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Original Scientific Paper

Genetic variation among different accessions of *Lathyrus sativus* (Fabaceae) revealed by RAPDs

Houshang Nosrati*, Mohammad-Ali Hosseinpour-Feizi, Maryam Nikniazi and Ahmad Razban-Haghighi

Department of Plant Science, University of Tabriz, Tabriz, Iran

ABSTRACT: The relationship between genetic similarity and larger geographical distance among populations of plant species has been attributed to a number of different factors mostly breeding systems. We assessed this relationship among five accessions of selfing legume *Lathyrus sativus* L. (grass peas, Fabaceae) using RAPDs by including 10 randomly selected individuals from each accession. Five primers produced 73 clear, reproducible and scorable polymorphic bands. The percentage polymorphic bands ranged from 20.6% in German to 60.3% in Polish accessions. The range of Nei's within-accession genetic diversity was wide, ranging from 0.075 in German to 0.25 in Polish accessions. Partitioning of total genetic diversity by AMOVA indicated 76.44% genetic diversity among accessions and 23.56% within accessions, indicating that *L. sativus* is a selfing species. The shortest genetic distance was detected between German and Iranian accessions (0.202), while the greatest genetic distance was revealed between Iranian and Polish accessions (0.5102), indicating that in selfing species genetic similarity among accessions is not correlated with geographical distance.

Key words: genetic diversity, grass peas, *Lathyrus sativus*, RAPDs, selfing

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INTRODUCTION

The genus *Lathyrus* (Fabaceae) consists of about 160 annual and perennial species (Allkin *et al.* 1986), some of which have agricultural importance. *Lathyrus sativus* L. (grass peas) is a forage crop and includes several inbred cultivars widely cultivated across the world (Duke 1981; Smartt 1990). This species has several ecological advantages including optimal growth in arid or semiarid environments, neutral to alkaline soils, heavy clays, and also high resistance to many pests compared to other forage legumes (Palmer *et al.* 1989).

Assessing the levels of genetic variation, and partitioning total genetic variation within and between populations in the cultivated accessions are major concerns in breeding programs, genecology and conservation genetics (Nybom & Bartish 2000). In addition, knowledge of the population genetic structure of a species is essential to make valid biological interpretations about its breeding system and reproductive biology (Smartt 1981; Bussell 1999). Studies using allozyme markers have indicated that breeding system and geographic distribution range are closely associated with both the amount of total genetic variation and partitioning of total genetic variation among and within populations (Hamrick & Godt 1989; 1996). In a comprehensive review of the studies based on Randomly Amplified Polymorphic DNA markers (RAPDs) Nybom & Bartish (2000) have shown that a strong negative association exists between sampling distances and between population diversity in the outcrossing taxa but was completely lacking in the selfing taxa.

This study aimed to assess the amount of genetic similarity among different accessions of *L. sativus* from large geographical distances, and to measure the levels of within- and between-population genetic variation of the accessions using RAPDs. These markers have been widely

*correspondence: hnosrati@tabrizu.ac.ir

applied to investigate population genetic structures, diversities and distances in plant taxa (Williams *et al.* 1990; Hollingsworth *et al.* 1999; Nybom 1999; Nybom & Bartish 2000), despite having some restrictions - e.g. a dominant nature which makes it impossible to distinguish homozygote and heterozygote genotypes at individual loci. In highly inbred species e.g. grass peas the dominance effect of RAPD markers is minimal, and monolocus approaches for RAPD data are generally considered appropriate for measuring the genetic structure of populations (Ferguson *et al.* 1998).

MATERIAL AND METHODS

Plant material. Five accessions of grass peas were included in this study from Germany, Poland, Hungary, Syria and Iran. Ten individual plants were randomly selected from each accession.

RAPD analysis and genetic variation. Genomic DNA was extracted from seeds and seedlings following Madden (2002). Concentration of the DNA samples was estimated by both gel electrophoresis and spectrophotometry to adjust the final concentration to 10ng/ml. In the first method, samples were run on 0.8% agarose gel electrophoresis and stained with ethidium bromide, and subsequently the staining intensity of RAPD bands was compared with size markers of known molecular weight. Secondly, DNA concentration of samples was measured by spectrophotometry at 260nm. As the concentrations of sample DNA were higher than 10ng/ml, the DNA samples were diluted with sterilised distilled water to get a final concentration of 10ng/ml. Ten decamer arbitrary RAPD primers (CinnaGen, Iran) were used, of which five primers produced clear and reproducible banding patterns, and these were selected for further analysis (Table 1). PCR amplification was carried out using 19µl Master Mix (CinnaGen PCR MasterKit, Cat. No. PR8251C) plus 5µl of 10ng template DNA and 1µl of 100pm/µl primer making a total volume of 25µl. Amplifications were performed in a Biometra thermal cycler for an initial 4 min denaturation at 94°C followed by 40 cycles of 1 min at 93°C (denaturation), 1 min at 40°C (annealing) and 1.5 min at 72°C (synthesis). All PCR products were separated by electrophoresis on 1.5% w/v agarose gels in 1 x TBE buffer, stained with ethidium bromide, viewed under ultraviolet light and photographed using a UV Transilluminator (UVP, USA). PCR reactions and electrophoresis were repeated at least twice in each case to ascertain the reproducibility of the bands. The banding patterns were scored as 1 for presence and 0 for absence of a band. Data obtained were entered into a binary matrix for cluster analysis using NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, ver. 2.02). The number and percentage of polymorphic RAPD bands were recorded for each accession. Genetic diversity within each accession was estimated using Nei's (1973) and Shanon's (Lewontin 1972) information index (Popgen ver. 1.32). To compare the relationship among the accessions, UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) dendrograms were generated based on Nei's distances among accessions obtained through SHAN (sequential, hierarchical, agglomerative and nested clustering of the NTSYS-pc). Total genetic variation was partitioned into within and among the accessions based on analysis of molecular variance (AMOVA) using Arlequin ver. 3.11 (Excoffier *et al.* 1992). AMOVA is the least biased method for apportioning variation among and within populations for RAPD data (Isabel *et al.* 1995). The significance level for F-statistics analogues was determined using 1023 bootstrap replicates to estimate the significance of genetic variation both among and within populations. The relationship among 43 individuals from the five accessions was also studied by obtaining a dendrogram using the Average Linkage (between-groups) cluster method based on the Euclidean square distance measure (SPSS, ver. 14).

Table 1. Primer sequences, number and percentage of polymorphic bands produced by five arbitrary RAPD primers on five different accessions of *L. sativus*.

Primercode	Sequences (5' to 3')	No. of polymorphic bands
A	TGGTCGCAGA	18
B	GGACACCACT	13
C	CCACACTACC	15
D	TGAGCCTCAC	13
E	ACTCCTGCGA	14

RESULTS

RAPD patterns and Genetic distance. The five primers used for RAPD amplifications produced a total of 73 clear, scorable and reproducible polymorphic bands, ranging in size from 200 to 2500 base pairs (Fig. 1). On average 14.6 polymorphic bands were produced by each primer, with a maximum of 18 bands for primer A and a minimum of 13 bands for primers B and D (Table 1). The percentage of polymorphic RAPD bands among accessions ranged from 20.6% in Germany to 60.3% in Poland. The highest within-population genetic diversity was detected in the Polish accession (0.25, Nei's; 0.36 Shannon's) and lowest was obtained in the German accession (0.075, Nei's; 0.11, Shannon's) (Table 2). The partitioning of total genetic

Table 2. Genetic diversity and percentage polymorphic RAPD bands within five accessions of *L. sativus* based on Nei's gene diversity and Shannon's information index. The highest and lowest genetic variations were detected within Polish and German accessions, respectively.

No.	Accession (code)	Nei's gene diversity (H)	Shannon's information index (I)	% Polymorphic RAPD bands
1	Poland (463)	0.2500	0.3620	60.27
2	Syria (587)	0.1481	0.2171	38.36
3	Iran (445)	0.1185	0.1815	38.36
4	Hungary (561)	0.1084	0.1584	27.40
5	Germany (453)	0.0745	0.1102	20.55

The plant materials codes were recorded by International Centre for Agricultural Research in Dry Areas (ICARDA, Aleppo

Table 3. AMOVA of total RAPD genetic variation partitioned to within- and among-accessions of *L. sativus*. Majority of total genetic variation was detected among accessions.

Source of variation	df	Sum of squares	Variance	%variance	*P**
Among accessions	4	311.24	7.548	76.44	<0.001
Within accessions	45	104.70	2.326	23.56	<0.001

*Significance test based on nonparametric method of randomly sampling 1023 bootstrap replications (F-statistics =0.764).

Table 4. The matrix of genetic distances between pairs of *L. sativus* accessions based on Nei's genetic distances. The shortest (0.2020) and largest (0.5102) genetic distances were detected respectively between Iranian and Germany, and Iranian and Polish accessions.

Accession	Germany	Poland	Syria	Hungary	Iran
Germany	0.0				
Poland	0.4370	0.0			
Syria	0.4569	0.3929	0.0		
Hungary	0.4215	0.4932	0.3324	0.0	
Iran	0.2020	0.5102	0.3504	0.2128	0.0

variation using AMOVA indicated 76.44% genetic diversity among accessions and 23.56% variation within accessions (Table 3). In the UPGMA dendrogram based on Nei's distances between pairs of populations (Table 4), German and Iranian accessions were nested within one cluster (Fig. 2). In addition, the Euclidean dendrogram also showed that all individuals of each accession were grouped within one cluster, and that the Iranian accession was closest to the German and Hungarian accessions (Fig. 3).

DISCUSSION

The levels of genetic diversity we measured within and among accessions of *L. sativus* are consistent with the range of values reported for herbaceous selfing legumes and other selfing taxa using the same markers. Our results showed that in *L. sativus* most of the total genetic variation was found among accessions (76.44%) rather than within accessions. This pattern of distribution of genetic diversity is a general rule in most selfing species studied to date, for example, 57% in *Hordeum spontaneum* Trusted. (Dawson et al. 1993), 63% in *Elymus fibrosus* (Schrenk) Tzvel. (Diaz *et al.* 2000), 60% in *E. alaskanus* (Scribn. and Merr.) A. Löve (Zhang *et al.* 2002), 53% in *Sinojackia dolichocarpa* C.J.Qi (Cao *et al.* 2006), 90% in *Phaseolus vulgaris* L. (Martins *et al.* 2006), and 80.67% in *Arbutus unedo* L. (Takrouni & Boussaid 2010), although in a few selfers total genetic diversity was reported to be either equally distributed within and among populations, e.g. both wild and domesticated populations of *Capsicum annuum* L. (Oyama *et al.* 2006), or the larger portion allocated to within-populations *e.g.* 55% in *Medicago truncatula*

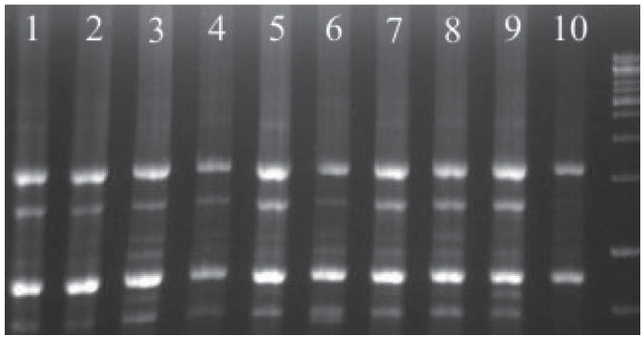

Fig. 1. RAPD patterns of 10 individuals of German accession of *L. sativus* produced by primer A. The first lane from the right is standard size markers (200-10000bp).

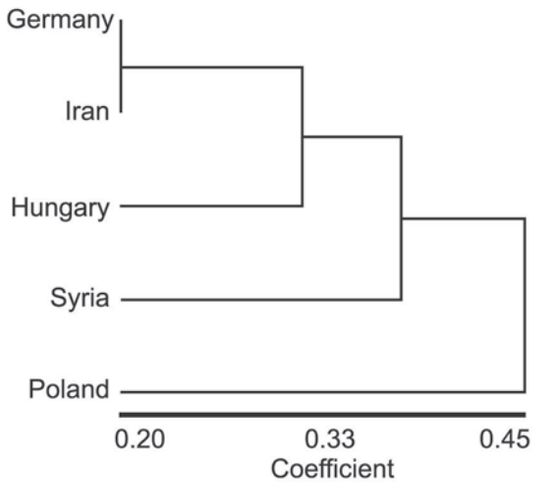

Fig. 2. UPGMA dendrogram based on Nei's distances showing the relationship among five accessions of *L. sativus* (Cogenetic coefficient: r = 0.). German and Iranian accessions show a close relationship, while the Polish accession separated from the all other accessions.

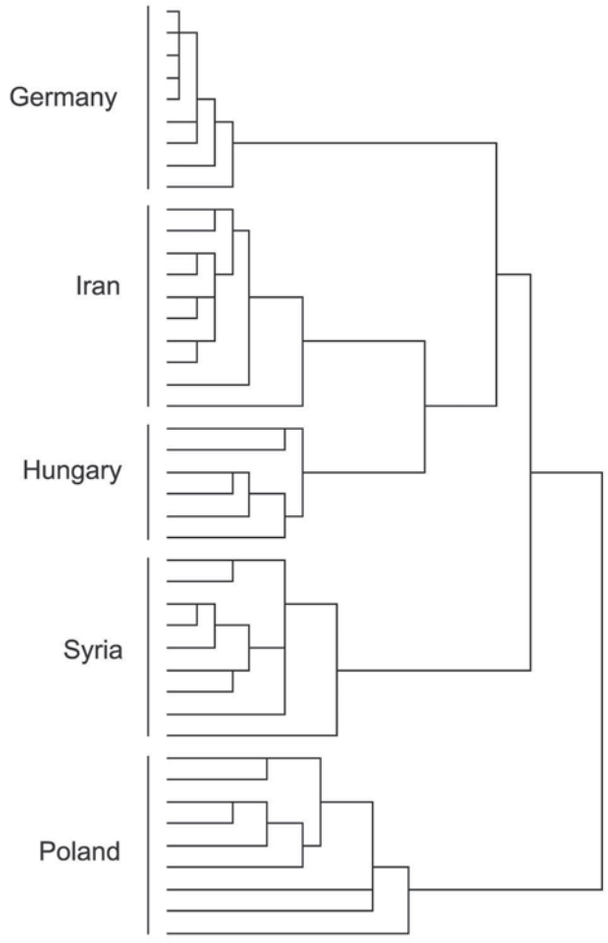

Fig. 3. The Dendrogram conducted using Average Linkage (Between-groups) based on Euclidean Square Distance measure showing the relationship among 43 individuals from five different accessions (The RAPD patterns of 7 out of 50 individual plants from one primer were missing, so excluded from analyses).

Gaertn. (Bonnin *et al.* 1996), *Lablab purpureus* L. (68.9%, Liu 1996), *Stylosanthes scabra* Vogel (66.6%, Liu 1997), and *Phaseolus vulgaris* L. (86.6%, Metais et al. 2000).

Our data revealed that in *Lathyrus sativus* the percentage of RAPD polymorphic loci and consequently the amounts of within-accession genetic variation were highly variable from accession to accession (20.55% - 60.27% and 0.0745% - 0.25%, Nei's distances, respectively). This greater population to population variation in genetic diversity is a characteristic of self-fertilizing species but is less-well understood (Brown & Schoen 1991). High among-accessions genetic differentiation has been reported among accessions of selfing *Geum urbanum* L. sampled from Estonia, Switzerland and Germany (Schmidt *et al.* 2009). The mean percentage of polymorphic loci for different populations of five *Lens* species was reported to vary from 27% to 88% (Ferguson *et al.* 1998). In the selfing legumes *Pueraria montana* (Lour.) Merr. and *P. phaseoloides* (Roxb.) Benth. the similarity among accessions based on Jacard's coefficient ranged from 0 - 0.87 with mean value of 0.35 for *P. montana*, and 0 - 1 with mean value of 0.52 for *P. phaseoloides* (Heider *et al.* 2007). In *Vigna unguiculata* (L.) Walp. the level of polymorphic loci of populations was 12% for domesticated lines (Menendez *et al.* 1997), and 80% for Malawian landraces (Nkongolo 2003). Similarly, a wide genetic variation was detected among different populations in other selfing species e.g. 0.05 to 0.21 in *Elymus fibrosus* (Schrenk) Tzvelev (Diaz *et al.* 2000), and 0.119 to 0.302 in *Amphicarpaea edgeworthii* (Zhang *et al.* 2005). However, in a few selfers the amount of genetic diversity was shown to be similar among various accessions and populations, for example, in annual *Linum*

usitatissimum L. the proportion of fixed recessive RAPD loci ranged from 0.427 to 0.529 (Fu *et al.* 2002), and in *Elymus alaskanus* (Scribn. & Merr.) A. Löve the mean genetic diversity based on RAPDs ranged from 0.142 to 0.262 (Zhang *et al.* 2002).

In our study, the Nei's distance-based UPGMA dendrogram constructed for accessions was consistent with the Euclidean-based dendrogram constructed for individuals as both dendrograms indicated a close relationship between German and Iranian accessions, and separated the Polish accession from all the others. These results are consistent with data obtained from selfing plant taxa using the same approaches. Nybom & Bartish (2000) in a comprehensive review on many studies based on RAPDs concluded that in outcrossing taxa, estimates of between-population diversity were closely correlated with maximum geographic distance between the sampled populations, but not in selfing taxa. They attributed these results to the fact that RAPDs can be a sensitive method for detection of genetic structuring according to the isolation-by-distance model. On the other hand, the genetic relationships among populations of a given species do not often accord with their geographical distance, especially for species with large distribution areas (Irwin 2001; Qiu *et al.* 2004). The relationship between genetic similarity and geographical distance might be distance-limited because population history and random genetic drift have a great influence on genetic variation (Le Corre *et al.* 1997).

In this study, the Polish population exhibited a high level of genetic diversity similar to values generally reported for outcrossing populations. The factors and mechanisms responsible for the high level of genetic diversity within populations of selfing species remain unclear (Brown & Schoen 1991). However, a self-fertilizing population under predominant selfing is expected to fracture into essentially isolated lineages, and thus likely to be subdivided into small neighbourhoods that consist of single differentiated lineages. Such subdivision within a selfing population could thus play a major role in the maintenance of genetic diversity at the whole population level (Whitlock & Barton 1997).

The floral biology in *L. sativus* favours inbreeding. However, environmental factors can affect the level of outcrossing. For example, the flower colours in this species are diverse e.g. blue, pink, red and white, and these can affect outcrossing frequency via differential rates of pollinator attraction (Rahman *et al.* 1995). In addition, male sterility due to the failure of pollen formation has been reported in *L. sativus* and this can increase the level of outcrossing (Srivastava & Somayajulu 1981), and consequently can result in higher genetic variation within a population. All these data indicate that the patterns of genetic variation in a given selfing species are diverse.

RAPDs proved to be a high-resolution technique for the detection of genetic variation among and within populations of *L. sativus* as, using only five primers, we managed to obtain high genetic variation (73 RAPD polymorphic bands) within and among populations with relatively small sample sizes (total of 50 plants from five accessions). The current study identified a high level of differentiation among accessions of *L. sativus*. We are currently examining the genetic relationship of the same material from these five accessions of *L. sativus* by ISSR markers (Inter Simple Sequence Repeats) to estimate the consistency of RAPDs and ISSRs in studying genetic similarity among accessions of a selfer species sampled from a larger geographical distance.

CONCLUSION

In *L. sativus* the majority of total genetic variation belongs to between populations, indicating that it is a selfing species. The levels of genetic variation across accessions were very variable, a common character for selfing species.

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REZIME

Genetička varijabilnost odabranih individual *Lathyrus sativus* (Fabaceae) na osnovu RAPD analize

Houshang Nosrati, Mohammad-Ali Hosseinpour-Feizi, Maryam Nikniazi, Ahmad Razban-Haghighi

Odnos genetičkog diverziteta i geografske distance izmedju populacija biljaka najčešće se objašnjava sistemima ukrštanja. U ovom radu procenjivano je pet populacija mahunarke *Lathyrus sativus* uz pomoć RAPD. Za svaku populaciju tretirano je deset individual odabranih po principu slučajnosti. Pet prajmera produkuje 73 jasnih polimorfnih bandova. Precentualno izražena polimorfnost iznosi od 20.6% kod nemačkih do 60.3% kod poljskih individual. Opseg Nei genetičkog diverziteta je širok, od 0.075 kod nemačkih do 0.25 kod poljskih individua. Ukupan genetički diverzitet na osnovu AMOVA ide do 76.44% izmedju populacija do 23.56% unutar populacija, što ukazuje na samooplodnju kod *L. sativus*. Najmanja genetička distance je izmedju nemačkih i iranskih uzoraka (0.202), dok je najveća izmedju iranskih i poljskih uzoraka (0.5102), što ukazuje da kod samooplodnih vrsta genetički diverzitet izmedju populacija nije korelisan sa geografskim distancama.

Ključne reči: genetički diverzitet, *Lathyrus sativus*, RAPD, samooplodnja