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Transferability of Genomic SSR Markers in *Lens* Species for Genus Characterization and Lentil (*Lens culinaris* Medik.) Breeding

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ABSTRACT

Simple sequence repeats (SSRs) are pivotal for germplasm characterization and crop improvement. However, lentil breeding faces challenges due to the limited availability of markers. This study assessed the transferability of genomic SSR markers developed in cultivated lentils to wild *Lens* species. A total of 100 SSR loci were analyzed in 21 accessions representing all the *Lens* genus using PowerMarker software v3.25, which identified 41 polymorphic loci. These loci generated 219 alleles, with an average of 5.34 per locus and a polymorphic information content (PIC) value of 0.61. Notably, 52.2% of the polymorphic loci were shared among all *Lens* species. Transferability rates varied across species: 41% for *Lens nigricans*, 40% for *Lens culinaris* subsp. *tomentosus*, 39% for *Lens culinaris* subsp. *orientalis*, 34% for *Lens culinaris* subsp. *odemensis*, 34% for *Lens ervoides*, and 31% for *Lens lamottei*. The identified polymorphic markers provide a valuable resource for exploring the genetic diversity of wild lentil relatives and the breeding of beneficial agronomic traits into new lentil cultivars.

1. INTRODUCTION

Lentil (*Lens culinaris* Medik.) is a widely cultivated legume crop, classified within the galegoid clade of the *Fabaceae* (*Leguminosae*), which is the third largest plant family (Konda & Annapragada, 2024). This autogamous diploid legume, with a chromosome number of 2n = 14 and a large genome of approximately 4 Gbp (Bett & Cook, 2016; Ates, 2019), is cultivated over 5.01 million hectares worldwide, yielding around 6.53 million tons annually. The leading producers include Canada, India, Australia, Türkiye, the USA, and Nepal (FAOSTAT 2023). Besides its role in soil fertility management by nitrogen fixation, lentil is an essential, affordable dietary staple. It is rich in carbohydrates (63.4%), protein (24.5%), essential minerals, vitamins, and dietary fiber, which is also beneficial for livestock nutrition (Dhull et al., 2022).

Despite ongoing debates regarding the classification of the *Lens* genus, the widely accepted taxonomy includes four species: *L. culinaris*, with its four subspecies (subsp. *culinaris*, subsp. *orientalis*, subsp. *odemensis*, and subsp. *tomentosus*), *L. lamottei*, *L. ervoides*, and *L. nigricans* (Wong et al., 2015; Koul et al., 2017). Phylogenetic analysis using Genotyping-by-Sequencing (GBS) has further grouped these species into distinct genetic pools. The primary gene pool encompasses *L. culinaris*, *L. orientalis*, and *L. tomentosus*; the secondary pool includes *L. lamottei* and *L. odemensis*, while the tertiary and quaternary pools consist of *L. ervoides* and *L. nigricans*, respectively. Among these, *L. culinaris* subsp. *orientalis* is considered the putative progenitor of cultivated lentils, whereas *L. nigricans* represents the most distant relative (Wong et al., 2015; Koul et al., 2017; Dissanayake et al., 2020; Liber et al., 2021).

The domestication of lentils that dates back to the Pre-Pottery Neolithic resulted in the co-occurrence of most *Lens* wild relatives in Southwestern Türkiye, the Mediterranean, and the Aegean regions (Ferguson et al., 1998). Although domestication contributed to the acquisition and/or loss of novel genes due to varying environmental stimuli, global warming has exacerbated the negative effects of stress on lentil cultivation. This trend has prompted the development of innovations to enhance yields on farmlands to counteract food scarcity (Caracuta et al., 2017).

Molecular markers and characterization of wild gene pools with extensive genetic variability are crucial to introduce target genes into cultivated lentil varieties. Precise identification of wild genetic resources increases the success of gene introgression into crops, and marker-assisted selection (MAS) using DNA markers such as simple sequence repeats (SSRs) can improve the efficiency of lentil breeding (Rubiales & Fondevilla, 2012; Dissanayake et al., 2020; Koboyi & Bakir, 2024). Simple Sequence Repeats (SSRs) are widely recognized as ideal molecular markers due to their utility in mapping and genetic diversity analyses (Saıdou et al., 2016). Their effectiveness has been demonstrated across various legumes, including lentil (Gutierrez-Gonzalez et al., 2022; Sharma et al., 2023), marama bean (Li, 2023), chickpea (Ningwal et al., 2023), mungbean (Mehandi et al., 2023), peanut (Daudi et al., 2021), Medicago (Zhao et al., 2024), and common bean (Ozkan et al., 2022). Although the development of genomic SSRs requires significant cost and labor, their numerous advantages include widespread occurrence throughout the genome, high polymorphism, locus specificity, co-dominant multi-allelic nature, high information content, reproducibility, and transferability (Cevik et al., 2015; Begna & Yesuf, 2021).

The conserved flanking regions of SSRs across related species or genera enable the assessment of their polymorphic potential and transferability, which is crucial for understanding phylogeny and genetic diversity (Datta et al., 2013). This transferability has been observed within the same legume species, as seen in *Lens* (Dikshit et al., 2015) and *Cicer* (Choudhary et al., 2009), as well as across species (Reddy et al., 2010; Datta et al., 2010; Gupta et al., 2012; Jingade et al., 2014; Verma et al., 2014; Bakir, 2019; Singh et al., 2020). Although relatively few sets of genomic SSR markers have been developed in lentil specifically, their polymorphism offers an invaluable resource for crop improvement. These markers hold significant potential for enhancing lentil breeding programs through marker-assisted selection. (Hamwieh et al., 2009; Verma et al., 2014; Andeden et al., 2015; Bakir & Kahraman, 2019; Demir & Bakir, 2022; Bakir et al., 2024).

This study aims to evaluate the transferability of genomic SSR markers developed in cultivated lentil to its wild relatives. The transferable SSR markers identified in this study will be invaluable for advancing lentil molecular research programs, providing a robust tool for understanding genetic variation, improving marker-assisted selection (MAS) strategies, and accelerating the development of enhanced lentil varieties. Furthermore, this research expands the limited genomic resources available for lentil improvement by uncovering additional genetic diversity within the *Lens* genus. It facilitates the identification of both intra- and interspecies candidate genes for future breeding efforts.

2. MATERIALS AND METHODS

Plant Materials and DNA Extraction

The plant material comprised 18 accessions of wild lentil species and subspecies obtained from Akdeniz University, Faculty of Agriculture, Department of Field Crops, and three cultivated cultivars of *L. culinaris* subsp. *culinaris* sourced from the GAP International Agricultural Research Center (Table 1). The seeds were grown in a growth chamber for three weeks at the Erciyes University Genome and Stem Cell Center, Türkiye, and the five pooled seedlings were used for DNA extraction. The genomic DNA isolation from the 21 samples was done using the CTAB method protocol of Lefort et al. (1998). The quantification and determination of the quality of the extracted DNA were performed using the NanoDrop®ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis (1%).

Table 1. List of the 21 genotypes used in the Transferability Analysis

No	Species	Accession number	Origin
1	*L. nigricans*	IG72545	ICARDA-Syria
2	*L. nigricans*	IG136636	ICARDA-Syria
3	*L. nigricans*	IG136639	ICARDA-Syria
4	*L. lamottei*	IG116009	ICARDA-Syria
5	*L. lamottei*	IG110812	ICARDA-Syria
6	*L. lamottei*	IG110811	ICARDA-Syria
7	*L. ervoides*	IG72862	ICARDA-Syria
8	*L. ervoides*	IG72914	ICARDA-Syria
9	*L. ervoides*	IG72920	ICARDA-Syria
10	*L. culinaris* subsp. *tomentosus*	IG72644	ICARDA-Syria
11	*L. culinaris* subsp. *tomentosus*	IG72830	ICARDA-Syria
12	*L. culinaris* subsp. *tomentosus*	IG72643	ICARDA-Syria
13	*L. culinaris* subsp. *orientalis*	IG135399	ICARDA-Syria
14	*L. culinaris* subsp. *orientalis*	IG136679	ICARDA-Syria
15	*L. culinaris* subsp. *orientalis*	IG136670	ICARDA-Syria
16	*L. culinaris* subsp. *odemensis*	IG116047	ICARDA-Syria
17	*L. culinaris* subsp. *odemensis*	IG136653	ICARDA-Syria
18	*L. culinaris* subsp. *odemensis*	IG116049	ICARDA-Syria
19	*L. culinaris* subsp. *culinaris*	Firat-87	GAPUTEM-Turkiye
20	*L. culinaris* subsp. *culinaris*	Tigris	GAPUTEM-Turkiye
21	*L. culinaris* subsp. *culinaris*	Seyran-96	GAPUTEM-Turkiye

SSR Amplification and Genetic Diversity Analysis

One hundred genomic SSRs were developed by Bakir and Kahraman (2019), Demir and Bakir (2022), and Bakir et al. (2024), and were used for amplification. This validation was performed in a total reaction volume of 15 µl, comprising 200 ng of genomic DNA, 1X DreamTaq Green Buffer (includes 2 mM of MgCl) (Thermo Scientific, Waltham, MA, USA), dNTPs (0.2 mM of each), 10 µM of each forward and reverse primer, DreamTaq DNA polymerase (0.5 u) (Thermo Scientific, Waltham, USA), and PCR water. The reactions were performed in a Bio-Rad MyCycler US device by an initial step of 3 min at 94°C, 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 amplified fragments were electrophoresed in 4% MetaPhor™ agarose for 4 hours and visualized in the Gel-Documentation Unit (Bio-Rad, Hercules, CA, USA). Markers exhibiting clear bands in the 100-500 bp range were selected. Genetic diversity analysis according to Scheulke (2000) to ascertain polymorphism in the 21 genotypes using an M13 tailed primer (M13 universal sequence (− 21), TGT AAA ACG ACG GCC AGT) attached to the 5' of the forward primers and tagged with fluorophores ROX, HEX, or 6-FAM at the 5' end to enable multiplexing was performed in a Bio-Rad Mycycler US device. The final reaction mixture of 15 µl consisted of 15 ng of genomic DNA, 1X DreamTaq Green Buffer (includes two mM of $MgCl_2$) (Thermo Scientific, Waltham, MA, USA), dNTPs (0.2 mM of each), 0.1 µM of each primer (forward and reverse), M13 labeled (-21) universal primer of 0.1 µM, DreamTaq DNA polymerase (Thermo, 0.5 U - Thermo Scientific, Waltham, MA, USA), and PCR water. The amplification program consisted of an initial step of 3 min at 94°C, 35 cycles of 1 min at 94°C and 1 min at 50-66 °C, 2 min at 72 °C, followed by eight 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. The amplicons were grouped in threes in a ratio of 1:1:2 for HEX, 6-FAM, or ROX fluorescent dyes, respectively, in a mixture with 0.5 µl GeneScan-600 LIZ size standards (Applied Bio-systems, Foster City, CA, USA) and 9.5 µl Hi-DiTM formamide (Applied Bio-systems, Foster City, CA, USA). The products were denatured at 95 °C for 5 min, immediately chilled on ice, and placed in an ABI

PRISM® 3500 Genetic Analyzer (Applied Bio-systems, Foster City, CA, USA) for capillary electrophoresis. The GENEMAPPER software v5.0 (Applied Bio-systems, Foster City, CA, USA) was used for fragment size detection.

Data Analysis

Genetic diversity analysis was carried out at the locus level for each species. Statistical analysis was implemented using PowerMarker software V3.25 (Liu and Muse, 2005) by calculating the genetic parameters; the polymorphism information content ($PIC=1-\sum pi^2-\sum \sum 2pi^2pj^2$) for each SSR marker according to Botstein et al. (1980), observed heterozygosity (Ho), expected heterozygosity ($He=1-\sum pi^2$) (Nei, 1973), and allele number (n) per locus. The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) from the genetic similarity matrix by using the MEGA6 (Tamura et al., 2007), and bootstrap analyses over the marker loci were performed and a consensus tree obtained using the consensus program in the Phylip package (Felsenstein, 1993).

3. RESULTS AND DISCUSSION

Transferability of SSR Markers to Wild Lentil Species

Wild species are an invaluable resource that harbors most of the gene repertoire for resistance/tolerance to biotic and abiotic stresses. Therefore, interspecies transferability using genomic SSRs offers great opportunities to identify and utilize this diversity for crop improvement. In the current study, the inter-specific amplification to wild *Lens* species yielded 31% in *L. lamottei*, 34% in *L. ervoides*, and 34% in *L. culinaris* subsp. *odemensis*, 39% in *L. culinaris* subsp. *orientalis*, 40% in *L. culinaris* subsp. *tomentosus*, and 41% in *L. nigricans*. In comparison to the former, these rates are considerably lower. Verma et al. (2014) reported 100% amplification of genomic DNA for all the subspecies of *L. culinaris*, while cross-species amplification ranged from 80.72% in *L. ervoides* to 87.88% in *L. lamottei* for 33 polymorphic SSRs. Singh et al. (2016) also reported successful amplification in Lens wild species, with transferability rates of 94.82% in *L. culinaris* subsp. *orientalis*, 94.82% in *L. culinaris* subsp. *tomentosus*, 95.4% in *L. nigricans*, 96.55% in *L. ervoides*, and 98.81% in *L. culinaris* subsp. *odemensis.* A cross-transferability study by Singh et al. (2020) using 46 polymorphic markers revealed an amplification of 58% in *L. nigricans*, 60% in *L. lamottei*, 69% in *L. odemensis*, and 71% in *L. ervoides*. Compared to our results, this variation could be attributed to the type of SSRs used in these studies. EST-SSRs are known to be highly transferable compared to genomic SSRs because they originate from conserved coding regions that control specific traits (Varshney et al., 2005). High cross-species transferability of EST-SSRs has been reported in other crops such as chickpea (Choudhary et al., 2009) and peanut (Oliveira et al., 2023).

The lentil cultivars Firat-87, Seyran-96, and Tigris were positive controls in PCR reactions for all 100 tested markers. A total of 41 SSRs showed polymorphism, with 21(51%) of these being commonly amplified in all the wild species (Table 2). This could imply that these markers originate from conserved genes across wild and cultivated lentil species. In addition, the transferability of these SSRs suggests that the flanking regions of amplified SSR motifs are conserved across *Lens spp*. (Tiwari et al., 2021). Whereas 23 of the 100 markers were unamplified (23%) and 19 were registered as monomorphic (19%), 17 markers produced unspecified bands (Table 2). The polymorphism rates in the tested wild lentil species were 31% (*L. lamottei*), 34% (L. *ervoides*), 34% (*L. culinaris* subsp. *odemensis*), 39% (*L. culinaris* subsp. *orientalis*), 40% (*L. culinaris* subsp. *tomentosus*), and 41% (*L. nigricans*). These 41 SSRs detected a total of 219 alleles in 21 genotypes, with an average of 5.34 alleles per locus (Table 3). The alleles ranged from 2 to 12, with the marker Lc_MCu56 detecting the lowest allele number and the Lc_MCu70 detecting the highest. While the *He* ratio of the polymorphic markers ranged from 0.216 (Lc_MCu26) to 0.897 (Lc_MCu70) with an average of 0.657, the *Ho* value range was from 0.000 for multiple markers to 1.000 (Lc_MCu56) with an average of 0.140. The PIC value varied between 0.20 for Lc_MCu26 and 0.88 for Lc_MCu70, with an average value of 0.61.

Table 2. Transferability of 41 genomic SSR markers across five wild *Lens* species.

Locus	*L. nigricans*	*L. culinaris* subsp. *tomentosus*	*L. culinaris* subsp. *orientalis*	*L. culinaris* subsp. *odemensis*	*L. ervoides*	*L. lamottei*
Lc_MCu1	+	+	+	-	+	-
Lc_MCu2*	+	+	+	+	+	+
Lc_MCu4	+	+	+	-	+	-
Lc_MCu5	+	+	+	+	+	-
Lc_MCu6	+	-	+	+	-	-
Lc_MCu9*	+	+	+	+	+	+
Lc_MCu10*	+	+	+	+	+	+
Lc_MCu15*	+	+	+	+	+	+
Lc_MCu17*	+	+	+	+	+	+
Lc_MCu18*	+	+	+	+	+	+
Lc_MCu19	+	+	+	+	-	+
Lc_MCu20	+	+	+	-	+	+
Lc_MCu21	+	+	+	+	-	+
Lc_MCu24	+	+	-	+	+	+
Lc_MCu26*	+	+	+	+	+	+
Lc_MCu28*	+	+	+	+	+	+
Lc_MCu31	+	+	+	-	+	+
Lc_MCu32*	+	+	+	+	+	+
Lc_MCu35*	+	+	+	+	+	+
Lc_MCu45	+	+	+	-	+	+
Lc_MCu47*	+	+	+	+	+	+
Lc_MCu49	+	+	+	+	-	+
Lc_MCu50	+	+	+	+	-	+
Lc_MCu53*	+	+	+	+	+	+
Lc_MCu56	+	+	+	+	+	-
Lc_MCu58	+	+	+	+	-	+
Lc_MCu62*	+	+	+	+	+	+
Lc_MCu69	+	+	+	+	+	-
Lc_MCu70*	+	+	+	+	+	+
Lc_MCu74	+	+	+	-	+	-
Lc_MCu79*	+	+	+	+	+	+
Lc_MCu80	+	+	-	+	+	+
Lc_MCu81*	+	+	+	+	+	+
Lc_MCu85*	+	+	+	+	+	+
Lc_MCu87*	+	+	+	+	+	+
Lc_MCu89*	+	+	+	+	+	+
Lc_MCu91*	+	+	+	+	+	+
Lc_MCu94	+	+	+	+	-	-
Lc_MCu96*	+	+	+	+	+	+
Lc_MCu98	+	+	+	-	+	-
Lc_MCu100	+	+	+	+	+	-

The primers marked with an asterisk (*) in the table successfully amplified across all wild species

Table 3. Number of alleles (n), expected heterozygosity (*He*), observed heterozygosity (*Ho)*, and polymorphic information content (PIC) values obtained after polymorphism analysis of 21 genotypes at 41 SSR loci.

Locus	n	*He*	*Ho*	PIC
Lc_MCu1	4	0.685	0.142	0.62
Lc_MCu2*	7	0.761	0.000	0.73
Lc_MCu4	4	0.685	0.142	0.62
Lc_MCu5	9	0.713	0.047	0.68
Lc_MCu6	4	0.577	0.047	0.51
Lc_MCu9*	4	0.500	0.142	0.44
Lc_MCu10*	9	0.800	0.095	0.77
Lc_MCu15*	3	0.391	0.238	0.35
Lc_MCu17*	7	0.789	0.000	0.76
Lc_MCu18*	7	0.770	0.000	0.74
Lc_MCu19	7	0.712	0.000	0.68
Lc_MCu20	4	0.693	0.000	0.64
Lc_MCu21	3	0.580	0.000	0.51
Lc_MCu24	6	0.780	0.000	0.74
Lc_MCu26*	3	0.216	0.238	0.20
Lc_MCu28*	3	0.321	0.285	0.29
Lc_MCu31	4	0.704	0.190	0.64
Lc_MCu32*	5	0.591	0.190	0.55
Lc_MCu35*	7	0.780	0.000	0.75
Lc_MCu45	8	0.784	0.523	0.75
Lc_MCu47*	6	0.693	0.000	0.65
Lc_MCu49	3	0.648	0.000	0.57
Lc_MCu50	4	0.507	0.000	0.46
Lc_MCu53*	3	0.471	0.000	0.39
Lc_MCu56	2	0.500	1.000	0.37
Lc_MCu58	3	0.571	0.190	0.48
Lc_MCu62*	4	0.607	0.000	0.56
Lc_MCu69	5	0.748	0.285	0.71
Lc_MCu70*	12	0.897	0.000	0.88
Lc_MCu74	7	0.718	0.238	0.67
Lc_MCu79*	6	0.739	0.000	0.69
Lc_MCu80	6	0.798	0.000	0.76
Lc_MCu81*	10	0.851	0.333	0.83
Lc_MCu85*	4	0.651	0.047	0.58
Lc_MCu87*	5	0.719	0.285	0.67
Lc_MCu89*	3	0.430	0.000	0.37
Lc_MCu91*	8	0.839	0.000	0.81
Lc_MCu94	5	0.647	0.285	0.60
Lc_MCu96*	5	0.729	0.428	0.68
Lc_MCu98	4	0.658	0.333	0.59
Lc_MCu100	6	0.667	0.047	0.61
Average	5.34	0.657	0.140	0.61
Total	219	26.94	5.761	25.0

*Polymorphic markers are commonly amplified in all the wild lentil species.

The low polymorphism of lentils among different molecular markers is mainly owed to its autogamous behavior. However, SSRs are frequently used to detect higher polymorphism because they are abundant throughout the genome, easy to detect with PCR, multi-allelic, and codominant (Saidi et al., 2022). Verma et al. (2014) reported a 100% polymorphism of all the tested loci, with an average PIC value of 0.66. Singh et al. (2020) validated 50 EST-SSRs to achieve 92% polymorphism with an average PIC value of 0.44. Out of 106 markers, 29 (27.4%) polymorphic markers were reported by Singh et al. (2016), with an average PIC value of 0.436. Our study observed a 41% polymorphic rate and an average PIC value of 0.61 from the 100 genomic SSR markers tested on wild lentil species. This result is similar to the findings by Dikshit et al. (2015), in which 75 EST-SSRs and 27 genomic SSRs were tested on 86 *Lens* genotypes to produce 42.2% polymorphism with 31 EST-SSRs (41.3%) and 12 (44.4%) genomic SSRs. Reddy et al. (2010) also reported a 43% polymorphism in the 30 lentil microsatellites tested on a wide range of genotypes, including wild subspecies. Our finding is, however, higher than 32.72% by Singh et al. (2019). Although polymorphism could be

attributed to the level of genetic variation among the tested individuals, the kind of SSRs utilized is also influential, as it has been reported that genomic SSRs are often more polymorphic than EST-SSRs. This is because genomic SSRs widely spread throughout the genome belong to the non-coding regions with greater variation in the flanking regions. On the other hand, EST-SSRs originate from conserved coding regions that control specific traits and are therefore under high selection pressure that limits the number of mutations (Varshney et al., 2005; Choudhary et al., 2009; Dikshit et al., 2015). The PIC is indispensable for SSR selection for gene tagging and diversity studies; the higher the value, the more informative and better for phylogenetic analyses. The current research exhibited high polymorphism with a high average number of alleles per locus (5.34) and a high PIC value (0.61) greater than 0.5. This indicates their effectiveness in detecting variation and relatedness among accessions (Peng & Lapitan, 2005; Rajendra et al., 2019; Saxena et al., 2019). Additionally, mostly dinucleotide repeats among the tested SSRs could have influenced the polymorphism. Dinucleotide repeats are located outside gene coding regions that are prone to mutations, making them highly polymorphic (Odeny et al., 2007; Oliveira et al., 2023; Topu et al., 2023)

Genetic Diversity Analysis among Different Lens Species

The UPGMA dendrogram was constructed based on the genetic similarity matrix using 41 SSR markers, which grouped the 21 *Lens* genotypes into two major groups that bifurcated into subclusters. Group I is split into subcluster I, comprising all *L. ervoides* accessions and *L. tomentosus* accessions except *L. tomentosus* (IG72830), and subcluster II, comprising all *L. odemensis* accessions, all *L. lamottei* accessions, two *L. nigricans* accessions (IG136639 and IG72545), and one *L. tomentosus* accession (IG72830). Group II was further grouped into two subclusters, with *L. culinaris* subsp. *culinaris* (Firat87, Seyran96, Tigris) in subcluster I, while subcluster II comprised all accessions of *L. orientalis* and one *L. nigricans* (IG36636). Pairwise genetic similarities to cultivated *L. culinaris* subsp. *culinaris* accessions were highest with *L. culinaris* subsp. *orientalis* and lowest between *L. ervoides* (IG72862) and *L. culinaris* subsp. *culinaris* (Tigris) (6%). However, among wild species *L. culinaris* subsp. *orientalis* and *L. nigricans* (81%) showed the highest similarity, while the lowest similarity was observed between *L. ervoides* and *L. nigricans* (10%).

Introgression of target traits into cultivated varieties is actualized by accurate evaluation of crop wild relatives (CRW) as a prerequisite to enhance genetic gains (Rajpal et al., 2023). In our study, the cultivars of *L. culinaris* subsp. *culinaris* (Firat-87, Seyran-96, and Tigris) clustered in Group II with all *L. culinaris* subsp. *orientalis* and *L. nigricans* (IG136636). And the genetic similarity of all wild subspecies/species to the cultivated subspecies showed *L. culinaris* subsp. *orientalis* as the closest. Our results are similar to Dissanayake et al. (2020), who, consistent with Wong et al. (2015), found *L. orientalis* to be the closest to *L. culinaris*, with *L. odemensis/L. Lamottei, L. ervoides, and L. nigricans,* respectively. Liber et al. (2021), using principal component analysis (PCA), discriminant analysis of principal components (DAPC), and phylogeny, also grouped *L. orientalis* and *L. culinaris* together from the four major *Lens* genera identified in concurrence with Dikshit et al. (2015). Additionally, Gutierrez-Gonzalez et al. (2022), using transcriptome analysis, identified *L. orientalis* as the most similar to *L. culinaris* and *L. nigricans* as the least similar. Similarly, Hamwieh et al. (2009) and Verma et al. (2014) reported the clustering of cultivated germplasm with *L. culinaris* subsp. *orientalis*. This indicates *L. culinaris* subsp. orientalis as the progenitor of cultivated lentils. In support of this, our findings align well with those reported by Reddy et al. (2010), Wong et al. (2015), Dikshit et al. (2015), and Liber et al. (2021), which also characterized wild *Lens* species and subspecies using molecular markers. Consistent with Sharma et al. (1995, 1996) and Hamwieh et al. (2009), our results affirm the close genetic relationship between *L. culinaris* and *L. orientalis*. The clustering of *L. odemensis* and *L. lamottei* observed in our dendrogram corresponds with their assignment to the secondary gene pool by Wong et al. (2015). Moreover, our finding that *L. tomentosus* grouped with *L. ervoides*, rather than *L. orientalis* and *L. culinaris*, aligns with Liber et al. (2021) but contrasts with the primary gene pool classification by Wong et al. (2015). Notably, *L. nigricans* accession IG136636 clustered away from the other *L. nigricans* lines, as also reported in studies by Wong et al. (2015) and Liber et al. (2021), which suggested this species belongs in a quaternary gene pool due to its divergence and hybridization limitations. The lowest similarity (10%) between *L. ervoides* and *L. nigricans* in our study matches Reddy et al. (2010) and Wong et al. (2015), confirming the high interspecific variation between these species. These comparisons further validate the utility of the SSR markers used and reinforce the reliability of the phylogenetic relationships presented in this study. Our phylogenetic analysis placed *L. odemensis* quite far from *L. culinaris* (Figure 1). Despite the controversy from crosses made between *L. odemensis* and *L. culinaris*, similar results showing the separation of these two species (Wong et al., 2015; Polanco et al., 2019; Dissanayake et al., 2020).

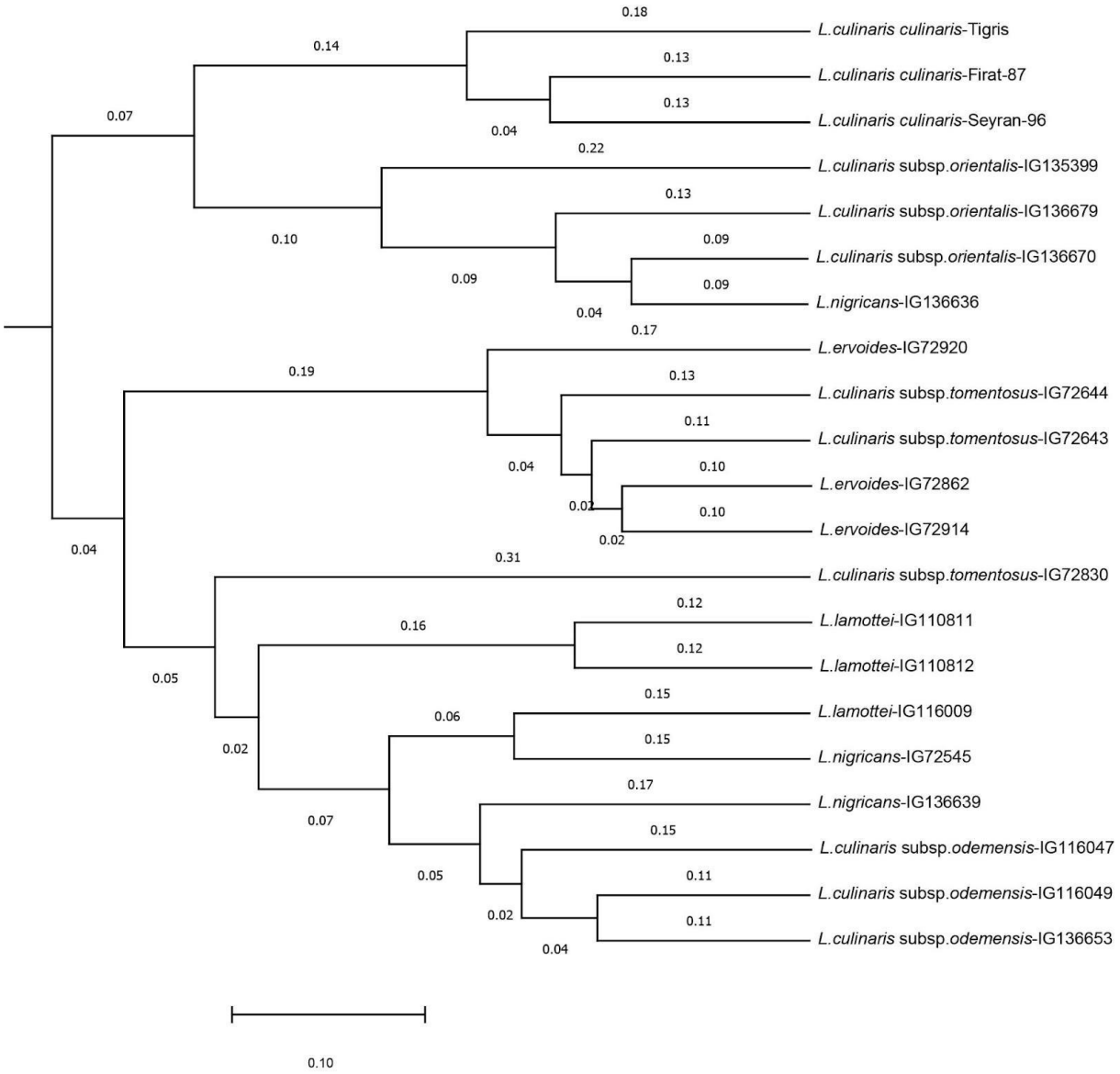

Figure 1. The UPGMA-based dendrogram of 21 lentil accessions analyzed using 41 genomic SSR markers.

The highest dissimilarity between cultivated and wild subspecies/species was reported between *L. ervoides* (IG72862) and *L. culinaris* subsp. *culinaris* (Tigris). Dissanayake et al. (2020) similarly reported *L. ervoides* (ILWL042) and *L. culinaris* (10H063L, 12HS3003) accessions as most divergent. A contradiction could arise due to successful offspring from a cross between *L. ervoides* and *L. culinaris* ssp. *culinaris*, as this could necessitate a restructuring of the primary gene pool to include *L. ervoides;* however, offspring can only be attained using in vitro embryo rescue. For this reason, *L. ervoides* was placed in a tertiary gene pool, thus concurring with the results in this study (Singh et al., 2022; Gutierrez-Gonzalez et al., 2022). According to cytogenetic and cross-compatibility assays of wild *Lens* with cultivated lentils, *L. culinaris* subsp. *orientalis* was placed in a primary gene pool, while *L. lamottei, L. ervoides, L. nigricans, L. odemensis*, and *L. tomentosus* were placed in a secondary gene pool (Muehlbauer and McPhee, 2005). Although the latter findings also concur with the current study, Wong et al. (2015) proposed a four-gene pool classification of the genus *Lens* based on the clustering of *L. lamottei/L. odemensis, L. culinaris/L. tomentosus, L. nigricans*, and *L. ervoides* from the population structure and phylogenetic analyses. The primary gene pool (*L. culinaris/L. orientalis/L. tomentosus*) was based on the successful seed production upon interspecific crossing of these species. Similarly, the present study grouped all *L. odemensis* and *L. lamottei* accessions in subcluster II. This is similar to Verma et al. (2014), who characterized them as sister clades, and Wong et al. (2015), who placed them in the same secondary gene pool. However, on the contrary, Group I subclustered *L. tomentosus* in a cluster distant from *L. orientalis* and *L. culinaris*, differing from the findings that reported a close relation of *L. tomentosus/L. orientalis/L. culinaris* in a primary gene pool.

An evaluation of the genetic relationships among wild subspecies/species revealed the highest similarity between *L. culinaris* subsp. *orientalis* and *L. nigricans* (81%), while the lowest was observed between *L. ervoides* and *L. nigricans*

(10%). The finding of *L. culinaris* subsp. *orientalis* and *L. nigricans* (81%) similarity could be specific to the accessions used, with an explanation from the refuted findings of Renfrew (1973) that had suggested *L. nigricans* as the progenitor of *L. culinaris*. The inferences from the electrophoretic studies of seed protein profiles found *L. culinaris, L. orientalis*, and *L. nigricans to be* closely related. Furthermore, reports from cytoplasmic studies of *Lens* showed three chromosome interchanges between the cultivated *L. culinaris* and *L. nigricans* and only one between the cultivated species and *L. orientalis* (Hoffman, 1988). These studies showed the evolutionary relatedness of these Lens species and provided more evidence for *L. orientalis* as a putative progenitor of cultivated lentils.

Ahmad et al. (1997) and Wong et al. (2015) reported results similar to this study, which found the highest genetic dissimilarity between *L. ervoides* and *L. nigricans*, indicating the highest interspecific variation among these wild species. Based on phylogenetic analyses and population genetics, Liber et al. (2021) observed *L. nigricans* and *L. ervoides* to be very different from the cultivated genus. Additionally, *L. c*. subsp. *odemensis, referred* to as the sister clade of *L. ervoides*, is also quite distinct from *L. nigricans* (Dissanayake et al., 2020). Intraspecific variations reflected by the scattered placement of L. nigricans and L. tomentosus accessions were also observed. Although Reddy et al. (2010) clustered cultivated lentil accessions with *L. orientalis* (ILWL 1) and *L. nigricans* (ILWL 14) in group I, the result contradicts the grouping according to gene pools that placed *L. culinaris, L. orientalis*, and *L. tomentosus* species together, while *L. nigricans* was placed in a distinct quaternary gene pool (Wong et al., 2015). Furthermore, the structure and phylogenetic analyses showed that *L. nigricans* is the most distant relative of cultivated lentils owing to the unsuccessful development of interspecific offspring with *L. culinaris* past the F1 generation (Wong et al., 2015). This within-species genetic variation could be a result of continuous genetic transmissions, since high genetic diversity at the species level has been reported in *Lens* (Sharma et al., 1996; Hamwieh et al., 2009; Reddy et al., 2010; Kumar et al., 2014). In addition, the observations of the genetic similarity among these accessions could be attributed to the limited number of markers, their dispersion in the genome, and the general genetic composition of the tested plant material (Tantasawat et al., 2011).

4. CONCLUSION

Conclusively, 41 genomic SSRs developed in cultivated lentils were successfully transferable to wild *Lens* and used to characterize the genus by assessing their genetic variation and phylogeny. These polymorphic SSRs are a valuable addition to lentils' limited genetic resources for further characterization of *Lens* germplasm to point out genetic relationships that would permit the discovery of novel genes and their utilization in agronomical advancements. Additionally, these markers will contribute to evolutionary studies, comparative mapping, linkage mapping, and quantitative trait loci (QTL) identification. Based on these findings, the assayed accessions could be utilized in breeding programs to enable the introgression of genes into cultivated lentils for yield improvement and/or tolerance to abiotic and biotic stresses.

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