

Morpho-genetic characterization of diploid and tetraploid taro (*Colocasia esculenta* L. Schott) cv. Kaliurang – An Indonesian local cultivar

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Abstract: Kaliurang is one of the local taro cultivars in Indonesia, potentially due to its high productivity and delicacy. This study analysed morpho-genetic variation between one diploid and three tetraploid Kaliurang taro clones from *in vitro* polyploid induction after plantlet acclimatization in the greenhouse for 6 weeks. Plants' morphological characters were analysed using the unweighted pair group method with arithmetic mean (UPGMA) method based on 50 characters according to the taro descriptor from the International Plant Genetic Resources Institute (IPGRI). Furthermore, a morphometric characterization based on the Euclidean distance of 17 digitations from mature leaves, was analysed with the Analysis Phylogenic and Evolution package in R software. DNA band pattern was also performed with ISSR to analyse the clone's genetic variance. The results showed morpho-genetic character diversity in diploid and tetraploid plants. A plant morphological study revealed that 22 out of 50 characters were distinct, with similarity coefficients ranging from 0.35 to 0.78. Leaf geometric morphometric analysis showed changes in five digitations and formed three clusters. Twelve ISSR primers out of twenty amplified the sequence of the Kaliurang taro genome to yield two clusters with a similarity coefficient of 0.71–0.83. Based on these three characterization approaches, tetraploid clones (K2 and K3) consistently differed morphologically from diploid clones (K0).

Keywords: clones; clusters; ISSR; morphology; morphometric

As a functional food, taro may support a food security program in Indonesia. In Indonesia, 20 cultivars of taro are inventoried by preliminary researchers through the Taro Network for South East Asia and Oceania (TANSO) project (Prana & Kuswara 2002). Twenty-three local varieties of taro and breeding achievement have also been listed on The Center for Crop Variety Protection and Agricultural Licensing

(PVTTP) with the year of registration from 2015 to 2021 (Pusat PVTTP 2021).

The breeding program of taro is very challenging due to the vastness of genetic variability, genetic instability, and low tolerance to environmental changes, thus leading to low productivity (Banjaw 2017). Polyploidy in tuber crops has high breeding value since the new cultivar may have larger yields, better

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quality, and greater resistance to biotic and abiotic challenges (Palanivel & Shah 2021). For instance, tetraploid cassava showed better photosynthetic capacities than the original diploid plants because significant differences in morphology and anatomy were found between the diploid and tetraploid plants (Zhou et al. 2017). Induction of tetraploids in other tuber crops, namely carmine radish plant, showed that the tetraploid plants displayed the “Gigas” effect, including the greater size of both vegetative (leaf, taproot) and reproductive organs (flower) (Pei et al. 2019). Induction of tetraploids in other tuberous plants belonging to the ornamental plant group, ‘Red Flash’ *Caladium* produced ten variants based on leaf shape colour differences and colouration (Zhang et al. 2020).

Polyploidy in taro has been documented in the Bentul cultivar when oryzalin was used (Wulansari et al. 2016) and in the Kaliurang cultivar when colchicine was used (Ermayanti et al. 2018). Kaliurang is one of the top Indonesian native cultivars named after the Kaliurang area in Yogyakarta province. Morphological characteristics are only described in general. They have extended leaves because of the stem curling outward. The leaf stalks are green mixed with reddish light purple and relatively high stature, reaching 150 cm or more. With such qualities, this plant is simple to identify in the field due to its distinct purplish-green leaf stalk that differs from the full green typical of other native Indonesian taro. This cultivar grows well in both the lowlands and the highlands, and the tubers range in size from oval to spherical, weighing 1 to 4 kg. Furthermore, the flesh of the tuber is white. Also, it tastes better and fluffier, is disease-tolerant, and is pest-resistant (Prana & Kuswara 2002).

Comprehensive information about the differences in the characteristics of diploid and tetraploid taro plants can be used as the basis for sustainable breeding efforts. The analysis of plant morphological, leaf geometric morphometrical, and molecular markers can be applied to know the characteristic diversity. Morphological analysis is a traditional technique used to know genetic variation within species referring to morphology differences (Acquaah 2012). Morphology is still helpful as a focal point in the determination, characterization, preparation operations, and primary data for evidence taxonomies utilized as a generic approach to genetic diversity (Beyene et al. 2005; Jingura & Kamusoko 2015; Rifai 2017). Furthermore, morphometric analysis is a new

method to detect genetic diversity represented by geographic localities, developmental stages, genetic effects, environmental effects, etc. It is a more effective and powerful statistical procedure in capturing shape differences (Rohlf 1990).

The inter simple sequence repeat (ISSR) marker may assess genetic diversity across plants. It is more reliable than other markers such as random amplified polymorphic DNA (RAPD) and amplified fragments of length polymorphic DNA (AFLP). ISSR is a genotyping technique that amplifies DNA segments between two identical microsatellites repeating regions in opposite directions (Ng & Tan 2015). Analysis of genetic variation of plants using ISSR has been successfully conducted to compare *Xanthosoma* (taioba) and *Colocasia* (taro) from Brazil (Sepúlveda-Nieto et al. 2017), on *Anthurium* one of the ornamental plants from family Araceae (Srisamoot & Padsri 2018) and melon (Daryono et al. 2019). This study analyses the morpho-genetic variation between the diploid and tetraploid Kaliurang taro clones.

MATERIAL AND METHODS

Plant material. In this study, one diploid (K0) and three tetraploid clones (K1, K2, K3) of Kaliurang taro (*Colocasia esculenta* cv. Kaliurang) were used. Ermayanti et al. (2018) confirmed the ploidy level of these clones by flow cytometric analysis and chromosome counting. Chromosome metaphase photographs were documented in Figure S1 in Electronic Supplementary Material (ESM). The K1 clone was made polyploid by immersing the *in vitro* culture in 0.1% colchicine solution for one day. Meanwhile, the K2 and K3 clones were made polyploid by immersing the *in vitro* culture in 0.05% colchicine solution. Finally, all clones (diploid control and tetraploids) were grown in the greenhouse for six weeks to reach the first leaf fully bloom, and the leaves are used to reconfirm the taro clones’ ploidy level using a BD Accuri C6 Plus flow cytometer (Biosciences, USA) (Figure S2 in the ESM).

Plant morphological analysis. Morphological characteristic analysis was carried out by observing vegetative organs such as roots, stem, sheaths, petioles, and leaves of 6-week-old plants of three different individuals for each clone. Data collection, literature study, morphology recording, plant descriptions, and clustering analysis were all used in the approach based on Rifai’s (2017) procedure. These characters’ observation was extracted from Taro Descriptor by

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The International Plant Genetic Resources Institute (IPGRI 1999), the Manual of herbarium taxonomy (Veldkamp 1987), and The Genera of Araceae (Mayo et al. 1997). Furthermore, morphological variety was observed based on 50 characters and applied binary scoring, while Sequential Agglomerative Hierarchical Nested (SAHN) cluster analysis from the SIMQUAL NTSYS (Ver. 2.11a) program, with the unweighted pair group method with arithmetic mean (UPGMA) method, resulted in a dendrogram based on similarity coefficient.

Leaf geometric morphometric analysis. According to the Viscosi and Cardini protocol (2012), the geometric morphometric analysis was carried out. Firstly, photographs of the abaxial leaf of four clones of Kaliurang taro were obtained using a digital camera, and three leaves were used for specimen repetition of each clone. The photographs were then digitized using the tpsDig application to generate coordinates with three replicates (Morais et al. 2019), and variations between specimens were analysed using nonaffine change. Furthermore, the abaxial leaf venation description was determined using 17 anatomical points (Figure 1) and the description digitation consensus (Table S1 in ESM), and the average of all coordinate species was calculated using the tpsRelw program (Rohlf 2003c). The result of the digitation species average was visualized using the tpsSuper program (Rohlf 2004b), and deformation from each species of Kaliurang taro was visualized using the tpsSpln program (Rohlf 2004a) to observe the variation. Phylogenetic relations between specimens were determined using R software based on relative warp value. A phylogenetic diagram to describe the relationship between specimen or clones in the morphometric

analysis was prepared using the Neighbour Joining method in Analysis Phylogenetics and Evolution package (Paradis & Schliep 2019) in R software (R Core Team 2021).

Plant genetic variation analysis. A quick-DNA plant miniprep kit (Zymo Research, USA) was used to extract DNA from 150 mg of finely sliced Kaliurang taro leaves. Beta-mercaptoethanol was added to the Genomic Lysis Buffer (Zymo Research) at a final dilution of 0.5% (v/v) to improve its performance. DNA extraction procedure follows the Zymo Research user manual. After extraction, subsequently, the eluted DNA was transferred to a prepared Zymo-Spin™ III-HRC Spin Filter (Zymo Research) in a clean 1.5 mL microcentrifuge tube and centrifuged at exactly $16\,000 \times g$ for 3 min. The DNA had been screened and was ready for PCR.

Twelve from twenty Sigma primers, based on Singh et al. (2012) were evaluated for preliminary ISSR analysis, producing repeatable bands. Initial denaturation was performed at $94\text{ }^{\circ}\text{C}$ for 3 min, followed by 35 cycles at $94\text{ }^{\circ}\text{C}$ for 30 s. Furthermore, primer annealing denaturation was performed for 45 s at different temperatures. A 30-s extension at $72\text{ }^{\circ}\text{C}$ was then performed, followed by a 5-min extension at $72\text{ }^{\circ}\text{C}$, and only reproducible products were considered for further data analysis.

The presence or absence of DNA bands based on electrophoresis data was evaluated. Molecular characterization on three replicates was arranged in a binary matrix and scored. In the absence of a band, the three replications yield a value of zero (0), but they yield a value of one (1) when there is a band. Similarity dendrograms were constructed using SAHN from the SIMQUAL NTSYS (Ver. 2.11a) program (Rohlf 1998).

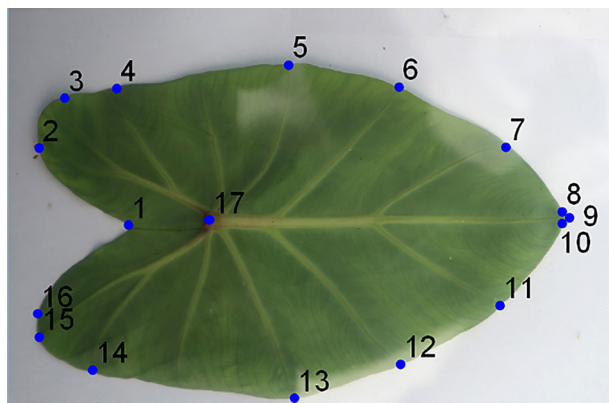


Figure 1. Digitation consensus of Kaliurang taro leaf (abaxial)

RESULTS AND DISCUSSION

Plant morphological analysis. Based on that three references, Kaliurang taro has a general or stable character of monocot, herbaceous, and terrestrial; fibrous root; end of midrib pointed, smooth texture, covered; petiole growing upright and straight; single leaves and complete with a midrib, petiole, and leaf blade, a paper-like texture, moderate posterior, arrowhead shape (sagittate), heart base, tip ribbed, bifacial surface since the colour of the adaxial is different from that of abaxial leaf side, glabrous, colocasioid vein, submarginal vein, number of main veins 8-10, leaf blade on petiole insertion, smooth

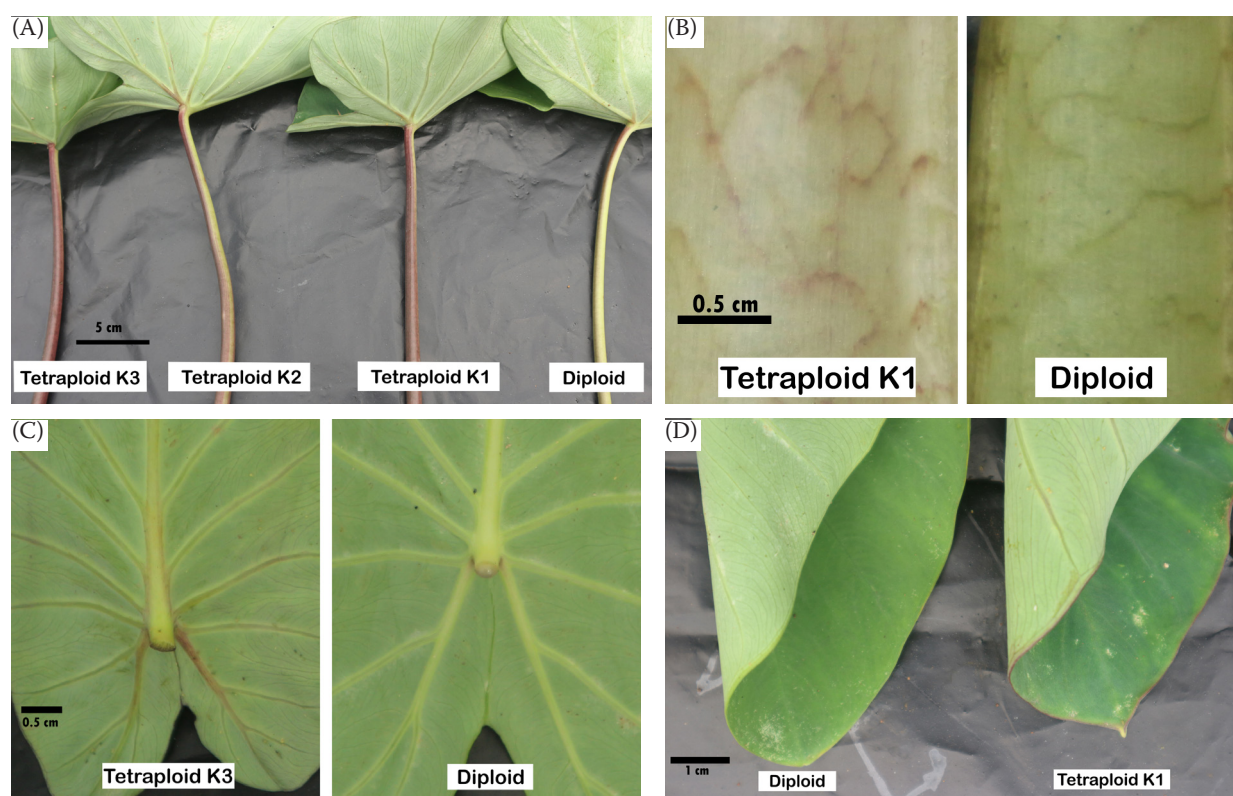


Figure 2. Variation of morphological appearances between tetraploid (K1, K2 and K3) and diploid (K0) taro Kaliurang: petiole colour (scale bar = 5 cm) (A), sheath pattern (scale bar = 0.5 cm) (B), colour of the abaxial vein on V pattern (scale bar = 0.5 cm) (C), colour of leaf margin (scale bar = 1 cm) (D)

surface, position predominantly downward, and young leaves curl.

Subsequently, 50 characters were studied for morphological traits, including habitus, sheath, petiole, and leaves. Several variations of morphological appearances between diploid and tetraploid Kaliurang taro were presented in Figure 2. Based on morphological characterization, 22 (Table S2 in ESM) out of 50 characters were diverse, indicating the morphological separation of diploid from tetraploid clones.

The qualitative and quantitative character of the four Kaliurang taro clones showed several variations. Phenetic analysis based on the morphological characters of the four clones produced two groups with a similarity coefficient of 0.35–0.78 as shown in Figure 3. The coefficient of similarity between clusters one and two is 0.35. This low value indicates the Kaliurang taro diploid and tetraploids have little similarity in character, while the similarity coefficient between sub-cluster A and sub-cluster B is 0.72. This

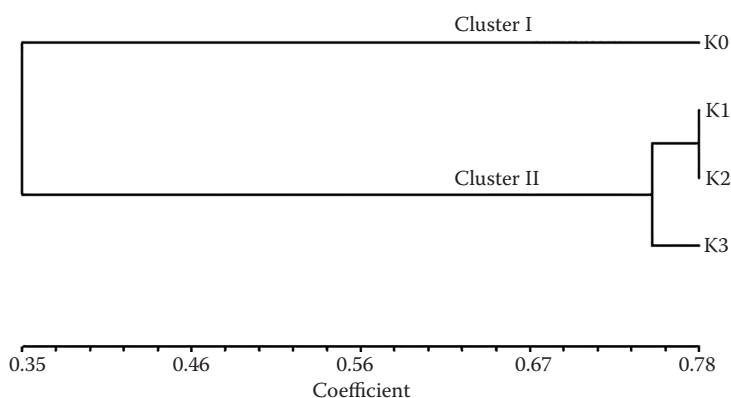


Figure 3. Dendrogram of Kaliurang taro clustering based on morphological characterisation

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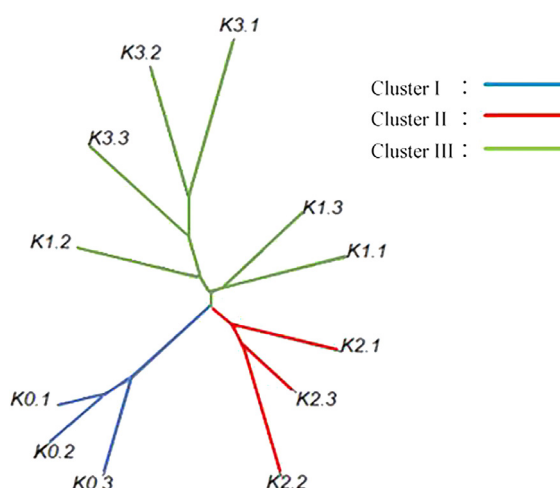


Figure 4. The clustering tree based on morphometric geometric analysis of leaf of clone diploid (K0) and tetraploid clones of Kaliurang taro (K1, K2 and K3)

result shows that taro tetraploids have many characters in common, especially between K1 and K2, shown by the coefficient of similarity value of 0.78. Morphology variations between diploid and tetraploid plants are in accordance with previous research on cassava (Zhou et al. 2017), carmine radish plant (Pei et al. 2019) and *Calladium* (Zhang et al. 2020).

Leaf geometric morphometric analysis. Geometric morphometric analysis on Kaliurang taro leaves was carried out to quantitatively describe the difference in their morphological forms. The digitization results showed average leaf shape based on the average value of 17 points on total of twelve leaf samples (Figure S3 in ESM).

The phylogenetic relation between Kaliurang taro clones using R software based on relative warp value is shown in Figure 4. Diploid Kaliurang taro is distinguished from tetraploid Kaliurang taro based on variation in shape or deformation.

Table 1. Specific band of the Kaliurang taro clone resulted from ISSR marker analysis

Clone	Specific band
K0 (diploid)	(CA) ₈ ART T ⁷⁰⁰ , (AC) ₈ YG T ⁵⁵⁰ , (CA) ₈ YC T ⁷⁰⁰
K1 (tetraploid)	(AG) ₈ YC T ²⁵⁰
K2 (tetraploid)	–
K3 (tetraploid)	(CA 8YC)T ⁴⁵⁰ , (AG 8YC)T ³⁵⁰ , (GGC 6)T ²⁵⁰

Figure 5 shows the differences in leaf typology between clusters I, II, and III. Cluster I has a typology that is longer than cluster II and III. This difference can be seen from the Ts points in clusters II and III, pointing downwards (inferior), which indicates that these two clusters have shorter leaf sizes than cluster I. Meanwhile, clusters II and III have wider leaf typologies, seen from the points Vn1, Vn2 Vn3 which are widened with variations at the point Vp.

A study on plant grouping based on morphometry was also carried out by Raisal Haq (2019) on 252 *Nepenthes* species. The shape was grouped into seven to distinguish each *Nepenthes* species using the flower bag's character. Previous researcher (Ariawan et al. 2020) identified *Shorea* species in the seedling phase from leaf shape with geometric morphometrics analysis. There are no species misidentifications; therefore, the conservation effort was not compromised.

ISSR analysis. The sequencing of the Kaliurang taro genome was amplified by twelve ISSR primers (Table S3 in ESM) from twenty primers, yielding 42 bands with an optimization temperature of 35–55 °C. Nineteen bands were polymorphic (45.2%), and the number amplified by each was 2–6 bands with lengths ranging from 250 to 1 000 bp (Table S4 in ESM).

Primer ((TC)₈RG) obtained a maximum number of DNA bands that is six bands measuring from 260–750 bp, and the three DNA bands produced were monomorphic at 250, 350, and 400 bp

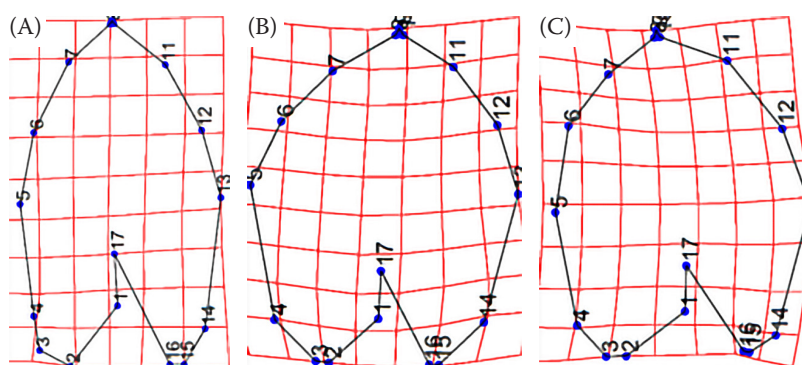


Figure 5. Grid deformation of the abaxial leaf venation of Kaliurang taro: cluster I (diploid K0) (A), cluster II (tetraploid K2) (B), cluster III (tetraploid K1 and K3) (C)

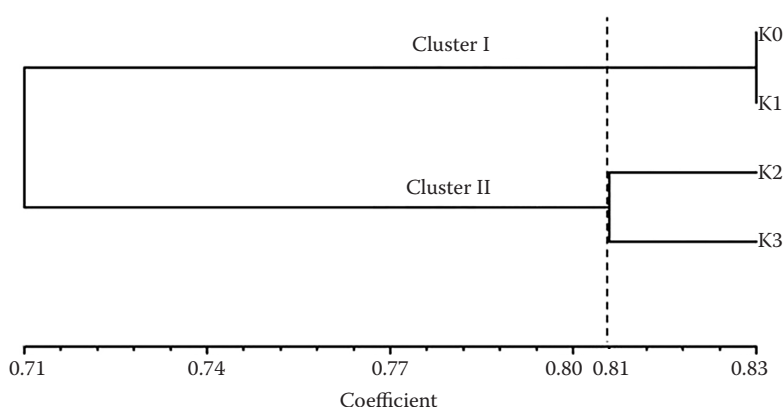


Figure 6. Dendrogram of 4 clones of the Kaliurang taro based on 42 ISSR locus from 12 primer markers with UPGMA analysis

UPGMA – unweighted pair group method with arithmetic mean

(Figure S4 in ESM). Three more DNA bands were polymorphic with 450, 550, and 700 bp. (AG)₈YA primers obtained 100% monomorphic DNA bands, which range from 375–625 bp (Figure S5 in ESM). These primers revealed a consistent DNA band pattern throughout all Kaliurang taro, indicating that the genotype has a stable character. (CTC)₆ primers obtained 100% polymorphic DNA bands, which ranged from 500–1 000 bp (Figure S6 in ESM). Furthermore, 500 bp was detected on K0, K1, and K2 clones, while 1 000 bp was discovered on K0 and K1 clones. Several primers produced distinct bands, which were only present at clones; therefore, primers can be distinguished by clones as shown in Table 1.

Clustering based on ISSR Analysis using SAHN with UPGMA method resulting in a dendrogram with a similarity coefficient of 0.71–0.83 as shown in Figure 6. The results revealed that diploid taro is still in the same cluster as tetraploid K1 clones but separate from tetraploid K2 and K3 clones. Therefore, the variations caused by polyploidization are random based on ISSR markers. This result is similar with Nurilmala et al. (2017), where mutation of Bogor taro showed diversity up to 51% compared to the parents based on AFLP analysis.

CONCLUSION

Three characterization methods of plant morphological, leaves geometric morphometric, and plant genetic analyses with ISSR marker proved that the tetraploid clone of Kaliurang taro had different characters from the diploid clone because of chromosomes doubling. Furthermore, morpho-genetics of tetraploid Kaliurang taro clones (K2 and K3) consistently differed with diploid clones (K0) based on the dendrogram resulting from those three characterization methods.

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