Development of SSR Marker Set to Identify Fourty Two Indonesian Soybean Varieties
(Pengembangan Set Marka SSR untuk Identifikasi Empat Puluh Dua Varietas Unggul Kedelai Indonesia)

Andari Risliawati*, Eny I. Riyanti, Puji Lestari, Dwinita W. Utami, and Tiur S. Silitonga
Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Jl. Tentara Pelajar 3A, Bogor 16111 Indonesia
Phone (+62-251) 8337975; Fax. (+62-251) 8338820; *E-mail: boendar@yahoo.co.id
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ABSTRACT
Profile of molecular marker can be used for variety identification, genetic purity monitoring of germplasm and additional requirement in proposing intellectual property protection. DNA fingerprinting of soybean had been applied at the ICABIOGRAD-IAARD since 2004 using simple sequence repeat (SSR) markers which were run automatically by CEQ 8000 Genetic Analyzer platform based on capillary electrophoresis system. This method had produced unique DNA fingerprints of the varieties tested, but the marker set to efficiently identify the varieties had not yet been developed. This study aimed to develop a set of SSR markers as a tool to identify the Indonesian soybean varieties. Fourty two soybean varieties were analyzed using 14 random SSR markers. A total of 168 alleles that were obtained from the polymorphism analysis. The average of polymorphic information content (PIC) value observed was 0.7337 per SSR locus. Based on marker reproducibility rate, PIC value, number of rare alleles, frequency of dominant alleles, and percentage of SSR fragment detected by genetic analyzer, we identified five SSR markers i.e. Satt414, Satt147, Satt308, Satt009, and Satt516 as a SSR marker set to be used for soybean variety identification purposes. This marker set was used to develop the identity (ID) of the 42 Indonesian soybean varieties.

Keywords: DNA fingerprinting, marker set for identification, soybean, SSR.
INTRODUCTION

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 (Krisdiana, 2014; PPVT, 2015). In order to preserve the genetic diversity of the Indonesian soybean, the ICABIOGRAD genebank coordinated under the IAARD have conserved them together with other local varieties (BB Biogen, 2010). However, managing a large number of germplasm collections need a huge effort particularly in preventing germplasm from seed mixture and collection duplication.

It is well known that the use of molecular marker for marker assisted selection (MAS) have shortened time period in a breeding program and increased the precision of obtaining gene target. Meanwhile in germplasm research area, some studies showed that the application of molecular marker can generate phylogeny tree more precisely and the possibility to predict evolution existed among species or germplasm (Priolli et al., 2002; Vu et al., 2013). This advantage can be utilized to discard redundant collection and decrease management cost for germplasm conservation. Moreover, molecular marker can be used for variety identification as complementary tool for distinctness, uniformity and stability (DUS) tests in releasing a new variety (Gunjaca et al., 2008; Hudcovicova and Kraic, 2003; Narvel et al., 2000; Song et al., 2004).

SSR is a type of molecular markers that has been used widely in genetic diversity analysis of some important crops such as rice, soybean, tomato, potato, wheat, and sweet potato (Bredemeijer et al., 2002; Corbett et al., 2001; Luce et al., 2001; Plaschke et al., 1995; Zhang et al., 2000; Yoon et al., 2009). Therefore, since 2004 ICABIOGRAD has applied SSR markers for genetic diversity analysis of soybean (Chaerani et al., 2009; Santoso et al., 2006; Septiningsih et al., 2004). SSR marker is highly reproducible, codominant, able to detect high variance of allele polymorphism, only few amount of sample needed and is free from environmental influences (Agarwal et al., 2008). Therefore, SSR marker is considered a better marker in distinguishing genotypes having a close genetic relationship.

The use of SSR marker can be automated by using a genetic analyzer platform based on capillary electrophoresis system (Diwan and Cregan, 1997). The running time also can be shortened though multiplex panels by labelling the 5’ ends of the primers using different fluorescent colors (Chaerani et al., 2009). Another important feature of this platform is that it can read DNA fragments differences up to 1 base pair (bp). The accurate reading is necessary for variety identification since most of the release varieties were genetically close and is very difficult to be distinguished phenotypically. The flexibility of adjusting the multiplex panel also eases the user to add the marker number anytime in order to increase the reliability of the analysis. Practically, this method can benefit the Center of Crop Variety Protection personnel in assigning the uniqueness of a new variety. The DNA fingerprinting information can also equip phenotypic data from variety that is being registered for plant protection.

The aim of the study was to develop SSR marker set as a specific identification for the Indonesian soybean varieties. The marker set was developed based on genetic diversity and relationship analysis among varieties tested. The developed SSR marker set is expected to be able to assign specific profiles of soybean varieties. Furthermore this study is a part of barcode database development for Indonesian soybean varieties in term of completing the barcode from phenotypic data.

MATERIALS AND METHODS

Genetic Materials and SSR Markers

A total of 42 improved soybean varieties originated from Indonesia was used as the genetic materials in this study, which 23 of them had been used previously (Table 1). The soybean genomic DNA were amplified with 14 SSR markers those were selected based on the result of soybean genetic diversity analysis as previously reported (Santoso et al., 2006; Song et al., 1999). All markers were labelled using three different fluorescent colors (black, green, and blue) and run to five multiplex panels in a genetic analyzer platform (Table 2).

DNA Extraction and Amplification

Seeds of soybean varieties were planted in pots at ICABIOGRAD green house. Three to four weeks after planting the seeds, DNA of young leaves from a single vigorous plant from each variety was extracted based on basic protocol from Doyle laboratorium in miniprepcp scale with few modification using extraction buffer of CTAB. The FastStart program for PCR was carried out with condition as follows: 1 cycle of 94°C for 4 min, then 40 cycles of 95°C denaturation for 45 sec, 55–60°C annealing for 45 sec, and 72°C elongation for 30 sec, with a final extension cycle of 72°C for 5 min and incubation at 4°C.
Table 1. List of Indonesian soybean varieties used in the study.

| Variety name   | Year released | Pedigree                                                                 |
|----------------|---------------|---------------------------------------------------------------------------|
| Anjasmoro      | 2001          | Mass selection from Mansuria pure line                                    |
| Argo Mulyo     | 1998          | Introduction from Thailand (var. Nakhon Sawan I)                          |
| Baluran        | 2002          | AVRDC crossing                                                           |
| Burangrang     | 1999          | Natural segregant from Jember local variety                              |
| Cikuray        | 1992          | No 630 x No 1343 (Orba)                                                  |
| Davros         | 1965          | Lines selection from Garut local variety                                 |
| Dempo          | 1984          | Introduction from Colombia (var. Amerikana)                              |
| Delam 1        | 2008          | Introduction line 9637 x Kawi                                            |
| Delam 2        | 2006          | Introduction line 9637 x Willis                                          |
| Dieng          | 1991          | Manalagi x Orba                                                          |
| Galunggung     | 1981          | Davros (No 1248) x TK-5 (No. 1291)                                        |
| Gepak Ijo      | 2008          | Lines selection from Ponorogo local variety                              |
| Gepak Kuning   | 2008          | Lines selection from Ponorogo local variety                              |
| Grobogan       | 2006          | Lines selection of Malabar Grobogan local variety                        |
| Gumitir        | 2005          | Introduction from Taiwan (GC 86019-190-IN)                               |
| Guntur         | 1982          | TK-5 (Gm 26) x Genjah Slawi (Gm 14)                                      |
| Ijen           | 2003          | Backcross of Wilis x Himeshirazu                                        |
| Kaba           | 2001          | Diallel cross of 16 parents                                              |
| Kawi           | 1996          | Introduction line from Taiwan MSC 9050-C-7-2 (G 10050) x MSC 8306-1-M     |
| Kipas Putih    | 1992          | Lines selection of Aceh local variety                                    |
| Leuser         | 1998          | Pasuruan local var (MLG 2621) x mutant B-1682                             |
| Lokon          | 1982          | TK-5 (Gm 26) x Genjah Slawi (Gm 14)                                      |
| Lompobatang    | 1989          | Sinyonya x No 1682                                                      |
| Lumajang Bewok | 1989          | Lines selection of Lumajang local variety                                |
| Malabar        | 1992          | No 1592 x Wilis                                                          |
| Menyapa        | 2001          | B-3034 x Lampung local variety                                           |
| Mera           | 1938          | Lines selection of East Java local variety                              |
| Merbabu        | 1986          | Orba x Sinyonya                                                          |
| Orba           | 1974          | Davros x Shakti                                                         |
| Panderman      | 2003          | Introduction from Taiwan                                                 |
| Pangrango      | 1995          | Lampung local variety x Davros                                           |
| Petek          | 1989          | Lines selection of Kudus local variety                                   |
| Rajabasa       | 2004          | Mutant No 214 x 23-D (irradiation of Guntur var)                          |
| Ratai          | 2004          | Wilis x No. 3465                                                        |
| Raung          | 1986          | Darvos x Shakti                                                         |
| Rinjani        | 1989          | Shakti x No. 1682                                                       |
| Sindoro        | 1995          | Dempo x Wilis                                                           |
| Slamet         | 1995          | Dempo x Wilis                                                           |
| Tambora        | 1989          | Introduction from Philipines                                             |
| Tanggamus      | 2001          | Kerinci x No.3911                                                       |
| Tidar          | 1987          | Mutant selection of B 1682 from Taiwan                                   |
| Wilis          | 1995          | Orba x No. 1682                                                         |

*Source: Puslitbangtan, 2009; 2015; PPVT, 2015.

Table 2. Multiplex panel set of soybean SSR markers used in this study.

| Multiplex panel | SSR markers* | Fluorescent color | Chromosome | Repeat motifs | Allele size (bp) |
|-----------------|--------------|-------------------|------------|---------------|------------------|
| 1               | Satt131      | D4-blue           | 18         | (TAT)13       | 111–320          |
|                 | Satt516      | D3-green          | 13         | (TAA)19       | 131–280          |
| 2               | Satt009      | D2-black          | 3          | (AAAT)3(AAT)13| 161–250          |
|                 | Satt038      | D3-green          | 18         | (ATA)17       | 161–200          |
|                 | Satt114      | D4-blue           | 13         | (AAT)17       | 71–120           |
| 3               | Satt242      | D2-black          | 9          | (TTA)26       | 131–160          |
|                 | Satt177      | D3-green          | 8          | (ATT)16       | 101–120          |
|                 | Satt294      | D4-blue           | 4          | (TAT)23       | 251–300          |
| 4               | Satt147      | D2-black          | 1          | (ATA)15       | 100–179          |
|                 | Satt305      | D2-black          | 7          | (TTA)22       | 180–330          |
|                 | Satt414      | D4-blue           | 16         | (ATT)23       | 160–380          |
| 5               | Satt191      | D4-blue           | 18         | (TAT)19       | 191–240          |
|                 | Satt534      | D3-green          | 14         | (TAT)30       | 101–230          |
|                 | Satt373      | D4-blue           | 19         | (TAT)21       | 151–170          |

*Soybean primers sequence can be accessed at http://soybase.org/resources/ssr.php.
Detection of SSR Fragment with CEQ8000 Genetic Analyzer

Procedure for sample preparation and running process in CEQ8000 Genetic Analyzer followed basic protocol of Thomson (Thomson, 2004). PCR products were diluted with sample loading solution (SLS) at ratio of 1:6 (v/v). For each multiplex panel set, the diluted PCR products from three different fluorescent SSR markers were loaded in the same well of CEQ sample plate. SLS liquid and 0.5 µl of CEQ internal standard size (400 bp) were added to the well until the volume reached 40 µl. To prevent the evaporation of sample-mixed during the fragment separation by CEQ8000, one drop of mineral oil was added to each well. In another plate (CEQ buffer plate), CEQ buffer was added up to three-quarters part of the well. Afterward, both plates were placed into CEQ8000 Genetic Analyzer platform and Frag-1 program was run with following condition: capillary temperature of 35°C, injection at 2.0 kV for 30 sec, denaturation at 90°C for 120 sec, and separation at 7.5 kV for 35 min. After 12 hours, DNA fragments can be seen in the monitor screen as peaks with different fluorescent color.

Data Analysis, Marker Set Development, and Varieties Coding

Allele sizes of each DNA fragment obtained from CEQ8000 Genetic Analyzer were analyzed by binning analysis using CEQ Fragment Analysis Software (Thomson, 2004). Binning is grouping the DNA fragments based on the number of repeat motifs of di-, tri-, or tetra- nucleotide repeats of SSR markers. Binning data then was analyzed by PowerMarker v3.25 program to obtain the genetic information and polymorphism rate of SSR markers, which can be accessed at http://statgen.ncsu.edu/powermarker/index.html (Liu and Muse, 2005). This information was used as selection criteria for SSR marker set candidate for variety identification.

Afterward we determined the number of groups and the group range of each SSR marker. The number of group was based on the repeat number of SSR motifs. As an example for Satt414 which has SSR motif of “(ATT)23”, we assigned 23 group for this locus. Meanwhile, group range represented the range of allele size in each group and was determined based on following standard grouping formula (https://www.wyzant.com/).

\[
\text{Group range} = \frac{\text{Maximum allele size} - \text{Minimum allele size}}{\text{The number of repeat motif of SSR marker}}
\]

The last step was to transform the allele size of the varieties into appropriate group code number which set in two numeric codes started from “00”. The transformation was done to all SSR marker set candidates and the final ID of a variety is a combination of two numeric codes from all SSR marker set candidates. Validation of possibly duplicated code or ID was conformed through cluster analysis using SPSS 21.0 statistical software.

RESULTS AND DISCUSSION

Polymorphism Analysis and SSR Characterization

Based on SSR fragment generated from genetic analyzer platform, each SSR marker from the multiplex panel resulted a variety of amplicons (Figure 1). Fluorescent signal from each marker was shown as different peak color and size that represented DNA fragment from each variety. Based on a general terminology of SSR marker, one marker represents one locus and fragments from a marker represent alleles within the locus (Agarwal et al., 2008). Therefore different sizes of peaks from a marker show polymorphism among genotypes that are being tested. This allele size can be assigned as a specific DNA fingerprinting band of the varieties and can be used as variety identification.

The characteristics of 14 SSR markers from allele calling and binning analysis of the 42 soybean varieties are shown in Table 3. Eleven markers among of them had fragments detection more than 85%. This can be used as an initial criterion to determine SSR marker candidate for variety identification. Total detected alleles observed from this study were 168 alleles with a range of 4–25 alleles per SSR locus. This was higher than the previous study that detected only 115 alleles with a range of 7–19 alleles per SSR locus (Santoso et al., 2006). The more diverse varieties tested, the higher polymorphism rate of the markers would be obtained.

Other criteria that can be used to select SSR marker candidate for variety identification are frequency of rare allele and PIC value. Rare allele is the allele whose presence less than 5% within a population. We identified three SSR markers that did not have rare alleles, i.e. Satt177, Satt242, and Satt373. These markers could not be used for variety identification since they will not be able to distinguish tested varieties (Karp et al., 1997).

In this study, the PIC value represents high variations of alleles. From 14 SSR markers tested four markers demonstrated high PIC value of more than 0.8, i.e. Satt131, Satt147, Satt414, and Satt516. As previously reported, molecular marker applied in fingerprinting system must reflect the genotype information, having high ability to discriminate...
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The development of SSR marker set for variety identification aims to maximize the ability in distinguishing varieties with a number of markers within the set as minimum as possible (Jones et al., 2010). Therefore some factors have to be considered when selecting marker set composition for variety identification. Some approaches were applied by other researchers abroad such as linear integer (Gale et al., 2005), multivariate (Song et al., 1999) and genetic algorithm (Jones et al., 2010).

In our study we considered some factors in developing the marker set. This includes: (1) criteria to select candidate for marker set, (2) method to assign ID for genotypes tested, and (3) method to validate the chosen marker set. In selecting a marker candidate for variety identification, we used some criteria such as detection rate of the SSR fragment, the presence of rare allele, and PIC value. Reproducibility of SSR markers can be considered as well, as a criterion to

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**Table 3. Allele characteristics of 14 SSR markers used in DNA fingerprinting studies of 42 Indonesian soybean varieties.**

| SSR Markers | Chromosome | Fragment detection rate* (%) | Number of alleles detected | Frequency of dominant allele** (%) | Number of rare alleles detected | PIC value | Marker reproducibility*** |
|-------------|------------|------------------------------|---------------------------|-----------------------------------|--------------------------------|-----------|--------------------------|
| Satt009     | 3          | 100.00                       | 12                        | 35.71                             | 9                              | 0.7817    | 3 times                  |
| Satt038     | 18         | 85.71                        | 7                         | 36.11                             | 2                              | 0.7277    | 3 times                  |
| Satt114     | 13         | 100.00                       | 8                         | 59.52                             | 3                              | 0.5794    | 3 times                  |
| Satt131     | 18         | 100.00                       | 25                        | 9.52                              | 18                             | 0.9466    | once                     |
| Satt147     | 1          | 80.95                        | 17                        | 17.65                             | 12                             | 0.9089    | 3 times                  |
| Satt177     | 8          | 100.00                       | 4                         | 39.29                             | 0                              | 0.6589    | 3 times                  |
| Satt191     | 18         | 95.24                        | 13                        | 62.50                             | 11                             | 0.5819    | once                     |
| Satt242     | 9          | 100.00                       | 5                         | 46.43                             | 0                              | 0.6728    | 3 times                  |
| Satt294     | 4          | 100.00                       | 11                        | 53.33                             | 5                              | 0.6195    | 2 times                  |
| Satt308     | 7          | 78.57                        | 12                        | 39.39                             | 9                              | 0.7856    | 3 times                  |
| Satt373     | 19         | 100.00                       | 6                         | 44.05                             | 0                              | 0.6411    | once                     |
| Satt414     | 16         | 85.71                        | 22                        | 36.11                             | 17                             | 0.8354    | 3 times                  |
| Satt516     | 13         | 83.33                        | 18                        | 18.57                             | 12                             | 0.8848    | once                     |
| Satt534     | 14         | 100.00                       | 8                         | 51.19                             | 3                              | 0.6480    | once                     |

*The percentage of successful detection of DNA fragment from 42 varieties by genetic analyzer platform; **the frequency of dominant allele throughout 42 varieties that are being tested; ***the number of repeated assays of using the same SSR locus in DNA fingerprinting analysis.
select a marker candidate for variety identification. From 14 SSR markers tested in this study, nine markers have been used 2–3 times in 2004, 2008, and 2011 (Table 3). The reproducibility means the use of same markers in different year of DNA fingerprinting analysis. Therefore considering those criteria, we selected four SSR markers as first candidates for variety identification, i.e Satt414, Satt147, Satt308, and Satt009.

Following step was determining the method for assigning variety ID that could be either original allele size-based or coding-based methods. Original allele size-based method can be performed qualitatively through DNA band image from gel electrophoresis and quantitatively in the form of allele size generated from genetic analyzer platform. The disadvantage of this method is difficulties in distinguishing alleles those have 1–3 bases differences. Whereas, coding-based method means the original allele size will be coded in another numeric value. To do so, series of groups have to be developed to distinguish alleles among the varieties and group range is calculated using a specific formula.

We determined the number of group for each SSR marker candidates based on their SSR motif repeat number. We calculated group range based on the formula in the materials and methods. This represented the allele size range within each group of each SSR marker candidates (Table 4). Afterward based on this code table, we transformed the allele size into codes and combine codes from all SSR marker candidates as the final ID of the varieties. As an example, Argomulyo variety had allele sizes of 296, 146, 190, and 213 for locus of Satt414, Satt147, Satt308, and Satt009, respectively. Based on the code table, this allele would be coded as 14, 09, 04, and 10 for Satt414, Satt147, Satt308, and Satt009 loci respectively. Therefore, the variety ID for Argomulyo would be "14090410". The same procedure then was applied to other varieties until all of them had specific ID. For those that did not successfully amplified in a particular marker, would be assigned as "**".

Afterward we checked the possible variety ID duplication among varieties tested through clustering analysis. We found that two soybean varieties had same ID, i.e. Rinjani and Slamet (Figure 2). Therefore we added another SSR marker into the marker set to distinguish these two varieties. We checked their allele sizes in the remaining SSR markers that have not been used in the set. We found only one marker (Satt516) that can discriminate these two varieties. We then applied the same coding procedure for Satt516 to all varieties and added two numeric codes of Satt516 in the last position of variety ID that were being assigned previously. Therefore the final marker set for soybean varieties identification consisted of five markers (Satt414, Satt147, Satt308, Satt009, and Satt516) and variety ID was comprised of 10 numeric codes (Table 5).

Assigning ID or numeric code for a variety using a marker set is an effort to give a specific identity that

| Code | Satt414 | Satt147 | Satt308 | Satt009 | Satt516 |
|------|---------|---------|---------|---------|---------|
| 00   | 166–174 | 100–104 | 160–166 | 171–174 | 141–147 |
| 01   | 175–183 | 105–109 | 167–173 | 175–178 | 148–154 |
| 02   | 184–192 | 110–114 | 174–180 | 179–182 | 155–161 |
| 03   | 193–201 | 115–119 | 181–187 | 183–186 | 162–168 |
| 04   | 202–210 | 120–124 | 188–194 | 187–190 | 169–175 |
| 05   | 211–219 | 125–129 | 195–201 | 191–194 | 176–182 |
| 06   | 220–228 | 130–134 | 202–208 | 195–198 | 183–189 |
| 07   | 229–237 | 135–139 | 209–215 | 199–202 | 190–196 |
| 08   | 238–246 | 140–144 | 216–222 | 203–206 | 197–203 |
| 09   | 247–255 | 145–149 | 223–229 | 207–210 | 204–210 |
| 10   | 256–264 | 150–154 | 230–236 | 211–214 | 211–217 |
| 11   | 265–273 | 155–159 | 237–243 | 215–218 | 218–224 |
| 12   | 274–282 | 160–164 | 244–250 | 219–222 | 225–231 |
| 13   | 283–289 | 165–169 | 251–257 | 223–226 | 232–238 |
| 14   | 292–300 | 170–174 | 258–264 | 227–230 | 239–245 |
| 15   | 301–309 | 175–179 | 265–271 | 231–234 | 246–252 |
| 16   | 310–318 | 180–186 | 272–278 | 235–238 | 253–259 |
| 17   | 319–327 | 185–191 | 279–285 | 240–242 | 260–266 |
| 18   | 328–336 | 190–196 | 286–292 | 247–251 | 267–273 |
| 19   | 337–345 | 195–201 | 293–299 | 254–258 | 274–280 |
| 20   | 346–354 | 200–206 | 300–306 | 261–265 | 281–287 |
| 21   | 355–363 | 205–211 | 307–313 | 268–272 | 288–294 |
| 22   | 364–372 | 210–216 | 314–320 | 275–280 | 295–301 |
| 23   | 373–381 | 215–221 | 321–327 | 282–288 | 302–308 |
|      |         |         |         |         | Additional**DNA fragment was not successfully read by genetic analyzer. |
can quantitatively differentiate them from others. It gives benefit in variety protection and breeder right, complement tools for DUS testing, and seed purity testing. In this study, 42 soybean varieties had unique profile ID and can be distinguished each other (Table 5). Two closest variety for example Rinjani and Slamet had ID of "1406040116" and "1406040117", respect-
The allele uniqueness of molecular marker for identification has been given specific identity of elite variety in some species. As reported by other researchers, 13 SSR loci were able to differentiate 66 American soybean varieties, 4 SSR markers differentiated 66 apple cultivars, 12 SSR markers differentiated 48 wheat cultivars, and 6 SSR markers differentiated 400 potato varieties (Galli et al., 2005; Reid and Kerr, 2007; Song et al., 1999). In Japan, identification marker consisted of random amplified polymorphic DNA (RAPD) was applied to protect Koshihikari rice from commercial seed forgery (Ohtsubo and Nakamura, 2007). Several japonica and indica rice have been identified as well using identification marker related to flavored rice quality trait (Lestari et al., 2009; 2012).

Therefore assigning an ID using a marker set can be implemented for releasing superior varieties in Indonesia. It can be applied as well for protecting local indigenous varieties of some crops. Marker composition within the marker set should be flexible in line with variety addition or alteration. However, method and technique used in developing the marker set should be consistent.

**CONCLUSIONS**

According to polymorphism and clustering analysis, a marker set for identification of Indonesian soybean varieties was successfully developed. This marker set comprised of five SSR markers (Satt 414, Satt147, Satt308, Satt009, and Satt516) and can differentiate 42 improved varieties of the Indonesian soybean. 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.

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