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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(1): 337-348. **OPEN ACCESS**

Phenotypic and molecular characterization of M_3 lentil lines selected from laser and gamma irradiated Egyptian cultivars

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Fifteen M_3 selected lentil families along to their original five cultivars were evaluated to explore the extent of variation among M_3 lentil mutations descended from irradiated these Egyptian cultivars by using laser and gamma rays. Various RAPD and ISSR molecular markers were used to elucidate the polymorphism among the investigated lentil genotypes. The M_3 lentil selected mutations were performed better than corresponding parental varieties for all studied traits. The extent of variability measured by CV% either among parental varieties or selected M_3 mutations showed different situations due to traits. The magnitudes of broad sense heritability were moderate (for tillers, plant dry wt and pods) and high for other traits. Cluster analysis segregated the investigated lentil genotypes (parents and families) into three groups. Group A has an intermediate performance between group C (the superior cluster) and group B (inferior one). The outstanding cluster, i.e C included only two M_3 mutations descended from G.51 variety by laser irradiation. The lesser performed group, i.e B comprised the five lentil parents plus three mutations. The intermediate group (as A) included the rest selected lentil mutations. Our results indicated that RAPD profile revealed lesser polymorphism (67.13 %) than ISSR profile (94 %). Thus, ISSR markers were more informative and strong than RAPD markers for exploring genetic variation in lentils. Moreover, the genetic similarity estimates ranged from 0.528 to 0.921 and agreed to some extent with cluster analysis. The obtained results proved that proper treatments of laser and gamma rays are useful for resulting encouraging lentil genetic variation and the pattern of DNA polymorphism could be detected by using proper molecular markers.

Keywords: Lentil*, Lens culinaris,* Laser irradiation, Gamma rays, M_3 Mutations, RAPD, ISSR

INTRODUCTION

Lentil (Lens culinaris Medikus ssp. culinaris) is the third most important cool-season food legume in the world due to seed richness in protein (27.5-31.7%) and its role in soil fertility (De Ron et al., 2017).The nutritional value of grain legumes make them important components of food security, in spite of their lower production than that of cereals (Muñoz et al., 2017). Numerous abiotic and biotic limitations affecting the production of this crop and the alleviations of such effects are of great role for upgrading the lentil production. Breeding of lentil suffers from narrow genetic variation that limited genetic advance. Different conventional procedures such as hybridization and/or induction of mutation may be recommended for induction useful variability (Tullu et al., 2013). Mutation induction has been employed successfully in

lentil. Gamma rays were the most used mutagen to change gene (s) in lentil due to their easy application (Tabti et al., 2015). The application of laser improved the yield performance of lentil plants in addition to be considered a promising and safe measure for inducing useful variation (Darwish et al., 2013). However, the indirect effects of laser irradiation, as mutation agent in the M_2 lentil plant varied greatly from lentil genotype to another. They suggested that laser may be recommended as mutagenic agent for widening variation in lentil cultivars. Genetic variation among lentil cultivars is an important for lentil breeding (El-Kadi et al., 2011; Erdoğan, 2015).Different molecular markers varied for evaluating DNA polymorphism in lentil and the patterns of variation are clearly affected by the genetic constituents (Seyedimoradi and Talebi, 2014). Molecular genetic techniques such as RAPD and ISSR have been used in crops for exploring genetic variability and the nature of polymorphism (Toklu et al., 2008 and Erdoğan 2015). The DNA markers could be used for identification of QTL regions linked to agronomic traits, and for marker-assisted selection in lentil (Gupta et al., 2012 and El-Kadi et al., 2017).

Therefore, the present studies are planned to explore the extent of variation among M_3 lentil mutations descended from irradiated five Egyptian cultivars by using laser and gamma rays. The elucidations of polymorphism among the obtained lentil mutations compared to parental genotypes by using RAPD and ISSR molecular markers are also objective.

MATERIALS AND METHODS

Fifteen M_3 lentil families descended of irradiation the five Egyptians lentil varieties (Sinia 1, Giza 9, Giza 29, Giza 51, Giza 370) were evaluated during 2016/2017 under pot experiment along to corresponding original varieties. The M_0 seed of the five varieties were treated during 2014 by using two wave lengths of He-Ne laser (594 nm and 632.8 nm) with three exposure periods (5, 10, 15 minutes) at the Lab of Laser Applications in Agriculture and Biotechnology, National Institute of Laser Enhanced Sciences (NILES), Cairo University. Three gamma ray doses (cobalt 60): 30 Gy (2 min), 60 Gy (4 min) and 90 Gy (6 min) at the National Center for Research and Radiation Technology, Nasr City, Cairo, Egypt were also used. M_1 and M_2 plants were evaluated during 2014/2015 and 2015/2016 and the superior performed individuals of high doses irradiations were considered for the present investigation to elucidated the extent of phenotypic variation compared to molecular characterization. A factorial CRD pot-experiment with four replications was used to evaluate the M_3 plants. Each replicates was represented by one plastic 30 cm pot filled with peat-vermiculite (1:1) mixture contains with five plants. Plant traits were recorded at maturity by using all harvested plants. These traits included dry weight, g (PDwt), height, cm (PHt), numbers of tillers (Tillers), number of pods (Pods), number of seeds (Seeds), seed index (S.I), seed yield, g (PSY) and harvest index (H.I). Regular analysis of variance of completely randomized design (CRD) was performed. The genotypic and phenotypic variances were estimated among the investigated stocks by partitioning the variances considered as random model. The most fully expanded leaves of M_3 pot grown lentil plants with 30 days old were used for molecular characterization. The total genomic DNA was isolated using the CTAB method (Sambrook and Russel, 2001).Thirteen RAPD primers and ten ISSR primers (Tables 3 & 4) obtained from Bio Basic Canada Inc. were used. PCR analysis were performed in 25 µl reaction volume containing 1 µl genomic DNA (50 ng/µl), 10µl Dream Taq green master Mix (Bio Basic Canada Inc.), 1 µl primer (100 ng/µl) and complete the total volume with nuclease free water to 25 µl. Amplification conditions (thermocycler UNO II, Biometra) were programmed with an initial step of 4 min at 94°C. After that, the amplification reaction was carried out using 40 cycles of 1 min at 94°C for denaturation, an annealing step of 1 min at 34 ^{o}C for RAPD and 54 ^{o}C for ISSR markers, and an elongation step of 2 min at 72°C and finally a terminal extension cycle at 72°c for 5 min. The PCR products were resolved with 1.5% agarose gel in 1x TAE buffer and; DNA bands visualized with ethidium bromide staining (0.5 µg/ml), and photographed under UV light using the gel documentation system (Bio-Rad® Gel Doc-2000). One kb ladder and 100 bp DNA were used as the molecular weight size markers. The product of RAPD and ISSR molecular markers were scored on the basis of presence (1) or absence (0) of bands for each sample. The data obtained using primers in the ISSR and RAPD analyses were then joined and used to measure the genetic similarity coefficient according to the Dice coefficient (Sneath and Sokal, 1973). The dendrogram was generated using the UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) (Sneath and Sokal, 1973). The

computer programs systat ver. 7 (SPSS Inc. 1997 SPSS Inc. 3/97 standard version) were used to calculate the pairwise difference matrices (Yang and Quiros, 1993).

RESULTS

Phenotypic Characterization

Lentil parental varieties recorded wide variability for all studied traits in spite of insignificant statistical differences among the check cultivars for PDwt, tillers, pods, seeds and seed yield (PSY), Table (1). The lacking of significance among recommended lentil varieties was recorded by El-Kadi et al. (2011). Sinai 1 (S.1) lentil variety possessed significantly short plants that beard highest number of pods that contain heavier seeds which resulted in high seed yield (PSY) and harvest index (HI). It is known that this variety belonged to the macrosperma seed type with earliest plant in flowering and maturity. The selected M_3 lentil families performed better than corresponding parental varieties for all studied traits (Table 1). However, the extent of variability measured by CV% either among original varieties or selected M_3 lentil families showed different situations due to traits. Pods and seed yield (PSY) per plant recorded similar CV% among both sets of genotypes. However, seed index (SI) and harvest index (HI) among selected families exhibited narrower variability than those among parental varieties. Wider variation could be observed among M_3families than corresponding parents for PDwt, PHt, tillers and seeds.

Encouraging magnitudes of broad sense heritability as moderate (of tillers, PDwt and pods) to high estimates were obtained. Cluster analyses are one of the appropriate tools for grouping the tested genotypes according to mean performance for several traits into intra homogeneous and inter distinct groups**.** At 5% level of significance, cluster analysis segregated the investigated lentil genotypes (parents and families) into three groups (Fig. 1 and Table 2). According to the averages of performance, group A may be considered as an intermediate performed cluster between the superior group (i.e C) and the inferior one (as B). Group C included G.51-3 and G.51-6 two M_3 families, which significantly outstanding their parental variety and other lentil genotypes for all traits (Tables 1 and 2). The inferiorly performed group (designated B) includes all five lentil parents plus G.51-9, G.70-3 and G.370-9. The intermediate group (as A) comprises the nine selected families from Sinai 1, G.9 and G.29 in addition to G.370-6 lentil mutation. These lentil genotypes seems performed intermediately in spite of all were selected from segregating irradiated mutations.

Molecular Characterization

RAPD Profile

Thirteen random primers were used for detection the polymorphism among the lentil selected families and corresponding cultivars (Fig.2). Among the thirteen tested primers, ten primers generated reproducible and easily scorable RAPD profiles with a number of amplified DNA fragments ranging from 5 to 10 amplicons (Table 3). The total number of fragments produced by the primers was 70 with an average number of 7 amplicons. The different primers revealed different levels of polymorphism. Primer AB-12 amplified the highest number of amplicons (10) with a polymorphism of 90 %, followed by primers AA-07, AA-12 and AE-16 with a polymorphism (87.5, 85.7 and 83.3, respectively).While, primer AC- 20 reproduce the lowest number of amplicons (5) with a polymorphism of 40%. RAPD profile revealed 67.13 % total polymorphism among studied lentils genotypes.

ISSR PROFILE

The ten ISSR primers (Table 4 and Fig. 3) generated a total 79 amplicons with a number of amplified DNA fragments ranging from 7 to 13 amplicons with an average of 8.77 bands per primer. Primers UBC817, UBC818, UBC843, UBC849, 844A and TA-3 revealed the high ratio of polymorphism 100 % among investigated lentil genotypes. Meanwhile, the primer 814 exhibited the low polymorphism (71.4 %). On average, ISSR primers showed 94 % total polymorphisms among lentil families and treatments.

Combined genetic relationships revealed by rapd and issr data

To assess the genetic relationships among the studied lentil genotypes, the data scored from the RAPD and ISSR primers were analyzed using the genetic similarity (GS) matrices based on the Dice coefficients. The combined data were illustrated in Table (5). The genetic similarity estimates ranged from 0.528 to 0.921.

Table (1): Mean performance of M_3 lentil families compared to corresponding parental varieties and estimates of coefficients of variability and heritability for studied traits.

Genotypes	PDwt	PHt	Tillers	Pods	Seeds	PSY	SI	HI
S.1	2.22a	20.1b	2.6a	27.2a	18.5a	0.588a	3.00a	25.6a
S.1-3	3.40	21.8	2.5	40.8	38.0*	1.263*	3.46	37.6*
S.1-6	2.35	19.3	2.3	32.3	28.0	0.945	3.47	40.8*
S.1-9	2.52	20.8	2.5	29.8	24.3	0.843	3.44	34.2*
G.9	2.07a	24.6ab	2.8a	11.8a	10.9a	0.233a	1.50b	11.9b
G.9-3	4.56*	28.3	3.0	38.5*	38.5*	0.953*	2.58*	25.3*
G.9-6	4.60*	30.0*	3.3	47.8*	48.8*	1.350*	2.99*	29.9*
G.9-9	3.22	28.5	3.8*	33.5*	33.8*	1.063*	3.18*	34.8*
G.29	2.90a	26.2a	3.0a	19.2a	13.3a	0.288a	1.48b	10.3b
G.29-3	4.60	30.3	3.0	54.5*	46.3*	1.073*	2.39*	23.9*
G.29-6	3.89	31.3	3.3	43.5*	45.3*	1.115*	2.42*	29.5*
G.29-9	6.26*	27.3	3.5	44.5*	38.5*	0.983*	2.16*	14.6
G.51	2.74a	24.4ab	3.0a	16.1a	13.7a	0.290a	1.32b	8.8b
G.51-3	5.91*	31.0*	3.8*	64.5*	67.3*	2.058*	3.06*	35.2*
G.51-6	6.37*	38.3*	4.0*	72.0*	75.0*	1.920*	2.57*	30.2*
G.51-9	3.64	26.3	3.5	17.5	16.3	0.303	1.94	9.1
G.370	2.89a	23.0ab	2.9a	20.4a	19.9a	0.423a	1.65b	15.6b
G.370-3	3.40	29.5*	3.3	29.3	25.5	0.618	2.34	17.3
G.370-6	4.17	27.8	3.5	38.0	36.0	1.010*	2.82*	23.8*
G.370-9	3.52	28.8*	3.0	32.0	24.5	0.563	2.33	15.1
$LSD_{0.05}$	*1.82*	*4.8*	*0.7*	*19.8*	*17.8*	*0.483*	*0.72*	*7.5*
Par- Mean	2.56	23.7	2.9	18.9	15.3	0.364	1.79	14.5
Fam-Mean	4.16	27.9	3.2	41.2	39.1	1.070	2.74	26.7
Par-CV%	15.3	9.6	5.6	29.9	24.9	39.3	38.4	46.7
Fam-CV%	29.91	16.8	15.7	34.3	40.9	43.0	18.2	35.2
Heritability	0.647	0.806	0.543	0.720	0.813	0.838	0.801	0.904

Mean of parental varieties followed by the same letter/s are not statistically different at 5% level of probability.
However, family designated by star (*) is differed significantly from its corresponding parental variety.

Table (2): Mean performance of formed groups at 5% level of probability of 20 lentil genotypes for yield components.

Group	No. entries	PDwt	PHt	Tillers	Pods	Seeds	PSY	SI	HI
A	10	3.96	26.5	3.1	40.3	37.7	1.060	2.89	29.4
B	8	2.92	25.4	3.0	21.7	17.8	0.413	1.95	14.2
C	2	6.14	34.6	3.9	68.3	71.1	1.989	2.81	32.7

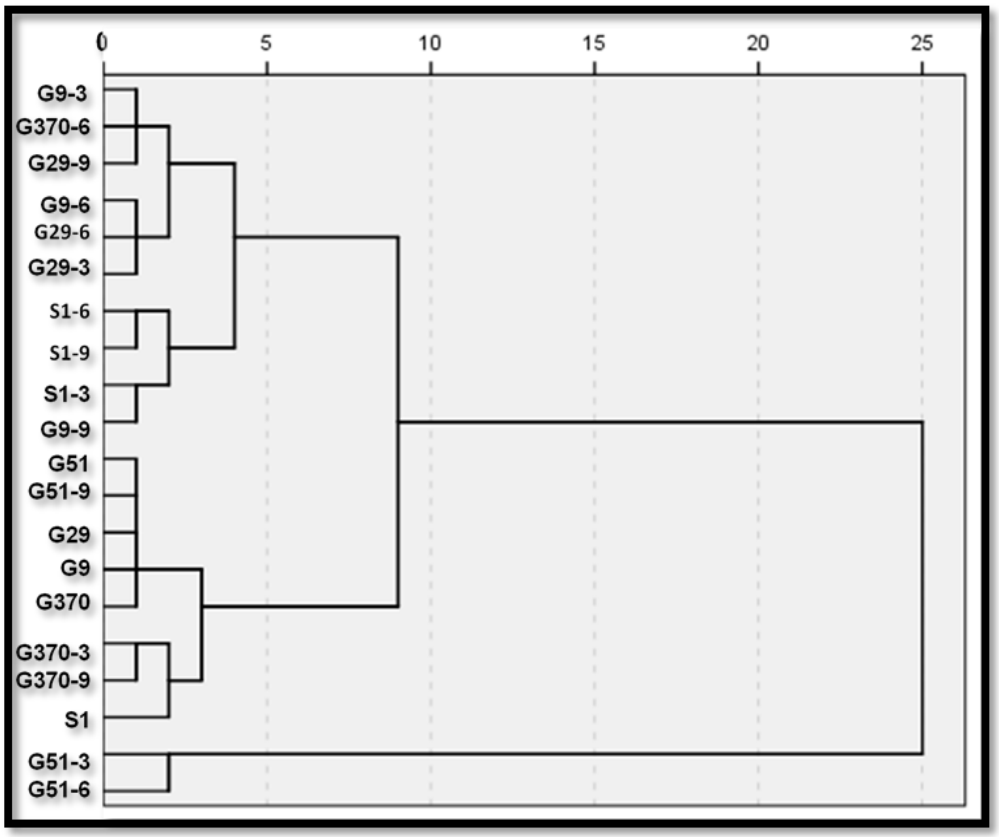

Figure (1): Dendrogram of cluster analysis of fifteen M_3 lentil families along to parental cultivars for seed yield and yield components.

Table (3): Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism revealed by RAPD markers among the investigated lentil genotypes.

Name of primer	Sequence 5`⟶3`	Total No. of amplicons	Mono-morphica-mplicons	Poly-morphicam-plicons	Percent of poly-morphism
AA-07	CTACGCTCAC	8	1	7	87.5
AA-12	GGACCTCTTG	7	1	6	85.7
AB-12	CCTGTACCGA	10	1	9	90
AB-17	TCGCATCCAG	7	2	5	71.4
AC-08	TTTGGGTGCC	7	4	3	42.8
AC- 20	ACGGAAGTGG	5	3	2	40
AE-04	CCAGCACTTC	8	3	5	62.5
AE-16	TCCGTGCTGA	6	1	5	83.3
OPAK-15	ACCTGCCGTT	5	2	3	60
OPAR-15	ACACTCTGCC	7	3	4	57.1
Total		70	21	49	-
Average		7	2.1	4.9	67.13

Table (4): Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism as revealed by ISSR markers among the lentil genotypes.

Name of primer	Sequence 5`⟶3`	Total No. of amplicons	Mono-morphica-mplicons	Poly-morphicam-plicons	Percent of poly-morphism
UBC811	(GA)8C	8	1	7	87.5
UBC817	(CA)8A	8	0	8	100
UBC818	(CA)8G	7	0	7	100
UBC822	(TC)8A	8	1	7	87.5
UBC843	(CT)8RA	10	0	10	100
UBC849	(GA)8T	9	0	9	100
814	(CT)8TG	7	2	5	71.4
844A	(CT)8AC	13	0	13	100
TA-3	(AGG)6	9	0	9	100
Total		79	4	75	-
Average		8.77	0.40	8.33	94

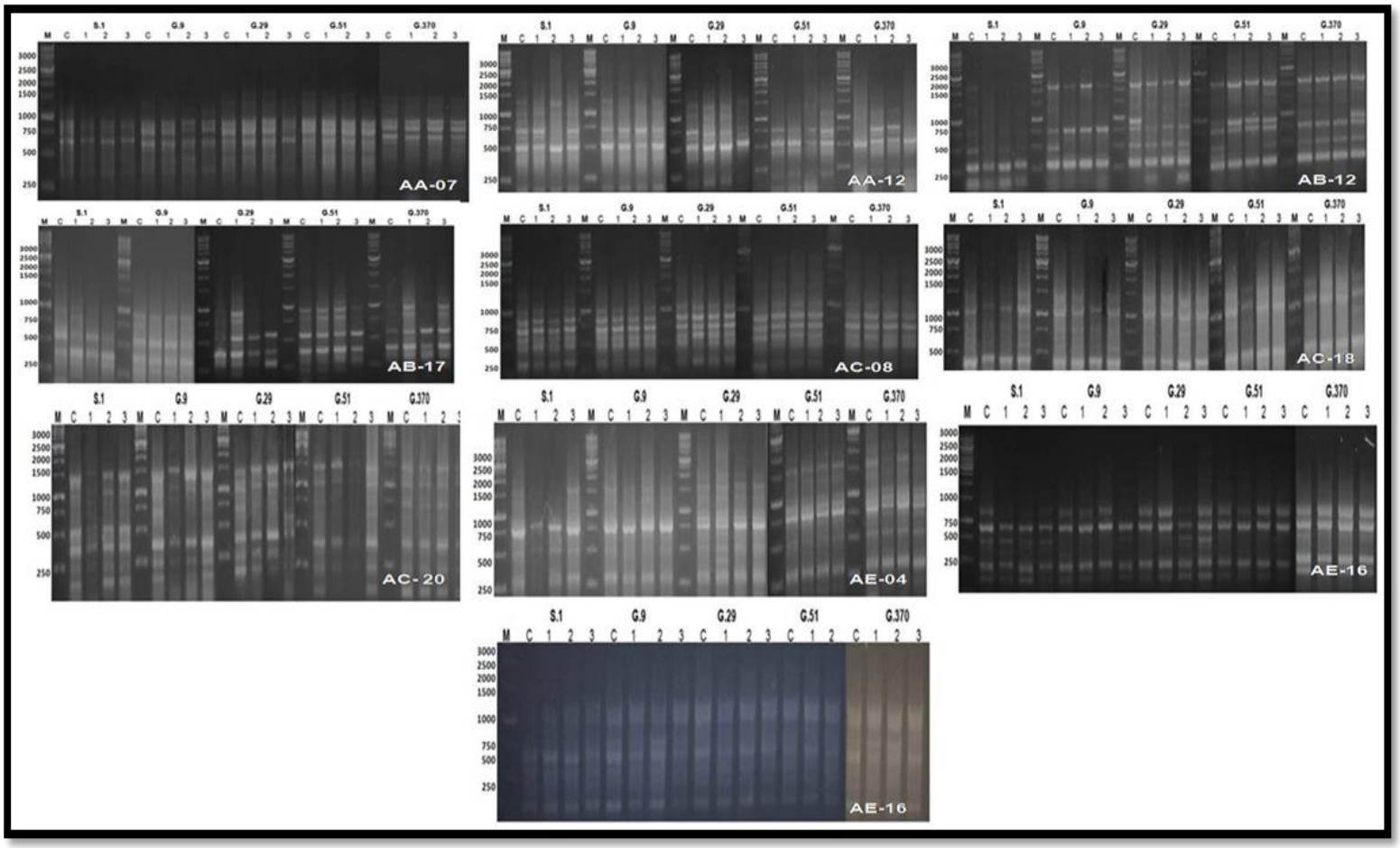

Figure (2): RAPD profiles of the lentil selected families and cultivars amplified with RAPD primers. M, molecular weight marker (1 Kb DNA ladder); C=check parent; 1=(family -3); 2=(family -6) and 3=(family -9) of each of the five Cultivars; S.1 (Sinai 1); G.9 (Giza 9); G.29 (Giza 29); G.51 (Giza 51) and G.370 (Giza 370).

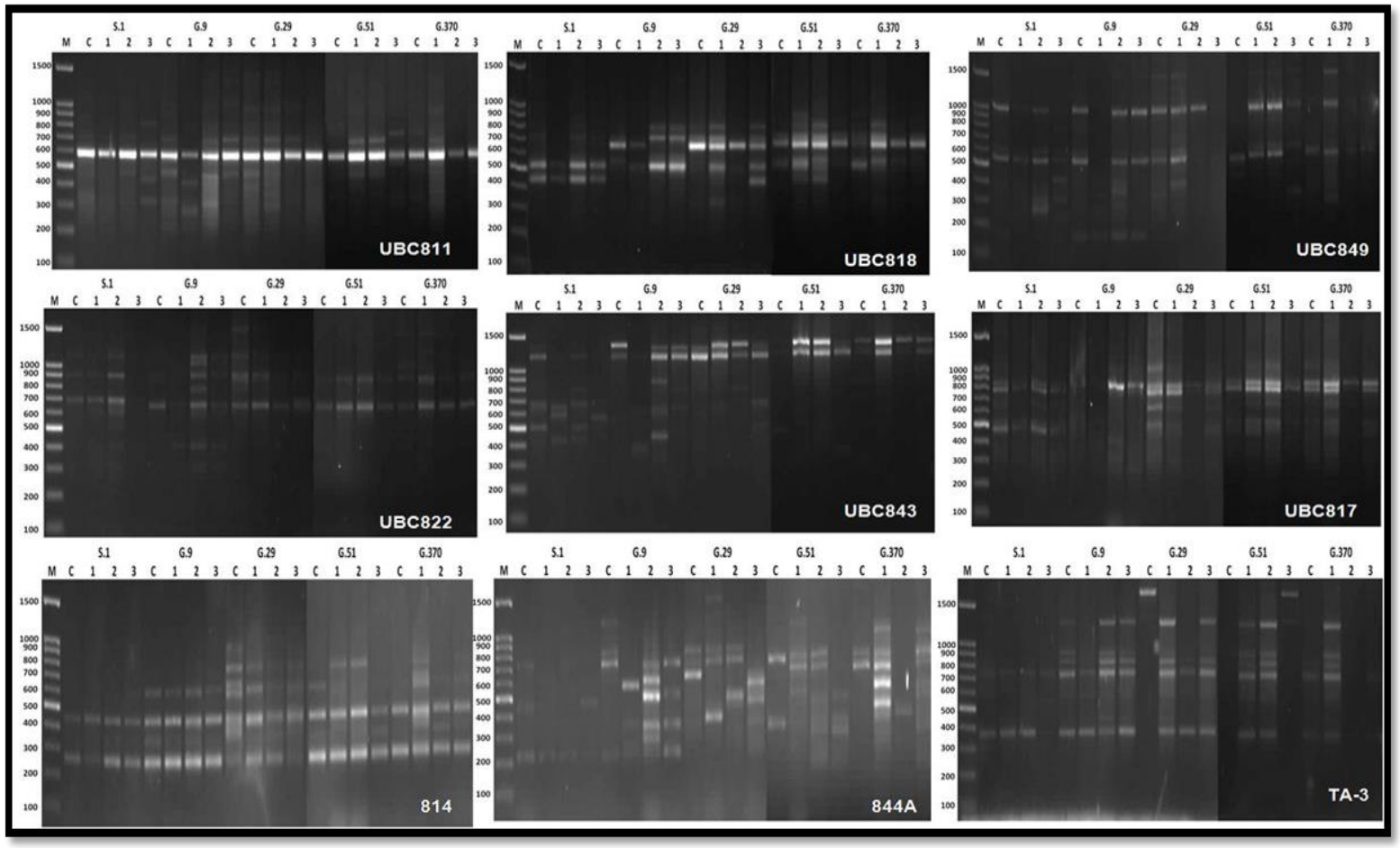

Figure (3): ISSR profiles of the lentil genotypes amplified with ISSR primers. M, molecular weight marker (100 bp DNA ladder); C= check parent; 1=(family -3) ; 2=(family -6) and 3=(family -9) of each of the five cultivars; S.1 (Sinai 1); G.9 (Giza 9); G.29 (Giza 29); G.51 (Giza 51) and G.370 (Giza 370).

Table (5): Genetic similarity (GS) matrices computed according to Dice coefficient from RAPD and ISSR combined data.

	S.1	S.1 -3	S.1 -6	S.1 -9	G.9	G.9 -3	G.9 -6	G.9 -9	G.29	G.29 -3	G.29 -6	G.29 -9	G.51	G.51 -3	G.51 -6	G.51 -9	G.370	G.370 -3	G.370 -6	G.370 -9
S.1	1																			
S.1-3	0.797	1																		
S.1-6	0.830	0.855	1																	
S.1-9	0.797	0.752	0.760	1																
G.9	0.761	0.702	0.755	0.750	1															
G.9-3	0.698	0.696	0.661	0.750	0.791	1														
G.9-6	0.716	0.657	0.685	0.690	0.808	0.743	1													
G.9-9	0.746	0.718	0.727	0.676	0.787	0.761	0.846	1												
G.29	0.667	0.618	0.649	0.610	0.723	0.619	0.683	0.684	1											
G.29-3	0.781	0.681	0.721	0.700	0.805	0.696	0.763	0.792	0.767	1										
G.29-6	0.731	0.683	0.711	0.672	0.817	0.746	0.716	0.761	0.762	0.863	1									
G.29-9	0.768	0.724	0.748	0.742	0.781	0.738	0.750	0.767	0.742	0.867	0.826	1								
G.51	0.636	0.595	0.602	0.619	0.657	0.645	0.616	0.586	0.690	0.694	0.712	0.721	1							
G.51-3	0.597	0.586	0.579	0.551	0.697	0.574	0.620	0.605	0.726	0.718	0.708	0.662	0.803	1						
G.51-6	0.606	0.580	0.573	0.559	0.680	0.567	0.628	0.600	0.723	0.714	0.704	0.685	0.757	0.921	1					
G.51-9	0.642	0.587	0.594	0.626	0.662	0.636	0.636	0.593	0.693	0.725	0.701	0.709	0.859	0.816	0.814	1				
G.370	0.643	0.589	0.596	0.627	0.716	0.621	0.662	0.608	0.693	0.724	0.729	0.667	0.855	0.840	0.797	0.853	1			
G.370-3	0.581	0.528	0.551	0.577	0.675	0.558	0.627	0.589	0.726	0.695	0.697	0.629	0.771	0.861	0.847	0.810	0.845	1		
G.370-6	0.598	0.603	0.609	0.645	0.652	0.639	0.596	0.593	0.643	0.647	0.677	0.641	0.816	0.745	0.696	0.800	0.842	0.797	1	
G.370-9	0.656	0.600	0.591	0.624	0.691	0.650	0.621	0.633	0.639	0.699	0.702	0.637	0.822	0.809	0.777	0.821	0.891	0.816	0.887	1

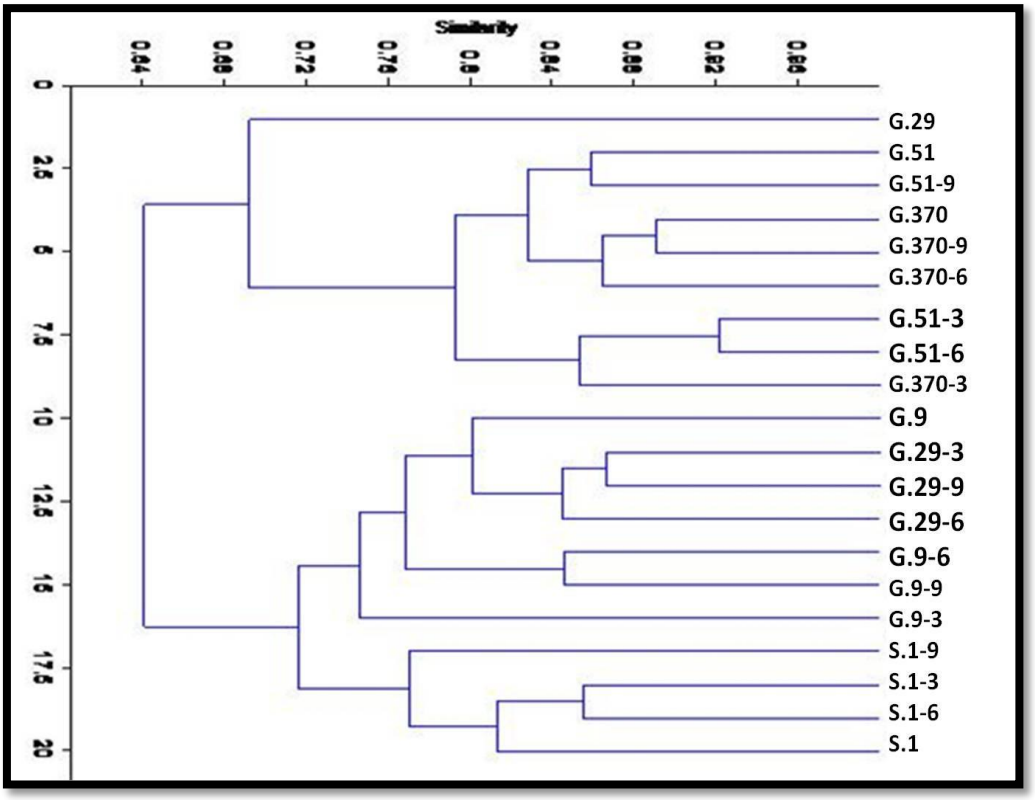

Figure (4): Dendrogram for the studied lentil genotypes constructed from RAPD and ISSR data using Unweighted Pair-Group Arithmetic Average (UPGMA) and similarity matrices computed according to Dice coefficients.

The highest genetic similarity (0.921) was observed between two lentil selected families G.51-3 and G.51-6 which proves that both families are distantly related and may be considered genetically similar. This finding could be supported by the previously detection of cluster analysis grouping (Fig. 1). Meanwhile, the lowest genetic similarity (0.528) was detected between lentil families S.1-3 and G.370-3. The dendrogram based on the UPGMA method using RAPD and ISSR combined data (Figure 4) was established to investigate the linkage distance between the studied lentil genotypes.The dendrogram grouped the lentil genotypes into two main clusters; the first one contains variety G.29 in separated sub-cluster. While, the second sub-cluster comprises G.51, G.51-9, G.370, G.370-9, G.370-6, G.51-3, G.51-6 and G.370-3. On the other hand, the second cluster includes the remaining lentil genotypes, particularly, Sinai 1 with derived families which indicated that the seed type is the most distinct effect.

DISCUSSION

From the above mentioned results, it's obvious that the investigated RAPD and ISSR markers could be used for access the genetic diversity and phylogenetic analysis among lentil mutations. However, the recorded polymorphism ratio of ISSR (94 %) is higher than the ratio of RAPD (67.13%). The average detected number of amplicons of ISSR (8.77) was higher than that of RAPD (7). This demonstrates that the ISSR markers were more informative and strong than RAPD markers for detecting lentil genetic diversity. In addition to, the both markers proved to be useful tools in discriminating between the lentil mutations and parental cultivars. These results are in agreement with previous studies that used RAPD and ISSR markers (Nagaoka and Ogihara, 1997; Fikiru *et al.,* 2007; and Baloch *et al.,* 2015). But Guasmi *et al.,* (2012) and Tanyolac *et al.,* (2010) found that RAPD marker was more efficient than ISSR. This may be due to the genotypic variation and the structure of markers.

Erdoğan (2015) used molecular and biochemical markers for characterization lentil genotypes. RAPD markers and SDS-PAGE were effective for determining polymorphism in lentil cultivars. Moreover, Sonnante and Pignone (2001) reported that RAPD and ISSR markers produced dissimilar clusters for lentil genotypes. Also, Toklu et al. (2008) characterized molecularly 38 lentil landraces six commercial varieties from southeast Turkey by using inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) with six primer combinations. The analysis amplified 119 polymorphic fragments. Even though the AFLP produced more bands per primer combinations, the ISSR detected more polymorphisms. Various molecular markers possessed variable efficiency for evaluating DNA polymorphism in lentil which clearly influenced by the used genetic marker used. The M_3 lines exhibited encouraging variation differed from parental varieties.

CONCLUSION

Regarding the results of this study, the induction of mutation using laser and gamma rays encourage the genetic variability in M_3 lines and solve the problem of the breeding of lentil which suffers from narrow genetic variation that limited genetic advance. Furthermore, the RAPD and ISSR markers are success for the detection of different DNA structural changes after laser and gamma treatments.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

The authors would thank the College of Agriculture, Cairo University for financing and supporting the activities of the present work.

AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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