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Linkage mapping of Lentil (*Lens culinaris* L.) genome using recombinant inbred lines revealed by AFLP, ISSR, RAPD and some morphologic markers

Bahattin TANYOLAC[1] Sehnaz OZATAY[1] Abdullah KAHRAMAN[2] Fred MUEHLBAUER[3]

[1] Ege University, Department of Bioengineering, 35100 Bornova-Izmir TURKEY

[2] Harran University, Department of Field Crops, S. Urfa, TURKEY

[3] Department of Crop and Soil Sciences, USDA-ARS, Washington State University, 303 Johnson Hall, Pullman, WA99164-6434, USA.

Correspondence Author
e-mail: bahattin.tanyolac@ege.edu.tr

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ABSTRACT

We have constructed a molecular linkage map of Lentil (*Lens culinaris* L.) in a RIL population (developed from the parents Precoz x WA8949041) of 94 plants with 166 markers. The resulting linkage map consists of 11 (varying between 50.9–436.5 cM) linkage groups covering 1396.3cM with an average map distance between framework markers of 8.4 cM. Most of the RAPD and ISSR markers were evenly distributed across the genome. By using 40 primer combinations, we were able to generate 391 AFLP markers in the RIL population. The majority of the AFLP markers clustered in linkage group 2. In the segregation distortion analysis, 185 markers deviated from Mendelian segregation. Of the 185 markers with skewed segregation, 108 markers were unlinked and the rest of the distorted markers mapped to linkage groups. LG2 contained the most distorted marker among the linkage groups.

Key Words: AFLP-RAPD-ISSR-Lentil-Segregation Distortion-Linkage map

INTRODUCTION

Lentil is an important crop for the Indian sub-continent and Mediterranean region. It is important for humans because of its high nutritional value and its straw is also used as livestock feed. Lentil can be grown as a secondary crop in dry land cereal-based rotations, because it is extremely good at nitrogen fixation. Lentil is self-pollinated, diploid (2n=2x=14) with a large genome size of approximately 4Gb [1]. To understand better the genetic structure of such a large genome, the genome needs to be characterized and mapped by molecular markers.

The construction of molecular linkage maps has become an essential tool for plant molecular genetics and breeding research. Recent developments in molecular genetics offer plant breeders a rapid and precise alternative approach to conventional selection schemes for improving cultivars for yield, adaptability, pest resistance, etc.

Such traits do not consistently fall into discrete classes because environmental conditions greatly modify their performance. Molecular markers are important tools for generating genetic linkage maps and have provided a significant increase in genetic information of plant species [2].
Several kinds of segregating populations (F_2, F_3, Backcross, Doubled Haploid, F1 population, RIL) are used in linkage mapping studies.

Among them, recombinant inbred lines (RIL) are a good genetic source for constructing linkage maps by facilitating the localization of quantitative characters and providing sufficient seed for replicated field trials [3].

In RILs, dominant and co-dominant markers have similar information content which allows the integration of dominant markers such as RAPD [4], DAFs [5], AFLP [6] and ISSRs [7] into a framework of codominant markers like RFLPs and SSRs. The first linkage map of lentil covering a small portion of the genome was constructed by Havey and Muehlbauer [8]. After that, Eujayl et al. [9] developed a *Lens* sp. linkage map with 86 recombinant inbred lines (RILs) and which consisted of 89 RAPDs and 79 AFLPs together with six co-dominant markers, most of the latter being RFLPs. Rubeena et al. [10] constructed a lentil genetic map using 100 RAPDs, 11 ISSRs and 3 resistance gene analog (RGA) markers. Hamwieh et al. [11] generated a lentil linkage map and they used 283 DNA markers covering the genome with an average marker distance of 2.6 cM and total coverage of about 751 cM. The most recent linkage map of lentil was developed by Tullu et al. [12] for mapping of earliness and plant height traits.

Their map consisted of 207 markers (AFLP, RAPD and SSRs) and the map covered the genome (1868 cM) with an average marker density of 8.9 cM.
The objectives of this study were to construct a genetic linkage map of lentil using RILs based on RAPD, AFLP, ISSR and some morphologic markers that can be used to detect the loci controlling traits that segregate in the population.

MATERIALS and METHODS

Plant Material

RILs were kindly provided from Washington State University Pulman, USA. The RILs were developed from a Precoz x WA8949041 cross with single seed descent until the F_7 generation. Ninety-four seeds of RILs were grown in a greenhouse.

DNA extraction

Young leaves from 4-6 weeks-old seedlings of 94 RILs and parents were sampled in aluminium foil and saved in liquid nitrogen. Leaf tissue from each individual was ground to a fine powder in liquid nitrogen with a mortar and pestle. Total genomic DNA was extracted following the procedure as described by Saghai-Maroof et al. [13]. DNA was treated with RNAse and Proteinase K. The purified DNA was quantified with ND-1000 (Nanodrop, Thermo Co.) spectrophotometer.

RAPD analysis

A total of 384 decamer primers were purchased from Operon Technologies, Alameda CA, USA. RAPD analysis was conducted using a PTC-225 Tetrad Thermocycler (MJ Research^{TM}). The reaction mixture(15 ul) contained 1 unit of Taq polymerase (Fermentas), 100 μM each of dNTP, 1X PCR reaction buffer (10 μM Tris-HCl, pH 8.3, 1.5 μM $MgCl_2$, 50 μM KCl, 0.1 mg/ml gelatin), 10 pmol primer (OPERON) and 20 ng genomic DNA [14]. The mixture was subjected to following conditions to amplify DNA: hold at 94^o C for 1 min, followed by 35 cycles of 30 sec at 94^o C, 45 sec at 35^o C, 30 sec at 72^o C.The final elongation segment was held for 2 min at 72^o C. The PCR products were resolved on 2% agarose gel in 1XTAE buffer at 100 to 120 volts for 3 to 3.5 hours. The gels were stained with EtBr visualized under ultraviolet light.

ISSR analysis

A total of 25 ISSR primers 15 to 23 nucleotides in length were purchased from The Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada and were used to screen the parents for polymorphism. The PCR reaction conditions used by Tanyolac [14] were used for ISSR analysis. The PCR products of some ISSR primers were resolved on 2% agarose gel in TAE buffer and stained with ethidium bromide and some ISSRs (UBC807, 808, 809, 822, 834, 841 and 842) products were resolved by 4.5% PAGE (Polyacrylamide gel electrophoresis) and silver stained (Promega, Madison, WI).

AFLP Analysis

Li-Cor AFLP Kit (cat#830-06197 AFLP 2-DYE Selective Amplification Kit) was used according to manufacturer recommendations. According to the kit, 100ng pure DNA was digested with EcoR I and MSe I restriction enzymes. The enzyme adaptors were ligated to the digested DNA. Selective amplification of restriction fragments was conducted using primers with three selective nucleotide labeled RD700/800 dyes. Forty primer combinations were used to screen polymorphism between parents. Amplification products were resolved on 6% acrylamide gel in 1XTBE buffer. LiCor 4300 S DNA Analyser machine was used to image, analyse and screen markers.

Linkage Analysis

Polymorphic bands were evaluated for presence or absence in the RIL individuals. Linkage analysis was performed using 3 different genetic linkage map software programs. MAPMAKER 2.0 [15] was used to construct initial linkage groups. Mapmanager [16] was used to verify Mapmaker results and also to visualize quality of marker data. Join Map [17] was used to compare results from Mapmaker and Mapmanager. Each software allowed us to examine data in different ways. For Mapmaker software, linkage criteria were set at LOD 3.0 with a recombination fraction of 30 cM. Because 1 to 1 segregation is expected in a given RIL population due to homozygosity of alleles at a locus, goodness of fit to the 1:1 ratio was calculated for each locus by Chi-square ($\chi 2$) test using Join Map. The maximum likelihood and estimates of recombination frequencies between linked loci were computed using MAPMAKER. Recombination values were converted into map distances (centimorgans) by applying the Kosambi function [18]. Pairwise comparisons and grouping of markers were carried out at a LOD score of 3.0 to identify linkage groups (LG). The marker order was confirmed with the ripple command.

RESULTS

Marker genotyping and analysis

A total of 384 RAPD primers were amplified in parents DNA to detect polymorphic markers. Of the 384 RAPD primers screened, 116 primers yielded 192 segregating bands (30.2%). The polymorphic bands per primer was 1.65 for RAPD analysis. Of the 192 segregating bands, 75 bands (39%) were clear and scorable markers. The scored markers were mapped on the lentil genome and the markers distributed on all linkage groups. The rest of the RAPD bands (89 locus) were unlinked. ISSR analysis yielded 18 polymorphic primers. The 18 polymorphic primers produced 23 polymorphic loci and the ISSR markers were mapped onto all the linkage groups. Polymorphic loci per ISSR primer were 1.27.

In AFLP analysis, forty EcoR I/Mse I primer combinations were used to detect polymorphism in the mapping population. Forty primer combinations produced 391 loci. Bands per primer combination were 9,7. Of these 391 loci, 180 were scorable polymorphic loci (% 46). Among the scorable locus, 64 loci (35%) were mapped into different linkage groups. But, most of the AFLP loci clustered within linkage group I. The average number of polymorphic loci per AFLP primer combination was 8.14 loci. The rest (116 scorable loci) of the AFLP loci were unlinked.

Table 1. Characteristics of the genetic linkage map of lentil.

Linkage groups	Length (cM)	Number of markers	Average distance between markers (cM)
LG 1	436.5	41(%28.48)	10.6
LG 2	134.2	24 (%7.66)	5.59
LG 3	199.8	19 (%19.48)	10.5
LG 4	134.1	19(%8.48)	7.05
LG 5	97.5	12(%5.59)	8.1
LG 6	117	11(%7.7)	10.6
LG 7	86.3	9 (%4.34)	9.58
LG 8	72.1	9 (%4.68)	8.01
LG 9	16.7	9 (%7.9)	18.38
LG 10	48.5	7 (%2.58)	6.9
LG 11	50.9	6 (%3.06)	8.48
Total	1396.3	166	Average:8.4 cM

Genetic map construction

A genetic map consisting of 11 linkage groups covering 1396.3 cM was constructed from the genomic data using a LOD score of 3 and maximum recombination value of 0.30 (Figure 1). The 166 markers across 1396.3 cM provide an average marker density of 1 marker per 8.4 cM.
There is great variability in these densities because many markers showed pronounced clustering (Figure 1).

The map characteristics are presented in Table 1. The length of LGs varied from 16 cM to 436.8 cM (Fig.1). The number of marker loci per LG ranged from 6 to 41 (Fig 1). LG1 was the group containing the most markers (41) with an average marker density of 10.6 cM in length. LG2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 consisted of 134.2, 199.8, 134.1, 97.5, 117, 86.3, 72.1, 16.7, 48.5, 50.9 cM, respectively.

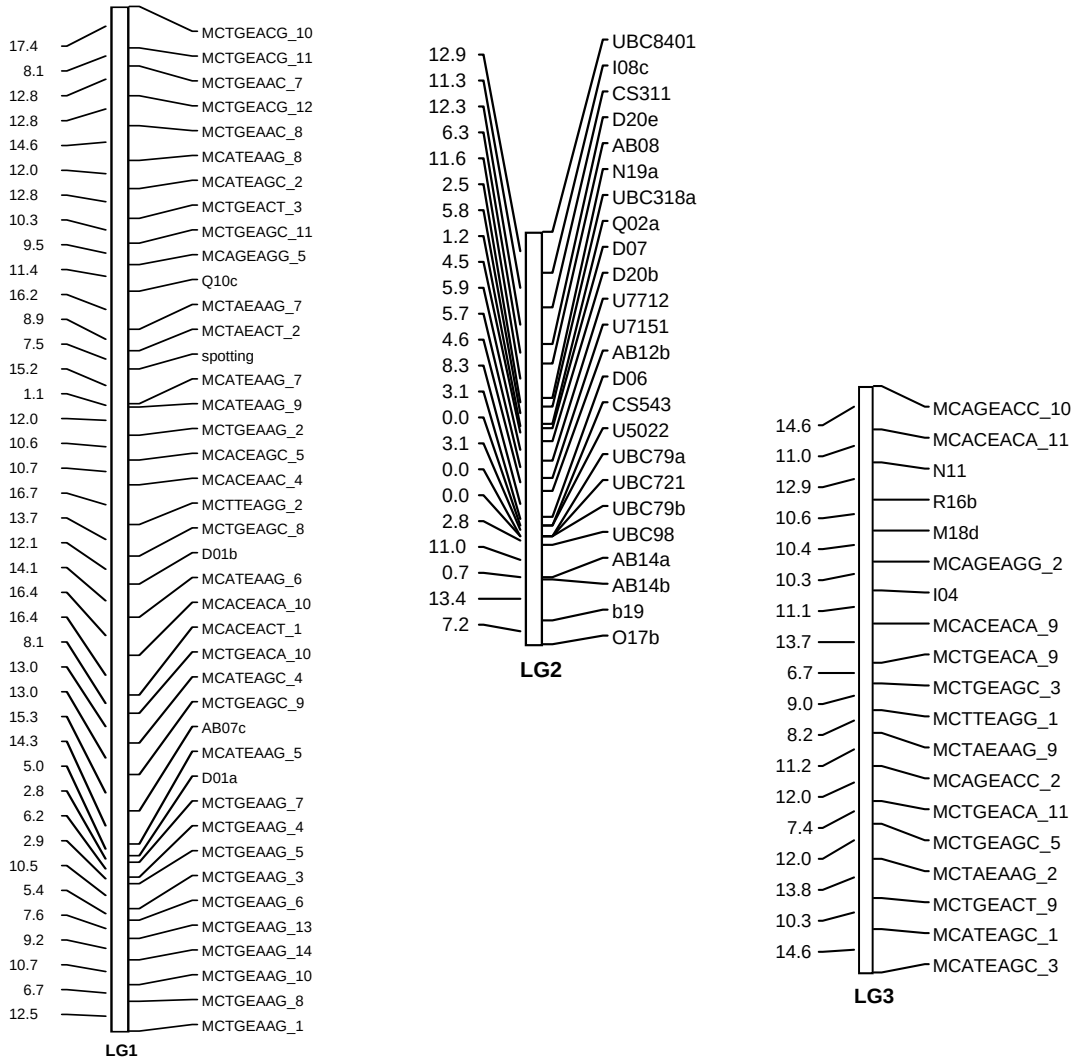

Figure 1. A Lentil linkage map from 94 individuals of RIL (developed from Precoz x WA8949041) population with 166 loci, 11 linkage groups, covering 1396.3 cM of the genome. Map distances between adjacent markers are in CentiMorgans.

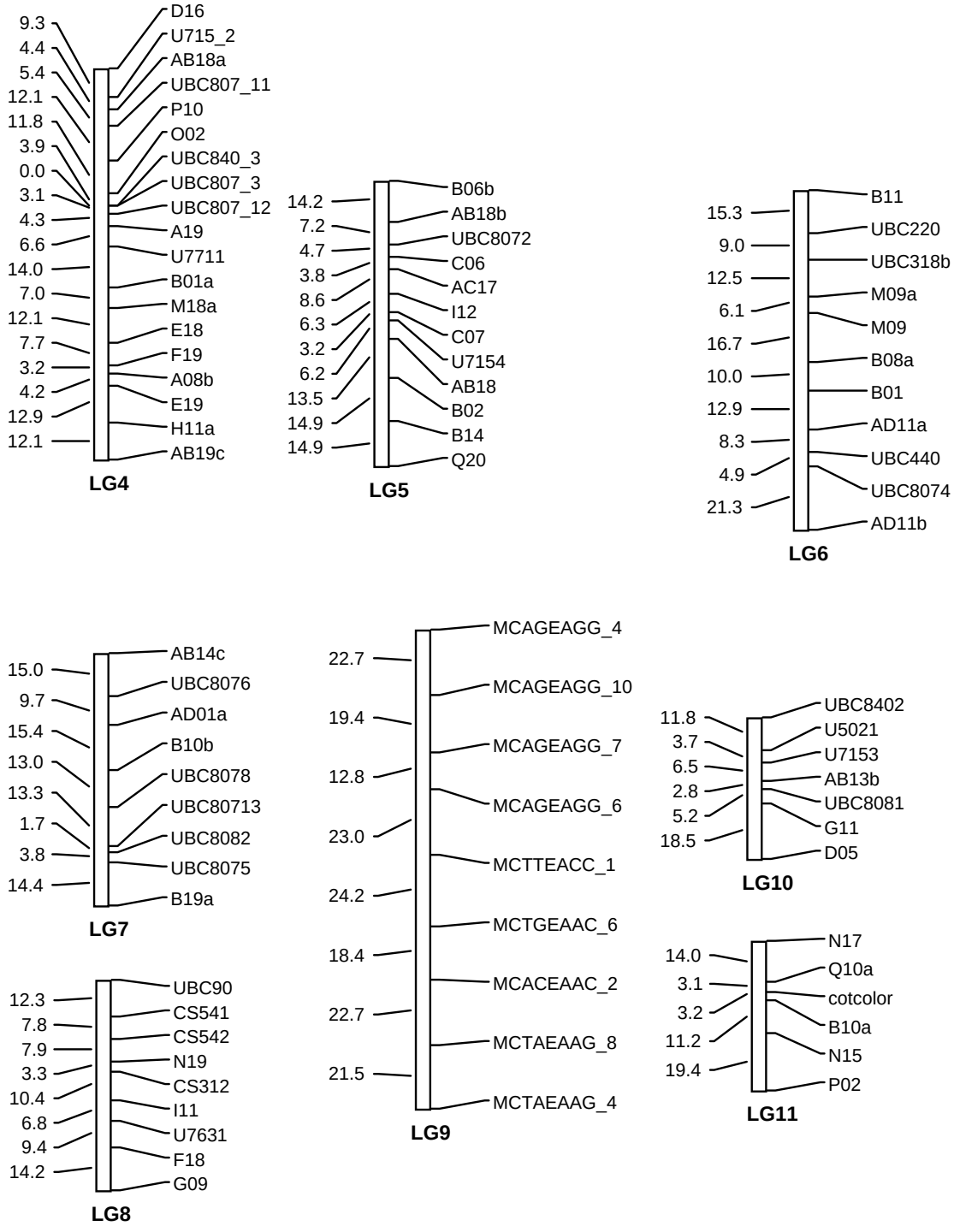

Figure 1. Continued.

DISCUSSION

In this study, 94 individuals of the RIL population were used to map the lentil genome. The map spanned 1393.6 cM with an average marker density of 8.4 cM. In comparison, a recent map of lentil was constructed by Ford and Taylor [19] in an intraspecific F_2 population covering the genome with 784.1 cM in length and another map was constructed by Eujayl et al. [9] covering the genome 1073 cM with 177 markers using RIL population. Eujayl et al. [19] had an average marker density of 15 cM in their genome mapping study which is approximately two times more than our average marker density results (8.4cM). The lentil map consisted of 11 linkage groups. Since the haploid genome of lentil consists of seven chromosomes, some of the linkage groups must join each other when new bridge markers are added to the linkage map. Duran et al. [20] obtained 10 linkage groups, four of which were small segments. Ford and Taylor [19] found nine linkage groups, two of which were small parts. The most recent linkage map of lentil was constructed by Tullu et al. [12] and they generated 12 linkage groups (three of which were small groups) with 207 markers covering the genome 1868 cM.

Molecular markers, RAPDs, AFLPs and ISSRs, have already been used for *Lens* genetic linkage mapping studies [9, 19, 20, 21]. Of the 384 RAPD primers tested, 116 primers (29.7%) produced 192 polymorphic bands. The degree of RAPD polymorphism agreed with Eujayl et al. (1998) of the polymorphic RAPD primer. Polymorphic band per RAPD primers were found to be 1.65. This result agreed with Ford and Taylor [19]'s results of 1,7 polymorphic band per RAPD primers.

ISSR primers produced fewer polymorphic markers. Of the ISSR primers screened, 18 were polymorphic and the polymorphic bands per primer were 1.27. These results support Ford and Taylor [19]'s result of 1.4 polymorphic bands per ISSR primer data in lentil. ISSRs can be used as highly informative markers for genome mapping and gene tagging [22].

In AFLP analysis, 40 primer combinations were used and these combinations produced 180 scorable polymorphic bands (4.7%). Eujayl et al. [9] found a total of 121 number of polymorphic AFLP markers using 17 primer combination and out of 121 polymorphic AFLP markers, they assigned 79 AFLP loci (4.6%) to linkage group in their study. Using AFLP markers in linkage mapping studies has been reported as a reliable and reproducible assay in many crops [20, 23]. The application of the AFLP technique has a major advantage in crops with a large genome. As compared with other legumes, lentil has a relatively large genome [1] and more markers are needed to merge the unlinked markers into linkage groups [9].

Dominant markers (RAPD, ISSR and AFLP) were used to map lentil genome in this study, because of the unavailability of co-dominant markers for lentil. Although dominant markers are unable to distinguish heterozygotes from homozygotes, however, they allow many polymorphic markers to be quickly identified. They have been used to construct linkage maps in various legume crops [9, 24, 25] and also to extend the existing linkage map of rye [26]. Molecular maps based on RAPD markers using F_2 populations have previously been successfully constructed [27, 28].

Among the type of markers mapped, AFLP markers were detected the most distorted markers type. Ninety-six AFLP markers were distorted from the expected ratio (data not shown). The most distorted AFLP markers were mapped to LG2. AFLP marker system is a powerful tool to generate polymorphic markers. Owing to these advantages, the AFLP system has been used for various studies such as genetic fingerprinting [29, 30] and genome mapping [9].

AFLP is a very useful [20] marker system for construction of genome maps, because it can cover the entire genome when sufficient markers are obtained. However, when we used the AFLP system, the segregation of markers was severely distorted. In our study, 185 markers (48%) in a total of 384 were distorted. The rest of the markers fit the Mendelian ratio. Although the segregation distortion ratio mainly depends on the specific cross performed, in comparison Eujayl et al. [9] found 26.6% segregation distortion in RIL population. In contrast, Nikaido et al. [23] found the distorted AFLP markers ratio as 41% in sugi. Eujayl et al. [21] reported segregation distortion of 83.3% in previous mapping study of lentil.

In conclusion, we have constructed a genetic linkage map of lentil using AFLP, ISSR, RAPD and some morphological markers. The map could be used in mapping of agronomically important genes, map based cloning of the genes, in marker assisted selection of biotic, abiotic resistance genes, further genetic studies and plant breeding. The map also could be used in comparative genome anlysis with other maps of legume species. However, additional markers, such as SNP, need to be added to saturate the linkage map.

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