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Citation	Theoretical and Applied Genetics, 113(3), 477-484 https://doi.org/10.1007/s00122-006-0312-4
Issue Date	2006-08
Doc URL	https://hdl.handle.net/2115/14566
Rights	The original publication is available at www.springerlink.com
Type	journal article
File Information	TAAG.pdf

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The Owen mitochondrial genome in sugar beet (*Beta vulgaris* L.): possible mechanisms of extensive rearrangements and the origin of the mitotype-unique regions

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Abstract The mitochondrial genomes of normal fertile and male-sterile (Owen CMS) cytoplasms of sugar beet are highly rearranged relative to each other and dozens of inversional recombinations and other reshuffling events must be postulated to interconvert the two genomes. In this paper, a comparative analysis of the entire nucleotide sequences of the two genomes revealed that most of the inversional recombinations involved short repeats present at their endpoints. Attention was also focused on the origin of the Owen CMS-unique mtDNA regions, which occupy 13.6% of the Owen genome and are absent from the normal mtDNA. BLAST search was performed to assign the sequences, and as a result, 7.6% of the unique regions showed significant homology to previously determined mitochondrial sequences, 17.9% to nuclear DNA, 4.6% to mitochondrial episomes, and 0.1% to plastid DNA. Southern blot analysis revealed that additional sequences of nuclear origin may be included within the unique regions. We also found that the copies of many short repeat families are scattered throughout the unique regions. This suggests that, in addition to the incorporation of foreign DNAs, extensive duplication of short repetitive sequences and continued scrambling of mtDNA sequences may be implicated in the generation of the Owen CMS-unique regions.

Introduction

The mitochondrial genomes of higher plants are distinguished from other mitochondrial genomes by their ability to undergo extensive recombination, as evidenced by the multitude of sequence rearrangements detected within them (Knoop 2004). Divergent mitochondrial DNA (mtDNA) arrangements are observed even between isolates of the same plant species. This is well exemplified in sugar beet (*Beta vulgaris* L.) where multiple structural differences were found between the mitochondrial genomes of normal fertile and male-sterile Owen cytoplasms (Kubo et al. 1999; Satoh et al. 2004). Owen cytoplasmic male-sterility (Owen CMS) was originally discovered in the cultivar

'US-1' (Owen 1945) and has been widely used as a convenient way to produce commercial beet hybrids (Bosemark 1993). The chloroplast DNAs from the normal and Owen cytoplasms are very similar to each other (99% identical) at the nucleotide sequence level (Ran and Michaelis 1995; Fenart et al. 2005; Y. Yanai, T. Kubo and T. Mikami, unpublished). This suggests that the Owen cytoplasm is derived from within *B. vulgaris* or its wild progenitors and has not originated through interspecific hybridization.

If a large number of rearrangement events between the normal and Owen mitochondrial genomes have taken place relatively recently, it is likely that there will be readily discernible vestiges of whatever sequences, if any, have caused them. We recently determined the entire nucleotide sequences of the normal and Owen mitochondrial genomes (Kubo et al. 2000; Satoh et al. 2004). Hence, a complete sequence comparison will constitute an essential phase in understanding about how the distinct mitochondrial genome configuration characteristic of Owen CMS was generated over the relatively short evolutionary times.

The comparison previously revealed that (1) the normal and Owen mitochondrial genomes (368,801 bp and 501,020 bp in size, respectively) differ radically in their sequence order and a number of inversion and/or rearrangement events must be postulated to align the two mtDNAs; (2) in addition to being highly rearranged, the Owen mitochondrial genome contains 13 regions with a total length of 68,263 bp that are not present in the normal mtDNA (Satoh et al. 2004). In this paper, we present the further analysis of extensive reorganizations in the Owen mitochondrial genome and of the origin of the mitotype-unique sequences.

Materials and methods

Plant materials

A male-sterile Owen cytoplasm line cv. TK81-MS (Kubo et al. 1999) and its maintainer line cv. TK81-O (Kubo et al. 2000) were used in this study.

Analysis of sequence data

The entire nucleotide sequences of TK81-O mtDNA (accession number BA000009) and TK81-MS mtDNA (accession numbers BA000024) were determined in our laboratory (Kubo et al. 2000; Satoh et al. 2004; BA000009 was recently updated). Nucleotide sequence was analyzed with Genetyx (SDC, Tokyo, Japan) and sequence alignments were generated using Sequencher 3.0 software (Gene Codes, Philadelphia, PA). Data base search was done with the BLAST network service (http://www.ddbj.nig.ac.jp/search/blast-j.html) with default parameter, as described by Kubo et al. (2000).

Nucleic acid isolation and hybridization

MtDNA was prepared from green leaves as described (Mikami et al. 1985). Total cellular DNA was isolated according to Doyle and Doyle (1990). DNA samples were digested with restriction enzymes (*Eco*RI and *Eco*RV: Takara Bio, Ohtsu, Japan) and electrophoresed in a 0.8% agarose gel. Size fractionated DNA fragments were transferred to Hybond N+ membrane (GE healthcare Bio-Sciences, Piscataway, NJ) according to the instruction manual. The sequence data of the TK81-MS-unique regions were used to synthesize oligonucleotides that were subsequently employed for PCR amplification of probe DNAs (Table 1). The probes were labeled with ^{32}P using Megaprime DNA labeling system (GE healthcare Bio-Sciences, Piscataway, NJ) and hybridization was done according to the instruction manual.

Results

Multiple genome rearrangements are associated with short repeated sequences

A comparative analysis of the entire sequences of the TK81-O and TK81-MS mtDNAs allowed the identification of 14 different sequence blocks (more than 500 nt in size) in the Owen genome in which nucleotide sequences are virtually identical to corresponding blocks in the normal genome. However, the order and orientation of these blocks relative to each other differed greatly between the two genomes (see Fig. 2 of Satoh et al. 2004)(Fig. 1).

In seeking possible bases of these mtDNA rearrangements, we found that the endpoints of the rearrangements are mostly associated with short repeated sequences (Fig. 1). For example, a 9 bp repeat (5'-TCTTTCTGG-3') occurs at the ends of two sequence blocks C and J that are 117 kb apart in TK81-O mtDNA (see Fig. 2 of Satoh et al. 2004). The repeats presumably participated in the recombination that caused the two blocks to be juxtaposed in TK81-MS mtDNA. This recombination also must have resulted in the inversion of Block J (or Block C) in the two genomes relative to each other. As shown in Fig. 1, identified at the ends of eight sequence blocks (D1/D5, E, G, H, I1, K, L1/L2 and M1) were three additional sets of short repeats (12, 135 and 376 nt, respectively) that could serve as sites of inversional recombination. It thus follows that the genomic regions described have been subjected to more than four inversions though the temporal order of these inversion events cannot be reliably inferred.

On the other hand, no repeated sequences were found at and near the endpoints of the remaining sequence blocks. This suggests that a number of mutations may have deleted or otherwise altered the sequences originally involved in promoting recombination.

Owen CMS-unique mtDNA regions

The sequence comparison also revealed that 13 regions (designated s1 - s13) with a total length of 68,263 nt in TK81-MS mtDNA are not conserved in TK81-O mtDNA (see Fig. 2 of Satoh et al. 2004)(Table 1), though they occasionally include short stretches homologous to TK81-O mtDNA (see below). Out of these 13 regions, six are reiterated in the mitochondrial master chromosome of TK81-MS so that the sequence unique to the Owen mtDNA actually totals 44,764 nt.

Similarity searches of the 13 regions in question were conducted using the BLASTN and BLASTX algorithms to the nuclear, plastid and mitochondrial data bases (Fig. 2 and data not shown). The searches showed that the Owen CMS-unique regions included at least 36 short integrated sequence segments (ranging in size between 28 and 1403 nt), of which 30 (12,224 nt in total) originated from the nuclear genome, five (3,119 nt in total) from mitochondrial episomes and one (50 nt) from the plastid genome. Almost all of the integrated nuclear sequences were identified as pieces of retroelements. However, we also found that *orf265a* in s1 contained a stretch of 795 nt which is homologous to the fourth exon of an *Arabidopsis* presumptive nuclear gene At5g13390.1 (Fig. 2).

In addition, 47 short segments (25 - 546 nt) with significant homology to previously determined mtDNA sequences proved to be scattered throughout the Owen CMS-unique regions. Of these segments, 12 (57-344 nt) are also present as homologous regions in TK81-O mtDNA. The remaining one segment (97 nt) lies within s10 and its homologous sequence stretch was found in *Arabidopsis* and rapeseed mtDNA (Unseld et al. 1997; Handa 2003) but missing from TK81-O mtDNA (Fig. 2). It seems likely that this segment may be present in the ancestral genome common to both TK81-MS. We also note that 69.8 % of the Owen CMS-unique regions could not be found in the extant databases.

Hybridization analysis indicates additional sequences of nuclear origin

In sugar beet, nuclear genome sequence data are rapidly increasing (Schulte et al. 2006) but still incomplete. With this in mind, Southern blot analysis was performed to determine whether any additional sequences of nuclear origin are present within the Owen CMS-unique regions. A total of 22 probes were prepared (Table 1) and hybridized to blots containing mtDNAs from TK81-MS and TK81-O as well as total cellular DNA from TK81-O. For example, probe s1-1 hybridized to two fragments in TK81-O total DNA but not with TK81-O mtDNA (Fig. 3). This is not unexpected because, as mentioned above, the probe contains the sequence homologous to an *Arabidopsis* nuclear gene At5g13390.1. The result also raises the possibility that At5g13390.1 homologue(s) is (are) encoded in the nuclear genome of sugar beet.

On the other hand, five probes carrying the retrotransposon-related sequences produced middle- to high-copy sequence signals on TK81-O total DNA, reflecting their repetitive nature. An example of such a hybridization pattern is shown in Fig. 3 (probes s8-2 and s11-1). Our observation is in line with the widespread occurrence of transposons in the nuclear genomes of sugar beet (Schmidt and Heslop-Harrison 1998; Jacobs et al. 2004; Schulte et al. 2006).

Sixteen out of 22 probe sequences exhibited no significant homology to any of the entries in the data bases (Table 1). Of these 16 probes, however four were found to give hybridization signals to TK81-O total DNA (Table 1; Fig. 3), which indicates that a significant fraction of these as yet unassigned sequences is of nuclear origin. The most noteworthy result was obtained when probe s5-1 was used for hybridization. As shown in Fig. 3, this probe exhibited multiple hybridization signals on TK81-O total DNA. This suggests strongly that the sequences contained within s5-1 occur at multiple locations in the nuclear genome of sugar beet. Interestingly, the s5-1 sequence was entirely included within the coding region of *preSatp6* (Fig. 2). The *preSatp6* sequence is most likely to be responsible for Owen CMS and is fused with

atp6 in a single continuous ORF termed *preSatp6/atp6* (Yamamoto et al. 2005). The *preSatp6/atp6* locus thus appears to be derived from fusion of a conserved mitochondrial gene with repetitive sequences of nuclear origin. It should also be noted that the other 12 probes failed to hybridize with both mtDNA and total DNA from TK81-O (Table 1), and therefore, the origin of these probe sequences is still unclear.

Repeated sequences in the Owen CMS-unique regions

We previously identified 45 families of short repeats (50 - 380 nt in size) in TK81-MS mtDNA (Satoh et al. 2004). Here, a short-repeat family was defined as sequences having at least 98% similarity and identical length. We noticed that a 201-nt- segment (Rep4) containing 5' upstream and first 12 nt of *nad3* was duplicated downstream from *atp6* in TK81-MS mtDNA, leading to the formation of an Owen CMS-unique region s6 (Fig. 4). A similar duplication was found in the corresponding location (the intergenic region between *atp6* and *orf114*) in TK81-O mtDNA (Fig. 4). Assuming that this duplication occurred in a mitochondrial genome ancestral to both TK81-MS and TK81-O mtDNAs, subsequent events would necessarily have involved sequence divergence by way of base substitutions and small insertions/deletions, in order to account for the differences now existing between the mitotype-unique regions, s6 and n16 (Fig. 4).

As summarized in Table 2, members of the 16 short-repeat families proved to reside within nine Owen CMS-unique regions. Interestingly, the segments with high similarity to ten out of 16 different short repeats were found in the mitochondrial genome of normal sugar beet (TK81-O) and/or other plant species (Table 2). Hence an additional contributing factor that might explain the origin of Owen-unique regions could be the duplication and reshuffling of pre-existing mtDNA sequences.

Discussion

The mitochondrial genomes of normal and Owen male-sterile cytoplasms of sugar beet are highly rearranged relative to each other with dozens of inversional recombinations and other reshuffling events being required to interconvert the two genomes (Satoh et al. 2004). As the two mitochondrial genomes also proved to contain an abundance of short repeats (Kubo et al. 2000; Satoh et al. 2004), one can suppose that some of these repeats acted as substrates for infrequent homologous recombinations, leading to generation of mtDNA rearrangements characteristic of Owen CMS. The present study actually provided evidence that most of the inversional recombinations involve short repeats at their endpoints, though in other instances no repeats were found associated with inversion endpoints. It is also possible of course that in the latter instances either the short repeats were lost as the consequence of a number of mutations, or additional inversion mechanisms were involved as well.

Another focus of this study was to understand how the Owen CMS-unique mtDNA regions, which are absent from the normal mtDNA, were generated. It is known that much less DNA from the plastid genome as well as mitochondrial episomes is recognizably part of the mitochondrial genome compared with the integrated nuclear DNA sequences (Marienfeld et al. 1999; Kubo et al. 2000; Notsu et al. 2002). The same holds true for the Owen-unique regions: our homology search revealed that only 0.1 and 4.6% of the unique regions were sequences imported from the plastid and mitochondrial episomes, respectively. On the other hand, the search enabled us to identify 30 sequence stretches of nuclear origin that represent 17.9% of the unique regions. Intriguingly, additional sequences of nuclear origin were further located within the unique regions by Southern blot analysis. Nevertheless, the precise size of such sequences can not be determined until more sequence information about the sugar beet nuclear genome becomes available.

We should also note that the copies of many short repetitive DNA families were scattered throughout the Owen CMS-unique regions. Bendich (1985) proposed that

recombination within plant mitochondrial genomes around repetitive sequences would result in scrambling of mtDNA sequences on a fine scale. This type of recombination could duplicate repetitive regions, but continued scrambling would lead to divergence of the duplicated regions and a loss of homology, thereby contributing to the creation of species-specific mitochondrial sequences and genome expansion (Lilly and Harvey 2001). Analogous reasoning may be applied to generation of the Owen CMS-unique regions.

The origin of Owen CMS is an interesting and open question. Bonavent et al. (1989) speculated that in the past, a cross occurred between a CMS plant of the old garden-beet cultivar ‘Crapaudine’ and a sugar-beet plant, and some individuals of the progeny were collected by Owen. The Owen cytoplasm was also reported to be rarely found in wild beet populations growing along the French coasts (Laporte et al. 2001). This raises the possibility that a fertile progenitor to the Owen cytoplasm and/or the derived sister cytoplasms may be discovered in garden-beet landraces or wild beet accessions. Such cytoplasms, if any, would be valuable materials to gain a better understanding of how and when the Owen mitochondrial genome was created.

Acknowledgements This work was done in part at the Research Center for Molecular Genetics, Hokkaido University, and was supported in part by Grants in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and in part by the grant from Agriculture, Forestry and Fisheries Technical Information Society, Japan, as Research project for utilizing advanced technologies in agriculture, forestry and fisheries.

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Table 1 List of the Owen CMS (cv TK81-MS)-unique mtDNA regions and probes used in this study.

Unique region	Location in TK81-MS mtDNA[*a]	Probe	Location in TK81-MS mtDNA[*a]	Sequence homology	Hybridization to nuclear DNA
s1	134,996-138,596; 327,512-331,112	s1-1	135,776-137,022; 328,292-329,538	*Arabidopsis* At5g13390.1	+
		s1-2	137,450-138,341; 329,966-330,857	None	-
s2	333,206-334,696	s2-1	333,796-334,485	None	-
s3	216,003-217,589; 399,105-400,691	s3-1	399,158-399,939; 216,748-217,536	None	-
s4	197,601-199,714; 416,980-419,093	s4-1	416,995-418,014; 198,680-199,699	None	+
s5	420,781-422,560	s5-1	421,441-421,578	None	+
s6	423,360-423,474				ND[*b]
s7	473,401-481,067	s7-1	477,122-478,054	None	-
		s7-2	478,811-479,895	None	-
s8	6,500-14,482	s8-1	7,899-8,950	Retroelement	-
		s8-2	9,520-9,931	Retroelement	+
		s8-3	11,436-12,902	None	-
		s8-4	13,362-13,734	None	-
		s8-5	14,070-14,474	None	-
s9	54,254-55,017; 246,770-247,533	s9-1	54,388-54,658; 246904-247426	None	-
s10	92,241-98,174; 284,757-290,690	s10-1	93,577-94,822; 286,093-287,338	None	+
		s10-2	95,004-95,231;	Retroelement	+

			287,520-287,744		
		s10-3	97,664-98,004; 290,180-290,520	None	-
s11	99,579-109,077; 292,095-301,593	s11-1	101,177-10,1581; 293,694-294,097	Retroelement	+
		s11-2	102,580-103,634; 295,096-296,150	None	-
		s11-3	107,268-108,348; 299,784-300,864	Retroelement	+
s12	156,020-156,256	s12-1	156,020-156,256	None	-
s13	161,105-163,096	s13-1	161,112-162456	None	+

*a, Numbers correspond to nucleotide sequence in BA000024; *b, not determined.

Table 2 Repeated sequences found in the Owen-CMS unique regions.

Repeat (unique region)	Length (nt)	Frequency in TK81-MS mtDNA (times)	Description
Rep1 (s1)	103	4	Homologous sequence is present in TK81-O mtDNA
Rep2 (s2)	57	3	Homologous sequence is present in TK81-O mtDNA Frequently found in the upstream region of angiosperm *nad5-A*
Rep3 (s2)	59	2	Tandem duplicated in *Scox2-2*
Rep4 (s2, s6)	201	2	Homologous repeat family is present in TK81-O mtDNA A part of Rep4 is found in the upstream region of angiosperm *nad3*
Rep5 (s4)	122	4	Homologous sequence is present in TK81-O mtDNA
Rep6 (s4, s11)	169	4	No homologous entry is fished by BLAST search
Rep7 (s7)	62	3	Homologous sequence is present in TK81-O mtDNA
Rep8 (s7, s11)	75	3	No homologous entry is fished by BLAST search
Rep9 (s7)	431	3	Homologous sequence is present in TK81-O mtDNA
Rep10 (s8)	81	3	Homologous repeat family is present in TK81-O mtDNA A part of Rep10 is found in the upstream region of pea *ccb248*
Rep11 (s8)	101	2	Homologous repeat family is present in TK81-O mtDNA A part of Rep11 is found in the upstream region of angiosperm *atp1*, *atp6*, *nad9* and *cox2*
Rep12 (s8, s11)	193	3	No homologous entry is fished by BLAST search
Rep13 (s8)	344	3	Homologous sequence is present in TK81-O mtDNA
Rep14 (s10, s11)	54	4	No homologous entry is fished by BLAST search
Rep15 (s10,	55	3	No homologous entry is fished by BLAST search

s13)			
Rep16 (s11)	191	3	Homologous repeat family is present in TK81-O mtDNA

Figure legends

Fig. 1 Schematic illustration of the representative sequence blocks in TK81-O and TK81-MS mitochondrial master chromosome (based on Fig. 2 of Satoh et al. 2004). Four repeated sequence families found at the boundaries of homologous sequence blocks are shown by red, green, blue and purple arrows. Possible recombination events through these repeated sequences are in parenthesis. Unique regions n4, n16, s2 and s6 (see Fig. 4) are shown by vertical arrows.

Fig. 2 Organization of five Owen CMS unique regions, s1 (panel a), s5 (b), s7 (c), s10 (d) and s11 (e). Scale bar is shown below. BLAST search revealed that the unique regions contain sequence segments homologous to nuclear DNA (shown in blue), previously characterized mtDNA sequences (red) and mitochondrial episome (yellow). The extent of ORFs is indicated by open boxes. Their direction is from left to right for those above lines and from right to left for those below lines. Probes for hybridization experiments are shown by black horizontal bars. Repeated sequence families are shown by horizontal arrows. A vertical line in panel d indicates a sequence segment homologous to *Arabidopsis* and rapeseed mtDNA but not to TK81-O mtDNA.

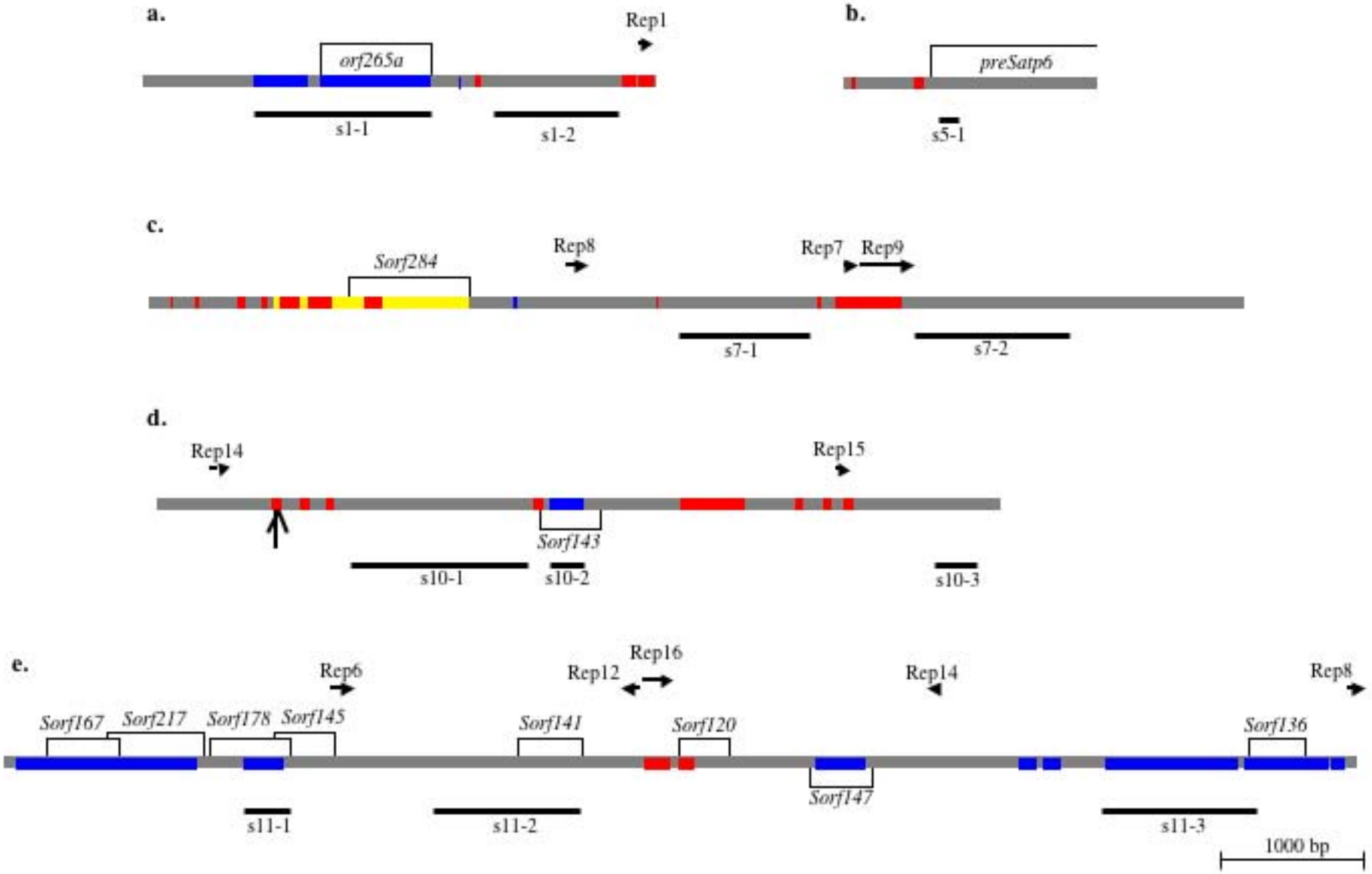
Fig. 3 DNA gel blot analysis using mtDNA of TK81-MS (lane a) and TK81-O (lane b), and total cellular DNA of TK81-O (lane c). Size marker is shown at the left of panels. One μg of mtDNA and five μg of total cellular DNA was electrophoresed. Restriction enzyme used in this experiment is *Eco*RI (s1-1, s8-2, s10-2 and s11-1) or *Eco*RV (s5-1 and s10-1).

Fig. 4 Duplication of a 201-nt-segment (Rep4) in TK81-MS mtDNA and generation of Owen CMS-unique region, s6. From top to bottom, schematic presentation of the upstream region of *nad3* in TK81-O, its corresponding region in

TK81-MS, the intergenic region between *atp6* and *orf114* in TK81-MS and its corresponding region in TK81-O. Sequence blocks are indicated by blue and green boxes. Shared sequences found upstream of *nad3* and in the *atp6/orf114* intergenic region from both TK81-MS and TK81-O mtDNAs are shaded. The location of repeated sequences , Rep4 is indicated by horizontal arrows. Scale bar is shown under the panel.

TK81-O

TK81-MS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Rep1
orf265a
s1-1
s1-2
b.
preSatp6
s5-1
c.
Rep8
Rep7
Rep9
Sorf284
s7-1
s7-2
d.
Rep14
Rep15
Sorf143
s10-1
s10-2
s10-3
e.
Rep6
Rep12
Rep16
Rep14
Rep8
Sorf167
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Sorf147
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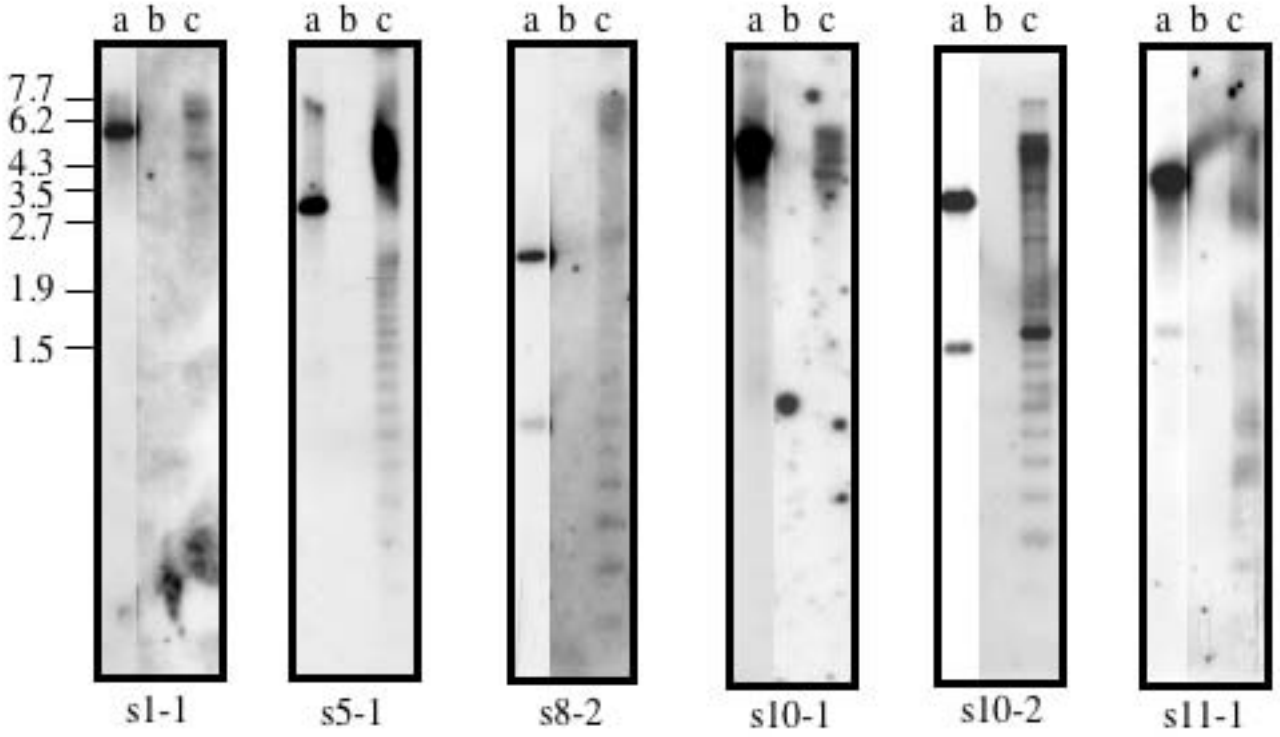
a b c
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6.2
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s5-1
s8-2
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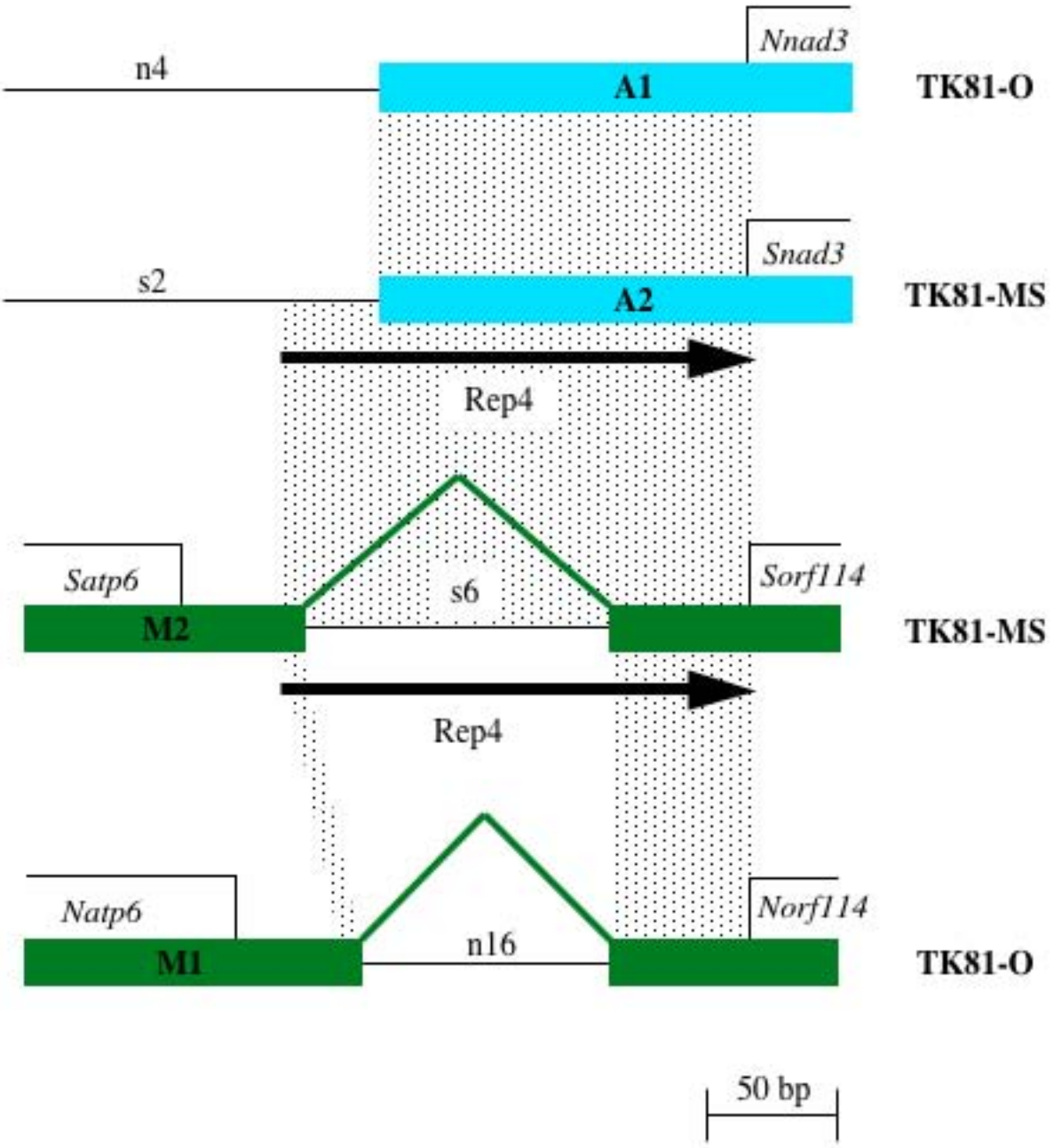
s11-1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A1
TK81-O
Snad3
s2
A2
TK81-MS
Rep4
Satp6
s6
Sorf114
M2
TK81-MS
Rep4
Natp6
n16
Norf114
M1
TK81-O
50 bp