

Clustering of Fifth Generation Mutants of Genetically - Improved *Kipas Putih* Soybean with High Protein Content (Pengelompokan Mutan Generasi Kelima Kacang Soya Kipas Putih Terubah Suai Genetik dengan Kandungan Protein Tinggi)

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ABSTRACT

Kipas Putih is one of the local varieties of soybean from Aceh, Indonesia. To improve the productivity of this variety, the irradiation mutation technique was applied. This study aimed to examine the level of genetic diversity in the M₅ generation of *Kipas Putih* soybean using SSR markers and to identify whether any used markers were associated with the loci that control the protein content in soybean. A total of 18 SSR markers were used to amplify the DNA of 11 mutant lines of *Kipas Putih*. The total alleles detected were 55 alleles which range from 1 to 7 alleles per locus. The average of major allele's frequency was 0.71 with the heterogeneity of 0.17. The PIC value ranged from 0.00 to 0.73 with an average of 0.35. Satt308 was identified as the most polymorphic marker among others since it had a PIC value greater than 0.70. The genetic distance analysis showed that the mutant lines B4, B10 and B13 had the furthest genetic distance (0.45) with the *Kipas Putih* (B0) soybean variety. The phylogenetic analysis showed *Kipas Putih* soybean and ten mutants of M₅ generation grouped into four main clusters at a similarity coefficient of 0.20. Furthermore, association analysis showed that only two SSR markers, Satt577 and Satt431 were significantly associated with protein content with an R² value of 0.7855 to 0.9643.

Keywords: Genetic diversity; mutant lines; phylogenetic; SSR markers

ABSTRAK

Kipas Putih adalah sejenis kacang soya tempatan yang berasal dari Aceh, Indonesia. Untuk meningkatkan produktivitinya, teknik mutasi penyinaran telah diaplikasikan. Kajian ini bertujuan untuk mengetahui tahap kepelbagaian genetik mutan kacang soya *Kipas Putih* generasi M₅ menggunakan penanda SSR dan untuk menentukan sama ada mana-mana penanda yang digunakan berkorelasi dengan lokus yang mengawal ciri kandungan protein dalam kacang soya. Sebanyak 18 penanda SSR digunakan untuk mengamplifikasi 11 titisan mutan kacang soya *Kipas Putih*. Jumlah alel yang dikesan adalah 55 alel dengan jarak 1 hingga 7 alel setiap lokus. Purata frekuensi alel dominan adalah 0.71 dengan tahap kepelbagaian 0.17. Nilai PIC berjulat antara 0.00 hingga 0.73 dengan purata 0.35. Satt308 dikenal pasti sebagai penanda yang paling polimorfik berbanding penanda SSR lain memandangkan nilai PICnya yang melebihi 0.70. Analisis jarak genetik menunjukkan bahawa titisan mutan B4, B10 dan B13 mempunyai jarak genetik terjauh (0.45) dengan kacang soya *Kipas Putih* (B0). Analisis filogenetik menunjukkan bahawa kacang soya *Kipas Putih* dan sepuluh mutan M₅ dikelompokkan menjadi empat kelompok utama dengan nilai pekali 0.20. Seterusnya, analisis perkaitan menunjukkan bahawa hanya dua penanda SSR iaitu Satt577 dan Satt431 berkait secara signifikan dengan kandungan protein dengan nilai R² 0.7855 hingga 0.9643.

Kata kunci: Filogenetik; kepelbagaian genetik; penanda SSR; titisan mutan

INTRODUCTION

Aceh has a famous soybean variety namely the *Kipas Putih* and has become a national variety since 1992. However, this variety is less desirable in home regions because of its low production and has smaller seeds than other national varieties such as Dega-1, Anjasromo, Biosoy-1, and Kemuning-2. The average weight of 100 *Kipas Putih* seeds is 12 g and the protein content is around 35%. For this reason, the *tempe* and *tofu* manufacturing industry does not want to utilize the *Kipas Putih* variety as a base for the production of *tofu* and *tempe*. However, *Kipas Putih* as a local variety is very suitable to be cultivated in Aceh Province compared to other existing national varieties. The results of the research conducted by the Syiah Kuala University (unpublished data) showed that the Dega-1 variety was not adaptive to the natural conditions of Aceh.

Mutation techniques either by chemical or physical mutagens have proved to increase the productivity of plants. Not only for plants that are vegetatively propagated but also for plants that propagated through their seeds. From 1930 to 2014, about 3,200 mutants has been released and this was occupied dominantly by food crops (75%). These mutant crops are cereals and are mostly cultivated in the Asia region (Foster & Shu 2012). Compare to other breeding techniques, the mutation can be applied chemically or physically, easy-handling and has minimum risk. In soybean, physical mutation through gamma irradiation has been significantly increased the grain yields and yield component (Mudibu et al. 2012). Other than yield characters, gamma irradiation also has been used to improve the soybean tolerance to abiotic stresses such as drought, salinity, and shading stress (Giono et al. 2014). Therefore, mutation technique through gamma irradiation was selected to increase the productivity and quality of the *Kipas Putih* variety.

Recently, the mutation breeding of *Kipas Putih* has reached M_5 generation. These mutants of gamma radiation from 100 to 1000 Gy, with LD_{50} between 200 Gy and 300 Gy. After mass selection for four generations, Zuyasna et al. (2020) found that some of the mutant lines have the potential for high production and higher protein content compared to the wild type *Kipas Putih* variety. However, it is necessary to check whether the mutants tested have a close or far kinship from their wild type, *Kipas Putih*. Plant breeding which deploys molecular technique was considered more accurate and effective in selecting a desirable feature because the selection is based on genetic rather than environmental influence (Nuraida 2012). Therefore,

molecular characterization gives more effective, fast, and accurate analysis than the morphological approach. The association mapping technique is an alternative method of determining the molecular marker associated with a particular trait. In this approach, a marker is defined based on the level of its association with a specific phenotype in a population (Chaerani et al. 2014). Reasonability using the SSR marker is based on the advantages such as wide distribution in the soybean genome, high allelic variation, ease to analyze using Polymerase Chain Reaction (PCR) technique, and high replication ability (McCouch et al. 2002).

Information derived from the molecular marker may complement the phenotypic information from the morphological characterization of the plant. During breeding activities, a molecular marker associated with a particular trait may aid the selection of the plant. Therefore, selection especially on the post-harvest character may be carried early to minimize costs. For example, the selection of seed protein content could be carried out from the vegetative phase, where no cost would be needed for analysis.

This research aimed to analyze the genetic variation and identify SSR markers that are presumed to be associated with high protein content in M_5 mutants of *Kipas Putih* variety. We consider in this report the clustering of those mutants to get clear information regarding their morphology characteristics. From our study, we have been able to group 10 tested mutants into 4 clusters based on their DNA-related morphology.

MATERIALS AND METHODS

MATERIALS

Selected 10 mutant lines from the M_5 generation of *Kipas Putih* soybean variety mutated by gamma irradiation were used in this study. The selection in every generation was primarily based on agronomic performance compare to the wild type, *Kipas Putih* variety. Specifically, for the M_5 generation, we selected the mutant lines which had 100 seed weights greater than 14 g.

LOCATION

The research was conducted in the greenhouse and molecular biology laboratory of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) in Bogor, West Java, Indonesia.

METHODS

DNA ISOLATION AND EXTRACTION

Seeds of each genotype were germinated directly into pot follows the standard cultivation protocol (Supriyadi 2011). The experiment was designed by randomized complete block design (RCBD) with three replications. The DNA was isolated from three-week-old seedlings. Extraction of DNA was carried out using a GeneEx™ Plant Kit (GeneALL, Korea). This kit consists of RNase A solution and three types of extraction buffer namely buffer PL, buffer PP, and buffer RE. About 100 mg of soybean leaves were collected from each mutant line and put into 2.0 mL Eppendorf tubes. Each tube was added with 250 µL buffer and the leaves were ground using a mortar until fine slurry was obtained. Then, 300 µL of PL buffer and 3.0 µL RNase A were added and the mixture was vortexed until homogenous. The mixture was incubated at 65 °C for 15 min. The lysate was then centrifuged at 14000 rpm for 5 min and 400 µL supernatant was transferred to the new 1.5 mL Eppendorf tube. 140 µL of buffer PL was directly added to the lysate and the mixture was vortexed for 15 s. Before centrifugation for 5 min at 14,000 rpm, the tube was incubated on ice for 5 min to slightly increase the quality of DNA. After centrifugation, 400 µL supernatant was transferred into the new tube, pre-filled with 300 µL of isopropanol, and incubated in a freezer at -20 °C for 1 h. The tubes were then centrifuged for 10 min at 14000 rpm. The supernatant was removed and 300 µL of 70% ethanol was added before centrifuged for 1 min at 14000 rpm. The ethanol was removed from the remaining DNA pellets by vacuum (Thermos-scientific Savant DNA 120) for 10 min. Finally, 100 µL of buffer RE was added to the DNA pellet and incubated at 65 °C for 20 min. The extracted DNA was stored at -20 °C until further use.

ANALYSIS OF DNA QUANTITY AND QUALITY

The extracted DNA was quantified using a spectrophotometer Nanodrop 2000^c with blank buffer RE, and the quantity was measured at wavelength of A260, A280, and A260/280. DNA quality was visualized by electrophoresis in 1% agarose gel. 3 µL DNA was mixed with 5.0 µL Blue Juice Gel Loading Buffer, 2.0 µL loading dye, 0.5 µL SYBRGold 100x, and 2.5 ddH₂O on a paraffin paper. For the DNA ladder, a mixture of 1 µL Trackit™ 100 bp DNA Ladder Invitrogen and 0.5 µL SYBR Gold was made. The gel was electrophoresized at 90 V and 320 mA for 90 min. The result of electrophoresis

was then observed under the UV Trans-Illuminator machine.

DNA AMPLIFICATION BY PCR

Based on our optimization, PCR was conducted in a total volume of 10.0 µL consisting of 10.0 ng DNA template 1.0 µL; 2xmytaq HS red mix 5.0 µL; 10.0 µM primer forward and reverse respectively 0.5 µL and sterile ddH₂O 3.0 µL. The amplification of soybean DNA was conducted by using 18 SSR primers (Table 1). Among these markers, seven SSR primers have been reported to be associated with high protein content of soybean (Casanadi et al. 2001; Jun et al. 2008; Li et al. 2011; Liang et al. 2010; Nichols et al. 2006; Panthee et al. 2005; Risliawati et al. 2015; Shi et al. 2010; Shibata et al. 2008).

PCR reaction was run in a PCR TI Thermalcycler (Biometra, German) with a program as follows: initial denaturation was carried out at 95 °C for 5 min followed by 34 denaturation process cycles at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. PCR reaction was completed by final extension at 60 °C for 15 min. PCR products were loaded into the 6% gel polyacrylamide before electrophoresized at 80 V, 500 mA for 90 min. The gel was then soaked using ethidium bromide color solution for 10 min, rinsed with Aquadest and observed using UV Trans- Illuminator Bio-Rad (UVP. UK) in Chemidoc XRS program.

DATA ANALYSIS

DNA band position from the electrophoregram was scored as 1/1 if DNA band was present and 0/0 if absent, aided by GelAnalyzer software (www.gelanalyzer.com). Scoring data was imported to Power Maker v3.25 before the genetic similarity and phylogeny tree among the samples were analyzed. Meanwhile, marker trait association was analyzed using Tassel v.5 software (<https://www.maizegenetics.net/tassel>).

RESULTS

THE QUANTITY AND QUALITY OF THE DNA TESTED

Table 2 shows the result of DNA isolation of the *Kipas Putih* variety and their M₅ mutants. All DNA samples were highly concentrated with average concentration of 55.79 ng/µL, ranging from 21.60 to 97.30 ng/µL. The B7 mutant had highest DNA concentration while B22 had the lowest. In addition, the DNA purity was also measured using spectrometer Nanodrop tool in A260

(measuring ratio of DNA/RNA) and A280 wavelengths (measuring ratio of protein). The DNA is determined as

pure when having ratio of A260/A280 ranging from 1.8 to 2.0. In this study, all DNA samples were pure ranging from 1.81 to 1.94.

TABLE 1. List of SSR markers used for genetic diversity analysis and association studies of M₅ generation mutants of *Kipas Putih* soybean

No	SSR Marker	Chrom.	SSR types	SSR motif	References
1.	Satt147	1	Universal	(ATA)15	Risliawati et al. (2015)
2.	Satt267	1	Protein content	(AAT)16	Casanadi et al. (2001); Li et al. (2011)
3.	Satt005	2	Oil content	(TTA)21	Li et al. (2011); Panthee et al. (2005); Shi et al. (2010)
4.	Satt009	3	Universal	(AAAT)3(AAT)13	Risliawati et al. (2015)
5.	Satt294	4	Universal	(TAT)23	Risliawati et al. (2015)
6.	Satt308	7	Universal	(TTA)22	Risliawati et al. (2015)
7.	Satt551	7	Protein content	(AAT)21	Jun et al. (2008)
8.	Satt177	8	Universal	(ATT)16	Risliawati et al. (2015)
9.	Satt242	9	Universal	(TTA)26	Risliawati et al. (2015)
10.	Satt243	12	Universal	(TTA)20	Risliawati et al. (2015)
11.	Satt114	13	Universal	(AAT)17	Risliawati et al. (2015)
12.	Satt516	13	Universal	(TAA)19	Risliawati et al. (2015)
13.	Satt577	14	Oil content	(ATT)12	Li et al. (2011); Shi et al. (2010)
14.	Satt414	16	Universal	(ATT)23	Risliawati et al. (2015)
15.	Satt431	16	Protein content	(AAT)21	Jun et al. (2008)
16.	Satt038	18	Universal	(ATA)17	Risliawati et al. (2015)
17.	Satt239	20	Oil content	(AAT)22	Li et al. (2011); Liang et al. (2010); Nichols et al. (2006); Shibata et al. (2008)
18.	Satt571	20	Protein content	(AAT)21	Jun et al. (2008)

TABLE 2. DNA quantity of *Kipas Putih* variety and their M5 mutant lines

No.	Name	Concentration of DNA (ng/ μ L)	A260	A280	A260/280
1.	<i>Kipas_Putih</i>	57.2	1.15	0.63	1.83
2.	B4	44.3	0.89	0.48	1.84
3.	B7	97.3	1.95	1.05	1.85
4.	B10	59.9	1.20	0.63	1.89
5.	B11	59.5	1.19	0.64	1.85
6.	B12	89.8	1.80	0.95	1.89
7.	B13	34.5	0.69	0.36	1.91
8.	B14	56.2	1.12	0.60	1.88
9.	B15	69.0	1.38	0.74	1.87
10.	B18	24.4	0.49	0.25	1.94
11.	B22	21.6	0.43	0.24	1.81
	Mean	55.79			

A260 = ratio of DNA/RNA, A280 = protein ratio, and A260/280 = DNA purity

ALLELE PROFILES BASED ON 18 SSR MARKERS

The SSR markers profile was shown in Table 3. A total of 55 alleles were successfully detected in 11 soybean genotypes based on 18 SSR markers. The average allele detected was three alleles per locus and ranging from 1 to 7 alleles per locus. Satt308 was the SSR marker that detected the high number of alleles (7 alleles), while Satt038, Satt147 and Satt571 detected the fewest allele (1 allele). The mean frequency of dominant alleles ranged from 0.32 (Satt308) to 1.00 (Satt038, Satt147 and Satt571)

with a mean value of 0.71. Meanwhile, heterozygosity values ranged from 0.00 to 1.00 with the mean value of 0.17. Gene diversity ranged from 0.00 (Satt038 and Satt147) to 0.76 (Satt308) with the mean value of 0.38. The polymorphism information content (PIC) values ranged from 0.00 (Satt038, Satt147 and Satt571) to 0.73 (Satt308) with mean value of 0.35. Satt308 and Satt431 were SSR markers with PIC values of more than 0.7, therefore both markers were considered as polymorphic markers and showed a different electrophoresis band pattern among mutant lines (Figure 1).

TABLE 3. Allele profile characteristic of *Kipas Putih* Variety and their M5 mutant lines

SSR marker	Size of product (bp)	Alleles number	Frequency of dominant alleles (%)	Gene diversity	Heterozygosity	PIC value
Satt147	180–182	1	1.00	0.00	0.00	0.00
Satt267	228–240	1	0.91	0.17	0.00	0.15
Satt005	151–173	2	0.45	0.67	1.00	0.61
Satt009	175–254	6	0.41	0.69	1.00	0.63
Satt294	292–298	3	0.73	0.43	0.00	0.39
Satt308	165–201	7	0.32	0.76	1.00	0.73
Satt551	244–248	7	0.82	0.30	0.00	0.25
Satt177	109–112	2	0.91	0.17	0.00	0.15
Satt242	184–211	4	0.64	0.55	0.00	0.50
Satt243	199–239	3	0.64	0.51	0.00	0.44
Satt114	102–116	2	0.91	0.17	0.00	0.15
Satt516	212–274	2	0.86	0.24	0.09	0.21
Satt577	110–116	14	0.64	0.51	0.00	0.44
Satt414	298–304	3	0.73	0.43	0.00	0.39
Satt431	223–267	16	0.36	0.74	0.00	0.70
Satt038	165–166	1	1.00	0.00	0.00	0.00
Satt239	189–202	20	0.55	0.56	0.00	0.48
Satt571	125–126	20	1.00	0.00	0.00	0.00
Mean		3.09	0.71	0.38	0.17	0.35
Total		55				

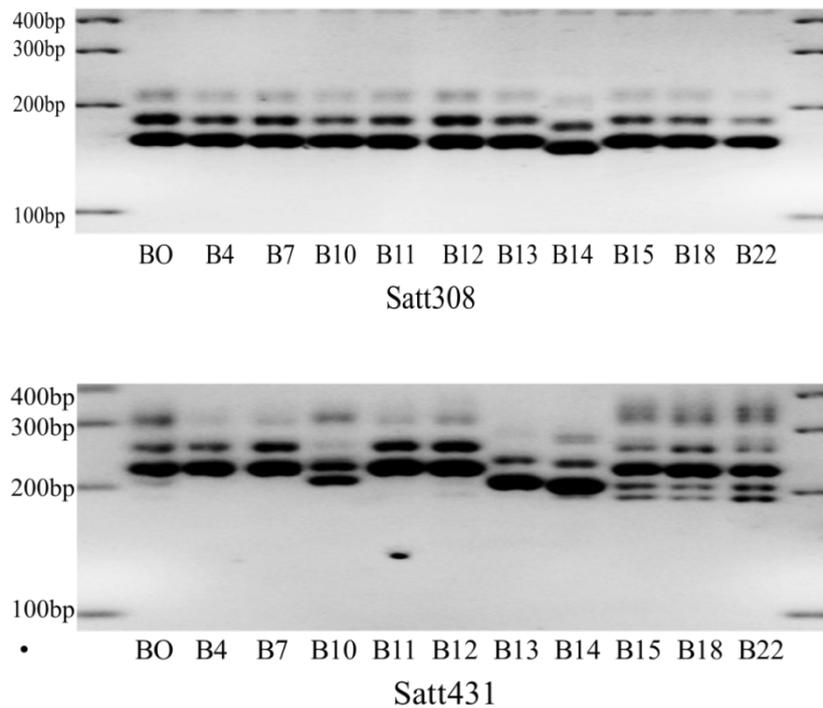


FIGURE 1. Patterns of electrophoresis bands for the *Kipas Putih* variety and M_5 mutant lines in polyacrylamide gel which amplified by two polymorphic markers, i.e. Satt308 and Satt431

ANALYSIS OF GENETIC DISTANCE AND PHYLOGENETIC

Initially, genetic distance and phylogenetic among ten mutants and their wild type were assessed using 11 universal SSR markers from the total of 18 SSR markers used. The results of genetic similarity analysis indicated that mutant B18 had the closest genetic similarity (0.05) with *Kipas Putih* variety (B0). Meanwhile, three mutants that had the farthest distance to *Kipas Putih* variety (B0), i.e. B4, B10 and B13, with a genetic distance value of 0.45 (Table 4).

Phylogenetic analysis indicated that the *Kipas Putih* variety and their M_5 mutant lines were grouped into four main clusters with a similarity coefficient of 0.20 based on 11 SSR markers universal (Figure 2). The first cluster consisted of two mutants (B10 and B13), while the second and the third clusters consisted of one genotype, B4 and B14, respectively. The fourth cluster had the most clusters' members, i.e. seven genotypes including the wild type *Kipas Putih* variety.

TABLE 4. Genetic distance matrix for *Kipas Putih* and 10 M_5 mutant lines

	B0	B4	B7	B10	B11	B12	B13	B14	B15	B18	B22
B0	0.00										
B4	0.45	0.00									
B7	0.18	0.41	0.00								
B10	0.45	0.41	0.27	0.00							
B11	0.18	0.32	0.18	0.27	0.00						
B12	0.18	0.41	0.18	0.45	0.18	0.00					
B13	0.45	0.68	0.45	0.36	0.45	0.45	0.00				
B14	0.36	0.55	0.45	0.59	0.45	0.27	0.55	0.00			
B15	0.23	0.50	0.36	0.55	0.27	0.36	0.64	0.55	0.00		
B18	0.05	0.45	0.14	0.41	0.14	0.14	0.41	0.36	0.23	0.00	
B22	0.09	0.41	0.09	0.36	0.09	0.09	0.36	0.36	0.27	0.05	0.00

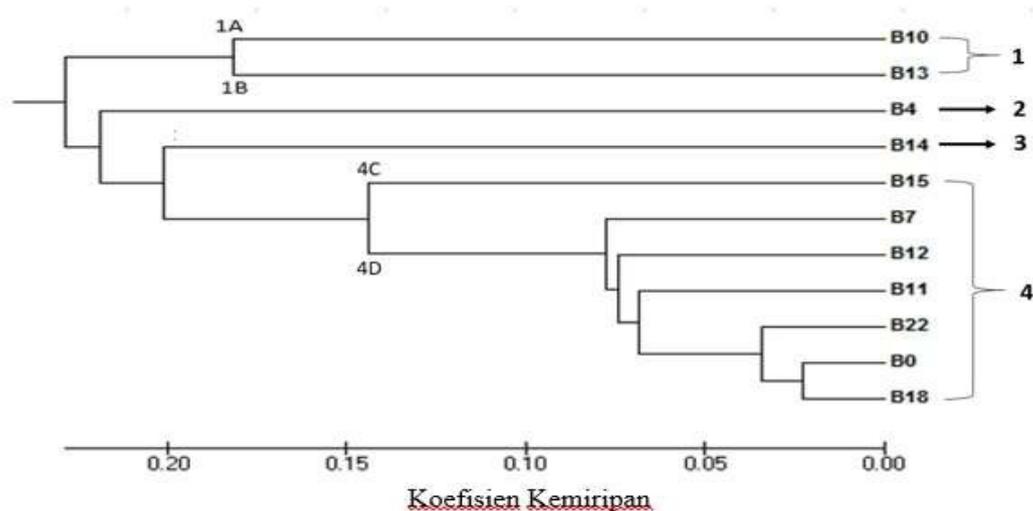


FIGURE 2. Dendrogram of *Kipas Putih* variety and their M_5 mutant lines using 11 universal SSR markers (UPGMA method with PowerMaker v3.25 Software)

Besides universal SSR markers, we also constructed the phylogenetic tree among mutants and their wild type based on seven functional SSR markers which were associated with loci controlling high protein content. The result showed that 11 genotypes were grouped into four major clusters (Figure 3). Three mutants had zero coefficient of similarity i.e. B7, B4, and B12, while one

mutant (B14) was the distinct genotypes from others. Based on the value of protein content, all mutants had higher values compared to their wild type (Figure 3). Mutants that had higher protein content were grouped clusters 1 and 2, whereas those that had lower protein content belong to clusters 3 and 4 (Figure 3).

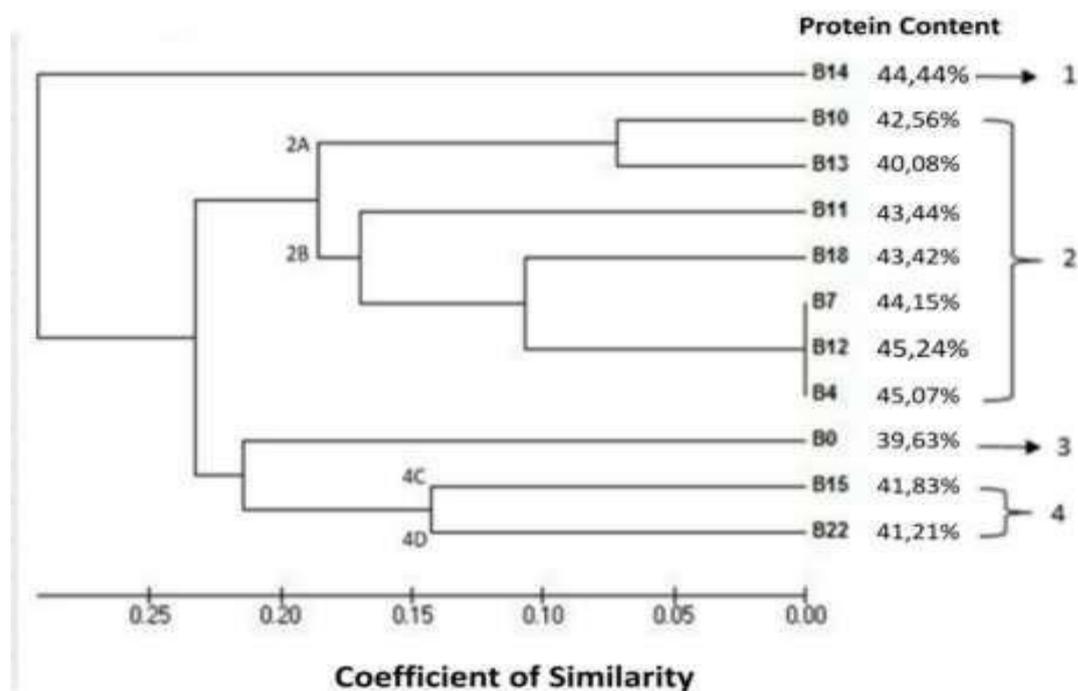


FIGURE 3. Dendrogram of *Kipas Putih* variety and its M_5 mutant lines using seven functional SSR markers

ASSOCIATION OF PROTEIN TRAIT MARKERS

Among seven SSR markers used for trait association, two SSR markers were associated significantly ($P \leq 0.05$) with the high protein content on soybean with R^2 values

ranging from 0.7855 to 0.9643 (Table 5). These markers were Satt577 and Satt431. In addition to both markers, there was another marker which is Satt239, also showing a high association rate ($R^2 = 0.8230$) although it was not significantly associated (p -value = 0.0585).

TABLE 5. Analysis of SSR markers associated with protein trait

Marker	Chromosome	Size of alleles (bp)	<i>P</i> value	R^2
Satt005	2	151	0.5822	0.1265
Satt239	20	189	0.0585	0.8230
Satt267	1	228	0.1828	0.3461
Sat431	16	187	0.0347	0.9643
Sat551	7	244	0.0836	0.7003
Sat571	20	125	0.9997	0.0000
Sat577	14	110	0.0097	0.7855

$P \leq 0.05$ = associating significantly

DISCUSSION

ALLELES PROFILE BASED ON 18 SSR MARKERS

The heterozygosity of SSR markers used in this study showed different values. The zero value (0.00) indicated the primer was homogenous and only detected one allele in each genotype (Safina 2017). Meanwhile, according to Johari et al. (2008), high heterozygosity rate increases the ability to distinguish among soybean accessions. The average gene diversity value was 0.38, indicating the selection of analyzed variety and genotypes had effects on gene diversity value (Bredemeijer et al. 2002).

The PIC value showed whether a high or low polymorphism rate existed in a marker. The obtained mean PIC value was 0.35. PIC and gene diversity values correlated directly. A small value of PIC was generally followed by a lower value of gene diversity (Widaningsih et al. 2014). Santosa et al. (2015) suggested the higher PIC value means the marker was assumed to be unique and different from each of individuals, whereas the lower PIC value implies that it was not specific or nearly all individuals contained the marker. Markers with PIC values of more than 0.7 are very informative, on the other hand, markers with low values of PIC is not that informative and should be eliminated in molecular analysis (Hildebrand et al. 1992 in Nugroho et al. (2017)). In this study, Satt308 and Satt431 can be defined as polymorphic SSR markers because they had

high PIC values. This is also supported by the pattern of electrophoresis bands among genotypes as shown in Figure 1.

ANALYSIS OF GENETIC AND PHYLOGENETIC DISTANCES

In this study, genotypes with the closest similarity were B0 and B18 with a genetic distance coefficient of 0.05, indicating a 95% genetic similarity between both genotypes. While the farthest distance with B0 was genotypes B4, B10, and B13 with a genetic distance coefficient of 0.45 meant that there was 55% genetic similarity and a difference of 45%. Therefore, these three genotypes could be developed further to be crossed with B0 with the expectation of a genetic improvement of their filial. These mutants could also be crossed with other genotypes such as B12 and B7, since they had a distant genetic relationship. Results of genetic similarity analysis of soybean could be the basis of characterizing the existing mutants and give information on utilization and selection in genetic improvement programs. The genetic distance could be considered as a reference to soybean improvement (Pavlov et al. 2016; Yi et al. 2017). Moreover, the phylogenetic analysis could represent clarity of identification for a species (Arian et al. 2016). A phylogenetic tree shows species relationships based on genetic similarity.

ASSOCIATION OF PROTEIN TRAIT MARKERS

Analysis of association was conducted to identify SSR markers that are potentially associated with high protein content. According to Chaerani et al. (2014), the association is considered significant with p -value ≤ 0.05 . Therefore, in this study, SSR markers Satt577 and Satt431 could be used to detect the protein content, although bias probability generated from population structure level could be ignored (Jun et al. 2008). These markers could also be evaluated further to other populations for identifying potential genotypes of high quality protein.

The identified SSR markers are the basis of understanding soybean genotypes with microsatellite locus mapped near or available to gene segments and QTL of high protein content. The markers, especially Satt577 and Satt431 needed further confirmation in the next derivative population (common by descending population) to ensure feasibility as selectors for high protein content.

CONCLUSION

Eleven universal SSR markers were successfully detected genetic variety among *Kipas Putih* local soybean variety and their ten M_2 mutant lines. A total of 55 alleles with a gene diversity of 0.38 and a PIC value of 0.35 were observed. Of the 11 genotypes, three genotypes (B4, B10, and B13) had the most distant genetic relationship with B0. The phylogenetic analysis suggested that mutant lines were highly genetically different from each other and this may facilitate further selection of the best mutants. Among the 11 universal SSR markers used, Satt308 showed a high polymorphism rate and could be used for genetic study and mapping of soybean. Moreover, the analysis of SSR markers associated with high protein traits showed two SSR markers (Satt577 and Satt431) that were significantly associated with high protein content. These markers could be developed for marker-assisted selection (MAS) after series of validation on segregated population.

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