

Genetic diversity of *Scopellaria marginata* (Cucurbitaceae) with varied fruit shapes based on random amplified polymorphic DNA (RAPD) molecular markers

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Abstract

Scopellaria marginata is a plant that produces fruit with various shapes. The diversity of fruit shapes can be influenced by genetic factors. One approach that can be used to analyze genetic variation is Random Amplified Polymorphic DNA (RAPD). The objective of this study was to analyze the genetic diversity of *S. marginata* with various fruit shapes based on RAPD markers. This study used *S. marginata* grown from seeds originating from round and ellipsoid fruit shapes. The RAPD primers used included OPA-1, OPA-2, OPA-3, OPA-4, OPA-5, OPA-7, OPA-8, OPA-10, OPA-11, OPA-16, OPA-17, OPA-18, OPA-19, OPA-20, OPB-1 and OPB-5. The data obtained were analyzed using GenALEX 6.51b2, NTSYSpc 2.10e, and STRUCTURE v2.3.4. Based on RAPD markers, *S. marginata* had high genetic diversity with a polymorphism level of $76.80 \pm 2.29\%$. The genetic diversity of *S. marginata* in round fruit (79.08%) was higher than ellipsoid fruit (74.51%). The genetic structure of *S. marginata* with varying fruit shapes was divided into 5 groups with 8 sub-groups, namely A, B1, B2, B3, B4, C, D, and E. Groups A, B3, B4 and E consisted of round fruit individuals, groups B2, C and D consisted of ellipsoid fruit individuals and group B1 consisted of round and ellipsoid fruit individuals. This study indicated that the fruit shape of *S. marginata* had a high level of genetic diversity. It found that genetic variation in the fruit shape of *S. marginata* could be valuable for breeding programs, especially in horticultural plants.

Keywords: ellipsoid, fruit shape, RAPD, round, *S. marginata*

1. Introduction

Scopellaria marginata is a wild species of the Cucurbitaceae that is spread throughout East Myanmar, China, Thailand, Laos, Cambodia, Vietnam dan Malesia (Sumatera, Malaysia Penninsula, Borneo, West Java, East Java, Central Sulawesi, and Philippines) (de Wilde and Duyfjes, 2010; Arumingtyas *et al.*, 2023). *S. marginata* has various fruit shapes, such as round, ellipsoid, and fusiform (de Wilde and Duyfjes, 2006a; de Wilde and Duyfjes, 2006b). Typically, an individual plant produces fruit of only one shape. However, it was reported that there were three variations in fruit shape in one individual *S. marginata* (Arumingtyas *et al.*, 2023). This species was first reported from East Java, Indonesia and confirmed by molecular identification based on the *trnL*-UAA and *trnL*-*trnF* intergenic spacer regions (Turhadi *et al.*, 2024). However, no studies have reported whether genetic diversity contributes to the observed variation in fruit shape in *S. marginata*.

Random Amplified Polymorphic DNA (RAPD) is one of the common and widely molecular markers used to identify genetic diversity in plants (Arif *et al.*, 2010a). RAPD serves as an effective molecular marker for assessing genetic diversity in fruit shape variation

(Iordăchescu *et al.*, 2022; Zahid *et al.*, 2022). RAPD is widely used because it is simple, easy and does not require target DNA sequence information (Rawashdeh *et al.*, 2011; Amiteye 2021; Slameto *et al.*, 2023; Anwar *et al.*, 2024). RAPD is often used in plant breeding and genetic studies to identify cultivars and map genomes (Arif *et al.*, 2010b). Especially in Cucurbitaceae family, RAPD has been used to genetically analyze several species, including various accessions of *Cucurbita pepo* with different agroecological origins (Ntuli *et al.*, 2015), melon (*Cucumis melo*) cv. Gama Melon Basket (GMB) (Huda and Daryono 2013) and watermelon (*Citrullus lunatus*) (Ebadi *et al.*, 2022).

Several studies are reported on fruit shape variation based on RAPD markers, including eggplant (*Solanum melongena*) (Nunome *et al.*, 2001), chili (*Capsicum annum*) (Cvikic *et al.*, 2009; Niklas and Olszewska 2021), date palm (*Phoenix dactylifera*) (Al Khalifah *et al.*, 2012), guava (*Psidium guajava*) (Li *et al.*, 2016; Usman *et al.*, 2020), mango (*Mangifera indica*) (Thakur *et al.*, 2017), and tomato (*Solanum lycopersicum*) (Badulescu *et al.*, 2020). The study of fruit shape can also provide knowledge that can help improve plant breeding and cultivation (Costa *et al.*, 2011; Monforte *et al.*, 2014; van der Knaap and Østergaard 2018). Moreover, the fruit shape is a complex trait determined by many genetic factors and

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regulatory networks (Li *et al.*, 2023). As a preliminary study on the genetics of fruit shape in *S. marginata*, this research is considered as basic information for further bioprospection.

As a newly recorded species in East Java with reported variations in fruit shape, *S. marginata* requires molecular analysis using RAPD markers. The objective of this research was to assess the genetic diversity of *S. marginata* with variable fruit shapes using RAPD markers. This approach will assess its genetic diversity related to rapid fruit shape variation, even without prior knowledge of the target DNA sequence. These molecular findings can offer a more complete understanding of the fruit shape characteristics previously described in *S. marginata*.

2. Material and Methods

2.1. Plant materials

All plant materials used in this study were obtained by planting seeds from round (Figure 1A1-A2) and ellipsoid (Figure 2B1-2) fruits of *S. marginata* from Malang, East Java, Indonesia (7°57'7.01420" S; 112°36'41.29880" E), nine of each fruit shape. The seeds are soaked in warm water (± 50 °C) for 60 minutes and then planted in a germination tray containing a mixture of garden soil: fertilizer: sand (2:1:1; v/v/v). Planting was carried out at the green house of the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, East Java, Indonesia.

2.2. DNA extraction

The genomic DNA was extracted from fresh leaf of round and ellipsoid fruit plants using 2% Cetyltrimethylammonium Bromide (CTAB) buffer (Orozco-Castillo *et al.*, 1994). The extracted DNA was checked for quality on gel electrophoresis with 0.8% agarose and run at 50 V for 50 minutes. Subsequently, the extracted DNA was also checked for its concentration and purity level using a NanoPhotometer[®] NPOS 6.6c (Implen, Inc., USA) at the wavelength (λ) of 260 and 280 nm.

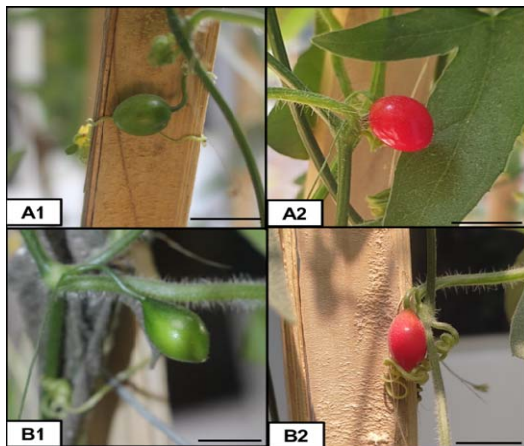


Figure 1. Fruit types of *S. marginata* with round (A1-A2) and ellipsoid (B1-B2) (Scale = 1 cm)

2.3. RAPD-PCR

The volume of PCR-RAPD was 10 μ L consisting of: 2.5 μ L DNA template (10 ng/ μ L), 0.5 μ L of RAPD primer (10 pmol), 5 μ L of Go Taq[®] Green PCR mix (Promega,

USA) and 2 μ L of nuclease free water (Promega, USA). The amplification process was carried out in 45 cycles with the following PCR program: pre-denaturation at 94°C for 4 minutes, denaturation at 94°C for 30 seconds, annealing at different temperatures for each primer (Table 1) for 1 minute, extension at 72°C for 30 seconds and post-extension at 72°C for 5 minutes. PCR-RAPD products were visualized in 1.5% agarose gel electrophoresis in 0.5X TBE buffer (Promega, USA) and SYBR DNA Stain (Jena Bioscience, Germany) at 50 V for 60 minutes. The result was documented using a Gel Documentation, UV Transilluminator (Major ScienceCo., Ltd., USA).

Table 1. RAPD primers used in this study

Primer code	Sequence	Ta (°C)	Reference
OPA-1	CAGGCCCTTC	41	Probojati <i>et al.</i> 2019)
OPA-2	TGCCGAGCTG	45	Probojati <i>et al.</i> 2019)
OPA-3	AGTCAGCCAC	39	Probojati <i>et al.</i> 2019)
OPA-4	AATCGGGCTG	40	Probojati <i>et al.</i> 2019)
OPA-5	GAAACGGGTG	37	Probojati <i>et al.</i> 2019)
OPA-7	GAAACGGGTG	35	Li <i>et al.</i> (2016)
OPA-8	GTGACGTAGG	36	Probojati <i>et al.</i> 2019)
OPA-10	GTGATCGCAG	35	Li <i>et al.</i> (2016)
OPA-11	CAATCGCCGT	41	Probojati <i>et al.</i> 2019)
OPA-16	AGCCAGCGAA	43	Probojati <i>et al.</i> 2019)
OPA-17	GACCGCTTGT	40	Probojati <i>et al.</i> 2019)
OPA-18	AGGTGACCGT	41	Probojati <i>et al.</i> 2019)
OPA-19	CAAACGTCCG	39	Probojati <i>et al.</i> 2019)
OPA-20	GTTGCGATCC	38	Probojati <i>et al.</i> 2019)
OPB-1	GTTTCGCTCC	35	Li <i>et al.</i> (2016)
OPB-5	TGCGCCTTC	35	Cvikic <i>et al.</i> (2009)

Note: Ta = annealing temperature

2.4. Data analysis

The amplified DNA band products appearing on the gel were assessed using a binary score to determine the polymorphism, present (1) or absent (0). From these data, the polymorphic information content (PIC) was calculated following Saldaña *et al.* (2021) (Equation 1) to determine the most informative RAPD primers in this study.

$$PIC = 1 - [fi^2 + (1 - fi)^2] \quad (1)$$

Where, fi is the frequency of present bands (1) and $(1-fi)$ is frequency of absence bands (0).

The obtained data were analyzed into GenAIEx v.6.51b2 (Peakall and Smouse, 2012) to estimate genetic diversity. Other genetic parameters, including the percentage of polymorphic loci (P), Shannon's index (I), expected heterozygosity (He), and expected heterozygosity without bias (uHe) were calculated.

Population structure and clustering analysis were determined using STRUCTURE v2.3.4 (Pritchard *et al.*, 2000) and NTSYSp2.10e (Rohlf, 2000) software, respectively. Population structure analysis was run with the test model following Naznin *et al.* (2024). The most reasonable number of clusters was determined using the Structure Selector (<https://lmme.ac.cn/StructureSelector/>) by plotting the LnP(K) value against the ΔK value (Li and Liu, 2018). The best K value was selected according to the Evanno test (Pandian *et al.*, 2020). Meanwhile,

dendrogram was reconstructed using the Unweighted Pair-Group Method Arithmetic Average (UPGMA) clustering algorithm (Huda and Daryono, 2013).

3. Results

3.1. Polymorphism level of *S. marginata* with varied fruit shapes

RAPD amplification showed that all sixteen primers used produced 153 bands in all eighteen samples, and all

bands were polymorphic and the percentage of polymorphic bands was 100% (Table 2). In addition, PIC analysis of 16 RAPD markers identified OPA-2, OPA-3, OPA-4, and OPA-5 as the most informative markers (Table 2).

Table 2. Genetic profile of 16 RAPD markers in 18 individuals of *S. marginata* with round and ellipsoid fruit shapes

Primer code	Total number of bands	Number of polymorphic bands	Polymorphic band percentage (%)	PIC
OPA-1	11	11	100	0,270
OPA-2	10	10	100	0,362
OPA-3	7	7	100	0,358
OPA-4	7	7	100	0,384
OPA-5	1	1	100	0,346
OPA-7	1	1	100	0,198
OPA-8	5	5	100	0,242
OPA-10	9	9	100	0,233
OPA-11	4	4	100	0,105
OPA-16	10	10	100	0,278
OPA-17	20	20	100	0,272
OPA-18	9	9	100	0,224
OPA-19	7	7	100	0,272
OPA-20	20	20	100	0,298
OPB-1	18	18	100	0,202
OPB-5	14	14	100	0,238
Total	153	153	-	-
Average	9,6	9,6	100	0,268
Standard deviation	5,9	5,9	0	0,070

Note: PIC = polymorphic information content

3.2. Genetic diversity of *S. marginata* with varied fruit shapes based on RAPD markers

The genetic diversity of *S. marginata* with varying fruit shapes based on RAPD markers showed the results as in Table 3. It is also known that genetic diversity in round fruit was higher than in ellipsoid fruit. Based on the percentage of genetic diversity and PhiPT values, it was known that the genetic diversity of *S. marginata* was higher within the group (Table 4). This indicated that genetic diversity with the same fruit shape was higher than diversity in different fruit shapes.

Table 3. Genetic diversity of *S. marginata* with varied fruit shapes based on RAPD markers

Parameters	Fruit types		
	All shape	Round	Ellipsoid
N	18	9	9
Na	1,539 ± 0,048	1,582 ± 0,066	1,497 ± 0,070
Ne	1,274 ± 0,017	1,279 ± 0,023	1,268 ± 0,024
I	0,0294 ± 0,013	0,303 ± 0,017	0,286 ± 0,018
He	0,180 ± 0,009	0,185 ± 0,012	0,176 ± 0,013
uHe	0,191 ± 0,009	0,196 ± 0,013	0,186 ± 0,014
P (%)	76,80 ± 2,29	79,08	74,51

Notes: N = number of samples, Na = number of different alleles, Ne = number of effective alleles, I = Shannon's information index, He = expected heterozygosity, uHe = unbiased expected heterozygosity, P (%) = percentage of polymorphic loci

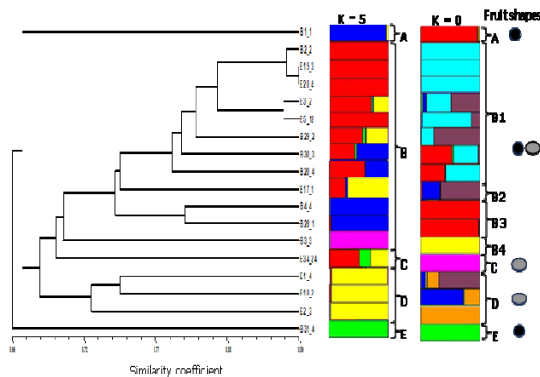
Table 4. Analysis of genetic diversity within and between *S. marginata* fruit groups using AMOVA

Source	df	SS	MS	Variance estimation	%	PhiPT	P
Among group	1	37,661	37,611	1,870	8		
Within group	16	332,444	20,778	20,778	92	0,083	0,003
Total	17	370,056		22,648	100		

Notes: df = degrees of freedom, SS = sum of squares, MS = mean of squares, % = percentage of genetic diversity, PhiPT = pairwise population differentiation, P: probability

3.3. Genetic population structure of *S. marginata* with varied fruit shapes based on RAPD markers

The genetic population structure of *S. marginata* classified 18 *S. marginata* samples with round and ellipsoid fruit shapes into 5 groups consisting of 8 sub-groups assessed based on the value of $K = 5$ and $K = 8$ as the optimum value. These results were also in line with the construction of the dendrogram at a similarity coefficient of 69% obtained 5 main groups of *S. marginata* with round and ellipsoid fruit shapes (Figure 2).

**Figure 2.** Population structure of *S. marginata* with round and ellipsoid fruit shapes based on 16 RAPD markers.

4. Discussion

4.1. Polymorphism level of *S. marginata* with varied fruit shapes

Eighteen samples of *S. marginata* with round and ellipsoid fruit shapes had high genetic diversity as seen from the high polymorphic bands that appeared (Table 2). The high level of polymorphism also indicated that the RAPD markers and 16 primers used in this study could be used as suitable markers and primers for similar studies to study genetic diversity and concluded the *S. marginata* genome (Paul and Saha, 2019; Probojati *et al.*, 2019). In addition, it was known that the PIC value of the 16 RAPD primers used in this study was classified as slightly informative to quite informative, ranging from 0.105-0.384 (Table 2). The OPA-2, OPA-3, OPA-4 and OPA-5 primers were the most informative primers compared to other primers used in this study, so they are recommended for

similar studies. A higher PIC value indicated that the primer was better when used to analyze genetic variation in a sample group (Probojati *et al.*, 2019).

4.2. Genetic diversity of *S. marginata* with varied fruit shapes based on RAPD markers

Analysis of 18 *S. marginata* individuals using RAPD markers demonstrated substantial genetic diversity in fruit shapes, showing $76.80 \pm 2.29\%$ polymorphism. The number of alleles (N_a) and the number of effective alleles (N_e) in this study were 1.539 ± 0.048 and 1.274 ± 0.017 , respectively (Table 3). N_a indicated the number of alleles observed in a population, meanwhile N_e indicated the estimated number of alleles with the same frequency to obtain heterozygosity. The higher the N_a and N_e values indicated higher genetic diversity in a population (Wang *et al.*, 2016). The N_a and N_e values of *S. marginata* were lower than the N_a and N_e values in cucumber, which ranged from 6.78 to 10.3 and 1.79 to 2.37 (Lv *et al.*, 2012). The Shannon's index (I), expected heterozygosity (H_e) and unbiased expected heterozygosity (uH_e) values in this study were relatively low, namely 0.0294 ± 0.013 , 0.180 ± 0.009 and 0.191 ± 0.009 (Table 3). The low Shannon's index value with a high percentage of polymorphic loci is thought to be due to uneven distribution of alleles and a low number of effective alleles (Nie *et al.*, 2022).

The percentage of polymorphism was higher in *S. marginata* with a round fruit shape (79.08%) compared to the ellipsoid fruit shape (74.51%). This percentage of polymorphism is in line with other parameters, namely the number of alleles (N_a), the number of effective alleles (N_e), Shannon's index (I), expected heterozygosity, and unbiased expected heterozygosity (Table 3). All of these parameters had higher values in *S. marginata* with a round fruit shape than ellipsoid. This is because the fruit shape is regulated by many genes (polygenic) so that it has higher genetic diversity. Fruit shape in Cucurbitaceae is controlled by many genes (polygenic), including *ethylene regulates transcription factors (E2F-DP)*, *OVATE*, and *TRM5* which determine round and ellipsoid fruit shapes (Boualem *et al.*, 2022; Goldman *et al.*, 2023).

Analysis of *S. marginata* populations showed greater genetic diversity within groups than between groups (Table 4). This pattern was consistent with polygenic control of fruit shape, where allelic variation at multiple loci maintains high diversity within populations (Monforte *et al.*, 2014; Goldman *et al.*, 2023). The genetic diversity within groups was found to be higher than among groups, also reported by Sari *et al.* (2018) in sapodilla (*Manilkara zapota*), Warburton *et al.* (2002) in corn (*Zea mays*), Maksylewicz and Baranski (2013) in carrots (*Daucus carota*), Biabani *et al.* (2013) in *Jatropha curcas*, Lorello *et al.* (2018) in *Citrullus lanatus* and *C. pepo*. The high genetic diversity within the group indicated the occurrence of gene flow in *S. marginata*. Gene flow enhances genetic variation within populations while simultaneously reducing differentiation among them. Continuous gene flow promotes allele sharing between populations, leading to increased genetic homogeneity across groups (Andrews, 2010; Smith *et al.*, 2020).

4.3. Genetic population structure of *S. marginata* with varied fruit shapes based on RAPD markers

The genetic population structure of *S. marginata* grouped 18 samples into 5 groups ($K=5$) with 8 subgroups

(K=8) (Figure 2). At K=5, 18 *S. marginata* samples were categorized into five groups, namely A, B, C, D, and E. Group I (A) with alleles dominated by blue color consisted of individuals from round fruit. Group II (B) with alleles dominated by red and dark blue colors consisted of individuals from round and ellipsoid fruit. Group III (C) with alleles dominated by red and yellow colors consisted of individuals from ellipsoid fruit. Group IV (D) with alleles dominated by yellow color consisted of individuals from ellipsoid fruit. Group V (E) with alleles dominated by green color consisted of individuals from round fruit.

Grouping based on the value of K=8 categorized 18 *S. marginata* samples into eight subgroups with group B divided into 4 subgroups, namely B1, B2, B3 and B4 (Figure 2). Subgroup B1 with alleles dominated by light blue color consists of individuals from round and ellipsoid fruits. Subgroup B2 with alleles dominated by grayish purple color consists of individuals from ellipsoid fruits. Subgroup B3 with alleles dominated by red color consists of individuals from round fruits. Subgroup B4 with alleles dominated by yellow color consists of individuals from round fruits. Groups A and E with alleles dominated by red and green color consist of individuals from round fruits. Our results highlight the value of RAPD markers in evaluating genetic variation within *S. marginata*, and it could be useful for breeding program in economically horticulture plants, especially Cucurbitaceae.

5. Conclusion

Scopellaria marginata displayed substantial genetic diversity, showing $76.80 \pm 2.29\%$ polymorphism. Interestingly, round fruits (79.08%) had greater genetic diversity than ellipsoid fruits (74.51%). Analysis of genetic variation showed significantly higher diversity within groups (92%) than between groups (8%). The population structure of *S. marginata* accessions with diverse fruit shapes was organized into five main clusters (k=5) and eight subclusters (k=8). Grouping into 5 groups divided 18 *S. marginata* samples into group A (round shape), B (round- and ellipsoid shape), C (ellipsoid shape), D (ellipsoid shape), and E (round shape). Grouping into 8 groups divided 18 *S. marginata* samples into groups A, B1, B2, B3, B4, C, D, and E. Groups A, B3, B4 and E consisted of individuals from round fruit; and groups B2, C and D consisted of individuals from ellipsoid fruit and group B1 consisted of individuals from round and ellipsoid fruit. Such important findings could support breeding programs, especially those targeting fruit shape traits.

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