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Intraspecific diversity in the fungal species *Chaunopycnis alba*: implications for microbial screening programs

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Intraspecific variation among 84 isolates of the anamorphic fungus *Chaunopycnis alba* from 26 different geographical locations was analyzed by investigating optimal growth temperatures, differences in the production of secondary metabolites and presence or absence of the cyclosporin synthetase gene. The genetic diversity was assessed using random amplified polymorphic DNA (RAPD). Analysis of these data showed high genetic, metabolic and physiological diversity within this species. Isolates from the Antarctic represented the most homogeneous group within *C. alba* and together with isolates from the Arctic these polar strains differed from alpine, temperate and tropical strains by low optimal growth temperatures and by low production of secondary metabolites. Isolates from tropical climes were characterized by high optimal growth temperatures and by the production of comparatively diverse metabolite spectra. Most of the isolates that were similar in the combination of their physiological and metabolic characters were also genetically related. Isolates from different geographical origins did not show many similarities, with the exception of the cyclosporin A-producing isolates, and large diversity could be observed even within a single habitat. This leads us to the suggestion that for pharmaceutical screening programs samples should be collected from a diversity of different geographical and climatic locations. For the selection of strains for screening the RAPD assay seems to be the most powerful tool. It reflected the highest intraspecific diversity and the results corresponded well with the other characteristics.

Keywords: intraspecific variation; RAPD; HPLC profile; temperature requirements; cyclosporin synthetase gene; creativity

Introduction

The utilization of genetic resources for pharmaceutical or biotechnological purposes has recently become a major interest [7,8], because wild species are probably the most important but least utilized resource for biotechnological and pharmaceutical innovations [50]. Most reports on biodiversity focus on taxonomic diversity [28], however metabolic diversity of the strains is of more relevance for industrial microbial screening programs. Our understanding is still scant in respect to functional and metabolic diversity and how it is affected by genetic and taxonomic diversity [11,52]. In conceiving a screening concept the questions arise as to what extent intraspecific variation should be considered and how relevant climatic, geographic and ecological aspects are for the productivity of fungal strains.

Chaunopycnis alba W Gams was selected for this study because we isolated this species frequently from antarctic, arctic and alpine lichens and mosses, as well as from tropical and temperate regions. This large collection of strains offered a good opportunity to compare the intraspecific variation between isolates from various climatic and geographic regions distributed all over the world and concurrently to examine the variation within one habitat. Some isolates of *C. alba* produce cyclosporin A (CyA) which is a potent immunosuppressive compound [4]. The industrially most important producer of CyA is *Tolypocladium inflatum* W Gams, but fungi belonging to other taxa also produce CyA [13].

In the case study presented here we focused on the intraspecific physiological, metabolic and genetic diversity in the cosmopolitan species *C. alba*. We analyzed optimal growth temperatures, examined the production of metabolites using HPLC, determined the biological activity of these metabolites in various biological assays, studied the sensitivity to CyA and examined the presence of the cyclosporin synthetase gene. In addition we considered the data from a RAPD analysis published recently [37]. Our goal was to obtain information about the overall variation and to find correlations among these different parameters and between the test results and ecological factors.

Materials and methods

Fungal isolates

The 84 isolates of *C. alba* examined in this study, their geographic and climatic origin and the substrates from which they were isolated are given in Table 1. The strains were used in a genetic diversity study [37]. Isolates with the prefix 'Ant' were isolated from lichen and moss samples collected on King George Island, Antarctica, in an area of about 300 m south-west from the Polish research station 'Arctowski'. Strains named 'Alp' were obtained from lichens and mosses from two different valleys in the Swiss Alps and those with the prefix 'Sp' were isolates of lichens and mosses from four locations on Spitsbergen (Norway) that were several kilometers away from each other and were separated by fjords. In the Alps and on Spitsbergen each sample collection site comprised an area of about 200 m^2.

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Table 1 *Chaunopycnis alba* isolates used in this study, their geographic and climatic origin and the substrates from which they originated. Isolates marked with * were used for detection of the cyclosporin synthetase gene

Isolate	Geographic provenance	Substrate	Climatic origin
Ant 32-07	King George Island (Antarctica)	lichen (*Caloplaca regalis*)	polar
Ant 32-27	King George Island (Antarctica)	lichen (*Caloplaca regalis*)	polar
Ant 32-28*	King George Island (Antarctica)	lichen (*Caloplaca regalis*)	polar
Ant 33-03*	King George Island (Antarctica)	lichen (*Xanthoria elegans*)	polar
Ant 34-15	King George Island (Antarctica)	lichen (*Physica caesia*)	polar
Ant 34-16	King George Island (Antarctica)	lichen (*Physica caesia*)	polar
Ant 35-05	King George Island (Antarctica)	lichen (*Ramalina terebrata*)	polar
Ant 35-09	King George Island (Antarctica)	lichen (*Ramalina terebrata*)	polar
Ant 37-03	King George Island (Antarctica)	lichen (*Haematomma erythromma*)	polar
Ant 37-12	King George Island (Antarctica)	lichen (*Haematomma erythromma*)	polar
Ant 39-26	King George Island (Antarctica)	moss	polar
Ant 39-30	King George Island (Antarctica)	moss	polar
Ant 40-01*	King George Island (Antarctica)	lichen (*Turgidosculum complicatulum*)	polar
Ant 40-07*	King George Island (Antarctica)	lichen (*Turgidosculum complicatulum*)	polar
Ant 43-03	King George Island (Antarctica)	lichen	polar
Ant 43-10 (CBS 701.92)*	King George Island (Antarctica)	lichen	polar
Ant 44-01	King George Island (Antarctica)	lichen (*Turgidosculum complicatulum*)	polar
Ant 44-07*	King George Island (Antarctica)	lichen (*Turgidosculum complicatulum*)	polar
Ant 45-02	King George Island (Antarctica)	lichen (*Caloplaca regalis*)	polar
Ant 45-09	King George Island (Antarctica)	lichen (*Caloplaca regalis*)	polar
Ant 48-18	King George Island (Antarctica)	lichen (*Ramalina terebrata*)	polar
Alp 5-01	Swiss Alps/Sustenpaß	moss	alpine
Alp 5-22*	Swiss Alps/Sustenpaß	moss	alpine
Alp 6-22	Swiss Alps/Sustenpaß	moss	alpine
Alp 32-08	Swiss Alps/Sustenpaß	lichen	alpine
Alp 73-01	Swiss Alps/Maighelstal	moss	alpine
Alp 73-04*	Swiss Alps/Maighelstal	moss	alpine
Alp 74-08	Swiss Alps/Maighelstal	lichen	alpine
Alp 77-08	Swiss Alps/Maighelstal	lichen	alpine
Alp 88-02	Swiss Alps/Maighelstal	moss	alpine
Alp 89-05*	Swiss Alps/Maighelstal	moss	alpine
Alp 90-01	Swiss Alps/Maighelstal	lichen	alpine
Apl 122-15*	Swiss Alps/Maighelstal	lichen (*Nephroma* sp)	alpine
S 21498	Swiss Alps/Leysin	lichen	alpine
S 21551	Swiss Alps/Leysin	lichen	alpine
Sp 13-12	Spitsbergen/Nansenbreen	moss	polar
Sp 19-21	Spitsbergen/Longyearbyen	moss	polar
Sp 22-01*	Spitsbergen/Longyearbyen	lichen (*Peltigera* sp)	polar
Sp 44-03	Spitsbergen/Ny Ålesund	moss	polar
Sp 48-08*	Spitsbergen/Ny Ålesund	moss	polar
Sp 50-09	Spitsbergen/Ny Ålesund	lichen (*Umbilicaria* sp)	polar
Sp 72-21	Spitsbergen/Cross-Fjord	moss	polar
Sp 88-01 (CBS 702.92)*	Spitsbergen/Cross-Fjord	lichen (*Umbilicaria* sp)	polar
Sp 88-03	Spitsbergen/Cross-Fjord	lichen (*Umbilicaria* sp)	polar
Sp 95-22*	Spitsbergen/Cross-Fjord	lichen (*Umbilicaria* sp)	polar
Sp 34-1/3	Spitsbergen/Cross-Fjord	lichen (*Umbilicaria* sp)	polar
Sp 34-1/5	Spitsbergen/Cross-Fjord	lichen (*Umbilicaria* sp)	polar
Sp 34-3/1	Spitsbergen/Cross-Fjord	lichen (*Umbilicaria* sp)	polar
Sp 34-9/7	Spitsbergen/Cross-Fjord	lichen (*Umbilicaria* sp)	polar
CBS 968.73B*	The Netherlands/Wageningen	Soil	temperate
CBS 968.73C*	The Netherlands/Rotterdam	soil	temperate
CBS 492.80B*	France	needles (*Abies alba*)	temperate
S 23074*	France/Petite Camarque	moss	temperate
S 23029	France	soil	temperate
CBS 830.73*	Sweden	soil	temperate
S 23032	Sweden	soil	temperate
S 23592*	Germany/Black Forest	lichen	temperate
S 23606*	Germany/Black Forest	lichen	temperate
S 23608	Germany/Black Forest	lichen	temperate
S 2465	Germany/Black Forest	lichen	temperate
S 2239*	England	lichen	temperate
S 2746	Scotland	lichen	temperate
S 2757-02	Scotland	lichen	temperate
S 2757-04	Scotland	lichen	temperate
S 21316*	Madagascar	organic material	tropical
CBS 492.80A*	Columbia/3200 m	soil	alpine

Table 1 Continued

Isolate	Geographic provenance	Substrate	Climatic origin
CBS 393.89*	Brazil	soil	tropical
S 2226*	Brazil/Belem	soil	tropical
S 2428*	Brazil/Belem	soil	tropical
S 3873-01*	Brazil/Belem	soil	tropical
S 3873-03*	Brazil/Belem	soil	tropical
S 4039	Brazil/Belem	soil	tropical
S 23239*	Brazil/Amazonas	moss	tropical
S 3930*	USA/Redwood Forest	plant (*Athecium*)	temperate
S 3954-03	USA/Redwood Forest	plant (*Sequoia*)	temperate
S 3954-04*	USA/Redwood Forest	plant (*Sequoia*)	temperate
S 4382*	Singapore	leaf litter	tropical
CBS 176.75*	Sri Lanka	soil	tropical
S 2035-01	Malaysia/Kinabalu	moss	alpine
S 2033-01	Malaysia/Kinabalu	organic material	alpine
S 2033-03*	Malaysia/Kinabalu	organic material	alpine
S 2033-04	Malaysia/Kinabalu	organic material	alpine
S 2634	?	?	
S 2636	?	?	

Isolates named 'S' were taken from the SANDOZ strain collection.

Isolates of *Chaunopycnis ovalispora* Möller & W Gams, *Sesquicillium microsporum* (Jaap) Veenbaas-Rijks & W Gams, *Tolypocladium cylindrosporum* W Gams and the ascomycete, *Thelebolus microsporus* (Berk & Br) Kimbr, were included in this study as outgroups (Table 2). All cultures were grown on 2% malt agar slants at 15°C and stored at 4°C.

Culture—media

The media used for the determination of optimal growth temperatures, the detection of secondary metabolites, their biological activities and the CyA sensitivity test were prepared in 1 L of deionized water as follows: **CE**: cerelose 20 g, malt extract 2 g, yeast extract 2 g, soya-peptone 2 g, $MgSO_4 \cdot 7H_2O$ 2 g, KH_2PO_4 2 g; **MYE I**: malt extract 20 g, yeast extract 4 g, agar 20 g; **MYE II**: malt extract 20 g, yeast extract 4 g, Bacto agar 1 g; **MYE III**: malt extract 20 g, yeast extract 4 g, agar 20 g, cyclosporin A dissolved in methanol 0.2 μg; **MAY**: maltose · H_2O 22 g, Bacto yeast extract 7.2 g; **TB** (test against bacteria): Bacto tryptose blood agar base 15 g, Bacto agar 8 g, (pH 7.0–7.5); **TF** (test against filamentous fungi): Bacto malt extract 10 g, Bacto agar 12 g, (pH 5.0–5.5); **TS** (test against *Saccharomyces cerevisiae*): Bacto yeast extract 10 g, Bacto peptone 20 g, D-glucose · H_2O 20 g, Bacto agar 20 g, (pH 5.0–5.5).

Table 2 Isolates of *Chaunopycnis ovalispora, Sesquicillium microsporum, Tolypocladium cylindrosporum* and *Thelebolus microsporus* used as outgroups

Isolate	Species
Ant 32-17 (CBA 138.93)	*Chaunopycnis ovalispora* Möller & W Gams
Ant 32-16 (CBS 700.92)	*Chaunopycnis ovalispora*
CBS 954.72	*Sesquicillium microsporum* (Jaap) Veenbaas-Rijks & Gams
CBS 788.85	*Sesquicillium microsporum*
CBS 718.70	*Tolypocladium cylindrosporum* W Gams
CBS 716.92	*Thelebolus microsporus* (Berk & Br) Kimbr

Optimal growth temperatures

C. alba strains were incubated in shake flasks (200 rpm) containing 50 ml CE-medium at 21°C in the dark for 7 days. The test cultures were grown in 14-well multi-screen plates (Petra-Plastic, Chur, Switzerland) containing 5 ml CE-medium per well. Each well was inoculated with 0.5 ml of the pre-cultures and incubated in the same manner. After 7 days each culture was homogenized, aliquots were transferred with a sterile metal stamp onto agar plates (MYE I) in triplicate and the plates were incubated at 4°C, 10°C, 16°C, 21°C, 24°C, 27°C and 30°C. The optimal growth temperatures were determined after 7 days incubation as the average of the measurements of the three plates.

Production of metabolites

Preparation of extracts: The production of secondary metabolites was examined analyzing culture broth extracts by HPLC and several biological assays. For the preparation of culture broth extracts, all the *C. alba* isolates and the outgroup strains were incubated in shake pre-culture (200 rpm) in 50 ml MYE II medium at 21°C in the dark. After 6 days, main-cultures were inoculated with 5 ml of the homogenized pre-cultures and incubated for 8 days in shake culture (200 rpm) containing 50 ml MAY medium at 21°C in the dark. The outgroup isolates of the psychrophilic *C. ovalispora* were incubated at 15°C. Twenty-five milliliters of each main-culture were lyophilized and extracted with 10 ml methanol for 20 h at room temperature. The extract was separated from the mycelium by centrifugation and stored at −20°C. Each isolate was incubated in two parallel main-cultures. In order to test the reproducibility of metabolite production, 20 strains were additionally inoculated in duplicate pre-cultures and for 12 isolates the

influences of two different temperatures (13°C and 21°C) and three incubation periods of the main-culture (7 days, 11 days, 14 days) were studied.

High performance liquid chromatography (HPLC): A Merck Hitachi L-6200A chromatograph with a 199 × 4 mm Lichrocart-ODS 4-μm column (Merck, Darmstadt, Germany) and a photodiode-array detector was used. Each extract was analyzed twice. For the first analysis, the solvent program was a 20-min linear gradient from 100% H_3PO_4 0.1% (v/v) to 100% MeCN and for the second analysis from 70% H_3PO_4/30% MeCN to 100% MeCN at a flow rate of 2 ml min^{-1}. A 20-μl sample volume was used for each analysis. For the second analysis of each extract internal standards of alkylphenones were added according to methods described by Hill [29] and Frisvad and Thrane [22]. Approximately 20-μmol ml^{-1} solutions of Aceto-, Propio-, Valero-, Octano- and Decanophenone were prepared in methanol and mixed in equal volumes; 50 μl of this mixture were added to 950 μl of each extract and 20 μl were analyzed. The UV-VIS spectra between 200 and 500 nm were recorded from each reproducible peak that was higher than 20 mAu (absorption units) at 210 nm.

Results of HPLC runs with internal standards were primarily used for the analysis. Converted retention times were calculated for each reproducible peak that was higher than 20 mAu at 210 nm. The retention time of the alkylphenone, which elutes before the metabolite, was subtracted from the retention time of the metabolite.

The presence and absence of each metabolite was determined and scored as 1 or 0, respectively for all the isolates. The chromatograms that were obtained in runs without internal standards were used for the detection of metabolites which had similar retention times as the alkylphenones.

In addition, the strains tested were grouped into 'creativity groups' according to the number of reproducible peaks higher than 20 mAu. Isolates yielding more than five peaks were regarded as being 'creative', those showing three to five peaks were classified as 'weakly creative' whereas strains with two or less peaks were classified as 'not creative'. The proportion of each creativity class is shown graphically for the different climatic origins of the isolates (tropical, temperate, alpine, polar).

Biological assays: For all the *C. alba* isolates the biological activity of one culture extract was analyzed in agar diffusion tests against fungi, Gram-positive and Gram-negative bacteria (Table 3) and in reporter-gene tests. For the agar diffusion tests filter disks of cellulose (6 mm diameter) were soaked with 25 μl culture extract, air-dried and placed in duplicate onto the test agar plates containing bacteria, hyphomycetes or yeasts (Table 3). The plates were incubated at the temperatures given in Table 3 and after 24 h inhibition zones were measured or morphological changes were recorded.

Table 3 Test organisms, their incubation media and incubation temperatures

Organism	Culture medium	Incubation temperature (°C)
Staphylococcus aureus	TB	37
Comamonas terrigena	TB	27
Saccharomyces cerevisiae	TS	27
Candida albicans	TF	37
Aspergillus niger	TF	37
Neurospora crassa	TF	27

For the preparation of the test agar plates one liter each of the media TB, TF, TS were dissolved and cooled to 48°C. Six milliliters of a suspension containing the test organism (Table 3) (containing approximately 10^6 cells) were added and carefully mixed with the medium. Plates were stored at 2°C. In addition to the agar diffusion tests, four different reporter-gene tests [1] were applied (IL-2, IL-6, TNF-α, Dr-α). The extracts were diluted 1 : 200 and analyzed in duplicate in our routine screening at SANDOZ. Averages were calculated from the two measurements. According to standard protocols the activity was regarded as positive if the inhibition was higher than 40% for the TNF-α assay, whereas for IL-2, IL-6 and Dr-α statistically reliable statements are only possible at an inhibition of at least 60%.

The analysis of the biological activity was similar to that carried out with the HPLC data. For the overall analysis the presence or absence of each biological activity of each isolate (agar diffusion test and reporter-gene assay) was scored as 1 or 0, respectively. Additionally, correlations between climatic origin and the number of positive test results were studied with correspondence analysis using SimCA 2.1 [25]. For this analysis the isolates were grouped into four different climatic origins (tropical, temperate, alpine and polar) and according to the number of biological activities.

Sensitivity to cyclosporin A

For the detection of sensitivity to CyA in culture media, two agar plates for each *C. alba* isolate with media MYE I and MYE III were inoculated with an agar plug of 0.5-cm diameter punched from a fresh agar culture. After 2 weeks incubation at 21°C the colony diameters were measured and growth rates between cultures with and without CyA (MYE I and MYE III) were compared. In addition, the edges of the colonies were examined under the microscope in order to detect changes in mycelial branching. An isolate was regarded as being sensitive to CyA if either the growth was significantly inhibited or the overall (micro- and macro-) morphology of the colony had changed.

Detection of the cyclosporin synthetase gene

We searched for specific gene fragments in selected *C. alba* strains with PCR and primers that were constructed according to the sequence of the cyclosporin synthetase gene and other genes from *T. inflatum* [46]. Representatives from each geographical region were randomly chosen for this examination (Table 1, marked with an asterisk). DNA was extracted as described in Möller *et al* [37].

Amplification conditions: Amplification reactions were carried out in volumes of 50 μl containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM of each dATP, dCTP, dGTP, dTTP (Promega, Madison,

WI, USA), 1 μM of each primer, 50 ng DNA, 1.25 U Taq DNA polymerase (Boehringer, Mannheim, Germany). The reactions were overlaid with 100 μl mineral oil (Sigma Chemical Co, St Louis, MO, USA). Amplifications were performed in a Biometra Trio thermoblock programmed for 1 cycle of 94°C for 120 s, 30 cycles of 55°C (primerpair 106/107, 104/105) or 60°C (primerpair 77/80, 95/97) for 30 s, 120 s of 72°C, 45 s of 94°C, 1 cycle of 72°C for 120 s. The composition of the four primer pairs is given in Table 4. Amplified products together with a 1-kb ladder (Gibco-BRL, Gaithersburg, MD, USA) were resolved by gel electrophoresis (6.7 V cm^{-1}) on a 1% agarose gel stained with ethidium bromide [40]. Gels were photographed over a UV transilluminator using a video camera and printed out on a video printer.

Southern blot hybridization: The amplification products of *C. alba* obtained with the primer pairs 77/80, 106/107, 95/97 and 104/105 were transferred to nylon membranes (Boehringer) in 20 × SSC according to Sambrook *et al* [40]. DNA fragments specific for *T. inflatum* and flanked by the listed primers were cloned and labelled with a DIG DNA labelling kit (Boehringer). They were used as probes in Southern blot hybridizations, following the hybridization conditions detailed in the kit protocol. Detection was performed with the DIG Luminescent Detection Kit (Boehringer) according to the supplier's protocol.

Random amplified polymorphic DNA (RAPD)

Analysis of the intraspecific genetic diversity has already been described in a recently published paper [37].

Comparison of RAPD results with the physiological and metabolic examinations

In order to test the correspondence between results previously obtained with the RAPD assay [37] and the physiological experiments, the data of all tests except the RAPD assay were combined in a matrix. For all 81 characters the presence or absence was scored as 1 or 0, respectively. For the optimal growth temperature, the lower temperature was considered if there were two with equal growth. The presence or absence of each peak detected with HPLC or of each biological activity in the bioassays was recorded, as well as the sensitivity against CyA and the presence or absence of specific fragments of the CyA synthetase gene. The resulting matrix was used to calculate Jaccard coefficients of similarity for each pairwise comparison. Unweighted pair-group arithmetic average (UPGMA) analysis was applied to construct a dendrogram using SYSTAT for windows 5 (SYSTAT Inc, Evanson, IL, USA). The dendrogram obtained by these results was then compared with the dendrogram based on the previous RAPD analysis [37].

Analysis of the overall variation

As a final step of this study, the overall genetic, physiological and metabolic variation in *C. alba* was demonstrated. The matrix used for the construction of the previous dendrogram was therefore combined with the matrix obtained with the RAPD assay [37]. The resulting matrix including 378 characters was used to calculate Jaccard coefficients for each pairwise comparison. UPGMA analysis was applied to construct the dendrogram. The analysis was carried out with SYSTAT for windows 5.

Results

Optimal growth temperatures

The optimal growth temperatures varied among the *C. alba* isolates between 16°C and 30°C (Table 5). Differences in the lowest value of the optimal range could be detected between isolates from the different climatic regions (Figure 1). The majority of polar isolates grew best at 16°C, whereas the optima for strains from alpine, temperate and tropical zones varied among different isolates and were less homogeneous. Isolates with optima at 30°C were only isolated from tropical regions, however.

Production of metabolites

As shown with two examples (Figure 2), the metabolite profiles detected by HPLC varied within *C. alba*. Some extracts showed a very low profile (Figure 2a) and no peak could be used for analysis, while in others up to 12 peaks were detected (Figure 2b, Table 5). The metabolite patterns were not significantly affected by incubation temperatures or longer incubation periods. In some cases the quantity of single metabolites was affected by different incubation conditions but the general pattern of metabolites for one isolate remained constant under the conditions tested (data not shown).

Table 4 Primer pairs used for the amplification of fragments within the cyclosporin synthetase gene

Region of the gene	Primer	Sequence (5′–3′)
Promotor region	77	GCC CAT GGT GAC CAA GAA AGC AA
	80	ATG CAG GAG AAT CGG CTG GGA TGG
Domain 4, methyltransferase domain	106	TGC TGG TGA TCG CTT CAG TAG C
	107	GTG TAT TGA GTT GAC GGC CTA GAC
Domain 7, central part	95	GTG AGA CTG AAA CCT TTG AGT CAC TAG
	97	CGT TGA CCT GTG CTT CCA CA
Region encoding for the C-terminal end of the synthetase	104	AGC GCG ATG TTG TAC CTC AGA T
	105	AGC ATG GTC TCG CAA AGT TCA T

Table 5 Optimal growth temperatures, number of HPLC-peaks, number of biological activities, production of CyA, sensitivity against CyA in culture media and occurrence of fragments of the CyA – synthetase gene of the *C. alba* isolates

Isolate	Optimal growth temperatures (°C)	HPLC-peaks	Biol activ	Prod CyA/sensit CyA	CyA-synth-gene
Ant 32-07	16–24	0	0	–/+	n.d.
Ant 32-27	16–21	0	1	–/+	n.d.
Ant 32-28	16–21	0	0	–/+	–
Ant 33-03	16–24	0	0	–/+	–
Ant 34-15	16–24	0	0	–/+	n.d.
Ant 34-16	16–24	0	0	–/+	n.d.
Ant 35-05	16–24	0	0	–/+	n.d.
Ant 35-09	16–21	1	2	–/+	n.d.
Ant 37-03	16–24	0	0	–/+	n.d.
Ant 37-12	16–21	1	2	–/+	n.d.
Ant 39-26	16–24	0	1	–/+	n.d
Ant 39-30	16–24	1	2	–/+	n.d.
Ant 40-01	16–21	0	2	–/+	–
Ant 40-07	16–24	0	2	–/+	–
Ant 43-03	16–21	0	1	–/+	n.d.
Ant 43-09	16–21	0	1	–/+	–
Ant 44-01	16–24	0	0	–/+	n.d.
Ant 44-07	16–24	0	0	–/+	–
Ant 45-02	16–21	0	0	–/+	n.d.
Ant 45-09	16–21	0	1	–/+	n.d.
Ant 48-18	16–24	0	0	–/+	n.d
Alp 5-01	24	0	3	–/+	n.d.
Alp 5-22	24	0	3	–/+	–
Alp 6-22	27	1	0	–/+	n.d.
Alp 32-08	16–24	2	2	–/+	n.d.
Alp 73-01	24	4	4	–/+	n.d.
Alp 73-04	24	4	4	–/–	–
Alp 74-08	24	3	4	–/+	n.d.
Alp 77-08	24	4	4	–/+	n.d.
Alp 88-02	16–21	5	4	–/+	n.d.
Alp 89-05	16–21	6	4	–/+	–
Alp 90-01	16–21	0	3	–/+	n.d.
Apl 122-15	16–24	2	5	–/+	–
S 21498	21	0	0	–/+	n.d.
S 21551	16–21	1	2	–/+	n.d.
Sp 13-12	24	0	0	–/–	n.d.
Sp 19-21	16–21	2	0	–/+	n.d.
Sp 22-01	16–21	0	1	–/+	–
Sp 44-03	16–21	1	0	–/+	n.d.
Sp 48-08	16–21	2	0	–/+	–
Sp 50-09	16	1	0	–/+	n.d.
Sp 72-21	24	1	3	–/+	n.d.
Sp 88-01	16–24	0	1	–/+	–
Sp 88-03	16–24	0	2	–/+	n.d.
Sp 95-22	16–21	2	1	–/+	–
Sp 34-1/3	24	1	3	–/+	n.d.
Sp 34-1/5	24	1	3	–/+	n.d.
Sp 34-3/1	24	0	1	–/+	n.d.
Sp 34-9/7	24	0	1	–/+	n.d.
CBS 968.73B	27	2	4	–/+	–
CBS 968.73C	27	0	1	–/+	–
CBS 492.80B	21–24	8	4	–/+	–
S 23074	24	3	6	–/+	–
S 23029	27	3	0	–/+	n.d.
CBS 830.73	27	4	8	–/+	–
S 23032	27	3	8	–/+	n.d.
S 23592	21–24	3	6	+/–	+
S 23606	16–24	2	6	+/–	+
S 23608	16–24	2	6	+/–	n.d.
S 2465	24	2	5	+/–	n.d.
S 2239	16–24	2	4	+/–	+
S 2746	21–27	4	4	–/+	n.d.
S 2757-02	27	4	3	–/+	n.d.
S 2757-04	27	3	2	–/+	n.d.
S 21316	27	3	2	–/+	–
CBS 492.80A	21–24	5	4	–/+	–
CBS 393.89	21–24	3	0	–/+	–
S 2226	24	7	7	+/–	+
S 2428	16	2	0	–/+	–
S 3873-01	21–24	2	6	+/–	+
S 3873-03	21–24	2	6	+/–	+
S 4039	30	4	4	–/+	n.d.
S 23239	30	7	5	–/+	–
S 3930	21	3	3	–/+	+
S 3954-03	16–21	1	4	+/–	n.d.
S 3954-04	16–21	2	4	+/–	+
S 4382	24	1	4	+/–	+
CBS 176.75	27	5	4	–/+	–
S 2035-01	21	5	2	–/+	n.d
S 2033-01	21	5	1	–/+	n.d
S 2033-03	21–24	12	4	–/+	–
S 2033-04	21–24	12	4	–/+	n.d.
S 2634	21–27	0	3	–/+	n.d.
S 2636	21–27	2	3	–/+	n.d.

Biol active, number of biological activities; prod CyA, production of CyA; sensit CyA, sensitivity against CyA. n.d. = not determined; + = positive; – = negative.

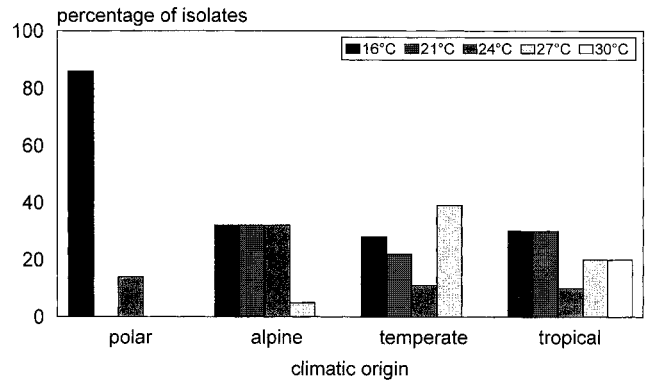

Figure 1 Proportions of isolates having their lowest optimal growth temperature at 16°C, 21°C, 24°C, 27°C or 30°C for the four different climatic regions (polar, alpine, temperate, tropical).

A total of 58 peaks with different retention times and UV-VIS spectra were considered for this analysis; 35 of these were detected only in the extract of a single isolate. No metabolites were found in the extracts of a large group of strains. Almost all antarctic and arctic isolates belonged to this group. Isolates with identical or very similar metabolic spectra normally were isolated from the same or nearby samples with the exception of some CyA-producing strains. Eleven CyA-producers were detected with the *Neurospora crassa* biological assay (Table 5). Seven of these (S 3873-03, S 3873-01, S 2465, S 23608, S 23606, S 23592, S 2239) had similar metabolite profiles although they originated from different habitats (Brazil, Germany, England, Table 1).

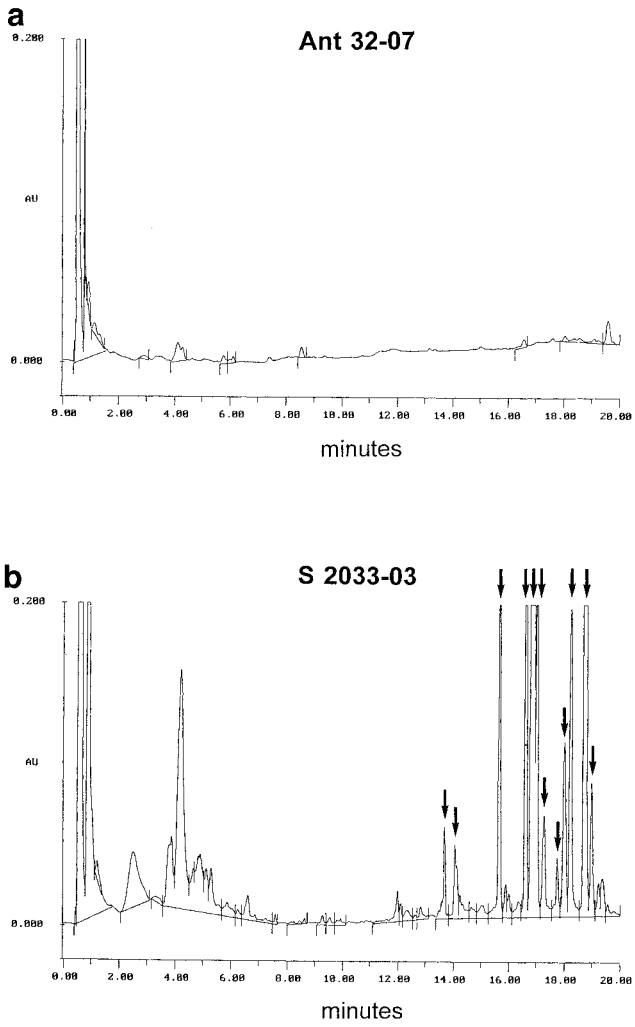

Figure 2 HPLC chromatograms of culture broth extracts of isolates Ant 32-07 (Antarctica; a) and S 2033-03 (Malaysia; b) without external standards. Arrows indicate peaks which were detected and reproducible and which could be considered for the analysis.

The differences in productivity among the *C. alba* isolates from different climatic origins became even clearer if the isolates were divided into 'creativity groups' according to the number of HPLC peaks detected. Figure 3 shows the proportion of the three creativity groups for each climatic region. Whereas 100% of the polar isolates were not creative, 16% of the alpine and 20% of the tropical strains produced a very variable metabolite pattern, only approximately 40% of these being 'not creative'. The percentage of 'creative' isolates from temperate regions was not as high (6%) as for the tropical and alpine zones but 50% of the temperate isolates were 'weakly creative' and therefore they also differed as a group from the polar isolates. These findings were supported by the results of the bioassays. Correspondence analyses (Figure 4) showed that polar strains differ from the others by having little or no biological activity, whereas tropical and temperate isolates were characterized by more positive test results (Table 5). Alpine fungi were intermediate between these extremes having 2, 3 or 4 biological activities more often than isolates from the other climatic regions. In this analysis 91% of the total inertia was reflected by the two-dimensional display.

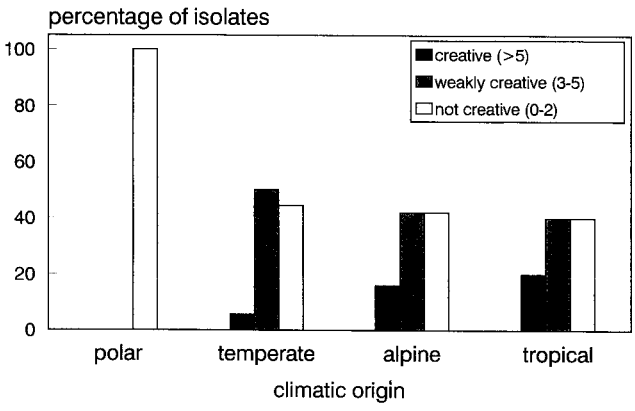

Figure 3 Proportions of creative, weakly creative and not creative isolates among the polar, temperate, alpine, and tropical *C. alba* isolates.

Sensitivity against cyclosporin A

Twelve isolates were not sensitive to CyA (Table 5) All eleven CyA-producing strains (Table 5) were not sensitive, and with one exception (Sp 13–12), inhibition or changes in morphology were visible for all non-producing isolates.

Detection of the CyA synthetase gene

The presence of four fragments of the cyclosporin synthetase gene was examined using PCR with 35 isolates of *C. alba* including eight CyA-producers and 27 non-producers. Parts of the synthetase gene were detected in the genome of all the tested CyA-producers (Table 5), but the amplification was successful only in one non-producing isolate (S 3930). In all nine positive isolates (S 3930 and the eight

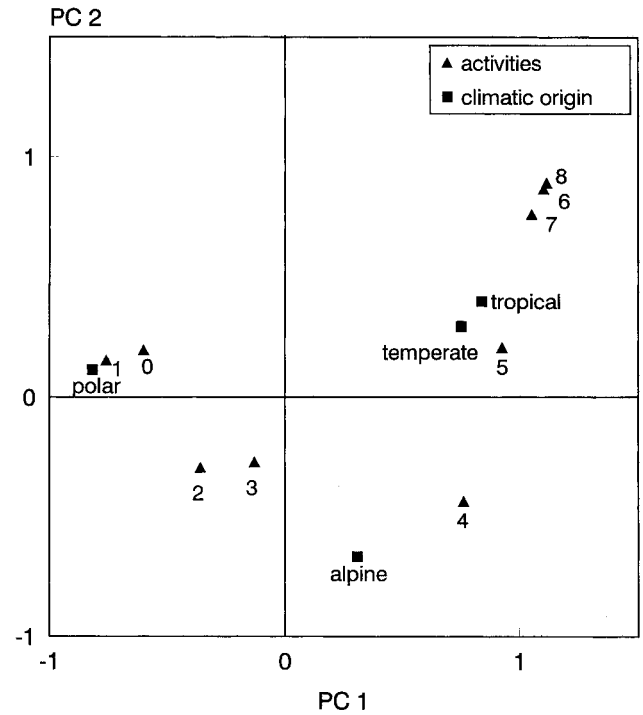

Figure 4 Results of the correspondence analysis for the climatic origin and the number of positive biological assays.

CyA-producers) the size of the bands obtained with the primer pairs 106/107, 95/97 and 104/105 was identical to that of the positive control (*T. inflatum*) and hybridization with marked fragments of the *T. inflatum* synthetase gene demonstrated high sequence homology. The fragment obtained with primer pair 77/80 was detected in only seven isolates (S 23592, S 23606, S 3873-01, S 3930, S 4382, S 2239, S 2226) and it was smaller than the amplification product of *T. inflatum*. However, the hybridization with *Tolypocladium* fragments was successful. In addition to these synthetase primers, two primer pairs were used which amplify fragments of the alanine-racemase gene and of the promotor of the nitrate-reductase gene in *T. inflatum*. Amplification products were not detected in any *C. alba* isolate using these primers.

Comparison of the genetic variation with the physiological and metabolic tests

The results obtained by the RAPD assay [37] were compared with the other data collected in this study. A dendrogram (Figure 5) was constructed using as similarity indices the Jaccard coefficients obtained by pairwise comparison of all test results except the RAPD data. At the first branch point of this dendrogram (Figure 5) the isolate Sp 13-12 is separated against all the other isolates. Remarkably, this strain was the only non-CyA producer which was not sensitive to CyA and therefore clearly differed from all the other *C. alba* isolates. In the RAPD dendrogram this strain was also different from most of the other *C. alba* isolates. At the second branch point two large groups are divided. The first contains isolates from various geographical locations whereas the second comprises all the antarctic and arctic and several alpine isolates. In this second cluster one alpine (S 21498) and a group of alpine and arctic isolates (group F) are separated from the others. The first and more heterogeneous group of isolates from various origins is split at the next branch points into three clusters (B–D) with one of these containing all the CyA-producers (B). In the RAPD analysis, CyA-producers were similar as well and they were more related to each other than to other isolates.

Thirty-eight different clusters of strains were formed at 40% similarity in the genetic analysis, whereas the variation among the isolates was less for the physiological and metabolic examinations. A similar separation among the isolates is obtained at 60% similarity in the dendrogram (Figure 5). Thirty-two groups are formed with twenty of these containing only a single isolate. Those which are formed by more than one strain, often consisted, as in the RAPD analysis, of isolates from the same geographical origin (cluster 8, 15, 17, 19, 20, 26, 28), although not all of them clustered together in the genetic analysis. The alpine isolate S 21551, for instance, belonged together with S 21498 to a group of other alpine strains (Alp 74-08 to Alp 77-08) in the RAPD assay. According to the physiological and metabolic results this isolate clusters together with different alpine strains (Alp 89-05, Alp 88-02, Alp 122-15).

Exceptions where isolates from different geographical regions are grouped together are cluster 2 which contains the CyA-producers, cluster 11 which is formed by isolates from the Swiss Alps and one French isolate and the clusters in the bottom section of the dendrogram which contain alpine and polar isolates (27–30). Only a few of these polar and alpine isolates were similar according to the RAPD assay analysis.

In general, most of the isolates that were similar in their combination of optimal growth temperature, HPLC profile, biological activity, CyA-sensitivity and presence of the CyA synthetase gene were also similar in their RAPD banding patterns, whereas not all the genetically similar isolates had corresponding physiological and metabolic features.

Analysis of the overall variation

For the overall analysis of the physiological, metabolic and genetic diversity in *C. alba*, all test results listed above were considered and combined with the RAPD data. The presence and absence of a total of 378 characters was determined, Jaccard coefficients were calculated and with UPGMA clustering a dendrogram was constructed (Figure 6).

At the first branch point of this dendrogram, *Sesquicillium microsporum* (CBS 954.72) and Sp 13-12 are separated from the other isolates. At the next branch points other outgroup isolates (*C. ovalispora, Thelebolus microsporus* and *Sesquicillium microsporum*) together with the *C. alba* strains Alp 90-01, S 2757-04, S 2757-02, Alp 6-22, CBS 393.89 and S 2428 are divided off. At a dissimilarity of –0.2 in the dendrogram, twelve groups are formed. Most of the isolates are grouped together in cluster E, F, G, H and I. Group E consists of alpine and arctic isolates, group F mainly of antarctic and some alpine and arctic isolates. Cluster G is composed only of three antarctic strains whereas in the heterogenous group H isolates from different origins are combined. Group I contains isolates from various geographical regions, but these isolates have the production of CyA or the occurrence of the cyclosporin synthetase gene in common. All clusters branched deeply within each group, so that at branching nodes of higher similarity (eg 40%) most of the groups contain only a single isolate. Clusters with more than one strain are with two exceptions composed of isolates from the same geographical region, in some cases even from the same sample. Only in groups 31 and 32 are isolates of different origin combined (31: Brazil, Germany, England; 32: USA, Singapore). They all produced CyA in culture media or fragments of the cyclosporin synthetase gene were detected. The strain 3930 which did not produce CyA but possessed fragments of the synthetase gene clearly belonged to this group of CyA-producers if all characteristics are taken together.

Discussion

Optimal growth temperatures

The optimal growth temperatures of the *C. alba* isolates tested varied between 16°C and 30°C. In other taxa similar differences were recorded as well [9,32,41]. We found correlations between the lowest optimal growth temperature and the climatic origin of the isolates (Figure 1). More polar isolates reached their maximal radial growth at 16°C whereas the tropical and temperate strains more frequently had maximal growth rates at 27°C or 30°C. Similar adaptations to the climatic conditions of the natural habitat were demonstrated by Latter and Heal [33] for two Mucorales.

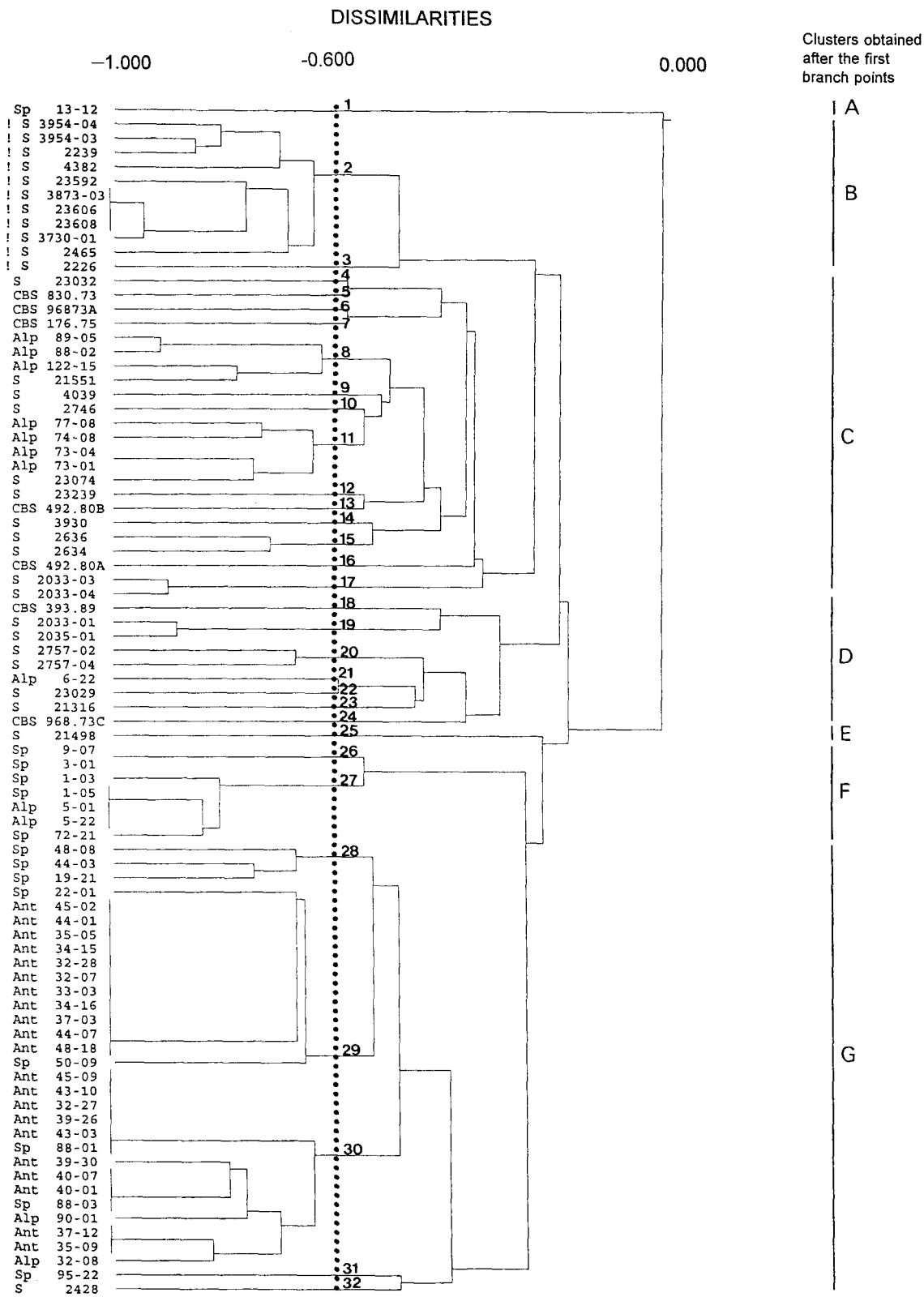

Figure 5 Dendrogram showing the results of the physiological and metabolic examinations (optimal growth temperatures, HPLC profiles, biological activities, presence of the cyclosporin synthetase gene, sensitivity against CyA) after UPGMA clustering (CyA-producers are marked by !). The origin of the strains is given in Table 1 (Ant = Antarctica, Alp = Swiss Alps; Sp = Spitsbergen, S = SANDOZ strain collection).

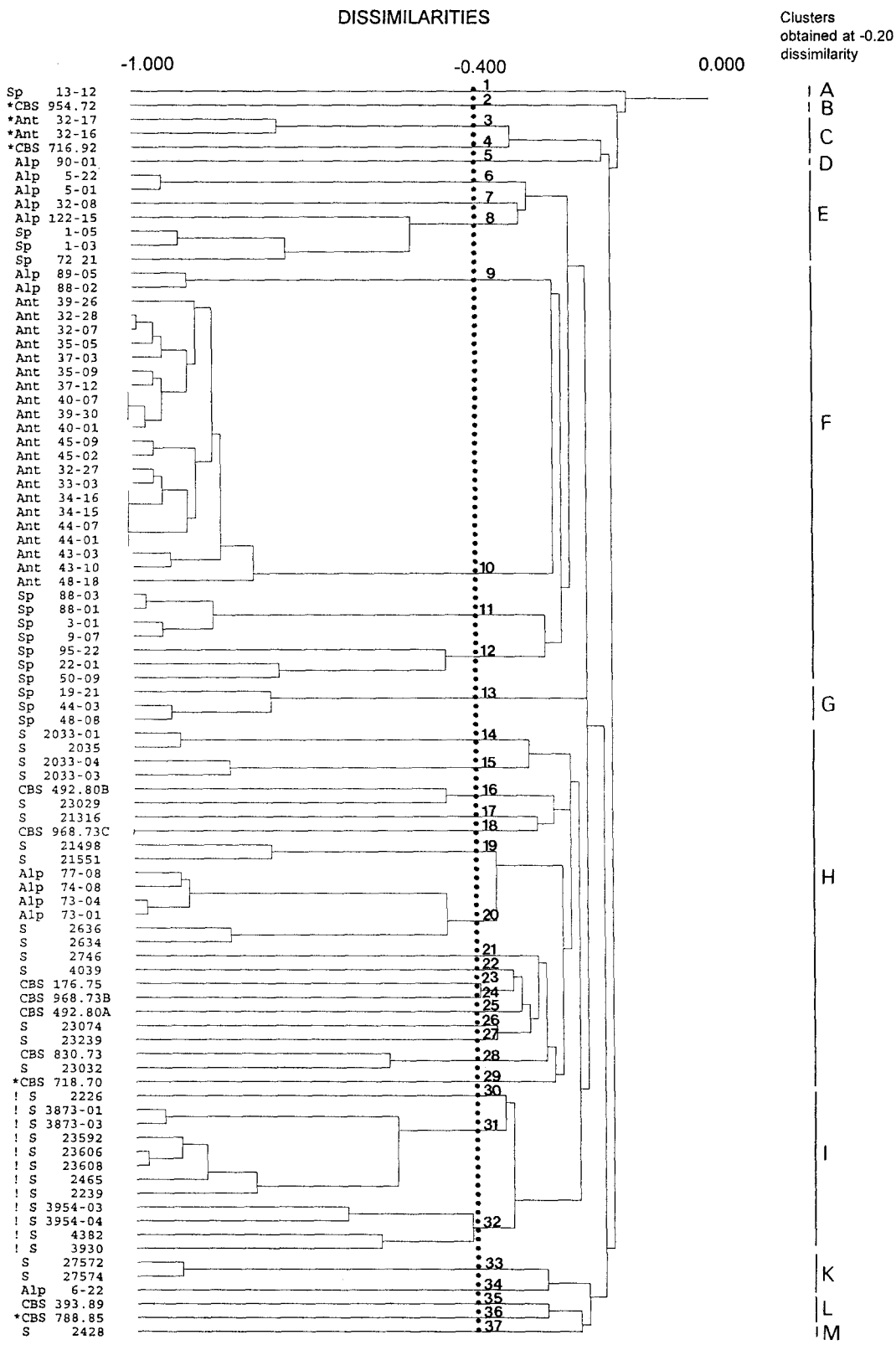

Figure 6 Dendrogram showing the results of all examinations including the data from the RAPD assay after UPGMA clustering (outgroup isolates are marked by *, CyA-producers or those having the cyclosporin synthetase gene are marked by !). The origin of the strains is given in Table 1 (Ant = Antarctica, Alp = Swiss Alps; Sp = Spitsbergen, S = SANDOZ strain collection).

In these taxa [33] antarctic isolates had a higher tolerance to low temperatures than isolates from temperate regions. Cline *et al* [9] showed for isolates of *Pisolithus tinctorius* (Pers) Coker & Couch from North America that strains from northern provinces had lower optima than those from southern regions.

Production of secondary metabolites

As shown for other taxa [21], the profiles of secondary metabolites are reproducible for *C. alba* in parallel cultures. The pattern was not dramatically affected when the incubation temperature or time was changed. It became evident that in *C. alba* the spectrum of detectable secondary metabolites is variable and not as constant as in other taxa where the metabolic pattern is species specific and can be used as a taxonomic character. The analysis of secondary metabolites in culture broth extracts of different penicillia [5,17,19,21,30,31,39] and of other mycotoxin-producing taxa like *Fusarium, Aspergillus, Alternaria, Trichothecium* and *Myrothecium* [12,20,22,34,42,43] make a classification possible which is consistent with morphological and other physiological data. In contrast, the profiles of metabolites in *C. alba* were not homogeneous and therefore cannot be considered to be species specific. Most of the *C. alba* isolates showed a very low profile but no peak was detected which could be used to separate *C. alba* against the outgroup isolates. No direct correlation between the origin of the isolates and identical HPLC profiles was detected although isolates with similar metabolite pattern often originated from the same geographical area of sampling site. In general, isolates from polar regions produced fewer metabolites than those from temperate, alpine or tropical regions and among these, alpine and tropical isolates were most creative. Differences in the production of secondary metabolites in dependence on climatic origin were also detected in *Trichoderma viride* Pers ex Gray [23].

Variations within *C. alba* isolates were also found with respect to their activities on biological assays. Extracts that had identical or very similar HPLC profiles were mostly concurrent in the spectra of biological activities although the number of peaks detected did not always correlate with the number of positive test results and in some cases extracts were active although no peak was recorded. These findings demonstrate that one method alone may reveal only a part of the whole metabolite pattern. Further on the choice of biological tests is restricted and the results are dependent on each other. CyA for instance is responsible for four positive test results. Therefore all CyA producers are similar in their activity pattern. In contrast to Dreyfuss and Chapela [13] who did not find tropical CyA-producing taxa, some more-recently isolated tropical strains of *C. alba* were CyA-producers.

As with the HPLC analysis, the results of the correspondence analysis of the biological activity and the climatic origin indicated that the polar isolates differ from those of temperate and tropical regions and were less active in the applied bioassays which we have carried out. It has been postulated that organisms from extreme habitats are potentially the most potent producers of secondary metabolites [10] and that for antarctic organisms the production of secondary metabolites might be important for survival in the Antarctic [15]. Polar strains of *C. alba* did not produce a great variety of secondary metabolites. Polar isolates of microorganisms which have adapted to extreme conditions during their evolution may have actually lost both their ability to adapt and the ability to produce secondary metabolites [51]. Under less-selective and non-extreme conditions the ability to produce and excrete antagonistic products might compensate for growth rate disadvantages between microbial competitors and would therefore be a selective advantage for survival [45]. This would explain the high metabolic creativity among the tropical isolates. In the tropics with their high species diversity, interactions with other organisms are inevitable and secondary metabolites might be involved in antagonistic and mutual communication mechanisms.

Occurrence of the synthetase gene and sensitivity to CyA

The coding region of the CyA synthetase gene was recently cloned and sequenced [46]. It contains an open reading frame of 45.8 kb and is therefore the largest known gene encoding for one enzyme. The primer pairs which were used for the detection of the gene amplify fragments in *T. inflatum* which are distributed over the whole gene. The amplification was successful in all CyA-producers while the fragments were not detected in non-producers with the exception of strain S 3930. This strain possessed all parts of the gene, but CyA was not detected in the culture broth extracts with the *Neurospora crassa* ramification tests, and in contrast to the CyA-producers it was clearly sensitive to CyA in the culture medium. In the genetic analysis using RAPD, S 3930 clearly belonged to the group of CyA-producers [37]. The absence of amplified fragments for the CyA-negative strains indicate that the synthetase gene with high sequence homology to the *Tolypocladium* gene is missing. As already demonstrated for other secondary metabolites [45], the production of CyA seems to be a strain specific character in *C. alba* and seems to be independent of climatic origin or ecological factors.

Successful amplification and hybridization with fragments of *T. inflatum* prove the high degree of sequence homology among the synthetase gene in *Tolypocladium* and *Chaunopycnis*. Homology was found only for the synthetase region whereas other genes of *Tolypocladium* were not detected in *Chaunopycnis*. Correspondence between genes encoding for enzymes of secondary metabolism were detected in other taxa [44,47]. Weigel *et al* [47] discussed that sequence homology of about 75–80% among fungal isopenicillin N synthetase genes is fairly typical for organisms that diverged several hundred million years ago whereas the high degree of similarity between procaryotes and eucaryotes is unusual. Possible explanations for this high homology are slow changes of the IPNS gene during evolution or horizontal gene transfer [47].

Comparison of the genetic variation with the physiological and metabolic diversity

The similarity of the dendrograms obtained by the RAPD assay [37] and by the other test results (Figure 5) demonstrates that most of the metabolically and physiologically similar isolates are genetically related. Exceptions were

some polar and alpine isolates which seemed to have corresponding physiological and metabolic features but did not cluster together in the RAPD analysis. They were characterized by only very few positive test results because they had low HPLC profiles, they were active in none or few biological assays and the CyA synthetase gene was not found. As positive common features, they were all sensitive to CyA and they had low optimal growth temperatures. Based on this correspondence they were grouped together in the bottom section of the dendrogram (Figure 5) although they might be very different in other physiological respects. Due to this small number of positive characters the opposite effect could be observed as well. Some antarctic isolates, for instance, which were genetically almost identical, appear to be physiologically distinct although the different grouping in the dendrogram is due to only one or two variations in the HPLC or activity pattern. Differences among strains in the bottom section of the dendrogram should therefore not be overestimated.

In general, there is a trend showing that similar strains on the basis of RAPD analysis originate from the same geographical origin or are those producing CyA and are also similar on the basis of physiological and metabolic characters.

Analysis of the overall variation

The overall intraspecific variation in *C. alba* is very high. Groups of isolates which are formed at a similarity of more than 40% consist, without exception, either of CyA-producers or of isolates from the same geographic origin. All the CyA-producers showed more homologies to each other than to any other *C. alba* isolate. The strain S 3930 which was the only non-producer of CyA in these clusters, possessed the synthetase gene and showed high genetic similarities with the CyA-producers from the same geographical location (USA, cluster 32). Genetic similarity in correspondence with production of secondary metabolites was also shown for other fungal species [2,3]. Besides these CyA-producers all other groups which showed similarities consisted of isolates from the same geographic location. Even isolates from different sources (SANDOZ strain collection and CBS, Baarn, The Netherlands) finally grouped (CBS 492.80B + S 23029, France; CBS 830.73 + S 23032, Sweden). We could not find out which geographic region strains S 2636 and S 2634 were isolated from but we can infer from the data of our examination that they could have originated from the Swiss Alps (cluster 20).

Strains from within a habitat were not always similar and could show considerable differences. The large diversity among the alpine isolates demonstrates such variability. In contrast, we found high similarity among all of the antarctic isolates. We assume that the species has been introduced relatively recently and therefore divergence has not yet progressed. For *Cryphonectria parasitica*, a parasitic ascomycete originally endemic to Japan and China, a comparably low diversity was also shown for the introduced population in America [36]. The antarctic *C. alba* strains were distinct from all the other isolates, but showed more similarities with some alpine or arctic strains than with those from tropical or temperate regions. This is due to their relatively low optimal growth temperature and their low activity and HPLC profiles. In other fungal taxa high intraspecific variation has been detected with a variety of molecular biological methods, and groups of similar isolates could often be correlated with ecological parameters [14,16,18,24,26,35]. Biochemical and physiological tests revealed intraspecific variation in different fungal taxa as well [27,38,49]. For *C. alba* we combined these two approaches and merged the results in order to demonstrate the overall intraspecific variation. This variation is mainly caused by the results of the RAPD analysis; all other tests showed high diversity as well and contributed to the variation among the tested isolates. As in the RAPD analysis alone, the characters failed to separate *C. alba* from the outgroup isolates probably because the RAPD assay is not useful for the classification of higher ranks of taxa [6] and might be too sensitive to analyze isolates of very diverse fungal species as well.

Implications for screening programs

This study clearly demonstrates that variation within a single fungal species can be large and shows that biodiversity is not only reflected by species diversity but also by ecologic, genetic and physiologic variability. For the biotechnological and pharmaceutical use of diversity [7,8] intraspecific diversity must therefore be considered as well. It is not sufficient for a routine screening to screen a single strain of a fungal species. Isolates from different geographical regions can be quite different from each other and high variation can be detected even within a single habitat. Our data suggest that collecting fungal isolates from different geographic and climatic regions increases the intraspecific metabolic diversity available for screening programs.

The results obtained with *C. alba*, however, indicate that polar strains might not be as useful for the production of secondary metabolites as tropical isolates are. Such a statement is not necessarily generally true and further investigations of other taxa are required. Results could be different under different incubation conditions, because secondary metabolism is often restricted to a small temperature range [48]. The cyclosporin synthetase gene with high sequence homology to the same gene from *Tolypocladium*, however, was present in only a few strains and non-production of CyA was not dependent on incubation conditions. The CyA-producing isolates were genetically more closely related and a correlation between the presence of the synthetase gene and ecological factors was not found.

The RAPD assay provided a reliable assessment of intraspecific diversity and the results correlated well with the other parameters investigated. The RAPD assay is therefore an efficient and powerful tool for the selection of strains for the microbial screening, as concluded by Fujimori and Okuda [23].

Acknowledgements

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