Theor Appl Genet (1991) 82:321–328

THEORETICAL AND APPLIED GENETICS

Mitochondrial genome size variation and restriction fragment length polymorphisms in three *Phaseolus* species

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Received January 25, 1991; Accepted March 7, 1991
Communicated by R. Hagemann

Summary. Restriction patterns of mitochondrial DNA (mtDNA) from three *Phaseolus* species were examined to estimate their relative genome sizes and to determine the level of interspecific variability and relatedness. Three restriction endonucleases that produced relatively simple profiles were identified and used to determine the genome size of the three species. Taking into account fragment stoichiometries, the average estimates across enzymes were 456, 324, and 400 kb, respectively, for *P. vulgaris*, *P. coccineus*, and *P. acutifolius*. Restriction fragment length polymorphisms (RFLPs) differentiated the species when the mtDNAs were digested with seven endonucleases and hybridized with five cosmid clones covering ca. 200 kb of mtDNA sequences. Proportions of shared restriction fragments between every two species were computed as *F*-values and demonstrated that *P. vulgaris* and *P. coccineus* are more related to each other than either is to *P. acutifolius*, and that the latter has a similar degree of relationship to the other two species.

Key words. Mitochondrial RFLPs – *Phaseolus* – Species relationships – Genome size

Introduction

The genus *Phaseolus* L. originated in the American continent and comprises about 30 species (Maréchal et al. 1978), of which approximately 90% occur in Mexico or adjacent areas of the USA and Central America (Smartt 1985). Four species have been most prominent as cultivated food crops: *P. vulgaris* L. (common bean, dry bean, snap bean), *P. coccineus* L. (runner or scarlet runner bean), *P. acutifolius* Gray (tepary bean), and *P. lunatus* L. (lima bean, Sieva bean). All four species are diploid (2n = 2x = 22) and have both domesticated and wild representatives. In the case of both *P. vulgaris* and *P. lunatus*, small-seeded and large-seeded forms are thought to have been domesticated independently in Mesoamerica and Andean South America (Kaplan 1965, 1981). The other two species appear to have been domesticated in Mesoamerica (Kaplan 1965), although Pratt and Nabhan (1988) suggest Aridoamerica (northwestern Mexico and the southwestern United States) as the area of domestication of tepary bean.

Several studies on morphological and pollen characteristics (Maréchal et al. 1978), interspecific hybridization (reviewed in Hucl and Scoles 1985; Mok et al. 1986), seed protein composition (Derbyshire et al. 1976; Sullivan and Freytag 1986) and their immunochemical reactions (Kloz et al. 1966; Kloz and Klozová 1974), and isozyme patterns (Bassiri and Adams 1978) have provided information on the relationships between the four cultivated species. The studies suggested that *P. vulgaris* and *P. coccineus* are most closely related, with *P. acutifolius* more distantly related and *P. lunatus* the most distant from the other three species. According to Harlan and de Wet's (1971) gene pool nomenclature system, the primary gene pool of *P. vulgaris* comprises the genetic resources of the wild and domesticated populations, its secondary gene pool contains *P. coccineus* (and vice versa), and the tertiary gene pool includes the other species (Smartt 1985).

Comparative analysis of restriction fragment length polymorphisms (RFLPs) of organelle DNAs has enabled the resolution of species relationships in such crops as *Brassica* (Palmer et al. 1983), *Coffea* (Berthou et al. 1983), *Daucus* (DeBonte et al. 1984), *Aegilops* (Terachi and Tsunewaki 1986), *Lycopersicon* and *Solanum* (Mc-

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Clean and Hanson 1986), and *Pennisetum* (Chowdhury and Smith 1988). The relatively small size of the chloroplast genome (120–180 kb) allows differences in restriction patterns to be seen clearly in agarose gels, permits restriction site differences to be mapped, and facilitates deduction of the events that resulted in the RFLPs. Due to the larger sizes and higher rates of rearrangements of plant mitochondrial genomes with respect to chloroplast genomes, measures of mitochondrial DNA (mtDNA) relatedness between plant species have been limited to the estimation of the proportion of shared restriction fragments between any two species. Plant phylogenies have been constructed using either the cladistic approach where chloroplast DNA (cpDNA) restriction sites are considered separate characters (e.g., Palmer et al. 1983), or the phenetic approach where relationships are expressed by a distance measure obtained from the number of shared restriction sites (more feasible with cpDNA) or shared restriction fragments (more common with mtDNA, e.g., Terachi and Tsunewaki 1986).

A study of mtDNA diversity within *P. vulgaris* indicated a high complexity (large size) of the genome and a very low level of variation (Khairallah et al. 1990). The infrequent RFLPs allowed the differentiation of cultivated common bean into its two major gene pools. In this study, we have utilized mtDNA RFLPs to determine the extent of mtDNA diversity among *Phaseolus* species in comparison to the diversity within the species. We have used these data to examine species relationships. For this reason, we have limited our study to the primary, secondary, and tertiary gene pool representatives: *P. vulgaris, P. coccineus,* and *P. acutifolius*. An additional objective of this research was to estimate the mitochondrial genome size of the three species because only rough estimates for *P. vulgaris* and *P. coccineus* are available.

Materials and methods

Plant materials

Small-seeded accessions of *P. vulgaris* were used to compare its mtDNA to that of the other two species because of the common Mesoamerican origin in contrast to the South American origin of large-seeded common beans. Because no RFLPs have been detected in mtDNA from small-seeded lines of *P. vulgaris* (Khairallah et al. 1990), two were used as the source of mtDNA. These were lines 5-2 and 5-8 collected in Malawi (see Khairallah et al. 1990). A single accession each of *P. coccineus* and *P. acutifolius* was examined. These consisted of local cultivars collected at Fransisco I. Madero, Durango, and San Pedro, Coahuila in Mexico, respectively, and were grown for seed increase at Durango, Durango.

Seeds were planted in vermiculite trays and allowed to germinate in darkness. The tepary bean seeds were first scarified to ensure a faster and more uniform germination.

Genome size estimation

The mtDNAs used for estimating the sizes of the three genomes were isolated as described in Khairallah et al. (1990), but were further purified through a cesium chloride (CsCl) gradient as follows. After overnight precipitation at −20 °C, the mtDNA pellet was resuspended in 2–3 ml $T_{10}E_{0.1}$ (10 m*M* TRIS, 0.1 m*M* EDTA, pH 8.0) and 1.2 g solid CsCl/ml was added and allowed to dissolve. Bisbenzimide was added to a final concentration of 10–11 μg/ml and more CsCl was included to a final density of 1.67 g/ml, which corresponded to a refractive index of 1.3970 ± 0.0008. The gradients were centrifuged for 18–20 h at 154,300 × *g* in a Sorvall TV 865 vertical rotor. The mtDNA band was removed and the dye was extracted with NaCl-saturated isopropanol. The mtDNA was then precipitated in 1/20 vol. of 5 *M* ammonium acetate and 1/2 vol. ice-cold isopropanol at −20 °C overnight.

Samples of the mtDNAs were digested at 37 °C for 5–7 h with *Nar*I, *Sal*I, or *Sst*II [Bethesda Research Lab, Inc. (BRL)]. The resulting fragments were separated by electrophoresis in 0.5–0.7% agarose gels at room temperature for 20–35 h at constant voltage (30–40 V), 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). Three sets of molecular size markers were used: lambda DNA digested with *Hin*dIII, lambda DNA digested with *Hin*dIII and *Eco*RI, and high-molecular-weight markers from BRL. The gels were photographed with Polaroid type 55 film under UV light using one or two different exposures. The negatives were then enlarged on 8 × 10 sheet film (Kodak TMAX 100) and were scanned on a Gilford Response II spectrophotometer using 500 nm visible light to read the percentage transmittance. The areas under the peaks were determined manually by counting the number of squares on graph paper in each peak.

Sizes of individual restriction fragments were estimated following the method of Schaffer and Sederoff (1981) using a computer program written in QuickBasic.

RFLP analysis

Procedures and conditions for mtDNA isolation, digestion, and electrophoresis, Southern blotting, nick translation, and hybridization were as described in Khairallah et al. (1990). Seven restriction endonucleases were used in this study: *Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, and *Xho*I. Southern blots were consecutively hybridized to five cosmid clones provided by Dr. C. D. Chase (University of Florida, Gainesville). The clones contain random inserts from the bean mitochondrial genome ranging in size from 29 to 38 kb.

For each enzyme × probe combination, the total number of fragments hybridizing per species (*Nx, Ny*) and the number of fragments shared by each pair of species (*Nxy*) were recorded. These were then added across clones for each enzyme and adjusted by counting fragments that hybridized to more than one clone only once. Using those figures, indices of relatedness (*F*-values) for the three pairs of species were computed according to Nei and Li (1979), where $Fxy = [2\ Nxy]/[Nx + Ny]$. Higher *F*-values indicate more relation between the species.

Results

Genome size estimation

Estimates of the mitochondrial genome size of the three bean species were obtained from restriction profiles of three enzymes by adding up lengths of individual fragments and accounting for their multiplicities. For these analyses, the enzymes *Nar*I, *Sal*I, and *Sst*II were selected from a group of 11 enzymes because they produced the

Table 1. Molecular weights and stoichiometries of *Nar*I, *Sal*I, and *Sst*II restriction fragments of *P. vulgaris* mtDNA

*Nar*I			*Sal*I			*Sst*II		
Number	Size[a] (kb)	Stoichiometry	Number	Size[a] (kb)	Stoichiometry	Number	Size[a] (kb)	Stoichiometry
1	39.56		1	29.11		1	45.62	
2	31.57		2	27.26		2	39.00	
3	27.02		3	24.88		3	37.77	×2
4	24.24		4	20.73	×3	4	32.96	
5	21.85	×3	5	17.16	×2	5	24.28	
6	19.19		6	15.11		6	22.14	×2
7	16.24		7	13.64		7	18.58	×2
8	14.67	×2	8	13.23		8	13.36	×2
9	14.10		9	12.67	×2	9	12.16	
10	12.54	×2	10	10.97	×3	10	11.70	×2
11	11.93		11	9.97	×2	11	8.92	
12	11.69		12	9.08	×3	12	8.59	
13	9.59	×2	13	8.43	×2	13	7.86	
14	9.28	×2	14	7.68		14	7.19	
15	8.60	×3	15	7.38		15	6.61	
16	7.63		16	7.11		16	6.00	
17	7.45		17	6.67	×4	17	5.28	
18	6.63		18	5.13	×2	18	5.18	
19	5.19	×2	19	4.61		19	4.24	×3
20	4.75		20	4.45	×5	20	4.11	
21	4.33		21	3.88		21	3.74	
22	3.80		22	3.50	×2	22	3.39	
23	3.58	×2	23	3.25		23	3.14	×2
24	2.94		24	3.05		24	3.04	
25	2.83		25	2.87	×2	25	2.78	
26	2.25		26	2.54		26	2.71	
27	2.05		27	2.42		27	2.55	
28	1.70		28	2.36		28	1.91	
29	1.56					29	1.70	
30	1.31					30	1.30	
Total	330.07 **445.82**[b]		Total	279.14 **458.24**[b]		Total	347.81 **462.98**[b]	

[a] Sizes of individual fragments are average values from three or four gels
[b] Numbers in bold are the sum of fragment sizes, taking into account their relative multiplicities

simplest profiles (see Fig. 1 A for *Sal*I). Each digest was repeated three to four times and those replications were used to determine presence/absence of a band, band sizes, and stoichiometries (Tables 1, 2, and 3).

Sheet film negatives scanned on a spectrophotometer allowed the identification of individual band peaks (e.g., Fig. 1 B). Band multiplicities were determined by considering the areas under peaks in the densitometric scans, as well as the band intensities in enlarged prints of the gels. Submolar bands or fragments not present in all replicates were not counted in the totals and are not mentioned in Tables 1, 2, and 3.

By averaging the values obtained with the three enzymes, we estimate the mitochondrial genome sizes of *P. vulgaris, P. coccineus,* and *P. acutifolius* to be 456, 324, and 400 kb, respectively.

RFLP analysis

In order to determine the level of mitochondrial interspecific variation, seven restriction endonucleases were used to digest the mtDNA of three *Phaseolus* species. The resulting profiles after electrophoresis were quite complex, but differences between the species were obvious for all enzymes. Figure 2 A shows the patterns observed with the enzyme *Eco*RI. For a more accurate analysis of the variation between species, the gels were blotted and the Southern filters were consecutively hybridized to five cosmid clones. The number of mtDNA fragments hybridizing to each clone varied between species and for each enzyme. Figure 2 shows only the case of the *Eco*RI digestion and the hybridizations with clones C2, C6, and C8. The number of fragments hybridizing per species and those common between two species were totaled across

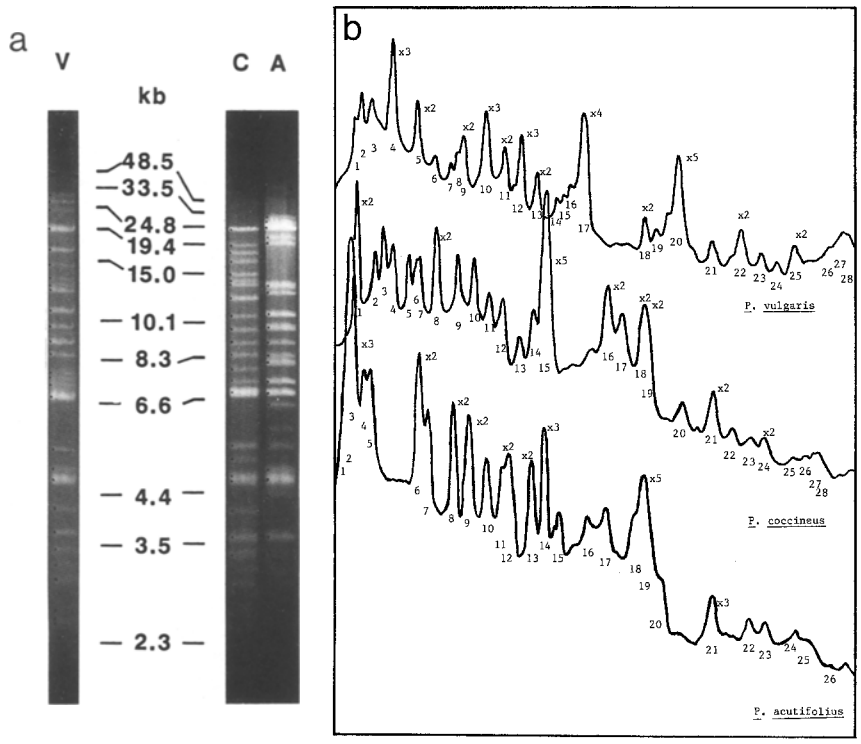

Fig. 1 a and b. *Sal*I digestion patterns of mtDNAs from three *Phaseolus* species. V = *P. vulgaris*, C = *P. coccineus*, and A = *P. acutifolius*. **a** Ethidium-bromide-stained gel; *dots* identify individual bands. **b** Densitometric scans of photographic negatives. Numbers under the peaks identify the individual restriction fragments, and above the peaks indicate the multiplicity of non-stoichiometric bands

Table 2. Molecular weights and stoichiometries of *Nar*I, *Sal*I, and *Sst*II restriction fragments of *P. coccineus* mtDNA

*Nar*I			*Sal*I			*Sst*II		
Number	Size[a] (kb)	Stoichiometry	Number	Size[a] (kb)	Stoichiometry	Number	Size[a] (kb)	Stoichiometry
1	40.07		1	25.80	×2	1	45.55	
2	34.78		2	20.93		2	33.92	×2
3	24.11	×2	3	19.42		3	18.60	
4	22.18	×2	4	17.32		4	13.64	×2
5	18.32		5	15.50		5	12.44	×2
6	16.91		6	14.59		6	11.88	×2
7	14.67		7	14.08		7	10.60	×2
8	9.86	×2	8	12.63	×2	8	9.02	
9	9.47		9	10.94		9	8.73	
10	7.81		10	9.97		10	6.65	×2
11	7.70		11	9.10		11	6.01	
12	6.91		12	8.41		12	5.34	
13	6.78		13	7.69		13	5.21	
14	5.31	×2	14	7.17		14	4.29	×2
15	4.82		15	6.77	×5	15	4.18	
16	4.38		16	5.19	×2	16	3.80	
17	4.27		17	4.88		17	3.43	
18	3.90		18	4.48	×2	18	3.21	
19	3.66		19	4.40	×2	19	3.04	
20	2.98		20	3.89		20	2.79	×2
21	2.87		21	3.51	×2	21	2.73	×2
22	2.20		22	3.26		22	1.96	
23	2.00		23	3.05		23	1.30	
24	1.66		24	2.86	×2			
25	1.50		25	2.62				
26	1.26		26	2.51				
			27	2.39				
			28	2.37				
Total	260.38 **321.84**[b]		Total	245.73 **331.68**[b]		Total	218.32 **317.26**[b]	

[a] Sizes of individual fragments are average values from three or four gels
[b] Numbers in bold are the sum of fragment sizes, taking into account their relative multiplicities

Table 3. Molecular weights and stoichiometries of *Nar*I, *Sal*I, and *Sst*II restriction fragments of *P. acutifolius* mtDNA

*Nar*I			*Sal*I			*Sst*II		
Number	Size[a] (kb)	Stoichio-metry	Number	Size[a] (kb)	Stoichio-metry	Number	Size[a] (kb)	Stoichio-metry
1	38.17		1	29.08		1	32.94	
2	32.40	×2	2	27.41		2	30.54	
3	25.11	×3	3	25.13	×3	3	28.45	
4	21.67		4	22.40		4	24.47	
5	18.04		5	21.19		5	22.43	×4
6	17.30		6	14.11	×2	6	16.48	×3
7	14.70	×2	7	13.15		7	13.63	
8	11.53		8	11.12	×2	8	13.03	
9	10.94		9	10.15	×2	9	12.60	
10	9.76		10	9.12		10	12.22	
11	9.22		11	8.47		11	8.91	
12	8.55		12	8.12	×2	12	7.84	
13	7.60	×3	13	7.28	×2	13	7.19	
14	6.67	×2	14	6.83	×3	14	6.58	
15	5.29		15	6.40		15	5.96	
16	4.80	×4	16	5.65		16	5.80	
17	4.34		17	5.17		17	5.15	
18	3.78		18	4.64		18	4.25	×2
19	3.30		19	4.43	×5	19	4.10	
20	2.98		20	4.16		20	3.75	
21	2.87		21	3.50	×3	21	3.39	
22	2.20		22	3.22		22	3.16	
23	2.00		23	3.05		23	3.07	
24	1.82		24	2.83		24	2.80	×2
25	1.66		25	2.51		25	2.73	
26	1.50		26	2.38		26	2.54	
27	1.26					27	1.97	
						28	1.58	
Total	269.46 **403.05**[b]		Total	261.50 **400.92**[b]		Total	287.56 **394.86**[b]	

[a] Sizes of individual fragments are average values from three or four gels
[b] Numbers in bold are the sum of fragment sizes, taking into account their relative multiplicities

Table 4. Proportion of mtDNA fragments shared between three pairs of *Phaseolus* species as obtained from hybridization experiments to five cosmid clones

Enzyme	Total fragments[a]			Common fragments[b]			*F*-values[c]		
	Nv	Nc	Na	Nvc	Nva	Nca	Fvc	Fva	Fca
*Bam*HI	36	33	36	24	20	19	0.70	0.56	0.55
*Dra*I	37	29	32	12	8	9	0.36	0.23	0.30
*Eco*RI	51	49	48	36	26	24	0.72	0.53	0.50
*Hin*dIII	49	41	45	33	35	30	0.73	0.74	0.70
*Pst*I	28	28	28	18	17	14	0.64	0.61	0.50
*Sal*I	21	20	18	10	9	9	0.49	0.46	0.47
*Xho*I	33	31	28	20	18	19	0.63	0.59	0.64
Totals	255	231	235	153	133	124	0.63	0.54	0.53

[a] Total number of restriction fragments hybridizing to the five clones for each species; v = *P. vulgaris*, c = *P. coccineus*, and a = *P. acutifolius*
[b] Total number of fragments across the five clones shared by two species
[c] Proportion of shared fragments between two species, $F_{xy} = \frac{2N_{xy}}{N_x + N_y}$ (Nei and Li 1979)

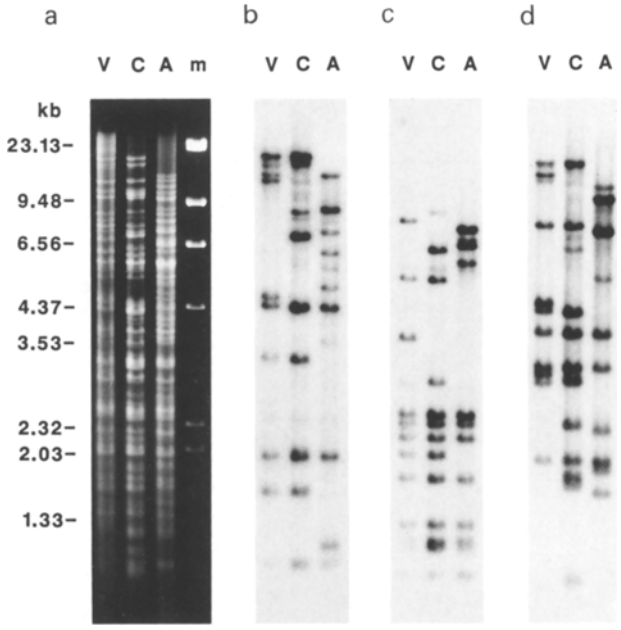

Fig. 2a–d. *Eco*RI digestion patterns of mtDNAs from three *Phaseolus* species. V = *P. vulgaris*, C = *P. coccineus*, and A = *P. acutifolius*. **a** Ethidium-bromide-stained gel m = lambda DNA digested with *Hin*dIII. **b+c+d** Southern blots hybridized respectively with clones C2, C6, and C8

the five clones for each species × enzyme combination, and the proportions of shared fragments (*F*-values) between two species were calculated according to Nei and Li (1979) for all the enzymes (Table 4).

F-values ranged from a low of 0.23 for *Dra*I to a high of 0.74 for *Hin*dIII. For five out of seven of the enzymes, the highest *F*-value was for the *P. vulgaris-P. coccineus* comparison, indicating that these species are most closely related. When the *F*-values were averaged across enzymes, the proportion of shared fragments was 0.63, 0.54, and 0.53 for the *P. vulgaris* /*P. coccineus*, *P. vulgaris*/*P. acutifolius*, and *P. coccineus*/*P. acutifolius* comparisons, respectively.

Discussion

Genome size estimation

Plant mitochondrial genomes vary remarkably in size (200–2400 kb) and are significantly larger than those from animals (15–18 kb) and fungi (18–78 kb) (Levings 1983; Newton 1988). The variation in size can be quite high within the same family of plants as Ward et al. (1981) showed for the cucurbits (320–2400 kb), or minimal (208–242 kb) as in the *Brassica*/*Raphanus* mitochondrial genomes (Lebacq and Vedel 1981; Palmer and Herbon 1988).

The most accurate method for estimating the mitochondrial genome size is by restriction mapping, which has been used for maize (Lonsdale et al. 1984), for *Brassica* (Palmer and Shields 1984; Palmer and Herbon 1986), and for spinach (Stern and Palmer 1986). However, the large sizes of mitochondrial genomes have limited both its use, as well as that of electron microscopy in assessing lengths of mtDNA molecules. Two other methods have been utilized more extensively, namely, reassociation kinetics and summation of fragment sizes from restriction profiles. The latter two methods have resulted in figures that agree, while the estimates from restriction maps are usually 15–30% larger. The restriction profile method was used in the present study to determine the genome size of three *Phaseolus* species. In order to address the problems of the complex banding patterns and nonstoichiometry of some of the fragments associated with this method, three endonucleases that produce relatively simple profiles were chosen, and from these, we have estimated the copy number of individual fragments. To optimize the resolution of the larger fragments obtained with the less frequent cutters, low percentage agarose gels were run at low voltages for long times. In addition, CsCl purification of mtDNAs helped minimize the background in the gels, three to four gels of each enzyme were run to increase the confidence of the estimates, and 30 molecular weight markers ranging in size from 48.5 to 0.56 kb were present in all gels.

For each species, the estimates obtained with the different enzymes were close (less than 5% difference) to each other (Tables 1, 2, and 3). The average respective values of 456, 324, and 400 kb for common bean, runner bean, and tepary bean fall within or close to the range of 290–430 kb for other leguminous crops (Ward et al. 1981; Bendich 1982; Stern and Palmer 1984). A smaller genome size for *P. coccineus* compared to *P. vulgaris* has previously been reported (Bannerot and Charbonnier 1988). Using *Sal*I digestion profiles, Bannerot and Charbonnier (1988) had estimated the mitochondrial genome size of *P. vulgaris* and *P. coccineus* to be about 320 kb and 240–260 kb, respectively. Their estimates did not take into account band multiplicities, and they recognized that the use of several enzymes will improve the estimation of the genome size. The totals obtained with our *Sal*I digests, disregarding individual band stoichiometries, were 40 kb less for *P. vulgaris* but agree well for *P. coccineus* when compared to these previous estimates (Tables 1 and 2). The difference in the common bean mtDNA estimate is probably due to variation in resolution in certain parts of their gels or ours.

Phaseolus species relationships

Considerable variation of mtDNA restriction profiles and hybridization patterns differentiated the three *Phaseolus* species, in contrast to the high homogeneity within *P. vulgaris* (Khairallah et al. 1990). Due to this

high level of mtDNA variation and because no restriction maps of the mitochondrial genomes of beans are as yet available, it was not possible to determine the nature of the mutations giving rise to the observed polymorphisms. However, the variability in genome size between the three species indicates that insertion/deletion/duplication events must have taken place during the evolution of the *Phaseolus* genus. We cannot rule out the possible past occurrence of rearrangements, especially in light of the recent study in *Brassica*, where Palmer and Herbon (1988) have clearly demonstrated the significant role of mtDNA rearrangements in the evolution of the mitochondrial genome in that genus.

Due to the probable rearrangements and length mutations, we are not using the *F*-values as measures of nucleotide divergence (Nei and Li 1979), but as indices of relatedness between every pair of species. For the same reason, the data from the different endonucleases are not pooled, since the same length mutation or rearrangement will be detected with several, if not all, of the enzymes used. *F*-values have also been used as indices of relatedness in studies of species relationships in *Daucus* (DeBonte et al. 1984; Ichikawa et al. 1989), *Aegilops* (Terachi and Tsunewaki 1986), and *Pennisetum* (Chowdhury and Smith 1988). These *F*-values agreed with the conventional classification methods but also provided unique insights into the phylogenetic relationships.

In the case of *Phaseolus,* the indices of mtDNA relatedness comply with the species relationships as defined by morphological, genetic, and biochemical data. The higher Fvc values as compared to Fva or Fca (Table 4) clearly demonstrate that *P. vulgaris* and *P. coccineus* are more related to each other than either is to *P. acutifolius*. A *P. vulgaris-P. coccineus* species complex that includes the wild *P. vulgaris* forms and the three other *P. coccineus* subspecies, *polyanthus, obvallatus,* and *formosus,* has been recognized by Maréchal et al. (1978) after an extensive study of morphological and pollen characteristics.

It was suggested that the close genetic relationship of the common and the scarlet runner bean results from either common descent or through species introgression (Bassiri and Adams 1978). Because of the maternal inheritance of mitochondria, introgression between those species could perhaps be detected as either the presence of a *P. vulgaris*-like chondriome (mitochondrial genome) in wild *P. coccineus* populations or a *P. coccineus*-like chondriome in wild *P. vulgaris* populations. Since most successful hybridizations between the two species occur when *P. vulgaris* is the female parent (Hucl and Scoles 1985), the first case is more probable than the second. It would be interesting to look at the mtDNA diversity within *P. coccineus,* especially in wild populations suspected or known to grow within the natural range of *P. vulgaris* wild populations.

P. acutifolius is often referred to as closely related to the *P. vulgaris-P. coccineus* complex rather than to either species. Our results support the same kind of relationship. The Fva and Fca values are not consistently larger or smaller when compared across the endonucleases used (Table 4). Their averages (Fva = 0.54 and Fca = 0.53) are not significantly different, indicating the same level of mtDNA divergence between *P. vulgaris* and *P. acutifolius* as between *P. coccineus* and *P. acutifolius.*

A different approach such as the examination of cpDNA variation and mapping of its various mutations, or the comparison of DNA sequence data, will probably provide a finer resolution of such relationships.

Acknowledgements. The authors thank J. A. Acosta for supplying seed samples of the tepary bean and the scarlet runner bean, C. D. Chase for providing the cosmid clones, J. F. Hancock and J. D. Kelly for reviewing the manuscript and allowing use of equipment, R. Mohtar for writing the program for determining DNA fragment sizes, and P.-O. Lim for advice and helpful comments during the course of this study. M.M.K. was supported by the Bean/Cowpea Collaborative Research Support Program, Malawi project. Funding from the Michigan State University Agriculture Research Station is acknowledged.

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