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Evaluation of hypotheses concerning the origin of *Lotus corniculatus* (Fabaceae) using isoenzyme data

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Summary. An isoenzyme survey was conducted for several geographically dispersed accessions of four diploid *Lotus* species, *L. alpinus* Schleich., *L. japonicus* (Regel) Larsen, *L. tenuis* Waldst. et Kit and *L. uliginosus* Schkuhr, and for the tetraploid *L. corniculatus* L., in order to ascertain whether isoenzyme data could offer additional evidence concerning the origin of *L. corniculatus*. Seven enzyme systems were examined using horizontal starch gel electrophoresis. These were PGI, TPI, MDH, IDH, PGM, 6-PGDH, and ME. *Lotus uliginosus* had monomorphic unique alleles, that were not found within *L. corniculatus*, at 7 loci. These loci and alleles are: *Tpi1*-112, *Pgm1,2*-110, *Pgm3*-82, *Mdh3*-68, *6-Pgdh1*-110, *6-Pgdh2*-98,95, and *Me2*-100. Other diploid taxa contained alleles found in *L. corniculatus* for these and other loci. The implications of the isoenzyme data to theories on the origin of *L. corniculatus* are discussed.

Key words: *Lotus corniculatus* – *Lotus* species – Fabaceae – interspecific hybridization – isoenzymes

Introduction

This is the third paper in a series resulting from an isoenzyme study of several species within the genus *Lotus*. Previous papers discribed the experimental procedures and the results of a study of segregation of isoenzyme alleles in interspecific hybrids (Raelson and Grant 1988). Segregation of alleles was also examined in synthetic allo- and autotetraploids, and in *L. corniculatus* (Raelson et al. 1988). This study was initiated as previous studies have shown that classical karyotype analyses were not a suitable method to investigate the parentage of the tetraploid species *L. corniculatus* because of the chromosomal repatterning which is believed to have occurred during the evolutionary development of the closely related diploid species (Grant 1986) and with newer molecular approaches being useful in elucidating species relationships (Grant 1984, 1987).

In this paper are reported the findings of a survey of isoenzyme loci for four diploid species, *L. alpinus* Schleich., *L. japonicus* (Regel) Larsen, *L. tenuis* Waldst. et Kit and *L. uliginosus* Schkuhr, and for the tetraploid *L. corniculatus* L.

Each of the four diploid species 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*, while Larsen (1954) proposed that it was an autotetraploid of *L. alpinus*. The synthetic autotetraploids of these species have been produced (De Lautour et al. 1978; Somaroo and Grant 1971 a) and they do not resemble *L. corniculatus* in morphology or fertility. In addition, we have provided evidence of quadruplication of *Pgi2* loci in both the synthetic allotetraploid $(L.\ japonicus \times L.\ alpinus)^2$ and in *L. corniculatus* that was not found for the synthetic autotetraploid $(L.\ alpinus)^2$. This fact suggests that *L. corniculatus* is indeed an allotetraploid.

Others have also suggested that *L. corniculatus* is a segmental allotetraploid (Stebbins 1950). Somaroo and Grant (1971 b, 1972) proposed that *L. japonicus* and *L. alpinus* could be ancestral species based upon the fact that the synthetic amphidiploid $(L.\ japonicus \times L.\ alpinus)^2$ can be easily crossed with *L. corniculatus* producing progeny with high fertility and a high degree of meiotic regularity. Recently, Ross and Jones (1985) reviewed the problem of the ancestry of *L. corniculatus* and proposed that either *L. alpinus* or *L. tenuis* could be the maternal parent of the original hybrid, since both species matched *L. corniculatus* for *Rhizobium* specificity

Table 1. Taxa of *Lotus* used in this study along with their accession number, chromosome number, and source

Taxon	Accession number	Chromosome number	No. individuals	Source
L. alpinus Schleich.	77	12	30	Origin: Rocky limestone pasture at the bottom of the Valley of Emaney, Swiss Alps. Collector, C. Favager. Seed from Institut de Botanique, Université de Neufchâtel. Received as *L. corniculatus* var. *alpinus* Ser.
	324	12	10	Origin: Lebanon. Collector, W. S. Edgecombe
	774*x*	24	10	Artificial autotetraploid (Somaroo and Grant 1971)
	828	12	10	Origin: Switzerland. Collector, K. Urbanska
L. corniculatus L.	106	24	10	Origin: Czechoslovakia, Australia, C.P.I. No. 24449
	247	24	10	Origin: France, Groupement National Interprofessionel de Production et d'Utilisation des Semences, Graines et plants Paris
	279	24	10	Plant Introduction Station, Geneva, N.Y. P.I. No. 161878, Origin, Argentina
	554	24	30	Introduction Center, Izmir, Turkey
	710	24	30	cv. 'Viking'
	764	24	30	cv. 'Leo', developed by J. S. Bubar, 1963, at Macdonald College, Ste. Anne de Bellevue, Quebec
	811	24	10	Plant Introduction Station, Geneva, N.Y. P.I. No. 380896, Origin, Iran
L. japonicus (Regel) Larsen	129	12	10	Origin: Gifu, Japan. Collector, I. Hirayoshi
	177	12	30	Plant Introduction Station, Geneva, N.Y. No. G-7359; Source, Korea
	541	12	10	Origin: Hiroshima, Japan. Collector, R. Tanaka
	581	12	10	Origin: Jima Islands, Japan. Collector, N. Satomi
	842	12	30	Origin: Shimizu, Japan. Source, D. A. Jones
L. tenuis Waldst. et Kit	109	12	30	Australia, C.P.I. No. 23788. Origin: Turkey
	131	12	30	U.S.D.A. Ames, Iowa. Origin: Turkey P.I. No. 206446
	145	12	30	U.S.D.A. Soil Conservation Service, Pleasanton, California. No. P-14496
	222	12	10	Plant Introduction Station, Geneva, N.Y. P.I. No. 246731, Origin, Spain
	296	12	10	Plant Intoduction Station, Geneva, N.Y. P.I. No. 229569, Origin, Greece
	297	12	10	Plant Introduction Station, Geneva, N.Y. P.I. No. 247898, Origin, France
	298	12	10	Plant Introduction Station, Geneva, N.Y. P.I. No. 251148, Origin, Yugoslavia
	826	12	10	Plant Introduction Station, Geneva, N.Y. P.I. No. 243222, Origin, Iran
	837	12	10	Origin: Buenos Aires, Argentina. Collector: A. M. Arambarri
L. uliginosus Schkuhr	110	12	10	U.S.D.A., PL 69-55. Origin unknown.
	193	12	30	Service de la Recherche Agronomique et de l'Experimentation Agricole, Rabat, Morocco
	120	12	10	Institut für Kulturpflanzenforschung Gatersleben, E. Germany
	201	12	10	Hortus Botanicus, Coimbra, Portugal
	289	12	30	Plant Introduction Station, Geneva, N.Y. P.I. No. 234493, Origin, Sweden
	290	12	10	Plant Introduction Station, Geneva, N.Y. P.I. No. 235527, Origin, Hungary
	293	12	10	Plant Introduction Station, Geneva, N.Y. P.I. No. 251529, Origin, Yugoslavia
	846	12	10	Origin: Caen, France. Source, D. A. Jones
	854	12	30	Origin: Niedersachsen, W. Germany

which is known to be inherited maternally. They also proposed that *L. uliginosus* was the pollen parent based upon the fact that this is the only other species in the *L. corniculatus* group that possesses tannins, and upon the fact that this species is similar to *L. corniculatus* for certain phenolics.

Previous studies have successfully used isoenzyme data to verify or discount theories of ancestral origin for species that had been based upon morphological or cytogenetic findings. These include such species as wheat (Nakai 1981), *Talinum teretifolium* (Murdy and Carter 1985) and *Tragopogon mirus* (Roose and Gottlieb 1976) to give but a few examples. Isoenzyme data provide a new approach for examining this problem in the genus *Lotus*.

The power of isoenzyme data to test such hypotheses is derived from the fact that an allotetraploid or hybrid species will display hybrid phenotypes for any of the codominant alleles that differ between true ancestral species. Alleles which are differentially monomorphic, or fixed, in putative parental species are particularly useful (Crawford 1985). The failure of monomorphic alleles of a species to appear in the polyploid is strong evidence against an ancestral role for that species.

Materials and methods

Various accessions of the five species were surveyed for isoenzyme phenotypes. These are presented in Table 1. The source of the material was from the world *Lotus* collection maintained by W. F. Grant at Macdonald College of McGill University. The number of accessions for which isoenzyme analyses were carried out reflected their availability within the collection. An attempt was made to select as wide a geographical range as possible for each species. Four accessions of *L. alpinus*, 5 accessions of *L. japonicus*, 9 accessions each of *L. tenuis* and *L. uliginosus*, and 7 accessions of *L. corniculatus* were sampled.

The fact that the genotypes came from a seed bank presented a certain problem. The authors had no control over the manner in which the seed was collected and cannot know whether the genotypes contained in each accession are truly a random sample of the original population or were merely collected from a limited number of individuals. Therefore, it cannot be assumed that genotype and allelic frequencies of the samples truly represent population parameters. To overcome this limitation, it was decided to sample fewer individuals from more accessions, rather than sample many individuals from fewer accessions. In view of these uncertainties, population samples and genetic identity indices such as that carried out by Nei (1972) would not have been meaningful and were not calculated.

Allelic frequencies for alleles at various isoenzyme loci were calculated from a sample of 30 individuals for three accessions of each species when enough seed was available, and allelic frequencies were also calculated for each entire species based upon all observed individuals. These allelic frequencies cannot be considered to represent allelic frequencies within populations but merely within seed samples in the collection. Therefore, standard errors and confidence intervals for allelic frequencies were calculated by simply using multinomial probability theory according to the formula C.I. 95% = $1.96\,(p_i/1-p_i)/n)^{-1/2}+\frac{1}{2}n)$ where C.I. = the 95% confidence interval for the multinomial approximation to the normal curve and where $p_i = xi/n$ or the frequency of a given allele (Moran 1968; Snedecor and Cochran 1980). No consideration was given to population parameters such as self compatibility as suggested by Brown and Weir (1983) when calculating standard errors.

During the analysis, emphasis was placed upon distinct fixed alleles characteristic of each species, rather than upon allelic frequencies because of the sampling problem. Nevertheless, it is of practical interest to determine whether allelic frequencies could be used to characterize different samples within a species. A seed sample of a cultivar such as Viking or Leo could be reasonably expected to be a random sample of the genetic diversity of the cultivar. The characterization of such samples by allelic frequencies would be useful for breeder protection. The sample calculations of allelic frequency for several accessions within each species was made in order to determine if such characterization was possible and which isozymes are most useful for this purpose.

Electrophoresis: The protocols of horizontal starch gel electrophoresis have been previously described (Raelson and Grant 1988). Isoenzyme systems examined for PGI and TPI were electrophoresed on a LiOH-borate buffer system, pH 8.1–8.4, IDH and MDH were analyzed on a tris-citrate system, pH 7.1, and PGM, 6-PGDH, and ME were analyzed on a histidine-citrate system, pH 6.5. Determination of individual phenotypes was made on the basis of three replications.

Results

A graphic summary of all observed isoenzyme zymograms for PGI, TPI, PGM, MDH, IDH, 6-PGDH, and ME are presented in Figs. 1–3 for the five diploid species and for *L. corniculatus*.

Data on the frequency of various alleles within the five species are presented in Table 2. Determination of the number of loci and alleles represented by the various phenotypes was based upon knowledge obtained through previous studies of phenotype segregation and pollen electrophoresis (Raelson and Grant 1988). Alleles for various loci were named according to their mobility relative to one common phenotype band which was arbitrarily designated as Relative Mobility 100. The loci designation for PGM was the most uncertain of all the enzymes. The upper region of the PGM phenotype appeared to have fixed heterozygosity (constant two-banded phenotype) for all individuals of *L. uliginosus*. We assumed that this reflected the presence of duplicated loci similar to that found in *Layia* by Warwick and Gottlieb (1985), rather than being an artifact, such as that observed in *Pteridium aquilinum* by Wolf et al. (1987). We have tentatively assigned two loci to the upper PGM isoenzymes, though no segregation studies were actually undertaken. The upper IDH zone and the lower zone for 6-PGDH have also been assumed to be controlled by duplicated loci. This assumption is based upon the fact that heterozygous individuals displayed unbalanced

Table 2. Characteristic alleles and allele frequencies for various isoenzyme loci for *Lotus tenuis*, *L. uliginosus*, *L. alpinus*, *L. japonicus* and *L. corniculatus*

Locus, allele	*Lotus tenuis*				*L. uliginosus*				*L. alpinus*				*Lotus japonicus*				*L. corniculatus*			
	No. accs.	No. allel.	Freq. No.	C.I.	No. accs.	No. allel.	Freq. No.	C.I.	No. accs.	No. allel.	Freq. No.	C.I.	No. accs.	No. allel.	Freq. No.	C.I.	No. accs.	No. allel.	Freq. No.	C.I.
Pgi1	9	300			9	300			4	120			6	200			8	552		
100	9		1				0.483	0.058			1		9		1				1	
95			–				0.517	0.058			–				–				–	
Pgi2																	8	1,104		
86			–				0.036	0.023			–				–				–	
80			–				0.153	0.042			0.037	0.038			0.850	0.052			0.261	0.026
72			0.433	0.058			0.803	0.047			0.228	0.079			0.086	0.041			0.385	0.029
62			0.256	0.051			–				0.713	0.085			0.001	0.006			0.146	0.021
52			0.297	0.053			–				0.022	0.030			0.045	0.031			0.208	0.024
Tpi1	9	300			9	300			4	120			6	200			8	552		
112			–				1				–				–				–	
100			1				–				1				1				1	
Tpi2																				
97			–				0.740	0.051			–				0.268	0.064			–	
90			1				0.260	0.051			1				0.732	0.064			1	
Pgm1, 2	9	600			9	600			4	240			6	400			8	1,109		
110			–				0.500	0.041			–				–				–	
100			0.530	0.041			–				0.441	0.065			0.500	0.050			0.529	0.029
98			–				0.500	0.041			–				–				–	
95			0.470	0.041			–				0.345	0.062			0.500	0.050			0.438	0.029
85			–				–				0.243	0.056			–				0.033	0.011
Pgm3	9	300			9	300							6	200			8	552		
82			–				1				–				–				–	
76			1				–				1				1				1	
Mdh1	9	300			9	300			4	120			6	400			8	552		
112			0.047	0.026			–				–				–				–	
100			0.953	0.026			1				1				1				1	
Mdh3																				
85			–				–				0.507	0.094			0.895	0.031			0.106	0.027
68			–				1				–				–				–	
66			1				–				0.493	0.094			0.105	0.031			0.894	0.027
Idh1, 2	9	600			9	600			4	240			6	400			8	1,104		
100			0.953	0.018			–				0.669	0.086			0.638	0.048			0.639	0.029
85			0.047	0.018			1				0.331	0.086			0.362	0.048			0.361	0.029
6-Pgdh1	9	300			9	300			4	120			6	200			8	552		
120			–				–				0.059	0.046			–				0.201	0.034
110			–				1				–				–				–	
100			1				–				0.941	0.046			1				0.798	0.034

Table 2. Continued

Locus, *allele*	*Lotus tenuis*			*L. uliginosus*			*L. alpinus*			*Lotus japonicus*			*L. corniculatus*		
	No. accs.	No. allel.	Freq. No.	C.I.	No. accs.	No. allel.	Freq. No.	C.I.	No. accs.	No. allel.	Freq. No.	C.I.	No. accs.	No. allel.	Freq. No.	C.I.
6-Pgdh2																
98			–				0.733	0.052			–				–	
95			–				0.267	0.052			–				–	
90			–				–				0.228	0.079			0.011	0.010
80			0.890	0.037			–				0.772	0.079			0.989	0.010
65			0.110	0.037			–				–				–	
Me2	9	300			9	300			75	120			6	200		
152			–				–				0.043	0.040			0.011	0.010
100			–				1				–				–	
85			1				–				0.957	0.040			0.989	0.010

C.I. 95% = multinomial 95% confidence interval $+(1.96\,(p_i\,(1-p_i)/n)^{-1/2}+1/2n)$

Fig. 1 A – E. Graphic summary of all isoenzyme phenotypes for PGI, TPI, and PGM; **A** *Lotus tenuis*, **B** *L. uliginosus*, **C** *L. alpinus*, **D** *L. japonicus*, **E** *L. corniculatus;* the six-banded phenotype of PGI for *L. alpinus* is from the autotetraploid accession 774*x*

Fig. 2 A – E. Graphic summary of all isoenzyme phenotypes for IDH, MDH, and 6-PGDH; lowest band of MDH phenotypes are truncated; **A** *Lotus tenuis*, **B** *L. uliginosus*, **C** *L. alpinus*, **D** *L. japonicus*, **E** *L. corniculatus;* open dots of IDH phenotypes represent consistent but poorly stained bands

Fig. 3 A – E. Graphic summary of all isoenzyme phenotypes for ME; **A** *Lotus tenuis*, **B** *L. uliginosus*, **C** *L. alpinus*, **D** *L. japonicus*, **E** *L. corniculatus;* five-banded phenotypes are most likely heterozygous for tetrameric enzyme

three-banded phenotypes. The distortion in band density ratios would result if polypeptide subunits of the dimeric molecules were not present in a 1 : 1 ratio which would be the case where two loci are present with only one locus heterozygous. Segregation has been studied for IDH and 6-Pgdh1 (Raelson and Grant 1988), but not for *6-Pgdh2*. All diploid taxa displayed balanced heterozygous phenotypes for all other zones of allozyme activity. This implies lack of duplication at these loci.

Allelic frequencies calculated for some accessions of each of the five species are displayed in Tables 3–7. It is again stressed that these frequencies do not necessarily reflect allelic frequencies in populations because of uncertainty of the method of population sampling during seed collection.

Pgi2 was found to be the most useful of all loci for distinguishing among accessions. Most accessions contained statistically distinct frequencies for the various *Pgi2* alleles.

Discussion

Crawford (1985) has stressed the value of isoenzyme data for documenting the origin of hybrid plants. He stated that the most desirable situation is that the two taxa under consideration be each monomorphic for mutually exclusive alleles at some locus and that both of these alleles be found within populations of the derived species. The most important criterion to be used in this analysis is based upon this concept. This criterion is as follows. If any of the taxa exclusively have certain unique alleles at a given locus that are never found in *L. corniculatus*, then this is good evidence against the role of that species in the ancestry of the tetraploid.

Several alleles meet this criterion for *L. uliginosus*. These are *Tpi1*-112, *Pgm1,2*-110, *Pgm3*-82, *Mdh3*-68, *6-Pgdh1*-110, *6-Pgdh2*-98,95, and *Me2*-100. Such exclusively unique alleles are not found at any locus for the other three species.

A much weaker criterion is the presence of a certain allele in *L. corniculatus* that is only found in one of the diploid species. This is the case for *6-Pgdh1*-120 and *Me2*-152 which are only found in *L. alpinus* and *L. corniculatus*. Such a criterion is not definitive because we have looked at only a few accessions of only four species of the *L. corniculatus* group (which is characterized by equal length calyx teeth, yellow flowers, $x = 6$). Five other species exist in this group, *L. boissieri*, *L. borbasii*, *L. filicaulis*, *L. krylovii*, and *L. schoelleri*, that may also contain such alleles.

According to the data that we have observed and presented here, the hybrid *L. japonicus* × *L. alpinus*, or the reciprocal cross, could contain all of the alleles found in *L. corniculatus*, but this is also true of the cross

Table 3. Allelic frequencies for various isoenzyme loci for three accessions of *Lotus tenuis*

Accession	109		145		131	
Locus, allele	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*
Pgi1						
100	1	60	1	60	1	60
95	–		–		–	
Pgi2						
86	–		–		–	
80	–		–		–	
72	0.883, 0.090	60	0.083, 0.078	60	0.417, 0.133	60
62	–		0.567, 0.134		0.150, 0.099	
52	0.117, 0.090		0.350, 0.129		0.350, 0.129	
Tpi1						
112	–	60	–	60	–	60
100	1		1		1	
Tpi2						
97	–		–		–	
90	1		1		1	
Pgm1, 2						
110	–	120	–	120	–	120
100	0.633, 0.090		0.400, 0.092		0.533, 0.093	
98	–		–		–	
95	0.367, 0.090		0.600, 0.092		0.467, 0.093	
85	–		–		–	
Pgm3						
82	–	60	–	60	–	60
76	1		1		1	
Idh1, 2						
100	1	120	1	120	1	120
85	–		–		–	
Mdh1						
112	–	60	–	60	0.100, 0.084	60
100	1		1		0.900, 0.084	
Mdh3						
85	–		–		–	
68	–		–		–	
66	1		1		1	
6-Pgdh1						
120	–	60	–	60	–	60
110	–		–		–	
100	1		1		1	
6-Pgdh2						
98	–	120	–	120	–	120
95	–		–		–	
90	–		–		–	
80	1		0.933, 0.049		1	
65	–		0.067, 0.049		–	
Me2						
152	–	60	–	60	–	60
100	–		–		–	
85	1		1		1	

N* = total number of alleles
C.I. 95% = multinomial 95% confidence interval + $(1.96 (p_i(1-p_i)/n)^{-1/2} + 1/2n)$

Table 4. Allelic frequencies for various isoenzyme loci for three accessions of *Lotus uliginosus*

Accession	193		289		854	
Locus, allele	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*
Pgi1						
100	0.767, 0.115	60	0.667, 0.128	60	1	60
95	0.233, 0.115		0.333, 0.128		–	
Pgi2						
86	–		0.167, 0.103		–	
80	0.117, 0.090		0.033, 0.054		0.067, 0.071	
72	0.883, 0.090		0.800, 0.110		0.933, 0.071	60
62	–		–		–	
52	–		–		–	
Tpi1						
112	1	60	1	60	1	60
100	–		–		–	
Tpi2						
97	1		0.533, 0.135		0.817, 0.106	
90	–		0.467, 0.135		0.183, 0.106	
Pgm1, 2						
110	0.500, 0.094	120	0.500, 0.094	120	0.500, 0.094	120
100	–		–		–	
98	0.500, 0.094		0.500, 0.094		0.500, 0.094	
95	–		–		–	
85	–		–		–	
Pgm3						
82	1	60	1	60	1	60
76	–		–		–	
Idh1,2						
100	–	120	–	120	–	120
85	1		1		1	
Mdh1						
112	–	60	–	60	–	60
100	1		1		1	
Mdh3						
85	–		–		–	
68	1		1		1	
66	–		–		–	
6-Pgdh1						
120	–	60	–	60	–	60
110	1		1		1	
100	–		–		–	
6-Pgdh2						
98	0.650, 0.090	120	0.750, 0.082	120	0.867, 0.065	120
95	0.350, 0.090		0.250, 0.082		0.133, 0.065	
90	–		–		–	
80	–		–		–	
65	–		–		–	
Me2						
152	–	60	–	60	–	60
100	1		1		1	
85	–		–		–	

N* = total number of alleles counted
C.I. 95% = multinomial 95% confidence interval + (1.96 $(p_i (1-p_i)/n)^{-1/2} + 1/2n$)

Table 5. Allelic frequencies for various isoenzyme loci for three accessions of *Lotus alpinus*

Accession	77		224		828	
Locus, allele	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*
Pgi1						
100	1	60	1	20	1	20
95	–		–		–	
Pgi2						
86	–		–		–	
80	–		–		–	
72	–	60	–	20	0.650, 0.234	20
62	1		1		0.350, 0.234	
52	–		–		–	
Tpi1						
112	–	60	–	20	–	20
100	1		1		1	
Tpi2						
97	–		–		–	
90	1		1		1	
Pgm1, 2						
110	–	120	–	40	–	40
100	0.500, 0.094		0.500, 0.167		0.500, 0.167	
98	–		–		–	
95	0.117, 0.062		0.500, 0.167		0.500, 0.167	
85	0.383, 0.091		–		–	
Pgm3						
82	–	60	–	20	–	20
76	1		1		1	
Idh1, 2						
100	1	120	1	40	1	40
85	–		–		–	
Mdh1						
112	–	60	–	60	–	60
100	1		1		1	
Mdh3						
85	0.800, 0.109		–		–	
68	–		–		–	
66	0.200, 0.109		1		1	
6-Pgdh1						
120	–	60	–	20	0.400, 0.240	20
110	–		–		–	
100	1		1		0.600, 0.240	
6-Pgdh2						
98	–	120	–	40	–	40
95	–		–		–	
90	0.467, 0.093		0.150, 0.123		–	
80	0.533, 0.093		0.850, 0.123		1	
65	–		–		–	
Me2						
152	–	60	0.050, 0.121	20	0.250, 0.215	20
100	–		–		–	
85	1		0.950, 0.121		0.750, 0.215	

N* = total number of alleles counted
C.I. 95% = multinomial 95% confidence interval + (1.96 $(p_i (1-p_i)/n)^{-1/2} + 1/2n$)

Table 6. Allelic frequencies for various isoenzyme loci for three accessions of *Lotus japonicus*

Accession	177		129		892	
Locus, allele	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*
Pgi1						
100	1	60	1	20	1	60
95	–		–		–	
Pgi2						
86	–		–		–	
80	1		1		–	
72	–		–		–	
62	–		–		–	
52	–		–		–	
Tpi1						
112	–	60	–	20	–	20
100	1		1		1	
Tpi2						
97	–		–		–	
90	1		1		1	
Pgm1, 2						
110	–	120	–	40	–	40
100	0.500, 0.094		0.500, 0.167		0.500, 0.094	
98	–		–		–	
95	0.500, 0.094		0.500, 0.167		0.500, 0.094	
85	–		–		–	
Pgm3						
82	–	60	–	20	–	60
76	1		1		1	
Idh1, 2						
100	0.688, 0.087	120	0.075, 0.094	40	0.717, 0.085	120
85	0.317, 0.087		0.925, 0.094		0.283, 0.085	
Mdh1						
112	–	60	–	20	–	60
100	1		1		1	
Mdh3						
85	–		1		0.983, 0.041	
68	–		–		–	
66	–		–		0.017, 0.041	
6-Pgdh1						
120	–	60	–	20	–	60
110	–		–		–	
100	1		1		1	
6-Pgdh2						
98	–	120	–	40	–	120
95	–		–		–	
90	–		–		–	
80	1		0.950, 0.080		1	
65	–		0.050, 0.080		–	
Me2						
152	–	60	–	20	–	60
100	–		–		–	
85	1		1		1	

N* = total number of alleles counted
C.I. 95% = multinomial 95% confidence interval + (1.96 $(p_i\,(1-p_i)/n)^{-1/2}+1/2n$)

Table 7. Allelic frequencies for various isoenzyme loci for three accessions of *Lotus corniculatus*

Accession	554 Wild population		710 'Viking'		764 'Leo'	
Locus, allele	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*
Pgi1						
100	1	120	1	112	1	120
95	–		–		–	
Pgi2						
86	–	240	–	224	–	240
80	0.183, 0.051		0.188, 0.053		0.325, 0.061	
72	0.417, 0.065		0.250, 0.059		0.445, 0.065	
62	0.092, 0.039		0.214, 0.056		0.087, 0.038	
52	0.308, 0.061		0.348, 0.065		0.142, 0.046	
Tpi1						
112	–	120	–	112	–	120
100	1		1		1	
Tpi2						
97	–		–		–	
90	1		1		1	
Pgm1, 2						
110	–	240	–	224	–	240
100	0.500, 0.065		0.500, 0.068		0.500, 0.065	
98	–		–		–	
95	0.400, 0.064		0.500, 0.068		0.500, 0.065	
85	0.100, 0.056		–		–	
Pgm3						
82	–	120	–	120	–	116
76	1		1		1	
Idh1, 2						
100	0.442, 0.065	240	0.454, 0.065	240	0.491, 0.066	232
85	0.558, 0.065		0.546, 0.065		0.509, 0.066	
Mdh1						
112	–	120	–	120	–	116
100	1		1		1	
Mdh3						
85	0.458, 0.093		–		–	
68	–		–		–	
66	0.542, 0.093		1		1	
6-Pgdh1						
120	0.067, 0.049	120	–	120	–	116
110	–		–		–	
100	0.933, 0.071		1		1	
6-Pgdh2						
98	–	240	–	240	–	232
95	–		–		–	
90	–		0.050, 0.030		–	
80	1		0.950, 0.030		1	
65	–		–		–	
Me2						
152	–	120	0.050, 0.043	120	–	116
100	–		–		–	
85	1		0.950, 0.043		1	

N* = total number of alleles counted
C.I. 95% = multinomial 95% confidence interval + (1.96 $(p_i\,(1-p_i)/n)^{-1/2}+1/2n$)

L. tenuis × *L. alpinus*. Again, because of the limited scope of the survey, we can only say that such hybrids were the possible ancestors of *L. corniculatus* but we cannot exclude other possibilities.

The exclusion of *L. uliginosus* from the ancestry of *L. corniculatus* by the enzyme data creates certain problems. Paramount among these is the fact that *L. uliginosus* is the only known source of tannins which are also found in *L. corniculatus* (Ross and Jones 1985). It is also the only known member of the *L. corniculatus* group to contain the phenolic delphinidin (on chromatographic band 14, Harney and Grant 1965). Can these discrepancies be resolved?

The *L. corniculatus* group is of relatively recent origin within the genus. Larsen and Zertova (1965) proposed that the course of evolution included the reduction of basic chromosome number from $x=8$ in ancient *Lotus* taxa, through $x=7$ to $x=6$. Furthermore, it appears that these events occurred independently in the Old World and New World (Grant and Sidhu 1967). The only certain fact is that the origin of *L. corniculatus* ($2n=4x=24$) occurred after the isolation of the *L. corniculatus* group $x=6$. It is possible that this polyploid occurred within a species complex prior to the isolation of distinct species.

It may be imagined that the loss of *L. uliginosus* alleles from the polyploid has occurred since the time of its formation, yet Gottlieb (1977) has pointed out that since there is not likely to be natural selection against electrophoretic mobility, isoenzyme data should strongly reflect phylogenetic origins. The only plausible method for the loss of *L. uliginosus* alleles from the pre-*corniculatus* hybrid would be extensive backcrossing to one of the parents in a hybrid swarm prior to the initial chromosome doubling event. This hypothesis would also have to assume that the presence of tannins and certain phenolics conferred a distinct advantage to progeny and therefore, was conserved.

The phenolic evidence presented by Harney and Grant (1965) does show many similarities between *L. uliginosus* and *L. corniculatus*. However, there is another source of data that shows dissimilarity between these taxa. Crompton (1982) conducted a survey of pollen morphology among species of the Loteae. He performed principal component analyses on several characters of pollen morphology for many taxa and found that *L. uliginosus* did not cluster with *L. tenuis* and *L. corniculatus* for these characters.

Crawford (1985) has stressed that isoenzyme data are most useful when viewed in the context of other biosystematic data. We have shown that *L. uliginosus* is distinct from *L. tenuis, L. alpinus, L. japonicus* and *L. corniculatus* for several isoenzyme alleles at several loci in a limited survey. Such a finding may be most useful as a warning that additional study using several types of data are required before the origin of *L. corniculatus* can be ascertained.

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