JJBS

Volume 8, Number 1, March .2015
ISSN 1995-6673
Pages 61 - 64

Jordan Journal of Biological Sciences

Evaluating the Genetic Relatedness within *Lupinus pilosus* L. Species Based on RAPD Analysis

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Received: December 26, 2014 Revised: January 13, 2015 Accepted: January 17, 2015

Abstract

Nine Random Amplified Polymorphic DNA (RAPD) primers showing polymorphic bands were used to determine the genetic relatedness among and within single individual plants of *Lupinus pilosus* and used for the construction of the dendrogram and the similarity matrix. A total of 1112 bands were obtained, 219 of which were polymorphic. Similarity values among the studied single individuals of *Lupinus pilosus* ranged from 0.450 to 0.02. The cluster analysis obtained by Unweighted Pair-Group Method with arithmetic averages (UPGMA) grouped the tested individuals into three clusters. A genetic variation was found within *Lupinus pilosus* based on RAPD markers. The variability emphasis the presence of genetic diversity within *Lupinus pilosus* species.

Keywords: Lupine, Genetic diversity, Polymorphism, RAPD.

1. Introduction

Lupinus, commonly known as Lupine or lupin, is a genus including more than 200 species; it is one of the most diverse and widespread taxonomic groups of flowering plants. Its wild species occur naturally in the Mediterranean region, including areas of Northern Africa and in North and South America and used as a sources of food protein (Wolko *et al*., 2011; Drummond *et al*., 2012). Sienkiewicz *et al*. (2008) reported that wild lupins, including perennial and annual species, are distributed across climatic ranges from subarctic Alaska, Mediterranean and semi desert climates, Africa, Mexico and USA. The chromosome numbers were determined in 22 accessions of 16 *Lupinus* species from Bolivia, Ecuador and Peru, all had 2n=48 chromosome except *L. bandelierae* (Camillo *et al*., 2006). *Lupinus pilosus*, like the Old World (Africa) rough-seeded lupines, has chromosome number 2n=42 as described by (Naganowska *et al*., (2003). Lupine oil could also be a valuable source of phosphatidylcholine in the human diet; the content of fatty acids in lupine oil is similar to that of oils from other oilseed crops (Borek *et al*., 2009). Many techniques proved useful for both inter- and intra-specific studies of diversity in *Lupinus*. Random Amplified Polymorphic DNA (RAPD) was used to study the molecular taxonomy among *Mentha spicata*, *Mentha longifolia* and *Ziziphora tenuior* populations (Al-Rawashdeh, 2011); also RAPD and Inter Simple Sequence Repeat (ISSR) markers were used to determine the genetic relationships among 20 Old World lupin genotypes from 3 lupin species (*Lupinus albus, L. angustifolius, L. luteus*) using 15 primers in each case (Yorgancilar *et al*., 2009). Talhinhas *et al*. (2003) used AFLP, ISSR and RAPD techniques to evaluate the genetic diversity among *L. albus, L. angustifolius, L. cosentinii, L. hispanicus, L. luteus, L. mutabilis, L. pilosus* and *L. polyphyllus*; they found low levels of similarity that ranged from 0.205 to 0.432. Aïnouche and Bayer (1999) studied 44 taxa of *Lupinus* genus using Internal Transcribed Spacer (ITS). DNA Amplification Fingerprinting (DAF) techniques were used to evaluate the genetic relationships among 24 randomly selected white lupin (*Lupinus albus* L.), accessions originating from four endemic regions (Qiu *et al*., 1995). The genetic diversity was studied in 94 accessions of white lupin (*Lupinus albus* L.) (Raman *et al*., 2014) and among different taxa (Sienkiewicz *et al*., 2008) as well as among and within Moroccan lupine species, using ISSR and AFLP (Sbabou *et al*., 2010) and molecular phylogeny which was reconstructed using nucleotide sequences of *rbc*L and nuclear ITS regions of 54 lupine species (Wink *et al*., 1999). The 2C nuclear DNA content was estimated for 18 species of genus *Lupinus* using propidium iodide (Naganowska *et al*., 2003). To the best of our knowledge, there are few studies on the genetic diversity of *Lupinus pilosus* using RADP markers, particularly in Jordan. The present study was undertaken to assess the genetic diversity and relatedness within some single individual plants of *Lupinus pilosus* using RAPD markers.

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2. Materials and Methods

2.1. Plant Material and DNA Isolation

The leaves of a single plant were collected from the wild field at As-salt governorate in Jordan (Plate 1) and then transferred into a lab of NCARE for DNA analysis. The total cellular DNA was extracted from a single individual of each sample according to the Doyle and Doyle (1987), Yorgancilar *et al.* (2009) and Talhinhas *et al.* (2003) with minor modifications. Twenty mg of fresh leaves were grounded in the presence of liquid nitrogen and the homogenate resuspended in extraction buffer with 750 µl of freshly and preheated 2x CTAB solution with 0.8g PVPP in 2 ml tubes and then placed at 65ºC for 1 hr. The mixture was added to 750 µl of chlorophorm/ isoamyl alcohol (24:1), vortexed for few seconds, and then centrifuged at 14,000g for 20 min. The supernatant was placed in 2 ml tubes with 750 µl isopropanol, and then mixed gently until the thread of DNA appeared; it is then centrifuged for 20 min at 14000 g. The solution is poured in tubes and left to dry; then 750 µl of cooled 70% ethanol was added to the solution and placed in the refrigerator (–20ºC) overnight. The following day, the ethanol was poured in the dried tubes and 100 µl of TE was added and the whole mixture was placed at 65ºC for 1 hr. Four micolitters of RNAase (10mg/ml) were added per tube and left for 1hr at 37ºC. The DNA concentration was measured using S2100 UV/VIS DIODE-Array-Spectrophotometer, machine Version 1.7.

Plate 1. Wild *Lupinus pilosus* L. species grown at Wadishueib in Jordan

2.2. PCR Amplification

In order to select primers with high level of polymorphism, the RAPD analysis was carried out using 40 of 10-mer primers, corresponding to kits A, B, D, T, W and Z (Opern Technologies), as previously described by Williams *et al.* (1990); those analyzed for polymorphism used in 4 populations. Each reaction was repeated twice. Only repeatable fragments with strong and medium intensity were used in the analysis. Nine RAPD markers showed a high polymorphism and were used in genetic diversity analysis. The PCR reaction was performed as described by Williams *et al.* (1990) with 10-mer ologonucleotides synthesized by Operon technologies (Almeda, Calif.). The final volume of 25µl contained 10 x buffer with $MgCl_2$, 20 ng of total genomic DNA, 0.25 mM dNTPs (Promega), 12 pmole of primers (Opern technologies, US), 1.5 mM $MgCl_2$ and 1U of *Taq* polymerase (Promega). Amplification was carried out in thermocycler (MJ Research, USA, Model PCT-200), one cycle of 1 min at 94°C followed by 44 cycles, each consisting of a denaturation step for 1min at 94°C, followed by an annealing step for 1min at 36°C and an extension step for 2 min at 72°C, followed by a further extension step for 5 min at 72°C. After the final cycle, the samples were cooled at 4°C. Samples of 10 µl RAPD-PCR product were analyzed by electrophoresis on 1.4% a garose gel and the amplified products were detected after staining by ethidium bromide.

2.3. Data Analysis

For subsequent statistical analysis, in order to obtain a binary matrix, polymorphic bands amplified by primers of RAPD were scored as present (1) or absent (0). Genetic similarities for RAPD markers were calculated by using the Jaccards' coefficient (Jaccard, 1908) and the dendrogram obtained by clustering according to the Unweighted Pair-Group Method with arithmetic averages (UPGMA) using the SPSS 2000 (v.11.0) software. The polymorphism percentage was estimated by dividing the number of polymorphic bands over the total number of bands.

3. Results

3.1. RAPD Markers Variations

A total of 1112 bands with an average of 124 polymorphic bands per primer were amplified by RAPD analysis (Table 1). Among those, 219 were polymorphic across the 40 single individual. The highest and lowest numbers of polymorphic bands per assay were 33 and 20 for (OPD14) and (OPB01 and OPB12) bands, respectively (Table 1). The highest percentage of polymorphism was obtained by OPD14, OPB12, OPT16 and OPB01, while OPA16 showed the lowest (%) of polymorphism.

Table 1: Total amplified bands, number of polymorphic bands and percent of polymorphic bands of RAPD primers used in *Lupinus pilosus* analysis.

Primer name	Total bands/primer	Number of polymorphic bands	% of polymorphism
OPA16	153	22	14
OPB01	095	20	21
OPB06	150	23	15
OPB12	083	20	24
OPB10	123	24	20
OPB17	144	28	19
OPD14	117	33	28
OPT16	183	28	24
OPT19	068	21	31
Total over loci	**1112**	**219**	**19.6**
Mean per primer	**124**	**24**	**-**

3.2. Estimation of Genetic Similarity

High similarity values, recorded between pair of individuals of *Lupinus pilosus*, were (17 and 19), (34 and 35), (31 and 32), (25 and 27) and (1 and 2), 0.43, 0.45, 0.43, 0.39 and 0.36, respectively; while the lowest similarity value (0.02) was recorded between individuals (3 and 35; 12 and 4; and 4 and 10) (Figure 1 and Table 2). A dendrogram was arbitrarily divided into three clusters. The first cluster was divided into two sub clusters; the first including 17 single individuals, the second cluster had 20 individuals (Figure 1). The second cluster included only one individual (number 21). The final cluster contained two individuals (number 39 and 40)

Table 2. Similarity matrix values among and within *Lupinus*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1	1																																							
2	0.36	1																																						
3	0.25	0.32	1																																					
4	0.20	0.20	0.18	1																																				
5	0.20	0.23	0.29	0.30	1																																			
6	0.08	0.11	0.10	0.10	0.25	1																																		
7	0.13	0.28	0.20	0.20	0.23	0.33	1																																	
8	0.17	0.15	0.20	0.09	0.18	0.29	0.25	1																																
9	0.15	0.25	0.18	0.07	0.21	0.26	0.29	0.37	1																															
10	0.11	0.14	012	0.02	0.13	0.24	0.14	0.17	0.29	1																														
11	0.17	0.15	0.21	0.14	0.19	0.05	0.11	0.19	0.21	0.14	1																													
12	0.06	0.15	0.11	0.02	0.09	0.15	0.11	0.14	0.25	0.28	0.24	1																												
13	0.08	0.18	0.14	0.08	0.10	0.11	0.09	0.19	0.28	0.23	0.20	0.32	1																											
14	0.09	0.09	0.14	0.07	0.14	0.16	0.10	0.29	0.21	0.20	0.23	0.25	0.31	1																										
15	0.04	0.06	0.07	0.09	0.21	0.27	0.09	0.18	0.17	0.22	0.13	0.21	0.19	0.35	1																									
16	0.14	0.12	0.08	0.06	0.13	0.25	0.15	0.28	0.31	0.20	0.16	0.14	0.14	0.31	0.26	1																								
17	0.09	0.11	0.12	0.10	0.17	0.18	0.14	0.27	0.20	0.22	0.20	0.13	0.16	0.22	0.30	0.32	1																							
18	0.14	0.12	0.11	0.11	0.14	0.22	0.11	0.22	0.25	0.20	0.19	0.14	0.26	0.20	0.28	0.28	0.42	1																						
19	0.12	0.10	0.16	0.12	0.16	0.20	0.11	0.25	0.15	0.15	0.19	0.10	0.15	0.20	0.26	0.23	0.43	0.35	1																					
20	0.13	0.16	0.12	0.14	0.22	0.07	0.16	0.07	0.12	0.14	0.15	0.09	0.09	0.19	0.15	0.10	0.18	0.11	0.11	1																				
21	0.04	0.10	0.11	0.14	0.09	0.04	0.06	0.06	0.05	0.06	0.10	0.08	0.06	0.13	0.14	0.06	0.13	0.10	0.17	0.13	1																			
22	0.09	0.17	0.20	0.13	0.18	0.08	0.17	0.11	0.12	0.06	0.18	0.07	0.14	0.11	0.11	0.15	0.12	0.19	0.11	0.16	0.11	1																		
23	0.09	0.14	0.15	0.15	0.21	0.12	0.20	0.09	0.10	0.12	0.14	0.05	0.10	0.18	0.04	0.08	0.12	0.05	0.11	0.21	0.11	0.26	1																	
24	0.08	0.13	0.14	0.15	0.25	0.12	0.14	0.13	0.16	0.08	0.15	0.11	0.11	0.10	0.15	0.12	0.21	0.13	0.13	0.18	0.13	0.22	0.24	1																
25	0.09	0.09	0.07	0.15	0.15	0.07	0.14	0.10	0.10	0.03	0.18	0.10	0.07	0.12	0.06	0.09	0.14	0.11	0.10	0.12	0.09	0.31	0.21	0.33	1															
26	0.05	0.09	0.09	0.11	0.11	0.03	0.09	0.06	0.06	0.10	0.10	0.010	0.16	0.14	0.08	0.02	0.09	0.10	0.10	0.16	0.11	0.13	0.17	0.23	0.31	1														
27	0.06	0.09	0.06	0.13	0.13	0.10	0.10	0.07	0.03	0.12	0.07	0.07	0.07	0.14	0.09	0.06	0.10	0..13	0.11	0.14	0.08	0.20	0.17	0.24	0.39	0.33	1													
28	0.08	0.16	0.14	0.17	0.19	0.14	0.23	0.09	0.10	0.06	0.09	0.02	0.05	0.12	0.09	0.08	0.12	0.11	0.09	0.18	0.08	0.27	0.27	0.34	0.38	0.27	0.38	1												
29	0.05	0.09	0.11	0.09	0.13	0.16	0.12	0.15	0.19	0.16	0.14	0.13	0.16	0.08	0.15	0.15	0.21	0.19	0.17	0.14	0.11	0.26	0.17	0.30	0.29	0.14	0.19	0.22	1											
30	0.08	0.09	0.04	0.12	0.10	0.09	0.08	0.09	0.10	0.10	0.11	0.05	0.14	0.12	0.09	0.17	0.12	0.13	0.11	0.11	0.04	0.17	0.14	0.16	0.28	0.28	0.30	0.26	0.30	1										
31	0.07	0.04	0.05	0.05	0.13	0.14	0.07	0.13	0.12	0.10	0.10	0.10	0.14	0.18	0.18	0.15	0.09	0.12	0.08	0.08	0.09	0.11	0.13	0.16	0.20	0.18	0.15	0.23	0.27	0.36	1									
32	0.09	0.11	0.08	0.08	0.15	0.08	0.15	0.09	0.08	0.04	0.09	0.04	0.08	0.16	0.07	0.10	0.06	0.03	0.02	0.10	0.06	0.17	0.10	0.17	0.24	0.19	0.20	0.21	0.11	0.27	0.41	1								
33	0.09	0.12	0.11	0.07	0.19	0.23	0.14	0.21	0.16	0.16	0.10	0.15	0.16	0.12	0.21	0.17	0.16	0.15	0.12	0.14	0.07	0.21	0.18	0.20	0.20	0.11	0.16	0.20	0.29	0.18	0.39	0.26	1							
34	0.09	0.09	0.04	0.08	0.21	0.12	0.10	0.16	0.12	0.10	0.10	0.12	0.08	0.19	0.12	0.08	0.10	0.09	0.09	0.12	0.06	0.13	0.15	0.28	0.27	0.15	0.13	0.22	0.22	0.10	0.27	0.21	0.38	1						
35	0.06	0.09	0.02	0.08	0.12	0.07	0.10	0.11	0.06	0.4	0.05	0.03	0.09	0.14	0.09	0.08	0.08	0.03	0.03	0.07	0.02	0.19	0.14	0.18	0.33	0.20	0.16	0.26	0.16	0.26	0.36	0.37	0.33	0.45	1					
36	0.17	0.14	0.06	0.06	0.13	0.08	0.13	0.16	0.12	0.08	0.08	0.06	0.06	0.09	0.02	0.11	0.06	0.05	0.07	0.10	0.06	0.18	0.10	0.15	0.19	0.05	0.10	0.17	0.17	0.15	0.27	0.29	0.29	0.30	0.31	1				
37	0.05	0.05	0.04	0.02	0.02	0.06	0.04	0.08	0.11	0.19	0.08	0.13	0.24	0.11	0.10	0.09	0.06	0.06	0.08	0.04	0.05	0.11	0.11	0.04	0.12	0.16	0.11	0.08	0.14	0.21	0.14	0.14	0.14	0.09	0.16	0.17	1			
38	0.07	0.08	0.03	0.07	0.11	0.09	0.05	0.06	0.12	0.13	0.06	0.06	0.10	0.09	0.12	0.09	0.13	0.12	0.08	0.14	0.11	0.09	0.09	0.16	0.20	0.15	0.13	0.14	0.25	0.16	0.19	0.13	0.18	0.22	0.16	0.17	0.27	1		
39	0.02	0.04	0.02	0.03	0.03	0.08	0.03	0.06	0.07	0.10	0.08	0.12	0.08	0.04	0.08	0.03	0.02	0.03	0.05	0.07	0.02	0.03	0.05	0.02	0.04	0.08	0.05	0.02	0.06	0.07	0.06	0.03	0.04	0.02	0.03	0.02	0.06	0004	1	
40	0.06	0.09	0.08	0.13	0.08	0.12	0.10	0.20	0.10	0.04	0.05	0.02	0.09	0.10	0.09	0.08	0.12	0.09	0.05	0.07	0.04	0.12	0.12	0.10	0.07	0.07	0.08	0.07	0.12	0.08	0.09	0.06	0.12	0.04	0.06	0.08	0.02	0.09	0.16	1

Figure 1: A dendrogram within *Lupinus pilosus* L. species using nine polymorphic RAPD primers based on Jaccards´ coefficient of similarit

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4. Discussion

Knowing the genetic diversity between and within the populations of *Lupinus* species is a prerequisite for the management, the monitoring and the conservation of genetic resources. The genetic diversity within and among the tested individuals of *Lupinus pilosus* was present under the present study. The forming separate cluster with one single individual confirmed that each individual had a unique DNA sequence, which could be due to the differences in the fatty acid compositions, protein content as well other gene expressions. Borek *et al*. (2009) reported that the main fatty acid in yellow lupine cotyledons was linoleic acid; in white lupine it was oleic acid, and, in Andean lupine, it was both linoleic and oleic acids. On the other hand, a low percentage of similarity could be due to a high, long diverging process concerning non-coding regions. Talhinhas *et al*. (2003) reported that the low genetic similarity among *Lupinus* spp. is most unlikely to be due to the differences in coding sequences. In this investigation, a high level of polymorphisms was found within single tested individuals of *Lupinus pilosus* L. This is due to its ploidy level. *Lupinus pilosus*, as the Old World (Africa) rough-seeded lupines, has chromosome number 2n=42 as described by Naganowska *et al*. (2003). A high level of genome diversity was found among the lupine accessions, but several others, from the Middle East and West Africa, tended to cluster together; the results support the future use of DAF markers for the characterization and identification of white lupine germplasm (Qiu *et al*., 1995). Sienkiewicz *et al*. (2008) indicated that the American species were characterized by the widest diversity with respect to ecological distribution and adaptive abilities. Pezhmanmehr *et al*. (2009) stated that a high level of polymorphisms (86%) was obtained by RAPD analysis, indicating the effectiveness of this marker for the evaluation of genetic diversity in Black cumin. Knowledge of wide genetic diversity observed in the *Lupinus pilosus* L. populations, using molecular markers, provides important information for the management of germplasm resources with regard to future domestication and breeding programs, in addition to selecting markers linked to a gene conferring disease resistance within *Lupinus* genus. Further molecular markers, such as ISSR, AFLP and SSRs, are needed to test the genetic relatedness between other *Lupinus* species (native or exotic) and compared to *Lupinus pilosus* in the future. Also, the individuals that showed diverse traits can be selected by breeders to use in the breeding programs and they can be invested in establishing propagate working for *Lupinus* species in Jordan.

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