Journal of Tropical Biodiversity and Biotechnology
Volume 08, Issue 01 (2023): jtbb77228
DOI: 10.22146/jtbb.77228

Short Communications

Genetic Variation of Butternut Squash (*Cucurbita moschata* Duchesne) based on Inter-Simple Sequence Repeat

Nabila Rizki Ananda Putri[1], Prima Sekti Kusnanda[1], Bunga Finovel Angellya[1], Dian Sartika[1], Purnomo[1], Budi Setiadi Daryono[1]*
1)Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Sekip Utara, Yogyakarta 55281, Indonesia
* Corresponding author, email: bs_daryono@mail.ugm.ac.id

Keywords:
butternut squash
genetic variation
ISSR
PCR
Submitted:
22 August 2022
Accepted:
19 November 2022
Published:
11 January 2023
Editor:
Miftahul Ilmi

ABSTRACT
Butternut squash (*Cucurbita moschata*) is a Cucurbitaceae plant that has been widely cultivated in Indonesia. Butternut squash is known to have various cultivars. A new cultivar introduced by the Faculty of Biology UGM is named 'Citra Laga' which is expected to be able to compete with the imported cultivars. The number of cultivars within a species may indicate genetic variation. This research was conducted to observe genetic variation and the phenetic relationship between 'Citra Laga' and the imported butternut squash cultivars based on the molecular marker ISSR. The ISSR analysis between 'Citra Laga' and the imported cultivars showed an average low polymorphism rate by 18.61% with a high similarity percentage of 83.7%. Thus, it can be said that the genetic variation is low and 'Citra Laga' is not genetically much different from the imported cultivars.

Indonesia is high biodiversity country, especially in the horticulture field. Squash or *waluh* (genus *Cucurbita)* is one of the abundant horticultural crops in Indonesia and has the potential to be developed into a food source. *Cucurbita moschata* or butternut squash is one type of squash that has been widely cultivated. Butternut squash has various fruit shapes, namely globular, flattened, cylindrical, turbinate, dumbbell, elongated, pyriform, and crooked neck (Purnomo et al. 2015). Butternut squash as a vegetable plant has a lot of nutrients that are good for the human body. The nutritional content includes fiber, protein, vitamins, fats, iron, phosphorus, potassium, beta carotene, amino acids, antioxidants, minerals, phenolics, and flavonoids (Marie-Magdeleine et al. 2011; Dari & Yaro 2016; Nopianasanti & Daryono 2018).

Butternut squash is known to have various cultivars. Nopianasanti & Daryono (2018) stated that various cultivars in *Cucurbita* species are based on their mitochondrial gene. One cultivar introduced by the Faculty of Biology, Universitas Gadjah Mada recently is the 'Citra Laga' butternut squash which was developed by Nopianasanti and Daryono (2018). This new cultivar is a result from a cross between 'Labu Madu' F3 and 'Hannah' F2 (Figure 1). 'Citra Laga' is known to have a faster harvest period, contains high beta carotene, and has a higher resistance to Begomovirus. The fruit shape of this cultivar is diverse, namely pyriform,

dumbbell, and globular (Figure 2). The diverse fruit shape happens because there is still segregation gene that produces genotypic variations so that the fruit shape is still not stable. Some of the imported cultivars that have been widely commercialized are 'Tiana', 'Waltham', and 'Jacqueline'. The imported cultivars are known to have a stable fruit shape. 'Tiana' and 'Jacqueline' have a blocky fruit shape meanwhile 'Waltham' has a pyriform fruit shape (Figure 2). However, the imported cultivars are known to have a relatively high price because the seeds are gained from limited cultivation in Australia, the Netherlands, and others. Therefore, the breeding research of 'Citra Laga' butternut squash is very important so that this cultivar can be recognized as a native cultivar of Indonesia. This cultivar is also expected to have superior traits and good competitiveness with imported cultivars but at a relatively cheaper price.

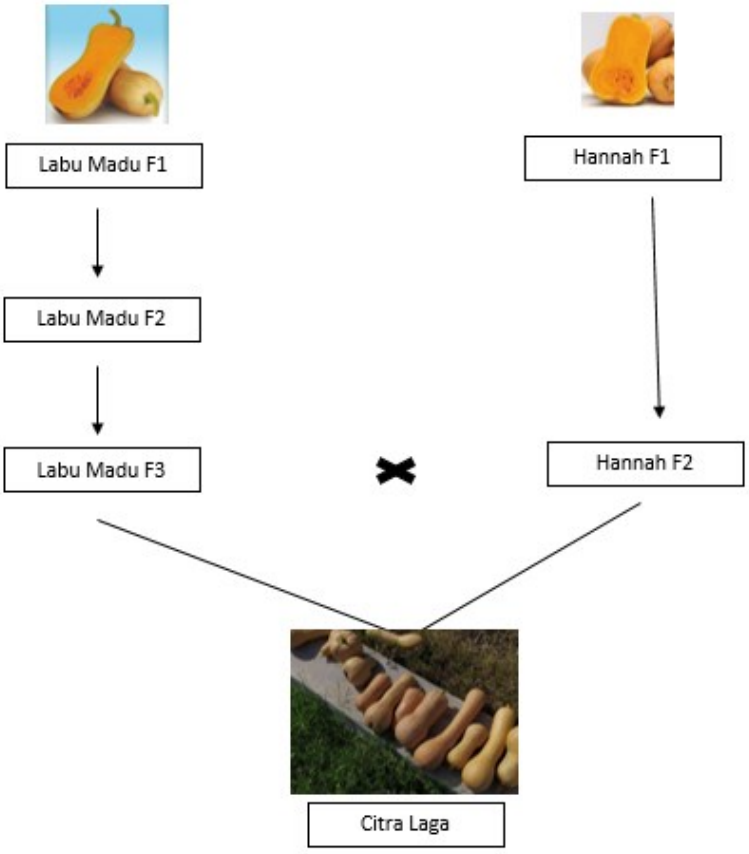

Figure 1. Parental history of 'Citra Laga' butternut squash.

A large number of cultivars in one species may indicate the presence of genetic variation within the species. Therefore, genetic variation research between 'Citra Laga' and imported cultivars specifically 'Tiana', 'Waltham', and 'Jacqueline' needs to be conducted. If the genetic variation level between 'Citra Laga' and imported cultivars is low, then it is likely that 'Citra Laga' is not genetically much different from imported cultivars. If the genetic variation level between 'Citra Laga' and imported cultivars is high, then it can be said that 'Citra Laga' has genetic differences as special characteristics for the 'Citra Laga' cultivar.

Genetic variation can be analyzed using DNA molecular markers. One of the molecular markers that can be used is the Inter-Simple Sequence Repeat (ISSR) (Abdein 2018). ISSR is a *Polymerase Chain Reaction* (PCR) based technique that uses microsatellite sequences for DNA amplification (Andriyani & Jadid 2021). The regions between these microsatellite sequences when amplified through PCR with a single primer will produce products that can be used as multilocal markers in the genetic

variation study (Ng & Tan 2015). The ISSR has the advantage of being simple and having a high degree of polymorphism. ISSR is also known to be reproducible for *fingerprinting*, genetic diversity studies, and kinship studies (Abdein 2018).

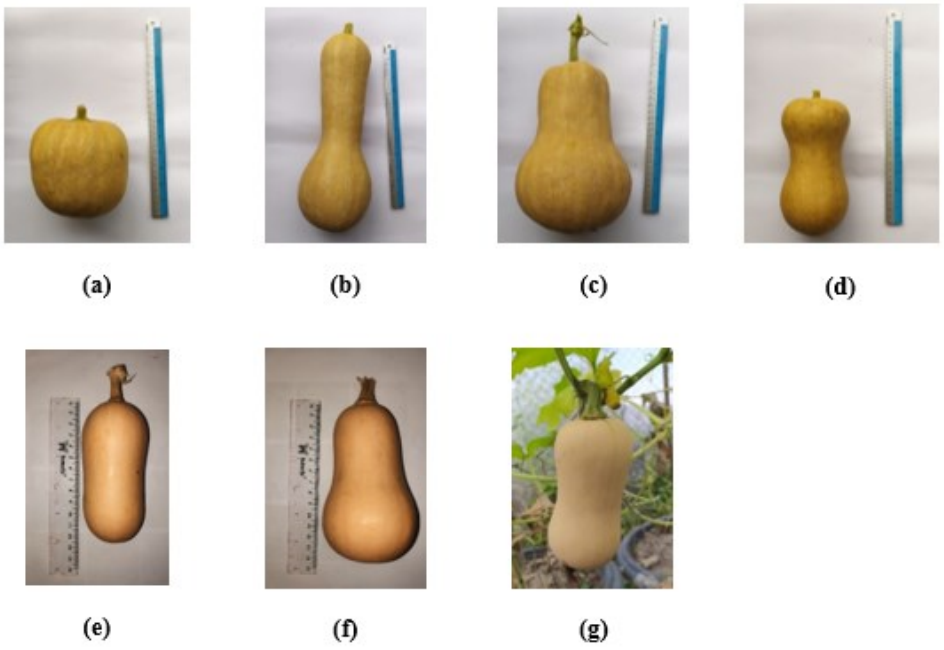

Figure 2. Fruit shape of (a-d) 'Citra Laga', (e) 'Tiana', (f) 'Waltham', and (g) 'Jacqueline'. ((a) globular, (b, c, f) pyriform, (d) dumbbell, and (e, g) blocky).

This study was done from April to July 2022 at the Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada. The sampling was carried out at Pusat Inovasi Agroteknologi of UGM Green House, Kalitirto, Berbah, Sleman, Yogyakarta. The samples were 3-4 weeks old leaves of 'Citra Laga', 'Tiana', 'Waltham', and 'Jacqueline' that were healthy and not infected with viruses to minimize contaminants. The too-young leaves generally contain high RNA, while the too-old leaves contain high secondary metabolites. DNA extraction as a first step in molecular analysis in plants was performed to separate DNA from various contaminants such as cell membranes, RNA protein, and other cell components so that pure DNA can be obtained (Gupta 2019). In general, the DNA extraction steps are the cell walls and membranes lysis, nucleolar lysis, and DNA precipitation (Sari et al. 2014). In this study, the DNA extraction method was done using Geneaid Genomic DNA mini kit for the plant according to Melani et al. (2018) with some modifications. The materials used were 100 mg leaves, 800 µL GP1 buffer, 100 µL GP2 buffer, 1,5X GP3 buffer, 400 µL W1 buffer, 600 µL wash buffer, and 50 µL elution buffer. The quality of the DNA extract obtained was then measured with Nanodrop UV-Vis Spectrophotometer.

Electrophoresis visualization of PCR products using ISSR primers can show the polymorphic and monomorphic DNA bands. If the primer is capable to amplify varied genome regions, then the DNA bands produced will also be polymorphic whereas if the primer did not amplify varied genome regions, it will produce monomorphic DNA bands. Thus, it can be said that polymorphic bands are DNA bands that do not always appear in all observed individuals. In contrast, monomorphic DNA bands are always found in all observed individuals (Munif et al. 2004). The ISSR primers used in this study were UBC 807, UBC 809, UBC 810, UBC 812, and UBC 841. The use of these 5 ISSR primers refers to the research of Inan et al. (2012) which examined the efficacy of the ISSR technique for

molecular characterization in squashes (*Cucurbita*). The UBC 807, UBC 809, UBC 810, UBC 812, and UBC 841 primers on Inan et al. (2012) research succeeded in producing 100% polymorphism level in *Cucurbita pepo*, *Cucurbita moschata*, and *Cucurbita maxima*. Therefore, those 5 primers were used to see whether UBC 807, UBC 809, UBC 810, UBC 812, and UBC 841 could also produce high levels of polymorphism for *the Cucurbita moschata* variety level.

The PCR composition for DNA amplification was 12,5 µL MyTaq HS Red Mix 2X Bioline PCR kit, 2 µL DNA template, 1 µL ISSR primer, and 9,5 µL ddH_2O. The protocols for DNA amplification were 3 minutes of pre-denaturation at 95°C, 1 minute of denaturation at 95°C, 1 minute and 30 seconds of annealing at 49,5-54,1°C (depend on primers in Table 1), 1 minute of extension at 72°C, 5 minutes of post-extension at 72°C, and infinity hold at 12°C. The denaturation, annealing, and extension processes were performed in 35 cycles. The amplified PCR-ISSR products were then separated by electrophoresis on 2% agarose gel with 1X TBE buffer for 60 minutes at 50V. DNA band patterns were visualized with gel doc UV-Transilluminator. The DNA band patterns were converted into binary data 0-1 based on the presence or the absence of the DNA band then they were analyzed using MVSP 3.1 program with the UPGMA method. The PCR visualization results are presented in Figure 3.

Table 1. ISSR primers.

Primer	Sequences (5'→3')	Annealing Temperature (°C)
UBC 807	AGAGAGAGAGAGAGAGT	52,3
UBC 809	AGAGAGAGAGAGAGAGG	54,1
UBC 810	GAGAGAGAGAGAGAGAT	45
UBC 812	GAGAGAGAGAGAGAGAC	47,5
UBC 841	GAGAGAGAGAGAGAGATC	49,5

The visualization using UBC 807 presented in Figure 3a shows a total of 9 DNA bands at size ±151 - ±1253 bp. The DNA bands consist of 2 polymorphic and 7 monomorphic DNA bands. Polymorphic band sizes were ±845 bp found in T and W, also 391 bp found in T, W, and J. Figure 3b shows a visualization using UBC 809 primer. The total DNA bands obtained were 12 bands with sizes of ±195 - ±1720 bp. The total polymorphic DNA bands were 4 bands with sizes of ±1191 bp found in CL, T, and W, ±1175 bp found in CL, W, and J, ±231 bp found in J, and ±195 bp found in CL. The visualization using UBC 810 primer in Figure 3c shows the total of DNA bands obtained were 8 with all of them being monomorphic. The size of the DNA bands was ±245 - ±1330 bp. The visualization using UBC 812 primer in Figure 3d shows 5 monomorphic bands in the size of ±271 bp - ±1290 bp without polymorphic bands. The absence of polymorphic DNA bands in both results means that the UBC 810 and UBC 812 primers produced low genetic variation. Figure 3e shows that the visualization using UBC 841 primer produced 8 DNA bands with sizes of ±357 bp - ±1861 bp that consist of 3 polymorphic bands and 5 monomorphic bands. The polymorphic bands sized ±1760 bp, ±539 bp, and ±359 bp were found in T, W, and J.

Table 2 shows that the total DNA bands amplified in this study were 42 bands with a total of 33 monomorphic bands and 9 polymorphic bands. The average number of polymorphic bands and the polymorphism percentage were 1.8 and 18.61%. Based on all the results above, it can be seen that the UBC 841 primer produced the highest genetic variation

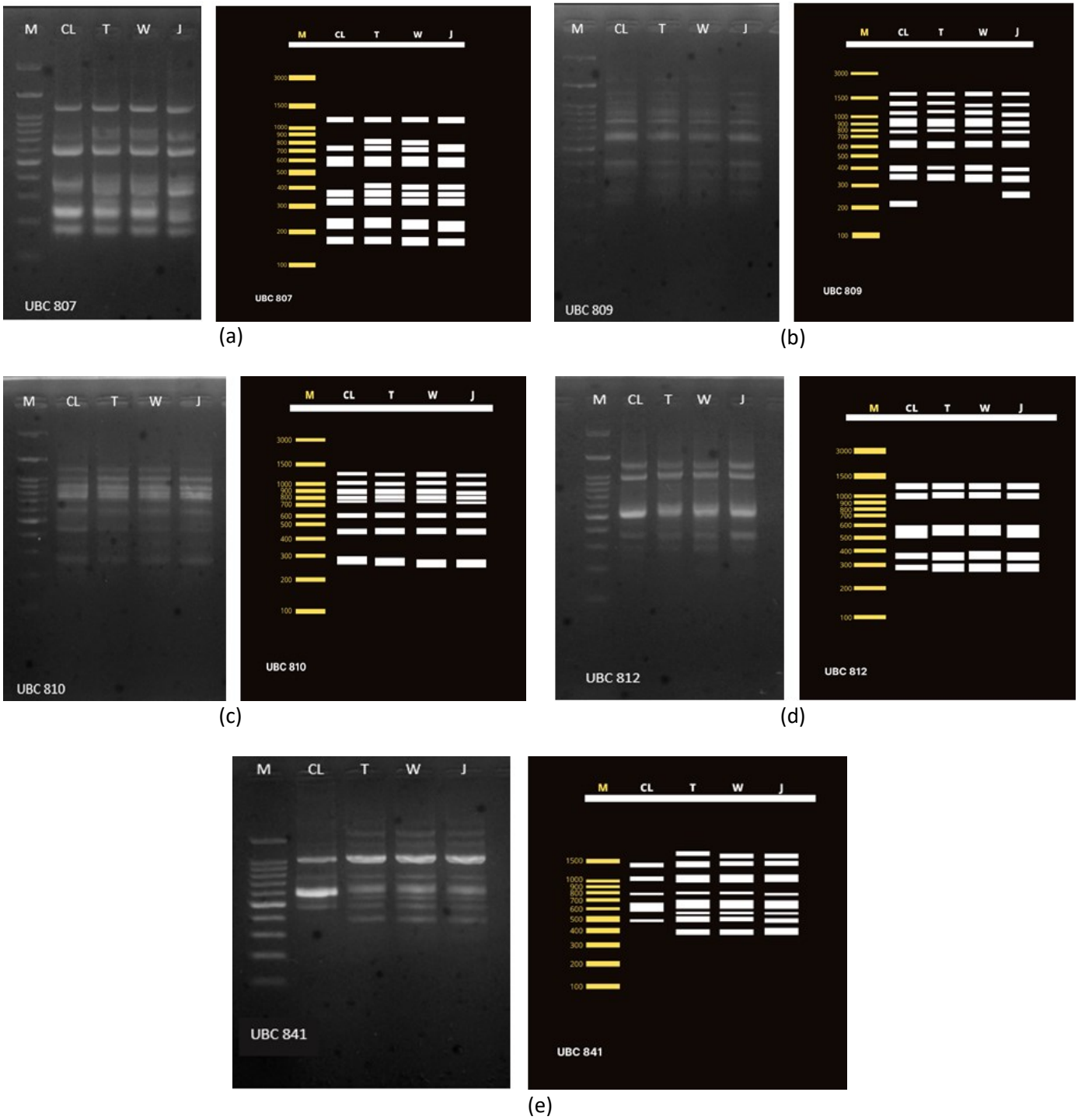

Figure 3. PCR products visualization of 4 butternut squash cultivars using (a) UBC 807 primer, (b) UBC 809 primer, (c) UBC 810 primer, (d) UBC 812 primer, and (e) UBC 841 primer. (M= Marker, CL= 'Citra Laga', T= Tiana, W= Waltham, and J= Jacqueline).

with a polymorphism percentage of 37.5%, followed by the UBC 809 primer with a percentage of 33.33%, and UBC 807 with a percentage of 22.22%. The UBC 810 and UBC 812 primers had the lowest genetic variation due to the absence of polymorphic DNA bands. Samiyarsih et al. (2020) stated that primers with a high level of polymorphism are those with percentage of polymorphism ≥50%. Therefore, it can be said that all primers used in this study had a low level of polymorphism.

The similarity index between the 4 cultivars of butternut squash may indicate their kinship. In phenetic analysis, the similarity of all existing characters will be compiled. If the similarity value is high, then the kinship relation will also be close (Rahayu & Jannah 2019). The results of

Table 2. Polymorphism percentage of 4 butternut squash cultivars based on 5 ISSR primers.

ISSR Primer	Total DNA Bands	Total Monomorphic Bands	Total Polymorphic Bands	Percentage of Polymorphism (%)	Size of DNA Bands (bp)
UBC 807	9	7	2	22.22	151-1253
UBC 809	12	8	4	33.33	195-1720
UBC 810	8	8	0	0	24-1330
UBC 812	5	5	0	0	271-1290
UBC 841	8	5	3	37.5	357-1861
Total	42	33	9	-	-
Average	8.4	6.6	1.8	18.61	-

the phenetic relationship analysis of 'Citra Laga', 'Tiana', 'Waltham', and 'Jacqueline' are presented in Figure 4.

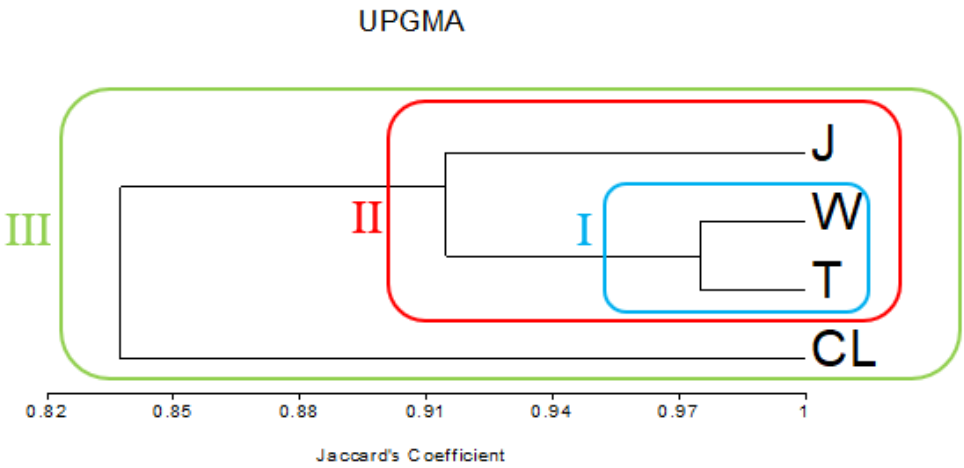

Figure 4. Dendrogram showing the clustering of 4 butternut squash cultivars based on the molecular character using the UPGMA methods with Jaccard's Coefficient (CL= 'Citra Laga', T= 'Tiana', W= 'Waltham', dan J= 'Jacqueline').

Figure 4 shows that the analysis of the phenetic relationship between 4 butternut squash cultivars resulted in a total of 3 clusters, namely I, II, and III. Cluster I consist of 'Waltham' and 'Tiana' with a similarity percentage of 97.5%. Furthermore ' Waltham' and 'Tiana' fused into one cluster with 'Jacqueline' forming cluster II with a similarity percentage of 91.5%. Next, 'Citra Laga' fused with 'Waltham', 'Tiana', and 'Jacqueline' forming cluster III with a similarity percentage of 83.7%.

Based on the clusters obtained, it can be seen that 'Tiana' had the closest phenetic kinship to 'Waltham', while 'Citra Laga' had the most distant phenetic kinship relationship compared to all the cultivars tested. Nonetheless, all cultivars have a high level of similarity because they are valued at ≥70% so it can be said that the phenetic kinship relationship between the 4 butternut squash cultivars tested is very close. The high similarity level is in line with the low polymorphism level and that means the genetic variation of 4 butternut squash cultivars in this study is molecularly low. The low polymorphism level and the high similarity level are due to the samples tested in this study and are still being included in one species, namely *Cucurbita moschata*. According to Hidzroh & Daryono (2021), the genetic components in the same species tend to be the same, so genetic variation may be low. The accuracy of the primers selection can also be the reason for the results obtained in this study because the number of polymorphic DNA bands depends on the ISSR primer and the sample used. Inan et al. (2012) in their study using the UBC 807, UBC 809, UBC 810, UBC 812, and UBC 841 primers resulted in high rate of poly-

morphism at the genus level of *Cucurbita*. Therefore, it can be said that the 5 primers used in this study are more precise to analyze polymorphism at the *Cucurbita* genus level, not for the *Cucurbita moschata* variety level. Despite this, some primers can still detect the presence of genetic variation between the 4 butternut squash cultivars. Genetic variations that appear in the same species can be caused due to mutations and recombination as a result of a long selection process (Daryono & Maryanto 2017).

This study concluded that 'Citra Laga' cultivar and 3 imported cultivars, specifically 'Tiana', 'Waltham', and 'Jacqueline', had a low polymorphism level and their phenetic kinship relationship was close. Thus, it can be said that their genetic variation is low. The results of this study are expected to be supporting data for the process of proposing 'Citra Laga' as an Indonesian local cultivar. The closeness genetic between 'Citra Laga' and the imported cultivars means that genetically 'Citra Laga' is not much different from the imported one, so the 'Citra Laga' will be able to compete as a local cultivar native to Indonesia.

AUTHORS CONTRIBUTION

N.R.A.P analyzed the data and wrote the manuscript. P.S.K. and B.F.A. collected the samples. D.S., P., and B.S.D. designed the research and supervised all research processes.

ACKNOWLEDGMENTS

This research was funded by MBKM (Merdeka Belajar Kampus Merdeka) Research Program by Faculty of Biology, Universitas Gadjah Mada, No: 823/UN1/FBI.1/KSA/PT.01.03/2022.

CONFLICT OF INTEREST

There is no conflict of interest in the research or in the research funding.

REFERENCES

Abdein, M.A.E-H., 2018. Genetic diversity between pumpkin accessions growing in the northern border region in Saudi Arabia based on biochemical and molecular parameters. *Egyptian Journal of Botany*, 58(3), pp.463-375. doi: 10.21608/EJBO.2018.3612.1171.

Andriyani & Jadid, N., 2021. Genetic diversity and morphological responses of *Capsicum annuum* varieties under alumunium stress. *Biodiversitas*, 22(5), pp.2576-2582. doi: 10.13057/biodiv/d220516.

Dari, L. & Yaro, N.S., 2016. Nutritional composition and storage of butternut squash. *Ghana Journal of Horticulture*, 12(1), pp.25-31.

Daryono, B.S. & Maryanto, S.D., 2017. *Keanekaragaman dan Potensi Sumber Daya Genetik Melon*, Yogyakarta: Gadjah Mada University Press.

Gupta, N., 2019. DNA extraction and polymerase chain reaction. *Journal of Cytology*, 36(2), pp.116-117. doi: 10.4103/JOC.JOC_110_18.

Hidzroh, F. & Daryono, B.S., 2021. Keseragaman dan kestabilan karakter tanaman melon (*Cucumis melo* L. 'Tacapa Gold') berdasarkan karakter fenotip dan Inter-Simple Sequence Repeat. *Biospecies*, 14(2), pp.11-19.

Inan, N. et al., 2012. Efficacy of ISSR and SRAP techniques for molecular characterization of some *Cucurbita* genotypes including naked (hull-less) seed pumpkin. *The Journal of Animal and Plant Sciences*, 22(1), pp.126-136.

Marie-Magdeleine, C., Mahie, M., & Archimede, H., 2011. Pumpkin (*Cucurbita moschata* Duchesne ex Poir.) Seeds as an Anthelmintic Agent?. In *Nuts and Seeds in Health and Disease Prevention*. Massachusetts: Academic Press, pp.933-939.

Melani, R., Adiartayasa, W., & Wijaya, I.N., 2018. Deteksi penyakit *Citrus Vein Phloem Degenation* (CVPD) dengan teknik *Polymerase Chain Reaction* (PCR) pada daun tanaman jeruk yang memiliki pola gejala kronis berbeda. *E-Jurnal Agroteknologi Tropika*, 7(2), pp.104-173.

Munif, A. et al., 2004. Polimorfisme genetik dari *Anopheles barbirostris* kaitannya dengan prevalensi malaria di Kecamatan Cineam, Kabupaten Tasikmalaya. *Buletin Penelitian Kesehatan*, 32(1), pp.1-16.

Ng, W.L., & Tan, S.G., 2015. Inter-simple sequence repeat (ISSR) markers: are we doing it right?. *ASM Science Journal*, 9(1), pp.30-39.

Nopianasanti, H. & Daryono, B.S., 2018. Kestabilan fenotip tanaman labu susu (*Cucurbita moshata* (Duchesne) Poir "Butternut") hasil budidaya di Sleman D.I Yogyakarta. *Biogenesis*, 6(2), pp.115-123. doi: 10.24252/bio.v6i2.4751.

Purnomo, Daryono, B.S., & Sentori, M.B., 2015. Variability and intraspecies classification of pumpkin (*Cucurbita moschata* (Duch. ex Lam.) Duch. ex Poir.) based on morphological characters. *KnE Life Sciences*, 2, pp.286-293. doi: 10.18502/kls.v2i1.160.

Rahayu, D.A. & Jannah, M., 2019. *DNA Barcode Hewan dan Tumbuhan Indonesia*, Jakarta: Yayasan Inspirasi Ide Berdaya.

Samiyarsih, S. et al., 2020. Anatomical profile and genetic variability of sweet potato (*Ipomoea batatas*) cultivars in Banyumas, Central Java, based on RAPD markers. *Biodiversitas*, 21(4), pp.1755-1766. doi: 10.13057/biodiv/d210460.

Sari, S.K. et al., 2014. Optimasi teknik isolasi dan purifikasi dna pada daun cabai rawit (*Capsicum frutescens* cv. Cakra Hijau) menggunakan genomic DNA mini kit (plant) Geneaid. *Prosiding Seminar Nasional XI Pendidikan Biologi FKIP UNS*, 11(1), pp.65-70.