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Full Length Research Paper

Phenotypic and molecular analysis of M7 generation of soybean mutant lines through random amplified polymorphic DNA (RAPD) marker and some morphological traits

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When genetic variability is diminished using traditional breeding methods, induced mutation is a good way to increase genetic diversity in soybean. Hence, genetic diversity amongst 33 M7 generation soybean mutant lines with high N2 fixation character and one non-irradiated cultivar (L17) was studied by using random amplified polymorphic DNA (RAPD) markers and some morphological traits. RAPDs established 104 major amplified products using 10 polymorphic primers. Out of 104 markers, 34 were monomorph and the remaining (70) were polymorph. Cluster analysis of studied lines in terms of RAPD markers, separated mutant lines group from parent cultivar. Both morphological and RAPD markers successfully detected genetic variation within induced mutant lines and the variation between irradiated and non irradiated lines which were morphologically indistinguishable also detected by RAPD. Therefore RAPD markers with average PIC = 0.80, can be more useful for detecting induced diversity among mutant lines. It can be inferred from the results that irradiation did induce significant genetic variability with regard to majority of studied traits such as number of nodule per plant and harvest index. Sequencing and cloning of band pattern (3 kb) obtained from parent cultivar with OPA09 primer and introducing it as a SCAR marker can be used in marker assisted selection.

Key words: diversity, morphological traits, mutation, random amplified polymorphic DNA, soybean.

INTRODUCTION

Mutation breeding in crop plants is an effective tool in hands of plant breeders especially in crops having narrow genetic base. Many mutants have been identified as donors of desirable traits in breeding program. Mutation breeding work in soybean crop has yielded in identification of many mutant lines with desirable traits like high germination and survival percent (Rahman et al., 1994). Despite the richness of the soybean germplasm collection the genetic base of the present day collection remains poor (Delannay et al., 1983). Because of this problem, induction of genetic diversity and its use in soybean breeding programs is essential to create performance mutant lines to meet the worldwide soybean demand.

It can be possible to increase the genetic variability by inducing many mutations in plants with ionized radiations at the *in vivo* and *in vitro* studies of mutation breeding. After the creation of mutant lines genetic diversity of these lines can be monitored using morphological as well as genetic based tools, DNA techniques (Bennici et al., 2003) and advanced molecular methods (Barazani et al., 2002; Shiran et al., 2007). The polymerase chain reaction (PCR) based method for DNA profiling, random amplified polymorphic DNA (RAPD) techniques (Li et al., 2001; Fracaro et al., 2005) has been extensively applied in

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evaluation of genetic diversity of different plant species and soybean cultivars. The RAPD assay is efficient for screening for nucleotide sequence polymorphism among individuals as each primer (on average) will direct the amplification of several discrete loci within a genome (Samal et al. 2003). As primers modified by even a single nucleotide produce different banding profiles, the RAPD technique can generate polymorphisms between very closely-related genotypes (Deng et al., 1995).

Present investigation was, therefore, undertaken to obtain information on effect of gamma irradiation on morphological traits of the M7 generation of soybean mutant lines. There are problems about soybean disease and pests such as cyst-nematode in Iran and also lack of genetic diversity in soybean, traditional breeding programs have not been resulted in favorable results. In our research the studied lines were previously established and has no genetic evaluation was not conducted on these lines. The objective of this study was mainly to evaluate the rate of genetic diversity amongst induced mutant plants and their parent cultivar (L17) and their classification using RAPDs and morphological markers. We also expected the mutant lines that are separated from parent cultivar allow them to create resistant varieties against pests and disease such as cyst-nematode. We can use these mutant lines in other soybean mutation breeding programs.

MATERIALS AND METHODS

Plant materials

This experiment containing *33* M7 generation soybean mutant lines which previously evolved by γ ray (cobalt - 60) from L17 cultivar irradiated with doses 150, 200 and 250 Gray (absorbed dose) and L17 cultivar. Seeds of mutant lines and parent cultivar were planted in pots in greenhouse of Nuclear Research Center for Agriculture and Medicine Karaj – Iran. Experiment was conducted in a completely randomized design (CRD) with three replications, in this research treatments were 33 mutant lines and one parent cultivar (L17).

Morphological characteristics

Each line was characterized using 11 traits taken at the different stages. Number of leafs per plant at vegetative stage, number of pods per plant, number of seed per pod, number of seed per plant, number of nodule per plant at R_8 stage, and 100-seed weight (g), plant dry weight (g), seed yield per plant (g), harvest index, root dry weight (g), nodule dry weight (g) after harvesting were measured.

The genomic DNA isolation and PCR

The DNA was extracted from leaves using the procedure described by Sharma et al. (2003). Ten random primers were used for PCR amplifications. Amplification for RAPD was carried out in 25 µl volumes containing 50 ng of template DNA, 400 ng primer, 2.5 U of *Taq* polymerase (Fermentas), 2.5 µl 10X PCR buffer, 300 µM dNTPs, 1.4 M $MgCl_2$ and sterile distilled water to 25 µl. The amplifications were performed in Biorad thermalcycle. It was programmed for two cycles at 94°C for 2 min, 35°C for 1 min, and 72°C , two cycles at 94°C for 1 min, 35°C for 30 s and 72°C for 1 min, and finally 40 cycles at 94°C for 15 s, 35°C for 30 s and 72°C for 1 min, and an additional elongation step at 72°C for 5 min. The products were held at 4°C until analyzed. Amplified samples were run on a 1% agarose gel stained with ethidium bromide at 100 V for up to 90 min with 1X TAE as running buffer and gels were visualized under UV light and photographed with Polaroid film. Duplicate reactions were performed to ensure reproducibility.

Data analysis

Amplification product profiles were scored for the presence (1) or absence (0) of bands. Molecular size of the amplified fragments was estimated using SM-0403 DNA ladder (Fermentas). RAPD assays generating weak or ambiguous amplification products were repeated to confirm the consistency of these markers. The NTSYS-pc software was used to estimate genetic similarities with the Jaccard's similarity coefficient. The generated matrix of similarities was analyzed by the complete linkage (CL) or farthest neighbor, using the sequential hierarchical agglomerative and nested clustering (SHAN) module. Analysis of variance have been carried out on the experimental data to evaluate the gamma irradiation effect on yield and other traits and their pertinent means were separated through the Duncan Multiple Range Test popularly known as Duncan's Test with SAS 9.0 software. Also morphological traits were analyzed by using Wards method and Euclidian distance coefficient with the SPSS-pc (ver. 16.0). The average polymorphic information content (PIC) was calculated for RAPD markers through following formula, given by Powell et al. (1996):

$H_e=1-\Sigma P_i^2$, where H_e is the expected hetrozygosity or PIC and P_i is the frequency of ith allele.

RESULTS

Variance analyses (ANOVA) of studied traits are presented in Table 1. There were highly significant differences ($P < 0.01$) for number of leaf per plant, number of grain per plant, number of pod per plant, plant dry weight (shoot dry weight), root dry weight, harvest index, number of nodule per plant, nodule dry weight, and significant differences ($P < 0.05$) for 100 seed weight and seed yield per plant among mutant lines and parent cultivar. Only for number of seed per pod there are no significant differences among mutant lines and parent cultivar. Means of number of nodule per plant, harvest index and shoot dry because of their importance are shown in Table 2. Majority of mutant lines such as M13 mutant line showed significantly ($P < 0.05$) increased number of nodule per plant as compared to parent L17. The mutant line M1showed higher harvest index and shoot dry weight as compared to parent L17 and other mutant lines.

Cluster analysis of the studied lines using 11 traits was carried out. The dendrogram and clustering pattern are observed in Figure 1. The ward dendrogram based on morphological characterization indicated that all lines were clustered into four major groups. According to the proximity matrix mutant lines number 24 (M24) and 26 (M26) were nearest lines to parent cultivar and fourth

Table 1. Analysis of variance of studied traits in M_7 generation of soybean mutant lines and parent cultivar M17.

MS								
Seed yield per plant	**100 seed weight**	**Shoot dry weight**	**Number of pod per plant**	**Number of grain per plant**	**Number of grain per pod**	**Number of leaf per plant**	**DF**	**Source of variation**
**308/0	**139/5	**0003/0	^{ns} 007/0	*01/0	*063/0	**0006/0	33	genotype
119/0	74/0	00008/0	005/0	005/0	035/0	0001/0	68	Error
46/17	42/7	17/7	75/17	67/17	58/8	87/10		%CV

MS					
Nodule dry weight	**Number of nodule per plant**	**Root dry weight**	**Harvest index**	**DF**	**Source of variation**
**0044/0	**188/0	**320/0	**53/1	**33**	genotype
00008/0	003/0	082/0	44/0	**68**	Error
89/11	62/9	21/5	08/16		%CV

*, ** = significant at the 5 and 1% of probability level, respectively; ns = not significant.

Table 2. Means of number of nodule per plant, harvest index, shoot dry weight in M_7 generation of mutant lines (M1-M33) and parent cultivar L17.

Number of line	Number of nodule	Harvest index	Shoot dry weight	Number of line	Number of nodule	Harvest index	Shoot dry weight
M1	14.77D	0.333A	8.33A	M18	11.76E	0.266ABC	8.3AB
M2	6.53KIJ	0.31AB	6.67D-J	M19	8.55GH	0.316AB	7.46A-F
M3	4.55MLN	0.283ABC	7.1C-I	M20	9.99GF	0.253ABC	7.56A-D
M4	14.89D	0.316AB	6.86D-J	M21	8.77GH	0.23BCD	6.43G-J
M5	3.44MN	0.283ABC	7.44A-G	M22	17.44C	0.303AB	7.46A-F
M6	14.5D	0.263ABC	6.45F-G	M23	4.99KML	0.23BCD	6.56D-J
M7	19.88B	0.3AB	6.7D-J	M24	11.22EF	0.253ABC	6.67D-J
M8	16.1CD	0.26ABC	7.13C-I	M25	10.88EF	0.29AB	7.36A-H
M9	4.66MLN	0.28ABC	6.23IJ	M26	8.66GH	0.26ABC	6.7D-J
M10	19.55B	0.293AB	7.33C-H	M27	3.21N	0.3AB	6.36H-J
M11	3.11N	0.313AB	6.86D-J	M28	5.44KLJ	0.253ABC	6.86D-J
M12	7.44HI	0.313AB	7.46A-F	M29	6.55KIJ	0.196CD	6.23IJ
M13	23.77A	0.29AB	8A-C	M30	10.66EF	0.26ABC	6.5F-J
M14	17.1C	0.313AB	7.9A-C	M31	8.77GH	0.24BCD	6.5E-J
M15	3.44MN	0.28ABC	7.23C-I	M32	6.77JL	0.303AB	7.1C-I
M16	5.88KILJ	0.316AB	7.53A-E	M33	11.87E	0.303AB	7.05C-J
M17	6.44KIJ	0.26ABC	6.06J	P(L17)	7.55HI	0.26ABC	7.23C-I

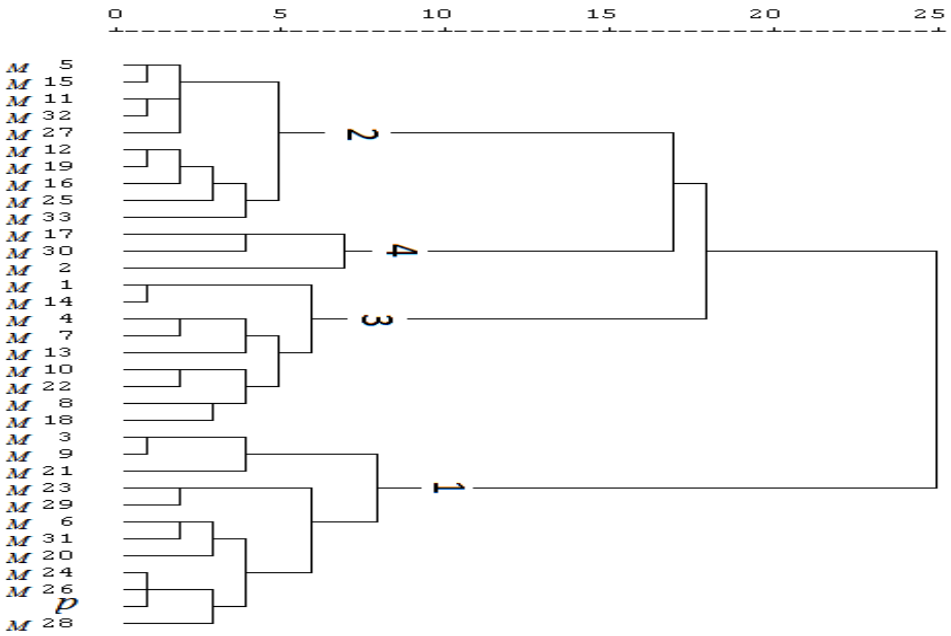

Figure 1. Dendrogram of 33 M_7 generation mutant lines and parent L17 cultivar (p) of soybean based on morphological traits.

Table 3. Means of studied traits for each group derived from cluster analysis of 33 M_7 generation mutant lines and L17 cultivar of soybean.

	Means of traits										
Group	Number of leaf	Grain per pod	Grain per plant	Pod per plant	Dry weight(g)	100 seed weight(g)	Yield per plant(g)	Harvest index	Root dry weight(g)	Nodule per plant	Nodule dry weight(g)
1	8.9	2.18	18.03	8.21	7.1	11.73	2.09	0.29	5.32	5.58	0.051
2	9.21	2.11	15.06	7.2	6.54	11.02	1.64	0.25	5.58	6.72	0.053
3	9.01	2.29	18.16	7.96	7.35	12.12	2.19	0.29	5.61	17.55	0.129
4	8.68	2.26	17.61	7.83	7.29	11.4	1.98	0.24	5.61	11.08	0.085

Underlined numbers have highest.

groups mutant lines including number 2 (M2), 17 (M17) and 30 (M30) were entirely different from parent cultivar. Means of each group are shown in Table 3. In terms of studied traits mutant lines group (2, 3 and 4) were higher as compared with parent cultivar group (1). Amplification patterns are performed twice in order to evaluate the reproducibility of the RAPD markers. The results showed that reproducible RAPD patterns can be obtained under the same amplification conditions for two replicates. The ten polymorphic primers resulted in 104 storable bands, ranging from 200 to 3100 bp in size. Of these, 70 (67%) bands were polymorphic (Table 4). The number of bands for each primer varied from 6 (OPA - 01) to 12 (OPA - 03, OPA - 07) with an average of 10.4 bands per primer. To estimate the similarities among studied lines, the Jacard coefficient provided similarity values ranging from 0.35 to 0.79. According to the CL dendrogram (Figure 2) the studied lines (mutant lines pulse parent cultivar) were clustered into 5 major clusters. According to the similarity matrix, the mutant line number 32 (M32) was closer to parent cultivar and the mutant line number 9 (M9) was entirely different from parent cultivar (L17). For each of the 10 RAPD primers, PIC were calculated and PIC values ranged 0.73 to 0.86 (Table 4). The mean PIC values for all loci were 0.80.

DISCUSSION

Morphological variation

In this research induct variation by gamma irradiation was assessed using morphological traits in m7 generation of soybean mutant lines. It can be inferred from the results that irradiation did induce significant genetic variability with regard to majority of studied traits such as number of

Table 4. Degree of polymorphism and information content for RAPD primers along with their sequences, applied to 33 M_7 generation mutant lines and L17 cultivar of soybean.

No.	Primers	Sequence	Total (T) fragment	Polymorphic (POL) fragment	% Polymorphism (POL/T*100)	PIC
1	OPA-01	CAGGCCCTTC	6	5	83	0.77
2	OPA-02	TGCCGAGCTG	11	7	64	0.79
3	OPA-03	AGTCAGCCAC	12	7	58	0.80
4	OPA-04	AATCGGGCTG	9	4	44	0.73
5	OPA-05	AGGGGTCTTG	11	8	73	0.87
6	OPA-07	GAAACGGGTG	12	10	83	0.83
7	OPA-08	GTGACGTAGG	11	9	82	0.86
8	OPA-09	GGGTAACGCC	11	7	64	0.80
9	OPA-10	GTGATCGCAG	10	6	60	0.74
10	OPA-11	CAATCGCCGT	11	7	64	0.82

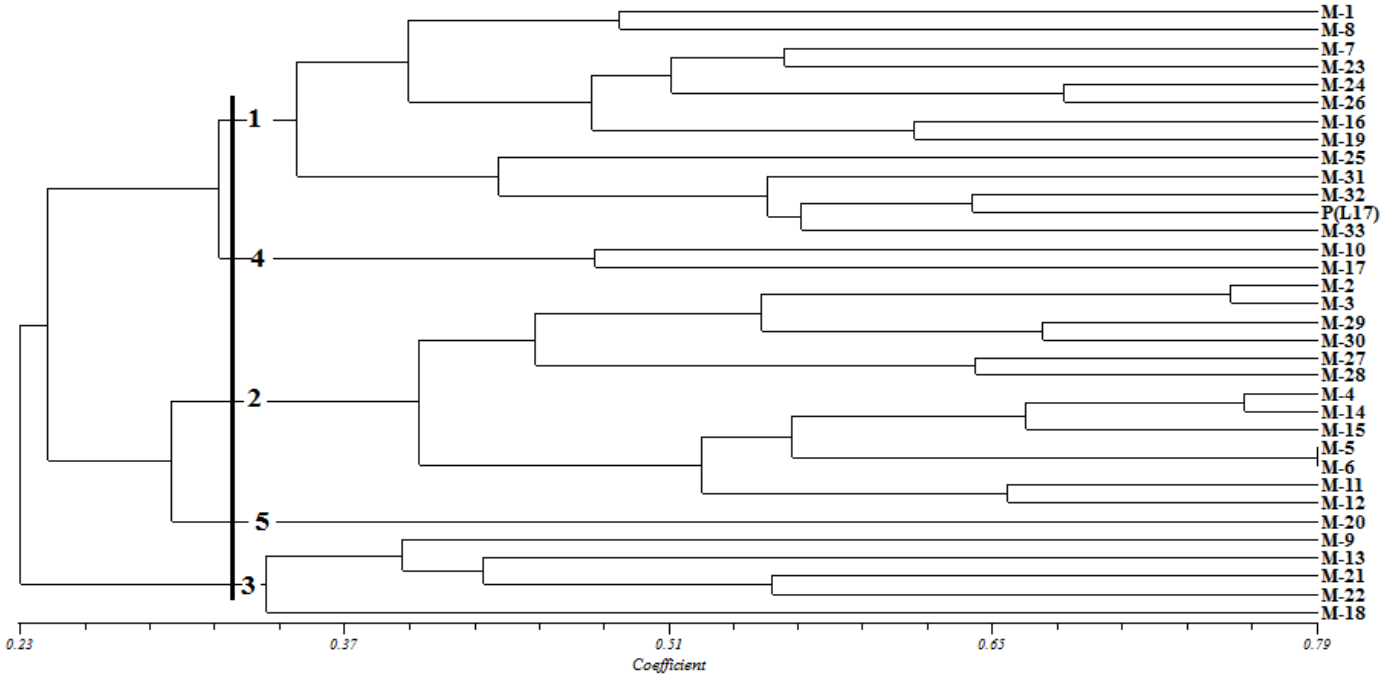

Figure 2. Dendrogram of 33 M_7 generation mutant lines and parent cultivar (L17) of soybean based on RAPD.

nodule per plant and harvest index. Also results demonstrate that radio mutation induction and selection could be successfully used in the soybean cultivars to improve the N_2 fixation and some of the agronomic characters, e.g. number of nodule per plant, harvest index and shoot dry weight. Similar results were also reported by Padavai and Dhanavel (2004) and Singh and Kole (2005). Increase in studied traits in mutant lines may be attributed to chromosomal damages. Also separation of mutant lines from parent cultivar shows gamma ray effect in creating genetic diversity that resulted into separate mutant lines from parent cultivar. According to the means of studied traits in each group (Table 2) mutant lines groups (second, third and fourth groups) in comparison with parent group (first group) have highest value. Since these traits are most important agronomical traits such as yield and yield components traits therefore relevant mutant lines can be selected from segregated group. Using radiation techniques, similar improvements in the agronomic characters of soybean and other crops were achieved (Rahman et al., 1994; Odeigah et al., 1998; Dubey et al., 2007; Arulbalachandran et al., 2010). The next step will be to verify the disease reaction of these materials and to test them for combining ability.

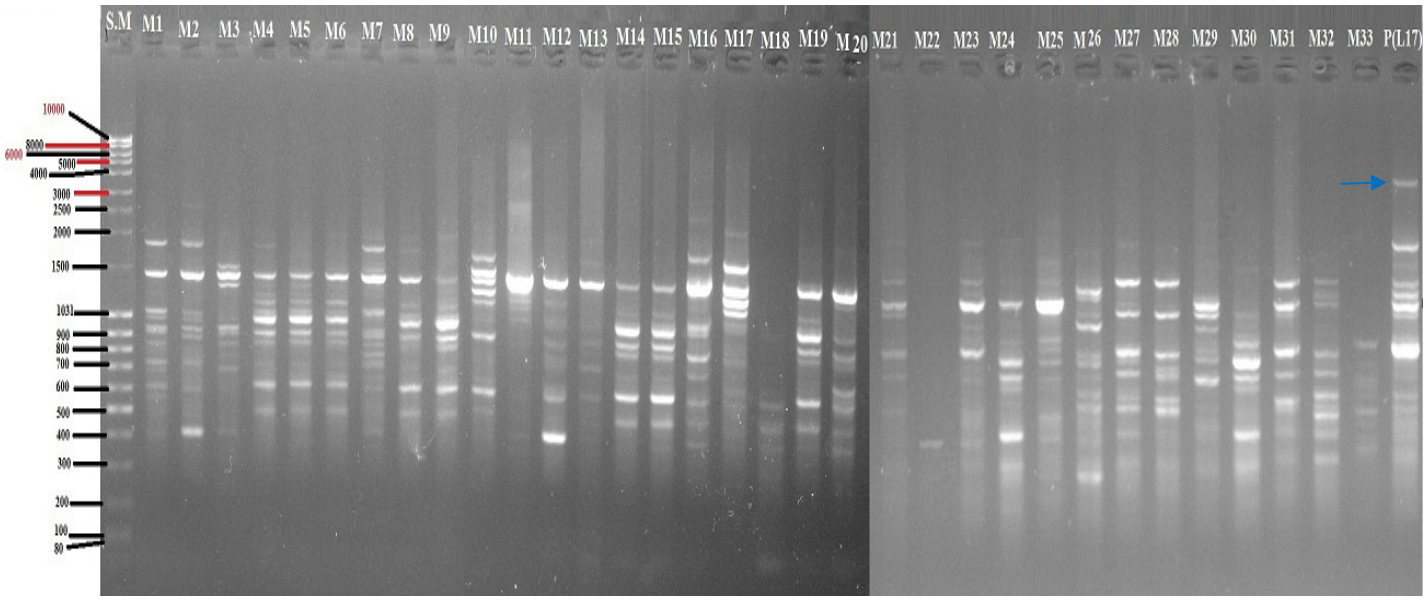

Figure 3. RAPD pattern of M_7 generation mutant lines (M1-M33) and parent cultivar (L17) obtained with primer OPA-09. S.M. demonstrated the size marker DNA (SM-0403). According to the figure parent cultivar (L17) have a band which this band deleted in mutant lines.

The best lines such as M13 and M1 will be used for hybrid production.

Molecular markers variation

Changes in DNA caused by mutagens result in genetic variation detected by RAPD analysis (Rani et al., 1995; Teparkum and Veilleux, 1998). Polymorphic amplification products which represent one allele per locus can result from changes in either the sequence of the primer binding site, such as point mutations, or from changes altering the size or preventing successful amplification of a target DNA such as insertions, deletions, and inversions (Rani et al., 1995). Mutations resulting in polymorphisms are those occurring on primer binding sites, leading to an increase or decrease in the total number of primer binding sites, and consequently the number of amplified fragments. In the present study, 10 RAPD primers have revealed polymorphic fragments among soybean mutant lines that are most likely due to treatment with the physical mutagen gamma. These polymorphisms indicate presence of genetic differences in soybean mutant lines. It is possible that these mutations have occurred in different loci, although it is not yet known whether these mutations and polymorphism bands have correlated with useful traits. RAPD polymorphisms observed in this study are likely due to alterations in the number of primer binding sites following mutagenesis. Point mutations at other regions of the genome that fall within the amplified fragments are likely to go undetected. In this study, detected out of 70 polymorph fragments indicate a high level of mutation due to gamma mutagenesis. Thus, RAPD markers are useful in detecting polymorphisms in soybean mutant lines as they provide sufficient numbers of DNA fragments for conducting assays. This approach can then be used to rapidly screen mutant lines in efforts to detect mutants with desirable agronomic traits.

This study showed molecular markers such as RAPD is more useful for analysis of genetic diversity because RAPD also detected variation between the irradiated and non irradiated lines, which were morphologically indistinguishable therefore RAPD markers can be more useful for detecting induced diversity among mutant lines. Sequencing of polymorphism bands between mutant lines and parent cultivar and additional research are needed for screening and detecting of new genes that are linked with desirable traits. The sequencing of unique band that produced in parent (Figure 3) and introducing it as a SCAR marker can be used for marker assisted selection.

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