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Physical map of chloroplast DNA of aerial yam, *Dioscorea bulbifera* L.*

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Summary. A physical map of chloroplast DNA (ctDNA) of aerial yam, *Dioscorea bulbifera* L. was constructed using three restriction endonucleases, *Pst*I, *Sal*I, and *Sma*I. In addition, a clone bank of the *Bam*HI-digested fragments were generated, and the locations of most *Bam*HI fragments on the map were also determined. The ctDNA of *D. bulbifera* was found to be a circular molecule with a total size of ca. 152 kb involving two inverted repeats of ca. 25.5 kb, and small and large single copy regions of ca. 18.5 and 83.4 kb, respectively. The genes for the large subunit of the ribulose 1,5-bisphosphate carboxylase (*rbcL*) and the ATP-synthase subunits β and ε (*atpB*/*atpE*) were mapped.

Key words: Aerial yam – *Dioscorea bulbifera* L. – Chloroplast DNA – Physical map – Clone bank

Introduction

The analysis of chloroplast DNA (ctDNA) has proven to be a powerful tool for elucidation of the phylogenetic relationship in many plant taxa. CtDNA analysis was most successfully applied in studies on interspecific or intergeneric relationships, because of its strong conservatism. However, in certain cases, intraspecific variations were also revealed, providing information on intraspecific differentiation as well as on the origin of a given species (Palmer et al. 1983, 1985; Clegg et al. 1984; Murai and Tsunewaki 1986; Palmer 1987). In some plant taxa, the phylogenetic relationships were already well established, and the phylogeny independently derived from examinations of ctDNA variation fit very well with existing data (Palmer and Zamir 1982; Enomoto et al. 1985; Jansen and Palmer 1987). Therefore, ctDNA analysis of plant taxa, for which traditional methods of phylogenetic studies have been difficult up to now, will provide a first-hand clue towards uncovering their phylogeny. In this context, the phylogeny or origin of some woody genera, which had been left largely unexamined, was subjected to ctDNA analysis, i.e., *Coffea* (Berthou et al. 1983), *Prunus* (Kaneko et al. 1986) and *Citrus* (Green et al. 1986). The phylogenetic study of vegetatively propagating crops generally poses the same difficulty and would be suitable for ctDNA investigation, as already shown in potato (Hosaka 1986, 1988; Heinhorst et al. 1988).

The genus *Dioscorea*, consisting of some 600 species, includes important vegetatively reproducing tuber crops, known as yam. Together with the *Colocasia* species, yams have played a significant role in the advent of agriculture in Southeast Asia and equatorial Africa (Coursey 1972, 1981). Apart from their importance in ancient time, species such as *D. alata* in Southeast Asia and Oceania, and the *D. cayenensis-D. rotundata* complex in West Africa make a major contribution to the staple diet of the region. Therefore, taxonomic and phylogenetic studies on *Dioscorea* are important, both from the ethnobotanical as well as agricultural points of view. With some *Dioscorea* species, attempts to clarify the phylogenetic relationship among the cultivars have been made by examining morphological (Martin and Rhodes 1978; Onyilagha and Lowe 1985), chemical (Miège 1982a), or cytological (Miège 1954, 1982b) characters. However, ambiguous results were obtained because of a high degree of variability in these characters. In the present study, effort was

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concentrated on constructing a physical map of ctDNA, forming a strong basis for an effective analysis of ctDNA variation.

An aerial yam, *Dioscorea bulbifera* L., was chosen as the first material to work with because of its relative ease for ctDNA preparation. This species is characterized by the formation of many axillary tubers (bulbils). Its distribution is pantropical along the equator, and is especially abundant in Southeast Asia and West Africa. In both of these areas, cultivated types with large bulbils are seen along with wild types bearing easily detached small bulbils. Taxonomic status and intraspecific classifications of *D. bulbifera* are diverse, according to Prain and Burkill (1936) and Chevalier (1936). As keys for the classification, most of them used highly variable characters such as the shape, color, and dimension of bulbils and leaves. The chromosome number, which is usually critically examined for the taxonomic purpose, was revealed to be very high and unstable even within a single plant for *D. bulbifera* (Terauchi unpublished), such that it could not be used for decisive examination of intraspecific variation in this species. Therefore, the only promising approach which could be relied upon turned out to be the DNA analysis. Though not included in the present report, restriction endonuclease analysis, using a small number of accessions of *D. bulbifera,* revealed abundant intraspecific ctDNA variations. Therefore, the physical map and clone bank of ctDNA generated here will serve to assess these variations and phylogenetic relationships within a convincing framework in forthcoming studies.

Materials and methods

Plant material

An accession, DBl of *D. bulbifera,* originally collected in Antananarivo, Madagascar, was used for ctDNA extraction and physical mapping of ctDNA. After the classification of Prain and Burkill (1936), this accession belongs to var. *anthropophagorum* because of its angular bulbils, which contrast to globular bulbils characterizing Asian varieties.

CtDNA preparation

Approximately 100 g of leaves from a single plant was used for ctDNA extraction. The extraction of intact chloroplasts were made after Ogihara and Tsunewaki (1982), with the following modifications: A leaf sample was homogenized with 1 l of a buffer containing 5 m*M* 2-mercaptoethanol and 0.6% Polyvinylpolypyrolidone (PVPP), in order to dilute out phenolic compounds and polysaccharides, the latter causing high viscosity in the homogenate. The homogenates were filtered only once with a single layer of cheesecloth to minimize the time during which oxidation of homogenates occurs. The discontinuous gradient used was made of 15%, 40%, and 60% sucrose solutions instead of 10%, 40%, and 75% Percoll solutions. From this chloroplast preparation, ctDNA was isolated after Kolodner and Tewari (1975).

Recovery of ctDNA from an agarose gel

After the digestion with *Pst*I, *Sal*I, and *Sma*I, electrophoretically separated ctDNA fragments were individually recovered from an agarose gel using the glass powder Geneclean (BIO 101, USA) following the supplier's instruction.

Cloning the ctDNA restriction fragments

CtDNA of the accession DBl was digested with restriction endonucleases *Bam*HI, *Sal*I, and *Pst*I and used for molecular cloning. For cloning the *Bam*HI restriction fragments, vectors pUC19 and pUC119 were used in different trials. In cloning the *Pst*I and *Sal*I fragments, only pUC19 was used. Ligation was carried out after Maniatis et al. (1982). Competent *E. coli* cells (JM 109), prepared after Hanahan (1985), were transformed with the ligated plasmids. Recombinants were screened on a plate containing ampicilline, Xgal, and Isopropyl-β-D-thiogalactopyranoside (IPTG), and finally checked for ct DNA insertion by the rapid plasmid screening method of Maniatis et al. (1982).

Southern blotting

Location of some cloned fragments and *rbcL* and *atpB/atpE* genes on the ctDNA map of *D. bulbifera* was determined by employing the molecular hybridization method of Southern (1975). Total ctDNA was digested with *Pst*I, *Sal*I, and *Sma*I, either solely or in combination, and was bi-directionally blotted to Biodine A membranes (Pall Ultrafine Filtration, USA). Recombinant clones serving as the probe were labelled by ^{32}P-labelled dCTP and nick-translated utilizing a kit purchased from Takara Shuzo Co., Japan. Autoradiography was carried out for 24–72 h at $-70\,^{\circ}$C, using Fuji X-ray film loaded in a cassette with lightening-plus intensifying screen.

Results

Restriction endonuclease analysis and genome size estimation

Figure 1 shows the electrophoretic patterns of the *D. bulbifera* ctDNA digested with *Bam*HI, *Pst*I, *Sal*I, and *Sma*I solely or in combinations of two enzymes. From the molecular sizes of individual restriction fragments and their copy number, the total genome size was estimated to range from 137.6 kb for the *Bam*HI to 152.1 kb for the *Sma*I digest (Table 1). From these results, the most reasonable estimate for the chloroplast genome size of *D. bulbifera* is ca. 152 kb.

Southern hybridization of the cloned PstI and SalI fragments to total ctDNA digested with PstI, SalI, and SmaI

Some of the *Pst*I and *Sal*I fragments were cloned. These were P9 (7.6 kb), P10 (4.9 kb), P11 (2.7 kb), and P12 (2.6 kb) of the *Pst*I fragments, and S6 (14.5 kb) and S7 (11.5 kb) of the *Sal*I fragments (see Tables 3 and 4). Three of these clones were labelled with ^{32}P and hybridized to the Southern (1975) blot of *D. bulbifera* ctDNA

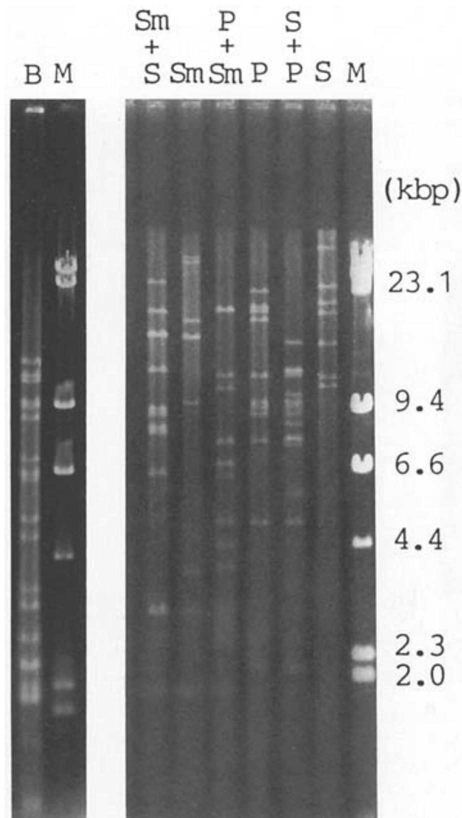

Fig. 1. Restriction fragment patterns of *D. bulbifera* ctDNA obtained by single and double digestion with *Bam*HI (B), *Pst*I (P), *Sal*I (S), and *Sma*I (Sm)

digested with either *Pst*I, *Sal*I, or *Sma*I, or in combinations of two. Two examples are shown in Fig. 2. Table 2 gives a list of the ctDNA fragments which hybridized to each probe.

Subfragments generated from the individual restriction fragments by digesting with another enzyme

The *Pst*I, *Sal*I, or *Sma*I restriction fragments were individually recovered from agarose gel after electrophoresis, and were further digested with a second enzyme, eventually generating double-digested fragments. Figure 3 shows the electrophoretic patterns of the individual *Sma*I fragments recovered and their subfragments generated by the additional *Sal*I digestion. The same sort of experiments were carried out, reversing the order of two enzymes in their use.

Many subfragments of similar sizes were generated from the individual *Pst*I fragments, when digested with *Sal*I. Therefore, identification of subfragments was incompletely done. Table 3 shows the molecular sizes of *Pst*I/*Sal*I subfragments unambiguously identified in the respective *Pst*I fragments. Three cloned *Pst*I fragments did not give rise to any subfragments when treated with *Sma*I (Table 3).

All *Sal*I fragments recovered individually from gels and two cloned fragments, S6 and S7, were further digested with *Bam*HI, *Pst*I, and *Sma*I. The sizes of the subfragments generated were estimated, as shown in Table 4.

Table 1. Molecular size and copy number of the restriction fragments of *D. bulbifera* ctDNA generated by single or double digestion with *Pst*I, *Sal*I, and *Sma*I, and by single digestion with *Bam*HI

*Pst*I		*Pst*I/*Sal*I		*Sal*I		*Sal*I/*Sma*I		*Sma*I		*Sma*I/*Pst*I		*Bam*HI	
No.	kb	No.	kb	No.	kb	No.	kb	No.	kb	No.	kb	No.	kb
P1	24.1	PS1	14.6	S1	46.0	SSm1	27.0	Sm1	40.0	SmP1	19.6 (×2)	B1	12.0
P2	20.2	PS2	12.1 (×2)	S2	26.0	SSm2	19.0	Sm2	37.0	SmP2	11.7	B2	11.0
P3	19.6	PS3	11.7	S3	20.5	SSm3	16.0 (×2)	Sm3	17.5	SmP3	10.5	B3	10.8
P4	17.7	PS4	10.2	S4	19.0	SSm4	12.6	Sm4	16.0 (×2)	SmP4	7.6 (×2)	B4	9.5
P5	11.7	PS5	9.8	S5	14.6	SSm5	9.2	Sm5	9.7	SmP5	6.7 (×2)	B5	9.4
P6	9.8	PS6	9.5	S6	11.5	SSm6	8.9	Sm6	3.9 (×2)	SmP6	6.2	B6	9.0
P7	9.5	PS7	9.1	S7	11.0	SSm7	8.3	Sm7	3.1	SmP7	4.9 (×2)	B7	7.0
P8	9.1	PS8	8.5			SSm8	8.0	Sm8	1.7 (×2)	SmP8	4.4 (×2)	B8	6.5
P9	7.6	PS9	7.8 (×2)			SSm9	6.4	Sm9	0.8 (×2)	SmP9	3.9 (×2)	B9	6.0
P10	4.9 (×2)	PS10	7.5			SSm10	3.1 (×4)			SmP10	3.1	B10	5.2
P11	2.7	PS11	5.5			SSm11	1.7 (×2)			SmP11	2.9	B11	4.8
P12	2.6	PS12	4.9 (×2)			SSm12	0.8 (×2)			SmP12	2.7	B12	3.8
P13	2.1 (×2)	PS13	2.6							SmP13	2.6 (×2)	B13	3.7 (×2)
P14	1.7	PS14	2.2							SmP14	2.4	B14	3.0 (×2)
		PS15	2.1 (×3)							SmP15	2.2	B15	2.5 (×4)
		PS16	1.7							SmP16	2.1	B16	2.3
		PS17	1.2							SmP17	1.8 (×3)	B17	2.1 (×4)
		PS18	0.8 (×2)									B18	1.8 (×2)
												B19	1.5
												B20	1.3
Total	150.3		151.6		148.6		148.8		152.1		148.6		135.5

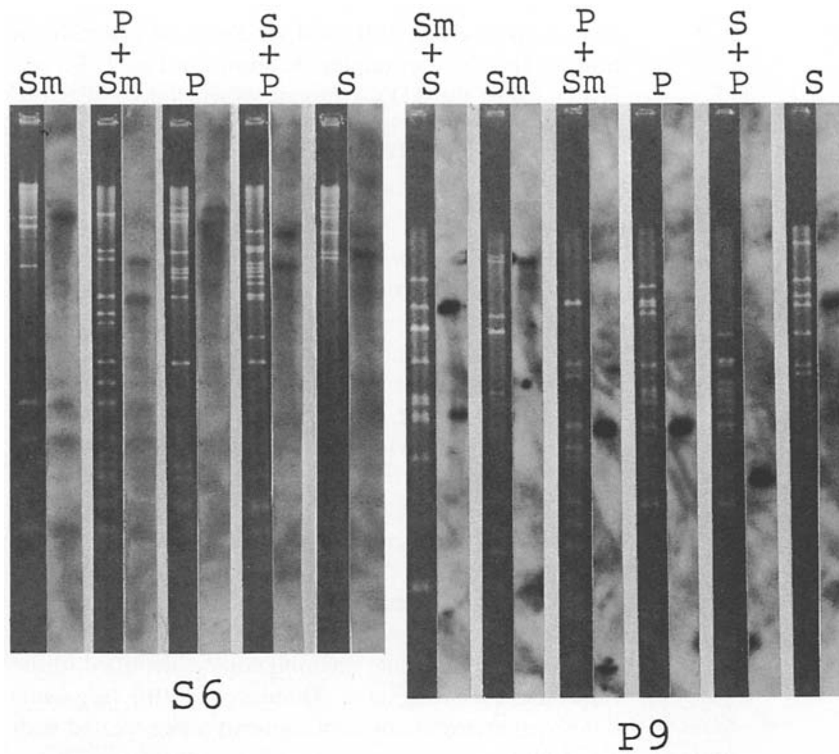

Fig. 2. Hybridization of the cloned S6 and P9 fragments as probe to *D. bulbifera* ctDNA digested with *Pst*I, *Sal*I, or *Sma*I alone, or in their combination

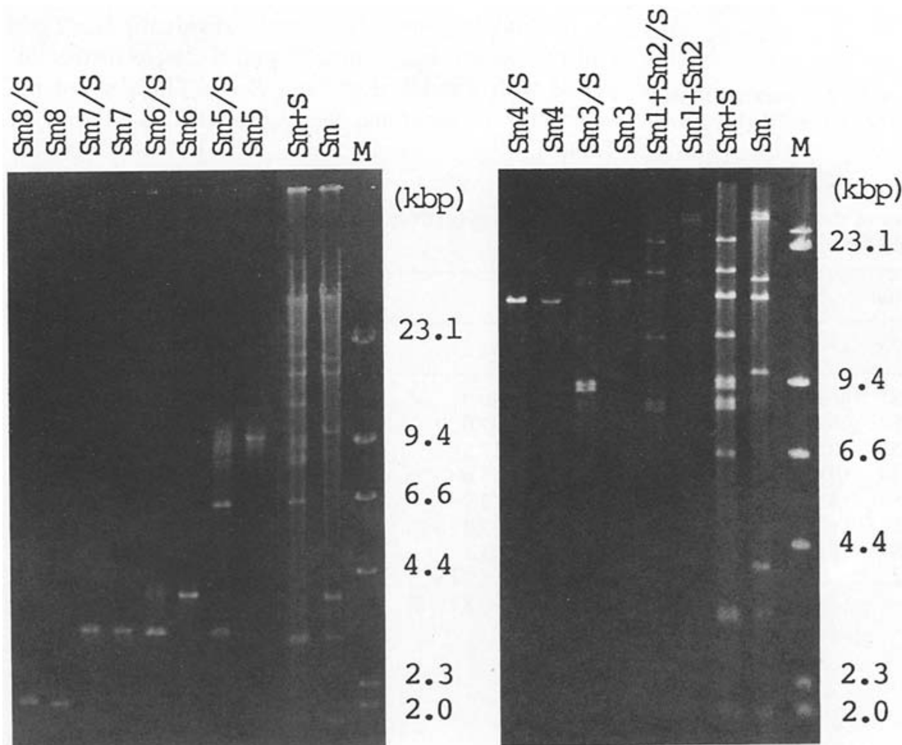

Fig. 3. Restriction fragment patterns of the individual *Sma*I fragments (Sm) recovered from gel and their subfragments generated by *Sal*I digestion (Sm/S)

As for the *Sma*I fragments, the two largest fragments, Sm1 and Sm2, could not be recovered separately, because of their similar molecular sizes. Furthermore, due to the limitation in the amount of the recovered *Sma*I fragments, they were only treated with *Sal*I and subfragment size was estimated (Table 5).

Mapping of the PstI, SalI, and SmaI sites

*Pst*I/*Sal*I site mapping

Cloned P9 was cleaved into 2.2- and 5.5-kb PS fragments (= *Pst*I/*Sal*I subfragments), and hybridized to S3 and S4 (Table 2), indicating that these two S fragments are next

Table 2. Southern hybridization of the cloned *Pst*I and *Sal*I fragments as probe to *D. bulbifera* ctDNA digested with *Pst*I, *Sal*I, or *Sma*I, alone or in combination

Probe (size, kb)	Size (kb) of hybridized fragment					
	*Pst*I	*Pst*I/*Sal*I	*Sal*I	*Sal*I/*Sma*I	*Sma*I	*Sma*I/*Pst*I
P9 (7.6)	7.6 (P9)	5.5 2.2	20.5 (S3) 19.0 (S4)	19.0 8.3	37.0 (Sm2)	7.6
P12 (2.6)	2.6 (P12)	2.1	26.0 (S2)	–	9.7 (Sm5)	2.6
S6 (11.5)	24.1 (P1) 17.7 (P4)	14.6 10.2	14.6 (S5) 11.5 (S6)	–	17.5 (Sm3) 3.9 (Sm6) 3.1 (Sm7) 1.7 (Sm8)	10.5 7.6 3.9 3.1 1.7

– Not examined

Table 3. Size (kb) of subfragments generated from the *Pst*I fragments of *D. bulbifera* ctDNA by *Sal*I and *Sma*I digestion

*Pst*I fragment (size, kb)	Subfragment (size, kb)	
	*Sal*I **	*Sma*I
P1 (24.1)	14.6	–
P2 (20.2)+P3 (19.5)	12.1 (×2)	–
P4 (17.7)	10.2, 7.8	–
P5 (11.7)	11.7	–
P6 (9.8)	9.8	–
P7 (9.5)	9.5	–
P8 (9.0)	9.0	–
P9 (7.6)*	5.5, 2.2	–
P10 (4.9) (×2)*	4.9	4.9
P11 (2.7)*	2.6, 0.2	2.7
P12 (2.6)*	2.1, 0.5	2.6

* Cloned fragment
** Only definitely identified subfragments are shown

to each other. S3 produced three other PS fragments of 9.8, 7.5, and 2.1 kb, besides the 2.2-kb fragment, whereas S4 gave rise to two other PS fragments of 12.1 and 1.7 kb, together with the 5.5-kb fragment (Table 4).

Of the four PS fragments of S3, the 9.8 kb (=P6) and 2.1 kb (=P13) must be located internally and the 7.5-kb fragment at an end of S3, because P6 and P13 did not have any internal *Sal*I site (Tables 1 and 4). The order of arrangement of P6 and P13 in relation to other fragments could not be determined here. P3 (19.5 kb) produced 12.1- and 7.4-kb PS fragments, and no other P fragments were likely to produce a 7.4–7.5-kb PS fragment. Therefore, the 7.5-kb PS fragment of S3 must be the same as the 7.4-kb PS fragment of P3. The distal 12.1-kb PS fragment of P3 should be the same as a 12.1-kb PS fragment of S1, because only S1 and S4 produced this size of PS fragments, and S4 was already located in another part of the map. S1 generated four other PS fragments (11.7, 9.5, 7.8, and 4.9 kb), of which the 11.7-kb (=P5), 9.5-kb (=P7), and 4.9-kb (=P10) fragments must be internally located in S1, because none of them had any

Table 4. Size (kb) of subfragments generated from the *Sal*I fragments of *D. bulbifera* ctDNA by *Bam*HI, *Pst*I, and *Sma*I digestion

*Sal*I fragment (size, kb)	Enzyme used	Subfragment (size, kb)
S1 (46.0)	*Bam*HI	9.2, 6.5, 4.8, 3.7, 3.0, 2.5
	*Pst*I	12.1, 11.7, 9.5, 7.8, 4.9
	*Sma*I	27.0, 16.0, 3.1
S2 (26.0)	*Bam*HI	7.5, 3.7, 3.0, 2.5
	*Pst*I	9.1, 7.8, 4.9, 2.1 (×2)
	*Sma*I	16.0, 6.4, 3.1
S3 (20.5)	*Bam*HI	9.4, 2.5, 2.3, 2.1
	*Pst*I	9.8, 7.5, 2.2, 2.1
	*Sma*I	12.6, 8.3
S4 (19.0)	*Bam*HI	10.0, 9.3
	*Pst*I	12.1, 5.5, 1.7
	*Sma*I	19.0
S5 (14.5)	*Bam*HI	8.5, 4.5
	*Pst*I	14.5
	*Sma*I	9.2, 3.1, 1.7
S6 (11.5)*	*Bam*HI	5.2, 3.8, 2.8
	*Pst*I	10.2, 1.2
	*Sma*I	8.9, 1.7, 0.8
	*Pst*I/*Sma*I	7.5, 1.7, 1.2, 0.8
S7 (11.0)*	*Bam*HI	5.2, 3.1, 1.6, 1.0
	*Pst*I	8.5, 2.6
	*Sma*I	8.0, 3.1
	*Pst*I/*Sma*I	8.0, 2.5, 0.6

* Cloned fragment

Table 5. Size (kb) of subfragments generated from the *Sma*I fragments of *D. bulbifera* by *Sal*I digestion

*Sma*I fragment (size, kb)	Subfragment (size, kb)
Sm1 (40.0)+Sm2 (37.0)	27.0, 19.0, 12.6, 8.3, 8.0
Sm3 (17.5)	9.2, 8.9
Sm4 (16.0) (×2)	16.0 (×2)
Sm5 (9.7)	6.4, 3.1
Sm6 (3.9) (×2)	3.1 (×2), 0.8 (×2)
Sm7 (3.1)	3.1
Sm8 (1.8) (×2)	1.8 (×2)

internal *Sal*I sites. The order of these three PS fragments within S1 could not be decided. It is possible for P1 (24.1 kb) to be cleaved into two pieces (14.6 and 9.5 kb), or into three pieces (14.6, 7.8, and 1.7 kb) with *Sal*I. The former case was unlikely to occur, because only a single 9.5-kb fragment was produced by *Pst*I/*Sal*I double digestion of the intact ctDNA, and it must correspond to P7. Thus, the 7.8-kb terminal PS9 fragment of S1 should overlap to the 7.8-kb PS fragment of P1, of which the 14.6-kb PS fragment (=S5, no internal *Pst*I site) must be located internally.

Of the three PS fragments of S4, the 5.5-kb fragment is at the end close to S3, and the 12.1-kb fragment is located at the other end, because no P fragment had the same size as this, and a 1.7-kb fragment (=P14) had no internal *Sal*I site. A 12.1-kb PS fragment from P2 should overlap this fragment, because only P2 and P3 generated this size of PS fragments, and P3 was already located in another part of the map. The P2 fragment generated another PS fragment of 8.1 kb (or a little larger, corresponding to an 8.5-kb PS fragment), to which the 8.5-kb PS fragment of S7 should correspond (Tables 3 and 4). The other PS fragment (2.6 kb) of S7 will overlap with a fragment of the same size produced from P11 (Tables 3 and 4). From these considerations, a partial *Pst*I/*Sal*I site map was constructed as shown in Fig. 4a.

P4 consists of 10.2- and 7.8-kb PS fragments (Table 3), the former being a part of S6, and the latter a part of S2 (Table 4). S2 contained 9.1-kb (=P8), 4.9-kb (=P10), and two 2.1-kb (=P13) PS fragments, of which P8, P10, and P13 had no internal *Sal*I site. The order of these three P fragments could not be determined. Therefore, the possible arrangement of these fragments can be drawn as shown in Fig. 4b.

The two maps shown in Fig. 4a, b cover the entire chloroplast genome, considering their fragment constitutions and molecular sizes. The two maps better fit by connecting P11 to P12, and P1 to P4. Thus the entire *Pst*I/*Sal*I site map is completed as shown in Fig. 4c.

*Sal*I/*Sma*I site mapping

A mixture of Sml (40.0 kb) and Sm2 (37.0 kb) produced five SSm fragments (*Sal*I/*Sma*I subfragments) of 27.0, 19.0, 12.6, 8.3, and 8.0 kb (Table 5), of which 27.0- and 12.6-kb fragments are assumed to have been derived from Sml, and the remaining three from Sm2, according to their molecular sizes. Because the 12.6- and 8.3-kb SSm fragments originated from S3 (Table 4), the 12.6-kb fragment from Sml and 8.3-kb fragment from Sm2 are adjacent to one another. The cloned P9 hybridized to two SSm fragments of 8.3 kb and 19.0 kb (=S4) (Table 2). From these data, the following order is suggested for the five SSm fragments: (Sm)–27.0 kb–(S)–12.6 kb–(Sm)–8.3 kb–(S)–19.0 kb–(S)–8.0 kb–(Sm), where (S) and (Sm) correspond to *Sal*I and *Sma*I sites, respectively. Among three SSm fragments (27.0, 16.0, and 3.1 kb) from S1, the 16.0-kb fragment (=one of Sm4) had no internal *Sal*I site, indicating its internal location in S1. Because a single 27.0-kb SSm fragment was produced by *Sal*I/*Sma*I double digestion of intact ctDNA (Table 1), the order of all these SSm fragments is assumed as follows: (S)–3.1 kb–(Sm)–16.0 kb–(Sm)–27.0 kb–(S)–12.6 kb–(Sm)–8.3 kb–(S)–19.0 kb–(S)–8.0 kb–(Sm). A terminal 8.0-kb SSm fragment could originate only from S7 (11.0 kb), together with another SSm fragment of 3.1 kb (Table 4). From this information, a partial *Sal*I/*Sma*I site map was constructed as shown in Fig. 4d.

An 8.9-kb SSm fragment of S6 and a 9.2-kb fragment of S5 belonged to Sm3 (17.5 kb) (Tables 4 and 5), indicating that S5 and S6 are adjacent to each other. Because the cloned S6 hybridized to both S5 and S6 (Table 2), their opposite ends are evidently located within the inverted repeats. S6 produced two other SSm fragments of 1.7 and 0.8 kb, whereas Sm6 (3.9 kb), which gave rise to 3.1- and 0.8-kb SSm fragments, hybridized to the cloned S6 (Table 2). Thus, the 0.8-kb SSm fragments of S6 and Sm6 should be the same. The 1.7-kb SSm fragment of S6 had no internal *Sma*I site and, therefore, corresponded to one of the two Sm8 fragments. From this information, the order of the SSm fragments of S6 and Sm6 was assumed as follows: (Sm)–3.1 kb–(S)*–0.8 kb*–(Sm)*–1.7 kb*–(Sm)*–8.9 kb–(S), where the asterisked fragments and restriction sites are the ones assumed to be located in the inverted repeats. S5 also produced two other SSm fragments of 3.1 and 1.7 kb. Because the opposite ends of S5 and S6 constitute the corresponding part of the inverted repeats, as stated above and, therefore, they must have symmetrical site arrangement, the arrangement of SSm fragments in S5 is assuemd as follows: (S)–9.2 kb–(Sm)–3.1 kb–(Sm)*–1.7 kb*–(Sm)*–0.8 kb*–(S)*–3.1 kb–(Sm), although the 0.8-kb fragment was not detected in the *Sma*I digest of S5 (Table 4). Because the 8.9-kb fragment of S6 and the 9.2-kb fragment of S5 are adjacent to each other, the above two sequences are combined into one map, as shown in Fig. 4e.

S2 produced three SSm fragments of 16.0, 6.4, and 3.1 kb, of which the 16.0-kb fragment had no internal *Sal*I site and, therefore, was assumed to be the same as one of the Sm4 fragments. Consequently, the order of the three SSm fragments is (S)–6.4 kb–(Sm)–16.0 kb–(Sm)–3.1 kb–(S). A single 6.4-kb SSm fragment was produced from *Sal*I/*Sma*I double digestion of the intact ctDNA (Table 1), and undoubtedly derived from Sm5 (Table 5), proving that the 6.4-kb end of S2 overlaps Sm5. These considerations lead to construction of a third, partial *Sal*I/*Sma*I site map shown in Fig. 4f.

Judging from both their molecular sizes and fragment constitutions, the three partial *Sal*I/*Sma*I site maps evidently cover the entire chloroplast genome. Because all

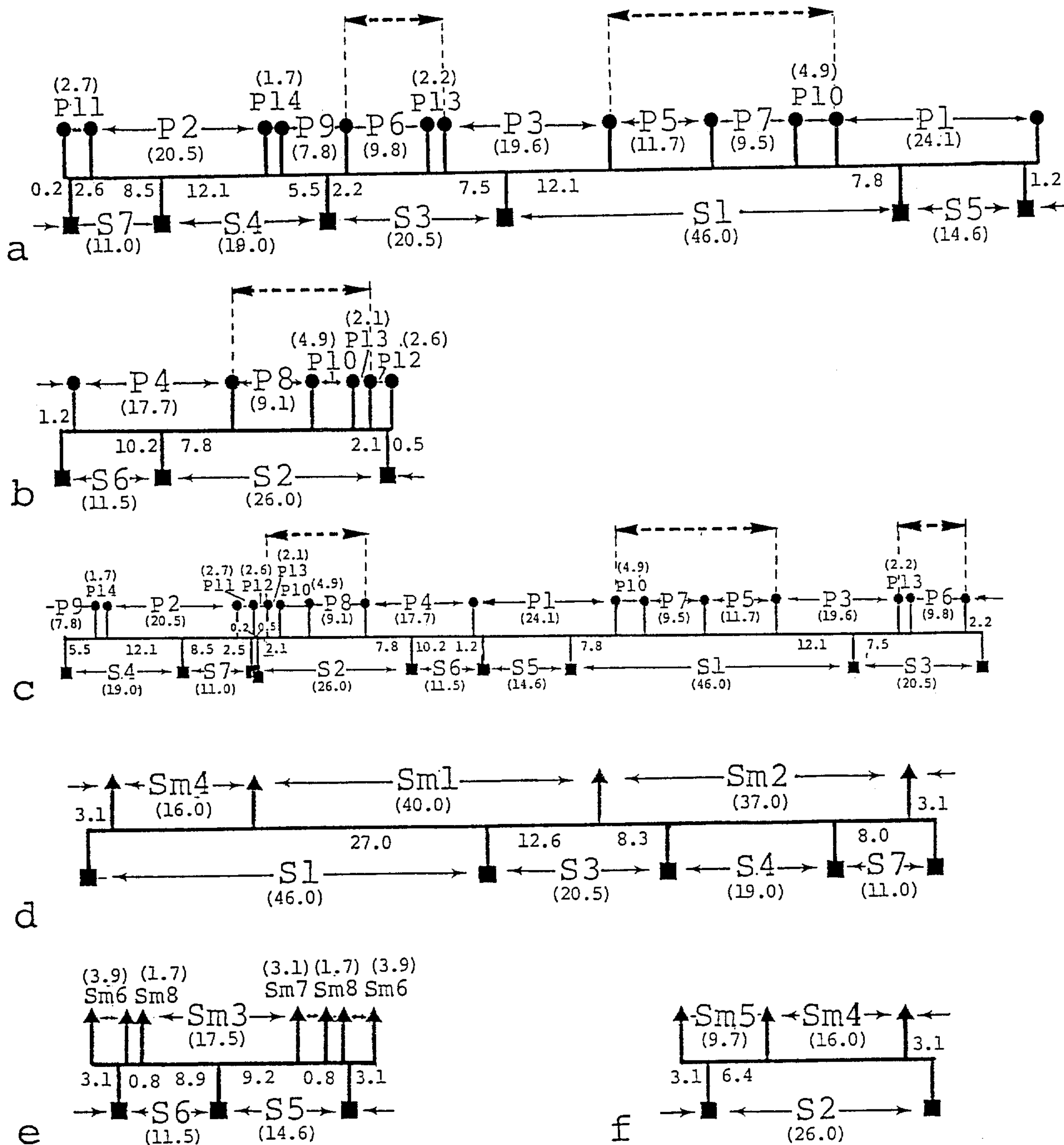

Fig. 4a–f. Partial *Pst*I/*Sal*I **a–c**, and *Sal*I/*Sma*I **d–f** site maps of *D. bulbifera* ctDNA. Numbers in parentheses indicate fragment length in kb. The scale is the same for **a**, **b**, **d**, and **e**, but reduced for **c**. The dotted lines indicate the fragments whose order could not be determined

three partial maps have a 3.1-kb SSm fragment at both ends, eight different unified maps can be constructed from these partial maps. However, only one of them is compatible with the previously constructed *Pst*I/*Sal*I map, as shown in Fig. 5.

In the *Pst*I/*Sal*I map, the order of a few *Pst*I fragments could not be determined. These are P6 and P13; P5, P7, and P10; and P8, P10, and P13. Now, their order is established as shown in Figure 5, referring to the *Sal*I/*Sma*I map, and to the results of *Pst*I/*Sma*I double digestion of the intact ctDNA, although the details of their determination procedures are not explained.

Clone bank construction and mapping of the BamHI fragments

The *Bam*HI fragments inserted into a plasmid pUC19 were compared, as regards their molecular sizes, to the

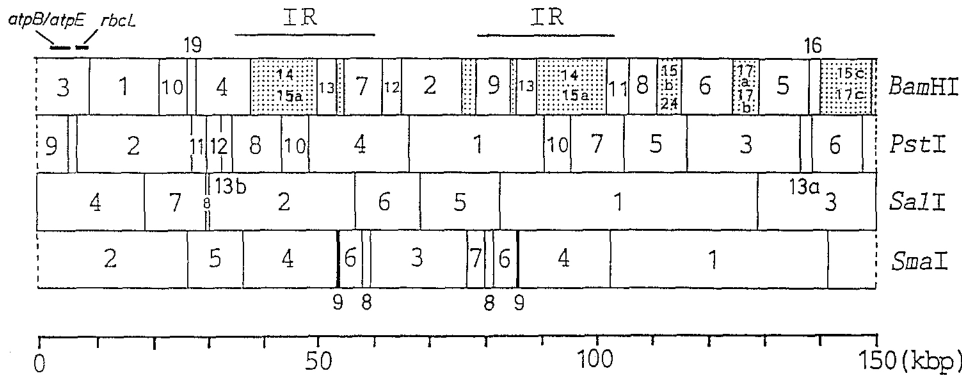

Fig. 5. An integrated physical map of *D. bulbifera* ctDNA in a linear form. *rbcL*: rubisco large subunit gene, *atpB*/*atpE*: genes for the β and ε subunits of ATP-synthase, respectively, IR: inverted repeats, shaded *Bam*HI fragment: location was not definitely determined

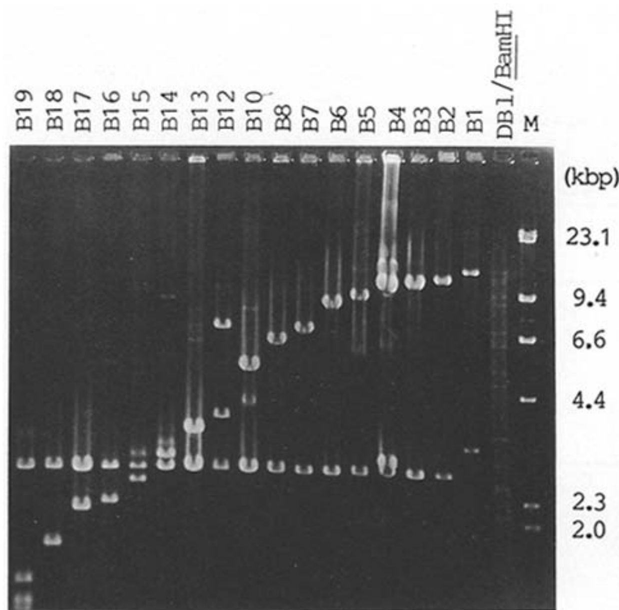

Fig. 6. Cloned *Bam*HI fragments of *D. bulbifera* ctDNA. B9 is not shown, though it has been obtained. B11 was not obtained

*Bam*HI fragments from the intact ctDNA (Fig. 6). All fragments larger than 1.0 kb, except the B11 fragment, were cloned. Molecular sizes of the subfragments generated from the individual *Bam*HI clones by *PstI*, *Sal*I, or *Sma*I digestion are given in Table 6.

Comparisons of Tables 4 and 6 revealed which *Sal*I and *Bam*HI fragments overlap each other, because of the production of common *Sal*I/*Bam*HI subfragments. The location of *Bam*HI fragments having a *Sal*I site(s) was determined by comparing the results of *Bam*HI/*Sal*I double digestion given in Tables 4 and 6. The location of *Bam*HI fragments which have no internal *Sal*I sites were determined by comparing the results of their digestion with *Pst*I or *Sma*I to the map of *Pst*I or *Sma*I sites shown in Fig. 5. Locations of five *Bam*HI fragments were fur-

Table 6. Size of subfragments generated from the *Bam*HI fragments of *D. bulbifera* ctDNA by *Pst*I, *Sal*I, or *Sma*I digestion

*Bam*HI fragment (size, kb)	*Pst*I	*Sal*I	*Sma*I
B1 (12.0)	12.0	9.3, 3.1	12.0
B2 (11.0)	10.0, 1.0	8.5, 2.8	11.0
B3 (10.8)	6.5, 2.5, 1.7	10.0, 0.6	10.8
B4 (9.5)	3.4, 2.6, 2.1, 1.7	7.5, 1.6, 0.6	8.6, 0.9
B5 (9.4)	7.6, 1.8	9.4	9.4
B6 (9.0)	8.0, 1.0	9.0	9.0
B7 (7.0)	7.0	5.2, 1.8	2.6, 2.5, 1.7
B8 (6.5)	6.5	6.5	6.5
B9 (6.0)	6.0	4.5, 1.7	2.5, 1.9, 1.7
B10 (5.2)	5.2	5.2	5.2
B12 (3.8)	3.8	3.8	3.8
B13 (3.7)	3.7	3.7	2.7, 0.6, 0.4
B14 (3.0)	3.0	3.0	3.0
B15 (2.5)	2.5	2.5	2.5
B16 (2.3)	1.9, 0.4	2.3	2.3

Table 7. Southern hybridization of five cloned *Bam*HI fragments to single or double digest of *D. bulbifera* ctDNA with *Pst*I, *Sal*I, and *Sma*I

Probe used (size, kb)	Fragment hybridized		
	*Pst*I	*Sal*I	*Sma*I
B1 (12.0)	20.5	19.0, 11.0	40.0/37.0
B5 (9.4)	19.6, 2.1	20.5	40.0/37.0
B6 (9.2)	19.6, 11.7	46.0	40.0/37.0
B8 (6.5)	11.7	46.0	40.0/37.0
B16 (2.3)	9.8, 2.1	20.5	40.0/37.0
	*Pst*I/*Sal*I	*Sal*I/*Sma*I	*Sma*I/*Pst*I
B1	–	19.0, 8.0	–
B5	–	12.6	–
B6	12.1/11.7	27.0	19.6, 11.7
B8	11.7	–	11.7
B12	–	12.6	–

/ Could not be determined to be either of the two fragments
– Not examined

ther confirmed by Southern (1975) hybridization to the total ctDNA digested either with or in combinations of *Pst*I, *Sal*I, and *Sma*I (Table 7). Nevertheless, the location of some *Bam*HI fragments included in large *Sal*I fragments, such as S1, could not be definitely determined (Fig. 5).

Location of the ribulose 1,5-bisphosphate carboxylase large subunit gene (rbcL) and ATP synthase subunits β and ε genes (atpB/atpE)

The recombinant plasmids containing wheat *rbcL* and *atpB/atpE* were Southern-blotted (1975) to *D. bulbifera* ctDNA digested with *Sal*I/*Sma*I and *Bam*HI. In this experiment, the SSm2 (19.0 kb) and B3 fragments (10.8 kb) hybridized to the plasmid DNAs. Subsequently, by hybridizing these genes onto cloned B3 fragment digested with several endonucleases, their more precise location was determined as shown in Fig. 5. The *rbcL* gene was revealed to be located inside the 2.3-kb long region where B3 and P2 fragments overlap. The *atpB/atpE* genes were found to stretch over the *Pst*I site, delimiting P9 and P14 fragments.

Discussion

The molecular size of *D. bulbifera* ctDNA was estimated to be about 152 kb, that is, slightly smaller than that (158 kb) of *Narcissus pseudonarcissus* (Thompson et al. 1981) in Liliales, the order closely related to Dioscoreales (Ayensu 1972). Similar to other angiosperm ctDNAs except those of some legumes (Palmer and Thompson 1981), the *D. bulbifera* ctDNA contains inverted repeat sequences (IRs) which divide a single copy sequence into a large and small single copy region. Presence of the IRs in *D. bulbifera* was evident from the fact that in all the *Bam*HI, *Pst*I, and *Sma*I maps, one to three fragments were found in duplicates, in the same regions of the integrated map (Fig. 5). In addition, the order of the *Bam*HI fragments was reversed in the duplicated regions. The left and right ends of the left IR (Fig. 5) should be somewhere between the left end of P8 and the left end of Sm4, and between the right end of B7 and the right end of Sm8, respectively. Therefore, the molecular size of the left IR should be somewhat between 22.4 and 27.0 kb. Similarly, the left and right ends of the right IR should be somewhere between the left end of B9 and the left end of Sm8, and between the right end of P7 and the right end of Sm4, respectively. Therefore, the molecular size of the right IR should be somewhere between 24.4 and 26.7 kb.

As to the extent of single copy regions, the left and right ends of the small single copy region were the same as the right end of the left IR and the left end of the right IR, respectively. Therefore, its size was estimated to be somewhere between 16.3 and 20.6 kb. Similarly, the left and right ends of the large single copy region were the same as the right end of the right IR and the left end of the left IR, respectively. Therefore, its size was estimated to be somewhere between 80.1 and 86.7 kb. Thus, the best estimates for the three regions are given as follows:

Inverted repeat $= 25.5 \pm 1.2$ kb
Small single copy region $= 18.5 \pm 2.2$ kb
Large single copy region $= 83.4 \pm 3.3$ kb.

As to the sizes of these regions and the location of the *rbcL* and *atpB/atpE* genes that partitions the large single copy region into two parts at an almost 1:2 ratio in their relative size, *D. bulbifera* ctDNA has similar structural characteristics to those of most other angiosperm ctDNAs.

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