Genetic diversity of Indonesian soybean (Glycine max L. Merrill) germplasm based on morphological and microsatellite markers

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Abstract. Genetic diversity on soybean germplasm will determine the success of the soybean breeding program. The purpose of this research was to study the diversity of Indonesian soybean (Glycine max L. Merrill) germplasm based on agronomic traits and microsatellite markers in order to know the genetic relationship among the accessions. Genetic material used was 45 soybean accessions consisting of 14 genotypes introduced from nine countries, 26 landrace from 10 provinces in Indonesia, and five Indonesian high yield varieties. Morphological characterization performed on nine qualitative and four quantitative traits. The genetic relationships were estimated using three SSR markers. The results showed that cluster analysis based on agronomic traits clearly separate the soybean accessions of black seeds into one group with genetic distance range from 0.61 to 1. Other groups for accessions with yellow, green-yellowish and brown seed have genetic distance ranging from 0.46 to 1. Based on microsatellite markers, three primers showed polymorphism banding pattern. The number of alleles that are formed these three primers between four and five alleles with sizes ranged from 200 bp to 800 bp. Cluster analysis based on microsatellites markers showed that the genetic distance of 45 accessions ranged from 0.61 to 1.

Keyword: Cluster analysis, genetic distance, similarity.

1. Introduction
Soybean (Glycine max L. Merrill) is one of the most important food crops in Indonesia, which ranks third after rice (Oryza sativa L.) and maize (Zea mays L.). Until 2016, the Indonesian government has released 85 soybean varieties [1]. Compared to rice and maize varieties (363 and 203 varieties, respectively), the number of soybean varieties in Indonesia is relatively small. This illustrates the slow increase in the number of soybean varieties that are assembled by Indonesian soybean breeders. Therefore, soybean breeding programs in Indonesia are still needed to increase the number of soybean varieties with the desired characteristics of soybean farmers.

Soybean breeding program to create new varieties require a population base with wide genetic diversity. According to Allard [2] genetic diversity in the population will determine the success of
plant breeding programs. One way that can be taken to increase the genetic diversity is by collecting germplasm. Until 2016, the number of soybean germplasm that have been collected by Indonesian Legumes and Tuber Crops Research Institute (ILETRI) has reached 660 accessions. Most of the germplasm collections were acquired through exploration in all provinces in Indonesia, and small portion obtained through the introduction from other countries.

Compared with Asian countries, the numbers of soybean accessions in Indonesia is relatively small. The collection of soybean germplasm in China totaled 6,172 accessions of annual wild soybean [3], and 22,637 accessions of cultivated soybean [4]. In Korea, no less than 7,000 accessions of soybean Korean landrace had been conserved at the Rural Development Administration (RDA) Genebank [5]. Meanwhile in Japan, soybean germplasm conserved at the National Institute of Agrobiological Sciences (NIAS) Genebank which has a total of germplasm approximately 11,300 accessions [6]. Agarwal et al. [7] reported that soybean germplasm collections in India amounted to 4,248 of cultivated soybean accessions and 36 of wild soybean accessions.

Information on the genetic distance among accessions can assist plant breeders in determining germplasm that will be used as parent in hybridization. The farther of genetic distance between the two parent that used in hybridization, then the greater of opportunity to obtain high genetic diversity in the offspring. Thus, the selection of desirable traits will become easier. Germplasm collection from different geographic origin is expected to have a difference in the genotypic and phenotypic. Therefore, genetic distance information needs to be known through morphological and molecular characterization. The objectives of the present study were to study the diversity of Indonesian soybean germplasm based on agronomics trait and microsatellite markers and to study the genetic relatedness among the soybean accessions.

2. Materials and methods

2.1. Plant genetic materials
A total of 40 accessions of soybean used to study genetic relationship of soybean germplasm collections of ILETRI. The 40 soybean accessions used consisted of 14 accessions introduced from nine countries (USA, Morocco, The Philippines, Taiwan, Venezuela, Japan, Mexico, Australia and Thailand) and 26 landrace accessions from 10 provinces in Indonesia. Total five soybean varieties used as a check, namely Grobogan, Wilis, Detam 1, Anjasmor, and Argomulyo.

2.2. Morphological characterization
This research was conducted at Kendalpayak Experimental Station in Malang District, Indonesia. Each accession were planted on plots measuring width of 2 m and length of 3 m, and laid out as augmented design. Planting space used was 40 cm between rows and 15 cm within rows, two plants per hole. Fertilize was given at the planting time with a dose according to the fertilizer recommendation, i.e. 50 kg ha⁻¹ Urea, 100 kg ha⁻¹ SP36, and 100 kg ha⁻¹ KCl. Irrigation was done four times, i.e. at planting time, on 3 wk after planting (WAP), during flowering and pods filling. Pest and disease control carried out intensively, starting from one wap until the pods are ready to be harvested. Weeding was done at three and six WAP.
Characterization on 13 agronomic traits, consists of nine qualitative traits and four quantitative traits, was conducted by following the guide books of soybean characterization [8]. For the purpose of data analysis, the observation of agronomic traits was given a score between 1 and 9. The scores are used shown in table 1.

2.3. Microsatellite characterization
Each genotype was planted with 25 cm × 20 cm × 5 cm plastic polybag. The planting medium used a mixture of soil, sand and manure in a ratio of 1:1:1. The leaves of soybeans will be extraction about 3 wk after planting. Procedures of DNA isolation were modified by Doyle and Doyle [in 9]. Whereas for testing the quantity of DNA was measured using a spectrophotometer nanodrop. Isolated DNA
(1 µL) was put into a nanodrop spectrophotometer and absorbance was measured at a wavelength of 230 nm, 260 nm and 280 nm, 1 µL of distilled water was used as blanco.

| No. | Agronomic traits | Score         | No. | Agronomic traits | Score         |
|-----|------------------|---------------|-----|------------------|---------------|
| 1   | Hypocotyl color  | 1 (green)     | 7   | Branche trichome| 1 (white)     |
|     |                  | 2 (purple)    |     |                  | 2 (light brown)|
| 2   | Flower color     | 1 (white)     | 8   | Seed color       | 1 (light yellow)|
|     |                  | 2 (purple)    |     |                  | 2 (yellow)    |
| 3   | Leaf shape       | 1 (lanceolate)|     |                  |               |
|     |                  | 2 (triangular)|     |                  |               |
|     |                  | 3 (pointed ovate)| | |               |
|     |                  | 4 (rounded ovate)| | |               |
| 4   | Leaf texture     | 1 (smooth)    | 9   | Hilum color      | 1 (white)     |
|     |                  | 2 (slightly smooth)| | | 2 (yellow)    |
|     |                  | 3 (rough)     |     |                  | 3 (light brown)|
|     |                  | 4 (very rough)|     |                  | 4 (dark brown) |
| 5   | Growth type      | 1 (determinate)|     |                  | 5 (nearly black)|
|     |                  | 2 (semi-determinate)| | | 6 (black)    |
|     |                  | 3 (semi-indeterminate)| | |               |
|     |                  | 4 (indeterminate)| | |               |
| 6   | Pod color        | 1 (brown yellowish) |     |                  |               |
|     |                  | 2 (brown)     |     |                  |               |
|     |                  | 3 (dark brown) |     |                  |               |

Quantitative characteristics

| No. | Time of 50 % flowering | Score | No. | Plant height* | Score |
|-----|-------------------------|-------|-----|---------------|-------|
| 1   | (early, ≤ 36 d)        | 1     | 3   | (very short, < 15 cm) | 1     |
|     | (medium, 37 d to 39 d) | 2     |     | (short, 15 cm to 50 cm) | 2     |
|     | (late, ≥ 40 d)         | 3     |     | (medium, 51 cm to 68 cm) | 3     |
| 2   | (very early, < 70 d)   | 4     |     | (tall, 69 cm to 86 cm) | 4     |
|     | (early, 70 d to 80 d)  | 5     |     | (very tall, > 86 cm) | 5     |
|     | (medium, 81 d to 85 d) | 6     |     | (small, < 10 g per 100 seeds) | 6     |
|     | (late, 86 d to 90 d)   | 7     |     | (medium, 10 g to 14 g per 100 seeds) | 7     |
|     | (very late, > 90 d)    | 8     |     | (large, ≥ 14 g per 100 seeds) | 8     |

Source: [8], *[10].

DNA quality testing is done by electrophoresis techniques from DNA isolation. Addition of loading dye as dye and Ethidium Bromide (EtBr) will bind DNA so that DNA migration can be detected with ultraviolet light. The results of DNA amplification with PCR can be determined by doing agarose gel electrophoresis. Electrophoresis gels was then soaked in a solution of ethidium bromide for 15 min to 25 min and then visualized on an UV transluminator and photographed with a Polaroid camera or digital camera.

DNA amplification was carried out in Gene Cycler with the SSR method to obtain the banding pattern resulting from amplification with SSR primers as shown in table 2. DNA amplification by PCR-SSR was carried out as many as 35 cycles at denaturation stage (50 °C for 40 s), annealing (50 °C for 40 s), and extension (72 °C for 1 min) and final extension (72 °C for 5 min) in DNA samples by mixing 1 µL forward SSR primer, 1 µL SSR reverse primer, 8.5 µL ddH2O, 12.5 µL Green kit PCR, and 2 µL sample of soybean plant DNA. The results of amplification were then visualized by electrophoresis using agarose gel. Electrophoresis gel was then soaked in a solution of ethidium
bromide for 15 min to 25 min then visualized on UV transluminator and photographed. The results of amplification were measured based on DNA markers (100 bp DNA ladder). DNA bands with the same migration rate are assumed as homologous loci. Analysis of the polymorphism between genotypes is done with a score or not the ribbon formed. Amplified fragments of different sizes were considered as different alleles. The DNA bands that were amplified by a given primer were scored as present (1) or absent (0) for all the samples.

Table 2. The name oligonucleotides and sequences of SSR primers used in this research.

| Oligonucleotides name | Forward primer sequence (5'-3') |
|-----------------------|---------------------------------|
| AT36                  | GCGGACAGTGTGCTC ATA TAA TAG     |
| ATT21                 | GCGAATATAGCCAAATTTAGGTTG AATGACA |
| ATT20                 | GCGGAATAATTGTTATTGTGAGAC         |

2.4. Data analysis

Cluster analysis using Minitab software is carried out on score data based on agronomic traits. Meanwhile, the grouping of DNA characters was analyzed using the Unweighted Pair Group Method Arithmetic (UPGMA) method through version 2.1 of the Numerical Taxonomy and Multivariate System (NTSYS) program [11].

3. Result and discussion

3.1. Morphological characterization

The results from observation of nine qualitative characters and four quantitative characters showed that there are differences in morphological characters among soybean germplasm tested. As many as 37 of the 40 accessions tested had purple hypocotyls, while three other accessions had green hypocotyls. In line with the color of hypocotyls, as many as 37 accessions have purple flowers and only three accessions have white flowers. Most of the soybean germplasm tested generally have pointed ovated leaves (31 accessions), with rough leaf surface texture (30 accessions). In this study, only two types of growth were observed, namely determinate (29 accessions) and semi-determinate (11 accessions). The diversity in the color of stem hair observed was brown stem hair (32 accessions) and white stem hair (eight accessions). In the character of ripe pods, 32 of them have brown pods. In the character of the seed color, the diversity among the 40 soybean accessions tested is increasingly visible. In this study, there were five observed seed colors, namely yellow (22 accessions), greenish yellow (one accession), yellowish green (six accessions), black (nine accessions), and brown (two accessions). Meanwhile, the diversity of hilum color in this study is soybean accession with hilum in brown (29 accessions), yellow (two accessions) and black (nine accessions).

Observations on plant height showed that soybean accession tested had a diversity of plant heights between 42.4 cm and 104.8 cm. The diversity was also found at flowering time, which was between 35 d and 45 d after planting (DAP), while the diversity of pod maturity varied between 70 DAP and 108 DAP. In the character of seed size shown by the weight of 100 seeds, the diversity among 40 soybean accessions ranged from 5.95 g to 12.88 g per 100 seeds.

By using the scores described in table 1, the genetic distance of 45 soybean genotypes can be seen in figure 1. In this study, the results of cluster analysis were able to classify black-seeded soybean accessions into one cluster. Nine accessions of black-seeded (MLGG 0019, MLGG 0388, MLGG 0476, MLGG 0489, MLGG 0610, MLGG 0638, MLGG 0653, MLGG 0169 and MLGG 0591) and Detam 1 variety clustered in one group with similarity coefficient from 0.61 to 1.00. Meanwhile, the other cluster groups consisted of soybean accessions with yellow, green-yellowish, and brown seed with genetic distance ranging from 0.46 to 1.

The difference between one genotype with other genotypes can be seen from the difference in phenotypic expression of each genotype. Morphological characters have been quite effective as a differentiator among genotypes in soybean germplasm collection. The geographical distribution and genetic diversity of soybean Chinese germplasm collections, both wild and cultivated soybean, have
been successfully performed using agronomic characters by [3, 4, 12] reported that 22 agronomic traits were used have succeeded in separating 18 soybean genotypes based on their type of growth. However, [13] found that the genetic relationship among genotypes using agro-morphological data is not always in line with the genetic closeness based on their pedigree. This occurs because of the soybean genotypes, especially soybean varieties that exist today, are generally obtained through conventional breeding by hybridization using parents with narrow genetic backgrounds. Therefore, other data are still needed to study the phylogenetic relationship among soybean germplasm collections, one of which is the molecular characterization obtained through DNA analysis. DNA markers can also be used as a tool in the selection, thus increasing the efficiency and effectiveness of conventional breeding [14].

![Figure 1](image_url)

Figure 1. The genetic relationship among 45 soybean genotypes based on agronomic traits.
3.2. Microsatellite characterization

Testing to 40 accessions and five check varieties showed the banding pattern of the amplification results using SSR primers. The three primers were selected, namely primary 1 (AT36), primary 3 (ATT21) and primary 6 (ATT20). These three primers showed polymorphism quite clearly so that they can distinguish among accessions (figure 2, figure 3 and figure 4). The use of molecular markers (AFLP and SSR) in soybeans for genetic diversity studies is more appropriate than RAPD. This has been proven by [15] to detect genetic diversity in 50 soybean genotypes in China. In this study, the various sizes of PCR fragment of alleles that appeared on the accessions tested ranged from 200 bp to 300 bp (base pair) on primer 1 (figure 2), while in primer 3 showed 300 bp, 325 bp and 800 bp (figure 3) and in primer 6 ranged from 275 bp to 300 bp (figure 4). As many as twenty accessions tested have five alleles and 15 alleles having four alleles. This information is needed to detect the existence of genetic relationship. Tantasawat et al. [16] reported genetic diversity and genetic relationships of 25 soybean accessions using SSR with 11 primers, the results showed a coefficient of genetic similarity between 0.73 to 1.00 with an average of 0.88.

![Figure 2](image1)

**Figure 2.** Representative gel photograph of 45 soybean genotypes using primer 1 (AT36).

One of the molecular markers that can be used to detect similarities and differences among germplasm collection is microsatellite markers or SSR (simple sequence repeats). These DNA markers have characteristics as co-dominant markers with the sequences repeated only 1 bp to 5 bp. In addition, the number of repeating units of base pairs and the repetition patterns are different for each genotype that makes it unique to each genotype. Santoso et al. [17] added that the main advantages of microsatellite markers is the ability to read DNA fragment more accurately with a precision of up to 1 bp. An et al. [15] reported that SSR markers are more suitable than RAPD markers in order to study the genetic diversity in soybean. Tantasawat et al. [16] found that the SSR markers showed clear polymorphism and recommended for use in detecting genetic diversity and phylogenetic relationship.
study on soybean. In order to protect soybean varieties, the results of molecular characterization using SSR markers can be used as an identifier of soybean varieties [18–20].

**Figure 3.** Representative gel photograph of 45 soybean genotypes using primer 3 (ATT21).

**Figure 4.** Representative gel photograph of 45 soybean genotypes using primer 6 (ATT20).
The genetic relationship based on SSR markers are showed on figure 5. Based on the three primers were used, then 45 genotypes (including five check varieties) were observed to be one group at coefficient of genetic similarity 0.60. The higher the genetic similarity coefficient between the two genotypes, the closer the genetic distance is. On the coefficient of 0.90, 45 soybean genotypes are divided into nine groups (Table 3). The results of cluster analysis showed that most of the accessions (15 accessions) clustered in one group, namely group 5 (table 3). Check varieties were generally not in one group, except for Detam 1 and Argomulyo varieties grouped in the same group. Two accessions were separate from others accessions, namely MLGG 0638 which is introduced from Australia and MLGG 0610 from West Kalimantan. In this study, the three primers used have not been able to separate soybean accessions based on their origin. This finding is in line with [21] research who found that 50 genotypes tested could be grouped into 10 groups, but the grouping was not related to the variety group or the origin of the location.

**Figure 5.** The genetic relationship among 45 soybean genotypes based on SSR markers.

Information about genetic relationship based on microsatellites markers can be used as a consideration determining the parents of hand-crossing pollination. The further the genetic distance between the two parents, the greater the chance to get a good hybrid. SSR markers have been widely applied in the genetic diversity of the soybean germplasm [22, 23] stated that by using SSR markers a breeder can choose two or more parents with high genetic distances. Chauhan et al. [24] added that SSR markers used effectively to detect genetic diversity in soybean cultivars.
Table 3. Clustering on coefficient similarity 0.90 baesd on SSR markers.

| Cluster | Genotype and its origin |
|---------|-------------------------|
| 1       | Grobogan, MLGG 0121 (East Java), MLGG 0393 (East Java), MLGG 0890 (North Sulawesi), MLGG 0603 (West Java), MLGG 0624 (The Philippines). |
| 2       | Wilis, MLGG 0113 (Taiwan), MLGG 0873 (Bali). |
| 3       | MLGG 0123 (Lampung), MLGG 0124 (Lampung), MLGG 0128 (Nangro Aceh Darussalam), MLGG 0614 (Mexico), MLGG 0400 (South Sulawesi), MLGG 0465 (Central Java), MLGG 0613 (Mexico). |
| 4       | Detam 1 and Argomulyo |
| 5       | MLGG 0104 (Central Java), MLGG 0598 (Japan), MLGG 0592 (Bali), MLGG 0489 (DIY), MLGG 0109 (Morocco), MLGG 0112 (The Philippines), MLGG 0392 (East Java), MLGG 0269 (East Java), MLGG 0857 (West Nusa Tenggara), MLGG 0856 (West Nusa Tenggara), MLGG 0372 (East Java), MLGG 0597 (Japan), MLGG 0591 (Venezuela), MLGG 0115 (USA), MLGG 0657 (Thailand). |
| 6       | Anjasmoro (Cec variety), MLGG 0160 (East Java), MLGG 0169 (East Java), MLGG 0233 (Taiwan), MLGG 0482 (Central Java). |
| 7       | MLGG 019 (East Java), MLGG 0653 (DIY), MLGG 0103 (USA). |
| 8       | MLGG 0388 (East Java) dan MLGG 0476 (Central Java). |
| 9       | MLGG 0638 (Australia). |
| 10      | MLGG 0610 (West Kalimantan). |

4. Conclusion

Based on the results of this study, it can be concluded that there is genetic diversity among Indonesian soybean germplasm. Genetic distance based on morphological characters ranged from 0.46 to 1.00, while based on microsatellite markers ranged from 0.60 to 1.00.

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