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M. J. Havey · J. D. McCreight
B. Rhodes · G. Taurick

Differential transmission of the *Cucumis* organellar genomes

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Abstract Although plants generally show maternal transmission of the organellar genomes, previous research has demonstrated that the mitochondrial (mt) genome of cucumber is paternally transmitted. In this study, we identified RFLPs in the organellar genomes of melon, squash, and watermelon to establish organellar DNA transmission. Serial dilutions of DNA demonstrated that our hybridizations revealed the presence of a polymorphic cytoplasm when it represented at least 1% of the DNA sample. At this level of sensitivity, the chloroplast genomes of melon, squash, and watermelon were maternally transmitted. The mitochondrial genomes of squash and watermelon were maternally transmitted; however, melon, like cucumber, showed paternal transmission of the mitochondrial genome. Because most angiosperms and the related genera *Cucurbita* and *Citrullus* show maternal transmission of the mtDNA, paternal transmission in *Cucumis* is likely the derived state. The *Cucumis* mitochondrial genomes are several-fold larger than those of other cucurbits. Based on 55 probe-enzyme combinations, mtDNA size differences could not be explained by duplication of the entire genome or partial duplication of regions hybridizing with the mitochondrial probes. Because the chloroplast, mitochondrial, and nuclear genomes of *Cucumis* are differentially transmitted, this genus is an excellent system to study the role of intergenomic transfer in the evolution of extremely large mitochondrial genomes.

Key words Cucumber · Melon · Mitochondria · Chloroplast · DNA

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M. J. Havey (✉)
USDA/ARS, Department of Horticulture, 1575 Linden Drive, University of Wisconsin, Madison, WI 53706, USA,
Fax: +1 6082624743, e-mail: mjhavey@facstaff.wisc.edu

J. D. McCreight
USDA/ARS, 1636 East Alisal St., Salinas, CA 93905, USA

B. Rhodes
E147 Poole Agricultural Center, Clemson, SC 29634-0375, USA

G. Taurick
Ferry-Morse Seed Co, PO Box 392, Sun Prairie, WI 53590, USA

Introduction

The major cultivated species of the Cucurbitaceae include cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), squash (*Cucurbita pepo* L.), and watermelon (*Citrullus lanatus* [Thunb.] Matsum. & Nakai). Although all are members of the subfamilia Cucurbitoideae, these cucurbits have evolved separately for millions of years; cucumber originated from India, melon and watermelon from Africa, and the squashes from Central America (Jeffrey 1990). Perl-Treves et al. (1985) proposed that African and Indian *Cucumis* spp. have been separated for at least 90 million years, based on divergence for isozymes and morphological traits. Strong interspecific crossing barriers separate *Cucumis* species of different chromosome numbers (Deakin et al. 1971; Singh and Yadava 1984). Hybrid progenies can be generated from interspecific crosses within *Cucurbita* (Erwin and Haber 1929; Pearson et al. 1951; Rhoades 1959) and *Citrullus* (Khoshoo and Viz 1963; Singh 1978). There are no reports of successful intergeneric crosses.

The cucurbits possess chloroplast (cp) genomes of approximately equal size (150–155 kb) and structure, based on the mapping of restriction enzyme sites in the cpDNA of cucumber (Palmer 1982), squash (Lim et al. 1990), and melon (Perl-Treves and Galun 1985). However, they have accumulated significant differences in the nuclear and mitochondrial (mt) genomes. Chromosome numbers are not multiples due to polyploidy, e.g., basic numbers of 7, 10, 11, and 12 exist for cucumber, squash, watermelon, and melon, respectively. Changes in these basic numbers likely resulted from

chromosomal fragmentation or centric fusions (Singh 1990). These cucurbits also have several-fold size differences in their mitochondrial genomes (Ward et al. 1981). Watermelon possesses a relatively small mitochondrial genome of 330 kb (220 MDa), squash has a larger mitochondrial genome of 840 kb (560 MDa), while cucumber and melon possess huge mitochondrial genomes of 1500 kb (1000 MDa) and 2400 kb (1600 MDa), respectively. These differences in mtDNA cannot be attributed to increased mitochondrial volume (Bendich and Gauriloff 1984) or accumulation of repetitive DNA (Ward et al. 1981). Larger mitochondrial genomes could result from partial or complete duplication of the genome, intergenomic sequence transfer (reviewed by Schuster and Brennicke 1988), or the accumulation of multiple introns (Stern et al. 1986).

Plants predominately show maternal transmission of the organellar genomes (Gillham 1978; Harris and Ingram 1991). However, paternal transmission of the mitochondria (Boynton et al. 1987) or chloroplast (Neale and Sederoff 1989) genomes is known. Biparental transmission of plastids (Medgyesy et al. 1986; Smith 1989b; Mason et al. 1994) or mitochondria (Erickson et al. 1989; Erickson and Kemble 1990) has been documented and may be under genetic control (Cornu and Dulieu 1988; Smith 1989a; Tilney-Bassett et al. 1992). Matuursa (1995) and Havey (1997) demonstrated paternal transmission of the mitochondrial genome of cucumber. Maternal transmission of chlorophyll deficiencies, presumably due to mutations in the chloroplast genome, has been demonstrated for the squash *Cucurbita maxima* Duch. (Hutchins and Youngner 1952) and melon (Ray and McCreight 1996). Corriveau and Coleman (1988) used a DNA fluorochrome and epifluorescence microscopy to demonstrate the physical exclusion of cpDNA from the male gametophyte of cucumber, indirect evidence for maternal inheritance. In this study, we identified restriction fragment length polymorphisms (RFLPs) in the organellar genomes of the major cultivated cucurbits and generated reciprocal crosses to establish their inheritance. Our analyses also provide insights about the evolution of the extremely large *Cucumis* mitochondrial genomes.

Materials and methods

Seed sources of cucurbit accessions are listed in Table 1. Total genomic DNA was isolated and purified through CsCl gradients (Havey 1991) using approximately 25 plants of each accession. We attempted to identify previously characterized polymorphisms in the chloroplast genomes of *C. pepo* (Wilson et al. 1992) and *C. melo* (Perl-Treves and Galun 1985). Total genomic DNA was digested at 10 units/μg with *Bam*HI, *Bg*III, *Bst*EII, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Pvu*II, *Xba*I, or *Xho*I according to the manufacturer's (Gibco-BRL, Gaithersburg, Md.) recommendations. Electrophoresis through 0.7% agarose gels, blotting, and autoradiography have been reported (Havey 1997). We individually nick translated and hybridized the five mitochondrial clones atp6 and atp9 (Dewey et al. 1985), cox1 (Isaac et al. 1985), cox3 (Hiesel et al. 1987), and cob (Dawson et al. 1984) and chloroplast fragments P1, P3, P4, P6, P8&P10, P14, P16&S6, and P18&S8 (Sytsma and Gottlieb 1986). RFLPs were scored within cucurbit species and approximate fragment sizes estimated (Schaffer and Sederoff 1981).

To identify nuclear polymorphisms, we hybridized a clone of the wheat nuclear 45S ribosomal DNA (Gerlach and Bedbrook 1979) to the above listed enzyme digests. We demonstrated segregation of polymorphisms in the 45S rDNA using F_2 families from *C. melo* subsp. *agrestis* (Naud.) Pangalo plant introduction (PI) 313970 crossed with the cultivated melon Topmark and *C. lanatus* var. *citroides* accession R309 crossed with the cultivated watermelon Dixielee, respectively, and tested goodness-of-fit with chi-squared analyses. For *C. pepo*, we evaluated Dark Green Zucchini (DGZ)

Table 1 Sources of cucurbit species evaluated for polymorphisms in organellar and 45S ribosomal DNA

Cucurbit	Abbreviation	Accession[a]	Source	Seed lot
Cucumis melo subsp. *melo*	M1	Topmark	USDA, Salinas, Calif.	
	M2	124113	USDA, Ames, Iowa	87ncsi01
	M3	161375	USDA, Ames, Iowa	87ncsi01
	M4	357775	USDA, Ames, Iowa	87ncsi01
	M5	414723	USDA, Ames, Iowa	92ncsi01
Cucumis melo subsp. *agrestis*	M6	313970	USDA, Salinas, Calif.	
Cucumis sativus var sativus	C1	Marketmore 76	Peto, Woodland, Calif.	
	C2	TMG1	USDA, Madison, Wis.	
	C3	Gy14	USDA, Madison, Wis.	
Cucumis sativus var hardwickii	C4	183967	USDA, Madison, Wis.	
Citrullus lanatus var lanatus	W1	Dixielee ⊗[b]	Clemson University	
	W2	New Hampshire Midget ⊗[b]	Clemson University	
Citrullus lanatus var citroides	W3	296341	Clemson University	
	W4	R309	Clemson University	
Cucurbita pepo	S1	Mexican pepo	HDW5457	PE406-1
	S2	Dark Green Zucchini	HDW5466	PE412
	S3	Golden Summer Crookneck	Rupp Seed Co, Wauseon, Ohio	13569
	S4	Shenot Crown of Thorns	Jung Seed Co, Randolph, Wis.	02345A

[a] Accessions with six-digit numbers were obtained from USDA plant introduction system. Those with a HDW prefix were the gift of Dr. H. Wilson, Texas A&M University (Wilson et al. 1992). Other accessions are commercially available or can be obtained from the authors
[b] ⊗ = S_1 family from self pollination of a single plant

and Golden Summer Crookneck (GSC) for isozymes shikimic dehydrogenase (SKDH), leucine aminopeptidase (LAP), and malate dehydrogenase (MDH) as described by Ignart and Weeden (1984).

Reciprocal crosses were generated between individual plants of PI313970 and Topmark, New Hampshire Midget (NHM) and PI296341, and DGZ and GSC. Seedlings were greenhouse-grown, and the stem tip and one-half of the expanded second true leaf harvested. DNA was isolated by grinding freeze-dried tissue to a fine powder in liquid nitrogen and incubation in sterile extraction buffer [100 m*M* TRIS (pH 8), 1.4 *M* NaCl, 20 m*M* EDTA, 2% CTAB, 0.2% β-mercaptoethanol] at 50°C for 1 h. The solution was extracted once with chloroform/isoamyl alcohol (24:1), precipitated with isopropyl alcohol, washed with 70% ethanol, vacuum-dried, and resuspended in 10 m*M* TRIS-HCl (pH 8.0) and 1 m*M* EDTA with RNase. Concentrations were determined by comparing to known λ concentrations on ethidium bromide-stained agarose gels. The following polymorphic probe-enzyme combinations were used to establish mtDNA transmission: cob/*Pvu*II and cox1/*Bst*EII for *C. pepo*, atp9/*Eco*RV for *Citrullus* spp., and atp9/*Eco*RI and cox1/*Hin*dIII for *C. melo*. To establish the transmission of the chloroplast genome, we evaluated the same reciprocal crosses for P8&P10/*Dra*I (*C. pepo* and *C. lanatus*) or P14/*Bam*HI and P14/*Bgl*II (*C. melo*). Hybridity was established by scoring additive patterns for polymorphic *Eco*RV (*C. lanatus*) or *Dra*I (*C. melo*) sites in the nuclear 45S rDNA. For *C. pepo*, the nuclear-encoded isozyme MDH (Ignart and Weeden 1984) and fruit type were scored. For all reciprocal crosses, RFLPs in F_1 progenies were compared to RFLPs revealed using DNA from the parental population, not the individual plant used to generated hybrid progenies.

To estimate the level at which we could detect occasional biparental transmission of the organellar DNAs, we created ten-fold dilution series (from 10^{-1} to 10^{-6}) using genomic DNA of GSC and PI313970. Two micrograms of genomic DNA from DGZ was mixed with 2.0 µg (undiluted) and with each of the serially diluted DNAs (0.2 µg, 0.02 µg, etc.) from GSC. The same mixtures were prepared for TM and PI313970. We evaluated the DGZ-GSC DNA mixtures for polymorphisms in the chloroplast (P8&P10/*Dra*I) and mitochondrial (cob/*Pvu*II) genomes. The TM-PI313970 DNA mixtures were evaluated for the chloroplast polymorphism revealed by P14/*Bam*HI. Two sets of dilution series of GSC and PI313970 were independently generated, mixed with DNA of DGZ or TM (respectively), digested with the appropriate restriction enzyme, and blotted to produce two replications of each dilution series. For all three polymorphisms, the serially diluted parental DNAs (GSC and PI313970) possessed unique restriction enzyme sites, generating smaller fragments to avoid confusing partial digestions with the presence of the polymorphic fragment.

Results and discussion

Identification of polymorphisms in the organellar and nuclear genomes

The cucurbit mitochondrial genomes have accumulated significant degrees of polymorphism both among and within the cultivated species. Among the cultivated cucurbits, we detected RFLPs for all of the 55 mitochondrial probe-enzyme combinations (five probes and 11 enzymes), as expected given the spatial and temporal separations (Jeffrey 1990) and potential recombination among repeated sequences (Palmer 1990). Within the individual cucurbit species, 16 mitochondrial probe-enzyme combinations revealed polymorphisms (Table 2).

Colinearity of cucurbit cpDNAs was established by comparing watermelon *Pst*I or *Pvu*II fragments with the maps of the *C. pepo* (Lim et al. 1990), *C. melo* (Perl-Treves and Galun 1985), and *C. sativus* (Palmer 1982). Polymorphic restriction enzyme sites in the cpDNA were detected among accessions of melon, squash, and watermelon (Table 3). Within *C. pepo*, we detected one polymorphism (P3/*Eco*RI) not previously reported by Wilson et al. (1992).

For watermelon, we detected polymorphisms in the nuclear 45S rDNA with *Dra*I (10.5 kb for W1 and W2 versus 10 kb for W3 and W4) and *Eco*RV (10.5 kb for W1 and W2 versus 6.0 + 4.5 kb for W3 and W4). Among accessions of melon, a polymorphism was revealed using *Dra*I (4.7 for M1, M3, and M4 versus 5.0 kb for M5 and M6; accession M2 possessed both fragments). The polymorphic *Eco*RV site in the 45S rDNA of watermelon segregated in F_2 families (autoradiogram not shown) and fit the expected 1:2:1 ratio ($p = 0.11$), establishing its nuclear origin. The polymorphic *Dra*I site in the 45S rDNA of melon segregated, but we were not able to confidently score segregation, possibly due to more than one rDNA repeat in melon. Nevertheless, we were able to establish additive patterns in the 45S rDNA to confirm hybridity of the melon progenies.

Although polymorphisms have been described in the intergenic spacer of squash (Ganal and Hemleben 1986), we were unable to identify RFLPs among the studied squash accessions using 11 restriction enzymes and the heterologous wheat probe. We evaluated the squashes for isozyme polymorphisms, of which MDH was polymorphic between DGZ and GSC (gels not shown).

Inheritance of the cucurbit organellar genomes

Polymorphisms in the nuclear 45S rDNA of watermelon and melon were biparentally transmitted and established the hybridity of all progenies (autoradiograms not shown). Hybridity of squash progenies were established using MDH and fruit type. Polymorphisms in the chloroplast [P8&P10/*Dra*I (Fig. 1A)] and mitochondrial [cob/*Pvu*II (Fig. 1B) and cox1/*Bst*EII] genomes of *C. pepo* were maternally transmitted for all of the 45 hybrid progenies. These chloroplast results agree with maternal transmission of a chlorophyll-deficient mutant of *C. maxima* (Hutchins and Youngner 1952). Maternal transmission of both the chloroplast [P8&P10/*Dra*I (Fig. 2A)] and mitochondrial [atp9/*Eco*RV (Fig. 2B)] genomes were demonstrated for 35 hybrid watermelon progenies. Melon showed maternal transmission of the chloroplast [P14/*Bam*HI (Fig. 3A)] and paternal transmission of the mitochondrial [cox1/*Hin*dIII (Fig. 3B) and atp9/*Eco*RI] genomes for all 38 hybrid progenies. For polymorphisms P8&P10/*Dra*I [GSC × DGZ (Fig. 1A)], P8&P10/*Dra*I [PI296341 ×

Table 2 Polymorphisms in the mitochondrial genome among accessions of cucurbit species

Cucurbit	Probe	Enzyme	Sizes (kb)	Accessions with fragment[a]
Cucumber	atp6	*Hin*dIII	8.5	C1, C2, C3, C4
			15.1	C2
	atp9	*Bam*HI	15.0	C1, C3[b], C4
			18.0	C2
	atp9	*Eco*RV	11.9	C1, C3, C4
			16.8	C2
	cob	*Xba*I	8.0	C4
			15.0	C3[b]
			20.0	C1, C2
Melon	atp6	*Eco*RI	2.7	M1, M2, M3, M4, M5[c], M6[c]
			5.3	M5[c], M6[c]
	atp6	*Hin*dIII	1.9	M2, M3, M4[c], M5
			20.0	M1, M4[c], M6
	atp9	*Eco*RI	2.6	M1, M2, M3, M4
			6.0	M6
			6.7	M5
	atp9	*Hin*dIII	13.0	M2, M3, M4, M5
			15.0	M1, M6
	cox1	*Eco*RV	3.5	M1, M2, M3, M4, M5
			3.3	M6
	cox1	*Hin*dIII	2.5	M1, M2, M3, M4, M5
			2.3	M6
Watermelon	atp9	*Eco*RV	18.6	W1, W2
			15.2	W3, W4
Squash	atp9	*Bgl*II	9.6	S1, S2
			6.6	S3, S4
	atp9	*Bst*EII	14.0	S1, S2
			11.0	S3
			17.0	S4
Squash	atp9	*Eco*RI	15.7/5.7	S1, S2
			6.7	S3, S4
	cob	*Pvu*II	22.0/3.6	S1, S2
			12.0	S3
			11.2	S4
	cox1	*Bam*HI	5.6	S1, S2
			9.6	S3
			10.4	S4
	cox1	*Bst*EII	10.0	S1, S2
			11.1	S3
			13.4	S4
	cox3	*Bst*EII	12.0	S1, S2
			11.0	S3
			13.5	S4

[a] For descriptions of accessions, see Table 1
[b] Fragment sizes were reported previously by Havey (1997) and are included here for convenience
[c] Both fragments present

NHM (Fig. 2A)], and P14/*Bam*HI [PI313970 TM (Fig. 3A)], the presence of the larger paternal fragments was due likely to partial digestions. In all cases, the smaller fragments were not visible in the reciprocal hybrids.

Reciprocal crosses established maternal transmission of the chloroplast genomes of melon, squash, and watermelon. Epifluorescence microscopy demonstrated the exclusion of the cpDNA from the male gametophyte of cucumber, supporting maternal transmission of the chloroplast genome (Corriveau and Coleman 1988). Because maternal transmission of the mitochondrial genome normally occurs in most plants (Gillham 1978) and in the related genera *Citrullis* and *Cucurbita* (Figs. 1 and 2), paternal transmission in *Cucumis* [Fig. 3 and Havey (1997)] is likely the derived state.

Occasional biparental transmission of the organellar genomes may occur. We evaluated the fidelity of Southern hybridizations to reveal genomic DNA mixtures and observed that, for mixtures of $1:1$, $1:10^{-1}$, and $1:10^{-2}$, restriction fragments were visible. However, we could not detect the presence of unique chloroplast or mitochondrial restriction fragments below the $1:10^{-2}$ dilution (autoradiograms not shown). Therefore, organellar transmission would not be detected below 1%, and our studies demonstrate the predominate mode of organellar transmission in these cucurbits.

Table 3 Polymorphisms in the chloroplast genome among accessions of individual cucurbit species

Probe	Enzyme	Fragment sizes[a]	State[b]	Accessions[c]
P3	*Eco*RI	4.7 = 4.6 + (0.1)	0	S1, S3, S4
			1	S2
P8&P10[d]	*Dra*I	7.9 = 4.3 + 3.6	0	S1, S2
			1	S3, S4
P8&P10	*Dra*I	7.9 = 7.0 + 0.9	0	W1, W2
			1	W3, W4
P8&P10	*Xba*I	13.0 = 9.0 + 4.0	0	W4
			1	W1, W2, W3
P6	*Xba*I	4.3 = 3.6 + (0.7)	0	W1, W2
			1	W3, W4
P14	*Bam*HI	5.0 = 3.0 + 2.0	0	M1, M4, S3[h]
			1	M2, M3, M5, M6, S1, S2, S3[h], S4
P14	*Bg*III	1.8 = 1.7 + (0.1)[g]	0	M2, M3, M5, M6
			1	M1, M4
P16&S6[e]	*Bg*III	10.9 = 6.4 + 4.5	0	S1, S2
			1	S3, S4
P16&S6	*Xba*I	4.2 = 3.5 + 0.7	0	W3, W4
			1	W1, W2
P18&S8[f]	*Eco*RI	6.0 = 5.3 + (0.7)	0	S3, S4
			1	S1, S2

[a] Fragment sizes given in kilobases. Fragment sizes in parentheses were not visible on autoradiograms
[b] 0 and 1, absence and presence of restriction enzyme sites, respectively
[c] M = melon, S = squash, and W = watermelon. For origin of accessions and numbers, see Table 1
[d,e,f] Characters nos. 31, 9, and 49 of Wilson et al. (1992), respectively
[g] Likely the insertion or deletion reported by Perl-Treves and Galun (1985)
[h] Accession S3 possessed both character states

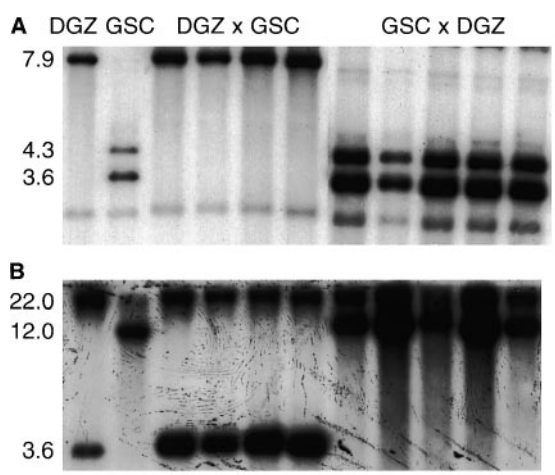

Fig. 1A,B Autoradiograms demonstrating maternal transmission of the chloroplast (**A** P8&P10/*Dra*I) and mitochondrial (**B** cob/*Pvu*II) genomes of *Cucurbita pepo* accessions Dark Green Zucchini (DGZ) and Golden Summer Crookneck (GSC). For description of clones, see Materials and methods. Fragment sizes in kilobases shown on left

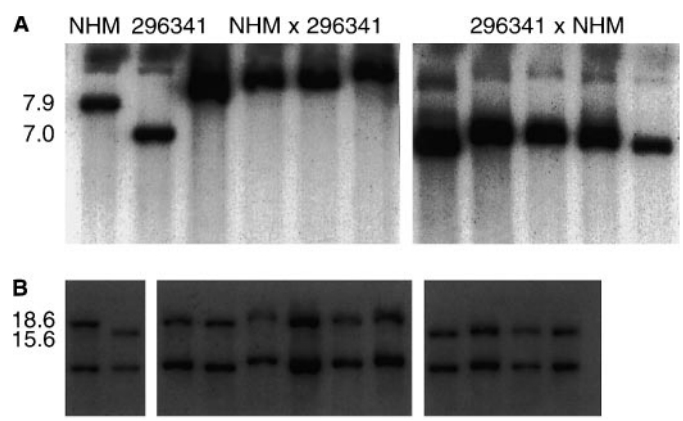

Fig. 2A,B Autoradiograms demonstrating maternal transmission of the chloroplast (**A** P8&P10/*Dra*I) and mitochondrial (**B** atp9/*Eco*RV) genomes of *Citrullus lanatus* accessions New Hampshire Midget (NHM) and USDA plant introduction 296341. For description of clones, see Materials and methods. Fragment sizes in kilobases shown on left

Evolution of the cucurbit organellar genomes

The large size differences in the cucurbit mitochondrial genomes could be the result of partial or complete duplication of the genome. Ward et al. (1981) demonstrated that the accumulation of repetitive DNA did not significantly contribute to these mitochondrial size differences. Because the plant mitochondrial genome evolves slowly in sequence and accumulates structural changes by recombination among direct repeats (Palmer and Hebron 1988; Palmer 1990), we would expect that duplication of the entire genome would yield greater mean numbers of fragments for cucumber and melon, than for squash and watermelon. To assess this possibility, we scored the numbers of fragments detected for each mitochondrial probe-enzyme combination. For cucumber, melon, squash, and watermelon, we detected an average of 1.1 $\pm$ 0.3, 1.2 $\pm$ 0.4, 1.2 $\pm$ 0.4, and

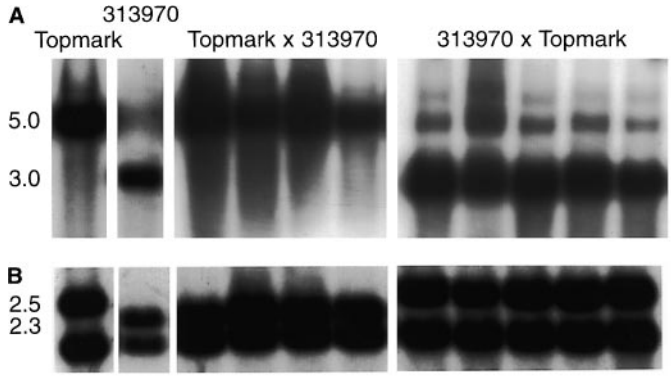

Fig. 3A,B Autoradiograms demonstrating maternal transmission of the chloroplast (**A** P14/*Bam*HI) and paternal transmission of the mitochondrial (**B** cox1/*Hin*dIII) genomes of *Cucumis melo* accessions Topmark and USDA plant introduction 313970. For description of clones, see Materials and methods. Fragment sizes in kilobases shown on left.

1.3 ± 0.5 fragments, respectively, which were not significantly ($P < 0.05$) different. These results and those of Ward et al. (1981) indicate that duplication of the entire genome, or partial duplication of the regions revealed by our probe-enzyme combinations, did not play a significant role in the evolution of mitochondrial size differences among these cucurbits.

Another possibility is that intergenomic transfers contributed to mitochondrial size differences. The larger cucumber and melon mitochondrial genomes could have resulted from the transfer of sequences from the nuclear (Schuster and Brennicke 1988) or chloroplast (Nugent and Palmer 1988; Schuster and Brennicke 1988; Nakazono and Hirai 1993) genomes. If intergenomic transfer of chloroplast sequences contributed significantly to the huge *Cucumis* mitochondrial genomes, we would expect that the hybridization of chloroplast clones would have revealed paternally inherited polymorphisms, which we did not detect. A second possibility is that the smaller mitochondrial genomes resulted from an exodus of mitochondrial sequences to the nuclear (Thorsness and Fox 1990; Fukuchi et al. 1991; Nugent and Palmer 1991; Sun and Callis 1993) or chloroplast (no examples known in plants) genomes. For both of these scenarios, there should exist polymorphisms paternally transmitted in cucumber or melon and biparentally or maternally inherited in watermelon or squash. Because the chloroplast, mitochondrial, and nuclear genomes are differentially transmitted (maternally, paternally, and biparentally, respectively) in cucumber and melon, these *Cucumis* species are an excellent experimental system to establish the role of intergenomic transfer in the evolution of extremely large mitochondrial genomes.

Disclaimer Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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