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# THE POTENTIAL USE OF SSR MARKERS TO SUPPORT THE MORPHOLOGICAL IDENTIFICATION OF INDONESIAN MUNGBEAN VARIETIES

## *Potensi Penggunaan Marka SSR dalam Mendukung Identifikasi Morfologi Varietas Kacang Hijau Indonesia*

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## ABSTRACT

Mungbean varieties were mainly characterized based on morphological traits. Molecular genetic approach is expected to help the breeder in identification of mungbean varieties in more detail and to protect intellectual property right. This study aimed to identify Indonesian mungbean varieties based on DNA fingerprint profile using a marker set to support morphological characters. A total of 22 Indonesian mungbean accessions were characterized based on 21 morphological traits and 55 simple sequence repeats (SSRs) primers. Of the total 22 mungbean varieties used in the present study, 16 varieties were improved varieties and remaining six varieties were local varieties originated from Java, Nusa Tenggara and Sulawesi collected in GeneBank of ICABIOGRAD. The results showed that the 21 morphological characters were not sufficient to differentiate 22 mungbean varieties, while SSR analysis revealed that eight multi-alleles markers and high polymorphic information content (PIC) values have been successfully selected for varietal identification. The selected markers enabled to differentiate each mungbean variety according to their genetic marker with the lowest distance of 0.125, demonstrating the robustness of the selected marker set as a tool to identify a specific DNA fingerprint profile as a varietal identity (ID). The genetic identity of a variety was shown by digital barcoding which represented a series of alleles produced by corresponding markers. The DNA fingerprint profile of each variety would be beneficial as reference identities of a mungbean variety.

[***Keywords*:** Mungbean, morphological characters, SSR markers, DNA fingerprint, varietal identity]

## ABSTRAK

*Karakterisasi kacang hijau pada umumnya dilakukan berdasarkan sifat-sifat morfologi. Pendekatan genetika molekuler diharapkan dapat membantu pemulia dalam mengidentifikasi kacang hijau dan melindungi hak kekayaan intelektual pemulia. Penelitian ini bertujuan untuk mengidentifikasi varietas kacang hijau Indonesia berdasarkan penampilan sidik jari DNA dengan menggunakan satu set marka untuk mendukung karakterisasi secara morfologi. Sebanyak 22 aksesi kacang hijau Indonesia telah dianalisis berdasarkan 21 karakter morfologi dan secara molekuler menggunakan 55 primer* simple sequence repeats *(SSR). Di antara 22 varietas kacang yang digunakan pada penelitian ini, 16 varietas merupakan varietas unggul dan enam varietas merupakan varietas lokal asal Jawa, Nusa Tenggara, dan Sulawesi yang dikoleksi di BankGen BB Biogen. Hasil penelitian menunjukkan bahwa 21 karakter morfologi yang digunakan belum cukup informatif untuk membedakan 22 varietas kacang hijau. Berdasarkan analisis SSR, delapan marka dan nilai informasi polimorfis yang tinggi telah terseleksi sebagai marka untuk identifikasi varietas kacang hijau. Set marka terseleksi tersebut mampu membedakan masing-masing varietas dengan jarak genetik terendah sebesar 0,125 dan secara spesifik dapat digunakan sebagai alat bantu yang andal untuk identitas varietas (ID). Identitas genetik dari varietas tersebut ditunjukkan oleh angka* barcoding *yang merupakan rentetan dari alel-alel yang dihasilkan oleh masing-masing marka. Profil sidik jari DNA dari masing-masing varietas kacang hijau akan sangat bermanfaat terutama sebagai referensi identitas varietas kacang hijau.*

[***Kata kunci*:** Kacang hijau, karakter morfologi, SSR, sidik jari DNA, identitas varietas]

## INTRODUCTION

Mungbean (*Vigna radiata* L. (Wilczek)), a second economically important legume crop in Indonesia after soybean, is an important source of protein, vitamin and mineral (Tomooka *et al*. 2002; Lambrides and Godwin 2007; Somta and Srinives 2007; Mondal *et al.* 2012). In spite of the best efforts for improving mungbean varieties, the yield of this crop remains low. According to the Central Bureau Statistics of Indonesia, the national mungbean productivity was 1.12 ton $ha^{-1}$ with the total area of 182,058 ha (BPS 2013). Therefore, the genetic improvement of mung-

bean should be based on the genetic information of the plant.

Conservation of the genetic resources of mungbean is vital for future breeding programs and food security, therefore, characterization and proper assignation of individual genotypes to species is required (Karp 1996; Vincent *et al*. 2013). The International Union for the Protection of New Varieties of Plants (UPOV) has provided guidelines on establishing the uniqueness of a variety through testing for distinctiveness, uniformity and stability (DUS) (UPOV 2011). Through the adoption of the UPOV system, a breeder is obliged to protect a variety for commercial exploitation. Protection can be granted if a variety of the crop is distinct from the existing ones supported with sufficiently uniform and stable genetic characteristics. Hence, the varietal identification of mungbean becomes a critical importance.

Traditionally, the accessions of mungbean in Indonesia are characterized based on their morphological and physiological traits (Van den Bosch 1987; Hakim 2008). Van den Bosch (1987) reported an intercropping of mungbean landraces originated from East Java with maize. After three cycles of selection, a yield of intercropped mungbean increased 24% as compared to the original mungbean landraces, and the yield of maize in the mixture was not affected by the increase in mungbean landrace yield. In another study, Hakim (2008) evaluated 350 mungbean accessions for their agronomic characters in the field. A number of these characters (days to maturity, plant height, pods per plant and seed size) were known to be significantly varied. Number of pods per plant and plant height were important characters that can be used as the selection criteria in mungbean breeding in the early generation stage (Hakim 2008). However, this traditional method is costly and time-consuming since numbers of existing varieties are quite large that require wide land and skilled personnel, and often subjective decisions (Cooke 1995; Kumar *et al.* 2009). Therefore, reliable and cost-effective methods for identifying varieties are desirable to differentiate the increasing numbers of new varieties and eliminate duplicates from germplasm collections. An effective method for variety identification such as DNA fingerprinting is essential for DUS testing of new varieties and for protection of intellectual property right of new varieties (Lu *et al*. 2009).

The use of molecular markers for differentiating mungbean varieties of Indonesia has been reported previously. In this study, a total of 30 newly SSR simple sequence repeat (SSR) markers which were developed from the genome of Korean mungbean varieties (Sunhwanokdu and Gyeonggijaerae 5) proved their application to detect genetic variability of 83 accessions of Indonesian mungbean to support breeding program and conservation strategy. Moreover, these markers were able to identify improved mungbean varieties that were genetically similar to some landraces from one of the main mungbean-producing regions in Indonesia (Lestari *et al*. 2014). Considering the prospecting SSR markers (Lestari *et al*. 2014), our current study applied those markers in addition to other markers for varietal identification rather than genetic diversity study. The use of molecular markers which discriminate varieties in nucleotide sequences are unaffected by environments and becomes more desirable in varietal identification and differentiation.

SSR markers have been the most widely-used genotyping markers in many plant species over the past decade due to their stability, ease of application, representing highly polymorphic, abundant presence in the genome, reproducible, co-dominant, and multiallelic types of variation (Minamiyama *et al*. 2006; Yi *et al.* 2006; Lorenzo *et al*. 2007; Portis *et al*. 2007; Agarwal *et al.* 2008; Senthilvel *et al.* 2008; Sundaram *et al.* 2008; Kumar *et al.* 2009). The working group on biochemical and molecular techniques of UPOV has identified SSR markers as the predominant markers for plant varietal characterization (UPOV 2011). A specific set of SSRs can be used in different sets of genotypes, making them particularly useful for DNA fingerprinting.

The objective of this study was to identify Indonesian mungbean varieties based on DNA fingerprint profile using a set of developed markers to support the morphological characters.

## MATERIALS AND METHODS

### Plant Materials

A total of 22 Indonesian mungbean varieties consisted of 16 improved varieties and six local varieties as a comparison were subjected to both morphological and molecular analyses (Table 1). All of the accessions belonged to the genebank of the Indonesian Center for Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor, West Java.

**Table 1. Twenty two Indonesian mungbean varieties used in this study together with their origin or genetic background.**

| Variety | Genetic background/origin |
|---|---|
| Si Walik | Selected from a population in Jeneponto |
| Arta Ijo | Selected from local varieties originating from Sumenep, Madura |
| Manyar | Introduced varieties from AVRDC (Taiwan) |
| Bhakti | Selected from introduced varieties from Sri Lanka |
| No. 129 | Selected from introduced varieties from Philippines |
| Nuri | Selected from introduced varieties from AVRDC (Taiwan) |
| Kenari | Introduced from AVRDC, Taiwan 1987, single cross from VC 1178B as male and VC 1624 as female |
| Betet | Selected from cross of MB 129 x Siwalik |
| Gelatik | Selected from introduced varieties from AVRDC (Taiwan) |
| Parkit | PHLV-18/VC.1177 1979 |
| Merpati | Selcted from F4 generation of introduced lines from Taiwan |
| Walet | Selected from introduced varieties from AVRDC (Taiwan) |
| Camar | Developed using Gamma irradiation of 0.1 kGy dose on Manyar variety |
| Merak | Selected from introduced varieties from Philippines |
| Perkutut | Introduced from AVRDC, Taiwan |
| Vima-1 | Synthetic crossing of VC 1973 A and 2750A |
| Nilon | Sulawesi |
| Lok Belu | Nusa Tenggara |
| Lok Garut | Java |
| Tecer Hijau | Java |
| Lok NTB | Nusa Tenggara |
| Lok Muntaha K2 | Sulawesi |

## Morphological Characterization

All genetic materials were grown at the Experimental Farm of Seoul National University in Suwon, Korea (altitude: 74 m, longitude: 127°362 E, latitude: 37°512 N) following the standard cultural practices. Two individual plants were grown in each pot with two replications. Twenty one qualitative morphological characters related to growth habit, leaf, stem and pod were observed and scored according to the guidelines for conducting tests for distinctness, homogeneity and stability criteria provided by the UPOV (UPOV 2011). All recorded characters were converted to numerical numbers which could be designed as digital morphological markers of each mungbean variety. Based on the qualitative morphological data, a phylogenetic tree was generated using PowerMarker software.

## DNA Isolation

Young and healthy leaves of three to four day-old mungbean seedlings were harvested for DNA extraction. The leaf tissues were ground into a fine powder in a liquid nitrogen by a pestle and mortar. The genomic DNA was extracted using a standard cetyltrimethylammonium bromide (CTAB) method (Gelvin and Schilperoort 1995). For an initial screening, a total of 55 SSR markers developed in the Crop Genomic Laboratory, Seoul National University were used on the basis of *de novo* sequencing data of Korean mungbean cultivars (Sunhwanokdu and Gyeonggijaere). Then the DNA polymorphisms of 22 Indonesian mungbean varieties were checked by a fluorescence-based capillary electrophoresis.

## PCR Amplification

PCR reactions were conducted in a total volume of 20 µl and contained 20 ng genomic DNA, 0.25 µM each primer (forward and reverse), 0.125 mM each dNTP, 0.16 units AmpliTaq Gold® DNA Polymerase (Applied Biosystem, Warrington, UK), and 1x AmpliTaq buffer with $MgCl_2$. The PCR amplification was performed using a 96-well plate in a tetrad thermal cycler (DNA Engine Tetrad, MJ Research). The amplification conditions were as recommended by the manufacturer's protocol (Applied Biosystem, Warrington, UK) that consisted of an initial denaturation step of 5 minutes at 94° C, followed by 35 cycles of 45 seconds at 94° C, 30 seconds at 55° C, 30 seconds at 72° C with a final extension at 72° C for 10 minutes. PCR products were first separated on 2% (w/v) agarose

gel containing GelRed (Biotium) in 0.5x TBE buffer. The amplicons were visualized under UV light, and the sizes were estimated relative to the 100 bp DNA ladder.

For further determination of polymorphism, the amplicons were run in a fluorescence-based capillary electrophoresis using Fragment Analyzer CE System (Advanced Analytical Technologies, Inc., USA). To ensure reproducibility of amplification products, the analyses were repeated at least twice. Allele sizes were determined for each SSR locus using fragment analysis software which was automatically offered by the capillary electrophoresis Fragment Analyzer CE System.

### Data Analysis

Genotypic data were subjected to PowerMarker software to analyze the polymorphic information content (PIC) values of the tested primers, for the calculation of allele number, allele frequency, heterozygosity, gene diversity, and probability of identity per locus. The PIC values of each SSR marker were calculated for the total population (Liu 2001). SSR markers that exhibit high value of PIC (> 0.5) were chosen to be recommended as valuable SSR markers on DNA fingerprinting of Indonesian mungbean varieties. Such information is important to be used as basic criteria to select molecular markers candidate for varietal identification. In parallel with selection of SSRs for marker set, estimated calculation of genetic distance through generating phylogenetic tree was performed to maximize the varietal differentiation efficiency.

The polymorphic bands of 22 mungbean varieties were scored as binary characters for their presence (1) or absence (0) and the resulting data were analyzed using NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System) version 2.1 (Rohlf 1998). Genetic similarity between cultivars was calculated based on the simple matching coefficient using the SIMQUAL subprogram. Cluster analysis was performed using the Unweighted Pair Group with Arithmetic Mean (UPGMA) method in the SAHN subprogram of NTSYS-PC. This phylogenetic analysis assisted to formulate SSR marker set to identify mungbean variety.

The genetic identity (ID) of mungbean variety was then determined by a digital value which represented a series of alleles produced by corresponding marker. In this step, the allele size was transformed to two digits of numerical number produced by each marker. The two digital codes were started from "01" for each SSR motif observed on each primer. This digital code transformation would be done for total SSR in the formulated marker set and the digital codes represented the ID of each mungbean variety (Risliawati *et al*. 2015).

## RESULTS AND DISCUSSION

### Variability of Mungbean Varieties Based on Morphological Characters

Twenty one qualitative morphological characters of 22 Indonesian mungbean varieties were presented on Table 2. The present data indicated that a low variation (0.28) was evidenced in all mungbean varieties evaluated. Seven morphological characters were uniform, whereas the remaining ones were considerably varied ranging from low (0.04) for mature pod color to the highest (0.81) for leaf pubescence character. A moderate degree of variation was observed in the leaf color, stem color and seed size. All varieties showed no lobe of leaf, ovate lanceolate shape of primary leaf, deltate of terminal leaf, drum of seed, yellow flower, pod pubescence and green color of premature pod. Majority (> 80%) of varieties had seed pubescence, straight of pod curvature and green seed. According to mature pod characteristics, only a local variety of Tecer Hijau from Java had a brown color in contrast the green one of the rest varieties.

The morphological character data showed common on both improved and local varieties, and some traits might be specific to genotypes as demonstrated in our study. The morphological characters of released varieties could be selected as important descriptor for breeding program (Stoilova *et al*. 2013). While, local varieties with better adaptation to local growing regions possessed valuable character for cultivation in different agroecological conditions (Stoilova and Sabeva 2006).

To simply identify, the total of 21 characters were scored and converted to numerical number (Table 1). When morphological characters were used as markers, it was noted that each genotype either improved variety or local variety had its own identity. However, these total morphological characters were enabled to differentiate each mungbean variety yet. As a representative, a variety named Parkit with morphological identity of "**2**212312111113211211" and a variety named No. 129 with its code of "**1**212312111113211211" seemed to be close and differed based on only one character, plant habit as

**Table 2. Morphological characteristics of 22 mungbean varieties used in present study.**

| Variety name | Reg. number | PH | GH | StC | SP | LP | LL | LC | LVC | PLS | TLS | SSh | PCv | BP | FC | SS | PP | PPC | MPC | SL | SC | H |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Si Walik | VR9-1 | 1 | 2 | 2 | 2 | 3 | 1 | 3 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 2 |
| Arta Ijo | VR26 | 1 | 1 | 1 | 2 | 4 | 1 | 3 | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 2 |
| Manyar | VR110 | 1 | 1 | 1 | 2 | 3 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 2 | 1 | 2 |
| Bhakti | VR116 | 2 | 1 | 1 | 2 | 3 | 1 | 3 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 2 | 1 | 1 | 2 | 1 | 2 |
| No.129 | VR129 | 1 | 2 | 1 | 2 | 3 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 3 | 2 | 1 | 1 | 2 | 1 | 1 |
| Nuri | VR137 | 2 | 2 | 1 | 2 | 3 | 1 | 2 | 2 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 2 |
| Kenari | VR196 | 1 | 1 | 2 | 2 | 4 | 1 | 3 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 3 | 2 | 1 | 1 | 2 | 1 | 1 |
| Betet | VR217 | 1 | 1 | 1 | 2 | 3 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 2 | 1 | 1 | 1 | 3 | 1 |
| Gelatik | VR218 | 2 | 1 | 1 | 2 | 3 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 3 | 2 | 1 | 1 | 1 | 1 | 2 |
| Parkit | VR219 | 2 | 2 | 1 | 2 | 3 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 3 | 2 | 1 | 1 | 2 | 1 | 1 |
| Merpati | VR220 | 1 | 2 | 1 | 2 | 4 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 2 | 1 | 1 |
| Walet | VR222 | 2 | 2 | 1 | 2 | 4 | 1 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 2 | 1 | 1 | 2 | 1 | 1 |
| Camar | VR224 | 2 | 2 | 1 | 2 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 2 | 1 | 1 |
| Merak | VR423 | 2 | 2 | 2 | 2 | 4 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 3 | 2 | 1 | 1 | 2 | 3 | 1 |
| Perkutut | VR1058 | 2 | 1 | 1 | 2 | 4 | 1 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 2 | 1 | 1 | 2 | 1 | 1 |
| Lima-1 | VR1074 | 2 | 1 | 1 | 2 | 4 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 3 | 2 | 1 | 1 | 1 | 1 | 1 |
| Nilon | VR997 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 3 | 2 | 1 | 1 | 2 | 1 | 2 |
| Lok Muntaha K2 | VR1005 | 1 | 1 | 2 | 1 | 3 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 2 |
| Lok Belu | VR1077 | 2 | 2 | 3 | 1 | 1 | 1 | 3 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 2 | 1 | 1 | 2 | 1 | 2 |
| Lok NTB | VR1079 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 2 | 1 | 2 |
| Lok Garut | VR1057 | 2 | 1 | 2 | 2 | 3 | 1 | 2 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 3 | 2 | 1 | 1 | 2 | 1 | 1 |
| Tecer Hijau | VR1069 | 1 | 1 | 2 | 3 | 2 | 1 | 3 | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 2 | 1 | 2 | 1 | 1 | 2 |

Abbreviations: PH = plant habit, GH = growth habit, StC = stem color, SP = stem pubescence, LP = leaf pubescence, LL = leaf lobe, LC = leaf color, LVC = leaf vein color, PLS = primary leaf shape, TLS = terminal leaf shape, SSh = seed shape, PCv = pod curvature, BP = branching pattern, FC = flower color, SS = seed size, PP = pod pubescence, PPC = premature pod color, MPC = mature pod color, SL = seed lusture, SC = seed color, H = hypocotyle

1. Plant habit: 1 = ID (indeterminate), 2 = D (determinate)
2. Growth habit: 1 = SE (semierect), 2 = SHE (semierect-horizontal)
3. Stem color: 1 = LG (light green), 2 = DG (dark green), 3 = GP (mixed green-purple)
4. Stem pubescence: 1 = G (globrous/absent of hair), 2 = P (pubescence/present of hair)
5. Leaf pubescence: 1 = VSP (very sparsely pubescence), 2 = SP (sparsely pubescence), 3 = MP (moderately pubescence), 4 = VMP (very moderately pubescence)
6. Leaf lobe: 1 = no lobe, 2 = having lobe
7. Leaf color: 1 = LG (light green), 2 = G (green), 3 = DG (dark green)
8. Leaf vein color: 1 = G (green), 2 = GP (greenish purple)
9. Primary leaf shape: 1 = OL (ovate lanceolate)
10. Terminal leaf shape: 1 = deltate
11. Seed shape: 1 = D (drum)
12. Pod curvature: 1 = S (straight), 2 = C (curvature)
13. Branching pattern: 1 = all, 2 = central
14. Flower color: 1 = yellow
15. Seed size: 1 = small, 2 = medium, 3 = big
16. Pod pubescence: 1 = globrous, 2 = pubescence
17. Premature pod color: 1 = green
18. Mature pod color: 1 = black, 2 = brown
19. Seed lusture: 1 = dull, 2 = shiny
20. Seed color: 1 = green, 2 = black, 3 = green yellowish, 4 = yellow
21. Hypocotyl color: 1 = green, 2 = purple

denoted by "2" on variety Parkit and "1" on variety No 129. While between Walet and Perkutut were not distinct each other as reflected by the same identity of "211241311111113211211". These results suggested that the numerical morphological characters which reflect the differences in the mungbean organs are not effective to discriminate among improved mungbean varieties along with the local ones. This results is in agreement and relevant with the previous report showing that the genetic differentiation based on morphological characters in the species level of *Vigna* is less sufficient (Tantasawat *et al*. 2010).

Similarity coefficients among the tested mungbean varieties were also calculated based on the 21 morphological traits to generate a dendrogram (Fig. 1). Most varieties belonged to main clade (clade 1) suggesting that they shared similar morphological features. Interestingly, all improved mungbean varieties were preferentially in the clade 1 including two local varieties Lok Garut from Java and Lok NTB from Nusa Tenggara. In particular, other local varieties appeared un-clustered, namely Tecer Hijau (Java) and Lok Muntaha K2 (Sulawesi). The second main clade grouped two local varieties (Nilon from Sulawesi and Lok Belu from Nusa Tenggara). These findings indicated that the genetic cluster of these varieties did not reflect the geographical origin of the mungbean accessions. Consistent to the same morphological identity, Parkit and No.129 had no genetic distance based on 21 morphological characters. Since this morphological data analysis was not sufficient for differentiating the Indonesian mungbean varieties, DNA fingerprinting analysis using molecular markers to identity Indonesian mungbean varieties is needed to complement this phenotypic characterization.

## Polymorphism Analysis of SSR

The genetic diversity among 22 mungbean varieties were assessed to select SSR markers to be candidate as SSR marker set. Fourteen out of 55 primers that produced polymorphic information content (PIC) higher than 0.5 were chosen for further analysis. Statistic summary of 14 SSR primers observed on 22 mungbean varieties is shown in Table 3. The selected 14 SSR loci with such PIC values are considered as very informative, leading to be powerful for differentiation of mungbean varieties surveyed. Of these, four SSR loci demonstrated relatively high PIC values of > 0.7 namely P89, P33, P36 and P7, and the highest PIC value (0.79) was detected on P87. This high PIC of the genomic-based SSR markers on our study confirmed the polymorphism detection efficiency to mungbean genetic diversity, as previously reported (Chen *et al*. 2015).

SSR allele numbers observed ranged from 3 to 8 with an average of 5.14 and a total number of 72 alleles in the 22 mungbean varieties. Loci of P7, P45 and P59 generated the lowest number of alleles (three alleles each) whereas other three SSR loci (P33, P87 and P17) had the highest number of alleles (eight alleles each), which could be useful as differentiators of varieties due to their multiple allele nature. Major allele frequency (higher than 30%) was found on the selected 14 SSR loci with the highest was observed on P74 (frequency of 59%). Approximately 43% of total varieties tested shared dominant alleles. Heterozygosity referring to specific variety was found only on one locus, P36 with an average of 0.003. This heterozygous allele existed in local mungbean rather than in improved varieties could well be understood.

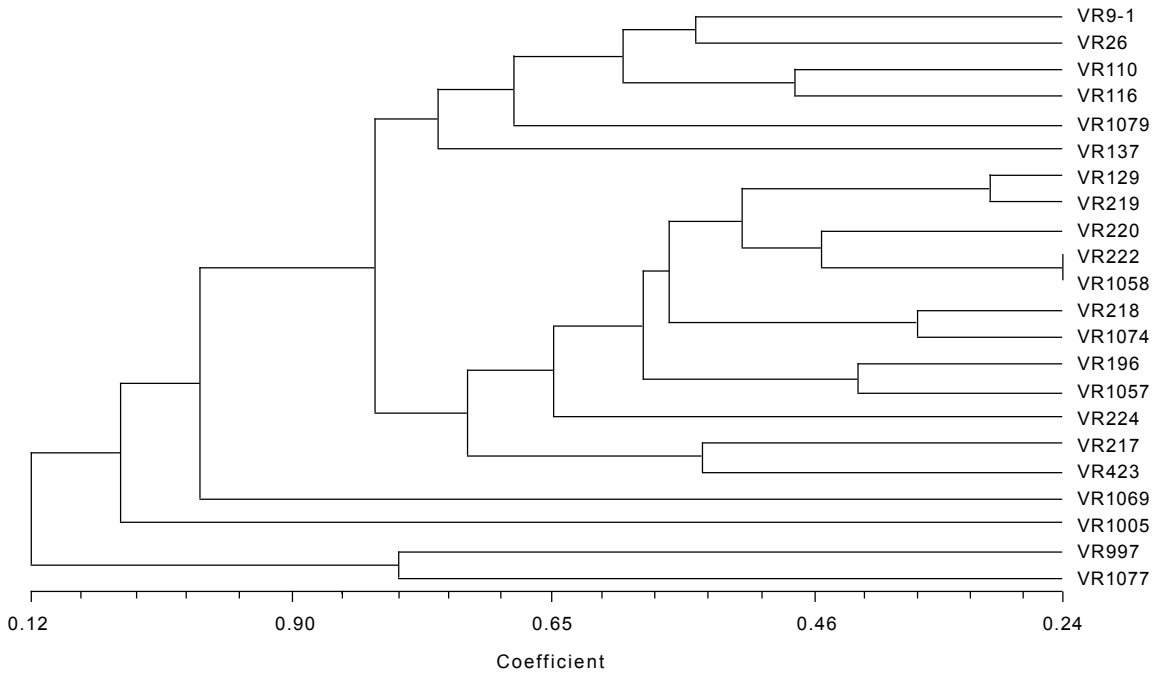


**Fig. 1.** Unweighted Pair Group with Arithmetic Mean (UPGMA) cluster analysis of 22 Indonesian mungbean varieties on the basis of 21 morphological characters.

**Table 3. Summary of statistics of 14 simple sequence repeat (SSR) markers observed in 22 Indonesian mungbean varieties.**

| Marker | Major allele frequency | Allele number | Gene diversity | Heterozygosity | PIC |
|---|---|---|---|---|---|
| P59 | 0.36 | 3 | 0.67 | 0.00 | 0.59 |
| P33 | 0.32 | 8 | 0.78 | 0.00 | 0.75 |
| P7 | 0.55 | 3 | 0.58 | 0.00 | 0.51 |
| P95 | 0.41 | 4 | 0.71 | 0.00 | 0.67 |
| P45 | 0.45 | 3 | 0.64 | 0.00 | 0.57 |
| P57 | 0.41 | 4 | 0.70 | 0.00 | 0.64 |
| P74 | 0.59 | 4 | 0.58 | 0.00 | 0.53 |
| P87 | 0.27 | 8 | 0.82 | 0.00 | 0.79 |
| P27 | 0.55 | 7 | 0.66 | 0.00 | 0.63 |
| P29 | 0.41 | 5 | 0.70 | 0.00 | 0.65 |
| P36 | 0.30 | 6 | 0.78 | 0.05 | 0.75 |
| P69 | 0.50 | 5 | 0.61 | 0.00 | 0.54 |
| P16 | 0.50 | 4 | 0.61 | 0.00 | 0.53 |
| P17 | 0.45 | 8 | 0.74 | 0.00 | 0.72 |
| Means | 0.43 | 5.14 | 0.68 | 0.003 | 0.63 |

Gene diversity of this mungbean collection was relatively high, 0.68. Loci P87 which had the highest PIC value, showed the highest gene diversity (0.82), suggesting a positive correlation between PIC and gene diversity. Discrepancy of genetic diversity based PIC demonstrated that the choice of markers and varieties affected the variation of molecular markers used. Markers with high PIC value and are informative turned out to be a marker set for DNA fingerprinting analysis for identification and differentiation of particular varieties (Bredemeijer *et al*. 2002).

## SSR Marker Set Development and Specific Identity of Mungbean Varieties

To design marker set to identify DNA fingerprint profile of variety as genetic identity needs several steps including selection of marker candidate, design method for marker set and creation of varietal ID in the digital barcode. In this study, based on PIC, gene diversity and allele number of each marker, finally 14 SSR loci candidate were selected from a total of 55 primers surveyed. Out of the chosen 14 SSR primers, further selection was done to obtain eight primers as a basis in the marker set formulation. These markers included those with high PIC values ranging from 0.63 to 0.79, namely P33, P95, P57, P87, P27, P29, P36 and P17. This simple SSR analysis containing minimized markers (eight) could be a suitable method for routine identification of mungbean varieties, which is in good agreement with the previous report (Prammanee *et al*. 2000).

Both morphological and molecular data demonstrated the diversity level of the Indonesian mungbean varieties. A phylogenetic analysis was generated to initial estimation of varieties differentiation, leading to the identification of each variety as a distinctive individual (Fig. 2). Therefore, a dendrogram of the 22 Indonesian mungbean varieties was performed using the genetic similarity matrix generated by the Nei 1973 similarity coefficient based on alleles produced by eight SSR markers. Two main clades appeared to group 10 varieties (clade I) and the remaining varieties (clade II). Both clades contained improved varieties together with three local varieties, in which Lok Belu (Nusa Tenggara), Lok Garut (Java), Tecer Hijau (Java) belonged to clade I, and Nilon (Sulawesi), Lok NTB (Nusa Tenggara) and Lok Muntaha K2 (Sulawesi) into clade II. Importantly, each variety was clearly distinct with each other without the same genetic distance.

Based on the allele size of DNA fingerprint profile using a marker set containing eight SSR loci, finally a barcoding in the form of digital ID of each mungbean variety was successfully developed (Table 4). For example an improved variety of Si Walik had ID "0401030804010503", while Nuri which was found to have heterozygote alleles automatically had two IDs depending on the harbored alleles, 0501040504020102 and 0101020705030207. Notably, local varieties from Java (LokGarut and Tecer Hijau) seemed to be discrepancy according to their barcoding. Two improved varieties (Walet and Perkutut) that were not able to be differentiated based on morphological characters, by using eight markers finally were

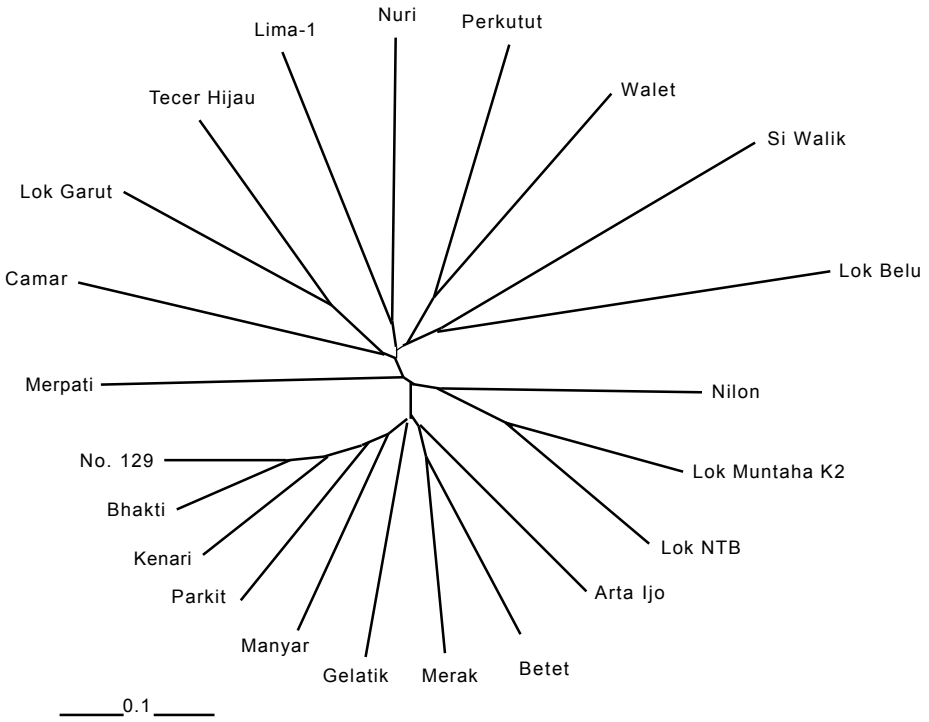


**Fig. 2.** Dendrogram of 22 Indonesian mungbean varieties based on Unweighted Pair Group with Arithmetic Mean (UPGMA) analysis using the genetic similarity matrix of the Nei coefficient generated with eight simple sequence repeat (SSR) markers.

**Table 4. The genetic identity of Indonesian mungbean varieties represented by digital values based on DNA fingerprint profile produced by eight simple sequence repeat (SSR) markers.**

| Varieties | Primers | | | | | | | | Digital ID |
|---|---|---|---|---|---|---|---|---|---|
| | P33 | P95 | P57 | P87 | P27 | P29 | P36 | P17 | |
| Improved varieties | | | | | | | | | |
| Si Walik | 04 | 01 | 03 | 08 | 04 | 01 | 05 | 03 | 0401030804010503 |
| Arta Ijo | 03 | 01 | 02 | 07 | 04 | 04 | 04 | 08 | 0301020704040408 |
| Manyar | 06 | 04 | 01 | 06 | 04 | 02 | 05 | 03 | 0604010604020503 |
| Bhakti | 05 | 03 | 03 | 05 | 04 | 04 | 01 | 03 | 0503030504040103 |
| No 129 | 05 | 03 | 03 | 04 | 04 | 04 | 01 | 03 | 0503030404040103 |
| Nuri | 04 | 02 | 01 | 02 | 03 | 02 | 02/03 | 04 | 04020‘0203020204 or 0402010203020304 |
| Kenari | 08 | 03 | 03 | 06 | 01 | 04 | 01 | 04 | 0803030601040104 |
| Betet | 01 | 03 | 03 | 07 | 07 | 04 | 04 | 05 | 0103030707040405 |
| Gelatik | 07 | 02 | 04 | 03 | 03 | 04 | 06 | 06 | 0702040303040606 |
| Parkit | 01 | 02 | 03 | 07 | 04 | 05 | 01 | 01 | 0102030704050101 |
| Merpati | 01 | 02 | 04 | 01 | 04 | 04 | 05 | 03 | 0102040104040503 |
| Walet | 04 | 04 | 03 | 03 | 04 | 02 | 02 | 08 | 0404030304020208 |
| Camar | 01 | 04 | 01 | 05 | 06 | 04 | 02 | 04 | 0104010506040204 |
| Merak | 01 | 03 | 01 | 05 | 04 | 04 | 02 | 08 | 0103010504040208 |
| Perkutut | 01 | 04 | 03 | 06 | 05 | 03 | 02 | 03 | 0104030605030203 |
| Lima-1 | 04 | 02 | 04 | 03 | 04 | 03 | 03 | 05 | 0402040304030305 |
| Local varieties | | | | | | | | | |
| Nilon (Sulawesi) | 05 | 02 | 04 | 03 | 05 | 03 | 04 | 03 | 0502040305030403 |
| Lok Belu (Nusa Tenggara) | 05 | 02 | 04 | 06 | 04 | 02 | 04 | 03 | 0502040604020403 |
| Lok Garut (Java) | 05 | 01 | 04 | 05 | 04 | 02 | 01 | 02 | 0501040504020102 |
| Tecer Hijau (Java) | 01 | 01 | 02 | 07 | 05 | 03 | 02 | 07 | 0101020705030207 |
| Lok NTB (Nusa Tenggara) | 02 | 02 | 01 | 07 | 02 | 03 | 04 | 03 | 0202010702030403 |
| Lok Muntaha K2 (Sulawesi) | 05 | 02 | 03 | 07 | 03 | 02 | 02 | 03 | 0502030703020203 |

*Barcoding for varieties based on DNA fingerprinting produced by SSR markers from left to right P33-P95-P57-P87-P27-P29-P36-P17

distinguished the two with genetic distance of 0.125. Thus, this analysis proved that the eight SSR markers enabled to create specific DNA fingerprint profile on each of the Indonesian mungbean varieties. These barcodes which possibly provided reference genetic identity (ID) along with marker set in our study could assist effective protection and management of mungbean germplasm/collection in genebank and market in Indonesia. Importantly, molecular ID could protect a variety for commercial exploitation and a plant breeder can be granted and also would be useful to protect local variety from claim of other countries and tracking their local origin.

The marker set developed in this study could be utilized as a recommended molecular test for identification of new mungbean varieties to support the phenotypic assay of DUS requirement in the plant variety protection in Indonesia. This study is in agreement with the previous study by Lestari *et al*. (2014) and has also confirmed the usefulness of the SSR molecular marker set to identify mungbean varieties which had a narrow genetic background (Sestili *et al*. 2011), suggesting to open new perspectives towards protection of released varieties and conservation of Indonesian mungbean genetic resources.

## CONCLUSION

A total of 22 improved and local varieties of Indonesian mungbean showed a common morphological characteristic with a low variation (0.28). Seven morphological characters, i.e. leaf lobe, primary leaf shape, terminal leaf shape, seed shape, flower color, pod pubescence and premature pod color were uniform and found a considerable level of variability ranging from low (0.04) for mature pod color to the highest (0.81) for leaf pubescence character. The use of the eight SSR markers proved usefulness for differentiating the Indonesian mungbean varieties. Eight of the SSR markers have facilitated a specific DNA fingerprint profile and could be used as the reference genetic identity for identification of Indonesian mungbean varieties.

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