Theor Appl Genet (1991) 81: 461–470

THEORETICAL AND APPLIED GENETICS

Intraspecific variation of chloroplast DNA in *Dioscorea bulbifera* L.

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Received September 11, 1990; Accepted September 19, 1990
Communicated by G. Wenzel

Summary. Restriction fragment length polymorphism (RFLP) analysis of chloroplast (ct) DNAs from 15 accessions of *Dioscorea bulbifera* collected from Africa and Asia was carried out using the Southern hybridization technique. Eight cloned ctDNA fragments of *D. bulbifera* and *D. opposita*, which cover 80% of the total chloroplast genome, were used as the probes to detect variation in ctDNA digested with nine restriction endonucleases. Ten variable sites, located in the large and small single-copy regions, were disclosed among the 15 accessions, of which six showed base substitution and four carried length mutation. Positions of the latter mutations were determined on the physical map of ctDNA. Based on these results, chloroplast genomes of the 15 accessions could be classified into nine types. Their phylogenetic relationships are assumed to be as follows: (1) African and Asian chloroplast genomes diverged from each other at the earliest point in time; (2) E-type chloroplast genome, occurring in the south-east edge of the Asian continent, appears to be the most ancient among all the Asian chloroplast genomes; and (3) four chloroplast genomes, found in Asian insular regions, are probably derived independently from the E-type genome. The discrepancy between the taxonomic relationship and the proposed chloroplast genome phylogeny of the present materials is noted.

Key words: *Dioscorea bulbifera* L. – Chloroplast DNA – RFLP analysis – Southern hybridization – Phylogeny

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Introduction

Tuber crops of the genus *Dioscorea*, collectively called yam, have contributed much to agriculture in the tropical areas of the Old World, i.e., in Southeast Asia, West Africa, and Polynesia (Coursey 1972). Especially in the latter two regions, interrelationships between man and yam have formed a particular agrocultural complex called "the yam civilization" (Miège 1954; Coursey 1972). In spite of the importance of yam in this economic and cultural context, there have been few investigations of this taxon even up to the present because of the difficulty in handling it with traditional taxonomic methods.

Aerial yam, *Dioscorea bulbifera* L., is a species belonging to the section Opsophyton Uline, with sinistrorse (left-handed) vines and alternate leaves. This species is characterized by the formation of many bulbils on the base of petioles. Differing from other cultivated *Dioscorea* species, the organ of *D. bulbifera* used as the foodstuff is in most cases the bulbil. *D. bulbifera* is the sole species of this genus that is distributed in the wild state in both Asia and Africa. Extreme polymorphism is found within the species and, as a consequence, its taxonomy is confusing. An apparent difference in the morphology of the bulbil between Asian and African forms is the globular bulbils in the former and the angular bulbils in the latter. In fact, Chevalier (1913) classified these forms into different species, i.e., the Asian form to *D. bulbifera* and the African form to *D. anthropophagorum* (after Miège 1982). On the other hand, Prain and Burkill (1936) treated them as separate varieties of the same species, leaving the African form as a single variety, *D. bulbifera* var. *anthropophagorum*, and subdividing the Asian form into nine varieties on the basis of morphological differences found in bulbils, tubers, and leaves. Chevalier (1936) described six varieties in *D. latifolia*, which is the

African form of *D. bulbifera*, in his nomenclature. Recently, Miège (1982) proposed the key to classifying *D. bulbifera* from the Ivory Coast into 11 varieties.

We are trying to clarify the phylogenetic relationships among these confusing taxa per se, and to understand the dispersal of this relic crop mediated by human migration in prehistoric times, by analyzing their chloroplast (ct) DNA variation. For this purpose, we first constructed a physical map of ctDNA of *D. bulbifera* (Terauchi et al. 1989). At the same time, a clone bank of ctDNA of this species was prepared in order to make critical and rapid assessment of the ctDNA variation in the *Dioscorea* species. In this paper, we report the intraspecific ctDNA variation disclosed among 15 *D. bulbifera* accessions from Africa, Asia, and Oceania by RFLP analysis.

Materials and methods

Plant materials

Fifteen accessions of *D. bulbifera* L. were studied (Table 1). High variability of their bulbil shape was observed (Fig. 1). They were classified into six varieties after Prain and Burkill (1936) (Table 1). The keys used for their classification and the accessions that belonged to each variety are as follows:

var. *anthropophagorum* – angular bulbils (DB1, DB3, and DB4);

var. *elongata* – elongated tubers and bulbils (DB6);

var. *heterophylla* – elongated and long cordate leaves and small nauceous bulbils (DB10, DB16, DB 17, and DB18);

var. *sativa* – edible large bulbils with smooth periderm (DB7 and DB8);

var. *suavior* – edible bulbils with warted dark periderm (DB5 and DB12);

var. *vera* – short cordate leaves and small nauceous bulbils (DB13, DB14, and DB15).

All the accessions were maintained vegetatively, using bulbils. A single clone was employed in each accession for ctDNA analysis.

Table 1. Accessions of *Dioscorea bulbifera* used in the present investigation

Accession[a]	Collection site	Cultivated or wild	Variety
DB1	Antananarivo, Madagascar	cultivated	*anthropophagorum*
DB3	Lushoto, Tanzania	cultivated	*anthropophagorum*
DB4	Jinka, Ethiopia	cultivated	*anthropophagorum*
DB5	Townsvill, Qld, Australia	wild	*suavior*
DB6	Nambayufa, Papua New Guinea	wild	*elongata*
DB7	Tonga	cultivated	*sativa*
DB8	Oahu Island, Hawaii	cultivated	*sativa*
DB10	Sandimen, Taiwan	wild	*heterophylla*
DB12	Wushe, Taiwan	cultivated	*suavior*
DB13	Lanyu Island, Taiwan	wild	*vera*
DB14	Tokushima, Japan	wild	*vera*
DB15	Oita, Japan	wild	*vera*
DB16	Cheng Mai, Thailand	wild	*heterophylla*
DB17	Cheng Rai, Thailand	wild	*heterophylla*
DB18	Cheng Sen, Thailand	wild	*heterophylla*

[a] Bulbils were supplied from the following: DB3 from H. Hawanga, Jangwani Secondary School, Dar Es Salaam, Tanzania; DB4 from M. Shigeta, Plant Germ-Plasm Institute, Kyoto University, Kyoto, Japan; DB5, DB6, and DB8 from P. Matthews, Dept. of Prehistory, ANU, Canberra, Australia (DB5 and DB6 are Australian Botanical Garden Code nos. 752178 and 8314749P, respectively); DB7 from S. Yazawa, Faculty of Agriculture, Kyoto Prefectural University, Kyoto, Japan; DB16, DB17, and DB18 from T. Ishii, Laboratory of Genetics, Faculty of Agriculture, Kyoto University, Kyoto, Japan. All the remaining are from our own collection

ctDNA extraction

Pure ctDNA could be extracted from only nine accessions (DB1, 3, 5, 6, 7, 10, 12, 13, and 15) by the method previously described (Terauchi et al. 1989). In other accessions, a high concentration of diosgenin (a kind of detergent) and phenolic compounds prevented the isolation of intact chloroplasts. For all the accessions, including those which provided pure ctDNA, crude ctDNA was isolated from the pellet of low-speed centrifugation, using a buffer specially prepared to preserve organellar structure intact; then it was purified by CsCl/ethidium bromide centrifugation.

Restriction endonuclease analysis of pure ctDNA samples

Pure ctDNAs of nine accessions were treated with a restriction endonuclease, BamHI, and electrophoresed, using 0.8% agarose slab gel. The electrophoretic patterns were analyzed after Terauchi et al. (1989).

RFLP analysis of crude ctDNA samples by Southern hybridization

Approximately 1 μg/accession of the crude ctDNA sample was digested with each of five six-base cutters, BamHI, EcoRI, EcoRV, HindIII, and XhoI, and three four-base cutters, HaeIII, MboI and MspI. Each sample was additionally double-digested with BamHI and DraI. The digested DNA was electrophoresed and bidirectionally blotted to the Hybond N membranes (Amersham) after Southern (1975). The transferred DNA was cross-linked to the membrane by UV light irradiation for a few minutes. The same membrane was used several times for the hybridization experiment, by removing the previously used probes.

Characteristics of the ctDNA probes

A ctDNA clone bank of *D. bulbifera* was previously constructed with its BamHI fragments, using the ctDNA of the accession, DB1 (Terauchi et al. 1989). From this bank, clones of the five fragments – B2 (11.0 kb), B5 (9.4 kb), B6 (9.0 kb), B8 (6.55 kb), and B16 (2.3 kb) – which showed discernible size variation among different accessions (see Results), were picked up. In addition, three clones of the SalI fragments of *D. opposita* ctDNA, namely, S2 (26.0 kb, DO no. 2), S5–6 (26.4 kb, DO no. 1), and S4–7 (30.0 kb, DO no. 3), were used as probes to detect intraspecific ctDNA variation in *D. bulbifera*. Their locations in the physical maps of the respective ctDNAs are given in Fig. 2. In fact, the physical map of the SalI sites is the same between *D. bulbifera* and *D. opposita*. Probe DO no. 1 covers the small, single-copy region entirely, probe DO no. 2 corresponds to most parts of the inverted repeat, and probe DO no. 3, together with the four probes, B5, B6, B8, and B16, covers about 70% of the large, single-copy region.

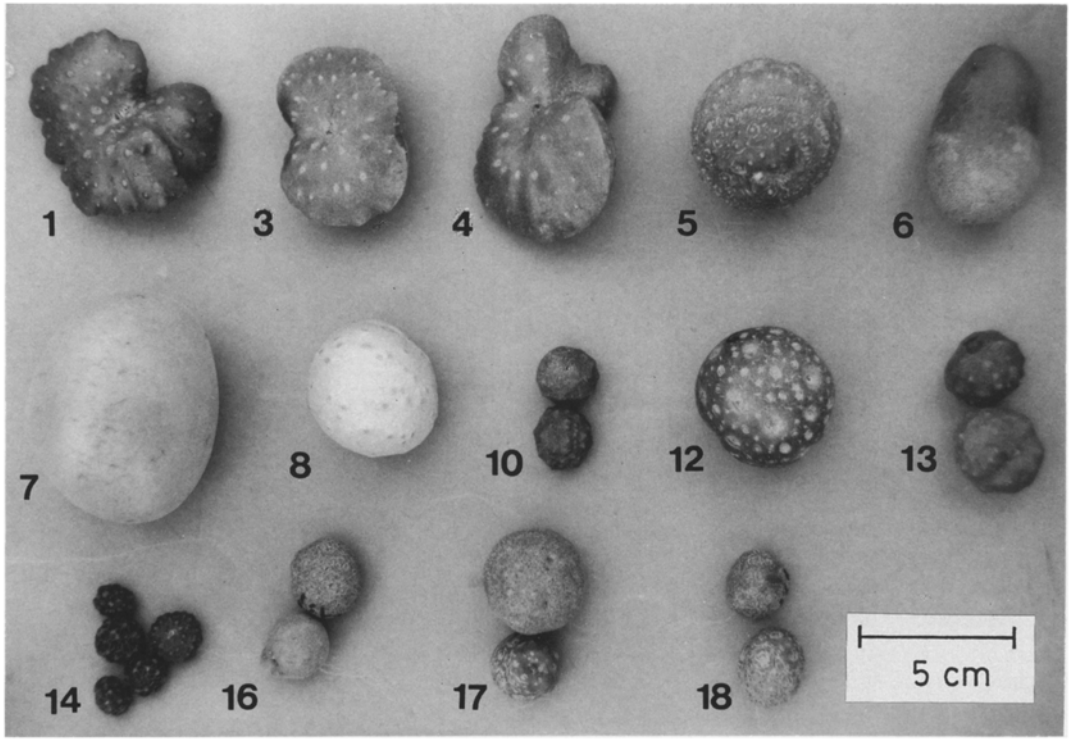

Fig. 1. Variation on bulbil shape of *D. bulbifera*. The numbers correspond to the accession numbers given in Table 1

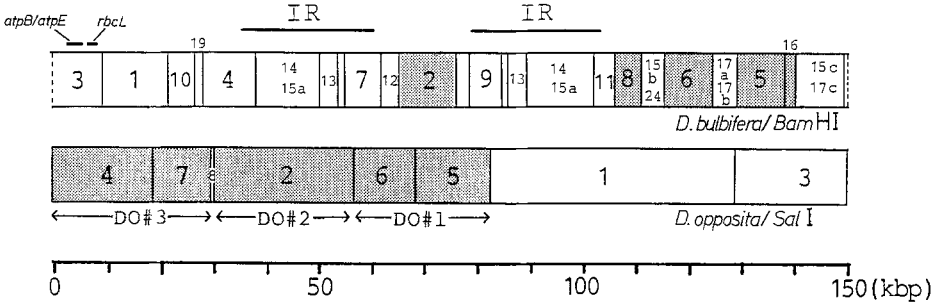

Fig. 2. The BamHI restriction map of *D. bulbifera* ctDNA (accession DB1) and the SalI map of *D. opposita* ctDNA. Hatched fragments are used as the probes for the Southern hybridization

DNA labelling and detection of hybrid bands

The ctDNA probes were chemically labelled by random-primed DNA labelling method with digoxigenin-dUTP, using the non-radioactive DNA labelling and detection kit (Boehringer Mannheim). Labelled DNA was repeatedly used to probe different membranes. Detection of the hybrid bands was made according to the supplier's instructions.

Results

Variation disclosed in the BamHI digests of pure ctDNAs

Seven different BamHI restriction fragment patterns were observed among the nine accessions, DB1, 3, 5, 6, 7, 10, 12, 13, and 15, for which pure ctDNA was available (Fig. 3). The ctDNAs that give rise to these patterns are tentatively called types I–VII (Table 2). The size variation was found in six fragments, B2, B5, B6, B8, B16, and B18 (Table 2). It could not be ascertained which BamHI fragment(s) of accessions DB5–19 (all of Asian and Oceanian origin) correspond to one of the doubled B18 fragments of DB1 and DB3 (Fig. 3 B). The locations of the B18 fragments in the ctDNA map are also not known. Southern hybridization proved that the B16 fragment (2.3 kb) of accession DB1 corresponds to the 2.0 kb BamHI fragment of the Asian and Oceanian accessions (data not shown). The B2 fragment, showing a difference only in type IV ctDNA (DB13), is located in the small, single-copy region. Four other variable fragments – B5, B6, B8, and B16 – are located in the large, single-copy region (Fig. 2).

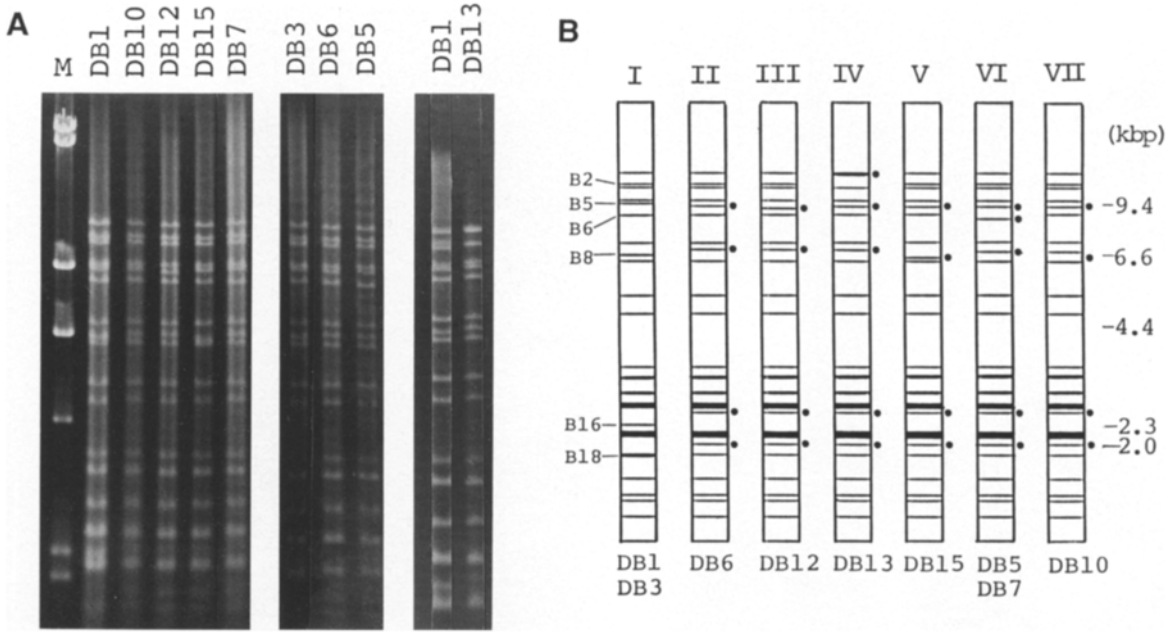

Fig. 3A and B. BamHI restriction fragment patterns of seven variant types of ctDNAs found among nine accessions of *D. bulbifera*. **A** BamHI restriction fragment patterns, **B** their schematic drawings. •: Fragments differing from the corresponding fragments of type I ctDNA

Table 2. Estimated molecular size (kb) of the five variable BamHI fragments observed among seven tentative ctDNA types of *D. bulbifera*

ctDNA type	Accession	Molecular size (kb)				
		B2	B5	B6	B8	B16
I	DB1 & DB3	11.0	9.40	9.0	6.55	2.3
II	DB6	11.0	9.30	9.0	6.65	2.0
III	DB12	11.0	9.25	9.0	6.65	2.0
IV	DB13	12.0	9.30	9.0	6.60	2.0
V	DB15	11.0	9.30	9.0	6.50	2.0
VI	DB5 & DB7	11.0	9.30	8.9	6.60	2.0
VII	DB10	11.0	9.30	9.0	6.60	2.0

Fine physical maps of variable BamHI fragments

To determine the mutations responsible for the size variations found in the B5, B6, B8, and B16 fragments, their fine physical maps were constructed using the cloned fragments of accession DB1 (Fig. 4). All four variable BamHI fragments have several DraI sites, which partition each fragment into subfragments smaller than 4.0 kb.

Characterization of the size mutations observed in the five BamHI fragments

The cloned B2 fragment of accession DB1 was Southern-hybridized as the probe to the crude ctDNAs of the 15 accessions, which were digested with BamHI. Four other cloned fragments – B5, B6, B8, and B16 – were hybridized as the probe to the crude ctDNAs, which were double-digested with BamHI and DraI, to visualize subtle length mutations within them.

B2 fragment. Southern hybridization patterns of BamHI-digested crude ctDNAs of the 15 accessions, which were probed with the B2 fragment of DB1, are classified into two types, a and b (Fig. 5). In both types, a single fragment was hybridized, whose size is 11.0 kb in type a and 12.0 kb in type b. Because no simultaneous size change was observed in the small, single-copy region of DB13 ctDNA with type b B2 fragment in the restriction fragment patterns from its digests with three other enzymes, MboI, MspI and HaeIII, the size mutation revealed in the B2 fragment of this accession is considered to be due to a base substitution in one of the BamHI sites bordering this fragment.

B5 fragment. Southern hybridization patterns of the double-digested crude ctDNAs of the 15 accessions, which were hybridized to the B5 fragment of DB1 as probe, are classified into four types, a–d (Fig. 6). Types b and c were previously grouped into the same 9.3-kb B5 fragment type (ref. Table 2). Two internal DraI-DraI subfragments and the right-end DraI-BamHI subfragment showed some difference. The type d B5 fragment had ca. 100 bp deletion in the internal DraI-DraI subfragment, as compared to all other types. In the right end of B5 fragment, a ca. 50 bp deletion, including the most right-

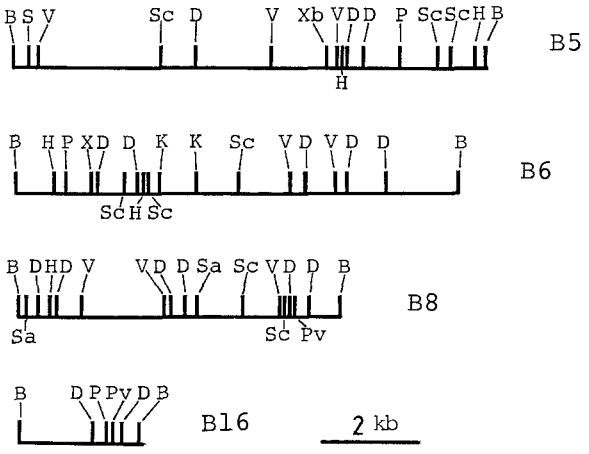

Fig. 4. Fine physical maps of the B5, B6, B8, and B16 fragments of a *D. bulbifera* accession, DB1. B, D, H, K, P, Pv, Sa, Sc, X, and Xb: BamHI, DraI, HindIII, KpnI, PstI, PvuII, SacI, ScaI, XhoI, and XbaI, respectively

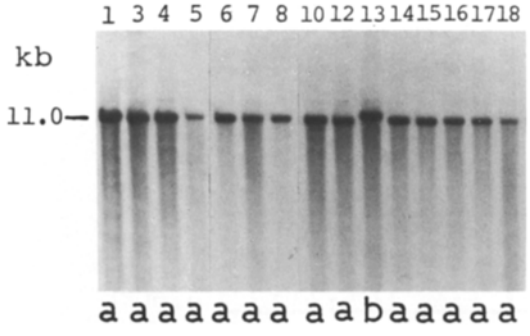

Fig. 5. Southern hybridization of the B2 probe to the BamHI-digested crude ctDNAs of 15 *D. bulbifera* accessions

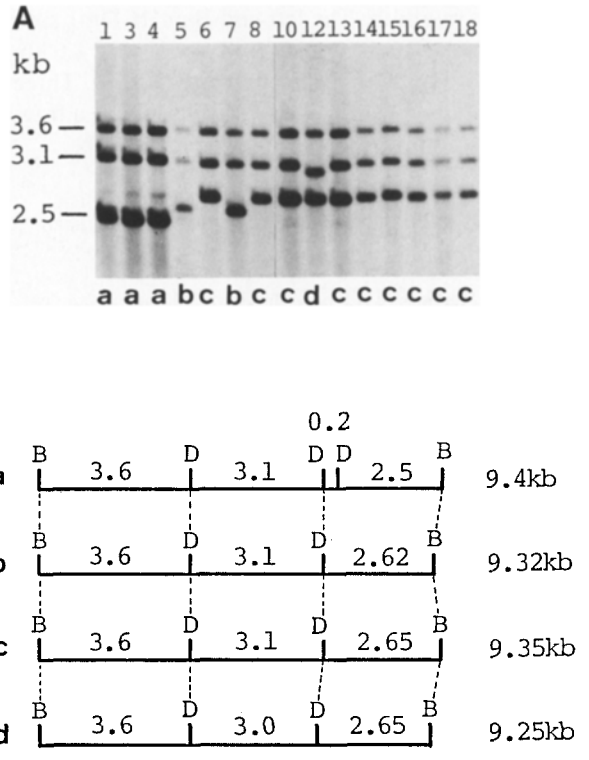

Fig. 6 A and B. Southern hybridization of the B5 probe to crude ctDNAs of 15 *D. bulbifera* accessions, which are double-digested with BamHI and DraI. **A** Southern hybridization patterns. Arabic numerals and lowercase letters indicate, respectively, the accession number and the hybridization pattern type of each sample. **B** Physical maps of the four variant B5 fragments deduced from the above patterns. B and D in the maps show the BamHI and DraI site, respectively. Size of the subfragments is given in kb

hand side DraI site in type a, was assumed for type c–d ctDNAs, which is responsible for the generation of a 2.65-kb DraI-BamHI subfragment. In this subfragment, an additional ca. 30 bp deletion was present in type b ctDNA. All African accessions possessed the type a fragment. Two accessions from Australia and Tonga (DB5 and DB7) were of type b, and one accession from Taiwan (DB12) was of type d. All the remaining accessions had the type c fragment.

B6 fragment. Southern hybridization patterns of the BamHI-DraI double-digested, crude ctDNAs of the 15 accessions, probed with the B6 fragment of DB1, are classified into two types, a and b (Fig. 7). Compared to type a, the B6 fragment of type b had ca. 100 bp deletion in the central portion, involving an internal DraI site. The type b fragment was found only in two accessions from Australia (DB5) and Tonga (DB7).

B8 fragment. Southern hybridization patterns of the BamHI-DraI double-digests and the HaeIII (a four-base cutter) digests of crude ctDNAs, probed to the B8 fragment of DB1, disclosed six variant types, a–f, of this fragment among the 15 accessions. In the BamHI-DraI patterns (Fig. 8), both the largest and second-largest DraI-DraI subfragments (2.2 and 2.05 kb, respectively, in DB1) showed variation in length. They were separated by a 0.3-kb intervening DraI-DraI subfragment. The size mutations of 50–200 bp appear to have occurred independently in these two subfragments.

The Southern hybridization patterns of the HaeIII digests (Fig. 9) are also classified into six types, a–f. Two accessions from Madagascar (DB1) and Tanzania (DB3) are of type a, one accession from Ethiopia (DB4) is of type b, seven accessions from Australia (DB5), Tonga (DB7), Taiwan (DB10 and DB13), and Thailand (DB16–18) are of type c, and four accessions from Papua New Guinea (DB6), the Hawaiian islands (DB8 and DB9), and Taiwan (DB12) are of type d. Two accessions from Japan are of type e (DB14) and type f (DB15).

B16 fragment. Southern hybridization patterns of the BamHI-DraI double-digested ctDNAs of the 15 accessions, probed with the B16 fragment of DB1 are classified

into two types, a and b. The left-end BamHI-DraI subfragment of the B16 fragment of type a was 1.6 kb, whereas that of type b was 1.3 kb in size (Fig. 4). Three African accessions (DB1, 3, and 4) possessed type a fragment, whereas all other accessions were of type b.

Variation of D. bulbifera ctDNA detected by Southern hybridization using heterologous probes

Three ctDNA clones, DO nos. 1–3, of *D. opposita* (ref. Fig. 2) were used to probe the crude ctDNAs of the 15 *D. bulbifera* accessions, which were treated with the following endonucleases; EcoRI, EcoRV, HaeIII, HindIII, MboI, MspI, and XhoI. The hybridization of MboI and MspI digests to probe Do no. 2 failed. Among the 19 successful hybridization experiments, five revealed ctDNA variation among the accessions tested. These are DO no. 1-HaeIII, DO no. 1-MboI, DO no. 1-MspI, DO no. 3-HaeIII, and DO no. 3-MspI combinations.

In the DO no. 1-MspI patterns (Fig. 10), a 3.9-kb fragment found in type a is split into two fragments of 2.2 and 1.7 kb in type b that is present in two accessions,

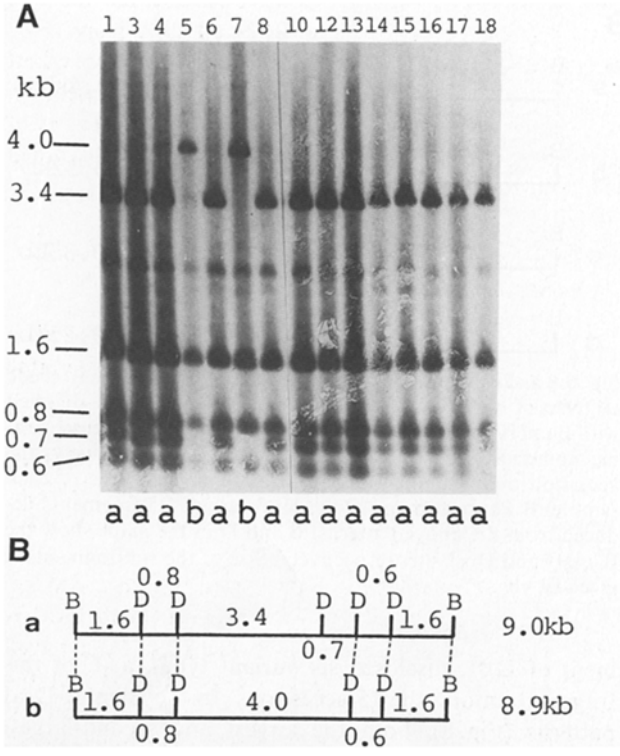

Fig. 7 A and B. Southern hybridization of the B6 probe to crude ctDNAs of 15 *D. bulbifera* accessions, which are double-digested with BamHI and DraI. **A** Southern hybridization patterns. **B** Physical maps of the two variant fragments, a and b. For the symbols used, refer to Fig. 6

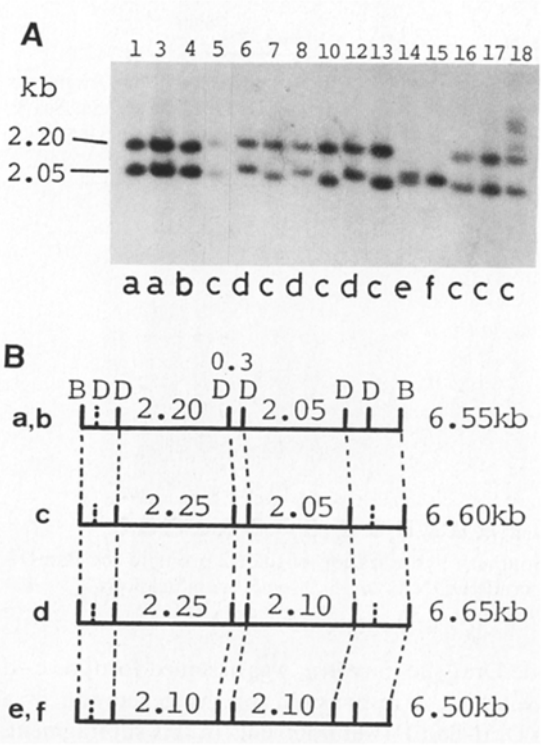

Fig. 8 A and B. Southern hybridization of the B8 probe to crude ctDNAs of 15 *D. bulbifera* accessions, which are double-digested with BamHI and DraI. **A** Southern hybridization patterns. **B** Physical maps of the six variant fragments, a–f. For the symbols used, refer to Fig. 6

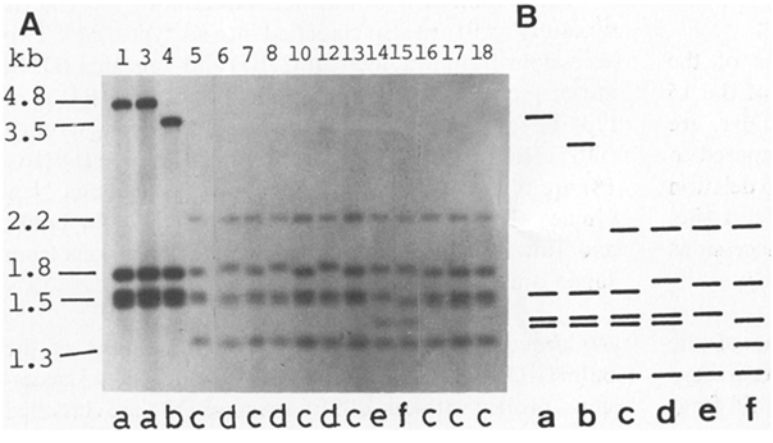

Fig. 9 A and B. Southern hybridization of the B8 probe to crude ctDNAs of 15 *D. bulbifera* accessions, which are double-digested with HaeIII. **A** Southern hybridization pattern, **B** their schematic drawings. For the symbols used, refer to Fig. 6

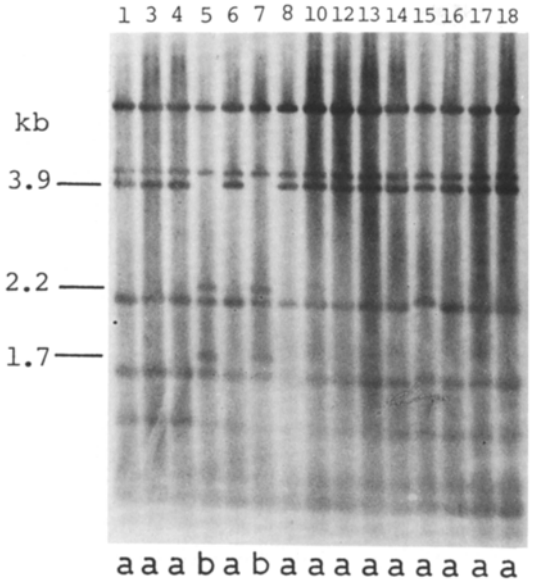

Fig. 10. Southern hybridization of the DO no. 1 probe to crude ctDNAs of 15 *D. bulbifera* accessions, which are digested with MspI. For the symbols used, refer to Fig. 6

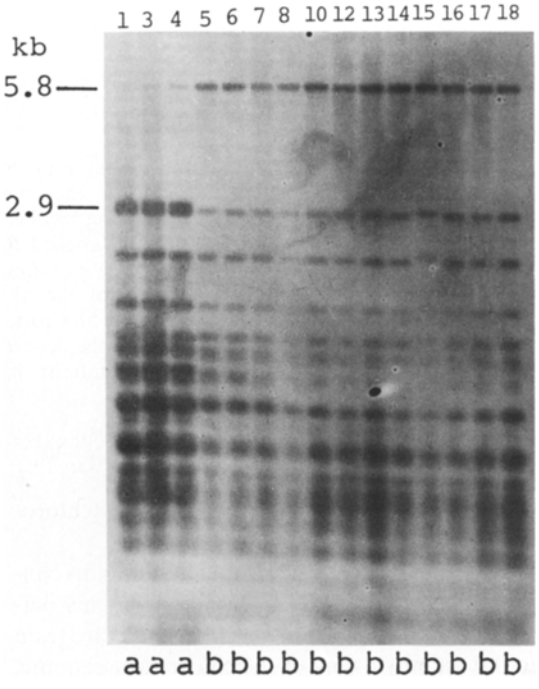

Fig. 11. Southern hybridization of the DO no. 3 probe to crude ctDNAs of 15 *D. bulbifera* accessions, which are digested with MspI. For the symbols used, refer to Fig. 6

DB5 and DB7. In the DO no. 3-MspI patterns (Fig. 11), two 2.9-kb fragments in type a pattern (DB1, DB3, and DB4) form a single 5.8-kb fragment in type b by the loss of a MspI site.

Three other probe-enzyme combinations each also revealed two variant types, a and b (data not shown). In all cases, the difference between types a and b could be ascribed to a unique base substitution, because other enzyme digests hybridized to the same probe did not reveal any simultaneous size changes in the same accession groups.

Discussion

Classification of the ctDNA types

By Southern hybridization using five homologous and three heterologous ctDNA clones as probe, ten variable sites have been identified among the ctDNAs from 15 *D. bulbifera* accessions. As stated above, two to six variant types are found in each site, which are collectively shown in Table 3. From this result, the chloroplast genomes of 15 accessions are classified into nine types, A-I, as shown in the last column of Table 3.

Minimum mutational steps required to give rise to the nine chloroplast genomes

The minimum number of mutations is inferred for each of ten variable sites of *D. bulbifera* ctDNA. They are one for all variable sites found in three BamHI fragments, B2, B6, and B16, and those disclosed by the five probe-enzyme combinations, DO no. 1-HaeIII, DO no. 1-MboI, DO no. 1-MspI, DO no. 3-HaeIII, and DO no. 3-MspI, because only two variant forms, types a and b, were detected in all of them.

The B5 and B8 fragments contained four and six variant types, respectively. By consulting with the hybridization profiles given in Figs. 6, 8, and 9, the least number of mutations existing between all pairs of the variant types was inferred (Table 4). Because the mutations observed in ten variable sites are all independent from each other, the least number of mutations detected between every pair of the nine chloroplast genomes is given by the sum of the least numbers of mutations revealed in the individual variable sites (Table 5).

Phylogenetic relationship between the nine chloroplast genomes of D. bulbifera

Using a computer program for the unweighted pair-group mean (UPGM) method of clustering (Sneath and Sokal 1973), a dendrogram showing the phylogenetic relationship between the nine chloroplast genomes was constructed (Fig. 12), based on the data presented in Table 5.

One of the two primary clusters consists of three African accessions having the chloroplast genomes, A and B. The other cluster is formed by 12 accessions from Asia, Polynesia, and Australia, having the C to I chloroplast genomes. In this cluster, three subclusters are recog-

Table 3. Variant fragment types revealed among ctDNAs of 15 *D. bulbifera* accessions by their Southern hybridization with ten probe-enzyme combinations

Accession	Probe-enzyme combination[a]										Chloroplast genome
	B2 Bm	B5 B+D	B6 B+D	B8 B+D	B16 B+D	DO no.1 Ha	DO no.1 Mb	DO no.1 Ms	DO no.3 Ha	DO no.3 Ms	
DB1	a	a	a	a	a	a	a	a	a	a	A
DB3	a	a	a	a	a	a	a	a	a	a	A
DB4	a	a	a	b	a	a	a	a	a	a	B
DB5	a	b	b	c	b	b	a	b	b	b	C
DB6	a	c	a	d	b	b	b	a	b	b	D
DB7	a	b	b	c	b	b	a	b	b	b	C
DB8	a	c	a	d	b	b	b	a	b	b	D
DB10	a	c	a	c	b	b	a	a	b	b	E
DB12	a	d	a	d	b	b	b	a	b	b	F
DB13	b	c	a	c	b	b	a	a	b	b	G
DB14	a	c	a	e	b	b	a	a	b	b	H
DB15	a	c	a	f	b	b	a	a	b	b	I
DB16	a	c	a	c	b	b	a	a	b	b	E
DB17	a	c	a	c	b	b	a	a	b	b	E
DB18	a	c	a	c	b	b	a	a	b	b	E

[a] Probe-enzyme combination refers to the DNA clone used as probe and restriction endonuclease used for ctDNA digestion in Southern hybridization. Bm, Ha, Mb, and Ms indicate BamHI, HaeIII, MboI, and MspI, respectively. B+D shows double-digestion with BamHI and DraI

Table 4. The least numbers of mutations estimated between all pairs of the variant types of the B5 and B8 fragments of *D. bulbifera* ctDNA

B5 fragment				B8 fragment					
Variant type	a	b	c	Variant type	a	b	c	d	e
b	1			b	1				
c	1	1		c	1	1			
d	2	2	1	d	2	2	1		
				e	3	3	2	2	
				f	3	3	2	2	1

Table 5. The least numbers of mutations estimated between all pairs of the nine chloroplast genomes of *D. bulbifera*

Chloroplast genome	A	B	C	D	E	F	G	H
B	1							
C	8	8						
D	8	8	5					
E	6	6	3	2				
F	9	9	6	1	3			
G	7	7	4	3	1	4		
H	8	8	5	3	2	4	3	
I	8	8	5	3	2	4	3	1

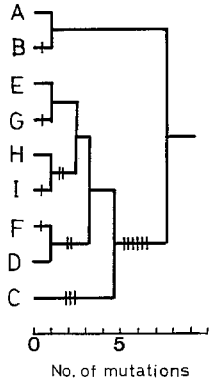

Fig. 12. A dendrogram showing a phylogenetic relationship among the nine chloroplast genomes. Clustering has been made by the UPGM method applied for the minimum numbers of the mutations estimated between all pairs of the nine chloroplast genomes (ref. Table 5). Crossed bars drawn in each branch of the dendrogram show the number of mutations that occurred on it

nized: one consisting of two accessions from Australia and Tonga (chloroplast genome C); the second containing three accessions from widely separated locations, namely, Taiwan, Papua New Ginea, and Hawaii (chloroplast genome D and F); and the third involving seven accessions from Thailand, Taiwan, and Japan (chloroplast genomes E, G, H, and I).

A single parsimonious network that assumes no convergent mutations was hand-drawn (Fig. 13). This network is more informative than the UPGM dendrogram in considering the phylogeny of the chloroplast genome, which does not undergo recombination, as demonstrated in the same type of study on animal mitochondrial genomes (Avise et al. 1987). The following three conclusions are drawn from Fig. 13, as to chloroplast genome differentiation in *D. bulbifera*: (1) African and Asian (including Oceanian and Polynesian) groups of the accessions were differentiated from each other at the earliest point in time; (2) among seven chloroplast genomes of the latter group, genome E appears to be the ancestral form of all other types; and (3) four subclusters of the genomes found in Asia, i.e., genome C, genomes D and

F, genome G, and genomes H and I, are assumed to have been derived independently from genome E. The first conclusion is in agreement with the suggestion by Burkill (1960) that African and Asian *D. bulbifera* were separated from each other in the beginning of the Tertiary Pliocene (about 10 million years ago) by the desiccation of southwestern Asia.

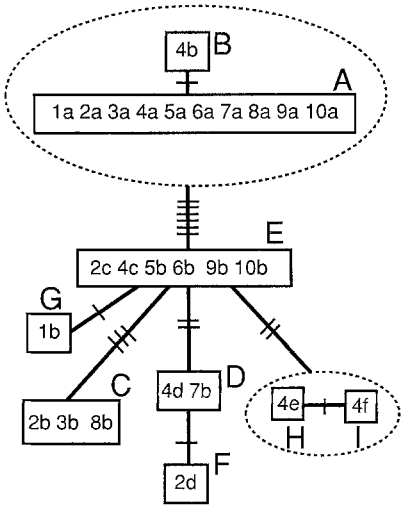

Fig. 13. A parsimonious network, summarizing phylogenetic relationships among the nine chloroplast genomes of *D. bulbifera*. Genome A is regarded as the standard type. Numbers 1–10 indicate ten variable sites as follows; 1=B2, 2=B5, 3=B6, 4=B8, 5=B16, 6=DO no. 1/HaeIII, 7=DO no. 1/MboI, 8=DO no. 1/MspI, 9=DO no. 3/HaeIII, and 10=DO no. 3/MspI. Number of bars drawn on each branch indicates the number of mutations assumed to have occurred in it

Geographical distribution of different chloroplast genomes in D. bulbifera

The geographical distribution of the nine chloroplast genomes is depicted in Fig. 14, together with their inferred evolutionary course. Genome A is distributed in Tanzania and Madagascar, and genome B in Ethiopia. Genome E, from which all Asian genomes are assumed to have been derived, is found in the southeast edge of the Asian continent. Two genomes, C and D, are distributed in Melanesia and Polynesia. It is interesting that genome F, derived by a single mutation from genome D, is found in Taiwan. Because the accession DB12 having genome F is a cultivated form, it seems to have been introduced to Taiwan from a southern Pacific island with past human immigration.

Need for reexamination of varietal classification

Table 6 gives the chloroplast genomes observed in each of the six varieties. Two accessions of *D. bulbifera* var. *suavior* have genomes C and F, which differ by six mutations from each other. Two accessions of *D. bulbifera* var. *sativa* also differ by five mutations because they have C and D genomes, respectively. Intravarietal differentiation of the chloroplast genome in these two varieties is extremely large for them to be treated as a single variety,

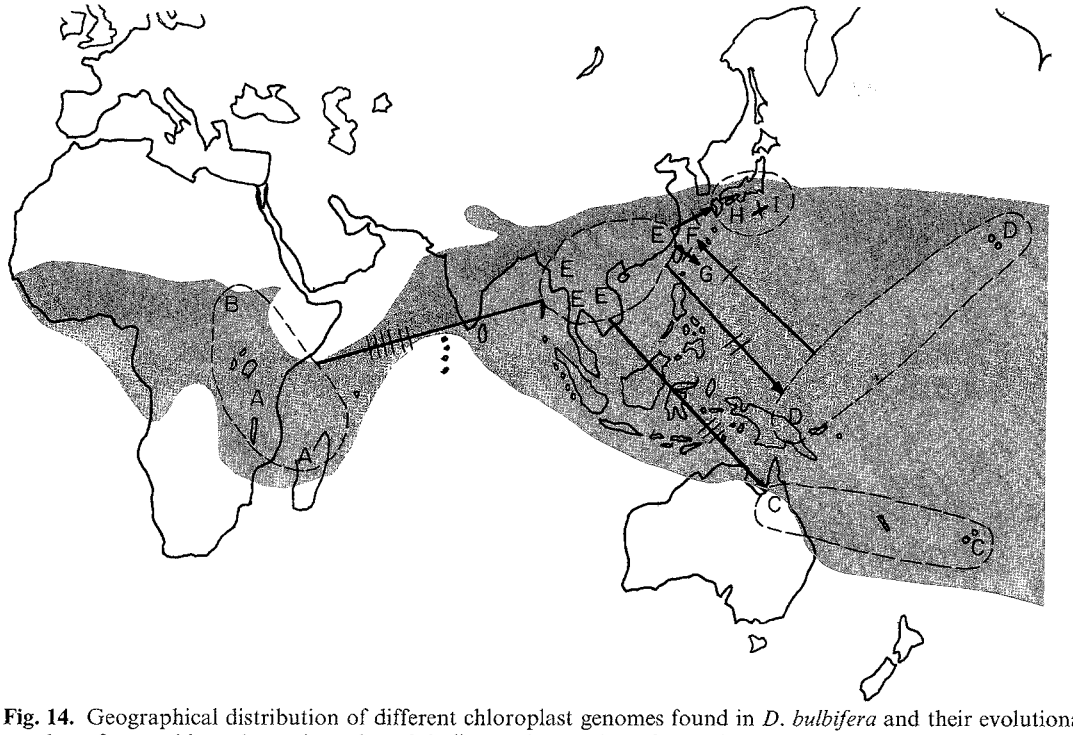

Fig. 14. Geographical distribution of different chloroplast genomes found in *D. bulbifera* and their evolutionary relationships. The number of crossed bars drawn in each path indicates the number of mutations assumed to have occurred in it. Shaded area indicates distribution of *D. bulbifera*

Table 6. Number of accessions having different chloroplast genomes, which are found in six varieties of *D. bulbifera*

Variety	No. accessions	Chloroplast genome								
		A	B	C	D	E	F	G	H	I
Anthropophagorum	3	2	1							
Suavior	2			1			1			
Elongata	1				1					
Sativa	2			1	1					
Heterophylla	3					3				
Vera	4					1		1	1	1
Total	15	2	1	2	2	4	1	1	1	1

when compared to three other varieties, *anthropophagorum, heterophylla*, and *vera*, in which three or four accessions belonging to each show zero, one, and three mutations, respectively, at most. This fact requires reexamination of the varietal classification in this species. Two accessions of *D. bulbifera* var. *sativa* are likely to have different origins. DB7 from Tonga and DB8 from Hawaii of *D. bulbifera* var. *sativa* are closely related to DB5 of *D. bulbifera* var. *suavior* from Australia and DB6 of *D. bulbifera* var. *elongata* from Papua New Guinea, respectively.

Acknowledgements. Contribution from the Plant Germ-Plasm Institute and the Laboratory of Genetics (No. 511), Faculty of Agriculture, Kyoto University, Japan. The work has been supported in part by a Grant-in-Aid (No. 01300012) from the Ministry of Education, Science, and Culture, Japan. The manuscript was improved after kind suggestions by Dr. M. Raj, IITA, Ibadan, Nigeria, to whom we would like to express our heartful appreciation.

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