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Unique plasmid-like mitochondrial DNAs from indigenous maize races of Latin America

(maize systematics/restriction endonuclease fragment analysis)

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ABSTRACT **Mitochondrial DNA from 81 races of Latin American maize were examined by agarose gel electrophoresis. Twelve South American races each contained two plasmid-like mtDNA molecules similar to those of the cytoplasmic male-sterile S type (*cms*-S). The plasmid-like elements from all 12 races, designated RU, appear to be identical. Both molecules appear *in vitro* as double-stranded linear DNAs terminated by repeated sequences arranged in reverse polarity (terminal inverted repeats). The larger molecule of the pair, R-1, contains about 7460 nucleotides. It shares considerable homology with the larger plasmid-like molecule of *cms*-S, S-1, but is about 1000 nucleotides longer than S-1, has a unique sequence of about 2576 nucleotides, and also contains a *Bam*HI recognition site not present in S-1. R-2, the smaller plasmid-like element, consists of about 5450 nucleotides and appears to share complete homology with S-2, the smaller plasmid-like molecule of *cms*-S. Neither pollen sterility nor any other trait has been associated with the R-1 and R-2 plasmid-like mtDNAs. The *Bam*HI restriction fragments of total mtDNA from the 12 RU cytoplasms display similar patterns, which differ only slightly but vividly from that of a normal maize standard, B73 × Mo17. *Bam*HI restriction analysis of 22 additional races produced arrays similar to those of the RU cytoplasms, but which lacked plasmid-like mtDNAs. The taxonomic significance of this digestion pattern and of the RU cytoplasms is discussed. One Mexican race, Conico Norteño, has been shown to contain the *cms*-S cytoplasm.**

The mitochondrial genome of maize, *Zea mays* L., contains a heterogeneous array of DNA molecules, probably existing as covalently closed circular molecules ranging from 0.6–90 × 10^6 daltons (1). The frequency and sequence arrangement of the variously sized molecules in the mtDNA complement varies among the four major cytoplasmic groups of fertile normal (N) and the C, S, and T male-sterile cytoplasms (1–8). Plasmid-like mtDNAs have been reported only in S cytoplasmic male-sterile (*cms*-S) maize (4, 7, 8). These DNAs have molecular weights of about 4.05 × 10^6 and 3.45 × 10^6 and are designated S-1 and S-2, respectively. Each of these molecules has a terminal inverted repeat in which identical DNA sequences lie in reverse polarity at either end of the molecule. Inverted repeats are common in the mechanism by which transposable elements are inserted into bacterial chromosomes (9).

In the absence of the dominant male fertility-restoring allele *Rf*3 in the nucleus, the S male-sterile cytoplasm normally results in male sterility (10). However, in certain cases a spontaneous reversion to pollen fertility occurs (11). Recently, Levings *et al.* (12) have shown that spontaneous reversion is associated with what appears to be a transpositional event in the mitochondrial genome involving the S-1 and S-2 plasmid-like molecules. In the revertant strains, these plasmid-like DNAs are no longer found as free elements, while new endonuclease restriction fragments, not found in *cms*-S, appear. It was suggested that the new sequences in the mitochondrial genomes arose by incorporation of the plasmid-like DNAs and that the plasmid-like DNAs might have arisen by excision from the mitochondrial genome, an event which could be linked with the origin of *cms*-S. The unique properties of these plasmid-like DNAs suggest their use as vehicles for inserting desirable DNA sequences into the maize genome (13).

The objectives of this study were to determine if (*i*) plasmid-like DNAs exist in indigenous races of maize, and (*ii*) the characteristics and distribution of the plasmid-like DNAs are associated with biosystematic relationships among these races.

MATERIALS AND METHODS

Purification of mtDNA. Mitochondria were isolated from dark-grown maize seedlings, and the mtDNA was isolated from Sarkosyl/proteinase-K lysates of mitochondria as described (2). The plasmid-like DNAs, S-1, S-2, R-1, and R-2, were purified by electrophoresing whole mtDNA preparations in 0.8% agarose gels and excising the appropriate DNA species. DNAs were electroeluted from gel segments (14).

Agarose Gel Electrophoresis. Approximately 1-μg samples of mtDNA were electrophoresed at room temperature in horizontal 0.8% agarose gels prepared as described (4). Samples were electrophoresed at 1.6 V/cm for 17 hr. Mitochondrial DNAs were digested with *Bam*HI endonuclease (New England BioLabs) for 1 hr at 37°C according to the supplier's protocol, and the restriction fragments were separated by gel electrophoresis. The gels were stained in water containing ethidium bromide (0.5 μg/ml) for 30 min, illuminated with short-wave UV light, and photographed through a Wratten 23A filter on Polaroid type 55 positive/negative film.

Determination of Molecular Size. DNA to be sized was mixed with *Hin*dIII-digested λ phage DNA (New England BioLabs) and fractionated by electrophoresis in 0.8% agarose gels. An extension of Southern's method (15) has been developed (16) to fit a hyperbolic relationship for migration distance and molecular weight.

Electron Microscopy. Bands containing plasmid-like DNAs were cut from electrophoretic gels, extracted by the "freeze-squeeze" technique (17), dialyzed against 10 mM Tris/0.5 mM EDTA, and mounted for electron microscopy by the formamide technique (as modified from refs. 18–20). In some cases, DNA was alkaline denatured and rehybridized before mounting for electron microscopy. Microscopy was performed on a Siemens Elmiskop 1A electron microscope at magnifications of ×10,000–20,000. Either φX174 RFII DNA (open circles with contour length of 5375 nucleotides) or the *cms*-S plasmid-like

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Abbreviation: *cms*, cytoplasmic male-sterile.

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DNAs S-1 and S-2 (with lengths of about 6450 and 5450 nucleotides, respectively) were used as internal size standards. Projected images of micrographs were traced and measured as described (4).

Determination of Pollen Fertility. Pollen was stained with 1% potassium iodide and examined microscopically. Plants that shed pollen were counted as partially or fully fertile.

Maize Stocks. Seed was produced by open pollination of collections of maize tracing back by direct female lineage to those originally classified in the "Races of Maize" bulletins (21–29). The single-cross hybrid B73 × Mo17 was used as the normal standard for comparisons and as a control in electrophoretic gels.

RESULTS

Electrophoretic Analysis. Agarose gel electrophoresis of mtDNAs (Fig. 1*A*) resolved a single, broad, slowly migrating band containing high molecular weight DNAs in all collections surveyed. In addition, all mtDNAs contained small highly mobile bands previously reported (1, 7, 8) to be small circular DNA molecules (minicircles) of less than 2000 nucleotides in length.

One collection, the race Conico Norteño of highland Mexico, contained plasmid-like DNAs with electrophoretic mobilities identical to those of the slowly (S-1) and rapidly (S-2) migrating plasmid-like elements of *cms*-S. *Bam*HI restriction of this mtDNA resulted in a fragment pattern identical to that of *cms*-S mtDNA (Fig. 2).

Twelve of the races screened, Marron (Argentina, ARG 468), Coroico (Bolivia, BOV 1063), Enano (BOV 1036), Kcello (BOV 948), Morotí (BOL II), Pollo (Colombia, CUN 443), Mishca (Ecuador, ECU 321), Racimo de Uva (ECU 398), Mochero (Peru, LBQ 14), Aragüito (Venezuela, VEN 628), Chirimito (VEN 529), and Guaribero (VEN 653), contained two plasmid-like DNAs, R-1 and R-2, very similar to those found in *cms*-S. This group of cytoplasms, designated "RU," was previously designated "S*" (30).

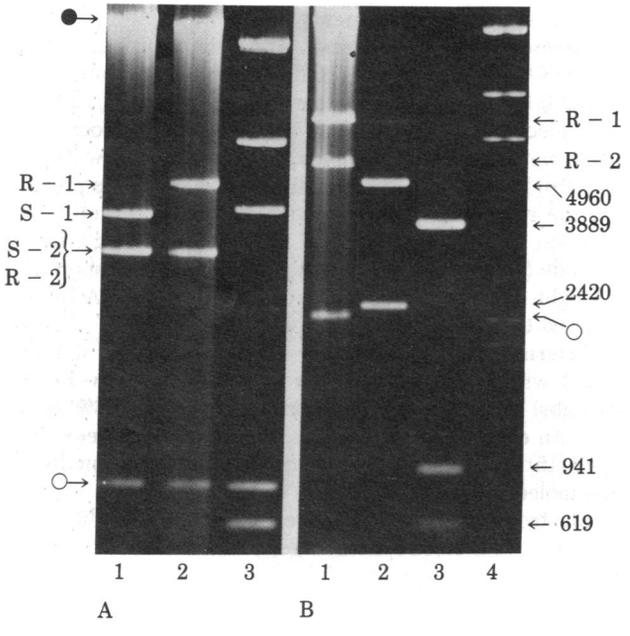

FIG. 1. (*A*) Agarose gel electrophoresis of DNA from *cms*-S maize mtDNA (lane 1); RU maize mtDNA (lane 2); and *Hin*dIII digestion fragments of λ phage DNA as size standards (lane 3). ●, High molecular weight chromosomal mtDNA; ○, low molecular weight circular mtDNA. (*B*) Agarose gel electrophoresis of undigested RU mtDNA (lane 1); isolated R-1 digested with *Bam*HI endonuclease (lane 2); isolated R-2 digested with *Bam*HI endonuclease (lane 3); and *Hin*dIII digestion fragments of λ phage DNA (lane 4). Numbers indicate approximate size of fragments expressed in nucleotides. ○, Low molecular weight circular mtDNA.

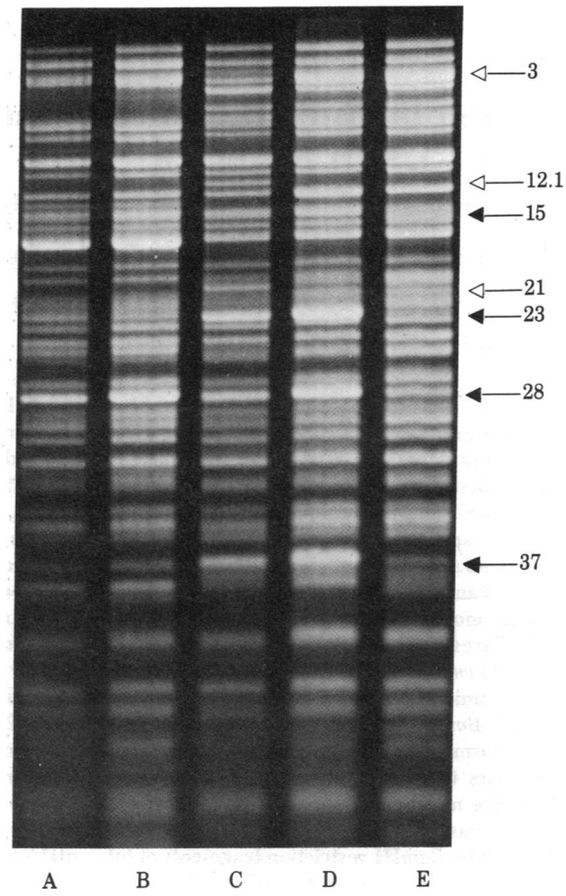

FIG. 2. Agarose gel electrophoretic patterns of *Bam*HI restriction endonuclease digests of maize mtDNAs. Lanes: A, *cms*-S; B, Conico Norteño (*cms*-S); C, Coroico (atypical RU); D, Chirimito (RU); E, single-cross hybrid (B73 × Mo17). Open arrows indicate fragments distinguishing Coroico digestion pattern from that of typical RU; solid arrow indicates distinct features of RU restriction fragments (i.e., absence of band 15; intense bands 23, 28, and 37).

The smaller of these newly discovered molecules, R-2 (originally designated S-2*), has an electrophoretic mobility (Fig. 1*A*) identical to that of S-2 of *cms*-S. Each molecule has a molecular length of about 5450 nucleotides. R-1 (formerly S-1*) migrated more slowly than R-2, S-2, or S-1. It has a molecular length of about 7460 nucleotides, ≈1000 nucleotides longer than S-1, which is composed of about 6450 nucleotides.

Electrophoretograms of *Bam*HI restriction products of total mtDNA from the 12 RU cytoplasms were all similar, but they differed markedly from that of *cms*-S mtDNA (Fig. 2). The RU restriction pattern also differed only slightly from that of the normal standard, B73 × Mo17; band 15 was present in the normal standard but was absent from all the RU cytoplasms. Another notable feature of the RU *Bam*HI restriction patterns was the presence of very brightly fluorescing bands 23, 28, and 37. These DNAs have lengths of about 4960, 3876, and 2420 nucleotides, respectively. There were three exceptions to the RU digestion pattern. Coroico differed by having an additional band, 12.1, and the displacement or fluorescing intensity of two other bands, 3 and 21, respectively (Fig. 2). Bands 23, 28, and 37 of Mishca had normal fluorescence. Pollo produced one additional band, 13.1 (not shown).

*Bam*HI cleavage of purified R-1 DNA produced fragments of about 4960 and 2420 nucleotides (Fig. 1*B*), corresponding to bands 23 and 37 in the electrophoretogram of the whole-mtDNA *Bam*HI cleavage fragments. Digestion of R-2 and S-2 DNAs with *Bam*HI each yielded fragments of about 3889, 941, and 619 nucleotides. The 3889-nucleotide fragment corresponded with band 28. The two lower molecular weight fragments of R-2 migrated to a position distant from the origin and were faint.

An additional 22 races, all but one of which were South American, (Table 1) produced *Bam*HI fragment patterns which were similar to those of the RU races but which had bands 23, 28, and 37 of only normal fluorescence. The lower intensity of these bands is accounted for by the fact that these 22 races do not possess the plasmid-like DNAs, R-1 and R-2. These 22 races plus the 12 RU races account for over half of the 58 South American races examined. This prevalent fragment pattern was found in only one Meso-American race, Nal-Tel ATB. All other races producing this pattern are indigenous to South America.

Electron Microscopy. When isolated R-1 DNA molecules (Fig. 3*B*) were alkaline denatured and then neutralized, stem-loop structures, the result of intramolecular hybridizing of the terminal inverted repeats, were produced (Fig. 3*D*). The double-stranded region of the stem-loop has a length of about 152 (± 2.5; $n = 51$) nucleotides. The two inverted repeat regions together contain about 300 nucleotides or about 4% of the total molecule.

Heteroduplexing studies of R-1 and S-1 molecules revealed partial homology (Fig. 3*E*). The S-1 and R-1 molecules have terminal homologous sequences measured to be about 165 (± 5.5; $n = 21$) at one end and about 4719 (± 40.0; $n = 21$) nucleotides at the other. This demonstrates homology of the terminal inverted repeats of about 152 nucleotides in the S-1 and R-1 molecules.

Isolated R-2 molecules (Fig. 3*A*), denatured and neutralized, also yielded stem-loop structures, (Fig. 3*C*). The double-stranded portion extends for about 155 (± 4; $n = 18$) nucleotides. Therefore, the inverted repeats occupy about 6% of the total sequence of the R-2 molecule.

Table 1. Maize races having the predominant South American mtDNA *Bam*HI restriction endonuclease fragment pattern but lacking R-1 and R-2

Country	Race	Accession number
Bolivia	Altiplano	BOV 903
	Camba	BOV 1131
	Chake-Sara	BOV 413
	Confite Puneño	BOV 1002
	Karapampa	BOV 961
	Niñuelo	BOV 1088
	Uchuquilla	BOV 303
Chile	Capio Chico Chileno	CHI 382
Colombia	Cacao	SAS 335
	Güirua	MAG 469
	Pira	BOY 462
	Sabanero	SAN 329
Ecuador	Blanco Blandito	ECU 523
	Chococeño	ECU 891
	Enano Gigante	ECU 969
	Huandango	ECU 623
Guatemala	Nal Tel ATB*	GUA 281
Peru	Chullpi	HCA 69
	Granada	ANC 57
	Huayleño	ANC 181
	Morado Canteño	LIM 34
Venezuela	Pira	VEN 485

* ATB, Amarillo, Tierra Baja.

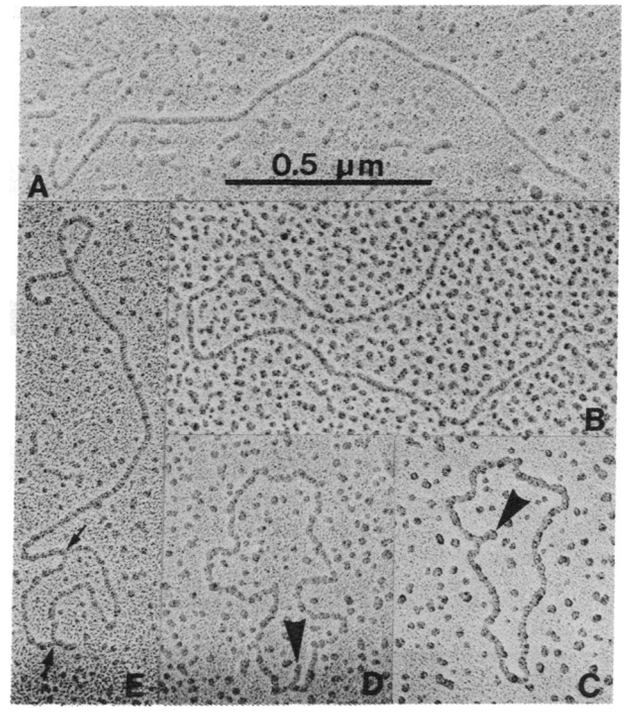

FIG. 3. Electron micrographs of double-stranded linear R-2 molecule (*A*), double-stranded linear R-1 molecule (*B*), stem-loop formed by denaturing and annealing R-2 (*C*), stem-loop formed by denaturing and annealing R-1 (*D*), and heteroduplex formed by denaturing and annealing isolated S-1 and R-1 DNAs (*E*). Region between arrows in *E* is the single-stranded, nonhomologous portion of molecule. Heavy arrows in *C* and *D* indicate the double-stranded "stem" portion of stem-loop molecules.

Heteroduplexing studies with S-2 and R-2 produced only stem-loop and double-stranded linear molecules. These results suggest that S-2 and R-2 DNAs are homologous, but they do not preclude the possibility that there are small heterologous regions whose size is below the resolving power of the heteroduplex technique.

The structural data for S-1, S-2, R-1, and R-2 are summarized in Fig. 4.

Pollen Fertility. All 472 plants of the RU cytoplasm races, Coroico, Guaribero, Kcello, Marron, Mochero, and Racimo de Uva were classified in the field as fully male fertile. Test crosses with a maintainer male carrying no restorer genes for any known cytoplasmic sterility and with a maintainer specific for *cms*-S failed to produce any male-sterile progeny.

The suspected *cms*-S Conico Norteño produced sterile plants and pollen in ratios consistent with those expected in the *cms*-S system. The *cms*-S was verified when male fertility was restored to the progeny of sterile Conico Norteño females by test crosses with the *cms*-S restorer male inbred A634, which contains the *Rf*3 allele.

DISCUSSION

We have examined mtDNAs from 81 diverse Latin American races of maize and have demonstrated in 12 of these, all of which are from South America, the presence of a unique pair of plasmid-like DNA molecules. mtDNAs from nine of these cytoplasms, designated RU, appear identical to one another based on *Bam*HI restriction analyses. The other three RU cytoplasms, Mishca, Coroico, and Pollo have mtDNA restriction patterns

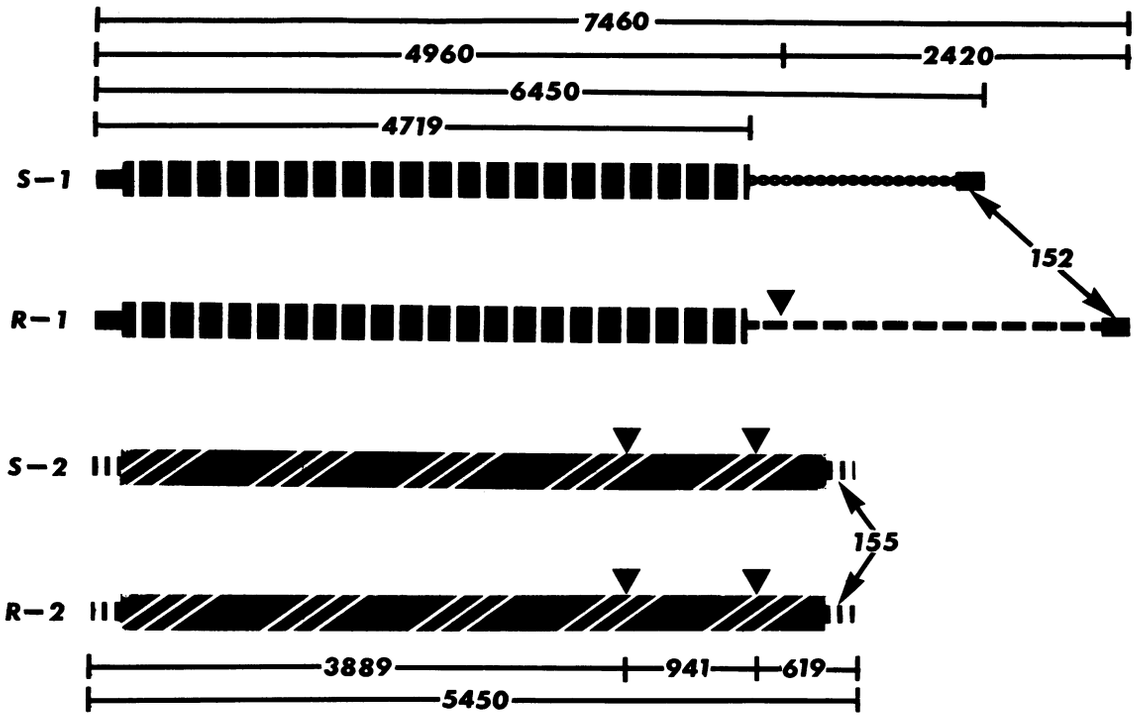

FIG. 4. Paired comparisons of schematic representations of the *cms*-S and RU plasmid-like DNA molecules, S-1 with R-1 and S-2 with R-2. The diagrams are drawn to scale, and numbers indicate the length in nucleotides; homologous regions are of like pattern, and nonhomologous regions of S-1 and R-1 are of unlike pattern. ▼, Position of *Bam*HI restriction endonuclease site.

that differ slightly from the other plasmid-bearing races. Twenty-two other South American cytoplasms have mtDNA restriction patterns similar to RU except that they do not contain R-1 and R-2. Apparently, we have identified a large group of South American maize races with similar mtDNAs that differ primarily in the presence or absence of the plasmid-like DNAs R-1 and R-2.

Another pair of plasmid-like mtDNAs was found only in the highland Mexican race Conico Norteño. These molecules appear identical in all respects to S-1 and S-2 of *cms*-S (4). We have concluded that Conico Norteño has the *cms*-S mtDNA. This observation establishes that a *cms* system can exist and be maintained in indigenous populations of maize.

Similarities between the plasmid-like mtDNAs of the RU and S cytoplasms are striking. In each case, the molecules occur in pairs and are isolated as linear molecules with terminal inverted repeats. R-2 and S-2 are identical in size, 5450 nucleotides, and share complete sequence homology as judged by heteroduplex analysis. The R-1 molecule consists of about 7460 nucleotides and is about 1000 nucleotides longer than S-1. S-1 and R-1 are approximately 70% homologous. R-1 has a unique region not found in S-1 of about 2556 nucleotides, including a *Bam*HI recognition sequence, whereas S-1 has a unique sequence of about 1546 nucleotides.

The S and RU cytoplasms differ greatly. *Bam*HI digestion of total mtDNA from *cms*-S produces an array of fragments markedly distinct from any other maize mtDNA examined. The upper 80% of the fragment pattern is distinguished from that of the normal standard, B73 × Mo17, by the absence, presence, or displacement of as many as 17 different bands. In contrast, mtDNA of RU differs from the standard by the presence or absence of only one of two bands, plus the plasmid-like DNAs. Moreover, the S and RU cytoplasms differ in geographic distribution and apparent correlation with cytoplasmic male sterility. The natural occurrence of *cms*-S thus far appears restricted to Meso- and North America, whereas the RU cytoplasm is essentially South American. Unlike *cms*-S, the RU cytoplasm is not associated with cytoplasmic male sterility or any other known phenotypic trait.

Origin of Plasmid-Like Mitochondrial DNAs. The structural similarities between the plasmid-like DNAs of the S and RU cytoplasms—i.e., common terminal inverted repeats, preponderant homologies of S-1 and R-1, and the apparent identity of S-2 and R-2—raise intriguing questions concerning the origins of these molecules. Although the mechanism by which these elements originated remains unknown, their similarities suggest that they may have arisen by a common mechanism. Furthermore, several of their characteristics are suggestive of models for origin and movement that are analogous to those observed with bacterial transposable elements (31).

Mitochondrial plasmid-like DNAs may have arisen by excision from the large mitochondrial chromosomes. Currently, this view is supported by only limited evidence from studies with S-1 and S-2 (12). Earlier investigations have demonstrated that sequences homologous to S-1 and S-2 are present in the large mitochondrial chromosomes from normal as well as *cms*-S maize (12, 32, 33). This discovery, coupled with the transposable nature of the plasmid-like DNAs, makes the excision explanation an attractive speculation. However, it does not rule out the alternative possibility of an exogenous origin (e.g., by incorporation of viral DNA).

Taxonomic Implications. No apparent relationship has been discovered between the presence of the R-1 and R-2 molecules and specific morphological features of the various maize races. Additionally, the predominant South American *Bam*HI restriction pattern (irrespective of presence or absence of R-1 and R-2) does not appear to be correlated with morphological characteristics, except for ovoid ear shape. However, none of the pointed popcorns examined, regardless of geographic origin,

exhibited this restriction pattern. This is in keeping with earlier findings from morphological (24, 26) and nuclear chromosomal knob (34) data, which established the Meso-American affinities of the South American pointed popcorns Canguil and Pisankalla.

Additional discussion of the role of the mtDNAs in the evolution of specific races or racial groupings would be premature. It is stressed, however, that differences observed among these mtDNAs are significant and have systematic meaning because they reflect differences in mtDNA sequence arrangements. Previous work (35) suggests that classification of taxa based upon similarities and differences of organelle DNAs correlates well with divisions arrived at by more traditional means.

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