Fatty Acid Composition of Symbiotic Cyanobacteria from Different Host Plant (*Azolla*) Species: Evidence for Coevolution of Host and Symbiont

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The total cellular fatty acid contents of 40 recently isolated cyanobacterial symbionts obtained from seven species of *Azolla* host plants were determined by gas-liquid chromatography–mass spectroscopy. A total of 63 fatty acids belonging to seven distinct chemical classes were identified. Fatty acid compositions varied among the cyanobacteria depending on the hosts species. Parameters that differed significantly (at the 99% level of probability) included the concentrations of the 16:0 and 18:3 fatty acids, the total concentrations of the polyunsaturated acids, the total concentrations of the 16-carbon and 18-carbon fatty acids, the ratios of unsaturated fatty acids to saturated fatty acids, and the total percentages of straight-chain even-carbon-number fatty acids, unsaturated fatty acids, and branched-chain unsaturated fatty acids. The results of an analysis of variance suggested statistical regression for the total percentages of these fatty acids and chemical classes according to the following linear alignment of cyanobacteria by host: *Azolla filiculoides*, *Azolla microphylla*, *Azolla caroliniana*, *Azolla mexicana*, *Azolla rubra*, *Azolla nilotica*, and *Azolla pinnata* (including *Azolla pinnata* subsp. *pinnata* and *Azolla pinnata* subsp. *imbricata*). The seven groups could be divided into two distinct clusters on the basis of the results of a dendrogram analysis of Euclidian distances. The symbionts obtained from *A. filiculoides*, *A. microphylla*, *A. mexicana*, and *A. caroliniana* constituted one cluster, and the symbionts obtained from *A. rubra*, *A. nilotica*, and *A. pinnata* constituted a second cluster. A minor dichotomy separated the *A. filiculoides* symbionts from the other members of the first cluster. The clustering of *Azolla* cyanobacterial symbionts based on the results of our fatty acid analysis correlates remarkably well with the taxonomic grouping of the American *Azolla* species. This correlation suggests that the cyanobacterial symbionts of *Azolla* spp. coevolved into distinct genetic groups with their hosts.

The cyanobacteria are a diverse group of oxygenic, photosynthetic prokaryotes which occur worldwide in marine, aquatic, and terrestrial habitats (8). Many genera of cyanobacteria are symbiotically associated with plants, fungi (lichens), animals, and bacteria. The genera *Anabaena* and *Nostoc* are the major genera whose members are found in plants (23).

Members of the water fern genus *Azolla* are hosts of cyanobacterial symbionts that occur in specially adapted cavities in the upper leaf fronds (7, 16, 18). There are seven *Azolla* species belonging to two subgenera, each of which has a distinct morphology; *Azolla caroliniana*, *Azolla filiculoides*, *Azolla mexicana*, *Azolla microphylla*, and *Azolla rubra* are members of the subgenus *Azolla*, and *Azolla nilotica* and *Azolla pinnata* are members of the subgenus *Rhizosperma*. *A. pinnata* contains two subspecies, *A. pinnata* subsp. *pinnata* and *A. pinnata* subsp. *imbricata* (13, 17). Using plant morphology, ecophysiology, isozyme, and DNA fingerprint criteria, Zimmerman et al. (25–27) found that *A. caroliniana*, *A. mexicana*, and *A. microphylla* are closely related phylogenetically and that *A. filiculoides* and *A. rubra* are not as closely related.

The *Azolla* symbionts are morphologically diverse, particularly in their cellular dimensions (1, 11) and the arrangement of their vegetative and reproductive cells in filaments (1). The cyanobacteria whose hosts are members of the two subgenera (subgenera *Azolla* and *Rhizosperma*, the latter including *A.*

rubra) have characteristic morphological differences related to the growth and development of their reproductive cells, such as hormogonia and akinetes (1, 2). In addition, analyses of DNA restriction fragment length polymorphisms in cyanobacterial DNAs have revealed differences between symbionts whose hosts are members of the two subgenera at the molecular level (9, 10, 15, 21, 22).

Fatty acid composition is a useful analytical tool in bacterial taxonomy, and the fatty acid compositions of a limited number of *Azolla* cyanobacterial symbionts have been determined previously; wide ranges of values have been obtained for some important components in these analyses (3, 6). It is not clear if the variability observed was due to strain differences or was related to the host species. In this study we examined the variations in the fatty acid compositions of 40 different strains of cyanobacteria isolated from the seven *Azolla* species and in particular tried to detect differences related to the hosts of the organisms.

MATERIALS AND METHODS

Media and growth conditions. *Azolla* plants were grown in defined mineral medium without nitrogen (3). The photon flux density was 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and was provided by a combination of cool white fluorescent and incandescent light; a 14-h light–10-h dark cycle was used (19). The temperature was $23 \pm 1^\circ\text{C}$ during the light periods and $17 \pm 1^\circ\text{C}$ during the dark periods. The cyanobacterial strains which we used and their geographic origins and sources are listed in Table 1.

Cyanobacterial symbionts were harvested from 14- to 18-day-old plants as previously described (3) and were stored at -20°C for subsequent fatty acid analysis.

Fatty acid analysis. Preparations of cyanobacterial cells were saponified, esterified, and then analyzed to determine their fatty acid contents by gas-liquid chromatography–mass spectroscopy, as previously described, using a Varian

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TABLE 1. Origins of *Azolla* host plants used in this study and designations of symbiotic cyanobacterial strains

Host ^a	Bacterial strain	Original designation	Geographic origin	Source ^b
<i>A. caroliniana</i>	C1	C1	Virginia	G.P.
	C2	3010	Rio Salimões, Ilha Paciência, Amazonas, Brazil	W.Z.
	C3	3014	Rio Negro, Catalão, Amazonas, Brazil	W.Z.
	C4		La Esperanza, Manatí, Puerto Rico	R.C.
<i>A. filiculoides</i>	C8		Río Chavón, Dominican Republic	R.C.
	F1	81FI	Lima, Peru	V.H.
	F8	6005	Kyoto Tanabe-Cho, Japan	W.Z.
	F10	103FI	Hidalgo, Mexico	V.H.
	F12	126FI	Karoo, South Africa	V.H.
	F13	159FI	Victorville, Calif.	V.H.
	F14	162FI	Tristan da Cunha, United Kingdom	V.H.
	F15	167FI	Pondolivado, Greece	V.H.
	F18	185FI	Montevideo, Uruguay	V.H.
	C9	208CA	Fyrish, Guyana	V.H.
<i>A. mexicana</i>	X1 (= BR-S-CF)	X1	California	G.P.
	X4		Mexico City, Mexico	R.O.
	X5	54	Guyana	W.R.
	X8	58	California	W.R.
	X10		Illinois	R.C.
<i>A. microphylla</i>	M1	65MI	Paraíso do Norte, Brazil	V.H.
	M2 (= TL-GI-CF)	M2	Galápagos, Ecuador	G.P.
	M4	104MI	Sinaloa, Mexico	V.H.
	M5	4032		W.Z.
	RF3 (= RC-B1-CF)	F3	Hawaii	G.P.
<i>A. rubra</i>	R1	163RU	Victoria, Australia	V.H.
	R2	002RU	North Island, New Zealand	V.H.
	R3	204RU	Nouvelle, New Zealand	V.H.
	R4	200RU	South Island, New Zealand	V.H.
	N1	82NI	Kosti, Sudan	V.H.
<i>A. nilotica</i>		N1	Kosti, Sudan	G.P.
		5001	Kosti, Sudan	W.Z.
	N1-SC	82NI	Kosti, Sudan	V.H.
		N1	Kosti, Sudan	G.P.
		5001	Kosti, Sudan	W.Z.
	N2	15NI	Burundi	V.H.
	I1 (= K2B-T-CF)	I1	Shoolong, People's Republic of China	G.P.
	I2	TDP15	Bankok, Thailand	W.Z.
	I3	JNPI1	Chisato Mie, Japan	W.Z.
	I4	73	Indonesia	W.Z.
<i>A. pinnata</i> subsp. <i>imbricata</i>	I5	36PI	Senegal	W.Z.
	P1 (= BR-M-CF)	P1	Malaysia	G.P.
	P1-SC (= BR-M-CF)	P1	Malaysia	G.P.
	P4	136PP	Sierra Leone	V.H.

^a *A. caroliniana*, *A. filiculoides*, *A. mexicana*, *A. microphylla*, and *A. rubra* are members of the subgenus *Azolla*, and *A. nilotica* and *A. pinnata* are members of the subgenus *Rhizosperma*.

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model 3700 gas chromatography equipped with a flame ionization detector and a capillary glass column (15 m by 0.25 mm) coated with SPB-1 (Supelco, Inc., State College, Pa.) as the nonpolar stationary phase (3, 5). Peaks were identified by cochromatography with authentic standards (Supelco, Inc.), by chemical confirmatory tests, and by mass spectroscopy, as described previously (5). Each cyanobacterial sample was analyzed once, and the average for each fatty acid component was determined by using the data for all isolates obtained from each host species. Cyanobacteria obtained from *A. nilotica* (strain N1), *A. rubra* (strain R1), and *A. pinnata* subsp. *pinnata* (strain P1) were analyzed seven, five, and three times, respectively, in order to determine the level of variability due to the experimental procedure used.

Statistical analysis. Statistical differences between values obtained for selected fatty acids and between ratios were determined by performing an analysis of variance, using Duncan's multiple-range test (24). Differences were considered significant at the 95% level of probability ($P \geq 0.05$). The fatty acid data for cyanobacterial isolates obtained from the two subspecies of *A. pinnata* were averaged in order to increase the total sample number for the group and because there were no statistically significant differences between the compositions of the two populations.

The variability in the values from repeated fatty acid analyses performed with

the same strain (i.e., variability due to experimental procedure) was determined by calculating proximity coefficients. These values were derived by constructing a model profile for 40 strains of cyanobacteria on the basis of the average values shown in Table 2 and calculating the aggregate levels of difference for the individual fatty acids obtained from each sample used for the model.

A cluster analysis of the Euclidian distances between taxonomic groups was performed and a dendrogram was constructed by using the STATPRO statistical software (Wadsworth Professional Software, Inc., Boston, Mass.). We selected fatty acid components that exhibited statistically significant differences as a function of host species. The values which we obtained were then used as input data for a statistical matrix in the cluster analysis, from which the dendrogram was constructed. The dendrogram was based on group distances obtained from a pair-weighted cluster analysis.

RESULTS

General features of fatty acid profiles. We identified 63 different fatty acids, and these fatty acids were present at levels that were consistent with the levels described in previously

TABLE 2. Cellular fatty acid contents (means and ranges) of cyanobacterial symbionts isolated from seven *Azolla* species

Fatty acid		ECL ^a	% of total fatty acids in cyanobacterial symbionts of:																			
			<i>A. filiculoides</i>			<i>A. microphylla</i>			<i>A. caroliniana</i>			<i>A. rubra</i>			<i>A. nilotica</i>			<i>A. pinmata</i>			All species	
			Mean	Range		Mean	Range		Mean	Range		Mean	Range		Mean	Range		Mean	Range		Mean	Range
Class A: saturated, even-carbon-number, straight-chain acids																						
8:0	8.0	0.03	0.00-0.13	0.02	0.01-0.05	0.04	0.00-0.15	0.06	0.02-0.12	0.03	0.00-0.07	0.10	0.06-0.17	0.03	0.00-0.08	0.04	0.00-0.17					
10:0	10.0	0.01	0.00-0.03	0.02	0.00-0.05	0.03	0.00-0.05	0.06	0.01-0.14	0.02	0.00-0.05	0.12	0.05-0.24	0.02	0.00-0.04	0.03	0.00-0.24					
12:0	12.0	0.13	0.07-0.23	0.14	0.08-0.22	0.24	0.15-0.38	0.49	0.10-0.91	0.42	0.19-0.86	0.75	0.36-1.40	0.24	0.08-0.71	0.30	0.07-1.40					
14:0	14.0	0.73	0.33-0.87	1.14	0.84-1.53	0.99	0.70-1.22	1.02	0.83-1.57	1.14	0.81-1.69	1.20	0.90-1.62	0.97	0.66-1.44	0.99	0.33-1.69					
16:0	16.0	31.16	30.29-32.47	33.90	30.95-38.00	34.33	31.34-37.12	32.27	28.89-34.05	36.48	34.33-39.25	36.83	34.69-38.51	39.81	36.40-46.16	34.94	28.89-46.16					
18:0	18.0	1.17	0.62-1.70	1.40	0.73-2.52	1.60	1.00-2.78	1.88	0.87-4.30	2.86	1.78-4.58	2.94	2.40-3.91	1.49	1.13-1.80	1.76	0.62-4.58					
20:0	20.0	0.10	0.01-0.16	0.13	0.00-0.20	0.19	0.10-0.27	0.15	0.06-0.31	0.28	0.13-0.64	0.22	0.13-0.31	0.36	0.19-0.91	0.21	0.06-0.91					
Class A total		33.33	31.61-35.09	36.75	33.86-40.74	37.42	33.83-41.20	35.93	31.61-38.36	41.24	38.80-40.09	42.15	38.72-44.93	42.92	39.82-49.69	38.26	31.61-49.69					
Class B: saturated, odd-carbon-number, straight-chain acids																						
9:0	9.0	0.01	0.00-0.03	0.02	0.00-0.05	0.01	0.00-0.07	0.07	0.00-0.20	0.02	0.00-0.07	0.10	0.06-0.14	0.02	0.00-0.05	0.03	0.00-0.14					
11:0	11.0	0.02	0.00-0.04	0.00	0.00-0.00	0.01	0.00-0.04	0.01	0.00-0.04	0.00	0.00	0.02	0.00-0.05	0.02	0.00-0.06	0.01	0.00-0.06					
13:0	13.0	0.01	0.00-0.03	0.02	0.00-0.06	0.02	0.00-0.08	0.05	0.00-0.22	0.00	0.00	0.03	0.00-0.08	0.01	0.00-0.04	0.02	0.00-0.08					
15:0	15.0	0.26	0.09-1.17	0.36	0.27-0.55	0.44	0.19-0.86	0.67	0.12-1.09	0.76	0.39-1.57	0.88	0.81-0.95	0.37	0.13-1.22	0.48	0.09-1.57					
17:0	17.0	0.25	0.06-0.41	0.29	0.14-0.55	0.32	0.17-0.56	0.22	0.15-0.34	0.27	0.10-0.36	0.29	0.24-0.39	0.19	0.12-0.33	0.25	0.06-0.56					
19:0	19.0	0.15	0.01-0.58	0.02	0.00-0.10	0.17	0.01-0.55	0.14	0.00-0.36	0.15	0.00-0.39	0.10	0.06-0.16	0.13	0.01-0.52	0.13	0.00-0.58					
Class B total		0.70	0.24-1.42	0.71	0.51-0.91	0.96	0.51-1.59	1.16	0.64-2.07	1.20	0.52-2.24	1.41	1.18-1.72	0.74	0.33-2.08	0.92	0.24-2.24					
Class C: unsaturated, straight-chain acids																						
12:1	11.8	0.33	0.18-0.98	0.62	0.32-1.42	0.70	0.07-2.03	1.80	0.25-6.14	1.96	0.85-5.31	1.49	1.18-2.02	0.35	0.12-0.76	0.90	0.07-6.14					
13:1	12.8	0.02	0.00-0.11	0.01	0.00-0.04	0.02	0.00-0.05	0.02	0.00-0.06	0.01	0.00-0.04	0.02	0.00-0.06	0.01	0.00-0.03	0.01	0.00-0.11					
14:1 <i>cis</i> -7	13.8	0.35	0.00-0.48	0.37	0.20-0.61	0.24	0.00-0.48	0.11	0.00-0.32	0.09	0.00-0.29	0.00	0.00-0.00	0.08	0.00-0.17	0.19	0.01-0.61					
14:1 <i>cis</i> -9	13.9	2.04	0.58-2.58	2.11	1.04-3.51	1.85	0.98-2.90	1.63	0.71-2.42	1.93	0.74-3.42	2.37	0.96-3.46	1.27	0.13-2.94	1.83	0.13-3.51					
15:1	14.9	0.00	0.00-0.02	0.08	0.00-0.25	0.04	0.00-0.21	0.04	0.00-0.00	0.00	0.00-0.00	0.00	0.00-0.00	0.05	0.00-0.32	0.03	0.00-0.32					
16:4(?) ^b	15.2	0.11	0.00-0.37	0.15	0.11-0.28	0.18	0.00-0.49	0.24	0.10-0.40	0.15	0.00-0.44	0.43	0.22-0.68	0.19	0.00-0.56	0.19	0.00-0.68					
16:1(?) ^b	15.5	0.85	0.00-2.02	1.31	0.91-1.73	1.02	0.00-1.89	1.20	0.66-2.56	1.30	0.82-1.79	1.96	1.61-2.39	1.42	0.49-1.98	1.23	0.00-2.56					
16:4 <i>cis</i> -4	15.55	0.82	0.00-2.27	0.61	0.30-0.84	1.32	0.39-2.56	1.36	0.44-2.22	0.76	0.22-1.32	0.59	0.09-1.51	1.18	0.54-1.58	0.98	0.18-2.56					
16:3 <i>cis</i> -6	15.6	1.29	0.00-3.48	2.32	1.49-3.25	1.60	0.00-3.29	2.72	1.08-4.42	2.33	1.49-3.18	3.76	2.64-4.33	2.45	0.86-3.46	2.19	0.00-4.32					
16:1 <i>cis</i> -7	15.75	0.26	0.00-2.07	1.17	1.00-1.54	0.51	0.00-1.46	0.72	0.00-1.40	0.77	0.00-1.01	2.05	1.00-2.96	1.25	0.00-2.44	0.86	0.00-2.96					
16:1 <i>cis</i> -9	15.8	6.81	2.64-9.09	5.31	4.65-6.15	4.69	1.64-6.48	6.90	3.76-13.07	5.38	3.99-7.15	4.55	3.78-5.01	3.45	0.12-6.54	5.30	1.64-13.04					
16:1 <i>trans</i> -9	15.85	1.67	0.00-7.68	0.02	0.00-0.05	1.51	0.00-5.4	0.63	0.00-1.86	0.17	0.00-0.85	0.10	0.00-0.30	0.58	0.00-2.37	0.79	0.00-7.68					
16:1 <i>cis</i> -11	15.9	0.13	0.00-0.93	1.95	1.32-2.94	1.94	0.00-3.80	2.07	1.17-3.04	1.17	1.33-2.31	2.54	2.36-2.72	3.20	1.04-4.32	1.87	0.00-4.32					
16:1 <i>trans</i> -3	15.95	1.13	0.00-2.80	0.36	0.00-0.90	0.64	0.00-2.19	0.78	0.39-1.15	0.66	0.48-1.00	0.70	0.40-1.06	0.76	0.00-2.00	0.75	0.00-2.80					
17:1(?) ^b	16.33	0.06	0.00-0.12	0.02	0.00-0.10	0.00	0.00-0.00	0.00	0.00-0.00	0.00	0.00-0.00	0.04	0.00-0.13	0.04	0.00-0.21	0.03	0.00-0.13					
17:1	16.8	0.20	0.11-0.52	0.21	0.10-0.21	0.17	0.00-0.56	0.18	0.06-0.24	0.15	0.00-0.69	0.13	0.00-0.20	0.23	0.10-0.30	0.19	0.00-0.69					
18:3 <i>cis</i> -6	17.2	1.25	0.71-1.92	0.41	0.34-0.50	0.62	0.39-0.87	0.86	0.48-1.24	0.60	0.27-1.00	0.76	0.61-1.02	0.79	0.33-1.32	0.79	0.27-1.92					
18:4 <i>cis</i> -6	17.5	0.03	0.00-0.19	0.28	0.06-0.89	0.16	0.06-0.23	0.21	0.03-0.39	0.26	0.15-0.36	0.27	0.20-0.32	0.24	0.11-0.56	0.19	0.00-0.89					
18:2 <i>cis</i> -9	17.7	7.73	5.00-15.00	9.72	6.84-11.47	11.92	5.00-27.15	11.18	8.08-20.00	7.94	5.27-14.00	8.42	6.80-10.40	7.38	5.06-11.01	9.05	4.66-27.15					
18:3 <i>cis</i> -9	17.75	27.41	14.69-33.06	24.98	18.05-31.30	19.47	10.00-25.79	12.67	8.76-20.00	16.95	8.55-27.17	9.95	9.24-10.58	13.36	8.00-17.44	18.64	8.00-33.06					
18:1 <i>cis</i> -9	17.8	1.56	0.00-3.00	2.41	1.28-3.68	2.87	1.43-5.18	3.02	1.72-3.68	3.58	3.01-4.46	3.29	2.64-3.69	3.37	1.00-5.11	2.79	0.00-5.18					
18:1 <i>trans</i> -9	17.85	0.83	0.00-3.75	1.71	0.72-4.10	1.76	0.44-4.08	2.67	1.64-4.48	3.04	1.27-6.34	2.69	2.41-3.44	2.47	0.90-3.83	3.04	0.04-6.63					
20:4 <i>cis</i> -5	19.2	0.98	0.47-1.50	0.42	0.27-0.58	0.83	0.40-1.39	1.41	0.40-2.18	1.03	0.60-1.48	0.98	1.34-2.52	1.12	0.50-1.84	1.03	0.27-2.52					
20:4 <i>cis</i> -8	19.4	0.36	0.18-0.62	0.33	0.05-1.23	0.20	0.00-0.63	0.45	0.20-0.77	0.19	0.01-0.34	0.34	0.20-0.52	0.47	0.12-0.89	0.19	0.00-1.23					
20:2 <i>cis</i> -11	19.6	0.25	0.16-0.59	0.16	0.10-0.23	0.13	0.06-0.30	0.31	0.15-0.65	0.18	0.01-0.42	0.59	0.40-0.88	0.39	0.10-1.06	0.18	0.01-1.06					
Class C total		56.47	53.29-58.51	57.07	53.32-62.54	54.37	50.87-58.48	53.11	50.24-55.50	51.23	46.30-57.26	49.01	47.54-49.96	46.08	42.68-51.18	52.52	42.68-58.51					

Class D: hydroxy-substituted

acids																	
2OH-10:0	11.15	0.05	0.01-0.10	0.03	0.00-0.07	0.07	0.00-0.19	0.24	0.00-0.71	0.07	0.00-0.28	0.23	0.15-0.33	0.07	0.00-0.37	0.09	0.00-0.71
3OH-10:0	11.4	0.14	0.04-0.62	0.10	0.04-0.15	0.12	0.00-0.25	0.17	0.09-0.24	0.39	0.08-1.49	0.21	0.11-0.32	0.09	0.02-0.22	0.16	0.00-1.49
iso-3OH-11:0	12.1	0.08	0.00-0.15	0.15	0.07-0.21	0.13	0.02-0.22	0.18	0.08-0.25	0.09	0.01-0.20	0.23	0.14-0.34	0.16	0.01-0.35	0.14	0.00-0.35
2OH-12:0	13.2	0.04	0.00-0.11	0.00	0.00-0.00	0.05	0.00-0.14	0.11	0.00-0.47	0.05	0.00-0.18	0.18	0.11-0.25	0.05	0.00-0.28	0.06	0.00-0.47
3OH-12:0	13.5	0.01	0.00-0.05	0.00	0.00-0.00	0.02	0.00-0.12	0.05	0.00-0.23	0.03	0.00-0.07	0.13	0.09-0.16	0.02	0.00-0.13	0.03	0.00-0.16
iso-3OH-13:0	14.1	0.02	0.00-0.12	0.00	0.00-0.00	0.08	0.00-0.50	0.00	0.00-0.01	0.00	0.00-0.00	0.00	0.00-0.00	0.04	0.00-0.26	0.03	0.00-0.26
iso-3OH-14:0	15.1	0.21	0.00-1.00	0.60	0.00-1.13	0.60	0.00-1.38	0.71	0.44-1.03	0.46	0.00-0.78	0.42	0.00-0.78	0.74	0.06-1.20	0.53	0.00-1.38
iso 3OH-15:0	16.1	0.03	0.00-0.15	0.02	0.00-0.10	0.02	0.00-0.12	0.12	0.00-0.27	0.06	0.00-0.18	0.00	0.00-0.00	0.02	0.00-0.16	0.04	0.00-0.27
iso-3OH-17:0	18.1	0.53	0.10-0.73	0.15	0.01-0.27	0.26	0.01-0.49	0.17	0.10-0.20	0.12	0.00-0.19	0.04	0.01-0.10	0.28	0.03-1.23	0.26	0.00-1.23
2OH-17:0	18.2	0.07	0.00-0.18	0.08	0.00-0.29	0.05	0.00-0.25	0.10	0.00-0.38	0.00	0.00-0.00	0.00	0.00-0.00	0.06	0.00-0.23	0.06	0.00-0.38
3OH-17:0	18.4	0.23	0.09-0.38	0.23	0.12-0.31	0.32	0.14-0.49	0.34	0.23-0.44	0.29	0.20-0.42	0.34	0.26-0.34	0.36	0.23-0.80	0.30	0.09-0.49
Class D total	1.42	0.91-2.74	1.35	0.76-2.17	1.71	0.66-2.54	2.19	1.51-2.74	1.57	0.88-2.80	1.80	1.50-2.02	1.88	0.95-3.00	1.69	0.41-3.00	

Class E: branched-chain acids

iso-13:0	12.6	0.06	0.01-0.16	0.07	0.01-0.14	0.11	0.08-0.14	0.17	0.10-0.22	0.07	0.00-0.16	0.12	0.08-0.16	0.10	0.05-0.19	0.10	0.00-0.07
iso-14:0	13.6	0.07	0.00-0.24	0.08	0.00-0.21	0.10	0.00-0.17	0.15	0.00-0.30	0.06	0.00-0.20	0.08	0.06-0.10	0.27	0.12-0.87	0.12	0.00-0.87
iso-15:0	14.6	0.11	0.01-0.59	0.03	0.01-0.09	0.04	0.01-0.80	0.12	0.00-0.23	0.09	0.00-0.35	0.04	0.00-0.13	0.10	0.01-0.18	0.08	0.00-0.59
anteiso-15:0	14.7	0.12	0.01-0.62	0.02	0.01-0.05	0.05	0.01-0.11	0.13	0.01-0.26	0.07	0.00-0.28	0.13	0.00-0.22	0.11	0.01-0.25	0.09	0.00-0.62
iso-16:0	15.6	2.09	0.12-5.22	1.79	0.40-3.90	2.38	0.44-4.47	2.77	1.50-4.68	1.69	0.99-3.15	0.69	0.09-1.57	3.68	1.53-5.20	2.35	0.09-5.46
iso-17:0	16.6	0.61	0.26-1.08	0.23	0.17-0.26	0.39	0.17-0.52	0.38	0.07-0.60	0.27	0.12-0.44	0.41	0.26-0.52	0.38	0.24-0.51	0.40	0.07-1.08
anteiso-17:0	16.7	0.29	0.08-0.44	0.23	0.18-0.30	0.20	0.12-0.34	0.29	0.21-0.35	0.22	0.05-0.54	0.36	0.19-0.70	0.12	0.06-0.21	0.23	0.05-0.70
iso-19:0	18.6	0.08	0.00-0.14	0.05	0.00-0.15	0.12	0.01-0.19	0.07	0.01-0.10	0.05	0.00-0.15	0.03	0.01-0.08	0.08	0.00-0.17	0.07	0.00-0.19
anteiso-19:0	18.7	0.09	0.00-0.28	0.09	0.00-0.20	0.05	0.00-0.27	0.12	0.01-0.23	0.08	0.00-0.19	0.15	0.01-0.23	0.11	0.00-0.30	0.10	0.00-0.30
Class E total	3.51	0.94-7.15	2.59	1.00-5.00	3.44	1.19-5.69	4.19	3.30-6.17	2.61	1.46-5.09	2.06	0.92-3.77	4.97	2.75-6.65	3.55	0.92-6.65	

Class F: cyclopropane acids

cyclo-17:0	16.9	0.15	0.01-0.55	0.05	0.00-0.10	0.14	0.02-0.42	0.20	0.06-0.29	0.07	0.00-0.25	0.19	0.11-0.26	0.17	0.01-0.39	0.14	0.00-0.55
cyclo-19:0	18.9	0.26	0.00-1.30	0.15	0.00-0.75	0.36	0.10-0.53	0.55	0.00-1.37	0.40	0.00-0.68	0.88	0.46-1.14	0.50	0.00-1.48	0.41	0.00-1.48
Class F total		0.41	0.01-1.38	0.20	0.00-0.85	0.50	0.12-0.67	0.74	0.29-1.62	0.47	0.00-0.92	1.07	0.66-1.30	0.67	0.02-1.59	0.55	0.01-1.62

Class G: unsaturated branched-chain acids

chain acids																	
iso-15:1	14.4	0.04	0.00-0.27	0.02	0.00-0.05	0.03	0.00-0.10	0.02	0.00-0.04	0.06	0.00-0.24	0.00	0.00-0.00	0.02	0.00-0.09	0.03	0.00-0.24
iso-17:1	16.4	0.42	0.20-0.59	0.16	0.12-0.22	0.28	0.13-0.38	0.32	0.21-0.37	0.26	0.12-0.44	0.28	0.18-0.37	0.29	0.18-0.42	0.30	0.12-0.59
anteiso-17:1	16.7	0.29	0.13-0.59	0.03	0.00-0.11	0.05	0.00-0.12	0.18	0.00-0.38	0.05	0.00-0.25	0.20	0.00-0.53	0.13	0.00-0.23	0.14	0.00-0.59
iso-18:1	17.4	2.34	0.87-3.70	0.40	0.01-1.31	0.49	0.15-1.45	0.40	0.01-0.72	0.13	0.00-0.46	0.39	0.03-0.78	0.47	0.15-1.22	0.78	0.00-3.70
Class G total	3.09	1.20-4.54	0.61	0.14-1.60	0.85	0.42-1.83	0.92	0.37-1.33	0.50	0.25-1.39	0.87	0.23-1.68	0.91	0.39-1.63	1.25	0.14-4.54	

Unidentified components

	0.74	0.21-1.63	0.71	0.00-1.70	1.07	0.41-2.91	1.76	0.28-2.63	1.20	0.00-2.62	1.64	1.04-2.50	1.82	0.95-3.34	1.27	0.00-3.34
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^a ECL, equivalent chain length (carbon chain length).^b A question mark indicates that the compound is unsaturated (as determined chemically), but the site (or sites) has not been determined.

TABLE 3. Variability in fatty acid analysis data obtained after repeated extractions of cyanobacteria from three different *Azolla* hosts

Host ^a	Symbiont strain	No. of replicates examined ^b	Avg % Proximity to group model ^c
<i>A. nilotica</i>	N1	7	80.1 ± 4.07
<i>A. rubra</i>	R1	5	70.8 ± 2.92
<i>A. pinnata</i> subsp. <i>pinnata</i>	P1	3	76.5 ± 1.68

^a Plants were grown in defined mineral medium without nitrogen as described in the text.

^b Cyanobacteria were extracted from 14- to 18-day-old plants harvested at different times.

^c Average ± standard deviation proximity values for individual fatty acid profiles compared with a group model composed of the profiles of 40 different strains. None of the values was statistically significantly different.

published reports, in which smaller numbers of cyanobacterial samples were analyzed. Sixteen major fatty acid components (each constituting more than 0.9% of the total fatty acids) accounted for 87.92% of the total fatty acids. These major components included the saturated 14:0 (0.99%), 16:0 (34.94%), and 18:0 (1.76%) fatty acids; the unsaturated straight-chain 12:1 (0.90%), 14:1 *cis*-9 (1.83%), 16:1 *cis*-9 (5.30%), 16:1 *cis*-11 (1.87%), 16:3 *cis*-6 (2.19%), 16:4 *cis*-4 (0.98%), 18:2 *cis*-9 (9.05%), 18:3 *cis*-9 (18.64%), 18:1 *cis*-9 (2.79%), 18:1 *trans*-9 (3.04%), and 20:4 *cis*-5 (1.03%) fatty acids; and the branched-chain iso-16:0 fatty acid (2.35%) (Table 2).

We detected and identified 44 minor fatty acid components which accounted for 12.04% of the total fatty acids and three components that were present at trace levels. These included hydroxy-substituted fatty acids (1.69%), branched-chain fatty acids other than the iso-16:0 fatty acid (which accounted for 2.35% of the total of 3.55% determined for this class), and cyclopropane acids (0.55%). We also detected an incompletely characterized unsaturated 16-carbon fatty acid (1.23%) with an equivalent chain length of 15.5. The most abundant fatty acid was the 16:0 fatty acid, which accounted for between 28.89 and 46.16% of the total fatty acids (Table 2).

Variability due to experimental procedures. The differences in the fatty acid compositions of individual strains due to the experimental procedures which we used were not significant statistically. The average proximity coefficient obtained when we compared the results of seven replicate analyses of cya-

nobacterial isolate N1 obtained from *A. nilotica* with the results obtained for the entire *A. nilotica* group (Table 2) was 80.1%, and the individual proximity coefficients ranged from 76.2 to 83.3% (all with a standard deviation of ±4.07%). Similarly, the differences in the data for the two other strains analyzed were not statistically significant (Table 3).

Variability due to host. We observed statistically significant differences in the fatty acid compositions of cyanobacteria obtained from different hosts. Differences in the following parameters were significant at a level of 99%: concentrations of the 16:0 and 18:3 fatty acids, total concentrations of the polyunsaturated fatty acids, 16-carbon and 18-carbon fatty acids, class A fatty acids (straight-chain saturated fatty acids), class C fatty acids (straight-chain unsaturated fatty acids), and class G fatty acids (branched-chain unsaturated fatty acids), and ratios of unsaturated fatty acids (classes C and G) to saturated fatty acids (classes A and B) (Table 4).

The results of an analysis of variance suggested that the cyanobacterial symbionts obtained from the seven hosts could be positioned along a continuum on the basis of the values obtained for the variable fatty acid parameters. The cyanobacterial symbionts obtained from *A. filiculoides* were at one end of the continuum, the symbionts obtained from *A. caroliniana*, *A. mexicana*, *A. microphylla*, and *A. rubra* were in intermediate positions, and the symbionts obtained from *A. nilotica*, *A. pinnata* subsp. *pinnata*, and *A. pinnata* subsp. *imbricata* (the latter two as a combined group) were at the other end. Segregation of the host species into clusters along the continuum was evident when the data were subjected to an analysis of Euclidian distances and a dendrogram was constructed.

The dendrogram which we constructed indicated that the cyanobacteria obtained from the seven *Azolla* host species segregated into two main clusters, with a secondary dichotomy in one of these clusters (Fig. 1). The symbionts obtained from *A. pinnata*, *A. nilotica*, and *A. rubra* constituted the first cluster, and the other *Azolla* symbiont cluster was composed of symbionts obtained from *A. filiculoides*, *A. microphylla*, *A. mexicana*, and *A. caroliniana*. The symbionts obtained from *A. filiculoides* were separated from the other symbionts by a secondary dichotomy.

DISCUSSION

Host species have been shown to be significant sources of differences in the fatty acid compositions of populations of

TABLE 4. Alignment of cyanobacterial symbionts obtained from seven *Azolla* species based on statistical differences in fatty acid parameters

Host species	% of the following fatty acids in cyanobacterial symbionts ^a :									Ratios ^a	
	Class A fatty acids		Class C fatty acids			Class G fatty acids	All PUFA	All C ₁₆ fatty acids	All C ₁₈ fatty acids	Class C/classes A and B	Classes C and G/classes A and B
	All class A fatty acids	16:0	All class C fatty acids	18:3 <i>cis</i> -9	C ₁₈ PUFA						
<i>A. filiculoides</i>	33.3 (a) ^b	31.2 (a)	56.5 (a)	27.4 (a)	36.4 (a)	3.1 (a)	40.2 (a)	44.2 (a)	40.0 (a)	1.7 (a)	1.8 (a)
<i>A. microphylla</i>	36.7 (ab)	33.9 (a)	57.1 (a)	25.0 (a)	35.4 (a)	0.6 (b)	39.4 (a)	47.1 (a)	40.9 (a)	1.5 (a)	1.6 (b)
<i>A. caroliniana</i>	37.4 (bc)	34.3 (ab)	54.4 (ab)	19.5 (b)	32.2 (b)	0.9 (b)	36.4 (b)	47.7 (a)	38.4 (ab)	1.4 (ab)	1.5 (b)
<i>A. mexicana</i>	35.9 (ab)	32.3 (a)	53.1 (b)	12.7 (bc)	24.9 (bc)	0.9 (b)	31.7 (c)	48.9 (ab)	32.5 (b)	1.5 (a)	1.5 (b)
<i>A. rubra</i>	41.2 (c)	35.5 (bc)	51.2 (b)	17.0 (bc)	25.8 (bc)	0.5 (b)	30.6 (c)	49.8 (ab)	35.2 (b)	1.2 (b)	1.2 (c)
<i>A. nilotica</i>	42.2 (c)	36.8 (bc)	49.0 (c)	10.0 (c)	19.4 (bc)	0.9 (b)	27.1 (d)	53.5 (b)	28.3 (c)	1.1 (b)	1.2 (c)
<i>A. pinnata</i> ^c	42.9 (c)	39.8 (c)	46.1 (c)	13.4 (c)	21.8 (c)	0.9 (b)	27.6 (d)	54.3 (b)	29.1 (c)	1.1 (b)	1.1 (c)

^a The values are the means for the fatty acid contents of the strains obtained from the hosts listed in Table 1. The class A fatty acids are the saturated, even-carbon-number, straight-chain fatty acids; the class B fatty acids are the saturated, odd-carbon-number, straight-chain fatty acids; the class C fatty acids are the unsaturated, straight-chain fatty acids; and the class G fatty acids are the unsaturated, branched-chain fatty acids. PUFA, polyunsaturated fatty acid.

^b In each column values that are not followed by the same letter are statistically different at least at the 0.05% level.

^c Combined data for all symbiont strains obtained from the two subspecies of *A. pinnata*.

FIG. 1. Dendrogram based on the results of a cluster analysis of Euclidian distances between cyanobacteria obtained from seven *Azolla* host species. In this analysis we used average fatty acid values that were statistically different in symbionts of different host species (see Table 3). The horizontal distances are Euclidian distances between groups. The *Azolla* host species used were *A. filiculoides*, *A. microphylla*, *A. caroliniana*, *A. mexicana*, *A. rubra*, *A. nilotica*, and *A. pinnata*.

cyanobacterial symbionts isolated from members of the genus *Azolla*. These differences may be due to differences in the ecological niches in which the symbiotic cyanobacteria develop, since each *Azolla* species may be assumed to provide a distinct physiological, nutritional, and genetic environment. Morphologically, the *Azolla* cyanobacterial symbionts appear to be highly variable within certain parameters and also exhibit differences as a function of the host species (1).

The clustering of *Azolla* cyanobacterial symbionts which we observed based on the results of a fatty acid analysis correlates remarkably well with genetic clustering of selected tropical and temperate American *Azolla* species. Zimmerman et al. (25–27) reported that *A. filiculoides* had a distinct DNA fingerprint that could be distinguished from the fingerprint of a group composed of *A. caroliniana*, *A. mexicana*, and *A. microphylla*. In addition, *A. rubra* was found to be distinct from the latter group. Similarly, the fatty acid composition of *A. filiculoides* symbionts was different from the fatty acid composition of symbionts of *A. caroliniana*, *A. mexicana*, and *A. microphylla*, all of which were separated from a secondary *Azolla* subgroup. As was found in the DNA analysis, *A. rubra* was a member of the second group (1). This correlation between cyanobacterial symbiont fatty acid composition and host genetics suggests that the *Azolla* cyanobacteria coevolved into distinct groups along with their hosts.

While it is difficult to detect and prove coevolution, there are other lines of evidence that strengthen the concept with regard to the *Azolla* cyanobacteria. Although there is no mention of symbiotic cyanobacteria in studies of the *Azolla* fossil record (12), the following characteristics suggest that the symbiotic relationship evolved at an early stage: (i) the obligate nature of the cyanobacterial partners in the symbiosis (18, 20); (ii) the

synchronization of cyanobacterial and plant life cycles (18); and (iii) the fact that cyanobacteria caused induction and buildup of carbohydrates in plants utilized for bacterial growth (4). In addition, coevolution could have been driven by geographic isolation of the ferns since some members of the subgenus *Azolla* (*A. caroliniana*, *A. mexicana*, *A. microphylla*, and *A. filiculoides*) are found in the Americas and *A. rubra* occurs in Australia and New Zealand. The subgenus *Rhizosperma* contains *A. nilotica* (which occurs in Africa) and *A. pinnata* (which occurs in Africa and Asia).

Analyses of the cellular fatty acid profiles of members of the closely related but free-living cyanobacterial genera *Anabaena* and *Nostoc* revealed similarities with, but also significant differences from, the fatty acid profiles of the *Azolla* cyanobacterial symbionts (3). The *Azolla* cyanobacteria, therefore, may be a distinct taxon and may even be composed of different subspecies, each of which correlates with one of the dichotomous groups detected in the fatty acid analysis.

There is restriction fragment length polymorphism evidence that *Azolla* cyanobacterial symbionts arose from a common ancestor (10). Some morphological data, such as the occurrence of double heterocysts and synchronized cell expansion, support the hypothesis that these organisms had a common ancestor (1). Furthermore, division of the cyanobacteria into subgenera (whose members occur in members of *Azolla* subgenera *Azolla* and *Rhizosperma*) has been confirmed by morphological characteristics, including early hormogonium cell growth and differences in the large akinetes, and G+C contents (1).

Regarding the evolution of the host species, Hills and Gopal (12) presented an evolutionary scheme in which the subgenus *Azolla* gave rise to the subgenus *Rhizosperma*. According to the available fossil record, the possible ancestor of the subgenus *Rhizosperma* is an unknown species (no fossil record) which lived during the Upper Cretaceous Period (100×10^6 to 80×10^6 years ago). Division into two subgenera, *Azolla* and *Rhizosperma*, may have started in the Mesozoic Era, with progressive changes in float number and glochidium (i.e., sexual organ) development continuing until relatively recent times (12). It is noteworthy that the cyanobacterial symbionts of *A. rubra* are considered more closely related to symbionts of species belonging to the subgenus *Rhizosperma* than to symbionts of species belonging to the subgenus *Azolla*. *A. rubra* is placed in the subgenus *Azolla* because of a general morphological resemblance to *A. caroliniana*, *A. filiculoides*, and *A. microphylla*. However, Mahabalé (14) found that *A. rubra* has characteristics of *Azolla primaeva* and *Azolla prisca*, which are extinct species which lived during the Eocene and Oligocene periods (49×10^6 to 30×10^6 years ago). *A. primaeva* and *A. prisca* are transitional species whose descriptions are based on reductions in the structure of the glochidia. Therefore, *A. rubra* may be a link between the subgenera *Azolla* and *Rhizosperma* or may belong to a new subgenus whose origin is different from that of the other subgenera.

ACKNOWLEDGMENTS

We acknowledge the contributions of Gerald Peters (Virginia Commonwealth University, Richmond), Charles Van Hove (Université Catholique de Louvain, Louvain-la-Neuve, Belgium), William Zimmerman (University of Michigan, Dearborn), Roxana Olvera (Mexico City, Mexico), William Rains (University of California, Davis), and Sandra Nierzwicki-Bauer (Rensselaer Polytechnic Institute, Troy, N.Y.), who provided some of the *Azolla* plants used in this study. We thank Thomas Hartman and Joseph Lech (Center for Advanced Food Technology, Rutgers-The State University of New Jersey, New Brunswick) and Larry G. Revear (U.S. Department of Agriculture) for technical assistance. We also thank Peter Anderson (Department of

Biochemistry and Microbiology, Rutgers-The State University of New Jersey, New Brunswick) for word processing.

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