Molecular Analysis of Genetic Diversity and Genetic Relationship of *Polygonatum kingianum* Samples Collected in Northern Mountainous Regions in Vietnam

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ABSTRACT

Polygonatum kingianum Coll. ex Hemsl is a medicinal plant belonging to the Convallariaceae family. This species is rarely found in some mountainous areas including Lao Cai province. It has been listed in the Red Book of Vietnam and has been assessed as critically endangered. This study investigated the genetic diversity and phylogenetic relationships of 21 plant samples of *P. kingianum* populations collected from some different areas in northern regions including Hoang Lien Son, National Park using chloroplast DNA *trn*L-F sequences. The results showed that the average nucleotide composition of the *trn*L-F region was 36.22% A, 12.7% C, 17.57% G, and 33.51% T, respectively. The genetic distance value varied from 0.000 to 0.003 according to the Kimura-2 parameter model. Phylogenetic analysis revealed a close relationship between the *P. kingianum* samples collected in Northern mountainous regions and formed a distinct phylogenetic clade from reference species. This study has provided useful information for further plant species identification, evolution, conservation, exploitation and effective use of this valuable genetic resource in this country.

Keywords: Genetic diversity, *Polygonatum kingianum*, genetic relationship, Hoang Lien Son National Park, chloroplast DNA *trn*L-F sequences

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Introduction

Polygonatum kingianum Coll. ex Hemsl is a member of the Convallariaceae family. This plant naturally grows in humid forests, on humus-rich soil in greenstone mountains of northern provinces Vietnam (predominantly in Sa Pa - Lao Cai province). This is a perennial herbaceous plant. The rhizome of *P. kingianum* is known as a medicinal part used as "Thuc hoang", a traditional medicine that has a long history of disease treatments in both Vietnam and China (Chi, 2012; Loi, 2004). "Thuc hoang" has a sweet flavor, neutral property, and functions of tonifying qi, nourishing yin, moistening the lungs, fortifying the spleen, benefiting the kidneys, and curing coughs caused by dehydration, heat, spleen deficiency, debility, fatigue, anorexia, dry mouth, thirst, internal heat, and blood stasis (Chi, 2012; Hong-Min *et al*., 2020). Modern medicine has shown that *P. kinganum* has anti-diabetic, lipid-regulating and disease-preventing effects (Pan-Yue *et al*., 2022; Zhang *et al*., 2021).

In Vietnam, this plant is only naturally harvested from some moutainous areas and has not been cultivated. A few years ago, hundreds of tons of medicinal materials were exploited annually, mainly for export to China. However, due to overexploitation, deforestation, conversion of forest land to agriculture, and other factors, this medicinal resource has become rare and endangered. The Red Book of Vietnam in 1996 listed *P. kinganum* as an endangered species (V category). In 2006, this species was included in the List of Endangered and Rare Forest Plants and Animals (Group 2) to limit its exploitation and commercial use. Nevertheless, illegal harvesting and export still persisted, along with the reduction of forest area, leading to the depletion of this medicinal resource. By 2007, *P. kinganum* was included in the Red Book of Vietnam and was rated as an endangered species (EN category) (Vietnam Red Data Book, 2007). According to our recent surveys, the rhizome of *P. kinganum* has become very scarce, and it is very difficult to find the populations of *P. kinganum* in the natural forests. Globally, the medicinal plant Polygonatum has also been recorded in the IUCN Red List as an endangered species (Gumber *et al*., 2023). In nature, *P. kinganum* grows under the forest canopy at an altitude of over 1000 meters. It is also one of the non-timber forest products that needs to be conserved and developed. Therefore, breeding and cultivation of *P. kinganum* for conservation, exploitation and development is an imperative work in this country.

Germplasm collection and identification are essential for resource conservation, variety selection, and genetic improvement of *P. kinganum* (Jiao *et al*., 2018). Phylogenetics is a powerful tool and a basis for many biological fields, such as developmental genetics, genomics, taxonomy and biogeography (Sevindik *et al*., 2020). Recently, various molecular markers have been developed to overcome the limitations of morphological and biochemical markers in plant phylogenetics, such as nuclear ribosomal DNA of the internal transcribed spacer (ITS), chloroplast DNA, inter-generic spacer (IGS), external transcribed spacer (ETS), and simple sequence repeat (SSR) technology which have been applied in plant classification (Ha *et al.*, 2020; Huong *et al*., 2022; Sevindik *et al*., 2020). Chloroplast DNA (cpDNA) sequence variations are widely used to investigate species-specific relationships among angiosperms and other plants. cpDNA sequences, especially the non-coding regions such as the *trn*L (UAA) intron and the intergenic spacer of *trn*L (UAA)-*trn*F (GAA), are used for their ability to reveal the phylogenetic relationships of the species (Sevindik *et al*., 2020). *trn*L-F region, consisting of the *trn*L intron and *trn*L-F spacer, has become one of the most common chloroplast markers for phylogenetic analyses in plants (Dong *et al*., 2012).

Currently, there are sporadical studies on *P. kinganum* plant in Vietnam, however, these reports have only focused on describing botanical or pharmacological insights. Therefore, in this study, we have made great effort to perform sequence analyses of 21 plant samples of *P. kinganum* samplespopulations collected in some northern mountainous regions using cpDNA *trn*L intron and *trn*L-F sequences to elucidate phylogenetic relationships and genetic diversity among the investigated taxa.

Materials and Methods

Material collection

A total of 21 samples of *P. kinganum* species were collected from different regions in the northern part of Vietnam. The sample information is listed in Table 1. The main rhizome (tubers) and leaves of 21 specimens were collected, labeled and stored in the freezer until processed.

Table 1: List of the collected *Polygonatum kinganum* samples used in this study

No	Sample code	Place of collection	Sequence length	Voucher accession
1	HTD 1	San Sa Ho Commune Hoang Lien National Park	399	OQ532971.1
2	HTD 2		400	OQ532971.1
3	HTD 3		401	OQ532971.1
4	HTD 4		398	OQ532971.1
5	HTD 5	Ban Khoang Commune Sa Pa, Lao Cai	400	OQ532971.1
6	HTD 6		399	OQ532971.1
7	HTD 7		402	OQ532971.1
8	HTD 8		405	OQ532971.1
9	HTD 9	Pa Cheo Commune, Bat Xat, Lao Cai	401	OQ532971.1
10	HTD 10		402	OQ532971.1
11	HTD 11		402	OQ532971.1
12	HTD 12		405	OQ532971.1
13	HTD 13	Trung Leng Ho Commune Bat Xat, Lao Cai	399	OQ532971.1
14	HTD 14		401	OQ532971.1
15	HTD 15		402	OQ532971.1
16	HTD 16	Sa Pa, Lao Cai	397	OQ532971.1
17	HTD 17		398	OQ532971.1
18	HTD 18		398	OQ532971.1
19	HTD 19	Liem Phu Commune, Van Ban, Lao Cai	403	OQ532971.1
20	HTD 20		397	OQ532971.1
21	HTD 21		397	OQ532971.1

Extraction of Genomic DNA

DNA was extracted from young leaves using a standard CTAB protocol with minor modifications (Doyle and Doyle, 1987). A total of 300 mg of the Polygonatum tissues were removed from storage and ground with a CTAB buffer (2% CTAB, 100 mM Tris–HCl pH 8.0, 20 mM ethylenediaminetetraaceticacid (EDTA), and 1.4 M NaCl) and transferred into 1.5 ml Eppendorf tubes. The tubes were vortexed and incubated at 60°C for 30 min. Then, cold chloroform: isoamyl alcohol (24:1) solution was added, mixed, and centrifuged at 13,000 rpm for 15 min. Cold isopropanol was added and mixed with the supernatant in a new tube and kept at -20°C for 30 min to precipitate the DNA. The tubes were centrifuged at 13,000 rpm for 15 min. The DNA precipitate was washed with 70% ethanol and left to air dry. The DNA was re-suspended in 100 µl of TE buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTA). The DNA concentration was estimated spectrophotometrically, and quality was checked by agarose gel electrophoresis (Cong *et al*., 2023). The DNA was diluted to a working concentration of 10 ng/ml.

PCR amplification

The *trn*L-*trn*F IGS regions were amplified by using universal primers *trn*-L (5'- GGT TCA AGT CCC TCT ATC CC-3') and *trn*-F (5'- ATT TGA ACT GGT GAC ACG AG -3') (Vijayan & Tsou, 2010). PCR amplifications were performed with an initial 5 min at 95 ^{o}C, followed by 35 cycles of 1 min at

95^{o} C, 1 min at 58^{o} C, 2 min at 72 ^{o}C and a final 5 min at 72 ^{o}C. Amplifications were performed in 25 µL volumes containing 50 ng of template DNA, 12.5 µL of PCR master mix buffer (2X) (Bioline, Germany), and 10 pmol for each primer. The PCR products were subjected to gel electrophoresis and were cleaned up using a PCR clean-up kit (Promega, USA). Purified PCR products were directly sequenced in two directions using the corresponding primers. The sequencing process was performed using ABI/Prism 377 automated sequencers. The sequence identification was confirmed by comparison with various published sequences available in GenBank.

Analysis of sequencing data

The sequences of the *trn*L-F gene region were processed using BioEdit programs (Hall, 1999). Contigs from forward and reverse sequences of each type were constructed. The obtained sequences were checked and then a bootstrap neighbor-joining tree was generated using MEGA version 5.1. Comparisons with sequences in the GenBank database were achieved through BLAST searches at the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov).

Results and Discussion

Identification of *Polygonatum kinganum* samples based on *trn*L-*trn*F IGS

The 1% agarose gel electrophoresis of PCR products from 21 *P. kinganum* species of Hoang Lien National Park showed a single band of 400 bp for all the 21 PCR products of *P. kinganum* species. All the samples showed the bands without failing, indicating that the PCR amplification and DNA sequencing success rate for *trn*L-*trn*F IGS of *P. kinganum* gene was 100%. The sequencing process was performed from both forward and reverse directions, and the results were successfully verified. The annotated sequences of the *trn*L-*trn*F spacer region varied in length from 397 to 405 bp (Table 1). The average nucleotide composition of *trn*L-F was 36.22% A, 12.7% C, 17.57%G, and 33.51% T. The nucleotide number in each sequence fragment was different among the samples. This difference might be due to InDels at some positions on the gene segment (Ha *et al*., 2022).

A total of 21 sequences were generated for the *trn*L-*trn*F spacer region of *P. kinganum* (Table 1). All of these sequences matched 100% with the only available sequence of this species at this locus in Genbank (Accession Number OQ532971.1). We compared the consensus sequences of 21 *P. kinganum* samples and detected the differences in Table 2. The comparison revealed only 15 SNPs among the sequences, which were verified by the chromatogram. This showed low genetic diversity in *P. kinganum* population. The sequence of sample HTD 3 had a G nucleotide at position 11 while the others had a C nucleotide. The chromatogram showed a peak overlap between G and C at this position, but the G peak was higher, indicating a possible contamination or heterozygous mutation. Similar cases occurred at positions 40 - 364 as shown in Table 2. This result also occurred in the study of Ha *et al*. (2022) who assessed the genetic diversity of 15 samples of *Disporopsis* species by amplifying the *trn*H-*psb*A locus in the chloroplast. In this study, by using the *trn*L-*trn*F primers, the polymorphisms of the 21 samples of *P. kinganum* were determined.

Table 2: The position of SNPs in the nucleotide sequence of *Polygonatum kinganum* samples.

Sample	Position of nucleotide														
	15	40	74	90	127	143	157	188	225	264	275	314	343	381	388
HTD 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 3	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 5	-	-	-	-	-	A	A	-	-	-	-	-	-	-	-
HTD 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 7	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-
HTD 8	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 9	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-
HTD 10	-	-	-	-	C	-	-	-	-	-	G	-	-	-	-
HTD 11	-	-	T		-	-	-	-	-	-	-	-	-	G	-
HTD 12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 15	-	-	-	A	-	-	-	-	-	A	-	-	-	-	C
HTD 16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 17	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-
HTD 18	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-
HTD 19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HTD 21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Genetic relationship analyses of 21 samples of *Polygonatum kinganum*

Genetic distances for 21 samples were calculated using the Kimura-2 parameter model. The genetic distances between the 21 *P. kinganum* genotypes ranged from 0.00 to 0.003, indicating very low nucleotide diversity (Table 3).

Table 3: Pairwise distance between aligned sequences using Kimura-2 parameter model of nucleotide substitution.

	HTD_1	HTD_2	HTD_3	HTD_4	HTD_5	HTD_6	HTD_7	HTD_8	HTD_9	HTD_10	HTD_11	HTD_13	HTD_14	HTD_15	HTD_16	HTD_17	HTD_18	HTD_19	HTD_20	HTD_21	OQ532971.1	MW568447.1	MZ286326.1	OQ532996.1	ON872712.1	MZ286324.1
HTD_1																										
HTD_2	0.000																									
HTD_3	0.000	0.000																								
HTD_4	0.000	0.000	0.000																							
HTD_5	0.000	0.000	0.000	0.000																						
HTD_6	0.000	0.000	0.000	0.000	0.000																					
HTD_7	0.000	0.000	0.000	0.000	0.000	0.000																				
HTD_8	0.000	0.000	0.000	0.000	0.000	0.000	0.000																			
HTD_9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000																		
HTD_10	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000																	
HTD_11	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000																
HTD_13	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000															
HTD_14	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000														
HTD_15	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000													
HTD_16	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000												
HTD_17	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000											
HTD_18	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000										
HTD_19	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.003	0.003	0.000	0.000	0.000	0.000	0.000	0.000									
HTD_20	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000								
HTD_21	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000							
OQ532971.1	1.033	1.037	1.044	1.029	1.022	1.033	1.050	1.041	1.045	1.053	1.048	1.037	1.045	1.048	1.022	1.015	1.026	1.045	1.022	1.022						
MW568447.1	1.033	1.037	1.044	1.029	1.022	1.033	1.050	1.041	1.045	1.053	1.048	1.037	1.045	1.048	1.022	1.015	1.026	1.045	1.022	1.022	0.000					
MZ286326.1	1.033	1.037	1.044	1.029	1.022	1.033	1.050	1.041	1.045	1.053	1.048	1.037	1.045	1.048	1.022	1.015	1.026	1.045	1.022	1.022	0.000	0.000				
OQ532996.1	1.030	1.034	1.041	1.026	1.019	1.030	1.047	1.038	1.043	1.050	1.045	1.034	1.043	1.045	1.019	1.012	1.023	1.043	1.019	1.019	0.003	0.003	0.003			
ON872712.1	1.030	1.034	1.041	1.026	1.019	1.030	1.047	1.038	1.043	1.050	1.045	1.034	1.043	1.045	1.019	1.012	1.023	1.043	1.019	1.019	0.003	0.003	0.003	0.000		
MZ286324.1	1.030	1.034	1.041	1.026	1.019	1.030	1.047	1.038	1.043	1.050	1.045	1.034	1.043	1.045	1.019	1.012	1.023	1.043	1.019	1.019	0.003	0.003	0.003	0.000	0.000	

Using the neighbor-joining method for phylogenetic analysis, the result obtained well-supported tree topologies. Figure 1 shows the neighbor-joining tree based on cpDNA *trn*L intron sequences. All genotypes of *P. kinganum* formed a monophyletic clade, while six reference species grouped into another clade. This indicates that *P. kinganum* from Hoang Lien National Park is endemic due to the distinct terrain and climatic conditions. The *trn*L intron sequences also revealed two clades within *P. kinganum* populations. Clade 1 consisted of HTD 17, which was collected in Ban Khoan commune, Sa Pa. Clade 2 comprised 20 samples, which were further divided into 2 subclades: 2.1 included HTD 21, which was collected in Liem Phu commune, Lao Cai province and 2.2 contained the remaining 19 samples, respectively.

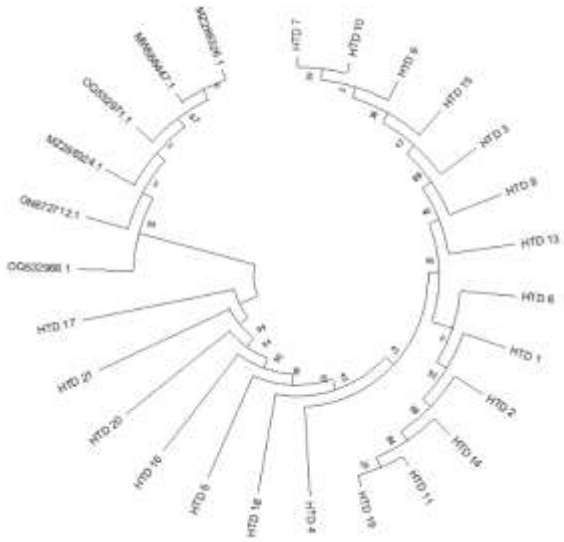

Figure 1: Neighbor-joining tree based on cpDNA trnL-trnF spacer sequences. Numbers near nodes represent bootstrap proportions.

P. kinganum is a medicinal plant with various applications in traditional medicine and health food. However, its morphological variation makes it hard to differentiate it from other verticillate leaf types

of *Polygonatum* species. We found that the molecular evolution rate at the plastid DNA *trn*L-*trn*F spacer locus for *P. kinganum* is very low but still detectable. This suggests a low evolutionary pressure on this medicinal plant at this locus in Hoang Lien mountains. A phylogenetic analysis showed that the chloroplast *trn*L-*trn*F sequence data can be used as a barcode to identify *P. kinganum* species in Hoang Lien National Park and its vicinity and compare them with other far regions. Molecular markers are powerful tools for assessing plant genetic diversity (Ismail *et al*., 2016; Huong *et al*., 2021). Molecular phylogenetics can reveal the genetic structure and the taxonomic relationships of plant varieties beyond the genetic diversity analysis (Sevindik *et al*.,2020). Previous studies have employed different molecular markers such as SSR (Liu *et al*., 2022), EST-SSR and SRAP (Feng *et al*., 2020), and SCoT (Liu *et al*., 2016) to evaluate the genetic diversity of *Polygonatum* species.

Chloroplast DNA has a small genome size and a slow mutation rate in plants, which makes it very suitable for plant phylogenetic studies and resolving taxonomic issues (Liang *et al*., 2020). The chloroplast DNA *trn*L-F region can be applied to address questions related to closely related species and genera (Choulak *et al*., 2017). Similar studies have been conducted in the past with sequences and populations of important plant species such as Turkish cotton (*Gossypium hirsutum* L.) lines (Hocaoglu-Ozyigit *et al*., 2022), Turkish *Prunus armeniaca* L. genotypes (Sevindik *et al*., 2020), *Laurus nobilis* L. (Lauraceae) populations (Sevindik *et al*., 2020), and *Alsophila spinulosa* in southern China (Su *et al*., 2005). Wang *et al*. (2022) analyzed the genetic diversity and phylogenetic structure of 19 Polygonatum species using eight regions of the cpDNA (*rps*16-*trn*Q-UUG, *trn*S-GCU-*trn*G-UCC, *rpl*32-*trn*L-UAG, *mat*K-*rps*16, *pet*A-*psb*J, trnT-UGU-*trn*L-UAA, *acc*D-*psa*I, and *trn*C-GCA-petN). They identified eight highly variable loci across the Polygonatum cp genome that could serve as potential markers for phylogenetic and population genetics studies. Their work showed that the cp genome within Polygonatum is highly conserved. Shi *et al*. (2023) performed a systematic analysis of six verticillate leaf types of *Polygonatum* species by de novo assembling nine complete chloroplast genomes. The newly sequenced genomes ranged from 155,437 to 155,977 bp in total length and exhibited well-conserved genomic structures and gene orders.This study has provided a valuable barcode for the development of molecular markers to distinguish *P. kingianum* Lao Cai from other related verticillate leaf types of *Polygonatum* species and for phylogenetic analyses of the *Polygonatum* Lao Cai species with verticillate leaf types.

In conclusion, this study is the first time to report on genetic stability of *P. kinganum* in Northern region including Hoang Lien National Park and its vicinity, Vietnam using *trn* IGS region analysis. The results displayed that the molecular evolution rate of this species at *trn*L-F locus is very low. Moreover, our analyses revealed a distinct clade of Vietnam *P. kingianum* isolates, which is very interesting. The results of this study offer valuable information for the development of molecular markers and phylogenetic analyses of the *P. kinganum* species in this country.

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