Theor Appl Genet (1996) 93:751–758

S. K. Sharma · M. R. Knox · T. H. N. Ellis

AFLP analysis of the diversity and phylogeny of *Lens* and its comparison with RAPD analysis

Received: 10 November 1995 / Accepted: 12 April 1996

Abstract AFLP and RAPD marker techniques have been used to evaluate and study the diversity and phylogeny of 54 lentil accessions representing six populations of cultivated lentil and its wild relatives. Four AFLP primer combinations revealed 23, 25, 52 and 48 AFLPs respectively, which were used to partition variation within and among *Lens* taxa. The results of AFLP analysis is compared to previous RAPD analysis of the same material. The two methods provide similar conclusions as far as the phylogeny of *Lens* is concerned. The AFLP technique detected a much higher level of polymorphyism than the RAPD analysis. The use of 148 AFLPs arising from four primer combinations was able to discriminate between genotypes which could not be distinguished using 88 RAPDs. The level of variation detected within the cultivated lentil with AFLP analysis indicates that it may be a more efficient marker technology than RAPD analysis for the construction of genetic linkage maps between carefully chosen cultivated lentil accessions.

Key words *Lens* · AFLP · RAPD · Phylogeny · Diversity

Introduction

Lentil (*Lens culinaris* Medik.) is an important diploid, self-pollinating, grain legume of the Old World. *Lens* comprises four species. One of these, *Lens culinaris*, has two sub-species: *culinaris* encompassing two cultivated varietal groups, the small-seeded (*microsperma*) and large-seeded (*macrosperma*); and *orientalis*, its presumed wild progenitor. The other three wild species are *L. odemensis*, *L. nigricans* and *L. ervoides*. The crop has been successfully introduced in North and South America but, unlike cereals, the productivity of cultivated lentil has not been markedly improved through conventional breeding. The use of molecular marker technology has provided the potential to speed up plant improvement for a variety of objectives including disease resistance. Consequently, the establishment of molecular marker technology and the generation of genetic maps are valuable tools for plant geneticists and breeders to undertake marker-assisted selection and positional cloning.

Attempts have been made in the past to examine the diversity and phylogeny of *Lens* using morphological, cytological, isozyme, seed protein and nuclear RFLP markers (Ladizinsky et al. 1984; Hoffman et al. 1986; Havey and Muehlbauer 1989). Recently, chloroplast (cp) DNA polymorphism has been used to examine the origin, diversity and phylogeny of the genus *Lens* (Muench et al. 1991; Mayer and Soltis 1994). These studies revealed low levels of genetic polymorphism in cultivated lentils. Therefore, the existing lentil linkage map, comprising morphological, isozyme, RFLP and other loci, is partial and based on intra- or inter-specific crosses (Simon et al. 1993; Tahir et al. 1993; Muehlbauer et al. 1995). The availability of a sufficient number of polymorphic markers in cultivated lentils is a pre-requisite for establishing a reasonably dense map of the lentil genome which can be of immediate relevance in lentil breeding.

The use of RAPD markers in studying intra- and inter-specific variation in *Lens* has been reported (Aboelwafa et al. 1995). The utility of RAPDs as an additional DNA marker technique in studying the diversity and phylogeny of cultivated lentils and its wild taxa has also been demonstrated (Sharma et al. 1995). Black (1993) has expressed reservation about the utility of arbitrary primed polymorphisms in species phylogenetic analyses. However, in the present paper we demonstrate

Communicated by G. Wenzel

S. K Sharma[1] · M. R. Knox · T H. N Ellis (✉)
Department of Applied Genetics, John Innes Centre, Colney Lane, Norwich NR4 7UH, England

Present address:
[1] Department of Plant Breeding and Genetics, H.P Agricultural University, Palampur-176062, India

that considerable variation at the varietal level is detectable by AFLP (Zabeau and Vos 1993; Vos et al. 1995) and the number of polymorphic products obtained with one primer combination can be several times higher than that obtained with RAPD markers (our unpublished results indicate that AFLP is more efficient at detecting informative markers than is RFLP analysis). Studies comparing marker techniques in *Lens* are beginning to appear in the literature; Havey and Muehlbauer (1989) found broad agreement in species relationships in *Lens* based on RFLPs and isozymes, but RFLPs detected a greater level of variation. Given the proliferation of genome marker technologies, a comparison of different marker techniques using the same DNA samples is timely. The objectives of the present study, therefore, were to investigate the use of AFLP in order: (1) to analyze the genetic diversity in the cultivated lentil and its wild taxa, (2) to discern the phylogeny of *Lens* to assess the utility of the AFLP approach and (3) to determine whether a sufficient number of AFLP markers could easily be detected among cultivated lentils to generate a detailed genetic linkage map. For comparative purposes, the AFLP data presented here can be compared directly to the RAPD data of Sharma et al. (1995).

Materials and methods

Plant materials and DNA samples

Fifty-four genotypes were used for the present study (Table 1). These included 26 genotypes of *L. culinaris* (13 each of vars. *macrosperma* and *microsperma*), seven genotypes each of the wild taxa, *L. culinaris* ssp. *orientalis*, *L. odemensis*, *L. nigricans* and *L. ervoides*. Of the cultivated genotypes, seven were from ICARDA, while 19 were selected from the Indian lentil breeding programme. All the wild sub-species were provided by ICARDA, Aleppo, Syria. DNA preparations from these materials as described by Sharma et al (1995) were used in this analysis.

DNA concentrations were estimated and standardised against known concentrations of lambda DNA on 1% agarose gels. Aliquots from the same DNA preparations were used for both RAPD and AFLP analyses.

AFLP analysis

AFLP analysis is a PCR-based strategy, by which a selection of restriction fragments of total DNA is detected by amplification using PCR (Zabeau and Vos 1993; Vos et al. 1995).

In these experiments, genomic DNA was digested with *Pst*I and *Mse*I and adapters ligated to the digested DNA. The *Pst*I adapter was 5′ biotinylated. The fragments with *Pst*I ends were subtracted from the ligation mixture using streptavidin beads (Dynal). The adapter sequences were:

*Pst*I 5′-ctcGTAGACTGCGTACAtgca-3′
*Mse*I 5′-gacgATGAGTCCTGAG**-3′

The sequence in upper case was double stranded and **indicates a 5′ overhang corresponding to the TA sticky end of *Mse*I fragments.

Primer labelling was performed by phosphorylating the 5′ end of selective primer with ^{33}P γ-ATP and polynucleotide kinase. Selective primers were complementary to the *Pst*I or *Mse*I adapters, but with the addition of two or three selective bases at the 3′ end to define the specificity of the selective amplification (see Gel analysis below).

PCR was performed using a thermal cycler PTC-100 (M.J. Research Inc.) with a regime of denaturation for 30 at 94 °C, annealing for 30 s at 65 °C, followed by an extension reaction of 60 s at 72 °C for one cycle. For a further 11 cycles the annealing temperature was lowered by 0.7 °C per cycle. For a final 24 cycles, the denaturation was 30 s at 90 °C, annealing for 30 s at 56 °C and extension for 60 s at 72 °C.

Table 1 Genotypes of lentil and the wild *Lens* taxa used for AFLP and RAPD analyses

Taxon	Genotype no.	Genotype Name	Source/Origin	Taxon	Genotype No	Genotype Name	Source/Origin
Var.	1	L-4163	India	*L. odemensis*	28	IL WL-36	Turkey
macrosperma	2	EC-158593	ICARDA, Syria		29	IL WL-153	Turkey
	3	EC-158917	ICARDA, Syria		30	IL WL-165	Turkey
	4	GL-259	India		31	IL WL-169	Turkey
	5	HPL-4	India		32	IL WL-170	Turkey
	6	Precoz	ICARDA, Syria		33	IL WL-322	Turkey
	7	EC-158918	ICARDA, Syria	Ssp. *orientalis*	34	IL WL-1	Cyprus
	8	EC-151516	ICARDA, Syria		35	IL WL-70	Iran
	9	EC-158856	ICARDA, Syria		36	IL WL-117	Syria
	10	L-4136	India		37	IL WL-181	Syria
	11	L-178	India		38	IL WL-146	Syria
	12	LH-82	India		39	IL WL-247	Syria
	13	L-4076	India		40	IL WL-257	Syria
Var.	14	EC-158867	ICARDA, Syria	*L. nigricans*	41	IL WL-14	France
microsperma	15	L-3044	India		42	IL WL-26	Former Yugoslavia
	16	DPL-119	India		43	IL WL-31	Spain
	17	LL-145	India		44	IL WL-38	Turkey
	18	L-4191	India		45	IL WL-111	Turkey
	19	L-4-81-8	India		46	IL WL-311	Turkey
	20	L-1662	India		47	IL WL-305	Turkey
	21	L-4134	India	*L. ervoides*	48	IL WL-45	Former Yugoslavia
	22	PL-406	India		49	IL WL-48	Former Yugoslavia
	23	L-830	India		50	IL WL-50	Former Yugoslavia
	24	L-259	India		51	IL WL-41	Turkey
	25	L-4661	India		52	IL WL-42	Italy
	26	L-303	India		53	IL WL-60	Turkey
L. odemensis	27	ILWL-35	Turkey		54	IL WL-251	Syria

Gel analysis

AFLP analysis was done using a single *Pst* primer (PstA) and four *Mse* primers (*Mse*2, *Mse*3, *Mse*4 and *Mse*5), as follows:

*Pst*A. 5′-GACTGCGTACATGCAGCC-3′

*Mse*2: 5′-GATGAGTCCTGAGTAA CAC-3′

*Mse*3: 5′-GATGAGTCCTGAGTAA TGC-3′

*Mse*4: 5′-GATGAGTCCTGAGTAA ATA-3′

*Mse*5: 5′-GATGAGTCCTGAGTAA TAT-3′

The primers for RAPD analysis were described by Sharma et al. (1995)

The AFLP reaction products were analyzed in 4.5% denaturing polyacrylamide gels run in 1 × TBE for 0 5 h before loading samples After electrophoresis, gels were dried on a standard slab gel drier for 2 h, and then exposed to X-ray film or phospho-imaging plates for variable lengths of time depending upon the signal. The X-ray films were developed, whereas imaging plates were scanned with a Bio-image Analyser (BAS 1000, Fuji Photo Films Co. Ltd.)

Data analysis

For each genotype, a binary matrix reflecting specific AFLP- or RAPD-band presence (1) or absence (0) was generated. Estimates of similarity were based on the number of shared amplification products (Nei and Li 1979) Product frequency was employed to estimate differentiation among grouped genotypes using BIOSYS-1 (Version 1.7. Swofford 1989) and an UPGMA (unweighted pair group with arithmetic average) clustering tree derived from Nei's (1973) estimates of genetic identity. Principal co-ordinate and single linkage cluster analyses among individuals (Kempton and McNicol 1990) were performed with the GENSTAT 5 (1987) Statistical Package. An unrooted tree of genetic distance was also prepared from the AFLP data generated by different primer combinations. The tree was derived from a table of all positive genetic distances by the neighbour joining method of Saitou and Nei (1987).

Results

The AFLP primer combinations *Pst*A-*Mse*2, *Pst*A-*Mse*3, *Pst*A-*Mse*4 and *Pst*A-*Mse*5 were used to analyze 54 genotypes of six *Lens* taxa; these yielded 23, 25, 52 and 48 AFLPs respectively. An example of the level of polymorphism detectable with the *Pst*A-*Mse*5 primer combination is presented in Fig. 1. The number of polymorphic loci detected with four AFLP primer combinations and RAPDs in six populations of *Lens* is given in Table 2. Nei's estimate of similarity, based on the number of shared amplifications (Nei and Li 1979), is shown in Table 3.

A dendrogram of six *Lens* populations was constructed using 23 AFLPs generated with primer *Pst*A-*Mse*2 reflecting these conclusions (Fig. 2). The primer combinations *Pst*A-*Mse*3, *Pst*A-*Mse*4 and *Pst*A-*Mse*5 gave similar results when analyzed in this way (data not shown; analysis of the *Pst*A-*Mse*2 combination is given in Figs. 2–5). The most parsimonious cladogram obtained for these 54 genotypes with 23 AFLP loci using primer combination *Pst*A-*Mse*2 was prepared (Fig. 3).

AFLP Products in Cultivated Lentils and its Wild Taxa Using Primer Combination Pst A and Mse 5

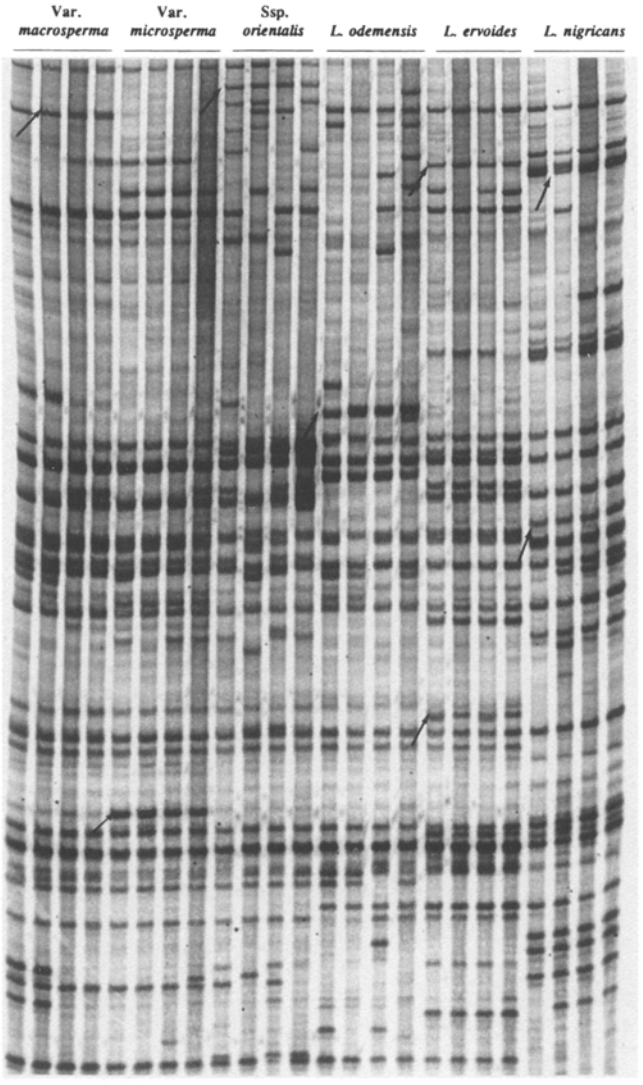

Fig. 1 AFLPs generated from genomic DNA of 54 genotypes of the genus *Lens* using primer combination *Pst*A-*Mse*5. *Arrows* indicate taxon-specific products

Fig. 2 Dendrogram of six populations of the genus *Lens* based on Nei's genetic identity for 23 AFLPs

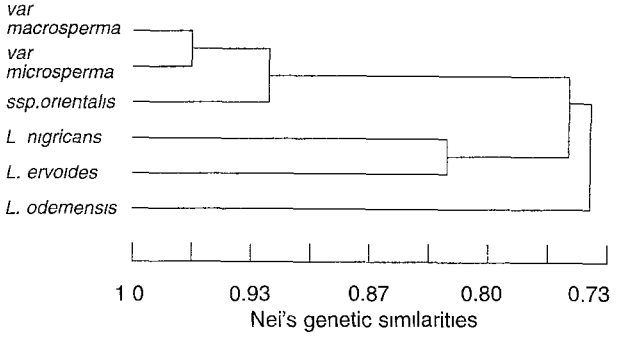

Table 2 Genetic variability identified with AFLP and RAPD analyses in six populations of the genus *Lens*

Population	AFLP primer combination/RAPD	Mean sample size/locus	Loci polymorphic (%)	Unbiased mean (± SE) heterogeneity per locus
Var. *macrosperma*	*Pst*A-*Mse*2	13 0	43.5	0.140 (0.041)
	*Pst*A-*Mse*3	13.0	20.0	0.073 (0.032)
	*Pst*A-*Mse*4	13.0	26.9	0.110 (0.026)
	*Pst*A-*Mse*5	13.0	43.8	0.157 (0.029)
	RAPD	12.8	33.3	0.104 (0.021)
Var. *microsperma*	*Pst*A-*Mse*2	13.0	47.8	0.151 (0.039)
	*Pst*A-*Mse*3	13.0	12.0	0.035 (0.022)
	*Pst*A-*Mse*4	13.0	19.2	0.063 (0.016)
	*Pst*A-*Mse*5	13 0	12.5	0.039 (0.017)
	RAPD	12 9	16.7	0.037 (0.011)
L. odemensis	*Pst*A-*Mse*2	7.0	30.4	0.126 (0.042)
	*Pst*A-*Mse*3	7.0	12.0	0.053 (0 030)
	*Pst*A-*Mse*4	7.0	7.7	0.031 (0.016)
	*Pst*A-*Mse*5	7.0	27.1	0.115 (0.029)
	RAPD	7.0	30.0	0.116 (0.021)
Ssp. *orientalis*	*Pst*A-*Mse*2	7.0	30.4	0.126 (0.042)
	*Pst*A-*Mse*3	7.0	8.0	0.021 (0.015)
	*Pst*A-*Mse*4	7.0	26.9	0.105 (0.025)
	*Pst*A-*Mse*5	7.0	41.7	0.167 (0.031)
	RAPD	7.0	38.3	0.150 (0 026)
L. nigricans	*Pst*A-*Mse*2	7.0	69.6	0.260 (0.041)
	*Pst*A-*Mse*3	7.0	80.0	0.292 (0 035)
	*Pst*A-*Mse*4	7.0	65.4	0.262 (0.029)
	*Pst*A-*Mse*5	7.0	77.1	0.311 (0.029)
	RAPD	7.0	63.3	0.229 (0.025)
L ervoides	*Pst*A-*Mse*2	7.0	56.5	0.214 (0.044)
	*Pst*A-*Mse*3	7.0	48 0	0.158 (0.037)
	*Pst*A-*Mse*4	7.0	40 4	0.111 (0.020)
	*Pst*A-*Mse*5	7.0	56.3	0.168 (0.024)
	RAPD	6.9	55.0	0.186 (0.024)

Table 3 Estimate of Nei's genetic identities based on AFLP and RAPD analyses in six populations of the genus *Lens*

Population	Primer combination/RAPD	2	3	4	5	6
1. Var. *macrosperma*	*Pst*A-*Mse*2	0.963	0.760	0.936	0.821	0.725
	*Pst*A-*Mse*3	0.957	0.633	0.934	0.605	0.663
	*Pst*A-*Mse*4	0.948	0.713	0.940	0.753	0.731
	*Pst*A-*Mse*5	0.925	0.770	0.884	0.728	0.720
	RAPD	0.897	0.662	0.894	0.750	0.596
2. Var. *microsperma*	*Pst*A-*Mse*2		0.719	0.911	0.774	0.743
	*Pst*A-*Mse*3		0.585	0.882	0.663	0.603
	*Pst*A-*Mse*4		0.652	0.901	0.714	0.689
	*Pst*A-*Mse*5		0.715	0.826	0.653	0.661
	RAPD		0.682	0.875	0.699	0.543
3. *L odemensis*	*Pst*A-*Mse*2			0.777	0.756	0.752
	*Pst*A-*Mse*3			0.711	0.729	0.817
	*Pst*A-*Mse*4			0.686	0.657	0 747
	*Pst*A-*Mse*5			0.701	0.729	0.710
	RAPD			0.715	0.604	0.547
4. Ssp. *orientalis*	*Pst*A-*Mse*2				0.820	0.711
	*Pst*A-*Mse*3				0.678	0.705
	*Pst*A-*Mse*4				0.749	0.716
	*Pst*A-*Mse*5				0.669	0.643
	RAPD				0.765	0.648
5 *L. nigricans*	*Pst*A-*Mse*2					0.817
	*Pst*A-*Mse*3					0.793
	*Pst*A-*Mse*4					0.818
	*Pst*A-*Mse*5					0.812
	RAPD					0.818
6. *L. ervoides*						

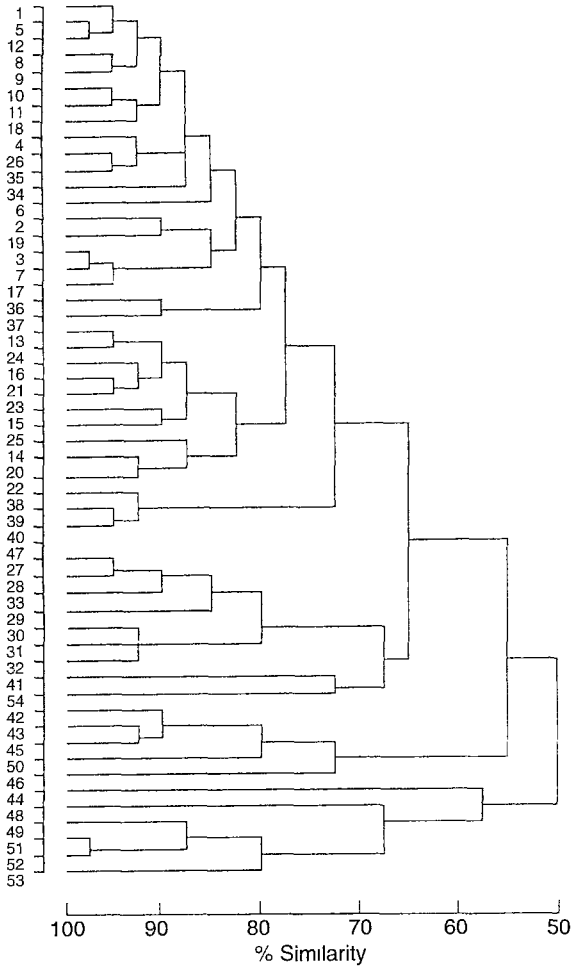

Fig. 3 Dendrogram of 54 genotypes of the genus *Lens* based on similarity for 23 AFLPs

In order to assess whether the grouping of individuals based on the 23 AFLPs could be further resolved, principal component analysis (PCA) was used to examine the shared-fragment data available for 54 genotypes (Fig. 4). The first two principal components accounted for 41% of the total variation observed.

An unrooted phylogenetic tree, prepared according to the neighbour joining method using 23 AFLPs derived from the *Pst*A-*Mse*2 primer (Fig. 5), supports our earlier conclusions based on cluster analysis and PCA (Figs. 3 and 4). The tree has four major branches representing *L. culinaris*, *L. odemensis*, *L. nigricans* and *L. ervoides*. In order to undertake further discrimination at the varietal or sub-species level, an unrooted tree was prepared using 148 AFLPs derived from the four primer combinations (Fig. 5).

Discussion

AFLP analysis generated an average of 37 informative bands per primer combination. Sharma et al. (1995) used the same DNA samples in an analysis of 100 ten-mer RAPD primers and found ten-fold fewer informative bands per primer.

The proportion of loci which are polymorphic, and the mean (unbiased) heterogeneity per locus, provides a good measure of genetic diversity within species, subspecies and varietal groups. From the data in Table 2 it can be seen that these AFLP primer combinations revealed different degrees of polymorphic loci within different *Lens* taxa and some demonstrated a higher level of polymorphism than did the RAPD analysis. There seems to be no simple relationship between the numbers of AFLPs or RAPDs generated and the proportion which correspond to polymorphic loci (data not shown). The most diverse group of accessions, as judged

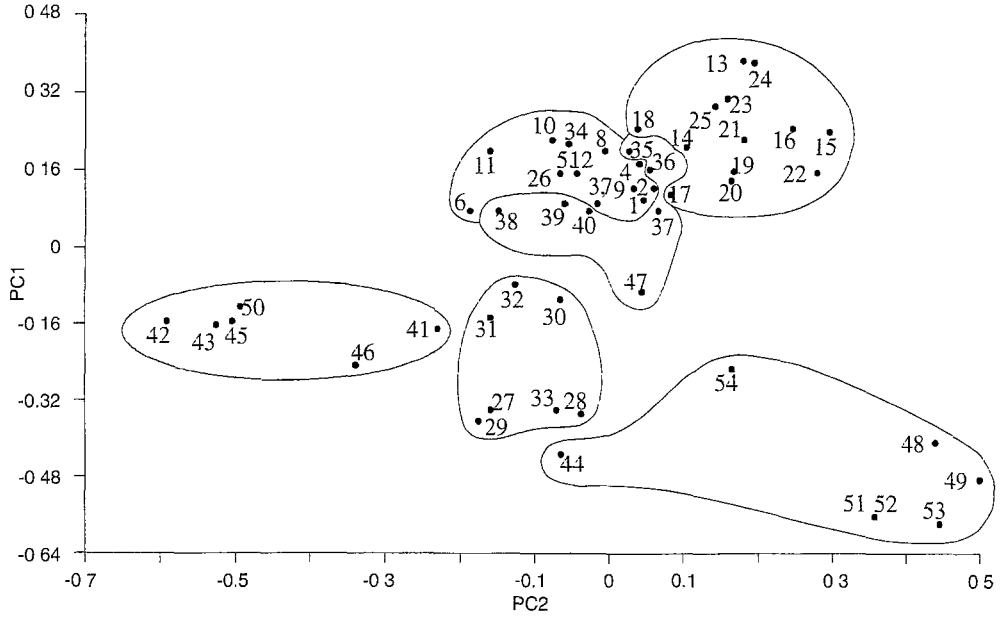

Fig. 4 Principal co-ordinate analysis of 54 genotypes of the genus *Lens* for 23 AFLPs. *Genotypes 1–13* = var. *macrosperma*. *14–26* = var. *microsperma*, *27–33* = *L. odemensis*. *34–40* = ssp. *orientalis*. *41–47* = *L. nigricans* and *48–54* = *L. ervoides*

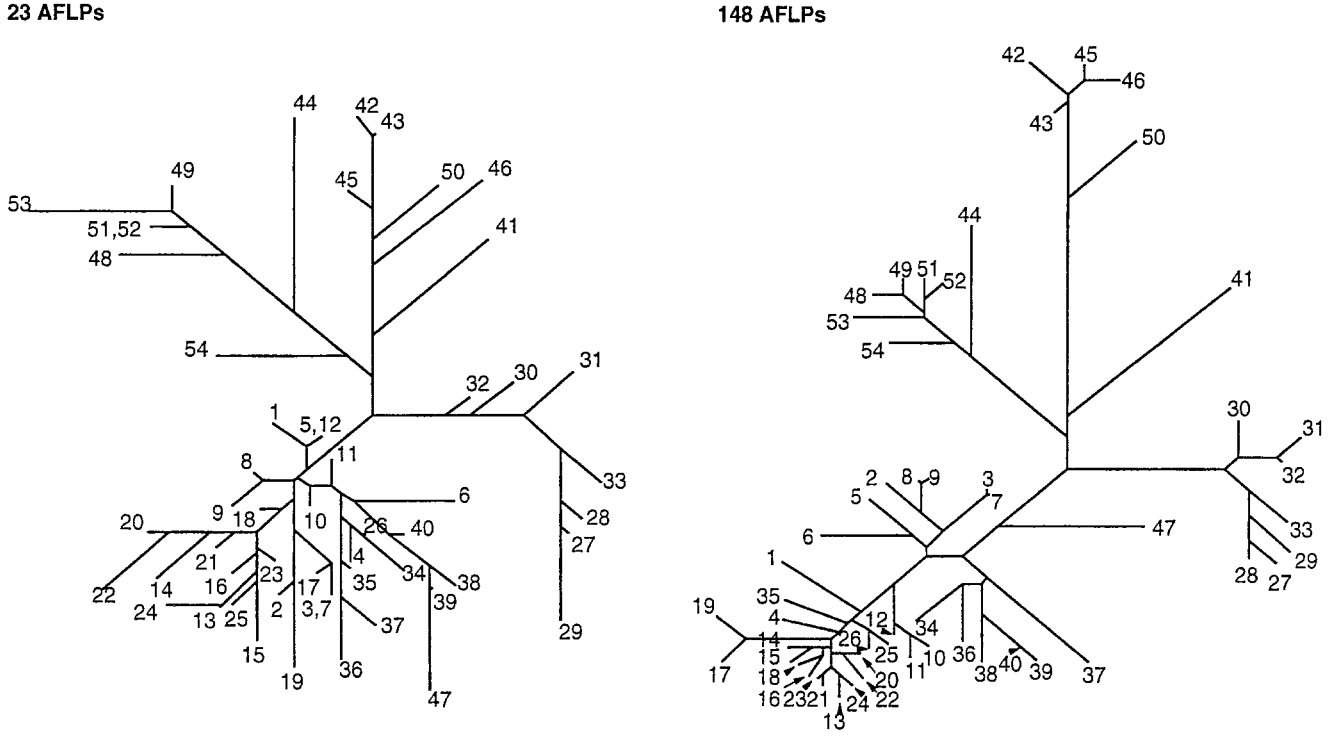

Fig. 5 Trees of genetic relatedness of the cultivated lentil and its wild taxa derived from 23 AFLPs using primer combination *Pst*A-*Mse*2, and from 148 AFLPs using primer combinations *Pst*A-*Mse*2, *Pst*A-*Mse*3, *Pst*A-*Mse*4 and *Pst*A-*Mse*5. *Genotypes 1–13* = var. *macrosperma*, *14–26* = var *microsperma*, *27–33* = *L. odemensis*, *34–40* = ssp. *orientalis*, *41–47* = *L. nigricans* and *48–54* = *L. ervoides*

from the proportion of polymorphic loci, was *L. nigricans*, while the least diverse group was var. *microsperma*.

Estimates of Nei's genetic similarities within *Lens* indicated that the greatest similarity was shown between ssp. *orientalis* and var. *macrosperma*. Likewise, ssp. *orientalis* exhibited a high degree of genetic identity to var. *microsperma*. These results show that ssp. *orientalis* is the best candidate for the progenitor of cultivated lentils. This observation supports the previous analyses based upon affinities in morphology and cytology (Ladizinsky 1979, Ladizinsky et al. 1984), isozymes and seed proteins (Hoffman et al. 1986), and nuclear and chloroplast RFLP markers (Havey and Muehlbauer 1989; Mayer and Soltis 1994). Abo-elwafa et al. (1995) used RFLP markers in *Lens* and suggested *L. culinaris* ssp. *orientalis* to be progenitor of cultivated lentils. The genetic identity between ssp. *orientalis* and var. *macrosperma* using AFLPs was slightly higher (I = 0.884–0.936) than with var. *microsperma* (I = 0.826–0.911). In the case of RAPD analysis, the genetic identity between ssp. *orientalis* and *macrosperma* (0.894) was comparable with *microsperma* (0.875), suggesting a near simultaneous evolution of these two varietal groups from ssp. *orientalis*, whereas AFLP analysis suggested a closer affinity between var. *macrosperma* and ssp. *orientalis*.

Among the wild species/subspecies, the highest genetic identities were discernible between *L. nigricans* and *L. ervoides* (Table 3), indicating them to be the most closely related wild taxa in *Lens*. RAPD analysis, presented previously (Sharma et al. 1995), also generated comparable dendrograms, but the phylogenetic placement of *L. odemensis* differed between the AFLP and RAPD analyses. In case of *Pst*A-*Mse*3, *Pst*A-*Mse*5 and RAPDs, *L. odemensis* was intermediate to *L. culinaris* on one hand and to *L. nigiricans* and *L. ervoides* on the other. However, in primer combinations *Pst*A-*Mse*2 and *Pst*A-*Mse*4, *L. odemensis* was the species farthest from the remaining taxa. Our phenetic analysis of AFLP-based data supports the classification of *Lens* based on crossability barriers (Ladizinsky et al. 1984), traditional views (Ladizinsky 1993), cpDNA phylogeny (Mayer and Soltis 1994) and RAPD-based data (Abo-elwafa et al. 1995; Sharma et al. 1995).

The phylogenetic tree clusters genotypes that are closely related and the topography of the tree portrays the relationship among individual genotypes. Previous RAPD analysis showed that the genotypes of var. *macrosperma* (1–13), var. *microsperma* (14–26) and ssp. *orientalis* (34–40) formed closely associated clusters (Sharma et al. 1995) and that the *macrosperma* genotypes 4 and 13 were associated with the *microsperma* (14–26) cluster. However, the analysis of 23 AFLPs (Fig. 3) did not reveal as distinct a clustering pattern of genotypes as that obtained with the 88 markers from the RAPD analysis. The seven genotypes of *L. odemensis* (27–33) formed a distinct cluster with both the RAPD and AFLP analyses. Barring genotypes 47 and 44, which clustered with the genotypes of ssp. *orientalis* and *L. ervoides* respectively, all the genotypes of *L. ervoides* (48–54) except 50 were clustered together; genotype 50 clustered with the genotypes of *L. nigricans*. Our unpublished results with three other separate AFLP primer combinations has led to similar conclusions. Genotypes

44 and 50 do not appear within the cluster of their supposedly related taxa either by AFLP or RAPD analysis, suggesting that these genotypes have been misidentified, or else that the groups to which they belong are very diverse, and that these accessions are atypical within the sample studied here.

In the principal component analysis (Fig. 4) the genotypes of var. *macrosperma*, var. *microsperma* and ssp. *orientalis* were so similar that it was difficult to cluster the genotypes of each *Lens* taxon. *L. nigricans* and *L. ervoides* revealed clear separation from each other. The exceptional genotype 47 (*L. nigricans*) was associated with ssp. *orientalis*, while 41 (*L. nigricans*) and 54 (*L. ervoides*) were associated with *L. odemensis*, 44 (*L. nigricans*) with *L. ervoides* and 50 (*L. ervoides*) with *L. nigricans*. Similar results were obtained with RAPD analysis (Sharma et al. 1995). In general, PCA using 23 AFLPs provided a clear separation of the different species and supported the conclusions from the cluster analysis. The relative utility of ordination for describing the relationships between accessions nevertheless depends upon the level of resolution desired. While clustering provided the best indication of relationship among closely related accessions, ordination, using only the first two principal co-ordi-nates, appeared to provide a more complete representation of the relationships among major groups.

The cluster and PC analyses of AFLP and RAPD data revealed comparable results in discriminating *Lens* species. Similar results were obtained with the other primer combinations (data not shown). The analysis of 148 AFLPs was able to detect variation within subspecies and even varietal groups. With major exceptions, it was possible to identify distinct groupings of vars. *macrosperma* and *microsperma* genotypes. Several *macrosperma* genotypes (2, 3, 5, 6, 7, 8 and 9) constituted one connected group, with three genotypes (10, 11 and 12) constituting another. All the *microsperma* genotypes (14–26) lay within a distinct group. *Macrosperma* genotypes 1, 4, and 13 were associated with the *microsperma* group. The grouping of genotypes 1, 4 and 13 with *microsperma* is not surprising as some of the large-seeded genotypes studied have been taken from the Indian lentil breeding programme incorporating the large-seed size of *macrosperma* into the *microsperma* genetic background. Genotypes of the ssp. *orientalis* formed a separate branch with the exception of genotype 35 which was peripherally associated with the genotypes of the *microsperma* group, indicating that this genotype is either misclassified or else is an atypical selection from ssp. *orientalis*, which is internally deverse in comparison to the differences between the accessions of the *L. culinaris* sub-groups which we have studied. Genotype 47 exhibited relatedness with the genotypes of ssp. *orientalis*, while 41 appears to be independent from the remaining genotypes of *L. nigricans*. Similar results were obtained with RAPD analysis (Sharma 1995). The data suggest that AFLP and RAPD techniques are useful in supplementing traditional taxonomic methods. However, the degree of resolution obtained with 148 AFLPs was higher than with RAPDs.

As far as the comparison of RAPD and AFLP marker techniques is concerned, 88 RAPDs were unable to discriminate between several genotypes (10 and 11; 15, 16, 25 and 26; 18 and 21; Sharma et al. 1995). The use of 23 AFLPs also failed to discriminate between genotypes 51 and 52, 3 and 7, and 5 and 12 (Fig. 3). However, using 148 AFLPs arising from four primer pairs, it was possible to discriminate between all the genotypes employed in the present study, and this increased resolution is the main difference between the two unrooted trees. The main groups identified in the 23 AFLP tree are retained in the 148 AFLP tree but, with the larger number of markers, the groups are more separate. If the probability that a marker detects a difference within a group (p_w) is comparable to, though smaller than, the probability that it detects a difference between groups (p_b), then with small numbers of markers the groups will tend to merge, but with larger numbers of markers the distinction between p_w and p_b will become clearer. This is simply a consequence of sampling error. The overall similarity of the two AFLP-derived trees suggests that this technique is recognizing distinct taxonomic groups, because the same groupings appear in both the 23 and 148 marker analyses, but the groups are more clearly distinct with the larger number of markers. The groupings which the AFLP technique identifies have a good correspondence to the recognized taxonomic divisions within the genus *Lens*. Furthermore, these results indicate the robustness of the AFLP technique in providing a higher degree of resolution for discriminating closely related germ plasm accessions than is available with RAPDs and other molecular techniques. This additional resolution is required to tease out the taxonomic relationships between internally diverse, but closely related, taxa.

Conclusion

The present AFLP analysis provided better resolution in discerning the phylogeny of *Lens* than has been obtained with isozyme (Pinkas et al. 1985; Hoffman et al. 1986), nuclear RFLPs (Havey and Muehlbauer 1989) or cpDNA RFLPs (Muench et al. 1991), indicating that AFLP markers have the potential of complementing both conventional and molecular markers in reconstructing the phylogenetic history of the genus *Lens*.

Based upon the results of the present study, it appears that the relationship estimated from AFLPs generated by a single primer combination was not at variance with the broad classification of *Lens* taxa as described by RAPD analysis. However, the most significant impact of the additional AFLP-based markers arising from four primer combinations, was the more accurate determination of relationships within and between the *macrosperma* and *microsperma* genotypes, which are too close to be accurately differentiated either by a single AFLP primer combination or with the 88 RAPDs. These re-

sults suggest that, in addition to providing important information on the evolutionary pattern of *Lens*, the increased resolution associated with the large numbers of markers available with the AFLP approach may provide sufficient markers to construct genetic linkage maps between carefully chosen cultivated lentil accessions. These maps will be of more immediate relevance in lentil breeding than those derived from wide crosses.

Acknowledgements S. K. Sharma acknowledges the receipt of an E U. Marie Curie fellowship (CI1-CT93-0251) which enabled this work to be undertaken. The John Innes Centre is supported by a grant in aid from the B.B.S.R.C. We thank C M. Thomas and S. Poyser for their help and advice, R. Casey and J. Hofer for their comments on the manuscript.

References

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

Black WCIV (1993) PCR with arbitrary primers: approach with care. Insect Mol Biol 2.1–6

Genstat 5 Committee (1987) Genstat 5 Manual. Clarendon Press, Oxford

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

Hoffman DL, Soltis E, Muehlbauer FJ, Ladizinsky G (1986) Isozyme polymorphism in *Lens* (Leguminosae). Syst Bot 11:172–176

Kempton R, McNicol J (1990) Graphical method for multivariate data. Scottish Agricultural Stastistics Service

Ladizinsky G (1979) The genetics of several morphological traits in the lentil. J Hered 70:135–137

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

Ladizinsky G, Cohen D, Muehlbauer FJ (1984) The biological species of the genus *Lens*. Bot Gaz 145:253

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

Muehlbauer FJ, Kaiser WJ Clement SL, Summerfield RJ (1995) Production and breeding of lentil Adv Agron 54:284–332

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 (1973) Analysis of gene diversity in sub-divided populations. Proc Natl Acad Sci USA 70·3321–3323

Nei M, Li W (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

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

Saitou N, Nei M (1987) The neighbour joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4·406–425

Sharma SK, Dawson IK, Waugh R (1995) Relationship among cultivated and wild lentils revealed by RAPD analysis. Theor Appl Genet 91:647–654

Simon CJ, Tahir M, Muehlbauer FJ (1993) Linkage map of lentil (*Lens culinaris*) 2n = 14. In: O'Brien SJ (ed) Genetic maps. Locus maps of complex genomes, 6th edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 6.96–6.100

Swofford D (1989) BIOSYS-1, Release 1.7. Illinois Natural History Survey, University of Illinois, Champaign, Illinois, USA

Tahir M, Simon CJ, Muehlbauer FJ (1993) Gene map of lentil: a review. *Lens* Newslett 20:3–10

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kupier M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Zabeau M, Vos P (1993) Selective restriction fragment amplification·a general method for DNA fingerprinting European Patent Application 924026 29.7 (Publication number:0534 858 A1)