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Deoxyribonucleic acid reassociation and interspecies relationships of the genus *Chlorella* (*Chlorophyceae*)

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Abstract: Phylogenetic relationships within the genus *Chlorella* were studied by means of DNA/DNA hybridization under both optimal and relaxed reassociation conditions as well as by determination of the thermal stability of hybrid DNA duplexes. The results indicate a relationship between *C. fusca* var. *fusca, C. fusca* var. *rubescens, C. fusca* var. *vacuolata,* and the genus *Scenedesmus.* In addition, the strains endosymbiotic with *Paramecium bursaria* seem to be related with the *C. vulgaris/sorokiniana* group. The relations between most other species, however, could not be sufficiently resolved by the above methods. This implies considerable phylogenetic divergency within the genus *Chlorella.*

The taxonomy of *Chlorella* has for a long time remained unsatisfactory, in spite of the great importance of these unicellular green algae in basic research as well as in biotechnology. Morphological and structural characteristics are not easily discernible in the light microscope and are not sufficient for an unequivocal classification. Early descriptions of strains of *Chlorella* therefore sometimes lacked morphological details but instead tried to use physiological features as a means of classification. Several sugars and organic nitrogen compounds, for instance, have been assayed for stimulating effects on heterotrophic growth. The results of these experiments, however, are hardly reproducible and based on such rather vague statements as good or bad growth. SHIHIRA & KRAUSS (1965) were the first to attempt a comprehensive treatment of the genus *Chlorella* from both a physiological and taxonomical viewpoint. They studied 41 isolates and defined 19 species and numerous varieties to give a whole of 30 taxa. A few years later FOTT & NOVÁKOVÁ (1969) in their monograph of the genus *Chlorella* criticized that three-quarters of these taxa were defined by the observation of a single strain and the majority of the characteristics used for species identification were expressed by degree and not by presence or absence. They instead combined morphological and structural features with some of the physiological characteristics observed by SHIHIRA & KRAUSS (1965) and chemotaxonomic data from KESSLER and coworkers (KESSLER & SOEDER 1962, KESSLER & al. 1963, KESSLER 1967). A key was given for distinguishing 9 species and 6 varieties, primarily based on cell morphology and

structure. The combination of ten biochemical and physiological characters, which proved to be species-specific, eventually led to an extension and partial rearrangement of the classification of Fott & Nováková (for reviews see Kessler 1982, 1984). 16 taxa are characterized at present by the criteria of Kessler (1987).

With the determination of the guanine + cytosine (G + C) content of the DNA and of DNA similarities by a hybridization procedure, Hellmann & Kessler (1974 a) and Kerfin & Kessler (1978) introduced modern molecular criteria for revealing a phylogenetic relationship into the taxonomy of *Chlorella*. As a result, five species proved to be homogeneous with respect to the DNA base composition while three were partly heterogeneous. The remaining taxa were each represented by only one strain. DNA hybridizations were carried out with only two reference strains, so most intra- and interspecific relations remained unknown.

Some *Chlorella* spp. have been reclassified and placed in other genera during the last years (cf. Kalina & Punčochářová 1987). In order to avoid any confusion we want keep the nomenclature used by Kessler (1987) until a comprehensive reorganization in a natural system on the basis of molecular data such as DNA homologies and rRNA sequences is possible.

In the preceding papers of this series, we determined DNA homologies within *C. sorokiniana* (Huss & al. 1986) and *C. saccharophila* (Huss & al. 1987 a), both heterogeneous species, as well as *C. fusca* var. *vacuolata, C. kessleri*, and *C. protothecoides* which are homogeneous species in all characters so far investigated (Huss & al. 1987 b, 1988). *C. fusca* varr. *fusca* and *rubescens*, however, have been shown to be not closely enough related to *C. fusca* var. *vacuolata* and to each other to justify the rank of subspecies or even varieties (Huss & al. 1987 b).

This paper presents evidence for a deep phylogenetic branching within the genus *Chorella* by means of DNA hybridization data obtained both under optimal and relaxed reassociation conditions, and thermal stability of hybrid DNA duplexes.

Materials and methods

A list of the organisms studied and their origin is given in Table1. The algae were grown in the media of Kessler & Czygan (1970) or Kessler & Zweier (1971, for mesotrophic strains). They were incubated in 6 l flasks for one week at 20 – 25 °C and gassed with air + 2% CO_2.

DNA was extracted from 20 l of algae and purified as described by Huss & al. (1986). DNA containing more than about 5% of organell DNA as estimated from DNA melting curves, was further purified by CsCl density gradient centrifugation (Huss & al. 1988).

DNA base composition and DNA homologies were determined according to Huss & al. 1987 a). However, only the membrane filter technique with DNA fragments labelled in vitro with deoxy-(1′,2′,5′-^{3}H)-cytidine-triphosphate (Amersham) according to Rigby & al. (1977) was used for the quantitative determination of DNA hybrids unless stated otherwise.

The thermal stability of DNA hybrids which have been formed during the 90 h reassociation period on the membrane filters was determined by a modified procedure after De Smedt & De Ley (1977). Dried filters were washed for 10 min at 25 °C in the same formamide-containing buffer system as used for reassociation reactions and subsequently incubated for each 10 min in 5-degree temperature steps from 30 °C to 75 °C. The radioactivity released from the filters at each temperature was determined by liquid scintillation counting. The thermal stability is characterized by the temperature at which 50% of the hybrid is eluted ($T_{M(e)}$).

Table 1. List of *Chlorella, Scenedesmus*, and *Prototheca* strains studied, their origin and DNA base composition. [a] Göttingen, Algensammlung des Pflanzenphysiologischen Instituts der Universität Göttingen, F.R.G.; Cambridge, Culture Centre of Algae and Protozoa, Cambridge, U.K.; Bethesda, culture collection at Bethesda, Maryland, U.S.A.; Austin, culture collection of algae, Austin, Texas, U.S.A. (formerly Bloomington, Indiana, U.S.A.); Andreyeva, V. M. Andreyeva, Leningrad, U.S.S.R.; Reisser, W. Reisser, Göttingen, F.R.G. [b] *Escherichia coli* B (52.0 mol% G+C; GILLIS & al. 1970) was used as reference; [c] *1* HUSS & al. (1986); *2* HUSS & al. (1987 a); *3* HUSS & al. (1987 b); *4* HUSS & al. (1988); [d] nuclear DNA, purified by CsCl density gradient centrifugation (HUSS & al. 1988); [e] endosymbiont of *Paramecium bursaria*; [T] Type strain

Species	Strain	Origin[a]	Mol% G+C[b]	Ref.[c]
Chlorella fusca var. *vacuolata* SHIHIRA & KRAUSS	211-8 b[T]	Göttingen	51.6	2
C. fusca var. *fusca* SHIHIRA & KRAUSS	343[T]	Austin	55.7	3
C. fusca var. *rubescens* (DANGEARD) KESSLER & al.	232/1[T]	Cambridge	56.8	3
C. homosphaera SKUJA	211/8 e[T]	Cambridge	73.0	
C. kessleri FOTT & NOVÁKOVÁ	211-11 g[T]	Göttingen	55.1	3
C. lobophora ANDREYEVA	750-I[T]	Andreyeva	60.6[d]	
C. luteoviridis CHODAT	211-2 a[T]	Göttingen	45.2	
C. minutissima FOTT & NOVÁKOVÁ	C-1.1.9	Bethesda	46.0	
C. mirabilis ANDREYEVA	748-I[T]	Andreyeva	57.1[d]	
	211-11 r	Göttingen	58.5[d]	
	211-30	Göttingen	56.3[d]	
C. protothecoides KRÜGER	211-7 a[T]	Göttingen	59.0	4
C. saccharophila var. *ellipsoidea* (GERNECK) FOTT & NOVÁKOVÁ	211-1 a[T]	Göttingen	57.3[d]	
C. saccharophila var. *saccharophila* [(KRÜGER) MIGULA] FOTT & NOVÁKOVÁ	211-9 a[T]	Göttingen	52.0	2
	211/1 f	Cambridge	48.7	2
	211-9 b	Göttingen	49.4	2
C. sorokiniana SHIHIRA & KRAUSS	211-8 k[T]	Göttingen	63.1	1
	211/11 d	Cambridge	61.5	1
	211-11 k	Göttingen	66.6	1
C. vulgaris BEIJERINCK	211-11 b[T]	Göttingen	61.2	2
C. zofingiensis DÖNZ	211-14 a[T]	Göttingen	50.1	
C. spec.	241.80[e]	Göttingen	66.4[d]	
C. spec.	211-6[e]	Göttingen	67.1[d]	
C. spec.	Pbi[e]	Reisser	67.1[d]	
C. spec.	211-18	Göttingen	51.3	1
Scenedesmus obliquus (TURPIN) KÜTZING	276-3 a	Göttingen	55.1	
S. armatus var. CHOD.	276-4 a	Göttingen	55.7	
Prototheca zopfii KRÜGER	263-1 a	Göttingen	69.5	4

Table 2. Interspecies DNA homology data matrix of the genus *Chlorella*. DNA homologies were temperature of the (^{3}H)-labelled strain). – Not determined; ^{N} nuclear DNA, purified by CsCl density (cf. Huss & al. 1987 a); ^{b} determined optically (cf. Huss & al. 1987 a)

Source of filter-bound DNA	Degree of binding (%D) with (^{3}H)-labelled DNA from									
	211-8 b	343	232/1	211-11 g	750-I^N	211-2 a	C-1.1.9	748-I^N	211-11 r	211-7 a
211-8 b	100	15	17	7	–	6	1	–	2	3
343	15	100	16	7	7	5	–	4	3	4
232/1	17	13	100	8	6	5	2	3	4	2
211/8 e	16	–	–	7	–	4	2	–	7	8
211-11 g	14	–	–	100	5	6	3	3	4	5
750-I^N	–	–	–	–	100	–	–	4	5	–
211-2 a	5	–	–	7	–	100	2	–	3	3
C-1.1.9	9	11	10	10	4	6	100	3	4	4
748-I^N	–	–	–	–	9	–	–	100	67^a	–
211-11 r	10	–	–	6	7	5	2	67^a	100	3
211-30	10	–	–	6	–	5	2	70^b	100^a	3
211-7 a	6	5	–	9	7	5	2	3	4	100
211-1 a^N	–	–	–	12	8	–	–	4	–	6
211-9 a	7	8	–	9	5	9	1	–	4	6
211-8 k	12	–	–	13	9	6	2	4	5	6
211-11 b	11	13	14	12	14	6	2	5	6	6
211-14 a	12	12	13	8	–	5	1	–	2	3
241.80	–	–	–	–	–	–	–	–	–	8
211-6	–	–	–	–	12	–	–	4	4	6
Pbi^N	–	–	–	–	–	–	–	–	–	–
211-18	–	–	–	–	–	–	–	–	–	–
276-3 a	14	19	21	–	–	–	–	–	–	–
276-4 a	13	18	14	–	–	–	–	–	–	–
263-1 a	5	4	–	3	–	2	–	–	4	5

Results

The DNA base composition of different species of the genus *Chlorella* shows a wide range from 45.2 Mol% G+C in *C. luteoviridis* to 72.7 Mol% in *C. homosphaera* (Table 1; Hellmann & Kessler 1974 a). The highest amount of guanine plus cytosine within *Chlorella* has been determined in a strain of *C. sorokiniana*, 211-40 a, with 75.2 Mol% (Huss & al. 1986). The base composition of both *Scenedesmus* strains investigated (55.1 Mol% and 55.7 Mol% G+C) is well within the range determined for different species of this genus by Hellmann & Kessler (1974 b) and Paschma & Hegewald (1986) and similar to that of *C. fusca* var. *fusca* (55.7 Mol%) which has been shown to belong to the genus *Scenedesmus* due to structural similarities of the cell wall (Fott & al. 1975, Hegewald & Schnepf 1979).

Table 2 shows a DNA homology data matrix of the genus *Chlorella* including two species of *Scenedesmus* and one of *Prototheca*. Most homologies between the different strains are too low to indicate a significant relationship. Some relations, however, are significant and should be emphasized. Strains 211-30 and 211-11 r which have not yet been assigned to any species by Kessler (1987) and which cannot be distinguished by physiological and biochemical characteristics are also

determined in 3 × SSC/formamide at 45 °C under optimal conditions (T_M – 25 °C, related to the melting gradient centrifugation (Huss & al. 1988); [a] mean value of membrane filter- and optical determination

211-1a^N	211-9a	211-8k	211-11b	211-14a	241.80	211-6	211-18	276-3a	276-4a	263-1a
–	6	9	9	8	–	–	–	22	26	4
–	4	11	10	8	–	–	–	23	27	5
–	3	11	10	8	–	–	–	30	20	–
–	3	14	14	7	–	–	–	–	–	–
3	6	9	11	6	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	7	3	5	5	–	–	–	–	–	–
–	5	6	8	6	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	3	8	9	4	17	18	–	–	–	–
11	3	9	8	5	19	20	–	–	–	–
5	8	9	7	4	13	14	7	–	–	7
100	8	9	16	–	18	17	–	–	–	–
5	100	5	6	5	–	–	–	–	16	–
5	5	100	15	6	31	30	8	–	–	–
6	6	12	100	7	28	23	–	22	24	–
4	4	6	6	100	–	–	–	16	15	–
–	–	17	16	–	100	53[a]	–	–	–	–
–	–	17	20	–	68[a]	100	–	–	–	–
–	–	–	–	–	65[b]	99[b]	–	–	–	–
5	–	10	–	–	–	–	100	–	–	–
–	11	–	–	7	–	–	–	100	24	–
–	7	–	–	7	–	–	–	25	100	–
–	–	6	4	4	–	–	–	–	–	100

identical regarding DNA homology (100%) and belong to *C. mirabilis* (67 – 70% D compared with the type strain 748-I). Strain 211-6, isolated by Loefer (1936) from *Paramecium bursaria*, was found to be highly related (99% D) with strain Pbi, which also was isolated from *Paramecium* by Reisser (1975). Both strains exhibit about 60% homology with strain 241.80, one more strain endosymbiotic with *P. bursaria* (Schlösser 1982).

The data from Table 2 indicate a relationship between *Scenedesmus* and *C. fusca* (about 20% D). This becomes more obvious from the data of Table 3 which were obtained under relaxed reassociation conditions (T_M – 35 °C). Under these reaction conditions DNA reassociation takes place even with sequences which are not perfectly complementary. This results in a strong increase of the degree of binding of DNA species too remotely related to react under optimal reassociation conditions, which are defined to be at 25 °C below the melting temperature, and allows to enlarge the resolution power of the DNA/DNA hybridization technique. Such an increase is most obvious in the binding of labelled *Scenedesmus*-DNA with filter-bound DNA of *C. fusca* (57 – 67% D). However, there are some inconsistencies concerning reciprocal values and the relation of *C. vulgaris* 211-11 b with *C. fusca* and *Scenedesmus*, which appears likewise to be high. For a plausible interpretation,

Table 3. DNA homologies of selected *Chlorella* strains under relaxed reassociation conditions in 3 × SSC/formamide at 45 °C (T_M − 35 °C), related to the melting temperature of the (^{3}H)-labelled strain. − Not determined

Source of filter-bound DNA	Degree of binding (%D) with (^{3}H)-labelled DNA from												
	211-8b	343	232/1	211-7a	211-9a	211-8k	211-11k	211-11b	211-14a	241.80	211-6	276-3a	276-4a
211-8b	100	26	45	−	−	−	−	11	16	−	−	−	−
343	29	100	48	−	−	−	−	−	21	−	−	60	57
232/1	28	28	100	−	−	−	−	−	23	−	−	67	57
211-30	17	19	25	−	−	−	−	−	16	−	−	36	−
211-7a	13	15	17	100	16	19	−	14	−	−	−	24	−
211-1a	−	−	−	14	20	−	−	23	−	−	32	−	−
211/1f	−	−	−	10	30	13	−	−	−	−	19	−	−
211-9a	11	16	6	7	100	11	14	13	7	15	15	21	14
211-9b	−	−	−	7	14	−	−	11	−	−	13	−	−
211-8k	23	−	−	17	18	100	28	30	12	51	53	−	−
211-11d	−	−	−	15	−	32	−	−	−	−	45	−	−
211-11k	−	−	−	18	−	32	100	−	−	50	54	−	−
211-11b	24	27	51	16	21	30	29	100	19	53	52	49	47
211-14a	21	21	28	−	−	−	−	13	100	−	−	34	34
241.80	−	−	−	13	16	28	24	27	−	100	60	−	−
211-6	−	−	−	14	20	28	26	30	−	68	100	−	−
276-3a	23	24	48	−	−	−	−	−	15	−	−	100	42
276-4a	18	21	31	−	−	−	−	−	15	−	−	34	100

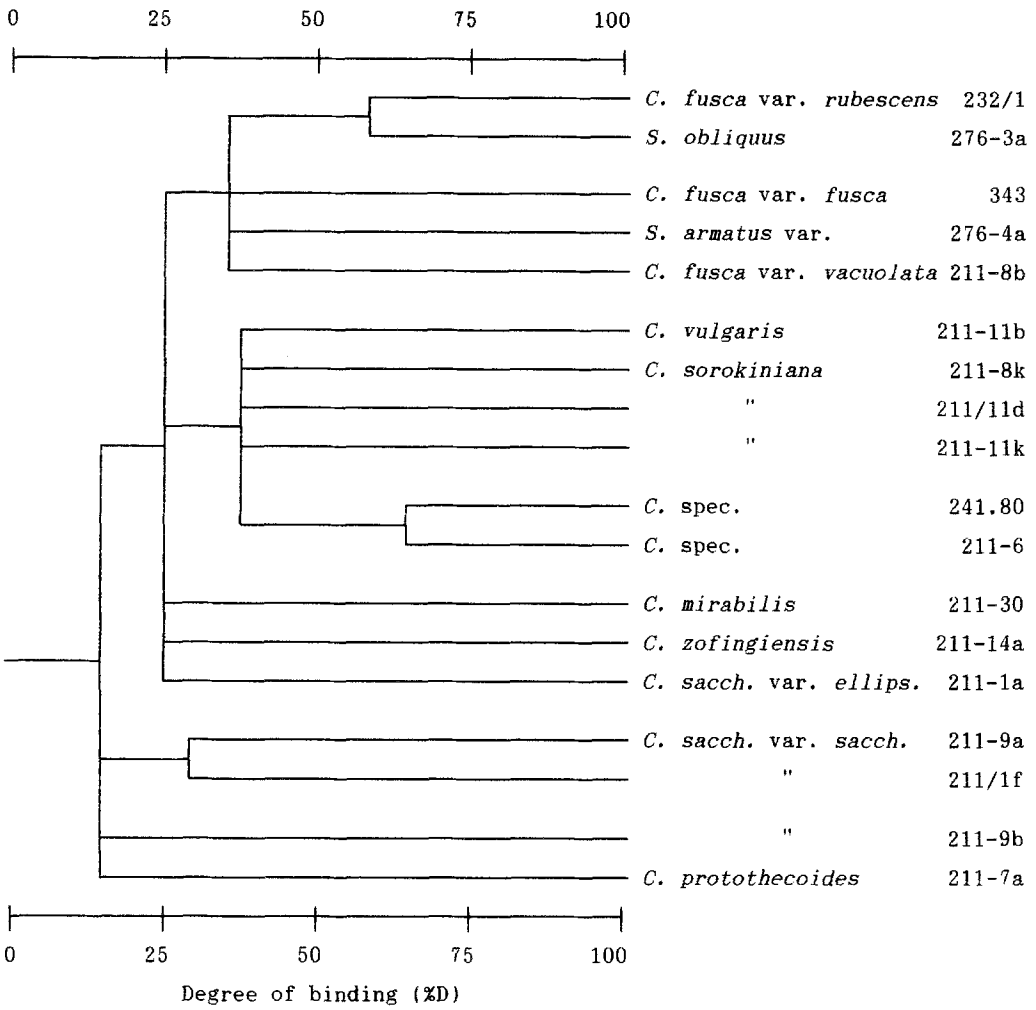

Fig. 1. Average linkage dendrogram of DNA homologies within the genus *Chlorella* using relaxed reassociation conditions ($T_M - 35\,°C$). Data are from Table 3

the data from Table 3 have to be taken as a whole, comparing relative binding within each row and column and considering that the DNAs of two related organisms should exhibit about the same low binding capacities when compared with a less related strain. Under these aspects the average linkage dendrogram shown in Fig. 1 was constructed from the data of Table 3 in an attempt to demonstrate interspecies relationships within the genus *Chlorella*. The scale of this dendrogram should be regarded in a more relative rather than quantitative way. The dendrogram confirms the relationship of *Scenedesmus* with *C. fusca* and shows the strains endosymbiotic with *Paramecium* to be most closely related with the *C. vulgaris/sorokiniana* group as has been suggested by REISSER (1984). It also shows a deep branching of other *Chlorella* species, especially of *C. protothecoides* and *C. saccharophila* var. *saccharophila*.

Another criterion for the measurement of base-pairing imperfections and mutational differences is the thermal stability of the DNA duplexes formed during

Table 4. Thermal stability of DNA hybrid duplexes of *Chlorella* spp. with *C. mirabilis* 748-I^T and 211-11 r, and *C. lobophora* 750-I^T reassociated for 90 h in 3 × SSC/formamide at 45 °C under optimal conditions (T_M — 25 °C). [a] Temperature at which 50% of the bound labelled DNA was eluted from the filters (see Materials and methods); [b] difference in thermal stability between homologous and heterologous hybrids; [N] nuclear DNA, purified by CsCl density gradient centrifugation (Huss & al. 1988)

Source of filter-bound DNA	(^{3}H)-labelled DNA from					
	C. mirabilis				*C. lobophora*	
	748-I^N		211-11 r^N		750-I^N	
	$T_{M(e)}$[a]	Δ[b]	$T_{M(e)}$	Δ	$T_{M(e)}$	Δ
C. mirabilis 748-I^N	62.5	0.0	59.1	3.1	47.3	14.8
C. mirabilis 211-11 r^N	59.3	3.2	62.2	0.0	48.8	13.3
C. lobophora 750-I^N	48.6	13.9	47.3	14.9	62.1	0.0
C. fusca var. *fusca* 343	49.3	13.2	47.5	14.7	47.6	14.5
C. fusca var. *rubesc.* 232/1	48.2	14.3	47.2	15.0	47.9	14.2
C. kessleri 211-11 g	47.2	15.3	47.1	15.1	47.3	14.8
C. minutissima C-1.1.9	44.0	18.5	45.0	17.2	48.0	14.1
C. protothecoides 211-7 a	48.2	14.3	48.1	14.1	48.2	13.9
C. sacchar. var. *ellips.* 211-1 a^N	49.8	12.7	–	–	48.3	13.8
C. sacchar. var. *sacchar.* 211-9 a	–	–	46.7	15.5	47.8	14.3
C. sorokiniana 211-8 k	48.7	13.8	46.8	15.4	48.1	14.0
C. vulgaris 211-11 b	46.4	16.1	46.4	15.8	48.5	13.6
C. spec. 211-6^N	47.7	14.8	46.3	15.9	48.5	13.6

the reassociation (Brenner & Cowie 1968, De Ley & al. 1973). As an example, the results of three experiments with *C. mirabilis* 748-I^T and 211-11 r as well as *C. lobophora* 750-I^T as reference organisms are shown in Table 4. The thermal stability of hybrids of *C. mirabilis* which are binding to a degree of 67% (Table 2) is only lowered by about 3 °C, whereas that of all other hybrids tested is lowered by about 15 °C. However, as all thermal stabilities of heterologous hybrids are decreased by about the same amount, no better resolution of interspecies relationships could be achieved. Moreover, the thermal stability of homologous hybrids varies from 49 °C to 62 °C on unknown reasons rendering impossible to compare the $\Delta T_{M(e)}$-values (difference in melting points between homologous and heterologous hybrids) of the different reference strains. What can be clearly shown, however, is an enormous decrease in thermal stability of heterologous hybrids indicating a high degree of variation in the primary structure of DNA of different *Chlorella* species.

Discussion

The heterogeneity of DNA base composition within the genus *Chlorella* has already been demonstrated by Hellmann & Kessler (1974 a) and taken as a basis for the discussion of a polyphyletic origin of these unicellular green algae (Kessler 1976, 1982). This variation can only be explained in two ways: Firstly, it could be due

to a different primary structure, i.e. base sequence of the whole DNA as a result of a long evolutionary distance since the splitting off into different lineages. This would result in altered or different gene products eventually leading to phenotypic variations (one has to keep in mind, however, the possible effect of the degeneracy of the genetic code; cf. CLARKE 1982, BERNARDI & BERNARDI 1985).

Secondly, the variation may be due to variable amounts of repetitive sequences which may influence the overall base composition in spite of homologous single copy genes. This explanation can be excluded as all *Chlorella* spp. have rather uniform and low contents (about 5–8%) of repetitive sequences (HUSS & al., unpubl.). Undoubtedly, therefore, the variation in DNA base composition reflects the phylogenetic divergency of *Chlorella*.

In this and our previous studies of this series we used the technique of DNA/DNA hybridization for a more quantitative assay of phylogenetic relationships within *Chlorella*. This technique, widely used in bacterial taxonomy, proved to be a very useful tool in the taxonomy of simple eukaryotic organisms such as *Chlorella*. We could demonstrate *C. fusca* var. *vacuolata*, *C. kessleri*, and *C. protothecoides* to be very homogeneous taxonomic entities even at the genetic level (HUSS & al. 1987b, 1988). Two species, *C. sorokiniana* and *C. saccharophila* turned out to be rather heterogeneous (HUSS & al. 1986, 1987a). The DNA/DNA hybridization technique, however, is only appropriate for identifying organisms on a species- and subspecies level. It cannot discern unequivocally whether two more remotely related organisms belong to the same or different genera. According to SCHLEIFER & STACKEBRANDT (1983) homology values above 60%, obtained under optimal conditions ($T_M - 25\,°C$), relate prokaryotic organisms at the species level, whereas values above 20% indicate that the organisms investigated are closely related members of one genus. The question arises, whether this system can be simply transferred to even "primitive" eukaryotes which might have quite a different genome organization such as different families of repetitive sequences as well as a mixture of different DNA species, i.e. nuclear-, plastid-, and mitochondrial DNA. It can be argued that DNA homologies of whole cell DNA mostly used in our studies, must not reflect homologies of single copy DNA. This is indeed the case. As will be shown elsewhere, however, both chloroplast DNA as well as repetitive sequences slightly increase homology values as compared to hybridization of single copy DNA. This effect is more obvious with low homology values (as long as its detection is not limited by the resolution power of the method employed) and thus even enhances the heterogeneity observed between different *Chlorella* spp. by DNA/DNA hybridization.

Genetic diversity in spite of phenotypic similarity is not a common phenomenon in eukaryotes. The opposite situation has been found for instance in different classes of fungi. Most authors investigating genetic relatedness among fungi report many cases of high DNA homologies between different species and sometimes even between genera of, for example, *Zygomycetes* (ELLIS 1985), *Deuteromycetes* (DAVISON & MACKENZIE 1984), *Ascomycetes* (CHAKRABARTTY 1977, DUTTA & al. 1976, KURTZMAN 1984), and *Basidiomycetes* (HORGEN & al. 1984). In yeasts, DNA homologies of about 80–100% have been suggested to comprise a species (PRICE & al. 1978) while this range would be about 25–100% for *Chlorella* considering the current taxonomy. If such differences in the estimation of species delimitation are not due to different methodology [most hybridization data in fungi have been

determined, in contrast to ours, either by hydroxylapatite chromatography (cf. Price & al. 1978) or optically by the method of Seidler & Mandel (1971)], they reflect the difference in phylogenetic divergency between the groups of organisms investigated. As a consequence, for each of such groups a distinct key relating DNA homologies to species delimitation should have to be worked out for sake of comparison.

In an attempt to raise the resolution power of the DNA/DNA hybridization technique for the detection of interspecies relationships, we determined DNA homologies under relaxed reassociation conditions allowing the renaturation of more divergent sequences. By this approach, it becomes evident that not only *C. fusca* var. *fusca* (Fott & al. 1975, Hegewald & Schnepf 1979) but also *C. fusca* var. *rubescens* and *C. fusca* var. *vacuolata* are closely related with the genus *Scenedesmus* (Fig. 1) (cf. Kümmel & Kessler 1980, Kessler 1982). In addition, the strains living in endosymbiosis with *Paramecium bursaria* (241.80 and 211-6) appear to belong to the *C. vulgaris/sorokiniana* group (cf. Reisser 1975, 1984). On the other hand, the previously suggested (Kerfin & Kessler 1978, Kümmel & Kessler 1980) relationships of *C. zofingiensis* to the *C. fusca* group and of *C. saccharophila* with the *C. vulgaris* group could not be corroborated in this study. According to our present data, *C. protothecoides* and *C. saccharophila* var. *saccharophila* seem to show the lowest degree of relationship with the other *Chlorella* species. However, even this technique as well as the thermal stability of hybrids (Table 4) do not give sufficient insight into the evolution of *Chlorella* as the phylogenetic divergency of these algae seems to be too large. To solve this problem, sequence comparisons of small subunit ribosomal RNAs are currently under investigation.

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