Theor Appl Genet (1989) 77:839–843

Variability for restriction fragment lengths and phylogenies in lentil

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Received December 1, 1988; Accepted January 4, 1989
Communicated by J. Mac Key

Summary. Thirty accessions of domesticated (*Lens culinaris* ssp. *culinaris*) and wild (*L. culinaris* ssp. *orientalis*, *L. culinaris* ssp. *odemensis*, *L. nigricans* ssp. *ervoides* and *L. nigricans* ssp. *nigricans*) lentil were evaluated for restriction fragment length polymorphisms (RFLPs) using ten relative low-copy-number probes selected from partial genomic and cDNA libraries of lentil. Nei's average gene diversity was used as a measure of genetic variability for restriction fragment lengths within subspecies and a dendrogram was constructed from genetic distance estimates between subspecies. The wild lentils *L. culinaris* ssp. *orientalis* and *L. culinaris* ssp. *odemensis* showed the greatest variability for restriction fragment lengths and were closely positioned to domesticated lentil in the dendrogram. Little variability for restriction fragment lengths was observed within accessions of *L. nigricans* ssp. *ervoides* and *L. nigricans* ssp. *nigricans*. This observation is consistent with a previously published proposal that *nigricans* may have been independently domesticated. Estimates of genetic variability based on RFLPs tended to be greater than estimates from isozymes.

Key words: *Lens culinaris* – *Lens nigricans* – RFLP – Wild lentil – Genetic distance

Introduction

Lentil (*Lens* Miller) is a diploid ($2n=2x=14$) self-pollinating cool-season grain legume. Historically, most cultivars of lentil have been selected from heterogeneous populations, such as land races, and have received little conscious breeding effort (Muehlbauer and Slinkard 1981). Today all principal lentil growing areas of the world support breeding programs. In the USA, lentil cultivars trace back to a few plant introductions and therefore have a restricted germplasm base. Introgression of germplasm from wild accessions of lentil has become a focus of breeding efforts in the USA.

Lentil has recently undergone taxonomic revision based on crossability studies and variation within and between species and subspecies for morphological traits (Williams et al. 1974) and isozyme markers (Ladizinsky 1979; Hoffman et al. 1986). Ladizinsky et al. (1984) proposed that two species, or crossability groups, exist in lentil: *Lens culinaris*, comprising the cultivated lentil, *L. culinaris* ssp. *culinaris*, and two wild subspecies, *L. culinaris* ssp. *orientalis* and *L. culinaris* ssp. *odemensis*; and *Lens nigricans*, containing two wild subspecies, *L. nigricans* ssp. *ervoides* and *L. nigricans* ssp. *nigricans*. Hereafter, the various groups will be referred to by their subspecies names. Partially to fully fertile hybrids can be generated between subspecies within a crossability group. Crosses between crossability groups result in embryo breakdown shortly after fertilization. However, hybrids between *culinaris* and *ervoides* have been generated by embryo rescue, but irregular meiosis indicated that some chromosomal interchanges were present (Cohen et al. 1984, Ladizinsky et al. 1985).

Biochemical markers are powerful tools in the assessment of genetic variability within and between plant populations and in the elucidation of relationships between domesticated and wild forms of plants (Gottlieb 1981). DNA markers, e.g., nuclear restriction fragment length polymorphisms (RFLPs) have recently received much attention as a plant breeding tool (Soller and Beckmann 1983) and as an estimator of phylogenetic relationships (Song et al. 1988). Isozymes and RFLPs have

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several advantages over morphological markers, e.g., multiple allelic forms, codominance, and absence of pleiotropic effects on economically important traits (Beckmann and Soller 1983). RFLPs have the added advantage over some isozymes, e.g. peroxidases, of developmental stability. Surveys of variability for isozymes and morphological markers in lentil have demonstrated that *culinaris* shares a large number of alleles with *orientalis* and *odemensis*, the former one presumed to be the progenitor of cultivated lentil (Williams et al. 1974; Pinkas et al. 1985; Hoffman et al. 1986). Although isozyme variants often differed between *nigricans* and *culinaris*, very few variants were detected within *L. nigricans* (Hoffman et al. 1986). The question remained whether the low level of variability for isozyme variants observed in *nigricans* is due to an inability to detect differences at the enzyme level or whether little variability exists in the germplasm collection for some subspecies of lentil. The purpose of this research was to study variability between and within lentil subspecies to ascertain: (1) if RFLPs detect greater variability than previously reported for isozyme markers, (2) the species and subspecies relationships based on DNA markers, and (3) whether sufficient numbers of RFLPs can be detected between cultivars of domesticated lentil to generate a detailed genetic map.

Materials and methods

Six accessions of each of the five subspecies of domesticated and wild lentil were randomly chosen from the germplasm collection of the USDA Grain Legume Genetics and Physiology Unit at Washington State University (Table 1). The seed was from the first increase of seed collected in the wild and, therefore, has had little opportunity for selection. DNA was isolated from the above-ground parts of seedlings as previously described (Havey and Muehlbauer 1989). One of four restriction enzymes with a six-base-pair recognition sequence (BglII, EcoRI, EcoRV or HindIII) was randomly chosen for each probe used in evaluations for RFLPs. Digestion, electrophoresis and blotting of DNA were carried out as previously described (Havey and Muehlbauer 1989). Ten probes were randomly selected from previously identified relatively low-copy-number fragments from EcoRI (E1, E14 and E25) or PstI (P37, P46, P68, P79 and P95) partial genomic libraries or a cDNA (C6 and C41) library of lentil (Havey and Muehlbauer 1989). Probe-enzyme combinations were as follows: C6-HindIII, C41-EcoRI, E1-EcoRV, E14-EcoRI, E25-EcoRI, P37-BglII, P46-EcoRV, P68-HindIII, P79-BglII, and P95-HindIII. Procedures for labeling of probes with P^{32}, hybridization to Southern blots, and autoradiography have been described (Havey and Muehlbauer 1989).

Fragment lengths were estimated by a computer program interpolating from a standard curve from the HindIII digest of the bacteriophage λ (cubic spline fit algorithm of C. Buckler, NIH, Bethesda, MD, USA). The restriction fragment lengths were assumed to be a random sample of the variability present in the germplasm collection. Estimates of total gene diversity (H_t) across all subspecies, partitioned into components within (H_s) and between (D_{st}) subspecies, and average gene diversity within subspecies were calculated from the frequencies of fragment sizes (Nei 1987). Genetic identities and distances (Nei 1987) were also calculated for all accessions. A hierarchical cluster analysis was then performed and a dendrogram based on estimates of genetic distance was generated using the unweighted pair-group mean method by a computer program made available by Dr. K. Ritland, Department of Botany, University of Toronto, Toronto, Canada. Estimates of H_t, H_s, D_{st} and Nei's identities were also calculated from the isozyme data of Hoffman et al. (1986).

Table 1. Accessions of wild and domesticated lentil evaluated for variability for restriction fragment lengths

Lens subspecies[a]	Accession or cultivar	Origin
culinaris	Brewer	USA
	Dupuy	France
	Giza 9	Egypt
	Laird	Canada
	Precoz	Chile
	Redchief	USA
orientalis	Lo4	Uzbekistan, USSR
	Lo6	Tehran, Iran
	Lo56	Denizli, Turkey
	Lo66	Nemrut, Turkey
	Lo77	Tokat, Turkey
	Lo78	Corum, Turkey
odemensis	Ld19	Golan Heights, Israel
	Ld20	Golan Heights, Israel
	Ld51	Edremit, Turkey
	Ld61	Iskanderum, Turkey
	Ld80	Belem Pass, Turkey
	Ld84	Beydag, Turkey
	Ld94	Beydag, Turkey
ervoides	Le12	Yalta, USSR
	Le44	Risan, Yugoslavia
	Le54	Yechian, Israel
	Le87	Gaziantep, Turkey
	Le97	Boz Dag, Turkey
	Le101	Anamur, Turkey
nigricans	Ln13	Livorno, Italy
	Ln15	Fontaine de Vaucluse, France
	Ln18	El Escorial, Spain
	Ln26	Makarska, Yugoslavia
	Ln34	Yalta, USSR
	Ln93	Boz Dag, Turkey

[a] For description of subspecies, see Introduction

Results

Under high stringency (68 C) washes, we found very little evidence for variability in restriction fragment lengths within a single lentil accession (data not presented). Lentil is self-pollinating and wild lentil accessions collected in the field trace back to a few plants which are probably closely related. Evidence of duplication of genetic material was found in the *odemensis* accession Ld80, which showed more bands than the other accessions for five of

Table 2. Restriction fragment lengths detected by ten probes in five subspecies of domesticated and wild lentil

Accession[a]	Fragment lengths detected by probe[b]									
	C6	C41	E1	E14	E25	P37	P46	P68	P79	P95
Brewer	1.8/1.4	3.7/2.0	11.5	1.2	1.4	7.7	17.9/6.1	7.0	7.8	3.4
Dupuy	1.8/1.4	3.7/2.0	21.4	1.2	1.4	7.7	17.9/6.1	7.0	7.8	3.4
Giza 9	1.8/1.4	3.0/2.0	21.4	1.2	1.4	7.7	8.1/6.1	7.0	11.0	3.4
Laird	1.8/1.4	3.7/2.0	11.5	1.2	1.4	7.7	17.9/6.1	7.0	7.8	3.4
Precoz	1.8/1.4	3.7/2.0	11.5	1.2	1.4	7.7	17.9/6.1	7.0	5.1	3.4
Redchief	1.8/1.4	3.7/2.0	11.5	1.2	1.4	7.7	17.9/6.1	10.4	7.8	3.4
Lo4	1.8/1.4	3.3/2.0	17.0	1.2	1.4	7.7	17.9/6.1	4.4	5.1	3.4
Lo6	1.8/1.4	3.0/2.0	21.4	1.2	1.4	7.7	17.9/6.1	9.4	5.1	3.6
Lo56	1.8/1.4	3.0/2.0	21.4	1.2	1.4	7.7	17.9/6.1	7.0	7.8	3.4
Lo66	1.8/1.4	3.7/2.0	21.4	1.2	1.4	7.7	21.0/6.1	2.2	5.1	3.4
Lo77	1.8/1.4	3.7/2.0	21.4	1.2	1.4	7.7	17.9/6.1	2.2	7.8	3.4
Lo78	1.8/1.4	3.7/2.0	21.4	1.2	1.4	7.7	17.9/6.1	8.6	7.8	3.4
Ld19	1.8/1.4	3.0/2.0	–	–	–	–	15.4/6.1	–	–	–
Ld20	–	–	16.1	6.3	1.4	7.7	3.7	5.5	5.1	4.2
Ld51	1.8/1.4	3.0/2.0	18.4	6.3	1.4	7.7	15.4/6.1	5.5	5.1	3.4
Ld61	1.8/1.4	3.0/2.0	18.4	6.3	1.4	7.7	15.4/6.1	5.5	5.1	3.4
Ld80	1.8/1.4	5.1/3.0/2.0	18.4/11.5	6.3/6.9	1.4	7.7	17.9/6.1	7.9/6.0	15.7/5.1	3.4
Ld84	2.0/1.8/1.4	3.0/2.0	18.4	6.3	1.4	7.7	15.4/6.1	5.5	5.1	3.4
Ld94	1.8/1.4	3.0/2.0	18.4	6.3	1.4	7.7	–	5.5	5.1	3.4
Le12	2.1/1.4	2.0	11.5	4.7	1.4	7.7	17.9/6.1	3.5	5.1	3.4
Le44	2.1/1.4	2.0	8.3	4.7	1.4	7.7	17.9/6.1	3.5	5.1	3.4
Le54	2.1/1.4	2.0	11.5	4.7	1.4	7.7	17.9/6.1	3.5	5.1	3.4
Le87	2.1/1.4	2.0	17.0	4.7	1.4	2.8	17.9/6.1	3.5	5.1	3.4
Le97	2.1/1.4	2.0	11.5	4.7	1.4	7.7	17.9/6.1	3.5	5.1	3.4
Le101	2.1/1.4	2.0	17.0	4.7	1.4	7.7	17.9/6.1	3.5	5.1	3.4
Ln13	2.1/1.1	4.0/2.0	null	2.9	1.4	7.7	7.0/6.1	2.6	10.2	3.4
Ln15	1.8/1.1	4.0/2.0	null	2.9	1.4	7.7	7.0/6.1	2.6	6.0	3.4
Ln18	1.8/1.1	4.0/2.0	null	2.9	1.4	7.7	7.0/6.1	2.6	6.0	3.4
Ln26	2.1/1.1	4.0/2.0	null	2.9	1.4	7.7	7.0/6.1	2.6	6.0	3.4
Ln34	2.1/1.1	4.0/2.0	null	2.9	1.4	7.7	7.0/6.1	2.6	6.0	3.4
Ln93	2.1/1.1	4.0/2.0	null	2.9	1.4	7.7	7.0/6.1	2.6	10.2	3.4

[a] For origin of accessions, see Table 1

[b] See Materials and methods for description of probes. Lengths reported in kilobases

the ten probes (Table 2). Allelic frequencies for restriction fragment lengths were calculated from the six accessions of each subspecies (Table 2). Nei's estimate of total gene diversity (H_t) was 0.510, of which 0.169 was due to variability within lentil subspecies (H_s) and 0.341 between subspecies (D_{st}). These estimates of gene diversity reflect heterogeneity between true-breeding accessions and not heterozygosity at a locus. Estimates of average gene diversity within the subspecies were low, 0.180 for *culinaris*, 0.274 for *orientalis*, 0.271 for *odemensis*, 0.061 for *ervoides* and 0.089 for *nigricans*. Although tests of significance are not possible for sample sizes reported in this paper, *orientalis* and *odemensis* nevertheless showed the greatest variability for restriction fragment lengths. The extremely low values observed in *ervoides* and *nigricans* may reflect a narrow germplasm base for these subspecies. Estimates of Nei's identities demonstrated that, although little variability was observed within *ervoides* and *nigricans*, the fragment lengths were often different from those found in subspecies within the culinaris crossability group (Table 3). This result was also reflected in the dendrogram of the five subspecies (Fig. 1). Domesticated lentil (*culinaris*) possessed many restriction fragment lengths in common with *orientalis* and *odemensis* and was placed between the two wild species in the dendrogram. Fragment lengths observed in subspecies *ervoides* and *nigricans* (crossability group 2) were more different from the subspecies of crossability group 1 than from each other. These results support previous reports proposing *orientalis* as the progenitor of cultivated lentil (Williams et al. 1974; Pinkas et al. 1985; Hoffman et al. 1986).

Estimates of total gene diversity and components within and between the subspecies were approximately equal for restriction fragment length and isozyme markers (estimated from the data of Hoffman et al. 1986); however, restriction fragment lengths tended to detect greater variability than isozymes: $H_t = 0.521$ and 0.422; $H_s = 0.175$ and 0.141; and $D_{st} = 0.346$ and 0.282 for

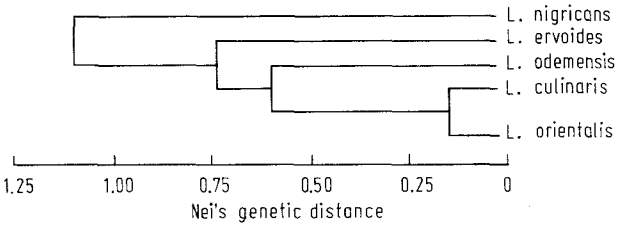

Fig. 1. Dendrogram of genetic distance (Nei 1987) between five subspecies of wild and domesticated lentil for restriction fragment lengths

Table 3. Estimates of Nei's identities between five subspecies of wild and domesticated lentil for restriction fragment lengths

Lentil sub-species[a]	Lentil subspecies				
	culinaris	*orientalis*	*odemensis*	*ervoides*	*nigricans*
culinaris	–	0.884	0.528	0.494	0.347
orientalis		–	0.598	0.511	0.348
odemensis			–	0.463	0.347
ervoides				–	0.324
nigricans					–

[a] For description of subspecies, see Introduction

RFLPs and isozymes, respectively. Subspecies *nigricans* was always the most distant from *culinaris* in this study, agreeing with the isozyme studies of Pinkas et al. (1985) and Hoffman et al. (1986).

Discussion

Isozymes and RFLPs are powerful tools in the assessment of genetic variability within a germplasm collection. Because of the narrow genetic base of the cultivated lentil in the USA, it is important to identify and collect wild lentils that are genetically divergent from the cultivated forms. This is especially important now that the habitat of some wild lentils is in danger of destruction (Solh and Erskine 1981). Estimates of average gene diversity within subspecies of wild lentil indicated that accessions of *orientalis* and *odemensis* possess the greatest variability for restriction fragment lengths, agreeing with data from morphological and isozyme markers (Williams et al. 1974; Pinkas et al. 1985; Hoffman et al. 1986). Assuming that the small sample sizes in this study are representative, the low average gene diversity observed in *ervoides* and *nigricans* may reflect a genetic bottleneck in the history of these two wild subspecies. This was surprising because of the wide geographic area represented by the accessions of *ervoides* and *nigricans* (Table 1). It is unlikely that accessions of *ervoides* and *nigricans* have undergone selection during increase and maintenance of seed, because the plants used in this study represent th first generation since collection.

Ladizinsky et al. (1983) observed that *nigricans* exis as small populations in disturbed habitats and propose that *nigricans* may have been independently dome ticated in southern Europe. The limited genetic variabil ty for isozymes and restriction fragment lengths amor accessions from a wide geographic area is consistent wit this observation. Although little variability was observe within *ervoides* and *nigricans*, the restriction fragmei lengths were often different from those of subspecies i crossability group 1 (Table 2, Fig. 1). These observatior agreed with isozyme results of Pinkas et al. (1985) an Hoffman et al. (1986). Flavell et al. (1986) have also r ported significant correlations between estimates of g netic diversity from allozyme and rDNA spacers. This in contrast to the discrepancies reported between ph logenies based on morphological characters and DN markers (Palmer et al. 1983; Sytsma and Schall 198 Sytsma and Gottlieb 1986).

There appears to be insufficient variability f RFLPs within the domesticated lentil to generate a d tailed genetic map. The estimate of average gene diversi within *culinaris* of 0.180 indicates that, on the averag one needs to evaluate approximately six probe-enzyn combinations to identify a single RFLP between tw cultivars. Therefore, it appears that crosses between *cu naris* and either *orientalis* or *odemensis* are necessary f identification of sufficient numbers of RFLPs for ma ping. However, problems may occur during mapping b cause translocations have been reported between *cu naris* and these wild subspecies (Ladizinsky et al. 198 and skewed allelic frequencies have been observed i interspecific lentil crosses (Zamir et al. 1986; Havey an Muehlbauer 1989).

References

Beckmann J, Soller M (1983) Restriction fragment length pol morphisms in genetic improvement: methodologies, ma ping and costs. Theor Appl Genet 67:35–43

Cohen D, Ladizinsky G, Meira Z, Muehlbauer F (1984) Resc of interspecific *Lens* hybrids by means of embryo cultur Plant Cell Tiss Org Cult 3:343–347

Flavell R, O'Dell M, Sharp P, Nevo E, Beiles A (1986) Variatio in the intergenic spacer of ribosomal DNA of wild whea *Triticum dicoccoides*, in Israel. Mol Biol Evol 3:547–558

Gottlieb L (1981) Electrophoretic evidence and plant popul tions. Prog Phytochem 7:1–46

Havey M, Muehlbauer F (1989) Linkages between restrictio fragment length, isozyme, and morphological markers i lentil. Theor Appl Genet 77:395–401

Hoffman D, Soltis D, Muehlbauer F, Ladizinsky G (1986) Is zyme polymorphism in *Lens* (Leguminosae). Syst B 11:392–402

Ladizinsky G (1979) Species relationships in the genus *Lens* indicated by seed-protein electrophoresis. Bot G 140:449–451

Ladizinsky G, Braun D, Muehlbauer F (1983) Evidence for domestication of *Lens nigricans* (M. Bieb.) Godron in S. Europe. Bot J Linn Soc 87:169–176

Ladizinsky G, Braun D, Goshen D, Muehlbauer F (1984) The biological species of the genus *Lens*. Bot Gaz 145:253–261

Ladizinsky G, Cohen D, Muehlbauer F (1985) Hybridization in the genus *Lens* by means of embryo culture. Theor Appl Genet 70:97–101

Muehlbauer F, Slinkard A (1981) Genetics and breeding methodology. In: Webb C, Hawtin G (eds) Lentils. Commonwealth Agricultural Bureaux, London, pp 69–90

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York, 512 pp

Palmer J, Shields C, Cohen D, Orton T (1983) Chloroplast DNA evolution and the origin of amphidiploid *Brassica* species. Theor Appl Genet 65:181–189

Pinkas R, Zamir D, Ladizinsky G (1985) Allozyme divergence and evolution in the genus *Lens*. Plant Syst Evol 151:131–150

Solh M, Erskine W (1981) Genetic resources. In: Webb C, Hawtin G (eds) Lentils. Commonwealth Agricultural Bureaux, London, pp 53–68

Soller M, Beckmann J (1983) Genetic polymorphism in varietal identification and genetic improvement. Theor Appl Genet 67:25–33

Song K, Osborn T, Williams P (1988) *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs). Theor Appl Genet 75:784–794

Sytsma K, Gottlieb L (1986) Chloroplast DNA evidence for the origin of the genus *Heterogaura* from a species of *Clarkia* (Onagraceae). Proc Natl Acad Sci USA 83:5554–5557

Sytsma K, Schaal B (1985) Phylogenetics of the *Lisianthius Skinneri* (Gentianaceae) species complex in Panama utilizing DNA restriction fragment analysis. Evolution 39:594–608

Williams J, Sanchez A, Jackson M (1974) Studies on lentils and their variation. I. The taxonomy of the species. SABRAO J 6:133–145

Zamir D, Tadmor V (1986) Unequal segregation of nuclear genes in plants. Bot Gaz 147:355–358