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Comparison of fatty acids of marine fungi using multivariate statistical analysis

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SUMMARY

Ten obligate marine fungi have as their principal fatty acids 16:0, 18:0, 18:1*n*9 and 18:2*n*6. The fatty acids ranged from 14 to 22 carbons, completely dominated by those with even numbers of carbons. The amount of unsaturated fatty acids varied between 35% and 80%. Each isolate contained small amounts of the acids 18:3*n*3 and 20:4*n*6. Branched, hydroxy- or cyclic fatty acids were not detected. Multivariate statistical, i.e. principal component analysis, showed that all ten strains could be distinguished on the basis of their fatty acid composition. These results indicate that the marine fungi do not have an unusual fatty acid composition and suggest that chemometric, multivariate analysis might be employed to confirm taxonomic relationships among these organisms.

INTRODUCTION

The obligate marine fungi complete their life cycles only in saline habitats. Marine fungi have been largely overlooked because routine enrichment and isolation procedures tend to select for terrigenous fungi such as *Penicillium* spp., which have been called geofungi [20]. The dormant spores of geofungi can predominate in nearshore waters and may overgrow true marine fungi on laboratory culture media [9,11,15], but geofungi cannot complete their life cycles in marine habitats [6,10,20] and their spores lack appendages which allow them to compete in an aquatic environment [15,16]. In contrast, the true marine fungi predominate on materials in situ within marine habitats [11,15]. Many species produce spores with appendages that facilitate colonization and dispersal in seawater [7,22] and they can both grow and reproduce in seawater [15]. Species prevalent on submerged wood are termed lignicolous, those prevalent on the stems of decaying marine plants caulicolous and those prevalent on beach sand arenicolous [15].

Many arenicolous marine fungi are able to grow using hydrocarbons as their sole source of organic carbon, possibly because they have been exposed for millennia to hydrocarbons in surface slicks and in sea foam. This ability is less prevalent among the lignicolous marine fungi and is rare among caulicolous marine fungi [12,13]. Because little is known of the physiology and biochemistry of marine fungi, because they may be sources of nutrients in marine food webs and because of their potential for participating in the degradation of spilled petroleum, we have begun to examine this interesting group of microorganisms.

This report describes the fatty acid composition of ten strains of marine fungi and presents the results of multivariate statistical analysis applied to the data in order to determine if the fatty acid composition can be useful for taxonomic purposes.

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MATERIALS AND METHODS

Ten marine fungi, eight arenicolous and two lignicolous strains including seven Ascomycetes and three Deuteromycetes and representing seven genera and eight species (Table 1) were maintained on agar slants of an artificial seawater medium [13] with 1% (w/v) glucose as carbon source. The medium also contained (g L^{-1} quartz-distilled H_2O): NaCl 17.66, $MgCl_2 \cdot 6H_2O$ 7.97, Na_2SO_4 2.94, NH_4NO_3 0.37, $NH_4H_2PO_4$ 0.07, NaBr 0.06, $SrCl_2 \cdot 6H_2O$ 0.03, H_3BO_3 0.02, $CaCl_2$ 1.10, KCl 0.56, (tris) hydroxymethylaminomethane 1.20; (mg L^{-1}): $FeCl_3$ 2.90, $ZnCl_2$ 0.63, $MnCl_2 \cdot 4H_2O$ 1.80, $CoCl_2 \cdot 6H_2O$ 0.04, $CuCl_2 \cdot 2H_2O$ 0.27, $(NH4)_6MoO_{24} \cdot 4H_2O$ 0.04, NaF 2.0; (μg L^{-1}): thiamine HCl 100, biotin 10, and pyridoxine 50. The vitamins were filter-sterilized and added aseptically to the minerals solution which had been autoclaved and adjusted to pH 8.1. The medium was dispensed in 100-ml quantities to 250-ml Erlenmeyer flasks.

TABLE 1

Organisms

Species and strain	Class	Ecological type	Region of isolation
Arenariomyces parvulus, Koch J34	Ascomycetes	Arenicolous	Tropical
Corrolospora lacera, (Linder) Kohlmeyer K50	Ascomycetes	Arenicolous	Virginia
Corrolospora maritima, Werdermann K3	Ascomycetes	Arenicolous	Virginia
Corrolospora maritima, Werdermann K14	Ascomycetes	Arenicolous	Virginia
Corrolospora maritima, Werdermann K30	Ascomycetes	Arenicolous	Virginia
Dendryphiella salina, (Sutherland) Pugh & Nicot F200	Deuteromycetes	Arenicolous	Virginia
Lulworthia sp. J46	Ascomycetes	Lignicolous	Tropical
Sigmoidea marina, Haythorne & Jones J33	Deuteromycetes	Arenicolous	Tropical
Varicosporina ramulosa, Meyers & Kohlmeyer K92	Deuteromycetes	Arenicolous	Virginia
Verruculina enalia, (Kohlmeyer) Kohlmeyer & Volkmann-Kohlmeyer K33	Ascomycetes	Lignicolous (intertidal)	Virginia

[1] Cultures are on deposit in the Department of Biology, Old Dominion University, Norfolk, VA. They are also maintained in the Environmental Sciences Program at the University of Massachusetts, Boston, MA.

To prepare a homogeneous inoculum, mycelium was teased from the surface of a slant and suspended in 5–10 ml of sterile artificial sea water. This suspension was homogenized aseptically in a Potter-Elvehjem glass homogenizer equipped with a Teflon pestle; 15 μl of the resulting cell suspension was used as inoculum for flask cultures which contained artificial seawater medium with glucose but without agar. Flasks were incubated without shaking at 20 ± 2 °C. When a confluent mat had formed or when large discrete colonies were present suspended in the medium, cells were harvested by filtering the culture through Whatman No. 1 paper on a Buchner funnel. Cells were washed three times on the filter with distilled water, frozen and stored at −70 °C until they were analyzed.

Cells were allowed to warm to room temperature and approximately 10 mg of material was transferred to a thick-walled glass tube, 0.5 ml anhydrous 2 N HCl in methanol was added and the tube was securely closed with a Teflon-lined screw cap. The solvent contained 26.5 μg of the fatty acid 21:0 as an internal standard which was used in quantifying total fatty acids. Methanolysis of the material was achieved by heating the tube to 100 °C for 15 h. The tube was allowed to cool and the methanol/HCl was evaporated in a stream of nitrogen gas to half the volume and 0.25 ml of water was added. The water/methanol phase was extracted twice with 0.5 ml hexane and 1 μl of the combined extracts was gas chromatographed on a 30 m × 0.32 mm fused silica column from J & W Scientific, Folsom, CA, with a 0.25-μm thick stationary phase of 50% cyanopropylmethyl and 50% methyl-phenyl polysiloxane, and with helium as the mobile phase. The components eluting from the column were detected by a flame ionization detector, and the detector output was coupled to a VG Multichrome lab data system (Fisons Instruments, Altrincham, Cheshire, England) for storage and treatment of the chromatograms. Fatty acid methyl esters were identified by comparing their retention time with the retention times of authentic compounds.

The four major and ten minor fatty acids were selected for multivariate, principal component computations. The areas of their methyl ester peaks were fed into the computer using the program SIRIUS [17]. The areas were first normalized by expressing each of them as a percentage of the total peak area of all methyl esters in the sample. This eliminates differences among the fungi in the total amount of fatty acids. They were then logarithmically transformed to give the minor fatty acids relatively greater importance. The computer positions the samples in a coordinate system with one coordinate for each of the 14 fatty acids. New coordinates are computed in the direction of the largest and second largest variance among the samples. The larger part of the systematic variance between the samples is described by these new coordinates, or principal components. In this manner the relationship between the samples, and thus the major features of the data set, can be displayed in two dimensions, i.e. a PC_1–PC_2 plot, without considerable loss of the total original variance.

The distances among the samples in the PC_1–PC_2 plot reflect the differences among the samples. Samples which are positioned farthest away from the origin in the plot have had the largest influence on the direction of the principal components. Since the PC_1–PC_2 plot is a projection of the samples on the plane described by the first two principal components, smaller differences between samples may be in other directions and therefore are not seen in the plot. To make such differences visible, a new principal component computation is carried out on samples which are located close together in the first plot, thereby excluding the influence of more remote samples. Such narrowing down into smaller groups is done stepwise. Principal component analysis has been described by Wold et al. [25].

TABLE 2a

Relative amounts of fatty acids, as percentage of sum ± SD, and total amounts of the fatty acids in μg mg^{-1} dry tissue weight, in marine fungi. All cells were harvested in trophophase (stationary phase). The cases with two columns are from two different growth experiments, and the second column represents results from slightly older cultures

Fatty acids	*A. parvulus*	*C. lacera*	*C. maritima* (K3)	*C. maritima* (K14)		*C. maritima* (K30)
	$n = 4$	$n = 3$	$n = 4$	$n = 4$	$n = 5$	$n = 4$
14:0	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.03	0.6 ± 0.05	0.3 ± 0.2	0.3 ± 0.03
16:0	17.0 ± 0.7	16.3 ± 0.3	16.6 ± 0.5	21.6 ± 0.7	16.9 ± 0.4	15.5 ± 1.0
16:1*n*7	0.6 ± 0.02	0.8 ± 0.01	0.9 ± 0.2	1.4 ± 0.2	0.8 ± 0.1	0.6 ± 0.03
17:0	0.6 ± 0.04	0.5 ± 0.01	0.5 ± 0.04	0.1 ± 0.01	0.5 ± 0.04	0.7 ± 0.06
18:0	4.9 ± 0.3	4.2 ± 0.02	4.4 ± 0.4	3.0 ± 0.4	4.1 ± 0.7	4.0 ± 0.3
18:1*n*9	20.6 ± 1.8	23.6 ± 0.3	23.6 ± 2.7	39.6 ± 2.1	19.4 ± 1.1	23.7 ± 2.2
18:1*n*7	0.1 ± 0.03	0.1 ± 0.01	0.2 ± 0.06	1.6 ± 0.3	0.6 ± 0.3	0.1 ± 0.01
18:2*n*6	54.0 ± 1.0	52.2 ± 0.6	51.3 ± 2.0	30.1 ± 3.4	55.2 ± 0.4	52.6 ± 0.9
18:3*n*3	1.1 ± 0.2	1.0 ± 0.05	1.1 ± 0.3	< 0.1	1.3 ± 0.4	1.2 ± 0.03
20:0	0.2 ± 0.01	0.1 ± 0.01	0.2 ± 0.03	0.3 ± 0.05	0.1 ± 0.02	0.1 ± 0.02
20:1*n*9	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.9 ± 0.5	0.1 ± 0.01	0.1 ± 0.02
20:4*n*6	0.3 ± 0.07	0.1 ± 0.04	0.3 ± 0.02	0.2 ± 0.04	0.3 ± 0.03	0.5 ± 0.05
22:0	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.01	0.7 ± 0.07	0.1 ± 0.01	0.1 ± 0.02
unid.	0.2 ± 0.09	0.3 ± 0.06	0.3 ± 0.05	0.1 ± 0.01	0.3 ± 0.08	0.4 ± 0.09
Total unsaturates	77	78	78	74	78	79
Total FA	33.6 ± 5.6		27.6 ± 3.3		27.5 ± 2.5	22.0 ± 7.1

Fatty acids	*D. salina*		*Lulworthia* sp.	*S. marina*	*V. ramulosa*	
	$n = 4$	$n = 3$	$n = 3$	$n = 3$	$n = 4$	$n = 4$
14:0	0.2 ± 0.01	0.2 ± 0.01	0.3 ± 0.02	1.5 ± 0.02	0.3 ± 0.03	0.4 ± 0.02
16:0	24.6 ± 0.4	26.6 ± 2.6	15.4 ± 0.5	42.6 ± 0.5	17.6 ± 1.5	19.1 ± 0.9
16:1*n*7	0.8 ± 0.03	0.7 ± 0.06	1.0 ± 0.08	0.6 ± 0.01	0.5 ± 0.1	0.3 ± 0.02
17:0	0.3 ± 0.04	0.2 ± 0.1	0.6 ± 0.05	0.4 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
18:0	12.3 ± 1.5	5.4 ± 0.4	4.2 ± 0.7	17.1 ± 0.5	13.9 ± 0.4	19.5 ± 0.6
18:1*n*9	21.6 ± 0.8	22.3 ± 2.6	24.1 ± 0.9	16.6 ± 1.7	41.0 ± 4.3	30.3 ± 0.4
18:1*n*7	0.9 ± 0.3	1.2 ± 0.04	0.6 ± 0.2	0.1 ± 0.01	0.4 ± 0.1	0.5 ± 0.02
18:2*n*6	36.3 ± 1.4	40.5 ± 2.7	51.3 ± 1.3	14.9 ± 2.7	23.2 ± 3.8	27.0 ± 1.1
18:3*n*3	0.6 ± 0.8	0.5 ± 0.1	1.4 ± 0.06	0.1 ± 0.01	< 0.1	0.1 ± 0.01
20:0	0.6 ± 0.1	0.2 ± 0.01	0.2 ± 0.03	1.4 ± 0.05	1.2 ± 0.2	0.8 ± 0.03
20:1*n*9	0.3 ± 0.4	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.01	0.6 ± 0.08	0.2 ± 0.02
20:4*n*6	0.5 ± 0.03	0.6 ± 0.5	0.2 ± 0.03	0.3 ± 0.03	0.1 ± 0.01	0.1 ± 0.01
22:0	0.7 ± 0.2	0.2 ± 0.06	0.1 ± 0.01	3.2 ± 0.1	1.1 ± 0.08	1.3 ± 0.1
unid.	0.3 ± 0.01	1.4 ± 1.2	0.3 ± 0.05	1.3 ± 0.2	0.2 ± 0.03	0.3 ± 0.04
Total unsaturates	61	66	79	33	66	58
Total FA		86.6	29.5 ± 3.0	29.8 ± 1.6		106.9 ± 8.4

RESULTS AND DISCUSSION

The total content of fatty acids in the fungi varied from about 20 μg mg^{-1} dry tissue weight to about 100 μg mg^{-1}. The fatty acid methyl esters derived from total cellular fatty acids are summarized in Tables 2a and 2b. In each organism the fatty acids detected ranged from 14 to 22 carbons. Palmitic (16:0), oleic (18:1*n*9) and linoleic (18:2*n*6) acids were the principal fatty acids in each organism, comprising 89% or more of the total peak areas for six organisms. The notation used for fatty acids is that recommended by Ackman [1] where 18:2*n*6 is equivalent to 18:2*w*6. The other three

TABLE 2b

Fatty acids	*V. enalia*	
	$n = 4$	$n = 3$
14:0	0.4 ± 0.03	0.3 ± 0.02
16:0	24.1 ± 1.5	23.6 ± 0.8
16:1*n*7	0.6 ± 0.04	0.7 ± 0.03
17:0	0.1 ± 0.01	0.1 ± 0.03
18:0	9.5 ± 0.4	9.7 ± 1.0
18:1*n*9	20.7 ± 1.8	20.0 ± 2.1
18:1*n*7	0.7 ± 0.01	0.8 ± 0.02
18:2*n*6	37.9 ± 2.0	39.0 ± 2.0
18:3*n*3	3.0 ± 0.6	3.4 ± 0.3
20:0	0.5 ± 0.06	0.4 ± 0.02
20:1*n*9	0.3 ± 0.02	0.2 ± 0.04
20:4*n*6	1.1 ± 0.1	0.4 ± 0.06
22:0	0.5 ± 0.1	0.5 ± 0.06
unid.	0.6 ± 0.03	0.7 ± 0.2
Total unsaturates	64	64
Total FA		58.1 ± 4.7

organisms, *S. marina*, *V. ramulosa* and *V. enalia*, had increased contents of stearic acid (18:0). The only odd-carbon fatty acid detected was 17:0 which constituted <1% of the total peak area in each organism. The total unsaturated fatty acids varied considerably, ranging from 33 to 79%. No hydroxy- or cyclopropyl fatty acids were detected.

Polyenoic acids are potentially useful in fungal taxonomy [18,19]. The *n*-3 and *n*-6 polyunsaturated fatty acids are of commercial interest [2,5,8]. Each of these ten fungi contained small amounts of linolenic (18:4*n*3) and arachidonic (20:4*n*6) acids. Eicosapentaenoic acid (20:5*n*3) was not detected.

The total cellular fatty acids of these marine fungi are similar to those of terrigenous filamentous fungi [2,4,5,19, 21,23]. They are also similar to the triglyceride fatty acids of two marine fungi, *Corollospora maritima* and *Zalerion maritimum* [3], which are closely related to some of the organisms examined in the present work. Thus, the fatty acid composition does not provide a clue to the ability to prosper in the marine environment.

The multivariate computations on the pattern of fatty acids showed that four of the species, *D. salina*, *S. marina*, *V. ramulosa* and *V. enalia*, were clearly different from each other and from the other six species (Fig. 1). Among these four species, the distances in the plot showed that *V. enalia* and *D. salina* had more similar fatty acid patterns than the others.

The patterns of the other six organisms were more similar, since they fall together in one group in the PC_1–PC_2 plot. To investigate if there still might be differences among them, they were recomputed without the influence of the first four species (Fig. 2). By this computation *C. maritima* (K14), *C. maritima* (K30) and *Lulworthia* sp. were distinguished from

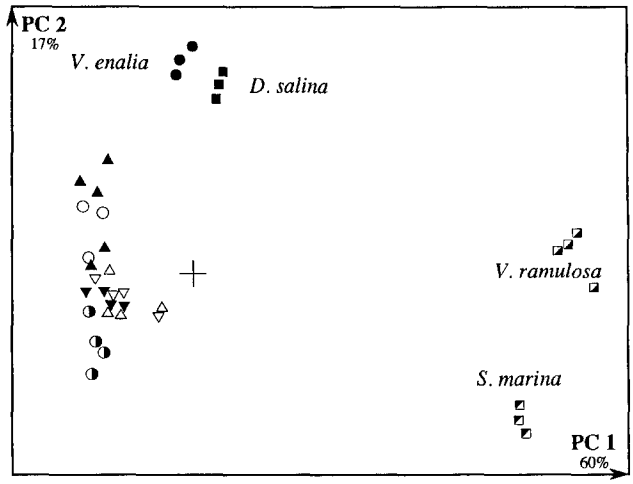

Fig. 1. Plot of all samples of fungi in a coordinate system of the first two principal components, PC_1–PC_2 plot. The origin of the axes is marked with a cross. The different species are marked with the symbols: ◑ – *Arenariomyces parvulus* J34; △ – *Corollospora lacera* K50; ▽ – *Corollospora maritima* K3; ▲ – *Corollospora maritima* K14; ▼ – *Corollospora maritima* K30; ■ – *Dendriphiella salina* F200; ○ – *Lulworthia* sp. J46; ◩ – *Sigmoidea marina* J33; ◪ – *Varicosporina ramulosa* K92; ● – *Verruculina enalia* K33.

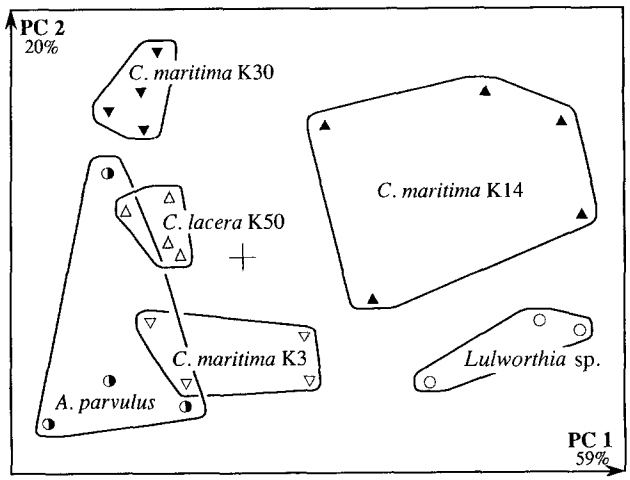

Fig. 2. Recomputed PC_1–PC_2 plot of the six organisms which fell in one group in Fig. 1. Symbols as in Fig. 1.

the remaining three. A new computation performed on these three showed that they were distinct.

These successive principal component analyses show that all ten strains had distinct fatty acid profiles, although with varying degrees of difference. The difference between replicates for each strain indicate analytical variance, but may also suggest that the fungal mat was not homogeneous.

Four of the organisms were cultured a second time to test the consistency of the fatty acid patterns. For *V. enalia*

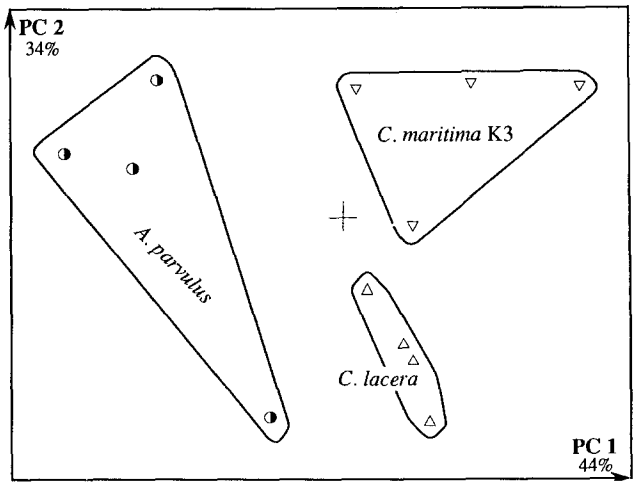

Fig. 3. Recomputed PC_1–PC_2 plot of the three organisms which fell closest together in Fig. 2. Symbols as in Fig. 1.

the pattern was the same in each case, while the other three had somewhat different patterns in the two cases. However, these differences were not large enough to cause overlap between the species, indicating that the fatty acid pattern, even with these variations, can be used to distinguish among marine fungi. The differences were most likely due to differences in harvesting time in relation to the growth cycle. All cultures were harvested in trophophase (stationary phase), which may give more reproducible fatty acid profiles than younger cultures [14], but there were differences in the age of cultures at the time of harvest. Where two data sets are shown for an organism in Tables 2a and 2b, the second set is for slightly older cultures and culture age can affect fatty acid composition [19,21]. In the first experiment cells were harvested after 55–63 days incubation, and in the second experiment after 64–70 days. Even for the organism which showed greatest variation from culture to culture, *C. maritima* K14, the patterns were still distinct.

Our results show that these marine fungi do not have an unusual fatty acid composition; they suggest that the fatty acid pattern is genotypic, and that this type of chemometric, multivariate analysis might be employed for taxonomic purposes. To evaluate the relationship between strains and species, more than five replicates of each strain/species should be analyzed, and the distance between samples and groups of samples should be estimated via further multivariate analysis such as SIMCA (Soft Independent Modelling of Class Analogy) [24].

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