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COMPARISON OF *rbc*L AND *trn*H-*psb*A DNA BARCODES IN DIVERSE *CAMELLIA* SPECIES COLLECTION IN VIETNAM

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SUMMARY

Camellia is a large genus in the tea family of Theaceae. In this genus, several species serve different purposes, such as, medicinal and ornamental plants and beverage production. Thus, country-wide cultivation of various species of *Camellia* genus sought to alleviate poverty and promote economic development in many regions. However, studies on evaluating its genetic resources as a foundation for the conservation and development of this plant are yet to start. Currently, using DNA barcoding often serves as a highly reliable approach to identifying and characterizing numerous plants. In the presented study, a total of 10 *Camellia* accessions collected from Dong Bua village, Tam Quan commune, District Tam Dao, and Vinh Phuc province, Vietnam, underwent study during 2020–2022 at Ho Chi Minh City University of Food Industry, Vietnam. Evaluation of two DNA barcoding regions, namely, *rbc*L and *trn*H-*psb*A, transpired for their ability to distinguish the *Camellia* accessions belonging to different species in Vietnam. The results revealed a significant difference in the DNA sequences of the *rbc*L and *trn*H-*psb*A regions among the *Camellia* species. In addition, the *trn*H-*psb*A barcode region also showed higher effectiveness versus the *rbc*L region in recognizing various species of *Camellia*. The results authenticated the potential of DNA barcoding in the management, conservation, and development of the genetic resources of *Camellia* in Vietnam.

Keywords: *Camellia* species, DNA barcode, identification, *rbc*L, *trn*H-*psb*A

Key findings: DNA barcodes were found as efficient tools for plant identification; however, the accuracy depends upon the utilized barcode regions. In this study, the *trn*H-*psb*A proved superior over *rbc*L for differentiating *Camellia* species.

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INTRODUCTION

The *Camellia* genus includes many species with different commercial values, such as, medicine, ornamentation, beverage, and cooking oil from seed extract. Although the plants can be found in many parts of the world, the species are most common in East and Southeast Asia regions (Lu *et al.,* 2012). *Camellia* species cultivation on a large scale provides raw materials for producing herbal drinks and processing products for preventing high blood pressure, cardiovascular diseases, diabetes, and tumors (Bhatt *et al.,* 2010; Lin *et al.,* 2013; Yoon *et al.,* 2017; Zhao *et al.,* 2022). With several uses and economic benefits, expanding growing *Camellia* enhances its production in Vietnam (Nguyen *et al.,* 2015) and worldwide (Soni *et al.,* 2015; Batta and Rajput, 2021).

A recent study showed 42 *Camellia* species in Vietnam, of which 32 are endemic to the country (Le and Luong, 2016). However, the research on *Camellia* in Vietnam focuses mainly on its propagation (Nguyen and Nguyen, 2017). Accurate identification and classification are vital for the assessment, conservation, and sustainable use of genetic resources in plants, in general, and *Camellia,* in particular. Traditionally, classifying this genus is mainly based on morphological characteristics, influenced by environmental conditions and plant growth stages, leading to poor classification and characterization (Hoi *et al.,* 2020; Azka *et al*., 2021).

Recently, molecular markers' application assessed genetic diversity in the tea family using markers, such as, RAPD and DNA barcode (Nguyen *et al.,* 2018). A DNA barcode is one of the molecular markers widely used in plants. Several genomic regions have been used as barcodes for plant taxonomic studies, such as, ITS, *mat*K, *rbc*L, *atp*F-*atp*H, *psb*K-*psb*I, and *trn*H-*psb*A (CBOL Plant Working Group*,* 2009). The current use of this method serves the taxonomy purpose, biodiversity assessment, and genetic resources' conservation. The barcode regions *rbc*L and *trn*H-*psb*A have been extensively used in differentiating plant species and cultivars (Skuza *et al.,* 2019) since *in silico* study supported that the combination of these two markers increases discriminating power among related land plant species (Kress and Erickson, 2007). Furthermore, *rbc*L is a suitable maker for identifying plants in variable conditions (Trujillo-Argueta *et al.,* 2022), and *trn*H-*psb*A is one of the most variable plastid regions and easy to amplify through PCR reactions in different land plants (Balkanska *et al.,* 2020).

Compared with traditional methods, molecular markers are easier to perform under laboratory conditions, giving fast results and high accuracy. Therefore, molecular markers become an effective tool to accurately assess the genetic diversity of medicinal plants to select and conserve the desirable plant species that better serve researchers for breeding purposes. The presented study aimed to evaluate the efficiency of two DNA barcoding regions, namely *rbc*L, and *trn*H-*psb*A, in discriminating common tea species collected in Vietnam.

MATERIALS AND METHODS

Genetic material

Collecting a total of 10 *Camellia* accessions with specific characteristics occurred from gardens in different areas, i.e., Dong Bua village, Tam Quan commune, Tam Dao district, and Vinh Phuc province, Vietnam, then studied during 2020–2022 at Ho Chi Minh City University of Food Industry, Vietnam. (Table 1). Preliminary identification of the *Camellia* accessions was according to the combination of the common names of the tea accessions in the various localities and the morphological traits, as described by Lu *et al.* (2012) and Le (2018). After collection, all the accessions were stored in a cool place before DNA extraction.

Methods

The DNA extraction from *Camellia* leaves used TopPURE® Plant DNA extraction KIT (ABT, Vietnam), according to the manufacturer's procedure. After extraction, the DNA quality check proceeded with electrophoresis on 1% agarose, using a spectrometer (Optima SP

Table 1. Molecular identification of 10 *Camellia* accessions based on *rbc*L and *trn*H-*psb*A barcode regions.

Sample ID	Morphological identification	Identification using NCBI BLAST with *rbc*L or *trn*H-*psb*A	Identification using BOLD with *rbc*L
BC	*Camellia* sp.	*Camellia* sp.	*Phlox longifolia*
CP	*Camellia cucphuongensis*	*Camellia* sp.	*Diospyros macrocarpa*
DO	*Camellia* sp.	*Camellia* sp.	*Diospyros macrocarpa*
LO	*Camellia hirsuta*	*Camellia* sp.	*Phlox longifolia*
NI	*Camellia nitidissima*	*Camellia* sp.	*Diospyros macrocarpa*
PE	*Camellia petelotii*	*Camellia* sp.	*Diospyros macrocarpa*
PH	*Camellia phanii*	*Camellia* sp.	*Phlox longifolia*
TD	*Camellia tamdaoensis*	*Camellia* sp.	*Phlox longifolia*
TI	*Camellia tienii*	*Camellia* sp.	*Phlox longifolia*
TY	*Camellia euphlebia*	*Camellia* sp.	*Phlox longifolia*

3000 nano UV-VIS, Japan) to determine the DNA concentration. Then, storing the DNA samples in a freezer was at -20 °C until usage for PCR reactions.

The two barcoded regions amplified by primer pairs had the following sequences: *rbc*L-F 5'- TGAAAACGTGAATTCCCAACCGTTT ATGCG-3'; *rbc*L-R: 5'- GCAGCAGCTAGTT CCGGGCTCCA-3' (Hasebe *et al.,* 1994); *trn*H-*psb*A-F: 5'-CGCGCATGGTGGATTCACAATCC-3'; *trn*H-*psb*A -R: 5'- GTTATGCATGAA CGTAATGCTC-3' (Costion *et al.,* 2011). The PCR reaction consisted of the following components: 12.5 µL 2X Mytaq Mix (Bioline, UK), 100 ng DNA, 1.7 µM per primer, and adding PCR water (Sigma-Aldrich, USA) to a final volume of 24 µl. The PCR mixture was run in a SureCycler 8800 Thermal Cycler (Agilent, USA) with the following thermal cycling: initial denaturation at 94 °C for 2 min; then repeat 35 cycles for 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C, and finally 10 min at 72 °C, to complete the reaction. The PCR products were then stained with GelRed 6X (Biotium, UK) and run on 1.5% agarose gel electrophoresis using a standard 1 kb ladder (Bioline, UK) to determine the length of amplified products. Then sequencing the PCR products employed the Sanger method at Nam Khoa Company (Ho Chi Minh City, Vietnam), with each sample sequenced for both forward and reverse directions. The DNA sequence quality check used FinchTV software (Digital World Biology Products, USA). Only regions with a quality index (PHRED) higher than 20 advanced for analysis. The reverse sequences were aligned with the forward sequences to ensure accuracy.

The additional 10 DNA sequences of *rbc*L and *trn*H-*psb*A regions belonging to different genera of the Theaceae family were retrieved from the nucleotide database of NCBI (http://www.ncbi.nlm.nih.gov). The species details of each locus with their accession numbers presented in Table 2. All sequences were evaluated carefully to remove low-quality ones, such as, strings without a species name or containing more than 3% ambiguous base 'N' (Suesatpanit *et al.,* 2017).

The DNA sequences in the *rbc*L and *trn*H-*psb*A barcode regions, determined for similarity with the published sequences, engaged the Basic Local Alignment Tools (BLAST) algorithm (NCBI, USA). Simultaneously, *rbc*L DNA sequences were also used to identify collected samples through the BOLD system (https://www.boldsystems.org/) at the dedicated tab for plant identification. The DNA sequences' comparison used the Muscle method in MEGA 6.0 software (https://www.megasoftware.net) Kimura-2-parameter (K2P) algorithm. Then, the genetic relationship trees' creation, based on the sequences, followed by the Neighbor-Joining method for each barcode region or a combination of sequences of both regions. Applying a bootstrap of 1000 times value for analysis ensued to increase the accuracy in phylogenetic construction.

Table 2. The DNA sequences with corresponding accession numbers used in this study.

Species	Accession number for *rbc*L region	Accession number for *trn*H- *psb*A region
Camellia angustifolia	KY807980.1	KX121729.1
Camellia limonia	KY807972.1	KX121713.1
Camellia salicifolia	KY807991.1	KX121757.1
Camellia cordifolia	KY807990.1	KX121708.1
Camellia macrosepala	KY807989.1	KX121717.1
Camellia danzaiensis	KY807988.1	KX121716.1
Camellia ptilosperma	KY807971.1	KX121726.1
Camellia parvisepala	KY807985.1	KX121758.1
Camellia fangchengensis	KY807983.1	KX121736.1
Camellia sinensis	KY807984.1	KX121761.1

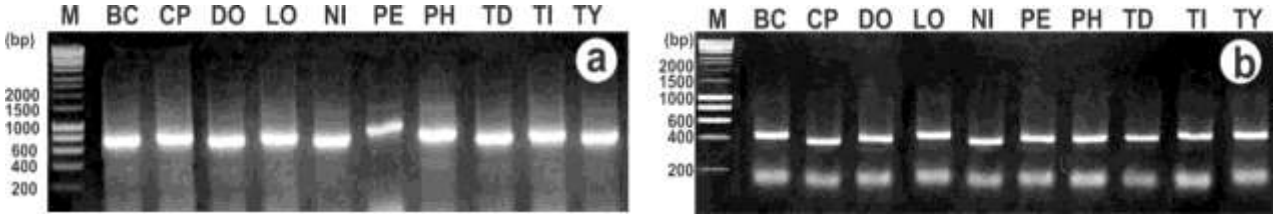

Figure 1. PCR amplification of a) *rbc*L and b) *trn*H-*psb*A in 10 *Camellia* accessions. M: 1000 bp DNA ladder (Bioline, UK).

RESULTS AND DISCUSSION

Gene sequencing and identification

The PCR reaction results showed clear bands (Figure 1), and the product sizes were coherent with the theoretical sizes, i.e., 742 bp for *rbc*L (Figure 1a) (Parmentier *et al.,* 2013) and about 450 bp for *trn*H-*psb*A region (Figure 1b) (Kress *et al.,* 2012). Afterward, the PCR products underwent purification and sequencing and were later calibrated to remove low-confidence sequences. The *rbc*L sequence regions ranged from 432 to 491 bp, while *trn*H-*psb*A ranged from 406 to 432 bp. Utilizing these sequences further obtained similarities using the BLAST program. The BLAST results revealed that the strings of these two barcode regions could only be determined at the genus level (Table 1). These results were consistent with the previous findings based on classifying various species of the genus *Cinnamomum* (Chandrasekara *et al.,* 2021).

The search results in the international barcode organization (BOLD) database with *rbc*L barcode yielded significant findings compared with the genus *Camellia*. Most obtained results were linked with *Diospyros macrocarpa*, with even some sequences identified as shrub plants (*Phlox longifolia)*. The reason could be the limitation of submitted barcode sequences in the BOLD database. Hence, species not yet studied much on this barcode are prone to misidentification since the identification software assigns the submitted sequences and the closest species (Parmentier *et al.,* 2013). With BOLD, the only support for species identification based on *mat*K and *rbc*L regions and *trn*H-*psb*A regions only receive analysis with NCBI BLAST.

Sequence analysis results

The sequence analysis enunciated the differences among the *rbc*L sequences ranged from 0.00 to 0.82, while the variations among the *trn*H-*psb*A regions ranged from 0.00 to 0.49. The values for specific differences are in Table 3. The obtained data revealed that several *Camellia* accessions, identified as distinct species based on morphological characteristics (data not shown), show high variability among the barcode sequences. The results further suggested that morphological identification was unreliable for identifying *Camellia* species. By using three types of DNA barcodes, including *mat*K, ITS2, and *rbc*L to analyze different species of genus *Camellia*, the previous studies reported that *Camellia chrysantha* species collected from Quang Ninh province, Vietnam, and *Camellia euphlebia* species procured from Bac Giang province, Vietnam were notably as only one species (*Camellia euphlebia*) (Nguyen *et al.,* 2019).

Differences, including transition among the purine and pyrimidine molecules, can reveal trends in the evolution of plants. In the presented study, the substitutions of different bases in the two DNA barcode regions got evaluated across the entire codon position (1^{st} + 2^{nd} + 3^{rd} nucleotides), with the data shown in Table 4. In general, transitions were found superior to transversion-type conversions. The predominance of the transition might be due to the change between chemically similar bases and having fewer effects on gene function. However, the transversion substitution may cause significant variations in the genetic makeup of the gene. The changes occur in the spatial structure of the DNA molecule, and the rate of amino acid sequence change in the gene might be higher, leading to a severe impact on the growth traits of the individual

Table 3. Evolutionary differences across sequence pairs between *rbc*L and *trn*H-*psb*A sequences in *Camellia* accessions.

Sample ID	BC	CP	DO	LO	NI	PE	PH	TD	TI	TY
BC		0.01	0.46	0.48	0.01	0.00	0.01	0.02	0.02	0.00
CP	0.79		0.45	0.47	0.00	0.01	0.00	0.01	0.00	0.01
DO	0.79	0.00		0.10	0.46	0.45	0.45	0.46	0.46	0.45
LO	0.01	0.79	0.79		0.48	0.47	0.47	0.49	0.48	0.47
NI	0.80	0.01	0.01	0.80		0.00	0.00	0.01	0.01	0.00
PE	0.79	0.00	0.00	0.79	0.00		0.01	0.02	0.01	0.00
PH	0.01	0.79	0.79	0.00	0.80	0.79		0.01	0.00	0.01
TD	0.80	0.01	0.01	0.80	0.01	0.01	0.80		0.00	0.02
TI	0.79	0.00	0.00	0.79	0.00	0.00	0.79	0.01		0.01
TY	0.01	0.81	0.81	0.01	0.82	0.81	0.02	0.81	0.81	

The base substitutions per site from between sequences of *rbc*L and *trn*H-*psb*A regions are shown below and above the diagonal, respectively.

Table 4. Analysis of the substitution rates (%) of nucleotides in the *rbc*L and *trn*H-*psb*A barcode regions in *Camellia* accessions.

	*rbc*L region				*trn*H-*psb*A region			
	A	T	C	G	A	T	C	G
A	-	*4.79*	*4.79*	15.43	-	*0.57*	*0.13*	19.57
T	*4.79*	-	15.43	*4.79*	*0.50*	-	3.35	*0.16*
C	*4.79*	15.43	-	*4.79*	*0.50*	15.23	-	*0.16*
G	15.43	*4.79*	*4.79*	-	59.13	*0.57*	*0.13*	-

Substitution patterns and rates were estimated under the Tamura-Nei model. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

plant, thereby reducing the transmission of heritability and accumulating these substitution mutations (Guo *et al.,* 2017).

Genetic relationship analysis results

In the current study, the obtained 10 sequences in each DNA barcode region gained combination with 10 corresponding ones from NCBI Genebank and targeted for carrying out the genetic relationship analysis. Generally, the DNA sequence samples collected from Vietnam were classified into two groups with high bootstrap values and separated with DNA sequences from NCBI Genbank (Figure 2). These results suggested the highest genetic distance of *Camellia* accessions collected in Vietnam and *Camellia* accessions from other countries based on *rbc*L and *trn*H-*psb*A regions. Vietnam is a tropical country, considered a hotpot for the *Camellia* populations. With several *Camellia* species previously described, the recent discovery of several new ones has been reported, such as *Camellia luteopalliada* (Luong *et al.,* 2016), *Camellia honbaensis* (Luu *et al.,* 2018), *Camellia pukhangensis* from central Vietnam (Do *et al.,* 2019), and *Camellia puhoatensis* (Nguyen *et al.,* 2019, 2020).

The variations among *rbc*L sequences were higher compared with the *trn*H-*psb*A region (Table 3), with an average of 0.428 and 0.172, respectively. Based on *rbc*L sequences, 10 Vietnamese *Camellia* accessions were divided into two groups and distinguished with 10 others collected from NCBI Genbank (Figure 2a). Meanwhile, based on the phylogenetic tree constructed on the *trn*H-*psb*A region, *Camellia* accessions from Vietnam were only partially separated from NCBI Genbank sequences. Nevertheless, two accessions, namely, DO (local name for "red flower tea") and LO (local name for "hairy yellow tea"), achieved clustering in distinct groups (Figure 2b), suggesting a significant consistency with the morphological variations between these two samples, in comparison with the eight remaining accessions in Vietnam.

Combining both sequence regions, the results showed a significant difference between the accessions, DO, and LO compared with other *Camellia* accessions (Figure 2c). However, the clustering pattern was relatively corresponding to Figure 2a, which may be due to the influence of the *rbc*L sequences. These results were consistent with the past findings that the *trn*H-*psb*A region was more effective than the *rbc*L region in distinguishing some yellow tea accessions collected from Tam Dao National Park of Vietnam (Hoang, 2016). Past studies revealed that *trn*H- *psb*A also has an effective barcode region to identify the Vietnam tea species (*Camellia vietnamensis*) (Dai *et al.,* 2021).

Recently, Zhu *et al.* (2022) also reported that the *trn*H-*psb*A region showed more effective in recognizing the varied ranges of taxonomic units applicable for plant taxonomy studies at the subspecies level. Conversely, *rbc*L and *mat*K, previously suggested as the core DNA barcodes for plant identification, were likely suitable for identifying at the genus level. Furthermore, combining this region with other barcode regions could significantly increase the ratio of plant identification (Pang *et al.,* 2012). Overall, the results revealed the *trn*H-*psb*A barcode's potential to authenticate, conserve genes, and breed *Camellia* species in Vietnam.

CONCLUSIONS

The presented study evaluated the discrimination efficiency of the *rbc*L and *trn*H-*psb*A barcode regions. The results showed the various accessions of *Camellia* at the species level were not distinguished by both barcode regions. However, compared with *rbc*L, the *trn*H-*psb*A barcode region showed a better ability to distinguish *Camellia* species. Since the limited number of analyzed sequences in this study may affect the obtained results' reliability, the study suggests larger samples for inclusion in future related research.

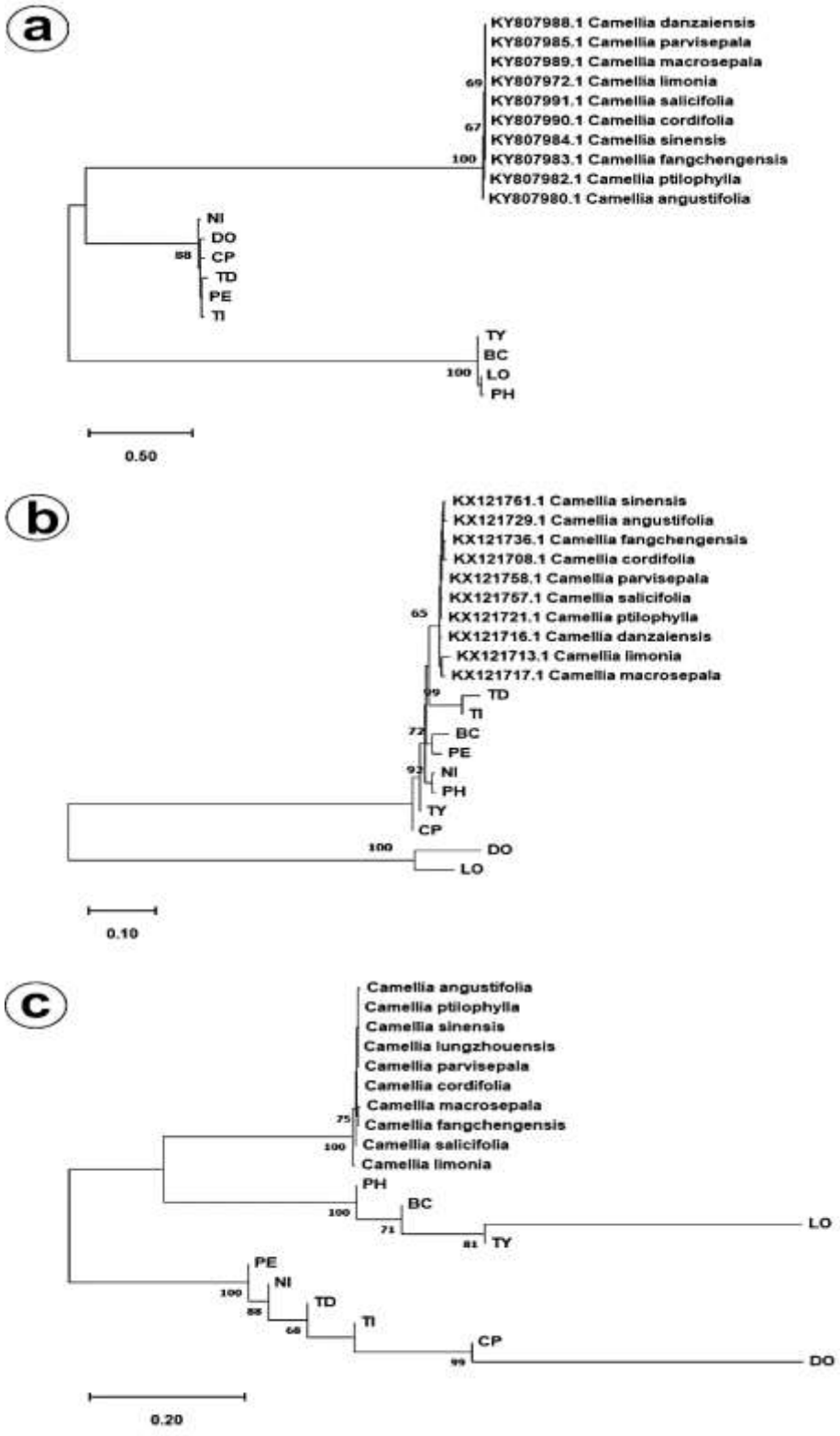

Figure 2. Genetic clustering tree based on 20 *Camellia* accessions by Maximum Likelihood method with a) *rbc*L, b) *trn*H-*psb*A, and c) combination of *rbc*L + *trn*H-*psb*A sequences.

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