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Chloroplast DNA as an evolutionary marker in the *Phaseolus vulgaris* complex

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Abstract We have analyzed the changes occurring in the chloroplast DNA (cpDNA) of taxa belonging to the *Phaseolus vulgaris* complex to help clarify relationships among species of this complex. Two restriction maps for 11 restriction enzymes comprising the whole chloroplast genome from a wild *P. vulgaris* and a wild *P. coccineus* accession were constructed. These maps allowed us to compare a total of 330 restriction sites between the two genomes in order to identify polymorphisms, assess the type of mutations detected, and identify regions of high variability. A region, located in the large single-copy region near the borders with the inverted repeats, accounted for a large portion of the variation. Most of the mutations detected were due to restriction sites gains or losses. Variable and conserved regions were then evaluated in 30 accessions belonging to taxa of the *P. vulgaris* complex. Phylogenetic analyses were made using parsimony methods. Conclusions obtained from such analyses were the following: (1) there was high cpDNA variability within *P. coccineus* but not in *P. vulgaris*. (2) *P. coccineus* subsp. *glabellus* showed a very distinct cpDNA type that strongly suggests that it actually belongs to a different but as yet undetermined section of the genus. Our cpDNA observations are supported by distinctive morphological traits and reproductive biology of this taxon. (3) In *P. coccineus* subsp. *darwinianus* (also classified as *P. polyanthus*), the cpDNA lineage was in disagreement with data obtained from nuclear markers and suggested a reticulated origin by hybridization between *P. coccineus* as the male parent and an ancestral *P. polyanthus* type, closely allied to *P. vulgaris*, as the seed parent. This initial cross was presumably followed by repeated backcrossing to *P. coccineus*. Our cpDNA studies illustrate the importance of molecular markers in elucidating phylogenetic relationships. They also indicate that accurate phylogenies will require analyses of both nuclear and cytoplasmic genomes.

Key words cpDNA · *Phaseolus vulgaris* *Phaseolus coccineus* · Phylogeny · Molecular evolution

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Introduction

Phaseolus vulgaris (common bean), an annual, self-pollinating species, is economically the most important species of the genus. It is a major source of proteins for human consumption in many parts of the world (FAO 1980). The perennial, cross-pollinated *P. coccineus* (runner bean) is a source of variability for several agronomic traits for the improvement of the primary gene pool of *P. vulgaris* (Gepts 1981). In spite of marked differences in pollination systems and life cycles, both species are closely related as evidenced by data on genetic compatibility and reproductive biology (e.g., Mendel 1865; Hucl and Scoles 1985; Guo et al. 1991), morphology (Maréchal et al. 1978), seed protein immunology (Kloz and Klozová 1974), isozymes (Bassiri and Adams 1978), electrophoresis of seed proteins (Sullivan and Freytag 1986), and mitochondria DNA (mtDNA) polymorphism (Khairallah et al. 1991). Consequently, the two taxa have been grouped into the *P. vulgaris* complex.

P. coccineus and *P. vulgaris* are partially intercrossable, with strong reciprocal differences. When *P. coccineus* is used as the female parent, hybrid embryos do not develop beyond the late-cotyledonary stage. Viable plants are obtained only when *P. vulgaris* is used as the seed parent (Wall 1970; Shii et al. 1982; Hucl and Scoles 1985). Furthermore, a number of dwarf and other abnormal plants are observed in the progeny, and the viable hybrids have typically only 25% viable pollen (Gepts 1981; Shii et al. 1982).

Several additional taxa with a wide range of morphological and reproductive characteristics have been included in the *P. vulgaris* complex. They have been classified as

Table 1 List of accessions sampled for cpDNA restriction site variation

Accession	Source[a]	Species	Subspecies	Status	Location
BAT93	1	*vulgaris*	——	Cultivated	Breeding line
Jalo EEP558	1	*vulgaris*	——	Cultivated	Brazil
G12873	1	*vulgaris*	——	Wild	Mexico
PG11A	1	*lunatus*		Cultivated	Colombia
ADS3	2	*coccineus*	*coccineus*	Cultivated	Mexico (Puebla)
ADS4	2	*coccineus*	*coccineus*	Cultivated	Mexico (Chiapas)
ADS5	2	*coccineus*	*coccineus*	Cultivated	Mexico (Chiapas)
ADS11	2	*coccineus*	*formosus*	Wild	Mexico (Oaxaca)
ADS14	2	*coccineus*	*glabellus*	Wild	Mexico (Hidalgo)
Delgado286	3	*coccineus*	*coccineus*	Wild	Mexico (Morelos)
Delgado550	2	*coccineus*	*coccineus*	Cultivated	Mexico (Puebla)
Delgado553	2	*coccineus*	*darwinianus*	Cultivated	Mexico (Puebla)
Delgado577	2	*coccineus*	*darwinianus*	Cultivated	Mexico (Puebla)
Delgado578	2	*coccineus*	*glabellus*	Wild	Mexico (Puebla)
Delgado584	2	*coccineus*	*darwinianus*	Cultivated	Mexico (Puebla)
Delgado602	2	*coccineus*	*coccineus*	Cultivated	Mexico (Puebla)
Delgado605	2	*coccineus*	*darwinianus*	Cultivated	Mexico (Puebla)
Delgado608	2	*coccineus*	*glabellus*	Wild	Mexico (Puebla)
DelgadoB	2	*coccineus*	*coccineus*	Wild	Mexico (Chihuahua)
DGD78/045	3	*coccineus*	*coccineus*	Cultivated	Mexico
G35651	3	*coccineus*	*glabellus*	Wild	Mexico
DGD2043	3	*coccineus*	*glabellus*	Wild	Mexico
DGD2374	3	*coccineus*	——	Wild	Mexico (Puebla)
DGD2445	3	*coccineus*	——	Wild	Guatemala
DGD2462	3	*coccineus*	——	Wild	Guatemala
HSG22522	3	*coccineus*	*darwinianus*	Cultivated	
DGD1661	3	*coccineus*	*darwinianus*	Cultivated	Guatemala
DGD1684	3	*coccineus*	*darwinianus*	Cultivated	Guatemala
OTCH1	3	*coccineus*	*coccineus*	Cultivated	Colombia
DGD713	3	*coccineus*	*darwinianus*	Cultivated	Peru (Cajamarca)
DGD1631	3	*coccineus*	*darwinianus*	Wild	Guatemala

[a] Sources referred to are: 1, O. Tori, S. Singh, and M. Iwanaga, CIAT, Cali, Colombia; 2, A. Delgado Salinas, Universidad Nacional Autónoma de México; 3, D.G. Debouck, IBPGR, c/o CIAT, Cali, Colombia

separate species or subspecies of *P. coccineus* (Piper 1926; Maréchal et al. 1978; Delgado Salinas 1985). According to Delgado Salinas (1985), they include: (1) *P. coccineus* subsp. *coccineus* L., (2) *P. coccineus* subsp. *formosus* (Kunth) Maréchal, Mascherpa and Stainier (= *P. formosus* Kunth), (3) *P. coccineus* subsp. *glabellus* (Piper) A. Delgado Salinas (= *P. glabellus* Piper), (4) *P. coccineus* subsp. *griseus* (Piper) A. Delgado Salinas (= *P. griseus* Piper), and (5) *P. coccineus* subsp. *darwinianus* Hdez. X and Miranda C. (= *P. polyanthus* Greenman).

Chloroplast DNA (cpDNA) has been extensively used as a molecular marker in evolution analysis, and its advantages have been discussed elsewhere (e.g., Dowling et al. 1990; Crawford 1990; Clegg and Zurawski 1992). The disadvantages and risk of generalizations based on the use of cpDNA as the only marker to infer phylogenetic relationships in plant species have also been reviewed (Rieseberg and Brunsfeld 1992; Harris and Ingram 1991).

In this report we describe our analysis of changes occurring in the cpDNA among different taxa of the *P. vulgaris* complex. The objectives of this analysis were: (1) to assess the mode of evolution of cpDNA based on the type and distribution of changes; (2) to use cpDNA as a molecular marker to clarify the systematics of the *P. vulgaris* complex; and (3) to evaluate the diversity of some of the taxa analyzed.

Materials and methods

DNA extraction

Plants from the accessions listed in Table 1 were grown in a greenhouse. Total DNA was isolated from young leaves according to the method described by Gepts et al. (1992). Harvested leaves (2–5 g per plant) were ground in liquid nitrogen and placed in 10 ml extraction buffer (50 mM TRIS, 700 mM NaCl, 1 mM Na_2EDTA, 1% SDS, 1% β-mercaptoethanol, 50 mg/ml polyvinyl polypyrrolidone, pH 9.0). The suspension was then incubated at 60 °C for 1 h, followed by two chloroform:isoamyl alcohol (24:1) extractions. DNA was precipitated with isopropanol, washed with 70% ethanol and resuspended in TE (10 mM TRIS, 1 mM Na_2EDTA). DNA concentration was estimated using a dedicated fluorometer TK-100 (Hoefer, San Francisco, Calif.).

DNA samples from some accessions presented digestibility problems and were further purified by ultracentrifugation in a cesium chloride-ethidium bromide gradient basically according to Sambrook et al. (1989). Samples were set in a 1.55 g/ml cesium chloride solution containing 250 g/ml ethidium bromide. After ultracentrifugation at 400,000 *g* for 12 h, the DNA was removed and ethidium bromide was extracted with TE-saturated butanol. Samples were dialyzed against TE (1000:1) for 10 h and then precipitated with ethanol. Purified DNA was resuspended in TE at a final concentration of 300 µg/ml.

Restriction mapping

The wild accession 'G12873' belonging to the Middle American gene pool (Gepts 1990) was used for the *P. vulgaris* map. The cpDNA map

for *P. coccineus* subsp. *coccineus* was constructed using a wild accession collected in Chihuahua, Mexico ('DelgadoB'). Additionally, partial maps were made from a wild *P. coccineus* subsp. *glabellus* accession ('DGD2043') and from a cultivated Andean *P. lunatus* accession ('PG11A').

In each case, 3 µg of total DNA was digested separately with the following 6-base restriction endonucleases: *Sal*I, *Pst*I, *Pvu*II, *Sma*I, *Kpn*I, *Xho*I, *Sst*I, *Hin*dIII, *Bam*HI, *Dra*I, and *Eco*RI (Bethesda Research Laboratories; New England Biolabs; Boehringer Mannheim). The reaction conditions were those specified by the supplier. In addition, 50 ng/µl RNase was added to the reaction mixture. Samples were incubated for 4–5 h using an eightfold enzyme excess to assure total digestion. In addition to the 11 single digestions, 44 double digestions were made. When optimal conditions were different for each of the 2 enzymes in a double digestion, the digestion with lower salt concentration was accomplished first, followed by addition of the second enzyme and KCl or NaCl to the recommended concentration.

For mapping, restriction fragments were separated by electrophoresis in a 20 to 25 cm-long, 0.5%–0.6% horizontal agarose gels in 1 × TAE (40 mM TRIS, 20 mM glacial acetic acid, 10 mM Na_2-EDTA), running at 1 V/cm, as described by Sambrook et al. (1989). Lambda phage DNA cut with *Hin*dIII was used as the size standard. For comparative analysis among accessions, gels and DNA transfer were made according to the same protocols. However, gels for comparative analysis were only 10 cm long and consisted of 0.8% agarose in 1 × TAE.

Hybridization was carried out initially according to the procedure described in Gepts et al. (1992). The procedure was subsequently modified as follows: DNA fragments from the agarose gels were denatured and transferred by capillarity onto nylon (Zetabind) membranes according to Sambrook et al. (1989) and then crosslinked with 150 mJ UV light using a Bio-Rad UV linker. Nylon membranes were prehybridized at 42 °C in 5 × SSPE, 1% SDS, 50% formamide for 30 minutes. The probe (10^5 cpm/cm^2) was then added to the same solution and incubated at 42 °C for another 20 h. The membrane was washed briefly in 2 × SSC, 0.1% SDS at room temperature and then for 30 min in 0.1 × SSC, 0.1% SDS at 60 °C. An X-ray film was subsequently exposed to the membrane for from 3–4 h to 1 day. This method gave excellent results and was inexpensive and faster than the previous method. Membranes were reused approximately 15 times. Prior to reuse, the probe was removed by washing in 0.4 *N* NaOH at 42 °C for 30 min, followed by a 30-min wash in 0.5 *M* TRIS, 0.1×SSC, 0.1% SDS at 42 °C.

DNA probes and labeling

Plasmids containing *Vigna radiata* cpDNA fragments (kindly provided by Dr. J.D. Palmer, University of Indiana at Bloomington; Palmer and Thompson 1981) were amplified in competent cells of *Escherichia coli* strain DH5α prepared according to the $CaCl_2$ method and transformed with the plasmids containing the fragments. Plasmids were isolated by the boiling miniscreen procedure (Sambrook et al. 1989) and then labeled with [^{32}P]-dCTP by nick translation (Rigby et al. 1977) or by random priming (Feinberg and Vogelstein 1983).

Phylogenetic analysis

Phylogenetic analysis was performed with the program PAUP (Phylogenetic Analysis Using Parsimony) Version 3.0 provided by D.L. Swofford (Illinois National History Survey, Champaign, Ill.). Mapped restriction sites were analyzed in a cladistic fashion using Wagner (Farris 1970), Dollo (LeQuesne 1974; Farris 1977), and weighted parsimony (Albert et al. 1992). For the weighted method we used a restriction site gain:loss ratio equal to 1:2.3, which is considered to be optimal for cpDNA restriction site analysis (Albert et al. 1992).

Initially, *P. lunatus* was used as the outgroup in order to polarize the mutations because it is phylogenetically distant from the *P. vulgaris* complex within the genus (Delgado Salinas et al. 1993; Mok et al. 1978). However, as explained later, *P. lunatus* turned to be an inadequate outgroup for our analyses and was subsequently included as an additional accession in the unrooted trees.

We estimated phylogenies using the whole set of accessions or representative accessions from each taxa. For the whole set of accessions (30 samples and 48 synapomorphies), we used heuristic searches using Tree Bisection-Reconnection (TBR) and ACCTRAN optimization in PAUP 3.0. For smaller phylogenies (e.g., 6 taxa and 46 synapomorphies), we were able to use the Branch-and-Bound search (for weighted parsimony) and the exhaustive search modes (for Dollo and Wagner parsimonies) and ACCTRAN optimization. For bootstrapping, we made 100 repetitions in the Phylip 3.01 programs Boot and Dolboot (Felsenstein 1988).

For the estimation of phylogeny under weighted parsimony, we used the Stepmatrix option of PAUP 3.0, with heuristic search using Tree Bisection-Reconnection (TBR) and ACCTRAN optimization. Random data acquisition sequence was used with heuristic search and Tree Bisection-Reconnection (TBR) swapping to find the most parsimonious trees. A 10:23 step matrix was employed as suggested by Albert et al. (1992).

Results

Development of a *Phaseolus vulgaris* cpDNA restriction map

Single and double digestions of total genomic DNA from the wild *P. vulgaris* accession 'G12873' were performed using 11 endonucleases. cpDNA was visualized by sequential Southern hybridization of total DNA to radioactively labeled heterologous cpDNA probes from mung bean (*V. radiata*), a species from a related genus (Palmer and Thompson 1981). Mubumbila et al. (1983) and Palmer et al. (1983) previously constructed a *P. vulgaris* cpDNA map for 3 enzymes (*Pst*I, *Xho*I and *Sal*I) and also demonstrated the colinearity and high similarity between this genome and that of *V. radiata* cpDNA.

A total of 11 simple and 44 double digestions were carried out. The mung bean cpDNA probes comprised 96% of the complete cpDNA genome. Three hundred and thirty 6-bp restriction sites were assigned to a restriction map. This corresponds to 1.3% of the total cpDNA sequence (on average, 1 site every 460 bp). The restriction enzymes *Sal*I, *Pst*I, *Sma*I, *Kpn*I, *Xho*I, *Pvu*II, and *Sst*I cut *P. vulgaris* cpDNA at a low frequency (5–20 sites), whereas the enzymes *Dra*I, *Hin*dIII, *Bam*HI, and *Eco*RI cut it at high frequencies (40–100 sites/genome) (Fig. 1).

The complete *P. vulgaris* cpDNA map is presented in Fig. 1. The map presents only one of two possible isomeric conformations present in similar proportions in *P. vulgaris* as demonstrated by Palmer (1983). The map is complete for 9 enzymes. For 2 high-frequency cutting enzymes (*Eco*RI and *Dra*I), there were some ambiguous regions that have been omitted in the map. The ambiguity is due to the presence of many small fragments and the absence of overlapping fragments in small non-probed regions.

An average size of *P. vulgaris* cpDNA of 152.4 kb was calculated using the 9 restriction endonucleases with the lowest cutting frequency because of the possibility of large amounts of undetected small fragments present in the high-

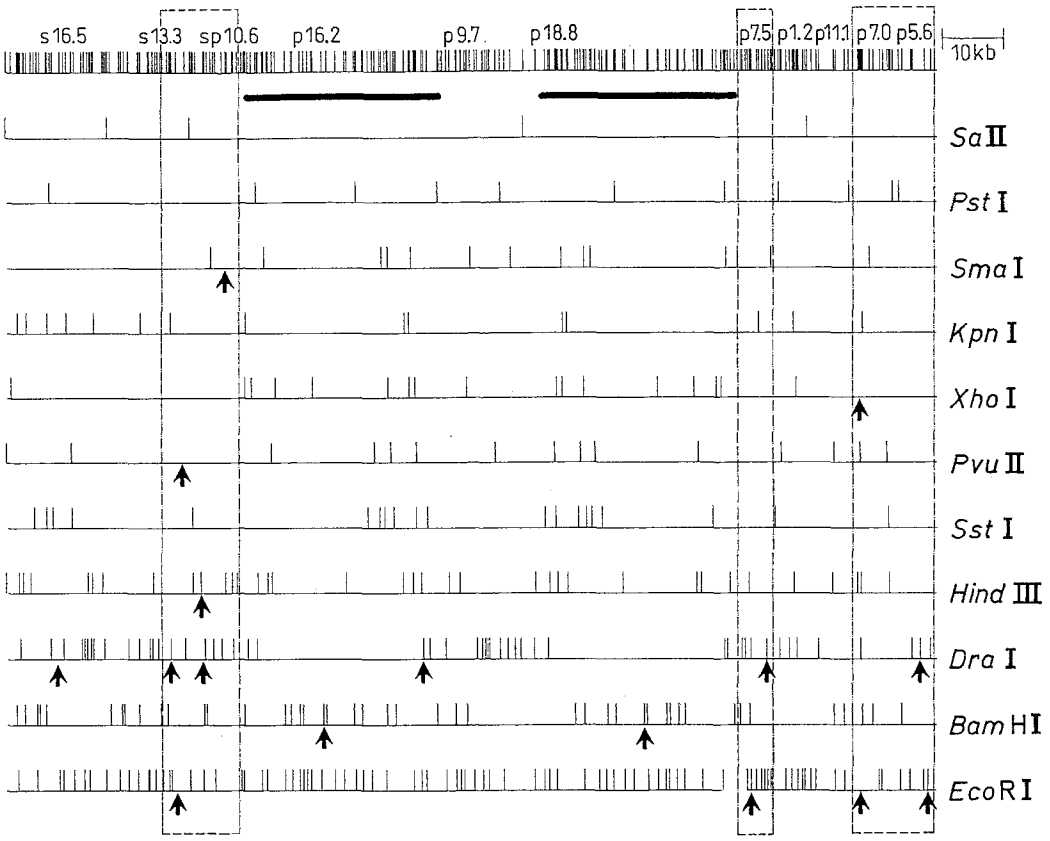

Fig. 1 High-resolution restriction map of *P. vulgaris* cpDNA. The *top row* shows the *Vigna radiata* cpDNA probes used in the Southern hybridizations. The *second row* is a composite restriction map showing all restriction sites detected with the 11 enzymes. The *following rows* are restriction maps for individual enzymes. The two *heavy horizontal bars* are the inverted repeats. The three *boxes* marked by *stippled lines* represent regions with a high frequency of polymorphism. *Arrows* indicate restriction site differences with the *P. coccineus* subsp. *coccineus* map. The *blank region* in the *Eco*RI map could not be mapped because of the high frequency of restriction sites.

frequency restriction digestions with *Dra*I and *Eco*RI. Minor discrepancies among size calculations for each enzyme were probably due to small fragments (0.3–0.05 kb) not being detected under our conditions. Estimates for some of the fragments sizes differed slightly (0.5–3%) from those previously published (Mubumbila et al. 1983). This is probably due to differences in the procedures followed in both works. We visualized restriction fragments after Southern hybridization of total DNA fragments separated in ethidium bromide-agarose gels instead of using purified cpDNA. Agarose gel overloading and the presence of ethidium bromide in the gel may retard DNA fragments in gel.

The small single copy region was approximately 19 kb long. Each repeat had a size of at least 25 kb, whereas the large single-copy region was approximately 83 kb long. These values are consistent with those obtained for *P. vulgaris* and other angiosperms with cpDNA containing inverted repeats (reviewed in Crouse et al. 1986; Palmer and Stein 1986; Palmer 1987).

Comparison among cpDNA restriction maps

A second cpDNA map was constructed for wild accession 'DelgadoB' of *P. coccineus* subsp. *coccineus* using the same restriction endonucleases as above. Similar results were obtained in *P. coccineus* subsp. *coccineus* and *P. vulgaris* with respect to the frequency of restriction sites recognized by the various restriction enzymes, but a total of 16 differences were found between their respective genomes, which corresponds to 5% of the sites analyzed. Figure 1 shows the differences found between *P. coccineus* subsp. *coccineus* and *P. vulgaris* cpDNA. All of the detected differences except one are probably simple restriction site gains or losses. The exception is most likely due to a small insertion-deletion mutation in region p11.1. No major rearrangements (large insertions, deletions, inversions, or translocations) were detected. However, additional insertions and inversions of up to 300 bp may have been overlooked by our methods in some cases.

All mutations except 3 were located in the large single-copy region. The distribution of mutations in this region was uneven: 1 single 10-kb region in s13.3 contained 6 site differences, that is, 40% of the total observed. The presence of monomorphic sites between polymorphic sites suggests that the former sites are not the product of a single event (e.g., insertion, deletion or inversion), but have been produced independently. An uneven distribution of mutations has been found in other species (Close et al. 1989). In our subsequent analyses, all mutations, except those of *Bam*HI p16.2 and *Bam*HI p17.2, were considered to be independent. The *Bam*HI p16.2 and *Bam*HI p17.2 mutations

are located in the inverted repeat, one in each, and may have resulted from sequence homogenization, as has been inferred in other plant species (Palmer 1985).

Partial maps based on 4 enzymes (*Dra*I, *Eco*RI, *Bam*HI and *Hin*dIII) were also generated for *P. lunatus* (PG11A) and *P. coccineus* subsp. *glabellus* ('DGD2043'). Such maps were required in order to assign the position of sites in regions where inference from the other maps was not possible because of the presence of multiple changes.

Phylogenetic analysis

We screened several regions of cpDNA in 30 accessions of the *P. vulgaris* complex. The samples included wild and cultivated accessions of (1) *P. coccineus* subsp. *coccineus*, (2) P. *coccineus* subsp. *darwinianus* (= *P. polyanthus*), (3) P. *coccineus* subsp. *formosus*, (4) *P. coccineus* subsp. *glabellus*, (5) the Middle American and Andean *P. vulgaris* gene pools, and (6) *P. lunatus*. The latter was included originally as the outgroup to polarize mutations within the *P. vulgaris* complex.

An analysis of 190 restriction sites revealed 60 mutations. All of these mutations except 9 were shared by at least 2 or more taxa, and consequently they were phylogenetically informative. Most of the mutations (56) could be explained by restriction site gain/loss events, whereas 4 were probably occasioned by small (ca. 0.1 kb) insertions or deletions. Length mutations were not included in our phylogenetic analysis. As observed in Fig. 2, cpDNA data analysis showed *P. lunatus* to be more closely related to *P. coccineus* subsp. *coccineus* and *P. coccineus* subsp. *darwinianus* than to *P. coccineus* subsp. *glabellus*. Surprisingly, the latter taxon was distantly related to all of the other taxa analyzed in this study.

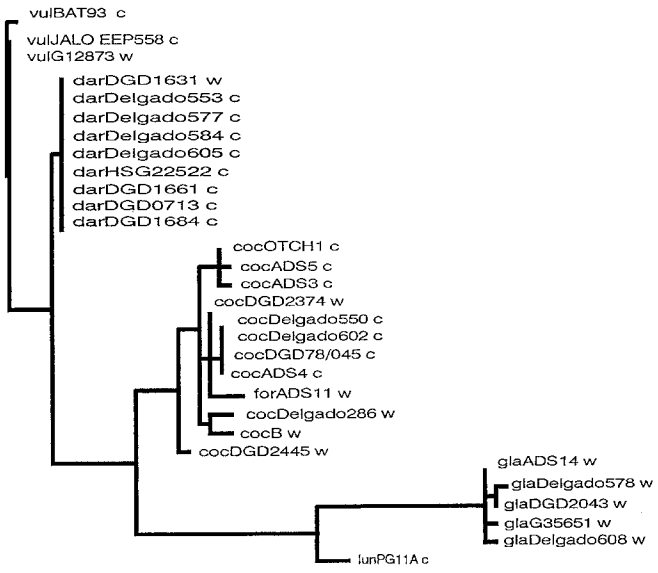

Fig. 2 Cladogram showing relationships from the restriction site data among accessions of the *P. vulgaris* complex. This is one of ten most parsimonious cladograms for the 30 separated accessions, with a length of 63 steps and a consistency index of 780 (Homoplasy index = 220). The *first three letters* of the label for each accession represent the taxon to which it belongs: (*coc P. coccineus* subsp. *coccineus*, *dar P. coccineus* subsp. *darwinianus*, *for P. coccineus* subsp. *formosus*, *gla P. glabellus*, *lun P. lunatus*, *vul P. vulgaris*). The *last letter* indicates whether the accession is wild-growing (*w*) or cultivated (*c*)

Discussion

Levels of polymorphism

We detected significant levels of polymorphism among species and within species in the *P. vulgaris* complex. Recent work indicates that moderate variation at the interpopulational (and even intrapopulational) level is common in at least several other species (see Soltis et al. 1992 and Crawford 1990 for a review). Our results show taxon-dependent differences in polymorphism. *P. coccineus* subsp. *coccineus* showed the highest number of polymorphic sites. On the other hand, *Phaseolus coccineus* subsp. *darwinianus* did not show polymorphism in any of the wild and cultivated accessions analyzed. Only 1 polymorphism was observed among the 3 accessions of *P. vulgaris* in spite of the marked divergence of nuclear DNA sequences between Middle American (e.g., 'BAT93') and Andean (e.g., 'Jalo') genotypes (Nodari et al. 1992). This polymorphism differentiates 'BAT93' and other genotypes from race Mesoamerica (Singh et al. 1991) from other Middle American genotypes and Andean genotypes (V. Llaca, V. Becerra, and P. Gepts unpublished results).

Phylogenetic implications for *P. coccineus* subsp. *darwinianus*

cpDNA from all of the accessions belonging to *P. coccineus* subsp. *darwinianus* was more similar to that of *P. vulgaris* than to that of *P. coccineus* subsp. *coccineus*. According to our most parsimonious trees, *P. vulgaris* is separated from *P. coccineus* subsp. *darwinianus* by only 3 changes, whereas at least 14 changes are required to explain the differences between the closest *P. coccineus* subsp. *coccineus* and *P. vulgaris*. A comparable number (13) of differences separates *P. coccineus* subsp. *coccineus* from *P. coccineus* subsp. *darwinianus*. Similar results were obtained by Schmit (1992).

These results suggest a discrepancy between the phylogenetic relationships suggested by cpDNA and nuclear DNA of *P. coccineus* subsp. *darwinianus*. Data from previous studies using nuclear markers such as enzymes and antibodies against seed proteins (Piñeiro and Eguiarte 1989; Kloz and Klozova 1974) and our own observations using restriction fragment length polymorphism (RFLP) analysis of random single-copy and highly repetitive nuclear clones as well as the polymerase chain reaction (PCR) amplification of phaseolin genes (V. Llaca and P. Gepts unpublished results) indicate a close relationship between *P.*

coccineus subsp. *darwinianus* and *P. coccineus* subsp. *coccineus*, whereas both are readily discernible from *P. vulgaris*. The similarity in the nuclear genomes of *P. coccineus* subsp. *darwinianus* and *P. coccineus* subsp. *coccineus* is consistent with the reduced reproductive isolation between these two subspecies (Gepts 1981).

The lack of coherence between nuclear and chloroplast lineages may be accounted for by a reticulated origin of *P. coccineus* subsp. *darwinianus*. The intermediate morphology of *P. coccineus* subsp. *darwinianus* compared with that of *P. vulgaris* and *P. coccineus* subsp. *coccineus* was first noted by Hernandez-Xolocotzin et al. (1959), who proposed a hybrid origin for this taxon, with *P. coccineus* subsp. *coccineus* as the seed parent and *P. vulgaris* as the pollen parent. Alternate views considered a gradual differentiation of *P. vulgaris* from *P. coccineus* subsp. *darwinianus* more likely (Smartt 1973, Schmit and Debouck 1991). Our work supports the first point of view but suggests that *P. vulgaris* or a taxon closely related to it would be the seed parent due to maternal inheritance of cytoplasmic DNA in *Phaseolus* (H. Bannerot personal communication). Recurrent unidirectional backcrosses with *P. coccineus* plants as male parents could explain the similarity of the nuclear genomes. Hybridization would have occurred before the split of the two major gene pools (Middle American and Andean) in *P. vulgaris* (Gepts 1990; Gepts and Debouck 1991) because all *P. coccineus* subsp. *darwinianus* accessions share the same apomorphies with the *P. vulgaris* accessions representing the two gene pools. According to the distribution of both the cultivated and few wild *P. coccineus* subsp. *darwinianus* accessions collected (Schmit and Debouck 1991; R. Ramirez personal communication), this taxon originated in Middle America (Mexico and Central America) and has only recently been introduced into South America.

Phaseolus glabellus cpDNA

The results obtained in this work strongly suggest that the chloroplast of *P. coccineus* subsp. *glabellus* is very distant from that of the other taxa analyzed. *P. coccineus* subsp. *glabellus* had been included in the *P. vulgaris* complex because of the presence of scarlet flowers, cross-pollination, and a perennial life cycle, which are all also observed in *P. coccineus* subsp. *coccineus* (Piper 1926; Maréchal 1978; Delgado-Salinas 1985). In this study, *P. coccineus* subsp. *glabellus* cpDNA restriction sites showed a considerable divergence from those of any of the other taxa. Furthermore *P. lunatus* cpDNA was intermediate between that of *P. coccineus* subsp. *glabellus* and that of the other taxa surveyed. These results confirm those obtained by Schmit (1992).

Several morphological traits, the presence of manifest reproductive barriers, and the great divergence at the molecular level suggest the reclassification of *P. glabellus* as a different species outside the *P. vulgaris* complex. Inflorescence number and structure, leaf structure, and seed morphology (Maréchal et al. 1978, Delgado-Salinas 1985) as well as the absence of natural or artificial hybrids (Delgado Salinas 1988; Sousa-Peña et al. unpubl. results) also argue against a close relationship with *P. coccineus*.

Variability of cultivated *P. coccineus* subs. *coccineus*

P. coccineus subsp. *coccineus* showed a high level of intraspecific polymorphism. All of the cultivated accessions were grouped in the same cluster, together with a wild accession from the Mexican state of Puebla, which suggests a single center of domestication. The reduction in diversity observed between wild and cultivated *P. coccineus* subsp. *coccineus* confirms observations made previously for cpDNA in other wild ancestor-cultivated descendant combinations (Doebley 1992). It also confirms observations made in other species of the *Phaseolus-Vigna* group with other molecular markers (*P. vulgaris*: Gepts et al. 1986; *P. acutifolius*: Schinkel and Gepts 1988, 1989; *Vigna unguiculata*: Panella and Gepts 1992).

We were unable to distinguish between the cpDNA of *P. coccineus* subsp. *formosus* and *P. coccineus* subsp. *coccineus*. Furthermore, some wild *P. coccineus* subsp. *coccineus* accessions were closer to *P. coccineus* subsp. *formosus* than to other *P. coccineus* subsp. *coccineus* accessions. Therefore, a very close relationship among accessions of both subspecies is inferred. Nevertheless, *P. coccineus* subsp. *formosus* seems to include a heterogenous group of populations. For example, individuals with high reciprocal compatibility to *P. vulgaris* and lilac flowers have been described within *P. coccineus* subsp. *formosus*, whereas common wild, red-flowered wild plants are also included in the same group (Delgado Salinas 1988). The *P. coccineus* subsp. *formosus* accession included in this work corresponds to the second type, that similar in morphology to wild *P. coccineus* subsp. *coccineus*. The analysis of cpDNA in lilac-flowered, *P. vulgaris*-compatible populations could show if *P. coccineus* subsp. *formosus* is in fact a monophyletic group or whether it consists of a heterogeneous group of populations.

Overall, our results set the stage for further analysis of phylogenetic relationships in the genus *Phaseolus* and related genera. They underscore the need to analyze cytoplasmic and nuclear genomes to establish more reliables phylogenies.

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References

Albert VA, Mishler BD, Chase MW (1992) Character-state weighing for restriction site data in phylogenetic reconstruction, with an example from chloroplast DNA. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman Hall, New York, pp 369–403

Bassiri A, Adams MW (1978) An electrophoretic survey of seedling isozymes in several *Phaseolus* species. Euphytica 27:447–459

Clegg MT, Zurawski G (1992) Chloroplast DNA and the study of plant phylogeny: present status and future prospects. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman Hall, New York, pp 1–13

Close PS, Shoemaker RC, Keim P (1989) Distribution of restriction site polymorphism within the chloroplast genome of the genus *Glycine*, subgenus *Soja*. Theor Appl Genet 77:768–776

Crawford DJ (1990) Plant molecular systematics. Wiley, New York
Crouse EJ, Mubumbila M, Stummann B, Bookjans G, Michalowski C, Bohnert H, Weil JH, Henningsen KW (1986) Divergence of chloroplast gene organization in three legumes: *Pisum sativum, Vicia faba* and *Phaseolus vulgaris*. Plant Mol Biol 7:143–149
Delgado Salinas A (1985) Systematics of the genus *Phaseolus* (Leguminosae) in North and Central America. PhD thesis, University of Texas, Austin
Delgado Salinas A (1988) Variation, taxonomy, domestication, and germplasm potentialities in *Phaseolus coccineus*. In: Gepts P (ed) Genetic resources of *Phaseolus* beans. Kluwer, Dordrecht, the Netherlands, pp 441–463
Delgado Salinas A, Bruneau A, Doyle JJ (1993) Chloroplast phylogenetic studies in New World Phaseolinae (Leguminosae:Papilionoideae:Phaseoleae). Syst Bot 18:6–17
Doebley J (1992) Molecular systematics and crop evolution. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman Hall, New York, pp 202–222
Dowling TE, Moritz C, Palmer JD (1990) Nucleic acids II: restriction site analysis. In: Hillis DM, Moritz C (eds) Molecular systematics. Sinauer, Sunderland, Mass., pp 250–317
FAO (1980) Food balance sheets: 1975–1977 average and per capita food supplies, 1961–1965 average, 1967–1977. Food and Agriculture Organization, Rome
Farris JS (1970) Methods for computing Wagner trees. Syst Zool 19:83–92
Farris JS (1977) Phylogenetic analysis under Dollo's law. Syst Zool 26:77–88
Feinberg AP, Vogelstein (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:9–13
Felsenstein J (1988) Phylogenies from molecular sequences: inference and reliability. Annu Rev Genet 22:521–565
Gepts P (1981) Hibridaciones interespecíficas para el mejoramiento de *Phaseolus vulgaris*. Internal seminar, SE-10–81, Centro Internacional de Agricultura Tropical, Cali, Colombia
Gepts P (1990) Biochemical evidence bearing on the domestication of *Phaseolus* beans. Econ Bot 44(3S):28–38
Gepts P, Debouck DG (1991) Origin, domestication, and evolution of the common bean, *Phaseolus vulgaris*. In: Voysest O, Van Schoonhoven A (eds) Common beans: research for crop improvement. CAB, Oxon, UK, pp 7–53
Gepts P, Osborn TC, Rashka K, Bliss FA (1986) Phaseolin-protein variability in wild forms and landraces of the common bean (*Phaseolus vulgaris*): evidence for multiple centers of domestication. Econ Bot 40:451–468
Gepts P, Llaca V, Nodari RO, Panella L (1992) Analysis of seed proteins, isozymes, and RFLPs for genetic and evolutionary studies in *Phaseolus*. In: Linskens H-F, Jackson JF (eds) Modern methods of plant analysis (new series): seed analysis. Springer, Berlin Heidelberg New York, pp 63–93
Guo M, Lightfoot DA, Mok MC, Mok DWS (1991) Analyses of *Phaseolus vulgaris* L. and *P. coccineus* Lam. hybrids by RFLP: preferential transmission of *P. vulgaris* alleles. Theor Appl Genet 81: 703–709
Harris SA, Ingram R (1991) Chloroplast DNA and biosystematics: the effects of intraspecific diversity and plastid transmission. Taxon 40:393–412
Hernandez Xolocotzi E, Miranda Colín S, Prwyer C (1959) El origen de *Phaseolus coccineus* L. *darwinianus* Hernandez X. & Miranda C. subspecies nova. Rev Soc Mex Hist Nat 20:99–121
Hucl P, Scoles GJ (1985) Interspecific hybridization in the common bean: a review. HortScience 20:352–357
Khairallah MM, Adams MW, Sears BB (1991) Mitochondrial genome size variation and restriction fragment length polymorphisms in three *Phaseolus* species. Theor Appl Genet 82:321–328
Kloz J, Klozová E (1974) The protein euphaseolin in Phaseolinae – a chemotaxonomical study. Biol Plant 16:290–300
LeQuesne WJ (1974) The uniquely evolved character concept and its cladistic applications. Syst Zool 18:201–205
Maréchal R, Mascherpa J-M, 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
Mendel G (1865) Experiments on plant hybrids. In: Stern C, Sherwood ER (eds) The origin of genetics. W. H. Freeman, San Francisco, pp 1–48
Mok DWS, Mok MC, Rabakoarihanta A (1978) Interspecific hybridization of *Phaseolus vulgaris* with *P. lunatus* and *P. acutifolius*. Theor Appl Genet 52:209–215
Mubumbila M, Gordon KJH, Crouse EJ, Burkard G, Weil JH (1983) Construction of the physical map of the chloroplast DNA of *Phaseolus vulgaris* and localization of ribosomal and transfer genes. Gene 21:257–266
Nodari RO, Koinange EMK, Kelly JD, Gepts P (1992) Towards an integrated linkage map of common bean. I. Development of genomic DNA probes and levels of restriction fragment length polymorphism. Theor Appl Genet 84:186–192
Palmer JD (1983) Chloroplast DNA exists in two orientations. Nature 301:92–93
Palmer JD (1985) Comparative organization of chloroplast genomes. Annu Rev Genet 19:325–354
Palmer JD (1987) Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. Am Nat 130:S6–S29
Palmer JD, Stein DB (1986) Conservation of chloroplast genome structure among vascular plants. Curr Genet 10:823–833
Palmer JD, Thompson WF (1981) Clone banks of the mung bean, pea and spinach chloroplast genomes. Gene 15:21–26
Palmer JD, Singh GP, Pillay DTN (1983) Structure and sequence evolution of three legume chloroplast DNAs. Mol Gen Genet 190:13–19
Panella L, Gepts P (1992) Genetic relationships within *Vigna unguiculata* (L.) Walp. based on isozyme analyses. Genet Res Crop Evol 39:71–88
Piñeiro D, Eguiarte L (1988) The origin and biosystematic status of *Phaseolus coccineus* ssp. *polyanthus*: electrophoretic evidence. Euphytica 37:199–203
Piper CV (1926) Studies in American Phaseolinae. Contrib US Nat Herb 22:663–701
Rieseberg LH, Brunsfeld SJ (1992) Molecular evidence and plant introgression. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman Hall, New York, pp 151–176
Rigby PWJ, Dieckman M, Rhodes C, Berg O (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase. J Mol Biol 113:237–251
Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Schinkel C, Gepts P (1988) Phaseolin diversity in the tepary bean, *Phaseolus acutifolius* A. Gray. Plant Breed 101:292–301
Schinkel C, Gepts P (1989) Allozyme variability in the tepary bean, *Phaseolus acutifolius* A. Gray. Plant Breed 102:182–195
Schmit V (1992) Etude de *Phaseolus polyanthus* Greenman et autres taxons du complexe *Phaseolus coccineus* L. PhD thesis, Faculté des Sciences Agronomiques de Gembloux, Belgium
Schmit V, Debouck DG (1991) Observations on the origin of *Phaseolus polyanthus* Greenman. Econ Bot 45:345–364
Shii CT, Rabakoarihanta A, Mok MC, Mok DWS (1982) Embryo development in reciprocal crosses of *Phaseolus vulgaris* and *Phaseolus coccineus*. Theor Appl Genet 62:59–64
Singh SP, Gepts P, Debouck DG (1991) Races of common bean (*Phaseolus vulgaris* L., Fabaceae). Econ Bot 45:379–396
Smartt J (1973) The possible status of *Phaseolus coccineus* L. ssp. *darwinianus* Hdez X et Miranda C. as a distinct species and cultigen of the genus *Phaseolus*. Euphytica 22:424–426
Soltis DE, Soltis PS, Milligan BG (1992) Intraspecific chloroplast DNA variation: systematic and phylogenetic implications. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematic of plants. Chapman Hall, New York, pp 117–150
Sullivan JG, Freytag G (1986) Predicting interspecific compatibilities in beans (*Phaseolus*) by seed protein electrophoresis. Euphytica 35:201–209
Wall JR (1970) Experimental introgression in the genus *Phaseolus*. 1. Effect of mating systems on interspecific gene flow. Evolution 24:356–366