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Terverticillate Penicillia Studied by Direct Electrospray Mass Spectrometric Profiling of Crude Extracts. I. Chemosystematics

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Key Word Index—*Penicillium*; ESMS; mass profile; chemotaxonomy; cluster analysis; secondary metabolites.

Abstract—A chemosystematic study of 339 isolates from all known terverticillate *Penicillium* taxa was performed using electrospray mass spectrometric analysis of extractable metabolites. The mass profiles were made by injecting crude plug extracts made from cultures grown on Czapek Yeast Autolysate agar (CYA) and Yeast Extract Sucrose agar (YES) directly into the electrospray source of the mass spectrometer. A data matrix was made from each substrate by transferring the complete centroid mass spectrum from 200 to 700 amu as 501 variables to individual columns. No attempt was made to identify ions in the mass profile, but a noise level was applied. Cluster analysis using the correlation coefficient resulted in dendrograms where approximately 75% of the included taxa could be considered segregated in distinct clusters. Standard normalized data (mass spectra) resulted in clear clusters, but grouped taxa dominated by a single intense ion together, whereas logarithmized data revealed finer detail but with a shorter distance between clusters. The overall results showed that substantial taxonomic information can be extracted from mass profiles even when the identity of ions is unknown. Ions corresponding to known secondary metabolites were, however, found in all mass profiles. © 1997 Elsevier Science Ltd. All rights reserved

Introduction

Classification of the terverticillate penicillia has always been difficult using classical morphological methods even though this group of fungi has been the subject of several comprehensive studies. A clear taxonomic segregation of the terverticillate penicillia remains a matter of some debate, and confusion is often encountered in literature due to misidentifications (Frisvad and Filtenborg, 1989). Several decades of taxonomical work has improved classification schemes but has also shown that taxonomy based on one type of character might be insufficient to provide a clear classification (Samson and Gams, 1984; Pitt and Samson, 1990; Samson, 1991; Pitt, 1991; Frisvad and Filtenborg, 1989; Svendsen and Frisvad, 1994; Lund and Frisvad, 1994; Larsen and Frisvad, 1995a,b; Bridge *et al.*, 1989b; Kozakiewicz *et al.*, 1993).

Classical morphological studies are laborious and very dependent on a taxonomic judgement and require considerable training. Growing the fungi on different substrates has proved to be helpful in morphological studies, but cannot always give a clear identification since batch differences in less well-defined substrate components like malt and yeast extracts can blur the results (unpublished). Classification on the basis of chemical analyses of one or several cell components, i.e. isozymes, has been used with some success (Pitt, 1991; Bridge *et al.*, 1989a; Paterson *et al.*, 1989) in *Penicillium* taxonomy, but they may also reflect the growth conditions and adaptations rather than differentiation of the organism. Only a few examples of the use of pyrolysis in combination with gas chromatography or mass spectrometry are found in the literature of *Penicillium*

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taxonomy (Irwin, 1982). During the last decade, different molecular biological methods (e.g. DNA-profiles) have been developed for use in classification, but the results and applications in every day fungal taxonomy are still limited (Pitt and Samson, 1990; Taylor and LoBuglio, 1994; Bruns *et al.*, 1991; Bainbridge, 1994; Peterson, 1993) although these methods are expected to possess large future potential. To date, the most successful single type of character in fungal taxonomy has been the secondary metabolite profile (Frisvad and Filtenborg, 1989; Culberson and Elix, 1989; Lund and Frisvad, 1994; Svendsen and Frisvad, 1994; Larsen and Frisvad, 1995a,b; Whalley and Edwards, 1995).

Only a few studies of the terverticillate penicillia have included a large group of taxa and isolates. In one comprehensive study of 348 isolates, at CAB International Mycological Institute, Kew, using all the above mentioned techniques, including morphology and physiology, about 90% of the isolates were considered to be classified into taxa, although several commonly accepted taxa could not be separated (Bridge, 1985; Bridge *et al.*, 1989a,b, 1990; Paterson *et al.*, 1989; Kozakiewicz *et al.*, 1993). A chemotaxonomic study by Svendsen and Frisvad (1994) showed that 279 isolates of terverticillate penicillia can be classified into taxa by cluster analysis using secondary metabolite profiles determined by HPLC.

Chemotaxonomic studies based on secondary metabolites are traditionally done either by using specific analysis for selected metabolites or by screening methods where a profile is determinated by TLC, HPLC or GC analysis (Filtenborg *et al.*, 1983; Frisvad and Thrane, 1987; Frisvad, 1987; Larsen and Frisvad, 1994, 1995a; Whalley and Edwards, 1995). Evaluation of TLC plates does require some training and needs at least some metabolites to be available as reference compounds. Furthermore, the analyst has to decide which spots on TLC plates or peaks in the HPLC or GC chromatograms shall be included in the analysis and also how the data should be processed to extract taxonomic information. This type of information is mostly used in a binary form. The study by Svendsen and Frisvad (1994) was based on transforming HPLC peak data into chromophore families based on UV spectra and retention times. A data matrix was constructed by assigning 1 if a peak that belonged to a specific chromophore family was found in the chromatogram from the analysis of an isolate. These authors (Svendsen and Frisvad, 1994) had, however, to decide which chromophore families to use and to assign membership to these families for each isolate examined. A similar approach was used in a chemotaxonomic study by Larsen and Frisvad (1995b) where selected volatile metabolites were used to distinguish between 132 isolates from 25 taxa.

A new and rapid tool for chemotaxonomic classification is to use electrospray mass spectrometry (ESMS) to determine a mass spectrum directly from crude extracts taking advantage of the high sensitivity and very limited fragmentation (Smedsgaard and Frisvad, 1996) in ESMS. These mass spectra will almost exclusively show protonated and/or sodiated ions from a broad range of the extractable metabolites and can be used as a mass profile of the sample (Smedsgaard and Frisvad, 1996). The analyst does not have to make an a priori decision which masses to include but can include the entire spectrum in the data analysis. Another advantage is that mass profiles (spectra) can be stored and retrieved very easily from computer systems and used in chemometric analysis. This can be done by cluster analysis of a matrix containing entire spectra thus considering each nominal mass in the scan range as variables and the entire spectrum as an object to obtain a classification within the group of isolates examined.

Mass profiles can furthermore be used to give an indication whether a specific metabolite is produced or not. It is, however, important to note that observing an ion corresponding to the protonated molecular mass of a specific metabolite is not an identification of that metabolite, as several secondary metabolites or fragments can have the same mass, but it can be used as an indication of production of a specific metabolite in combination with other information (LC, LC-MS, etc.) (Smedsgaard and Frisvad, 1996).

The aim of this study was to evaluate the chemosystematic possibility using mass profiles to segregate the complex group of subgenus *Penicillium* species including a large number of taxa and isolates.

Materials and Methods

A total of 339 isolates from different habitats and geographic regions of the genus *Penicillium* were selected from the IBT culture collection at the Department of Biotechnology, DTU. The isolates were selected to represent the taxa described in subgenus *Penicillium* with a predominantly terverticillate conidiophore (Pitt, 1979; Frisvad and Filtenborg, 1989; Svendsen and Frisvad, 1994; Lund and Frisvad, 1994). Other taxa not yet described and *Eupenicillium tularense* were also included.

The isolates were cultivated using three point inoculations on Czapek Yeast Autolysate agar (CYA) and Yeast Extract Sucrose agar (YES), see pp. 307–311 in (Samson *et al.*, 1995) for formulation (the substrates were prepared with the addition of a trace element solution). A conidial suspension made from seven day-old cultures grown on CYA was used for inoculation. The isolates were inoculated on to 9 cm Petri dishes and grown for 7 days in the dark at 25°C.

All the isolates were examined and identified using morphology and physiology before chemical analysis. The isolates were reinoculated in cases of contamination or other irregularities. Inoculations were done following the IBT collection numbers to obtain a partial randomization of taxa during analyses. All inoculation and chemical analyses were done within a 5 month period.

Extractions and analyses were done as previously described (Smedsgaard, 1997). The method is based on extraction of three plugs (6 mm diameters) cut from seven day-old cultures with 0.5 ml of a mixture of dichloromethane: methanol: ethyl acetate (2:1:3) acidified to 1% formic acid for 60 min in 1.5 ml vials using an ultrasonic bath. The extracts were transferred to clean vials and the solvent evaporated under a stream of nitrogen and the residues were redissolved in 0.4 ml 75% methanol acidified to a final concentration of 0.6% (v/v) formic acid and 0.02% (v/v) hydrochloric acid. The redissolved samples were analysed after filtration through a 0.45 µm filter. All sample manipulations were done using 1.5 ml disposal sample vials and disposable pasteur pipettes and chemicals of analytical grade.

The ESMS analyses were performed as described in (Smedsgaard and Frisvad, 1996) by injecting the filtered extracts into a Fisons/VG TRIO 2000 (Micromass, UK) single stage quadropole mass spectrometer with an electrospray source. The carrier was 90% methanol (HPLC grade) in double distilled water at a flow-rate of 6 µl/min delivered from a Phoenix 20 syringe pump (Fisons Instruments). Injections were done automatically using an FAMOS autoinjector (LC-Packings) in full loop microflow mode (5 µl loop). Instrumental parameters were selected to reduce fragmentation and in-source reactions and to optimize sensitivity. Mass spectra were collected from 200 to 700 amu as 3 s scans and all scans collected during the eluting time (approximately 1.3 min corresponding to 24 scans) were summarized to one continuum spectrum. A background subtraction and Savitzky-Golay smoothing (three times with a peak width of 0.75 amu) was performed on all spectra, and the centroid was calculated.

The peak intensity (height) from all mass peaks (at nominal masses) in each centroid spectrum was transferred to a column (as an object) in a data matrix with the nominal masses from 200–700 amu as the rows (501 variables). The intensity was set to zero for mass peaks with an intensity below 10,000 counts corresponding to the background noise level in the spectra. Data collected from isolates cultivated on CYA and YES were kept separately resulting in a 344 object by 501 variables matrix from CYA and a 342 object times 501 variables matrix from YES including five substrate blanks in each matrix (two isolates were missing in the YES matrix).

Cluster analyses where done using NT-SYS ver. 1.80 (Applied Biostatistics, New York, U.S.A.). All peak data in the matrices were included in the analyses in order to perform an unbiased cluster analysis. Each object (i.e. spectrum) was normalized by dividing the intensities of all variables (mass peaks) by the highest intensity for that object and then multiplying by 1000 to compensate for differences in absolute concentration of the extracts and for drift in instrument sensitivity. This process resulted in objects comparable to the usual representation of mass spectra. Two transformations were included: (1) the logarithm (base 10) was calculated for all elements to reduce the numerical difference (of approximately three orders of magnetude); (2) the matrices

were transformed to binary matrices by setting all intensities above 100 (10%) in the normalized matrices to 1 and all other elements to 0.

All the relevant similarity/dissimilarity coefficients available in the NT-SYS program were tested on the quantitative data as no previous experience of cluster analysis of large groups of complete mass spectra was available. The Yule coefficient was used for the binary matrices based of previous experience (Frisvad, 1994; Svendsen and Frisvad, 1994) although there were a large number of zeros in the matrices. UPGMA linkage was used in all cases.

All extracts were, as reference, analysed by HPLC as described in Smedsgaard (1997). The analyses were done using a reversed phase gradient system with diode array detection. Separations were done on a Hypersil BDS-C_{18} column (100 mm * 4 mm id.) (Hewlett Packard) with a gradient going from 15% acetonitrile in water to 100% acetonitrile in 40 min then maintaining 100% acetonitrile for 3 min with a flow of 1 ml/min. Both eluents contained 0.05% (v/v) tri-fluoroacetic acid (TFA). All UV spectra from 200 to 600 nm with a sampling interval of approximately 0.5 s were stored during the 43-min runtime.

Results and Discussion

The lowest IBT numbers are the oldest isolates, some of these have been stored in the IBT collection for more than 5 years. From our experience some of the old isolates tend to be less productive and have a slower growth than more recent isolates (unpublished). This is reflected in the mass spectra where the total ion currents are quite low and have pronounced ions from solvent clusters. The HPLC analyses with diode array detection confirmed the production of most known secondary metabolites for all isolates studied and also unknown metabolites in agreement with Svendsen and Frisvad (Frisvad and Filtenborg, 1989; Svendsen and Frisvad, 1994). Several of the isolates included in this study could not be fitted into one of the described taxa and represent taxa not yet described or single isolates (i.e. only one isolate of that particular species). These isolates are labelled P. sp. followed by a reference number. Approximately 35% of the isolates were re-inoculated at least one month later to check identity and stability of the mass profiles. Almost identical mass profiles were obtained from the re-analysis of these isolates compared with the first analysis. A few isolates were reclassified after morphological and chemical re-examinations.

Evaluation of mass spectra

Examples of mass spectra (mass profiles) obtained from *P. coprophilum* cultivated on CYA and YES are shown in Fig. 1 as smooth continuum spectra. The mass profiles obtained from the two different substrates are in this case very similar. *Penicillium coprophilum* produces the following secondary metabolites (with the nominal mass in brackets): dechlorogriseofulvin (318 amu), griseofulvin (352 amu), roquefortine C (389 amu), meleagrin (433 amu) and oxaline (447 amu) (Frisvad and Filtenborg, 1989). Ions corresponding to the protonated molecular mass (M + 1) of these secondary metabolites can easily be recognized in the spectra as peaks at 319, 353/355, (390 small), 434 and 448 amu. The chlorine containing metabolite griseofulvin is particularily easy to recognize due to the isotopic pattern from the chlorine isotopes giving mass peaks at a relative intensity of 3 to 1 seen at 353 amu (relative intensity 100%) and 355 amu (relative intensity 35%). The production of these metabolites was confirmed by HPLC analyses of the same extracts and comparing retention times and UV spectra with those obtained from analyses of standards. Several other distinct peaks are seen in the mass profiles and in the chromatograms corresponding to unknown metabolites or fragmentation. The ion seen at 436 amu is found only in spectra from *P. coprophilum* and not in spectra from any other taxon.

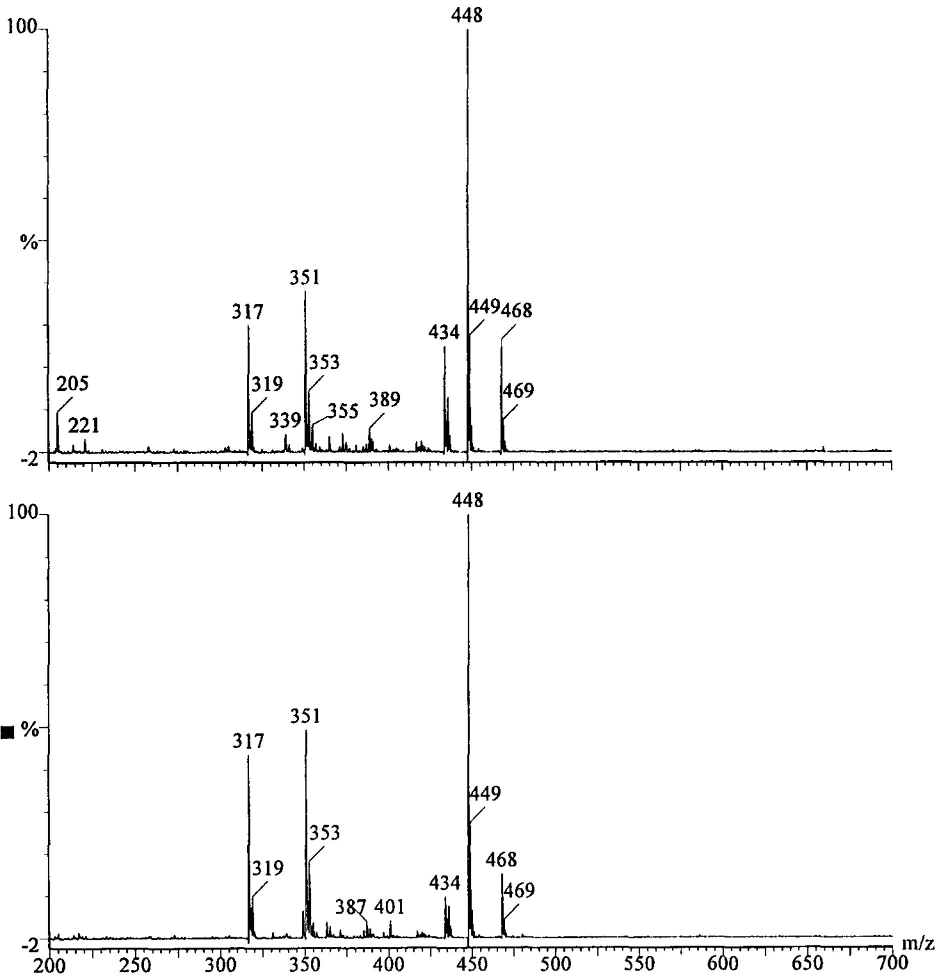

FIG. 1. SIMILAR MASS PROFILES OBTAINED FROM *P. COPROPHILUM* (IBT 12992) ARE SEEN FROM GROWTH ON CYA (TOP) AND YES (BOTTOM). Ions seen in the mass profiles correspond to metabolites produced by *P. coprophilum* as follows: dechloro-griseofulvin at 319 amu, griseofulvin at 353 and 355 amu (a very small roquefortine C at 390 amu), meleagrin at 434 amu, oxaline at 448 amu.

Mass profiles from *P. discolor* are shown in Fig. 2 as an example of the influence of growth substrate on the metabolite production seen as the difference in mass profiles obtained from CYA and YES. Ions corresponding to the production of the following metabolites by *P. discolor* (with nominal mass in brackets): viridicatin (237 amu), viridicatol (253 amu), palitantin (254 amu), dehydro-cyclopeptin (278 amu), cyclopeptin (280 amu), cyclopenin (294 amu), cyclopenol (310 amu) chaetoglobosin A–D (528 amu), and chaetoglobosin E–F (530 amu) can be seen as the protonated and sodiated ions (M + 1 and M + 23) in the mass profile from CYA. Several unidentified ions are seen in the mass profiles from cultivation on CYA. A considerably different profile is, however, found from the cultivation on YES and this is confirmed by HPLC analysis. The ions observed above 500 amu in the CYA mass profiles are not seen in the YES profiles whereas several new ions are found in the 300–400 amu range that cannot be assigned to

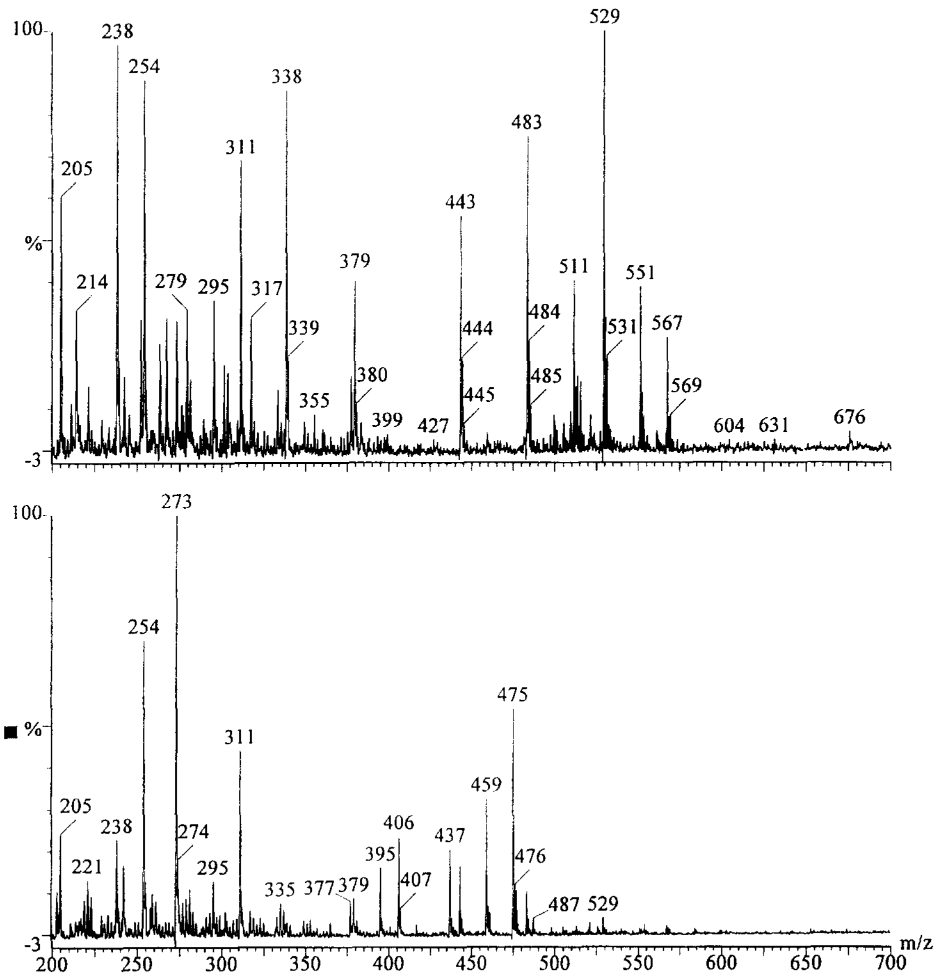

FIG. 2. MASS PROFILE FROM *P. DISCOLOR* (IBT 11512) GROWN ON CYA (TOP) AND YES (BOTTOM). Ions corresponding to the following metabolites produced by *P. discolor* can be seen in the CYA mass profile at the top: viridicatin at 238 amu, viridicatol at 254 amu, dehydro-cyclopeptin at 279 amu, cyclopeptin at 281 amu, cyclopenin at 295 amu, cyclopenol at 311 amu, chaetoglobosin A–D at 529 amu($M+H^+$) and 551 amu ($M+Na^+$), chaetoglobosin E–F at 531 amu ($M+H^+$) and at 567 amu ($M+Na^+$). Only a few of these metabolites can be found in the mass profile from YES.

known metabolites. Similar differences between the profiles obtained from CYA and YES are seen in mass and HPLC profiles from approximately half of the examined isolates.

The examples shown on Figs 1 and 2 represent average mass profiles obtained in this study. Very dilute extracts or extracts from taxa (e.g. *P. verrucosum, P. nalgiovense, P. camemberti*) producing few metabolites determinated by ESMS were dominated by solvent clusters in the low mass range. Other examples of mass profiles can be found in (Smedsgaard and Frisvad, 1996; Frisvad and Smedsgaard, 1997). It is, however, important to note that a mass profile will occasionally be dominated by a single very intense ion like terrestric acid (molecular mass 210 amu) or meleagrin (molecular mass 433 amu) which has to be considered when analysing data from taxa that produce these metabolites.

Cluster analysis of the mass profile data

Each mass profile contained between 100 and 300 mass peaks above 10.000 counts with an average number around 250 values. All these mass peaks were transferred to the data matrices. The cosine and correlation coefficients (Sneath and Sokal, 1973) gave similar and most resolved dendrograms containing all 342/344 isolates and blanks. Only the dendrogram calculated using the correlation coefficient is considered in the following discussion.

The data matrices contained a large number of non-informative noise variables resulting in a low distance between clusters, but a clear clustering could be identified. The general picture is that segregation into clusters is more pronounced in the dendrogram calculated from data obtained from CYA than from YES. This observation is probably due to a higher production of alkaloids from cultivation of the penicillia on CYA (Filtenborg *et al.*, 1983) as the ESMS method has a high sensitivity to alkaloids. The reversed situation was the case for the HPLC analysis, where production of metabolites in general was better on the rich YES substrate, evaluated as the number of peaks in the chromatograms.

Of the more than 50 taxa accepted within the terverticillate penicillia included in this study, the following 36 taxa were considered as grouped by cluster analysis using data from CYA: *P. aethiopicum, P. albocoremium, P. allii, P. atramentosum, P. aurantiogriseum, P. brevicompactum, P. carneum, P. confertum, P. coprobium, P. coprophilum, P. crustosum, P. digitatum, P. dipodomyicola, P. dipodomyis, P. discolor, P. echinulatum, P. expansum, P. freii, P. griseofulvum, P. hirsutum, P. hordei, P. italicum, P. lanosum, P. melanoconidium, P. neoechinulatum, P. olsonii, P. oxalicum, P. palitans, P. paneum, P. polonicum, P. roqueforti, P. scabrosum, P. sclerotigenum, P. tricolor, P. venetum* and *P. vulpinum*. Isolates of the taxa *P. flavigenum* and *P. glandicola* were found in a common cluster as were the isolates of *P. aurantiovirens* and *P. cyclopium*. The isolates from taxa not yet described, *P. sp. 7, P. sp. 8, P. sp. 10* and *P. sp. 22*, were all grouped as separate clusters.

Taxa of which only a single isolate were available for the study was placed rather arbitrarily in the dendrograms. Based on an evaluation of the distinct mass profiles from the single isolates they are expected to form clear clusters if more isolates were available. Very weak isolates, with a very limited production of metabolites as determined by direct ESMS, will be dominated by solvent, matrix and noise ions and were placed randomly in the dendrograms. Typically weak taxa were *P. camemberti, P. commune, P. nalgiovense* and *P. verrucosum* as well as some old isolates.

The normalized data (i.e. the standard spectra calculated as permille) will in some cases be dominated by a few intense ions in mass profiles with a peak hight that is between two and three orders of magnitude higher than any other mass peak in the spectra. The clusters will thus be separated by these few intense ions within a relative high distance coefficient. Some finer details are, however, lost when compared with the dendrograms calculated using logarithmized data. This is most clearly seen within taxa dominated by a few metabolites such as meleagrin and terrestric acid, both metabolites that will produce very intense ions. Taxa producing metabolites (ions) not seen in other taxa are found in the most distinct clusters using the normalized data.

A part of the dendrogram calculated from the normalized data from CYA is shown in Fig. 3 where *P. olsonii* and *P. hirsutum*, taxa that produce intense ions not seen from any other taxa, form clear separate clusters, together with a larger cluster. This cluster is

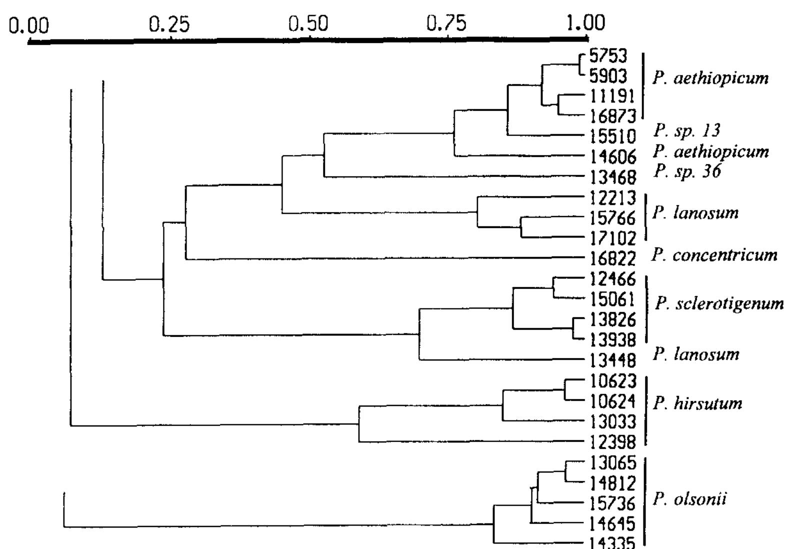

FIG. 3. HIGH SEGREGATION OF TAXA IN SEPARATE CLUSTERS IS FOUND IF THE MASS PROFILES ARE DOMINATED BY ION NOT SEEN IN OTHER TAXA; ILLUSTRATED BY SEGREGATION OF *P. OLSONII* AND *P. HIRSUTUM*. Part of the full dendrogram calculated from normalized data from the CYA mass profiles using the correlation coefficient and UPGMA linkage.

grouping some of the griseofulvin producing taxa. *Penicillium sp 13* and *P. sp 36* are single isolates which do not fit in any described taxa. Most of the taxa that produce terrestic acid when cultivated on YES are grouped in one cluster with very little substructure in the dendrogram calculated from normalized data (Fig. 4). Isolates *P. sp 2* and *P. sp 3* are similar to *P. albocoremium*, but do not fit within the description of that taxon. Using logarithmized data from YES as shown in Fig. 5, when compared to Fig. 4, does reveal some of the differences in metabolite production, with *P. hordei* (except one isolate), *P. crustosum* and *P. venetum* as separate clusters.

Figure 6 shows part of the dendrogram calculated from the logarithmized data from CYA which includes several taxa from the *Penicillium aurantiogriseum* complex (Lund and Frisvad, 1994). The first cluster includes *P. solitum* and a single isolate *P. sp* with a weak profile that does not fit with a described taxon. *Penicillium aurantiovirens* and *P. cyclopium* are grouped in one cluster, but can be distinguished from each other by the presence of xanthomegnin and viomellein (e.g. by TLC analysis), which are only produced by *P. cyclopium* (Lund and Frisvad, 1994). Both these metabolites give a weak response under the ESMS conditions used. The production of xanthomegnin and viomellein was confirmed by HPLC analysis. One isolate, IBT 16692, identified as *P. freii*, is as close to *P. neoechinulatum* as to the other *P. freii* isolates, but these two taxa can easily be distinguished morphologically by the rough-wall conidia of *P. neoechinulatum* whereas *P. freii* has smooth-wall conidia (see also Figs 7 and 8). One isolate (IBT 12879) is missing from the *P. echinulatum* cluster probably due to an intense 211 amu ion originating from a cross contamination (terrestric acid) during analysis. This isolate is placed correctly in the *P. echinulatum* cluster (Fig. 7) calculated from logarithmized data from YES. All isolates of *P. freii* are grouped in one cluster in the dendrogram shown on Fig. 7. A combined cluster of *P. aurantiovirens* and *P. cyclopium* is also found in the dendrogram from logarithmized YES data similar to the one in the CYA

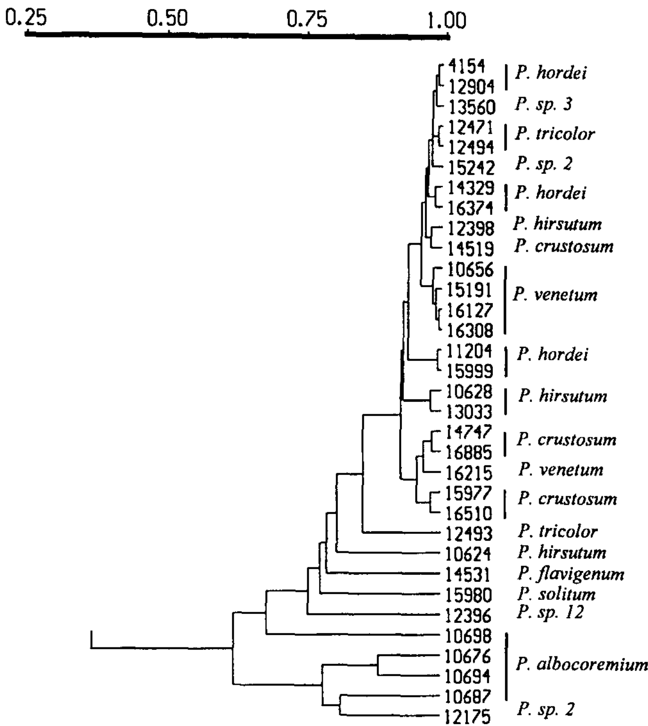

FIG. 4. THE INFLUENCE OF A STRONG ION ON THE DENDROGRAM IS SEEN BY THE GROUPING OF SEVERAL TERRESTRIC ACID PRODUCING TAXA WITH A LITTLE SUBSTRUCTURE USING NORMALIZED DATA DIRECTLY. Part of the full dendrogram calculated from normalized data from YES mass profiles using correlation coefficient and UPGMA linkage.

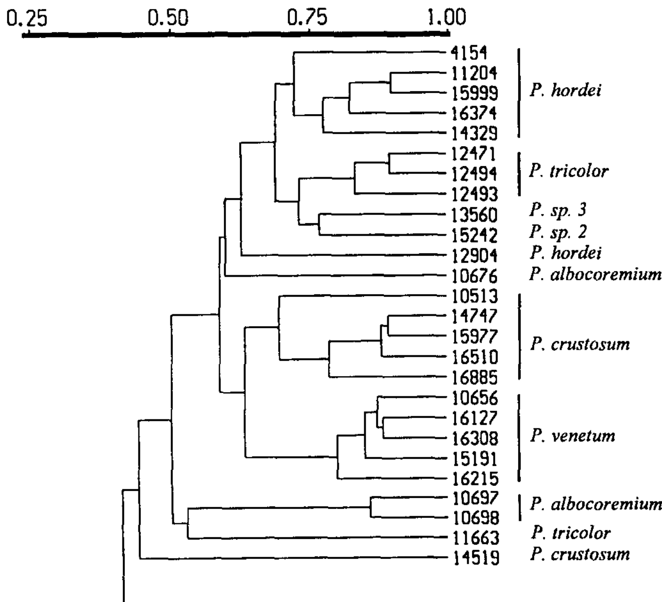

FIG. 5. LOGARITHMIZED DATA REVEAL MINOR DIFFERENCES BETWEEN TAXA DOMINATED BY STRONG IONS AS SEEN BY SEPARATION OF SOME TAXA THAT COULD NOT BE SEPARATED USING NORMALIZED DATA (COMPARE TO FIG. 4). Part of the full dendrogram calculated from logarithmized data from YES mass profiles using correlation coefficient and UPGMA linkage.

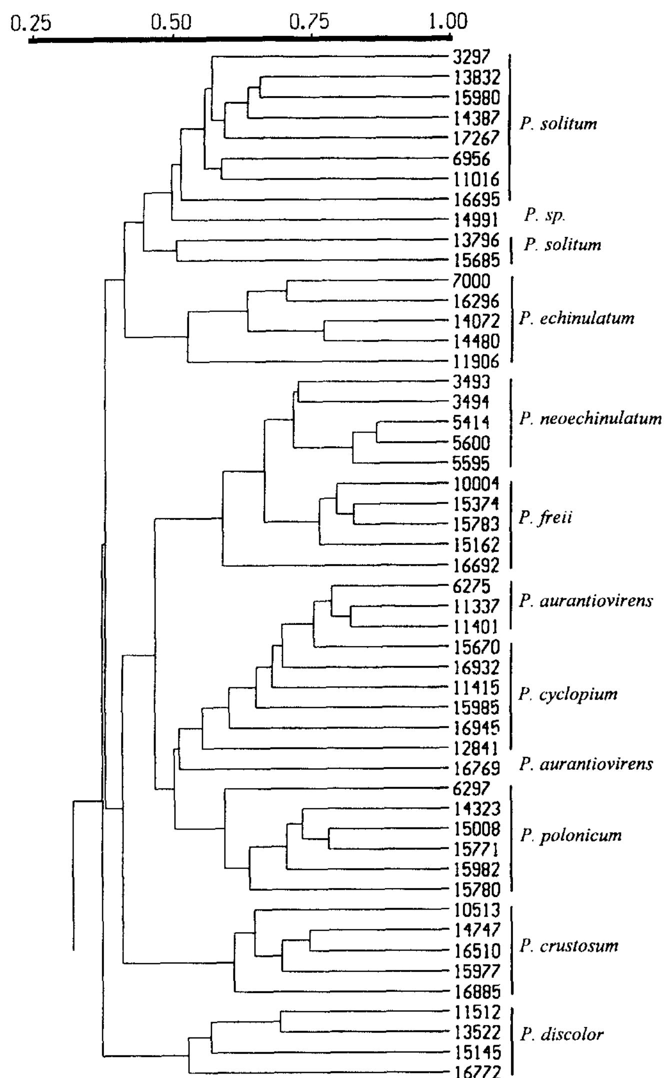

FIG. 6. SEVERAL OF THE CLOSELY RELATED TAXA IN THE *P. AURANTIOGRISEUM* COMPLEX ARE SEPARATED. This is part of the full dendrogram calculated from logarithmized data from CYA mass profiles using correlation coefficient and UPGMA linkage.

dendrogram shown in Fig. 6. One isolate of *P. cyclopium* falls in the related *P. polonicum* cluster probably due to large ions from the 3-methoxy-viridicatin group of metabolites (Lund and Frisvad, 1994).

Part of the dendrogram calculated from normalized data from CYA is shown in Fig. 8. *Penicillium neoechinulatum* and *P. polonicum* are grouped into clearly separate clusters, whereas *P. cyclopium* and *P. aurantiovirens* are grouped in one cluster. Compared to the dendrogram in Fig. 6, calculated from logarithmized data, the distance seen in Fig. 8 between *P. neoechinulatum* and the other clusters is larger. *Penicillium neoechinulatum* therefore can be well separated from all other taxa, including *P. freii.*

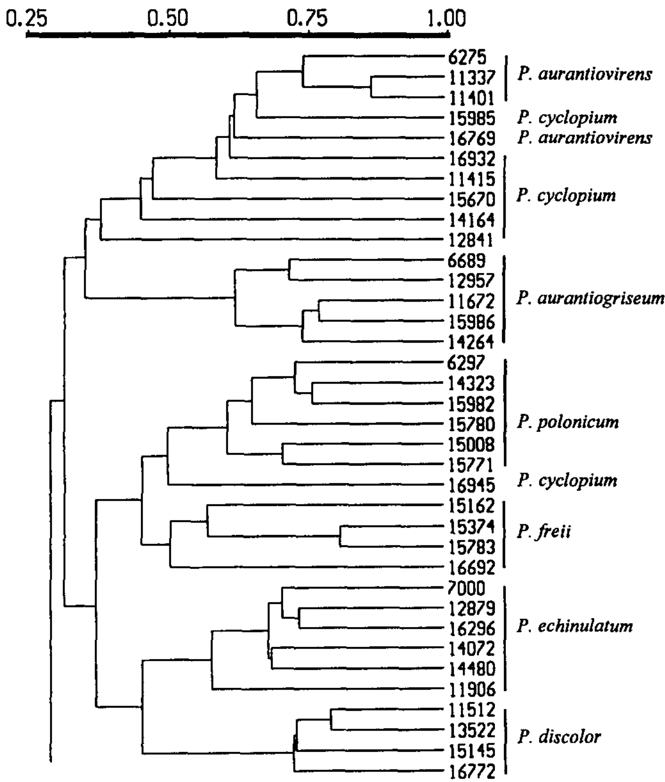

FIG. 7. SEPARATION SIMILAR TO THAT SHOWN IN FIG. 6 CAN BE FOUND IN PART OF THE FULL DENDROGRAM CALCULATED FROM LOGARITHMIZED DATA FROM YES MASS PROFILES USING CORRELATION COEFFICIENT AND UPGMA LINKAGE.

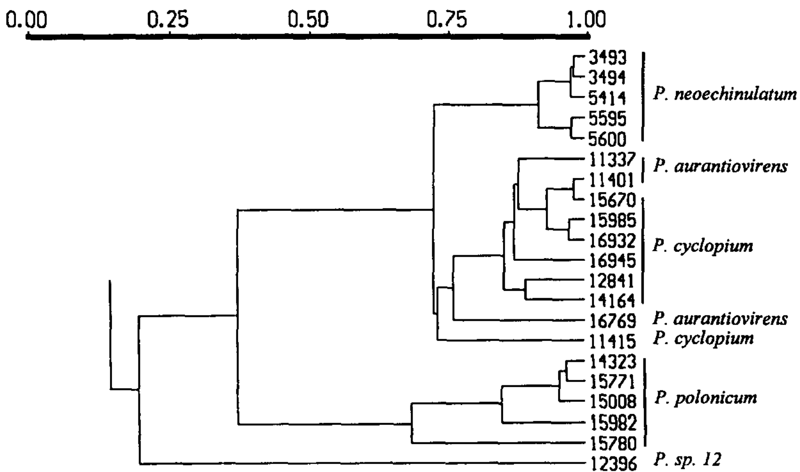

FIG. 8. A LARGER DISTANCE BETWEEN CLUSTERS USING NORMALIZED DATA CAN BE SEEN FOR SOME TAXA INCLUDED IN FIG. 6 IN THIS PART OF THE FULL DENDROGRAM CALCULATED FROM NORMALIZED DATA FROM CYA MASS PROFILES USING CORRELATION COEFFICIENT AND UPGMA LINKAGE.

The binary data matrices constructed with a threshold of 10% resulted in dendrograms (Yule coefficient and UPGMA clustering) with strong groupings into a few large clusters. One of these clusters was linked by a few ions (i.e. metabolites) corresponding to the roquefortine C—meleagrin—oxaline family, all metabolites that give strong responses in ESMS. Almost all griseofulvin producing taxa were grouped in another cluster. *Penicillium olsonii* formed a very distinct cluster probably due to the occurrence of the basepeak at 285 amu, an ion not seen in the mass profiles from any other isolate and a dominant 507 ion, both of unknown structure. Several other taxa are also separated in clusters using the binarized data, particularly the taxa dominated by several intense ions. The overall picture is, however, that the threshold level at 100 (10%) is very restrictive and will eliminate usable information. The 10% level will leave only one informative variable for some good meleagrin producing taxa (the 434 amu variable as a 1 and the other 500 variables as zeros) although ions corresponding to other metabolites can be found. It is difficult to select a suitable level for the very large group of isolates and taxa studied. Including all data as a quantitative variable are, however, preferable. Another approach is to select a limited number of specific ions that allow segregation into taxa when determined in a binary fashion as demonstrated by Smedsgaard and Frisvad (1996).

General comments

Systematic classification of a group of organisms can be based on any set of characters, such as traditional morphological, secondary metabolite or molecular data. The problem is whether the characters have taxonomic meaning or not, thus turning the classification into a general taxonomy with biological significance. Production of secondary metabolites occupies a large part of the metabolism and genome in the fungi. They can thus be regarded as a significant expression of the *Penicillium* species and should therefore be included in taxonomic keys.

This chemosystematic study has shown that around 75% of the taxa known to exist in the terverticillate penicillia can be classified into taxa by ESMS analyses of secondary metabolites and relative simple data analysis. Combining the use of mass profile data with other characters, such as simple morphological or physiological characters or the production of volatile metabolites (Larsen and Frisvad, 1995b) will present a clear taxonomic classification of the terverticillate penicillia.

Few other studies within the genus *Penicillium* have attempted to include as many isolates and taxa in one data matrix as in this study. Extensive work at International Mycological Institute, which included 348 isolates representing 37 taxa and using 100 characters (Bridge *et al.*, 1989b), revealed a comparable segregation as obtained by this study using one type of character. A study by Svendsen and Frisvad (1994), including 279 isolates, gave also a similar segregation based on much more laborious HPLC analysis and data evaluations, but their study included a separation of *P. aurantiovirens* and *P. cyclopium*.

The advantage of using direct electrospray mass spectrometric analysis of crude plug extracts is the speed and easy handling of the analysis from the same cultures inoculated for more traditional studies. Compared to nonspecific chemical methods like pyrolysis, the mass profiles can be correlated to the production of specific metabolites and used as a basis for further studies.

The data processing approach used in this study (using the cluster analysis), has shown that direct ESMS analysis can produce chemotaxonomically significant mass profiles. There is, however, little doubt that simple cluster analysis using the correlation coefficient (developed in the 1930s according to Sneath and Sokal (1973), p. 137) used in this study cannot reveal all the information contained in the data. Other chemometric methods such as principal component analysis could be used, but the programs commercially available are not suited for processing such large data matrices as those generated in this study. An improved method should also include a method to identify variables that contribute to clustering and to discharge noise. Knowledge about structure of MS data like isotopic patterns could be important a priori information to include in a future method.

The use of complex and expensive equipment like a mass spectrometer will normally demand an experienced operator. The stability of the method is high and several maintenance operations were carried out during the 5-month period in which this study was carried out. The instrument used in this study is a first generation electrospray instrument with lower sensitivity, laborious operation and higher overall cost than the new generation of bench-top instruments. Instrumental developments have already resulted in small relative cheap benchtop mass spectrometers with a variety of ionization methods and very simple operation.

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