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GENETIC VARIATION IN EGYPTIAN WHITE LUPIN (*Lupinus albus* L.) GENOTYPES BASED ON COMBINED DATA OF ISSR AND FLUORESCENCE-BASED AFLP MARKERS

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The genus *Lupinus* comprises more than 300 species, but only four of them have gained agronomic importance. These are *Lupinus albus* (white lupin), *L. angustifolius* (blue lupin) and *L. luteus* (yellow lupin) of the "Old World" lupin species, and one "New World" species namely *L. mutabilis* (Pearl lupin) (Hondelmann, 1984; Yorgancilar *et al*., 2009). *Lupinus albus* L. (2n=50 chromosomes) belongs to the tribe Genisteae, subfamily Papilionoideae, family Fabaceae (Wolko *et al*., 2011). White lupin is cultivated in a wide range of environments across Egypt. It is suitable for highly variable soil types (El-Attar *et al*., 1987). Egypt ranks the ninth among the world in lupin annual production (3077 metric tons). However, Egypt's lupin high quality is the fourth according to international prices (FAO, 2010).

A landrace is an important source of alleles for improving some yield properties in local cultivars (Raza and Jornsgard, 2005).Since the conservation of landraces as genetic resources for breeding programs is crucial, a large number of accessions have been collected, stored and maintained in gene banks

There has been a significant increase in the application of molecular genetics methods for assessing the conservation and use of plant genetic resources (Badr *et al*., 2002; Mondini *et al*., 2009). Among molecular markers, AFLP technique provides a very powerful DNA fingerprinting technique for DNAs of any origin or complexity. However, the number of fragments that can be analyzed simultaneously is dependent on the resolution of the detection system (Vos *et al*., 1995). Fluorescence-labeled PCR products are best analyzed on an automated DNA sequencer (Genetic Analyzer) that replaced the standard polyacrylamide gel electrophoresis equipment (Karudapuram and Larson, 2005; Weising *et al*., 2005). Capillary electrophoresis (CE) has some advantages over slab gels (polyacrylamide gels) in resolution, speed and the availability of quantitative information in an electronic format following the completion of a run (Butler, 2005).

Another molecular technique based on microsatellite loci is inter simple sequence repeat ISSR-PCR, which involves the use of microsatellite sequences as primers in a polymerase chain reaction to generate multi-locus markers. ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Reddy *et al*., 2002).

This study was conducted in an attempt to address the following objectives: (1) to estimate the genetic polymorphism among eighteen lupin genotypes, including cultivars and landraces, using ISSR and AFLP; (2) to identify unique DNA markers and determine a specific fingerprint for each genotype that acts as a unique identity when conserved in the Egypt's National Gene Bank (NGB) and (3) to assess the genetic relationships between these genotypes thus helping in choosing the most likely parents from a selection of candidates for future breeding programs.

MATERIALS AND METHODS

Plant material

Eighteen *Lupinus albus* L. accessions were provided by the National Gene Bank (NGB), Agriculture Research Center (ARC), Giza, Egypt. Accession numbers, collection dates and sites are listed in Table (1). The two cultivars Giza 1 and Giza 2 were developed through individual selection from local landraces; Giza 1 is adapted for cultivation in northern region of Egypt, whereas Giza 2 is adapted for Upper Egypt region planting. Both cultivars are tolerant to wilt disease (Hefny, 2011). The local commercial varieties, lines 3, 15 and 21 are breeding lines under investigation that are developed by Agriculture Research Center (ARC), Giza, Egypt.

Methods

Genomic DNA extraction, purification and quantification

The CTAB protocol was adapted, according to Doyle and Doyle (1990) to

extract total genomic DNA from the eighteen different accessions. The quantity and quality of the extracted DNA were determined using spectrophotometric measurement of UV absorbance at 260 nm and 280 nm in a Thermo Scientific NanoDrop 2000™ spectrophotometer.

Inter simple sequence repeat (ISSR) technique

ISSR-PCR reactions were conducted using twelve anchored primers which were synthesized by Eurofins, Germany. The primer names, sequences and annealing temperatures are shown in Table (2). The reaction conditions were optimized and the following reagents were mixed in a final volume of 25 µl: 200 µM of dNTPs, 2 mM of $MgCl_2$, 1 X of green GoTaq® Flexi buffer, 20 pM of primer, 30 ng of template DNA, 1 U of GoTaq® Flexi DNA Polymerase and distilled H_2O up to 25 µl; (Taq DNA Pol., Buffer, $MgCl_2$ and dNTPs were all supplied from Promega). Amplification was carried out in a Gene Amp® PCR System 9700 thermal cycler (Applied Biosystems) programmed as follows: 94°C/5 min (1 cycle); [94°C/1 min, 40, 42, 45, 46 or 50°C/1 min, 72°C/2 min] (40 cycles); 72°C/7 min (1 cycle) and 4°C (infinitive). A volume of 10 µl of the ISSR-PCR product was resolved using (1.5%) agarose gel electrophoresis according to Sambrook *et al*. (1989). A 1 Kb DNA marker (Fermentas Gene Ruler) was used as a DNA molecular weight standard. Results were visualized on a UV transilluminator at 302 nm and photographed by Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software, Bio-Rad™. Only the clearest and strongest ISSR bands were scored manually as present (1) or absent (0) to be used for further analysis.

Amplified fragment length polymorphism (AFLP) technique

AFLP procedure was applied according to the AFLP Plant Mapping protocol - Applied Biosystems, 2010.

AFLP assay

Genomic DNA was digested with the restriction enzymes *Eco*RI and *Mse*I (New England BioLabs) and then ligated with adaptors using T4 DNA ligase (New England BioLabs) to produce modified restriction fragments to be used in pre-selective amplification. Selective amplification was performed using two AFLP primers; one Fluorescence-labeled primer: *Eco*RI [Dye-primer-AXX] and unlabeled *Mse*I [Primer–CXX]. Four primer combinations were used: E-ACT (FAM)/M-CAT, E-ACG(JOE)/M-CAT, E-ACG(JOE)/M-CAG and E-ACC(NED)/M-CTA.

One µl of selective amplified product was mixed with 12 µl of Hi-Di formamide and 0.5 µl of GeneScan500 ROX internal size standard (Applied Biosystems, Foster City, California, USA). The mixture was denatured and loaded on the single capillary of the Applied Biosystems 310 Genetic Analyzer. POP-4 polymer (Applied Biosystems) was used as a molecular sieve. GeneMapper®

Analysis Software Version 4.1 (Applied Biosystems) was used in analyzing the generated electropherograms in raw data according to AFLP System Analysis Getting Started Guide (Applied Biosystems). The software was configured to perform binary scoring of the AFLP alleles from 50 to 500 base pairs (bp). A peak height of 100 RFU was set as a threshold.

Data analysis

A number of genetic diversity parameters were evaluated from ISSR and AFLP fingerprinting data to obtain a measure of the usefulness of the marker systems. Number of observed alleles (Na), number of effective alleles (Ne) (Hartl and Clark, 1989) and Shannon index (I) that estimates Shannon's information index as a measure of gene diversity (Shannon, 1949) are basic parameters for genetic diversity that were calculated in the POPGENE program version (1.32) software (Yeh *et al*., 1997). The Polymorphic Information Content (PIC), as a value of a marker for detecting polymorphism within a population, was estimated by the Power Marker program version (3.25) software according to Botstein *et al*. (1980).

Other parameters were also evaluated; the multiplex ratio (MR) was calculated, according to Powell *et al*. (1996), representing the total number of loci simultaneously detected per assay. The effective multiplex ratio (EMR) was defined as the average number of polymorphic loci detected per assay (Powell *et al*., 1996) and the marker index (MI) was used to calculate the overall utility of a marker system depending on the formula; MI = EMR x PIC (Powell *et al*., 1996; Tonk *et al*., 2011). Mantel test (Mantel, 1967) was estimated by the Power Marker program, to determine the significance of correlation between the two genetic distance matrices revealed by both marker systems. The pairwise comparisons between the tested genotypes were used to calculate the genetic similarity using the Bio-Rad diversity database software package according to Nei and Li coefficient (Nei and Li, 1979). Cluster analysis was presented as a dendrogram based on similarity estimates using the un-weighted pair-group method with arithmetic average (UPGMA) (Sokal and Michener, 1958).

RESULTS AND DISCUSSION

ISSR analysis

In the present study twelve ISSR primers were used to investigate the genetic polymorphism among the 18 lupin genotypes. In general, these primers varied in their ability to diagnose lupin accessions. As shown in Fig. (1), the size of ISSR amplified fragments among the 12 primers ranged from 250 to 4000 bp with a total number of 168 bands; 100 of these (59.5%) were polymorphic across the 18 genotypes (Table 2). The number of amplicons/primer ranged from eight with the primer (BEC) to 20 with the primer (HB-15). While the number of polymorphic amplicons varied from three with the primer (ISSR-2) to 14 with primers (ISSR-1 and UBC-815). Thus, the average number of polymorphic fragments per primer was 8.3. This polymorphism is of

major importance in germplasm management: as a means of identification, to detect genetic diversity and to reveal genetic relationships.

The present findings are in partial agreement with those obtained by Gilbert *et al*. (1999) who found that 37 accessions of *Lupinus albus* gave reliable banding patterns with ten ISSR primers in which 137 total bands were scored including 122 polymorphic bands, showing a percentage of polymorphism of 89%. They detected the genetic variability within and between accessions of lupin germplasm and reported that diagnosis of all the 37 lupin accessions was possible using any two of the ten ISSR primers. Similar results were also reported by Sbabou *et al*. (2010) who tested 15 ISSR primers to assess intraspecific polymorphism among 10 accessions of *L. albus* that showed a high level of polymorphism (97.7%). In addition, sixty-four specific fragments (15.9%) were scored.

Positive and negative unique markers were used in genotype identification and in generating a unique fingerprint for each genotype. Results presented in Table (2) showed a total of 18 unique ISSR markers generated by the 12 primers, including nine positive and nine negative unique markers. The positive unique markers (PUMs) were characterized by seven primers, while, the negative unique markers (NUMs) were recorded by five primers. Primers ISSR-4 and BEC characterized three genotypes each, primers ISSR-1, UBC-809 and UBC-815 characterized two genotypes each, while primers ISSR-3, HB-15, 17899-A, 17898-B and UBC-807 identified only one genotype each. On the other hand, primers ISSR-2 and UBC-808 did not show any unique markers. The genotype 17 was identified by two PUM in addition to one NUM, while the genotype 1 was characterized by one PUM and two NUMs. Moreover, the genotypes 4, 5 and 14 were characterized by one PUM and one NUM each. Six genotypes were identified by only one PUM (genotypes 2, 3 and 16) or one NUM (genotypes 7, 10 and 13).

AFLP analysis

AFLP electropherograms of three *Lupinus albus* genotypes (9, 10 and 11) selectively amplified by the primer combination E-ACC(NED)/M-CTA (as an example) are illustrated in Fig. (2). The grey bars represent bins "alleles" assigned to the peaks. The quality flags of Off-scale (OS) and Sizing quality (SQ) process quality values (PQV) are shown on top of each sample. Peaks highlighted in black indicate common peaks present in all three genotypes. AFLP electropherograms of three *Lupinus albus* genotypes (13, 14 and 18) amplified selectively by the primer combination E-ACT(FAM)/M-CAT are demonstrated in Fig. (3). Red arrow indicates example of polymorphic peaks that are present in genotype 13 and absent in the other two genotypes.

In order to inspect polymorphic peaks and allele calls more efficiently for all genotypes present in each of the four AFLP primer combinations, an overlay

view of the eighteen AFLP sample plots was generated for each primer combination. Figure (4) represents a section of a sample plot where peaks amplified for the eighteen *Lupinus albus* accessions were overlaid on top of each other with the primer combination E-ACT (FAM)/M-CAT. The grey bars indicate the bins "called alleles" which were assigned to the peaks. The calculated size of the fragment (in bp), and the calculated height of the peak (in RFU) are also shown. Red arrow indicates an example of monomorphic peaks that are present in all of the 18 samples; these peaks are not called as "delete common alleles" is set in the GeneMapper software.

The four primer combinations tested for the selective amplification of DNA fragments of the 18 lupin genotypes produced a total of 638 well-resolved AFLP peaks of which 604 were polymorphic (Table 3). This corresponds to a level of polymorphism of 94.6%. The different primer combinations revealed different levels of polymorphism among the 18 lupin genotypes. The highest number of amplified DNA fragments was 222 with the primer combination E-ACG(JOE)/M-CAT, while the lowest number was 119 with the primer combination E-ACC(NED)/M-CTA. The number of polymorphic amplicons per primer combination ranged from 113 (94.9%) with the primer combination E-ACC (NED)/M-CTA to 221 (99.5%) with the primer combination E-ACG (JOE)/M-CAT. On the average, the number of amplicons per primer across the 18 genotypes was 159.5 and for polymorphic amplicons were 151. These results are in partial agreement with a study aiming to explore the genetic diversity of 122 lupin lines in the United States Department of Agriculture (USDA) germplasm collection using eighteen primer combinations that amplified a total of 2277 fragments. Fluorescent labeled AFLP fragments were separated on CEQ 8800 genetic analyzer (Iqbal *et al*., 2008).

Talhinhas *et al*. (2003) evaluated the genetic diversity among *L. albus* and seven related species using twelve AFLP primer combinations (detected by denaturing polyacrylamide gel), resulting in 1340 bands (an average of 112 bands per primer), of which only 12 were monomorphic (0.9% of total). Further, they used two more AFLP primer combinations in an intra-specific genetic diversity assay between five accessions belonging to *L. albus*. Comparable results were also reported by Sbabou *et al*. (2010) who used ten AFLP primer combinations to study inter-specific genetic diversity between four Moroccan *Lupinus* germplasms on denaturing polyacrylamide gel. They detected 510 bands (with an average of 51 bands by primer), 457 (89.6%) polymorphic fragments and 322 (30.26%) unique bands. In the present study, a high level of polymorphism (94.6%) with an average of 159.5 peaks per primer was recorded. This may be attributed to the difference in the detection method; as the automated fluorescent-AFLP is a more accurate and sensitive technique than the manual denaturing polyacrylamide gel.

AFLP positive and negative unique markers were successful in distinguishing a number of lupin genotypes among the four primer combinations. In the E-ACT (FAM)/M-CAT primer combination, 15 lupin genotypes were distinguished by 55 positive and/or negative AFLP unique markers (Table 3). Each of the 15 genotypes revealed one or more unique marker. These markers ranged in size from 57 to 491 bp. While the primer combination E-ACG(JOE)/M-CAT was able to distinguish 14 lupin genotypes by 80 positive and negative AFLP unique markers that ranged between 57 and 498 bp. The E-ACG (JOE)/M-CAG primer combination characterized 11 lupin genotypes by 44 positive and/or negative AFLP unique markers ranging from 67 to 498 bp. Finally, the primer combination E-ACC (NED)/M-CTA succeeded to distinguish 13 genotypes by 27 positive and/or negative AFLP unique markers.

Both ISSR and AFLP markers succeeded to produce positive and negative unique markers that helped in genotype identification. This result matched some related lupin studies where a number of species-specific and accession-specific markers were detected, thus assisting in molecular identification of lupin genotypes (Gilbert *et al.*, 1999; Sbabou *et al*., 2010) and is useful in plant breeding programs.

The result showed different levels of polymorphism between primers and primer combinations ranging from 99.5% polymorphism for AFLP primer combination E-ACG(JOE)/M-CAT to 25% of polymorphism for ISSR primer HB-15. Variations in the level of polymorphism detected by either AFLP or ISSR assays could be due to different primers or primer combinations used, in addition to the different lupin genotypes included in the different investigation.

Genetic relationships

Information about the genetic relationships among accessions of the same species and between different species has valuable applications in crop improvement programs. Knowledge of genetic relationships among genotypes permits the organization of germplasm, including elite lines, and provides more efficient parental selection. Furthermore, exotic germplasm is an important source of genes with highly quantitative effects on traits.

In this study, the investigated *L. albus* genotypes showed a relatively high genetic similarity using ISSR markers, ranging from 0.77 to 0.935 (Data not shown). On the other hand, AFLP markers showed a relatively lower genetic similarity range from 0.29 to 0.751 (Data not shown). In this respect, similar results were reported by Sbabou *et al*. (2010) who found similarity values ranging from 0.70 to 0.82 among 10 accessions of *L. albus* tested by 15 ISSR primers. However, the results presented in this study disagree with Talhinhas *et al*. (2003) who recorded similarity values ranging between 0.908-0.955 among five *L. albus* accessions tested by two AFLP primer combinations. This can refer to the high

discriminatory power of AFLP especially the automated fluorescent-AFLP. However, by combining AFLP and ISSR data to generate more accurate relatedness among the 18 lupin genotypes, it was found that the range of pair similarity coefficient was from 0.530 to 0.805 (Table 4). Genetic similarity values may vary among different studies in terms of evaluation of *Lupinus albus* genotypes collections. This is due to the difficulty to compare genetic distances between different materials in the different studies, different markers used and number of genotypes analyzed.

Because landraces are genetically dynamic populations, being affected by the surroundings, the genetic similarity may be related to some extent to the location where a landrace is cultivated. Landraces 13 and 14 (both cultivated in Upper Egypt; Qena and Sohag, respectively) recorded the highest genetic similarity (0.935) among the 18 lupin genotypes tested by ISSRs. However, AFLPs revealed the highest genetic similarity (0.751) between landraces 6 and 8 that share the same location (Fakous-Sharkeya) (Data not shown). The results from the combined data supported the AFLP results as exhibiting the highest genetic similarity (0.805) which was recorded between landraces 6 and 8. On the other hand, genotypes showing the lowest genetic similarity are of great importance to plant breeders to be further selected as parents. Weising *et al*. (2005) mentioned that it is mandatory that genetically divergent parents be chosen that exhibit sufficient polymorphisms, but are not so distant as to cause sterility of the progeny. ISSRs revealed the lowest genetic similarity (0.77) between landrace 1 (Belbies-Sharkeya) and cultivar 17 (Giza 2-adapted for Upper Egypt region), while AFLPs detected the lowest genetic similarity (0.294) between landrace 4 (Belbies-Sharkeya) and the foreign germplasm 18 (from France). The combined data again agreed with the AFLP results as the lowest genetic similarity (0.533) was found between landraces 4 and 18. This could be attributed to the fact that the two genotypes were adapted to different regions or because they may originally descended from different ancestors. It is thought that the higher multiplex ratio (i.e. the total number of loci simultaneously detected per assay) of AFLP in comparison to ISSR markers is the reason behind that AFLP and combined data share similar results for the highest and lowest similarity values among genotypes. In addition, this study suggests some other promising genotypes could be used for parental selection. Genotype 18 is presented as it is a foreign germplasm with a genetic similarity ranging (from 0.533 to 0.706) when compared with any of the other investigated genotypes.

The results of the present investigation revealed that different types of markers expressed different levels of genetic similarity among the 18 lupin genotypes. This could be due to the different mechanisms of polymorphism detected by both markers. This result agreed with Powell *et al*. (1996) who stated that a different estimate of the relationships may be

obtained depending on the type of sequence variation detected with each marker system. The accuracy of genetic similarity estimates based on molecular data depends on several variable factors such as the number of markers analyzed, their distribution over the genome, and the accuracy in scoring the markers (Schut *et al*., 1997). Therefore, data obtained from the different types of markers were combined to increase the genome coverage with higher resolution and consequently reveal more informative genetic relationships.

Cluster analysis

The cluster analysis indicated that all the 18 accessions could be distinguished by AFLP and ISSR markers. In the present study, results obtained from the ISSR UPGMA cluster analysis were in partial agreement with the data obtained from AFLP analysis. However, a degree of inconsistency between the two dendrograms was also reported (Fig. 5). This variation in the markers data might be due to different markers representing different genome regions.

Thus, the dependence on a general dendrogram that combines data from both markers used, provided larger genome coverage, became the method of choice and widely used in recent studies concerning clustering analyses. Therefore, the collective dendrogram constructed from both marker systems presented in this study (Fig. 6) is considered to express the genetic relatedness between the 18 *Lupinus albus* accessions used with a better resolution of the relationships for most of the genotypes, according to their geographic area of diffusion, due to the higher number of markers included compared to data for each marker separately.

Dendrograms of data from each type of molecular marker (Fig. 5) and that of combined data from both markers (Fig. 6), showed some differences in the graphic representations of the genetic relatedness among the eighteen *Lupinus albus* accessions. Nevertheless, in spite of the variations found in the dendrograms, some constant results emerged. Some pairs of species showed very stable clustering and relatively higher genetic similarities, such as: (genotypes 13 and 14), (genotypes 11, 3, 4 and 5) and (genotypes 6 and 9).

Consequently and by inspecting the dendrogram from combined data, it was found that some genotypes represent a relation to their distribution position, for instance; landraces 3 and 4 (from Belbies-Sharkeya) and landrace 5 (from Abo Hammad-Sharkey); as well as landraces 6, 7 and 8 (cultivated in Fakous-Sharkeya) are in close genetic distances. Furthermore, the Upper Egypt group with genotypes 13 and 14 from Qena and Sohag, respectively, together with genotype 17 (Giza 2 cultivar, that is planted in Upper Egypt) and a landrace from Sohag (15) are also clustered together. This may refer to sharing the same environmental and agricultural conditions, the issue that make them genetically related as they have similar adapting circumstances. While geno-

type 18, the French variety, was partially separated from the other genotypes.

Meanwhile, the genetic relatedness of some accessions did not give an absolute indication about their geographical origin. Although landraces 1, 2, 3 and 4 belong to the same location (from Belbies-Sharkeya), they were separated into two distant groups; one having genotypes 1 and 2, while the other comprising 3 and 4. Examination of the AFLP electropherograms and ISSR profiles for these samples did not indicate problems with the number or quality of fragments. However, increasing the number of primers or primer combinations might be necessary to confirm whether these results were indicative of possible biological divergence. In this regard, Gilbert *et al.* (1999) postulated that it was not possible to relate the clustering of the investigated *Lupinus albus* accessions to their geographical origin. This is due to the poor documentation associated with the lines in their study, coupled with widespread transportation of stocks that may have been moved away from their point of origin. Moreover, Sbabou *et al*. (2010) confirmed this conclusion in their study on the classification of Moroccan lupin germplasm accessions showing that the grouping of accessions was independent from their geographical origin. Furthermore, they attributed the similarity between accessions of different geographicalorigin to the autogamy and allogamy reproduction system of lupin. Thus, a possible gene flow between populations of the same species may take place. In this context, it was recorded that, although white lupin is primarily a self-pollinated species, its insect-mediated outcrossing rate reaches a range of 5-10% (Faluyi and Williams, 1981; Williams, 1987; Huyghe, 1997).

Comparison between the efficiency of AFLP and ISSR markers in the lupin genome analysis

Two different types of markers (AFLP and ISSR) were used in the present investigation to screen the 18 lupin genotypes. The usefulness and informativeness of each marker system were compared through applying a number of diversity indices. A summary of the results is given in Table (5). Four primer combinations for AFLP and twelve primers for ISSR revealed a different total number of amplicons (bands or peaks). The total number of amplicons revealed by AFLP was 638 AFLP peaks, while 168 bands were produced by ISSR primers; with a multiplex ratio of 159.5 and 14, respectively (Table 5). AFLP produced a higher number of polymorphic amplicons (604 corresponding to 100 for ISSR). Consequently, AFLP exhibited higher percentage of polymorphism (94.6%) as compared with ISSR (59.5%). Furthermore, the number of observed alleles (Na), effective multiplex ratio (EMR) and polymorphic information content (PIC) indices, all recorded elevated values for AFLP (1.95 ± 0.22, 151 and 0.21, respectively) when compared to ISSR (1.59 ± 0.49, 8.3and 0.15, respectively). Moreover,

Shannon index (I) was slightly higher for AFLP (0.34 ± 0.22) than that of ISSR (0.32 ± 0.30). However, the number of effective alleles (Ne) was slightly higher for ISSR (1.38 ± 0.40) than that of AFLP (1.33 ± 0.31). In addition, the marker index (MI) highlighted the distinctive nature of AFLP where MI (31.44) was significantly high as compared to ISSR (1.22).

The compatibility and the degree of correlation among the similarity matrices revealed by both AFLP and ISSR was estimated through correlation coefficient produced from the Mantel test showed a non significant correlation value (0.086) between them at (P-value = 0.3). Thus, genetic distances in the matrix revealed by ISSRs for the 18 lupin genotypes, are independent of the distances in the AFLP matrix. The mechanism of polymorphism detection is dependent on the type of marker used. However, different marker systems may selectively screen complementary, rather than overlapping regions of the lupin genome. This prominent from the relatively low correlation coefficient (0.08) between AFLP and ISSR detected by the Mantel test, thus emphasizing the importance of using both techniques together. Finally, it could be concluded that AFLPs and ISSRs could be considered as suitable markers for the evaluation of the genetic diversity among different plants and as criteria in future genotype identification and germplasm conservation. Phylogenetic relationships among lupin genotypes may enhance the efficiency of breeding programs in Egypt by selecting desirable parents and development of new cultivars.

SUMMARY

Genetic relationships among eighteen white lupin (*Lupinus albus* L.) genotypes, including 12 Egyptian landraces were studied using ISSR and AFLP markers. Twelve ISSR and four AFLP primers/primer combinations were used to assay the polymorphism levels among the lupin accessions. These molecular markers revealed high levels of polymorphism, 94.6% for AFLP and 59.5 % for ISSR. A total of 180 AFLP peaks were scored as positive unique markers "PUMs" and 26 peaks as negative unique markers "NUMs". Eighteen unique ISSR markers were detected, including 9 PUMs and 9 NUMs. The estimated similarities produced from combined data for both markers among the 18 lupin genotypes ranged between 53.3 and 80.5. Cluster analysis was presented as a dendrogram based on similarity estimates using the un-weighted pair-group method with arithmetic average (UPGMA). Through a comparison study, AFLP exhibited significantly higher multiplex ratio (159.5), number of observed alleles (1.946), effective multiplex ratio (151), polymorphic information content (0.208) and marker index (31.44) when compared to those of ISSR. The use of AFLPs and ISSRs allowed for the genetic analysis spanning the lupin genome and revealed the high genetic variations found among accessions that make them useful tools for the breeder to

decide the best combinations to be chosen for breeding programs.

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Table (1): List of the eighteen *Lupinus albus* L. genotypes and their collection information. *Accession number in the Egyptian National Gene Bank.

Serial	*Accession no.	Accessions	Collection	
			Date	Site
1	FLLP 1	Egyptian landrace	May 1996	Belbies-Sharkeya-Egypt
2	FLLP 3	Egyptian landrace	May 1996	Belbies-Sharkeya- Egypt
3	FLLP 4	Egyptian landrace	May 1996	Belbies-Sharkeya- Egypt
4	FLLP 6	Egyptian landrace	May 1996	Belbies-Sharkeya- Egypt
5	FLLP 11	Egyptian landrace	May 1996	Abo Hammad-Sharkeya-Egypt
6	FLLP 14	Egyptian landrace	May 1996	Fakous-Sharkeya- Egypt
7	FLLP 15	Egyptian landrace	May 1996	Fakous-Sharkeya- Egypt
8	FLLP 16	Egyptian landrace	May 1996	Fakous-Sharkeya- Egypt
9	FLLP 24	Egyptian landrace	May 1996	Kantara-Ismailia- Egypt
10	FLLP 63	Local commercial variety (Line3)	Cultivated in Agriculture Research Center (ARC), Giza, Egypt (under investigation)	
11	FLLP 64	Local commercial variety (Line15)	Cultivated in Agriculture Research Center (ARC), Giza, Egypt (under investigation)	
12	FLLP 65	Local commercial variety (Line21)	Cultivated in Agriculture Research Center (ARC), Giza, Egypt (under investigation)	
13	FLLP 68	Egyptian landrace	May 1993	Qena- Egypt
14	FLLP 93	Egyptian landrace	May 1993	Sohag- Egypt
15	FLLP 94	Egyptian landrace	May 1993	Sohag- Egypt
16	FLLP 99	Local commercial variety (Giza-1)	Adapted for cultivation in northern region of Egypt	
17	FLLP 100	Local commercial variety (Giza-2)	Adapted for Upper Egypt region cultivation	
18	FLLP 103	French variety	1996	Foreign germplasm-from France

Table (2): ISSR primers name, sequences, annealing temperatures (Ta), number of bands and the genotypes revealing positive (PUM) and negative (NUM) unique markers as revealed by ISSR analysis.

Primer name	Primer sequence (5'→3')	Ta°C	No. of bands	Polymorphic bands (%)	PUM		NUM	
					No. of PUM /primer	Genotypes showing PUM	No. of NUM/ primer	Genotypes showing NUM
ISSR-1	$CAC(TCC)_5$	50	18	14 (78.7%)	2	5, 14	-	-
ISSR-2	$AGA(TCC)_5$	50	10	3 (30.0%)	-	-	-	-
ISSR-3	$TGTA(CA)_7$	46	11	5 (45.5%)	1	17	-	-
ISSR-4	$(CA)_8AT$	46	15	11 (73.3%)	1	16	2	5, 13
HB-15	$(GTG)_3GC$	40	20	5 (25.0%)	1	3	-	-
17899-A	$(CA)_6AG$	40	12	9 (75%)	1	4	1	4
17898-B	$(CA)_6GT$	40	17	10 (58.8%)	1	17	-	-
BEC	$(CA)_7TC$	42	8	6 (75.0%)	-	-	3	14, 1 , 7
UBC-807	$(AG)_8$ T	45	12	7 (58.3%)	-	-	1	1
UBC-808	$(AG)_8C$	45	13	7 (53.8%)	-	-	-	-
UBC-809	$(AG)_8G$	45	14	9 (64.2%)	-	-	2	10, 17
UBC-815	$(CT)_8G$	45	18	14 (77.7%)	2	2, 1	-	-
Total			168	100 (59.5%)	9	-	9	-
Average			14	8.3	-	-	-	-

Table (3): Number of peaks, number of polymorphic peaks, number of unique alleles and genotypes identified by each primer combination as revealed by AFLP analysis, Lupin genotypes are numbered (1-18) as listed in Table (1).

Primer combination name	No. of peaks	No. of polymorphic peaks (%)	Genotypes showing positive unique alleles	Genotypes showing negative unique alleles	No. of unique alleles
E-ACT(FAM)/M-CAT	157	147 (93.6%)	1, 2, 3, 4, 5, 7, 8, 10, 11, 12, 13, 15, 16, 17, 18	2, 11, 12, 16, 18	55
E-ACG(JOE)/M-CAT	222	221 (99.5%)	3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16, 17, 18	5	80
E-ACG(JOE)/M-CAG	140	123 (87.8%)	4, 5, 6, 7,11, 12, 15, 16 & 17	5, 9,15,16, 17 & 18	44
E-ACC(NED)/M-CTA	119	113 (94.9%)	2, 4, 5, 6, 7, 8, 9, 10, 12, 13, 16, 17	4, 13, 18	27
Total	638	604 (94.6%)			

Table (5): Levels of polymorphism and comparison of informativeness with AFLPs and ISSRs markers in the 18 lupin genotypes.

Parameter	Value	
	AFLP	ISSR
Number of assay units	4 primer combinations	12 primers
Total number of amplicons	638.00	168.00
Multiplex ratio (MR)	159.50	14.00
Number of polymorphic amplicons	604.00	100.00
Polymorphism % per assay	94.6%	59.5%
Number of observed alleles (Na)	1.95 ± 0.22	1.59 ± 0.49
Number of effective alleles (Ne)	1.33 ± 0.31	1.38 ± 0.40
Shannon index (I)	0.34 ± 0.22	0.32 ± 0.30
Effective multiplex ratio (EMR)	151.00	8.30
Polymorphic information content (PIC)	0.21	0.15
Marker index (MI)	31.44	1.22

Table (4): Genetic similarity matrix among the 18 *Lupinus albus* genotypes as computed according to Nei and Li's coefficient from combined data of ISSR and AFLP Lupin genotypes are numbered (1-18) as listed in Table (1).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
2	73.4																
3	65.7	64.4															
4	67.5	65.7	70.4														
5	65.5	64.1	68.6	69.1													
6	73.0	72.8	67.7	65.8	70.5												
7	69.0	69.5	68.6	63.6	67.1	77.8											
8	76.8	76.5	73.0	70.7	71.4	***80.5***	78.7										
9	73.7	73.5	71.2	70.8	69.1	79.5	77.0	79.4									
10	75.5	72.2	64.2	66.9	67.0	77.2	71.8	78.4	73.7								
11	70.3	64.9	62.9	62.2	61.1	65.0	67.2	70.1	67.2	68.1							
12	67.2	65.3	62.8	62.6	63.8	70.4	68.3	72.4	67.4	66.7	68.1						
13	65.7	64.8	66.4	63.9	65.5	70.3	69.9	71.5	69.9	67.9	70.5	74.1					
14	62.9	60.4	62.1	63.2	61.3	66.8	63.4	66.3	66.0	65.6	70.8	71.6	78.0				
15	65.5	72.3	63.7	59.3	63.4	70.0	69.0	72.8	66.4	67.2	66.4	69.4	67.7	66.2			
16	59.8	60.7	55.3	59.2	58.5	61.4	59.8	61.9	59.3	64.2	63.5	61.9	64.7	64.1	63.1		
17	66.2	69.1	61.5	60.2	63.4	68.1	69.2	71.2	68.6	67.6	61.9	64.9	66.6	63.4	73.5	62.8	
18	63.6	64.4	54.5	***53.3***	55.6	63.6	62.4	65.6	61.3	60.9	61.1	70.1	68.6	66.3	70.6	60.4	66.5

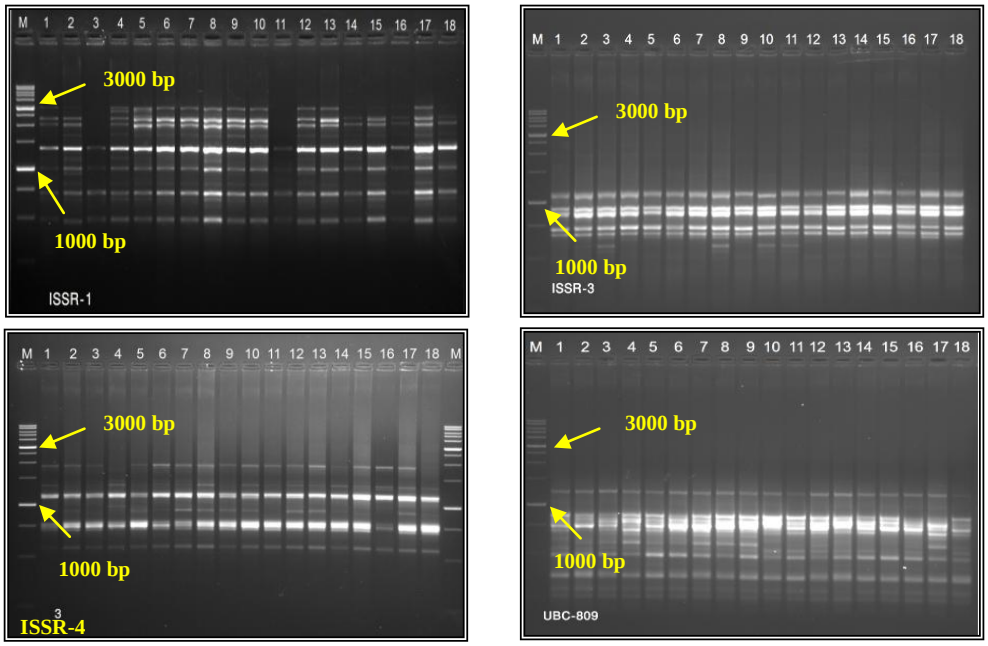

Fig. (1): ISSR profiles of the eighteen *Lupinus albus* genotypes as detected by different ISSR primers: ISSR-1, ISSR-3, ISSR-4 and UBC-809, respectively. M is 1 Kb DNA marker, 1-18 genotypes' numbers according to the list in Table (1).

Fig. (2): AFLP electropherograms of three *Lupinus albus* genotypes (9, 10 and 11), amplified selectively by the primer combination E-ACC(NED)/M-CTA.

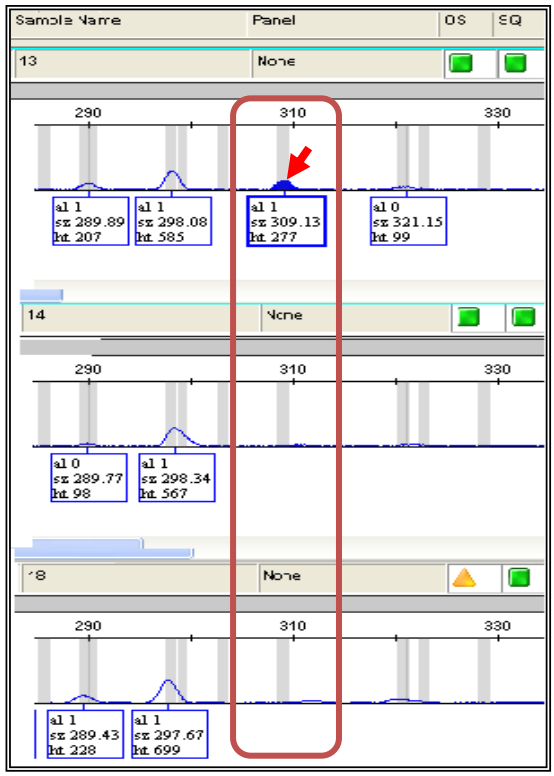

Fig. (3): AFLP electropherograms of three *Lupinus albus* genotypes (13, 14 & 18), amplified selectively by the primer combination E-ACT(FAM)/M-CAT. Red arrow indicates a polymorphic peak example that is present in genotype 13 and absent in the other two genotypes (14 & 18).

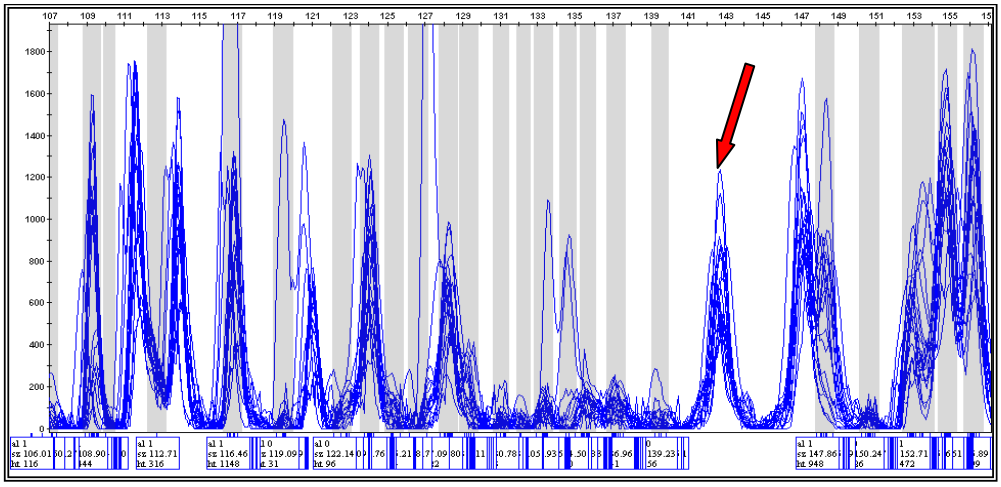

Fig. (4): Overlaying multiple plots to view polymorphic peaks and allele calls. The x-axis represents size in bp and the y-axis shows the intensity of the fluorescence in rfu. Arrow indicates an example of monomorphic peaks that are present in all of the 18 samples amplified selectively by the primer combination E-ACT(FAM)/M-CAT, these peaks are not called as "delete common alleles" option is set.

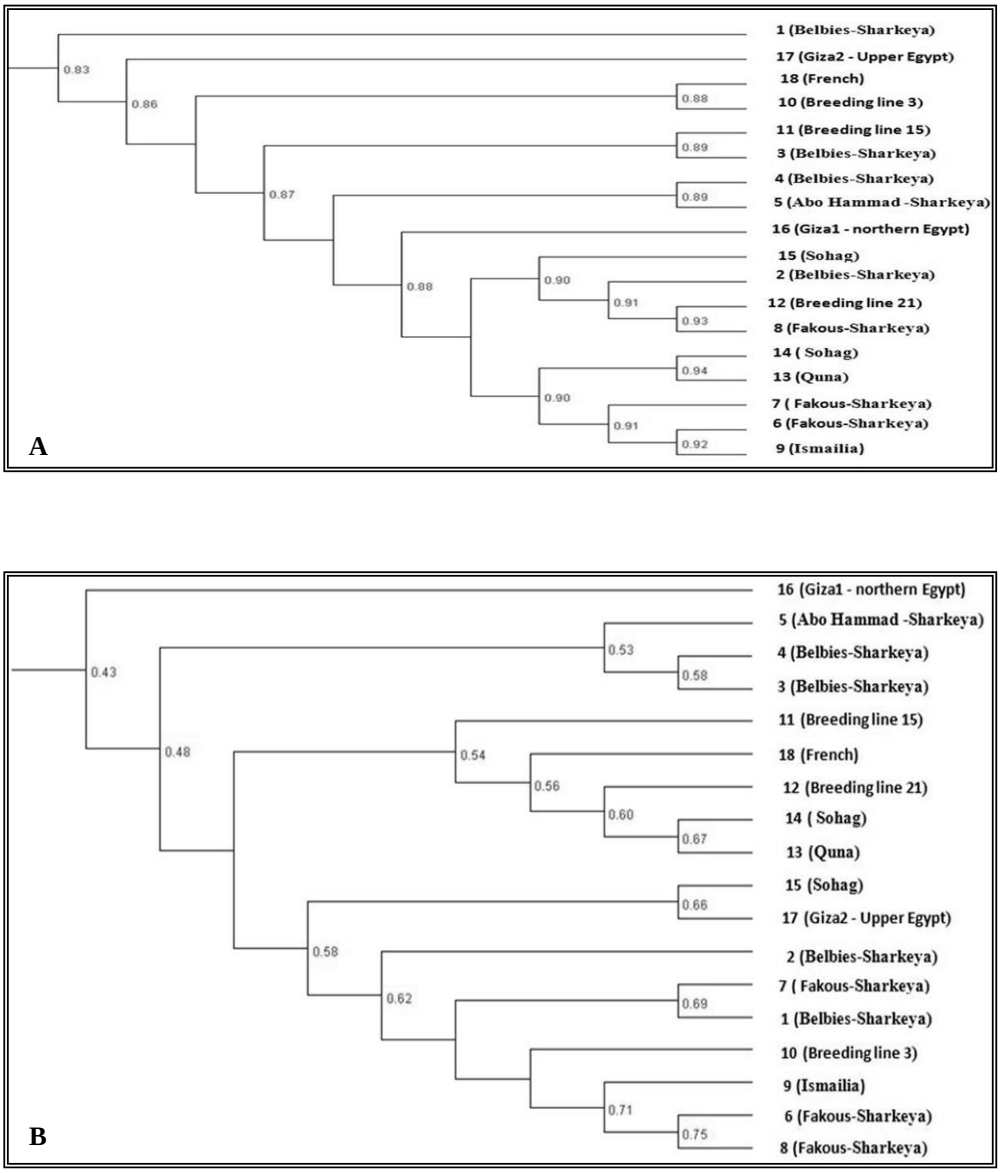

Fig. (5): Dendrogram for the 18 *Lupinus albus* genotypes using Unweighed Pair-group Arithmetic Average (UPGMA) and similarity matrix computed according to Nei and Li's coefficient (Nei and Li, 1979). A) constructed from the ISSR data, B) constructed from the AFLP data. Lupin genotypes are numbered (1-18) as listed in Table (1).

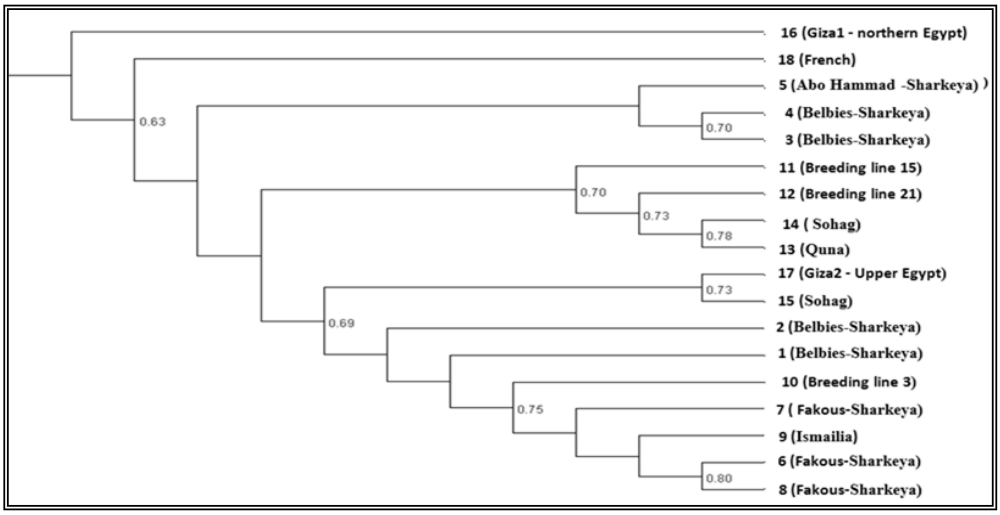

Fig. (6): Dendrogram for the 18 *Lupinus albus* genotypes constructed from the combined data of ISSRs and AFLPs using Unweighed Pair-group Arithmetic Average (UPGMA) and similarity matrix computed according to Nei and Li's coefficient. Lupin genotypes are numbered (1-18) as listed in Table (1).