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POPULATION GENETIC DIVERSITY OF *HUPERZIA CHINENSIS* (HERTER EX NESSEL) CHING. IN NORTHWEST REGION OF VIETNAM REVEALED BY ISSR AND SCOT MARKERS

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SUMMARY

Genetic diversity of natural *Huperzia chinensis* population in Northwest region of Vietnam was analyzed based on the DNA fingerprint data induced by ISSR and SCoT methods separately and combined data from both methods. The genetic diversity parameters and genetic distances among individuals based on SCoT technique were higher than ISSR technique [expected heterozygousity (H_{eS}) was 0.2812; Shannon index (I_S) was 0.4153; Percentage of polymorphic bands (PPB_S) was 75.44%; Genetic similarity coefficients (GSC_{SS}) were in range of 0.561 - 0.947 with the average value ($AGSC_S$) of 0.758 revealed by SCoT technique compared to H_{eI} was 0.2394; I_I was 0.3515; PPB_I was 60.87%, GSC_{SI} were 0.551 - 0.986 and $AGSC_I$ was 0.860 revealed by ISSR technique. Combined data showed that genetic diversity level of investigated population (H_e was 0.2583; I was 0.3804; PPB was 67.46%, higher than genetic diversity level of *Huperzia serrata* population in the same distribution area which was previously evaluated by the same markers. Combined data also indicated the genetic similarities among individuals (GSCs) were 0.579 - 0.960 with the average ($AGSC$) of 0.814. ISSR method reflected more loci in the investigated plants than SCoT method but SCoT method could differentiate the tested samples better and reflects genetic diversity at higher level than ISSR method.

Keywords: ISSR, SCoT, *Huperzia chinensis*, genetic diversity, population, Vietnam

INTRODUTION

In traditional and modern medicine, club mosses have been known as a group of medicinal plants used for treatment of Alzheimer's and other diseases due to the high content of Huperzin A. The most famous club moss is *Huperzia serrata* (Thunb. ex Murray) Trevis., however *Huperzia chinensis* (Herter ex Nessel) Ching. is also popular one of club mosses (Ma *et al.* 2006). These species are phylogenetically close together and sharing many botanical, phenotypic and ecological characteristics. The difference between them is the plant size, i.e *H. chinensis* is smaller than *H. serrata*. The phenotypic similarity has made the two species become confused to identify and even used to be considered as the same species. Recently, our field investigation showed that *H. serrata* and *H. chinensis* also share the same habitat in Northwest of Vietnam and could be identified by molecular characteristics (the Genbank accession numbers are MH464763 and MH464764 for *rbc*L gene, MH464749 and MH464750 for *psb*A-*trn*H intergenic region).

Because of the excessive rumors about the pharmacological effects, *H. chinensis* has been overexploited in recent years. Besides, *H. chinensis* plants only grow well in cool, shadowy, wet and high humidity, rich organic matter condition. This habitat makes *H. chinensis* population in Northwest region of Vietnam become small with scanty number of individuals and narrow distribution area.

High genetic diversity helps the population of certain taxon can adapt to the change of ecological factors and exist through generations (Hogbin, Peakall 1999). The knowledge about population genetic diversity is very important for plant conservation, development and sustainable exploitation. Based on the genetic diversity parameters of a plant population, the suitable strategies will be established and applied in that population for *in situ* and *ex situ* conservation and development (Hogbin and Peakall, 1999). This approach has been executed for large range of plant species including several *Huperzia* species such as *H. serrata* var. *longipetiolata* (Zhang *et al.*, 2011), *H. serrata* (Huang and He, 2010; Chen *et al.*, 2014; Ho *et al.*, 2018, Minh *et al*., 2019). However, the population genetic diversity of *H. chinensis* has not been conducted in Vietnam. Therefore, a thorough understanding of population genetic diversity of *H. chinensis* in Vietnam is desirable, even that there is only one population found in Northwest region of the country.

Nowadays, molecular markers are considered as powerful tools for genetic diversity analysis (Ouborg *et al*., 1999). Various approaches are available for DNA fingerprinting, such as RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), SSR (Simple Sequence Repeat), ISSR (Inter Simple Sequence Repeat) etc. (Kumar *et al.*, 2009) and SCoT (Start Codon Targeted) (Collard, Mackill, 2009). Each of them possesses the own advantages and disadvantages. Of which, ISSR and SCoT have been proved to be simple, low-cost and easy to implement and worthy for population genetic diversity estimation and considered as dominant markers. ISSR technique targets DNA regions between the simple sequence repeats (microsatellites) which can belong to either the transcribed regions or the non transcribed regions of the genome, while SCoT technique targets the start codon of functional genes. However, in principle, these techniques are similar when using a single primer as the forward and the reverse primer (Collard, Mackill, 2009).

This study was performed to evaluate the genetic diversity level of natural *H. chinensis* population in Northwest region of Vietnam and also the level of genetic similarity among individuals of this population. The results here will create the fundamental information for sustainable conservation and utilization of this medicinal plant. In addition, the current research showed the potential and the limitation of ISSR and SCoT techniques for population genetic diversity analysis.

MATERIALS AND METHODS

Materials

Thirty plants were randomly collected to represent for the *H. chinensis* population naturally distributed in Hoang Lien National Park (Lao Cai Province) for genetic diversity assessment. Sampled individuals were signed from HuS1 to HuS30 and collected with basal soil and transferred to Plant Breeding Technology Laboratory at Da Lat University for next studies, including genetic analysis and acclimatization research also. Fresh leaves were used for DNA extraction. DNA sample signs were same to the sign of collected individuals

DNA extraction

Total genomic DNA was extracted from fresh leaves using CTAB protocol I (Weising *et al*., 2005) with a modification of adding 10% SDS to the extraction buffer. Two DNA samples were extracted from each collected plant. Extracted DNA was dissolved in TE buffer. Then DNA concentration and quality were evaluated by Spectrophotometry method (Weising *et al.*, 2005) using the NanoScan2

system (Analytik Jena, Germany). Good DNA samples with value of OD_{260}/OD_{280} from 1.7 to 1.9 and closer with 1.8 were chosen and kept in -20°C for further analysis.

DNA fingerprinting

DNA fingerprinting of samples were conducted by ISSR and SCoT techniques. ISSR primers for this study were synthesized by PhuSa Biochem Ltd. Company (Vietnam), according to the primer set in the report by Admed (2005), Collard and Mackill (2009). Twenty random ISSR and SCoT primers were initially screened. Of which, 10 ISSR primers and 9 SCoT primers that yielded bright, clear bands and at least possessed one polymorphic band in five randomly tested *H. chinensis* samples were chosen for DNA fingerprinting.

PCRs were performed in 50 µL reactions containing 25 µL My Red HS Taq mix (Bioline), 0.2 µM primer and approximately 30 ng DNA templates. The reactions were performed in a Mastercycler® nexus thermocycler (Eppendorf, Germany) with the following programs:

In ISSR method: initial denaturation at 94 °C for 5 min; 10 cycles of 94 °C for 45 s, annealing temperature +5 (T_a +5) °C (Table 3) for 45s, decreased 0.5 °C/cycle, 72°C for 1min 30 s; 36 cycles of 94 °C for 45 s, annealing temperature for 45 s, 72°C for 1 min 30 s; Final extension at 72°C for 15 min.

In SCoT method: initial denaturation at 94 ^{0}C for 5 min; 36 cycles of 94 ^{0}C for 45 s, 50^{0}C for 45 s, 72^{0}C for 1 min 30 s; Final extension at 72^{0}C for 15 min.

The PCR products were separated in 2.5% agarose gel, using TBE buffer at 60 V for 3 h, stained with Ethidium bromide (0.5 µg/mL), and photographed under 254/312 nm wavelength lights using UVP GelStudio Plus System (Analitik Jena, Germany).

Data analysis

Since ISSR and SCoT markers are dominantly inherited, each observed band was assumed to represent the genotype at a single biallelic locus (Williams *et al*., 1990). The basic parameters for genetic diversity were calculated with the POPGENE software application. These parameters comprise the percentage of polymorphic bands (*PPB*), mean Nei's gene diversity index (H_e), and the Shannon index (I) (Yeh *et al.*, 1999).

Genetic similarity coefficients (*GSC*s) between pairs of samples and UPGMA dendrogram for genetic relationship among tested samples were calculated and established by using NTSYSpc 2.1 (Numerical Taxonomy and Multivariate Analysis System) software (Rohlf, 2004). The average of these genetic similarity coefficients (*AGSC*) was also calculated.

Population genetic diversity and genetic similarity among individuals of the investigated population assessment was executed based on ISSR-PCR data (through corresponding parameters singed as H_{eI}, I_I, PPB_I, GSC_{SI}, $AGSC_I$) and SCoT-PCR data (corresponding parameters singed as H_{eS}, I_S, PPB_S, GSC_{SS}, $AGSC_S$) separately for technical comparison. Based on the combined data from both methods, the adequate level of population genetic diversity and genetic similarity among individuals were evaluated.

The criteria to compare usability of two techniques in this study on investigated population are the rate of the chosen primers for DNA fingerprint inducing to initially tested primers (1); amount of achieved information as number of revealed loci per used primer (2); level of population genetic diversity reflection through *PPB*, H_e, and I parameters respectively (3); and level of genetic difference among investigated samples reflection through their pairwise genetic similarity coefficients (4).

Besides, for each used DNA fingerprinting technique, discriminatory ability was calculated as Polymorphism Information Content (PIC); number of revealed loci was recognized through efficiency of the primer-marker system as Effective Multiplex Ratio (EMR); efficiency of used method was measured by calculated

Marker Index (MI); and ability of the primer combination to detect the differences between investigated samples was expressed by Resolving power (Rp). PIC, EMR, MI and Rp values based on ISSR technique-induced data and SCoT technique-induced data were separately calculated by iMEC - an Online marker efficiency calculator tools which is available at https://irscope.shinyapps.io/ iMEC/ (Amiryousefi *et al*., 2018). These values then were used to compare the two DNA fingerprinting methods.

Table 1. Characteristics of the used primers in the study.

No.	Primer code	Sequence 3' to 5'	T_a (°C)	Chosen after screening	Number of induced bands	*PPB* (%)
1	UBC 807	5'-$(AG)_8$ T-3'	54	×	6	66.67
2	ISSR 814	5' –$(CT)_8$ TG-3'	51.5	×	8	75
3	UBC 842	5'-$(GA)_8$ T/C G -3'	51.5	×	9	66.67
4	UBC 844	5' –$(CT)_8$ AC-3'	52	×	9	33.33
5	UBC856	5'-$(AC)_8$ T/C A-3'	52	×	7	71.43
6	UBC873	5'-$(GACA)_4$ -3'	52	×	7	100
7	HB 8	5'-$(GA)_6$ GG-3'	52	×	5	60
8	HB12	5'-$(CAC)_3$ GC-3'	52	×	6	50
9	HB 15	5'-$(GTG)_3$GC-3'	52	×	7	42.86
10	UBC 17899	5'-$(CA)_6$ A/G G-3'	54	×	5	40
11	UBC 808	5' –$(AG)_8$ C-3'	52			
12	HB 9	5'-$(GT)_6$ GG-3'	52			
13	UBC 17898A	5'-$(CA)_6$ AG-3'	54			
14	HB 11	5'-$(GT)_6$ CC-3'	52			
15	UBC 17898B	5'-$(CA)_6$ GG-3'	54			
16	UBC 826	5'-$(AC)_8$ C-3'	54			
17	UBC 809	5'-$(AG)_8$G-3'	52			
18	UBC 862	5'- $(AGC)_6$ -3'	53.5			
19	UBC 830	5'-$(TG)_8$ G-3'	52			
20	UBC 813	5'-$(CT)_8$ T-3'	52			
	Total of ISSR technique based on selected primers				69	
	Average of ISSR technique based on selected primers				6.9	60.87
21	SCoT 1	CAACAATGGCTACCACCA	50	×	6	50
22	SCoT 3	CAACAATGGCTACCACCG	50	×	6	100
23	SCoT 12	ACGACATGGCGACCAACG	50	×	5	80
24	SCoT 13	ACGACATGGCGACCATCG	50	×	8	87.5
25	SCoT 18	ACCATGGCTACCACCGCC	50	×	8	62.5
26	SCoT 19	ACCATGGCTACCACCGGC	50	×	7	71.43
27	SCoT 22	AACCATGGCTACCACCAC	50	×	7	85.71
28	SCoT 29	CCATGGCTACCACCGGCC	50	×	5	80
29	SCoT 30	CCATGGCTACCACCGGCG	50	×	5	60
30	SCoT 9	CAACAATGGCTACCAGCA	50			
31	SCoT 2	CAACAATGGCTACCACCC	50			
32	SCoT 5	CAACAATGGCTACCACGA	50			
33	SCoT 21	ACGACATGGCGACCCACA	50			
34	SCoT 23	CACCATGGCTACCACCAG	50			
35	SCoT 33	CCATGGCTACCACCGCAG	50			
36	SCoT 25	ACCATGGCTACCACCGGG	50			
37	SCoT 36	GCAACAATGGCTACCACC	50			
38	SCoT 8	CAACAATGGCTACCACGT	50			

39	SCoT 27	ACCATGGCTACCACCGTG	50	
40	SCoT 17	ACCATGGCTACCACCGAG	50	
	Total of SCoT technique based on selected primers		57	
	Average of SCoT technique based on selected primers		6.33	75.44

RESULTS AND DISCUSSION

Genetic diversity and structure parameters of investigated population generated by ISSR method

Based on ISSR technique, the percentage of polymorphic bands of *H. chinensis* population in Northwest of Vietnam was PPB_I = 60.87%. The expected heterozygousity of this population was H_{el} = 0.2394 and its' Shannon index was I_I = 0.3515.

Genetic similarity coefficients between pairs of tested samples in investigated population were in range of $GSCs_I$ = 0.551 - 0.986. The average value of $AGSC_I$ = 0.860. Pairs of HuS8 – HuS9 and HuS11 – HuS12 were the highest genetically similar while the highest genetic distance was observed in pairs of HuS1 – HuS29 and HuS1 – HuS20.

Based on the achieved genetic similarity coefficients, the UPGMA dendrogram for genetic relationship among investigated samples was established as Figure 1.

Genetic diversity and structure parameters of investigated population generated by SCoT method

Results from Table 1 showed that based on SCoT technique, the percentage of polymorphic bands (PPB_S) of *H. chinensis* population in Northwest of Vietnam was 75.44%. The expected heterozygousity (H_{eS}) of this population was 0.2812 and its' Shannon index (I_S) was 0.4153.

Genetic similarity coefficients ($GSCs_S$) between pairs of samples in investigated population were in range of 0.561–0.947 with the average value ($AGSC_S$) of 0.758. Pair of HuS8 – HuS10 was the highest genetically similar while the highest genetic distance was observed in pair of HuS1 – HuS16.

Based on the genetic similarity, the UPGMA dendrogram for genetic relationship among studied individuals was established as Figure 2.

Genetic diversity and structure of investigated population based on combined data

Based on combined data, the percentage of polymorphic bands (*PPB*) of studied population was 67.46%. The expected heterozygousity (H_e) of this population was 0.2538 and Shannon index (*I*) was 0.3804.

Genetic similarity coefficients (*GSC*s) between pairs of individuals in investigated population were in range of 0.579-0.960 with the average value (*AGSC*) of 0.814. Pair of HuS28 – HuS29 was the highest genetically similar while the highest genetic distance was observed in pair of HuS1 – HuS27. UPGMA dendrogram for genetic relationship among studied individuals was established based on the genetic similarity coefficients and shown Figure 3.

For genetic diversity assessment, it is better and more exact when using more data and investigating more loci in genome (Carling and Brumfield, 2007). Thus, to exploit all achieved data in this study, the ISSR technique-induced data and SCoT technique-induced data were combined to show the genetic diversity of *H. chinensis* in Northwest of Vietnam.

There are several studies on wide range of plants which share same life history traits to *H. chinensis*, i.e. short-lives perennial, narrow distribution area, breeding system of mixed matting, spore dispersal by wind and water. Two remarkable from these are the studies of Hamrick and Godt (1996) and Nybom and Bartish (2000). As comparing to previous study using Isozyme technique implemented by Hamrick and Godt (1996), combined data-based percentage of polymorphic bands and heterozygousity in the current study were relatively high but still in estimated range.

However. As compared to study of Nybom and Bartish (2000) these genetic diversity parameters were clearly higher.

Huperzia chinensis has been recorded in Yunan (China) and Northwest of Vietnam, mainly in Hoang Lien National Park (Lao Cai Province) but no study on population genetic diversity of this species has been reported. Some previous studies on genetic diversity were performed in the same genus species, mainly in *H. serrara*.

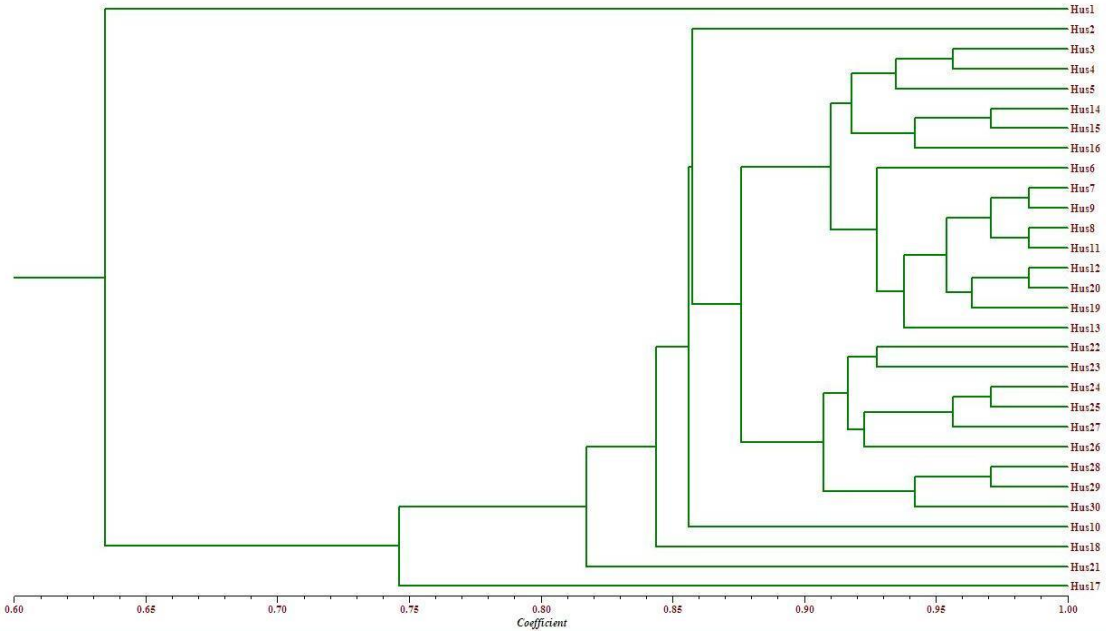

Figure 1. ISSR technique-based UPGMA dendrogram for genetic relationship among individuals belong to studied *H. chinensis* population.

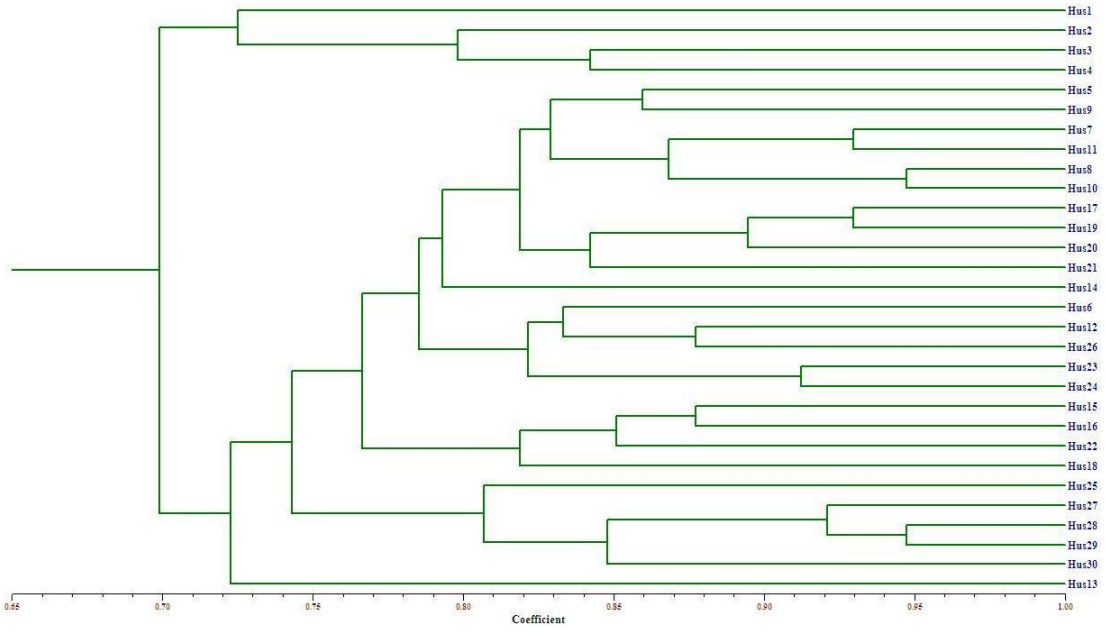

Figure 2. SCoT technique-based UPGMA dendrogram for genetic relationship among individuals belong to studied *H. chinensis* population.

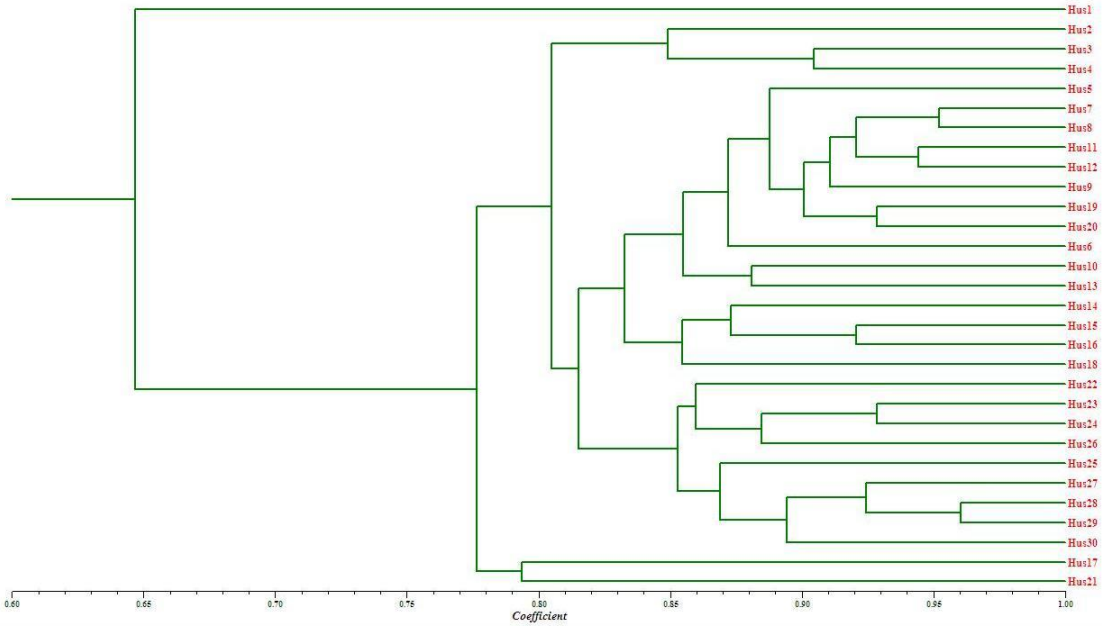

Figure 3. Combined data-based UPGMA dendrogram for genetic relationship among individuals belong to studied *H. chinensis* population.

In the current study, *H. chinensis* population was relatively less diverse (H_e = 0.2538 and I = 0.3804) than the *H. serrata* var. *longipetiolata* populations in Wuyi mountains of China which estimated by Zhang *et al.* (2011) using AFLP technique. The average of heterozygousity and Shannon index in these populations was 0.272 and 0.392, respectively.

The average of heterozygousity and Shannon index of *H. chinensis* population in Vietnam was higher than the most genetic diverse *H. serrata* populations of ten natural population around China according to Huang *et al.* (2010) and *H. serrata* populations/species level in Wuling mountain using AFLP technique Chen *et al.* (2014). However, the percentage of polymorphic bands recorded in *H. chinensis* population in Vietnam was lower than the *H. serrata* populations in China (Huang *et al.* 2010, Chen *et al.* 2014).

Recently, Minh *et al*. (2019) studied on the *H. serrata* population which has shared the same habitat with *H. chinensis* population investigated in the current study using the same DNA fingerprinting methods. From the genetic diversity of these two populations, the *H. chinensis* population was more diverse than *H. serrata* population (H_e = 0.2583; I = 0.3804; *PPB* = 67.46% for *H. chinensis* population and H_e = 0.1436; I = 0.2161; *PPB* = 45.45% for *H. serrata* population). Even these two populations were overexploited, but *H. serrata* plants were more harvested due to their larger size and high possibility of being found out.

Figure 3 for UPGMA dendrogram of genetic relationship among individuals in studied *H. chinensis* population showed that almost samples was clustered in a main group, while there were three individuals established three own groups. These separated individuals may be the consequence of the migration from other same species population distributed in China and/or the human's activities-formed habitat separation.

Comparison of usability between ISSR and SCoT methods

DNA fingerprinting ability and characteristics of induced data from ISSR and SCoT techniques in investigated population were described in Table 2.

Table 2 showed that the rate of the chosen primers for DNA fingerprint inducing to initially tested primers in ISSR technique was about 5% higher than in SCoT technique. For amount of achieved information, the average number of revealed loci per a chosen primer in ISSR technique was 1.09 fold higher than SCoT technique, indicating that ISSR technique reveals more loci in the investigated plants than SCoT technique.

Table 2. DNA fingerprinting ability of ISSR and SCoT techniques in studied population.

Criterion	ISSR technique	SCoT technique
Rate of the chosen primers for DNA fingerprint inducing to initially tested primers	10/20	9/20
Average number of revealed loci per chosen primer	6.9	6.33
Percentage of polymorphic bands (*PPB*)	60.87%	75.44%
Expected heterozygousity (H_e)	0.2394	0.2812
Shannon index (*I*)	0.3515	0.4153
Average of pairwise genetic similarity coefficient (*AGSC*)	0.860	0.758

Table 3. Effect of used primers and techniques in genetic diversity estimation of studied population.

No.	Primer code	Polymorphism information content (PIC)	Effective multiplex ratio (EMR)	Marker index (MI)	Resolving power (Rp)
1	UBC 807	0.1913	7.1667	0.0056	1.6667
2	ISSR 814	0.1646	7.3667	0.0081	2.6000
3	UBC 842	0.1952	6.3667	0.0050	1.1333
4	UBC 844	0.2070	8.7333	0.0019	0.5333
5	UBC856	0.2074	4.8667	0.0017	0.2667
6	UBC873	0.2072	5.8333	0.0018	0.3333
7	HB 8	0.1472	3.8667	0.0090	0.6667
8	HB12	0.1820	5.2000	0.0067	1.6000
9	HB 15	0.1736	5.9000	0.0074	2.2000
10	UBC 17899	0.1538	5.5333	0.0087	1.4667
	Average of ISSR technique	0.1829	6.0833	0.0056	1.2467
11	SCoT 1	0.2981	4.9667	0.0097	1.8000
12	SCoT 3	0.3279	5.7667	0.0097	3.0000
13	SCoT 12	0.3207	4.9000	0.0098	2.8667
14	SCoT 13	0.3152	4.1000	0.0099	1.4000
15	SCoT 18	0.3069	3.9333	0.0099	3.7333
16	SCoT 19	0.3142	3.4000	0.0099	2.2667
17	SCoT 22	0.3540	5.5333	0.0087	1.2000
18	SCoT 29	0.3348	3.7000	0.0095	1.8000
29	SCoT 30	0.3844	4.3667	0.0064	1.2667
	Average of SCoT technique	0.3285	4.5185	0.0093	2.1481

However, the ability to reflect population genetic diversity of SCoT technique was significantly higher than ISSR technique. These were recognized more clearly based on the values of the percentage of polymorphic bands (1.23-fold) than based on the values of the expected heterozygousity and Shannon index (1.17- and 1.18-fold, respectively).

The average pairwise genetic similarity coefficient among tested samples by SCoT method was 1.35 fold lower than by ISSR method. This indicated that SCoT method reflected the genetic difference among investigated samples better than ISSR method.

Above results can be explained by the nature of the used techniques. ISSR method reveals the DNA regions between the simple sequence repeats (DNA microsatellites) which can belong to either the transcribed regions or the non transcribed regions of the genome while SCoT technique targets the start codon of functional genes which exit in transcribed regions only. As another explanation, the genetic diversity of DNA microsatellites in investigated population could be high but it was higher the functional genes.

Using the same markers, Minh *et al*. (2019) studied on the *H. serrata* population sharing the same habitat with *H. chinensis* population in this study and found that 7/20 SCoT primers and 10/20 ISSR SCoT primers were chosen based on the same criteria to screening the primer with the current study. In addition, the genetic diversity parameters were relatively similar by using ISSR and SCoT techniques (H_{eS} = 0.14; I_S = 0.22; PPB_S = 45.00% based on SCoT technique compared to H_{eI} = 0.15; I_I = 0.23; PPB_I = 45.83% based on ISSR technique). In consistent to Minh *et al*. (2019), our results indicated that DNA fingerprinting ability and characteristics by ISSR and SCoT techniques also depend on the plant taxon.

Our comparative results could be demonstrated by the average values of Polymorphism Information Content (PIC), Effective multiplex ratio (EMR), Marker index (MI) and Resolving power (Rp) which achieved from ISSR technique-induced and SCoT technique-induced data analysis in Table 3.

The average values of PIC, MI and Rp using SCoT method were significantly higher than using ISSR method. This indicated that discriminatory ability, efficiency and ability of the primer combination when using SCoT method were better than using ISSR method. In addition, the average effective multiplex ratio (EMR) achieved from ISSR technique was higher than from SCoT technique indicating more loci revealed. According to Chesnokov and Artemyeva (2015), PIC value reflects the discriminatory ability of the marker; the higher the value of EMR means the more efficient the "primer-marker system". Moreover, the higher MI means the better the method. Furthermore, Rp is a parameter used to characterize the ability of the primer/marker combination to detect the differences between investigated genotypes.

The results from the current study are significantly consistent to the report of Shahlaei *et al.* (2014) about the efficacy of SCoT and ISSR techniques in assessment of tomato genetic diversity in Iran. Shahlaei and colleagues also showed that ISSR technique induced more loci and caused higher value of EMR than SCoT technique while PIC and Rp values achieved by SCoT technique also were higher than those achieved by ISSR technique.

CONCLUSIONS

The genetic diversity of the natural *H. chinensis* population in Northwest region of Vietnam was slightly high in comparison to the same life history traits plant populations and same genus populations and significantly higher than *H. serrata* population in the same habitat.

In technical aspect, the obtained results for investigated population indicated that ISSR method reflected more loci than SCoT method. However, SCoT technique could differentiate the individuals better and reflect genetic diversity at higher level than ISSR technique.

The achieved genetic diversity parameters

based on combined data from both ISSR and SCoT methods should be considered as fundamental information for *in situ* conservation of *H. chinensis* species in Northwest region of Vietnam.

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