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RESEARCH ARTICLE

ESTIMATION OF GENETIC DIVERSITY IN LENTIL (*LENS CULINARIS*) USING PROTEIN PROFILING AND RAPD

Sheikh, G.N., Showkat Ahmad and Rajdeep Kudesia

Institute of Basic Science, Department of Botany, Bundelkhand University , Jhansi(U.P)-284128, India

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ABSTRACT

Electrophoretic SDS-PAGE and RAPD analysis were performed to established genetic diversity for five accessions (IPL-406, IPL-81,DPL-62, DPL-15, & Sehore-74-3) and to elucidate their genetic relationships. The resulted protein banding pattern showed high level of polymorphism(i.e., 95.6% and could be used to discriminate completely among the five accessions under study. However, the protein profile could be considered as general biochemical finger print of the lentil.During present study the polymorphism was high at protein level. One decamer primer(OPA-17) having sequence CACCGCTTGT was used to estimate genetic diversity in *Lens culinaris* accessions.Atotal of 43 bands were scored , 39 bands showed polymorphism(90.6%) and the rest 4 bads showed 9.30% monomorphism during RAPD analysis. Number of bands produced in each accession varied during RAPD and SDS-PAGE analysis .Similarity index reveals maximum similarity between accessions IPL-406(L-1) and DPL-15(L-4) , DPL-15(L-4) and Sehore-74-3(L-5) i.e., 35.71% and 35.29% respectively while distantly related accessions were IPL-81(L-2) and DPL-62(L-3) with similarity index 23.8% when RAPD profile was observed but when protein profile of five accessions of lentil was observed, show highest similarity index value between accessions IPL-406(L-1) and Sehore-74-3(L-5) i.e., 36.6% while distantly related accessions show lowest similarity index were IPL-406(L-1) and DPL-15(L-4) i.e., 15%. Two dendrograms constructed based on Jaccard's method and UPGMA using oth SDS-PAGE and RAPD profiles. The resulting dendrogram for protein profile revealed two main seperate genetic clusters ; the first comprises the accession IPL-81(L-2), The first cluster was further subdivided into IPL-406 and Sehore-74-3 while second cluster comprises accessions DPL-62 and DPL-15.The dendrogram constructed for RAPD profile revealed main separate genetic clusters ; the first cluster comprises the accession DPL-62, the first cluster was further subdivided IPL-406 and DPL-15 while as second cluster comprises accession IPL-81 and Sehore-74-3.

INTRODUCTION

Lentil is a self pollinated diploid,(Alabboud *et al*.,2000) , selender , annual bushy, much branched and herbaceous plant.It is a cool season grain legume with relative genome of 41063 Mbp (Arumuganthan and Earle,1991). Lentil is an important seed legume crop cultivarted world wide as human food (Ertugrul *et al.,*2004).It belongs to family Fabaceae. This crop contains 26% 0f protein content.Lentils are always on the list of production as one of the important rabi crops**.** The plant likely originated in the Near East and is believed to have originated from *lens orientalis.* It has been part of human diet since the aceramic (non pottery producing) Neolithic times, being one of the first crops domesticated in the Near East and is an important part of diet in many parts of the world, especially in the Middle East the Indian sub continents(Ahmad *et al*.1996). The ancient pulse crop was domesticated in the Fertile Crecent where it has been cultivated since at least the seventh century B.C. (Ladizinsky,1979).

Corresponding author: ghbot119@gmail.com

Indian production of this crop hovers around 10 lakh metric tons per year that cultivated on about 14 lakh hectares of land.Around 90% of the production comes the Eastern and the Western part of the country. Canada is the largest exporter of lentil in the world contributing about 1043200 tonnes / year. Among biochemcal techniques SDS-PAGE is widely used due o its simplicity and effectiveness for describing the genetic structures of crop gerplasm (Murphy *et al*., 1990 ; Javaid *et al*., 2004 ; Anwar *et al.,* 2003). The main objective of our research was to estimate the potential of SDS-PAGE technique to assess genetic diversity and relatedness among 5 accessions of *Lens culinaris* based on seed storage protein profile and to develop an optimized and efficient operational system for their use.Knowledge of cytological and molecular relationships between plant accessions is very useful in planning effective breeding strategies to transfer desirable genes or genes clusters from one species to another, thereby producing fruitful genomic reconstructions and disease free plants.Estimation of genetic diversity of any crop species is suitable precursor for improvement of the crop because it generates baseline data to guide selection of parental lines and

design of a breeding scheme. Genetic diversity refers to any variation in the nucleotides, genes, chromosomes or genome of the organisms. Thus , each gene comprises a hereditary selection of DNA that occupies a specific place of the chromosomes and controls a particular characteristics of an organism(Welsh *et al*.,1990).Each allele codes for the production of aminoacids that string together to form proteins. Thus, differences in the nuclotide sequence of alleles result in the production of slightly different strings of aminoacids of the proteins. These proteins code for the development of the anatomical and physiological characteristics of the organisms (Shibata, 2005). Randomly amplified polymorphic DNA (RAPD) assay, on the other hand which detects nucleotide sequence polymorphism by means of the polymerase chain reaction (PCR). A single primer of arbitrary nucleotide sequence is a useful method for generating molecular markers and provides accurate finger prints at the molecular level (Williams *et al*.,1990 ; Welsh & McClelland, 1990).DNA markers give a higher degree of polymorphism and reproducibility.The PCR products that are not shared between some individuals act as polymorphic markers.The development of DNA markers have been recently introduced in plant discriminations and being employed for the improment of intractable traits such as drought tolerance , resistance to foliar feeding insects and combination of high protein / high yield (Linc *et al.*, 1996; Lefebvre *et al*.,2001; Hassan, 2002, Lu,& Myers 2002 ; Abdel-Tawab *et al.,* 2003)

MATERIALS AND METHODS

Materials (lenil accessions) for protein profiling and RAPD studies were collected from IIPR, Kanpur,(U.P), India. Total seed proteins were also extracted from 1g seed flour using 400 micro litre of extraction buffer that contained 25 Mm tris Hcl Ph-8.3, 12% SDS. 5M urea and 10% mercapto ethanol. Seed flour was thoroughly mixed with buffer by vertexing. The extracted protein was separated by centrifuging the sample at the rate of 1500 rpm for 10 minutes electrophoresis was carried out in discontinuous SDS-PAGE using 7% acrylamide gel. Electrophoresis was run at 50v. The gels were stained in the staining solution containing 40ml methanol, coomassie blue 1% (1g) and galacial acetic acid (10ml) was made up to 100ml by adding distilled water. Destaining was done in a solution containing 30ml methanol, 6ml galacial acetic 74ml of distilled water until the back ground colour disappeared, and protein bands were clearly visible.

DNA Isolation Protocol

- Grind 0.5g of seed material in .liquid nitrogen to a fine powder using pre-chilled mortar and pestle.
- Transfer the powder to a 15ml .polypropylene centrifuge tube containing 5ml of pre warmed extraction buffer, use spatula to disperse the material completely.
- Incubate samples at 60°c for 30 minutes with occasional mixing by gentle swirling.
- Add 30ml. of chloroform iso-amyl alcohol and mix by inversion to emulsify.
- Spin at 15000 rpm for 10 minutes at room temperature.
- Remove the aqueous phase with a wide bore pipette, transfer to a clean tube, Add 2/3n volume of iso-propanol and mix by quick gentle inversion.
- Wash the DNA pellet in 70% ethanol (5-10 ml) for 20 minutes.
- Dry the pellet and dissolve in 500 µl TE buffer.

PRIMER SELECTION

The primer OPA-17 has been selected on the basis of high polymorphism (approx.69.2%), detected in nine genotypes of *Lens culinaris*, Alabboud *et. al*, 2009.

POLYMERASE CHAIN REACTION

REQUIREMENTS

- Taq DNA polymerase (1 units/µl)
- Oligonucleotide primers (2 µl)
- Deoxy nucleotide triphosphate (2µl)MgCl2 5.5µl
- Agarose 2%
- Ethidium bromide (10mg/ml)
- Gel loading dye (6x)
- 0.25% bromophenol blue.O.25 % xylene cyanol,15 % ficoll type in 400 ml water.
- TBE buffer (5x)
- 0.45 tris-borate, 0.01 EDTA
- DNA size standard

Polymerase chain reaction (PCR) is a technique used to selectively amplify *in vitro* a specific segment of the total genomic DNA a billon fold·. The PCR involves three basic steps which constitute single cycle.

- Denaturation of the target DNA at 92- 94°c.
- Annealing of the primers to the template DNA at 35-60°c.
- Primer extension by addition of the nucleotides to the 3' end of the primers at 72°c by the enzyme DNA polymerase

Sterilized water	17 µl
Buffer $mgCl_2$	5.5 µl
dNTPs	1 µl (200 µM) each
Primer	2 µl (300 ng) each
Enzymes	1 unit
Template DNA	2 µl (200ng) each
Sterile water	To a final volume of 25 µl each

Table 1. Presence and absence of bands in protein profile

S.No.	Rf value	Molecular wt.	L1	L2	L3	L4	L5
1.	0.01	99	-	-	+	-	-
2.	0.025	98	+	-	-	-	+
3.	0.03	97	-	-	+	+	-
4.	0.05	95	+	-	+	-	+
5.	0.06	94	+	+	+	+	+
6.	0.08	92	+	-	-	+	+
7.	0.1	90	-	+	-	-	-
8.	0.11	89	+	-	-	-	-
9.	0.12	88	-	+	+	-	-
10.	0.13	87	+	-	-	-	+
11.	0.25	75	+	+	+	+	+
12.	0.26	74	+	-	-	-	-
13.	0.35	65	-	-	+	+	-
14.	0.37	63	-	-	+	+	-
15.	0.4	60	+	+	-	-	+
16.	0.42	58	+	-	-	-	+
17.	0.46	54	+	-	-	-	-
18.	0.56	44	+	+	+	-	+
19.	0.6	40	-	-	-	+	-

Table 2:Showing Jaccard's similarity coefficient

	L1	L2	L3	L4	L5
L1	0.000				
L2	0.28571				
L3	0.23529	0.36364			
L4	0.18750	0.18182	0.45455		
L5	0.83333	0.33333	0.26667	O.21429	0.000

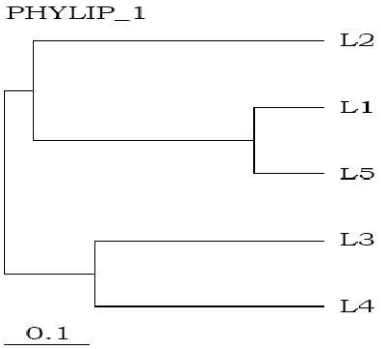
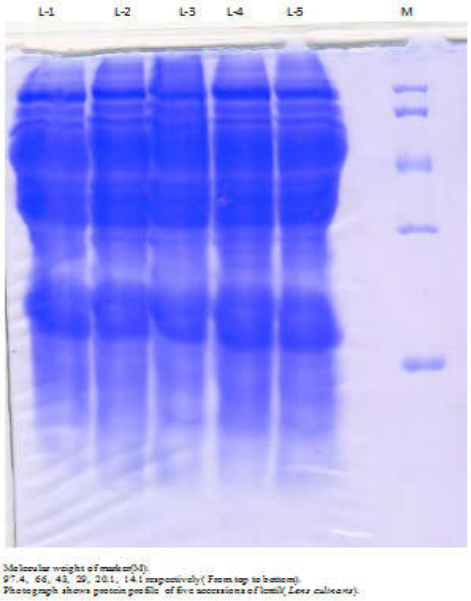


Fig1. UPGMA & Jaccard's method dendrogram showing the genetic relationship among 5 accessions of lentil revealed by SDS PAGE cluster analysis



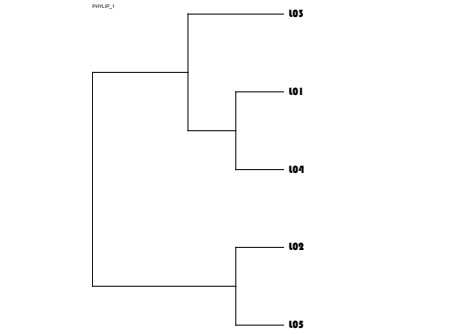
Molecular weight of marker(kD): 97.4, 66, 43, 29, 20.1, 14.1 respectively (From top to bottom). Photograph shows protein profile of five accessions of lentil(*Lens culinaris*).

Table 3: Presence and absence of bands in RAPD profile

S.NO	Rf Value	Fragment length(bp)	L-1	L-2	L-3	L-4	L-5
1	0.39	610	-	-	+	+	-
2	0.4	600	-	+	-	-	+
3	0.46	540	-	-	-	+	-
4	0.47	530	-	-	+	-	-
5	0.49	510	-	+	-	-	-
6	0.51	490	-	-	+	-	-
7	0.55	450	-	+	-	+	+
8	0.56	440	-	+	-	-	-
9	0.57	430	-	-	+	-	-
10	0.60	400	+	+	+	+	-
11	0.61	390	-	-	-	-	+
12	0.64	360	-	-	+	+	+
13	0.65	350	-	+	-	-	-
14	0.71	290	+	+	+	+	+
15	0.76	240	+	+	+	+	+
16	0.80	200	+	+	+	+	+
17	0.87	130	-	-	+	-	-
18	0.95	70	+	+	+	+	+

Table 4 : Showing Dice's similarity coefficient

	L1	L2	L3	L4	L5
L1		0.50000	0.45455	0.55556	0.44444
L2	0.50000		0.31250	0.46154	0.50000
L3	0.45455	0.31250		0.53846	0.35714
L4	0.55556	0.46154	0.53846		0.54545
L5	0.44444	0.50000	0.35714	0.54545	



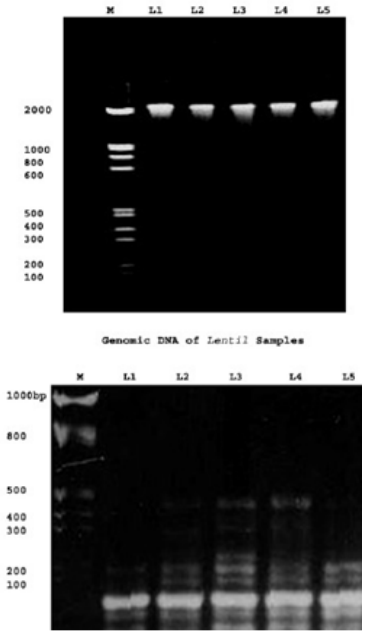


Fig.2 : UPGMA based dendrogram showing genetic relationship among lentil accessions for RAPD data

RESULTS AND DISCUSSION

Great variations were observed in qualitative,biochemical and molecular characters among five experimental accessions of *Lens culinaris* belongs to family Fabaceae.To find out diversity among accessions of lentil, cytological and molecular analysis through protein profiling and RAPD revealed correlation between five experimental accessions of *Lens culinaris.* To find out intervarietal correlation between cultivars, several earlier workers e.g., Jha and Ohri(1996), Ladizinsky(1979) made protein prifiling study through SDS-PAGE and find almost same observations. Present investigation revealed that protein profiling is one of the basic and reliable method to detect intervarietal genetic diversity and study phylogenetic relationship among the five selected experimental accessions of *Lens culinaris.* When bands of all accessions were compared, we obtained a total of 46 bands .Out of them 44 were polymorphic with 95.6% polymorphism.(table – 1). The near polymorphism percentage

i.e., 100% was found in *Oryza sativa* L. Galani *et al*. (2010). Bhat and Kudesia(2011), found 100% in different five species of Solanaceae. This little change may be due to crop change.

Dendrogram was constructed based on unweighted pair group method using arithmetic averages.Cluster analysis of data placed five accessions of lentil into two main clusters.Cluster first comprised one accession IPL-81(L2) and cluster first further subdivided into two subclusters comprising IPL-406(L1) and Sehore-74-3(L5).Cluster second comprised DPL-62(L3) , and DPL-15(L4).This is given in(Figure-1).
RAPD analysis , one of the molecular approaches was used for identification of cultivars in a number of plant species (Rajasegar *et al*.(1997) , Galderisi *et al.(*1991) , Sant *et al.*(1999) ,Lefebvre *et al*.(2001) , Fernandez *et al*.(2002) , Yu *et al.(*2002).When bands of all five accessions were calculated , we obtained a total of 43 bands. Out of them 39 bands were polymorphic with a total of 90.6% polymorphism and 4 bands were monomorphic showed 9.3% monomorphism(Table-3).Almost similar results were reported using RAPD analysis by several workers.They reported genetic polymorphism almost similar i.e., 90% or around this value.

Sami *et al.* (2009) reported 92%, Padmesh *et al*. (2006) observed 90.1% , Bibi *et al.*(2009) reported 89.2% , Iqbal *et al*.(1996) 89.1% , Ikbal *et al*. (2009), reported 93.9% polymorphism, Deshmukh *et al.*(2009) observed 90.7% and Asante *et al.(*2003) seen 90-100% polymorphism. Similarly Jan et al.(2011) got ninety five RAPD fragments of which ninety were polymorphic with 94.7% polymorphism. The change might be due to the number of primers used. The number of amplified fragment sizes ranged from 70-610 bp.(Table 3). The Dice's similarity coefficient ranged from 0.31250-0.55556(Table 4). Dendrogram was constructed based on unweighted pair group method using arithmetic averages. Cluster analysis of data placed five accessions of lentil two main clusters . The first cluster includes accessions IPL-81(L02) and Sehore-74-3 (LO5) and cluster second includes 1PL-406(L01) and DPL-15(LO4).DPL-62(L03) were placed separate in the dendrogram (Fig. 2).

Conclusion

Seed protein profiling through SDS-PAGE is the most important tool in determining the molecular polymphism and genetic-homology. Seed storage proteins helps in cultivars identification by avoiding the external environmental influences. During present study 5 accession of lentil (i.e., IPL-406 (4) and Sehore-74-3; DPL-62 (L_3) with DPL-15 (L_4) show highest value of Jaccard's similarly index (i.e.,0.83333 in IPL-406 and Sehore-74-3) respectively which revealed that these accession are phylogenetically close to each other while as accession IPL-81 and DPL-15 having Jaccard's similarly index (0.18182) which means that these accession are distantly related from each other. This has been proved at molecular level by using protein profiling among accession of lentil (*Lens cutlinaris*). Although these has been great advancement in the market technology with the advent of different DNA markers like Amplified fragment length polymorphism (AFLP), Simple Sequence repeats (SSR), Single Nucleotide Polymorphisms (SNPs) etc. Still RAPD is quite convenient to apply provided the problems of reproducibility is minimized.

From the present investigation, it is clear that RAPD analysis revealed enough polymorphism in lentil. The only option left over is to validate by the samples provided. The preliminary work carried out with one random primer selected from literature revealing the genetic diversity between accession viz., IPL-51 (L_2), DDL-62 (L_3) and DPL-62 (L_3), Sehore-74-3 (4) age distantly related as per dendrogram constructed on the basis of UPGMA on the basis of RAPD data, could be exploited further by increasing the number of random primers and validating it with other available DNA markers. The accession which are distantly related either at protein or gene level should be used for plant breeding program in future.

Abreviations

SDS= sodium dodecyl sulphate
UPGMA=Unweighted pair group method with arithmetic averages
PAGE= Polyacrylamide gel electrophoresis
RAPD=Randomly amplified polymorphic DNA
IGFRI=Indian grass land and fodder research institute
CTAB=Cetyltrimethyl ammonium bromide
SSR=Simple sequence repeats
RFLP=Restriction length polymorphim
AFLP= Amplified fragment length polymorphism
SNPs =Single nucleotide polymorphism.

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