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P. Gauthier · R. Lumaret · A. Bedecarrats

Chloroplast-DNA variation in the genus *Lotus* (Fabaceae) and further evidence regarding the maternal parentage of *Lotus corniculatus* L.

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Abstract To resolve the maternal parentage of the tetraploid *Lotus corniculatus*, restriction-site variation of chloroplast DNA (cpDNA) was studied in several accessions of that species, in the four putative parental diploid species, *L. tenuis*, *L. alpinus*, *L. japonicus* and *L. uliginosus*, and in four phylogenetically more distant diploid species, *L. hispidus*, *L. edulis*, *L. ornithopodoides* and *Tetragonolobus maritimus* var. *siliquosus*. Evidence of cpDNA maternal inheritance was obtained by using reciprocal controlled crosses between plants of *L. corniculatus* and natural tetraploid individuals of *L. alpinus* showing very distinct restriction patterns. Interspecific cpDNA variation in the eight *Lotus* species and *T. siliquosus* was analysed by comparing cpDNA fragment patterns produced by five restriction endonucleases and totalling 304 distinct fragments. Genetic differentiation in cpDNA was very high between the *L. corniculatus* group and *L. hispidus* on the one hand, and the three other species on the other hand. Sixteen restriction-site mutations and eight length polymorphisms were identified among the five species of the *L. corniculatus* group and *L. hispidus*, *Lotus uliginosus*, *L. alpinus* and *L. japonicus* showed at least six DNA changes with regard to the molecule of *L. corniculatus*. Accordingly, these species should be excluded as maternal progenitors of *L. corniculatus*. Conversely, the cpDNA of *L. tenuis* differed from that of *L. corniculatus* by only two small-length mutations. As also suggested previously from an analysis of several nuclear markers, the results reported here show decisively that *L. tenuis* may be considered as the most probable maternal ancestor of *L. corniculatus*.

Key words *Lotus corniculatus* · *Fabaceae* · cpDNA variation · Maternal inheritance · RFLP

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P. Gauthier (✉) · R. Lumaret
Centre Louis Emberger, CEFE/CNRS, F-34293, Montpellier Cedex 05, France

P. Gauthier · A. Bedecarrats
CEMAGREF, Groupement de Grenoble, B.P. 76, F-38402 Saint Martin d'Héres Cedex, France

Introduction

In the genus *Lotus* (*Fabaceae*) the *L. corniculatus* group, with a basic chromosome number equal to six, represents a polyploid complex comprising ten species, most of them diploid. One taxon, *L. corniculatus* L. (Birdsfoot trefoil), is particularly abundant occurring from Europe to Asia and North Africa. Cultivars of this taxon have been widely used as a forage legume, mostly in cool wet climate conditions. Several conflicting theories have been proposed to explain the phylogenetic origin of the widespread natural tetraploid ($2n = 4x = 24$) *L. corniculatus* L. Most of the arguments supporting the various hypotheses have been reviewed in detail by Campos et al. (1994), Grant (1995) and Grant and Small (1996). Diploid individuals ($2n = 2x = 12$) of four species in the *L. corniculatus* group, namely *L. alpinus* (D.C.) Schleich., *L. tenuis* Waldst et Kit, *L. japonicus* (Regel) Larsen and *L. uliginosus* Schkuhr, have been suggested as the most putative progenitors of tetraploid *L. corniculatus*. Several authors have proposed that *L. corniculatus* is an autotetraploid of either *L. tenuis* (e.g. Dawson 1941) or *L. alpinus* (Larsen 1954; Blaise et al. 1991). However, the synthetic autopolyploids of both these species (Somaroo and Grant 1972) and the natural tetraploids observed in *L. alpinus* (Grant 1995 and the present paper) do not resemble *L. corniculatus* morphologically. Stebbins (1950) proposed that *L. corniculatus* is a segmental allotetraploid, and results from further studies (Somaroo and Grant 1972; Ross and Jones 1985) suggest that *L. corniculatus* may

be of allotetraploid origin. Somaroo and Grant (1972) proposed that *L. japonicus* and *L. alpinus* could be the ancestral species whereas Ross and Jones (1985) suggested that *L. alpinus* or *L. tenuis* could be the maternal parent, and *L. uliginosus* the pollen parent, of *L. corniculatus*. More recently, isozyme data (Realson and Grant 1988) and total DNA using RAPD markers (Campos et al. 1994) in several accessions of the four putative diploid ancestor species and the tetraploid *L. corniculatus*, suggest that the diploid *L. uliginosus* is not an ancestor of *L. corniculatus*. From an extensive review based on morphological, geographic, genetic and biochemical data, Grant and Small (1996) confirmed that *L. corniculatus* may be of allotetraploid origin. In addition, based on evidence from the maternal inheritance of flower colour intensity and interactions of rhizobium strains, these authors concluded that *L. tenuis* could have been the female parent of *L. corniculatus*.

Cytosolic isozymes and RAPD markers are mostly dependent on nuclear genes. They are inherited biparently and are subject to recombination. Using these markers, identification of the diploid ancestor(s) of *L. corniculatus* may be difficult because several complex genetic modifications may have occurred subsequent to the polyploidization event. In contrast, chloroplast DNA (cpDNA) is a highly conserved cytoplasmic molecule which is clonal (without recombination). Restriction-site analysis of cpDNA is thus a powerful tool in plant systematics for phylogenetic reconstruction at both inter- and intra-specific levels (Palmer 1987). It has been used successfully to document the parentage of allopolyploids (e.g. Erickson et al. 1983; Song and Osborn 1992) and of autopolyploids (e.g. Lumaret et al. 1989). However, because cpDNA is maternally inherited in most angiosperms, the use of that marker is limited to identification of the maternal parentage.

In the present paper, we studied intra- and interspecific cpDNA variation using restriction fragment length polymorphism (RFLP) in the tetraploid *L. corniculatus* and in its four putative diploid relatives, *L. alpinus* (which was also analysed at the tetraploid level) *L. tenuis*, *L. japonicus* and *L. uliginosus*. In addition, three other *Lotus* species, namely *L. hispidus* Desf., *L. edulis* L. and *L. ornithopodoides L.*, and one species of *Tetragonolobus* (a genus close to *Lotus*), namely *T. maritimus* var. *siliquosus* (L.) Dominquez and Galiano, were also analysed for cpDNA variation and were considered as outgroup species. The objectives of the study were: (1) to check for the mode of inheritance of cpDNA in *Lotus* using controlled crosses between plants showing distinct chlorotypes; (2) to assess cpDNA variation in *Lotus*, more particularly among the five species of the *L. corniculatus* group; and (3) to use information from cpDNA in an attempt to resolve the maternal diploid parentage of tetraploid *L. corniculatus*.

Materials and methods

Origin of plant material

Chloroplast DNA analyses were carried out on 101 individual plants from 19 natural populations of eight *Lotus* species and of *Tetragonolobus maritimus* var. *siliquosus* (see Table 1). Because they may be of artificial origin, cultivars were not included in the sampling design. The three French populations of *L. corniculatus* were located several hundred kilometers apart. Species identification was according to the Flora Europea (Tutin and Webb 1976). For *L. alpinus*, *L. japonicus* and *L. hispidus* seeds were collected in natural sites and were grown in uniform conditions in a greenhouse for 2 months prior to analysis. The plants were maintained under short day, non-flowering conditions, with natural day light. For *L. corniculatus* from Orsay (near Paris) and Oulches (central France), *L. tenuis* from Orsay, and *L. uliginosus* from the forest of Grimbosq (Normandy), fresh leaves were obtained from adult plants of the collection at the Paris XI University. For *L. corniculatus* from St-Martial (Cévennes, southern France), *L. tenuis* from Cortona (center of Italy), *L. uliginosus* from La Londes (Provence, southern France), *L. ornithopodoides*, *L. edulis* and *T. siliquosus*, adult plants were collected directly and grown in the greenhouse for 1 month under the same conditions as the plants grown from seeds. In *L. alpinus*, chromosome counts were made in root-tip squashes using the Feulgen technique to determine the ploidy level.

Controlled crosses

Two natural tetraploid individuals of *L. alpinus* from "La Sassière" (French Alps) were crossed with three plants of a *L. corniculatus* cultivar which showed a very distinct cpDNA restriction pattern (haplotype) from that of the *L. alpinus* individuals. Plants were isolated in an insect-free greenhouse. Two reciprocal crosses were made, each plant being considered alternatively as male or female by using distinct floral stems. Flowers of the female parent were emasculated by removing the stamens with a forceps. Two days later, pollen of the male parent was applied to the stigmas of the emasculated flowers. Seeds were collected when pods became brown (between 30 and 40 days after pollination).

Preparation and restriction endonuclease analysis of cpDNA

The plants were placed in the dark for 36 h to de-starch the leaves before the leaf tissue was harvested. The leaves were ground in liquid nitrogen and freeze-dried. Chloroplasts were isolated from 1.2-g aliquots of the freeze-dried powder using a non-aqueous procedure as described by Michaud et al. (1995) and adapted to herbaceous material. A single modification was used here, namely that no PVP was added during the chloroplast re-suspension.

Aliquots of 25 μg of chloroplast DNA were incubated with the restriction endonucleases listed in Table 1, according to the recommendation of the suppliers (Appligene, Boehringer). The digestion products were fractionated by electrophoresis on horizontal 0.85 and 1.2% agarose-slab gels in order to separate, and be able to identify, a large range of fragment sizes. Out of six restriction enzymes five, namely *Bam*HI (Boehringer), *Ava*I (Appligene), *Hin*dIII (Appligene), *Dra*I (Boehringer) and *Eco*RI (Appligene), were six-cutter restriction enzymes and one, *Cfo*I (Appligene), was a four-cutter restriction enzyme. Gels were stained with ethidium bromide and photographed under UV light. Lambda DNA digested with *Hin*dIII and *Eco*RI and a 1-Kb Ladder DNA were used as size standards. Gels with 1.2% agarose were used more particularly for *Cfo*I, *Eco*RI and *Dra*I which produced many small fragments.

For each cpDNA restriction endonuclease pattern, DNA restriction fragment sizes were determined using "Bande" software (Duggleby et al. 1981).

Study of cpDNA variation in all the taxa

The cpDNA restriction endonuclease patterns of individual plants were scored for fragment-length differences. This method has been advocated when the cpDNA sequences have diverged to the extent that direct restriction-site mutation analysis can not be used (Sandbrinck et al. 1989). When intraspecific variation was observed, the most frequent pattern that occurred in the several geographical areas studied was considered as representative of the species. The different fragments obtained from the restriction patterns using several enzymes were scored as presence/absence data, and pooled to compute a similarity matrix using the method of Nei and Li (1979) adapted to fragment analysis instead of restriction site. The fraction of shared fragments was estimated by $F = 2Nxy/(Nx + Ny)$, where Nxy is the number of bands taxon x and taxon y have in common, and Nx and Ny are the total number of bands found for taxon x and y, respectively. The distance matrix (1-F values) was then analysed by the UPGMA method using the Kitsch option of PHYLIP 3.5 (Felsenstein 1993).

CpDNA variation in *L. corniculatus* and its putative ancestors, namely *L. tenuis*, *L. alpinus*, *L. japonicus* and *L. uliginosus*.

The distance matrix (1-F values) corresponding to the five species was analysed by the UPGMA method as described above. In addition, all the cpDNA changes were identified as either length or site mutations. The detection of specific changes, each revealed from individual plant by several restriction enzymes, suggests that alterations in the length of the fragments may be due to DNA length mutations rather than site mutations. By scoring those length mutations arbitrarily as the same mutations, we avoided counting the same addition/deletion several times and, therefore, of over-estimating the number of distinct mutations. However, this does not completely exclude the possibility that they may represent independent mutations. The observed cpDNA changes were scored as presence/absence data and analysed cladistically by enumeration of all the most-parsimonious unrooted trees using the Wagner parsimony method (MIX option of PHYLIP 3.5, Felsenstein 1993). Moreover, the SEQBOOT option of PHYLIP was used to place bootstrap-based confidence limits on branching points in the parsimony tree (Felsenstein 1993). Finally, considering *L. hispidus* as outgroup species because its cpDNA was close to that of the five species of the *L. corniculatus* group, these DNA changes were also analysed cladistically by enumeration of all parsimonious rooted trees using the Dollo parsimony method (DOLLOP option of PHYLIP 3.5, Felsenstein 1993). The SEQBOOT option of PHYLIP was also used with DOLLOP to place confidence limits on branching points in the parsimony tree (Felsenstein 1993).

Results

Inheritance of cpDNA

The chlorotypes of the five progenies of the two reciprocal crosses and of the cross involving a male-sterile plant were identified. Between 30 and 50 individuals per progeny were analysed. Identification of the chlorotype was based on at least three restriction enzymes (*Ava*I, *Dra*I and *Cfo*I) which very clearly discriminated both chlorotypes. All the individuals of the five progenies showed the maternal chlorotype indicating a maternal inheritance in *L. corniculatus* and *L. alpinus*.

Chloroplast-DNA variation in all the taxa studied

Several data were lacking for cpDNA analysis using *Eco*RI. Therefore, the results obtained with that enzyme were not taken into account for all the taxa.

When cpDNA from the 101 individuals listed in Table 1 was analysed by digestion with the five other restriction endonucleases, 35 different banding patterns were observed giving a total of 304 different fragments of which 206 (68%) varied among the nine *Lotus* and *Tetragonolobus* species. When *L. edulis*, *L. ornithopodoides* and *T. siliquosus* were excluded, due to the high dissimilarity of their restriction patterns compared to the other species, the total number of fragments was reduced to 212 of which only 51 (24%) varied among the six *Lotus* species. Restriction banding patterns obtained for *Bam*HI, *Dra*I and *Cfo*I are illustrated in Figs. 1 and 2. In the nine taxa, the restriction endonucleases *Bam*HI, *Hin*dIII, *Ava*I, *Dra*I and *Cfo*I generated an average of 34.38, 30.75, 34.14, 38.63 and 47.13 fragments, respectively. In the five taxa of the *L. corniculatus* group, *Eco*RI generated an average of 39.3 fragments of which an only one double fragment was more than 5.0 kb. 24% of the individuals were analysed on at least two identical gels per enzyme and no variation was observed among replicate samples. Other than the single mutation found for one individual of *L. alpinus* no other polymorphism was detected for this species. The diploid and the tetraploid individuals of *L. alpinus* showed identical restriction patterns.

Chloroplast DNA molecular size was estimated by adding together the size of the fragments generated by each endonuclease, particularly those produced by *Ava*I, *Bam*HI and *Hin*dIII which provided fewer and larger-sized fragments. The molecular size of cpDNA was estimated to range between 132 and 133 kb for *L. corniculatus*, *L. tenuis*, *L. alpinus*, *L. japonicus*, *L. uliginosus* and *L. hispidus*. The cpDNA molecular size was estimated to be 135, 137 and 135 kb for *T. siliquosus*, *L. edulis* and *L. ornithopodoides*, respectively.

The proportion of fragments shared by all nine species was 47.1, 34.8, 43.1, 6.7 and 44.9% for *Bam*HI, *Hin*dIII, *Ava*I, *Dra*I and *Cfo*I respectively. When *T. siliquosus*, *L. edulis* and *L. ornithopodoides* were excluded the previous percentages increased to 69.1, 93.3, 75.0, 48.2 and 67.2. The percentage of shared fragments (F-values) calculated from all the restriction patterns obtained with the five restriction enzymes decreased from 97.6% between *L. corniculatus* and *L. tenuis* to 64.5% between *L. alpinus* or *L. japonicus* and *L. ornithopodiodes* (Table 2).

When the distance matrix obtained from the F-values was subject to the UPGMA analysis, three main

Table 1 Geographic origin and chromosome number of the 101 plants of eight *Lotus* species analysed; and of *T. maritimus* var. *siliquosus*; and the cases where RFLP analyses were performed (X) for six restriction enzymes

Species Geographic origin[a]	Collector[b]	2n	Sample size	Restriction enzymes					
				*Ava*I	*Bam*HI	*Cfo*I	*Dra*I	*Hind*III	*Eco*RI
L. corniculatus									
1 Orsay (F)	2	24	5	X	X	X	X	X	X
2 Oulches (F)	2	24	5	X	X	X	X	X	X
3 St-Martial (F)	1	24	5	X	X	X	X	X	—[c]
L. tenuis									
4 *Orsay* (F)	2	12	5	X	X	X	X	X	X
5 Cortona (I)	1	12	5	X	X	X	X	X	—
L. alpinus									
6 Bozel (F)	1	12	6	X	X	X	X	X	X
7 La Sassière (F)	1	12	5	X	X	X	X	X	X
8 La Sassière (F)	1	24	5	X	X	X	X	X	X
9 Les Arcs (F)	1	24	5	X	X	X	X	X	—
10 Val d'Isère (F)	1	24	10	X	X	X	X	X	X
L. japonicus									
11 Fukui (J)	3	12	5	X	X	X	X	X	X
12 Nishinasumo (J)	3	12	5	X	X	X	X	X	X
13 Awaji (J)	4	12	4	X	X	X	X	X	X
L. uliginosus									
14 Grimbosq Forest (F)	2	12	5	X	X	X	X	X	X
15 La Londes (F)	1	12	6	X	X	X	X	X	—
L. hispidus									
16 Nuret le Ferron (F)	2	12	5	X	X	X	X	X	—
L. ornithopodoides									
17 La Londes (F)	1	14	5	X	X	X	X	X	—
L. edulis									
18 La Londes (F)	1	14	5	X	X	X	X	X	—
T. maritimus var. *siliquosus*									
19 Le Liganio (F)	1	14	5	X	X	X	X	X	X

[a] Origins: F = France, J = Japan, I = Italia,
[b] Collectors: 1 = Authors of the present paper. 2 = S. Blaise, A. Bazin, U. P. S. Paris Sud-91405-Orsay (France). 3 = M. Tajimi, T. Takamizo, N.G.R.I.-Nishinasumo (Japan). 4 = H. Sato.
[c] no data

groups of species could be distinguished (Fig. 3). The first cluster included *L. ornithopodoides* and *L. edulis*, the second one was made up of *T. siliquosus*, and the third one corresponded to the other *Lotus* species, namely *L. corniculatus*, *L. tenuis*, *L. alpinus*, *L. japonicus*, *L. uliginosus* and *L. hispidus*.

cpDNA restriction-site variation in *L. corniculatus* and its putative progenitors

When data from the second matrix (Table 2, bold type numbers) were subjected to a UPGMA analysis, *L. corniculatus* was observed to be very close to *L. tenuis* (Fig. 4 a). These two species were grouped with *L. uliginosus*. In comparison with these three species, *L. alpinus* and *L. japonicus* showed a higher number of changes in their cpDNA molecule.

In the four species of the *L. corniculatus* group, namely *L. tenuis*, *L. alpinus*, *L. japonicus* and *L. uliginosus*, as well as in *L. hispidus* which is phylogenetically close to that group, the mutations responsible for cpDNA variation as compared to the restriction pattern observed in *L. corniculatus*, could be identified and are listed in Table 3.

A total of sixteen site mutations and eight length mutations were obtained. For cpDNA changes D, E, F, G and H (10–30 bp), it could not unequivocally be determined whether the variability was due to site or length mutations since length variation could not be confirmed using the other endonucleases. Because the probability that a small length change is detected decreases when the fragment's size increases, changes D, E, F, G and H were considered as addition/deletions which are known to involve many small sized changes (Systma and Gottlieb 1986).

From the whole mutations obtained with the five endoncleases, six main haplotypes corresponding to the six species were obtained (Table 4). An additionnal haplotype due to a punctual intraspecific length mutation (50 pb addition) was observed in a single plant of *L. alpinus* out of 31 plants analysed. *Lotus tenuis* differed from *L. corniculatus* by two length mutations. Six mutations discriminated *L. japonicus* and *L. ulignosus* from *L. corniculatus*. Finally, nine and ten mutations opposed *L. alpinus* and *L. hispidus* respectively to

L. corniculatus. *Lotus japonicus* and *L. alpinus* shared four mutations (2, 11, G, H). *Lotus uliginosus* shared one mutation (13) with *L. alpinus*.

Analysis of presence/absence of the mutations observed in the five species of the *L. corniculatus* group (haplotypes I, II, III, IV, VI) with the Wagner Parsimony method resulted in the same general clustering pattern as obtained above, using UPGMA (Fig. 4b). Three most parsimonious trees were obtained which each required 19 steps to account for the 11 site and 7 length changes. 100 bootstraps cycles were performed. A consensus tree was obtained with confidence values being 100% and 86% for the two major branches.

The same general clustering pattern was also observed using the Dollo parsimony method and by using the same five haplotypes and that of *L. hispidus*, which was considered as an ancestral species (Fig. 5). The tree required 25 steps to account for eight length and 16 site changes. Confidence limits decreased from 100% to 93% for the three major branches.

Discussion

Results from the controlled crosses provided evidence for the maternal inheritance of cpDNA in

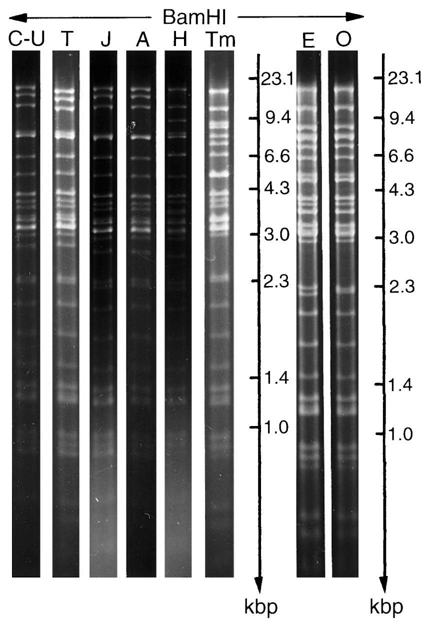

Fig. 1 Restriction fragment patterns obtained by digestion of cpDNA with *Bam*HI in the eight *Lotus* species, *L. corniculatus* (*C*), *L. tenuis* (*T*), *L. alpinus* (*A*), *L. japonicus* (*J*), *L. uliginosus* (*U*), *L. hispidus* (*H*), *L. edulis* (*E*), *L. ornithopodoides* (*O*) and in *T. maritimus* var. *siliquosus* (*Tm*). The agarose concentration in the original gels was 0.8%

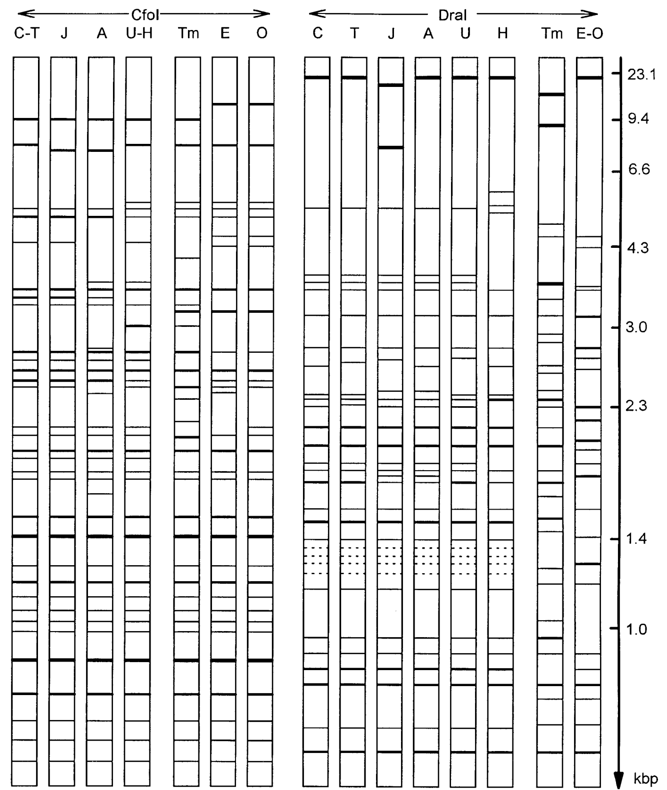

Fig. 2 Diagrammatic representation of the restriction fragment patterns obtained by digestion of cpDNA with *Dra*I and *Cfo*I in the eight *Lotus* species, *L. corniculatus* (*C*), *L. tenuis* (*T*), *L. alpinus* (*A*), *L. japonicus* (*J*), *L. uliginosus* (*U*), *L. hispidus* (*H*), *L. edulis* (*E*), *L. ornithopodoides* (*O*), and in *T. maritimus* var. *siliquosus* (*Tm*). High-intensity bands indicate the occurrence of two or three identical fragments. The agarose concentration of the original gels was 0.8%

Table 2 Chloroplast DNA data matrix for the eight *Lotus* species and for *T. maritimus* var. *siliquosus* from RFLPs using *Bam*HI, *Ava*I, *Hin*dIII, *Dra*I and *Cfo*I restriction enzymes, showing the proportion of shared fragments (F-values, %). Data for the five species of the *L. corniculatus* group are indicated in bold type

Species	1	2	3	4	5	6	7	8	9
1 *L. corniculatus*	**100**								
2 *L. tenuis*	**97.6**	**100**							
3 *L. alpinus*	**92.7**	**91.9**	**100**						
4 *L. japonicus*	**93.8**	**94.1**	**91.4**	**100**					
5 *L. uliginosus*	**95.9**	**95.1**	**91.4**	**91.4**	**100**				
6 *L. hispidus*	89.9	91.3	85.3	86.4	91.8	100			
7 *L. ornithopodoides*	66.3	66.5	64.5	64.5	67.0	65.7	100		
8 *L. edulis*	67.0	67.2	65.8	65.2	67.8	66.5	97.2	100	
9 *T. siliquosus*	69.7	69.9	66.3	67.9	69.9	69.2	72.8	71.9	100

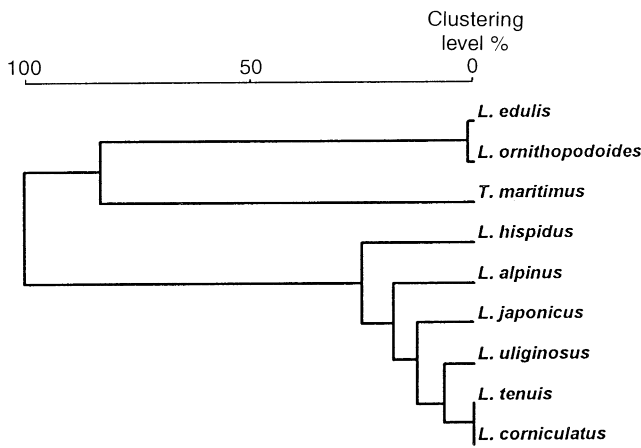

Fig. 3 UPGMA phenogram, for the eight *Lotus* species and *T. maritimus* var. *siliquosus*, based on the proportion of shared fragments scored for *Bam*HI, *Ava*I, *Hin*dIII, *Dra*I and *Cfo*I cpDNA RFLPs

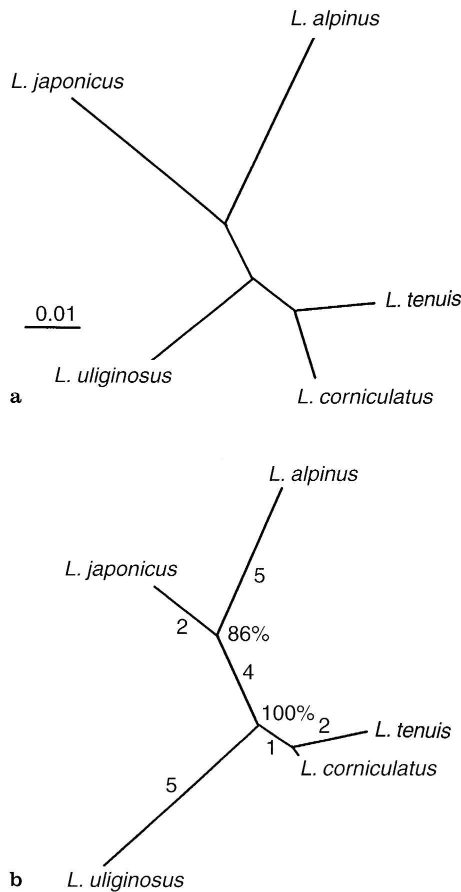

Fig. 4 UPGMA phenogram (**a**) and Consensus Wagner tree (**b**) for the five species of the *L. corniculatus* group (*L. corniculatus*, *L. tenuis*, *L. alpinus*, *L. japonicus* and *L. uliginosus*) scored for *Bam*HI, *Ava*I, *Hin*dIII, *Dra*I and *Cfo*I cpDNA RFLPs. *Numbers* on the branches indicate the minimum number of mutation steps. For each major branch, the percentage of times that the defined group occurred in 100 bootstrap samples is indicated

L. corniculatus and *L. alpinus*. This result is consistent with the prediction of Corriveau and Coleman (1988), who did not observe proplasmids in the pollen of *L. corniculatus*. Proplasmids have been localised in the pollen of others legumes such as *Medicago sativa* which is now well known to show a biparental cpDNA inheritance (Lee et al. 1988; Fitter et al. 1996). However, whereas Corriveau and Coleman (1988) did not find proplasmids in the pollen of *Lens culinaris* Medik (Fabaceae), Rajora and Mahon (1995) observed a biparental inheritance of cpDNA with maternal overdominance in this species.

Analysis of cpDNA variation in the nine taxa showed that *T. maritimus* var. *siliquosus*, *L. edulis* and *L. ornithopodoides* are clearly discriminated from the other *Lotus* species. *Lotus edulis* and *L. ornithopodoides* are both annuals, have the same basic chromosome number ($n = 7$), which is distinct from that of the other studied *Lotus* species ($n = 6$), and often co-exist in the same habitat. The present work illustrates that these two species also possess a very similar cpDNA molecule. Based on cpDNA variation, *L. hispidus*, which is annual, is located closely to the species of the *L. corniculatus* group, which are all perennials, and more particularly to *L. uliginosus*.

In the *L. corniculatus* group, a high consistency was obtained from the several data treatments which provided exactly the same general phyletic pattern. Among the four putative maternal ancestors of *L. corniculatus*, *L. uliginosus* seems to be the least likely. Such a result is also in agreement with previous conclusion based on nuclear markers, namely allozymes (Realson and Grant 1988) and RAPDs (Campos et al. 1994). However, because *L. uliginosus* and *L. corniculatus* shared several characters in common such as specific flavonoids and tannins, which were not found in any of the other diploid *Lotus* species, several authors considered that *L. uliginosus* which often grows in sympatry with *L. corniculatus* may have played a key role in the emergence of that species (Ross and Jones 1988; Grant and Small 1996). Our data do not support this claim. In the present study, we found that *L. alpinus* and *L. japonicus*, which differ from *L. corniculatus* by a large number of mutations, have a very low probability of being the maternal ancestor of that species. The similarity of cpDNA restriction patterns in diploid and tetraploid *L. alpinus* individuals which are also morphologically similar, except for organ size and flower number per stems, supports the occurrence of a direct, at least maternal, relationship between plants of the two ploidy levels as already suggested by Urbanska-Worytkiewicz and Schwank (1980). Further studies using nuclear markers are necessary to confirm the autopolyploid origin of the tetraploids in *L. alpinus*. From morphological data, these tetraploids were sometimes confounded with individuals of *L. corniculatus* (Urbanska-Worytkiewicz 1979). From morphological (Grant and Small 1996), isozymic (Realson and Grant

Table 3 Restriction fragment length changes (kb) as compared to the fragment of *L. corniculatus* and the type of mutation (site or length) observed from restriction patterns in the *L. corniculatus* group (*L. tenuis*, *L. japonicus*, *L. alpinus* and *L. uliginosus*) and *L. hispidus*. Indices with the same letter are changes attributable to the same mutation

Restriction enzyme	Mutation					
	Code	Site		Code	Length	
		Other species	*L. corniculatus*		Other species	*L. corniculatus*
*Bam*HI						
	1	9.40 + 4.00	→ 13.40	A	4.06	→ 4.01
	2	7.00 + 0.30	→ 7.30	B	2.97	→ 2.93
	3	6.00	→ 5.80 + X[a]	C	2.89	→ 2.93
	4	2.83 + X[a]	→ 2.93			
*Hin*dIII						
				C	6.21	→ 6.25
*Ava*I						
	5	5.47 + 4.57	→ 10.04	C	4.69	→ 4.73
	6	4.96	→ 4.73 + X[a]	A	3.87	→ 3.82
	7	3.30 + X[a]	→ 3.50			
*Dra*I						
	8	14.90 + 7.10	→ 22.00	D	2.62	→ 2.59
	9	5.90	→ 3.90 + 2.00	E	2.61	→ 2.59
	10	5.50	→ 3.71 + 1.79	F	2.60	→ 2.59
				G	2.40	→ 2.38
				A	2.35	→ 2.30
				B	2.34	→ 2.30
				H	1.80	→ 1.79
*Cfo*I						
	11	2x(7.20 + X[a]	→ 7.40)	C	2.50	→ 2.54
	12	5.40	→ 5.10 + 0.30			
	13	3.70	→ 3.50 + X[a]			
	14	3.10 + 0.40	→ 3.50			
	15	3.10	→ 2.60 + 0.50			
	16	1.65 + 2.85	→ 4.50			

[a] X: fragment not visualized because of size

Table 4 Presence (X) of mutations as compared to *L. corniculatus* in the four species of the *L. corniculatus* group (*L. tenuis*, *L. japonicus*, *L. alpinus* and *L. uliginosus*) and in *L. hispidus*

Species	Haplotype	Ploidy level	Mutations																							
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	A	B	C	D	E	F	G	H
L. tenuis	II	2x																		X				X		
L. japonicus	III	2x		X						X			X										X		X	X
L. alpinus	IV	2x/4x		X			X		X				X		X			X			X				X	X
L. alpinus	V	2x		X			X		X				X		X			X							X	X
L. uliginosus	VI	2x						X						X	X	X	X					X				
L. hispidus	VII	2x	X		X	X					X	X		X	X	X	X		X							

1988) and molecular (Campos et al. 1994) markers, *L. alpinus* was often considered as one of the nearest species to *L. corniculatus*, *L. alpinus* may be either the diploid paternal ancestor of *L. corniculatus* or may have introgressed genetically into this species by pollen flow from the tetraploid populations.

The combined results of the several phylogenetic analyses suggest that *L. tenuis*, which differed by only two length mutations from *L. corniculatus*, is the most likely putative maternal ancestor of the latter species. Based on a large range of characters including several which were maternaly inherited, *L. tenuis* was already considered as the most probable maternal ancestor of *L. corniculatus* by Grant and Small (1996). The results of the present study further support this claim. Individual plants of *L. tenuis* have been shown to produce 2n gametes and to generate tetraploid individuals when they were crossed with *L. corniculatus* (Negri and Veronesi 1989). Sexual polyploidisation is likely to be the origin of *L. corniculatus*, as is the case for many other polyploid species (Bretagnolle and Thompson 1995). However, complex multiple scenarios, involving

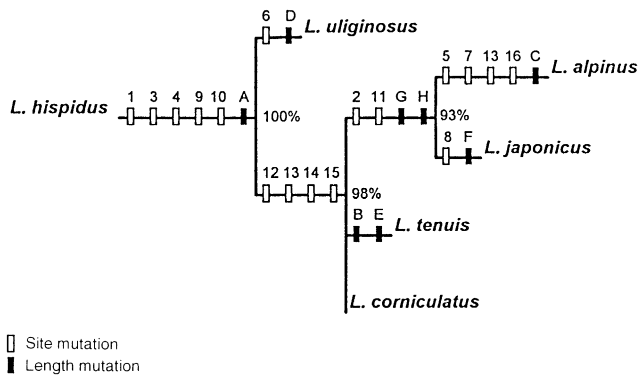

Fig. 5 Consensus tree obtained from the Dollo parsimony method and bootstrap-based confidence limits for the five species of the *L. corniculatus* group (*L. corniculatus*, *L. tenuis*, *L. alpinus*, *L. japonicus* and *L. uliginosus*), using *L. hispidus* as an outgroup species. The mutations are identified on the branches of the tree. For each major branch, the percentage of times that the defined group occurred in 100 bootstrap samples is indicated

either allopolyploidy between individuals of distinct diploid species or multiple autopolyploidy occuring in several diploid species followed by multi/interspecific crosses at the polyploid level, may account for the present complex situation observed in that species. Such hybridisation among tetraploids may be a major component of the widespread distribution of polyploid *L. corniculatus* (Stebbins 1985). The present study provides a further illustration of how analysis of cpDNA variation represents a powerful tool to document the parentage of naturally occurring polyploids.

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