Genetic relationships between yardlong bean (*Vigna unguiculata* ssp. *sesquipedalis*) F1 progenies and their parents based on RAPD markers

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**Abstract.** Yardlong bean is a very popular vegetable with moderately high nutritional value and versatile. The genetic relationships among yardlong beans should be explored for long-term success in its breeding programs. The aim of the study was to evaluate genetic relationships among yardlong bean F1 crossbred progenies and their parents based on DNA markers. RAPD was performed on five genotypes of F1 yardlong bean progenies from diallelic crosses and three cultivars of parental plants. Five selected primers generated 16 scorable bands with their size ranging from 280 bp to 1160 bp, and the average number of bands per primer of 3.20. Cluster analyses using UPGMA procedure were performed using MVSP 3.1 software and produced a dendrogram with two clusters. Jaccard similarity matrices among yardlong bean parents and their F1 progenies varied between 0.40 – 0.73. The results suggest that RAPD markers can be an efficient, fast, and inexpensive way for differentiation of yardlong bean F1 progenies and their parents, as well as to evaluate their genetic relationships.

1. Introduction

The leguminosae genus *Vigna* is comprising around 100 species mainly found in Africa and Asia: include the cowpea (*Vigna unguiculata* L. Walp) and yardlong bean (*Vigna unguiculata* L. Walp ssp. *unguiculata* cv. –gr. *sesquipedalis*) which are differ phenotypically as result of divergent selection during the crop evolution [1]. Yardlong bean is grown extensively in West Africa, South China, and Southeast Asia [2]. In Indonesia, yardlong bean is known as “kacang Panjang” which produces delicious crisp and tender pods that can be eaten both fresh and cooked [3], and it is containing high digestible protein (23.52 – 26.27 %) along with phosphorus, sodium, potassium, magnesium, calcium, iron, zinc, manganese, cobalt, thiamin, riboflavin, vitamin A, and vitamin C, [4]. Annual production at 461239 tons covering about 84798 ha (average 5.5 tons ha⁻¹) [5]. Moreover, breeder try to increase the yields because of yardlong bean economical important to meet consumers high demand [6]. Genetic diversity has been considered as important factor and pre-requisite for successful breeding program [7]. Yardlong bean has wide polymorphism in respect of flower color, fruit color, fruit size and weight, pod size, color, texture, etc. [8]. They are important source for yardlong bean breeding, because the narrow genetic diversity is a main problem restricting the progress of it [9]. Many scientific researches looking into the nature of yardlong bean genetic diversity, as well as in the uses of genetic data in its breeding strategies [10 - 13]. Over the years, the methods for evaluating the diversity of genetic have
expanded from discrete morphological to biochemical and molecular characters analysis, and then several DNA-based markers have developed [14]. Therefore, breeder consider marker assisted selection (MAS) a useful way in breeding program, because: the direct phenotypic selection is time-consuming or more expensive, the genes expression requires specific conditions, the heritability is low and the phenotypic selection is less efficient, and multiple genes for the same character are cumulatively under selection [15]. Genetic diversity in yardlong bean were evaluated by using different types of DNA markers: AFLP [16], RAPD [8], SSR [17], SNP [18], ISSR and SCoT [19].

Among DNA-based markers, RAPD markers are informative, fast, and inexpensive, which despite the dominant and low reproducible, allows polymorphism analysis in many individuals with good coverage of the entire genome [20]. RAPD have been used to determine the genetic relationships among germplasm of yardlong bean would be very useful to preservation, as well as for efficient utilization of the germplasms, especially for breeding purposes. Previously, we have assessed F1 progenies and their parents based on their morphological markers. Since the information about the germplasm diversity and the relationships of breeding materials are important for breeding program, the aim of the study was to evaluate genetic relationships among yardlong bean F1 progenies and their parents based on RAPD markers. The information obtained represent the early evaluation of the potential use of the RAPD markers as an efficient, fast, and inexpensive way for diversity screening, and possibility of applying MAS in yardlong bean breeding programs.

2. Materials and methods

The yardlong bean cultivars: “Fagiola Ungu” (FG), “Aura Hijau” (AH) and “Super Putih” (SP), were chosen based on their divergent morphological characters (Table 1) and used as parents in a complete diallel with reciprocals crosses (FG x AH, FG x SP, AH x FG, AH x SP, SP x FG, SP x AH). The method proposed by [21], which mechanical emasculation of the female parent using forceps on flower buds was done a day before flowering followed by crossed pollination using ripe pollen from open flowers of the male parents, and 5 F1 hybrids were obtained (AH x FG was not included because no seed was available). The populations consisting of five F1 progenies and three parents, totaling 8 treatments were evaluated in the greenhouse at the Laboratory of Physiology and Crop Breeding, Diponegoro University, Semarang in early 2018. The design of the experiment was a completely randomized design (CRD) with three replicates. Four seeds were grown in 25 cm³ pot containing substrate, and thinned seven days after emergence, then two plants were left in each pot. Two plants per pot made up an experimental unit. Crop management was as recommended for the yardlong bean crop [22].

Table 1. Important morphological characters and sources of three yardlong bean cultivars

| Name of cultivar | Pod color | No. of pods per plant | Pod length (cm) | No. of seeds per pod | Sources |
|------------------|-----------|-----------------------|-----------------|----------------------|---------|
| Fagiola Ungu     | Purple    | 5.78                  | 32.81           | 11.65                | Benih Dramaga - IPB |
| Aura Hijau       | Green     | 2.67                  | 47.98           | 12.58                | CV. Aura Seed Indonesia |
| Super Putih      | White     | 4.89                  | 36.63           | 10.07                | PT. BISI International, Tbk. |

Yardlong bean DNA was extracted from 1 g of healthy leaves, harvested from plants grown in the greenhouse, using the Plant Genomic DNA Kit (DP305; Tiangen Biotech-Beijing Co., Ltd., Beijing, China) according to the manufacturer’s protocol. The extracted DNA samples were quantified with NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Massachusetts, USA). Next, they were frozen at -20°C and kept until they were used for RAPD amplification by PCR. Five decamer primers (OPC-06: 5'-GAACGGACTC-3', OPR-12: 5'-ACAGTGCGT-3', OPZ-03: 5'-CGCACCCGCA-3', OPZ-08: 5'-GGGTGGGTAA-3', Sigma-Aldrich Co., Tokyo, Japan) were dissolved in nuclease free water according to the technical data sheet. They were used for RAPD
amplification as described by [2]. Briefly, the PCR amplification reaction was carried out in a 25 μl reaction volume containing 22 μl PCR Master Mix (12.5 μl AmpliTaq Gold® 360 DNA Polymerase, 1 μl 360 GC Enhancer, 8.5 μl nuclease free water), 1 μl primer (working solution 15 μM), and 2 μl of DNA template (concentration approximately 50 ng/μl). The conventional PCR was performed in a controlled thermal cycler (TC9610; MultiGene™ OptiMax Thermal Cycler, New Jersey, USA).

The first cycle consisted of activation of PCR Master Mix at 95 °C for 10 min and denaturation of DNA template at 95 °C for 1 min, followed by primer annealing at 37 °C for 1 min and primer extension at 72 °C for 2 min. In the next 44 cycles, the period of denaturation, annealing and extension time remained as in the first cycle. The last step was primer final extension at 72 °C for 5 min and final hold at 4 °C. PCR products were separated by electrophoresis (Mupid®-exu; Mupid Co., Ltd., Tokyo, Japan) at 100 volts for 50 min on 1.75 % agarose containing SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Invitrogen™, USA) using 1 x TAE buffer. The sizes of the amplified fragments were determined by using GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, Thermo Scientific™, USA). DNA fragments were visualized and photographed using gel documentation system (Uvidoc HD6; UVItec Ltd., Cambridge, UK). The interpretation of band patterns was coded binarily by the visual scores [23]. Jaccard’s similarity coefficient was used for clustering F1 progenies and their parents by unweighted pair-group method for arithmetic average analysis (UPGMA) cluster method. Dendrogram was drawn using cluster analysis options as available in MVSP 3.1 software.

3. Results and discussion

In the present study, we evaluated 8 yardlong bean genotypes consisted of five F1 progenies and three parentals. RAPD-PCR revealed the present of amplicons and the diversity was observable based on the number of bands (Figure 1). In the analysis of samples, five selected primers generated 16 scorable bands (loci) with their size ranging from 280 to 1160 bp (Table 2), and the average number of bands per primer of 3.20. It is lower than a number of bands used in [2] and [23]. Primer OPC-06 produced 6 bands, primer OPZ-13 produced 4 bands, while primers OPR-12, OPZ-03, and OPZ-08 produced 2 bands, respectively. Four new bands, which were not present in the parents, were detected in the certain progenies. Interestingly, some of them occurred in only one progeny: band 520 bp OPC-06 which was specific for the progeny FG x SP; band 310 bp OPC-06 was specific for the progeny AH x SP and band 280 bp OPC-06 was specific for the progeny SP x FG. The other band was detected in three progenies: band 460 bp OPZ-13 was present in SP x FG, FG x AH and SP x AH. This might indicate the existence of specific loci in the genotypes studied. Furthermore, detection of the band 500 bp generated by primer OPZ-08 in the parentals SP and AH, that were absent in the progenies, indicates the primer has potential to be used in identification of yardlong bean breeding materials.

Since variation occurs in different types of mutational events, as well as in the primer annealing site and between two adjacent sites which responsible for amplification [25], the presence or absence of bands indicates the genetic changes in the hybrids’ genome, through the loss or rearrangement of its nucleotides [26]. It might affect the types and number of the amplified DNA bands (Table 3). Five RAPD primers yielded a total of 15 polymorphic bands out of 16 bands, while primer OPR-12 revealed a monomorphic band. The common or monomorphic bands among the yardlong bean F1 progenies and their parents likely represent highly conserved regions in the genome. All primers revealed discriminating patterns which the number of bands ranged from 2 to 6. However, it shows the presence genetic polymorphism in these genotypes which may be used in yardlong bean breeding. On the contrary, there were some unique bands only in three progenies generated by primer OPC-06, indicates the presence of specific loci in the genotypes studied. Although not all genotypes gave unique bands with every primer, certain primer, i.e. OPC-06, revealed more polymorphism than others. The present study revealed the average polymorphism of 90 % indicating the presence of greater genetic difference among the genotypes. Moreover, the RAPD markers was found to be suitable for use with yardlong bean because of its consistent polymorphic markers. Therefore, the primers have used can be chosen to conduct a test of marker segregation in F1 progenies.
Figure 1. Amplification profiles of yardlong bean parentals and their F1 progenies DNA samples using RAPD markers. M = 1000 bp DNA ladder. White arrows indicate unique bands.

The genetic distance was calculated based on the Jaccard’s similarity coefficient. They ranged from 0.40 to 0.73, showing there was a high amount of genetic diversity among the genotypes. UPGMA dendrogram was drawn to visualize relationships among yardlong bean parentals and their F1 progenies (Figure 2). The genetic relationship with high coefficient (> 0.60) indicates the close relationship among the genotypes [27]. Two clusters were formed, the largest cluster consisted of 5 genotypes including parentals and hybrids, while the second cluster contained 3 genotypes including only hybrids. They were
separated in different clusters in the coefficient of 0.40. The first cluster was separated on the coefficient of 0.45 into cluster A that includes FG x SP, and cluster B. Cluster B was separated on the coefficient of 0.63 into cluster i and ii. Cluster i was separated on the coefficient of 0.67 into cluster a and b. Interestingly, SP x AH (cluster a) is closer to FG (cluster a) than AH (cluster b) or SP (cluster ii). It explains that hybridization fall outside the range of parental variation but might have advantage of transient hybrid vigor, move desirable variation, and generate novel characters, however it should be assessed in the F2 generation and later to distinct it from heterosis or transgressive segregation [28].

Table 2. Bands appearance generated by five RAPD primers in yardlong bean parentals and their F1 progenies

| Primer   | Genotypes | Marker (bp) | FG x SP | SP x FG | FG x AH | SP x AH | AH x SP | FG | SP | AH |
|----------|-----------|-------------|---------|---------|---------|---------|---------|----|----|----|
| OPC - 06 |           | 780         | +       | +       | +       | +       | +       | +  | +  | +  |
|          |           | 520         | +       |         |         |         |         | +  | +  | +  |
|          |           | 460         |         | +       | +       | +       | +       | +  | +  | +  |
|          |           | 370         |         |         | +       | +       | +       | +  | +  | +  |
|          |           | 310         |         |         |         | +       |         | +  | +  | +  |
|          |           | 280         |         |         |         |         |         | +  | +  | +  |
| OPR- 12  |           | 800         | +       | +       | +       | +       | +       | +  | +  | +  |
|          |           | 480         |         | +       | +       |         | +       | +  | +  | +  |
| OPZ-03   |           | 600         | +       | +       | +       | +       | +       | +  | +  | +  |
|          |           | 524         |         |         |         | +       |         | +  | +  | +  |
| OPZ-08   |           | 500         |         |         |         |         |         | +  | +  | +  |
|          |           | 400         |         |         |         |         |         | +  | +  | +  |
|          |           | 1160        | +       | +       | +       | +       | +       | +  | +  | +  |
|          |           | 690         |         |         |         |         |         | +  | +  | +  |
| OPZ-13   |           | 460         | +       | +       | +       | +       | +       | +  | +  | +  |
|          |           | 390         |         |         |         |         |         | +  | +  | +  |

Table 3. Types and number of the amplified DNA bands as well as the percentage of the total polymorphism revealed by five RAPD primers in yardlong bean parentals and their F1 progenies

| Primer | Monomorphic band | Polymorphic bands | Total band | Polymorphism (%) |
|--------|------------------|-------------------|------------|------------------|
| OPC-06 | 0                | 3                 | 3          | 6                |
|        |                  |                   |            | 100              |
| OPR-12 | 1                | 0                 | 1          | 2                |
|        |                  |                   |            | 50               |
| OPZ-13 | 0                | 0                 | 4          | 4                |
|        |                  |                   |            | 100              |
| OPZ-03 | 0                | 0                 | 2          | 2                |
|        |                  |                   |            | 100              |
| OPZ-08 | 0                | 0                 | 2          | 2                |
|        |                  |                   |            | 100              |

Reciprocal hybrids from crosses FG x SP (cluster A) and SP x FG (cluster iii) or SP x AH (cluster a) and AH x SP (cluster E), both were separated with its reciprocal on the coefficient of 0.40. Reciprocal differences are caused by unequal contribution of cytoplasmic determinants from female and male gametes to the zygote [29]. Possible explanations are nuclear-cytoplasmic interactions or parent-of-origin effects, which the differential transmission of the organelles allows contrasting chloroplast, and mitochondrial combinations, and identical nuclear genotypes [30]. If crosses and their reciprocals are
included, these estimated effects were not separated, indicating the contribution of each parent to the cross combination when this particular parent is used as a female or male [31]. However, whether this substandard performance is attributable to the genotypes used as the paternal parents or due to the maternal parents’ genotypes is not evident from this study. So, it is important to point out that the genetic contribution of the parental parentals to various components of the yardlong bean F1 progenies are equal in future studies. Moreover, the results indicate that evaluation of genetic relationships among closely related genetic materials, i.e. F1 progenies and their parents, can be very efficient using RAPD markers.

Figure 2. Dendrogram of yardlong bean parentals and their F1 progenies based on RAPD markers

4. Conclusions
Five primers generated 16 scorable bands with their size ranging from 280 to 1160 bp, and the average number of bands per primer of 3.20. Cluster analyses using UPGMA procedure were performed using MVSP 3.1 software and produced a dendrogram with two clusters. Jaccard similarity matrices among yardlong bean parental and their F1 progenies varied between 0.40 – 0.73. The results obtained revealed that use of RAPD markers can be an efficient, fast and inexpensive way for differentiation of yardlong bean F1 progenies and their parents, as well as to evaluate their genetic relationships.

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