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Multivariate analysis of fatty acids in spores of higher basidiomycetes: a new method for chemotaxonomical classification of fungi☆

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Abstract

The aim of the present work was to study the possibility of using the fatty acid content in the basidiospores as a taxonomic tool. Basidiospores of *Armillaria borealis*, *Amanita muscaria*, *Agaricus sylvicola*, *Hypholoma capnoides*, *Cortinarius nemorensis* and *Russula delica* were used. The content of fatty acids as well as other substances may vary to a certain degree depending on the part (pileus, stipe, lamella) or stage of development of the actual basidiocarp analysed. Moreover, substances from fungivorous invertebrates, parasitic fungi or bacteria may be found in the chemical analyses of the basidiocarps. Chemotaxonomic conclusions may, therefore, be burdened with serious uncertainties. On the other hand, the ripe basidiospores are terminated structures and belong to the most homogenous structures encountered from a basidiocarp. Their shape, size, colour and ornamentation are considerably homogenous within an actual species. Therefore, the basidiospores are often used as a reliable differentiating characteristic separating species as well as taxa of higher categories. From a practical point of view, ripe spores are easy to obtain in relatively large quantities with simple techniques, and they are not so prone to decay as the carpophore tissue. In the present study, gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS), after methanolysis of the fungus spores, were used to map essential fatty acids in basidiomycetes. Gas chromatography and gas chromatography–mass spectrometry revealed the presence of fatty acids of C12:0–C24:0 size in the basidiospores of these higher basidiomycetes. The major fatty acid in *H. capnoides* is C18:2, and the major fatty acid in the other species is C18:1. The basidiospores proved to be a good source of fatty acids for chemotaxonomic investigations of agarics.
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1. Introduction

The content of fatty acids has been a useful tool in taxonomy and chemotaxonomy for single-celled organisms such as bacteria and yeasts [1]. Fatty acids were analysed by our research group since 1980 for chemotaxonomy of bacteria, yeasts, spirochetes, and moulds belonging to Ascomycota [2]. The analysis of fatty acids in moulds has been performed using asexual conidiospores [2]. Among the Basidiomycota, the agarics (the orders Agaricales, Boletales and Russulales) constitute one of the most conspicuous part of the fungal kingdom. They embrace a vast number of species and are the most visible element of the fungal biodiversity.

Several groups of these species or the genera of agarics have been characterised by their pigment composition, and even evolutionary tales have been outlined wholly or partly based on pigments, e.g. for Boletales [3]. On the other hand, chemotaxonomic works based on chemical compounds other than the pigmented ones are more scanty. Among these, the fatty acids constitute important parts of the chemical composition in the tissue of basidiocarps. In the agarics, several fatty acids have previously been recorded from the basidiocarp tissue [4–7].

However, we have not seen any analysis from the basidiospores although they may serve as a better source of fatty acids than the basidiocarp tissue. The content of fatty acids, as well as other substances, may vary to a certain degree depending on the part (pileus, stipe, lamella) or stage of development of the actual basidiocarp analysed. Moreover,

☆ To Larysa Karaliova. This article marks 25 years in the development of chemotaxonomy.

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substances from fungivorous invertebrates, parasitic fungi or bacteria may be found in the chemical analyses of the basidiocarps.

Chemotaxonomic conclusions may, therefore, be burdened with serious uncertainties. On the other hand, the ripe basidiospores are the terminated structures and belong to the most homogenous characteristics encountered in a basidiocarp. Their shape, size, colour and ornamentation are considerably homogenous within an actual species.

Therefore, the characteristics of basidiospores are often used as a reliable differentiating tool separating basidiomycete species as well as taxa of higher categories. From a practical point of view, ripe spores are easy to obtain in relatively large quantities with simple techniques, and they are not so prone to decay as the carpophore tissue. Chemotaxonomy based on fatty acids has recently attracted much interest as a more precise method for distinction between, for instance, closely related bacteria [1].

As chemotaxonomy has developed, it has become possible to perform analyses with a small amount of material and also in conidiospores [2]. The fatty acids may prove to be good chemotaxonomic markers for agarics, especially if we want to reveal the relationships between the different species, genera, families, or even orders.

In the present study, gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS), after methanolysis of the fungus spores, were used to map essential fatty acids in basidiomycetes.

2. Materials and methods

2.1. Spores

Basidiocarps of the following species were selected in September 1997 on the peninsula of Bygdøy in Oslo, SE Norway: *Armillaria borealis* Marxmuller and K. Korhonen (Tricholomataceae, Agaricales), *Amanita muscaria* (L.: Fr.) Hook. (Amanitaceae, Agaricales), *Agaricus sylvicola* (Vitt.) Peck (Agaricaceae, Agaricales), *Hypholoma capnoides* (Fr.) Kumm. (Strophariaceae, Agaricales), *Cortinarius nemorensis* (Fr.) Lange (Cortinariaceae, Agaricales), and *Russula delica* Fr.(Russulaceae, Russulales).

The pileus of the basidiocarps were put on white writing paper and covered by plastic baskets to prevent desiccation. Spores were allowed to precipitate for 3 days in shadow at an outdoor temperature about 10 °C. Then the caps were removed, and the spore prints allowed to dry for 12 h at 20 °C. The spore powder was collected with a teaspoon of stainless steel in glass tubes prior to analysis and stored at −20 °C.

2.2. Methanolysis

The spores (10 mg) were methanolysed in triplicate at 95 °C in 3 ml of 3 M HCl in anhydrous methanol (Fluka) for at least 24 h. The reaction mixture was cooled on ice diluted with 5 ml distilled water and extracted twice with 3 ml hexane spectroscopic grade Uvasol (Merck, Darmstadt, Germany). The hexane extract was dried with anhydrous $MgSO_4$ (Merck) and evaporated on ice under N_2 to a volume of 50 μl. The three parallel analyses were made from each sample.

2.3. Reference compounds

In this study, reference fatty acid methyl esters were C9:0–C20:0, purchased from Sigma (St. Louis, MO, USA). Bacterial fatty methyl ester mixture, CP (catalogue no. 4-7080), gas chromatography standard mixture, GLC 70 (catalogue no. 4-7044), American Oil Chemists' Society oil reference mixture, RM-1 rapeseed (catalogue no. 4-7019), National Institute of Health's reference mixtures (catalogue nos. A-NHI-C 4-7010, A-NHI-D 4-7011 and A-NHI-F 4-7013) were obtained from Supelco (Bellefonte, PA, USA). 13-Methylmyristic acid methyl ester, 12-methylmyristic acid methyl ester, methyl 15-methylhexadecanoate mixture, FO 1,3-hydroxyhexadecanoic acid, 3-hydroxydecanoic acid, 3-hydroxytridecanoic acid, 3-hydroxytetradecanoic acid methyl ester, 3-hydroxytetradecanoic acid, 3-hydroxypentadecanoic acid, and 3-hydroxyhexadecanoic acid were purchased from Larodan Fine Chemicals (Malmø, Sweden). No. 1200—a calibration standard—was obtained from Microbial ID (Newark, DE, USA). Free fatty acids were methylated before assessment of fatty acid recovery.

3. Gas chromatography

Gas chromatography was performed by a model 8600 gas chromatograph (Perkin-Elmer (PE), Norwalk, CT, USA). The column used was an AT-225 (Alltech Associates, Inc., Deerfield, IL), 10 m length and 0.53 mm i.d. Helium was used as a carrier gas at 5 ml/min. A flame ionisation detector (FID) which operated at 320 °C was used for the detection of fatty acids. The injector was operated at 230 °C. The sample volume was 2 μl. The temperature was 200 °C. Integration was performed by means of the PE software TorboChrom (Perkin-Elmer (PE), Norwalk, CT, USA). The attenuation was set to 4. The three parallel analyses were made from each methanolysed sample.

4. Gas chromatography–mass spectrometry

In the GC–MS interface, the temperature was 285 °C and helium was used as the carrier gas. The flow rate was around of 1 ml/min. The capillary column was introduced directly into the ion source of the mass spectrometer. The MS instrument was operated in the electron impact (EI) mode at 70 eV, with an ion source temperature of 170 °C. The pressure in

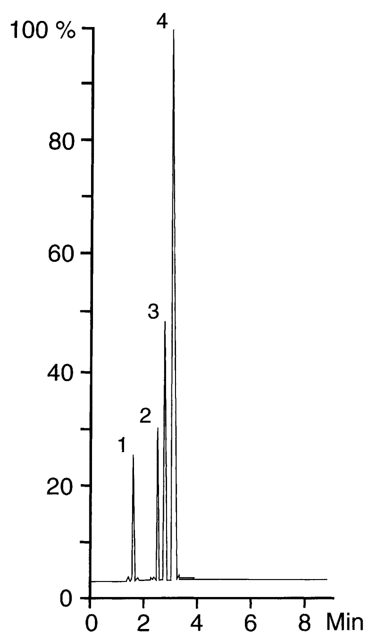

Fig. 1. A characteristic chromatogram for *Hypholoma capnoides* spores: 1, C16:0 (hexadecanoic acid); 2, C18:0 (octadecanoic acid); 3, C18:1 (9-*cis*-octadecenoic acid); 4, C18:2 (9,11-*cis*-octadecenic acid).

the source was 6.6×10^{-4} Pa. Chromatographic separation was achieved by temperature programming: 80 °C for 3 min, then 5 °C/min until 235 °C and for 1 min at 235 °C. Chromatography was performed on the AT-225 capillary column.

The injector was a split/splitless injector operated in the split mode. GC–MS data were collected by using a Carlo Erba high-resolution gas chromatograph (HRGC) (Carlo Erba, Milan, Italy) attached to a double-focussing mass spectrometer, EB geometry, JEOL DX303 (JEOL, Tokyo, Japan). The total ion chromatograms (TIC) and the mass spectra were recorded using a Dell personal computer. Interface and software were part of the Shrader System (Shrader Analytical & Consulting Laboratories Inc., Detroit, MI, USA).

5. Numerical methods

A data set was made with the species as objects and the various fatty acids as variables expressed to show their presence (value 1) or absence (value 0). It was analysed by means of principal component analysis (PCA) [8], run by the program CANOCO, Version 3.15 (Microcomputer Power, Ithaca, USA).

6. Results and discussion

A characteristic chromatogram for *H. capnoides* spores is shown in Fig. 1. The composition of the fatty acids in the spores is shown in Table 1. All species investigated contained 16.5–82.0% 9-*cis*-octadecenoic acid, 7.6–17.5% hexadecanoic acid (C16:0), and 2.1–8.7% octadecanoic acid (C18:0). *H. capnoides*, *C. nemorensis*, *R. delica*, and *A. borealis* also contained 14.8–72.5% 9,11-*cis*-octadecenic acid (C18:2), while *A. sylvicola* and *A. muscaria* only contained 0.4 and 1.4% traces of this acid, respectively. When present, the other fatty acids were only encountered in minor amounts (Table 1). *R. delica* had the highest number of fatty acids and had 10 different types. *A. borealis* and *A. muscaria* had seven different types, *Agaricus silvicola* had six, and *H. capnoides* and *C. nemorensis* had five different types. The PCA analysis based on the presence or absence of fatty acids gave the following eigenvalues for the four first axes: PC1 = 0.54, PC2 = 0.27, PC3 = 0.12, PC4 = 0.06. The first axis explains more than half of the variation, the second more than a quarter, while the remaining axes do not contribute much.

An ordination diagram for PC1 and PC2 for the species is shown in Fig. 2. The main separation is between *R. delica* with a high positive score on PC1 and the rest of the species with negative or low positive scores on PC1. However, these species exhibit a good separation with PC2. *A. muscaria* and *A. sylvicola* have both negative scores on PC2 and form a loose couple.

Table 1
Content (%) of fatty acids

	H. capnoides	*C. nemorensis*	*R. delica*	*A. borealis*	*A. sylvicola*	*A. muscaria*
C12:0	–	–	0.2	1.5	–	–
C14:0	–	–	0.2	2.7	–	–
C15:0	–	–	0.3	–	0.8	0.6
C16:0	8.9	15.0	8.1	13.3	17.5	7.6
C17:0	–	–	–	–	1.1	–
C18:0	2.1	3.0	2.7	3.8	3.8	8.7
C18:1	16.5	55.4	71.6	43.7	75.4	82.0
C18:2	72.5	26.6	14.8	27.4	1.4	0.4
C20:0	–	–	0.5	–	–	0.5
C22:0	–	–	0.7	–	–	–
C24:0	–	–	0.8	7.6	–	–

$n = 9$, R.S.D. = 3%. C12:0, dodecanoic acid; C14:0, tetradecanoic acid; C15:0, pentadecanoic acid; C16:0, hexadecanoic acid; C17:0, heptadecanoic acid; C18:0, octadecanoic acid; C18:1, 9-*cis*-octadecenoic acid; C18:2, 9,11-*cis*-octadecenic acid; C20:0, eicosanoic acid; C22:0, doeicosanoic acid; C24:0, tetraeicosanoic acid.

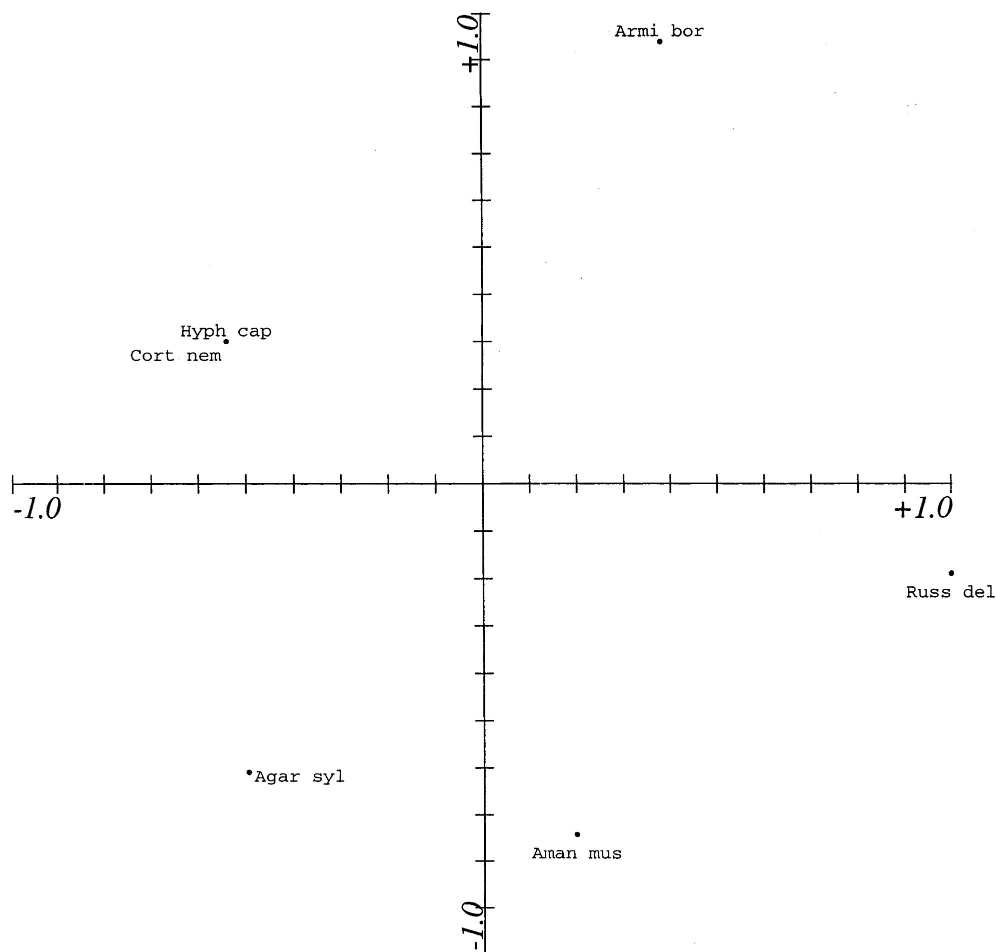

Fig. 2. Principal component analysis (PCA) showing the position of the various species on the first two ordination axes, PC1 as abscissa and PC2 as ordinate. The species are *Hypholoma capnoides*, *Cortinarius nemorensis*, *Agaricus sylvicola*, *Amanita muscaria*, *Russula delica*, and *Armillaria borealis*.

H. capnoides and *C. nemorensis* are grouped together with a low positive score on PC2. *A. borealis* has a high positive score on PC2.

Judged from the fact that it contained the highest number of fatty acids and from the position given in the PCA analysis (Fig. 2), *R. delica* deviates most from the other species investigated. This is in accordance with recent phylogenetic studies using nuclear ribosomal DNA or mitochondrial DNA [9,10]. Here, *Russula* and *Lactarius* are placed in a separate cluster (clade) together with many non-gilled fungi as, e.g. the polypores *Bondarzewia* and *Heterobasidion* and the corticoid fungi *Stereum* and *Gloeocystidiellum*. The rest of the species we investigated form a loose group, which can be placed among the so-called euagarics, comprising manly gilled fungi, with the exception of *Russulaces* and Boletales [10]. It is interesting that *C. nemorensis* in Cortinariaceae and *H. capnoides* in Strophariaceae contain exactly the same kinds of fatty acids. These two families are often regarded as related because of similarities in morphology and pigment chemistry [11].

7. Conclusion

The content of fatty acids as well as other substances may vary to a certain degree depending on the part (pileus, stipe, lamella) or stage of development of the actual basidiocarp analysed. Moreover, substances from fungivorous invertebrates, parasitic fungi or bacteria may be found in the chemical analyses of the basidiocarps. Basidiospores are terminated structures and belong to the most homogenous structures encountered from a basidiocarp. Basidiospores are not so prone to decay as the basidiocarp tissue. In corresponding analyses of ascomycetous moulds, the conidiospores were chosen for fatty acid analysis in chemotaxonomy [2].

The basidiospores seem to be a useful source of fatty acids for chemotaxonomic investigations of fungi in general and of agarics in particular. The difference between the occurrence of various fatty acids followed, to a certain degree, the evolutionary lines within groups of higher basidiomycetes considering recent phylogenetic analyses. More investigations will show whether the content of fatty acids in the ba-

sidiospores will serve as a stable phylogenetic character in these fungi.

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