J. Phycol. **35,** 587–598 (1999)

BIOCHEMICAL TAXONOMY AND MOLECULAR PHYLOGENY OF THE GENUS *CHLORELLA* SENSU LATO (CHLOROPHYTA)[1]

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A multimethod approach was used to characterize unicellular green algae that were traditionally assigned to the genus *Chlorella* Beijerinck and to resolve their phylogenetic relationships within the *Chlorophyta*. Biochemical, physiological, and ultrastructural characters, together with molecular data such as DNA base composition and DNA hybridization values, were compared with a molecular phylogeny based on complete 18S rRNA sequences. Our results show that *Chlorella* taxa are dispersed over two classes of chlorophytes, the *Trebouxiophyceae* and the *Chlorophyceae*. We propose that only four species should be kept in the genus *Chlorella (Chlorophyta, Trebouxiophyceae): C. vulgaris* Beijerinck, *C. lobophora* Andreyeva, *C. sorokiniana* Shih. et Krauss, and *C. kessleri* Fott et Nováková. Common characteristics of these taxa are glucosamine as a dominant cell wall component and the presence of a double thylakoid bisecting the pyrenoid matrix. Norspermine, norspermidine, and secondary carotenoids are never produced. Other *"Chlorella"* species belong to different taxa within the *Trebouxiophyceae* (*"C." protothecoides* = *Auxenochlorella protothecoides* [Krüger] Kalina et Punčochářová, *"C." ellipsoidea, "C." mirabilis, "C." saccharophila,* and *"C." luteoviridis*) and *Chlorophyceae* (*"C." zofingiensis* and *"C." homosphaera* = *Mychonastes homosphaera* Kalina et Punčochářová). The latter taxa can easily be recognized by the production of secondary carotenoids under nitrogen-deficient conditions.

Key index words: **18S rRNA; chemotaxonomy; *Chlorella;* Chlorophyta; DNA base composition; DNA/DNA hybridization; molecular systematics; *Muriella;* phylogeny; *Prototheca; Scenedesmus***

Members of the genus *Chlorella* Beijerinck are among the best-studied unicellular green algae. Since Warburg (1919) introduced mass cultures of *Chlorella* for basic research, these algae have served as model organisms for pioneering plant physiological and biochemical studies of, for example, photosynthesis and nitrate reduction (Warburg and Negelein 1920, Pirson 1937, Kessler 1953, Falkowski and Raven 1997). In agriculture and biotechnology, they are extensively used in some countries for feeds (single cell protein) or for human consumption as protein-rich ''health'' food, in waste treatment and water recovery, as a gas exchange system, and for microbial energy production (Golueke and Oswald 1964, Fogg 1971, Soeder 1976, 1980, Abbott and Cheney 1982 and references therein, Dunahay et al. 1992). *Chlorella* extracts have even been ascribed to possess antitumor (Konishi et al. 1985, Miyazawa et al. 1988) and antimicrobial effectiveness (Pratt and Spoehr 1944, Tanaka et al. 1986).

The lack of obvious morphological characters combined with an exclusively asexual reproductive cycle by means of autospores has caused considerable problems in the taxonomic description and identification of *Chlorella* species (Kessler and Huss 1992). However, the choice of suitable strains or species is crucial for many of the previously mentioned applications (Kessler 1986, 1992). In the past, numerous methods have been applied to the taxonomy of *Chlorella.* Many of them were based on nutritional requirements of *Chlorella* strains when cultivated under autotrophic or heterotrophic conditions (Shrift and Sproul 1963, Shihira and Krauss 1965). Also, numerical classification strategies have been applied for the evaluation of taxonomic conclusions (Cullimore 1969, DaSilva and Gyllenberg 1972). In their monograph of the genus *Chlorella,* Fott and Nováková (1969) combined morphological and structural features with some physiological characteristics, resulting in the description of nine species and six varieties. Others have used serological cross-reactions (Sanders et al. 1971, Maruyama 1977, Kümmel and Kessler 1980) for the identification and classification of *Chlorella* strains. A third categorization has been based on the ultrastructure and chemical composition of the cell wall (Atkinson et al. 1972, Conte and Pore 1973, Yamada and Sakaguchi 1982, Blumreisinger et al. 1983, Takeda 1991, 1996a) and pyrenoid ultrastructure (Ikeda and Takeda 1995). The most acknowledged and practicable system for species delimitation in *Chlorella* proved to be a chemotaxonomical classification scheme combining biochemical and physiological characters (Kessler and Soeder 1962, Kessler 1982, 1984, 1992). This work led to the characterization of 19 taxa, some of which exhibited striking differences (Table 1). In contrast, other important genera of green algae, such as *Scenedesmus* and *Ankistrodesmus/Monoraphidium,* appeared physiologically and biochemically much more uniform (Hellmann and Kes-

[1] Received 17 June 1998. Accepted 15 January 1999.

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TABLE 1. Biochemical and physiological characters of 19 *Chlorella* taxa (including three taxa belonging to the genus *Scenedesmus*). Hydr. = hydrogenase, Sec. car. = secondary carotenoids, NO_3^- = nitrate reduction, Thiam. = thamine requirement, B_{12} = vitamin B_{12} requirement, Mann. = growth on mannitol, pH = limit of growth at pH, %NaCl = limit of growth, °C = upper limit of growth, %GC = mol% G+C of the DNA.

Species	No. of Strains	Hydr.	Sec. car.	NO_3^-	Thiam.	B_{12}	Mann.	pH	% NaCl	°C	%GC
C. vulgaris	20	−	−	+	−	−	−	3.5–4.5	3–4	28–32	58–63
C. lobophora	1	−	−	+	−	−	−	4.0	1	30	61
C. sorokiniana	17	+	−	+	−	−	−	3.5–5.0	<1–3	36–42	62–68, 73–75
C. spp. (''*paramecii*'')	3	+	−	+(−)	+	+	−	5.5	<1	26–30	66–67
C. spp. 211–18	1	+	−	+	−	−	−	5.0	1	28	51
C. kessleri	10	+	−	+	−	−	−	2.5–3.0	1–2	34–36	54–57
C. minutissima	2	−	−	+	−	−	−	5.5	1	32	46
''*C.*'' *protothecoides* (= *Auxenochlorella protothecoides*)	16	−	−	−	+	−	−	3.5–4.0	3–4	28–34	58–62
''*C.*'' *ellipsoidea*	2	−	−	+	−	−	−	2.0–3.0	2	28–30	56–57
''C.'' mirabilis	3	−	−	+	−	−	−	4.0	<1–1	26–28	56–58
''*C.*'' *luteoviridis*	6	−	−	+	−	−	+	3.0	3–5	28	44–45
''*C.*'' *saccharophila*	6	−	−	+	−	−	+	2.0–3.0	4–6	26–30	49–52
''*C.*'' *saccharophila* 211-9b (= *Watanabea reniformis*)	1	−	−	+	−	−	+	3.0	3	26	49
''*C.*'' *homosphaera* (= *Mychonastes homosphaera*)	1	+	+	+	−	−	−	6.0	<1	28	73
''*C.*'' *zofingiensis*	3	−	+	+	−	−	−	5.0–5.5	1	28	50
''*C.*'' *zofingiensis* C-1.2.1 (= SAG 4.80) (=*Muriella aurantiaca*)	1	−	+	+	−	−	−	4.5	1	28	63
''*C.*'' *fusca* var. *fusca* (= *Scenedesmus abundans*)	1	+	+	+	−	−	−	4.0	2	34	55
''*C.*'' *fusca* var. *rubescens* (= *Scenedesmus rubescens*)	1	+	+	+	−	−	−	4.5	3	30	57
''*C.*'' *fusca* var. *vacuolata* (= *Scenedesmus vacuolatus*)	11	+	+	+	−	−	−	3.0–3.5	3	32–36	50–52

sler 1974b, Kessler 1982; Kessler et al. 1997). These results suggested substantial heterogeneity within the genus *Chlorella*.

Comparative studies of nucleic acids are indispensable for tracing the phylogenetic relationships of these algae. Previous work on the DNA base composition revealed an amazingly broad range (44%–75%) of the molar guanosine + cytidine (GC) content within the genus *Chlorella* (Fig. 1, Table 1). This heterogeneity was subsequently confirmed by extensive quantitative DNA/DNA hybridization studies (Kerfin and Kessler 1978, Huss et al. 1986, 1987a, b, 1988, 1989a, b). Under ''optimal'' DNA reassociation conditions, no significant DNA hybridization could be detected between any species and, in some cases, not between strains of a single species. Extended studies using ''relaxed'' reassociation conditions (Huss et al. 1989a) revealed an interspecific relationship between *C. vulgaris* and *C. sorokiniana,* a species formerly called *C. vulgaris* forma *tertia* by Fott and Nováková (1969). Both species appear morphologically identical, and *C. sorokiniana* could be separated from *C. vulgaris* only by its possession of hydrogenase activity and thermophily (Table 1; Kessler 1982). More surprisingly, a similar close relationship could be shown for the three varieties of *C. fusca* and species of the morphologically different genus *Scenedesmus.* A possible relationship between *C. fusca* and *Scenedesmus* has been proposed before on evidence of similarity of sterols (Patterson 1974), ribosomal proteins (Götz and Arnold 1980a, b), and cytochrome c-553 (Kümmel and Kessler 1980). On the basis of submicroscopical structures of the cell wall, Fott et al. (1975) recognized only *C. fusca* var. *fusca* as a unicellular member of the genus *Scenedesmus* (later described as *S. abundans* by Hegewald and Schnepf 1991), whereas such a relation was not acknowledged for the varieties *vacuolata* and *rubescens.*

The resolution of DNA/DNA reassociation studies allows only the detection of closely related species of a given genus (cf. Schleifer and Stackebrandt 1983). To reveal the relationships between all *Chlorella* species and to determine their phylogenetic position within the Chlorophyta, comparative sequence analyses of conserved genes, such as the 18S ribosomal RNA genes, are especially useful. Previous work based on that gene supported the proposed heterogeneity of the genus *Chlorella* (Huss and Sogin 1990) and showed that the three former varieties of *''C. fusca''* are different species of the genus *Scenedesmus* (Kessler et al. 1997). Here we present a comprehensive 18S rRNA based phylogeny of the genus *Chlorella* sensu lato, including species of *Prototheca, Muriella,* and *Scenedesmus.*

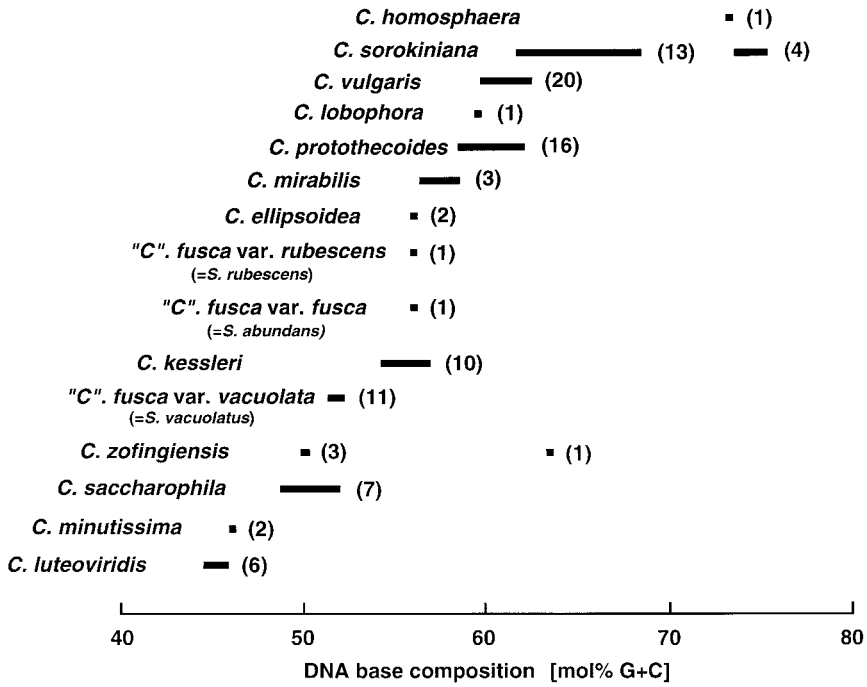

FIG. 1. Summary of the nuclear DNA base composition in the genus *Chlorella* sensu lato. The bars represent the range of molar guanosine + cytidine (GC) contents determined for the number of strains shown in brackets. Modified after Huss and Jahnke (1994).

MATERIALS AND METHODS

All organisms used in this investigation, their strain numbers, origins, and the GenBank/EMBL accession numbers for their 18S rRNA gene sequences are listed in Table 2. The sequences of 17 strains of *Chlorella* and related taxa that were determined in this study are indicated by an asterisk. Other organisms taken for reference were chosen to show the relationships of *Chlorella* species to other genera in the Trebouxiophyceae/Chlorophyceae. The ulvophycean algae *Ulothrix zonata* and *Gloeotilopsis planctonica* were used as ''outgroups'' in the phylogenetic analyses.

Mass culturing of algae, DNA isolation and purification, and determination of the DNA base composition (GC content) have been described previously (Huss et al. 1986). DNA reassociation kinetics for the quantitative determination of DNA similarities were determined optically in a UV/VIS spectrophotometer (Gilford Response™) equipped with a thermoprogrammer as described by Huss et al. (1987b). Nucleic acid similarities of heterologous DNAs, expressed as degree of binding (%D), were calculated using the equation of De Ley et al. (1970). Mean values were taken from at least three independent determinations of the thermal melting points and DNA similarities.

The 18S rRNA genes were amplified from total genomic DNA or from isolated nuclear DNA (Huss et al. 1988) by the polymerase chain reaction (PCR) as described by Huss and Sogin (1990) with eukaryote-specific synthetic oligonucleotide amplification primers (Table 3). The amplified DNA fragments were either cloned into bacteriophages M13mp18 and M13mp19 (Medlin et al. 1988) or directly sequenced taking advantage of the Dynabeads-280 streptavidin system (Hultman et al. 1991). Sequences of the coding and noncoding strand were determined by the dideoxynucleotide chain-terminating sequencing method (Sanger et al. 1977) with oligonucleotide primers that are complementary to evolutionary conserved regions of the 18S rRNA gene (Table 3).

All sequences used in this work were manually aligned on a MicroVAX computer with the sequence editor program distributed by G. Olsen (Olsen et al. 1992). For the phylogenetic analyses, the sequences for organisms listed in Table 2 were extracted from a larger alignment that included about 250 sequences of green algae as well as several sequences from land plants and other groups of organisms. To improve the alignment of the data set, secondary structure models that combined features of the models proposed by Huss and Sogin (1990) and Neefs and De Wachter (1990) were constructed for all sequences. Highly variable regions that could not be aligned unambiguously for all sequences used were excluded from the analyses, resulting in a total of 1751 positions. The alignment is available from the authors on request.

Phylogenetic trees were inferred from the aligned sequence data by the neighbor-joining (NJ), the maximum parsimony (MP), and the maximum likelihood (ML) method. Neighbor-joining and MP bootstrap analyses (Felsenstein 1985) were conducted with the PHYLIP package 3.572c of Felsenstein (1995) on a Silicon Graphics Indy computer. For the NJ analysis (Saitou and Nei 1987), the correction of Kimura (1980) was used to convert pairwise sequence similarities into evolutionary distances, the addition of taxa was jumbled, and a transition/transversion ratio of 2.0 was selected. The same ratio was used for the MP analysis with random addition of taxa repeated three times for each individual bootstrap replication. A total of 1000 bootstrap resamplings was used for each method.

For the ML analyses, the fastDNAml program of Olsen et al. (1994) was used to infer the tree topology shown in Figure 2. This topology has the largest Ln likelihood that could be achieved in 10 independent analyses using the generalized two-parameter model of evolution (Kishino and Hasegawa 1989), empirical base frequencies, and random addition of taxa. In addition, 100 bootstrap replications were carried out with a varying input order of taxa until the best Ln likelihood score was reached three times in a maximum of eight independent searches within each bootstrap replication.

RESULTS

Table 1 shows a chemotaxonomic classification system that allows assignment of unidentified strains of *Chlorella*-like algae to the indicated taxa. Data are gathered from the work of Kessler (cf. Kessler and Huss 1992) with new information added. The advantage of this system is that most of the biochemical and physiological characters considered can easily be determined without special equipment. In addition to the DNA base compositions of more than

TABLE 2. List of organisms used in the analyses, their origin, and their GenBank/EMBL accession numbers for 18S rRNA gene sequences. ALCP = L'Algothèque, Laboratoire de Cryptogamie, Paris; Andreyeva = V.M. Andreyeva, St. Petersburg, Russia; Baslerová = M. Baslerová, Praha, Czech Republic; Bethesda = Culture Collection at Bethesda, Maryland, USA; CCAP = Culture Centre (now Collection) of Algae and Protozoa, Cambridge (now Ambleside), UK; CCHU = Culture Collection Hiroshima University; CBS = Carolina Biological Supply; IB = Culture Collection of the Botanical Institute at Innsbruck, Austria; Pore = R.S. Pore, Morgantown, West Virginia, USA; SAG = Sammlung von Algenkulturen der Universität Göttingen, Germany (Schlösser 1994); UTEX = University of Texas Culture Collection (Starr and Zeikus 1993).

Taxonomic position	Species	Strain	GenBank/EMBL acc. no.	Reference[a]
Chlorophyta				
Chlorophyceae[b]	*Ankistrodesmus stipitatus* (Chodat) Komárková-Legnerová	SAG 202-5	X56100	A
	Characium hindakii Lee et Bold	UTEX 2098[c]	M63000	B
	Chlamydomonas humicola Lucksch	UTEX 225	U13984	C
	Chlamydomonas reinhardtii Dangeard	Unknown	M32703	D
	''*Chlorella*'' *homosphaera* Skuja (= *Mychonastes homosphaera* [Skuja] Kalina et Punčochářová)	CCAP 211/8e[c]	X73996	*
	''*Chlorella minutissima*'' Fott et Nováková (= *Mychonastes homosphaera* [Skuja] Kalina et Punčochářová)	Lefèvre ALCP no. 87	Y13761	*
	''*Chlorella*'' *zofingiensis* Dönz	SAG 211-14[c]	X74004	*
	''*Chlorella*'' *zofingiensis* Dönz (= *Muriella aurantiaca* Vischer)	Bethesda C-1.2.1	X74005	*
	Hydrodictyon reticulatum (Linné) Lagerheim	CBS	M74497	E
	Muriella aurantiaca Vischer	SAG 249-1[c]	X91268	*
	Neochloris aquatica Starr	UTEX 138	M62861	B
	Pediastrum duplex Meyen	Isolated by L.W.W.	M62997	B
	Scenedesmus abundans (Kirchn.) Chod. (= ''*Chlorella fusca* var. *fusca*'' Shih. et Krauss)	UTEX 343	X73995	F
	Scenedesmus costato-granulatus Skuja	SAG 18.81	X91265	F
	Scenedesmus obliquus (Turpin) Kützing	SAG 276-3a	X56103	A
	Scenedesmus producto-capitatus Schmula	SAG 21.81	X91266	F
	Scenedesmus rubescens (Dangeard) Kessler et al. (= ''*Chlorella fusca* var. *rubescens*'' [Dangeard] Kessler et al.	CCAP 232/1[c]	X74002	F
	Scenedesmus vacuolatus (Shih. et Krauss) Kessler et al. (= ''*Chlorella fusca* var. *vacuolata*'' Shih. et Krauss)	SAG 211-8b[c]	X56104	F
	Spermatozopsis similis Preisig et Melkonian	SAG 1.85	X65557	G
	Volvox carteri f. *nagariensis*	UTEX 1885	X53904	H
Trebouxiophyceae[b]	''*Chlorella*'' *ellipsoidea* Gerneck	SAG 211-1a[c]	X63520	*
	Chlorella kessleri Fott et Nováková	SAG 211-11g[c]	X56105	A
	Chlorella lobophora Andreyeva	Andreyeva 750-I[c]	X63504	*
	''*Chlorella*'' *luteoviridis* Chodat	SAG 211-2a[c]	X73997	*
	Chlorella minutissima Fott et Nováková	Bethesda C-1.1.9	X56102	A
	''*Chlorella*'' *mirabilis* Andreyeva	Andreyeva 748-I[c]	X74000	*
	''*Chlorella*'' *protothecoides* Krüger (= *Auxenochlorella protothecoides* [Krüger] Kalina et Punčochářová)	SAG 211-7a[c]	X56101	A
	''*Chlorella*'' *saccharophila* (Krüger) Migula	SAG 211-9a[c]	X63505	*
	''*Chlorella*'' *saccharophila* (Krüger) Migula (= *Watanabea reniformis* Hanagata et al.)	SAG 211-9b	X73991	*
	Chlorella sorokiniana Shihira et Krauss	SAG 211-8k[c]	X62441	*
		SAG 211-40a	X73993	*
		Baslerová Prag A14	X74001	*
	Chlorella spp.	SAG 211-18	X73992	*
	Chlorella vulgaris Beijerinck	SAG 211-11b[c]	X13688	A
	Dictyochloropsis reticulata (Tschermak-Woess) Tschermak-Woess	CCHU 5616	Z47207	I
	Leptosira terrestris (Fritsch et John) Friedl	SAG 463-3	Z28973	J, K
	Myrmecia bisecta Reisigl	IB T74	Z47209	I
	Nanochlorum eucaryotum Menzel et Wild	SAG 55.87	X06425	L
	Prototheca wickerhamii Tubaki et Soneda	SAG 263-11	X74003	*
		Pore 1283	X56099	A
	Prototheca zopfii Krüger	SAG 263-1	X63519	*
	Trebouxia impressa Ahmadjian	UTEX 892	Z21551	J
Ulvophyceae	*Gloeotilopsis planctonica* Iyengar et Philipose	SAG 29.93	Z28970	J
	Ulothrix zonata (Weber et Mohr) Kützing	SAG 38.86	Z47999	K

[a] *: This work; A: Huss and Sogin (1990); B: Lewis et al. (1992); C: Gordon et al. (1995); D: Gunderson et al. (1987); E: Wilcox et al. (1992); F: Kessler et al. (1997); G: Sensen et al. (1992); H: Rausch et al. (1989); I: Friedl (1995); J: Friedl and Zeltner (1994); K: Friedl (1996); L: Sargent et al. (1988).

[b] *sensu* Friedl (1995).

[c] Type species.

TABLE 3. Oligonucleotide primers used for PCR and sequencing reactions of SSU rRNA genes of green algae.

Primer name	Annealing site[a]	Sequence[b]	Reference
PCR primers			
5′-PCR[c]	1–21	CCGAATTCGTCGACAACCTGGTTGATCCTGCCAGT[d]	Medlin et al. 1988
5′-PCR[e]	1–21	WACCTGGTTGATCCTGCCAGT[f]	This work
3′-PCR[c]	1774–1798	CCCGGGATCCAAGCTTGATCCTTCTGCAGGTTCACCTAC[d]	Medlin et al. 1988
3′-PCR[e]	1774–1798	GATCCTTCYGCAGGTTCACCTAC[f]	This work
Sequencing primers[g]			
M13 Universal		GTAAAACGACGGCCAGT	Pharmacia, USB
1>	4–20	CTGGTTGATCCTGCCAG[h]	Gunderson et al. 1986
82>	84–100	GAAACTGCGAATGGCTC	M.L. Sogin, pers. comm.
300>	371–387	AGGGTTCGATTCCGGAG	Elwood et al. 1985
528>	576–591	CGGTAATTCCAGCTCC	Gunderson et al. 1986
690>	899–913	YAGAGGTGAAATTCT	Elwood et al. 1985
920>	1132–1148	GAAACTTAAAKGAATTG	Elwood et al. 1985
960>	1187–1202	TTTGACTCAACACGGG	Gunderson et al. 1986
1055>	1269–1283	GGTGGTGCATGGCCG	M.L. Sogin, pers. comm.
1200>	1428–1443	CAGGTCTGTGATGCCC	Modified after Gunderson et al. 1986
1400>	1629–1645	TGYACACACCGCCCGTC	Elwood et al. 1985
108<	110–96	CTGATTTAATGAGCC	Modified after Gunderson et al. 1986
300<	398–382	TCAGGCTCCCTCTCCGG	Elwood et al. 1985
536<	584–567	GWATTACCGCGGCKGCTG	Gunderson et al. 1986
690<	913–899	AGAATTTCACCTCTG	Elwood et al. 1985
920<	1146–1132	ATTCCTTTRAGTTTC	Elwood et al. 1985
1055<	1283–1269	CGGCCATGCACCACC	Elwood et al. 1985
1200<	1443–1428	GGGCATCACAGACCTG	Gunderson et al. 1986
1400<	1644–1630	ACGGGCGGTGTGTRC	Elwood et al. 1985
1490<	1767–1749	CTTGTTACGACTTCTCC[h]	This work
1520<	1789–1774	CYGCAGGTTCACCTAC[h]	Gunderson et al. 1986

[a] Annealing positions refer to the SSU rRNA sequence of *Chlorella vulgaris* (Huss and Sogin 1989).
[b] Ambiguous nucleotides are abbreviated according to the IUB-standard: K = G/T, R = A/G, W = A/T, Y = C/T.
[c] PCR primers used for ligating PCR-fragments into M13 vectors.
[d] Primer contains additional restriction sites at the 5′-end to facilitate ligation into M13 vectors.
[e] PCR primers used for the Dynabeads-280 streptavidin sequencing system.
[f] Primer is biotinylated at the 5′-end to allow binding of strepavidin covalently linked to the surface of the Dynabeads.
[g] Designation of the sequencing primers refer to approximate annealing sites of *Escherichia coli* SSU rRNA. Forward (>) primers are complementary to the coding, and reverse (<) primers to the noncoding DNA strand.
[h] Optional sequencing primers close to the 5′- or 3′-end of the SSU rRNA gene that were not routinely used.

100 *Chlorella* strains indicated in Table 1 and summarized in Figure 1, we determined the nuclear GC content from *Chlorella homosphaera* CCAP 211/8e and *C. minutissima* Lefèvre ALCP 87 as 72.0 mol% each and from *Muriella aurantiaca* SAG 249-1 as 64.1 mol%.

We have reconfirmed DNA hybridization between *Prototheca wickerhamii* strains SAG 263-11 and Pore 1283 and determined a DNA similarity of 83%D ± 3.5%D (mean ± SD, n = 12). This value indicates conspecificity of both strains and is consistent with the finding that the 18S rRNA genes of both strains are identical. The DNA similarity of only 6%D erroneously reported earlier for the same strains (Huss et al. 1988) was probably caused by confusion of DNA samples.

A partial 18S rRNA gene sequence of 1164 nucleotides from *Chlorella minutissima* strain Lefèvre that included the most variable regions differed by only one nucleotide compared with the type strain CCAP 211/8e of *C. homosphaera*. The DNA similarity between both strains was determined as 90.2%D ± 14.0%D (mean ± SD, n = 8), thus confirming conspecificity. As a control, 28.8%D ± 13.1%D (mean ± SD, n = 8) were obtained between *C. minutissima* strain Lefèvre and *C. sorokiniana* Prag A14, which has a similar GC content.

The phylogenetic tree in Figure 2 contains the information of four independent analyses (see legend to Fig. 2). It demonstrates the problematic state of *Chlorella* taxonomy. *Chlorella* species are distributed over two classes of green algae: the Trebouxiophyceae and the Chlorophyceae. Each class is supported by high bootstrap values, confirming that the Trebouxiophyceae are a sister class to the Chlorophyceae (Friedl 1995). The type species of *Chlorella, C. vulgaris,* belongs to the Trebouxiophyceae and is closely associated with *C. lobophora, C. sorokiniana,* and *C. kessleri*. The next most closely related clusters contain *C. protothecoides* together with species of the genus *Prototheca,* and *C. minutissima* together with *Nanochlorum eucaryotum*. However, the branching order of these groups could not be resolved.

The heterotrophic genus *Prototheca* (including *C. protothecoides*) is characterized by an accelerated mutation rate of its 18S rRNA gene. This is most obvious for *P. zopfii* with its unusually long branch. The *P. zopfii* sequence contains several base substitutions

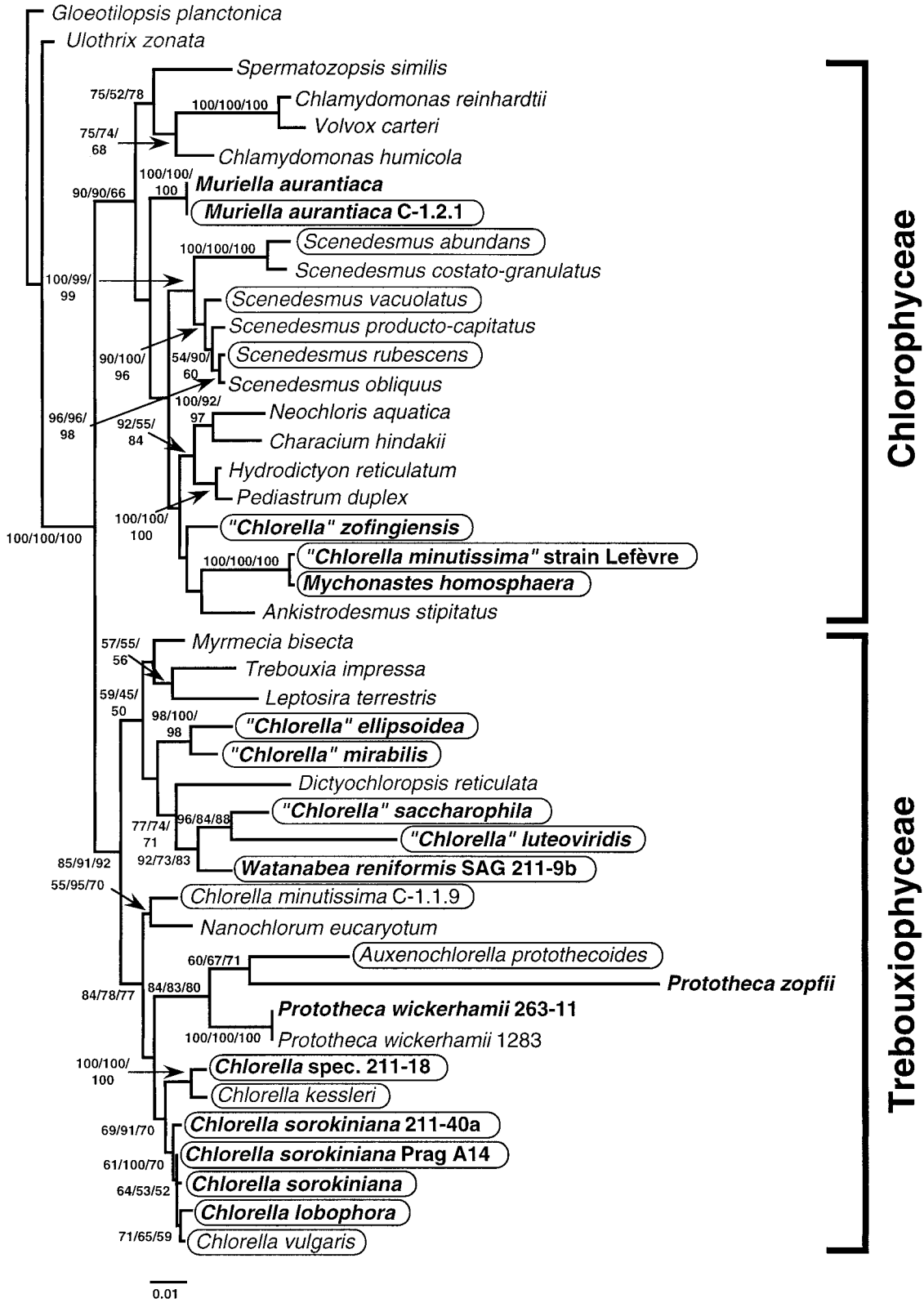

FIG. 2. Phylogenetic tree inferred from 18S rRNA gene sequences showing the polyphyly of the genus *Chlorella* within the Trebouxiophyceae/Chlorophyceae. Taxa that were traditionally assigned to *Chlorella* are circled. Sequences determined in this study are typed in boldface. The tree topology is derived from a maximum likelihood analysis (based on 1751 positions) that yielded the best Ln likelihood (−11625.86) in 10 independent searches with jumbled taxon addition. Bootstrap values are shown at the internal nodes for maximum likelihood (ML; 100 replications), neighbor joining (NJ; 1000 replications), and maximum parsimony (MP; 1000 replications), respectively, if the node is supported by at least two bootstrap values of 50% or above. Branch lengths correspond to evolutionary distances. A distance of 0.01 is indicated by the scale.

at positions that are otherwise conserved within the green algae, and the 18S rRNA gene of *C. prototheco*ides contains a unique insertion in the variable helix E21-1 (Huss and Sogin 1990, Huss et al. 1994).

A second group within the Trebouxiophyceae is supported only by very low bootstrap values of 45% to 59% and might actually consist of several independent lineages. It contains zoospore-forming coccoid green algae that are often found as phycobionts of lichens, such as species of *Trebouxia* de Puymaly, *Myrmecia* Printz, and *Dictyochloropsis* Geitler (Friedl 1995). Several *Chlorella* species fall within this group, and some of them are also known to be symbionts. Strain HHG of *C. saccharophila* (Huss et al. 1987b) is an endosymbiont of *Heterostegina depressa* (Foraminifera; Lee et al. 1982), and strain SAG 3.80 of *C. ellipsoidea* (Kessler 1987) was isolated from the lichen *Trapelia coarctata* (Tschermak-Woess 1978). A close relationship of *C. ellipsoidea* with *C. mirabilis* and of *C. saccharophila* with *C. luteoviridis* is supported by high bootstrap values. In contrast, strain SAG 211-9b of *C. saccharophila* is more distinct and takes a position ancestral to both *C. saccharophila* and *C. luteoviridis*.

The remaining *Chlorella* species studied belong to a different class, the Chlorophyceae (Fig. 2). This class comprises several independent lineages whose relationships cannot be resolved reliably by the 18S rRNA data. One of these lineages is represented by the Chlamydomonadales (sensu Melkonian 1990). The genus *Scenedesmus* represents another lineage that contains the former varieties of *Chlorella fusca,* namely, var. *vacuolata* (= *Scenedesmus vacuolatus*), var. *fusca* (= *S. abundans*), and var. *rubescens* (= *S. rubescens*) (Kessler et al. 1997). Other lineages include the Hydrodictyaceae plus Characiaceae, the Ankistrodesmaceae represented by *Ankistrodesmus stipitatus; C. homosphaera* together with strain Lefèvre of *C. minutissima;* and *C. zofingiensis*. However, strain C-1.2.1 of *C. zofingiensis* is conspecific with *Muriella aurantiaca,* and both algae form an unresolved lineage within the Chlorophyceae as well.

The taxonomic conclusions that follow from this and a similar study by Hanagata and Chihara (1997; Hanagata, pers. comm.) are being considered by Hanagata et al. (pers. comm.).

DISCUSSION

Tracing the evolutionary history of a group of organisms with such few morphological and ultrastructural features as those found in the genus *Chlorella* is not feasible on the basis of morphological criteria alone. Therefore, we combined several phenotypic and genotypic features to characterize *Chlorella* taxa with respect to their interspecific relationships. Although the phenotypic characters listed in Table 1 are not sufficient alone for deriving phylogenetic relationships, it is possible to deduce their phylogenetic significance by comparing these data with the molecular phylogeny. Whenever necessary and applicable, such a strategy could solve a problem that arose when molecular trees took an increasingly predominant role in systematics: We frequently are confronted with the fact that the molecular and the ''classical'' morphology-based systematics are not congruent for a given group of organisms. The identification of phylogenetically significant phenotypic characters and their application to the systematics of such critical groups might allow the assignment of strains into a natural system without the need for determining a molecular sequence. For the genus *Chlorella,* this was clearly not possible before. In addition to its systematic value, the knowledge of biochemical and physiological properties might be of practical importance in basic research or biotechnology (Kessler 1986, 1992).

Concerning the molecular part of such an approach, several methods are available with differing resolution that can be used for different taxonomic levels. From the methods that we have applied to the systematics of the genus *Chlorella,* the determination of the DNA base composition is indicative of the heterogeneity of the organisms studied. As shown in Figure 1, the nuclear base composition within the genus *Chlorella* extends over a wide range, covering almost the entire GC spectrum found in eukaryotes. This criterion alone shows that *Chlorella* in its present definition cannot represent a natural genus. De Ley (1969) has estimated that prokaryotes separated by 16 mol% GC can share at most 4% of their nucleotide sequences. The situation is more complicated with eukaryotes, as the different quantity of repetitive sequences could have an influence on the nuclear base composition. Likewise, DNA base modifications can influence the thermal melting point of DNA, which is obtained for calculating the GC content (cf. Ehrlich et al. 1975, Rae and Steele 1978). However, both factors are likely not responsible for the observed heterogeneity in base composition in *Chlorella* because large differences were observed neither in the amount of repetitive sequences nor in DNA base modification (Dörr and Huss 1990, Huss and Jahnke 1994).

It is especially intriguing that within strains of a single species, *C. sorokiniana,* the DNA base composition varies by up to 15 mol% GC. Despite these differences and despite some heterogeneity also in biochemical and physiological properties (Table 1), our rRNA sequence analyses in Figure 2 as well as DNA/DNA hybridization studies (Huss et al. 1986, 1989a) show that the different strains of *C. sorokiniana* with RNA homologies of 99.5% to 99.8% have to be regarded at least as closely related species. Such a large discrepancy between genomic base composition and rRNA similarity is unprecedented as far as we know. An extreme bias in the codon usage of the different strains could provide an explanation but has not yet been studied.

Although DNA/DNA hybridizations, in contrast to sequence comparisons, are of only semiquantita-

tive value, they provide a superior method for delimiting species and identifying closely related species within a genus. In a comparison of 16S rRNA homologies and respective DNA hybridization values in prokaryotes, several groups of organisms could be identified that share almost identical 16S rRNA sequences but in which DNA hybridization may be as low as 25%, thus indicating that they represent individual species (Stackebrandt and Goebel 1994). Above an RNA homology of about 97.5%, DNA reassociation values can either be low or as high as 100%. Each method is strong in those areas of relationships in which the other method fails to depict such relationships reliably. On the basis of our extensive DNA hybridization studies in the genus *Chlorella* (Huss et al. 1986, 1987a, b, 1988, 1989a, b), it is therefore unnecessary to determine rRNA sequences of strains with more than about 40% DNA similarity to a reference strain.

Since the publication of the first 18S rRNA tree that included *Chlorella* species (Huss and Sogin 1990), numerous sequences of different chlorophyte lineages have become available, allowing observation of the heterogeneity of these algae in a much broader phylogenetic context. On the basis of rRNA sequence analyses, Friedl (1995) established the new class Trebouxiophyceae as a sister group to the Chlorophyceae. *Chlorella* species are scattered over both classes with the type species *C. vulgaris* together with the most closely related taxa, *C. lobophora, C. sorokiniana,* and *C. kessleri,* belonging to the Trebouxiophyceae. (*"C. pyrenoidosa"* [Chick 1903], a taxon often mentioned in the literature, could not be recognized according to morphological characters [Shihira and Krauss 1965, Fott and Nováková 1969]. Most of the strains so labeled seem to belong to *"C. fusca,"* which was assigned to the genus *Scenedesmus* [Kessler et al. 1997].) According to the molecular data it seems appropriate to restrict the genus *Chlorella* to these species. *"C." protothecoides* (= *Auxenochlorella protothecoides* [Krüger] Kalina et Punčochářová) is more closely related to the heterotrophic genus *Prototheca* and already displays a considerable evolutionary distance to *C. vulgaris*. However, it must be taken into account that the long branches leading to *"C." protothecoides* and especially *P. zopfii* indicate a much higher mutation rate of their 18S rDNAs compared to the other algae (cf. Huss and Sogin 1990). This tachytelic behavior complicates estimating the time that passed after these algae separated from the *C. vulgaris* group and obscures the real relationships. The same applies for the relationship between *"C." protothecoides* and *Prototheca*. Although the rRNA tree indicates a closer relationship of *"C." protothecoides* with *P. zopfii* instead of clustering the two *Prototheca* species, the bootstrap support is relatively poor, and ''long branch attraction'' (Felsenstein 1988) could be responsible for this topology. With respect to the complete loss of autotrophy in the genus *Prototheca,* it would be more parsimonious if *"C." protothecoides,* which is auxotrophic and mesotrophic (Table 1), were ancestral to both *Prototheca* species. Then, the ability to synthesize chlorophyll could have been lost only once, whereas otherwise the reappearance of autotrophy has to be postulated for *"C." protothecoides,* or a twofold independent loss for *P. wickerhamii* and *P. zopfii* must have occurred.

The microalgae *C. minutissima* and *Nanochlorum eucaryotum,* which are placed in our phylogeny into a moderately supported common cluster, are characterized by their small cell size of about 2 μm (Fott and Nováková 1969, Wilhelm et al. 1982) and by a genome size that is among the smallest found so far within eukaryotes (Wilhelm et al. 1982, Zahn 1984, Dörr and Huss 1990). The taxonomic status of both algae is rather confusing. Following a comparative ultrastructural study with autospore-forming *Nannochloris* species including *N. coccoides* SAG 251-1, *Nanochlorum eucaryotum* was transferred by Menzel and Wild (1989) to the genus *Nannochloris*. This transfer is incorrect in two respects. First, *N. eucaryotum* with a GC content of 48 mol% (Zahn 1984) is not related to *N. coccoides* (66 mol% GC; Huss and Jahnke 1994) according to the rRNA phylogeny (Krienitz et al. 1996). Second, according to the original description by Naumann (1921), *Nannochloris* propagates by binary division and not by autosporulation and belongs to the Ulotrichales (Ulvophyceae). Therefore, the autospore-forming *"N. coccoides"* has been transferred to the genus *Choricystis* (Skuja) Fott as *C. minor* (Krienitz et al. 1996). The revised name *Nannochloris eucaryotum* (Wilhelm et al.) Menzel et Wild is untenable, as would be a transfer to the genus *Choricystis*. We suggest maintenance of the original genus name *Nanochlorum* Wilhelm, Eisenbeis, Wild et Zahn, to avoid further confusion. On the other hand, the description of *Chlorella minutissima* by Fott and Nováková (1969) was based on strain Lefèvre no. 87 of the Paris collection as the type culture and included, among others, strain C-1.1.9 of the Cambridge collection. Our molecular data, in concordance with observations made by Kalina et Punčochářová (1987), show that the type culture of *"C." minutissima* is conspecific with *"C." homosphaera* Skuja 1948 (= *Mychonastes homosphaera* Kalina et Punčochářová), a species name that has to be given priority over *C. minutissima* Fott et Nováková 1969. For strain C-1.1.9, which is unrelated to *"C." homosphaera* (Fig. 2), we propose keeping the name *C. minutissima* provisionally until more information becomes available.

For a long time, *Chlorella saccharophila* and *C. ellipsoidea* were not distinguishable according to the chemotaxonomic criteria of Kessler and were combined into a single species, *C. saccharophila* (Kessler 1967). Differences in the DNA base composition (Hellmann and Kessler 1974a), salt tolerance (Kessler 1974a), and fermentation products (Vinayakumar and Kessler 1975) later allowed the separation of

some strains. The observation that these algae are further characterized by an extreme cadmium sensitivity and by the inability to grow on mannitol as a carbon source in the dark led to the designation of these strains as *C. saccharophila* var. *ellipsoidea* (Kessler 1986, 1987) according to Fott and Nováková (1969; but see Punčochářová 1994). DNA hybridization studies by Huss et al. (1987b, 1989a) finally led to a complete delimitation of both taxa into two species: *C. saccharophila* and *C. ellipsoidea* (Kessler and Huss 1992). The rRNA phylogeny not only confirms this separation but also justifies the transfer into two distinct genera apart from *Chlorella* (Hanagata and Chihara, pers. comm.). Moreover, strain SAG 211-9b of *"C." saccharophila* is also distinct in several respects from the type culture SAG 211-9a. Acid and salt tolerance is less pronounced (Kessler 1965, 1974a), DNA base composition is slightly lower, and DNA hybridiziations between both taxa showed no significant values (Huss et al. 1987b, 1989a). Accordingly, the phylogenetic tree in Figure 2 shows a separate position of *"C." saccharophila* SAG 211-9b (= *Watanabea reniformis* Hanagata, Karube, Chihara et Silva; Hanagata et al. 1998) more distant than *"C." luteoviridis*. The close relationship of *"C." luteoviridis* with *"C." saccharophila* is further substantiated by the ability to grow on mannitol (Kessler 1987), by a high acid and salt tolerance (Table 1), and by a similar cell wall composition (Takeda 1991, 1993a, 1996a). On the other hand, *"C." ellipsoidea* is closely related to *"C." mirabilis*.

"Chlorella fusca," "C." homosphaera, and *"C." zofingiensis* belong to a different class, the Chlorophyceae. The varieties of *"C. fusca"* have already been transferred to the genus *Scenedesmus* on the basis of not only the high rRNA similarities but also biochemical, physiological, serological, and molecular data (Kessler et al. 1997). *"Chlorella" homosphaera* (= *Mychonastes homosphaera* [Skuja] Kalina et Punčochářová) has always been an isolated taxon within the genus *Chlorella* with no apparent affinity to any other species (Table 1). The 18S rRNA phylogeny confirms this view and indicates an independent lineage of *"C." homosphaera* within the Chlorophyceae. Another independent lineage is represented by *"C." zofingiensis*. This alga has been transferred to the genus *Muriella* (Boye-Peters) Vischer by Hindák (1982) and later to the genus *Mychonastes* Simpson et Van Valkenburg by Kalina and Punčochářová (1987) on the basis of morphological studies, but neither transfer is supported by our analyses. Only strain C-1.2.1 of *"C." zofingiensis,* which could be discerned from this taxon only after molecular methods were applied (Huss et al. 1989b), is related to the genus *Muriella* and conspecific with *M. aurantiaca* (Fig. 2). However, the type culture of *"C." zofingiensis* (strain SAG 211-14) represents an independent lineage within the Chlorophyceae. Further studies have to show whether *"C." zofingiensis* can be assigned to an existing genus (such as strain C-1.2.1) or whether it has to be described as a new taxon. Therefore, *"C." zofingiensis* is a prominent example demonstrating the problems and pitfalls in the systematics of chlorococcalean algae when based on phenotypic criteria alone.

The close relationship between the autosporic and zoosporic taxa included in this study can be explained by multiple complete loss of motility in different chlorophycean lineages (Wilcox et al. 1992); therefore, autosporulation cannot be used as a phylogenetic marker. Likewise, the almost complete 18S rRNA identity of the unicellular *"C. fusca* var. *rubescens"* (= *Scenedesmus rubescens*) with the coenobial *S. obliquus* and other examples within the genus *Scenedesmus* show that coenobial versus unicellular is not a reliable characteristic. Therefore, the molecular phylogeny can be used to recognize phylogenetically relevant and unreliable nonmolecular properties. This recognition is useful for the modification of existing phenotypically based classification schemes toward a natural system.

When the molecular phylogeny is compared with the chemotaxonomical classification scheme of *Chlorella* in Table 1, many features, although in their combination very useful for species delimitation, are not relevant in a phylogenetic sense per se. For example, the presence or absence of hydrogenase activity, the first criterion that allowed discrimination between the closely related species *C. vulgaris* and *C. sorokiniana* (Kessler and Soeder 1962), is found not only in the Trebouxiophyceae but also in the Chlorophyceae. As hydrogenase is active only under strictly anaerobic conditions, it might represent a relic from the early, anaerobic phase of life on earth (Kessler 1974b). It is unlikely to play a functional role in algae and apparently was independently lost several times during evolution. Other features have proved to be phylogenetically more reliable markers for systematic purposes. Thermophily within *Chlorella* and *"Chlorella*-like'' algae is restricted so far exclusively to strains of *C. sorokiniana* (Table 1). Growth on mannitol in the dark is indicative for the related taxa *"C." saccharophila* and *"C." luteoviridis* and cadmium sensitivity for *"C." ellipsoidea* (Kessler 1987). Norspermine is produced only by *"C." saccharophila, "C." luteoviridis, "C." ellipsoidea,* and *"C." mirabilis,* all members of one of the two subgroups within the Trebouxiophyceae, whereas norspermidine was found in *Scenedesmus, "C." zofingiensis,* and *"C." homosphaera,* all members of the Chlorophyceae (Hegewald and Kneifel 1982). Members of the second subgroup of the Trebouxiophyceae, which contains the ''true'' *Chlorella* species as well as *C. minutissima* and *"C." protothecoides,* produce neither norspermine nor norspermidine.

Perhaps the most significant and easily determined feature of phylogenetic relevance is the production of secondary carotenoids. These ketocarotenoids, mainly astaxanthin, canthaxanthin, and echinenone, are produced under conditions of ex-

treme nitrogen deficiency in most members of the Chlorophyceae but so far have never been observed in the Trebouxiophyceae (cf. Czygan 1968, 1982). The production of secondary carotenoids seems to be related to the ability to synthesize sporopollenin and to the development of the outer, trilaminar cell wall found in these algae (Atkinson et al. 1972; but see Burczyk et al. 1995). The sugar composition of the cell wall is another character that has been shown to be useful for the systematics of *Chlorella* by the work of Takeda (1991, 1993a, b, 1996a, b). Here, the ''true'' *Chlorella* species *C. vulgaris, C. lobophora, C. sorokiniana,* and *C. kessleri* are characterized by glucosamine as the exclusive component of the rigid cell wall, making them completely different from all other *''Chlorella''* species. Similarly, in a study of pyrenoid ultrastructure, the same species could be discerned from others by the presence of a double thylakoid bisecting the pyrenoid matrix (Ikeda and Takeda 1995).

For a century, the taxonomy of the genus *Chlorella* has been problematic and the phylogenetic position of different species within the chlorophytes was unknown. Molecular data as well as a combination of physiological, biochemical, and ultrastructural characters now make it possible to identify *Chlorella*-like strains and place them into a natural system. Obviously, however, characters that have proved to be of phylogenetic significance in *Chlorella* might not necessarily have the same importance in other groups of algae and have to be determined independently for each group.

We wish to thank Mrs. C. Holweg, Mrs. G. Steingräber, and Mrs. E. Weitemeyer for excellent technical assistance. We are also grateful to Drs. N. Hanagata and M. Chihara for providing their new classification system of *Chlorella* prior to publication. This work was supported by the Deutsche Forschungsgemeinschaft and the Universitätsbund Erlangen-Nürnberg.

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