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Genetic Diversity Analysis of Aluminum-toxicity Tolerant and Sensitive Soybean Genotypes Assessed with Microsattelite Markers

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ABSTRAK

Analisis Diversitas Genetik Genotipe Kedelai Toleran dan Peka Keracunan Aluminium Menggunakan Marka Mikrosatelit. ***I Made Tasma dan Ahmad Warsun*****.** Persilangan dua genotipe kedelai dengan jarak genetik jauh menghasilkan progeni dengan polimorfisme tinggi pada banyak lokus yang memfasilitasi keberhasilan program pemuliaan dan pemetaan karakter agronomi penting kedelai. Tujuan penelitian ini untuk mengetahui diversitas genetik genotipe kedelai toleran dan peka keracunan aluminium (Al), informasi diversitas alel dan tingkat polimorfisme marka SSR dari genotipe kedelai yang diuji, menentukan genotipe dengan jarak genetik jauh sebagai tetua dalam pembentukan populasi pemetaan karakter toleran Al, dan informasi diversitas genetik dalam pemilihan tetua untuk program pemuliaan kedelai toleran keracunan Al. Dua puluh empat genotipe kedelai toleran dan peka keracunan Al dianalisis menggunakan 15 marka SSR. Marka SSR lokasinya menyebar pada 14 kromosom kedelai. Dendrogram dikonstruksi menggunakan *Unweighted Pair-Group Method Arithmatic* (UPGMA) melalui program *Numerical Taxonomy and Multivariate System* (NTSYS) versi 2.1-pc. Diversitas genetik antara dua genotipe kedelai berkisar antara 2-33,2%. Pada diversitas 33,2% uji klaster UPGMA membagi genotipe menjadi 2 kelompok masing-masing terdiri dari 19 dan 5 genotipe untuk kelompok 1 dan 2. Jumlah alel SSR total 81dengan rata-rata jumlah alel per lokus SSR 4,4 dan rata-rata tingkat polimorfisme 0,55. Menggunakan diversitas tertinggi 33,2% dua genotipe paling peka Al (B3293 dan B3442) dari kelompok 1 dan dua genotipe paling toleran Al (B3462 dan B3851) dari kelompok 2 dipilih untuk membentuk populasi pemetaan karakter toleran Al. Berdasarkan nilai diversitas genetik tertinggi 33,2% banyak kemungkinan kombinasi persilangan dapat dilakukan antara genotipe toleran Al untuk pemuliaan kedelai toleran Al.

Kata kunci: Diversitas genetik, marka SSR, keracunan aluminium, kedelai.

INTRODUCTION

Aluminum (Al) toxicity limits soybean [*Glycine max* (L.) Merr.] production in acid soils. Indonesia has over 47.5 millions hectares of red yellowish podsolic soils (Syarifuddin and Abdurachman 1993). This kind of soil is the typical acid soils generally used for a national food and estate crop extensification program including for soybean. Cultivated plants fully adapted to these environments, therefore, should posses a tolerance to toxic Al and efficient in nutrients utilization for deep root growth (Spehar 1995). Traditionally, the problem of acid soils has been corrected by liming and fertilization. The ploughed layer benefits mostly from such an amendments, and only in the long run do nutrients leach down into the subsoil, mostly caused by vicious tillage. The above method of fixing acid soils, however, is not always practically feasible especially in the remote rural areas as the supporting infrastructures might not always be readily available. Development of Al tolerant cultivars is one of the strategies to enable cultivation of soybean on acid soils. There is a considerable genetic variation for Al tolerance in soybean and this has been exploited by conventional breeding methods (Arsyad *et al*. 2002, 2005) to improve productivity and quality of soybean grown in acidic soils.

The genetics of Al tolerance has been actively studied in several crop species. Significant intraspecific variation for Al tolerance is well known in many plant species (Foy 1988). The genetic control of Al-toxicity tolerance is varied across plant species, being monogenic in some species but polygenic in others. In wheat (*Triticum aestivum*) for example, the majority of variation for Al tolerance occurs at a single locus (Delhaize *et al*. 1993). In other species, such as maize (*Zea mays*) and rice (*Oryza sativa*), Al tolerance appears to be quantitative (Pellet *et al*. 1995, Wu *et al*. 2000, Nguyen *et al*. 2001). Similar quantitative inheritance was also reported for soybean and that at least three to five genes were predicted to control Al-toxicity tolerance in the model crop legume (Bianchi-Hall *et al*. 1998, 2000).

Microsattelite DNA marker, also 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. Santoso *et al.* (2006) reported that as many as 19 alleles per SSR locus were observed among a group of 96 Indonesian soybean accessions assayed with 10 SSR loci. Using 21 Indonesian soybean varieties and seven SSR markers,

an average of 3.4 alleles per SSR locus was reported by Trustinah *et al.* (2006). Akkaya *et al.* (1992) demonstrated as many as 8 alleles per SSR locus among 43 US cultivated and wild soybean genotypes. In addition, an average of 10.1 alleles per SSR locus was shown using 20 SSR loci against 35 soybean genotypes (Diwan and Cregan 1997). The SSR marker has also been successfully used in the construction of the soybean genetic linkage map (Cregan *et al*. 1999, Tasma *et al*. 2001, Song *et al*. 2004). The polymorphism level of the SSR markers in the intraspecific and interspecific populations is generally no lower than 50% (Cregan *et al*. 1999, Tasma *et al*. 2001, Song *et al*. 2004).

The objectives of this study were to: (1) determine genetic diversity of the 24 soybean genotypes tolerance and sensitive to Al toxicity as well as their DNA fingerprints, (2) determine allelic diversity and polymorphism levels of the 15 SSR markers against the 24 soybean genotypes, (3) determine the parent pairs having the highest SSR marker diversity levels to be used for development of F_2 mapping populations, and (4) information on the available genetic diversity for selecting parent candidates for possible used in a soybean Al-toxicity tolerant breeding program.

MATERIALS AND METHODS

Plant Materials

A total of 24 soybean genotypes were used. Among those 15 genotypes were known to be tolerance and 9 genotypes were sensitive to Al toxicity. The complete list and genetic backgrounds of the soybean genotypes used in this study are as previously reported (Tasma *et al*. 2007).

SSR Marker Selection

SSR markers were selected to be distributed well across the soybean genome. The primer pairs must show a good segregation ratio on the reference interspecific *G. max* x *G. soja* population (Cregan *et al*. 1999, Song *et al*. 2004). The SSR loci selected were the ones that were previously well-tested for soybean genetic diversity analyses (Diwan and Cregan 1997, Trustinah *et al*. 2006). The characteristics of the SSR markers used in this study are shown in Table 1 and the primer sequences of each SSR locus are shown in Table 2. The SSR primers were synthesized by Invitrogen (Invitrogen, California, USA).

Genomic DNA Isolation, PCR, Electrophoresis, and SSR Data Scoring

Plant materials used in this study were grown in pots in a glass house of BB-Biogen. Leaf was harvested from a two-week old plant collected from six plants per genotype. The collected young leaf was used to isolate genomic DNA using the method of Keim *et al.* (1988) using CTAB extraction buffer. The isolated DNA was diluted in TE buffer, their quality was tested using the agarose gel electrophoresis standard method and the relative DNA quantity of each DNA sample was measured using the standard Lambda DNA (Sambrook *et al*. 1989). Genomic DNA was then diluted using sterile water to make DNA concentration of 30 ng/µl. SSR amplification was conducted using the method of Akkaya *et al.* (1995) by using the PCR Core System of Promega (Promega, Wisconsin, USA). A PCR reaction of 20 µl total volume per reaction was used. The reaction contains 30 ng DNA, 1 x PCR buffer (1 x PCR buffer consisted of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Gelatine), 100 µM dNTP mix, 0.25 µM each of forward and reverse primers and one unit of Taq DNA polymerase Core System from Promega. PCR reaction was conducted in an MJ Research 96-well PCR machine (MJ Research, New Jersey, USA) for 45 cycles. A total of 15 SSR markers (Table 1) were assayed through the PCR study. The primer sequences are shown in Table 2. PCR products for each SSR primer pairs were then separated in a 5% polyacrylamide gel electrophoresis using 0.5 x TBE buffer using a Vertical SesquiGen GT 38 x 30 electrophoresis apparatus from Bio Rad (Bio Rad, California, USA). Gels were then stained using the silver staining method by following the standard protocol (Sambrook *et al*. 1989). The separated DNA was scanned, scored, and analyzed.

Data Analysis

The DNA bands were scored. Each DNA band in a gel represents DNA fragment of a plant genotype and was given a value of 1 when the band exists and 0 when no band appears. Genetic similarity was calculated using the method of Rohlf (2000). The polymorphism information content (PIC) values for each SSR marker was calculated using the formula of Smith *et al.* (1997) as shown below:

$$PIC = 1-\sum_{1}^{n} fi^2;\ i = 1, 2, 3, ..., n$$

fi^2 = the i^{th} allele frequency

A dendrogram then was constructed using the Unweighted Pair Group Method Arithmetic (UPGMA) method through the Numerical Taxonomy and Multivariate System (NTSYS) program version 2.1-pc (Rohlf 2000).

Table 1. Gene bank accession number, repeated type, genomic location, and segregation ratio of the selected microsattelite markers used in this study.

SSR marker	Chromosome/linkage group	Genebank accession number	Repeated type	The segregation ratio in the reference F_2 population *G. max* x *G. soja*[a]
Sat_022	D2	BH126254	(AT)27	13A : 28H : 11B
Sat_043	K	CC453677	(AT)21	12A : 32H : 15B
Sat_069	D1b	CC453679	(AT)29	17A : 30H : 14B
Satt012	G	BH146213	(ATT)19	14A : 34H : 12B
Satt070	B2	BH126318	(ATT)24	16A : 29H : 12B
Satt100	C2	BH126330	(ATT)33	11A : 28H : 14B
Satt405	J	CC453963	(ATT)9	15A : 32H : 10B
Satt300	A1	BH126491	(ATT)19	13A : 33H : 13B
Satt409	A2	BH126589	(ATT)27	13A : 32H : 13B
Satt230	E	BH126431	(ATT)15	14A : 34H : 11B
Satt131	G	BH126345	(ATT)13	18A : 26H : 14B
Satt222	H	BH126425	(ATT)20	17A : 28H : 14B
Satt516	F	BH126686	(ATT)19	11A : 27H : 17B
Satt446	L	BH126621	(ATT)21	11A : 33H : 12B
Satt285	J	CC453960	(ATT)19	12A : 32H : 14B

[a]Segregations ratios of the primer pairs used was determined in an F_2 population of an inter-specific cross between *G. max* breeding line A7861 and *G. soja*. The null hypothesis of the test was that markers segregated in a 1 : 2 : 1 ratio of alleles originated from *G. max*, from both parents (*G. max* and *G. soja*) and *G. soja*, respectively (Cregan *et al*. 1999, Song *et al*. 2004). The size of the *G. max* x *G. soja* population was 60 individual F_2. All the selected primers followed the segregation ratio 1 : 2 : 1 of a single locus segregation ratio.

Table 2. Primer sequences of the SSR markers used in the genetic diversity study.

SSR Primer	Forward	Reverse
Sat_022	5'-GCGGCCTTTTCTGACTGTTAA-3'	5'-GCGCAGTGACTAAAACTTACTAT-3'
Sat_043	5'-GCGGTCCGTCAATGAATATTAAATTAAAA-3'	5'-GCGAAAGCGGCAGAGAGAGAAAGGT-3'
Sat_069	5'-CGACCAGCTGAAGAAA-3'	5'-CTGAATACCCATCATTACTTAA-3'
Satt012	5'-GCAATTAGTTTTAAAATGTTTC-3'	5'-AGAATAGAGCCTACATATAATCATA-3'
Satt070	5'-TAAAAATTAAAATACTAGAAGACAAC-3'	5'-TGGCATTAGAAAATGATATG-3'
Satt100	5'-ACCTCATTTTGGCATAAA-3'	5'-TTGGAAAACAAGTAATAATAACA-3'
Satt300	5'-GCGCCCACACAACCTTTAATCTT-3'	5'-GCGGCGACTGTTAACGTGTC-3'
Satt409	5'-CCTTAGACCATGAATGTCTCGAAGATA-3'	5'-CTTAAGGACACGTGGAAGATGACTAC-3'
Satt230	5'-CCGTCACCGTTAATAAAATAGCAT-3'	5'-CTCCCCCAAATTTAACCTTAAAGA-3'
Satt131	5'-AATTTCCCATTATCATTTAGAA-3'	5'-GGCCTTCATTCCAAAAC-3'
Satt222	5'-GCGTGTTTTGTGAAAATAATAATTAAAGATG-3'	5'-GCGCCACAAGTAACTAATGTAATAGGTGTT-3'
Satt516	5'-GCGTTAGCACTATTTTTTTACAAGA-3'	5'-GCGCCGTTCCTCTTTACTTTAT-3'
Satt405	5'-GCGGAAATTGTGAAAAATGTCCTAGTAA-3'	5'-GCGTTTTAGTCGTTAAATTTATGAAATG-3'
Satt446	5'-CCGCATAAAAAACACAACAAATTA-3'	5'-GCGGGCAAATTTGACCTAACTCACAAC-3'
Satt285	5'-GCGACATATTGCATTAAAAACATACTT-3'	5'-GCGGACTAATTCTATTTTACACCAACAAC-3'

RESULTS AND DISCUSSION

Genetic distance study of the 24 soybean genotypes by using 15 SSR markers (Table 3) was conducted at the Molecular Biology Laboratory of ICABIOGRAD. Examples of the SSR banding pattern are shown in Figure 1. The total SSR alleles observed was 81. The average allele per locus was 4.4 (ranges from 2 to 8) and the allele sizes range from 115 to 270 bp (Table 3). The average polymorphism level (PIC) value of the 15 SSR markers tested in the 24 soybean genotypes was 0.55 (ranges from 0.17 to 0.82) (Table 3).

The high allelic diversity and polymorphism level observed in this study are generally very similar to the ones previously reported (Akkaya *et al*. 1992, Diwan *et al*. 1997, Santoso *et al*. 2006, Trustinah *et al*. 2006). This finding also further indicated that the polymorphism level of SSR markers is much higher than that shown by RFLP markers that generally show polymorphism level ranges from 20 to 30% (Cregan *et al*. 1999, Tasma *et al*. 2001).

Cluster analysis using the highest genetic diversity level of 33.2% divided the accessions into two groups (Figure 2). The first group includes accessions B4377, B4374, B4408, B4372, B3866, B4378, B4368, B4412, B4409, B4410, B4411, B4376, B3776, B3442, B3898, B4403, B3293, and B4311. The second group includes accessions B1958, B3614, B3851, B3614, B3531, and B3462 (Figure 2).

Cluster analysis showed that neither the Al-toxicity tolerant nor the sensitive soybean genotypes were consistently grouped together. They were,

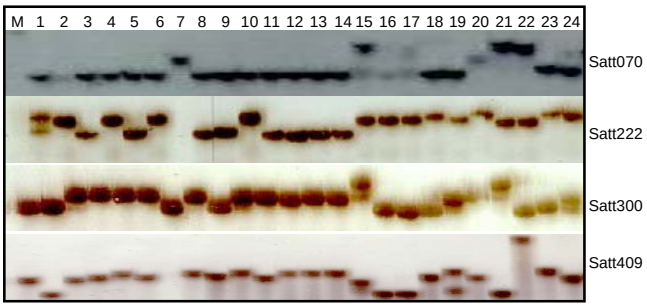
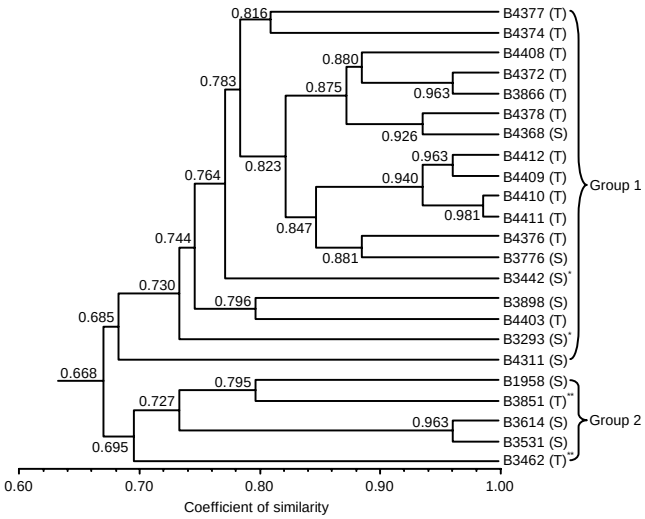


Figure 1. The banding pattern of four SSR markers used to genotype 24 soybean accessions. M = 100 bp DNA ladder, 1 = B4377, 2 = B4374, 3 = B4408, 4 = B4372, 5 = B4412, 6 = B3866, 7 = B4311, 8 = B4409, 9 = B4378, 10 = B4376, 11 = B3898, 12 = B3776, 13 = B4410, 14 = B4411, 15 = B1958, 16 = B3614, 17 = B3531, 18 = B4368, 19 = B3442, 20 = B4403, 21 = B3851, 22 = B3462, 23 = B3293, 24 = B4375. DNA band sizes range from 100 to 300 bp.

Table 3. Profiles of 15 SSR markers assayed in 24 aluminum-toxicity tolerant and sensitive soybean genotypes.

SSR marker	Number of alleles detected	Range of allele size (bp)	Polymorphism level (PIC value)
Sat_022	6	237-271	0.78
Sat_043	5	250-270	0.64
Sat_069	8	162-189	0.82
Satt012	6	115-185	0.68
Satt070	4	162-187	0.42
Satt100	2	150-158	0.41
Satt300	3	245-265	0.57
Satt409	6	125-280	0.74
Satt230	3	216-227	0.50
Satt131	4	185-210	0.35
Satt222	3	237-260	0.57
Satt516	3	225-260	0.42
Satt043	5	250-270	0.64
Satt285	3	200-240	0.17
Mean	4.4 (2-8)	-	0.55 (0.17-0.82)

however, mixed each other (i.e., the Al tolerant and the Al sensitive soybean genotypes exist at the same group) (Figure 2). This indicates that soybean accession grouping based on phenotypic trait Al-toxicity tolerance is different from the one derived from SSR markers. A similar result was reported by Dahlberg *et al.* (2003) on a comparative assessment of variation among sorghum germplasm accessions using seed morphology and RAPD measurements. They found that clustering of 94 sorghum accessions were quite different between those based on seed morphology and the ones derived from RAPD markers. In many respects such findings were not unexpected. Finding a high correlation between agronomic descriptors such as seed morphology and aluminum-toxicity tolerance and the RAPD, SSR or any other current DNA techniques may be difficult. Most phenotypic traits, including the soybean aluminum-toxicity tolerance and sorghum seed morphology are controlled by several genes which most likely be highly influenced by the environment. Most of these traits also sample a very small region of the plant genome. In many cases, DNA profiling, given the current technology, is a refine of random evaluation of the genome. In the case of SSR assay used in this study the SSR primers applied were originated from 14 out of 20 soybean chromosomes. The selected SSR markers used in this study, therefore, are randomly distributed throughout the soybean genome. Finding a high correlation between these different sampling techniques (small genome sampling of phenotypic traits and random and wide genome sampling of DNA marker assays) would be very difficult. It may require that the full genome of a crop species be identified and genes sequenced before a true correlation between the agronomic traits and DNA marker measures can precisely be determined.



T = tolerance, and S = sensitive to Al toxicity. * and ** are the most Al-sensitive and the most Al-tolerant soybean genotypes, respectively, that were selected as parents for developing four F_2 mapping populations (Tasma *et al*. 2007).

Figure 2. Dendrogram of 24 Al-toxicity tolerant and sensitive soybean genotypes assayed with 15 SSR markers.

Ten of the soybean genotypes tested showed high genetic similarity (92.6 to 98.1%) (Figure 2). This happens mainly because of their similar genetic backgrounds. For example, accessions B4409, B4410, B4411, and B4412 are all somaclonal variants derived from a common ancestor Sindoro. A pair (B4409 and B4412) showed 96.3% similarity and another pair (B4410 and B4411) showed 98.1% similarity and as a whole the all four demonstrated 94% genetic similarity (Figure 2). This means that there were some DNA sequence changes might occur when the calli were

treated with radioactive materials in addition to the effect of the *in vitro* treatment itself during the somaclonal variation development (Mariska *et al*. 2004). B4372 and B3866 (96.3% similarity), however, are sister lines as both were derived from a single cross population Dempo and Willis (Arsyad *et al*. 2002). The interesting one is the high genetic similarity (96.3%) between B3614 (a landrace from Malang) and B3531 (a landrace from Pasuruan) both were sensitive to Al toxicity. It is possible that the two genotypes were actually the same genetically but they were given different names as they were collected from different areas although both regions were actually neighbours. Similar observation was reported by Santoso *et al.* (2006) showing that some soybean accessions with different names were genetically identical based on SSR marker genotyping. This information becomes crucial to soybean curators to combine the identical accessions for efficient storage and handlings.

The two most tolerant genotypes (B3462 and B3851) and two most sensitive genotypes (B3293 and B3442) to Al toxicity (Tasma *et al*. 2007) appear at different clusters (Figure 2). Genetic distance between B3462 and B3851 was 30.5% (69.5% similarity). Genetic distance of B3293 and B3442 was 25.6% (74.4% similarity). Genetic distance between the most tolerant genotypes (B3462 and B3851) and the most sensitive genotypes (B3293 and B3442) was 33.2% (66.8% similarity) (Figure 2). This is the most genetic distance observed in this study. Based on these results for the purposes of Al-toxicity tolerant QTL studies, the four genotypes were used as parents for developing four F_2 mapping populations (Tasma *et al*. 2007). One of the populations (B3462 x B3293) showed over 57% polymorphism among more than 200 SSR markers already assayed so far (Tasma *et al*. 2008). This finding further supports the result of this study showing that the average of polymorphism level (PIC value) observed across the 24 soybean genotypes was 0.55 (Table 3).

As most Al-toxicity tolerant genotypes exist in the first group and three (including two most tolerant genotypes) are in the second group, at diversity level of 33.2%, there are many possible crossing combinations that can be done between the Al-toxicity tolerant genotypes to form segregating populations to be used in a breeding program to develop new Al-toxicity tolerant soybean cultivars. From genetic diversity point of view, at diversity 33.2% there should be a very good chance available for manipulating heterosis between the Al-toxicity tolerant parents of the two groups to support Al-toxicity tolerant soybean breeding program.

CONCLUSION

Diversity levels between two soybean genotypes range from 2 to 33.2%. At the diversity level 33.2% UPGMA cluster analysis divided the soybean accessions into 2 groups each consisted of 19 and 5 genotypes, respectively.

The total number of SSR alleles detected was 81 with the average of 4.4 alleles per SSR locus and the average polymorphism level (PIC values) of 0.55. SSR markers showing PIC values over 30% should be very useful for an efficient soybean Al-toxicity diversity study as well as for a genetic map construction.

Using the highest diversity level 33.2% the two most Al-toxicity tolerant genotypes (B3462 and B3851) from group 1 and the two most Al-toxicity sensitive genotypes (B3293 and B3442) from group 2 were selected for parents to form four F_2 mapping populations.

At the highest diversity level of 33.2%, many possible crossing combinations between Al-toxicity tolerant genotypes can be done for breeding new soybean Al tolerant.

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