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Relationships among cultivated and wild lentils revealed by RAPD analysis

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Abstract RAPD markers were used to distinguish between six different *Lens* taxa representing cultivated lentil and its wild relatives. Twenty-four arbitrary sequence 10-mer primers were identified which revealed robust and easily interpretable amplification-product profiles. These generated a total of 88 polymorphic bands in 54 accessions and were used to partition variation within and among *Lens* taxa. The data showed that, of the taxa examined, ssp. *orientalis* is most similar to cultivated lentil. *L. ervoides* was the most divergent wild taxon followed by *L. nigricans*. The genetic similarity between the latter two species was of the same magnitude as between ssp. *orientalis* and cultivated lentil. In addition, species-diagnostic amplification products specific to *L. odemensis*, *L. ervoides* and *L. nigricans* were identified. These results correspond well with previous isozyme and RFLP studies. RAPDs, however, appear to provide a greater degree of resolution at a sub-species level. The level of variation detected within cultivated lentils suggests that RAPD markers may be an appropriate technology for the construction of genetic linkage maps between closely related *Lens* accessions.

Key words *Lens* · RAPD · Cluster analysis · Diversity · Phylogeny

Introduction

Lentil ranks as one of the most important grain legumes of the Old World. Traditionally cultivated from the Atlantic coast of Spain and Morocco in the west, to India in the east, it acts as a valuable meat substitute in many subsistance communities. More recently, the crop has been successfully introduced to the New World, where the leading producers are Argentina, Chile and the USA. The cultivated lentils and their wild relatives are self-pollinating diploids ($2n = 2x = 14$). Although lentil breeding has in the past received little attention relative to other major pulses, such as beans or peas (Muehlbauer and Slinkard 1981), all major lentil-growing countries are currently supporting a lentil-breeding programme.

A pre-requisite for the efficient use of genetic resources in all plant-breeding programmes is a detailed understanding of the extent and distribution of the genetic variation available within a genus. During the past 15 years numerous attempts have been made to examine the diversity and phylogeny of *Lens* (Ladizinsky 1979; Zamir and Ladizinsky 1984; Pinkas et al. 1985; Hoffman et al. 1986; Havey and Muehlbauer 1989a; Muench et al. 1991; Mayer and Soltis 1994). These studies recognised *Lens* as comprising of two interfertile groups or species which were classified as *L. culinaris* and *L. nigricans*. The former included ssp. *culinaris* (cultivated lentils), ssp. *orientalis* and ssp. *odemensis*, while the latter included ssp. *nigricans* and ssp. *ervoides*. In a recent study, Ladizinsky (1993) revised the taxonomy of the genus *Lens* into four species: *L. culinaris*, *L. odemensis*, *L. nigricans* and *L. ervoides*. In this classification, *L. culinaris* Medikus has two subspecies: ssp. *culinaris*, encompassing the cultivated lentils, and ssp. *orientalis*, its closest wild relative. Within cultivated lentils scores of distinct lines have been described (Barulina 1930). Conventionally, these are divided into two groups: (1) small-seeded lentils (var. *microsperma*), with small convex pods (6–15 mm length by 3.5–7 mm width) and small (3–6 mm diameter) seed, and (2) large-seeded lentils (var. *macrosperma*), with larger flattened pods (15–20 mm length by 7.5–10.5 mm width) and seeds attaining a diameter of 6–9 mm. Hereafter, in this paper, varietal or sub-specific taxa will be referred to by their respective epithets.

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Assessing genetic variation at the DNA level, using techniques such as RFLPs (Botstein et al. 1979) or RAPDs (Williams et al. 1990), is now commonly accepted as the method of choice for examining relationships among plant taxa. Despite uncertainty regarding the exact nature (and therefore utility) of polymorphism (Black 1993), RAPDs have been widely adopted because of the simplicity of the assay and its immediate applicability to a wide range of species. RAPDs have been used to analyse genetic diversity and phenetic relationships in *Brassica oleracea* (Kresovich et al. 1992), *Sorghum bicolor* (Tao et al. 1993), *Apium graveolens* (Yang and Quiros 1993), *Beta* (Jung et al. 1993), *Allium* (Susan et al. 1993), *Lotus* (Campos et al. 1994), *Avena sterilis* (Heun et al. 1994) and *Coffea* (Orozco-Castillo et al. 1994). To-date, however, RAPDs have not been used to address the genetic diversity and phenetics of *Lens*. Furthermore, to our knowledge, no study has yet taken into consideration the varietal classification of cultivated lentils. The objective of this current work was therefore (1) to analyse and quantify the genetic diversity in cultivated and wild lentils using RAPDs; (2) to compare phenetic relationships within *Lens* derived using data obtained with RAPD markers with those based on alternative markers in order to assess the utility of the RAPD approach; and (3) to determine whether a sufficient number of RAPD markers to generate detailed genetic linkage maps could be easily detected among cultivated lentils.

Materials and methods

Plant material

Fifty-four lentil genotypes were used in the present study (Table 1). These included 26 cultivated genotypes (13 each of vars. *macrosperma* and *microsperma*), and seven genotypes each of the wild taxa ssp. *orientalis*, *L. odemensis*, *L. nigricans* and *L. ervoides*. Of the cultivated genotypes, seven were introduced by ICARDA, while 19 were selected from an Indian lentil-breeding programme. The wild sub-species/species were provided by ICARDA, Allepo, Syria.

DNA extraction and RAPD analysis

Seeds of each genotype were grown in the glasshouse at SCRI and DNA was extracted from fresh leaves of single 2-week-old seedlings using a modification of the method described by Edwards et al. (1991). DNA concentrations were estimated and then standardised against known concentrations of uncut λ DNA on 1% agarose gels. PCR reactions were performed exactly as described by Chalmers et al. (1992). The arbitrary sequence 10-mer primers used were either obtained from Operon Technologies Inc. (Almeda Calif.), or were synthesised at SCRI on an Applied Bio-Systems 392 PCR-mate oligonucleotide synthesiser. The 24 primers finally chosen for analysis are listed in Table 2. DNA fragments generated by amplification were separated according to size on 2% agarose gels, stained with ethidium bromide and visualised by illumination with UV light (312 nm).

Data analysis

For each genotype, a binary matrix reflecting specific RAPD band presence (1) or absence (0) was generated. Estimates of similarity were

Table 1 Genotypes of lentils and the wild taxa used for RAPD analysis

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

Table 2 Primers used in the detection of polymorphism in the genus *Lens*

SC10-22	5′ GTAGGCGTCG 3′
SC10-23	5′ GGCTCGTACC 3′
SC10-38	5′ GACCCCGGCA 3′
SC10-43	5′ GCCTGGTTAC 3′
SC10-48	5′ CTGGTATGCG 3′
SC10-53	5′ CAGGGGACGA 3′
SC10-65	5′ CAGGGGTGAT 3′
SC10-76	5′ CGCAGACTTG 3′
SC10-77	5′ AGATAGCGGG 3′
SC10-79	5′ CGCCACGTTC 3′
SC10-82	5′ GCCGTGAAGT 3′
SC10-91	5′ CTCGACTAGG 3′
SC10-93	5′ GCCTCCTACC 3′
SC10-94	5′ GGGGTCGATT 3′
SC10-95	5′ GGCGTCGGGG 3′
SC10-99	5′ CAGGGCCGCT 3′
OPB-4	5′ GGACTGGAGT 3′
OPG-9	5′ CTGACGTCAC 3′
OPG-13	5′ CTCTCCGCCA 3′
OPH-9	5′ TGTAGCTGGG 3′
OPH-12	5′ ACGCGCATGT 3′
OPR-3	5′ ACACAGAGGG 3′
OPU-16	5′ CTGCGCTGGA 3′
OPW-6	5′ AGGCCCGATG 3′

based on the number of shared amplification products (Nei and Lei 1979). Principal co-ordinate and single linkage cluster analyses among individuals (Kempton and McNicol 1990) were performed with the GENSTAT 5 (1987) Statistical Package. In addition, product frequency was used 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) estimate of genetic identity.

Results and discussion

One hundred 10-mer primers were screened on a representative range of individuals from each taxa. Twenty-four primers showing consistently reproducible polymorphisms were selected and used to analyse all 54 genotypes. A total of 88 clear polymorphic products (0.2–2.0 kb in size) were scored in all individuals tested. An example of the level of polymorphism detected is shown in Fig. 1.

The proportion of RAPD loci which are polymorphic, and the mean (unbiased) heterogeneity (H) per locus calculated by BIOSYS-1, provide good measures of genetic diversity within species, sub-species and varietal groups. BIOSYS-1 version 1.7 is restricted to 60 polymorphic loci, which were chosen as a random subset of the total number of polymorphisms available. *L. nigricans* exhibited the highest genetic variability, followed by *L. ervoides* and ssp. *orientalis* (Table 3). Vars. *macrosperma* and *microsperma* displayed the lowest levels of genetic variability (H = 0.104 and 0.037 respectively), suggesting a relatively narrow genetic base among the cultivated lentils tested.

Estimates of Nei's genetic identities among species, sub-species and varietal groups within *Lens* (Table 4) calculated by BIOSYS-1 indicated that of the wild taxa ssp. *orientalis* showed the greatest similarity to vars. *macrosperma* and *microsperma* (identities of 0.894 and 0.875 respectively), suggesting that it may be the progenitor of cultivated lentils. This observation correlates well with previous analyses based upon affinities in morphology and cytology, as well as isozyme, seed protein, nuclear and chloroplast RFLP marker studies (Ladizinsky 1979; Ladizinsky et al. 1984; Hoffman et al. 1986; Mayer and Soltis 1994; Havey and Muehlbauer 1989a). While an increase in seed size is considered to be one of the most conspicuous trends under domestication, with *macrosperma* forms traditionally regarded as the most advanced cultivars (Zohary 1972), the present study revealed almost equal similarities between ssp. *orientalis* and vars. *macrosperma* and *microsperma*. This raises the possibility that these two varietal groups simulataneously evolved from a common ssp. *orientalis* progenitor, followed by geographical isolation and local adaptation, leading to fragmentation into different varietal groups. In addition, the almost equal level of similarity between varietal groups on the one hand (I = 0.897) and between varietal groups and ssp. *orientalis* on the other (I = 0.894 and 0.875) indicates a significant degree of differentiation between vars. *macrosperma* and *microsperma* ascribable by RAPDs. *L. odemensis* appeared to be somewhat intermediate between *L. culinaris* (I = 0.628–0.715), *L. nigricans* (I = 0.604) and *L. ervoides* (I = 0.547) justifying its elevation to an independent species (Ladizinsky 1993). The similarity co-efficient between *L. nigricans* and *L. ervoides* (I = 0.818) is similar to that between ssp. *culinaris* and ssp. *orientalis* (I = 0.875–0.897) which may again reflect a relatively recent split between these species. Cluster analysis, presented in the form of a dendrogram (Fig. 2), reflects these conclusions. Overall, the present phenetic analysis of RAPD data in *Lens* supports both

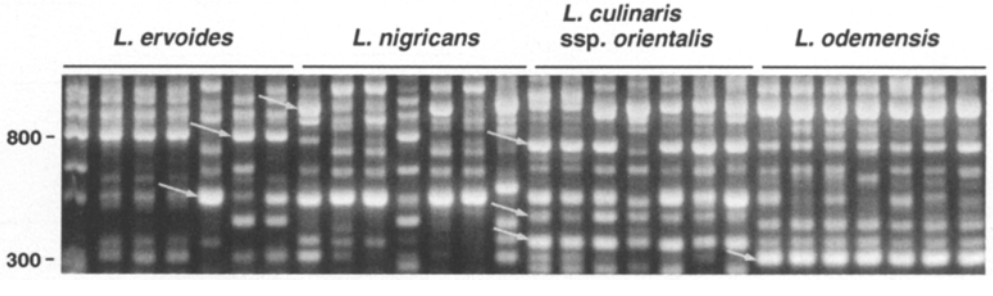


Fig. 1 Amplification products generated from genomic DNA of wild taxa in the genus *Lens* using arbitrary sequence primer SC10-23. *Arrows* indicate polymorphic products scored in the present study

Table 3 Genetic variability identified with 60 RAPD loci in six populations of the genus *Lens*

Population	Mean sample size per locus	Percentage of loci polymorphic	Unbiased mean heterogeneity, H, per locus (SE)
Var. *macrosperma*	12.8	33.3	0.104 (0.021)
Var. *microsperma*	12.9	16.7	0.037 (0.011)
Ssp. *orientalis*	7.0	38.3	0.150 (0.026)
L. odemensis	7.0	30.0	0.116 (0.02)
L. nigricans	7.0	63.3	0.229 (0.025)
L. ervoides	6.9	55.0	0.186 (0.024)

Table 4 Estimate of Nei's genetic identities based on 60 RAPD loci in six populations of the genus *Lens*

Population	1	2	3	4	5	6
1. Var. *macrosperma*						
2. Var. *microsperma*	0.897					
3. Ssp. *orientalis*	0.894	0.875				
4. *L. odemensis*	0.662	0.628	0.715			
5. *L. nigricans*	0.750	0.699	0.765	0.604		
6. *L. ervoides*	0.596	0.543	0.648	0.547	0.818	

the traditional taxonomic views of the genus (Ladizinsky 1993) and cpDNA-based phylogeny (Mayer and Soltis 1994), indicating that, despite reservations regarding the utility of arbitrary primed polymorphisms in species phylogenetic analyses (Black 1993), RAPD markers can complement both conventional and established molecular markers in reconstructing the history of the genus *Lens*. Moreover, at the sub-species level, including between-varietal groups, RAPDs provide a degree of resolution unavailable with isozymes (Pinkas et al. 1985; Hoffman et al. 1986), nuclear RFLPs (Havey and Muehlbauer 1989a) or chloroplast RFLPs (Muench et al. 1991; Mayer and Soltis 1994).

A dendrogram obtained from individual pairwise comparisons of 88 RAPDs among all 54 genotypes is shown in Fig. 3. As expected, accessions from particular species, sub-species or varietal groups generally cluster tightly. With the exception of genotypes 4 (GL-259) and 13 (L-4076), the *macrosperma* genotypes formed a single cluster as did the *microsperma* genotypes which incorporated the former two genotypes 4 and 13. Placement of these two *macrosperma* genotypes with *microsperma* is not surprising as the introgression of large seed size into *microsperma* genotypes has been an objective of the Indian lentil-breeding programme from which many of these accessions were obtained. Genotype pairs 10/11 (L-4136/L-178), and 18/21 (L-4191/L-4134) were found to be genetically identical, as were genotypes 15 (L-3044), 16 (DPL-119), 25 (L-4661) and 26 (L-303). There are several potential explanations for this observation. The 'All India Pulses Improvement Programme', of which lentil breeding is a part, currently evaluates and selects the same material at several agro-climatic zones in the country, followed by the development of cultivars or breeding lines according to local needs. It is likely, therefore, that genotypes with different names may have a high degree of common ancestry and therefore cannot be differentiated in the current study. Alternatively, genotypes with different numbers may represent duplications in the germplasm collections or simply mislabelled accessions.

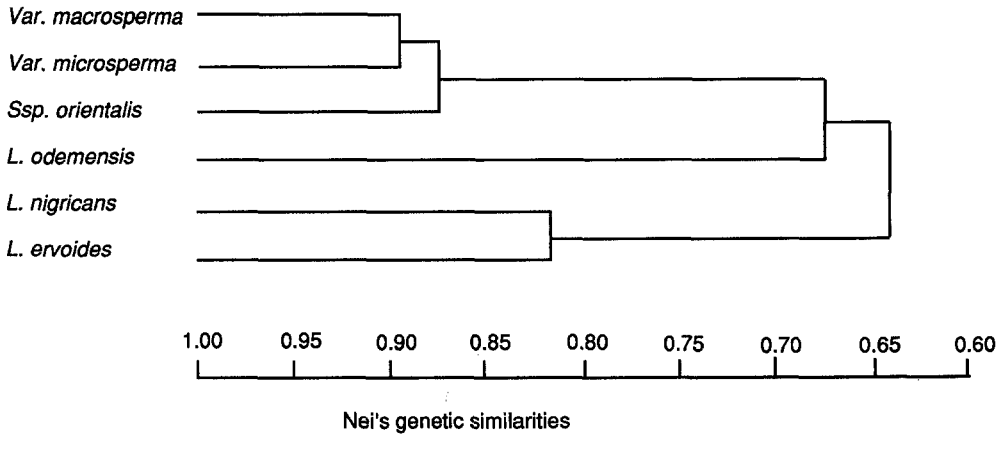


Fig. 2 Dendrogram of six populations of the genus *Lens* based on Nei's genetic identity for 60 RAPD products

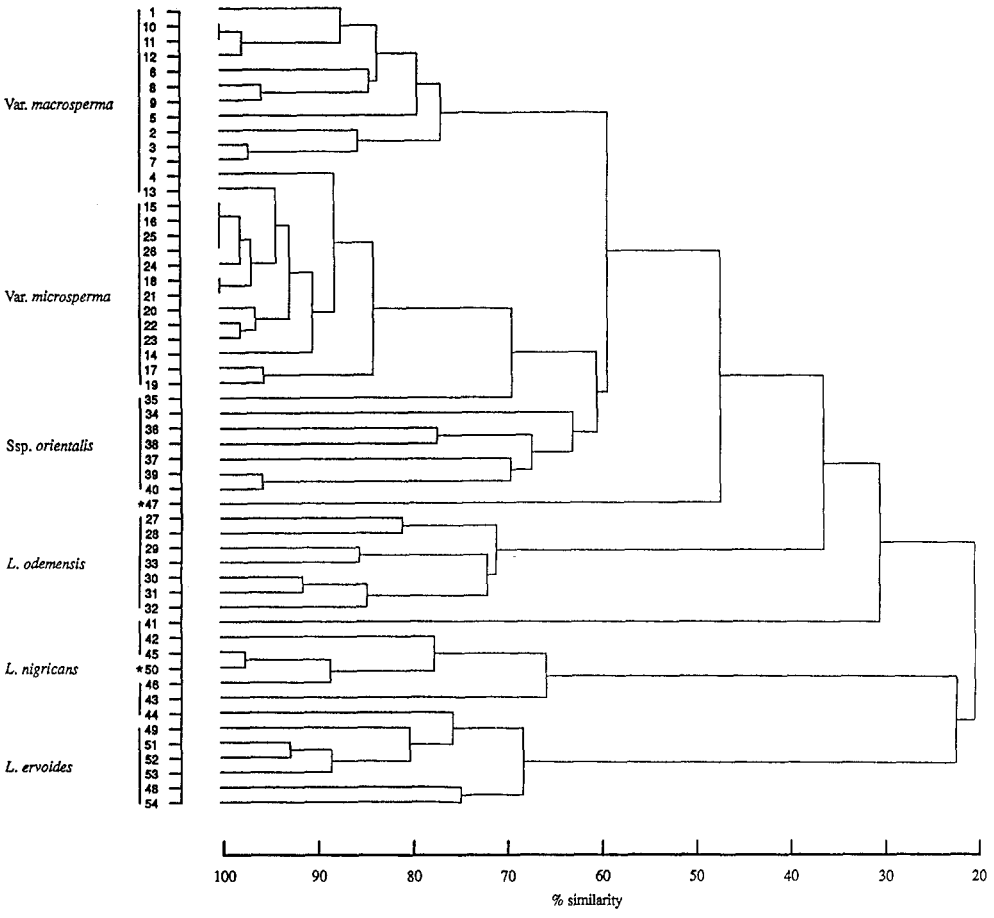


Fig. 3 Dendrogram of 54 genotypes of the genus *Lens* based on similarity for 88 RAPD products. *47 = *L. nigricans*; *50 = *L. ervoides*

Of the seven genotypes sampled from ssp. *orientalis* (34–40), genotype 35 clustered with *microsperma* at 70% similarity, while the remaining six genotypes formed a separate cluster joining vars. *macrosperma* and *microsperma* at 60% similarity. These results provide further credence to our earlier conclusion that both varietal groups of cultivated lentil and ssp. *orientalis* may have evolved simultaneously from a common ancestor. The seven genotypes of *L. odemensis* (27–33) formed a distinct cluster which joined the *L. culinaris* cluster at 35% similarity, confirming grouped analysis (Fig. 2) which supported its elevation to an independent species. Barring genotypes 41, 44 and 47, the remaining *L. nigricans* genotypes (42, 43, 45 and 46) formed one cluster, which joined the *L. ervoides* cluster and then the *L. odemensis* cluster at 20% similarity. This correlated with the greater degree of similarity between *L. nigricans* and *L. ervoides* than with *L. odemensis* observed from the cluster analysis of frequency data (Fig. 2). The disparity between clustering levels in the two analyses indicates that, despite overall levels of product commonality among related groups, individual accessions taken from different groups share only a subset of these common products; this applies particularly in *L. nigricans* and *L. ervoides* where interspecific variation appears relatively high (Table 1). This is reflected in the placement of individual accessions such as genotypes 47 and 41 of *L. nigricans*, which remain independent from all major clusters. In addition, *L. nigricans* genotype 44 associated with the *L. ervoides* cluster and *L. ervoides* accession 50 with the *L. nigricans cluster.*

In order to assess whether the grouping of individuals based on RAPDs could be further resolved, principal component analysis (PCA) was used to examine the shared-fragment data available among individual genotypes (Fig. 4). The first two principal components accounted for 43% of the total variation observed. Apart from accession 50, genotypes generally grouped according to their supposedly related taxa, although the distinctness of the groupings varied. As anticipated, var. *microsperma*, var. *macrosperma* and ssp. *orientalis* displayed close similarity. In addition the *L. nigricans* and *L. ervoides* groups appeared relatively close, while *L. odemensis* appeared distinct. In general terms, PCA provided a clear separation of the different species, sub-species and varietal groups, and supported the conclusions drawn from the previous forms of analysis. The relative utility of ordination for describing relationships between accessions nevertheless depends on the level of resolution desired. While clustering provided the best indication of relationship among closely related accessions, ordination (here using only the first two principal

co-ordinates) appeared to provide a more complete representation of the relationships among major groups.

"Band map", an alternative graphical method for presenting DNA marker data, which facilitates the identification of markers specific to certain taxa, is shown in Fig. 5. The advantages of this form of data presentation have been described previously. (Powell et al. 1991). Band frequencies are given in one margin and corresponding band numbers in the other. The presence of an amplification product is represented by a filled box and the genotype re-ordering is exactly that generated on single linkage cluster analysis (Digby and Kempton 1987) (i.e. as in Fig. 3). Thus, 'band map' graphically illustrates the actual product frequencies and their occurrence in the different accessions studied. Although further experimentation on a greater number of individuals would be required to determine the absolute specificity of loci to particular taxa, the following information is presented heuristically to demonstrate how RAPDs may be applied for discrimination purposes. Within cultivated lentils one locus, SC10-79-L1000 (band 63), was specific to var. *microsperma* (except for including genotype 13 of *macrosperma*), while another, OPG-13-L600 (band 4), was speicific to var. *macrosperma*, excluding genotypes 4 and 13. Among all accessions tested, the loci OPU-16-L500 (band 74), SC10-76-L250 (band 44), OPG-9-L1050 (band 47), SC10-77-L300 (band 9), SC10-53-L650 (band 23) and SC10-23-L300 (band 37) were specific to *L. odemensis*, while loci SC10-93-L900 (band 80), SC10-65-L600 (band 45), SC10-23-L800 (band 34) and OPR-3-300 (band 88) were specific to all genotypes of *L. ervoides* except accession 50, but including accession 44 of *L. nigricans*. Ssp. *orientalis* shared many loci with *macrosperma* and *microsperma*, but no loci specific to ssp. *orientalis* were noted. Similarly, no loci specific to *L. nigricans* were observed. The main reason in the latter case was that three (41, 44 and 47) out of the seven genotypes were not grouped closely within a supposedly related taxon. The presence of loci SC10-93-L900 (band 80), SC10-65-L600 (band 45), SC10-23-L800 (band 34) and OPR-3-L300 (band 88) in genotype 44, specific to *L. ervoides*, indicated that this accession should be grouped with the former rather than with *L. nigricans*. Conversely, the presence of the RAPD loci SC10-94-L900 (band 82) and SC10-84-L450 (band 19) in genotype 50, which are specific to four *L. nigricans* genotypes, suggests that this genotype belongs to the *L. nigricans* group rather than *L. ervoides*. Genotype 47 of *L. nigricans* possessed several RAPD loci shared by ssp. *orientalis* and other varietal groups of cultivated lentils, suggesting an origin in ssp. *orientalis* rather than *L. nigricans*. RAPD loci present in genotype 41, however, were not found to be consistently matched with any of the taxa studied and may therefore suggest an independent origin.

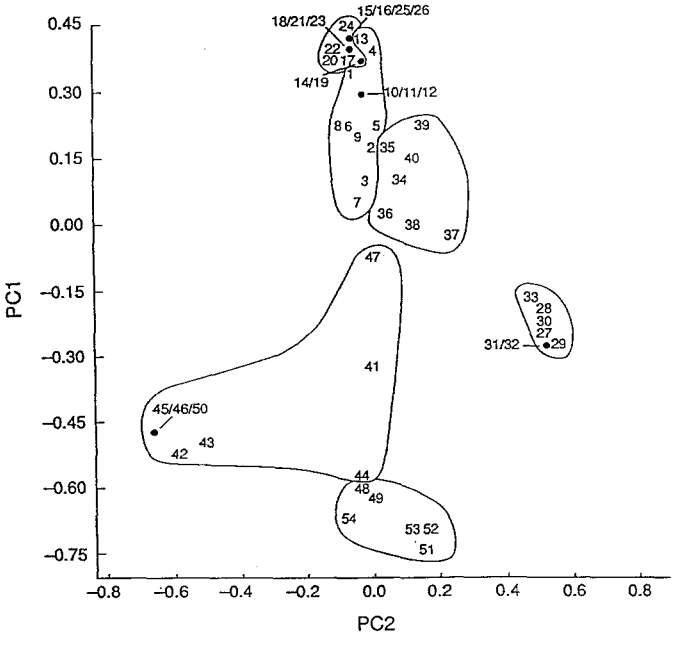
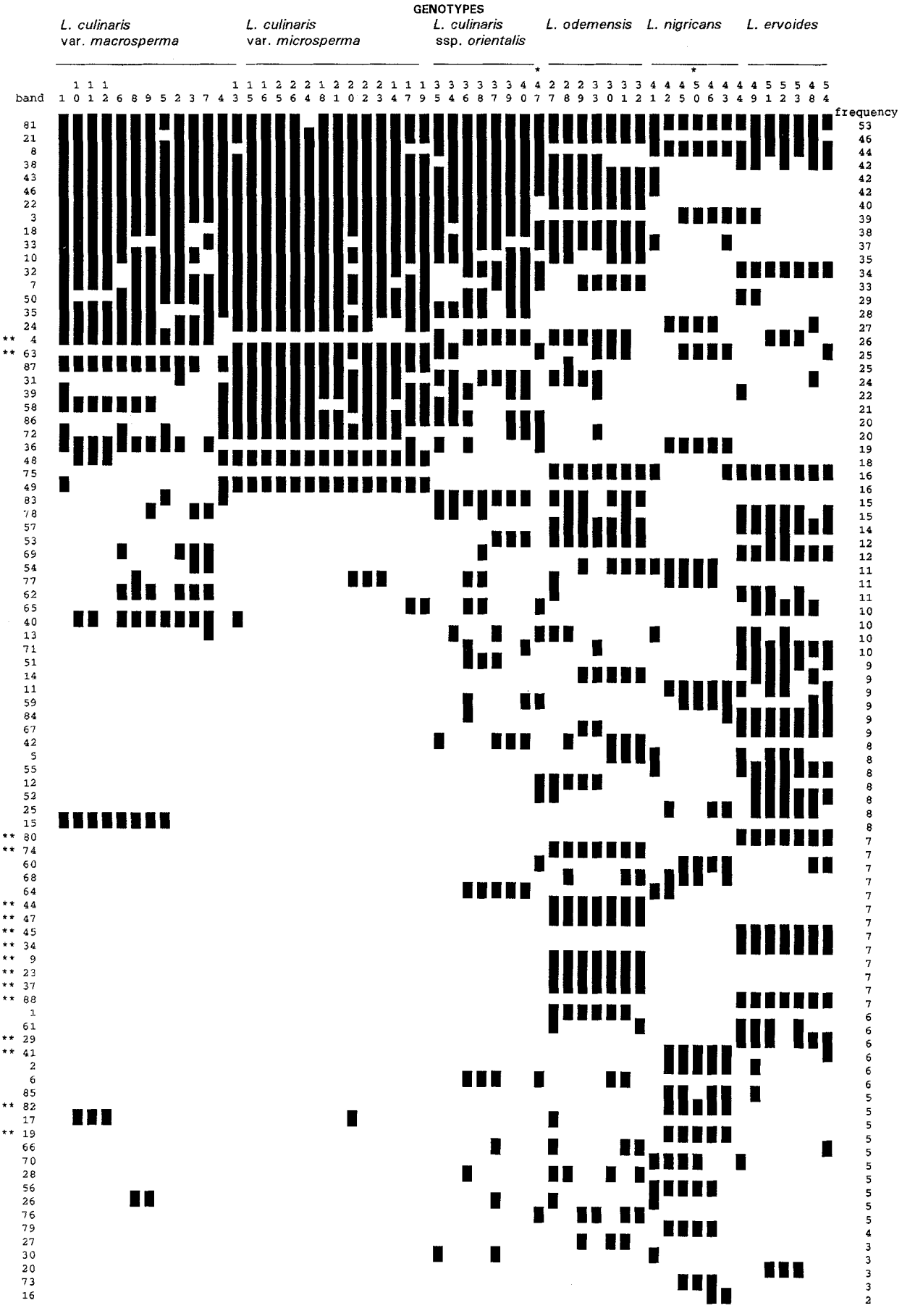


Fig. 4 Principle co-ordinate analysis of 54 genotypes of the genus *Lens* for 88 RAPD products. Genotypes *1–13* = var. *macrosperma*, *14–26* = var. *microsperma*, *27–33* = *L. odemensis*, *34–40* = ssp. *orientalis*, *41–47* = *L. nigricans* and *48–54* = *L. ervoides*

Fig. 5 Band map of shared RAPD products among 54 accessions from the genus *Lens*. *47 = *L. nigricans*; *50 = *L. ervoides*. **Bands mentioned in the text

The presence of unique composite RAPD genotypes among the majority of *Lens* accessions indicates the utility of the approach for fingerprinting purposes. RAPD fingerprinting has a number of potential applications and includes the determination of cultivar purity, efficient use and management of genetic resource collections, particularly the identification of mis-labelled accessions, and the establishment of property rights (plant variety protection and patenting).

A major goal in lentil research remains the construction of detailed genetic linkage maps. However, in order to detect sufficient polymorphism, previous work using RFLPs, isozymes and morphological markers has focused on interspecific crosses between cultivated lentils and wild species or sub-species (Zamir and Tadmor 1986; Havey and Muehlbauer 1989b; Muehlbauer et al. 1989; Weeden et al. 1992). In these crosses the segregation of markers often deviated significantly from expected ratios. The results described here suggest that, in addition to providing important information on the evolutionary history of *Lens*, the increased resolution associated with the larger number of potential arbitrary primers available to the RAPD approach may provide sufficient markers to construct genetic linkage maps between carefully chosen cultivated lentil accessions. Skewed segregation could thus be avoided and linkage data of more immediate relevance to lentil breeding be provided.


GENOTYPES
L. culinaris var. macrosperma
L. culinaris var. microsperma
L. culinaris ssp. orientalis
L. odemensis
L. nigricans
L. ervoides
band
frequency

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