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Original scientific paper

MOLECULAR CHARACTERIZATION OF *L. sativus* L. COLLECTION BASED ON ISSR MARKERS

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Inter simple sequence repeat (ISSR) marker system was used to evaluate the genetic diversity of local and introduced grasspea (*Lathyrus sativus* L.) genotypes. A total of 144 bands were amplified using 10 ISSR markers, of which 122 were polymorphic among the accessions. The percentage of DNA polymorphism varied from 66.6% to 96%, with a mean of 86%. UBC 857, UBC 810 and UBC 835 with high effective multiplex ratio (EMR), marker index (MI) and resolving power (RP) values were estimated as the most informative primers for distinguishing *L. sativus* genotypes. High genetic diversity was detected in the grass pea germplasm. The genetic diversity index (GDI) ranged between 0.42 and 0.92, with an average value of 0.75 for the whole collection. The cluster analysis with 5000 bootstrapping value divided grasspea accessions into four major clusters. Most accessions were placed into the same cluster close to each other with regard to their botanical varieties. Principal coordinate analysis (PCoA) agreed with the cluster analysis and clearly discriminated the accessions into two genetically distinct groups. The first three coordinate axes accounted for 84.3% of the total variation. The result of the cluster and PCoA analyzes underline the importance of consideration of botanical variety traits in grasspea breeding programs. It can be concluded that the present germplasm constitutes an important pool of diversity for further genetic analysis, linkage mapping and breeding activities.

Keywords: grasspea; ISSR; genetic diversity; PCoA; cluster analysis

INTRODUCTION

The grasspea (*Lathyrus sativus* L.) is an annual, predominantly self-pollinated and diploid (2n=14) legume plant commonly cultivated for food, feed and fodder. The plant contains a high

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level of proteins and starch, which makes it an excellent candidate for human diets, especially in Near East, North Africa, West Asia, Indian subcontinent and China (CHINNASAMY *et al*., 2005; SAMMOUR, 2014). The grasspea is highly adapted to arid conditions and can be grown on a wide range of soil types, including very poor soil and heavy clays, however, it can be grown on land subject to flooding. Archaeobotanical and phytogeographical evidence indicates the Balkan peninsula as an origin of *L. sativus*, where it was cultivated since the beginning of the 6th millennium BC. Remains of *L. sativus* also have been reported in India dating back to 2000-1500 BC (DIXIT *et al*., 2016).

Eighteen species of *Lathyrus* genus, including *L. sativus* are distributed in Azerbaijan; the cultivated forms mainly can be found in the southern region of the country. In the collection of the Genetic Resources Institute (GRI) of Azerbaijan National Academy of Sciences 103 accessions of *Lathyrus*, including 75 accessions of *L. sativus* from almost all areas of its cultivation are being conserved and have been involved into breeding. The comprehensive characterization and the study of this genetic diversity will enable their effective involvement in the breeding process in order to broaden the genetic base, to obtain new grasspea varieties, well adapted to the different conditions of the country.

In recent years, DNA based molecular markers have shown promise for assessment of genetic diversity, owing to their several advantages including abundance, independence from the environment, suitability for early and rapid evaluation, and non-tissue specific characteristics (ZONG *et al*., 2015). The different types of molecular markers are applied in plant genomic analysis, among which the inter simple sequence repeats (ISSR) have been used successfully as tools for molecular characterization (KUMARI, 2016). The stability of ISSR markers over different environments, no stage specificity and the advent of rapid workable techniques make this molecular technology convenient for genetic diversity studies.

The objective of the present study was to describe the molecular diversity and assess the genetic relationship in grasspea collection using ISSR markers.

MATERIALS AND METHODS

Plant material and molecular analysis

A set of 34 accessions of *L. sativus* of diverse origin from National Gene Bank of GRI was used as a research material (Table 1). The studied genotypes belonged to two subspecies (ssp. *asiaticus* and ssp. *europaeus*) and four botanical varieties. Genomic DNA was extracted from fresh leaves using CTAB (ELIAS *et al*., 2004) method with some modifications and quantity and quality were checked by Nanodrop (Thermo Scientific, 2000). Nine 3' anchored and one unanchored ISSR primers were employed for intraspecific genetic diversity analysis in *L. sativus* (Table 2). Amplification reactions were performed in a total volume of 20 µl containing 2 µL 10x PCR buffer; 2 µL mixture dNTP (5 mM); 1.5 µL $MgCl_2$ (50mM); 2 µL of each primer (15 pmol/µL); 0.1 µL of Taq-polymerase enzyme (1 U/µL) and 2 µL of template DNA (50 ng/µL). PCR program consisted of initial denaturation for 30 s at 94°C, followed by 35 cycles of 1 min at 94°C, 45 s at temperature proper to the primer, 1 min at 72°C, and final extension for 5 min at 72°C (Applied Biosystems, USA). Amplification products were separated in 2% agarose gel, stained with ethidium bromide and visualized under UV transillumination (BioRad).

Table 1. The list of grasspea genotypes used in the study

№	Genotypes	Origin	Subspecie	Botanical variety	Seed size
1	İFLA-1870	Bangladesh	*asiaticus*	*coeruleus*	minor
2	İFLA-274	Canada	*asiaticus*	*coeruleus*	minor
3	İFLA-1720	Morocco	*asiaticus*	*coeruleus*	minor
4	İFLA-479	Ethiopia	*asiaticus*	*rubiginosus*	minor
5	İFLA-2475	Bangladesh	*asiaticus*	*coeruleus*	minor
6	İFLA-1795	Pakistan	*asiaticus*	*coeruleus*	minor
7	İFLA-276	Hungary	*asiaticus*	*coeruleus*	minor
8	İFLA-2026	Bangladesh	*asiaticus*	*albus*	minor
9	İFLA-2636	Bangladesh	*asiaticus*	*albus*	minor
10	İFLA-2282	Bangladesh	*asiaticus*	*rubiginosus*	major
11	İFLA-143	Greece	*europaeus*	*rubiginosus*	major
12	İFLA-2973	Bangladesh	*europaeus*	*rubiginosus*	minor
13	İFLA-242	Afghanistan	*asiaticus*	*rubiginosus*	minor
14	İFLA-160	Germany	*asiaticus*	*rubiginosus*	minor
15	İFLA-157	Greece	*asiaticus*	*rubiginosus*	minor
16	İFLA-148	Greece	*asiaticus*	*rubiginosus*	minor
17	İFLA-142	Greece	*asiaticus*	*rubiginosus*	minor
18	İFLA-169	Greece	*europaeus*	*rubiginosus*	minor
19	İFLA-123	Greece	*europaeus*	*coeruleus*	major
20	İFLA-151	Greece	*europaeus*	*coeruleus*	major
21	İFLA-128	Greece	*europaeus*	*coeruleus*	major
22	İFLA-176	Greece	*asiaticus*	*coeruleus*	major
23	İFLA-134	Greece	*europaeus*	*rubiginosus*	minor
24	İFLA-240	Afghanistan	*europaeus*	*rubiginosus*	minor
25	GP-73	ICARDA	*europaeus*	*rubiginosus*	minor
26	GP-53	ICARDA	*asiaticus*	*coeruleus*	minor
27	GP-77	ICARDA	*asiaticus*	*coeruleus*	minor
28	GP-65	ICARDA	*europaeus*	*coeruleus*	major
29	GP-88	ICARDA	*europaeus*	*coeruleus*	major
30	GP-96	ICARDA	*asiaticus*	*rubiginosus*	minor
31	GP-58	ICARDA	*europaeus*	*rubiginosus*	minor
32	GP-51	ICARDA	*europaeus*	*rubiginosus*	minor
33	ST	AZE	*asiaticus*	*cyanescens*	major
34	Zirve	AZE	*europaeus*	*albus*	major

Data analysis

The presence and absence of bands were scored as 1 or 0, respectively. The generated binary data matrix was used to calculate the Nei's distance coefficients. Cluster and PCoA analyzes were performed using UPGMA method and DARwin version 6 software (PERRIER and JACQUEMOUD-COLLET, 2006). The performance of the markers was measured using genetic

diversity index (GDI) (WEIR, 1990), polymorphic information content (PIC) (ROLDAN-RUIZ *et al*., 2000), effective multiplex ratio (EMR), marker index (MI) (POWELL *et al*., 1996), resolving power (RP) and mean resolving power (MRP) (PREVOST and WILKINSON, 1999) parameters.

RESULTS AND DISCUSSION

As for all genetic resources a major goal of conservation strategy on *L. sativus* is to conserve a wide representation of extant genetic variations. In comparing with other legume crops, such as chickpea, lentil, faba bean the grasspea has received less attention for molecular genetic studies (YANG *et al*., 2014).

In the current study 10 highly polymorphic ISSR primers were used to characterize and evaluate the genetic diversity and relationship among 34 grasspea accessions. The number of amplification products had a wide range and varied from 8 for UBC 812 and UBC 823 to 25 for UBC 857 with an average of 14.4 per primer (Figure 1). The total number of alleles for the entire collection was 144; 127 (88.2%) of which were polymorphic between the samples (Table 2). The range for the number of polymorphic bands was between 6 and 24, averaged 12.7. The number of total and polymorphic bands in the present study is much superior to those found by AMBADE *et al*. (2015), who found 118 bands using 12 ISSR primers in 48 *L. sativus* accession. The percentage of DNA polymorphism in grasspea accessions in the current study varied from 66.6% to 96%, with a mean of 86%. The maximum polymorphism was recorded for $(AC)_8YG$ sequence. The data provided evidence of high molecular polymorphism within the studied grasspea collection. The variability within *Lathyrus sativus* collection was previously investigated using various genetic marker systems, where a different level of variability was revealed (CHOWDHURY and SLINKARD, 2000; POLIGNANO *et al*., 2005; BELAID *et al*., 2006; SAMMOUR *et al*., 2007).

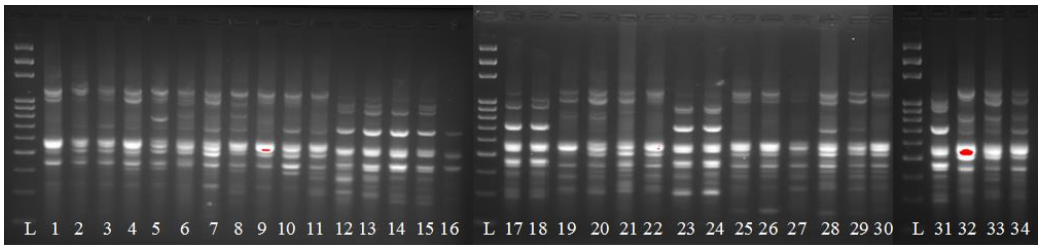

Fig. 1. Amplification products for 34 grasspea genotypes using ISSR primer UBC 857.

Effective multiplex ratio, marker index and resolving power were calculated in order to evaluate the overall utility of the marker system. The EMR values ranged between 4.0 and 23.04. High EMR values were scored with primers UBC 857, UBC 835 and UBC 810, while lowest scores were obtained for primers IS16 and UBC 812. The primers with higher EMR had also higher MI values (Table 2). Mean EMR and MI for ISSR analysis in grasspea collection was estimated at 11.3 and 3.5, respectively. RP determines the efficiencies and discriminatory potential of the primers and provides an accurate estimate of the number of genotypes identified by a primer (NAJAPHY *et al*., 2011). In our assessment, RP varied from 2.9 to 9.7 (average 5.7)

and mean resolving power was reported in the range of 0.37-0.61 (average 0.47). UBC 857, UBC 810 and UBC 835 with high RP values were estimated as the most informative primers for distinguishing the grasspea genotypes. Polymorphism information content values ranged from 0.28 to 0.42. The average PIC value for the ten primers was identified 0.32. The highest PIC index was recorded for primer IS 02, while the lowest PIC was noted for primer UBC-841. All primers had moderate or high PIC values, which on one hand indicates the usefulness of these ISSR primers for evaluation of polymorphism among *L. sativus* genotypes and on the other hand proves the diverse nature of the studied grasspea accessions.

Table 2. Marker parameters of genetic variation for grasspea collection based on ISSR data

Primers	Primer sequence, 5`~3`	Total number of bands	Number of polymorphic bands	Percentage of polymorphic	Genetic diversity index	PIC	EMR	MRP	RP	MI
IS 02	$(ACC)_6G$	9	8	88.8	0.42	0.42	7.1	0.61	4.88	3.01
IS 16	$(AC)_8CCT$	9	6	66.6	0.67	0.30	4.0	0.58	3.48	1.17
UBC 810	$(GA)_8T$	20	18	90	0.89	0.30	16.2	0.46	8.3	4.8
UBC 812	$(GA)_8A$	8	6	75	0.6	0.31	4.5	0.48	2.9	1.4
UBC 818	$(CA)_8G$	16	15	93.7	0.88	0.30	14.06	0.42	6.38	4.3
UBC 823	$(TC)_8C$	8	7	87.5	0.56	0.39	6.1	0.56	3.9	2.4
UBC 835	$(AG)_8YC$	21	19	90.5	0.89	0.29	17.19	0.43	8.11	4.9
UBC 840	$(GA)_8YT$	16	15	93.7	0.83	0.30	14.06	0.43	6.39	4.2
UBC 841	$(GACAC)_5$	12	9	75	0.84	0.28	6.75	0.37	3.4	1.9
UBC 857	$(AC)_8YG$	25	24	96	0.92	0.29	23.04	0.40	9.69	6.4
Total		144	127							
Mean		14.4	12.7	85.7	0.75	0.32	11.3	0.47	5.7	3.5

Note: Y= C, T

A wide range of genetic diversity was detected in the grasspea germplasm, as also expressed by higher values of GDI. The genetic diversity index was found to be within the range of 0.42 and 0.92, resulting in an average value of 0.75 for the whole collection. GDI for 8 out of ten primers was higher than 0.6. These findings once again support that the studied set of primers can be included into a collection of highly polymorphic ISSR markers for diversity analysis and cultivar identification in grasspea. Self-pollinated crops often possess low variability (NKONGOLO, 2003). Grasspea is a self-pollinated crop having cross-pollination up to some extent (up to 36.8%), which can rapidly increase the intraspecific heterogeneity. In addition, despite a low number of the accessions the studied collection includes genotypes of diverse origin that belong to two subspecies and four botanical varieties. All these facts may probably contribute to the high genetic diversity within the studied grasspea collection. ISSR markers have already been successfully applied to the evaluation of the genetic diversity and genetic relationship in grasspea (KAUR, 2014; AMBADE *et al.*, 2015). Similarly, in the study of diversity in *Lathyrus* genus using ISSR method BELAID *et al.* (2006) revealed large genetic diversity among and within

L. sativus, L. cicera and *L. ochrus* species. Summarizing the results of diversity analysis in *L. sativus* based on ISSR markers it can be concluded that the present germplasm constitutes a wide genetic diversity.

Nei genetic distance indices for genotype pairs ranged from 0.01 to 0.79 and averaged 0.46. In their study of 23 grasspea genotypes using ISSR markers, KAUR (2014) found similarity coefficient ranging from 0.47 to 0.90. The highest similarity in the current study was recorded between IFLA-274 (Canada) and IFLA-1720 (Morocco), while two genotype pairs (IFLA-160 (Germany)/GP-73 (ICARDA) and IFLA-160 (Germany)/GP-53 (ICARDA)) were the most distantly related genotypes. The UPGMA cluster analysis with 5000 bootstrapping value divided 34 accessions into four major clusters (Figure 2). The cluster analysis based on ISSR data was able to distinguish all studied genotypes from each other and confirmed a wide genetic variation among the studied accessions. GP-96 from ICARDA and IFLA-2475 from Bangladesh constituted independent clusters III and IV, respectively. In general, the topology of dendrogram based on the ISSR-analysis showed that most of the accessions were divided into groups corresponding to botanical varieties. Thus cluster II was homogeneous and comprised of 10 genotypes of var. *rubiginosus* with 100% bootstrapping value. Cluster I subsequently divided into three subclusters. The first subcluster contained a total of 17 grasspea genotypes. Six genotypes of var. *coeruleus* were assigned to separate homogeneous group within the subcluster. Two other genotypes of var. *coeruleus* (IFLA-274 and IFLA-1720) and two genotypes of var. *rubiginosus* (IFLA-143 and GP-51) were grouped very closely in the same subcluster. The genetic distance between these pairs of accessions were 0.01 and 0.099, respectively which indicates their genetic similarities. In addition, out of 10 large-seeded grasspea genotypes 9 were placed in the first subcluster of Cluster I; among them, 5 genotypes formed a separate group together with one small-seeded accession. One and four genotypes fell into the second and third subclusters. In general, the vast majority (63%) of var. *rubiginosus* genotypes included in the study was assigned to Cluster II, while all var. *coeruleus* accessions grouped into clusters I and IV. All three var. *albus* genotypes also fell into same (first) subcluster of cluster I, indicating a certain portion of shared alleles. The only genotype of var. *cyanescens* from Azerbaijan was most similar to another local variety Zirve (var. *albus*) with 0.05 genetic distance index. In addition, both genotypes of Afghanistan origin grouped together in cluster II. No other relationship between clustering of genotypes and their origin country was revealed. It was noted that life form, geographic range, breeding system and taxonomic status had significant effects on the partitioning of genetic diversity within and among plant populations (AI *et al*., 2014). In the current study, the grouping of introduced and local *L. sativus* accessions was in accordance with botanical varieties; subspecies and geographic origin did not affect the dendrogram topology. The genotypes from different clusters and subclusters might be used as potential parents in grasspea breeding programs.

Principal coordinate analysis (PCoA) was used to illustrate the multiple dimensions of the distribution of the genotypes in a scatter-plot (Figure 3). The first three coordinate axes accounted for 84.3% (first axis = 75.2%) of the total variation. PCoA analysis agreed with the cluster analysis and displayed more clearly the relationships among the botanical varieties. The genotypes belonging to a particular cluster were also grouped together in the PCoA plot. Clear differentiation of studied grasspea collection into two genetically distinct groups can be observed

in the plot. Group 1 only represented by var. *rubiginosus* that formed a tight set in the left part of the scatter plot, whereas group 2 contained remaining accessions spread along the right part of the PCo plot.

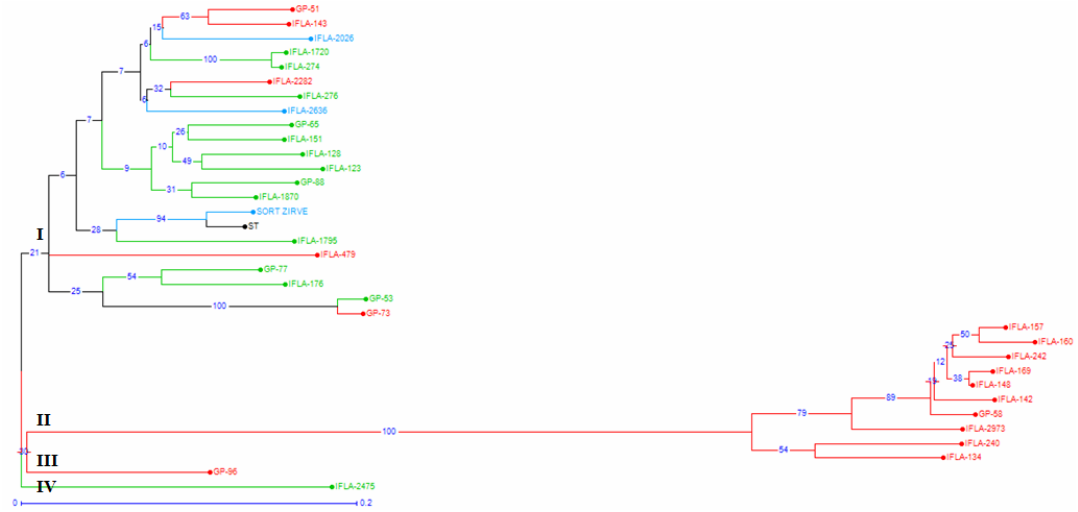

Fig 2. Dendrogram based on Nei genetic distance coefficient for 34 grasspea genotypes (bootstrap values were indicated).

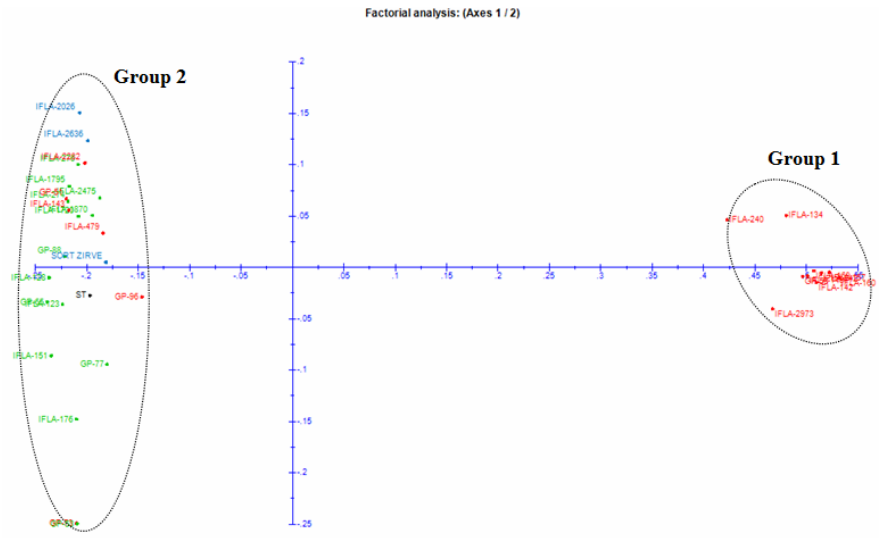

Fig 3. Principle coordinate analysis for 34 grasspea genotypes based on ISSR data.

CONCLUSION

In conclusion, our results demonstrate that the studied *L. sativus* collection constitutes an important pool of diversity and can be confidently used for further genetic analysis and breeding activities. ISSR markers were efficient to assess genetic diversity within the collection and were able to identify samples with the same botanical varieties. The results of the cluster and PCoA analyzes obtained in the current experiment for the first time, underline the importance of consideration of botanical variety traits in grasspea breeding programs. Information on a high level of genetic diversity and existence of genetically separated groups in the current grasspea collection can be applied to the development of linkage maps, in the identification of QTLs for traits of agronomic importance, including botanical variety traits. In addition, crosses between genetically distant genotypes will enable to obtain populations with high levels of segregation and will accelerate the creation of new varieties with high agronomic performance and low ODAP content.

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MOLEKULARNA KARACTERIZACIJA KOLEKCIJE *L. sativus* L. ISSR MARKERIMA

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Izvod

Sistem markera ISSR korišćen je za procenu genetičkog diverziteta lokalnih i introdukovanih genotipova sastrice (*Lathirus sativus* L.). Ukupno je 144 traka amplifikovano korišćenjem 10 ISSR markera, od kojih je 122 bilo polimorfno. Procenat DNK polimorfizma varirao je od 66,6% do 96%, sa srednjim nivoom od 86%. UBC 857, UBC 810 i UBC 835 sa visokim efektivnim multipleksnim odnosom (EMR), markerskim indeksom (MI) i razlučujućom snagom (RP) procenjeni su kao najinformativniji prajmeri za razlikovanje genotipova *L. sativus*. U germplazmi sastrice otkrivena je velika genetska raznolikost. Indeks genetske raznolikosti (GDI) kretao se u rasponu od 0,42 do 0,92, sa prosečnom vrednošću od 0,75 za celu kolekciju. Analiza klastera sa 5000 vrednosti podelila je uzorke sastrice u četiri glavna klastera. Većina uzoraka je smeštena u isti klaster blizu jedan drugom na osnovu njihove botaničke srodnosti. Analiza glavnih koordinata (PCoA) složila se sa analizom klastera i jasno je razdvojila uzorke u dve genetski različite grupe. Prve tri koordinatne osi činile su 84,3% ukupne varijacije. Rezultat klaster i PCoA analiza podvlači važnost razmatranja botaničkih osobina u programima oplemenjivanja sastrice. Može se zaključiti da je ova germplazma važan izvor diverziteta za dalju genetsku analizu, mapiranje i oplemenjivačke aktivnosti.

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