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Assessment of genetic variation and species relationships in a collection of *Lens* using RAPD and ISSR

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Abstract

The genetic variation of a collection of twenty-two *Lens* accessions was assessed using RAPD and ISSR markers. The collection included accessions of the cultivated lentil, *Lens culinaris* ssp. *culinaris*, and its wild ancestor *L. c.* ssp. *orientalis*, and the other wild species of the genus: *L. odemensis*, *L. ervoides*, *L. nigricans*, *L. tomentosus*, and *L. lamottei*. Both types of markers produced a relatively high number of polymorphic markers in the whole collection, although the degree of variation within the cultivated materials was lower. ISSR markers produced on average more bands and useful polymorphisms than RAPD markers. The Fitch-Margoliash dendrogram, based on Jaccard indices taking jointly RAPD and ISSR into account, showed that the cultivated materials were grouped according to their macro- and microsperma type and their geographical origin, and was compatible with the hypothesis of the existence of six different species in the genus *Lens*, with *L. tomentosus* being the closest species to *L. culinaris*.

Key words: lentil, phylogeny, molecular markers, polymorphism.

Resumen

Estimación de la variación genética y de las relaciones entre especies en una colección de *Lens* mediante el uso de RAPD e ISSR

Se ha estimado la variación genética de una colección de veintidós accesiones de *Lens* mediante el uso de marcadores RAPD e ISSR. La colección incluía accesiones de lenteja cultivada, *Lens culinaris* ssp. *culinaris*, y de su ancestro silvestre *L. c.* ssp. *orientalis,* y otras de las especies silvestres de este género: *L. odemensis*, *L. ervoides*, *L. nigricans*, *L. tomentosus* y *L. lamottei*. Ambos tipos de marcadores rindieron un número relativamente alto de marcadores polimórficos en el conjunto de la colección, aunque el grado de variación entre los materiales cultivados fue menor. Los marcadores ISSR produjeron en promedio más marcadores y más polimorfismos por cebador que los marcadores RAPD. Un dendrograma según el método de Fitch-Margoliash, basado en índices Jaccard, y considerando conjuntamente los datos de RAPD e ISSR mostró que los materiales cultivados se agrupaban según el tipo micro- o macrosperma y su origen geográfico, y era compatible con la hipótesis de la existencia de seis especies diferentes en el género *Lens*, siendo *L. tomentosus* la especie más cercana a *L. culinaris*.

Palabras clave: lenteja, filogenia, marcadores moleculares, polimorfismos.

Introduction

Lentil is included in the cool season food legume group of pulses and is an important source of protein and fiber in human diet. Moreover, they are also valuable as feed and fodder for livestock, and play an important role in crop rotations because their nitrogen fixing capability. This crop was domesticated in the Fertile Crescent where it has been cultivated since at least the seventh century B.C. (Ladizinky, 1979), and its cultivation area expanded around the Mediterranean Basin, Middle East, Ethiopia and the Indian Subcontinent. The species is currently an important pulse grown in many areas of temperate climate such as the Mediterranean Basin, Central and Western Asia, and some areas in North and South America and Australia, where the crop is grown in semi-arid regions, usually in rotation with cereals.

The species *Lens culinaris* Medik. is included in the genus *Lens* Miller, tribe *Vicieae*, subfamily *Papilionacea*, family *Leguminosae*. The tribe *Vicieae* includes another three additional genera of agronomic interest,

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namely, *Vicia* L., *Lathyrus* L., and *Pisum* L. All the species of the genus *Lens* are diploid with 2n = 14 chromosomes. The number of species within the genus *Lens* and their phylogenetic relationships were reviewed several times on the basis of different criteria: morphological, hybridization ability, biochemical and molecular markers, chloroplast DNA (Ladizinsky, 1979, 1993; Cubero, 1984; Hoffman *et al.*, 1986; Rosa and Jouve, 1992; Mayer and Soltis, 1994; Ahmad and McNeil, 1996; Ferguson *et al.*, 2000). The species *L. tomentosus* has been recently added to the genus (Ladizinsky, 1997). The most generally accepted current taxonomy of the genus *Lens* includes six species, namely: *L. culinaris* Medik., with two subspecies *L. c.* ssp. *culinaris* (the cultivated lentil) and its wild ancestor *L. c.* ssp. *orientalis* (Boiss.) Ponert, *L. odemensis* (Godr.) Ladiz., *L. ervoides* (Bring.) Grande, *L. nigricans* (Bieb.) Godr., *L. tomentosus* Ladiz., and *L. lamottei* Czfr. (Van Oss *et al.*, 1997). However, Ferguson *et al.* (2000) only differentiated four species including *odemensis* and *tomentosus* as subspecies of *L. culinaris*. *L. lamottei* was first described by Czefranova (1971) and further identified by Ladizinsky among materials previously considered *L. nigricans* (Ladizinsky, 1997). Cultivated lentil, *L. culinaris* spp. *culinaris* Medik. (synonymous, *Lens esculenta* Moench), is classified on the basis of seed size as macrospermas or microspermas (Barulina, 1930), categories which have been considered as subspecies, races or varieties by different researchers and breeders.

Lentil is a naturally self-pollinated species due to its cleistogamous flowers, with a level of natural cross-pollination amounting to as low as 0.8% (Wilson and Law, 1972). Therefore, most of the individuals of this species are homozygous for most, if not all, of their genes, although wild populations and cultivated landraces can be formed by a combination of different genotypes (Alvarez *et al.*, 1997).

One of the most widely used DNA markers are RAPDs (random amplified polymorphic DNA), generally obtained by the random amplification of DNA sequences using 10-mer primers (Pérez de la Vega, 1997). Inter-simple sequence repeats (ISSR) are DNA markers which involve the direct use of microsatellite sequences as primers in the PCR (Gupta *et al.,* 1994). According to Zietkiewicz *et al.* (1994) and Ratnaparkhe *et al.* (1998) this technique is more reliable than the RAPD technique and generates larger numbers of polymorphisms per primer. ISSRs have been used in many crop species including legumes (Ratnaparkhe *et al.,* 1998; Bornet and Branchard, 2001; Iruela *et al.,* 2002; Rajesh *et al.,* 2003), and have proved their usefulness in genetic mapping and marker assisted selection (Zietkiewicz *et al.,* 1994; Ratnaparkhe *et al.,* 1998; Rubeena *et al.,* 2003). ISSR markers have already been used in lentil to evaluate genetic variation in collections of cultivated lentils (Závodná *et al.,* 2000; Sonante and Pignone, 2001).

Material and Methods

Plant material and DNA extraction

The materials used in this work are listed in Table 1. They include modern cultivars, land races and samples of wild species, hereafter all of them referred to as accessions. Since we were mainly interested in the degree of differentiation between accessions, we pooled equal amounts of DNA from each of 4 seedlings per sample to amplify RAPDs and ISSRs. This was based on the previous knowledge that the level of variation within accessions was relatively low in cultivated lentils (Alvarez *et al.*, 1997), and in that this method of pooling DNA is an appropriate strategy in assessing genetic variability in plant germplasm collections (Yu and Pauls, 1993; Gilbert *et al.*, 1999). DNA was extracted from fresh leaf tissue of 15-day-old seedlings as described by Edwards *et al.* (1991) with minor modifications.

Random amplified polymorphic DNA (RAPD) analysis

A total of 160 decamer primers (Operon Technologies, Alameda, Calif., USA; Kits A, B, C, D, G, H, P and W) were assayed, and 36 of them which produced easily observable and repeatable fragments were used in further assays. PCR amplifications were carried out following the procedure described by Abo-Elwafa *et al.* (1995) for lentil. RAPD products were separated on 1.8% agarose gels and bands were visualized with ethidium bromide.

Inter-simple sequence repeats (ISSR) analysis

One hundred ISSR primers (University of British Columbia Biotechnology Laboratory, primer set #9) were used to screen for polymorphisms. Finally, 14

Table 1. *Lens* materials used

Species	Variety/Accession	Status	Origin
L. culinaris ssp. *culinaris*	Alpo (m)	Cultivar	León (Spain)
	Armuña (M)	Land race	Salamanca (Spain)
	Castellana (M)	Land race	León (Spain)
	Diskiai (m)	Cultivar	Lithuania
	Lupa (m)	Cultivar	León (Spain)
	Mala (m)	Land race	Lanzarote (Canary Islands, Spain)
	Mosa (M)	Cultivar	León (Spain)
	Pardina (m)	Land race	León (Spain)
	Paula (m)	Cultivar	León (Spain)
	Tetir (m)	Land race	Fuerteventura (Canary Islands, Spain)
	Smelinuliai (m)	Cultivar	Lithuania
	Verdina (m)	Land race	León (Spain)
	ILL 5588 (m)	Land race	Jordan
L. culinaris ssp. *orientalis*	ILWL-7[2]	Wild	ICARDA (Syria)
	BG-16880[1]	Wild	Israel
L. odemensis	ILWL-39[2]	Wild	ICARDA (Syria)
	ILWL-235[2]	Wild	ICARDA (Syria)
	ILWL-252[2]	Wild	ICARDA (Syria)
L. nigricans	BG 16873[1]	Wild	Yugoslavia
*L. ervoide*s	BG 16877[1]	Wild	Israel
L. lamottei	244[3]	Wild	Morocco
L. tomentosus	133[3]	Wild	Turkey

M: macrosperma. m: microsperma. Samples: [1] from the Spanish Germplasm Bank, [2] from the Germplasm Bank of the International Centre for Agricultural Research in Dry Areas (ICARDA), [3] kindly provided by Prof. G. Ladizinsky.

primers which produced amplification patterns with the highest number of intense bands were chosen. The ISSR technique was performed as described by Ratnaparkhe *et al.* (1998) with minor modifications as follows: the reaction mixture (25 µl) contained 40 ng of template DNA, 10 pmol of oligonucleotide primer, *Taq*-DNA polymerase buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), 2 mM of $MgCl_2$, 0.25 mM each of the four dNTPs and 1 U of *Taq* DNA Polymerase (GIBCO BRL). The DNA was first denatured for 1 min at 94°C, followed by 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 50°C and 2 min elongation at 72°C, with a final elongation of 10 min at the same temperature. The reaction products were separated in 2.0% agarose gels and bands were visualized with ethidium bromide.

Statistical methods

The relationship between accessions was estimated using the Jaccard's similarity index (*SI*) (Sneath and Sokal, 1973), and transformed in distance as 1-*SI*. Dendrograms were obtained with the Fitch and Margoliash (1967) method. Bootstrap resampling (Efron and Gong, 1983) was used to estimate branch-length standard errors and the support values of nodes.

Results and Discussion

The estimation of the degree of differentiation between materials included in a crossing program is useful since it can help in selecting the different parents. However, for this purpose, the use of a high number of markers represents a considerable amount of work. We decided to use two types of dominant markers (RAPD and ISSR) to estimate the variation and the differences between a set of lentil material included in a crossing program and some samples of wild materials, some of which are also included in the crossing program. Both techniques yielded a high number of markers (Table 2) with a high degree of polymorphism in the whole collection: 91.3% of the RAPD bands and the 98.8% of the ISSR bands were polymorphic. Band sizes ranged

Table 2. Polymorphism of the two types of molecular markers

	Type of marker	
	RAPD	**ISSR**
Number of primers used	36	14
Number of bands	321	166
Average of bands per primer	8.9	11.8
Number of polymorphic bands	293	164
Average of polymorphic bands per primer	8.1	11.7

between 300 and 2,000 bp for RAPDs, and between 400 and 4,000 bp for ISSRs. The average of RAPD polymorphic markers per primer was 8.1, which is almost twice higher than the values described by Abo-Elwafa *et al.* (1995) and Sharma *et al.* (1995). This difference is most probably due to a more adequate selection of primers. On average ISSR markers produced more markers and more polymorphisms per primer than RAPD markers (Table 2). Thus, this result supports the previous reports of the suitability of ISSR in the assessment of plant genetic variation (Zietkiewicz *et al.,* 1994; Ratnaparkhe *et al.*, 1998). ISSR markers have been used to evaluate genetic variation within collections of cultivated lentils (Závodná *et al.,* 2000; Sonante and Pignone, 2001), but they have not been used in the assessment of the variability between wild *Lens* materials. The polymorphisms generated either by RAPDs or ISSRs were enough to differentiate each accession. The level of polymorphism is much lower in the cultivated materials than in the wild ones, as indicated by RAPD markers in previous works (Abo-Elwafa *et al.,* 1995; Sharma *et al.*, 1995; Alvarez *et al.*, 1997) and in this work. For instance, only a 40% of the RAPDS were polymorphic in Spanish lentil accessions (Alvarez *et al.*, 1997).

Both kinds of markers were used to estimate the similarity between accessions by the Jaccard similarity index. For RAPDs the similarity values ranged from 0.909 between Mosa and Castellana, two Spanish macrosperma samples, to 0.208 between *Lens nigricans* and *L. ervoides*; for ISSRs the values ranged from 0.929 between Pardina and Verdina, two microsperma samples from León (Spain), to 0.157 between *L. nigricans* and the sample ILWL 39 of *L. odemensis*. The correlation between the similarity value matrices calculated for RAPDs and for ISSRs was 0.969 ($p < 0.001$). This high correlation value points out that both kinds of markers are probably estimating the same type of genetic variability within the lentil genomes. The average RAPD and ISSR similarities between cultivated lentil accessions were similar, 0.768 and 0.758, respectively (Tables 3 and 4). The similarities between cultivated lentil for RAPDs ranged from 0.909 (between Mosa and

Table 3. Average similarity between accessions within and between species based on RAPDs

	culinaris	***orientalis***	***odemensis***	***nigricans***	***ervoides***	***tomentosus***	***lamottei***
culinaris	0.768 (0.075)						
orientalis	0.525 (0.028)	0.591					
odemensis	0.376 (0.019)	0.390 (0.015)	0.715 (0.020)				
nigricans	0.290 (0.011)	0.300 (0.011)	0.251 (0.006)	NA			
ervoides	0.345 (0.016)	0.367 (0.032)	0.324 (0.015)	0.208	NA		
tomentosus	0.461 (0.021)	0.472 (0.023)	0.358 (0.012)	0.256	0.339	NA	
lamottei	0.385 (0.021)	0.379 (0.003)	0.422 (0.006)	0.254	0.353	0.426	NA

Standard deviation in parentheses. NA: not applicable as there was a single accession.

Table 4. Average similarity between accessions within and between species based on ISSRs

	culinaris	***orientalis***	***odemensis***	***nigricans***	***ervoides***	***tomentosus***	***lamottei***
culinaris	0.758 (0.073)						
orientalis	0.571 (0.027)	0.657					
odemensis	0.301 (0.029)	0.306 (0.035)	0.702 (0.020)				
nigricans	0.261 (0.024)	0.300 (0.008)	0.168 (0.009)	NA			
ervoides	0.313 (0.015)	0.325 (0.020)	0.290 (0.030)	0.225	NA		
tomentosus	0.425 (0.024)	0.437 (0.011)	0.267 (0.016)	0.209	0.298	NA	
lamottei	0.251 (0.018)	0.305 (0.030)	0.280 (0.012)	0.222	0.306	0.255	NA

Standard deviation in parentheses. NA: not applicable as there was a single accession.

Castellana) to 0.586 (between Pardina and ILL 5588), and for ISSRs from 0.929 (between Pardina and Verdina) to 0.615 (also between Pardina and ILL 5588, as for RAPDs). The lowest similarities observed between samples of cultivated lentils were always higher than any similarity between the cultivated lentils and accessions of the wild ancestor *L. culinaris* ssp. *orientalis*.

Distances were used to obtain dendrograms. First, RAPD and ISSR distances were used separately obtaining dendrograms with similar clustering (not shown). In both cases there was a perfect discrimination at the sub-specific and specific levels. Considering the cultivated materials, *Lens culinaris* ssp. *culinaris*, RAPD marker data generated two clear clusters, one including the microsperma accessions and the other the macrosperma accessions, while ISSR marker data differentiated Spanish lentil accessions (both, from Continental Spain and from the Canary Islands) from

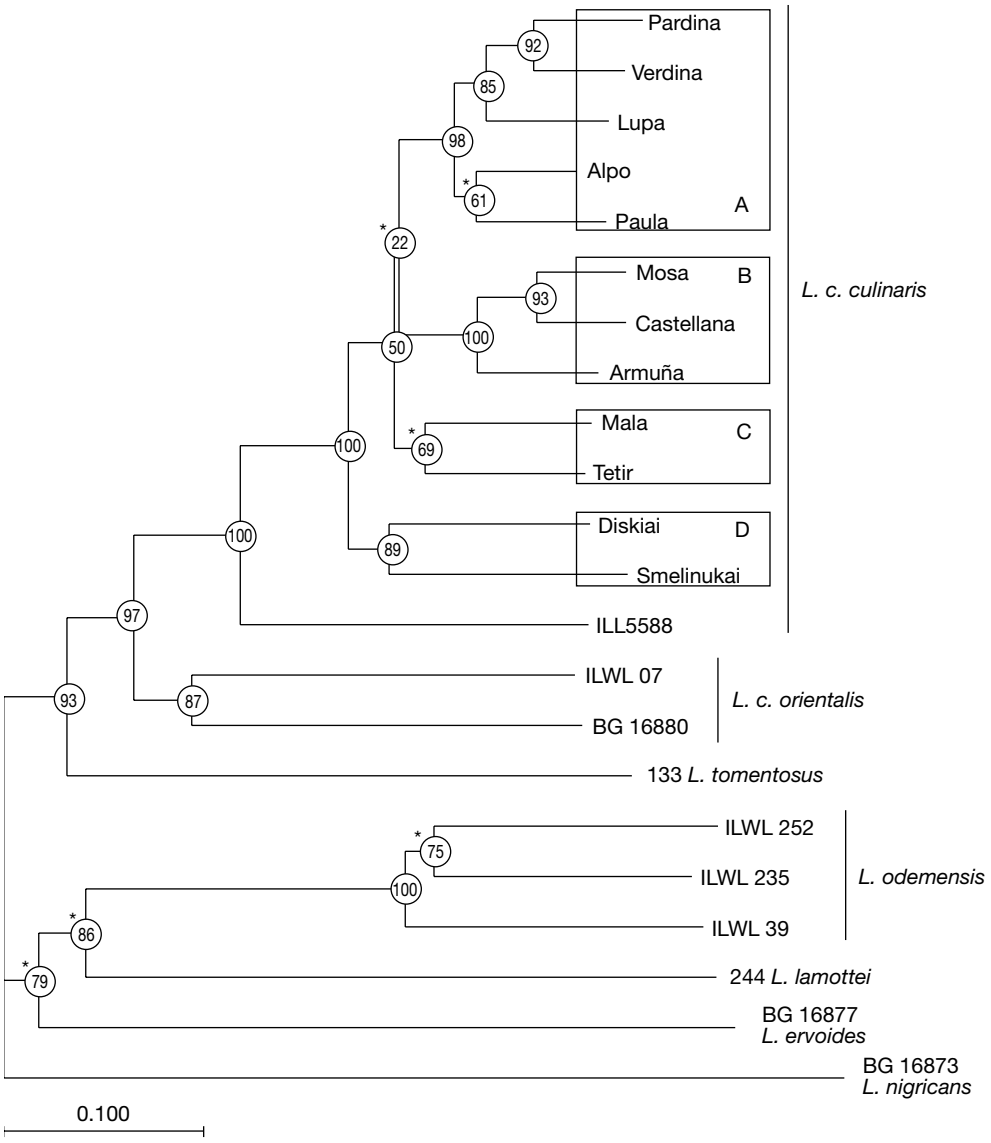

Figure 1. Dendrogram obtained using the Fitch-Margoliash method on the basis of Jaccard distances calculated considering jointly RAPDs and ISSRs. Figures on the nodes indicate the bootstrap values. Branches marked with and asterisk did not have a significantly different length from 0 with $p < 0.05$. All the samples inside box A are microspermas from mainland Spain, those in B are macrospermas from mainland Spain, those in C are microspermas from the Canary Islands, and those in D are microspermas from Lithuania.

non-Spanish accessions. The ability of RAPDs to discriminate between microsperma and macrosperma accessions has already been pointed out by Alvarez *et al.* (1997). Figure 1 shows the dendrogram obtained considering RAPD and ISSR marker data together. It can be observed that species and subspecies are differentiated in the dendrogram, that cultivated materials are clearly differentiated from the wild accession of *L. c*. ssp. *orientalis*, and that both *culinaris* subspecies are grouped in the same cluster together with *L. tomentosus*, the latter being a species morphologically similar to *L. c*. ssp. *orientalis* (Ferguson *et al*., 2000). The cultivated materials were grouped according to their macro- and microsperma type and their geographical origin (Fig. 1), although this result must be considered as provisional because some branch lengths were not significantly different from 0 and the values of some nodes were low. Ferguson (2000) pointed out that conflicting specific relations have emerged which appear to depend on the accessions and the techniques used in measuring genetic variation. The clustering of *L. tomentosus* with *L. culinaris* agreed with previous reports pointing out the taxonomic proximity between both species (Van Oss *et al.*, 1997; Ferguson *et al*., 2000). Morphologically *L. lamottei* is closely related to *L. odemensis*, it is practically equally associated with *L. odemensis* and *L. culinaris* on the basis of isozyme data, but it appears to be the most distantly related taxon to all other *Lens* species according to RAPDs (Ferguson, 2000). However, our data taking into account RAPD, ISSR or both together, clearly differentiated *L. lamottei* from *L. culinaris*, even if it always appeared as a sister species of *L. odemensis*. Although the dendrogram is unrooted (Fig. 1), our data suggest that *L. nigricans* is the most differentiated taxon in the genus, according to previous results based on chloroplast DNA and other polymorphisms (Van Oss *et al*., 1997; Ferguson, 2000).

To sum up, both RAPD and ISSR markers contribute with a significant number of polymorphic markers which could be useful in identifying lentil cultivars, contributing to saturate genetic maps, in marker-assisted selection, but which also could contribute useful data in phylogenetic analyses in the genus *Lens*.

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