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GENOTYPING OF A NEW STRAIN OF LENTIL (*LENS CULINARIS* MEDIK.) BY DNA FINGERPRINTING

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Abstract

The objective of the present study was to find out the extent of genetic diversity among the 4 lentil cultivars viz., Masur-85, Masur-93, an exotic variety Precos (introduced in NWFP, Pakistan as Mansehra-89) and a newly released strain NIAB-Lentil. Random amplified polymorphic DNA (RAPD) analysis was used to evaluate the genetic diversity among the four lentil cultivars which were analysed with 20 random decamer primers. Out of 20, thirteen primers detected polymorphism in all varieties and six primers amplified the monomorphic DNA bands only a single primer could not amplify the genomic DNA of all the varieties. Similarity matrix was obtained after multivariant analysis using Nei and Li's coefficients. The coefficients of similarity matrix were used to construct dendrogram by the unweighted pair group method of arithmetic means (UPGMA). Three varieties viz., Masur-85, Masur-93 and NIAB-Lentil can be clustered in one group and the Precos in the second group. The similarity between the exotic variety Precos and the other three viz., NIAB-Lentil, Masur-93 and Masur-85 ranges from 45.15 to 64.25%. However, the similarity between the local adapted cultivars ranges from 77.88 to 86.36%. The analysis revealed that the inter-varietal genetic relationships of the varieties are related to their place of origin. It is therefore, proposed that the local varieties have narrow genetic base. The information collected can be utilised to map novel genes for different traits. Moreover, the information will provide a guideline to draw future breeding strategies and also help to enforce the intellectual breeders rights in the country.

Introduction

Lentil (*Lens culinaris* Medik.) an important pulse crop is cultivated over 3.21 million hectares in the subtropical as well as in temperate regions of the world with an annual production of 2.56 million tonnes (Bahl *et al.*, 1993). Lentil is the second most important pulse crop of Pakistan and contributing 30,000 t grain (Malik *et al.*, 1988). It is the best source of protein after meat. Most of the lentil cultivars in Pakistan have been developed through hybridization followed by selection of superior genotypes sharing the common gene pool of local land races. Hence, it results in narrow genetic base of the varieties. The varieties with narrow genetic could not provide any buffer against the outbreak of any natural disasters.

Similarly, the evolution of so many cultivars, varieties, strains and germplasm, makes it difficult even for breeders to identify a variety on the basis of morphological as well as physiological features of crop plants. These features based on visual observations are not true representative of the total genetic make up of the plants as it has been very well documented that many traits are polygenic in nature, which are susceptible to environmental conditions. The morphological traits are indicative of a genotype but only at few loci. A large number of polymorphic markers are required to estimate genetic relationships and genetic diversity to tag any variety.

With the advances in the field of molecular biology, it is possible to see the differences among the crop plants at molecular level. Molecular markers such as isozymes and restriction fragment length polymorphisms (RFLPs) have been extensively used for genetic studies and plant identification. Isozymes are biochemical markers, have been used to differentiate homozygous individuals from heterozygous and to estimate the extent of genetic variability in plant populations (Melchinger, 1990). Stuber (1992) also used isozyme markers to identify QTLs in maize, tomato and soybean. However, isozymes are available only in small number. RFLP is a powerful technique, generates large number of markers and have been used extensively for mapping and genetic studies (Wang *et al.*, 1995) but the analysis is time consuming, requires a large quantity of DNA (02 to 10 ug). Additionally, the risk to use radioactivity is also involved.

With the advent of polymerase chain reaction (PCR) technology, it has made it possible to study the genetic differences in plants and animals. DNA fingerprinting, gene mapping and phylogenetic studies have tremendously benefited from PCR. One variation of PCR is the random amplified polymorphic DNA (RAPD), which generates DNA fingerprints with a single synthetic oligo-nucleotide primer (Williams *et al.*, 1990). RAPDs are inherited in a simple Mendelian fashion and are usually dominant markers. Gene mapping using RAPD markers have several advantages over RFLPs. RAPD procedure is less expensive, faster, requires small amount of DNA (0.5 to 50ng) and does not involve the radioisotopes. Due to these advantages, RAPDs have proven useful in genotype identification and gene mapping (Robert *et al.*, 1999; Swantson *et al.*, 1999). The technique has been successfully used for the estimation of genetic similarities and cultivar analysis of various plant species including rice (Yu & Nguyen, 1994; Mackill 1995), *Brassica* (Jain *et al.*, 1994) and *Lycopersicon* (Williams & St-Clair, 1993). Iqbal *et al.*, (1997) have used RAPD analysis for DNA fingerprinting of different cotton varieties in Pakistan. Multani & Lyon (1995) studied 114 Australian cotton varieties by RAPD markers using silver staining and demonstrated that very closely related varieties can also be distinguished. The genetic distance obtained from RAPD markers of 16 elite US cotton varieties were compared with the taxonomic distances measured from morphological characters. The classification of varieties based on the two methods produced similar results (Tatineni *et al.*, 1996).

The objective of the present study was to find out genetic diversity and genetic relationship among the four varieties of lentil. The DNA fingerprinting study will provide an additional proof for the varieties under study. Moreover, DNA fingerprints will provide documentary evidence of breeder rights for a particular variety.

Material and Methods

Seeds of four lentil varieties viz., Masur-85 (male parent), Masur-93, Precos (female parent, introduced from Argentina) and NIAB-Lentil (candidate variety). The Precos was released for general cultivation in NWFP, Pakistan and now recognised as Mansehra-89. The seeds of the lentil genotypes were supplied by Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad. Plants were developed in pots and supplied with optimum amount of water and nutrition through Hoagland solution.

DNA Extraction: After two week of germination, the leaf samples were collected in liquid nitrogen for DNA extraction. DNA was extracted from the leaves by a method proposed by

Iqbal *et al.*, (1997). Concentration of the DNA was measured by spectrophotometer. Absorbance ratio of the extracted genomic DNA at 260 nm and 280 nm was 1.76. Quality of DNA was checked by running 25 ng DNA on an agarose gel. The DNA samples giving smear in the gel were rejected.

Condition Optimisation: DNA concentration in the working solution of approximately 2ng/ul in ddH_2O was confirmed by spectyrophotometric analysis at 260 nm. For RAPD analysis, concentration of the genomic DNA of all the varieties, 10 X buffer without $MgCl_2$, $MgCl_2$, dNTPs (dATP, dCTP, dGTP, dTTP), 10-mer random primer, and Taq DNA polymerase were optimised, respectively. 10-base oligonucleotide primers were used from Operon Technologies, Inc. USA. Taq polymerase, together with 10 X PCR buffer, $MgCl_2$, dNTPs and gelatin were of Perkin Elmer.

PCR was performed in volumes of 25 ul containing 10mM Tris-Cl (pH 8.3), 50mM KCl, 3mM $MgCl_2$, 100uM each of dATP, dCTP, dGTP, dTTP, 0.2uM primer, 5 ng of genomic DNA, and 1 unit of Taq polymerase. A total of 20 primers were used for the analysis. Amplification was performed in Perkin Elmer DNA thermal cycler 480 programmed for 40 cycles of 1 minute at 94°C, 1 minute at 36°C, 2 minutes at 72°C, using the fastest available transitions between each temperature regime.

Scoring and Analysis of RAPD Data: Amplification products were analysed by electrophoresis in 1.2% agarose gel and detected by staining with ethidium bromide (10 ng/100 ml of agarose solution in TBE), examined under ultra-violet transilluminator and photographed using the Stratagene Eagle Eye still video system. All visible and unambiguously scorable fragments amplified by primers were scored under the heading of total scorable fragments.

Analysis of amplification profiles: Amplification profile of four lentil varieties was compared with each other and bands of DNA fragments were scored as present (1) or absent (0). The data of the primers were used to estimate the similarity on the basis of number of shared amplification products (Nei & Li, 1979). Using the unweighted pair group method of arithmetic means (UPGMA) generated a dendrogram based on similarity coefficients.

Results and Discussion

A total of 20 primers of 10-mer oligonucleotide were used in PCR. Out of 20 primers, 19 primers amplified the genomic DNA in all the varieties, 13 primers amplified polymorphic DNA bands among all the varieties and 6 produced similar banding pattern. The DNA bands amplified were in the range 250 to 3000 bp (Fig. 1). Approximately 26% of the total reactions could not amplify genomic DNA. Contamination in a reaction mixture may cause primer degeneration that results into complete failure of amplification. Such kind of contamination can be avoided by preparing the reaction mixture in a sterile laminar air flow cabinet and by the careful sterilization of distilled water and eppendorf tubes (Malik, 1995). Occasionally a smear was observed. However, the cause of DNA smear is difficult to explain. The amount of DNA used in the amplification reaction (5ng) is so small that it can not be visible on a gel unless it is amplified (Malik, 1995).

Fig. 1. Amplification profile of 4 lentil genotypes with primers OPJ-11, OPJ-12, OPJ-13 and OPJ-14. M= kb ladder, A= Precos/Mansehra-89, B= Masur-85, C= Masur-93, D= NIAB-Lentil.

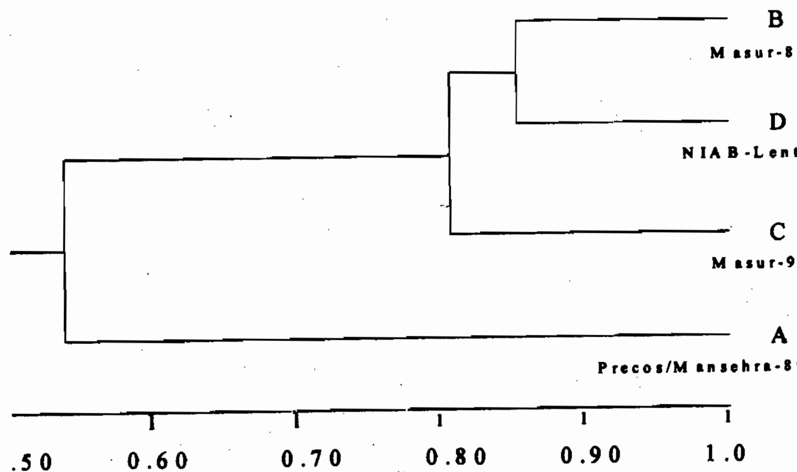

Fig. 2. A dendrogram of four lentil varieties.

In the PCRs, few primers amplified faint DNA bands and few were very bright. The extremely bright bands may be produced by the amplification of high copy number sequences in the genomic DNA. Consequently, large number of fragments will be produced from high copy number sequences than those with low copy number sequences. But sometimes, the bright bands may be subdivided in two fragments when agarose gel of high concentration (2.5%) was used (Malik, 1995). Faint bands may be produced due to mismatch of primers at complementary sites, ultimately the primer can not sit properly resulting in a poor amplification of the genomic DNA region. So these fragments will be quantitatively less, resulting in faint bands (Williams *et al.*, 1990).

The level of DNA polymorphism was different with different primers. After scoring the results, similarity matrix was developed after multivariant analysis using Nei & Li's (1979) coefficients (Table 1). The similarity coefficients were used to develop a dendrogram (Fig. 2) by UPGMA analysis in order to determine the clustering of the varieties.

Table 1. Similarity Matrix for Nei and Li's coefficient of four varieties obtained from RAPD analysis.

Variety	Precos/Mansehra-89	Masur-85	Masur-93	NIAB-Lentil
Precos/ Mansehra-89	1			
Masur-85	0.6429	1		
Masur-93	0.5107	0.7788	1	
NIAB-Lentil	0.4519	0.8636	0.8523	1

The exotic parent, Precos/Mansehra-89 is the least similar variety. It is genetically 45.19% similar with NIAB-lentil (a candidate variety), 64.29% with Masur-85 and 51.07% with Masur-93. The low similarity of Precos/ Mansehra-89 with the other varieties is due to the fact that Precos/Mansehra-89 is an exotic variety, possessing diverse blood and thus revealed different genetic make up at many loci from the local varieties. The NIAB-Lentil is 86.36% similar with Masur-85, which is a male parent. Hence, it is evident from the results that the plants were selected from the segregating population that was genetically very close to Masur-85. Similarly, the candidate variety (NIAB-Lentil) is 85.23% similar to Masur-93. Moreover, Masur-85 and Masur-93 are 77.88% similar. That is why in dendrogram, these three local varieties clustered in one group (A) and the exotic variety the second group (B). The close genetic kinship among these three varieties is due to the fact that the varieties have been developed by mixing the blood of land races or their derivatives with the exotic germplasm followed by selection to recover the characters of the local race. Thus, the selection of desirable segregating genotypes similar to the local adapted race brings these cultivars genetically close. Tatineni *et al.*, (1996) compared the genetic distance of 19 cotton genotypes obtained from RAPD data with taxonomic data. In their analysis 33.8% primers did not reveal any polymorphism. In our studies, 30% primers amplified monomorphic DNA bands. These results are very close to the findings of Tatineni *et al.*, (1996). It has also been concluded that the local lentil varieties are genetically very close and different from each other at few loci.

In the light of these findings it may be suggested that breeders are working with very narrow genetic base to tailor lentil cultivars. The results of our findings are indicative of their genetic relationship. The genetic similarities obtained from the analysis can be exploited for the selection of parents to raise a mapping population. Moreover, it will also help a lot to choose parents for future breeding program.

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