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Morphological Traits and Nuclear Genetic Diversity of *Coptis sp.* in Hoang Lien National Park, Lao Cai Province, Vietnam

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Abstract

Coptis is a medicinal plant genus in the Ranunculaceae family, and is also known as "Hoang Lien" in Vietnamese. It is a perennial herb that grows in some regions of the world. However, Coptis is endangered and faces global threats. This study aimed to characterize some main morphological characteristics and performed a phylogenetic analysis of 11 samples of Coptis sp. collected from Hoang Lien National Park using nuclear DNA sequence analyses. All sample species have unique morphological traits with distinct yellow rhizomes and basal leaves with five segments. The petiole measures 13-25 cm and is smooth, while the leaf blade is ovate, ranging from 7 to 15.5 cm in length and 5.5 to 14 cm in width. The leaves are subleathery, glabrous on the underside, and sparsely puberulous on the veins on the upper side. The molecular characterization of Coptis sp. genotypes was determined by ITS markers. The length of the ITS1-ITS2 sequences varied from 363 to 371 nucleotides. The average nucleotide composition was 17.11% A, 31.25% C, 32.247% G and 19.41% T, respectively. The comparison with the GenBank database showed that the samples had 95.71- 96.37% similarity with the species Coptis quinquesecta. The genetic distance among the 11 Coptis samples fluctuated from 0.00 to 0.017. A neighbor-joining tree was constructed to show the genetic relationships among Coptis samples. The results indicated that this endangered species had low levels of genetic diversity. The study has provided valuable information for genetic-based conservation of this rare endemic species and suggested some conservation strategies.

Keywords: *Coptis sp., Genetic diversity, ITS, Hoang Lien National Park, ITS*

1. Introduction

Coptis, also known as "goldthread or canker root" and "Hoang Lien" in Vietnamese, is a genus containing 15 species and is an herbaceous perennial plant in the Ranunculaceae family. These plant species were reported to have valuable medicinal properties. *Coptis* is a genus of endangered plants that are globally threatened according to the IUCN Red List [1]. In Vietnam, *Coptis* species are native to some mountain provinces in North areas and are typically found in high-altitude montane habitats ranging from 1000 - 2500 m, with high precipitation and humidity levels [2]. The rhizome of *Coptis* is rich in alkaloids (5 - 8%), predominantly berberine, and also contains palmatin, worenin, *Coptis*in, jatrorrhizin, magnoflorin, columbamin, epiberberin, epiberberin, etc. The alkaloid content in different plant parts varies according to the phenological stages and seasons. Furthermore, the rhizome of *Coptis* also comprises starch and organic acids such as ferulic acid [3-5]. *Coptis* has a bitter taste, cold properties, and acts on the heart, liver, gallbladder, and small intestine meridians. It has the following pharmacological effects: clearing heat and purging fire, mainly treating intestinal heat disorders leading to dysentery, and diarrhea; clearing the mind and relieving restlessness: heart fire causes restlessness, insomnia, mouth and tongue ulcers; clearing heat and detoxifying boils, high fever, dizziness, delirium, madness, red tongue; clearing liver and brightening eyes, treating eye diseases caused by liver fire such as eye

pain, red eyes, watery eyes[2]. Currently, *Coptis* is extensively cultivated in China, mainly in Sichuan, accounting for about 80% of the total production in the country. Meanwhile, *Coptis* in Vietnam is mainly exploited from nature for many years and is being exploited in a destructive way, so it is facing the risk of extinction [6].

The genetic diversity and differentiation of a species determine its potential for adaptation to environmental changes and its distribution area. Genetic diversity not only affects the adaptive potential of a species but also underpins the long-term stability of the entire ecosystem. Therefore, the assessment of genetic diversity is crucial for understanding the evolutionary history of endangered species and designing effective conservation and management methods[7-9]. The genetic diversity of a species plays a crucial role in determining its evolutionary and ecological success and serves as the foundation for the preservation and utilization of germplasm resources in the field of medicinal plant research [10-11]. The nuclear genome of eukaryotes typically contains tandemly repeated clusters of ribosomal genes. Each cluster comprises the genes for the 18S, 5.8S, and 28S rRNA, two internal transcribed spacers (ITS1 and ITS2) that separate these genes, and a non-transcribed intergenic (IGS) spacer that isolates the transcriptional units. ITS is located between the highly conserved 18S, 5.8S, and 28S genes of the rDNA gene cluster. Thus, rDNA sequences tend to be nearly identical within a given organism and well-differentiated between species [12].

Hoang Lien National Park is located in Lao Cai province and is part of the Hoang Lien Son range and is 1,000 to 3,143 m above sea level. This area has a distinctive variation in climate, weather, and topography depending on its altitude, hence, it has a vibrant ecosystem of plants and animals with many characteristics. Notably, plant diversity and plant species richness with over 2,100 species were recorded [13]. Hoang Lien National Park contains numerous plant species with proven uses as medicine, ornament, food, or timber. A large number of reports assessing climate differentiation as well as ecological surveys and plant distribution according to elevation belts have been reported [14-15]. However, there is a scarcity of data on genetic diversity and resources of *Optis.* Therefore, in this study aimed to evaluate the morphological traits and nucleotide sequence of the *ITS* gene region from natural *Coptis sp.* samples collected in Hoang Lien National Park in order to supplement detailed and accurate scientific data on the genetic characteristics of *Coptis sp.* for conservation and development this species in this country.

2. Materials And Methods

Collection of material samples

Three populations of *Coptis sp.* were collected at Hoang Lien National Park from three regions of San Sa Ho, Ban Khoang, and Pa Cheo communes. In total, 11 leaf samples were randomly selected from three populations (as shown in Figure 1 and Table 1). Samples were labeled from HL1 to HL11 and stored at −20 ◦C until used for DNA purification.

Morphological evaluation

The main morphological characteristics were presented based on previously described[18] with some minor modifications[11]. After collection, all samples were immediately transferred to the laboratory and were maintained at -20ºC for DNA extraction.

DNA extraction

A modified CTAB method was used to isolate total genomic DNA from fresh leaves of a single individual with some minor modifications[16-17]. Electrophoresis was performed to check the total DNA obtained on 1% agarose gel.

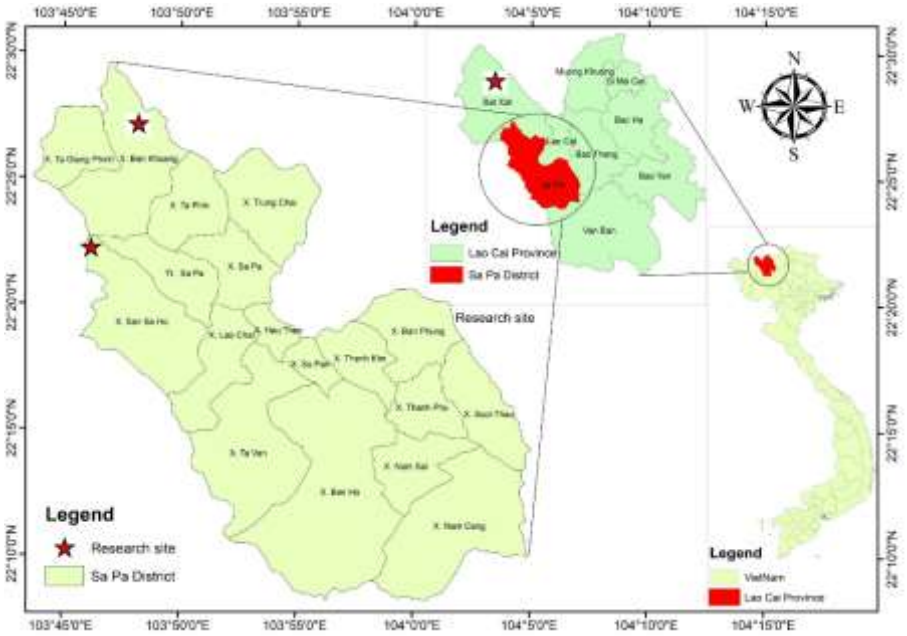

Figure 1. The map of the sample collection. The star symbol implies the typical samples collected from 3 different communes in Lao Cai province

Table 1. List of *Coptis sp*. samples information used in this study

No.	Code	Collection site	Length of sequence	Accession number
1	**HL 1**	San Sa Ho commune, Sa Pa, Lao Cai	364	KX714731.1
2	**HL 2**		367	KX714731.1
3	**HL 3**		369	KX714731.1
4	**HL 4**		368	KX714731.1
5	**HL 5**		363	KX714731.1
6	**HL 6**	Ban Khoang commune, Sa Pa, Lao Cai	371	KX714731.1
7	**HL 7**		359	KX714731.1
8	**HL 8**		370	KX714731.1
9	**HL 9**	Pa Cheo commune, Bat Xat, Lao Cai	362	KX714731.1
10	**HL 10**		370	KX714731.1
11	**HL 11**		363	KX714731.1

Genetic diversity analysis

The PCR reaction was performed with the primer pair ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3'. The PCR reaction components consisted of: 1 µL total DNA (100 ng/µL), 0.5 µL of each primer (10 pmol), 1 µL 10X GoTaq Green Master Mix (Promega, USA), and 7 µL of nuclease-free water. The total reaction volume was 10 µL. The PCR reaction was carried out in a thermal cycler (Cleave Scientific, UK) with the following program: 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 58°C for 45 seconds, and 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The PCR products were purified and sent for nucleotide sequencing analysis at the company 1st BASE (Apical Scientific Sdn. Bhd., Malaysia).

Data analysis

The DNA sequencing results were analyzed by Bioedit software (v7.2.5). The similarity and coverage of the DNA sequences were assessed by comparing them with the available sequences in the NCBI database using the BLAST tool (Basic Local Alignment Search Tool, [https://blast.ncbi.nlm.nih.gov/Blast.cgi]). The DNA sequences were aligned by the Clustal W function on MEGA 5.1 software with the default parameters. The phylogenetic tree was constructed based on the Neighbor-joining method with a bootstrap value of 500. The genetic analyses were performed using the ITS gene region sequences. The genetic diversity among populations was estimated by the total number of haplotypes, haplotype diversity (Hd) and nucleotide diversity (π) using DnaSP v6.12 software [19].

3. Results and Discussion

Morphological characteristics and growth traits of the samples

In this investigation, a total of 11 plant samples were gathered, each distinguished by unique morphological features. The most notable characteristics include a distinct yellow rhizome and basal leaves with 3-5 segments, setting it apart from other *Coptis* species. The petiole measures 13-25 cm and is smooth, while the leaf blade is ovate, ranging from 7 to 15.5 cm in length and 5.5 to 14 cm in width. The leaves are subleathery, glabrous on the underside, and sparsely puberulous on the veins on the upper side. The base is cordate, and the lateral segments are smaller, either unequally 2-lobed or parted. The central segment is petiolulate, rhombic-lanceolate, 5.5-14 cm in length, and pinnately divided. There are four pairs of segments, each with an acute serrate margin and an attenuate apex. The scapes, reaching a height of 23-28 cm, are glabrous. The inflorescences consist of about six flowers, accompanied by oblong to lanceolate bracts that are 3-lobed or acutely serrate. Unfortunately, the characteristics of the flower remain unknown. The follicles measure 3-6 mm in length, and the stipe is nearly as long as the follicle. Fruit development annually occurs in May (Figure 1.)

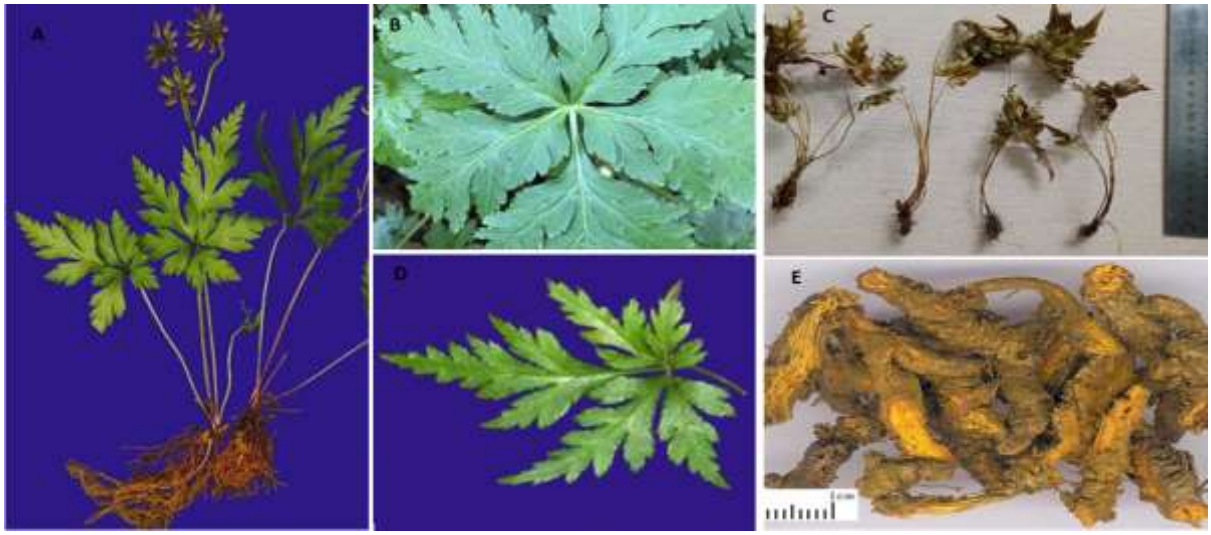

Figure 2. Main morphological traits of *Coptis* samples. A: whole plants including flowers, leaves, stems and roots; B: Leaves with 5 segments from atop view; C: whole plants in dry samples; D: leaves in horizontal shot; D: roots (photos were taken by Nguyen Hoang and [20].

Amplification of ITS gene region and analysis of the nucleotide sequence

The results of electrophoresis on 1% agarose gel of PCR products from 11 samples of *Coptis sp*. showed a single band of about 400 bp for all 11 PCR products. All samples showed no error bands, indicating a 100% success rate of the PCR process. The sequencing process was successfully performed from both directions. The sequences obtained ranged in size from 363 to 371 bp (Table 1)

The comparison with the sequences available on Genbank showed that the samples of *Coptis sp*. obtained had a similarity of 95.71 - 96.37% with the species *Coptis quinquesecta* isolate CO10 (KX714731.1). This indicated that the samples collected in the field were not contaminated by other samples, and the sample preservation process was good and achieved high reliability. It also confirms that the samples of *Coptis sp.* collected in Hoang Lien National Park have a close relationship with the species *C.quinquesecta*. The genus *Coptis* comprises 15 species that are diverse at the morphological level [21]. According to the survey of the National Institute of Medicinal Materials [2], in Vietnam, *Coptis* genus has two species, *Coptis chinensis* Franch and *C. quinquesecta* W.T.Wang, which are distributed in high mountain areas from 1000 - 2500 m, where we collected our samples. Thus, the samples collected in Lao Cai in this study are likely to be the species *C.quinquesecta*. Recently, Xiang *et al*[22] used universal primers to amplify two plastid regions (*trnL-F* and *trnH-psbA*) and one ITS region (primer pair ITS1/ITS4) to distinguish 21 individuals representing 15 species in the genus *Coptis*. Therefore, to accurately identify the exact subspecies of our samples, it is necessary to perform further analysis of both the nuclear ITS gene region and other chloroplast gene regions such as *ITS1/ITS4, ITS1/ITS8, matK, atpB, ndhF, rbcL, tnrH – tnrK*, etc.

Sequence analysis of the ITS region from 11 *C. quinquesecta* samples revealed an average nucleotide composition of 17.11% A, 31.25% C, 32.247% G and 19.41% T. Six SNPs were identified in different positions of the ITS region (Table 2) and verified by chromatogram peaks. Samples HL1, HL2, HL3,

HL4, and HL5 had nucleotide A at position 42 and nucleotide G at position 76, while the rest had nucleotide T and A, respectively. Samples HL6, HL7, HL8 had nucleotide G at position 104, while the rest had nucleotide C. Samples HL9, HL10, HL11 had nucleotide C at position 139, while the rest had nucleotide G. Samples HL6, HL7, HL8 had nucleotide T at position 187, and the rest had nucleotide G. Samples HL1, HL3, HL4, HL5, HL8, HL9 and HL11 had nucleotide C at position 246, while samples HL2, HL6, HL7, and HL10 had nucleotide A. The chromatogram peaks showed no noise signals below the SNPs, suggesting that they were caused by transversion mutations. The sequence differences (Table 2) showed that samples HL1, HL2, HL3, HL4, and HL5 from San Sa Ho commune in Hoang Lien National Park were 100% identical; samples HL6 and HL7, HL9, and HL11 were identical within each group and differed by one nucleotide between groups.

Table 2: The position of SNPs in the nucleotide sequence of *Coptis sp.* samples.

Sample code	Position of nucleotide					
	42	**76**	**104**	**139**	**187**	**246**
HL 1	A	G	C	G	G	C
HL 2	A	G	C	G	G	A
HL 3	A	G	C	G	G	C
HL 4	A	G	C	G	G	C
HL 5	A	G	C	G	G	C
HL 6	T	A	G	G	T	A
HL 7	T	A	G	G	T	A
HL 8	T	A	G	G	T	C
HL 9	T	A	C	C	G	C
HL 10	T	A	C	C	G	A
HL 11	T	A	C	C	G	C

Genetic diversity of Coptis sp. population in Hoang Lien National Park

Genetic relationship analysis of 11 *Coptis sp.* samples using the Kimura-2 parameter model revealed a low genetic distance (0.00 - 0.017) among the samples collected in Hoang Lien National Park, indicating that the population under study had almost no difference, which implied the genetic relationship among the samples are very small difference. This showed a high level of genetic similarity within the comparison population in Hoang Lien National Park. However, the genetic distance between these samples and the reference samples on GenBank was high (0.00 - 1.748), indicating a high nucleotide diversity between them (Table 3). Further analysis of other molecular markers might help to clarify the taxonomic status and phylogenetic relationship of these samples.

Table 3. Pairwise genetic distance between samples of *Coptis sp.* using the Kimura-2 parameter model

	KX714731.1	KX714729.1	X714727.1	KX714733.1	KY780750.1	KC815158.1	HL1	HL2	HL3	HL4	HL5	HL6	HL7	HL8	HL9	HL10	HL11
KX714731.1																	
KX714729.1	0.003																
X714727.1	0.003	0.007															
KX714733.1	0.007	0.010	0.010														
KY780750.1	0.045	0.049	0.049	0.053													
KC815158.1	0.045	0.049	0.049	0.053	0.000												
HL1	1.685	1.711	1.661	1.599	1.546	1.546											
HL2	1.720	1.748	1.695	1.629	1.574	1.574	0.003										
HL3	1.685	1.711	1.661	1.599	1.546	1.546	0.000	0.003									
HL4	1.685	1.711	1.661	1.599	1.546	1.546	0.000	0.003	0.000								
HL5	1.685	1.711	1.661	1.599	1.546	1.546	0.000	0.003	0.000	0.000							
HL6	1.685	1.711	1.661	1.599	1.546	1.546	0.017	0.013	0.017	0.017	0.017						
HL7	1.685	1.711	1.661	1.599	1.546	1.546	0.017	0.013	0.017	0.017	0.017	0.000					
HL8	1.652	1.676	1.629	1.570	1.519	1.519	0.013	0.017	0.013	0.013	0.013	0.003	0.003				
HL9	1.685	1.711	1.661	1.599	1.546	1.546	0.010	0.013	0.010	0.010	0.010	0.013	0.013	0.010			
HL10	1.720	1.748	1.695	1.629	1.574	1.574	0.013	0.010	0.013	0.013	0.013	0.010	0.010	0.013	0.003		
HL11	1.685	1.711	1.661	1.599	1.546	1.546	0.010	0.013	0.010	0.010	0.010	0.013	0.013	0.010	0.000	0.003	

Sliding window analysis in DnaSP revealed six haplotype types in Hoang Lien National Park with a high haplotype diversity (Hd) of 0.85455 (Table 4). The nucleotide diversity (π) of *Coptis sp.* (π = 0.00957) was relatively high compared to its close relative, wild and cultivated *Coptis chinensis* in China with an average π of 0.00197 [20]. Haplotype diversity (Hd) measured the occurrence of different haplotypes in a population, with Hd = 1 indicating that all individuals had unique haplotypes. Nucleotide diversity (π) measured the average proportion of nucleotide differences between pairs of sequences compared in each survey[21]. The genetic diversity (haplotype and nucleotide) of the population was influenced by various factors such as sample size, sampling time, sampling location,

natural selection, mutation rate, gene flow between populations and human activities [25-26]. Generally, medicinal plants with a long history of cultivation and use tended to have a relatively low level of genetic diversity[27]. Species with narrow geographic ranges usually exhibited lower genetic diversity than widespread congeners [28]. In this study, we found that the wild populations of *Coptis sp.* had relatively low nuclear genetic diversity. This might explain the reason why they can only grow in some areas in the Northern mountain's areas of Vietnam.

Table 4. The genetic diversity indices of *Coptis sp.* population

Population	Number of accessions	Nucleotide diversity (π)	Number of haplotype	Haplotype diversity
San Sa Ho	5	0.00132	2	0.4
Ban Khoang	3	0.00219	2	0.67
Pa Cheo	3	0.00219	2	0.67
Total	11	0.00957	6	0.85

Phylogenetic tree construction

Phylogenetic analysis of the ITS region from 11 *Coptis sp*. samples and reference samples of the genus *Coptis* (Figure 2) revealed two distinct groups with high bootstrap support (98%). This confirmed the close relationship of the samples with the genus *Coptis* and suggested that *Coptis sp.* is an endemic species in Lao Cai province. The ITS region also showed two subgroups within the *Coptis sp.* samples. Sample HL8 from San Sa Ho commune formed one subgroup, while the other 10 samples formed another subgroup with moderate bootstrap support (64%). The latter subgroup was further divided into two clusters: HL6 and HL7 from San Sa Ho commune, and the remaining eight samples from Ban Khoang and Pa Cheo communes. This indicated that natural conditions influenced the genetic variation of *Coptis sp.* in Lao Cai and reflected its evolutionary diversity. The ITS region is a common molecular marker for phylogenetic studies of plants, as it can resolve the relationships among closely related species. This study used the ITS region to assess the genetic diversity of some Coptis sp. samples for conservation and development purposes of this medicinal plant. The ITS region sequence was obtained and compared with other sequences from Genbank to infer the evolutionary history and variation of the samples. This approach has been widely applied in plant molecular studies [27]. Other molecular markers, such as nuclear and chloroplast regions, can also be used to complement the ITS region data and improve the taxonomic and phylogenetic resolution of plant species. For example, Liu *et al* [29] used five chloroplast regions (*mat*K, *rbc*L, *psb*A-*trn*H and *ycf*5) and one nuclear region (ITS2) to identify species in the Araliaceae family and found that ITS2 was a robust barcode for this family. Tuong *et al* [30] used the ITS region to analyze the genetic relationship of 23 native rose samples in Vietnam and found that they were highly diverse and influenced by environmental adaptation.

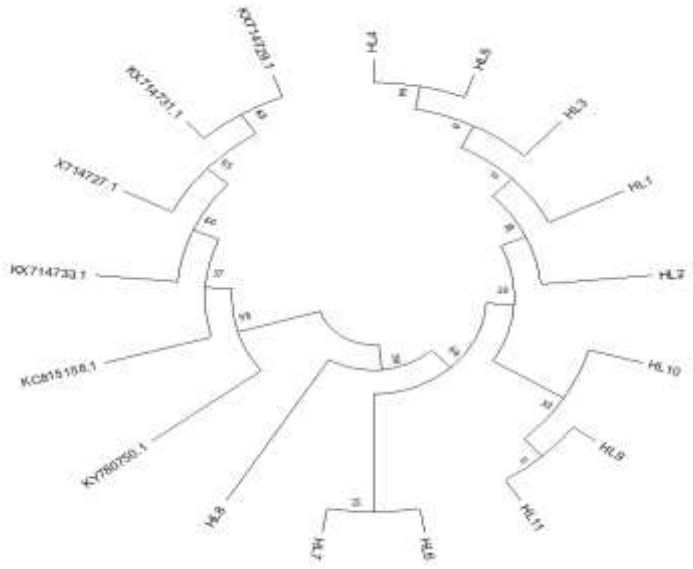

Figure 2. Phylogenetic relationship of 11 *Coptis sp*. samples based on ITS sequence. The numbers near the nodes indicate bootstrap values.

The genetic diversity and structure of rare, endemic, and endangered species are important for understanding their limited distribution and designing effective conservation strategies[7]. In this study, we used nuclear DNA markers to analyze the wild populations of *Coptis sp.* and detected relatively low nuclear genetic diversity, which supports its restricted distribution in the Hoang Lien Son mountains. Therefore, *in situ* conservation measures are needed for this medicinal plant.

4. Conclusion

In conclusion, this is the first report to use nuclear markers to analyze the genetic diversity of *Coptis* species in Hoang Lien National Park. The morphological characteristics of the collected samples exhibited unique morphological traits with distinct yellow rhizomes and basal leaves. Our findings showed that the *Coptis sp.* population had low levels of genetic diversity within the population and high levels of genetic differentiation among the populations in the *Coptis* genus. However, *Coptis sp.* could not be identified using ITS-rDNA sequence analyses. The analysis only suggested that these samples were closely related to *C. quinquesecta* species. A conservation strategy of *in situ* conservation and germplasm collection is recommended for this species. This research is a preliminary step to understanding the genetic patterns of *Coptis* in Hoang Lien Mountain. The data from this study enriched the database system on the genetic diversity of medicinal plant species in Vietnam.

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