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Evaluation of genetic diversity in *Stephania brachyandra* Diels from Thai Nguyen province, Vietnam

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ABSTRACT

While *Stephania brachyandra* Diels represents a unique native medicinal plant in the north of Vietnam, this study aimed to investigate the variation in the morphological characteristics, genetic diversity, and molecular identification of *S. brachyandra* populations collected from five particular districts in Thai Nguyen Province, a mountainous region of Vietnam. Though differences in stem length (1.3–4.5 m), fruit size (0.6–0.8 cm), and leaf dimensions could be noticed, morphological observations revealed that the collected samples were generally similar to reported taxonomic descriptions, probably reflecting the changes in local environmental conditions such as elevation and slope. Genetic diversity was quantified using six polymorphic inter-simple sequence repeat (ISSR) primers, which generated 52 bands with an overall polymorphism rate of 53.46%. Genetic diversity was evaluated using six polymorphic ISSR primers, which generated 52 bands with an overall polymorphism rate of 53.46%. The mean genetic diversity indices were $h = 0.194$ and $I = 0.290$, indicating a moderate level of genetic diversity. Analysis of molecular variance revealed that 57% of the total genetic variation occurred within populations, whereas 43% occurred among populations ($\Phi PT = 0.432$, $p \leq 0.001$), demonstrating critical genetic distinction. Substantial variation in genetic diversity was detected across populations, in which Phu Luong and Dai Tu showed the greatest genetic variability, and Dinh Hoa exhibited the lowest. Patterns of genetic distance revealed a strong genetic association among the Dinh Hoa, Dai Tu, and Dong Hy populations, while Vo Nhai was clearly differentiated from the remaining populations. Subsequent DNA barcode analyses corroborated the species-level identification of all collected specimens. The rbcL sequences showed 99.85%–100% similarity to the reference sequence of *S. brachyandra* Diels in GenBank, while internal transcribed spacer sequences showed 98.47%–99.25% similarity. Phylogenetic analyses consistently clustered all samples with *S. brachyandra* and clearly separated them from the closely related species *Stephania cephalantha*. The present study confirms the identity of *S. brachyandra* populations distributed in Thai Nguyen Province and indicates moderate genetic diversity characterized by considerable genetic variation within populations. The findings offer valuable scientific evidence to support conservation planning, sustainable resource management, germplasm conservation, and future genetic improvement programs for this valuable medicinal species.

1. INTRODUCTION

Vietnam is known globally as a biodiversity hotspot, and it has a rich flora of significant medicinal, ecological, and economic value. Among many plant species, *Stephania brachyandra* Diels (Bình vôi) has been extensively utilized as a medicinal plant in Vietnamese traditional medicine because its tubers contain the alkaloid rotundine, which possesses sedative properties that have been employed to cure insomnia symptoms as well as anxiety disorders. On the other hand, *S. brachyandra* Diels has also been used to support vegetation and prevent soil erosion, contributing to stabilize ecological systems in mountainous areas. These dual functionalities of medicinal and ecological values make it an important plant species that should be conserved.

Vietnam is known globally as a biodiversity hotspot, and it has a rich flora of significant medicinal, ecological, and economic value. Amid many plant species, *S. brachyandra* Diels (Bình vôi) has been extensively utilized as a medicinal plant in Vietnamese traditional medicine because its tubers contain the alkaloid rotundine, which possesses sedative properties that have been employed to cure insomnia symptoms as well as anxiety disorders [1,2]. On the other hand, *S. brachyandra* Diels has also been used to support vegetation and prevent soil erosion, contributing to stabilize ecological systems in mountainous areas [3,4]. These dual functionalities of medicinal and ecological values make it an important plant species that should be conserved.

However, *S. brachyandra* Diels populations are decreasing because of overexploitation, habitat degradation, and genetic degradation,

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especially in mountainous areas such as Thai Nguyen province [2]. Conservation becomes even more complicated by taxonomic ambiguity, including morphological characters, stem morphology, and tuber form, which are conventionally used to distinguish *S. brachyandra* Diels from closely related taxa [4]. The appearance of various morphological types in the same area has raised concerns regarding taxonomic misidentification and the introduction of non-native germplasm. Such uncertainties can hinder accurate conservation planning and the sustainable use of medicinal plant resources [4].

The genetic diversity assessment is therefore crucial. Genetic diversity not only reflects the adaptive potential and evolutionary resilience of species but also provides a basis for long-term conservation strategies [5–7]. Techniques based on retrotransposon markers, such as inter-simple sequence repeat (ISSR), have been widely applied to reveal polymorphisms and assess genetic variation even in species lacking complete genome sequences [8–10]. These marker systems, developed from the stable chromosomal integration patterns of retrotransposons, have demonstrated their effectiveness in various plant taxa, including *Triticum turgidum* ssp. *dicoccoides* (wild emmer wheat), where they successfully revealed extensive genetic variability [11]. In addition, improved DNA isolation and purification methods, such as the optimized acidic cetyltrimethylammonium bromide (CTAB) protocol for complex biological samples [12,13], have enhanced the reliability of cetyltrimethylammonium bromide (CTAB), polymerase chain reaction (PCR)-based genotyping by minimizing contamination from polysaccharides and phenolic compounds.

In Vietnam, 11 species of *Stephania* have been recorded [2,14,15]. Although some studies have examined the chemical composition and pharmacological properties of *S. brachyandra* Diels [4,16–19], and some research has applied DNA barcoding or chloroplast genome analysis to related *Stephania* species, comprehensive studies integrating both morphological and population-level genetic data for *S. brachyandra* Diels in Vietnam remain lacking. Particularly, there is still limited information on intraspecific genetic variation and population structure of *S. brachyandra* Diels throughout its natural distribution.

To this end, the present study integrates morphological verification with ISSR markers and DNA barcoding [(rbcL, internal transcribed spacer (ITS)] to evaluate genetic diversity and clarify taxonomic relationships among *S. brachyandra* Diels populations in Thai Nguyen Province. ISSR markers were selected due to their cost-effectiveness, reproducibility, and suitability for detecting polymorphism in non-model species where genomic resources are limited, making them appropriate for preliminary population genetic assessment.

This study represents the first population-level genetic assessment of *S. brachyandra* Diels in Vietnam and the first to integrate both morphological and molecular data for this species in the country. By revealing patterns of morphological variation and genetic differentiation among populations, the study provides essential baseline information for conservation planning, population prioritization, and the sustainable utilization of this medicinal plant.

2. RESEARCH METHODOLOGY

2.1. Materials

Stephania brachyandra Diels samples were collected from five districts in Thai Nguyen Province, namely Dai Tu, Dong Hy, Dinh Hoa, Vo Nhai, and Phu Luong. These sampling sites were selected to represent the major natural distribution areas of the species within the province and to capture potential ecological and geographic variation among populations.

In each district, 12 mature leaf samples were collected from spatially separated individuals, with a minimum distance of 20–30 m between sampled plants, to reduce the likelihood of repeatedly sampling clonal individuals and to better represent independent plants within each population. In total, 60 samples were collected. However, because *S. brachyandra* Diels may reproduce vegetatively, the possibility that some sampled individuals were clonally related cannot be completely excluded, and this should be considered a limitation of the sampling design.

The geographic distances among populations ranged from approximately 15 to 70 km, reflecting moderate spatial separation across the study area. Fresh leaf samples were kept in cool gel packs during transport and subsequently stored in the laboratory for genomic DNA extraction. Detailed information on sample codes and collection sites is provided in Table 1.

Genetic diversity was assessed using six ISSR primers, with sequences and annealing temperatures listed in Table 2.

2.2. Research Methods

2.2.1. Morphological identification

Species identification was conducted based on the morphological characteristics of *S. brachyandra* Diels samples collected from Thai Nguyen Tables 3 and 4. Key traits, including leaf shape and color, stem morphology, flower structure, and tuber appearance, were recorded and analyzed. The data collected were then compared with taxonomic descriptions provided by [20–23] to confirm species identity. Samples within each population were collected at intervals of approximately 20–30 m to reduce the likelihood of repeatedly sampling the same genet. However, because *S. brachyandra* Diels may reproduce vegetatively, the possibility of collecting clonally related individuals cannot be completely excluded, and this should be considered a limitation of the sampling design Figure 1.

Table 1. Collection sites and sample codes of *S. brachyandra* Diels in Thai Nguyen.

No	Origin	No. of samples	Sample codes
1	Dai Tu	12	DT1-DT12
2	Dong Hy	12	DY1-DY12
3	Dinh Hoa	12	DH1-DH12
4	Vo Nhai	12	VN1-VN12
5	Phu Luong	12	PL1-PL12
Total		60 samples	

Table 2. ISSR primers used in this study.

No	Primer ID	Sequence (5′–3′)	Annealing temperature (°C)
1	UBC808	AGAGAGAGAGAGAGAGC	48.8
2	UBC811	GAGAGAGAGAGAGAGAC	46.8
3	UBC818	CACACACACACACACAG	51.0
4	UBC856	ACACACACACACACACYA	52.8
5	UBC881	GGGTGGGGTGGGGTG	59.3
6	ISCS14	AGTGAGTGAGTGAGTGAGTGA	55.0

2.2.2. Genomic DNA extraction (summary)

Genomic DNA was extracted from mature leaf samples of *S. brachyandra* Diels using a modified CTAB protocol [24]. DNA quality and concentration were assessed by agarose gel electrophoresis and NanoDrop spectrophotometry, and only samples with ≥20 ng/µl and an A260/280 ratio of 1.8–2.0 were used for further analyses.

2.2.3. ISSR-PCR amplification

ISSR-PCR amplification was carried out following the method originally described by Zietkiewicz *et al.* [25], with minor modifications. Species identity of 12 samples was first confirmed using rbcL and ITS DNA barcoding, while ISSR markers were used for genetic diversity analysis. Phylogenetic analysis of rbcL and ITS sequences was performed using the neighbor-joining method under the Kimura 2-parameter model with 1,000 bootstrap replicates. PCR reactions were carried out in 25 µl volumes containing 25 ng DNA template, 1× PCR buffer (1.5 mM MgCl2), 0.4 µM primer, 0.2 mM dNTPs, and 1 U Taq DNA polymerase (Thermo Scientific, USA). Amplification was performed in a GeneAmp PCR System 9,700 (Applied Biosystems, USA). The thermal cycling program consisted of initial denaturation at 94°C for 5 minutes; 40 cycles of 94°C for 45 seconds, annealing at 46.8°C–59.3°C for 45 seconds, and 72°C for 90 seconds; followed by a final extension at 72°C for 7 minutes. Fifteen ISSR primers were screened, and six primers (UBC808, UBC811, UBC818, UBC856, UBC881, and ISCS14) showing clear and reproducible polymorphic patterns were selected. These primers generated 52 scorable bands (100–1,600 bp), with 53.46% polymorphism. PCR products were separated on 1.8% agarose gels in 1× TAE buffer, stained with RedSafe, and visualized under UV light. Fragment sizes were estimated using a 1 kb DNA ladder (Thermo Scientific, USA) [26,27].

2.3. Data Analysis

Morphological data were analyzed using one-way analysis of variance (ANOVA) in SPSS 20.0 (IBM Corp., USA) to test for significant differences among populations. Mean values of morphological traits were compared, and significance levels were assessed at $p < 0.05$. ISSR bands were scored as binary data (1/0) and analyzed in GenAlEx 6.5 to calculate genetic diversity indices percentage of polymorphic bands (PPB, He, I) and perform analysis of molecular variance (AMOVA) with 999 permutations. Cluster analysis (UPGMA based on Jaccard's coeffcient) and Principal Coordinates Analysis (PCoA) for five population-level points were conducted using NTSYS 2.11 and GenAlEx, respectively. DNA sequences of rbcL and ITS were aligned and analyzed in MEGA 7.0 for phylogenetic confirmation of species identity. Sequence alignment and comparative analysis were performed using ClustalW algorithm in BioEdit software. NCBI GenBank database was employed to perform the comparison of nucleotide sequences of *S. brachyandra* Diels samples with available sequences of *S. brachyandra* Diels.

3. RESULTS AND DISCUSSION

3.1. Morphological Characteristics of *S. brachyandra* Diels in Thai Nguyen

Although several changes in morphologies are observed due to the difference of environmental conditions, *S. brachyandra* Diels still exhibits morphological appearances that are well-matched with previous descriptions [22,28,29]. This species typically grows at elevations of 180–297 m with slopes ranging from 20° to 39°, and it has climbing, twining green stems from 1.3 to 4.5 m in length and 0.5–0.8 cm in diameter at the root collar. From the samples collected in Thai Nguyen province, it can be noted that the shortest stems in length from 1.3 to 3.5 m were observed in the Dinh Hoa area, meanwhile, the longest stems in length from 1.8 to 4.5 m were revealed in the Vo Nhai area. This result confirms that geographic variation certainly influences the morphological appearance of *S. brachyandra* Diels [29]. Besides, leaves taken from different areas are generally alternate, ovate, and pointed tips with an average length of 9–12 cm and a width of 5–7.5 cm. The petiole ranges from 3.3 to 5.4 cm.

In the flowering season from May, *S. brachyandra* Diels has pale yellow flowers, and fruits ripen between September and November. The sizes of fruits vary from 0.6 to 0.8 cm, while the average weight of a single fruit is 65–82 mg, with approximately 1,360–1,650 fruits per 100 g. In each fruit, dark-brown colored seeds with 6.0 × 5.1 × 1.5 mm dimensions are measured, and around 3,900–4,000 seeds can be found per 100 g. The tube has an external dark-brown color with a whitish to pale yellow interior when scraped. The findings in morphological variation, including stem length and fruit size, further suggest that the environmental conditions may influence the characteristics of *S. brachyandra* Diels. [16,27]. Therefore, based on these results, it should be noted that local studies providing appropriate strategies for conservation and sustainable utilization of this medicinal plant are critically important.

3.2. Genetic Diversity Assessment Results of *S. brachyandra* Diels Samples

3.2.1. Genomic DNA quality and ISSR-PCR results

Prior to genetic analysis, 12 collected samples were verified using rbcL and ITS DNA barcoding sequences to ensure their identity as *S. brachyandra* Diels. These markers were used individually for species



Figure 1. Morphological features of *S. brachyandra* Diels (leaves, tuber, flowers, and ripe fruits).

Table 3. Summary of morphological traits of *S. brachyandra* Diels across five districts in Thai Nguyen.

District	Elevation (m)	Slope (°)	Stem length (m)	Stem diameter (cm)	Stem surface	Leaf type/ arrangement	Petiole length (cm)	Leaf length (cm)	Leaf width (cm)	Flower type/color	Fruit type	Seed
Dai Tu	190–295	20–39	2.0–3.6	0.6–0.8	Smooth	Ovate, alternate	3.3–4.7	10–12	6–7	Cluster/pale yellow	Ovate	1
Dong Hy	190–297	20–39	1.6–3.9	0.5–0.8	Smooth	Ovate, alternate	4.1–4.6	10–12	6–7	Cluster/pale yellow	Ovate	1
Dinh Hoa	180–292	24–39	1.3–3.5	0.5–0.8	Smooth	Ovate, alternate	4.2–4.6	10–12	6–7.5	Cluster/pale yellow	Ovate	1
Vo Nhai	180–290	25–39	1.8–4.5	0.5–0.8	Smooth	Ovate, alternate	4.1–4.4	11–12	6–7	Cluster/pale yellow	Ovate	1
Phu Luong	190–297	25–39	1.5–3.3	0.6–0.8	Smooth	Ovate, alternate	4.1–5.4	9–12	5–7	Cluster/pale yellow	Ovate	1

Table 4. Statistical summary (one-way ANOVA).

Trait	*F*-value	*p*-value
Stem length	1.27	0.31
Stem diameter	0.94	0.44
Petiole length	1.52	0.22
Leaf length	0.88	0.47
Leaf width	1.11	0.36

Note: One-way ANOVA ($p < 0.05$) showed no significant differences among districts for all measured traits ($p > 0.05$); therefore, no post hoc grouping letters are presented. Values in the table are expressed as observed ranges.

identification, whereas ISSR markers were employed to determine genetic diversity. In the case of phylogenetic analysis, rbcL and ITS sequences were aligned with GenBank references using standard multiple sequence alignment. Phylogenetic trees were constructed using the neighbor-joining method under the Kimura 2-parameter model, utilizing 1,000 bootstrap replicates for evaluating node support. In the case of ISSR analysis, 15 primers were first screened, and 6 polymorphic primers with clear amplification patterns were selected. PCR reactions were performed in 25 µl volumes. The thermal cycling conditions were identical for all primers except for annealing temperature (46.8°C–59.3°C), and consisted of initial denaturation at 94°C for 5 minutes, followed by 40 cycles of 94°C for 45 seconds, annealing for 45 seconds, and extension at 72°C for 90 seconds, with a final extension at 72°C for 7 minutes.

Six chosen primers produced 52 reproducible bands varying from 100 to 1,600 bp with a polymorphism rate of 53.46%, showing possible genetic variation among *S. brachyandra* Diels populations. This polymorphism level is considered a proper level for assessing genetic diversity, which is consistent with reported ISSR-based studies in the literature [2,4]. ISSR bands were scored based on clear and reproducible amplification profiles, and only distinct fragments were included in the analysis. Representative amplification patterns are shown in Figure 2.

It can be noted that the banding profiles unveiled variation among populations, indicating the presence of genetic differentiation among provenances. All ISSR data were sufficiently clear and reproducible for reliable scoring and subsequent statistical analysis. Together, DNA barcoding and ISSR results provide molecular evidence for species authentication and preliminary assessment of genetic variation in *S. brachyandra* Diels, supporting future conservation and breeding studies of this medicinal species.

3.2.2. Results of genetic diversity assessment across provenances

The processed data yielded results on the genetic diversity of *S. brachyandra* Diels populations, as presented in Table 5. Key indicators of genetic diversity—including the number of observed alleles (Na), the number of effective alleles (Ne), Shannon's information index (I), Nei's gene diversity index (h), and the PPB—were compared among the populations of *S. brachyandra* Diels collected from different localities within Thai Nguyen Province.

The analysis of genetic diversity in *S. brachyandra* Diels populations from Thai Nguyen revealed a mean Nei's gene diversity of $h = 0.194$, Shannon's information index of $I = 0.290$, and PPB = 53.46%, indicating a moderate level of genetic diversity at the population level. This value is lower than that reported for several other medicinal plants analyzed using ISSR markers, such as *Gymnema sylvestre* ($H = 0.26$, $I = 0.40$, PPL = 80.89%) and *Mallotus oblongifolius* (mean $h = 0.223$, mean $I = 0.334$, mean PPB = 65.14%), both of which showed comparatively higher within-species variation. By contrast, the diversity observed in *S. brachyandra* Diels is clearly higher than that reported for the endangered medicinal species *Breonadia salicina*, for which ISSR analysis detected very low diversity ($h = 0.086$, $I = 0.125$, PPL = 21.3% at the population level) [30]. In the same genus, a recent study on *Stephania rotunda* in Vietnam reported 70.76% polymorphic loci based on ISSR markers and high genetic dissimilarity among accessions, suggesting that genetic variation in *Stephania* may differ substantially depending on species biology, sampling scale, and habitat conditions [31].

The medium level of genetic diversity found in *S. brachyandra* Diels populations may be attributed to the combination of various factors such as gene flow restriction, local seed dispersal, and habitat fragmentation. Since the examined populations were primarily taken from mountainous areas, the spatial isolation may decrease the exchange between pollen and seed, which eventually leads to the genetic variation among populations. Similar findings have also been reported for other medicinal plant species, in which geographic barriers and limited dispersal play important roles in shaping population structure. For example, high population differentiation in *Euphorbia resinifera* has been linked to low gene flow caused by mountain barriers, discontinuous distribution, limited pollinator movement, and restricted seed migration.

The level of genetic diversity observed in *S. brachyandra* Diels appears to be intermediate when compared with that reported for other medicinal plant species analyzed using dominant molecular markers. This result implies that though this medicinal species still maintains acceptable genetic preservation, the variation may still be

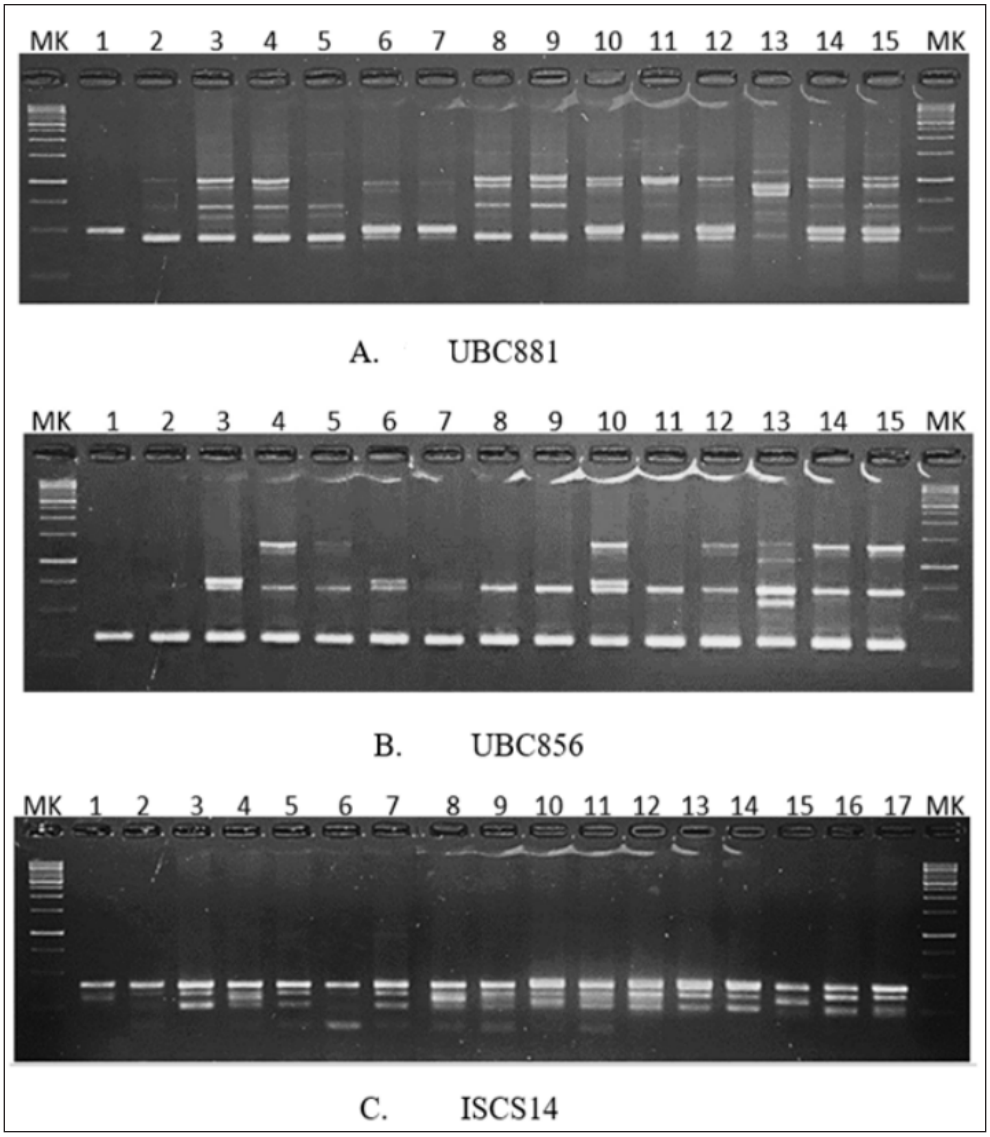

Figure 2. ISSR-PCR results of selected *S. brachyandra* Diels samples analyzed in this study.

Table 5. Genetic diversity parameters of *S. brachyandra* Diels across different provenances.

	Genetic diversity indices				
Provenance	**Na**	**Ne**	***I***	***h***	**PPB**
Dai Tu	1.269	1.350	0.311	0.207	59.62
Dinh Hoa	1.058	1.244	0.217	0.145	40.38
Dong Hy	1.231	1.305	0.270	0.182	48.08
Phu Luong	1.288	1.424	0.354	0.242	61.54
Vo Nhai	1.346	1.336	0.298	0.198	57.69
Mean	1.238	1.332	0.290	0.194	53.46
Standard error (SE)	0.055	0.023	0.018	0.012	4.01

vulnerable to ongoing habitat disturbance and population isolation. In addition, as *S. brachyandra* Diels reproduces via either vegetative propagation or short-distance seed dispersal, the aforementioned biological characteristics could further restrict allele exchange among populations. This may help explain the observed differentiation among populations and the relatively higher diversity detected in some populations, such as Phu Luong and Dai Tu, compared with others. In summary, these results suggest that the current genetic resources of *S. brachyandra* Diels are still sufficient for conservation planning; however, the moderate diversity level also indicates vulnerability to continued habitat disturbance and overharvesting. Therefore, high diversity populations should be critically considered for *in-situ* conservation, while genetically representative specimens from diverse populations should be included in future *ex-situ* conservation and gene resource management programs [32].

3.2.3. Genetic variation among S. brachyandra Diels provenances

AMOVA was utilized to verify the genetic differentiation among and within the *S. brachyandra* Diels provenances. As displayed in Table 6, the results revealed that 43% of the total genetic variation was attributed to differences among provenances, while 57% belonged to the variation among individuals within the same provenance. This differentiation was found to be statistically significant ($p \leq 0.001$), indicating a strong population structure among populations.

The genetic distances between five *S. brachyandra* Diels provenances varied from 0.049 to 0.283, showing a moderate level of genetic differentiation in Thai Nguyen Province. This level is compatible with reported studies for medicinal plants in the literature, which have reported the degrees of genetic divergence among geographically separated populations [18,22]. As shown in Table 7, a strong correspondence was observed between genetic distance and similarity coefficients across all provenances. The closest genetic relationship recorded was between Dai Tu and Dinh Hoa, with the lowest genetic distance of 0.049 and the highest similarity coefficient of 0.952. Dai Tu and Dong Hy also showed a comparatively closed genetic relationship (distance = 0.098; similarity = 0.907), demonstrating a high level of genetic relatedness among these populations. By contrast, Vo Nhai's provenance exhibited the greatest genetic divergence compared to other populations. Particularly, the genetic distance to Dinh Hoa, Dai Tu, and Dong Hy was 0.283, 0.259, and 0.234, respectively, whereas lower similarity coefficients of 0.753, 0.772, and 0.792 were also measured. This result proves that the Vo Nhai population possessed high genetic distinctness, probably due to the geographical isolation or distinct environmental conditions. Besides, Phu Luong showed intermediate genetic relationships with the remaining provenances, in which a genetic distance ranging from 0.143 to 0.208 and similarity coefficients from 0.812 to 0.867 were detected. Overall, genetic similarity coefficients ranged from 0.753 to 0.952 (Table 7), reflecting a generally moderate to high level of genetic relatedness among the studied populations. The relatively high ΦPT value of 0.432 further validates limited gene flow among provenances, which may be possibly caused by natural geographic barriers such as mountainous terrain and habitat fragmentation in Thai Nguyen province. Restricted pollen, seed dispersal, and local environmental heterogeneity may have reinforced this genetic structure over time.

Table 6. AMOVA results for five *S. brachyandra* Diels provenances.

AMOVA	Percentage of total variation (%)
Among provenances	43
Within provenances	57
PhiPT	0.432; $p \leq 0.001$

These results further demonstrated that geographic isolation and environmental variation represent pivotal features in genetic structure in plant populations, which is also reported elsewhere [12,14,18]. It is suggested that *S. brachyandra* Diels populations in Thai Nguyen province could be capable of adapting to different ecological conditions, indicating the importance of conserving these genetic resources for sustainable management and future utilization.

The analysis was performed with MEGA X software, which generated dendrograms illustrating the genetic relationships among the five *S. brachyandra* Diels provenances and among 60 individual samples from these provenances, as shown in Figure 3.

The dendogram in Figure 3 showed that five *S. brachyandra* Diels provenances were divided into two groups, reflecting their genetic differentiation within Thai Nguyen Province. While the first group contains Vo Nhai and Phu Luong provenances with higher genetic divergence from the other populations, the second group includes the remaining three provenances, which are then subdivided into two subgroups, including Dong Hy individually, and Dinh Hoa and Dai Tu. This grouping methodology is well-matched with the above-mentioned genetic distances, supporting the hypothesis that local environmental factors and geographical isolation may play a significant role in shaping the genetic structure of *S. brachyandra* Diels populations in Thai Nguyen.

Cluster analysis using the UPGMA method from ISSR data revealed the genetic relationships among 60 individual samples as presented in Figure 4. The dendrogram shows that individuals had a grouping tendency based on their geographic origins, though some mixing of

Table 7. Genetic distance (below diagonal) and genetic similarity (above diagonal) among five *S. brachyandra* Diels provenances in Thai Nguyen province.

Dai Tu	Dinh Hoa	Dong Hy	Phu Luong	Vo Nhai	
-	0.952	0.907	0.849	0.772	Dai Tu
0.049	-	0.936	0.812	0.753	Dinh Hoa
0.098	0.066	-	0.823	0.792	Dong Hy
0.163	0.208	0.195	-	0.867	Phu Luong
0.259	0.283	0.234	0.143	-	Vo Nhai

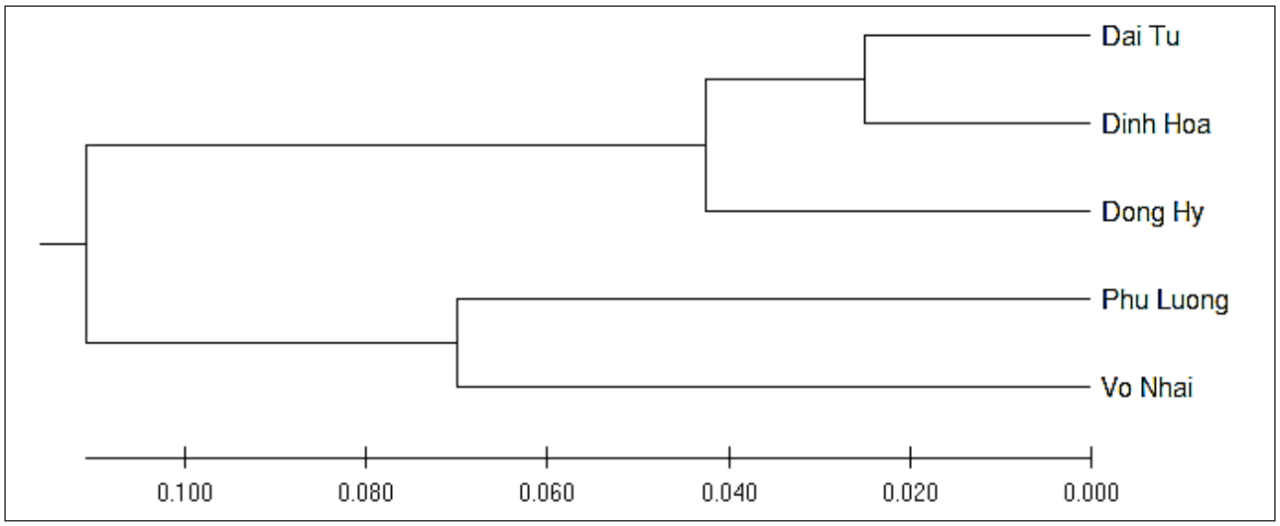

Figure 3. Dendrogram of genetic relationships among *S. brachyandra* Diels samples from five provenances.

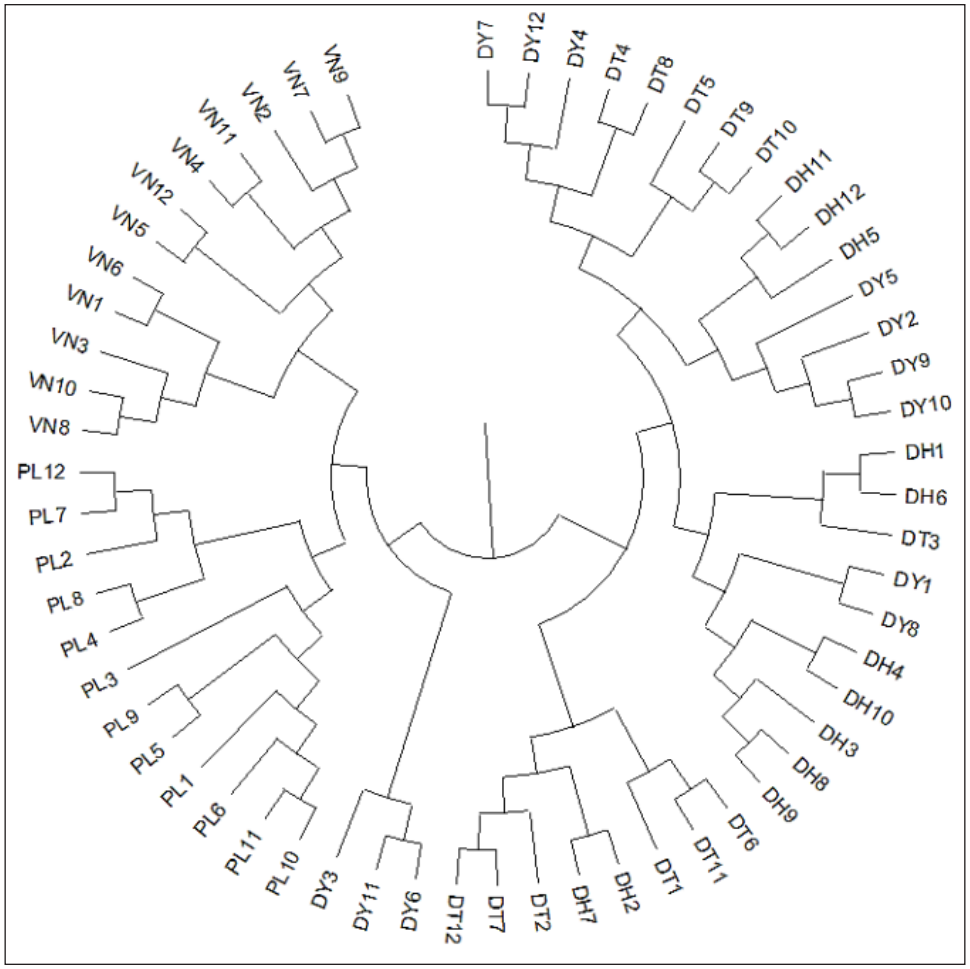

Figure 4. Dendrogram of genetic relationships among 60 *S. brachyandra* Diels samples from five provenances.

clusters was observed. This cluster pattern suggests that populations are not completely isolated; however, genetic differentiation is still observed. This could be due to the restricted gene flow and spatial separation among provenances that have contributed to the observed genetic structure.

PCoA was subsequently performed to visualize the genetic relationships among five population-level points (Fig. 5). PCoA results displayed a moderate separation for all individuals according to their provenances, which is compatible with the above UPGMA clustering pattern. Although overlap among groups could be seen, individuals from the same geographic origin tended to cluster more closely in the multivariate space. This strongly supports the presence of partial genetic structuring within *S. brachyandra* populations in Thai Nguyen Province.

3.3. Results of Gene Sequencing at Selected Loci in *S. brachyandra* Diels

3.3.1. Nucleotide sequence analysis of gene regions in S. brachyandra Diels samples

The nucleotide sequences of 12 *S. brachyandra* Diels samples from *rbcL* and *ITS* were analyzed, which is then compared with available sequences of *S. brachyandra* Diels in NCBI GenBank database (including CB1 and CB2 as reference specimens). Phylogenetic trees were then constructed using tools from the NCBI database to visualize genetic relationships among the sequences.

The phylogenetic clustering indicated that samples from the same provenance showed a tendency to group closely together, validating a correlation between genetic similarity and geographic origin. It is noticeable that samples taken from Vo Nhai formed a distinct subclade, possibly reflecting local environmental variation or limited gene flow in comparison with other areas. This confirms that both geographic separation and ecological conditions significantly contribute to the genetic differentiation among populations.

3.3.2. rbcL gene region

Following the sequencing of the *rbcL* gene region in the *S. brachyandra* Diels samples, the results are presented in Table 8, which includes nucleotide sequence length and AT–GC composition. The analysis of the *rbcL* region indicated that all studied samples exhibited a higher AT content compared to GC content. Specifically, the average AT composition was 55.67%, while the average GC content was 44.33%. The mean sequence length for the *rbcL* region was approximately 661 base pairs (bp).

This AT-rich composition is typical for chloroplast genes and indicates good sequencing quality. The high similarity in sequence length and composition among samples suggests low inter-population divergence

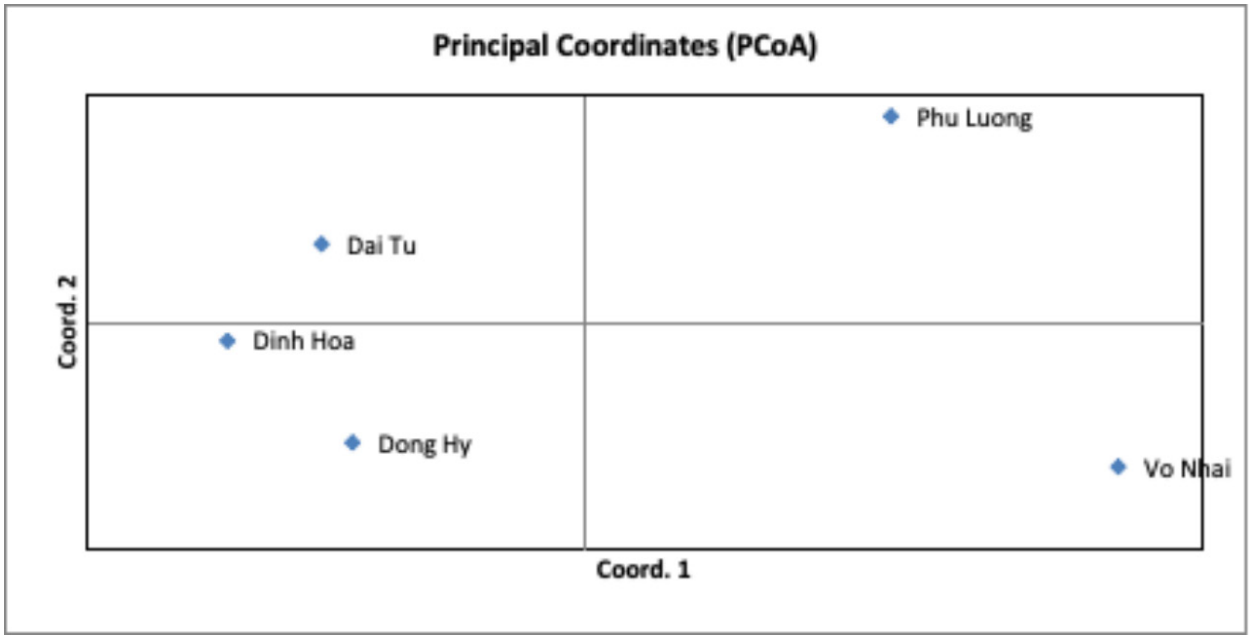

Figure 5. PCoA results of five *S. brachyandra* Diels provenances from Thai Nguyen province.

Table 8. Nucleotide composition of 12 *S. brachyandra* Diels samples in the rbcL gene region.

No.	Sample	Percentage (%)		Size (bp)
		A + T	G + C	
1	DT3	55.87	44.13	664
2	DT6	55.71	44.29	666
3	DY9	55.71	44.29	666
4	DY12	55.96	44.04	663
5	DH8	55.56	44.44	657
6	DH12	55.74	44.26	662
7	PL2	55.49	44.51	656
8	PL12	55.49	44.51	656
9	VN3	55.64	44.36	665
10	VN9	55.49	44.51	656
11	CB1	55.74	44.26	662
12	CB2	55.56	44.44	667
Average		55.67	44.33	661.67

Table 9. Sequence comparison results of the rbcL gene region in the GenBank database.

No.	Sample	Similarity coefficient (%)	
		Stephania brachyandra (Accession No. JN051690.1)	*Stephania cephalantha* (Accession No. JN051691.1)
1	DT3	100	99.85
2	DT6	100	99.85
3	DY9	100	99.85
4	DY12	99.85	99.85
5	DH8	100	99.85
6	DH12	100	99.85
7	PL2	100	99.85
8	PL12	100	99.85
9	VN3	100	99.85
10	VN9	100	99.85
11	CB1	100	99.85
12	CB2	100	99.85

at the *rbcL* locus, supporting the use of this marker for reliable species authentication rather than population-level differentiation.

After sequence editing and comparison with reference sequences in the NCBI GenBank database, the *rbcL* gene region of the studied samples showed high similarity with the published sequences of *S. brachyandra* Diels (accession no. JN051690.1) and *Stephania cephalantha* (accession no. JN051691.1) (Table 9) [2,33]. Specifically, when compared to *S. brachyandra* Diels (JN051690.1), 11 samples exhibited 100% sequence identity, while DY12 showed 99.85% identity. In comparison with *S. cephalantha* (JN051691.1).

These results indicate that the *rbcL* gene sequences of the analyzed samples are highly similar (99.85%–100%) to those of *S. brachyandra* Diels (JN051690.1), strongly confirming that the genetic identity of the samples is consistent with *S. brachyandra* Diels. The slight variation observed among samples likely reflects minor intraspecific polymorphism rather than taxonomic divergence, which is consistent with the relatively stable chloroplast genome of this species [11]. Moreover, the close clustering of samples from geographically proximate sites suggests limited differentiation at the *rbcL* locus, supporting the conclusion that *rbcL* is suitable for species authentication but not for resolving population-level structure.

A phylogenetic tree was constructed based on the *rbcL* gene sequences of the studied *S. brachyandra* Diels samples, along with two reference sequences: *S. brachyandra* Diels (JN051690.1) and *S. cephalantha* (JN051691.1). The tree clearly indicates that the studied *S. brachyandra* Diels samples cluster together with *S. brachyandra*

Diels (JN051690.1) in the same branch, which is consistent with previous studies on the genetic relationships within the *Stephania* genus [34,35]. In contrast, *S. cephalantha* (JN051691.1) forms a separate branch, distinct from the *S. brachyandra* Diels samples, confirming the genetic distinction between the two species, a result that aligns with previous phylogenetic studies that identified clear genetic differentiation between *S. brachyandra* Diels and *S. cephalantha* [17]. Notably, samples from geographically closer provenances tended to cluster together, suggesting that genetic similarity partially reflects spatial proximity and local ecological conditions. This pattern supports the view that limited gene flow and environmental variation among districts may influence the observed phylogenetic relationships within *S. brachyandra* Diels populations.

3.3.3. ITS gene region

The nucleotide composition of the ITS gene region for the studied *S. brachyandra* Diels samples was analyzed, and the results for the AT-GC content and sequence length are presented in Table 10.

The analysis of the *ITS* gene region revealed that the studied samples exhibited a lower AT content compared to GC content. Specifcally, the average AT content was 48.54%, while the average GC content was 51.46%, with an average nucleotide length of 536.50 bp. This finding is consistent with other studies on the genetic composition of plant species, where GC content often exceeds AT content in certain plant genomes [36,37].

After sequence processing, the *ITS* gene region sequences were compared with published sequences in the NCBI GenBank database. The comparison results (Table 11) demonstrated a high similarity to *S. brachyandra* Diels (accession no. KJ566126.1) [2] and *S. cephalantha* (accession no. KJ566127.1) [2]. Specifically, the similarity to *S. brachyandra* Diels (KJ566126.1) ranged from 98.47% to 99.25%, while the similarity to *S. cephalantha* (KJ566127.1) was slightly lower, ranging from 94.47% to 95.35%. These findings support the conclusion that the studied samples are genetically consistent with *S. brachyandra* Diels and confirm the distinct genetic separation between *S. brachyandra* Diels and *S. cephalantha* [4,38]. Moreover, minor sequence variation among populations may reflect local environmental adaptation or restricted gene flow, as samples from nearby districts tended to show slightly higher sequence similarity. This suggests that while *ITS* is effective for species-level identification, it also captures subtle intraspecific variation influenced by geographic and ecological factors.

Table 10. Nucleotide composition of 12 *S. brachyandra* Diels samples in the ITS gene region.

No.	Sample	Percentage (%)		Size (bp)
		A + T	G + C	
1	DT3	48.40	51.60	531
2	DT6	47.87	52.13	539
3	DY9	49.14	50.86	523
4	DY12	48.15	51.85	542
5	DH8	48.06	51.94	541
6	DH12	48.15	51.85	542
7	PL2	48.62	51.38	543
8	PL12	48.97	50.84	535
9	VN3	48.41	51.09	535
10	VN9	48.61	51.39	541
11	CB1	49.15	50.85	531
12	CB2	48.97	51.03	535
Average		48.54	51.46	536.50

Table 11. Sequence similarity analysis of the region of the *S. brachyandra* Diels compared with NCBI GenBank references.

No.	Sample	Similarity coefficient (%)	
		Stephania brachyandra (Accession No. KJ566126.1)	*Stephania cephalantha* (Accession No. KJ566127.1)
1	DT3	98.87	95.11
2	DT6	98.89	95.19
3	DY9	98.47	94.66
4	DY12	98.71	94.86
5	DH8	98.89	95.26
6	DH12	98.71	94.86
7	PL2	98.90	95.05
8	PL12	98.88	94.47
9	VN3	99.07	95.34
10	VN9	99.06	95.39
11	CB1	99.25	95.30
12	CB2	99.07	95.34

A phylogenetic tree was constructed based on the combined ITS sequences of the studied *S. brachyandra* Diels samples and two reference sequences: *S. brachyandra* Diels (KJ566126.1) and *S. cephalantha* (KJ566127.1). The samples under investigation clustered together within the same clade and grouped with *S. brachyandra* Diels (KJ566126.1), whereas *S. cephalantha* (KJ566127.1) was located in a distinct clade. This strongly supports the identification of the studied *S. brachyandra* Diels samples as belonging to *S. brachyandra* Diels.

Notably, samples from nearby collection sites tended to group more closely within the *S. brachyandra* Diels clade, suggesting that geographic proximity and local environmental conditions may contribute to subtle genetic differentiation within this species. Such clustering patterns further support the view that ITS variation can reflect both taxonomic identity and limited spatial genetic structure among populations.

4. CONCLUSION

This study provides a comprehensive assessment of the morphological characteristics and genetic diversity of *S. brachyandra* Diels populations collected from five districts in Thai Nguyen Province, Vietnam. Morphological characterization showed that all studied specimens were consistent with the diagnostic features previously reported for *S. brachyandra* Diels, while variations in stem length, leaf dimensions, and fruit traits among populations suggest the influence of local environmental conditions. Genetic diversity analysis based on ISSR markers revealed a moderate level of genetic variation, with an overall polymorphism rate of 53.46%. Among the studied populations, Phu Luong and Dai Tu exhibited the highest levels of genetic diversity, whereas Dinh Hoa showed the lowest. AMOVA indicated that 57% of the total genetic variation occurred within populations and 43% among populations, demonstrating both substantial within-population diversity and notable genetic differentiation among provenances. Genetic distance and similarity analyses further supported this pattern, with the Vo Nhai population showing greater genetic distinctiveness

compared with the remaining populations. Molecular identification using DNA barcoding markers confirmed the taxonomic identity of all samples as *S. brachyandra* Diels. The rbcL sequences showed very high similarity (99.85%–100%) to the reference sequence of *S. brachyandra* (JN051690.1), while ITS sequences exhibited similarity values ranging from 98.47% to 99.25% relative to the reference sequence (KJ566126.1). Phylogenetic analyses based on ITS sequences clearly grouped all studied samples with *S. brachyandra* and separated them from the closely related species *S. cephalantha*, providing strong evidence for species identification. Overall, the results highlight the importance of conserving the genetic resources of *S. brachyandra* Diels in Thai Nguyen Province, particularly in populations exhibiting high genetic diversity, such as those in Phu Luong and Dai Tu. The findings provide a valuable scientific basis for future conservation strategies, sustainable utilization, germplasm management, and breeding programs for this medicinally important species.

5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to the 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 present journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work.

6. FUNDING

No.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study on *Stephania brachyandra* Diels followed national regulations on biodiversity conservation and medicinal plant use. No endangered populations were disturbed; plant materials were responsibly sourced from permitted areas or research plots. Fieldwork was conducted with local consent, and all techniques aligned with sustainable practices. As no human or animal subjects were involved, IRB approval was not required.

9. DATA AVAILABILITY

The data supporting the findings of this study on *Stephania brachyandra* Diels are available from the corresponding author upon reasonable request. The dataset includes the ISSR binary band matrix used for genetic diversity and population structure analyses, as well as DNA barcoding sequences (rbcL and ITS) generated during this study. Researchers interested in accessing these data for verification or further analysis are encouraged to contact the corresponding author.

10. PUBLISHER'S NOTE

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