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SSR Marker-based Molecular Characterization of Lentil (*Lens culinaris* Medik.) Genotypes

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ABSTRACT

Background: Lentil is an important legume crop that plays a vital role in sustainable agriculture and human health. However, the restricted genetic foundation or parentage of contemporary cultivars has arisen as a serious challenge for lentil development. Therefore, determining the genetic diversity and yield-related characteristics is crucial for the breeder to broaden the genetic base and aid in selecting the desirable parents for hybrid development programmes.

Methods: In the current study, microsatellite markers were used for diversity analysis among 37 lentil genotypes. Morphological and molecular systems both were able to differentiate lentil genotypes. Among applied SSR primers 10 were able to produce successful amplifications with template DNA of all the studied genotypes of lentil.

Result: A sum of 357 scoreable bands were produced, 148 of which accounted for 41.45% polymorphism. The UPGMA dendrogram grouped 37 lentil genotypes into 2 groups. PIC values ranged from 0.37 to 0.77. For this experiment, SSR 80, SSR 130, SSR 34-2 and SSR 33 were highly informative polymorphic markers, demonstrating the efficacy and higher resolution in exposing molecular genetic diversity among lentil genotypes. The highest numbers of alleles (5) were produced by SSR 80 primer which was selected as extremely polymorphic. This study reveals the variation across lentil genotypes, which might be employed further in breeding efforts for lentils that result in strong heterosis in the segregating generation. The SSR markers found as polymorphic may also be utilized for further polymorphism analysis among different set of lentil genotypes.

Key words: Dendrogram, Genetic diversity, Lentil, PIC value, Polymorphism, SSR markers, UPGMA.

INTRODUCTION

Lentil (*Lens culinaris* Medik.) is one of the pulse crops to be primarily domesticated; it thrives largely in rain-fed regions (Liber *et al*., 2021). It is an autogamous, diploid (2n = 2X = 14) crop with a genomic size of roughly 4 Gbp (Arumuganathan and Earle, 1991). India is the world's biggest producer of pulses. After chickpea and field pea, lentil is the world's third leading winter legume and it covered an area of 1.56 million hectares in India, producing 1.23 million tonnes and yielding 901 kg per hectare (FAOSTAT, 2021). However, it is often grown under challenging biotic and abiotic circumstances. Due to susceptibility to diseases, severe droughts, heat stress and poor soil fertility (Sharpe *et al*., 2013), it is not under main line cultivation in many emerging nations (Kumar *et al*., 2013). Furthermore, the restricted genetic foundation or parentage of contemporary cultivars has surfaced as a serious challenge for lentil development. As a result, prospective genetic advances in lentil output could not be realized. Lentil's pace of genetic advance is slower than that of other important legumes like pigeonpea and chickpea (Kumar *et al*., 2014). In contemporary plant breeding, selecting suitable and diverse parents for hybridization operations is critical before planning crop improvement activity (Tripathi *et al*., 2022).

Among different molecular markers, microsatellite markers have been employed in several studies to characterize genotypes in various crop species (Tiwari *et al.,* 2019; Singh *et al.,* 2022), however, their usage for genotypes of lentils has been limited due to their paucity (Wang *et al*., 2009; Weising *et al*., 2005). This method, which employs simple sequence repeat (SSR) markers in lentil genotypes, might be used as a quick and dynamic tool for finding polymorphism at the DNA level and analyzing genetic variation. Determining yield-related characteristics and genetic diversity is crucial for the breeder to develop qualitative traits, broaden the genetic base and aid the plant breeder in selecting the correct parents in breeding operations for desired high-yielding varieties (Mishra *et al.,* 2022). In view of the above background, the goal of this study was to assess genetic diversity among lentils genotypes based on agro-morphological traits and SSR markers.

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MATERIALS AND METHODS

Plant materials

The experiment composed primarily of 37 different lentil advanced breeding lines (Table 1) obtained from AICRP on MULLaRP, JNKVV, Jabalpur. Following a randomized complete block design (RCBD) with three replications, the genotypes were characterized during the *Rabi* season of 2021-2022 at Research Farm, JNKVV, Jabalpur. Six rows were planted for each genotype. The row-to-row width was 22.5 cm, while among plants the distance was 8-10 cm. Five plants were randomly chosen from each replication to acquire data on various traits such as days to flower initiation, days to maturity, plant height (cm), number of primary branches per plant, number of pods per plant, number of seeds per plant, 100 seed weight (g), biological yield per plant (g), harvest index (%) and seed yield per plant (g).

Genomic DNA extraction

Young leaf samples were obtained from a month-old seedling for DNA isolation. The collected leaves were crushed and homogenized in liquid nitrogen followed by genomic DNA extraction using the NucleoSpin® Plant II kit according to the instructions provided in the manual. Qualitative evaluation of extracted DNA was done on 1% agarose gel through electrophoresis in 1X TAE buffer. Uncut λ*Hind* III DNA ladder was used as a control during electrophoresis.

Microsatellite based diversity analysis

The SSR markers were initially tested with template DNA of four lentil genotypes to select polymorphic markers for further analysis. Among all the tested markers only 10 SSR markers (Table 2) were able to produce polymorphic alleles and further processed with all the genotypes of lentil. The Mastercycler Nexus PCR machine was used for amplification. A total of 14 µl of the PCR master mix was made, which included 2 µl of genomic DNA, 7 µl of master mix, 4 µl of Nuclease free water and 1 µl of forward and reverse primer. The PCR programming was done for 3 minutes of denaturation at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds of annealing at 52-, 55°C (depending on the primer) and 1 minute of elongation at 72°C. The final extension was set at 72°C for 5 minutes. The amplified products were resolved on 2.5% agarose gel electrophoresis and visualized under the Syngene gel documentation system. Amplicon size was determined with a 100 bp DNA ladder.

Data analysis

Power marker version 3.25 software (Liu and Muse, 2005) was used to compute the average number of alleles, gene diversity and polymorphic information content (PIC) values. To quantify molecular diversity and create a dendrogram, the UPGMA (Unweighted Pair Group Approach with Arithmetic Mean) method was applied based on Jaccard's dissimilarity coefficient (Jaccard, 1908). Molecular data was examined in accordance with Peakall and Smouse, 2012 using GenAlEx version 6.503 to explore parameters such as Shannon's information index (I), observed (Ho) and expected (He) heterozygosity.

RESULTS AND DISCUSSION

Morphological diversity

Morphological data obtained for all the traits during the study were analyzed using ANOVA. It revealed statistically significant differences between the genotypes (Table 3). The findings imply that there is significant variation among the lentil genotypes investigated and selection might be used effectively taking into account these traits in practical lentil breeding programmes as suggested in earlier study by Gupta and Sharma (2006). Similar findings were reported

Table 1: List of lentil genotypes used for morphological and molecular diversity analysis.

Genotypes coding	Varieties names
Asha	ASHA
DPL 15	DPL 15
JL 1	JL 1
JL 3	JL 3
NDL 1	NDL 1
PL 406	PL 406
PL 5	PL 5
SUBRATA	SUBRATA
VL4	VL 4
VL103	VL 103
LLS 21-193	RVL 20-5
LLS 21-194	L 4727
LLS 21-195	RLG 318
LLS 21-197	IPLRS 704
LLS 21-198	IPL 349
LLS 21-199	RKL 607-1
LLS 21-200	RLG 327
LLS 21-202	RKL 3-91
LLS 21-133	RKL 14-37
LLS 21-204	LL 1769
LLS 21-205	PL 348
LLS 21-206	IPLRS 703
LLS 21-207	PLL 20-2
LLS 21-209	RKL 14-201
LLS 21-211	RKL 58 F 111
LLS 21-215	IPL 350
LLS 21-216	IPL 316
LLS 21-218 A	RKL 20-11
LLS 21-124	RKL 603-11-1
LLS 21-125	RKL 20-26
LLS 21-126	PL 366
LLS 21-127	IPL 801
LLS 21-128	VL 535
LLS 21-129	RVL 18-4
LLS 21-130	RKL 20-21
LLS 21-131	RVL 11-6
LLS 21-132	RLG 280

earlier on morphological traits-based diversity in lentil including plant height, days to flower initiation, days to maturity, numbers of primary branches per plant, number of pods per plant *etc.* (Sharma *et al.,* 2022; Pawar *et al*., 2022). According to these findings the number of pods and the number of branches per plant are the important morphological traits that can be used for selecting high-yielding lentil germplasm (Sakthivel *et al*. (2019).

Molecular diversity

All 37 lentil genotypes were subjected to SSR marker analysis. Only clear and sharp alleles (Fig 1) were considered

Table 2: List of sequences of SSR primer pairs.

Marker	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
SSR 33	CAAGCATGACGCCTATGAAG	CTTTCACTCACTCAACTCTC
SSR 207	GAGAGATACGTCAGAGTAG	GATTGTGCTTCGGTGGTTC
SSR 80	CCATGCATACGTGAACTGC	GTTGACTGTTGGTGTAAGTG
SSR 336	GTGTAACCCAACTGTTCC	GGCCGAGGTTGTAACAC
SSR19	GACTCATACTTTGTTCTTAGCAG	GAACGGAGCGGTCACATTAG
SSR 99	GGGAATTTGTGGAGGGAAG	CCTCAGAATGTCCCTGTC
SSR 130	CCACGTATGTGACTGTATG	GAAAGAGAGGCTGAAACTTG
SSR 213	CACTCGCACCTCTTATG	GAAATTGTCTCTTAGCAAG
SSR 90	CCGTGTACACCCCTAC	CGTCTTAAAGAGAGTGACAC
SSR 34-2	CGGCGGATGAAACTAAAG	CATTTCCTTCACAAACCAAC

Table 3: ANOVA for ten quantitative traits of 37 lentil genotypes.

Traits	Sources of variation		
	Replication (df=2)	Genotype (df=36)	Error (df=72)
Days to flower initiation	12.87	50.47***	1.08
Days to maturity	11.36	124.12***	1.18
Plant height (cm)	0.06	1.92***	0.10
Number of primary branches per plant	23.54	52.46***	2.35
Number of pods per plant	4.48	1304.92***	15.77
Number of seeds per plant	0.002	0.075***	0.001
100 seed weight (g)	0.01	1.78***	0.01
Biological yield per plant (g)	0.31	83.17***	0.83
Harvest index (%)	1.70	245.79***	2.83
Seed yield per plant (g)	0.07	20.47***	0.05

Note: ***Significant at 0.001%; **Significant at 0.005%.

Table 4: Allelic variation revealed by SSR markers in lentil genotypes.

Locus	N	Na	Ne	I	Ho	He	GD	PIC	TNB	Tm
SSR33	37.00	4.00	1.65	0.73	0.14	0.39	0.73	0.69	37.00	56.27
SSR 207	37.00	2.00	1.58	0.56	0.32	0.37	0.50	0.37	35.00	55.59
SSR 80	37.00	5.00	3.80	1.42	0.97	0.74	0.79	0.77	37.00	55.96
SSR336	37.00	4.00	2.81	1.12	0.62	0.64	0.50	0.37	33.00	54.43
SSR19	37.00	3.00	1.81	0.69	0.22	0.45	0.50	0.37	35.00	58.21
SSR99	37.00	4.00	2.35	0.98	0.19	0.58	0.67	0.59	34.00	54.30
SSR 130	37.00	4.00	2.99	1.18	0.84	0.67	0.75	0.70	37.00	54.88
SSR 213	37.00	2.00	1.34	0.42	0.14	0.25	0.67	0.59	36.00	51.48
SSR 90	37.00	4.00	1.66	0.75	0.14	0.40	0.50	0.37	36.00	54.77
SSR 34-2	37.00	4.00	3.67	1.34	0.65	0.73	0.75	0.70	37.00	53.44
Mean	37.00	3.60	2.37	0.92	0.42	0.52	0.63	0.55	357.00	54.93

Where,

Na = No. of different alleles, Ne = No. of effective alleles = 1 / (Sum pi^2), I = Shannon's Information Index = -1* Sum [pi * Ln (pi)], Ho = Observed heterozygosity = No. of hets/N, He = Expected Heterozygosity = 1 - Sum pi^2, GD= Gene diversity, PIC= Polymorphic information content, TNB= Total number of bands, Tm= Annealing temperature.

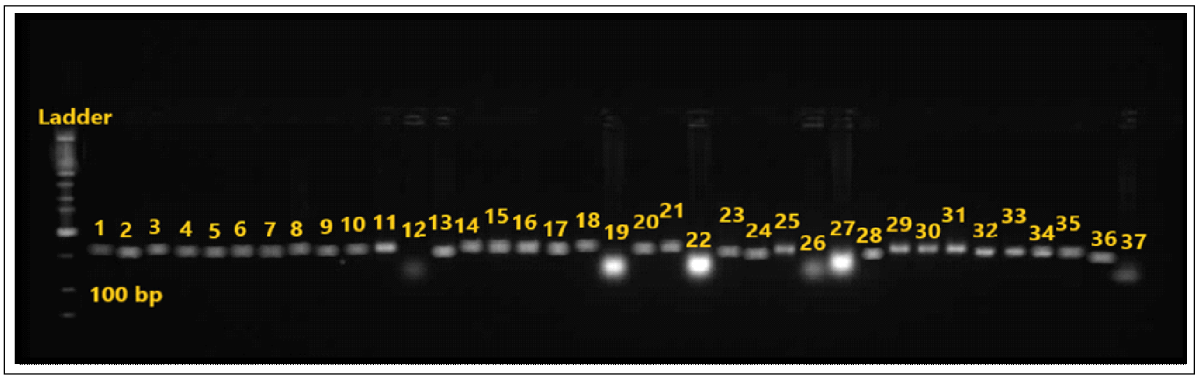


Fig 1: A representative gel picture of lentil genotypes with marker SSR 80.

for scoring. Total 31 alleles (Table 4) were obtained with a mean of 3.60 alleles per locus. The highest gene diversity was shown by the marker SSR 80 (0.79) followed by SSR 130 and SSR 34-2 (0.75), SSR 33 (0.73), SSR 99 and SSR 213 (0.67) SSR 19, SSR 90, SSR 336 and SSR 207 (0.50) with a mean value of 0.63. To ascertain the information of every marker and its capability for differentiation, the polymorphic information content (PIC) was used for each locus. The PIC value is said to be evidence of diversity among the evaluated varieties (Pervaiz *et al*., 2009). The PIC value can also be evaluated based on its alleles and can be different for every SSR locus. In the present study, the highest PIC value was observed for SSR 80 (0.77) followed by SSR 130 and SSR 34-2 (0.70), SSR 33 (0.69), SSR 99 (0.59) and SSR 213 (0.59) and lowest by SSR 207, SSR 19, SSR 90 and SSR 336 (0.37) with an average of 0.67. The results of genetic diversity and PIC values are consistent with the conclusions of Saidi *et al*. (2022) where SSR markers were used to evaluate genetic diversity among lentil genotypes. The studied markers were divided into three categories based on PIC values. The markers with a PIC value greater than 0.50 was considered highly informative whereas the markers with a PIC value between 0.26-0.49 were considered moderately informative and the markers with PIC values less than 0.25 were considered less informative. This variation helps assess the diversity of a marker/gene/DNA segment in a population which will help in understanding the evolutionary pressure and mutations on the locus over a while.

The number of alleles per primer varied from 2 to 5 with an average of 3.60 alleles per primer similar to the results of Gleridou *et al*. (2022). The sizes of scoring bands ranged from 179 to 244 bp. The maximum numbers of alleles (5) were produced by the marker SSR 80. Most of the other primers SSR 33, SSR 336, SSR 99, SSR 130, SSR 90 and SSR 34-2 produced four alleles each. Three alleles were produced by SSR 19 marker while, SSR 207 and SSR 213 produced only two alleles respectively. The number of alleles produced per primer depends on multiple factors like the primer used, the genotype of the plant and the resolution of the amplified product. Shannon's information index revealed the values in a range of 0.42 (SSR 213) to 1.42 (SSR 80) with an average of 0.92. Expected heterozygosity revealed a range of values from 0.25 (SSR 213) to 0.74 (SSR 80) with an average of 0.52. Observed heterozygosity showed values ranging from 0.14 for SSR 33, SSR 213 and SSR 90 to 0.97 in SSR 80 with an average of 0.42 per primer. The same outcomes have been documented by Yadav *et al*. (2016) and Dikshit *et al*. (2015).

Scored alleles were used to prepare data input file for the software and a dendrogram was created using the UPGMA method (Fig 2). To choose suitable genotypes for breeding programmes, it is crucial to consider the most diverse genotypes. Dendrogram grouped all thirty-seven lentil genotypes into two main clusters *i.e*., cluster I and cluster II. Cluster I was further divided into two sub-groups IA and IB and IA was differentiated into IA-1 and 1A-2. Cluster IB was further subdivided into two subgroups i.e., IB-1 and IB-2. ASHA, LLS 21-126, LLS 21-216, LLS 21-128, LLS 21-124, LLS 21-132, LLS 21-130 and VL 4 genotype belongs to IA-1 sub-cluster and in IA-2 sub-cluster include genotype LLS 21-207, LLS 21-205, LLS 21-202, LLS 21-211, LLS 21-199, LLS 21-197, LLS 21-194 and VL 103. IB- cluster is divided into two sub-clusters IB-1 includes JL 3, DPL 15, PL 406 and SUBRATA genotypes while, IB-2 includes genotypes LLS 21-204, LLS 21-200 and LLS 21-133 respectively. This demonstrated that the genotypes under study exhibited significant diversity. Out of thirty-seven genotypes, 27 genotypes belong to cluster I. Cluster II was divided into two subgroups IIA and IIB. Cluster II A includes 8 genotypes, while cluster II B includes 6 genotypes. Cluster IIA 1 comprises genotype PL 5, NDL 1, LLS 21-215, LLS 21-209 and LLS 21-206. Cluster II A 2 includes LLS 21-198, LLS 21-195 and LLS 21-193. Cluster II B includes 6 genotypes, Cluster II B is sub-divided into Cluster IIB 1 comprises genotypes LLS 21-125, LLS 21-218 A, LLS 21-129, LLS 21-127 and JL 1. Cluster II B 2 has a single genotype *i.e*., LLS 21-131 genotypes. Similar grouping based on SSR markers was reported earlier by Mekonnen *et al*. (2016)

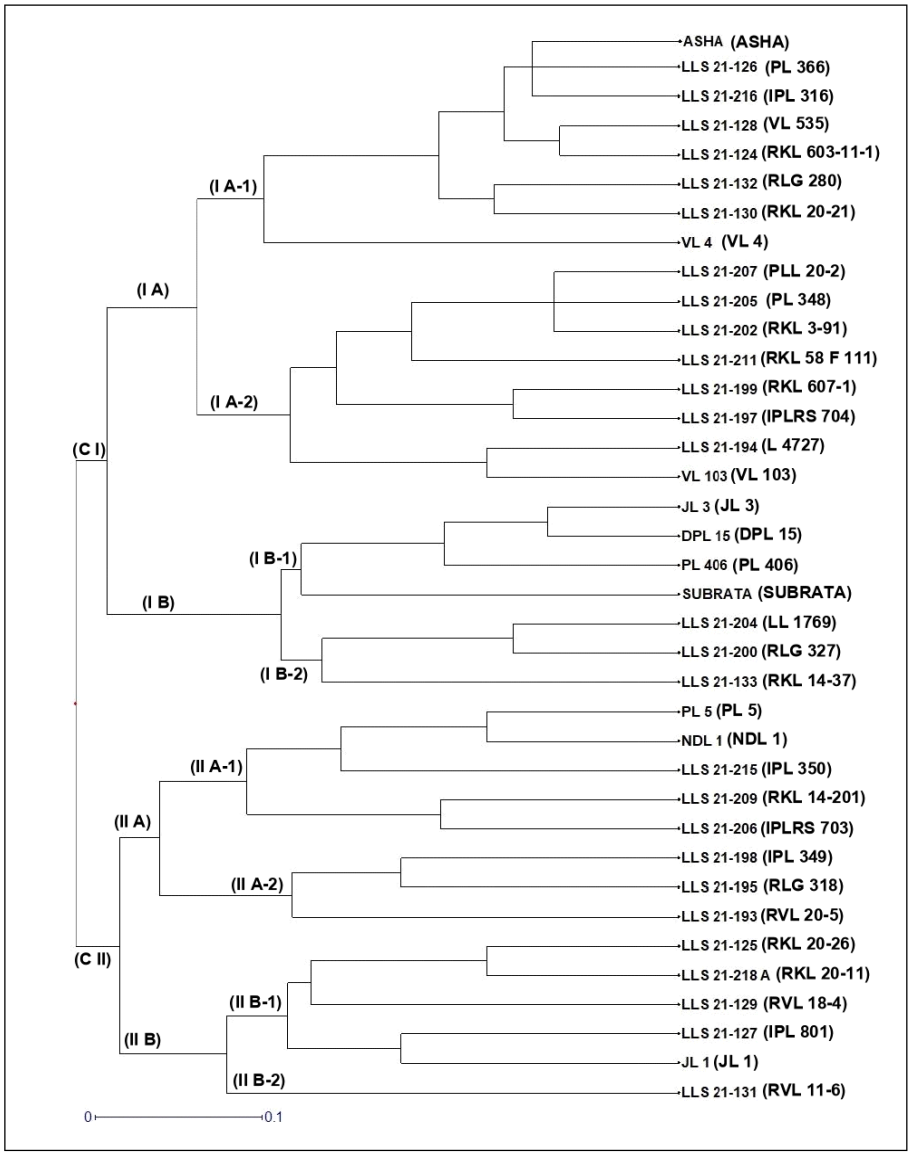


Fig 2: Dendrogram of 37 lentil genotypes based on SSR markers data.

compared the dissimilarity indices across lentil cultivars employing SSR markers and found nearly identical results.

CONCLUSION

From the findings presented above, it can be concluded that there is significant diversity among the analyzed genotypes for the agronomically desirable traits like days to flower initiation, days to maturity, plant height (cm), number of primary branches per plant, number of pods per plant, number of seeds per plant, 100 seed weight (g), biological yield per plant (g), harvest index (%) and seed yield per plant (g). SSR markers profiling showed that SSR 80, SSR 130, SSR 34-2 and SSR33 for this analysis were incredibly insightful and traceable polymorphic markers indicating the effectiveness of a marker in revealing molecular genetic diversity. According to this, a dendrogram was created using highly polymorphic 10 SSR markers for 37 genotypes of lentils, which revealed two groups. The genotypes varied greatly depending on the source of origin and pedigree. Based on molecular characterization, genotypic variations showed that genotypes fit in various clusters as a result of their underlying genetic components. Hence, it may be utilized for future breeding programmes of lentil, particularly for hybridization and selection from several clusters that will provide the most heterosis in favour of yield. This study revealed a significant amount of heterogeneity and divergence between lentil accessions that can be employed in breeding strategies in the future. This study demonstrated the diversity across lentil genotypes, which can be applied furthermore in breeding initiatives for lentils causing the segregating generation to exhibit a highly heterotic reaction.

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Conflict of interest: None.

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