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OPTIMIZATION OF DNA PROTOCOLS AND MARKERS FOR POLLEN DNA BARCODING FROM FLOWERING PLANTS

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ABSTRACT. *Pollen identification using DNA barcoding offers a precise and reliable approach that is independent of morphological traits. However, challenges such as the presence of a robust exine layer encapsulating DNA, inhibitors, and contaminants necessitate improved DNA extraction methods from flowering plant pollen. Additionally, the minute quantity of DNA available in a single pollen grain complicates extraction and subsequent analysis. This study aimed to optimise DNA protocols and selection of the ideal marker for pollen DNA barcoding. Nine species of flowering plants; Acacia auriculiformis, Melastoma malabatricum, Rhodomyrtus tomentosa, Ixora chinensis, Tetracera indica, Mischocarpus sundaicus, Acronychia pedunculata, Melaleuca cajuputi, and Gmelina asiatica were obtained from Taman Rimba Ilmu Tanah BRIS UniSZA Kampus Besut (TRIBE). DNA was extracted from 50-100 mg of pollen grains and flowers using two different kits. Three plant DNA markers, the rbcL, ITS2 and trnL were used for PCR optimization. DNA extraction from 100 mg of plant tissue (pollen with flower) yielded more distinct bands compared to 50 mg, indicating improved DNA quality with increased sample mass. Among the eight DNA samples obtained through optimised extraction, six demonstrated successful amplification with at least one marker; rbcL, ITS2, or trnL, as evidenced by distinct bands at the expected sizes. While rbcL and ITS2 consistently produced clear and specific bands, amplification with trnL resulted in multiple bands across several samples, suggesting non-specific binding or primer-dimer formation. This non-specific amplification may complicate downstream sequencing and species identification, indicating that trnL may be less suitable for these plant tissues under the current PCR conditions. BLAST analysis of six plant samples amplified with the rbcL marker identified four to the species level: Ixora chinensis, Tetracera indica, Acronychia pedunculata, and Rhodomyrtus tomentosa which based on high sequence identity (≥92.59%) and significant E-values. The remaining two samples were resolved only to the genus level (Acacia sp. and Mischocarpus sp.), indicating limitations in species-level resolution for certain taxa. These findings underscore the importance of DNA extraction quality in achieving efficient PCR amplification and highlight that marker selection, particularly the use of rbcL plays a critical role in accurate species-level identification. Further optimisation of the PCR conditions for the trnL marker is recommended to improve amplification specificity.*

INTRODUCTION

Malaysia received the recognition as the megadiverse country that home 15 300 species of vascular plant where the number can be divided to 12 000 and 9 030 plant species that occur in East Malaysia and West Malaysia respectively (Chua et al., 2023). These plants species commonly identify based on morphological characteristics of the leaves, flowers, and pollen. However, identification of plants especially from pollen of flowering plants can be challenging due to variation in morphology between closely related species (Naim & Mahboob, 2020). Pollen is a collection of microspores that are found in a seed plant and typically take the form of a fine dust which transported from the male structures plant to the female structure for the fertilization to occur. Insects, wind, and water are just some of the ways that pollen can be carried from one place to another (Meeuse and JD, 2025). Additionally, traditional methods for identification may not always provide accurate results and require experts. Therefore, pollen identification using DNA barcoding is a reliable method that uses short DNA fragments to identify species at the molecular level and is not limited to morphological traits (Abubakar et al., 2018).

Utilizing DNA barcoding and DNA metabarcoding for pollen identification is more efficient than traditional methods, as it does not necessitate advanced expertise, resulting in time savings (Bayram, 2021). DNA barcoding is a technique that requires at least one short DNA region, which the accuracy and reliability is getting enhanced with the high technologies such as next-generation sequencing (NGS) (Yong et al., 2024). Unlike animal kingdom, currently there are no existing ideal DNA barcodes in plants but there are some single and multiple barcodes that have been used. DNA barcode for pollen identification can be categorised into two groups which are the nuclear and chloroplast genome. Nuclear genome such as Internal Transcribed Spacer 2 (ITS2) was suggested as the best nuclear barcode marker due to its high accuracy in distinction (Khan et al., 2019). Meanwhile, RuBisCo, Ribulose-1,5-bisphosphate carboxylase oxygenase, large subunit barcode (rbcL) is one of the commonly used chloroplast genomes due to ability to achieve high percentage in identification at the genus level (Cahyaningsih et al., 2022). Combining several markers is suggested to overcome the limitations in universality and discriminatory power (Teklemariam et al., 2023).

One of the most crucial procedures before DNA barcoding is DNA extraction. DNA extraction is important because it will affect the downstream process such as qPCR and sequencing. Sequencing is considered sensitive and will be affected by the quality of extracted DNA (Yang et al., 2021). By analysing the purity and concentration of DNA samples, the rate of success can be determined. The range of acceptable concentration is usually between 716 to 1034 ng/µL and the purity at 260/280 is between 1.7 to 2.0 (Sajali et al., 2018).

Optimisation of DNA extraction can help DNA barcoding to complement traditional methods of identification of pollen. It is recommended that DNA barcoding and DNA metabarcoding become a routine technique for ecologists, as they have the potential to provide answers to ecological inquiries, such as investigating the variety range of floral visitation by analysing flower-visiting insects (Lowe et al., 2022). Not only that, but other fields of study also gain many benefits from utilizing this method. For instance, forensic can analyse clinical samples and other samples presence in the scene such as soil, animal and plant tissue (Dairawan and Shetty, 2020). Hence, this study focused on the (1) optimisation of DNA protocols and (2) selection of the ideal marker for DNA barcoding utilising pollen of the flowering plants.

MATERIALS AND METHODS

The methodology must be clearly stated and described in sufficient detail or with sufficient references. The author shall explain the research question, describe the research framework, and the methods applied in detail. It should be furthermore highlighted why the research question is relevant to theory and practice, and why the chosen method(s) are suited for the problem.

Pollen and flowers sampling and processing

Approximately 50-100 grams of pollen grains along with its flowers from ten plant species which grow at Taman Rimba Ilmu Tanah Bris (TRIBE) were collected in September 2024. These samples were stored in separate plastic bags with silica gel and stored at -20°C until further use. All tools used during

pollen isolation were placed in 10% bleach for 1 minute prior to use and in between each step of pollen isolation to avoid cross contamination (Gorki et al., 2024). The samples (pollen and flowers) were homogenized in liquid nitrogen and grind using mortar and pestle and stored at –20℃ until extraction. Between each usage, the mortar and pestle were washed and sanitised with a solution of 90% ethanol and 10% bleach.

Optimization of DNA Extraction with PrimeWay Plant DNA Extraction kit and SPINeasy DNA Kit for Plant.

Fifty milligrams to 100 mg of the pollen samples were extracted using two different kits. The first kit was the PrimeWay Plant DNA extraction kit (1st BASE, Singapore) using cetyltrimethylammonium bromide (CTAB) method and the second kit was SPINeasy DNA Kit for Plant (MP Biomedicals, California). The extraction steps were done according to the manufacturer's protocols except for lysis incubation step which was increased from 10 mins to 30 mins in 65℃ waterbath.

Determination of DNA Quality Using Agarose Gel

The quality of DNA was assessed using a 1% agarose gel was used by mixing a tablet of TopVision agarose tablets (ThermoFisher, USA) in 50 ml of 1X TBE buffer (ThermoFisher, USA). Ten microlitres of DNA sample was mixed with 2 µl of loading dye and loaded onto the gel. 1kb DNA ladder was used as standard. The gel was set to run at 80 V for about an hour and observed under UV illumination in a gel documentation system (Simel et al., 1997). The intensity of each band in the gel was corresponded to the amount of DNA present in each sample, indicating the presence of DNA in the samples.

Optimization of plant DNA markers using gradient PCR

There were two chloroplast genomes used, the rbcL and trnL, and a nuclear ribosomal barcode regions ITS2 used for the amplification of plant DNA (Table 1). The PCR was performed in a 25 µl volume containing 1 µl of DNA, 1.25 µl of forward and reverse primers at different concentrations of 5 µM, 1 µM, and 0.5 µM and 12.5 µl GoTaq® Green Master Mix (Promega, USA). The amplification reaction commenced with an initial denaturation at 95°C for 2 min, followed by 30 cycles consisting of 1 min at 95°C for denaturation, 30 s at 50°C-60°C for annealing, 1 min at 72°C for extension and final extension at 72°C for 5 min. However, the amplification used six different annealing temperatures and different time set for the final extension step aimed for optimization. The temperature for gradient PCR ranged between 50 to 60°C which consist of 53.2°C, 54.4°C, 55.6°C, 56.8°C, 58.0°C, and 59.0°C.

Table 1. Primers used for amplification of DNA regions

Gene region	Name	Sequence	Product size	Reference
rbcL	rbcLF	ATGTCACCACAAAGAGACTAAAGC	~600 bp	(Amin et. al, 2019)
	rbcLR	AGGGGACGACCATACTTGTTCA		
ITS2/3	ITS2F	ATGCGATACTTGGTGTGAAT	~350-450 bp	(Cahyaningsih et al., 2022)
	ITS3R	GACGCTTCTCCAGACTACAAT		
trnL	trnL-F	ATTTGAACTGGTGACACGAG	~400-600bp	
	trnL-c	CGAAATCGGTAGACGCTACG		

DNA sequencing

Samples with successful amplification (S1, S3, S4, S5, S6, S7) of the rbcL marker were submitted to sequencing company for Sanger sequencing. The resulting chromatograms were manually inspected for quality, trimmed, and assembled using MEGA software (ver.12). The consensus sequences were then subjected to BLASTn analysis against the NCBI GenBank database for species identification. The nucleotide sequences obtained from this study have been successfully deposited in the NCBI GenBank database and assigned the accession numbers PZ351577 (for sample S1) and PZ399243 to PZ399247 for samples S3, S4, S5, S6, and S7, respectively.

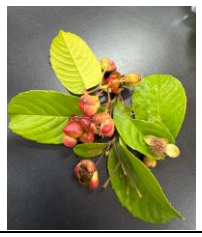
RESULTS AND DISCUSSION

Species morphological identification and DNA extraction

A total of nine samples from different species of flowering plants were collected from Taman Rimba Ilmu Tanah Bris, UNISZA, Terengganu. These samples represented nine distinct species from various families, serving as germplasm resources from the eastern region of Peninsular Malaysia. Table 2 showed the list of plant name based on morphology observations. Plant identification was done using PlantNet application (Li et al., 2022) and verified by botanist Dr. Liliwiarinis Md Nawi from Faculty of Applied Sciences, UiTM Pahang, Kampus Jengka. There were nine species collected from different families of Fabaceae, Melastomataceae, Myrtaceae, Rubiaceae, Dilleniaceae, Sapindaceae, Rutaceae, Myrtaceae, and Lamiaceaa.

Table 2. List of flowers from plants sampled in TRIBE.

Sample	Family	Species	Local name	location
S1	Fabaceae	*Acacia auriculiformis* 	Akasia	5.76076°N, 102.63342°E
S2	Melastomataceae	*Melastoma malabatricum* 	Senduduk	5.76469°N, 102.6400°E
S3	Myrtaceae	*Rhodomyrtus tomentosa*	Kemunting, lidah katak laut	5.76503°N, 102. 63980°E

				
S4	Rubiaceae	*Ixora chinensis* 	Siantan cina, ayam hutan, jarum-jarum, pecah periuk	5.76453°N, 102.63948°E
S5	Dilleniaceae	*Tetracera indica* 	Mempelas, mempelas paya, mempelas paya, akar mempelas	5.76453°N, 102.63948
S6	Sapindaceae	*Mischocarpus sundaicus* 	Sugi, Ludai bulan, Medang serai	5.77051°N, 102.63424
S7	Rutaceae	*Acronychia pedunculata* 	Sejagung	5.76522°N, 102.63829
S8	Myrtaceae	*Melaleuca cajuputi* 	Gelam, kayu putih, gelam tikus	5.77051°N, 102.63424

S9	Lamiaceae	*Gmelina asiatica* 	Bulangan, tangginang, kutang, taring pelandok, belingkot, rukan, bulang	5.76252°N, 102.63796°E

DNA extraction

The preliminary DNA extraction utilised the initial sample of *Acacia auriculiform* (S1) pollen to ascertain the ideal quantity of pollen for DNA extraction. Results showed that 100 mg pollen gave higher DNA band intensity compared to 50 mg. Hence, 100 mg was used for the rest of the samples. Previous research stated that, utilisation of materials weighing 100 mg or more was effective during DNA extraction (Abdel-Latif & Osman, 2017; Aggarwal et al., 2022; Izham et al., 2023), however, each kit specifies an optimal sample weight range to ensure efficient DNA extraction, as using excessive samples may clog the column, hinder proper filtration, and ultimately reduce DNA yield and quality (Russo et al., 2022; Wei et al., 2018). Figure 1 showed gel electrophoresis of DNA extracted from *A.auriculiform* (S1). So, the remaining samples 2-9 were extracted using 100 mg of starting materials (Figure 2). From the extraction using the PRIMEway kit, a few samples showed lower quality and less intense DNA band. As observed in Figure 2, only sample S1, S4, S6, S7 and S8 were able to be extracted via this kit. Hence, DNA extracted were repeated using SPINeasy DNA Kit for Plant for samples S2, S3, S5, S8 and S9. From eight of the nine samples, DNA was successfully extracted using both kits. It is possible that utilizing the wrong tissue sample, in this case, the anthers omitting the floral elements may lead to the inability to recover DNA from sample S9. Furthermore, the existence of worms inside the flowers suggested that the material had deteriorated. Because some plant species do not have readily available free pollen, Prudnikow et al. (2023) suggested desiccating the anther to aid in the release of internal pollen. Apart from the absence of extracted DNA of sample S9, other samples showed a clear band depict the genomic DNA that is sufficiently pure and unsheared for further molecular studies (Tüfekçi, 2023). Hence, S9 was excluded from PCR amplification.

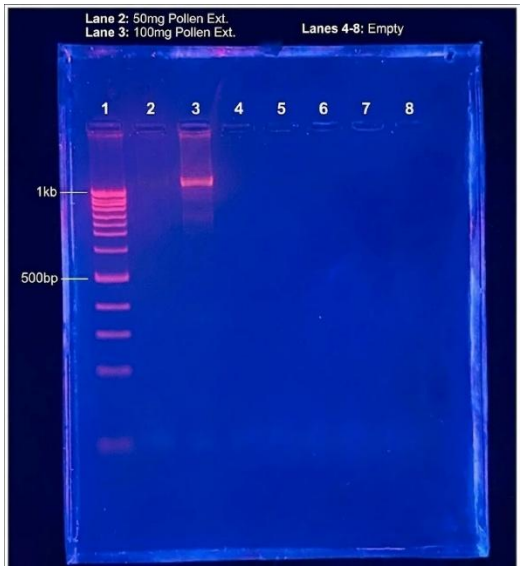


Figure 1. Gel electrophoresis of extracted DNA from 50 mg (lane 2) and 100 mg (lane 3) of S1. 100 mg of samples showed higher DNA band intensity as shown in circle.

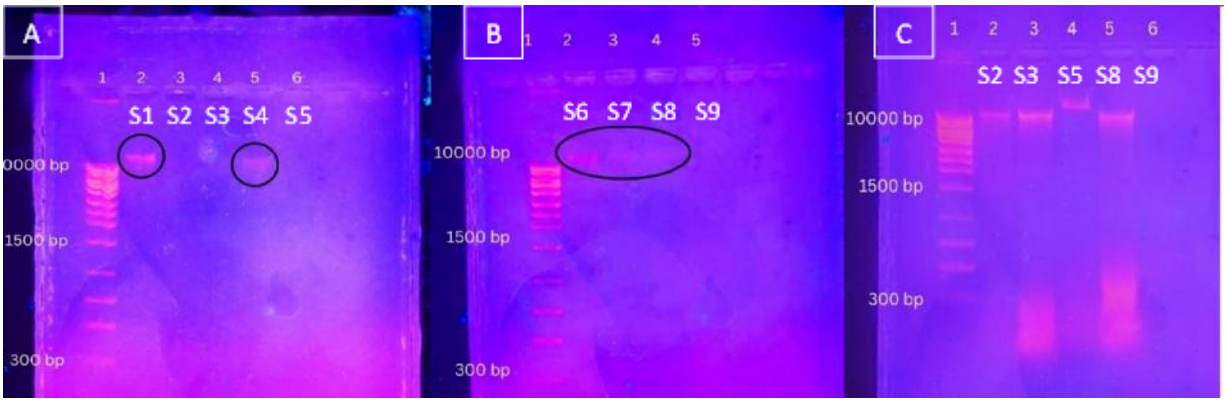


Figure 2. (A) Gel electrophoresis of extracted DNA from S1-S5 (lane 2-6), (B) S6-S9 (lane 2-5) using PrimeWay kit. (C) DNA is extracted from S2, S3, S5, S8 and S9 (lane 2-6) using SPINeasy DNA Kit for Plant.

Optimisation of DNA amplification using rbcL, ITS2 and trnL for plant DNA

During optimisation, primer concentration and annealing temperature were adjusted for each DNA marker. Among the primer concentrations used, 1 µM showed the best primer concentration to be used. It is because higher primer concentration leads to the formation of primer dimer. Primer dimer formation happens when two primers hybridise, indicating the interaction of one oligonucleotide with other oligonucleotides, such as forward and reverse primers, during a PCR reaction (Hendling & Barišić, 2019). Furthermore, the annealing temperature for *rbcL, ITS, and trnL* was set between 53.2°C and 59°C during gradient PCR. For *rbcL*, all temperatures successfully amplified the target region, yielding bands of the anticipated size between 400-600 bp (Figure 3(A); for *ITS*, a temperature of 53.2°C resulted in a band at 400 bp with less smearing (Figure 4(A). Consequently, an annealing temperature of 60°C was chosen for *rbcL*, and all samples showed successful amplification except for S2 and S8 (Figure 3B). Additionally, an annealing temperature of 50°C was selected for *ITS* and all samples also showed successful amplification except for S2 and S8 (Figure 4B). Failed to amplify S2 and S8 might be due to degradation of the extracted DNA which might be caused by mechanical grinding and potentially lead to DNA fragmentation, making DNA more susceptible to degradation (Ceccherini et al., 2003). Nonetheless, for *trnL* marker, the gel electrophoresis revealed triple amplicons across the amplification lane (Figure 5). The expected target size for S1 (*Acacia sp*) is approximately 550 bp, which corresponds closely to the major intense band observed around 600 bp. The other two non-specific extra bands appeared lower down on the gel at approximately 400 bp and 200 bp, respectively. This may be due to

the non-specific binding occurs when primers bind to the template, because of the two extra amplicons appear unintendedly. Multiple bands following PCR amplification may result from many reasons, including elevated marker concentration and inappropriate annealing temperature (Utama et al., 2024). Further optimisation of the remaining samples using *trnL* was not conducted due to time constraints hindering the experiment's progression.

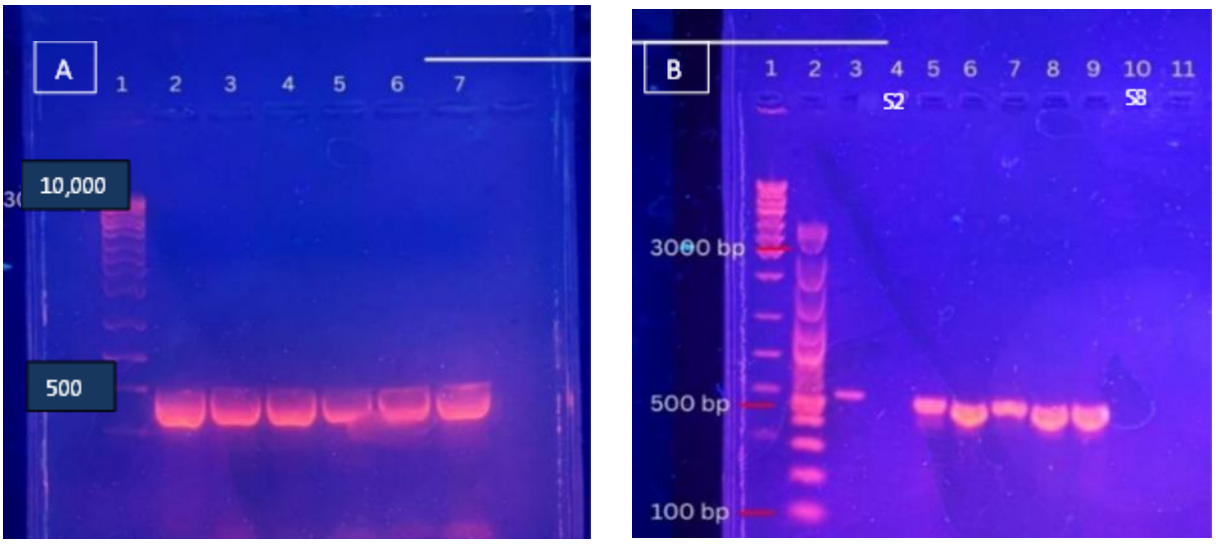


Figure 3. (A) PCR products of rbcL marker of S1 of gradient PCR (53.2-59°C), (B) PCR product of rbcL marker of S1, S2, S3, S4, S5, S6, S7, S8 and non-template control (NTC), S2 and S8 showed absence of band.

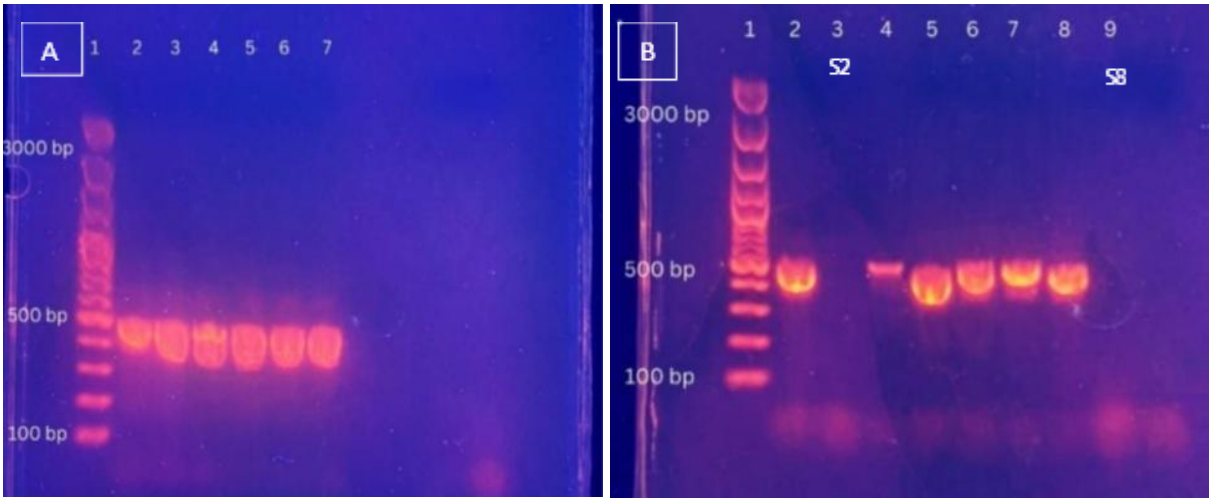


Figure 4. (A) PCR product of ITS2 marker of S1 of gradient PCR (53.2-59°C), (B) PCR of ITS2 marker of S1, S2, S3, S4, S5, S6, S7, S8 and non-template control (NTC), S2 and S8 showed absence of band.

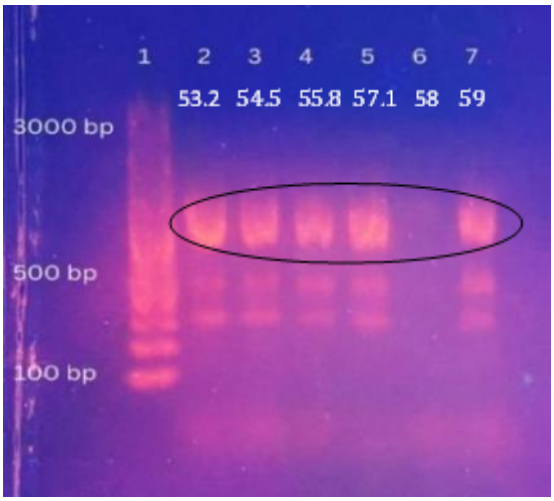


Figure 5. Gradient PCR amplification of the trnL marker from *Acacia sp*. (S1), across an annealing temperature range of 53.2–59°C. Lane 1: 1 kb DNA ladder; Lanes 2–5 and 7: PCR products showing multiple bands, with the main target trnL amplicon located around 600 bp; Lane 6: Absence of amplification at 58°C.

DNA sequencing and species determination

The results of nucleotide blast (blastn) for six plant samples amplified using the *rbcL* marker are summarized in **Table 3**. Each sample was matched against the NCBI nucleotide database to determine the closest species-level identification based on sequence similarity. Among the six samples, four were successfully identified to the species level with high sequence identity (≥98%) and Expect-values of 0, indicating highly significant matches. These include *Ixora chinensis* (S4), *Tetracera indica* (S5), and *Acronychia pedunculata* (S7). Sample S3, identified as *Rhodomyrtus tomentosa*, showed a slightly lower identity (92.59%) but still had a highly significant E-value (3×10^{-178}), supporting its species-level identification. In contrast, samples S1 and S6 could only be identified to the genus level (*Acacia sp*. And *Mischocarpus sp*., respectively), with identity values below the typical species-level threshold. This limitation may be attributed to the relatively conserved nature of the *rbcL* gene, which can lack sufficient variability to distinguish closely related species within certain genera (Cahyaningsih et al., 2022). These findings are consistent with previous studies that have reported the *rbcL* marker as reliable for higher-level taxonomic identification but sometimes insufficient for resolving species-level differences, especially in taxa with low interspecific divergence (Turk et a.,2023). Despite this, the *rbcL* marker proved effective for initial screening and identification in most of the samples analysed. To improve resolution and achieve more accurate species-level identification, it is recommended to complement *rbcL* with additional markers such as *ITS* and *trnL*, which offer higher variability and discriminatory power. Future work should include sequencing of these markers to enhance the robustness of plant DNA barcoding in this study.

Table 3. BLAST Results for Plant Samples Using rbcL Marker.

Sample ID	Identified species	Query cover	E-value	% identity	Accession number Genbank (closest match)
S1	*Acacia sp.*	96%	0	94.95%	ON594118.1
S3	*Rhodomyrtus tomentosa*	88%	3×10^{-178}	92.59%	HQ415191.1
S4	*Ixora chinensis*	99%	0	96.81%	MZ221832.1
S5	*Tetracera indica*	95%	0	98.50%	KU853139.1
S6	*Mischocarpus sp.*	85%	0	96.96%	AY724363.1
S7	*Acronychia pedunculata*	87%	0	98.65%	HQ415180.1

CONCLUSION

The integration of DNA sequencing in this study enabled accurate species identification through comparison with reference databases. This study highlights the importance of DNA extraction quality and marker selection in successful PCR amplification and species identification. Flower and pollen tissues proved viable alternatives to leaves for DNA isolation, though optimisation of extraction protocols and PCR conditions remains essential. Among the tested markers, rbcL and ITS2 showed higher amplification success than trnL, underscoring the need for careful primer design and thermal cycling adjustments. Further refinement of in-house extraction methods may enhance yield and purity, offering a cost-effective approach for diverse plant taxa. To further enhance species resolution and phylogenetic insights, future research could explore additional markers such as matK and trnH-psbA. Combining multiple markers may improve identification accuracy and PCR efficacy, particularly in complex or mixed samples. The success of this study contributes to the development of a DNA barcode library for flowering plants in Terengganu, supporting biodiversity research and conservation efforts. The optimised protocols presented here may be adapted for other plant taxa, promoting broader applications in plant taxonomy and ecological studies.

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