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Selection in M2 Generation of Black Soybean Using RAPD Markers Associated with Salt Tolerance

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ABSTRACT

The black soybean was mutagenized with gamma rays to increase the genetic variation in our previous article. In this paper, the RAPD markers of black soybean in M2 generation were further analyzed. A total of 9 M2 genotypes were investigated for molecular variations. Furthermore, our PCR results demonstrated that the all markers, associated with salt tolerance, produced different-sized fragments. RAPD analysis detected a total of 32 amplification products, among which 23 were found to be polymorphic. Primer OPAA-01 produced the maximum number of bands (10), while the least number of bands (1) was recorded for primer OPAA-03. The amplified DNA fragments ranged from 303 to 1,931 bp. The maximum polymorphism (100%) was recorded for the primers OPAA-02 and OPAA-14, while the minimum polymorphism (0%) was produced by the primer OPAA-03. Analysis of genetic relationship showed there were two groups in the coefficient of 0.55. The first group inluded Detam 3 Prida as salt-sensitive black soybean cultivar and it is closely related with BSMG-160, BSMG-304, BSMG-256, BSMG-352, BSMG-400, BSMG-448, BSMG-496, and BSMG-592 genotypes, sequentially. The second group has only BSMG-208 genotype as an effective out-group. In conclusion, contruction of genetic relationships can be done using RAPD markers and the use of molecular markers will allow a quick selection. Together, our results indicated that, M2 generation showed molecular variations and the strong potential usage for further advances in black soybean breeding.

Key Words: *Gamma-irradiation, Glycine max, Mutant, Mutation, Stress.*

1. INTRODUCTION

Black soybean is an important dicot crop due has many advantages, both in terms of health as well as economic [1]. It containing higher protein and lower fat is needed as the material of soy sauce industry, and result in healthier soy sauce with better color and taste due to the high content of glutamate and anthocyanin [2]. Soybean is generally considered as a salt-sensitive glycophytes, which salinity stress inhibits its germination and growth [3], reduces nodules formation, and decreases accumulation of biomass and seed yield [4]. High salinity is one of the most widespread abiotic constraints and constitutes the most serious factor limiting plant distribution and productivity [5]. Salt stress also has various effects on plant ecophysiological processes, such as changes in protective enzyme activities, properties of photosynthesis, mineral distribution, and membrane permeability [6]. Salinity is responsible for ionic and osmotic stresses, firstly ionic stress resulting from salt enters the plant reaching toxic levels, and secondly leads to decreased ability in water uptake, which is reffered to as the water-deficit effect or osmotic stress [7]. Therefore, the demand to develop black soybean cultivar having tolerance to salt-stress is unavoidable.

The black soybean-producing regions in Indonesia are throughout East Java province, and separate breeding programs developed the black soybeans were cultivated in these areas [8]. Detailed characterization of breeding germplasm is a crucial prerequisite for cultivars improvement [9]. DNA markers provide a powerful tool for the cultivar development programs; they are the most widely used in crop breeding and its genetic evaluation, especially for marker assisted evaluation, marker assisted backcrossing, and marker assisted piramiding [10]. Many molecular genetic markers have been used in detecting population genetic structure since 1966, and random amplified polymorphic DNA (RAPD) is the easiest method [11]. RAPD can amplify a large number of loci simultaneously, thereby producing a more representative genome sample than allozymes, so it has become an

increasingly important tool in genetic analysis [12]. Previously, evaluation of genetic diversity using RAPD markers have been used in Soybean [13; 14]. Apart from using them in diversity analysis, RAPD markers have been shown to be associated with salt tolerance [11; 15].

Mutations induced by gamma-irradiation shows abundant variations, but only the dominant mutation characters are visualized in the M1 generation [16]. In addition, the most identified is lethality at various stages of growth, and its significance is a source for M2 generation, whereas the recessive characters are identified in the M2 generation [17]. Therefore, M2 generation is the most efficient time to screen the mutants, although mutant phenotypes may not be inherited in the next generation due to DNA self-repair mechanism, consequently, we should analyze mutants in the M3 or M4 generations [18]. Compared with other species, the mutant libraries research on black soybean is relatively limited. In our previous study, a black soybean cultivar Detam 3 Prida was treated by gamma-irradiation, and the molecular variations in M1 generation were analyzed [19]. We further investigate the M2 generation in this gamma-irradiation mutagenized line, and analyzed the RAPD variation in this study. In recent years, DNA polymorphism assays have been used for marker assisted selection. Our works may have considerable significance in salt-tolerant black soybean breeding research.

2. MATERIALS AND METHODS

2.1 Plant Materials and Growth Conditions

In our previous study, we used 160, 208, 256, 304, 352, 400, 448, 496, 544, and 592 Gy gamma rays-mutagenized black soybean seeds (200 seeds/doses), and 42 genotypes of M2 generation seeds were harvested. Nine of 42 genotypes were analyzed in this study, and 180 M2 individual plants were transplanted in the field located in the Agrotechnopark - Faculty of Animal and Agricultural Sciences - Diponegoro University (-7^{o} 3'15.27"S; 110^{o} 26'31.7"E). Seedlings were allowed to grow in the field followed method of Kawasaki *et al*. [20]. The M2 generation molecular traits were investigated and recorded.

2.2 DNA Isolation and PCR

Black soybean DNA was extracted from 1 g leaf tissue, harvested from healthy leaves of the 30 days after planting, using a modified manufacturer's protocol of the Plant Genomic DNA Kit DP305 (Tiangen Biotech-Beijing Co., Ltd.; China). Extracted DNA samples were tested for quantity and quality with a UV-Vis spectrophotometer NanoDrop™ 2000 (Thermo Fisher Scientific, Inc.; U.S.). Tested DNA samples were then stored at -21^{o}C and kept until used by PCR for RAPD amplification. Six decamer primers viz. OPAA-01: 5'-AGACGGCTCC-3', OPAA-02: 5'-GAGACCAGAC-3', OPAA-03: 5'-TTAGCGCCCC-3', OPAA-09: 5'-AGATGGGCAG-3', OPAA-14: 5'-AACGGGCCAA-3', OPAA-15: 5'-ACGGAAGCCC-3' (Sigma-Aldrich Co., Japan) were dissolved into nuclease free water Invitrogen™ (Thermo Fisher Scientific, Inc.; U.S.). They were used for RAPD amplification as described by Khan *et al.* [11]. PCR reactions were carried out in a 25 µl of reaction volume containing 22 µl AmpliTaq Gold™ 360 Master Mix (8.5 µl nuclease free water, 1 µl 360 GC-Enhancer, 12.5 µl AmpliTaq Gold® 360 DNA Polymerase) (Thermo Fisher Scientific, Inc.; U.S.), 1 µl primer (15 µM working solution), and 2 µl of DNA template (approximately 50 ng/µl). For DNA amplification, a controlled thermocycler TC9610 MultiGene™ OptiMax Thermal Cycler (Labnet International, Inc.; U.S.) was initially programmed.

In the first cycle of PCR, activation of PCR Master Mix was carried out at 94 ^{o}C for 10 min and denaturation of DNA template at 94 ^{o}C for 30 sec, followed by primer annealing at 38 ^{o}C for 1 min and primer extension at 72 ^{o}C for 2 min. The next 44 cycles, the period of denaturation, annealing and extension time remained as in the first cycle. Lastly, PCR cycle was the primer final extension at 72 ^{o}C for 8 min and hold at 4 ^{0}C. The amplified products were separated by electrophoresis multiSUB Mini, Mini Horizontal Electrophoresis System (Cleaver Scientific, Ltd.; U.K.) on 1.5 % agarose gel in 1 x TAE buffer. The gel was run at 100 V constant voltage for 30 min, and then stained with an ethidium bromide solution for 15 min. GeneRuler 100 bp DNA-Ladder Thermo Scientific™ (Thermo Fisher Scientific, Inc.; U.S.) was used to determine the sizes of the amplified fragments. Next, gel documentation system Uvidoc HD6 (UVItec Ltd., U.K.) were used for the visualization and photography. Band patterns were analyzed by Gel Analyzer software and they were then scored in the binary code. Jaccard's similarity coefficient was used to cluster the genotypes by unweighted pair-group method for arithmetic average analysis (UPGMA) as a simple agglomerative hierarchical clustering method. Finally, dendrogram was drawn using sequential, agglomerative, hierarchical and non-overlaping (SAHN) clustering method as available in NTSYSPc 2.1 Software.

3. RESULTS AND DISCUSSION

Six RAPD markers associated with salt tolerance [11, 15,19] were tested on black soybean in M2 generation (Figure 1 and 2). A total of 32 amplification products were generated by these primers, among which 23 were found to be polymorphic (Table 1). Primer OPAA-01 obtained maximum numbers of 10 amplification products, whereas primer OPAA-03 generated minimum numbers of 1 amplification product, so the number of amplification products ranged from 1 to 10 from 6 primers. Interestingly, the molecular weight of the generated bands in the present study ranged from 303 to 1,931 bp. Six RAPD products were recorded

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as unique or genotype specific. These resulted in 335% polymorphism with an average of 55.83%. However, different result were reported by earlier researchers in soybean. Polymorphic amplification products were showed in the present study, which were relatively lower than previous reports.

Table 1. Fragment size range, types and number of the amplified DNA bands as well as the polymorphism percentage revealed by six RAPD primers of Detam 3 Prida and black soybean in M2 generation

Primer	Fragment size (bp)	Monomorphic band	Polymorphic bands		Total bands	Polymorphism (%)
			Unique	Shared		
OPAA-01	303 – 1,931	1	4	5	10	90
OPAA-02	319 – 1,273	0	1	5	6	100
OPAA-03	821 – 821	1	0	0	1	0
OPAA-09	346 – 862	4	0	1	5	20
OPAA-14	346 – 1,167	0	1	5	6	100
OPAA-15	363 – 754	3	0	1	4	25
Total		9	6	17	32	335
Mean		1.50	1.00	2.83	5.33	55.83

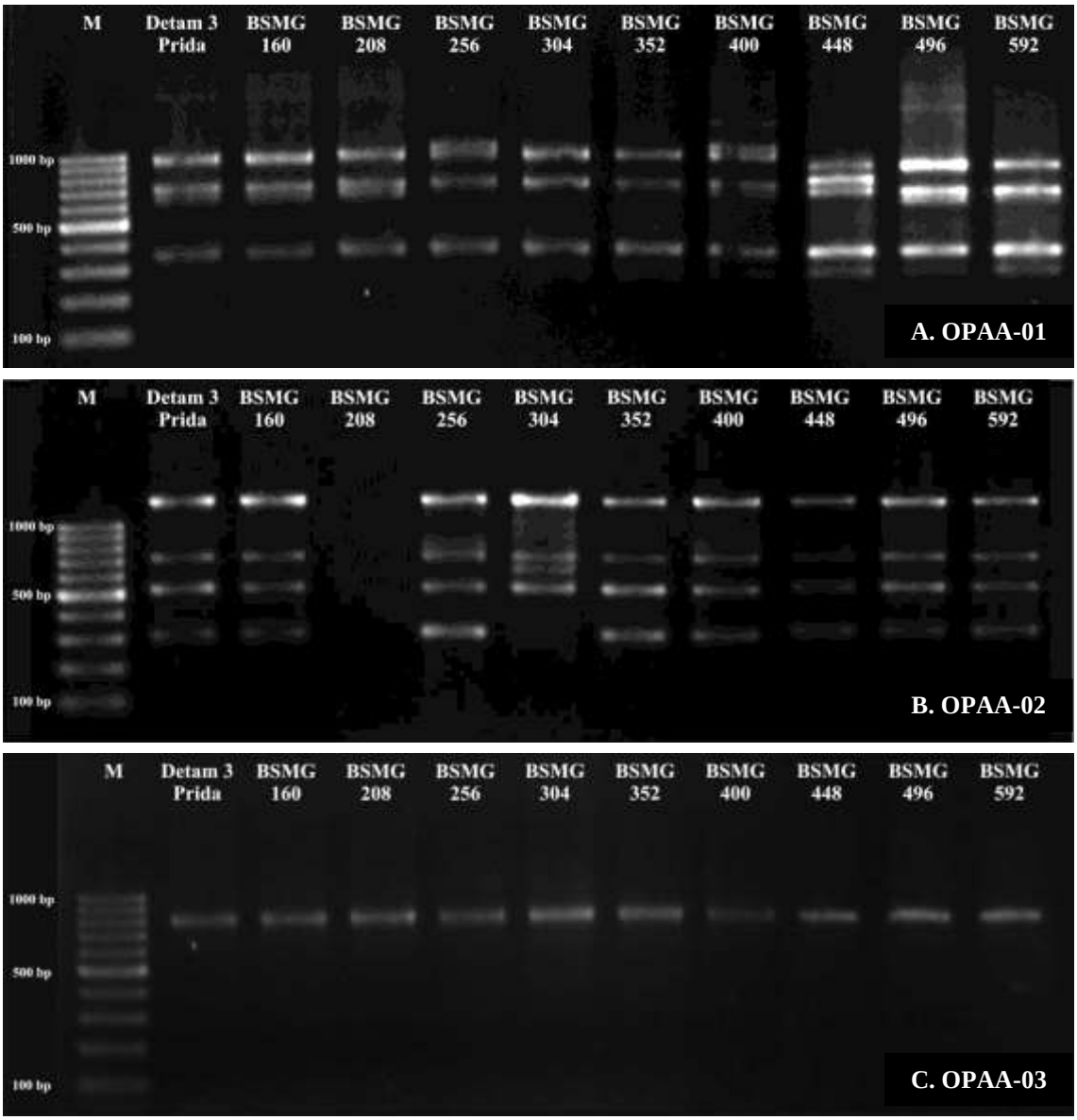

Figure 1. Amplification profiles of Detam 3 Prida and M2 generation of black soybean DNA samples using RAPD primers (A = OPAA-01, B = OPAA-02, and C = OPAA-03). M= 1000 bp DNA ladder

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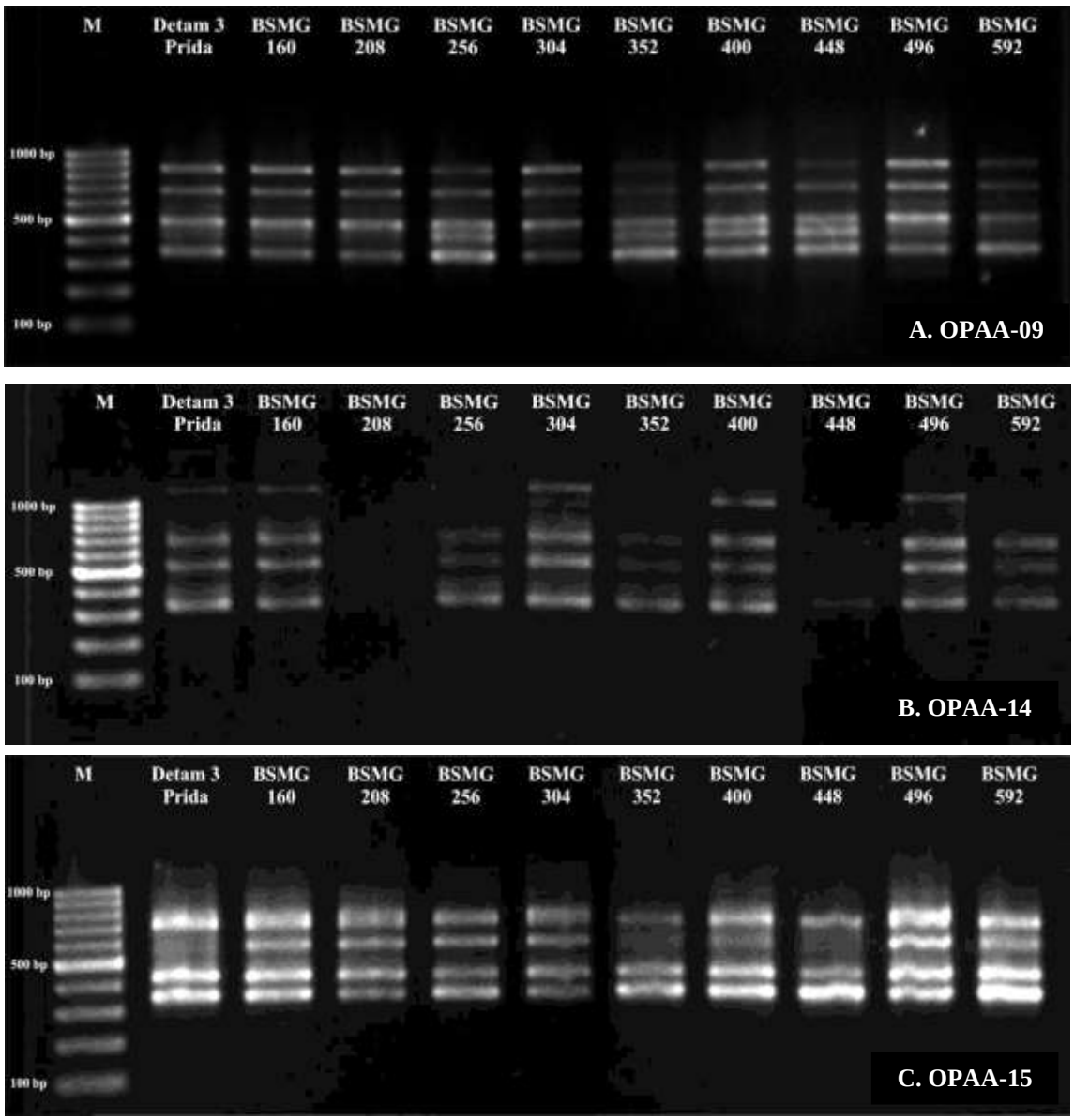

Figure 2. Amplification profiles of Detam 3 Prida and M2 generation of black soybean DNA samples using RAPD primers (A = OPAA-09, B = OPAA-14, and C = OPAA-15). M= 1000 bp DNA ladder

Khan *et al.* [11], Mahgoub *et al.* [15], Tidke and Jadhav [21], Choudhury *et al.* [22], and Tidke *et al.* [13] reported 98.17 %, 87.74%, 84.67%, 83.57%, and 73.69% polymorphism among soybean genotypes in their RAPD study, respectively. Hamzekhanlu *et al.* [23] observed a lower level (67.50%) of DNA polymorphism among 33 M7 generation soybean mutant lines after analysis with RAPD markers. Different reports on the levels of observed polymorphism in their soybean studies could be attributed to the precise nature of the genetic materials that was from different geographical regions, thus having relatively large genetic variation, and also many different sequences of the primers were used under investigation. However, our results are in agreement with our previous study which also reported very low polymorphism (38.00%) of black soybean in M1 generation [19].

The RAPD based genetic relation clearly formed a very divergent group (Figure 3) and genetic relation of Detam 3 Prida and black soybean in M2 generation are presented in the (Table 2). According to Saeed *et al.* [24], the genetic relation with coefficient that was more than 0.60 showed the close relation among the genotypes. In our study, relationship among the genotypes were separated into two groups in the coefficient of 0.55, namely group I and II. Group I on the coefficient of 0.70 was separated into group A and B. The analysis of group A was started by considering Detam 3 Prida as salt-sensitive black soybean cultivar [25]; it is closely related with M2 generation derived from 160 – 400 Gy of gamma rays. Meanwhile, group B consisted of M2 generation derived from 448 – 592 Gy of gamma rays. It has been explained that increasing of mutagen doses increased the genetic distances coefficient, and predicted the previous mutagen treatments are not contribute much to salt tolerance. On the contrary, group II has only BSMG-208 genotype as an effective out-group.

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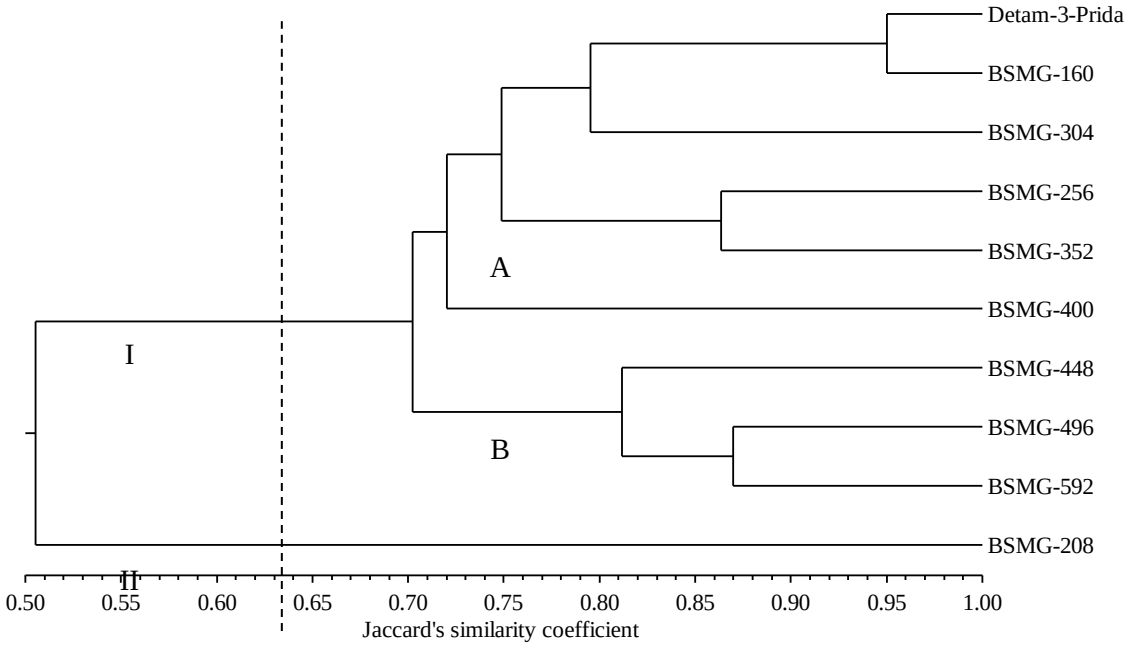

Figure 3. Dendrogram of Detam 3 Prida and black soybean in M2 generation based on RAPD

RAPD markers have been efficiently used for evaluation of genetic relationship between soybean cultivars [26, 27, 28] and among different lines of soybean mutants [23, 29]. In addition, they have also been used for evaluation under salt stress in rice [30], wheat [31], barley [32], cotton [33], maize [34], soybean [35], and petunia [36]. Salt tolerance differences among the genotypes could be related to the difference in the genetic distances coefficient. So, the less different in salt tolerance are indicated by the more genetically similar genotypes. Our data supports that genetic instability induced by gamma-rays in M2 generation was reflected by changes in RAPD profile: disappearance of bands and appearance of new bands occurred in the profiles in comparison to control. Nevertheles, it is evident that RAPD markers can efficiently distinguish all of the genotypes which associated with salinity tolerance.

Table 2. Genetic similarity matrix of Detam 3 Prida and black soybean in M2 generation generated by RAPD

	Detam-3-Prida	**BSMG-160**	**BSMG-208**	**BSMG-256**	**BSMG-304**	**BSMG-352**	**BSMG-400**	**BSMG-448**	**BSMG-496**	**BSMG-592**
Detam-3-Prida	1.000									
BSMG-160	0.950	1.000								
BSMG-208	0.550	0.600	1.000							
BSMG-256	0.667	0.708	0.435	1.000						
BSMG-304	0.773	0.818	0.524	0.708	1.000					
BSMG-352	0.773	0.818	0.524	0.864	0.818	1.000				
BSMG-400	0.720	0.760	0.500	0.731	0.630	0.760	1.000			
BSMG-448	0.739	0.708	0.435	0.750	0.640	0.783	0.667	1.000		
BSMG-496	0.680	0.720	0.458	0.630	0.593	0.654	0.679	0.760	1.000	
BSMG-592	0.773	0.818	0.524	0.708	0.667	0.739	0.692	0.864	0.870	1.000

Determination of RAPD-specific markers were enabled by polymorphism of RAPD bands. However, further experimentation required to be done to determine the linkage between the genes responsible for salt tolerance and RAPD markers in the next

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generation of black soybean. This make it possible that these markers have the potential to be mapped onto the soybean genome for locating the positions of corresponding chromosomal regions, where the salt-sensitive and salt-tolerance genes are situated. Evaluation of salinity tolerance in soybean is often difficult and needs long time, but tolerance to salinity is different in growth stages, thus breeding and screening for salinity tolerance require a rapid and reliable technique. The evaluation of genetic distance together with salt tolerance ability provides some useful information for assisting plant breeders in selecting interested genotypes.

4. CONCLUSIONS

Detam 3 Prida was considering as salt-sensitive black soybean cultivar, and it is closely related with BSMG-160, BSMG-304, BSMG-256, BSMG-352, BSMG-400, BSMG-448, BSMG-496, and BSMG-592 genotypes, sequentially. The black soybean in M2 generation derived from previous study might not contribute much to improvement in salt tolerance. In addition, BSMG-208 genotype is an effective out-group. Our data suggest that RAPD is a good molecular markers to assess genetic diversity of black soybean in M2 generation and examine these genetic relationships. Therefore, this study may help breeders in selecting genetically diverse seed source for salt tolerance and strong potential usage for next step of breeding in black soybean.

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