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Genomic SSR marker development in lentil (*Lens culinaris* Medik.) and assessment of cross-species/genera transferability to related legumes

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Abstract: Simple sequence repeat (SSR) markers are valuable genetic and genomic research tools and are extensively used in major crops. However, a paucity of available molecular markers in lentils (*Lens culinaris* Medik.) has significantly hindered genomic studies in this vital legume crop. In this study, we developed 33 new SSR markers for lentils using an enriched genomic library and tested their polymorphism in 10 lentil cultivars. We found that 16 (48.5%) SSR markers were polymorphic. The expected heterozygosity values of the polymorphic SSR markers ranged from 0.095 to 0.820, while observed heterozygosity values varied between 0.100 and 1.00. In addition, we tested the transferability of 86 SSR markers, including the 33 newly developed ones, to other legume species. Transferability rates of lentil SSR markers to other species varied between 13.76% (*Vicia sativa* L.) and 2.58% (*Phaseolus vulgaris* L. and *Trifolium pratense* L.). These new SSR markers could be used in further molecular breeding, population genetics, and genetic mapping studies in lentils and other legume species.

Keywords: cross-transferability; genomic library; legumes; simple sequence repeats

Lentil (*Lens culinaris* Medik.) is among the oldest cultivated crop plants (Bahl et al. 1993; Rehman & Altaf 1994). Lentil is a self-pollinated annual cool-season crop (Arumuganathan & Earle 1991; Muehlbauer 1991) and is extraordinarily rich in dietary protein (22–35%), making the crop widely used in human nutrition and animal feeding (Hamwieh et al. 2005). World lentil production was recorded at 5.6 million tons in 5.5 million hectares (FAOSTAT 2023). Canada, India, Australia, and Türkiye are some of the significant lentil producers in the world (FAOSTAT 2023).

Simple Sequence Repeat (SSR) markers have become an essential tool in genetic and genomic research due to their co-dominant inheritance, high degree of polymorphism, and multi-allelic nature (Gupta et al. 1996; Powell et al. 1996; Parida et al. 2009). They are widely used in various applications, such as genetic diversity studies, phylogenetic analysis, population structure analysis, QTL mapping, and marker-assisted selection (MAS) (Hendre & Aggarwal 2007). The limited availability of molecular markers explicitly developed for lentils has hindered the progress of molecular genetics and genomic studies in this crop species (Gupta et al. 2012).

Developing species-specific SSR markers is time-consuming and expensive, limiting their development

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and use in some species (Rossetto 2001; Squirrell et al. 2003; Oliveira et al. 2006). As an alternative strategy, cross-transferability of SSR markers can be used when conserved homologous DNA flanking regions are present (Peakall et al. 1998; Oliveira et al. 2006; Reddy et al. 2010; Huang et al. 2014). Successful cross-amplification of SSR markers has been demonstrated in many species, and the success of cross-transferability is often inversely related to the evolutionary distance between the two species. Studies have shown that SSR markers can be transferred into different legume species, such as *Vicia sativa* L., *Phaseolus vulgaris* L., and *Trifolium pratense* L. Successful transfer of SSR markers can facilitate further molecular breeding, population genetics, and genetic mapping studies in legume species including lentil (Almeida et al. 2014; Jingade et al. 2014; Wang et al. 2015; Min et al. 2017; Bakır 2019; Singh et al. 2020) and other species (Cipriani et al. 1999; Roa et al. 2000; Zucchi et al. 2002; Satya et al. 2016; Li et al. 2021).

Lentil suffers from poor SSR resources as compared to other legumes. There are only 389 SSR genomic markers currently available for lentils, which significantly limits their use in molecular breeding technologies for improving lentil crops (Duran et al. 2004; Hamwieh et al. 2005, 2009; Saha et al. 2010; Verma et al. 2014; Andeden et al. 2015; Bakır & Kahraman 2019). Therefore, developing new functional SSR markers is essential for advanced lentil breeding programs. Additionally, identifying transferable SSR markers between different genera is crucial for comparative genomic studies, offering significant economic and time savings. This study aimed to develop and characterize new functional SSR markers and determine their transferability to legume species. The outcomes of this study will provide significant contributions to enhancing the number of polymorphic SSR markers in cultivated lentils and further comparative genomic studies in other legume species.

MATERIAL AND METHODS

Plant material and DNA extraction. Ten registered lentil cultivars were used to assess the polymorphism rates of the developed markers. Additionally, 17 registered cultivars from 11 distinct legume species, including at least one cultivar per species, were employed to evaluate the transferability of these markers to the other legume species (Table 1). Genomic DNA was extracted from lentil seedlings using the protocol specified by Lefort et al. (1998), with minor modifications. The quality and quantity of the extracted DNA were evaluated using a spectrophotometer (NanoDrop Technologies, Wilmington, USA) and agarose gel electrophoresis (1%).

Construction of genomic library and analysis of microsatellite-containing sequences for primer design. A genomic library was constructed from *L. culinaris* cv. Kafkas using a hybridization-based capture technique with minor modifications to the protocol described by Techen et al. (2010). Biotinylated $(AG)_{12}$ and $(AC)_{12}$ oligoprobes and streptavidin-coated magnetic beads were used for the construction, and the detailed protocol can be found in Bakır and Kahraman (2019).

Microsatellite-containing colonies were identified through a colony polymerase chain reaction (PCR) (Bloor et al. 2001), and the PCR products were run on a 2% agarose gel electrophoresis. The PCR products with two or more bands were considered microsatellite-containing plasmids.

Positive plasmids were amplified using the TempliPhi Amplification kit (GE Healthcare, Wauwatosa, USA) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit in an Applied Biosystems Prism 3500 Genetic Analyzer System (Applied Biosystems, Foster City, USA). Vector sequences were removed using Vecscreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/), and redundancy was eliminated using CAP3 (https://doua.prabi.fr/software/cap3) software (Huang & Madan 1999). The location of microsatellite repeats in the sequences was determined using the SSRIT software (https://archive.grame ne.org/db/marke rs/ssrto ol; Temnykh et al. 2001), and BioEdit software (Hall 1999) was used to identify and remove duplicated sequences. Primers were designed using BatchPrimer3 software (You et al. 2008) based on a minimum of 5 dinucleotide and three trinucleotide repeat-containing sequences.

Genetic diversity and transferability analysis of microsatellites. The newly developed SSR markers were validated using Tigris and Seyran-96 lentil cultivars. PCR amplification (15 µL) was conducted with the following components: 90 ng genomic DNA, 10 µM of each primer, 2.5 mM of each deoxynucleotide triphosphate (dNTP), 5× Dream*Taq* Green Buffer (2 mM $MgCl_2$), and 0.5U Dream*Taq* DNA Polymerase (Thermo Scientific, Waltham, USA). PCR reactions were performed using a Bio-Rad thermocycler. The amplification process included the following steps: 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min

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at 50–66 °C, and 2 min at 72 °C, followed by a final extension step of 5 min at 72 °C. The PCR products were analyzed via 3% agarose gel electrophoresis, and only those products with one or two bands and within the amplification range of 100–500 bp were considered for further analysis.

The optimized SSR markers were evaluated for polymorphism ratios in 10 lentil cultivars using M13-tailed primer. Each forward primer had a tail (M13 universal sequence (–21), TGT AAA ACG ACG GCC AGT) added to its 5' end. PCR amplification (15 µL) was performed using the following components: 90 ng genomic DNA; 1.5 µM of each SSR primer; 1.5 µM labeled M13 (–21) universal primer; 2.5 mM of each dNTP; 5× Dream*Taq* Green Buffer (2 mM $MgCl_2$); 5U Dream*Taq* DNA Polymerase (Thermo Scientific, Waltham, USA). The amplification process involved the following steps: 3 min, 94 °C; 35 cycles, 1 min, 94 °C; 1 min, 50–66 °C; 2 min, 72 °C; 8 cycles, 1 min, 94 °C; 1 min, 53 °C; 2 min, 72 °C; 10 min, 72 °C. The M13 (–21) universal primer was 5'-fluorescently tagged with HEX, 6-FAM or ROX. A set of three PCR products (0.5 µL each) was supplemented with 0.5 µL GeneScan-600 LIZ size standards and 9.5 µL Hi-Di™ formamide, denatured at 95 °C for 5 min, chilled on ice, and electrophoresed on the Applied Biosystems Prism 3500 Genetic Analyzer System. Fragment size was determined with the aid of GENEMAPPER software (Ver. 5.0).

The transferability of 33 newly developed and 53 previously developed markers (Bakır & Kahraman 2019) was evaluated across 12 species and 27 cultivars, as listed in Table 1, using the same PCR conditions described above. Amplified products were analyzed

Table 1. Plant material used in the study

Species	No.	Cultivar	Registration center
Lens culinaris Medik.	1	Tigris	GAP International Agricultural Research and Training Center
	2	Seyran-96	GAP International Agricultural Research and Training Center
	3	Altın Toprak	GAP International Agricultural Research and Training Center
	4	Yerli Kirmizi	GAP International Agricultural Research and Training Center
	5	Kafkas	Field Crops Central Research Institute
	6	Ankara Yesili	Field Crops Central Research Institute
	7	Ceren	Field Crops Central Research Institute
	8	Erzurum-89	East Anatolian Agricultural Research Institute
	9	Emre-20	East Anatolian Agricultural Research Institute
	10	Sazak-91	Transitional Zone Agricultural Research Institute
Phaseolus vulgaris L.	11	Berrak	Field Crops Central Research Institute
	12	4F-89 Fransiz	Transitional Zone Agricultural Research Institute
Vicia faba L.	13	Eresen-87	Aegean Agricultural Research Institute
	14	Filiz-99	Aegean Agricultural Research Institute
Glycine max (L.) Merrill	15	Nazlican	Eastern Mediterranean Agricultural Research Institute
	16	Turksoy	Eastern Mediterranean Agricultural Research Institute
Pisum sativum	17	13	Akdeniz University, Department of Field Crops
	18	14	Akdeniz University, Department of Field Crops
Vicia sativa L.	19	Yucel	Eastern Mediterranean Agricultural Research Institute
	20	Ankara Moru-08	Field Crops Central Research Institute
Vicia narbonensis L.	21	Tarman-2002	Field Crops Central Research Institute
Vicia pannonica Crantz.	22	Kansur	Field Crops Central Research Institute
Vicia villosa Roth	23	Selcuklu-2002	Field Crops Central Research Institute
	24	Segmen-2002	Field Crops Central Research Institute
Onobrychis sativa L.	25	Ozerbey	Field Crops Central Research Institute
Trifolium pratense L.	26	Dadas Cayir Ucgulu	East Anatolian Agricultural Research Institute
Medicago sativa L.	27	Sazova	Transitional Zone Agricultural Research Institute

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using 3% agarose gel electrophoresis, and those with additional bands or outside the amplification range of 100–500 bp were excluded from the analysis.

Data analysis. Each developed lentil SSR locus was analyzed for expected heterozygosity using the equation (He = 1 – Σpi^2) (Nei 1973). Observed heterozygosity was determined through direct counting, and the polymorphism information content (PIC) was calculated using the equation (PIC = 1 – Σpi^2 – $\Sigma\ \Sigma 2pi^2pj^2$) (Botstein et al. 1980). The UPGMA (unweighted pair-group method using arithmetic average) was used to construct a dendrogram from the genetic similarity matrix by using the MEGA6 (Tamura et al. 2007) and PowerMarker software programs (Liu & Muse 2005). Bootstrap analyses with 100 replicates were performed, and a consensus tree was obtained to measure the confidence levels for the clusters.

RESULTS

Identification of positive clones containing microsatellites and characterization of microsatellite patterns. A total of 250 clones were generated, and 37 were found to contain SSR motifs using colony PCR (Bloor et al. 2001). These 37 SSR-containing clones were then sequenced, and four duplicate sequences were identified and excluded, resulting in 33 unique sequences with 120 SSR motifs suitable for primer design. Most identified microsatellite repeats were dinucleotide repeats, with few trinucleotide repeats observed. Tetranucleotide repeats were not used for primer design due to their small number of repeats. Of the 33 SSR markers selected, 78.8% exhibited imperfect repeats, 15.2% contained perfect dinucleotide repeats, and 6% contained compound repeats (Table S1 in Electronic Supplementary Material (ESM)).

Determination of polymorphism ratio and genetic diversity analysis. Out of the 33 developed SSR markers, 9% (3 pairs) failed to amplify, 18.2% (6 pairs) produced non-specific bands, 48.5% (16 pairs) were found to be polymorphic, and 24.2% (8 pairs) were found to be monomorphic. After excluding the markers that failed to amplify and produced non-specific bands, the remaining 24 markers were evaluated on 10 lentil cultivars to determine their polymorphism rates and suitability for genetic analysis.

Among the 16 polymorphic SSR markers tested, 57 alleles were generated in the studied cultivars, ranging from 2 to 8 alleles per locus (average 3.56). The highest number of alleles was obtained from the Lc_MCu87 marker with 8, followed by Lc_MCu79 with 7 alleles. The expected heterozygosity ratios ranged from 0.095 (Lc_MCu90) to 0.820 (Lc_MCu87) with an average of 0.541, while the observed heterozygosity ratios ranged from 0.100 (Lc_MCu90) to 1.000 with an average of 0.363. The (PIC values ranged from 0.09 (Lc_MCu90) to 0.79 (Lc_MCu87) with an average of 0.46 (Table 2).

A dendrogram was constructed based on the 57 loci, and the cultivars were grouped into two main clusters, Cluster I and II, containing 7 and 3 cultivars, respectively. Each cluster was further divided into two subgroups (Figure 1). Cluster I included Sazak-91, Emre-20, Ceren, Ankara Yesili, Eruzurm-89, Kafkas, and Yerli Kirmizi cultivars, while Cluster II included Seyran-96, Altin Toprak, and Tigris cultivars. The highest genetic similarity of 80% was observed between Ankara Yesili and Erzurum-89, as well as between Tigris and Altin Toprak cultivars. Sazak-91 and Altin Toprak were the most distant cultivars, with a genetic similarity of only 24%.

Transferability of lentil SSR markers to other legume species. The transferability of SSR markers to other species is an essential aspect of their utility in genetic studies. The study tested the transfer-

Table 2. Genetic parameters for simple sequence repeat markers

Locus	*n*	He	Ho	PIC
Lc_MCu68	4	0.740	1.000	0.69
Lc_MCu69	2	0.320	0.200	0.26
Lc_MCu70	6	0.710	0.900	0.67
Lc_MCu74	2	0.255	0.300	0.22
Lc_MCu79	7	0.850	0.600	0.77
Lc_MCu80	3	0.460	1.000	0.41
Lc_MCu81	5	0.715	0.300	0.66
Lc_MCu83	2	0.420	0.200	0.33
Lc_MCu84	3	0.395	0.200	0.34
Lc_MCu85	3	0.565	0.700	0.48
Lc_MCu86	3	0.460	1.000	0.41
Lc_MCu87	8	0.820	0.500	0.79
Lc_MCu88	2	0.500	1.000	0.37
Lc_MCu90	2	0.095	0.100	0.09
Lc_MCu94	3	0.645	0.700	0.57
Lc_MCu98	2	0.320	0.200	0.26
Mean	3.56	0.541	0.363	0.46

n – number of alleles; He – expected heterozygosity; Ho – observed heterozygosity; PIC – polymorphism information content

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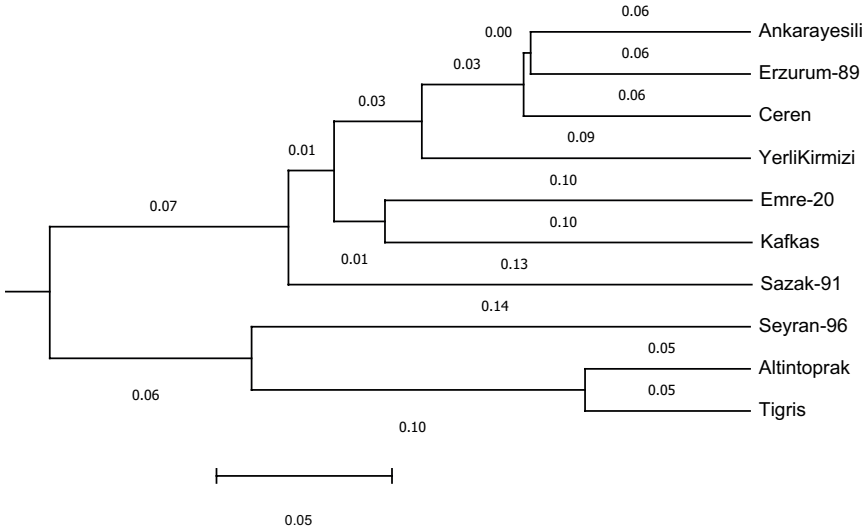


Figure 1. Genetic similarity dendrogram generated from genetic similarity matrix using unweighted pair group method with arithmetic mean in 10 lentil cultivars with newly developed simple sequence repeat markers

ability of 86 lentil SSR markers, including 33 newly developed markers and 53 markers previously developed by Bakır and Kahraman (2019), to several other legume species (Table 1). The transferability of amplified markers varied among species. The highest transferability rate was found in the *Vicia* species, with 18.06% (21 pairs) in *V. sativa*, followed by *V. pannonica* with 13.76% (16 pairs), *V. villosa* with 12.09% (15 pairs), *V. faba* with 12.09% (15 pairs), and *V. narbonensis* with 11.18% (13 pairs). In all, 42.8% of the amplified markers worked across all *Vicia* species. The transferability rate in other species was 12.09% (15 pairs) for *P. sativum*, 8.6% (10 pairs) for *M. sativa*, 6.88% (8 pairs) for *O. sativa*, 5.16% (6 pairs) for *G. max*, and 2.58% (3 pairs) for *T. pratense* and *P. vulgaris* (Table S2a, b in ESM).

DISCUSSION

The lack of sufficient molecular markers in lentils has hindered progress in genomic studies and molecular breeding efforts. The present study aimed to develop new lentil SSR markers using enriched genomic libraries to address this need. The study yielded a higher number of clones containing the repeat motif compared to Hamwieh et al. (2009), who did not use the enriched method, and a lower number compared to Verma et al. (2014), Andeden et al. (2015), and Bakır and Kahraman (2019), who used the enriched method. Moreover, 89% of the sequences containing SSR motifs were found to be suitable for primer design, which is higher than rates reported by Hamwieh et al. (2009), Verma et al. (2014), Andeden et al. (2015), and Bakır and Kahraman (2019). However, microsatellite detection studies have shown that the results may be influenced by the selection of restriction enzymes during library creation, the type of SSR motifs selected for enrichment, methodological approaches, or the low availability of selected SSR motifs in the relevant plant genome (Cuc et al. 2008). Therefore, further research is needed to optimize genomic SSR marker development for lentils.

Bakır and Kahraman (2019) tested 31 SSR markers in 24 cultivars and found 144 alleles with a range of 2 to 15 alleles per locus (average 4.64). The PIC values ranged from 0.194 to 0.895 (average 0.520). Verma et al. (2014) used 33 SSR markers in 46 genotypes of *Lens culinaris* sub-species and eight different legumes and reported a total of 123 alleles with a range of 2 to 5 alleles per locus (average 3.73). The PIC values ranged from 0.13 to 0.99 (average 0.66). Andeden et al. (2015) used 78 polymorphic SSR markers in 15 genotypes and found 400 alleles with a range of 2 to 11 alleles per locus (average 5.1). The PIC values ranged from 0.07 to 0.89 (average 0.58). Hamwieh et al. (2009) experimented on *Lens culinaris* sub-species using SSR markers and reported 182 alleles with an average of 13 per locus. The total number of alleles for *L. culinaris* subsp. *culinaris* was reported as 128, and the range of alleles per locus was between 2 and 16 (average 9.14).

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The present study used 16 polymorphic SSR markers to test 10 lentil cultivars. The number of alleles per locus and the average number of alleles were lower than those of Hamwieh et al. (2009), Andeden et al. (2015), and Bakır and Kahraman (2019) and similar to the findings of Verma et al. (2014). The PIC values were close to each other, and the differences were attributed to the number and diversity of genotypes used in these studies (Bakır & Kahraman 2019).

Evolutionary proximity and adequate homology between species make the transferability of SSR markers possible (Doyle & Luckow 2003; Choi et al. 2004). Taxonomically, the closest genera to *Lens* are *Vicia* and *Pisum*, *Trifolium,* and *Medicago*. *Phaselous* and *Glycine* genera are far from *Lens* (Doyle & Luckow 2003; Choi et al. 2004). In this study, based on evolutionary proximity, the highest transferability rate was observed in closely related species identified as *Vicia* (18.06%), followed by *P. sativum* (12.09%) and *M. sativa* (8.6%). In contrast, transferability rates were lower in more distant species, such as 5.16% for *G. max* and 2.58% for *P. vulgaris*. Transferability rates also varied among *Trifolium* species (2.58%.), with lower rates observed than other closely related genera.

Studies on cross-transferability of SSR markers in lentil to other legume species have reported variable rates ranging from 10.9% to 87.88%. Verma et al. (2014) tested 33 SSR markers in *L. culinaris* subspecies, wild species, and eight legume species. The transferability of SSR markers between the species varied, with rates ranging from 80.72% (*L. ervoides*) to 87.88% (*L. lamottei*) among *Lens*, and 72.73% for *T. alexandrinum*, 69.70% for *P. sativum*, 60.61% for *Cicer reticulatum*, 54.55% for *M. truncatula*, 48.48% for *C. cajan*, 45.45% for *G. max*, and 12.12% for *V. radiata* in the other species. Similarly, Singh et al. (2020) tested the cross-transferability of 46 newly developed EST-SSR markers in lentils to 12 legume species. The transferability rates ranged from 54.3% to 10.9%. Cluster bean, rice bean, lobia bean, horse gram, pea, and pigeon pea had cross-transferability rates of greater than 50%, while moth bean, urdbean, yard long bean, mungbean, chickpea, and cowpea had cross-transferability rates of less than 50%. Reddy et al. (2010) tested the transferability of markers developed from *T. pratense*, *M. truncatula*, and *P. sativum* species to lentils due to insufficient microsatellite markers. The transferability rates were 62% for *Trifolium* SSRs, 36% for *Medicago* SSRs, and 25% for *Pisum* SSRs. However, these rates were lower than the transferability rates reported by Verma et al. (2014) and Singh et al. (2020), although the genera or species studied were not the same. In this study, the transferability rate among the legume species was found to be higher than that of Reddy et al. (2010), Verma et al. (2014), and Singh et al. (2020). Choudhary et al. (2009) suggested that genus-specific evolutionary events resulted in variable marker transferability rates. The choice of loci and the total number of markers analyzed could also contribute to the differences in transferability rates (Choudhary et al. 2009).

The lack of available molecular markers in lentils has significantly hindered genomic studies. Thus, molecular studies need robust and informative genetic markers for different purposes (Andeden et al. 2015). This study has developed new lentil SSR markers that will significantly contribute to further lentil molecular breeding studies. The transferable lentil SSR markers can be utilized in interspecies genetic association and mapping studies.

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