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EXAMINING THE GENETIC DIVERSITY AND GENETIC STRUCTURE OF *Dysosma difformis* IN THE NORTHERN REGION OF VIETNAM USING INTER-SIMPLE SEQUENCE REPEAT MARKERS (ISSR)

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ABSTRACT

The restricted distribution range of rare medicinal plants can largely be attributed to unscientific harvesting practices and the high societal demand for pharmaceuticals and health supplements. One of the outstanding cases is the *Dysosma* genus, which is reported to have numerous species endemic to China. Many studies indicate that these species typically have small wild populations, leading to significant challenges. These small populations face intense pressure from natural selection, genetic drift, and a potential reduction in genetic diversity. In this research, we assess the genetic diversity of *Dysosma difformis* populations by applying Inter-Simple Sequence Repeat markers (ISSR). The findings reveal considerable outcomes, with Shannon's genetic index (*I*) of 0.538, Nei's genetic diversity (*H*) of 0.3658, gene flow (*N*m) of 1.0604, and genetic differentiation among populations of G_{ST} = 0.3204. The genetic distances among populations varied, with a minimum of 0.0645 (Phu Tho and Ba Vi) and a maximum of 0.3901 (Phu Tho and Lai Chau). The study's findings revealed that *D. difformis* in northern Vietnam exhibits significant genetic diversity, even with a small population size. Furthermore, we discovered that the *D. difformis* populations in Phu Tho and Ba Vi are genetically closely related, suggesting they likely share a common evolutionary origin despite being located in two distinct geographical areas that are adjacent to each other. Furthermore, this study provides insights into the genetic structure and evolutionary origin of *D. difformis* populations in northern Vietnam. Additionally, this information is also crucial for conservationists to support genetic breeding initiatives and preserve the diversity of *D. difformis*.

Keywords: Conservation, *Dysosma difformis*, genetic diversity, genetic structure, ISSR.

INTRODUCTION

Several *Dysosma* species are assessed by the IUCN and classified as vulnerable (VU), including *Dysosma veitchii, Dysosma aurantiocaulis, Dysosma tsayuensis*, and *Dysosma versipellis*. The Vietnamese Red Data Book highlights the risk of *Dysosma difformis*, warning about the decline of its genetic resources. Plant species in the *Dysosma* genus are recognized as traditional medicinal herbs in Southeast Asia, used for treating ulcers, snakebites, and more. According to previous reports, *D. difformis* is a native species in both China and northern regions of Vietnam. This species has been utilized in traditional medicine in China for many years due to its antibacterial properties and effectiveness in treating snake bites. Recently, the genus *Dysosma* has gained increased attention because it contains podophyllotoxin, a natural resource used in the production of anti-cancer drugs (Ardalani *et al.*, 2017). A recent report shows that *D. difformis* in Vietnam contains endophytic fungi that are capable of producing podophyllotoxin (PTOX), an important discovery for sourcing raw materials to produce the anti-cancer drug PTOX (Hoa *et al.*, 2022). Podophyllotoxin is known as a significant biologically active compound found in *D. difformis*. It has been scientifically proven to inhibit the proliferation of various human cancer cell lines by stimulating their apoptosis cycle. Compounds with similar effects derived from flavonoids and lignans with antioxidant properties, which inhibit tumors, are also contained by *D. difformis* (Zheng *et al.*, 2016).

People use molecular markers to assess the genetic diversity and population structure of various plant species. ISSR ones are commonly used for this purpose because they are simple to use, require minimal knowledge of the species genome, and yield highly reproducible results. There are several previous reports that have used molecular markers to evaluate genetic diversity in several species within the genus *Dysosma*. In Vietnam, the medicinal species *Dysosma tonkinense*, which is listed in the Vietnam Red Data Book and has low reproductive capacity, was evaluated with Inter Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) markers and showed high genetic diversity. The data illustrated a genetic similarity of 72% among 20 accessions collected from 13 geographical regions of Vietnam (Khanh *et al.*, 2021). *Dysosma pleiantha*, a significant medicinal species native to southeastern China, was sampled from five populations to assess genetic diversity using 12 ISSR markers. The assessment revealed low genetic diversity and notable genetic differences between populations. The study also presented a G_{ST} value of 0.465 for *D. pleiantha*, indicating high genetic differentiation among the populations (Zong *et al.*, 2008). Moreover, *D. versipellis*, one of the endemic species, is found in southern China. Researchers examined six existing populations of this species and assessed their genetic diversity using 11 ISSR markers. Of the 144 total bands detected, 57.64% were polymorphic. The paper reported that *D. versipellis* exhibited a low level of genetic diversity and low heterozygosity (Qiu *et al.*, 2006). The former demonstrates a remarkable *D. versipellis* showing a significant deficiency in heterozygosity across all analyzed populations based on allozyme data. The genotype frequencies within these

populations suggest severe inbreeding, which could explain the low seed production observed in this species as reported in this study (Qiu *et al.*, 2005).

MATERIALS AND METHODS

Materials

D. difformis was collected from four locations in northern Vietnam, including Lai Chau, Ha Giang, Xuan Son National Park and Ba Vi National Park (Figure 1). Sampling coordinates are shown in Table 1.

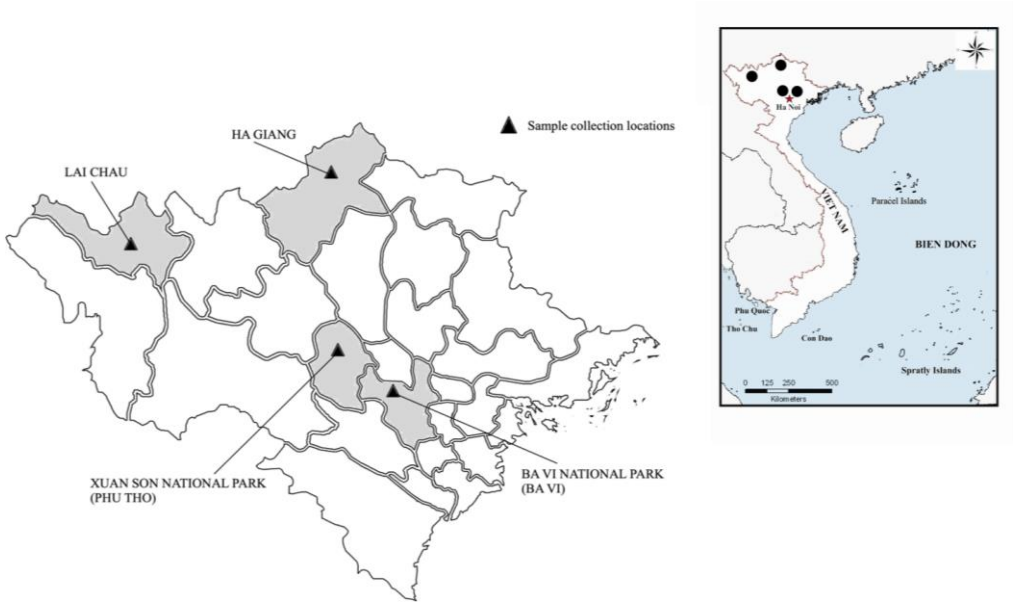

Figure 1. The geographical location of four *D. difformis* populations in the northern region of Vietnam.

Table 1. Original site and coordinates of the studied accessions.

Location	No of Individuals	Latitude (N)	Longitude (E)	AMSL (m)
Ha Giang province	31	23°00'40.14" N	104°53'06.92" E	1074
Lai Chau province	11	22°13'02.55" N	103°35'00.58" E	1890
Ba Vi National Park (Ba Vi province)	20	21°03'36.02" N	105°21'43.04" E	1089
		21°03'34.05" N	105°21'40.04" E	1033
		21°03'35.05" N	105°21'44.05" E	1051
		21°03'35.00" N	105°21'45.03" E	1061
		21°03'34.03" N	105°21'45.05" E	1068
		21°03'34.07" N	105°21'45.02" E	1074
		21°03'33.06" N	105°21'45.03" E	1072
		21°03'34.07" N	105°21'46.04" E	1091
Xuan Son National Park (Phu Tho province)	8	21°06'52.01"N	104°57'37.01" E	548

DNA extraction

The leaf tissue was ground into a fine powder in liquid nitrogen. Genomic DNA was isolated from leaves, following the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987). The quality and quantity of isolated DNA were checked on 1% agarose gel. The gel was visualized with RedSafe. Stocks of DNA were stored in TE buffer.

PCR amplification using ISSR markers

Reaction mixture of 20 µl. The DNA amplifications were carried out with an initial denaturation at 94ºC for 5 minutes. The temperature profile of each cycle was 1 minute denaturation at 94ºC, 30 seconds annealing at 52ºC and 2 minutes extension at 72ºC. Each reaction was of 36 cycles followed by a 10 minutes final extension at 72ºC. Eight ISSR primers (Biotechnology laboratory, The University of Columbia, Canada) were used for a full survey of all 70 individuals: UBC 824 = (TC)8G, UBC 815 = (CT)8G, UBC 854 = (TC)8RG, UBC 853 = (TC)8RT, UBC 845 = (CT)8RG, UBC 846 = (CA)8RT, UBC 848 = (CA)8RG, UBC 834 = (AG)8YT.

Scoring of PCR profiles and data analyses

All individuals were compared based on the banding pattern of ISSR primers, scoring the presence of a band as "1" and the absence of a band as "0". Genetic differentiation coefficient (G_{ST}) (Nei, 1973), Shannon index (I), gene flow (N_m), expected heterozygosity (H_e), unbiased expected heterozygosity (uH_e), observed number of alleles (N_a), effective number of alleles (N_e), calculated by Genepop 1.32 software. Principal Coordinates (PCoA) visualized by GenAlEx 6.5 (Peakall *et al*., 2012). Population structure was computed using Bayesian algorithms, with similarities and differences in the population visualized by STRUCTURE software (Porras *et al*., 2013). The result file from STRUCTURE software then has to be processed by applying CLUMPAK (Kopelman *et al*., 2015) to choose the appropriate delta K.

RESULTS AND DISCUSSION

ISSR markers informativeness

The results of the primer annealing temperature screening and the details of polymorphic bands are illustrated in the following Table 2.

The electrophoresis of ISSR primer products and subsequent analysis produced 104 ISSR bands. Among these, 8 bands were monomorphic, representing 7.7%; while 96 bands were polymorphic, making up 92.3% (Figure 2). Besides, the annealing primer annealing temperature optimization for all primers in the PCR reactions was above 50ºC, with temperatures ranging from 50ºC to 55ºC.

Genetic variation within populations

From Table 3, it is seen that Ha Giang population exhibited the highest genetic diversity, with an N_a of 1.740, N_e of 1.452, H_e of 0.266, and uH_e of 0.27. Besides, the percentage of polymorphic loci varied between populations, ranging from 54.81% in Phu Tho population to 84.62% in Ha Giang population, with an average of 59.38%. Moreover, Lai Chau population demonstrated the lowest genetic diversity, with N_e = 1.194, H_e = 0.113, and $\%P$ = 31.73%. In addition, the Shannon index (I) was the lowest in Lai Chau population at 0.113 and the highest in Ha Giang population at 0.4.

Table 2. Characteristics of eight ISSR marker used for *Dysosma difformis*.

No	Primer	Size range	Ta	TB	MB	PB	%PPB
1	UBC 815	250 – 2500	50	17	2	15	88.23
2	UBC 834	150 – 2300	52	14	3	11	78.57
3	UBC 845	200 – 2500	50	16	0	16	100
4	UBC 848	100 – 2300	53.5	12	0	12	100
5	UBC 846	150 – 2000	55	12	1	11	91.66
6	UBC 824	400 – 2000	52	11	0	11	100
7	UBC 854	300 – 1500	55	11	0	11	100
8	UBC 853	250 - 2000	52	11	2	9	81.81

Ta: annealing temperature, TB: total band, MB: monomorphic band, PB: polymorphic band, %PPB: percentage of polymorphic band.

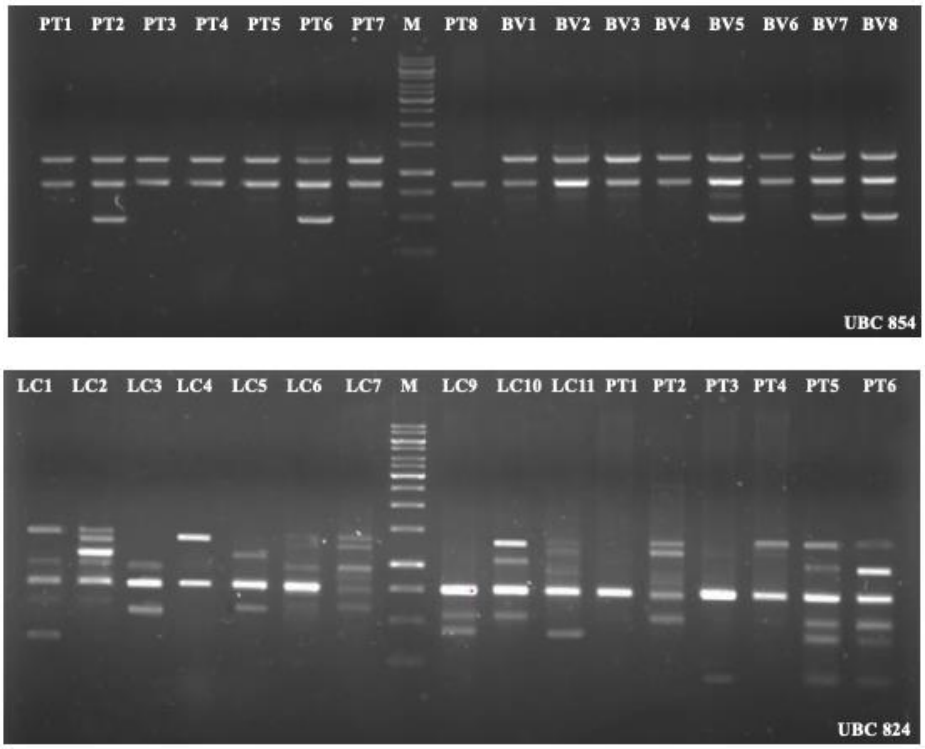

Figure 2. Genetic profile of different cultivars of individuals using UBC 854 and UBC 824 primers. M: marker 1 kb; Lane PT1-PT8 represented for Phu Tho population; Lane BV1-BV8 represented for Ba Vi population; Lane LC1-LC11 represented for Lai Chau population.

Table 3. Genetic variation between 4 populations of *D. difformis*.

Population	*N*	N_a	N_e	*I*	H_e	uH_e	%*P*
Ha Giang	31	1.740	1.452	0.400	0.266	0.270	84.62
Lai Chau	11	1.096	1.194	0.113	0.113	0.118	31.73
Ba Vi	20	1.529	1.441	0.252	0.252	0.259	66.35
Phu Tho	8	1.375	1.325	0.191	0.191	0.203	54.81
Mean	17.250	1.435	1.353	0.306	0.205	0.213	59.38

Mean over loci for each population N_a (observed number of alleles), N_e (effective number of alleles), H_e (expected heterozygosity), I (Shannon's information index), %P (the percentage of polymorphic loci), and uH_e (unbiased expected heterozygosity).

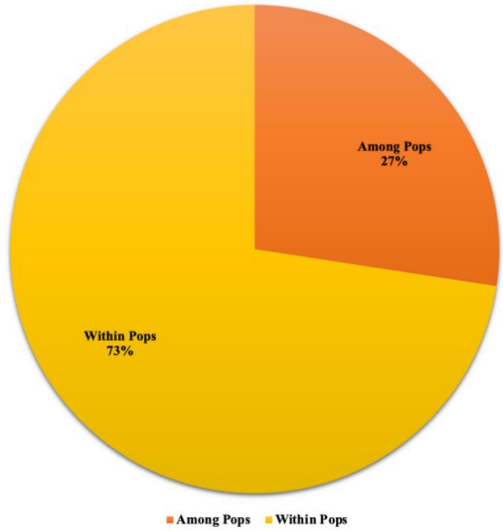

Figure 3. Percentages of molecular variance (AMOVA analysis).

AMOVA analysis uncovered significant genetic diversity within populations, accounting for 73% of the total variation, while indicating comparatively lesser genetic variance among populations at 27% (Figure 3).

Population structure

The gene flow index N_m = 1.0604, implying a stable level of gene flow, as an N_m value above 1 suggests adequate genetic exchange between populations. The G_{ST} index of 0.3204 (where values above 0.25 denote significant genetic differences) (Boyle *et al*., 2000) pointed out a remarkable differentiation among populations. The Shannon diversity coefficient I = 0.5380 reflected high genetic diversity within the *D. difformis* population. Nei's genetic diversity index was 0.3658, with total genetic diversity (H_T) at 0.3686, and genetic diversity within populations (H_S) at 0.2505 (Table 4).

Table 4. Genetic diversity of *D. difformis* populations based on ISSR markers.

	N_m	G_{ST}	H_T	H_S	H	I	N_a	N_e
Mean	1.0604	0.3204	0.3686	0.2505	0.3658	0.5380	1.9615	1.6442

H_T: total genetic diversity, H_S: genetic diversity within populations, G_{ST}: genetic differentiation coefficient, N_m: geneflow, H: Nei's genetic diversity, I: Shannon's information index, N_a: observed number of alleles, and N_e: effective number of alleles.

The G_{ST} value for *D. difformis* in this study is lower than reported for other species in the genus *Dysosma*. For instance, G_{ST} values for *D. versipellis* (Qiu *et al*., 2006) and *D. pleiantha* species in another study (Zong *et al*., 2008) were 0.6 and 0.465, respectively, indicating substantial genetic differentiation. In comparison, *D. difformis* has a G_{ST} of 0.3204, still large but lower than species exhibiting prolonged genetic isolation with independent evolutionary trajectories. Despite this genetic differentiation, *D. difformis* maintains genetic exchange between populations, as shown by a gene flow index (N_m) of 1.0604. A gene flow above 1 signifies stable genetic exchange, promoting population balance and resilience against genetic drift, which can unpredictably eliminate alleles (Higgins *et al*., 2015). Therefore, it leads to a challenge for them to recover once they are lost.

Like other *Dysosma* species in China, *D. difformis* encounters difficulties due to its limited size, which makes it more vulnerable to natural selection and genetic drift, resulting in its decline. Therefore, increasing population size through breeding is crucial for long-term stability and sustainability, particularly for species like *D. difformis*. In addition, individual density within populations could also facilitate greater interactions and genetic exchange among individuals.

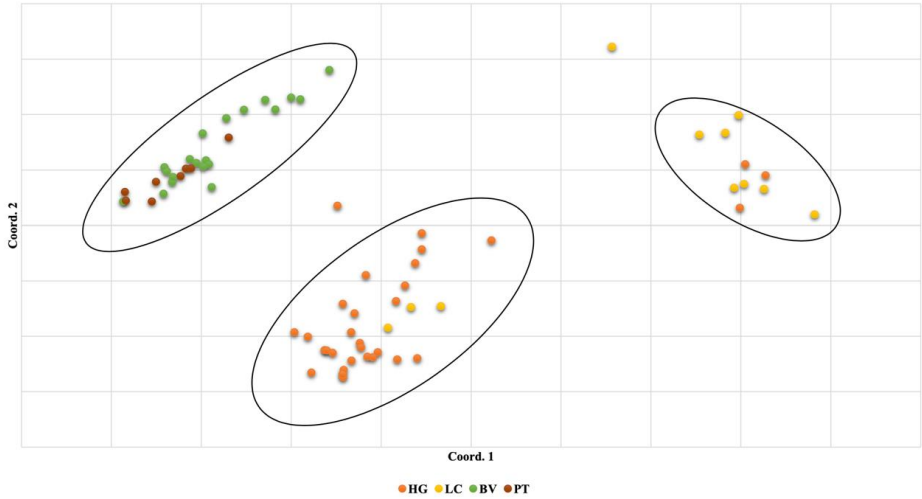

Figure 4. Principal Coordinates (*PCoA*) illustrating genetic relationships among different samples of *D. difformis* using ISSR markers. (HG: Ha Giang population; LC: Lai Chau population; BV: Ba Vi population; PT: Phu Tho population)

The *PCoA* analysis using GenAlEx 6.5 software on binary data from 8 ISSR primers classified the research samples into three main groups: Group I (Lai Chau population), Group II (Ha Giang population), and Group III (Phu Tho and Ba Vi populations). Lai Chau population is distributed separately from the other two groups. Besides, the Ba Vi and Phu Tho populations have overlapping distributions, presenting a shared genetic origin and evolutionary background. Furthermore, *PCoA* analysis further confirms that these two populations have the closest genetic relationship among the four populations studied.

Table 5. Nei's genetic diversity between four populations.

Population	Ha Giang	Lai Chau	Ba Vi	Phu Tho
Ha Giang	0.0000	0.8488	0.7966	0.7700
Lai Chau	0.1640	0.0000	0.7047	0.6770
Ba Vi	0.2273	0.3499	0.0000	0.9376
Phu Tho	0.2523	0.3901	0.0645	0.0000

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Phu Tho and Ba Vi populations have the smallest genetic distance (0.0645), while Phu Tho and Lai Chau populations exhibit the largest genetic distance (0.3901). The Nei's genetic identity between the Phu Tho and Ba Vi populations is 0.9376, demonstrating that these two populations have highly similar genetic sources and share the closest ancestral evolutionary origin. In contrast, 0.677 was represented for the genetic identity between the Phu Tho and Lai Chau populations, which suggested that they had the greatest genetic distance from each other among the four groups, reflecting the most distant evolutionary origin. Furthermore, Ba Vi and Phu Tho populations exhibit the closest genetic relationship, indicated by a genetic distance of 0.0645, considered as quite minimal (Table 5). This suggests a shared genetic evolutionary origin, likely due to plant dispersal influenced by humans or animals, as *D. differmis* has limited natural dispersal abilities. Geographically, these two populations are the closest among the four studied, making human-driven plant propagation highly plausible.

The STRUCTURE software performs genetic structure analysis using a Bayesian algorithm, and the results are visualized by the CLUMPAK website. Prob(K) is a value used to state the probability of the number of distinct genetic groups that represent the genetic structure observed. Prob(K) helps determine the highest possibility of genetically distinct groups in the analyzed data. With K = 9 (similarity score = 0.682) and K = 3 (similarity score = 0.991), the genetic structure is divided into three main clusters (Figure 5). This result aligns with the *PCoA* analysis, which also divides the four populations into three clusters. Individuals within the group show high homogeneity according to Bayesian analysis, with those having a similarity greater than 0.682 considered homogeneous for K = 9, and those with a similarity greater than 0.991 considered homogeneous for K = 3. Delta K helps identify the genetic structure with the

highest probability of occurrence in genetic analysis using Bayesian algorithms. Applying a Bayesian algorithm with K = 3 or K = 9, the genetic structure of *D. difformis* in northern Vietnam was classified into three primary groups, which showed significant genetic differences from one another.

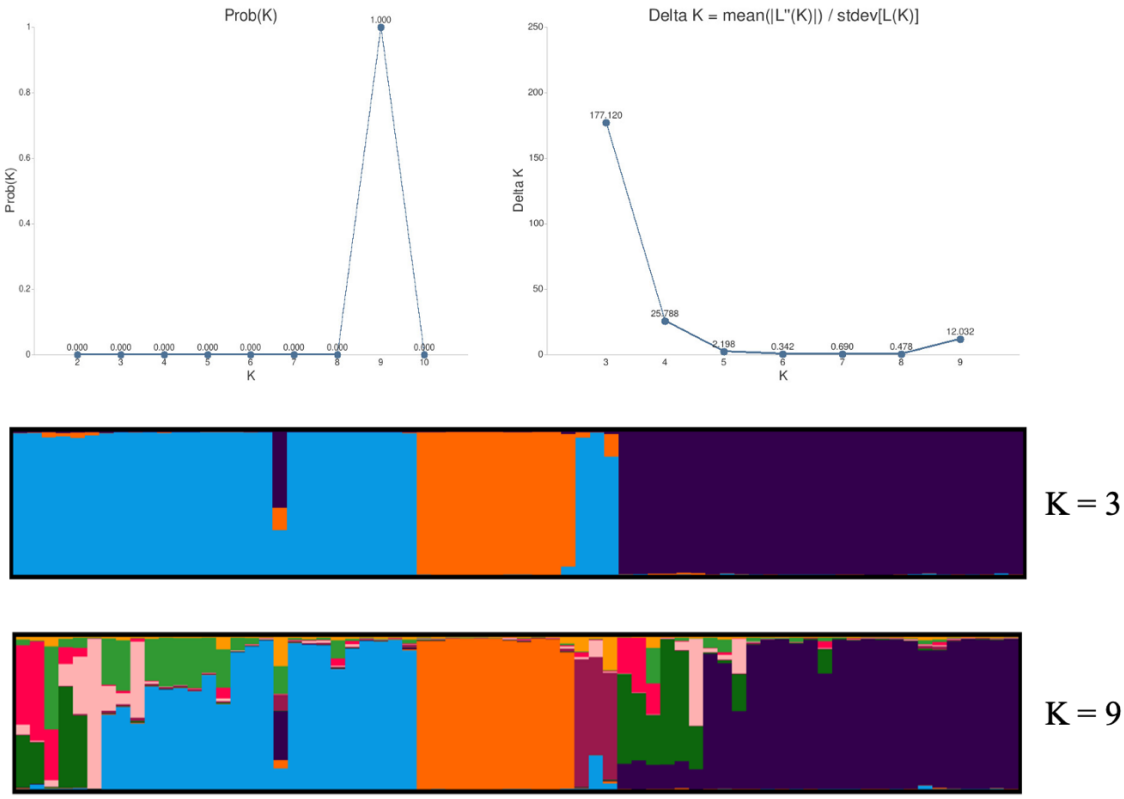

Figure 5. Genetic structure plot *D. difformis* by STRUCTURE software.

CONCLUSION

The study indicates that the genetic distances of the Ba Vi and Phu Tho populations were significantly small, thus implying the possibility that these two populations share a common genetic origin. The Ha Giang population showed the greatest genetic diversity, while the Lai Chau population had the lowest one. The diversity of the Ha Giang population needs to be conserved. Besides, propagating the number of individuals in small populations could sustainably maintain population growth.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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