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INTEGRATION OF NOVEL SSR MARKERS INTO THE LENTIL (*Lens culinaris* Medik.) GENOME

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ABSTRACT

The development of simple sequence repeat markers (SSRs) for lentils has played a pivotal role in enhancing the comprehension of the lentil (*Lens culinaris* Medik.) genome through genetic mapping. The study aimed to determine the relative positions of newly developed microsatellites to the lentil genome using an F_7-derived recombinant inbred lines (RIL) population of 71 individuals developed from a cross between Eston and PI320937. Molecular analysis was performed with 100 newly developed lentil SSR markers and a linkage map was constructed using MapMaker/EXP 3.0b and MapChart 2.2 software. Among the 100 SSR markers, 12 markers exhibited polymorphism, 54 markers were identified as monomorphic, and 34 markers remained unamplified. While 10 out of the 12 polymorphic markers successfully integrated into two linkage groups, covering a cumulative length of 19.2 cM, two markers remained unlinked. Linkage group-1, comprised of 8 markers, spanned 4.8 cM, and linkage group-2 extended over a length of 14.4cM with two markers. Despite only partially representing 2 out of the 7 chromosomes in the lentil genome, this map holds promise for future mapping studies. Through the addition of markers, it could facilitate marker-assisted selection and the identification of QTLs associated with specific agronomic traits.

Keywords: Linkage map, Microsatellites, Polymorphism, Recombinant Inbred Lines, Lentil.

INTRODUCTION

Lentil (*Lens culinaris* Medik.), documented as one of the oldest legumes ever cultivated is an annual autogamous cool season legume with a diploid chromosome number of 2n =2x =14 and a ~4.2 Gb genome size (Bett and Cook, 2016). The global production area of lentil spans 5.01 million hectares and yields 6.53 million tons of lentils, with leading producers including Canada, India, Australia, Türkiye, USA, Nepal, Syria, Bangladesh, and China (FAOSTAT 2022). In addition to contributing 35-53% starch, 23-31% protein, and 18% fiber to the human diet, its low cholesterol establishes it as a nutritious and health-conscious choice (Devos, 1998). Moreover, the nitrogen fixation from root nodulation, combined with its capability for carbon sequestration, underscores the significant role lentils play in enhancing soil fertility and optimizing crop management practices (Quereshi et al., 2010).

Currently, the advancement of lentil improvement is hindered by the limited developed molecular tools for genomic analyses (Gupta et al., 2012). This underscores the pressing need for increased development of molecular markers that can hasten the accurate applicability of biotechnological approaches to enhance marker assisted selection (MAS) in lentil breeding. Despite the reported lower genetic variation in lentil compared to other plants, the use of microsatellites remains unparalleled in maximizing polymorphism for the creation of high-density maps that facilitate the identification of QTLs and target genes (Sonante and Pignone, 2001; Saha et al., 2010).

Microsatellites, also known as SSRs or short tandem repeats (STR), consist of 1-6 nucleotides that are unevenly distributed throughout the entire prokaryotic and eukaryotic genomes (Asp et al., 2007). Utilizing microsatellites in lentil has immensely permitted analysis of linkage and agronomical traits due to their co-dominance inheritance, high polymorphic rate, transferability and information content, locus specificity, reproducibility, relatively simple and safe detection procedure (Begna and Yesuf, 2021). Despite the impediment to the use of microsatellites in crop breeding and molecular studies due to their high cost of development, they are still considered ideal for map construction (Avise, 2012). While the initial lentil genetic maps were constructed using morphological markers (Zamir and Ladizinsky, 1984), isozyme markers (Tadmor, 1987), and DNA-based markers (Havey and Muehlbauer, 1989), the significant improvement in lentil genome mapping started with using SSR, AFLP, ISSR and morphological loci (Duran, 2004). Subsequently, a series of maps followed (Hamwieh et al., 2005; Tullu et al., 2008; Andeden et al., 2015; Verma et al., 2015; Dikshit et al.,

2016; Kumar et al., 2018; Singh et al., 2019; Kahraman et al., 2019; Gupta et al. 2023; Topu et al. 2023.). Simultaneously, other molecular tools were utilized in lentil mapping studies (ITAP (intron-targeted amplified polymorphic) (Phan et al., 2007), ISSR (Rubeena and Taylor, 2003; Tanyolac et al., 2010), SNPs (Ates et al., 2016; Bhadauria et al. 2017, Vijayan, et al., 2017; Sudheesh, Rodda, et al., 2016; Temel et al., 2014, Ates et al., 2018).

Given the current global climate change crisis resulting in the escalation of biotic and abiotic stresses of disease, drought, floods, and salinity (Jain et al., 2023), it becomes crucial to innovate strategies to mitigate their effects on lentil cultivation on a genetic basis. Because of the foregoing, increasing the marker repertoire by availing novel SSRs is essential for creating detailed lentil maps. Linkage maps based on gene recombination are classical tools for genetic analyses that enable the localization of targeted genomic regions and hence primal to lentil trait development. This research aimed to pinpoint the positions of novel genomic SSR markers on the genome that will later be used for gene identification on respective chromosomes. This will in turn contribute to effective plant breeding, MAS and further mapping studies of major lentil genes.

MATERIALS AND METHODS

Plant Materials and DNA Extraction

An F_7-derived LR39 RIL mapping population of 71 individuals developed from the cultivated lentil parents "Eston" × "PI320937" by the University of Saskatchewan, Canada was used. This population was developed to ascertain the genetic background of resistance to anthracnose (*Colletotrichum lentis*) in PI320937 (Tullu et al., 2003). Genomic DNA was isolated from fresh 3-week-old seedlings at the Erciyes University, Genome and Stem Cell Center, Türkiye by Lefort et al. (1998). The quantity and quality of the extracted DNA was determined using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and 1% agarose gel electrophoresis.

PCR Reactions

A total of 100 genomic SSR markers (Bakir and Kahraman, 2019; Demir and Bakir, 2022; Bakir et al., 2023), developed from enriched genomic libraries of AC and AG repeats in *Lens culinaris* cv. Kafkas, were used. The PCR amplification was performed in a final volume of 15 ul in the presence of 15 ng of genomic DNA, 10 pmol of each primer (Forward & Reverse), 2 mM of $MgCl_2$, dNTP (0.5 ul), Buffergreen (10X), 0.35-unit Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA). The PCR program consisted of an initial step of 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 50-66 °C, 2 min at 72 °C and a final extension of 10 min at 72°C. The PCR reaction was checked in 3% metaphor agarose to evaluate the polymorphism of the SSR markers in the parents.

Successfully amplified polymorphic markers in parents were used to ascertain polymorphism according to Schuelke (2000) using an M13-tailed primer (M13 universal sequence (−21), TGT AAA ACG ACG GCC AGT) that was 5'- tagged with fluorophores ROX, HEX or 6-FAM to enhance multiplexing and added to the 5' end of each forward primers. The total reaction mixture was 15 ul comprising of 15 ng of genomic DNA, 10 pmol of each primer (Forward & Reverse), 2 mM of $MgCl_2$, dNTP (0.5 ul), Buffer green (10X), 0.1 µM labeled M13 (-21) universal primer, 0.35-unit Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA). The amplification program consisted of an initial step of 3 min at 94°C, followed by 35 cycles of 1 min at 94 °C, 1 min at 50-66 °C, 2 min at 72 °C, followed by 8 cycles of 1 min at 94 °C, 1 min at 53 °C, 2 min at 72 °C, and a final extension of 10 min at 72 °C. In a ratio of 1:1:2 for HEX, 6-FAM or ROX respectively, amplicons were grouped in threes and mixed with 9.5 µl Hi-Di™ formamide (Applied Bio-systems, Foster City, CA, USA) and 0.5 µl GeneScan-600 LIZ size standards (Applied Bio-systems, Foster City, CA, USA). The final mixture was run through a denaturation process at 95 °C for 5 min, immediately chilled on ice and then electrophoresed on the Applied Bio-systems Prism 3500-Genetic Analyzer System (Applied Bio-systems, Foster City, CA, USA). The fragment size was determined using GENEMAPPER software v5.0 (Applied Bio-systems, Foster City, CA, USA).

Linkage Mapping

The chi-square (χ^2) goodness of fit test was used to calculate the presence of segregation distortion at each locus ($p < 0.05$) by comparing the observed segregation ratios to the 1:1 expected Mendelian ratios of RIL populations and a genetic linkage map was constructed using the MAPMAKER/EXP V.2.0 program (Lander et al., 1987). In determining the genetic linkage between two random markers, the linkage criteria of LOD = 3 score was used and the Kosambi mapping function was used to convert recombination frequencies into genetic distances (Kosambi, 2016). Mapchart software V.2 was then used to visualize the output (Voorrips, 2002).

RESULTS AND DISCUSSION

The RIL population derived from "Eston" × "PI320937" lentil parents was used in other studies to construct a consensus map using 9,793 DArT markers, identify QTLs associated with earliness, plant height using (AFLP, SSRs and RAPD markers), QTL analysis for selenium uptake (4 SSRs, and 1,780 SNPs), resistance to anthracnose (*Colletotrichum lentis*) and ascochyta blight (*Ascochyta lentis*) (RAPD and AFLP markers) and stemphylium blight resistance (*Stemphylium botryosum*) (Tullu et al., 2002; Tullu et al., 2006; Tullu et al., 2008; Podder, 2012; Ates et al., 2016; Ates et al., 2018; Ates, 2019).

SSR Analysis

In the tested population, 12 SSR markers that accounted for 12% of the total 100 tested markers were found

polymorphic. A proportion of these same markers were tested on 24 lentil cultivars (Bakir and Kahraman, 2019), 23 lentil cultivars (Demir and Bakir, 2022), and 10 lentil cultivars (Bakır et al., 2023). However, higher polymorphic rates of 58.4%, 26.6% and 48.5% respectively were observed. In a like manner, Kahraman et al. (2019) reported dissimilar polymorphic rates for the same SSR primers used by Duran et al. (2004), Hamwieh et al. (2005), and Rajesh et al. (2008) yielding polymorphic rates of 20%, 6.45% and 34.85%, respectively. Whereas Kahraman et al. (2019) used WA8649041 × Precoz, mapping populations from ILL 5588 x L 962-16-1 and *Lens culinaris* ssp. *culinaris* cv. Lupa × *L. culinaris* ssp. *orientalis* Boiss. (BG 16880) were used by Hamwieh et al. (2005) and Duran et al. (2004), respectively. This could suggest population genetic makeup as the source of the significant polymorphic dissimilitude. With the source of the population's genetic makeup being parental combination, polymorphism could be affected by either interspecific or intraspecific combinations (Sari et al., 2023) https://doi.org/10.1038/s41598-023-37268-w). Although generally in the genus *Lens* Mill., intersubspecific populations produce higher polymorphic rates (Tahir and Muehlbauer 1994). The subspecies combination of the Eston × PI320937 (cultivated lentil × cultivated lentil) was found similar to several studies. Jha et al. (2017) reported a polymorphic rate of 5.79% from an intraspecific cross of *Lens culinaris* ssp. *culinaris* (WA8649090 × Precoz), while Rubeena et al. (2003) observed a rate of 19.2% in an F_2 intraspecific population (ILL5588 × ILL7537). Additionally, Phan et al. (2007) found a rate of 15.7% from 626 ITAP markers tested on the cultivars Digger (ILL5722) and Northfield (ILL5588) parents of an F_5 RIL population as well as Radhika et al. (2007) observed polymorphism of 9.5% in JV (JG62 × Vijay) and 11.6% in VI (Vijay × ICC4958) intraspecific $F_{8:9}$ RIL populations of chickpea. Other similar studies include Septiningsih et al. (2012) and Koyama et al. (2001) that used a mapping population from a combination of indica × indica parents in rice. Septiningsih et al. (2012) reported 10.5% polymorphism out of the 1,074 SSR markers used. Besides the parental crosses, polymorphism could be attributed to the plant's mode of reproduction (cross- or self-pollinated), type of mapping population (RILs, F_2, BC, DH, NILs) and the type of markers used for genotyping (Vaillancourt and Slinkard, 1993; Eujayl et al., 1998).

Genetic Mapping

Segregation distortion analysis showed nine markers (75%); Lc_Mcu5, Lc_Mcu6, Lc_Mcu14, Lc_Mcu19, Lc_Mcu20, Lc_Mcu45, Lc_Mcu70, Lc_Mcu87 skewed towards the genotype Eston and Lc_Mcu26 towards the genotype P1320937 (Table 1). A 1:1 normal segregation was exhibited by three markers (Lc_Mcu79, Lc_Mcu47 and Lc_Mcu2). All the polymorphic markers were used in the mapping to eliminate the loss of any genetic information that could be linked to distorted markers (Takumiet et al., 2013, Kirungu et al., 2020). Although segregation distortion depends on the specific cross of parents (intra- or interspecific), in comparison; Eujayl et al. (1997) observed 83.3% in lentil, Eujayl et al. (1998) 26.6% in a RIL population, 14% by Rubeena et al. (2003) in lentil, (9.5% SSRs and 17.8% AFLP in a lentil RIL population) Hamwieh et al. (2005), 48% Tanyolac et al. (2010), 5.6% Ates et al. (2018) and 38.4% Winter et al. (2000) in *Cicer* sp. The underlying factors of this phenomenon could be selective elimination and gametic selection which includes preferential fertilization and pollen tube competition (Paterson et al., 2000).

Table 1. Chi-square test (χ^2) for segregation distortion of genomic SSRs in RIL population

		Genotype				
Marker	Genetic characteristic	A/A	B/B	A/B	χ^2	Direction of distortion
Lc_Mcu2	codominant	43	27	1	3.61	-
Lc_Mcu5	codominant	42	0	25	35.66**	Eston
Lc_Mcu6	codominant	49	20	0	12.18**	Eston
Lc_Mcu14	codominant	46	0	25	38.60**	Eston
Lc_Mcu19	codominant	22	2	46	35.94**	Eston
Lc_Mcu20	codominant	36	2	32	31.14**	Eston
Lc_Mcu26	codominant	2	69	0	63.22**	P1320937
Lc_Mcu45	codominant	44	0	3	41.38**	Eston
Lc_Mcu47	codominant	25	23	2	0.16	-
Lc_Mcu70	codominant	22	0	48	39.82**	Eston
Lc_Mcu79	codominant	27	39	3	2.21	-
Lc_Mcu87	codominant	38	13	9	11.76**	Eston

** :Significant at the 0.05 probability level

A total of 10 markers accounting for 83.3% of the polymorphic markers were mapped (Figure 1). Despite the low polymorphic rate, the majority of the markers were mapped with only two markers Lc_Mcu2 and Lc_Mcu26 (16.7%) unlinked. A similar scenario was reported by Gupta et al. (2012) and Andeden et al. (2015) that observed 4.43% and 18% polymorphism but mapped 82.35% and 79% of the polymorphic markers, respectively. Additionally, Singh et al. (2021) successfully genotyped RILs with only eight polymorphic markers out of 389 SSRs (2.05%) and revealed markers LcSSR440 and LcSSR606 that co-segregated with rust resistance. A comparison with

Gupta et al. (2012) - 66.7%, Verma et al. (2015) - 33.3% and Kahraman et al. (2019) - 56.5% found this mapping rate higher, but to the contrary, lower than Saha et al. (2013) and Qureshi et al. (2010) with 100% and 91.5%, respectively.

This linkage map (Figure 1) which covers a total length of 19.2 cM is one of the smallest microsatellite maps created. Albeit the consideration of having the same number of LGs as the haploid chromosome numbers of the species under study is ideal, this study only had two of the seven expected LGs. The insufficient number of polymorphic markers coupled with the small mapping population could be a reason for this. This is not only accentuated by Ferreira et al. (2006) that an insufficient mapping population number produces inaccurate ordering of loci on LGs and/or imprecise fragmentation but also by Pootakham et al. (2015) that asserts the negative effects of few markers on the calculation of marker order. Therefore, increasing marker density could decrease the number of unlinked markers to attain better coverage of the genome (Gupta et al., 2012).

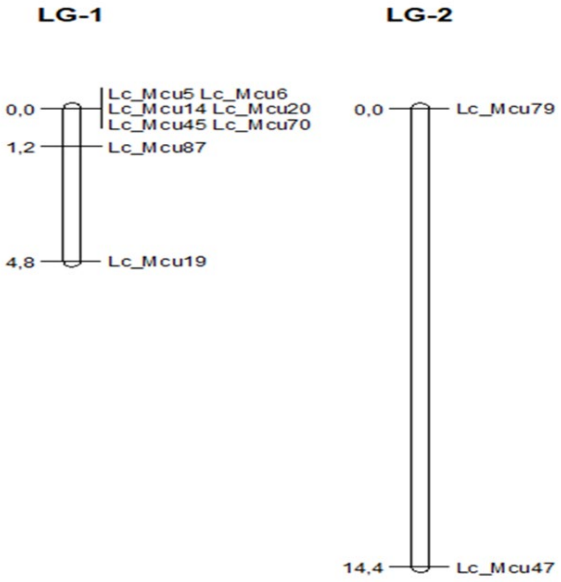

Figure 1. Linkage group 1 (LG-1) that included 8 markers and Linkage group 2 (LG-2) mapped two markers.

Using RIL populations in mapping is purposed to increase linkage breakdown since the probability of recombination is two-fold higher than in F_2 or BC_1 populations (Biswas et al., 2010). However, an appraisal of the linkage map (Figure1) depicts an uneven distribution of markers to the distal points of the LGs indicating a difference in the crossing-over frequency on chromosomes as the possible cause for the observed marker density. The markers are situated at the telomeres which are recombination-suppressed regions that have an extremely low recombination rate than the regions of the centromeres (Tanksley et al., 1992).

CONCLUSION

In spite of the low number of genomic SSRs used for constructing the linkage map in this study, we envisage that the partial coverage of two out of the seven chromosomes of the lentil genome could still be used for further mapping studies. Owing to the profound applicability of microsatellites, addition of new markers or an amalgamation with other lentil maps is imperative for its future utilization in breeding and crop advancement strategies. Besides enabling complete genome coverage by saturation with markers, using a larger mapping population will counteract rare recombination events caused by the presence of centromeric heterochromatin or limited recombination at the telomeres and in turn produce a fine map that will be used as a genetic framework for future qualitative and quantitative trait analysis for lentil.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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