Mitochondrial DNA Phylogeny of Cultivated and Wild Beets: Relationships Among Cytoplasmic Male-Sterility-Inducing and Nonsterilizing Cytoplasms

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ABSTRACT

Cytoplasmic male sterility (CMS), the maternally inherited failure to produce functional pollen, has been used in the breeding of sugar beet (*Beta vulgaris* ssp. *vulgaris*). At least three different sources of CMS can be distinguished from one another as well as from normal fertile cytoplasm by polymorphisms in their mitochondrial genomes. Here we analyzed 50 accessions of cultivated and wild beets to investigate the phylogenetic relationships among male-sterility-inducing and normal cytoplasms. The haplotypes were characterized by the nucleotide sequence of the mitochondrial *cox2-cox1* spacer region and mitochondrial minisatellite loci. The results indicated that (1) a normal cytoplasm line, cv. TK81-O, was situated at the major core node of the haplotype network, and (2) the three sterilizing cytoplasms in question derived independently from the core haplotype. The evolutionary pathway was investigated by physical mapping study of the mitochondrial genome of a wild beet (*B. vulgaris* ssp. *orientalis*) accession BGRC56777 which shared the same mitochondrial haplotype with TK81-O, but was not identical to TK81-O for the RFLP profiles of mitochondrial DNA. Interestingly, three sets of inverted repeated sequences appeared to have been involved in a series of recombination events during the course of evolution between the BGRC56777 and the TK81-O mitochondrial genomes.

THE involvement of cytoplasmic factors has been reported in >150 plant species in which male sterility occurs (LASER and LERSTEN 1972). This type of male sterility, called cytoplasmic male sterility (CMS), is usually attributed to chimeric ORFs in the mitochondrial genome (CHASE 2007). In some plant species, multiple forms of CMS are found (FAURON and CASPER 1994; SCHNABLE and WISE 1998). These forms can be distinguished by their unusual associated mitochondrial ORFs and by their response to nuclear-encoded, restorer-of-fertility (*Rf*) genes that suppress the male-sterile phenotype.

Several different sources of male-sterile cytoplasms have been described in cultivated beets (*Beta vulgaris* ssp. *vulgaris*) and their wild progenitor, all of which are assigned into the section Beta (syn. *Vulgares*) of the genus Beta (MIKAMI *et al.* 1985; SAUMITOU-LAPRADE *et al.* 1993). The first identified was the Owen cytoplasm, discovered in the cultivar "US-1" (OWEN 1945). Up to now hybrid seed production in the sugar beet has exclusively relied on Owen CMS (BOSEMARK 1993).

We have recently found that a mitochondrial 35-kDa protein is most likely responsible for Owen CMS (SATOH *et al.* 2004; YAMAMOTO *et al.* 2005). The protein proved to be the product of a 387-codon mitochondrial ORF (designated *preSatp6*), which is fused in frame with the downstream *atp6* ORF (YAMAMOTO *et al.* 2005). Interestingly, this protein was not detectable in the anther tissue of two different sources of CMS [I-12CMS(2) and I-12CMS (3)] from wild Beta beets collected in Turkey and Pakistan; instead, a CMS-associated protein of 12 kDa was identified (HALLDEN *et al.* 1992; M. P. YAMAMOTO, Y. ONODERA, T. KUBO and T. MIKAMI, unpublished results). These three male-sterile cytoplasms showed different patterns of male-sterility maintenance and male-fertility restoration when crossed with the same pollen parents (T. KUBO, T. KINOSHITA and T. MIKAMI, unpublished data). Moreover, DUCOS *et al.* (2001) reported that male sterility caused by the G cytoplasm of wild Beta beets was associated with a mitochondrial *cox2* gene lacking eight highly conserved, C-terminal amino acids. Beta beets thus appear to maintain several distinct CMS cytoplasms, each capable of conferring male sterility by an apparently different mechanism.

In this article, we attempted to obtain information on the evolutionary relationships among these male-sterility-inducing and nonsterilizing, normal cytoplasms. For this purpose, we determined the nucleotide sequence of the *cox2-cox1* intergenic spacer region from a number of

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TABLE 1

Summary of haplotypes defined by the *cox2-cox1* intergenic spacer and minisatellite loci in the mitochondrial genomes of cultivated and wild beets

Species	Accession	Sequence type	Indel type	Minisatellite	Chondriome group	Origin[a]	Source[b]
			Section Beta (syn. *Vulgares*)				
B. vulgaris ssp. *vulgaris*	TK81-O	seq01	ind05	min19	A	Japanese cultivar	NARCH
B. vulgaris ssp. *vulgaris*	TK81-MS	seq07	ind02	min04	B	Japanese cultivar	NARCH
B. vulgaris ssp. *vulgaris*	I-12CMS(2)	seq08	ind05	min06	A	Turkey	GW
B. vulgaris ssp. *vulgaris*	I-12CMS(3)	seq09	ind05	min06	A	Pakistan	GW
B. vulgaris ssp. *vulgaris*	BGRC58265-MS	seq05	ind02	min04	B	Turkey	cpro-dlo
B. vulgaris ssp. *vulgaris*	BGRC58265-MF	seq01	ind05	min11	A	Turkey	cpro-dlo
B. vulgaris ssp. *vulgaris*	BGRC56743	seq14	ind03	min17	F	Greece	FAL
B. vulgaris ssp. *vulgaris*	BETA98/80	seq10	ind05	min16	A	—	IPK
B. vulgaris ssp. *vulgaris*	BETA78/90	seq02	ind05	min06	A	—	IPK
B. vulgaris ssp. *vulgaris*	BETA77/86	seq01	ind05	ND	A	—	IPK
B. vulgaris ssp. *vulgaris*	BETA35/85	seq01	ind05	min15	A	Tunisia	IPK
B. vulgaris ssp. *vulgaris*	BETA31/81	seq05	ind02	min04	B	—	IPK
B. vulgaris ssp. *vulgaris*	BETA29/82	seq01	ind05	ND	A	—	IPK
B. vulgaris ssp. *vulgaris*	BETA192/92	seq01	ind05	ND	A	—	IPK
B. vulgaris ssp. *vulgaris*	BETA184/82	seq01	ind05	ND	A	USSR	IPK
B. vulgaris ssp. *vulgaris*	BETA179/73	seq01	ind05	ND	A	China	IPK
B. vulgaris ssp. *vulgaris*	753994	seq01	ind05	ND	A	—	IPK
B. vulgaris ssp. *vulgaris*	753962	seq13	ind02	min04	B	—	IPK
B. vulgaris ssp. *vulgaris*	753932	seq12	ind05	min06	A	—	IPK
B. vulgaris ssp. *vulgaris*	753931	seq11	ind05	min10	A	—	IPK
B. vulgaris ssp. *orientalis*	BGRC56777	seq01	ind05	min06	A	—	FAL
B. vulgaris ssp. *maritima*	PI546530-337	seq04	ind05	min12	A	Italy	USDA
B. vulgaris ssp. *maritima*	PI518369-311	seq01	ind05	min10	A	England	USDA
B. vulgaris ssp. *maritima*	PI504186-119	seq22	ind05	min06	A	Italy	USDA
B. vulgaris ssp. *maritima*	FR4-31	seq06	ind05	min01	C	France	USDA
B. vulgaris ssp. *maritima*	BGRC57715-MS	seq24	ind05	min06	A	Portugal	cpro-dlo
B. vulgaris ssp. *maritima*	BGRC57713-MS	seq02	ind05	min06	A	Portugal	cpro-dlo
B. vulgaris ssp. *maritima*	BGRC57712	seq21	ind05	min13	A	Portugal	FAL
B. vulgaris ssp. *maritima*	BGRC57705-MS	seq03	ind05	min12	A	—	cpro-dlo
B. vulgaris ssp. *maritima*	BGRC57705-MF	seq23	ind05	min12	A	—	cpro-dlo
B. vulgaris ssp. *maritima*	BGRC56773	seq01	ind05	min10	A	Italy	FAL
B. vulgaris ssp. *maritima*	BGRC54842	seq20	ind04	min18	D	Portugal	FAL
B. vulgaris ssp. *maritima*	BGRC54841	seq01	ind05	min10	A	Ireland	FAL
B. vulgaris ssp. *maritima*	BGRC54772	seq19	ind05	min07	A	Spain	FAL
B. vulgaris ssp. *maritima*	BGRC48810-MS	seq03	ind05	min14	A	Tunisia	cpro-dlo
B. vulgaris ssp. *maritima*	BGRC48810-MF	seq03	ind05	min14	A	Tunisia	cpro-dlo
B. vulgaris ssp. *maritima*	BGRC36478	seq18	ind05	min07	A	Greece	FAL
B. vulgaris ssp. *maritima*	BGRC35289	seq01	ind05	ND	A	Turkey	FAL
B. vulgaris ssp. *maritima*	BGRC32396	seq01	ind05	min10	A	Greece	FAL
B. vulgaris ssp. *maritima*	BGRC28901	seq05	ind02	min04	B	Italy	FAL
B. vulgaris ssp. *maritima*	BETA280/91	seq01	ind05	min19	A	—	IPK
B. vulgaris ssp. *maritima*	BETA156/86	seq17	ind05	min12	A	Belgium	IPK
B. vulgaris ssp. *maritima*	BETA140/83	seq16	ind05	min02	A	—	IPK
B. vulgaris ssp. *maritima*	BETA(29)18/82	seq01	ind05	ND	A	—	IPK
B. vulgaris ssp. *maritima*	BETA(29)17/85	seq15	ind05	min10	A	France	IPK
B. vulgaris ssp. *maritima*	AM4247-295	seq01	ind05	min10	A	Spain	USDA
B. vulgaris ssp. *maritima*	AM4223-230	seq01	ind05	ND	A	Greece	USDA
B. vulgaris ssp. *macrocarpa*	Canary Island	seq25	ind05	min05	A	Canary Islands	NARCH
B. vulgaris ssp. *adanensis*	Egypt	seq04	ind05	min09	A	Egypt	NARCH
			Section *Corollinae*				
B. trigyna	WB47	seq26	ind01	min03	F	—	NARCH

Dashes indicate unknown

[a] Original place of collection.

[b] The places from which we obtained seed samples: NARCH, National Agricultural Research Center for Hokkaido Region, Japan; cpro-dlo, Centre for Plant Breeding and Reproduction Research; FAL, BA Zuchtungsforschung Genbank; USDA, USDA Agriculture Research Service, U. S. Agricultural Research Station; GW, The Great Western Sugar Company; IPK, Institüt für Pflanzengenetik und Kulturpflanzenforschung Gatersleben.

cultivated and wild beets as well as CMS accession lines. In the sugar beet mitochondrial genome, the *cox2* gene was shown to reside 1498 bp upstream of *cox1* and no genic sequences (ORFs) were found within the intergenic spacer (KUBO *et al.* 2000; SATOH *et al.* 2004). This linkage proved to be well conserved in the mitochondrial genomes of Beta species belonging to sections Beta and *Corollinae* (SENDA *et al.* 1995), making the *cox2-cox1* spacer a likely site for an approximation of the neutral point-mutation rate. Furthermore, we previously identified variable number tandem repeats (VNTRs) in the mitochondrial genomes of Beta beets (NISHIZAWA *et al.* 2000). Here the VNTRs were also used to evaluate relationships among the mitochondrial genomes of Beta beets. During these analyses and the mitochondrial DNA (mtDNA) RFLP assay, we became aware of the existence of the mitochondrial genome (*B. vulgaris* ssp. *orientalis* acc. BGRC56777), which resembled that of the normal cytoplasm more than any other characterized mtDNAs. We present the physical map of this genome and a detailed comparison with the normal and Owen mtDNA physical maps.

MATERIALS AND METHODS

Plant materials and nucleic acids isolation: Fifty accessions used in this study are listed in Table 1. TK81-MS and TK81-O are Owen CMS and its maintainer (known in sugar-beet terminology as type-O, see BOSEMARK 1993) sugar-beet lines, respectively. They are near isogenic except for cytotype (male sterile *vs.* male fertile) and their mitochondrial genomes were completely sequenced in our laboratory (KUBO *et al.* 2000; SATOH *et al.* 2004). I-12CMS(2) and I-12CMS(3) possess the male-sterile cytoplasms derived from wild beets collected in Turkey and Pakistan, respectively (MIKAMI *et al.* 1985). Total cellular DNA was isolated according to DOYLE and DOYLE (1990). MtDNA was isolated according to MIKAMI *et al.* (1985).

PCR and sequencing: The primers used to amplify the *cox2-cox1* intergenic spacer are 5′-AAGAAGCTTCGCTCGCTCG CTCTAACGC-3′ and 5′-CTGGATCCGCTGATAGATCTGCGC CTCG-3′ (*Hin*dIII and *Bam*HI sites were introduced to facilitate cloning and are underlined). The sequences correspond to nucleotide 237,196–237,223 and 237,966–237,993 in TK81-O mtDNA (BA000009), respectively. The sequences of four mitochondrial tandem repeat loci (TR1, TR2, TR3, and TR4) were amplified using primers described in (NISHIZAWA *et al.* 2000). Primer sets for the amplification of *B. vulgaris* ssp. *orientalis* acc. BGRC56777 mtDNA regions were: 5′-CCTCAG ATCTATGACCTCCC-3′ and 5′-CCGCAAACGAAGGCTAAC CC-3′ for region 1, 5′-AGTTCTTAAAGTTCCATGCG-3′ and 5′-TAAACAACCCTTTCTCTCAC-3′ for region 3, 5′-TTCGA GAGGCGAAGAATAGC-3′ and 5′-GTACTGAGTTTCCTCCT CCC-3′ for region 4, 5′-TTCGAATCCCTCTCTTTCCGTA-3′ and 5′-TGGTACAGTAGCATCCTGCC-3′ for region 5, and 5′-TCAAAACCTTTGCGCTTCAG-3′ and 5′-GGATTAGCCGA TCGCTTTCG-3′ for region 6. PCR was done according to McPHERSON *et al.* (1991). The PCR products were sequenced either directly or after cloning into the pBluescript vector (Stratagene, La Jolla, CA). The lambda clone was sequenced by shotgun-sequencing strategy (KUBO *et al.* 2000). The sequence was determined on both strands using ABI377 (Applied Biosystems, Foster City, CA) or Li-COR4000L (Li-COR, Lincoln, NE) DNA sequencers.
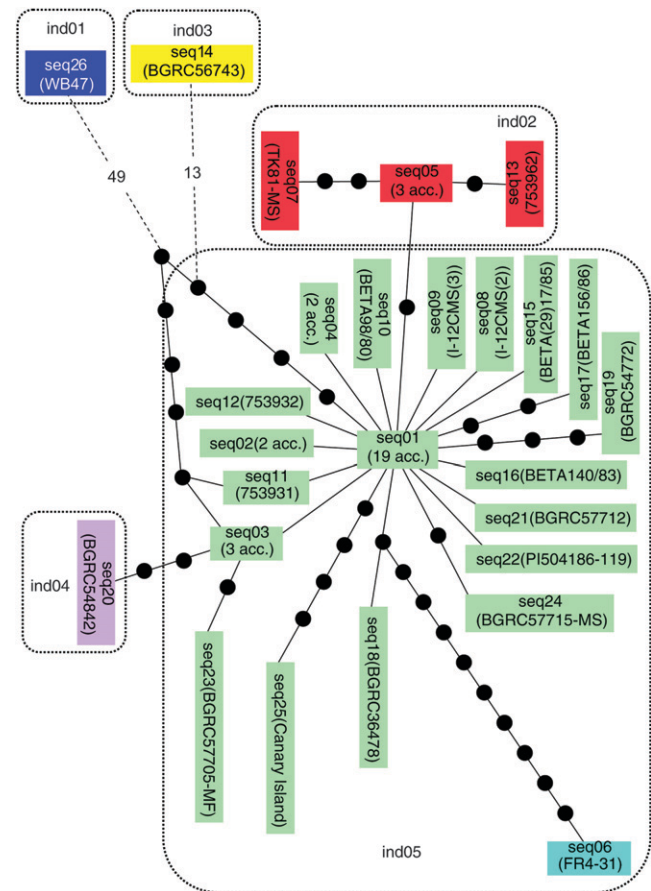

FIGURE 1.—The minimum spanning network relating 26 haplotypes defined on the basis of mitochondrial *cox2-cox1* spacer sequences in cultivated and wild beets. The haplotypes are enclosed by dotted lines according to the type of indels. These 26 haplotypes were tentatively assigned into six chondriome groups: A (green), B (red), C (sky blue), D (purple), E (yellow), and F (deep blue). Haplotypes are represented by rectangles, and substitutions by black dots on the solid black lines connecting the rectangles. Dashed lines with numbers indicate the steps separating the haplotypes.

Data analysis: Nucleotide sequences were aligned with the aid of ClustalW software at http://www.ddbj.nig.ac.jp/E-mail/homology-j.html then manually adjusted in the most parsimonious way. Ambiguous sites were excluded from analysis. A statistical parsimony network was constructed using TCS (ver. 1.21) software (CLEMENT *et al.* 2000). The initial network was drawn with default parameters, and then the connection limit was increased to 55 steps to connect Seq14 (BGRC56743) and seq26 (WB47).

Construction of genomic library and physical mapping of mtDNA: MtDNA prepared from *B. vulgaris* ssp. *orientalis* acc. BGRC56777 was partially digested with *Mbo*I and then overlaid on continuous sucrose density gradients (SAMBROOK *et al.* 1989). DNA fragments of ~18 kbp were fractionated by centrifugation and cloned into the Lambda DASH II vector (Stratagene) according to standard procedures. All other library manipulations were as described by KUBO *et al.* (1995, 1999). DNA fragments were labeled using Gene Images (GE Healthcare Bio-Sciences, Piscataway, NJ). Families of overlapping phages were identified by screening the acc. BGRC56777 mtDNA library with the subfragments of TK81-O mtDNA (KUBO *et al.* 1995) or TK81-MS mtDNA (KUBO *et al.* 1999). The

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ind01   341 CTAAGATCATCAGATAAGGAGACAAGCCACAGCTCGTATTCATTCAGTCATTTTACCCCT
ind02   341 -----------------------------------------------------------
ind03   341 -----------------------------------------------------------
ind04   341 -----------------------------------------------------------
ind05   341 -----------------------------------------------------------


ind01   401 TCTTCATTCTTGGACTTTGCTTCTTAATATTGTGACTTTTCTGTACGTACGGTAGAGGTT
ind02   341 -----------------------------------------------------------
ind03   341 -----------------------------------------------------------
ind04   341 -----------------------------------------------------------
ind05   341 -----------------------------------------------------------


ind01   461 GATTGAAG--------------------------------------------ATGATGTTA
ind02   341 --------TATATAGGAGCTATAGCCGTTTCATTCCTATTCGTTGGTTGTTATGATGTTC
ind03   341 --------TATATAGGAGTTATAGCCGTTTCATTCCTATTCGTTG----TTATGATGTTC
ind04   341 --------TATATAGGAGCTATAGCCGTTTCATTCCTATTCGTTG----TTATGATGTTC
ind05   341 --------TATATAGGAGCTATAGCCGTTTCATTCCTATTCGTTG----TTATGATGTTC


ind01   478 CGCAGCGTT------        689 GATTTCCACTTTAGT---
ind02   393 CACAGCGTT------        604 CCTTTTTACTTTAGTAGG
ind03   389 CACAGTGTT------        600 CCTTTTTACTTTAGTAGG
ind04   389 CACAGCGTTAGCGT-        605 CCTTTTTACTTTAGTAGG
ind05   389 CACAGCGTTAGCGTT        606 CCTTTTTACTTTAGTAGG
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FIGURE 2.—Nucleotide sequence alignment of the portions of *cox2-cox1* spacer sequences from WB47 (ind01), TK81-MS (ind02), BGRC56743 (ind03), BGRC54842 (ind04), and TK81-O (ind05), representing five indel-based haplotypes. Dashes are introduced for the maximum matching.

restriction maps of the phage clones identified were determined and further extended by genome walking from the extremities of the isolated groups of phages. In this approach, distal restriction fragments of a family of clones were used to probe the phage library of BGRC56777 from which new overlapping clones were identified and mapped.

RESULTS AND DISCUSSION

Sequence variation of the *cox2-cox1* spacer region: Total DNA was prepared from 49 accessions of the section Beta as well as from one accession of the section *Corollinae*, and was subjected to PCR-amplification using a set of primers targeting a part (~800 bp) of the *cox2-cox1* spacer region. The amplified DNA fragments showed a clear single-band product on an agarose gel, which was subsequently sequenced either directly or after cloning. The sequence alignment yielded 102 site mutations (nucleotide substitutions) and six indels (1–128 bp) when all accessions were considered.

Phylogeny of the *cox2-cox1* spacer sequences: *Nucleotide substitutions:* The use of indels as characters in parsimony analysis is logical, but their weighting relative to nucleotide substitutions is problematic. Here we presented results from analysis only when indels were coded as missing data. Informative polymorphisms allowed us to define a total of 26 distinct haplotypes of mitochondrial *cox2-cox1* spacer among 50 accessions examined (Table 1). A normal cytoplasm line, TK81-O, was found to share the same haplotype (named seq01) with 8 *B. vulgaris* ssp. *vulgaris*, 9 *B. vulgaris* ssp. *maritima,* and 1 *B. vulgaris* ssp. *orientalis* accessions. Seq01 was the most frequently observed haplotype. The number of accessions having each of the other 25 haplotypes ranged from 1 to 3 (Table 1).

Statistical parsimony applied to our data led to the construction of the haplotype phylogeny depicted in Figure 1. The most abundant haplotype, seq01, was found to occupy the central position of the haplotype network. Eighteen terminal haplotypes (seq02, seq03, seq04, seq08, seq09, seq10, seq11, seq12, seq15, seq16, seq17, seq18, seq19, seq21, seq22, seq23, seq24, and seq25) coalesced to the internal seq01 with one to five nucleotide substitutions; phylogenetically, this group of haplotypes (provisionally named chondriome group A) in the network branched into a structure with a star-like pattern. Interestingly, two haplotypes, seq08 and seq09, were associated with male-sterile cytoplasms, I-12CMS(2) and I-12CMS(3), respectively, and our data indicate a close relationship among these two sterilizing cytoplasms and normal ("TK81-O-type") cytoplasm.

TABLE 2

Insertions/deletions found in the mitochondrial *cox2-cox1* spacer region

Haplotype	Species	Accession
ind01	*B. trigyna*	WB47
ind02	*B. vulgaris* ssp. *vulgaris*	TK81-MS
	B. vulgaris ssp. *vulgaris*	BETA31/81
	B. vulgaris ssp. *vulgaris*	753962
	B. vulgaris ssp. *maritima*	BGRC28901
	B. vulgaris ssp. *vulgaris*	BGRC58265-MS
ind03	*B. vulgaris* ssp. *vulgaris*	BGRC56743
ind04	*B. vulgaris* ssp. *maritima*	BGRC54842
ind05	The others	

TABLE 3

Number of the repeat units in the four mitochondrial minisatellite (TR) loci of cultivated and wild beets

Haplotype	Chondriome group	Species	Accession	Sequence type	TR1	TR2	TR3	TR4
min01	C	*B. vulgaris* ssp. *maritima*	FR4-31[a]	seq06	2	3	1	3
min02	A	*B. vulgaris* ssp. *maritima*	BETA140/83	seq16	3	3	1	3
min03	F	*B. trigyna*	WB47	seq26	3	3	3	3
min04	B	*B. vulgaris* ssp. *vulgaris*	BETA31/81	seq05	4	3	2	4
	B	*B. vulgaris* ssp. *vulgaris*	BGRC58265-MS	seq05	4	3	2	4
	B	*B. vulgaris* ssp. *maritima*	BGRC28901	seq05	4	3	2	4
	B	*B. vulgaris* ssp. *vulgaris*	TK81-MS[a]	seq07	4	3	2	4
	B	*B. vulgaris* ssp. *vulgaris*	753962	seq13	4	3	2	4
min05	A	*B. macrocarpa*	Canary Island	seq25	4	3	3	3
min06	A	*B. vulgaris* ssp. *orientalis*	BGRC56777	seq01	5	3	2	3
	A	*B. vulgaris* ssp. *vulgaris*	BETA78/90	seq02	5	3	2	3
	A	*B. vulgaris* ssp. *vulgaris*	I-12CMS(2)	seq08	5	3	2	3
	A	*B. vulgaris* ssp. *vulgaris*	I-12CMS(3)[a]	seq09	5	3	2	3
	A	*B. vulgaris* ssp. *maritima*	PI504186-119	seq22	5	3	2	3
	A	*B. vulgaris* ssp. *maritima*	BGRC57715-MS	seq24	5	3	2	3
	A	*B. vulgaris* ssp. *vulgaris*	753932	seq12	5	3	2	3
	A	*B. vulgaris* ssp. *maritima*	BGRC57713-MS	seq02	5	3	2	3
min07	A	*B. vulgaris* ssp. *maritima*	BGRC36478	seq18	5	3	3	3
	A	*B. vulgaris* ssp. *maritima*	BGRC54772	seq19	5	3	3	3
min08	A	*B. vulgaris* ssp. *adanensis*	Egypt	seq04	6	3	2	3
min09	A	*B. vulgaris* ssp. *maritima*	AM4247-295[a]	seq19	6	3	3	3
	A	*B. vulgaris* ssp. *maritima*	BETA(29)17/85	seq15	6	3	3	3
	A	*B. vulgaris* ssp. *vulgaris*	753931	seq11	6	3	3	3
	A	*B. vulgaris* ssp. *maritima*	PI518369-311	seq01	6	3	3	3
	A	*B. vulgaris* ssp. *maritima*	BGRC56773	seq01	6	3	3	3
	A	*B. vulgaris* ssp. *maritima*	BGRC54841	seq01	6	3	3	3
	A	*B. vulgaris* ssp. *maritima*	BGRC32396	seq01	6	3	3	3
min10	A	*B. vulgaris* ssp. *vulgaris*	BGRC58265-MF	seq01	7	3	2	3
min11	A	*B. vulgaris* ssp. *maritima*	BGRC57705-MS	seq03	7	3	3	3
	A	*B. vulgaris* ssp. *maritima*	BGRC57705-MF	seq23	7	3	3	3
	A	*B. vulgaris* ssp. *maritima*	PI546530-337	seq04	7	3	3	3
	A	*B. vulgaris* ssp. *maritima*	BETA156/86	seq17	7	3	3	3
min12	A	*B. vulgaris* ssp. *maritima*	BGRC57712	seq21	7	3	3	4
min13	A	*B. vulgaris* ssp. *maritima*	BGRC48810-MF	seq03	8	3	3	5
	A	*B. vulgaris* ssp. *maritima*	BGRC48810-MS	seq03	8	3	3	5
min14	A	*B. vulgaris* ssp. *vulgaris*	BETA35/85	seq01	8	6	3	1
min15	A	*B. vulgaris* ssp. *vulgaris*	BETA98/80	seq10	9	3	3	3
min16	E	*B. vulgaris* ssp. *vulgaris*	BGRC56743	seq14	9	3	2	5
min17	D	*B. vulgaris* ssp. *maritima*	BGRC54842	seq20	11	3	3	3
min18	A	*B. vulgaris* ssp. *vulgaris*	TK81-O[a]	seq01	13	3	3	3
	A	*B. vulgaris* ssp. *maritima*	BETA280/91	seq01	13	3	3	3

[a] Data are from NISHIZAWA *et al.* (2000).

The seq01 haplotype connected to seq05, beyond which was placed haplotype seq07 (TK81-MS, Owen-sterilizing cytoplasm). The Owen CMS mtDNA haplotype was distinguished from seq05 and seq01 by three and five mutations, respectively. This indicates that the TK81-O-type normal cytoplasm is more similar to the I-12CMS(2) and I-12CMS(3) cytoplasms than to the Owen cytoplasm.

The seq05 and seq07 haplotypes formed a separate group (chondriome group B) together with seq13; this is also supported by both indel and minisatellite analyses (see below). Similarly, it might be better to consider
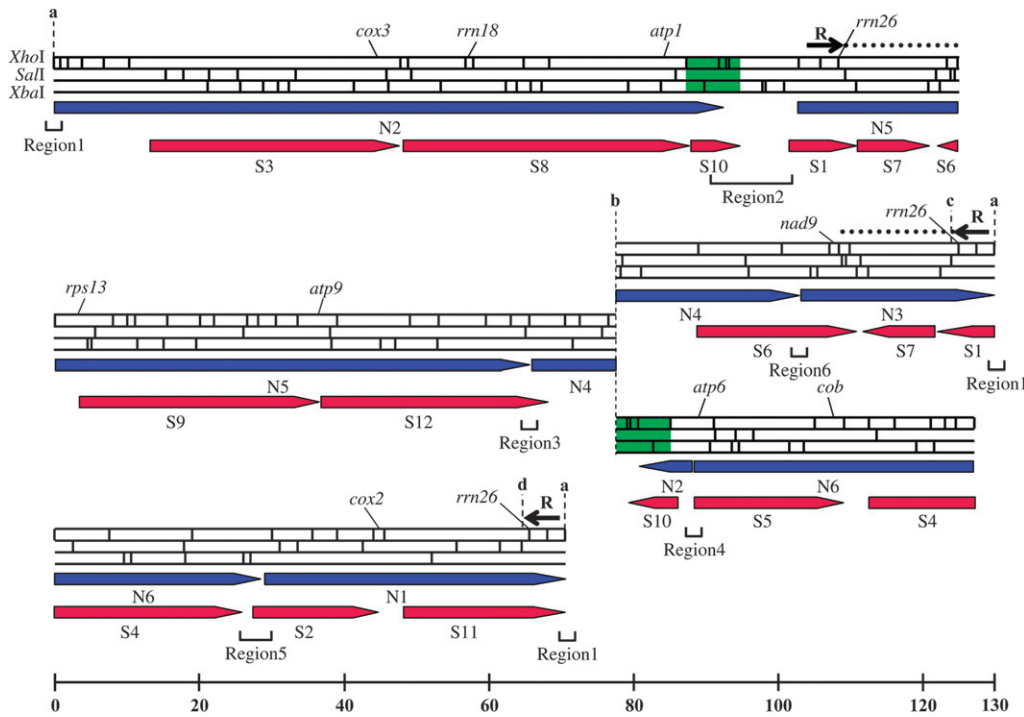
FIGURE 3.—A physical map of the mitochondrial genome of *B. vulgaris* ssp. *orientalis* acc. BGRC56777. Three rearrangement points (designated b, c, and d) were identified. The 47.0-kb region from b to c and the 113.7-kb region from b to d do not have features in common. The ends of these two single-copy regions can be connected to the end (top left) of the map. Black horizontal arrows labeled by the letter R indicate the active recombination repeated sequences containing *rrn26*. Dotted lines show the extension of the R repeat. Green-boxed sequences are repeated sequences but not recombinogenic. The regions of homology between the BGRC56777 and the TK81-O mtDNA and between the BGRC56777 and the TK81-MS mtDNAs are indicated by blue arrows designated N1–N6 and red arrows designated S1–S12, respectively. The sequenced regions (regions 1–6) are indicated by brackets.

seq06, seq20, seq14, and seq26 as four separate groups—chondriome groups C, D, E, and F, respectively. Since the outgroup seq26 (*B. trigyna* acc. WB47) was connected to seq01, seq11, and seq03, chondriome groups B, C, and D may be derived from chondriome group A. From seq01, three of the lineages gave rise to the Owen, I-12CMS(2) and I-12CMS(3) sterilizing mitotypes. This finding is consistent with the results of chloroplast DNA analysis (FENART *et al.* 2006), which also revealed that the four different sources of beet CMS (E, G, H, and Owen types) did not constitute a single evolutionary lineage but occurred independently from an ancestral nonsterilizing cytoplasm.

Indels: Six indels could distinguish five haplotypes (ind01–ind05), as detailed in Figure 2 and Table 2. As might have been expected, almost all indels were
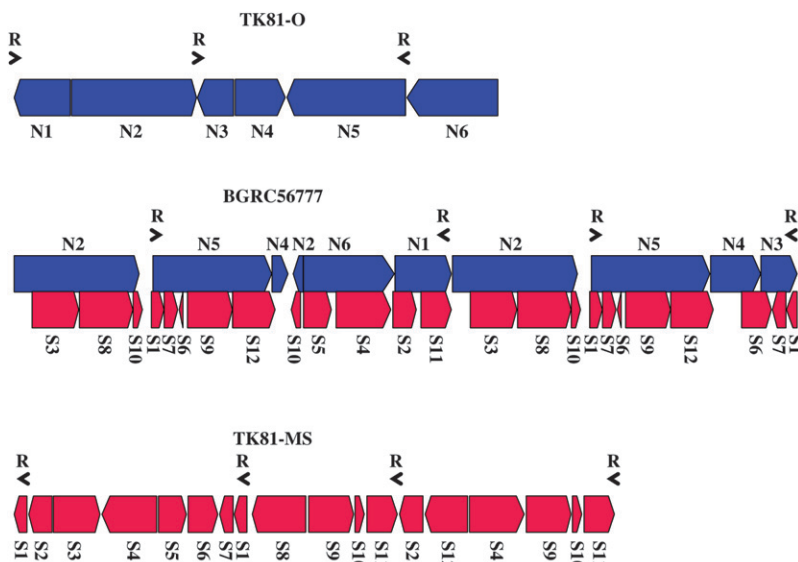

FIGURE 4.—Schematic of the rearrangements among the mitochondrial genomes of BGRC56777, TK81-O, and TK81-MS. The order and relative orientation of the homologous regions (designated N1–N6 and S1–S12, see Figure 3) between the BGRC56777 and the TK81-O mtDNAs and between BGRC56777 and TK81-MS mtDNAs are shown. Black horizontal arrowheads labeled by the letter R indicate the active recombination repeated sequences. In the TK81-MS mitochondrial genome, two other recombinogenic repeated sequences (KUBO *et al.* 1999) are not identified.

correlated with an outgroup, *B. trigyna* (ind01). Most of the accessions examined shared the ind05 haplotype, which showed four indel differences from the ind01 haplotype. The ind02 haplotype was found in the five members of chondriome group B (TK81-MS, BETA31/81, 753962, BGRC28901, and BGRC58265-MS) and differed from the three other haplotypes (ind01, ind03, and ind04) by one or two indels. The ind03 and ind04 corresponded to chondriome groups E (BGRC56743) and D (BGRC54842), respectively.

MtDNA minisatellite polymorphism: Four tandem repeat loci (TR1, TR2, TR3, and TR4) were shown to be variable in the mitochondrial genomes of beets (NISHIZAWA *et al.* 2000; FIEVET *et al.* 2007). These minisatellite sequences were used to study genetic relationships among 41 accessions representing all the haplotypes of mitochondrial *cox2-cox1* spacer. All four loci analyzed were polymorphic in the material examined, with the number of alleles per locus ranging from 2 to 10 (Table 3). Heteroplasmy, or the presence of multiple-size classes of minisatellites within individual mtDNA samples, was not detected in any samples.

Eighteen multi-locus haplotypes (min01–min18) were identified, of which 13 were observed in the accessions assigned into the chondriome group A (Tables 1 and 3). Chondriome group B was monomorphic for haplotype min04, which was not found in any other chondriome groups and this group thus proved to be clearly separated from the others. Chondriome groups C, F, E, and D were characterized by unique min01, min03, min16, and min17, respectively, none of which was found in the other chondriome groups.

A physical map of the mitochondrial genome of *B. vulgaris* ssp. *orientalis* acc. BGRC56777: The seq01 haplotype has attracted our interest because it most likely represents the progenitor common to the Owen, I-12CMS(2) and I-12CMS(3) sterilizing mitotypes. One can expect that more intensive comparative analysis on the mitochondrial genomes of beet accessions carrying the seq01 haplotype would further subdivide this haplotype, allowing the maternal lineage of the three sterilizing mitotypes to be unambiguously traced. As a first step toward this end, we constructed a physical map of the mitochondrial genome of *B. vulgaris* ssp. *orientalis* acc. BGRC56777 (seq01 haplotype) and compared it with the other known sugar beet mtDNA maps (KUBO *et al.* 1995, 1999). BGRC56777 was chosen for mapping because this accession was found to be similar but not identical to cv. TK81-O for the RFLP profiles of mtDNA and was also considered to possess the fertile cytoplasm (S. NISHIZAWA, T. MIKAMI and T. KUBO, unpublished data).

Two sets of overlapping phage clones, each spanning the entire mitochondrial genomes of cv. TK81-O and cv. TK81-MS (KUBO *et al.* 1995, 1999), respectively, were sequentially used as probes against Southern blots prepared with total genomic DNA from BGRC56777. This enabled us to identify a number of regions that were
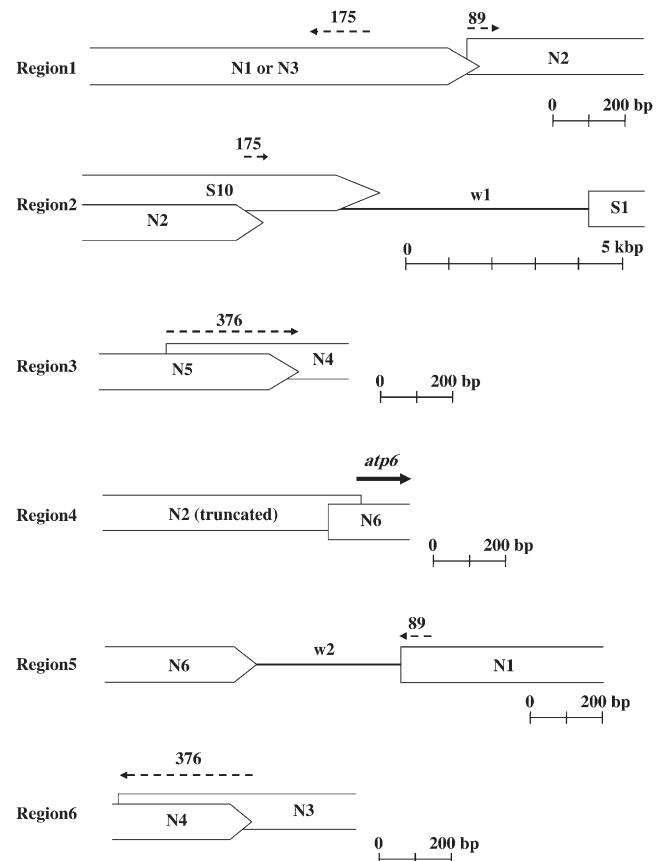

FIGURE 5.—Schematic of the six regions (1–6) covering the rearrangement breakpoints in the BGRC56777 mitochondrial genome (see Figure 3). Scale bars are shown under each illustration. Part of the *atp6* coding region is shown by a solid arrow. Dashed arrows indicate three sets of small repeated sequences mentioned in the text. Boxes are sequences shared between the BGRC56777 and the TK81-O mitochondrial genomes or between the BGRC56777 and the TK81-MS mitochondrial genomes (see Figure 3). Solid horizontal lines between the boxes represent sequences unique to the BGRC56777 mitochondrial genome, labeled w1 and w2.

unchanged in hybridization patterns either between BGRC56777 and TK81-O or between BGRC56777 and TK81-MS, but that were delimited by rearrangement breakpoints. We further adopted the genome-walking technique where fragments located at the extremities of the homologous sequence regions were used to probe a BGRC56777 phage library from which overlapping clones were identified and mapped. In this manner, the entire sequence complexity was mapped onto two circular chromosomes of 257.6 and 324.3 kb, which share 210.6-kb of homologous sequence. A master circle (581.9 kb) can also be constructed with the assumption that recombination may happen via the 210.6-kb repeats (Figure 3).

We also found a single family of recombination-active repeats; the region common to all repeat copies was 6.1 kb in length and harbored the *rrn26* gene (Figure 3). All possible combinations of flanking sequences around the repeats were detected either by restriction mapping
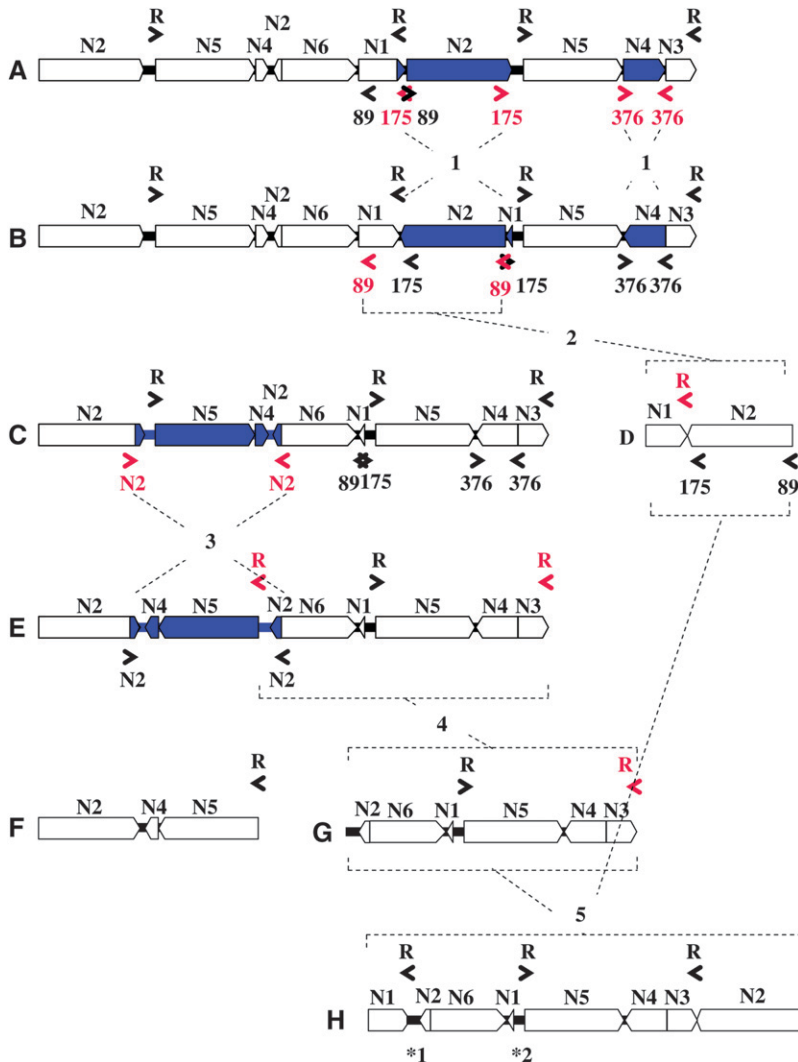
Figure 6.—A proposed model for the evolution of the BGRC56777 mitochondrial master chromosome toward the TK81-O-like mitochondrial master chromosome. Three sets of inverted repeated sequences (89, 175, and 376 bp) were identified at the rearrangement breakpoints within the BGRC56777 mitochondrial genome (see also Figure 5), which are depicted in the linealized master chromosome A (581.9 kb). At the initial step (shown by 1), 175- and 376-bp repeated sequences (highlighted in red) recombined to introduce two inversions of the blue-colored regions, resulting in the isomeric master chromosome B. At the next step (shown by 2), 89-bp repeated sequences (highlighted in red) recombined to loop out 135.2-kb subgenomic molecule D. The remaining 446.7-kb molecule C was further converted to the isomeric molecule E by the recombination via truncated N2 repeated sequences (highlighted in red) at step 3. The molecule E was then split into two molecules, F (195.5 kb) and G (251.2 kb), by the recombination-active repeats R containing *rrn26* (highlighted in red) at step 4. The molecules D and G were fused by the recombination mediated by R (highlighted in red) to yield TK81-O-like master chromosome H (386.4 kb) at step 5. *1 and *2 (parts of region 2) indicate the position of sequences unique to the 386.4-kb molecule.

of repeat-containing phages or by hybridizations of flanking-region probes to genomic DNA, which indicates that they are recombinogenic (data not shown).

Comparison of the mitochondrial genomes among BGRC56777, fertile line TK81-O, and Owen CMS line TK81-MS: We compared the linear arrangement of the homologous restriction enzyme fragments among BGRC56777, TK81-O, and TK81-MS mtDNAs on the basis of phage clone analysis and cross-hybridization studies. The BGRC56777 mitochondrial genome could be divided into regions where boundaries or breakpoints were defined by an interruption in the restriction maps of the BGRC56777, TK81-O, or TK81-MS master chromosomes (Figure 3). When BGRC56777 was compared with TK81-O, six such regions (N1–N6) were identified in the TK81-O genome, each of which had at least one counterpart in the BGRC56777 mitochondrial genome. As shown in Figure 4, a segment of the BGRC56777 genome contains regions N2–N5 in the same order as the TK81-O genome, although the regions have different orientations. A possible evolutionary pathway

for interconverting the two genomes will be presented later.

Twelve regions (S1–S12) were delimited by comparing BGRC56777 and TK81-MS. We should note that 5 regions (S1, S2, S6, S10, and S12) spanned the organizational breakpoints between TK81-O and BGRC56777, indicating that the 5 regions are conserved between the BGRC56777 and the TK81-MS mitochondrial genomes. Figure 4 illustrates the large differences in order and orientation of the 12 regions between the two mitochondrial genomes. In addition to the rearrangements of homologous sequences, the BGRC56777 mtDNA sustained several deletions (as many as five) and insertions (as many as five) relative to the TK81-MS mtDNA. The complexity of the differences does not offer a straightforward explanation of the evolutionary relationship of the two genomes.

Rearrangements found between the mitochondrial genomes of BGRC56777 and TK81-O: events and evolutionary pathways: What we next describe is the possible mechanism of rearrangements detected between

the mitochondrial genomes of BGRC56777 and TK81-O. The mitochondrial genomes of higher plants are characterized by an abundance of short dispersed repeats, some of which are associated with various rearrangements, including inversions, insertions/deletions, and transpositions (Fauron *et al.* 1995; Lilly and Havey 2001; Kubo and Mikami 2007). A complete sequence comparison of the normal and Owen CMS mtDNAs revealed in fact that most, if not all, rearrangements involved short repeats present at the recombination endpoints (Satoh *et al.* 2006). With this in mind, we determined the nucleotide sequences of the six regions covering the rearrangement breakpoints in the BGRC56777 mitochondrial genome and analyzed these sequences to search for repeats (see Figure 3); regions 1, 3, 4, 5, and 6 were PCR amplified, whereas the insert of a lambda phage clone (347) was used to sequence region 2 (accession numbers AB303857–AB303862). A comparison of the sequences determined here with the TK81-O mtDNA sequences successfully identified the exact boundary of regions N1–N6 (Figure 5).

As shown in Figures 5 and 6, three sets of inverted repeats (89, 175, and 376 bp in length) were found flanking the breakpoints. Region N2 was flanked on one side by a copy of the 89-bp repeat and on the other side by a copy of the 175-bp repeat, whereas region N1 was bounded by another 89-bp repeat copy and another 175-bp repeat copy. In addition, two copies of the 376-bp repeat bracketed the region N4. These data were used to postulate the most parsimonious evolutionary pathway for interconverting the mitochondrial genomes of BGRC56777 and TK81-O.

The evolutionary scenario presented in Figure 6 was constructed on the basis of the assumption that the mitochondrial genome of TK81-O was derived from a BGRC56777-like ancestral genome by a succession of five major sequence rearrangements:

1. Recombination across the 175-bp repeat resulted in the inversion of N2, causing two copies of the 89-bp repeat to be directly oriented. The 376-bp inverted repeat mediated recombination to invert region N4.
2. The 89-bp direct repeat subsequently recombined to produce two subgenomic circular molecules of 135.2 and 446.7 kb, although we have no evidence bearing on the temporal order of the inversion of N4 and the generation of the 446.7-kb subgenomic molecule.
3. Recombination between region N2 and its truncated copy inverted the segment (128 kb) of the 446.7-kb molecule between the repeated sequences, which included one copy of the *rrn26* gene and placed the two copies in direct orientation.
4. This was followed by recombination through *rrn26*, which gave rise to two subgenomic circular molecules of 195.5 and 251.2 kb.
5. The 135.2- and 251.2-kb subgenomic molecules underwent intermolecular recombination through the

rrn26 repeat to give a molecule of 386.4 kb. The resultant master chromosome is almost identical to that of TK81-O in terms of gene order and restriction sites, but differs from the latter in having additional sequences derived from region 2 (indicated by *1 and *2 in Figure 6).

The five-step rearrangements described above seem to be the simplest and most parsimonious hypothesis to explain the evolutionary relationship of the mitochondrial genomes of BGRC56777 and TK81-O. However, we have no definite evidence supporting this progenitor (BGRC56777-like genome)/derivative (TK81-O genome) relationship. It should also be borne in mind that consideration of parsimony or simplicity may be inadequate for determining whether the proposed model may closely represent actual evolutionary events. It may be best to use comparative mapping data by examining a number of related mitochondrial genomes. We are now attempting to construct the physical maps of mitochondrial genomes of I-12CMS(2) and I-12CMS(3), as well as different sources of normal cytoplasms carrying seq01 haplotype, with the aim of gaining a better understanding of how the three sterilizing cytoplasms in question evolved.

LITERATURE CITED

Bosemark, N. O., 1993   Genetics and breeding, pp. 66–119 in *The Sugar Beet Crops*, edited by D. A. Cooke and R. K. Scott. Chapman & Hall, London.

Chase, C. D., 2007   Cytoplasmic male sterility: a window to the world of plant mitochondrial-nuclear interactions. Trends Genet. **23:** 81–90.

Clement, M., D. Posada and K. A. Crandall, 2000   TCS: a computer program to estimate gene genealogies. Mol. Ecol. **9:** 1657–1660.

Doyle, J. J., and J. L. Doyle, 1990   Isolation of plant DNA from fresh tissue. Focus **12:** 13–15.

Ducos, E., P. Touzet and M. Boutry, 2001   The male sterile *G* cytoplasm of wild beet displays modified mitochondrial respiratory complexes. Plant J. **26:** 171–180.

Fauron, C. M.-R., and M. Casper, 1994   A second type of normal maize mitochondrial genome: an evolutionary link. Genetics **137:** 875–882.

Fauron, C. M.-R., B. Moore and M. Casper, 1995   Maize as a model of higher plant mitochondrial genome plasticity. Plant Sci. **112:** 11–32.

Fenart, S., P. Touzet, J.-F. Arnaud and J. Cuguen, 2006   Emergence of gynodioecy in wild beet (*Beta vulgaris* ssp. *maritima* L.): a geneal-

ogical approach using chloroplastic nucleotide sequences. Proc. R. Soc. London B **273:** 1391–1398.

Fievet, V., P. Touzet, J.-F. Arnaud and J. Cuguen, 2007 Spatial analysis of nuclear and cytoplasmic DNA diversity in wild sea beet (*Beta vulgaris* ssp. *maritima*) populations: Do marine currents shape the genetic structure? Mol. Ecol. **16:** 1847–1864.

Hallden, C., C. Lind and I. M. Moller, 1992 Variation in mitochondrial translation products in fertile and cytoplasmic male-sterile sugar-beets. Theor. Appl. Genet. **85:** 139–145.

Kubo, T., Y. Satoh, T. Muro, T. Kinoshita and T. Mikami, 1995 Physical and gene organization of mitochondrial DNA from the fertile cytoplasm of sugarbeet (*Beta vulgaris* L.). Curr. Genet. **28:** 235–241.

Kubo, T., S. Nishizawa and T. Mikami, 1999 Alterations in organization and transcription of the mitochondrial genome of cytoplasmic male sterile sugar beet (*Beta vulgaris* L.). Mol. Gen. Genet. **262:** 283–290.

Kubo, T., S. Nishizawa, A. Sugawara, N. Itchoda, A. Estiati *et al.*, 2000 The complete nucleotide sequence of the mitochondrial genome of sugar beet (*Beta vulgaris* L.) reveals a novel gene for tRNA^{Cys}(GCA). Nucleic Acids Res. **28:** 2571–2576.

Kubo, T., and T. Mikami, 2007 Organization and variation of angiosperm mitochondrial genome. Physiol. Plant. **129:** 6–13.

Laser, K. D., and N. R. Lersten, 1972 Anatomy and cytology of microsporogenesis in cytoplasmic male sterile angiosperms. Bot. Rev. **38:** 425–454.

Lilly, J. W., and M. J. Havey, 2001 Small, repetitive DNAs contribute significantly to the expanded mitochondrial genome of cucumber. Genetics **159:** 317–328.

McPherson, M. J., P. Quirke and G. R. Taylor, 1991 *PCR: A Practical Approach.* Oxford University Press, New York.

Mikami, T., Y. Kishima, M. Sugiura and T. Kinoshita, 1985 Organelle genome diversity in sugar beet with normal and different sources of male sterile cytoplasms. Theor. Appl. Genet. **71:** 166–171.

Nishizawa, S., T. Kubo and T. Mikami, 2000 Variable number of tandem repeat loci in the mitochondrial genomes of beets. Curr. Genet. **37:** 34–38.

Owen, F. V., 1945 Cytoplasmically inherited male-sterility in sugarbeet. J. Agr. Res. **71:** 423–440.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual,* Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Satoh, M., T. Kubo, S. Nishizawa, A. Estiati, N. Itchoda *et al.*, 2004 The cytoplasmic male-sterile type and normal type mitochondrial genomes of sugar beet share the same complement of genes of known function but differ in the content of expressed ORFs. Mol. Genet. Genomics **272:** 247–256.

Satoh, M., T. Kubo and T. Mikami, 2006 The Owen mitochondrial genome in sugar beet (*Beta vulgaris* L.): possible mechanisms of extensive rearrangements and the origin of the mitotype-unique regions. Theor. Appl. Genet. **113:** 477–484.

Saumitou-Laprade, P., G. J. A. Rouwendal, J. Cuguen, F. A. Krens and G. Michaelis, 1993 Different CMS sources found in *Beta vulgaris* ssp *maritima*: mitochondrial variability in wild population revealed by a rapid screening procedure. Theor. Appl. Genet. **85:** 529–535.

Schnable, P. S., and R. P. Wise, 1998 The molecular basis of cytoplasmic male sterility and fertility restoration. Trends Plant Sci. **3:** 175–180.

Senda, M., Y. Onodera, T. Kinoshita and T. Mikami, 1995 Mitochondrial gene variation and phylogenetic relationships in the genus *Beta.* Theor. Appl. Genet. **90:** 914–919.

Yamamoto, M. P., T. Kubo and T. Mikami, 2005 The 5′-leader sequence of sugar beet mitochondrial *atp6* encodes a novel polypeptide that is characteristic of Owen cytoplasmic male sterility. Mol. Genet. Genomics **273:** 342–349.

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