

Full Length Research Paper

Botanical and molecular evidences of landraces from the germplasm exclusively collected from Baluchistan, a centre of diversity for *Lens culinaris*

Abdul Ghafoor^{1*} and Tayyaba Sultana²

¹Plant Genetic Resources Program, Institute of Agricultural Biotechnology and Genetic Resources, National Agricultural Research Centre, Islamabad, Pakistan.

²GC University, Faisalabad, Pakistan.

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Botanical descriptors, total seed proteins, isozymes and RAPD markers were applied to identify landraces from indigenous lentil germplasm exclusively collected from the province of Baluchistan, Pakistan. The Germplasm revealed the prevalence of landraces, especially on the basis of isozymes and RAPD markers. Diversity explored through various techniques revealed validity irrespective of the sample size from a particular district, RAPD being the best choice for investigating both inter and intra-accession variation that is needed to extend to more germplasm study along with botanical descriptors.

Key words: DNA marker, genetic resources, isozymes, lentil, SDS-PAGE, RAPD markers.

INTRODUCTION

Lentils are one of the oldest food crops of mankind that researchers have traced back to 7000 - 8000 BC and probably originated from fertile crescent from where it spread to adjacent regions of west Asia and Africa and later to Europe and north America (Hawtin et al., 1980; Anonymous, 2003). Major lentil producing regions are Asia and north Africa region. The crop has the ability to grow under water stress conditions and survive under high range of drought and cold (Cubero, 1981). Plant descriptors coupled with molecular markers provide a valid evidence of diversity as these are least affected by environmental fluctuations (Ahmad et al., 1997; Jha and Ohri, 1996; Margale et al., 1995).

Electrophoresis for various biochemical and molecular markers along with field evaluation adds information to taxonomy and should not be disassociated from morphological, anatomical and cytological observation (de Vries, 1996; Piergiovanni and Taranto, 2003; Sultana et al., 2006; Sarker and Erskine, 2006; Sultana and Ghafoor 2008). Among biochemical techniques, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), isozymes and randomly amplified polymorphism

DNA (RAPD) are widely used due to their validity and simplicity in describing the genetic structure of crop germplasm (Murphy et al., 1990). Seed protein profiles and molecular markers obtained by electrophoresis have been successfully used to study taxonomical and evolutionary relationships of several crop plants (Gepts et al., 1989; Rao et al., 1992; Ghafoor and Arshad, 2008).

With the advancement in biological research, DNA-based markers provide powerful and reliable tools for discerning variations within crop germplasm and for studying evolutionary relationships (Virk et al., 1995). For better management of genebank, a precise comprehensive knowledge of agricultural and biochemical data (protein and DNA) is essential. The molecular markers have been used for estimating genetic variation at population level and among closely related species (Nienhuis et al., 1995). No single method is adequate for assessing genetic variation because the different methods sample genetic variation at different levels and differ in their power of genetic resolution as well as in the quality of information content. The present study was undertaken to assess inter and intra-accession variation to investigate the extent of landraces in lentil germplasm exclusively collected from Baluchistan that is known as centre of diversity for many crop species including lentil (Salamini et al., 2002).

*Corresponding author. E-mail: ghafoor59pk@yahoo.com.

MATERIALS AND METHODS

17 bulk populations/accessions exclusively collected from farmers' fields in the province of Baluchistan were studied for plant descriptors; total seed proteins, 6 isozymes [Aspartate amino transferase (AAT), Leucine aminopeptidase (LAP), malic enzyme (ME), shikimate dehydrogenase (SKDH), phosphoglucose mutase (PGM) and phosphoglucose isomerase (PGI)] and random amplified polymorphic DNA (RAPD). For all the 4 types of data (botanical descriptors, seed proteins, isozymes and RAPD) ten plants were sampled at random to study inter and intra-accession variation within each accession. For isozymes, 2 to 3 foliage leaves of young seedlings were crushed to extract protein in about 0.2 to 0.3 ml of the appropriate buffer (Tris-HCl extraction buffer for all system 1 ml, 2 mercaptoethanol 1 μ l) with a glass rod. For electrophoresis, the gels were prepared using 23.5 g of hydrolyzed potato starch (Sigma S-4501) in 220 ml of gel buffer (Tris citrate buffer pH - 8.4). Paper wicks were soaked in crude extract of leaves and were loaded on starch gel. The electrophoresis was carried out at 50 milliamps for the first 10 min, then wicks were removed and electrophoresis was continued for 4 h at 4°C. After completion of electrophoresis, the gel was sliced horizontally in 4 or more sections, depending on the thickness of the gel. The gel slices were placed into individual staining trays and processed individually according to the isozyme to be studied. The RAPD analysis was performed for each accession sown in small plastic pots under green house. Approximately 0.1 g of primary leaves was cut with disinfected scissors. After washing the leaves samples with distilled water, leaves were crushed in the 1.5 ml appendorf with pellet mixers. Extraction of DNA was performed by using isoplant kit. The DNA pellets were dried in vacuum for 2-3 min at room temperature then dissolved in the solution of TE buffer and RNase and finally stored at 4°C. The DNA dilution for PCR was determined by visual comparison with lambda DNA (λ Hind III/ECORI) of known concentration.

The PCR was performed in the 0.5 ml reaction tubes with 5 μ l of DNA solution containing 2.5 ng of genomic DNA, 1 μ l of primer, 0.2 μ l of 20 mM dNTP, 2 μ l of 10 x buffer, 1.6 μ l $MgCl_2$ and 0.2 μ l of Taq polymerase. The volume in the tube was made 20 μ l by adding 10 μ l of H_2O . 20 operon oligonucleotide primers were used for PCR. The reaction mixture was overlaid with a drop of mineral oil to avoid evaporation. Amplification reaction was carried out at the Perkin Elmer thermocycler with 94°C for 1 min, 30°C for 1 min and 72°C for 2 min for 45 cycles. Prior to storage at 4°C annealing was completed at 72°C. The amplified fragments were recorded after the electrophoresis on 1% agarose gels and electrophoresis was conducted at 100 constant voltage was conducted for 70 min. The molecular marker \times 174/Hae III digest was used as 5 μ l. After the completion of electrophoresis, the gels were stained in ethidium bromide (200 ml TE and 10 μ l ethidium bromide). Amplification was observed on the UV illuminator and Polaroid 667 was used for photographs.

Data on stem colour, flower colour, pedicle colour, growth habit, tendrils, hairiness, leaf pubescence, leaflet size, pod pigmentation, pod indehiscence, presence/absence of beak, seed coat colour, seed coat pattern, seed coat pattern colour and cotyledon colour were recorded according to IPGRI descriptors (IBPGR, 1985). The data for seed proteins, isozymes and RAPD markers were "1" for the presence and "0" for the absence of a band. To avoid taxonomic weighing, the intensity of bands was not taken into consideration, only the presence of the bands was taken as indicative. Since DNA samples consisted of bulk sample of DNA extracted from individual plants, a low intensity for any particular fragment may be explained by the lesser representation of that specific sequence in the bulk sample of DNA. Therefore, the intensity of bands was not taken into account and the fragments with the identical mobility were considered to be the identical fragments. Only major bands were scored and faint bands were not

considered. The molecular size of the amplification products was measured with Lambda DNA (λ Hind III/ECORI). Polymorphic bands were scored and used for further analysis. Intra and inter-accession diversity was estimated for each accession on 10 plant samples for all the techniques. On the basis of data, percent deviation for each trait was computed and then aggregated for each set of accessions. The variation was classified as low (< 25% plants differed from each other within one collection site to 100%) and then termed as high (> 50% plants differed within one accession), medium (< 50% plants differed within one accession) and low (< 25% plants differed within one accession) genetic diversity.

RESULTS

Lentil germplasm explored for genetic diversity based plant descriptors; SDS-PAGE, isozymes and RAPD markers revealed differences among various techniques that indicated the validity of each technique for one or the other locus. Table 1 presents polymorphism for all the 4 techniques used in the present study. 15 plant descriptors were recorded and variation was observed for stem colour, presence of beak and tendril tendency. For total seed protein, among 8 polymorphic bands, 3 (B_3 , B_{15} , B_{21}) were polymorphic in 2 accessions only. Intra-accessions diversity for 2 isozymes (AAT and ME) was observed in 8 accessions, whereas for RAPD all the accessions exhibited polymorphism for one or the other DNA marker which were 28 out of 102. Seed protein profiles and molecular markers obtained by electrophoresis have been successfully used to study taxonomical and evolutionary relationships in crop plants (Popelka et al., 2004; Yüzbaşıoğlu et al., 2008).

Among all the accessions, 55 protein bands were recorded ranging from the molecular weight of 14 to 66 kDa and out of these 8 were polymorphic. Out of 6 isozymes, 2 (AAT and ME) exhibited variation and hence could be used for exploring genetic diversity in lentil germplasm, whereas all others did not reveal much polymorphism. The accessions (PAK66301 and PAK66304) were found with maximum variation. Overall, a low level of genetic variation was disclosed by SDS-PAGE indicating that genetic variation within lentil germplasm collected from Pakistan is narrow, which may necessitate the use of novel techniques for creation of genetic variability in lentil. Due to low utilization of the information obtained from the plant descriptors, SDS-PAGE protein profiles and isozymes analysis, the RAPD analysis was considered powerful and efficient technique to explore genetic diversity and phylogenetic relationship. The extent of genetic diversity by different techniques for geographic distribution was observed irrespective of the sample size or geographic pattern. In the present investigation 10 primers, namely, OPA-01 (CAGGCCCTTC), OPA-06 (GGTCCCTGAC), OPA-07 (GAAACGGGTG), OPA-08 (GTGACGTAGG), OPA-10 (GTGATCGCAG), OPA-11 (CAATCGCCGT), OPA-16 (AGCCAGCGAA), OPA-17 (GACCGCTTGT), OPA-18 (AGGTGACCGT), OPA-20 (GTTGCGATCC), were poly-

Table 1. Polymorphism in lentil germplasm collected from Baluchistan for botanical descriptors, SDS-PAGE, isozyme and RAPD markers.

Accession	District	Heterogeneous/polymorphic for			
		Qualitative traits	SDS-PAGE	Isozyme	DNA
PAK66140	Nag	Stem colour, Beak	None	AAT-2,	OPA11 (L1*), OPA07 (L1)
PAK66141	Nag	None	None	AAT-2, AAT-4	OPA11 (L1), OPA20 (L1), OPA01 (L1)
PAK66152	Kalat	Stem colour	None	AAT-2	OPA11 (L1)
PAK66154	Khuzdar	None	None	AAT-1, AAT-2	OPA20 (L1)
PAK66156	Turbat	Tendril	None	None	OPA11 (L1), OPA07 (L1), OPA01 (L1)
PAK66157	Khuzdar	Stem colour, beak	B ₃ , B ₁₅	None	OPA11 (L1), OPA20 (L1), OPA01 (L1), OPA10 (L1)
PAK66158	Khuzdar	Tendril, Beak	None	None	OPA11 (L1), OPA01 (L1), OPA16 (L1)
PAK66301	Chaghi	Stem colour, Beak	None	AAT-1, ME-2, ME-3, ME-4	OPA11 (L1), OPA20 (L1), OPA06 (L2), OPA16 (L1, L2), OPA18 (L1), OPA08 (L1, L3), OPA17 (L1, L2, L3)
PAK66304	Kharan	Stem colour	B ₃ , B ₂₁	AAT-1, AAT-2, ME-3, ME-4, ME-5	OPA11 (L1, L2), OPA07 (L1, L2, L3, L6), OPA06 (L1, L2, L3), OPA16 (L1, L2), OPA18 (L1), OPA10 (L1, L2, L3, L4, L5), OPA08 (L2, L3), OPA17 (L1, L3)
PAK66305	Kharan	Stem colour	None	None	OPA01 (L1), OPA16 (L2), OPA08 (L1), OPA17 (L1, L3)
PAK66307	Kharan	Tendril	None	None	OPA07 (L4, L5), OPA01 (L1), OPA16 (L2), OPA08 (L1)
PAK66308	Kharan	Tendril	None	None	OPA07 (L4, L5), OPA06 (L3), OPA18 (L1), OPA08 (L1), OPA17 (L1, L3)
PAK66309	Kharan	Stem colour, Tendril	None	AAT-1	OPA07 (L4), OPA20 (L1), OPA01 (L1), OPA16 (L1), OPA18 (L1), OPA08 (L1), OPA17 (L1, L3)
PAK66311	Kharan	Tendril	None	None	OPA11 (L1), OPA07 (L4, L5), OPA06 (L2), OPA16 (L2), OPA08 (L1), OPA17 (L3)
PAK66321	Khuzdar	Stem colour	None	None	OPA11 (L1), OPA01 (L1)
PAK66324	Mastung	Tendril	None	None	OPA11 (L2), OPA20 (L1), OPA01 (L1), OPA16 (L1), OPA10 (L2)
PAK66325	Mastung	None	None	AAT-4	OPA11 (L1), OPA07 (L1), OPA07 (L5), OPA01 (L1)

*Polymorphism for the locus amplified for particular primer from Operon series A. The primer code and its sequence have been included in the text.

morphic, which generated 102 fragments. Out of these 28, (27.5%) were polymorphic in one or another of the lentil accessions.

Use of 28 polymorphic RAPD markers for 17 accessions (10 samples for each accession) generated information to establish genetic relationship and thus inter and intra-accession diversity was analyzed for cluster analysis (Nei and Li 1979). It was obvious from the dendrogram presented in Figure 1 that diversity was observed for both within as well as between accessions and it was higher for inter rather than intra-accession. 2 main groups were observed and most of the accessions indicated intra-accession variation of varying degrees. The landrace PAK66304 was the most diverse and all the samples of this accession were grouped together with low level of similarity.

Diversity observed for all the techniques was quantified as mentioned in materials and methods part was depicted in the Figure 2. Within Baluchistan, varying degrees of diversity were observed; the accessions collected from Turbat gave low diversity for plant descriptors and RAPD, whereas no polymorphism was observed for total seed proteins and isozymes. On the contrary the material collected from Kharan gave high diversity for RAPD and medium for other evaluation techniques and this was followed by the material collected from Khuzdar. Inter and intra-accession diversity for the germplasm collected exclusively from Baluchistan indicated the validity of RAPD to investigate intra-accession diversity where other techniques failed. It was concluded that SDS-PAGE could not be used for identification of land-

aces from heterogeneous populations. Apparently accessions with intra-specific variation for botanical descriptors remained less diverse in SDS-PAGE. The RAPD markers were even able to disclose intra-accession variation that was not possible by other techniques.

DISCUSSION

Variation between and within populations of crop species is useful for analyzing and monitoring germplasm during the maintenance phase and predicting potential genetic gain in a breeding programme (Hayward and Breese, 1993; Toklu et al., 2009). Intra-accession variation for stem colour, tendril and beak on the pod gave

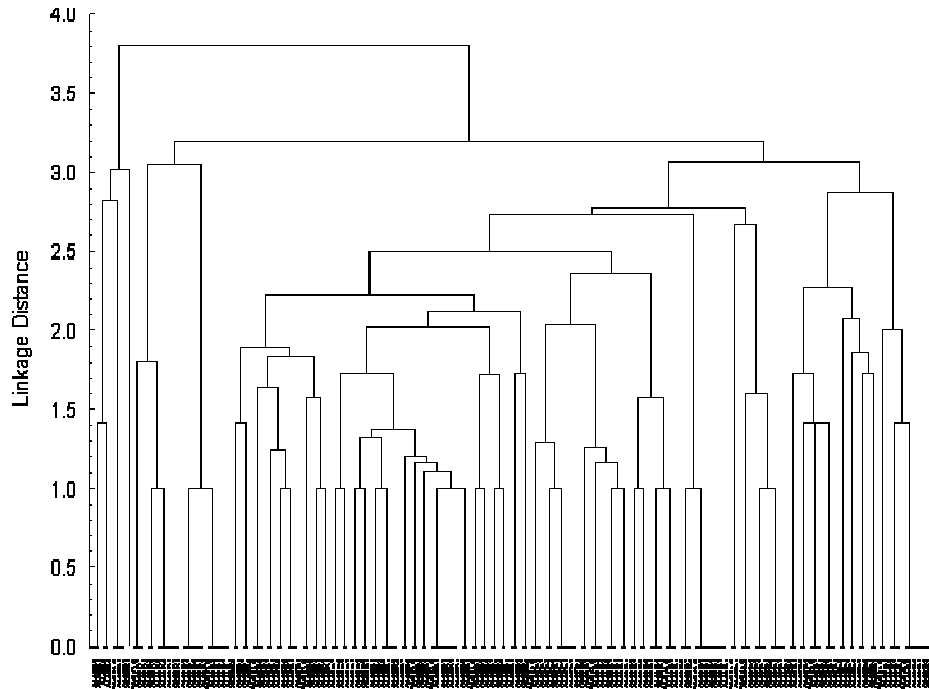


Figure 1. Cluster diagram of 17 indigenous *Lens culinaris* landraces based on RAPD markers. Ten samples were analyzed for each sample.

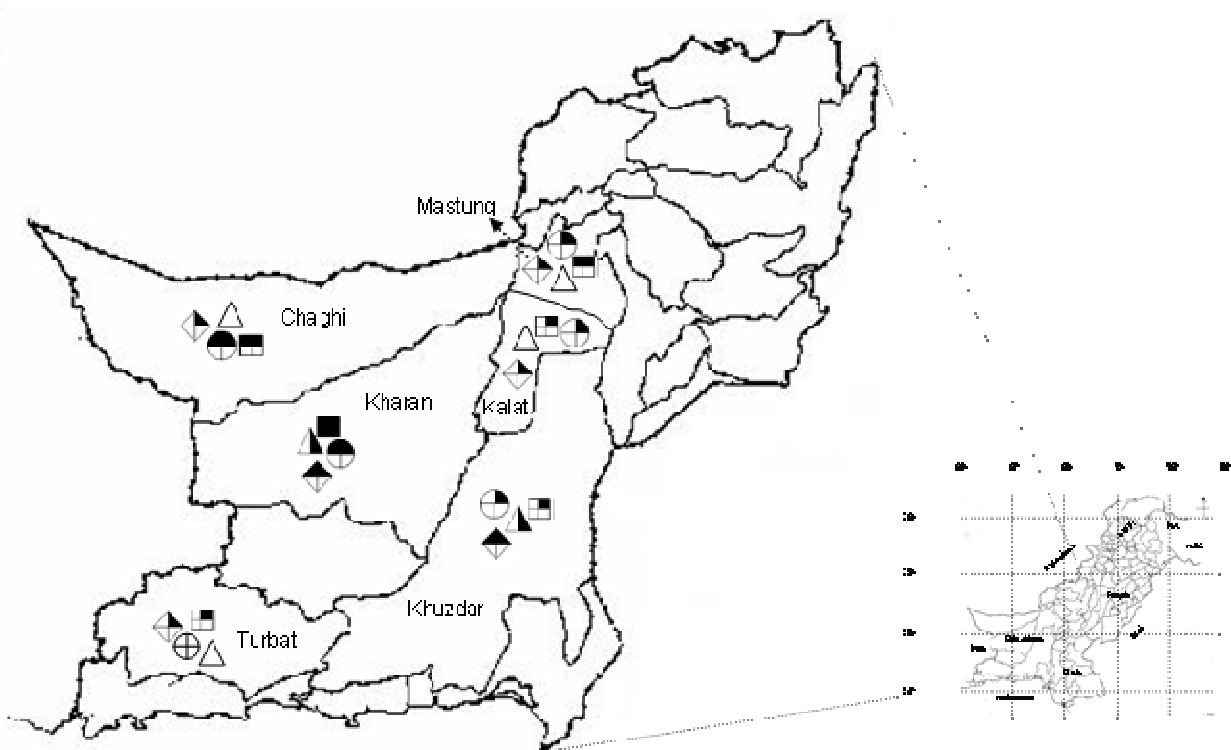


Figure 2. Genetic diversity in indigenous lentil germplasm exclusively collected from Baluchistan for plant descriptors (◊), total seed protein profile (△), isozymes (⊕) and RAPD markers (⊞). The symbols completely filled represent high genetic diversity, half medium, one fourth low and blank represent no diversity for particular technique.

indication for the prevalence of landraces. Muehlbauer and Slinkard (1981) reviewed the genetics of Lens and listed 12 genes which account for morphological and seed variation in lentil. Seed proteins have been successfully used to study the variation of seed storage protein based on geographic distribution (Erskine and Muehlbauer, 1991; Piergiovanni and Taranto, 2003; Sultana et al., 2006; Yüzbaşıoğlu et al., 2008). In our study intra-specific variation was limited and it was concluded that SDS-PAGE alone did not exhibit high level of intra-specific variation (Thiellement et al., 1999). Diverse accessions based on SDS-PAGE are suggested to be acquired from various sources to build a broad based gene pool with maximum variability. It has already been observed by many researchers that isozymes give less polymorphism as compared to DNA markers (Erskine and Muehlbauer, 1991). In most of the cases previous literature is available on isozyme especially on inheritance and linkage due to co-dominance nature rather for genetic diversity (Tahir et al., 1993, 1995).

The RAPD has been found important to resolve various levels of inter-and intra-specific polymorphism, which facilitates assessment of genetic relationships, definition of regional grouping and identification of individual accessions (Skroch and Nienhuis, 1995; Virk et al., 1996; Babayeva et al., 2009). Our study has shown that the RAPD is very efficient in the production of DNA polymorphism in lentil for studying intra-accession variation. The methodology is relatively simple to perform, rapid and amenable to automation. The protocol is ready to use in breeding, for registration and control of the distribution of commercial cultivars, the control of seed purity and for the cataloguing of accessions in germplasm collection as well (Masood et al., 2003). In future prospects, the results of this study can lead to a creation of group/variety-specific probes, using PCR primers (Paran and Michelmore, 1993) or RFLP (Schulz et al., 1994). The results can also be used for mapping purpose (Kiss et al., 1993) as well as the rapid generation of markers for important agronomic traits (Timmerman et al., 1994).

Polymorphic fragments were the highest in PAK66304 and were followed PAK66301 that indicated nature of diversity in various accessions used in the present study. The accessions with diverse pattern for RAPD are suggested for use in further study and to select parents for inheritance or linkage groups (Eujayl et al., 1997, 1998). Grouping germplasm into geographical entries and elucidating affinities among these groups can define gene pools and determine gene flow among populations. Variation on the basis of isozyme could identify even intra-accession variation if a particular isozyme is used with polymorphic nature for a particular locus. This enhances the validity for studying segregating populations for gene mapping (Gutierrez et al., 2001). Varying degrees of diversity in the material collected from various districts of Baluchistan indicated the presence of indigenous landraces especially in the material collected from Kharan and Khuzdar districts.

Genetic variation obtained by isozyme markers was not enough, though lentil germplasm chosen for isozyme studies were from most important region of diversity. Kongkiatngam et al. (1995) reported that all naturally occurring populations of self-fertilizing subterranean clover were found to be isozymically homogeneous. In routine variety identification, the problems can arise with the RAPD pattern reproducibility and the evaluation of fingerprints when the assays are performed in various laboratories and by different scientists (Samec and Nasinec, 1996). Sonnante and Pignone (2007) investigated eleven landraces from Italy for ISSR markers on 15 randomly chosen individuals for each landrace, whereas we used 10 random samples for each accession /landrace. Therefore, it is always necessary to perform control reactions with standard genomic DNA and sampling protocols investigate genetic diversity within plant species.

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