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Comparative Analysis of Genetic Diversity among Grass Pea Landraces as Detected by Random, Semi Random and Morphological Markers

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Abstract: This study was carried out to evaluate morphological traits of the grass pea landraces using a randomized complete block design with 3 replications at Research Farm of Isfahan University of Technology and evaluation genetic diversity of 20 grass pea landraces from various locations in Iran were investigated using 32 RAPD and ISJ primers at Institute of Agriculture of Biotechnology of University of Zabol. Analysis of variance indicated a highly significant differences among 20 grass pea landrace for the morphological traits. Average of polymorphism percentage of RAPD primer was 73.9%. Among used primer, 12 random primers showed polymorphism and a total of 56 different bands were observed in the genotypes. Jafar-Abad and Sar-Chahan genotypes with similarity coefficient of 66% and Khoram-Abad 2 and Khoram-Abad 7 genotypes with similarity coefficient of 3% were the most related and the most distinct genotypes, respectively. Fourteen primers out of 17 semi random primers produced 70 polymorphic bands which included 56% of the total 126 produced bands. Genetic relatedness among population was investigated using Jacard coefficient and Unweighted Pair Group Mean Analysis (UPGMA) algorithm. The result of this research verified possibility of use of RAPD and ISJ markers for estimation of genetic diversity, management of genetic resources and determination of repetitive accessions in grass pea.

Key words: Grass pea, genetic diversity, RAPD, ISJ, morphological traits

INTRODUCTION

Genetic diversity is the basis for the genetic improvement. Different methods have been used to assess genetic diversity. This information can be obtained from pedigree analysis, morphological traits or using molecular Marker (Pejic *et al*., 1998). The genus lathyrus is a member of the visia tribe (family fabaceae). The genus consists of about 160 annul and perennial species (Campbell, 1997). The species are separated into 13 sections based on morphological traits (Campbell, 1997). The grass pea (*Lathyrus sativus*) has over the past decade received increased interest as a plant that is adapted to arid and semi arid condition and contains high levels of protein, a component that is increasingly becoming hard to acquire in many developing area. Many works based on morphological characters, cytology and enzyme electrophoresis have been used to study the diversity and phylogeny of species of the Grass pea (Hossaert and Valero, 1986; Yunus and Jackson, 1991).

There have been a number of evaluations of *L. sativus* germplasm to study the variation in it and related or wild species (Jackson and Yunus, 1984). There also have been evaluations of the nutritional value (Dutta *et al*., 1982; Deshpande and Campbell, 1992), of which several have focused on the forage, fodder or feed value (Ghobrial *et al*., 1983; Somaroo, 1988; Keatinge *et al*., 1991). Several of these studies compared *L. sativus* collected locally with collections from other areas. It should be noted that in some of the studies only one or two lines have originated from a given country while a large number of lines have been evaluated from another area or country. While these evaluations provide very important data on the geographic variability that exists one must also be aware that a single line may or may not be representative of an area and therefore exercise caution about the conclusions that are drawn. A strong epidemiological association is known to exist between the consumption of grass pea and a motor neuron disease called lathyrism (paralysis of lower limbs). A neurotoxin, β-N-oxalyl-L-a, β-diaminopropionic acid (ODAP also known as BOAA) has been identified as the causative principle for lathyrism and is present in all parts of the plant (Campbell *et al*., 1994). Random Amplified polymorphism DNA (RAPD) markers represent amplification products from a Polymerase Chain

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Reaction (PCR), utilizing arbitrary primers and genomic DNA (Karp *et al*., 1997). RAPD markers have been demonstrated to be useful for the studies of taxonomic identities, systematic relationships, population genetic structure, species hybridization and parentage identifications (Fahima *et al*., 1999). The RAPD system, which is useful for many crops, is not suitable for the genetic analysis of the large and complex genomes of such as cereals (Devos and Gale, 1992). The main reason for this is the low rate of polymorphisms detected by RAPD primers, along with the difficulties with the reproducibility of the results. Some approaches aimed at improving the RAPD analysis, such as the selection of a large number of primers or changes in detection techniques, markedly increase the time and cost of PCR analysis. Therefore, the development of alternative PCR procedures that use single primers would seem to be an important step. Another PCR based system with semi-random primers targeting the Intron-Exon Splice Junction (ISJ), proposed by Weining and Henry (1995) and developed by Rafalski *et al*. (2002).

Genetic diversity assessments of numerous crop species have been conducted with DNA markers alone, or in tandem with morphological analyses (Karp *et al*., 1996; Yee *et al*., 1999).

In this study, we compare the productivity of molecular markers (RAPD and ISJ) and morphological marker for examining the levels of genetic variation within 20 landraces from a wide range of geographical origins of Iran.

MATERIALS AND METHODS

Plant material and morphological trait: The germplasm used in this study consisted of twenty landraces varieties of altitude region of Iran (Table 1). These landraces were provided by Center of Agricultural Research in Zabol. The field experiment was carried out in agricultural research of Isfahan University of Technology at 12th March 2006. The experiment field was arranged as a complete randomized block design with 20 landraces in 3 replications. Irrigation was applied to maintain soil moisture through the growing season. Weeds were controlled through weeding. Twelve morphological traits were evaluated including days to 50% flowering, days to maturity, plant height, No. of branches per plant, No. of pods per plant, pods length, pods width, No. of seed per pod, biological yield, yield per hectare, ODAP% and 1000 seed weight. Results data were subjected to statistical analysis using SAS/PC version 9.1 (SAS, 1999) and NTSYS pc2.0 (Rohlf, 1998). Principal components

Table 1: Names, city, origin and elevation for 20 grass pea landraces used in this study

Names	Origin	Elevation (m)
LS 11001	Baoonat	1250
LS 11201	Shiraz	1500
LS 11312	Eghlid	1400
LS 5001	Khoramabad	2300
LS 5002	Khoramabad	2300
LS 5003	Khoramabad	2300
LS 5004	Khoramabad	2300
LS 5005	Khoramabad	2300
LS 5006	Khoramabad	2300
LS 5007	Khoramabad	2300
LS 17131	Naghade	1900
LS 1312	Semirom	2450
LS 1313	Semirom	2450
LS 1663	Safashahr	1300
LS 1299	Safashahr	1300
LS 1913	Sanandaj	2150
LS 1471	Norabad	1500
LS 1133	Ardebil	2600
LS 1222	Moghan	1600
LS 1456	Sedeh	1450

grouping of the traits was employed to examine the percentage contribution of each trait to total genetic variation. Cluster analysis based on similarity matrices was also employed on agro-botanical data using the Un-Weighted Pair Group Method with Arithmetic mean (UPGMA) to obtain a dendrogram. Analysis of variance was carried out for all traits using PROC GLM of SAS 8.1 (SAS, 1999). The model applied to field and greenhouse data separately was:

$$Y_{ij} = l+p_i+b_j+E_{ij}$$

where, Y_{ij} is the value recorded on the plant from population i in replication j, p_i is the fixed population effect, b_j is the fixed replication effect and E_{ij} is the residual random term including experimental error and individual effect of replication j in population i. The F statistic for the population effect tested against the residual term was used to check whether the experimental design was able to discriminate the populations for the trait submitted to the analysis of variance. A Pearson correlation matrix, including was computed using PROC CORR (SAS, 1999). A principal component analysis was computed from the correlation matrix of traits exhibiting significant population effect in analysis of variance (PROCPRINCOMP of SAS 8.1), using data from the 20 landraces.

The 20 plant subjected to molecular analysis were germinated, then transferred to the greenhouse at research form of Zabol University and installed in pots with 10 cm^3 soil capacity. Young leaflets (500 mg) of the 20 plants were harvested and DNAs were extracted according to describe by Dellaporta *et al*. (1983). DNA concentration and quality were measured in

1.2% concentration agarose gel using phage λ DNA as a standard. The same DNA samples used in the RAPD and ISJ analyses.

RAPD: A total of 10 primers were selected according to another study on the grass pea (Chtourou *et al*., 2001) (Table 2).

Each amplification was performed in a reaction volume of 25 μL containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mg mL^{-1} gelatin, Triton×100 0.1%, 0.1 mM of each dATP, dCTP, dGTP and dTTP (Sinagene), 15 ng of random primer, 50 ng of genomic DNA and 1 unit of Taq polymerase (Sinagene). Amplification was carried out in a mastercycler (eppendorf) as follows: 1 cycle of 5 min at 94°C, 1 min at 36°C and 2 min at 72°C; 40 cycles of 50 sec at 94°C, 1 min at 36°C and 2 min at 72°C; 1 cycle of 50 sec at 94°C, 1 min at 36°C and 10 min at 72°C.

Table 2: Nucleotide sequence of selected primers with the number of amplified products for RAPD primers

Cod	Sequence 5' to 3'	No. of amplified products	Percentage of polymorphism
OPG11	GACCGCTTGT	10	40
OPG08	GTGACGTAGG	10	70
OPG13	CAAACGTCGG	9	77
OPG10	TCCGCTCTGG	9	73
OPG07	CCGCATCTAC	6	66
OPG04	GTGCCTAACC	5	60
OPG03	TGCCCGTCGT	5	60
OPG02	CTCTCCGCCA	4	100
OPG14	GGAAGTCGCC	4	100
OPG17	CTGCATCGTG	4	100

Table 3: Nucleotide sequence of selected primers with the number of amplified products for semi random primers

Cod	Sequence 5' to 3'	No. of amplified products	No. of polymorphic bands
ET32	ACTTACCTGGGCACG	3	2
ET34	ACCTACCTGGGCGAG	6	4
ET35	ACCTACCTGCCGCAG	7	6
ET38	ACTTACCTGAGGCGCGAC	8	4
ET39	ACTTACCTGCTGGCCGGA	8	5
ET40	ACTTACCTGGCCAGCTGC	8	4
ET41	ACTTACCTGCCTGCCGAG	4	4
ET42	ACTTACCTGGCACGCCTC	7	4
IT1	CCGGCAGGTCAGGTAAGT	5	4
IT3	GCAGAGGGCCAGGTAAGT	8	7
IT4	CTGCGGCCACAGGTAAGT	5	4
IT5	GGCGGAGAGCAGGTAAGT	8	7
IT31	GAAGCCGCAGGTAAG	7	7
IT33	GATGCCCCAGGTAAG	7	7

ISJ: Amplification was performed in a mastercycler (eppendorf) programmed for 40 cycles of 20 sec at 94°C, 40 sec at 37°C and 1 min at 72°C. PCR primed with semi-random oligomers was carried out in 25 mL reaction mixtures containing 40 ng of template DNA, 1X PCR buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 200 mM dNTP, 1.2 mM primer and 1 Unit DNA polymerase. Two thermocycling programs were used for two different groups of primers (ET12 and ET15) (Table 3). The amplification conditions for the ET12 primers were: 7 cycles of 40 sec at 94°C, 1 min at 40°C, 2 min at 72°C, followed by 5 min at 72°C and 33 cycles of 40 sec at 94°C, 1 min at 44°C, 2 min at 72°C, followed by 10 min at 72°C. All the plants were analyzed at least twice with the same primer. The amplification products were separated by electrophoresis on 1.4% (RAPD) or 1.5% (Semirandom) agarose gel in 1X TAE buffer. The DNA was stained with ethidium bromide and visualized under UV light. For each primer, the consistent amplified products were recorded. Each RAPD and ISJ markers was assumed to correspond to a locus with two alleles (presence and absence of the band).

RESULTS

The landraces in each replicate differed significantly to all trait except 1000 seed weight No. of pod per plant (Table 4).

Landrace LS11001 that belongs to Baoonat recorded the earliest flowering day (53 day), earliest maturity date (105 day) as well as longest pod width (1.9 cm) and ODAP% (0.87%). However landrace LS5007 originated from Lorestan province recorded lowest number of pods per plant (10), number of seed per pod (1.92), biological yield (0.6 g) and yields in hectare (989.3 kg) whereas this landrace had longest pods (7.13 cm). There was a significant difference between landrace LS11312 belongs to Eghlid and another population for yields in hectares (3245.2 kg ha^{-1}). It should be noted that most of the khoramabad populations originated from Lorestan province had short plant height and high ODAP% with all traits. The number of pod per plant had a negatively significant correlation with seed index and biological yield (Table 5). The analysis of correlation for the data from field indicated that the yield per hectare had positive and

Table 4: Mean square from analysis of variance for random complete block design for 13 traits measured in 20 grass pea landrace

SOV	df	Day to 50% flowering	Day to maturity	Plant height	Branch/ plant	Pod/ plant	Pod/ length	Pod/ width	Seed/ pod	Biological yields	Yield/ (ha)	ODAP (%)	1000 seed weight
Replication	2	0.15	0.172	1.19	0.27	0.11	0.00	0.66	0.29	0.15	0.11	0.15	0.14
Landrace	19	83.40	112.150	607.25	84.76	1690.20	1.04	0.89	151.16	21.76	208420470.01	0.011	1573.06
Error	38	11.50	41.030	9.49	35.50	96.11	0.00	0.00	0.11	0.98	1581.2	0.00	1891.20

Table 5: Correlation coefficient of 13 traits used in characterizing 20 grass pea landraces

SOV	Day to 50% flowering	Day to maturity	Plant height	Branch/ plant	Pod/ plant	Pod/ length	Pod/ width	Seed/ pod	Seed index	Biological yield	Yield/ (ha)
Day to 50% flowering	1	0.67**	0.51**	-0.43*	0.22	0.06	-0.12	0.19	0.29	0.57**	0.66**
Day to maturity		1.00	0.61**	-0.29	0.17	0.28	0.18	-0.18	0.33	0.46*	0.75**
Plant height			1.00	0.26	-0.37	0.18	-0.39	0.21	-0.16	0.51**	0.55**
Branch/plant				1.00	0.61	-0.29	0.34	-0.12	0.18	0.65**	0.63**
Pod plant					1.00	0.31	-0.26	-0.47*	-0.48*	0.14	0.19
Pods length						1.00	0.69**	0.49**	0.39*	0.14	0.17
Pod width							1.00	0.45*	0.36	0.11	0.12
Seed/pod								1.00	-0.47	0.17	0.31
Seed index									1.00	-0.24	0.11
Biological yield										1.00	0.55**
Yields/ (ha)											1.00
ODAP (%)											
1000 seed weight											

**p<0.05, *p<0.01

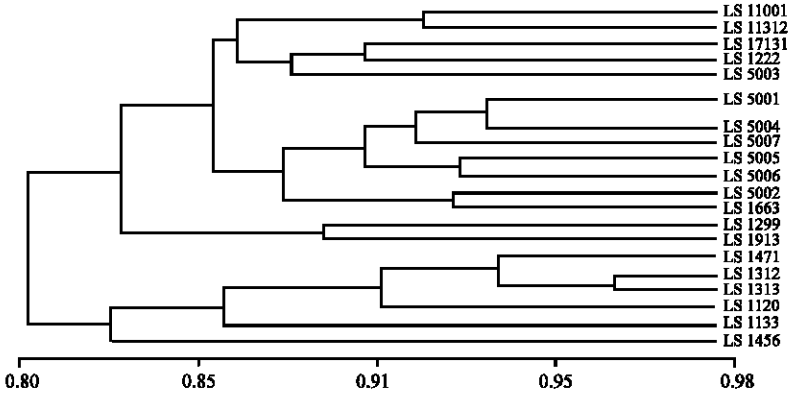

Fig. 1: UPGMA dendrogram based on average taxonomic distances among landrace means of morphological traits

significant correlation with maturity data, days to 50% flowering data, plant height, number of branch per plant and seed index. There were no significant association between percentage of antinutrition ODAP and any other traits. The principle component analysis was realized with means of 13 trait indicated that the first four principle components were accounting for a cumulative 76% of the variance. All the traits included in the principle component analysis contributed significantly to the loading of one or several of four eigenvectors with eigenvalues equal or above 4. The statistics of cluster analysis based on morphological traits showed that 20 landrace clustered to 7 groups when dendrogram cutting in 0.7 similarity coefficient in which Ardebil and Sede individually formed the separate cluster.

According to this analysis, the highest similarity belongs to Semirom 1 and Semirom 2 originated from Isfahan province. The least similarity belonged to Naghade and Shiraz (Fig. 1). Among 25 primers (Sinagene), 10 were selected that generated PCR products with a clear pattern for the all studied landraces studied and showed a repeatable pattern in separate amplification experiments. These primers generated 56 polymorphic PCR products whose size varied from 250 to 2500 bp (Table 2). The degree of polymorphism within each landrace varied depending on the primer tested from 1-6. A band (locus) was considered as polymorphic if the band differentiates at least any 2 of 20 landraces. The highest number of band was obtained with primers OPG11 and OPG 08 while the lowest number was obtained with primer OPG 17 (Table 2). In semi specific PCR (ISJ), 17 primers were used belonging to four groups of Intron Targeting (IT), Exon Targeting (ET) 15 and 18 bases in length (Table 3). These primers produced 126 DNA fragment (70 polymorphic bands) with 5.6 bands per primer. The majority of primers revealed polymorphism between landraces and only four of them generated non-polymorphic band pattern (Table 3) the highest and lowest of the polymorphic band belonged to IT5 and Et32, respectively. The average number of band for each semi random primer was 4.86.

There was no significant difference between number of polymorphic band obtained from 15 and 18- mer primer using q^2-test.

The most satisfactory result was obtained using primers with 18 bases in length.

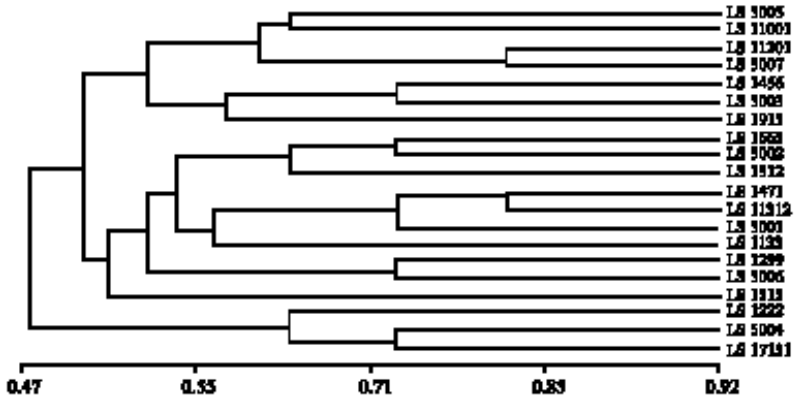

Fig. 2: Dendrogram of 20 grass pea landraces from ISJ-GS

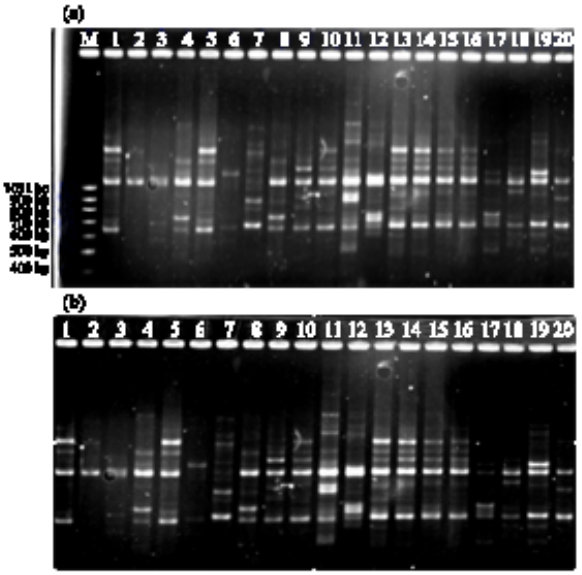

Fig. 3: RAPD profiles generated by OPJ08 (a) and IT5 (b) primers from individuals of a Iranian landraces of *L. sativus*

All the 56 bands, generated from 10 RAPD primers, were subjected to calculation of the genetic similarity index (RAPD-GS) among the 20 landraces (Fig. 3).

The RAPD-GS value ranged from 0.13 to 0.66 with the mean of 0.47. The highest genetic similarity was found between LS 1312 and 1313 landraces originated from Semirom while the lowest was observed between LS 1471 (Norabad) and LS 1133 (Ardebil).

The ISJ-derived data were subjected to calculate the genetic similarity (ISJ-GS) (Fig. 2). The genetic similarity coefficient varied between 0.28 and 0.71, with the average of 0.52. The minimum GS value was between LS1456 and LS 1133 landraces while the maximum GS value was between LS 5004 and LS 5007 landraces originated from Lorestan. The ISJ-GS among significantly lower than RAPD-GS. The average genetic similarity value based on ISJ markers was higher than of than that of RAPD markers approximately among the all landraces. These result suggested that higher genetic diversity could be detected by ISJ markers than of RAPD markers among the 20 grass pea landrace from Iran. Using RAPD data six groups were clustered LS 1471 landrace belonged to Fras province formed one group, separately (Fig. 2). According to this clustering, the landraces belongs to Lorestan are more closely related to each other than to other that were examined. The correlation of pairwise distances between all pairs of groups for RAPD compared to morphological was r = 0.43. The correlation of pair wise distances among all pairs of grass pea for RAPD and ISJ was r = 0.39. The cluster analysis using ISJ markers reveal five group when dendrogram cutting in 0.6 coefficient similarity. Landraces belongs to Lorestan province in this clustering were segregated among another landrace.

DISCUSSION

In this study, we analyzed 20 landraces of grass pea and evidenced the high significant variation for same morphological traits.

Levels of variability for all traits analyzed in this study were similar to those found in the 1187 grass pea germplasm by Pandey *et al*. (1995).

No significant correlation was detected between the observed patterns of morphological variation and RAPD-based variation ($p = 0.41$, $r = 0.21$) while Genetic diversity of grass pea measured using ISJ and RAPD marker exhibited highly significant association with morphological classification ($p<0.01$). There may be several reasons for the discrepancy between result based on morphology and RAPD. First, RAPD are considered to be neutral and thus to provide no direct assessment of fitness. The forces that cause differentiation for these

markers would be the result of mutation, genetic drift and low gene flow and no selection. Conversely, morphological traits are generally believed to be subject to natural and their expression is partially under the influence of environmental factors. Secondly, in contrast to morphological traits, RAPD variation is based directly on DNA sequence variation. The study has evaluated ISJ and RAPD markers as tools for assessing genetic variation among landrace from wide range geographical origins. Polymorphisms obtained with ISJ and RAPD marker have different underlying causes at molecular level and thus may differ for their informativeness in the analysis of genetic relationship.

In this study, it was obvious that dendrogram based on RAPD markers was not in accord with the dendrogram based on ISJ markers. The dendrogram generated by the ISJ matrix agree better with the morphological and geographical variation.

On the other hand, the relationship observed using molecular markers may provide information on the history and biology of cultivars, but it does not necessarily reflect what may be observed with respect to agronomic traits (Zoghlami *et al*., 2007). The selection process leads to an accumulation of best alleles for the traits under selection. RAPDs and ISSRs are dispersed throughout the genome and their association with agronomic traits is influenced by the breeder only in the region under selection pressure. The other loci are subjected to random genetic drift (Fernández *et al*., 2002). Another explanation could be the putatively similar bands originating for RAPDs in different bulked samples were not necessarily homologous although they shared the same size in base pairs (Karp *et al*., 1997). This situation might lead to wrong results when calculating genetic relationships (Fernández *et al*., 2002). Both ISJ and RAPD markers have unprecedented utility for analysis of landrace genetics and phylogenetic diversity of sorghum. Because they do not require radioactive isotopes, these methods can be used efficiently by researcher in developing countries.

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