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Genome relationships among *Lotus* species based on random amplified polymorphic DNA (RAPD)

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Abstract The ability of random amplified polymorphic DNA (RAPD) to distinguish among different taxa of *Lotus* was evaluated for several geographically dispersed accessions of four diploid *Lotus* species, *L. tenuis* Waldst. et Kit, *L. alpinus* Schleich., *L. japonicus* (Regel) Larsen, and *L. uliginosus* Schkuhr and for the tetraploid *L. corniculatus* L., in order to ascertain whether RAPD data could offer additional evidence concerning the origin of the tetraploid *L. corniculatus*. Clear bands and several polymorphisms were obtained for 20 primers used for each species/accession. The evolutionary pathways among the species/accessions presented in a cladogram were expressed in terms of treelengths giving the most parsimonious reconstructions. Accessions within the same species grouped closely together. It is considered that *L. uliginosus* which is most distantly related to *L. corniculatus*, may be excluded as a direct progenitor of *L. corniculatus*, confirming previous results from isoenzyme studies. *Lotus alpinus* is grouped with accessions of *L. corniculatus*, which differs from previous studies. With this exception, these findings are in agreement with previous experimental studies in the *L. corniculatus* group. The value of the RAPD data to theories on the origin of *L. corniculatus* is discussed.

Key words *Lotus corniculatus* · *Lotus* species · Fabaceae · interspecific hybridization · RAPD

Introduction

The genus *Lotus* (Leguminosae) is a large polymorphic group comprising approximately 200 annual and perennial species (Grant 1965). One of these, Birdsfoot Trefoil (*Lotus corniculatus* L.), is a tetraploid forage legume that has distinct advantages for forage production (Grant and Marten 1985). Various authors have proposed different species as the progenitors of tetraploid *L. corniculatus* ($2n = 4x = 24$). The arguments favoring different hypotheses have been based upon the inheritance of the few genetic markers available or upon meiotic analyses of hybrids (Beuselinck and Mosjidis 1991; Bubar and Miri 1965; Donovan and McLennan 1964; Wernsman et al. 1964). Several diploid ancestors in the *L. corniculatus* group have been proposed as progenitors, and these include *L. alpinus* Schleich., *L. japonicus* (Regel) Larsen, *L. tenuis* Waldst. et Kit, and *L. uliginosus* Schkuhr.

This study was initiated because previous studies have shown that classical karyotype analysis is not a suitable method by which to investigate the parentage of the tetraploid species *L. corniculatus*: firstly because of chromosomal repatterning, which is believed to have occurred during the evolutionary development of the closely related diploid species (Grant 1986), and secondly, because new molecular approaches have been shown to be useful in elucidating species relationships (Grant 1987; Crawford 1990; Soltis et al. 1992).

Each of the four diploid species (*L. alpinus*, *L. japonicus*, *L. tenuis*, and *L. uliginosus*) has been proposed as a possible ancestor for the tetraploid species by various authors. Dawson (1941) proposed that *L. corniculatus* was an autotetraploid of *L. tenuis*, Wernsman et al. (1964) agreed with this hypothesis based on the meiotic behavior found in crosses between 4x *L. tenuis* and *L. corniculatus*. Since diploid *L. alpinus* closely resembles *L. corniculatus*, Larsen (1954) proposed that *L. corniculatus* was an autotetraploid of *L. alpinus*. Synthetic autotetraploids of these species have been produced, but they do not resemble *L. corniculatus* in morphology or fertility (Tome and Johnson 1945; De Lautour et al. 1978; Somaroo and Grant 1971).

Stebbins (1950) proposed that *L. corniculatus* is a segmental allotetraploid. Other evidence suggests that *L. corniculatus* is an allotetraploid involving at least two species. Somaroo and Grant (1972a) proposed that *L. japonicus* and *L. alpinus* could be the ancestral species, based on the fact that the artificial amphidiploid (*L. japonicus* × *L. alpinus*)^2 can be easily crossed with *L. corniculatus* producing progeny with high fertility and meiotic regularity. Ross and Jones (1985) have proposed that either *L. alpinus* or *L. tenuis* could be the maternal parent of *L. corniculatus*, since the hybrid matches both species for *Rhizobium* specificity, a character that is

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inherited maternally. They also proposed that *L. uliginosus* was the pollen parent based upon the fact that it is the only other species in the *L. corniculatus* group that possesses tannis and that this species is similar to *L. corniculatus* for certain phenolics. Raelson and Grant (1989) carried out an isoenzyme survey for different accessions of four diploid *Lotus* species, *L. alpinus*, *L. japonicus*, *L. tenuis*, and *L. uliginosus*, and the tetraploid *L. corniculatus*, to determine whether isoenzyme information could provide enough evidence to elucidate the origin of *L. corniculatus*. They found that the hybrids *L. japonicus* × *L. alpinus* and *L. tenuis* × *L. alpinus*, or their reciprocals, contained all of the alleles found in *L. corniculatus*, whereas *L. uliginosus* was distinct from all of the other species for several isoenzyme alleles at several loci. Although these studies have shed some light on the question of the origin of the tetraploid, it is evident that further studies are necessary to solve some of these controversies. Recently, genome relationships of various species and hybrids have been resolved through the analyses of DNA polymorphisms, and it is anticipated that some of the systematic discrepancies in the *Lotus corniculatus* group may be elucidated by this procedure.

Welsh and McClelland (1990) and Williams et al. (1990) have described simple methods for assessing genomic variability and for constructing genomic maps, based on the amplification of genomic DNA with single primers of arbitrary nucleotide sequence. These primers have been shown to detect polymorphisms in the absence of specific nucleotide sequence information in DNA. The terms RAPD (random amplification of polymorphic DNA; Williams et al. 1990) and AP-PCR (arbitrary primed polymerase chain reaction) fingerprinting (Welsh and McClelland 1990) have been applied to identify these techniques. In RAPD, the oligonucleotides are randomly produced and do not require any DNA target information. The observed fingerprints for a given DNA sample will depend on the length and sequence of the primer, as well as on the optimization of reaction conditions such as reagent concentrations and annealing temperature. Since the likelihood of a primer binding to genomic DNA is (1/4), where *n* is the length in bases of the primer, oligonucleotides with shorter sequences of base pairs, such as those used in RAPD, will provide a higher probability for priming events. Anollés et al. (1991) have reported DNA amplification with arbitrary primers as short as five nucleotides.

In the past 2 years several analyses have been performed using RAPD to solve genome relationships among different species (Crowhurst et al. 1991; Welsh et al. 1991; Yao and Sink 1991; Hu and Quiros 1991; Quiros et al. 1991; Weeden 1991). The polymorphisms observed are visualized in acrylamide and agarose gels, providing unique information that is complementary to other traditional approaches of classification (e.g., see Song et al. 1988; Dally and Second 1990; Wang et al. 1992). A variety of computer programs have also become available for generating phylogenetic trees based on DNA information from different individuals, making the task of studying genome relationships more efficient and accurate. The purpose of this paper is to make use of DNA polymorphisms to study genome relationships among different *Lotus* species in the *Lotus corniculatus* group and to use this information to aid in resolving the origin of the tetraploid species, *Lotus corniculatus*.

Materials and methods

The source of material for the present study was obtained form the world *Lotus* collection maintained by W. F. Grant on the Macdonald Campus of McGill University. A complete list of the species and accession numbers are presented in Table 1. The molecular analyses were performed according to the following procedures.

DNA extraction

The method described in Appels and Dvorak (1982), modified by Doyle and Beachy (1985), was used for DNA extraction. In this procedure, 0.2 g fresh leaf tissue is ground in liquid nitrogen in a mortar and pestle. Then 1 ml extraction buffer (0.1 *M* NaCl, 0.1 *M* EDTA, 50 m*M* Tris-HCl pH 7.0) is added. The slurry is extracted with two volumes of phenol- chloroform (1 : 1) and precipitated with ethanol. RNA is eliminated from the precipitate by treatment with DNAse-free pancreatic RNAse. Ethanol precipitation is used to collect RNA-free DNA. In soybean, this procedure yielded from 50–100 mg DNA per 0.19 g fresh leaf tissue (Doyle and Beachy 1985).

Table 1 List of species studied, their accession number, source and somatic chromosome number

Species	Accession number	Chromosome number	Origin
L. tenuis Waldst. et Kit.	131	12	USDA, Ames, Iowa Origin: Turkey
	222	12	Plant Introduction Station, Geneva, N.Y. P.I. no. 246731 Origin: Spain
	296	12	P.I.S., Geneva, N.Y. P.I. no. 229569 Origin: Greece
	298	12	P.I.S., Geneva, N.Y. P.I. no. 251148 Origin: Yugoslavia
L. uliginosus Schkuhr	865	12	Museum of Natural History, Paris, France. Origin: St. Côme d'Olt, Aveyron, France
	854	12	Origin: Niedersachsen, FRG
	201	12	Hortus Botanicus, Coimbra, Portugal
	858	12	Origin: Hessen, FRG
L. corniculatus L.	554	24	P.I.S., Geneva, N.Y. Origin: Samsun, Turkey
	279	24	P.I.S. Geneva, N.Y. P.I. no. 161878, Argentina
	247	24	Origin: Loir-et-Cher, Herbault, France
	811	24	P.I.S., Geneva, N.Y. P.I. no. 380896 Origin: Iran
L. japonicus (Regel) Larsen	129	12	Origin: Gifu, Japan Collector: I. Hirayoshi
	557	12	Origin: Iwaya, Awaji, Hyogo Prefecture, Japan Collector: Y. Huziwara
	581	12	Origin: Jima Islands, Japan Collector: N. Satomi
L. alpinus Schleich.	77	12	Origin: Valley of Emaney, Swiss Alps Collector: C. Favarger
L. corniculatus cv Leo	764	24	Emile Lods Research Station, Macdonald Campus, McGill University

Taq and DNA optimum concentrations

A series of assays were carried out to evaluate both Taq polymerase and DNA concentration. For each species/accession, several assays were carried out to determine DNA concentrations in order to obtain between 20 and 25 ng genomic DNA. This was accomplished by comparing the dillutions to a known genomic DNA standard after separation on 1.2% agarose gel and staining with ethidium bromide. After the optimum DNA concentrations were established for each species/accession, the effect of the following Taq polymerase concentrations in *Lotus* were evaluated 1.0, 1.3, 1.5 and 2.3 units.

Primers

The arbitrary decamer oligonucleotides were obtained from Operon Technologies (Alameda, Calif.). The primers were used for the amplification of random DNA sequences. The nucleotide sequence of each primer is shown in Table 2. The criteria used to select these primers was the CG content, which was between 50% and 70%.

Amplification conditions

The amplification reactions were performed under conditions similar to those used by Williams et al. (1990). A volume of 25 µl was used which contained 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 2 m*M* $MgCl_2$, 100 µ*M* each of dATp, dCTp, dGTp, and genomic DNA, and 1.0 unit of Taq DNA polymerase (GIBCO BRL). The amplfications were carried out in a Hybaid DNA Thermal Cycler programmed for 45 cycles of 1 min at 94 °C to denature, 1 min at 35 °C for the annealing primer, and 2 min at 72 °C for extension. Tubes containing all of the reaction components except for the genomic DNA were included as a control when a new primer was used. After the cycling was completed, 15 µl of each sample was run in 1% agarose gel and visualized in the presence of ethidium bromide. The molecular standard used was the lambda DNA digested by *Eco*RI/*Hin*dIII. The gels were photographed under UV light with Polaroid film 655 or 667.

Table 2 List of primers used and their respective oligonucleotide sequence

Primer	Oligonucleotide sequence 5′ to 3′
H-03	AGACGTCCAC
H-04	GGAAGTCGCC
H-05	AGTCGTCCCC
H-07	CTGCATCGTG
H-12	ACGCGCATGT
H-13	GACGCCACAC
H-14	ACCAGGTTGG
H-19	CTGACCAGCC
G-02	GGCACGGAGG
G-08	TCACGTCCAC
G-09	CTGACGTCAC
G-10	AGGGCCGTCT
G-12	CAGCTCACGA
G-13	CTCTCCGCCA
G-14	GGATGAGACC
G-16	AGCGTCCTCC
G-17	ACGACCAGCA
G-18	GGCTCATGTG
G-19	GTCAGGGCAA
FCP4	TCCTAAGCGG

Data analyses

The occurrence of a specific band of amplified DNA was scored as 1 and absences as 0 for all prominent bands within a fingerprint. Therefore, a sequence of 0's and 1's was generated for each primer/species to form a data matrix. The evolutionary pathways among the species/accessions were expressed in terms of treelengths to give the most-parsimonious reconstructions of ancestral states (Maddison and Maddison 1992). The treelength statistics were calculated between all 17 taxa to determine the shortest treelengths to give the best fit. The most parsimonious cladogram was generated using the MacClade program version 3.1 (Maddison and Maddison 1992).

Results and discussion

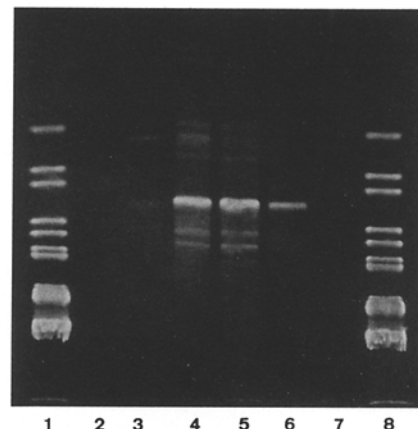
Optimum DNA and Taq concentration

Approximately 120 assays were carried out to obtain the optimum DNA concentration for each species. Figure 1 shows the results of Taq concentration for the species *L. corniculatus* accession 811. It can be observed that the concentrations of 1.3, 2.3 and 1.0 units give clear bands. This result was generally consistent for all of the species/accessions, and therefore the concentration of 1.0 unit was chosen arbitrarily for the analyses.

Polymorphic bands

RAPD polymorphisms were evaluated using twenty, 10-base primers for each species/accession (see Table 2). None of these primers had been previously selected to be used in *Lotus*. In order to detect if the amplification products were reliable polymorphics, three replications were performed for each primer. Several polymorphic bands were observed for each primer. Most primers produced multibanded 'fingerprints' with sizes ranging from 300 bp to 3000 bp. Figures 2, 3 and 4 illustrate results obtained for primers H-03, H-04 and H-05,

Fig. 1 Visualization of optimum Taq concentration in 1.2% agarose gel. *Lanes 1 and 8*: lambda DNA, *lanes 2 and 7*: empty, *lane 3*: 1.3 units, *lane 4*: 2.3 units, *lane 5*: 1.0 unit, *lane 6*: 1.5 units



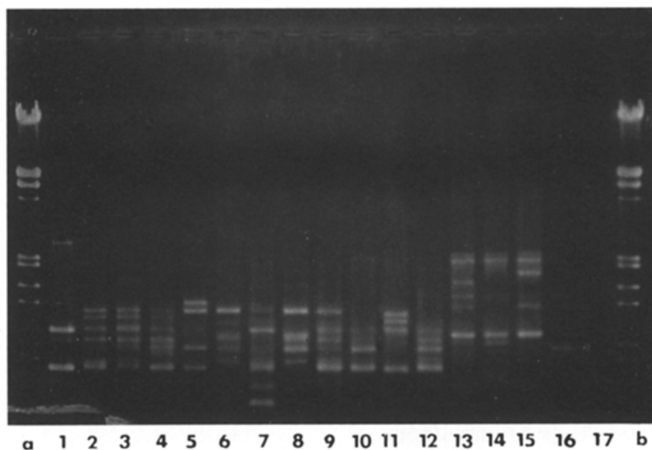


Fig. 2 Fingerprinting analysis of several accessions of *Lotus* using the primer H-03. Labels for each lane are as follows: *a*, *b* = Markers; *1* = *Lotus alpinus*, *2–5* = *Lotus corniculatus*, *6–8* = *Lotus japonicus* *9–12* = *Lotus tenuis*, *13–16* = *Lotus uliginosus*, *17* = negative control. Additional information is given in Table 1

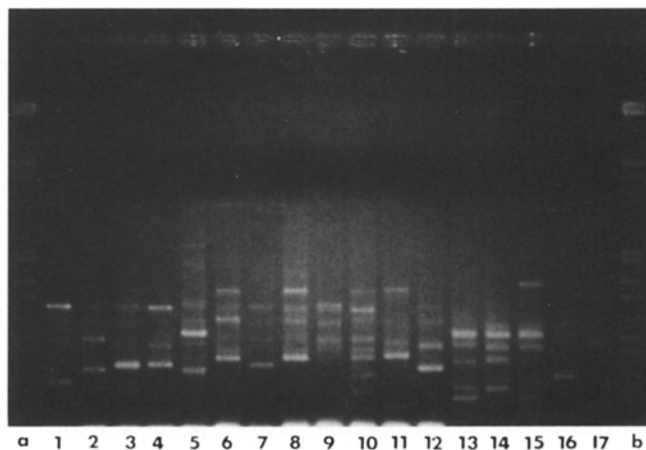


Fig. 4 Fingerprinting analysis of several accessions of *Lotus* using the primer H-05. Labels for each lane are as follows: *a*, *b* = Markers; *1* = *Lotus alpinus*, *2–5* = *Lotus corniculatus*, *6–8* = *Lotus japonicus* *9–12* = *Lotus tenuis*, *13–16* = *Lotus uliginosus*, *17* = negative control. Additional information is given in Table 1

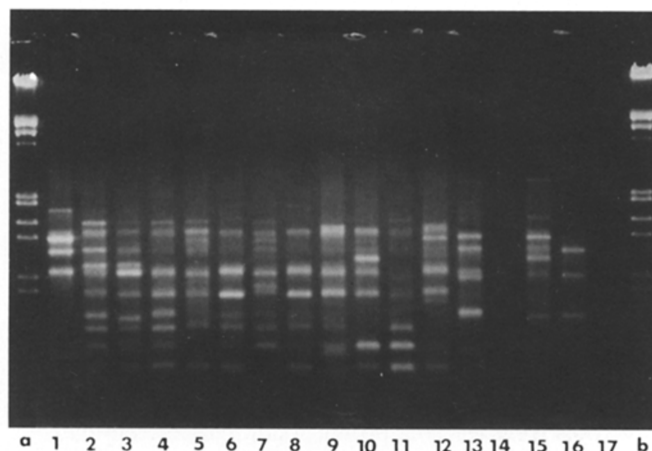


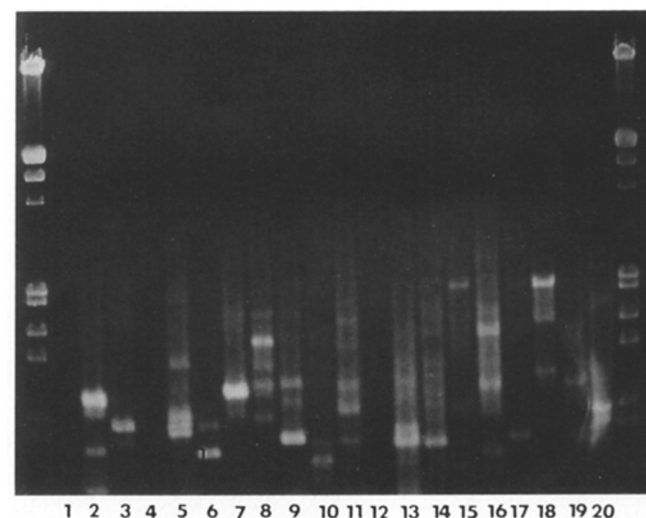
Fig. 3 Fingerprinting analysis of several accessions of *Lotus* using the primer H-04. Labels for each lane are as follows: *a*, *b* = Markers; *1* = *Lotus alpinus*, *2–5* = *Lotus corniculatus*, *6–8* = *Lotus japonicus* *9–12* = *Lotus tenuis*, *13–16* = *Lotus uliginosus*, *17* = negative control. Additional information is given in Table 1

respectively. Primers H-03 (Fig. 2) and H-05 (Fig. 4) reproduced bands for all accessions analyzed, whereas primer H-04 did not reproduce any band for the accession 854 of *L. uliginosus*. The same results were obtained for the three replications performed for each primer. No bands were produced by the negative control (lane 17; Figs. 2 and 3), but a very weak band was observed as shown in Fig. 4. However, the latter band did not prevent the interpretation of the polymorphisms of the species. One clear difference can be detected between the accessions for the species *L. uliginosus* (lanes 13–16) and the other species. These results are in agreement with the observations of the isoenzyme study of Raelson and Grant (1988), who excluded this species from the ancestry of *L. corniculatus*.

Polymorphisms were also detected within the species. For example, on lane 16, the accession 865 of *L. uliginosus* appears distinct from the other three accessions for this species on both Figs. 2 and 4. Similarly, on Figs. 2 and 4, lane 7 indicates a clear polymorphism for the accession 557 of *L. japonicus*, which differs from the other two accessions for this species. It would be risky to infer that such accessions are wrongly classified based on the analyses of just a few primers. A more general analysis based on the performance of several primers combined is presented in the next section.

A series of assays including all primers for each species/accession was also carried out. Figure 5 illustrates the results obtained for *L. corniculatus* B764 using 20 different primers. It can be observed that primers

Fig. 5 Fingerprinting analysis of *Lotus corniculatus* cv 'Leo' using 20 different primers as follows: *lane 1* = G-02, *lane 2* = FCP-4, *lane 3* = G-08, *lane 4* = G-09, *lane 5* = G-10, *lane 6* = G-11, *lane 7* = G-12, *lane 8* = G-14, *lane 9* = G-17, *lane 10* = G-18, *lane 11* = G-19, *lane 12* = G-20, *lane 13* = G-13, *lane 14* = H-03, *lane 15* = H-05, *lane 16* = H-15, *lane 17* = H-14, *lane 18* = H-16, *lane 19* = H-04, *lane 20* = H-13



G-02, G-09 and G-20 failed to produce bands. However, analyses performed for all species/accessions did not show any primer consistently yielding smaller or large numbers of bands.

Genetic distance analysis

The most parsimonius caldogram obtained for all primers combined is presented in Figure 6. The phylogenetic tree clusters species that are closely related and the topology of the tree portrays the relationships among the species/accessions. *Lotus uliginosus* appears as the most distant species from *L. corniculatus*, followed by *L. tenuis* and *L. japonicus*. Accessions within the same species appear to be closely affiliated, which is good evidence for the caliber of the experimental data. *Lotus alpinus* is grouped with the *L. corniculatus* accessions which is a variance from previous studies (Somaroo and Grant 1972a, b; Raelson and Grant 1988).

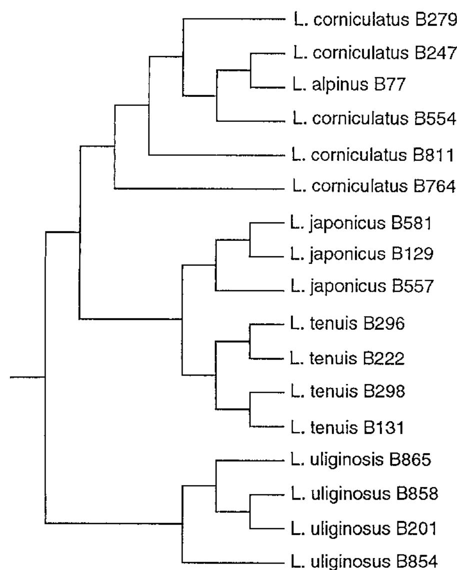
Lotus uliginosus shows sufficient differences to be excluded as an ancestral form of *L. corniculatus*, which confirms previous results from isoenzyme studies (Raelson and Grant 1988). The fact that *L. uliginosus* and *L. corniculatus* are the only known species possessing tannins in the *L. corniculatus* group (Ross and Jones 1985) and the phenolic compound delphinidin (Harney and Grant 1965) would imply that they are more closely related than the evidence provided by both isoenzyme and RAPD data indicates. However, evidence that agrees with the isoenzyme and RAPD data comes from an electron micrographic survey of pollen morphology among species of the Loteae. Through a principal component analysis of several morphological pollen characters for a number of taxa, it was conclude/d that *L. uliginosus* is dissimilar to *L. tenuis* and *L. corniculatus*, again indicating the distinctness of these two species (Crompton and Grant 1993).

The close relationship of *L. tenuis* with *L. corniculatus* is in agreement with the meiotic chromosome behavior observed in crosses between 4x *L. tenuis* and *L. corniculatus* (Wernsman et al. 1964). Likewise, the close relationship of *L. japonicus* with *L. corniculatus* would agree with hybridization studies in which *L. japonicus* has been considered to be one of the progenitor species (Somaroo and Grant 1971). While other studies have shown *L. alpinus* to be more closely related to the diploid species (Somaroo and Grant 1972a, b), Raelson and Grant (1988) from their isoenzyme study found an allele present in *L. corniculatus* that was only found in *L. alpinus*. This shows the close relationship between *L. corniculatus* and *L. alpinus* and gives further evidence for *L. alpinus* as an ancestral species.

Overall, the power of RAPDs to reveal polymorphisms among *Lotus* species is clearly revealed, with our results closely paralleling those from both meiotic chromosome analyses, crossing behavior, and isoenzyme studies. The results presented here are not intended to be a final analysis of the genomic relationships among the *Lotus* species that have been studied. This study represents first step in using RAPD markers as a tool to implement studies of molecular systematics in this large genus (Grant 1965). The inclusion of additional accessions of these species and several other species in the *L. corniculatus* group not considered in the present study (*L. boissieri*, *L. borbasii*, *L. filicaulis* and *L. schoelleri*) and the use of an increased number of primers may provide a greater resolution of the relationships of these taxa. Until these studies are carried out, further speculations on the origin of *L. corniculatus* are premature.

Acknowledgements Financial support from the Natural Sciences and Engineering Research Council of Canada to W.F. Grant for studies in the genus *Lotus* is gratefully acknowledged.

Fig. 6 The most parsimonious cladogram showing the relationships among several species/accessions of *Lotus*



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