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Genetic Variability of Eggplant (*Solanum melongena* L. 'Nasubi') Based on ISSR Markers and Phenotypic Characters

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Abstract
'Nasubi' eggplant plants produce fruit phenotypic characteristics that are different from those planted before the planting period in 2018. These factors cause the decline in harvest rates and the current high increase in production costs. Observation of variations in phenotypic and molecular characters with ISSR molecular markers between seeds before 2018 and after 2018 became the first step for the research team to reveal information on the uniformity of phenotypic and molecular characters in eggplant 'Nasubi' from seeds purchased by farmers before 2018 and post-2018. The results of the molecular analysis with ISSR primary molecular markers, namely UBC 809, UBC 815, IBC 880, UBC 888, and UBC 892, showed that the six samples had a high similarity index of 90% and resulted in a low polymorphism average of 12%. The results of the phenotypic analysis showed that eggplants from the seeds before 2018 and sources after 2018 in this study had variations in the character of the leaf tip angle, leaf base, flower crown color, stamen color, young fruit color, fruit curve, and fruit tip shape.

Keywords: crop; diversity; horticulture; morphology; uniformity

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Introduction

Eggplant (*Solanum melongena* L.) has long been an essential commodity in Asia, Africa, Europe, and America (1; 2; 3). In India, this plant is called the 'King of Vegetables.' Eggplant fruit can be consumed as food, and its roots and leaves can be used as ingredients for traditional medicine (1). Eggplant is a horticultural commodity with economic value and excellent development prospects in Indonesia and the world. These commodities have relatively high prices for both the domestic and export markets. The increased public interest in eggplant makes eggplant one of the leading agribusiness commodities. The countries with the most significant eggplant production in 2020, respectively, are China, India, Indonesia, and Japan (4). Eggplant production in Indonesia continued to increase from 2015 to 2019. In 2019, eggplant production in Indonesia reached 575.392 tons; the harvest number showed an increase of 4.33% compared to 2018 (5). In 2020, eggplant production in Indonesia reached 618,000 tons (4). The high consumption and production of eggplant are expected to increase farmers' income, provide more employment opportunities, and meet the community's nutritional needs.

'Nasubi' eggplant is the only type of eggplant from Japan. All 'Nasubi' eggplant and superior-quality seeds are imported (6). This eggplant has a characteristic: the skin is more glossy and purple-black, the shape is larger, some are more rounded, and the flesh is slightly thicker (7).' Nasubi' eggplant fruit which can be exported is known to have black skin characteristics. As one of the export commodities, eggplant has a drawback, the shelf life is very short. Fruit freshness can be maintained for several days at 7.8°C-12.2°C and relative humidity of 90% (8). Therefore, according to the information from the exporter, 'Nasubi' eggplant is exported half-cooked, where after frying, the fruit's skin does not turn brown but remains black.

Seed stability is essential in the cultivation of export crops, where the exported fruit has strict qualifications. Changes in fruit phenotype due to segregation, mutation, or environmental influences can produce fruit with substandard quality so that it cannot be exported. The presence of impure seeds is very detrimental to farmers because farmers only know of differences in fruit phenotypes after the fruit reaches a certain age. One way that can be done to determine the purity of seeds is by analyzing genetic variation using molecular markers. Molecular markers are specific segments of DNA that represent differences at the genome level. Compared to morphological markers, molecular markers are stable, can be detected in all plant tissues, and are not influenced by the environment (9). One of the molecular markers, ISSR, can produce high polymorphisms (10) and is effective in detecting genetic variations ranging from 0.10-0.51 (11). this study was conducted on the diversity in the eggplant cultivar 'Nasubi' based on describing morphological and molecular characters with the ISSR (Inter Simple Sequence Repeat) marker.

Material and Methods

The type of research was experimental research. This research was conducted in March-August 2022. Eggplant planting and phenotypic data collection were carried out in Ngentak RT. 67, Argorejo, Sedayu, Bantul, D. I. Yogyakarta with conventional cultivation. Molecular analysis was carried out at the Genetics and Breeding Laboratory, Faculty of Biology, Gadjah Mada University. The seeds used in this study included 'Nasubi' eggplant seeds obtained from eggplant farming communities on Java Island.

Eggplant planting

The seeds used in this study include 'Nasubi' eggplant seeds obtained from eggplant farming communities on Java Island. The seeds used are seeds purchased before 2018 and seeds purchased after 2018. The seeds are called the 'Money Maker No. 2', produced by Takii Seed Co., Japan, and distributed by PT. Tani Murni Indonesia. Eggplant seeds are germinated in a tray with a tissue moistened with water. The media is moistened once a day until all the seeds germinate. The 5 cm plantlet were transferred to 5x5 cm polybags containing a mixture of fine soil and planting media (1:1). The 30 days old plants were transferred to 20x20 cm polybags containing a variety of soil, planting media, and manure (2:5:2). The plants are watered twice a day in the morning and evening. Fertilization is done once a week with Phonska NPK fertilizer (15% N:15% P2O5:15% K2O:10% S) as much as one tablespoon on the edge of the polybag. After flowering, the plants are fertilized with Pearl NPK 16-16-16 once a week with as much as one tablespoon on the edge of the polybag. Pesticides and fungicides are applicated every two weeks and as needed.

DNA Isolation

Leaf samples for DNA isolation can be collected from 4-5 weeks old plants by taking the 5th leaf from the tip of the plant (12). The leaves were put in a ziplock bag and stored in the freezer at -20°C. DNA was extracted using the Geneaid Plant Genomic DNA Mini Kit (Geneaid Biotech Ltd). Each leaf sample was weighed as much as 100 mg and then crushed using a mortar and pestle. GP1 buffer (200 µl) can be added to facilitate grinding. The samples were poured into a 1.5 ml tube, then added with 400 µl of GP1 buffer and homogenized using a vortex for 5 seconds. The boxes were incubated at 60°C for 10 minutes in a heat block.

In the lysis stage, 100 µl of GP2 buffer was added into the tube and homogenized using a vortex for 5 seconds, then placed in the freezer for 5 minutes. The filter column was assembled on a 2 ml collection tube; the sample was poured into the filter column from the 1.5 ml tube. The mixture was centrifuged at 1000 x g for 1 minute; then, the filter column was removed. The supernatant in the 2 ml collection tube was transferred to a new 1.5 ml tube. Next, 1.5 volume of GP3 buffer was added and homogenized using a vortex for 5 seconds.

The GD column was assembled in a 2 ml collection tube in the DNA binding stage. After that, 700 µl of the mixture was added and centrifuged at 16,000 x g for 2 minutes. The flow-through in the 2 ml collection tube was discarded, then the process was repeated for the remaining mixture. The W1 buffer (400 µl) was added to the GD column and centrifuged at 12,300 x g for 1 minute. Then, wash buffer (600 µl) was added and centrifuged at 12,300 x g for 1 minute. The flow-through in the collection tube was discarded, then centrifuged again at a speed of 12,300 x g for 4 minutes to dry the column matrix. The GD column was air-dried on a tissue for 1 minute before the elution stage.

In the DNA elution stage, the GD column was attached to a 1.5 ml tube. Then, 50 µl of pre-heated elution buffer (60°C) was poured into the center of the column matrix. It

was left to stand for 5 minutes before being centrifuged at 12,300 x g for 1 minute. Another 50 l of elution buffer was added and then centrifuged again at 12,300 x g for 1 minute. The DNA samples obtained were then stored at -20°C.

PCR-ISSR amplification and gel electrophoresis

The isolated DNA was tested for purity and concentration using Nanodrop UV-Vis (NanoVue 4282 V2.0.4 Beckman). DNA purity was seen by comparing the OD (optical density) values at 260 nm and 280 nm wavelengths. Good virtue is between 1.8 to 2 (13). PCR reaction was carried out in a volume of 24 µl containing 1 µl DNA, 12.5 µl Meridian MyTaq Red Mix, 9.5 µl of ddH2O, and 1 µl ISSR primer. Five primers of the UBC primer set (University of British Columbia) were used for ISSR analysis based on Isshiki et al. (14) and are listed in Table 1.

PCR reaction was performed in the BOECO Thermal cycler TC-PRO PCR which was set according to the PCR profile (protocol) for each primer in Table 1. A total of 6 µl of PCR product (ISSR) were separated by electrophoresis on 2% agar gel in 1.0 x TBE buffer containing 3 µl FloroSafe DNA stain (Sybr Safe DNA Gel Stain Invitrogen, Thermo Fisher Scientific) and running at 50 v for 60 minutes using electroporator machine MUPID-exU. A 3 µl DNA ladder (100 bp DNA ladder, Geneaid, and Promega) was loaded to estimate the sizes of ISSR markers in base pairs. Gel Documentation visualized DNA bands.

Table 1. Polymerase chain reaction mix composition

Material	Volume (µl)	Concentration
ddH_2O	9.5	-
Meridian 2x *MyTaq Red Mix*	12.5	-
ISSR Primer	1	20 µM/ µl
DNA Template	1	100 ng/ µl
Total	24	

Phenotypic character analysis

Phenotypic data were analyzed descriptively. Phenotypic data were collected based on descriptors for eggplant by the International Board for Plant Genetic Resources (15) and research on eggplant diversity and classification by Husnuddin (12), with some modifications as needed.

Scoring and analysis

Molecular data in the form of ISSR results were analyzed numerically. Each ISSR marker fragment was scored as present (1) or absent (0). Similarity coefficients with Jaccard Coefficient (16) and dendrogram construction based on the UPGMA (Unweighted Pair-Group Method Using Arithmetic Average) algorithm cluster method were done using the MVSP (Multivariate Statistical Program) software version 3.1.

Results and Discussion

Analysis of polymorphism by ISSR

The ISSR profile produced by primers for pre-2018 (A1, A2, and A3) and post-2018 (K1, K2, and K3) 'Nasubi' eggplants is shown in Figure 1. The reproducible polymorphic bands generated by five primers shown in Table 2 were detected in the 200-2400 bp range, with a total of 67 amplified bands. Amplified polymorphic bands and polymorphic rate by each primer are shown in Table 3. The five primers produce 67 bands with nine polymorphic and 58 monomorphic bands. The UBC 815, UBC 880, and UBC 892 produce higher polymorphic bands than the UBC 809 and UBC 888, which produce none of the polymorphic bands. PCR results with UBC 815 primer show 3 DNA bands only owned by post-2018 (K1, K2, and K3) 'Nasubi' eggplants at the size 2314-2391 bp, 546–558 bp, and 446-461 bp. Those bands become the specific band markers of the post-2018 'Nasubi' eggplants.

Table 2. ISSR primers and their nucleotides base sequence used in this study

Primer	**Sequence 5'-3'**	**Annealing temp (°C)**
UBC 809	AGAGAGAGAGAGAGAGG	50,8
UBC 815	CTCTCTCTCTCTCTCTG	42
UBC 880	GGAGAGGAGAGGAGA	49,6
UBC 888	BDBCACACACACACACA	51,6
UBC 892	TAGATCTGATATCTGAATTCCC	43,4

Note: B = (C, G, T); D = (A, G, T)

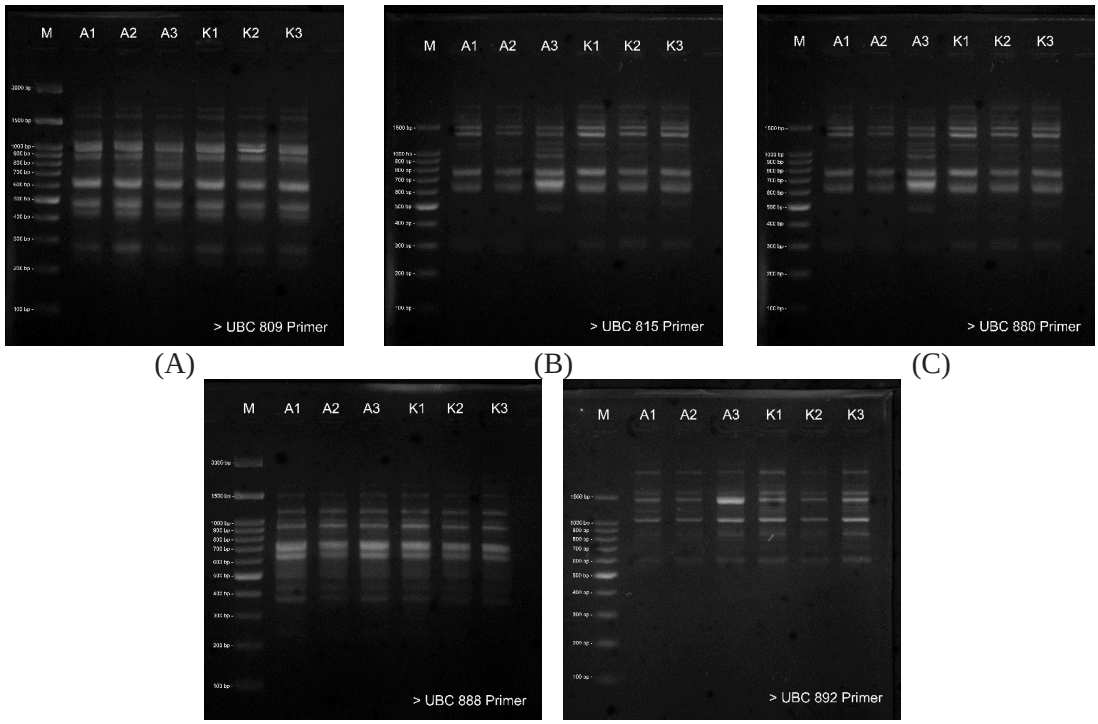


Figure 1. ISSR marker profiles of eggplants: (A) UBC 809 primer, (B) UBC 815 primer, (C) UBC 880 primer, (D) UBC 888 primer, (E) UBC 892 primer

Table 3. Polymorphic band markers from eggplant A and K with 5 primers ISSR

Primer	Sequence	ISSR fragment	Polymorphic fragment	Polymorphic rate (%)	DNA band (bp)
UBC 809	$(AG)_8G$	11	0	0	256 – 2045
UBC 815	$(CT)_8G$	15	6	40	289 – 2351
UBC 880	$(GGAGA)_3$	15	1	6,67	208 – 2129
UBC 888	$BDB(CA)_7$	11	0	0	361 – 1923
UBC 892	TAGATCTGATAT CTGAATTCCC	15	2	13,33	589 – 2155
Average		13,4	1,8	12	-
Total		67	9	60	-

Note: B = (C, G, T); D = (A, G, T)

Genetic variability of 'Nasubi' eggplants

Based on the similarity matrix for pre-2018 (A1, A2, and A3) and post-2018 (K1, K2, and K3) 'Nasubi' eggplants shown in Table 4., with the UPGMA cluster analysis method, the 6 'Nasubi' eggplant samples formed a dendrogram shown in figure 2. The dendrogram clearly distinguished the six samples into two main clusters in pre-2018 (A1, A2, and A3) and post-2018 (K1, K2, and K3) clusters with Jaccard's similarity coefficient ranging from 0.91-1. The Jaccard's similarity coefficient of the pre-2018 and post-2018 are 0.92 and 1.00; all plants have a similarity value of 90%.

Table 4. Similarity matrix of eggplant A and K

	Pre 2018			Post 2018		
	A1	A2	A3	K1	K2	K3
A1	1,00					
A2	1,00	1,00				
A3	0,92	0,92	1,00			
A4	0,90	0,90	0,91	1,00		
A5	0,90	0,90	0,91	1,00	1,00	
A6	0,90	0,90	0,91	1,00	1,00	1,00

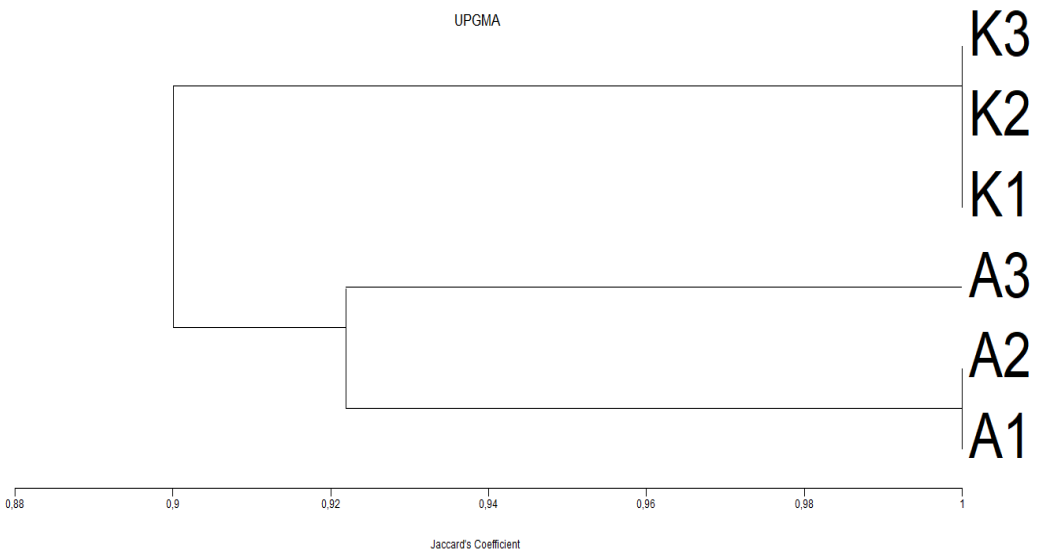


Figure 2. Dendrogram illustrating the variability and relationship among the samples of *Solanum melongena* L. 'Nasubi' based on ISSR markers

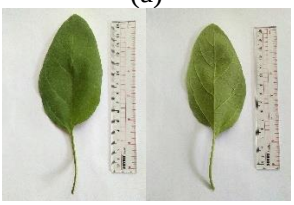
Phenotypic variability of 'Nasubi' eggplants

Data were analyzed descriptively to illustrate the plant morphology based on each character. All plants were observed using 31 morphological characters based on the descriptor list (Table 5) adopted from the International Board for Plant Genetic Resources (15) and research on eggplant diversity and classification by Husnudin (12), with some modifications as needed. Morphological characteristics were observed from vegetative characters (leaf and stem) and reproductive characters (flower and fruit), according to Husnudin et al. (17). The results showed that of the 31 characters observed, six characters were polymorphic and 25 characters were monomorphic. The evaluated 'Nasubi' eggplant in this study has variations in the character of the angle of the leaf tip, leaf base, flower crown color, stamen color, young fruit color, fruit curves, and fruit tip shape.

There were differences in leaf morphology in the form of leaf shape, leaf tip angle, and leaf base on the observed plants. In this study, the pre-2018 eggplant plants had oval and rhombus-shaped leaves, with rounded and pointed leaf tips and pointed and asymmetrical leaf bases. Meanwhile, the post-2018 eggplant plants have ovate, oval, and rhombus-shaped leaves, with rounded, tapered, and intermediate leaf tips, and leaf bases that are symmetrically pointed, asymmetrically pointed, symmetrically rounded, and asymmetrically rounded. Eggplant flowers in pre-2018 (A) and post-2018 (K) have different flower colors: brilliant purple on eggplant A and light purple on eggplant K.

Table 5. Comparison of phenotypical characters of eggplant seeds A and K.

Phenotypic Character	**A (Pre-2018 seeds)**	**K (Post-2018 seeds)**
Plant morphology		
Habitus	Upright	Upright)
Anthochyanin in stem	Present	Present

Leaf morphology	 (a)	 (a)
	 (b)	 (b)
	 (c)	 (c)
		 (d)
Leaf stalk color	Greenish purple	Greenish purple
Leaf top surface color	RHS 137 A (Moderate olive green)	RHS 137 A (Moderate olive green)
The color of the underside of the leaf	RHS 138 B (Moderate yellow green)	RHS 138 B (Moderate yellow green)
Leaf tip angle	(a) Obtuse (~110°) (b. c) Acute (~45°)	(a) Obtuse (~110°) (b, d) Acute (~45°) (c) Intermediate (~75°)
Thorns on leaves	Absent	Absent
Leaf edge nicks	(a) Flat (b) Curvy (c) Strong leaf blade lobing	(a, b) Flat (b) Curvy (c) Strong leaf blade lobing
Leaf lobe tip	Obtuse	Obtuse
Leaf base	(a) Symmetrical pointed (b, c) Asymmetric rounded	(a) Symmetrical pointed (b) Asymmetric pointed (c) Symmetrical rounded (d) Asymmetric rounded

Flower morphology	 (a)	 (a)
	 (b)	 (b)
	 (c)	 (c)
		
Amount of flower per inflorescence	1-2	1-2
Flower crown color	RHS N87 C (Brilliant purple)	RHS N87 D (Light purple)
Flower petal color	RHS N186 A (Dark greyish purple)	RHS N186 A (Dark greyish purple)
Stamen color	RHS 7 A (Brilliant yellow)	RHS 7 B (Brilliant yellow)
Pistil head color	RHS 143 C (Strong yellow green)	RHS 143 C (Strong yellow green)
Number of crown leaves	5-7	5-7
Number of petals	5-7	5-7
Flower petal thorns	Present	Present
Fruit morphology		
Fruit shape	Obovate	Obovate
Fruit type	Seed	Seed
Young fruit color	RHS NI16 A (Dark greyish purple)	RHS NI16 B (Dark greyish purple)
Ripe fruit color	RHS NI16 A (Dark greyish purple)	RHS NI16 A (Dark greyish purple)
Length and width ratio	Length three times width	Length three times width
Curves on the fruit	Absent	Slightly curved
Fruit position	Hang up	Hang up
Fruit tip shape	Pointed	Pointed
Rind	Shiny	Shiny
Fruit line	Absent	Absent
Stripes on fruit	Absent	Absent
Fruit flesh texture	Solid	Solid
Flesh color	White	White

Discussion

Several molecular markers have been used to study the relationships between the cultivated *Solanum melongena* L. and related species. The earlier markers used in the study of

eggplant genetic diversity were allozyme (18), RAPD (19; 20), SRAP markers (21), etc. ISSR markers have been used for assessing the genetic diversity relationship between eggplant and related species (14; 22; 20; 23; 17). The primers with the annealing temperatures used in this research are listed in Table 2. There is a difference in annealing temperature in each primer. This is due to each primer's melting temperature (Tm) difference. Melting temperature is the temperature required to dissociate a duplex primer. Primer annealing temperature (Ta) estimates the temperature at which the primer can bind to the DNA template stably (24).

Based on the result of this study, ISSR primers are effective in showing polymorphism of pre-2018 (A1, A2, and A3) seeds and post-2018 seeds (K1, K2, and K3) eggplants' Nasubi' with an average of polymorphic rate by 12% and a total of 60% (Table 3). The lowest percentage of polymorphisms was produced by UBC 809 and UBC 888, with a value of 0%, and the highest was produced by UBC 815, with 40%. In this study, all primers showed low polymorphism (≤ 50%), indicating a high similarity value (Table 4). The low level of polymorphism indicates low genetic variation between eggplants. Nunome et al. (25) assumed that this low level of polymorphism was caused by the narrow genetic background of the cultivated eggplant and the very small gene pool from which the cultivated forms arose (19). Previously, low levels of molecular polymorphisms were also shown in a study by Isshiki et al. (18) observed Japanese cultivars of eggplant using allozyme markers showing low-level polymorphisms. Later, Isshiki et al. (14) found that 8 Japanese eggplant cultivars were categorized into a single group from the related *Solanum* with ISSR markers.

The evaluated 'Nasubi' eggplant in this study, shown in Table 5, has variations in the character of the angle of the leaf tip, leaf base, flower crown color, stamen color, young fruit color, fruit curves, and fruit tip shape. These morphological differences can occur due to the expression of certain genes. This is according to the research of Doganlar et al. (26) through genetic linkage mapping, it has been shown that fruit size, shape, color, and plant spines in eggplant are controlled by a small number of genetic loci with large phenotypic effects and that most of these loci have orthologs on tomatoes, potatoes, chili peppers and other members of the Solanaceae.

An eggplant is a shrub (herb) in the form of a bush. This plant can reach a height of 0.3-1.5 m (27). Sympodial branching forms with many branches. The evaluated 'Nasubi' eggplant in this study has a greenish brown main stem (primary stem) at the base, a gradually purplish green towards the tip, and a purple branching stem (secondary stem). The surface of the stem can be purplish due to the presence of anthocyanins. The stem is not thorny; on the surface of the stem, there are accessories in the form of hair (trichomes). The stem morphology of the observed plants was uniform. Hairs (trichomes) on the stems have a role as stem protection from external factors such as herbivores, pathogens, and environmental conditions (light intensity, extreme temperatures), reducing excess water and salt secretion (28). Eggplant plants have trichomes with a distinctive shape, namely the shape of a star (29; 30).

Eggplant leaves are single scattered arrangements and are located alternately. Leaves consist of petioles and leaf blades (lamina) (27). The leaf stalks of the 'Nasubi' eggplant were purplish green to purple on the upper surface and green on the lower surface. The color difference is caused by differences in the proportion of pigments such as carotenoids, chlorophyll and flavonoids such as anthocyanins (31).

Eggplant flowers on eggplant A and K have single and compound flowers (2 flowers per inflorescence) in one individual. Both also have petals and corolla numbering 5-7 leaves per flower. The number of flowers per inflorescence is an important character in eggplant breeding. This is because, in eggplant breeding, a large number of flowers per inflorescence will be reduced to increase the uniformity of fruit size (Kaushik et al., 2016). Eggplants A and K each have one thorn on the flower petals. These thorns can be a form of plant protection against animal disturbances that can damage plant organs (32).

Eggplant A and K have a uniform obovate fruit shape, eggplant A not having a dent in the fruit while eggplant K is slightly curved. Eggplant A has a pointed tip, while eggplant K is rounded. The diversity of fruit shape in eggplant is strongly influenced by fruit elongation activity controlled by SUN and OVATE ortholog genes found in eggplant (33; 31). Eggplant A and K have the same ripe fruit color: dark greyish purple (RHS NI16 A). In contrast, the color of the young fruit has a different color, namely

dark greyish purple (RHS NI16 A) on eggplant A and dark greyish purple (RHS NI16 B) on eggplant K. Purple skin color is caused by the presence of anthocyanin pigments, where the difference in color level can be caused by differences in anthocyanin levels in the fruit skin (31).

Conclusion

The molecular character of the eggplant 'Nasubi' from seeds purchased by farmers before 2018 and seeds after 2018 is based on molecular characters with molecular markers of 5 ISSR primers, namely UBC 809, UBC 815, IBC 880, UBC 888, and UBC 892 have a high similarity index. i.e., 90% and the resulting low polymorphism average of 12%. 'Nasubi' eggplant from seeds purchased by farmers before 2018 and seeds after 2018 in this study had variations in the character of the leaf tip angle, leaf base, flower crown color, stamen color, young fruit color, fruit curve, and fruit tip shape.

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Conflict of Interest

All authors declare no conflicts of interest.

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