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Minicircle variation in *Beta* mitochondrial DNA

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Summary. Mitochondrial DNAs from nine male fertile and eight cytoplasmic male sterile (cms) accessions of wild and cultivated *Beta* beets were investigated for the presence of low molecular weight DNA molecules. Five different supercoiled DNA molecules were detected, varying in size from 1.33 to 1.63 kb. Southern hybridizations revealed multimeric forms and sequence homologies between the minicircles. The occurrence of the different minicircles among the 17 accessions was investigated by agarose gel electrophoresis and Southern hybridization using minicircle specific probes. The 1.33 and 1.63 kb minicircles were found in most accessions, the other three minicircles were found in one or two of the wild *Beta* beet accessions. The presence of a low number of small, more or less homologous, minicircles in all investigated plants makes these molecules a general characteristic of *Beta* mtDNA. No association is found between the presence or absence of specific minicircles and the expression of male sterility. Neither does the distribution of the different minicircles in *Beta* beets indicate any essential biological role of these minicircles.

Key words: Cytoplasmic male sterility – Mitochondrial DNA – Plasmids – Sugar beet – *Beta*

Introduction

Many different low molecular weight (LMW) linear or circular DNA molecules have been found in the mitochondria (mt) of higher plants (see the review by Pring and Lonsdale 1985). These LMW molecules exist in addition to the high molecular weight (HMW) mt-genome and are thought to replicate autonomously of the HMW genome. An association between the presence of these plasmid-like molecules and the expression of cytoplasmic male sterility (cms) has been noted in some species, e.g. maize (Schardl et al. 1984, 1985), faba bean (Goblet et al. 1985), *Brassica* (Palmer et al. 1983), rice (Mignouna et al. 1987) and sugar beet (Powling 1981). However, a causal relationship between specific minicircles and male sterility has not yet been established in any species.

In sugar beet, studies of the minicircular (mc) complement of the mtDNA have concentrated on the differences between the male fertile (mf) and the cms cytoplasms used in sugar beet breeding today. Some cms cytoplasms originating from wild beets have also been compared with the previously known types (Mikami et al. 1986; Halldén et al. 1988). All cultivated cms lines examined possess a particular minicircle species, mc *a*, which is also present in most mf breeding lines. A second molecule mc *c*, is present in all mf lines, together with mc *d* in most cases and with mc *b* in some cases (Powling 1981; Powling and Ellis 1983). There is no homology between the minicircles and the main mitochondrial genome, while the minicircles show homology with each other (Hansen and Marcker 1984; Thomas 1986). Little is known about the origin of these molecules (see, however, Thomas 1986) and their function, if any, is unknown.

In this paper we describe the mtDNA minicircle composition and some basic characteristics of the mc-molecules in nine male fertile and eight male sterile cytoplasms originating from wild and cultivated *Beta* beets. Three of the male fertile sugar beet lines are old German cultivars and were chosen to represent the cytoplasmic variation present in fertile cultivated beets. Similarly, the five male fertile populations of wild *Beta* beets show a large morphological variation and were chosen from different locations in the eastern part of the Mediterranean. The seven male sterile lines with origins in wild *Beta* beets come from a survey of a large number of different lines

Table 1. Minicircle composition of mtDNA in different *Beta* beet materials

Line	Fertile/ sterile	Origin of cytoplasm	Minicircles (kb)				
			1.63	1.57	1.51	1.45	1.33
			a			*c*	*d*
SEK 81458	F	cv. Ero from Dippe	+			+	+
SEK 48521	F	cv. Optima from Dieckman	+			+	+
SEK 97962	F	cv. Erta from Klein Wanzleben	+			+	+
C8682	F	*Beta* sp. from Creta	+				+
C8731	F	*B.* sp. from Greece	+				+
C5634	F	*B.* sp. from Sardinia	+				+
C8735	F	*B.* sp. from Greece					+
C25261	F	*B.* sp. from Italy	+	+		+	+
I-12-75L	F	Maintainer line to Owen's cms	*			*	
I-12CMS (2)	S	*B.* sp. from Turkey; PI 164747		*	*		*
I-12CMS (3)	S	*B.* sp. from Pakistan; PI 177272	*				*
I-12CMS (4)	S	*B.* sp. from Turkey; PI 120704	*				
I-12CMS (5)	S	*B.* sp. from Turkey; PI 120705	*				
I-12CMS (6)	S	*B.* sp. from Iran; PI 140357	*				
I-12CMS (7)	S	*B.* sp. from Manchuria; PI 141919	*				*
I-12CMS (8)	S	*B.* sp. from Turkey; PI 169027	*				
I-12CMS (R)	S	Breeding line with the Owen-type cytoplasm	*				

Minicircles were classified by Southern blot hybridization (*) or by size, estimated from the linear forms (+). The Plant Introduction (PI) number refers to the USDA, Ames, Iowa collection

kept in the USDA, Ames, Iowa collection. The principal object of the study was to investigate the minicircle variation in fertile and cms cytoplasms of wild beets belonging to the section *Beta* and to evaluate the possible role of these molecules in the development of male sterility.

Materials and methods

Plant material

The following sugar beet lines, obtained from N.O. Bosemark, Hilleshög AB, Sweden, were studied: (a) the hybrid variety Primahill, carrying the *a* minicircle in the Owen male sterile cytoplasm and LNSZ 1, a normal fertile, multigerm population carrying minicircles *a*, *c* and *d* (Halldén et al. 1988); (b) three male fertile varieties – Ero, Optima and Erta from Germany; (c) five male fertile populations of wild *Beta* beets, C5634, C8735, C8682, C8731 and C25261. Included in the study were also the nine near-isogenic male sterile lines, I-12CMS (2) to (8), 1-12CMS (R) and 1-12-75L, which were kindly provided by Dr. R.K. Oldemeyer, Mono-Hy Sugar Beet Seed, USA. The information on the different lines is summarized in Table 1.

Analysis of mtDNA

Mitochondrial DNA was isolated as described in Halldén et al. (1988). Digestion with nuclease S1 was performed in 0.3 *M* NaCl, 0.03 *M* sodium acetate pH 4.6 and 0.001 *M* zinc sulphate. RNase treatment was performed in 0.01 *M* Tris pH 7.4, 0.001 *M* EDTA, and DNase treatment in 0.01 *M* Tris pH 7.4, 0.001 *M* EDTA, 0.01 *M* magnesium chloride. The mtDNA samples were electrophoresed (1.7 V/cm) through 1.5% agarose gels at room temperature for 14–16 h in 0.04 *M* Tris-acetate pH 7.8, 0.002 *M* EDTA. Plasmid pBR 322 DNA digested with HinfI or BglI and bacteriophage lamda DNA digested with HindIII were used as molecular size markers. Separated mtDNA samples were depurinated, denatured and transferred to Gene Screen membranes by electroblotting, as described previously (Halldén et al. 1988). Hybridization was performed as before (Halldén et al. 1988) but with a stringent wash at 68 °C in 1 × SSC. The probes were: (1) cloned minicircles *a* and *d* (Hansen and Marcker 1984), kindly given to us by M. Lund, Department of Molecular Biology and Plant Physiology, University of Aarhus, Denmark; (2) open circular forms of the 1.57 kb and the 1.51 kb minicircles and covalently closed circular forms of the *c* and *d* minicircles, all excised from low melting point agarose gels. The plasmid containing minicircle *a* was labelled by nick translation using ^{32}P dCTP to a specific activity of approximately $1-5 \times 10^7$ cpm/μg and used in the hybridization experiment described in Fig. 1. All other probes were labelled by multiprime labelling to a specific activity of $1-5 \times 10^9$ cpm/μg as described by the supplier (Amersham). Autoradiography was carried out at −80 °C for 4–24 h using Amersham MP Hyperfilm and intensifying screens.

Results

The molecular configurations of mc a

Gel electrophoresis of total mtDNA from sugar beet in 1.5% agarose gels separates the main mt-genome from a set of low molecular weight (LMW) DNA molecules. These LMW molecules are resistant to digestion with RNase A but disappear when treated with DNase I (Fig. 1, lanes 2 and 3). They are double-stranded, circular DNA molecules which are transformed from a covalently closed circular (c.c.c) form to open circular (o.c.) and linear forms upon treatment with nuclease S1 or with ultraviolet light in the presence of ethidium bromide.

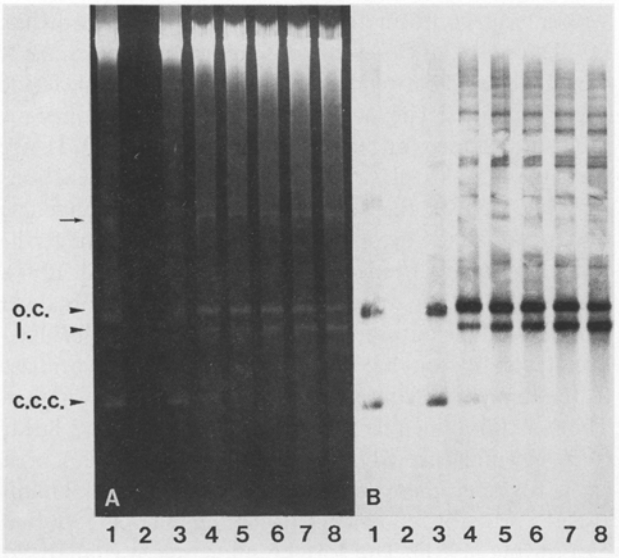

Fig. 1 A and B. Analysis of mtDNA from the cms line I-12CMS (R) by agarose gel electrophoresis **A** and hybridization to cloned mc *a* **B**. *Lane 1* – untreated; *lane 2* – treated with 1 μg DNase for 30 min at 20 °C; *lane 3* – treated with 1 μg RNase for 30 min at 20 °C; *lane 4* – treated with 5 U S1 nuclease for 10 min at 20 °C; *lanes 5–8* – treated with 5 U, 20 U, 40 U and 80 U, respectively of nuclease S1 for 30 min at 37 °C. *Arrow* indicates the RNA molecule

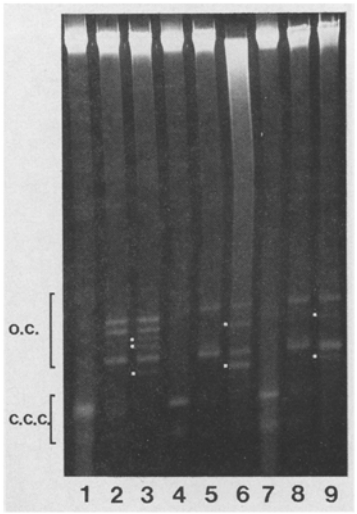

Fig. 2. Electrophoresis in an agarose gel of mtDNA from the cms lines I-12CMS (2): *lanes 1–3*; I-12CMS (3): *lanes 4–6*; I-12CMS (7): *lanes 7–9*. *Lanes 1, 4* and *7* represent untreated samples; *lanes 2, 5* and *8*, treatment with 5 U nuclease S1 for 10 min at 22 °C; *lanes 3, 6* and *9*, treatment with 100 U nuclease S1 for 30 min at 37 °C. Linear forms are indicated by *dots*

All cms breeding lines examined by Powling (1981) and Powling and Ellis (1983) possessed a single 1.63-kb minicircle, which they named mc *a*. Figure 1 shows mtDNA samples from another cms line, I-12CMS (R), digested with differing amounts of nuclease S1. The size of the resulting linear form of the only minicircular species was determined to be 1.63 kb by comparison with a set of marker fragments. A mild treatment with nuclease S1, 5U for 10 min at 20 °C (lane 4) transformed almost all of the DNA-molecules from their c.c.c. form to their o.c. form or, in low amounts, to their linear form. When the digestion was more extensive, 5–80 U for 30 min at 37 °C (lanes 5–8), the c.c.c. form disappeared completely and the number of linear molecules increased. However, it was not possible under these conditions to transform all molecules to a linear form by nuclease S1 treatment.

Southern blot hybridization of the gel in Fig. 1 A with an mc *a* specific clone (Hansen and Marcker 1984) confirmed that the studied molecule was mc *a* (Fig. 1 b). The hybridization also demonstrated the presence of molecules homologous to mc *a* but of higher molecular weight. These molecules disappeared after DNase treatment but were present in samples treated with nuclease S1 and, to a lesser extent, in samples treated with RNase A. We suggest that these molecules are oligomeric forms of minicircle *a* that poorly transferred to the membrane unless they were nicked by nuclease S1.

In the middle of the gel illustrated in Fig. 1 A (arrow) a distinct band can be seen which could be removed by RNase A but not by nuclease S1. It was also removed by the DNase preparation used in obtaining Fig. 1, but not when an RNase-free DNase preparation was used instead (data not shown). We presume that this band represents an RNA species of viral origin. This suggestion is compatible with the fact that the band could be seen in only some preparations and that the band never hybridized with any of the mc probes studied.

Variation in minicircle composition in mf and cms Beta beets

Eight male sterile breeding lines with cytoplasmic origins in wild beets and a male fertile type from the collection of Dr. R. K. Oldemeyer (lines labelled with I in Table 1) were screened for the presence of minicircles using gel electrophoresis and Southern blot hybridization with mc-specific probes. Another five breeding populations of wild beets and three varieties, all male fertile, were analysed by gel electrophoresis only. The results of the studies are summarized in Table 1 and Figs. 2, 3 and 4.

The mitochondria of five of the cms lines contained only mc *a* (Table 1). The mtDNAs of the other three cms lines, I-12CMS (2), I-12CMS (3) and I-12CMS (7) are shown in Fig. 2. The linear monomers found were size-determined to 1.63 kb and 1.33 kb for I-12CMS (3) and I-12CMS (7), which make them equivalent in size to mc *a* and *d*, respectively (Hansen and Marcker 1984; Thomas 1986). That the bands corresponded to mc *a* and mc

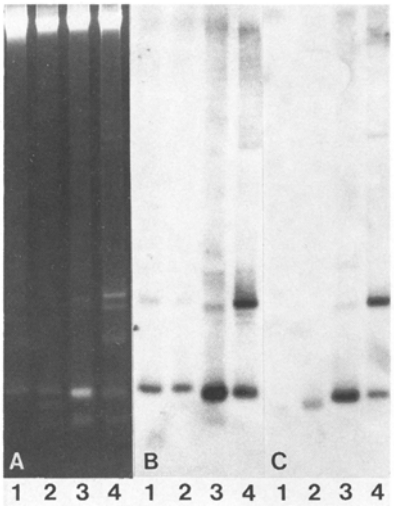

Fig. 3. **A** Analysis of the 1.57-kb and 1.51-kb minicircles by agarose gel electrophoresis, **B** hybridization to the isolated 1.57-kb minicircle or **C** to the 1.51-kb minicircle. *Lane 1*–cv. Primahill (mc *a*); *lane 2*–LNSZ 1 (mc *a, c, d*); *lane 3*–I-12CMS (2) carrying the 1.57-, 1.51-kb and *d* minicircles; *lane 4*–I-12CMS (2) treated with 5 U nuclease S1 for 10 min at 22 °C

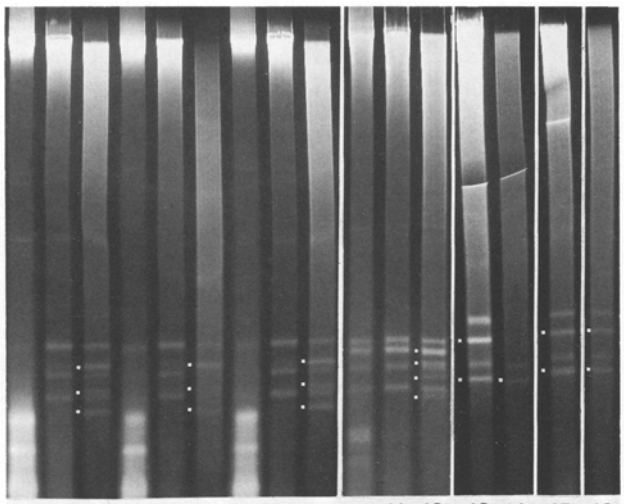

Fig. 4. Agarose gel electrophoresis of mtDNA from the male fertile lines SEK 8145 *lanes 1–3*; SEK 48521 *lanes 4–6*; SEK 97962 *lanes 7–9*; C 25261 *lanes 10–12*; C 5634 *lane 13*; C 8735 *lane 14*; C 8682 *lane 15* and C 8731 *lane 16*. *Lanes 1, 4, 7* and *10* represent untreated samples; *lanes 2, 5, 8* and *11*, treatment with 5 U nuclease S1 for 10 min at 22 °C; *lanes 3, 6, 9, 13–16*, treatment with 100 U nuclease S1 for 30 min at 37 °C. Linear forms are indicated by *dots*

d was confirmed by Southern blot hybridization with minicircle-specific clones (data not shown).

The cms line I-12CMS (2) contained, in addition to mc *d*, two mtDNA species of size 1.57 kb and 1.51 kb (Fig. 2, lane 3). Their reaction to S1 nuclease indicated that they are double-stranded, circular DNA molecules with a c.c.c. conformation in their native state. Powling (1981) reported the presence of a minicircle species, mc *b*, which migrated between mc *a* and mc *c* in two male fertile sugar beet lines. The mc *b*, cross-hybridized to cloned mc *a* but not to mc *c* or mc *d* (Powling and Ellis 1983). We used the 1.57-kb plasmid as a probe in hybridizations to I-12CMS (2), the variety Primahill which have the Owen male sterile cytoplasm with mc *a* and a male fertile type, LNSZ 1, with mc *a, c* and *d* (Halldén et al. 1988). Figure 3A shows the electrophoretic pattern obtained and Fig. 3B the autoradiograph that followed hybridization. It can be seen that the 1.57-kb probe only hybridizes to the minicircle from which it originated and to mc *a*. The 1.57-kb minicircle is transformed to o.c. and linear forms by nuclease S1, and multimeric molecules of it exist. Together, these data indicate that the 1.57-kb minicircle is probably equivalent to the *b* minicircle reported by Powling (1981). The 1.51-kb minicircle was analysed in a similar way. When used as a probe in Southern blot hybridization, it hybridized only to itself (c.c.c. and o.c. forms) and to mc *c* (Fig. 3C).

The minicircle composition in the lines I-12CMS (2) to I-12CMS (8) and I-12CMS (R) have been analysed earlier by Mikami et al. (1986). Although the electrophoretic pattern presented in Fig. 3 of their paper correspond to the equivalent patterns obtained by us, our interpretation of the results is different due to the added information we have from the investigations of nuclease S1 transformation of c.c.c. forms to o.c. and linear forms and from the minicircle-specific hybridizations. Mikami et al. (1986) were presumably under the impression that the nuclease S1 treatment they used (20–30 U nuclease S1 for 40 min at 37 °C) completely transformed the c.c.c. and o.c. molecules to linear forms. Consequently, they interpreted o.c. and linear forms of the same molecule as being different minicircles. However, the titration studies carried out by us and illustrated in Figs. 1, 2 and 4 showed conclusively that nuclease S1 does not fully transform *Beta* minicircles to linear molecules under the conditions used by Mikami et al. (1986). Instead our Southern blot hybridizations show the 1.2-kb and 1.1-kb molecules interpreted by Mikami et al. to be different minicircles [Mikami et al. (1986), Fig. 3, lanes 3 and 4] and are, in fact, the o.c. linear forms of mc *d* (Fig. 2, lane 6).

The male fertile line I-12-75L of the Oldemeyer collection was determined by hybridization to contain mc *a* and mc *c* (Table 1). The remaining three fertile varieties and five male fertile populations of wild beet origin were analysed for the presence of minicircles by gel electrophoresis only (Fig. 4). Minicircle sizes were compared with the linear forms of mc *a* (cv. Primahill) and mc *a, c* and *d* (breeding line LNSZ 1). Lines with three or more minicircles were also titrated with increasing amounts of nuclease S1 as described in Fig. 2.

The line C8735 contains only mc *d* (Fig. 4, lane 14). It has earlier been reported that there are lines that contain only one minicircle (Powling 1981; Thomas 1986). However, in all such cases mc *a* has been the single minicircle present. The other cytoplasms investigated here contain two or three different mc molecules, except the cytoplasm of C25261 which contains four minicircles, mc *a, c, d* and the 1.57-kb minicircle probably equivalent to mc *b* (Fig. 4, lanes 10–12). Lines with two types of minicircles all possess *a* in addition to *c* (I-12-75L) or *d* (C5634, C8682, C8731, Fig. 4, lanes 13, 15 and 16). The commercial varieties Ero, Optima and Erta all contain three minicircles: *a, c* and *d* (Fig. 4, lanes 1–9).

Characteristics of the minicircular mtDNA species

When mtDNA from *Beta* beets is titrated with nuclease S1, the c.c.c. forms of the different minicircles are gradually transformed to their corresponding o.c. and linear forms. Also oligomeric molecules are transformed by nuclease S1, as seen in Figs. 1 and 2. The lines I-12CMS (R) (Fig. 1), I-12CMS (3) (Fig. 2, lanes 4–6) and I-12CMS (2) (Fig. 2, lanes 1–3) contain one, two and three species of minicircles, respectively. When they are treated with nuclease S1, their oligomeric band patterns are changed and an increase in band complexity is seen. We believe that also in nature all minicircles exist in c.c.c. oligomeric forms. This was investigated by Southern blot hybridizations using minicircle-specific probes (Fig. 1 and results not shown), but it was not possible to determine which band corresponded to which conformation and meric number.

The *a* minicircle appeared to be more abundant than mc *c* and mc *d*, especially in untreated samples (Fig. 2, lanes 4–7). As this was less marked when o.c. and linear forms of the different minicircles were compared, the observations may be an artefact or may result from conformational differences between the different minicircles. This is currently under investigation.

The relative copy number of the different minicircles compared to the main mt-genome did not vary notably among the 17 investigated cytoplasms. Different cytoplasms in the same nuclear background (I-label in Table 1) contained different sets of minicircles, but the copy numbers of the different minicircles did not vary relative to the main mitochondrial genome. In some cases, the same cytoplasm, as determined by restriction analysis but in combination with different nuclear backgrounds, contained different sets of minicircles, e.g. SEK 81458 (*a, c* and *d*) and I-12-75L (*a, c*). Also, in these cytoplasms the relative copy numbers of the minicircles and the main mt-genome did not vary. We have, thus, not found any evidence that nuclear genes directly influence the presence of different mc or their copy number.

Discussion

Variation in minicircle composition

It has been suggested that the minicircles *c* and *d* are involved in the maintenance of pollen fertility in sugar beet (Powling 1981), since they at one time were absent from all investigated cms lines and present in all (mc *c*) or most (mc *d*) fertile lines investigated. However, we have previously shown that there are cms cytoplasms that contain the *c* and *d* minicircles (Halldén et al. 1988). And in the present paper two cms lines containing mc *d* in addition to mc *a* (Table 1) and one cms line containing the 1.57-kb and the 1.51-kb minicircles in addition to mc *d* are described. This means that with the exception of the 1.51-kb mc, to date detected in only one cms line, all types of minicircles found in male fertile beets have also been found in cms beets (*a*, 1.57 (*b*), *c* and *d*). Hence, there is no general correlation between the presence of any specific minicircle and the expression of male fertility or sterility in *Beta*.

All sugar beet plants investigated so far contain at least one minicircle. Mc *a* is most common and was found in 15 of the 17 accessions of wild and cultivated beets studied here. Powling (1981) found mc *a* in 16 out of 18 analysed sugar beet lines. Three out of the five cytoplasms which are known to lack mc *a* (Table 1, Powling 1981; Halldén et al. 1988) have mc *b* (1.57 kb). The two exceptions are the fertile line C8735 that has mc *d* only and the cms line C8640 (Halldén et al. 1988) that contains both mc *c* and mc *d*. In cytoplasms coming from wild *Beta* beets, mc *c* is the most rarely detected minicircle. We have not been able to see any clear geographic pattern in the distribution of the different minicircles.

There is a variation in minicircle composition among different lines of cultivated fertile beets, e.g. between I-12-75L (*a, c*) and cv. Ero, cv. Optima and cv. Erta (*a, c, d*). Powling (1981) also found the combination *b, c, d* in two different lines. Restriction analysis of these fertile lines (to be published) detected no differences between their HMW DNA. Thus, even if the normally highly variable main mt-genome shows no differences between lines, the mc complements may be different. In contrast, no minicircle variation has been detected in cultivated male sterile beets, all contain minicircle *a* only. This is a reflection of the common origin of all cultivated cms beets. The data collected on the distribution of the different mtDNA minicircles in wild and cultivated, male sterile and male fertile *Beta* beets, seem to us to not indicate any essential biological role of these minicircles.

Characteristics of the minicircular mtDNA species in Beta beets

Five different mitochondrial minicircles of *Beta* beets have been characterized. Monomeric c.c.c. and o.c.

forms were predominant in all preparations, but oligomeric forms were also detected. The different oligomeric molecules were readily transformed to o.c. and linear forms by treatment with nuclease S1. Multimeric forms of mc *a*, *c* and *d* were also found by Powling and Ellis (1983) and Thomas (1986). Multimeric forms of mitochondrial minicircles have also been detected in rice (Shikanai et al. 1987) and may thus be a general feature of mt minicircles. Sequencing of mc *a*, *d* (Thomas 1986) and *c* (Hansen and Marcker 1984) has revealed sequences common to all three DNA molecules. These homologies have also been demonstrated with Southern blot hybridizations (Thomas 1986; Halldén et al. 1988). In the present investigation, we have shown that mc *c* and the 1.51-kb mc are homologous. Thus, all minicircle species found in *Beta* beets show sequence homologies indicating either a common origin or that recombination has occurred between the different minicircle species. The occurrence of a low number of small (1.3–1.6 kb), more or less homologous minicircles in all accessions investigated makes these molecules a general characteristic of *Beta* mtDNA.

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