Fatty Acid Composition of Lipids Present in Selected Lichenized Fungi: A Chemotyping Study

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ABSTRACT: The total-lipid composition of 21 lichens of the ascomycetous genera *Cladonia* (11) and *Cladina* (1) of the family Cladoniacea, *Cladia* (1), *Parmotrema* (3), *Ramalina* (2), *Leptogium* (1), *Cetraria* (1), and the basidiomycetous genus *Dictyonema* (1) was determined. Analyses of those of *Dictyonema glabratum* were carried out with a total extract and those obtained after successive extractions with various solvents. Each extract was partitioned between *n*-heptane/isopropanol and 1 M sulfuric acid, giving triglycerides (TG) in the upper phase. Extracts were methanolyzed and the resulting methyl esters were analyzed by gas chromatography–mass spectrometry. Methanolyzates of TG unexpectedly contained esters of 9-oxo-decanoic, 9-methyl-tetradecanoic, 6-methyl-tetradecanoic, 3-hydroxy-decanoic, nonanedioic, and decanedioic acids, as well as common fatty acids. Fatty acid methyl ester profiles from the lichens were submitted to cluster analysis, and the resulting dendogram showed a cluster consistent with *Cladonia* spp., suggesting an efficient aid to lichen taxonomy. The total carbohydrate content of each lipid extract was determined by a modified phenol-sulfuric acid method, which compensated for the presence of pigments.

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Many different types of lipids occur in lichens, including fatty acids, phospholipids, and glycolipids (1–6). A β-galactosyl ceramide and digalactosylglyceride were found in *Ramalina celastri* (5,6), the latter being isolated from a *Sticta* sp. after deacylation (7). Each component was analyzed in terms of carbohydrates and lipids. More recently, a family of galactolipids was obtained from the basidiomycetous lichen *Dictyonema glabratum* and completely characterized (8). The presence of fatty acids in fungi has long been observed (9–11) and summarized in a review by Wassef (12), who described the composition of lipids in many fungal species and their utilization for taxonomic purposes. This review proposed a separation into phycomycetes (>$C_{18:3}$, $C_{20:0}$, and $C_{22:0}$), ascomycetes, and deuteromycetes (fungi imperfecti), which contained little or no polyenoic acids and >$C_{16:1}$, and basidiomycetes with α- rather than γ-linoleic acid: all groups contained $C_{16:0}$, $C_{18:1}$, and $C_{18:2}$ as major fatty acids.

Investigations that involve cladistic analysis of fatty acids as an aid in the taxonomy of fungi gave rise to positive results for correlation of fungal species (13–15). The most important class of fungal lipids is the triglycerides (TG), which represent the major component found in the lipid extracts of fungi. These are mainly esterified by unsaturated fatty acids such as oleic and linoleic at the HO-2 position of the glycerol moiety and at HO-1 and HO-3 by saturated fatty acids. The presence of TG in terms of fatty acid composition in lichens has not been described to date.

There is a complex relationship between lichen phytobionts and mycobionts, but as the latter comprise ~90% of the total biomass (16), the fatty acids of total lipid might be expected to be those of the mycobiont. We now study the composition of the lipid extracts of 21 selected lichens from various genera, having ascomycetes and a basidiomycete as mycobionts, in terms of carbohydrates, fatty acids that arose from TG, and total fatty acid, which could serve as taxonomic aid, including a modification of the phenol-sulfuric acid method (17), modified to compensate for the presence of pigments.

EXPERIMENTAL PROCEDURES

Lichens. Lichens of the genus *Cladonia (C. clathrata*, *C. imperialis, C. signata, C. furcata*) and *Cladia aggregata* were collected during May 1993 in the Serra da Mantiqueira, Itamonte, State of Minas Gerais, Brazil, and *C. connexa, C. crispatula*, *C. ibitipocae*, *C. miniata*, *C. penicillata*, *C. salmonea*, and *C. substellata* in the Serra da Ibitipoca, Lima Duarte, State of Minas Gerais, 1994. *Cladina rangiferina* was collected in Uusimaa, Finland, August 1998. *Dictyonema glabratum* was harvested September 1998 from an embankment close to the 47-km sign of the National Highway (BR) 277, at an altitude of 900 m, in the proximity of Curitiba, State of Paraná, Brazil. *Ramalina usnea, R. celastri,* and *Leptogium phyllocarpum* were collected in 1998, in the Serra da Graciosa, PR, Brazil. *Cetraria islandica* (Iceland moss) was obtained from S.S. Penick and Co. (New York, NY; material obtained in 1984). *Parmotrema delicatum, P. mantiqueirense,* and *P. shindler* were collected in 1998, Lapa, State of Paraná, Brazil. Brazilian lichens were identified by Prof. Marcelo Marcelli and the Finnish one by Dr. Teuvo

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Abbreviations: B, blank; EI, electron ionization; FAME, fatty acid methyl esters; GC–MS, gas chromatography–mass spectroscopy; OD, optical density; S, standard; TFA, trifluoroacetic acid; TG, triglycerides; TLC, thin-layer chromatography.

Ahti. All lichens were each cleaned, dried, and powdered prior to the lipid extraction procedures.

General experimental procedures. Lipid extracts obtained from each sample were obtained by three successive extractions with 10 vol/wt of refluxing $CHCl_3$/MeOH (2:1, vol/vol) and (1:1, vol/vol), for 2 h. *Dictyonema glabratum* (15 g) was similarly extracted in order to compare yields with: Me_2CO, $CHCl_3$/MeOH (2:1, vol/vol), $CHCl_3$/MeOH (1:1, vol/vol), EtOH/H_2O (9:1, vol/vol), and $CHCl_3$/MeOH/H_2O (7:10:3, by vol), except that the Me_2CO extraction was carried out at room temperature. All extracts were evaporated at <40°C under reduced pressure, dried, and stored in sealed tubes maintained below −10°C. Thin-layer chromatography (TLC) was performed on silica gel G plates from Merck (Darmstadt, Germany); solvent: $CHCl_3$/MeOH/H_2O (65:25:4, by vol) and isopropyl ether/HOAc (96:4, vol/vol).

In order to obtain TG, each total lipid extract was partitioned between *n*-heptane/isopropanol/1 M H_2SO_4 (33.6:59.1:7.3, by vol) following vortex homogenization for 1 min. The upper organic phase, which contained TG, was evaporated at <40°C under reduced pressure and stored in a sealed tube below −10°C: the lower one was discarded. TG were developed with one-dimensional, two-step TLC using (i) isopropyl ether/HOAc, 96:4, vol/vol) and (ii) *n*-heptane/Et_2O/HOAc, 90:10:1, by vol) and detected with a 40% aqueous H_2SO_4 spray with heating at 120°C (18). The TG samples were estimated by colorimetric determination of the glycerol component. They were dissolved in $CHCl_3$ (5 mL), and an aliquot (0.1 mL) was mixed with of 0.1 mL of 7 M KOH in EtOH/H_2O (3:1, vol/vol). The mixture was maintained at 56°C for 15 min, after which was added 0.5 mL of water, followed by periodic acid/0.35 M H_2SO_4 (0.35 mL), which resulted in the formation of formaldehyde. For estimation of liberated formaldehyde, the solution was treated with 3.0 mL of the acetylacetone/NH_4OAc/sodium arsenate/water reagent (4 mL), and the mixture was maintained at 56°C for 5 min, resulting in an yellow complex that absorbed at 410 nm.

In order to analyze the fatty acid composition of the lichens, each total lipid extract and TG (5 mg) was methanolyzed by refluxing in 3% HCl in MeOH for 2 h (19). The resulting fatty acid methyl esters (FAME) were extracted from water with $CHCl_3$, and these were analyzed by their gas chromatography–mass spectrometry (GC–MS), R_t values, and electron ionization–mass spectrometry (EI–MS) profiles and compared with those of standards (Sigma products for lipids) on a DB-23 capillary column (30 m × 0.25 mm i.d. and 60 m × 0.25 mm i.d.), programmed from 50 to 180°C and 200°C (40°C·min^{-1}), then held. The sugar components were examined by GC–MS as alditol acetates, on a DB-225 capillary column (30 m × 0.25 mm i.d), programmed from 50 to 220°C (40°C·min^{-1}), then held.

Colorimetric estimation of sugars in the pigment-containing lipid extracts using a modified phenol-sulfuric acid method. Lipid extracts were added to a tube (1 to 3 mg) containing M trifluoroacetic acid (TFA) (1 mL) and hydrolyzed at 100°C for 8 h, and to these mixtures were added $CHCl_3$ (1 mL) and the extracted lipids discarded. An aliquot (0.5 mL) of components soluble in the acid solution was added to a tube containing 0.5 mL of 5% aqueous PhOH-H_2SO_4 (2.5 mL). The optical density (OD) at 490 nm was determined against a blank (B) containing 0.5 m; of M TFA, plus reagent. Another tube (S) containing 24 µg of galactose and 2 mg of lipid extract (from *C. islandica*) was submitted to the above procedure as a control and gave an OD of 0.702. For calibration, standards used were of 10, 30, 50, and 70 µg of galactose; 35 µg of galactose gave an OD of 0.608.

Aqueous acid hydrolysis of lipid extracts. Hydrolyses were performed with 1 M TFA at 100°C for 8 h (8), followed by evaporation to dryness, and the residue was partitioned between $CHCl_3$ and water. The aqueous phase was reduced with $NaBH_4$ and the product acetylated with Ac_2O/NaOAc at 120°C for 1 h. The Ac_2O was destroyed with ice water, and the resulting alditol acetates were extracted with $CHCl_3$ (20) and analyzed by GC–MS, as described above.

Numerical analysis of FAME data. Total lipids were extracted, and the FAME data from 21 lichen species (GC–MS) were submitted to cluster analysis. Dendograms were constructed using the unweighted pair group with mean average from the pairwise Euclidean distance units. The analysis was performed using an NTSys program (Exeter Software, Setauket, NY).

RESULTS

Relationship between organic solvent extracts with yields and fatty acid composition of the lichen species after successive extractions. *Dictyonema glabratum* was extracted separately with the following solvents in order to compare the yields of extracts and the fatty acid composition of each one: Me_2CO (ext. A), $CHCl_3$/MeOH (2:1, vol/vol, ext. B), $CHCl_3$/MeOH (1:1, vol/vol, ext. C), EtOH/H_2O (9:1, vol/vol, ext. D), and $CHCl_3$/MeOH/H_2O (7:10:3, by vol, ext. E), as shown in Table 1. The principal methyl esters obtained after methanolysis of the total extracts were from the saturated fatty acids, 14:0, 16:0, and 18:0, but the longer-chain 20:0, 22:0, and 24:0 derivatives were also observed. Odd-number esters were observed in smaller proportions, 17:0 being found in 13 lichens. Unsaturated fatty acids were present, the most abundant and common being 18:1 (Table 2).

Eleven species of the genus *Cladonia* showed similarities in their fatty acid composition in terms of the chain length, but with some percentage variation. Observed were 16:0, 18:0, 17:0, 23:0, and 24:0 in six lichens, 22:0 in eight, 14:0 and 18:1 in nine, and 20:0 in ten. The presence of other fatty acids is shown in Table 2. *Cladina rangiferina* components were compared with those of *Cladonia* spp., since it also belongs to the family Cladoniaceae. The fatty acid composition was quite similar, showing 14:0, 16:0, 17:0, 18:0, 18:1, 20:0, 22:0, and 23:0, the only difference being the proportion of each one. The fatty acid composition agrees with the data obtained by Dembitsky *et al*. (3). *Parmotrema delicatum, P. mantiqueirense,* and *P. shindler* did not give rise to long-chain

TABLE 1
Fatty Acid Composition Arising from Different Types of Extraction (Ext) of *Dictyonema glabratum*

Fatty acid	R_t[a]	Ext (A)	Ext (B)	Ext (C)	Ext (D)	Ext (E)
		Percentage yields of the different types of extraction				
		0.6	2.7	4.9	4.3	6.3
10:0 (caproic)	5.49	5.3	1.6	—	—	—
12:0 (lauric)	7.00	9.4	3.0	6.3	5.0	5.1
14:0 (myristic)	8.32	8.45	3.75	6.3	4.7	5.8
16:0 (palmitic)	10.47	61.5	55.0	74.0	50.2	56.0
16:2 (hexadecadienoic)	11.13	—	—	—	2.7	—
17:0 (heptadecanoic)	12.16	—	1.2	—	—	—
18:0 (stearic)	14.01	15.5	8.2	9.4	9.0	7.9
18:1 (oleic)	14.29	—	8.0	—	10	—
18:2 (linoleic)	15.29	—	19.1	4.0	18.1	19.2
18:3 (linolenic)	18.13	—	0.3	—	—	—
22:0 (behenic)	25.20	—	—	—	—	6.0
24:0 (lignoceric)	28.27	—	—	—	0.3	—

[a]Retention time (R_t) in minutes. Ext (A) Me_2CO, Ext (B) $CHCl_3$/MeOH (2:1), Ext (C) $CHCl_3$/MeOH (1:1, vol/vol), Ext (D) EtOH/H_2O (9:1, vol/vol), and Ext (E) $CHCl_3$/MeOH/H_2O (7:10:3, by vol).

or odd-numbered fatty acid esters. The acids 16:0 and 18:0 were observed in all of the lichens examined as were unsaturated fatty acids 18:1 and 18:3. The fatty acid composition of the basidiolichen, *D. glabratum* (21), showed the presence of short-chain fatty acids such as 10:0, 12:0, and 16:2.

Fatty acid composition of TAG extracts. TG were extracted with a solution of *n*-heptane/isopropanol/1 M sulfuric acid (33.6:59.1:7.3, by vol) as described above, and the resulting upper phase contained TG, whose yields ranged from 11.4 to 37.3%: its respective fatty acid composition is shown in Table 3. The fatty acids were obtained *via* methanolysis and analyzed by GC–MS. The main saturated FAME were: 14:0, 16:0, and 18:0, along with smaller proportions of 20:0, 22:0, 22:1, 22:2, 23:0, and 24:0. Detected in lower quantities were the odd-number fatty acids, 15:0 and 17:0, which were found in 13 of 15 lichens. The unsaturated fatty acids 16:1, 18:1, 18:2, 18:3, and 22:2 were also observed (Table 3).

Unusual lipids such as dicarboxylic fatty acids, a long-chain alcohol, branched- and hydroxy-fatty acids, and keto-fatty acids were unexpectedly detected (Table 4). The resulting GC–MS profile of each sample was analyzed on the basis of a EI–MS library, incorporating the NIST/NBS Library of Masslinks Library Service, to determine chemical structures. The main mass fragments are shown in Table 4. The most frequently encountered unusual FAME was nonanedioic acid methyl ester, this compound being present in 15 species. Its main fragments (*m/z*) and intensities (%) were, respectively, 185 (27), 152 (70), 143 (30), 111 (45), 83 (63), 74 (100), 69 (47), 55 (81), 43 and 41 (40), and 40 (2), although its ion peak did not appear. Also present were esters of 9-oxo-decanoic and 9-oxo-nonanoic. The latter showed fragments with *m/z*: 155 (16), 143 (35), 115 (7), 111 (33), 87 (53), 74 (100), 55 (47), 43 and 41 (35), and 40 (2).

Dendograms of fatty acid profiles: evaluation of lichen relationships. The FAME profiles of the 21 lichen species were submitted to cluster analysis. Twenty different FAME, varying in chain length from 10 to 24 carbons (saturated or unsaturated), were identified (Table 2). The resulting dendogram resolved species most consistent with their taxonomic position. Ten *Cladonia* species are grouped in two related subclusters (I and II, Fig. 1). However, one *Cladonia* sp., *C. miniata*, was distantly related, clustering with *P. mantiqueirense. Cladina rangiferina* clustered within subcluster II and, surprisingly *P. delicatum* was clustered in this same group. The dendrogram was reinforced by a cophenetic coefficient of 0.89. This value gives an idea of the consistency of the dendrogram, when related to the distance matrix.

Modification of the PhOH-H_2SO_4 method to estimate carbohydrates in the presence of pigments and monosaccharide composition. The total carbohydrate content of the lipid extracts, obtained from 21 lichen species, was initially determined using a conventional PhOH-H_2SO_4 procedure (17), using 1000 µg to 50 µg of each extract. All the values obtained for the samples obeyed the Lambert-Beer's law over a range of 0–80 µg hexose. Good results were obtained with total hydrolysis using 1 M TFA at 100°C. This procedure produced free sugars, the lipids being removed by partition between chloroform and an aqueous solution. Another control of the method (S) was carried out using 24 µg of galactose (OD of 0.417) and 2 mg of the lipid extract from *C. islandica*; this control containing 40.4 µg of total sugar, giving an OD of 0.702. The total carbohydrate content of 2 mg for *C. islandica* was 16.92 µg corresponding to an OD of 0.294, such controls confirming the validity of the modified method. The standard curve and the total sugar yields are represented in Figure 2 and Table 5, respectively.

The yields in terms of total carbohydrates varied from 0.2 to 3.3% for all lichens of the genus *Cladonia*, whereas those of the genus *Parmotrema* varied from 0.9 to 1.9% and those of *Ramalina* from 0.8 to 1.3%. Values for other species are presented in Table 5.

The sugar compositions were determined as derived alditol

TABLE 2
Fatty Acid Composition of Total Lipid Extracts, Obtained from 21 Lichens

		Fatty acid																			
Lichen	Yield (%)	10:0	12:0	14:0	15:0	16:0	16:1	16:2	17:0	17:1	17:2	18:0	18:1	18:2	18:3	19:0	20:0	22:0	22:1	23:0	24:0
Cetraria islandica	6.5	—	—	3.15	2.5	67.6	—	—	4.4	—	—	17.7	—	—	—	—	3.6	—	—	—	1.1
Cladonia clathrata	3.5	—	—	—	—	23.3	—	—	—	—	—	45.0	16.5	2.4	—	—	1.3	5.4	1.4	—	4.8
C. connexa	3.6	—	—	1.5	—	50.6	—	—	—	0.9	—	30.1	4.8	3.5	—	—	3.2	—	—	4.0	2.2
C. crispatula	3.9	—	—	3.1	—	46.1	—	—	—	—	2.2	19.1	20.8	—	—	—	2.8	3.6	—	—	2.0
C. furcata	4.2	—	1.2	1.2	—	43.5	—	—	2.8	—	—	39.8	7.2	—	—	—	2.3	—	—	1.1	1.0
C. ibitipocae	3.4	—	—	1.2	—	43.8	—	—	—	—	—	26.6	14.0	—	—	—	5,6	4.6	—	4.1	—
C. imperialis	2.9	—	—	0.8	—	31.8	—	—	2.6	—	—	29.4	16.9	—	—	11.3	1.1	0.7	—	2.5	2.4
C. miniata	4.6	—	—	48.7	—	31.6	—	—	—	—	—	19.6	—	—	—	—	—	—	—	—	—
C. penicillata	3.2	—	—	2.4	—	42.5	—	—	4.2	—	—	45.4	0.9	—	—	—	1.4	1.9	—	—	1.2
C. salmonea	7.9	—	—	—	—	40.7	—	—	2.3	—	—	26.2	22.3	—	—	—	3.6	1.7	—	3.2	—
C. signata	2.7	—	—	0.5	—	43.6	—	—	3.7	—	—	37.5	8.3	—	—	—	2.8	2.6	—	1.0	—
C. substellata	6.4	—	—	2.9	—	15.3	—	—	4.0	—	—	23.2	—	—	—	—	0.4	3.2	—	—	—
Cladia aggregata	5.2	—	—	—	—	28.4	—	—	12.2	—	—	15.5	10.3	33.5	—	—	—	—	—	—	—
Cladina rangiferina	6.3	—	—	0.3	—	33.3	—	—	3.1	—	—	19.0	21.2	3.0	—	—	5.8	11.5	—	2.7	—
Dictyonema glabratum	4.9	1.6	3.0	3.2	—	49.2	—	2.1	0.8	—	—	8.2	7.9	18.8	0.9	—	4.7	—	—	—	0.3
Leptogium phyllocarpum	10.6	—	—	13.3	—	15.2	—	—	7.7	—	—	21.0	—	—	—	—	—	19.0	—	—	23.7
Parmotrema delicatum	13.2	—	—	—	—	35.3	—	—	—	—	—	30.2	34.5	—	—	—	—	—	—	—	—
P. mantiqueirense	8.1	—	—	35.9	—	19.3	—	—	—	—	—	44.7	—	—	—	—	—	—	—	—	—
P. shindler	12.2	—	—	7.6	—	18.1	—	—	—	—	—	16.5	12.8	—	44.9	—	—	—	—	—	—
Ramalina celastri	3.2	—	—	0.8	—	18.9	2.3	—	0.8	—	—	7.2	14.7	36.2	19.0	—	—	—	—	—	—
R. usnea	3.2	—	—	—	1.3	65.2		—	4.3	—	—	29.1	—	—	—	—	—	—	—	—	—

TABLE 3
Fatty Acid Composition of the Lipids Present in the *n*-Heptane-Isopropanol Phase from 21 Lichens[a]

	Triglycerides	Fatty acid																
Lichen	Yield (%)	12:0	13:0	14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:0	21:0	22:0	22:2	23:0	24:0
C. islandica	14.0	2.0	1.0	7.9	4.0	62.3	—	2.0	17.8	—	—	—	3.0	—	—	—	—	—
C. clathrata	23.7	—	—	3.4	5.3	49.5	—	2.7	35.5	—	—	—	3.4	—	2.8	—	—	—
C. connexa	22.5	—	—	10.7	—	61.5	—	—	27.7	—	—	—	—	—	—	—	—	—
C. crispatula	21.5	—	—	2.3	5.0	60.5	—	3.6	24.2	—	—	—	2.1	0.8	1.5	—	—	—
C. furcata	37.3	—	—	—	—	66.2	—	—	33.8	—	—	—	—	—	—	—	—	—
C. ibitipocae	26.6	—	—	3.6	6.0	60.0	—	3.6	23.4	—	—	—	1.8	—	1.5	—	—	—
C. imperialis	35.0	—	—	8.2	4.9	41.1	—	5.2	38.6	—	—	—	—	—	1.9	—	—	—
C. miniata	29.8	—	—	5.0	8.7	53.4	—	4.2	25.3	—	—	—	1.7		1.7	—	—	—
C. penicillata	37.0	—	—	3.5	2.7	57.9	—	2.9	26.1	—	—	—	2.9	—	3.9	—	—	—
C. salmonea	26.4	—	—	2.9	3.0	60.5	—	2.8	27.9	—	—	—	1.6		1.2	—	—	—
C. signata	32.0	—	—	1.8	1.7	49.7	—	4.6	35.2	—	—	—	3.0	—	1.7	—	1.3	0.8
C. substellata	26.6	—	—	4.5	4.5	39.2	—	1.8	51.8	—	—	—	—	—	—	—	—	—
C. aggregata	22.1	—	—	—	—	33.9	—	3.2	19.0	11.6	29.7	—	—	—	—	2.8	—	—
C. rangiferina	29.3	—	—	2.2	1.6	46.6	—	2.4	22.4	—	—	—	6.0	—	14.2	—	2.2	2.4
D. glabratum	21.6	—	—	2.1	3.3	75.0	—	3.6	16.1	—	—	—	—	—	—	—	—	—
L. phyllocarpum	20.8	—	—	—	—	100	—	—	—	—	—	—	—	—	—	—	—	—
P. delicatum	11.9	—	—	—	—	69.4	—	—	30.5	—	—	—	—	—	—	—	—	—
P. mantiqueirense	27.3	—	—	—	—	74.6	—	—	25.3	—	—	—	—	—	—	—	—	—
P. shindler	16.0	—	—	2.1	—	67.0	—	—	30.8	—	—	—	—	—	—	—	—	—
R. celastri	18.3	—	—	—	—	17.3	3.0	1.0	8.1	18.6	38.1	13.7	—	—	—	—	—	—
R. usnea	11.4	—	—	3.1	5.0	51.8	—	5.2	30.1	—	—	—	1.3	—	2.0	—	1.3	—

[a]See Table 2 for lichen abbreviations.

TABLE 4
Unusual Lipids Present in the *n*-Heptane-*iso*propanol Phase from 19 Lichens

Lipid[a]		Lichen[c]																	
m/z in parentheses	T_M[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Hexanedioic acid (143, 114, 111, 101, 74, 55, 54)	0.82	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	7.0	—	—	—
Octananedioic acid (171, 138, 129, 97, 74, 69, 55)	0.92	0.6	—	—	—	—	—	1.1	—	—	—	—	—	—	—	1.5	—	—	—	—
Nonanedioic acid (185, 152, 143, 111, 83, 74, 69, 55)	0.98	2.4	3.6	1.4	3.1	—	1.5	—	4.7	2.5	2.6	9.7	3.2	1.3	—	3.6	6.7	1.3	—	1.4
Decanedioic acid (199, 166, 138, 125, 98, 74, 69, 55)	1.05	—	—	—	1.1	—	—	—	—	0.8	0.6	—	—	—	—	—	—	—	—	—
Undecanedioic acid (213, 171, 152, 139, 98, 74, 69, 55)	1.11	—	1.1	—	1.2	1.1	—	—	1.5	—	—	1.3	—	—	—	—	—	—	—	—
10-Nonadecanol (265, 264, 215, 201, 171, 157, 109, 83)	1.36	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3-Hydroxy-decanoic acid (155, 129, 103, 71, 69, 57, 43)	0.85	—	—	—	—	2.6	—	—	—	—	—	—	—	—	—	—	—	—	1.5	—
9-Oxo-nonanoic acid (159, 158, 143, 109, 87, 69, 43)	0.98	—	—	—	—	—	—	—	—	—	—	4.4	—	—	—	—	—	—	—	—
9-Oxo-decanoic Acid (185, 169, 143, 111, 87, 83, 69, 43)	1.04	—	—	—	—	1.1	—	—	—	—	—	1.3	—	—	—	—	—	—	—	—
6,10,14-Me_3-2-pentadecanone (250, 210, 109, 85, 71, 58, 43)	0.99	—	—	—	—	—	0.6	—	—	—	—	—	—	—	1.0	—	—	—	—	—
6-Me-heptanoate (171, 137, 130, 115 , 103, 87, 74, 55)	0.86	—	—	—	—	—	—	—	—	—	—	0.6	—	—	1.4	—	—	—	—	—
9-Me-tetradecanoate (256, 227, 199, 129, 101, 87, 74)	0.92	—	—	—	—	—	—	1.3	—	—	—	—	—	—	—	—	—	—	—	—
11,14-Eicosadienoic acid (294, 263, 220, 215, 95, 81, 67)	1.19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.2	—	—

[a]Lipids determined as total. 1, *C. islandica*; 2, *C. clathrata*; 3, *C. connexa*; 4, *C. crispatula*; 5, *C. furcata*; 6, *C. ibitipocae*; 7, *C. imperialis*; 8, *C. penicillata*; 9, *C. salmonea*; 10, *C. signata*; 11, *C. substellata*; 12, *C. rangiferina*; 13, *C. aggregata*; 14, *D. glabratum*; 15, *P. delicatum*; 16, *P. mantiqueirense*; 17, *P. shindler*; 18, *R. celastri*; 19, *R. usnea*.
[b]Retention time (T_M) relative to palmitic acid methyl ester.
[c]All the lichens were studied (21), but only 19 species contained these types of lipid. See Table 2 for lichen abbreviations.

acetates, which were analyzed by GC–MS. The monosaccharides obtained after total hydrolysis (Table 5) show the presence of glucose, mannose, galactose, and xylose in all cases. Fucose was observed only in extracts of *C. islandica*, *P. mantiqueirense*, and *P. shindler*. Rhamnose was found in most lichens, but not in those of *C. aggregata*, *P. shindler, P. mantiqueirense*, and *R. celastri*.

DISCUSSION

Extractions with various solvents gave rise to different yields and fatty acids; the best yields were obtained with extracts D, C, and E, although all of them contained different fatty acids. The procedure of Extract A was the most efficient for extracting short-chain fatty acids. The advantages of using EtOH/H_2O (9:1, vol/vol) under reflux were apparent since it gave a high yield, was cheaper and less toxic. It is capable of extracting polar glycolipids, according to Leeden and Yu (22). However, for a more reliable examination of total fatty acids in tissues, a battery of different solvents is necessary. *Dictyonema glabratum* was chosen as the best candidate, as its fresh tissues were more readily obtainable in greater amounts.

Total lipids were examined, and palmitic and stearic acids were present in all studied lichens. This observation was supported by the natural biosynthetic route of lipids in fungi and by the fact that most organisms produce fatty acids with various chain lengths (C_{12}–C_{20}), as described by Wassef (12). Since lichens contain ~90% mycobiont (16), similar results would be expected. The presence of odd-number fatty acids was shown by Lynen (cited in Ref. 12), and these arose using propionate as primer in the substitution of acetate and the use of isobutyrate, leucine, valine or isoleucine, which gave rise to iso- and anteiso-branched fatty acids (Baraud, cited in Ref. 12). In our upper organic phases, other unusual lipids were detected, namely they were aldehyde, keto, hydroxy, and dicarboxylic fatty acids. These have not been previously described in lichens. We also showed the presence of dicarboxylic acids, and the main one found was nonanedioic (azelaic) acid (Table 4); these are probably formed by ω-oxidation (23). Nonanedioic and hexanedioic acids have a cytotoxic effect against squamous carcinoma affecting cell proliferation (24). However, why are these compounds present in lichens? Other types of unusual lipids were the aldehyde and keto-derivatives, 9-oxo-nonanoic and 9-oxo-decanoic acids, which can be associated with cold-acclimation (25). Another reason to justify this hypothesis is the presence of large amounts of unsaturated fatty acids in lichens when they are submitted to cold conditions (4).

The family Cladoniaceae includes 11 genera with polyphyletic assemblage (26). We have now investigated lichens of the *Cladonia* and *Cladina* genera for their carbohydrate and FAME compositions, both of total fatty acids and isolated TG. Although the relationship of these genera is not totally clear (27), the dendogram derived from FAME data shows that they have similar profiles on fatty acid analysis. The cluster analysis evaluated for *Cladonia*, *Cladina*, and other

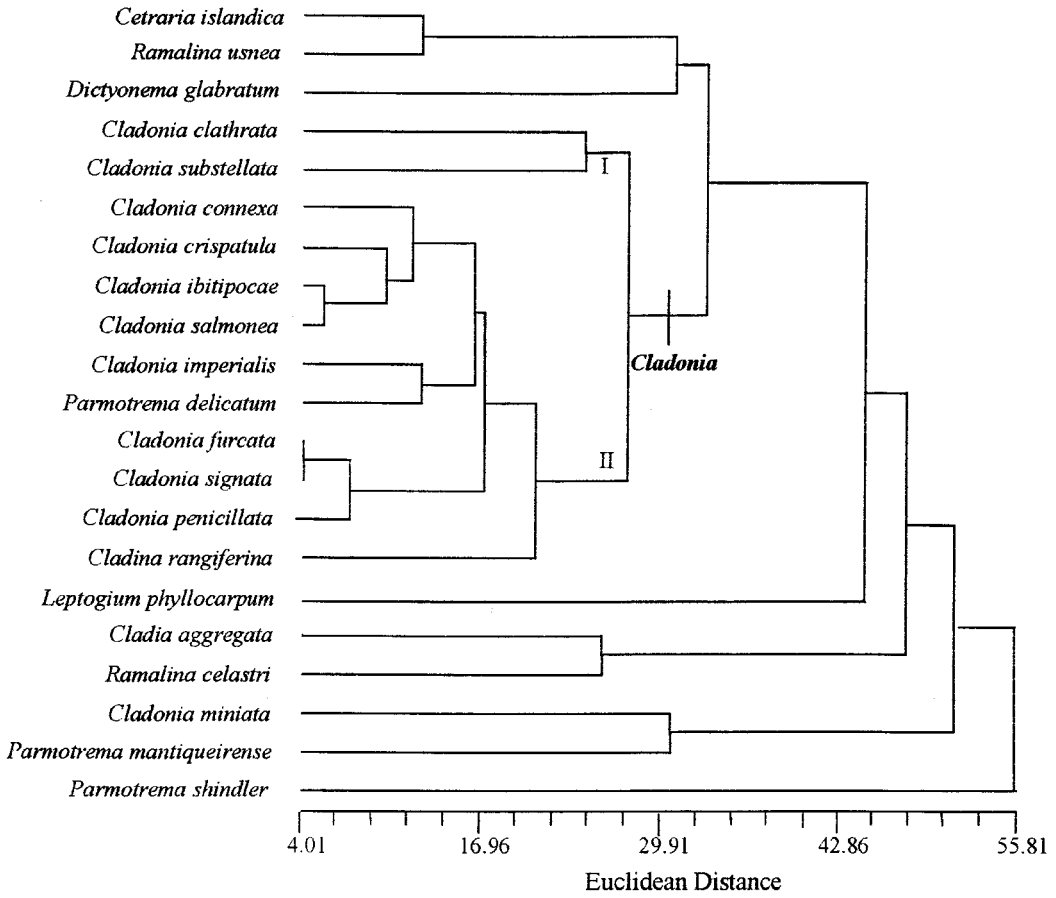

FIG. 1. Unweighted pair group with mean average from pairwise calculated distances obtained comparing fatty acid methyl ester profiles from 21 lichen species performed using the NTSys program. (I and II) *Cladonia* subclusters.

lichens from these data enabled the consistent conjunction of *Cladonia* species. Although these lichens were subjected to different growth conditions, this criterion is important: the option of laboratory culture in synthetic media of so many lichens is very laborious. The data suggest a similar profile of fatty acids for the *Cladonia* genus. *Cladia miniata* showed a different fatty acid composition and was positioned outside the group. This agrees with morphological, chemical, and ecological characteristics evaluated by Stenroos *et al.* (27), who carried out cluster analysis of *Cladonia* and *Cladina* genera. On the other hand, *C. rangiferina* clustered with *Cladonia* species, emphasizing a pronounced relationship between these genera. It should be observed that the genus *Cladina* is not well established. In the Americas, Asia, Australasia, and Russia, it is now usually recognized, while in Europe most lichenologists consider it a subgenus of *Cladonia* (27). *Cladia aggregata* did not show the same pattern of fatty acids as obtained for the lichens of the genera *Cladonia* and *Cladina*. This observation could have taxonomic value (12), since *C. rangiferina* belongs to the same family as those of the genus *Cladonia*. The basidiolichen *D. glabratum* appeared to be interesting for comparison of its fatty acid profile, which was quite different from that of lichenized ascomycetes (28), with the presence of the short-chain fatty acids, 10:0, 12:0, and 16:2. The lichen *L. phyllocarpum* is ascomycetous and with a cyanobacterium symbiosis, and its fatty acids were similar to those of other ascolichens. These observations (ascomycetous and basidiomycetous) showed that the fatty acids might be derived from the mycobionts, since they are different and phycobionts are cyanobacteria.

It is possible that some lichen fatty acids could be formed directly by the phytobiont and modified by the mycobiont. An

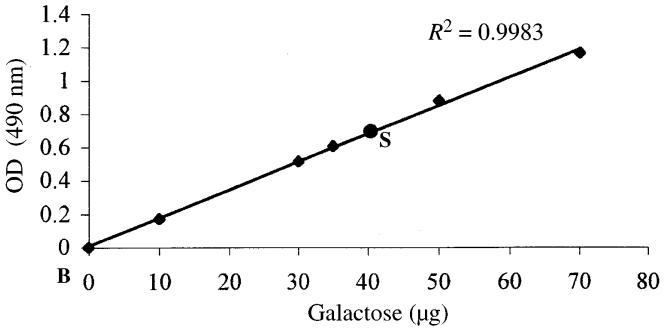

FIG. 2. Blank tube (B) containing 0.5 mL of M trifluoroacetic acid and the phenol-sulfuric acid mixture. Standard (S) containing 24 µg of galactose and 2 mg of lipid extract *(Cetraria islandica)*. Calibration was carried out with standards of 10, 30, 50, and 70 µg of galactose. OD, optical density.

TABLE 5
Monosaccharide Composition and Carbohydrate in Total Lipid Extracts, Obtained from 21 Lichen Species

Lichen	Yield (%)[a]	(%) Monosaccharide content							
		Glycerol	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
C. islandica	0.9	1.2	2.5	3.1	32.4	2.1	5.4	9.1	40.6
C. clathrata	0.9	1.8	3.9	—	43.0	0.8	11.5	12.3	20.8
C. connexa	0.5	0.9	2.5	—	18.0	5.9	15.6	23.3	34.4
C. crispatula	0.2	1.0	6.1	—	33.9	7.1	17.0	12.3	20.3
C. furcata	0.8	0.6	2.5	—	22.2	3.3	23.0	29.6	18.8
C. ibitipocae	1.0	0.8	0.9	—	10.9	5.3	27.6	25.0	29.8
C. imperialis	0.9	0.5	19.8	—	47.0	14.2	4.3	2.8	10.1
C. miniata	2.5	0.3	0.4	—	3.2	0.2	38.4	22.9	34.0
C. penicillata	0.8	1.0	1.8	—	28.2	4.2	22.0	13.6	29.2
C. salmonea	0.9	0.3	1.7	—	15.4	9.8	16.9	19.1	36.8
C. signata	0.8	3.6	13.6	—	37.6	11.8	4.3	7.2	20.8
C. substellata	0.6	1.1	0.8	—	7.7	3.8	38.2	18.8	29.4
C. aggregata	0.8	0.8	—	—	91.3	3.3	2.6	1.0	1.0
C. rangiferina	2.3	2.1	14.6	—	34.4	12.0	13.9	4.6	15.1
D. glabratum	3.3	3.8	8.0	—	25.3	3.1	7.1	28.5	17.1
L. phyllocarpum	1.9	0.9	2.7	—	3.2	2.4	49.7	18.2	22.6
P. delicatum	0.9	0.5	9.2	—	36.5	7.7	15.9	10.1	20.0
P. mantiqueirense	1.1	0.8	—	1.8	1.7	1.6	64.2	15.3	10.1
P. shindler	1.9	2.6	—	5.8	—	19.3	15.8	25.1	26.2
R. celastri	0.8	0.5	—	—	93.2	0.5	3.6	1.2	0.8
R. usnea	1.3	1	2.8	—	24.5	5.5	6.5	47.6	12

[a]SD (standard deviation), triplicates of total sugar concentration varied from ± 0.009 to ± 0.021. See Table 2 for lichen abbreviations.

example of this process occurs in the transformation of free aldoses to polyalcahols as suggested by Ahmadjian (29), although evidence has not yet been observed in lichens that fatty acids, or any other metabolite, can be synthesized in the mycobiont and later modified or transferred to the phycobiont (16,29). It appears likely that lichen fatty acids should arise from the mycobiont, which comprises ~90% of the mycelial biomass and which is formed in large amounts in fungi (16). Many of the fatty acids are similar to those in lichenized fungi (16,29); these observations may show the lichen fatty acids as markers for taxonomical study just like those observed in fungi (13). For better results, more cladistic analyses should be carried out on FAME profiles in other well-classified genera, although the value of the FAME profiles appears to be an important additional parameter that can be used in lichen taxonomy.

The colorimetric estimation of the total sugar in pigmented material is a common problem. The use of direct TFA-hydrolyzed material instead of water in the conventional method did not interfere with the optical densities, enabling accurate determinations with the phenol-sulfuric acid method.

Monosaccharide analysis of the 21 lichens showed high levels of total galactose, expected since the major phycobiont glycolipids are galactolipids, but the presence of high levels of arabinose, mannose, and glucose was not. It may be significant that the presence of their corresponding polyalcohols in lichens has been observed (29). The presence of arabinitol and mannitol could be explained by the carbohydrate movement (29), the glucose produced by the phycobiont being rapidly converted to mannitol and arabinitol by the mycobiont. Although *C. aggregata* showed high levels of arabinose (91.3%), when compared to total monosaccharide, this suggests a difference in taxonomical position, in relationship to the genus *Cladonia* (Table 5). The presence of high levels of polyalcohols and saccharide derivatives would be expected, since they are more soluble in hot organic solvents. Another possible explanation is that aldoses were liberated by hydrolysis from lipopolysaccharides, since this polymer is produced by some algae and fungi (30,31), and is soluble in organic solvents.

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