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The plastome of a brown alga, *Dictyota dichotoma**

I. Physical properties and the Bam HI/Sal I/Bgl II cleavage site map

M. Kuhsel & K. V. Kowallik**
Botanisches Institut der Universität Düsseldorf, Universitätsstraße 1, D-4000 Düsseldorf, FRG

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Summary

Plastids of the brown alga *Dictyota dichotoma* contain a single homogeneous DNA species which bands at a buoyant density of 1.693 g/cm^3 in neutral CsCl equilibrium density gradients. The corresponding nuclear DNA has a density of 1.715 g/cm^3. The molecular size of the plastid DNA is 123 kbp as calculated by both electron microscopy of spread intact circular molecules and gel electrophoresis following single and double digestions with various restriction enzymes. A restriction map has been constructed using the endonucleases Sal I, Bam HI, and Bgl II which cleave the *Dictyota* plastome into 6, 12, and 17 fragments, respectively. No large repeated regions, as found in chlorophycean and *Euglena* plastid DNAs, were detected. *Dictyota dichotoma* is the first member from the chlorophyll c-line of the algal pedigree for which a physical map of plastid DNA has been established.

Introduction

Physical maps of chloroplast DNAs have been constructed mainly from dicots (reviewed in 15, 42), whereas only few reports refer to monocots (3, 7, 13, 35, 38). Plastomes from Archegoniates have been analyzed even more sporadically (29, 31). The most striking feature of the plastomes from the majority of vascular plants investigated thus far is their highly conserved anatomy including an inverted repeat sequence of about 20 kbp separated by two single copy regions of different size. Only in the *Fabaceae*, some major variations with respect to the inverse duplication carrying the genes for the ribosomal RNAs have occurred (23, 32, 33).

Restriction maps from algal plastomes have been constructed from only two genera (*Euglena gracilis*: 16; *Chlamydomonas reinhardii*: 39), both of which belong to the chlorophyll b-line of the algal kingdom. The plastome of *Chlamydomonas* exceeds that of most higher plants by some 45 kbp corresponding to about 30 Md (4). Nevertheless, its anatomy is basically similar to that of plastomes from higher plants regarding the inverse duplication of the rDNA although the orientation of transcription differs with respect to the position of the gene for the large subunit of ribulose biphosphate carboxylase/oxygenase (40). On the other hand, the plastome of *Euglena* – though similar in size to that of higher plants – differs by its unique organization of tandemly arranged ribosomal RNA genes (24, 43).

Information about plastomes from algae containing chlorophyll c remain sparse or even controversial (1, 9, 10, 14). Restriction maps of the latter do not exist. Nevertheless, this evolutionary branch with properties quite different from the chlorophycean line appears to be most interesting for studying plastid DNA for at least two reasons: (i) The considerable heterogeneity among the *Chromophyta* provides a reliable condition to investigate plastome evolution on a large scale. (ii) A comparison

* Dedicated to Professor Dr. W. Stubbe on the occasion of his 65th birthday.

** To whom correspondence should be addressed.

of plastomes from algae belonging to more closely related phyla as well as the molecular analysis of plastomes from related genera or species should lead to a better understanding of the small scale evolution of plastomes.

In this paper we present the first restriction endonuclease cleavage map from a member of the chlorophyll c-line, the brown alga *Dictyota dichotoma*.

Material and methods

1. Cultivation of *Dictyota dichotoma*

Small lateral branches of *Dictyota* were dissected from *thalli* of stock cultures and grown in artificial seawater medium according to Wiedemann-Kramer (cited in 19), modified by decreasing nitrate from 1.2 mM to 0.6 mM, by adding both $10 \times Fe^{3+}$ and trace elements PII (36), and vitamins SII (37), and by adjusting the pH to 7.6 using Tris/HCl buffer. The cultures were maintained at 24 °C and 14:10 h light:dark. After four to six weeks the individual plants grew up to 2.5 cm (Fig. 1a, b). 50–80 l of culture medium yielded up to 50 g fresh material. Three days before isolation of plastids the algae were kept in the dark at room temperature to reduce both liquid carbohydrates and the number of physodes with their tannic compounds (8) which adversely affect subcellular fractionation.

2. Isolation of plastids

The algae were repeatedly washed in isotonic NaCl solutions to remove iron and magnesia from mucilage and cell walls. NaCl was then replaced by washing the material with isolation medium containing 0.4 M mannitol, 80 mM Na_2EDTA, 50 mM Tris, 4 mM $MgCl_2$, 4 mM mercaptoethanol, 0.5% polyvinylpyrrolidone (PVP) and 0.1% bovine serum albumin (BSA), pH 7.6. The cells were broken using a Waring blendor (30 sec, high speed), and the homogenate was filtered through 50 μm and 2×5 μm nylon gauze. The filtrate was centrifuged briefly at $1{,}500 \times g$ to remove crude debris. Plastids were pelleted by centrifuging the supernatant for 30 min at $1{,}500 \times g$. They were then separated from contaminating cellular components by several steps of differential centrifugation in resuspension medium (isolation medium without PVP and BSA). The maintainance of a relatively high concentration of Na_2EDTA was essential to avoid clumping of plastids (Fig. 1c).

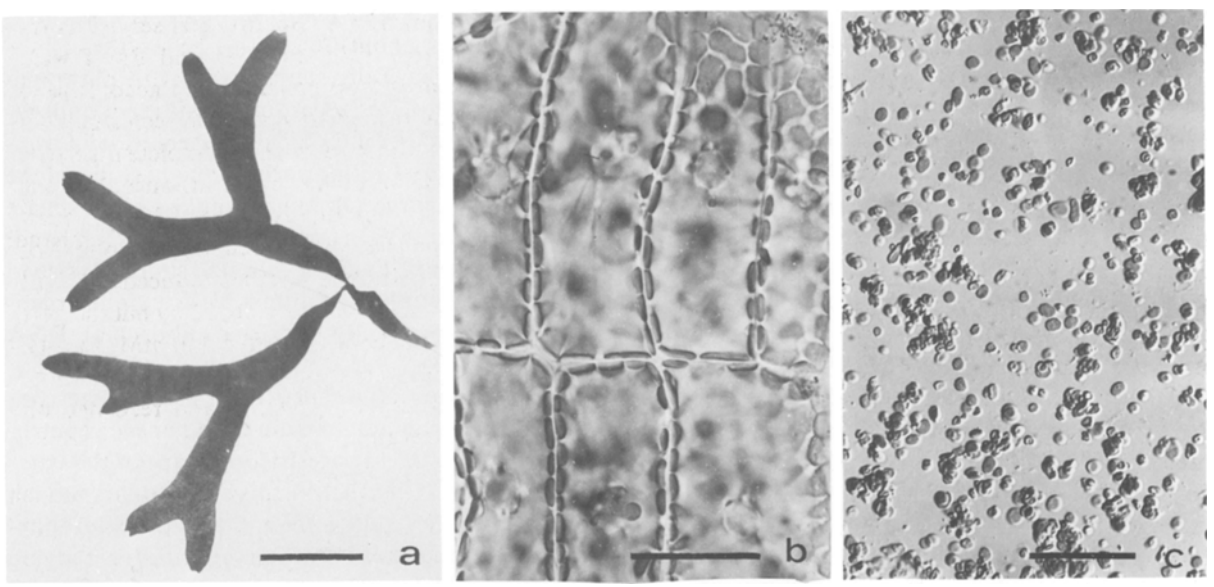

Fig. 1. (a) Four weeks old branched thallus of *Dictyota dichotoma* grown in artificial seawater medium at 24 °C, 16:8 h, 600 lux (scale 0.5 cm). (b) Differentiated cells of the epidermal layer showing plastids in front (upper right) and profile view. Note the different size of the nuclei in the center of the cells (scale: 25 μm). (c) Typical aspect of isolated plastids purified by differential centrifugation (scale: 50 μm).

3. Preparation of plastid DNA (ptDNA)

The plastids were lysed by adjusting the suspension to 1.5% SDS and 0.7% N-lauroyl-sarcosine, and allowed to stand for 2 h at 55 °C in the presence of proteinase K (100 μg/ml). This treatment appears to be necessary for obtaining plastid DNA of *Dictyota* that can be digested with restriction enzymes. The DNA was extracted by adding an equal volume of phenol/chloroform (1:1) equilibrated with 50 mM Tris, 100 mM Na_2EDTA, pH 8.3, followed by chloroform treatment and dialysis against Tris/EDTA buffer (10 mM/1 mM), pH 8.0. For further purification the DNA was centrifuged in neutral CsCl gradients ($n = 1.3880$) in the presence of ethidium bromide using a Beckmann VTI 80 rotor at 146,000 × *g* for 16–18 h. The individual DNA bands were removed with a syringe and microdialyzed against Tris/EDTA buffer (10 mM/1 mM) at pH 8.0. The DNA was concentrated by ethanol precipitation and redisolved in a small volume of Tris/EDTA buffer (10 mM/2 mM) at pH 8.0. The yield is between 10 and 50 μg of pure plastid DNA.

4. Preparation of whole cell DNA

Axenically maintained cultures initially prepared by growing small young *thalli* in the presence of antibiotics (ampicillin 100 mg/l; kanamycinsulfate 100 mg/l) were used to isolate total cellular DNA. Tests for sterility were carried out by transferring parts of a *thallus* from each culture vessel into sterile seawater medium enriched with peptone (1 mg/ml), sugar (1 mg/ml) and beef extract (1 mg/ml). The inoculum was incubated for several days at 30 °C. Five to 10 g of material was homogenized in the above mentioned isolation medium. The homogenate was lysed and extracted with phenol/chloroform as described above. Since purification of DNA by ethanol precipitation proved to be impossible due to contaminating high viscosity carbohydrates the DNA was purified by electro-elution utilizing the different electrophoretic mobility of both mucilage and DNA. After a subsequent phenolization step the DNA was precipitated by ethanol. The redissolved total cellular DNA was used to determine the buoyant densities in a Beckman Model E analytical centrifuge.

5. Electron microscopy of DNA

For visualization of intact ptDNA molecules phenol extraction was replaced by centrifuging the DNA from the lysate into a CsCl cushion ($n = 1.400$) using a RPS-40 T swinging bucket rotor for 16 h at 130,000 × *g* (21). The gradient was fractionated and the DNA microdialyzed against Tris/EDTA buffer (10 mM/1 mM), pH 8.0. Spreading of DNA was carried out according to Davis *et al.* (11) but with distilled water as a hypophase. ØX 174 RF DNA was used as an internal standard. The DNA/cytochrome c-film was picked up with parlodion covered grids, stained with uranyl acetate and rotary shadowed with Pt/C using a Balzers electron beam source.

6. Restriction enzyme analysis

With the exception of Bgl II and Sal I which were prepared according to Duncan *et al.* (12) and Arrand *et al.* (2), respectively, the restriction enzymes Bgl I, Pst I, Eco R I, Pvu II and Bam HI were purchased from Boehringer (Mannheim). The enzymes were used as recommended by the supplier. The fragments were separated on 0.4–1.0% horizontal slab gels (SeaKem ME Agarose). The Sal I fragment pattern of spinach ptDNA (22) and Hind III digested lambda DNA (Boehringer) served as internal standards. Sal I, Bam HI and Bgl II were selected to construct a physical map according to the SeaPlaque low gelling agarose technique described in (22). Since in general complete digestion of *Dictyota* ptDNA did not occur in concentrations higher than 1 μg/100 μl, digestion of 10 μg, necessary for this analysis, was carried out in a volume of about 1 ml. This volume was then reduced to 100 μl under vacuum and the salt removed by microdialysis against Tris/EDTA buffer (10 mM/1 mM), pH 8.0. The gels were transilluminated with a short wave UV light source (254 nm) and recorded on Ilford HP5 sheet film.

Results

1. Analytical ultracentrifugation

Scans of whole cell DNA exhibit a main peak of density 1.715 g/cm^3 and two smaller peaks of densi-

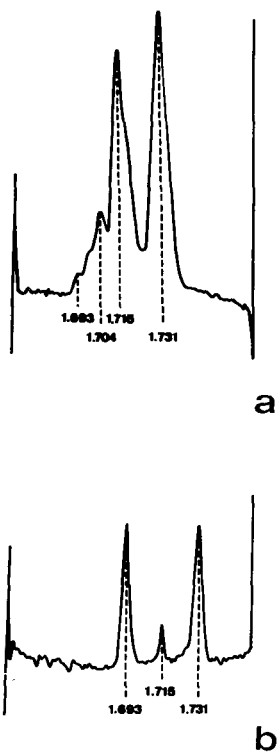

Fig. 2. Buoyant density distribution of whole cell DNA of axenically grown *Dictyota* (a) and of DNA from isolated plastids (b). ρ = 1.693 g/cm^3 = plastid DNA, ρ = 1.715 g/cm^3 = nuclear DNA, ρ = 1.731 g/cm^3 = *Micrococcus luteus* DNA. The DNA of density 1.704 g/cm^3 is of undetermined origin.

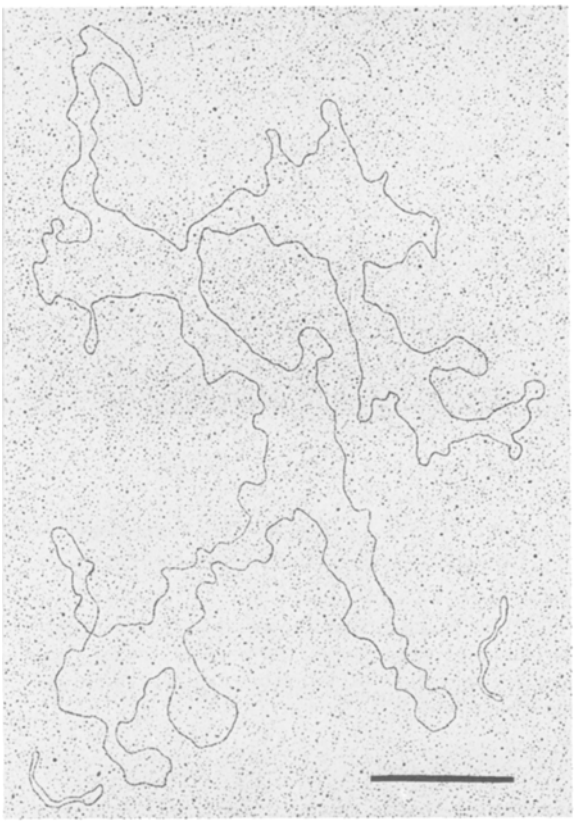

Fig. 3. Open circular molecule of cytochrome c-spread *Dictyota* ptDNA, co-spread with øχ 174 RF DNA (scale: 1 μm).

ties 1.704 and 1.693 g/cm^3, respectively. A slight shoulder on the light flank of the 1.704 peak may occur in some preparations (Fig. 2a). Purified ptDNA extracted from plastid preparations showed only one main peak of density 1.693 g/cm^3, accompanied by an indication of a peak at density 1.715 g/cm^3 (Fig. 2b). The third peak of medium density is no longer detectable. It is thus obvious that the peak of density 1.693 g/cm^3 is of plastid origin, whereas the main peak can be attributed to nuclear DNA. No further attempt has been made to clarify the nature of the intermediate peak of density 1.704 g/cm^3.

2. *Electron microscopy*

DNA prepared from gently lysed plastids by centrifugation into CsCl contained up to 20% intact molecules. With the exception of a few covalently closed molecules, these were encountered in the open circular form (Fig. 3). From 10 different preparations a total number of 81 molecules has been recorded. The circumference of 79 molecules ranged from 35.6–43.7 μm (mean size 40.1 μm, corresponding to about 128 kbp) indicative of a single homogeneous size class (Fig. 4). Only one molecule was found to be of nearly double length (68.9 μm, corresponding to 218 kbp) and is interpreted as dimeric. The origin of another circular molecule of 14.9 μm circumference (about 48 kbp) remains undetermined.

3. *Restriction endonuclease analysis*

Since the DNA prepared for electron microscopy was not accessible to restriction enzymes, and most of the DNA did not migrate into the CsCl cushion, probably due to the presence of remaining DNA-protein complexes, we removed most of the proteinaceous components by phenolyzing the lysate prior to banding in neutral CsCl. Normally there

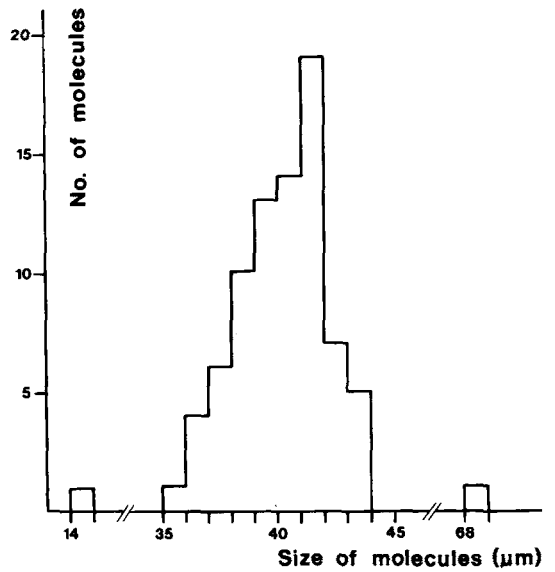

Fig. 4. Length distribution of open circular molecules isolated from *Dictyota* plastid preparations. One circular molecule of 14.9 µm circumference is of unknown origin whereas a second molecule of 68.9 µm circumference may be considered as a dimeric molecule of ptDNA.

were three clearly distinguishable DNA bands, of which the two uppermost usually gave a stronger fluorescence under longwave UV than the lower one. In some preparations only two fluorescing bands were discernable. Only the DNA extracted from the two uppermost bands or from the upper band, respectively, yielded clear restriction patterns. No differences have been observed among the restriction patterns of either of these bands. Irrespective of the restriction enzyme used, the DNA of the lower band, though totally digested, generates no defined fragments. This is not due to previous degradation as could be demonstrated by gel electrophoresis of enzymatically untreated DNA. The density of this DNA corresponds to that of the main peak in analytical CsCl density centrifugations of whole cell DNA preparations (Fig. 2a) and can thus be identified as nuclear DNA.

With the exception of Sal I which cleaves the DNA into 6 fragments, all other enzymes of the six base recognition type (Bam HI, Pvu II, Bgl II, Pst I, Eco RI) yield a moderate number of fragments ranging from 12–17. Only two recognition sites are present for Bgl I. The fragment patterns of both Bam HI and Eco RI include one fragment appearing in twofold stoichiometry. Two fragments of double intensity are visible in fractionation patterns generated after digestion with Bgl II and Pvu II; three double fragments may be observed after digestion with Pst I, whereas Sal I and Bgl I give rise only to fragments appearing singularly.

Double digestion with Pvu II/Sal I produces one subfragment of twofold intensity, and two double subfragments may be encountered after Bam HI/Sal I digests. Six double fragments are included in the restriction patterns of both Bam HI/Pst I and Bam HI/Bgl II double digestions (Table 1, Fig. 5). The sizes of the fragments generated by each of the restriction enzymes used add up to about 123 kbp.

The combination of Bam HI/Sal I seemed to be most suitable for constructing a physical map of the *Dictyota* plastome since both enzymes produced fragments not smaller than 1.4 kbp (Bam HI) and 6.6 kbp (Sal I), respectively, and the subfragments of the double digestion are additive and clearly distinguishable. Since, however, a sequential ordering of all primary fragments is usually not possible using only one pair of restriction enzymes, we used Bgl II as the third enzyme to eliminate ambiguities and to further characterize the 56 kbp Sal I fragment S1.

4. Sequential determination of primary and secondary fragments (Fig. 6)

Serial ordering of individual fragments of primary digestions resulting from a pair of enzymes was achieved by determining overlapping regions which may be detected as identical subfragments in reciprocal redigestions. Table 2 summarizes the fragments from primary digestions of Sal I and Bam HI together with the subfragments from reciprocal redigestions. The six Sal I fragments (S1–S6) are redigested by Bam HI into 18 subfragments of which 7 are part of S1 and 4 of S2, whereas S4 has no Bam HI cleavage site and is thus identical to SB3. S3 gives rise to SB6 and SB7, the former of which is also part of B6. The second subfragment (SB7) of 7.4 kbp is part of the redigested bimolar fragment B3/4. Upon redigestion with Sal I the resulting four subfragments of this bimolar B3/4 fragment are clearly distinguishable from each other and add up to 29 kbp which is twice the size of either B3 or B4. Hence it follows that B3 and B4 are different from each other. B3 has been defined as

Table 1. Size (kbp) of restriction fragments from *Dictyota* ptDNA.

Fragment	Sal I	Sal I Bam HI	Bam HI	Bam HI Bgl II	Bgl II	Pvu II	Pvu II Sal I	Bam HI Pst I	Bgl I	Pst I*	Eco RI*
1	56.0	19.4	22.9	17.7	27.1	22.8	22.8	14.6	> 75	19.5	27.7
2	24.0	13.9	19.4	14.2	20.6	22.8	10.2	9.1	> 40	17.7	21.2
3	15.7	12.6	14.5	10.2	15.7	13.2	9.2	9.1		13.9	18.6
4	12.6	10.3	14.3	10.0	10.2	13.2	8.8	8.5		11.9	14.6
5	9.1	8.6	13.9	8.8	8.8	8.8	8.8	8.5		10.5	9.2
6	6.6	8.3	9.7	7.5	6.0	5.8	8.2	7.7		8.9	7.8
7		7.4	8.6	5.8	5.5	4.6	6.5	7.2		8.5	7.8
8		7.1	7.1	5.2	5.5	4.3	5.8	5.8		5.1	6.0
9		7.1	5.5	5.1	5.2	3.5	5.7	5.8		5.1	4.6
10		5.7	3.7	4.6	4.5	3.4	4.6	5.4		4.9	4.0
11		5.5	2.2	4.3	3.0	3.2	4.5	4.8		4.3	3.4
12		4.9	1.4	4.1	2.3	2.8	4.3	4.8		4.3	2.6
13		4.0		2.3	1.6	2.7	3.5	3.5		3.9	
14		3.4		2.0	1.6	2.6	3.4	3.5		3.9	
15		2.2		2.0	1.55	2.3	2.8	3.0		2.3	
16		1.7		1.7	1.55	2.0	2.7	3.0		2.0	
17		1.4		1.6	1.25	1.0	2.5	2.5			
18		x		1.6			2.5	2.3			
19				1.55			2.3	2.1			
20				1.55			2.0	2.1			
21				1.55			1.0	1.6			
22				1.55			x	0.6			
23				1.45			x	x			
24				1.4				x			
25				1.4				x			
26				1.3				x			
27				1.3				x			
28				1.25				x			
29				x							
Sum of kbp of identified fragments	124.0	123.5	123.2	122.8	121.9	119.0	122.1	115.5	>115	126.7	127.5
Number of identified/ calculated fragments	6	17/18	12	28/29	17	17	21/23	22/28	2	16	12

* Not illustrated in Fig. 5.

the sum of subfragments SB7 and SB8, whereas B4 consists of the two remaining subfragments SB4 and SB13. Thus, B3 adjoins B6.

S6 is cut into SB12 and SB16, the latter of which is also part of B6. Thus, S3 and S6 are connected to each other. Since SB12 is also part of B1, this fragment overlaps S6. B1 also contains SB3 and one of the bimolar band SB10/11. Since SB3 is identical to S4, it must be positioned between SB12 and one of the subfragments SB10/11. On the other hand, one of the SB10/11 double fragment (in the following defined as SB11) is identical with B9 and can therefore not be considered as a flanking fragment. Hence it follows that SB10 is a terminal subfragment of B1. Moreover, SB10 and SB11 are also part of S5 and S2. The question as to whether S2 or S5 join S4 could be answered by scrutinizing the subfragments of both Sal I fragments. Since S5 consists of only two subfragments, SB10 is the overlap between S5 and B1. The sequential order of Sal I fragments considered hitherto is therefore S3–S6–S4–S5.

S5 also contains SB14 which is part of B10 although its second subfragment which has a size of

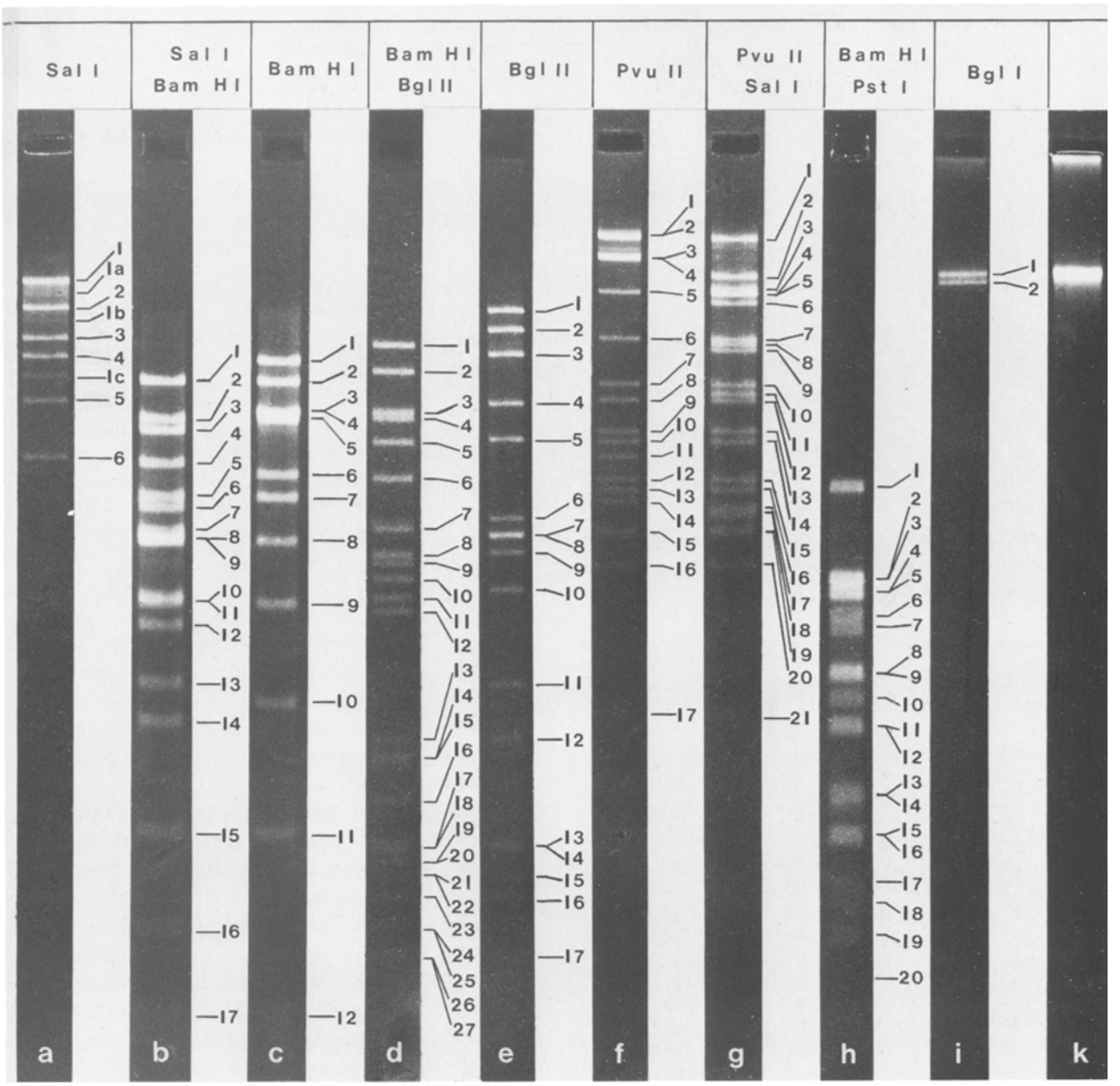

Fig. 5. Separation of restriction endonuclease fragments of *Dictyota* ptDNA by agarose (SeaKem) gel electrophoresis. Samples were digested with (a) Sal I (0.7% agarose), (b) Sal I/ Bam HI (0.5% agarose), (c) Bam HI (same gel as in b), (d) Bam HI/ Bgl II (0.6% agarose), (e) Bgl II (same gel as in d), (f) Pvu II (0.9% agarose), (g) Pvu II/ Sal I (same gel as in f), (h) Bam HI/ Pst I (0.4% agarose), (i) Bgl I (0.8% agarose). (k) demonstrates undigested high molecular weight ptDNA (same gel as in i).

less than 1 kbp was not detectable and may have migrated out of the gel. Since in Bam/Sal double digests 17 fragments can easily be identified and attributed to secondary fragments of redigests, this small subfragment of B10 is the missing 18th fragment of Bam/Sal double digests. The partial sequence of Bam HI fragments is therefore B3–B6–B1–B10. Since SB8 is part of both B3 and S1, the position of S1 is adjacent to S3, and thus S2 has to fill the gap between S1 and S5. The orientation of S2 can be deduced by its subfragment SB13 which is also part of B4. As long as B4 shares its other

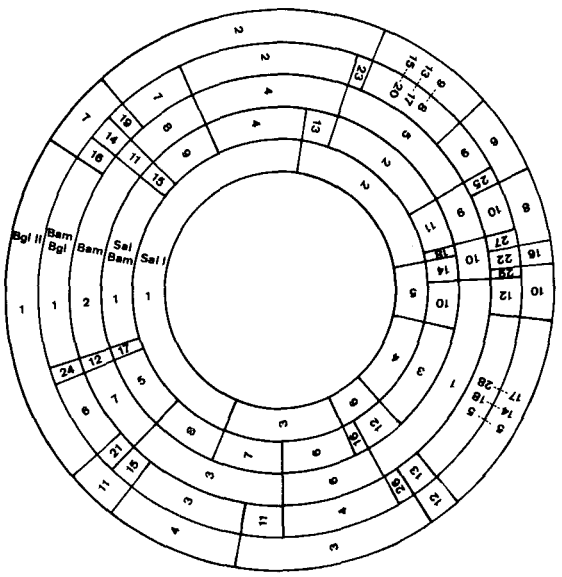

Fig. 6. Physical map of the *Dictyota* plastome showing the restriction sites for the endonucleases Sal I, Bam HI, and Bgl II, respectively.

Table 2. Relations between primary and secondary fragments of Sal I/Bam HI digests.

Primary Sal I fragments	Subfragments after rediges-tion with Bam HI	Primary Bam HI fragments	Subfragments after rediges-tion with Sal I
S1	SB1, 4, 5, 8, 9, 15, 17	B1	SB3, 10, 12
		B2*	SB1
S2	SB2, 11, 13, 18	B3, 4	SB4, 7, 8, 13
S3	SB6, 7	B5*	SB2
S4*	SB3	B6	SB6, 16
		B7*	SB5
S5	SB10, 14	B8*	SB9
S6	SB12, 16	B9*	SB11
		B10	SB14, 18
		B11*	SB15
		B12*	SB17

* Primary fragments lacking recognition sites for the reciprocal enzymes.

subfragment SB4 with S1, B4 is the overlapping fragment between S1 and S2. Thus, the final sequence of Sal I fragments is S2–S1–S3–S6–S4–S5.

The annexation of S2 to S5 means that B10 overlaps S2, and thus SB18 has to be considered as the second marginal fragment of S2. SB2 and SB11 are intervening fragments of S2 and are equivalent to B9 and B5, respectively. The remaining Bam HI fragments B2, B7, B8, B11, B12 are mid fragments of S1. They correspond to subfragments SB1, SB5, SB9, SB15, SB17.

Table 3. Relations between primary and secondary fragments of Bam HI/Bgl II digests.

Primary Bam HI fragments	Subfragments after rediges-tion with Bgl II	Primary Bgl II fragments	Subfragments after rediges-tion with Bam HI
B1	BBg5, 12, 13, 18, 26, 28	Bg1	BBg1, 6, 24
B2	BBg1, 16	Bg2	BBg2, 7, 23
B3, 4	BBg2, 3, 11, 15	Bg3	BBg4, 11, 26
B5	BBg8, 9, 17, 20, 23	Bg4*	BBg3
B6*	BBg4	Bg5*	BBg5
B7	BBg6, 21	Bg6	BBg9, 25
B8	BBg7, 19,	Bg7, 8	BBg10, 14, 16, 19, 27
B9	BBg10, 25	Bg9*	BBg8
B10	BBg22, 27, 29	Bg10	BBg12, 29
B11*	BBg14	Bg11	BBg15, 21
B12*	BBg24	Bg12*	BBg13
		Bg13*, 14*	BBg17, 18
		Bg15*	BBg20
		Bg16*	BBg22
		Bg17*	BBg28

* Primary fragments lacking recognition sites for the reciprocal enzymes.

To order these mid fragments, we used Bgl II as the third enzyme (Table 3). Some difficulties were to be expected because of the increased number of primary Bgl II fragments compared to the Bam HI pattern and the occurrence of a bimolar band (Bg7/8). However, redigestion with Bam HI clearly showed that this bimolar band is not composed of homologous fragments, since it generates five subfragments of which two (BBg10, BBg27) and three (BBg14, BBg16, BBg19) each add up to the size of the primary fragments. Starting with B8, which upon redigestion with Bgl II gives rise to the subfragments BBg7 and BBg19, the former of which is also the overlap to Bg2, we may deduce its direct connection to B4. This position is confirmed because B4 is identical to BBg2, the mid fragment of Bg2. Redigestion with Bgl II also confirms the heterogeneity of the B3/4 double band which has already been deduced by considering the sizes of their Sal I subfragments.

The second subfragment of B8 is part of the bimolar band BBg19/20 in double digestions. Since

this bimolar band is composed of one terminal and one intervening fragment we defined the terminal fragment as BBg19. This is also the overlapping subfragment to Bg7 which, in addition, is subdivided into BBg16 and one fragment of the bimolar band BBg14/15. Since BBg16 is the overlapping fragment to B2, thus clarifying the sequential position of B2, at least one of the two subfragments BBg14/15 is intervening. The second subfragment of BBg14/15 can be elucidated as one of the two redigestion products of Bg11. Hence it follows that the second fragment of BBg14/15 must be placed at a terminal position. We therefore defined the mid fragment as BBg14 which is then identical to B11, the position of which is between B8 and B2.

The second subfragment of B2, BBg1, overlaps Bg1 which furthermore is subdivided into the fragments BBg6 and one of the bimolar band BBg24/25. The distinction between the fragments BBg24 and BBg25 is possible on the basis of one being at the terminal position and the other being a mid fragment. Thus we defined the mid fragment of Bg1 as BBg24, corresponding to B12. The gap between B12 and B3 may unambiguously be closed in as much as BBg6 is part of B7. The connection of B7 to B3 is confirmed by the two subfragments of Bg11, the one (BBg21) overlapping B7, the other (BBg15) overlapping B3. The overlap between B7 and Bg11 is unequivocal because of BBg21 being the only terminal fragment of the bimolar band BBg21/22.

In order to clarify the sequential position of B5 and B9 we have to consider the second terminal subfragment of Bg2, BBg23. This small subfragment may also be identified as one of the five subfragments of B5. The second terminal subfragment is BBg9, overlapping Bg6, whereas the order of the mid fragments BBg8, BBg17, and BBg20, corresponding to Bg9, Bg13, and Bg15, remains unclear.

Since upon redigestion both Bg6 and B9 give rise to only two subfragments, BBg25 can easily be identified as the overlap of both B9 and Bg6. Therefore the complete sequence of the Bam HI fragments can be laid down as B9–B5–B4–B8–B11–B2–B12–B7–B3–B6–B1–B10.

Although the entire complement of Bam HI fragments has been brought into a sequential order, it remains to be demonstrated that B9 and B10 are neighbouring fragments by clearly identifying their overlap. Starting with BBg10 as the second subfragment of B9, it can be shown that it also represents one of the two subfragments of Bg8. The second subfragment of Bg8, BBg27, comigrates with BBg26. Since both fragments are terminal fragments of either B1 or B10, B1 could theoretically be next to B9. However, since the position of B1 has unequivocally been determined by its three Sal/Bam subfragments, B10 is the only remaining fragment adjacent to B9. This connection also facilitates the discussion of the subfragments of both B10 and Bg10. The second clearly recognizable subfragment of B10 is part of the bimolar band 21/22 in Bam/Bgl double digestions. One of the two subfragments corresponds to Bg16 and is therefore not overlapping. This means that a third subfragment of B10 of a size less than 0.9 kbp is the unidentified but necessarily present 29th subfragment of Bam/Bgl double digests. This supposition is furthermore substantiated by considering the sizes of the subfragments of B10. Finally, this missing BBg29 subfragment provides evidence of the connection of Bg10 to Bg16 since upon redigestion of Bg10 a slightly smaller subfragment, BBg12 is generated, whereas the second subfragment of 0.5 kbp is missing. It is thus evident that this missing subfragment BBg29 is the overlap to B10.

The sequence of the subfragments generated after redigestion of B1 remains uncertain since B1 includes five Bgl II recognition sites which give rise to the four intervening BBg subfragments 5, 13, 18, 28, equivalent to Bgl II fragments 5, 12, 14, 17, respectively. However, the serial position of both BBg13 and the corresponding B12 has been clarified by hybridization experiments (Kuhsel & Kowallik, in preparation). BBg26 as the second subfragment of B1 has already been discussed.

To decide the orientation of the remaining fragments Bg3 and Bg4, of which only the former has Bam HI recognition sites, we have to consider the subfragments BBg11 and BBg26 which are overlaps of B3 and B1, respectively. The intervening subfragment BBg4 corresponds to B6. Finally, Bg4 can be shown to be equivalent to the mid fragment BBg3 and thus clarifies its neighbouring position to Bg11.

Discussion

Our investigations have clearly demonstrated that the plastome of *Dictyota dichotoma* is repre-

sented by circular molecules belonging to a single size class. In this respect it does not differ from plastomes of other chlorophyll c-containing algae investigated so far (*Olisthodiscus*: 1; *Vaucheria, Botrydium, Tribonema, Odontella*: 26). Preliminary observations on the plastome from both male and female gametophytes of the brown alga *Laminaria hyperborea* show similar results with respect to size homogeneity and circularity although the plastome of *Laminaria* exceeds that of *Dictyota* by about 10 kbp (Kuhsel, unpublished).

However, in contrast to our investigations, Dalmond *et al.* (10) consider heterogeneous plastome populations as a peculiar feature of brown algae. This statement is based on size determinations of plastomes from *Pylaiella* and *Sphacelaria* by electron microscopy and is further substantiated by few results from restriction enzyme analysis.

The question arises as to whether the plastomes of both *Pylaiella* and *Sphacelaria* do indeed represent a type of organization which hitherto has been unknown from lower and higher plants and which in some way is similar to the situation in plant mitochondria (25). However, the characterization of an organellar DNA population may be afflicted with certain inconsistencies: First, despite cell fractionation, the origin of individual DNA molecules is by no means unequivocal. Linear molecules exceeding the size of the largest circular DNA molecules are to be considered as candidates for nuclear DNA (21, 41), even after DNase treatment of the organellar fraction (1, 5). Correspondingly, circular molecules deviating considerably from the mean value of a plastome population do not necessarily originate from plastids, since such molecules may be encountered even in spread preparations of plastomes for which physical restriction maps exist (21, 41). Second, the characterization of a plastome by restriction endonuclease analysis presupposes the complete and specific fractionation of the DNA. This does not necessarily depend on the ratio enzyme/DNA, but may also be affiliated upon the star activity of certain restriction enzymes including Bam HI. According to our data, restriction patterns of the *Dictyota* plastome may yield different values if the DNA is still contaminated with substances that partially inhibit restriction enzymes. On the other hand, even with highly purified plastid DNA of *Dictyota*, certain restriction enzymes including Sal I do not uniformly recognize their cleavage sites. Irrespective of the ratio enzyme/DNA three subfragments (Sa, Sb, Sc) of weak fluorescent intensity are regularly encountered. If one neglects the understoichiometry of these three fragments, the total molecular size as calculated by summing up all visible Sal I fragments would considerably exceed the size of the plastome. Yet, complete digestion patterns produced by other enzymes and combinations of enzymes yielding various numbers of fragments unequivocally confirm the uniform plastome size of 123 kbp. We thus do not deal with a mixture of different molecules (cf. 10), although the nature of the Sal I fragments Sa, Sb, and Sc still remains to be discussed: These three fragments may not be considered as incompletely cleaved fragments but are themselves subfragments of S1. This interpretation is substantiated by the fact that the only possible cleavage products of Sc, S5 and S6, are not neighbouring fragments, that the molecular size of both Sc (10.8 kbp) and S5 + S6 (15.7 kbp) are different and that upon redigestion with Bam HI the subfragments of both Sc and S5/S6 are not identical. A possible explanation for the incomplete cleavage of S1 could be a modification inside the nucleotide sequence of the Sal I recognition site. The true reason, however, still remains unknown since the use of restriction endonucleases does not permit a direct measure of DNA sequence modification (34).

While our results differ fundamentally from those of Dalmond *et al.* (10) with respect to both the plastome size and the interpretation of restriction fragment patterns, there are remarkable similarities among the densities of the plastomes from *Dictyota*, *Pylaiella*, and *Sphacelaria*. A relatively uniform density of 1.691–1.694 g/cm^3 corresponding to about 31–35% d(G + C) appears to represent a common feature among the members of the chlorophyll c line (1, 9, 26, unpublished observations). Although there are comparable values for plastomes of some chlorophyll b-containing algae as well (*Chlamydomonas*: 4; *Codium*: 20) the densities of plastid DNAs from both *Acetabularia* (17, 18) and *Euglena* (28) indicate a greater variation within the green algal line. This variability is further accompanied by the observation that the plastomes of chlorophycean algae differ in size from 86 kbp for *Codium* (20) to about 200 kbp for *Chlamydomonas* (4), and the significance of the variability may even be greater if *Acetabularia* is taken into considera-

tion (27).

The fragment patterns of the *Dictyota* plastome did not exhibit true identical double fragments of a size large enough to be compared with the inverted repeats of the plastomes of most higher plants. Repeats, however, remain unidentified as long as they do not include at least two recognition sites for a given restriction enzyme although the probability of detecting fragment duplication increases with the number of fragments produced. The fragments of twofold stoichiometry generated after digestion with Bam HI (B3/4) or Bgl II (Bg7/8), respectively, have been shown to be different according to their subfragments after reciprocal redigestion. Likewise, the two large fragments of Pvu II digests (Pv1/2 and Pv3/4), which may be regarded as possible candidates for sequence repetitions, prove to be different after redigestion with Sal I.

As known from plastomes of both land plants (e.g. 29, 30, 41) and algae (6, 39, 43, 44), repeats harbour the genes for the ribosomal RNAs of approximately 5.5 kbp. Although the rRNA genes still remain to be identified on the plastome of *Dictyota*, a sequence duplication inside one fragment, if present, could only be suggested for SB1–3 and Bg1/2. On the other hand, there are additional fragments, of course, which may carry the rDNA in singular.

The few results from chlorophyll c-containing algae are certainly not suitable for drawing further conclusions, and the situation in this important evolutionary branch of algae will appear more sophisticated as soon as additional information from other genera are available. However, it seems that – according to certain physical data like buoyant density and size – the plastomes of members of the chlorophyll c-line including *Xanthophyceae*, *Chrysophyceae*, *Bacillariophyceae*, and *Phaeophyceae* (1, 2, 6) are more uniform than those of the chlorophyll b-branch of the algal kingdom (16, 17, 20, 26, 39).

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