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Potential DNA Barcoding for Identification of Large Leaf Homalomena (*Homalomena pendula* (Blume) Bakh.f.)

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Le Nguyen Thoi Trung[1,2], Hoang Tan Quang[3], Truong Thi Bich Phuong[1], Tran Nam Thang[4,*]

1. University of Sciences, Hue University, 77 Nguyen Hue Str, Hue City, Vietnam
2. The Coastal of Nature Museum, Xuan Thuy, Vi Da, Hue City, Vietnam
3. Institute of Biotechnology, Hue University, Nguyen Dinh Tu Str., Hue city, Vietnam.
4. University of Agriculture and Forestry, Hue University, 102 Phung Hung Str., Hue city, Vietnam

* trannamthang@huaf.edu.vn

ABSTRACT

Background: *Homalomena* is a genus of the Araceae family, which consists of many medicinal plants used in Vietnamese traditional medicine. However, this genus shows relatively similar morphological characteristics across its species, so classification by morphological comparison has several limitations, and the scientific names and classifications of some species remain controversial. *H. pendula* is an endangered medicinal herb, highly valued for its tonic, analgesic, and anti- inflammatory properties, but wild populations are threatened by overharvesting. In addition, the DNA barcodes for *H. pendula* have not been well studied. Thus, developing DNA barcodes for *H. pendula* is necessary to identify and conserve this species.

Methods: This study used four DNA barcodes (*trnL-trnF, rbcL, trnH-psbA,* and *trnQ- rps16*) to determine the most effective DNA barcode sequences for distinguishing *H. pendula*.

Results: Among 4 *Homalomena* species, one SNP was found in the *trnL-trnF* sequence at position 16 (A > G), and three SNPs were found in the *rbcL* sequence at positions 126 (C > T), 272 (G > A), and 278 (T > A). Five different *H. pendula* samples collected from Hue city, Vietnam, distributed in a separate branch from *trnL-trnF* or *rbcL* on the phylogenetic tree. Both *trnH-psbA* and *trnQ-rps16* fragments have lots of changes in nucleotide sequences, however, these are random differences and are not significant in *H. pendula* identification, only useful for genetic diversity studies.

Conclusion: Based on the nucleotide sequences and phylogenetic tree analysis, the results show that *trnL- trnF* and *rbcL* markers can be used to distinguish *H. pendula* from other closely related species.

INTRODUCTION

The large leaf Homalomena (LLH-*Homalomena pendula* (Blume) Bakh.f.), commonly known as *Homalomena gigantea* Engl., belongs to the genus *Homalomena* , a large genus within the Araceae family consisting of approximately 150 species worldwide [1]. This species is a fairly large herbaceous plant; leaf blade up to 50 cm long; large spathe, longer than 10 cm. *Homalomena pendula* roots have long been popular as a digestive stimulant, rheumatism treatment, and for anti-inflammatory and tonic purposes [2]. This medicinal plant is currently facing overexploitation by residents for therapeutic purposes, leading to its depletion. This species was listed in the *Vietnam Red Book* (2007) at an endangered level (VU Alc, Bl+2b,c) due to its limited distribution range. The progressively shrinking distribution of this species poses a risk of extinction unless effective conservation measures are implemented. In Hue city, Vietnam, natural populations of *Homalomena pendula* species have been found at Phong Dien, A Luoi, Phu Loc districts, where they grow naturally in the forest with different morphology characteristics [3].

Homalomena spp. species have relatively similar morphological characteristics, so classification by the morphological comparison method has several limitations. DNA barcode analysis helps to increases the accuracy. Most plant DNA barcodes are found in the chloroplast genome, either within coding sequences (such as *rbcLa* and *matK*) or in intergenic regions (such as *trnL-trnF* or *trnH-psbA*). Additionally, some nuclear loci, like the non-coding internal transcribed spacer of ribosomal DNA (ITS), have also been utilized as DNA barcodes. Typically, multiple barcodes per plant individual are sequenced and employed for taxonomic assignments [4]. For *Homalomena* species, several DNA barcodes were studied and deposited to GenBank (NCBI database), such as *matK*, *trnL-trnF*, *rbcL*, *trnH-psbA*, and *trnQ-rps16*. However, only the DNA barcodes for several *Homalomena* species were reported; the DNA barcode for *H. pendula* was limited.

The present study aimed to determine the most effective DNA barcode sequences for *Homalomena* species by sequencing four DNA barcodes (*trnL-trnF, rbcL, trnH-psbA,* and *trnQ-rps16*) from five different samples collected in natural habitats at Hue city, Vietnam.

METHODS

Materials

The young leaves of *Homalomena pendula* were collected from the natural forest of A Luoi and Phong Dien districts, Hue city, Vietnam in 2023-2024 (Table 1). Fresh leaf samples were stored in sealed nylon bags at a cool temperature for subsequent DNA extraction.

Molecular maker to identify the *Homalomena pendula* species

Whole genomic DNA of LLH samples was extracted using the TopPURE Plant DNA Extraction Kit (ABT, Vietnam) following the standard protocol. Extracted DNA was quantified and stored at -20°C [5]. The universal primers are used to amplify the region (Table 2).

Polymerase chain reaction (PCR) was conducted in a total volume of 50 µL, consisting of 100 ng of DNA, 20 pg of each primer, 25 µL of 2× Go Taq® Green Master Mix (M7502, Promega, USA), and distilled water to reach the final reaction volume. The amplification protocol included an initial denaturation step at 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute, concluding with a final extension at 72°C for 10 minutes (SimpliAmp, ThermoFisher Scientific, USA).

The PCR products were analyzed using electrophoresis on a 1% agarose gel containing SafeView Classic Nucleic Acid Stains (abm, Canada). Gel images were visualized using an Ultra Slim LED Illuminator and analysed by a standard DNA ladder (GeneRuler 1kb DNA Ladder, #SM0313). Purified products were sent for sequencing in both forward and reverse directions to a DNA sequencing company in Can Tho, Vietnam.

Sequencing results were analysed by Bioedit (v7.2.5) and MEGA11 software. The similarity and coverage of the DNA sequences were evaluated by comparing them to the sequences available in NCBI's NR-NT database using the BLAST tool on GenBank (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/blast.cgi) with default parameters. *Philodendron hederaceum*

and *Aglaonema costatum* were used as outgroups (Table 3).

Phylogenetic tree construction

MEGA 11 software was utilized to align the sequences using the MUSCLE algorithm and to construct a phylogenetic tree. Phylogenetic tree analysis of each gene and fragments (*trnL-trnF, rbcL, trnH-psbA,* and *trnQ-rps16*) was created by Maximum Likelihood (ML). The confidence level of the phylogenetic tree was assessed using 1,000 replications [9].

RESULTS

BLAST-based identification

In this study, we use 4 gene fragments to identify 5 samples of *Homalomena pendula* from Hue city, Vietnam (the *trnL-trnF, rbcL, trnH-psbA,* and *trnQ-rps16* intergenic spacer regions). Genomic DNA was successfully isolated from all five different samples and amplified for four barcode regions (Table 1). The nucleotide sequences, along with all BLAST results for Homalomena, were aligned and trimmed to the same length.

With the *trnL-trnF* fragment, five samples were amplified and sequenced, and the 5 sequences were 100% identical, with no differences found between the 5 samples. These sequences were 99.43% similarity to the *trnL-trnF* intergenic spacer region of *H. pendula* (GenBank accession number: KU727660.1, with the coverage length of 340 bp), 98.62% similarity with *H. occulta* (NC_054336.1). In the retained total nucleotide alignment (929 bp), 3 single- nucleotide polymorphisms (SNPs) were found between 6 *Homalomena* species, including sites: 16 (A > G), 224 (C > A in *H. tenuispadix*), and 330 (deletion of G in several species) (Figure 2A). Similarly, five sequences of the *rbcL* gene fragment were 100% identical. These sequences were 99.65% similar to the *rbcL* partial gene of *H. occulta* (NC_054336.1), 99.63% of *H. aromatica* (MW091545.1). In the 570 bp of coverage sequence, 3 SNPs were found between 4 *Homalomena* species, including sites: 126 (C > T in *H. magna*), 272 (G > A), and 278 (T > A) between *H. pendula* and others (Figure 2B).

In contrast, in the nucleotide sequence of *trnH-psbA* fragments, 22 different sites were found between 5 samples, 21 of them belong to TNK2 and one belongs to TNK6. The sequences of TNK1, TNK2, and TNK6 shared 92.35%, 92.09%, and 91.93% similarity, respectively, with the *trnH-psbA* sequence of *H. occulta* (NC_054336.1). The coverage sequence (817 bp) has 4 main different sequences between samples, including nucleotide sequences from 52-58, 295-308, 457-473, and 515-545 (Figure 2C). Only three *trnQ-rps16* fragments from five samples were amplified and sequenced (TNK2, TNK5, and TNK6); three sequences were 100% identical. These sequences were 98.73% similarity to the *trnQ-rps16* sequence of *H. occulta* (NC_054336.1), 96.71% with *H. deltoidea* (MH089240.1), 95.73% with *H. rubescens* (MH089243.1), and 95.55% with *H. aromatica* (MH089239.1). In the coverage sequence (1352 bp), 4 main different sequences were found between species, including nucleotide sequences from 34-38, 214, 669-680, and 1273-1319 (Figure 2D).

Phylogeny-Based Identification

A maximum likelihood tree was constructed and visualized as a rooted cladogram based on the aligned sequences (Figure 3). For the *rbcL* partial gene fragment, five samples of *H. pendula* were distributed in a separate branch of the phylogenetic tree. The *H. pendula* species was clearly identified with other *Homalomena* species with a bootstrap value of 90% (Figure 3B). Similarly, five samples of *H. pendula* were also located in a branch based on the *trnL-trnF* region with a bootstrap value of 64% (Figure 3A). The *H. pendula* species was also clearly identified with other *Homalomena* species based on *trnQ-rps16* sequences with a bootstrap value of 52%. However, only 3/5 samples were amplified and sequenced with *trnQ* and *rps16* primers (Figure 3D). In contrast, *trnH-psbA* region not suitable for *H. pendula* identification, *H. pendula* and *H. occulta* present in the same branch (Figure 3C).

Figures

Figure 1: Morphology of Homalomena pendula.

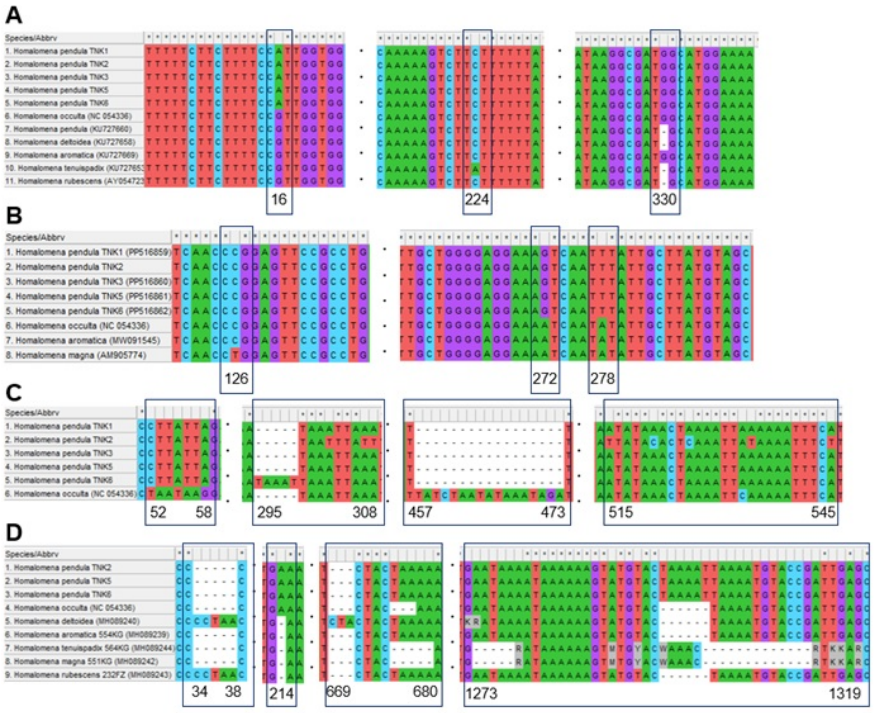

Figure 2: Main differences in nucleotide sequences between Homalomena pendula and other Homalomena species. A. trnL-trnF, B. rbcL, C. trnH-psbA, D. trnQ-rps16.

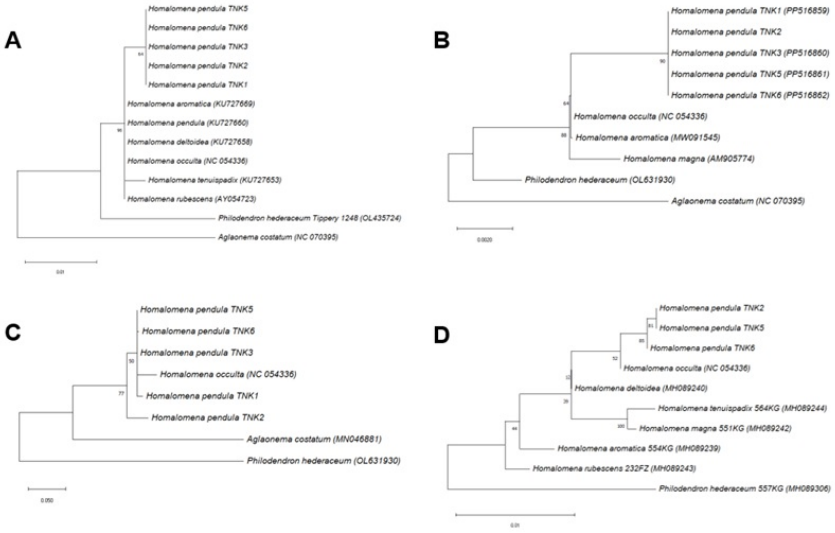

Figure 3: Main differences in nucleotide sequences between Homalomena pendula and other Homalomena species. A. trnL-trnF, B. rbcL, C. trnH-psbA, D. trnQ-rps16.

Tables

Samples	Districts	Coordinates
TNK1	A Luoi	16°09'24''N; 107°41'02''E
TNK2	A Luoi	16°19'11''N; 107°12'46''E
TNK3	A Luoi	16°08'09"N; 107°48'15"E
TNK5	Phong Dien	16°24'54" N; 107°11'55"E
TNK6	Phong Dien	16°26'59"N; 107°14'46"E

Table 1: Sampling sites of Homalomena pendula.

Regions	Primer names	Primer sequences	References
trnL-trnF	UniC UniF	CGAAATCGGTAGACGCTACG ATTTGAACTGGTGACACGAG	[6]
rbcL	1F 724R	ATGTCACCACAAACAGAAAC TCGCATGTACCTGCAGTAGC	[7]
trnH-psbA	psbA trnH	GTTATGCATGAACGTAATGCTC CGCGCATGGTGGATTCACAATCC	[8]
trnQ-rps16	trnQ rps16	GCGTGGCCAAGYGGTAAGGC GTTGCTTTYTACCACATCGTTT	[7]

Table 2: Details of primer pairs used to amplify the gene fragments.

Samples/species	*trnL-trnF*	*rbcL*	*trnH-psbA*	*trnQ-rps16*
TNK1	This study	PP516859	This study	nd
TNK2	This study	This study	This study	This study
TNK3	This study	PP516860	This study	nd
TNK5	This study	PP516861	This study	This study
TNK6	This study	PP516862	This study	This study
Homalomena occulta	NC_054336	NC_054336	NC_054336	NC_054336
Homalomena pendula	KU727660	-	-	-
Homalomena deltoidea	KU727658	-	-	MH089240
Homalomena aromatica	KU727669	MW091545	-	MH089239
Homalomena tenuispadix	KU727653	-	-	MH089244
Homalomena magna	KU727614	AM905774	-	MH089242
Homalomena rubescens	AY054723	-	-	MH089243, LT996286
Philodendron hederaceum	OL435724	OL631930	OL631930	MH089306
Aglaonema costatum	NC_070395	NC_070395	MN046881	-

Table 3: DNA barcodes used to construct a phylogenetic tree. nd: none PCR product detected.

DISCUSSION

In recent decades, DNA barcoding has become a widely used tool for herb identification, promoting safety and innovation in the field of herbal medicine [10]. DNA barcodes are either organelle or nuclear loci that exhibit a high level of conservation at the species level. By comparing newly sequenced DNA barcodes to reference databases, researchers can accurately assign an unknown biological sample to its correct taxonomic classification [4]. Standard and high-species coverage DNA barcode reference libraries have been developed to offer reference sequences for species identification, thereby enhancing the accuracy and reliability of species discrimination based on DNA barcoding [10]. Both single loci and multiple loci have been extensively used, offering sufficient resolution for the identification of most herbs. While it is currently the primary method for molecular identification, it still has some limitations. Taking the morphology, physical, and chemical properties of the species into account can overcome the deficiency of conventional DNA barcodes for the identification of closely related species [10].

Following a comprehensive inventory of gene regions in the mitochondrial, plastid, and nuclear genomes, the nuclear ITS region and the chloroplast genes rbcL and/or matK have emerged as the preferred standard DNA barcodes. The Consortium for the Barcode of Life (CBOL) has recommended these as a standard two-locus barcode for global plant databases due to their combined effectiveness in species discrimination [11]. For *Homalomena* species, using DNA barcodes was studied, such as ITS, *matK*, *trnL-trnF*, *rbcL*, *trnH-psbA*, and *trnQ-rps16* [12-14].

Based on the nucleotide sequences analysis, the results show that *trnL-trnF* and *rbcL* markers are effective in distinguishing *H. pendula* from other species. Four SNPs were found between *H. pendula* with other species, including one SNP at position 16 (A > G) in the *trnL-trnF* sequence and three SNPs at positions 126 (C > T in *H. magna*), 272 (G > A), and 278 (T > A) in the *rbcL* sequence (Figure 2). Both *trnH- psbA* and *trnQ- rps16* fragments have lots of changes in nucleotide sequences, however, these are random differences and have no significant impact on *H. pendula* identification. The *trnH-psbA* and *trnQ-rps16* markers are useful for genetic diversity studies.

Using the phylogenetic tree, *H. pendula* samples were distributed in a separate branch from *trnL-trnF* or *rbcL*, similar to the results of the sequencing analysis. Thus, *trnL-trnF* or *rbcL* sequences are suitable for *H. pendula* identification (Figure 3).

In previous literature, rbcL was established as the standard marker for barcoding plants due to its effectiveness for PCR amplification and sequencing. However, this region evolves slowly and exhibits the lowest divergence among flowering plants, limiting its discriminatory power primarily to the family and genus levels. Despite these limitations, rbcL has still been suggested as one of the best candidate barcodes [11]. In contrast, the trnL-trnF intergenic spacer and trnL intron have been utilized effectively for both intra- and interspecific analyses, as well as for assessments at the subfamilial and tribal levels [15]. The *trnL- trnF* intergenic spacer, which separates the second exon of the trnL(UAA) gene from the exon of the trnF(GAA) gene, exhibits remarkable length variation in angiosperms. [16,17]. Van et al. [13] used two DNA markers (*trnL* intron and *trnL-trnF* sequences) to distinguish *H. occulta* and *H. pierreana*. The comparison of the *trnL* intron and *trnL-trnF* regions proved that *H. occulta* and *H. aromatica* were two distinct species.

TrnH-psbA is among the most commonly used plastid markers. It displays significant sequence divergence and has elevated rates of insertion and deletion [11]. Although *trnH-psbA* potential use as a second-tier marker after the *rbcL* gene, this marker is not suitable for *H. pendula* identification in our study.

Based on the nucleotide sequences and phylogenetic tree analysis, *trnL-trnF* and *rbcL* markers can be used to distinguish *H. pendula* from other species. Four SNPs were found between *H. pendula* and other *Homalomena* species, including one SNP at position 16 (A > G) in the *trnL-trnF* sequence and three SNPs at positions 126 (C > T), 272 (G > A), and 278 (T > A) in the *rbcL* sequence. In the phylogenetic trees constructed from *trnL-trnF* and *rbcL* sequences, *H. pendula* samples are distributed in a separate branch from *trnL-trnF* or *rbcL* on the phylogenetic tree.

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

Conceptualization: Le Nguyen Thoi Trung, Truong Thi Bich Phuong, Methodology: Le Nguyen Thoi Trung, Hoang Tan Quang, Formal analysis and investigation: Le Nguyen Thoi Trung, Hoang Tan Quang, Writing – original draft preparation: Le Nguyen Thoi Trung, Hoang Tan Quang; Writing – review and editing: Truong Thi Bich Phuong, Tran Nam Thang, Supervision: Tran Nam Thang. All authors have read and agreed to the published version of the manuscript.

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