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Cellular fatty acid profiles for the differentiation of *Penicillium* species

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Abstract

Cellular fatty acid composition of eighteen species of *Penicillium* was studied to investigate its taxonomic usefulness. Many fungi included in this study displayed the same fatty acid composition but differed in relative concentration. Several test species presented the same fatty acid composition but differed in relative concentration. The principal fatty acids were palmitic (16:0), oleic (18:1) and linoleic (18:2). The amount of unsatured fatty acids varied between 68.5% and 78.5%. Multivariate analyses of data showed that it is possible to differentiate some species that belonged to different *Penicillium* groups. The level of agreement of long chain fatty acids with morphological taxonomy was acceptable.

Keywords: *Penicillium* spp.; Fatty acid composition; Taxonomy; Multivariate analysis

1. Introduction

It is a common practice to use cellular fatty acid analysis to identify bacterial genera and species [1–3]. Although fungi possess less different fatty acids than bacteria, recent work has shown that cellular fatty acid profiles can be used [4,5]. The conventional methods for classification and identification of fungi are mentioned in the literature as time consuming and requiring a great deal of taxonomic expertise which is not always available [5]. Physiological data provided by fatty acid profiles, when used in association with morphological features in conventional methods, gives a higher accuracy and resolution in the identification of unknown yeast isolates [6].

The identification of filamentous fungi is almost exclusively based on keys derived from morphological characteristics. Such keys are available for the identification of *Penicillium* isolates, although little attention has been given to the reliability of the diagnostic characters. Pitt [7] suggested that 70–80% of isolates from common sources could be identified with some reliance, and that identification of the

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Abbreviations: IMI, International Mycological Institute; CCMI, Colecção de Culturas de Microrganismos Industriais (Industrial Microorganisms Cultures Collection); ATCC, American Type Culture Collection

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remainder must increasingly rely on the skill and experience of the taxonomist. *Penicillium* is one of the most economically important genera among filamentous fungi. Much of their economic impact is deleterious, with food spoilage, mycotoxin production and biodeterioration heading the list, but in fact their potential for economic utility is equally important. Dart et al. [8] analyzed the fatty acid composition of seventeen *Penicillium* spp. Cluster analysis was performed and two dendrograms were obtained. These dendrograms showed that the 17 species of *Penicillium* used could be divided into three major groups but their subgroups did not correspond to those defined by [9], on the basis of morphological and ecological data. However, most of the *Penicillium* species studied by these authors are now included in other fungial genera. Our study reports the fatty acid composition of 18 *Penicillium* species and presents results of multivariate statistical analysis applied to the data in order to determine its importance.

2. Materials and methods

2.1. Organisms and cultural conditions

The following *Penicillium* species were included: *P. atramentosum* Thom IMI 39752, *P. aurantiogriseum* Dierckx CCMI 630, *P. brevicompactum* Dierckx CCMI 190, *P. camembertii* Thom CCMI 57, *P. chrysogenum* Thom IMI 24314, *P. clavigerum* Demelius CCMI 238, *P. crustosum* Thom CCMI 629, *P. digitatum* (Pers. ex Fr.) Sacc. ATCC 34919, *P. expansum* Link IMI 39761, *P. hirsutum* Dierckx IMI 296066, *P. viridicatum* Westling IMI 39758, *P. caseicolum* Bain. CCMI 276, *P. citreonigrum* Dierckx CCMI 168, *P. oxalicum* Currie and Thom CCMI 120, *P. corylophilum* Dierckx CCMI 61, *P. citrinum* Thom IMI 92196, *P. cyaneofulvum* Biourge CCMI 121, *P. amagasakiense* Kusai ATCC 28686.

The cultures were maintained on slants of Malt Extract Agar (Difco) at 25°C. Fungal biomass of a slant was transferred into 500-ml Erlenmeyer flasks containing 300 ml of Czapek Dox Liquid medium (Oxoid). The pH was adjusted to 5.0 before autoclaving. The culture was grown for 10 days on an orbital shaker at 120 rpm at 25°C.

The mycelium was harvested by filtration, during stationary phase, according to [10], washed with distilled water, freeze-dried and ground.

2.2. Analytical methods

Fatty acid extraction and the preparation of methyl esters were carried out according to the method of [11]. The methyl esters were analyzed by gas-liquid chromatography, on a Hewlett-Packard chromatograph model 5880 A, equipped with dual flame ionization detectors. Identification of esters was based on the comparison of sample retention times with known standards. All analyses were carried out using steel columns (2 mm i.d. $\times$ 2 m), packed with diethyleneglycol succinate on Chromosorb W (80–100 mesh). The detector and injector temperatures were 250°C and 230°C, respectively. The running conditions were: starting temperature 150°C, until 220°C, with 4°C min^{-1}. The flow rate of the carrier gas (nitrogen) was 17 cm^3 min^{-1}.

2.3. Statistical analysis

Principal component analysis (PCA)[1] of the quantitative fatty acids data was performed as described by [12]. In this case, new variables are raised as linear combinations of the initial variables and the results were plotted graphically in two dimensions, i.e. a PC1-PC2 plot. We have established that the fungi (single points) may be projected in a factorial axes system in the central area and the co-ordinates of the fatty acids composition should be read in terms of correlation.

The graphic representation reveals differences in fatty acid composition. Samples which are positioned farthest away from the origin in the plot have the largest influence on the direction of the principal components.

To make visible some of the smaller differences between the analyzed samples (samples may be in other directions and therefore not seen in the plot), a stepwise principal component computation was carried out on samples which are located close to-

[1] The Principal Components Analysis is a classic tool used to analyze data matrices. In this study the data matrix is composed of 18 rows (fungi) and 16 columns (fatty acids).

Table 1
Fatty acid composition of the 18 *Penicillium* species

Fungi	% of total fatty acid content (mean ± S.D.)															
	14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18::3ω6	18:3ω3	20:0	20:1	20:2	22:0	24:0
P. viridicatum	0.27 ± 0.05	1.02 ± 0.25	12.77 ± 0.86	2.02 ± 0.12	0.95 ± 0.10	0.53 ± 0.05	4.07 ± 0.31	16.45 ± 1.09	55.13 ± 1.21	–	4.10 ± 1.49	0.28 ± 0.10	0.27 ± 0.08	0.52 ± 0.17	0.17 ± 0.12	1.08 ± 0.25
P. cyanofulvum	0.32 ± 0.04	1.07 ± 0.15	14.48 ± 0.94	0.95 ± 0.16	0.55 ± 0.05	0.53 ± 0.08	5.68 ± 0.82	33.68 ± 1.42	37.58 ± 1.64	–	1.38 ± 0.42	0.40 ± 0.13	0.27 ± 0.08	0.33 ± 0.10	0.42 ± 0.10	1.00 ± 0.14
P. coryliphilum	0.32 ± 0.04	0.25 ± 0.05	16.82 ± 0.71	0.62 ± 0.10	0.55 ± 0.05	0.18 ± 0.04	8.08 ± 0.45	18.17 ± 1.45	47.83 ± 1.32	–	2.92 ± 0.42	0.53 ± 0.10	0.22 ± 0.08	0.37 ± 0.10	0.48 ± 0.08	1.08 ± 0.12
P. crustosum	0.40 ± 0.06	1.28 ± 0.21	14.18 ± 1.21	1.65 ± 0.10	1.15 ± 0.29	0.73 ± 0.14	5.13 ± 0.68	15.27 ± 1.02	54.35 ± 0.94	–	2.88 ± 0.85	0.28 ± 0.08	0.22 ± 0.04	0.37 ± 0.08	0.23 ± 0.05	1.10 ± 0.20
P. digitatum	0.52 ± 0.08	2.18 ± 0.16	16.62 ± 0.88	2.48 ± 0.33	0.82 ± 0.08	0.58 ± 0.12	4.27 ± 0.47	21.85 ± 1.51	45.92 ± 1.85	–	1.23 ± 0.12	0.33 ± 0.05	0.22 ± 0.04	0.40 ± 0.11	0.20 ± 0.06	0.87 ± 0.08
P. citrinum	0.23 ± 0.05	0.45 ± 0.05	16.78 ± 0.23	0.50 ± 0.00	0.50 ± 0.06	0.12 ± 0.04	10.52 ± 0.62	24.57 ± 1.03	42.23 ± 1.23	–	0.93 ± 0.14	0.62 ± 0.08	0.13 ± 0.05	0.25 ± 0.05	0.60 ± 0.13	1.07 ± 0.23
P. citreonigrum	0.33 ± 0.05	0.35 ± 0.08	15.77 ± 0.38	0.67 ± 0.10	0.37 ± 0.05	0.22 ± 0.08	8.58 ± 0.95	37.10 ± 1.13	30.87 ± 0.70	0.82 ± 0.12	0.20 ± 0.06	0.6 ± 0.06	0.25 ± 0.05	0.32 ± 0.10	0.57 ± 0.16	1.62 ± 0.20
P. expansum	0.63 ± 0.05	0.87 ± 0.15	16.83 ± 0.38	1.42 ± 0.19	0.88 ± 0.08	0.48 ± 0.10	7.02 ± 0.76	20.73 ± 1.89	44.33 ± 2.38	–	2.48 ± 0.38	0.42 ± 0.08	0.18 ± 0.04	0.42 ± 0.08	0.33 ± 0.19	2.25 ± 0.34
P. hirsutum	0.48 ± 0.04	1.97 ± 0.19	16.95 ± 1.21	2.12 ± 0.21	0.73 ± 0.10	0.65 ± 0.10	4.47 ± 0.59	22.10 ± 1.00	46.63 ± 0.67	–	1.48 ± 0.37	0.30 ± 0.06	0.22 ± 0.08	0.42 ± 0.12	0.20 ± 0.00	0.82 ± 0.10
P. amagasakiense	0.28 ± 0.04	0.32 ± 0.10	19.13 ± 0.52	0.83 ± 0.05	0.57 ± 0.08	0.42 ± 0.08	4.97 ± 0.61	24.47 ± 1.23	43.48 ± 0.57	1.40 ± 0.35	–	0.35 ± 0.05	0.22 ± 0.08	0.48 ± 0.10	0.38 ± 0.12	0.47 ± 0.14
P. crysogenum	0.23 ± 0.05	0.95 ± 0.14	13.00 ± 1.56	0.97 ± 0.20	2.03 ± 0.87	0.98 ± 0.64	4.12 ± 0.69	7.23 ± 0.29	59.93 ± 1.29	–	7.62 ± 1.04	0.32 ± 0.12	0.13 ± 0.05	0.52 ± 0.13	0.13 ± 0.05	0.90 ± 0.11
P. caseicolum	0.33 ± 0.05	0.85 ± 0.08	14.88 ± 0.44	2.08 ± 0.28	0.68 ± 0.12	0.53 ± 0.08	3.10 ± 0.22	19.93 ± 0.94	51.77 ± 1.76	–	1.68 ± 0.66	0.23 ± 0.08	0.32 ± 0.10	0.50 ± 0.13	0.30 ± 0.09	0.68 ± 0.15
P. clavigerum	0.80 ± 0.09	0.87 ± 0.16	14.75 ± 0.56	1.62 ± 0.43	0.90 ± 0.21	0.45 ± 0.10	8.30 ± 0.57	23.68 ± 1.64	43.65 ± 1.98	–	1.22 ± 0.21	0.50 ± 0.09	0.20 ± 0.09	0.32 ± 0.10	0.45 ± 0.16	1.10 ± 0.30
P. aurantiogriseum	0.63 ± 0.05	0.87 ± 0.08	16.82 ± 0.43	1.48 ± 0.29	0.88 ± 0.88	0.37 ± 0.05	7.00 ± 0.80	18.83 ± 1.22	47.03 ± 1.99	–	2.57 ± 0.66	0.43 ± 0.08	0.25 ± 0.05	0.50 ± 0.14	0.38 ± 0.08	1.47 ± 0.18
P. atramentosum	0.55 ± 0.05	0.32 ± 0.12	15.78 ± 0.72	1.68 ± 0.21	0.62 ± 0.18	0.70 ± 0.17	4.88 ± 0.60	51.23 ± 1.92	18.15 ± 0.84	–	2.77 ± 0.57	0.33 ± 0.08	0.48 ± 0.08	0.13 ± 0.19	0.23 ± 0.05	1.40 ± 0.21
P. camemberti	0.42 ± 0.08	1.73 ± 0.19	15.40 ± 0.74	1.92 ± 0.25	0.83 ± 0.12	0.60 ± 0.14	4.77 ± 0.69	15.92 ± 0.71	54.15 ± 1.19	–	1.45 ± 0.37	0.27 ± 0.08	0.18 ± 0.08	0.37 ± 0.27	0.22 ± 0.04	0.98 ± 0.41
P. oxalicum	0.28 ± 0.12	0.62 ± 0.12	16.23 ± 1.14	1.18 ± 0.25	1.62 ± 0.60	0.43 ± 0.05	5.63 ± 0.36	14.52 ± 0.88	51.55 ± 1.29	–	5.07 ± 0.69	0.35 ± 0.08	0.22 ± 0.15	0.42 ± 0.15	0.22 ± 0.10	0.62 ± 0.17
P. brevicompactum	0.45 ± 0.05	0.78 ± 0.08	14.90 ± 1.24	1.05 ± 0.19	0.48 ± 0.04	0.47 ± 0.10	5.75 ± 0.43	44.93 ± 1.76	25.90 ± 1.66	–	1.07 ± 0.08	0.47 ± 0.08	0.62 ± 0.15	0.27 ± 0.17	0.43 ± 0.10	1.52 ± 10.16

Each species was analysed on six occasions.

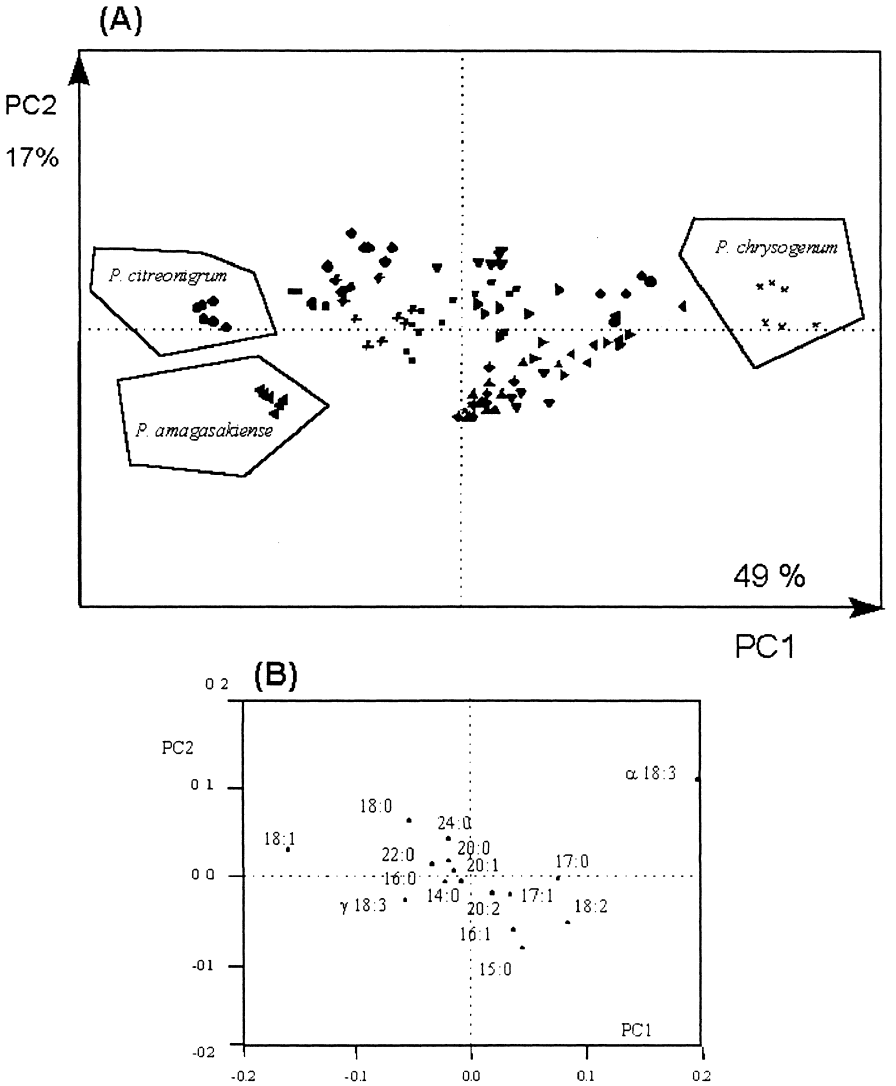

Fig. 1. Each species was represented by six entries in the PC analysis, corresponding to different extractions of the same species. A: Graphic representation of the fungi in a plan defined by the first two axes (PC1 and PC2) of the principal components. Symbols: ○, *P. oxalicum*; ●, *P. citreonigrum*; +, *P. cyaneofulvum*; ▽, *P. camembertii*; ▼, *P. corylophilum*; ◀, *P. amagasakiense*; □, *P. brevicompactum*; ■, *P. clavigerum*; ▲, *P. caseicolum*; ▲, *P. digitatum*; ×, *P. chrysogenum*; ◁, *P. viridicatum*; ■, *P. expansum*; ◇, *P. hirsutum*; ◆, *P. atramentosum*; •, *P. citrinum*; ▷, *P. crustosum*; ▶, *P. aurantiogrireum*. B: Graphic representation of the variables (fatty acid composition) in the same plan.

gether on the former plot, thereby excluding the influence of more remote samples.

In all cases, projections of more than the first two principal components provided no additional information on the relationship between species, only plots of the first two principal components are presented.

3. Results and discussion

Mycelia and conidia are two different stages of the vegetative life cycle of filamentous fungi. Although the ultrastructural morphology of the two stages is very similar, relevant differences in their lipid, ribosome and protein composition have been described

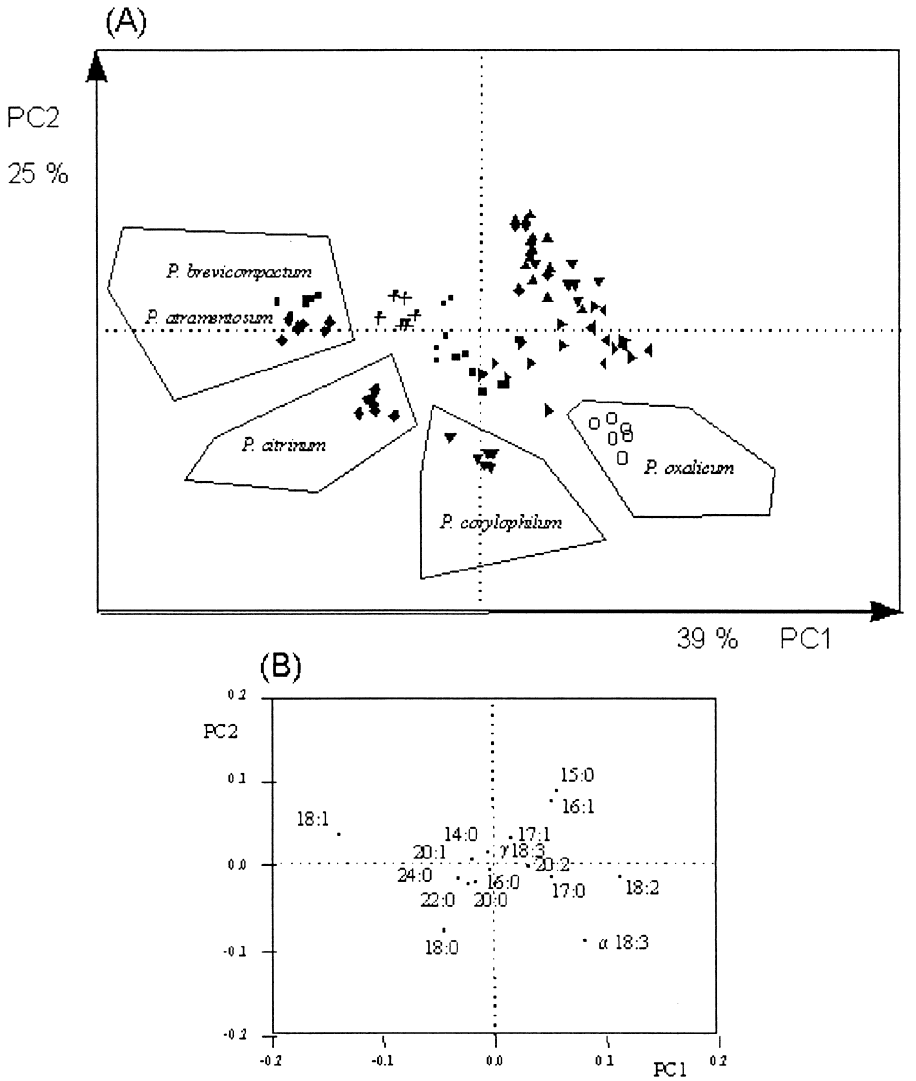

Fig. 2. A: Recomputed PC1-PC2 plot of the 15 fungi which fell in one group in Fig. 1 (symbols as in Fig. 1). B: Graphic representation of the variables (fatty acid composition) in the same plan.

[13]. In order to obtain microorganisms in the mycelial form, the cultures were carried out at slow agitation. Microscopic examination of the cultures revealed absence of conidia.

Fatty acid profiles for each microorganism are summarized in Table 1. The growth conditions were kept uniform to minimize variation in fatty acid composition. In each organism, the fatty acids ranged from 12 to 24 carbons. Most fungi possess a similar fatty acid composition, but showed different concentration. The most common and abundant fatty acids extracted were palmitic (16:0), oleic (18:1) and linoleic (18:2), comprising 84% or more of the total peak areas for 18 *Penicillium* spp studied. In most cases, linoleic acid (18:2) was present in a higher percentage than oleic acid (18:1), except for *P. citreonigrum*, *P. atramentosum* and *P. brevicompactum*. The total unsatured fatty acids ranged from 68.5 to 78.5%.

The first projection of the two PCA axes (Fig. 1), named PC_1 and PC_2, described 49% and 17%, respectively, of the total variance, and showed that

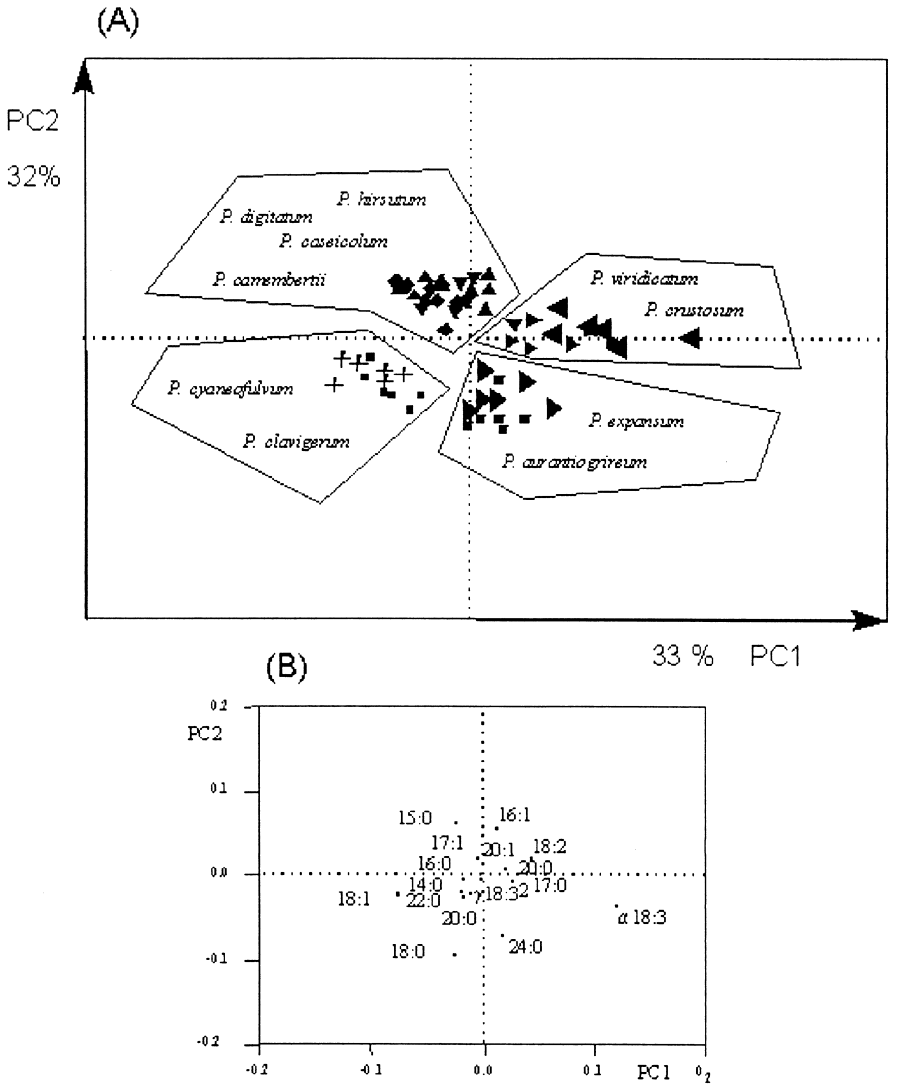

Fig. 3. A: Recomputed PC1-PC2 plot of the 11 fungi which fell in one group in Fig. 1 (symbols as in Fig. 1). B: Graphic representation of the variables (fatty acid composition) in the same plan.

three of the organisms – *P. chrysogenum, P. citreonigrum* and *P. amagasakiense* – have clearly different patterns and could be considered apart from the other 15 species. *P. amagasakiense* and *P. citreonigrum* are the only two species which possess γ-linoleic acid (18:3ω6) and *P. chrysogenum* is the species which contains the highest percentage of α-linolenic acid (18:3ω3) (Fig. 1B). This separation is in agreement with the morphological taxonomy described in the literature [14]. *P. citreonigrum* belongs to the subgenus *Aspergilloides* Dierckx with monoverticillate penicilli and ampulliform phialides in verticils. *P. chrysogenum* is classified in the subgenus *Penicillium* Pitt. In this fungus the penicilli are mostly terverticillate. The taxonomic position of *P. amagasakiense* remains uncertain. According to the description, this fungal species might be similar to *Paecilomyces marquandii* (Massee) Hughes, but without examination of the type culture its taxonomic status could be doubtful [15]. Among these three

species, the distances in the plot showed that *P. citreonigrum* and *P. amagasakiense* had a similar fatty acid profile, and opposite to *P. chrysogenum*.

To test if there were any differences among the 15 microorganisms that fell in the central group, another PCA was performed (Fig. 2), and the projection PC_1-PC_2 described 64% (39%+25%) of total variance. In order to determine possible differences among them, they were recomputed without the influence of the first three species. This analysis showed that *P. brevicompactum*, *P. atramentosum*, *P. oxalicum*, *P. citrinum* and *P. corylophylum* were separated from the remaining species. This plot showed that *P. brevicompactum* and *P. atramentosum* were included in the same group which is characterized by a significantly higher mean percentage of oleic acid (18:1). Both species are morphologically related, they belong to the subgenus *Penicillium* [14]. *P. oxalicum*, *P. citrinum* and *P. corylophylum* are included in the subgenus *Furcatum* Pitt, and were different from each other and from the other species. From Fig. 2B, it can be inferred that *P. oxalicum* was separated from the others, because it is the species which encloses the highest percentage of α-linolenic acid (18:3ω3).

Another PCA was performed on the remaining 10 species (Fig. 3). The two axes, PC_1 and PC_2, described 65% (33%+32%) of the total variance in the data. In this case, there was no clear differentiation between the species, but it was possible to match the species according to their relative distances. These species can be divided into four groups. A first group included four species, *P. digitatum*, *P. hirsutum*, *P. caseicolum* and *P. camembertii*. All species belong to the subgenus *Penicillum* Pitt, and *P. caseicolum* is now considered a synonym of *P. camembertii* [7]. A second group was composed by *P. crustosum* and *P. viridicatum*. These species were also included in the subgenus *Penicillum* Pitt and they have phylogenetic affinities based on morphological properties: conidial color, stipe dimensions and tendency to produce detachable masses of conidia on Malt Extract Agar medium [14]. A third group, composed of *P. cyaneofulvum* and *P. clavigerum*, was separated from the fourth group which included *P. expansum* and *P. aurantiogriseum*. All species were classified into the subgenus *Penicillium* Pitt except *P. cyaneofulvum*, which was placed in the subgenus *Furcatum* Pitt.

These successive PCA showed that: (i) it was possible to differentiate subgenus (subgenus *Aspergilloide* Dierckx, from the subgenus *Penicillium* Pitt, 1st PCA); (ii) even in the same subgenus – *Furcatum* – it was possible to separate species (2nd PCA, *P. oxalicum*, *P. citrinum* and *P. corylophilum*). It was more difficult to differentiate the species that belonged to the subgenus *Penicillium*. However, there were three species in this subgenus that were readily separated from the others (in the 1st PCA *P. chrysogenum* and in the 2nd PCA *P. atramentosum* together with *P. brevicompactum*). As it has been pointed out, this subgenus has proved to be taxonomically difficult. Nevertheless, it was possible to create groups which suggest similar fatty acid patterns. This could be valuable data in the identification and characterization of these fungi.

Additional studies should be made including strains of different species to investigate the relation between strains and species, using multivariate analysis. The development of simple extraction and transesterification method, together with the capability to obtain reproducible profiles, may offer an underexploited source of taxonomic information at species and subspecies levels, as well as other approaches complementary to the conventional methods to *Penicillium* taxonomy, like profiles of mycotoxins and other secondary metabolites, visualized by thin layer chromatography, and the use of electrophoretic patterns of certain isoenzymes.

References

[1] Mukwaya, G.M. and Welch, D.F. (1989) Subgrouping of *Pseudomonas cepacia* by cellular fatty acid composition. J. Clin. Microbiol. 27, 2640–2646.

[2] Veys, A., Callewaert, W., Waelkens, E. and Van Den-Abeele, K. (1989) Application of gas-liquid chromatography to the routine identification of non-fermenting Gram-negative bacteria in clinical specimens. J. Clin. Microbiol. 27, 1538–1542.

[3] Dykes, G.A., Cloete, T.E. and von Holy, A. (1995) Taxonomy of lactic acid bacteria associated with vacuum-packaged processed meat spoilage by multivariate analysis of cellular fatty acids. Int. J. Food Microbiol. 28, 89–100.

[4] Tredoux, H.G., Kock, J.L., Lategan, P.M. and Muller, H.B. (1987) A rapid identification technique to differentiate between *Saccharomyces cerevisiae* strains and other yeast species in the wine industry. Am. J. Enol. Vitic. 38, 161–164.

[5] Stahl, P.D. and Klug, M.J. (1996) Characterization and differentiation of filamentous fungi based on fatty acid composition. Appl. Environ. Microbiol. 62, 4136–4146.
[6] Oosthuizen, A., Kock, J.L., Botes, P.J. and Lategan, P.M. (1987) The long-chain fatty acid composition of yeasts used in the brewing industry. Appl. Microbiol. Biotechnol. 26, 55–60.
[7] Pitt, J.I. (1986) A Laboratory Guide to Common *Penicillium* Species. CSIRO, North Ryde, Australia.
[8] Dart, R.K., Stretton, R.J. and Lee, J.D. (1976) Relationships of *Penicillium* species based on their long-chain fatty acids. Trans. Br. Mycol. Soc. 66, 525–529.
[9] Pitt, J.I. (1973) An appraisal of identification methods for *Penicillium* species. Mycologia 65, 1135–1157.
[10] Viljoen, B.C., Kock, J.L.F. and Lategan, P.M. (1986) Long-chain fatty acid composition of selected genera of yeasts belonging to the Endomycetales. Antonie Van Leeuwenhoek 52, 45–51.
[11] Lepage,G. and Roy, C.C. (1986) Direct transesterification of all classes of lipids in a one-step reaction. J. Lipid Res. 27, 114–119.
[12] Cooney, J.J., Doolittle, M.M., Grahl-Nielsen, O., Haaland, I.M. and Kirk, P.W. Jr. (1993) Comparison of fatty acids of marine fungi using multivariate statistical analysis. J. Ind. Microbiol. 12, 373–378.
[13] Chattopadhyay, P., Banerjee, S.K., Sen, K. and Chakrabarti, P. (1987) Lipid profiles of conidia of *Aspergillus niger* and a fatty acid auxotroph. Can. J. Microbiol. 33, 1116–1120.
[14] Pitt, J.I. (1979) The Genus *Penicillium*. Academic Press, London.
[15] Samcon, R.A. (1974) *Paecilomyces* and some allied Hyphomycetes. Stud. Mycol. 6, 1–119.