Floribunda 9 (1) 2026: 27 — 33
https://ejournal.brin.go.id/floribunda/
DOI: 10.55981/floribunda.2026.15889
P-ISSN : 0215 - 4706, E-ISSN : 2460 - 6944

DNA Barcoding and Taxonomic Placement of *Alocasia flemingiana* Yuzammi & A. Hay Based on *trnL-trnF* Intergenic Spacer Region

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ABSTRACT

Siti Latipah, Miftahul Jannah & Muhammad Rifqi Hariri 2026. DNA Barcoding dan Posisi Taksonomi *Alocasia flemingiana* Yuzammi & A. Hay berdasarkan sekuen *trnL-trnF* intergenic spacer. Floribunda 9(1) 27 – 33 — *Alocasia flemingiana* Yuzammi & A. Hay merupakan salah satu spesies dari famili Araceae yang masih memiliki keterbatasan data molekuler. Kemiripan morfologi antarspesies *Alocasia* yang begitu tinggi kerap mempersulit proses pengidentifikasian spesies maupun pengklasifikasian taksonomi. Studi ini ditujukan untuk mengkategorisasikan sekuens daerah intergenik *trnL-trnF* (IGS) pada *A. flemingiana* serta mengevaluasi posisi filogenetiknya memakai pendekatan DNA barcoding. Sekuen *trnL-trnF* IGS sepanjang 450 bp berhasil diamplifikasi serta dianalisis. Hasil analisis BLAST memperlihatkan kemiripan sekuens yang tinggi dengan sejumlah spesies *Alocasia*, dengan cakupan kueri senilai 98%-100%, nilai E-value 0,0, serta persentase identitas senilai 99,12%-100%. Kemiripan tertinggi ditemukan pada *Alocasia* sp. PSA280 (100% identitas; 98% query coverage), sedangkan sejumlah aksesi *A. longiloba* pun memperlihatkan taraf kemiripan yang sangat tinggi (>99% identitas). Rekonstruksi filogenetik memakai metode Maximum Likelihood dengan model Tamura 3-parameter dan 1.000 ulangan bootstrap memposisikan *A. flemingiana* dalam satu klad yang didukung kuat bersama *A. longiloba* (ON777914.1), dengan nilai bootstrap senilai 92%. Hasil studi ini memperlihatkan bahwasanya daerah *trnL-trnF* IGS berguna dalam mengidentifikasi spesies dan analisis filogenetik pada genus *Alocasia*, sekaligus memberi bukti molekuler yang mendukung penempatan taksonomi *A. flemingiana*. Penelitian ini berkontribusi pada karakterisasi molekuler *A. flemingiana* serta menekankan pentingnya DNA barcoding pada kajian taksonomi tumbuhan serta penelitian keanekaragaman hayati.

Kata kunci : Aroid, Filogeni, Jawa, Maximum Likelihood, MEGA 11

Siti Latipah, Miftahul Jannah & Muhammad Rifqi Hariri 2026. DNA Barcoding and Taxonomic Placement of *Alocasia flemingiana* Yuzammi & A. Hay based on *trnL-trnF* intergenic spacer region. Floribunda 9(1) 27 – 33 — *Alocasia flemingiana* Yuzammi & A. Hay is a species of the family Araceae for which molecular data remain limited. The high morphological similarity among *Alocasia* species often complicates species identification and taxonomic classification. This research tended to characterize the *trnL-trnF* intergenic spacer (IGS) sequence of *A. flemingiana* and examine its phylogenetic position employing DNA barcoding. A 450 bp *trnL-trnF* IGS sequence was successfully amplified and examined. BLAST analysis revealed high sequence similarity with several *Alocasia* species, with query coverage ranging from 98% to 100%, E-values of 0.0, and percent identities of 99.12% to 100%. The highest similarity was observed with *Alocasia* sp. PSA280 (100% identity, 98%

query coverage), while several accessions of *A. longiloba* also showed high sequence similarity (>99% identity). Phylogenetic reconstruction using the Maximum Likelihood method under the Tamura 3-parameter model with 1,000 bootstrap replicates placed *A. flemingiana* in a well-supported clade with *A. longiloba* (ON777914.1), supported by a bootstrap value of 92%. These results demonstrate that the *trnL-trnF* IGS region is useful for species identification and phylogenetic inference in *Alocasia*, while providing molecular evidence supporting the taxonomic placement of *A. flemingiana*. This study contributes to the molecular characterization of *A. flemingiana* and highlights the value of DNA barcoding in plant taxonomy and biodiversity research.

Keywords: Aroid, Java, Maximum Likelihood, MEGA 11, Phylogeny

INTRODUCTION

Indonesia is one of the 17 megadiverse countries of the world with extremely high flora diversity. Among these Alocasia(Schott) G. Don, from, the Araceae family, contains about 90 species found in Southeast Asia, Malesia and Australia. (POWO, 2026). Because the Indonesian climate is ideal for growing, the diverse of species in Alocasia can be growing and well-adapted to the hot climate and can be cultivated as plants for food supplementation or a main meal source as ornamental plants, too. Several species are commonly found on Java Island, including *A. longiloba* Miq., *A. flemingiana* Yuzammi & A.Hay, *Alocasia alba* Schott, and *Alocasia puber* (Hassk.) Schott, while numerous other species occur outside Java (Hay, 1991; Asih *et a*l., 2023).

Modern DNA technology has allowed the use of barcoding to unmask gene diversity and for promoting biodiversity studies and for taxonomic support (Peninal *et al*., 2017; Rahayu & Jannah, 2019).

In plants reliable molecular marker are necessary to assure correct molecular identity. Among non-coding regions trnL-trnF IGS (intergenic spacer) the trnL-trnF spacer region is probably the one most often adopted because the region is easily sequenced and can be used to distinguish among species and identify new species. (Lestari *et al*., 2018). Consequently, the *trnL-trnF* IGS marker plays a significant role in plant taxonomy and evolutionary studies.

Alocasia flemingiana is an endemic species restricted to Java Island, with a distribution ranging from West Java to Central Java (POWO, 2026). It was first described by Yuzammi and Hay (Hay, 1998) and is characterized by glossy, heart-shaped leaves, an upright stem, and tuber-like roots, with a height ranging from 90 to 180 cm (Imran *et al*., 2022). This species is monoecious, meaning male and female flowers appear on unisexual inflorescences. The species commonly occurs in tropical forests, ranging from both primary and secondary forests, in swamp regions, in disturbed habitat and in low elevation to 1,000 m above sea level. (Hay, 1991; Nisyawati & Mustaqim, 2017). Moderate levels of humidity and seasonal rain and in fertile soil like the high organic latosols in which this tree lives.

The trnL-trnF IGS is a common marker which have been widely used for studying molecular variation of Alocasia, for example in the case of A. Longiloba where diversity in forms of insertions, deletion, transition and transversions were noted (Asih *et al*., 2023). However, there has no DNA barcoding research of *A. flemingiana* in the world utilizing this DNA marker. Molecular Data of the DNA Barcoding loci *trnL-trnF* Intergenic Spacer in the Genus A. To supplement Alocasia flemingiana and construct a molecular phylogeography as far as our knowledge of the material permits.

MATERIALS AND METHODS

Sample Collection and Preparation

Leaf samples of *A. flemingiana* were obtained from Pangandaran Regency, West Java, Indonesia (roadside habitat) by Arifin Surya Dwipa Irsyam in 2023. The leaf material was decontaminated in sterile 70% ethanol and the lamina of the leaves were removed from the midrib. Roughly 0.5g fresh weight of leaf material was weighed out.

DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted using the TIANGEN Plant DNA Extraction Kit following the manufacturer's protocol, except that the initial incubation step was extended to 30 minutes in a 65°C heat block. Leaf samples were cut into small pieces and homogenized in a 2 ml microcentrifuge tube containing two beads and 700 µl of GP1 buffer using a GenoGrinder 2010 at 1500 rpm for 5 minutes. The homogenate was incubated at 65°C for 30 minutes with periodic mixing every 5 minutes. After cooling at room temperature, 700 µl of chloroform was added, followed by centrifugation at 12,000 rpm for 5 minutes. The supernatant was transferred to a new tube and mixed with 700 µl of GP2 buffer, then centrifuged at 12,000 rpm for 1 minute. Subsequently, 500 µl of GD buffer was added and centrifuged under the same conditions, and this step was repeated twice. The DNA bound to the spin column was eluted using 200 µl of TE buffer and stored at −4°C.

PCR amplification was performed to amplify the *trnL-trnF* IGS region using specific forward and reverse primers (Taberlet *et al*., 1991). The PCR reaction mixture had a total volume of 50 µl, consisting of 10 ng template DNA, 25 µl MyTaq HS Red Mix, 5 µM primer for each forward and reverse, and 16 µl ddH_2O. Amplification was carried out using a Max Thermal Cycler PCRMax under standard cycling conditions, including 94□C initial denaturation for 5 min, 35× cycle consisting of 94□C denaturation for 15 s, 52□C annealing for 15 s, 72□C extension for 15 s, and 72□C final extension for 5 min. The expected fragment size was approximately 400 bp.

PCR Products Analysis The resulting PCR products were detected using a 1% agarose gel electrophoresis that prepared with 1X TAE buffer that add DNA stain. Electrophoresis was run in 100 V for 35 minutes. 100 bp DNA ladder as DNA size marker run in parallel to the samples.DNA products can be visualized under UV transilluminator. Selected DNA product results were then outsourced to 1st BASE (Malaysia) for sequencing under the service of PT Genetika Science Indonesia.

Data Analysis

Sequencing results in the form of forward and reverse chromatograms were processed using BioEdit version 7.2.5 for trimming and contig assembly. The assembled sequences were compared with reference sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) (Boratyn *et al*., 2013). Sequence alignment was performed using MEGA version 11 to determine sequence similarity among samples and reference data (Tamura *et al*., 2021). A phylogenetic tree was reconstructed using the Maximum Likelihood (ML) method with the Tamura 3-parameter (T92) model and 1000 bootstrap replicates (Felsenstein, 1985; Tamura 1992). The analysis included 21 Alocasia species obtained from NCBI, with *Colocasia esculenta* (L.) Schott and *Arisaema amurense* Maxim. used as outgroups. The resulting tree was used to infer the evolutionary relationships of *A. flemingiana* with closely related species.

RESULTS AND DISCUSSION

The amplified trnL-trnF IGS region was successfully detected in A. Flemingiana after electrophoresis. 1% agarose gel electrophoresis clearly demonstrated that a DNA band was found to have an estimated size of around 450 bp (Taberlet *et al*., 1991), which was in agreement with size range predicted (Figure 1). There was no smearing or other bands found, which demonstrates that the PCR amplification was very specific and uncontaminated by anything else. (Mazlan *et al*., 2024). This represents high quality DNA and optimized PCR amplification. An amplicon appearing as a single sharp band is considered a marker for success.1 This is relatively short when compared to the size of some trnL-trnF IGS regions already available in the literature, but is expected to be of this length given the size variability of IGSs in different plant species. (Fitmawati *et al*., 2022; Yilmaz *et al*., 2025).

ISSN 0215-4706 (print) | e-ISSN 2460-6944 (online) |

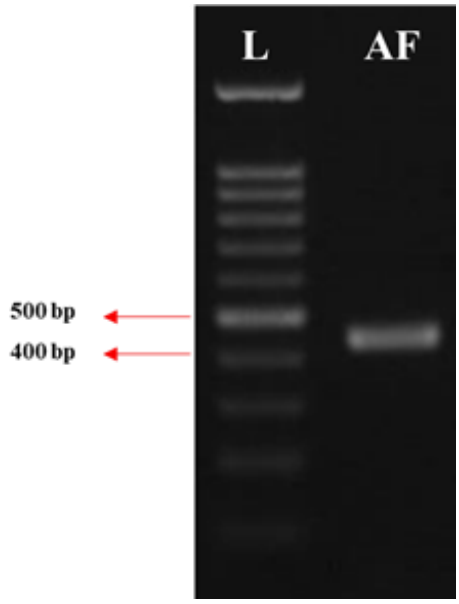

Figure 1. Agarose gel electrophoresis of PCR amplification products of the *trnL-trnF* IGS in *Alocasia flemingiana*. A single distinct DNA band is observed at approximately 450 bp, indicating successful amplification of the target fragment. L=100 bp DNA ladder; AF=*Alocasia flemingiana* sample.

After amplification, sequencing was performed and the resultant high-quality forward and reverse chromatograms were produced with good clear peaks of each nucleotide. The chromatograms were edited, compiled into a consensus contig sequence by assembling with BioEdit and trimming any low quality sequences was carried out. BLAST analysis of the consensus contig against the GenBank database demonstrated very similar match to other species in the same genus (Table 1), with more than 99.56% sequence identity in the case of Alocasia longiloba, and 100% query cover was achieved with this species which meant that no sequence information was lost.(Jain *et al*., 2020).

Additionally, several sequences exhibited percent identity values exceeding 95% and E-values of 0, indicating highly significant matches that are unlikely to have occurred by chance. The highest similarity was observed with *Alocasia* sp. PSA280, which shared 100% sequence identity with *A. flemingiana*. However, despite this complete sequence similarity, phylogenetic analysis placed the two taxa in separate clades, suggesting that the trnL-trnF IGS region may have limited resolution for distinguishing among closely related *Alocasia* species. This result highlights the difference between pairwise sequence similarity and phylogenetic relationships inferred from multiple sequence comparisons. Overall, these findings demonstrate that the trnL-trnF IGS region is sufficiently conserved for reliable identification at the genus level while retaining enough variation to resolve major evolutionary lineages within *Alocasia*.

Table 1. BLAST analysis results of the *trnL-trnF* IGS sequence of *Alocasia flemingiana* compared with top 5 reference sequences in GenBank.

Species name	Accession number	Max Score	Total Score	Query cover (%)	E. value	Percent Identity (%)
Alocasia longiloba	ON777915.1	828	828	100	0.0	99.56
Alocasia sp. PSA280	ON736877.1	826	826	98	0.0	100
Alocasia longiloba	ON745538.1	821	821	98	0.0	99.78
Alocasia longiloba	ON777912.1	819	819	100	0.0	99.12
Alocasia longiloba	ON777913.1	817	817	98	0.0	99.55

Phylogenetic analysis further supported the molecular identification of *A. flemingiana* by providing insight into its evolutionary relationships. The Maximum Likelihood tree constructed under the Tamura 3-parameter (T92) model placed *A. flemingiana* in a well-supported clade with *A. longiloba* ON777914, with a bootstrap value of 92% (Figure 2). Bootstrap values above 70% are generally considered indicative of strong support, suggesting that this relationship is robust and reliable (Yu *et al*., 2019). The clustering of these taxa is consistent with their low genetic distance and high sequence similarity, indicating a close evolutionary relationship and possible shared ancestry (Pearson, 2013).

In contrast, species such as *A. cuprea* (K.Koch & C.D.Bouché) K.Koch, *A. sanderiana* W.Bull, *and A. wongii* A.Hay formed distinct lineages, reflecting greater genetic divergence within the genus. Several internal nodes, including those involving *A. nebula* and *A. reginae*, received moderate bootstrap support (approximately 54%), indicating lower confidence in these relationships and suggesting that additional molecular markers may improve phylogenetic resolution (Yu *et al*., 2019).

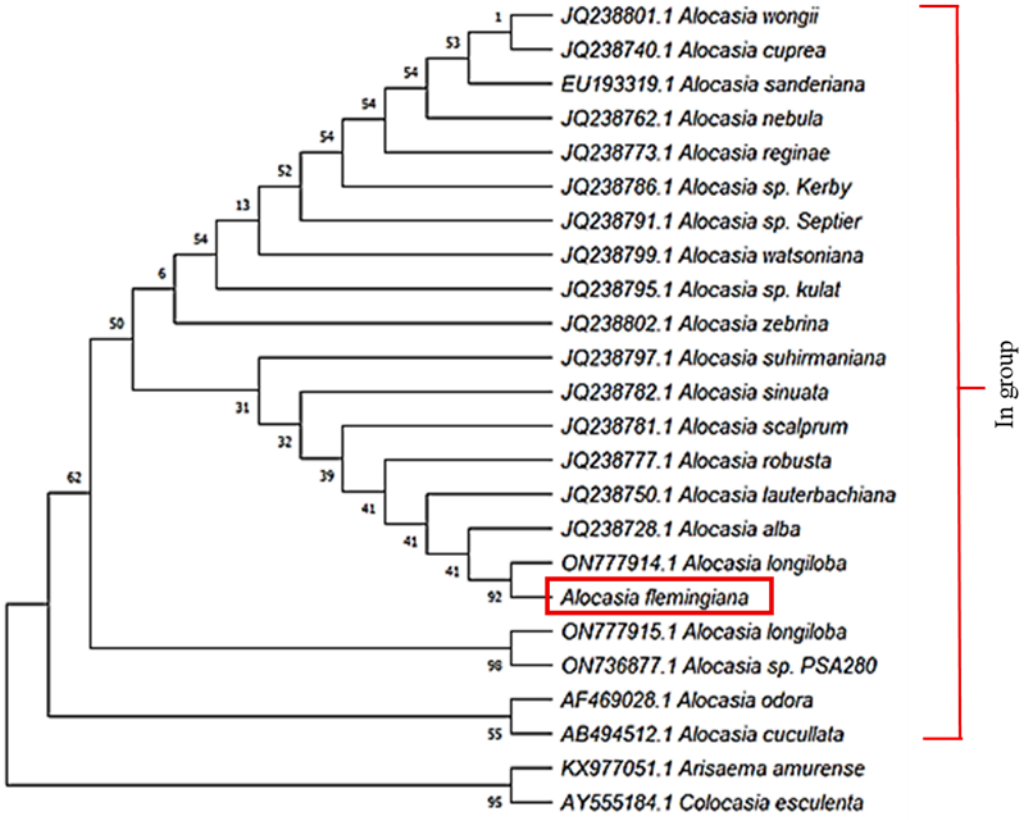

Figure 2. Phylogenetic tree of *Alocasia flemingiana* based on *trnL–trnF* intergenic spacer (IGS) sequences constructed using the Maximum Likelihood method with the Tamura 3-parameter (T92) model and 1000 bootstrap replicates. Bootstrap values are indicated at each node. *Alocasia flemingiana* clusters closely with *Alocasia longiloba* (ON777914.1), while *Colocasia esculenta* and *Arisaema amurense* are used as outgroups to root the tree.

Interestingly, although *Alocasia* sp. PSA280 showed high sequence similarity to *A. flemingiana* in the BLAST analysis, the two taxa were recovered in separate clades. This discrepancy highlights the difference between pairwise sequence similarity and phylogenetic inference (Dwivedi & Gadagkar, 2009). While BLAST seeks out best matching pairs of sequences, phylogenetic reconstruction uses all sites within a sequence alignment which

ISSN 0215-4706 (print) | e-ISSN 2460-6944 (online)

carry meaningful information in the comparison. That the sequences are clearly separate in phylogenetic analysis is perhaps an indication that the trnL-trnF intergenic spacer is not particularly variable, and may be insufficient to fully resolve relationships amongst a group of highly related Alocasia sequences..

Addition of *Colocasia esculenta* and *Arisaema amurense* as outgroups effectively separated the main *Alocasia* clade, confirming their suitability for rooting the phylogenetic tree and distinguishing ancestral and derived character states following Henriquez *et al.* (2014). This result contrasts with previous studies in which *A. suhirmaniana* and *Amorphophallus muelleri* Blume were used as outgroups (Asih *et al.*, 2023). In those analyses, *A. suhirmaniana* was nested within the *A. longiloba* clade, while *A. longiloba* POC PSA388 showed a close relationship with the outgroup (*A. muelleri*), indicating less appropriate outgroup selection. These differences highlight the importance of selecting phylogenetically distinct outgroups to ensure reliable tree topology and accurate interpretation of evolutionary relationships.

Genetic distance analysis provides further quantitative support for the phylogenetic relationships observed. The genetic distance values between *A. flemingiana* and other *Alocasia* species ranged from 0.000 to 0.024, indicating relatively low nucleotide divergence (Lin *et al.*, 2015). The lowest genetic distance (0.000) was observed between *A. flemingiana* and *A. longiloba*, *A. lauterbachiana* (Engl.) A. Hay, and *Alocasia* sp. PSA280, suggesting very close genetic relationships. However, identical *trnL-trnF* IGS sequences do not necessarily indicate taxonomic equivalence and may reflect recent divergence or the conserved nature of the marker.

Interestingly, *A. flemingiana*, which belongs to the Macrorrhizos Group, clustered closely with *A. longiloba* of the Longiloba Group, suggesting that plastid phylogeny may not fully correspond to morphology-based classifications. In addition, *A. longiloba* ON777915 and *A. watsoniana*, both associated with the Longiloba Group, were recovered in separate clades, indicating possible incongruence between morphological grouping and chloroplast-based phylogenetic relationships.

The greatest genetic distance was observed between *A. flemingiana* and *A. sanderiana*, indicating a comparatively more distant relationship within the genus. The clear separation of the outgroup species further supported the phylogenetic structure of the tree. Overall, these results demonstrate that the *trnL-trnF* IGS marker is useful for DNA barcoding and phylogenetic analysis in *Alocasia*. Although the marker provides sufficient resolution to distinguish major lineages (Tsai *et al.*, 2006), additional molecular markers may be required to resolve relationships among closely related species and clarify discrepancies between molecular and morphological classifications. Furthermore, the inclusion of more representatives from the Macrorrhizos Group is necessary to better assess the evolutionary relationships of *A. flemingiana* and to provide stronger support for its taxonomic placement within *Alocasia*.

ACKNOWLEDGMENT

The authors would like to express their sincere gratitude to Arifin Surya Dwipa Irsyam (Herbarium Bandungense, SITH ITB) for kindly providing the plant material used in this study. The authors also acknowledge financial support from the National Research and Innovation Agency through the CfJRC program under the Rumah Program Organisasi Riset Hayati dan Lingkungan (2025 Contract No. B-3605/III.5/PR.03.06/12/2024).

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ISSN 0215-4706 (print) | e-ISSN 2460-6944 (online)