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Phylogenetic Analysis of the *Aglaonema* Putative MYB Gene for Anthocyanin Regulation in Plants

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Abstract. The red colour in *Aglaonema* leaves is influenced by anthocyanin accumulation, which is synthesized through a series of reactions catalyzed by specific enzymes. These proteins are encoded by genes regulated by transcription factors, among which the MYB gene family plays a vital role. The research aimed to identify a putative MYB gene in *Aglaonema* that encodes an MYB-transcription factor and analyze its relationship with several previously reported MYB genes involved in anthocyanin regulation. We aligned six gene sequences from Araceae and used a highly conserved region among five sequences to design the AMYB primer. Phylogenetic analysis of the *Aglaonema* putative MYB gene (amplified by the AMYB primer) and three previously characterized MYB genes from other plant species revealed that the AMYB primer was successfully constructed with optimal properties. The *Aglaonema* putative MYB gene sequence shares the highest similarity with HvMYB, known to activate HvMYC2 – a gene strongly associated with anthocyanin expression in plants, highlighted by a bootstrap value of 97% and a genetic distance of 0.3. The findings provide a reference for primer design and gene identification in *Aglaonema*, contributing valuable data for genetic conservation and plant breeding programs. This research supports the Sustainable Development Goals of Good Health and Well-Being (number 3) and Life on Land (number 15). *Aglaonema* plants, developed to produce unique leaf color patterns, can improve farmers' economic well-being and, in turn, enhance their overall well-being. Furthermore, *Aglaonema* development can enrich ornamental germplasm collections, thereby contributing to increased biodiversity in Indonesia.

Keywords: *Aglaonema*; MYB gene; primer; anthocyanin; transcription factor.

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INTRODUCTION

Aglaonema is an established ornamental potted plant in Indonesia and a major source of income for farmers (Haryanto et al., 2023). Revenue from *Aglaonema* cultivation can reach several million rupiahs, highlighting its strong market value in the ornamental plant industry (Nugrahani et al., 2024). *Aglaonema* farming can generate a total annual income of IDR 39,230,000, with a net income of IDR 10,453,772 (Apriany et al., 2023). The substantial increase in *Aglaonema* cultivation shows its potential for further development (Wibowo et al., 2024). For example, *Aglaonema commutatum* 'Red Valentine' is widely used for exterior and interior because it is easy to cultivate and manage (Li et al., 2023). In 2022, 1,396,552 *Aglaonema* plants were produced in Indonesia, with red coloured plants emerging as the favourite (Haryanto, 2024).

Aglaonema is often referred to as "the queen of leaves" due to its attractive leaf phenotypic variations (Sugiharti et al., 2025). These leaf colour traits highlight the species' uniqueness and enhance its desirability. The primary pigments that produce red, purple, or blue colours are anthocyanins, which are glycosylated anthocyanidins characterised by one or more sugar groups (Khoo et al., 2017; Li et al., 2022a). Glycosylated anthocyanidins are categorised into

malvidin, petunidin, pelargonidin, peonidin, delphinidin, and cyanidin (Li et al., 2021). Each of these compounds contributes to a specific colour in plants; for example, delphinidin aglycone results in blue tones, cyanidin aglycone in red, and pelargonidin aglycone in pink (Zhao & Tao, 2015).

Anthocyanins are widely distributed across plant organs and play key roles in the vegetative and generative phases of plant growth and development. In the vegetative phase, they can protect photosynthetic cells from high light intensity (Hughes et al., 2005). Anthocyanin biosynthesis consists of several steps involving various genes, namely structural genes such as PAL (phenylalanine ammonia-lyase), CHS (chalcone synthase), F3H (flavanone 3-hydroxylase), DFR (dihydroflavonol 4-reductase), and ANS (anthocyanin synthase), as well as genes encoding transcription factors (TFs) MYB (Myeloblastosis), bHLH (basic helix-loop-helix), and WDR (WD-repeat) (Zhao & Tao, 2015; Naing et al., 2018; Li et al., 2022a). MYB transcription factors are important regulators within plant metabolic networks (Yan et al., 2021). MYB genes have been reported to regulate the expression of structural genes involved in anthocyanin biosynthesis at the transcriptional level, with overexpression leading to high anthocyanin accumulation in leaves (Zhao & Tao, 2015). Moreover, variations in MYB5 can result in differences in flower colour (Sun et al., 2016).

Genetic information onanthocyanin biosynthesis in *Aglaonema* remains limited, particularly regarding the transcription factor genes that regulate this pathway. *Aglaonema commutatum* MYB TFs (AcMYB1 and AcMYB2) are homologues of *Anthurium andraeanum* MYB TFs (AaMYB1 and AaMYB2) (Li et al., 2023). The AaMYB1 and bHLH genes have been associated with anthocyanin biosynthesis (Albert et al., 2010). However, the molecular mechanisms underlying the reduced proportion of red foliage in *A. commutatum* remain unclear (Li et al., 2023). The molecular basis of leaf colour variation in *A. commutatum* was explored using mRNA transcript sequencing. The results showed high anthocyanin content in the mRNA transcript per million (TPM) value and detected the positive regulation of transcription factors responsible for modulating anthocyanin biosynthesis in *Aglaonema*.

Research on the MYB gene in *Aglaonema* may serve as a foundation for advancing the genetic study of this species. This study aimed to identify the sequences derived from the amplification of the putative MYB gene encoding the MYB transcription factor. Information related to the MYB gene sequence in *Aglaonema* can be used as preliminary data to improve anthocyanin expression in the vegetative and generative phases.

METHODS

Procedures

Primer Design

Primers were designed using the PrimerQuest tool from Integrated DNA Technologies (IDT; https://sg.idtdna.com/pages). Primers specific to the *MYB* gene stage were designed based on the method described by Wardi et al. (2021). DNA sequences were downloaded from the National Center for Biotechnology Information (NCBI) and saved in FASTA format. The candidate AMYB primers were designed by comparing six gene sequences, namely, the promoter region of *Lemna aequinoctialis MYB11* (MT771646.1), *Philodendron hederaceum* complete chloroplast genome (NC_064988.1), *Philodendron hederaceum* complete chloroplast genome (OL631930.1), *Anthurium andraeanum* cultivar *Alabama voucher* HZ0711 complete chloroplast genome (NC_072558.1), *Alocasia macrorrhizos* chloroplast partial genome (KR296655.1), and *Homalomena occulta* complete chloroplast genome (NC_054336.1). These sequences were aligned against five different sequences using ClustalW in BioEdit. A high level of site conservation indicates a close relationship amongthe sequences being analyzed (Lin et al. 2021). Sequences with highly conserved regions relative to the other five comparative sequences were used to design the AMYB primers using the Integrated DNA Technologies (IDT) PrimerQuest tool. Parameters including GC (guanine-cytosine) content, Tm (melting temperature), and primer interactions (hairpin and dimer formation) were analyzed using the IDT Oligoanalyzer tool.

Genomic DNA Extraction of Aglaonema

Young *Aglaonema* leaves were ground using liquid nitrogen and transferred into a 2-mL tube (Li et al., 2022b). Genomic DNA was extracted using the CTAB protocol (Sukma et al., 2020). Briefly, 800 µL of extraction buffer [2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 2% (m/v) PVP, and 2% (v/v) β-mercaptoethanol] was added to each ground leaf sample. The suspension was incubated at 65°C for

30 minutes. The suspension was extracted twice with an equal volume (800 µL) of chloroform : isoamyl alcohol (24:1), then centrifuged at 13,000 rpm for 15 minutes. Discard the supernatant and replace it with an equal volume of isopropanol and 0.3 M ammonium acetate (1/10 of the supernatant volume). The suspension was centrifuged at 13,000 rpm for 10 minutes. The supernatant was discarded, and the pellet was washed with 500 µL of 70% ethanol. The pellet was centrifuged at 13,000 rpm for 5 minutes, dried, and dissolved in 100 µL of TE buffer. DNA concentration and purity were determined using a nanophotometer.

DNA Amplification Using Candidate Primers

DNA amplification was conducted using MyTaq™ HS Red Mix. The polymerase chain reaction (PCR) cycles were: (1) initial denaturation at 95°C for 3 minutes, (2) 35 cycles, each consisting of denaturation at 95°C for 30 seconds, annealing at 52°C for 1 minute, and extension at 72°C for 30 seconds, and (3) final extension at 72°C for 7 minutes. Qualitative PCR products were checked on a 2% agarose gel by electrophoresis for 30 minutes at 100 volts. The PCR amplicons were sequenced at 1st Base.

Data Analysis

The separate forward and reverse sequencing results were combined into a single consensus sequence of the PCR product (i.e., a contig) using the BioEdit software. The *Aglaonema* plant sequence was aligned with three reported *MYB* gene sequences involved in anthocyanin biosynthesis:*Ipomoea purpurea* anthocyanin gene transcription factor (*myb1*) gene, complete CDS (AY986898.1), *Hordeum vulgare subsp. vulgare* cultivar Clipper *MYB (MYB4H)* gene, complete CDS (MH618661.1), and *Gossypium arboreum* MYB transcription factor (*MYB*) gene, complete CDS (GU244349.1). Further validation was obtained via phylogenetic analysis of the alignment results using the maximum-likelihood tree method in MEGA 11.

RESULTS AND DISCUSSION

Primer Design

Selecting genes from families closely related to *Aglaonema* was expected to improve primer design effectiveness. The alignment results show that the complete chloroplast genome of *Anthurium andraeanum* var. Alabama voucher HZ0711 (NC_072558.1) has the highest level of conservation among the five gene sequences. The sequence from 147,951–148,248 in *Anthurium andraeanum* (NC_072558.1) is CGATTAACCTCTGGATTTGAAATCACTGC TTATATACCTGGTATTGGCCATAATTCAC AAGAACATTCTGTAGTATTAGTAAGAGGA GGAAGGGTTAAGGATTTACCCGGTGTGA GATATCACATTGTTCGAGGAACCCTAGAT GCTGTCGGAGTAAAGGATCGTCAACAAG GGCGTTCTAGTGCGTTGTAGATTCTTATC CAAGACTTGTATCATTTGATGATGCCATG TAAATCGCTAGAAACATGTGAAGTGTATG GCTAACCCAATAACGAAAGTTTCGTAAGG GGACTGGAG. This sequence is the initial 297-bp sequence in *Anthurium andraeanum* and was used to design the AMYB primers (Table 1).

PCR Product Visualisation from Candidate Primers

The AMYB primer pair as candidate primer successfully amplified the target sequence in *Aglaonema*, resulting in amplicons of 204 bp, 209 bp, and 209 bp (Figure 1). AMYB primers showed a monomorphic pattern accross the three *Aglaonema* genotypes, indicating no allelic variation at the amplified locus.

Table 1. Characteristics of the candidate primers.

Primer	Type	Sequence	Primer Length (bp)
AMYB	Forward	ACCTGGTATTGGCCATAATTCA	22
	Reverse	GGGTTAGCCATACACTTCACA	21

Primer Characteristics					
Type	**Tm (˚C)**	**GC (%)**	**Self-complementary**	**Self 3′s complementary**	**Hairpin**
Forward	62.0	40.90	6	2	2
Reverse	61.9	47.61	4	0	1

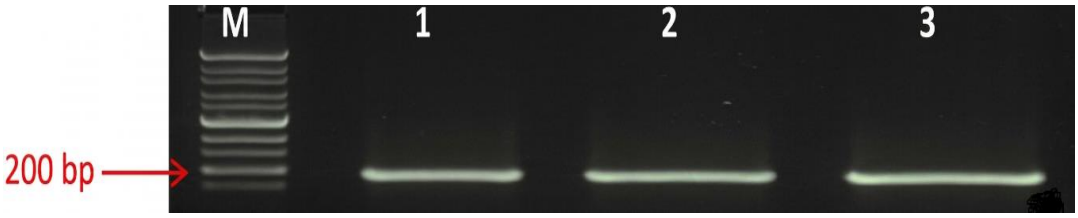

Figure 1. Visualization of the PCR amplicon using a UV transilluminator. Lane 1: *Aglaonema* sp. var. Lipstick, lane 2: *Aglaonema* sp. var. Wulandari, lane 3: *Aglaonema* sp. var. Dona Carmen, and lane M: 100-bp ladder.

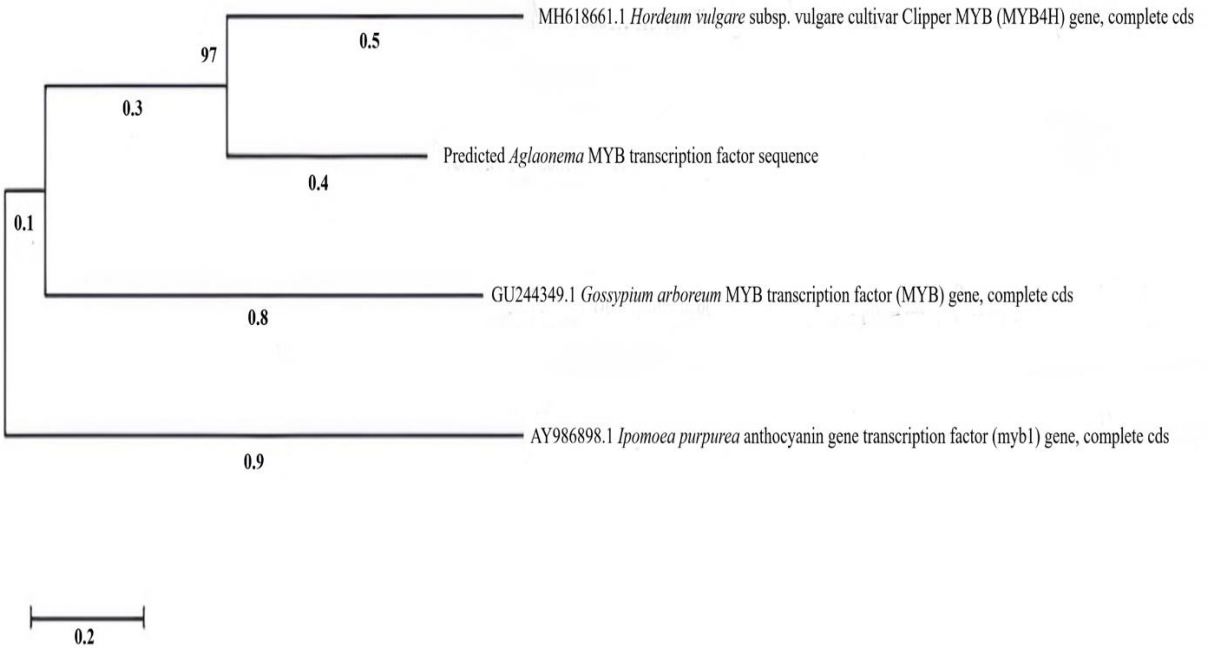

Figure 2. Phylogenetic tree construction using the MEGA 11 software-derived consensus sequence of *Aglaonema* samples with three reported *MYB* gene sequences.

Analysis of Putative DNA Sequences

PCR fragments amplified by AMYB primers were sequenced to obtain the nucleotide base sequence of the AMYB locus allele, which was used in subsequent analyses. Three sequences were generated :DNA sequences of *Aglaonema* sp. var. Lipstick, *Aglaonema* sp. var. Wulandari, and *Aglaonema* sp. var. Dona Carmen. The contig from the three PCR samples showed the same base length of 232 bp. This base length matched the length estimated by IDT following primer design. The three consensus sequences were aligned using MEGA 11 and showed 100% similarity (identic); therefore, the AMYB primer pair amplified the same gene in the three samples.

Phylogenetic analyses indicated that the *Aglaonema* gene sequence *Aglaonema*showed the highest similarity to the *Hordeum vulgare MYB* gene sequence, while displaying the lowest similarity to the *Ipomoea purpurea* (*myb1*) gene sequence. Furthermore, the *Ipomoea purpurea myb1* gene showed the lowest similarity compared to the other two reported *MYB* gene sequences, suggesting that the *Ipomoea purpurea* (*myb1*) gene is the most distantly related compared to the *Hordeum vulgare MYB (MYB4H*) gene and *Gossypium arboreum MYB* gene.

The candidate primers successfully amplified the putative *MYB* gene from all samples.Table 1 lists the characteristics of the markers obtained. Primer design effectiveness is influenced by parameters such as primer length, the number of single-base stretches, melting temperature, and GC content (Asif et al., 2021). In this study, the primer candidate is 22 bp long. The optimal primer length is 18–25 bp. The length parameter can be used to determine the annealing time during PCR. Primers that are too long or too short can affect DNA amplification efficiency (Anika et al., 2019).

The primer Tm is the temperature at which

half of the base pairs denature, and the primer becomes single-stranded DNA, affecting its stability. Here, Tm analysis shows that the primer temperature ranges from 61.904°C to 62.545°C. The optimal Tm range is typically between 50°C and 60°C, ideally between 65°C and 75°C with a ≤ 5°C difference between primers. The GC percentages for the AMYB primers are 40.90% (forward) and 47.61% (reverse). Ideally, according to Khaira et al. (2023), GC content ranges from 40-60%; however, 40-50% is often considered optimal for primer stability and PCR specificity. A high GC percentage can increase primer Tm and PCR specificity. However, a GC percentage that is too high may lead to undesirable secondary structures, such as hairpins, which can interfere with PCR efficiency. If the Tm exceeds 70°C, the primers may function effectively at lower temperatures and form excessively strong bonds with the DNA template, reducing PCR product. Our results indicate that all the designed primers possess the optimal characteristics expected from an effective primer.

The GC percentages range from 40% to 60%, with the forward AMYB primer showing the lowest GC percentage of 40.909%. The primer is promising because primers should ideally have a GC percentage within the 40–60% range (Sharma, 2021). GC content plays a key role in stabilising bonds between DNA strands, and an optimal GC percentage contributes to stronger DNA bonds because more nucleotide bonds influence Tm (Violita et al., 2024). Primers with a GC percentage below 50% must be longer than 18 bp to ensure that the Tm remains above the minimum threshold (Khaira et al., 2023). This stability benefits the combination. A GC percentage that is too high can inhibit primer-template separation. At the same time, a GC value that is too low can interfere with primer function because the primer may struggle to compete effectively to bind to the target area (Assal & Lin, 2021).

Candidate primers contained 1–3 hairpins. Although hairpin formation should be avoided during primer design, obtaining primers without hairpins or loops can be challenging. In primer design, hairpin formation refers to a primer's ability to form a secondary structure (a loop) with itself (Rianti et al., 2021). These hairpins can interfere with primer attachment to the DNA target and reduce PCR efficiency (Masnaini et al., 2023). Hairpin formation can be avoided by ensuring a balanced base composition, especially in terms of GC percentage and primer length. Based on the results, all designed primers meet the ideal characteristics expected of a primer.

The AMYB primer pair successfully amplified the gene in all tested DNA samples, as evidenced by the amplicons separated via gel electrophoresis. Sample 1 showed the faintest band compared to the other samples. The DNA band produced by Sample 3 was the thickest. Band thickness correlated with DNA concentration in the sample. The thickness of the DNA band is typically influenced by several factors, including the thickness of the agarose mould comb, the temperature and duration of annealing, the number of PCR cycles, and the duration of the extension cycle (Arslan et al., 2021; Silalahi et al., 2021; Martinez et al., 2015). Higher DNA concentration corresponds to a thicker DNA band, and vice versa. Therefore, sample 1 had the lowest DNA concentration, whereas the other samples had the highest. The amplicon length was approximately 200 bp. The AMYB primers revealed a monomorphic pattern across the three *Aglaonema* genotypes analysed, indicating no allelic variations at the amplified locus. The amplicons were sequenced to obtain the nucleotide base sequence of the *MYB* gene locus allele for subsequent analysis.

The sequencing analysis generated putative DNA sequences for *Aglaonema* sp. var. Lipstick, *Aglaonema* sp. var. Wulandari, and *Aglaonema* sp. var. Dona Carmen, which showed high similarity. Sequence diversity analysis was carried out by aligning these putative DNA sequences with several reported gene sequences. Biological sequences analysis cannot be separated from sequence alignment, which is essential for identifying homologous characteristics (Leiwakabessy et al., 2023). The predominant alignment technique is multiple sequence alignment, where more than two sequences are aligned to create a phylogenetic tree that explains their relationship (Amiroch et al., 2017). Most phylogenetic analysis methods assume that each protein or nucleic acid sequence position changes independently of the others (except during RNA sequence evolution). A phylogenetic tree is a taxonomic classification of organisms based on their historical genealogy (i.e., their phylogeny). It is an integral part of systematic science aimed at determining the phylogeny of living things based on their characteristics (Leiwakabessy et al., 2023).

The phylogenetic outcome of the alignment results was further validated using the maximum-likelihood tree method. Zou et al. (2024) stated that this method employs clear model assumptions

and a statistical approach based on evolutionary models, with benefits such as robustness, the ability to compare different trees, comprehensive use of original data within a statistical framework, and statistical consistency. Phylogenetic analyses use DNA sequences to describe the taxonomic classification of living organisms based on their evolutionary history.

The constructed phylogenetic tree (Figure 2) showed that the *Hordeum vulgare MYB (MYB4H*) gene and the consensus sequence of the *Aglaonema* sample are most closely related, as indicated by the shortest branch length. *Hordeum vulgare MYB (MYB4H*) gene and *Aglaonema* consensus sequences show synapomorphic characteristics and are in a monophyletic group. Synapomorphic characteristics are inherited traits classed in a monophyletic group (Hidayat & Pancoro, 2008). *Hordeum vulgare MYB (MYB4H*) gene and *Aglaonema* consensus sequences have a bootstrap value of 97%, indicating that the clade is reliable. A bootstrap value of 70% or more may indicate that a classification can be trusted (Hillis & Bull, 1993).

The role of *HvMYB* in regulating anthocyanin biosynthesis aligns with that of the *Aglaonema MYB* gene. Xu et al. (2022) stated that in *H. vulgare*, the *MYB (MYB4H*) gene triggers the activation of *HvMYC2*, which is involved in anthocyanin expression in plants. The strong relationship between *HvMYC2* and genes associated with anthocyanin biosynthesis makes *HvMYC2* a key player in anthocyanin accumulation.

Phylogenetic analysis is strengthened by considering genetic distance, specifically pairwise distance. The genetic distance for the *Aglaonema* consensus sequence, *Hordeum vulgare MYB (MYB4H*) gene, *Gossypium arboreum* MYB transcription factor (*MYB*) gene, and *Ipomoea purpurea* (*myb1*) gene is 0.4, 0.5, 0.8, and 0.9, respectively. The genetic distance between the *Aglaonema* consensus sequence and the *Hordeum vulgare MYB (MYB4H*) gene is 0.3. A greater genetic distance indicates a more distant relationship. Thus, the *Aglaonema* consensus sequence is most closely related to the *Hordeum vulgare MYB (MYB4H*) gene. The *Gossypium arboreum MYB* gene sequence remains in the same group as the consensus sequences of *Aglaonema* and *Hordeum vulgare MYB (MYB4H*) but is distinct from the *Ipomoea purpurea* (*myb1*) gene. Furthermore, phylogenetic analysis shows that the *Ipomoea purpurea* (*myb1*) gene is an outgroup. Outgroups are essential when constructing phylogenetic trees as they illustrate the polarisation of characteristics (apomorphic and plesiomorphic), resulting in rooted trees with clear limitations (Hidayat & Pancoro, 2008). Based on our findings, we estimate that the *Aglaonema* consensus sequence obtained using the aMYB1 primer is a partial *MYB* gene sequence.

Phylogenetic studies of MYB gene sequences in several plant commodities have never been conducted, particularly in Indonesia. To date, genetic studies of *Aglaonema* have focused on fundamental molecular mechanisms underlying anthocyanin biosynthesis (Li et al., 2022a; Li et al., 2023) and on the influence of environmental factors on *Aglaonema* anthocyanin levels (Jasmine et al., 2023; Zhu et al., 2025)--furthermore, Salama (2021) conducted in vitro micropropagation of *Aglaonema*.Phylogenetic study of *Aglaonema* have been conducted on one green species *Aglaonema*and 5 variegated cultivars*Aglaonema*, using chloroplast genome data (Li et al., 2022b).

Phylogenetic analyses of gene sequences, particularly those involved in the biosynthesis of transcription factor proteins that regulate anthocyanin biosynthesis, have significantly contributed to identifying genes associated with changes in the morphology, physiology, and development of the studied traits (Dunn et al., 2013) within the context of evolutionary change between species. Furthermore, this study is crucial for understanding the mechanisms and factors underlying evolutionary change, such as mutation, selection, and others (Jacques et al., 2023). If genes involved in protein synthesis related to anthocyanin biosynthesis are identified, this information could serve as a basis for developing ornamental *Aglaonema* cultivars with high anthocyanin content through various plant breeding programs, such as physical mutation by gamma irradiation (Lestari et al., 2018) and chemical mutation by Ethyl Methane Sulfonate (EMS) (Martha et al., 2023).

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CONCLUSION

In conclusion, the AMYB primer pair as candidate primer was successfully designed with suitable parameters. Sequence analysis via alignment and phylogenetic diagrams has indicated that the *Aglaonema* consensus sequence (amplified using the candidate primer pair) is likely a partial sequence of the *MYB* gene in *Aglaonema* plants. This primer successfully amplified DNA fragments from *Aglaonema* sp. var. Lipstick*, Aglaonema* sp. var. Dona Carmen*,* and *Aglaonema* sp. var. Wulandari samples. The resulting consensus sequence is most closely related to the *MYB* gene from *Hordeum vulgare MYB (MYB4H*), with a genetic ratio of 0.3. Furthermore, this information may be utilised in marker construction and genetic diversity studies to support plant breeding programs.

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AUTHOR CONTRIBUTION STATEMENT

WY conceived and designed the study; AHN conducted DNA extraction and gel electrophoresis; KN conducted and supported data visualization; S conducted the data analysis; S assisted in the literature review; AM proofread the manuscript; EO supervised the research and provided substantial feedback during the manuscript drafting process. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that Grammarly tool was used to proofread this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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