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MOLECULAR BASED IDENTIFICATION AND PHYLOGENETIC RELATIONSHIP BY USING *ITS-rDNA* AND *YCF1* GENE OF *Cinnamomum parthenoxylon* (JACK) MEISN. IN NORTHERN VIETNAM

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ABSTRACT

Accurate species identification plays a critical role in conservation, evolutionary studies, and taxonomic classification. Traditional morphological identification is often unreliable, particularly at immature developmental stages, whereas DNA barcoding offers a rapid and precise alternative. In this study, we applied two DNA barcoding regions, the nuclear ribosomal DNA (*ITS-rDNA*) and the chloroplast gene (*ycf1*), to identify 15 samples of *Cinnamomum parthenoxylon* species collected from five geographic regions in northern Vietnam (Quang Ninh, Phu Tho and Thanh Hoa), and to investigate their phylogenetic relationships within the genus *Cinnamomum*. PCR amplification and sequencing success rates were 100% for both markers. The obtained sequence lengths were 588 bp for *ITS-rDNA* and 795 bp for *ycf1*. Base composition analysis showed that *ITS-rDNA* sequences contained, on average, T (12.1%), C (35.9%), A (16.3%), and G (35.7%), whereas *ycf1* sequences comprised T (39.9%), C (15.1%), A (29.9%), and G (15.1%). *ITS-rDNA* and *ITS + ycf1* sequence analysis revealed high similarity among the 15 *C. parthenoxylon* samples, differing by only one nucleotide, and strongly clustered with *C. parthenoxylon* (MLBS = 92-96%, BPP = 88-98% with the *ITS* gene region and MLBS = 93-98%, BPP = 94-97% with the *ITS + ycf1* gene region). In contrast, *ycf1* sequences showed five nucleotide differences among the samples and indicated a close relationship with both *C. parthenoxylon* and *C. balansae* (MLBS = 67-80%; BPP = 55-74%). Genetic divergence among *Cinnamomum* species averaged 6% (range 0-13%) for *ITS-rDNA* and 2% (range 0-4%) for *ycf1*. These findings

suggest that *ITS-rDNA* or combined *ITS + ycf1* are suitable markers for species identification within *Cinnamomum* genus, whereas the *ycf1* region has lower resolution and is less effective for distinguishing among closely related species in this genus.

Keywords: *Cinnamomum parthenoxylon*, DNA barcoding, ITS-rDNA, phylogenetic tree, *ycf1*.

INTRODUCTION

Cinnamomum parthenoxylon (Jack) Meisn. is a medium-sized evergreen tree belonging to the Lauraceae family, reaching heights of up to 30 meters. This species is broadly distributed across South and Southeast Asia, including China, India, Malaysia, Bhutan, Cambodia, Indonesia, Laos, Myanmar, Nepal, Pakistan, Thailand, and Vietnam (Li *et al.,* 2008; Nguyen, 2017; VAST, 2024). Within Vietnam, *C. parthenoxylon* primarily inhabits the Northeastern region, the North and South-Central regions, and the Central Highlands (Nguyen, 2017; Ha *et al.,* 2021; Vu *et al.,* 2022; Ha *et al.,* 2022; Pham *et al.,* 2024; Vu *et al.,* 2024; VAST, 2024). Morphologically, the species is characterized by alternate, elliptic-ovate leaves, axillary or pseudo-terminal panicles, small white or yellowish flowers, and spherical fruits that transition from green to black upon ripening (Ha *et al.,* 2021; Vu *et al.,* 2024).

In addition to its ecological role, *C. parthenoxylon* is of significant commercial value. Its leaves and bark are widely used as spices, and its essential oils serve as flavoring agents in the food and beverage industries. Moreover, the species is exploited for timber, oils, plastics, and pharmaceutical products (Nguyen *et al.,* 1995; Tangjitjaroenkun *et al.,* 2020; Adfa *et al.,* 2020; Qiu *et al.,* 2023). However, decades of overexploitation and unsustainable harvesting, particularly for timber and medicinal use, have led to a marked decline in wild populations. Compounded by its poor natural regeneration capacity, the species is now classified as *Critically Endangered* [CR A2acd] in the Vietnam Red Data Book (VAST, 2024) and is listed for urgent conservation under Decree No. 84/2021/ND-CP. Given these threats, accurate species identification and taxonomic clarification are vital for developing effective conservation strategies. Traditional morphological methods are often unreliable for species-level identification within *Cinnamomum* due to high morphological similarities among closely related taxa (Liu *et al*., 2017; Liu *et al*., 2022). In this context, molecular tools provide more reliable and objective approaches for species delimitation and phylogenetic inference.

DNA barcoding has emerged as a powerful tool for species identification and biodiversity assessment (Liu et al., 2017; Pham *et al.,* 2021; Zhu *et al.,* 2022; Letsiou *et al.,* 2024). An ideal barcode region must exhibit high interspecific variation while remaining conserved within species and should be easily amplified and sequenced using standard PCR-based methods. In plants, both nuclear and plastid genomic regions have been employed for barcoding, including nuclear ribosomal internal transcribed spacer (*ITS-rDNA*) and large subunit 28S rDNA, as well as chloroplast genes such as *matK*, *rbcL*, *trnH–psbA*, *rpoB*,

trnL–trnF, and *ycf1* (Huang *et al.,* 2016; Liu *et al.,* 2017; Liu *et al.,* 2021; Liu *et al.,* 2022).

Within the Lauraceae family, Liu *et al.* (2021) undertook a case study comparing standard DNA barcoding to plastid genome sequencing for species discrimination in the ecologically and economically important family Lauraceae, using 191 plastid genomes for 131 species from 25 genera, representing the largest plastome data set for Lauraceae to date. Results showed that the plastome sequences were useful in correcting some identification errors and finding new cryptic species. However, plastome data overall were only able to discriminate 60% of the species. Beyond species discrimination, the plastid genome sequences revealed complex relationships in the family, with 12/25 genera being non-monophyletic and with extensive incongruence relative to nuclear ribosomal DNA. Liu *et al.* (2017) assessed the performance of four DNA barcode markers (*matK*, *rbcL*, *trnH–psbA*, and *ITS*) for 409 individuals representing 133 species and 12 genera in Lauraceae from China. Their results indicated that *ITS* was the most efficient for identifying species (57.5%), and genera (70%) were ineffective for species discrimination in Lauraceae. In Vietnam, Ha *et al.* (2021) and Vu *et al.* (2024) evaluated three cpDNA regions (*matK*, *rbcL*, and *trnH–psbA*) in *C. parthenoxylon* populations from Tam Dao National Park and Song Hinh Protection Forest. All three regions supported species identification, though *trnH-psbA* was found to be less effective than *matK* and *rbcL*. Similarly, Sudmoon *et al.* (2014) analyzed *rpoB*, *rbcL*, and *matK* in multiple *Cinnamomum* species, including *C. aromaticum*, *C. camphora*, *C. bejolghota*, *C. tamala*, *C. zeylanicum*, and *C. burmannii*. Despite observable genetic distances ranging from 0.00 to 0.52 for *matK*, 0.00 to 0.36 for *rbcL*, and 0.00 to 0.30 for *rpoB*, none of these markers provided sufficient resolution to reliably separate closely related taxa.

Among available markers, *ITS-rDNA* has consistently demonstrated high discriminatory power at both genus and species levels in Lauraceae (Liu *et al*., 2017; Liu *et al*., 2022). The plastid gene *ycf1* has also emerged as a promising marker due to its high variability and success rates in PCR amplification (97.28%) and sequencing (93%) (Liu *et al*., 2017; Liu *et al*., 2022). This makes ycf1 particularly valuable for resolving closely related species within Lauraceae. Therefore, combining nuclear (*ITS*) and plastid (*ycf1*) markers provides a complementary dual-genome strategy for accurate species identification and phylogenetic reconstruction.

In this study, we employed the nuclear *ITS-rDNA* region and the plastid *ycf1* gene to confirm the taxonomic identity of *C. parthenoxylon* using sequence alignment and phylogenetic analysis. The results aim to contribute molecular evidence to support species delimitation and inform conservation strategies for this critically endangered species.

MATERIALS AND METHODS

Plant materials

The inner barks or leaves of adult trees were collected from 5 natural populations of *C. parthenoxylon* (3 individuals for one population), representing the range of its geographical distribution in Vietnam (Figure 1 and Table 1). A total of 15 adult trees (3 individuals for one population) were randomly sampled and preserved in marked

plastic bags with silica gel in the field, then transferred to the Molecular Laboratory, Joint Vietnam-Russia Tropical Science and Technology Research Center, and stored at -80°C until used for DNA extraction.

Figure 1. Adult plant of the *C. parthenoxylon* species collected in Xuan Son National Park, Phu Tho Province.

Table 1. List of sampled species, locations, and GenBank accessions.

Species	**Collected location**	**Samples**	**GenBank accession number**	
			ITS	***ycf1***
C. parthenoxylon	Yen Tu National Park, Quang Ninh Province, 155m, 21°08'23"N, 106°43'5"E	XXQN01-XXQN03	This study	This study
	Tam Dao National Park, Vinh Phuc Province, 368m, 21°24'23"N, 105°34'10"E	XXVP01-XXVP03		
	Xuan Son National Park, Phu Tho Province, 536m, 21°06'04"N, 104°56'29"E	XXPT01-XXPT03		
	Thuong Tien Nature Reserve, Hoa Binh Province, 356m, 20°25'15.8"N,106°44'31.2" E	XXHB01-XXHB03		
	Xuan Lien Nature Reserve, Thanh Hoa Province, 880m, 19°59'5"N, 104°59'06"E	XXTH01-XXTH03		

	GenBank	MT628624	MZ289715
C. bejolghota	GenBank	KX546413	MZ289690
C. camphora	GenBank	KX546414	MZ289695
C. glanduliferum	GenBank	KX546415	OR264222
C. iners	GenBank	KX546417	MZ289705
C. longepaniculatum	GenBank	KX546419	MZ289708
C. pittosporoides	GenBank	KX546422	MZ289713
C. wilsonii	GenBank	KX546423	MZ289723
C. micranthum f. kanehirae	GenBank	KY271521	KR014245
C. balansae	GenBank	PQ048881	PQ576718
C. burmanni	GenBank	FM957802	
C. mairei	GenBank	KU139859	
C. tamala	GenBank	KX822091	MZ289718
C. verum	GenBank	KX509827	MZ289722
C. agasthyamalayanum	GenBank	MH232437	
C. appelianum	GenBank	KP092853	MZ289689
C. aromaticum	GenBank	KY238310	MN173819
C. wightii	GenBank	MH232489	
C. walaiwarense	GenBank	MH232490	
C. tsangii	GenBank	KU139900	
C. travancoricum	GenBank	MH232479	
C. tenuifolium	GenBank	KU139892	
C. chago	GenBank	KU139830	MZ289697
Cryptocarya acutifolia	GenBank	KX546424	MZ289725

DNA isolation and amplification

A plant DNA isolation kit (Norgenbiotek, Canada) was employed to extract the total genomic DNA. The Nanodrop™ ND-2000 spectrophotometer (NanoDrop Technologies, DE, USA) was used to assess the purity and integrity of the total DNA. Subsequently, the DNA was diluted to a concentration of 20 ng/µL. The two *ITS-rDNA* and *ycf1* gene regions were amplified (Table 2).

Polymerase chain reaction (PCR) was performed in 25 µL reaction volumes comprising 2 µL of 20 ng template DNA, 12.5 µL of 2 x Taq Master Mix, 9 µL of deionized water, 1.25 µL of each primer, and 5 U of Taq DNA polymerase using the Applied Biosystems GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). PCR amplification of the *ITS* and *ycf1* barcoding regions was conducted using specific primer pairs for each region.

Table 2. The primers, reaction processes of the PCR amplification protocol, and sequencing of two specific barcodes.

Gene or spacer region	Primer pairs	Sequence (5′-3′)	PCR Reaction Process	References
ITS	ITS5P	GGAAGGAGAAGTCGTAACAAGG	Procedure: 95°C, 4 min; 35 x (94°C, 45 s; 56°C, 1 min; 72°C, 1 min); 72°C, 10 min; 72°C, hold at 4°C.	Möller & Cronk, 2001
	ITS8P	CACGCTTCTCCAGACTACA		
ycf1	ycf1F	CCACTCCAAA(T/A)ATTTTCTAT	Procedure: 95°C, 4 min; 35 x (94°C, 45 s; 50°C, 1 min; 72°C, 1 min); 72°C, 10 min; 72°C, hold at 4°C.	Liu *et al.*, 2022
	ycf1R	GAAAGAATATACAT(G/A)(G/C)ATA		

Sequencing and sequence editing

PCR products were visualized using electrophoresis through a 1.5% agarose gel, a 100 bp DNA ladder, 1X TAE, stained with RedSafe™ Nucleic Acid Staining Solution and photographed under UV light of the GelDoc system (Quantum CX5, Villber, France). Successful amplifications were purified to remove PCR components and nonspecific amplifications using an innuPREP gel extraction Kit (Analytikjena, Germany) for two genes (*ITS-rDNA* and *ycf1*). Purified PCR products were sent to FirstBase (Malaysia) for sequencing in both directions. Sequencing was performed on an Avant 3100 automated DNA sequencer using the Dye Terminator Cycle sequencing kit (PE Applied Biosystems). Sequencing of the 15 studied samples used the primers ITS5P/ITS8P and ycf1F/ycf1R.

Raw DNA sequences were edited, and low-quality signal peaks were removed using ChromasPro v.2.1.6 (Technelysium, Brisbane, Australia). The edited sequences were compared with existing sequences in GenBank using the BLAST algorithm available at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments were performed using BioEdit v.7.0.5.2 (Hall, 1999). Ambiguously aligned or highly variable regions were excluded from subsequent analyses to ensure alignment accuracy and phylogenetic reliability.

Phylogenetic analysis

Phylogenetic relationships were inferred using two approaches: Maximum Likelihood (ML) implemented in Treefinder v.2011 (Jobb, 2011), and Bayesian Inference (BI) performed with MrBayes v.3.2.1

(Ronquist & Huelsenbeck, 2003). Before ML and BI analyses, nucleotide sequence data were examined for base composition and evaluated for evolutionary model assumptions using Kakusan v.4.0 (Tanabe, 2011), based on the corrected Akaike Information Criterion (AICc).

For the *ITS-rDNA* gene region, the best-fitting substitution model selected for both ML and BI analyses was the General Time Reversible model (GTR) with a gamma distribution to account for among-site rate variation (gamma shape parameter G = 0.579 for ML and 0.151 for BI). Similarly, the GTR model was selected as optimal for the *ycf1* gene region, with gamma shape parameters of 0.585 (ML) and 0.209 (BI), respectively. The GTR model was selected as optimal for the combined *ITS* + *ycf1* gene region, with gamma shape parameters of 0.353 (ML) and 0.809 (BI), respectively. Convergence diagnostics and parameter estimation reliability were assessed using Tracer v.1.5 (Rambaut and Drummond, 2009). Node support for the ML tree was evaluated through 1,000 bootstrap replicates (ML bootstrap support, MLBS), while posterior probabilities (Bayesian posterior probability, BPP) were estimated from 1,000 replicates in the BI analysis. Pairwise genetic distances (*p*-distances) among species within the genus *Cinnamomum* were calculated using MEGA v.11.0 (Tamura *et al.,* 2021).

RESULTS

Sequence characteristics of the *ITS-rDNA* and *ycf1* gene regions

In this study, we successfully amplified and analyzed the nuclear DNA regions (*ITS-rDNA*) and plastid regions (*ycf1*) from 15 individuals of *C. parthenoxylon*, representing five distinct populations (three individuals per population). PCR amplification for both markers yielded high-quality results, achieving a 100% sequencing success rate across all samples. Sequence chromatograms were edited and assembled using ChromasPro v.2.1.6, and ambiguous bases were curated manually. After trimming low-quality regions at both termini, the final sequence lengths were standardized to 588 bp for *ITS-rDNA* and 795 bp for *ycf1*. Sequence identity was verified using BLASTn against the GenBank database, showing a 100% match to *C. parthenoxylon*, thus validating the taxonomic identification of the studied material.

In the two loci examined, all three samples from each of the regions (Thanh Hoa, Hoa Binh, Phu Tho, and Vinh Phuc) exhibited completely identical sequences. In which, the *ITS-rDNA* region was highly conserved, exhibiting only a single polymorphic site at position 149 (T↔C), distinguishing the samples from Quang Ninh province from those of the remaining four populations (Thanh Hoa, Hoa Binh, Phu Tho, and Vinh Phuc) (Figure 2A). In contrast, the *ycf1* sequences displayed higher variability, with five polymorphic sites identified at nucleotide positions 132 (A↔C), 207 (G↔A), 210 (A↔G), 566 (T↔C), and 649 (G↔T) (Figure 2B). The presence of multiple variable sites in the *ycf1* region underscores its potential as a complementary plastid barcode for intraspecific and population-level studies in *Cinnamomum*.

Nucleotide analysis revealed distinct base composition profiles between the two regions. For the *ITS-rDNA* sequences, the average nucleotide frequencies were as follows: T (12.1%), C (35.9%), A (16.3%), and G (35.7%). The region exhibited a

relatively high GC content of 71.6%, compared to a lower AT content of 28.4% (Table 3). In contrast, the *ycf1* sequences were AT-rich, with mean nucleotide frequencies of T (39.9%), A (29.9%), C (15.1%), and G (15.1%). The overall GC content of the *ycf1* region was markedly lower (30.2%) than the AT content (69.8%). Codon position-specific analysis of the *ycf1* sequences further demonstrated an AT bias at all three codon positions, with AT content values of 32.3%, 37.2%, and 35.3% for the 1st, 2nd, and 3rd codon positions, respectively (Table 3). This compositional asymmetry suggests selective constraints or mutational biases acting upon the *ycf1* coding region and highlights the evolutionary dynamics of plastid genes in *Cinnamomum*.

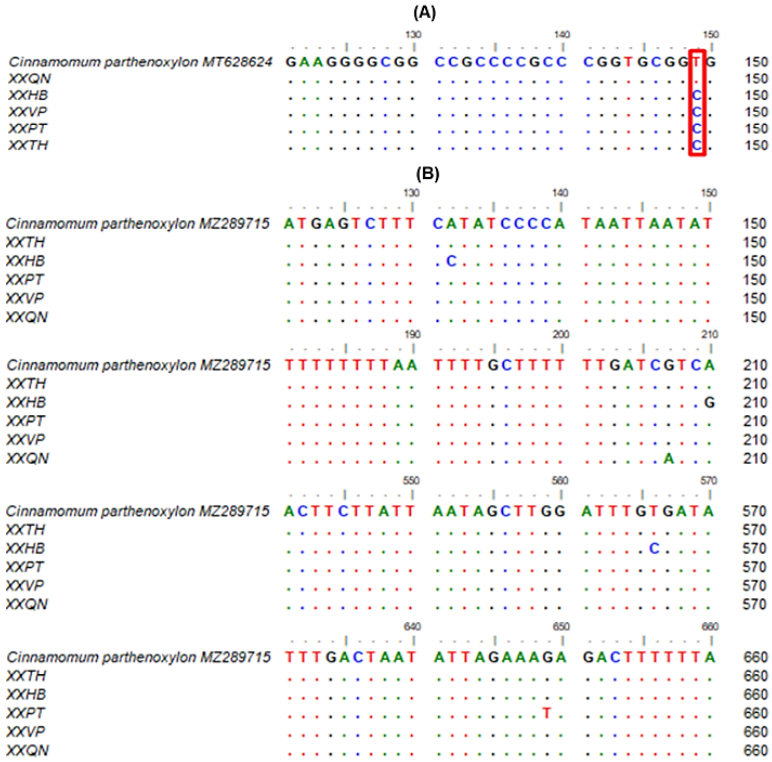

Figure 2. Nucleotide differences in the *ITS-rDNA* gene region (A) and the *ycf1* gene region (B) among *C. parthenoxylon* samples from five populations in northern Vietnam.

Table 3. Nucleotide base compositions (%) for the *ITS-rDNA* and *ycf1* sequences of *C. parthenoxylon.*

Codon positions	***ITS-rDNA* gene region**					***ycf1* gene region**				
	T	**C**	**A**	**G**	**Total**	**T**	**C**	**A**	**G**	**Total**
All positions	12.1	35.9	16.3	35.7	588	39.9	15.1	29.9	15.1	795
1st positions	ns	ns	ns	ns	ns	38.1	18.5	26.4	17.0	265
2nd positions	ns	ns	ns	ns	ns	36.2	14.3	38.1	11.3	265
3rd positions	ns	ns	ns	ns	ns	45.3	12.5	25.3	17.0	265

Note: ns = not significant

Genetic divergence based on *ITS-rDNA* and *ycf1* gene regions

In the ITS-rDNA gene region: The nucleotide sequences of the *ITS-rDNA* region from 15 samples were compared with sequences from 23 species in the genus *Cinnamomum* from GenBank. After removing all positions containing gaps, the remaining aligned sites were subjected to further analysis. Among the 588 aligned positions, 263 nucleotides were found to be variable, of which 84 nucleotides were parsimony-informative. Genetic distance analysis based on the *p*-distance model revealed a relatively broad range of interspecific divergence within the genus *Cinnamomum*, with an average divergence of approximately 6% (range: 0-13%) (Table 4). Notably, the genetic divergence between the XXQN, XXTH, XXPT, XXVP, and XXHB samples from five populations (Quang Ninh, Thanh Hoa, Phu Tho, Vinh Phuc, and Hoa Binh) and the *C. parthenoxylon* reference sequence (GenBank accession MT628624) was remarkably low, ranging from 0 to 1%, indicating a high degree of genetic similarity. These results suggest that the studied specimens most likely belong to *C. parthenoxylon*. To support this conclusion, phylogenetic analyses were subsequently conducted.

In the ycf1 gene region: The ycf1 gene nucleotide sequences of 15 collected samples were compared with those of 15 *Cinnamomum* species retrieved from GenBank. After removing all positions containing gaps, a total of 795 aligned positions were analyzed. Among them, 124 nucleotides were variable, and 24 nucleotides were parsimony-informative. Genetic distances estimated using the *p*-distance method ranged from 0% to 4%, with a relatively low average divergence of 2% (Table 5). Sequence divergence between the XXQN, XXTH, XXPT, XXVP, and XXHB samples and several species, including *C. parthenoxylon* (MZ289715), *C. longepaniculatum* (MZ289708), *C. balansae* (PQ576718), *C. glanduliferum* (OR264222), *C. micranthum f. kanehirae* (KR014245), *C. aromaticum* (MN173819), *C. tamala* (MZ289718), and *C. verum* (MZ289722) was remarkably low (0-1%). This low level of divergence indicates that species-level identification based solely on the *ycf1* region is not feasible. Therefore, further phylogenetic analyses are required to accurately determine the taxonomic placement of the studied samples.

Table 4. Genetic distance of the studied samples with species in the genus *Cinnamomum* based on nucleotide sequence analysis of the *ITS-rDNA* gene region.

Voucher/ species name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1. XXQN	-																											
2. XXHB	0.01	-																										
3. XXVP	0.00	0.00	-																									
4. XXPT	0.00	0.00	0.00	-																								
5. XXTH	0.00	0.00	0.00	0.00	-																							
6. *C. parthenoxylon* MT628624	0.00	0.01	0.00	0.00	0.00	-																						
7. *C. bejolghota* KX546413	0.08	0.08	0.08	0.08	0.08	0.08	-																					
8. *C. camphora* KX546414	0.02	0.02	0.02	0.02	0.02	0.02	0.09	-																				
9. *C. glanduliferum* KX546415	0.02	0.02	0.02	0.02	0.01	0.02	0.08	0.02	-																			
10. *C. iners* KX546417	0.09	0.09	0.09	0.09	0.09	0.09	0.02	0.10	0.10	-																		

11. *C. longepaniculatum* KX546419	0.01	0.01	0.01	0.01	0.01	0.01	0.08	0.02	0.00	0.08	-						
12. *C. pittosporoides* KX546422	0.06	0.06	0.06	0.06	0.06	0.06	0.05	0.07	0.06	0.06	0.05	-					
13. *C. wilsonii* KX546423	0.09	0.09	0.10	0.10	0.09	0.09	0.09	0.10	0.09	0.09	0.09	0.07	-				
14. *C. micranthum f. kanehirae* KY271521	0.03	0.03	0.03	0.03	0.02	0.03	0.08	0.04	0.02	0.09	0.01	0.06	0.09	-			
15. *C. balansae* PQ048881	0.01	0.01	0.01	0.01	0.01	0.01	0.07	0.02	0.01	0.08	0.01	0.06	0.05	0.02	-		
16. *C. burmanni* FM957802	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.06	0.05	0.07	0.05	0.03	0.05	0.06	0.05	-	
17. *C. mairei* KU139859	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.06	0.05	0.07	0.05	0.03	0.05	0.06	0.05	0.00	-
18. *C. tamala* KX822091	0.06	0.06	0.06	0.06	0.05	0.06	0.05	0.07	0.06	0.05	0.05	0.04	0.12	0.07	0.02	0.05	0.05
19. *C. verum* KX509827	0.09	0.10	0.10	0.10	0.08	0.09	0.03	0.11	0.09	0.04	0.08	0.06	0.09	0.09	0.07	0.08	0.07

20. *C. agasthyamalayanum* MH232437	0.08	0.08	0.09	0.09	0.09	0.08	0.02	0.09	0.09	0.03	0.09	0.05	0.05	0.08	0.09	0.05	0.05	0.00	0.00	-					
21. *C. appelianum* KP092853	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.06	0.05	0.06	0.05	0.03	0.05	0.04	0.05	0.01	0.00	0.05	0.06	0.05	-				
22. *C. aromaticum* KY238310	0.08	0.08	0.06	0.06	0.07	0.08	0.08	0.09	0.07	0.08	0.07	0.06	0.08	0.07	0.08	0.04	0.03	0.06	0.09	0.08	0.03	-			
23. *C. wightii* MH232489	0.08	0.08	0.09	0.09	0.09	0.08	0.02	0.09	0.08	0.04	0.08	0.04	0.04	0.07	0.09	0.04	0.04	0.00	0.01	0.01	0.04	0.07	-		
24. *C. walaiwarense* MH232490	0.09	0.09	0.09	0.09	0.09	0.09	0.02	0.09	0.09	0.04	0.08	0.05	0.04	0.08	0.09	0.05	0.05	0.00	0.02	0.02	0.04	0.08	0.02	-	
25. *C. tsangii* KU139900	0.11	0.11	0.11	0.11	0.11	0.11	0.12	0.12	0.12	0.13	0.11	0.09	0.12	0.12	0.13	0.08	0.08	0.08	0.13	0.15	0.08	0.08	0.15	0.16	-
26. *C. travancoricum* MH232479	0.09	0.09	0.09	0.09	0.09	0.09	0.02	0.09	0.09	0.03	0.08	0.05	0.04	0.08	0.09	0.05	0.05	0.00	0.01	0.01	0.04	0.07	0.01	0.01	0.15
27. *C. chago* KU139830	0.06	0.06	0.06	0.06	0.06	0.06	0.05	0.07	0.06	0.05	0.05	0.01	0.08	0.07	0.06	0.03	0.03	0.03	0.07	0.04	0.03	0.05	0.04	0.05	0.09
28. *C. tenuifolium* KU139892	0.05	0.05	0.05	0.05	0.04	0.05	0.06	0.06	0.05	0.06	0.04	0.03	0.08	0.06	0.02	0.02	0.02	0.06	0.09	0.05	0.02	0.04	0.05	0.05	0.04

Table 5. Genetic distance of the studied samples with species in the genus *Cinnamomum* based on nucleotide sequence analysis of the *ycf1* gene region.

Voucher/species name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. XXTH																				
2. XXHB	0.00																			
3. XXPT	0.00	0.01																		
4. XXVP	0.00	0.01	0.00																	
5. XXQN	0.00	0.01	0.00	0.00																
6. *C. parthenoxylon* MZ289715	0.00	0.00	0.00	0.00	0.00															
7. *C. longepaniculatum* MZ289708	0.01	0.01	0.01	0.01	0.01	0.01														
8. *C. balansae* PQ576718	0.01	0.01	0.01	0.01	0.01	0.01	0.01													
9. *C. glanduliferum* OR264222	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01												
10. *C. micranthum f. kanehirae* KR014245	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.00											

11. *C. aromaticum* MN173819	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01									
12. *C. bejolghota* MZ289690	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.01	0.01	0.01								
13. *C. iners* MZ289705	0.01	0.02	0.01	0.01	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.01							
14. *C. pittosporoides* MZ289713	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01						
15. *C. wilsonii* MZ289723	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.01	0.02	0.01	0.01					
16. *C. tamala* MZ289718	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01				
17. *C. verum* MZ289722	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01			
18. *C. appelianum* MZ289689	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01		
19. *C. chago* MZ289697	0.01	0.02	0.01	0.01	0.02	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	
20. *C. camphora* MZ289695	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04

Phylogenetic tree construction based on *ITS-rDNA* and *ycf1* gene regions

In the ITS-rDNA gene region: Phylogenetic trees were constructed using ML and BI methods, incorporating the 15 samples (XXQN, XXTH, XXPT, XXVP, and XXHB) and 23 other *Cinnamomum* species (Figure 3). Both methods produced congruent topologies, with log-likelihood scores of –lnL = 2722.375 (ML) and –lnL = 2764.032 (BI), respectively. All the samples from Quang Ninh (XXQN), Thanh Hoa (XXTH), Phu Tho (XXPT), Vinh Phuc (XXVP), and Hoa Binh (XXHB), together with *C. parthenoxylon* (MT628624), formed a distinct clade with high genetic similarity (99-100%) and strong statistical support (MLBS = 92-96%, BPP = 88-98%). Additionally, *C. parthenoxylon* and *C. balansae* (PQ048881) exhibited a close relationship with 99% sequence similarity, supported by MLBS = 62% and BPP = 65%. These findings further support the hypothesis that the 15 samples from Quang Ninh (XXQN), Thanh Hoa (XXTH), Phu Tho (XXPT), Vinh Phuc (XXVP), and Hoa Binh (XXHB) share a common origin with *C. parthenoxylon*.

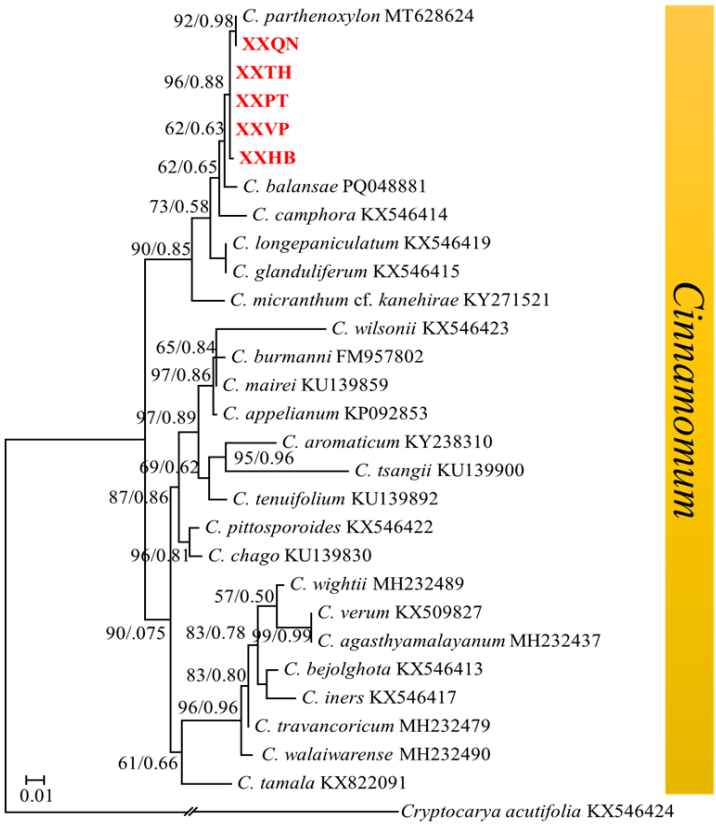

Figure 3. The phylogenetic relationship of the studied samples with species in the genus *Cinnamomum* was based on the *ITS-DNA* sequence using ML and BI methods. The numbers on the branches represent bootstrap support values. XXQN (*C. parthenoxylon* in Yen Tu National Park, Quang Ninh Province), XXTH (*C. parthenoxylon* in Xuan Lien Nature Reserve, Thanh Hoa Province), XXPT (*C. parthenoxylon* in Xuan Son National Park, Phu Tho Province), XXVP (*C. parthenoxylon* in Tam Dao National Park, Vinh Phuc Province) and XXHB (*C. parthenoxylon* in Thuong Tien Nature Reserve, Hoa Binh Province). *Cryptocarya acutifolia* (KX546424) outgroup.

In the ycf1 gene region: The phylogenetic trees constructed using ML and BI methods based on the *ycf1* gene region yielded consistent topologies, with log-likelihood values of -lnL = 1850.953 (ML) and -lnL = 1881.629 (BI). The 15 collected samples from Quang Ninh (XXQN), Thanh Hoa (XXTH), Phu Tho (XXPT), Vinh Phuc (XXVP), and Hoa Binh (XXHB), together with *C. parthenoxylon* (MZ289715) and *C. balansae* (PQ576718), formed a distinct clade with high sequence similarity (99-100%). This clade received moderate support in both ML and BI analyses, with bootstrap values ranging from 67–80% (MLBS) and posterior probabilities from 55–74% (BPP) (Figure 4). These results suggest that the 15 samples collected from northern Vietnam may originate from either *C. parthenoxylon* or *C. balansae*. However, the low interspecific genetic divergence observed in the *ycf1* region indicates that this marker alone is insufficient for accurate species delimitation within the genus *Cinnamomum*.

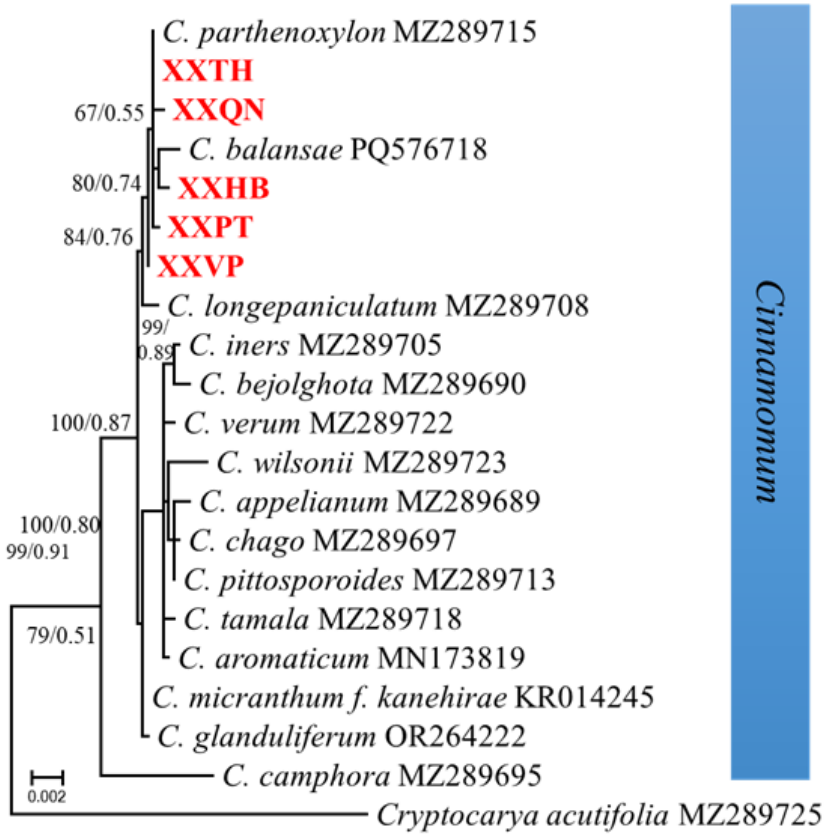

Figure 4. The phylogenetic relationship of the studied samples with species in the genus *Cinnamomum* was based on the *ycf1* sequence using ML and BI methods. The numbers on the branches represent bootstrap support values. XXQN (*C. parthenoxylon* in Yen Tu National Park, Quang Ninh Province), XXTH (*C. parthenoxylon* in Xuan Lien Nature Reserve, Thanh Hoa Province), XXPT (*C. parthenoxylon* in Xuan Son National Park, Phu Tho Province), XXVP (*C. parthenoxylon* in Tam Dao National Park, Vinh Phuc Province) and XXHB (*C. parthenoxylon* in Thuong Tien Nature Reserve, Hoa Binh Province). *Cryptocarya acutifolia* (MZ289725) outgroup.

Combine ITS + ycf1 sequences: Phylogenetic trees were constructed using ML and BI methods, incorporating the 15 samples (XXQN, XXTH, XXPT, XXVP, and XXHB) and 15 other *Cinnamomum* species (Figure 5). Both methods produced congruent topologies, with log-likelihood scores of (–lnL) = 4639.861 (ML) and (–lnL) = 4659.609 (BI), respectively. All the samples from Quang Ninh (XXQN), Thanh Hoa (XXTH), Phu Tho (XXPT), Vinh Phuc (XXVP), and Hoa Binh (XXHB), together with *C. parthenoxylon* (MT628624 + MZ289715), formed a distinct clade with high genetic similarity and strong statistical support (MLBS = 93-98%, BPP = 94-97%). Additionally, *C. parthenoxylon* and *C. balansae* (PQ048881 + PQ576718) exhibited a close relationship with strong support (MLBS = 100% and BPP = 97%).

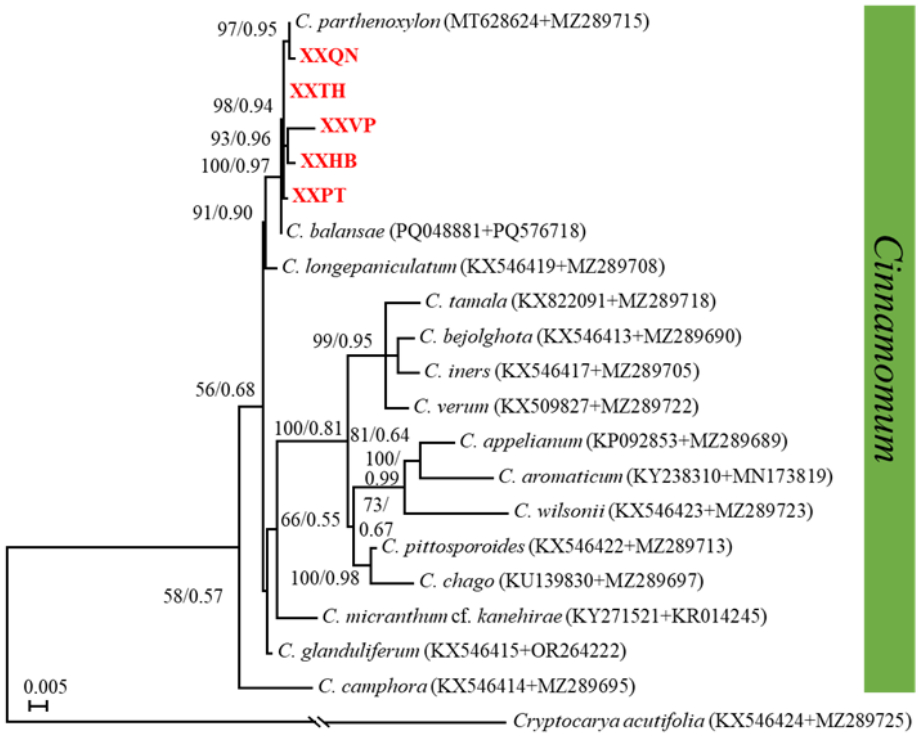

Figure 5. The phylogenetic relationship of the studied samples with species in the genus *Cinnamomum* was based on combined *ITS* + *ycf1* sequences using ML and BI methods. The numbers on the branches represent bootstrap support values. XXQN (*C. parthenoxylon* in Yen Tu National Park, Quang Ninh Province), XXTH (*C. parthenoxylon* in Xuan Lien Nature Reserve, Thanh Hoa Province), XXPT (*C. parthenoxylon* in Xuan Son National Park, Phu Tho Province), XXVP (*C. parthenoxylon* in Tam Dao National Park, Vinh Phuc Province) and XXHB (*C. parthenoxylon* in Thuong Tien Nature Reserve, Hoa Binh Province). *Cryptocarya acutifolia* (KX546424 + MZ289725) outgroup.

DISCUSSION

DNA barcoding can act as a tool for detecting errors in species identifications (Gonzalez *et al*., 2009, Gostel & Kress, 2022). The tree-based and similarity-based approaches using DNA barcoding in combination with morphology are thus very useful to address identification mistakes based only on morphology (Huang *et al*., 2015; Zhu *et al.,* 2022). Examination of the

initially misidentified samples showed that misidentifications were most likely to occur when the samples were only flowering or fruiting and their morphological characters and geographical distributions were similar. Once the morphology-based errors listed above were taken into account, mistakes in individual identifications were then only detectable through DNA sequencing (Liu *et al*., 2017; Mo *et al*., 2017).

Recent advancements in whole plastome sequencing, such as the work by Song *et al.* (2020), demonstrate that whole plastome sequencing provides more robust evolutionary relationships compared to traditional Sanger sequencing of individual loci. While the overarching phylogeny of Lauraceae is generally well understood, species relationships within many genera remain poorly defined. Most studies to date have relied on single individuals per taxon, and have shown that sampling multiple individuals across various species is crucial for testing species-level monophyly and achieving better species discrimination (Ji *et al.,* 2019; Pham *et al*., 2021).

The accurate identification of *C. parthenoxylon*, a species of ecological and medicinal importance, is essential due to the existence of closely related taxa within the genus *Cinnamomum*. This study utilized two molecular markers, the nuclear *ITS-rDNA* region, and the chloroplast *ycf1* gene, to evaluate their effectiveness in distinguishing *C. parthenoxylon* from other species within the genus. In the *ITS-rDNA* region, genetic distance analysis based on the *p*-distance model revealed considerable interspecific divergence within *Cinnamomum*, with an average divergence of approximately 6% (0-13%). This level of divergence affirms the suitability of *ITS-rDNA* as a reliable marker for species discrimination within the genus. Additionally, *ITS-rDNA* sequence analysis revealed slight genetic variation among *C. parthenoxylon* samples from five different populations, with a single nucleotide polymorphism observed in samples from Thanh Hoa, Hoa Binh, Phu Tho, and Vinh Phuc compared to those from Quang Ninh. This finding may reflect the impact of geographic isolation or environmental factors on genetic differentiation among populations. Specifically, this genetic differentiation could be attributed to environmental factors, such as habitat fragmentation or ecological and geographical variation within these regions. Phylogenetic analysis of the *ITS-rDNA* gene revealed that all *C. parthenoxylon* samples from the five populations formed a distinct clade, showing high genetic similarity with other *C. parthenoxylon* samples. This supports the hypothesis that these populations share a common origin and belong to the same species, despite exhibiting minor genetic differences.

In contrast, the *ycf1* gene demonstrated more pronounced variation, with five polymorphic sites, suggesting a greater degree of evolutionary change. This higher variability may reflect genetic changes that are necessary for the species' adaptation to the distinct environmental conditions of each population. Genetic distances estimated using the p-distance method ranged from 0% to 4%, with a relatively low average divergence of 2%. Phylogenetic analysis of the *ycf1* gene revealed a relatively consistent evolutionary relationship among species, with *C. parthenoxylon* samples clustering together but showing close relationships with other species (*C. longepaniculatum, C. balansae, C. glanduliferum, C. micranthum f. kanehirae, C. aromaticum, C. tamala,* and *C. verum*). However, the low interspecific

genetic divergence observed in the *ycf1* region indicates that this marker alone is insufficient for accurate species delimitation within *Cinnamomum*, highlighting the need for complementary markers to achieve clearer species resolution within the genus.

Combining DNA barcodes is generally considered to improve species identification (Liu *et al*., 2017; Liu *et al*., 2022) and in this study, the combined dataset of *ITS* and *ycf1* sequences produced a single phylogenetic tree with a well-resolved topology, clearly delineating 15 samples from the five populations in northern Vietnam and species *C. parthenoxylon* in the GenBank with strong support values (Figure 5). These results highlight that although useful for improving phylogenetic resolution in the genus *Cinnamomum* and providing some species-level insights, plastome sequences only partially improve species discrimination, and suggest here that using *ITS* as a single barcode or a combination of barcode markers (*ITS* + *ycf1*) would be the most suitable approach for barcoding in the genus *Cinnamomum*.

CONCLUSION

In the current study, we sequenced two gene regions (*ITS-rDNA* and *ycf1*) to identify *C. parthenoxylon* in northern Vietnam and suggested that *ITS* or *ITS* + *ycf*1 regions are an effective marker for distinguishing species within *Cinnamomum*, providing higher resolution than other markers, such as the cpDNA region (*ycf1*). The findings will be significant in the study of evolution, systematics, and conservation of the species.

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CONFLICT OF INTEREST

The authors declared that there is no potential conflict of interest.

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