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ORGANIZATION OF THE CHLOROPLAST GENOME OF THE FRESHWATER CENTRIC DIATOM *CYCLOTELLA MENEGHINIANA*[1]

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ABSTRACT

We constructed a complete physical map and a partial gene map of the chloroplast genome of Cyclotella meneghiniana *Kützing clone 1020-1a (Bacillariophyceae). The 128-kb circular molecule contains a 17-kb inverted repeat, which divides the genome into single copy regions of 65 kb and 29 kb. This is the largest genome and inverted repeat found in any diatom examined to date. In addition to the 16S and 23S ribosomal RNA genes, the inverted repeat contains both the* ndh*D gene (as yet unexamined in other diatoms) and the* psb*A gene (located similarly in one of two other examined diatoms). The* Cyclotella *chloroplast genome exists as two equimolar populations of inversion isomers that differ in the relative orientation of their single copy sequences. This inversion heterogeneity presumably results from intramolecular recombination within the inverted repeat. For the first time, we map the* ndh*D,* psa*C,* rpo*B,* rpo*C1, and* rpo*C2 genes to the chloroplast genome of a chlorophyll* c*-containing alga. While the* Cyclotella *chloroplast genome retains some prokaryotic and land plant gene clusters and operons, it contains a highly rearranged gene order in the large and small single copy regions compared to all other examined diatom, algal, and land plant chloroplast genomes.*

Key index words: Bacillariophyceae; chloroplast DNA; Cyclotella meneghiniana; *gene mapping; inverted repeat; restriction site mapping*

The origins and evolution of land plants and algae and their chloroplasts are of considerable historical and continuing interest. The discovery of chloroplast DNA (cpDNA) (reviewed in Kirk 1986) and the application of the tools of molecular biology have increased the size of the relevant data base for comparative evolutionary studies. While accumulated molecular data point toward the chloroplast being ". . . a reduced remnant of a cyanobacterium-like endosymbiont . . ." (Gray 1989), debate continues over whether the diversity of extant chloroplast genomes is the result of one endosymbiotic event or a number of primary and even secondary endosymbiotic events (Gray 1989, Whatley 1989, Douglas et al. 1991, Penny and O'Kelly 1991). Clearly, we need more data and we must study more taxa to resolve the nature of cpDNA evolution.

Most cpDNA studies have featured the chlorophyll *a*/*b*-containing land plants and chlorophyte algae (Palmer 1991). Among the Chromophyta (sensu Christensen 1964, 1989), chloroplast genomes are mapped in only eight isolated representatives of five algal groups. These include two chrysophytes, *Olisthodiscus luteus* (Reith and Cattolico 1986) and *Ochromonas danica* (Cattolico and Loiseaux-de Goër 1989), two distantly related brown algae, *Dictyota dichotoma* (Kuhsel and Kowallik 1987) and *Pylaiella littoralis* (Loiseaux-de Goër et al. 1988), several strains of a xanthophycean alga, *Vaucheria sessilis* (Linne von Berg and Kowallik 1988), the cryptomonad *Cryptomonas* Φ (Douglas 1988), and two distantly related centric diatoms, *Coscinodiscus granii* and *Odontella sinensis* (Kowallik 1989). From these studies it is already clear that chromophyte algal cpDNA has retained several prokaryotic operons but is more variable in gene organization and gene order than the cpDNA of land plants (Kowallik 1989, Palmer 1991). Since it is the conservative character of land plant cpDNA that has proven useful in phylogenetic studies at several taxonomic levels (Palmer et al. 1988), we do not know whether or not the structurally more variable cpDNA of chromophyte algae would also be useful in phylogenetic studies. Linne von Berg and Kowallik (1988) suggested a reexamination of algal species concepts relative to those of land plants based on a study of intraspecific cpDNA variation in *Vaucheria sessilis*. More recently, Stabile et al. (1990) examined intraspecific variation in three strains of the diatom *Skeletonema costatum* using heterologous

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cpDNA and nuclear DNA probes. Fain et al. (1988) studied intergeneric cpDNA variation in two orders of brown algae (Phaeophyta). However, there are no detailed studies of interspecific cpDNA variation in closely related chlorophyll *a/c* algae. Therefore, we do not know how useful cpDNA restriction site studies might be in chromophyte algal phylogenetics.

We have constructed a cpDNA clone bank and both a detailed restriction site map and a partial gene map for "*Cyclotella meneghiniana*" clone 1020-1a (Schlösser 1982), an ecologically important (Stoermer and Yang 1970, Stoermer and Ladewski 1976) freshwater centric diatom in the family Thalassiosiraceae. Because of environmentally related polymorphisms (Schultz 1971) and related complexities of morphological character analyses, *Cyclotella* is in need of taxonomic revision (Theriot and Kociolek 1986, Theriot et al. 1987). We have chosen the chloroplast genome of *C. meneghiniana* clone 1020-1a as a reference genome in comparative cpDNA studies of closely related species in the *meneghiniana* subgroup of *Cyclotella* (C. Bourne, E. Stoermer, and J. Palmer, unpubl.). This clone is readily available in axenic culture, grows dependably to high cell densities, and yields sufficient quantities of high molecular weight DNA to facilitate initial studies. In this paper, we present methods for extraction and purification of *Cyclotella* cpDNA from total DNA. We report on the size, physical structure, gene content, and gene organization of the *Cyclotella* chloroplast genome and compare it to other chloroplast genomes in land plants and algae.

MATERIALS AND METHODS

The axenic clonal culture *Cyclotella meneghiniana* Kützing 1020-1a, isolated by Pringsheim in 1959, was obtained from the Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Göttingen (Schlösser 1982). The culture was maintained in sterile WC medium, pH 6.8–7.0 (Guillard 1975) or modified WC medium (WC with the following substitutions: 18.38 $mg \cdot l^{-1}$ $CaCl_2 \cdot 2H_2O$, 4.36 $mg \cdot L^{-1}$ K_2HPO_4, 21.2 $mg \cdot L^{-1}$ $NaNO_3$, 56.84 $mg \cdot L^{-1}$ $NaSiO_3 \cdot 9H_2O$). Cultures were grown at 20° C, with a 16:8 h LD photoperiod, and illuminated at 16–25 $\mu E \cdot m^{-2} \cdot s^{-1}$ as measured with a Li-Cor Model LI-185 Quantum/Radiometer/Photometer. Inocula (100 $mL \cdot L^{-1}$) were transferred and maintained aseptically in 1.5 L WC or modified WC in cotton-plugged, sterile, 2.8-L Fernbach lo-form flasks on rotary platform shakers illuminated at 35–60 $\mu E \cdot m^{-2} \cdot s^{-1}$. Cultures in 8 L WC were grown in cotton-plugged, sterile, 9-L Nalgene polycarbonate carboys on magnetic stirrers and bubbled with air through tubing fitted with in-line Acrodisc (Gelman Sciences) 0.2-μm filters. Carboys were illuminated at 80–100 $\mu E \cdot m^{-2} \cdot s^{-1}$, and all cultures were grown at 19–20° C and at a 16:8 h LD photoperiod. Cell densities were monitored with a Neubauer Hy-Lite Batch Counting Chamber (Hausser Scientific, Blue Bell, PA 19422) on a light microscope, and cells were harvested in late log phase at a concentration of 2.5×10^5 cells $\cdot mL^{-1}$.

Cells from 5 to 20 L were collected by centrifugation at 4000–9000 rpm at 4° C for 10–15 min in a Sorvall GS-3 or GSA rotor. Pellets were resuspended on ice in 50 mM Tris (pH 8.0), 25 mM EDTA, and 50 mM NaCl to a maximum volume of 20 mL and evenly suspended with a Vortex mixer.

Cells were broken (breakage averaged 75%) in a cold French pressure cell press by one slow pass at 2000–2500 psi. To every 20 mL of cells, 1 mL Pronase (20 $mg \cdot mL^{-1}$) was added and the solution was mixed by gentle inversion for 5 min at room temperature. Next, 4 mL of lysis buffer (5% sodium sarcosinate, 50 mM Tris-HCl (pH 8.0), and 25 mM EDTA) were added and mixed by gentle rocking for 15–20 min. To each 10–15 mL of the resulting lysate, 24.75 g of CsCl and 0.5 mL of ethidium bromide (10 $mg \cdot mL^{-1}$ in 50 mM Tris (pH 8.0), 10 mM EDTA) were added and brought to a final volume of 33 mL with distilled water. The gradients were centrifuged in a TV-850 rotor (Sorvall) for 12–14 h at 42,000–44,000 rpm at 20° C. The top dark-green layer was removed, and the broad DNA band containing all cellular fractions of DNA was centrifuged one or two more times to remove cellular debris in 5-mL gradients at 58,000 rpm for 5 h or at 35,000 rpm for 14 h in a TV-865 rotor (Sorvall).

Ethidium bromide was extracted with NaCl- and H_2O-saturated isopropanol, and the DNA-containing CsCl phase was adjusted to a density of 1.63 $g \cdot mL^{-1}$ (n = 1.3960). The DNA was distributed in 2–3-mL aliquots to 5-mL centrifuge tubes, brought to a volume of 4.5 mL with stock 1.63 $g \cdot mL^{-1}$ CsCl in buffer (50 mM Tris (pH 8.0), 10 mM EDTA) and 40 μL of bisbenzimide stock (10 $mg \cdot mL^{-1}$ H_2O) (Hoechst 33258 dye, Sigma). The gradients were centrifuged at 50,000–58,000 rpm for 6–20 h at 20° C in a TV-865 rotor (Sorvall). The uppermost band of presumptive cpDNA was collected, pooled, and spun one or two more times as earlier without further addition of dye. The final uppermost band was collected in a small volume and bisbenzimide extracted as earlier for the ethidium bromide. The DNA-CsCl phase was dialyzed overnight against three changes of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

Purified *Cyclotella* clone 1020-1a cpDNA was digested with *Pst*I, *Sal*I, and *Bam*HI restriction enzymes in single and double combinations and ligated with 10 ng of the correspondingly digested pUC9, pUC12, or pBS SK+ plasmid vectors. *Escherichia coli* strains JM83, JM103, or XL-1 Blue were transformed with the ligation mixture, and white recombinant colonies were selected on ampicillin, X-gal plates. Plasmid DNA was prepared according to the alkaline miniprep methods of Birnboim and Doly (1979). The sizes and identities of the inserted DNA fragments were determined by restriction enzyme digestion and gel electrophoresis analysis. Large cpDNA inserts were subcloned in the vector pUC12 and transformed into *E. coli* strain JM103 or XL-1 Blue and analyzed as earlier. Seven fragments were separated by gel electrophoresis of cpDNA in 1% SeaPlaque (FMC) low melting temperature agarose gel. Excised fragments were ligated into 10 ng pBS SK+ and radiolabeled with ^{32}P by nick-translation. Three other uncloned fragments were gel-isolated and directly radiolabeled with ^{32}P by nick-translation in low melting agarose.

Complete restriction site maps of *Cyclotella* clone 1020-1a cpDNA were constructed for 10 enzymes using the overlap hybridization approach of Palmer (1986). Mapping digests consisted of single digests with each of 10 enzymes, double digests with *Pst*I and each of the nine other enzymes, and double digests with *Pvu*II and each of *Eco*RI, *Eco*RV, and *Hin*dIII. Digests were loaded into a 1.0% agarose gel, separated by electrophoresis until the bromphenol-blue dye had run 20 cm, and photographed. Replica blot transfers were prepared by bidirectional transfer of DNA to Zetabind membrane filters (AMF-Cuno) according to the alkaline blot method of Smith and Summers (1980). Filters were washed in 2× SSC (1× SSC = 0.15 M NaCl, 15 mM trisodium citrate), dried under vacuum at 80° C for 1 h, and washed with 0.1× SSC and 0.5% SDS at 65° C in a shaking water bath for 1 h. Individual filters were prehybridized (at 60–65° C) and hybridized in plastic boiling bags in either 20 mL 4× SSC, 5× Denhardt's solution, 10 mM EDTA, 0.5% SDS, and 250 $\mu g \cdot mL^{-1}$ carrier DNA (10 $mg \cdot mL^{-1}$) or 4× SSC, 0.5% nonfat dry milk, and 1% SDS. Plasmid clones or gel-isolated restriction fragments were radiolabeled with ^{32}P by nick-translation and hybridized overnight to the filters

in a shaking water bath at 65° C. Filters were washed four times in 2× SSC and 0.5% SDS (twice at 20° C and twice at 65° C) and autoradiographed. Filters were reprobed after old probe was removed either with 0.4 N NaOH at 42° C or with four washes of boiling 0.1× SSC.

Southern blots of *Cyclotella* clone 1020-1a cpDNA digested with *Cla*I, *Pvu*II, *Eco*RI, and *Hin*dIII were probed with a total of 60 heterologous cpDNA fragments from tobacco, pea, spinach, and *Chlamydomonas reinhardtii* obtained from the clone banks maintained in J. Palmer's laboratory and one fragment from *Cryptomonas* Φ provided by S. Douglas. Procedures were as described earlier, except the gels were blotted by single-sided capillary wick transfers and hybridizations were carried out at 55–60° C.

RESULTS AND DISCUSSION

Physical mapping. Centrifugation of total DNA from *Cyclotella* in bisbenzimide-CsCl gradients resulted in three DNA bands. The upper band contained cpDNA in high concentration as determined by the simplicity of restriction enzyme digestion patterns and the positive hybridization of heterologous 16S rDNA and 3′*psb*A cpDNA gene probes. We cloned a total of 31 overlapping fragments and subfragments ranging in size from 1.5 to 20 kilobase pairs (kb) and representing 86% of the genome (Table 1). We also gel-isolated three fragments representing 12% of the genome. In all, we radiolabeled 23 fragments covering 98% of the genome as hybridization probes to produce a restriction site map (Fig. 1). Table 2 lists the sizes of the restriction fragments shown in Figure 1.

Physical organization of chloroplast DNA in diatoms. At 128 kb, the circular chloroplast genome of *Cyclotella meneghiniana* clone 1020-1a is 10 kb larger (Fig. 2) than the only other two mapped diatom genomes (Kowallik 1989, Fig. 2). All three diatoms are in the order Centrales and are similar to most land plants and green algae in that their circular chloroplast genomes contain a large inverted duplication. This duplication, termed the inverted repeat (IR), contains the rRNA-encoding genes. The 17-kb IR of *Cyclotella* divides the genome into a 29-kb small single copy region (SSC) and a 65-kb large singly copy region (LSC) (Fig. 1). The *Cyclotella* IR is nearly double the size of the 8.7-kb IR of *Odontella sinensis* (Kowallik 1989) and larger than the "slightly enlarged" IR of *Coscinodiscus granii* (Kowallik 1989, Fig. 2). Thus, some of the overall genome size differences between *Cyclotella* and the other two diatoms (whose genomes are the same size) can be accounted for by differences in IR size rather than by differences in genome complexity.

Molecular heterogeneity. A *Sac*I digest of *Cyclotella* cpDNA yielded fragments of varying intensity raising the possibility that *Sac*I did not cut within the IR and that there was more than one molecular arrangement of the *Cyclotella* chloroplast genome (Palmer 1983, 1986). Southern blot analysis (data not shown) confirmed this inference. A radiolabeled 4.7-kb *Sal*I/*Bam*HI fragment (clone No. 20B) that overlapped the SSC end of the IR (Fig. 1) hybridized to two half-molar bands of 33 kb and 29 kb and a unimolar band of 31 kb. A radiolabeled 8.4-kb *Eco*RV fragment (clone No. 16) that contains a *Sac*I site and is wholly within the SSC (Fig. 1) hybridized to fragments of 31 kb, 29 kb, and 9 kb in approximately equal intensity. The most plausible explanation of these data is that the probe that contains the end of the IR hybridized to four half-molar fragments (33 kb, 31 kb, 31 kb, 29 kb), each of which contains the entire IR, and that the probe from within the SSC hybridized to the single copy portion of these half-molar fragments (31 kb, 29 kb). Maps of two inversion isomers of the *Cyclotella* chloroplast genome are consistent with the data and differ in the relative polarity of their SSC and LSC regions due to intramolecular exchange in the IR (Fig. 3). This phenomenon is also characteristic of chloroplast genome populations of all land plants (Palmer 1983,

TABLE 1. Cyclotella meneghiniana *clone 1020-1a cpDNA clone bank. The coordinates are according to the kilobase scale at the bottom of Figure 1. Both sets of coordinates are given for the three cloned fragments (12, 13, and 14A) that are internal to the IR.*

No.	Size (kb)	Enzymes	Coordinates	Vector
1	5.0	*Pst*I	0.0–5.0	pBS SK+
2	5.0, 1.0	*Pst*I	0.0–6.0	pBS SK+
3	11.2	*Pst*I	6.0–17.2	pUC9
3A	4.2	*Pst*I-*Pvu*II	6.0–10.2	pBS SK+
3B	7.0	*Pvu*II-*Pst*I	10.2–17.2	pBS SK+
4	1.6	*Eco*RV-*Pst*I	17.2–18.8	[a]
5	8.4	*Eco*RV	18.8–27.2	pBS SK+
6	7.5	*Eco*RV	27.2–34.7	[a,b]
7	6.0	*Pst*I	33.9–39.9	pUC9
8	1.6	*Pst*I	39.9–41.5	pUC12
9	3.0	*Pst*I	41.5–44.5	pBS SK+
9.1	0.6	*Pst*I	44.5–45.1	[a]
10	2.2	*Pst*I	45.1–47.3	pBS SK+
11	17.0	*Pst*I/*Sal*I	47.3–64.3	pUC12
11A	2.0, 2.1	*Pst*I/*Eco*RI	47.3–51.4	pUC12
11B	2.0	*Pst*I/*Eco*RI	47.3–49.3	pUC12
11C	2.1	*Eco*RI	49.3–51.4	pBS SK+
11D	6.5	*Eco*RI	51.4–57.9	pBS SK+
11.1	0.2	*Eco*RI/*Bgl*II	57.9–58.1	[a]
12	5.7	*Bgl*II/*Sal*I	58.1–63.8	pUC12
			108.8–114.5	
13	6.0	*Sal*I/*Pst*I	63.8–69.8	pUC12
			102.8–108.8	
14	3.4	*Pst*I	69.8–73.2	pBS SK+
14A	1.9	*Pst*I/*Bam*HI	69.8–71.7	pUC9
			100.9–102.8	
14B	1.5	*Bam*HI/*Pst*I	71.7–73.2	pUC9
15	0.5	*Eco*RV/*Pst*I	73.2–73.7	[a]
16	8.4	*Eco*RV	73.7–82.1	pBS SK+
17	1.9	*Eco*RV	82.1–84.0	[a,b]
18	6.2	*Eco*RV	84.0–90.2	[a,b]
19	5.9	*Sal*I/*Bam*HI	90.2–96.1	pUC12
20A	2.3	*Pst*I/*Bam*HI	93.8–96.1	pUC12
20B	4.7	*Sal*I/*Bam*HI	96.1–100.8	pUC12
21	20.0	*Bam*HI	100.8–120.8	pUC9
22	7.9	*Bam*HI/*Sal*I	100.8–108.7	pUC12
24	12.0	*Sal*I/*Bam*HI	108.7–120.7	pUC12
24B	6.3	*Bgl*II/*Bam*HI	114.4–120.7	pBS SK+
25	18.0	*Sal*I/*Pst*I	108.7–126.7	pUC12
25A	6.0	*Bam*HI/*Pst*I	120.7–126.7	pUC12

[a] Not cloned.
[b] Gel isolated and used as probe.

TABLE 2. *Sizes of* Cyclotella meneghiniana *clone 1020-1a cpDNA fragments.*

Fragment number	Fragment size (kb)									
	*Pst*I	*Eco*RV	*Pvu*II	*Hin*dIII	*Eco*RI	*Bam*HI	*Sal*I	*Cla*I	*Ban*II	*Bcl*I
1	24.0	24.0	19.0	11.6	10.3	40.0	42.0	19.0	23.5	20.5
2	22.5	15.0	15.6	8.5	7.1	38.0	40.0	12.7	20.0	14.8
3	20.6	14.0	14.6	7.0	6.5	25.0	18.0	9.5	17.0	10.0
4	14.0	12.5	13.2	6.5	6.0	20.0	12.6	9.3	10.8	8.5 (2×)
5	11.2	8.4	11.0	6.4	5.8	4.8	9.0	8.9	10.8	8.2
6	9.0	8.4	7.0	5.7	5.6		6.0	7.0	8.8	6.0
7	6.0	8.4	6.5	4.8	5.2			6.9	7.4	5.5
8	5.0	7.5	5.6 (2×)	4.7	4.4			6.5	6.2	5.4
9	3.4	6.2	3.5	4.6 (2×)	4.4			6.0	4.3	5.2
10	3.0	4.5	3.0	4.5	4.2			3.4	4.2	4.8
11	2.7	4.1	3.0	4.0	4.0			3.0	3.4	4.4
12	2.2	2.9 (2×)	2.7	3.6	3.8 (2×)			3.0 (2×)	2.7	3.9
13	1.6	2.8 (2×)	2.3	3.6	3.8			2.9	2.3	3.0
14	1.0	1.9	2.2	3.6	3.3			2.8 (2×)	2.1	3.0
15	0.6	0.7	1.8	3.6	3.1			2.6	2.0	2.2
16			1.8	3.5	2.9			2.1	1.6	1.8
17			1.8	2.8	2.8			2.0		1.7 (2×)
18			1.7	2.4	2.7			1.8		1.4 (2×)
19			1.3	2.3	2.5			1.7		1.3 (2×)
20			1.2	2.1	2.5			1.6		0.5
21			0.7	2.0	2.3			1.6 (2×)		0.3 (2×)
22			0.6 (2×)	1.9	2.2			1.4 (2×)		
23			0.6	1.8	2.1					
24				1.7	2.1 (2×)					
25				1.6	2.0 (2×)					
26				1.5	1.6					
27				1.5	1.4					
28				1.3 (2×)	1.4 (2×)					
29				1.1	1.4					
30				1.1	1.3					
31				1.0	1.2					
32				1.0	1.2					
33				0.9	1.0					
34				0.7	0.8					
35				0.7	0.7 (2×)					
36				0.6	0.6 (2×)					
37				0.4	0.5					
Sum	126.8	127.0	129.9	122.5	125.3	127.8	127.6	124.5	127.1	125.6

Stein et al. 1986) and algae (Linne von Berg and Kowallik 1988, Kowallik 1989) examined in this regard.

Gene mapping. Between 17 and 24 genes were mapped to the chloroplast genome of *Cyclotella* (Fig. 1) by heterologous hybridization with 60 probes. These included 58 probes from the land plants tobacco, spinach, and pea and one each from *Chlamydomonas reinhardtii* and *Cryptomonas* Φ. Only 27 of the 60 gene probes tested gave positive hybridization to *Cyclotella* cpDNA (Table 3). These signals are considered strong evidence for the presence of these genes in *Cyclotella* cpDNA. One set of gene mapping digests and three representative gene mapping blots of *Cyclotella* cpDNA are shown (Fig. 4).

In addition to the highly conserved rRNA genes, most of the probes from tobacco, spinach, and pea that hybridized to *Cyclotella* cpDNA included genes coding for proteins that have an amino acid sequence identity of greater than 87% between tobacco and the liverwort *Marchantia polymorpha* chloroplasts (Wolfe and Sharp 1988). These included some ATP synthase subunit genes and genes coding for photosystem I and II polypeptides. Most of the NADH dehydrogenase and RNA polymerase probes that hybridized had amino acid sequence identities of 60–70%.

Thirty-three probes containing genes for proteins with amino acid sequence identities between *Marchantia polymorpha* and tobacco of between 50% and 85% failed to hybridize to *Cyclotella* cpDNA at moderately low stringency (55–60° C, 4× SSC). These included genes and portions of genes encoding ribosomal proteins (*rps*2, *rps*3, *rps*7, *rps*8, *rps*16, *rps*18, and *rps*19, *rpl*12, *rpl*14, *rpl*16, *rpl*20, *rpl*22, *rpl*23, *rpl*33), photosynthetic proteins (*psa*D, *psb*E, *psb*F, *psb*G, *psb*L, *psb*M, *pet*A, *atp*E, *atp*F, *atp*H, *atp*I), NADH dehydrogenase subunits (*ndh*A, *ndh*B, *ndh*C, *ndh*E, *ndh*F, *ndh*G, *ndh*H), and RNA polymerase subunits (*rpo*B, *rpo*C1, *rpo*C2). The *rbc*S probe from *Cryptomonas* Φ did not hybridize.

There are some important limitations to drawing inferences from hybridization signals with heterologous probes. In some instances, we used multiple probes that contained different parts of the same gene (e.g. *rbc*L, *psb*B 23S rDNA, *rps*7, *ndh*B, *atp*H,

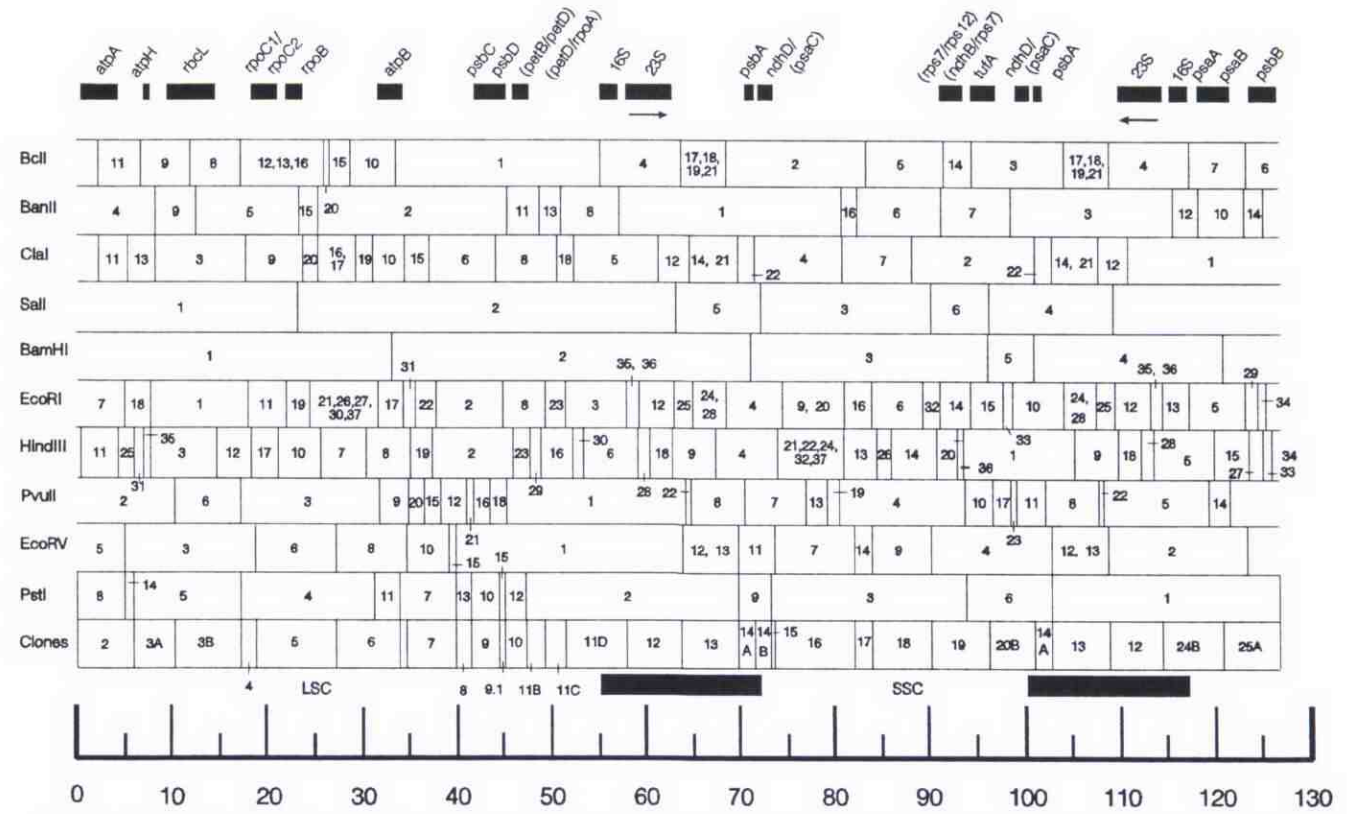

FIG. 1. Restriction site, clone, and gene map of cpDNA of *Cyclotella meneghiniana* clone 1020-1a. Restriction fragment numbers correspond to those given in Tables 1 and 2. Unordered fragments are separated by commas. The scale at the bottom of the map is in kb. The circular map has been linearized at the *Pst*I site separating fragments #8 and #1. The two long, heavy black lines below the map indicate the 17 kb maximum possible extent of the inverted repeat (IR). The row of fragments above the heavy black lines and below the *Pst*I map show the positions and numbers of the cloned cpDNA fragments listed in Table 1 that were used as probes to produce the map. The short, black boxes show the position and represent the maximum extent of hybridization of 24 of the genes listed in Table 3. The arrows indicate the orientation of the 23S rRNA genes from 5′ to 3′. A slash [/] between two genes indicates that the order of the two genes is undetermined, and parentheses [( )] indicate uncertainty as to which gene or genes of a double gene probe hybridized.

*rpo*B, *rpo*C1, and *rpo*C2). In other instances, we used probes that contained parts of two or more genes. In both cases, negative results must be interpreted with caution. We interpret any positive hybridization as evidence for the presence of a gene, even if a probe containing another portion of the same gene did not hybridize. Absence of hybridization signals in either case may indicate either that the gene (or portion of the gene) is there but too divergent to hybridize or that the gene is not there at all. Thus, while we cannot map genes from lack of hybridization, we cannot conclude with certainty that the genes are completely absent. Furthermore, positive hybridization signals from probes that contain parts of two or more genes (e.g. *psa*A and *psa*B, *psb*D and *psb*C, *pet*B and *pet*D, *pet*D and *rpo*A, *ndh*D and *psa*C, *ndh*B and *rps*7, *rps*7 and *rps*12 in Table 3) must be interpreted with caution because the signal may be due to either the presence of only one gene or the other or of both genes in the *Cyclotella* chloroplast genome (Fig. 1). We discuss specific instances of these occurrences later as appropriate.

Chloroplast gene order in diatoms. Several gene clusters found in land plant cpDNA are also present in

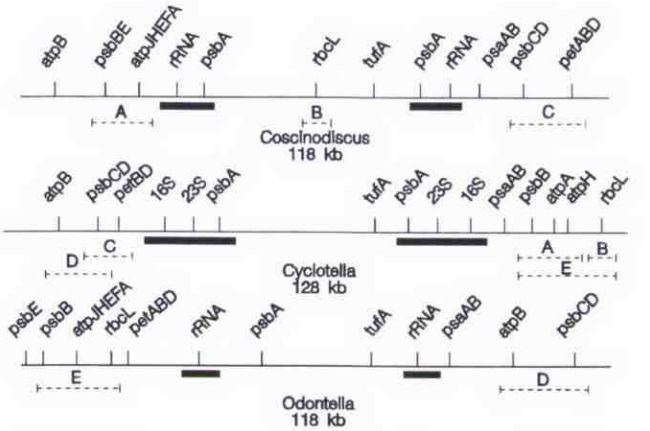

FIG. 2. Linear maps comparing chloroplast gene order in three diatoms. The data for *Cyclotella meneghiniana* clone 1020-1a are from this paper, while those for *Coscinodiscus granii* and *Odontella sinensis* are from Kowallik (1989 and pers. commun.). The thick bars below each map represent the inverted repeats. Lettered, broken lines below each map indicate the gene clusters that are rearranged between *Cyclotella* and the other two diatoms.

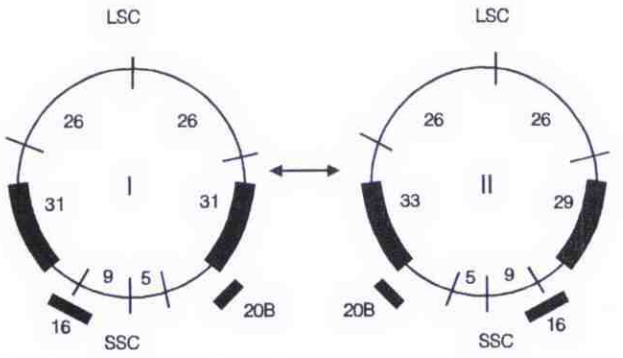

FIG. 3. Two different circular forms of the *Cyclotella* chloroplast genome. *Sac*I fragment sizes are indicated in kb. The curved, wide black bands on the circle indicate the 17-kb inverted repeat (IR). The straight wide bands mark the positions of the cloned *Cyclotella* cpDNA fragments (Table 1 and clone map in Fig. 1) used as probes in a *Sac*I hybridization. The 8.4-kb *Eco*RV fragment (clone No. 16) lies fully within the small single copy region (SSC). The 4.7-kb *Sal*I/*Bam*HI fragment (clone No. 20B) overlaps the IR and the SSC. The two 31-kb *Sac*I fragments of isomer I span the 17-kb IR and cut the genome asymetrically in the large single copy region (LSC) and SSC, resulting in unequal sizes of the single copy portions of the 31-kb fragments. Recombination within the IR results in the reassociation of the SSC and LSC portions of the two 31-kb fragments into a 33-kb and a 29-kb fragment containing the IR as in isomer II. As a result, the polarity of the SSC is flipped relative to the LSC.

Cyclotella cpDNA (the 16S and 23S rRNA genes in the IR; the *psa*A and *psa*B tandem pair; *psb*C and *psb*D; *pet*B and *pet*D; *atp*A and *atp*H; *rps*7 and *rps*12; *rpo*B, *rpo*C1, and *rpo*C2; and, perhaps, *ndh*D and *psa*C). Many of these clusters are also present in the other two centric diatoms, *Coscinodiscus granii* and *Odontella sinensis*, whose cpDNAs are mapped (Kowallik 1989 and pers. commun.). Several genes and gene clusters (in particular, the rRNA genes, the *psa*AB cluster, *tuf*A, and *psb*A) are arranged in a similar order in the three diatom chloroplast genomes (Fig. 2). However, other genes are rearranged relative to the other two diatoms. Three clusters of genes (A, B, and C) are rearranged in the *Coscinodiscus/Cyclotella* comparison and another two clusters (D and E) are rearranged in the *Odontella/Cyclotella* comparison (Fig. 2). We estimate that less than a quarter of the protein genes are mapped in each diatom genome. Therefore, since the gene maps are incomplete, it is not possible to deduce with certainty the precise nature of the events responsible for these gene order rearrangements. However, since inversions are known to account for major cpDNA gene rearrangements in many land plants (Palmer 1991), it is likely that some of the diatom gene order rearrangements are also due to inversions.

Inverted repeat gene organization. The *Cyclotella* cpDNA IR contains not only the highly conserved 16S and 23S rRNA genes found in the IR of most land plants, algae, and diatoms, but also the photosystem II *psb*A gene (Fig. 1). The *Cyclotella* rRNA genes as a whole map near the LSC end of the IR, similar to the situation in another centric diatom, *Coscinodiscus granii* (Kowallik 1989, Fig. 2). The 16S gene maps closer to the LSC, and the 23S gene maps closer to the SSC, similar to the arrangement in most land plants (e.g. Palmer and Stein 1986, Palmer et al. 1988, reviewed in Palmer 1991) and other chlorophyll *a*/*c*-containing algae (Douglas 1988, Loiseaux de Goër et al. 1988).

The *psb*A gene, which codes for the 32-kDa thylakoid membrane herbicide binding protein of photosystem II, maps in the IR closer to the SSC in *Cyclotella* (Figs. 1, 2) as it does in the IR of the centric diatom *Coscinodiscus granii* (Kowallik 1989, Fig. 2),

TABLE 3. *Gene probes that hybridized to* Cyclotella meneghiniana *clone 1020-1a cpDNA. Gene names are those used in Sugiura (1989). Tobacco coordinates are from Shinozaki et al. (1986). For pea, spinach, and* C. reinhardtii *genes, "+x" indicates gene probe either starts or ends x bp following the initiation codon; "++x" indicates gene probe ends x bp following the termination codon. Probes that contain parts of two genes in two categories are listed in each category.*

Gene probe	Source	Coordinates
Bioenergetic genes		
RuBisCO		
5′*rbc*L	Tobacco	56836–58047
3′*rbc*L	Tobacco	58047–59305
PSI and PSII		
*psa*A; 5′*psa*B	Spinach	+50/++277
3′*psa*B	Spinach	+598/++80
*psa*C; 5′*ndh*D	Tobacco	118604–119520
*psb*A	Tobacco	419–1731
5′*psb*B	Tobacco	73736–75822
3′*psb*B	Tobacco	75822–76545
*psb*C internal	Tobacco	35839–36455
3′*psb*D; 5′*psb*C	Pea	+227*psb*D/+36
Cytochrome b_6/f complex		
*pet*B; 5′*pet*D	Tobacco	77375–79188
3′*pet*D; 3′*rpo*A	Tobacco	79486–80652
ATPase		
*atp*A	Tobacco	10600–12060
5′*atp*B	Tobacco	55921–56836
5′*atp*H	Tobacco	13913–14471
NADH dehydrogenase		
5′*ndh*B; 3′*rps*7	Tobacco	98527–99726
3′*ndh*D; ORF313	Tobacco	116171–118604
5′*ndh*D; *psa*C	Tobacco	118604–119520
Genetic apparatus genes		
Ribosomal RNA		
16S rDNA	Tobacco	101532–104801
5′23S rDNA	Tobacco	105372–107136
23S rDNA internal	Tobacco	107136–108816
3′23S rDNA	Tobacco	108816–109722
Ribosomal protein		
3′*rps*7; 5′*ndh*B	Tobacco	98527–99726
5′*rps*7; 3′*rps*12	Tobacco	99726–100952
RNA polymerase		
3′*rpo*A; 3′*pet*D	Tobacco	79486–80652
5′*rpo*C2	Tobacco	20287–21488
*rpo*C1 internal	Tobacco	21947–23937
*rpo*B internal	Tobacco	25128–26191
5′*rpo*B	Tobacco	26291–28590
Elongation factor EF-Tu		
5′*tuf*A	*C. reinhardtii*	+110/+489

the marine chromophyte *Olisthodiscus luteus* (Reith and Cattolico 1986), and three *Chlamydomonas* species (Turmel et al. 1987, Woessner et al. 1987). In *Chlorella ellipsoidea*, the *psb*A gene is located in the IR close to the LSC (Yamada 1991). However, it is present in the SSC in three other chlorophyll *a/c* algae (Douglas 1988, Kowallik 1989, Fig. 2) and in the LSC of the brown alga *Dictyota dichotoma* (Kuhsel and Kowallik 1987) and land plants (e.g. Palmer and Stein 1986, Palmer et al. 1988, reviewed in Palmer 1991). *Psb*A maps to both cpDNA molecules of *Pylaiella littoralis* and in both the LSC and SSC in the larger molecule (Loiseaux de Goër et al. 1988). The presence of a rRNA operon in all examined IRs may be due to either strong selective pressures to increase rRNA gene numbers and thus to multiple parallel origins of the IR or to its presence in one chloroplast genome ancestor (Palmer 1991). However, differences in IR presence, size, and gene content may be due to a combination of multiple independent gains or losses of the IR and processes of IR expansion (or contraction) into single copy regions of the genome (reviewed in Palmer 1991).

Mapping data suggest that portions of the NADH dehydrogenase gene, *ndh*D, and perhaps the photosystem I *psa*C gene may either be wholly internal to the IR or overlap the SSC end of the IR (Fig. 1). This is the first known mapping of these genes in a diatom. Since *psa*C is included in the 5′*ndh*D probe, the evidence is less strong for its presence than it would be with a single gene probe. However, as we discussed earlier, we cannot say with certainty that it is not there. Thus, its presence in the *Cyclotella* chloroplast genome is tentative (Fig. 1). The *ndh*D and *psa*C genes code for proteins that have amino acid sequence identities of 70% and 91%, respectively, between tobacco and *Marchantia polymorpha* (Wolfe and Sharp 1988) and are co-transcribed in maize cpDNA (Schantz and Bogorad 1988). These facts tend to support the inference that both genes may also occur next to one another in *Cyclotella* cpDNA.

Small single copy gene organization. The *Chlamydomonas tuf*A gene, which codes for the chloroplast protein elongation factor EF-Tu, hybridizes to the SSC region of *Cyclotella* cpDNA (Fig. 1). The other two centric diatoms investigated have the gene in much the same relative position (Kowallik, pers. commun., Fig. 2). The *tuf*A gene, absent from land plant cpDNAs, was apparently transferred from the chloroplast to the nucleus in the green algal ancestors of land plants and retained in the chloroplast of the chromophyte *Cryptomonas* Φ (Baldauf and Palmer 1990, Baldauf et al. 1990, Douglas 1991) and the rhodophyte *Porphyra yezoensis* (Shivji 1991).

Since each of the two ribosomal protein gene-containing probes that hybridized contains parts of two genes (Table 3), we are uncertain as to which gene or genes in each probe is responsible for the positive signals. However, two probes containing

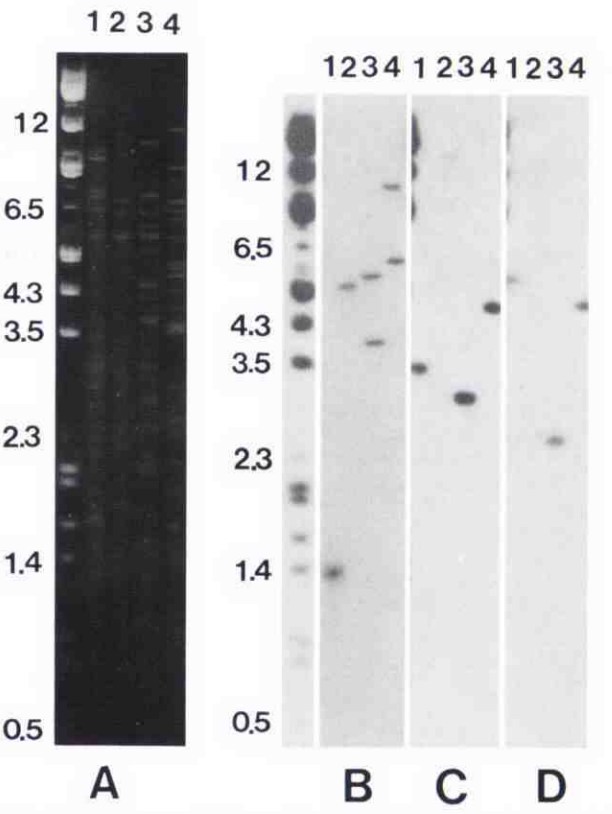

FIG. 4. Agarose gel electrophoresis of *Cyclotella* cpDNA and corresponding autoradiographs of hybridizations of selected heterologous probes. A) A 1.0% agarose gel of cpDNA of *Cyclotella meneghiniana* clone 1020-1a digested with *Cla*I (lane 1), *Pvu*II (lane 2), *Eco*RI (lane 3), and *Hin*dIII (lane 4). Size in kb is indicated for selected lambda DNA reference fragments shown in the far left lane. Autoradiographs of hybridization of three heterologous gene probes (listed in Table 3 and mapped in Fig. 1) to Southern transfers of replicates of the gel in Fig. 4A. Size in kb is indicated for selected lambda DNA reference fragments shown in the lane to the left of the autoradiographs: B) tobacco *psb*A gene probe, C) tobacco *atp*B gene probe, D) tobacco 5′*rpo*B gene probe.

portions of *ndh*B adjacent to the *ndh*B portion of the 5′*ndh*B/3′*rps*7 probe did not hybridize. This may indicate that the signal is due to either the 5′*ndh*B or 3′*rps*7 portion of the probe or to portions of both genes (Fig. 1). A single probe containing the same 5′*rps*7 sequence as in the double 5′*rps*7/3′*rps*12 probe did not hybridize. This is slightly stronger evidence that the signal is due to 3′*rps*12 rather than to 5′*rps*7. The *rps*7 and *rps*12 genes have been mapped in the chloroplast genome of only one other chromophyte alga, *Cryptomonas* Φ, in which they are adjacent and co-transcribed (Douglas 1991). Since each hybridizing gene probe contains parts of one or both genes, it is likely that both are also present in the *Cyclotella* SSC region.

Large single copy gene organization. The LSC of *Cyclotella* contains the *rbc*L gene coding for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Fig. 1), as is true in the centric diatom *Odontella sinensis* (Kowallik, pers. commun., Fig. 2) and in *Cryptomonas* Φ (Douglas and

Durnford 1989). In contrast, *rbc*L maps to the SSC in *Coscinodiscus granii* (Kowallik, pers. commun., Fig. 2) and appears to map to a repeated sequence in the pennate diatom *Cylindrotheca* (Hwang and Tabita 1989).

Unfortunately, since the *Cryptomonas* Φ *rbc*S gene probe did not hybridize to *Cyclotella* cpDNA, we cannot ascertain whether this gene is present in the cpDNA of *Cyclotella*, as it is in the chloroplast genomes of several chlorophyll *a/c* algae (Reith and Cattolico 1986, Boczar et al. 1989, Douglas and Durnford 1989, Hwang and Tabita 1989) and rhodophytes (Valentin and Zetsche 1989), or in the nucleus, as in land plants and green algae. The *rbc*L and *rbc*S genes are closely associated and co-transcribed in the chloroplast genome of the marine diatom *Cylindrotheca* sp. strain N1 (Hwang and Tabita 1991). We do not know if a similar arrangement occurs in *Cyclotella* or would be detected with a less divergent *rbc*S gene probe.

Three pairs of closely linked genes map to the LSC in *Cyclotella* (Fig. 1) as they do in land plants and the other two mapped centric diatoms (Kowallik 1989, Fig. 2). These include the *psa*A and *psa*B genes, encoding subunits A1 and A2, respectively, of the P700 chlorophyll *a* apoprotein of photosystem I; the *psb*C and *psb*D genes, coding for the 43-kDa and 32-kDa-like proteins of photosystem II, respectively; and the *pet*B and *pet*D genes, which encode cytochrome b_6 and subunit IV of the cytochrome b_6/f complex, respectively. Differential hybridization of probes allowed ordering of *psa*A and *psa*B and of *psb*C and *psb*D, whereas the relative order of *pet*B and *pet*D could not be determined.

Three of the six genes for ATP synthase subunits present in land plant cpDNA (Sugiura 1989) hybridize to the LSC of *Cyclotella* cpDNA (Fig. 1). *Atp*A and *atp*H map near one another and distantly from *atp*B (Fig. 4), as also found in land plants, other chlorophyll *a/c* algae (Kowallik 1989, Fig. 2), and cyanobacteria (Palmer 1991).

This is the first time that RNA polymerase genes have been mapped in any chlorophyll *a/c* algae. Probes specific for three of the four RNA polymerase subunit genes (*rpo*A, *rpo*B, *rpo*C1, and *rpo*C2) found in spinach and all sequenced genomes (Sugiura 1989) map next to one another in the LSC of *Cyclotella* cpDNA (Figs. 1, 4). If their order is the same as that in land plants, then the same operon may exist in *Cyclotella*. The *rpo*A gene presence is tentative because this gene is contained in a double gene probe.

CONCLUSIONS

In conclusion, diatom cpDNA gene order is proving to be highly variable and much less conservative than that of land plant cpDNA. Within one diatom order, the Centrales, there is an apparent retention of the rRNA-encoding IR and gene clusters and operons found in prokaryotes and land plants. However, there is also a highly rearranged LSC gene order between diatom taxa. More detailed restriction site mapping and sequencing will reveal the endpoints of individual rearrangements and clarify the actual processes that led to these gene order changes. It is currently unknown if these characteristics will prove useful in phylogenetic analyses and what significance they hold for evolutionary studies of diatom cpDNA.

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