Theor Appl Genet (1989) 77:595–607

An isoenzyme study in the genus *Lotus* (Fabaceae). Experimental protocols and genetic basis of electrophoretic phenotype

J.V. Raelson and W.F. Grant

Department of Plant Science, P.O. Box 4000, Macdonald College of McGill University, Ste. Anne de Bellevue, Quebec H9X 1C0, Canada

Received September 20, 1988; Accepted September 28, 1988
Communicated by H.F. Linskens

Summary. An isoenzyme survey of some taxa in the genus *Lotus* (Fabaceae) was undertaken to increase the number of genetic markers available to breeders and to students of *Lotus* phylogeny. Twenty-one enzymes were examined using starch gel electrophoresis and nine buffer systems. Clear, consistent banding patterns were obtained for PGI, TPI, MDH, IDH (NADP), PGM, 6-PGDH, and ME. Clear but inconsistent banding patterns were obtained for FDP, G_3PDH (NADP), β-EST, LAP, MDH, DIA, and NADHDH. Phenotypes of the seven consistently resolved enzyme systems were obtained for different tissues for each of several genotypes at different stages of development. Variation in enzyme phenotypes of the same individuals under different growth conditions indicated the presence of different isozymic forms of these enzymes. Shoot tissue of plants over 6 weeks of age was found to be suitable material for further genetic studies, since phenotype for this tissue was constant despite changes in growing conditions. A formal genetic analysis of segregation and/or recombination of allozymes for the enzymes PGM, TPI, MDH, IDH, and 6-PGDH was undertaken. Isoenzyme phenotypes were examined for the diploids *L. alpinus* Schleich., *L. burttii* Sz. Borsos, *L. conimbricensis* Brot., *L. ornithopodioides* L., *L. tenuis* Waldst. et Kit., and *L. uliginosus* Schkuhr; and for the diploid interspecific hybrids *L. alpinus* × *L. conimbricensis*, *L. burttii* × *L. ornithopodioides*, and *L. japonicus* × *L. alpinus*. Several new loci were identified for *Lotus*, namely, *Idh1*, *Idh2*, *Mdh3*, *Pgi1*, *Pgi2*, *Tpi1*, *Tpi2*, and *6-Pdgh1*. Duplications of loci of *IDH*, *MDH*, *PGI*, and *6-PGDH* were detected in the diploid ($2n = 12$) interspecific hybrid *L. japonicus* × *L. alpinus*.

Key words: *Lotus corniculatus*

Introduction

Lotus corniculatus L. ($2n = 4x = 24$), Birdsfoot Trefoil, is a forage legume with many advantages for use in cool, wet climates. However, it has certain disadvantages that limit its use. Perhaps the most serious of these is seed-pod dehiscence (Seaney and Henson 1970; Grant and Marten 1985). Attempts have been made to overcome these limitations by means of selection within the species (Peacock and Wilsie 1957, 1960) and by interspecific hybridization (Phillips and Keim 1968; O'Donoughue and Grant 1988).

Breeding of birdsfoot trefoil is difficult due to a lack of qualitative genetic markers and due to the small chromosome size and similarity among karyotypes for different species (Zandstra and Grant 1968; Grant 1986). The few characters for which formal genetic analyses have been undertaken have been found to segregate in an imperfect tetrasomic manner in *L. corniculatus*. Qualitative characters that have been noted include cyanogenesis, leaf size, leaf color, keel tip color, flower striation, pubescence, self-incompatibility, phenolics, presence of tannins, and *Rhizobium* specificity (Dawson 1941; Donovan and McLennan 1964; Poostchi and MacDonald 1961; Buzzell and Wilsie 1963; Bubar and Miri 1965; Harney and Grant 1965; Ross and Jones 1985).

The purpose of the study reported here was to discover polymorphic isoenzyme loci that may be useful in characterization of *Lotus* genotypes and, thus, to increase the number of genetic markers available within the genus. Isoenzymes have provided a large number of genetic markers for other taxa (Tanksley and Orton 1983), but their study in *Lotus* has been limited. One of the few reports on isoenzyme phenotypes in *Lotus* was that of De Lautour et al. (1978). They examined *L. tenuis* and *L. corniculatus* for phenoloxidase, phosphatase, esterase

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

Taxon	Accession no.	Genotype	Use	Source
L. alpinus Schleich.	77	several	hybrid and parental species	Grant et al. (1962)
L. alpinus × *L. conimbricensis*	(77 × 126)	1	hybrid and parental species	Synthetic interspecific hybrid (O'Donoughue and Grant 1988)
L. burttii Sz. Borsos	303	several	hybrid and parental species	Royal Botanic Gardens, Edinburgh; Collector: B.L. Burtt; Origin: Peshawar, Pakistan
L. burttii Sz. Borsos × *L. ornithopodioides* L.	(303 × 100)	1	hybrid and parental species	Synthetic interspecific hybrid (O'Donoughue and Grant 1988)
L. conimbricensis Brot.	126	several	hybrid and parental species	Commonwealth Scientific and Industrial Research Organization, Canberra, Australia; Origin: Portugal
L. japonicus (Regel) Larsen × *L. alpinus*	(129 × 77)	23 28	pollen and progeny from selfing	Synthetic interspecific hybrid (Somaroo and Grant 1971)
L. ornithopodioides L.	100	several	hybrid and parental species	Commonwealth Scientific and Industrial Research Organization, Canberra, Australia, C.P.I. No. 19526, Collector: J.F. Miles; Origin: Tunisia
L. tenuis Waldst. et Kit	109	21 20	standard	Australia, C.P.I. no. 23788 Origin: Turkey
L. uliginosus Schkuhr	193	52	standard buffer assay life stages	Service de la Recherche Agronomique et de l'Experimentation Agricole, Rabat Morocco

and peroxidase enzymes. No attempt was made to determine segregation of phenotypes or the genetic basis for the isoenzyme patterns.

The findings of a series of experiments designed to define the protocols for obtaining reproducible isoenzyme phenotypes in *Lotus* and to examine the genetic basis for isoenzyme phenotype are presented in this paper. In the first experiment, 21 enzymes were examined using horizontal starch gel electrophoresis with 9 different buffer systems. Changes in enzyme phenotype associated with different life-cycle stages and different tissues were examined in a second experiment, to determine which differences can be associated with ontogenic factors. In a third experiment, the segregation of enzyme phenotypes among progeny of selfed heterozygous interspecific hybrids was examined to determine whether enzyme phenotype conformed to Mendelian expectation.

Materials and methods

Plant material

Isoenzyme electrophoresis was performed on both sporophyte leaf tissue and on pollen. The various plant taxa and genotypes and their use in this study are listed in Table 1. All material was obtained from the world *Lotus* collection maintained by W. F. Grant at Macdonald College of McGill University. Accession numbers and/or genotypes are given in parentheses following the name of the taxon.

Several individuals of *Lotus uliginosus* (accession no. 193), which originated in Morocco, were used for the initial enzyme-buffer experiment. These plants were grown in the greenhouse under short-day, non-flowering conditions with natural daylight. Different individuals from the same seed accession were used for the second life-stage experiment. These plants were grown under controlled conditions in a growth cabinet as follows:

Short-day regime: 12 h under cool white fluorescent light at an intensity of 83.5 μEinsteins sec^{-1} m^{-2} with a light temperature of 23 °C and a dark temperature of 20 °C. Electrophoresis was performed on entire seedlings (with two leaves), and on both shoots and roots of 6-week old plants.

Long-day (flowering) regime: 18 h under cool white fluorescent light with approximately 10% incandescent light at a total light intensity of 275 μEinsteins sec^{-1} m^{-2} with a light temperature of 24 °C and a dark temperature of 20 °C. Electrophoresis was performed on both shoots and roots of plants that were 12 weeks old.

Electrophoretic phenotypes for the enzymes PGI, MDH, IDH, and 6-PGDH were examined for two interspecific hybrid genotypes (23 and 28), derived from the cross *L. japonicus* × *L. alpinus*, and for the progeny obtained from selfing these individuals. Plants were selfed by tripping the flower keels with the tip of a toothpick to which a small piece of sand-paper was attached. Self-fertilized plants were then kept in a netted cage to avoid contamination by foreign pollen carried by insects. Seed was collected when pods became brown (approximately 35 days after pollination). From each parent, 125 seeds were planted in flats in the greenhouse. Electrophoresis was performed when the plants were approximately 2 months old.

Genetic recombination of electrophoretic phenotypes for the enzymes PGM and TPI was studied by examining two inter-

Table 2. Buffers used during preliminary experiments

Buffer	pH	Composition		Electrophoretic amperage	Reference
A	5.0	Electrode:	0.05 *M* L-histidine (free base) 0.024 *M* citric acid (monohydrate), adjusted to pH	25 mA	Cardy et al. (1981)
		Gel:	1:12 dilution of electrode buffer		
B	5.7	Electrode:	0.065 *M* L-histidine (free base) 0.02 *M* citric acid (monohydrate), adjusted to pH	25 mA	Cardy et al. (1981)
		Gel:	1:6 dilution of electrode buffer		
C	6.1	Electrode:	0.04 *M* citric acid (anhydrous), adjusted to pH with N-(3-amino propyl)-morpholine	50 mA	Clayton and Tertiaak (1972)
		Gel:	0.007 *M* citric acid (anhydrous), adjusted to pH with N-(3-amino propyl)-morpholine		
D	6.5	Electrode:	0.065 *M* L-histidine (free base) 0.002 *M* citric acid (anhydrous), adjusted to pH	25 mA	Cardy et al. (1981)
		Gel:	1:3 dilution of electrode buffer		
E	7.0	Electrode:	0.41 *M* citrate (trisodium salt) 0.41 *M* citrus acid (anhydrous), adjusted to pH	30 mA	Fildes and Harris (1966)
		Gel:	0.005 *M* histidine (hydrochloride monohydrate), adjusted to pH with NaOH		
F	7.1	Electrode:	0.1 *M* tris, 0.05 *M* citric acid (anhydrous), adjusted to pH, 0.001 *M* EDTA (disodium salt)	60 mA	Modified from Ayala et al. (1973)
		Gel:	0.1 *M* tris, 0.003 *M* citric acid (anhydrous), 0.001 *M* EDTA (disodium salt), adjusted to pH		
G	7.8	Electrode:	Same as in F, except pH	40 mA	Modified from Ayala et al. (1973)
		Gel:	Adjusted to pH 7.8 with tris or citric acid		
H	8.0	Electrode:	0.16 *M* tris, 0.05 *M* citric acid (anhydrous)	40 mA	Modified from Ayala et al. (1973)
		Gel:	7% electrode buffer		
I	8.1	Electrode:	0.06 *M* LiOH, 0.3 *M* boric acid, adjusted to pH 8.1	40 mA	Ridgway et al. (1970)
	8.4	Gel: 90%	0.03 *M* tris, 0.05 *M* citric acid (anhydrous), adjusted to pH 8.4; 10% electrode buffer		
J	8.1	Electrode:	0.3 *M* boric acid, adjusted to pH 8.2 with 0.1 *M* NaOH	40 mA	Schaal and Anderson (1974)
	8.8	Gel:	0.15 *M* tris, 0.005 *M* citric acid (monohydrate), adjusted to pH 8.8		

specific hybrids (produced by L. S. O'Donoughue), *L. burttii* × *L. ornithopodioides* and *L. alpinus* × *L. conimbricensis* (O'Donoughue and Grant 1988) and comparing their phenotype to that of their parents. Since these hybrids were infertile, it was not possible to study segregation of phenotypes among the progeny.

Two diploid individuals, *Lotus uliginosus* (193-52) and *L. tenuis* (109-21), were included in electrophoretic gels as standards to which the banding pattern of other samples could be compared.

Electrophoresis

Enzymes were extracted from young shoot tips and leaves and from roots by grinding approximately 100 mg (fresh weight) of tissue in 350 µl of an extraction buffer consisting of 0.1 *M* tris-HCl (ph 7.5), 1.0 m*M* EDTA (disodium salt), 10 m*M* $MgCl_2 \cdot H_2O$ and 10 m*M* KCl, to which 100 mg of polyvinyl pyrrolidone per mL of buffer were added (Gottlieb 1981). Just prior to grinding, ten µl of 2-mercaptoethanol were added. Tissue samples were kept cold during this procedure by placing on ice. This crude homogenate was applied directly to gels by means of small wicks made of Whatman No. 4 filter paper.

Pollen enzymes were extracted in a buffer of 0.1 *M* K_2HPO_4 (dibasic anhydrous) adjusted to pH 7.0 with HCl (Weeden and Gottlieb 1980), to which was added 50 µl glycerol and 50 mg of polyvinyl pyrrolidone per mL of buffer. 2-Mercaptoethanol was not used with the pollen phosphate buffer. Pollen was collected into microanalyzer vials by gently rubbing the keel tips of the flowers with a toothpick tipped with a small piece of sandpaper. Fifty µl of chilled buffer was added to approximately 20 µl of pollen, which was then allowed to soak overnight at 4 °C. Prior to application of the gels, the soaked pollen was either applied directly to the starch gels using filter paper wicks or else crushed with a teflon pestle designed to fit the microanalyzer vial.

In the initial experiment, each of 21 enzymes was assayed on 9 different buffer systems. The buffer systems ranged from pH 5.0–pH 8.8 (Table 2). The 21 enzymes are described in Table 3. The staining recipes for each enzyme have been detailed in various publications. Those used in this experiment were published by O'Malley et al. (1980), Vallejos (1983), and Cheliak and Pietel (1984).

Table 3. Quality of banding phenotypes for various enzymes using different electrophoresis buffer systems for *L. uliginosus* accession no. 193

Enzyme	Electrode/Gel buffer system (Table 1)									
	A	B	C	D	E	F	G	H	I	J
ACO	–	1	2	2	1	1	1	–	–	–
ALD	2	2	1	1	1	1	1	–	–	–
AAT	–	1	1	–	–	1	1	–	3	1
DIA	–	–	3	3	1	2	1	1	2	1
β-EST	2	1	3	3	1	2	2	2	–	–
FDP	1	1	3	1	1	2	1	1	2	1
G_3-PDH	–	3	1	2	2	2	2	1	1	–
G_2-DH	–	–	–	2	1	2	1	1	1	–
GDH	–	–	–	–	2	2	1	2	2	2
IDH	1	1	2	2	2	4	2	1	1	1
LDH	2	1	2	–	2	2	1	1	–	–
LAP	–	1	2	3	1	1	2	1	2	1
MDH	1	1	1	1	1	4	2	2	2	–
ME	–	2	2	4	2	2	1	–	–	–
MR	1	1	3	3	1	2	2	2	1	2
NADHDH	1	1	3	3	2	2	2	2	1	2
PGM	1	2	1	4	1	1	1	1	1	–
6-PGDH	1	2	2	4	2	2	1	–	–	–
PGI	1	1	1	1	1	1	2	1	4	1
SKDH	–	1	2	2	1	2	1	–	–	1
TPI	1	1	2	2	2	1	1	1	4	1

– No staining activity observed
1 Staining, but blurred, no distinct bands; 2 bands appear, but faint and difficult to read; 3 distinct, clear bands sometimes occur but not consistently; 4 distinct, clear bands consistently obtained

Genetic models

The formal description of loci and alleles is as follows: Each locus is named by giving the enzyme an abbreviation followed by a number which refers to the locus, for example, *Pgi2* or *Idh3*. Electrophoretic bands are denoted by their mobility relative to one commonly occurring band, which is arbitrarily assigned the relative mobility of 100. If, for example, the band designated as 100 migrates 25 mm from the origin during electrophoresis and a second band migrates 15 mm, then the second band is designated as $15/25 \times 100 = 60$. An allele at a given locus is designated by giving the locus name and number followed by a dash and the relative mobility of the band that is produced when that locus is homozygous for that allele, for example, *pgi2*-60. These formal allelic designations of loci are abbreviated for simplicity during much of the discussion. Informally, the most anodally migrating allele of a given set of alleles is called fast (*F*) and the most cathodally migrating allele is called slow (*S*). A third allele that migrates between *F* and *S* is called middle (*M*). A further abbreviation is used in figures and tables: the alleles of a genotype are numbered. If three alleles are present within a single genome, the genotype is given three numbers, if two are present, it is given two numbers. For three alleles the numbers designate the number of copies of *F, M,* and *S*, present. For example, the genotype *FFMS* would be coded as 211, and the genotype *FMSS* as 112. When only two alleles are present, only two numbers are used, for example, *FSSS* becomes 13.

The enzyme products of separate isozymic loci are typically isolated in separate subcellular compartments and are often found in distinct zones of the zymogram (Weeden 1983). Apart from the distinct loci that encode isolated subcellular forms of the same enzyme, there may also occur duplication of a given locus that encodes a given isozymic form. These duplications have been found in diploids but are most often associated with polyploidy and have been used as a criterion for ascertaining basic chromosome number (Gottlieb 1981). For dimeric enzymes such as PGI, the products of these duplicated loci can interact and form interlocus heterodimers (Tanksley et al. 1981; Weeden and Gottlieb 1980).

If such a duplicated locus is present in a genome, the 1:2:1 ratio of band density associated with the heterozygous genotype of a dimeric enzyme may be distorted because the alleles, and thus the polypeptide subunits, may not be present in a 1:1 ratio. Three or four different alleles may be present within a single genome. An individual with duplicated loci and heterozygous for three alleles will produce six different types of dimers, three homodimers and three heterodimers (all of these may not be distinguishable on the electrophoretic gel). Østergaard and Nielsen (1981) have demonstrated such complex phenotypes for PGI in tetraploid ryegrass (*Lolium perenne* L.).

The band density of any phenotype of any level of enzyme quaternary structure (dimer, trimer, tetramer, etc.) can be predicted from the genotype by the multinomial expansion:

$$[(f+m+s+\ldots)!/(f!\,m!\,s!\ldots)] \quad (F^f M^m S^s \ldots)$$

where upper case letters refer to the number of copies of a given allele: *F, M, S,* etc., within the genome and where the lower case letters refer to the number of polypeptides of a given type: *f, m, s,* etc., within a particular active enzyme molecule (May 1980).

The expected electrophoretic phenotypes for individuals with various doses of distinct alleles for a dimeric enzyme contained within their genomes have been calculated according to the formula and are presented graphically in Figs. 1 and 2. An allelic dose is equivalent to the number of copies of any allele; a diploid with a nonduplicated locus would have two allelic doses, a tetraploid or a diploid with a duplicated locus would have four, etc. Expected phenotypes when two distinct alleles are present are shown in Fig. 1, and those phenotypes expected when three distinct alleles occur are shown in Fig. 2. Only five bands, rather than the theoretical six, are shown for individuals that are heterozygous for three alleles because the heterodimer, *fs*, generally migrates the same distance as the homodimer, *mm*, and is, thus, not distinguishable on electrophoretic gels. The top of each diagram corresponds to the anodal end of a gel and the bottom corresponds to the cathode and origin of electrophoretic migration.

Pollen electrophoresis

It is possible to distinguish between isozyme components that occur in the cytoplasm and those that are sequestered within organelles using pollen electrophoresis (Weeden and Gottlieb 1980). If pollen is allowed to soak in extraction buffer only, cytosolic enzymes will be released. When the pollen is crushed in the same buffer, organelle-sequestered enzymes will also be released and the electrophoretic phenotype will show all isozymic forms. Comparison of zymograms between soaked and crushed pollen can distinguish between isozymic forms of an enzyme.

Results

Enzyme and buffer experiment

Seven enzymes produced clear consistent isoenzyme bands (Table 3). These are IDH (NADP) and MDH (tris-

citrate buffer, F, pH 7.1), Me, 6-PGDH, and PGM (histidine-citrate buffer, D, pH 6.5) and PGI and TPI (LiOH-borate buffer, I, pH 8.1–8.4). All subsequent research was confined to these seven enzyme systems. However, it should not be interpreted that the other enzymes could not necessarily be made to produce useful zymograms by adjusting experimental conditions.

Other enzymes, besides the seven mentioned above, did produce well-banded phenotypes, but these were not consistent. These enzymes were AAT, β-Est, FDP, G_3PDH (NADP), LAP, DIA, MR, and NADHDH. These enzymes can be considered to be useful but difficult to work with.

DIA, MR, and NADHDH, though theoretically distinct enzymes, produced identical banding phenotypes with our material, that is, the staining recipes that were used did not distinguish among the enzymes. MR was the easiest of these to use, and since they all produced the same phenotype, this enzyme is recommended for use in electrophoretic studies of *Lotus*.

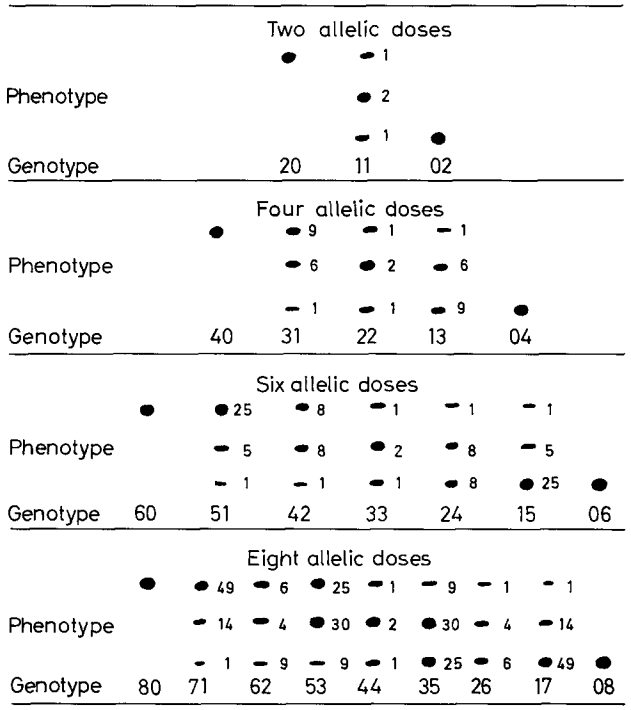

Fig. 1. Expected isoenzyme banding phenotypes for individuals with various doses of two alleles with distinct electromobility for a dimeric enzyme

Life cycle stage and tissue type

Graphic summaries of the different phenotypes found for individuals of *L. uliginosus* (acc. no. 193) are presented in Figs. 3a and 3b. In these diagrams, the most anodal band is arbitrarily assigned a relative mobility of 100, and all other bands are indicated as having a relative mobility which is a percentage of this most anodal distance. Phenotypes of both roots and shoots are shown for both short-day and long-day growing conditions.

Two types of variation were associated with ontogenic change. The first of these was the absence of a particular zone from the zymogram such as the lower bands of MDH (absent in seedlings and roots), IDH (absent in seedlings), and PGM (absent in roots). This variation with type of tissue suggests differential regulation of distinct loci. The second type of variation was the displacement of the position of certain entire groups of bands for root enzymes while the positions of the bands within the group remain constant.

The uppermost band for PGI phenotypes of roots is more anodal, as is the thick band for ME, while for MDH phenotypes, the three uppermost bands are all shifted towards the anode in roots. The mechanisms of

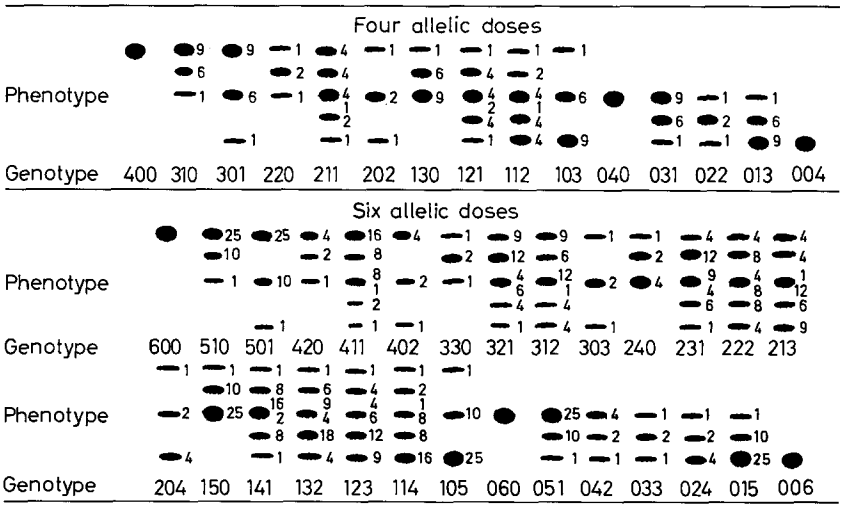

Fig. 2. Expected isoenzyme banding phenotypes for individuals with various doses of three alleles with distinct electromobility for a dimeric enzyme

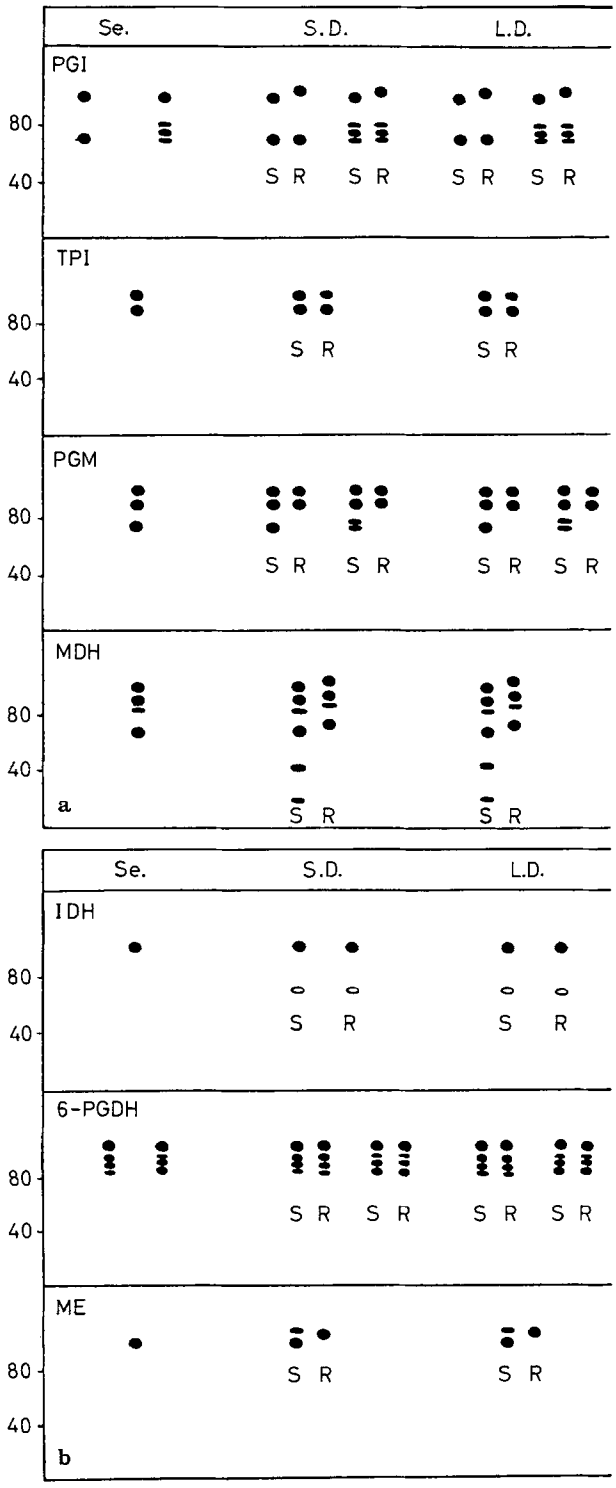

Fig. 3 a and b. Summary of isozyme phenotypes for *Lotus uliginosus* at different stages of development and in different tissues. **a** Isozymes PGI, TPI, PGM, and MDH. **b** Isozymes IDH, 6-PGDH, and ME. Se = seedling (two true leaves). S. D. = after 6 weeks of short-day growing conditions. L. D. = after 12 weeks with final 6 weeks under long-day growing conditions. S = shoot. R = root. The y axis shows relative mobility

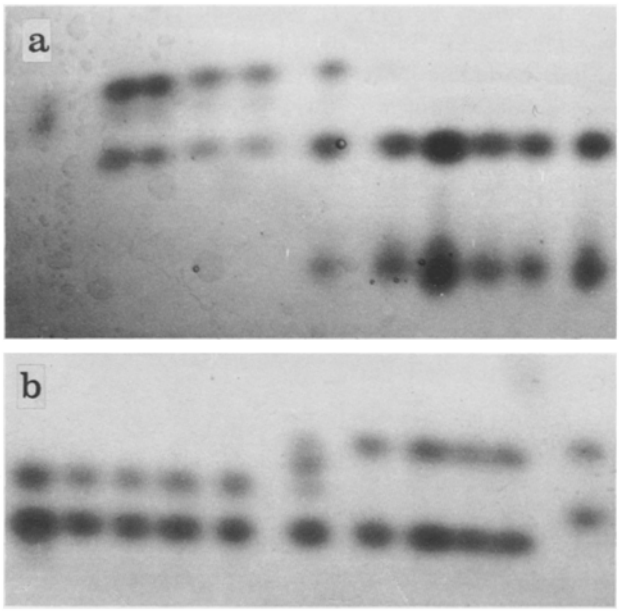

Fig. 4 a and b. Photographs of isoenzyme banding phenotypes. **a** For PGM for diploids *L. burttii* (acc. no. 303; *lanes 2–5*), *L. ornithopodioides* (acc. no. 100; *lanes 7–11*), and for the interspecific hybrid *L. burttii* × *L. ornithopodioides* (*lane 6*). *Lane 1* is *L. tenuis* (109-21), which is used as a standard. **b** For TPI for diploids *L. alpinus* (acc. no 77; *lanes 1–5*), *L. conimbricensis* (acc. no. 126; *lanes 7–10*), and for the interspecific hybrid *L. alpinus* × *L. conimbricensis* (*lane 6*). *Lane 11* is *L. uliginosus* (193-52), which is used as a standard

these shifts are uncertain (perhaps variation in non-peptide protein groups, glycosolation, or conformational change in distinct chemical environments). Of concern in this study is the fact that they are associated with ontogenic and not genetic factors. Shoot material of plants older than 6 weeks displayed consistently complete and invariable isoenzyme phenotypes for any individual regardless of day-length. Consequently, such material was used in subsequent genetic studies without further concern for day-length.

Pollen electrophoresis

Successful staining was obtained for pollen for the enzymes PGM and TPI. Isoenzyme phenotypes for sporophytes, and for soaked and crushed pollen for PGM and TPI for two genotypes of the interspecific hybrid *L. japonicus* × *L. alpinus*, are shown in Fig. 5a, b. The lowest or most cathodal band fails to appear in the zymogram for soaked pollen. It can be concluded from these results that the upper zone for PGM and TPI represent cytosolic isozymes, whereas the other zones for these enzymes correspond to organelle sequestered enzymes.

Genetic recombination and segregation of isoenzyme markers

PGM. The interspecific hybrid *L. burttii* × *L. ornithopodioides* was the only individual available that was heterozygous for PGM. This individual was infertile so that no segregation studies could be performed. The recombination of PGM alleles in the interspecific hybrid is shown in Fig. 5c. Each of the parents displayed a common PGM band (relative mobility 100) in addition to another band which was not shared. The hybrid phenotype contains all three parental bands but no new hybrid bands occur. This implies that PGM is a monomeric enzyme and agrees with previous reports (Warwick and Gottlieb 1985; Gottlieb 1981; Kahler and Lay 1985; Wolf et al. 1987).

TPI. The interspecific hybrid *L. alpinus* × *L. conimbricensis* was heterozygous for the more anodal (cytosolic) locus of TPI. This hybrid is also infertile, precluding segregation studies. The parental species each possessed distinct bands for this locus (relative mobility 100 and 110). The hybrid phenotype contains both of these bands plus a hybrid heterodimeric band between them (Fig. 5d). The hybrid band indicates that the cytosolic TPI enzyme is dimeric, a fact which also agrees with previous reports. This hybrid is the only individual that we have observed that is heterozygous for the cytosolic TPI locus.

PGI. The two genotypes (23 and 28) of the interspecific diploid hybrid *L. japonicus* × *L. alpinus* were heterozygous for the more cathodal (cytosolic) isozyme of PGI. These hybrids are fertile and were selfed to produce progeny. The segregation of phenotypes among these progeny was analyzed. The phenotypes and the number of each that were observed among the progeny of selfed *L. japonicus* × *L. alpinus* (23) are shown in Fig. 6. The parent displayed a five-banded phenotype indicating the unexpected presence of three alleles in at least two duplicated cytosolic loci in a diploid hybrid.

The more anodal (chloroplast-sequestered) locus of PGI has been called *Pgi1* and the cytosolic locus *Pgi2* in previous studies (Østergaard and Nielsen 1981; Gottlieb 1981). In compliance with this precedent, we name the duplicated cytosolic loci *Pgi2* and *Pgi3*. The three alleles of *Pgi2* and *Pgi3* are identified by the relative mobilities of their homodimeric bands. The most anodal (chloroplast or *Pgi1*) band has been arbitrarily assigned the relative mobility of 100 and the three alleles of the cytosolic loci become 72, 62, and 52. The presence of three alleles makes it possible to test for the segregation of the hypothesized two cytosolic loci separately (Fig. 7). The genetic model proposes segregating duplicated heterozygous loci (*F/S, F/M*). The choice of which locus is *Pgi2* and which is *Pgi3* is arbitrary. Figure 7 is a contingency table that allows the calculation of three Chi-square values:

$$\chi_1^2 = \sum[(o_i - e_1)^2/e_i]$$
$$\chi_2^2 = \sum[(o_j - e_j)^2/e_j]$$
$$\chi_3^2 = \sum[(o_{ij} - e_{ij})^2/e_{ij}]$$

χ_1^2 with 2 df tests whether the first locus is segregating according to the expected 1:2:1 Mendelian ratio, χ_2^2 also with 2 df tests whether the second locus is segregating according to a 1:2:1 ratio, and χ_3^2 with 4 df tests whether the two loci are segregating independently in a 1:2:1:2:4:2:1:2:1 ratio. The results of these tests are in accordance with a model of two linked duplicated loci.

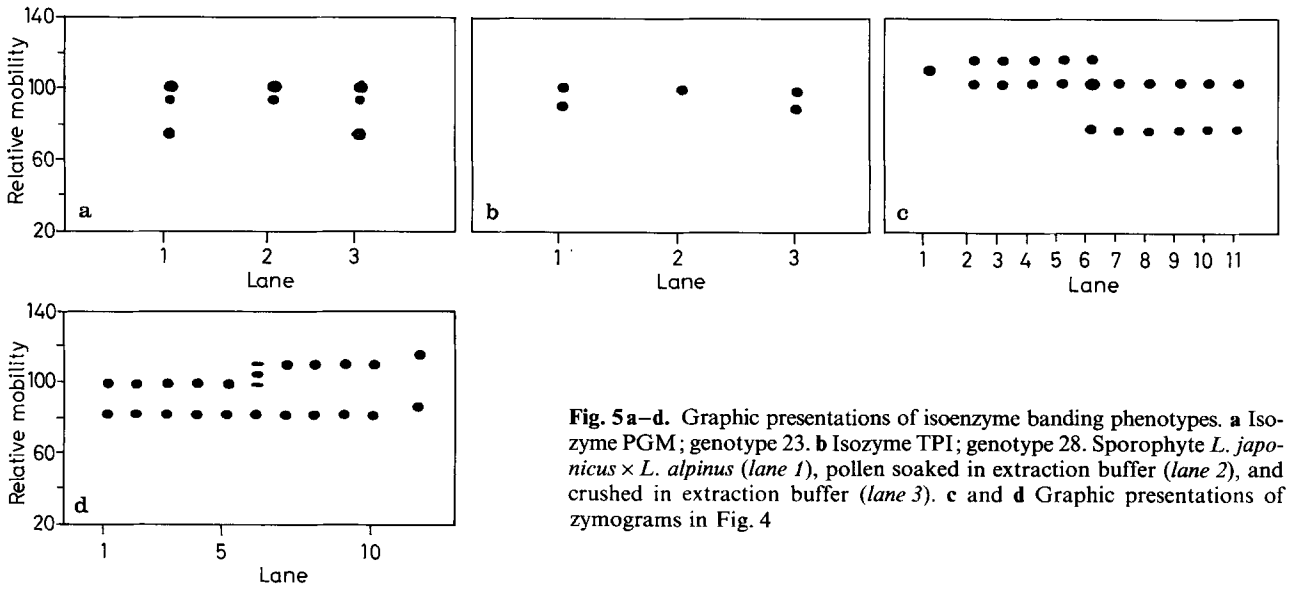

Fig. 5a–d. Graphic presentations of isoenzyme banding phenotypes. **a** Isozyme PGM; genotype 23. **b** Isozyme TPI; genotype 28. Sporophyte *L. japonicus* × *L. alpinus* (*lane 1*), pollen soaked in extraction buffer (*lane 2*), and crushed in extraction buffer (*lane 3*). **c** and **d** Graphic presentations of zymograms in Fig. 4

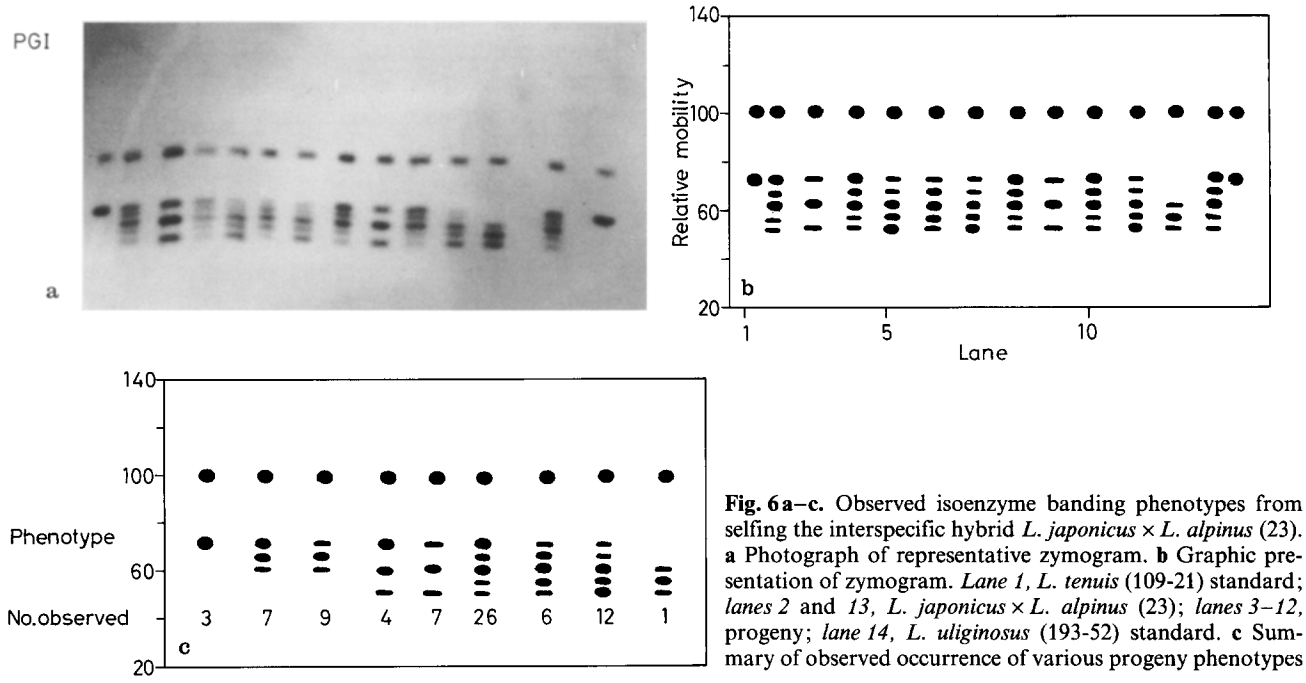

Fig. 6a–c. Observed isoenzyme banding phenotypes from selfing the interspecific hybrid *L. japonicus* × *L. alpinus* (23). **a** Photograph of representative zymogram. **b** Graphic presentation of zymogram. *Lane 1, L. tenuis* (109-21) standard; *lanes 2* and *13, L. japonicus* × *L. alpinus* (23); *lanes 3–12,* progeny; *lane 14, L. uliginosus* (193-52) standard. **c** Summary of observed occurrence of various progeny phenotypes

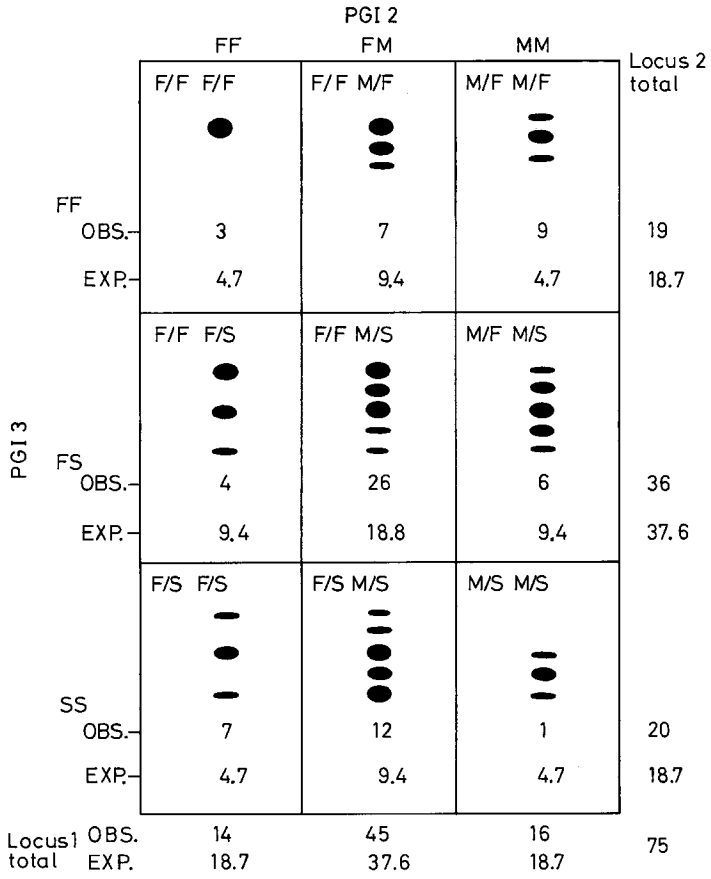

Fig. 7. Genetic model for segregation of duplicated PGI alleles in progeny from selfing the interspecific hybrid *L. japonicus* × *L. alpinus* (23). The expected frequencies and genotypes are those that would be produced if two independent loci, heterozygous for three distinct alleles (*F*/*M* and *F*/*S*), were segregating. Chi-square analysis indicated that the observed values corresponding to each locus occur in the expected Mendellian 1:2:1 ratio, but that the two loci are not independent. See text for further explanation. (*F* = PGI-72, the fastest or most anodal allele; *M* = PGI-62, the allele that migrates to the middle between fast and slow; *S* = PGI-52, the slowest or most cathodal allele). Chi-square 1 = 3.026 N.S.; Chi-square 2 = 0.082 N.S.; Chi-square 3 = 17.008, $0.01 > P > 0.001$

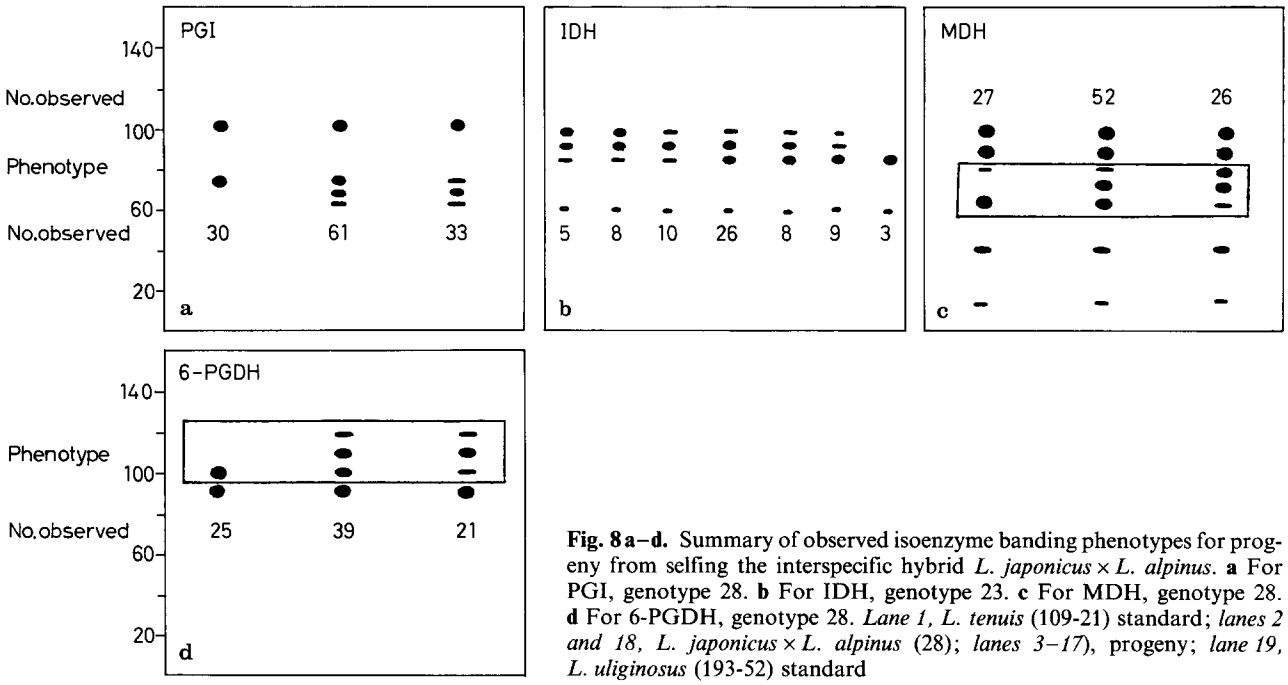

Fig. 8a–d. Summary of observed isoenzyme banding phenotypes for progeny from selfing the interspecific hybrid *L. japonicus* × *L. alpinus*. **a** For PGI, genotype 28. **b** For IDH, genotype 23. **c** For MDH, genotype 28. **d** For 6-PGDH, genotype 28. *Lane 1, L. tenuis* (109-21) standard; *lanes 2 and 18, L. japonicus* × *L. alpinus* (28); *lanes 3–17*), progeny; *lane 19, L. uliginosus* (193-52) standard

The progeny phenotypes of selfed *L. japonicus* × *L. alpinus* (28) and the observed number of progeny phenotypes are shown in Fig. 8a. The parent displayed a three-banded phenotype with a distorted band density ratio. This ratio was more in the range of 9:6:1 than 1:2:1. The latter would be expected for a heterozygote with only one cytosolic PGI locus. There are three progeny phenotypes: a single banded phenotype, a parental phenotype, and a three-banded phenotype with a 1:2:1 band density ratio. These results are consistent with a parental genotype with a duplicated locus, of which only one locus was heterozygous (*F/M, F/F*).

IDH. The interspecific hybrid *L. japonicus* × *L. alpinus* (23) was heterozygous for anodal loci of IDH. The progeny phenotypes from this selfing are presented in Fig. 8b, along with the observed occurrence of each progeny phenotype. The zymograms display two zones of enzyme activity, and upper, clearly stained zone (relative mobility, 100-85) and a lower, more cathodal zone (relative mobility, 60–62) which is poorly stained at this buffer pH. Allelic segregation was confined to the upper zone.

The progeny phenotypes observed from the selfing of the hybrid were complex. The parental phenotype was three-banded and unbalanced with denser cathodal bands. This fact again suggests the presence of two distinct alleles (100 and 85) in duplicated loci. This pattern for IDH segregation is complex and suggests that reported by Kiang and Gorman (1985) for soybean (*Glycine max* (L.) Merr.).

MDH: Parental and progeny phentoypes for the selfed interspecific hybrid *L. japonicus* × *L. alpinus* (28) for MDH were complex as would be expected for an enzyme with several isozymic forms (Weeden 1983). There was a single zone of segregation between relative mobilities 82 and 66 (Fig. 8c). This zone is characterized by a changeable three-banded phenotype, thus implying the presence of a dimeric molecule. The parental phenotype is unbalanced with the most cathodal band being more dense, and again suggesting interaction with a second locus. Given the complexity of the phenotype and the lack of knowledge concerning interaction among loci, it is not possible to hypothesize an exact genotype for the parent. The presence of three progeny phenotypes in a frequency ratio of 1:2:1, with the parental phenotype being most common among the progeny, would suggest the segregation of a single locus in the presence of a second homozygous locus.

6-PGDH. The interspecific hybrid *L. japonicus* × *L. alpinus* (28) was also heterozygous for the upper zone of 6-PGDH. The progeny phenotypes from the selfing of this hybrid are presented in Fig. 8d, along with the observed frequency of occurrence of each progeny phenotype. The observations are in accordance with a model that assumes two loci, one heterozygous for the alleles *6-Pgdh1*-120 and *6-Pgdh1*-100, and the other homozygous for the cathodal allele (*S/S*).

Independence of PGI, MDH, and 6-PGDH loci

The interspecific hybrid *L. japonicus* × *L. alpinus* (28) was heterozygous for loci of PGI, MDH, and 6-PGDH. If the

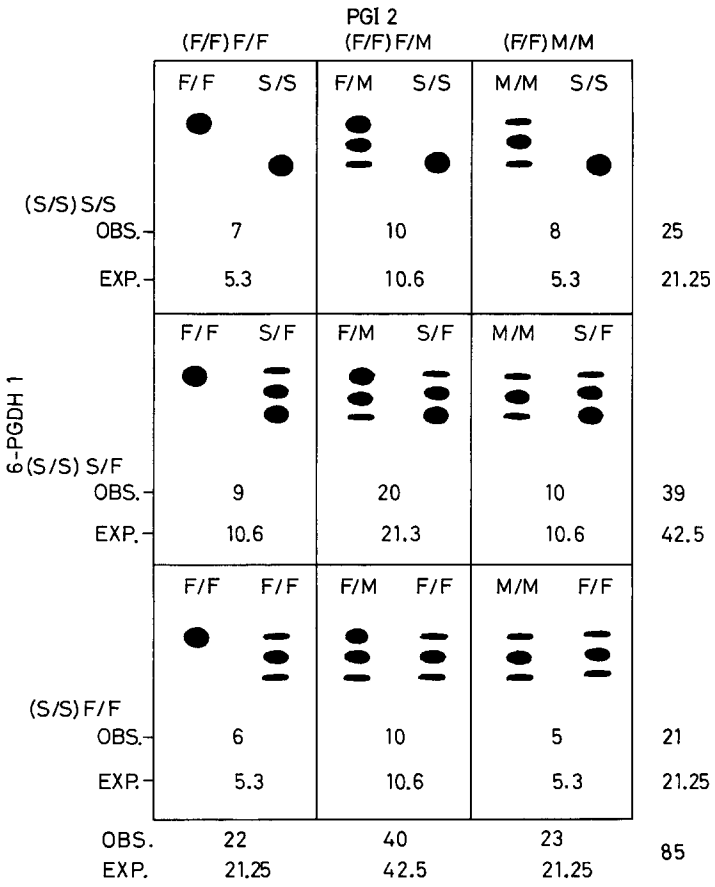

Fig. 9. Models for independent segregation of loci for PGI and 6-PGDH for progeny from selfing the interspecific hybrid *L. japonicus* × *L. alpinus* (28). The expected genotypes and frequencies are those that would be produced if two independent loci were controlling the phenotypes for the two enzymes. Chi-square analysis indicates no significant differences in observations from that expected under such a model. (PGI alleles: *F* = fast or most anodal, PGI-72; *M* = middle, PGI-62; 6-PGDH alleles: *F* = fast, 6-PGDH-118; *S* = slow or most cathodal, 6-PGDH-100). Chi-square 1 = *Pgi 2* 1:2:1, 2 D.F., 0.318 N.S.; Chi-square 2 = 6-PGDH 1:2:1, 2 D.F., 0.953 N.S.; Chi-square 3 = *Pgi 2* and 6-PGDH1 segregate independently, 4 D.F., 2.453 N.S.

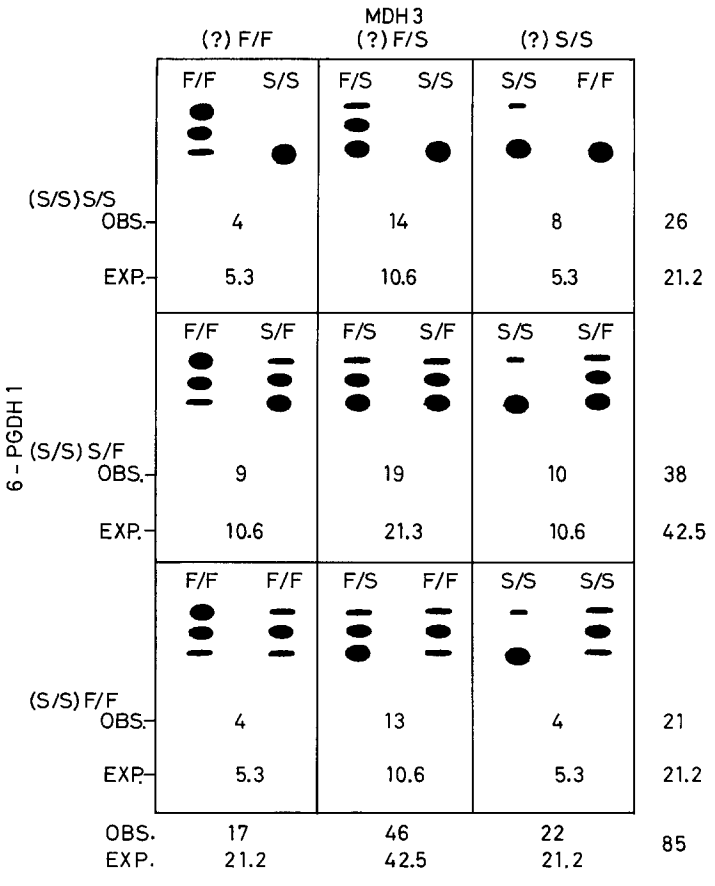

Fig. 10. Models for independent segregation of loci for MDH and 6-PGDH for progeny from selfing the interspecific hybrid *L. japonicus* × *L. alpinus* (28). The expected genotypes and frequencies are those that would be produced if two independent loci were controlling the phenotypes for the two enzmyes. Chi-square analysis indicates no significant differences in observations from that expected under such a model. (MDH alleles: *F* = fast, MDH-82; *S* = slow, MDH-66; 6-PGDH alleles: *F* = fast, 6-PGDH-118; *S* = slow, 6-PGDH-100). Chi-square 1 = MDH3 1:2:1, 2 D.F., 1.151 N.S.; Chi-square 2 = 6-PGDH 1:2:1, 2 D.F., 1.565 N.S.; Chi-square 3 = MDH3 and 6-PGDH1 segregate independently, 4 D.F., 4.490 N.S.

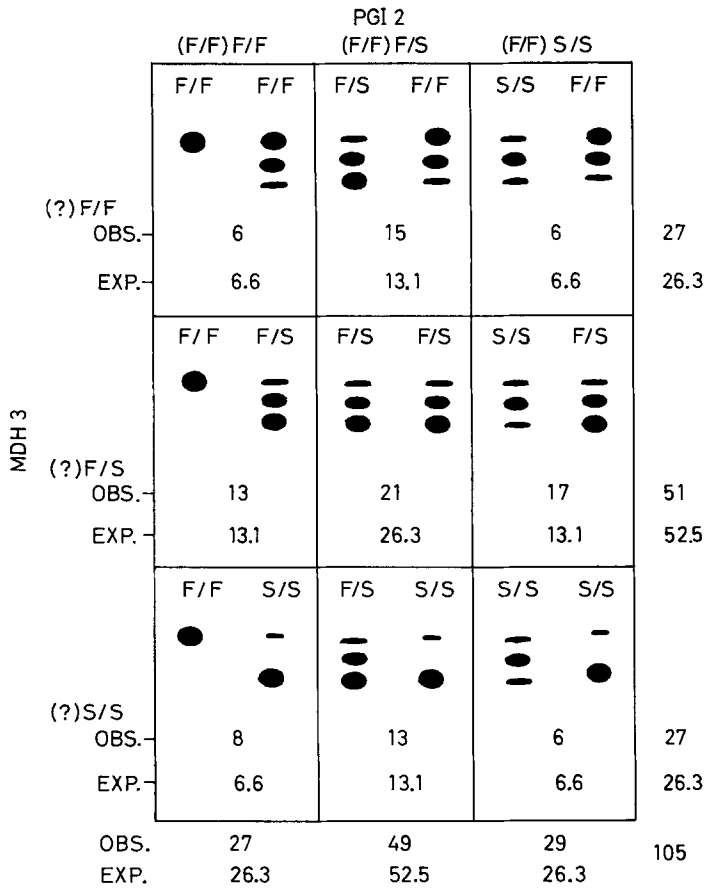

Fig. 11. Models for independent segregation of disomic loci for PGI and MDH in the selfed interspecific hybrid *L. japonicus* × *L. alpinus* (28.) The expected genotypes and frequencies are those that would be produced if two independent disomic loci were controlling the phenotypes for the two enzymes. Chi-square analysis indicated no significant differences in observations from that expected under such a model. (PGI alleles: *F*=fast or most anodal, PGI-72; *M*=middle, PGI-62; MDH alleles: *F*=fast, MDH-82; *S*=slow, MDH-66). Chi-square 1=*Pgi2* 1:2:1, 2 D.F., 0.529 N.S.; Chi-square 2=MDH3 1:2:1, 2 D.F., 0.080 N.S.; Chi-square 3=*Pgi 2* and MDH3 segregate independently, 4 D.F., 2.967 N.S.

plausible models for duplicated loci are assumed for each of these enzymes, then it is possible to test for the independence of segregation of these loci by comparing phenotypes for each enzyme among progeny. Independence of segregation among these loci was tested using Chi-square analysis (Figs. 9–11). All Chi-square values were nonsignificant. The analysis suggests a model of three unlinked loci for these enzymes. It was not possible to determine linkage relationships between the heterozygous and homozygous loci for each enzyme.

Discussion

Characterization of loci

The enzymes PGM and TPI possess distinct cytosolic and organelle sequestered zones of activity. The cytosolic enzymes are represented by upper, more anodal bands of PGM and TPI. These results agree with those reported by Weeden and Gottlieb (1980) for pea (*Pisum sativum* L. cv. Alaska).

The complex phenotypes of IDH and MDH were not resolved into their organelle and cytosolic components. However, a single zone of allozymic activity was identified for each. Two isozymes of IDH were identified, corresponding with results presented for soybean by Kiang and Gorman (1985). They demonstrated that the more anodal zone of the phenotype was cytosolic and controlled by two loci that they named *Idh1* and *Idh2*, and that the lower zone represented an enzyme form sequestered within mitochondria.

Two isozymes of 6-PGDH were identified with segregation confined to the anodal region for the individuals studied here.

Allozymic recombination and/or segregation was displayed for the cytosolic loci of TPI, for two loci of PGM, for the cytosolic loci of PGI, for the presumably cytosolic loci of IDH, for the most anodal region of 6-PGDH, and for the middle zone of MDH. The newly identified loci and alleles for *Lotus* are designated as follows: *Tpi1*-100 and -110, *Pgi2,3*-82, -72, -62, and -52; *Idh1,2,3,4*-100 and -85; *Mdh3*-82 and -66; and *6Pgdh1*-120 and -100.

Duplicated loci

The presence of duplicated isoenzyme loci for PGI, 6-PGDH, and IDH at the diploid level in the two genotypes of the interspecific hybrid *L. japonicus* × *L. alpinus* was an unexpected finding that leads to interesting hypotheses concerning the origin of the duplication.

Gottlieb (1982) reviewed the subject of duplication of isoenzyme loci and pointed out that duplication may arise from translocations between chromosomes in diploid genomes or from ancient polyploid events.

It can be asked whether such mechanisms were responsible for the duplications found during this study, that is, do the duplications represent ancient polyploidy in *Lotus*, or might they have arisen from an inherited duplication from one of the diploid parents? The answer to both of these questions would appear to be negative. Firstly, basic chromosome numbers within the tribe Loteae are 5, 6, 7, and 8 (Darlington and Wylie 1955; Grant 1986). Within the entire Papilionaceae subfamily, the lowest reported chromosome number is 5, which has been found in certain species of *Vicia* and *Hedysarum* (Darlington and Wylie 1955). Secondly, no evidence exists for duplication of the *Pgi2* locus in any of the diploid species of *Lotus* (data not presented here), including *L. japonicus* and *L. alpinus*. All heterozygotes observed among the diploid species displayed the 1:2:1 band density ratio expected for a single, unduplicated locus coding for a dimeric enzyme.

If the interspecific hybrids could not have inherited the duplications from parental species, then the duplications must have arisen as a result of the hybrid condition. An example of a possible mechanism to explain duplication of loci in an interspecific hybrid would be unequal crossing-over between structurally rearranged homoelogous chromosomes. These hypothesized structural differences are not enough to prevent synapsis of homoeologues in the hybrid (as the fertility of the hybrid attests). A pericentric inversion, for example, could rearrange the *Pgi2* locus along with adjacent chromosomal material. Unequal crossing-over within the inversion would produce duplicated as well as inviable deficient chromosomes. Recombination would result in viable progeny with two chromosomes with duplications.

It might be objected that this model would explain the presence of only two alleles for the duplicated loci but could explain the presence of three distinct alleles for *Pgi2* within the hybrid. In reply to this objection, we point out that the material that we examined was descendant from parents representing several distinct crossing events (Somaroo 1970).

Acknowledgements. Financial support from the Quebec "Fonds pour la formation de chercheurs et l'aide la recherche" for a Postgraduate Fellowship to J. V. Raelson, and an operating grant from the Natural Sciences and Engineering Research Council of Canada to W. F. Grant for cytogenetic studies on the genus *Lotus* are gratefully acknowledged.

References

Ayala FJ, Hedgecock DG, Zumwalt S, Valentine JW (1973) Genetic variation in *Tridacna maxima*, an ecological analog of some unsuccessful evolutionary lineages. Evolution 27: 177–191

Bubar JS, Miri RK (1965) Inheritance of self-incompatibility and brown keel tip in *Lotus corniculatus* L. Nature 205: 1035–1036

Buzzell RI, Wilsie CP (1963) Genetic investigations of brown keel tip color in *Lotus corniculatus* L. Crop Sci 3:128–130

Cardy BJ, Stuber CW, Goodman MM (1981) Techniques for starch gel electrophoresis of enzymes from maize (*Zea mays* L.). Institute fo Statistics Mimeographed Series No. 1317, North Carolina State University, Raleigh/NC, 31 pp

Cheliak WM, Pitel JA (1984) Techniques for starch gel electrophoresis of enzymes from forest tree species. Information Report PI-X-42, Petawawa National Forestry Institute, Can For Serv, 49 pp

Clayton JW, Tertiaak DN (1972) Amine citrate buffers for pH control in starch gel electrophoresis. J Fish Res Board Can 29:1169–1172

Darlington CD, Wylie AP (1955) Chromosome atlas of flowering plants. Hafner, New York, 519 pp

Dawson CDR (1941) Tetrasomic inheritance in *Lotus corniculatus* L. J Genet 42:49–73

De Lautour G, Jones WT, Ross MD (1978) Production of interspecific hybrids in *Lotus* aided by endosperm transplants. N Z J Bot 16:61–68

Donovan LS, McLennan HA (1964) Further studies on the inheritance of leaf size in broadleaf birdsfoot trefoil, *Lotus corniculatus* L. Can J Genet Cytol 6:164–169

Fildes RA, Harris H (1966) Genetically determined variation of adenylate kinase in man. Nature 209:261–263

Gottlieb LD (1981) Electrophoretic evidence and plant populations. Prog Phytochem 7:1–46

Gottlieb LD (1982) Conservation and duplication of isozymes in plants. Science 216:373–380

Grant WF (1986) The cytogenetics of *Lotus* (Leguminosae). J Nat Hist 20:1461–1465

Grant WF, Marten GC (1985) Birdsfoot trefoil. In: Heath ME, Metcalfe DS, Barnes RF (eds) Forages: Science of grassland agriculture, 4th ed. Iowa State University Press, Ames/IA, pp 98–108

Grant WF, Bullen MR, Nettancourt D de (1962) The cytogenetics of *Lotus*. I. Embryo-cultured interspecific diploid hybrids closely related to *L. corniculatus*. Can J Genet Cytol 4:105–128

Harney PM, Grant WF (1965) A polygonal presentation of chromatographic investigations on the phenolic content of certain species of *Lotus*. Can J Genet Cytol 7:40–51

Kahler AL, Lay CL (1985) Genetics of electrophoretic variants in the annual sunflower. J Hered 76:335–340

Kiang YT, Gorman MB (1985) Inheritance of NADP-active isocitrate dehydrogenase isozymes in soybeans. J Hered 76:279–284

May B (1980) The salmonid genome. Evolutionary restructuring following a tetraploid event. PhD Thesis, Pennsylvania State University, State College/PA

O'Donoughue LS, Grant WF (1988) New sources of indehiscence for birdsfoot trefoil (*Lotus corniculatus*, Fabaceae) produced by interspecific hybridization. Genome 30:459–468

O'Malley D, Wheeler NC, Guries RP (1980) A manual for starch gel electrophoresis. University of Wisconsin, Department of Forestry, Staff paper no. 11, Madison/WI, 16 pp

Østergaard H, Nielsen G (1981) Cultivar identification by means of isoenzymes. I. Genotypic survey of *Pgi2* locus in tetraploid ryegrass. Z Pflanzenzücht 87:121–132

Peacock HA, Wilsie CP (1957) Selection for resistance to seed pod shattering in birdsfoot trefoil (*Lotus corniculatus* L.). Agron J 49:429–431

Peacock HA, Wilsie CP (1960) Selection for vegetative vigor and seed setting in birdsfoot trefoil (*Lotus corniculatus*). Agron J 52:321–324

Phillips RL, Keim WF (1968) Seed pod dehiscence in *Lotus* and interspecific hybridization involving *L. corniculatus* L. Crop Sci 8:18–21

Poostchi I, MacDonald HA (1961) Inheritance of leaf color in broadleaf birdsfoot trefoil. Crop Sci 1:327–328

Ridgway GJ, Sherburne SW, Lewis RD (1970) Polymorphisms in the esterases of Atlantic herring. Trans Am Fish Soc 99:147–151

Ross MD, Jones WT (1985) The origin of *Lotus corniculatus*. Theor Appl Genet 71:284–288

Schaal BA, Anderson WW (1974) An outline of techniques for starch gel electrophoresis of enzymes from the American oyster, *Crassostrea virginica* Gmelin. Georgia Marine Sci Ctr Tech Rept Ser 74-3, 17 pp

Seaney RR, Henson PR (1970) Birdsfoot trefoil. Adv Agron 22:119–157

Somaroo BH (1970) A cytogenetic study of interspecific diploid hybrids and amphidiploids in the genus *Lotus*. PhD Thesis, McGill University, Montreal, Quebec, Canada 295 pp

Somaroo BH, Grant WF (1971) Interspecific hybridization between diploid species of *Lotus* (Leguminosae). Genetica 42:353–367

Tanksley SD, Orton TJ (1983) Isozymes in plant genetics and breeding, part B. Elsevier, Amsterdam New York, 472 pp

Tanksley SD, Zanier D, Rick CM (1981) Evidence for extensive overlap of sporophytic and gametophytic gene expression in *Lycopersicon esculentum*. Science 213:453–455

Vallejos CE (1983) Enzyme activity staining. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, part A. Elsevier, Amsterdam New York, pp 469–516

Warwick SI, Gottlieb LD (1985) Genetic divergence and geographic speciation in *Layia* (Compositae). Evolution 39: 1236–1241

Weeden NF (1983) Plastid isozymes. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, part A. Elsevier, Amsterdam New York, pp 139–156

Weeden NF, Gottlieb LD (1980) Isolation of cytoplasmic enzymes from pollen. Plant Physiol 66:400–403

Wolf PG, Haufler CH, Sheffield E (1987) Electrophoretic evidence for genetic diploidy in the bracken fern (*Pteridium aquilinum*). Science 236:947–949

Zandstra II, Grant WF (1968) The biosystematics of the genus *Lotus* (Leguminosae) in Canada. I. Cytotaxonomy. Can J Bot 46:557–583