GENETIC DIVERSITY EVALUATION OF *Cinnamomum parthenoxylon* (Jack) MEISN IN SOME NATURE RESERVES IN NORTH VIETNAM USING SSR MARKERS

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ABSTRACT

Cinnamomum parthenoxylon (Jack) Meisn is one of the valuable wood trees indigenous to the lowland tropical forests of Vietnam, which is in danger of extinction due to habitat fragmentation and overexploitation. Furthermore, the genetic diversity of this species has not been investigated to date. This study evaluated the genetic diversity in five natural populations from 101 individuals representing the *C. parthenoxylon* distribution range in natural reserves in North Vietnam using nine SSR markers. All of the nine loci showed polymorphic. Genetic diversity in population level was moderate (*Na* = 3.82; *Ne* = 2.23; *I* = 0.9, *He* = 0.38 and *Ho* = 0.5). The lowest levels of genetic diversity were detected in the Quang Ninh population (*Ho* = 0.32, *He* = 0.0.47), and the highest levels were detected in the Vinh Phuc population (*Ho* = 0.46, *He* = 0.52). Analysis of molecular variance determined a significant 66% of genetic variation within individuals. All five populations were a positive inbreeding coefficient (*Fis* > 0). These results revealed the excess of homozygotes and deficiency of heterozygotes in all populations. Cluster analysis using the unweighted pair group method with arithmetic average (UPGMA) with genetic similarity coefficients indicated that two genetic clusters were significantly correlated with the geographical distributions. Group I included individuals collecting from two provinces (Thanh Hoa and Ninh Binh), while group II included plants from 3 areas (Phu Tho, Vinh Phuc, and Quang Ninh). Our results provide valuable information for further studies in the conservation, management, and restoration of this species.

Keywords: *Cinnamomum parthenoxylon*, genetic diversity, habitat disturbance, species conservation, SSR.

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1. INTRODUCTION

Cinnamomum parthenoxylon (Jack) Meisn is a species having broad evergreen leaves, which are considered a big tree, distributed on a large scale in eighteen provinces in four different ecological regions of Vietnam: Northeast (Cao Bang, Lang Son, Bac Kan, Tuyen Quang, Thai Nguyen, Phu Tho, Vinh Phuc, Bac Giang, and Quang Ninh), North Central region (Quang Binh, Quang Tri, and Thua Thien Hue), South Central Region (Da Nang, Phu Yen, and Quang Nam) and Central Highlands (Kon Tum, Gia Lai and

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Lam Dong) [1, 2]. Moreover, this species is not only economically significant within the ecosystem of the forest, but they also have a large commercial value, particularly in the fields of woodworking, plastic manufacturing, essential oil extraction, and medicinal research [3, 4, 5, 6]. In addition, studies on the species cinnamon showed biological activity in the inhibition and treatment of many diseases such as cancer, antimicrobial, anti-fungal, and anti-diabetes [6, 7, 8, 9]. However, there are only some species, such as *C. verum*, *C. cassia*, *C. zeylanicum*, and *C. camphora* were deeply researched in biological activity, ingredients, and molecular mechanisms. Unfortunately, over the past decades, many populations of *C. parthenoxylon* and *C. cambodianum* have been endangered as a result of excessive exploitation and deforestation for medicinal materials and timber. Thus, the natural populations of the two species have experienced a sharp decline. Meanwhile, these species' regeneration is not good [10]. In Vietnam, these species are listed as endangered: *C. parthenoxylon* - CR A1a,c,d [2]. They are currently protected by both the central and local governments in Vietnam and need urgent conservation and restoration. Up to now, we lack information on genetic variation at population and species levels and the mating system within populations, especially the harmful effects of human activities.

DNA molecular marker techniques have been widely applied in plants' genetic diversity studies in recent decades [11, 12]. Of these, the microsatellite markers, i. e. simple sequence repeats (SSRs), which are dispersed throughout all genomes, are codominant and multiallelic markers and are considered highly sensitive for detecting polymorphisms Investigations on plant genetic diversity, population structure, gene flow, and mating systems have benefited greatly from the application of SSRs [13, 14]. Previous studies have been undertaken to investigate the genetic variation and verify the taxonomic status of the *Cinnamomum* species at the molecular level [15, 16, 17, 18, 19, 20, 21, 22, 23, 24]. Exploration of genetic variability within and among populations of *C. parthenoxylon* is of crucial importance and priority for species conservation, management, and restoration. To date, the genetic variability of this species is unknown. Therefore, the objective of the present study was to evaluate genetic variation within and among *C. parthenoxylon* populations using SSRs as genetic markers and to provide a platform for the conservation of the endangered species in Vietnam.

2. MATERIALS AND METHODS

2.1. Plant materials

Figure 1. Adult plant of *C. parthenoxylon* species collected in Xuan Lien Nature Reserve

[Photo: Pham Mai Phuong]

The inner bars or leaves of adult trees were collected from 5 natural populations of *C. parthenoxylon,* representing the range of its geographical distribution in Vietnam (Figure 1; Table 1). A total of 101 trees were randomly sampled and preserved in marked plastic bags with silica gel in the field, then transferred to Molecular Laboratory, Join Vietnam - Russia Tropical Science and Technology Research Centre and stored at - 30°C until used for DNA extraction.

Table 1. Information of sampled populations of *C. parthenoxylon* in Northern Vietnam

Population code	Sample size	Collection locality	Altitude	Longitude
Quang Ninh	20	Yen Tu National Forest, Quang Ninh province	2336326.03	391401.64
Vinh Phuc	13	Tam Dao National Park, Vinh Phuc province	2371699.59	569664.79
Phu Tho	19	Xuan Son National Park, Phu Tho Province	2338550.91	515494.31
Hoa Binh	19	Hang Kia - Pa Co Nature Reserve, Hoa Binh province	2293578.64	389485.28
Thanh Hoa	30	Xuan Lien Nature Reserve, Thanh Hoa province	2194294.51	524572.19

2.2. DNA extraction

Total genomic DNA was isolated from the inner bars using a DNA extraction kit (Norgenbiotek, Canada). Samples were ground in liquid nitrogen using a Mixer mill MM 400 (Retsch). The total amount of DNA was checked by electrophoresis on a 1.2% agarose gel and spectrophotometer using NanoDrop 2000 (Thermo Sci., Waltham, MA, USA) and then diluted to a concentration of 10 ng/µL and stored at -20°C for microsatellite analysis.

2.3. Microsatellite amplification

A total of nine SSR primers were chosen for this analysis from *C. parthenoxylon* species was performed by polymerase chain reaction (PCR) (Table 2). Reaction mixtures of 25 µL solution volumes containing 17.5 µL of ddH_2O, 2.5 µL of 1 X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.4), 1.0 µL of $MgCl_2$ (2.5 mM), 0.5 µL of dNTPs (0.2 mM each), 1.5 µL of genomic DNA (10 ng), 1.0 µL of Tag DNA polymerase at 1.25 units and 0.5 µL of each primer at 10 pmol. PCR amplification was implemented in the GeneAmp PCR System

9700 (Applied Biosystems) as follows: an initial denaturing step at 94°C for 5 min, 35 cycles of 94°C for 1 min, annealing temperature for each primer pair at 54-56°C for 30 s and 1 min extension at 72°C. After the final cycle, samples were incubated at 72°C for 10 min and held at 4°C until used for analysis. The amplification products were determined using a Sequi-Gen®GT DNA electrophoresis system with 6% polyacrylamide gels in 1 x TAE buffer and then visualized by GelRed™ Nucleic Acid Gel Stain. PCR fragment sizes were detected using Gel-Analyzer software of GenoSens1850 (Clinx Science Instruments Co., Ltd) with a 25 bp DNA ladder (Invitrogen, Carlsbad, CA, USA).

Table 2. Characteristics of nine SSR markers used in this study

Locus	Primer sequences	Tm (°C)	Size (bp)	References
SSR1	F: 5'- ACGAGTTTCCACCGATTACG -3'	55	268	[15]
	R: 5'- ACTCCTTTCAGCACCGATTG - 3'			
SSR2	F: 5'- TTGTTAAAAACACCAACCCCA - 3'	55	200	
	R: 5'- CAGTGGGCCAAGTGTATCCT - 3'			
SSR3	F: 5'- ACGTGAATGTGAATGGGGTT- 3'	55	190	
	R: 5'- TAGGCAAAGACTCCGAAGGA - 3'			
SSR4	F: 5'- ATTCGGGTCTCTCTCCCTGT - 3'	55	250	
	R: 5'- CTCTCTCGCTGTCTCTGCCT - 3'			
SSR5	F: 5'- TGGAGAACAACTTTGGGAGG - 3'	55	120	
	R: 5'- TGTTCCATGTTACAGATACAG - 3'			
SSR6	F: 5'- ATATGGTCCCAACTCCCTCC - 3'	55	200	

	R: 5'- ACCGTCACCAGATCATCCAT - 3'			
SSR7	F: 5'- TCCCAACTGGACGAAGTTCT - 3'	55	240	
	R: 5'- TTTGCTCGCTGTTATGATGC - 3'			
SSR8	F: 5'- ATCCTCCCAAGGACGCTTAT - 3'	55	130	
	R: 5'- CCTTCAAGGAAAGAAGGGCT - 3'			
SSR9	F: 5'- CACCACCTTCTCCTTCCAAA - 3'	55	230	
	R: 5'- TTGTTGGGGTCTCCAAACTC - 3'			

2.4. Genetic diversity analysis

The presence of null alleles for microsatellite data was tested using the MICRO-CHECKER v.2.2.3 [25]. We estimated genetic diversity, including the number of alleles per locus (Na), the average number of effective alleles per locus (Ne), Shannon's information index (*I*), observed heterozygosity (Ho) and expected heterozygosity (He), the inbreeding coefficient (*Fis*), an analysis of molecular variance (AMOVA), genetic distance and genetic identity using the program GenAlEx v.6.5 [26]. A UPGMA (Unweighted Pair Group Method) tree was constructed for genetic association among populations using POPTREE [27]

3. RESULTS

3.1. Evaluation of genetic diversity

Nine SSR loci were amplified consistently under standard conditions and used to assess the population's genetic structure. All primers were submitted to amplification cycles with primer annealing at 55°C and were polymorphic in *C. parthenoxylon*. Out of the nine SSR markers, five loci showed evidence for a null allele. This population is possibly in Hardy Weinberg equilibrium with loci SSR1, SSR2, SSR3, SSR4, and SSR9, indicated by signs of a null allele. The number of alleles per locus (Na) ranged from 3.33 in the Vinh Phuc population to 4.56 in the Thanh Hoa population, an average of 1.5, and the effective alleles per locus (*N*e) within populations varied from 2.1 (Phu Tho) to 2.47 (Thanh Hoa), with an average of 2.23. The mean observed (*H*o) and expected heterozygosity (*H*e) for each population were 0.38 and 0.5, respectively. The lowest heterozygotes were found in the Quang Ninh population (*H*o = 0.32 and *H*e = 0.47), while the highest heterozygotes were observed in the Vinh Phuc population (*H*o = 0.46 and *H*e = 0.52). The inbreeding coefficient (*Fis*) varied among populations, from 0.01 in Vinh Phuc to 0.33 in Quang Ninh with an average of 0.2, suggesting a mixed breeding system of both inbreeding and outbreeding (Table 3).

Table 3. Genetic diversity estimates nine populations of *C. parthenoxylon*

Population	N	Na	Ne	I	Ho	He	Fis
Quang Ninh	20	3.89	2.24	0.93	0.32	0.47	0.33
Vinh Phuc	13	3.33	2.16	0.85	0.46	0.52	0.01
Phu Tho	19	3.44	2.10	0.83	0.35	0.47	0.26
Hoa Binh	19	3.89	2.18	0.89	0.39	0.49	0.14
Thanh Hoa	30	4.56	2.47	1.02	0.36	0.54	0.30
Mean		3.82	2.23	0.90	0.38	0.50	0.20

Note: N: sample size; I: Shannon's information index; Na: alleles per locus; Na: effective alleles; Ho and He: observed and expected heterozygosities; Fis: inbreeding coefficient.

3.2. Genetic differentiation and AMOVA

The analysis of molecular variance revealed genetic variation among and within populations of the *C. parthenoxylon* (Table 4). For *C. parthenoxylon*, the majority of the overall variance could be attributable to differences that occurred among individuals (66%, $p<0.001$), within populations (28%, $p<0.001$), and among populations (7%, $p<0.001$).

Table 4. Analysis of molecular variance of *C. parthenoxylon*

Source of variation	d.f.	Sum of squares	Variance component	Total variation (%)	P value
Among populations	4	38.34	9.59	7%	<0.001
Within populations	96	292.35	3.05	28%	<0.001
Within individuals	101	167.50	1.66	66%	<0.001
	201	498.19			

The mean value of Nei's genetic distance between the studied populations of *C. parthenoxylon* ranged from 0.03 (Phu Tho/Quang Ninh) to 0.20 (Vinh Phuc/Hoa Binh) (Table 5). Low values of Nei's genetic distance were found between population pairs of Phu Tho/Quang Ninh (0.03), Vinh Phuc/Quang Ninh (0.08), and Thanh Hoa/Phu Tho (0.08). The highest values of Nei's genetic distance were found between population pairs of Vinh Phuc/Hoa Binh (0.20). Similarly, the Genetic identity of *C. parthenoxylon* varied from 0.82 (Hoa Binh/Vinh Phuc) to 0.97 (Quang Ninh/Phu Tho).

Table 5. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) within populations of *C. parthenoxylon*

	Thanh Hoa	Vinh Phuc	Phu Tho	Quang Ninh	Hoa Binh
Thanh Hoa	-	0.84	0.92	0.94	0.91
Vinh Phuc	0.18	-	0.90	0.92	0.82
Phu Tho	0.08	0.10	-	0.97	0.84
Quang Ninh	0.06	0.08	0.03	-	0.89
Hoa Binh	0.10	0.20	0.18	0.12	-

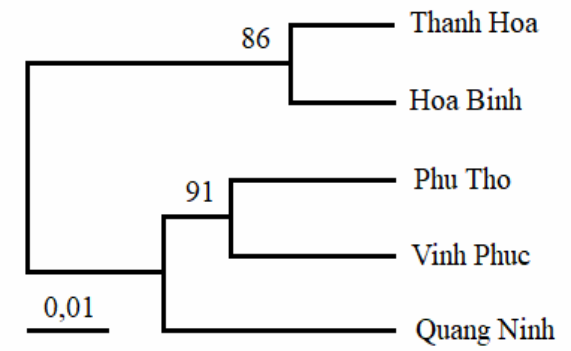

Figure 2. UPGMA dendrogram based on Nei's (1972) genetic distance among populations.

The studied populations were clustered using the UPGMA dendrogram based on pairwise genetic distances (Figure 2). The geographically closed populations were clustered together. Two different clusters were generated in the phylogenetic tree. The first cluster contained two populations (Thanh Hoa and Hoa Binh). The second included three populations (Phu Tho, Vinh Phuc, and Quang Ninh). However, the Quang Ninh population was separated clearly from geographically close populations (Phu Tho and Vinh Phuc).

4. DISCUSSION

The high level of genetic diversity within a species reflects its evolutionary history and eco-geographical environment. Species related to wide distribution patterns, large population size, long lifespan, predominantly outcrossing,

and successional stages often maintain high genetic diversity [28, 29]. Our research found that *C. parthenoxylon* (*Na* = 0.38, *Ne* = 2.23) had lower than *C. camphora* in Japan (*Na* = 13.37, *Ne* = 3.78) and in China (*Na* = 21.73, *Ne* = 9.51) [21, 22] but higher than *C. balansae* (*Na* = 1.9, *Ne* = 1.5) [15]. The results obtained in the present study indicate *C. parthenoxylon* exhibited a moderate level of genetic diversity (*Ho* = 0.38 and *He* = 0.5). High levels of genetic diversity in other *Cinnamomum* species based on SSR markers have been reported, such as *Ho* = 0.53-0.6 and *He* = 0.55-0.68 analysis from 104 adult trees of *C. camphora* collection three populations (Meiji Jingu (Shinto Shrine), Kajiya Plantation, and Manazuru Peninsula) in Japan [21], genetic diversity estimates of *C. camphora* (300 trees) in China and Taiwan with *Ho* = 0.728 and *He* = 0.878 and *C. Camphora* (504 trees) with *Ho* = 0.614 and *He* = 0.714) [22]. However, low genetic diversity has been reported in some other *cinnamomum*, such as *C. balansae* (*Ho* = 0.244, *He* = 0.264) [15], *C. camphora* (*Ho* = 0.3449, *He* = 0.4254) [30]. *Cinnamomum* species are widely distributed and have a long life. They are predominantly outcrossed via gene flow within and between populations. Pollen dispersal plays an important role in the transfer from one generation to the next. Species with a narrow distribution or endangered species tend to reduce genetic variability and maintain low genetic diversity [28, 31]. Our results indicated that *C. parthenoxylon* is endangered and has medium genetic diversity. Its habitats are degraded and isolated. Deforestation and over-exploitation of *C. parthenoxylon* may have a major impact on genetic variation and lead to low heterozygotes within populations and species while also affecting the number of alleles in all studied populations. The decrease in allelic diversity may be the result of a small population size. In this study, the two populations of Phu Tho and Quang Ninh had low genetic diversity compared to the remaining populations. These results suggested that high habitat disturbance and fragmentation had a negative effect on genetic variability within populations.

Similarly, AMOVA analysis indicated that most of the genetic variation was distributed among individuals (66%). This can be a consequence of the decrease in genetic divergence among populations. *C. parthenoxylon* is a long-living and outcrossing species. The species is insect-pollinated by insects and predominantly outcrossed. Pollen dispersal could contribute mainly to the gene flow and population structure of *C. parthenoxylon*. Moreover, its seeds can be dispersed by animals (rodents and birds). UPGMA tree showed two different groups of genetically mixed individuals of *C. parthenoxylon*. Two populations, Thanh Hoa and Hoa Binh were closed together and formed a genetic cluster. Quang Ninh population was separated from two populations (Phu Tho and Vinh Phuc) and formed one cluster by geographical distances.

Furthermore, our results showed that the inbreeding coefficient (*Fis*) was significantly positive in all populations. These factors suggested that the existence of biparental inbreeding can reduce genetic diversity within the *C. parthenoxylon* populations. This might be mainly attributed to limited pollen dispersal. To prevent inbreeding depression and gradually restore the *C. parthenoxylon* population size in the future, ex situ conservation through seed collection from natural populations into local gardens will be the material source to increase the variability of progenies in natural populations.

5. CONCLUSION

In five provinces of North Vietnam, *C. parthenoxylon* showed moderate genetic diversity. Two genetic groups connected to habitat degradation and regional separation were discovered using clustering studies.

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