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DNA fingerprinting and genetic diversity in Lentil Germplasm using SSR markers

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ABSTRACT

Molecular markers are useful tools for evaluating genetic diversity and DNA fingerprinting. The purpose of this study was to evaluate the genetic diversity within lentil germplasms microsatellite markers. The observed variability from morphological and SSR analysis among 110 lentil accessions is important for varietal improvement in Bangladesh. However, such information is not available for Bangladesh lentils. The genetic diversity in 121 lentil accessions comprising landraces, modern popular cultivars, selected advanced lines and phenologically adapted exotic accessions was assayed by nine primer combinations. A total of 104 alleles were detected across all microsatellite loci and the number of alleles per locus ranged from 4 (SSR340) to 22(SSR33), with an average of 11.56 alleles. The frequency of the most common allele at each locus ranged from 12.50% (SSR33) to 86.49% (SSR340). On an average, 44.21 % of the 121 lentil accessions shared a common major allele at any given locus. The genetic diversity varied from 0.24 (locus SSR 340) to 0.93 (locus SSR33) with a mean of 0.69. Polymorphism Information Content (PIC) values ranged from 0.23 to 0.93 with an average of 0.66. Cluster analysis based on SSR amplification products grouped genotypes into three clusters with 54, 40 and 27 accessions each and substantial association between molecular diversity and origin was evident.

Key words: Fingerprinting, Lentil, diversity, germplasm

INTRODUCTION

Lentil (*Lens culinaris* ssp. c*ulinaris*) is a diploid (2n=2x=14), autogamous species and is one of the oldest crops in the world, which originated in the Near East. Bangladesh is one of the major lentil producing countries of the South Asia with 77,330 hectares of land and 71,000 tones of production (BBS, 2010). Domestic pulse production is much less than the country's need which only satisfies about 30% of the country's demand. The rest, some 140,000 tons, is imported at a cost of about US$32.2 million per annum (Sarker *et al.,* 2004). Moreover, the area and production of lentil has been continuously declined over the recent past and its cultivation is mainly confined to marginal land. Because farmers are eager to replace lentil with more remunerative crops like wheat, boro rice and winter vegetables. Lentils are genetically low yield potential; sensitive to inputs, diseases as well as yield is instable. Evidence of the paucity of variation within Indian germplasm comes from a study of the sensitivity of a world lentil collection to temperature and photoperiod (Erskine *et al*., 1994). Several important traits, such as biomass yield, pod shedding, nitrogen fixation and susceptibility to aphids cannot be addressed by breeding because of insufficient genetic variation. Thus lentil improvement programmes in South Asia is being handicapped due to poor access to sufficient genetic variability (Sarker and Erskine, 2006).

There is not much information available on the diversity of lentil germplasm of Bangladesh. Extensive collections of lentil germplasm now exist in various gene banks around the world including ICARDA. This germplasm including wild *Lens* species has been used in plant introduction strategies and in efforts to widen the potential sources of increasing genetic diversity in the breeding programmes of lentil. Improved techniques are emerging to overcome hybridization barriers between species and as a result, interspecific hybrids have been successfully obtained between species. Several interspecific recombinant inbred line populations have been developed. Selected and backcrossed lentil lines are currently in advanced yield trial stages, and desirable traits such as yield, disease resistance and agronomic traits have been incorporated into cultivated lentil especially from *Lens ervoides*, generating a wider spectrum of variability. Further expansion of the overall pool of germplasm and examination of allelic variation at the nucleotide level will benefit lentil-breeding programmes by augmenting phenotype-based variation to further advance cultivar development Knowledge of genetic diversity among existing cultivars of any crop is essential for long-term success in breeding programme and to maximize the exploitation of the germplasm resources (Belaj *et al;* 2002). Particular combination of characters required is often not found and in such cases known diversity for that particular character is needed

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to generate desired character. Broadening of genetic base and systematic exploitation of heterosis in cultivated lentil requires reliable information on genetic diversity in the germplasm. Viewing this in mind assessment of genetic diversity at molecular level among 121 lentil accessions was carried out using SSR markers. On the other hand, Simple Sequence Repeats (SSR) can presently be short motif nucleotides. DNA fingerprinting using SSR markers is playing an important role to identify gene for tolerance. They have become a popular type of co-dominant molecular marker in genetic analysis and plant breeding application and also been useful in integrating genetics, physical and sequence based maps of rice and provides breeders and geneticists with efficient tool to link phenotypic and genotypic variations.

MATERIALS AND METHODS

The experiment was carried out at ICARDA, Aleppo, Syria. and used to analyze the molecular characteristics and relationships among them**.** The present study was comprised of 121 landraces, modern varieties, phenologically adapted exotic lines of lentil (*Lens culinaris* Med.) of diverse origin listed in Table 1. The seventy accessions were selected from the collection of ICARDA germplasm bank and the others were provided by BINA and BARI included released popular cultivars and selected advanced lines. The material was subjected to molecular evaluations for determining their DNA based diversity using SSR primers and DNA was extracted from four weeks young leaves of lentil seedlings grown in the plastic house of ICARDA, Aleppo, Syria.

DNA extraction

Genomic DNA was extracted using modified CTAB procedure. The concentration of extracted DNA was estimated by DNA confirmation test by (1 %) Agarose gel electrophoresis with lamda DNA and PCR analysis for microsatellite markers.

Table 1. Source and country of origin of 121 lentil accessions used in the experiments

Accessions	Source of collection	Origin/Developed
ILL 1922, ILL 4605, ILL 8109 and ILL 8108, ILL 8107	ICARDA, Syria	Argentina
ILL 5888, ILL 8147, 8406-122, 955-167, and 40-50134-5	ICARDA, Syria	Bangladesh
ILL 1712 and ILL 2501, ILL 1959, ILL 2032, ILL 2069	ICARDA, Syria	Ethiopia
ILL 9995, ILL 10020, ILL 10066, ILL 10067, ILL 10068, ILL 10069, ILL 10070, ILL 10071, ILL 10072, ILL 10073 and ILL 10077, ILL 6994 and ILL 6994	ICARDA, Syria	ICARDA
ILL 2532, ILL 2581, ILL 2815, ILL 3312, ILL 3597, ILL 4147, ILL 7556, ILL 2493, ILL 7558, ILL 7715, ILL 3614, ILL 2684 and ILL 2580, ILL 8008, ILL 2565	ICARDA, Syria	India
ILL 8009, ILL 7253, ILL 3485 , ILL 4611 and ILL 8010	ICARDA, Syria	Nepal
ILL 4402, ILL 7163, ILL 7164, ILL 8114, ILL 88527, ILL 91517 and ILL 9836	ICARDA, Syria	Pakistan
BINA-2, BINA-3, N1I- 424, N1M-134, N1M-149, N2M-119, N2M-214, N2M -715, N4M-402, N4M-423, N4M-433, N5M-507, N5M-338, N5M-564, E1M-606, E1M-617, E4M-941, E5M-229, E5M-501 and N5M-573	BINA, Bangladesh	Bangladesh
BARI-1, BARI-3, BARI-6, BLx98002-3, ILLx87040, Lx98002-4, BLx98004-3, BLx98006-3, BLx98008,10741-87012	BARI, Bangladesh	Bangladesh
ILL 4703, ILL 5072, ILL 5098, ILL 5102, ILL 5108, ILL 5143, ILL 6305, ILL 7656	BARI, Bangladesh	ICARDA
ILL 2460, ILL 2475, ILL 2493, ILL 2507, ILL 2527, ILL 5113, DPL-44, 128xE28,	BARI, Bangladesh	India
BARI-2 and BARI-4	BARI, Bangladesh	Nepal
ILL 6308	BARI, Bangladesh	Pakistan
P114E14-136	BARI, Bangladesh	USA
ILL 4400, ILL 4401	ICARDA, Syria	Syria
ILL 759, ILL 1051, ILL 1106, ILL 2392	ICARDA, Syria	Iran
ILL 96, ILL 1936, ILL 4788	ICARDA, Syria	Morocco
ILL 1878, ILL 590, ILL 5604	ICARDA, Syria	Turkey
ILL 465, ILL 975, ILL 1005, ILL 1828	ICARDA, Syria	Chile
ILL 5883	ICARDA, Syria	Jordan

Note: USA= the United States of America, Bangladesh accessions were developed either mutation or hybridization at BINA or BARI

Table 2. Sequences, motif, locus name and nature of inheritance of the twenty primers used Hamwieh *et al.* (2005)

Primer	Primer sequence (5 – 3)	motif	Locus name	Nature of inheritance
SSR336	GTGTAACCCAACTGTTCC GGCCGAGGTTGTAACAC	$(TAA)_6AGA(TAA)_4$	SSR336	Co-dominant
SSR323	AGTGACAACAAAATGTGAGT	$(AT)_{22}(CA)_4$	SSR323	Co-dominant
SSR309-2	GTATGTCGTTAACTGTCGTG	(AT)3GT(TA)3T(TAT)6	SSR309-2	Co-dominant
SSR302	CAAGCCACCCATACACC GGGCATTAAGTGTGCTGG	$(TA)_{15}(CA)_{11}$	SSR302A SSR302B	Co-dominant Dominant
SSR213	CACTCGCACCTCTTATG	$(TA)_8((TG)_5$ $TA(GT)_5$	SSR213	Co-dominant
SSR204	CACGACTATCCCACTTG CTTACTTTCTTAGTGCTATTAC	$(TG)4 +(AC)^b_7$	SSR204	Co-dominant
SSR199	GTGTGCATGGTGTGTG CCATCCCCCTCTATC	$(GT)_4GC(GT)_8GC(GT)_3$	SSR199A SSR199B	Co-dominant Dominant
SSR167	CACATATGAAGATTGGTCAC	$(TA)_{16}(TG)_{21}$	SSR167	Co-dominant
SSR156	GTACATTGAACAGCATCATC	$(TC)_2(TG)_{13}$	SSR156	Co-dominant
SSR130	CCACGTATGTGACTGTATG GAAAGAGAGGCTGAAACTTG	$(GT)_9$	SSR130	Co-dominant
SSR119	GAACTCAGTTTCTCATTG GAACATATCCAATTATCATC	(TA)TT(TA)11(TG)19	SSR119	Co-dominant
SSR113	CCGTAAGAATTAGGTGTC GGAAAATAGGGTGGAAAG	$(AC)_{17}(AT)_{13}$	SSR113	Co-dominant
SSR99	GGGAATTTGTGGAGGGAAG CCTCAGAATGTCCCTGTC	$(TG)_8TC(TG)_2$	SSR99	Co-dominant
SSR80	CCATGCATACGTGAACTGC GTTGACTGTTGGTGTAAGTG	$(TC)_{14}(AC)_{12}(AT)_2$	SSR80	Co-dominant
SSR48	CATGGTGGAATAGTGATGGC CTCCATACACCACTCATTCAC	$(TG)_{13}$	SSR48	Co-dominant
SSR33	CAAGCATGACGCCTATGAAG CTTTCACTCACTCAACTCTC	$(CA)_{21}(GA)_{25}$	SSR33	Co-dominant
SSR19	GACTCATACTTTGTTCTTAGCAG GAACGGAGCGGTCACATTAG	$(TG)_{14}$	SSR19	Dominant

Primer design and PCR amplification of microsatellites

Primer pairs were designed close to the microsatellite repeats in flanking regions as described by Hamwieh *et al*. (2005). PCR amplification reactions of microsatellites were performed in a final volume of 20 µl in presence of 10ng of template DNA, 10 pmol of each primer, 0.2 mM of each deoxynucleotide, 1xPCR buffer (Invitrongen, Carlsbad, CA), and 1 unit Taq polymerage (Invitrogen). The forward primer was 5'-labeled with one of three florophores (6FAM, NED, or TET). PCR was carried out using a PE 9600 thermo cycler (Perkn-elmer, Foster City,CA). After 3 minutes at 94ºC, 30 cycles were performed with 30 s at 94º C, 30 s at either 52ºC, 53ºC, 54º C or 55º C (depending on the locus), and 1 min at 72º C, followed by final extension step of 5 min at 72º C. Amplified products were detected on a MegaBACE 500 Capillary System (Amersham Pharmacia Biotech, Piscataway, NJ). Samples were prepared by adding 1µl of diluted PCR products to 9µl formamide and samples included 1% (v/v) ET-Rox900 bp DNA size standard (Amersham Bioscience). Microsatellite fragment sizes were estimated using the MageBACE Genetic Profile version 2.0 (Amersham Pharmacia Biotech).

Electrophoresis of the amplified products and documentation

The amplified products were separated electrophoretically on 1.5% agarose gel. Then the PCR products were mixed with 5 µl of 10X gel loading dye. Eight µl of the mixture was loaded slowly per well on the gel. The molecular weight marker (1kb+ DNA ladder) was loaded at the first well on the gel. Agarose gel electrophoresis was conducted in 0.5X TBE buffer at 120 V for 1 hr. The separation process was monitored by the migration of the dyes in the loading buffer. After electrophoresis, the gel was taken out carefully from the gel chamber and transferred in a prepared ethidium bromide solution for staining and placed on the UV transilluminator in the dark chamber of the Gel Documentation System to observe DNA and photographed by a Gel Cam Polaroid camera.

Data analysis

Analysis of microsatellite diversity was conducted at locus level in lentil accessions. For each locus, we estimated the number of alleles, range of fragment sizes, mean of fragment size and gene diversity using power marker V3.25. The genetic relationship was further estimated at accession level with 100 bootstrap values using the unweighted pair-group method with arithmetic average (UPGMA) and neighbor-joining methods (PAST software version 1.62.

Polymorphic information content (PIC) was used to measure the relative value of each accession with respect to the amount of polymorphism it exhibits. Genetic diversity measure of each accession was calculated based on (Botstein *et al.,* 1980):

$$PIC= 1- \sum X_i^{\ 2}$$

X_i is the proportion of the genotypes carrying i^{th} allele, calculated for each microsatellite locus.

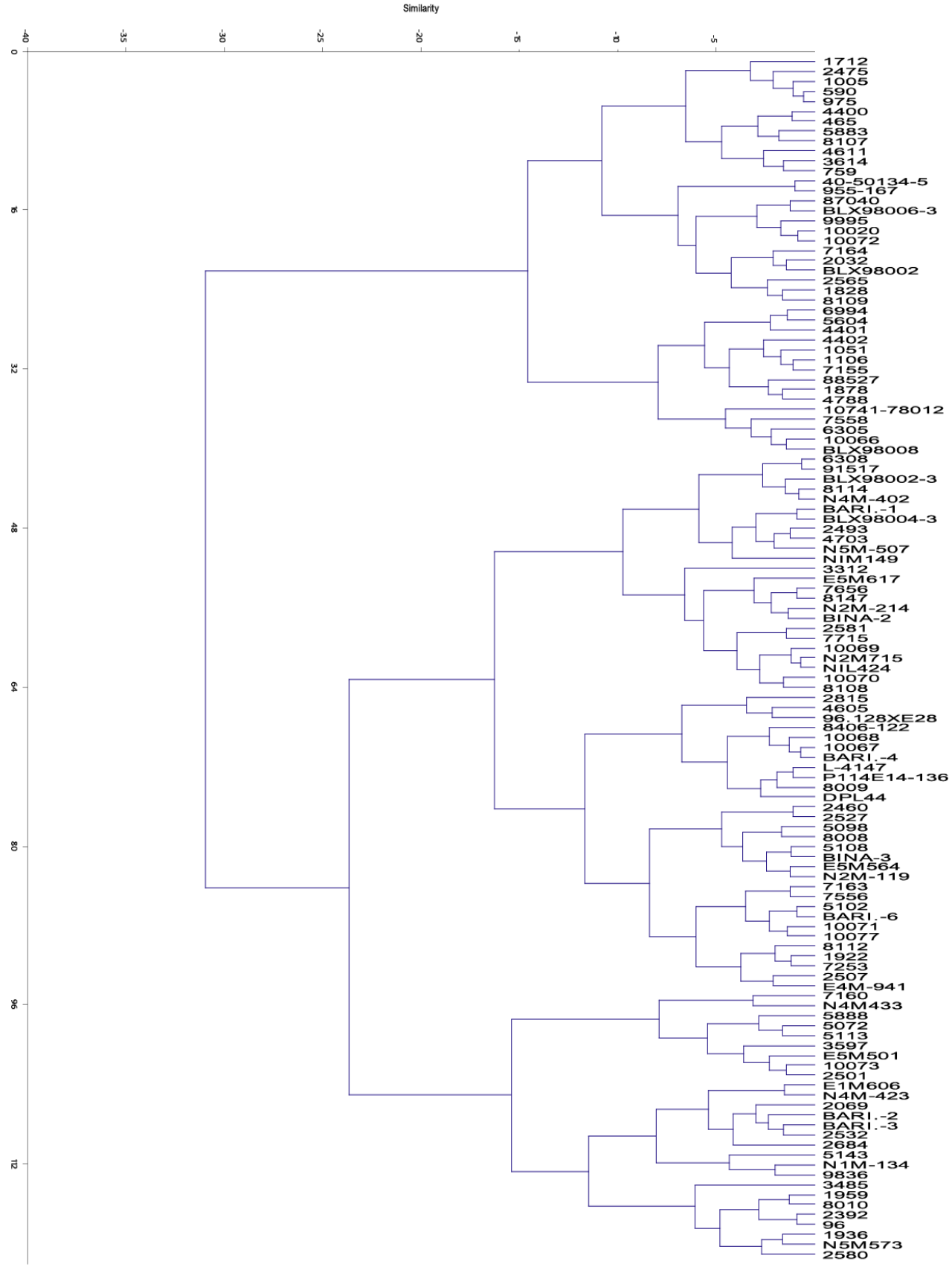

Figure 2. A UPGMA cluster dendogram showing the genetic relationships among 110 lentil accessions based on alleles detected by microsatellite markers (SSRs)

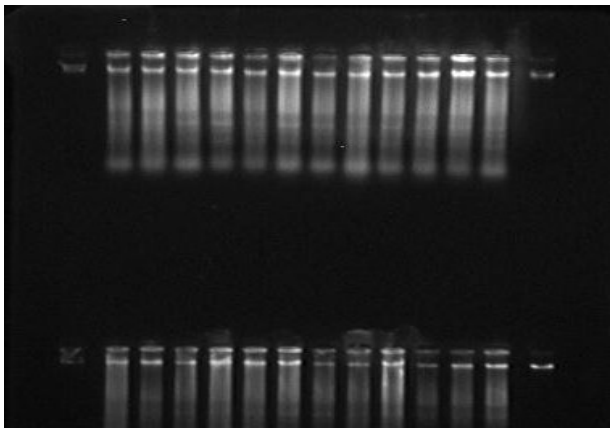

Fig. 1a

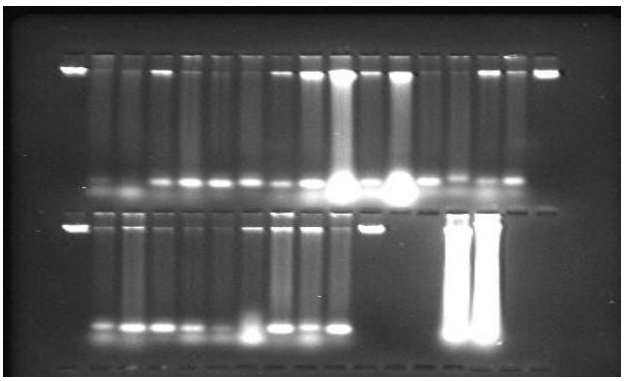

Fig. 1b

Figure 1 (a &b). Electrophoregram of ethidium bromide stained genomic DNA samples of 47 different lentil accessions for confirmation of DNA. Lane M: Molecular weight marker (λ DNA)

RESULTS AND DISCUSSION

A total of 121 accessions of lentil were used to produce molecular data for diversity study and 9 microsatellite markers (SSRs) were used for amplification of genomic DNA. Morphological and phenological studies have revealed relevant variations in lentil population by many researchers whereas few studies have evaluated genetic variation at molecular level (Sonnante and Pignone, 2001). Morphological characters, limited in number, often do not reliably portray genetic relationships because of environmental interactions, epistatic interactions and the largely unknown genetic control of the traits (Smith and Smith, 1989). Morphological and phenological traits are often controlled by multiple genes and are subjected to the action of environmental factors and differences between closely related species are not always absolute (Ahmad *et al,* 1996). Genetic markers such as simple sequence repeats (SSR) represent genetic variation at the DNA level allowing the estimation of the degree of relatedness between individuals without the influence of environmental variation. Quantification of genetic variation between individuals could enhance the level of variation in breeding population.

The genetic similarity analysis using UPGMA clustering system (Fig. 2) generated three clusters. Cluster B was the largest and included 54 accessions followed by cluster A with 40 accessions and cluster C comprising of 27 accessions. These clusters could be subdivided. Cluster A could be subdivided into three sub-clusters as A1, A2 and A3. These three sub-clusters contained 12, 13 and 15 accessions, respectively. All the 40 accessions belonged to the cluster A were provided by ICARDA. The first sub-cluster presented by 12 accessions was majority of Turkey, Iran, Jordan and Syria origin. There inclusion in the same cluster is very justified. Major accessions of sub-cluster A2 belonged to ICARDA and Ethiopia. Similarly, accessions of A3 sub-clusters were originated from ICARDA, Turkey, Syria, Iran and Jordan. So the inclusion of the 15 accession of close origin in the same cluster is very much justified.

Cluster B could be subdivided into five sub cluster B1, B2, B3, B4 and B5. All the accessions of sub cluster B1 originated from India, Nepal and Pakistan. Some accessions of B2 sub cluster were from Bangladesh, some were from India, Argentina and ICARDA. Accessions of sub cluster B3 originated from Bangladesh, India, Pakistan, Nepal, Argentina and ICARDA. All the accessions of sub-cluster B4 originated from India. Sub-cluster B5 comprised of 11 accessions. Of them two originated from Bangladesh, three were from India, two was from Pakistan, two were from ICARDA and the remaining two were from Nepal and Argentina, respectively. Most of the accessions of this cluster originated from diverse origins which justify existence of diversity among lentil accessions.

Cluster C could be subdivided into three sub clusters. Sub cluster C1 comprised of nine accessions. Out of these nine accessions most of them were from Bangladesh and India. Similarly, most of the accessions of sub cluster C2 were from Bangladesh and India. All eight accessions of sub cluster C3 originated from diverse origin as two from Nepal, two from Morocco, one from Bangladesh, one from India, one from Ethiopia and one from Iran. Most of the lines of this cluster originated from diverse origins which justify existence of diversity among lentil accessions of this investigation. Similar results were reported by Babayeva *et al.* (2009) and they found that cluster analysis using the unweighted pair group method with arithmetic mean classified accessions into six major groups.

One hundred and four alleles were detected at the loci of nine microsatellite markers across 121 lentil accessions. The number of alleles per locus ranged from 4 (SSR340) to 22(SSR33), with an average of 11.56 alleles across the nine loci (Table 3). The frequency of the most common allele at each locus ranged from 12.50% (SSR33) to 86.49% (SSR340). On an average, 44.21 % of the 110 lentil accessions shared a common major allele at any given locus. Polymorphism Information Content (PIC) values ranged from 0.23 to 0.93 with an average of 0.66. Babayeva *et al.* (2009) found 33 alleles determined ranging from 3-8 per locus estimated gene diversity value for 33 loci was 0.66 in lentil. Genetic similarity indices among 39 accessions ranged from 0.24 to 1.0. The observed variability from SSR analysis among 110 lentil accessions is important for varietal improvement in Bangladesh.

Genotypic variations based on molecular characterization indicated that genotypes belonging to

Table 3. Data on major allele frequency, genotype number, allele number, gene diversity, heterozygosity and polymorphic information content (PIC) found among 110 lentil accessions for nine microsatellite markers

Marker	Major Allele Frequency	Geno-type No	Sample Size	No. of obs.	Allele No	Gene Diver-sity	Heterozy-gosis	PIC
SSR33	0.1250	21.0000	124.0000	48.0000	22.0000	0.9355	0.0625	0.9319
SSR80	0.5281	15.0000	124.0000	89.0000	15.0000	0.6944	0.0337	0.6789
SSR99	0.4494	11.0000	124.0000	89.0000	7.0000	0.6243	0.4045	0.5522
SSR130	0.4798	5.0000	124.0000	99.0000	5.0000	0.5929	0.0101	0.5097
SSR156	0.2500	12.0000	124.0000	36.0000	12.0000	0.8673	0.0000	0.8546
SSR213	0.2444	20.0000	124.0000	90.0000	18.0000	0.8467	0.2000	0.8306
SSR317	0.5238	12.0000	124.0000	84.0000	12.0000	0.6898	0.0119	0.6693
SSR340	0.8649	5.0000	124.0000	74.0000	4.0000	0.2451	0.0811	0.2347
SSR19	0.5139	9.0000	124.0000	72.0000	9.0000	0.6736	0.0000	0.6393
Mean	0.4421	12.2222	124.0000	75.6667	11.5556	0.6855	0.0893	0.6557

different clusters depend on their genetic components itself, but not at geographical origin at all. Therefore, it could be concluded that for further research program, especially for hybridization, genotype could be selected from different clusters will be provided maximum heterosis regarding yield.

CONCLUSION

In conclusion, SSR markers have been provide to be powerful tools for molecular genetic analysis of rice cultivars for plant breeding programme to assess genetic diversity available to allow for the production of new varieties that are aimed towards the improvement of crop productivity and able to withstand from biotic and abiotic factors.

Divergence between any two parents expresses the allelic differences between them. The accessions grouped into the same cluster presumably diverge very little from one another. Crossing of accessions belonging to the same cluster is not expected to yield desirable sergeants. Therefore, crosses between the members of clusters separated by inter-cluster distances are likely seemed to be beneficial for further improvement. Significant differences among the accessions for different characters indicated variations for their use in the breeding programme. Crosses between parents with maximum divergence would be more responsive to improvement since they are likely to produce higher heterosis and desirable genetic recombination.

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