Theor Appl Genet (1996) 92:1091–1098 © Springer-Verlag 1996

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Genetic relationships in *Lens* species and parentage determination of their interspecific hybrids using RAPD markers

Received: 19 September 1995 / Accepted: 3 November 1995

Abstract Broadening of the genetic base and systematic exploitation of heterosis in cultivated lentils requires reliable information on genetic diversity in the germplasm. The ability of random amplified polymorphic DNA (RAPD) to distinguish among different taxa of *Lens* was evaluated for several geographically dispersed accessions/cultivars of four diploid *Lens* species. This study was carried out to assess whether RAPD data can provide additional evidence about the origin of the cultivated lentil and to measure genetic variability in lentil germplasm. Three cultivars of *Lens culinaris* ssp. *culinaris*, including one microsperma, and two macrosperma types, and four wild species (*L. culinaris* ssp. *orientalis*, *L. odemensis* and *L. nigricans*) were evaluated for genetic variability using a set of 1 11-mer and 14 random 10-mer primers. One hundred and fifty-eight reproducible and scorable DNA bands were observed from these primers. Genetic distances between each of the accessions were calculated from simple matching coefficients. Split decomposition analysis of the RAPD data allowed construction of an unrooted tree. This study revealed that (1) the level of intraspecific genetic variation in cultivated lentils is narrower than that in some wild species. (2) *L. culinaris* ssp. *orientalis* is the most likely candidate as a progenitor of the cultivated species, (3) *L. nigricans* accession W6 3222 (unknown) and *L. c.* ssp. *orientalis* W6 3244 (Turkey) can be reclassified as species of *L. odemensis* and (4) transmission of genetic material in *Lens* interspecific hybrids is genotypically specific, as identified by the RAPD markers in our study.

Key words *Lens culinaris* · Wild species · RAPD · Intra- and interspecific variation · Genetic distance · Split decomposition

Communicated by J. Mac Key

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Introduction

Lentil (*Lens culinaris* ssp. *culinaris*) is one of the oldest West Asian crops and is still of considerable economic importance in the Indian subcontinent, the Middle East, southern Europe, and eastern and northern Africa. Lentils are also grown in the New World, including New Zealand, although on a much smaller scale. Using data from crossability and cytological studies, Ladizinsky et al. (1984) applied the biological species concept to the genus *Lens* and concluded that there were only two species, *L. culinaris* and *L. nigricans*. *L. culinaris* contained the three subspecies *culinaris*, *odemensis* and *orientalis*, while *L. nigricans* consisted of two subspecies, *nigricans* and *ervoides*. Ladizinsky (1993) recently revised *Lens* taxonomy according to information derived mainly from studies in which isozyme markers were used (Pinkas et al. 1985; Hoffman et al. 1986) and nuclear DNA restriction fragment length polymorphisms (RFLPs) (Havey and Muehlbauer 1989). In his new proposal, all of the subspecies except *culinaris* and *orientalis* were elevated to species status; the two exceptions were retained as subspecies under *L. culinaris*. This new classification was not confirmed by a SDS-PAGE analysis (Ahmad et al. 1995a), which indicated that *L. odemensis* is a subspecies of *L. culinaris* and was more closely related to cultivated lentils than *L. c.* ssp. *orientalis* (Sammour 1994).

Patterns of genetic diversity have been studied in cultivated crop species using a variety of molecular, chemical and morphological traits. However, as indicated above, there is a potential that depends on the method different conclusions will be reached with regard to genetic relationships. It may be necessary to assess the utility of different methods to determine species relationships. This knowledge of relative genetic

distances among individuals or populations determined by various comparative techniques is useful in a breeding programme because it permits organization of the germplasm and provides far more efficient sampling of genotypes (Nienhuis et al. 1992) and because knowledge of genetic similarity between genotypes may facilitate the choice of individuals to cross in hybrid combinations by optimizing the expression of heterosis (Godshalk et al. 1990; Melchinger et al. 1990; Moll et al. 1965; Smith et al. 1990).

Detection and exploitation of naturally occurring DNA sequence polymorphisms represents one of the most significant recent developments in molecular biology. In the genus *Lens*, Havey and Muehlbauer (1989) analysed phylogenetic relationships using nuclear DNA RFLPs, Ahmad et al. (1995a) determined phylogenetic relationships on the basis of SDS-PAGE analysis, while chloroplast DNA (cpDNA) phylogeny was clarified by Muench et al. (1991) and Mayer and Soltis (1994). Their results established *L.c.* ssp. *orientalis* as a progenitor of the cultivated lentil; a conclusion supported by morphological and cytological studies (Ladizinsky 1979a; Ladizinsky et al. 1984), suggesting a high level of consistency between new and old methods. The development and application of DNA amplified by the polymerase chain reaction (PCR) using arbitrary primers has resulted in an alternate source of molecular markers for the detection of genomic DNA polymorphisms (randomly amplified polymorphic DNA, RAPD) (Welsh and McClelland 1990; Williams et al. 1990). The technical simplicity of the RAPD technique has facilitated its use in the analysis of phylogenetic relationships in several plant genera, e.g. *Hordeum* (Gonzalez and Ferrer 1993), *Allium* (Wilkie et al. 1993) and *Populus* (Castiglione et al. 1993). Furthermore, RAPD markers also detect genetic variation at the intraspecific level between closely related cultivars (Kresovich et al. 1992).

In the study present here, we have used RAPD markers to detect intra- and interspecific genetic variations in genus *Lens* and to establish the genetic relationships among *Lens* species and their interspecific hybrids. This is part of a larger programme that will analyse the same results using morphological and seed protein markers in order to assess the relative conclusions drawn from these assessment procedures.

Materials and methods

Plant material

Three commercially grown New Zealand bred cultivars (one microsperma and two macrisperma to achieve morphological diversity) of cultivated lentil (*L.c.* ssp. *culinaris*), and twelve different accessions of wild species (*L.c.* ssp. *orientalis*, *L. odemensis*, *L. nigricans*, *L. ervoides*) (Table 1) and their interspecific hybrids (Ahmad et al. 1995a) (Table 2) were used.

Genomic DNA extraction and preparation for RAPD analyses

Lentil DNA was isolated using the procedure reported by Appels and Lagudah (1990). Fresh leaf tissue (1.0 g) devoid of necrotic region was ground to a fine powder in liquid nitrogen; mixed with 4.6 ml of extraction buffer [0.05 *M* Tris-HCl (pH 8.0), 0.3 *M* EDTA], 60 ul proteinase K (10 mg/ml) and 300 ul of 10% SDS; and incubated at 37 °C for 1 h. Sodium perchlorate (1 g) was added to percipitate proteins and the samples were centrifuged at 8000 rpm for 5 minutes. Two volumes of the ethanol perchlorate were added to the supernatant to precipitate the DNA. The DNA was resuspended in 500 ul of TE buffer [10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA] for 2–3 h at 4 °C. Four hundred milliliters of phenol/chloroform [phenol: chloroform: isoamy alcohol (25:24:1, v/v), Gibco BRL Cat. No. 15593-031] was added to the samples, and these were vortexed and left on a shaker for at least one h. Samples were centrifuged at 10 000 rpm for 3 min, and DNA was recovered from the aqueous phase by adding 50 ul 2 *M* sodium acetate (pH 5.2) and 950 ul absolute ethanol (stored at 4 °C). The DNA pellet was cleaned with 70% ethanol (stored at 4 °C) and resuspended in 250 ul TE buffer. Twenty microliters of RNAse A (Sigma, USA; 10 ug/ml) was added to the samples, which were then incubated (37 °C) for 30 min. The DNA was purified

Table 1 Cultivated and wild *Lens* accessions used in RAPD studies

Number	Species	Subspecies	Type [a]	Cultivar/accession	Source [b,c]
1	*Lens culinaris*	*orientalis* [e]		W6 3244	Turkey [b]
2	*Lens nigricans*			W6 3208	Italy [b]
3	*Lens nigricans* [d]			W6 3222	Unknown [b]
4	*Lens nigricans*			W6 3210	Yugoslavia (former) [b]
5	*Lens nigricans*			W6 3218	Spain [b]
6	*Lens nigricans*			W6 3221	Russian Federation [b]
7	*Lens ervoides*			W6 3173	Russian Federation [b]
8	*Lens ervoides*			W6 3176	Yugoslavia (former) [b]
9	*Lens ervoides*			W6 3192	Turkey [b]
10	*Lens culinaris*	*orientalis*		W6 3241	Turkey [b]
11	*Lens culinaris*	*orientalis*		W6 3261	Turkey [b]
12	*Lens culinaris*	*orientalis*		W6 3248	Turkey [b]
13	*Lens culinaris*	*culinaris*	Microsperma	Titore	Rakaia, NZ [c]
14	*Lens culinaris*	*culinaris*	Macrosperma	Invincible	Rakaia, NZ [c]
15	*Lens culinaris*	*culinaris*	Macrosperma	Olympic	Rakaia, NZ [c]

[a] Microsperma: small-seeded type; macrosperma: large-seeded type
[b] Western Regional Plant Introduction Station, Washington, USA
[c] Whenuapai Farm, Rakaia, South Island, New Zealand.
[d] Reclassified as *Lens odemensis* by Western Regional Plant Introduction Station (WRPIS), Washington, USA and by Plant Science Department, Lincoln University, New Zealand
[e] Reclassified as *Lens odemensis* by Plant Science Department, Lincoln University, New Zealand

Table 2 *Lens* interspecific hybrids used in this research

A	*L. c.* ssp. *culinaris* cv invincible × *L. c.* ssp. *orientalis* W6 3241
B	*L. c.* ssp. *culinaris* cv invincible × *L. c.* ssp. *orientalis* W6 3261
C	*L. c.* ssp. *culinaris* cv titore × *L. odemensis* W6 3222
D	*L. c.* ssp. *culinaris* cv olympic × *L. c.* ssp. *orientalis* W6 3261
E	*L. c.* ssp. *culinaris* cv titore × *L. c.* ssp. *orientalis* W6 3261
F	*L. c.* ssp. *culinaris* cv titore × *L. ervoides* W6 3192
H	*L. c.* ssp. *culinaris* cv olympic × *L. ervoides.* W6 3192

by phenol/chloroform extraction, and the final DNA pellet was dissolved in 250 ul TE buffer. DNA was estimated from the A_{260} and diluted to 25 ng/ul for PCR. The working stock was heated in a water-bath at 95 °C for 5 min and then frozen at −20 °C until required.

RAPD, PCR

Concentrations of the Mg^{2+} (1.5 and 2.5 m*M*), *Taq* polymerase (1.0 and 2.0 U) and DNA (25, 50 and 100 ng) and the number of cycles required (35 and 42) were optimized in pilot assays. The final assay conditions were 25-ul reactions consisting of 100 u*M* each dNTP (Boehringer Mannheim, Germany), 10 u*M* primer (Table 3), 1.0 unit *Taq* polymerase (Boehringer Mannheim, Germany), 100 u*M* *Taq* buffer [50 m*M* KCL, 10 m*M* Tris-HCl (pH 9.0), 0.1% Triton X-100], 2.5 m*M* $MgCl_2$ and 25 ng genomic DNA. Reaction mixtures were overlaid with 20 ul of mineral oil and incubated in a thermal cycler (Autogene II, Grant, USA). Thermal cycle parameters were an initial denaturation period of 30 s at 95 °C, then 42 cycles of 60 s at 93.5 °C denaturation, 60 s at 37 °C annealing, 30 s at 72 °C extension, followed by a final extension of 7 min. The amplification products were electrophoresed in 1.8% agarose (type 1-A: low EEO, Sigma A-0169) gels and detected by staining with ethidium bromide.

Data analysis

RAPD assays were performed in duplicate, and only those bands obtained twice very clearly were scored. Pair-wise comparisons of accessions, based on the presence and absence of bands, were used to generate simple matching coefficients (S_{sm}). The genetic distance (*d*) was calculated applying GENSTAT Computer package (GENSTAT 1989) as

$$d = \text{square root } (1 - S_{sm})$$

The distances were used in a split decomposition analysis to construct unrooted trees (Bandelt and Dress 1992). The index proposed by Nei and Li (1979) was used to calculate the similarities, S_{ij} between the species/accessions *i* and *j*:

$$S_{ij} = \frac{2N_{ij}}{(N_i + N_j)}$$

where N_{ij} = the number of bands in common between species/accessions *i* and *j* and N_i and N_j are the number of bands for species/accessions *i* and *j*, respectively.

Table 3 Sequences of oligonucleotides used as primers in RAPD assays

Primer [a,b]	Mer	5'-3' Sequence
OPA 02	10	TAG CGA GCT G
OPB 08	10	GTC CAC ACG G
OPB 11	10	GTA GAC CCG T
OP 01	10	CCT GTA GTG G
OP 02	10	TAC CTT CCG T
OP 03	10	GTC CGT TGG G
OP 04	10	GTT AGG TCG T
OP 05	10	TCT CTG TCC C
OP 06	10	TCG CCC CAT T
OP 07	10	CGT GGT TCC C
OP 08	10	GTC CCG TTA C
OP 09	10	ACG CCC TAG T
OP 10	10	TTT CAC ATG G
OP 11	10	CTG TGC TGT G
OP 12	11	TGG TGG ATG TA

[a] Primers were obtained from Operon Technologies, USA. The first three are contained in standard kits. OP01–OP12 were custom-made based on the sequence of Abbo et al. (1992)
[b] All the primers were reconstituted to a 20 m*M* working concentration in sterilized deionized water and stored at −20 °C

Results

Means and maximum values of interspecific variation are shown in Table 4. The mean genetic distance ranged from 0.51 for the closely related species (*L. c.* ssp. *culinaris*:*L. c.* ssp. *orientalis*) to 0.68 for the distantly related species (*L. c.* ssp. *culinaris*:*L. ervoides*; *L. ervoides*: *L. nigricans*). Means and maximum values of genetic distances within *Lens* species as revealed by RAPD analysis are shown in Table 5. In comparison with the intraspecific genetic variation of cultivated lentil (*L. c.* ssp. *culinaris*: 0.44), the wild lentils, *L. odemensis* and *L. nigricans* showed a wide range of intraspecific variation. In contrast, *L. c.* ssp. *orientalis* and *L. ervoides* (both wild lentils), with maximum genetic distances of 0.55 and 0.45, respectively, exhibited a low level of intraspecific variation.

Genetic relationships of *Lens* species and their F_1 interspecific hybrids based on a similarity matrix of the RAPDs

A genetic similarity matrix of 15 accessions of the *Lens* species based on 158 RAPD bands is presented in Table 6. Genetic similarities ranged from 50% for *L. ervoides* W6 3176:*L. nigricans* W6 3210 and *L.c.* ssp. *orientalis* W6 3261:*L. ervoides* W6 3173 to 96.8% for *L. nigricans* W6 3221:*L. nigricans* W6 3218. Minimum genetic similarities within species were 89.2% for *L. odemensis*, 82.3% for *L. nigricans*, 79.7% for *L. ervoides*, 70.3% for *L.c.* ssp. *orientalis* and 81.0% for *L.c.* ssp. *culinaris*. The most closely related accessions were found in *L. nigricans*, and this same species also showed a wide range of genetic variation among different accessions.

A genetic similarity matrix for 2 *Lens* interspecific hybrids and their respective parents based on 164 RAPD bands is presented in Table 7. In the 'Titore' × W6 3222 F_1 interspecific hybrid, the hybrid was 71.3% and 72.0% genetically similar to the female and male parents, respectively. In the 'Olympic' × W6 3261 cross, the F_1 progeny was 73.8% similar to the female parent

Table 4 Means (above the diagonal) and ranges (below the diagonal) of genetic distance between *Lens* species based on 158 RAPD bands

Species/subspecies	*culinaris*	*orientalis*	*odemensis*	*nigricans*	*ervoides*
culinaris	—	0.51	0.63	0.65	0.68
orientalis	0.46–0.57	—	0.61	0.63	0.66
odemensis	0.60–0.65	0.53–0.67	—	0.52	0.65
nigricans	0.63–0.68	0.58–0.68	0.36–0.61	—	0.68
ervoides	0.67–0.70	0.62–0.71	0.63–0.69	0.64–0.71	—

Table 5 Genetic distance within *Lens* species based on 158 RAPD bands

Species	Subspecies	Mean	Range
L. culinaris	*culinaris*	0.41	0.37–0.44
	orientalis	0.49	0.44–0.55
L. odemensis		0.51	0.33–0.61
L. ervoides		0.40	0.36–0.45
L. nigricans		0.33	0.18–0.42

Table 7 Similarity matrix (%) of *Lens* interspecific crosses based on 164 RAPD bands

		1	2	3	4	5	6
L. culinaris cv Titore	1	100					
Titore × W6 3222	2	71.3	100				
L. odemensis W6 3222	3	48.2	72.0	100			
L. culinaris cv Olympic	4	68.9	56.1	46.3	100		
Olympic × W6 3261	5	65.9	62.8	43.3	73.8	100	
L. culinaris W6 3261 ssp. *orientalis*	6	59.1	46.3	41.5	59.8	68.9	100

Table 8 Similarity matrix (%) of *Lens* interspecific crosses based on 138 RAPD bands

		1	2	3	4	5	6
L. culinaris W6 3241 ssp. *orientalis*	1	100					
Invincible × W6 3241	2	73.2	100				
L. culinaris cv Invincible	3	62.3	74.6	100			
L. culinaris W6 3261 ssp. *orientalis*	4	63.8	57.2	63.8	100		
Invincible × W6 3261	5	50.7	65.9	65.2	78.3	100	
L. culinaris cv Invincible	6	62.3	74.6	100	63.8	65.2	100

68.9% to the male parent. The 2 interspecific hybrids were 62.8% genetically similar to each other, even without any mutual parents. The hybrid between the 'Invincible' × W6 3241 cross was 73.2% and 74.6% genetically similar to its male and female parents, respectively, based on 138 RAPD bands, as shown in Table 8. The hybrid of the 'Invincible' × W6 3261 cross was 78.3% and 65.2% genetically similar to its male and female parents, respectively. Both hybrids have the same female parent ('Invincible') and shared 65.2% genetic similarity.

The hybrid of the 'Titore' × W6 3261 cross was 85.5% and 74.6% genetically similar to its male and female parents, respectively, as presented in Table 9. The hybrid of the 'Titore' × W6 3192 cross was 51.4% similar to its male parent and 72.3% to its female parent. These 2 hybrids have a common female parent, 'Titore', and were 87.3% genetically similar to each other. The hybrid of the 'Olympic' × W6 3192 cross showed a genetic relationship of 47.4% to its male parent and

Table 6 Similarity matrix (%) of *Lens* species based on 158 RAPD bands

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
L. odemensis	W6 3244	1	100														
L. nigricans	W6 3208	2	62.7	100													
L. odemensis	W6 3222	3	89.2	65.8	100												
L. nigricans	W6 3210	4	63.3	86.7	70.3	100											
L. nigricans	W6 3218	5	63.6	82.9	66.5	82.3	100										
L. nigricans	W6 3221	6	62.7	83.5	65.8	84.2	96.8	100									
L. ervoides	W6 3173	7	58.2	52.5	58.9	50.6	54.4	55.1	100								
L. ervoides	W6 3176	8	57.6	53.2	57.0	50.0	53.8	54.4	84.2	100							
L. ervoides	W6 3192	9	60.1	57.0	60.8	58.9	53.8	57.0	86.7	79.7	100						
L. culinaris ssp. *orientalis*	W6 3241	10	68.4	63.9	71.5	65.8	59.5	61.4	58.2	61.4	60.1	100					
L. culinaris ssp. *orientalis*	W6 3261	11	56.3	55.7	59.5	58.9	53.8	57.0	50.0	53.2	51.9	80.4	100				
L. culinaris ssp. *orientalis*	W6 3248	12	59.5	65.2	60.1	64.6	57.0	58.9	58.2	56.3	58.9	75.9	70.3	100			
L. culinaris ssp. *culinaris*	Titore	13	60.8	58.9	62.7	58.2	57.0	57.6	55.7	53.8	55.1	78.5	76.6	72.2	100		
L. culinaris ssp. *culinaris*	Invincible	14	60.8	60.1	63.9	60.8	57.0	57.6	54.4	51.3	55.1	75.9	71.5	70.9	82.3	100	
L. culinaris ssp. *culinaris*	Olympic	15	59.5	57.6	62.7	59.5	53.2	53.8	50.6	52.5	51.3	74.7	67.7	73.4	81.0	86.1	100

Table 9 Similarity matrix (%) of *Lens* interspecific crosses based on 173 RAPD bands

L. culinaris W6 3261 ssp. *orientalis*	1	100								
Titore × W6 3261	2	85.5	100							
L. culinaris cv Titore	3	68.2	74.6	100						
L. ervoides W6 3192	4	45.1	50.3	46.8	100					
Titore × W6 3192	5	82.1	87.3	72.3	51.4	100				
L. culinaris cv Titore	6	68.2	74.6	100	46.8	72.3	100			
L. ervoides W6 3192	7	45.1	50.3	46.8	100	51.4	46.8	100		
Olympic × W6 3192	8	67.6	72.8	64.7	47.4	76.3	64.7	47.4	100	
L. culinaris cv Olympic	9	61.8	65.9	75.1	43.9	67.1	75.1	43.9	74.6	100
		1	2	3	4	5	6	7	8	9

74.6% to its female parent, as shown in Table 9. These last 2 hybrids share a mutual wild male parent and showed 76.3% genetic similarity to each other.

Genetic relationships of *Lens* species and their F_1 interspecific hybrids based on a Split Decomposition Tree constructed by genetic distances

Split decomposition analyses of the genetic distance values were performed to generate an unrooted tree showing genetic relationships within and between different *Lens* species (Fig. 1). In Fig. 1, the location of bifurcations separating different accessions is a measure of the genetic distance between them. Three commercial cultivars of *L. c.* ssp. *culinaris* (13,14,15) and three wild accessions of *L. c.* ssp. *orientalis* (10,11,12) clustered in a single large group, suggesting that ssp. *orientalis* is the closest genetically to cultivated lentil. In the cultivated lentil, macrosperma types ['Invincible' (14) and 'Olympic' (15)] joined together before clustering with the microsperma type ['Titore' (13)]. The three accessions of *L. c.* ssp. *orientalis* (10,11,12) were closely related to *L. c.* ssp. *culinaris*, an indication that *L. c.* ssp. *orientalis* is a subspecies of *L. culinaris*. *L. ervoides* accessions (7,8,9) clustered together with a long branch before joining the cultivated lentil cluster, indicating its distinctiveness from cultivated lentil. The accession of *L. nigricans* (2,4,5,6) formed a monophyletic group.

A accession of *L. odemensis*, W6 3222 (3), (previously described as *L. nigricans*) clustered with an accession of *L. c.* ssp. *orientalis*, W6 3244 (1), and formed an independent group.

Split decomposition analysis of the genetic distance values was performed to construct an unrooted tree by which to indicate genetic relationships between parents and their F_1 progenies (Fig. 2). The hybrids (A) between the 'Invincible' (14) and W6 3241 (10) parents shared 50% genetic material with each parent and was genetically placed between them. In contrast, biased similarities were observed for the hybrid (B) between 'Invincible' (14) W6 3261 (11), which showed more genetic similarity based on RAPD analysis to its wild male parent, W6 3261, than to its female parent, *L. c.* ssp. *culinaris* cv 'Invincible'. Both these interspecific hybrids have the same female parent in common, but two different accessions of *L. c.* ssp. *orientalis* were used as the male parent. The hybrids (A, B) showed different genetic relationships to their respective parents. This also indicated, by the net formation, that some DNA exchange had occurred between parents and hybrids, a result which is obviously required and confirms that A and B were in fact true hybrids. Other interspecific hybrids (with different species cross-combinations) appeared to be more closely related to the female parent based on RAPD analysis; for example the hybrid (C) of 'Titore' (13) and *L. odemen-*

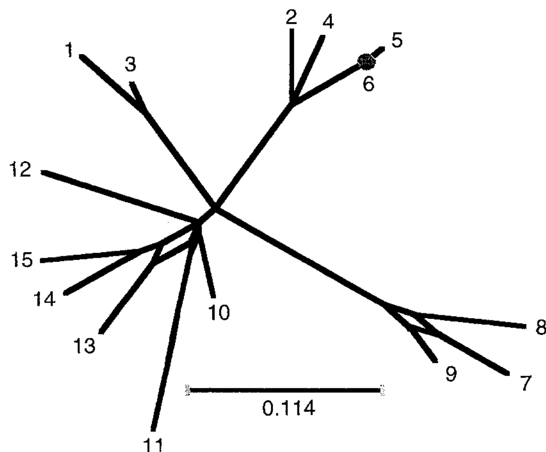

Fig. 1 Tree based on genetic distances demonstrating the clustering of parental lines. For taxon *numbers* refer to Table 1

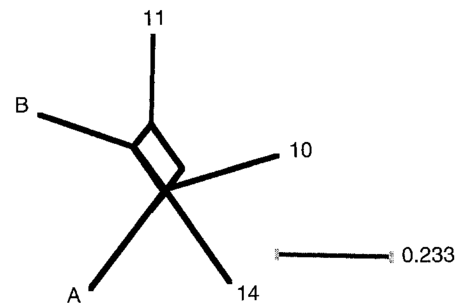

Fig. 2 Tree based on genetic distances demonstrating the clustering of parents and their hybrids. For taxon *numbers* refer to Tables 1 and 2

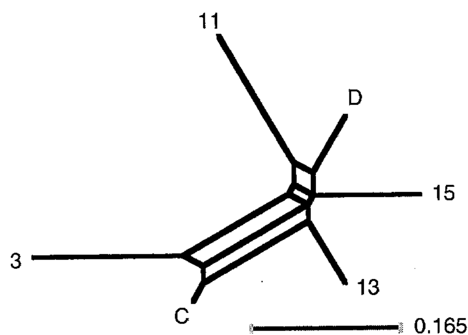

Fig. 3 Tree based on genetic distances demonstrating the clustering of parents and their hybrids. For taxon *numbers* refer to Tables 1 and 2

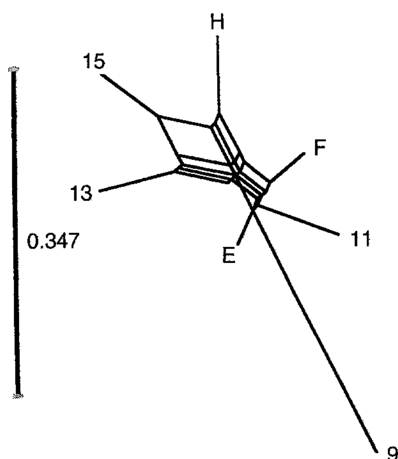

Fig. 4 Tree based on genetic distances demonstrating the clustering of parents and their hybrids. For taxon *numbers* refer to Tables 1 and 2

sis W6 3222 (3). However, when a *L. c.* ssp. *orientalis* W6 3261 (11) male was crossed with an 'Olympic' (15) female (Fig. 3), the hybrid (D) was genetically midway between the both parents. Figure 3 also indicates, by the net formation, that the expected DNA exchange between parents and hybrids had taken place. The same trend was observed for the hybrid (F) of 'Olympic' (15) × W6 3192 (9) where *L. ervoides* was used as the male parent (Fig. 4), and also when this male was crossed with 'Titore' (13), the resultant F_1 (H) showed the same genetic trend of a closer relationship to the male parent. On the basis of RAPD analysis the hybrid (E) of 'Titore' (13) × W6 3261 (11) showed a greater genetic similarity to its male parent (11).

Discussion

The main objective of this study was to develop RAPD markers for measuring hybrid, intra- and interspecific genetic variation and relationships in the genus *Lens* that could be used for comparison with the same data obtained by other methods (Ahmad et al. 1995a). Together these data would estimate the utility of RAPDs as a tool for determining genetic relationships within *Lens*. RAPD markers have been highly valuable tools for measuring intraspecific variation in a range of plant species such as *Ribes nigrum* L. (Lanhan et al. 1995), *Brassica oleracea* (Kresovich et al. 1992), *Lycopersicon esculentum* (Williams and Clair 1993), *Brassica juncea* (Jain et al. 1994) and the genus *Lens* (Abo-elwafa et al. 1995). Our research within genus *Lens* supports this. The within-species variation was determined on relatively few accessions and is therefore only approximate. However, some impressions can still be presented provided they are interpreted with caution. Compared with the wild species (except for *L. c.* ssp. *orientalis* and *L. ervoides*), there was a relatively low level of intraspecific variation in cultivated lentil (Ahmad et al. 1995c). This species may therefore have passed through a genetic bottleneck during its domenstication and evolution. Data from a much larger sample of *L. c.* ssp. *culinaris* accessions tested with RAPDs is consistent with this conclusion (Ford et al. 1995). The cluster net formation at joining points of *L. c.* ssp. *culinaris* and ssp. *orientalis* suggested that some degree of natural cross pollination has occured between both these subspecies during the process of evolution and crop domestication.

The low level of intraspecific variation postulated in *L. c.* ssp. *culinaris* is supported by previous studies using cpDNA analysis, where 112 out of 114 cultivated accessions produced identical RFLP patterns (Mayer and Soltis 1994). The low values of genetic distances observed in *L. c.* ssp. *orientalis* and *L. ervoides* indicates that these species (or at least available collections) may also have a narrow genetic base. These results are in agreement with those of Abo-elwafa et al. (1995) who found a low range of genetic variability in four accessions of *L. ervoides*. Measurement of RAPD markers enabled us to construct an unrooted tree showing intra- and interspecific genetic relationships in *Lens* species. The data for between-species relationships based on a larger number of accessions are therefore more reliable. These trees indicated that *L. c.* ssp. *orientalis* is the species most closely related to the cultivated lentil (*L. c.* ssp. *culinaris*); for example, W6 3248 (12) clustered near the cultivated lentils. These findings clearly suggest that *L. c.* ssp. *orientalis* is a candidate for being the progenitor of *L. c.* ssp. *culinaris*, a conclusion also reached in previous studies using other techniques; morphological, cytological (Ladizinsky 1979a; Ladizinsky et al. 1984), isozyme markers (Pinkas et al. 1985; Hoffman et al. 1986), RFLPs of nuclear DNA (Havey and Muehlbauer 1989), cpDNA (Muench et al. 1991; Mayer and Soltis 1994), RAPD markers (Abo-elwafa et al. 1995), SDS-PAGE analysis (Ahmad et al. 1995a,c) and crossability trends (Ahmad et al. 1995b).

Interspecific hybrids that shared cv 'Titore' as female parent were shown by our analysis of RAPD bands to be more closely related to the female than to the male parent. When cv 'Invincible' was used as the female

parent, this asymmetric form of relationship was not as apparent. Conversely, hybrids which shared *L. ervoides* W6 3192 as the male parent appeared to be more closely related to the male parent than hybrids which had 'Invincible' as their female parent. The results obtained from similarity matrix and phylogenetic analyses enabled us to conclude that: (1) interspecific hybrids which share 'Olympic' as a common female parent are, based on RAPD band presence, more similar to 'Olympic' than to the wild male parents (*L. ervoides*, *L. c.* ssp. *orientalis*); (2) *L. ervoides* W6 3192 when used as male parent, transmits fewer RAPD bands to its F_1 progeny than the female parent [*L. c.* ssp. *culinaris*, macrosperma or microsperma types ('Olympic', 'Titore')]; (3) female 'Titore' when crossed with different wild species (W6 3222, W6 3261 and W6 3192) contribute fewer RAPD bands to its progeny; (4) female 'Invincible' when crossed with different accessions of the same wild species (*L. c.* ssp. *orientalis* W6 3241, W6 3261) did not contribute the same RAPD bands to its progenies; (5) and male *L. c.* ssp. *orientalis* W6 3261 when crossed with different females ('Invincible' and 'Titore') contributed more RAPD bands to its F_1 hybrids, although they transmitted fewer RAPD bands when crossed with 'Olympic'. Theoretically, it would be expected that the F_1 hybrid would inherit all of the RAPD bands present in both parents. Thus, the observed genetical relationship would put the hybrid closer to the parent that contained the larger number of RAPD bands. However, this was not always the case with F_1 hybrids when inheriting the absence of a band. This unexpected inheritance of RAPD bands by F_1 hybrids supported the concept of genotypic specification of genetic transmission. The genetic relationship trends of parents to their hybrids might have been influenced by chromosomal rearrangements in the interspecific hybrids; the amount of DNA transmitted from parents to F_1 progeny or interference between RAPD bands in their amplification. For example, the introduction of a RAPD band that perfectly matches both end primers might starve a less-perfect match of dNTPs and prevent its amplification. These findings confirm previous results of Rajora and Mahon (1994, 1995) where different patterns of mitochondrial DNA (mtDNA) and chloroplast DNA inheritance were found. However, at present there is no adequate explanation of the differences in RAPD band transmissions to F_1 progeny.

The cluster pattern of cultivated lentil cultivars indicates that genetic variation within this species is very narrow, which may necessitate the use of novel techniques/procedures for the creation of genetic variability in *L. c.* ssp. *culinaris*. The cluster pattern also revealed that the previously described *L. nigricans* W6 3222 and *L. c.* ssp. *orientalis* W6 3244 are both *L. odemensis* accessions. The reclassification of W6 3222 is consistent with a new classification put out by the WRPIS, Washington. While our study indicates some genetic differences between microsperma and macrosperma types (Barulina 1930), the small sample size precludes any convincing separation. This disagrees with the studies of Williams et al. (1974), Ladizinsky (1979b) and Ahmad et al. (1995) who using morphological and seed protein comparisons, found no biological basis for separating small- and large-seeded types into subspecies. Our results also support the new reclassification of *Lens* species proposed by Ladizinsky (1993) in which all subspecies are elevated to species status except for *L. c.* ssp. *culinaris* and *L. c.* ssp. *orientalis*, which are retained as subspecies under *culinaris*. The new proposed genus *Lens* taxonomy now consists of *L. odemensis*, *L. ervoides*, *L. nigricans*, *L. c.* ssp. *culinaris* (cultivated lentil) and *L. c.* ssp. *orientalis*.

Acknowledgements The authors gratefully acknowledge the statistical assistance provided by Dr. R. Sedcole during the course of this work. This work was funded by Lincoln University Plant Science Project No. 90107, New Zealand.

References

Abbo S, Ladizinsky G, Weeden NF (1992) Genetic analysis and linkage studies of seed weight in lentil. Euphytica 58:259–266

Abo-elwafa A, Murai K, Shimada T (1995) Intra- and inter-specific variation in *Lens* revealed by RAPD markers. Theor Appl Genet 90:335–340

Ahmad M, Fautrier AG, Burritt DJ (1995a) Genetic diversity and relationships in *Lens* species and their interspecific hybrids as determined by SDS-PAGE. Plant Breed (in press)

Ahmad M, Fautrier AG, McNeil DL, Burritt DJ, Hill GD (1995b) Attempts to overcome postfertilization barrier in interspecific hybrids of the genus *Lens*. Plant Breed 114: 558–560

Ahmad M, McNeil DL, Fautrier AG, Burritt DJ (1995c) Use of RAPD's and seed proteins for determining relationships between lentil species. In: Kriedemann P (ed) Proc 35th Annu Gen Meet NZ Soc Plant Physiol 17th Annu Gen Meet. Australian Society of Plant Physiologists Inc. Publishers, Sydney p 19

Appels R, Lagudah ES (1990) Notes for molecular cytogenetics/RFLP course. CSIRO, Australia

Bandelt HJ, Dress AWM (1992) Split decomposition: a new and useful approach to phylogenetic analysis of distance data. Mol Phylogenet Evol 1:242–252

Barulina H (1930) Lentils of the USSR and of other countries. Bull Appl Bot Plant Breed [Suppl]40:1–319

Castiglione S, Wang G, Damiani G, Bandi C, Bisoffi S, Sala F (1993) RAPD fingerprintts for identification and for taxonomic studies of elite poplar (*Populus* ssp.) clones. Theor Appl Genet 87:54–59

Ford R, Pang E, Taylor P (1995) Developments of molecular markers for the genetic improvement of lentiles. In: Kriedemann P (ed) Proc 35th Annu Gen Meet NZ Soc Plant Physiol 17th Annu Gen Meet. Australian Society of Plant Physiologists Inc. Publishers, Sydney p 18

GENSTAT (1989) GENSTAT 5 reference manual. Oxford University Press, Oxford

Godshalk EB, Lee M, Lampkey KR (1990) Relationship of restriction fragment length polymorphisms to single-cross hybrid performance in maize. Theor Appl Genet 80:273–280

Gonzalez JM, Ferrer E (1993) Random amplified polymorphic DNA analysis in *Hordeum* species. Genome 86:497–504

Havey MJ, Muehlbauer FJ (1989) Variability for restriction fragment lengths and phylogenesis in lentil. Theor Appl Genet 77:839–843

Hoffman DL, Solits DE, Muehlbauer FJ, Ladizinsky G (1986) Isozyme polymorphism in *Lens* (Leguminosae). Syst Bot 11: 392–402

Jain A, Bhatia S, Banga SS, Prakash S, Lakshmikumaran M (1994) Potential use of random amplified polymorphic DNA (RAPD)

technique to study the genetic diversity in Indian mustard (*Brassica juncea*) and its relationship to heterosis. Theor Appl Genet 88:116–122

Kresovich S, Williams JGK, McFerson JR, Routman EJ, Schael BA (1992) Characterization of genetic identities and relationships of *Brassica oleracea* L. via a random amplified polymorphic DNA assay. Theor Appl Genet 85:190–196

Ladizinsky G (1979a) The origin of lentil and its wild genepool. Euphytica 28:179–187

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

Ladizinsky G (1993) Wild lentil. Crit Rev Plant Sci 12:169–184

Landizinsky G, Braun D, Goshen D, Muelbauer FJ (1984) The biological species of the genus *Lens* L. Bot Gaz 145:253–261

Lanham PG, Brennan RM, Hackett C, McNeil RJ (1995) RAPD fingerprinting of blackcurrent (*Ribes nigrum* L.) cultivars. Theor Appl Genet 90:166–172

Mayer MS, Soltis PS (1994) Chloroplast DNA phylogeny of *Lens* (Leguminosae): origin and diversity of the cultivated lentil. Theor Appl Genet 87:773–781

Melchinger AE, Lee M, Lamkey KR, Hallauer AR, Woodman WL (1990) Genetic diversity for restriction fragment length polymorphisms: relation to estimated genetic effects in maize inbres. Crop Sci 30:1033–1040

Moll RH, Lonnquist JH, Fortuna JV, Johnson CE (1965) The relationship of heterosis and genetic divergence in maize. Genetics 52:139–144

Muench DG, Slinkard AE, Scoles GJ (1991) Determination of genetic variation and taxonomy in lentil (*Lens* Miller) species by chloroplast DNA polymorphism. Euphytica 56:213–218

Nei M, Li W (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5256–5273

Nienhuis J, Slocum MK, Devos DA, Muren R (1992) Genetic similarity among *Brassica oleracea* genotypes as measured by restriction fragment length polymorphisms. J Am Soc Hortic Sci 118: 298–303

Pinkas R, Zamir D, Ladizinsky G (1985) Allozyme divergence and evolution in the genus *Lens*. Plant Syst Evol 151:131–140

Rajora OP, Mahon JD (1994) Inheritance of mitochondrial DNA in lentil (*Lens culinaris* Medik.). Theor Appl Genet 89:206–210

Rajora OP, Mahon JD (1995) Paternal plastid DNA can be inherited in lentil. Theor Appl Genet 90:607–610

Sammour RH (1994) Species relationships in genus *Lens* as indicated by elecrophoresis – a reappraisal. Lens Newsl 21:1–4

Smith OS, Smith JSC, Bowen SL, Tenborg RA, Wall SJ (1990) Similarities among a group of elite maize inbreds as measured by pedigree, F_1 grain yield, grain yield, heterosis, and RFLPs. Theor Appl Genet 80:833–840

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18:7213–7218

Wilkie SE, Isaac PG, Slater RJ (1993) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. Theor Appl Genet 86:497–504

Williams CE, Clair DAS (1993) Phenetic relationships and levels of variability detected by restriction fragment length polymorphisms and random amplified polymorphic DNA analysis of cultivated and wild accessions of *Lycopersicon esculentum*. Genome 36:619–630

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Williams JT, Sanchez AMC, Jackson MT (1974) Studies on lentils and their variation.1. The taxonomy of the species. SABRAO J 6: 133–145