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Genetic Diversity Analysis of Lentil (*Lens culinaris* L.) Germplasm Using DNA Based SSR Markers

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Abstract

Lentil (*Lens culinaris* Medik.subsp. *culinaris*) is an important principal cool season pulse crop of Nepal. To estimate genetic diversity, one hundred eighty five diverse lentil germplasm were collected from National Grain Legume Research Program, Rampur; Regional Agricultural Research Station, Nepalgunj and National Agriculture Genetic Resource Center, Khumaltar. Thirty polymorphic microsatellites marker were used for PCR analysis and finger printing. The results obtained from microsatellite profiling revealed that maximum alleles were amplified from SSR 19, SSR 99, SSR 113, SSR 156 and SSR 202 with amplicon size 180-395. Highly informative and detectable polymorphic markers for this study, foundwere SSR 34-2, SSR 90 and SSR 207 which indicate the power and higher resolution of those marker systems in detecting molecular diversity. The dendrogram constructed by highly polymorphic 30 SSR markers from 185 lentil accessions showed ten major groups from Group I to X based on source of origin and their pedigree. Groups III and VI genotypes were totally different from other groups whereas group X genotypes were from Nepal cross lines. This study show the divergency among lentil genotypes which can be further used in lentil breeding programs. All genotypes involved in this study exhibited wide range of genetic variability due to different center of origin, different genetic constitution. The level of genetic relatedness largely depends on the type of molecular markers used in the study, nature of SSR repeat motif, number of SSR markers and the genetic relatedness of the lentil germplasm to be analysed. The genetic relatedness detected in this study may constitute the foundation for future systematic lentil breeding programs.

Keywords

Germplasm, Lentil Characterization, Genotypes, Marker

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1. Introduction

Creation of variation and selection is the basis of plant breeding. The knowledge of genetic diversity and association of characters with yield is of great importance to the breeder for making an improvement of quantitative characters. Molecular marker is used for estimating genetic variation at population level and among closely related species [1]. Different types of molecular markers have been developed showing that lentil has relatively low levels of genetic variation [2, 3]. Plant descriptors coupled with molecular markers provide a valid evidence of diversity as these are least affected by environmental fluctuations [4, 5, 6].

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Knowledge of genetic variation and genetic relationship between lentil genotypes is important for efficient utilization of germplasm resources and is more important to know which marker techniques and how many of the markers represent variation in the entire genome and should be used in order to derive reliable diversity pattern [7].

Lentil (*Lens culinaris* Medik.subsp. *culinaris*) is an important principal cool season pulse crop of the Indian Subcontinent, the Middle East, North America, North Africa and West Asia [8]. Lentil is the major winter pulse crop of Nepal which occupy about 60% of pulse growing area. Nepal has altogether 2,06,522 ha area of lentil with 2,26,931 metric ton productivity and 1,099 kg yield per hectare [9]. The crop has developed into a range of varieties adapted to diverse growing areas and cultural preferences, and containing unique nutritional compositions, colors, shapes and tastes. Lots of lentil land races, primitive races, indigenous races and wild races are still available in Nepal but they have not been studied properly. The genetic diversity of lentil based on molecular level has not been properly studied yet in Nepal. Thus the yield attributing traits, disease resistance traits, insect pest resistance traits, abiotic stress tolerance traits and quality traits have not been identified and, cause delay in breeding for developing elite lines. Now a day the importance of lentil in Nepal is increasing due to its high nutritive value, important components of Nepalese diet, increased internal consumption and exportable commodity to foreign countries. Thus, there is an urgent need to increase the overall production and productivity of this crop through varietal improvement and suitable agronomic practices under rice-maize cropping systems in Nepal. Before initiation of lentil breeding activities there is urgent need to characterize, evaluate lentil germplasm available to us. Therefore present study was conducted with an objective of selecting divergent parents based on genetic distance for future lentil breeding programme.

2. Main Body

2.1. Materials and Methods

Diverse lentil germplasm were collected from National Grain Legume Research Program (NGLRP), Rampur; Regional Agricultural Research Station (RARS), Nepalgunj and National Agriculture Genetic Resource Center (NAGRC), Khumaltar (Table 1). Thirty polymorphic microsatellites marker were used for PCR analysis based on the results of previous report by [10, 11]. The list of polymorphic markers, their name, sequence information, annealing temperature and amplification size are given (Table 2). DNA fingerprinting was conducted with SSR markers in Biotechnology unit, Nepal Agricultural Research Council, Khumaltar, Lalitpur. Similarly, lentil DNA extraction was done by Modified CTAB method [12] using standard protocol followed by DNA quantification, PCR amplification, gel separation and scoring of gel separated bands using standard protocol. The amplified products were scored as bands on visualization on gel in UV illuminator. Only the reliable bands were included in analysis. The presence of bands was scored as "1" and absence of band was scored as "0". The respective data analysis, data entry and processing was carried out by using Microsoft Excel 2007.

The gene diversity was calculated by Nei's (1993) formula: Gene diversity=1-$\sum Pi^2$, where Pi is the frequency of i^{th} microsatellite allele present in the examined accessions. Polymorphic information content of each marker was calculated. The number of alleles per locus, size range, gene frequency and genetic distance were estimated. The PIC value index is the function of gene frequencies.

Table 1. List of lentil accessions used in this study based on source of origin.

SN	Variety name	Source of origin	SN	Variety name	Source of origin
1	LN-0135	Nepal Local	93	khajura-2	Nepal Local
2	RL-45	Nepal Cross	94	RL-28	Nepal Cross
3	RL-67	Nepal Cross	95	RL-78	Nepal Cross
4	RL-49	Nepal Cross	96	RL-13	Nepal Cross
5	RL-79	Nepal Cross	97	ILL-6256	ICARDA
6	ILL-3338	ICARDA	98	ILL-7220	ICARDA
7	RL-56	Nepal Cross	99	ILL-6025	ICARDA
8	RL-68	Nepal Cross	100	NRX9801-1	Nepal Corss
9	RL-8	Nepal Cross	101	39-S-66L	ICARDA
10	X94s-48	Nepal Cross	102	ILL-6408	ICARDA
11	ILL-2712	ICARDA	103	ILL-6468	ICARDA
12	ILL-1970	ICARDA	104	Flip2006-55L(ILL-10134)	ICARDA
13	ILL-10071	ICARDA	105	FLIP05-44L(ILL-10065)	ICARDA
14	ILL-9924	ICARDA	106	X94 S-43	ICARDA
15	ILL-6465	ICARDA	107	ILL-2716	ICARDA
16	ILL-9926	ICARDA	108	ILL-8186	ICARDA
17	ILL-6458	ICARDA	109	PL-639	ICARDA

SN	Variety name	Source of origin	SN	Variety name	Source of origin
18	ILL-1920	ICARDA	110	F2003-49L	ICARDA
19	ILL-6811	ICARDA	111	ILL-9990	ICARDA
20	HUL-57	ICARDA	112	ILL-7980	ICARDA
21	Sagun	ICARDA	113	RL-9	Nepal Cross
22	M-Bharatai	ICARDA	114	RL-12	Nepal Cross
23	ILL-7162	ICARDA	115	PL-406	ICARDA
24	ILL-7723	ICARDA	116	ILL-3490	ICARDA
25	LN-0136	Nepal Local	117	RL-83	Nepal Cross
26	ILL-3768	ICARDA	118	ILL-6821	ICARDA
27	DPL-62	India	119	ILL-6447	ICARDA
28	ILL-7537R	ICARDA	120	ILL-2373	ICARDA
29	WBL-77	India	121	RL-11	Nepal Cross
30	IL-1	ICARDA	122	ILL-9943	ICARDA
31	ILL-7979	ICARDA	123	ILL-9996	ICARDA
32	ILL-7715	ICARDA	124	RL-55	ICARDA
33	RL-4	Nepal Cross	125	PL-4402	Nepal Cross
34	ILL-6467	ICARDA	126	ILL-3280	India
35	ILL-7164	ICARDA	127	Khajura-1	Nepal Local
36	ILL-3490	ICARDA	128	ILL-6829	ICARDA
37	ILL-6256	ICARDA	129	ILL-6024	Nepal Local
38	LG-12	India	130	ILL-8132	ICARDA
39	ILL-3111	ICARDA	131	ILL-7990	ICARDA
40	ILL-2527	ICARDA	132	ILL-8605	ICARDA
41	FLIP-2006-99L	ICARDA	133	RL-84	ICARDA
42	FLIP 95-1L	ICARDA	134	ILL-9949	ICARDA
43	RL-60	Nepal Cross	135	LN-0137	Nepal Cross
44	FLIP2009-60L	ICARDA	136	Simrik	ICARDA
45	FLIP04-60L(ILL-10013)	ICARDA	137	ILL-9927	Nepal Local
46	RL-70	Nepal Cross	138	ILL-1672	ICARDA
47	RL-73	Nepal Cross	139	ILL-3496	ICARDA
48	ILL-6021	ICARDA	140	RL-51	ICARDA
49	FLIP05-24L(ILL-10045)	ICARDA	141	ILL-8187	ICARDA
50	FLIP05-24L(ILL-10065)	ICARDA	142	ILL-7986	Nepal Cross
51	RL-71	Nepal Cross	143	ILL-9992	ICARDA
52	NRx2001-72-3	Nepal Cross	144	ILL-8191	ICARDA
53	FLIP2008-7L	ICARDA	145	ILL-590	ICARDA
54	FLIP 2009-54L	ICARDA	146	ILL-2501	ICARDA
55	RL-75	Nepal Cross	147	NRX2001-71-3	ICARDA
56	RL-35	Nepal Cross	148	Cumara	ICARDA
57	RL-43	Nepal Cross	149	ILL-27001-1	Nepal Cross
58	RL-69	Nepal Cross	150	ILL-8188	ICARDA
59	RL-44	Nepal Cross	151	ILL-4139	ICARDA
60	RL-42	Nepal Cross	152	ILL-2573	ICARDA
61	RL-76	Nepal Cross	153	Shikhar	ICARDA
62	RL-26	Nepal Cross	154	X49s-48	Nepal Cross
63	RL-41	Nepal Cross	155	ILL-1704	ICARDA
64	RL-39	Nepal Cross	156	Arun	Nepal Local
65	RL-58	Nepal Cross	157	Sindur	ICARDA
66	RL-62	Nepal Cross	158	NRX - 99S -95-95-1	Nepal Cross
67	RL-47	Nepal Cross	159	Simal	ICARDA
68	RL-80	Nepal Cross	160	ILL-7978	ICARDA
69	RL-21	Nepal Cross	161	Shisir	Nepal Local
70	RL-23	Nepal Cross	162	ILL-9932	*ICARDA*
71	FLIP05-52(ILL-10073)	ICARDA	163	ILL-7163	ICARDA
72	ILL-6260	ICARDA	164	ILL-3885	ICARDA
73	RL-94	Nepal Cross	165	Jutpani	Nepal Local
74	X39S-66L	ICARDA	166	Mangal Bazar	Nepal Local
75	ILL-10134	ICARDA	167	RL-77	Nepal Cross
76	NRX2001-71-4	Nepal Cross	168	ILL-I970	ICARDA

SN	Variety name	Source of origin	SN	Variety name	Source of origin
77	RL-74	Nepal Cross	169	ILL-9885	ICARDA
78	RL-20	Nepal Cross	170	RL-6	Nepal Cross
79	RL-25	Nepal Cross	171	ILL-7157	ICARDA
80	RL-95	Nepal Cross	172	ILL-9993	ICARDA
81	ILL-10068	ICARDA	173	ILL-6818	ICARDA
82	RL-22	Nepal Cross	174	ILL-7538	ICARDA
83	RL-38	Nepal Cross	175	Baitadi 6A	Nepal Local
84	RL-15	Nepal Cross	176	ILL-2526	ICARDA
85	ILL-7664	ICARDA	177	ILL-9881	ICARDA
86	DIGGER	ICARDA	178	ILL-9976	ICARDA
87	Bari Masuro-4	ICARDA	179	ILL-3236	ICARDA
88	NRX9901-1	Nepal Cross	180	PL-4	India
89	Aarial	Nepal Local	181	RL-85	Nepal Cross
90	ILL-6458	ICARDA	182	RL-81	Nepal Cross
91	X95S-83	ICARDA	183	LN-0111	Nepal Local
92	FLIP2009-59L	ICARDA	184	ILL7616	ICARDA
			185	NRx-99S-95-1-12	Nepal Cross

Table 2. List of forward and reverse SSR primers used for lentil characterization, with annealing temperature, expected size.

S.N.	SSR No.	Forward	Reverse	Annealingtemp.(Tm) used for PCR(°C)	Xpected size(bp)
1	SSR 34-2	CGGCGGATGAAACTAAAG	CATTTCCTTCACAAACCAAC	53	185
2	SSR 66	GGTAGTGGTGAGGAATGAC	GCATCACTGCAACAGACC	55	253
3	SSR 90	CCGTGTACACCCCTAC	CGTCTTAAAGAGAGTGACAC	55	181
4	SSR 132RN	CCAGAACAAACGTAAACC	CTATCGCATATGAGTGAAC	52	330
5	SSR 191	GCAAATTTCTTGGTCTACAC	GGGCACAGATTCATAAGG	53	238
6	SSR 197	CACCAATCACCAACACAC	GAGCTGTGAAGTCTTATCTG	54	173
7	SSR 207	GAGAGATACGTCAGAGTAG	GATTGTGCTTCGGTGGTTC	55	227
8	SSR 33	CAAGCATGACGCCTATGAAG	CTTTCACTCACTCAACTCTC	56	289
9	SSR 19	GACTCATACTTTGTTCTTAGCAG	GAACGGAGCGGTCACATTAG	58	250
10	SSR 48	CATGGTGGAATAGTGATGGC	CTCCATACACCACTCATTCAC	57	165
11	SSR 96	GTTATCTTCCAGCGTC	GATATACAATCAGAGATG	49	210
12	SSR 99	GGGAATTTGTGGAGGGAAG	CCTCAGAATGTCCCTGTC	57	161
13	SSR 107	GCGGCGAGCAAATAAAT	GGAGAATAAGAGTGAAATG	51	161
14	SSR 113	CCGTAAGAATTAGGTGTC	GGAAAATAGGGTGGAAAG	51	211
15	SSR 124	GTATGTGACTGTATGCTTC	GCATTGCATTTCACAAACC	52	174
16	SSR 130	CCACGTATGTGACTGTATG	GAAAGAGAGGCTGAAACTTG	55	196
17	SSR 156	GTACATTGAACAGCATCATC	CAAATGGGCATGAAAGGAG	53	176
18	SSR 167	CACATATGAAGATTGGTCAC	CATTTATGTCTCACACACAC	54	160
19	SSR 199	GTGTGCATGGTGTGTG	CCATCCCCCTCTATC	51	182
20	SSR 213	CACTCGCACCTCTTATG	GAAATTGTCTCTTAGCAAG	51	151
21	SSR 309-2	GTATGTCGTTAACTGTCGTG	GAGGAAGGAAGTATTCGTC	50	182
22	SSR 317-1	GTGGGTGTAATTATTGCTAC	GTATCAAACTTATGGTGAAATC	53	308
23	SSR 317-2	CACGTAACATCTTGCTTATG	GTAGCAATAATTACACCCAC	53	120
24	SSR 323	AGTGACAACAAAATGTGAGT	GTACCTAGTTTCATCATTG	51	250
25	SSR 336	GTGTAACCCAACTGTTCC	GGCCGAGGTTGTAACAC	54	253
26	SSR 183	GCTCGCATTGGTGAAAC	CATATATAGCAGACCGTG	52	119
27	SSR 202	CAACCTCACTTACCTTAC	GCTCTTTATCATCATTCTAC	52	220
28	SSR 28	GAGGGCATAAATTCAGATTC	GGACAACGCACATTTGATG	53	383
29	SSR 72	CAAACAGTACAAGGAAAGGAG	CTGACTGAGCTGCTTGAAC	55	253
30	SSR 230	CCAACAACAATTCACCATAC	AACATTGTACTGAGAGGTG	53	251

2.2. Results and Discussion

2.2.1. Microsatellite Profiling

Thirty polymorphic SSR markers were used to study 185 lentil accessions during this study. On an average 160 alleles per markers and 1 alleles per locus were amplified from 185 lentil accessions from 30 SSR markers. Maximum alleles (178) were amplified from SSR 19, SSR 99, SSR 113, SSR 156 and SSR 202 and minimum alleles (72) from SSR 90 and SSR 207 with amplicon size 180-395. The allelic frequency ranged from 0.4 to 0.98 with an average 0.88. Only 16.6%

markers were able to amplify maximum number of alleles and 6.6% markers gave minimum alleles per locus. SSR 317-1 showed heterozygosity with double alleles per locus which showed that this marker may be made between cross of two related accessions. Similar results were obtained by Salahvarzi et al 2013[13] who reported that based on molecular data,165 bands were detected and 117 bands were polymorph. The mean number of bands was 9.1 bands per primer using ISJ, the fragment size varied withen a significantaly narrower range (150-2500bp). Similarly, Verma et al (2014) [14] also reported that a total of 123 alleles were amplified at 33 loci ranging from 2-5 alleles with an average of 3.73 alleles per locus.

Table 3. Primer's name and their potential to detect the genetic polymorphism in 185 selected lentil accessions.

SN	Marker name	No. Of alleles/locus	Allelic frequency	PIC Value
1	SSR 34-2	73	0.40	0.84
2	SSR 66	176	0.97	0.059
3	SSR 90	72	0.4	0.84
4	SSR132RN	176	0.97	0.059
5	SSR 191	173	0.96	0.078
6	SSR 197	164	0.91	0.17
7	SSR 207	72	0.4	0.84
8	SSR 33	151	0.83	0.31
9	SSR 19	178	0.98	0.039
10	SSR 48	176	0.97	0.059
11	SSR 96	175	0.97	0.059
12	SSR 99	178	0.98	0.039
13	SSR 107	172	0.95	0.097
14	SSR 113	178	0.98	0.039
15	SSR 124	177	0.98	0.039
16	SSR 130	173	0.96	0.078
17	SSR 156	178	0.98	0.039
18	SSR 167	160	0.88	0.22
19	SSR 199	177	0.98	0.039
20	SSR 213	152	0.84	0.29
21	SSR 309-2	168	0.93	0.13
22	SSR 317-1	166	0.92	0.15
23	SSR 317-2	168	0.93	0.13
24	SSR 323	169	0.93	0.13
25	SSR 336	154	0.85	0.27
26	SSR 183	160	0.88	0.22
27	SSR 202	178	0.98	0.039
28	SSR 28	175	0.97	0.059
29	SSR 72	171	0.95	0.097
30	SSR 230	172	0.95	0.097
	Average	160.4	0.886	0.185

2.2.2. Polymorphic Information Content

The polymorphic information content (PIC) value ranged from 0.039 to 0.84 for SSR based 30 selected markers. The PIC highest value was 0.84 for SSR 34-2, SSR 90 and SSR 207 and the lowest value 0.039 was for SSR 19, SSR 99, SSR 113, SSR 124, SSR 156, SSR 199 and SSR 202. Several research showed that markers with PIC value between 0.4 to 0.8 is considered good and informative with high polymorphism [15]. The average PIC value was 0.185 for 30 markers used in this study. Highly informative and detectable polymorphic markers for this study were SSR 34-2, SSR 90 and SSR 207. Similarly, markers SSR 19, SSR 99, SSR 113, SSR 124, SSR 156, SSR 199 and SSR 202 had low PIC value which was less informative. Similar results have been reported by several authors using SSR, RAPD and ISSR profiling in the literature [11, 16]. Hamweih et al. (2009) [11] observed that cultigens accessions are less diverse comparing to wild accessions. Their study revealed that the diversity index for same set of SSR markers ranged from 0.03 to 0.87 with a mean of 0.65 in 109 accessions from 15 countries representing 57 cultigens and breeding lines from eight countries. Unlike their observation on PIC value, the present study revealed that relatively little polymorphism in 180 lentil breeding lines being used in this study. Polymorphism information content (PIC) values for SRAP and AFLP markers were higher than 0.8, indicating the power and higher resolution of those marker systems in detecting molecular diversity [17]. Similiar results were also evidenced bySeyedimoradi and Talebi, 2014 and Kushwaha et al, 2013 [18, 15].

2.2.3. Dendrogram Construction

Dendrogram constructed by highly polymorphic 30 SSR markers from 185 lentil accessions showed ten major groups from Group I to X based on source of origin and their pedigree. Group I was the largest one followed by group II, group IX, group VIII, group VII, group V, group IV and group X. Group III and group VI consisted only one genotypes. Group I was the largest one and it contains lentil genotypes of different source of origin, which can be again divided into different sub groups. Groups III and VI genotypes were totally different from other groups whereas group X genotypes were from Nepal cross lines. This study show the divergency among lentil genotypes which can be further used in lentil breeding programs. The present results based on SSR profiling are much similar to the earlier reports of Fikiru et al. (2010) [19] and Hamweih et al. (2009) [11]. Fikiru et al. (2010) [19] observed five groups of lentil by analysing 70 Ethiopean lentil accessions using set of ISSR markers. Likewise, Babayeva et al. (2009) classified 39 lentil accessions from Central Asia and Caucasian region and found six cluster using five pair of SSR markers. Other research also reported five to nine cluster using SSR markerin diverse lentil accessions. On contrary to this, Alabboud et al. (2009) [16] reported very low level of genetic diversity with only two groups signifies low level of dectective power of RAPD comparing to SSR marker system. Hamwieh et al. (2009) [11] observed the highest genetic diversity in wild and cultivated species using SSR-66 in contrary to the present result reflecting the lentil accessions included in this study are relatively diverse and in most of the accessions displayed heterozygosity for that locus. The heterozygosity in this locus probably due to the

introgression of genes or duplication of microsatellite motif during the breeding and or the course of lentil line evolution. Thus the level of genetic diversity detection largely depends on the type of molecular markers, nature of SSR repeat motif, number of SSR markers and the genetic relatedness of the lentil germplasm to be analysed. All genotypes involved in this study exhibited wide range of genetic variability due to different center of origin, different genetic constitution. The genetic relatedness detected in this study may constitute the foundation for future systematic lentil breeding programs.

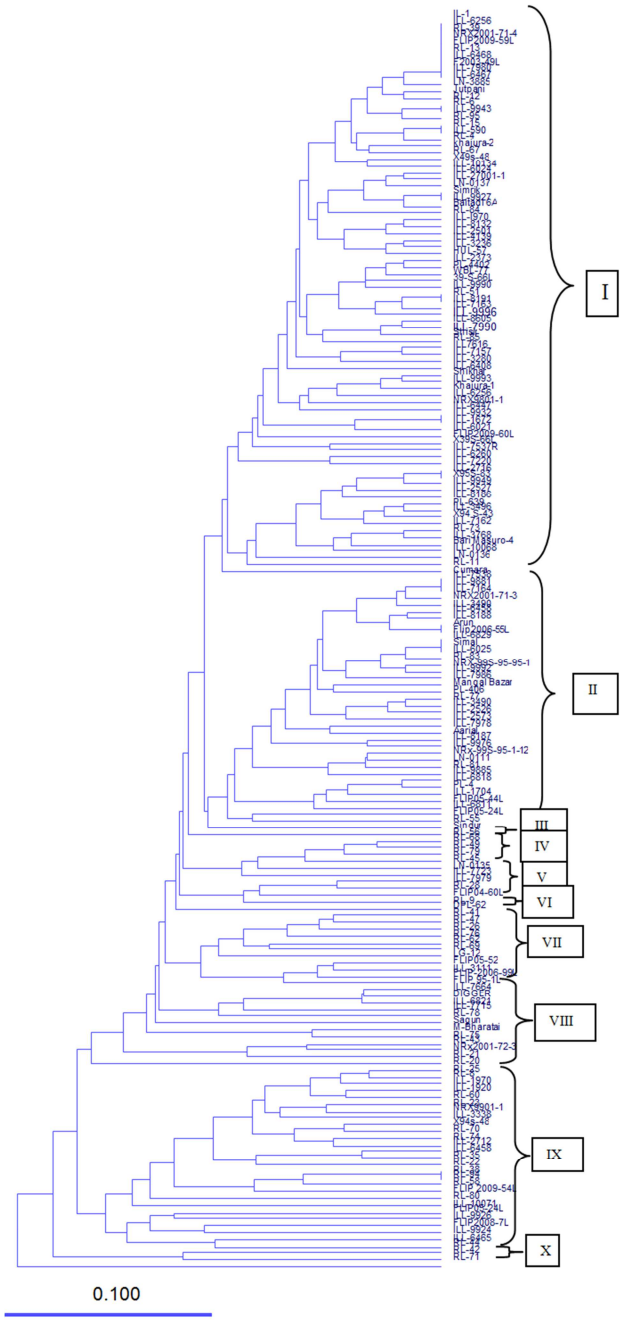

Figure 1. Dendrogram showing genetic diversity of lentil germplasm constructed by 30 selected polymorphic SSR markers from 185 lentil accessions collected from different geographical regions. This figure accession numbers and lentil full names are shown in table 1.

3. Conclusion

Microsatellite profiling revealed maximum alleles were amplified from SSR 19, SSR 99, SSR 113, SSR 156 and SSR 202 with amplicon size 180-395. Highly informative and detectable polymorphic markers for this study were SSR 34-2, SSR 90 and SSR 207 which indicate the power and higher resolution of those marker systems in detecting molecular diversity. In the same way, dendrogram constructed by highly polymorphic 30 SSR markers from 185 lentil accessions showed ten major groups from Group I to X based on source of origin and their pedigre. Groups III and VI genotypes were totally diffrent from other groups whereas group X genotypes were from Nepal cross lines. This study show the divergency among lentil genotypes which can be further used in lentil breeding programs. The level of genetic relatedness largely depends on the type of molecular markers used in the study, nature of SSR repeat motif, number of SSR markers and the genetic relatedness of the lentil germplasm to be analysed. All genotypes involved in this study exhibited wide range ofgenetic variability due to different center of origin, different genetic constitution. The genetic relatedness detected in this study may constitute the foundation for future systematic lentil breeding programs.

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