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Morpho Genetic Variability and Anthocyanine (Cyanidin-3-O-Glucoside) Concent of Indonesia Roselle (*Hibiscus sabdariffa* L.)

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***Abstract*— Roselle (*Hibiscus sabdariffa* L.) is a popular plant due to its colored calyces for herbal medicines and its stem for fibers. The development of new varieties of roselle with high-accumulating anthocyanins and productivity has become one of the breeding targets. Local varieties and selected accession roselle may be used as potential sources of important traits for breeding. Therefore, this study aimed to evaluate the phenotypes, genetics, and anthocyanin (cyanidin-3-O-glucoside) levels of four varieties and roselle accession with high yields that were grown at Cibinong, West Java, Indonesia. Results on phenotypic characterization in all genotypes showed variations in the red color intensities in flower organs. The color intensities of calyces and corollas are related; calyces with darker color intensities have darker color intensities in corollas. Genetic analysis using AFLP and RAPD markers revealed a high genetic diversity index, with average heterozygosity and diversity levels of 1 and 0.71 for AFLP and 0.995 and 0.63 for RAPD, respectively. The anthocyanin of cyanidin-3-O-glucoside levels ranged from 0.07 mg/100 g flower dry weight (FDW) in green roselle genotypes to 446 mg/100 g FDW in Roselindo 4. Two accession roselles, RUB1 and RUA7, showed the highest potential yields of dry calyx, reaching up to 600 kg/ha. Roselindo 4 has higher anthocyanins and RUB1 and RUA7 have higher potential yields. These are the parents we will use in the future for breeding new roselle cultivars that have higher anthocyanins and higher yields.**

***Keywords*— Rosella; morphology; AFLP; RAPD; Cyanidin-3-O-glucoside.**

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I. INTRODUCTION

Roselle (*Hibiscus sabdariffa* L./HS) is a member of the Malvaceae family of fiber plants. Roselle plants are typically annuals but can be perennials in tropical and subtropical regions. All parts of the roselle plant, from the bark and stems to the leaves, seeds, and calyx, can be utilized, but the calyx is the most commonly [1]. Roselle flower calyx is a common ingredient in herbal medicine and natural red food coloring. In the structure of the flower, the calyx covers the fruit. According to Mahunu [2], the morphological structure of the roselle calyx is distinguished by the various characteristics ranging from green with red stripes but inedible, such as *bhagalpuriensi*, to greenish-yellow, edible, and fiber-producing *intermedius* and *albus*, as well as red calyces that can be consumed fresh or dried.

All parts of roselle contain phytochemicals that have the potential to exert pharmacological effects including groups of anthocyanins, phenols, alkaloids, tannins, flavonoids, saponins, and organic acids. Roselle calyx is rich in vitamin C and antioxidants including anthocyanins, flavonoids, gossypetine, hibiscetine, and sabdaretine, while the seeds contain phytosterols, tocopherols, and amino acids [3]–[6]. Furthermore, roselle compound extracts have antibacterial, antifungal, anti-inflammatory, anti-diabetic, antioxidant, nephro- and hepato-protective, renal/diuretic, anticancer, and antihypertensive properties. The pharmacological effects are elicited by strong antioxidant activity, inhibition of a-glucosidase, a-amylase, and angiotensin-converting enzyme

(ACE), as well as direct vaso-relaxant effect or calcium channel modulation.

Anthocyanins are water-soluble polyphenol pigments that change color from red to blue [7]. The anthocyanins found in roselle are a source of antioxidants with anti-inflammatory, hepatoprotective, and cancer-cell growth-inhibiting properties [8]. Several varieties that have been identified from the plant's callus and calyx include Cyanidin-3-O-glucoside, Delphinidin-3-O-glucoside, Cyanidin-3-O-sambubioside, Delphinidin-3-O-sambubiosid, Malvidin-3-O-glucoside, and Petunidin-3-O-glucoside [9].

Roselle is highly viable for cultivation in Indonesia due to its adaptability to tropical climates [2]. Several Indonesian roselle varieties have been developed and released, including Roselindo 1, Roselindo 2, Roselindo 3, and Roselindo 4, which have a potential yield range of 471,448 to 554,73 kg/ha dry weight. On the other hand, our previous study found 13 genotypes with yield potentials ranging from 497.5 to 656.31 kg/ha dry weight [10] that can be used to improve varieties.

Roselle is an important medicinal plant, and it is essential to provide superior roselle types to meet the growing demand for its pharmacologic effects. To create new and superior varieties, natural or artificial genetic diversity in germplasm is required [1], [11]. Genetic diversity is the foundation for crop improvement. Diversity of Plant Genetic Resources (PGRs) allows plant breeders to develop or improve plant varieties with desired qualities, such as crops with high yield and anthocyanin content. Diversity in germplasm can be characterized by morpho-agronomic, biochemical/ physicochemical, and molecular genetic characteristics. Morpho-agronomic characters in roselle are distinguished based on the calyx color and quantitative agronomic features [12]

Meanwhile, the genetic variety or diversity can be analyzed using amplification-based molecular markers include Randomly Amplified Polymorphic DNA (RAPD), Direct amplification of minisatellite DNA (DAMD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), and Inter simple sequence repeat (ISSR) [13]. The AFLP method was utilized on *Hibiscus rosasinensis* and *Hibiscus sabdariffa* from Nigeria [14], [15]. In the genus *Hibiscus*, RAPD genetic analysis was used to compare *H. sabdariffa* with *H. cannabinus* [16], [17]. Furthermore, three single primary amplification reactions namely SPAR: RAPD, ISSR, and DAMD were compared to determine the genetic diversity of roselle based on in-vitro subculture frequency [13]. Molecular marker-based genetic analysis has been shown to identify diversity more precisely.

The combination of morpho-agronomy, physicochemical, and molecular diversity data, together with potential yield data, can serve as a database for selecting or improving high-yielding genotypes/lines/varieties through breeding. Breeding plays an important role in breeding crops including Roselle (*Hibiscus sabdariffa*). Through breeding, desirable traits can be enhanced or introduced into roselle varieties to meet specific agricultural, nutritional and market needs. Therefore, this study aimed to examine phenotypes, genetics, and anthocyanins (cyanidin-3-O-glucoside) levels of 17 roselle genotypes, including four varieties and 13 accession of high yield roselle grown at Cibinong, West Java, Indonesia.

This study is essential important for selecting genetic material for future roselle breeding, with the main target of new cultivars with higher anthocyanin and higher yields.

II. Material and Methods

A. Location and Time of Study

This study was conducted in 2021 at the Research Center for Genetic Engineering as well as Vaccines and Drugs-National Research and Innovation Agency-Indonesia.

B. Genetic Material

The genetic materials used were of four roselle varieties namely Roselindo 1, Roselindo 2, Roselindo 3, Roselindo 4, and 13 lines previously selected from seeds due to high potential yields. These were RUB 1, RUB 22, RUB 21, RUB 11, RUB 5, RUA 13, RUA 10, RUA 7, RUA 9, RUA 23, Green 11, Green 12, and Green 13 (Hartati et al., 2021). The seeds of roselle were planted for seven days in a nursery tub, then the healthy seedlings obtained were potted and kept in a greenhouse. The experiment was repeated three times for each genotype. The planting medium consists of a mixture of soil, manure, husk, and lime with a volume of approximately 10 kg per pot.

C. Phenotyping Analysis

Five replicates were used to observe the vegetative and generative phenotypes of the plant. The qualitative phenotypic characteristics of the vegetative phase included stem color, petiole, leaf blade, length, shape, and margins. As for the generative organs, the color of the corolla and calyx were also observed. The color observation was carried out using Munsell's Plant Tissue Color Book (PN: M50150B-2012, Michigan USA).

D. Genotyping Analysis

1) AFLP (amplification fragment length polymorphism): DNA isolation referred to the method of [18] wherein a total of 400 ng roselle sample DNA was digested with *Eco*R1 enzyme (5 units) at 37ºC for 2 hours and followed by *Tru*I1 (5 units) at 65ºC for 2 hours. The results of digested genomic DNA were ligated with *Eco*R1 adapters (reverse and forward, 50 pmol each per reaction) and *Tru*I1 adapters (reversed and forward, 50 pmol each per reaction). Ligation reaction occurred at room temperature overnight and 5 ul of the solution was used as template DNA for preamplification using the MyTaq Red mix kit (Bioline) with the primers *Eco*R1-A and *Tru*I1-C, 40 pmol each per reaction. The preamplification conditions were 21 cycles at 94ºC, 30 S; 56ºC, 60 S; and 72ºC, 60 S. The PCR results were diluted in a ratio of 1:7 and used as a DNA template for the selective amplification reaction with the MyTaq Red mix kit (Bioline) and 8 pairs of primers E-AAG/M-CAC; E-AAT/M-CTG, E-ACT/M-CTG, E-ACT/M-CTT, E-AGC/M-CTT, E-AAG/M-CAG, E-ACT/M-CAG, E- ACA/M-CTC 40 pmol each per reaction. The selective amplification conditions were 13 cycles at 94ºC, 30 S; 65ºC, 30 S; 72ºC, 60 S and followed by 25 cycles at 94ºC, 30 S; 56ºC, 60 S; 72ºC, 60 S. Subsequently, the PCR product was electrophoresed on 3% agarose superfine resolution (SFR) (Amresco) at 50 Volts for 90 minutes. Image

visualization was then performed on a Syngeneic G: Box Gel Image Analysis System machine.

2) RAPD (random amplified polymorphism DNA): Approximately 100 mg of the isolated DNA was analyzed by RAPD using 25 primers, including OPA-7, OPA-13, OPB-10, OPB-14, OPC-05, OPN-12, OPA-01, OPA-02, OPA-4, OPA-10, OPA-11, OPB-7, OPB-11, OPG-5, OPG-13, OPG-18, OPG-8, OPA-06, OPA-12, OPD-7, OPG-17 [19], [20]. OPAF-08, OPP-01, OPQ-20, and OPM-01 which have not been previously reported. The 12.5 µl PCR reaction volume contained 6.25 µl of MyTag HS RedMix buffer, 1 µl of 100 ng of DNA, 1 µl of 10x primer, and 4.25 µl of dH2O. The following conditions were used for amplification: pre-denaturation at 94°C for 3 minutes, amplification at 94°C for 20 S, annealing temperature adjusted for 40 S, and synthesis at 72°C for 60 S in 40 cycles. Furthermore, primer elongation was performed for 7 minutes at 72°C. The 2% agarose gel was stained with 4% SYBR DNA Stain (Firstbase: Florosafe BIO-5170) at 50 Volts for 90 minutes, and the PCR results were visualized on the Syngeneic G: Box Gel Image Analysis System.

E. Analysis of Diversity

DNA bands from PCR products were scored to generate binary data, then the value of PIC (Polymorphism Information Content) and the diversity of each sample were analyzed using Gene Calc website https://gene-calc.pl/pic. The primary informative level for the PIC value was adjusted according to [21]. Subsequently, a variation analysis was performed using Past software and the Jaccard coefficient. The Jaccard coefficient is used to measure the genetic distance between two genotypes. The principal component analysis (PCA) was also performed.

F. Anthocyanin Analysis/Cyanidin-3-O-Glucoside Content

Roselle extraction referred to the method described by [22] wherein 100 g dry weight of calyx was used. The combined filtrates were frozen at -20°C, freeze-dried, and weighed. The anthocyanin marker compound, cyanidin-3-O-glucoside chloride, was analyzed using HPLC instruments with a modified technique. The following conditions were applied to the UFLC Shimadzu Prominence LC-20AD pump, SIL-20A autosampler, SPD-M20A photodiode array detector, and CBM-20A controller: Column C18, mobile phase comprising 225 mL methanol, 225 mL acetonitrile, 100 mL acid format, and 400 mL aquadest in degas for 20 minutes, injection volume of 10 µL and 20 µL for the standard and extract respectively, room temperature, flow rate of 1 mL/minute, and wavelength of 535 nm.

III. Results and Discussion

A. Agronomical Traits and Phenotyping Analysis

The 17 roselle genotypes population (4 varieties and 13 accession) have different dry calyx potential yields ranging from 364 to 656.3 kg/Ha (Table 1). This data can be combined with morphology, metabolites, and genetics to select breeding strategies for roselle.

TABLE I
ACCESSION NAME, POPULATION TYPE AND POTENTIAL YIELD OF DRY CALYX OF 17 INDONESIAN ROSELLE (H. SABDARIFFA) ACCESSIONS

No	Roselle Accession	Population	Potential yield of dry calyx (kg/ha)	References
1	RUA 7	Accession	656.3	[10]
2	RUA 9	Accession	511.6	[10]
3	RUA 10	Accession	497.5	[10]
4	RUA 13	Accession	510.5	[10]
5	RUA 23	Accession	557.9	[10]
6	RUB 1	Accession	612.2	[10]
7	RUB 5	Accession	532.6	[10]
8	RUB 11	Accession	469.5	[10]
9	RUB 21	Accession	577.2	[10]
10	RUB 22	Accession	575.7	[10]
11	Green 11	nd	364	Unpublished data
12	Green 12	nd	523.4	Unpublished data
13	Green 13	nd	316.3	Unpublished data
14	Roselindo 1	Variety	544.97	http://balitas.litbang.pertanian.go.id/index.php
15	Roselindo 2	Variety	478.59	http://balitas.litbang.pertanian.go.id/index.php
16	Roselindo 3	Variety	471.45	http://balitas.litbang.pertanian.go.id/index.php
17	Roselindo 4	Variety	554.73	http://balitas.litbang.pertanian.go.id/index.php

Characterization of the phenotypes including accessions and varieties of the 17 genotypes for the color of the petioles revealed that they were predominantly red (5R) with 10 distinct gradients (Table 2). A total of 6 exhibited color consistency, while the remaining did not. There were 5 color gradients in the yellowish green (7.5 GY) leaves of roselle. The corolla of the flower was red and green, with 5R and 2.5R being the dominant colors of all tested species and only Roselindo 3 was green (5Y 8/2). Furthermore, the Green genotypes 11, 12, and 13 were lighter in color or tend to be reddish-white/2.5R 8/2 than other genotypes whose leaves were red.

TABLE II
COLOR CHARACTER SCORES FOR THE PETIOLE, LEAF BLADE, COROLLA, AND CALYX OF 17 INDONESIAN ROSELLE

No	Genotype	Petiole					Leaf					Corolla					Calyces				
		Replication																			
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1	RUA 7	2	2	2	2	1	1	1	3	3	1	7	2	2	2	7	5	5	5	5	5
2	RUA 9	1	1	1	1	1	3	1	3	3	3	7	7	7	7	7	4	4	4	4	4
3	RUA 10	2	2	2	2	2	3	3	3	1	1	2	2	2	2	2	3	3	3	3	3
4	RUA 13	2	2	2	2	2	3	1	1	1	5	3	3	3	3	3	2	2	2	2	2
5	RUA 23	4	7	1	4	1	1	1	1	1	3	9	9	4	4	4	6	6	6	6	5
6	RUB 1	1	1	9	9	9	1	1	2	3	3	1	1	1	1	1	1	1	1	1	1
7	RUB 5	1	1	1	1	1	3	1	1	1	1	7	7	7	7	7	4	4	4	4	4
8	RUB 11	1	1	1	1	2	3	1	1	1	1	8	8	8	8	8	1	1	1	1	1
9	RUB 21	2	2	1	2	1	1	1	1	1	3	8	8	8	7	7	3	3	3	3	3
10	RUB 22	1	1	1	1	1	3	1	1	3	1	2	2	2	2	2	5	5	5	5	5
11	Green 11	5	5	2	6	5	1	1	3	1	6	10	10	10	10	10	8	8	8	8	8
12	Green 12	5	6	6	10	5	1	4	1	1	6	10	10	10	10	10	8	8	8	8	8
13	Green 13	6	6	6	6	6	1	4	1	1	6	10	10	10	10	10	8	8	8	8	8
14	Roselindo 1	3	1	2	1	2	1	1	1	1	3	2	2	2	2	2	1	1	1	1	1
15	Roselindo 2	2	2	2	1	1	1	1	1	3	1	6	6	6	6	6	3	3	3	3	3
16	Roselindo 3	4	4	4	4	4	1	1	1	1	1	5	5	5	5	5	7	7	7	7	7
17	Roselindo 4	1	8	2	2	2	1	1	3	1	6	7	7	7	7	7	4	4	4	4	4

Note:

- R = Red; GY = Green yellow; Y = Yellow,
- Numeric notation of gradient color scores according to the Munsell Plant Tissue Color Book (PN: M50150B-2012, Michigan USA).
- Code for the petiole: 1=5R 3/6; 2=5R 3/4; 3=5R 3/1; 4=5R 5/6; 5=5R 4/6; 6=5R 4/4; 7=5R 6/8; 8=5R 3/4; 9=5R 3/8; 10=5R 4/2
- Code for the leaf blade: 1 = 7.5 GY 4/4; 2 = 7.5 GY 3/2; 3= 7.5 GY 3/4; 4= 7.5 GY 4/2; 5= 7.5 GY 5/4; 6= GY 4/4
- Code for the corolla: 1=5R 8/4; 2=5R 7/4; 3=5R 7/6; 4=5R 8/2; 5=5Y 8/2; 6=5R 6/4; 7=2.5R 6/6; 8=2.5R 6/8; 9=2.5R 5/8; 10= 2.5R 8/2
- Code for the calyx: 1=5R 4/10; 2=5R 6/4; 3=5R 3/6; 4=5R 3/8; 5=5R 3/10; 6=5GY 6/6; 7=2.5 GY 7/6; 8= 2.5R 5/1

The plant's flower calyx is frequently consumed and appears to be a phenotypic dominant marker, while the corolla tends to be divided into red and green. There are 7 levels of red, and one green genotype, specifically, 11, 12, and 13 were between red and green with a brighter red level than the other genotypes. Figure 1 and Table 2 show a representation of the phenotypic performance obtained.

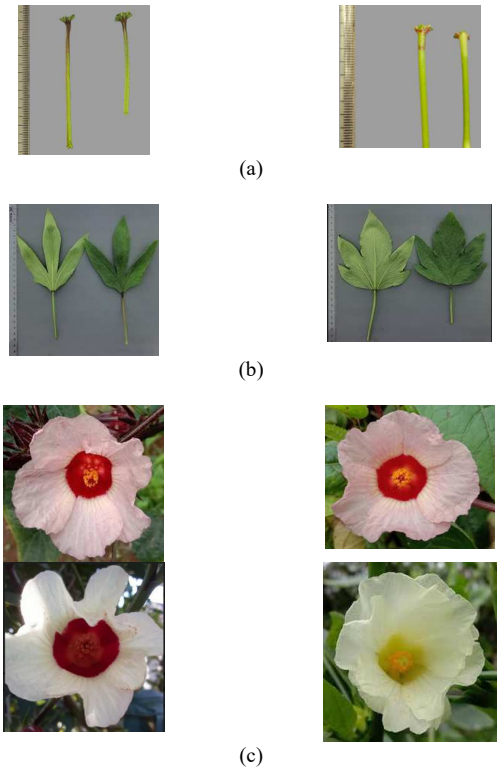


Fig. 1 Phenotypic representation of the color diversity roselle of the petiole (A), lamina (B), corolla (C) and calyx (D

The color characteristics of the corolla and calyx of flowers seemed to be related meaning that when the color of the corolla tends to be darker, the calyx will also be darker, and vice versa. The predominant calyx colors of the 17 identified roselle species were red (76.5%), green (5.7%), and a combination of both (17.5%). The color distribution of the calyx differs from those of 60 roselle collections from Nigeria, in which 41.7% were green, 20% were dark red, and 6.7% were pink [12]. Similarly, compared to 55 roselle collections from Ghana, there was a high proportion of green calyxes [23].

The single leaf of the Roselle plant typically has three to five lobes with varying depths and a slender, elongated appearance as shown in Table 2. For instance, the left leaf lobes are more substantial than the right as depicted in Figure 1b. There was no difference between the tested genotypes in terms of leaf shape between the genotypes tested and the leaf margins of every genotype were jagged.

The quantitative characteristics tested with ANOVA revealed significant differences between the shortest and longest leaves of each genotype, with Green 11 and 12 having the shortest leaf size, while Roselindo 1 and 3 had the longest. Similarly, the shortest genotype for leaf width was Green 11 and the longest was Roselindo 1, while the number of leaf lobes ranged from three to five. The average genotypes of plants with three lobes were RUB 1, RUB 22, RUB 11, RUA 7, and green 13, which was significantly different from Roselindo 3 with an average of five lobes as shown in Table 3.

TABLE III
SIZE AND NUMBER OF LEAF LOBE OF 17 ROSELLE

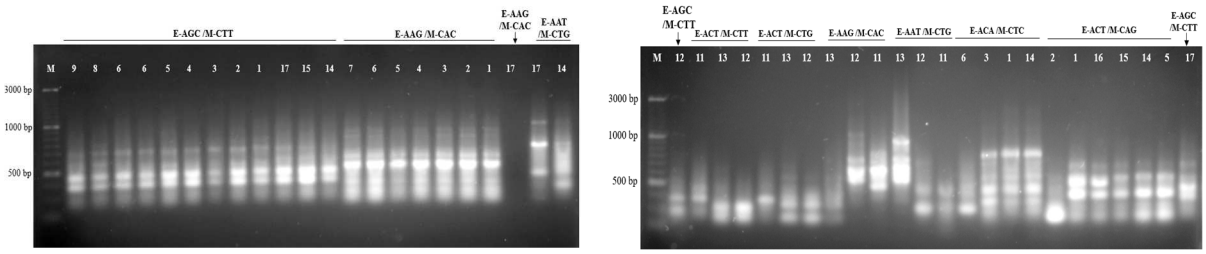
No	Genotype	Leaf length (cm)	Leaf width (cm)	Number of leaf lobes
1	RUA 7	12,20±0.35 bcde	12,13±1,01 ab	3±0.0 a
2	RUA 9	13,48±0.10 f	14,10±0.69 cdef	4,25±1 bcde
3	RUA 10	12,60±0.33 bcdef	14,33±1,03 def	4,5±0.6 cde
4	RUA 13	11,70±0.75 cdef	12,28±0.65 abc	3,5±0.6 abc
5	RUA 23	11,70±0.75 cdef	12,28±0.65 abc	3,5±0.6 abc
6	RUB 1	13,35±0.24 f	13,98±0.50 cdef	3±0.0 a
7	RUB 5	11,70±0.91 bc	12,28±1,69 ab	3,5±0.6 abc
8	RUB 11	11,60±0.99 b	11,73±0.89 ab	3±0.0 a
9	RUB 21	12,13±0.50 bcde	13,43±0.30 bcde	4,75±0.5 de
10	RUB 22	12,85±0.52 def	14,08±0.74 cdef	3±0.0 a
11	Green 11	10.55±0.45 a	11,65±1,14 a	4,5±1 cde
12	Green 12	9,95±1,14 a	12,18±1,22 ab	3,75±0.0 abcd
13	Green 13	11,88±0.39 bcd	12,53±0.50 ab	3±0.9 a
14	Roselindo 1	12,23±0.53 g	12,93±0.82 f	4,25±0.5 bcde
15	Roselindo 2	12,23±0.83 bcde	12,93±0.85 abcd	3,25±0.0 ab
16	Roselindo 3	14,78±0.68 g	14,43±0.71 ef	5±1 e
17	Roselindo 4	12,83±0.56 def	13,00±0.50 abcde	4,25±1 bcde

Note: The F test was statistically significant at the 0.05 level for character Leaf length, Leaf width and Number of leaf lobes

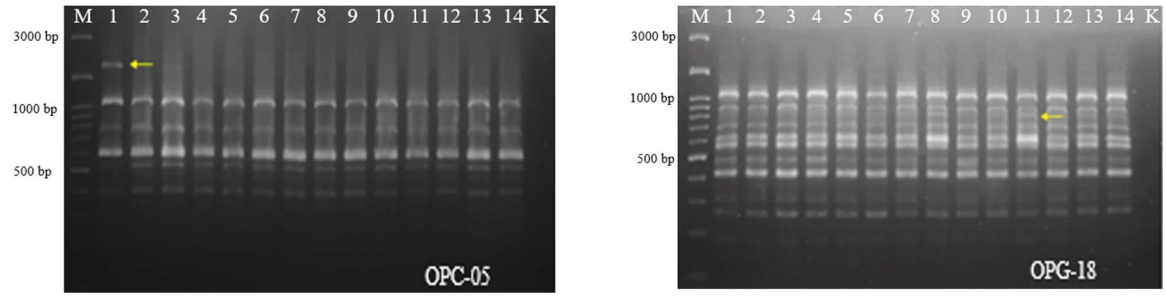
B. Genotyping Analysis

The PCR electropherogram of DNA using ALFP and RAPD markers in Figure 2 showed differences in banding patterns between primers and genotypes. AFLP genetic analysis using eight pairs of primers revealed that all samples could be amplified and were polymorphic. Table 4 presents the diversity index of the 17 roselle genotypes tested with RAPD and AFLP markers. AFLP analysis using 8 pairs of primers yielded an average of 11,625 alleles with a range of 7-13 and a main frequency of 0.5. Based on the results, the gene level was 0.71, indicating that all AFLP primer pairs used were able to distinguish heterozygous alleles in roselle samples.

Furthermore, the average PIC value ranged from 0.622 to 0.712, which is higher than the values of 109 *Hibiscus* species including 94 *H. rosasinensis* and 11 other *Hibiscus* species analyzed with 8 AFLP primers, with a PIC value of 0.203 [14]. The RAPD analysis on 25 primers yielded an average of 6,375 alleles, with a primary allele of 0.5. The most dominant and the highest main allele frequency in primer OPB14 were 16 and 0.758, respectively, while the average level of gene diversity in a population was 0.63. Based on the results, all RAPD primers used were able to discriminate heterozygous alleles in roselle samples as indicated by the gene level value of 0.99. One of the parameters used to assess the level of genetic diversity in a population is the heterozygosity value. The heterozygosity value of 23 primers was 1, while that of the other two primers was 0.941. The OPC5 primer produced the highest PIC value of 0.49 and according to [24], the PIC value is classified as low, moderate, or high when it is less than 0.25, between 0.25-0.50, and > 0.5. The more informative the primer used in differentiating roselle genotypes, the higher the PIC value. The dendrogram of 17 roselle genotypes based on ALFP and RAPD markers showed the same grouping with a similarity range of 54-96%. Both techniques produced two genotypic groups which separated the first group consisting of roselle Green 11, Green 12, and Green13 with the second group comprised 14 other genotypes as shown in Figure 3. Therefore, the genotypic analysis with both techniques produced consistent results. The consistency of phenotypic and genotypic results might simplify the selection of genetic material for future breeding. However, even though all genotypes are divided into two groups, the AFLP markers give a smaller similarity range (60-96%) in group two compared to RAPD (84-96%). These genetic analysis results appear to represent the phenotypic characters in group I. The epicalyx, calyx, and corolla of the green genotypes 11, 12, and 13 have very distinctive characteristics. In general, the three genotypes have a green epicalyx, the flower corolla tends to be white with a red tinge, and the calyx has a mixture of red and green colors. In Group II, all Roselle varieties (Roselindo 1, Roselindo 2, Roselindo 3, and Roselindo 4) were placed together with selected genotypes from high yield accession together with selected genotypes from high yield accession lines identified from previous works [10]. This indicates, that roselle varieties and accession genotypes are genetically close related, especially based on RAPD markers. However, of all the accession, cv Roselindo 3 has very distinctive corolla and calyx characters of yellow and yellowish green respectively with a similarity range of 54-96%. Based on these results, it is suggested that the AFLP markers used are closely related to the phenotypic character markers on the corolla and calyx.



(a)



(b)

Fig. 2 AFLP (A) and RAPD (B) banding patterns of roselle generated using several markers

TABLE IV
GENETIC INFORMATION GENERATED BY 8 PRIMER COMBINATIONS TO DETECT ALFP AND RAPD POLYMORPHISM AMONG 17 ROSELLE

Markers	Number of alleles	Frequency of main alleles	Genetic diversity	Heterozygosity	PIC
ALFP					
E-AAG/M-CAC	13	0.5	0.724	1	0.712
E-AAT/M-CTG	13	0.5	0.724	1	0.712
E-ACT/M-CTG	12	0.5	0.721	1	0.707
E-ACT/M-CTT	11	0.5	0.697	1	0.670
E-AGC/M-CTT	7	0.5	0.666	1	0.622
E-AAG/M-CAG	11	0.5	0.719	1	0.704
E-ACT/M-CAG	13	0.5	0.723	1	0.709
E-ACA/M-CTC	13	0.5	0.723	1	0.709
Mean	11.625	0.5	0.713	1	0.693
RAPD					
OPC-05	10	0.5	0.709	1	0.4906
OPQ-20	4	0.5	0.556	1	0.4844
OPB-11	11	0.5	0.718	1	0.4815
OPA-04	5	0.5	0.578	1	0.4808
OPA-02	5	0.5	0.618	1	0.4757
OPB-14	16	0.471	0.758	0.941	0.4358
OPA-10	5	0.5	0.578	1	0.4225
OPA-07	10	0.5	0.708	1	0.4045
OPG-05	4	0.5	0.576	1	0.4041
OPA-11	2	0.5	0.500	1	0.3784
OPAF-08	8	0.5	0.668	1	0.3739
OPG-18	5	0.5	0.606	0.941	0.3238
OPB-07	6	0.5	0.656	1	0.2766
OPP-01	7	0.5	0.689	1	0.2666
OPN-12	11	0.5	0.720	1	0.2645
OPG-08	3	0.529	0.611	1	0.2509
OPA-01	7	0.5	0.685	1	0.2471
OPA-06	3	0.5	0.558	1	0.2368
OPG-17	5	0.5	0.578	1	0.1705
OPD-07	7	0.5	0.662	1	0.1653
OPB-10	7	0.5	0.678	1	0.1646
OPG-13	4	0.5	0.756	1	0.0916
OPA-12	4	0.5	0.576	1	0.0754
OPA-13	4	0.5	0.576	1	0.0460
Mean	6.375	0.5	0.631	0.995	0.309

C. PCA Analysis

PCA analysis for qualitative color characters including stalk, leaf, corolla, and calyx and molecular markers (AFLP and RAPD) showed that the 17 roselle genotypes were distributed across four quadrants, with green genotypes 11, 12, 13 and Roselindo 3 in a separate quadrant from the other 14 (Figure 4). Green 11, Green 12, Green 13, and Roselindo 3 are clearly distinct from the other 14 genotypes which cluster in one large group. The results of the PCA analysis showed that the non-green genotypes were genetically closely related. It can be seen from the genotypes that are clustered around the adjacent quadrant axis, although when viewed phenotypically based on PCA, there are quite large morphological variations in the members of each quadrant.

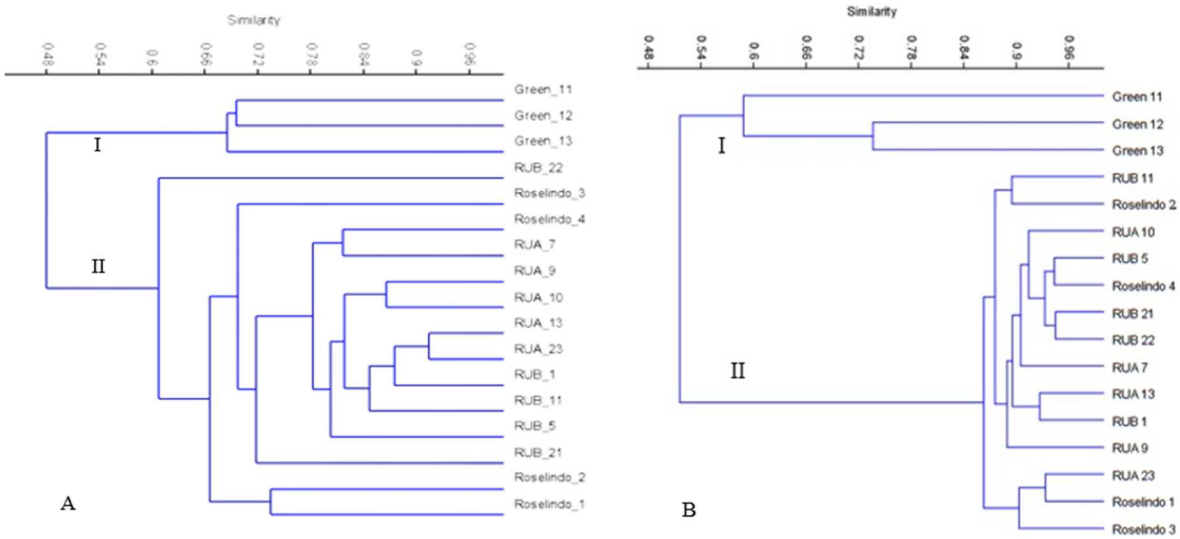


Fig. 3 Dendrogram based on AFLP (A) and RAPD (B) markers of 17 roselle genotypes

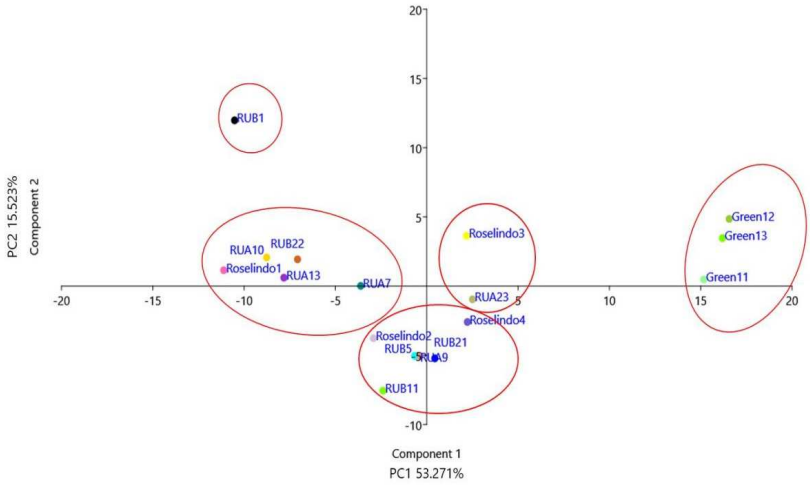


Fig. 4 PCA distribution of 17 roselle genotypes based on color qualitative characteristics (stalk, leaf, and calyx), ALFP and RAPD markers

D. Content of Cyanidine 3-O-glucoside

HPLC chromatograms taken at a wavelength of 535 nm yielded a retention time of 2.523 minutes for cyanidin 3-O-glucoside as standard. The peak retention time for each genotype was determined in comparison to the cyanidin 3-O-glucoside standard and the results are presented in Table 5.

The HPLC results showed that Roselindo 4 had the highest levels of cyanidin 3-o-glucoside at 446 mg/100 g dry weight, while Green 13 had the lowest at 0.07 mg/100 g dry weight. According to a previous study, cyanidin 3-o-glucoside levels in Mexican roselle ranged from 17.6 ±1.71 - 32.19 ± 0.27 mg/100 g dry weight [43]. Meanwhile, the cyanidin-3-O-glucoside content of calyx and callus roselle samples was 02.40 ± 0.02 and 08.01 ± 0.04 (mg.g^{-1}dm), respectively [9]. Based on these results, the content of the red anthocyanin compound in Roselindo 4 appears to be higher than that found by [25] and [9].

In general, among the 17 samples tested, there was no correlation between calyx color and anthocyanin content. However, among accession varieties that have high anthocyanin levels (Roselindo 4) have the same calyx color score as accession accession groups with high anthocyanin levels (RUA 9 and RUB 5). The calyx phenotype in Roselindo 3 was green, and it had cyanidin3-o-glucoside levels of 44 mg/100 g dry weight, while the purplish-red petals of Roselindo 1 have low levels amounting to 0.26 mg/100 g dry weight. According to previous reports, Roselindo-1 anthocyanin pigment is not derived from cyanidin-3-o-glucoside, delfinidin-3-glucoside, cyanidin-3-sambubioside, Delphinidin-3-O-sambubioside, Malvidin-3-O-glucoside, or Petunidin-3-O-glucoside [9]. Therefore, additional testing is

required using a variety of antioxidant and anthocyanin standards to identify the species and test their activity. This will help to obtain a more complete picture of their potential as herbal medicines as well as breeding and development strategies.

TABLE V
THE CYANIDIN 3-O-GLUCOSIDE CONTENT IN 17 GENOTYPES OF ROSELLE FROM 100 G OF DRY WEIGHT

No	Genotype	Cyanidin 3-O-glucoside content (mg)	No	Genotype	Cyanidin 3-O-glucoside content (mg)
1	RUA7	5.95	10	RUB22	2.27
2	RUA9	9.85	11	Green11	0.17
3	RUA10	13.68	12	Green12	0.09
4	RUA13	4.28	13	Green13	0.07
5	RUA23	4.25	14	Roselindo1	0.26
6	RUB1	1.52	15	Roselindo2	20.77
7	RUB5	5.37	16	Roselindo3	44.06
8	RUB11	1.59	17	Roselindo4	446.14
9	RUB21	1.55			

Production of information related to genetic background, morphological and agronomical characters is very important for the assembly of superior crops. All of information can be used to build a valuable plant genetic resources database for breeding in the future. From our study, the number of valuable data related to morphological characters, marker-based genetic information, and several important agronomic characters were generated to complement the existing Indonesian roselle germplasm database. A previous study conducted by [26] has built a more complete Roselle database information which is then used to conduct genetic divergence analysis using the D2 multivariate statistic and the Tochner method for grouping genotypes based on characters to obtain the information about the characters that most influence genetic diversity. Genetic diversity information is particularly important for assembling new superior varieties, because hybrid between lines of diverse origin generally display greater heterosis than between closely related lines. So far, the data collected from our study is limited to few varieties and selected accessions. To complete a broader genetic background, we need collections and information related to local roselle plants in Indonesia. We found that the varieties and the selected accessions used in our study, except for green Roselle, were genetically close related based on AFLP and RAPD genetic markers, and PCA analysis, although morphologically, they were quite diverse and had several superior characters. More specifically, the relationship between agronomic information and anthocyanin content, for example Roselindo 4 which has the highest anthocyanin content and several accessions that have high potential yields of calyx (RUA 7 and RUB 1) and supported by morphological diversity data, and molecular markers is valuable information for breeding strategy of roselle.

IV. CONCLUSION

The phenotypic characterization of 17 Roselle genotypes from Indonesia showed significant variation in flower corolla and calyx, as well as leaf length, color, width, and the number of lobes. The calyx color is a phenotypic dominant character or marker identity. Based on the genetic diversity analysis results of national roselle varieties and accessions selected using RAPD and AFLP markers, a relatively high genetic variation was found as indicated by the genetic diversity index, DNA fingerprint variation, and cluster analysis. But if we look more deeply at the varieties and selected accession lines without green roselle, all of them are genetically close related. Anthocyanin marker compound, namely cyanidin 3-O-glucoside level, was found to differ between the genotypes. The high genetic and metabolite diversity among the tested roselle samples suggests that they could be used as breeding material.

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