ACADEMIA JOURNAL OF BIOLOGY 2018, 40(4): 85–90
DOI: 10.15625/2615-9023/v40n4.10848

EVALUATION OF THE GENETIC DIVERSITY OF *Huperzia serrate* BY RAPD MARKERS

Ho Thi Huong[1], Ngo Thi Thuy Linh[1], Le Thi Lan Anh[2], Le Thi Bich Thuy[1, 2*]

[1]Institute of Biotechnology, VAST, Vietnam
[2]Graduate University of Science and Technology, VAST, Vietnam

ABTRACT

Huperzia serrata, a valuable pharmaceutical plant distributed in Lao Cai (Northwest Vietnam) and Lam Dong (Central Highlands of Vietnam), is known as pharmaceutical material for the treatment of Alzheimer's disease. As the result of over-exploitation and habitat fragmentation, *H. serrata* has become a threatened plant in Vietnam, China and other countries around the world. For the understanding of population dynamics, adaptation, evolution and conservation of plant species in general and *H. serrata* in particular, the evaluation of genetic diversity is very important. In this study, the total of 16 RAPD primers were used for evaluation of the genetic diversity of eight *H. serrata* samples collected from different locations of Sa Pa and Da Lat. Overall, 16 markers generated a total of 70 fragments in eight collected samples of *H. serrata*. Of 70 generated fragments, 44 were found to be polymorphic. The number of fragments of each primer ranged from 1 to 8 with an average of 4,4 fragments per primer. The OPB20 primer revealed the highest polymorphism, while the OPB15, OPB17 and OPC12 primers gave the lowest. Polymorphism information content (PIC) values varied from 0.5266 to 0.8624 with an average of 0.7182, in which the highest value belonged to OPB11 primer while the OPC10 primer showed the lowest PIC value. The dendrogram created based on RAPD data sheet divided the investigated strain into two major groups corresponding to their habitat: Group I included three Da Lat samples (DL1, DL2, DL3) and Group II consisted of five samples from Sa Pa (SP1, Sp2, SP3, Sp4, and SP5). In conclusion, the results of our study could be useful for the conservation and development of *H. serrata.*

Keywords: Huperzia serrata, genetic diversity, pharmaceutical plant, polymorphism, RAPD marker.

Citation: Ho Thi Huong, Ngo Thi Thuy Linh, Le Thi Lan Anh, Le Thi Bich Thuy, 2018. Evaluation of the genetic diversity of *Huperzia serrate* by rapd markers. *Academia Journal of Biology*, 40(4): 85–90. https://doi.org/10.15625/2615-9023/v40n4.10848.

**Corresponding author email*: ltbthuy@ibt.ac.vn

Received 2 November 2017, accepted 15 December 2018

INTRODUCTION

Huperzia serrata, a typical member of the genus *Huperzia* belonging to the family *Lycopodiaceae*, comprises about 500 species in the world (Christenhusz et al., 2011; Kitajima & Takayama, 2011). Known for medicinal value since 739 (the Tang Dynasty), the whole plant of *H. serrata*, named Qian Ceng Ta (in Chinese), is one of the oldest Chinese herbs and has been extensively used in the clinic to treat contusion, swelling, strains, schizophrenia, myasthenia gavis and organophosphate poisoning (Ma et al., 2007). *H. serrata* produces various types of bioactive lycopodium alkaloids, including huperzine A, a potent drug for AD. Huperzine A was first isolated from the whole plant of *H. serrata* by

Chinese scientists Liu and his co-worker (Liu et al., 1986a, b). Huperzine A, under the trade name "Cerebra", is currently used in the United States as a supplementary drug for patients with memory impairment. Nowadays it is marketed as a "smart drug" to boost brain power.

H. serrata is mainly found in sub-tropical to temperate forests at an altitude of 900 to 3500 m in many countries, such as Australia, China, Cuba, Fiji, India, Indonesia, Japan, Korea, Mexico, Myanmar, Nepal, Peninsular Malaysia, Russia, Samoa, Sri Lanka, Thailand, Taiwan, USA and Vietnam (Singh & Singh, 2010, Dixit, 1984). In Vietnam, *H. serrata* is distributed in Sa Pa (Lao Cai) and Da Lat (Lam Dong), where the elevation is above 1000 m, and it frequently grows in shaded, damp habitats with moist, acidic humus soil. The whole plant of *H. serrata* has been found to be the major source of huperzine A, a potent, reversible and selective acetylcholine esterase inhibitor (AChEI). Synthesis of huperzine A has not been industrialized yet, and it is primarily extracted from natural resources (Ma et al., 2005). The rapidly growing demand and the high price of the raw materials are increasing pressure on the natural habitats. Furthermore, these plants grow extremely slowly (Ma et al., 2007; Ma & Gang, 2004). As the result of over-exploitation and habitat fragmentation, *H. serrata* has become a threatened plant in Vietnam, China and other countries around the world (Tran Hop, 2005; Ma et al., 2007).

Studying the genetic diversity of rare plants not only enhances our understanding of population dynamics, adaptation and evolution, but also provides useful information for biological conservation (Schaal et al., 1991).

Molecular markers are powerful tools for analyzing genetic diversity (Ouborg et al., 1999). DNA fingerprinting can distinguish species rapidly using a small amount of DNA, thus providing useful information for phylogenetic analysis. Various approaches are available for DNA fingerprinting, such as AFLP (Amplified Fragment Length Polymorphism), SSR (Simple Sequence Repeats) and RAPD (Random Amplified Polymorphic DNA). RAPD is convenient and simple, hence the most common method to check genetic similarity and phylogenetic relationship (Gepts, 1993). Once DNA fragments are amplified by polymerase chain reaction (PCR) using primers with arbitrary sequences, a rapid screening for polymorphism can be performed, enabling diversity analysis of several plants simultaneously (Williams et al., 1990). In this study, we will evaluate the genetic diversity of eight *H. serrata* samples representing for eight populations in Sa Pa and Da Lat based on RAPD markers.

MATERIALS AND METHODS

Sampling sites and plant materials

The plant materials used in this study were collected from eight populations across the main niches of *H. serrata* in Sa Pa (Lao Cai) and Da Lat (Lam Dong), Vietnam. Locations of the eight natural populations were listed in table 1.

Table 1. Locations of the eight natural populations

No	Location	Population code (Sample)
1	Bidoup-Nui Ba	DL1
2	Don Duong-Lam Dong	DL2
3	Nam Ban-Lam Ha	DL3
4	Nam Cang-Sa Pa	SP1
5	Ban Ho-Sa Pa	SP2
6	Ta Van-Sa Pa	SP3
7	Lao Chai-Sa Pa	SP4
8	Ta Phin-Sa Pa	SP5

RAPD primers

16 RAPD primers used in RAPD-PCR reactions were purchased from Operon, USA (Table 2).

Table 2. List of RAPD primers used

No	RAPD primer	Nucleotide sequence (5'-3')	No	RAPD primer	Nucleotide sequence (5'-3')
1	OPB1	GTTTCGCTCC	9	OPC1	TTCGAGCCAG
2	OPB4	GGACTGGAGT	10	OPC5	GATGACCGCC
3	OPB8	GTCCACACGG	11	OPC6	GAACGGACTC
4	OPB11	GTAGACCCGT	12	OPC10	TGTCTGGGTG
5	OPB13	TTCCCCCGCT	13	OPC12	TGTCATCCCC
6	OPB15	GGAGGGTGTT	14	OPC13	AAGCCTCGTC
7	OPB18	CCACAGCAGT	15	OPC17	TTCCCCCCAG
8	OPB20	GGACCCTTAC	16	OPC19	GTTGCCAGCC

Extraction of genomic DNA

DNA was extracted from young leaves of *H. serrata* using CTAB method. 20 mg of plant tissues were ground, mixed with 600 µL of freshly-prepared and preheated 2X CTAB solution in a 1.5 mL tube, and incubated at 65°C for 60 mins. The supernatant was added to 600 µL of chloroform/isoamyl alcohol (24:1), vortexed for a few seconds and centrifuged at 12,000 g for 15 mins. DNA was then precipitated overnight with an equal volume of ice-cold isopropanol. Next day, DNA was pelleted by 15 mins centrifugation at 12,000 g and decanting before letting air dry. DNA pellet was washed by adding 600 µL of cooled (4°C) 70% ethanol and centrifuging for 15 mins at 12,000 g. After decanting ethanol, 100 µL of TE buffer (10 mM Tris-HCl, 1 M MEDTA, pH 8.0) was added and the mixture was incubated at 65°C for 60 min. 4 µL of RNAase (10 mg/mL) was, followed by a 60 mins incubation at 37°C. The purity DNA was measured by the ratio of the absorbance at 260 and 280 nm. DNA of five plants from a population was pooled to form the sample representing for appropriate population.

PCR amplification

PCR reaction was performed as described by Williams et al. (1990). The final volume of 25 µL contained 10X buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl2, and 0.001% (w/v) gelatin), 50 ng of template DNA, 0.25 mM dNTPs (Thermo Scientific), 0.025 mM of primers, 1.5 mM MgCl2 and 1U of Taq polymerase. Reaction mixtures were amplified in PTC-100 (MJ Research Inc., USA), each reaction was performed using an initial step of 94°C, followed by 35 cycles of 1-min denaturation at 94°C, 1-min annealing at 37°C and 2-mins extension at 72°C, and terminated by 5-mins extension at 72°C. At PCR completion, samples were kept at 4°C for analysis. RAPD products after PCR were separated by electrophoresis on 1% agarose gel in 1X TBE (Tris boric acid EDTA) buffer using ethidium bromide staining and visualized under UV light.

Data analysis

Target DNA amplicons were scored (present = 1, absent = 0) for each appropriate marker (bp). Faint bands were considered. Results were then entered in NTedit1.2a software accordingly. A phylogenetic tree was constructed using NTSYSpc 2.1 (Numerical Taxonomy and Multivariate Analysis System version 2.1) software (Rohlf, 2000).

RESULTS AND DISCUSSION

PCR-RAPD products were analyzed by 16 RAPD primers on 1% agarose gel electrophoresis for DNA polymorphism analysis of eight samples of *H. serrata* (Table 3). Of 16 primers, 15 were polymorphic, only one (OPB13) showed no polymorphism. The number of amplified fragments by each primer varied from 1 (OPB13 primer) to 8 (OPB4 and OPB11 primer) with an average of

4.4. A total of 70 amplified fragments were obtained. Among these 70 fragments, 44 were found to be polymorphic. The average PIC coefficient of the primers was quite high (0.7182). In particular, OPB11 primer (Fig. 1) had the highest PIC value (0.8624) and OPC10 primer (Fig. 2) had the lowest PIC value (0.5266).

Table 3. Primer sequences, total number of polymorphic bands, percentage of polymorphism and PIC value of the primers used

No.	Name of Primers	Total fragments	Polymorphic fragments	Percentage of polymorphic fragments	PIC values
1	OPB1	6	5	83,3	0.8164
2	OPB4	8	7	87,5	0.8356
3	OPB8	4	2	50	0.7407
4	OPB11	8	4	50	0.8624
5	OPB13	1	0	0	0
6	OPB15	6	2	33,3	0.8286
7	OPB17	3	1	33,3	0.5926
8	OPB20	2	2	100	0.6600
9	OPC1	5	4	80	0.7300
10	OPC5	7	4	57,1	0.8245
11	OPC6	4	3	75	0.7107
12	OPC10	2	1	50	0.5266
13	OPC12	3	1	33,3	0.6205
14	OPC13	3	2	66,7	0.6044
15	OPC17	4	3	75	0.7050
16	OPC19	4	3	75	0.7150
	Total	**70**	**44**		
	Average	**4.4**	**2.8**		**0.7182**

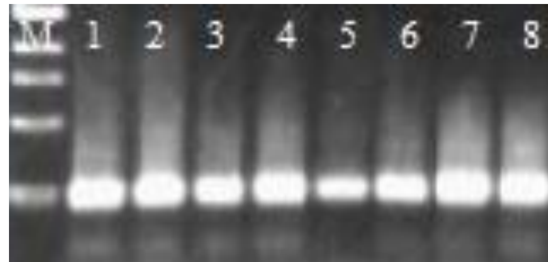

Figure 1. RAPD patterns of eight *H. serrata* samples generated by primer OPB10
(M: marker Thermo Scientific 1kb, 1–8: 8 sample of *H. serrata*)

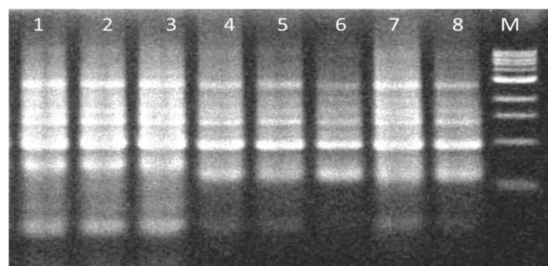

Figure 2. RAPD patterns of eight *H. serrata* samples generated by primer OPB11
(M: marker Thermo Scientific 1 kb, 1–8: 8 sample of *H. serrata*)

Results of genetic relationship analysis between the eight samples

Based on the data obtained by analyzing eight samples of *H. serrata* with 16 RAPD primers and using NTSYSpc version 2.1 software to calculate the genetic similarity coefficients between samples, the results are shown in table 4.

The genetic similarity coefficient of eight *H. serrata* samples ranged from 0.5217 to 0.9420. Among eight samples belong to eight locations, DL2 and DL3, SP1 and SP2 had the highest similarity coefficient (0.9420). DL1 and SP2 had the most distant genetic relationship, revealed by the lowest coefficient (0.5217).

Table 4. Similarity coefficient of eight different *H. serrata* samples

	DL1	DL2	DL3	SP1	SP2	SP3	SP4	SP5
DL1	1.0000000							
DL2	0.8405797	1.0000000						
DL3	0.8115942	0.9420290	1.0000000					
SP1	0.5507246	0.5652174	0.5942029	1.0000000				
SP2	0.5217391	0.5362319	0.5362319	0.9420290	1.0000000			
SP3	0.5942029	0.5797101	0.5797101	0.8695652	0.8405797	1.0000000		
SP4	0.5652174	0.5507246	0.5797101	0.8695652	0.8695652	0.9130435	1.0000000	
SP5	0.6666667	0.6521739	0.6521739	0.8260870	0.7971014	0.8115942	0.8115942	1.0000000

In the diagram of genetic relationship in figure 3, eight samples of *H. serrata* were divided into two major groups (I and II). Group I consisted of *H. serrata* samples collected from Da Lat (DL1, DL2, DL3), which were further sub-divided by their genetic similarity. DL2 and DL3 showed the highest genetic correlation (0.9420), indicating they were closer to each other than to DL1. Group II comprised of five samples of *H. serrata* from Sa Pa. This group could also be split up in to sub-group II.1 and II.2. The former was made up of SP1, SP2, SP3, SP4, in which SP1 and SP2 were most closely related with a similarity coefficient of 0.9420. SP5 sample had the most distant genetic relationship from the rest of Sa Pa samples, hence constituting a distinct branch.

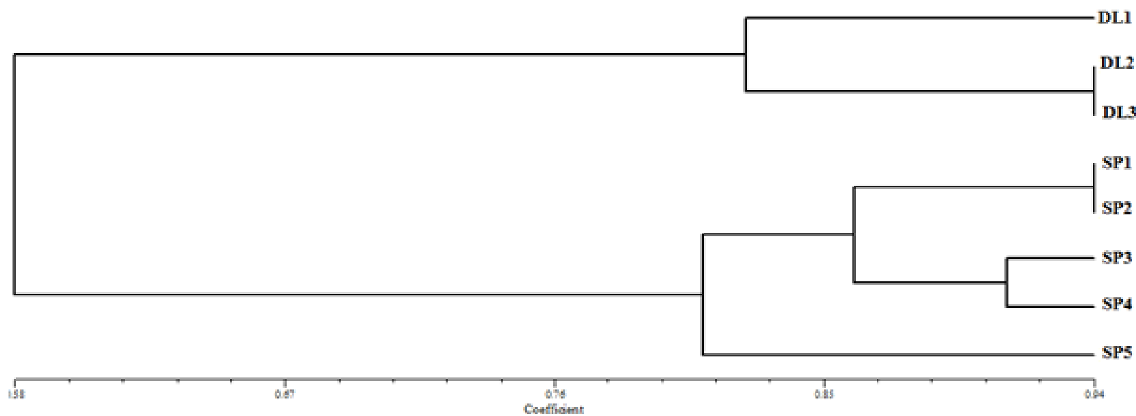

Figure 3. UPGMA dendrogram of cluster analysis of RAPD markers illustrating the genetic relationships among eight investigated samples of *H. serrata*

DNA fingerprinting analysis of eight *H. serrata* samples collected from Sa Pa and Da Lat showed that the genetic correlations among them were quite high. In terms of geography, we concluded that samples from neighboring areas, such as Nam Cang-Sa Pa, Ban Ho-Sa Pa or Ta Van-Sa Pa, Lao Chai-Sa Pa were closely related and it was possible that they evolved from a common ancestor.

CONCLUSION

Out of 16 analyzed RAPD primers, 15 were polymorphic and generated 70 DNA fragments, 44 of which were polymorphic. The number of amplified DNA fragments ranged from 1 to 8. The average PIC value of primers was 0.7182. In particular, OPB11 primer had the highest PIC value (0.8624) and OPC10 primer had the lowest PIC value (0.5266). Based on their genetic similarity, eight *H. serrata* samples were divided into two main groups corresponding to their habitat: Group I included three Da Lat samples (DL1, DL2, DL3) and Group II consisted of five samples from Sa Pa (SP1, Sp2, SP3, Sp4 and SP5). The results of our study could be useful for the conservation and development of *H. serrata.*

REFERENCES

Christenhusz M., Zhang X., Schneider H., 2011. A linear sequence of extant families and genera of lycophytes and ferns. *Phytotaxa*, 19(1): 7–54.

Gepts P., 1993. The use of molecular and biochemical markers in crop evolution studies. *Evol Biol*, (27): 51–94.

Dixit R.D., 1984. A census of the Indian Pteridophytes. Botanical survey of India, Howrah.

Kitajima M., Takayama H., 2011. Lycopodium alkaloids: isolation and asymmetric synthesis. Springer, 1–31.

Liu J. S., Yu C. M., Zhou Y. Z., Han Y. Y., Wu F. W., Qi B. F., Zhu Y. L., 1986a. Study on the chemistry of huperzine-A and huperzine-B. *Acta Chimica Sinica*, (44): 1035–1040.

Liu J. S., Zhu Y. L., Yu C. M., Zhou Y. Z., Han Y. Y., Wu F. W., Qi B. F., 1986b. The structures of huperzine A and B, two new alkaloids exhibiting marked anticholinesterase activity. *Canadian Journal of Chemistry*, (64): 837–839.

Ma X., Tan C., Zhu D., Gang D. R., Xiao P., 2007. Huperzine a from huperzia species-an ethnopharmacolgical review. *J Ethnopharmacol*, 113(1): 15–34.

Ma X. Q., Gang D. R., 2004. The lycopodium alkaloids. *Nat. Prod. Rep*., 21: 752–772.

Ouborg N.J., Piquot Y., Groenendael J. M., 1999. Population genetics molecular markers and the study of dispersal in plants. *J. Ecol*, (87): 551–568.

Rohlf F. J., 2000. Programa NTSYS-pc: numerical taxonomy and multivariate analysis system: version 2.1. Exeter Software. New York, 83p.

Schaal B. A., Leverich W. J., Rogstad S. H., 1991. Comparison of methods for assessing genetic variation in plant conservation biology. In: Falk, D.A., Holsinger, K.E. (Eds.), Genetics and Conservation of Rare Plants. Oxford University Press. New York, pp. 123–134.

Singh H., Singh M., 2010. *Huperzia serrata*: a promising medicinal pteridophyte from northeast India. *NeBIO*, 1(1): 27–34.

Tran Hop, 2000. Vietnamese medicinal plants. Agriculture publishing house, pp 110.

Williams J. G., Kubelik A. R., Livak K. J., Rafalski J. A., Tingey S. V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*., 18(22): 6531–6535.