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F. Hervieu · H. Bannerot · G. Pelletier

A unique cytoplasmic male sterility (CMS) determinant is present in three *Phaseolus* species characterized by different mitochondrial genomes

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Abstract Previous results have shown that cytoplasmic male sterility (CMS) in lines from *Phaseolus coccineus* and *Phaseolus vulgaris* contain the same CMS-specific sequence, raising the question of whether this sequence rearrangement arose before divergence of the two species or afterward with subsequent transfer by introgression. Hybridization patterns of total DNA from eight *P. vulgaris* lines with cytoplasm from *P. coccineus* and three *P. vulgaris* lines were examined in order to analyze the mitochondrial DNA (mtDNA) diversity within each species and to determine differences between CMS lines derived from the two species. Three restriction enzymes and 17 heterologous mtDNA sequences were used. The analysis of the different hybridization patterns revealed a considerable diversity in mtDNA organization particularly within *P. coccineus*. We obtained distinctive hybridization patterns for the five CMS lines tested. The resulting classification showed that mitochondrial genomes from *P. coccineus* CMS lines group with those of fertile *P. coccineus* but not with CMS lines from *P. vulgaris*. The groupings concur with the taxonomic classification of these lines. The results support the hypothesis of a single ancient origin of the CMS determinant and exclude the transfer of cytoplasm by introgression from *P. vulgaris* to *P. coccineus* and *P. coccineus* ssp *polyanthus*.

Key words RFLP · mtDNA diversity · *Phaseolus coccineus* · *P. polyanthus* · *P. vulgaris* · cytoplasmic male sterility

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F. Hervieu (✉) · G. Pelletier
Laboratoire de Biologie Cellulaire, INRA, route de St Cyr, 78026 Versailles Cedex, France

H. Bannerot
Laboratoire de Génétique et Amélioration des plantes, INRA, route de St Cyr, 78026 Versailles Cedex, France

Introduction

Restriction fragment length polymorphisms (RFLPs) in mitochondrial DNAs (mtDNAs) and chloroplast DNAs (cpDNAs) have been analysed for several plant species and diversity has been detected that has provided interesting information about relationships between and within species. RFLP analysis is a simple and rapid approach as illustrated by the number of published examples. Estimates of either cpDNA or mtDNA variation based on RFLP analysis have been reported for maize and teosinte (Timothy et al. 1979), *Brassica* (Palmer and Herbon 1988), *Helianthus* (Rieseberg et al. 1991), and *Aegilops* and *Triticum* (Terachi and Tsunewaki 1992). Such an analysis has resulted in the determination and characterization of cytoplasms associated with a cytoplasmic male sterility (CMS) phenotype in maize (Pring and Levings 1978), wheat (Ricard et al. 1986), *Helianthus* (Crouzillat et al. 1987) and *Brassica* (Bonhomme et al. 1991).

At least five sources of CMS have been found in the genus *Phaseolus*, the first case, in common bean, being observed at CIAT (Centro Internacional de Agricultura Tropical, Cali Colombia) in field trials of the accession line G08063 (Singh et al. 1980). The cytoplasmic nature of this source was confirmed later by Bassett and Shuh (1982). The CMS-Sprite, well characterized at the genetic and molecular level, was derived from a cross between *P. vulgaris* genotype G08063 and a relatively stable maintainer line "Sprite" (Mackenzie et al. 1988; Mackenzie and Chase 1990; Mackenzie 1991).

The four other CMS-inducing cytoplasms were identified at INRA (Institut National de la Recherche Agronomique), Versailles, by crossing different *P. coccineus* (or *P. coccineus* ssp. *polyanthus*) genotypes as female with *P. vulgaris*. The hybrids were successively backcrossed to the same male parent to obtain a *P. vulgaris* nuclear background (Bannerot and Charbonnier 1987). The CMS plants obtained have the same characteristic male-sterile phenotype irrespective of the

origin of their cytoplasm, i.e., whether from *P. vulgaris* or *P. coccineus*. Male sterility is expressed late in pollen development. To-date, all the different CMS lines accept the same set of maintainer and restorer genotypes (Bannerot, unpublished data). However, a complete (and reversible) fertility restoration in the F_1 generation has not been reported in bean. The introduction of fertility restorer gene candidates to CMS lines results in a higher percentage of pollen stainability than in sterile controls, but lower than in fertile ones, and the pollen grains remain associated in tetrads (Bannerot 1988).

Molecular studies on *P. vulgaris* CMS-Sprite (Chase and Ortega 1992; Johns et al. 1992) have resulted in the identification of a complex structure specific to CMS plants and flanking an *atp*A gene sequence. Our mitochondrial DNA analyses of the four CMS-inducing cytoplasms developed at Versailles suggest that they contain the same CMS determinant (Hervieu et al. 1993). The work described in the present paper identifies mtDNA differences between the five CMS-inducer cytoplasms identified in the genus *Phaseolus* and defines CMS-specific RFLPs for the CMS lines originating from interspecific crosses. We have classified the different cytoplasms using RFLP analyses of mtDNA to test if the occurrence of the CMS-specific determinant in two *Phaseolus* species was due to a transfer of cytoplasm from one species to the other. Cytoplasmic transfer would result in discrepancies between the classification based on mtDNA RFLP data and the traditional botanical classification. To test this, we have also analysed mtDNA from several fertile lines in order to classify the different cytoplasms according to their species origin.

Materials and methods

Plant material

The *Phaseolus* lines analysed (with CMS-inducing or non-inducing cytoplasms) are presented in Table 1. Most plants were crossed and successively backcrossed to the same maintainer (*P. vulgaris* MM3) for at least three (for fertile lines) or over ten (for sterile lines) generations to obtain a set of plants with a similar nuclear background.

The CMS line named Ci was derived from G08063 cytoplasm (kindly provided by S. Mackenzie) and may be considered as an equivalent of CMS-Sprite. This CMS line was sometimes unstable and spontaneous fertile revertant plants from CMS-Ci were obtained. Two of these revertant plants, (GM10 or Gitana), with a *P. vulgaris* sterility maintainer, were included in the analysis. It is noteworthy, however, that reversion to fertility was never observed with CMS-inducing cytoplasms other than Ci (H. Bannerot, personal communication). MoGit × KHR1 is a semi-fertile restored line resulting from the cross between a CMS line with the Mo cytoplasm and the line KHR1 derived from a multicross hybrid (*P. vulgaris* × *P. coccineus*).

Probes

Twelve different mtDNA gene sequence probes (see Table 2) and five anonymous lambda clones from an Ogura radish (Ogura 1968) mtDNA library (kindly provided by D. Lancelin) were used to detect RFLPs.

Isolation and analysis of nucleic acids

Total DNA was isolated from 4-week-old plant leaves according to Dellaporta et al. (1983). After precipitation, total DNA was purified by CsCl-ethidium bromide gradient centrifugation. Restriction digests, gel electrophoresis and blotting were as described in Sambrook et al. (1989). Ten micrograms of genomic DNA per sample were digested with the restriction enzymes *Bam*H1, *Eco*RI or *Eco*RV according to the supplier's instructions (Bethesda Research Laboratory). Prehybridization and hybridiztion was conducted according to Amersham. The probes used were labelled with the multiprime DNA labelling system (Amersham, Bucks, UK) and purified on Sephadex G50 columns (Sambrook et al. 1989).

Analysis of data

The presence of specific restriction fragments on autoradiograms was scored in digital form for all genotypes and all fragment positions checked. The data was analysed using the Restsite v1.1 program (Miller 1990). The pairwise similitude coefficient (F-values) and the distance between all individual genotypes were calculated using the shared fragments method of Nei and Li (1979); the F-value being calculated from the RFLP data using $F(x, y) = 2n_{xy}/[n_x + n_y]$, where n_x was the number of mtDNA fragments analysed in one cytoplasmic type, n_y the number of mtDNA fragments analysed in a second cytoplasmic type and n_{xy} the number of fragments shared between the two cytoplasmic types. The phenograms were obtained using the

Table 1 Description and origin of some genotypes and hybrids used as female parents (MM3, GM10 and Gitana are sterility maintainer genotypes; KHR1 is a fertility restorer candidate; CMS implies sterility inducing when combined with sterility maintainer genotypes)

Cytotype	Description and origin
Ci	*P. vulgaris* G08063 – CMS-inducing cytoplasm
Da	*P. polyanthus* (Greenman) NI785 – cultivar Guatemala-CMS-inducing cytoplasm
Hq	*P. coccineus* NI889-wild (Guatemala) – CMS-inducing cytoplasm
Mo	*P. coccineus* Morelos 662-wild (Mexico) – CMS-inducing cytoplasm
Sp	*P. coccineus* NI756-cultivar Patzun (Guatemala) – CMS-inducing cytoplasm
C	*P. coccineus* CL4x – selection maaterial (Chili)
L	*P. coccineus* LTE8 – selection material (Europe)
P	*P. coccineus* cultivar Petaco (Colombia)
T	*P. coccineus* ssp. *purpurascens* Tres Cumbres – wild (Mexico)
Rev1	*P. vulgaris* revertant fertile line of CMS line Ci Gitana
Rev2	*P. vulgaris* revertant fertile line of CMS line Ci GM10
Rest1	*P. coccineus* (MoxGM10) × KHR1-Semi restored F1 hybrid of CMS line Mo
MM3	*P. vulgaris* cultivar Emerite

Table 2 List of probes used

Probe		Origin	Reference
Cytochrome c oxidase subunit I	*coxI*	Wheat	Gift B. Lejeune
Cytochrome c oxidase subunit II	*coxII*	Wheat	Gift B. Lejeune
Cytochrome c oxidase subunit III	*coxIII*	Wheat	Gualberto et al. 1990
NADH ubiquinone reductase subunit 2	*nad2*	Wheat	Gift V. Patell
NADH-ubiquinone reductase subunit 3	*nad3*	Wheat	Gualberto et al. 1988
NADH ubiquinone reductase subunit 4	*nad4*	Wheat	Lamatina et al. 1989
NADH ubiquinone reductase subunit 5 intron IV	*nad5-IV*	Wheat	Peirera et al. 1991
rRNA subunit 18*s* and 5*s*	*rrn*18*s*, 5*s*	Wheat	Lejeune et al. 1987
F_1-ATPase subunit α	*atpA*	*Nicotiana*	Gift M. Boutry
F_0-ATPase subunit 6	*atp6*	Tobacco	Bland et al. 1987
F_0-ATPase subunit 9	*atp9*	Sunflower	Gift H. Recipon
Apocytochrome subunit b	*cob*	Maize	Dawson et al. 1984

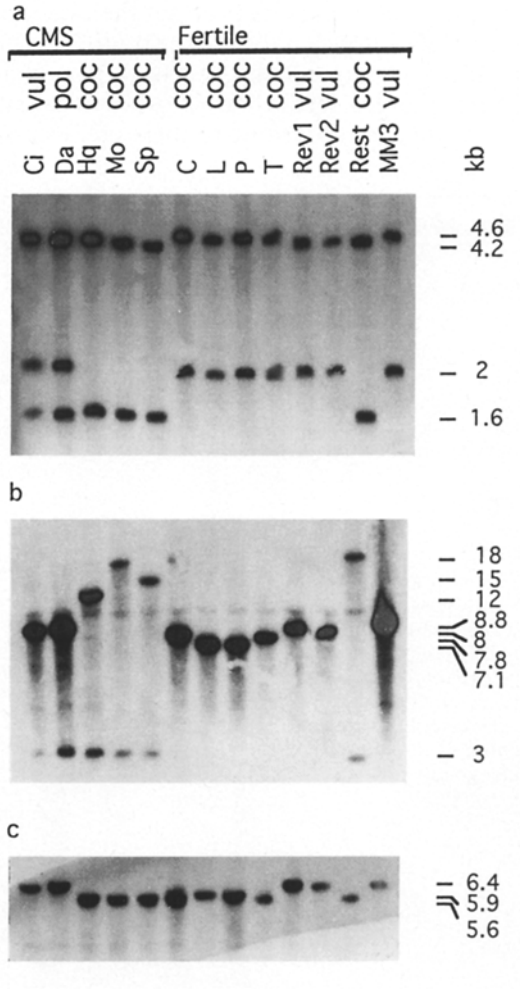
UPGMA program v2.0 (Miller 1990) performing Sneath and Sokal's UPGMA (unweighted pair-group method analysis) on matrices of distance values.

Results

RFLP analysis

Gene data. Studies using three restriction endonucleases were conducted on 12 total DNA preparations of either *P. coccineus* or *P. vulgaris* cytoplasms from CMS and male-fertile plants. The restriction fragment profiles of *Phaseolus* mtDNA are complex, due to the large size of *Phaseolus* mitochondrial genomes (350–400 kb; Kairallah et al. 1991), and relatively uniform across the different genotypes and species. We found that hybridization experiments provided more conclusive results. In order to simplify the procedure we have used total DNA; total DNA extraction is quicker and simpler than mtDNA preparation and can be done on much less plant material. Twelve mitochondrial gene probes were used in hybridization experiments. For example, Fig. 1 presents the hybridization patterns obtained with the mitochondrial probes *atpA* (Fig. 1a), *cob* (Fig. 1b) and *nad5* intron IV (Fig. 1c). The *atpA* and *cob* probes distinguished the CMS-inducing cytoplasms and non-inducing cytoplasms, whatever the enzyme used. In addition, *cob* combined with *Eco*RV revealed eight different cytoplasms among the 12 studied. The *nad5* intron IV probe distinguished cytoplasms according to their species-origin, *P. coccineus* or *P. vulgaris*, with the exception of the *P. polyanthus* cytoplasm, which was similar to that of *P. vulgaris*. A summary of the different combinations, and definitions of the different RFLP patterns obtained are given in Table 3. In all, 135 fragment positions were scored for 36 probe × enzyme combinations. No polymorphisms were detected among the 12 cytoplasms using *nad4*, *coxI*, or six other probe × enzyme combinations (*rrn*18*s*-5*s* × *Bam*HI, *Eco*RI; *atp9* × *Bam*HI, *Eco*RI; *nad2* × *Eco*RV and *nad5* intron IV × *Eco*RI). Six probe × enzyme combinations (*coxIII* × *Bam*HI, *coxIII* × *Eco*RV, *atpA* × *Eco*RI, *atpA* × *Eco*RV, *cob* × *Bam*HI and *cob* × *Eco*RI) provided redundant data. We have chosen to ignore non-polymorphic probes and redundant combinations [e.g., selecting only one *atpA* combination (*atpA* × *Bam*HI)

Fig. 1a–c Southern hybridization analyses of total DNA from different *Phaseolus* cytotypes (either CMS-inducing or non-inducing). Total DNA was digested and probed with; **a** *Bam*HI and the *atpA* gene; **b** *Eco*RV and the *cob* gene; **c** *Eco*RV and intron IV of the *nad5* gene *COC*, *P. coccineus; pol*, *P. polyanthus*; *vul. P.vulgaris*; *Rev*, revertant lines of CMS line Ci and *Rest*, fertile restored line of the CMS line Mo



and not counting the other which showed no additional differences between cytoplasms] thus leaving 20 probe × enzyme combinations which identified 82 polymorphic fragments. Figty-one percent of the fragments were specific for *P. coccineus* cytoplasms, less than 4% for *P. vulgaris*, and less than 4% for CMS cytoplasms other than those due to species differences. The results obtained are summarized (Table 3) and presented (Table 4) as F-values calculated with the 20 selected probe × enzyme combinations.

Anonymous sequence data. A second set of results were obtained using anonymous lambda clones from an Ogura radish mtDNA library as probes. These probes, larger than the gene-specific ones, were used to obtain further information by covering a larger portion of the mitochondrial genomes. The number of the fragments which hybridized was consequently higher per pattern. As for the known probes we have selected only polymorphic data for the phenogram analysis and have excluded probes which gave hybrid-

Table 3 Summary of patterns which show RFLPs obtained when total DNA from 12 bean cytoplasms was digested with three restriction endonucleases and probed with 12 mtDNA gene sequence probes together with definition of each pattern type indicated in kb (Numbers in bold indicate the presence of patterns type no. 1, 2... for each enzyme × probe combination. Description of polymorphic fragments from each probe × enzyme combination are indicated in kb)

Probe × enzyme		CMS					Fertile						
		Ci	Da	Hq	Mo	Sp	C	T	L	P	MM	Rev	Rest
rrn18s,5s	*Eco*RI	**1**	**2**	**1**	**1**	**1**	**3**	**3**	**4**	**4**	**4**	**1**	**1**
	kb										no1:6; no2:8.6; no3:4.3; no5:5		
coxIII	*Bam*H1	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**2**	**1**	**1**	**1**
	kb											no1:5.8; non2:3.6	
coxIII	*Eco*RV	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**2**	**1**	**1**	**1**
	kb											no1:6.8; no2:11	
coxIII	*Eco*RI	**1**	**2**	**1**	**1**	**1**	**3**	**3**	**4**	**4**	**1**	**1**	**1**
	kb										no1:9; no2:14; no3:11; no4:13.5		
coxII	*Bam*H1	**1**	**1**	**2**	**2**	**2**	**2**	**3**	**1**	**1**	**1**	**1**	**2**
	kb											no1:17; no2:14.5; no3:16	
coxII	*Eco*RV	**1**	**1**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**1**	**1**	**2**
	kb												nol:4; no2:4.5
cox*II*	*Eco*RI	**1**	**1**	**2**	**2**	**2**	**3**	**4**	**5**	**5**	**1**	**1**	**2**
	kb								no1:12; no2:19; no3:10.5; no4:8.6; no5:7.9				
atp9	*Eco*RV	**1**	**3**	**2**	**4**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**4**
	kb							no1:18 + 11; no2:21 + 20 + 11; no3:19 + 11; no4:11					
atp6	*Bam* H1	**1**	**2**	**1**	**2**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**2**
	kb										no1:13.5 + 3.5; no2:12.5 + 7.9		
atp6	*Eco* RV	**1**	**2**	**3**	**2**	**4**	**5**	**5**	**5**	**5**	**1**	**1**	**2**
	kb						no1:11.5; no2:6.8; no3:10.5; no4:14.5 + 6.8; no5:9.8 + 6.8						
atp6	*Eco*RI	**1**	**2**	**5**	**3**	**3**	**3**	**3**	**4**	**4**	**1**	**1**	**3**
	kb					no1:7.8 + 4; no2:8 + 1.35; no3:5 + 4; no4:7.2 + 2 + 1.35; no5:7.2 + 1.35							
atpA	*Bam* HI	**1**	**1**	**2**	**2**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**2**
	kb								no1:4.2 + 2 + 1.6; no2:4.2 + 1.6; no3:4.6 + 2				
atpA	*Eco*RV	**1**	**1**	**2**	**2**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**2**
	kb										no1:12 + 8; no2:8; no3:12		
atpA	*Eco*RI	**1**	**1**	**2**	**2**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**2**
	kb										no1:3.5 + 3; no2:3; no3:3.5		
cob	*Bam*H1	**1**	**1**	**2**	**2**	**3**	**4**	**4**	**4**	**4**	**4**	**4**	**2**
	kb							no1:6.5 + 3.8; no2:5.6 + 3.8; no3:3.8 + 3.5; no4:6.5					
cob	*Eco*RV	**1**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**7**	**8**	**8**	**3**
	kb			no1:8.8 + 3; no2:12.5 + 3:no.3:18 + 3; no4:15 + 3; no5:8;no6:7.8; no7:7.1; no8:8.8									
cob	*Eco*RI	**1**	**1**	**2**	**2**	**3**	**4**	**4**	**4**	**4**	**4**	**4**	**2**
	kb							no1:2.6 + 2.2; no2:2.6 + 1.2; no3:2.6 + 1.4; no4:2.2					
nad2	*Bam*H1	**1**	**1**	**1**	**1**	**1**	**2**	**2**	**1**	**1**	**1**	**1**	**1**
	kb											no1:3.9; no2:5.8	
nad2	*Eco*RI	**1**	**2**	**1**	**1**	**1**	**3**	**3**	**1**	**1**	**1**	**1**	**1**
	kb											no1:8; no2:11; no3:5	
nad3	*Bam*H1	**1**	**1**	**2**	**3**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**3**
	kb										nol:1.9; no2:2.8 + 2.6; no3:1.9 + 2.1		
nad3	*Eco*RV	**1**	**2**	**3**	**4**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**4**
	kb							nol:15 + 11.5; no2:7.6; no3:11.5 + 1.1; no4:5.4 + 1.1					
nad3	*Eco*RI	**1**	**1**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**
	kb											no1:0.9; no2:1.35 + 0.9	
nad5IV	*Bam*H1	**1**	**1**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**1**	**1**	**2**
	kb											no1:11 + 9; no2:7	
nad5IV	*Eco*RV	**1**	**1**	**2**	**2**	**2**	**2**	**2**	**3**	**3**	**1**	**1**	**2**
	kb										no1:6.4; no2:5.6; no3:5.9		

Table 4 F-values (lower section) and shared fragments (upper section) between pair of cytotypes ($F = 2n_{xy}/(n_x + n_y)$ where n_{xy} is the number of common RFLP for two cytotypes x and y, n_x and n_y are the numbers of total RFLP markers for cytoplasms x and y respectively)

	MM	rev	Ci	Da	Hq	Sp	Mo	Res	C	T	L	P
MM	55	52	52	30	26	21	22	22	22	22	22	22
rev	0.928	57	56	32	30	24	25	25	20	20	21	21
Ci	0.906	0.941	62	37	35	29	30	30	22	22	22	22
Da	0.495	0.520	0.578	66	27	35	36	36	31	32	30	30
Hq	0.437	0.496	0.555	0.415	64	45	50	50	30	29	24	24
Sp	0.365	0.410	0.475	0.555	0.725	61	56	56	40	39	24	24
Mo	0.360	0.403	0.465	0.541	0.763	0.881	67	67	40	40	33	33
Res	0.360	0.403	0.465	0.541	0.763	0.881	1.000	67	40	40	33	33
C	0.400	0.357	0.376	0.512	0.504	0.696	0.656	0.656	54	52	39	39
T	0.396	0.354	0.373	0.525	0.483	0.672	0.650	0.650	0.937	56	39	39
L	0.440	0.378	0.379	0.500	0.407	0.579	0.545	0.545	0.715	0.715	54	54
P	0.440	0.378	0.379	0.500	0.407	0.579	0.545	0.545	0.715	0.715	1.000	54

ization patterns identical to those already obtained with the gene probes.

Mitochondrial DNA diversity in the genus Phaseolus. Based on a comparison of hybridization patterns of different cytoplasms, either CMS-inducers or non-inducers, mtDNA variability from the two *Phaseolus* species showed that most of the cytoplasms studied were different and that each cytoplasm could be characterized by at least one fragment specific to that cytoplasm. The five CMS-inducers known in the genus *Phaseolus* are clearly distinct but all contain common CMS-specific fragments detected with a mitochondrial probe (either *atpA* or *cob*). These fragments were never observed in the mtDNA of non-inducers. CMS-inducer cytoplasms could be separated into two distinct groups based on their hybridization patterns using the *atpA* gene as a probe. The first contained the cytotype Ci from *P. vulgaris* and the cytoplasm from *P. polyanthus* (Da), and is characterized by two mitochondrial *atpA* loci, one of which is associated with a CMS-specific fragment corresponding to the CMS-Sprite *atpA* gene (Mackenzie and Chase 1990; Chase and Ortega 1992). The second group, which included the three other cytoplasms from *P. coccineus* (Hq, Mo and Sp), was characterized by only a single copy of the *atpA* gene associated with a CMS-specific fragment similar to the one observed in the first group (Fig. 1a; Hervieu et al. 1993).

Wide mtDNA diversity was observed in *P. coccineus* in comparison to the homogeneous mtDNA organization described in *P. vulgaris* (present study and Kairallah et al. 1990). The maximum number of mtDNA differences within *P. coccineus* (i.e., Hq vs C) is comparable to the number of mtDNA differences that can be observed between the cytoplasms of two distinct species (i.e., MM3 vs C). Little mtDNA diversity was discerned in *P. vulgaris*; the differences being mainly associated with the CMS phenotype. The sterility maintainer MM3 and the spontaneous revertant lines of Ci could be distinguished only by a single polymorphism using *rrn18s/5s* as a probe.

Most of the probe × enzyme combinations could be used to distinguish the cytoplasms studied, but generally without any correlation with the CMS phenotype. However, this analysis also revealed similarities between several cytoplasms. Within non-inducing cytoplasms from *P. coccineus*, LTE8 and Petaco could not be distinguished whatever the enzyme × probe combination used. Furthermore, no difference was observed between the hybridization patterns of the CMS-inducer cytotype Mo and the cytoplasm of the partially-restored combination resulting from the cross of another CMS-Mo line with a restorer candidate (KHR1). In this case, the fertility restoration occurred without any mitochondrial genome alteration and the nuclear gene involved is probably similar to the restorer gene *Fr2* described by Mackenzie (1991). This is in contrast with the reversion phenomenon which is associated with the loss of the CMS-specific mitochondrial region flanking the *atpA* gene (Mackenzie and Chase 1990). In the case of CMS-Sprite, this irreversible phenomenon is controlled by a nuclear gene designated *Fr* (Mackenzie et al. 1988; Mackenzie and Chase 1990).

Phenogram of fertile and sterile cytoplasms using hybridizing fragments of mtDNA

In order to construct dendrograms based on mtDNA diversity, RFLP patterns were generated by hybridizing total DNA digested independently with three restriction endonucleases and hybridized with 12 known heterologous mithochondrial probes and five anonymous probes. Four distance matrices were calculated from F-values based on data generated either by gene probes, anonymous probes, the total of 17 mtDNA sequences, or gene probes excluding CMS-specific fragments observed with *atpA* and *cob*. The last analysis was made in order to determine the effect of CMS-specific markers on the cytoplasm classification. The resulting phenograms had identical topology, but with minor differences in branch lengths (Fig. 2). A separation of the two species *P. coccineus* and *P. vulgaris* was evident. Within *P. coccineus* cytoplasms there is a clear separation of CMS inducers (Mo, Sp and Hq) from the four non-

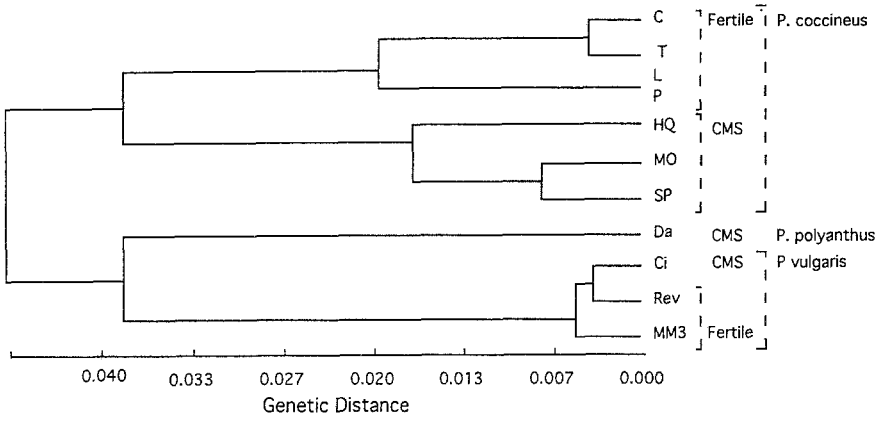


Fig. 2 UPGMA clustered tree, based on RFLP genetic distances, for 12 *Phaseolus* cytotypes either CMS-inducing, non-inducing, restored or revertant

inducer (C, L, P and T) cytoplasms. A homogeneous group contains the *P. vulgaris* cytoplasms which revealed lower intragroup mtDNA diversity. An intermediate position of Da was observed.

Discussion

Marked variation was present among mtDNA from five CMS-inducing cytoplasms from *P. coccineus*, *P. polyanthus* or *P. vulgaris*, which have not been earlier distinguished by genetic analyses. All five CMS lines accept the same set of maintainer genotypes and respond similarly to the different restorer candidates which have been tested (leading only to partial fertility restoration). Each of the five cytoplasms is characterized by at least four specific mtDNA polymorphisms among the 20 enzyme × gene combinations analysed. Based on this analysis, we have classified these cytoplasms into three groups; the first contains the three cytoplasms (Hq, Mo and Sp) from *P. coccineus*, the second (Da) a cytoplasm from *P. polyanthus* and the third (Ci) a cytoplasm from *P. vulgaris*. The F-values obtained between each of these groups ranged from 0.4 to 0.6. These values were comparable with those computed when the corresponding non-CMS-inducing cytoplasms from the different species were compared with each other. We need, however, to be prudent because it is difficult to use plant mitochondrial RFLP data to construct phylogenetic classifications. Due to the mode of evolution of plant mitochondrial genomes, which is essentially by intragenomic recombination and insertion of foreign sequences rather than point mutations, mitochondrial RFLPs violate the assumptions on which all mathematical treatments of RFLP analyses are based; i.e., that RFLPs are independent and occur randomly in time and location in the genome. In practice, however, if care is taken to avoid counting the same event more than once (by using widely-spaced, well-characterized probes and eliminating all redundant probe × enzyme combinations, as in the present work), the results are generally consistent and give valuable insights into closely-related cytoplasms (Palmer 1992); for example, examination of intra-specific or intra-generic variation.

In spite of such a wide mtDNA diversity observed within and between *P. coccineus* and *P. vulgaris* species, only the patterns of *atpA* and *cob* were associated with the CMS phenotype. These results, combined with those of genetic (Bannerot 1988) and molecular analyses of CMS-inducer cytoplasms from *P. coccineus* (Hervieu et al. 1993), strongly suggest the presence of an identical system of CMS induction in both species. The results of DNA sequence analyses (Chase and Ortega 1992; Johns et al. 1992; Hervieu et al. 1993) indicate that the CMS-specific fragment is flanked on one side by a complete *atpA* gene and on the other by a short sequence (111 bp) nearly identical to the carboxy-terminal of the *cob* gene so explaining the correlation of the *atpA* and the *cob* patterns with the CMS phenotype.

It was also interesting to note that results obtained with the CMS line with a cytoplasm (Da) from *P. polyanthus* revealed no close relationship to either of the two other species, *P. coccineus* and *P. vulgaris*. *Phaseolus polyanthus* has been classified as a subspecies of *P. coccineus* by Maréchal et al. (1978) and suggested to be a hybrid resulting from a cross between *P. vulgaris* and *P. coccineus* (Miranda-Colin 1979). The number of fragments specific to mtDNA from the CMS line with a cytoplasm (Da) from *P. polyanthus* was however higher than the number of mtDNA-specific fragments observed for any other cytoplasm. These results are not consistent with the classification suggested above but agree with the alternative hypothesis (Schmit and Debouck 1991) of a common phylogeny for the three taxa (*P. vulgaris*, *P. polyanthus* and *P. coccineus*) with a progressive separation of each of them.

Considering the complexity of the CMS determinant located in the proximity of the *atpA* gene, and the fact that the CMS-specific sequence is 99.8% identical in *P. polyanthus* and in *P. vulgaris* (Chase and Ortega 1992; Johns et al. 1992; Hervieu et al. 1993), we suggest that this CMS determinant had a single origin. In addition, the mtDNA diversity between the five CMS-inducers originating from the three species, *P. vulgaris*, *P. coccineus*

and *P. polyanthus*, and the absence of real discrepancies between the cytoplasm classification based on mtDNA RFLP and botanical data suggests that the CMS-determinant was not transferred between *Phaseolus* species through natural introgression (reviewed in Rieseberg and Soltis 1991) but rather that the CMS trait was present in the common ancestor of all three species.

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References

Bannerot H (1988) The potential of hybrid bean. In: Beebe S (ed) Current topics in breeding of the common bean. Working document no. 47. Bean program. CIAT, Cali, Colombia, pp 111–134

Bannerot H, Charbonnier L (1987) Induction de stérilités mâles cytoplasmiques par alloplasmie dans le genre *Phaseolus*: étude génétique et moléculaire. In: Bervillé A (ed) Variabilité génétique cytoplasmique et stérilité mâle cytoplasmique. INRA Editions, pp 181–198

Bassett MJ, Shuh DM (1982) Cytoplasmic male sterility in common bean. J Am Soc Hort Sci 107(5): 791–793

Bland MM, Levings III CS, Matzinger DF (1987) The ATPase subunit 6 gene of tobacco mitochondria contains an unusual sequence. Curr Genet 12:475–481

Bonhomme S, Budar F, Férault M, Pelletier G (1991) A 2.5-kb *Nco*I fragment of Ogura radish mitochondrial DNA is correlated with cytoplasmic male-sterility in *Brassica* cybrids. Curr Genet 19:121–127

Chase C, Ortega V (1992) Organisation of *atpA* and 3′ flanking sequences associated with cytoplasmic male sterility in *Phaseolus vulgaris* L. Curr Genet 22:147–153

Crouzillat D, Leroy P, Perrault A, Ledoigt G (1987) Molecular analysis of the mitochondrial genome of *Helianthus annuus* in relation to cytoplasmic male sterility and phylogeny. Theor Appl Genet 74:773–780

Dawson A, Jones VP, Leaver CJ (1984) The apocytochrome b gene in maize mitochondria does not contain introns and is preceded by a potential ribosome binding site. EMBO J 3:2107–2113

Dellaporta SL, Wood J, Hicks JP (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21

Gualberto JM, Wintz H, Weil JH, Grienenberger JM (1988) The genes coding for subunit 3 of NADH dehydrogenase and for ribosomal protein S12 are present in the wheat and maize mitochondrial genomes and are co-transcribed. Mol Gen Genet 215:118–127

Gualberto JM, Weil J-H, Grienenberger J-M (1990) Editing of the wheat *coxIII* transcript: evidence for twelve C to U and one U to C conversion and for the sequence similarities around editing sites. Nucleic Acids Res 18:3771–3776

Hervieu F, Charbonnier L, Bannerot H, Pelletier G (1993) The cytoplasmic male sterility (CMS) determinant of common bean is widespread in *Phaseolus vulgaris* L. and *Phaseolus coccineus* L. Curr Genet 24:149–155

Johns C, Meiquing L, Lyznik A, Mackenzie S (1992) A mitochondrial DNA sequence is associated with abnormal pollen development in cytoplasmic male-sterile bean plants. Plant Cell 4:435–449

Khairallah MM, Adams MW, Sears BB (1990) Mitochondrial DNA polymorphism of Malawian beaan lines: further evidence for two major gene pools. Theor Appl Genet 80:753–760

Khairallah MM, Adams MW, Sears BB (1991) Mitochondrial genome size variation and restriction fragment length polymorphism in three *Phaseolus* species. Theor Appl Genet 82:321–328

Lamattina L, Weil JH, Grienenberger JM (1989) RNA editing at a splicing site of an NADH dehydrogenase subunit IV gene transcript in wheat mitochondria. FEBS Lett 258:79–83

Lejeune B, Delorme S, Delcher E, Quetier F(1987) Recombination in wheat mitochondrial DNA: occurence of nine different genomic contexts for the 18*s*-5*s* genes. Plant Physiol Biochem 25:227–233

Mackenzie SA (1991) Identification of a sterility-inducing cytoplasm in a fertile accession line of *Phaseolus vulgaris* L. Genetics 127:411–416

Mackenzie S, Chase C (1990) Fertility restoration is associated with loss of a portion of the mitochondrial genome in cytoplasmic male-sterile common bean. Plant Cell 4:905–912

Mackenzie SA, Pring DR, Bassett MJ, Chase CD (1988) Mitochondrial DNA rearrangement associated with fertility restoration and reversion to fertility in cytoplasmic male sterility in *Phaseolus vulgaris* L. Proc Natl Acad Sci USA 85:2714–2717

Maréchal R, Mascherpa J, Stainier F (1978) Etude taxonomique d'un groupe complexe d'espèces des genres *Phaseolus* et *Vigna* (*Papilionaceae*) sur la base de données morphologiques et polliniques, traitées par l'analyse informatique.Boissiera 28:1–273

Miller JC (1990) Concerning Restsite package v1.1. University of Wisconsin

Miranda-Colin S (1979) Evolucion de *Phaseolus vulgaris y P. coccineus*. In Colegio de Postgraduados (eds) Contribuciones al conocimiento del frijol (*Phaseolus*) en Mexico. Chapingo, Mexico pp 83–99

Nei M, Li W-H (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

Ogura H (1968) Studies on the new male sterility in japanese radish, with special reference to the utilisation of this sterility towards the practical raising of hybrid seeds. Mem Fac Agric Kagoshima University 6:39–78

Palmer JD (1992) Mitochondrial DNA in plant systematics: applications and limitations. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics in plants. Chapman and Hall, New York, pp 36–49

Palmer JD, Herbon LA (1988) Plant mitochondrial DNA evolves rapidly in structure but slowly in sequence. J Mol Evol 28:87–97

Pereira de Souza A, Jubier M-F, Delcher E, Lancelin D, Lejeune B (1991) A trans-splicing model for the expression of the tripartite *nad5* gene in wheat and maize mitochondria. Plant Cell 3:1363–1378

Pring DR̀ Levings III CS (1978) Heterogeneity of maize cytoplasmic genomes among male-sterile cytoplasms. Genetics 89: 121–136

Ricard B, Lejeune B, Araya A (1986) Studies on wheat mitochondrial DNA organization. Comparison of the mitochondrial DNA from normal and cytoplasmic male-sterile varieties of wheat. Plant Sci 43:141–149

Rieseberg LH, Beckstrom-Sternberg SM, Liston A, Arias D (1991) Phylogeny and systematic inferences from chloroplast DNA and isozyme variation in *Helianthus* sect. *Helianthus* (*Asteraceae*). Systematic Bot 16:50–76

Rieseberg LH, Soltis DE (1991) Phylogenetic consequences of cytoplasmic gene flow in plants. Evol Trends Plants 5:65–84

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Schmit V, Debouck DG (1991) Observations on the origin of *Phaseolus polyanthus* Greenman. Econ Bot 45 (3):345–364

Singh S, White J, Gutierrez (1980) Male sterility in dry beans. Annu Rep Bean Improv Coop 23:55–57

Terachi T, Tsunewaki K (1992) The molecular basis of genetic diversity among cytoplasms of *Triticum* and *Aegilops*. VIII. Mitochondrial RFLP analyses using cloned genes as probes. Mol Biol Evol 9:917–931

Timothy DA, Levings III CS, Pring DR, Conde MF, Kermicle JC (1979) Organelle DNA variation and systematic relationships in the genus *Zea*: Teosinte. Proc Natl Acad Sci USA 76:4220–4224