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Genetic polymorphism in *Lens culinaris* collected from Malakand division Khayber Pakhtunkhwa, Pakistan

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Abstract

A major part of the human di *et al* over the world consists of cereals and legumes. Legumes are however, considered as the major source of protein and dietary amino acid for man and farm animals. The detailed evolutionary relations and cluster analysis in this group of plants may help in further manipulations and modifications of plants leading to enhanced nutritional supplementation for consumption. To estimatethe picture of genetic diversity in lentil (*Lens culinaris)*the proteomics assay was carried out. A total of 60 cultivars were collected from 60 lentil growing areas of Malakand Division Khyber Pakhtunkhwa, Pakistan. The obtained lines were biochemically evaluated for the purpose to check the genetic heterogeneity. Based on proteomics homology dendrogram and cluster plottingsorted 60 cultivars into 4 clusters i.e. Cluster-1 (sorted forty cultivars), cluster-2 (grouped single cultivar), cluster-3 (had seventeen genotypes) and cluster-4 (grouped two cultivars).Thirteen loci (bands) were spotted in the collected germplasm of lentils through SDS-PAGE**.** Intra species locus contribution toward genetic diversity (*LCTGD*) was 84.61. Out of thirteen loci, locus-1, 2, 3,4,5, 6,7,8, 9, 10 and 11 showed polymorphic.The results showed that SDS-PAGE displayed a low level Genetic polymorphism in our accessions but higher than in other legumes, and it can be increased by the addition of more extensively sourced germplasm.

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Introduction

Lentil is annual and herbaceous plant.It belongs to the genus *Lens* of the *Viceae*tribe in the *Legumnosae* (*Fabaceae*) family, commonly known as the legume family. The cultivated lentil, *Lens culinaris*spp. *culinaris*, has two varietal types: *micro-sperma* (small seeded) and*macro-sperma* (Larg seeded) (Cokkizgin and Shtaya, 2013).All members of lentils are self-pollinating, diploid (2n = 2× = 14) grain legume crop with a haploid genome size 4063 Mbp (Fikiru *et al.,* 2007).Lentil is supposed to have initiated in the Fertile Crescent of the Mediterranean region in Western Asia, dating back to the initial stages of agriculture, from where it spread to rest of the world (Duke, 1981; Sultana *et al.,* 2006). It is currently grown widely throughout the Indian Subcontinent, Middle East, Northern Africa, Southern Europe, North and South America, Australia and West Asia (Ford and Taylor, 2003). Lentil ranks seventh among grain legumes and is grown on 3.5 million hectares in more than 48 countries with a total production of over 3 million metric tons. The major lentil producing regions are Asia (58% of the area) and the West Asia-North Africa region (37% of the acreage of developing countries) (Fikiru *et al*., 2007). Lentil is matchless because it can be grown in marginal environments in which other crops cannot be cultivated (Cubero, 1981). The high protein content in lentil ranging from 19% to 36%, 55% starch, low levels of toxic and anti-nutrient factors,and its capability tosurvive underhigh range of drought and cold,are the main qualities that make this an important crop (Bhatty, 1998;Anonymous, 2003). Moreover, lentil plays a significant role in crop rotations due to its capability to fix atmospheric nitrogen (Durn *et al*.,2004;Ganjali *et al*.,2004).

Calculation of genetic diversity in lentil is required for prospective future breeding activities, in terms of broadening and maintaining the diversity of the genetic base, improving opportunities for selection of improved genetics and cultivar identification.Morphological traits are widely used to examine genetic diversity in crop species, but adversely affected by environmental fluctuations, thereby constraining the analysis of genetic variation (Nisar *et al.,* 2009). On the other hand biochemical techniques are more accurate and provide correct result because free of environmental fluctuations (Bretting *et al.,* 1995; Nisar *et al.,* 2009). Among biochemical methods, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is the most economically simple, reliable and extensively used biochemical technique in description of the genetic structure of crop germplasm (Hameed *et al*., 2009). Over the last two decades, huge interest has been focused on the use of electrophoretic procedures for reliable judgment and identification of plant varieties (Przybylska and Zimniak- Przybylska, 1995; Sultana *et al*., 2006). Electrophoresis adds information to taxonomy and should not be dissociated from morphological, anatomical and cytological observations (Piergiovanni and Taranto, 2003). SDS-PAGE is as an important genetic marker in some species, mainly in cereals in which their variability is related to technological properties of the species (Amar *et al.,* 2014).Seed storage proteins have been used as genetic markers in five major areas: 1; Analysis of genetic diversity within and between species, 2; Plant domestication in relation to genetic resources conservation and breeding, 3; Genome relationship, 4; a tool in crop improvement 5;to study taxonomical and evolutionary relations of several crop plants (Ghafoor *et al.,* 2002).In view of the importance of the crop, and the need to improve the scope and usefulness of its genetic resources, in this study it was focused to access and evaluate intra specific genetic diversity through SDS-PAGE in lentil *(lens culinaris)* collected from Malakanad Division KhayberPakhtunkha Pakistan.

Material and methods

Exploration and collection

During 2014 and 2015 different exploratory trips were arranged to 60 different lentil growing areas including Naloo, Rokhan, Ganori, Bekarai, Tallo, Randaish, Sundarawal, Shaga, Alakol, Dogar, Gandigar, Jar, Paloso, Karedara, Seratai, Sherengal,

Mano Banda, Jabar, Selkass, Karpat, Chakyatan, Patrak, Tarpatar, Jabalook, Sahib Abad, Unkar, Charagali, Kandao, Babyawar, Doroo, Barabanda, Katan-payeen, Satal, Wari, Cheragalibala, Amloknar, Darora, Dugdara, Kotkai, Rokhan, Sumkot, Ranzra, Bondaish, Barawal Mamondo, Nawagai, Zorbanda, Trakai, Loisam, Inzari, Ghondo, Smassai, Rashakai, Tangi, Khazana, Sadiq Abad, Inayat Pilla, Shenkot, Qausar and Mandal, of Malakand division, Khyber pakhtunkhwa Pakistan. A total of 60 genotypes were collected from local farmersand selected for SDS-PAGE analysis.

SDS-PAGE characterization

To estimate the picture of genetic diversity in *lens culinaris,* SDS-PAGE was carried out. For SDS-PAGE analysis single seed of each accession was ground to fine powder with the help of mortar and pestle for the extraction of proteins. About 400µl of protein extrication buffer (PEB) was added to 0.01 g of seed flour taken in 1.5ml effendorf tube. The effendorf tube was then vortexed thoroughly to homogenize the mixture. Bromophenol blue was then added as atracking dye to see the movement of protein in the gel. The homogenated samples were centrifuged at 13,000 rpm for 12 minutes at 10°C temperature.The electrophoretic procedure was then carried out using 12.75% polyacrylamide gel, resolving gel (3.0M Tris-HCl) pH9, 0.4% SDS and 4.5% stacking gel (0.4M Tris-HCl pH 7.0, 0.4% SDS). Electrode buffer (0.025 M Tris, 129 M Glycine, 0.125 % SDS) was added to the top pool of the apparatus. A 15 µl of the supernatant was loaded with the help of micropipette into the wells of the gel. Apparatus was connected with uninterrupted electric supply (100 V) until the bromophenol blue (BPB) was reached to the bottom of gel plate. The gels were then stained for an hour with the solution containing 0.2% Commassie Brilliant Blue dissolved in 10% glacial acetic acid, 40% methanol and water in the ratio of 10:40:50. Gels were de-stained in a solution containing 5% acetic acid and 20% methanol. The data was recorded on the basis of presence and absences of protein bands from the destained gel i.e. the presence of band was denoted by 1 and 0 for the absence of bands. The data obtained was subjected to Cluster analysis using software PC-ORD.

Results

SDS-PAGE was run in various combinations, and it turned out that a 12.75% acrylamide gel concentration and 15 µl samples showed the best resolution. The banding patterns of *Lens culinaris* are shown in Figure 1.Protein bands showed the cases of genetic diversity situations, which indicates that difference of each genotype from each other in a number of appeared or absent bands. It was very clear that the differences reflect the genetic polymorphism caused by genetic structure which expressed seed protein.

Two-way cluster analysis

To investigate the phylogenetic relationship and genetic polymorphism in sixty accessions of lentils cultivated in Malakand Division the Two-ways cluster analysis of molecular traits was constructed by using Ward's method represented in Fig (2). Based on the presence and absence of bands, the constructed dendrogram, divided 60 cultivars into two linkages. The Linkage-I (L-I) and linkage-II (L-II) at 25% genetic similarity or 75% disagreement level. Linkage-I bifurcated into two clusters at genetic similarity index 84% or 16% disagreement. Cluster-1 consists thirty nine genotypes, including Le-1, Le-2, Le-3, Le-5, Le-6, Le-7, Le-8, Le-9, Le-10, Le-11, Le-12, Le-13, Le-14, Le-15, Le-16, Le-17, Le-18, Le-19, Le-20, Le-21, Le-22, Le-23, Le-26, Le-27, Le-28, Le-29, Le-31, Le-32, Le-33, Le-34, Le-35, Le-36, Le-37, Le-38, Le-39, Le-40, Le-41, Le-46, Le-50, Le-58, and cluster-2 contains single genotype the Le-53 (Fig. 3.2). Linkage-II grouped 19 cultivars and subdivided into two clusters at 50% genetic similarity index (Fig. 3.2). Out of two clusters i.e. 3 and 4, the Cluster-3 clustered seventeen cultivars namely Le-4, Le-51, Le-54, Le-25, Le-24, Le-30, Le-42, Le-47, Le-48, Le-52, Le-57, Le-59, Le-60, Le-43, Le-44, Le-56 and Le-49. While cluster-4 contain only two genotypes the Le-45 and Le-55. Range of genetic diversity was in 0-84%. Maximum in Le-1, Le-2, Le-3, Le-5, Le-6, Le-7, Le-8,

Le-9, Le-10, Le-11, Le-12, Le-13, Le-14, Le-15, Le-16, Le-17, Le-18, Le-19, Le-20, Le-21, Le-22, Le-23, Le-26, Le-27, Le-28, Le-29, Le-31, Le-32, Le-33, Le-34, Le-35, Le-36, Le-37, Le-38, Le-39, Le-40, Le-41, Le-46, Le-50, Le-58, lines;while, it was minimum in Le-45 and Le-55 lines.The same pattern of phylogenetic relationship was confirmed by scattered plot. All genotypes were grouped into four clusters. Cluster-1 plotted forty genotypes, cluster-2 plotted only one genotype, cluster-3 enfolded seventeen genotypes and cluster-4 enfolded two genotypes (Fig. 3).

Locus variation

During present study intra-specific locus variation in 60 genotypes of *lens culinaris* was also identified. Among all the genotypes, thirteen loci (L-1 to L-13) were detected; out of which L-12 and L-13 were monomorphic. Furthermore, the lociL-1, L-2, L-3, L-4, L-5, L-6, L-7, L-8, L-9, L-10 and L-11spotted as polymorphic with30%, 1.67%, 10%, 3.33%, 1.67%, 3.33%, 1.67%, 8.33%, 5%, 1.67% and 8.33% variation respectively. The intra-specie comparative locus contribution toward genetic disagreement *(LCTGD)* was 84.62 in the collected cultivars (Table 1).

Table 1. Intra-specific locus variation among 60 genotypes of *Lens culinaris.*

LOCUS (L)	PRESENT	ABSENT	VARIATION	STATUS	GENETIC DISAGREEMENT (bands present)
L-1(band-1)	42(70%)	18(30%)	30%	poly	0.70
L-2(band-2)	59(98.33%)	1(1.67%)	1.67%	poly	0.98
L-3(band-3)	54(90%)	6(10%)	10%	poly	0.90
L-4(band-4)	58(96.67%)	2(2.33%)	3.33%	poly	0.96
L-5(band-5)	59(96.33%)	1(1.67%)	1.67%	poly	0.98
L-6(band-6)	58(96.67%)	2(3.33%)	3.33%	poly	0.96
L-7(band-7)	59(98.33%)	1(1.67%)	1.67%	poly	0.98
L-8(band-8)	55(91.67%)	5(8.33%)	8.33%	poly	0.91
L-9(band-9)	57(95%)	3(5%)	5%	poly	0.95
L-10(band-10)	59(98.33%)	1(1.67%)	1.67%	poly	0.98
L-11(band-11)	55(91.67%)	5(8.33%)	8.33%	poly	0.91
L-12(band-12)	60(100%)	0.00%	*NIL*	mono	1.00
L-13(band-13	60(100%)	0.00%	*NIL*	mono	1.00
locus contribution toward genetic disagreement GD=(poly loci/total loci)100	84.61				

Discussion

Electrophoresis is a powerful tool for population genetics and SDS-PAGE technology is considered as a reliable way because seed storage proteins are largely independent of environmental fluctuations (Iqbal *et al.*, 2005; Nisar *et al.,* 2007). Biochemical markers evaluate accurate genetic diversity index (Akhtar, 2001; Rabbani *et al.*, 2001).The use of genetic markers and protein profiling is also successfully used to resolve the taxonomic and evolutionary problems of several crop plants (Ghafoor *et al.*, 2002). Genetic diversity of seed storage proteins has been reported for many crops; *Lima* bean (Lioi *et al.*, 1999), *Phaseolus vulgaris* (Ferreira *et al.*, 2000), Chickpea (Ghafoor *et al.,* 2003; Nisar *et al.,* 2007;Hameed*et al.*, 2009)*Pisum sativa*(Nisar *et al.,* 2009) and *Lens culinaris* (Sultan *et al.,* 2006). As the SDS-PAGE has been used as a practical and reliable method therefore, the present study was conductedto access and evaluate intra specific genetic diversity of seed storage proteins in *lens culinaris* through SDS PAGE.

According to the results revealed by SDS-PAGE,out of total thirteen Loci (protein bands) eleven lociwere polymorphic. Out of polymorphic Loci;Locus 2, 5, 7 and 10 showed 1.67% variation, while Locus 4 and 6 showed 3.33% variation. Similarly, Locus-8 showed 8.33% and Locus-3 showed 10% variation. High intra-specific locus variation was observed in Locus-1 i.e. 30%. On the other hand Locus-12 and 13 contain 100% protein polypeptide bandstherefore marked as monomorphic loci.

Similarity of banding patterns between genotypes may be due toduplications in the germplasm, but this should be confirmed with two-dimensional electrophoresis (Picard *et al.*, 2005; Thiellement *et al.,* 1999).

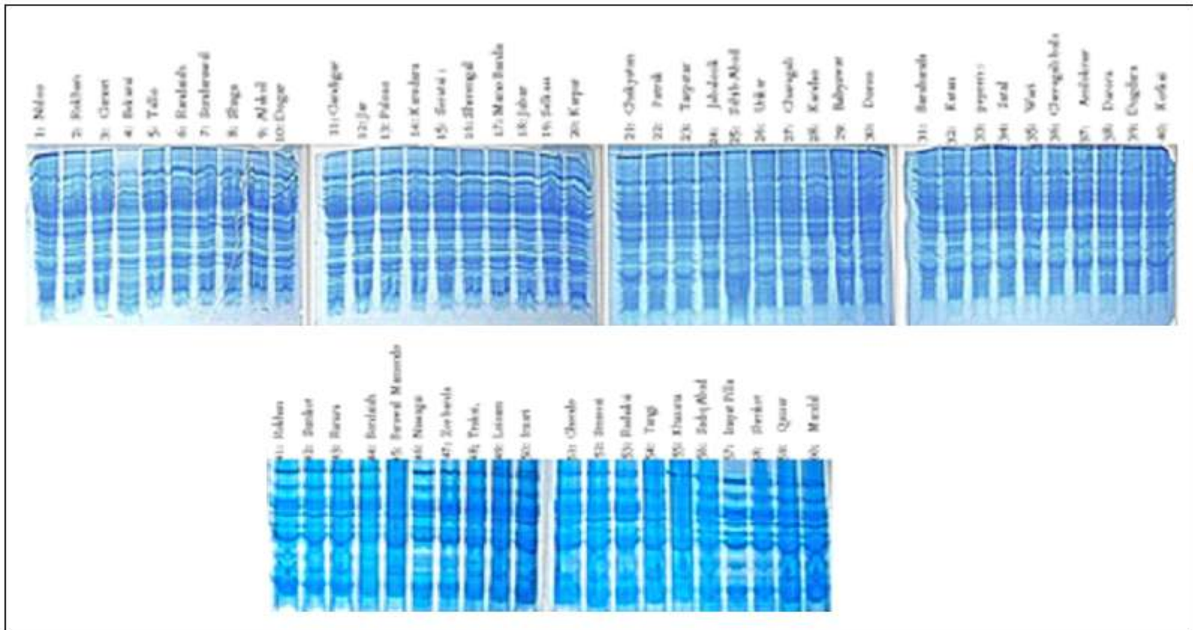

Fig. 1. Gel documentation of the 60*Lenusculinaris* genotypes collected from Malakand division Khayber Pakhtunkhwa Pakistan.

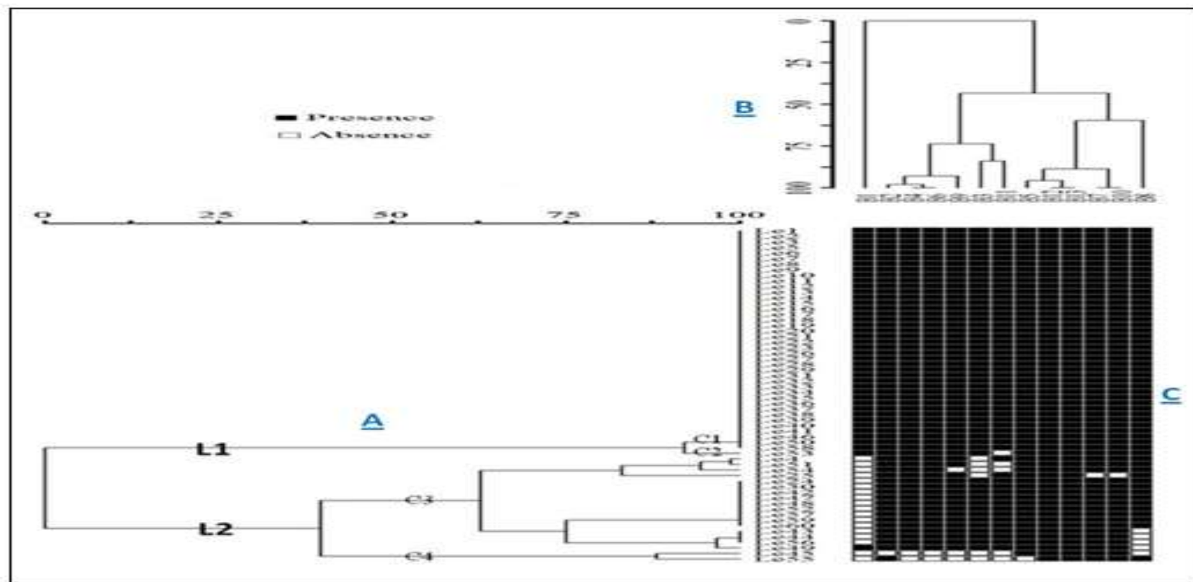

Fig. 2. Two way Cluster Analysis of Molecular Traits Matrix coding indication the presence and absence of protein bands using PCA: A; Cluster analysis of 60 among 60 cultivars of *Lens culinaris*. B; Genetic polymorphism based on protein polypeptide distributed in 60 cultivars of *Lens culinaris*. C; Zygomorph of 13 bands reported in indigenous *Lens culenaris* cultivars.

The numerical analysis (Phylogenetic tree and cluster plotting)sorted all the accessions into four clusters. It was found that each cluster had slight discriminative protein banding. The cluster analysis shows similarities amongLe-1(Naloo),Le-2(Rokhan),Le-3(Ganori),Le-5(Tallo),Le-6(Rondaish),Le-7(Sundarawa),Le-8(Shaga),Le-9 (Alakoo),Le-10(Dogdara),Le-11(Gandigar),Le-12(Jar),Le-13(paloso),Le-14(Kareda),Le-15(Seratai),Le-16(Sherenga),Le-17(ManoBanda),Le-18(Jabar),Le-19(selkas),Le-20(Karpat),Le-21(Chakyatan),Le-22(Patrak),Le-23(Tarpatar),Le-26(Unkar),Le-

27(Charagali),Le-28(Kandao),Le-29(Babyawar),Le-31(Barabanda),Le-32(katanpayeen),Le-33(Satal),Le-34(Wari),Le-35(Cheragalibala),Le-36(Amloknar),Le-37(Darora),Le-38(Dugdara),Le-39(Kotkai),Le-40(Rokhan),Le-41(Sumkot),Le-46(Nawagai),Le-50 (Inzari) and Le-58(Shenkot) genotypes. However, some minute differences in seed protein profile could be observed due to probably different environmental and climatic conditions of two regions.The accessions in one cluster are mostly identical in their protein profile and have less genetic diversity.In this study SDS-PAGE displayed low intraspecific variation as describe by sultana *et al* in 2006. SDS-PAGE should be used to select varied accessions from many sources, preferably from the center of diversity, so that a broad-based gene pool can be acquired. For improved management of the gene bank, accurate and broad knowledge of agricultural and biochemical data (proteins and DNA) is essential so that duplicates can be eradicated; this will help in compiling a core collection of lentil germplasm.

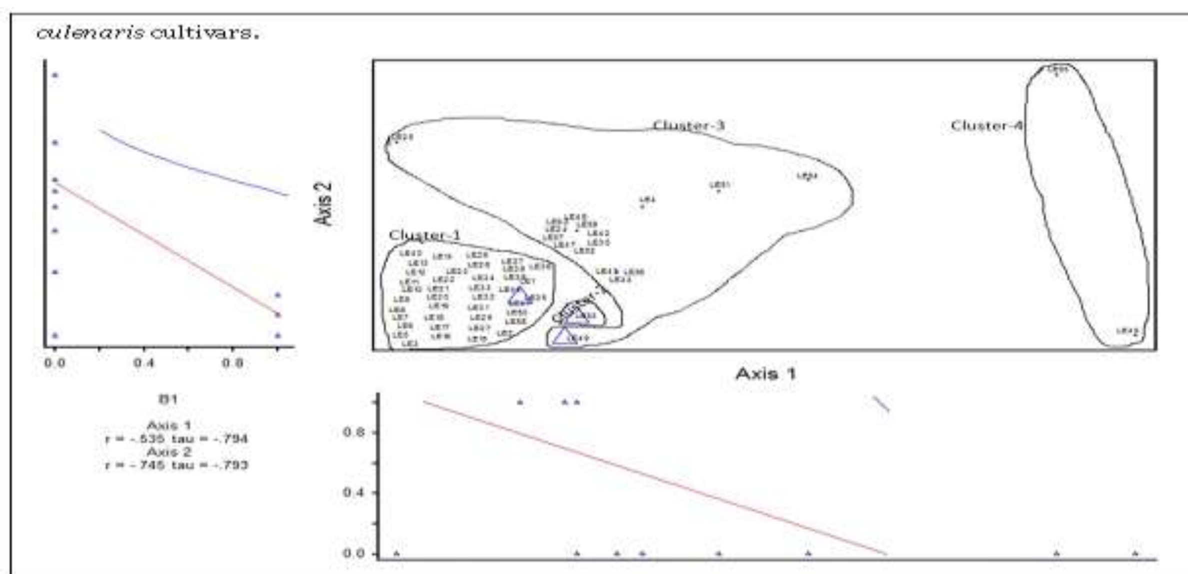

Fig. 3. Confirmation of phylogenetic relationship by scattered plot detected through cluster analysis in 60 cultivars of *Lens culinaris* collected from Malakand Division, Khyber Pakhtunkhwa, Pakistan.

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