Genetic variation and gene flow in Alpine diploid and tetraploid populations of *Lotus* (*L. alpinus* (D.C.) Schleicher/*L. corniculatus* L.). II. Insights from RFLP of chloroplast DNA

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RFLP of chloroplast DNA was studied in two diploid (2x) and five tetraploid (4x) populations of *Lotus alpinus* (D.C.) Schleicher in the French Alps. This was compared with four non-Alpine populations and two cultivars of *Lotus corniculatus* L. (4x) introduced to the Alpine valleys. Based on the 13 site and 11 length mutations detected, seven distinct haplotypes could be identified, which clustered in two groups, corresponding to the two *Lotus* species. With only one exception, the diploid and the tetraploid *L. alpinus* plants had the same haplotype. This is to be expected, because morphological variation and tetrasomic inheritance suggest that the tetraploids were derived from the diploids by autopolyploidy. In *L. corniculatus*, two very similar haplotypes were found in the natural very distant populations. In contrast, the chloroplast DNA (cpDNA) haplotypes of the two cultivars differed considerably from each other and were more related to those observed in *L. alpinus*, suggesting that these cultivars may be derived maternally from *L. alpinus* individuals. In one tetraploid *L. alpinus* population, a single cpDNA haplotype was identified. As this haplotype was related to those observed in the *L. corniculatus* group, local genetic introgression may have occurred between 4x *L. alpinus* plants and cultivated *L. corniculatus*.

Keywords: chloroplast DNA restriction fragment length polymorphisms, genetic introgression, *Lotus alpinus/corniculatus*, polyploid complex.

Introduction

In the Alps, diploid and tetraploid *Lotus* plants belonging to the *L. corniculatus* L. group have been reported to grow at high and intermediate elevation respectively (Favarger, 1969). This paper is the second of a pair presenting results of detailed genetic studies that have been carried out in Alpine *Lotus* populations to clarify the identity and origin of the Alpine tetraploid *Lotus* and to assess more precisely the genetic relationships occurring between plants of the two cytotypes.

In the first paper (Gauthier *et al.*, 1998), evidence for the autotetraploid origin of the tetraploid cytotype was obtained from tetrasomic inheritance at two allozyme loci in the tetraploids and from a

comparison of morphological and allozyme variation between plant populations of the two cytotypes. The tetraploids were therefore identified as *L. alpinus*. However, a substantial proportion of individuals from one of the tetraploid populations exhibited morphological characters typical of *L. corniculatus* cultivars, suggesting that hybridization and introgression may have occurred between tetraploid *L. alpinus* and *L. corniculatus*. Unfortunately, the lack of diagnostic alleles between the two species prevented allozymes being used to support this hypothesis (Realson & Grant, 1988; Gauthier *et al.*, 1998). A preliminary study of chloroplast DNA (cpDNA) variation in several *Lotus* species of the *L. corniculatus* group (Gauthier *et al.*, 1997) showed that cpDNA may be suitable for clarifying genetic relationships between *Lotus* taxa in the Alps. Restriction site analysis of cpDNA is a powerful tool

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in plant systematics for phylogenetic reconstruction at both inter- and intraspecific levels (Palmer, 1987).

CpDNA is a highly conserved cytoplasmic molecule, which is clonal (without recombination) and has been shown to be maternally inherited in the *L. corniculatus* group (Gauthier *et al.*, 1997). It has been used successfully to document the parentage of allopolyploids (e.g. Song & Osborn, 1992) and of autopolyploids (e.g. Lumaret *et al.*, 1989), to clarify patterns of hybridization and introgression between wild intra- or interspecific taxa (e.g. Palmer *et al.*, 1988; Rieseberg & Soltis, 1991) or between native and recently introduced species or subspecies (e.g. Lowe & Abbott, 1996) and also for contrasting gene flow through pollen and seeds (Ennos, 1994; McCauley, 1995).

In the present paper, intra- and interpopulation cpDNA variation were studied using RFLP in diploid and tetraploid *Lotus* populations from the French Alps and, for comparison, distant natural *L. corniculatus* populations from non-Alpine regions and several cultivars that have been introduced as a crop to Alpine valleys. CpDNA RFLPs are used as complementary markers to allozymes studied in the first paper to provide further evidence concerning: (i) the possible autopolyploid origin of the Alpine tetraploid plant material from diploid *L. alpinus*; and (ii) the occurrence of genetic introgression between tetraploid Alpine *Lotus* plants and introduced *L. corniculatus* cultivars.

Materials and methods

Plant material

Chloroplast DNA analysis was carried out on 75 individual plants from 13 distinct geographical origins (Table 1). Individual plants were sampled along two altitudinal transects, at 'La Sassièrè' and 'Mount Jovet', located 22 km apart in the Tarentaise valley in the French Alps (Savoy). For both transects, sampling was made in diploid populations (1 and 2) and in tetraploid populations (3 and 4) situated at the highest (2500–2700 m) and lowest (1800 m) elevations respectively. Plants of these four populations were also sampled for morphological and allozyme studies, the results of which are presented in the accompanying paper (Gauthier *et al.*, 1998) along with habitat and climate characteristics along the transects. Additional tetraploid individuals were sampled at Mount Jovet at an intermediate elevation (population 7). Plant material was also collected from two additional tetraploid populations from the French Alps (Val d'Isère, 5, and Les Arcs, 8). Plants of populations 1–5 and 7 and 8 were identified as *L. alpinus*. However, as reported previously (Gauthier *et al.*, 1998), population 4 also included plants that were morphologically intermediate between *L. alpinus* and *L. corniculatus* cultivars. Of the eight individuals analysed for cpDNA in population 4, six showed the *L. alpinus* morphological type and two had an intermediate

Table 1 Geographical source and ploidy level of the *Lotus* plants analysed for cpDNA RFLPs

Species	Source	No.	Altitude (m)	2n	Sample size
<i>Lotus alpinus</i>	La Sassièrè	1	2600	12	5
	Mt Jovet	2	2500	12	6
	La Sassièrè	3	1800	24	8
	Mt Jovet	4	1800	24	8
	Val d'Isère	5	1800	24	10
	Mt Jovet	7	2400	24	3
	Les Arcs	8	2000	24	5
<i>Lotus corniculatus</i>	Cultivars				
	San Gabriel	6	—	24	5
	NZ1	9	—	24	5
	Natural populations				
	Neuchâtel†	10	1800	24	5
	Orsay‡	11	150	24	5
	Oulche‡	12	100	24	5
	St Martial	13	500	24	5

†Material collected by F. Bretagnolle (Neuchâtel, Switzerland).

‡Material provided by S. Blaise from the University of Paris XI collection (Orsay, France).

morphotype. In addition, cpDNA variation was analysed using *L. corniculatus* individuals from cultivars San Gabriel (6) produced in Italy and NZ1 (9) produced in New Zealand (probably from European material), which were both known to have been introduced in the Alpine valleys, and from four natural populations, from Neuchâtel, Orsay, Oulches and St Martial (10–13). These localities were situated outside the Alps, in the Swiss Jura and in the north, centre and south of France respectively (Table 1).

For populations 1–7, seeds were grown under uniform conditions in a greenhouse at the CEFÉ/CNRS experimental garden in Montpellier for two months before analysis. Plants were maintained under short day, nonflowering conditions with natural daylight. For the other populations (8–13), adult plants were collected *in situ* and grown in the greenhouse for 1 month under the same conditions as the plants from seeds.

CpDNA analysis

Plants were placed in the dark for 36 h to destarch the leaves before leaf tissue was harvested. Leaves were ground in liquid nitrogen and freeze dried. Chloroplasts were isolated from aliquots of 1.2 g of the freeze-dried powder using a nonaqueous procedure, as described by Michaud *et al.* (1995) and adapted to herbaceous material (Gauthier *et al.*, 1997).

Of the five restriction enzymes, four, namely *Bam*HI (Boehringer), *Ava*I (Appligene), *Hind*III (Appligene) and *Dra*I (Boehringer), were six base cutters and one, *Cfo*I (Appligene), was a four base cutter. Gels were stained with ethidium bromide and photographed under UV light. Lambda DNA digested with *Hind*III, *Eco*RI and 1 kb ladder DNA were used as size standards. Because *Cfo*I and *Dra*I produced many small fragments, gels with 1.2% agarose were used preferentially. For each cpDNA restriction endonuclease pattern, DNA restriction fragment sizes were determined using BANDE software (Duggleby *et al.*, 1981).

CpDNA changes were identified as either length or site mutations. The observed cpDNA changes were scored for presence/absence and pooled to compute a similarity matrix (*F*-values) as indicated by Nei & Li (1979). The distance matrix (1–*F*-values) was then analysed by the UPGMA method using the Kitsch option of PHYLIP 3.5 (Felsenstein, 1993).

In addition, the several length and site mutations, again scored as presence/absence, were analysed

cladistically by enumeration of the most parsimonious unrooted trees using the Wagner parsimony method (MIX option of PHYLIP 3.5; Felsenstein, 1993). The SEQBOOT option of PHYLIP was used to place bootstrap-based confidence limits on branching points in the parsimony tree (Felsenstein, 1993).

Results

CpDNA RFLPs

When cpDNA from the 75 individuals listed in Table 1 was analysed by digestion with the five restriction endonucleases, 22 different banding patterns were observed, giving a total of 213 different fragments, of which 53 (25%) varied with source. Overall, the restriction endonucleases *Bam*HI, *Hind*III, *Ava*I, *Dra*I and *Cfo*I generated means of 33.86, 29.00, 34.57, 39.14 and 48.57 fragments, respectively. Twenty-four percent of the individuals were analysed on at least two identical gels per enzyme, and no variation was observed between replicate samples. Apart from a single mutation observed in one individual from population 2, no intrapopulation polymorphism was detected.

Chloroplast 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 *Hind*III, because they provided fewer and larger sized fragments. As before (Gauthier *et al.*, 1997), the molecular size was estimated to range between 132 and 133 kb for both *L. alpinus* and *L. corniculatus*.

The proportion of fragments shared by material from all sources was 83.78%, 93.33%, 64.29%, 65.96% and 75.44% for *Bam*HI, *Hind*III, *Ava*I, *Dra*I and *Cfo*I, respectively. The percentage of shared fragments calculated from all the restriction patterns obtained with the five restriction enzymes decreased from 96.3% between *L. alpinus* and cultivar 6 to 90.9% between population 4 and the same cultivar.

The 'mutations' responsible for cpDNA variation were identified by comparison with *L. alpinus*. They are listed in Table 2. A total of 13 site mutations and 11 length mutations were obtained. For cpDNA changes B to K, it could not be determined unequivocally whether the variability was caused by site or length mutations, because length variation could not be confirmed using the other endonucleases. Because the probability that a small length change will be detected decreases as the fragment size increases, the changes B to K were interpreted as addition/deletions, which are known to be

involved in many small changes (Systma & Gottlieb, 1986).

From the whole set of mutations observed with the five endonucleases, six main haplotypes (I, III, IV, V, VI and VII) were identified (Table 3). An additional haplotype (II) was caused by the two intrapopulation length mutations observed in a single plant of population 2. With the exception of

plants from tetraploid population 4, all individuals from diploid and tetraploid populations (1–3 and 5, 7 and 8) located in the French Alps showed identical restriction patterns for all the enzymes studied. These patterns were, therefore, considered to constitute the typical '*L. alpinus*' haplotype. Eleven and 12 mutations distinguished natural *L. corniculatus* populations 10 and 11, and 12 and 13, respectively,

Table 2 Restriction fragment length changes (kb) compared with the fragments of *Lotus alpinus* (2x) and type of mutation (site or length) observed from restriction patterns in several *Lotus* populations and distinct cultivars of *L. corniculatus*

Restriction enzyme	Mutation no.	Site		Mutation	Length	
		<i>L. alpinus</i>	Other species		<i>L. alpinus</i>	Other species
<i>Bam</i> HI	1	7.00 + 0.30 → 7.30		A	2.89 → 2.93	
	2	5.90 → 3.10 + 2.80				
<i>Hind</i> III				A	6.21 → 6.25	
<i>Ava</i> I	3	10.04 → 7.72 + 2.32		B	4.82 → 4.92	
	4	8.9 + 1.14 → 10.04		C	4.82 → 4.75	
	5	5.47 + 4.57 → 10.04		D	4.67 → 4.75	
	6	3.30 + X → 3.50				
<i>Dra</i> I	7	3.90 + 2.00 → 5.90		E	2.59 → 2.63	
	8	3.80 → 3.20 + 0.60		F	2.59 → 2.60	
				G	2.40 → 2.38	
				H	2.30 → 2.35	
				I	1.80 → 1.79	
				J	1.60 → 1.66	
<i>Cfo</i> I	9	(7.20 + X) × 2 → (7.40) × 2		K	2.50 → 2.54	
	10	(7.20) × 2 → (6.90 + 0.30) × 2				
	11	3.70 → 3.50 + X				
	12	3.70 → 3.20 + 0.50				
	13	1.65 + 2.85 → 4.50				

X, not visualized because of size.

Table 3 Presence (X) of mutations compared with the most common haplotype observed in *L. alpinus* in natural populations of *L. alpinus* and *L. corniculatus*, and in *L. corniculatus* cultivars

Populations	Haplotype	Ploidy level	Mutations																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	A	B	C	D	E
1, 2, 3, 5, 7, 8	I	2x/4x																		
2	II	2x														X	X			
11, 12, 13	III	4x	X				X	X			X	X		X	X	X	X		X	X
10	IV	4x	X				X	X			X	X		X	X	X	X		X	X
4	V	4x					X	X	X	X		X		X		X	X	X		X
9	VI	4x					X												X	X
6	VII	4x		X	X														X	X

from *L. alpinus*. Moreover, in *L. corniculatus*, population 10 differed from the three other natural populations (11, 12 and 13) by only three length mutations. Chloroplast DNA in population four showed the highest number of changes (13) compared with the cpDNA molecule of *L. alpinus*. Finally, only five and six mutations distinguished the *L. corniculatus* cpDNA molecule of cultivars 6 and 9, respectively, from that of *L. alpinus*.

When the distance matrix obtained from the *F*-values on the whole set of mutations (site and length) was subject to the UPGMA analysis, two groups of haplotypes could be distinguished (Fig. 1). The first cluster included the two haplotypes (I and II) observed in *L. alpinus* and the two haplotypes (VI and VII) of the cultivars, and the second one was made up of the two haplotypes (III and IV) observed in natural *L. corniculatus* and the haplotype (V) observed in the population from Mount Jovet.

Analysis of presence/absence of the mutations observed in all plants (haplotypes I, III, IV, V, VI and VII) with the Wagner parsimony was also applied exclusively to site changes. Three most parsimonious trees were found, which each required 13 steps to account for the 13 site changes (ignoring reversion). A consensus tree was obtained with confidence values of 100% and 93% for the two major branches (Fig. 2). The general clustering pattern was exactly the same as that obtained for the UPGMA analysis.

Discussion

Chloroplast DNA variation

In plants from the eight *L. alpinus* and the six *L. corniculatus* geographical sources, substantial cpDNA variation was observed compared with other plant species or genera analysed previously (Palmer

et al., 1988; Harris & Ingram, 1991). Within-population cpDNA variation was very low, only one population having more than one cpDNA haplotype. Use of larger sample sizes per population might increase the detection of rare mutations, but this would not substantially modify the whole cpDNA variation in each of the *Lotus* species. Within species, haplotypes differed from one another by at least two and, most often, by a large number of site and length mutations. This constitutes the most striking result of the present study. High interhaplotype differentiation may not reflect long periods of isolation between *Lotus* populations, because, with few exceptions (discussed below), within each species even geographically distinct populations had the same haplotype (e.g. populations 11, 12 and 13). These differences between haplotypes could in fact be caused by others factors, such as genetic disturbance, related to the multiple origins of the cultivars and/or to their putative genetic exchanges with various local *Lotus* material. High levels of cpDNA variation in inter- and intraspecific hybrid zones have already been reported in several taxa (e.g. Lumaret *et al.*, 1989; Harris & Ingram, 1991; Soltis & Soltis, 1993).

Origin of the Alpine tetraploid natural populations

In a previous study based on morphology, tetrasomic inheritance at allozyme loci and allele frequency variation, it has been suggested that the tetraploid Alpine populations are likely to be derived by autopolyploidy from diploid *L. alpinus* (Gauthier *et al.*, 1998). This conclusion is also clearly supported by the results obtained in the present work. Except in population 4 (see below), all the plants from the tetraploid populations identified morphologically as *L. alpinus* had the same cpDNA haplotype as diploid *L. alpinus*. In addition, this haplotype differed substantially from haplotypes observed in

Fig. 1 UPGMA phenogram based on the proportion of shared site and length mutations for the 13 *Lotus* populations/cultivars scored for *Bam*HI, *Ava*I, *Hind*III, *Dra*I and *Cfo*I cpDNA RFLPs. Species identified according to morphology are also indicated.

plants identified as *L. corniculatus*. In a previous study of cpDNA variation in several diploid and tetraploid species of the *L. corniculatus* group, Gauthier *et al.* (1997) suggested that the diploid species, *L. tenuis* L., which grows at lower elevation than *L. alpinus*, may be considered as the most probable maternal ancestor of tetraploid *L. corniculatus*. However, from morphological (Grant & Small, 1996), isozymic (Realson & Grant, 1988) and molecular (Campos *et al.*, 1994) evidence, *L. alpinus* was often considered as one of the nearest species to *L. corniculatus*. The possibility that *L. alpinus* may be the diploid paternal ancestor of *L. corniculatus* cannot be ruled out. In *L. alpinus*, autopolyploidization is likely to have occurred through crosses between diploid plants producing $2n$ gametes, as described in several other legume species, including red clover (Parrott *et al.*, 1985) and alfalfa (Veronesi *et al.*, 1986). In addition, Negri & Veronesi (1989) observed a substantial proportion of $2n$ gametes in several diploid plants of *L. tenuis*, which, like *L. alpinus*, belongs to the *L. corniculatus* group.

In *L. corniculatus*, the situation is complex, because natural populations possess cpDNA haplo-

types that are either identical (11, 12 and 13) or very similar (10) to one another, whereas the two cultivars (6 and 9) had haplotypes differing by five mutations from each other and were both closer to the haplotypes observed in *L. alpinus* than to those of the *L. corniculatus* populations. The possibility that these two cultivars, which appear to be well adapted to Alpine conditions, may be related maternally to *L. alpinus* ancestors cannot be ruled out completely.

Evidence for genetic introgression between tetraploid L. alpinus and L. corniculatus

In the present study, the eight plants analysed from the Alpine tetraploid population 4, located at 1800 m, had the same cpDNA haplotype (V) whatever their morphotype, so placing them with the haplotypes observed in *L. corniculatus* populations. Population 4 was predominantly composed of plants morphologically similar to *L. alpinus* with approximately 25% showing combinations of morphological characters similar or close to those observed in *L. corniculatus* cultivars (Gauthier *et al.*, 1998). In contrast, along the same transect but at a higher

Fig. 2 Wagner consensus tree from haplotypes observed in the seven diploid (1, 2, 3, 5, 7, 8) and tetraploid *L. alpinus* populations, the four natural *L. corniculatus* populations and the two *L. corniculatus* cultivars scored for *Bam*HI, *Ava*I, *Hind*III, *Dra*I and *Cfo*I cpDNA RFLPs. The numbers on branches indicate the minimum number of mutation steps. For each major branch, the percentage of time that the defined group occurred in 100 bootstrap samples is indicated. Species identified according to morphology are also shown.

elevation (2400 m), tetraploid *L. alpinus* plants possessed the same cpDNA haplotype as that observed in the diploid individuals of the same species. The morphological and cytoplasmic molecular results together suggest that the plants in population 4 were derived from local hybridization and subsequent introgression between tetraploid *L. alpinus* and *L. corniculatus*. The two taxa show substantial overlap in flowering periods at this elevation (P. Gauthier and R. Lumaret, unpublished data).

Because the cpDNA haplotype observed in population 4 was distinct from haplotypes found in *L. corniculatus* natural populations 10–13, the *L. corniculatus* material in population 4 is likely to be one of the several cultivars that were introduced as a forage crop in the studied region (usually below 1800 m) on several occasions during the last few decades. The identity of most of these cultivars is not known by local farmers. Although the sample size was low in population 4, the occurrence of a *L. corniculatus* cultivar haplotype in all the individuals analysed, despite the morphological resemblance to *L. alpinus*, suggests that, during the initial hybridization and subsequent back-crosses, the introduced plants were the female parents, whereas the indigenous plants, which were initially more numerous, were probably the male parents. This interpretation is further justified by the fact that the *L. corniculatus* cultivars produce more seed than the tetraploid *L. alpinus* individuals (P. Gauthier, unpubl. data). Consequently, introgression is found to be more important (and could even be restricted to a single direction) from *L. corniculatus* into *L. alpinus*, when measured with a maternally inherited cytoplasmic marker such as cpDNA in *Lotus* (Gauthier *et al.*, 1997), and to be predominantly in the reverse direction, when measured using nuclear markers such as those responsible for many morphological traits. At 1800 m, environmental conditions are very restrictive (because of low temperatures) for *L. corniculatus* plants, so that the unbalanced gene flow described above may be emphasized further, because cpDNA is likely to be a neutral marker, whereas morphological variation may be subject to natural selection under such conditions. A similar scenario has been observed in *Dactylis glomerata* L. in Galicia, where native populations have introgressed genes from introduced cultivars (Lumaret *et al.*, 1989). In the French Alps, environmental isolation may have prevented hybridization of *L. alpinus* by *L. corniculatus* until the recent introduction of *L. corniculatus* cultivars, which are probably derived from various species of the *L. corniculatus* group and thus

enlarge, although artificially, the genetic base of *L. corniculatus*.

The results of the present study are consistent with those based on morphological, phenological and allozyme variation on the same plant material (Gauthier *et al.*, 1998). Tetraploid *Lotus* populations, which grow from 1800 m to 2200 m, are probably derived from the diploid *L. alpinus* individuals found above 2200 m. In *L. alpinus*, diploid and tetraploid populations, which occupy distinct elevation ranges and are subjected to distinct habitat conditions, show narrow contact areas where both cytotypes are observed. However, evidence obtained from allozyme variation suggests that gene flow across these contact zones may be very limited. Results from the study of morphological and cpDNA variation in one of the Alpine populations of *Lotus* revealed that, at intermediate elevation, hybridization may occur between tetraploid *L. alpinus* and introduced *L. corniculatus*. Results from this study also support the conclusion that, at the tetraploid level, multiple genetic exchanges may have occurred in secondary contact areas between the two *Lotus* species, suggesting that they belong to the same polyploid complex, even if they were not derived originally from the same diploid parental species. Our results also suggest that the origin of *L. corniculatus* may be polyphyletic.

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