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DNA base modification in *Chlorella*

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The nuclear DNA base composition of 26 strains of green algae belonging to the Chlorococcales was analysed by reversed-phase high-performance liquid chromatography (HPLC). 5-Methyldeoxycytidine was the only modified nucleoside detected in addition to the standard nucleosides deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine. The amount of 5-methyldeoxycytidine varied from 1·5 mol% to 12·0 mol% and was correlated with the deoxyguanosine + deoxycytidine (G + C) content of the DNA. No significant influence of deoxycytidine methylation on the thermal stability of the DNA double strand was found. The variation of DNA base composition previously observed within the genus *Chlorella* and, in particular, within *C. sorokiniana*, could not be explained by the influence of variable amounts of modified bases on the DNA melting temperature from which the G + C contents were derived.

Key words: *Chlorella*, DNA base composition, Green algae, HPLC, Methylcytosine, Modified DNA bases, Taxonomy

Introduction

When assessing natural relationships between organisms by molecular methods, the DNA base composition is a first, relatively easily determined character. Whereas two organisms with a similar base composition may or may not be closely related, a different DNA deoxyguanosine + deoxycytidine (G + C) content reflects the divergence of DNA primary structure as a result of a series of mutation events separating these organisms. For prokaryotes it has been estimated that two bacteria separated by 16 mol% G + C can share at most 4% of their nucleotide sequences (De Ley, 1969).

Following these considerations it seems unlikely that the unicellular green alga *Chlorella* Beijerinck, comprising species with DNA base compositions from 45 mol% G + C up to 75 mol% G + C (Fig. 1), represents a natural genus (Kessler, 1976; Huss *et al.*, 1986, 1989*a*). Alternatively, the broad DNA G + C range might be caused by different amounts of repetitive sequences or by a variable degree of DNA base modification such as methylation of cytidine or adenine residues (Rae & Steele, 1978). While the former explanation can be excluded (Dörr & Huss, 1990), the reported DNA base compositions of *Chlorella* strains were determined by thermal melting according to Marmur & Doty (1962), and thus the derived DNA G + C contents are dependent on the amounts of modified nucleosides which may appreciably influence the DNA melting temperature (Ehrlich *et al.*, 1975; Rae & Steele, 1978). In a corresponding study of dinoflagellates, we demonstrated that the actual DNA G + C content may differ up to about 15 mol% from that calculated from the melting temperature, T_M (Blank *et al.*, 1988).

In this study we analysed the nuclear DNA of various strains of *Chlorella* and related taxa by high-performance liquid chromatography (HPLC) for their actual base composition and amount of modification.

Materials and methods

Axenic cultures of 26 strains of *Chlorella* and related chlorococcalean green algae were grown under the conditions described by Kessler (1977) and Kessler & Zweier (1971). Strain designations and the origin of the algae are listed in Table 1. Nuclear DNA from *Symbiodinium microadriaticum* (Blank *et al.*, 1988) and salmon sperm DNA (Sigma) were used as references. Total DNA was isolated according to Huss *et al.* (1986) and nuclear DNA separated and purified by subsequent caesium chloride (CsCl) density gradient centrifugation (Huss *et al.*, 1988). Approximately 20 μg DNA were hydrolysed with P1 nuclease and dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). Nucleosides were separated by reversed-phase HPLC.

The HPLC equipment consisted of an LKB 2150 high-pressure pump with a UV detector LKB 2151 (270 nm) connected to a SHIMADZU CR-3A integrator. The analytical column was a NUCLEOSIL 100–5C18 (250 × 4 mm; particle size 5 μm) equipped with a pre-column NUCLEOSIL 100–5C18 (20 × 4 mm; MELZ VDS, Berlin). Chromatography was carried out in 0·3 M $NH_4H_2PO_4$/acetonitrile [80 : 6 (v/v); pH 4·8] at 26°C and approximately 130 bar, resulting in a flow rate of 0·7 ml min^{-1} (modified after Tamaoka & Komagata, 1984).

Retention times of the nucleosides were determined with the following synthetic compounds obtained from Sigma: adenosine (A), guanosine (G), cytidine (C), uridine (U), thymidine (T), 2′-deoxyadenosine (dA), 2′-deoxycy-

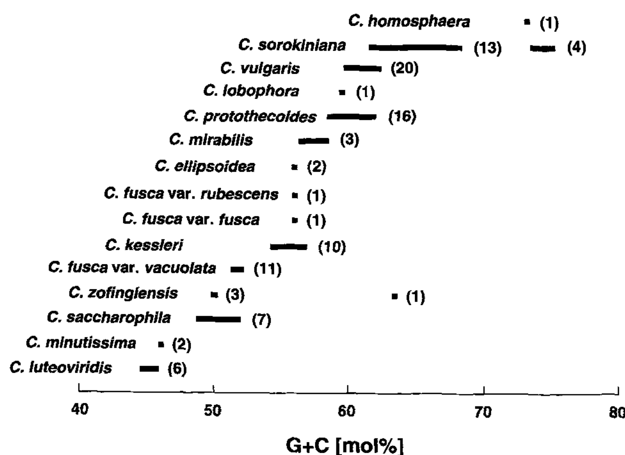

Fig. 1. Base composition of *Chlorella* species derived from T_M (data are taken from the literature as shown in Table 1). The bars indicate the range of G + C found for different strains (numbers in brackets) within a given species.

tidine (dC), 2′-deoxyguanosine (dG), 2′-deoxythymidine (dT), 5-methyl-2′-deoxycytidine (m^5dC), N6-methyl-2′-deoxyadenosine (m^6dA) and 5-hydroxymethyl-2′-deoxyuridine (hm^5dU). The instrument was calibrated with non-methylated lambda phage DNA (Sigma) with a DNA G + C content of 49·858 mol% (Sanger *et al.*, 1982). The overall DNA G + C content and the concentration of the rare bases m^5dC and hm^5dU were calculated automatically by the SHIMADZU integrator using the computer program GC.BAS (Jahnke, unpublished data) according to the formulae G + C = (dG/dG + dA)·100, m^5dC = (dG − dC)·100, and hm^5dU = (dA − dT)·100, where dC, dG, dT, dA, m^5dC and hm^5dU are the molar fractions of the corresponding nuclear bases. The calibration was checked at intervals during each measuring period by analysing methylated salmon sperm DNA (Sigma) of known base composition.

Table 1. HPLC-derived base ratios and modification of nuclear DNA from chlorococcalean algae

Species	Strain	Origin[a]	G + C by T_M[b] (mol%)	Ref.[c]	G + C by HPLC[d] (mol%) ± SD	m^5dC[e] (mol%) ± SD	m^5dC[f] (%)	N[g]
Chlorella ellipsoidea (Gerneck) Fott & Nováková	211–1a[T]	SAG	57.3[N]	5	59·1 ± 0.1	2·4 ± 0·2	8·12	3
C. fusca var. *fusca* Shihira & Krauss	343[T]	UTEX	55.7	3	57.1 ± 0·4	ca. 3·1	10·86	3
C. fusca var. *rubescens* (Dangeard) Kessler *et al.*	232/1[T]	CCAP	56·8	3	57·4 ± 0·1	2·3 ± 0·5	8.01	3
C. fusca var. *vacuolata* Shihira & Krauss	211–8b[T]	SAG	51·6	2	51·6 ± 0·2	2·9 ± 0·2	11.24	8
C. homosphaera Skuja	211/8e[T]	CCAP	73·0	5	73·1 ± 0·2	5·8 ± 0·8	15·87	3
C. kessleri Fott & Nováková	211–11g[T]	SAG	55·1	3	58·9 ± 0·5	4·4 ± 0·1	14·94	4
C. luteoviridis Chodat	211–3	SAG	45·0	6	46·0 ± 0·5	1·6 ± 0·3	6.96	6
C. minutissima Fott & Nováková	C-1.1.9	Bethesda	46.0	6	47·0 ± 0·3	2·2 ± 0·1	9·36	4
C. mirabilis Andreyeva	748–1[T]	Andreyeva	57·1[N]	5	57·5 ± 0·1	5·4 ± 0·5	18·78	4
C. protothecoides Krüger	211–7a[T]	SAG	59·0	4	64·1 ± 0·3	5·9 ± 0·6	18·41	4
C. saccharophila (Krüger) Migula	211–9a[T]	SAG	52·0	2	53·8 ± 0·2	3·1 ± 0·1	11·52	4
C. sorokiniana Shihira & Krauss	211–8k[T]	SAG	63·1	1	64·1 ± 0·2	6·3 ± 0·1	19·66	4
C. sorokiniana	211/11d	CCAP	61·5	1	65·2	4·8	14·72	2
C. sorokiniana	211–31	SAG	68·3	1	69·1	5·4	15·63	2
C. sorokiniana	211–40a	SAG	75·2	1	75·2	9·8	26·06	2
C. sorokiniana	1–9–30	Sorokin	64·9	1	66.9 ± 0·2	7·3 ± 0·1	21·82	3
C. sorokiniana	Prag A14	Baslerová	73.7	1	76·2	12.0	31·50	2
C. vulgaris Beijerinck	211–11b[T]	SAG	61·2	2	62·0 ± 0·2	6·0 ± 0·3	19·35	4
C. zofingiensis Dönz	211–14a[T]	SAG	50·1	6	51·4 ± 0·2	1·5 ± 0·1	5·84	3
Chlorella sp.	211–6	SAG	67·1[N]	5	67·7 ± 0·2	7·7 ± 0·4	22·75	5
Chlorella sp.	211–18	SAG	51·3	1	56·4 ± 0·2	2·8 ± 0·1	9·93	3
Monoraphidium braunii Komárková-Legnerová	202–7a	SAG	65·5	—	65·6	8·9	27·13	2
Nannochloris coccoides Naumann	251–1	SAG	69·1[N]	—	66·0 ± 0·2	4·3 ± 0·2	13·03	8
Prototheca wickerhamii Tubaki & Soneda	1283	Pore	60·3	4	64·4 ± 0·1	6·1 ± 0·1	18·94	4
P. zopfii Krüger	263–1a	SAG	69·5	4	73·0 ± 0·4	3·7 ± 0·3	10·13	3
Scenedesmus obliquus (Turpin) Kützing	276–3a	SAG	55·1	5	57·4 ± 0·4	4·4 ± 0·2	15·33	3

HPLC, high-performance liquid chromatography; T_M, melting temperature.
[a]Andreyeva, V.M. Andreyeva, St Petersburg, GUS; Baslerová, M. Baslerova, CFR; Bethesda, Culture Collection at Bethesda, Maryland, USA; CCAP, Culture Centre of Algae and Protozoa at Cambridge, UK; Pore, R.S. Pore, Morgantown, West Virginia, USA; SAG, Sammlung für Algenkulturen in Göttingen, Germany; Sorokin, C. Sorokin, College Park, Maryland, USA; UTEX, University of Texas Culture Collection of Algae at Austin, Texas, USA.
[b]Derived from T_M by the equation of Owen *et al.* (1969).
[c]1, Huss *et al.* (1986); 2, Huss *et al.* (1987*a*); 3, Huss *et al.* (1987*b*); 4, Huss *et al.* (1988); 5, Huss *et al.* (1989*a*). 6, Huss *et al.* (1989*b*); —, authors' data.
[d]Total amount (mol%) of dG + dC + m^5dC derived form HPLC analyses.
[e]Total amount (mol%) of m^5dC derived from HPLC analyses.
[f]Percentage of dC that is replaced by m^5dC: m^5dC/(dC + m^5dC) · 100.
[g]Number of determinations.
[N]Nuclear DNA, purified by CsCl density gradient centrifugation.
[T]Type strain.

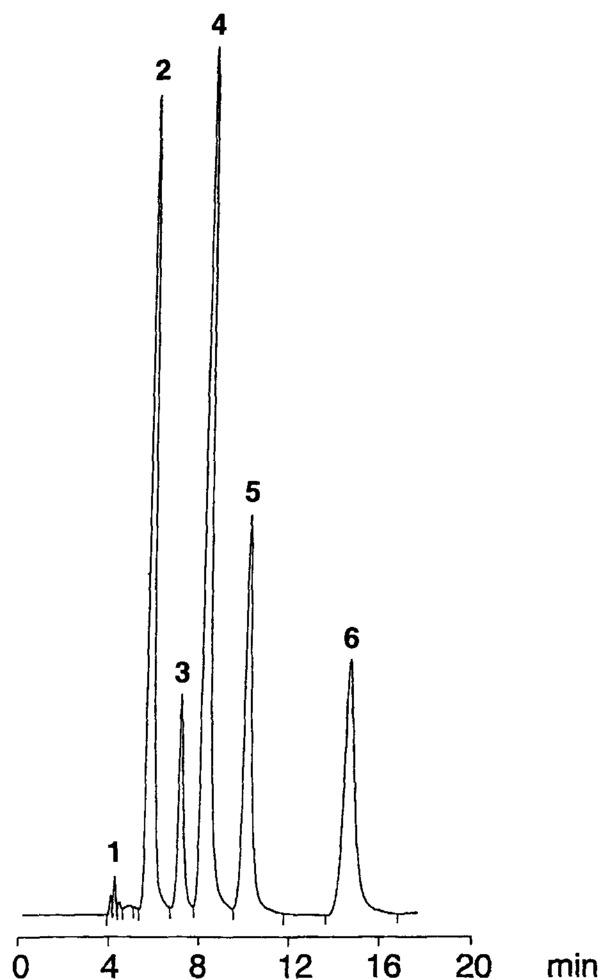

Fig. 2. HPLC chromatogram of the DNA hydrolysate of *Chlorella* sp. 211–6. The x-axis refers to the retention time, and the y-axis gives the relative absorbance at 270 nm. 1, injection peak; 2, dC; 3, m^5dC; 4, dG; 5, dT; 6, dA.

Results

The retention times of the deoxyribonucleosides increased in the order dC > hm^5dU > m^5dC > dG > dT > dA ≫ m^6dA (Fig. 2). The methylated DNA bases m^5dC and hm^5dU could be detected and quantified only in the absence of ribonucleosides. m^6dA, on the other hand, with a retention time of about 35 min, was well separated from all other nucleosides tested (not shown). For the separation of hm^5dU and m^5dC, a pH of 4·6–4·8 in the solvent system was favourable. The original high-salt solvent system containing 0·6 M $NH_4H_2PO_4$ could be replaced by 0·3 M $NH_4H_2PO_4$ without substantial loss of separating properties (T. Nagel & J. Tamaoka, personal communication). The reproducibility of the system within one series of runs was usually better than ± 0·4 mol%. The maximum standard deviation between different series of measurements over a period of several months was estimated to be ± 0·8 mol%. The high reproducibility of ± 0·1 mol% reported by Mesbah *et al.* (1989) could not be achieved with our equipment.

Methylated deoxycytidine was found in nuclear DNA of all green algal species examined, whereas m^6dA and hm^5dU could not be detected (Fig. 2; Table 1). The amount of m^5dC ranged from 1·5 mol% up to 12·0 mol% and was highly correlated ($R = 0{\cdot}77757$) with the G + C content of the nuclear DNA (Fig. 3). hm^5dU was found only in the dinoflagellate *Symbiodinium microadriaticum* (not shown). The DNA base compositions of this chromophyte alga and of salmon sperm, which served as controls, were in good concordance with literature values (Table 2).

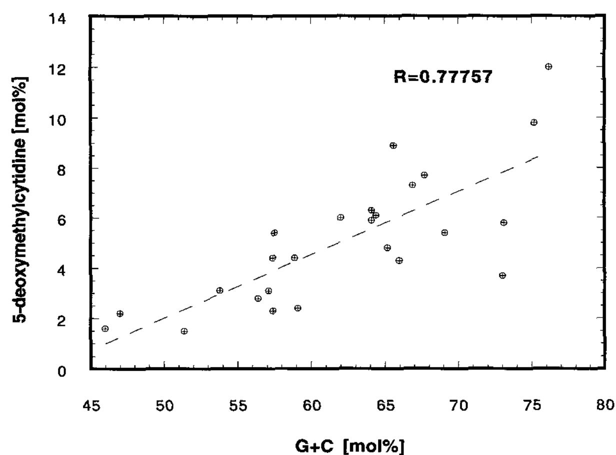

Fig. 3. Plot of 5-deoxymethylcytidine versus G + C content. Values are taken from Table 1. The dashed line shows the linear least squares fit. The correlation coefficient $R = 0{\cdot}77757$ indicates a high correlation between m^5dC and G + C content.

Discussion

The molar base composition of DNA is an important character in microbial systematics. With the development of HPLC technology, direct analysis of unusual and rare nucleosides not detected by classical methods such as determination of melting temperature (Marmur & Doty, 1962) or buoyant density in CsCl gradients (Schildkraut *et al.*, 1962) is possible (cf. Gehrke *et al.*, 1984). By influencing both the thermal stability and buoyant density of the DNA double strand, modified nucleotides may cause significant errors in the estimation of the DNA G + C content derived from such methods (Kirk, 1967; Rae, 1973, 1976).

The DNA of higher eukaryotes usually contains m^5dC in small amounts of the order of 10% or less of the total cytidine (Rae & Steele, 1978). In plant DNA, up to 30% of the cytidine may be replaced by m^5dC (Shapiro, 1976). Additionally, unicellular eukaryotes sometimes contain m^6dA or hm^5dU, the latter a thymidine analogue found in large amounts in the DNA of dinoflagellates (Rae, 1973; Blank *et al.*, 1988). For green algae the amount of DNA base modification has not often been examined, but recently Jarvis *et al.* (1992) studied the DNA nucleoside composition and methylation of several microalgae including four chlorophytes. Our data from chlorococcaean algae agree with their results in that significant but variable amounts of m^5dC only were detected (Table 1). There is, however, a discrepancy in the two values of the

Table 2. Nuclear base composition of reference DNAs

Species	G + C[a] (mol%) ± SD	m^5dC[b] (mol%) ± SD	hm^5dU[c] (mol%) ± SD	N[d]
Symbiodinium microadriaticum[e] Freudenthal emend. Trench & Blank	51·1 ± 0·4	2·2 ± 0·1	13·1 ± 0·2	3
Salmon sperm[e]	43.7 ± 0·5	2·0 ± 0·7	—	16
Symbiodinium microadriaticum[f]	50·5	1·0	11·4	
Salmon sperm[g]	43·4 ± 0·04	1·16 ± 0·09	—	
Salmon sperm[h]	44	1·57	—	

[a]Total amount (mol%) of dG + dC + m^5dC.
[b]Total amount (mol%) of m^5dC.
[c]Total amount (mol%) of hm^5dU.
[d]Number of determinations.
[e]This study.
[f]Blank *et al.* (1988).
[g]Mesbah *et al.* (1989).
[h]Kuo *et al.* (1980).

DNA G + C content of *Chlorella ellipsoidea*, which was examined in both studies. The figure obtained by Jarvis *et al.* (1992) is 7·5 mol% lower, which may be explained in two ways. Firstly, the CCAP strain 211/1a used in their study may not be identical to our strain 211–1a from the Göttingen collection SAG, as has previously been demonstrated for strains CCAP 211/8e and SAG 211-8e (Kessler & Zweier, 1971). Secondly, Jarvis and coworkers used total cell DNA in their HPLC analysis, with plastid (pt) DNA and, as a minor component, mitochondrial DNA, still present. Our whole-cell DNA preparations from *C. ellipsoidea* contained remarkably high amounts of pt DNA (of the order of 20%) prior to CsCl density gradient centrifugation. Considering the low DNA G + C content of *C. ellipsoidea* pt DNA (35·7 mol%; Yamada, 1982), the contaminating pt DNA may also be responsible for the low value determined by Jarvis *et al.* (1992).

The total amount of m^5dC increased with the G + C content of the DNA. The high correlation coefficient shown in Fig. 3 becomes even more significant ($R = 0{\cdot}82213$) if *Prototheca zopfii* with only 3·7 mol% m^5dC, and *Chlorella sorokiniana* Prag A14, with an extraordinarily high m^5dC content of 12 mol%, are excluded from the regression analysis. The increase in m^5dC with dC is, however, not directly proportional and might in fact be an expression of the increased probability of CG or CXG sequences, the preferential sites of cytidine methylation (Gruenbaum *et al.*, 1981, 1982).

The percentage of cytidine methylation ranges from 5·8% in *Chlorella zofingiensis* up to 31.5% in *C. sorokiniana* Prag A14 (Table 1). The evolutionary constraints causing such differences in DNA methylation (just as in base composition) within a relatively closely related group of organisms are not clear.

From the data of Ehrlich *et al.* (1975) on *Xanthomonas* phage XP-12, it was calculated that each 1% methylated cytidine produces an increase in T_M of 0.18°C thus raising the apparent mol% G + C by 0·32 (Coene & Cocito, 1985). Similar observations have been reported by Gill *et al.* (1974) and Klump & Löffler (1985) on poly(dG)·poly(m^5dC) versus poly(dG)·poly(dC). If DNA methylation stabilises the DNA double helix then the apparent G + C content calculated from T_M minus the actual DNA G + C content determined by HPLC should increase with the amount of dC that is replaced by m^5dC. For our data, however, this is not the case. In contrast, there is an almost total lack of correlation ($R = 0{\cdot}041468$) between the two parameters, indicating no significant influence of deoxycytidine methylation on the thermal stability of the DNA double strand. Our results thus contradict the observations made on methylated phage DNA and synthetic homopolymers. Although plastid DNA was not removed by CsCl density gradient centrifugation from most of our samples used for the thermal denaturation studies, this cannot account for the observed discrepancy. The effect of pt DNA on T_M was kept as low as possible by discarding the initial absorbance rise of biphasic melting curves and by evaluating the T_M from the derivative plots (Sly *et al.*, 1986). Moreover, similar results were obtained when purified nuclear DNAs were used (Table 1). At present we do not have any explanation for this inconsistency. The influence of DNA modification on thermal stability of dinoflagellate DNA shown in a previous paper (Blank *et al.*, 1988) was apparently based solely on the content of hm^5dU.

It was the main purpose of this study to demonstrate whether nucleoside modification may be responsible at least in part for the highly variable DNA base composition observed in previous taxonomic studies on the chlorococcalean green alga *Chlorella* (see Table 1 for references). The broad range of G + C covered by different species of *Chlorella* (Fig. 1) can largely be explained by the results of comparative small subunit ribosomal RNA (SSU rRNA) sequence analysis which show that *Chlorella* is not monophyletic (Huss & Sogin, 1990; unpublished results). Several species are more

closely related to other genera and some even belong to a different family, the Scenedesmaceae. The phylogenetic distances of these species are expressed at the phenotypic level by the heterogeneity of physiological and biochemical characters (Kessler, 1976, 1982). Strains which cannot be discriminated by such criteria are assigned to a single species. Some of these species, however, still comprise strains with varying DNA G + C contents. The most striking example is *C. sorokiniana*, a species uniquely characterised within the genus *Chlorella* by growing at high temperatures of 38–42°C (Kessler, 1976, 1985). Whereas DNA of the type strain 211–8k contains 63 mol% G + C, up to 75 mol% were found for other strains (Fig. 1; Table 1), indicating that some of these strains may be misclassified.

DNA/DNA hybridisation studies have shown that 16 strains of *C. sorokiniana* split into six branches with DNA similarities of only about 25% among each other (Huss *et al.*, 1986). Nevertheless, these branches are part of a single cluster separating *C. sorokiniana* from other *Chlorella* species inlcuding the most closely related *C. vulgaris* (99·6% SSU rRNA homology between the type strains; unpublished data). Therefore, if the observed DNA similarities of 25% are significant and not below the resolution limit of the membrane filter DNA hybridisation method, all strains of *C. sorokiniana* may in fact be closely related in spite of the different base composition. This appears unlikely if the melting temperature from which the DNA G + C content was derived is not strongly influenced by a different degree of base modification.

Our HPLC data, however, confirm that *C. sorokiniana* strains vary greatly in their DNA base composition and exclude DNA base modification as the cause of this heterogeneity. Whether a close phylogenetic relationship exists, as indicated by the DNA hybridisation studies, could be demonstrated by comparative rRNA sequence analyses.

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References

Blank, R.J., Huss, V.A.R. & Kersten, W. (1988). Base composition of DNA from symbiotic dinoflagellates: a tool for phylogenetic classification. *Arch. Microbiol.*, **149:** 515–520.

Coene, M. & Cocito, C. (1985). A microanalytical procedure for determination of the base composition of DNA. *Eur. J. Biochem.* **150:** 475–479.

De Ley, J. (1969). Compositional nucleotide distribution and the theoretical prediction of homology in bacterial DNA. *J. Theor. Biol.*, **22:** 89–116.

Dörr, R. & Huss, V.A.R. (1990). Characterization of nuclear DNA in 12 species of *Chlorella* (Chlorococcales, Chlorophyta) by DNA reassociation. *BioSystems*, **24:** 145–155.

Ehrlich, M., Ehrlich, K. & Mayo, J.A. (1975). Unusual properties of the DNA from *Xanthomonas* phage XP-12 in which 5-methylcytosine completely replaced cytosine. *Biochim. Biophys. Acta*, **395:** 109–119.

Gehrke, C.W., McCune, R.A., Gama-Sosa, M., Ehrlich, M. & Kuo, K. (1984). Quantitative reversed-phase high performance liquid chromatography of major and modified nucleosides in DNA. *J. Chromat.*, **301:** 199–219.

Gill, J.E., Mazrimas, J.A. & Bishop, C.C.Jr. (1974). Physical studies on synthetic DNAs containing 5-methylcytosine. *Biochim. Biophys. Acta*, **335:** 330–348.

Gruenbaum, Y., Naveh-Many, T., Cedar, H. & Razin, A. (1981). Sequence specificity of methylation in higher plant DNA. *Nature*, **292:** 860–862.

Gruenbaum, Y., Cedar, H. & Razin, A. (1982). Substrate and sequence specificity of a eukaryotic DNA methylase. *Nature*, **295:** 620–622.

Huss, V.A.R. & Sogin, M.L. (1990). Phylogenetic position of some *Chlorella* species within the Chlorococcales based upon complete small-subunit ribosomal RNA sequences. *J. Mol. Evol.*, **31:** 432–442.

Huss, V.A.R., Dörr, R., Grossmann, U. & Kessler, E. (1986). Deoxyribonucleic acid reassociation in the taxonomy of the genus *Chlorella*. I. *Chlorella sorokiniana*. *Arch. Microbiol.*, **145:** 329–333.

Huss, V.A.R., Schwarzwälder, E. & Kessler, E. (1987*a*). Deoxyribonucleic acid reassociation in the taxonomy of the genus *Chlorella*. II. *Chlorella saccharophila*. *Arch. Microbiol.*, **147:** 221–224.

Huss, V.A.R., Hehenberger, A. & Kessler, E. (1987*b*). Deoxyribonucleic acid reassociation in the taxonomy of the genus *Chlorella*. III. *Chlorella fusca* and *Chlorella kessleri*. *Arch. Microbiol.*, **149:** 1–3.

Huss, V.A.R., Wein, K.H. & Kessler, E. (1988). Deoxyribonucleic acid reassociation in the taxonomy of the genus *Chlorella*. IV. *Chlorella protothecoides* and its relationship to the genus *Prototheca*. *Arch. Microbiol.*, **150:** 509–511.

Huss, V.A.R., Huss, G. & Kessler, E. (1989*a*). Deoxyribonucleic acid reassociation and interspecies relationships of the genus *Chlorella* (Chlorophyceae). *Plant Syst. Evol.*, **168:** 71–82.

Huss, V.A.R., Scharpf, T.K. & Kessler, E. (1989*b*). Deoxyribonucleic acid reassociation in the taxonomy of the genus *Chlorella*. V. *Chlorella vulgaris*, *C. luteoviridis*, *C. minutissima*, and *C. zofingiensis*. *Arch. Microbiol.*, **152:** 512–514.

Jarvis, E.E., Dunahay, T.G. & Brown, L.M. (1992). DNA nucleoside composition and methylation in several species of microalgae. *J. Phycol.*, **28:** 356–362.

Kessler, E. (1976). Comparative physiology, biochemistry, and the taxonomy of *Chlorella* (Chlorophyceae). *Plant Syst. Evol.*, **125:** 129–138.

Kessler, E. (1977). Physiological and biochemical contributions to the taxonomy of the genus *Prototheca*. I. Hydrogenase, acid tolerance, salt tolerance, thermophily, and liquefaction of gelatin. *Arch. Microbiol.*, **113:** 139–141.

Kessler, E. (1982). Chemotaxonomy in the Chlorococcales. In *Progress in Phycological Research* (Round, F.E. & Chapman, D.J., editors), vol. 1, 111–135. Amsterdam, Elsevier.

Kessler, E. (1985). Upper limits of temperature for growth in *Chlorella* (Chlorophyceae). *Plant Syst. Evol.*, **151:** 67–71.

Kessler, E. & Zweier, I. (1971). Physiologische und biochemische Beiträge zur Taxonomie der Gattung *Chlorella*. V. Die auxotrophen und mesotrophen Arten. *Arch. Mikrobiol.*, **79:** 44–48.

Kirk, J.T.O. (1967). Effect of methylation of cytosine residues on the buoyant density of DNA in caesium chloride solution. *J. Mol. Biol.*, **28:** 171–172.

Klump, H.H. & Löffler, R. (1985). Reversible helix/coil transitions of left-handed Z-DNA structures. *Biol. Chem. Hoppe-Seyler*, **366:** 345–353.

Kuo, K.C., McCune, R.A., Gehrke, C.W., Midgett, R. & Ehrlich, M. (1980). Quantitative reversed-phase high performance liquid chromatographic determination of major and modified deoxyribonucleosides in DNA. *Nucleic Acids Res.*, **8:** 4763–4776.

Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.*, **5:** 109–118.

Mesbah, M., Premachandran, U. & Whitman, W.B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Inst. J. Syst. Bacteriol.*, **39:** 159–167.

Owen, R.J., Hill, L.R., & Lapage, S.P. (1969). Determination of DNA base compositions from melting profiles in dilute buffers. *Biopolymers*, **7:** 503–516.

Rae, P.M.M. (1973). 5-Hydroxymethyluracil in the DNA of a dinoflagellate. *Proc. Natl. Acad. Sci. U.S.A.* **70:** 1141–1145.

Rae, P.M.M. (1976). Hydroxymethyluracil in eukaryote DNA: a natural feature of the Pyrrhophyta (Dinoflagellates). *Science*, **194:** 1062–1064.

RAE, P.M.M. & STEELE, R.E. (1978). Modified bases in the DNAs of unicellular eukaryotes: an examination of distributions and possible roles, with emphasis on hydroxymethyluracil in dinoflagellates. *BioSystems*, **10:** 37–53.

SANGER, F., COULSON, A.R., HONG, G.F., HILL, D.F. & PETERSEN, G.B. (1982). Nucleotide sequence of bacteriophage λ DNA. *J. Mol. Biol.*, **162:** 729–773.

SCHILDKRAUT, C.L., MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *J. Mol. Biol.*, **4:** 430–443.

SHAPIRO, H.S. (1976). Nucleic acids. In *Handbook of Biochemistry and Molecular Biology* (Fasman, G.V., editor), vol. II, 241–283. Cleveland, CRC Press.

SLY, L.I., BLACKALL, L.L., KRAAT, P.C., TIAN-SHIEN, T. & SANGKHOBOL, V. (1986). The use of second derivative plots for the determination of mol% guanine plus cytosine of DNA by the thermal denaturation method. *J. Microbiol. Methods*, **5:** 139–156.

TAMAOKA, J. & KOMAGATA, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol. Lett.*, **25:** 125–128.

YAMADA, T. (1982). Isolation and characterization of chloroplast DNA from *Chlorella ellipsoidea*. *Plant Physiol.*, **70:** 92–96.

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