Indonesian Journal of Biotechnology, December, 2014 Vol. 19, No. 2, pp.136-145

The Use of DNA Microsatellite Markers for Genetic Diversity Identification of Soybean (*Glycine max* (L) Meriil.) as a Supplementary Method in Reference Collections Management

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Abstract

Large number of new soybean varieties are mostly derived from crosses of elite genotypes resulted in a narrowing of both the genetic diversity and the phylogenetic relationship between soybean varieties. Thus, discrimination among soybean varieties is becoming more difficult, especially when morphological traits were applied. In Plant Variety Protection (PVP) system, new varieties of soybeans including granted PVP right, local and breeding varieties registered in PVP office were frequently increased, implicate on increasingly the number of soybean varieties collections. To assist the management of varieties collections, a standard fingerprinting data is further needed. In comparison to the management of plant collection in the field, molecular marker systems which are rapid, reliable, informative and relatively simple are continually sought for practical applications in germplasm conservation, management and enhancement. This study aimed to identify the genetic diversity and phylogenetic relationship of soybean varieties that have earned PVP Right as well as local varieties and breeding varieties registered in the PVP office using microsatellite or simple sequence repeats (SSR) markers.

This study was conducted in Molecular Biology laboratory, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) Bogor, from February to May 2013. The data were analyzed using the genetic analysis package NTSYSpc 2.02i and PowerMarker V3.25. The result showed a relatively narrow genetic diversity among 45 varieties of soybean analyzed in present study which were indicated by the small number of genotypes and total number of alleles (N_A), and the low value of gene diversity and PIC values (<0.75). Cluster analysis showed that the grouping varieties are not related to morphological characters but related to phylogeny relationship between varieties. Despite the group of varieties were not clustered in accordance with morphological characteristics, SSR marker can be a powerful tool for discriminating varieties, so that it could be useful for initial varieties identity in conjunction with genetic diversity analysis.

Keywords: *Soybean, microsatellites, genetic diversity, PVP.*

Introduction

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. However, the increase in demand is not accompanied by an increase in domestic soybean production. These challenges make the Indonesian government launched a program of self-sufficiency in soybeans in 2014 (Ministry of Agriculture, 2009).

Increased demand for soybean will further encourage plant breeders to produce new high-yielding soybean varieties and high-quality soybean products. However, these

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activities have an effect on the narrowing of the genetic diversity of soybean plants and make the phylogenetic relationships between soybean varieties become closer. Most commercial soybean varieties were derived from crosses among elite soybean genotypes which generally exhibited a very narrow in genetic variation among genotypes. As a consequence, new released varieties often can not be easily distinguished by morphological characters (Diwan and Cregan, 1997).

In PVP system, more new varieties of soybeans that granted PVP right and also both local and breeding varieties registered in PVP office, would give practical problems especially in terms of managing the soybean crop varieties collection. Differentiation of new varieties based on morphology characters with existing varieties will be more difficult. Moreover, many descriptors related to phenotypic characters, especially expression of traits controlled by many genes (quantitative) were strongly influenced by environmental factors, hence planting plant materials in the greenhouse and field trials are extensive and necessary needed (Bredemeijer *et al*., 2002). It is a must in the PVP system to ensure that there are no identic varieties under different names .

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 or simple sequence repeat (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, co-dominant, 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 (Powel *et al.*, 1996; Diwan and Cregan, 1997; Garcia *et al*., 2004; UPOV, 2010).

The objective of present study was to identify the genetic diversity and phylogenetic relationship of soybean varieties that have earned PVP rights as well as local varieties and breeding that has been registered at PVP Office.

Materials and Methods

The study was conducted at the Laboratory of Molecular Biology, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) Bogor, in February to May 2013. A total of 45 soybean varieties consisting of one protected variety, 38 breeding varieties registered, 3 local varieties registered and 3 example varieties listed in the soybean DUS test guideline were used (see table 1). All soybean varieties were genotyped using eight functional microsatellite markers specific for soybean and the sequence of each primer was presented in table 2 .

Plant materials for DNA isolation was prepared by planting 10 seeds per variety into a polybag. The seeds were grown for 1-2 week-old until the first leaves appeared. 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

Table 1. List of 45 soybean varieties used in the study

NO	VARIETY NAME	OWNER	STATUS
1	Mulyowilis	Sigit Prastowo SPt	PVP Right granted
2	Tanggamus	Balitkabi	Registered (122/PVHP/09)
3	Nanti	Balitkabi	Registered (124/PVHP/09)
4	Ratai	Balitkabi	Registered (125/PVHP/09)
5	Seulawah	Balitkabi	Registered (126/PVHP/09)
6	Muria	Batan	Registered (184/PVHP/09)
7	Rajabasa	Batan	Registered (187/PVHP/09)
8	Lawit	Balitrawa	Registered (9/PVHP/2010)
9	Menyapa	Balitrawa	Registered (10/PVHP/2010)
10	Sindoro	Unsoed	Registered (16/PVHP/2010)
11	Slamet	Unsoed	Registered (17/PVHP/2010)
12	Dieng	Balitkabi	Registered (140/PVHP/2010)
13	Jayawijaya	Balitkabi	Registered (141/PVHP/2010)
14	Kawi	Balitkabi	Registered (142/PVHP/2010)
15	Leuser	Balitkabi	Registered (143/PVHP/2010)
16	Sinabung	Balitkabi	Registered (144/PVHP/2010)
17	Kaba	Balitkabi	Registered (145/PVHP/2010)
18	Mahameru	Balitkabi	Registered (146/PVHP/2010)
19	Anjasmoro	Balitkabi	Registered (147/PVHP/2010)
20	Ijen	Balitkabi	Registered (148/PVHP/2010)
21	Panderman	Balitkabi	Registered (149/PVHP/2010)
22	Gumitir	Balitkabi	Registered (150/PVHP/2010)
23	Argopuro	Balitkabi	Registered (151/PVHP/2010)
24	Detam 2	Balitkabi	Registered (153/PVHP/2010)
25	Shr W C 60 (Gema)	Balitkabi	Registered (155/PVHP/2010)
26	Bromo	BPTP Jatim	Registered (60/PVHP/2011)
27	Krakatau	Puslitbangtan	Registered (61/PVHP/2011)
28	Rinjani	Puslitbangtan	Registered (62/PVHP/2011)
29	Lompobatang	Puslitbangtan	Registered (63/PVHP/2011)
30	Burangrang	BPTP Jatim	Registered (64/PVHP/2011)
31	Tidar	Puslitbangtan	Registered (66/PVHP/2011)
32	Merbabu	Puslitbangtan	Registered (67/PVHP/2011)
33	Raung	Puslitbangtan	Registered (68/PVHP/2011)
34	Wilis	Puslitbangtan	Registered (71/PVHP/2011)
35	Argomulyo	BPTP Jatim	Registered (72/PVHP/2011)
36	Bibestanang	Sigit Prastowo SPt	Registered (67/PVHP/2012)
37	Dancip	Sigit Prastowo SPt	Registered (68/PVHP/2012)
38	Larisan	Sigit Prastowo SPt	Registered (70/PVHP/2012)
39	Grobogan	Kab. Grobogan	Registered (21/PVL/2008)
40	Gepak Ijo	Kab. Ponorogo	Registered (63/PVL/2008)
41	Gepak Kuning	Kab. Ponorogo	Registered (64/PVL/2008)
42	Cikuray	Balitkabi	Example variety
43	Petek	Balitkabi	Example variety
44	Pangrango	Balitkabi	Example variety
45	Sibayak	Balitkabi	Registered (123/PVHP/09)

Table 2. List of microsatellite markers, the composition of the primary bases, and the annealing temperature of each primer used in the study

NO	CHARACTER	MARKERS	COMPOSITION OF THE PRIMARY BASES	ANNEAL. TEMP.
1	Flower color	GmF35H	F-TAGAAAGCACCCTTCAACAC R-TTTATGTAGCCACAGCCACA	60^{0}C
2	Pubescence color	SoyF3H	F-GTCATAAAATATCATTATTATTATATCTATTAA R-CACTCCCAAAAGCTTTTAAGTGT	52^{0}C
3	Growth type	Sat_286	F-GCGTTGCTTGCTAAGTAGTGTTTTTTAATCCT R-GCGTCTCCCATCATGCAACTTCAATA	62^{0}C
4	Pod color	GMES1173	F-TATGGGACATCAAAGCCACA R-CGCACTGCCATATGAAGAGA	58^{0}C
5	Asian rust resistance	Satt288	F-GCGGGGTGATTTAGTGTTTGACACCT R-GCGCTTATAATTAAGAGCAAAAGA	58^{0}C
6	Seed coat color	Satt125	F-CAAATAAAAACATATACCTCTTGT R-TGCCTTACTCTACTCTGTTTC	52^{0}C
7	Seed coat color	Satt100	F- ACCTCATTTTGGCATAAA R- TTGGAAAACAAGTAATAATAACA	50^{0}C
8	Seed coat color	Satt333	F- GCGAATGGTTTTTGCTGGAAAGTA R- GCGCAACGACATTTTCACGAAGTT	60^{0}C

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 quality and quantity of DNA for each sample was estimated using a spectrophotometer (Nanodrop 2000) and electrophoresis techniques.

PCR analysis was performed with a total of 20 µL reaction for each sample. PCR reaction was consisted of 1X PCR buffer (KAPA), 160 µM dNTPs, 0.4 µM primer, 0.04 U/µL enzyme *Taq DNA polymerase* (KAPA) and 2 µL of DNA sample. Amplification reaction was performed using the *DNA Engine Tetrad Peltier Thermal Cycler 2 MJ Research* PCR machine. The PCR program consisted of : 1). Pre-denaturation at a temperature of 94^{0}C for 10 min; 2). Denaturation at a temperature of 94^{0}C for 25 sec; 3). Annealing at a temperature of 50-62^{0}C (depending upon the annealing temperature of each primer) for 30 seconds; and 4). Extension at temperature of 72^{0}C for 45 seconds. Step 2-4 was repeated for 35 times. In the last step of the PCR process was at temperature of 72^{0}C for 7 minutes for final extension and incubation at 15^{0}C for 3 minutes.

The PCR products were separated by electrophoresis technique using 6-8% polyacrylamide gel (depend on size difference in PCR product), using a mini-vertical electrophoresis apparatus. A total of 3 µL PCR product and 1 µL of loading dye is incorporated into each of the wells and separated with a current of 80 volts for 90-120 minutes. In the process included 50 bp or 100 bp DNA ladder as a marker to estimate the size of the PCR product. Furthermore, amplified products were stained by immersing the gel in a solution of ethidium bromide (1 mg/L) for 10-20 minutes. DNA fragments amplification products were then visualized under UV light and documented by Chemi-doc transluminator (Biorad).

DNA bands score was done based on the presence of bands at specific size, where 0 was the code for the absence and 1 for existence of bands at the same locus. Genetic similarity and phylogenetic tree were generated based on the SAHN method in the software NTSYS version 2.02. Similarity analysis was performed using SimQual function. The data matrix was calculated by DICE coefficients. 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).

Furthermore, to obtain numerical parameters that can clarify the ability of each marker to analyze the genetic diversity of a collection of plants studied, all binary data were also subjected to basic statistics calculation using the genetic analysis package PowerMarker for measurements of diversity at each microsatellite locus, including the total number of alleles (N_A), heterosigosity values (H), allele frequency, major allele frequency, gene diversity (GD), and polymorphism information content (PIC) (Liu, 2001). .

Results and Discussion

Alleles number

From 45 soybean varieties analyzed in this study, a total of 50 alleles were detected with vary in number of alleles at each marker, ranged from 4-9 alleles with an average of 6 alleles per marker (see Table 3). The lowest number of alleles were detected at the marker GmF35H, GmES1173 and Satt125 (4 alleles), and the highest one was detected by marker Satt288 (9 alleles). This finding indicated that the level of polymorphism of SSR primers genotyped to soybean varieties in present study was not too high .

A previous study reported by Chaerani *et al*. (2011) was able to detect a high number of detected alleles, of which 86 alleles among 50 soybean varieties were found with an average of 9 alleles per marker, whereas Santoso *et al*. (2006) successfully detected 116 alleles with an average of 11.6 alleles per marker. Both of these studies demonstrated a high degree of polymorphism in terms of the number of alleles since the genetic materials used in their studies were derived from soybean germplasm collection comprising of high yielding varieties, accessions of local and introduced varieties, so the genetic diversity between varieties was quite high .

However, present study used the improved varieties in the assembly using elite genotypes that have lower genetic diversity. Therefore, the low level of diversity of genetic materials used in present study might be a reason for the low number of both detected alleles and polymorphism level. Accordingly, low number of alleles was also obtained by Ibarra *et al*. (2011) that using 8 microsatellite markers to analyze 35 soybean varieties. The study resulted in 23 alleles with an average of 3.13 alleles per marker .

The gene diversity and Polymorphism Information Content (PIC)

The diversity of genes is defined as the possibility that two alleles drawn at random from the population are different. The diversity of genes is also often interpreted as the expected heterozygosity (Liu, 2001). The value of gene diversity in our soybean varieties ranged from 0.24 (GmF35H) to 0.83 (Satt333) with an average of 0.66 (see table 3). This value is directly proportional to the value of the PIC in which the PIC values was just smaller than the gene diversity values. The lowest PIC value was shown by GmF35H (0.22) and the highest one by the

Table 3. Major allele frequency, number of genotypes, the number of alleles, gene diversity, heterozygosity and polymorphism rate of 45 soybean varieties

Marker	Major Allele Freq.	Genotype number	Allele number	Gene Diversity	Heterozygosity	PIC
GmF35H	0,8667	3	4	0,24	0,02	0,22
SoyF3H	0,5000	7	8	0,69	1	0,66
Sat_286	0,4667	7	7	0,71	0	0,68
GmES1173	0,4778	3	4	0,62	1	0,55
Satt288	0,3111	9	9	0,76	1	0,72
Satt333	0,2000	5	6	0,83	1	0,80
Satt100	0,3000	4	8	0,78	1	0,75
Satt125	0,5000	3	4	0,67	1	0,62
average	**0,4528**	**5**	**6**	**0,66**	**0,75**	**0,63**

marker Satt333 (0.80) with an average of 0.63. Chaerani *et al*. (2011) 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). High and low 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. Considering these categories GmF35H is likely a less informative marker, however, since this marker is designed as one of the functional SSR markers which is associated with certain trait so that it can be useful and utilized in the analysis. GmF35H that provided low PIC value is the marker associated with flower color character which only consisted of two types of flowers, purple and white colors. Another seven markers can be recommended to further analyze in identifying soybean varieties, although only two SSR markers (Satt333 and Satt100) were categorized as informative markers according to Chaerani *et al*. (2011).

Heterozygosity

Heterozygosity is a comparison of heterozygous individuals in the population (Liu, 2001). Seven out of 8 microsatellite markers used in this study showed a high heterozygosity values (H), with an average value of 0.75. Of these, the markers SoyF3H, GMES1173, Satt288, Satt333, Satt100 and Satt125 exhibited the H value of 1. This finding indicates that alleles resulting from the analysis of these markers to soybean varieties tested in present study are 100% heterozygous alleles. GmF35H is among seven SSR markers which have a smaller value of H value (0.02), which is owned by only heterozygous alleles Rajabasa varieties.

The remaining SSR marker, Sat_286 has H value of zero implying that there was no heterozygous allele produced by genotyping of this marker on the soybean varieties. It was affirmed by Hairiansyah (2010) that homozygous locus gave rise only one band/allele per primer per individual. If the primers provided more than one allele then the loci is considered as heterozygous. Heterozygosity and homozygosity are crucial in plant selection avoiding disappearance genotypes with high levels of heterozygosity in which phenotypically is undetectable due to environmental factors.

The proportion of heterozygosity and degree of polymorphism at the level of individuals, populations and species are the two parameters that can be used to clarify the genetic diversity. The diversity will determine how the variations spread. This condition is related to the number of polymorphic loci that appear or the number of alleles at a locus. (Hairiansyah, 2010).

Major Allele Frequencies

Average frequency of major allele (dominant) found in this study was 45.28%, with the lowest value of 20% identified by marker Satt333 and the highest value of 86.67% identified by GmF35H. Despite, GmF35H showed the lowest values of both PIC and gene diversity among the other markers, it showed the highest value of major allele frequency. GmF35H is known as a marker that is connected with flower color trait in which the description of the varieties analyzed mention that flower color observed only purple and white (Ibarra *et al*., 2011). On the contrary, Satt333 provided the smallest value of dominant allele frequency (20%) and showed the largest values in both gene diversity and PIC. Since the Satt333 marker known as as functional SSR markers that tightly-linked with seed coat color trait, we have come to the conclusion that seed coat color genotypes have a more heterogeneous with a low level of dominant alleles.

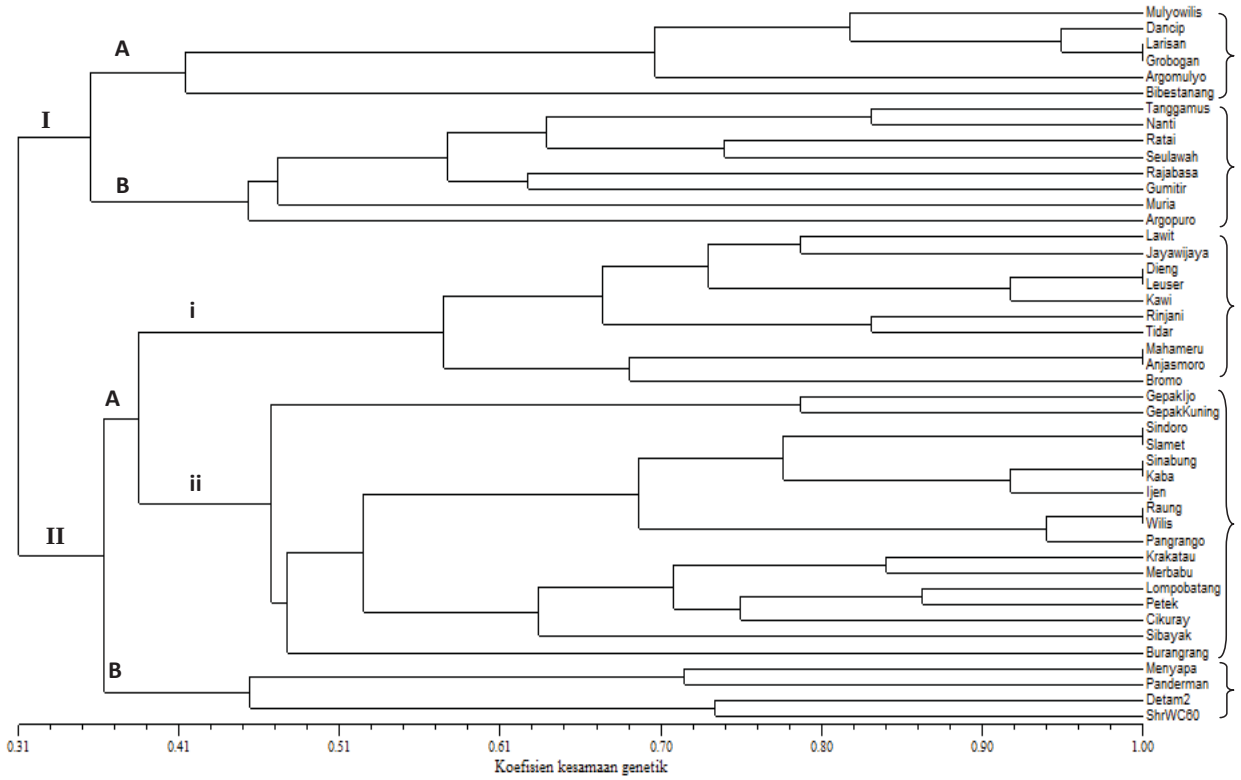

Figure 1. Dendrogram of forty-five soybean varieties derived from eight microsatellite markers

Cluster analysis

Analysis of the genetic profile using UPGMA analysis presented the 45 soybean varieties into two major clusters (I and II) at 0.31 similarity level (see Figure 1). Each cluster was further divided into two sub-clusters (A and B). Of these, cluster II A showed the highest density and consisted of 27 varieties, so it can be further divided into two sub-clusters (i and ii).

Grouping of varieties does not seem to be related to the morphological characteristics. As shown in dendrogram, several varieties which has white flower (Menyapa, Muria, Panderman and Argopuro) were clustered in two clusters. Of these, Muria and Argopuro were clearly grouped the cluster IB, and Menyapa and Panderman were in the cluster II B. In the case of the pubescence color, varieties which has white pubescence color (Mahameru, Anjasmoro, Argopuro, Bromo, Lompobatang, Bibestanang and Petek) spread over 4 different clusters and mixed with brown pubescence varieties.

In regards to growth type characters, soybean varieties which belong to semi-determinate types (Ratai, Lawit, Menyapa, Rinjani, Lompobatang and Merbabu) mingle with the determinate varieties. In relation to the seed coat color trait, the soybean varieties which morphologically showed black seed coat color (Cikuray and Detam 2) lies in the different cluster, namely cluster II A ii and II B for Cikuray and Detam2, respectively, with a similarity coefficient of 0.11. The absence of a clear grouping pattern showed by the functional markers used in this study reflecting that the functional marker can not be used to classify varieties based on morphologic characters. Nevertheless microsatellite analysis can describe phylogeny relationship between varieties.

In the dendrogram (Figure 1), 6 groups of soybean varieties have coefficient of

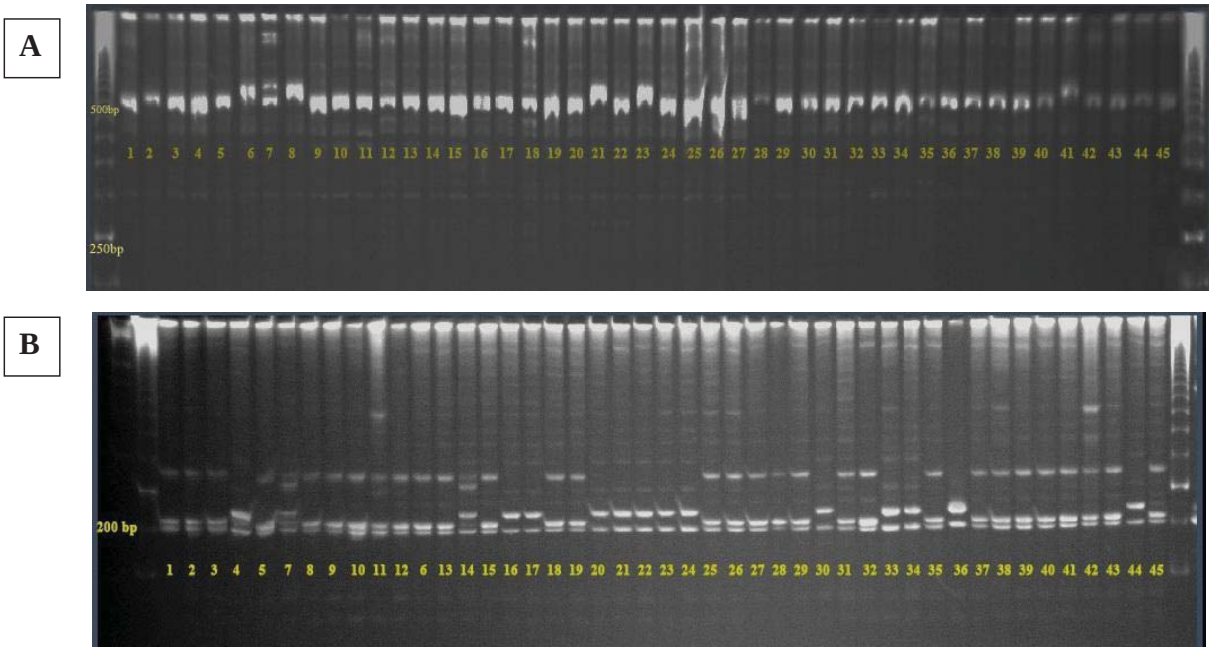

Figure 2. Polymorphism for SSR marker GmF35H (A), and GMES1173 (B). The amplified products were separated on 6.0% polyacrylamide gels. Numbers 1-45 refer to the list of varieties in Table 1.

similarity value of 1, such as the group of Larisan and Grobogan, Dieng and Leuser, Mahameru and Anjasmoro, Sindoro and Slamet, Sinabung and Kaba, and Raung and Willis. The level of similarity value of 1 revealed that those soybean varieties genotyped by microsatellite markers used in this study were genetically the same background at such loci. Therefore, to find the difference between the varieties, additional microsatellite markers genotyped to those varieties is required. By the increase of the number of SSR loci used in genotyping analysis, the effort to identify and prove whether the collection of soybean varieties especially those collected in PVP is clearly accpected as two different varieties or just the same varieties with two different names will be more easily. In order to identify the differences between varieties with higher validation, the increase of the number of markers used in the analysis should be considered. As performed by Bredemeijer *et al*. (2002) that used two additional markers to distinguish two varieties of tomato that originally could not be distinguished among 521 commercial varieties were analyzed using 20 microsatellite markers.

From tracking the plant description, varieties that have a similarity level 1 have close phylogeny relationship. Both Anjasmoro and Mahameru were derived from mass selection of pure strains MANSURIA population. Slamet and Sindoro have the same parents namely Dempo and Wilis. Sinabung and Kaba derived from double crosses 16 parents.

Mulyowilis varieties descended from crosses between Willis and Argomulyo, genetically showed a closer relationship with Argomulyo (male parent), with genetic similarity coefficient of 0.75. Dancip varieties was closer to the female parent (Burangrang) which showed the genetic similarity coefficient of 0.31, compared with its parental males (Slamet) which have a genetic similarity coefficient of 0.26. Sindoro and Slamet showed a close genetic resemblance to their male parent with a coefficient of 0.74. Shr W C 60 varieties also have male parent derived from Wilis, but the coefficient of genetic similarity between the two was only 0.27, as well as

Detam 2 (0.29), the other descendants of Wilis. In addition, Seulawah and Ratai were also a descendant of Wilis (female parent) and showed a genetic similarity coefficient of 0.47 and 0.46, respectively.

Taken together, we summarized that the analysis of forty-five soybean varieties collected in PVP using eight functional SSR markers showed a relatively narrow genetic diversity that indicated by the small number of genotypes and alleles detected, and the low value of both gene diversity and PIC (<0.75). Cluster analysis showed that the groups of soybean varieties were not related to morphological characters but they were related to phylogeny relationship between varieties. Nevertheless, for applications in the management of reference collections in PVP system, the results of microsatellite analysis in this study can be used primarily for genetic data input in a database. UPOV/INF/18/1 document recommends the use of molecular analysis for the purposes of managing the collection of plant varieties do not based approach to character by character, but seen from the genetic distance between varieties.

Acknowledgement

High appreciation conveyed to Dr. Muchlis Adie (Balitkabi) and Mr Sigit Prastowo S.Pt. for providing soybean seeds, as well as to the reviewers above constructive suggestions for the improvement of this paper.

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