Estimation of Genetic Diversity in Six Lentil (*Lens culinaris* Medik.) Varieties using Morphological and Biochemical markers

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Abstract-Morphological characters and Electrophoretic SDS-PAGE analysis were performed to established genetic diversity for six important lentil varieties of Bangladesh and to elucidate their genetic relationships. Analyses of variance of morphological characters showed significant differences among varieties. The resulted protein banding pattern showed 57.12% polymorphism and could be considered as general biochemical finger print of the lentil. Dissimilarity index reveals maximum dissimilarity between BARI masur-3 and BARI masur-6 when morphological traits were observed but when seed storage protein profile was observed, distantly related varieties showed the lowest similarity were BARI masur-1 and BARI masur-5. Two dendrograms constructed based on UPGMA using both morphological traits data and SDS-PAGE profiles, revealed two main separate genetic clusters. Principal component analyses supported the result of dendrogram, which proved that BARI masur-1 and BARI masur-5 is the most distant variety among six. So these two varieties can be considered as valuable gene resources for further breeding programs.

Index Terms- Genetic diversity, Lentil, Morphological marker, Protein profiling, UPGMA

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1 INTRODUCTION

Lentils are one of the oldest and an important seed legume crops, cultivated worldwide as human food. It is a self pollinated diploid (2n=14) [1] crop with a relatively large genome of 41063 Mbp [2]. It is cultivated mainly for its seed and only red cotyledon type is used as food in Bangladesh. Lentil seeds are valued as a food of both high quality plant proteins (26%) and fiber, in addition, the remaining plant residues can be used as animal feed and fodder for livestock and play an important role in crop rotations because their nitrogen fixing capability. Besides that sprouted lentils now have a reputation as “human food”.

Epidemiological studies suggested that lentils have antioxidant, anticancer and probiotic activity which confer protection against some important chronic diseases [3, 4]. In Bangladesh, production of major food crops such as rice wheat does not meet the present requirements of countries population of about 134 million. Agricultural scientists are faced with the complex and urgent task of bringing the “population-food supply” equation into rational balance. Rice and wheat have been the focus of concerned government effort in research and development. Similar attention was long overdue for the pulse crops, commonly known as poor man’s meat.

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Pulses mainly lentils are vital components in diversification of Bangladesh are predominantly rice-based cropping system. Genetic variation between and within populations of crop species is a major interest of plant breeders and geneticists [5] because it facilities the efficient sampling and utilization of germplasm resource [6]. The breeders must have the idea of choosing the accession that most likely possesses the trait of interest. The knowledge of genetic variation and relationships between populations is important to understand the available genetic variability and its potential use in breeding programs. There are several methods to study genetic diversity such morphological, biochemical and molecular markers. Morphological characterization is the first step in the classification and description of any crop germplasm [7, 8] which is a traditional and one of easiest method for traditional plant breeders in selecting the desirable traits. Considerable variations among the characters for use in breeding and selection programmes have been reported for various morphological characters [9, 10 and 11]. Many workers have been reported on genetic variation in lentil through morphological characters [12, 13] and seed storage protein profile [14, 15, 16 and 17]. Among biochemical techniques SDS-PAGE is widely used due to its simplicity and effectiveness for describing the genetic structures of crop gerplasm [18, 19 and 20]. Seed storage proteins profiling provides aid for identification and characterization of diversity in crop varieties and their wild varieties and phylogenetic relationship of the varieties, generating pertinent information to complement evaluation and passport data [21]. Traditional breeding method is very much important for crop improvement but they proved to be slow in targeting complex trait like grain yield, grain quality and drought or salinity tolerance. Plant descriptors coupled

with biochemical markers as well as seed storage protein provide a valid evidence of diversity as these are least affected by environmental fluctuations [22, 23 and 24]. The main objective of our research was to estimate the potential of SDS-PAGE technique and morphological characters analysis to assess genetic diversity and relatedness among six lentil varieties grown in Bangladesh based on seed storage protein profile and morphological characters to develop an optimized and efficient operational system for their use.

2 MATERIALS AND METHODS

2.1 Plant Materials

In this study, we used six lentil varieties such as BARI masur-1(Fig 1A), BARI masur-2 (Fig1b), BARI masur-3 (Fig 1C), BARI masur-4 (Fig 1D), BARI masur-5 (Fig 1E) and BARI masur-6 (Fig 1F) as a plant material for morphological characters analysis which were collected from Biometrical Genetics Laboratory, University of Rajshahi, Bangladesh. Matured seeds of BARI masur-1 (Fig 1G), BARI masur-2 (Fig 1H), BARI masur-3 (Fig 1I), BARI masur-4 (Fig 1J), BARI masur-5 (Fig 1K) and BARI masur-6 (Fig 1L) were used for total seed storage protein profile, which were collected from six lentil varieties after harvesting.

2.2 Morphological Analysis

The plants were sown in the field following Randomized complete block design in the year 2012 in order to obtain the morphological parameters were measured for each lentil varieties viz. date of first flower (DFF), plant height at first flower (PHFF), number of primary branches at first flower (NPBFF), number of secondary branches at first flower (NSBFF), date of maximum flowers (DFF), plant height at maximum flowers (PHFF), number of secondary branches at maximum flowers (NSBMF), individual plant weight (IPW), pod number per plant (PdNPP), pod weight per plant (PdWPP), seed number per plant (SNPP) and seed weight per plant (SWPP).

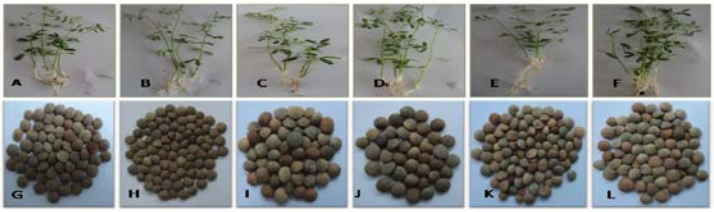

Fig 1: Young plants of (A) BARI masur-1 ; (B) BARI masur-2 ; (C) BARI masur-3; (D) BARI masur-4; (E) BARI masur-5; (F) BARI masur-6; Mature seeds of (G) BARI masur-1 ; (H) BARI masur-2 ; (I) BARI masur-3; (J) BARI masur-4; (K) BARI masur-5; (L) BARI masur-6.

2.3 Electrophoresis Analysis (SDS-PAGE)

Total seed proteins were extracted from 2 gm matured seeds using protein extraction buffer that containing 0.1 M Tris and pH 7.5 was adjusted. First of all seed coats were removed and seeds were crushed with buffer in morter pestle. Transfer it to eppendorf tube and supernatant was collected and sample was taken in a water bath at 94^0 C for 4-5 min. The extracted protein was separated by centrifuging the sample at the rate of 13000rpm gradually for 20 min; 45 min and 60 min and finally supernatants were heated in water bath for at 90^0 C for 5 min prior to loaded on gel. Samples were prepared by mixing 15 µl of extracted protein with loading dye of mercaptoethanol, 0.1% bromophenol blue, 1.5 M tris-Hcl (pH 6.8) containing 15% glycerol, 10% SDS and 8 M urea. The SDS-PAGE was carried out [25] and protein staining was performed using Coomassie Blue [26]. SDS-PAGE was performed with12% separating gel and 5% tacking gel. A vertical slab gel electrophoresis system (SLAB GEL SYSTEM, BIOTECH, YERCAUD-636601) was used. Electrophoresis was carried out at 15v/c for staking gel and 12 v/c for resolving ge. After complete the electrophoresis the gel was stained in Coomassie Brilliant Blue G-250 (1%) and 10% glacial acetic acid for 1 hour and destaining was done in a solution containing 35% methanol and 10% glacial acetic acid by gently shaking until the deep blue background colour disappeared and protein bands were clearly visible. A standard Acculadder molecular weight marker (low range) consisting of albumin (66kDa), ovalbumin (45 kDa), carbonic anhydrase (29kDa), trypsin inhitor (20.1 kDA), lysozyme (14.4 kDa) and aprotinin (6.5 kDa) was for calculating the molecular weights of different protein bands.

Photographs were taken of gels and zymograms were drawn manually by relative mobility (Rm) of each of the band. Relative mobility (Rm) was measured by the following formula.

$$\text{Relative mobility (Rm)} = \frac{\text{Migration distance of band}}{\text{Migrataion distance of dye front}}$$

2.4 Morphological Data Analysis

The collected data were analyzed by two way analysis of variance (ANOVA) and with the obtained values we calculated a matrix of Euclidian distance between the six lentil varieties from the standaadized trait mean values over each varieties using NTSYSpc software version 2.11 [27].Similarly, the standardized trait mean values of each variety were used to perform cluster analysis with the same software.

2.5 Protein Data Analysis

The bands were visually scored as present (1) or absent (0) for seed storage protein gel. Genetic similarities were calculated by Jaccard method [28]. The Jaccard similarity coefficient was used to build an unweighted pair-group method with arithmetic means (UPGMA) clustering procedure of Nested (SHAN) clustering methods [29] and NTSYSpc version 2.11T [27] was used for genetic similarity computing, dendrogram construction and principal component analysis (PCA).

3 RESULTS AND DISCUSSION

Great variations were observed both in qualitative and biochemical characters among six experimental varieties of lentil belong to family Fabaceae. In this present investigation, analysis of variance (ANOVA) of twelve morphological characters showed significant result of six lentil varieties for all the characters (**Table 1**), which indicating varieties are significantly different from each other. The same result reported in lentil [30, 31 and 32]. Significant variation was found both in cultivated and in wild species of sesamum [33, 34] found. The result of morphological evaluation of the characters showed significant genetic variation of different yield and yield contributing characters in the varieties indicating the scope and their warranty to use in the breeding programmes [35]. Genetic dissimilarity was calculated as Euclidian distance to study genetic variability among varieties for morphological characters and it was ranged from 0.10 to 0.87 (**Table 2**). Based on Euclidian distance, BARI was noted to be closely related with BARI masur-6 and showed the highest dissimilarity value 0.87. On the other hand, lowest genetic dissimilary relation could be noticed between BARI masur-1 vs. BARI masur-4. Eucliance distance also reported to construct dendrogram in lentil [30]. The genetic distance analysis using unweighted pair group method of arithmetic means (UPGMA) dendrogram was constructed for measuring genetic diversity and relatedness among the accessions (**Fig 2**). Cluster analysis indicated the extent of genetic diversity that is a practical use in plant breeding [14]. According to morphological dendrogram, it was observed that the varieties were grouped in two clusters: cluster-I and cluster-II (**Fig 2**). The biggest group cluster-II contained five varieties viz. BARI masur-2, BARI masur-3, BARI masur-4, BARI masur-5 and BARI masur-6, while cluster-I comprised only one varieties (BARI masur-1). From the dendrogram it was cleared that highest variation was formed between BARI masur-3 and BARI masur-6. The results indicate that low or high genetic distance exists respectively between varieties with similar or different origins. UPGMA dendrogram was also used for measuring genetic distance in lentil [30, 31].

Seed proteins have been successfully used to study the variation of seed storage protein in lentil [14, 36]. To find out intervarietal correlation between cultivars, several earlier workers [23, 37] made protein profiling study through SDSPAGE and find almost same observations. Present investigation revealed that protein profiling is one of the basis and reliable methods to detect intervarietal genetic diversity and study phylogenetic relationship among the six lentil varieties. When bands of all varieties were compared, we obtained a total of twenty one bands. Out of them twelve were polymorphic with 57.12% polymorphism (**Fig 4**). The bands were detected at approximately molecular ranging between 6.5 and 66 kDa and was divided into six regions with intervals of molecular markers (**Fig 3**).Region I was for albumin protein, had seven bands of more than 66 kDa of which with were polymorphic. Region II was for ovalbumin protein, ranged from 45 kDa to 66 kDa with three protein peptides, of which two were polymorphic. Region III was for carbonic anhydrase protein, ranged from 29 kDa to 45 kDa with three protein subunits, of which one were polymorphic. Region IV was trypsin inhibitor, ranging from 20.1 kDa to 29 kDa, had four protein bands of which three were polymorphic. Region V was for lysozyme ranged from 14.4 to 20.1 kDa, had two bands and all are polymorphic. Region VI was for aprotinin, ranging from 6.5 kDa to 14.4 kDa had two bands, which was monomorphic. From six type of seed storage protein, albumin protein was abundant in quantity in all the varieties and as well as all the varieties were polymorphic for lysozyme protein.

44 bands were polymorphic among 46 bands with 95.6% polymorphism in lentil [17], 100% polymorphic were reported in oryza sativa L. [38] and 100% polymorphism were found in different five species of Solanaceae [39]. 24 bands were found in lentil [36] in which only five bands were polymorphic with molecular masses ranging from 35 to 116 kDa where 55 protein bands were recorded in lentil [15] ranging from the molecular mass of 14-66 kDa. Out of them 13 bands were polymorphic in nature and they concluded that SDS-PAGE alone did not exhibit high level of intra-specific variation. The relative mobility of seed storage protein was measured ranging from 0.02 to 0.92 (**Fig 5**). The highest polymorphic bands were found in BARI masur-5 at the position of relative mobility 0.02, 0.12, 0.30, 0.67 and the lowest polymorphic bands were found in BARI masur-4 which exhibited only two polymorphic bands at position 0.07 and 0.12. BARI masur-1 was similar to BARI masur-2 and BARI masur-3 was similar to BARI masur-6 in band number.

Genetic similarity was analyzed based on Jaccard's similarity coefficient and it was ranged from 0.57 to 0.90. BARI masur-2 was noted be closely related with BARI masur-6 with the highest similarity coefficient 0.90. On the other hand the highest distant relation i.e. the lowest similarity coefficient could be noticed between BARI masur-1 vs. BARI masur-5, proceeded by BARI masur-2 vs. BARI masur-3 and BARI masur-2 vs. BARI masur-5. Dendrogarm was constructed and which showed two main clusters of six lentil varieties. First cluster comprised BARI masur-1, BARI masur-2 and BARI masur-6 and second cluster comprised BARI masur-3, BARI masur-4 and BARI masur-5.Finally Principal component analysis was done to confirm the result of dendrogram. The first three principal components from PCA accounted for 93.21% of the total variation among varieties. The proportion of principal components PC1, PC2 and PC3 were 77.85%, 9.95% and 5.41% respectively. The two dimensional (2D) and three dimensional (3D) distribution of PCA supported the results of dendrogram i.e. the highest genetic distance was found between BARI masur-1 and BARI masur-5.

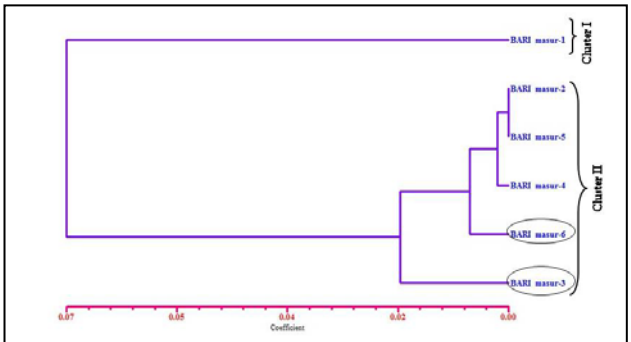

Fig 2: Dendrogram of six lentil varieties based on twelve morphological characters using genetic distance and UPGMA method

plant	Error	15	20.03	1.34	

N.B.: * and ** represent significant result at 5% and 1% significance level

TABLE 1

ANOVA FOR THE TWELVE MORPHOLOGICAL CHARACTERS MEASURED IN SIX LENTIL VARIETIES

Morphological Characters	Source	Degree of freedom	Sum of square	Mean of Square	F value
Date of first flower	Variety	5	413.55	82.71	5.01**
	Replication	3	277.94	92.64	5.61**
	Error	15	247.38	16.49	
Plant height at first flower	Variety	5	104.44	20.88	6.99**
	Replication	3	0.32	0.10	0.03 ^{NS}
	Error	15	44.89	2.99	
Number of primary branches at first flower	Variety	5	8.73	1.74	3.35*
	Replication	3	0.32	0.10	0.20 ^{NS}
	Error	15	7.82	0.52	
Number of secondary branches at first flower	Variety	5	13.33	2.66	3.54*
	Replication	3	0.45	0.15	0.20^{NS}
	Error	15	11.277	0.7518	
Date of maximum flowers	Variety	5	394.54	78.90	7.13**
	Replication	3	158.39	52.79	4.77**
	Error	15	165.8207	11.05471	
Plant height at maximum flowers	Variety	5	115.11	23.02	6.60**
	Replication	3	2.91	0.97	0.27 ^{NS}
	Error	15	52.28143	3.485429	
Secondary branches at maximum flowers	Variety	5	26.95	5.39	11.26**
	Replication	3	0.73	0.24	0.51^{NS}
	Error	15	7.180812	0.478721	
Individual plant weight	Variety	5	43.30	8.66	3.46*
	Replication	3	10.72	3.57	1.43 ^{NS}
	Error	15	37.46868	2.497912	
Pod number per plant	Variety	5	23903.69	4780.73	3.63*
	Replication	3	792.37	264.12	0.20 ^{NS}
	Error	15	19750.29	1316.686	
Pod weight per plant	Variety	5	35.71	7.14	3.04*
	Replication	3	5.47	1.82	0.77 ^{NS}
	Error	15	35.17255	2.344836	
Seed number per plant	Variety	5	44471.9	8894.38	3.66*
	Replication	3	1427.549	475.84	0.196^{NS}
	Error	15	36394.11	2426.27	
Seed weight per	Variety	5	23.52	4.70	3.52*
	Replication	3	1.45	0.49	0.36 ^{NS}

TABLE 2

EUCLIDEAN DISSIMILARITY MATRIX AMONG SIX LENTIL VARIETIES BASED ON MORPHOLOGICAL CHARACTERS

	BARI masur-1	BARI masur-2	BARI masur-3	BARI masur-4	BARI masur-5	BARI masur-6
BARI masur-1	0.00					
BARI masur-2	0.78	0.00				
BARI masur-3	0.24	0.15	0.00			
BARI masur-4	**0.10**	0.34	0.29	0.00		
BARI masur-5	0.85	0.14	0.19	0.29	0.00	
BARI masur-6	0.53	0.50	**0.87**	0.13	0.42	0.00

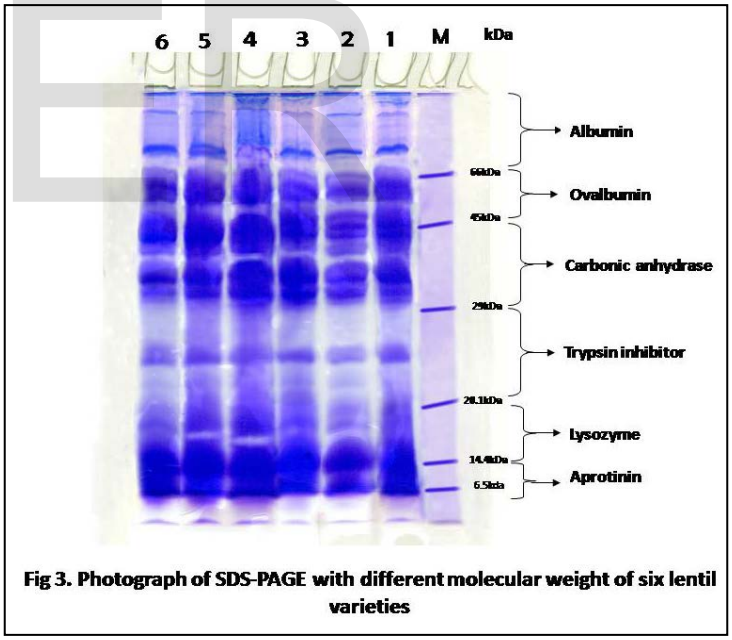

Fig 3. Photograph of SDS-PAGE with different molecular weight of six lentil varieties

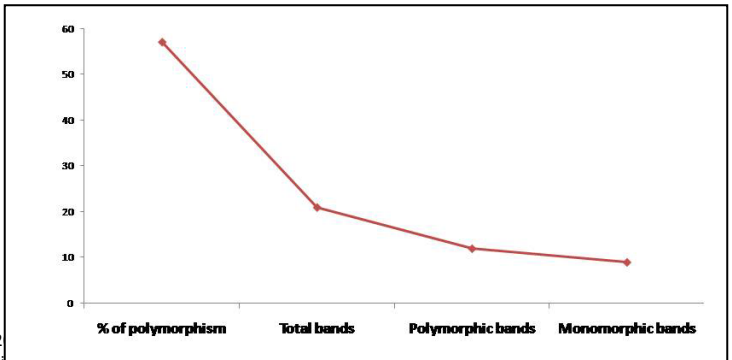

Fig 4. Number of total bands, polymorphic bands and monomorphic bands and Percentage of polymorphisms for seed storage protein markers

TABLE 3
JACCARD'S SIMILARITY COEFFICIENT MATRIX AMONG SIX LENTIL VARIETIES BASED ON SEED STORAGE PROTEINS PROFILING

	BARI masur-1	BARI masur-2	BARI masur-3	BARI masur-4	BARI masur-5	BARI masur-6
BARI masur-1	1.00					
BARI masur-2	0.76	1.00				
BARI masur-3	0.76	0.61	1.00			
BARI masur-4	0.71	0.66	0.76	1.00		
BARI masur-5	**0.57**	0.61	0.80	0.76	1.00	
BARI masur-6	0.85	**0.90**	0.71	0.76	0.71	1.00

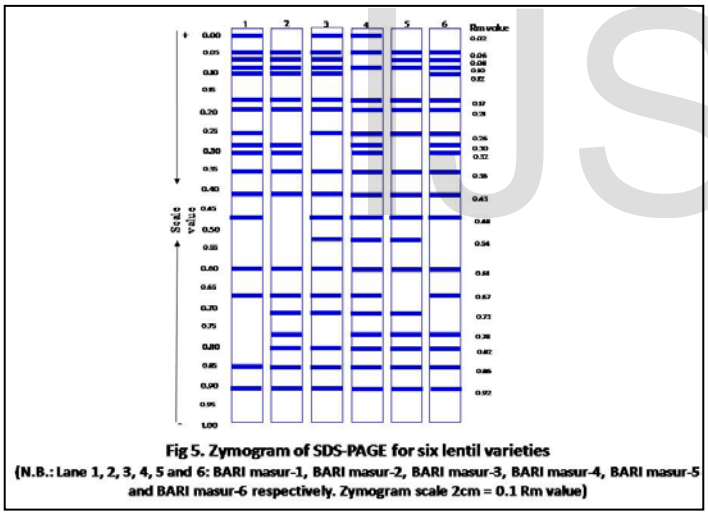

Fig 5. Zymogram of SDS-PAGE for six lentil varieties
(N.B.: Lane 1, 2, 3, 4, 5 and 6: BARI masur-1, BARI masur-2, BARI masur-3, BARI masur-4, BARI masur-5 and BARI masur-6 respectively. Zymogram scale 2cm = 0.1 Rm value)

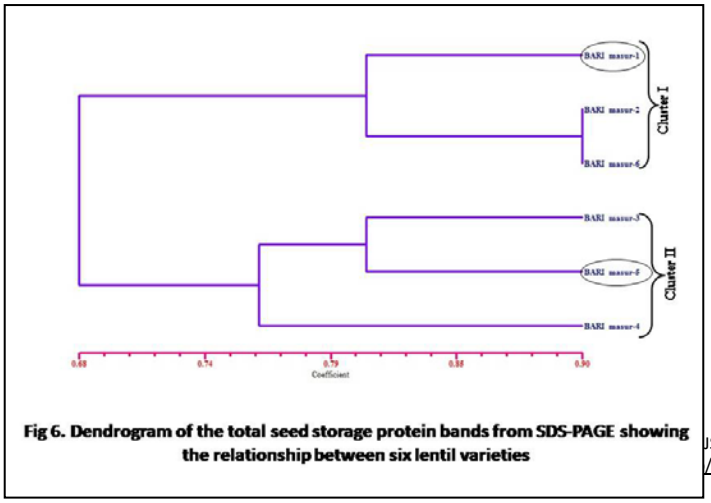

Fig 6. Dendrogram of the total seed storage protein bands from SDS-PAGE showing the relationship between six lentil varieties

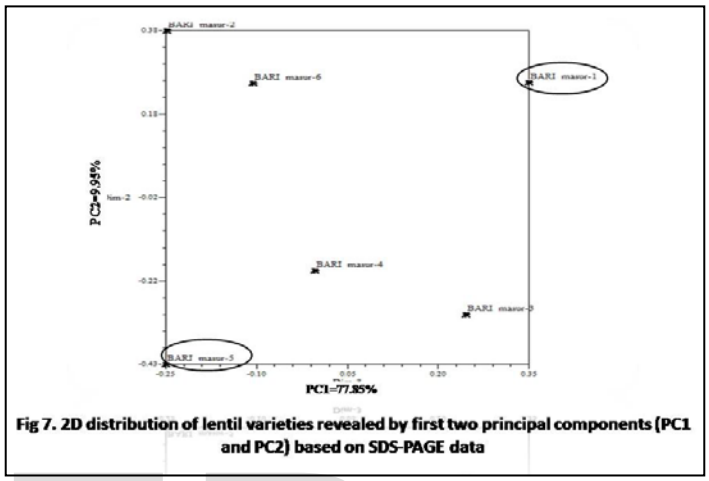

Fig 7. 2D distribution of lentil varieties revealed by first two principal components (PC1 and PC2) based on SDS-PAGE data

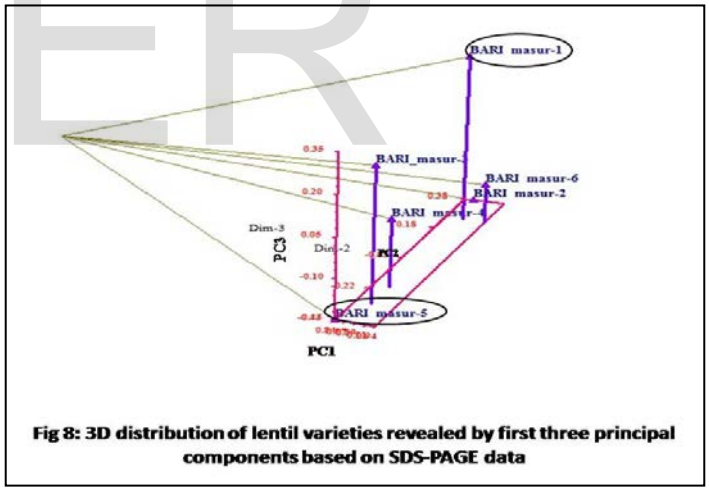

Fig 8: 3D distribution of lentil varieties revealed by first three principal components based on SDS-PAGE data

4 CONCLUSION

After all, the two dendrograms based on morphological traits and seed storage protein profiling revealed that BARI masur-3 and BARI masur-6 as well as BARI masur-1 and BARI masur-5 was the distant variety. This difference between two dendrograms can be due to fact the fact that the morphological traits can be influenced by many factors such as: environmental conditions, the sample size, the time of making the measures etc. This study has shown significant genetic variability among the varieties on both morphological and biochemical analysis but the biochemical analysis is more reliable because the varieties are not

influenced by the environmental conditions. So this study demonstrated that determining of genetic variability among six lentil varieties, seed storage protein marker is more precise and reliable than the morphological markers.

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