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DNA barcode of *matK* combined with *ITS* effectively distinguishes the medicinal plant *Stephania brachyandra* Diels collected in Laocai, Vietnam

Nhan Thi Thanh Pham[1], Dung Phuong Le[1], Khanh Thi Ngoc Pham[1,2], Xaykham Thipphavong[1,3], Mau Hoang Chu[1*]

[1]School of Biology, Thai Nguyen University of Education, Thai Nguyen, Vietnam.
[2]Specialized Upper Secondary School, Department of Education and Training, Bacgiang, Vietnam.
[3]Department of Biology Education, Luangnamtha Teacher Training College, Luang Namtha, Laos.

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ABSTRACT

In Vietnam, some species in the genus *Stephania* are being overexploited and recorded in the Red Data Book of Vietnam. In this article, we present the identification results of *Stephania* spp. collected in Lào Cai province using morphological characteristics and the DNA barcode method to contribute to the conservation and exploitation of genetic resources and pharmaceuticals of this species in Vietnam. The analysis of the data shows that all morphology characteristics and comparative anatomy of the petioles, stems, and leaves are typical of *Stephania brachyandra* Diels species. The *matK* gene and *ITS* region isolated from *Stephania*_Laocai have base lengths of 879 bp and 423 bp, respectively. The (Bbasic Llocal Aalignment Ssearch Ttool) analysis of the *matK* gene and *ITS* region obtained in this study has the highest similarity, 99.37% and 98.97%, respectively, to *S. brachyandra* species. The *matK* sequences were highly conserved and had low variable sites for 747 nucleotides (84.98%) and 132 nucleotides (15.29%), respectively, whereas the short *ITS* region was less conserved and had variable sites for 78 (18.44%) and 345 (81.56%), respectively. The results of the molecular phylogenetic analysis of the *matK* gene by the maximum likelihood method for the *Stephania*_Laocai sample showed that the *matK* sequence is suggested for better phylogenetic resolution than the *ITS* region and the combination of the *matK* gene and *ITS* region can be used to identify *S. brachyandra* species. Based on the combination of the characteristics of morphology and nucleotide sequences of the *matK* gene and *ITS* region, *Stephania* spp. collected in Lào Cai province of Vietnam were determined as *S. brachyandra* Diels.

1. INTRODUCTION

Stephania is the largest genus of the family Menispermaceae with about 60 species distributed in the tropical and subtropical regions of Asia and Africa. Some species are also found in Oceania. Recently, 37 species were recorded in China and 15 species in Thailand [1–3]. Because their tubers contain a number of important alkaloids, such as L-tetrahydropalmatine (rotundin), stepharin, roemerin, and cycleanin, *Stephania* spp. have long been used in traditional medicine to treat various diseases such as sedation, blood pressure stabilization, asthma, tuberculosis, dysentery, hyperglycemia, malaria, and cancer [4–6].

In Vietnam, this genus comprises 20 species with the similar dioecious flower [4], including several medicinal species such as *Stephania cepharantha* Hayata, *Stephania rotunda* Lour., and *Stephania japonica* Miers which are being overexploited and listed in the Red Data Book of Vietnam with a level of "going to be endangered" (V). However, the conservation of genetic resources of the species in the genus *Stephania* is still difficult due to the misidentification of species with similar morphological and anatomical characteristics.

Plant species can be identified by many different analytical methods. The current methods such as analysis and comparison

*Corresponding Author
Mau Hoang Chu, School of Biology, Thai Nguyen University of Education, Thai Nguyen, Vietnam. E-mail: chuhoangmau @ tnu.edu.vn

of morphological, anatomical, physiological, or biochemical characteristics have been reported successfully in a number of crops such as *Fallopia multiflora* (Thunb.) Haraldson [7], *Albizia myriophylla* Benth. [8], and *Pelargonium hortorum* L. H. Bailey [9]. However, it is difficult to efficiently identify plants, especially closely related species which belong to the same subgenus or their parts are not intact.

Recently, DNA barcode data have been widely and regularly used to provide additional evidence at the molecular level for plant taxonomic studies. The trend of combining morphological characteristics and chemical and genetic markers into a dataset for species identification becomes very important for systematic studies, in which DNA barcoding has become one of the most efficient tools for species identification of medicinal plants. Several barcoding loci including *matK*, *rpoC1*, *trnH-psbA*, *ITS*, and *rbcL* have been studied and applied effectively in the identification of medicinal plants [10–12]. The *matK* gene found in chloroplasts has been successfully applied to plant identification [11]. The *ITS* gene region located in the cell nucleus, including the *ITS*1-5.8S-*ITS*2 sequence, has achieved high identification rates at the species level. The studies determining the phylogenetic relationships between plant species based on *ITS* genome sequencing in *Dalbergia tonkinensis*, *Dalbergia cochinchinensis*, and *Dalbergia oliveri* [13] or genera *Erica* L. [14], *Scrophularia* [15], and *Potamogeton* [16] and many other plant species demonstrate the role of the *ITS* gene region in plant identification [17,18]. In this study, the *Stephania brachyandra* collected in Lào Cai (Vietnam) was identified using the comparative morphological method and supported the DNA barcode method with *mat*K and *ITS*.

2. MATERIALS AND METHODS

2.1. Materials

Stephania spp. samples were collected in Lào Cai province, Vietnam, and these samples were classified at the Laboratory of Botany and Genetics and grown in the Experimental Garden at the School of Biology, Thai Nguyen University of Education, Vietnam.

2.2. Morphological Analysis

The morphological features of *Stephania* spp. were studied following the protocol of Nguyen et al. [19], Flora of Vietnam by Pham [20], and Paris Linnaeus by Liang and Soukup [21]. Key indicators used for analysis include the height of the main stem and stem color; the number of leaves, leaf shape, and leaf size; and the shape, color, and number of calyxes, corollas, stamens, and pistils.

2.3. DNA Extraction, Polymerase Chain Reaction (PCR) Amplification, and Sequencing

Total genomic DNA was extracted from young fresh leaves material following the protocol of Shaghai-Maroof et al. [22]. The sequences of the *matK* gene and *ITS* region in *Stephania* spp. plants were amplified by PCR using the primer pairs presented in Table 1.

Table 1: Characteristics of *matK* and *ITS* primer pairs for the PCR.

Primer name	Nucleotide sequence 5′ → 3′	Theoretical length (bp)
matK-F	CGATCTATTCATTCAATATTTC	900
matK-R	TCTAGCACACGAAGTCGAAGT	
ITS-F	ACGAATTCATGGTCCGGTGAAGTGTTCG	500
ITS-R	TAGAATTCCCCGGTTCGCTCGCCGTTAC	

The PCR amplification was carried out using a final volume of 25 µl with 1.5 µl forward primers (10 pmol μl^{-1}), 1.5 µl reverse primers (10 pmol μl^{-1}), 12.5 µl 2× Master Mix, 1.0 µl template genomic DNA (500 ng ml^{-1}), and 8.5 µl deionized water. The PCR amplification profiles consisted of 4 minutes at 94°C for initial denaturation, 30 cycles of 1 minute at 94°C, annealing for 1 minute at 54°C, 1 minute 30 seconds at 72°C for extension, and a final extension step for 10 minutes at 72°C. The PCR products were detected by 1.0% agarose gel electrophoresis.

The *matK and ITS* sequences were identified by the machine ABI PRISM® 3100 Avant Genetic Analyzer with Kit BigDye® Terminator v3.1 Cycle Sequencing and a specific primer pair. The data were analyzed by the basic local alignment search tool (BLAST) tool.

2.4. Phylogenetic Analysis

The evolutionary history was inferred by using the maximum likelihood (ML) method and the Tamura and Nei model [23]. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed [24]. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). Evolutionary analyses were conducted in MEGA X [25].

3. RESULTS

3.1. Plant Sample Collection and Morphological Identification of *S. brachyandra*

The *Stephania* sample collected in Lào Cai (Vietnam) is perennial with herbaceous climbing vines up to 2–3 m long and a woody stem base; stem, leaves, and flowers are usually hairless (Fig. 1). The analysis of morphological characteristics of the collected *Stephania*_Laocai has indicated that this species is *S. brachyandra* Diels. It is a simple and alternate leaf, and petioles about 5–10 cm long are attached to the leaf blade at about one-third to one-sixth of the leaf length. The leaf blade is thin, glabrous, and egg- shaped to triangular or rounded, 515 cm, with the entire margins being smooth, base flat, or slightly convex. The leaf veins are propeller-shaped, consisting of 8–11 veins originating from the top of the

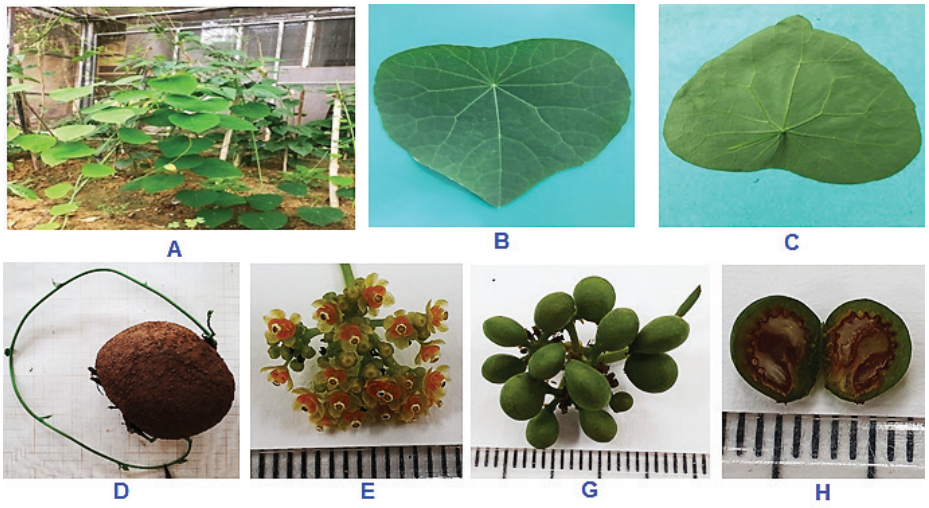

Figure 1: The morphology of *S. brachyandra* Diels collected in Lào Cai, Vietnam. (A) *S. brachyandra* plants; (B and C) upperside and underside of leaves, respectively; (D): stem and tuberous tuber root; (E): flowers; (G): fruits; and (H): cross-section of fruit. Scale bar: 1.0 mm.

petiole. The leaf tip is pointed or nearly rounded; the leaf base is rounded or heart- shaped. The front side of the leaf blade is dark green, while a pale green or slightly silver color is seen at the backside.

Shoots and young stems are usually smooth, dark green, light green, or glossy green. The outer layer of the bark has cracks along the stem or rough warts. The stems are ash gray, dark brown, and light brown. Tuberous roots are very diverse, usually spherical, lean, and brown. The size and weight of the tuber are very different, ranging from about 1–3 kg up to 80 kg. The inner part of the tuber is light yellow or lemon yellow, ivory white, or reddish-brown.

The flowers are unisexual, with the inflorescences compound umbelliform cymes, double canopy, single canopy, or head- shaped [26]; the inflorescences have peduncles, solitary or clustered on the primary inflorescence branches; the terminal branches sometimes irregular or the cymes gather into a disk that is head- shaped [27]. Male inflorescences are slightly slender: peduncle 2–4 cm, six sepals arranged in two rings, three yellow-orange petals,m and disk-shaped anthers. Female inflorescences have shorter stalks than the male inflorescences because of 8–9 small cymes, tightly arranged in a headed shape. Inflorescence peduncle is 2–3 cm, with the apex slightly swollen. Flowers are densely arranged. Therefore, it is hard to see the flower stalk. Female flowers are usually small: sepal one, pale green; two petals arranged on the same side of the flower, yellow-orange, with the shape of an inverted ovate. The ovary is ovate and curved, with a short peduncle; the stigma has – four to five small spiny lobes. Flowers are cross-pollinated mainly by several insect species [27,28].

The fruit has only one seed, 0.7–0.8 cm, ovate to nearly round, flattened on both sides. The outer skin is usually orange-red, smooth, and shiny when ripe. The ovary has two ovules, but only one develops into the seed, whereas the other one degenerates. Seeds are horseshoe-shaped, inverted ovate, amputated heads, membranous connected to the semicircular ring; in the middle of the seeds, there is an inverted ovoid hole. Along the dorsal and ventral edge of the seed, there are four rows of spines with bulging heads that swell up into the shape of a nail cap [26].

3.2. Analysis of *matK* and *ITS* Sequences of *S. brachyandra*

3.2.1. Total gGenomic DNA eExtraction and PCR aAmplification of the matK gGene and ITS rRegion

The purification of total genomic DNA extracted from leaves tissues of *S. brachyandra* was assessed via agarose gel electrophoresis and measured using a spectrophotometer. The result showed that the specific band was clean and had no contamination of RNA and protein (data not shown). The *matK* gene and *ITS* region of the genomic DNA were amplified by PCR using primer pairs *matK*-F/*matK*-R and *ITS*-F/*ITS*-R, respectively. The PCR products detected by 1.0% agarose gel electrophoresis revealed a DNA fragment of the *matK* gene and *ITS* region with the expected sizes of approximately 900 and 500 bp, respectively (Fig. 2).

The PCR products of the *matK* and *ITS* sequence were purified and sequenced on an ABI PRISM® 3100 automated sequencer, and the results showed that *matK* is 879 bp in size and *ITS* is 423 bp in size. The BLAST analysis showed that the *matK* and *ITS* sequences of *Stephania*_Laocai were close to *S. brachyandra*, and the *matK* sequence provided for 74% of query coverage and showed relatedness to *S. brachyandra* with 879 nucleotides of a total BLAST score and with a 99.37% sequence identity and the *ITS* provided for 100% of query coverage and showed relatedness to *S. brachyandra* with 423 nucleotides of a total BLAST score and with a 98.97% sequence identity.

The results of comparing 28 *matK* sequences by BLAST showed that the isolated *matK* sequence was close to species of the *Stephania* genus and provided for 53%–74% of query coverage and relatedness to the *Stephania* species with 342–571 of a total BLAST score and with 86.35%–99.37% sequence identity (Table 2). The aligned sequence of the *matK* gene showed 879 bp length, and

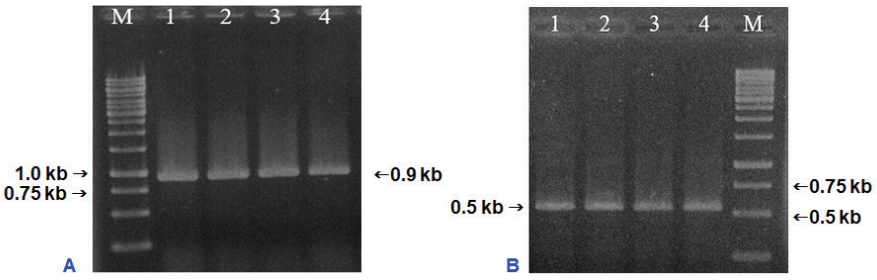

Figure 2: PCR amplification of the *matK* gene (A) and *ITS* region (B). M: Marker 1 kb; 1–4: PCR products of *matK*/*ITS*.

Table 2: Twenty-eight species in the top 100 BLAST hits of *matK*.

Species	Accession	Query cover	Total score	% identity
*Stephania*_Laocai				
S. brachyandra	KJ566126.1	74%	571	99.37%
Stephania intermedia	KJ566141.1	74%	569	99.37%
Stephania dicentrinifera	KJ566130.1	74%	568	99.05%
Stephania viridiflavens	KJ566153.1	74%	566	99.05%
Stephania sinica	KJ566150.1	74%	564	98.74%
Stephania officinarum	KJ566149.1	74%	564	98.75%
S. kwangsiensis	KY189283.1	74%	562	98.74%
Stephania macrantha	KJ566147.1	74%	562	98.74%
Stephania hainanensis	KJ566138.1	74%	556	98.42%
Stephania dielsiana	KJ566131.1	74%	553	98.41%
Stephania ebracteata	KJ566133.1	72%	547	99.02%
Stephania longipes	KY189285.1	74%	544	97.78%
Stephania dentifolia	KJ566129.1	74%	536	97.15%
Stephania lincangensis	KJ566144.1	74%	532	97.15%
Stephania dolichopoda	KJ566132.1	71%	529	98.03%
Stephania yunnanensis	KJ566154.1	74%	523	96.54%
Stephania excentrica	KJ566136.1	72%	510	96.74%
Stephania epigaea	KJ566135.1	74%	505	95.57%
S. cepharantha	KJ566127.1	74%	505	95.58%
Stephania mashanica	KY189280.1	62%	462	98.12%
Stephania kuinanensis	KY189281.1	62%	460	98.11%
Stephania micrantha	KY189279.1	62%	457	97.74%
Stephania succifera	AY017403.1	60%	444	98.05%
Stephania longa	MG730325	91%	444	87.40%
Stephania chingtungensis	AY017397.1	60%	444	98.05%
Stephania suberosa	KY189278.1	53%	409	99.12%
Stephania tetrandra	FJ609735.1	73%	342	86.35%

the *matK* sequences were highly conserved and had low variable sites for 747 nucleotides (84.98%) and 132 nucleotides (15.29%), respectively.

The results of comparing 20 *ITS* sequences by BLAST showed that the isolated *ITS* sequence was close to 13 species of the *Stephania* genus and seven species of the other genus and provided for 100% of query coverage and relatedness to the *Stephania* species with 1,378–1,567 of a total BLAST score and with 95.10%–98.97% sequence identity (Table 3). For the short *ITS* region, the aligned sequence showed 423 bp length and was less conserved and had variable sites for 78 (18.44%) and 345 (81.56%), respectively.

3.3. Phylogenetic Analysis

Recently, the ML method has been applied for DNA sequence analysis [29]. The results of the molecular phylogenetic analysis of the *matK* gene by the ML method (Fig. 3) for the *Stephania*_Laocai sample showed the ML tree from the *matK* locus. DNA barcode alignment was carried out with the highest log likelihood (−2,148.48). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 20 nucleotide *matK* sequences including a *Stephania*_Laocai sample and 19 *matK* sequences in GenBank. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 862 positions in the final dataset. In Figure 3, 13 species of the *Stephania* genus are grouped in 1 clade, which is divided into 2 subclades with 99%–100% support, in which the *Stephania*_Laocai sample and *S. brachyandra* (EF143871.1) [30] are in 1 subclade (bootstrap values = 76%). In contrast to the *matK* sequence, the *ITS* region dataset yielded less phylogenetic resolution than the bootstrap value which was 59% at the clade of the genus *Stephania* (Fig. 4). Additionally, the genus *Stephania* was separated into two main branches (bootstrap values = 99%). The second major branch further divides into two secondary branches and the second secondary branch divides into many clades and subclades. Thus, in the case of barcoding among species of the *Stephania* genus and to identify the *S. brachyandra* species, the *matK* sequence is suggested for better phylogenetic resolution.

Among the species of the genus *Stephania*, only a few species with both the *mat*K gene and the *ITS* region sequences were found in GenBank. The phylogenetic tree was established based on the *matK* and *ITS* sequence combinations (*matK/ITS*) shown and the *Stephania*_Laocai and *S. brachyandra* samples distributed in a clade with a bootstrap value of 97% (Fig. 5). Thus, the combination of *matK* and *ITS* can be used to identify *S. brachyandra*.

4. DISCUSSION

Along with this approach, Chinh et al. [31] used the chloroplast *rbcL* gene to clarify the relationship between three species of the genus *Stephania* (Menispermaceae) from Vietnam, *S. japonica*,

Table 3: Twenty species in the top 100 BLAST hits of *ITS*.

Species	Accession	Query cover	Total score	% identity
Stephania_Laocai				
S. brachyandra	EF143871.1	100%	1567	98.97%
Stephania delavayi	AF542589.2	100%	1555	98.63%
S. chingtungensis	EF143873.1	100%	1544	98.52%
S. cepharantha	EF143872.1	100%	1533	98.29%
S. dielsiana	NC_054337.1	100%	1528	98.18%
S. succifera	EF143876.1	100%	1522	98.06%
S. kwangsiensis	NC_048524.1	100%	1511	97.84%
Stephania abyssinica	JN051854.1	100%	1411	95.79%
S. tetrandra	EF143877.1	100%	1400	95.56%
Stephania elegans	EF143874.1	100%	1389	95.34%
S. japonica	KC494050.1	100%	1384	95.22%
S. longa	EF143875.1	100%	1378	95.10%
Cissampelos pareira	KX384057.1	100%	1373	94.99%
Antizoma angustifolia	KX384049.1	100%	1373	94.99%
Cyclea atjehensis	KX384065.1	100%	1367	94.87%
Cissampelos capensis	KX384055.1	100%	1356	94.65%
C. pareira	KX384059.1	100%	1345	94.42%
Strychnopsis thouarsii	JN051858.1	100%	1330	94.10%
Cocculus trilobus	DQ478611.1	100%	1330	94.10%

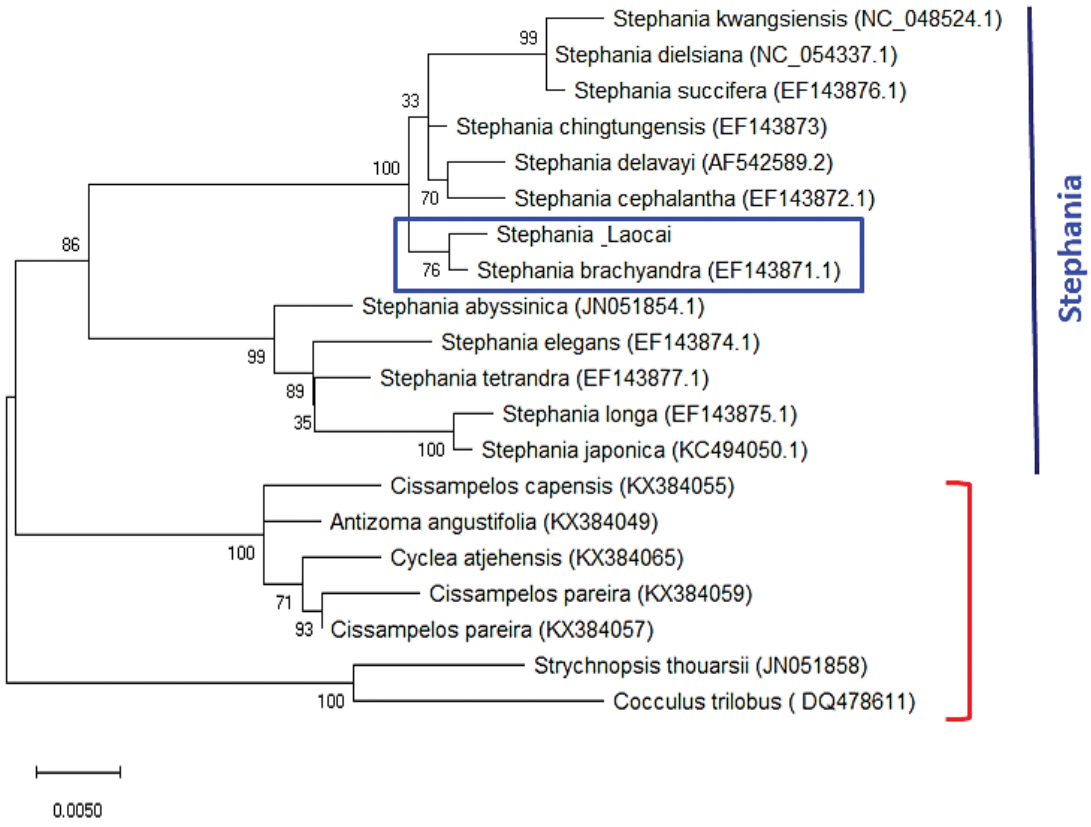

Figure 3: Molecular phylogenetic analysis of the *matK* gene. The 20 *matK* sequences were obtained including a *Stephania*_Laocai sample and 19 *matK* sequences in GenBank, in which there are 13 *matK* sequences belonging to the *Stephania* genus. Bootstrap values are above the nodes of the branches. The capital letters and numbers in parentheses are accession numbers of *Stephania* species published in the GenBank.

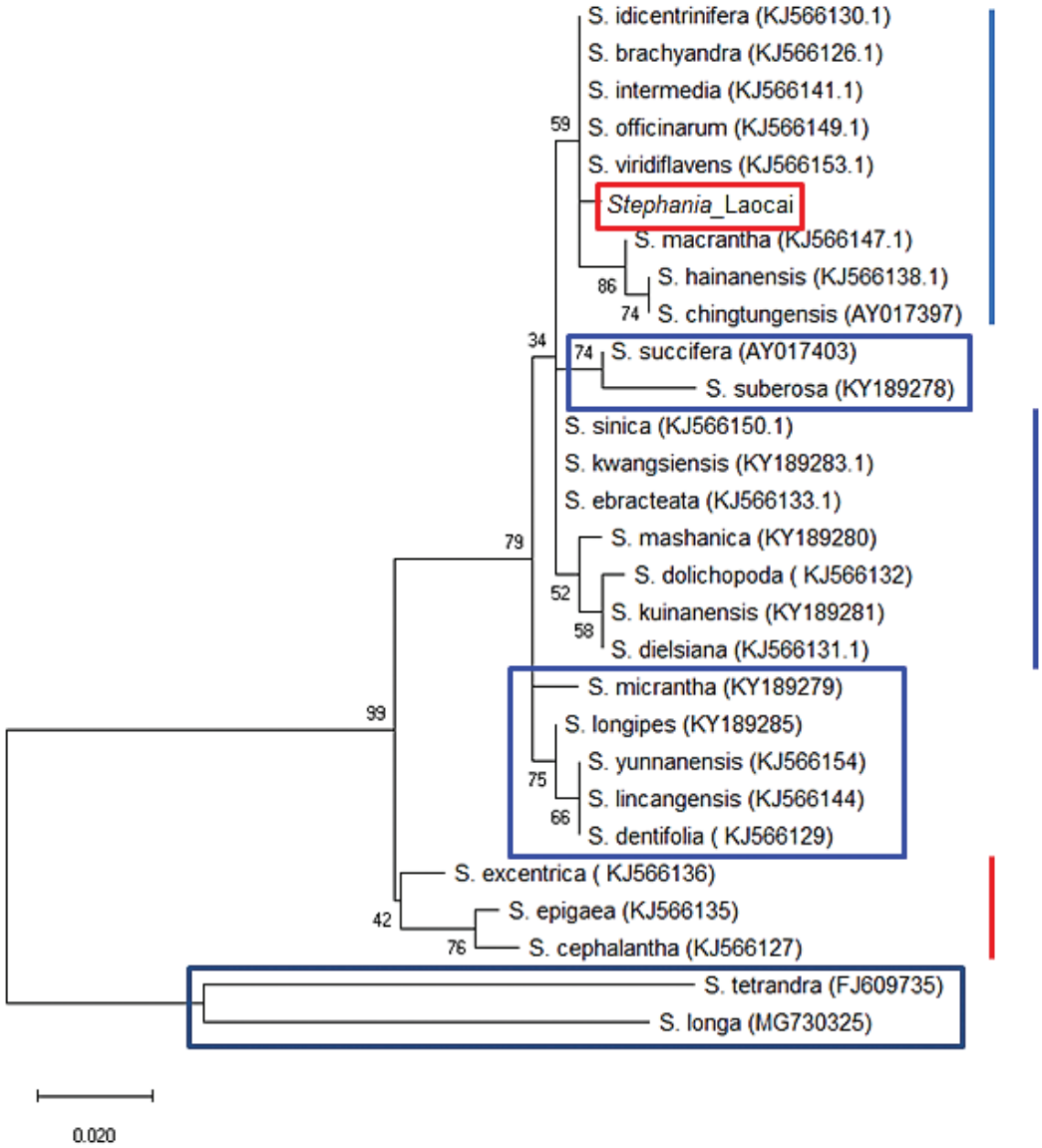

Figure 4: Molecular phylogenetic analysis of the *ITS* region. The 28 *ITS* sequences were obtained including a *Stephania*_Laocai sample and 27 *ITS* sequences in the GenBank. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 250 positions in the final dataset. Bootstrap values are above the nodes of the branches. The capital letters and numbers in parentheses are accession numbers of the *Stephania* species published in the GenBank.

Stephania polygona, and *S. rotunda*, and one subspecies, *S. japonica* var. discolor. According to these authors, the *rbcL* gene of the chloroplast genome is widely used as additional data for the study of species origin, molecular evolution, and phylogeny. Molecular analysis was carried out on the 523 bp segment of the *rbcL* genes. The dataset consists of 22 sequences used to reconstruct the evolutionary tree using two methods: Bayesian inference and mlML. The results indicated that *S. rotunda* was able to distinguish between *S. japonica* and *S. polygona*, while *S. japonica*, *S. japonica* var. discolor, and *S. polygona* could not distinguish each other. However, the *rbcL* gene also has its limitations. Previous studies showed that 58.5% of sister species were not identifiable by the *rbcL* gene sequence because of 100% similarity [12]. Hence, they suggested that other loci such as nuclear *ITS* and chloroplast *trnH-psbA* space should be examined further or a combination of multiple gene loci for the genus *Stephania* should be studied. Wang et al. [29] confirmed that the genus *Stephania* is polyphyly, which has been grouped but does not share an immediate common ancestor based on phylogeny and morphological evolution of the tribe Menispermeae (Menispermaceae) inferred from chloroplast and nuclear sequences. The inconsistency between the molecular system and the traditional classification system has been pointed out in the genera of Menispermaceae [5]. Therefore, a combination of morphological and molecular characteristics is needed to rearrange the classification system of the *Stephania* genus. DNA barcodes (or molecular markers) are an effective tool to support morphology in species identification and rearrangement of the classification system.

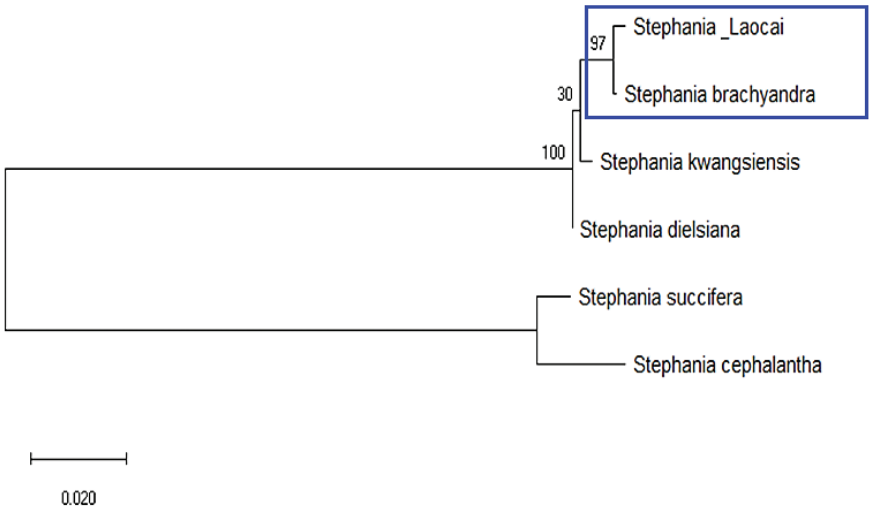

Figure 5: Phylogenetic tree of the *Stephania* species constructed on the matK combined with ITS (matK/ITS). Bootstrap values are above the nodes of the branches.

5. CONCLUSION

In this study, the morphological features of a *Stephania*_Laocai sample, as well as two DNA barcodes *matK* and *ITS*, were analyzed to identify this species. Our results demonstrate that the *Stephania* spp. sample collected in Lào Cai province, Vietnam, is *S. brachyandra* Diels and is proposed to use the *matK* gene or combine *matK* with *ITS* to identify *S. brachyandra* species.

6. ACKNOWLEDGMENT

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

8. ETHICAL APPROVAL

This article does not contain any studies involving animals or human participants performed by any of the authors.

9. CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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