AN ISOENZYME STUDY IN THE GENUS LOTUS (FABACEAE)

by

John Verner Raelson, B.A., B.Sc., M.Sc.

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

John Verner Raelson ©
Department of Plant Science
Macdonald College
McGill University
Montreal

November, 1987

ISBN 0-315-45972-7

ABSTRACT

Ph.D. JOHN V. RAELSON Plant Science

AN ISOENZYME STUDY IN THE GENUS LOTUS (FABACEAE)

An isoenzyme survey of several taxa within the genus Lotus was undertaken to provide markers for genetic research and to test hypotheses concerning the phylogenetic origin of Lotus corniculatus L. A preliminary study identified seven enzyme systems PGI, TPI, PGM, MDH, IDH, 6-PGDH and ME, that produced consistent clear phenotypes in L. uliginosus Schkuhr. Variation in phenotype with tissue type and stage of development suggested the presence of several isozyme zones in the phenotypes. Enzyme phenotype was constant for shoot tissue of plants older than six weeks. A second study examined recombination and segregation of isoenzyme phenotypes in interspecific hybrids, allo- and autopolyploids, and in L. corniculatus. Duplication and quadruplication of pgi2 loci in hybrids, amphidiploids, and in L. corniculatus was used as evidence that the latter is a segmental allotetraploid. A third study surveyed the occurrence of various isoenzyme alleles in L. alpinus Schleich., L. japonicus (Regel) Larsen, L. tenuis Waldst. & Kit., L. uliginosus and L. corniculatus. Lotus uliginosus had unique distinct alleles for several enzymes that did not occur in the other species. This evidence argues against the involvement of L. uliginosus in the origin of L. corniculatus.

RESUME

Ph.D. JOHN V. RAELSON Plant Science

UNE ETUDE D'ISOENZYMES DANS LE GENRE LOTUS (FABACEAE)

Un examen d'isoenzymes de plusieurs taxa du genre Lotus a été entrepris afin de fournir des marqueurs génétiques et de vérifier certaines hypothèses concernant l'origine phylogénétique de Lotus corniculatus L. Une étude préliminaire a permis d'identifier sept systèmes d'enzymes: PGI, TPI, PGM, MDH, IDH, 6-PGDH et ME, lesquels ont produit des phénotypes clairs avec régularité chez L. uliginosus Schkuhr. Des variations phénotypiques sont apparues selon le stade de développement et le type de tissu utilisés suggérant la présence de plusieurs zones d'isozymes dans les phénotypes. Le phénotype enzymatique fut régulier dans les tissus des pousses de plantes agées de plus de six semaines. Une deuxième étude a permis d'examiner la ségrégation et la recombinaison des phénotypes d'isoenzymes dans des hybrides interspécifiques, dans des allo- et autotétraploïdes ainsi que chez L. corniculatus. La duplication et la quadruplication des loci Pgi2 chez les hybrides, les amphidiploïdes et L. corniculatus ont mis en évidence la nature allotétraploïde segmentaire de ce dernier. Une troisième étude a permis l'examen de plusieurs allèles d'isoenzymes chez L. alpinus Schleich., L. japonicus (Regel) Larsen, L. tenuis Waldst. & Kit., L. uliginosus and L. corniculatus. Lotus uliginosus a présenté des allèles distincts pour plusieurs enzymes, lesquels étaient absents chez les autres espèces. Cette dernière constatation rend peu probable la participation de L. uliginosus quant à l'origine de L. corniculatus.

ACKNOWLEDGMENTS

I would, first of all, like to give heartful thanks to Dr. W. F. Grant, my supervisor. Dr. Grant has continually provided advice and support, not only during the course of my doctoral studies, but throughout the years I have been at Macdonald College. He has gone well beyond that which is expected in the student-supervisor relationship in providing assistance and understanding.

I would also like to thank Dr. Dan Schoen of McGill University, Biology Department, and Dr. Suzanne Warwick of the Biosystematics Research Institute, Agriculture Canada, Ottawa, who generously assisted me in establishing the electrophoretic protocols. Thanks also to Dr. John Bain who provided workspace in his already busy and crowded laboratory. I would especially like to thank Dr. Marcia Waterway who acted as my mentor for isoenzyme electrophoresis and who freely offered many good suggestions and stimulation with her contagious enthusiasm for research. I would also like to thank Dr. Miles Bullen who was a very pleasant boss when I was a Teaching Assistant for the Genetics Laboratory course.

Thank you also Philippe Lemaître and Karen Starkie for your able assistance in the daily rigors of research. I also give special thanks to Pierre St. Marseille, a fellow Graduate Student, for his good humor, helpfulness, and interest in theoretical discussion.

Finally, I sincerely thank my parents, Margaret and Verner Raelson, for their continued understanding, love, and support throughout my academic career.

CLAIM TO ORIGINAL RESEARCH

Isoenzyme electrophoresis is a well established technique which has been used as an aid in the biosystematic analyses of a number of taxa. However, to the best of the author's knowledge, it has not previously been applied on a systematic basis in the genus Lotus. The only previously reported use of isoenzymes in the genus Lotus that the author could find was that of a study by De Lautour et al. (1978). These researchers obtained phenotypes of phenoloxidase, phosphatase, esterase, and peroxidase for tetraploid Lotus tenuis and L. corniculatus, and for hybrids between these taxa. They used the isozyme phenotypes to ascertain that hybrid plants were obtained and no attempt was made to analyze segregation of phenotypes or to establish the genetic basis of these patterns.

Original findings made in the course of this research include the following:

1. Determination of correct buffers for obtaining consistent isoenzyme phenotypes for PGI, PGM, IDH, MDH, ME, 6-PGDH, and TPI within the Lotus corniculatus group.

2. Analysis of the genetic basis for phenotype for PGI, IDH, MDH, and 6-PGDH.

3. Determination of cytosolic and organelle sequestered isozyme forms of PGM, and TPI within the genus Lotus.

4. Discovery of duplication of the Pgi2 locus within the interspecific

hybrid L. alpinus X L. japonicus that did not occur within the other diploid taxa, and of discovery of the quadruplication of this locus within L. corniculatus.

5. Presentation of additional isoenzyme evidence that L. corniculatus is a segmental allotetraploid.

6. Presentation of isoenzyme evidence that appears to discount L. uliginosus as a possible diploid ancestor of L. corniculatus.

7. Demonstration that PGM and PGI phenotypes can be used to confirm the hybrid nature of the putative hybrids within Lotus.

8. Demonstration that PGI phenotypes can be used to distinguish between different accessions or cultivars of L. corniculatus based upon frequencies of Pgi2 alleles.

TABLE OF CONTENTS

FORWARD

This thesis is submitted under the form of three manuscripts according to the conditions outlined in the Guidelines Concerning Thesis Preparation which are as follows:

"The Candidate has the option, subject to the approval of the Department, of including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication. In this case the thesis must still confore to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (experimental and design data as well as descriptions of equipment) must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported. Abstract, full introduction and conclusion must be included, and where more than one manuscript appears, connecting texts and common abstracts, introduction and conclusions are required. A mere collection of manuscripts is not acceptable; nor can reprints of published papers be accepted.

While the inclusion of manuscripts coauthored by the candidate and others is not prohibited by McGill, the candidate is warned to make an explicit statement on who contributed to such work and to what extent, and supervisors and others will have to bear witness to the accuracy of such claims before the Oral Committee. It should also be noted that the task of the External Examiner is made much more difficult in such cases, and it is in the Candidate's interest to make authorship responsibilities perfectly clear."

All three manuscripts submitted within this thesis were coauthored by Dr. W. F. Brant, the candidate's research supervisor. The second manuscript was also coauthored by P. C. Lemaître and K. M. Starkie.

Dr. Brant undertook the administrative aspects of the research, obtaining funding and laboratory space, as well as being available for consultation concerning the theoretical aspects of the research. Mr. P. C.

Lemaître and Ms. K. M. Starkie were summer student technicians working in the Genetics Laboratory of Macdonald College. Mr. Lemaître provided assistance in loading gels and other routine tasks in the daily performance of electrophoresis. Ms. Starkie provided assistance in the photography and graphic presentations within the manuscripts. It was to thank them, and to acknowledge their assistance, that their names were included in the second manuscript.

The Candidate was solely responsible for all design and establishment of experimental procedure, as well as of analysis and presentation of results. The manuscripts were written entirely by him and all graphics are of his design.

The first two manuscripts will be submitted for publication to Genome and the third manuscript will be submitted to Theoretical and Applied Genetics.

Enzymes used in this study

Enzyme
ACO, Aconitase (EC 4.2.1.3)
ALD, Aldolase (EC 4.1.2.13)
AAT (GOT), Aspartate aminotransferase (EC 2.6.1.1)
β-EST, β-Esterase (EC 3.1.1.1)
DIA, Diaphorase (EC 1.6.4.3)
FDP, Fructose 1,6-diphosphatase (EC 3.1.3.11)
G_3PDH, Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)
G_2DH, Glycerate-2-dehydrogenase (EC 1.1.1.29)
GDH, Glutamate dehydrogenase (EC 1.4.1.2)
IDH, Isocitrate dehydrogenase (EC 1.1.1.42)
LDH, Lactate dehydrogenase (EC 1.1.1.27)
LAP, Leucine aminopeptidase (EC 3.4.11.1)
MDH, Malate dehydrogenase (EC 1.1.1.37)
ME, Malic enzyme (EC 1.1.1.40)
MR, Menadione reductase (EC 1.6.99.2)
NADH, Nicotinamide adenine dinucleotide dehydrogenase (EC 1.6.99.3)
PGM, Phosphoglucomutasetase (EC 2.7.5.1)
6-PGDH, 6-Phosphogluconate dehydrogenase (EC 1.1.1.44)
PGI, Phosphoglucose isomerase (EC 5.3.1.9)
SKDH, Shikimic dehydrogenase (EC 1.1.1.25)
TPI, Triosephosphate isomerase (EC 5.3.1.1)

GENERAL INTRODUCTION

Birdsfoot Trefoil (Lotus corniculatus L.) is a tetraploid (2n = 24) forage legume which has distinct advantages for forage production on wet, acid, or shallow soils. On such sites, it outproduces alfalfa (Medicago sativa L.) and is longer lived than alternative legume crops such as red and white clover. The agronomic advantages of birdsfoot trefoil have been well reviewed (Seaney and Henson 1970; Grant and Marten 1985).

Despite these advantages, birdsfoot trefoil has two serious disadvantages that limit its use. The first of these is poor seedling vigor, making stand establishment difficult, the second is poor seed yield resulting from indeterminate growth and seed pod dehiscence. Some progress has been achieved in breeding for increased vigor through recurrent selection (Twamley 1971, 1974; Draper and Wilsie 1965), and a genetic component for determinate growth has been identified (Buzzell and Wilsie 1964. However, no complete resistance to pod dehiscence has been found within the species. Because of this fact, much emphasis has been placed upon interspecific hybridization for the improvement of seed shattering in birdsfoot trefoil (Gershon 1961; Phillips and Keim 1968; Somaroo and Grant 1972; O'Donoughue 1986).

Genetic improvement of birdsfoot trefoil has been hindered by a lack of qualitative genetic markers. The few genetically controlled characters that have been studied include cyanogenesis (Dawson 1941), large versus small leaf size (Donovan 1959; Donovan and McLennan 1964), leaf color (Poostchi and MacDonald 1961), keel tip color (Buzzell and Wilsie 1963; Bubar and Miri

1965) and self incompatibility (Buzzell and Wilsie 1963). These characters have all been found to segregate in a tetrasomic manner. This paucity of characters hinders research in two distinct ways. Firstly, with few qualitative characters, selection can be made only on the desired quantitative characters such as vigor or seed yield. Thus, selection can only be made after a growing season in the field, and the shortcut of selecting for some correlated qualitative character can not be carried out. Secondly, the lack of genetic markers has hindered the determination of the phylogenetic origin of the tetraploid cultivated species. The determination of the origin of Lotus corniculatus would be useful in improving birdsfoot trefoil through interspecific hybridization.

The task of introducing the desired genetic material into the cultivated species becomes complex, because birdsfoot trefoil is a tetraploid and all known Lotus species with non-dehiscent pods are diploids. The strategy for such a transferal of genetic material would be to cross the wild non-dehiscent species with one of the diploids in the Lotus corniculatus group, and then double the chromosome number of this hybrid with colchicine and cross the resulting amphidiploid with birdsfoot trefoil (O'Donoughue 1986; Somaroo and Grant 1972). If one of the diploid parents of the hybrid was ancestral to L. corniculatus this task would be easier because meiotic regularity and fertility of the final product would be more likely (Somaroo and Grant 1972). Furthermore, much of the selection for desired characters and meiotic regularity could be achieved at the simpler diploid level through backcrossing before the desired germplasm was introduced at the tetraploid level.

Various authors have proposed different species as the progenitors of the tetraploid cultivated plant. It has been proposed that Lotus corniculatus is an autotetraploid of L. tenuis (Dawson 1941) and alternatively, that it is an allotetraploid of two species (Stebbins 1950; Harney and Brant 1965; Bosaroo and Brant 1972; Ross and Jones 1985). The various arguments have been based upon the few genetic markers available or upon meiotic analyses of hybrids. Various diploid ancestors have been proposed including L. alpinus, L. japonicus, L. tenuis, and L. uliginosus. However, there is no consensus on the subject because there is limited evidence upon which to base the various proposals.

In view of the above considerations, it was decided to undertake an isoenzyme survey within the genus Lotus. A knowledge of various isoenzyme loci and alleles would increase the data available for genetic study of the genus. Firstly, on a practical level, a knowledge of isoenzyme markers would provide perhaps a twofold increase in available characters that could be useful for such tasks as selection in the greenhouse and positive identification of putative interspecific hybrids. Secondly, a study of the segregation of isoenzyme markers could clarify the controversy surrounding the autotetraploid nature of Lotus corniculatus which was based upon observed tetrasomic inheritance. Thirdly, a comparison of isoenzyme phenotypes for Lotus corniculatus, and the various putative diploid ancestors, might be able to eliminate some of the proposed ancestors, if their phenotypes were not compatible with the role of possible ancestors.

Isoenzyme data are powerful as genetic tools. Information is obtained by observing the electromobility of specific proteins on a gel. Because of the colinearity of information between DNA and protein, electromobility

which is a function of primary protein structure is a reflection of nucleotide sequence within the gene. A change in mobility reflects mutation within the gene. In addition, isoenzyme alleles are codominant, that is, heterozygotes can be distinguished from homozygotes so that isoenzyme data are more precise than information obtained from phenotypes controlled by dominant alleles. Because of these facts, genotypes can be directly deduced from isoenzyme phenotypes (Gottlieb 1977).

The purpose of this isoenzyme study was specifically to answer four questions. These are:

1. What are the laboratory protocols that will produce distinct reproducible isoenzyme phenotypes in Lotus for as many enzymes as possible given constraints of time and expense?

2. Can it be demonstrated that these isoenzyme phenotypes segregate according to Mendelian expectation and, thus, that they accurately reflect genotype.

3. Is segregation of established isoenzyme markers tetrasomic or disomic in Lotus corniculatus?

4. Is the isoenzyme phenotype of any of the putative diploid ancestral species L. alpinus, L. japonicus, L. tenuis, and L. uliginosus incompatible with the possibility that it contributed genetic materials to the tetraploid Lotus corniculatus?

The results of the study follow and are presented in the form of three manuscripts. The first of these presents the findings of a study of laboratory techniques that produce the clearest isoenzyme phenotypes. This

initial study also examined the consistency of these phenotypes during various life cycle stages of the diploid L. uliginosus, in order to verify that differences in phenotype represented true genetic differences rather than ontogenetic differences. The second manuscript presents findings from a study of genetic segregation of isoenzyme markers in diploid interspecific hybrids in order to address question number 2, and in artificial autotetraploids, artificial allotetraploids, and in L. corniculatus, in order to answer question number 3. The third manuscript presents the results of a survey of isoenzyme phenotypes in several accessions of the various diploids, and in L. corniculatus, in order to address question number 4.

The use of horizontal starch gel electrophoresis may be questioned, and I conclude this introduction by a justification of its use. It can be argued that polyacrylamide electrophoresis provides finer resolution of enzyme differences than the older starch gel methods. I would answer this criticism by making three points. First, a large body of data already exist for starch gel electrophoresis in the literature in the field of plant taxonomy and genetics. The use of starch gel electrophoresis in this study provides results that are comparable with existing data. Secondly, though admittedly less precise, starch gel electrophoresis provides results with ample resolution to address theoretical questions, and is much easier and less expensive to use. Thirdly, the acrylamide monomer is a neurotoxin, whereas starch gels are relatively non-toxic. The enzyme staining chemicals are toxic but use of starch decreases the exposure to toxins while still providing adequate and reliable information.

REFERENCES

Bubar, J. S., and R. K. Miri. 1965. Inheritance of self incompatibility and brown keel tip in L. corniculatus L. Nature, 205: 1035-1036.

Buzzell, R. I., and C. P. Wilsie. 1963. Genetic investigation of brown keel tip color in Lotus corniculatus L. Crop Sci. 3: 128-130.

Buzzell, R. I. and Wilsie, C. P. 1964. Seed production potential of flowering types in birdsfoot trefoil. Crop Sci. 4: 436-437.

Dawson, C. D. R. 1941. Tetrasomic inheritance in Lotus corniculatus L. J. Genet. 42: 49-73.

Donovan, L. S. 1959. The inheritance of leaf size in broadleaf birdsfoot trefoil, Lotus corniculatus L. Can. J. Plant Sci. 39: 141-157.

Donovan, L. S. and McLennan, H. A. 1964. Further studies on the inheritance of leaf size in broadleaf birdsfoot trefoil, Lotus corniculatus L. Can. J. Genet. Cytol. 6: 164-169.

Draper, A. D. and Wilsie, C. P. 1965. Recurrent selection for seed size in birdsfoot trefoil, Lotus corniculatus L. Crop Sci. 5: 313-315.

Gershon, D. 1961. Breeding for resistance to pod dehiscence in birdsfoot trefoil, (Lotus corniculatus L.), and some studies of the anatomy of pods, cytology and genetics of several Lotus species and their interspecific hybrids. Ph.D. Thesis, Cornell University, Ithaca, New York.

Gottlieb, L. D. 1977. Electrophoretic evidence and plant systematics. Ann. Mo. Bot. Gard. 64: 161-180.

Grant, W. F. and Marten, G. C. 1985. Birdsfoot trefoil. In Forages: Science of grassland agriculture, 4th ed. M. E. Heath, D. S. Metcalfe and R. F. Barnes, eds. Iowa State Univ. Press, Ames, Iowa. pp. 98-108.

Harney, P. M. and Grant, W. F. 1965. A polygonal presentation of chromatographic investigations on the phenolic content of certain species of Lotus. Can. J. Genet. Cytol. 7: 40-51.

O'Donoughue, L. S. 1986. A study of seed dehiscence in the genus Lotus (Fabaceae) using interspecific hybridization and embryo culture. M.Sc. Thesis, Macdonald College of McGill University, Montreal, Quebec, Canada. 190 pp.

Phillips, R. L. and Keia, W. F. 1968. Seed pod dehiscence in Lotus and interspecific hybridization involving L. corniculatus L. Crop Sci. 8: 18-21.

Pootschi, I, and MacDonald, H. A. 1961. Inheritance of leaf color in broadleaf birdsfoot trefoil. Crop Sci. 1: 327-328.

Ross, M. D., and W. T. Jones. 1985. The origin of Lotus corniculatus. Theor. Appl. Genet. 71: 284-288.

Seaney, R. R. and Henson, P. R. 1970. Birdsfoot trefoil. Adv. Agron. 22: 119-157.

Sosaroo, B. H., and W. F. Grant. 1972. Crossing relationships between synthetic Lotus amphidiploids and L. corniculatus. Crop Sci. 12: 103-105.

Stebbins, G. L. 1950. Variation and evolution in plants. Columbia Univ. Press, New York and London. 643 pp.

Twamley, B. E. 1971. Selection methods for seedling vigor in birdsfoot trefoil. Can. J. Plant Sci. 51: 229-235.

Twamley, B. E. 1974. Recurrent selection for seedling vigor in birdsfoot trefoil. Crop Sci. 14: 87-90.

An isoenzyme study in the genus Lotus (Fabaceae). I. Establishment of experimental protocols

J. V. RAELSON, and W. F. GRANT

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

Received

Key words: Lotus corniculatus, Lotus species, Fabaceae, interspecific hybridization, isozymes, autotetraploids, allotetraploids, genetic ratios

Abstract

An isoenzyme survey of some taxa in the genus Lotus (Fabaceae) was undertaken in order to increase the number of genetic markers available to breeders and to students of Lotus phylogeny. The results of a preliminary study using the species L. uliginosus Schkuhr are presented. Twenty-one enzymes were examined using starch gel electrophoresis and nine buffer systems. Clear, consistent banding patterns were obtained for PGI, TPI (LiOH-borate buffer pH 8.1-8.4), MDH, and IDH (tris-citrate buffer, pH 7.1) and PGM, 6-PGDH, and ME (histidine-citrate buffer pH 6.5). Clear but inconsistent banding patterns were obtained for FDP (morpholine-citrate buffer, pH 6.1) G3PDH (histidine-citrate buffer, pH 5.7) and for DIA, β-EST, LAP, MDR and NADHDH (histidine-citrate buffer pH 6.5). Phenotypes of the seven consistent enzyme systems were obtained for different tissues for each of several genotypes at different stages of development. Variation in phenotypes of the same individuals under different conditions indicated the presence of different isozymic forms of the enzyme. Shoot tissue of plants over six weeks of age was found to be suitable material for further studies, since phenotype for this tissue was constant despite day-length changes.

Introduction

Lotus corniculatus L., Birdsfoot Trefoil, is a forage legume with many advantages for use in the cool, wet climate of north-eastern North America, however, it has certain disadvantages that limit its use. Perhaps the most serious of these is seed-pod dehiscence which makes seed harvest difficult, and thus, makes seed expensive (Seaney and Henson 1970; Brant and Marten 1985). Attempts have been made to overcome this limitation by means of selection within the species (Peacock and Wilsie 1957, 1960), however, the most promising route to improvement appears to be interspecific hybridization using certain species within the genus with non-shattering seed pods (Phillips and Keim 1968; O'Donoughue and Brant 1987).

The fact that L. corniculatus is a tetraploid (2n = 24), whereas species that lack pod dehiscence are all diploids, complicates the task of interspecific hybridization. It has been suggested that the identification of a diploid species ancestral to L. corniculatus would provide a bridge for transporting the foreign diploid germplasm into the cultivated tetraploid (Somaroo and Brant 1972). The non-shattering diploid species would be crossed with the diploid ancestral species and subsequent to (following?) chromosome doubling, the interspecific hybrid would then be crossed with L. corniculatus. Use of the ancestral species would result in less meiotic irregularity in progeny of the tetraploid cross.

Several species have been proposed as putative progenitors of L. corniculatus. These include L. tenuis Waldst. & Kit. (Dawson 1941; Ross and Jones 1985), L. alpinus Schleich. and L. japonicus (Regel) Larsen (Somaroo and Brant 1972) and L. uliginosus Schkuhr (Ross and Jones 1985). It is

difficult to test the correctness of the various hypotheses concerning the origin of L. corniculatus because of the paucity of genetic data.

Few qualitative genetic characters have been studied in the genus and the cytology is difficult 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, large vs. small 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 present study is to discover polymorphic isoenzyme loci that can 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 reported uses of isoenzymes in Lotus was reported by De Lautour et al. (1978). They examined L. tenuis and L. corniculatus for phenoloxidase, phosphatase, esterase and peroxidase enzymes. No attempt was made to determine phenotype segregation and the genetic basis for the isoenzyme patterns, nor did they attempt a systematic study of the taxa from the isoenzyme data.

It is anticipated that such a long delayed study of isoenzymes of Lotus will produce new genetic markers that will prove useful, both practically

for breeding and selection, and for testing the various hypotheses concerning the origin of L. corniculatus.

In this paper are presented the findings of a preliminary study designed to define the protocols for obtaining isoenzyme phenotypes using L. uliginosus. Twenty-one enzymes were examined using horizontal starch gel electrophoresis with nine different buffer systems. In addition, the enzyme phenotypes were examined during different life-cycle stages and for different tissues in order to verify that differences in phenotype reflected genetic rather than ontogenic differences.

Materials and methods

Plant material

All of the preliminary isoenzyme experiments reported here were performed on several genotypes of Lotus uliginosus (Acc. No. 193) which were obtained from the Lotus world seed collection maintained by W. F. Grant at Macdonald College of McGill University. Accession number 193 originated in Morocco and was obtained from the Service de la Recherche Agronomique et de l'Experimentation Agricole in Rabat.

Plants that were used in the initial experiment to determine optimal electrophoretic buffers for various enzymes were grown in the greenhouse under short-day, non-flowering, conditions with natural daylight. Plants used for the life-cycle experiments were grown under controlled conditions in a growth cabinet as follows:

Short-day regime: Twelve hours under cool white fluorescent light at an intensity of 83.5 uEinsteins sec^{-1} m^{-2} with a light temperature of 23°C

a dark temperature of 20°C. Electrophoresis was performed on entire seedlings (with two leaves), and on both shoots and roots, separately, for six-week old plants.

Long-day (flowering) regime: Eighteen hours under cool white fluorescent light with approximately 10% incandescent light at a total light intensity of 275 uEinsteins 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.

Electrophoresis

Enzymes were extracted from young shoot tips and leaves and from roots by grinding approximately 100 mg (fresh weight) of tissue in 350 uL of an extraction buffer consisting of 0.1 M tris-HCl, adjusted to pH 7.5, 1.0 mM EDTA (disodium salt), 10 mM $MgCl_2.H_2O$ and 10 mM KCl to which 100 mg of polyvinyl pyrolidone per mL of buffer were added (Gottlieb 1981). Ten uL of 2-mercaptoethanol were also added just prior to grinding. 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.

All electrophoresis was performed using horizontal starch gels. Gels were prepared by suspending 40 g of hydrolyzed starch (Connaught Laboratories, Willowdale, Ontario) in 125 mL of cold buffer, 175 mL of boiling buffer was then added to the slurry and the mixture was heated approximately 1 min until a decrease in viscosity indicated that the starch had dissolved (total w/v starch/buffer ratio was 13.3%). The starch solution was degassed by applying a vacuum for approximately 2 min, and then poured into plexiglass frames. Gels were then cooled to 4°C prior to

loading. Electrode buffer was placed into reservoirs each containing a platinum wire. These were attached to a constant power supply. The gel frames were placed above the reservoirs and electrode buffer was transported to the gels by means of Handy Wipe wicks. The electrophoretic apparatus is described by O'Malley et al. 1980).

In the initial experiment, each of 21 enzymes were assayed on nine different buffer systems. The buffer systems ranged from pH 5.0 to pH 8.8 and are described in Table 1. The 21 enzymes are described in Table 2. The staining recipe for each is given. These recipes have been detailed in various publications. Those listed in Table 2 were published by O'Malley et al. (1980), Vallejos (1983) and Cheliak and Pietel (1984). All staining chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri.

All enzyme stains using the tetrazolium staining (MTT) system (with the exception of Diaphorase) employed the agarose overlay method. 100 mg of agarose were added to 10 mL of the appropriate staining buffer. The suspension was brought to boil to dissolve the agarose and then stored in an oven at 80°C until needed. The staining substrates for the enzyme were dissolved in another 15 mL aliquot of buffer which was then added to the agarose solution. The resulting 25 mL solution was poured over the appropriate starch gel where it quickly cooled to form a superficial agarose gel over the starch. The gels were incubated at 37°C until the enzyme banding patterns emerged. These could easily be read through the agarose.

Results

Enzyme and buffer experiment

The results of the preliminary study to determine the appropriate buffer systems for the various enzymes are presented in Table 3. Seven enzymes were found to produce clear consistent isoenzyme bands for the plant material under study. These are IDH 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 further research in this study was confined to these seven enzyme systems. However, it should not be interpreted that the other enzymes necessarily could not be made to work better by adjusting experimental conditions. The data in Table 3 merely reflect our findings after a certain amount of effort and considerable replication of experiments. Perhaps, some of the other enzymes would work better on acrylamide systems.

Other enzymes besides the seven mentioned above did produce well banded phenotypes, but these were not consistent. These enzymes are AAT, β-Est, FDP, G_3PDH, LAP, and DIA, MR, and NADHDH. These enzymes can be considered as useful but difficult to work with. Perhaps with greater adjustment of techniques, they could also be used. However, due to limitations of time, it was decided to concentrate upon the enzymes which were consistently reproducible.

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 allow us to distinguish between the enzymes. MR was the easiest to use of the three, and since they all produced the same

phenotype, this enzyme is recommended for use in electrophoretic studies of Lotus.

Life cycle and tissue type experiment

Photographs of representative zymograms for the seven enzymes found to be consistently reproducible, namely, PGI, TPI, PGM, MDH, IDH, 6-PGDH, and ME are shown in Figs. 1 and 2. These zymograms represent phenotypes for three individuals of L. uliginosus (Acc. No. 193) grown under long-day flowering conditions. Phenotypes for both root and shoot tissue of each plant are shown. Summaries of the electrophoretic phenotypes found for each enzyme at each of the stages of growth are given in Figs. 3 and 4. The isoenzyme bands for each enzyme are located on the diagrams by their relative mobility. In each phenotype, the uppermost (most anodal) band is called Rm 100. All other bands are expressed by their distance from the origin of electrophoresis as a percentage of the distance of the most anodal band. The relative mobilities presented here are not necessarily the as same those that are given in other papers (Raelson et al. 1987; Raelson and Grant 1987) where phenotypes of L. uliginosus are being compared to those of other species.

In these latter instances, the standard band (Rm = 100) is not necessarily the most anodal band, but rather some common band that is arbitrarily chosen from among phenotypes of all species. It can be seen from Figs. 3 and 4 that phenotype changes with both stage of development and type of tissue. There are basically two types of change. One type is the disappearance of bands from one particular zone of the zymogram, such as the lower bands of MDH (for seedlings and roots), IDH (for seedlings) and PGM (for roots). The second type of change is a displacement of position of

certain entire groups of bands while their position with respect to each other remains 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 shifted towards the anode in root phenotypes. Finally, the uppermost TPI phenotype band becomes less intense in roots. Two other changes may be artifacts. In some zymograms for shoots, the lowermost zone of PGM is seen as two bands, whereas in other electrophoretic replicates of the same individuals this zone is seen as just one thicker band at the same location. Likewise, the upper thin band in shoot phenotypes for ME does not occur in all replicates.

Discussion

Variation in isoenzyme phenotype can arise from several sources. Different banding patterns can be allelic (allozymes) resulting from alternative DNA sequences at a given locus which segregate genetically or they may represent completely distinct loci (isozymes in the strict sense; Gottleib 1977). Often the enzyme products of distinct isozyme loci are sequestered in separate subcellular compartments. These distinct loci are all located on the nuclear DNA and translation of all isozyme mRNAs has been shown to occur on cytosolic ribosomes (Newton 1983). But, post translational events result in subcellular isolation of the various isozymes. Among the enzymes examined here, PGM, PGI, and 6-PGDH have been shown to possess two isozymic forms, one of which is located in the plastids, and one in the cytoplasm. MDH has been found to possess at least three isozyme forms, one each in the mitochondria, in microbodies, and in the cytoplasm. Experiments of dissociation and reassociation using different purified subcellular isolates of the dimeric enzyme PGI, from

several divergent species showed that monomer subunits of the enzyme can cross associate between species, but not between subcellular forms from the same species (Weeden and Gottlieb 1982). These distinct isozymic loci do not interact.

Many studies have also shown that distinct isozymic loci are differentially regulated in different stages of development. Catalase and alcohol dehydrogenase are two enzyme systems that have been well studied from the perspective of differential regulation, though evidence of such regulation has been found for most enzymes (Freeling 1983). Different loci may be regulated by inducible modifier genes. Other variations in phenotype banding patterns can arise from post-translational modificiation of polypeptides by addition of NAD^+-carboxyl groups (Freeling 1983), or by conformational change of polypeptides in different cellular environments (Newton 1983).

Two distinct insights can be gained from the examination of isoenzyme phenotypes in different tissues and at different stages in development. Firstly, this experiment can determine which conditions will produce invariable phenotypes for a given genotype, so that ontogenetic factors will not be confused with genetic factors. Our results show that the most complete and consistent phenotypes for the enzymes used here are obtained from examining shoot tissue in plants that are at least six-weeks old regardless of day-length conditions.

A second insight that was obtained from this study is a preliminary analysis of isozymic forms of the enzyme phenotypes. Differential activity in different tissues and life stages indicate that PGI, TPI, PGM, MDH and IDH have at least two isozymically distinct loci which determined the

phenotypes that we observed. This interpretation is also suggested by variation in one of the phenotypic zones for PGI and 6-PGDH. In the case of PGI (6 PGDH shows no development or tissue specific variation) this variation is associated with distinct individuals and is independent of tissue specific variation. This suggests that it reflects true genetic (or allozyme) variation. This hypothesis can be confirmed by the study of allozyme segregation. The results of such a study are reported in Raelson et al. (1987).

Acknowledgments

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.

AYALA, F. J., HEDGECOCK, D. G., ZUMWALT, S., and VALENTINE, J. W. 1973. Genetic variation in Tridacna maxima, an ecological analog of some unsuccessful evolutionary lineages. Evolution, 27: 177-191.

BUBAR, J. S., and MIRI, R. K. 1965. Inheritance of self-incompatibility and brown keel tip in Lotus corniculatus L. Nature, 205: 1035-1036.

BUZZELL, R. I., and WILSIE, C. P. 1963. Genetic investigations of brown keel tip color in Lotus corniculatus L. Crop Sci. 3: 128-130.

CARDY, B. J., STUBER, C. W., and GOODMAN, M. M. 1981. Techniques for starch gel electrophoresis of enzymes from maize (Zea mays L.). Institute of Statistics Mimeographed Series No. 1317, North Carolina State University, Raleigh, N.C. 31 pp.

CHELIAK, W. M., and PITEL, J. A. 1984. Techniques for starch gel electrophoresis of enzymes from forest tree species. Information Report PI-X-42, Petawawa National Forestry Institute, Can. Forestry Serv. 49 pp.

CLAYTON, J. W., and TERTIAAK, D. N. 1972. Amine citrate buffers for pH control in starch gel electrophoresis. J. Fisheries Res. Board Can. 29: 1169-1172.

DAWSON, C. D. R. 1941. Tetrasomic inheritance in Lotus corniculatus L. J. Genet. 42: 49-73.

DE LAUTOUR, G., JONES, W. T., and ROSS, M. D. 1978. Production of interspecific hybrids in Lotus aided by endosperm transplants.

DONOVAN, L. S., and McLENNAN, H. A. 1964. Further studies on the inheritance of leaf size in broadleaf birdsfoot trefoil, Lotus corniculatus L. Can. J. Cytol. 6: 164-169.

FILDES, R. A., and HARRIS, H. 1966. Genetically determined variation of adenylate kinase in man. Nature, 209: 261-263.

FREELING, M. 1983. Isozyme systems to study gene regulation during development: A lecture. In Isozymes in Plant Genetics and Breeding, Part A. Edited by S. D. Tanksley and T. J. Orton. Elsevier Science Publ., Amsterdam, New York. pp. 61-83.

GOTTLIEB, L. D. 1977. Electrophoretic evidence and plant systematics. Ann. Mo. Bot. Gard. 64: 161-180.

GOTTLIEB, L. D. 1981. Electrophoretic evidence and plant populations. Progr. Phytochem. 7: 1-46.

GRANT, W. F. 1986. The cytogenetics of Lotus (Leguminosae). J. Nat. Hist. 20: 1461-1465.

GRANT, W. F., and HARTEN, G. C. 1985. Birdsfoot trefoil. In Forages: Science of grassland agriculture, 4th ed. M. E. Heath, D. S. Metcalfe and R. F. Barnes, eds. Iowa State Univ. Press, Ames, Iowa. pp. 98-108.

HARNEY, P. M., and GRANT, W. F. 1965. A polygonal presentation of chromatographic investigations on the phenolic content of certain species of Lotus. Can. J. Genet. Cytol. 7: 40-51.

NEWTON, K. J. 1983. Genetics of mitochondrial isozymes. In Isozymes in Plant Genetics and Breeding, Part A. Edited by S. D. Tanksley and T. J. Orton. Elsevier Science Publ., Amsterdam, New York. pp. 157-173.

O'DONOUGHUE, L. S., and W. F. GRANT. 1987. A study of seed dehiscence in the genus Lotus (Fabaceae) using interspecific hybridization and embryo culture. Manuscript in preparation.

O'MALLEY, D., N. C. WHEELER, and R. P. GURIES. 1980. A manual for starch gel electrophoresis. University of Wisconsin, Department of Forestry, Staff Paper No. 11, Madison, WI. 16 pp.

PEACOCK, H. A., and WILBIE, C. P. 1957. Selection for resistance to seed pod shattering in birdsfoot trefoil (Lotus corniculatus L.). Agron. J. 49: 429-431.

PEACOCK, H. A., and WILSIE, C. P. 1960. Selection for vegetative vigor and seed setting in birdsfoot trefoil (Lotus corniculatus). Agron. J. 52: 321-324.

PHILLIPS, R. L., and KEIM, W. F. 1968. Seed pod dehiscence in Lotus and interspecific hybridization involving L. corniculatus L. Crop Sci. 8: 18-21.

POOTSCHI, I., and MACDONALD, H. A. 1961. Inheritance of leaf color in broadleaf birdsfoot trefoil. Crop Sci. 1: 327-328.

RAELSON, J. V., LEMAITRE, P. C., STARKIE, K. M., AND GRANT, W. F. 1987a. An isoenzyme study in the genus Lotus (Fabaceae). II. Genetic analyses of isoenzyme loci in interspecific hybrids, artificial allo- and autotetraploids, and in L. corniculatus. To be submitted to Genome.

RAELSON, J. V., AND GRANT, W. F. 1987b. An isoenzyme study in the genus Lotus (Fabaceae). III. Evaluation of hypotheses concerning the origin of L. corniculatus using isoenzyme data. To be submitted to Theo. Appl. Genet.

RIDGWAY, G. J., SHERBURNE, S. W. and LEWIS, R. D. 1970. Polymorphisms in the esterases of Atlantic herring. Trans. Am. Fisheries Soc. 99: 147-151.

ROSS, M. D., and JONES, W. T., 1985. The origin of Lotus corniculatus. Theor. Appl. Genet. 71: 284-288.

SCHAAL, B. A., and ANDERSON, W. W., 1974. An outline of techniques for starch gel electrophoresis of enzymes from the American oyster, Crassostrea virginica Gmelin. Georgia Marine Sci. Centr. Tech. Rept. Ser. 74-3. 17 pp.

SEANEY, R. R. and HENSON, P. R. 1970. Birdsfoot trefoil. Adv. Agron. 22: 119-157.

SOMAROO, B. H., and W. F. GRANT. 1972. Crossing relationships between synthetic Lotus amphidiploids and L. corniculatus. Crop Sci. 12: 103-105.

TANKSLEY, S. D., and ORTON, T. J. 1983. Isozymes in Plant Genetics and Breeding, Part B. Elsevier Science Publ., Amsterdam, New York. 472 pp.

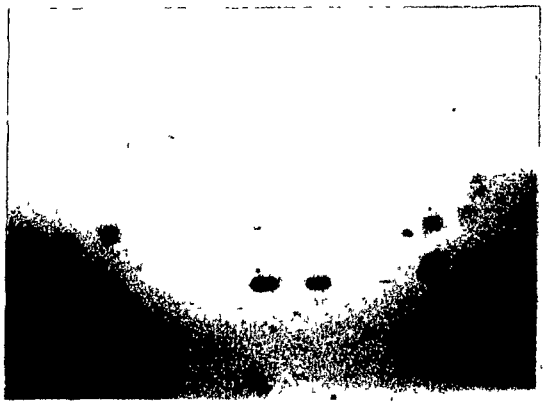
VALLEJOS, C. E. 1983. Enzyme activity staining. In Isozymes in Plant Genetics and Breeding, Part A. Edited by S. D. Tanksley and T. J. Orton. Elsevier Science Publ., Amsterdam, New York. pp. 469-516.

WEEDEN, N. F., and L. D. GOTTLIEB. 1982. Dissociation, reassociation, and purification of plastid and cytosolic phosphoglucose isomerase isozymes. Plan. Physiol. 69: 717-723.

ZANDSTRA, I. I., and GRANT, W. F. 1968. The biosystematics of the genus Lotus (Leguminosae) in Canada. I. Cytotaxonomy. Can. J. Bot. 46: 557-583.

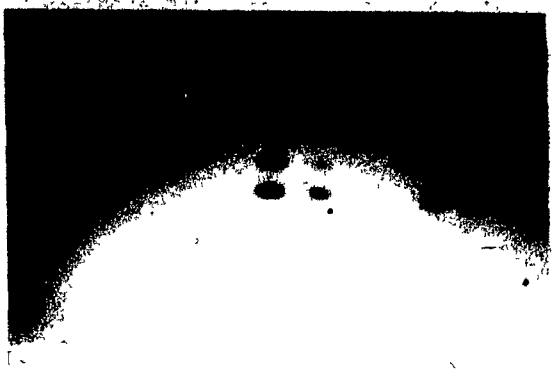
Fig. 1. Photographs of representative zymograms for three genotypes of Lotus uliginosus grown under long-day conditions. A. PGI. B. TPI. C. PGM. D. MDH. S = shoot. R = root.

A



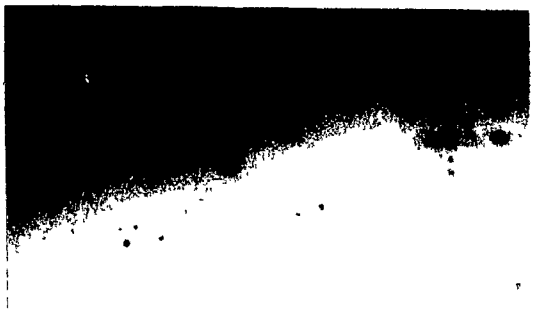
S R S R S R

B



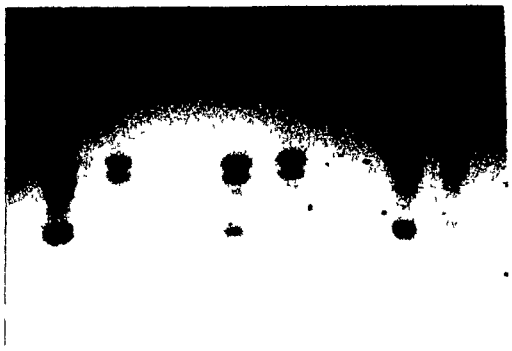
S R S R S R

C



S R S R S R

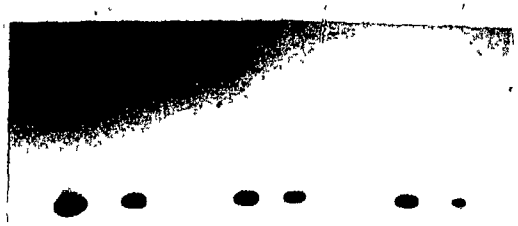
D



S R S R S R

Fig. 2. Photgraphs of representative zymograms for three genotypes of Lotus uliginosus grown under long-day conditions. E. IDH. F. 6-PGDH. G. ME. S = shoot. R = root.

E

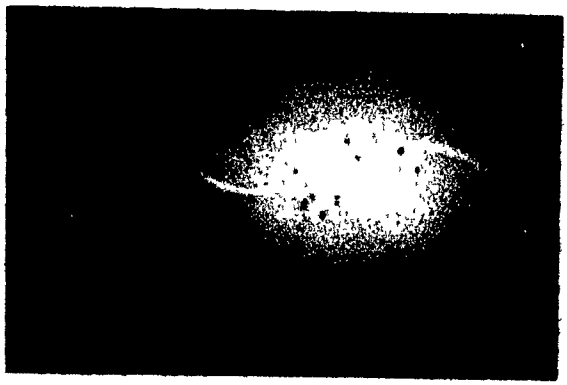


S R S R S R

F

S R S R S R

G



S R S R S R

Fig. 3. Summary of PGI, TPI, PGM, and MDH phenotypes for Lotus uliginosus at different stages of development and in different tissues. Se = seedling (two true leaves). S. D. = after six 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.

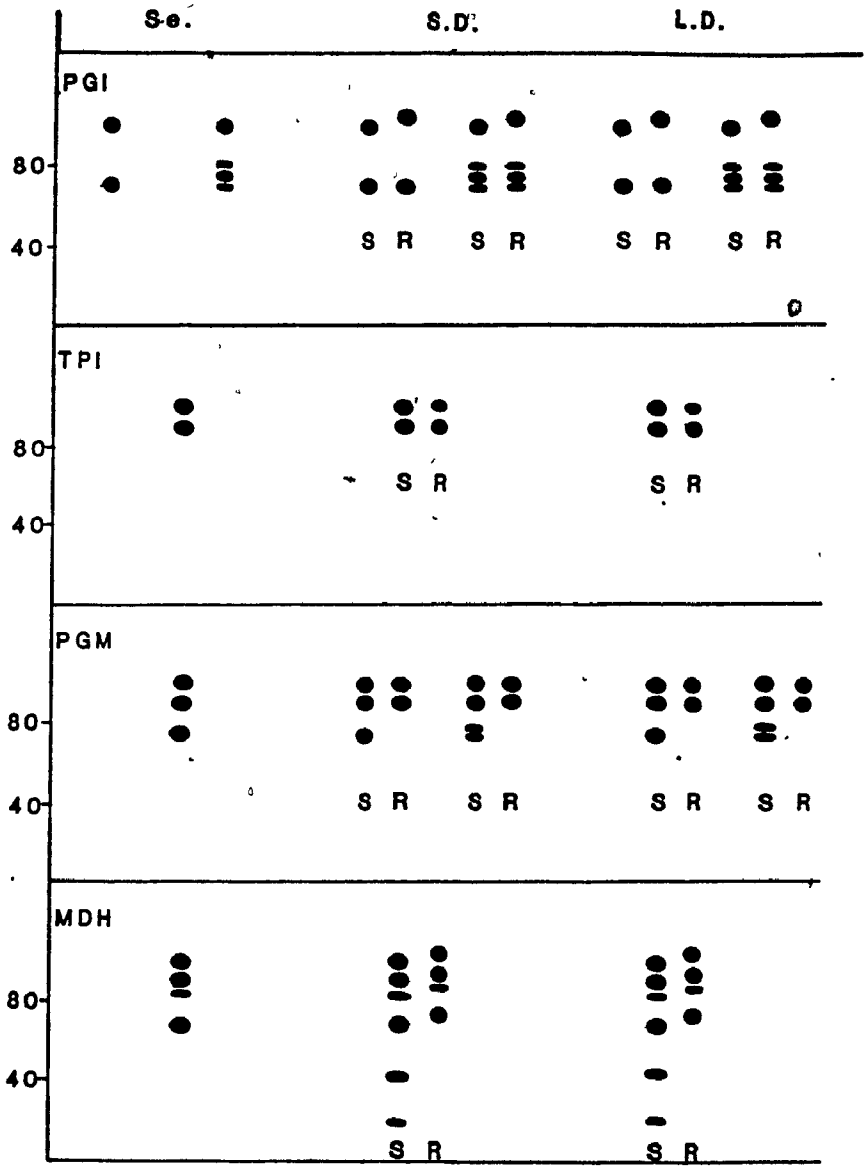

S.e.
S.D.
L.D.
PGI
80
40
S R S R S R S R
TPI
80
40
S R S R
PGM
80
40
S R S R S R S R
MDH
80
40
S R S R

Fig. 4. Summary of IDH, 6-PGDH, and ME phenotypes for Lotus uliginosus at different stages of development and in different tissues. Se = seedling (two true leaves). S. D. = after six 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.

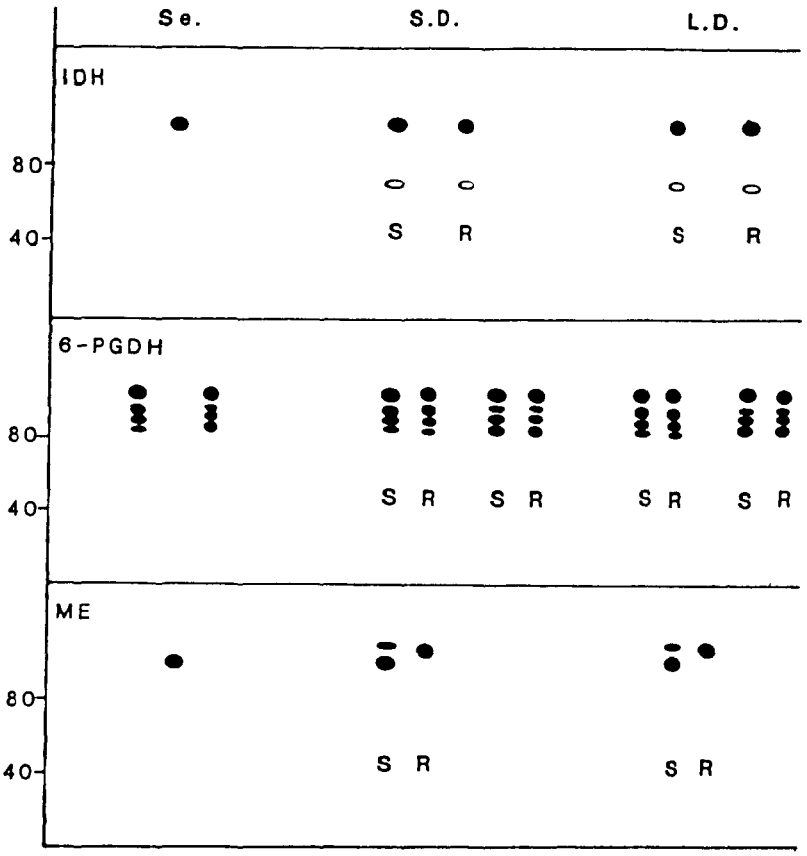

Se.
S.D.
L.D.
IDH
80
40
S
R
S
R
6-PGDH
80
40
S R S R S R S R
ME
80
40
S R
S R

TABLE 1. 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 Gel: 1:12 dilution of electrode buffer	25mA	Cardy et al. (1981)
B	5.7	Electrode: 0.065 M L-histidine (free base) 0.02 M citric acid (monohydrate), adjusted to pH Gel: 1:6 dilution of electrode buffer	25mA	Cardy et al. (1981)
C	6.1	Electrode: 0.04 M citric acid (anhydrous), adjust to pH with N-(3-amino propyl)-morpholine Gel: 0.007 M citric acid (anhydrous), adjust to pH with N-(3-amino propyl)-morpholine	50mA	Clayton and Tertiak (1972)
D	6.5	Electrode: 0.065 M L-histidine (free base) 0.002 M citric acid (anhydrous), adjust to pH Gel: 1:3 dilution of electrode buffer	25mA	Cardy et al. (1981)
E	7.0	Electrode: 0.41 M citrate (trisodium salt) 0.41 M citric acid (anhydrous), adjust to pH Gel: 0.005 M histidine (hydrochloride monohydrate), adjusted to pH with NaOH	30mA	Fildes and Harris (1966)

... TABLE 1. Cont'd.

TABLE 1. Continued

Buffer	pH	Composition	Electrophoretic amperage	Reference
F	7.1	Electrode: 0.1 M tris, 0.05 M citric acid (anhydrous), adjusted to pH, 0.001 M EDTA (disodium salt)	60mA	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	40mA	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)	40mA	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	40mA	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	40mA	Schaal and Anderson (1974)
	8.8	Gel: 0.15 M tris, 0.005 M citric acid (monohydrate), adjusted to pH 8.8		

TABLE 2. Enzyme recipes

Enzyme	Staining recipe
Aconitase (ACO, EC 4.2.1.3)	25 mL 0.1 M tris, 30 mg of cis-aconiticanhydride, 3 mg β-$NADP^+$, adjust to pH 8.0 with 1 M tris, 10 units of isocitrate dehydrogenase, 2 mL 0.1 M $MgCl_2.6\ H_2O$, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS
Aldolase (ALD, EC 4.1.2.13)	25 mL 0.1 M tris-HCl buffer at pH 8.0, 125 mg fructose-1-6-diphosphate (tetrasodium salt), 38 mg sodium arsenate, 20 mg β-NAD^+, 130 units glyceraldehyde 3-phosphate dehydrogenase, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS
Aspartate aminotransferase (AAT (GOT), EC 2.6.1.1)	50 mL 0.1 M tris, 0.5 mg pyridoxal-5'-phosphate, 130 mg L-aspartic acid, 100 mg α-ketoglutaric acid, adjust pH to 8.0, 50 mg fast blue BB salt
Diaphorase (DIA, EC 1.6.4.3)	50 mL 0.1 M tris-HCl buffer at pH 8.0, 0.5 mg 2,6-dichlorophenol-indophenol, 20 mg β-NADH (reduced), 0.5 mL NBT (10 mg/mL) (no PMS)

... TABLE 2. continued

TABLE 2. Contuinued

Enzyme	Staining recipe
β-Esterase (EST, EC 3.1.1.1)	50 mL 0.2 M tris-HCl buffer at pH 6.4, 50 mg β-napthyl acetate, 50 mg fast blue BB salt. Dissolve β-napthyl acetate in 5 mL acetone before adding buffer
Fructose 1,6-diphosphatase (FDP, EC 3.1.3.11)	25 mL 0.1 M tris-HCl buffer at pH 8.0, 25 mg fructose-1-6-diphosphate (tetrasodium salt), 3 mg β-NADP⁺, 35 units phosphoglucoisomerase, 35 units glucose-6-phosphate dehydrogenase, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS
Glyceraldehyde-3-phosphate dehydrogenase (G_3PDH, EC 1.2.1.12)	25 mL 0.1 M tris-HCl buffer at pH 8.0, 40 mg fructose-1-6-diphosphate (tetrasodium salt), 33 units of aldolase. Incubate solution 30 min at 37°C, then add 3 mg β-NADP⁺, 40 mg sodium arsenate, 60 mg EDTA (disodium salt), 2 mL 0.1 M $MgCl_2.6\ H_2O$, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS
Glycerate-2-dehydrogenase (G_2DH, EC 1.1.1.29)	25 mL 0.1 M tris-HCl buffer at pH 8.0, 75 mg DL-glycerate (hemicalcium salt) 20 mg β-NAD⁺, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS

... TABLE 2. continued

TABLE 2. Contuinued

Enzyme	Staining recipe
Glutamate dehydrogenase (GDH, EC 1.4.1.2)	25 mL 0.1 M tris-HCl buffer at pH 8.5, 3 g L-glutamate (monosodium salt) 25 mg β-NAD^+, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS
Isocitrate dehydrogenase (IDH, EC 1.1.1.42)	25 mL 0.1 M tris-HCl buffer at pH 8.0, 25 mg DL-isocitrate (trisodium salt), 3 mg β-$NADP^+$, 2 mL 0.1 M $MgCl_2.6\ H_2O$, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS
Lactate dehydrogenase (LDH, EC 1.1.1.27)	25 mL 0.1 M tris-HCl buffer at pH 7.5, 10 mL 85% DL-lactate, 30 mg β-NAD^+, 2 mL 0.1 M $MgCl_2.6\ H_2O$, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS
Leucine aminopeptidase (LAP, EC 3.4.11.1)	Soak gel 20 min in 0.25 M boric acid; drain, and add 100 mL solution of 365 mg maleic anhydride, 528 mg NaOH, 70 mg L-leucyl-napthalamide-HCl and 30 mg black K salt
Malate dehydrogenase (MDH, EC 1.1.1.37)	25 mL 0.1 M tris, 100 mg DL-malic acid, 20 mg β-NAD^+, adjust to pH 7.8 with 1 M tris, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS

... TABLE 2. continued

TABLE 2. Contuinued

Enzyme	Staining recipe
Malic enzyme (ME, EC 1.1.1.40)	25 mL 0.1 M tris, 210 mg DL-malic acid, 3 mg β-$NADP^+$, adjust to pH 7.2 with 1 n tris, 2 mL 0.1 M $MgCl_2.6\ H_2O$, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS
Menadione reductase (MR, EC 1.6.99.2)	25 mL 0.2 M tris-HCl buffer at pH 7.0, 25 mg menadione (sodium bisulfite salt), 25 mg β-NADH (reduced), 0.5 mL MTT (10 mg/mL) (no PMS)
Nicotinamide adenine dinucleotide dehydrogenase (NADHDH, EC 1.6.99.3)	25 mL 0.1 M tris-HCl buffer at pH 8.0, 15 β-NADH (reduced), 0.5 mL MTT (10 mg/mL) (no PMS)
Phosphoglucomutasetase (PGM, EC 2.7.5.1)	25 mL 0.1 M tris-HCl buffer at pH 8.0, 17 mg glucose-1-phosphate, 3 mg β-$NADP^+$, 0.1 mg glucose 1-6-diphosphate, 30 units of glucose-6-phosphate dehydrogenase, 2 mL 0.1 M $MgCl_2.6\ H_2O$, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS
6-Phosphogluconate dehydrogenase (6-PGDH, EC 1.1.1.44)	0.1 M tris-HCl buffer at pH 8.0, 30 mg 6-phosphogluconate (barium salt), 3 mg β-$NADP^+$, 2 mL 0.1 M $MgCl_2.6\ H_2O$, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS

... TABLE 2. continued

TABLE 2. Contuinued

Enzyme	Staining recipe
Phosphoglucose isomerase (EC 5.3.1.9)	25 mL 0.1 M tris-HCl at pH 8.3, 23 mg fructose-6-phosphate, 3 mg β-NADP$^+$, 30 units of glucose-6-phosphate dehydrogenase, 2 mL of 0.1 M $MgCl_2.6\ H_2O$, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS
Shikimic dehydrogenase (SKDH, EC 1.1.1.25)	25 mL 0.1 M tris, 25 mg shikimic acid, 3 mg β-NADP$^+$, adjust to pH 8.5 with 1 M tris, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS
Triosephosphate isomerase (TPI, EC 5.3.1.1)	25 mL 0.1 M tris-HCl at pH 8.0, 7 mg dihydroyacetone phosphate (lithium salt), 20 mg EDTA (disodium salt), 15 mg β-NAD$^+$, 230 mg sodium arsenate, 133 units glyceraldehyde, 3-phosphate dehydrogenate, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS

TABLE 3. Quality of banding phenotypes for various enzymes using different electrophoresis buffer systems for L. uliginosus accession number 193.

Enzyme	Electrode/Gel buffer system (See Table 1)									
	A	B	C	D	E	F	G	H	I	J
ACO	-	‡	‡‡	‡‡	‡	‡	‡	-	-	-
ALD	‡‡	‡‡	‡	‡	‡	‡	‡	-	-	-
AAT	-	‡	‡	-	-	‡	‡	-	‡‡‡	‡
DIA	-	-	‡‡‡	‡‡‡	‡	‡‡	‡	‡	‡‡	‡
β-EST	‡‡	‡	‡‡‡	‡‡‡	‡	‡‡	‡‡	‡‡	-	-
FDP	‡	‡	‡‡‡	‡	‡	‡‡	‡	‡	‡‡	‡
G-3-PDH	-	‡‡‡	‡	‡‡	‡‡	‡‡	‡‡	‡	‡	-
G-6-PDH	-	-	-	‡‡	‡	‡‡	‡	‡	‡	-
GDH	-	-	-	-	‡‡	‡‡	‡	‡‡	‡‡	‡‡
IDH	‡	‡	‡‡	‡‡	‡‡	‡‡‡‡	‡‡	‡	‡	‡
LDH	‡‡	‡	‡‡	-	‡‡	‡‡	‡	‡	-	-
LAP	-	‡	‡‡	‡‡‡	‡	‡	‡‡	‡	‡‡	‡
MDH	‡	‡	‡	‡	‡	‡‡‡‡	‡‡	‡‡	‡‡	-
ME	-	‡‡	‡‡	‡‡‡‡	‡‡	‡‡	‡	-	-	-
MR	‡	‡	‡‡‡	‡‡‡	‡	‡‡	‡‡	‡‡	‡	‡‡
NADHDH	‡	‡	‡‡‡	‡‡‡	‡‡	‡‡	‡‡	‡‡	‡	‡‡
PGM	‡	‡‡	‡	‡‡‡‡	‡	‡	‡	‡	‡	-

... TABLE 3. Continued

Table 3. Continued

Enzyme	Electrode/Gel buffer system									
	A	B	C	D	E	F	G	H	I	J
6-PGDH	‡	‡‡	‡‡	‡‡‡‡	‡‡	‡‡	‡	-	-	-
PGI	‡	‡	‡	‡	‡	‡	‡‡	‡	‡‡‡‡	‡
SKDH	-	‡	‡‡	‡‡	‡	‡‡	‡	-	-	‡
TPI	‡	‡	‡‡	‡‡	‡‡	‡	‡	‡	‡‡‡‡	‡

\- No staining activity observed.

‡ Staining, but blurred, no distinct bands.

‡‡ Bands appear, but faint and difficult to read.

‡‡‡ Distinct, clear bands sometimes occur but not consistently.

‡‡‡‡ Distinct, clear bands consistently obtained.

An isoenzyme study in the genus Lotus (Fabaceae). II. Genetic analyses of isoenzyme loci in interspecific hybrids, artificial allo- and autotetraploids, and in L. corniculatus

J. V. RAELSON, P. C. LEMAÎTRE, K. M. STARKIE, and W. F. GRANT

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

RAELSON, J. R., P. LeMAITRE, K. M. STARKIE, and W. F. GRANT. 1987. An isoenzyme study in the genus Lotus (Fabaceae). II. Genetic analyses of isoenzyme loci in interspecific hybrids, artificial allo- and autotetraploids, and in L. corniculatus. Genome

A formal genetic analysis of segregation and/or recombination of allozymes for the enzymes PGM, TPI, PGI, MDH, IDH, and 6-PGDH was made using several taxa within the genus Lotus. Isoenzyme phenotypes were examined for the diploids L. alpinus Schleich., L. burttii Sz. Borsos, L. coimbricensis Brot., L. ornithopodioides L., L. tenuis Waldst. et Kit, and L. uliginosus Schkuhr, for the interspecific hybrids L. alpinus X L. coimbricensis, L. burttii X L. ornithopodioides, and L. japonicus X L. alpinus, and for the tetraploid L. corniculatus L., the autotetraploid (L. alpinus)^2, and the amphidiploid (L. japonicus X L. alpinus)^2. Both pollen and sporophyte phenotypes were examined, as were progeny phenotypes for L. corniculatus, L. japonicus X L. alpinus, for the autotetraploid and for the amphidiploid.

Several new loci were identified for Lotus, namely, Idh1, Idh2, Mdh3, Pgi1, Pgi2, Tpi1, Tpi2, and 6-Pdgh1, with duplications of Idh1, Idh2, Mdh3, Pgi2 and 6-Pdgh1. It is also shown that for Lotus PGM is monomeric and that the other enzymes are dimeric as has been previously reported for other genera. Duplication of several loci was found in the diploid interspecific hybrid L. japonicus X L. alpinus, and evidence of the original diploid duplication was found in the amphidiploid. Quadruplication of loci was also found in L. corniculatus, but not in the autotetraploid (L. alpinus)^2. It is argued that this duplication resulted from unequal crossing over between homoeologues and that it provides evidence that L. corniculatus is a segmental allotetraploid. Quadruplication of loci in L. corniculatus may explain previously reported distorted tetrasomic ratios for characters in this species.

Introduction

This paper is the second in a series to report on an isoenzyme study of several species in the genus Lotus. One purpose of the study was to test several conflicting theories on the phylogenetic origin of the cultivated tetraploid Lotus corniculatus L. (Birdsfoot Trefoil). It has been proposed that L. corniculatus is an allotetraploid involving hybridization between two various diploid species. Somaroo and Grant (1972) proposed the species L. japonicus (Regel) Larsen and L. alpinus Schleich. as likely ancestral species, whereas Ross and Jones (1985) suggested that L. uliginosus Schkuhr was the pollen parent and that either L. tenuis Waldst. et Kit or L. alpinus was the female parent of the original hybrid.

Dawson (1941) observed tetrasomic inheritance of cyanogenesis in L. corniculatus from the putative cross AAaa X aaaa (duplex cyanogenic X nulliplex acyanogenic). He observed a 5:1 cyanogenic to acyanogenic ratio among the progeny which would correspond to a 1 AA : 4 Aa : 1 aa ratio for gamete segregation in the duplex parent. On the basis of these and cytological results, he proposed that L. corniculatus was an autotetraploid of L. tenuis.

The isoenzyme study intended to obtain as many electrophoretic phenotypes as possible for the pertinent diploid species, and for L. corniculatus, in order to determine whether any of the diploids could not have provided the alleles contained within the tetraploid species. In order to make such comparisons, it is necessary to characterize the genetics of multiple isoenzyme loci so that it is certain that homologous loci are being compared and that allozymic and isozymic differences may be distinguished

(Crawford 1985; Gottlieb 1977). It is the purpose of this paper to report the results of such a formal genetic analysis.

Segregation of four enzymes PGI, MDH, IDH, and 6-PGDH was studied among the progeny of selfed heterozygous interspecific hybrids of L. japonicus X L. alpinus. The interspecific hybrids were chosen because they are likely to be heterozygous for many loci and, thus, decrease the number of crosses that must be made (Tanksley 1983). Recombination of loci for PGM and TPI in the interspecific hybrids L. burttii Sz. Borsos X L. ornithopodioides L. and L. alpinus X L. conimbricensis Brot. were also examined and sporophyte and pollen phenotypes were compared for PGM, TPI, and PGI.

A second purpose of the genetic analysis was to test segregation models at the tetraploid level. Because isoenzyme alleles are codominant, more powerful tests of segregation are possible than are available with analyses of segregation of morphological characters which can not distinguish between heterozygotes and homozygous dominants (Gottlieb 1977). Analyses of isoenzyme segregation can be expected to provide new insights into the reported tetrasomic nature of segregation in L. corniculatus and the controversy concerning the allo- vs. autotetraploid origin of the species. Consequent to this second purpose, segregation of PGI and MDH loci was analysed in the artificial allotetraploid (L. japonicus X L. alpinus)^2, in the artificial autotetraploid (L. alpinus)^2, and in the tetraploid L. corniculatus.

Materials and methods

Plant Material

Isoenzyme electrophoresis was performed on both sporophyte leaf tissue and on pollen. In Table 1 are listed the various plant taxa and their use in this study. 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 brackets following the name of the taxon.

The diploids *Lotus uliginosus* (193-52) and *L. tenuis* (109-21) were included in all electrophoretic gels as standards to which the banding pattern of other samples could be compared. This practice allowed comparison of patterns among different gels. Electrophoresis of pollen was performed on *L. tenuis* (109-20), on the interspecific hybrid *L. japonicus* X *L. alpinus* (28), and on the artificial autotetraploid (*L. alpinus*)^2 (774x-5). The hybrid and the autotetraploid were all descendent from the original material produced by B. H. Somaroo (1970).

The sporophytes of the interspecific hybrids *L. japonicus* X *L. alpinus* (23 and 28), of the amphidiploid (*L. japonicus* X *L. alpinus*)^2 (28) and of the autotetraploid (*L. alpinus*)^2 (774x-5) were also analyzed, as were the progeny obtained from selfing these taxa. Plants were selfed by tripping the flower keels with the tip of a tooth pick to which a small piece of sand paper was attached. Self-fertilized plants were then kept in a netted cage to avoid contamination of foreign pollen carried by insects. Seed was collected when pods became brown (approximately 35 days after pollination). The pods were placed in small envelopes and allowed to dry several days.

After the pods dehisced, the seed was hand cleaned and 125 seed from each taxon were planted in flats in the greenhouse. Electrophoresis was performed when the plants were approximately two months old. A cross was also made between two plants from different accessions of the self-infertile L. corniculatus. Flowers of the female parent (554-5) were emasculated by removing the keels with a forceps and the plants were then sprayed with 10 ppm of 2,4,5-trichlorophenoxy propionic acid to discourage dropping of the injured flowers. Two days after emasculation, pollen from the male parent (Leo-1) was applied to the stigmas of the emasculated flowers. Seed collection was the same as that described above.

Electrophoretic analyses were also performed on several diploid species L. burttii (303), L. ornithopodioides (100), L. alpinus (77), L. conimbricensis (126) and on the interspecific hybrids L. burttii X L. ornithopodioides, and L. alpinus X L. conimbricensis. These hybrids were produced by L. S. O'Donoughue (1986).

Electrophoresis

The horizontal starch gel electrophoresis technique employed in this study has been described elsewhere (Raelson and Grant 1987a). 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 uL glycerol and 50 mg of polyvinyl pyrolidone 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 tooth pick tipped with a small piece of sand paper. 50 uL of chilled buffer was added to approximately 20 uL of pollen which was then allowed to soak overnight at 4°C. 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, prior to application to the gels.

Six enzymes were examined. Phosphoglucose isomerase (PGI, EC 5.3.1.9) and triosephosphate isomerase (TPI, EC 5.3.1.1.) were electrophoresed on a LiOH-borate buffer system (Ridgway et al. 1970). The electrode buffer consisted of 0.06 M LiOH and 0.3 M boric acid adjusted to pH 8.1. Gels were prepared using a buffer of 0.03 M tris, 0.05 M citric acid (anhydrous) adjusted to pH 8.4, and 10% electrode buffer. NADP-dependent isocitrate dehydrogenase (IDH, EC 1.1.1.42) and malate dehydrogenase (MDH, EC 1.1.1.37) were electrophoresed using a tris-citric acid system (modified from Ayala et al. 1973). The electrode buffer consisted of 0.1 M tris, 0.05 M citric acid (anhydrous) and 0.001 M EDTA (disodium salt) adjusted to pH 7.1. The gel buffer consisted of 0.01 M tris, 0.003 M citric acid (anhydrous) and 0.001 M EDTA (disodium salt) adjusted to pH 7.1. Phosphoglucomutase (PGM, EC 2.7.5.1) and 6-phosphogluconate dehydrogenase (6-PGDH, EC 1.1.1.44) were electrophoresed on a histidine-citrate buffer system (Cardy et al. 1981). The electrode buffer consisted of 0.065 M L-histidine (free base) and 0.007 M citric acid (anhydrous) adjusted to pH 6.5. The gel buffer was 33.3% electrode buffer. The LiOH-borate system was electrophoresed at 40 mA, the tris-citrate system at 60 mA, and the L-histidine-citrate system at 25 mA. All enzymes examined in this study moved towards the anode.

Staining recipes for analysis of different enzymes are described in several sources. The following are from Vallejos (1983). All enzyme stains employed the agarose overlay method.

TPI: 25 mL 0.1 M tris-HCl buffer at pH 8.0, 7 mg dihydroxyacetone phosphate (lithium salt), 20 mg EDTA (disodium salt), 15 mg β-NAD^+, 230 mg sodium arsenate, 133 units glyceraldehyde-3-phosphate dehydrogenase, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS.

PGI: 25 mL 0.1 M tris-HCl buffer at pH 8.3, 23 mg fructose-6-phosphate, 3 mg β-$NADP^+$, 5 units of glucose-6-phosphate dehydrogenase, 2 mL of 0.1 M $MgCl_2 \cdot 6 H_2O$, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS.

IDH: 25 mL 0.1 M tris-HCl buffer at pH 8.0, 25 mg of DL-isocitrate (trisodium salt), 3 mg of β-$NADP^+$, 2 mL 0.1 M $MgCl_2 \cdot 6 H_2O$, 0.5 mL MTT, (10 mg/mL) and 0.5 mg PMS.

MDH: 25 mL 0.1 M tris, 100 mg DL-malic acid, 20 mg β-NAD^+, buffer then adjusted to pH 7.8 with 1 M tris, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS.

PGM: 25 mL 0.1 M tris-HCl buffer at pH 8.0, 17 mg glucose-1-phosphate, 3 mg β-$NADP^+$, 0.1 mg glucose-1,6,-diphosphate, 30 units of glucose-6-phosphate dehydrogenase, 2 mL of 0.1 M $MgCl_2 \cdot 6 H_2O$, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS.

6-PGDH: 25 mL 0.1 M tris-HCl buffer at pH 8.0, 30 mg 6-phosphogluconate (barium salt), 3 mg β-$NADP^+$, 2 mL of 0.1 M $MgCl_2 \cdot 6 H_2O$, 0.5 mL MTT (10 mg/mL) and 0.5 mg PMS.

Genetic models

The nomenclature used to describe loci in the following discussions is as follows. Every locus is named by giving the enzyme 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 number 100. If, for example, the band designated as 100 migrates 25 millimeters from the origin during electrophoresis and a second band migrates 15 millimeters, then the second band is designated as 15/25 X 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 will be abbreviated when it is obvious to which locus and allele reference is being made. The most anodally migrating allele will be called F (for fast) and the most cathodal allele will be called S (for slow). A third allele that migrates between F and S will be called M (for middle). A further abbreviation that is used in the tables and figures is to number the alleles when referring to a genotype. If three alleles are present, the genotype is given three numbers, if two are present, it is given two numbers. When three alleles are present the numbers designate the number of copies of F, M, S, to show the number of alleles that are present. For example, the genotype FFMS is coded as 211, and the genotype FMSS is 112. When only two alleles are present, only two numbers are used, for example, FSSS becomes 13.

Variation in isoenzyme phenotype can arise from several sources. It may be allelic (Allozyme), resulting from alternative DNA sequences at a given locus, or isozymic in the strict sense, representing different sequences at separate loci (Gottlieb 1977). Often, the products of separate isozymic loci are isolated in separate subcellular compartments (Weeden 1983).

Apart from the distinct loci that encode isolated subcellular forms of the same enzyme which have been found in many diploid plants, there may also occur duplication of a given locus which encodes for 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 numbers (Gottlieb 1981). The products of these duplicated loci can interact and form interlocus heterodimers for dimeric enzymes such as PGI (Tanksley et al. 1981; Weeden and Gottlieb 1980).

All of the enzymes used in this study, with the exception of PGM, have been found to be dimeric (Tanksley et al. 1981; Weeden and Gottlieb 1980; Kahler and Lay 1985; Fernandez and Jouve 1987), that is, they are composed of two polypeptide subunits that are themselves the gene products and that randomly associate after translation to form the active enzyme. Three types of dimers will be formed in a diploid individual that is heterozygous for such a dimeric enzyme. Two of these will be distinct homodimers made up of identical subunits and the third type will be a heterodimer made up of different subunits, each encoded by a different allele. Because the association occurs randomly, the three types of dimers will occur in a 1:2:1 ratio and can usually be separated by electrophoresis. The ratio of occurrence is reflected in differential density of the enzyme bands.

If more than one diploid locus is present, the 1:2:1 ratio of band density may be distorted because the alleles, and thus the polypeptide subunits, may no longer be present in a 1:1 ratio. There can even exist three or more different alleles present within the same 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 electrophoresis gel.) Figure 1 is a summary of data presented by Ostergaard and Nielsen (1981). They show electrophoretic phenotypes associated with different genotypes of tetraploid ryegrass with duplicated Pgi2 (cytosolic) loci. The four left lanes show the phenotypes of individuals that are homozygous at all loci for each of four distinct alleles which they labeled A, B, C, and D. The individual in lane 5 contains all four alleles in its genotype. The phenotype shows only 7 of 10 possible bands due to overlap between various homo- and heterodimers. The individuals in lanes 6, 7, and 8 are heterozygous for two of the alleles but they contain varying doses of each. The individuals in lanes 9 and 10 are heterozygous for three of the alleles.

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

$$\left((f + m + s + \ldots) \,!/ \, (f!\, m!\, s! \ldots) \right) \; (F^f M^m S^s \ldots)$$

where upper case letters refer to the copies of a given allele: F, M, S, etc., within the genome and 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). For example, the genotype FFFM for a dimer

enzyme will have the following phenotype. The band density of the homodimer ff will be ((2) !/ (2!)) ($3^2 1^0$) = 9. The band density of the heterodimer fs will be ((1 + 1) !/ (1!1!)) ($3^1 1^1$) = 6, and the band density of the homodimer ss will be ((2!) / (2!)) ($3^0 1^2$) = 1. The expected phenotype will be three bands in a 9:6:1 ratio.

Figures 2 and 3 present the expected electrophoretic phenotypes for individuals with various doses of distinct alleles in their genomes. An allelic dose is equivalent to one copy of any allele; a diploid with a nonduplicated locus would have two allelic doses, a tetraploid four, etc. Figure 2 presents expected phenotypes when two distinct alleles are present, and Fig. 3 shows the phenotypes expected when three distinct alleles are present within the same genome. The ratio of band intensities is given for each phenotype. There are only five bands, rather than the theoretical six, shown for individuals that are heterozygous for three alleles because the heterodimer, fs, generally migrates the same distance as the homodimer, ss, on electrophoretic gels. The middle band in such phenotypes shows two numbers for its density ratio which correspond to the expected number of copies of the ss homodimer and the fs heterodimer, respectively. The top of a diagram corresponds to the anodal end of a gel and the bottom corresponds to the cathode and origin of electrophoretic migration. In the five banded phenotypes, the top band represents the homodimer ff, the second band represents the heterodimer fs, the middle band corresponds to the homodimer ss, and the heterodimer fs, the fourth band represents the heterodimer ss, and the bottom, band the homodimer ss.

It is doubtful that starch gel electrophoresis possesses enough resolution to distinguish among phenotypes when more than eight allelic

doses of replicated loci with two alleles, or more than six allelic doses of replicated loci with three alleles, are present. In addition, the actual density of bands must remain theoretical, since no densitometric measurements of gels were made. However, at these levels of loci replication and below, phenotypes can be distinguished visibly by noting the number and relative size and position of bands. For example, the difference in phenotypes between genotypes 510 and 420 can be seen visually on the starch gels.

In Table 2 are Punnett squares which illustrate the method for calculating the expected numbers for the various phenotypes among progeny of selfed heterozygotes. Two basic genetic models are used; that of independent disomic inheritance, and that of tetrasomic inheritance. Disomic inheritance is associated with segregation in the classical genomic allotetraploid. The duplicated loci are considered to represent two independently segregating diploid loci. Tetrasomic inheritance is the model of segregation associated with the classical concept of autotetraploidy. The duplicated loci are considered one locus with four allelic positions. The essential difference between the two models is that within a tetrasomic locus, any chromosome can pair with any other chromosome giving a gametic ratio of 1:4:1 (FF:FS:SS) for an individual that is duplex (FFSS) for two alleles; while a given chromosome can only pair with its homologue for duplicated disomic loci so that the gametic ratio is 1:2:1 (FF:FS:SS) for an individual with two heterozygous disomic loci (Schulz-Schaefer 1980).

Complications with the tetrasomic model arise with a heterozygous individual that is triplex (FFFS) or simplex (FSSS) or when the tetrasomic locus is far enough from the centromere so that crossing over results in

double reduction and chromatid segregation. A simplex or duplex individual produces only two types of gametes that recombine to give a 1:2:1 (FFFF:FFFS:FFSS or FFSS:FSSS:SSSS) ratio of progeny phenotypes. This ratio is indistinguishable from the segregation ratio of a single disomic locus. When chromatid tetrasomic segregation is in effect, any chromatid can be paired with any other and the gametic ratio becomes 3:8:3 (FF:FS:SS), rather than 1:4:1 for a duplex heterozygote.

When three alleles are present the pattern of segregation becomes quite complex, however, the basic principle remains that the rules of chromosome pairing are different for disomic and various tetrasomic models. These principles are the basis of the genetic models presented in Tables 3 and 4.

Table 3 presents examples of the expected frequencies of genotypes among selfed progeny of various heterozygotes with various numbers of alleles coding for a dimeric enzyme when independent disomic inheritance is in effect. An important assumption of this model is that the loci are segregating independently. Linkage among loci will distort these phenotypic ratios, and there is no reason to exclude the possibility of linkage among duplicated loci. This limitation is continually considered in the analysis to follow. Table 4 presents examples of the expected distribution of progeny genotypes when the duplicated loci segregate according to the various models of tetrasomic inheritance.

Pollen models

Much genetic information can be obtained through the comparison of isoenzyme phenotypes of sporophytic (diploid, somatic) tissue with those of pollen (haploid, gametophytic). If pollen is allowed to soak in extraction buffer only certain isoenzyme bands, corresponding to cytosolic enzymes, will appear. When the pollen is crushed in the same buffer, it displays a similar isoenzyme phenotype to that of sporophytic tissue. Weeden and Gottlieb (1980) demonstrated this fact by comparing zymograms of diploid sporophytic tissue of soaked and crushed pollen, and of purified, isolated chloroplasts for several different enzymes. The bands that failed to appear on the zymograms of soaked pollen were identical to those of chloroplasts. Crushed pollen contained the chloroplast associated bands. A comparison of zymograms of soaked and crushed pollen is, thus, one way to distinguish the cytoplasmic from the organelle sequestered isozymes. This is illustrated in Fig. 4A.

Other genetic information can be obtained from the comparison of sporophytic and pollen zymograms for heterozygotes. Differences in banding for either cytosolic or organelle loci may occur. Because of the haploid condition of the pollen, no intralocus heterodimers will occur (Weeden and Gottlieb 1979; Tanksley et al. 1981). If a diploid is heterozygous for only one nonduplicated locus, any one pollen grain will contain only one copy of either allele so that only homodimers will occur in a sample of pollen. However, if duplicated loci exist which are occupied by different allelic forms, the pollen can possess two different alleles and interlocus heterodimers will be produced. The presence of hybrid bands in pollen

zymograms is considered to be evidence of duplicated loci. This is illustrated in Fig. 4B.

Pollen zymograms may also distinguish between disomic and tetrasomic segregation when duplicated loci are present. The band density ratio may be different for pollen resulting from these different types of segregation because gamete frequency is different. This fact is illustrated in Fig. 4C.

Various models can be made to predict phenotype based upon the fact that no intralocus heterodimers can form in pollen of plants with duplicated disomic loci and upon the fact that band intensity ratios will be different for disomic and tetrasomic segregation. Several examples of such models are illustrated in Figs. 4 D and 4 E. It is doubtful that all of the different phenotypes can be distinguished visibly with the resolution available to starch gel electrophoresis. For example, it may not be possible to visually distinguish between the 2,2,8,2,2 and the 3,4,10,4,3 phenotypes in Fig. 4E. However, it should be possible to distinguish either of these from the 1,2,2,2,1 phenotype.

Results

Pollen Analysis

Successful isoenzyme staining was obtained for pollen with the enzymes PGM, TPI, and PGI. Figures 5 and 6 show isoenzyme phenotypes for sporophytes and for soaked and crushed pollen for PGM and TPI of L. japonicus X L. alpinus. 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, while the other zones for these enzymes correspond to organelle sequestered enzymes. Other

studies have shown that the lower zone of the PGI phenotype represents cytosolic loci (Ostergard and Nielsen 1981; Weeden and Gottlieb 1980).

Figure 7 shows the phenotypes of PGI for sporophytes and crushed pollen for several taxa. Lanes two and three contain the phenotypes for the sporophyte and pollen, respectively, of the diploid species L. tenuis (109-20). The pollen phenotype displays no hybrid band, indicating that this individual contains only one nonduplicated cytosolic locus for PGI. Lanes four and five contain the sporophyte and pollen for the interspecific hybrid L. japonicus X L. alpinus (23). Both phenotypes display five bands indicating the presence of three alleles within duplicated loci. It should be noted that this taxon is a diploid interspecific hybrid ($2n = 2x = 12$) and the evidence of duplicated loci at the diploid level is of interest. The pollen phenotype also contains five bands which implies that both loci are heterozygous (F/M, F/S). If the M and S alleles were both at the same locus (F/F, M/S), the ss heterodimer (second from the bottom) would not appear.

Lanes 6 and 7 contain sporophyte and pollen phenotypes for the artificial autotetraploid (L. alpinus)^2 (774x-5). The sporophyte phenotype is five banded with the middle three bands approximately equal in intensity and darker than the two extreme bands. This implies that it contains at least two duplicated cytosolic loci with three alleles. The pollen phenotype also possesses five bands with its middle band much darker than the others. This fact eliminates the possibility that the genotype is F/S, M/M which would have a band intensity ratio of 1,2,2,2,1 (see Fig. 4E). Whether the pollen phenotype has a band density of 2,2,8,2,2 (disomic

segregation of two loci F/M, M/S) or a ratio of 3,4,10,4,3 (tetrasomic locus FMMS) can not be determined with the resolution available with this gel.

Diploid sporophytes

PGM: The interspecific hybrid L. burttii X L. ornithopodioides was the only individual available that was heterozygous for PGM. This hybrid is infertile so that no segregation studies could be performed. Figure 8 shows the recombination of PGM alleles in the interspecific hybrid. Each of the parent species contains a common PGM band in its phenotype (relative mobility 100) in addition to another band which is not shared. The hybrid contains all three parental bands but no new hybrid bands occur. This fact implies that PGM is a monomeric enzyme and agrees with previous reports from other studies (Warwick and Gottlieb 1985; Gottlieb 1981; Kahler and Lay 1985; Wolf et al. 1987).

TPI: The interspecific hybrid L. alpinus X 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. 9). The hybrid band indicates that the cytosolic TPI enzyme is dimeric. This hybrid is the only individual that we have observed that is heterozygous for the cytosolic TPI locus.

PGI: The two interspecific hybrids L. japonicus X L. alpinus (23 and 28) 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 X L. alpinus (23) are shown in Fig. 10. The parent displayed five bands indicating the presence of three alleles in at least two duplicated cytosolic loci.

The upper (chloroplast sequestered) locus of PGI has been called pgi1 and the cytosolic locus pgi2 in previous studies (Ostergaard and Nielsen 1981; Gottlieb 1981). In compliance with this precedent, we will name the duplicated cytosolic loci pgi2 and pgi3. The three alleles of pgi2 and pgi3 are denoted by the relative mobilities of their homodimeric bands. The most anodal (chloroplast or pgi1) band has been arbitrarily designated as pgi1-100 and the three alleles become 72, 62, and 52.

The presence of three loci makes it possible to test for the segregation of the two distinct loci separately. Figure 11 presents the results of this test. The genetic model proposes two duplicated disomic heterozygous loci (F/S, F/M). The choice of which locus is pgi2 and which is pgi3 is arbitrary. Such a double heterozygous genotype has already been suggested by the pollen analyses. Figure 11 presents a contingency table that allows the calculation of three chi-square values.

The value Chi-square$_1$ = $\sum \frac{(o_i - e_i)^2}{e_i}$, 2 df, tests whether locus 1 is segregating according to the expected 1:2:1 Mendelian ratio,

and the value Chi-square$_2$ = $\sum \frac{(o_j - e_j)^2}{e_j}$, 2 df, tests whether

locus 2 is segregating according to a 1:2:1 ratio; and the value

Chi-square$_3$ = $\sum \frac{(o_{ij} - e_{ij})^2}{e_{ij}}$, 4 df, tests whether the two loci are segregating independently in a 1:2:1:2:4:2:1:2:1 ratio. The results are in accordance with a model of two linked duplicated disomic loci.

Although not likely for a diploid interspecific hybrid, the possibility that the two loci may segregate in a tetrasomic manner cannot be discounted. Table 5 presents the expected number of each phenotype in the progeny of a segregating tetrasomic heterozygote (FMMS) undergoing either chromosome or chromatid segregation. The Chi-square value for chromosome segregation is not significant. This illustrates the difficulty in distinguishing between the two models when linkage may be involved.

The parental and progeny phenotypes of selfed L. japonicus X L. alpinus (28) and the observed number of progeny phenotypes are shown in Fig. 12. The parent displays a three-banded phenotype with a distorted band density ratio. The ratio is more in the range of 9:6:1 than 1:2:1 which 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 those which would be expected if the selfed parent possessed two duplicated loci of which only one was heterozygous (F/M, F/F). The one segregating locus would give three genotypes in a frequency ratio of 1:2:1, while the homozygous locus would distort the band densities towards the more anodal bands. However, such a distribution of progeny phenotypes

could also be observed if the parents were triplex tetrasomic (FFFM). Two gametes would be equally frequent in such a triplex (FF and FM) and would recombine to give the same phenotypic frequency. One way to distinguish between these alternative modes of segregation would be to self any of the progeny with the 1:2:1 band-density phenotype. If the disomic model was correct, these individuals would be homozygous (F/F, M/M) and no segregation of PGI would occur among their progeny. If the tetrasomic model was correct, these individuals would be duplex (FFMM) and segregation would occur to produce phenotypes in the frequency of 1:8:18:8:1.

IDH: The interspecific hybrid L. japonicus X L. alpinus (23) was heterozygous for anodal loci of IDH. The parental and progeny phenotypes from this selfing are presented in Fig. 13, along with the observed occurrence of each progeny phenotype. The phenotypes display two zones of enzyme activity, an 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 heavier cathodal bands. This suggests that it possessed two alleles (100 and 85) with more slow alleles than fast in its genotype. Seven phenotypes were observed among the progeny suggesting the presence of three segregating loci (see Table 3). The simplest disomic parental genotype that is compatible with these observations would be (F/S, F/S, F/S, S/S), that is a genotype of four replicated loci of which three were heterozygous. Various tetrasomic models could also fit these observations. The observed progeny

frequencies along with those expected for disomic inheritance and for several models of tetrasomic inheritance are presented in Table 6. The chi-square value for goodness of fit is statistically significant for the independent disomic loci model (perhaps because linkage is distorting the ratio). Chi-square values are also significant for the tetrasomic models which fit even less well with observed values.

MDH: Parental and progeny phenotypes for the selfed interspecific hybrid L. japonicus X L. alpinus (28) for MDH as well as the observed numbers of progeny phenotypes are presented in Fig. 14. The zymograms are complex as would be expected for an enzyme with three isozymic forms. There is a single zone of segregation between relative mobilities 82 and 66. This zone is characterized by a changeable three-banded phenotype, thus implying the presence of a dimeric molecule. The parental phenotype is unbalanced with heavier weighting given to the lower bands suggesting the presence of at least one other homozygous locus controlling the phenotype in this region. Given the complexity of the phenotype and the lack of knowledge concerning interaction of this dimer with other loci, it is not possible to hypothesize an exact genotype for the parent, however, the presence of three progeny phenotypes in a frequency ratio of 1:2:1 and with the parental phenotype being most common among the progeny, it would suggest either the segregation of a single disomic locus, or of a triplex or a simplex tetrasomic locus. Selfing of the progeny with the third phenotype could distinguish between a disomic model with two loci homozygous for different alleles or a duplex tetrasomic locus for these individuals.

6-PGDH: The interspecific hybrid L. japonicus X L. alpinus (28) was also heterozygous for the upper zone of 6-PGDH. The parental and progeny

phenotypes from the selfing of the hybrid are presented in Fig. 15, along with the observed frequency of occurrence of each progeny phenotype. The observations are in accordance with a disomic model with two loci, one heterozygous for the alleles 6-PGDH1-120 and 6-PGDH1-100, and the other homozygous for the slower allele 6-PGDH2-100. This model again can not be distinguished from a simplex tetrasomic model (FSSS) except by selfing the progeny with the three-banded 1:2:1 band-density phenotype.

Independence of PGI, MDH, and 6-PGDH loci

The interspecific hybrid L. japonicus X L. alpinus (28) was heterozygous for Pgi2, Mdh3, and 6-Pgdh1. If the plausible model for duplicated disomic inheritance is 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 in each offspring. The results of this analysis are presented in contingency tables in Figs. 16, 17, and 18. Segregation of Pgi2 is compared to that of 6-Pgdh1 in Fig. 16, Mdh3 is compared to 6-Pgdh1 in Fig. 17, and Pgi2 is compared to that of Mdh3 in Fig. 18. All chi-square values are nonsignificant. These results are consistent with a model of three unlinked disomic loci for these enzymes. It is not possible to determine linkage relationships among the second homozygous loci for each enzyme nor between the two duplicated loci of each.

Tetraploid sporophytes

In view of the unexpected complexity found within the diploid interspecific hybrids, it is not surprising that the tetraploid genetics becomes quite complex. Cytosolic IDH enzymes were found to be controlled by at least four loci in the diploid hybrid. Thus, it could be expected to be

affected by eight loci in the artificial allotetraploid. This level of complexity goes beyond the resolution available with starch gel electrophoresis. Even the enzymes, PGI, MDH, and 6-PGDH which could be described by a model of two duplicated loci at the diploid level, would possess four loci in the allotetraploid. Any linkage among these loci could seriously distort ratios from those based upon independent disomic inheritance. Nevertheless, the number of different kinds of progeny phenotypes that are observed can indicate at least the number of segregating loci and an attempt has been made to analyze the tetraploids. The tetraploids (L. japonicus X L. alpinus)^2 (28), (L. alpinus)^2 (774x-5) and L. corniculatus were all heterozygous for PGI and MDH and were used in this analysis.

Allotetraploid (L. japonicus X L. alpinus)^2 (28)

A representative zymogram and observed progeny phenotypes for the selfed amphidiploid are presented in Figs. 19 and 20 for the enzymes PGI and MDH, respectively. The PGI progeny phenotypes are complex and it is difficult to ascertain an exact genotype for each. However, there are seven phenotypes found among the progeny, and the parental phenotype is unbalanced with the upper bands being of greater density. These facts suggest one homozygous locus and three heterozygous loci. Moreover, the observed progeny phenotypic ratio is not significantly different from the genotypic ratio that is predicted by a model for three segregating disomic loci (see Table 7). The only tetrasomic model that would provide for seven phenotypes among the progeny would be the independent segregation of one simplex and one duplex locus. The phenotypic ratios predicted by such a model do not fit the observed values (Table 7).

The progeny displayed five phenotypes for MDH. This is compatible with a model of two segregating disomic loci or one segregating tetrasomic locus. Tests of goodness of fit for these models are shown in Table 8. Chi-square values for all three models, disomic, tetrasomic, chromosome segregation, and tetrasomic chromatid segregation, are nonsignificant.

Artificial autotetraploid (L. alpinus)^2 (774x-5)

PGI: Parental and progeny phenotypes and observed occurrences of progeny phenotypes for the selfed artificial tetraploid are shown in Fig. 21. The parental phenotype has five bands and is balanced. This fact suggests a genotype with three alleles with two doses of the middle allele (relative mobility 62). The genotype could be (FMMS) for a tetrasomic model or F/M, M/S for a disomic model. There were eight phenotypes observed. This number of phenotypes could be produced by the segregation of one tetrasomic locus or two disomic loci if it is assumed that one of the rarer genotypes (202) did not occur within our sample (see Tables 3 and 4). Observed and expected frequencies of progeny phenotypes for disomic and tetrasomic models are shown in Table 9. All chi-square values were significant. The model with tetrasomic chromosome segregation provided the best fit with the observed data.

MDH: Results for the segregation of MDH among progeny of the selfed autotetraploid are shown in Fig. 22. The parental phenotypes are more dense for the lower segregating bands, indicating either a simplex tetrasomic locus (FSSS) or one homozygous and one heterozygous disomic locus (F/S, S/S). The progeny phenotypic ratio is approximately 1:2:1 which would be consitent with either model. Again, selfing the progeny with the phenotype with a band density ratio of 1:2:1 could test these alternatives.

It should be noted that the presence of only four allelic doses need be hypothesized to explain segregation in the autotetraploid, unlike the eight allelic doses needed for segregation in the amphidiploid. These would correspond to only one tetrasomic locus or two disomic loci and thus, only one nonduplicated locus for each enzyme in the diploid L. alpinus (77).

Lotus corniculatus L.

This cultivated tetraploid is not self-fertile. Thus, it was necessary to perform the more difficult task of artificial cross pollination. Since an outcross was necessary in any case, the crosses were chosen so that a heterozygote was crossed with a homozygote, an equivalent of the testcross. The phenotypes for PGI and MDH for the parents of this cross are shown in Fig. 23. The testcross is more precise than selfing because segregation occurs only in the heterozygote. Thus, gamete frequency from only one individual (the heterozygote) can be directly observed.

Results of the cross for PGI are shown in Fig. 24. There were five progeny phenotypes which indicate the presence of five gamete types in the heterozygotic parent (554-4). This number is consistent with a model for four segregating disomic loci (F/M, F/M, F/M, F/M) or else two tetrasomic loci (FFMM, FFMM). Results from tests of goodness of fit for disomic and tetrasomic models are presented in Table 10. All chi-square values were significant, indicating lack of fit. Again linkage among some of the four loci could distort disomic ratios. The model for four independent disomic loci produced the best fit of those tested.

The segregation of MDH loci within the heterozygotic parent produced two progeny phenotypes in an approximate 1:1 ratio (see Fig. 25). This is consistent with the presence of one heterozygous disomic locus or else a

triplex tetrasomic locus. The phenotype of the heterozygous parent was unbalanced implying the presence of at least one other homozygous locus for the disomic model.

Discussion

Characterization of loci

The first purpose of this study, to characterize and separate distinct loci, has been satisfied. 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 phenotype of MDH was not resolved into its organelle and cytosolic components, however, a single zone of allozymic activity was identified (relative mobility 82-66). IDH was found to contain two isozymic zones. This two-zoned phenotype corresponds with results presented by Kiang and Gorman (1985) for IDH in soybean (*Glycine max* (L.) Merr.). They demonstrated that the upper zone of the phenotype was cytosolic and controlled by two duplicated loci that they named *Idh1* and *Idh2*, and that the lower zone represented an enzyme form sequestered within the mitochondria.

The phenotype of 6-PGDH was resolved into two isozymic zones with segregation confined to the anodal region for the taxa 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

and IDH, for the most anodal region of 6-PGDH and for the middle zone of MDH mentioned above. The newly identified loci and alleles for Lotus are named 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. Evidence of heterozygosity for three other loci was discovered in an electrophoretic survey of the diploid species and will be reported elsewhere (Raelson and Grant 1987b). These loci are Tpi2 and 6-pgdh2 (both the more cathodal zones of the phenotypes) and Me1. These loci were not heterozygous in the taxa reported in this paper.

Duplicated loci and allo- and autotetraploidy

The presence of duplicated isoenzyme loci at the diploid level in the interspecific hybrids L. japonicus X L. alpinus (23 and 28) 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. Gottlieb and Weeden (1979) found evidence of duplicated Pgi2 loci in certain sections of the genus Clarkia. Because these loci segregated independently, they proposed a translocation between nonhomologous chromosomes as the source of the duplication, and considering such a translocation a unique event, they rearranged the classification of Clarkia by placing together those taxa with duplicated loci. Gottlieb (1982) presented samples where duplication reflected ancient polyploidy. He used the example of Zea mays for which the presence of duplicated loci for several enzymes along with other evidence suggested $x = 5$ rather than $x = 10$ as the correct basic chromosome number for the species.

It can be asked whether such a mechanism was responsible for the duplication of PGI, MDH, 6-PGDH, and IDH loci in the interspecific hybrid examined in this study, that is, do the duplications represent ancient polyploidy in Lotus or, might they have been inherited from one of the diploid parents of the hybrid (L. japonicus X L. alpinus)?

The answer to both of these questions appears to be no. Firstly, all basic chromosome numbers within the Loteae tribe are either 5, 6, 7, or 8 (Darlington and Wylie 1955; Grant 1986). Within the entire Papilionaceae subfamily the lowest reported chromosome number is 5 which has also been found in certain species of Vicia and Hedysarum (Darlington and Wylie 1955). Secondly, no evidence exists for duplication of Pgi2 in any of the Lotus diploid species that were examined during the course of this isoenzyme study. The pollen phenotype of the diploid L. tenuis (109-21, see Fig. 7) showed no heterodimeric band, suggesting the presence of a single locus for pgi2. Several accessions of the diploids L. tenuis, L. uliginosus, L. japonicus, L. alpinus were examined for electrophoretic phenotype. The results will be reported elsewhere (Raelson and Grant 1987b). All heterozygotes among these species possessed three-banded phenotypes with band density ratios of 1:2:1 for Pgi2 and 6Pgdh1 (for IDH, four rather than two, duplicated loci were found in the hybrids).

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. In this context, it is interesting to note that evidence for the initial duplication was found not only in the diploid hybrids (2 loci for Pgi2, Mdh2, 6-Pgdh1 and four loci for Idh1), but also for the artificial autotetraploid (L. japonicus)^2 (28) (four pgi2 loci) and in

L. corniculatus (four Pgi2 loci), while in the artifical autopolyploid (L. alpinus)^2 (774x-5) segregation could be explained by a model with only two PGI and MDH loci which would result simply from chromosome duplication. In short, the duplications provide additional evidence that L. corniculatus is indeed a segmental allotetraploid, produced by a doubling of the chromosome number of an interspecific hybrid between two species whose genomes possessed homoeologous chromosomes as was first proposed by Stebbins (1950).

In order to develop this argument, it is necessary to recall the various models of autotetraploidy, allotetraploidy, and segmental allotetraploidy. The classical concepts of auto- and allopolyploidy were perhaps best expressed by Darlington (1937). These models are illustrated in Fig. 26A and B. Doubling of the homologous chromosomes of a diploid species results in four homologous chromosomes and, therefore, a tetrasomic locus in the autotetraploid. A genomic allotetraploid results from doubling the chromosomes of an interspecific hybrid whose parental species are phylogenetically remote so that bivalent formation between structurally distinct chromosomes is greatly reduced. Chromosome doubling restores two distinct sets of homologues, one homologue in each pair is a copy of the other so that the resulting two disomic loci are homozygous for different allelic forms resulting in fixed heterozygosity. Stebbins (1947) elaborated on these classical concepts by introducing the idea of segmental alloploidy. A segmental allotetraploid is derived from the doubling of chromosomes of an interspecific hybrid between species that are phylogenetically close so that their chromosomes retain a high degree of homology but which may differ by small structural changes (homoeology). Upon chromosome doubling, such polyploids may display tetrasomic or disomic inheritance depending on

whether pairing is homogenic (between exact homologues) or heterogenic (between homoeologues).

An example of one possible mechanism that can explain how duplicated loci in the interspecific hybrid can be associated with segmental allotetraploidy follows (this mechanism is illustrated in Fig. 26C). Chromosomal rearrangement associated with speciation may have resulted in structural differences between homoeologues of L. japonicus and L. alpinus. These differences are not enough to prevent pairing 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 produce viable progeny with two chromosomes with duplications, and thus, with duplicated Pgi2 loci. Doubling the chromosome number of these individuals would produce the segmental allotetraploid with either four disomic or two tetrasomic loci. Subsequent diploidization of the allotetraploid would involve genetic factors such as the Ph locus in wheat that would lead to bivalent formation and disomic inheritance (Jackson 1982). This model applies by extension to L. corniculatus, since it also displays duplicated Pgi2 loci though we do not mean to imply that L. alpinus and L. japonicus were necessarily the diploid parents.

It might be objected that this model would explain the presence of two alleles in the interspecific hybrids and artificial amphidiploid but that it can not explain the presence of three distinct alleles in L. japonicus and L. alpinus (23). In reply to this objection, we point out that the hybrid

material was descendent from parents representing several distinct crossing events (Somaroo 1970).

Data concerning the meiotic regularity of the various taxa are presented in Table 11. The diploid species are all meiotically regular with no multivalents at diakinesis and MI and with high pollen stainability. The interspecific hybrid has a much lower pollen viability but retains a high level of bivalent formation which implies a high degree of homology, and therefore, supports the model of duplicated, linked, disomic inheritance. Doubling the chromosome number of the hybrid increased fertility, but also resulted in the formation of more multivalents. With respect to pollen stainability and multivalent formation, the amphidiploid is similar to the autotetraploid (L. alpinus)[2]. Tetrasomic loci within the amphidiploid are possible. Lotus corniculatus has a much higher level of pollen stainability and fewer multivalents. This could reflect genetic diploidization since its formation. Therrien and Grant (1984) found the frequency of quadrivalent formation to be less than previously reported for this species suggesting some selection for increased diploidization had occurred. The meiotic data do not contradict the above hypothesis.

The evidence for eight allelic doses or four loci of Pgi2 in L. corniculatus is interesting with respect to previous reports of tetrasomic inheritance in this species. As mentioned earlier, Dawson (1941) proposed tetrasomic inheritance of cyanogenesis based upon observations of a 5:1 ratio of cyanogenic to acyanogenic progeny from the putative duplex X nulliplex cross (AAaa X aaaa). He proposed that the nulliplex represented the acyanogenic genotype. The ratio that Dawson examined assuming the dominance of cyanogenesis is analogous to examining the ratio of all other

genotypes to either of the homozygous genotypes for the codominant Pgi2 alleles for the cross L. corniculatus (554-5 X Leo-1). These homozygous genotypes are 80 or 08 in Table 10. For two tetrasomic loci undergoing random chromosome segregation the expected ratio to either homozygote would be 36:1, for four independent disomic loci, the expected ratio would be 16:1, while the ratios for the observed homozygote (80), is essentially (and perhaps coincidentally) the 5:1 ratio found by Dawson.

Other workers have also reported tetrasomic inheritance for other characters in L. corniculatus. Perhaps the most studied of these characters is the brown vs. yellow color of the keel tips. Several studies determined that the character was inherited in a tetrasomic manner based upon the observation of a low frequency of homozygotes to heterozygotes. However, in some crosses the frequency of homozygotes was even lower than that expected from tetrasomic inheritance. Hart and Wilsie (1959) proposed a second locus beside that controlling keel-tip color that was lethal in the nulliplex condition in order to explain the unusually low number of homozygotes. Buzzell and Wilsie (1963) tested this hypothesis and found it to be incorrect. They suggested that meiotic irregularities were the explanation for the lower homozygote frequency as did Bubar and Miri (1965).

It is interesting to note that linkage among disomic loci would be sufficient to explain the distortion of expected ratios if these loci are present in four copies, as is the Pgi2 locus, rather than in the previously assumed two copies.

Acknowledgments

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, F. J., D. B. HEDGECOCK, S. ZUMWALT, and J. W. VALENTINE. 1973. Genetic variation in Tridacna maxima, an ecological analog of some unsuccessful evolutionary lineages. Evolution, 27: 177-191.

BUBAR, J. S., and R. K. MIRI. 1965. Inheritance of self-incompatibility and brown keel tip in Lotus corniculatus L. Nature, 205: 1035-1036.

BUZZELL, R. I., and C. P. WILSIE. 1963. Genetic investigations of brown keel tip color in Lotus corniculatus L. Crop Sci. 3: 128-130.

CARDY, B. J., C. W. STUBER, and M. M. GOODMAN. 1981. Techniques for starch gel electrophoresis of enzymes from maize (Zea mays L.). Institute of Statistics Mimeographed Series No. 1317, North Carolina State University, Raleigh, N.C. 31 pp.

CRAWFORD, D. J. 1985. Electrophoretic data and plant speciation. Syst. Bot. 10: 405-416.

DARLINGTON, C. D. 1937. Recent advances in cytology. 2nd. ed. Blakeston, Philadelphia. 671 pp.

DARLINGTON, C. D., and A. P. WYLIE. 1955. Chromosome atlas of flowering plants. Hafner, New York. 519 pp.

DAWSON, C. D. R. 1941. Tetrasomic inheritance in Lotus corniculatus L. J. Genet. 42: 49-73.

FERNANDEZ, J. A., and N. JOUVE. 1987. Chromosomal location of structural genes controlling isozymes in Hordeum chilense. 1. 6-Phosphogluconate dehydrogenase and malate dehydrogenase. Theor. Appl. Genet. 73: 433-439.

GOTTLIEB, L. D. 1977. Electrophoretic evidence and plant systematics. Ann. Mo. Bot. Gard. 64: 161-180.

GOTTLIEB, L. D. 1981. Gene number in species of Astereae that have different chromosome numbers. Proc. Natl. Acad. Sci. U.S.A. 78: 3726-3729.

GOTTLIEB, L. D. 1982. Conservation and duplication of isozymes in plants. Science, 216: 373-380.

GOTTLIEB, L. D., and N. F. WEEDEN. 1979. Gene duplication and phylogeny in Clarkia. Evolution, 33: 1024-1039.

GRANT, W. F. 1986. The cytogenetics of Lotus (Leguminosae). J. Nat. Hist. 20: 1461-1465.

GRANT, W. F., M. R. BULLEN, and D. NETTANCOURT, de. 1962. The cytogenetics of Lotus. I. Embryo-cultured interspecific diploid hybrids closely related to L. corniculatus. Can. J. Genet. Cytol. 4: 105-128.

JACKSON, R. C. 1982. Polyploidy and diploidy: New perspectives on chromosome pairing and its evolutionary implications. Am. J. Bot. 69: 1512-1523.

HART, R. H., and C. P. WILSIE. 1959. Inheritance of a flower character, brown keel tip in Lotus corniculatus L. Agron. J. 51: 379-380.

KAHLER, A. L., and C. L. LAY. 1985. Genetics of electrophoretic variants in the annual sunflower. J. Hered. 76: 335-340.

KIANG, Y. T., and M. B. GORMAN. 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. Ph.D. Thesis. The Pennsylvania State University, State College, PA.

O'DONOUGHUE, L. S. 1986. A study of seed dehiscence in the fenus Lotus (Fabaceae) using interspecific hybridization and embryo culture. M.Sc. Thesis, Macdonald College of McGill University, Montreal, Quebec, Canada.

O'DONOUGHUE, L. S., and W. F. GRANT. 1987. A study of seed dehiscence in the genus Lotus (Fabaceae) using interspecific hybridization and embryo culture. Manuscript in preparation.

OSTERGAARD, H., and G. NIELSEN. 1981. Cultivar identification by means of isoenzymes. I. Genotypic survey of Pgi-2 locus in tetraploid ryegrass. Z. Pflanzenzuechtg. 87: 121-132.

RAELSON, J. V., AND GRANT, W. F. 1987a. An isoenzyme study in the genus Lotus (Fabaceae). I. Establishment of experimental protocols. To be submitted to Genome.

RAELSON, J. V., AND GRANT, W. F. 1987b. An isoenzyme study in the genus Lotus (Fabaceae). III. Evaluation of hypotheses concerning the origin of L. corniculatus using isoenzyme data. To be submitted to Theo. Appl. Genet.

RIDGWAY, G. J., SHERBURNE, S. W. and LEWIS, R. D. 1970. Polymorphisms in the esterases of Atlantic herring. Trans. Am. Fisheries Soc. 99: 147-151.

ROSS, M. D., and JONES, W. T., 1985. The origin of Lotus corniculatus. Theor. Appl. Genet. 71: 284-288.

SCHULZ-SCHAEFFER, J. 1980. Cytogenetics: plants, animals, humans. Springer-Verlag, New York, Heidelberg, Berlin. 446 pp.

SOMAROO, B. H. 1970. A cytogenetic study of interspecific diploid hybrids and amphidiploids in the genus Lotus. Ph.D. Thesis, McGill University, Montreal, Quebec, Canada. 295 pp.

SOMAROO, B. H., and W. F. GRANT. 1971. Meiotic chromosome behavior in induced autotetraploids and synthetic amphidiploids in the L. corniculatus group. Can. J. Genet. Cytol. 13: 663-671.

SOMAROO, B. H., and W. F. GRANT. 1972. Crossing relationships between synthetic Lotus amphidiploids and L. corniculatus. Crop Sci. 12: 103-105.

STEBBINS, G. L. 1947. Types of polyploids: their classification, and significance. Adv. Genet. 1: 403-429.

STEBBINS, G. L. 1950. Variation and evolution in plants. Columbia Univ. Press, New York and London. 643 pp.

TANKSLEY, S. D. 1983. Gene mapping. In Isozymes in Plant Genetics and Breeding, Part A. Edited by S. D. Tanksley and T. J. Orton. Elsevier Sci. Publ., Amsterdam, New York. pp. 109-138.

TANKSLEY, S. D., LANIER, D., and C. M. RICK. 1981. Evidence for extensive overlap of sporophytic and gametophytic gene expression in Lycopersicon esculentum. Science, 213: 453-455.

THERRIEN, M. C., and W. F. GRANT. 1984. Meiotic chromosome aberrations and quadrivalent formation in Lotus corniculatus over four generations after seed treatment with EMS. Cytologia, 49: 27-32.

VALLEJOS, C. E. 1983. Enzyme activity staining. In Isozymes in Plant Genetics and Breeding, Part A. Edited by S. D. Tanksley and T. J. Orton. Elsevier Science Publ., Amsterdam, New York. pp. 469-516.

WARWICK, S. I., and L. D. GOTTLIEB. 1985. Genetic divergence and geographic speciation in Layia (Compositae). Evolution, 39: 1236-1241.

WEEDEN, N. F. 1983. Plastid isozymes. In Isozymes in Plant Genetics and Breeding, Part A. Edited by S. D. Tanksley and T. J. Orton. Elsevier Science Publ., Amsterdam, New York. pp. 139-156.

WEEDEN, N. F., and L. D. GOTTLIEB. 1979. Distinguishing allozymes and isozymes of phosphoglucose isomerases by electrophoretic comparisons of pollen and somatic tissues. Biochem. Genet. 17: 287-296.

WEEDEN, N. F., and L. D. GOTTLIEB. 1980. Isolation of cytoplasmic enzymes from pollen. Plant Physiol. 66: 400-403.

WOLF, P. G., C. H. HAUFLER, and E. SHEFFIELD. 1987. Electrophoretic evidence for genetic diploidy in the bracken fern (Pteridium aquilinum). Science, 236: 947-949.

FIGURE 1. Summary of phenotypes found by Ostergaard and Nielsen (1981) for duplicated pgi2 loci in tetraploid ryegrass. See text for further explanation.

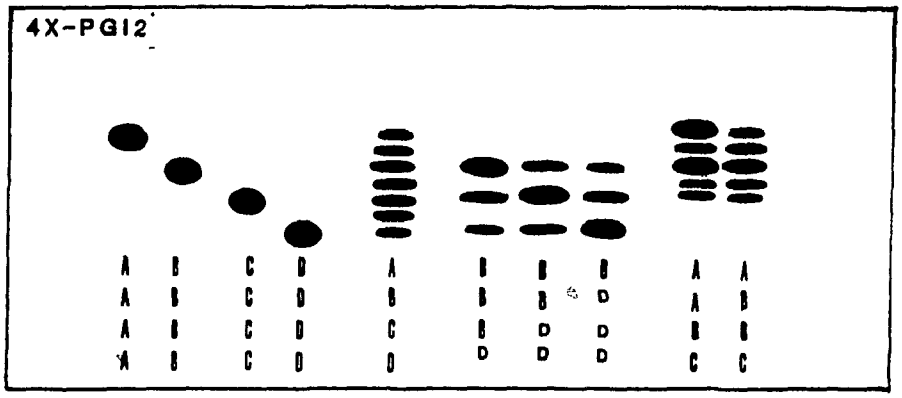

4X-PGI2
PHENOTYPE
GENOTYPE
A A A A
B B B B
C C C C
D D D D
A B C D
B B B D
B B D D
B D D D
A A B C
A B B C

FIGURE 2. Expected isoenzyme banding phenotypes for individuals with various doses of two alleles with distinct electromobility for a dimeric enzyme. See text for further explanation.

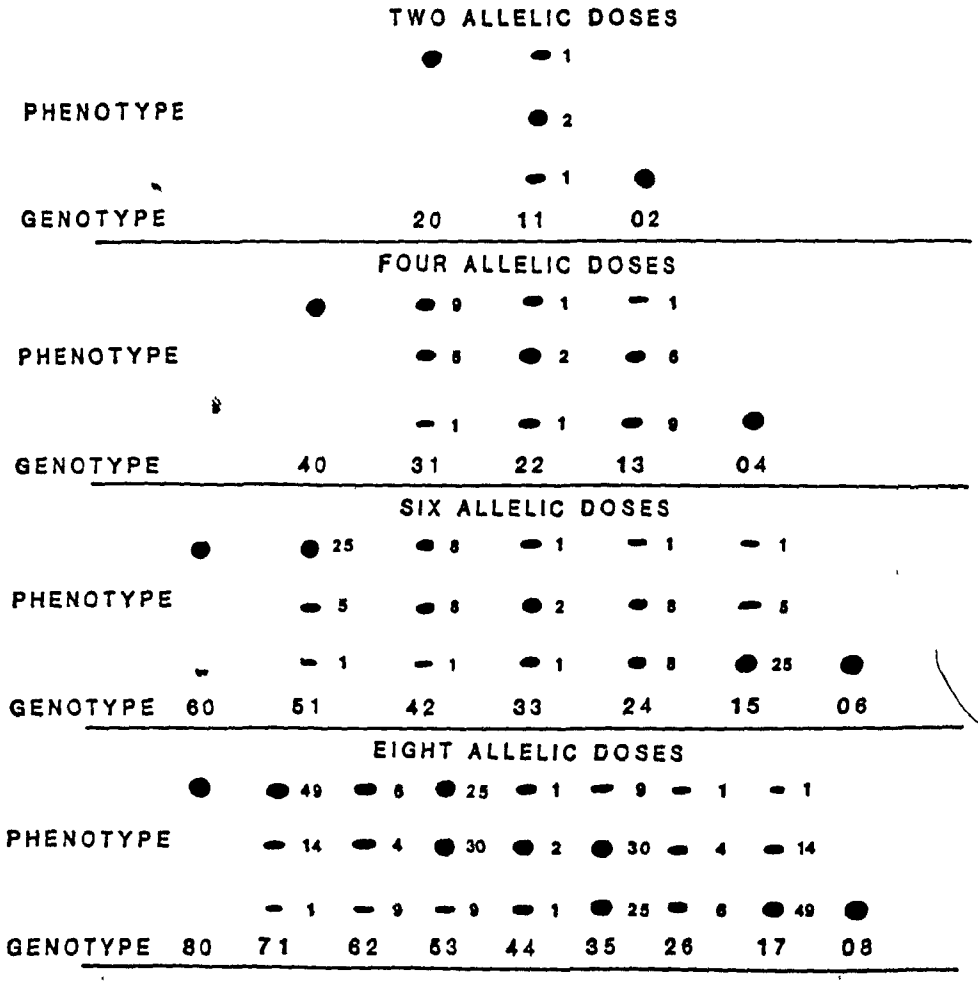

TWO ALLELIC DOSES
PHENOTYPE
GENOTYPE
20
11
02
FOUR ALLELIC DOSES
PHENOTYPE
GENOTYPE
40
31
22
13
04
SIX ALLELIC DOSES
PHENOTYPE
GENOTYPE
60
51
42
33
24
15
06
EIGHT ALLELIC DOSES
PHENOTYPE
GENOTYPE
80
71
62
53
44
35
26
17
08

FIGURE 3. Expected isoenzyme banding phenotypes for individuals with various doses of three alleles with distinct electromobility for a dimeric enzyme. See text for further explanation.

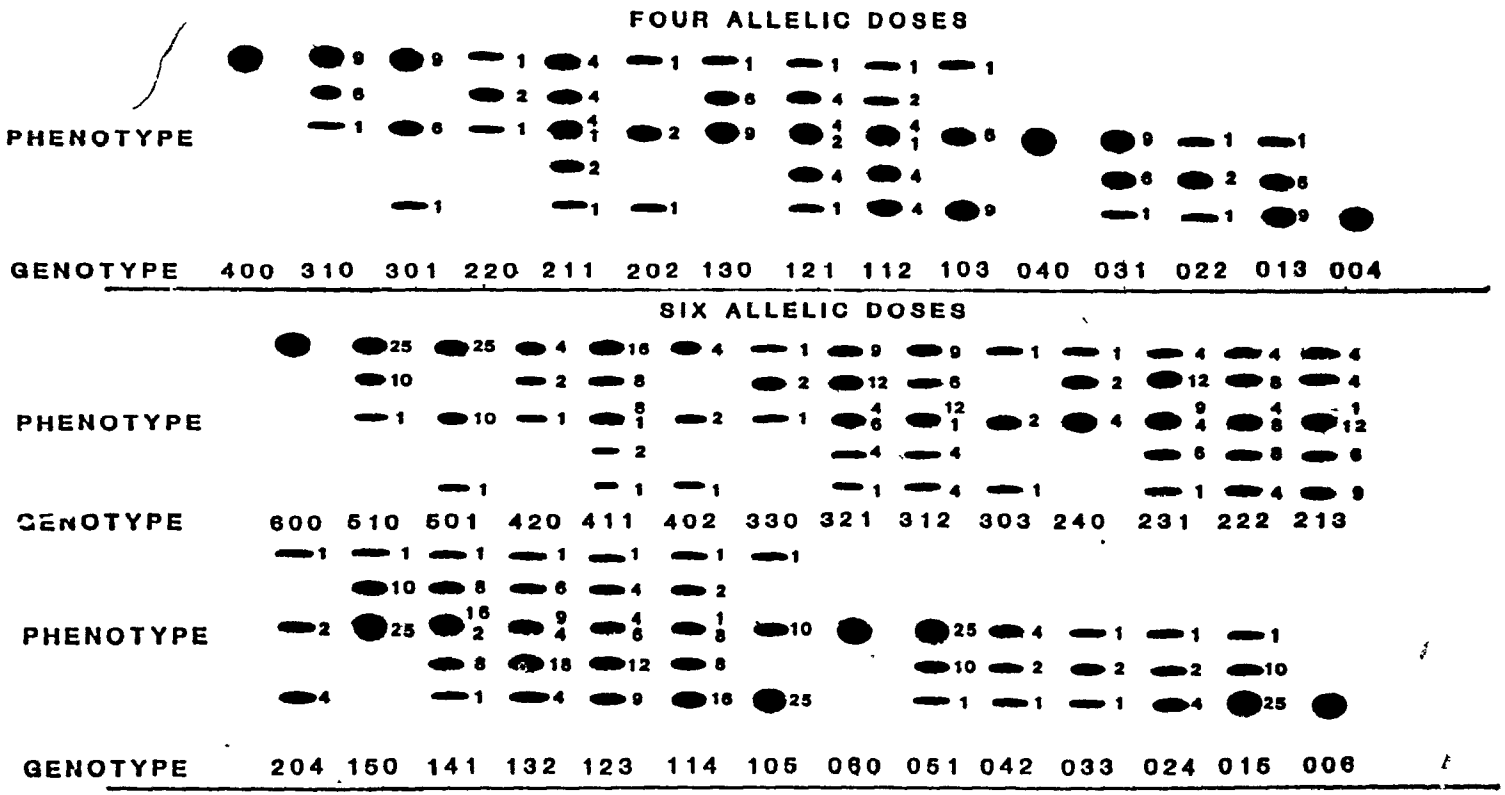

FOUR ALLELIC DOSES
PHENOTYPE
GENOTYPE 400 310 301 220 211 202 130 121 112 103 040 031 022 013 004
SIX ALLELIC DOSES
PHENOTYPE
GENOTYPE 600 510 501 420 411 402 330 321 312 303 240 231 222 213
PHENOTYPE
GENOTYPE 204 150 141 132 123 114 105 060 051 042 033 024 015 006

FIGURE 4. Expected isoenzyme banding phenotypes for sporophytes and pollen. A. Soaked vs. crushed pollen. B. Single vs. duplicated loci. C. Disomic vs. tetrasomic segregation. D. and E. Various models to ascertain genoptypes. See text for further explanation. S = sporophyte, P = pollen, F = fast, M = middle, S = slow. F7S F/M = duplicated disomic loci, FMMS = tetrasomic locus.

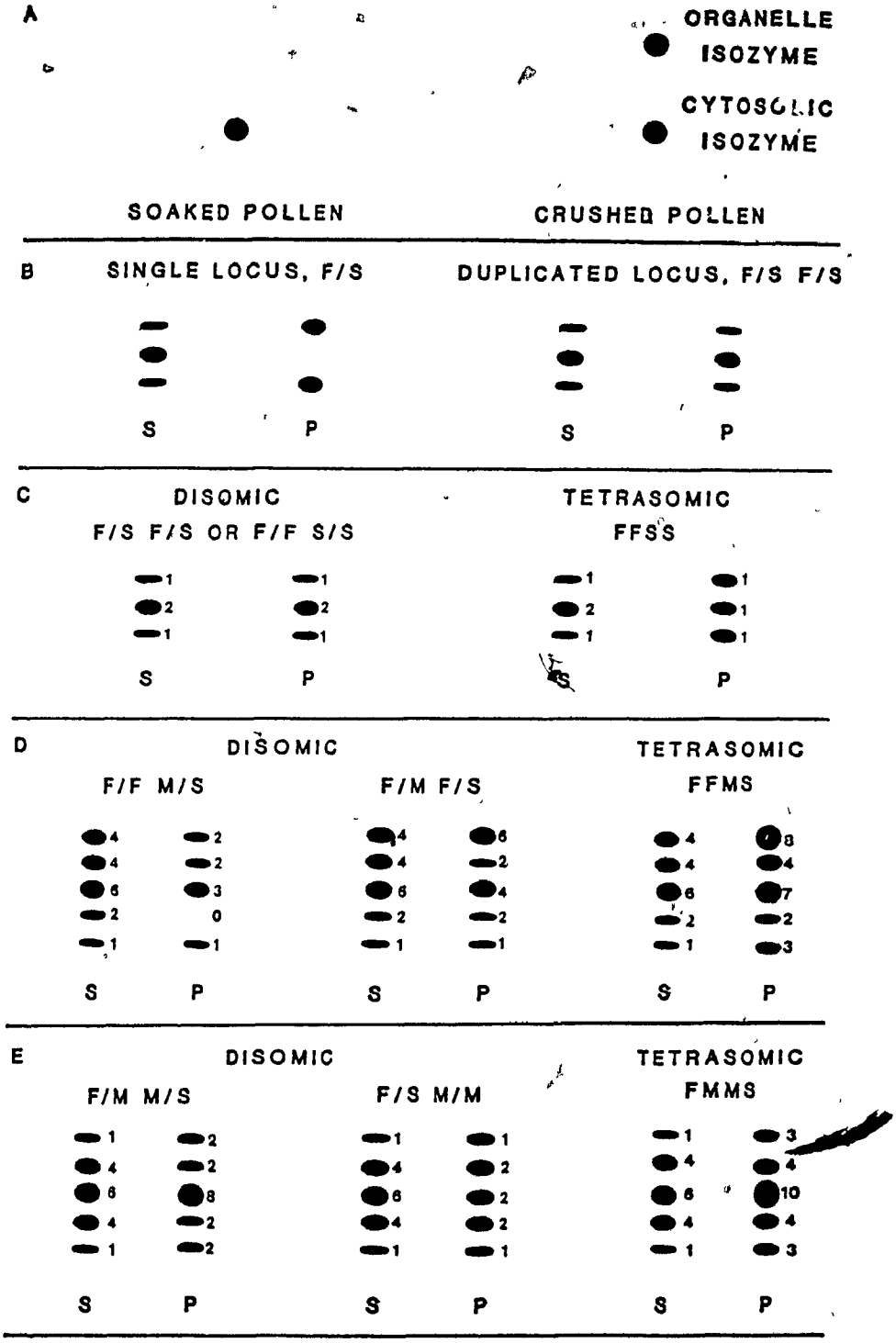
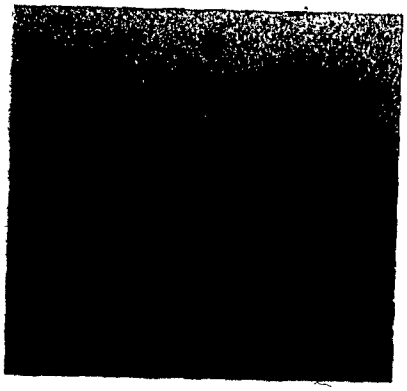

A
ORGANELLE ISOZYME
CYTOSOLIC ISOZYME
SOAKED POLLEN
CRUSHED POLLEN
B
SINGLE LOCUS, F/S
DUPLICATED LOCUS, F/S F/S
S
P
C
DISOMIC
F/S F/S OR F/F S/S
TETRASOMIC
FFSS
D
DISOMIC
F/F M/S
F/M F/S
TETRASOMIC
FFMS
E
DISOMIC
F/M M/S
F/S M/M
TETRASOMIC
FMMS

FIGURE 5. Observed isoenzyme banding phenotypes for PGM for sporophyte L. japonicus X L. alpinus (23; lane 1), and for pollen soaked in extraction buffer (lane 2), and crushed in extraction buffer (lane 3). (A) Photograph of zymogram. (B) Graphic presentation of zymogram.

PGM

A



B

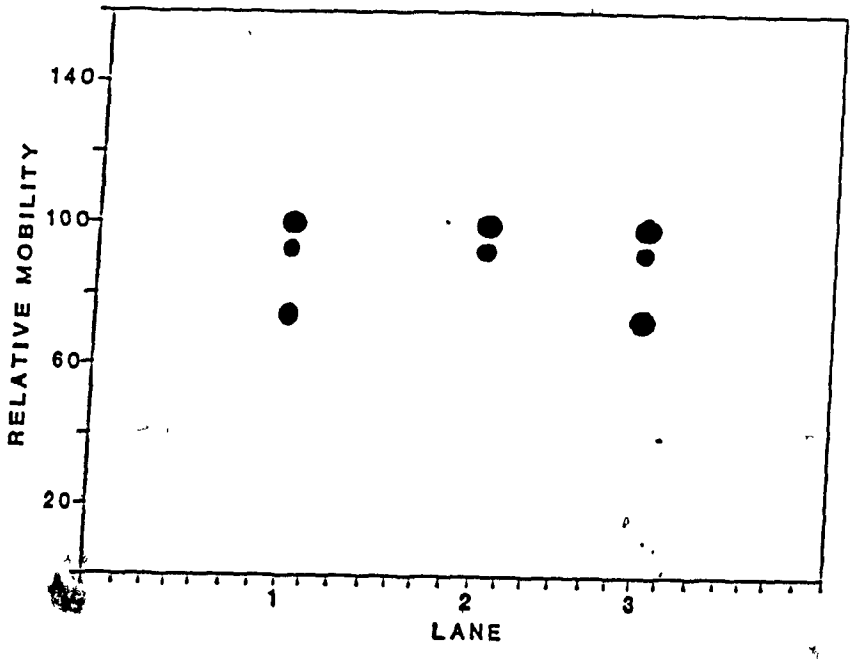
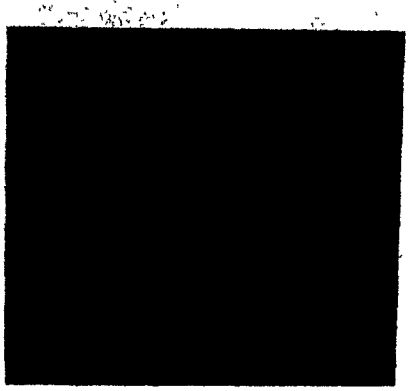


FIGURE 6. Observed isoenzyme banding phenotypes for TPI for sporophyte L. japonicus X L. alpinus (28; lane 1), and for pollen soaked in extraction buffer (lane 2), and crushed in extraction buffer (lane 3). (A) Photograph of zymogram. (B) Graphic presentation of zymogram.

TPI

A



B

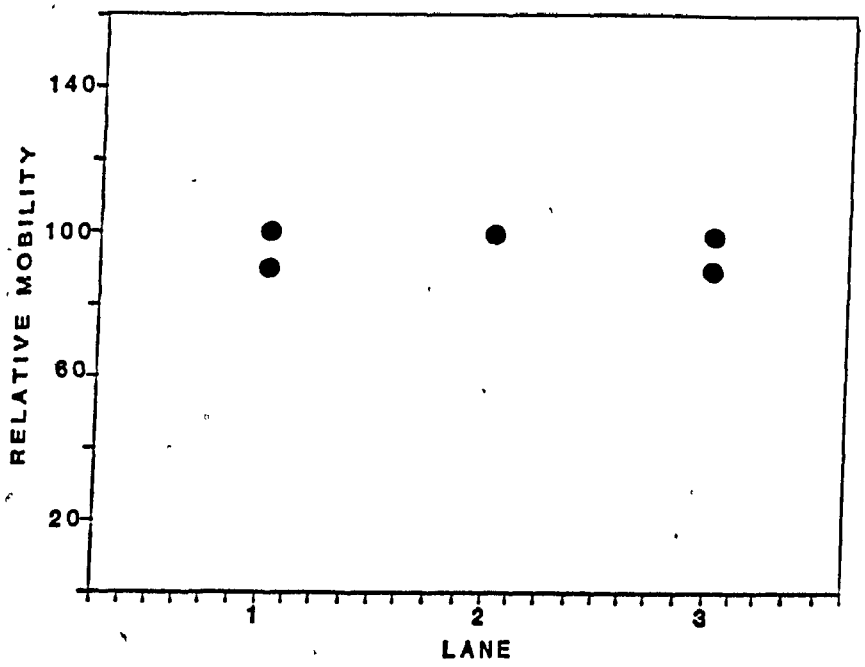
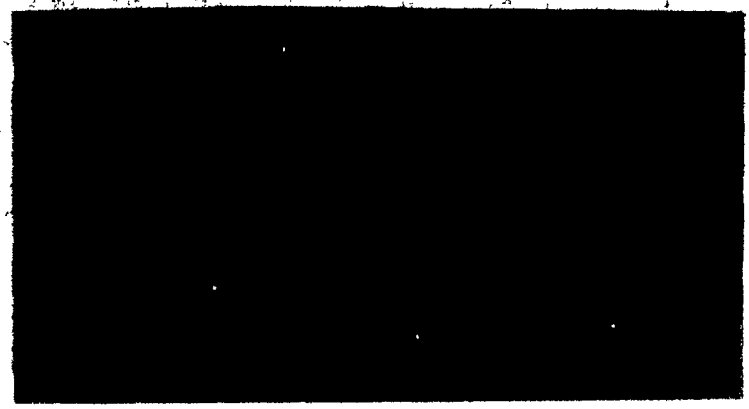


FIGURE 7. Observed isoenzyme banding phenotypes for PGI for various sporophytes and for pollen crushed in extraction buffer. Lane 1, L. tenuis (109-21) standard; lane 2, sporophyte of L. tenuis (109-20); lane 3, pollen of L. tenuis (109-20), lane 4, sporophyte of L. japonicus X L. alpinus (23); lane 5, pollen of L. japonicus X L. alpinus (23); lane 6, sporophyte (L. alpinus)^2 (774x-5); lane 7, pollen (L. alpinus)^2 (774x-5). (A) Photograph of zymogram. (B) Graphic presentation of zymogram.

PGI

A



B

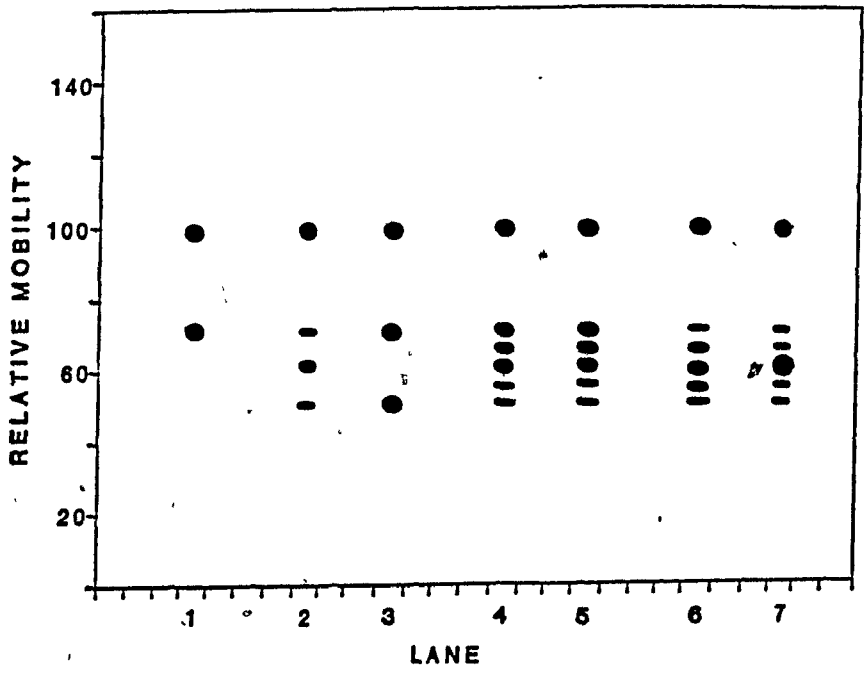
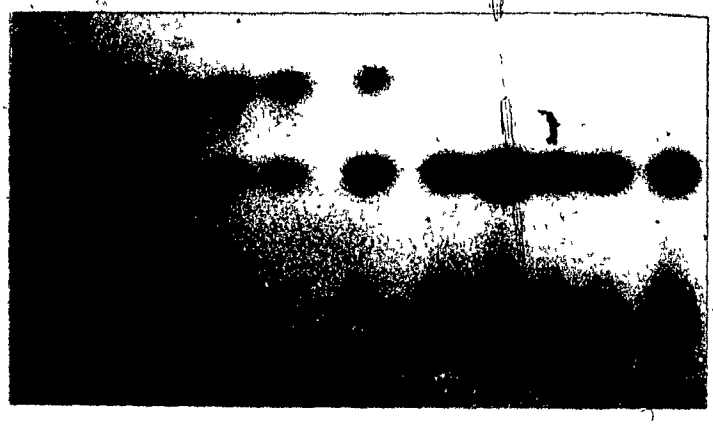


FIGURE 8. Observed isoenzyme banding phenotypes for PGM for diploids L. burttii (accession number 303; lanes 2 to 5), L. ornithopodioides (accession number 100; lanes 7 to 11), and for the interspecific hybrid L. burttii X L. ornithopodioides (lane 6). Lane 1 is L. tenuis (109-21) which is used as a standard. (A) Photograph of zymogram. (B) Graphic presentation of zymogram.

PGM

A



B

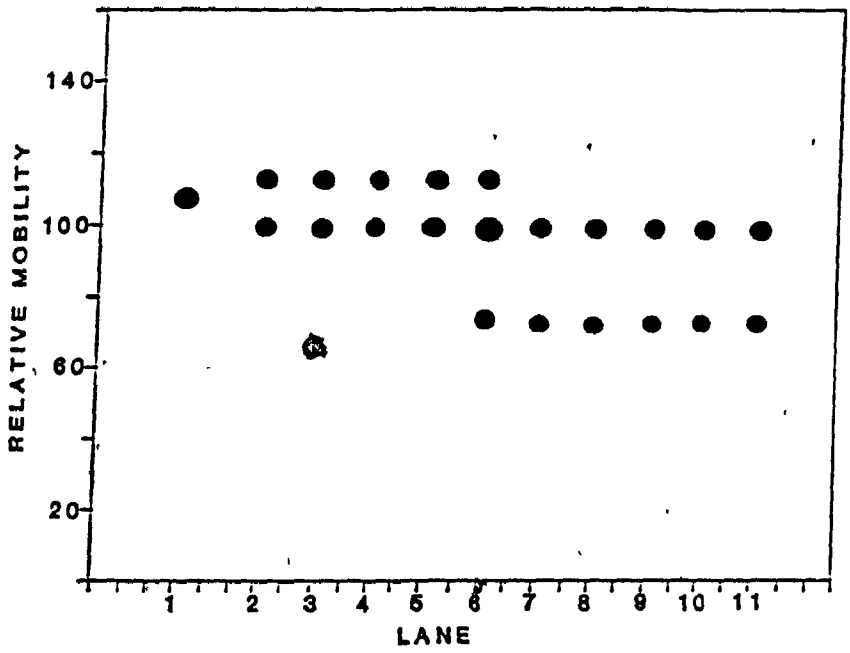


FIGURE 9. Observed isoenzyme banding phenotypes for TPI for diploids L. alpinus (accession number 77; lanes 1 to 5), L. conimbricensis (accession number 126; lanes 7 to 10), and for the interspecific hybrid L. alpinus X L. conimbricensis (lane 6). Lane 11 is L. uliginosus (193-52) which is used as a standard.
(A) Photograph of zymogram. (B) Graphic presentation of zymogram.

TRI

A

B

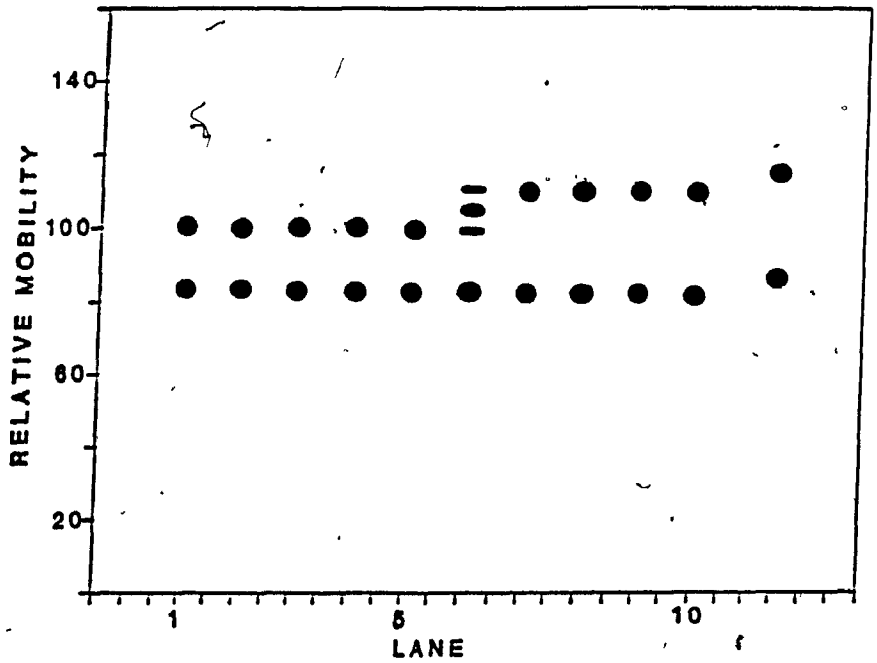
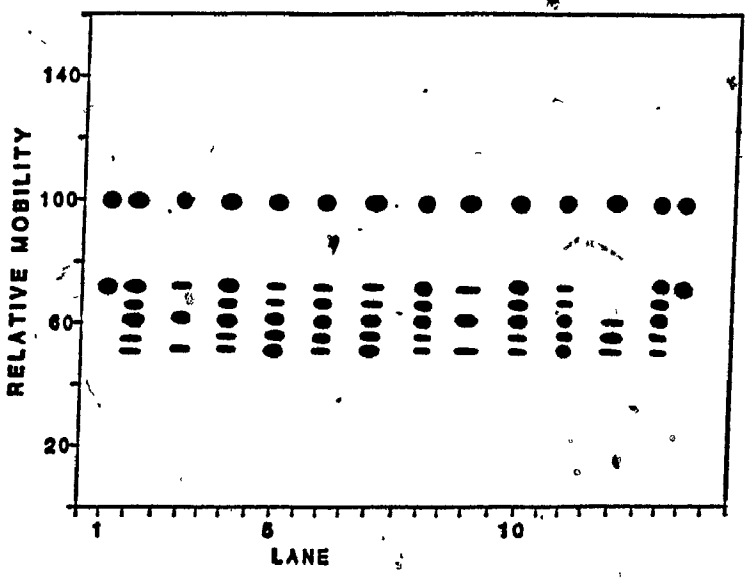


FIGURE 10. Observed isoenzyme banding phenotypes from selfing the interspecific hybrid L. japonicus X L. alpinus (23). (A) Photograph of representative zymogram. (B) Graphic presentation of zymogram. Lane 1, L. tenuis (109-21) standard; lanes 2 and 13, L. japonicus X L. alpinus (23); lanes 3 to 12, progeny; lane 14, L. uliginosus (193-52) standard. (C) Summary of observed occurrence of various progeny phenotypes.

PGI

A

B


140
100
60
20
RELATIVE MOBILITY
1
5
10
LANE

C

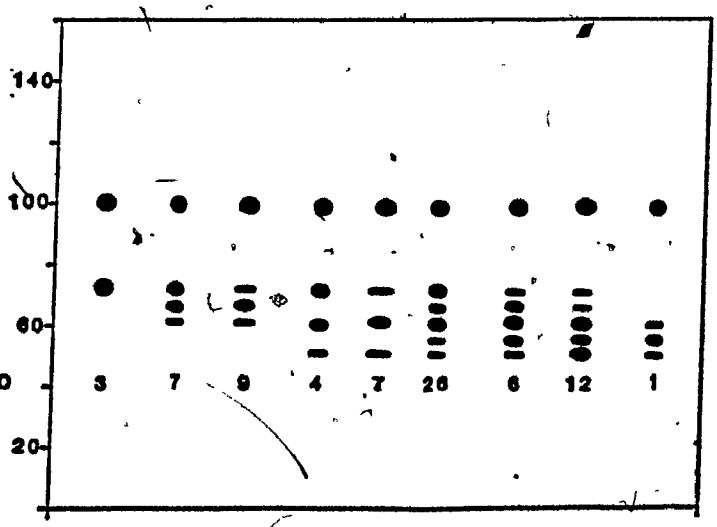

140
100
60
20
PHENOTYPE
NO. OBSERVED
3
7
9
4
7
26
6
12
1

FIGURE 11. Disomic genetic model for segregation of duplicated PGI alleles in progeny from selfing the interspecific hybrid L. japonicus X L. alpinus (23). The expected frequencies and genotypes are those that would be produced if two independent disomic loci, heterozygous for three distinct alleles (F/M and F/S), were segregating. Chi-square analysis indicated that the observed values correspond 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).

PGI2

PGI3		FF	FM	MM	LOCUS 2 TOTAL
		F/F F/F	F/F M/F	M/F M/F	
FF	OBS.	3	7	9	19
	EXP.	4.7	9.4	4.7	18.7
		F/F F/S	F/F M/S	M/F M/S	
FS	OBS.	4	26	6	36
	EXP.	9.4	18.8	9.4	37.6
		F/S F/S	F/S M/S	M/S M/S	
SS	OBS.	7	12	1	20
	EXP.	4.7	9.4	4.7	18.7
LOCUS 1 TOTAL	OBS.	14	45	16	75
	EXP.	18.7	37.6	18.7	

CHI-SQUARE 1 = 3.026 N.S.

CHI-SQUARE 2 = 0.082 N.S.

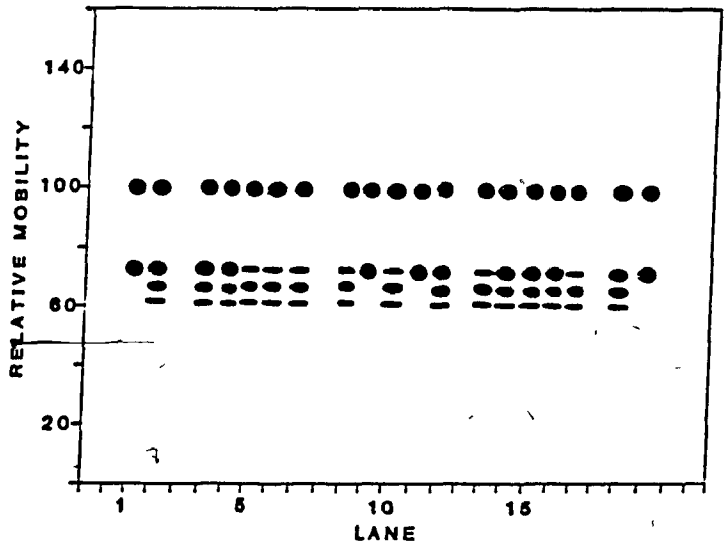
CHI-SQUARE 3 = 17.008 $0.01 > p > 0.001$

FIGURE 12. Observed isoenzyme banding phenotypes for PGI for progeny from selfing the interspecific hybrid L. japonicus X L. alpinus (28). (A) Photograph of representative zymogram. (B) Graphic presentation of zymogram. Lane 1, L. tenuis (109-21) standard; lanes 2 and 18, L. japonicus X L. alpinus (28); lanes 3 to 17, progeny; lane 19, L. uliginosus (193-52) standard. (C) Summary of observed occurrence of various progeny phenotypes.

PGI

A

B


140
100
60
20
RELATIVE MOBILITY
1
5
10
15
LANE

C

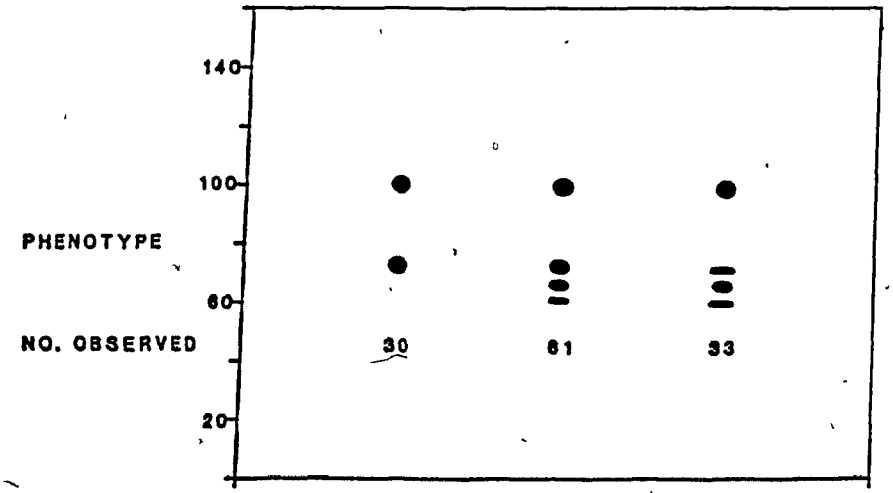
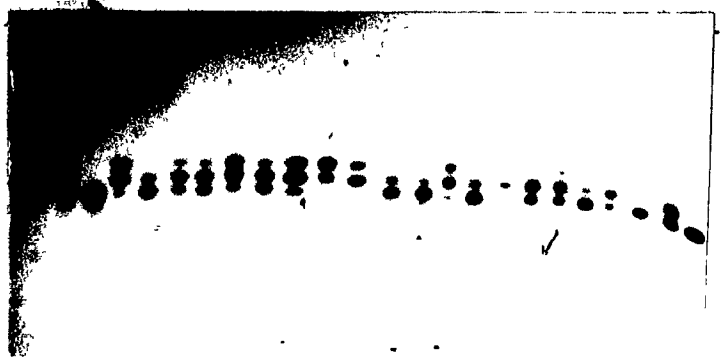

140
100
60
20
PHENOTYPE
NO. OBSERVED
30
61
33

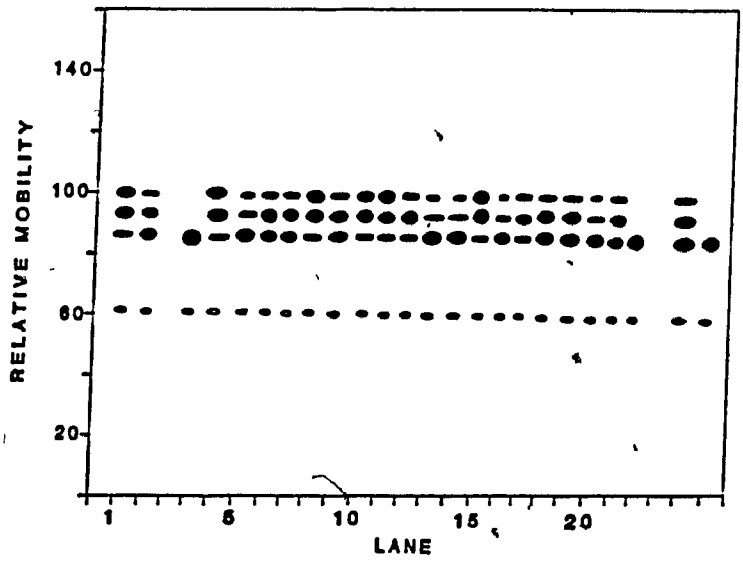
FIGURE 13. Observed isoenzyme banding phenotypes for IDH from selfing the interspecific hybrid L. japonicus X L. alpinus (23). (A) Photograph of representative zymogram. (B) Graphic presentation of zymogram. Lane 1, L. tenuis (109-21) standard; lanes 2 and 23, L. japonicus X L. alpinus (23); lanes 3 to 22, progeny; lane 24, L. uliginosus (193-52) standard. (C) Summary of observed occurrence of various progeny phenotypes.

IDH

A



B


RELATIVE MOBILITY
140
100
60
20
1
5
10
15
20
LANE

C

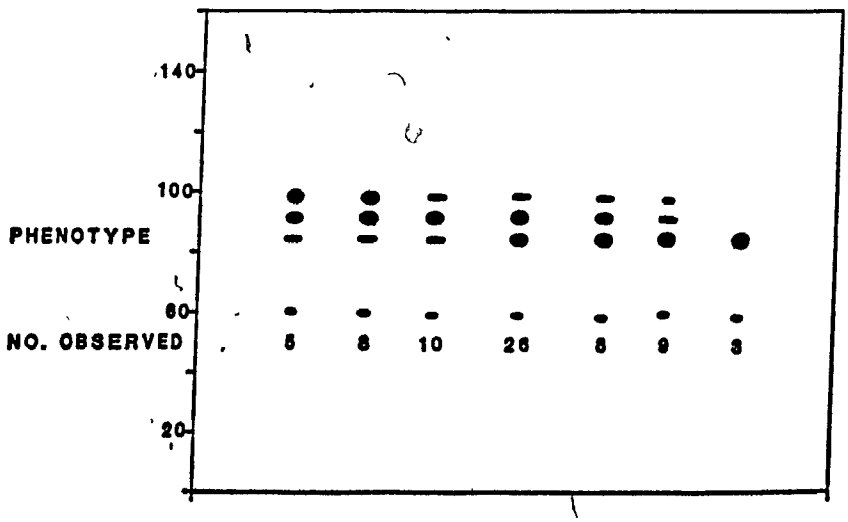
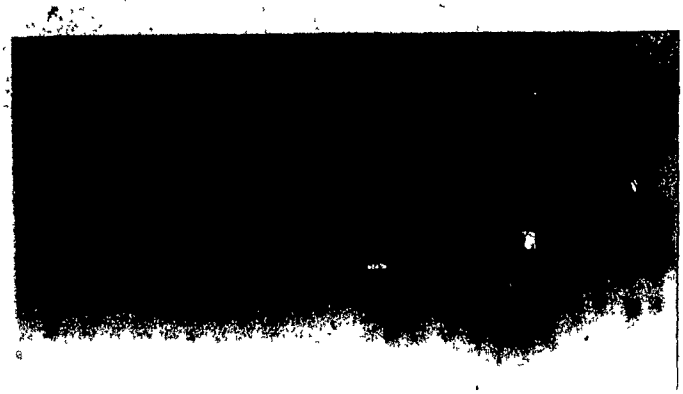

140
100
60
20
PHENOTYPE
NO. OBSERVED
5
8
10
26
8
9
3

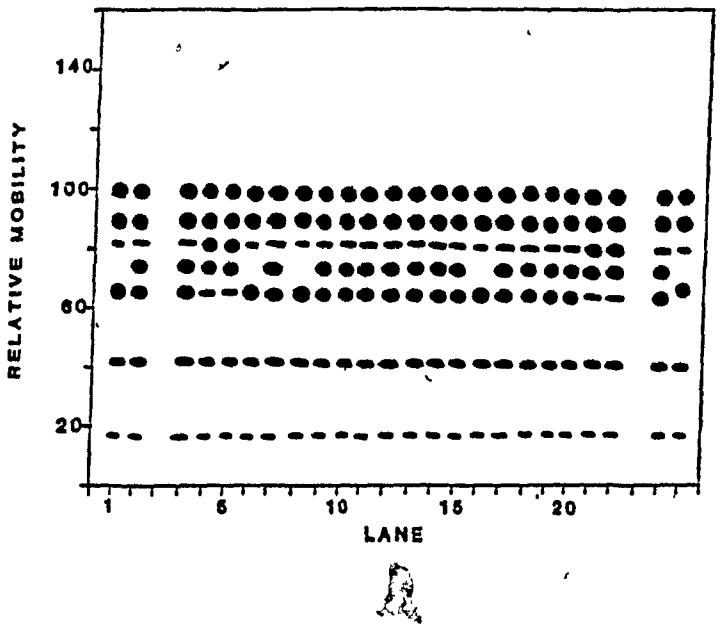
FIGURE 14. Observed isoenzyme banding phenotypes for MDH for progeny from selfing the interspecific hybrid L. japonicus X L. alpinus (28). (A) Photograph of representative zymogram. (B) Graphic presentation of zymogram. Lane 1, L. tenuis (109-21) standard; lanes 2 and 23, L. japonicus X L. alpinus (23); lanes 3 to 22, progeny; lane 24, L. uliginosus (193-52) standard. (C) Summary of observed occurrence of various progeny phenotypes.

MDH

A



B


140
100
60
20
RELATIVE MOBILITY
1
5
10
15
20
LANE

C

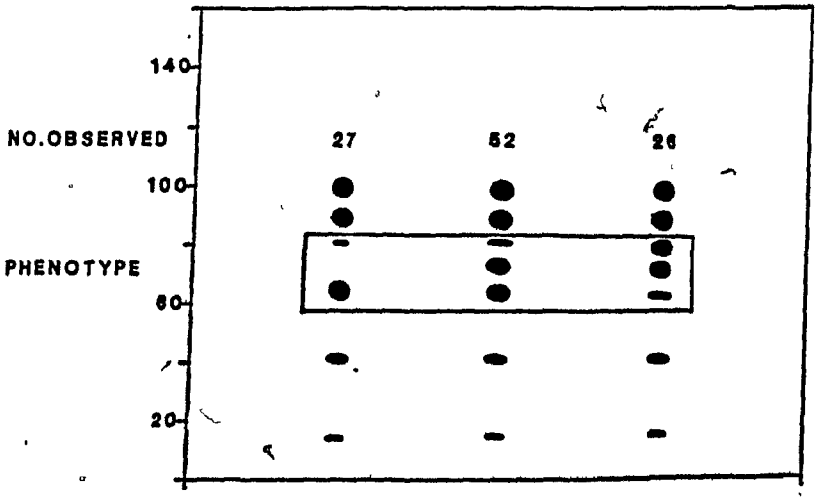
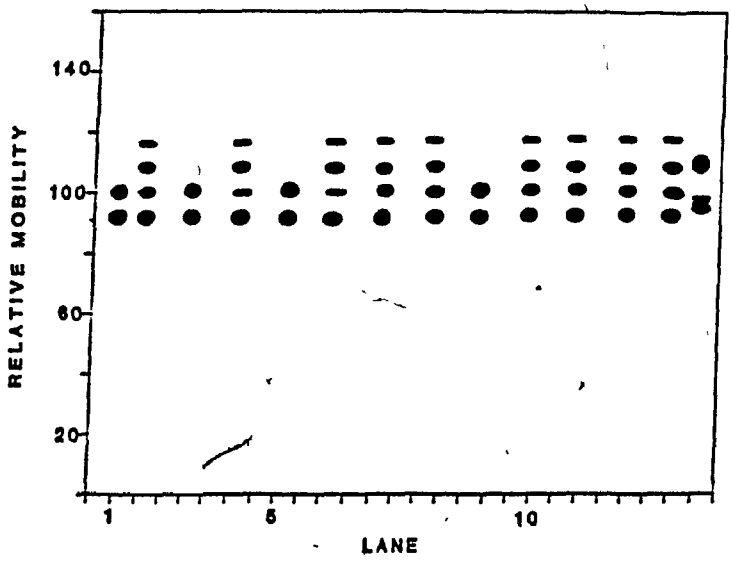

140
NO.OBSERVED
27
52
26
100
PHENOTYPE
60
20

FIGURE 15. Observed isoenzyme banding phenotypes for 6-PGDH for progeny from selfing the interspecific hybrid L. japonicus X L. alpinus (28). (A) Photograph of representative zymogram. (B) Graphic presentation of zymogram. Lane 1, L. tenuis (109-21) standard; lanes 2 and 13, L. japonicus X L. alpinus (28); lanes 3 to 12, progeny; lane 14, L. uliginosus (193-52) standard. (C) Summary of observed occurrence of various progeny phenotypes.

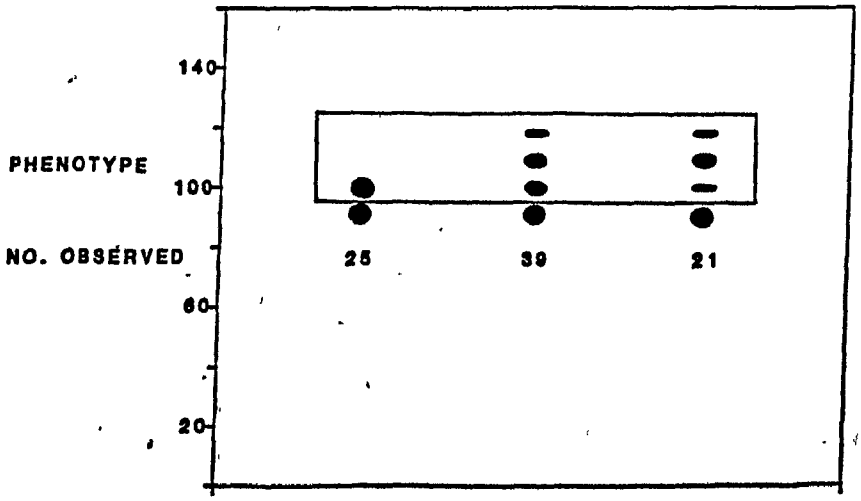
6-PGDH

A

B


140
100
60
20
RELATIVE MOBILITY
1
5
10
LANE

C


140
100
60
20
PHENOTYPE
NO. OBSERVED
25
39
21

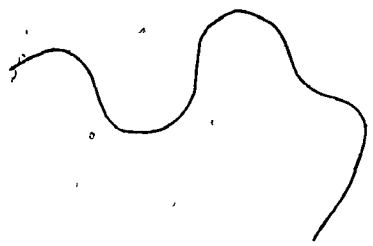
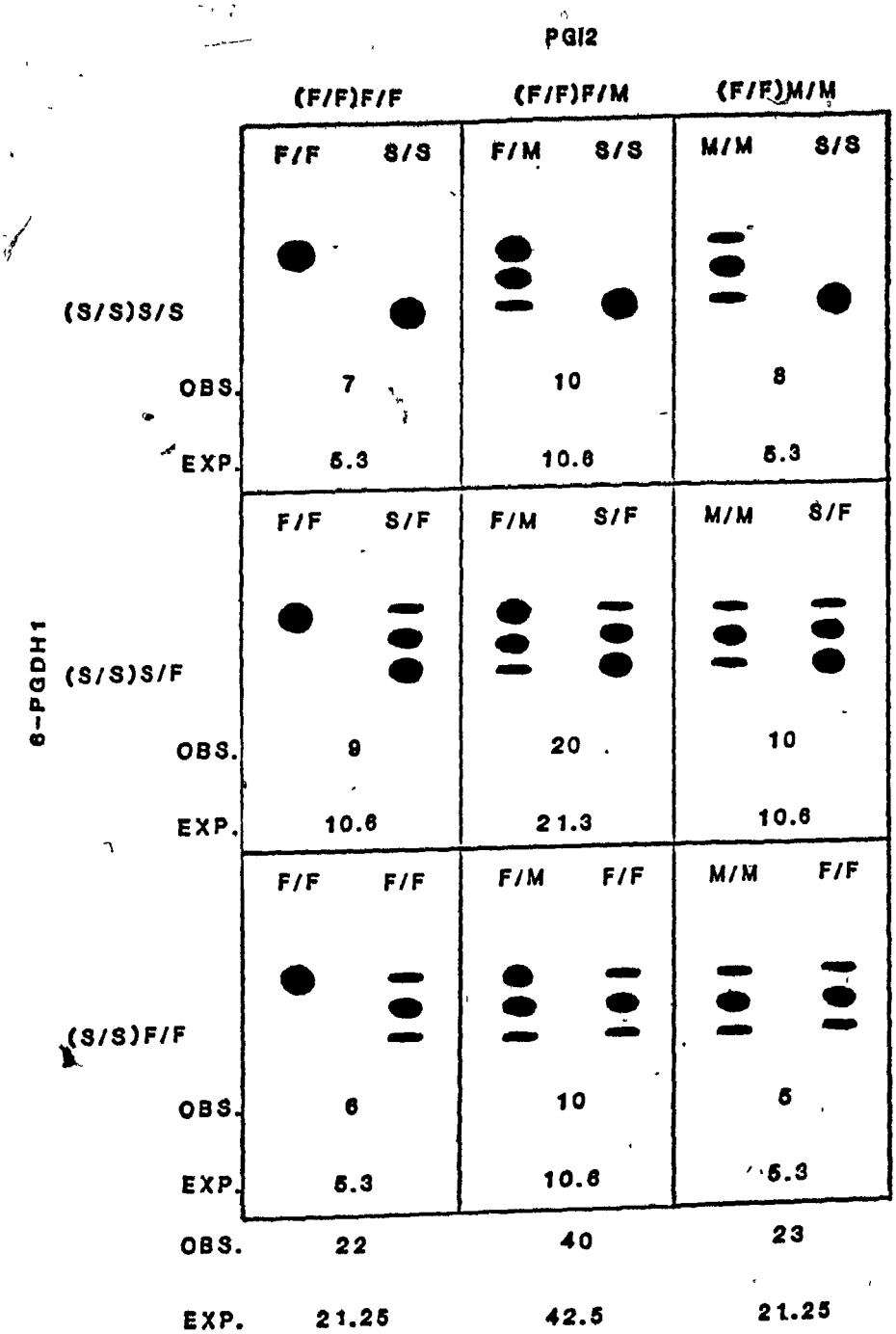


FIGURE 16. Models for independent segregation of disomic loci for PGI and 6-PGDH for progeny from selfing the interspecific hybrid L. japonicus X L. alpinus (28). The expected genotypes and frequencies are those that would be produced if two independent disomic loci were controlling the phenotypes for the two enzymes. Chi-square analysis 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).



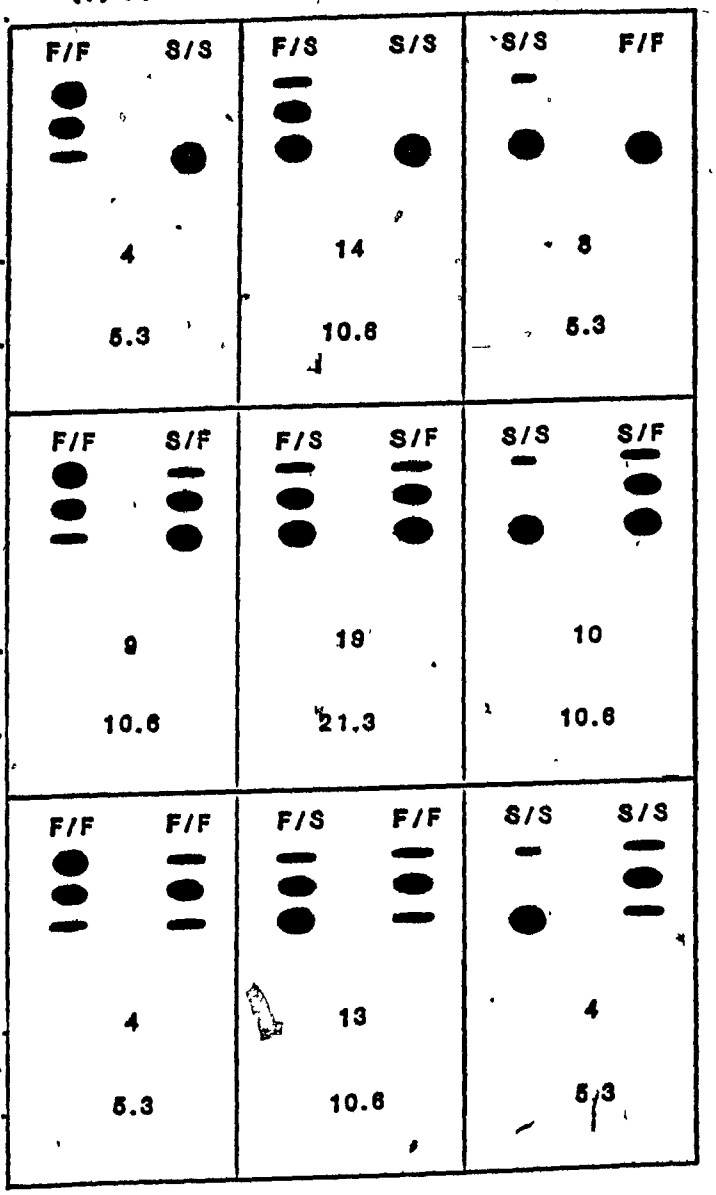
CHI-SQUARE 1 : PGI2 1:2:1, 2 D.F., 0.318 N.S.

CHI-SQUARE 2 : 6-PGDH 1:2:1, 2 D.F., 0.953 N.S.

CHI-SQUARE 3 : PGI2 AND 6-PGDH1 SEGREGATE INDEPENDENTLY, 4D.F., 2.453 N.S

FIGURE 17. Models for independent segregation of disomic loci for MDH and 6-PGDH for progeny from selfing the interspecific hybrid L. japonicus X 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 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-[redacted]S = slow, 6-PGDH-100;).

MDH3



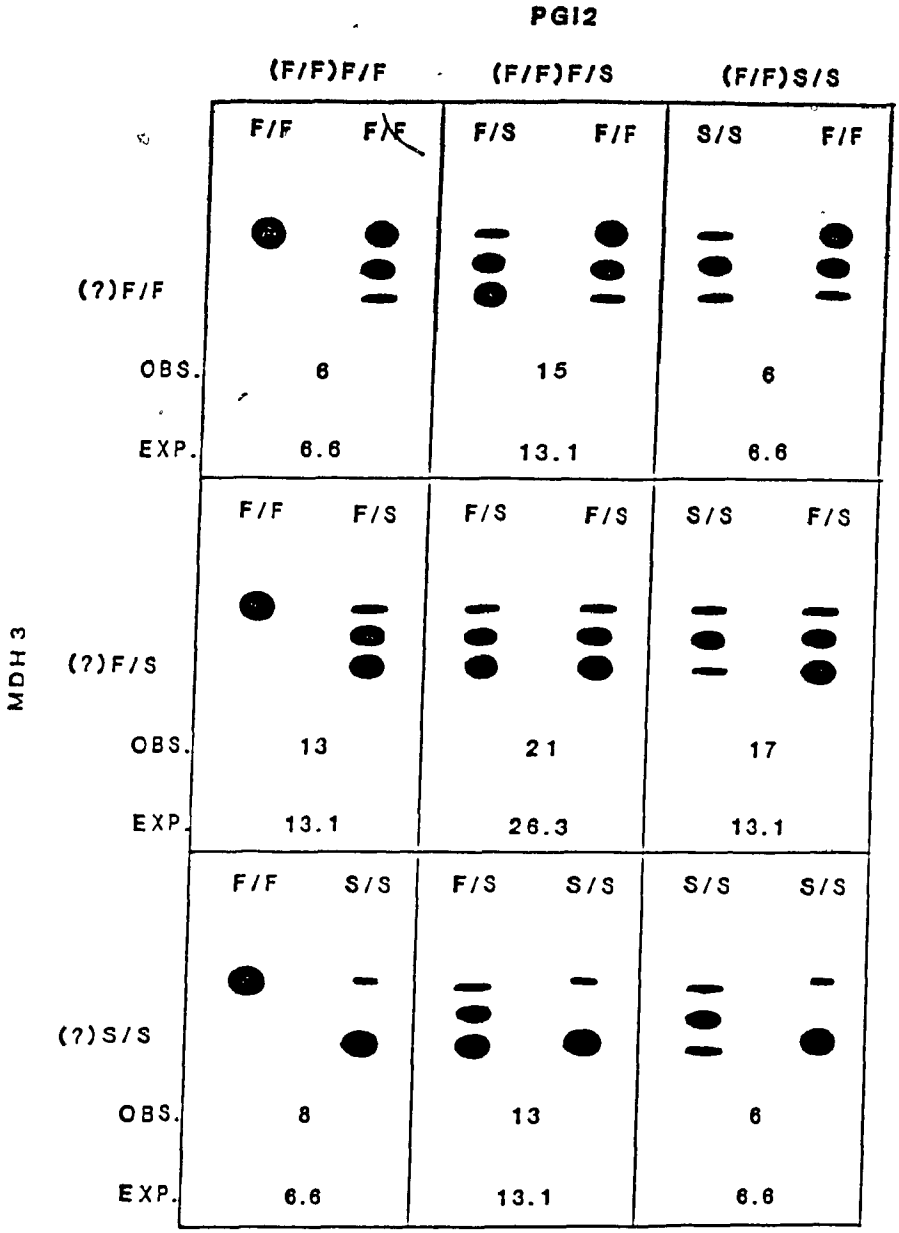
6-PGDH1		(?) F/F	(?) F/S	(?) S/S	
(S/S) S/S	OBS.	4	14	8	26
	EXP.	5.3	10.6	5.3	21.2
(S/S) S/F	OBS.	9	19	10	38
	EXP.	10.6	21.3	10.6	42.5
(S/S) F/F	OBS.	4	13	4	21
	EXP.	5.3	10.6	5.3	21.2
	OBS.	17	46	22	85
	EXP.	21.2	42.5	21.2	

CHI-SQUARE 1 : MDH3 1:2:1, 2 D.F., 1.151 N.S.

CHI-SQUARE 2 : 6-PGDH 1:2:1, 2D.F., 1.565 N.S.

CHI-SQUARE 3 : MDH3 AND 6-PGDH1 SEGREGATE INDEPENDENTLY, 4D.F., 4.490 N.S

FIGURE 18. Models for independent segregation of disomic loci for PGI and MDH in the selfed interspecific hybrid L. japonicus X 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.

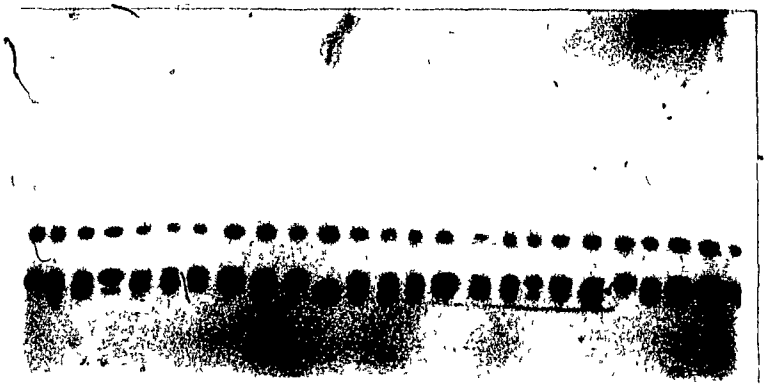
CHI-SQUARE 2 : MDH3 1:2:1, 2 D.F., 0.080 N.S.

CHI-SQUARE 3 : PGI2 AND MDH3 SEGREGATE INDEPENDENTLY, 4 D.F., 2.967 N.S.

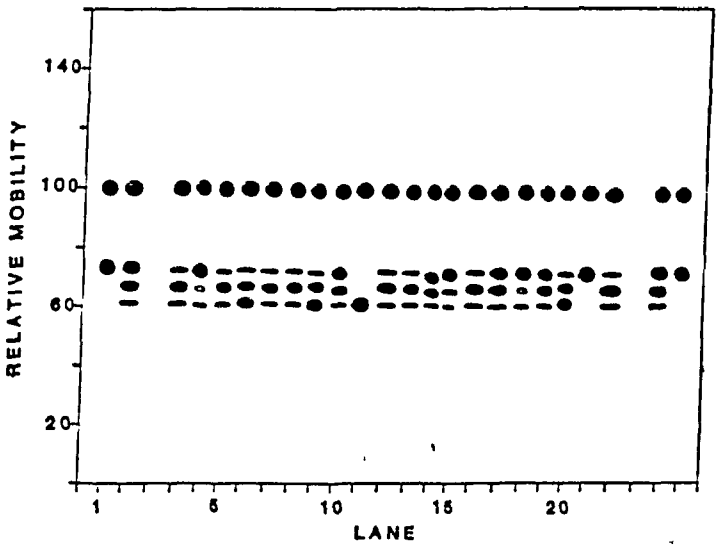
FIGURE 19. Observed isoenzyme banding phenotypes for PGI for progeny from selfing the artificial allotetraploid (*L. japonicus* X *L. alpinus*)^2 (28). (A) Photograph of representative zymogram. (B) Graphic presentation of zymogram. Lane 1, *L. tenuis* (109-21) standard, lanes 2 and 23, (*L. japonicus* X *L. alpinus*)^2 (28); lanes 3 to 22, progeny; lane 24, *L. uliginosus* (193-52) standard. (C) Summary of observed occurrence of various progeny phenotypes.

PGI

A



B


140
100
60
20
RELATIVE MOBILITY
1
5
10
15
20
LANE

C

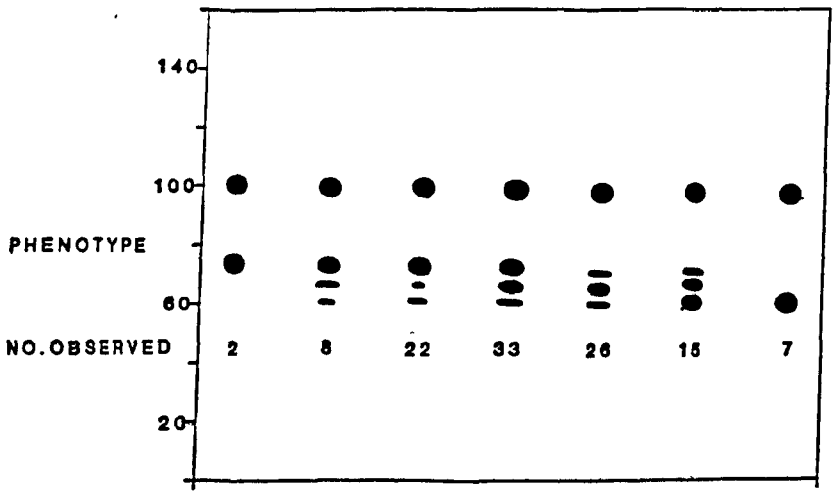
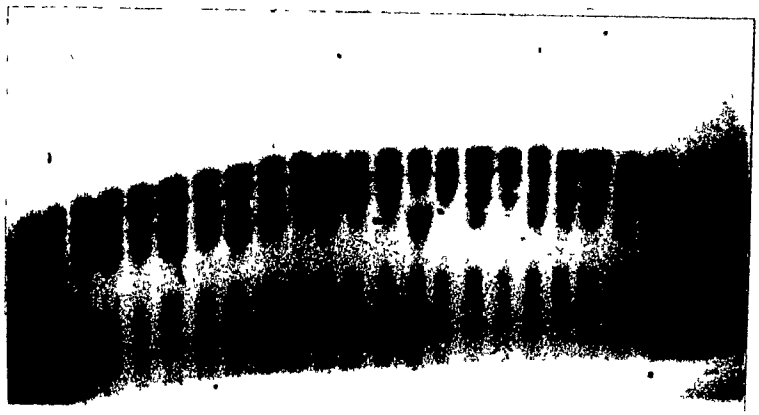

140
100
60
20
PHENOTYPE
NO.OBSERVED
2
8
22
33
26
15
7

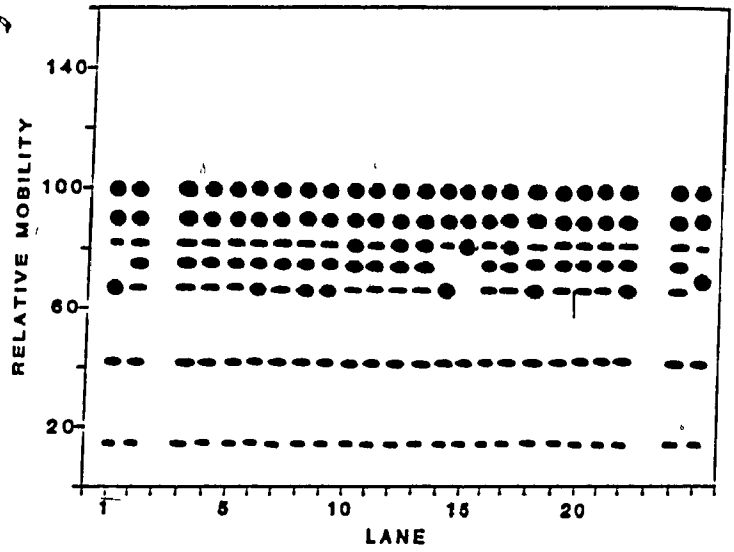
FIGURE 20. Observed isoenzyme banding phenotypes for MDH for progeny from selfing the artificial allotetraploid (L. japonicus X L. alpinus)^2 (28). (A) Photograph of representative zymogram. (B) Graphic presentation of zymogram. Lane 1, L. tenuis (109-21) standard; lanes 2 and 23, (L. japonicus X L. alpinus)^2 (28); lanes 3 to 22, progeny; lane 24, L. uliginosus (193-52) standard. (C) Summary of observed occurrence of various progeny phenotypes.

MDH

A



B


140
100
60
20
RELATIVE MOBILITY
1
5
10
15
20
LANE

C

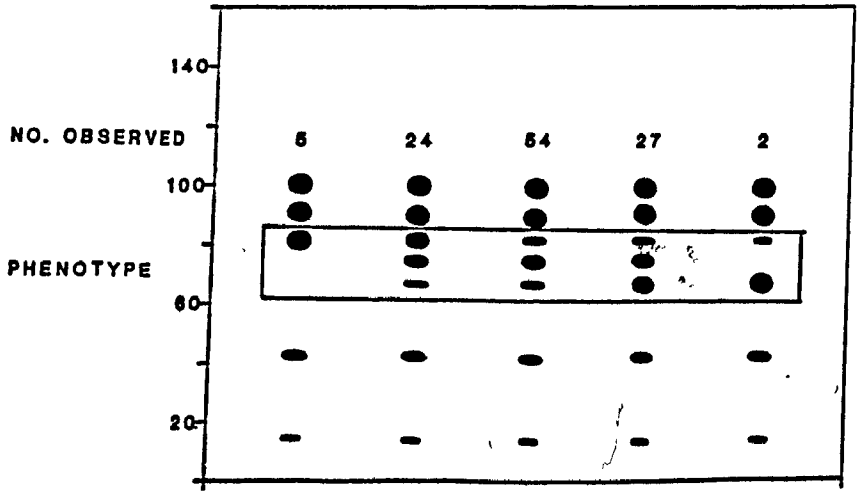
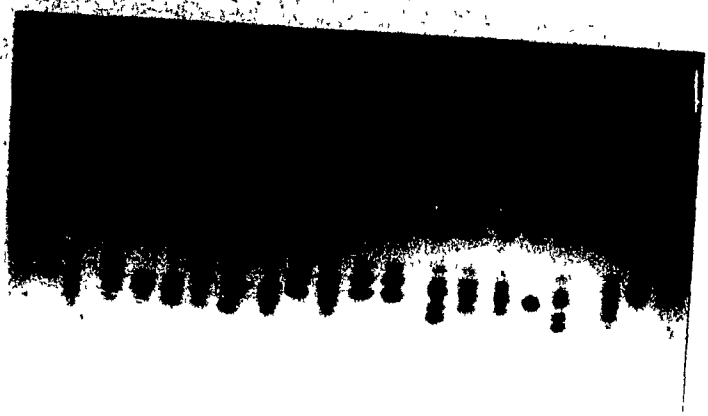

140
100
60
20
NO. OBSERVED
5
24
54
27
2
PHENOTYPE

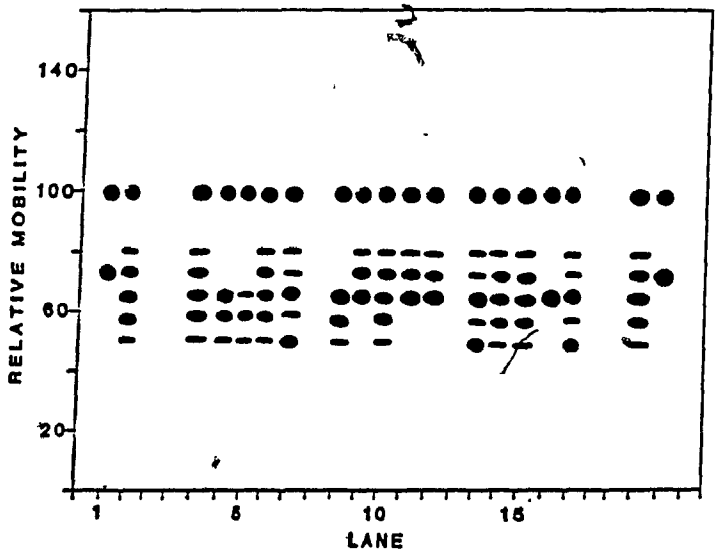
FIGURE 21. Observed isoenzyme banding phenotypes for PGI for progeny from selfing the artificial autotetraploid (*L. alpinus*)^2 (774*x*-5). (A) Photograph of representative zymogram. (B) Graphic presentation of zymogram. Lane 1, *L. tenuis* (109-21) standard; lanes 2 and 18, (*L. alpinus*)^2 (774*x*-5); lanes 3 to 17, progeny; lane 19, *L. uliginosus* (193-52) standard. (C) Summary of observed occurrence of various progeny phenotypes.

PGI

A



B


140
100
60
20
RELATIVE MOBILITY
1
5
10
15
LANE

C

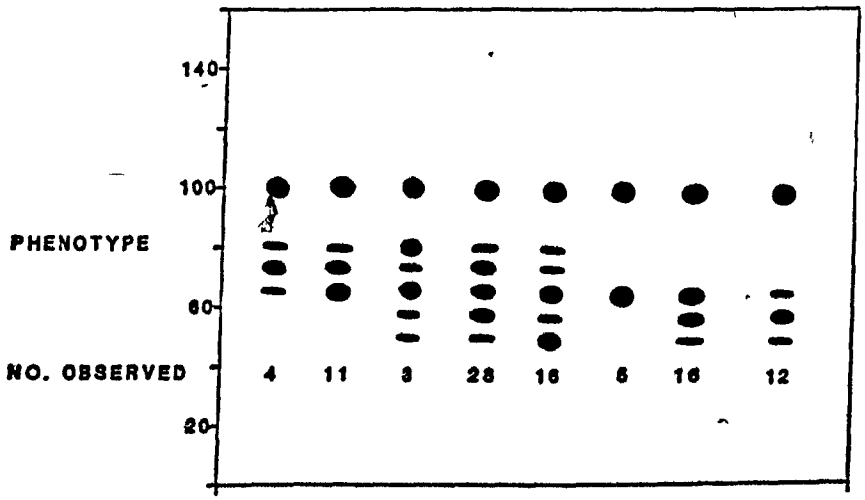
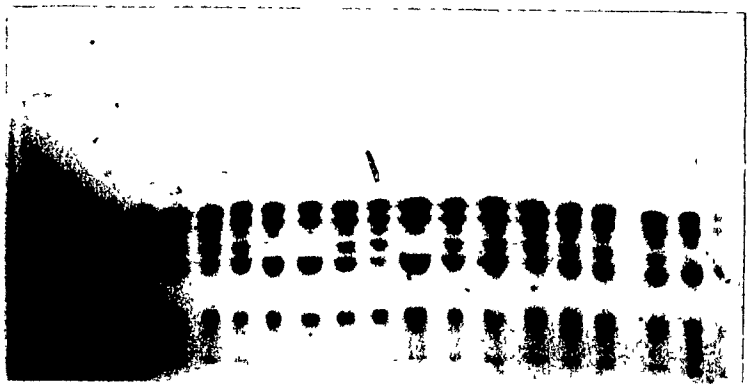

140
100
60
20
PHENOTYPE
NO. OBSERVED
4
11
3
28
16
5
16
12

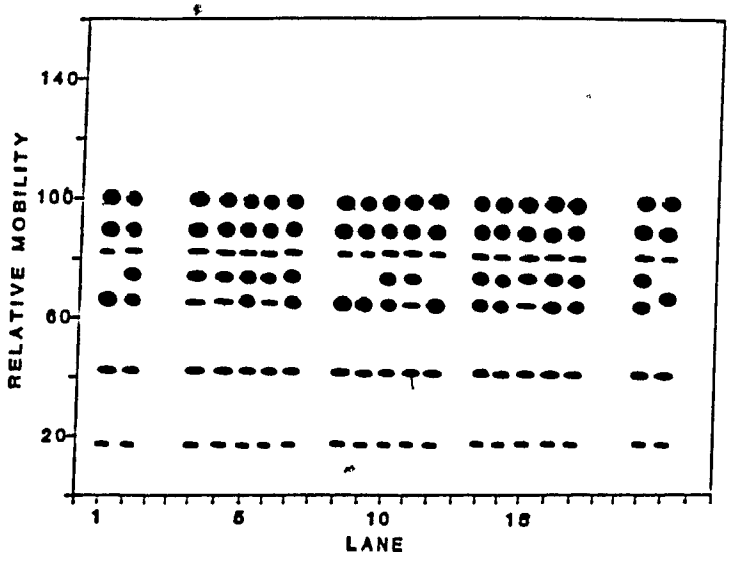
FIGURE 22. Observed isoenzyme banding phenotypes for MDH for progeny from selfing the artificial autotetraploid (L. alpinus)^{2} (774x-5). (A) Photograph of representative zymogram. (B) Graphic presentation of zymogram. Lane 1, L. tenuis (109-21) standard; lanes 2 and 18, (L. alpinus)^{2} (774x-5); lanes 3 to 17, progeny; lane 19, L. uliginosus (193-52) standard. (C) Summary of observed occurrence of various progeny phenotypes.

MDH

A



B


140
100
60
20
RELATIVE MOBILITY
1
5
10
15
LANE

C

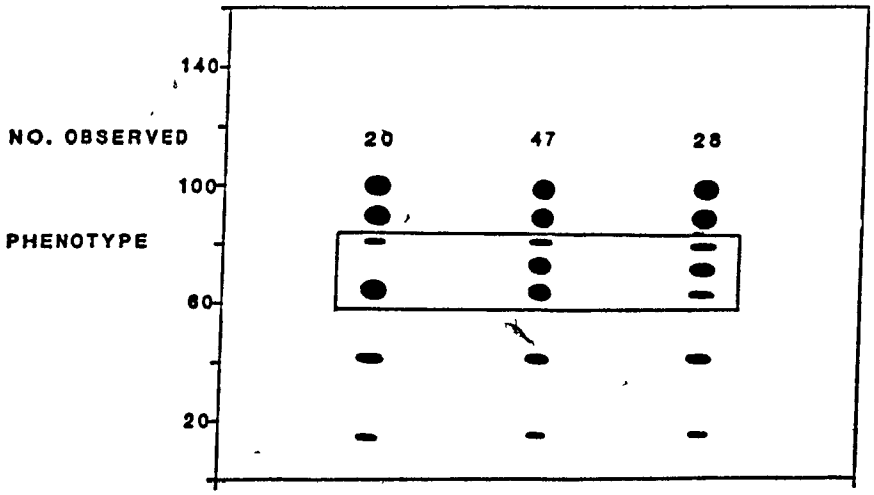

140
100
60
20
NO. OBSERVED
PHENOTYPE
20
47
28

FIGURE 23. Isoenzyme phenotypes for Lotus corniculatus (554-5) and cultivar 'Leo' (Leo-1) used as parents in a test cross for PGI and MDH.

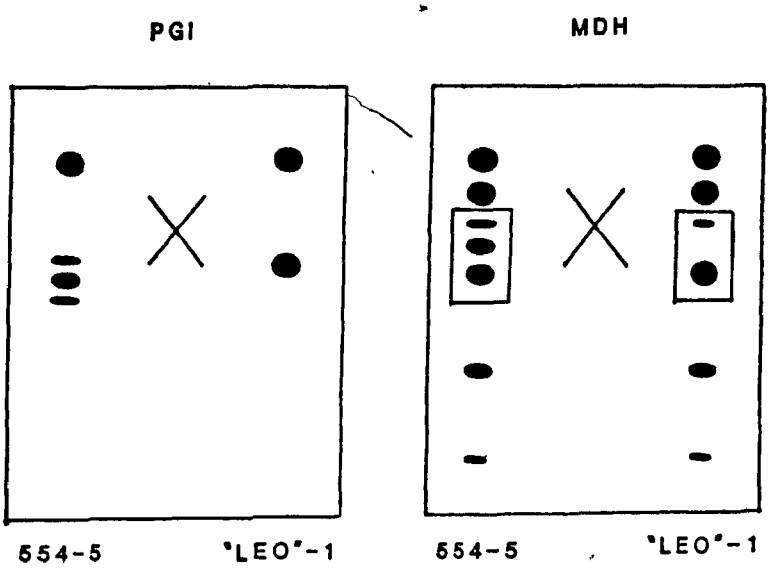
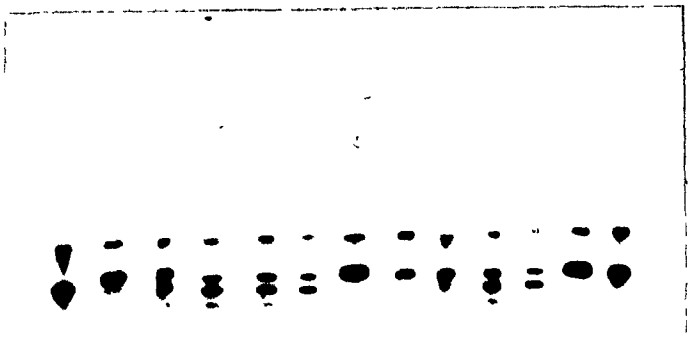

PGI
MDH
554-5
"LEO"-1
554-5
"LEO"-1

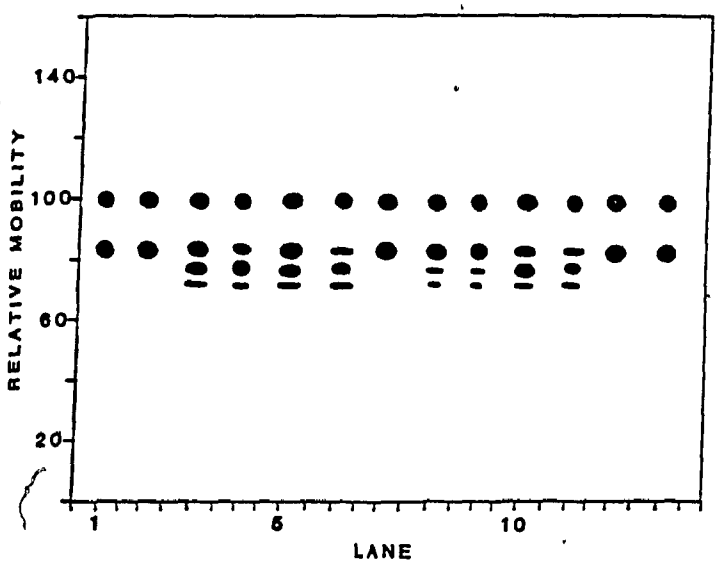
FIGURE 24. Observed isoenzyme banding phenotypes for PGI for progeny from a test-cross of Lotus corniculatus (554-5) X 'Leo' (Leo-1). (A) Photograph of representative zymogram. (B) Graphic presentation of zymogram. Lane 1, L. tenuis (109-21) standard; lane 2, L. corniculatus (554-5) standard; lanes 3 to 11, progeny; lane 12, Leo-1; lane 13, L. uliginosus (193-52) standard. (C) Summary of observed occurrence of various progeny phenotypes.

PGI

A



B


140
100
60
20
RELATIVE MOBILITY
1
5
10
LANE

C

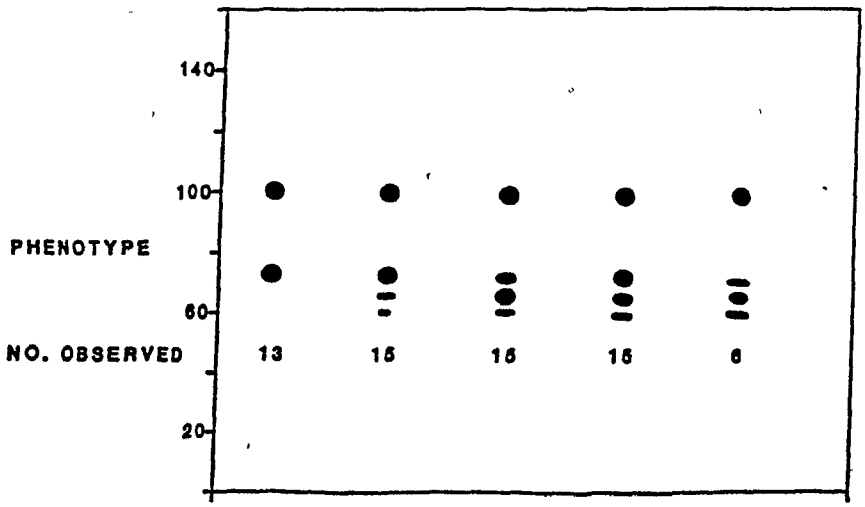
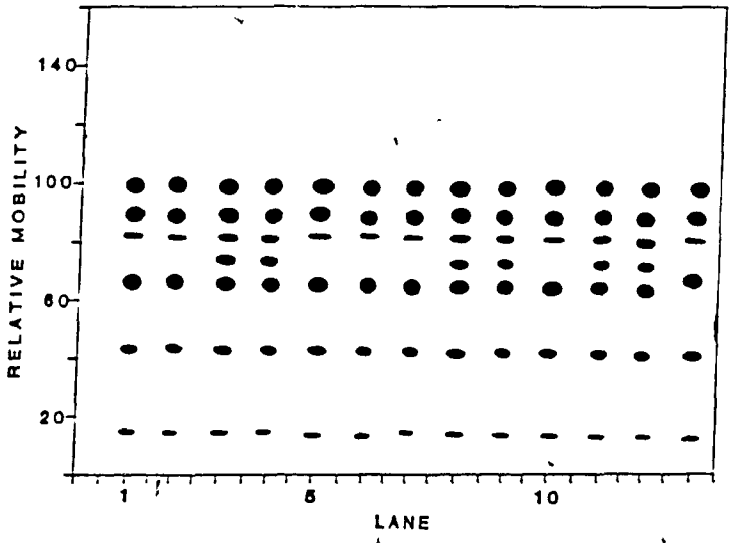

140
100
60
20
PHENOTYPE
NO. OBSERVED
13
15
15
15
6

FIGURE 25. Observed isoenzyme banding phenotypes for MDH for progeny from a test-cross of the artificial autotetraploid (L. alpinus)^2 (774x-5). (A) Photograph of representative zymogram. (B) Graphic presentation of zymogram. Lane 1, L. tenuis (109-21) standard; lanes 2 and 18, (L. alpinus)^2 (774x-5); lanes 3 to 17, progeny; lane 19, L. uliginosus (193-52) standard. (C) Summary of observed occurrence of various progeny phenotypes.

MDH

A

B


140
100
60
20
RELATIVE MOBILITY
1
5
10
LANE

C

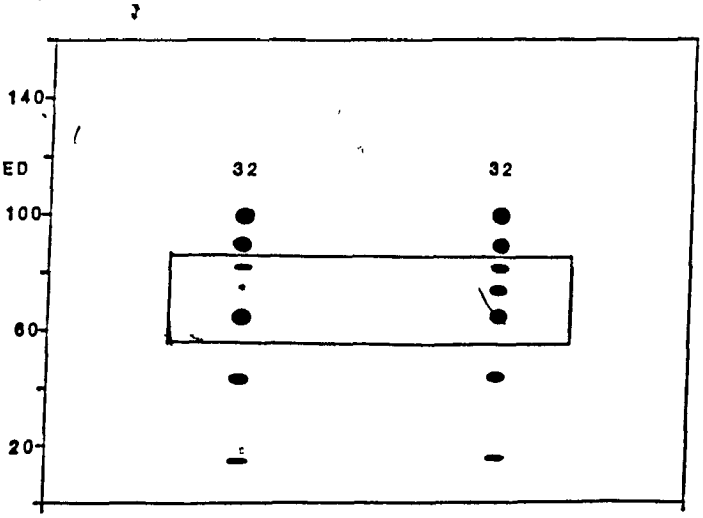

140
100
60
20
NO. OBSERVED
PHENOTYPE
32
32

FIGURE 26. Models of various types of tetraploidy. A. The classical concept of autotetraploidy producing a tetrasomic locus. B. The classical model of allotetraploidy producing duplicated disomic loci. C. Possible model for segmental allotetraploidy. Chromosomal rearrangement such as a pericentric inversion leading to displaced loci in homoeologous chromosomes. Unequal crossing over may result in a duplication deficiency. Deficient chromosomes are inviable so that viable progeny contain homologous duplicated chromosomes. Subsequent doubling may lead to eight allelic doses of the locus in an allotetraploid. Genetic factors may determine whether loci segregate in a disomic or tetrasomic manner.

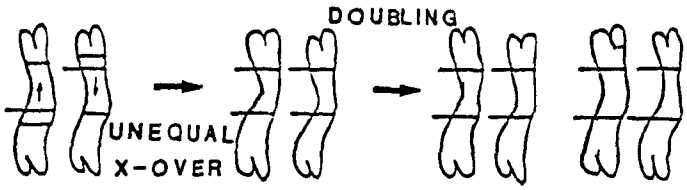
A AUTOTETRAPLOID

2 HOMOLOGS 4 HOMOLOGS

B GENOME ALLOTETRAPLOID

HETEROLOGS 2 SETS OF HOMOLOGS

C SEGMENTAL ALLOTETRAPLOID MODEL



HOMOEOLOGS DUPLICATED LOCI QUADRUPLICATED LOCI

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

Taxon	Accession number	Genotype	Use	Source
L. alpinus Schleich.	77	several	hybrid and parental species	See Grant et al. (1962)
(L. alpinus)^2	774x	5	pollen and selfed progeny	Artificial autotetraploid (Sonaroo and Grant 1971)
L. alpinus X L. conimbricensis	(77 X 126)	1	hybrid and parental species	Artificial interspecific hybrid (O'Donoughue and Grant 1987)
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 X L. ornithopodioides L.	(303 X 100)	1	hybrid and parental species	Artificial interspecific hybrid (O'Donoughue and Grant 1987)
L. conimbricensis Brot.	126	several	hybrid and parental species	Commonwealth Scientific and Industrial Research Organization, Canberra, Australia; Origin: Portugal
L. corniculatus L.	554	5	progeny of cross (554-5 X Leo-1)	Plant Introduction Center, Izmir, Turkey; Origin: Samsun, Turkey, No. 1465-64
L. corniculatus L.	cv. 'Leo'	1	progeny of cross (554-5 X Leo-1)	Cultivar developed at Macdonald College by J. S. Bubar 1963
L. japonicus (Regel) Larsen X L. alpinus	129 X 77	23 28	pollen and progeny from selfing	Artificial interspecific hybrid (Sonaroo and Grant 1971)

... TABLE 1 Cont'd.

TABLE 1. Cont'd.

Taxon	Accession number	Genotype	Use	Source
(*L. japonicus* X *L. alpinus*)^2	(129 X 77)^2	2B	pollen and progeny from selfing	Artificial amphidiploid (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 pollen	Australia, C.P.I. No. 23788. Origin: Turkey
L. uliginosus Schkuhr	193	52	standard	Service de la Recherche Agronomique et de l'Experimentation Agricole, Rabat, Morocco

TABLE 2. Disomic and tetrasomic models of allele segregation for selfed genotypes with various duplicated loci. F = fast, most anodal, S = slow, most cathodal, M = medium.

Disomic, 2 alleles

F F / S S		1 FF	2 FS	1 SS
	1	1	2	1
1	FF	FFFF	FFFS	FFSS
	2	2	4	2
2	FS	FFFS	FFSS	FSSS
	1	1	2	1
1	SS	FFSS	FSSS	SSSS

Disomic, 3 alleles

M M / F S		1 FF	2 FM	1 MM
	1	1	2	1
1	SS	FFSS	FMSS	MMSS
	2	2	4	2
2	MS	FFMS	FMMS	MMMS
	1	1	2	1
1	MM	FFMM	FMMM	MMMM

Tetrasomic chromosome segregation

Duplex model FFSS

		1 FF	4 FS	1 SS
	1	1	4	1
1	FF	FFFF	FFFS	FFSS
	2	2	16	2
2	FS	FFFS	FFSS	FSSS
	1	1	4	1
1	SS	FFSS	FSSS	SSSS

Triplex model FFFS

		1 FF	1 FS
	1	1	1
1	FF	FFFF	FFFS
	1	1	1
1	FS	FFFS	FFSS

TABLE 2, Cont'd.

TABLE 2, Cont'd.

	Tetrasomic chromosome segregation					Tetrasomic chromatid segregation			
	3 alleles FFMS					Duplex model FFSS			
	1 FF	2 FM	2 FS	1 MS		3 FF	8 FS	3 SS
1 FF	1 FFFF	2 FFFM	2 FFFS	1 FFMS	3 FF	9 FFFF	24 FFFS	9 FFSS
2 FM	2 FFFM	4 FFMM	4 FFMS	2 FMMS	8 FS	24 FFFS	64 FFSS	24 FSSS
1 FS	2 FFFS	4 FFMS	4 FFSS	2 FMSS	3 SS	9 FFSS	24 FSSS	9 SSSS
1 MS	1 FFMS	2 FMMS	2 FMSS	1 MMSS				

TABLE 3. Expected distribution of progeny genotypes for selfed plants with various levels of independently segregating duplicated disomic loci. F = fast, most anodal, S = slow, most cathodal, M = medium.

Parental genotype									No. of genotypes
$\frac{F}{S}$ (X)	1 locus, 2 alleles								3
Ratio (11)	1		2		1				
Genotype (FS)	20		11		02				
$\frac{F}{S}\frac{F}{S}$ (X)	2 independent loci, 2 alleles								5
Ratio (22)	1	4	6	4	1				
Genotype (FS)	40	31	22	13	04				
$\frac{F}{S}\frac{F}{S}\frac{F}{S}$ (X)	3 independent loci, 2 alleles								7
Ratio (33)	1	6	15	20	15	6	1		
Genotype (FS)	60	51	42	33	24	15	06		
$\frac{F}{S}\frac{F}{S}\frac{F}{S}\frac{F}{S}$ (X)	4 independent loci, 2 alleles								9
Ratio (44)	1	8	28	56	70	56	28	8	1
Genotype (FS)	80	71	62	53	44	35	26	17	08
$\frac{F}{M}\frac{F}{S}$ (X)	2 independent loci, 3 alleles								9
Ratio (211)	1	2	1	4	2	1	2	1	
Genotype (FMS)	400	301	202	211	112	220	121	022	

TABLE 3, Cont'd.

TABLE 3, Cont'd.

		No. of genotypes
$\frac{M}{F}\frac{M}{F}\frac{M}{S}\frac{M}{S}$ (X)	4 independent loci, 3 alleles	25
Ratio (242)	1 4 4 6 16 6 4 24 24 4 1	
Genotype (FMS)	800 710 701 620 611 602 530 521 512 503 440	
Ratio	16 36 16 1 4 24 24 4 6 16 6 4 4 1	
Genotype (FMS)	431 422 413 404 341 332 323 314 242 233 224 143 134 044	

TABLE 4. Expected distribution of progeny genotypes for selfed plants with various types of segregating tetrasomic loci. F = fast, most anodal, S = slow, most cathodal, M = medium.

Parental genotype						No. of genotypes
FFSS (X) FFSS (22)	1 duplex tetrasomic locus, 2 alleles chromosome segregation					5
Ratio	1	8	18	8	1	
Genotype (FS)	40	31	22	13	04	
FFSS (X) FFSS (22)	1 duplex tetrasomic locus, 2 alleles chromatid segregation					5
Ratio	9	48	64	48	9	
Genotype (FS)	40	31	22	13	04	
FFFS (X) FFFS (31)	1 triplex locus, 2 alleles chromosome segregation					3
Ratio	1	2	1			
Genotype (FS)	40	31	22			
FFFS (X) FFFS (31)	1 triplex tetrasomic locus, 2 alleles, chromatid segregation					4 frequent 1 rare
Ratio	225	360	174	24	1	
Genotype (FS)	40	31	22	13	04	

TABLE 4, Cont'd.

TABLE 4, Cont'd.

Parental genotype										No. of genotypes
FFSS (44)	⊗	FFSS (44)	2 independent duplex tetrasomic loci, 2 alleles; chromosome segregation							9
Ratio			1	16	100	304	454	304	100	16	1
Genotype (FS)			80	71	62	53	44	35	26	17	08
FFMS (211)	⊗	FFMS (211)	1 tetrasomic locus, 3 alleles chromosome segregation								9
Ratio			1	4	4	4	10	4	4	4	1
Genotype (FMS)			400	310	301	220	211	202	121	112	022

TABLE 5. Models for tetrasomic segregation of duplicated Pgi loci for selfed L. japonicus X L. alpinus (23)

Parent L. japonicus X L. alpinus (23)

Pgi2-72	Pgi2-72	Pgi2-62	Pgi2-52	Selfed
(FFMS or 211)				

Genotype	400	310	301	220	211	202	121
Observed	3	7	4	9	26	7	6
Expected[a],[b] chromosome segregation	2.2	8.3	8.3	8.3	21	8.3	8.3
Expected[a],[c] chromatid segregation	3.5	9.4	9.4	7.4	17.2	7.4	7.8

Genotype	112	130	022	031	013	040	004
Observed	12	0	1	0	0	0	0
Expected chromosome segregation	8.3	0	2.2	0	0	0	0
Expected chromatid segregation	7.8	1.6	1.7	0	0.8	0.1	0.1

[a]All expected categories less than 5.0 were lumped.

[b]Chi-square$_1$: chromosome segregation, 6 df = 6.207. NS

[c]Chi-square$_2$: chromatid segregation, 6 df = 13.722. S

NS = not significant at P = 0.05; S = significant at P = 0.05.

TABLE 6. Genetic models for duplicated IDH loci for selfed (L. japonicus X L. alpinus) 23. All expected categories less than five have been grouped for Chi² calculation.

Genotype (FB)	80	71	62	53	44	35	26	17	08
Observed	0	0	5	8	10	26	8	9	3
Expected for									
3 disomic loci segregating	0	0	1	6.5	16.2	21.6	16.2	6.5	1
					Chi² 5 df = 17.035**				
one duplex tetrasomic locus and one triplex locus, chromosome segregation	0	0	0.5	4.8	16.8	24.8	16.8	4.8	0.5
					Chi² 5 df = 29.033***				
duplex and simplex loci, chromatid segregation	0	0.3	1	6.9	18.1	19.3	19.8	2.6	1.2
					Chi² 4 df = 90.632***				

**Significant at p <0.01.

***Significant at p <0.001.

TABLE 7. Genetic models for segregation of PGI loci in the artificial allotetraploid (*L. japonicus* X *L. alpinus*)[2]. All expected categories less than five (underscored) have been lumped with adjacent category for Chi[2] calculation.

Phenotype number	1	2	3	4	5	6	7
Observed	2	8	22	33	26	15	7
Expected for							
Three independent disomic loci	1.8	10.6	26.5	35.3	26.5	10.6	1.8
			Chi[2] 4 df = 8.91 NS				
One duplex locus chromosome segregation			5 phenotypes				
One duplex and one triplex locus, chromosome segregation	0.7	7.3	25.7	45.5	25.7	7.3	0.7
			Chi[2] 4 df = 28.9**				
Duplex and simplex loci, chromatid segregation			9 phenotypes				
Two duplex loci			9 phenotypes				

NS = not statistically significant.

**Significant at $p < 0.01$.

TABLE 8. Genetic models for segregation of MDH loci in the artificial allotetraploid (*L. japonicus* X *L. alpinus*)[2].

Genotype (FS)	(?) 40	(?) 31	(?) 22	(?) 13	(?) 04
Observed	5	24	54	27	2
Expected for					
Two disomic loci	7	28	42	28	7
		Chi² 4 df = 8.18 NS			
Tetrasomic locus, chromosome segregation	3	25	56	25	3
		Chi² 4 df = 1.9 NS			
Tetrasomic locus, chromatid segregation	6	30	40	30	6
		Chi² 4 df = 9.23 NS			

NS = not statistically significant.

TABLE 9. Genetic models for duplicated PGI loci in artificial autotetraploid (*L. alpinus*)[2]. Categories with expected values less than five (underscored) have been grouped together for calculation of Chi[2].

Genotype (FMB)	202	130	220	211	121	112	031	022	040	Other
Observed	0	11	4	3	28	16	16	12	5	0
Expected for										
Two independent disomic loci	6.0	11.8	6.0	11.8	23.8	11.8	6.0	11.8	6.0	0
					Chi^2 8 df = 32.24***					
tetrasomic locus chromosome segregation	2.7	10.5	10.5	10.5	26.6	10.5	10.5	10.5	2.7	0
					Chi^2 7 df = 15.62*					
tetrasomic locus chromatid segregation	2.2	11.6	9.2	9.7	21.3	9.7	11.6	9.2	4.4	5.3
					Chi^2 8 df = 26.79***					

*Significant at $p < 0.05$.

***Significant at $p < 0.001$.

TABLE 10. Genetic models for segregation of PGI loci in progeny of cross L. corniculatus (Acc. no. 554-5) X cultivar 'Leo'-1. Categories with expected values less than five (underscored) have been grouped together for calculation of Chi².

554-5 gametes (F M)	40	31	22	13	04
Leo-1 gametes (F M)	40	40	40	40	40
Progeny genotype (F M)	80	71	62	53	44
Observed	13	15	15	15	6
Expected for					
Four independent disomic loci	4	16	24	16	4
		Chi² 3 df = 18.63***			
Two tetrasomic duplex loci, chromosome segregation	1.8	14.2	32	14.2	1.8
		Chi² 3 df = 75.00***			
Two tetrasomic duplex loci, chromatid segregation	2.9	15.7	26.8	15.7	2.9
		Chi² 3 df = 35.29***			

***Significant at p <0.001.

TABLE 11. Meiotic regularity of various taxa used in this study

Taxon	Accession number	Diakinesis and MI (%)			Anaphase I II's as			Pollen stainability (%)	Source
		II's	I's	IV's	II's	Bridges	Laggards		
L. alpinus	77	98.38	1.62	0.0	97.58	0.0	3.42	67.19	(1)[1]
(L. alpinus)[2]	774X	74.03	13.68	10.83	66.00	0.0	33.00	44.57	(1, 2)
L. corniculatus L.	554	91.98	3.93	3.62	87.27	-	12.73	78.81	(1, 2)
L. japonicus	129	99.02	0.98	0.0	99.64	0.0	0.36	96.43	(1)
L. japonicus X L. alpinus	129 X 77	96.13	2.53	0.93	91.11	3.7	5.19	26.77	(1, 2)
(L. japonicus X L. alpinus)[2]	(129 X 77)[2]	81.75	8.83	9.13	54.29	16.43	29.99	44.57	(1, 2)
L. tenuis	109	99.23	0.77	0.0	96.91	0.0	3.08	82.41	(1)

[1]Somaroo (1970)

[2]Somaroo and Grant (1971)

An isoenzyme study in the genus Lotus (Fabaceae). III. Evaluation of hypotheses concerning the origin of L. corniculatus using isoenzyme data

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)

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, isozymes, isoenzymes

Introduction

This is the third paper in a series resulting from an isoenzyme study of several species within the genus Lotus. Previous papers described the experimental procedures (Raelson and Grant 1987) and the results of a study of segregation of isoenzyme alleles in interspecific hybrids, in artificial allo- and autotetraploids, and in L. corniculatus (Raelson et al. 1987). In this paper is 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 have been proposed as possible ancestors 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 artificial autotetraploids of these species have been produced (De Lautour et al. 1978; Somaroo and Grant 1971a) and they do not resemble L. corniculatus in morphology or fertility. In addition, the authors have provided evidence of quadruplication of Pgi2 loci in both the artificial allotetraploid (L. japonicus X L. alpinus)^2 and in L. corniculatus that was not found for the artificial 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 (1971b, 1972) proposed that L. japonicus and L. alpinus could be ancestral species based upon the fact that the artificial amphidiploid (L. japonicus X L. alpinus)^2 can be

Introduction

This is the third paper in a series resulting from an isoenzyme study of several species within the genus Lotus. Previous papers described the experimental procedures (Raelson and Grant 1987) and the results of a study of segregation of isoenzyme alleles in interspecific hybrids, in artificial allo- and autotetraploids, and in L. corniculatus (Raelson et al. 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 have been proposed as possible ancestors 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 artificial autotetraploids of these species have been produced (De Lautour et al. 1978; Somaroo and Grant 1971a) 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 artificial allotetraploid (L. japonicus X L. alpinus)^2 and in L. corniculatus that was not found for the artificial 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 (1971b, 1972) proposed that L. japonicus and L. alpinus could be ancestral species based upon the fact that the artificial amphidiploid (L. japonicus X 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 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.

The isoenzyme data provide a new, previously unavailable approach, for examining this problem. 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.

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 a few 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 can not 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 $(p_i (1 - p_i)/n)^{-1/2} + 1/2n)$ 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 were 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 1987). 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

Representative isoenzyme zymograms for PGI, TPI, PGM, MDH, IDH, 6-PGDH, and ME are presented in Figs. 1-7, respectively. Each figure contains both a photograph and a graphic representation which is based upon three replicated electrophoretic experiments. A summary of all isoenzyme phenotypes for all accessions of all species for the seven isoenzymes is presented in Figs. 8-10.

Data on the frequency of various alleles within the five species is 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 et al. 1987). Alleles for various loci are named according to the mobility relative to one common phenotype band which is arbitrarily designated as Relative Mobility 100. The loci designation for PGM is the most uncertain of all the enzymes. The upper PGM locus appears to have fixed heterozygosity (constant two-banded phenotype) for all individuals of L. uliginosus. We assume that this reflects 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 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 (Raelson et al. 1987), 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 were also calculated for some accessions of each of the five species. These frequencies are displayed in Tables 4-7. 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

It was not meaningful to construct a genetic similarity index for the taxa examined in this study because of the uncertainty of the population samples obtained from the world Lotus collection. In the absence of such an index, two other criteria were chosen for the purpose of testing whether a given species was likely to be involved in the origin of L. corniculatus. These criteria are not of equal importance and dependability.

Crawford (1985) 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, 16-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 X L. alpinus could contain all of the alleles found in L. corniculatus, but this is also true of the cross L. tenuis X 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 possiblities.

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 the ancient Lotus, 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, and L. japonicus 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.

Acknowledgment

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.

Literature

Brown AHD, Weir BS (1983) Measuring genetic variability in plant populations. In: Tanksley SD, Orton TJ (eds) Isozymes in Plant Genetics and Breeding, Part A. Elsevier Science Publ., Amsterdam, New York. pp 219-239.

Crompton CW (1987) Pollen morphology of the tribe Loteae (Leguminosae) by light and scanning electron microscopy. M.Sc. Thesis, McGill University, Montreal, Canada.

Crawford DJ (1985) Electrophoretic data and plant speciation. Syst Bot 10: 405-416.

Dawson CDR (1941) Tetrasomic inheritance in Lotus corniculatus L. J Genet 42: 49-72.

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.

Gottlieb LD (1977) Electrophoretic evidence and plant systematics. Ann Mo Bot Gard 64: 161-180.

Grant WF, Sidhu BS (1967) Basic chromosome number, cyanogenetic glucoside variation, and geographic distribution of Lotus species. Can J Bot 45: 639-647.

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.

Larsen K (1954) Cytotaxonomical studies in Lotus. I. Lotus corniculatus L. sens. lat. Bot Tidsskr 51: 205-211.

Larsen K, Zertova A (1965) The Australian Lotus species. Feddes Repertorium 72: 1-18.

Moran PAP (1968) An introduction to probability theory. Clarendon Press, Oxford.

Murdy WH, Carter MEB (1985) Electrophoretic study of the allopolyploidal origin of Talinum teretifolium and the specific status of T. appalachianum (Portulacaceae). Am J Bot 72: 1590-1597.

Nakai Y (1981) D genome donors for Aegilops cylindrica (CCDD) and Triticum aestivum (AABBDD) deduced from esterase isozyme analysis. Theor Appl Genet 60: 11-16.

Nei M (1972) Genetic distance between populations. Am Nat 106: 283-292.

Raelson JV, Grant WF (1987) An isoenzyme study in the genus Lotus (Fabaceae). I. Establishment of experimental protocols. To be submitted to Genome.

Raelson JV, Lemaitre PC, Starkie KM, Grant WF (1987) An isoenzyme study in the genus Lotus (Fabaceae). II. Genetic analyses of isoenzyme loci in interspecific hybrids, artificial allo- and autotetraploids, and in L. corniculatus. To be submitted to Genome.

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

Roose, ML, Gottlieb LD (1976) Genetic and biochemical consequences of polyploidy in Tragopogon. Evolution 30: 818-830.

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

Somaroo BH, Grant WF (1971b) Meiotic chromosome behavior in induced autotetraploids and amphidiploids in the Lotus corniculatus group. Can J Genet Cytol 13: 663-671.

Somaroo BH, Grant WF (1972) Crossing relationships between synthetic Lotus amphidiploids and L. corniculatus. Crop Sci 12: 103-105.

Stebbins GL (1950) Variation and evolution in plants. Columbia Univ. Press, New York and London. 643 pp.

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

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

Figure 1. Representative zymogram for PGI. A. Photograph of zymogram. B. Graphic presentation. Lanes 1-4 L. tenuis, 5-8 L. uliginosus, 9-12 L. alpinus, 13-16 L. japonicus and 17-20 L. corniculatus.

PGI

A

B

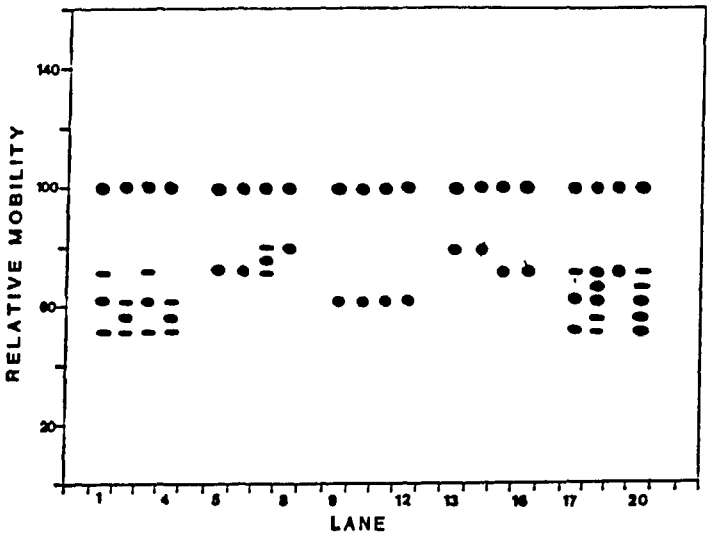
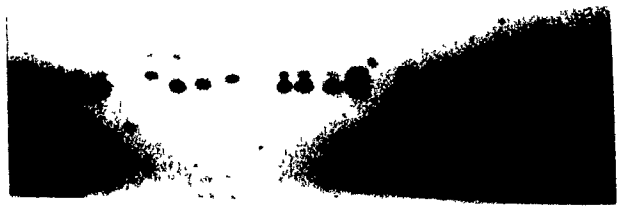


Figure 2. Representative zymogram for TPI. A. Photograph of zymogram. B. Graphic presentation. Lanes 1-4 L. tenuis, 5-8 L. uliginosus, 9-12 L. alpinus, 13-16 L. japonicus and 17-20 L. corniculatus. Solid dot represents consistent band for all replications. Open dot represents artifact of this particular gel.

TPI

A



B

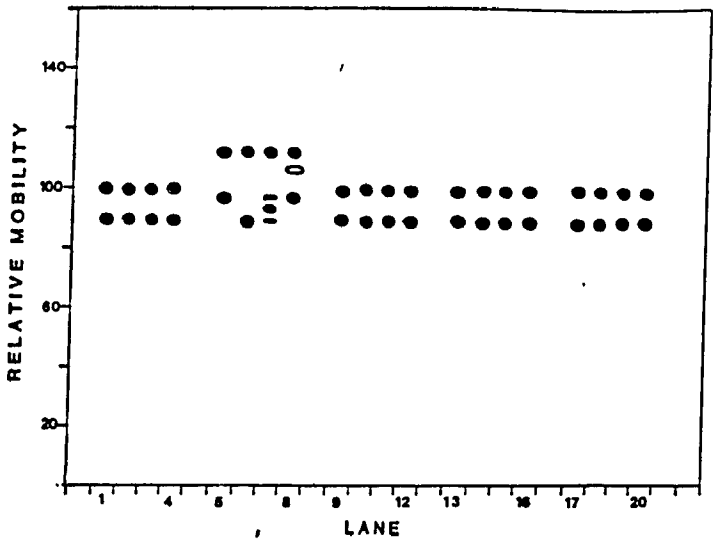


Figure 3. Representative zymogram for PGM. A. Photograph of zymogram. B. Graphic presentation. Lanes 1-4 L. tenuis, 5-8 L. uliginosus, 9-12 L. alpinus, 13-16 L. japonicus and 17-20 L. corniculatus. Solid dot consistent well-stained band. Open dots represent consistent but poorly stained bands. Open dot with line represents artifact of this particular gel.

PGM

A

B

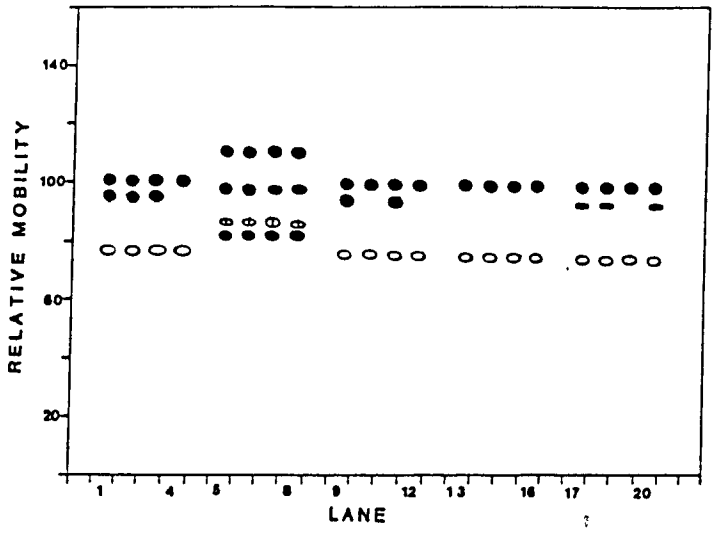


Figure 4. Representative zymogram for MDH. A. Photograph of zymogram. B. Graphic presentation. Lanes 1-4 L. tenuis, 5-8 L. uliginosus, 9-12 L. alpinus, 13-16 L. japonicus and 17-20 L. corniculatus.

MDH

A

B

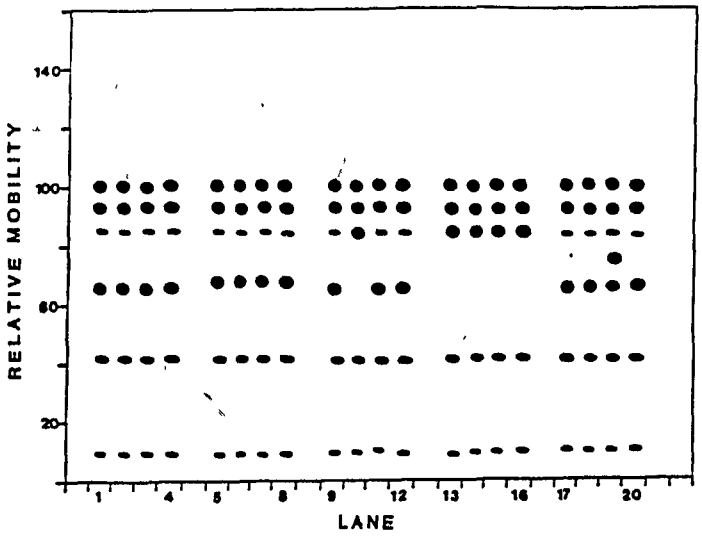


Figure 5. Representative zymogram for IDH. A. Photograph of zymogram. B. Graphic presentation. Lanes 1-4 L. tenuis, 5-8 L. uliginosus, 9-12 L. alpinus, 13-16 L. japonicus and 17-20 L. corniculatus. Solid dot consistent well-stained band. Open dot consistent but a poorly stained band.

IDH

A

B

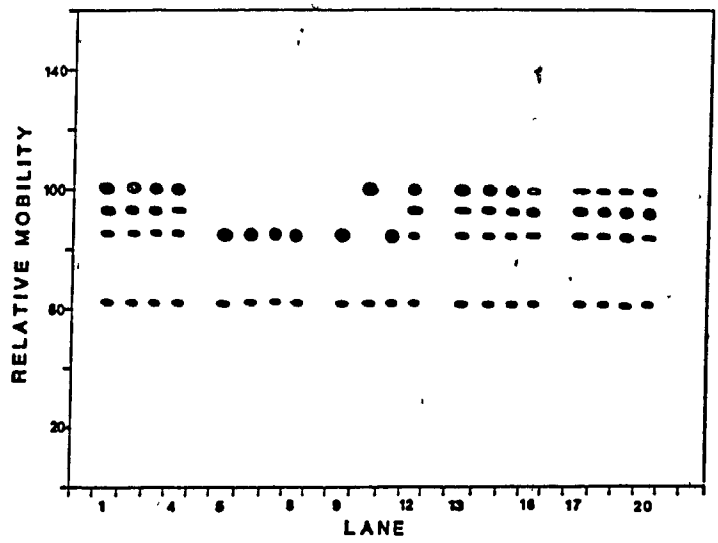


Figure 6. Representative zymogram for 6-PGDH.
A. Photograph of zymogram. B. Graphic presentation.
Lanes 1-4 L. tenuis, 5-8 L. uliginosus,
9-12 L. alpinus, 13-16 L. japonicus and
17-20 L. corniculatus.

6-PGDH

A

B

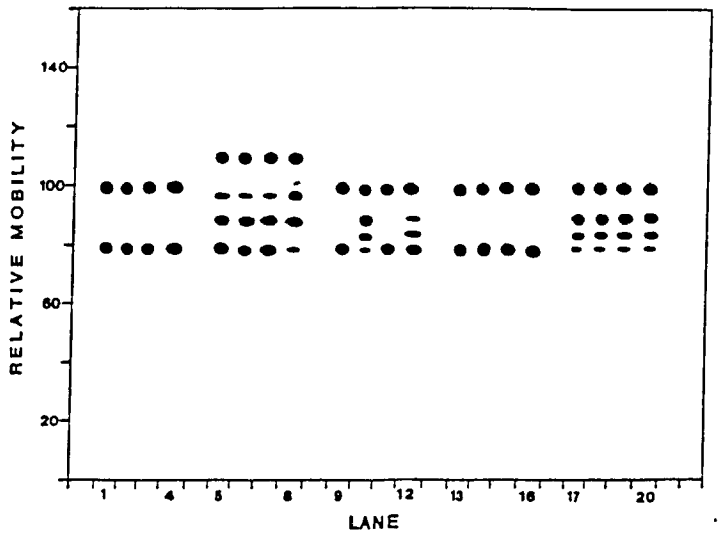


Figure 7. Representative zymogram for ME. A. Photograph of zymogram. B. Graphic presentation. Lanes 1-4 L. tenuis, 5-8 L. uliginosus, 9-12 L. alpinus, 13-16 L. japonicus and 17-20 L. corniculatus. Solid dots consistent tetrameric band. Open dots are probably breakdown of the tetrameric enzyme into monomers, dimers and trimers.

ME

A

B

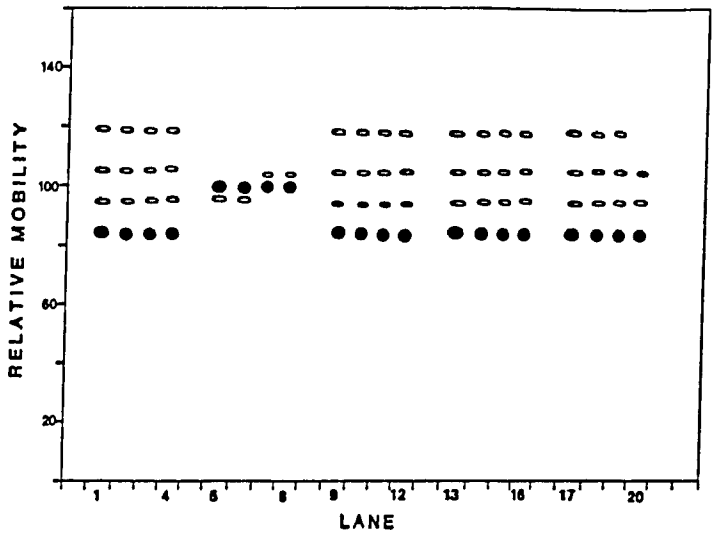


Figure 8. Graphic summary of all isoenzyme phenotypes for PGI, TPI, and PGM. A. Lotus tenuis. B. L. uliginosus. C. L. alpinus. D. L. japonicus. E. L. corniculatus. The six-banded phenotype of PGI for L. alpinus is from the autotetraploid accession 774x.

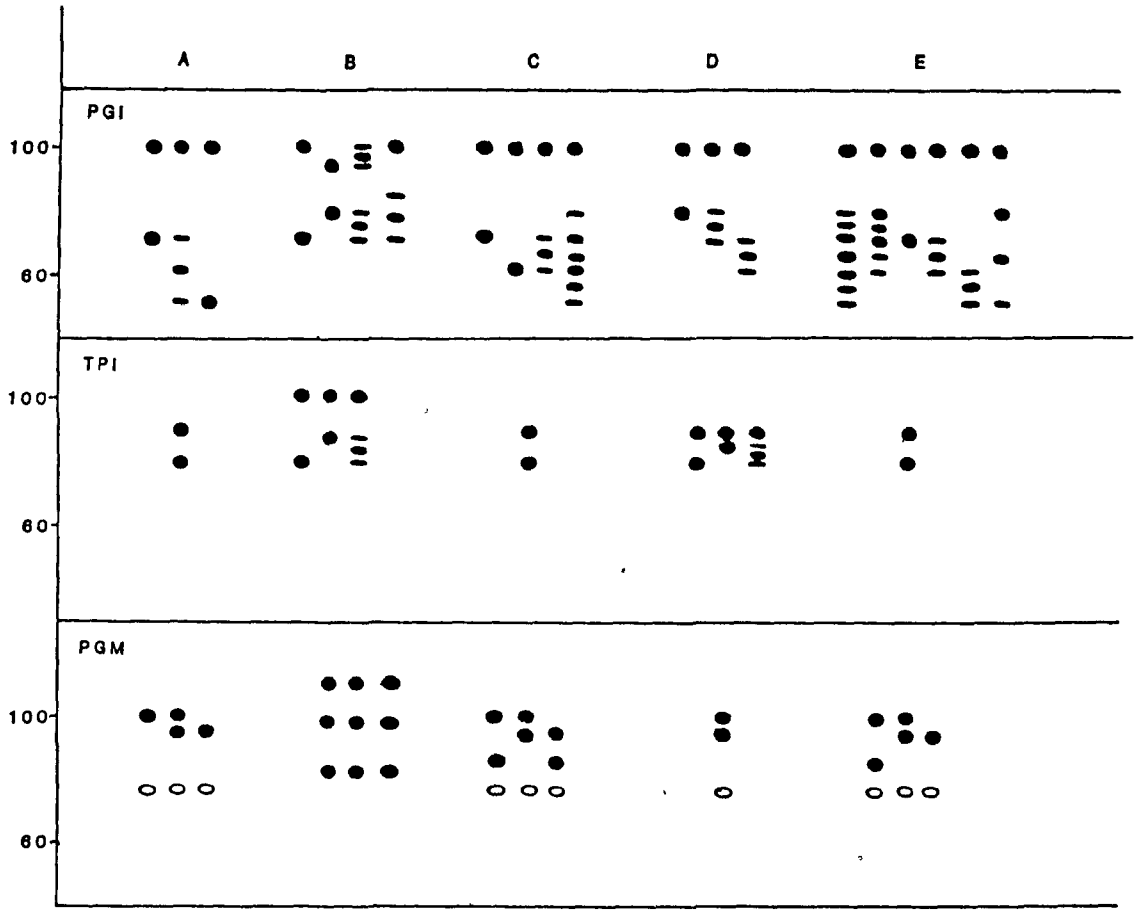

A
B
C
D
E
PGI
100
60
TPI
100
60
PGM
100
60

Figure 9. 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.

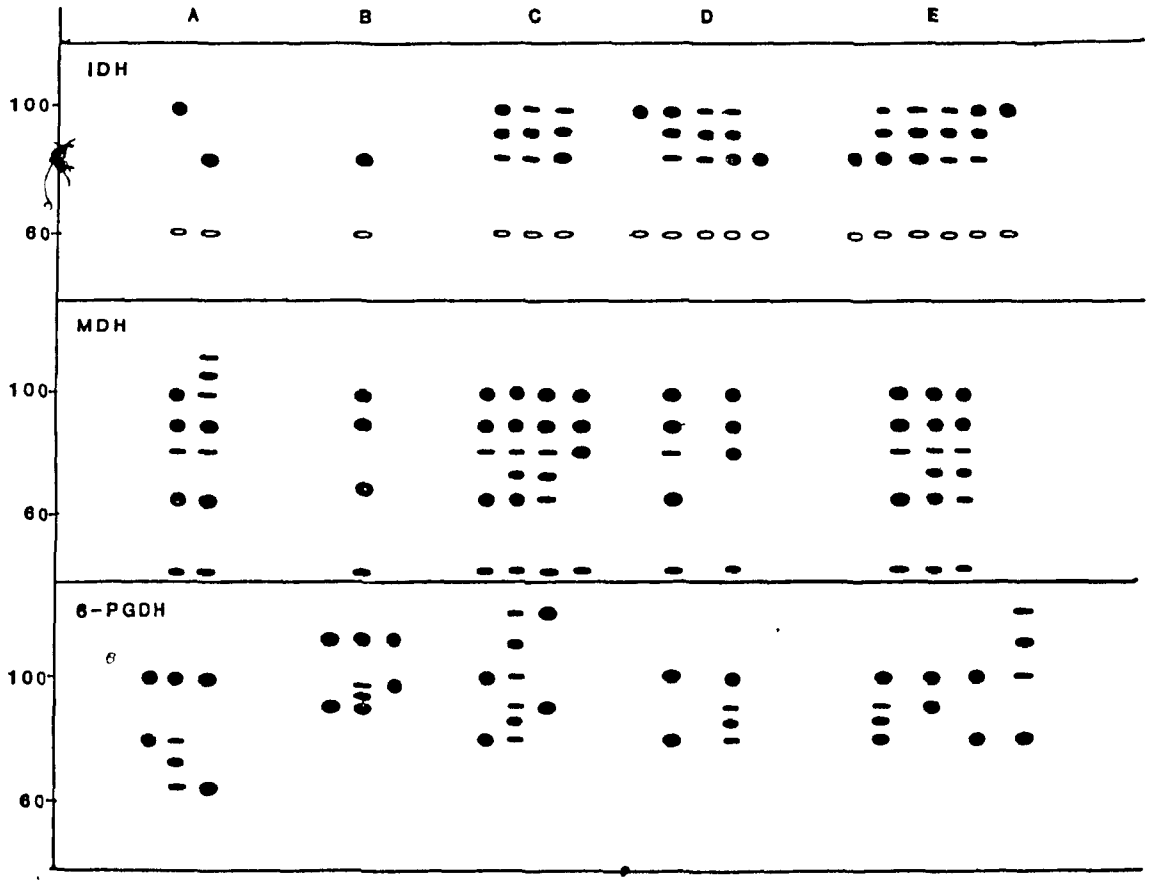

A
B
C
D
E
IDH
100
60
MDH
100
60
6-PGDH
100
60

Figure 10. 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.

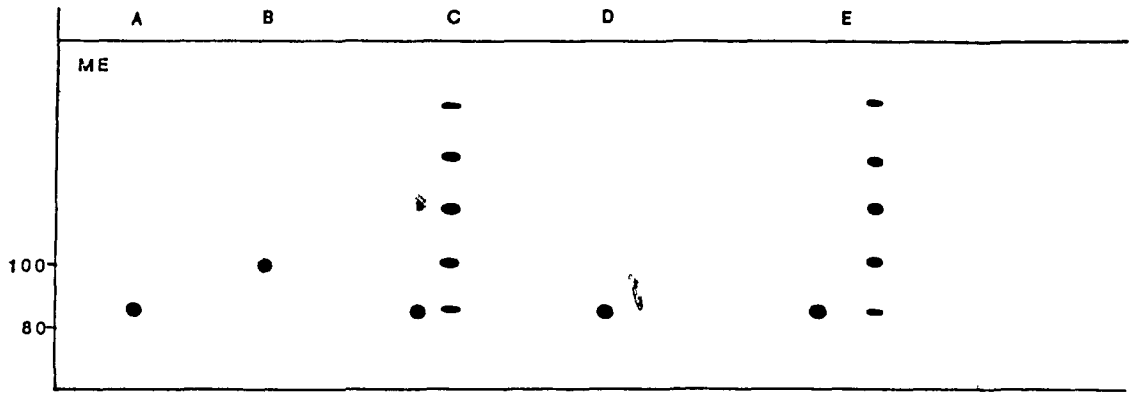

A
B
C
D
E
ME
100
80

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, Universite de Neuchatel. Received as L. corniculatus var. alpinus Ser.
	324	12	10	Origin: Lebanon. Collector, W. S. Edgecombe
	774x	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, Izair, 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

... Table 1. Cont'd.

Table 1. Continued

Taxon	Accession number	Chromosome number	No. individuals	Source
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 Introduction 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

... Table 1. Cont'd.

Table 1. Continued

Taxon	Accession number	Chromosome number	No. individuals	Source
L. uliginosus Schkuhr	110	12	10	Service de la Recherche Agronomique et de l'Experimentation Agricole, Rabat, Morocco
	193	12	30	U.S.D.A., PL 69-55. Origin unknown
	120	12	10	Institut fur Kulturpflanzenforschung Gatersleben, 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, Germany

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

	Lotus tenuis				L. uliginosus				L. alpinus				Lotus japonicus				L. corniculatus			
Locus, allele	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				.483	.058			1		9		1				1	
95			-				.517	.058			-				-				-	
Pgi2																	8	1104		
86			-				.036	.023			-				-				-	
80			-				.153	.042			.037	.038			.850	.052			.261	.026
72			.433	.058			.803	.047			.228	.079			.086	.041			.385	.029
62			.256	.051			-				.713	.085			.001	.006			.146	.021
52			.297	.053			-				.022	.030			.045	.031			.208	.024
Tpi1	9	300			9	300			4	120			6	200			8	552		
112	-		-				1				-				-				-	
100			1				-				1				1				1	

... Table 2. Cont'd.

Table 2. Cont'd.

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.
Tpi2																				
97			-				.740	.051			-				.268	.064				
90			1				.260	.051			1				.732	.064			1	
Pgm1,2	9	600			9	600			4	240			6	400			8	1109		
110			-				.500	.041			-				-				-	
100			.530	.041			-				.441	.065			.500	.050			.529	.029
98			-				.500	.041			-				-				-	
95			.470	.041			-				.345	.062			.500	.050			.438	.029
85			-				-				.243	.056			-				.033	.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			.047	.026			-				-				-				-	
100			.953	.026			1				1				1				1	

... Table 2. Cont'd.

Table 2. Cont'd.

Locus, allele	Lotus tenuis No. accs.	No. allel.	Freq. No.	C.I.	L. uliginosus No. accs.	No. allel.	Freq. No.	C.I.	L. alpinus No. accs.	No. allel.	Freq. No.	C.I.	Lotus japonicus No. accs.	No. allel.	Freq. No.	C.I.	L. corniculatus No. accs.	No. allel.	Freq. No.	C.I.
Mdh3																				
85			-				-				.507	.094			.895	.031			.106	.027
68			-				1				-				-				-	
66			1				-				.493	.094			.105	.031			.894	.027
Idh1,2	9	600			9	600			4	240			6	400			8	1104		
100			.953	.018			-				.669	.086			.638	.048			.639	.029
85			.047	.018			1				.331	.086			.362	.048			.361	.029
6-Pgdh1	9	300			9	300			4	120			6	200			8	552		
120			-				-				.059	.046			-				.201	.034
110			-				1				-				-				-	
100			1				-				.941	.046			1				.798	.034

... Table 2. Cont'd.

Table 2. Cont'd.

Locus, allele	*Lotus tenuis* No. accs.	No. allel.	Freq. No.	C.I.	*L. uliginosus* No. accs.	No. allel.	Freq. No.	C.I.	*L. alpinus* No. accs.	No. allel.	Freq. No.	C.I.	*Lotus japonicus* No. accs.	No. allel.	Freq. No.	C.I.	*L. corniculatus* No. accs.	No. allel.	Freq. No.	C.I.
6-Pgdh2																				
98			-				.733	.052			-				-				-	
95			-				.267	.052			-				-				-	
90			-				-				.228	.079			-				.011	.010
80			.890	.037			-				.772	.079			.005	.012			.989	.010
65			.110	.037			-				-				.995	.012			-	
Me2	9	300			9	300			75	120			6	200			8	552		
152			-				-				.043	.040			-				.011	.010
100			-				1				-				-				-	
85			1				-				.957	.040			1				.989	.010

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

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	
Pgai,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	-		-		-	

... Table 3. Cont'd.

Table 3. Cont'd.

Accession	109		145		131	
Locus, allele	Freq. (95% C.I.)	N°	Freq. (95% C.I.)	N°	Freq. (95% C.I.)	N°
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	

... Table 3. Cont'd.

Table 3. Cont'd.

Accession	109		145		131	
Locus, allele	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*
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	-		-		-	

... Table 4. Cont'd.

Table 4. Cont'd.

Accession	193		289		854	
Locus, allele	Freq. (95% C.I.)	N°	Freq. (95% C.I.)	N°	Freq. (95% C.I.)	N°
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	-		-		-	

... Table 4. Cont'd.

Table 4. Cont'd.

Accession	193		289		854	
Locus, allele	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*
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	
Pgmi,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		-		-	

... Table 5. Cont'd.

Table 5. Cont'd.

Accession	77		224		828		
Locus, allele	Freq. (95% C.I.)	N°	Freq. (95% C.I.)	N°	Freq. (95% C.I.)	N°
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	

... Table 5. Cont'd.

Table 5. Cont'd.

Accession	77		224		828	
Locus, allele	Freq. (95% C.I.)	N°	Freq. (95% C.I.)	N°	Freq. (95% C.I.)	N°
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		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	-		-		-	

... Table 6. Cont'd.

Table 6. Cont'd.

Accession	177		129		892	
Locus, allele	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*
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	

... Table 6. Cont'd.

Table 6. Cont'd.

Accession	177		129		892	
Locus, allele	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*
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		710		764	
	wild population		'Viking'		'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	

... Table 7. Cont'd.

Table 7. Cont'd.

Accession	554		710		764	
	wild population		'Viking'		'Leo'	
Locus, allele	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*
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		-		-	
81	-		-		-	
66	0.542, 0.093		1		1	

... Table 7. Cont'd.

Table 7. Cont'd.

Accession	554		710		764	
	wild population		'Viking'		'Leo'	
Locus, allele	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*	Freq. (95% C.I.)	N*
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 + $(1.96\ (p_i\ (1 - p_i)/n)^{-1/2} + 1/2n)$

GENERAL CONCLUSIONS

Isoenzyme data present a new field of information available to Lotus genetics. The research presented here has accomplished much of the detailed study which is necessary to place such data on a sound theoretical foundation. The major tasks of such preliminary research are developing effective laboratory protocols, and establishing the genetic basis of isoenzyme phenotypes through segregation studies. Having achieved these basics, the research went on to address an important theoretical question, that of the phylogenetic origin of Lotus corniculatus, using the newly available data. There are, however, other research applications for the isoenzyme methodology. In the remainder of this conclusion, I will suggest further research that could be undertaken.

The hybrids used in this study had been previously synthesized by former students. Because of this, it was not known whether different heterozygous alleles for different loci were in cis or trans position in the hybrid genotypes. If this information were known, linkage intensity for the various loci could be determined by means of the maximum likelihood methods used by Tanksley and Rick (1980) and Allard (1956). An interspecific hybrid between L. uliginosus and either L. alpinus or L. tenuis would be useful for this purpose, since such a cross is likely to be somewhat fertile with many heterozygous loci. A knowledge of the parental genotypes would provide a knowledge of their relationships within the hybrid. The segregation of other characters, such as cyanogenesis and keel-tip color could be simultaneously studied and the first linkage map for Lotus could be constructed.

A hybrid of L. uliginosus has not yet been obtained, however, it should not be too difficult using embryo culture techniques. The author was able to obtain hybrid seed pods using L. uliginosus as a female parent and L. tenuis as a male parent. Unfortunately, these were destroyed by insects before the embryos were ready for culture and could not be used in this study.

Another fruitful area of research would be to extend the isoenzyme survey of the L. corniculatus group to more accessions of the species studied and to the remainder of the diploid species within the group. The enzyme AAT would be of interest in such a study. This isoenzyme is able to distinguish between L. alpinus and L. japonicus (The phenotypes for AAT of the four diploid species used in this study are shown in Figure 1. The author was not able to obtain consistent staining with this enzyme in L. corniculatus, so it was not used in the studies reported here. There is some evidence that AAT becomes weaker in older plants. Some experimenting with techniques might produce consistent results that could detect the presence of japonicus or alpinus alleles in L. corniculatus.

Another use for the electrophoretic techniques would be to identify aneuploids for various Lotus species. Three of the enzymes studied in Paper II were found to be not linked. This does not guarantee that the loci are on different chromosomes, but it does suggest that enough loci are available to identify at least some of the aneuploids in a series.

There exists another small project that remains to be done which could provide answers to questions that remain from the segregation studies. The offspring of several selfed taxa segregated in a 1:2:1 ratio with three phenotypes. These phenotypes were generally a single band, an unbalanced

parental phenotype, and a balanced (1:2:1) band density phenotype. Such segregation could occur for either two disomic loci with only one of the loci heterozygous or for a simplex or triplex tetrasomic locus. Selfing the individuals with the balanced band density phenotype could show which of these two models is correct. If there were two disomic loci in the original parent, then the offspring of the selfed progeny would not segregate for the balanced phenotype. The heterozygosity would be fixed within two distinct loci each homozygous for different alleles. If the tetrasomic model were correct, the individuals with the balanced phenotype would be duplex heterozygous (FFSS) and their progeny would segregate in a 1:8:18:8:1 ratio. Such a 1:2:1 phenotype segregation was found among progeny of the following crosses for the following loci: selfed L. japonicus X L. alpinus (28), Pgi2, Mdh3, 6-PGDH1; selfed (L. alpinus)^2 (774x-5) and L. corniculatus (554-5) X Leo-1, Mdh3. Progeny of these crosses with the balanced band density phenotypes will be maintained in the greenhouse. Selfing these would not be difficult. This would be a relatively simple experiment suitable for an undergraduate honors research project, and would provide a definitive answer to the question of whether segregation in L. corniculatus is disomic or tetrasomic.

There remains enough further research with the isoenzyme technique for, certainly, a Masters' degree project, and perhaps for another Ph.D. project. Considerable expense and effort has been spent on setting up this system and in laying the theoretical foundation. It would be regrettable if these tools were not used for further Lotus research.

References

Tanksley, S. D. and Rick, C. M. 1980. Isogenic gene linkage map of the tomato. Applications in genetics and breeding. Theor. Appl. Genet. 57: 161-170.

Allard, R. W. 1956. Formulas and tables for calculating linkage intensities. Hilgardia 24: 232-278.

Figure 1. Zymogram for AAT for four diploid Lotus species. A. Photograph. B. Graphic presentation. Lanes 1-5, L. tenuis. Lanes 6-10, L. uliginosus. Lanes 11-15, L. alpinus. Lanes 16-20, L. japonicus. Banding was inconsistent for L. corniculatus.

AAT

A

B