Genetic diversity based on RAPD markers of third-generation (M₃) soybean mutant at saline soil

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Abstract. Genetic diversity is very important in plant breeding. One way to create genetic diversity is through induced mutation. The purpose of this study was to evaluate genetic diversity based on RAPD markers of third-generation (M₃) soybean mutant at saline soil. Seed of soybean cv. Detam was induced mutation by gamma rays at 0 (control), 160, 208, 256, 304, 352, 448, 496, 544 and 592 gy in Centre for Application of Isotope and Radiation – National Nuclear Energy Agency of Indonesia. First generation (M₁) of those seed was planted at saline soil and selected based on plant production at saline soil. Those selected plants from M₁ generation were planted at non saline soil as M₂ generation. Selection on M₂ was done based on plant production. There were 13 genotypes selected from M₂ as plant material for third-generation (M₃) that were grown at saline soil. Genetic diversity based on RAPD markers using 6 primers namely G1, G2, OPAA-01, OPAA-02, OPAA-09, and OPAA-14. These markers were specific markers for soybean tolerance in salinity stress. The results showed that six markers generated 389 bands, ranging in size from 304 to 1710 bp. Cluster analysis using UPGMA procedure in the NTSYS PC Software program showed dendogram with two clusters. Genotypes of BSMG 02-256-7 and BSMG 02-592-9 had genetic distance of 15 % with the other eleven genotypes and control variety. It can be concluded that there was genetic diversity based on RAPD markers of third generation (M₃) soybean mutant genotypes at saline soil.

1. Introduction
Soil salinity is one of the environmental stresses that has affected the production of many crops. Saline soil is defined as soil that has a high concentration of soluble salts or when the ECₑ (electrical conductivity of the saturation extract) is more or equal to 4 dS/m and has 15 % of exchangeable sodium [1]. The effect of salinity is seen as a result of complex interactions between morphological, biochemical and physiological processes that cause ion toxicity, osmotic stress, nutrient deficiency and oxidative stress in plants, and then limit the absorption of water from the soil. The negative effect of saline soil on plant growth can be reduced by salt leaching from root zone, amelioration of salinity and salt-tolerant plants [2]. The development of salt-tolerant crops has been a major objective of plant
breeding programs for decades to maintain crop productivity in semiarid and saline lands. A prerequisite and important factor for successful plant breeding program is genetic diversity.

Progress of saline stress tolerance soy bean breeding is restricting by narrow genetic diversity in the population. There are various techniques to increase genetic diversity such as hybridization and mutation. Mutation can be induced by chemical mutagen such as ethyl methane sulfonate and physical mutagens such as gamma rays. An induced mutation is successfully increased morphological and anatomical characteristic diversity in soy bean [3]; okra [4], mangosten [5].

Crop genetic diversity has traditionally been analyzed using morphological traits, particularly those agro-morphological traits of interest to users. To minimize the impact of environmental factors in the analysis, biochemical techniques such as isozyme and protein electrophoresis were later employed. Since 1990, various molecular techniques such as RAPD, AFLP, and SSR, have been used to measure genetic variation [6]. Molecular markers using RAPD technique is simple, fast, reliable and effective methods for detecting polymorphisms so it can be used to assess genetic diversity between genotypes [7]. RAPD markers have been used to identify corn mutant [8]. In soy bean, Twenty-two RAPD marker was used for assessing genetic diversity among 7 varieties of soybean. Eleven primers among 22 primer RAPD showed polymorphism [9]. Twenty RAPD markers affirmed high polymorphism and genetic diversity of 10 soybean cultivars under hydroponic salinity stress (NaCl). The primers used were OPAA-1, OPAA-2, OPAA-3, OPAA-4, OPAA-5, OPAA-6, OPAA-7, OPAA-8, OPAA-9, OPAA-10, OPAA-11, OPAA-12, OPAA-13, OPAA-14, OPAA-15, G-1, G-2, G-3, G-4 and G-5 [10]. RAPD markers (OPA) were used to determine molecular response of three soybean cultivars (Mahameru, Slamet and Detam) due to salt stress [11].

DNA extraction and proper primer selection will determine the successful identification and genetic diversity of plant. The use of RAPD markers to identify genetic diversity caused by gamma-ray induced mutations and selection in saline soil has never been carried out. The purpose of this study was to evaluate genetic diversity based on RAPD markers of third-generation (M3) soybean mutant at saline soil.

2. Materials and Methods
The research was conducted at saline soil in Kaliori sub-district area, Rembang Regency, Central Java Province, Indonesia. Rembang regency is located on the northeast coast of Central Java Province with annual rainfall is 1323 mm/year. The soil type was sandy loam. C-organic, total nitrogen, P2O5 available and K2O were 0.78 %, 0.11 %, 23.81 ppm and 97.94 mg/100 g. Soil pH was 6.69 (neutral) and the maximum electrical conductivity (EC) was 4.2 dS/m. According to saline soil classification [1], saline soil at that area classifies as moderately saline.

The seed of soybean cv. Detam was induced mutation by gamma rays at 0 (control), 160, 208, 256, 304, 352, 448, 496, 544 and 592 gy in Centre for Application of Isotope and Radiation – National Nuclear Energy Agency of Indonesia. First-generation (M1) of those seed was planted at saline soil and selected based on plant production at saline soil. Those selected plants from M1 generation were planted at non-saline soil as M2 generation. Selection on M2 was also done based on plant production. There were 13 genotypes selected from M2 as plant material for third-generation (M3) that were grown at saline soil.

Thirteen genotypes of mutant (BSMG 02-256-7, BSMG 02-448-9, BSMG 02-448-5, BSMG 02-448-10, BSMG 02-256-10, BSMG 02-352-2, BSMG 02-208-8, BSMG 02-496-2, BSMG 02-448-3, BSMG 02-448-7, BSMG 02-208-9, BSMG 02-400-3, BSMG 02-592-9) and cv. Detam as control were planted in plot (0.6 m x 10 m) or 40 plants/plot for each genotypes. Organic fertilizer was applied before planting. Anorganic fertilizer were 75 kg urea/ha, 100 kg SP36/ha and 100 kg KCl/ha. Pest and disease control were controlled by pesticides.
Leaf sample from 3 weeks old plant of each genotype and control variety was taken for DNA extraction and isolation. DNA extraction and isolation were carried out using Tiangen plant genome kit. Each DNA sample was diluted in a new PCR-tube at a concentration of 15 ng/μL in 100 μL. The sample was amplified in a total reaction of 25 μL containing 22 μL of Master mix (12.5 μL Amplitag Gold 360, 1 μL 360 GC Enhancer, 8.5 μL dd H2O), 1 μL Primer (15 μM working solution) and 2 μL DNA sample. DNA amplification was performed using 6 primers namely G-1, G-2, OPAA-01, OPAA-02, OPAA-09, and OPAA-14 (Table 1). These markers were specific markers for soybean tolerance in salinity stress.

PCR reaction used a PCR-Thermal Cycler machine with a PCR program based on optimization [10] as follows: initial denaturation was carried out at 94°C for 10 minutes, followed by 45 cycles of denaturation process at 94°C for 30 seconds, annealing at 38°C for 1 minute, and extension at 72°C for 2 minutes. The PCR reaction was ended with a final extension cycle at a temperature of 72°C for 8 minutes. PCR products were separated on a 1% agarose gel containing ethidium bromide using 1 x TAE buffer. The electrophoresis device was run at a voltage of 100 volts for 45 minutes. DNA fragment were visualized and photographed using gel documentation systems. Analysis of DNA bands was assisted by Gel analyzer software to assist in detecting DNA bands. The bands seen in the visualization results were considered as one allele. DNA bands having the same migration rate were considered to be the same loci.

Table 1. Sequence 5’ to 3’ of Six Primers

| No. | Primer     | Sequence 5’ to 3’     |
|-----|------------|-----------------------|
| 1.  | G-1        | AAGCCTCGTC            |
| 2.  | G-2        | TGCGTGCTTG            |
| 3.  | OPAA-01    | AGACGGCTCC            |
| 4.  | OPAA-02    | GAGACCAGAC            |
| 5.  | OPAA-09    | AGATG GCCA            |
| 6.  | OPAA-14    | AACGGGCCAA            |

RAPD products were scored as absent (0) or present (1) for each primer-genotype combination. The data entry was done into a binary data matrix as discrete variable. Matrices of pairwise similarity were generated using Jaccard’s coefficient of similarity. This matrix was subjected to unweighted pair-group method for arithmetic average analysis (UPGMA) procedure in the NTSYS PC software program to generate a dendogram using average linkage procedure.

3. Results and Discussion
Six primers were tested on 13 soybean genotypes and control variety. These primer generated a total of 389 bands. Maximum number of bands was obtained with primer OPAA-09. The number of bands ranged from 35 to 79 bands from six primers. The molecular weight of generated band in the present study ranged from 304 – 1533 bp (Illustration 1). Amplification with RAPD primers G-1 generated 71 bands with fragment size 360 – 1490 bp. G-2 primers resulted 35 bands with fragment size 435 – 1710 bp. While OPAA-01, OPAA-01, OPAA-09 and OPAA-14 generated 70 bands with fragment size 389 – 1439 bp; 65 bands with fragment size 304 – 1533 bp; 79 bands with fragment size 319 – 858 bp and 69 bands with fragment size 341 – 1258 bp.
Illustration 1. RAPD fingerprinting produced by six primer (a. G-1, b. G-2, c. OPAA-01, d. OPAA-02, e. OPAA-09, f. OPAA-14) of 13 genotypes and control variety at saline soil

DNA analysis of soybean mutant at saline soil with primer G-1 showed 6 loci, two locus showed polymorphism (33 %). The percentage of polymorphism in G-2, OPAA-01, OPAA-02, OPAA-09 and OPAA-14 were 60 %, 0 %, 20 %, 33 % and 33 %, respectively. The highest percentage of polymorphism was shown by G-2 primer, namely 60 %. Soybean DNA analysis using OPAA-1 primer did not show any polymorphism (Table 2). Mean polymorphic percentage of six primer on 13 genotypes and control variety was 29.83 %.

Table 2. DNA analysis of polymorphic bands using six random primers

| No. | Primer   | Number of Locus | Number of Polymorphic Locus | Polymorphic percentage |
|-----|----------|-----------------|-----------------------------|------------------------|
| 1.  | G-1      | 6               | 2                           | 33                     |
| 2.  | G-2      | 5               | 3                           | 60                     |
| 3.  | OPAA-01  | 5               | 0                           | 0                      |
| 4.  | OPAA-02  | 5               | 1                           | 20                     |
| 5.  | OPAA-09  | 6               | 2                           | 33                     |
| 6.  | OPAA-14  | 6               | 2                           | 33                     |
|     | Total    | 33              | 10                          |                        |
The percentage of polymorphisms in this study was lower than 98.17% polymorphism in various soybean cultivars with NaCl stress [10], the percentage of polymorphisms of 56% [12], 53.9% [13] and polymorphism of 36% [14]. Mean polymorphic percentage of six primer (G-1, G-2, OPAA-2, OPAA-10, OPAA-14 and OPAA-15) on six soybean cultivars under salt stress was 87.74% [15]. The differences in some of the results of these studies are related to the nature of the soybean genetic material investigated and its primary sequence.

Primer G-1 did not expose the amplified band with 1490 bp on genotype BSMG 02-496-2 and BSMG 02-592-9. While other genotype and control variety exposed amplified band with 1490 bp. One induced band (360 bp) was observed in genotype BSMG 02-256-7, 02-448-3 and BSMG 02-592-9, which did not appear in control variety and other genotypes (Table 3). Primer G-2 induced band with 1710 bp at BSMG 02-448-9 and 863 bp at BSMG 02-256-10, which did not appear in control variety and other genotypes. Induced band with molecular size of 435 bp only exposed at BSMG 02-256-7, BSMG 02-448-5, BSMG 02-448-10 and BSMG 02-496-2. Primer OPAA-02 did not induce band with 1175 bp at BSMG 02-448-10, BSMG 02-256-10, BSMG 02-208-8, BSMG 02-496-2 and BSMG 02-448-7. Primer OPAA-09 did not display band with molecular size of 563 bp at BSMG 02-256-7, BSMG 02-256-10, BSMG 02-208-9 and BSMG 02-592-9. Band with 319 bp from primer OPAA-09 did not expose only at genotype BSMG 02-448-10. Primer OPAA-14 did not produce band with 857 bp at BSMG 02-496-2 and BSMG 02-208-9. Primer OPAA-14 exposed band with 394 bp only at genotype BSMG 02-448-10 and BSMG 02-592-9. Appearance of new bands and disappearance of bands occurred in the profiles in comparison to the control variety.

It is interesting to note that the 13 genotypes varied at least one single band in comparison to control variety. Six primers used in this study are specific primers to identify soybean salinity tolerance in many research [10, 15]. Thirteen genotypes of soybean mutant and control variety at this study were genetically different to salinity tolerance. RAPD technique is used to identify specific molecular markers for important traits to differentiate different genotypes or cultivars. RAPD markers were developed for salt tolerance in sorghum [16], cultivated and wild barley [17] and maize [18]. Twenty RAPD primers were used to study the genetic variation among ten soybean genotypes [10]. RAPD technique is one of the useful methods to identify specific markers for selecting salinity tolerance cultivar in soybean breeding programs [15].

| Primer | Base pair | Genotype 1 | Genotype 2 | Genotype 3 | Genotype 4 | Genotype 5 | Genotype 6 | Genotype 7 | Genotype 8 | Genotype 9 | Genotype 10 | Genotype 11 | Genotype 12 | Genotype 13 | Genotype 14 |
|--------|-----------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| G1     | 1490      | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          |
|        | 1114      | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          |
|        | 983       | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          |
|        | 704       | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          |
|        | 452       | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          |
|        | 360       | +          | +          |             |             |             |             |             |             |             |             |             |             |             |             |             |
| G2     | 1710      |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
|        | 1013      |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
|        | 863       |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
|        | 511       | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          |
|        | 435       |             |             |             |             |             |             |             |             |             |             |             |             |             |             |             |
| OPAA-* | 1439      | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          | +          |
behaved similarity in their response to salinity tolerance distance between group A and B was 15% or BSMG 02 and control variety (Figure 2).

The results of cluster analysis in the form dendograms showed the relationship between 13 genotypes and control variety (Figure 2). There were 2 groups, namely group A and B. Group A were genotypes of BSMG 02-256-7 and BSMG 02-592-9. Group B were eleven genotypes and control variety. Genetic distance between group A and B was 15% or 85% similarity. Closer soybean cultivars in the cluster behaved similarity in their response to salinity tolerance [10]. Genotypes of BSMG 02-256-7 and BSMG 02-592-9 had different response to salinity tolerance compared to other genotypes and control variety.

Note: 1 = BSMG 02-256-7, 2 = BSMG 02-448-9, 3 = BSMG 02-448-5, 4 = BSMG 02-448-10, 5 = BSMG 02-256-10, 6 = BSMG 02-352-2, 7 = BSMG 02-208-8, 8 = BSMG 02-496-2, 9 = BSMG 02-448-3, 10 = control variety, 11 = BSMG 02-448-7, 12 = BSMG 02-208-9, 13 = BSMG 02-400-3, 14 = BSMG 02-592-9. + = present of amplified bands.

Figure 2. Dendogram illustrating genetic similarity among 13 genotypes and control variety (Detam-3) generated by UPGMA cluster analysis from 389 bands produced by 6 primers.
Conclusion
Six markers of RAPD (G-1, G-2, OPAA-01, OPAA-02, OPAA-09 and OPAA-14) generated 389 bands, ranging in size from 304 to 1710 bp. Cluster analysis using UPGMA procedure in the NTSYS PC Software program showed dendogram with two clusters. Genotypes of BSMG 02-256-7 and BSMG 02-592-9 had genetic distance of 15 % with the other eleven genotypes and control variety. There was genetic diversity based on RAPD markers of third-generation (M₃) soybean mutant genotypes at saline soil.

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