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Mitochondrial DNA polymorphisms of Malawian bean lines: further evidence for two major gene pools

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Summary. Intraspecific mitochondrial DNA (mtDNA) diversity was determined in 23 *Phaseolus vulgaris* genotypes, and compared to previously observed variability of morphoagronomic characters and isozyme loci. Twenty of the lines were collected from Malawian landraces; the other three were pure-bred cultivars. The mtDNAs were digested with eight restriction endonucleases, revealing complex banding patterns. Southern hybridization using cosmid clones covering about 200-kb of the genome showed a considerable amount of uniformity of the mtDNA banding patterns. However, five restriction fragment length polymorphisms (RFLPs) were detected, dividing the bean lines into two groups corresponding to the previously known Mesoamerican and Andean gene pools of *P. vulgaris*. The cultivar 'Mecosta' was separated from the rest of the lines by an additional RFLP. At least two out of the six RFLPs are believed to be due to base-pair mutation events. Our results provide the first evidence that the cytoplasms of the two major germ plasm pools of beans are distinct.

Key words: *Phaseolus vulgaris* – Mitochondrial RFLPs – Gene pools – Genetic diversity – Malawi

Introduction

The common bean, *Phaseolus vulgaris* L., is native to the American continent, where the wild ancestral forms are still distributed from west central Mexico to northwestern Argentina. Botanical, archaeological, and biochemical evidence indicate two major and independent domestication events in Mesoamerica and the Andes (reviewed by Gepts 1988a; Debouck and Tohme 1989), which resulted in two primary centers of genetic diversity. Consequently, two major gene pools are recognized in the common bean (for a review, see Gepts 1988b). These are distinguished by their seed sizes and growth habits (Evans 1976), environmental adaptation (Ghaderi et al. 1982; Kelly et al. 1987), disease resistance (Gepts and Bliss 1985), isozyme variants (Bassiri and Adams 1978; Sprecher 1988), and phaseolin types (Gepts et al. 1986). In addition, the two germ-plasm pools are separated by partial fertility barriers (Singh and Gutierrez 1984; Gepts and Bliss 1985; Sprecher 1988).

As beans from these previously separate gene pools spread to the Old World, secondary centers of diversity were formed. One such area is eastern Africa where Andean beans predominate, although strains from Mesoamerica are also grown. Recent germ plasm collections in Malawi have drawn attention to the abundant variability in farmers' fields (Adams 1983; Martin 1984). Studies on 375 landrace lines collected from 15 sites in northern Malawi revealed extensive morphological, phenological and agronomic variation (Martin and Adams 1987a, b), but showed that the lines cluster into only two major groups in terms of variability at isozyme loci (Sprecher 1988). These correspond to the small-seeded Mesoamerican and the large-seeded Andean gene pools. In this investigation, we selected a subsample of these lines to assess the variability at an additional level, that of the mitochondrial DNA (mtDNA). Our objectives were: (1) to compare and contrast the amount and pattern of mtDNA diversity with the morphoagronomic and isozyme variation; and (2) to explore the possibility that the two major gene pools of beans have evolved distinct mitochondrial genomes.

An increasing number of studies have compared organelle DNA restriction patterns as a measure of diversi-

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ty and to assess phylogenetic relationships within and among plant species. Because of its higher rate of rearrangement, mtDNA appeared to be more useful than chloroplast DNA for studies below the species level. Indeed, studies in maize and teosinte had revealed a considerable level of mtDNA intraspecific variability (Timothy et al. 1979; Kemble et al. 1983; Weissinger et al. 1983). However, in most other species examined subsequently, limited variation has been observed (e.g., Holwerda et al. 1986; Rines et al. 1988; Palmer 1988), rendering intraspecific comparisons of mtDNA less informative. In *P. vulgaris*, as in other species, mtDNA restriction fragment differences were observed when cytoplasmic male-sterile and fertile lines were compared (MacKenzie et al. 1988). Another problem is that the mitochondrial genome is relatively complex and RFLPs can be difficult to resolve. In our analysis of mtDNA variability from 23 bean lines, we addressed these difficulties by using eight restriction endonucleases and by Southern hybridizations to mtDNA probes covering almost half the genome.

Our results show that the mitochondrial genome of *Phaseolus vulgaris* is characterized by an appreciable degree of uniformity. In addition, this study provides the first evidence that the cytoplasms of the two major bean gene pools are distinct as defined by five mtDNA RFLPs.

Materials and methods

Bean lines

Bean landraces were collected in 1983 from 15 farm sites in northern Malawi. Twenty-five lines were extracted from each site by allowing self-pollination of plants derived from 25 individual seeds picked at random from the on-site collection (Martin and Adams 1987a). Of those 375 lines, our investigation used 20, originating at two sites in the Misuku Hills, Farms no. 4 and 5 in Martin and Adams (1987a). Based on genetic distances derived from a principal component analysis of morphoagronomic characters (Martin 1984; Martin and Adams 1987a), both "related" and "distant" lines were included. The lines differ in seed characteristics and include six isozyme genotypes (Sprecher 1988), and three phaseolin types (Fig. 1).

Three pure-line cultivars, 'Mecosta,' 'Sanilac,' and 'Tendergreen', were included as controls. The latter two are the cultivar

Bean Line	Isozyme Genotype	Gene Pool	Phaseolin	Bean Line	Isozyme Genotype	Gene Pool	Phaseolin
4-1	1	A	T	5-1	7	M	S
4-4	7	M	S	5-2	7	M	S
4-6	1	A	T	5-4	7	M	S
4-7	1	A	T	5-5	7	M	S
4-10	0	H	S	5-6	1	A	T
4-11	7	M	S	5-7	9	A	T
4-20	4	A	T	5-10	1	A	T
4-22	1	A	T	5-14	1	A	C
Mecosta	-	A	S	5-16	1	A	C
Sanilac	7	M	S	5-18	7	M	S
Tendergreen	1	A	T	5-20	11	A	C
				5-25	0	H	T

Fig. 1. Characteristics of the bean lines assayed for mtDNA variability. Isozyme genotype was based on the analysis of six isozyme loci (Sprecher 1988). Genotypes 1 and 7 denote Andean (**A**) and Mesoamerican (**M**) zymotypes, respectively; genotypes 4, 9, 11 fit the Andean zymotype except for one locus; genotype 0 segregated at one or more loci, indicating heterozygosity (**H**). Phaseolin types were determined at the International Center of Tropical Agriculture (CIAT); *T* Tendergreen, *S* Sanilac, and *C* Contender banding patterns (Brown et al. 1981). *T* and *C* are associated with the Andean gene pool and *S* with the Mesoamerican one (Gepts 1988b)

types for the phaseolin variants 'S' and 'T,' respectively (Brown et al. 1981).

The Malawian lines were grown in the field at Michigan State University for two summers in order to obtain enough seeds for the mtDNA extractions.

MtDNA isolation

Using 100–200 g of 10- to 21-day old seedlings grown in the dark, mtDNA was isolated according to the procedure followed by Mackenzie et al. (1988). This procedure is itself a combination of steps from McNay et al. (1983) and Dellaporta et al. (1983). It consists basically of a set of differential centrifugations following tissue homogenization to separate mitochondria from nuclei and plastids, a DNaseI treatment to digest any remaining extramitochondrial DNA, lysis of the mitochondria after DNaseI inactivation, precipitation of the protein-carbohydrate complexes, and ammonium acetate/isopropanol precipitation of the mtDNAs at −20 °C overnight. Except for our initial preparations, we did not use cetyl trimethylammonium bromide (CTAB) to reprecipitate nucleic acids, because the DNA was harder to dissolve when CTAB was used. The mtDNAs were washed twice with 70% ethanol, vacuum dried, and resuspended in $T_{10}E_{0.1}$ (10 m*M* TRIS, 0.1 *M* EDTA, pH 8.0). The mtDNAs that did not cut well with restriction endonucleases were phenol-chloroform extracted.

Restriction endonuclease analysis

The mtDNAs were digested with individual restriction endonucleases for 4–7 h at 37 °C using the buffers recommended by the manufacturers (Bethesda Research Lab, Boehringer Mannheim Biochemicals, and New England Biolabs). The fragments were separated by electrophoresis in 0.7% (DraI, PstI, SalI, XhoI), 0.8% (BamHI), 0.9% (EcoRI, HindIII), or 1.3% (HaeIII) agarose gels (Sigma, Type I: low EEO) using the TAE buffer system (0.04 *M* TRIS, 0.02 *M* sodium acetate, 0.001 *M* EDTA, pH 8.0, and 0.5 μg/ml ethidium bromide). Molecular size markers were obtained by digesting, DNA with HindIII alone and in combination with EcoRI. Gels were run at room temperature for 18–22 h at a constant 35–45 V.

After the gels were photographed, the restriction fragments were transferred to nylon hybridization membranes (0.45 μm, Micron Separations, Inc.) as described by Maniatis et al. (1982). The DNA was fixed on the membrane by either overnight baking at 80 °C or UV crosslinking for 3–5 min.

The membranes were consecutively hybridized to three to four different probes after the previous radioactive probe was stripped away by incubating the filters in 0.5 *M* NaOH at 50 °C for 1 h (Nugent and Palmer 1988).

Nick-translation and hybridization

The probes used in this study (651-12-C2, -C3, -C4, -C6, and -C8) are random DNA sequences of the bean mitochondrial genome cloned into the cosmid pHC79, kindly provided by Dr. C.D. Chase (University of Florida, Gainesville). The insert sizes are as follows (all in kb): C2-37.9; C3-33.2; C4-35.5; C6-27.7; and C8-34.6.

The clones were nick-translated using ^{32}P-labeled nucleotides (New England Nuclear) based on the procedure described by Maniatis et al. (1982), except that the nick-translation buffer was that of Rigby et al. (1977). The Southern filters were hybridized at 65 °C overnight and then washed of excess label according to Maniatis et al. (1982). However, in order to facilitate the later stripwashing of the probe, the membranes were not allowed to dry before they were exposed. We also gradually decreased the stringency of the third wash for the second, third, and fourth probe by lowering either the temperature or the time of incubation, or a combination of both. Filters were used for autoradiography with Kodak XAR-5 X-ray film in cassettes containing one or two intensifying screens at −70 °C.

Results

The mitochondrial genome of beans is large and its restriction patterns are complex

Mitochondrial DNAs from the 23 bean lines were digested with the eight restriction endonucleases chosen for this study. The patterns of fragments resulting after electrophoresis were complex, with at least 30 bands in the simplest profile (SalI) and a minimum of 60 bands with HindIII. The patterns of the different lines were very similar and it was hard to identify RFLPs with certainty in the stained gels. However, we were able to detect a few differences in the digestion patterns from two enzymes, DraI (Figs. 2A and 4A) and EcoRI (Fig. 3A). We ruled out the possibility of partially digested fragments by always adding excess enzyme and allowing long digestion times. Furthermore, the same RFLPs were seen in at least six gels for each enzyme.

Besides the six fragment differences indicated on the examples of the ethidium-bromide-stained gels (four in DraI and two in EcoRI), no other RFLPs were seen. Recognizing that because of the complexity of the restriction patterns, additional variability could go undetected in the stained gels, we decided to screen for more RFLPs by Southern analysis.

Little variation exists in the mitochondrial genome of bean

By transferring mtDNAs from the gels to nylon membranes for Southern hybridizations, we were able to more clearly examine specific regions of the mitochondrial genome. The screening was started with the use of cloned mitochondrial coding sequences from maize (cytochrome

Table 1. Number of bean mtDNA fragments to which the five cosmid clones hybridize

Enzyme	Recognition sequence	Total no. of fragments	Adjusted no. of fragments[a]	No. of polymorphic fragments[b]
HaeIII	GGCC	61	ND	0
BamHI	GGATCC	54	38	0
DraI	TTTAAA	48	35	6
EcoRI	GAATTC	62	46	5
HindIII	AAGCTT	63	46	0
PstI	CTGCAG	37	23	0
SalI	GTCGAC	29	20	0
XhoI	CTCGAG	44	32	0

[a] Adjusted by subtracting the number of overlapping fragments between clones

[b] Detected by Southern hybridization

ND – not determined

Table 2. Intraspecific RFLPs in bean mtDNA following digestion with eight restriction endonucleases

RFLP no.	Enzyme	Revealed by	Definition of pattern no. 1	Definition of pattern no. 2	No. of polymorphic fragments
1	DraI	stained gel	12.7 kb	no 12.7 kb	1
2	DraI	stained gel	no 3.7 kb	3.7 kb	1
3	DraI	clones C3, C6[a]	3.45 kb	3.37 kb	2
4	EcoRI	clone C2[a]	18.6+4.3 kb	22.3 kb	3
5	EcoRI	clone C4	6.2 kb	11.2 kb	2
6	DraI	clone C2	3.3 kb	no 3.3 kb	1
7	DraI	clones C4, C8[a]	4.5+3.3 kb	8.5 kb	3

[a] Also detected in the stained gel

oxidase subunit II gene region provided by T.D. Fox, Cornell University, Ithaca NY, and the 26S rDNA and 5S–18S rDNA obtained from C. S. Levings III, North Carolina State University, Raleigh NC). With these relatively small probes, one to four fragments per enzyme hybridized and no RFLPs were detected. We then decided to use larger cosmid clones (29–38-kb) consisting of random sequences of the bean mitochondrial genome. Each clone hybridized to 3–17 fragments per enzyme, with a total of 29–64 fragments per enzyme being revealed with five cosmid clones. These numbers were adjusted to 20–46 fragments per enzyme by accounting for possible overlapping sequences between the different clones (Table 1).

The Southern hybridizations showed very uniform patterns among the bean lines and, as Table 1 indicates, only two enzymes, DraI and EcoRI, produced polymorphic fragments (Figs. 2B, 3B, C, and 4B, C). Four of the polymorphic bands seen in the stained gels hybridized to the probes. The two nonhybridizing fragments and the ones revealed by hybridization totaled 13 polymorphic fragments, representing seven RFLPs (Table 2). Among all 23 bean lines, only two alternative patterns were observed for each of the seven RFLPs, patterns no. 1 and 2, defined in Table 2. For both RFLP 3 and 7, two different clones revealed the same polymorphic bands.

In order to estimate the proportion of the bean mitochondrial genome that was covered by the cosmid clones, the size of the fragments hybridizing to each clone was determined and added up for individual enzymes, overlapping fragments being counted only once. The estimates obtained were 200.0, 198.6, and 203.5-kb for DraI, EcoRI, and PstI, respectively.

Two distinct mitochondrial genomes are present in the Malawian beans, representing the two major gene pools

Most bean lines were screened for all enzyme × probe combinations and the results are summarized in Table 3. In contrast to RFLPs 6 (DraI with C2) and 7 (DraI with C4 or C8), which differentiate the cultivar Mecosta from all other lines, RFLPs 1–5 separate the bean lines into two groups. Among the Malawian lines, seven out of the eight pattern no. 1 (Table 2) are small-seeded ($\bar{x}=25$ g/100 seeds), have S phaseolin, and show the Mesoamerican isozyme allele complement (Fig. 1). Eleven out of 12 Malawian lines with pattern no. 2 are medium to large-seeded ($\bar{x}=40$ g/100 seeds), have T or C phaseolin, as well as the Andean isozyme genotypes. This indicates that the two major gene pools of beans, the Mesoamerican small-seeded and the Andean large-seeded, have differentiated mitochondrial genomes as defined by five RFLPs.

Two of the Malawian lines, 4–10 and 5–25, considered as products of recent gene pool recombination based on isozyme heterozygosity (Sprecher 1988), showed patterns no. 1 and 2, respectively, for all five RFLPs. The mtDNAs of the three control cultivars fit the gene pool affiliation seen among Malawian lines only with respect to RFLP 1. Sanilac is a small-seeded Mesoamerican cultivar with pattern no. 1, while Mecosta and Tendergreen are large-seeded Andean types, with pattern no. 2. However, for each of the other four RFLPs, all three cultivars exhibited pattern no. 1, associated with small seed size and Mesoamerican origin.

Nature of the mutations giving rise to the RFLPs

RFLPs 1 and 2 were detected only in the stained gels of the DraI digests (Fig. 2A). Since they did not hybridize to any of the probes, we do not know whether they are related to each other or what kind of mutation event caused them.

Both clones C3 and C6 hybridized to a DraI polymorphism, RFLP 3, consisting of a slight difference in migration of one fragment. This RFLP is likely to be due to a small insertion/deletion event on the order of 100 bp (Fig. 2). Such an event could possibly go undetected in digest patterns from other enzymes, if it was contained in a larger fragment.

RFLP 4 appeared in the EcoRI digest as a difference in migration of the largest fragment that could be interpreted as a large insertion or deletion event (Fig. 3A). Furthermore, when the filter was hybridized with clone

Table 3. Summary of results obtained when mtDNA from 23 bean lines was digested with eight restriction endonucleases and probed with five different mtDNA clones (C2, 3, 4, 6, 8)

Detected in gel or probe	Enzyme[c]	Bean Line[a]																							
		Farm site 4								Farm Site 5														Cultivar[b]		
		1 A	4 M	6 A	7 A	10 H	11 M	20 A	22 A	1 M	2 M	4 M	5 M	6 A	7 A	10 A	14 A	16 A	18 M	20 A	25 H	Me A	S M	T A
Gel	DraI[1]	2	1	2	2	1	1	2	2	1	1	1	1	2	2	2	2	2	1	2	2	2	1	2
	DraI[2]	2	1	2	2	1	1	2	2	1	1	1	1	2	2	2	2	2	1	2	2	1	1	1
C_2	HaeIII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	BamHI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	DraI[6]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1
	EcoRI[4]	2	1	2	2	1	1	2	2	1	1	1	1	2	2	2	2	2	1	2	2	1	1	1
	HindIII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	PstI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	SalI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	XhoI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C_3	HaeIII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	BamHI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	DraI[3]	2	1	2	2	1	1	2	2	1	1	1	1	2	2	2	2	2	1	2	2	1	1	1
	EcoRI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	HindIII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	–	1	1	1	1	1	1
	PstI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	SalI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	XhoI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C_4	HaeIII	1	1	–	1	1	1	–	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	BamHI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	DraI[7]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1
	EcoRI[5]	2	1	2	2	1	1	2	2	1	1	1	1	2	2	2	2	2	1	2	2	1	1	1
	HindIII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	–	1	1	1	1	1	1
	PstI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	SalI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	XhoI	1	1	1	1	1	1	1	1	1	1	1	–	1	1	1	1	1	1	–	1	1	1	1
C_6	HaeIII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	BamHI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	DraI[3]	2	1	2	2	1	1	2	2	1	1	1	1	2	2	2	2	2	1	2	2	1	1	1
	EcoRI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	HindIII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	PstI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	SalI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	XhoI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C_8	HaeIII	1	1	–	1	1	1	–	1	1	1	1	1	1	1	1	1	1	1	1	1	–	1	1
	BamHI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	DraI[7]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1
	EcoRI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	HindIII	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	PstI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	–	1	1	1	1	1	1	1
	SalI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	XhoI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Numbers indicate the presence of either patterns no. 1 or 2 for each probe × enzyme combination (see Table 2)

[a] A, M, and H – Andean, Mesoamerican, and heterozygous isozyme genotypes, respectively (Sprecher 1988)

[b] Me – Mecosta, S – Sanilac, T – Tendergreen

[c] Superscripts indicate the RFLP number from Table 2

– Not determined

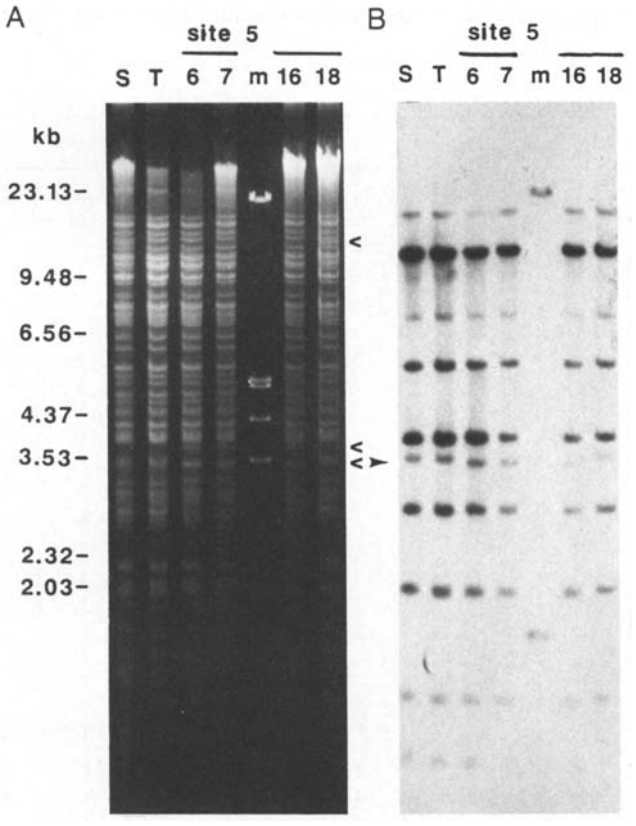

Fig. 2 A and B. DraI digestion patterns of six bean mtDNAs: Sanilac (*S*), Tendergreen (*T*), and four bean lines from site 5. **A** Ethidium-bromide-stained gel. **B** Southern blot hybridized with clone C6. *m* molecular size markers. Polymorphic bands in the gel are indicated by *open arrows* (from the top, RFLPs no. 1, 2, 3) and in the blot by a *closed one* (RFLP no. 3)

C2 (Fig. 3 B), a third polymorphic fragment of 4.3-kb appeared in the lanes of Mecosta and line 5–18 which, if added to the 18.6-kb fragment in those lanes, sums approximately to the 22.3-kb fragment present in the other lanes (lines 5–6, 5–7, 5–10, 5–16). This RFLP is thus most probably due to a base-pair mutation in an EcoRI recognition site within the 22.3-kb sequence.

RFLP 5 was revealed by the hybridization of clone C4 to a 6.2- and an 11.2-kb EcoRI fragment in patterns no. 1 and 2, respectively (Fig. 3C). These fragment differences were not detectable in the stained gel (Fig. 3 A). The polymorphism is probably not due to a large insertion/deletion event, or it would have been evident in DNA cut with the other enzymes, when hybridized with the same probe. It is possible that this RFLP also results from a base mutation in an EcoRI site, whereby the 11.2-kb fragment would give a 6.2- and a 5.0-kb fragment, and clone C4 does not extend into the 5.0-kb section, thus not hybridizing to it.

We believe that RFLPs 6 and 7, which differentiate Mecosta from the rest of the lines, are due to a single mutation event (Fig. 4). In RFLP 6, Mecosta lacks a 3.3-kb fragment, and in RFLP 7 (most probably due to a base mutation), it lacks both a 3.3- and a 4.5-kb fragment apparent in the other lines, but it shows a unique 8.5-kb band. The 3.3-kb fragment seen with probe C2 (RFLP 6) appears to be the same as that detected by C4 and C8 (RFLP 7). If the sequences contained in clone C2 do not extend into the area of the 4.5-kb fragment, C2 should still hybridize to an 8.5-kb band in Mecosta. As shown in Fig. 4 B, all lines hybridize to a fragment of that

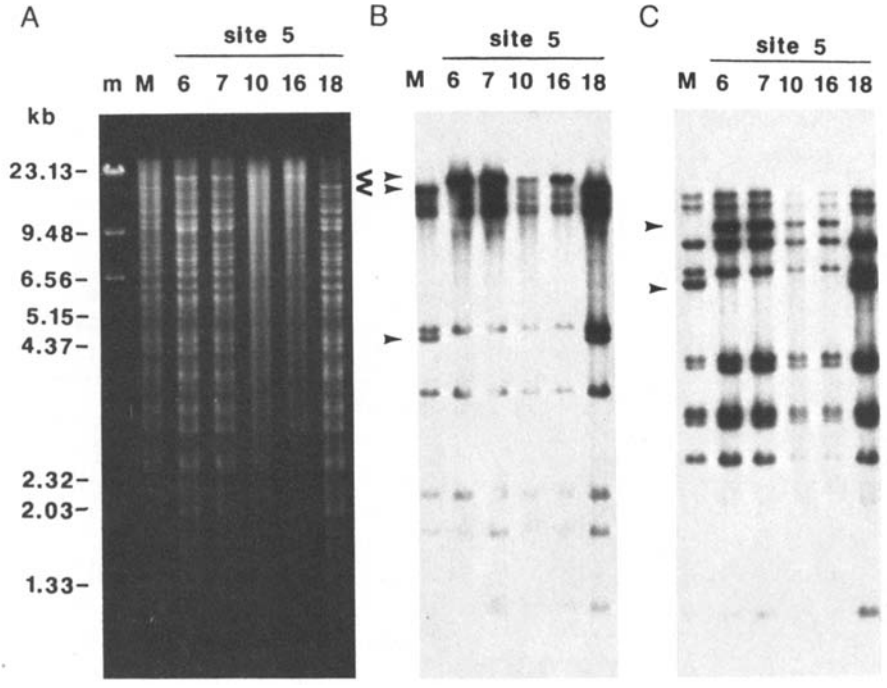

Fig. 3 A–C. EcoRI digestion patterns of six bean mtDNAs: Mecosta (*M*) and five bean lines from site 5. **A** Ethidium-bromide-stained gel; *m* molecular size markers. **B + C** Southern blots hybridized with clones C2 and C4, respectively. Polymorphic bands in the stained gel are indicated by *open arrows* (part of RFLP no. 4) and in the blots by *closed arrows* (RFLP no. 4 in **B** and no. 5 in **C**)

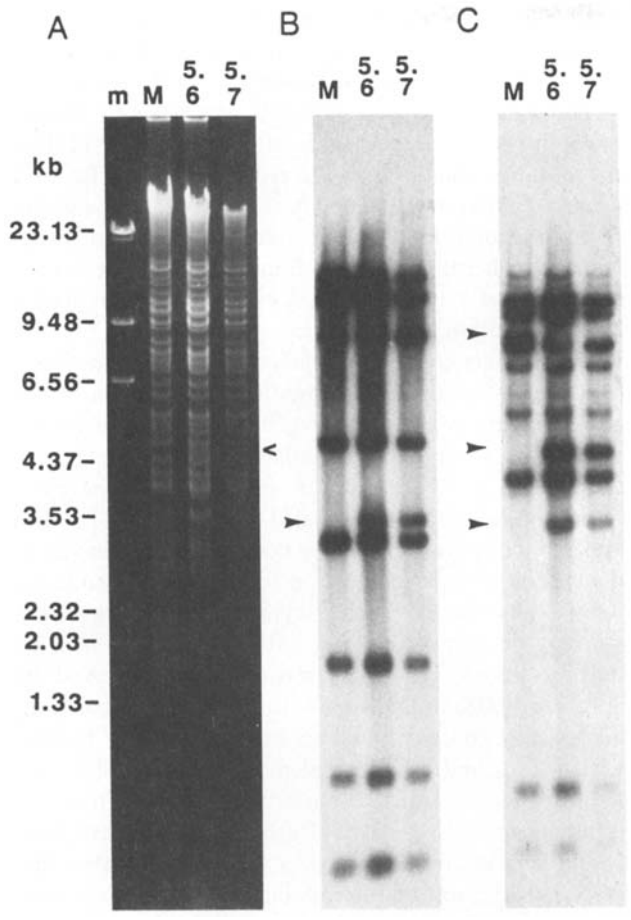

Fig. 4A–C. DraI digestion patterns of three bean lines: Mecosta (*M*) and two bean lines from site 5. **A** Ethidium-bromide-stained gel; *m* molecular size markers. **B+C** Southern blots hybridized with clones C2 and C4, respectively. Polymorphic bands in the gel are indicated by an *open arrow* (part of RFLP no. 7) and in the blots by *closed arrows* (RFLP no. 6 in **B** and no. 7 in **C**)

size, but this common fragment may mask the polymorphic one.

Discussion

Low levels of bean mtDNA variation

These analyses, using eight restriction endonucleases to digest mtDNA and cosmid probes covering about 200-kb of random mtDNA sequences, showed a high level of mitochondrial genome homogeneity among all 23 lines tested; this was in spite of their variability for seed types and sizes, growth habits, and other morphoagronomic characters, as well as seed storage protein patterns and isozymes (Fig. 1) (Martin 1984; Sprecher 1988). Similar low levels of mitochondrial intraspecific variability have been reported in soybean (Sisson et al. 1978), barley (Holwerda et al. 1986), tomato (McClean and Hanson 1986), oat (Rines et al. 1988), pearl millet (Chowdhury and Smith 1988), and eight *Brassica* species (Palmer 1988).

The kind of mutations revealed in this study contrast with the findings of Palmer (1988), who investigated the level of intraspecific mtDNA variation in eight *Brassica* species. The limited variation he found among 2–5 lines per species consisted entirely of structural changes: two deletions and an inversion. Out of 140 mapped restriction sites (or 840-bp), no base-pair mutation was detected. In our case, out of 247 restriction sites (excluding HaeIII fragments) representing 1,482-bp, at least two sites have undergone a base mutation (RFLPs 4 and 7). This gives an estimate of 0.14% nucleotide substitution within *P. vulgaris*. Holwerda et al. (1986) and McClean and Hanson (1986) reported estimates of nucleotide divergence of 0.098% in barley and 0.37% in tomato, repsectively. These estimates were determined using the shared fragment method, which assumes that all fragment changes are due to base mutations; however, in both cases this assumption was not met.

Apart from sequence comparisons, the most accurate method of studying nucleotide divergence is that followed by Palmer (1988). By mapping all restriction sites, he was able to determine the exact nature of every polymorphism. The larger size of the *P. vulgaris* mitochondrial genome (probably double that of the *Brassica*) makes this approach difficult in bean. It may also explain the higher estimate of nucleotide divergence, since the larger bean mitochondrial genome must contain more noncoding sequences than that of the *Brassica* species. Noncoding sequences are in general more prone to mutations, and they also may be richer in AT content relative to coding sequences, as is the case in the yeast mitochondrial genome (Gray 1982). This may be relevant to our findings since only two out of the eight enzymes revealed polymorphisms, and these two are EcoRI and DraI, which have a stretch of four and six ATs in their respective recognition sequences. The HindIII recognition site also contains four ATs, but these are divided by a GC pair in the middle (see Table 1).

Whether or not structural rearrangements play a more important role than sequence divergence in the evolution of the mitochondrial genomes of plants (Palmer and Herbon 1988) cannot be confirmed from this study. Without mapping, the hybridization analysis cannot reveal possible inversions, and the clones we used did not cover all of the genome. Considering the bean mt genome to be close to 450-kb in size (Khairallah 1990), and since the five cosmid clones have covered ca. 200-kb, we suspect that we have explored about 44% of the genome. This proportion should be representative of the whole genome, since the cosmid clones were randomly selected from a bean mtDNA library (C.D. Chase, personal communication).

Comparison of Malawian bean mtDNA variation and genetic diversity determined by other methods

The considerable homogeneity of the mitochondrial genome of the Malawian bean lines used in this study contrasts with the appreciable variability observed in the analysis of phenological, morphological, and agronomic traits (Martin and Adams 1987a), but not with the paucity of variation at six isozyme loci (Sprecher 1988). Out of the 375 lines used in both previous studies, the 20 analyzed here were from two farm sites and included related as well as distant lines, as determined by a principal component analysis (PCA) of 21 quantitative traits. The variability in mtDNA did not correlate with the PCA distances between the bean lines, and it did not effectively differentiate them based on seed types per se, but rather on the basis of their gene pool affiliation: the small-seeded Mesoamerican or the large-seeded Andean. Similarly, isozyme analysis characterized two major gene pools by the presence of contrasting alleles at six isozyme loci (Sprecher 1988). Both studies suggest that divergence between the gene pools preceded domestication. This hypothesis has been supported by investigations of phaseolin variability (Gepts et al. 1986) and, more recently, of allozyme diversity (Koenig and Gepts 1989) among wild bean populations. In both case, the wild beans of Mesoamerica were distinguished from those of the southern Andes by their different alleles and/or allelic frequencies at the protein loci investigated.

The usefulness of examining diversity of beans from Malawi comes from the fact that lines from both gene pools have been cocultivated for at least three centuries. Lack of inter-gene pool recombination in the mitochondrial genome may be due to its strict maternal inheritance. Two of the lines used in this study were isozyme heterozygotes, i.e., recent gene pool recombinants (Sprecher 1988). Lines 4–10 (phaseolin S) and 5-25 (phaseolin T) exhibited the Mesoamerican pattern no. 1 and the Andean no. 2, respectively, for all five mtDNA RFLPs. This suggests that in the original hybridizations, the female parents were small-seeded for 4–10 and large-seeded for 5–25, and it demonstrates the usefulness of mitochondrial RFLPs in tracing maternal lineages.

In the same context, the control cultivars Mecosta and Tendergreen, both large-seeded beans, followed the Mesoamerican pattern no. 1 for four out of five of the inter-gene pool RFLPs, contrary to our expectations. This strongly indicates that they must have had a small-seeded maternal parent in their pedigree. Mecosta does indeed have a Mesoamerican Great Northern (GN) bean in its background, [(GN × Commerical light red kidney) × Charlevoix kidney] however, the direction of the first cross is unknown. Moreover, Mecosta exhibited the S phaseolin pattern typical of small-seeded beans (Brown et al. 1981). The snap bean cultivar Tendergreen was developed in the mid 1940s, and although we do not know its entire pedigree, the data suggest that a small-seeded line was used as a maternal parent in its development.

Our interpretation of why both Mecosta and Tendergreen showed the large-seeded pattern for RFLP 1 is that the mutation that gave rise to pattern no. 1 for RFLP 1 occurred in the small-seeded gene pool at a later stage of domestication than the other mutations seen here. Strict maternal inheritance of mitochondria would rule out the possibility of a recombination event. Thus, this RFLP does not truly separate the gene pools.

Our results consolidate the concept of separate and presumably independent domestication events in *P. vulgaris*, leading to two major gene pools, as previously defined by morphological, adaptive, and biochemical traits. This study contributes evidence that the cytoplasms of the gene pools are also differentiated. This has important implications for the taxonomy and genetics of the species and the genus, and for efforts to recombine the two gene pools. Nuclear-cytoplasmic incompatibilities may be part of the reason for the presence of male sterility among gene pool recombinants observed by Sprecher (1988) in the Malawian lines. Such incompatibilities may contribute, along with the nuclear Dosage Lethal (DL_1 and DL_2) complementary genes (Shii et al. 1980), to the F_1 and F_2 hybrid weakness symptoms resulting from inter-gene pool crosses, which have been observed by many bean breeders. Knowledge of the exact kind and location of the mitochondrial genome mutations differentiating the gene pools and a better understanding of nuclear-cytoplasmic interactions in *P. vulgaris* will help resolve these questions.

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