

Diversity Analysis of 15 *Hibiscus* Accessions Based on RAPD Marker

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ABSTRACT

Indonesia has many species of *Hibiscus* genus such as *Hibiscus rosa-sinensis*, kenaf, rosella, waru, sharon, and others. These species have similar flower morphology despite their different benefits. Flower morphology can be used as morphological marker characters to identify the genetic relationship in one genus of *Hibiscus*. However, morphological markers are less accurate because they are strongly influenced by the environment. Therefore, the molecular markers using plant DNA are considered to be more accurate than morphological characters. Random Amplified Polymorphic DNA (RAPD) analysis was applied to 15 *Hibiscus* genus. The result showed that 10 primers operon produced polymorphic bands. The high diversity was observed in control population i.e., *H. cannabinus* (K1), *H. sabdariffa* (K2), *H. mutabilis* (K3), *H. syriacus* (K4), *H. schizopetalus* (K5), while in *H. rosa-sinensis*, the diversity was low especially on the genotypes with the same color of flower. These results suggest that RAPD can be used to identify the diversity in plant population. The control species [*H. cannabinus*, *H. sabdariffa*, *H. mutabilis*, *H. syriacus*, and *H. schizopetalus*] showed high heterozygosity, suggesting high diversity were observed in these species. Based on principal coordinate analysis (PCA), two clusters were formed, the control species were clustered depend on life cycle of plant growing season either perennial or annual, while *H. rosa-sinensis* accessions were clustered based on the flowers color. The variance within population of *H. rosa-sinensis* accessions was higher than among population, suggesting that *H. rosa-sinensis* is cross pollinated plants due to the position of stigma is higher than anthers.

Keywords: Diversity, *Hibiscus*, RAPD

INTRODUCTION

Hibiscus belongs to the *Malvaceae* family. The *Hibiscus* genus consists of 300 species with different growth characteristics ranging from small plants, shrubs, perennial crops, and annual crops spread over sub-tropical and tropical areas (Prasad, 2014). *Hibiscus rosa-sinensis* is able to grow and bloom throughout the year in both dry and rainy seasons, and even in a poor nutrient soil (Akpan, 2006; Kuligowska *et al.*, 2016). It has great variation of morphological characters such as flower colors and sizes, and the leaf shapes. The great variation in *Hibiscus rosa-sinensis* was observed because of crosses and mutation (Kadve *et al.*, 2012). Another variation is observed in the flower shape either single and double petals (Hammad, 2009). Indonesia has many species of *Hibiscus* genus such as *H. rosa-*

sinensis, kenaf, rosella, waru, sharon, etc. These species have similar flower morphology and different benefits such as traditional medicine (Pekamwar *et al.*, 2013) or ornamental plant because of flower variation (Harvey, 2004). *H. rosa-sinensis* has been used for traditional medicine in India because its flower contains antioxidant, antifungal, anti-infectious, antimicrobial, anti-inflammatory, anti-diarrheic, and antipyretic compounds (Patel *et al.*, 2012). *Hibiscus* consists of 300 species implying high diversity of this genus. Therefore, morphological and molecular marker can be used to reveal their phenotype and genotype diversities.

Flower morphology can be used as morphological marker characters to identify the genetic relationship in one genus of *Hibiscus*. However, morphological markers are less accurate because they are strongly influenced by the environment, requiring quite

amount of time, and showing limited and inconsistent diversity (Zulfahmi, 2013). Then, molecular markers are preferable to utilize because plant DNA is stable and not affected by environmental factors. One of the most used molecular marker is Random Amplified Polymorphic DNA (RAPD). RAPD is easy and uses random primer DNA sequences as marker (Kadve *et al.*, 2012). RAPD usually uses 10 bases short primer sequences to randomly amplify DNA genom. DNA fragmen amplification is performed using PCR machine at low annealing temperature (William *et al.*, 1990). The DNA bands amplified from PCR can be scored to identify the genetic relationship among 15 *Hibiscus* genus using dendogram.

MATERIALS AND METHODS

This research was conducted in June–November 2017 at the Plant Breeding Laboratory, Agriculture Department, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta. It used leaves of 15 *Hibiscus* genus consist of *H. cannabinus* (K1), *H. sabdariffa* (K2), *H. mutabilis* (K3), *H. syriacus* (K4), *H. schizopetalus* (K5) and *H. rosa-sinensis* (R1–R10). *H. rosa-sinensis* consist of 10 accessions with different flower colors i.e., red (R1–R3), white (R4–R6), yellow (R7–R8) and orange (R9–R10). Material used for DNA extraction is CTAB buffer solution, chloroform, isoamyl alcohol, sodium acetate 3M, isopropanol, and ethanol 70%.

DNA was extracted from a 0.1 g fresh and healthy leaf using CTAB method (Doyle and Doyle, 1990). Afterward, DNA pelette was diluted with ddH₂O. DNA concetration was measured with Gene Quant Spectrophotometer, then each sample of DNA was diluted with ddH₂O for temperature and primer optimization in PCR reaction. Ten optimized primers were used in this research i.e., OPA 13, OPB 18, OPC 2, OPD 2, OPD 5, OPD 7, OPD 8, OPD 18, OPD 20 and OPM 6. Optimization of annealing temperature was carried out at 35°C–45°C, then suitable temperature which produced brightest band was selected as annealing temperature.

DNA amplification was performed with PCR (Thermal Cycler BIORAD). The touchdown method was used by reducing annealing temperature from 41°C to 37°C. The amplification reaction stage was 95°C pre-heating for 5 min; 15 cycles of 95°C denaturation for 30 sec and specific annealing temperature for 30 sec; 72°C elongation for 90 sec; and 72°C final elongation for 7 min.

Gel electrophoresis was performed in 100 volts for 55 min using 1% agarose gel in TBE

buffer, visualized using UV light, and the image was captured with a digital camera. The bands were scored to form a binary data, 1 = the band was amplified, and 0 = no band.

The data was analyzed using Sequential, Agglomerative, Hierarchical, and Nested (SAHN) with Unweight pair-group method, arithmetic average (UPGMA) method for cluster analysis, in NTSYS-PC version 2.02 programs. Principal Coordinate Analysis (PCA) was performed using the same program, to illustrate the distribution of 15 *Hibiscus* genera. The number of specific alleles in each population, analysis of molecular variance (AMOVA), heterozygosity (H), the number of observed alleles (Na), and the number of effective alleles (Ne) were analyzed with GenAEx 6.1. The specific alleles was determined by the appearance of a specific band in one specific population and that was not found in other populations. Analysis of molecular variance was used to explain the distribution and magnitude of genetic diversity in and/or between populations.

RESULTS AND DISCUSSION

H. rosa-sinensis, one of the species in *Hibiscus* genus, the most common species found in Indonesia. Because of its flower variation, it was used as an ornamental and fence plant. Therefore, the purpose of this study was proposed to identify the diversity and genetic relationship of 15 *Hibiscus* genus. All of the species showed high diversity of flower morphologies as shown in figure 1. The variation of flower shapes was observed either within *H. rosa-sinensis* accessions or among other *Hibiscus* species

Hibiscus genus consists of annual crops group such as *H. cannabinus* (K1) and *H. sabdariffa* (K2), and perennial crops group such as *H. mutabilis* (K3), *H. syriacus* (K4), *H. schizopetalus* (K5) and *H. rosa-sinensis* (R1–R10). *H. rosa-sinensis* accessions was separated by the flower color i.e., red flower (R1–R3), white flower (R4–R6), yellow flower (R7–R8), and orange flower (R9–R10). All *Hibiscus* genera were generally had similar utility for ornamental, medicinal, and fiber source plants. Genetic relationship analysis was performed in accordance to the similarity of morphology, lifespan, and utility among several types of *Hibiscus*. Five from ten primers showed 100% of polymorphic bands (Table 1). OPB 18, OPD 7, OPD 18, OPD 20, and OPM 6 showed 100% of polymorphic bands suggesting that 15 *Hibiscus* genera had high diversity.

Genetic relationship of 15 *Hibiscus* genera using 10 RAPD primers was identified by dendogram

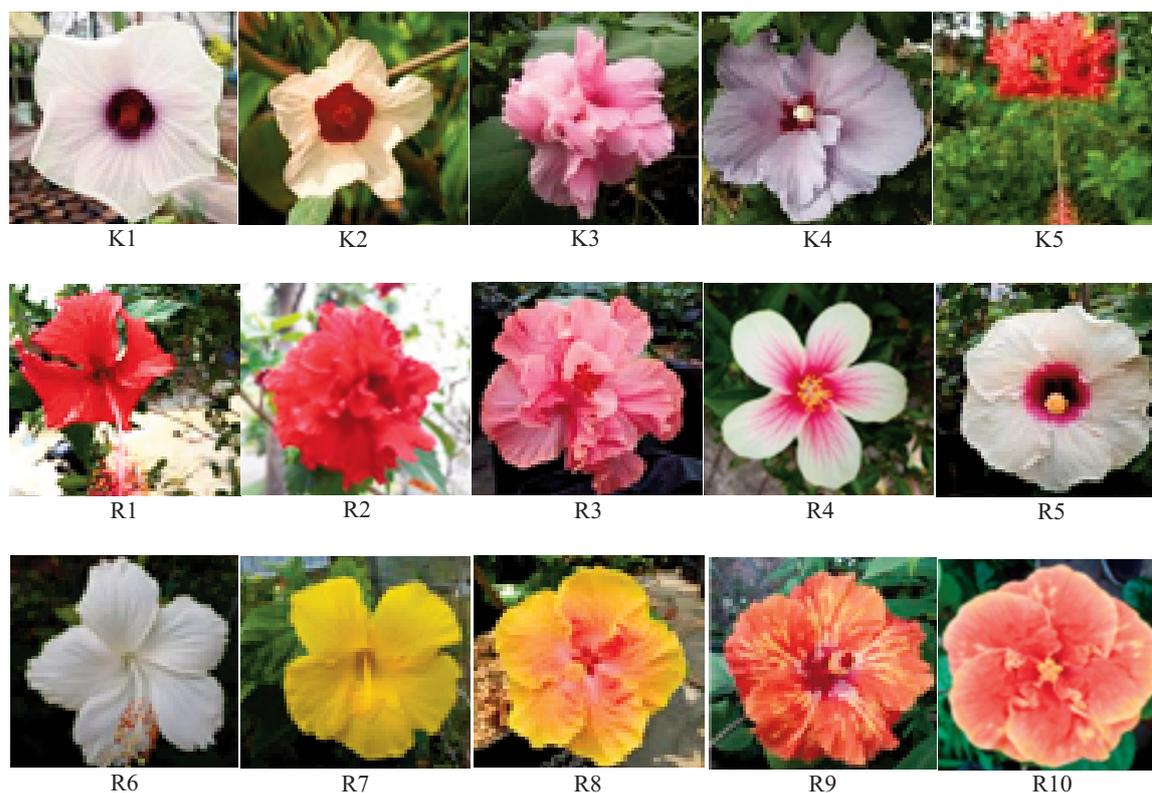


Figure 1. 15 genus hibiscus diversity

Note: K1 : *H. cannabinus*, K2 : *H. sabdariffa*, K3 : *H. mutabilis*, K4 : *H. syriacus*, K5 : *H. schizopetalus*, R1-R10: *H. rosa-sinensis*

Table 1. Polymorphic Bands 15 Hibiscus Genus in 10 Primer RAPD

Primer	Total No. of bands	Total No. of polymorphic bands	Total No. of monomorphic bands	Polymorphism (%)	Monomorphism (%)
OPA 13	15	14	1	93.33	6.67
OPB 18	21	21	0	100	0
OPC 2	19	18	1	94.74	5.26
OPD 2	16	14	2	87.5	12.5
OPD 5	13	12	1	92.31	7.69
OPD 7	17	17	0	100	0
OPD 8	17	16	1	94.12	5.88
OPD 18	21	21	0	100	0
OPD 20	19	19	0	100	0
OPM 6	17	17	0	100	0
Total	175	169	6	96.2	3.8

(Fig. 2). The result showed that the genetic relationship coefficient was 0.78 (the closest) and 0.36 (the farthest). R9 and R10 were the two most similar genotypes, due to their genetic relationship coefficient (0.78) and their similar phenotype appearances. Dendrogram categorized 15 genotypes into two main groups. The first group consisted of *H. cannabinus* (K1), *H. sabdariffa* (K2), and *H. mutabilis* (K3); the second group consisted of *H. syriacus* (K4), *H. schizopetalus* (K5) and *H. rosa-sinensis* (R1–R10). In the first group (A), K1 was separated from K2 and

K3 with 0.42 similarity coefficient, while K2 and K3 had similarity coefficient (0.47). The second group was categorized into two smaller groups, B1 and B2. B1 group consisted of 2 genotypes, K4 and R6, with 0.41 similarity coefficient, and B2 group was categorized into two groups, B.2.1 and B.2.2, where K5 was separated from the other 9 genotypes with the similarity coefficient is 0.43. B.2.2 group was categorized into two small groups, B.2.2.1 and B.2.2.2. B.2.2.1 consisted of three R1 genotypes with 0.63 similarity coefficient, and separated from

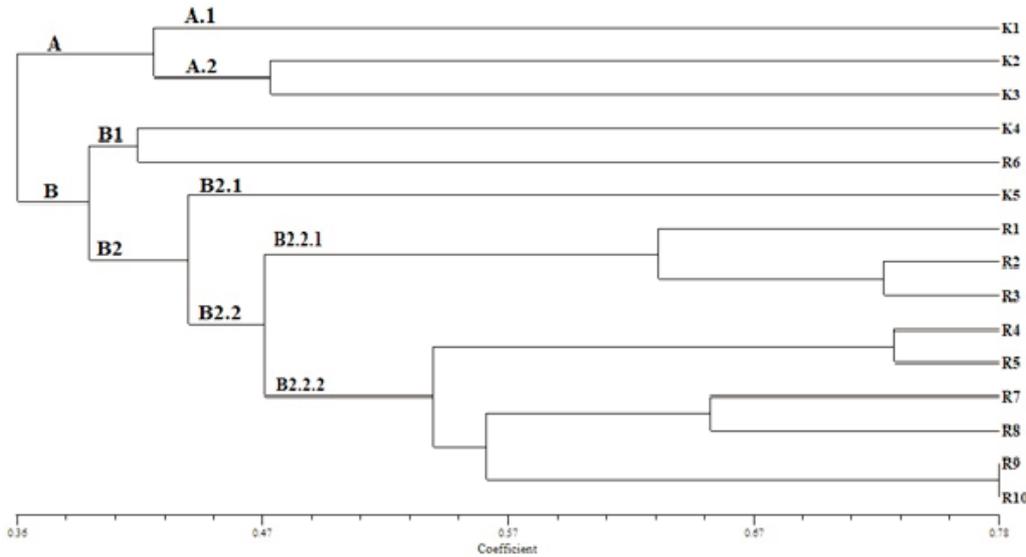


Figure 2. Dendrogram Diversity Analysis using RAPD Marker

R2 and R3 genotypes with the similarity coefficient was 0.72. Then B.2.2.2 consisted of 6 genotypes where R9 and R10 have the closest genetic relationship (similarity coefficient 0.78) compared with other genotypes (R7 and R8 with similarity coefficient 0.65, and R4 and R5 with similarity coefficient 0.73). *H. rosa-sinensis* group was tend to gather, separated from the other *Hibiscus* species.

Generally, the 15 *Hibiscus* accessions were grouped based on their population. The PCA result showed that all *Hibiscus* species were organized according to the flowers color (Fig. 3). Populations of the red *H. rosa-sinensis* are gathered into one group, as well as the orange one. While on the yellow, white, and control group, there were separated from those genotypes. K4 and K5, the perennial crops, were separated from K1 and K2, the annual crops. However, the white and yellow *H. rosa-sinensis* species were assembled in one group. These results suggest that all of 15 *Hibiscus* accessions were

assembled in accordance with their flower colors.

Several variables such as observed alleles, effective alleles, heterozygosity, and Shannon index, were used to evaluate genetic diversity in a population (Pereira *et al.*, 2015) and calculated from the three population's data (Table 2). Heterozygosity values of each population was 0.21 (control), 0.13 (red *H. rosa-sinensis*), 0.15 (white *H. rosa-sinensis*), 0.1 (yellow *H. rosa-sinensis*), and 0.06 (orange *H. rosa-sinensis*). These results suggest that the control group, consist of *H. cannabinus*, *H. sabdariffa*, *H. mutabilis*, *H. syriacus* and *H. schizopetalus* had the highest genetic diversity, whereas the orange group had the lowest genetic diversity. The observed allele value (N_a) and Shannon Index (I) analysis, control group and orange group were exhibited the highest and the lowest genetic diversity, respectively. Effective allele value (N_e) is usually smaller than N_a value in control population because the population consist of different kinds of *Hibiscus* species. However, in other population, N_e

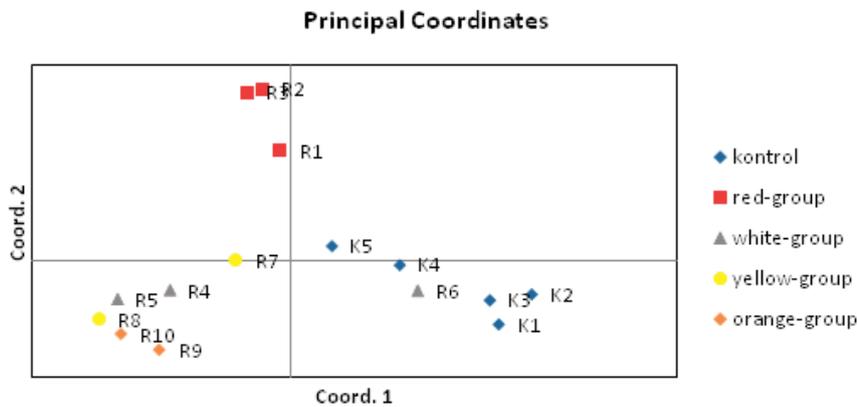


Figure 3. PCA Result of 15 *Hibiscus* Accessions

Table 2. Average of observed allele (Na), Effective allele value (Ne), Shannon Index (I) and heterozygosity (h)

Population	N	Na	Ne	I	H
Control	5	1.49 ± 0.064	1.31 ± 0.021	0.33 ± 0.017	0.21 ± 0.012
Red-group	3	0.82 ± 0.068	1.22 ± 0.027	0.19 ± 0.021	0.13 ± 0.014
White-group	3	0.92 ± 0.072	1.25 ± 0.026	0.23 ± 0.021	0.15 ± 0.014
Yellow-group	2	0.74 ± 0.063	1.18 ± 0.023	0.15 ± 0.020	0.1 ± 0.014
Orange-group	2	0.56 ± 0.056	1.11 ± 0.019	0.09 ± 0.016	0.06 ± 0.011
Average	3	0.91 ± 0.031	1.21 ± 0.011	0.20 ± 0.009	0.13 ± 0.006

Table 3. AMOVA

Source	df	Sum Square (SS)	Mean Square (MS)	Est. variance	Variance (%)
Among Populations	4	226.80	56.7	8.69	22
Within Populations	10	315.07	31.51	31.51	78
Total	14	541.87		40.19	100

value is bigger than Na because of the random mating was occurred in *H. rosa-sinensis*, and it was a cross pollinated plant.

Table 3 showed the value of genetic diversity within and between populations. Diversity value between different flowers of *H. rosa-sinensis* flower was varied. AMOVA showed the same pattern of genetic diversity within and between populations and it was used to identify the distribution of genetic diversity within and between populations. AMOVA result (Table 3) showed that 78% of the total genetic diversity was diversified within genotype population, while the rest was diversified between populations. Distribution patterns of genetic diversity, as well as the percentage of diversity within population, was higher than between populations, which commonly found in cross-pollinated plants.

Genetic diversity within population is resulted from its sexual reproduction (Pereira *et al.*, 2015). Then, the great diversity found in the cross pollinated crops was caused random mating. Meanwhile, population diversity is the average of genetic diversity from entire genotypes within the population. In cross-pollinated plants, although the genotypes generating each population vary, diversity value between one population and the other tend to be small, so the diversity between populations is small. This is in accordance with Hamrick *et al.* (1992), that wood species with cross-breeding system and seed dispersal caused by wind or animal will maintain more variation within species and population than other species, but the diversity between populations is smaller.

CONCLUSIONS

The molecular marker is a good marker to reveal the diversity in a population. Highest heterozygosity value in control group genotypes showed that there was a high diversity in control plants. Based on dendrogram result, *H. rosa-sinensis* had a close genetic relationship with *H. schizopetalus*. Based on the PCA result, the grouping in control plants was assembled according to their growth cycles (perennial and annual), while in *H. rosa-sinensis* the grouping was based on the flowers color. *H. rosa-sinensis* is cross pollinated plants because the stigma position is higher than anthers, and the varians within population of *H. rosa-sinensis* was higher than among population.

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