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Molecular evidence for an Andean origin and a secondary gene pool for the Lima bean (*Phaseolus lunatus* L.) using chloroplast DNA

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Abstract Chloroplast DNA (cpDNA) diversity has been examined using PCR-RFLP and RFLP strategies for phylogenetic studies in the genus *Phaseolus*. Twenty-two species, including 4 of the 5 cultivated species (*P. lunatus L.*, the Lima bean; *P. vulgaris* L., the common bean; *P. coccineus* L., the runner bean and *P. polyanthus* Greenman, the year-bean), represented by 86 accessions were included in the study. Six PCR primers designed from cpDNA and a total cpDNA probe were used for generating markers. Phylogenetic reconstruction using both Wagner parsimony and the neighbor-joining method was applied to the restriction fragment data obtained from each of the molecular approaches. *P. vulgaris* L. was shown to separate with several species of largely Mesoamerican distribution, including *P. coccineus* L. and *P. polyanthus* Greenman, whereas *P. lunatus* L. forms a complex with 3 Andean species (*P. pachyrrhizoides* Harms, *P. augusti* Harms and *P. bolivianus* Piper) co-evolving with a set of companion species with a Mesoamerican distribution. Andean forms of the Lima bean are found to be more closely related to the 3 Andean wild species than its Mesoamerican forms. An Andean origin of the Lima bean and a double derivative process during the evolution of *P. lunatus* are suggested. The 3 Andean species are proposed to constitute the secondary gene pool of *P. lunatus*, while its companion allies of Mesoamerican distribution can be considered as members of its tertiary gene pool. On the basis of these data, an overview on the evolution of the genus *Phaseolus* is also discussed.

Key words Intergenic regions · Molecular markers · Plant genetic resources · Phylogeny · Mesoamerica

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Introduction

In the context of conservation of plant genetic resources, a better knowledge of the phylogenetic relationships within genera of crop species is of great importance to germplasm curators and plant breeders. Phylogenetic investigations are useful for identifying the wild progenitors of domesticated species (Doebley 1992) and suggesting putative members of their secondary and tertiary gene pools, which may help to define priorities in sampling for ex situ collections as well as in the management of in situ conservation programs (Frankel et al. 1995).

Phylogenetic studies on the origin of cultivated plants are classically based on evidence from morphology (Piper 1926; Maréchal et al. 1978; Delgado 1985), seed-protein electrophoresis (Johnson 1972; Sullivan and Freytag 1986; Gepts et al. 1986) and allozyme variation (Doebley et al. 1984; Second 1982). From the late 1980s, molecular markers involving chloroplast DNA (cpDNA) variation have been extensively used to resolve conflicting phylogenies in cultivated taxa (Doebley et al. 1987; Neale et al. 1988; Ogihara and Tsunewaki 1988; Wolf et al. 1997). The reason for

focusing on cpDNA variation lies in its conservative rate of evolution, both in terms of genome size and structure (Palmer 1987), its maternal inheritance, the availability of cpDNA probes (Llaca et al. 1994; Jack et al. 1995) and, more recently, universal primers for polymerase chain reaction (PCR) amplification of cpDNA sequences (Ogihara et al. 1991; Taberlet et al. 1991; Demesure et al. 1995; Fofana et al. 1997a). The molecular techniques commonly applied to the study of cpDNA variation include (1) isolation of cpDNA followed by digestion with restriction enzymes and electrophoretic separation; (2) restriction digestion of total DNA followed by Southern analysis using cpDNA-specific probes [hereafter called the probed-restriction fragment length polymorphism (RFLP) method]; and (3) direct sequencing of cpDNA regions. These methods are powerful, but they restrict most analyses to small sample sizes due to their inherently high cost and time investments (Weatherhead and Montgomerie 1991). Recently, some PCR-based methods like the amplification of coding and/or non-coding sequences followed by restriction digestion (hereafter called the PCR-RFLP method) have increasingly been used in phylogenetic studies (Pérez de la Rosa and Farjon 1995; Tsumura et al. 1995). Furthermore, because non-coding sequences of the chloroplast genome are expected to evolve more rapidly than coding sequences (Wolfe and Sharp 1988; Wolfe et al. 1987), primers have been designed for the amplification of intergenic regions of cpDNA (Taberlet et al. 1991; Demesure et al. 1995; Dumolin-Lapègue et al. 1997; Fofana et al. 1997a).

Phaseolus is a large, diverse genus of at least 50 species that grow naturally in warm tropical and subtropical regions of the New World, from Sinaloa, Mexico, to Salta, Argentina (Debouck et al. 1987; Delgado 1985). Phylogenetic relationships among *Phaseolus* species have been investigated using morphological (Maréchal et al. 1978; Debouck 1991), biochemical (Sullivan and Freytag 1986; Jaaska 1996; Pueyo and Delgado 1997) as well as molecular markers (Delgado et al. 1993; Hamann et al. 1995; Schmit et al. 1993; Llaca et al. 1994; Vekemans et al. 1998). These studies identified a complex of species including the cultivated *P. vulgaris* L., *P. coccineus* L. and *P. polyanthus* Greenman (hereafter called the *P. vulgaris–P. coccineus* complex) and showed that *P. lunatus* L., the Lima bean, which is ranked second in economical importance among the cultivated species of the genus *Phaseolus*, was very distantly related to that complex. Furthermore, detailed studies in *P. lunatus* showed convincingly that the whole primary gene pool of the Lima bean is divided into two main groups: a Mesoamerican group and an Andean group (Debouck et al. 1989; Maquet et al. 1990; Nienhuis et al. 1995, Fofana et al. 1997b). Each group comprises both wild and cultivated forms, but the evolutionary relationships between these two groups and companion species of the Lima bean remain poorly understood. According to Maréchal et al. (1978), Baudoin (1988) and Debouck (1991), two groups of wild species are suspected to belong to the clade of *P. lunatus*: (1) wild species of Mesoamerican distribution such as *P. ritensis* Jones, *P. maculatus* Scheele, *P. jaliscanus* Piper, *P. marechalii* Delgado, *P. salicifolius* Piper and a specimen currently not determined referred to here as *P. sp.*; and (2) wild species of Andean distribution such as *P. augusti* Harms, *P. bolivianus* Piper and *P. pachyrrhizoides* Harms. These two groups of species will be referred to as the Mesoamerican and the Andean wild allies of *P. lunatus*, respectively. In a recent study, Maquet (1995) concluded that on the basis of seed protein patterns and allozyme variation *P. lunatus* was more closely related to its Andean wild allies than to the Mesoamerican ones. This evidence was used by the author to suggest an Andean origin for the species *P. lunatus*. However, phylogenetic information on several wild species of Mesoamerican distribution such as *P. xolocotzii* Delgado, *P. filiformis* Benth., *P. angustissimus* A. Gray, *P. oligospermus* Piper, *P. hintonii* Delgado, *P. grayanus* Woot, Standley, *P. microcarpus* Mart., *P. pedicellatus* Benth., *P. leptostachyus* Benth. and the eastern United States species *P. polystachyus* B.S.P. is still scarce. All of the above-mentioned species belong to the section *Phaseolus* of the genus with the exception of *P. hintonii* that belongs to section Xanthotricha (Delgado 1985). In addition, no phylogenetic studies based on molecular data have been carried out with both Mesoamerican and Andean wild species.

In this study, we investigate phylogenetic relationships among 22 species belonging to the genus *Phaseolus*, with special emphasis on the group of species currently described as wild allies of *P. lunatus*. We specifically address the issues whether the putative wild allies of *P. lunatus* are phylogenetically closer to *P. lunatus* than to the *P. vulgaris–P. coccineus* complex, whether the Andean wild allies are more closely related to *P. lunatus* than the Mesoamerican wild allies and whether the two gene pools of *P. lunatus* form together a monophyletic group with respect to other taxa. We use variation in cpDNA assessed by two distinct techniques, i.e. PCR-RFLP of intergenic regions (IGRs) and total cpDNA probed-RFLP, and two phylogenetic reconstruction methods, i.e. neighbor-joining and Wagner parsimony.

Materials and methods

Plant material and DNA extraction

Young leaves were collected in the greenhouse from 52 accessions of *P. lunatus* including 40 wild accessions, 1 weedy, and 11 landraces as well as from 34 accessions corresponding to 21 wild species including *P. vulgaris*, *P. coccineus* and *P. polyanthus* (Table 1). These plant materials were chosen either in the world seed bank of the Genetic Resource Unit of the CIAT, Cali, Colombia (for codes G, DGD, or PL) or in the base collection of the Belgium National Botanic

Table 1 List, biological status and origin of different species and ecotypes of the Lima bean

	Accession Number	Species	Status	Origin[a]
1	G25221	*P. lunatus*	Wild	MEX, Veracruz
2	G25224	*P. lunatus*	Wild	CRI, Guanacaste
3	G25225	*P. lunatus*	Wild	CRI, Guanacaste
4	G25227	*P. lunatus*	Wild	CRI, heredia
5	G25294C	*P. lunatus*	Wild	CUB, Matanzas
6	G25385A	*P. lunatus*	Wild	CRI, San Jose
7	G25411	*P. lunatus*	Cult[b]	ECU, Los Rios
8	G25551	*P. lunatus*	Cult[b]	MEX, Chiapas
9	G25583	*P. lunatus*	Wild	CRI, Guanacaste
10	G25915	*P. lunatus*	Wild	PER, Cajamarca
11	G26302	*P. lunatus*	Cult[b]	GTM, Suchitepequez
12	G25979	*P. lunatus*	Cult[b]	BOL, Cochabamba
13	G25585	*P. lunatus*	Wild	CRI, Heredia
14	G25913	*P. lunatus*	Wild	PER, Cajamarca
15	G25837	*P. lunatus*	Cult[b]	PER, Amazonas
16	G26348	*P. lunatus*	Wild	PER, Cajamarca
17	G25881	*P. lunatus*	Cult[b]	COL, Nariño
18	G25965	*P. lunatus*	Wild	CRI, San Jose
19	G25968	*P. lunatus*	Wild	CRI, San Jose
20	DGD-2884	*P. lunatus*	Wild	ECU, Loja
21	DGD-2804	*P. lunatus*	Weed	ECU, Piura
22	G26404	*P. lunatus*	Wild	ARG, Chacõ
23	G25844	*P. lunatus*	Wild	GTM, Sacatepequez
24	G26309	*P. lunatus*	Wild	COL, Magdalena
25	G25908	*P. lunatus*	Cult[b]	COL, Putumayo
26	G25805	*P. lunatus*	Cult[b]	COL, Hulla
27	DGD-2885	*P. lunatus*	Wild	ECU, El oro
28	G25818	*P. lunatus*	Wild	PAN, Panama
29	G25977	*P. lunatus*	Wild	GTM, Escuintla
30	G25226	*P. lunatus*	Wild	CRI, Guanacaste
31	G25290	*P. lunatus*	Wild	GTM, Sacatepequez
32	G25914	*P. lunatus*	Wild	PER, Cajamarca
33	G25819	*P. lunatus*	Wild	COL, magdalena
34	G25704	*P. lunatus*	Wild	MEX, Jalisco
35	G25916	*P. lunatus*	Wild	PER, Cajamarca
36	G25956	*P. lunatus*	Cult[b]	PER, Cajamarca
37	G25825	*P. lunatus*	Cult[b]	PER, Cajamarca
38	DGD-2880	*P. lunatus*	Wild	ECU, Loja
39	G26294	*P. lunatus*	Wild	CRI, Heredia
40	DGD-2113	*P. lunatus*	Wild	CRI, San Jose
41	DGD-2887	*P. lunatus*	Wild[b]	ECU, Azuay
42	G25230	*P. lunatus*	Wild	MEX, Colima
43	G25785	*P. lunatus*	Wild	MEX, Campeche
44	DGD-2094	*P. lunatus*	Wild	CRI, San Jose
45	DGD-2092	*P. lunatus*	Wild	CRI, Cartago
46	DGD-2875	*P. lunatus*	Wild	ECU, Loja
47	DGD-2106	*P. lunatus*	Wild	CRI, Ajajuela
48	DGD-2888	*P. lunatus*	Wild	ECU, Chimborazo
49	G25222	*P. lunatus*	Wild	GTM, Zacapa
50	G25364	*P. lunatus*	Cult[b]	SLV, La Libertad
51	NI814	*P. lunatus*	Wild	ARG, Chaco
52	G25390	*P. lunatus*	Wild	CRI, San Jose
53	NI788	*P. angustissimus*	Wild	USA, Arizo., S. Carter
54	DGD-2482	*P. augusti*	Wild	BOL, Cochabamba, C.
55	PL-8B	*P. bolivianus*	Wild	PER, Maccu piccu ruins
56	NI890[c]	*P. coccineus*	Wild	GTM
57	NI1354	*P. coccineus*	Wild	MEX, Oaxaca, Lach. V.
58	NI1223[c]	*P. filiformis*	Wild	MEX, Baja Cal. Sur
59	NI690	*P. filiformis*	Wild	USA, Arizona, Apache
60	NI1236	*P. grayanus*	Wild	MEX, S.L. Potosi
61	NI804	*P. grayanus*	Wild	MEX, Durango, Sombr.
62	NI806	*P. hintonii*	Wild	MEX, Durango, Frans.I.
63	NI707	*P. hintonii*	Wild	MEX, Durango, Mezq.
64	NI699	*P. leptostachyus*	Wild	MEX, Zacatecas, Corr.
65	NI696	*P. maculatus*	Wild	MEX, Zacatecas, Corr.

Table 1 Continued

	Accession Number	Species	Status	Origin[a]
66	NI729	*P. maculatus*	Wild	MEX, Zacatecas, Sain A.
67	NI560	*P. marechalii*	Wild	MEX
68	NI402	*P. marechalii*	Wild	MEX, Morelos
69	NI1049	*P. microcarpus*	Wild	MEX, Chiapas
70	NI709	*P. microcarpus*	Wild	MEX, Jalisco
71	NI1116	*P. oligospermus*	Wild	GTM, San Juan Acat.
72	NI896	*P. oligospermus*	Wild	MEX
73	DGD-2185	*P. pachyrrhizoides*	Wild	PER, Apurimac
74	DGD-2296	*P. pachyrrhizoides*	Wild	PER, Apurimac
75	NI766	*P. pedicellatus*	Wild	MEX, Mexico, Cuern.
76	NI1123	*P. polyanthus*	Wild	GTM, Solola, Patzicia
77	NI1044	*P. polystachyus*	Wild	USA, Florida, Gainsv.
78	NI563[c]	*P. polystachyus*	Wild	USA, Florida
79	NI796	*P. ritensis*	Wild	USA, Arizona, Santa L.
80	NI1132	*P. salicifolius*	Wild	MEX, Sinaloa, Villa U.
81	NI702	*P. sp*	Wild	MEX, Durango, Dur.
82	NI1470[c]	*P. vulgaris*	Wild	MEX, Guerrero
83	NI1433	*P. vulgaris*	Wild	MEX, Chiapas
84	NI1[c]	*P. vulgaris*	Cult	MEX
85	NI555	*P. vulgaris*	Wild	GTM, Alta V., Coban
86	NI1046	*P. xolocotzii*	Wild	MEX, Jalisco, Tepa. Pal

[a] MEX, Mexico; ECU, Ecuador; GTM, Guatemala; COL, Colombia; CRI, Costa-Rica; ARG, Argentina; PAN, Panama; SLV, Salvador; BOL, Bolivia; USA, United States of America
[b] Specifically included in PCR-RFLP
[c] Specifically included in probed-RFLP

Table 2 Heterologous PCR primers sequences deduced from cpDNA

Numbering of primers	Sequences 5′ → 3′		Sources
	Forward	Reverse	
Primers 1 (atpB-rbcL)	GTGTCAATCACTTCCATTCC	GTAAAATCAAGTCCACCGCG	Fofana et al. 1997a
Primers 2 (rps14-psaB)	CATTTCACGAAGTATGTGTCCG	TGGCGTGGATATTGGCAGGA	Fofana et al. 1997a
Primers 3 (PetA-psbE)	GCATCTGTTATTTTGGCACA	TACCTTCCCTATTCATTGCG	Fofana et al. 1997a
Primers 4 (PsbC-tRNAser)	GGTCGTGACCAAGAAACCAC	GGTTCGAATCCCTCTCTCTC	Demesure et al. 1995
Primers 5 (tRNAser-tRNAfmet)	GAGAGAGAGGGATTCGAACC	CATAACCTTGAGGTCACGGG	Demesure et al. 1995
Primers 6 (tRNAthr-tRNAphe)	CATTACAAATGCGATGCTCT	ATTTGAACTGGTGACACGAG	Demesure et al. 1995

Garden of Meise (for codes NI). For each accession, leaves were collected from two plants and bulked. Total DNA was extracted from 2 g of leaves following the CTAB method described by Chandelier (1995), with a minor modification which consists of the substitution of one of the two steps of chloroform/isoamylalcohol (24 : 1) extraction by one step of phenol chloroform (1 : 1) extraction.

PCR reactions and RFLP analysis

The design of cpDNA-derived primers is described elsewhere (Fofana et al. 1997a and Demesure et al. 1995). Six of these primers were used to amplify cpDNA intergenic regions (Table 2). We adjusted and homogenised the annealing temperature of primers designed by Demesure et al. (1995) to 55°C. PCR reactions were performed in 100-μl aliquots including 10 μl of total DNA (100 ng), 10 μl buffer 10× (Pharmacia, the Netherlands), 2 μl $MgCl_2$ (100 m*M*), 2.5 μl dNTP (2 m*M*), 2 μl of each primer (50 pmoles/μl), 0.5 μl *Taq* DNA polymerase (1U/0.2 μl) and 71.0 μl of sterile water. Reaction mixtures were overlaid with mineral oil and subjected to amplification in a Techne PHC-3 thermocycler for 45 cycles, each cycle consisting of a denaturation step for 1 min at 94°C, an annealing step for 1 min at 55°C (for primers 2, 4, 5, 6), at 53°C (for primer 1) or at 56°C (for primer 3) and an extension step for 2 min at 72°C. An initial denaturation and a final extension were conducted respectively at 94°C for 5 min and at 72°C for 10 min. PCR products were digested using the restriction enzymes *Hin*fI, *Alu*I, *Taq*I, *Rsa*I, *Hpa*II, *Sph*I, *Nde*I, *Kpn*I, *Hha*I, *Sau*3AI. The restricted fragments were then electrophoresed on 2% agarose gel and stained with ethidium bromide. A mix of λDNA/*Hin*dIII and $\varphi \times 174$RFDNA/*Hae*III digests was loaded in each gel as a molecular size standard. Banding patterns were visualised under UV light and photographed on a Polaroid film. The restriction fragments were scored for presence (1) or absence (0) and the data stored in a 0–1 matrix data set.

Additionally, a Southern blot analysis using a total cpDNA purified from a Lima bean accession (G25294c) as probe was performed. The cpDNA was [^{32}P]-labeled and used to probe under high stringency hybridisation conditions [65°C overnight, 3 × SSC, 5 × Denhardt, 0.1% (w/v) SDS] on total DNA digested with restriction enzymes (*Eco*RI, *Bam*HI, *Hin*dIII and *Eco*RV) and transferred on a nylon membrane (Hybond N+, Amersham). A small amount (10 ng) of the molecular size standard mix was included and radiolabeled together with the cpDNA. After high-stringency washes

[three times in 2 × SSC; 0.1% (w/v) SDS for 15 min each and three times in 0.1 × SSC; 0.1% (w/v) SDS for 15 min each] and exposure to Fuji RX films (for) (1–6 days), the visualised fragments were scored for presence (1) or absence (0) as in PCR-RFLP.

Data analysis

The proportion of shared fragments between each pair of haplotypes is determined as $F = (2m_{xy})/(m_x + m_y)$, where m_x and m_y are the total number of fragments scored in haplotypes x and y, respectively, and m_{xy} is the number of shared fragments between x and y (Nei and Li 1979). The matrix of pairwise $(1 - F)$ values, i.e. the proportion of fragments which are not shared, is directly used to reconstruct phylogenetic relationships among haplotypes by the neighbor-joining method (NJ) using procedure NEIGHBOR from the PHYLIP package (Felsenstein 1993). One thousand sets of bootstraps are performed by (1) sampling, with replacement, individual fragments from the original data set, (2) computing a new $(1 - F)$ matrix for each bootstrap, (3) reconstructing trees as described above, and (4) summarising the results using the procedure CONSENSE from PHYLIP. The phylogenetic trees obtained were rooted according to the midpoint criterion using procedure RETREE from PHYLIP. Wagner parsimony, including a bootstrap analysis, is applied to the original data matrix using the procedures MIX, SEQBOOT and CONSENSE from the PHYLIP package. All trees were drawn using the program TREEVIEW (Page 1992).

Results

Patterns of cpDNA variation

Using the PCR-RFLP technique, we amplified six intergenic regions (IGRs) from which only one, corresponding to the tRNAthr(UGU)-tRNAphe(GAA) region, revealed length polymorphism, ranging from 1840 to 1950 bp (Fig. 1). In total, around 8600 bp of cpDNA was amplified. Using ten restriction enzymes and the six IGRs, 191 distinct restriction fragments were scored, of which 150 (78.5%) were found to be polymorphic throughout the genus. The proportion of fragments shared by pairs of haplotypes ranges between 0.586 and 0.995. When *P. lunatus* was compared with the three Andean wild allies *P. pachyrrhizoides*, *P. augusti* and *P. bolivianus*, only 25 (13.1%) restriction fragments were polymorphic, whereas *P. lunatus* with the Mesoamerican wild allies showed 85 (44.5%) polymorphic fragments. Within *P. lunatus* 11 (5.8%) polymorphic fragments determining six different haplotypes were scored. Distinct patterns between haplotypes belonging to the Andean and Mesoamerican groups of the Lima bean were observed, for instance in tRNAser(UGA)-tRNAfmet(CAU) and tRNAthr(UGU)-tRNAphe(GAA) regions when digested with *Hin*fI (Fig. 2A), but no difference appeared in the restriction pattern between wild forms and landraces within each group. The average number of pairwise restriction fragment differences between accessions were estimated as 1.29 ± 0.83 in the Andean group of the Lima bean and 3.25 ± 1.73 in the Mesoamerican group. A one-way AMOVA (Excoffier et al. 1992) was performed to test

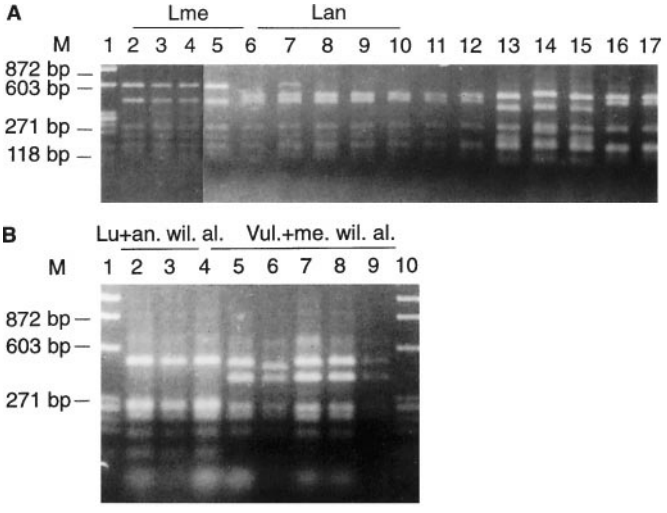
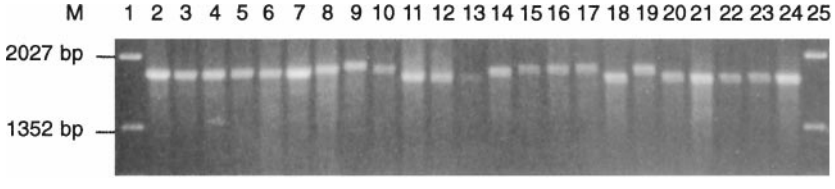


Fig. 2A, B Restriction pattern of PCR-amplified cpDNA IGRs observed on 2% agarose gel. **A** *Hin*fI-digested tRNAthr-tRNAphe intergenic region. *Lane 1* Molecular size marker (*M*), *lanes 2–5, P. lunatus*-Mesoamerican forms (*Lme*) G25551, G25704, G25785, G25221 respectively, *lanes 6–9 P. lunatus*-Andean forms (Lan) G26348, G25915, S32398 respectively, *lane 10 P. pachyrrhizoides*, *lane 11 P. augusti*, *lane 12 P. bolivianus*, *lane 13 P. vulgaris*, *lane 14 P. coccineus*, *lane 15 P. polyanthus*, *lane 16 P. polystachyus*, *lane 17 P. ritensis* **B** Representative pattern of *Hin*fI-digested atpB-rbcL intergenic region. *Lanes 1 and 10* Molecular size marker (*M*). *lun + an. wil. al. P. lunatus* and Andean wild allies: *lane 2 P. lunatus* (both Mesoamerican and Andean forms), *lane 3 P. pachyrrizoides, lane 4 P. bolivianus. vul. + me. wil. al. P. vulgaris* and mesoamerican wild allies: *lane 5 P. vulgaris*, *lane 6 P. coccineus*, *lane 7 P. polyanthus*, *lane 8 P. ritensis*, *lane 9 P. xolocotzii*

Fig. 1 PCR amplification products observed in IGR 6 (tRNAthr-tRNAphe). *Lanes 1 and 25* Molecular size marker (*M*), *lane 2 P. lunatus*-Mesoamerican forms (Lme), *lane 3 P. lunatus*-Andean forms (Lan), *lane 4 P. pachyrrhizoides*, *lane 5 P. augusti*, *lane 6 P. bolivianus*, *lane 7 P. polystachyus*, *lane 8 P. vulgaris*, *lane 9 P. coccineus*, *lane 10 P. polyanthus*, *lane 11 P. sp*, *lane 12 P. ritensis*, *lane 13 P. xolocotzii*, *lane 14 P. microcarpus*, *lane 15 P. filiformis*, *lane 16 P. oligospermus*, *lane 17 P. angustissimus*, *lane 18 P. maculatus*, *lane 19 P. hintonii*, *lane 20 P. pedicellatus*, *lane 21 P. salicifolius*, *lane 22 P. marechalii*, *lane 23 P. grayanus*, *lane 24 P. leptostachyus*



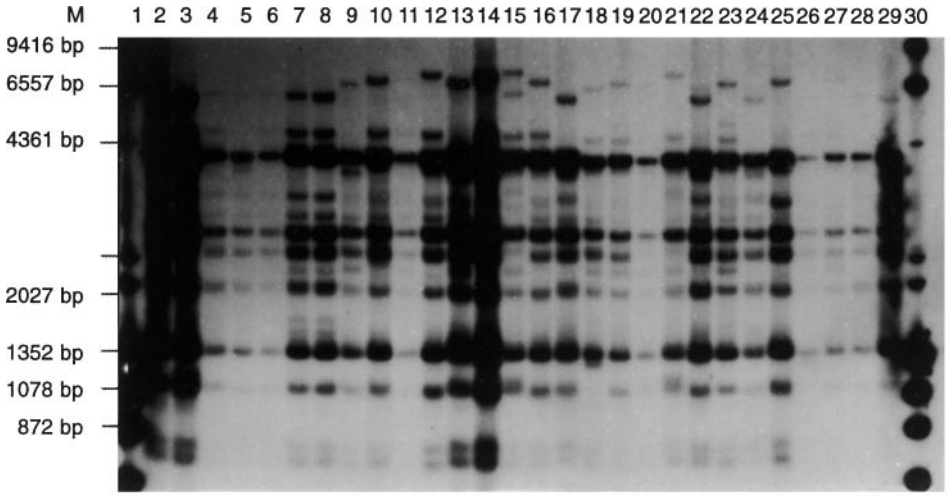


Fig. 3 Southern-blotting analysis in the genus *Phaseolus* using an *Eco*RI digest of total DNA and probed by the purified cpDNA. *Lane 1* Molecular size marker (*M*) *lane 2 P. bolivianus*, *lane 3 P. pachyrrhizoides*, *lane 4 P. augusti*, *lane 5 P. polystachyus*, *lanes 6–8 P. lunatus* (G25225, G25583, G25294 C), *lane 9 P. microcarpus*, *Lane 10 P. leptostachyus*, *lane 11 P. filiformis*, *lane 12 P. vulgaris*, *lane 13 P. filiformis*, *lane14 P. vulgaris*, *lane 15 P. oligospermus*, *lane 16 P. angustissimus*, *lane 17 P. maculatus*, *lane 18 P. hintonii*, *lane 19 P. pedicellatus*, *lane 20 P. microcarpus*, *lane 21 P. oligospermus*, *lane 22 P. salicifolius*, *lane 23 P. grayanus*, *lane 24 P. marechalii*, *lane 25 P. coccineus*, *lane 26 P. hintonii*, *lane 27 P. grayanus*, *lane 28 P. marechalii*, *lane 29 P. maculatus*

differentiation between Andean and Mesoamerican accessions. The results showed that the two groups are significantly differentiated ($P < 0.001$), 67.5% of the variation between groups and 32.5% within-group. Andean forms of the Lima bean showed a pattern similar to that of the Andean species (Fig. 2A, B).

Using a probed-RFLP technique, we obtained a pattern in genomic DNA restricted with *Eco*RI and hybridised with the purified cpDNA probe that depicts interspecific polymorphism (Fig. 3). Using four restriction enzymes combined with the total cpDNA probe, we scored 92 distinct fragments, of which 67 (72.8%) were polymorphic over the genus. The proportion of fragments shared by pairs of haplotypes ranges between 0.621 and 0.978. When *P. lunatus* was compared with the Andean wild allies *P. pachyrrhizoides*, *P. augusti* and *P. bolivianus*, only 47 (51.1%) restriction fragments were polymorphic, whereas *P. lunatus* with the Mesoamerican wild allies showed 55 (59.8%) polymorphic fragments. Within *P. lunatus* 40 (43.5%) polymorphic fragments were scored. The average number of pairwise restriction fragment differences between accessions was estimated as 8.93 ± 4.80 in the Andean group of the Lima bean and 14.29 ± 6.94 in the Mesoamerican group. A one-way AMOVA was performed to test differentiation between Andean and Mesoamerican accessions: a significant differentiation ($P < 0.05$) was observed between groups.

Phylogenetic reconstructions

Based on 191 fragments scored from the six intergenic regions, we computed a phylogenetic tree using the Wagner parsimony and the neighbor-joining methods. The resulting trees from both methods were globally in agreement (Fig. 4A, B).

When the neighbor-joining method was used two main divergent clusters supported by a bootstrap index of 736 were observed (Fig. 4A). Within one of these clusters (lower part of the tree), Andean and Mesoamerican forms of *P. lunatus* are monophyletic (bootstrap index of 922) and appear to be closely related to the Andean allies *P. augusti*, *P. pachyrrhizoides* and *P. bolivianus* forming together a large monophyletic group (bootstrap index of 954). Additionally, Andean forms of the Lima bean are closer to the Andean wild allies than are their Mesoamerican counterparts (Figs. 2A, 4A). Within the same main cluster, 1 species, *P. polystachyus*, distributed throughout the south-east of USA, appears as a sister taxa closely related to the group of Andean species that includes the Lima bean. Six other Mesoamerican species, namely *P. maculatus*, *P. xolocotzii*, *P. sp.*, *P. ritensis*, *P. marechalii* and *P. salicifolius*, are also members of this first main cluster.

In the other main cluster, *P. vulgaris*, *P. coccineus* and *P. polyanthus* are found together with 8 companion species of largely Mesoamerican distribution. The observed variation in this group and within each IGR in terms of gains or losses of restriction sites is very high compared to the previous one, and phylogenetic relationships among species are not well resolved. *P. vulgaris*, *P. filiformis*, *P. angustissimus* and *P. polyanthus* form a monophyletic group (bootstrap index of 680), closer (but not supported by bootstraps) to a clade formed by *P. coccineus*, *P. oligospermus*, *P. hintonii* and *P. microcarpus*. *P. pedicellatus* and *P. grayanus* are closely related. *P. leptostachyus* and the accession NI1049 of *P. microcarpus* cannot be attached to any group. In *P. microcarpus*, accession NI1049 shows

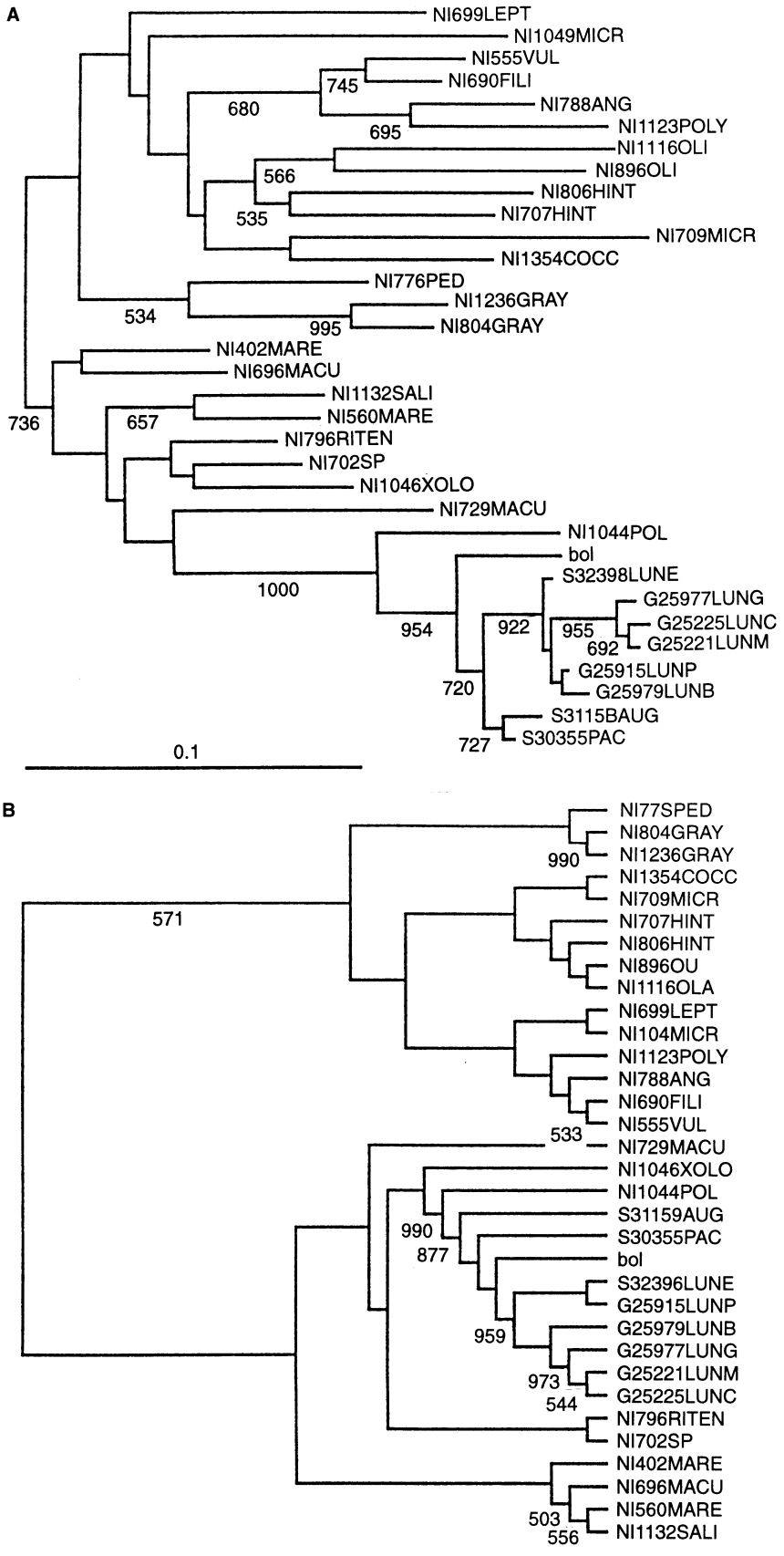


Fig. 4 A Phylogenetic tree obtained using neighbor-joining from PCR-RFLP of cpDNA IGRs in the genus *Phaseolus*. *LEPT P. leptostachyus*, *MICR P. microcarpus*, *VUL P. vulgaris*, *FILI P. filiformis*, *ANG P. angustissimus*, *POLY P. polyanthus*, *OLI P. oligospermus*, *HINT P. hintonii*, *COCC P. coccineus*, *PED P. pedicellatus*, *GRAY P. grayanus*, *MARE P. marechalii*, *MACU P. maculatus*, *SALI P. salicifolius*, *RITEN P. ritensis*, *SP P. sp*, *XOLO P. xolocotzii*, *POL P. polystachyus*, *bol P. bolivianus*, *LUN* (the fourth letter refers to the initial of the country of origin: *E* Ecuador, *G* Guatemala, *C* Costa Rica, *M* Mexico, *P* Peru, *B* Bolivia) *P. lunatus*, *AUG P. augusti*, *PAC P. pachyrrhizoides*. **B** Phylogenetic tree obtained using Wagner parsimony from PCR-RFLP of cpDNA IGRs in the genus *Phaseolus*. The abbreviations used are the same as those in **4A**

a very different pattern in each combination enzyme × IGRs compared to its other accession NI709.

From the Wagner parsimony analysis, the two main clusters involving either *P. lunatus* or *P. vulgaris* still emerge even if they are not strongly supported by bootstrapping (Fig. 4B). By this method we obtained a tree length of 437 steps. As in the neighbor-joining tree, the Lima bean accessions form, in the lower part of the tree, a monophyletic group (bootstrap index of 959) which is closely related to the 3 Andean wild allies and *P. polystachyus* (bootstrap index of 990). *P ritensis* and *P. sp.*, on the one hand, and *P. marechalii*, *P. maculatus* and *P. salicifolius*, on the other hand, appear closely related. These species, together with *P. xolocotzii*, are clustered with *P. lunatus* and its Andean allies.

Similarly, both neighbor-joining and Wagner parsimony were applied on data obtained from the probed-RFLP study. As described above, two main clusters were observed (data not shown). In the first, *P. lunatus* and the 3 Andean wild allies occur as a monophyletic group, as in the case of the PCR-RFLP analysis, with the exception of the Mexican accession NI699 belonging to *P. leptostachyus*, which also clusters with Lima bean accessions. The 3 Andean species are more closely related to the Andean forms of the Lima bean than to the Mesoamerican forms. Bootstrap indices obtained here were lower than indices with the PCR-RFLP analysis. In the second cluster, *P. vulgaris*, *P. coccineus*, *P. polyanthus*, *P. filiformis* and *P. xolocotzii* form a monophyletic group. All other species of Mesoamerican distribution are scattered in one or the other cluster, their position being therefore unresolved.

Discussion

From these results, three points related to the methodology, taxonomy and phylogeny, respectively, are worth discussing. First, we used PCR-RFLP and RFLP techniques to determine phylogenetic relationships between *P. lunatus* and its Andean and Mesoamerican wild allies with respect to the *P. vulgaris – P. coccineus – P. polyanthus* complex. From an experimental viewpoint, the PCR-RFLP technique of intergenic regions is technically more flexible and faster relative to the probe-RFLP method. Nevertheless, its main difficulty lies in the detection of all of the small fragments for an accurate mapping of the restriction sites. On the other hand, the interpretation of the cpDNA-probe RFLP pattern is not easy because of the variation in band intensities, the complexity of the pattern and the high risk of homoplasy (Straney 1981; Dowling et al. 1996; Forcioli et al. 1994). Some of these difficulties can be overcome by increasing the ratio scored fragments/Operational Taxonomic Units (OTUs). This means that 10–20 restriction enzymes should be used to produce highly resolved trees (Dowling et al. 1996). We used here only four restriction enzymes; this can partly explain the slight discrepancy between the two approaches. Thus, the two techniques are complementary and, using both neighbor-joining and parsimony methods, their results converge in the determination of the phylogenetic relationships in *Phaseolus*.

Second, by using two methods of phylogenetic inference on data obtained from two molecular approaches, we obtained results indicating two main lineages in the genus: one involving *P. vulgaris–P. coccineus–P. polyanthus*, and the other *P. lunatus*. Morphological (Maréchal et al. 1978) and molecular data (Schmit et al. 1993; Llaca et al. 1994; Jacob et al. 1995) have also shown these two lineages in spite of scanty and different taxa examined. Our results and others (Delgado et al. 1993; Schmit et al. 1993; Llaca et al. 1994) would indicate that in addition the genus *Phaseolus* would not be monophyletic and that some cpDNA, variation exists at the intraspecific level. Such variation is present within taxa such as *P. maculatus*, *P. microcarpus*, which authors (Delgado 1985; Debouck 1991) consider as coherent entities, thus limiting somewhat the power of cpDNA markers in the taxonomy of *Phaseolus*. Contrary to the findings of Delgado et al. (1993), *P. hintonii* would not form a separate clade. In the upper part of the tree, *P. leptostachyus*, a species with $2n = 2x = 20$ while the chromosome number is 22 for the others (Maréchal et al. 1978; Delgado 1985), separates clearly. *P. grayanus* and *P. pedicellatus* appear to be related, but perhaps not up to the level to be merged as proposed by Delgado (1985). *P. vulgaris*, *P. angustissimus* and *P. filiformis* appear to be related, in sharp contrast with hybridological data (Belivanis and Doré 1986; Maréchal and Baudoin 1978, respectively). Confirming earlier observations (Schmit and Debouck 1991; Schmit et al. 1993), *P. polyanthus* appears as a distinct taxon from *P. coccineus*; in addition, it would be closer to *P. vulgaris* than *P. coccineus*. The lower part of the tree groups together the Lima bean with several taxa, which are likely related according to hybridological data (Katanga and Baudoin 1990). Our results confirm the identity of *P. marechalii* and *P. xolocotzii* as distinct species, as revealed by protein polymorphism studies (Pueyo and Delgado 1997). *P. sp.* (NI702, DGD-409) could be different from *P. ritensis*, although the intraspecific cpDNA variation within *P. maculatus* raises doubts about such a difference; one could perhaps make the same statement about *P. augusti* and *P. pachyrrhizoides*. Interestingly, the Mesoamerican accessions of Lima bean would slightly separate from the Andean ones, evidencing further the gene pools shown elsewhere (Gutiérrez et al. 1995; Nienhuis et al.1995; Fofana et al. 1997b), but they form a coherent cluster together with the Andean allies of *P. lunatus*.

Third, our observations suggest that the two main lineages could have arisen from two ancestral forms (called A and B, involving *P. vulgaris* and *P. lunatus*,

respectively). Such forms might have evolved in Central America, centre of origin or speciation of the genus, where approximately 40 species occur today (Delgado 1985; Delgado et al. 1993). *Phaseolus* species are indeed numerous along the mountainous systems of Sierra Madre Occidental and the Eje Volcánico Transversal of Mexico, which were formed during the Oligocene-Miocene and Late Tertiary or Pliocene, respectively (Ferrusquía-Villafranca 1993). Therefore, the current *Phaseolus* diversity may stem from the Oligocene or later (Sousa and Delgado 1993). Glaciation cycles (Bernard 1962; Servant et al. 1993) have played important roles in this speciation process favouring the appearance of refugia. The ancestral form (B) would have migrated towards South America leaving behind it several variants such as *P. maculatus*, *P. marechalii*, *P. ritensis*, *P. salicifolius P. polystachyus* and *P. xolocotzii*. Thousands of years afterwards these variants would turn into the species of the tertiary gene pool of Lima bean, evidenced by Katanga and Baudoin (1990). Data based on cpDNA nucleotide sequences from these species (Fofana, unpublished) would support this hypothesis. Such migration events have been reported for elements of the Neotropical flora during the Pliocene or early Pleistocene (Haffer 1987; van der Hammen 1992). The ancestral form (B) would have reached the Andean highlands to undertake a sympatric speciation leading to *P. augusti*, *P. pachyrrhizoides*, *P. bolivianus* and *P. lunatus*. These trends in evolution fit well with our observations on the different cpDNA haplotypes, the model proposed by Maquet (1995) and one of the alternative hypotheses of Debouck (1996). In fact, for the latter, *P. lunatus* has either migrated in the Andes or evolved there with companion species such as *P. augusti*, *P. pachyrrhizoides* (if different from *P. augusti*) and *P. mollis* (which is restricted to the Galapagos Islands). So, an Andean origin of the Lima bean is highlighted by the closest relationships between its Andean forms and the Andean wild allies. The Mesoamerican form of *P. lunatus* could have derived from one of the Andean wild allies, since the seed protein patterns of both groups of materials are very similar (Maquet and Baudoin 1996). Preliminary results from our interspecific hybridisation experiments between the Lima bean and the 3 Andean species (unpublished) and the evidence from Maquet (1995) indicate that the 3 Andean species constitute the secondary gene pool of *P. lunatus*.

Due to higher fitness (Harding et al. 1966; Allard and Workmann 1963), the Lima bean spread from the highland centre of speciation to colonise new habitats in Neotropical savannahs both Southeast and northwards, while its highland ecotype concentrates in montane dry forests of the north-western Andes as suggested by Debouck (1996). Such a scenario led us to propose a double derivative evolution for the Lima bean from the Latin American highlands, first in Central America, then in the Andes. This scenario could be valid because of geographically separate processes of speciation for the long experienced genetic incompatibility between the Lima bean and the common bean (Debouck and Smartt 1995).

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References

Allard RW, Workmann PL (1963) Population studies in predominantly self-pollinated species. IV. Seasonal fluctuations in estimated values of genetic parameters in Lima bean populations. Evolution 17:470–480

Baudoin JP (1988) Genetic resources, domestication and evolution of the Lima bean, *Phaseolus lunatus*. In: Gepts P (ed) Genetic resources of *Phaseolus* beans. Kluwer Academic Publ, Dordrecht, the Netherlands, pp 393–407

Belivanis T, Doré C (1986) Interspecific hybridization of *Phaseolus vulgaris* L. and *Phaseolus angustissimus* A. Gray using in vitro embryo culture. Plant Cell Rep 5:329–331

Bernard EA (1962) Le caractère tropical des paléoclimats à cycles conjoints de 11 et 21,000 ans et ses causes: migrations des pôles ou dérives des continents. J. Duculot, Gembloux, Belgium

Chandelier A (1995) Structure du génome mitochondrial de l'épicéa commun (*Picea abies* (L) Karst) lors de l'embryogenèse somatique in vitro. PhD thesis, Faculté Sci Agric, Gembloux, Belgium

Debouck DG (1991) Systematics and morphology. In: Voysest O, Schoonhoven A (eds) Common beans: research for crop improvement. CAB Int, Wallingford, UK, pp 55–118

Debouck DG (1996) Colombian common and Lima beans: views on their origin and evolutionary significance. Revista CORPOICA 1:7–15

Debouck DG, Smartt J (1995) Beans, *Phaseolus spp.* (Leguminosae-Papilionoidae). In: Smartt J, Simmonds NW (eds) Evolution of crop plants. Longman Scientific and Technical, London, UK, pp 287–294

Debouck DG, Linan Jara JH, Campana SA, de la Cruz RJH (1987) Observations on the domestication of *Phaseolus lunatus* L. FAO/IBPGR Plant Genet Res Newsl 70:26–32

Debouck DG, Maquet A, Posso CE (1989) Biochemical evidence for two different gene pools in Lima bean, *Phaseolus lunatus* L. Annu Rep Bean Improv Coop 32:58–59

Delgado SA (1985) Systematics of the genus *Phaseolus* (Leguminosae) in North and Central America. PhD thesis, University of Texas, Austin, Tex., USA

Delgado SA, Bruneau A, Doyle JJ (1993) Chloroplast DNA phylogenetic studies in New World Phaseolinae (Leguminosae: Papilionoideae:Phaseoleae). Syst Bot 18:6–17

Demesure B, Sodzi N, Petit RJ (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol Ecol 4:129–131

Doebley J (1992) Molecular systematics and crop evolution. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman and Hall, New York, pp 202–222

Doebley J, Goodman, Stuber CW (1984) Isoenzymatic variation in *Zea* (Gramineae). Syst Bot 9 : 203–218

Doebley J, Renfroe W, Blanton A (1987) Restriction site variation in the *Zea* chloroplast genome. Genetics 117 : 139–147

Dowling TE, Moritz C, Palmer JD, Rieseberg LH (1996) Nucleic acids III: analysis of fragments and restriction sites. In: Hillis DM., Moritz C, Mable BK (eds) Molecular systematics. Sinauer Assoc, Sunderland, Mass, USA, pp 249–320

Dumolin-Lapègue S, Pemonge MH, Petit RJ (1997) An enlarged set of consensus primers for the study of organelle DNA in plants. Mol Ecol 6:393–397

Excoffier L, Smouse PE, Quarttro JM (1992) Analysis of molecular variance infered from metric distances among DNA restriction data. Genetics 131 : 479–491

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package). Distributed by the author, University of Washington, Seattle, Wash., USA

Ferrusquía-Villafranca, I (1993) Geology of Mexico: a synopsis. In: Ramamoorthy TP, Bye R, Lot A, Fa J (eds) Biological diversity of Mexico: origins and distribution. Oxford University Press, Oxford, UK, pp 3–107

Fofana B, Harvengt L, Baudoin JP, du Jardin P (1997a) New primers for polymerase chain amplification of cpDNA intergenic spacers in *Phaseolus* phylogeny. Belg J Bot 129 : 118–122

Fofana B, Vekemans X, du Jardin P, Baudoin JP (1997b) Genetic diversity in Lima bean (*Phaseolus lunatus* L.) as revealed by RAPD markers. Euphytica 95 : 157–165

Forcioli D, Saumitou-Laprade P, Michaelis G, Cuguen J (1994) Chloroplast DNA polymorphism revealed by a fast, non-radioactive method in *Beta vulgaris* ssp. *maritima*. Mol Ecol 3 : 173–176

Frankel OH, Brown AHD, Burdon JJ (1995) The conservation of plant biodiversity. Cambridge University Press, Cambridge, UK

Gepts P, Osbon TC, Rashka K, Bliss FA (1986) Phaseolin protein variability in wild forms and landraces of the common bean (*Phaseolus vulgaris* L.): evidence for multiple centers of domestication. Econ Bot 40 : 451–468

Gutiérrez SA, Gepts P, Debouck DG (1995) Evidence for two gene pools of the Lima bean, *P. lunatus* L., in the Americas. Genet Resources Crop Evol 42 : 15–28

Haffer J (1987) Quaternary history of tropical America. In: Whitmore TC, Prance GT (eds) Biogeography and quaternary history in tropical America. Oxford University Press, Oxford, UK, pp 1–18

Hamann A, Zink D, Nagl W (1995) Microsatellite fingerprinting in the genus *Phaseolus*. Genome 38 : 507–515

Harding J, Allard RW, Smeltzer DG (1966) Population studies in predominately self-pollinated species. IX. Frequency-dependent selection in *Phaseolus lunatus*. Genetics 56 : 99–104

Jaaska V (1996) Isoenzyme diversity and phylogenetic affinities among the *Phaseolus* beans (Fabaceae). Plant Syst Evol 200 : 233–252

Jack PL, Dimitrijevic TAF, Mayes V (1995) Assessment of nuclear, mitochondrial and chloroplast RFLP markers in oil palm (*Elaeis guineensis* Jacq). Theor Appl Genet 90 : 643–649

Jacob M, Zink D, Nagl W (1995) RFLPs of the rRNA genes in the genus *Phaseolus*. Genet Resources Crop Evol 42 : 97–106

Johnson BL (1972) Seed protein profiles and the origin of the hexaploid wheats. Am J Bot 59 : 952–960

Katanga K, Baudoin JP (1990) Analyses méiotiques des hybrides F_1 et études des descendances F_2 chez quatre combinaisons interspécifiques avec *P. lunatus*. Bull Rech Agron Gembloux 25 : 237–250

Llaca V, Delgado S A, Gepts P (1994) Chloroplast DNA as an evolutionary marker in the *Phaseolus vulgaris* complex. Theor Appl Genet 88 : 646–652

Maquet A (1995) Etude de la diversité génétique de la légumineuse alimentaire *Phaseolus lunatus* L. par l'analyse de caractères morphophysiologiques et protéiques. PhD thesis, Faculté Sci Agric Gembloux, Belgium

Maquet A, Baudoin JP (1996) New lights on phyletic relations in the genus *Phaseolus*. Annu Rep Bean Improv Coop 39 : 203–204

Maquet A, Gutiérrez A, Debouck DG (1990) Further biochemical evidence for the existence of two gene pools in Lima bean. Annu Rep Bean Improv Coop 33 : 128–129

Maréchal R, Baudoin JP (1978) Observations sur quelques hybrides dans le genre *Phaseolus*. IV. L' hybride *Phaseolus vulgaris* × *Phaseolus filiformis*. Bull Rech Agron Gembloux 13 : 233–240

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

Neale DB, Saghai-Maroof MA, Allard RW, Zhang Q, Jorgensen RA (1988) Chloroplast DNA diversity in population of wild and cultivated barley. Genetics 120 : 1105–1110

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

Nienhuis J, Tivang J, Skroch P (1995) Genetic relationships among cultivated and landraces of Lima bean (*Phaseolus lunatus* L.) as measured by RAPD markers. J Am Soc Hortic Sci 120 : 300–306

Ogihara Y, Tsunewaki K (1988) Diversity and evolution of chloroplast DNA in *Triticum* and *Aegilops* as revealed by restriction fragment analysis. Theor Appl Genet 76 : 321–332

Ogihara Y, Terachi T, Sasakuma T (1991) Molecular analysis of the hot spot region related to length mutation in wheat chloroplast DNAs. I. Nucleotide divergence of genes and intergenic spacer regions located in the hot spot region. Genetics 129 : 873–884

Page RDM (1992) An application to display phylogenetic trees on personal computer. Computer Applic Biosci 12 : 357–358

Palmer JD (1987) Chloroplast DNA evolution and biosystematic use of chloroplast DNA variation. Am Nat 130 : s6-s29

Pérez de la Rosa JSA, Farjon HA (1995) Noncoding chloroplast DNA variation in Mexican pines. Theor Appl Genet 91 : 1101–1106

Piper CV (1926) Studies in American Phaseolinae. Contrib US Natl Herb 22 : 663–701

Pueyo JJ, Delgado SA (1997) Presence of α-amylase inhibitor in some members of the subtribe Phaseolinae (Phaseoleae: Fabaceae). Am J Bot 84 : 79–84

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

Schmit V, du Jardin P, Baudoin JP, Debouck DG (1993) Use of chloroplast DNA polymorphism for the phylogenetic study of seven *Phaseolus* taxa including *P. vulgaris* and *P. coccineus*. Theor Appl Genet 87 : 506–516

Second G (1982) Origin of the genic diversity of cultivated rice (*Oryza* spp.): study of the polymorphism scored at 40 isozyme loci. Jpn J Genet 57 : 25–57

Servant M, Maley J, Turcq B, Absy M-L, Brenac P (1993) Tropical forest changes during the late Quaternary in African and South American Lowlands. Global Planet Change 7 : 25–40

Sousa SM, Delgado SA (1993) Mexican Leguminosae: phytogeography, endemism, and origins. In: Ramamoorthy TP, Bye R, Lot A, Fa J (eds) Biological diversity of Mexico: origins and distribution. Oxford University Press, Oxford, UK, pp 459–511

Straney DO (1981) The stream of heredity. In: Joule MHSaJ (ed) Mammalian population genetics. University of Georgia Press, Athens, Ga., USA, pp 100–138

Sullivan JG, Freytag G (1986) Predicting interspecific compatibilities in beans (*Phaseolus*) by seed protein electrophoresis. Euphytica 35 : 201–209

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 17 : 1105–1109

Tsumura Y, Yoshimura K, Tomaru T, Ohba K (1995) Molecular phylogeny of conifer using RFLP analysis of PCR-amplified specific chloroplast genes. Theor Appl Genet 91 : 1222–1236

van der Hammen T (1992) Historia de la vegetación de América del Sur. In: van der Hammen T (ed) Historia, ecologia y vegetación. Corporacion Colombia para la Amazonia "Araracuara", Bogota, Colombia, pp 77–104

Vekemans X, Hardy O, Berken B, Fofana B, Baudoin JP (1998) Use of PCR-RFLP on chloroplast DNA to investigate phylogenetic relationships in the genus *Phaseolus*. BASE (in press)

Weatherhead PJ, Montgomerie RD (1991) Good news and bad news about DNA fingerprinting. Trends Ecol Evol 6:173–174

Wolf PG, Murray RA, Sipes SD (1997) Species-independent, geographical structuring of choroplast DNA haplotypes in a montane herb *Ipomopsis* (Polemoniaceae). Mol Ecol 6:283–291

Wolfe KH, Sharp PM (1988) Identification of functional open reading frames in chloroplast genomes. Genes 66:215–222

Wolfe KH, Li WH, Sharp PM (1987) Rates of nucleotides substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc Natl Acad Sci USA 84: 9054–9058