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Intra- and Inter-specific variations in *Lens* revealed by RAPD markers

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Abstract Randomly amplified polymorphic DNA (RAPD) markers were used to estimate intra- and inter-specific variations in the genus *Lens* (lentil). Twenty cultivars of *L. culinaris* ssp. *culinaris*, including 11 microsperma (small-seeded) and nine macrosperma (large-seeded) types, and 16 wild relatives (four accessions each of *L. culinaris* ssp. *orientalis*, *L. odemensis*, *L. nigricans* and *L. ervoides*), were evaluated for genetic variability using a set of 40 random 10-mer primers. Fifty reproducibly scorable DNA bands were observed from ten of the primers, 90% of which were polymorphic. Genetic distances between each of the accessions were calculated from simple matching coefficients. A dendrogram showing genetic relationships between them was constructed by an unweighted pair-group method with arithmetical averages (UPGMA). This study revealed that (1) expect for *L. ervoides*, the level of intraspecific variation in cultivated lentil is lower than that in wild species, (2) *L. culinaris* ssp. *orientalis* is the most likely candidate for a progenitor of the cultivated species, and (3) microsperma and macrosperma cultivars were indistinguishable by the RAPD markers identified here.

Key words *Lens culinaris* · Lentil · RAPD Genetic distance · Intra- and inter-specific variation

Introduction

Lentil (*Lens culinaris* ssp. *culinaris*) is a grain legume that is valued as a high-protein food in the Middle East, northern Africa, southern Europe, and North and South America. Two types of seed size have been recognized in lentil, i.e., the large-seeded (macrosperma) and small-seeded (microsperma) types (Barulina 1930). Based on crossability and cytological studies, the genus *Lens* was classified into two species, *L. culinaris* and *L. nigricans* by Ladizinsky et al. (1984). The former contains three subspecies, ssp *culinaris* (cultivated lentil), ssp. *orientalis* and ssp. *odemensis*, while the latter includes two subspecies, ssp. *nigricans* and ssp. *ervoides*. Wild species are known to be distributed over western Asia, northern Africa and the Mediteranean region (Ladizinsky 1993). Ladizinsky (1993) has recently revised the taxonomy of *Lens* according to additional information derived mainly from studies on isozyme markers (Pinkas et al. 1985; Hoffman et al. 1986) and nuclear DNA restriction fragment length polymorphisms (RELPs) (Havey and Muehlbauer 1989). In his proposal, all subspecies are elevated to species status except for *culinaris* and *orientalis*, which are retained as subspecies under *L. culinaris*.

RFLP technology on nuclear or organellar DNA is a powerful tool in the estimation of phylogenetic relationships in plant species, e.g., nuclear DNA in *Brassica* (Song et al. 1988) and organellar DNA in *Avena* (Murai and Tsunewaki 1986). In *Lens*, Havey and Muehlbauer (1989) analyzed phylogenetic relationships using nuclear DNA RFLPs, while chloroplast DNA (cpDNA) phylogeny was clarified by Muench et al. (1991) and Mayer and Soltis (1994). Their results established *L. culinaris* ssp. *orientalis* as a progenitor to the cultivated lentil; a conclusion supported by morphological and cytological studies (Ladizinsky 1979a; Ladizinsky et al. 1984).

The development and application of randomly amplified polymorphic DNA (RAPD) markers generated by the polymerase chain reaction (PCR) using arbitrary primers has resulted in alternative molecular markers for the detection of nuclear DNA polymorphisms (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 et al.

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1993), *Allium* (Wilkie et al. 1993), and *Populus* (Castiglione et al. 1993). Furthermore, RAPD markers can be used to detect genetic variation at the intraspecific level between closely related cultivars (Kresovich et al. 1992).

In the present study, we have used RAPD markers to detect intra- as well as inter-specific variations in the genus *Lens*. Based on the intra- and inter-specific relationships of *Lens* accessions, a dendrogram has been constructed to obtain definitive information on the origin of the cultivated lentil, and to discriminate between cultivars.

Materials and methods

Plant material

Twenty cultivars (11 microsperma and nine macrosperma) of cultivated lentil (*L. culinaris* ssp. *culinaris*), and four accessions of wild lentil (*L. culinaris* ssp. *orientalis*, *L. odemensis*, *L. nigricans* and *L. ervoides*), were used in this study (Table 1).

Genomic DNA isolation

DNA was isolated from fresh leaf material using the modified CTAB procedure reported by Murray and Thompson (1980). Leaf material (0.5–3.0 g) was ground to a fine powder in liquid nitrogen, mixed with 10 ml of 2 x CTAB [20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 2% cetyl-trimetyl ammonium bromide and 0.2% 2-mercaptoethanol], and incubated at 60 °C for 30 min. The samples were then mixed with an equal volume of chloroform-isoamyl alcohol (24 : 1) for 15 min and centrifuged at 3,500 rpm for 15 min. The resulting supernatant was recovered and mixed with a 2/3 vol of cold isopropyl alcohol. The crude DNA was spooled out with a glass rod, suspended in 1 ml of TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA], and treated with RNase A (Sigma, USA; 10 µg/ml at the final concentration) at 37 °C for 45 min. The DNA was purified by the phenol-chloroform extraction method and precipitated with ethanol. The final pellet was dissolved in TE buffer. A set of DNA stock solutions was prepared for PCR at a final concentration of 20 ng/µl.

RAPD amplification

Forty 10-mer oligonucleotides (OPA-01 to 20 and OPB-01 to 20), obtained from Operon Technologies Inc. (USA), were tested as single primers for the amplification of RAPD sequences. Ten primers that showed relatively clear RAPDs were then used for further experiments (Table 2). Amplification reaction volumes were 20 µl, each containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton x-100, 1.5 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP (Takara-shuzo, Japan), 200 nM primer, 0.5 units of *Taq* DNA polymerase (Toyobo, Japan) and 10 ng of genomic DNA. Reaction mixtures were overlaid with 20 µl of mineral oil, and incubated in a thermal cycler (Program Temp Control System PC-700, Astec, Japan) programmed as follows: initial strand separation at 94 °C

Table 1 Cultivated and wild *Lens* accessions used in this study

No.	Species	Subspecies	Type[a]	Cultivar/line	Source[b]
1	*L. culinaris*	*culinaris*	Micro	Giza 9	Assiut
2	*L. culinaris*	*culinaris*	Micro	Giza 9m[c]	Assiut
3	*L. culinaris*	*culinaris*	Micro	Giza 9y[c]	Assiut
4	*L. culinaris*	*culinaris*	Micro	Eston	Saskatch.
5	*L. culinaris*	*culinaris*	Micro	ILL6004	ICARDA
6	*L. culinaris*	*culinaris*	Micro	ILL6005	ICARDA
7	*L. culinaris*	*culinaris*	Micro	ILL5753	ICARDA
8	*L. culinaris*	*culinaris*	Micro	ILL5698	ICARDA
9	*L. culinaris*	*culinaris*	Micro	Syrian Local Check	ICARDA
10	*L. culinaris*	*culinaris*	Micro	Crimson	USDA1
11	*L. culinaris*	*culinaris*	Micro	WA8649090	USDA1
12	*L. culinaris*	*culinaris*	Macro	Laird	Saskatch.
13	*L. culinaris*	*culinaris*	Macro	Brewer	USDA1
14	*L. culinaris*	*culinaris*	Macro	Chilean	USDA2
15	*L. culinaris*	*culinaris*	Macro	Redchief	USDA2
16	*L. culinaris*	*culinaris*	Macro	VW000421	USDA2
17	*L. culinaris*	*culinaris*	Macro	VW000440	USDA2
18	*L. culinaris*	*culinaris*	Macro	VW000504	USDA2
19	*L. culinaris*	*culinaris*	Macro	VW000563	USDA2
20	*L. culinaris*	*culinaris*	Macro	PY370630	USDA2
21	*L. culinaris*	*orientalis*		ILWL#3	ICARDA
22	*L. culinaris*	*orientalis*		ILWL#11	ICARDA
23	*L. culinaris*	*orientalis*		Lo3	USDA1
24	*L. culinaris*	*orientalis*		Lo10	USDA1
25	*L. odemensis*			ILWL#36	ICARDA
26	*L. odemensis*			ILWL#322	ICARDA
27	*L. odemensis*			Ld61	USDA1
28	*L. odemensis*			Ld84	USDA1
29	*L. nigricans*			ILWL#14	ICARDA
30	*L. nigricans*			ILWL#19	ICARDA
31	*L. nigricans*			Ln27	USDA1
32	*L. nigricans*			Ln34	USDA1
33	*L. ervoides*			ILWL#2	ICARDA
34	*L. ervoides*			ILWL#42	ICARDA
35	*L. ervoides*			Le35	USDA1
36	*L. ervoides*			Le52	USDA1

[a] Micro: microsperma (small-seeded) type
Macro: macrosperma (large-seeded) type

[b] Assiut: Agronomy Department, Assiut University, Egypt
Saskatch: Department of Crop Science and Plant Ecology, Univesity of Saskatchewan, Canada
ICARDA: The International Center for Agricultural Research in the Dry Areas
USDA1: Grain Legume Genetics and Physiology Research, USA
USDA2: The Association of Legume Breeding and Production Station, USA

[c] Two mutant lines of Giza 9 showing black mottle (m) and yellow (y) in seed color

Table 2 10-mer oligonucleotides used as primers in the RAPD assay

No.	Sequence (5′–3′)	No. of informative RAPDs
OPA-01	CAGGCCCTTC	3
OPA-10	GTGATCGCAG	5
OPA-11	CAATCGCCGT	4
OPA-12	TCGGCGATAG	3
OPA-17	GACCGCTTGT	4
OPB-05	TGCGCCCTTC	4
OPB-07	GGTGACGCAG	7
OPB-08	GTCCACACGG	8
OPB-10	CTGCTGGGAC	4
OPB-11	GTAGACCCGT	8
Total		50

(3 min), 44 cycles of 36 °C (1 min)/72 °C (2 min)/94 °C (1 min), final extension at 72 °C (5 min). Amplification products were electrophoresed in 1.5% agarose gels and detected by staining with ethidium bromide. DNA fragment mixtures of *Hin*dIII/*Eco*RI-digested lambda DNA and *Hae*III-digested ϕx174 DNA (Nippon gene, Japan) were used as size markers.

Data analysis

RAPD assays were performed in duplicate and only those patterns obtained clearly twice were scored. Pair-wise comparisons of accessions, based on the presence or absence of unique and shared fragments, were used to generate simple matching coefficient (S_{sm}). The genetic distance (d) was calculated as

$$d = \sqrt{1 - S_{sm}}$$

and was used to construct a dendrogram by an unweighted pair-group method with arithmetical averages (UPGMA) (Sneath and Sokal 1973).

Results

The effects of different template DNA concentrations (5, 10 and 20 ng/20 µl reaction mixture) and annealing temperatures (36, 37 and 38 °C) were examined using five *Lens* accessions, nos. 1, 12, 21, 25, 29 and 33, which represent each species or subspecies/types. Based on the criterion of showing simple clear banding patterns, optimum conditions of template DNA concentration (10 ng/20 µl reaction mixtures) and annealing temperature (36 °C) were selected.

A total of 40 10-mer primers (OPA-01 to 20 and OPB-01 to 20) were tested in each of five *Lens* accessions, nos. 1, 12, 21, 25, 29 and 33, to examine RAPD patterns. Ten primers (Table 2) that produced relatively simple banding patterns were ultimately selected for further evaluation. Using these ten primers, RAPD assay was performed in 36 *Lens* accessions. A total of 50 reproducible fragments were amplified, 90% of which showed polymorphism. Examples of typical RAPD patterns are shown in Fig. 1. The number of polymorphisms identified varied with the primer used, e.g., primer OPB-08 yielded high polymorphism for many accessions (Fig. 1 A) while primer OPB-11 detected a high degree of polymorphism in wild accessions but no polymorphism between the cultivars (Fig. 1 B).

Means and ranges of genetic distances within *Lens* species, as revealed by RAPD analysis, are shown in Table 3. In comparison with the intraspecific variation of cultivated lentil (0.000–0.316), the wild lentils, *L. culinaris* ssp. *orientalis*, *L. odemensis* and *L. nigricans*, showed wide intraspecific variation. In contrast, *L. ervoides* (wild lentil) exhibited a low level of intraspecific variation (0.000–0.200), indicating that the *L. ervoides* accessions tested here are genetically similar to each other. Means and ranges of interspecific variation are shown in Table 4. The genetic distance ranged from 0.324 for the most closely related species, *L. culinaris* ssp. *culinaris* (cultivated lentil) and ssp. *orientalis*, to 0.630 for the most distantly related species, cultivated lentil, and *L. ervoides*.

Cluster analysis of the genetic distance values was performd to generate a dendrogram showing genetic relationships within and between *Lens* species (Fig. 2). In this dendrogram, the location of bifurcations separating different accessions is a measure of the genetic distance between them. *L. culinaris* ssp. *culinaris* (cultivated lentil) and ssp. *orientalis* clustered on a single large branch, suggesting that ssp. *orientalis* has a genetic similarity to cultivated lentil. In the cultivated lentil, microsperma and macrosperma types were clustered in the same group, indicating that they were not differentiated in genetic background, as revealed by RAPD markers.

Discussion

The main objective of this study was to detect intra- as well as inter-specific variations in *Lens*. RAPD markers can be of great value in the measurement of intraspecific variation as revealed in *Brassica oleracea* (Kresovich et al. 1992), *Lycopersicon esculentum* (Williams and Clair 1993) and *Brassica juncea* (Jain et al. 1994). Our study demonstrated that RAPD markers can be also used to identify intraspecific polymorphisms within *Lens* species. As compared with wild species (except for *L. eovoides*), the low degree of intraspecific variation in the cultivated lentil suggests that the cultivated species has passed through a genetic bottleneck during domestication. This conclusion is supported by previous results using cpDNA analysis, which demonstrated that 112 out of 114 cultivated accessions showed identical cpDNA RFLP patterns (Mayer and Soltis 1994). The low values of genetic distance observed in *L. ervoides* may reflect narrow intraspecific variation in this species. Mayer and Soltis (1994) studied cpDNA diversity of three other *L. ervoides* accessions and found that the three accessions were differentiated from each other by three mutations in cpDNA. In contrast to our observation, their result indicates that *L. ervoides* has wide genetic variation. Because the sample size of our study

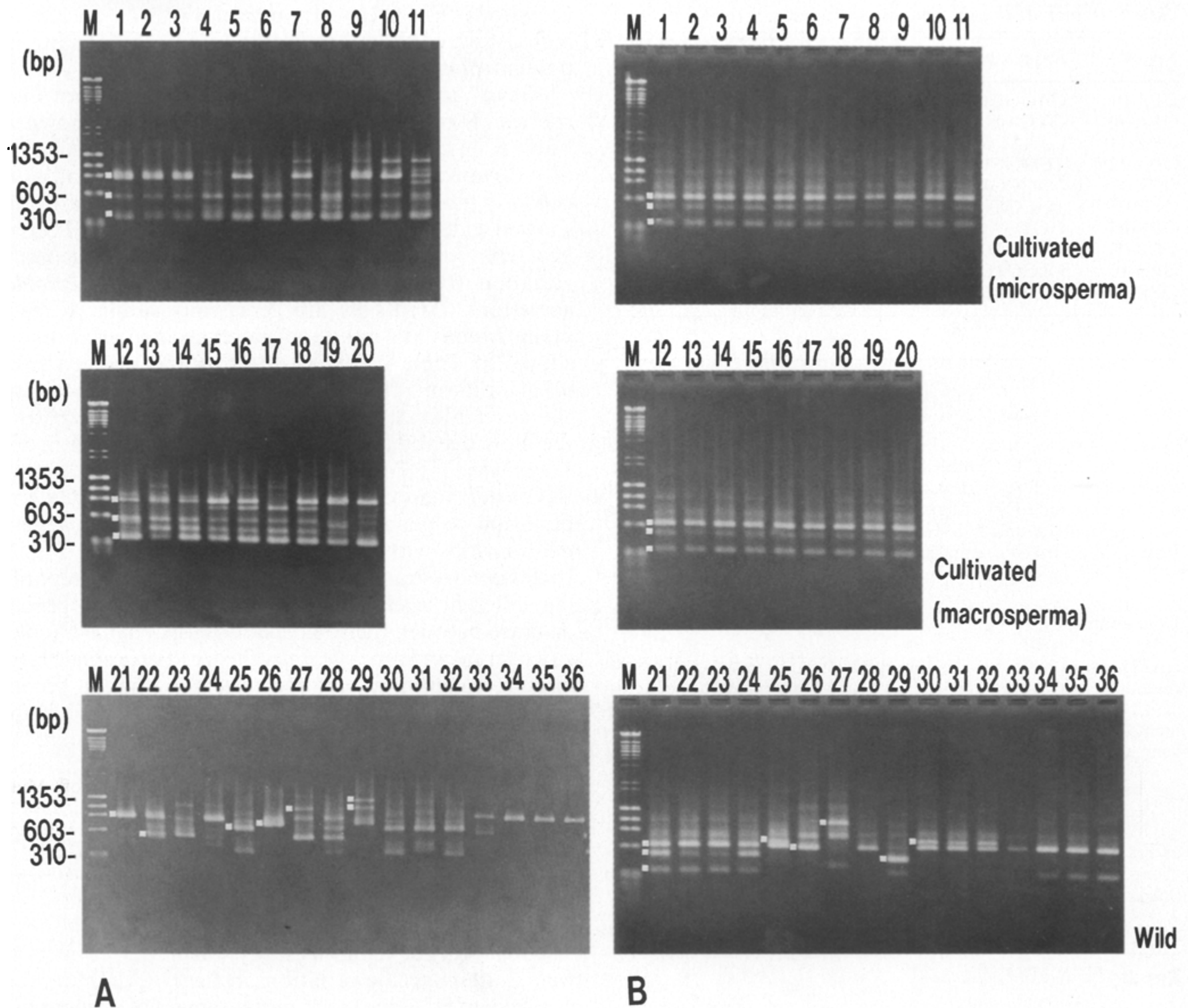

Fig. 1 RAPD patterns of 36 *Lens* accessions generated by primers OPB-08 (**A**) and OPB-11 (**B**). Numbers 1–36 represent the accessions listed in Table 1. *M* indicates molecular-size marker. Scorable RAPDs are marked by ■ in the accession with the youngest number at each panel

Table 3 Genetic distance among *Lens* species based on RAPD markers

Species	Subspecies	Mean	Range
L. culinaris	*culinaris*	0.191	0.000–0.316
	(microsperma	0.202	0.000–0.316)
	(macrosperma	0.157	0.000–0.245)
L. culinaris	*orientalis*	0.297	0.245–0.346
L. odemensis		0.450	0.316–0.566
L. nigricans		0.353	0.000–0.548
L. ervoides		0.167	0.000–0.200

was small, a more detailed analysis using a larger number of accessions is necessary to clarify intraspecific variation in *L. ervoides*.

RAPD analysis enabled us to construct a dendrogram showing intra- and inter-specific relationships in genus *Lens*. The dendrogram indicates that *L. culinaris* ssp. *orientalis* is most closely related to the cultivated lentil (ssp. *culinaris*), e.g., accession Lo10 of ssp. *orientalis* is clustered within the cultivated species. These results definitely demonstrate that ssp. *orientalis* is the progenitor of cultivated lentil, a conclusion that is supported by the results of previous studies on morphological, cytological (Ladizinsky 1979a; Ladizinsky et al. 1984), as well as isozyme, markers (Pinkas et al. 1985; Hoffman et al. 1986) and RFLPs of nuclear DNA (Havey and Muehlbauer 1989) and cpDNA (Muench et al. 1991; Mayer and Soltis 1994). Cluster analysis also revealed that all accessions belonging to the wild species, *L. culinaris* ssp. *orientalis*, *L. odemensis* or *L. nigricans*, were not clustered on the same branch, suggesting a greater degree of intraspecific variations within these wild species.

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

Species/ subspecies	*culinaris*		*orientalis*	*odemensis*	*nigricans*	*ervoides*
	microsperma	macrosperma				
culinaris micro	–	0.197	0.341	0.570	0.581	0.630
macro	0.000–0.316	–	0.324	0.562	0.575	0.623
orientalis	0.200–0.400	0.200–0.400	–	0.499	0.509	0.552
odemensis	0.510–0.616	0.510–0.616	0.447–0.548	–	0.479	0.581
nigricans	0.529–0.616	0.529–0.616	0.447–0.548	0.316–0.548	–	0.580
ervoides	0.583–0.663	0.583–0.663	0.510–0.600	0.548–0.616	0.469–0.632	–

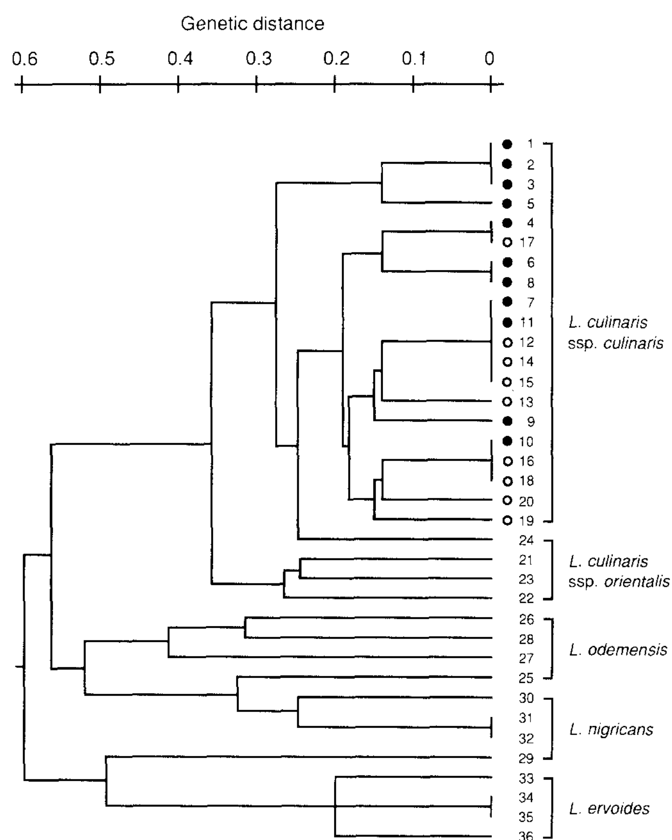

Fig. 2 Dendrogram of genetic relationships between 36 cultivated and wild *Lens* accessions. Numbers 1–36 represent the accessions listed in Table 1. ● and ○ indicate microsperma (small-seeded) and macrosperma (large-seeded) cultivars, respectively. The scale indicates the genetic distance

RAPD markers are useful tools to discriminate among cultivars in several crops, e.g., rice (Fukuoka et al. 1992; Yu and Nguyen 1994), papaya (Stiles et al. 1993), apple (Koller et al. 1993), barley (Tinker et al. 1993), potato (Mori et al. 1993), and rapeseed (Mailer et al. 1994). Our study has also demonstrated that the RAPD technique can be applied to discriminate among lentil cultivars. However, several cultivars derived from different sources, e.g., ILL5753 (ICARDA), Laird (Canada) and Redchief (USDA), showed identical RAPD patterns. These facts indicate that genetic variation within the cultivated lentil is narrow, and it will be necessary to search for other RAPD markers to discriminate between them. Barulina (1930) distinguished two subspecies within the cultivated lentil, i.e., ssp *macrosperma*, large-seeded type, and ssp *microsperma*, the small-seeded type. Based on morphological (Williams et al. 1974) and seed-protein (Ladizinsky 1979b) comparisons of the two types of *Lens* cultivars, there was no biological basis for separating the small- and large-seeded types into two subspecies. Our results clearly demonstrate that no genetic variability exists between microsperma and macrosperma except for the trait of seed size.

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