International Journal of Agriculture and Biosciences 2026 15(4): 1716-1727.
https://doi.org/10.47278/journal.ijab/2026.083

RESEARCH ARTICLE
eISSN: 2306-3599; pISSN: 2305-6622

Characterization and Primer Design for Cloroplast Gen rbcL Sequences Amplification in AFO Clove (*Syzygium aromaticum* L.var AFO) as The Oldest Clove from Ternate Island

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ABSTRACT

AFO cloves (*Syzygium aromaticum* L. var AFO) are the oldest cloves in the world originating from Ternate Island. The phenomenon of cloves' ability to live long and be the oldest is one of the genetic expressions. This study aims to design specific primers for the amplification of the gene encoding the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) as a target gene in AFO cloves, to support further molecular studies. Primer design in the PCR process is very important because the primer will bind to the DNA template and then amplify the target sequence. Primer design was carried out in silico using MEGA 11, Primer 3 Plus, and Geneus software. The rbcL gene sequence of AFO cloves was obtained from DNA extraction and the NCBI BlastN database. The results of the primer design obtained primer pair Forward (5'-CTCCTGACTATGAAACCAAAGATAC-3') and Reverse (5'-GACATTCATAAACTGCTCTACCGT-3') did not find a hairpin structure and self-complementarity, so the primer will not form a secondary structure that can trigger dimerization, the designed primer pair also meets the criteria of length, melting temperature, and ideal GCcontent for PCR reactions. The primers that have been obtained can be used as a basis for developing molecular markers on AFO cloves from Ternate Island which are useful for variety identification, phylogenetic analysis, and breeding programs.

Keywords: AFO clove; Chloroplast rbcL; Primer design; In silico PCR; DNA barcoding.

Article History
Article # 26-012
Received: 04-Jan-26
Revised: 15-Feb-26
Accepted: 04-Mar-26
Online First: 05-Apr-26

INTRODUCTION

Clove (*Syzygium aromaticum* L.) is one of the most economically important spice crops in the world and has long been associated with the Maluku Islands, particularly Ternate, Indonesia, which is widely recognized as one of the original centers of clove diversity (Kamsurya et al., 2023; Pergola et al., 2024; Rahman, 2024). Among the known clove populations, the AFO clove is historically regarded as one of the oldest and most distinctive clove types and is strongly associated with Ternate Island (Bermawie et al., 2024). This clove population is believed to represent a native genetic resource originating from Ternate and has been officially recognized by the Indonesian government through the Decree of the Minister of Agriculture Number 3680/Kpts/SR.120/11/2010 concerning the Release of the AFO Clove Population as a Superior Variety (Singh et al., 2024). The conservation and molecular characterization of this historically important clove germplasm are essential for understanding its genetic identity, evolutionary background, and potential use in breeding programs (Picek et al., 2023).

Cite this Article as: Sundari, Sapsuha Y, Wahab IHA, Mas'ud A, Watuguly TW and Sakti AS, 2026. Characterization and primer design for cloroplast gen rbcL sequences amplification in AFO clove (*Syzygium aromaticum* L.var AFO) as the oldest clove from Ternate Island. International Journal of Agriculture and Biosciences 15(4): 1716-1727. https://doi.org/10.47278/journal.ijab/2026.083

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Genetic diversity studies in clove are increasingly important due to the global demand for high-yielding, disease-resistant and climate-adapted varieties (Marasabessy et al., 2024). Clove is a perennial woody spice crop with relatively long breeding cycles, making molecular tools essential for accelerating germplasm identification, phylogenetic analysis and marker-assisted selection (Ouadi et al., 2022; Darwati et al., 2026). Chloroplast DNA markers have been widely used in plant systematics and genetic diversity studies because of their conserved structure, maternal inheritance and relatively slow mutation rate (Tamura et al., 2021; Meloni et al., 2023). In spice crops and woody plants, chloroplast markers are particularly useful for tracing evolutionary relationships, identifying plant varieties, and supporting germplasm conservation programs (Fakih et al., 2025). Recent advances in chloroplast genomics have further strengthened the use of plastid genes as molecular barcodes for plant identification and phylogenetic reconstruction across a wide range of taxa, including aromatic and medicinal plants (Kalendar et al., 2024; Kumar et al., 2024).

One of the most widely used chloroplast genes in plant molecular systematics and DNA barcoding is the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (rbcL) (Cahyaningsih et al., 2022; Mahadani et al., 2022). The rbcL gene encodes the large subunit of RuBisCO, a key enzyme in photosynthesis that plays a central role in carbon fixation in the Calvin cycle (Bolay et al., 2022). RuBisCO catalyzes the carboxylation of ribulose-1,5-bisphosphate with carbon dioxide, initiating the Calvin cycle in chloroplasts, and is therefore essential for plant growth and productivity (Meloni et al., 2023). Because of its conserved sequence and universal presence in photosynthetic plants, the rbcL gene has become one of the standard molecular markers used globally in plant phylogenetics, DNA barcoding and evolutionary studies (Thaenkham et al., 2022; Kumar et al., 2024). Numerous studies have demonstrated the effectiveness of rbcL in identifying plant species, analyzing genetic relationships, and supporting breeding and conservation strategies, particularly in woody plants and economically important crops (Cahyaningrum et al., 2021; Lating et al., 2024).

In recent years, the integration of chloroplast genomics and DNA barcoding has significantly advanced molecular identification in spice and woody crops (Mahima et al., 2022). Chloroplast genome-based markers, including rbcL, matK, and trnH-psbA, have been widely used to resolve taxonomic relationships and detect genetic variation among closely related plant species (Kalendar et al., 2024; Fakih et al., 2025). In clove and related *Myrtaceae* species, chloroplast markers provide valuable insights into phylogenetic structure, population differentiation, and genetic conservation, particularly for historically important germplasm such as AFO clove (Zhang et al., 2023; Huynh et al., 2024). Furthermore, advances in bioinformatics and genome sequencing technologies have enabled the development of more accurate and specific molecular markers derived from chloroplast DNA, which are essential for breeding programs and germplasm authentication (Kumar et al., 2024; Fakih et al., 2025).

A critical step in molecular genetic analysis is primer design, which determines the success of DNA amplification and downstream applications such as sequencing, barcoding, and marker development (Soroka et al., 2021). The development of primer design techniques is fundamental in molecular biology, particularly in the amplification and analysis of specific DNA segments (Amaniyah et al., 2023). In polymerase chain reaction (PCR), primer specificity is crucial because primers bind to the DNA template and determine the region to be amplified (Amiteye, 2021). A pair of oligonucleotide primers, consisting of forward and reverse primers, hybridizes to complementary sequences on the target DNA during the annealing stage (Chukwuemeka et al., 2020). Accurate primer binding requires high sequence complementarity between the primer and the target DNA region (Bu et al., 2024). Poorly designed primers can lead to nonspecific amplification, weak PCR signals, or complete amplification failure, thereby compromising molecular analysis (Sharma et al., 2021; Tripathi & Rathinam, 2025).

The use of bioinformatics and in silico approaches has significantly improved primer design efficiency and accuracy (Guo et al., 2020). In silico studies combine biological data and computational tools to design and evaluate candidate primers before laboratory validation (Kalendar et al., 2024; Fakih et al., 2025). One of the main roles of bioinformatics in primer design is to generate primer sequences with optimal characteristics, including appropriate length, melting temperature, GC content and minimal secondary structure formation (Tamura et al., 2021; Thaenkham et al., 2022; Kumar et al., 2024). MEGA 11 (Molecular Evolutionary Genetics Analysis) is widely used for DNA sequence analysis and primer design because it provides tools for sequence alignment, evolutionary analysis, and primer parameter evaluation (Chukwuemeka et al., 2020; Keklik, 2023; Kumar et al., 2024). Through in silico validation, candidate primers can be screened for specificity, stability, and amplification potential, reducing experimental errors and improving PCR success rates (Sharma et al., 2021).

Primers are short DNA fragments composed of specific nucleotide sequences that initiate DNA synthesis during PCR (Li et al., 2022). The designed primer sequence must match the region of DNA to be amplified to ensure successful replication (Rodriguez et al., 2023). Primer sequences may be derived from previously published studies or generated using specialized software such as Primer3, BioEdit, NCBI tools and Genamics Expression (Abellan-Schneyder et al., 2021; Indradewi et al., 2022; Vaulot et al., 2022). However, primer optimization often requires sequence modification, including the addition of restriction sites or adjustment of thermodynamic parameters, to produce efficient and specific primers (Syamsidi et al., 2021; Kalendar, 2022). The effectiveness of a primer in amplifying a gene fragment depends on several factors, including sequence specificity, primer length, annealing temperature, and GC content composition (Asif et al., 2021). Errors in primer design may result in nonspecific PCR products or incomplete amplification of the target gene (Lee & Fried, 2021).

Despite the historical and economic importance of AFO clove from Ternate, molecular studies focusing on its

chloroplast genetic markers remain limited. The development of specific primers targeting the rbcL gene is essential to support molecular identification, phylogenetic reconstruction, and genetic diversity analysis of this unique clove population (Algarni, 2022). Furthermore, such primers can facilitate the development of DNA barcoding tools and molecular markers for breeding and conservation programs, particularly for preserving historically significant clove germplasm (Cahyaningrum et al., 2021; Lating et al., 2024).

Therefore, this study aims to design specific primers for the amplification of the rbcL gene in AFO clove from Ternate Island using MEGA 11 software. The resulting primers are expected to support further molecular research on AFO clove, including genetic identification, phylogenetic analysis, chloroplast marker development, and molecular breeding applications, thereby contributing to the conservation and scientific understanding of one of the oldest clove germplasm resources in the world.

MATERIALS & METHODS

This study employed a structured and widely recognized workflow for primer design. The process began with DNA sequence preparation followed by an initial examination of the corresponding protein structures. The retrieved nucleotide sequences were translated into amino-acid sequences using the ExPASy Translate server, and subsequently aligned in MEGA 11 using the ClustalW algorithm to identify conserved motifs. To further evaluate structural conservation, a homology-based three-dimensional protein model was constructed using the SWISS-MODEL platform. The workflow then proceeded to primer design and validation. The DNA sequences were re-aligned in MEGA 11 to refine conserved regions, which served as the basis for constructing primer candidates using Primer3Plus. This stage closely followed previously established methodological recommendations (Padhi et al., 2020).

The resulting primer set was subjected to in silico validation using Geneious Primer to confirm target-site specificity, evaluate potential off-target interactions, and predict amplification performance. For this analysis, the complete chloroplast genome of *Glycine max* (Accession No. NC_007942.1) was used as the PCR template. The selection of *G. max* as a reference genome was based on its well-annotated, high-quality, and complete chloroplast sequence, which provides a reliable framework for evaluating primer binding efficiency and amplification behavior in conserved plastid regions. Additionally, its extensive use in molecular and chloroplast genomic studies allows consistent benchmarking and reproducibility across in silico PCR analyses (Syamsurizal et al., 2021; Yosilia et al. 2024).

The rbcL gene was selected as the primary molecular marker due to its high universality, conserved coding region, and extensive representation in plant barcode databases, making it particularly suitable for primer design and cross-species amplification. Compared with other chloroplast markers such as matK and trnH-psbA, rbcL exhibits more stable primer-binding sites and higher amplification success across diverse taxa, while still providing sufficient phylogenetic and taxonomic resolution for species identification and molecular characterization. These characteristics make rbcL a preferred marker in chloroplast-based molecular studies and DNA barcoding frameworks (Syamsurizal et al., 2021; Yosilia et al. 2024).

RESULTS

Study Area and Geographic Location of Sampling Sites

To accurately document the geographic origin of the AFO clove samples, a detailed location map of Ternate Island was prepared and is presented in Fig. 1. The map

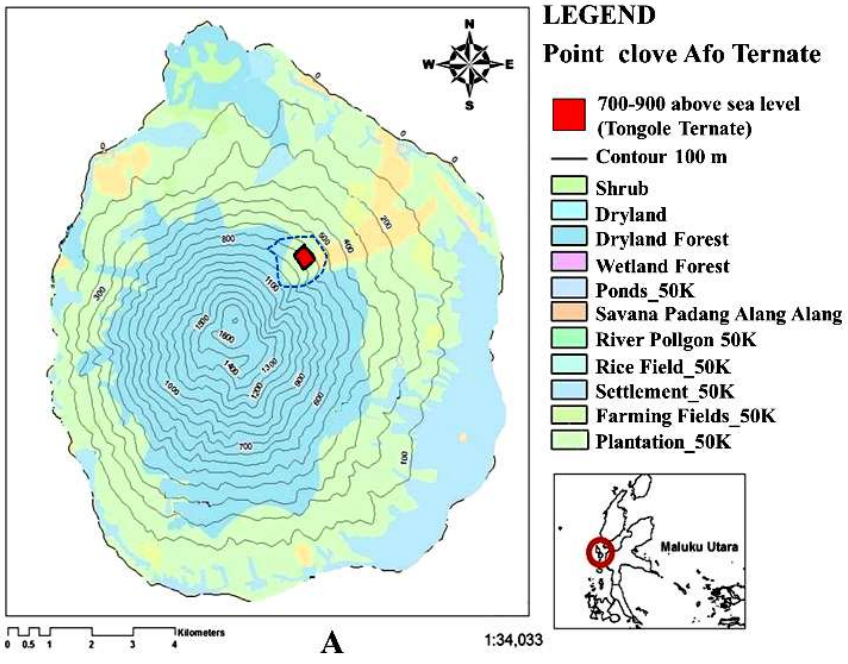


Fig. 1: Geographic distribution of AFO clove (*Syzygium aromaticum* L. var. AFO) sampling sites on Ternate Island, North Maluku, Indonesia.

identifies the precise sampling sites where AFO clove (*Syzygium aromaticum* L. var. AFO) specimens were collected on Ternate Island, North Maluku, Indonesia. All sampling points are situated within the traditional clove-growing region historically recognized as the native habitat of the AFO clove population.

The spatial distribution shown in the map indicates that the sampling sites are located approximately between 0°47′-0°51′ N latitude and 127°19′-127°23′ E longitude, representing the central zone of AFO clove occurrence on Ternate Island. Ternate Island is a small volcanic island dominated by Mount Gamalama, and the mapped sampling area corresponds to the historically documented ecological range of AFO clove trees. The spatial verification provided by this map reinforces the geographic authenticity of the sampling origin and supports the recognition of AFO clove as an indigenous genetic resource of Ternate Island.

Sequence Preparation and Protein Visualization

The DNA sequences obtained in this study were initially translated into their corresponding amino-acid sequences using the ExPASy Translate webserver. After translation, the protein sequences were aligned in MEGA 11 using the ClustalW algorithm to identify regions that were consistently conserved across the samples. These conserved amino-acid residues are illustrated in Fig. 2, which summarizes the results of the multiple-sequence alignment.

These conserved regions then served as a foundation for constructing the protein model. Homology-based modelling was carried out using the SWISS-MODEL automated modelling server, which predicted the three-dimensional structure of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) protein. The resulting structural model was subsequently examined in Discovery Studio Visualizer to evaluate its architecture and ensure the overall integrity of the predicted fold. The homology model and its structural visualization are shown in Fig. 3.

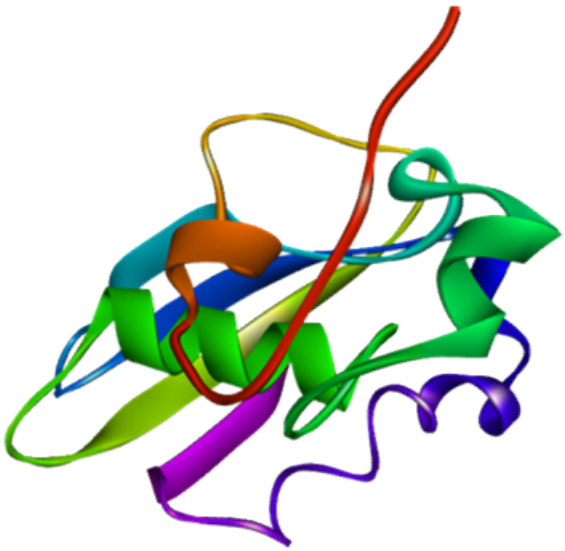
KDQAGVKIINLIILLTMKPKILISWQHSELLNLEFLLRKQGLRLLLNL
LLVHGQLCGPMGLPALIVIKEDATTSSLLLEKKINIYVMLLTLLTFLK
KVLLLICLLPLWVMYLGSKPCALYVWRICESLLPIRKLSKARLMAS
KLREINTSMAVPYWDVLLNLNWGYPLRTTVEQFMNVFAVDFK

Fig. 2: Conserved amino-acid regions identified from ClustalW multiple-sequence alignment of translated rbcL gene sequences.

Primer Design and Validation

The DNA sequences were aligned using the ClustalW algorithm implemented in MEGA 11. The conserved nucleotide regions identified from this alignment were subsequently used as the basis for primer construction. Primer design was carried out using the Primer3Plus webserver (https://www.primer3plus.com/index.html), ensuring that each primer met the required thermodynamic and structural criteria for PCR amplification. The conserved DNA regions generated from the alignment are presented in Fig. 4.

(A)



(B)

Fig. 3: Homology modelling and structural visualization of the predicted RuBisCO protein based on SWISS-MODEL using template PDB ID: 1RBO.

AAAGTGGATCAAGCTGGTGTTAAGATTATAAACTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGA
TATCTTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCCTCCTGAGGAAGCAGGGGCTGCGGTAGCTG
CTGAATCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGCCTTGATCGTTATAAAGGAA
GATGCTACCACATCGAGCCTGTTGCTGGAGAAGAAAATCAATATATATGTTATGTAGCTTACCCTTTAGACCTT
TTTGAAGAAGGTTCTGTTACTAATATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAAGCCCTGCGCG
CTATACGTCTGGAGGATCTGCGAATCCCTACTTCCTATACGAAAACTTTCCAAGGCCCGCCTCATGGCATCCA
AGTTGAGAGAGATAAATTGAACAAGTATGGCCGTCCCCTATTGGGATGTACTATTAAACCTAAATTGGGGTT
ATCCGCTAAGAACTACGGTAGAGCAGTTTATGAATGTCTTCGCGGTGGACTGATTTTACAAA

Fig. 4: Conserved nucleotide regions identified from ClustalW alignment of rbcL gene sequences used for primer design.

Table 1: Design specifications and thermodynamic characteristics of ten candidate primer pairs targeting conserved regions of the rbcL gene

No	Primer	Sequence (5′→3′)	Length (bp)	Tm	%GC	Amplifications Size
1	Foward	AAGTGGATCAAGCTGGTGTT	20	57.5	45.0	556
	Reverse	TCCACCGCGAAGACATTCAT	20	59.8	50.0	
2	Foward	AGTGGATCAAGCTGGTGTTAAGA	23	59.7	43.5	562
	Reverse	AAATCAGTCCACCGCGAAGA	20	59.7	50.0	
3	Foward	CTGATATCTTGGCAGCATTCCG	22	59.5	50.0	501
	Reverse	TTTGTAAAATCAGTCCACCGCG	22	59.8	45.5	
4	Foward	GTGGATCAAGCTGGTGTTAAGATTA	25	59.1	40.0	556
	Reverse	AGTCCACCGCGAAGACATT	19	59.3	52.6	
5	Foward	ACTCCTGACTATGAAACCAAAG	22	55.7	40.9	512
	Reverse	CCACCGCGAAGACATTCATAAA	22	59.3	45.5	
6	Foward	TACTCCTGACTATGAAACCA	20	52.7	40.0	509
	Reverse	CGCGAAGACATTCATAAACTGCT	23	59.9	43.5	
7	Foward	AAAGTGGATCAAGCTGGT	18	53.5	44.4	533
	Reverse	TGCTCTACCGTAGTTCTTAGCG	22	59.6	50.0	
8	Foward	CTCCTGACTATGAAACCAAAGATAC	25	57.2	40.0	501
	Reverse	GACATTCATAAACTGCTCTACCGT	24	58.9	41.7	
9	Foward	ATCTTGGCAGCATTCCGAGT	20	59.7	50.0	406
	Reverse	CCAATAGGGGACGGCCATAC	20	60.0	60.0	
10	Foward	TTGGCAGCATTCCGAGTAACT	21	60.0	47.6	401
	Reverse	AATAGGGGACGGCCATACTTG	21	59.6	52.4	

A total of ten primer pairs were successfully designed from conserved regions identified through ClustalW alignment. Their characteristics are summarized in Table 1. The primers ranged from 18–25 bp, which fits the optimal length for maintaining binding specificity and efficient annealing. Primers shorter than 18 bp risk non-specific binding, while those longer than 30 bp may form secondary structures; thus, all candidates meet the basic structural criteria.

The melting temperatures (Tm) of the forward and reverse primers ranged from 52.7°C to 60.0°C, with most pairs showing ≤ 2°C difference ideal for synchronized annealing and reducing amplification failure due to temperature mismatch. GC content ranged from 40–60%, the standard optimal range that ensures adequate thermodynamic stability without promoting hairpins or primer–dimers.

Predicted amplicon sizes varied from 401–562 bp, a range well-suited for routine PCR and clear resolution on agarose gels. Primer sets 9 and 10 produced the shortest amplicons (406 and 401 bp), which may be advantageous for degraded templates, while sets 1, 2, and 4 generated >550 bp products, offering broader sequence coverage for downstream analyses.

The in silico amplification analysis confirmed that all ten primer pairs generated clear, specific amplicons with no predicted off-target binding, indicating high target specificity. Most primers showed Tm values within the optimal 55–60°C range, with forward–reverse differences ≤2–3°C, supporting synchronized annealing. Although primer sets 5, 6, and 7 exhibited slightly wider Tm gaps due to lower GC content in their forward primers, the differences remained acceptable for routine PCR optimization.

Secondary-structure assessment further supported primer suitability. Several reverse primers (sets 1, 4, 5, 9, and 10) displayed modest hairpin Tm values (32–35°C), well below annealing temperatures, indicating low structural interference. Self-dimer and pair-dimer formation were also weak, with the highest self-dimer value (primer 4R, Tm 20.2°C) still insufficient to hinder amplification.

Predicted amplicons ranged from 401 to 562 bp, an ideal size window for efficient PCR and clear resolution on agarose gels. Primer sets 9 and 10 produced the smallest fragments (406 and 401 bp), advantageous for degraded DNA, whereas sets 1, 2, and 4 amplified >550 bp regions that provide broader sequence coverage.

Considering Tm compatibility, GC content, minimal secondary structures, and absence of off-target sites, primer sets 3, 8, 9, and 10 stand out as the strongest candidates for laboratory validation. However, all primer sets meet essential PCR design standards and can be selected according to specific experimental goals, such as targeting defined genomic regions or maximizing sequence coverage.

The translated amplicon sequences from all ten primer pairs show a consistently high level of conservation across the amplified protein regions. As summarized in Table 3, every primer set generated amino-acid sequences corresponding to the RuBisCO large subunit (rbcL), a protein well known for its highly conserved functional motifs. This conservation is clearly reflected in the amplicons, regardless of differences in primer positions or product length.

All primer sets produced translated sequences containing nearly identical conserved regions, including the recurring motif "TPDYETKDTDILAAFRVTPQPGVPPEEAGAAVAAESSTGTWTT VWTDGLT," which forms part of the catalytic core essential for substrate stabilization and enzymatic activity. The repeated presence of these motifs confirms that the primers successfully targeted functionally important regions of rbcL.

Longer amplicons produced by primer sets 1, 2, 4, and 7 included additional upstream residues such as "SGSSWC-DYKLTYYT," while shorter products from sets 9 and 10 captured truncated portions of the gene. Despite these differences in coverage, all essential motifs remained intact, indicating minimal sequence divergence across amplified regions.

The sequences also preserved conserved glycine-rich and proline-rich segments associated with key β-strand and loop structures in RuBisCO, supporting the structural reliability of the amplified regions. These segments are

evolutionarily constrained, and their consistent presence across all amplicons reflects accurate targeting of structurally important domains.

Alignment with the SWISS-MODEL homology structure further confirmed the validity of the translated sequences. Catalytic regions involved in ribulose-1,5-bisphosphate binding and Mg^{2+} coordination were present within the amplified fragments, demonstrating strong agreement between sequence-level conservation and the predicted 3D protein model.

The electrophoresis results in Fig. 5 show that all ten primer candidates produced clear, single PCR bands with no smearing, nonspecific products, or primer dimers, indicating efficient and highly specific annealing to the target region. The observed band sizes closely match the in silico predictions in Table 2: primer sets 1, 2, and 4 yielded ~550–560 bp products, sets 3, 5, 6, 7, and 8 produced ~500 bp fragments, and sets 9 and 10 generated the expected ~400 bp amplicons (406 bp and 401 bp). This strong agreement between predicted and experimental results validates both the primer design strategy and the conserved region selection.

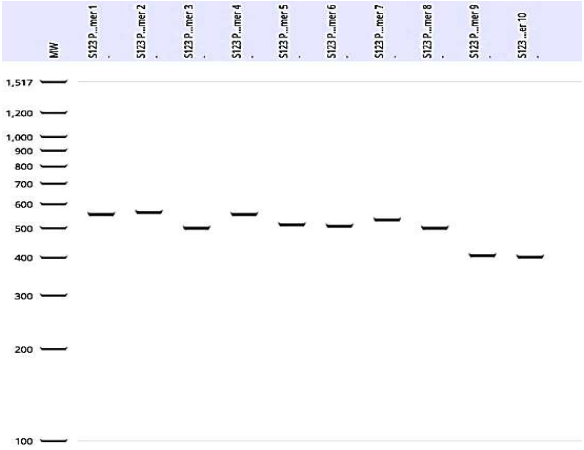
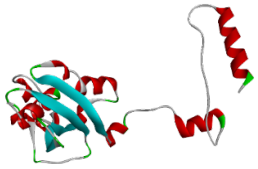
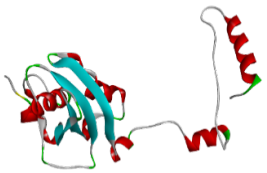
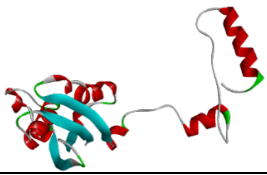


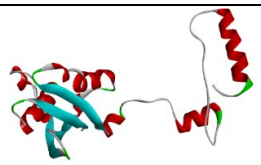
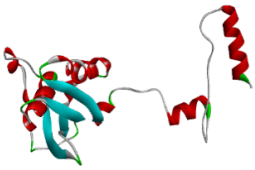
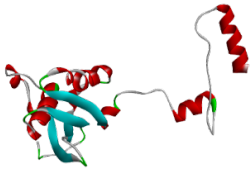
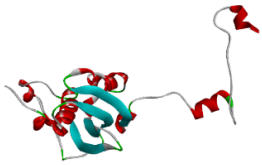
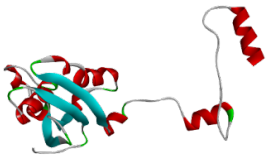
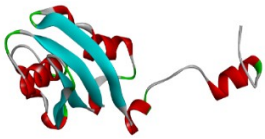
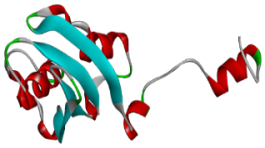
Fig. 5: Electrophoresis of PCR products generated from ten designed primer pairs targeting the rbcL gene.

Table 2: In silico amplification performance and structural characteristics of ten primer pairs

Primer	Amplified Product (bp)	Forward Primer Characteristics	Reverse Primer Characteristics
Primer 1	556	Length: 20 bp; GC: 45.0%; Tm: 57.3°C; Hairpin: None; Self-dimer: None; Pair-dimer: None; Off-target: 0	Length: 20 bp; GC: 50.0%; Tm: 59.8°C; Hairpin Tm: 34.9°C; Self-dimer Tm: 8.2°C; Pair-dimer: None; Off-target: 0
Primer 2	562	Length: 23 bp; GC: 43.5%; Tm: 59.7°C; Hairpin: None; Self-dimer: None; Pair-dimer Tm: 3.0°C; Off-target: 0	Length: 20 bp; GC: 50.0%; Tm: 59.7°C; Hairpin: None; Self-dimer Tm: 8.2°C; Pair-dimer Tm: 3.0°C; Off-target: 0
Primer 3	501	Length: 22 bp; GC: 50.0%; Tm: 59.5°C; Hairpin: None; Self-dimer Tm: 4.0°C; Pair-dimer: None; Off-target: 0	Length: 22 bp; GC: 45.5%; Tm: 59.8°C; Hairpin: None; Self-dimer Tm: 4.0°C; Pair-dimer: None; Off-target: 0
Primer 4	556	Length: 25 bp; GC: 40.0%; Tm: 59.1°C; Hairpin: None; Self-dimer: None; Off-target: 0	Length: 19 bp; GC: 52.6%; Tm: 59.3°C; Hairpin Tm: 32.8°C; Self-dimer Tm: 20.2°C; Off-target: 0
Primer 5	512	Length: 22 bp; GC: 40.9%; Tm: 55.7°C; Hairpin: None; Self-dimer: None; Off-target: 0	Length: 22 bp; GC: 45.5%; Tm: 59.3°C; Hairpin Tm: 34.9°C; Self-dimer Tm: 8.2°C; Off-target: 0
Primer 6	509	Length: 20 bp; GC: 40.0%; Tm: 52.7°C; Hairpin: None; Self-dimer: None; Off-target: 0	Length: 23 bp; GC: 43.5%; Tm: 59.9°C; Hairpin Tm: 34.9°C; Self-dimer: None; Off-target: 0
Primer 7	533	Length: 18 bp; GC: 44.4%; Tm: 53.6°C; Hairpin: None; Self-dimer: None; Off-target: 0	Length: 22 bp; GC: 50.0%; Tm: 59.6°C; Hairpin: None; Self-dimer Tm: 5.5°C; Off-target: 0
Primer 8	501	Length: 25 bp; GC: 40.0%; Tm: 57.2°C; Hairpin: None; Self-dimer: None; Off-target: 0	Length: 24 bp; GC: 41.7%; Tm: 58.9°C; Hairpin: None; Self-dimer: None; Off-target: 0
Primer 9	406	Length: 20 bp; GC: 50.0%; Tm: 59.7°C; Hairpin Tm: 34.2°C; Self-dimer: None; Off-target: 0	Length: 20 bp; GC: 60.0%; Tm: 60.0°C; Hairpin Tm: 35.2°C; Self-dimer Tm: 11.0°C; Off-target: 0
Primer 10	401	Length: 21 bp; GC: 47.6%; Tm: 60.0°C; Hairpin: None; Self-dimer: None; Off-target: 0	Length: 21 bp; GC: 52.4%; Tm: 59.6°C; Hairpin Tm: 35.2°C; Self-dimer Tm: 11.0°C; Off-target: 0

Table 3: Translated amino-acid sequences of amplified rbcL gene fragments and corresponding structural conservation

Primer	Translated Amplicon Sequence	Protein Structure
Primer 1	SGSSWC-DYKLTYYTPDYETKDTDILAAFRVTP QPGVPPEEAGAAVAAESSTGTWTTVWTDGLT SLDRYKGRCYHIEPVAGEENQYICYVAYPLDLF EEGSVTNMFTSIVGNVFGFKALRAIRLEDLRIPT SYTKTFQGPPHGIQVERDKLNKYGRPLLGCTIK PKLGLSAKNYGRAVYECLRGG	
Primer 2	SGSSWC-DYKLTYYTPDYETKDTDILAAFRVTPQ PGVPPEEAGAAVAAESSTGTWTTVWTDGLTSLD RYKGRCYHIEPVAGEENQYICYVAYPLDLFEEGS VTNMFTSIVGNVFGFKALRAIRLEDLRIPTSYTKT FQGPPHGIQVERDKLNKYGRPLLGCTIKPKLGLS AKNYGRAVYECLRGGLI	
Primer 3	DILAAFRVTPQPGVPPEEAGAAVAAESSTGTWTT VWTDGLTSLDRYKGRCYHIEPVAGEENQYICYVA YPLDLFEEGSVTNMFTSIVGNVFGFKALRAIRLEDL RIPTSYTKTFQGPPHGIQVERDKLNKYGRPLLGCT IKPKLGLSAKNYGRAVYECLRGGLILQ	

Primer 4	GSSWC-DYKLTYYTPDYETKDTDILAAFRVTPQP GVPPEEAGAAVAAESSTGTWTTVWTDGLTSLD RYKGRCYHIEPVAGEENQYICYVAYPLDLFEEG SVTNMFTSIVGNVFGFKALRAIRLEDLRIPTSYTK TFQGPPHGIQVERDKLNKYGRPLLGCTIKPKLGL SAKNYGRAVYECLRGG	
Primer 5	TPDYETKDTDILAAFRVTPQPGVPPEEAGAAVAA ESSTGTWTTVWTDGLTSLDRYKGRCYHIEPVAG EENQYICYVAYPLDLFEEGSVTNMFTSIVGNVFG FKALRAIRLEDLRIPTSYTKTFQGPPHGIQVERDK LNKYGRPLLGCTIKPKLGLSAKNYGRAVYECLRG	
Primer 6	TPDYETKDTDILAAFRVTPQPGVPPEEAGAAVA AESSTGTWTTVWTDGLTSLDRYKGRCYHIEPVA GEENQYICYVAYPLDLFEEGSVTNMFTSIVGNVF GFKALRAIRLEDLRIPTSYTKTFQGPPHGIQVER DKLNKYGRPLLGCTIKPKLGLSAKNYGRAVYECLR	
Primer 7	SGSSWC-DYKLTYYTPDYETKDTDILAAFRVTPQPG VPPEEAGAAVAAESSTGTWTTVWTDGLTSLDRYKG RCYHIEPVAGEENQYICYVAYPLDLFEEGSVTNMFT SIVGNVFGFKALRAIRLEDLRIPTSYTKTFQGPPHGI QVERDKLNKYGRPLLGCTIKPKLGLSAKNYGRA	
Primer 8	PDYETKDTDILAAFRVTPQPGVPPEEAGAAVAAESS TGTWTTVWTDGLTSLDRYKGRCYHIEPVAGEENQYI CYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALRAIRL EDLRIPTSYTKTFQGPPHGIQVERDKLNKYGRPLLG CTIKPKLGLSAKNYGRAVYEC	
Primer 9	ILAAFRVTPQPGVPPEEAGAAVAAESSTGTWTTVWT DGLTSLDRYKGRCYHIEPVAGEENQYICYVAYPLDL FEEGSVTNMFTSIVGNVFGFKALRAIRLEDLRIPTSY TKTFQGPPHGIQVERDKLNKYGRPLL	
Primer 10	LAAFRVTPQPGVPPEEAGAAVAAESSTGTWTTV WTDGLTSLDRYKGRCYHIEPVAGEENQYICYVA YPLDLFEEGSVTNMFTSIVGNVFGFKALRAIRLE DLRIPTSYTKTFQGPPHGIQVERDKLNKYGRPL	

The complete absence of secondary bands further reflects favorable primer thermodynamics. The computationally predicted low hairpin and dimer formation aligns with the laboratory outcome, demonstrating that the primers had appropriate annealing temperatures and GC content to ensure efficient binding without unintended secondary interactions.

Primer Product Testing and In Silico PCR Extraction

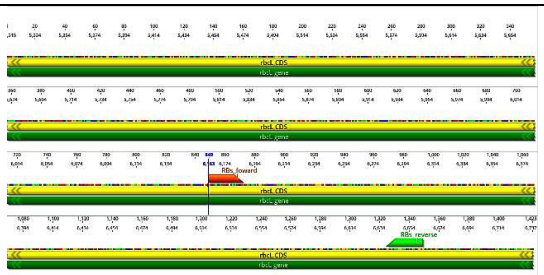
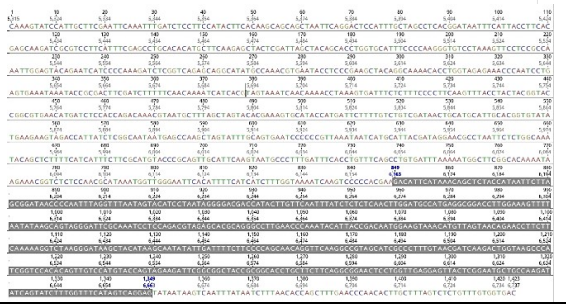
In silico amplification using Geneious Prime was performed to identify the precise genomic regions targeted by the designed primers. The complete chloroplast genome of *Glycine max* (Accession NC_007942.1) served as the PCR template, enabling visualization of primer binding sites, predicted amplicon boundaries, and verification of the amplified gene region.

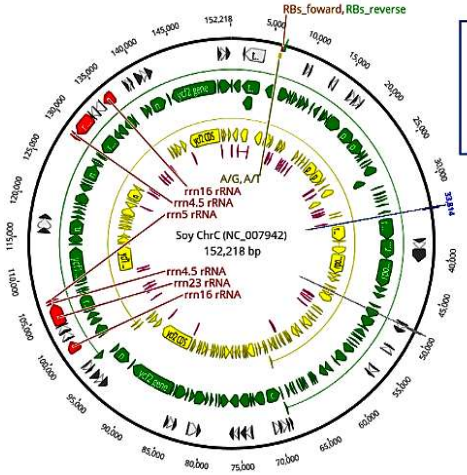
The analysis showed that the selected primer pair annealed specifically to the rbcL gene, a highly conserved chloroplast coding region. The forward and reverse primers were correctly positioned around the rbcL CDS, producing a predicted amplicon fully consistent with the size estimated using Primer3Plus. No off-target binding sites were detected, confirming high primer specificity.

A circular chloroplast genome map was generated to display the primer positions relative to neighboring genes, while a zoomed linear view further demonstrated that both primers annealed within conserved regions of the rbcL sequence. These results validate the accuracy of the primer design and confirm its suitability for downstream PCR amplification.

Both primers showed zero predicted off-target amplification events, confirming highly specific targeting of the soyChrC/*rbcL* genomic region. The bioinformatic analyses identified a validated primer pair targeting the conserved *rbcL* region of *Syzygium aromaticum* L. (clove). Sequence alignment, primer quality assessment, and in silico amplification collectively confirmed that the selected primers possess optimal physicochemical characteristics

Table 4: Primer binding characteristics and in silico mapping on the chloroplast genome template

No	Primer Characteristics	Binding Characteristics		Visualization
1	Primer Foward	Name	RBs_forward	
		Interval	6.163 → 6.186	
		Sequence (5′→3′)	GACATTCATAAACTGCTCTACCGT	
		Mismatch Positions	============T========G=	
		Number of Mismatches	2	
		Product Size	501 bp	
		Local Off-target Sites	0	
2	Primer Reverse	Name	RBs_reverse	
		Interval	6.663 → 6.639	
		Sequence (5′→3′)	CTCCTGACTATGAAACCAAAGATAC	
		Mismatch Positions	None	
		Number of Mismatches	0	
		Product Size	501 bp	
		Local Off-target Sites	0	



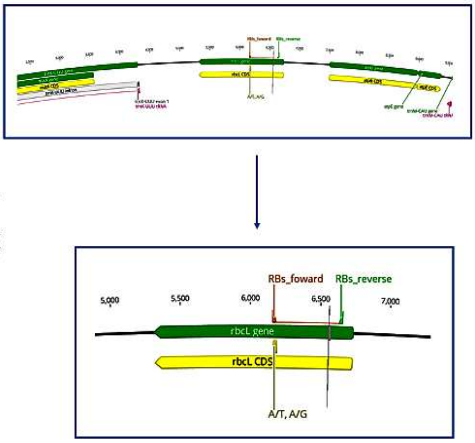


Fig. 6: In silico mapping of forward and reverse primer binding sites on the complete chloroplast genome of Glycine max (NC_007942.1).

and strong specificity to the target sequence. Using the *Glycine max* chloroplast genome (NC_007942.1) as a conserved reference, alignment highlighted stable *rbcL* regions suitable for primer binding (Fig. 6). Homology visualization showed that RBs_forward and RBs_reverse annealed precisely to the intended target without off-target binding. The circular chloroplast genome map and zoomed-in view further validated accurate placement within the *rbcL* coding region. As summarized in Table 4, RBs_forward binds at positions 6.163–6.186, producing a 501 bp amplicon. Although it contains two mismatches (T and G substitutions), these did not reduce specificity, as no off-target sites were predicted. RBs_reverse binds at positions 6.663–6.639 with zero mismatches, ensuring strong and selective annealing. Both primers showed zero predicted off-target amplification events, confirming highly specific targeting of the soyChrC/*rbcL* genomic region.

DISCUSSION

The present study developed and validated a highly specific primer pair targeting the chloroplast rbcL gene of AFO clove (*Syzygium aromaticum* var. AFO), a historically unique and genetically important clove population originating from Ternate Island. Rather than merely confirming primer suitability, the results provide deeper biological insights into chloroplast marker development, molecular stability, and genetic applications for perennial spice crops. The absence of hairpin loops, self-dimerization, and cross-complementarity indicates a structurally stable primer configuration capable of efficient hybridization, supporting the fundamental thermodynamic principles governing PCR amplification (Garafutdinov et al., 2020). These structural characteristics are not only technical indicators but also biologically relevant, as stable primer template interactions are essential for accurately amplifying conserved chloroplast coding regions such as rbcL (Li et al., 2020).

Compared with recent international studies on chloroplast primer development, the present work demonstrates comparable or improved thermodynamic balance. Studies in woody and medicinal plants have

emphasized that primer GC content between 40–60% and minimal melting temperature differences are crucial for amplification efficiency and specificity (Kalendar et al., 2024; Kumar et al., 2024). The primer pair identified here falls precisely within these optimal ranges, similar to chloroplast rbcL primers successfully applied in phylogenetic and DNA barcoding studies across angiosperms (Yan et al., 2023). Notably, the GC composition observed in this study ensures sufficient duplex stability without promoting nonspecific binding, a limitation reported in several primer design studies where excessive GC content caused amplification bias or incomplete denaturation (Deng et al., 2020; Wang et al., 2021).

A key methodological strength of this study lies in the integrated three-stage in silico workflow, combining sequence alignment, structural protein validation, and computational PCR simulation (Kalendar, 2022). Unlike conventional primer design approaches that rely solely on nucleotide conservation, the incorporation of protein-level structural visualization ensures that the selected primer-binding region corresponds to a functionally stable domain of the RuBisCO enzyme (Casola & Li, 2022). Similar integrative strategies have been recommended in modern molecular studies to enhance primer reliability and reduce amplification failure (Kalendar et al., 2024). The structural conservation observed in the translated amplicon sequences further confirms that the primers target biologically meaningful regions, rather than random conserved motifs, reinforcing the functional relevance of the amplified fragment (Zhao et al., 2023).

The predicted amplicon length (~500 bp) represents a biologically optimal fragment size for chloroplast markers (Travadi et al., 2022). Previous studies have shown that fragments between 400–600 bp provide a balance between amplification efficiency and phylogenetic resolution, particularly in partially degraded plant DNA samples (Mahadani et al., 2022; Travadi et al., 2022). Shorter amplicons may reduce phylogenetic informativeness, while excessively long fragments are prone to amplification failure in field-derived DNA samples (Liu et al., 2023). Therefore, the fragment size obtained in this study is well suited for downstream applications such as sequencing, molecular identification, and comparative genomics of clove germplasm (Regalado et al., 2020).

Specificity analysis using Primer-BLAST confirmed that the selected primer pair accurately targets the conserved rbcL region without off-target amplification (Siswanto et al., 2022). This level of specificity is critical in chloroplast marker development, as nonspecific amplification from nuclear plastid DNA (NUMTs) has been reported as a major limitation in plant molecular studies (Yan et al., 2023). The absence of nuclear off-target binding in this study indicates that the primer set can reliably amplify genuine chloroplast DNA, thereby improving the accuracy of phylogenetic and molecular identification analyses (Kang et al., 2021; Zhang et al., 2022). Similar high-specificity primer performance has been documented in recent chloroplast genome studies of *Syzygium* species and other woody crops (Zhang et al., 2023; Huynh et al., 2024), supporting the robustness of the primer design strategy employed here.

One of the most important contributions of this study is the novel development of a specific chloroplast primer for AFO clove, a historically significant and genetically distinct clove population. While previous molecular studies have investigated chloroplast markers in clove and related *Myrtaceae* species (Picek et al., 2023; Marasabessy et al., 2024), targeted primer development for AFO clove has remained limited. The present primer therefore represents a new molecular tool that can facilitate genetic identification and authentication of this unique germplasm (Bunjkar et al., 2024). Given the historical recognition of AFO clove as one of the oldest clove genotypes, the availability of a reliable chloroplast marker provides an important foundation for conservation genetics and germplasm preservation (Snoussi et al., 2022; Salgotra & Chauhan, 2023; Bidyananda, 2024).

From a breeding perspective, chloroplast markers such as rbcL are particularly valuable due to their maternal inheritance and structural stability (Chu et al., 2025; Goswami, 2025). In perennial woody crops like clove, long breeding cycles make molecular markers essential for early-stage selection and genetic tracking (Amiteye, 2021). The primer developed in this study can support marker-assisted breeding by enabling accurate identification of AFO clove lineage and genetic purity. Furthermore, the conserved nature of chloroplast DNA allows cross-population comparison, which is important for detecting genetic divergence, introgression, and population structure in breeding programs (Sun et al., 2023; Chung, 2025).

The conservation implications of this study are equally significant. AFO clove represents a historically and genetically important germplasm resource associated with the origin of global clove cultivation. Molecular characterization using chloroplast markers can support conservation strategies by distinguishing authentic AFO genotypes from hybrid or introduced populations (Lu et al., 2020; Lu et al., 2021). Recent studies in plant conservation genetics emphasize the importance of chloroplast DNA markers for identifying evolutionary lineages and preserving genetically unique populations (Mahima et al., 2022; Kumar et al., 2024). The primer developed here therefore contributes to the molecular toolkit required for safeguarding this historically important clove germplasm.

In addition to breeding and conservation, the primer set has strong potential for phylogenetic and evolutionary studies (Yang et al., 2021). The rbcL gene is one of the most widely used molecular markers in plant phylogenetics due to its universal presence, conserved coding sequence, and moderate mutation rate (Mahadani et al., 2022). The successful amplification of conserved functional motifs observed in this study indicates that the primer targets phylogenetically informative regions (Ross et al., 2021). This enables comparative evolutionary analysis of AFO clove with other *Syzygium* species and related *Myrtaceae* taxa, contributing to a better understanding of clove domestication, diversification, and evolutionary history (Low et al., 2022; Souza Neto et al., 2022).

Although the in silico results demonstrate strong primer performance, experimental validation remains essential. Laboratory PCR will confirm amplification efficiency, annealing stability, and reproducibility under real experimental conditions (Ruijter et al., 2021). Environmental DNA quality, template concentration, and PCR optimization parameters may influence amplification success, as reported in previous molecular studies (Tripathi & Rathinam, 2025). Future experimental validation will also determine whether the primer can be applied across multiple AFO clove populations and related clove varieties, thereby expanding its applicability in molecular breeding and phylogenetic research.

Overall, this study provides not only a technically validated primer pair but also a biologically meaningful molecular tool for studying one of the oldest clove genotypes in the world. The integration of structural validation, thermodynamic optimization, and specificity analysis ensures high reliability, while the novelty of primer development for AFO clove highlights its importance for breeding, conservation, and evolutionary research. The findings contribute to advancing molecular characterization of clove germplasm and establish a foundation for future genomic and phylogenetic studies in this economically and historically important spice crop.

Conclusion

This study successfully designed and validated a highly specific primer pair targeting the rbcL gene of *Syzygium aromaticum* var. AFO through an integrated in silico workflow. The primers demonstrated optimal thermodynamic properties and precise annealing to conserved regions, producing a predicted ~500 bp amplicon without off-target binding. These findings highlight the primer pair's strong potential for varietal identification, phylogenetic studies, and molecular-marker development in AFO clove. Overall, the results provide a reliable foundation for downstream molecular applications, with laboratory PCR validation required to confirm performance under experimental conditions.

DECLARATIONS

Funding: This project was funded by the Institute for Research and Community Service (LPPM), Universitas Khairun, for Hibah Penugasan Profesor Scheme with contract no. 234/UN44/PP.01/2024.

Acknowledgement: The researcher expresses his high appreciation and thanks to the Institute for Research and Community Service, Universitas Khairun, for providing financial support to this research with research contract number: No. 234/UN44/PP.01/2024.

Conflict of Interest: The authors have declared no conflict of interest.

Data Availability: The data presented in this study are available in this article and upon reasonable request.

Ethics Statement: This study did not involve humans or animals; therefore, it was not required.

Author's Contribution: Sundari and Abdu Mas'ud, arranged the experimental design and wrote the original manuscript. Iis Hamsir A.W, Theopilus Wutuguly and Yusri Sapsuha conducted the experiment in the laboratory. Sundari and Aditya S Sakti analyzed the data and finalized the draft. The final version of the manuscript was revised and approved by all authors.

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