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Comparison of secondary metabolite production by *Penicillium crustosum* strains, isolated from Arctic and other various ecological niches

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Abstract

Penicillium crustosum is common in food and feed both in subtropical and temperate regions. Recently, it has also been found occurring frequently in glacier ice, sea ice and sea water of Arctic regions of Svalbard. The aim of the study was to compare isolates of the same fungal species from widely different habitats and geographic regions to see if the nutritional physiology and the profile of secondary metabolites were consistent or depended on the isolation source. All 121 strains examined produced the following families of secondary metabolites: penitrems (100%), roquefortines (100%), terrestric acids (99.2%) and viridicatols (100%), whereas 81 of 83 Arctic isolates additionally produced andrastin A. However, only 8 of 38 non-Arctic isolates produced detectable andrastin A. The quantitative profiles of 96 strains were compared using cluster, principal component and correspondence analyses. There was no clear grouping of Arctic versus non-Arctic, creatine positive versus creatine negative strains.

Keywords: *Penicillium crustosum*; Penitrems; Roquefortine C; Andrastin A; Arctic; Glacier ice

1. Introduction

The terverticillate filamentous fungus *Penicillium crustosum* was first described by Thom in 1930 and is one of 225 accepted species of *Penicillium*, the major group in the *Trichocomaceae* family (Eurotiales) [1]. Its taxonomy has been problematic as it has been synonymized with *Penicillium verrucosum* var. *cyclopium* [2] or reduced to a variety of *Penicillium expansum* by Fassatiová [3]. It is therefore difficult to produce an accurate literature survey of this species.

Penicillium crustosum is a food-borne ubiquitous fungal species, frequently isolated from nuts, meat, cheese, feeds, vegetables, pomaceous and stone fruits. It is also common in the soil rhizosphere of vegetables [4]. It has been reported under the name *Penicillium cyclopium* and found on Svalbard [5] and in eastern Siberia [6]. Under the name of *P. crustosum* it has rarely been found in extremely cold environments. Recently, a study of mycobiota in the extreme Arctic environment showed a high occurrence of *P. crustosum.* It was isolated from seawater and sea ice, and was dominating in glacier ice, representing up to half of all *Penicillium* isolates [7].

Penicillium species are important not only due to their widespread occurrence but also because of their ability to produce mycotoxins and other secondary metabolites [8]. Classification of *Penicillium* species

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using traditional morphological features is often very difficult because of limited or variable data. Chemotaxonomic studies are therefore often used as additional parameters [9]. For many terverticillate Penicillia the secondary metabolite profile in itself enables conclusive classification [10]. These metabolite profiles are species-specific and usually consistently expressed in different isolates of the same species, regardless of geographic origin or habitat. Nevertheless, particular isolates may show strain-specific secondary metabolite profiles [9].

Penicillium crustosum produces many volatile and non-volatile metabolites. The most characteristic are penitrems, viridicatins, terrestric acid and roquefortine C [4,11]. Due to their potential as mycotoxin producers, secondary metabolite profiles of common ubiquitous isolates have been frequently determined [4,12]. Kawai et al. [13] proposed two chemotypes of *P. crustosum* and Rundberget et al. [14] suggested that secondary metabolite profiles were not consistent in this species. Isolation of a large number of *P. crustosum* strains from different niches in extreme Arctic environments enabled comparison of secondary metabolite profiles of these strains with isolates obtained from warmer environments.

The goal of the present study was to reveal potential differences in secondary metabolite profiles among strains of *P. crustosum*, isolated from different locations and habitats.

2. Materials and methods

2.1. Cultures used

The analysis included 83 strains of *P. crustosum*, isolated from Arctic environments and 38 strains form other environments (Table 1). The strains are maintained in the EX-F Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia and/or in IBT Culture Collection of The Centre for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark. The strains were identified to the species level by micro- and macro-morphology, and physiology [15].

2.2. Description of the Arctic isolation site

Kongsfjorden is one of the large Arctic fjords found on the western coast of Spitsbergen, Svalbard, located at 79°N, 12°E. It is 26 km long and 8 km wide and stretches from east–southeast to west–northwest at the Greenland Sea. The majority of the drainage basin is covered by glaciers. Annual mean temperature is around −5 °C [16–18]. Samples of seawater were taken in the fjord, snow samples on its coastline, while glacial ice originated from Conwaybreen and Kronebreen glaciers and directly from Kongsvegen and Austre Lovénbreen glaciers. Sampling was performed in June and August 2001 [7].

2.3. Cultivation, growth rate determination and morphological characterisation

For the determination of morphological characteristics and HPLC analyses, strains were inoculated in three point cultures on malt extract agar (MEA), Czapek yeast autolysate agar (CYA), yeast extract sucrose agar (YES) and creatine sucrose agar (CREA) and grown for seven days at 25 °C in the dark [19]. Growth rate was determined by measurement of colony diameter from hyphal tip to hyphal tip of the largest colony on MEA, CYA and YES. On the basis of morphological appearance of colonies, the growth rate was described on CREA medium as very good (++), good (+) or weak (−). The extent of acid production on CREA medium was also determined [20]. For the determination of micro-morphological characteristics of cultures MEA and CYA media were used and analysed with Olympus BX51 microscope with DP12 digital camera and DP-SOFT 3.2 application software.

2.4. Extraction for HPLC analysis

For HPLC extraction plugs (6 mm in diameter) were cut from the centre and close to the centre of a *P. crustosum* colony growing on CYA (2 plugs) and YES (2 plugs). All four plugs were transferred to a 1.5-ml disposable autosampler screw-cap vial and 500 μl of the solvent mixture, methanol–dichloromethane–ethyl acetate (3:2:1) containing 0.5% (v/v) formic acid, was added. The plugs were extracted ultrasonically for 60 min. The extracts were transferred to a clean vial and the organic phase was evaporated to dryness in fume hood and with centrifugation in vacuum (RVC). The residues were re-dissolved ultrasonically for 10 min in 500 μl methanol. All samples were filtered through 0.45 μm Minisart RC4 filters (Sartorius, Germany) into clean vials before analyses [21].

2.5. HPLC analyses

The HPLC analyses were based on the methods of Frisvad and Thrane [22,23], as modified by Smedsgaard [21]. The analyses were performed on an A1100 HPLC (Agilent, Germany) using 5 μl injections. The metabolites were detected at 210 nm, but each peak detected was characterized by its UV spectrum from 200-600 nm (diode array detection) with a peak width of 0.2 min. Separations were done on a 2×100 mm Luna2 OOD-4251-BO-C_{18} column (Phenomenex, Germany) with a C_{18} precolumn, both packed with 3 μm particles. The column was maintained at 40 °C. A linear gradient

Table 1
List of 121 strains used in the HPLC analyses, their growth on CREA medium and metabolite production according to substratum and geographic origin

Isolate	Substratum	Origin	C	R	P	V	T	A	M
EX-F 887, IBT 23312	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 896, IBT 23313	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 899, IBT 23309	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 900, IBT 23303	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 902, IBT 23345	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 904, IBT 23349	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 905, IBT 23311	Sea water	Svalbard	–	+	+	+	+	+	+
EX-F 906, IBT 23310	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1001, IBT 23882	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1003, IBT 24304	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 1005, IBT 23873	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1009, IBT 24309	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 1012, IBT 24310	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 1016, IBT 23375	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1018, IBT 23382	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 1019, IBT 24123	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1027, IBT 24081	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1036, IBT 24303	Melted snow	Svalbard	–	+	+	+	+	+	+
EX-F 1037, IBT 23875	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1045, IBT 23383	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 1048, IBT 23876	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1050, IBT 24311	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 1099, IBT 24093	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1103, IBT 24092	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1110, IBT 24348	Sea water	Svalbard	++	+	+	+	+	+	+
EX-F 1113, IBT 24352	Glacial water	Svalbard	++	+	+	+	+	+	+
EX-F 1116, IBT 24345	Glacial water	Svalbard	++	+	+	+	+	+	+
EX-F 1118, IBT 24349	Glacial water	Svalbard	++	+	+	+	+	+	+
EX-F 1125, IBT 23893	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1134, IBT 23894	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1141, IBT 24343	Glacial water	Svalbard	++	+	+	+	+	+	+
EX-F 1144, IBT 23376	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 1145, IBT 24350	Sea water	Svalbard	++	+	+	+	+	+	+
EX-F 1147, IBT 24346	Sea water	Svalbard	++	+	+	+	+	+	+
EX-F 1151, IBT 24126	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1157, IBT 23900	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1160, IBT 23386	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 1161, IBT 24300	Sea water	Svalbard	++	+	+	+	+	+	+
EX-F 1167, IBT 24264	Glacial ice	Svalbard	++	+	+	+	+	–	+
EX-F 1169, IBT 24231	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1171, IBT 24232	Glacial ice	Svalbard	++	+	+	+	+	–	–
EX-F 1181, IBT 24301	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1189, IBT 23377	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 1193, IBT 24353	Sea water	Svalbard	++	+	+	+	+	+	+
EX-F 1194, IBT 24261	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1199, IBT 24133	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1204, IBT 24129	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1209, IBT 24136	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1213, IBT 24132	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1218, IBT 24131	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1222, IBT 24135	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1231, IBT 23886	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1235, IBT 23891	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1240, IBT 24259	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1248, IBT 24234	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1252, IBT 24258	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1254, IBT 24262	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1303, IBT 24079	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1308, IBT 23883	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1315, IBT 23892	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1324, IBT 23890	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1329, IBT 24094	Glacial ice	Svalbard	++	+	+	+	+	+	+

(*continued on next page*)

Table 1 (*continued*)

Isolate	Substratum	Origin	C	R	P	V	T	A	M
EX-F 1330, IBT 23884	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1335, IBT 23901	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1339, IBT 23899	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1344, IBT 24089	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1349, IBT 24128	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1362, IBT 24302	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 1372, IBT 24260	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1375, IBT 24233	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1380, IBT 24263	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1381, IBT 24238	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1382, IBT 24237	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1383, IBT 24236	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1385, IBT 24235	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1396, IBT 24096	Glacial ice	Svalbard	++	+	+	+	+	+	+
EX-F 1398, IBT 23387	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1399, IBT 23880	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1400, IBT 23841	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1402, IBT 23336	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1407, IBT 23871	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1409, IBT 23878	Glacial ice	Svalbard	–	+	+	+	+	+	+
EX-F 1490, IBT 24305	Sea ice	Svalbard	–	+	+	+	+	+	+
EX-F 2025, IBT 24776	Saline water	Slovenia	++	+	+	+	+	–	+
IBT 10106, EX-F 1438	Ugli fruit	From tropics imported to Denmark	++	+	+	+	+	–	–
IBT 3422	Indoor air	Denmark	++	+	+	+	+	+	+
IBT 3423, G. Engel 6842	Cheese	Germany	++	+	+	+	+	–	+
IBT 3424, Leistner Sp. 2309	Salami	Germany	++	+	+	+	+	–	+
IBT 3425, IMI 285510	Thyme	Imported to Denmark	++	+	+	+	+	–	+
IBT 3426, ATCC 32014	Weevil damaged pecan	Georgia, USA	++	+	+	+	+	–	+
IBT 4131, EX-F 1429	Salt marsh soil	Egypt	++	+	+	+	+	–	+
IBT 5528, EX-F 2021, FRR 1669, (ex neotype)	Lemon	Scotland	++	+	+	+	–	+	+
IBT 6578, FRR 2223	Weevil damaged pecan	Georgia, USA	++	+	+	+	+	–	+
IBT 6580, EX-F 1430, FRR 1387	Cork	Portugal	++	+	+	+	+	–	+
IBT 10513	Air contaminant	Denmark	++	+	+	+	+	–	+
IBT 10529	Air contaminant	Denmark	++	+	+	+	+	–	+
IBT 11135, EX-F 1436, IMI 296059	Air in cave	England	++	+	+	+	+	+	–
IBT 11436, EX-F 1437	Leather harness	Saudi Arabia	++	+	+	+	+	–	+
IBT 13426, VKM F-261	Unknown	Russia	++	+	+	+	+	+	+
IBT 13427, VKM F-267	Unknown	Russia	++	+	+	+	+	+	+
IBT 13602, EX-F 1432, DAOM 215342	Forest soil	Canada	++	+	+	+	+	–	–
IBT 14519	Luchuiguilla cave	New Mexico, USA	++	+	+	+	+	–	+
IBT 14747, CBS 101025	Cheese	Portugal	++	+	+	+	+	–	+
IBT 15977, CBS 110075	Mixed feed	Bulgaria	++	+	+	+	+	–	+
IBT 16510	Soil under Betula sp.	Victoria, Canada	++	+	+	+	+	–	+
IBT 16885	Mouldy onion	Denmark	++	+	+	+	+	+	+
IBT 18099, FRR 1513	Deteriorated wood stakes	Queensland Australia	++	+	+	+	+	–	+
IBT 18359, CCRC 32633	Unknown	Taiwan	++	+	+	+	+	+	+
IBT 19390, EX-F 1434	Mangostan fruit	From Tropics imported to Denmark	++	+	+	+	+	–	+
IBT 20631, EX-F 1435	Soil, tropical ZOO	Victoria, Canada	++	+	+	+	+	–	–
IBT 22026	Unknown	Denmark	++	+	+	+	+	–	+
IBT 22482, EX-F 1439	Waste compost	Germany	++	+	+	+	+	–	+
IBT 23710, IMI 206159	Soil	New Zealand	++	+	+	+	+	–	+
IBT 23739	Cheese	Germany	++	+	+	+	+	–	+
IBT 23815, IMI 229034	Unknown	Unknown	++	+	+	+	+	+	+
IBT 25243, NRRL 968	Rice	China	++	+	+	+	+	–	+
IBT 26244, 1582 P2	Food or food waste	Norway	++	+	+	+	+	–	+
IBT br241	Air, ryebread factory	Denmark	++	+	+	+	+	–	+
IBT D210f	Green coffee bean	Indonesia	++	+	+	+	+	–	+
IBT VIA4, IMI 293182	Grape	Denmark	++	+	+	+	+	–	+
IBT X1983, CBS 747.74	Unknown	New York, USA	++	+	+	+	+	–	+

C, degree of growth on CREA medium; production of secondary metabolites (extrolites): R, roquefortine C; P, penitrem A; V, viridicatols; T, terrestric acid; A, andrastin A; M, metabolite Q.

starting from 85% water (A) and 15% acetonitrile (B) going to 100% acetonitrile in 20 min, then maintaining 100% acetonitrile for 5 min, was used at a flow rate of 0.4 ml min^{-1}. Both eluents contained 0.005% (v/v) trifluoroacetic acid (TFA). Chemicals used were Merck or Riedel-de Hoen (acetonitrile) analytical grade. An alkylphenone retention index was calculated for each peak detected. The metabolites were identified by comparison to standards and by their characteristic UV spectra. All peaks were quantified as their peak area and the logarithm of those values were used in the multivariate statistical analyses.

2.6. Multivariate statistical analyses

The quantitative secondary metabolite data were analysed with principal component analysis using the programs UNSCRAMBLER (CAMO, Oslo, Norway) and correspondence and UPGMA cluster analysis using NT-SYS (numerical taxonomy and multivariate analysis system, version 2.10, Exeter Software, New York). The logarithms of the peak areas of all the metabolites measured were used as variable values and the data were centred and weighted with the inverse of the standard deviation (autoscaling). The data used for correspondence analysis were not autoscaled, but used as the log peak area values.

3. Results

3.1. Macro- and micro-morphology

High growth rates were observed for all strains on MEA, CYA and YES media. Colony diameters on MEA (average ± SD = 32 ± 5; range: 18–46), CYA (average ± SD = 38 ± 4; range: 23 (just one strain) – 46) and YES (average ± SD = 49 ± 4; range: 39–66) confirmed that as well. All strains had two-stage branched conidiophores, rough-walled conidiophore stipes and smooth-walled, globose to subglobose conidia with an average diameter of 3.5–3.8 μm (on CYA medium). All strains produced the typical crusts of conidia on MEA after 7–9 days of growth, except the ex neotype culture, which was due to deterioration more floccose. All the measurements and colony colours were in concordance with the description of the species [19]. All non-Arctic isolates (38) grew very well on CREA medium (CREA-positive strains) and these had good (majority) to very good acid production. 46 of the Arctic isolates also grew well on CREA and produced acid (two isolates had very good acid production). However, unexpectedly, 37 of 83 Arctic isolates showed only weak growth on the same medium (CREA-negative strains) (Table 1). Most of these isolates (22) revealed even better acid production than creatine-positive strains. Thirteen of the CREA-negative strains showed very little or no acid production.

3.2. Secondary metabolite profile of P. crustosum

All 121 *P. crustosum* strains produced penitrem A. This is in agreement with earlier analyses based on other isolates of *P. crustosum* [4,15,24] and 42 isolates, mostly from Norwegian foods, examined by Rundberget et al. [14]. Based on evidence of UV spectra and polarity, eight further penitrems were produced by most isolates, as well as one or two thomitrems. The characteristic UV spectrum similar to that of paspalinine, found in three compounds, indicated that some of the PC-M4, PC-M5, PC-M5' and PC-M6 metabolites [45] were also produced. Finally, four further compounds with the chromophore of penitrem A were produced in low amounts, therefore they were often undetectable. None of the isolates produced metabolites with penitremone chromophores. In all strains 18 secondary metabolites referable to the penitrem biosynthetic family could be detected with some confidence. Of these metabolites 13 have their structure elucidated (Table 2).

In agreement with earlier observations on other strains of this species [4,14] all 121 strains of *P. crustosum* produced roquefortine C. Roquefortine D production was not confirmed, but this metabolite is an obligatory precursor of roquefortine C, so it could have been detected in younger cultures of *P. crustosum*. Another compound with a roquefortine C chromophore consistently detected in our analyses could have been roquefortine E [25] but this has to be confirmed by mass spectrometry.

All strains produced the viridicatols. Usually, the whole spectrum of known compounds in this biosynthetic family: cyclopeptin, dehydrocyclopeptin, cyclopenol, cyclopenin, viridicatol and viridicatin were produced, but as could be expected, the two precursor metabolites cyclopeptin and dehydrocyclopeptin were only present in low amounts. Frisvad and Filtenborg [4] also found that *P. crustosum* consistently produced cyclopenin, cyclopenol and viridicatin.

Terrestric acids were produced by all strains of *P. crustosum*, except one strain (IBT 5528). This strain did produce some other terrestric acid analogues, however. This consistent production is in accordance with the results of Frisvad and Filtenborg [4].

Andrastin A was found for the first time in *P. crustosum*, but this metabolite was most prominent in Arctic isolates of the species. It was produced by most isolates collected in the Arctic, with only two strains apparently not producing it, while it was produced by eight out of 38 strains isolated from warmer climates.

A family of unknown metabolites (provisionally named metabolite Q) with a characteristic chromo-

Table 2
Production of known mycotoxins and other secondary metabolites by strains of *Penicillium crustosum*

Metabolite	Reference	Species	Retention index (RI)
Roquefortines (biosynthezised from tryptophan, histidine and a terpene dimethylallyl unit)			
Roquefortine C	[24,40]	*P. crustosum*	RI = 769
Roquefortine D	[41], this report	*P. roqueforti*, *P.crustosum*	
Roquefortine E	[25]	*P. verrucosum* var. *cyclopium*	RI = 932
Penitrems (biosynthezised from tryptophan and a diterpene unit)			
Penitrem A	[42]	*P. crustosum*	RI = 1290
Penitrem B	[43]	*P. crustosum*	
Penitrem C	[43]	*P. crustosum*	
Penitrem D	[43]	*P. crustosum*	
Penitrem E	[43]	*P. crustosum*	
Penitrem F	[43]	*P. crustosum*	
Penitrem G	[44]	*P. crustosum*	
PC-M4	[45]	*P. crustosum*	
PC-M5	[45]	*P. crustosum*	
PC-M5′	[46]	*P. crustosum*	
PC-M6	[46]	*P. crustosum*	
Thomitrem A	[47]	*P. crustosum*	RI = 1235
Thomitrem E	[47]	*P. crustosum*	RI = 1139
Viridicatols (biosynthezised from anthranilic acid, phenylalanine and molecular oxygen)			
Cyclopeptin	[48]	*P. cyclopium* (was *P. solitum*)	RI = 818
Dehydrocyclopeptin	[48]	*P. cyclopium* (was *P. solitum*)	RI = 857
Cyclopenol	[49]	*P. cyclopium* (was *P. solitum*) and *P. viridicatum* (was *P. crustosum*)	RI = 723
Cyclopenin	[50]	*P. cyclopium* (was *P. solitum*)	RI = 797
Viridicatin	[51]	*P. crustosum*	RI = 919
Viridicatol	[49]	*P. cyclopium* (was *P. solitum*) and *P. viridicatum* (was *P. crustosum*)	RI = 810
Terrestric acids (biosynthezised from the tricarboxylic acid cycle)			
Viridicatic acid	[52]	*P. viridicatum*	RI = 650, broad peak
Terrestric acid	[53]	*P. terrestre* (syn. of *P. crustosum*)	RI = 707, broad peak
Andrastins (biosynthezised from terpene units)			
Andrastin A	[54], this report	*Penicillium* sp., *P. crustosum*	RI = 1088
Sterols (biosynthezised from terpene units)			
Ergosterol	[55]	*P. crustosum*	RI = 2109
Compound with ergosterol chromophore	This report	*P. crustosum*	RI = 1250
Metabolites of undetermined structure			
Metabolite Q chromophore family	This report	*P. crustosum*	RI = 760, 791, 900, 913, 1033, 1390
Metabolite BIS, possibly a bisanthrone	This report	*P. crustosum*	RI = 1100
Secondary metabolites reported from P. crustosum, but not produced by this species			
α-Cyclopiazonic acid	[56]	was *P. commune*	
Festuclavine	[13,14]	was *P. palitans*	
Fumigaclavine A	[13,14]	was *P. palitans*	
Patulin[a]	[57]	*P. crustosum*	
Penitremones A–C	[58] as claimed in [14]	was a new species	
Pyroclavine	[13]	was *P. palitans*	
Xanthomegnin and viomellein	[59]	was *P. freii*	

[a] Not confirmed in any strain of *P. crustosum*.

phore close to that of the polyketide asperentin was produced by 116 of the 121 isolates. Most isolates produced up to six different compounds with that chromophore.

One compound had a UV spectrum similar to that of the bisanthrones of *Aspergillus wentii*, but its exact identity has to be confirmed by structure elucidation. It was produced by at least 90 of 121 isolates.

3.3. Principal component and correspondence analyses of quantitative amounts of secondary metabolites in P. crustosum

The quantitative amounts of secondary metabolites as detected by HPLC were analysed by principal component analysis and correspondence analysis to see whether any grouping was possible. The first two prin-

cipal components described approximately 50% of the variation of the data and there was no obvious grouping of the isolates (data not shown). The relative quantitative amounts of the major secondary metabolites were very similar in all isolates examined, whether from Arctic or warmer areas, indicating that there is a rather tight regulation of secondary metabolism in *P. crustosum*. Because of the larger peak of andrastin A in most of the Arctic isolates there was a weak tendency of those isolates to group, but the groups of cold and warm climate isolates were very clearly overlapping. Creatine-positive and creatine-negative isolates could not be separated based on differences in secondary metabolism either.

4. Discussion

Penicillium crustosum is known to grow on rich substrates, such as meat, cheese, corn and nuts. Therefore, it was surprising to find it with high occurrence in oligotrophic Arctic habitats. It was mainly isolated from glacial ice, characterized by extremely low temperature and little biologically available water, due to ice crystal formation. One of the important taxonomic features of *P. crustosum* is its consistent ability to grow on media based on creatine as nitrogen source [19,20]. For the first time, however, we observed that certain Arctic isolates were not able to utilize creatine as the sole nitrogen source.

Otherwise, all *P. crustosum* isolates were very consistent in their properties irrespective of geographic origin or habitat. They all had smooth globose to subglobose hydrophobic conidia, rough conidiophore stipes, high growth rates on all common substrates and a bright yellow reverse on YES agar. They also all produced many conidia, in accordance with the name of the species signifying the ability to form a conidial crust on malt extract agar after 7–9 days of incubation at 25 °C. Pringle and Taylor [26] suggested that the extent of conidia production is a sign of high fitness in filamentous fungi, and this may explain the frequent occurrence of *P. crustosum*. Interestingly this property does not depend on the habitat in which the fungus originates, since all isolates produce copious amounts of conidia [12,14,15,27,28]. Pitt [15,27] estimated that *P. crustosum* produces approximately 100 times more conidia than other terverticillate *Penicillium* species. This could be one reason for the prevalence of this species in seawater and ice from the Arctic region, while another could be its recognised ability to grow on substrates rich in lipids and proteins. It can be considered as a halotolerant and psychrotolerant species, since selected strains from Arctic (EX-F 1005, EX-F 1329, EX-F 1339, EX-F 1383 and EX-F 1399) and non-Arctic (IBT 4131 and IBT 19390) habitats grew with up to 20% NaCl at 25 °C and even up to 14% NaCl at 2 °C. The same selected *P. crustosum* strains produced urease, esterase and proteases at both tested temperatures (data not shown) and therefore we speculate that *P. crustosum* may be involved in the degradation of dead Arctic animals.

The simultaneous production of penitrem A and roquefortine C has been reported by several authors, albeit under different names such as *Penicillium commune* [29], *P. cyclopium* [30] and *Penicillium lanosocoeruleum* [31], but these isolates were all shown to be *P. crustosum* [32]. Kawai et al. [13] and Rundberget et al. [14] indicated that some strains of *P. crustosum* (chemotype II) do not produce penitrem A and roquefortine C, but rather fumigaclavine A and festuclavine. These strains (1601 P4 [14] and I28, I29 and I30 [13]) were all shown to be *Penicillium palitans* (Table 2). The strains of this species grow slower than *P. crustosum*, produce less conidia (no conidial crust formation) and have a more pure green colour of conidia. Furthermore *P. palitans* produces no metabolites in common with *P. crustosum* except occasional production of the viridicatols. Rather *P. palitans* produces cyclopiazonic acid, palitantin, fumigaclavine A, festuclavin, terpenes typical of the aspereynones and occasionally the viridicatols [33]. It is interesting that the first strain reported to produce the tremortins (=the penitrems), was identified as *P. palitans* also. This strain (NRRL 3468 [34]) was later shown to be *P. crustosum* [12,15]. The other strain reported to be unable to produce penitrem A and roquefortine C (1582 P2 [14]) produced large amounts of both compounds in our hands. Our conclusion is that all strains of *P. crustosum* produce these two metabolites. It is evident from the article of Rundberget et al. [14] that strains of *P. crustosum* also produce thomitrem A and E, and the other known penitrems. This has been confirmed in all our strains of *P. crustosum*, as they all produced compounds with chromophores like those of the penitrems, their precursors (PC-M4 etc.) and the thomitrems.

Penicillium crustosum also produces terrestric acids and the viridicatols consistently as originally described by Frisvad [12] and Frisvad and Filtenborg [4,24]. Both viridicatic acid from the first secondary metabolite family and viridicatin and viridicatol from the second secondary metabolite family were named after *Penicillium viridicatum*, but none of these are produced by *P. viridicatum* [33].

Andrastin A was detected for the first time in *P. crustosum* in this study. The function of this sterol-like compound is unknown, but it may be important for regulating the sterol profile of the species. *P. crustosum* has been reported to be efficient in transformation of testosterone into 5α-dihydrotestosterone [35,36].

The sterol metabolism of this fungus may therefore be unique. Apart form the pure terpene derived compound andrastin A, terpene units are also used in the biosynthesis of both roquefortine C and the penitrems.

Polyketides have not been reported yet from *P. crustosum*. However, we found two candidates that may turn out to be polyketides, a series of metabolites with an asperentin chromophore and a compound with a chromophore similar to that of the bisanthrones of *A. wentii* [37].

Similarly as it has been shown for *P. expansum* [38], also *P. crustosum* is a consistent producer of a characteristic profile of secondary metabolites. Even though these species were placed in the same series by Raper and Thom [28], and *P. crustosum* was reduced to a variety of *P. expansum* by Fassatiová [3], they only share the apple rot potential, high growth rate and production of roquefortine C. The profile of secondary metabolite families found consistently in *P. crustosum* is unique and consists of penitrems, roquefortine C, viridicatols, terrestric acids and andrastin A. We believe the secondary metabolism of this species is under tight genetic control. Even at low temperature (10 °C) the same qualitative secondary metabolite profiles were detected for individual non-Arctic strains after three weeks of incubation. However, no andrastin A production by individual Arctic strains was detected under those conditions (data not shown).

The only property that was varying was the efficiency to use creatine as a sole nitrogen source. Strains unable to use creatine as a sole nitrogen source were isolated from seawater sea ice, as well as from glacial ice. Future studies will show if this property is associated with protease or lipase production that may have an adaptive function.

In conclusion *P. crustosum* is a species with highly conserved properties. It seems to have a highly efficient tool box for coping with different ecological situations rather than being adapted to any particular habitat. We therefore believe that a varied phenotypic reaction pattern [39] rather than just adaptation of individual communities is the cause of the success of this species.

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