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An intersubspecific genetic map of *Lens*

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Abstract A *Lens* map was developed based on the segregational analysis of five kinds of molecular and morphological genetic markers in 113 F_2 plants obtained from a single hybrid of *Lens culinaris* ssp. *culinaris* × *L. c*. ssp. *orientalis*. A total of 200 markers were used on the F_2 population, including 71 RAPDs, 39 ISSRs, 83 AFLPs, two SSRs and five morphological loci. The AFLP technique generated more polymorphic markers than any of the others, although AFLP markers also showed the highest proportion (29.1%) of distorted segregation. At a LOD score of 3.0, 161 markers were grouped into ten linkage groups covering 2,172.4 cM, with an average distance between markers of 15.87 cM. There were six large groups with 12 or more markers each, and four small groups with two or three markers each. Thirty-nine markers were unlinked. A tendency for markers to cluster in the central regions of large linkage groups was observed. Likewise, clusters of AFLP, ISSR or RAPD markers were also observed in some linkage groups, although RAPD markers were more evenly spaced along the linkage groups. In addition, two SSR, three RAPD and one ISSR markers segregated as codominant. ISSR markers are valuable tools for *Lens* genetic mapping and they have a high potential in the generation of saturated *Lens* maps.

Introduction

Lentil (*Lens culinaris* Medik.) is a self-pollinated diploid (2n=14) legume species with a relative large genome of 4,063 Mbp (Arumuganathan and Earle 1991). This species is an important pulse crop grown in many temperate areas, such as the Mediterranean Basin, Central and Western Asia and some areas in North and South America and Australia, where it is grown in semi-arid regions, usually in rotation with cereals. Lentil is valued as a source of human food as it provides relatively high amounts of healthy and good-quality plant proteins, and also for its residues, which are used in animal fodder. Nevertheless, despite their obvious economic and nutritional value, lentils have received less attention research-wise than other legume crops. The genus *Lens* Miller is included in the tribe Vicieae (Papilionaceae, Leguminosae) in which another three genera with pulse crop species are included, namely, *Lathyrus* L., *Pisum* L., and *Vicia* L. Lentil (*L. culinaris*) is the only cultivated species of the genus *Lens* and was most probably domesticated in the earliest stages of applied agriculture in eastern Turkey and northern Syria from *L. orientalis* (Ladizinsky 1999). Both taxa are currently considered to be subspecies: *Lens culinaris* ssp. *culinaris* Medik. and *L. c*. ssp. *orientalis* (Boiss) Ponert (Ladizinsky 1993).

In recent years, genetic maps have become keystones in both basic genetic research and plant breeding. Biochemical and molecular markers have revealed that lentil has relatively low levels of genetic variation compared with other plant species (Álvarez et al. 1997; Eujayl et al. 1997; Ford et al. 1997; Ferguson et al. 1998; Sonante and Pignone 2001). Likewise, this species has received less attention from researchers than other legume species, such as dry bean, pea or soybean. A low genetic variability and insufficient genetic information are the reasons why until recently genetic maps of this species consisted of a relatively small number of markers, mainly isozymes and restriction fragment length polymorphisms (RFLPs) that covered an also relatively small portion of the lentil genome (Havey and Muehlbauer 1989; Weeden et al. 1992; Tahir et al. 1993). The recent development of a large number of molecular markers, especially RAPDs (randomly amplified polymorphic DNA) and AFLPs (amplified fragment length polymorphisms), is now providing the basis for constructing saturated maps in this species (Eujayl et al. 1997, 1998; Rubeena et al. 2003), which consequently enables for the localization of

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genes of agronomic interest and facilitates a faster improvement in breeding programs (Tullu et al. 2003). Inter-simple sequence repeats (ISSRs) are a relatively new type of DNA marker that involves the direct use of microsatellite sequences as primers in PCR analyses (Gupta et al. 1994) to obtain DNA markers. According to Ratnaparkhe et al. (1998) and Zietkiewicz et al. (1994), this technique is more reliable than the RAPD technique and generates larger numbers of polymorphisms per primer. ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Reddy et al. 2002). They have been used in many crop species, including legumes (Ratnaparkhe et al. 1998; Ajibade et al. 2000; Bornet and Branchard 2001; Chowdhury et al. 2002; 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; Winter et al. 2000; Rubeena et al. 2003). ISSR markers have already been used in lentil to evaluate genetic variation in a collection of cultivated lentils (Závodná et al. 2000; Sonante and Pignone 2001).

Simple sequence repeats (SSRs) or microsatellites show most of the ideal characteristics for a genetic marker: they are abundant, highly polymorphic and reproducible, distributed throughout genomes and, in addition, they usually fit a codominant Mendelian inheritance. These characteristics make them ideal markers for applications in genetic mapping and genetic map comparisons. Microsatellite markers have been developed in several main crop species, including legume species such as lentil (Závodná et al. 2000), common bean (Hamann et al. 1995; Yu et al. 1999), *Vigna* (Yu et al. 1999), chickpea (Huttel et al. 1999), *Medicago* (Baquerizo-Audiot et al. 2001) and soybean (Akkaya et al. 1992; Powell et al. 1996). Microsatellite loci have been extensively used in genome mapping (Weising et al. 1998) and, in particular, they have been used in the construction of genetic maps in some legume species: soybean (Morgante et al. 1994; Akkaya et al. 1995; Cregan et al. 1999), common bean (Yu et al. 2000) and chickpea (Winter et al. 1999, 2000).

In order to maximize polymorphism for map construction in lentil, inter-subspecific hybrid populations have been used (Havey and Muehlbauer 1989; Patterson et al. 1990; Eujayl et al. 1997). The genetic map presented here, based on a hybrid between *L. culinaris* ssp. *culinaris* and *L. c.* ssp. *orientalis*, includes RAPD, ISSR, AFLP, SSR and morphological markers. It can serve as the basis for the development of a high-density map that can be used for mapping quantitative trait loci (QTLs) of agronomic interest, marker-assisted selection, and is a first step in the map-based cloning of resistance genes and other genes of interest.

Materials and methods

Plant material

The genetic map was constructed from a population of 113 F_2 individuals of the cross between *Lens culinaris* ssp. *culinaris* Medik. cv. Lupa and the wild relative *L. culinaris* ssp. *orientalis* Boiss. (BG 16880). A single F_1 plant was selfed to produce the F_2 population. The F_2 plants were grown under controlled environmental conditions in a greenhouse and used for segregation analyses.

DNA extraction

DNA isolation was carried out with two different protocols depending on the required level of DNA purity. Total genomic DNA to be used in RAPD and ISSR techniques was isolated from fresh leaves of 2-week-old plants following the procedure described by Edwards et al. (1991) with minor modifications. For the AFLP and SSR techniques, DNA of higher purity is required, thus total genomic DNA was isolated from fresh leaves of 2-week-old plants using the Dneasy Plant Mini kit (QIAGEN, Valencia, Calif.). In both cases, the extract was treated with DNase-free RNase (Roche Diagnostics, Germany) to degrade the RNA and subsequently quantified in agarose gels by comparison with standard lambda DNA and in a Gene Quant (Pharmacia Biotech, UK).

RAPD analysis

The protocol of Williams et al. (1991) was used with minor modifications for RAPD analysis. A total of 160 decamer primers (Operon Technologies, Alameda, Calif.; kits A, B, C, D, G, H, P and W) were used to screen the parents for polymorphism, and those primers which produced scorable, repeatable and distinct fragments were selected. 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 the bands visualized with ethidium bromide.

ISSR analysis

One hundred ISSR primers (University of British Columbia Biotechnology Laboratory, Vancouver, B.C.; primer set no. 9) were used to screen the parents for polymorphism. The 15 primers that 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 $MgCl_2$, 0.25 mM each of the four dNTP and 1 U *Taq* DNA polymerase (GibcoBRL, Gaithersburg, Md.). The DNA was first denatured for 1 min at 94°C, followed by 30 cycles of 30 s at 94°C (denaturation), 30 s at 50°C (annealing) and 2 min at 72°C (elongation), with a final elongation of 10 min at the same temperature. The reaction products were separated on 2.0% agarose gels and the bands visualized with ethidium bromide.

AFLP analysis

The protocol for the AFLP analysis was that suggested in the AFLP Plant Mapping Kit for regular plant genomes [PE Applied Biosystems, Foster City, Calif. (1995)], which is based on Vos et al. (1995). Total genomic DNA was digested with a combination of *Eco*RI and *Mse*I restriction enzymes and specific double-stranded adaptors were ligated to fragment ends. Pre-amplification was performed with 1-bp extension primers (*Mse*I primer+C; *Eco*RI primer+A). Selective amplification of restriction fragments was

conducted using primers with 3-bp selective nucleotides. Sixty-four primer combinations were used to screen for polymorphism between the parents, of which 12 were selected as obtaining the best ratio of polymorphic fragments/primer combination. The *Eco*RI primers were end-labeled with one of three fluorochromes—namely, FAM, NED or JOE. The AFLPs were analyzed by means of capillary electrophoresis in an ABI PRISM 310 Genetic Analyzer and GeneScan Program (PE Applied Biosystems).

SSR analysis

Primer sequences (Table 1) complementary to regions flanking some lentil microsatellite loci were used. Microsatellites were identified in our laboratory. The MS5S is included in the intergenic spacer of the ribosomal 5S of the cultivated lentil (Fernández 2002). SSR amplifications were performed in a total volume of 15 μl with 50 ng of template DNA, 10 μM of both specific primers, *Taq*-DNA polymerase buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, Triton X-100 1%, 1.5 mM $MgCl_2$), 0.3 mM each of the four dNTP, 1 U *Taq* DNA polymerase (Promega). The amplification program consisted of 1 min at 94°C, followed by 35 cycles of 15 s at 94°C (denaturation), 15 s at 50°C (annealing) and 1 min at 72°C (elongation), with a final elongation of 5 min at the same temperature. Forward primers were labeled with a fluorochrome—FAM or HEX. The protocol used for analyzing the PCR products was similar to that used for AFLP.

Band nomenclature

RAPD and ISSR markers were designated first with the commercial serial number of the primer used followed by a number that indicated the order by molecular weight (from highest to lowest) (i.e. OPA10-2; UBC808-2). AFLP markers were designated with a number and letter from each primer combination followed by a number that indicated the estimated size (in basepairs) of the fragment (i.e. 7F-221).

Morphological markers

The F_2 plants were scored individually for five morphological traits: color (orange vs. yellow) of the cotyledon (*Yc*), presence or absence of anthocyanin in the stem (*Gs*), seed coat pattern or spotting (*Scp*) and pod dehiscence-indehiscence (*Pi*) (Muehlbauer and Slinkard 1981) and ground color (brown vs. tan) of the seed (*Ggc*) (Vandenberg and Slinkard 1990).

Statistical analysis

Polymorphic bands were evaluated for presence or absence in the F_2 individuals, and the goodness of fit to Mendelian segregations for each locus was examined using the chi-square test. The maximum-likelihood and estimates of recombination frequencies between linked loci were computed using MAPMAKER/EXP version 3.0 (Lander et al. 1987). So as to identify linkage groups, pairwise comparisons and grouping of markers were performed at a LOD=3.0 and a maximum distance of 30 cM. The marker order was confirmed with the "ripple" command. Final map distances were calculated by applying the Kosambi function (Kosambi 1944) provided by the program.

Results

The preliminary analysis yielded a high number of polymorphisms for the four types of molecular markers tested. A summary of the results is presented in Table 2. Comparing the three types of dominant markers (AFLP, ISSR, RAPD), we observed the efficiency of the AFLP technique in generating a high number of polymorphic markers in a single assay. Whereas an average of 2.9 polymorphisms per primer was obtained with RAPDs, and 4.2 with ISSRs, this value rose to 11.8 for AFLPs (per primer pair in this case).

Table 1 Characteristics of the SSR loci analyzed in this study

Locus	Forward primer (5′–3′) Reverse primer (5′–3′)	Motif of repeat	Fragment size (bp)
MS1	TTACGAAAAAGGCAAACATA ATCTTCTTCTTCTTCTTCTCA	$(TC)_{20}$	118
MS3	TTGTGTTTTTCTACCCTTCA CCATTAGAGAGTTGAGTAGGC	$(TC)_{20}$	126
MS6	GAGATTTCCAATACTGAATA TTCTACTTCTTCTTTCTTGA	$(TC)_{12}$	203
MS11	ACTCTAGCCTTTTCAACG TTCTTCTTTTTCTTCTCTTG	$(TC)_{17}$	256
MS5S	TGGTAGCGTAAAAAAGTGTC TTTAGTGCTGGTATGATCGC	$(TA)_{21}$	181

Table 2 Polymorphism of the four types of molecular markers

Type of marker	Number of primers tested	Number of primers used in analyses	Number of polymorphic bands	Average number of polymorphisms per primer(s)
RAPDs	74	32	94	2.9
ISSRs	15	13	55	4.2
AFLPs	64[a]	12[a]	141	11.8
SSRs	5[b]	3[b]	3[c]	1[b]
Total	158	57	293	

[a] Combinations of primer pairs
[b] Primer pairs
[c] Number of segregating loci

Table 3 Number of markers and percentage of each type of segregation observed in the analysis of dominant markers

Type of marker	Polymorphic markers	Segregation			Distorted segregating markers
		3:1	15:1	1:2:1	
RAPD	94	68 (72.3%)	3 (3.2%)	3×2[a] (6.4%)	17 (18.1%)
ISSR	55	38 (69.1%)	2 (3.6%)	1×2[a] (3.6%)	13 (23.7%)
AFLP	141	83 (58.9%)	17 (12.0%)	–	41 (29.1%)
Total	290	189 (65.2%)	22 (7.6%)	4 (2.7%)	71 (24.5%)

[a] Two bands or markers per codominant locus

Table 4 Number and type of markers included in the linkage analysis

Type of marker	Dominant (3:1)		Codominant (1:2:1)	Total
	Dominant allele from P1[a]	Dominant allele from P2[b]		
RAPD	31	37	3	71 (35.5%)
ISSR	16	22	1	39 (19.5%)
AFLP	34	49	–	83 (41.5%)
SSR	–	–	2	2 (1.0%)
Morphological	–	5	–	5 (2.5%)
Total	81 (40.5%)	113 (56.5%)	6 (3.0%)	200

[a] ♀Parental *Lens culinaris* ssp. *culinaris* cv. Lupa
[b] ♂Parental *L. culinaris* ssp. *orientalis* (BG 16880)

A total of 286 RAPD, ISSR and AFLP polymorphic markers were scored as either present or absent in the parents and the F_2 (Table 3). All polymorphic bands were present in this and other F_1 plants of the same cross. A total of 189 markers (65.2%) (68 RAPD, 38 ISSR and 83 AFLP) fitted a 3:1 Mendelian ratio as expected for dominant single-locus inheritance. Three pairs of RAPD fragments (OPA15-2/1, OPC2-4/3, OPW8-2/1) and one pair of ISSRs (UBC864-4/3) were considered to be codominant alleles since bands of each pair had similar sizes, the simultaneous absence of both bands of each pair was not observed in any of the 113 F_2 individuals ($P<6.8\times10^{-4}$) and they fitted the 1:2:1 segregation pattern. Thus, 2.7% of the bands of these generally dominant markers were considered to be codominant alleles, and the 94 polymorphic RAPD and the 55 polymorphic ISSR bands were scored as 91 and 54 loci, respectively. On the other hand, 22 markers (7.6%) fitted a 15:1 segregation, suggesting that the band was the sum of two fragments of equal or similar sizes. Finally, 71 markers (24.5%) did not fit any Mendelian segregation and were classified as distorted markers (Table 3). The three polymorphic SSR markers fitted a codominant 1:2:1 segregation, except for MS1 that showed a distorted segregation. Morphological markers fitted the 3:1 monogenic dominant mode of inheritance.

The number and type of markers included in the linkage analysis, as well as the distribution of dominant alleles between the two parents, is shown in Table 4. For the construction of the genetic map 200 markers (71 RAPD, 39 ISSR, 83 AFLP, two SSR and five morphological) that fitted Mendelian 3:1 or 1:2:1 segregations were used (Table 4). Ten linkage groups including 161 markers (62 RAPD, 29 ISSR, 65 AFLP, one SSR and four morphological markers) were found (Fig. 1). The map covered 2,172.4 cM of the lentil genome, with a mean distance between markers of 15.9 cM, although there was a wide variation between individual distances. The pair of AFLP markers 6B-371 and 2B-247 showed no recombination. Based on the parameters used in the map construction, we were unable to assign 39 markers to any of the linkage groups.

The seed-color pattern locus (*Scp*) was linked to the OPG10-4 marker, at 8 cM, and to the codominant marker UCB864-4/3, at 13.3 cM, and mapped in group I. The cotyledon color locus (*Yc*) was flanked by OPW8-2/1, a codominant marker, at 5.6 cM, and the OPG3-1 marker, at 13.5 cM, and mapped in group II. The stem pigment locus (*Gs*) was mapped in group IV and flanked by the UBC842-4 and OPD7-1 markers, at 10.8 cM and 5.9 cM, respectively. The seed ground color locus (*Ggc*) and microsatellite marker MS5S (one of the two 5S loci in lentil; Fernández 2002) were assigned to group V; this is the first time both loci have been mapped in lentil. These morphological markers allowed us to tentatively relate the linkage groups found in this study to linkage groups in previous publications (Table 5).

The distribution of markers between linkage groups was unequal. There were six large groups that contained 12 or more markers and four small groups that contained two or three markers. Group V had the largest number of markers—49 (30.4%). The number and type of markers in each of the ten groups obtained are shown in Table 6. Likewise, the distribution of markers was not random. There was a tendency to cluster at central chromosomal regions. In the larger linkage groups (I–VI), chromosomal areas flanking regions of high marker density were often poorly marker-covered. Secondly, several clusters of markers of the same type were observed; for instance, linkage group V showed a cluster of six AFLP markers

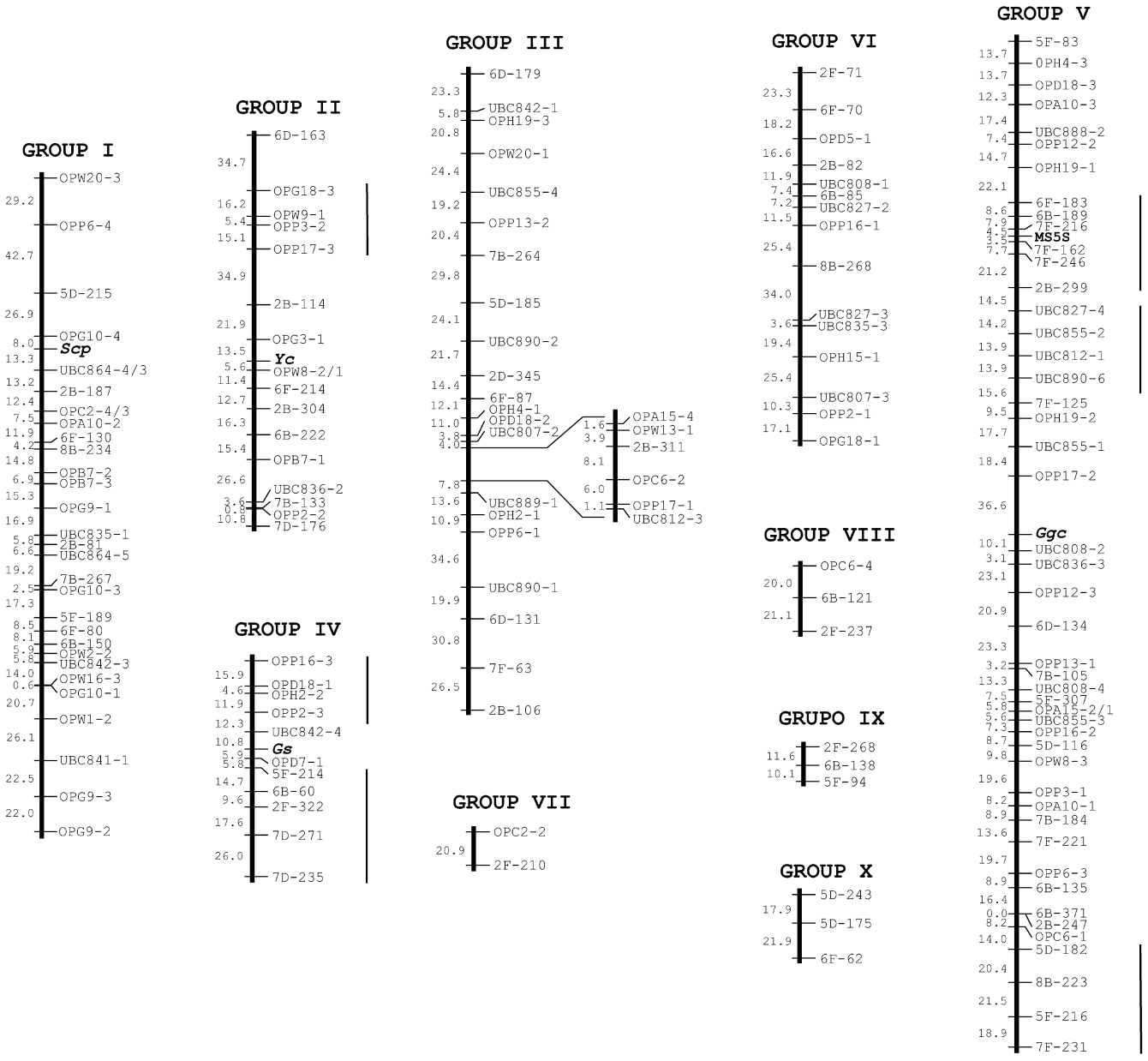

Fig. 1 Genetic linkage map of lentil showing the distribution of different markers at LOD 3.0. Genetic distances, on the *left side* on the maps, are in centiMorgans (Kosambi function). *Vertical lines* indicate clusters of RAPD, AFLP or ISSR markers

Table 5 Correspondence between linkage groups obtained in the present study and those obtained in previous studies on the basis of morphological markers

Marker locus	Present study	Tahir et al. 1993	Eujayl et al. 1998
Scp	I	V	III
Yc	II	II	-
Gs	IV	I	-
Pi	?	IV	II
Ggc	V	-	-

that covered about 53.4 cM, although the MS5S microsatellite was also located in the cluster. This cluster was linked at a distance of 14.5 cM to a group of four ISSR markers that spanned 42 cM. Another cluster of four AFLPs loosely linked markers was observed in one of the ends of linkage group V. Two sets of four RAPDs were observed in groups II and IV, respectively; in this last linkage group an additional cluster of five AFLPs was also observed (Fig. 1). However, the intermingling of different markers types was also observed. The RAPD markers were more evenly spaced along the linkage groups.

Table 6 Number and type of markers assigned to the linkage groups in lentil

Linkage group	Marker					Total
	RAPD	ISSR	AFLP	SSR	Morphological	
I	15	5	9	–	1	30 (18.6%)
II	8	1	7	–	1	17 (10.6%)
III	11	7	9	–	–	27 (16.8%)
IV	5	1	5	–	1	12 (7.4%)
V	16	10	21	1	1	49 (30.4%)
VI	5	5	5	–	–	15 (9.3%)
VII	1	–	1	-	–	2 (1.2%)
VIII	1	–	2	–	-	3 (1.8%)
IX	–	–	3	–	–	3 (1.8%)
X	–	–	3	–	–	3 (1.8%)
Total	62 (38.5%)	29 (18.0%)	65 (40.4%)	1 (0.6%)	4 (2.5%)	161

Discussion

Molecular markers, RAPDs, AFLPs and ISSRs, have already proven their usefulness for *Lens* genetic mapping (Eujayl et al. 1997, 1998; Rubeena et al. 2003), but microsatellite markers have not been previously used in lentil mapping. AFLPs have provided a robust method for producing linkage maps in breeding populations that have a relatively narrow genetic base (Powell et al. 1997). In the present study, the AFLP technique showed the highest efficiency in generating a large number of polymorphic markers in a single assay. The mapping of AFLPs increased the number of polymorphic markers and strengthened the reliability of the framework of this map, and AFLP markers had major advantages over RAPD markers due to their robustness and transferability (Powell et al. 1997).

There were more ISSR polymorphisms (4.2 per primer) than RAPDs (2.9 per primer). Ratnaparkhe et al. (1998) reported that ISSRs can be used as highly informative markers for genome mapping and gene tagging. In lentil, as in other species such as chickpea (Ratnaparkhe et al. 1998; Winter et al. 2000), ISSR markers represent a useful set of markers for genetic mapping purposes. The ISSR technique has been reported to be a reliable and reproducible assay in many crops, and previous investigators have demonstrated that ISSR analysis usually detected a higher level of polymorphism than that detected with RFLPs and RAPDs (Zietkiewicz et al. 1994; Ratnaparkhe et al. 1998).

Three out of the five SSR markers characterized in the enriched genetic library were polymorphic. Although the number of SSRs used was small, their high level of polymorphism and transferability across accessions and species of *Lens* (Durán 2002) ensured that SSR markers had extensive applications in *Lens* genetic mapping.

Our genomic map of lentil comprises 161 markers covering 2,171.4 cM. The previous most extensive lentil maps obtained from inter-subspecific and intraspecific crosses, respectively, cover 1,073 cM with an average distance between markers of 6.0 cM (Eujayl et al. 1998), and 784.1 cM with a density of 6.9 cM between adjacent markers (Rubeena et al. 2003). As the physical size of the lentil genome was estimated to be 4,086 Mpb (Arumuganathan and Earle 1991), 1 cM in the present map would represent 1.88 Mpb on average, which is half the value of 3.8 Mpb/cM calculated in the inter-subspecific map obtained by Eujayl et al. (1998). This value is far from the one calculated for the high-density map of tomato (Tanksley et al. 1992).

The present lentil map consists of ten linkage groups: six with 12 or more markers and four with two or three markers. Since the haploid genome of this species is made up of seven chromosomes, some of the ten linkage groups must merge. Eujayl et al. (1998) observed 15 linkage groups, eight of which were small segments. Unfortunately, the number of coinciding markers between the *Lens* maps was low (Tahir et al. 1993; Eujayl et al. 1998), thus only some linkage groups could be tentatively related to each other on the basis of morphological markers (Table 5).

Marker density varies within each linkage group. On the larger linkage groups (I–VI), the marker density was higher in the central regions than in the distal regions (Fig. 1). Other authors have reported a non-random distribution of markers—often due to centrally located clusters—in chickpea (Winter et al. 2000), sugar beet (Halldén et al. 1996) or barley (Langridge et al. 1995). Sometimes the apparently random marker distribution is due to a low number of markers; when more markers are added to the map, clusters became evident, such as SSRs in soybean (Akkaya et al. 1995; Cregan et al. 1999). The existence of differences in the crossing-over frequency could influence marker density in a linkage group. Tanksley et al. (1992) explained the uneven marker distribution with the reasoning that centromeres and centromeric heterochromatin, and in some instances telomeres, experience up to tenfold less recombination than other areas of the genome. For instance, in cereals, in which cytogenetic markers are available, the crossing-over frequency in the distal regions of the chromosomes has been shown to be higher than in the regions proximal to the centromere (Lukaszewski 1992; Alonso-Blanco et al. 1993).

ISSR and AFLP markers tended to generate small subclusters in the larger linkage groups. Clustering of AFLP markers has also been observed in the *Lens* genetic map (Eujayl et al. 1998). Similar observations concerning

marker type-specific subclustering were reported in the chickpea map (Winter et al. 2000) and the barley map (Becker and Heum 1995).

Of the 293 polymorphic markers obtained, 72 (24.6%; 17 RAPDs, 13 ISSRs, 41 AFLPs and one SSR) deviated from Mendelian segregations. An elevated degree of distortion has been previously described in crosses between *L. c. culinaris* and *L. c. orientalis* (Eujayl et al. 1997), although the value seems to depend mainly on the specific cross performed, since it can vary from 10% to 83%. Marker-distorted segregation may have been due to the presence of chromosomal reorganizations or linkage to deleterious alleles in the gametes. For the three marker classes used in the present investigation (RAPDs, ISSRs, AFLPs), distorted markers did not significantly deviate from a random distribution with respect to their origin. This means that approximately one-half of the alleles observed at higher frequencies than those expected under Mendelian segregation came from the *culinaris* parent and the other half from the *orientalis* parent. This result contrasts with previous reports indicating a predominance of *culinaris* alleles among the distorted markers in *L. c. culinaris* × *L. c. orientalis* (Havey and Muehlbauer 1989), and *L. c. culinaris* × *L. odemensis* crosses (Tadmor et al. 1987). AFLP markers showed a higher percentage of distortion (29.1% of the markers), which coincides with the results of Eujayl et al. (1998), who compared AFLPs with RAPDs and RFLPs. Eujayl et al. (1997) concluded that segregation distortion was not due to the marker technique but rather to a segregation distortion of the gametes or zygotes leading to the F_2 progenies. Winter et al. (2000), in a genetic map of chickpea, reported that RAPD markers exhibited a higher percentage of distortion than AFLP markers, thereby reaching the same conclusion that segregation distortion was less related to the class of affected markers than to the genomic region where they resided.

Our linkage analysis of distorted markers showed that 40 (20 from each parent) were included in linkage groups, with the remaining ones behaving as independent loci. Among those included in linkage groups, we observed a tendency to form small clusters in the distal part of the groups or between two loosely linked markers previously located in the map. For example, four markers (two RAPDs and two ISSRs) were located between OPP6-1 and UBC890-1, separated by 34.6 cM in group III, and three markers were added distal from the AFLP marker 7D-235, located in one of the presumed ends of group IV. It was also noticeable that six distorted AFLPs were added to group VIII, which resulted in this group having eight AFLPs and one RAPD. The clustering of distorted loci has been reported within the linkage groups of some crop species (Graner et al. 1991; Kammholz et al. 2001). Since lentil is a self-pollinated species, linkage to incompatibility loci cannot be the cause of the distortion (Wricke and Wehling 1995); thus, linkage to gametic lethal alleles (Pillen et al. 1992) or chromosomal reorganizations must be the cause. In lentil, Vaillancourt and Slinkard (1992) suggested that disturbed segregations were in most cases caused by the preferential elimination of a piece of chromosome carrying these markers as a result of translocations between parental karyotypes. The same phenomenon was also observed in *Capsicum* by Tanksley (1984).

In summary, the results presented here represent a significant contribution towards the construction of saturated genetic maps in lentil, and they will be useful in further genetic studies and in plant breeding. However, given the unequal distribution of the markers, the fact that 39 markers behaved independently and that more linkage groups were detected than the haploid number of chromosomes, the necessity to include more markers in the construction of a more saturated map covering the entire genome is quite clear. To do so, it will be necessary to increase the number of markers and to use more informative markers, such as SSR.

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