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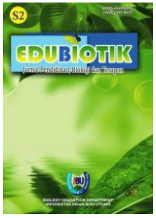

Research Article

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Development of primers amplifying DNA barcoding genes matK and rbcL of Legundi (*Vitex trifolia*)

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ABSTRACT

Legundi (*Vitex trifolia*) is one of the crucial ethno-pharmacological plants. However, the genetic exploration of these plants in Indonesia remains limited. Moreover, a set of primers can be an initial important step to explore the genetics of *V. trifolia* as well as to molecularly identify the species of Legundi. This study aimed to develop the two pairs of primers of maturase-K (matK) and ribulose bisphosphate carboxylase large subunit (rbcL) genes. This type of research is exploratory research using a quantitative approach. The research sampling are the leaves of the *V. trifolia* species, which were collected from Makassar, Indonesia, and the purposive sampling method. The data were obtained through DNA barcoding, and the data were analyzed using bioinformatics analysis. This study found two pairs of developed primers, matK and rbcL, successfully amplified both matK and rbcL target genes of V. trifolia. The newly developed species-specific primers successfully amplified the matK and rbcL genes of V. trifolia, and sequence analysis revealed high similarity values in BLAST and BOLD databases ranging from 99.2 to 100%, with PCR amplification of the matK marker showing particularly high DNA concentration and specificity for species-level identification. This study supports the genetic exploration and identification of useful ethno-pharmacy plants, *V. trifolia*. The conclusion of the study shows that two primer pairs of maturase-K (matK) and ribulose bisphosphate carboxylase large subunit (rbcL) genes have been developed from *V. trifolia* plants. This study supports the genetic exploration and identification of ethnopharmaceutical plants of *V. trifolia* and the role of bioinformatics tools in molecular studies of medicinal plants.

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INTRODUCTION

Vitex trifolia, commonly known as legundi plant, is one of the species of the Vitex genus (Verbenaceae) whose active compounds are commonly used as traditional medicine. *Vitex trifolia* is widely used by various countries in Asia, such as China (Wee et al., 2020), Malaysia (Abas et al., 2020), India (Naikwad & Jeedi, 2021), and Indonesia (Arpiwi et al., 2020; Ikawati et al., 2022) to reduce fever. reduce pain, reduce migraines, treat wounds (anti-bacterial), asthma, and eye pain (Goh, 2024; Nisa et al., 2023). All parts of this plant can be used as medicine, including seeds, roots, stems, flowers, and leaves (Zulkifli et al., 2021). Recently, many studies have been interested in discovering new pharmacological activities of Vitex plants (Kamal et al., 2022). From studies related to the Vitex genus, extracts and fractions from the leaves and fruits of the Vitex genus contain various types of secondary metabolites, including flavonoids, terpenoids, steroids, and iridoids (Bello et al., 2018; Gupta et al., 2022; Mottaghipisheh et al., 2024; Purwitasari et al., 2020) that significantly exhibit pharmacological activities such as anti-cancer, anti-oxidant, and anti-inflammatory (Bao et al., 2018; Ghafari et al., 2022; Liou et al., 2018).

Vitex trifolia is distributed and sold as herbal medicine in Asia under various local names, but the genus Vitex L. is difficult to identify and delineate because it has a high morphology that is unclear and controversial (Salvaña et al., 2019). In Indonesia, *Vitex trifolia* leaves have been demonstrated to possess anti-inflammatory properties, effective against both immunological and non-immunological inflammatory responses (Ikawati, 2022). *Vitex trifolia* extract could enhance cancer treatment efficacy by overcoming chemotherapy resistance (Lubis, 2025). Vitex sp as anti-COVID-19 plants in East Kalimantan (Noor'an, 2021). Legundi leaf essential oil has good potential to be explored further, especially to better understand the chemical activities of its compounds, since it shows toxic properties (Setianingsih et al., 2022). Therefore, it is necessary to carry out molecular identification to identify species that have potential as herbal medicines so that the public can avoid counterfeiting and errors in the use of herbal products (Su'udi et al., 2022).

Recently, DNA barcoding has developed into an effective taxonomic classification technique for species identification. It is considered a promising technique for accurate species identification by effectively using short regions of specific DNA sequences (Bhavana et al., 2021). DNA barcoding is a molecular technique used to identify species using DNA code-based similarities combined with morphological characteristics, which minimizes errors from conventional identification (Wardani et al., 2022). DNA barcoding can be used for the characterization of new species, cryptic species, or unknown species at the species level (Letsiou et al., 2024). Chloroplast DNA (cpDNA) is a specific type of DNA barcode that is commonly used in plants (Sundari et al., 2019). Chloroplast DNA sequences that are likely to be used for DNA barcoding are the genes atpF-H, matK, ndhF, rpoC1, trnH-psbA, rbcL, rpl32-trnL, and rpoB (Paksoy et al., 2022). The matK and rbcL genes have been used as standard coding for plant DNA barcoding. Therefore, in this study, these two genes were selected for DNA coding in *Vitex trifolia* plants.

The matK and rbcL genes can be used to distinguish different types of plant species and subspecies due to sequence variability, including acacia species (Suriani et al., 2021), orchids (Worthy et al., 2022), bamboo (Yong et al., 2024), Cucurbitaceae family (Ho & Nguyen, 2020), and Coelogyne genus (Pratiwi et al., 2023). The matK gene marker has a total sequence length of 1500 bp, and rbcL has about 1400 bp, which has high accuracy for DNA barcoding (Utama et al., 2024). The matK and rbcL genes are crucial in the authentication of medicinal plants, ensuring the right species is used in medicinal ingredients, which is important for conservation and treatment effectiveness (Cahyaningsih et al., 2022). In addition, the combination of matK and rbcL genes can discriminate about 90% of flowering plants (CBOL 5).

However, more conserved genomic regions can be identified and used to detect adulteration of medicinal materials (Wattoo et al., 2016).

The use of appropriate primers is one aspect that can affect the success of DNA barcoding. Primers are short nucleotide or oligonucleotide polymers composed of DNA or RNA that play an important role in the PCR (polymerase chain reaction) process (Salsabila et al., 2021). The main cause of unsuccessful PCR is the selection of primers that have low quality and are not appropriate (Fulghum et al., 2024). Primers are an important component during the amplification process, which consists of forward and reverse primers, where the first step of successful DNA sequencing of a gene is the use of appropriate primer design. Inaccuracy in primer design can cause the resulting PCR product to be non-specific and unable to amplify the entire DNA sequence (Indradewi, 2022). In some studies, it was found that universal primers failed to amplify various groups of species (Bahram et al., 2019; Dahl et al., 2022; Cräutlein et al., 2011). In addition, both primers matK and rbcL failed to discriminate Calligonum species (Kipkiror et al., 2023). In the study Corvalán et al. (2025), the rbcL primer covering more than 99% of the sequence while the matK primer less than 85% despite optimization, thus emphasizing the limitations of universal primers and the need for the development of taxa-specific primers. Several studies reported that no single universal primer set can successfully amplify all land plants, and matK primers, although informative, frequently fail to amplify consistently across taxa without optimization (Yu et al., 2011). Therefore, this study aims to design and evaluate species-specific primers based on the matK and rbcL genes using an in silico DNA barcoding approach, and to assess their effectiveness for accurate species-level molecular identification of *Vitex trifolia*.

RESEARCH METHODS

The research was conducted from August to November 2024 at the Biology Laboratory Research Department of Biology Education, Faculty of Teacher Training and Education, Muhammadiyah University of Surakarta, Indonesia. Data were obtained through DNA barcoding by including several stages such as DNA extraction, PCR, DNA visualization and qualification, and DNA sequencing. Then, the data were processed using bioinformatics analysis, which can be seen in Figure 1.

Plant material in the form of leaves from the *Vitex trifolia* tree was collected from Makassar, Indonesia. The plants are maintained in a controlled greenhouse environment with natural light. The full sequences of *Vitex trifolia* were obtained through the database from NCBI (National Center of Biotechnology Information). In the genomic DNA extraction and DNA concentration stage, the genomic DNA was extracted from 150 g of *Vitex trifolia* leaves. This consideration is carried out based on the criteria of the plants needed in the study, which are the leaves of the Vitex trifolia species with the morphology of three-fingered compound leaves, slightly ovoid, and grayish-green colour (Gentallan et al., 2024). Samples were sterilized with 70% alcohol and grinded it with nitrogen liquid using a pestle and mortar, and extraction was done using a GeneAid plant genomic DNA mini kit. The extracted DNA was quantified using the BioDrop spectrophotometer, which was used for measuring the extracted DNA concentrations in the samples. DNA was visualized using gel electrophoresis on a 1% agarose gel to verify the quality and quantity of the extracted DNA. The DNA was stored at –20°C for subsequent amplification experiments.

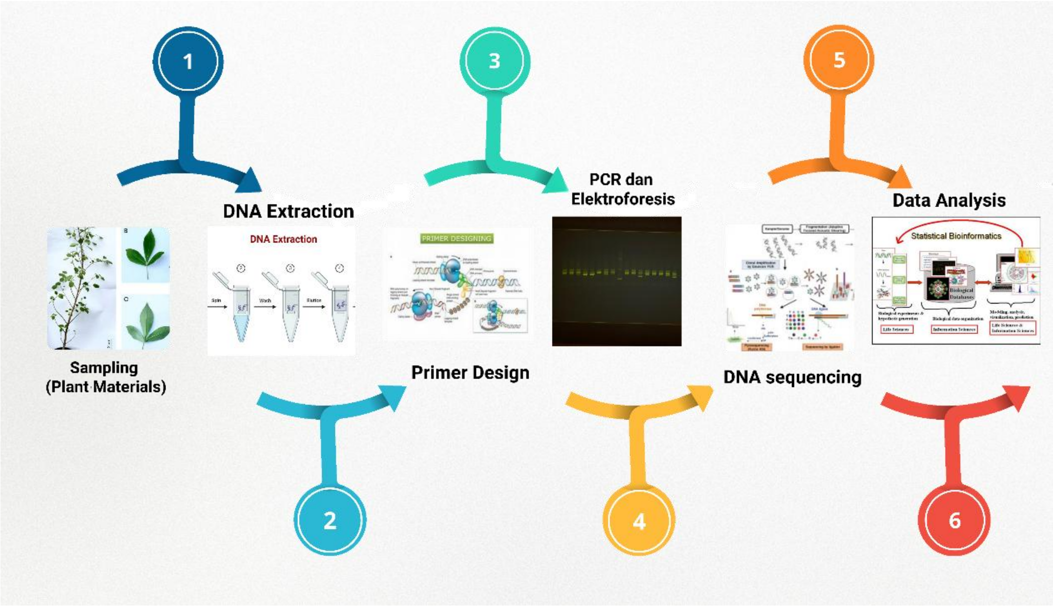


Figure 1. Workflow of DNA Barcoding and Species-Specific Primer Development in *Vitex trifolia*

The primer design stage. The primer design development was carried out by selecting the *Vitex trifolia* matK and rbcL gene sequences from the NCBI database (accession number NC_062602.1), downloaded in FASTA format, and aligned using MEGA version 11 to assess nucleotide similarity and sequence identity (Patel et al., 2020). Forward primers (18–30 bp) were manually designed in the 5'→3' direction, while reverse primers were generated from the terminal region and reverse-complemented using bioinformatics tools (https://www.bioinformatics.org/sms/rev_comp.html). Primer specificity was evaluated in silico by analyzing hairpin formation, melting temperature (Tm), self-annealing, and GC content (Sen et al., 2017) and optimized using the PCR primer stats tool (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) to meet optimal criteria, including primer length of 18–30 bp (Batubara et al., 2024), GC content of 40–60% (Messe et al., 2020), and a maximum Tm difference of 5°C between primer pairs (Praja & Rosalina, 2021).

Polymerase chain reaction (PCR) and DNA visualization stage. PCR was used to amplify the isolated DNA through repeated cycles of denaturation, annealing, and extension (Zhang et al., 2019; Shahzad et al., 2020), and amplification success was evaluated by gel electrophoresis (Dzikrina et al., 2022). Each reaction was prepared in a total volume of 50 µL containing 25 µL MyTaq Red Mix, 22 µL ddH$_2$O, 1 µL of each primer, and 1 µL DNA template. The PCR program for matK was as follows: initial denaturation at 94°C for 300 s; 35 denaturing cycles at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 60 s; and final extension step at 72°C for 300 s. The DNA was visualized by gel electrophoresis using 1% agarose gel. Furthermore, 4 µL of the sample was inserted into the well. After all the samples were put into the wells, 4 µL of DNA ladder was run in electrophoresis at 100 volts for 30 minutes. In the final step, the DNA bands that appeared on the agarose gel 1% under UV light using BluPAD Dual LED Blue/White Light Transilluminator (Bio-Helix).

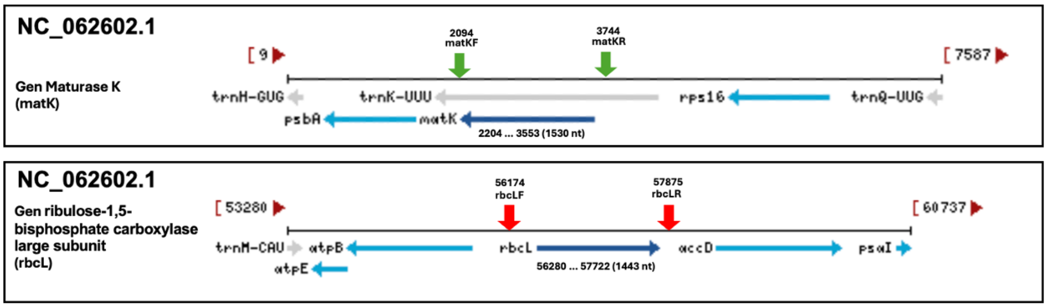
The DNA sequencing stage. DNA fragments obtained after electrophoresis were sequenced at PT Genetics Science Indonesia using the Sanger method. The resulting sequences were assembled into contigs with DNA Baser Assembler and then analyzed using BLAST on the NCBI website (www.ncbi.nlm.nih.gov) to determine sequence similarity with reference data in the database. The data analysis, primer candidates that have been obtained in the previous stage, are tested for primer specifications in-silico using the PCR primer stats tool from the Bioinformatics website (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) to determine the secondary structure of each of the candidate primers that exist. The DNA sequencing products are qontique using DNA baser software. In the contiq process, the ambiguous sequence was cut. The results of the nucleotide sequence were aligned with MEGA version 11, used for the alignment of nucleotides with reference to *Vitex trifolia* sequences that used matK and rbcL gene from NCBI (Budiman et al., 2020). Then the alignment results were analyzed for the highest sequence similarity using the BLAST (Basic Local Alignment Search Tool) tools on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). can find regions that have similarities with other sequences in existing data centers, so it can be known that there is a genetic relationship between sequences with other species or just a coincidence (Budiarsa et al., 2022). In addition, to validate the BLAST results, the data were analyzed using the BOLD website (https://v3.boldsystems.org/index.php/IDS_BlastRequest). BOLD can identify by matching sequences with the most closely related individuals available in the database by generating inter and intraspecific genetic distance graphs, barcode gaps, reconstruction trees, and haplotype distributions (Imtiaz et al., 2017).

FINDING AND DISCUSSION

Based on the results of a DNA sequence search using the NCBI database, one of the *Vitex trifolia* trnK gene sequences was obtained and then downloaded in FASTA format. The length of the V. trifolia trnk gene sequence obtained is around 2,580 bp (base pair), then converted into FASTA format as a template to facilitate the design of primer design manually, where this format is a text-based format to show nucleotide sequences without numbering (Sihotang et al., 2021). In the trnK gene sequence, there is a matK gene that overlaps the intron part of the trnK gene with a size of around 1500 bp.

The matK gene is a gene located within the intron of the trnK gene, where it overlaps with the intron of the trnK gene (Figure 2). MatK is a chloroplast coding gene that is nested between exons 5' and 3' of the trnK (tRNA-lysine) gene (Udensi et al., 2017). To obtain the full-length sequence of the matK gene, a portion of the trnK gene sequence can be used so that the entire matK sequence can be amplified. The length of the trnK gene region overlapping with matK is in the order of 2214 to 3744 bp (1.530 bp). Primer design to get the desired primer candidates previously needed to be done manually. Based on the DNA consensus sequence obtained, the primer candidate was obtained with the Forward and reverse primers (Figure 2). Furthermore, the obtained primer candidates can be tested in silico using PCR primer stats tools from the Bioinformatics website (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) to determine the secondary structure of each candidate primer. The secondary structures observed include melting temperature (Tm), GC count (%), GC clamp, self-annealing, and hairpin formation (Table 1). From these tests, it will be known whether the primer candidates meet the requirements of a good primer so that the resulting primer is specific. Each candidate is given a special naming to make it easier to recognize each primer, namely the forward primer with the letter symbol “F” and the reverse primer with the letter symbol “R”.

The results of the test show that the primer candidate that meets the criteria of a good primer is the matK primer candidate (forward and reverse), where a good primer meets the criteria of the prime parameter (Aulia et al., 2023). Good primer parameters include primer lengths ranging from 18-30 bp, g 40-60% guanine (G) and cytosine (C) bases, and the melting temperature (Tm) between the forward and reverse primers is not too far apart (Nuryady et al., 2025). Meanwhile, the rbcL primer candidate obtained low GC content results, namely 35% and 25%, where the amount is <40 - 60% as the ideal limit of the amount of guanine (G) and cytosine (C) contained in a primer to ensure the stability of the bond between the primer and the template (Suwarny et al., 2025). A low amount of GC content can reduce primer specificity because it leads to a low optimal annealing temperature (So et al., 2020). In addition, unspecific and too low primer annealing temperatures can lead to amplification of unwanted DNA segments (Mubarak et al., 2020).



The full length *mat*K gene was amplified using primers matKF (5' TGTCCCCGAGGTATCTATTCTTAC 3') and matKR (5' CATTGCACACGGCTTTCCCTA 3').

The full length *rbc*L gene was amplified using primers rbclF 5' TTGTGAAAGATTCCTGTGAA 3' rbclR 5' TTCTATTTCCAATCTTCTAT 3'

Figure 2. The Developed Primers of matK and rbcL of *Vitex trifolia* based on Full-Length Mitochondrial Sequences

Table 1. The Output of Primer-Stats for Sequence

No	Accession No	Primers	Name	Length (bp)	Melting Temperature (Tm ℃)	GC Content (%)	GC Clamp	Self-Annealing	Hairpin Formation
1	NC_062602.1	matK	F	24	63.87	45.83	Pass	Pass	Pass
	NC_062602.1	matK	R	21	65.80	52.38	Pass	Pass	Pass
2	NC_062602.1	rbcL	F	20	57.95	35.00	Pass	Pass	Pass
	NC_062602.1	rbcL	R	20	51.41	25.00	Pass	Pass	Pass

Visualization of electrophoresis results from Vitex trifolia DNA extraction using 1% agarose gel with staining using Florovue gel stain that allows DNA to fluoresce when illuminated with UV light. DNA bands were compared with a DNA ladder (Figure 3) and showed fragment sizes of approximately 10,000 bp. DNA concentration was measured with a NanoDrop spectrophotometer, giving a value of 100 µg/mL for the Makassar sample, a method widely recognized for accurately assessing DNA concentration and purity (García-Alegría et al., 2020). Three main factors affect the success of the DNA extraction process, namely the quality of the plant tissue used, the quantity of plant tissue, and the technique of destroying plant tissue (Nugroho et al., 2019). Good DNA quality obtained from extraction results is a basic requirement that must be met in molecular studies (Jayanti & Mushlih, 2021). Good DNA quantity can affect the success of subsequent DNA barcoding steps such as PCR, DNA sequencing, and electrophoresis (Sittenthaler et al., 2023).

Vitex trifolia DNA sequences were tested for concentration and purity by PCR using both candidate primers, namely matK and rbcL, obtained DNA amplification results that have been visualized by electrophoresis using 1% agarose gel (Figure 4) proving that the DNA was successfully amplified by looking at the presence of fluorescent DNA bands and measuring more than 1500 bp (Anissa et al., 2023). The size of the DNA band formed can determine the high and low concentration of the DNA sample, the thicker the DNA band produced, the higher the concentration and vice versa (Setiati et al., 2020). In addition, good quality DNA looks thick, solid, and has no smears (Ramlan et al., 2024). In the amplification results of the two primer candidates, it is known that both have high DNA concentrations and sizes ranging from 1500 to 2000 bp, but the quality of the DNA is low, marked by smears. Smears that appear at the electrophoresis stage can be caused by degradation or contamination of the DNA sample (Kusuma, 2022).

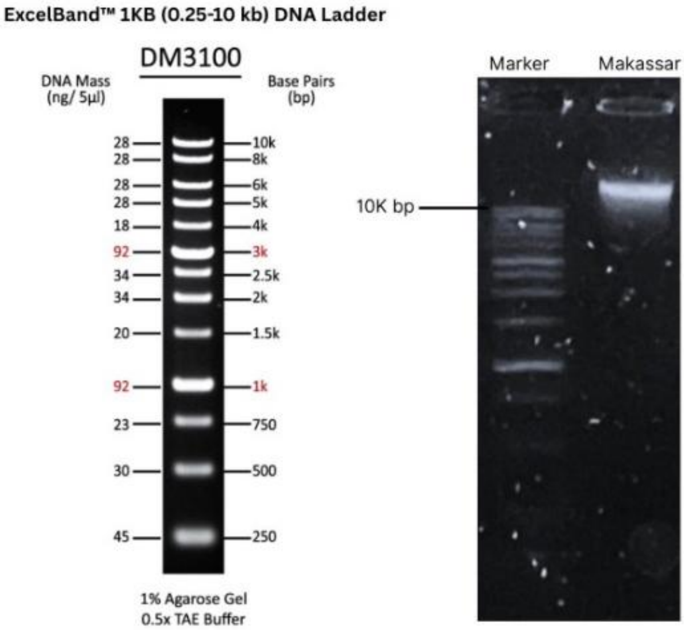


Figure 3. The Result of gDNA Extraction

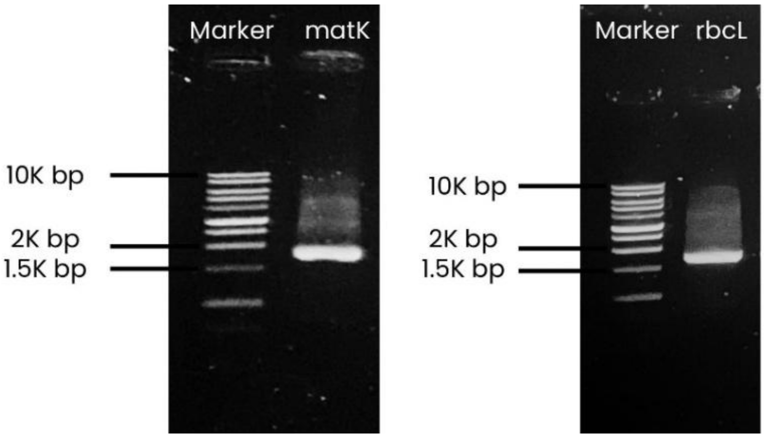


Figure 4. The Amplification Result of *Vitex trifolia* matK and rbcL Genes

Considering the quality of the matK primer against the in-silico test and the amplification results showing thick DNA bands and thinner smears than the rbcL primer, the matK gene will be used as the candidate primer tested in the next stage. *Vitex trifolia* matK gene sequences were tested using BLAST tools on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with various parameters such as species name, max score, total score, etc (Table 2). To determine the similarity/compatibility of a DNA sequence with the available database, the percent identity value is used; the higher the percent identity, the more significant the match, so that it can estimate biological similarity (Samal et al., 2021). In the table, the highest percent identity is 100.00% in the species *Vitex rotundifolia*, *Vitex trifolia*, and *Vitex bicolor*. So from these results, it is known that the matK primer successfully amplified the *Vitex trifolia* sequence with a percent identity of 100.00%. The BOLD result details of the *Vitex trifolia* matK sequence can be seen in Table 3.

Table 2. NCBI Blast Result Detail of *Vitex trifolia matK* Sequence

No	Species	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
1	*Vitex rotundifolia*	3330	3330	100%	0.0	100.00%	154446	OQ942922.1
2	*Vitex trifolia*	3330	3330	100%	0.0	100.00%	154472	ON711030.1
3	*Vitex trifolia*	3330	3330	100%	0.0	100.00%	154444	NC_062602.1
4	*Vitex bicolor*	3330	3330	100%	0.0	100.00%	154460	NC_065871.1
5	*Vitex rotundifolia*	3319	3319	100%	0.0	99.89%	154370	NC_05091.1
6	*Vitex negudo var cannabifolia*	3319	3319	100%	0.0	99.89%	154470	OP581016.1
7	*Vitex negudo*	3319	3319	100%	0.0	99.89%	154496	PP584504.1
8	*Vitex negudo*	3319	3319	100%	0.0	99.89%	154438	NC_057235.1
9	*Vitex tripinnata*	3319	3319	100%	0.0	99.89%	154166	MT473785.1
10	*Vitex negudo var cannabifolia*	3314	3314	100%	0.0	99.83%	154520	PQ541021.1

Table 3. The BOLD Result details of the *Vitex trifolia matK* Sequence

No	Phylum	Class	Order	Family	Genus	Species	Score	Similarity
1	Tracheophyta	Magnoliopsida	Lamiales	Lamiaceae	*Vitex*	*trifolia*	1298	99.92
2	Tracheophyta	Magnoliopsida	Lamiales	Lamiaceae	*Vitex*	*rotundifolia*	1296	99.85
3	Tracheophyta	Magnoliopsida	Lamiales	Lamiaceae	*Vitex*	*negundo*	1294	99.77
4	Tracheophyta	Magnoliopsida	Lamiales	Lamiaceae	*Vitex*	*Agnus-castus*	1294	99.77
5	Tracheophyta	Magnoliopsida	Lamiales	Lamiaceae	*Vitex*	*Agnus-castus*	1294	99.77
6	Tracheophyta	Magnoliopsida	Lamiales	Lamiaceae	*Vitex*	*glabrata*	1264	98.62
7	Tracheophyta	Magnoliopsida	Lamiales	Lamiaceae	*Vitex*	*quinata*	1264	98.62
8	Tracheophyta	Magnoliopsida	Lamiales	Lamiaceae	*Vitex*	*limonifolia*	1235	97.62
9	Tracheophyta	Magnoliopsida	Lamiales	Lamiaceae	*Vitex*	*carbuncolorum*	1235	97.62
10	Tracheophyta	Magnoliopsida	Lamiales	Lamiaceae	*Vitex*	*canescens*	1232	96.97

Furthermore, the same sequences and primer candidates were tested again using a different website known as BOLD (https://v3.boldsystems.org/index.php/IDS_BlastRequest). In the test, there are parameters that can be observed, including phylum, class, order, family, genus, species, score, and similarity (Table 3). When compared to the BLAST Bioinformatics tool, the BOLD system is easier to analyze for sequence similarity with the database and is more specific in taxonomy. The similarity percentage for identifying the *Vitex trifolia* matK gene sequence showed a similarity of around 99.92% with the Vitex trifolia species. Thus, it is known that the matK primer candidate successfully amplified the *Vitex trifolia* sequence. The similarity percentage of the *Vitex trifolia* matK gene sequence from both BLAST Bioinformatics and BOLD websites was successful in identifying up to the species level with a percentage of 99.92-100%, so the matK primer candidate was specific in amplifying the *Vitex trifolia* sequence. This finding is consistent with previous studies, where Wei et al. (2024) reported that amplification with the designed matK primer was more successful. Korotkova et al. (2011) observed successful matK amplification in Cactaceae, while Sahin et al. (2025) noted moderate performance for matK and lower reliability for rbcL in species-level resolution. Mishra et al. (2022) indicated that the matK gene exhibits higher sequence variability than the rbcL region. Overall, the findings demonstrate that the matK primers developed in this study exhibit higher reliability and specificity than the rbcL primers for molecular identification of *Vitex trifolia*, thereby supporting the use of matK as an efficient DNA barcode for genetic exploration and accurate species-level identification of ethnopharmacologically important plants.

CONCLUSION

The selection of specific primers is a success factor for DNA barcoding, especially in the PCR (polymerase chain reaction) stage. The specific primer must meet the criteria as a good primer so that it is optimal in amplifying the target DNA sequence. In this study, it is known that primer candidates that qualify as good primers are matK primer candidates consisting of forward primers (5 'TGTCCCCGAGGTATCTATTCTTAC 3') and reverse primers (5 'CATTGCACACGGCTTTCCCTA 3'). The results demonstrate that the designed matK primers show higher reliability and specificity than the rbcL primers for molecular identification of this species. Successful amplification and high sequence similarity (99.92-100%) confirmed the effectiveness of the matK marker in achieving species-level resolution. These findings imply that the matK primer can serve as a reliable molecular tool for accurate identification and genetic exploration of *Vitex trifolia*, supporting future studies in ethnopharmacology, biodiversity assessment, and conservation of medicinal plants.

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