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Mitochondrial DNA duplication/deletion events and polymorphism of the C group of male sterile maize cytoplasms*

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Summary. Five accessions of members of the C group of male sterile maize cytoplasms (BB, C, ES, PR, and RB) in two nuclear backgrounds (A619 and A632) were examined to elucidate the nature of mitochondrial genome diversity within a related group of cytoplasms. Cosmid and plasmid clones carrying single copy and recombinationally active sequences from N and S cytoplasms of maize were used as probes. Although restriction patterns are quite similar, each of the five could be discriminated by evidence of sequence duplication and recombination, deletion of recombinationally active sequences of N, normal cytoplasm, population of minicircular DNAs, and by restriction patterns. Each member of the group carried a 1,913 bp minicircular mtDNA, while all entries but RB carried a 1,445 bp minicircular mtDNA. Members of the C group clearly are not molecularly identical; evolution of the group included principal genome reorganization involving sequence duplication/deletion events, apparently independent of the cms trait.

Key words: Cytoplasmic male sterility – Mitochondrial DNA – Recombination

Introduction

Sources of cytoplasmic male sterility (cms) in maize (*Zea mays* L.) are categorized into three major groups, T, C, and S, based on nuclear gene fertility restoration characteristics (see review by Laughnan and Gabay-Laughnan 1983). Each cytoplasm can also be identified by a characteristic mitochondrial DNA (mtDNA) restriction endonuclease pattern (Pring and Levings 1978).

Genetic, biological, and molecular studies indicate variation within the C and S cms groups. C cytoplasms (BB, C, ES, PR, and RB) are restored to fertility by the single dominant *Rf4* gene (Kheyr-Pour et al. 1981). Cytoplasm C (Charrua) is of Brazilian origin, while RB is from Paraguay, ES is from El Salvador, and BB and PR (Parana) are from Brazil. Members of the C group were subdivided into three subgroups by restriction endonuclease patterns: C cytoplasm; RB, BB and E cytoplasms; and ES cytoplasm (Pring et al. 1980). "Late-breaking", or pollen abortion late in microsporogenesis, was noted in the W182BN-RB source of RB (Kheyr-Pour et al. 1981). Five major subgroups within 25 accessions of the S group can be identified on the basis of a variable extent of fertility restoration in a series of nuclear backgrounds and by restriction endonuclease patterns (Sisco et al. 1985).

Several mtDNA regions of the N and S cytoplasms of maize have been identified which play a role in genome alteration. In Wf9(N), a 5 kb repeat adjacent to R1/R2 sequences within the principal genome was shown to participate in recombination, generating sub-genomic molecules (Lonsdale et al. 1981, 1983, 1984; Houchins et al. 1986). In the S cytoplasm, two copies of a sequence homologous to part of the R1 episome form a recombinationally active repeat. In addition these sequences recombine with free S1/S2 DNAs, generating linear mtDNA molecules (Schardl et al. 1984, 1985).

The five C cytoplasm accessions, related by restoration to fertility by *Rf4* and by very similar mtDNA restriction patterns, can be regarded as closely related, yet displaying limited molecular diversity. An examination of recombinationally active and single copy mtDNA regions was undertaken to elucidate characteristics of mtDNA evolution within this group.

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Matrials and methods

Genetic stocks

Cytoplasms C, RB, BB, ES, and PR in the inbred lines A619 and A632 were prepared by backcrossing at least six generations. A619 fully restores these cytoplasms to fertility, while A632 maintains sterility or restores partial fertility to selected members (Sisco et al. 1982). Wf9(C), B37(C)×NC236, and W182BN(C) were included in most experiments.

Preparation of MtDNA

MtDNAs were prepared, restricted, and electrophoresed according to previously published methods (McNay et al. 1983). MtDNA was difficult to prepare from A619 lines unless diethyldithiocarbamate was added to 5 mM in homogenization buffer.

Probes

Cosmids 2c11, 2c44, cSA61, and cSA51 were utilized as described (Lonsdale et al. 1981; Schardl et al. 1984, 1985). Selected fragments of these cosmids were subcloned in pUR2, pBR322, or pUC8. Clones of the maize minicircular mtDNAs of 1,913 bp and 1,445 bp were in pUC8 and pBR322, respectively (Ludwig et al. 1985; Smith and Pring, unpubl.). Nick translation, separation of labelled DNAs, and hybridization were as described (Maniatis et al. 1982; McNay et al. 1983).

Results

BamHI restriction endonuclease patterns

*Bam*HI restriction of mtDNAs of the five entries from the C group revealed only subtle visual differences (Fig. 1), compared to digestion with *Xho*I or *Hind*III (Pring et al. 1980). As observed previously, ES was visually distinguished from other entries by the shift of two closely spaced *Bam*HI fragments at 6.5 kb (Fig. 1, G, H). All entries in the A619 background are characterized by a lower copy number of a fragment at 2.3 kb (Fig. 1 A, C, E, G, I) compared to other backgrounds. This fragment is a 2.3 kb minilinear DNA, which carries no *Bam*HI site, and is discussed below. No other nuclear genotype effects on mtDNA organization were apparent within the entries.

Cosmid hybridization patterns

A series of cosmids from Wf9(N) and Wf9(S) mtDNAs (Lonsdale et al. 1981, 1984; Schardl et al. 1984, 1985) were used as probes. Each cosmid readily distinguishes N, C, S, and T mtDNAs (not shown) and thus span regions of the genomes which vary among cytoplasms. A

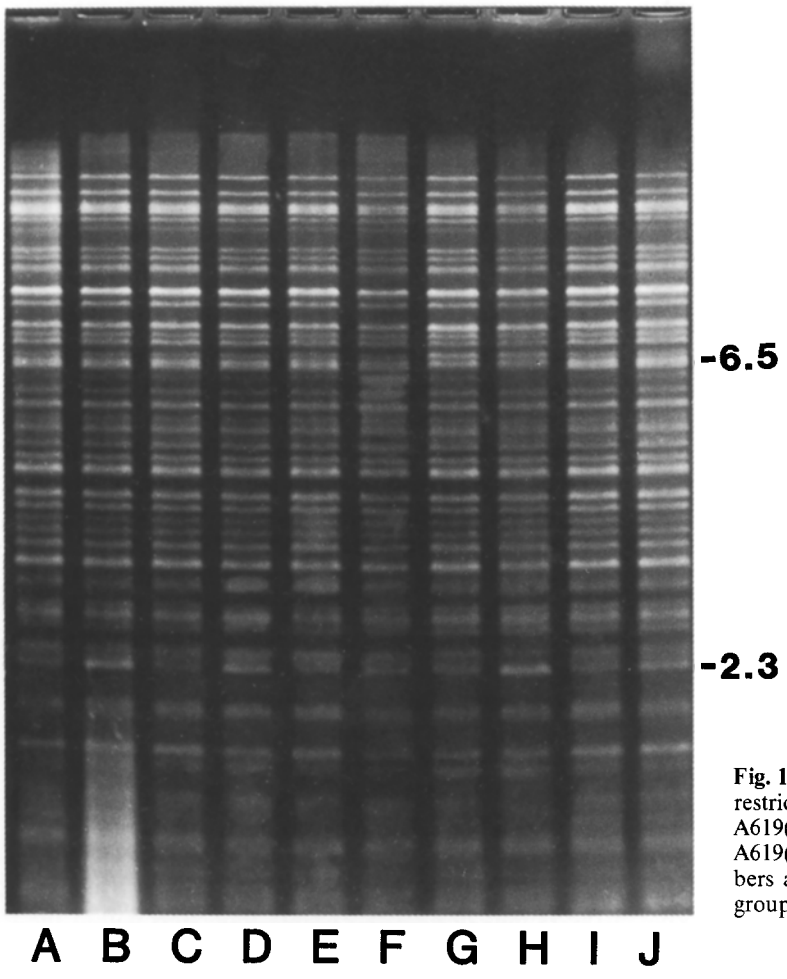

Fig. 1. Agarose gel electrophoresis patterns of *Bam*HI restricted mtDNAs of *A* A619(BB), *B* A632(BB), *C* A619(PR), *D* A632(PR), *E* A619(RB), *F* A632(RB), *G* A619(ES), *H* A632(ES), *I* A619(C), and *J* Wf9(*C*). Numbers are kb values of fragments which very within the group

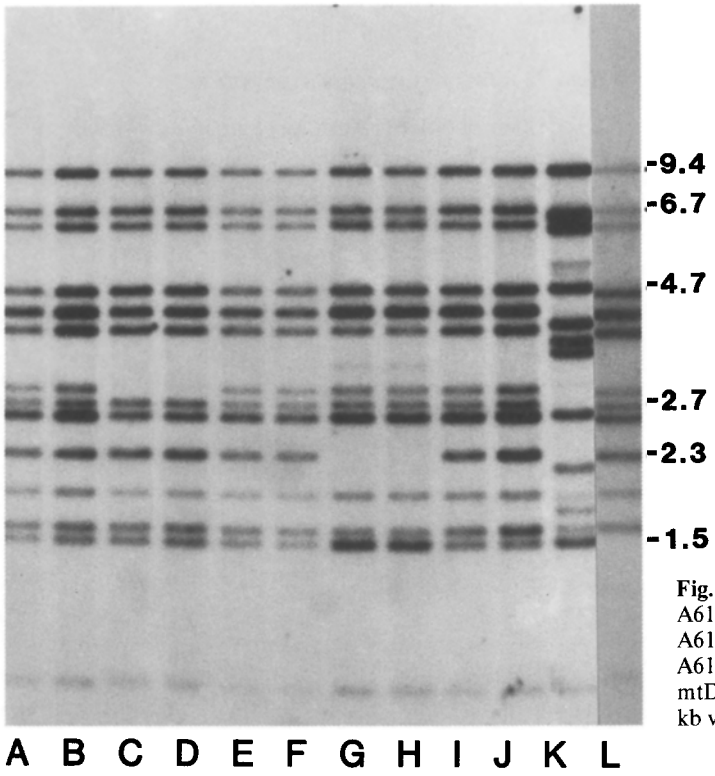

Fig. 2. Hybridization of cosmid 2c11 to *Bam*HI digests of *A* A619(BB), *B* A632(BB), *C* A619(PR), *D* A632(PR), *E* A619(RB), *F* A632(RB), *G* A619(ES), *H* A632(ES), *I* A619(C), *J* Wf9(C), *K* Wf9(N) and *L* B37(C)×NC236 mtDNAs. Slot *L* is from separate experiment. Numbers are kb values of selected fragments

detailed *Bam*HI map of cosmids 2c11 and 2c44 has been published (McNay et al. 1983).

Cosmid 2c11 was isolated from Wf9(N) mtDNA (Lonsdale et al. 1981), and carries a partial copy of the R1 episome, the probable progenitor of the S1 DNA molecule (Levings et al. 1983). The cosmid spans the "alpha-R1" site, carries the 5 kb repeat (coordinates 566.07-2.69; Lonsdale et al. 1984; Hodge and Lonsdale 1986), and a 1.72 kb *Bam*HI fragment which carries part of a 2 kb repeat (coordinates 15.9–17.14; Lonsdale et al. 1984; Hodge and Lonsdale 1986). Cosmid 2c11 is a recombinant through this region, and is from a 48 kb circle derived from recombination involving the 2 kb repeat. Hybridization of 2c11 to members of the C group (Fig. 2) indicated that PR (Fig. 2C, D) is characterized by the absence of a hybridizing fragment at 2.9 kb, and that ES (Fig. 2G, H) is characterized by the absence of hybridization at 2.3 kb, with a new, weakly hybridizing fragment at 3.2 kb. Other quantitative differences are observed, such as increased hybridization of ES (Fig. 2G, H) at 1.5 kb, and of Wf9(C) (Fig. 2J) at 1.8 kb. Wf9(N) mtDNA hybridization (Fig. 2K) was distinguishable from all C's examined. B37(C)×NC236 (Fig. 2L) is unique in the absence of the 1.5 kb fragment, with a minor fragment of ca. 1 kb apparent upon prolonged exposure.

Cosmid 2c44 carries most of the R2 DNA, the 5 kb repeat, and flanking regions, and is derived from the "alpha-R2" site (Lonsdale et al. 1981, 1983; Houchins et al. 1986). The alpha-R2 cosmids are recombinants, and do not exist in the master circle configuration (Lonsdale et al. 1984; Houchins et al. 1986). Hybridization of 2c44 to digests of C cytoplasm mtDNAs (Fig. 3) revealed that ES (Fig. 3G, H) is characterized by the absence of hybridizing fragments of 1.6 and 2.3 kb. Cytoplasm PR (Fig. 3C, D) is distinguished from other entries by the absence of a fragment hybridizing at 2.9 kb, also evident with other probes (Fig. 2C, D and Fig 5A, slot 6). The uppermost hybridizing band in A632(BB) (Fig. 3B) and Wf9(C) (Fig. 3J) is probably a partial digestion product.

Cosmid cSA61, which carries the sigma-sigma' sequences of S cytoplasm mtDNA (Schardl et al. 1984, 1985) and the gene cytochrome C oxidase subunit I (Isaac et al. 1985), distinguished only Cytoplasm ES (Fig. 4G, H) by the rearrangment of one *Bam*HI fragment at 6.7 kb. Cosmid cSA51 from S cytoplasm, which carries the psi-psi' sequences, did not differentiate within the C group (not shown). The two probes comprise ca. 60 kb of the S cytoplasm mtDNA, and hybridized to at least 25 C cytoplasm *Bam*HI fragments. Both cosmids readily distinguish N, C, T, and S cytoplasm mtDNAs.

Hybridization with selected plasmid clones

The origins of new or variable fragments within the C group were addressed by hybridization with cloned

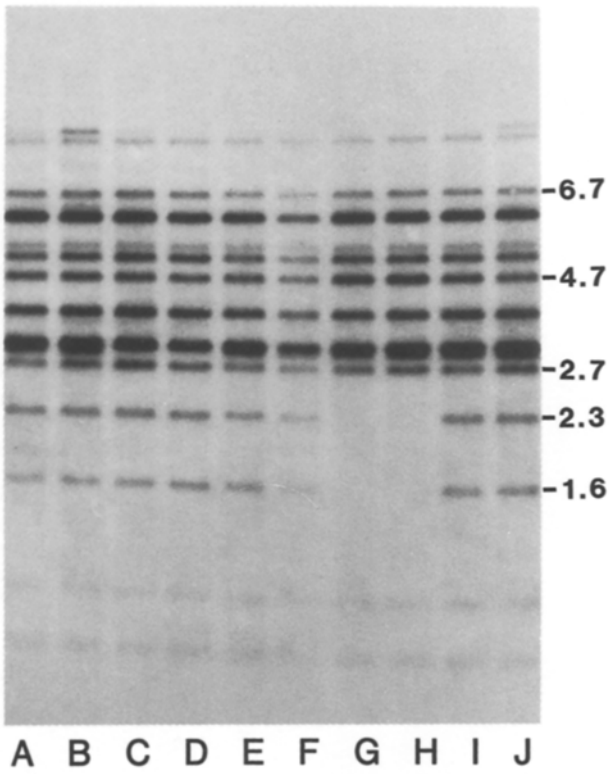

Fig. 3. Hybridization of cosmid 2c44 to *Bam*HI digests of *A* A619(BB), *B* A632(BB), *C* A619(PR), *D* A632(PR), *E* A619(RB), *F* A632(RB), *G* A619(ES), *H* A632(ES), *I* A619(C), and *J* Wf9(C) mtDNAs. Numbers are kb values of selected fragments

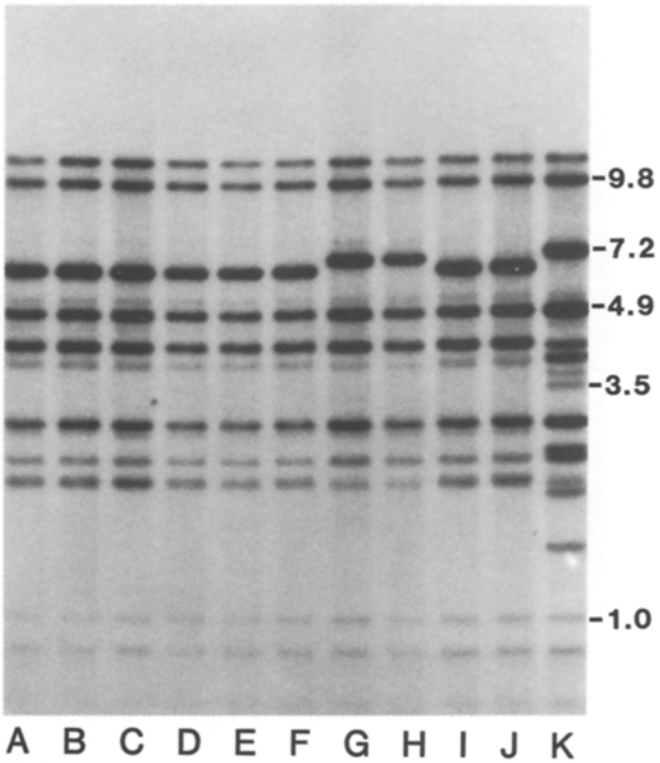

Fig. 4. Hybridization of cosmid cSA61 to *Bam*HI digests of *A* A619(BB), *B* A632(BB), *C* A619(PR), *D* A632(PR), *E* A619(RB), *F* A632(RB), *G* A619(ES), *H* A632(ES), *I* A619(C), *J* Wf9(C), and *K* Wf9(N) mtDNAs. Numbers are kb values of selected fragments

fragments from the 2c11 and 2c44 cosmids (Lonsdale et al. 1981; McNay et al. 1983). Fifteen *Bam*HI clones were hybridized to digests of N, C, T, and S mtDNAs, and to C, ES, RB, BB, and PR mtDNAs. We include here examples which are not conserved among cytoplasms, or vary within the C group. Fragments of 4.4 and 0.95 kb in N cytoplasm include homologies to S1/R2, and S2 (Lonsdale et al. 1981). Since major sequences of S1/S2 are not observed in these regions in C, S, or T cytoplasms (unpubl.), these clones are diagnostic for the four major cytoplasms, and are not included here. In that no nuclear genotype effects were detected, only representative data are presented.

*Bam*HI *3.5.* This fragment carries part of the 5 kb repeat, and over 90% of the fragment is homologous with the repeat (Houchins et al. 1986). The fragment spans the junction with the unique "alpha" sequence. Hybridization to Wf9(N) mtDNA (Fig. 5 A, slot 1) reveals homology to the fragment of origin and to a 3.6 kb fragment, which represents the "beta" copy of the 5 kb repeat. The 3.5 kb fragment is conserved in the S cytoplasm (Fig. 5 A, slot 2), and minor hybridization is apparent at 2 kb. T cytoplasm carries a single fragment of 6.7 kb (Fig. 5 A, slot 3). Fragments of 2.9, 2.7, and 1.6 kb are observed in BB, C, or RB (Fig. 5 A, slot 4). The 2.9 and 1.6 kb fragments were variable within the group, as indicated by hybridization with cosmids, and as shown by loss of the 1.6 kb fragment in ES (Fig. 5 A, slot 5), and of the 2.9 kb fragment in PR (Fig. 5 A, slot 6; Fig. 2 C, D).

*Bam*HI *2.1.* This fragment is contiguous with the 3.5 kb fragment in the cosmid 2c44, carries the remainder of the 5 kb repeat and approximately 600 bp of sequences homologous to the S2 episome (Levings and Sederoff 1983; Houchins et al. 1986). Sequences homologous to this clone were absent in ES (Fig. 5 B, slot 1) or occurred at 2.3 kb in BB, C, PR, or RB (Fig. 5 B, slot 2). The probe hybridized to the junction fragment of the 5 kb repeat at 3.9 kb in N (Fig. 5 B, slot 3). The missing fragment in ES is also apparent with the 2c11 probe, which carries the 3.9 kb *Bam*HI fragment, and with the 2c44 probe, which carries the 2.1 kb *Bam*HI fragment (Figs. 2 and 3). Presence of only one hybridizing fragment, or absence of the sequence, indicates lack of participation of these sequences in recombination in the C group.

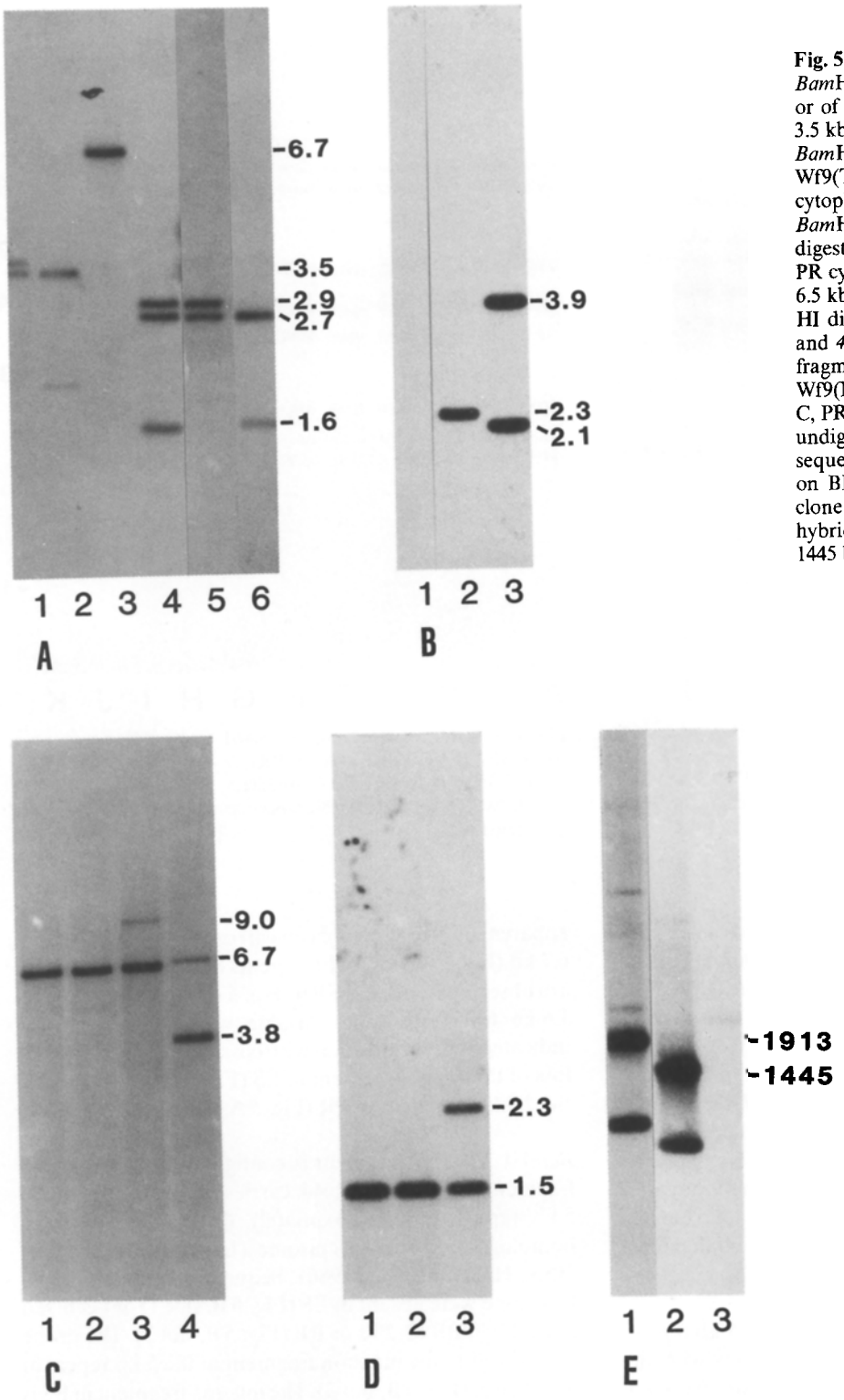

Fig. 5A–E. Hybridization of clones of *Bam*HI fragments of cosmids 2c11 or 2c44, or of minicircular mtDNAs, to mtDNAs. **A** 3.5 kb *Bam*HI fragment hybridized to *Bam*HI digested *1* Wf9(N), *2* Wf9(S), *3* Wf9(T), *4* BB, C, or RB cytoplasms, *5* ES cytoplasm, and *6* PR cytoplasm. **B** 2.1 kb *Bam*HI fragment hybridized to *Bam*HI digested *1* ES cytoplasm, *2* BB, C, RB, or PR cytoplasms, and *3* Wf9(N) cytoplasm. **C** 6.5 kb *Bam*HI fragment hybridized to *Bam*-HI digested *1* Wf9(N), *2* Wf9(S), *3* Wf9(T) and *4* Wf9(C) cytoplasms. **D** 1.5 kb *Bam*HI fragment hybridized to *Bam*HI digested *1* Wf9(N), Wf9(T), or Wf9(S), *2* ES, and *3* BB, C, PR, or BB cytoplasms. **E** hybridization of undigested mtDNAs to *1* a clone carrying sequences of the 1913 bp minicircular DNA on BB, C, ES, PR or RB cytoplasms, *2* a clone of the 1445 bp minicircular DNA hybridized to BB, C, ES, or PR, and *3* the 1445 bp clone hybridized to RB cytoplasms

*Bam*HI *6.5 kb.* This fragment is a single copy region in N cytoplasm, and is conserved in T and S cytoplasms (Fig. 5 C, slots 1–3); in the former, part of this fragment and adjacent sequences are duplicated (Dewey et al. 1986; Wise et al. unpubl.). The gene *atp6* (Dewey et al. 1985) is located within the 6.5 kb fragment but not within the portion which is duplicated in T cytoplasm. Recombination through this repeat in T generates a 9 kb fragment, detected by the 6.5 kb probe (Fig. 5 C, slot 3). C cytoplasms carry two hybridizing fragments; major homology is in a fragment of 3.8 kb and less homology occurs at 6.7 kb (Fig. 5 C, slot 4). A 2.7 kb *Hind*III probe, which includes approximately 1.2 kb of duplicated region of the 6.5 kb *Bam*HI fragment, hybridized to the 6.5 and 9.0 kb fragments of T mtDNA, and to the 3.8 and 6.7 kb fragments of C mtDNA. It is therefore clear that unique sequences associated with this region in N mtDNA are duplicated in C and T cytoplasm mtDNAs. No variation was observed within the C group.

*Bam*HI *1.5.* This fragment is represented as a single copy fragment in N, T, and S cytoplasms (Fig. 5 D, slot 1), and ES cytoplasm (Fig. 5 D, slot 2) but is duplicated at 2.3 kb in BB, C, PR, and RB cytoplasms (Fig. 5 D, slot 3).

Other probes. Clones carrying sequences of the 1 kb repeat (Lonsdale et al. 1984) showed no variation within the C group. A clone carrying part of the 2 kb repeat revealed variation from N, but not within the C group (not shown).

Minicircular and minilinear DNAs

Visual examination of gels of undigested C cytoplasm mtDNAs indicated the presence of both minicircular and minilinear DNAs. To survey the distribution of the minicircular DNAs, a *Bam*HI clone comprising 1.72 kb of the 1,913 bp minicircular DNA (Ludwig et al. 1985) and an *Eco*RI clone of the 1,445 bp minicircular DNA were hybridized to non-digested mtDNAs. The 1,913 bp DNA was present in all lines examined (Fig. 5 E, slot 1) with supercoiled, open circular and linear, and multimer forms of the DNAs detected. The 1,445 bp DNA was represented in all cytoplasms but RB (Fig. 5 E, slots 2, 3). We did not observe hybridization of the probe to the principal mtDNA. Prolonged exposure detected minor homology at 1.6 kb; the two minicircular DNAs share 62 bp of perfect homology (Smith and Pring in prep.), but we cannot ascribe this hybridization to the 1,913 bp DNA. No genotype effects on copy number of these DNAs was observed.

Visual examination of gels of undigested mtDNAs and *Bam*HI restricted DNAs suggested variation in copy number of a 2.3 kb linear DNA (Fig. 1), which has no *Bam*HI site. This variation was correlated with nuclear genotype; BB, ES, PR, and RB were available in A632 and A619, while C was examined in A619 and Wf9. In each case, the apparent copy number of the 2.3 kb DNA was higher in A632 than in A619. To quantitate copy number, laser densitometry of negatives from undigested, ethidium bromide-stained gels was performed. In the four examples of cytoplasms in A632 and A619, A632 yielded ca. 2.5-fold higher copy number of the 2.3 minilinear DNA than did A619. Wf9(C) was similar to A632, which suggests that the A619 background is associated with a lowered copy number of the molecule.

Discussion

The five entries of the C cytoplasm group can be differentiated on the basis of mtDNA restriction endonuclease patterns, hybridization with specific sequence probes, and population of minicircular DNAs. Cytoplasm ES, which seems particularly divergent, is distinct from all others with cosmids 2c11, 2c44, and cSA61, and with clones of the 3.5, 2.1, and 1.5 kb *Bam*HI fragments. Cytoplasm PR is distinct from all others with cosmids 2c11 and 2c44, and with the 3.5 kb BamHI fragment. Cytoplasms RB and BB can be distinguished from all others by *Hind*III digests (Pring et al. 1980); cytoplasm RB can be distinguished from BB and all others by the absence of a 1,445 bp minicircular DNA in the former. Cytoplasm C can be distinguished from others by *Hind*III or *Xho*I digestion (Pring et al. 1980), and by the altered 1.5 kb fragment in B37(C) × NC236. It seems possible that additional entries could be differentiated by other enzymes and probes.

The cumulative data establish that at least five subgroups of the C cytoplasm, and one variant designated C, are not repeated introductions of the same cytoplasms in diverse lines. Male sterile cytoplasms are identified among collections by crossing entries as female with pollen parents of known fertility restoration genotype, such as described by Beckett (1971) and Gracen and Grogan (1974). Entries responding to *Rf4* are categorized as "C", but can be of diverse geographic origin. It is not surprising to detect genomic structure variation even within one group of male sterile cytoplasms in consideration of the size of the genome and the recombinations documented for N cytoplasm mtDNA (Lonsdale et al. 1984). MtDNA polymorphisms within the S group of male sterile cytoplasms correlate with genetic and biological variation (Sisco et al. 1985). Conceptually, these variations may be considered analogous to variation among mtDNAs of normal, male fertile cytoplasms (Levings and Pring 1977; McNay et al. 1983).

The 1,913 (Ludwig et al. 1985) and 1,445 (Smith and Pring in prep.) bp mtDNAs represent two of 10 major extrachromosomal minicircular or minilinear mtDNAs described in *Zea* to date, and each of the 10 is absent within one or another entry (Pring and Smith 1985). Since neither of the two minicircular DNAs is universally present in *Zea*, they may be considered optional or dispensable. Certainly, absence of the 1,445 bp DNA in RB, with no evidence of hybridization to the principal mtDNA, indicates that the molecule is not associated with C cms. In agreement with observations of polymorphism within the principal

mtDNA, we interpret the deletion of the 1,445 bp DNA as evidence of evolution of the group independent of sequences which are associated with the cms trait. We did not observe nuclear genotype effects on principal mtDNA configuration, population or copy number of the minicircular mtDNAs, but did detect reduced copy number of the 2.3 kb minilinear DNA in the line A619. This line is a restorer of C cytoplasms (Sisco et al. 1982), but no correlation with copy number of the 2.3 kb DNA is inferred.

This study surveyed only approximately 100 kb of the C mtDNA genome, and included sequences of the 5 kb repeat, flanking regions, and regions carrying homology to the terminal inverted repeats of the S1/S2 DNAs of S cytoplasm. Only one C entry, ES, displayed variation with the cSA61 and cSA51 S cytoplasm cosmids, showing rearrangement of one 7 kb *Bam*HI fragment of 25 hybridizing fragments in the ca. 60 kb represented by the two cosmids. Six major repeats characterize the Wf9(N) mtDNA (Lonsdale et al. 1984), and the repeats not examined here may also may be rearranged in C cytoplasms. We cannot interpret these data to indicate a genomic region which is particularly pivotal to the evolution of maize mtDNA, but it is clear that the 5 kb repeat and flanking regions have been actively involved in generating polymorphism in the species. Normal, male fertile cytoplasm mtDNAs exhibit variation in the "Beta" copy of this repeat (Lonsdale et al. 1983; McNay et al. 1983). Sequencing of the repeat (Houchins et al. 1986) revealed no striking sequence motifs, and a general recombination mechanism was invoked for the role of the repeat in genomic recombination.

The five entries of the C group, in addition to sharing restoration of the cms trait by the nuclear *Rf4* gene, display similar restriction profiles. Thus they can be considered as a related collection of cytoplasms, and could provide insight into evolutionary processes which generate diversity of maize mtDNA. In the absence of a physical map of the C mtDNA genome, we cannot determine genome organization and an assessment of sub-genomic circles. The data does allow the limited conclusion that recombination events involving sequences known to participate in recombinational events in N cytoplasm are similarly involved in the evolution of, and within, the C cms group. It is evident from these data and related studies, that each male sterile cytoplasm, and subgroups within a cms group, may have evolved through recombination events resulting from repeated sequences found in N cytoplasm, and additionally, through duplication and recombination of apparent single copy sequences in N. I. E., the C subgroups vary in sequences detected with the 1.5 and 6.5 kb *Bam*HI fragments, which are not duplicated in the N cytoplasm master chromosome. Conversely, repeated sequences in N cytoplasm may be represented as single copy sequences in other cytoplasms. Thus, the evolution of the genomes may involve duplication of a sequence with resultant recombination, or deletion of one copy of a repeat with resultant "fixing" of a genomic configuration. In comparing a number of such probes on N, C, T, and S cytoplasms, we have observed numerous situations where apparent single copy sequences in N are dispersed and/or duplicated in male sterile cytoplasms, similar to data from randomly chosen N mtDNA clones hybridized to other *Zea* entries (Sederoff et al. 1981).

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