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Genetic Characterization and Relationships among Egyptian Lentil Cultivars as Revealed By Biochemical and Molecular Markers

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LENTIL (*Lens culinaris* Medikus) is an important seed legume crop cultivated worldwide as human food. Little is known about the molecular structure of the Egyptian lentil. Inter-varietal variation and cultivar identification are crucial for breeding and gene bank conservation of this plant worldwide. The SDS–PAGE and SRAP-PCR techniques were used to detect some biochemical and molecular markers of six Egyptian lentil genotypes (Sinai 1, Line 9, Giza 1, Giza 4, Giza 51 and Giza 370). The genetic distance between cultivars was also estimated from banding patterns. The results of SDS-PAGE revealed a total of 21 bands with molecular weight ranging from about to 6-102 KD. The maximum number of bands (14) appeared in Giza 4, while the minimum number of bands (10) appeared in Giza 1. Electrophoresis analysis among six lentil cultivars showed 80.95% polymorphism, while DNA variations were explored using ten combinations of SRAP markers. SRAP analysis among six lentil cultivars showed 61.54% polymorphism. The highest similarity index (0.869) was found between Giza 51and Giza 370. The lowest similarity index (0.603) was found between Giza 1 and Giza 4. The markers used in this study created polymorphic bands among the different cultivars that can be utilized as molecular markers for their differentiation. The obtained data indicated that SRAP and SDS-PAGE systems can efficiently identify and differentiate between the selected cultivars. Also these results, enhancing the available knowledge of lentil genetic resources in Egypt, may contribute to their conservation and utilization in breeding programs.

Keywords: Egyptian lentil, Genetic diversity, Phylogenetic tree, SDS–PAGE, SRAP marker.

Introduction

Lentil (*Lens culinaris* Medikus) is an annual diploid (2× = 2*n* = 14 chromosomes) species belonging to the Fabaceae. It is one of the most substantial grain legumes in the world after chickpea (*Cicer arietinum* L.) and pea (*Pisum sativum* L.). Lentils contain high levels of proteins (22 %-34.6%), including some important amino acids such as lysine, arginine and leucine (Thavarajah et al., 2011). It also has high levels of carbohydrates (55%–59%) and appreciable levels of micronutrients and vitamins (USDA 2013). Most of the lentil growing countries face certain sets of abiotic and biotic stresses causing considerable reduction in crop growth, yield and production. Lentil breeders have applied conventional plant breeding methods of selection to promote improved cultivars (Kumar et al., 2015).

Traditional techniques of plant breeding have made a substantial contribution to crop improvement but they proved to be slow in targeting a complex trait like grain yield and drought or salinity tolerance. Biotechnology offers novel and powerful tools to assist and complement the breeding efforts (Frederick et al., 2006). Seed protein patterns gained by gel electrophoresis have been successfully applied to distinguish cultivars of a particular crop species (Karihaloo et al., 2002 and Sammour, 2014). It provides a powerful, relatively appropriate and rapid method of identification and classification of gene bank collection that is used to distinguish between genotypes (Pedalino et al., 1992 and Berber & Yasar, 2011).The technique is economical, simple and rapid. This kind of information will enable breeders to develop highly sustainable crops through a well-organized breeding plan.

Few molecular genetics and genomic resources existed for lentil, partly due to its relatively large genome size (Khazaei et al., 2016). Assessment of genetic diversity of different crop species is appropriate precursor for improvement of the crop because it generates baseline data to guide selection of parental lines. Evaluation of crop genetic variation is critical to understanding the available genetic variability and potential use for varietal improvement through breeding programs (Hayward & Breese, 1993 and Tello-Ruiz et al., 2016).

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Molecular biology has contributed to the development of DNA- based markers that can be used for genotype identification, fingerprinting, genetic mapping and diversity assessment (Sonnante & Pignone, 2001; Zaccardelli et al., 2012 and Khazaei et al., 2016). The genetic diversity of *Lens culinaris* Medik. has been examined with a number of molecular marker systems, including: restriction fragment length polymorphism (Havey & Muehlbauer, 1989), random amplified polymorphic DNA assay (Alvarez et al., 1997), amplified fragment length polymorphism (Toklu et al., 2009), Inter simple sequence repeat (El-Nahas et al., 2011), and simple sequence repeat markers (Hamwieh et al., 2009; Kaur et al., 2011 and Kushwaha et al., 2015). Sequence-related amplified polymorphism (SRAP) is an easy and functional molecular marker technique used for genetic diversity and phylogenetic studies in different legume crops **(**Li et al., 2009; Baloch et al., 2010; Castonguay et al., 2010 and Alghamdi et al., 2012). SRAP markers are more potent for revealing genetic diversity among closely related cultivars than SSR, ISSR, or RAPD markers (Budak et al., 2004) and can be utilized for linkage map construction (Yeboah et al., 2007), genomic fingerprinting (Li & Quiros, 2001), hybrid identification (Mishra et al., 2011) and sex determination (Zhou et al., 2011). The SRAP primer system can be used without previous knowledge of DNA sequence.

Knowledge of molecular relationships between plant genotypes is useful in planning effective breeding strategies. Therefore the main objectives of this work are: (1) Determination of the molecular genetic fingerprints for the six lentil cultivars grown under the Egyptian environment using biochemical and molecular markers, (2) Determine the inter-varietal genetic polymorphism among those cultivars, and (3) Establish hypothetical molecular phylogenetic relationship between them using SRAP marker technique in order to assign suitable molecular markers for their future selection and potential breeding. To our knowledge, this is the first implementation of SRAP markers for the assessment of genetic diversity among Egyptian cultivars of *Lens culinaris* Medik.

Materials and Methods

Plant materials

Six Egyptian cultivars of (*Lens culinaris* Medikus) have been obtained from Agricultural Research Center (ARC), Giza, Egypt. The code of these six cultivars is shown in Table 1.

SDS-PAGE

A study of the total soluble protein in lentil seeds was made for the six genotypes by the SDS-polyacrylamide gel electrophoresis technique according to the method of Laemmli (1970). Gels were stained according to the silver staining method of Blum et al. (1987). The gel was photographed and analyzed using BIO-RAD video documentation system, Model Gel Doc, 2000.

DNA extraction

Total DNA was extracted from 100 mg young and healthy leaf tissue of 2 week old seedlings according to the modified CTAB procedure by Doyle & Doyle (1990). All the DNA samples were subjected to RNase treatment and further purified. The quality and quantity of different DNA samples was estimated using UV absorption spectrophotometer at 260 nm (Quawell Q5000 UV-Vis spectrophotometer, V2.1.4, USA) and diluted to 10 ng/µl for further use in PCR amplifications. DNA samples were stored at −20°C until use for PCR analyses.

DNA bands were photographed under UV light. The size of each amplification product was automatically estimated using UV soft image analyzer system.

The SRAP analysis was performed as described by Li & Quiros (2001). SRAP primer was screened using 10 different combinations of four forward and four reverse primers. The sequence of SRAP primers used in this work is shown in Table 2. All reagents and buffers were supplied for primers by Thermo Scientific Inc, (Germany). PCR reaction mixture was formed of 20 µl reaction volume containing 10 × PCR buffer (containing 25 mM $MgCl_2$), 200 µM of dNTPs, 0.6 µM of primers (forward and reverse), 1U Taq DNA Polymerase and 25 ng DNA. PCR conditions included initial denaturation at 94°C for 4 min for 1 cycle and10 cycles of denaturation at 94°C for 1 min, primer annealing at 35°C for 1 min, and primer extension at 72°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and primer extension at 72°C for 1 min. The amplification was completed with a 5 min final extension at 72°C for 1cycle. Amplified products were stored at −20°C till further use. DNA ladder was used as molecular standard in order to confirm the appropriate SRAP markers. The amplification products were separated by electrophoresis on 1.8% agarose gel with 1× TBE buffer and stained with ethidium bromide. Electrophoresis was carried out at 60 V and 40 mA and PCR amplified products were visualized under Bio-Rad Gel Documentation system.

TABLE 1. List of SRAP primer combinations used in the study.

Primers No.	SRAP combinations primers	Sequence of primers (5' – 3')
1	PC1(Em1+Me1)	GACTGCGTACGAATTAAT TGAGTCCAAACCGGATA
2	PC2(Em2+Me1)	GACTGCGTACGAATTTGC TGAGTCCAAACCGGATA
3	PC3(Em3+Me1)	GACTGCGTACGAATTGAC TGAGTCCAAACCGGATA
4	PC4(Em4+Me1)	GACTGCGTACGAATTTGA TGAGTCCAAACCGGATA
5	PC5(Em1+Me2)	GACTGCGTACGAATTAAT TGAGTCCAAACCGGAGC
6	PC6(Em2+Me2)	GACTGCGTACGAATTTGC TGAGTCCAAACCGGAGC
7	PC7(Em3+Me2)	GACTGCGTACGAATTGAC TGAGTCCAAACCGGAGC
8	PC8(Em4+Me2)	GACTGCGTACGAATTTGA TGAGTCCAAACCGGAGC
9	PC9(Em1+Me3)	GACTGCGTACGAATTAAT TGAGTCCAAACCGGAAT
10	PC10(Em2+Me4)	GACTGCGTACGAATTTGC TGAGTCCAAACCGGACC

TABLE 2. SDS-PAGE analysis of protein patterns of six Egyptian lentil cultivars. Lanes 1, 2, 4, 5 and 6 represent cultivars Sinai 1, Line 9, Giza 1, Giza 4, Giza 51 and Giza 370, respectively.

Band no.	M (kD.)	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Polymorphism
		Sinai 1	Line 9	Giza 1	Giza 4	Giza 51	Giza 370	
1	102	0	0	0	1	0	1	Polymorphic
2	98	1	1	0	1	0	1	Polymorphic
3	95	1	1	1	1	1	1	Monomorphic
4	85	0	0	0	0	1	1	Polymorphic
5	78	0	0	0	1	0	0	Polymorphic (+ve unique band)
6	75	1	1	0	1	1	1	Polymorphic (-ve unique band)
7	68	0	0	1	0	0	0	Polymorphic (+ve unique band)
8	66	1	1	0	1	0	0	Polymorphic
9	60	1	1	1	0	1	1	Polymorphic (-ve unique band)
10	58	0	0	0	1	0	0	Polymorphic (+ve unique band)
11	52	1	1	1	0	0	0	Polymorphic
12	48	0	0	0	1	1	1	Polymorphic
13	40	1	1	1	0	0	0	Polymorphic
14	38	0	0	0	1	1	1	Polymorphic
15	36	1	1	1	0	0	0	Polymorphic
16	30	1	1	0	0	0	0	Polymorphic
17	25	1	1	1	1	1	1	Monomorphic
18	20	0	1	1	1	1	1	Polymorphic (-ve unique band)
19	16	1	1	1	1	1	1	Monomorphic
20	12	1	1	1	1	1	1	Monomorphic
21	6	0	0	0	1	1	1	Polymorphic
Total = 21		12	13	10	14	11	13	% of polymorphism = 80.95%

Band scoring and data analysis

The SDS–PAGE and SRAP-PCR bands were visually scored as present (1) or absent (0). Only the clearest and strongest bands were recorded and used for the analysis. Data were analyzed using Sørensen's original formula (Sørensen, 1948) intended for binary data.

Results and Discussion

Seed storage proteins analysis

The SDS–PAGE for water-soluble protein was used to investigate the biochemical differences between the tested genotypes. The bands pattern (Table 2 and Fig. 1) indicates the differences among the tested genotypes in number, intensity and position of the bands. The results of SDS-PAGE revealed a total of 21 bands with molecular weight ranging from about 6 to 102 KD. The maximum number of bands (14) appeared in cultivar Giza 4, while the minimum number of bands (10) appeared Giza 1. Three positive unique bands were detected in the cultivars Giza 4 (78 & 58 kD) and Giza 1 (68 kD). Binary data obtained for absence (0) and presence (1) from protein gel electrophoresis among 6 lentil cultivars showed 80.95% polymorphism which denote the high level of protein polymorphism. Yüzbaşioğ et al. (2008) recorded 24 bands in some lentil cultivars in which only five bands were polymorphic with molecular masses ranging from 35 to 116 kD. Madina et al. (2013) stated that seed protein bands of six lentil varieties were detected at approximately molecular masses ranging between 6.5 and 66 kD and was divided into six regions with intervals of molecular markers. Region I was for albumin protein where bands of more than 66 kD. Region II was for ovalbumin protein, ranged from 45 kD to 66 kD. Region III was for carbonic anhydrase protein, ranged from 29 kD to 45 kD. Region IV was trypsin inhibitor, ranging from 20.1 kD to 29 kD. Region V was for lysozyme and ranged from 14.4 to 20.1 kD. Region VI was for aprotinin, ranging from 6.5 kD to 14.4 kD.

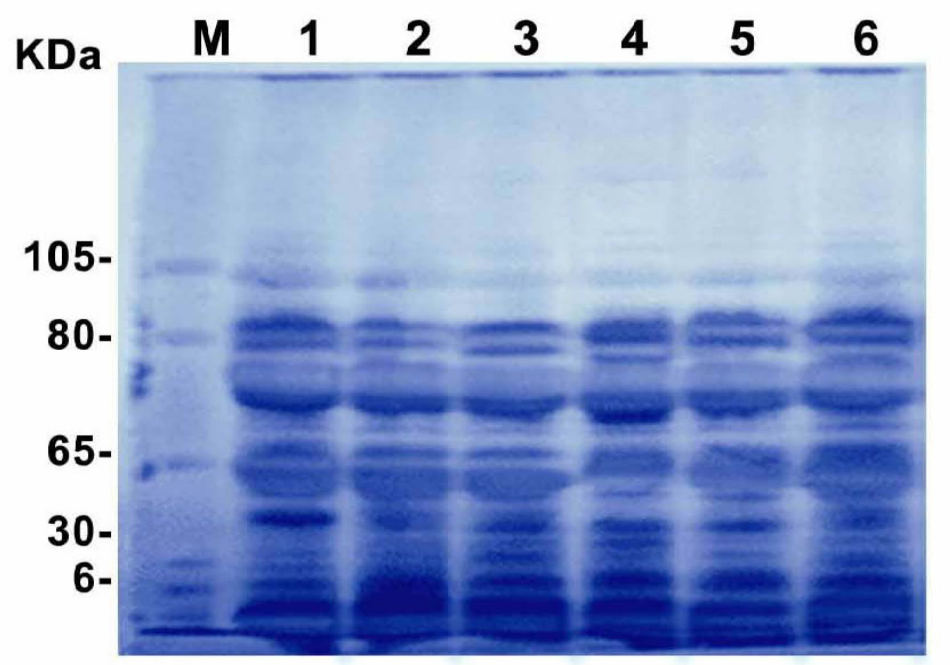

Fig. 1. SDS-PAGE analysis of protein patterns of six Egyptian lentil cultivars. M = marker. Lanes 1, 2, 4, 5 and 6 represent cultivars Sinai 1, Line 9, Giza 1, Giza 4, Giza 51 and Giza 370, respectively.

SRAP data analysis

Data obtained showed that the ten combinations of SRAP primer pairs generated 39 bands in total out of which 24 bands were polymorphic among the six lentil cultivars used in this study (Tables 3 , 4 and Fig. 2). On average, each primer gave around 2-9 bands/primer with ~ 850-75 bp size range. All primers used in this analysis were variably polymorphic with 77.78-33.33% range. The combination SRAP-PC1 (Em1+Me1) exhibited the highest polymorphic percentage (77.78%), while both combinations SRAP-PC5 (Em1+Me2) and SRAP- PC6 (Em4+Me2) recorded the lowest polymorphism percentage (33.33%).

TABLE 3. SRAP analysis of six Egyptian lentil cultivars. Lanes 1, 2, 3, 4, 5 and 6 represent cultivars Sinai 1, Line 9, Giza 1, Giza4, Giza 51 and Giza370, respectively.

SRAP primer code	Band size (bp)	Sinai 1	Line 9	Giza 1	Giza 4	Giza 51	Giza 370	Polymorphism %
PC1	835	0	0	0	0	1	0	77.77
	825	0	0	0	0	1	0	
	790	0	0	0	0	1	1	
	725	0	0	0	1	1	1	
	650	0	0	0	1	1	1	
	510	0	0	0	0	1	1	
	320	0	0	0	1	1	1	
	195	1	1	1	1	1	1	
	75	1	1	1	1	1	1	
PC2	700	0	0	1	0	0	1	50
	645	1	1	1	1	1	1	
	515	1	1	1	1	1	1	
	220	0	0	0	0	1	0	
PC3	750	1	0	0	1	0	0	75
	710	1	0	1	1	0	0	
	600	0	0	0	1	0	0	
	300	1	1	1	1	1	1	
PC4	520	0	0	1	1	1	0	50
	210	1	1	1	1	1	1	
PC5	700	1	0	0	0	0	0	33.33
	690	1	1	1	1	1	1	
	300	1	1	1	1	1	1	
PC6	720	0	1	0	0	0	0	33.33
	490	1	1	1	1	1	1	
	200	1	1	1	1	1	1	
PC7	715	1	0	0	0	0	0	66.67
	620	1	1	0	1	1	0	
	310	1	1	1	1	1	1	

TABLE 3. Cont.

SRAP primer code	Band size (bp)	Sinai 1	Line 9	Giza 1	Giza 4	Giza 51	Giza 370	Polymorphism %
PC8	850	1	0	0	0	0	0	**66.67**
	800	0	1	0	0	0	0	
	680	0	1	0	0	0	1	
	510	1	**1**	1	1	1	1	
	505	1	**1**	1	1	1	1	
	400	0	**0**	0	0	1	1	
PC9	520	1	0	0	1	1	0	**66.67**
	480	1	0	0	1	1	0	
	200	1	1	1	1	1	1	
PC10	750	0	1	0	0	0	0	**50.00**
	400	1	1	1	1	1	1	
Total	39	23	20	18	25	28	23	**61.54%**

TABLE 4. Total number of bands, monomorphic and polymorphic bands and percentage of polymorphism as revealed by SRAP markers among six lentil cultivars.

SRAP primer code	Total No. of bands	Average band size (bp)	Monomorphic bands	Polymorphic bands		Polymorphism %	No. of bands In each cultivar
				Unique	Non unique		
PC1	9	835-75	2	2	5	77.78	23 (Sinai 1)
PC2	4	700-220	2	1	1	50.00	20 (Line 9)
PC3	4	750-300	1	1	2	75.00	18 (Giza 1)
PC4	2	520-210	1	0	1	50.00	
PC5	3	700-300	2	1	0	33.33	25 (Giza4)
PC6	3	720-200	2	1	0	33.33	
PC7	3	715-310	1	1	1	66.67	28 (Giza 51)
PC8	6	850-400	2	1	3	66.67	
PC9	3	520-200	1	0	2	66.67	23 Giza370)
PC10	2	750-400	1	1	0	50.00	
Total	39		15	9	15	61.54%	

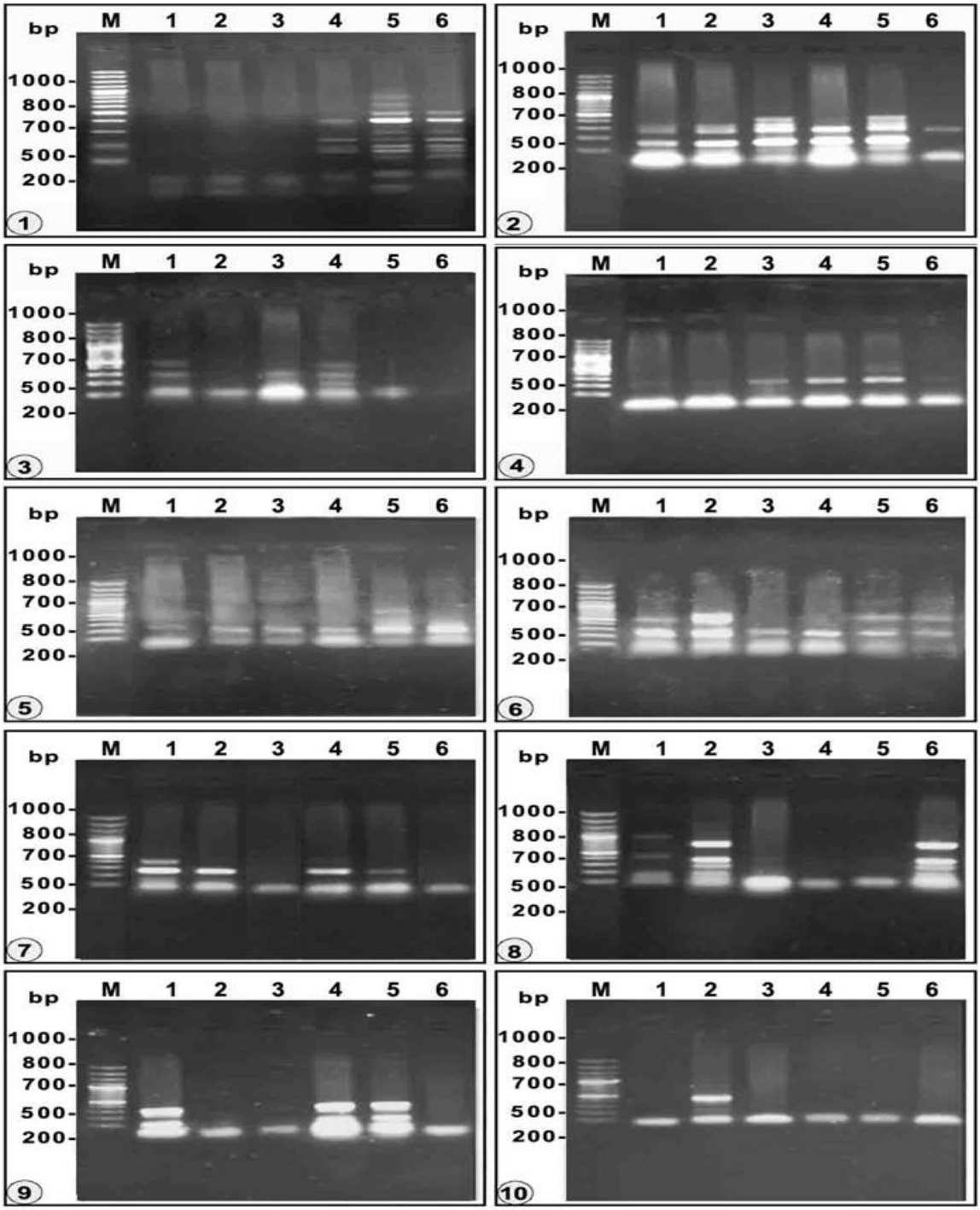

Fig. 2. The separation pattern of the SRAP products on 1.8% agarose gel using the primers: PC 1, PC 2, PC 3, PC 4, PC 5, PC 6, PC1 7, PC1 8, PC9 and PC 10. M: 1 kb ladder DNA marker. Lanes 1, 2, 4, 5 and 6 represent cultivars: Giza 51, Line 9, Giza 1, Giza 4, Sinai 1 and Giza 370, respectively.

SRAP-PC1 (Em1+Me1) generated two unique bands with Giza 51. SRAP-PC2 (Em2+Me1) generated one unique band with Giza 51. SRAP-PC3 (Em3+Me1) generated one unique band with Giza 4. SRAP-PC5 (Em1+Me2) generated one unique band with Sinai 1. SRAP-PC6 (Em2+Me2) generated one unique band with Line 9. SRAP-PC7 (Em3+Me2) generated one unique band with Sinai 1. SRAP-PC8 (Em4+Me2) generated two unique band with Sinai 1 and Line 9. SRAP-PC10 (Em2+Me4) generated one unique band with Line 9.

The average of overall polymorphism percentage generated with all primers was 61.54%. SRAP exhibited highe polymorphism (61.54%) compared to (80.95%) in SDS-PAGE analysis. The overall result gives insight about the potential of Egyptian lentil cultivars to modify their DNA particularly in the presence of a high selection pressure. The current lentil breeding programs are limited in their capability to perform marker assessed selection (MAS) due to a lack of genomic resources. In comparison to prime legume crops such as soybean, common bean, pigeon pea, and chickpea, the speed of development of genomic resources is late in lentil (Kumar et al., 2015). Large genome size, narrow genetic base, low density linkage map, and the difficulty in identifying beneficial alleles are the central limiting agents in genomics-enabled improvement in lentil. In the past, the restricted availability of genomic resources in lentil could not permit breeders to employ these tools in main stream breeding program (Kumar et al., 2015). Molecular tools have been used by lentil breeders and geneticists to realize the genetic basis of a few traits linked to biotic and abiotic stresses (Kumar et al., 2014**).** Genomics-assisted breeding is relatively potent and fast to develop high yielding varieties more suited to adverse environmental conditions. New tools such as molecular markers are predictable to create new awareness and improve our understanding of the genetics of complex traits.

Genetic similarity analysis based on SDS-PAGE and SRAP

The results generated from seed protein and SRAP profiles were pooled for drawing the relationships among the six lentil cultivars under study. The similarity indices among these cultivars were estimated for each pair-wise group (Tables 5, 6 and 7). The similarity relationships based on protein analysis ranged from 0.960 to 0.416 (Table 5). The highest similarity index (0.960) was found between Sinai 1 and Line 9, while the lowest similarity index (0.416) was found between Giza 1 and Giza 4.

TABLE 5. Genetic similarity matrices among six lentil cultivars as computed according to Sorensen coefficient from SDS-PAGE analysis.

	Sinai 1	**Line 9**	**Giza 1**	**Giza 4**	**Giza 51**	**Giza 370**
Sinai 1	0.00					
Line 9	0.960	0.00				
Giza 1	0.727	0.782	0.00			
Giza 4	0.538	0.592	0.416	0.00		
Giza 51	0.521	0.583	0.571	0.720	0.00	
Giza 370	0.560	0.615	0.521	0.814	0.916	0.00

TABLE 6. Genetic similarity matrices among six lentil cultivars as computed according to Sorensen coefficient from SRAP analysis.

	Sinai 1	Line 9	Giza 1	Giza 4	Giza 51	Giza 370
Sinai 1	0.00					
Line 9	0.744	0.00				
Giza 1	0.780	0.789	0.00			
Giza 4	0.833	0.711	0.790	0.00		
Giza 51	0.705	0.666	0.695	0.830	0.00	
Giza 370	0.652	0.744	0.780	0.750	0.823	0.00

TABLE 7. Genetic similarity matrices among six lentil cultivars as computed according to Sorensen coefficient from SDS-PAGE and SRAP analysis.

	Sinai 1	Line 9	Giza 1	Giza 4	Giza 51	Giza 370
Sinai 1	0.00					
Line 9	0.852	0.00				
Giza 1	0.753	0.785	0.00			
Giza 4	0.685	0.651	0.603	0.00		
Giza 51	0.613	0.624	0.633	0.775	0.00	
Giza370	0.606	0.679	0.650	0.782	0.869	0.00

Similarity indices among the 6 lentil cultivars based on SRAP analysis is given in Table 6. The highest similarity index 0.833 was recorded between the two cultivars Giza 4 and Sinai 1. The lowest similarity index (0.652) was recorded between the two cultivars Giza 370 and Sinai 1 indicating that these cultivars are genetically distant (Table 6). The similarity coefficient which resulted from the combined data of SDS- PAGE and SRAP techniques ranged from 0.869 to 0.603 (Table 7). The lowest similarity index (0.603) was observed between two cultivars Giza 1 and Giza 4 while, highest similarity index (0.869) was recorded between the two cultivars Giza 370 and Giza 51. The higher the similarity coefficient between two genotypes, the more the similarity between them based on protein bands (Aghili & Yousef, 2012). Sharma et al. (1996) obtained similar results using AFLP and RAPD marker to appreciate and examine the genetic diversity and phylogeny of 54 lentil accessions. On the other hand**,** Kushwaha et al. (2015) and Dharmendra et al. (2016) using SSR markers showed a broad range of genetic variability between ninety-six lentil genotypes due to their different origin and different genetic constitution. Cluster analysis indicated the dimension of genetic diversity that is a practical use in plant breeding (Sultana et al., 2006).

Conclusions

Considering all the gained data, it is evident that molecular marker SRAP is a good tool in assessing genetic variation among the six cultivars of lentil. These markers provide interesting tool for breeding new varieties in Egyptian lentil. In conclusion, although all of the techniques facilitate to resolve issues related to genetic diversity in lentil, both seed proteins and SRAP gave high levels of genetic diversity. Also, the results of this investigation provided some SRAP molecular markers associated with lentil genotypes productivity.

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الخصائص والعلاقات الوراثية بين اصناف العدس المصرى باستخدام الدلائل البيوكيميائية والجزيئية

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يعتبر محصول العدس من أهم المحاصيل الغذائية للإنسان ولكن حتى الآن لم يعرف إلا القليل عن تركيبه الجزيئي. إن الدراسة فى هذا البحث اعتمدت على إستخدام طريقتين من التحاليل الجزيئية لل د.ن.أ والبروتين على الترتيب (SRAP- PCR, SDS-PAGE), لتحديد بعض الصفات الجينية لـ 6 أصناف من العدس (سينا1 ولاين9 وجيزة 1 وجيزة 4 وجيزة 51 وجيزة 370) وتم تفسير النتائج بإستخدام الحزم الوراثية-banding pat terns كالأتى:

على مستوى البروتين:SDS-PAGE ظهور ٢١ حزمة (bands) بوزن جزيئي يتراوح بين 102-6 كيلودالتون وكان العدد الأكبر من الحزم الوراثية يتمثل في صنف جيزة4 حيث سجل 14 حزمة وعلى العكس كان جيزه1 أقلهم عدداً وسجل 10 حزم فقط وتم حساب التباين بين الأصناف (polymorphism) حيث سجل 80.95 %.

على مستوى د.ن.أ : كانت الإختلافات والتباين بين الأصناف عن طريق إستخدام ١٠ دلائل (markers SRAP) بنسبة 61.54% وسجل أعلى معدل تشابه (0.833) بين صنفي سينا1 وجيزة 4 وأقل معدل كان بين صنفي سينا1 و جيزة 370 بمقدار (0.652). ومن هذه النتيجة يتضح أن تحليل SRAP- PCR له كفاءة في تعريف وتحديد الإختلافات بين هذه الأصناف للعدس والذى يمكن أن تستخدم في برنامج تربية النباتات.