy sp. nov.

Penicillio lanoso simile, sed crescentia lentissima (0.5–7 mm diametro, conidias absentibus post 7 dies 25 °C), ramis valde divergentibus distinctum. Acidum penicillicum formatur, neque sclerotigeninum et compactinum. Typus: DAOM 234087^T (=IBT 21984^T=IBT 24411^T=CBS 102888^T), isolatus ex solo, *Jamesonlandii* in Groenlandia. Herbarium specimen: C 60164^T.

Description of *Penicillium jamesonlandense* Frisvad & Overy sp. nov. (Fig. 3)

Penicillium jamesonlandense (ja.me.son.lan.den'se. N.L. neut adj. *jamesonlandense* pertaining to Jamesonland, Greenland from where the type strain was isolated).

After 1 week of growth on CYA agar at 25 °C, colonies are 0.5–7 mm diameter. No conidia present; mycelium colour is white, reverse is cream–white to cream–yellow. Colonies on MEA after 1 week of growth at 25 °C are 0–4 mm diameter, with weak or no sporulation, reverse creamish yellow to light-yellow. Colonies on YES agar after 1 week of growth at 25 °C are 2–12 mm diameter, with no sporulation, reverse colour is cream–yellow, occasionally turning brownish. No growth at 30 or 37 °C. Growth on CREA is moderate with good acid production, 3–5 mm diameter. Conidiophores produced from aerial hyphae on MEA and OAT at 20 and 15 °C. Penicilli are biverticillate or twice biverticillate, not genuinely terverticillate as the ramus is borne divergently, usually with an angle of >60° and far below the terminal penicillus. Stipes are smooth-walled to finely roughened, measuring 60–200 × 3.5–5 µm, bearing smooth-walled to finely roughened, long (22–32 µm) rami. Metulae are borne quite divergently in verticils of (2–) 3 to 4, smooth-walled, measuring 8.2–16.2 (mean = 13.3) × 2.9–4.2 (mean = 3.5) µm and are weakly apically swollen (5.5–6.5 µm). Phialides are smooth-walled, flask-shaped, 8.1–10.9 (mean = 9.3) × 2.8–3.7 (mean = 3.2) µm, with rather long collula (1.5–3 µm). Conidia are green, rough-walled and borne in small irregular chains, oval- to limiform-shaped when young, becoming globose to subglobose when mature, 2.7–3.3 (mean = 2.9) µm. The species belongs in Section *Ramosum*, series *Lanosa* (Stolk & Samson, 1985).

The type strain, DAOM 234087^T (=IBT 21984^T=IBT 24411^T=CBS 102888^T), was isolated from a soil sample from Greenland.

Latin diagnosis of *Penicillium ribium* Frisvad *et* Overy sp. nov.

Penicillio lanoso simile, sed stipitibus conidiophorum longissimis, rugosis, conidiis levibus distinctum. Psychrophilinum et asperfuranum formantur, neque sclerotigeninum, compactinum et pyripyropeum. Typus: DAOM 234091^T (=IBT 16537^T=IBT 24431^T), isolatus ex solo alpino sub Ribes sp., Wyoming, USA. Herbarium specimen: C 60165^T.

Description of *Penicillium ribium* Frisvad & Overy sp. nov. (Fig. 4)

Penicillium ribium (rib'i.um. N.L. adj. *ribium* of *Ribes*, isolated from around *Ribes* spp. growing in tundra soil).

After 1 week of growth at 25 °C on CYA agar, colonies are 19–30 mm diameter, show good sporulation, produce clear exudate droplets and the mycelium is white with a greyish orange–brown reverse. Colonies on MEA after 1 week of growth at 25 °C are 21–24 mm in diameter, show good sporulation and the reverse of the mycelium is pale-yellow. Colonies on YES agar after 1 week of growth at 25 °C are 26–31 mm in diameter, show good sporulation and the reverse colour of the mycelium is creamish yellow, often with a brown centre. Weak growth on CREA; colonies are 7–16 mm in diameter with no acid production. No growth on CYA at 37 °C. Penicilli are biverticillate (terverticillate), with rough stipes, 150–2000 µm in length, with rather divergent asymmetric branching of finely roughened rami measuring 15.2–27.0 (mean = 20.8) × 2.8–3.8 (mean = 3.4) µm. Three rami are often present. Metulae are slightly roughened, sometimes smooth-walled, cylindrical, 9.6–13.5 (mean = 11.5) × 2.9–3.9 (mean = 3.3) µm. Phialides are smooth-walled, flask-shaped with short, visible collula, 7.8–9.7 (mean = 8.8) × 2.4–2.9 (mean = 2.7) µm. Conidia are green, smooth-walled, subglobose to ovoid, 2.1–2.8 (mean = 2.5) × 2.7–3.5 (mean = 3.1) µm. The species belongs in Section *Ramosum*, series *Lanosa* (Stolk & Samson, 1985).

The type strain, DAOM 234091^T (=IBT 16537^T=IBT 24431^T), was isolated from around plants of *Ribes* spp. growing in tundra soil in Wyoming, USA.

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References

- Amade, P., Mallea, M. & Bouaicha, N. (1994). Isolation, structural identification and biological activity of two metabolites produced by *Penicillium olsonii* Bainier and Sartory. *J Antibiot* **47**, 201–207.
- Boysen, M., Skouboe, P., Frisvad, J. & Rossen, L. (1996). Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles. *Microbiology* **142**, 541–549.
- Cole, R. J. & Cox, R. H. (1981). *Handbook of Toxic Fungal Metabolites*. New York: Academic Press.
- Dalsgaard, P. W., Larsen, T. O., Frydenvang, K. & Christophersen, C. (2004). Psychrophilin A and cycloaspeptide D, novel cyclic peptides from the psychrotolerant fungus *Penicillium ribeum*. *J Nat Prod* **67**, 878–881.
- Frisvad, J. C. & Filtenborg, O. (1989). Terverticillate penicillia: chemotaxonomy and mycotoxin production. *Mycologia* **81**, 837–861.
- Frisvad, J. C. & Filtenborg, O. (1990a). Revision of *Penicillium* subgenus *Furcatum* based on secondary metabolites and conventional characters. In *Modern Concepts in Penicillium and Aspergillus Classification*, pp. 159–170. Edited by R. A. Samson & J. I. Pitt. New York: Plenum.
- Frisvad, J. C. & Filtenborg, O. (1990b). Secondary metabolites as consistent criteria in *Penicillium* taxonomy and a synoptic key to *Penicillium* subgenus *Penicillium*. In *Modern Concepts in Penicillium and Aspergillus Classification*, pp. 373–384. Edited by R. A. Samson & J. I. Pitt. New York: Plenum.
- Frisvad, J. C. & Thrane, U. (1987). Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (diode array detection). *J Chromatogr* **404**, 195–214.
- Frisvad, J. C. & Thrane, U. (1993). Liquid column chromatography of mycotoxins. In *Chromatography of Mycotoxins. Techniques and Applications*, pp. 253–372, Journal Chromatography Library, vol. 54. Edited by V. Betina. Amsterdam: Elsevier.
- Frisvad, J. C., Smedsgaard, J., Larsen, T. O. & Samson, R. A. (2004). Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. *Stud Mycol* **49**, 201–241.
- Glass, N. L. & Donaldson, G. C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol* **61**, 1323–1330.
- Gunde-Cimerman, N., Sonjak, S., Zalar, P., Frisvad, J. C., Diderichsen, B. & Plemenitaš, A. (2003). Extremophilic fungi in arctic ice: a relationship between adaptation to low temperature and water activity. *Phys Chem Earth* **28**, 1273–1278.
- Haugland, R. A., Varma, M., Wymer, L. J. & Vesper, S. J. (2004). Quantitative PCR analysis of selected *Aspergillus*, *Penicillium* and *Paecilomyces* species. *Syst Appl Microbiol* **27**, 198–210.
- Hocking, A. D. & Pitt, J. I. (1980). Dichloran-glycerol medium for enumeration of xerophilic fungi from low-moisture foods. *Appl Environ Microbiol* **39**, 488–492.
- Kobayashi, R., Samejima, Y., Nakajima, S., Kawai, K. & Udagawa, S. (1987). Studies on fungal products. XI. Isolation and structures of novel cyclic pentapeptides from *Aspergillus* sp. NE-45. *Chem Pharm Bull (Tokyo)* **35**, 1347–1352.
- McRae, C. F., Hocking, A. D. & Seppelt, R. D. (1999). *Penicillium* species from terrestrial habitats in the Windmill Islands, East Antarctica, including a new species, *Penicillium antarcticum*. *Polar Biol* **21**, 97–111.
- O'Donnell, K. & Cigelnik, E. (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Mol Phylogenet Evol* **7**, 103–116.
- Peterson, S. W. (2000). Phylogenetic analysis of *Penicillium* species based on ITS and LSU-rDNA nucleotide sequences. In *Integration of Modern Taxonomic Methods for Penicillium and Aspergillus*, pp. 163–178. Edited by R. A. Samson & J. I. Pitt. Amsterdam: Harwood Academic.
- Pitt, J. I. (1979). *The Genus Penicillium and its Teleomorphic States Eupenicillium and Talaromyces*. London: Academic Press.
- Pointing, S. B. & Hyde, K. D. (2001). *Bio-exploitation of Filamentous Fungi*. Hong Kong: Fungal Diversity Press.
- Pringle, A. & Taylor, J. E. (2002). The fitness of filamentous fungi. *Trends Microbiol* **10**, 474–481.
- Rakeman, J. L., Bui, U., LaFe, K., Chen, Y.-C., Honeycutt, R. J. & Cookson, B. T. (2005). Multilocus DNA sequence comparisons rapidly identify pathogenic molds. *J Clin Microbiol* **43**, 3324–3333.
- Samson, R. A., Hoekstra, E. S. & Frisvad, J. C. (2004a). *Introduction to Food- and Airborne Fungi*, 7th edn. Utrecht: Centraalbureau voor Schimmelcultures.

- Samson, R. A., Seifert, K. A., Kuijpers, A. F. A., Houbraken, J. A. M. P. & Frisvad, J. C. (2004b).** Phylogenetic analysis of *Penicillium* subgenus *Penicillium* using partial β -tubulin sequences. *Stud Mycol* **49**, 175–200.
- Schadt, C. W., Martin, A. P., Lipson, D. A. & Schmidt, S. K. (2003).** Seasonal dynamics of previously unknown fungal lineages in tundra soils. *Science* **301**, 1359–1361.
- Skouboe, P., Frisvad, J. C., Lauritsen, D., Boysen, M., Taylor, J. W. & Rossen, L. (1999).** Nucleotide sequences from the ITS region of *Penicillium* species. *Mycol Res* **103**, 873–881.
- Smedsgaard, J. (1997).** Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *J Chromatogr A* **760**, 264–270.
- Stolk, A. C. & Samson, R. A. (1985).** A new taxonomic scheme for *Penicillium* anamorphs. In *Advances in Penicillium and Aspergillus Systematics*, pp. 163–190. Edited by R. A. Samson & J. I. Pitt. New York: Plenum.
- Swofford, T. (1999).** PAUP*: Phylogenetic analysis using parsimony (* and other methods), version 4.0b10. Sunderland, MA: Sinauer Associates.
- Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S. & Fisher, M. C. (2000).** Phylogenetic species recognition and species concepts in fungi. *Fungal Genet Biol* **31**, 21–32.
- Weinstein, R. N., Palm, M. E., Johnstone, K. & Wynn-Williams, D. D. (1997).** Ecological and physiological characterization of *Humicola marvinii*, a new psychrophilic fungus from fell field soils in maritime Antarctic. *Mycologia* **89**, 706–711.
- White, T. J., Bruns, T., Lee, S. & Taylor, J. W. (1990).** Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications*, pp. 315–322. Edited by M. A. Innis, R. H. Gelfand, J. J. Sninsky & T. J. White. New York: Academic Press.