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Research Article

Molecular Characterization of High Concentration of Iron (Fe) and Zinc (Zn) biofortified Nepalese Lentil (*Lens culinaris Medikus Subspecies culinaris*) Accessions and Their Genetic Diversity Analysis through Using Simple Sequence Repeat (SSR) Markers

Rajendra Darai[1*], Ashutosh Sarker[2], Krishna Hari Dhakal[3], Madhav Prasad Pandey[3], Surya Kant Ghimire[3], Shiv Kumar[4], Tara B.Ghimire[5], Jitendra Maharjan[5] and DB Thapa[6]

[1]Senior Scientist (S-4), Nepal Agricultural Research Council (NARC), Grain Legumes Research Program (GLRP), Khajura, Nepalgunj, Banke, Nepal

[2]Coordinator & Food legume Breeder, ICARDA South Asia and China Regional Program, NASC Complex, New Delhi, India

[3]Assist Professor and Professors, Faculty of Agriculture, Department of genetics and Plant breeding, Agriculture and Forestry University, Rampur, Chitwan, Nepal

[4]Principal Scientist (Lentil breeder), International Center for Agricultural Research in the Dry Areas (ICARDA), Rabat, Morocco

[5]Principal Scientist and Technical Assistant, Seed Science Research and Technology Division, NARI, Khumaltar, Lalitpur, Nepal

[6]Senior Scientist(S-4), Agri-Botany Division, NARI, Khumaltar, Lalitpur, Nepal

Abstract

Nepalese lentils are comparatively rich in iron (Fe) and zinc (Zn) in South Asian countries, making lentil a potential crop of whole food solution to aid in the global battle against the micronutrient malnutrition. Knowledge of genetics underlying the uptake of grain Fe and Zn from soils is required to increase their stable concentrations in lentils. Therefore, in present study, 25 accessions of lentil were characterized using 40 simple sequence repeat (SSR) markers in order to characterize genetic variation available among genotypes having high Fe and Zn concentrations. Out of the 40 SSR markers, 23 markers were found polymorphic while 12 markers were monomorphic and 5 markers were null. These 23 polymorphic markers produced a total of 584 alleles, of which total number of polymorphic alleles was 52 and average alleles per locus was 11.49. The allele number for each SSR locus varied between two to four with an average of 2.97 alleles per marker. Markers PLC 16, SSR 124, SSR 156, SSR 113, SSR 28 and SSR 107 showed higher level of polymorphism indicating the power and higher resolution of those marker systems in detecting molecular diversity. The polymorphic information content (PIC) values for the SSRs loci ranged from 0.14 to 0.57. The pair wise genetic similarity among 25 lentil accessions varied from 0.16 to 0.83. The dendrogram constructed based on genetic similarities among 25 lentil accessions identified five major clusters. Maximum seven accessions were grouped in cluster II followed by six in cluster III while cluster IV contained lowest number of accessions i.e. three accessions indicating their higher genetic similarity. Our result showed that significant genetic variability at molecular level on the basis of SSR markers that can be used towards the development of lentil cultivars having high concentration of Fe and Zn.

***Corresponding Author:** Rajendra Darai, Senior Scientist (S-4), Nepal Agricultural Research Council (NARC) Grain Legumes Research Program (GLRP), Khajura, Nepalgunj, Banke, Nepal, E-mail: rajendra5042@yahoo.co.uk

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Keywords: Lentil; SSR Marker; Accessions; Genetic Diversity; Allele; Molecular Characterization

Introduction

Lentil (*Lens culinaris Medikus*) is an autogamous diploid (2n = 2x = 14) species with haploid genome size of 4 063 Mbp. It is the most important pulse crop in Nepal and often called the "poor man's meat" due to its richness in protein (28%) for human consumption. Its straw is a valued animal feed having 2% minerals and 59% carbohydrates [1].Nepalese food items includes dal, bhat and tarkari(pulse, rice and vegetables) which is the pertinent sources of nutrition. In addition to this, lentil is a low fat food i.e. low-glycaemic carbohydrate that helps to prevent chronic diseases like diabetics and heart diseases. Globally, it is cultivated for its protein-rich grains in as many as 53 countries on 6.65 million ha area with annual production of 7.97 million ton [2]. However, top ten countries, namely Canada, India, Turkey, Nepal, Australia, China, Iran, USA, Syria, and Ethiopia contribute about 95% of the global production. Currently, annual world lentil production is approximately 4 million tones (MT) and its more than 85% is produced in five specific regions including India, Nepal, and Bangladesh (32%); western Canada (29%); Turkey and northern Syria (18%); Australia (4%). In Nepal, lentil shares about 62% and 64% of the total legume area and production, respectively[3]. Quick cooking quality, tasty pink red cotyledons and high micronutrient contents make Nepalese lentil highly preferable to the international consumers and popular in the international market [4]. Bangladesh, Singapore, SriLanka, Germany, Korea, UK, Indonesia are the major export markets for Nepalese lentils [5]. Also, nutritional profile of lentil grains makes it'sa potential candidate for mineral biofortification [6,7]. Evaluation of 35 advanced breeding lines of ICARDA at Saudi Arabia over two seasons showed significant variation for Fe, Zn, Cu, Ca, Mg, P, K, and Mn concentrations [8]. Iron concentration in cultivated and wild lentils mean Fe conc. was 61 mg kg-1 across all 26 lentil genotypes. Among the 20 L. culinaris genotypes, Fe conc. ranged from 26 (IG72830) to 92 mg kg^{-1} (CDC Red Rider) with a mean of 58 mg kg-1.Prior knowledge of genetic variability and characterization of germplasm available at the station has an important implication for future utilization to identify areas of major priority for conservation and improvement programs [9]. Quantitative traits provide an estimate of genetic diversity, and various numerical taxonomic techniques have been successfully used to classify and measure the pattern of genetic diversity in germplasm, as in lentil [10,11].

Knowledge of level of genetic diversity available in germplasm is prerequisite for any breeding programs that genetic gain could not be limited due to the availability of narrow genetic variability between parental lines involved in crossing programs [12]. In the past years, breeders used morphological data and pedigree information to assess genetic diversity among the lentil varieties. However, these studies could not make much contribution to our knowledge due to limited phenotypic diversity, high genotype x environment interaction, and paucity of accurate record of ancestry. DNA markers provide an opportunity for precise characterization of genotypes and measurement of genetic relationships over morphological and isozyme markers [13,14]. In earlier studies, molecular markers such as Simple Sequence Repeat (SSR), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Random Amplified Polymorphic DNA (RAPD) have been preferred for genetic diversity analysis in lentil [15-25] and gene mapping [26-30].

Kaur et al. (2014) developed a number of SSR markers in lentil [31]. Transferability of SSR markers from legume genera/species to lentil also reported earlier by several workers [32-38] suggesting high level of sequence conservation of microsatellite markers in legumes. SSRs offer several advantages over other marker systems such as locus specificity, multi-allelic and co-dominance nature and high repeatability. Also, they are inexpensive and hence these markers are being utilized routinely for germplasm fingerprinting and evaluation of genetic relationship between accessions or genotypes [39,40,41] and construction of genetic linkage maps [42]. In lentil, 32 polymorphic SSR markers have been used for molecular diversity of genotypes with micronutrients concentration. These markers amplified a total of 130 bands with PIC value ranging from 0.138 to 0.798. [43]. In the present investigation, SSR markers used to assess the diversity and genetic relatedness among Nepalese, Indian and exotic lentil genotypes having high concentration of Fe and Zn and to identify genotype specific markers.

Materials and Methods

Plant Material

In the present study, 25 accessions of cultivated lentil species (*L. culinaris* subsp. *Culinaris*) having richness in Fe and Zn used. These accessions represented the diversity of the entire collection available at NGLRP on the basis of passport, laboratory and morphological data (Table 1). These accessions originated from SAARC countries (14 accessions: Nepal-7, India-6, Bangladesh-1)and ICARDA(11 accessions).

DNA Extraction and PCR Amplification

Genomic DNA was extracted using the Cetyltrimethyl Ammonium Bromide Method (CTABmethod), described by Rogers and Bendich (1985) with minor modifications [44]. For this, 100mg young fresh leaf tissue of 2 weeks old seedlings of lentil was used to isolate genomic DNA from each accession using CTAB buffer consisting 50mM Tris-HCL, 25mM EDTA, 1M NaCl, 1% CTAB, 0.15% 2-mercaptoethanol. The DNA extract in the form of pellet was suspended in 100µl of TE buffer and prepared the 5% working DNA solution with deionized water and used for Polymerase Chain Reaction (PCR) amplification of SSRs. Forty four SSR markers reported by Hamweieh et al., 2005, fifty eight SSR markers reported by [45] and 18 EST SSR markers developed in IARI's laboratory exhibiting polymorphosim across Lens species were assayed for identification of polymorphic SSR markers. Forty SSRs were initially screened for polymorphism across the 25 accessions of lentil used in this study. The details of SSR markers, their sequences and motifs are given in supplementary table 2.

PCR was carried out using a PE 9600 thermo cycler (Perkin-Elmer, Foster City, CA), which was programmed for an initial denaturation of 3 min at 940C, followed by 30 cycles performed for 30 s at 94°C, annealing for 30 s at either 52°C, 53°C, 54°C or 55°C (depending on the locus) and elongation for 1 min at 72°C. The final extension step was programmed for 5 min at 72°C. A Mega BACE 500 Capillary System (Amersham Pharmacia Biotech, Piscataway, NJ) was used to separate amplified products in 3.5 % agarose gel. Samples were prepared by adding 1 µl of diluted PCR products to 9 µl formamide. 1% (v/v) ET-Rox 900 bp DNA size standard was used as marker ladder (Amersham Bioscience). Microsatellite fragment sizes were estimated using the MegaBACE Genetic Profiler Version 2.0 (Amersham Pharmacia Biotech). The amplified SSR products were scored by visualizing gel on UV illuminator. Only the prominent bands were scored for analysis.

SSR Allele Scoring and Data Analysis

The presence or absence of SSR fragments in each accession was recorded for all the polymorphic SSR markers. The SSR bands appearing without ambiguity were scored as 1(present) and 0(absent) for each SSR marker. The size of the amplified product was calculated on the basis of its mobility relative to molecular mass of the marker (50bp DNA ladder, Thermo Scientific, USA). The polymorphism information content (PIC) was determined by using the following formula for a measuring the allelic diversity at a locus [46].

$$PIC = 1-\Sigma P_i 2$$

Where, P_i is the frequency of the ith allele for its marker in the set of accessions analyzed, calculated for SSR locus. The genetic similarity among accessions was estimated based on Jaccard's similarity coefficient [47]. The resulting similarity matrix was further used to construct the dendogram based on the un weighted pair-group method arithmetic average (UPGMA) clustering algorithm. These computations were carried out using MINITAB Inc. File version 14.13.0.0 software.

Table: 1 Details about pedigree, type of materials and origin/source of 25 lentil accessions used in the present study.

Accession Code no	Accessions	Pedigree	Types of materials	Source/Origin
1	ILL-8006	ILL5888xILL5782	Breeding lines	Bangladesh
2	RL-6	ILL8008xILL5888	Nepal cross	Nepal
3	RL-12	Sindur x Khajura-2	Nepal cross	Nepal
4	ILL-7715	FLIP95-59L		ICARDA
5	ILL-7164	PAK86591		ICARDA
6	ILL-3490			ICARDA
7	Khajura-2(PL639)	L9-12xType 8		GPPUAT, Pantanagar
8	Simal(LG7)	Land race from India		India
9	Shital(ILL2580)	L1278	Cultivar	ICARDA/India
10	Sagun(ILL6829)	ILL4907(Pakistan)x ILL4605 (Argentine)		ICARDA
11	HUL-57	Mutant of HUL1	Cultivar	BHU, Banaras, India
12	LG-12	Local landrace from India		India

13	PL-4	UPL175x(PL184xP228)	Cultivar	GPPUAT, Pantanagar
14	RL-11		Nepal cross	Nepal
15	RL-4	ILL6037xILL8007	Nepal cross	Nepal
16	ILL-2712			ICARDA
17	Black Masuro	Local landrace from Rasuwa, Nepal		Nepal
18	RL-79	99S95-2-1	Nepal cross	Nepal
19	ILL-6467	ILL4605xILL2582		ICARDA
20	ILL-7979	FLIP1996-47L		ICARDA
21	ILL-6819			ICARDA
22	ILL-7723	Sel89503		ICARDA/Pakistan
23	WBL-77	Mutant of BR25	Cultivar	RAU, Dholi, India
24	ILL-4605	ILL5888x ILL5782	Cultivar	USA
25	RL-49	NR9901-1-17	Nepal cross	Nepal

Table 2: Forward and reverse primer sequences and annealing temperatures used for amplifications of 40 SSR markerloci

S/N	Name of Primers	Forward primer sequences	Reverse primer sequences	Annealing temperature(°C)
1	PLC10	TGCAACAAAGGACACTAGAGGTT	ATTTCTTTCTCCCTAACCAGCC	59
2	PLC16	CGTTTGATCTTCTAAGCCCCTA	AAGGGAAAGGATGTTTGACTTG	59
3	PLC17	AAGCTGAAGGAAATCAAAGTGG	TCAACACACTCCATGTTTAGAGC	59
4	PLC21	AACTCGCATCCTCTTCACAACT	GGACCTTTCCCTTGTAGTCACC	59
5	PLC22	TACACTGAAGGAGATGCACTGG	TAACAACAAAACACAGCTTCGC	60
6	PLC5	CATTGCAGCTTATTCTCACAGC	TGACCCATCCTCATCCTTAAAT	60
7	PLC35	TTGCTTCCTCCTCTTCTCACTC	AGCCTCAGTACCCTCCTCTTTT	60
8	SSR 124	GAACATATCCAATTATCATC	GTATGTGACTGTATGCTTC	52
9	SSR 154	GGAGCAAGAAGAAGCAG	GGAATTTATCACACTATCTC	51
10	SSR 66	GGTAGTGGTGAGGAATGAC	GCATCACTGCAACAGACC	55
11	SSR 90	CCGTGTACACCCCTAC	CGTCTTAAAGAGAGTGACAC	55
12	SSR 207	GAGAGATACGTCAGAGTAG	GATTGTGCTTCGGTGGTTC	55
13	SSR 72	CAAACAGTACAAGGAAAGGAG	CTGACTGAGCTGCTTGAAC	55
14	SSR 132RN	CCAGAACAAACGTAAACC	CTATCGCATATGAGTGAAC	52
15	SSR 107	GCGGCGAGCAAATAAAT	GGAGAATAAGAGTGAAATG	51
16	SSR 113	CCGTAAGAATTAGGTGTC	GGAAAATAGGGTGGAAAG	51
17	SSR 33	CAAGCATGACGCCTATGAAG	CTTTCACTCACTCAACTCTC	56
18	SSR 19	GACTCATACTTTGTTCTTAGCAG	GAACGGAGCGGTCACATTAG	58
19	SSR 48	CATGGTGGAATAGTGATGGC	CTCCATACACCACTCATTCAC	57
20	SSR 46-2	CACTAAACATGGAAAATAGG	CTTATCTTTGTTTGAAGCAA	50
21	SSR 28	GAGGGCATAAATTCAGATTC	GGACAACGCACATTTGATG	53
22	SSR 183	GCTCGCATTGGTGAAAC	CATATATAGCAGACCGTG	52
23	SSR 253	GAAGAAGCATTCACGGTG	GAGGGACTACTATATCAG	53
24	SSR 34-2	CGGCGGATGAAACTAAAG	CATTTCCTTCACAAACCAAC	53
25	SSR 191	GCAAATTTCTTGGTCTACAC	GGGCACAGATTCATAAGG	53

26	SSR 230	CCAACAACAATTCACCATAC	AACATTGTACTGAGAGGTG	53
27	SSR 202	CAACCTCACTTACCTTAC	GCTCTTTATCATCATTCTAC	52
28	SSR 197	CACCAATCACCAACACAC	GAGCTGTGAAGTCTTATCTG	54
29	SSR 99	GGGAATTTGTGGAGGGAAG	CCTCAGAATGTCCCTGTC	57
30	SSR 130	CCACGTATGTGACTGTATG	GAAAGAGAGGCTGAAACTTG	55
31	SSR 323	AGTGACAACAAAATGTGAGT	GTACCTAGTTTCATCATTG	51
32	SSR 156	GTACATTGAACAGCATCATC	CAAATGGGCATGAAAGGAG	53
33	PBALC0353	CCATAACAGACAAAACCCTACT	ATTCTCAAAGCCCATTTAGTT	59
34	GLLC 106	ACGACAATCCTCCACCTGAC	AACAAGGAAGGGGAGAGGAG	56
35	GLLC 511	ATTGAGAGGAGGCGGAGAA	CGCGTGTCTCTCTCTCTCAC	56
36	GLLC 563	ATGGGCTCATTGAACAAAAG	CCCCCTCTAAGAGATTTTCCTC	56
37	GLLC 598	TGGGCTCATTGAACCAAAAG	CCCCCTTCTAAGTGATTTTCC	56
38	PLC60	TGCTTGGACCCTAAATTTGC	AAGAAAAGGGCAACCACTGA	60
39	PBALC13	GCAGCAGCATGAGAAAATGA	ATTACTCGACGCCCCCTAGT	60
40	PLC64	ATTGGTGGGGAGTTTGAGTG	AAACAACTCATGATGTGCCCT	61

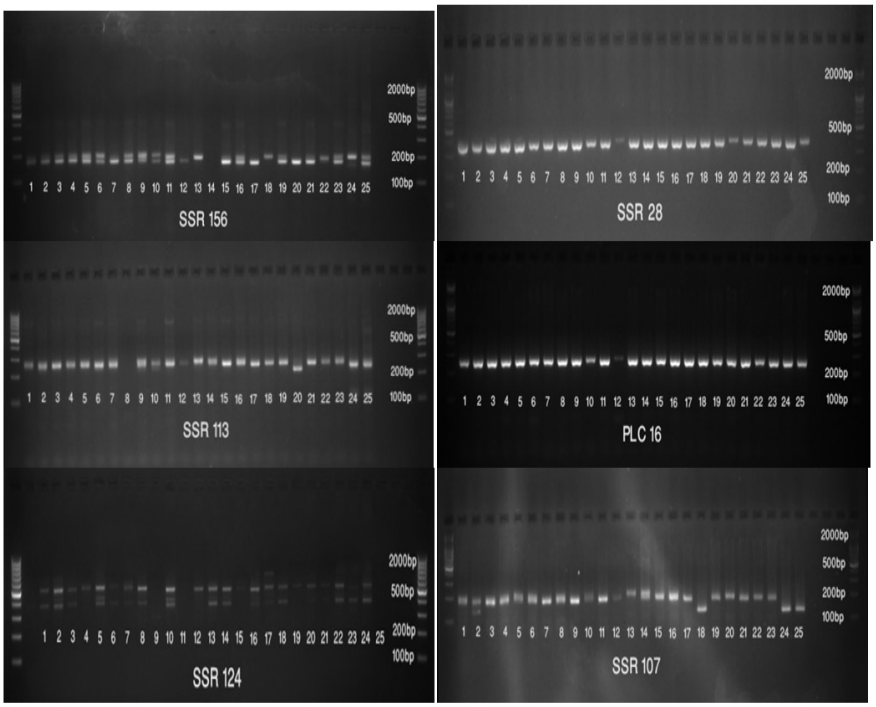

Figure 1: DNA amplification profile of 25 lentil accessions with SSR markers

Results And Discussion

Molecular Characterization and Genetic Diversity

Molecular markers help to know the underlying allelic diversity and genetic base of genotypes in the study. In the present study, 40 SSR markers have been used to know allelic diversity present among 25 accessions of lentil. Out of these 40 SSR markers, 23 were found polymorphic among studied genotypes. A representative of DNA profile of polymorphic SSR marker is presented in Figure 1. These polymprphic SSR markers are highly informative for genetic studies and can be used to distinguish the polymorphism rate at a specific locus in lentil accessions. In the present study, these 23polymorphic SSR markers produced 584 alleles and allele number, PIC values and allele size range produced by these SSR marker loci has been listed in table 3. Number of alleles per SSR locus was varied from 2 to 4 with an average of 2.26 suggesting a significant genetic diversity at molecular level among the studied genotypes. The fragment size of these 584

alleles was ranged from 100bp to 600bp. Four SSR loci produced unique alleles in 25 accessions of present study (Table 3). PBALC0353 SSR alleles were found rare with a frequency of 0.14 in the studied genotypes (Table 3). SSR 107 marker locus generated unique alleles as well as rare alleles in three accessions namely, RL-79, ILL4605 and RL-49. Therefore, these three accessions may serve as good sources for new alleles of the genes controlling agronomically important traits and may be of immense importance in intellectual property right issues [47]. Earlier SSR markers (PBALC 13, PBALC 206, and GLLC 563; PBALC 353, SSR 317–1, PLC 62, and PBALC 217) that also used in the present study have been shown to be associated with Fe (PVE: 9% to 11%) and Zn (PVE: 14%, to 21%) content in lentil [48].

Efficiency of markers and their utility in terms of polymorphism and quantitative estimation could be expressed in mean heterozygosity and marker index [49]. PIC values, a measure of the allelic diversity of SSRs, ranged from 0.14 in PBALC0353 to 0.57 in SSR 28 with an average PIC value of 0.46. Markers having PIC values ≥ 0.5 are more useful due to greater discriminating ability to distinguish the genotypes at a specific locus. These results showed that present SSR markers have high level of polymorphisms and similar to other studies conducted earlier in lentil. For example, in lentil, 33 alleles detected with a range of 3-8 per locus and estimated a gene diversity value 0.66 for these alleles [50]. A wide range of variation has been observed in term of allele numbers and gene diversity among lentil species when used SSR markers in lentil [53]. In the present study, a wide range of genetic variability has been shown among studied genotypes probably due to their different center of origin and genetic constitution. Similar results have been reported by several authors [51,52,53,54]. This study revealed the divergence among lentil accessions which can be further used in lentil breeding programs.

Table : 3 Analysis of the DNA profiling (fingerprinting)/genetic diversity among various lentil accessions

S/N	Primer code	Molecular wt. range (bp)	Total no. of alleles	No. of polymorphic alleles	Alleles per locus	Polymorphism information content (PIC)
1	PLC 10	300-400	24	2	12	0.45
2	PLC 16	200-300	25	3	8.33	0.5
3	PLC 17	300-400	25	2	12.5	0.46
4	PLC 21	300-400	25	2	12.5	0.48
5	PLC 22	300-400	23	2	11.5	0.43
6	PLC 35	200-300	25	2	12.5	0.49
8	SSR 124	400-600	38	3	12.66	0.55
9	SSR 154	400-600	19	2	9.5	0.5
14	SSR 132 RN	400-500	25	2	12.5	0.46
15	SSR 107	100-200	25	3	8.33	0.54
16	SSR 113	200-300	25	3	8.33	0.53
17	SSR 33	200-230	25	2	12.5	0.4
18	SSR 19	200-300	25	2	12.5	0.48
19	SSR 48	200-300	24	2	12	0.49
20	SSR 46	100-200	25	2	12	0.48
21	SSR 28	400-500	25	3	8.33	0.57
24	SSR 34	300-400	25	2	12.5	0.48
30	SSR 130	200-300	24	2	12	0.49
32	SSR 156	100-200	37	2	18.5	0.5
33	PBALC0353	100-200	25	2	12.5	0.14
34	GLLC 106	100-200	25	3	8.33	0.33
35	GLLC 511	100-200	23	2	11.5	0.5
36	GLLC 563	100-200	22	2	11	0.47
			Total alleles =584	Total no. of polymorphic alleles=52	Average alleles per locus=11.49	Average PIC value= 0.46

Molecular Diversity Analysis (Cluster Analysis)

Knowledge of genetic distance on the basis of available genetic diversity among the targeted germplasm lines helps to select desirable parents for crossing in the breeding program. This results in identification of desirable recombinants in segregation population leading to development of high yielding varieties. Use of molecular markers for estimating the genetic diversity has several advantages over morphological markers because molecular markers do not influence by environmental factors, they are to be highly reproducible and also widely distributed throughout the genome. Therefore, an insight into molecular diversity is necessary for studying the genetic base of lentil accessions. In the present study, genetic relationships have been established among the present accessions on the basis of Jaccard's similarity coefficients calculated over 40 SSR marker alleles. The pair wise genetic similarity among 25 accessions varied from 0.16 to 0.83. UPGMA cluster analysis resulted in adendogram or cladogram on the basis of genetic similarities between accessions. Twenty five accessions of present study were grouped i to five major clusters (Figure ii & iii).The cluster I contains four accessions namely ILL-8006, RL-6, RL-12, and ILL-7715. These accessions have their origin/source from Bangladesh, Nepal and ICARDA. The cluster II contains largest maximum number of accessions (i.e. 7 accessions), namely, ILL-7164, ILL-3490, Khajura-2(PL639), Simal(LG7), Shital(ILL2580), Sagun (ILL6829),and LG-12. These seven accession has their origin from ICARDA and India . The cluster III is the second largest group that contains six accessions namely HUL-57,PL-4, RL-11, RL-4, ILL-2712, Black Masuro representing from India, Nepal and ICARDA origin. The Cluster IV contains three accessions (i.e. RL79, ILL-4605, RL-49). These accessions belong to USA and Nepal country .The Cluster V contains five accessions (ILL-6467,ILL-7979, ILL-6819,ILL-7723, and WBL-77) of ICARDA, Pakistan and Indian origin(Fig iii).These lentil accessions were grouped into five clusters based on the neighbor-joining cluster analysis with dissimilarity min value = 0.028 and dissimilarity max value = 0.55 (Fig ii). These results demonstrated that accessions of a particular country did not cluster together and they were found to be distributed over the different clusters indicating genetic diversity among the accession on the basis of SSR markers. Highest similarity coefficient (0.83) was observed between RL-6 and RL-12accessions indicating that these two accessions are very closely related to each other. Lentil accessions ILL-3490 was closely related toKhajura-PL639 at similarity coefficient of 0.72 while popular and released variety Simal (LG7) was observed closely related to Shital (ILL2580) at the similarity coefficient of 0.70. Genetic distance between Cluster IV and Cluster V was highest (4.66-4.74) from the centroids that determined the possible candidates with Cluster I or other clusters for hybridization program (Table 4). This cluster pictorial indicated that there was genetic diversity among the high grain Fe and Zn concentration lentil accessions due to the different sources of origin and diverse genetic formation. These diverse genetic materials may be used for genetic improvements of lentil accessions.

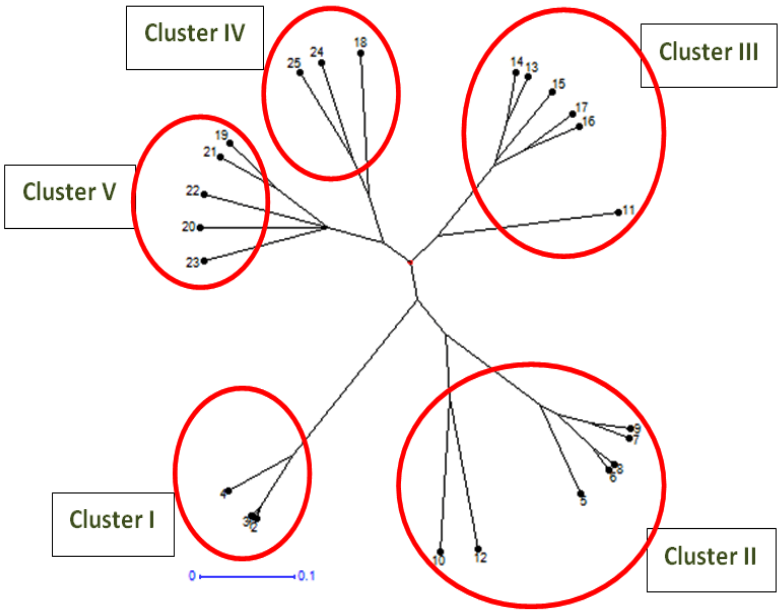

Figure 2: Genetic relationship among 25 lens accessions (1.ILL-8006,2. RL-6,3. RL-12,4.ILL-7715,5. ILL-7164,6.ILL-3490, 7.Khajura-2(PL639), 8.Simal(LG7), 9.Shital(ILL2580),10.Sagun(ILL6829),11.HUL-57,12.LG-12,13. PL-4, 14.RL-11, 15.RL-4, 16.ILL-2712,17.BlackMasuro, 18.RL-79,19. ILL-6467,20.ILL-7979, 21.ILL-6819,22.ILL-7723, 23.WBL-77,24. ILL-4605, 25.RL-49)using Unbiased neighbouring joining dendrogram of 23 SSR markers developed by Darwin software.

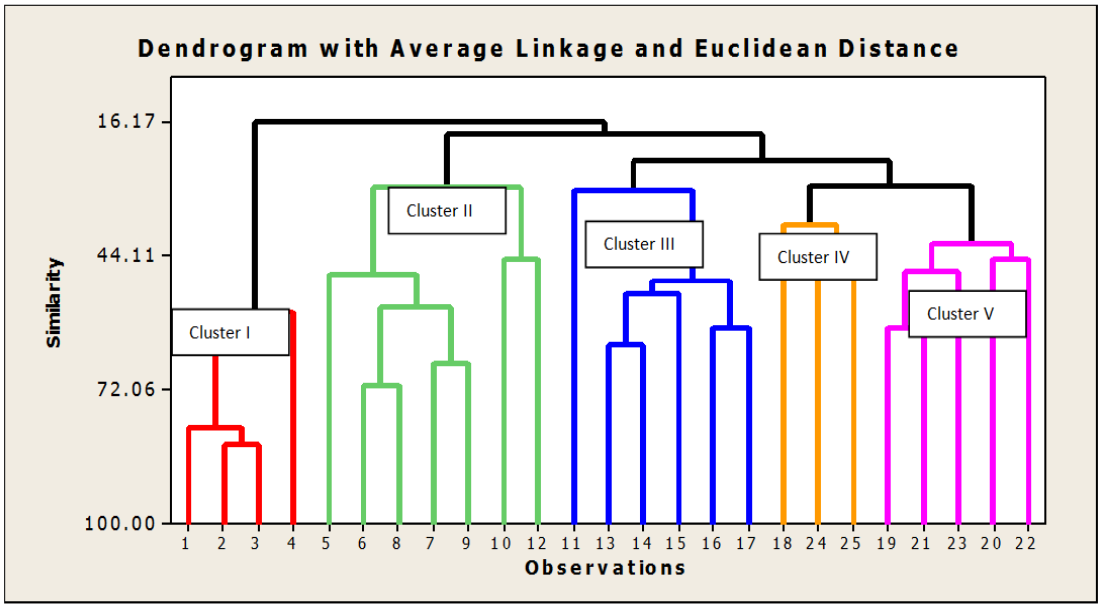

Figure 3: Cluster analysis of 25 high grain Fe and Zn lentil accessions using 23 SSR markers constructed by MINITAB

Table 4 : Distances between Cluster Centroids

	Cluster I	Cluster II	Cluster III	Cluster IV	Cluster V
Cluster I	0.00	4.14	4.11	4.66	4.74
Cluster II	4.14	0.00	3.75	4.23	3.34
Cluster III	4.11	3.75	0.00	3.69	3.20
Cluster IV	4.66	4.23	3.69	0.00	3.07
Cluster V	4.74	3.34	3.20	3.07	0.00

The information on genetic diversity among these lentil accessions will be helpful to lentil breeders in selection of appropriate hybridizing parents in developing superior accessions.

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