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Linkages between restriction fragment length, isozyme, and morphological markers in lentil

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Summary. A genetic linkage map of lentil comprising 333 centimorgans (cM) was constructed from 20 restriction fragment length, 8 isozyme, and 6 morphological markers segregating in a single interspecific cross (*Lens culinaris* × *L. orientalis*). Because the genotypes at marker loci were determined for about 66 F_2 plants, linkages are only reported for estimates of recombination less than 30 cM. Probes for identification of restriction fragment length polymorphisms (RFLPs) were isolated from a cDNA and EcoRI and PstI partial genomic libraries of lentil. The cDNA library gave the highest frequency of relatively low-copy-number probes. The cDNAs were about twice as efficient, relative to random genomic fragments, in RFLP detection per probe. Nine markers showed significant deviations from the expected F_2 ratios and tended to show a predominance of alleles from the cultigen. Assuming a genome size of 10 Morgans, 50% of the lentil genome could be linked within 10 cM of the 34 markers and the map is of sufficient size to attempt mapping of quantitative trait loci.

Key words: *Lens culinaris* – *Lens orientalis* – Restriction fragment length polymorphisms – cDNA – Random genomic fragments

Introduction

Detailed genetic maps have recently received much attention as a plant breeding tool (Tanksley and Rick 1980; Soller and Beckmann 1983; Helentjaris et al. 1985). It is, however, often difficult to identify a large number of morphological or isozyme markers segregating in a cross of interest. Recent advances in molecular biology have revealed a new class of genetic markers, restriction fragment length polymorphisms (RFLPs), which often occur in sufficient quantities to generate detailed genetic maps (Botstein et al. 1980; Soller and Beckmann 1983). The two primary advantages of RFLPs and isozyme markers over morphological markers are codominance and absence of pleiotropic effects; RFLPs have the added advantage of developmental stability (Beckmann and Soller 1983). RFLP, isozyme, and morphological markers have been used in practical plant breeding programs to map quantitative trait loci (QTLs) (Tanksley et al. 1982; Edwards et al. 1987; Stuber et al. 1987; Weller et al. 1988) and to monitor response to recurrent selection (Stuber et al. 1980, 1982).

Lentil (*Lens culinaris* Medik.) is a self-pollinating diploid ($2n = 2x = 14$) grain legume that is grown widely on the Indian subcontinent and in the Middle East, northern Africa, southern Europe, and North and South America. Lentil breeding has received little attention relative to other major pulses, such as dry bean or pea (Muehlbauer and Slinkard 1981). The genetic map of lentil is very rudimentary; thus far, 8 morphological, 1 disease resistance, and 22 isozyme loci have been assigned to 1 linkage group of 4 loci, 1 of 3 loci, and 7 of 2 loci (Muehlbauer and Slinkard 1981; Zamir and Ladizinsky 1984; Muehlbauer et al. 1989). In this paper, an expanded linkage map of lentil is presented using isozyme, morphological, and RFLP markers. This map can be used to identify linkages between markers and QTLs conditioning yield, biomass production or stress tolerances and, ultimately, to synthesize breeding populations of lentil with greater genetic variability for these traits.

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Materials and methods

Isolation, digestion, and blotting of lentil DNA

An initial study indicated that insufficient variability for RFLPs exists among accessions of cultivated lentil for detailed linkage studies (Havey and Muehlbauer, unpublished results). Cultivars Redchief and Brewer and two accessions of wild lentil [*Lens orientalis* (Boiss.) Hand.-Mazz.], Lo4 (Uzbekistan, USSR) and Lo56 (Denizli-Tavas, Turkey), were used in RFLP evaluations. DNA was isolated from the above-ground parts of 4- to 6-week-old seedlings using cTAB extraction (Murray and Thompson 1980), with the exception that CsCl was removed by dialysis against 10 m*M* Tris (HCl), pH 8.0, and 1 m*M* EDTA. Restriction enzymes with six-base-pair recognition sequences were chosen. Digestions of DNA with enzymes BglII, EcoRI, EcoRV, and HindIII (3 units/μg DNA) were completed using the manufacturer's (BRL, Gaithersburg/MA, USA) buffer and instructions. After 4 h, the restriction enzyme was denatured by heating at 65 °C for 10 min and gel-loading buffer (buffer type III, Maniatis et al. 1982) was added. Electrophoresis of digested DNA was carried out in 0.7%-agarose gels in 1x TBE (Maniatis et al. 1982) at 15 mA for 14 h. Gels were stained in ethidium bromide (0.5 μg/ml) and photographed over ultraviolet light. The DNA was denatured by soaking in 0.4 *N* NaOH with 0.6 *M* NaCl for 20 min and blotted overnight to Genescreen filters (NEN, Wilmington/DE, USA) by capillary transfer (Southern 1975) in the same solution. DNA was fixed to filters by illumination with UV light at 15 cm for 5 min.

Sources of probes

Random genomic probes were selected from PstI, a methylation-sensitive enzyme, and EcoRI partial genomic libraries of DNA from the cv Brewer. The libraries were constructed to assess the efficacy of the two restriction enzymes in producing low-copy-number probes from lentil for RFLP evaluations. Digestion of DNA with the respective restriction enzyme and electrophoresis was completed as described above. Fragments between 0.6 and 2.0 kb were electroeluted from the gel (Maniatis et al. 1982), extracted with phenol and chloroform, and ethanol precipitated in the presence of 2.5 *M* ammonium acetate. The eluted fragments were cloned into the EcoRI or PstI site in the pUC-19 plasmid after dephosphorylation with calf intestinal alkaline phosphatase (Maniatis et al. 1982). Strain DH5α of *Escherichia coli* was transformed with the plasmids (Hanahan 1983) and plated on LB media (Maniatis et al. 1982) with 100 μg/ml of penicillin-G and the indicator dye X-gal (Messing 1983). Colorless colonies containing recombinant plasmids were individually grown overnight in LB broth with antibiotic. Plasmids were isolated using the minipreparation procedure of Riggs and McLachlan (1986), digested with the appropriate restriction enzyme, and subjected to electrophoresis to identify plasmids with inserts. Potentially low-copy-number inserts were identified by the dot-blot procedure of Landry and Michelmore (1985). One μg of plasmids with lentil inserts were individually spotted onto a Genescreen filter. Filters were prehybridized in 20 ml buffer [1% BSA, 1 m*M* EDTA, 0.5 *M* Na_2HPO_4, pH 7.2 and 7% SDS (Church and Gilbert 1984)] at 68 °C for 60 min. Seventy ng of total sheared lentil DNA was labelled with P^{32} after random priming (Feinberg and Vogelstein 1983) to a specific activity of $3-5\times10^8$ cpm/μg. Probe DNA was heat-denatured and hybridized overnight to filters at 68 °C. Filters were washed twice in 0.5% BSA, 40 m*M* Na_2HPO_4 (pH 7.2), 1 m*M* EDTA and 5% SDS and three times in 40 m*M* Na_2HPO_4, 1 m*M* EDTA and 1% SDS at 68 °C for 20 min. Filters were rinsed in 0.1 *M* Na_2HPO_4 and placed on X-ray film (Kodak XAR-5) with DuPont Cronex Lightning Plus intensifying screens at −80 °C for 2–3 days.

Lentil inserts showing weak signals on the dot-blot were individually labelled and hybridized to EcoRI-digested lentil DNA. Fragments hybridizing to six bands or less were considered to be of low-copy-number and were retained for RFLP evaluations. The limit of six bands or less was arbitrarily chosen to allow easy identification of fragments. It is, however, recognized that some valuable probes may have been discarded. Probes from the EcoRI and PstI partial genomic libraries were designated as EMH-1, EMH-2, etc., and PMH-1, PMH-2, etc., respectively, following the nomenclature of Myers and Weeden (1988).

A cDNA library of lentil also served as a source of probes for RFLP evaluations. Total RNA was extracted from 3-week-old Brewer seedlings using the procedure of Chirgwin et al. (1979) and polyadenylated mRNA was isolated by three passes through an oligo-dT cellulose column (Bantle et al. 1976). cDNA molecules were synthesized using a kit (BRL) according to provided instructions. Blunt ends were synthesized with DNA polymerase I, internal EcoRI sites methylated (Promega methylase, Madison/WI, USA), EcoRI linkers added, and the cDNAs cloned into EcoRI-digested dephosphorylated pUC-13. Transformation and plating of DH5α were completed as previously described. cDNAs were labelled with P^{32} by random priming and hybridized to EcoRI-digested Brewer DNA; those hybridizing to six bands or less were considered to be low-copy and were used in RFLP evaluations. Clones from cDNA library were designated as CMH-1, CMH-2, etc.

Mapping

Lentil is highly self-pollinating and no evidence of heterogeneity for markers within an accession was found (Havey and Muehlbauer, unpublished results). F_1 plants were generated by crossing a Brewer or Redchief plant as the female parent with a single plant from Lo4 or Lo56. Hybrids between domesticated lentil and *L. orientalis* are fertile with generally normal meiosis, although translocations between the species have been reported (Ladizinsky et al. 1984). The F_1 was allowed to self-pollinate to generate the F_2 family. F_2 plants from Redchief by Lo4 segregated for eight isozyme loci [Asparate aminotransferase (*Aat-m* and *Aat-p*), leucine aminopeptidase (*Lap-1*), 6-phosphogluconate dehydrogenase (*Pgd-p*), shikimate dehydrogenase (*Skdh*), β-galactosidase pH 8.5 (*Gal-1*) and β-galactosidase pH 4.5 (*Gal-2*)], evaluated as described by Muehlbauer et al. (1989). Malic enzyme (*Me-2*) was assayed according to the procedure of Soltis et al. (1983). Plants were scored for six morphological markers: epicotyl color (*Gs*), growth habit (*Gh*), pod indehiscence (*Pi*) and seed coat spotting (*Scp*) as described by Muehlbauer and Slinkard (1981) and seed ground color (*Tgc*) as described by Vandenberg (1987). The presence and absence of violet stripes on pods were assumed to be conditioned by dominant and recessive alleles, respectively, at the pod pigmentation (*Pdp*) locus. Redchief was homozygous recessive for all morphological markers.

For mapping of RFLPs, above-ground parts from 16 to 25 F_3 plants from each of 66 F_2 plants of a single Redchief × Lo4 cross were harvested at 4 weeks and DNA was extracted using a minipreparation (Saghai-Maroof et al. 1984). Tissue from F_3 plants was bulked because of the difficulty in isolating adequate amounts of DNA from a single F_2 lentil plant. The DNA was digested, subjected to electrophoresis, transferred to Genescreen filters and probed as previously described. The genotype of the F_2 parent was classified as homozygous for the *culinaris* allele, heterozygous or homozygous for the *orientalis* allele from the genotype observed in the F_3 family. Goodness-of-fit to the ex-

pected F_2 segregations of 1:2:1 or 3:1 was tested by Chi-square analysis. Linkage between the RFLPs, isozyme, and morphological markers was estimated from F_2 segregations using maximum-likelihood formulas by the LINKAGE-1 program (Suiter et al. 1983). Linkage groups were constructed from the estimates of recombination between pairs of markers. Because the genotypes of only 66 F_2 plants were determined, independent segregation was assumed for linkage estimates greater than 30 centimorgans (cM).

Results

Quality of Probes from Libraries

Fifty-one PstI and 24 EcoRI random genomic fragments were evaluated for relative copy number. The random genomic libraries contained high frequencies of identical clones. Twenty-four of the 51 PstI fragments were the same clone and 10 of the 24 EcoRI fragments were identical. Of the remaining fragments, 11 PstI (41%) and 5 EcoRI (38%) were of relatively low-copy-number, i.e., 6 bands or less when hybridized to EcoRI-digested Brewer DNA.

The average size of the cDNA fragments was 1.1 kb, estimated from 90 clones. The cDNA library was a much more efficient source of relative low-copy-number probes. Of 25 cDNA clones, 22 (88%) hybridized to less than 6 bands on EcoRI-digested Brewer DNA. Signal intensities from hybridizations of cDNAs were not weaker than the low-copy random genomic fragments, as reported by Helentjaris et al. (1986).

The cDNA clones were about twice as efficient as random genomic fragments in detecting RFLPs between Redchief and Lo4. Fourteen RFLPs were identified with the 22 low-copy cDNAs (0.64 RFLP/cDNA). Six RFLPs were mappable with this cross from the 16 low-copy PstI and EcoRI fragments (0.38 RFLP/genomic fragment).

Segregation analyses and mapping

Initial evaluations identified 20 RFLP, eight isozyme, and six morphological markers segregating in the F_2 progeny of Redchief by Lo4. For RFLPs, the genotype of the F_2 plant was easily determined from the Southern blots of F_3 family DNA (Fig. 1). Observed segregations for 15 of 20 RFLP, 5 of 8 isozyme, and 5 of 6 morphological markers fit the expected F_2 ratios of 1:2:1 or 3:1 (Tables 1–3). The RFLP detected by probe PMH-14 segregated 3:1 because Redchief possessed two bands and the Lo4 parent had only the fast band. To our knowledge, this is the first report of segregation of *Me-2* in a lentil cross. *Me-2* exists as a tetramer and genotypes were easily scored (Fig. 2), but a poor fit to the expected ratio was found (Table 2). Of the nine deviant F_2 segregations, five showed an over-representation of the *culinaris* allele (Tables 1–3). Allelic frequencies for loci segregating 1:2:1

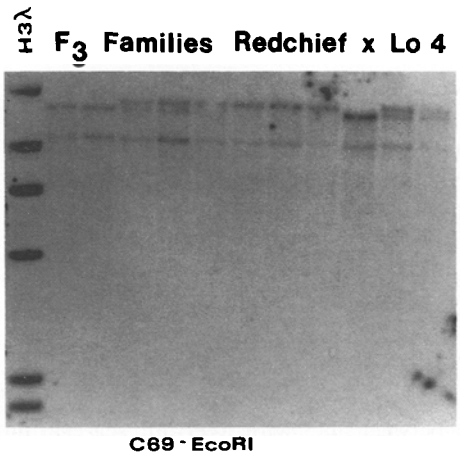

Fig. 1. Segregation of restriction fragment length polymorphism detected by probe CMH-69 hybridized to EcoRI-digested F_3 family DNA. H3λ is the HindIII digest of λ and served as size markers

Table 1. F_2 segregations of restriction fragment length polymorphisms markers in the cross Redchief by Lo4 and goodness-of-fit to expected segregations of 1:2:1 or 3:1

Probe[a]	Enzyme	Observed segregations			χ^2	Prob.[b]
		homozygous *culinaris*	heterozygote	homozygous *orientalis*		
CMH-03	EcoRV	19	26	11	2.57	0.28
CMH-32	HindIII	11	26	18	1.95	0.38
CMH-33	EcoRV	24	35	4	13.48	0.01*
CMH-34	EcoRV	18	28	11	1.747	0.42
CMH-41	EcoRI	18	30	18	0.55	0.76
CMH-45	HindIII	11	32	20	2.59	0.27
CMH-49	EcoRI	17	30	13	0.53	0.77
CMH-51	EcoRI	13	33	9	2.78	0.25
CMH-52	HindIII	11	26	17	1.41	0.50
CMH-58	BglII	15	26	15	0.29	0.87
CMH-69	EcoRI	20	32	12	2.00	0.37
CMH-71	EcoRV	27	17	22	16.27	<0.01*
CMH-81	EcoRV	12	33	20	1.99	0.37
CMH-93	EcoRI	12	42	10	6.38	0.04*
EMH-1	EcoRV	11	35	8	5.07	0.08
EMH-14	EcoRV	17	27	20	1.84	0.40
PMH-14	EcoRI	–54–		9	3.86	0.05*
PMH-68	HindIII	17	29	16	0.29	0.87
PMH-79	BglII	19	17	13	6.06	0.05*
PMH-119	EcoRI	16	27	23	3.67	0.16

[a] For nomenclature of probes, see 'Materials and methods'
[b] * indicates significant ($P < 0.05$) deviation from expected F_2 segregation

in the F_2 were 0.524 for the *culinaris* and 0.476 for the *orientalis* alleles. The predominance of *culinaris* alleles was evident from partitioning total Chi-square values into pooled and heterogeneity components for goodness-of-fit to homozygous *culinaris*, heterozygous, and homo-

zygous *orientalis* genotypes. The pooled Chi-square component was significant (9.07 with $df=2$; $P<0.05$) and the heterogeneity component was highly significant (124.03 with $df=54$; $P<0.01$) for loci segregating 1:2:1 in the F_2. The highly significant heterogeneity component indicated that a few marker loci showed large deviant segregation ratios and may be linked to chromosome regions conditioning differential fertility or viability. Loci deviating significantly from expected monogenic segregation ratios in the F_2 of Redchief × Lo4 did not show the same deviations in other interspecific crosses (Table 4).

A map was constructed from linkage estimates between individual loci (Fig. 3); however, estimates of recombination between loci with aberrant segregation ratios may not be accurate. The linkage groups are small and it is likely that continued mapping will join some of the groups. Six markers segregated independently of the others (Fig. 3). The RFLP detected by probe PMH-14 mapped to two locations, but only one position was confirmed by linkage to other markers (Fig. 3). Although continued mapping may reveal more than one map location for RFLPs detected by a single probe, there was little evidence of duplication. Linkages among the 28 loci comprise a total genetic distance of 333 cM. Assuming

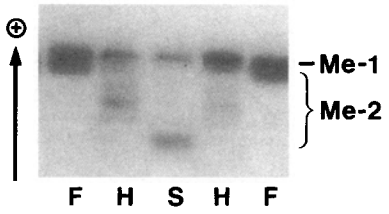

Fig. 2. Segregation of tetrameric malic enzyme 2 (Me-2) in F_2 progeny of lentil cross Redchief by Lo4. Malic enzyme 1 (Me-1) was monomorphic

Table 2. F_2 segregations of isozyme markers in the cross Redchief by Lo4 and goodness-of-fit to expected segregations 1:2:1

Enzyme[a]	Observed segregations			χ^2	Prob.[b]
	homozygous *culinaris*	heterozygote	homozygous *orientalis*		
Aat-m	41	47	10	19.78	<0.01*
Aat-p	14	53	31	6.55	0.04*
Gal-1	20	51	28	1.38	0.50
Gal-2	15	55	29	5.18	0.08
Lap-1	30	44	24	1.76	0.42
Me-2	42	48	8	23.63	<0.01*
Pgd-p	26	47	26	0.25	0.88
Skdh	29	48	21	1.35	0.51

[a] For nomenclature of isozymes, see 'Materials and methods'
[b] *indicates significant ($P < 0.05$) deviation from expected F_2 segregation

Table 3. F_2 segregations of morphological markers in the cross Redchief by Lo4 and goodness-of-fit to expected segregations

Marker[a]	Observed segregation			Expected	χ^2	Prob.[b]
	homozygous *orientalis*	heterozygous	homozygous *culinaris*			
Gh	19	53	27	1:2:1	1.79	0.41
Gs	–80–		19	3:1	1.78	0.18
Pi	–67–		21	3:1	0.06	0.81
Pdp	–46–		44	3:1	27.39	<0.01*
Scp	–13–		8	3:1	1.92	0.17
Tgc	–67–		22	3:1	0.01	0.95

[a] For description of morphological markers, see 'Materials and methods'
[b] * indicates significant ($P < 0.05$) deviation from expected F_2 segregation

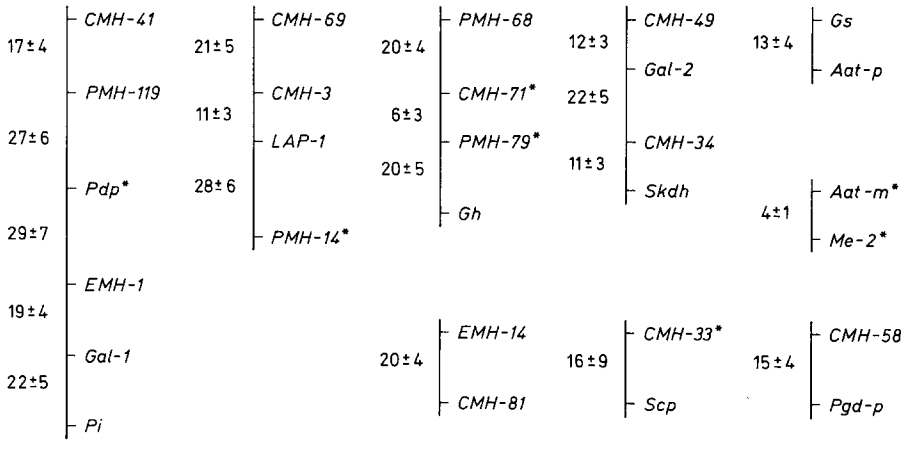

Fig. 3. Linkage map of 28 markers in Lentil. Estimates of recombination in centimorgans ± standard errors are shown on left. For description of loci, see 'Materials and methods'. *Asterisks* designate loci with aberrant F_2 segregation ratios

Table 4. Observed F_2 segregations for four markers demonstrating unacceptable goodness-of-fit to expected ratios in the cross Redchief × Lo4 and acceptable fits in other interspecific lentil crosses

Marker[a]	Observed segregations and Goodness-of-fit														
	Redchief × Lo4					Brewer × Lo4					Redchief × Lo56				
	cul[b]	het	ori	χ^2	Prob.[c]	cul	het	ori	χ^2	Prob.	cul	het	ori	χ^2	Prob.
Aat-p	14	53	31	6.55	0.04*	27	50	23	0.32	0.85					
Aat-m	41	47	10	19.78	<0.01*	28	56	16	4.32	0.12					
Pdp	44	–46–		27.39	<0.01*	30	–67–		1.82	0.18	44	–43–		27.68	<0.01*
CMH-93	12	42	10	6.38	0.04*						15	21	9	1.80	0.41

[a] For description of markers, see Materials and methods
[b] cul = homozygote for *culinaris* allele; het = heterozygote; and ori = homozygote for *orientalis* allele
[c] * indicates significant ($P < 0.05$) deviations from expected F_2 segregation

that the size of the lentil genetic map is approximately 10 Morgans, about 50% of the genome could be linked within 10 cM of the 34 markers (Beckmann and Soller 1983).

Discussion

The PstI and EcoRI partial genomic libraries gave approximately equal frequencies of relative low-copy-number probes. Although the experiments were not repeated due to the high cost of synthesizing and evaluating libraries, our results indicate no obvious advantage in generating probe libraries from hypomethylated regions in lentil. This is in contrast to results of Figdore et al. (1988) and Helentjaris et al. (1988); both observed a higher frequency (>75%) of low-copy-number probes from PstI partial genomic libraries of *Brassica* species and maize (*Zea mays* L.), respectively. The primary problem with the partial genomic libraries was the high frequency of identical clones. Forty-seven percent (24 of 51) of the PstI fragments and 42% (10 of 24) of the EcoRI fragments were the same clones. Because of their high frequency, the fragments may be from the plastid genomes. Cloning of chloroplast or mitochondrial fragments could be avoided by first isolating nuclei before digesting and cloning random fragments.

The cDNA library has proven to be an excellent source of probes for RFLP evaluations. The high frequency of low-copy-number probes (88%) eliminates the extra step of identifying relative low-copy-number clones, resulting in a significant reduction in time and materials. Thus, cDNA probes can be used without prior evaluation for relative copy number. The cDNAs were also twice as efficient as random genomic fragments in detecting RFLPs between Redchief and Lo4. This increased efficiency was observed between other intra- and interspecific lentil accessions (data not presented). Landry et al. (1987b) have also reported a 2.5-fold increase in RFLP detection efficiency of cDNAs over random genomic fragments in lettuce (*Lactuca sativa* L.).

The cDNAs were more efficient in detecting RFLPs than random genomic fragments. Although cDNAs correspond to the functional parts of genes (introns removed), this increased efficiency may be due to polymorphisms in flanking sequences outside the coding regions, or the cDNAs may encompass a greater length of nuclear DNA than obvious from their size [the average size of the cDNA clones (1.1 kb) used in RFLP evaluations was equal to that of the random genomic fragments], because introns are spliced out of polyadenylated mRNA. cDNAs from lentil and lettuce show greater efficiency in detecting RFLPs and both plants have relatively large amounts of DNA, 14.5 pg/cell for lettuce and 9.2 pg/cell for lentil (Bennett and Smith 1976). No mention could be found in the literature of increased efficiency of cDNAs over random genomic fragments in detecting RFLPs in plants with relatively less DNA, e.g., maize or tomato (*Lycopersicon esculentum* Mill.).

Little evidence of duplication of mapped loci was found. The RFLP identified by probe PMH-14 showed linkage to more than one independently segregating marker, although only a single location could be confirmed by other linked markers. These results indicate little redundancy of markers in lentil and agree closely with observations in tomato (Bernatzky and Tanksley 1986).

Linkages between *Aat-p* and *Gs*, *Gal-1* and *Pi*, and *Gal-1* and *Pdp* were observed in this research and have been reported in other lentil crosses (Zamir and Ladizinsky 1984; Weeden et al. 1988; Muehlbauer et al. 1989). The linkage group of *Pi-Gal-1-Pdp* is especially interesting because pod indehiscence and pigmentation are linked in pea (Blixt 1972), suggesting that some conservation of linkage groups may occur between pea and lentil.

Markers deviating significantly from the expected F_2 ratios often showed a predominance of the homozygote of the *culinaris* allele. Overrepresentation of a specific homozygous genotype in the F_2 has been reported for two interspecific crosses of tomato, although in both cases the allele from the wild parent was prevalent (Helentjaris et al. 1986; Bernatzky and Tanksley 1986). Detailed maps from intraspecific crosses of maize (Helentjaris et al. 1986) and lettuce (Landry et al. 1987a) have not revealed a predominance of alleles from one parent. Zamir and Tadmor (1986) observed a greater proportion of loci showing aberrant segregation ratios in interspecific than intraspecific *Lens* crosses. However, if a *culinaris* allele has a selective advantage, it does not appear consistent across other interspecific crosses (Table 4). This differential fertility, viability or environmental effect complicates the mapping of QTLs linked to markers showing significant deviations from expected ratios. Therefore, it appears advisable to generate detailed genetic maps using intraspecific crosses, if sufficient numbers of markers are detectable. Nevertheless, the present map is of sufficient size to attempt mapping of QTLs in lentil. However in trait-based analyses involving selection of a specific phenotype (Lebowitz et al. 1987), the genotypes of a random sample of plants may have to be determined to assure that deviant segregation ratios at marker loci are due to linked QTLs affecting the trait of interest and not due to differential fertility or viability of another linked allele.

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