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Genetics Diversity Analysis of Progeny F_3 Soybean (Glycine max L.) Tolerant Salinity Using Microsatellite Markers

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Abstract
Salt stress is one of the abiotic stresses that significantly reduce the yield of soybean. One of the strategies of plant breeding to produce soybean varieties that are resistant salinity is using artificial crosses that aimed to combine the properties of both parent genetics. SSR molecular markers can help to identify the genetic distribution that brings the character of salinity resistant high yielding of soybean. The purpose of this study were to determine the diversity of the progeny of soybean saline-tolerant and high yielding, information level of polymorphism using four SSR markers, determined the distance genotype progeny soybean with the elders and information on the available genetic diversity for selecting progeny candidates for possible use in a soybean breeding program of saline-tolerant soybean. Studies of genetic distance of 60 progeny soybean and 8 female individual numbers (61-64) and male (65-68) used four primary SSR markers was conducted in the Laboratory of Biomolecular Sucofindo Seed Production. This research used seeds were planted in crops with salinity levels of 5-6 dS / m-1 in a glass house at the Faculty of Agriculture, University of Sumatera Utara. Methods of SSR markers was applied with four SSR primers, 60 progeny soybean hybridization and eight numbers of female and male elders. The results showed the fourth primer is polymorphism with PIC average of 0:58. Cluster analysis divides the two groups. Only individuals who were outside 52 male group of male elders. There were 59 females and the progeny soybean traits such as female parent and male. F_3 progeny soybean derivative Anjasmoro hybridization with saline resistant genotypes potential for genetic improvement to produce high saline resistant crops in order to support the expansion planting to support the improvement of soybean production in Indonesia.
Keywords: soybean, progeny soybean hybridization,saline-tolerant, microsatellite markers

Introduction

Soybean (*Glycine max* L.) plays an important role in world agriculture because of its oil content and protein. It contain about 20% oil and 40% protein, it was devoted the highest cultivated lands in the world for soybean. Genetic variation for parental selection in breeding programs and protein polymorphisms are of great importance and concern in plant breeding. Majority of protein bands of soybean including (11S) glycine and beta-conglycinin (7S), which itself consist of several subunits. The oil produced from soybean is highly digestible and contains no cholesterol (Brooks and Morr, 1985). The quality of soybean seeds (the primary agricultural product) is affected by salt stress. In general, salt stress reduces the protein contents in soybean seeds (Wan *et al*., 2002). According to Ashraf (1994), soybean is classified as a moderately salt-tolerant crop and the final yield of soybean will be reduced when soil salinity exceeds 5 dS/m^{-1}. High salt imposes damages in the whole life cycle of soybean. Therefore, soybean, which is ranked as the fourth largest crop in terms of global yield, is a major food, fuel and feed crop, and has been classified as sensitive to salinity. Salt stress is one of the abiotic stresses that significantly reduce the yield of soybean. (Phang *et al*., 2008).

Until now there has been no found soybean varieties tolerant to salinity stress. One of the strategies of plant breeding to produce soybean varieties that are resistant salinity is using artificial crosses that aimes to combine the properties of both parent genetics (Barnawi *et al*., 2013). Utilization of soybeans by Indonesian society increasingly widespread both for household consumption, industrial raw materials and livestock. It gives consequences of the increasing demand for soybeans. Increased demand for soybean will further encourage plant breeders to produce new high-yielding soybean varieties and high quality soybean products (Widaningsih *et al.*, 2014). Krisdiana (2014) reported that soybean as an edible legume crop which contains high protein and vegetable oil is the third main crop in Indonesia. During the past 30 years its breeding program achieved a significant progress and more than 70 varieties have been released to farmers. Around twenty of the released varieties have been adopted by the farmers in soybean central production areas of Indonesia.

Management of reference varieties collection in the form of living collection takes a huge place, costly, time-consuming and great effort. Molecular markers can be used as an alternative for the management of reference varieties in the form of molecular data that can be stored in the database. Protected and registered has become a collection of reference and must be managed

on regular basis in order to properly recorded and can be utilized in the future. Molecular analysis with microsatellite markers can be a solution in identifying the genetic diversity of soybean varieties collection, so it can be a supplementary method to support the management of soybeans reference collections, especially in the PVP system in Indonesia. Identification based on DNA markers can provide unique profiles or fingerprints of

every variety are very necessary for the protection of breeder's rights (Diwan and Cregan, 1997).

Microsatellite DNA marker, known as Simple Sequence Repeat (SSR) is composed of a 1-to 6-base pairs (bp) DNA sequence that is repeated in a variable number of times. SSR markers demonstrate high levels of length polymorphism in soybean. Microsatellite or SSR markers were chosen and used to great advantage in studying of diversity, genetic structure, and classification. SSR markers over other molecular markers provided appropriate technology for laboratories and have several advantages of being based on simple PCR assay, are highly polymorphicmultiallelic, codominant, abundant presence in the genome, genome wide coverage, inexpensive to use and easily amplified by PCR, few DNA samples required and high reproducibility between laboratories (Diwan and Cregan, 1997; Garcia *et al*., 2004). In general, the microsatellite has been the most used molecular marker to address genetic diversity worldwide in many articles (Table 1). This marker has been applied for the germplasm conservation, phylogenetic analyses, plant and animal breeding programs, constructing linkage maps, mapping economically important quantitative traits and identifying genes responsible for desired traits (Hoshino *et al*., 2012).

Table 1. The microsatellite has been the most used molecular marker to address genetic diversity worldwide in many articles (Hoshino *et al*., 2012: 150).

Science Category	Molecular marker				
	Microsatellite or SSR	SNP	RAPD	AFLP	RFLP
Biochemistry Molecular Biology	1178	185	83	96	38
Evolutionary Biology	989	33	18	78	11
Ecology	989	23	17	49	16
Genetics Heredity	1134	493	124	131	32
Biodiversity Conservation	405	4	3	8	3
Total	4690	1.269	925	668	531

Risliawati *et al*. (2015) developed a set of SSR markers as a tool to identify the Indonesian soybean varieties. 42 soybean varieties were analyzed using 14 random SSR markers. A total of 168 alleles that were obtained from the polymorphism analysis, and recommended that this marker set can be used as a complementary tool in DUS test and can be continuously adjusted in line with the release of new soybean varieties.

The objective of present study were (1) to determine genetic diversity of the 60 progeni soybean from hibridisation Var. Anjasmoro with genotype tolerant salinity, (2) to identify polymorphism levels of the 4 SSR markers against 60 progeny soybean, to study the parent pairs used for development of F_3 mapping population, and (3) to inform on the available genetic diversity for selecting progeny candidates for possible use in a soybean tolerants salinity breeding program.

Materials and Methods

This study uses 60 F_3 progeny soybean Anjasmoro of crossbred varieties with resistant genotypes using 4 seed salinity resistant and female elders var. Anjasmoro and 4 seeds salinity resistant genotypes of male elders. Seeds were planted in pots with levels of salinity 5-6 dS/m^{-1} in a glass house at Faculty of Agriculture, University of Sumatera Utara. SSR Markers were selected to distributed well across the soybean genom. The primers pairs must show a good segregation recommended by Guan *et al*. (2014). The SSR loci for soybean genetic diversity analyses and the SSR primer were synthesized by Marcrogen (Table 1; Diwan and Cregan, 1997).

Genomic DNA Isolation, PCR, Electrophoresis and SSR Data Scoring

Plant materials used in this study were grown in pots in the glass house. Leaf was harvested from a two-week old plant collected from all progeny. Leaves samples were collected from young leaf tissue from five plants per variety and inserted into the 2 mL micro tubes. Micro tubes containing the sample is then put in a container and pour liquid nitrogen. DNA was isolated in miniprep scale according to protocol described by Doyle and Doyle (1990) with slight modification. The isoloated DNA was diluted in the buffer, and their quality was tested using the agarose gel electrophoresis standard method and the relative DNA quantity each sample was using the standard Lambda DNA (Sambrook *et al*., 1989). Genomic DNA was then diluted using sterile water to make DNA concentration of 50ng/µl. SSR amplification was conducted using the method of Thermo Scientific (Guan *et al*., (2014).

PCR reactions were carried out in 20 µl volumes and contained 1 × PCR buffer (200 µmol/ L^{-1} MgC_{12}, 150 µmol/ L^{-1}dNTPs, 150 µmol/ $^{L-1}$ of each primer, 50ngDNA, and 1.0 U of Taq-DNA polymerase (Thermo). PCR was programmed for initial denaturation 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, and 5 min at 72°C. The PCR products were separated on 1,7% agarose gels with 1 x Tris-acetate-EDTA (TAE) buffer at 80 V for 4 hours and stained with red gel. The primer sequences are presented in Table 2.

Table 2. Profiles from 5 primer of SSR markers for 60 F_3 progeny soybean Anjasmoro hibridisation.

Nama Marker	Sequence (5'→3')
QS08064	ACGTAAGTGGTTGAAGGCGTT(F)
	GGGCAAGGGATATGAAAA(R)
QS080465	ACTCAAGAGCAACTCACAAC(F)
	GCTAACGACTACCTCAATGC(R)
QS1101	CTTACCTTCACGGACGGAGA(F)
	CCCATCTCCCAATCCTAACA(R)
QS100011	TTTGATATTGCAGGGATGACA(F)
	AACTGACGGACCAATGGAAG(R)

Data Analysis

Molecular data to be analyzed must be converted into binary data based on the presence or absence of band amplification results. Polymorphic DNA segments amplified with each microsatellite primer were considered as different alleles, assigned a letter and each allele was scored as present (1) or absent (0). The polymorphism information content (PIC), a measure of the allelic diversity at a locus, was determined as PIC = 1-Σ*P*i2 where *P*i is the frequency of the ith allele in the examined test lines. NTSYSpc version 2.2
(Rohlf, 2000).

The genetic diversity of each microsatellite locus was obtained from the allele frequency using the following formula:

$$Polymorphism\ information\ content\ (PIC) = 1 - j = 1 - \sum_{j=1}^{n} p_{ij}^2$$

where p is the frequency of the jth allele for the primer i (Anderson *et al*., 1993).

Botsein *et al*. (1980) classified the value of the PIC into 3 classes:

PIC > 0.5 : very informative
0:25 > PIC < 0.5 : moderate
PIC < 0.25 : Low

Based on the value of genetic similarity, the data matrix was grouped and presented in the form of dendogram relationship using the UPGMA (Unweighted Pair Group Method Arithmetic). The results of the molecular banding pattern that has been converted into binary data was processed using computer program multivariate version 3.2 statistical software in order to get the genetic distance matrix and dendogram (phylogenetic) of the sample.

Results and Discussion

Studies of genetic distance of 60 progeny soybean and 8 female individual numbers (61-64) and male (65-68) used four primary SSR markers (Table 2) was conducted in the Laboratory of Biomolecular Sucofindo Seed Production. Example of SSR banding pattern shown in Figure 1. Rongwen *et al*. (1995) suggested that a gene diversity value higher than 0.8 is common for soybean microsatellites and this provides a good basis for DNA profiling of soybean. The average level PIC of the four SSR markers in our study were used to test 60 genotype was 0.58 (range 0.03 to 0.86; Table 2). Polymorphisms were observed in this study are also generally very similar to those reported previously by Chaerani *et al*. (2011), who obtained a PIC value of 0.58, while by using a high number of soybean genotypes and an increase of the number of markers in genotyping analysis, Santoso *et al*. (2006) detected a higher PIC value (0.70). Low and high values were influenced by the selection marker diversity and number of varieties analyzed (Bredemeijer *et al*., 2002). Chaerani *et al*. (2011) categorized microsatellite markers with gene diversity and PIC value of < 0.4 as a less informative markers and those of > 0.75 as informative one and such markers can be used to distinguish or discriminate the soybean accessions.

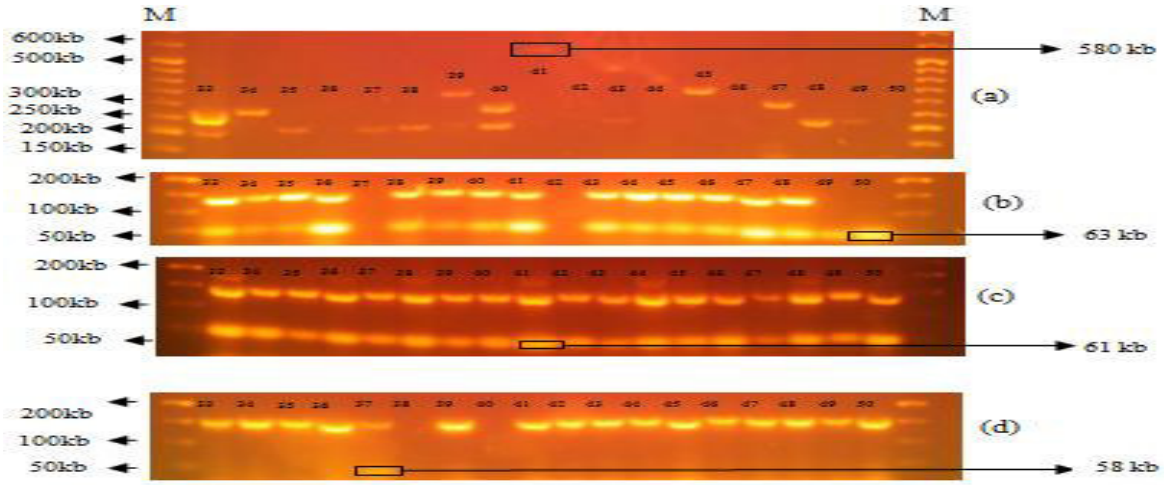

Figure. 1. Patterns of band amplification few progeny soybean hybridization with primer 5(a), Primer 1(b), Primer 2(c), Primer 3(d), the 4 primer using DNA ladder 50 kb were separated by 1.7 % agarose electrophoresis .

Data visualization of the amplification band patterns do scoring data to know each progeny soybean cluster Anjasmoro hybridization with saline resistant genotypes. UPGMA cluster analysis was calculated using the MVSP, as well as the genetic matrix calculated to determine the genetic distance of each progeny soybean. The genetic distance was used to determine the progeny-progeny that have good genetic similarities in common with the female elders, the elders and the similarities between the male and the hybridization results. The results for the 68 cluster sample number of soybean hybridization Anjasmoro with saline resistant genotypes is shown in Figure 2. Based on a cluster analysis of genetic distance of 0.3 divide progeny into two large groups. In the first group only contains an individual number 52, while the two other individuals sided started 1-68 except for number 52 (Figure 2). The data indicated that many individuals who potentially resistant salinity and high production, as many individuals are in one group with elders females and males.

PIC is used to select markers that can extinguishing between the progeny of soybean. Quantitative PIC is the number of alleles that can be generated by a marker and the frequency of each allele is determined by the frequency of occurrence of alleles. Profile fourth SSR primer used to differentiate 68 genotypes of soybean hybridization Anjasmoro with saline resistant genotypes can be seen in Table 2. The results show the fourth primer is polymorfis but to varying degrees. PIC level of the fourth ranging between 0:03 to 0.86. The highest PIC value contained in the primer 3 (QS1101) of 0.86. This indicates that the primary good enough to extinguishing hybridization Anjasmoro progeny of soybean genotypes resistant to saline. But the primer 5 (QS100011) with the lowest PIC values only by 0:03. This indicates that less suitable primer used to extinguishing the progeny soybean hybridization Anjasmoro with the saline resistant genotypes. High primary grades PIC demonstrated the ability to extinguishing the progeny soybean with the SSR markers (Diwan and Cregan, 1997; Garcia *et al*., 2004; Hoshino *et al*., 2012; Risliawati *et al*., 2015).

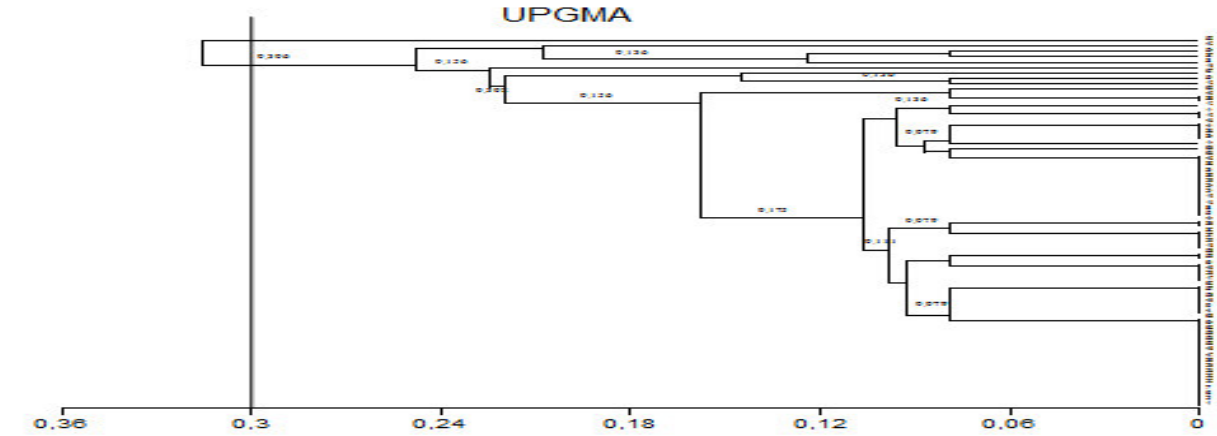

Figure 2. UPGMA cluster analysis of 68 individual (elder females, all males and progeny hybridization) soy -based genetic similarity using four SSR markers polymorphisms. Number of individual progeny hybridization start 1-60; Female elders (Anjasmoro) in individuals 61-64; male elders (genotypes resistant copy) in individuals 65-68 .

Data on Figure 2 showed that the characterization based on SSR markers progeny of hybridization Anjasmoro with saline resistant genotypes with genetic similarity coefficient ranged from 0:06 to 0:30, or the genetic distance of 0.079 to 0.306. The greater the value of the coefficient of genetic similarity between clusters, the greater the resemblance. But the greater the genetic distance showed that the more distant genetic distance between progeny.

The results of the research on the highest genetic similarity coefficient of 0.3, showed there were two cluster groups of all individuals, the first group only contains 52 individual numbers and group 2 contained all individuals other than the individual numbers 52 (individual numbers 1-51; 53-68). This showed that many progeny of soybean that has a character close to the female parent and the male individual. Only individual number 52 has a character beyond the nature of the parent, but it can still be changed because of the level of segregation is still high for the next planting. Based on the cluster analysis known that the resulting progeny soybeans still have a high level of diversity, seen from the genetic similarity coefficient is still are relatively low (0.3), still showing a high genetic diversity in this F_3. This is beneficial for breeders, because of the high genetic diversity is very useful in the selection of progeny to the purpose breeders of soybean (Rongwen *et al*. (1995).

From the results of cluster analysis at similarity coefficient of 0.3 divide into two groups, only one outside the group of different individuals . While the progeny of other individual numbers from 1 to 51; 53 to 60 is a group with both elders. The results showed many progeny that carry the character of the two parents. The results of SSR markers in this study suggested the potential for saline-resistant progeny high yield. This is supported by the analysis of genetic clusters that split into Several groups, were in a group of individuals there are numbers for the two parents. One was seen in the cluster analysis with 0:12 similarity coefficient is split into nine groups (Figure 2). In the group there were numbers and numbers of individual progeny individuals the two parents, the which showed both characters contained in the elder progeny numbers in this group. There were 49 known progeny Potentially high-yielding resistant copy because it has a genetic distance of the which is close to the two parents. With similarity coefficient of 0:12 showed numbers of individuals potentially in the order in culster analysis were 12, 11, 10, 45, 41, 35, 34, 14, 43, 33, 31, 30, 29, 24, 21, 19, 15, 8, 5, 44, 36, 32, 27, 20, 18, 58, 50, 49, 28, 17, 57, 56, 46, 9, 4, 59, 55, 48, 47, 39, 26, 25, 23, 22, 7, 6, 2 and number 1 (Figure 2). The results of cluster analysis at similarity coefficient 0,06 proved number of progeny genetic distance closer to the female elders (Anjasmoro) were 49, 28.17, 57, 56, 46, 9 and 4. While the progeny are closer to the genetic distance male elders (genotipa copy) was 43, 33, 31, 30, 29, 24, 21, 19, 15, 8 and 5. As well as the number of progeny that genetic distance close to the female and male elders was 59, 55.53, 48, 47, 39, 26, 25, 23, 22, 7, 6, 2 and 1 (Figure 2). The greater the genetic similarity coefficient, the greater the genetic similarity (Rodrigues *et al*., 2008).

From both the results of agronomic research and molecular markers obtained, we found that the derivative F_3 progeny soybean hybridization Anjasmoro saline resistant genotypes with potential to develop saline resistant crops produce high F_3. At have seen individuals with genetic distance close to the two parents, this is a great potential to improve soybean genetics. But there is still need for some further research to produce progeny soybean stable. Therefore , it can support the expansion of planting area to sub-optimal land (land saline) to support the improvement of soybean production in Indonesia. This is in accordance with by Risliawati *et al*. (2015), who has also developed a SSR marker set to be used for soybean variety identification purposes which was used to develop the identity of the 42 Indonesian soybean varieties.

Conclusions

Fourth SSR markers used were polymorphism markers with PIC mean value of 0.58. SSR markers that indicated the value of the PIC more than 30% was very useful to study the efficiency of selection salinity tolerant soybean. UPGMA cluster analysis of progeny soybean split into two groups, the group consists of only one individual 52 and the second group consists of more progeny. Molecular known F_3 numbers of individuals potentially resistant saline and high production, namely 12, 11, 10, 45, 41, 35, 34, 14, 43, 33, 31, 30, 29, 24, 21, 19, 15, 8, 5, 44, 36, 32, 27, 20, 18, 58, 50, 49, 28, 17, 57, 56, 46, 9, 4, 59, 55, 48, 47, 39, 26, 25, 23, 22, 7, 6, 2 and 1 with number sequence corresponding to the analysis culster. F_3 progeny soybean derivative Anjasmoro hybridization with saline resistant genotypes potential for genetic improvement to produce high saline resistant crops in order to support the expansion of planting area to support the improvement of soybean production in Indonesia.

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