

GENETIC DIVERSITY OF NATURALLY DISTRIBUTED *Rhododendron moulmainense* Hook. f. POPULATIONS IN LAM VIEN PLATEAU, VIETNAM REVEALED BY ISSR AND SCoT MARKERS

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ABSTRACT

Genetic diversity and variation of three naturally distributed *Rhododendron moulmainense* populations in the Lam Vien Plateau of Vietnam were analyzed using ISSR and SCoT markers separately and data from the two types of markers were then combined to show the overall results. Based on combined data, the genetic diversity parameters of the Tuyen Lam population were percentage of polymorphic bands (PPB) = 28.79%; genetic heterozygosity (H_e) = 0.1041; Shannon index (I) = 0.1552. The corresponding parameters were PPB = 37.88%; H_e = 0.1434; I = 0.2131 in the Hon Nga population, and PPB = 46.21%; H_e = 0.1857; I = 0.2709 in the Bidoup population. At the species level in Lam Dong province, the corresponding parameters were PPB = 62.12%; H_e = 0.1747; I = 0.2756. The average of pairwise genetic similarity coefficients among investigated individuals of Tuyen Lam population was 0.973, of Hon Nga population was 0.911, and of Bidoup population was 0.895. The genetic differentiation and the number of migrants per generation among investigated populations were G_{STs} = 0.1735 and N_m = 2.3824 individuals, respectively. Genetic distance between Tuyen Lam and Hon Nga populations was 0.0399, between Tuyen Lam and Bidoup populations were 0.0621, and Bidoup and Hon Nga populations were 0.0670. Data generated by both marker types and the combined revealed similar trends of genetic diversity levels among the studied populations. The SCoT data showed higher values of genetic diversity and population gene differentiation than did the ISSR data, but the latter showed higher gene differentiation among sampled individuals in the study populations. Comparing the values of Polymorphism Information Content (PIC) for discriminatory ability; Effective Multiplex Ratio (EMR) for several revealed loci and/or efficiency of the primer-marker system; Marker Index (MI) for the efficiency of used method; and Resolving Power (Rp) between used techniques indicated that discriminatory ability, efficiency, and ability of the ISSR primer combinations were higher but using SCoT marker system was more efficient than ISSR marker system for evaluating genetic diversity.

Key words: *Rhododendron moulmainense*, ISSR, SCoT genetic diversity, Vietnam

INTRODUCTION

The genus *Rhododendron* belongs to Ericaceae and comprises about 1000 species (Fang & Stevens, 2005). In Vietnam, 44 species of this genus have been recorded (Huong *et al.*, 2012). Many *Rhododendron* species have been cultivated as medicinal plants and as ornamental plants for their beautiful flowers. *Rhododendron moulmainense*

Hook. is one of these species (Chi, 2012). Recently, the exploitation of precious plants from their natural habitats for ornamental plant/bonsai making purposes has been intensifying in Lam Dong province, in the Lam Vien plateau of Vietnam, and changes in the local ecology have been intensifying. This has exposed *R. moulmainense* to a loss in genetic diversity and if there is no conservation solution, the extinction of these species populations is only a matter of time.

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Research on the genus *Rhododendron* in Lam Dong province by Duy *et al.* (2014) revealed was difficult to find seedlings and young plants of *R. moulmainense*. Populations were small and the number of individuals had continuously declined by more than 50% over 10 years. The total area of the species' distribution in Lam Dong was less than 100 km² (Duy *et al.*, 2014). Collating with IUCN standards, *R. moulmainense* had been listed as Vulnerable level, i.e. VU.A2a; B2b; C2b (iii, iv); D2b (iii, iv), and these researchers concluded that improved conservation measures were required to protect the species.

Genetic diversity enables plant species to evolve and adapt to climate change and changing environmental conditions. An understanding of population genetic diversity is essential for long-term conservation, development, and sustainable exploitation. Genetic diversity and genetic variation within and among populations of a species are assessed to create the basis to establish and apply suitable strategies for *in-situ* and *ex-situ* conservation and development of those populations (Hogbin & Peakall, 1999). This approach has been applied in many plant species including several *Rhododendron* species such as *R. trifolium* and *R. nivale* in Tibet (Xu *et al.*, 2017a; 2017b), *R. aureum*, *R. protistum* var. *giganteum* in China (Liu *et al.*, 2012; Wu *et al.*, 2015) and *R. ferrugineum* populations in Northern Apennines (Bruni *et al.*, 2012). However, the population genetic diversity of *R. moulmainense* has not yet been studied in Vietnam.

Based on the rapid development of biotechnology, molecular markers are considered powerful tools for analysis of population genetic diversity and variation (Muhammad & Muhammad, 2014). 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) (Kumar *et al.*, 2009), and SCoT (Start Codon Targeted) (Collard & Mackill, 2009). Each of those approaches possesses their, own advantages and disadvantages, but ISSR and SCoT have proved to be low-cost, simple, and easy to execute and worthy markers for population genetic diversity assessment and are considered as dominant techniques. While

the ISSR technique targets DNA regions between the microsatellites which distribute either the transcribed regions or the non transcribed regions of the genome. SCoT technique targets the start codon of functional genes. However, these techniques are technically similar when using a single oligonucleotide as forward and reverse primers in PCR (Collard & Mackill, 2009). ISSR and SCoT techniques were proposed to be used in conjunction or with other DNA fingerprinting techniques for various applications such as genetic analysis and quantitative trait loci mapping, especially in laboratories with a preference for agarose gel electrophoresis (Nagoaka & Ogihara, 1997; Collard & Mackill, 2009).

The main purpose of our study was to assess the genetic diversity, variation, and structure of three naturally distributed *R. moulmainense* populations in the Lam Vien plateau of Vietnam, to provide basic data for conservation and sustainable utilization of this medicinal/ornamental plant species. Also, we compared the performance of ISSR and SCoT techniques for assessing the genetic diversity of the investigated populations.

MATERIALS AND METHODS

Twenty individuals were chosen for sampling from each other at a distance of at least 200 m from each of three natural populations of *R. moulmainense* on the Lam Vien plateau (Lam Dong province). Fresh leaves from chosen individuals were labeled and collected separately to bring to the Plant Breeding Technology Laboratory of Dalat University for DNA extraction. Information for the collected samples is presented in Table 1.

DNA extraction

CTAB protocol I (Weising *et al.*, 2005) was modified by adding 10% SDS to the extraction buffer, which was used to extract total genomic DNA from fresh leaves. Two to three DNA samples were extracted from each collected sample. Extracted DNA was dissolved in the TE buffer. DNA concentration and purity were measured by the Spectrophotometry method (Weising *et al.*, 2005) using the NanoScan2 system (Analytik Jena). The DNA samples with a value of OD₂₆₀/OD₂₈₀ from

Table 1. Collected samples used in this research

Sample code	Population	Geographic localities	Longitude/Latitude
RhC1, RhC2, ..., RhC20	Tuyen Lam	Tuyen Lam lake	108°26'22.79"E, 11°52'13.16"N
RhB1, RhB2, ..., RhB20	Hon Nga	Hon Nga mountain	108°14'10.43"E, 12° 1'16.64"N
RhA1, RhA2, ..., RhA20	Bidoup	Bidoup mountain	108°34'57.43"E, 12° 9'12.35"N

1.7 to 2.0 and closer to 1.8 were chosen for analysis in the study. DNA samples were stored at -20°C for subsequent use.

DNA fingerprinting

ISSR and SCoT techniques were used to create DNA fingerprints. ISSR primers used in this study were synthesized by PhuSa Biochem Ltd. Company (Vietnam), according to the primer set suggested by the University of British Columbia and Zagazig University (Egypt) (Admed, 2005), while SCoT primers were the set published by Collard and Mackill (2009), respectively.

For each DNA fingerprinting technique, twenty randomly chosen primers were used on five randomly tested *R. moulmainense* samples to determine which yielded bright, clear bands and displayed at least one polymorphic band. Nine ISSR and nine SCoT primers met the set-out criteria and were chosen and described in Table 2. These selected primers were applied to the full set of 60 *R. moulmainense* samples.

PCRs were performed in 50 µL-volume, which each contained 25 µL My Red HS Taq mix (Bioline), 0.2 µM primer, and approximately 30 ng DNA templates. The amplifications were with the following programs:

- For ISSR techniques: initial denaturation at 94°C for 5 min; 10 cycles of 94°C for 15 sec, annealing temperature +5 (T_a+5)°C (Table 2) for 15 sec, decreased 0.5°C/cycle, 72°C for 45 sec; 30 cycles of 94°C for 15 sec, annealing temperature for 15 sec, 72°C for 45 sec; final extension at 72°C for 10 min.
- For SCoT technique: initial denaturation at 94°C for 5 min; 36 cycles of 94°C for 15 sec, 50°C for 15 sec, 72°C for 45 sec; final extension at 72°C for 10 min.

The PCR products were examined for specificity via 2.5% agarose gel electrophoresis, using TBE buffer at 70 V for 2.5 hr, stained with Ethidium bromide (0.5 µg/mL), and photographed under 254 and 312 nm wavelength lights using UVP GelStudio Plus System (Analytik Jena, Germany).

Data analysis

Since ISSR and SCoT markers are dominantly inherited, each band was assumed to represent the phenotype at a single biallelic locus (Williams *et al.*, 1990). The bands were scored as presence (1) or absence (0) characters to construct the binary data matrix.

Table 2. ISSR and SCoT primers used in this study

No.	Primer code	Sequence 3' to 5'	T_a (°C)	Chosen after screening	Number of induced bands	PPB (%)
ISSR primers						
1	UBC 17899	5'-(CA) ₆ A/G G-3'	54	×	10	80.00
2	HB 8	5'-(GA) ₆ GG-3'	52	×	9	44.44
3	HB12	5'-(CAC) ₃ GC-3'	52	×	9	55.56
4	UBC 807	5'-(AG) ₈ T-3'	54		6	50.00
5	UBC 808	5'-(AG) ₈ C-3'	52	×	5	40.00
6	ISSR 814	5'-(CT) ₈ TG-3'	51.5	×	6	50.00
7	UBC 842	5'-(GA) ₈ T/C G -3'	51.5	×	10	50.00
8	UBC856	5'-(AC) ₈ T/C A-3'	52	×	5	60.00
9	UBC873	5'-(GACA) ₄ -3'	52	×	6	83.33
Total of ISSR technique based on selected primers					66	
Average of ISSR technique based on selected primers					7.3	57.58
SCoT primers						
10	SCoT 1	CAACAATGGCTACCACCA	50	×	10	60.00
11	SCoT 9	CAACAATGGCTACCAGCA	50		7	71.43
12	SCoT 12	ACGACATGGCGACCAACG	50	×	7	85.71
13	SCoT 13	ACGACATGGCGACCATCG	50	×	6	50.00
14	SCoT 18	ACCATGGCTACCACCGCC	50	×	7	57.14
15	SCoT 19	ACCATGGCTACCACCGGC	50	×	6	83.33
16	SCoT 22	AACCATGGCTACCACCAC	50	×	7	85.71
17	SCoT 29	CCATGGCTACCACCGGCC	50	×	7	42.86
18	SCoT 30	CCATGGCTACCACCGGCG	50	×	9	77.78
Total of SCoT technique based on selected primers					66	
Average of SCoT technique based on selected primers					7.3	68.22

The basic parameters for genetic diversity were calculated with the Popgene 32 software application. These parameters comprise the percentage of polymorphic bands (*PPB*), genetic heterozygosity/Nei's gene diversity index (H_e), the Shannon index (I), Nei's coefficient of gene differentiation (G_{ST}), gene flow (Nm), and genetic distance between populations (D) (Yeh *et al.*, 1999). Based on genetic distances between pairs of investigated populations, the dendrogram expressing the genetic relationship among these populations was established using NTSYSpc 2.1 (Numerical Taxonomy and Multivariate Analysis System) software (Rohlf, 2004). Analysis of molecular variance (AMOVA) was carried out using the GenAlEx 6.0 program (Peakall & Smouse, 2006) to describe the distribution of genetic variability among and within investigated populations. Pairwise genetic similarity coefficients ($GSCs$) among investigated samples, their average ($AGSC$), and UPGMA dendrogram for the genetic relationship among studied samples were calculated and established by using NTSYSpc 2.1 software (Rohlf, 2000).

Genetic parameters of three investigated populations were calculated based on ISSR technique-induced data (corresponding parameters coded as PPB_I , H_{eI} , I_I , G_{STI} , Nm_I , D_I , $GSCs_I$, $AGSC_I$) and SCoT technique-induced data (corresponding parameters coded as PPB_S , H_{eS} , I_S , G_{STS} , Nm_S , D_S , $GSCs_S$, $AGSC_S$) separately for comparison of the two DNA fingerprinting techniques. Using a data set combined from both techniques, final genetic parameters were calculated and coded as PPB , H_e , I , G_{ST} , Nm , D , $GSCs$, $AGSC$.

The criteria to compare the usability of the used techniques comprised the Polymorphism Information Content (PIC) for discriminatory ability; Effective Multiplex Ratio (EMR) for number of revealed loci and/or efficiency of the primer-marker system; Marker Index (MI) for the efficiency of used method; and Resolving Power (Rp) for the ability of the primer combination to detect the differences between investigated samples. PIC, EMR, MI, and Rp values for all samples were calculated based on ISSR and SCoT techniques separately using iMEC-an online marker efficiency calculator tools which is available at <https://irscope.shinyapps.io/iMEC/> (Amiryousefi *et al.*, 2018).

RESULTS AND DISCUSSION

ISSR technique-induced data for genetic diversity and genetic variation among and within investigated populations

A total of 66 bands were recorded with an average of 7.3 bands per primer. Genetic diversity parameters based on ISSR markers are shown in Table 3.

The pairwise genetic similarity coefficients ($GSCs_I$) among investigated individuals of the Tuyen Lam population were between 0.758 and 0.985 with an average ($AGSC_I$) of 0.893. The $GSCs_I$ were between 0.697 and 1.000 with an average ($AGSC_I$) of 0.906, and between 0.727 and 1.000 with an average ($AGSC_I$) of 0.883 was obtained for Hon Nga and Bidoup populations, respectively. At the species level, the pairwise genetic similarity coefficients ($GSCs_I$) of 60 *R. moulmainsense* individuals investigated in this study were between 0.667 and 1.000 with an average ($AGSC_I$) of 0.880. However, 5 pairs of individuals could not be genetically distinguished.

Analyzing the DNA fingerprints induced by the ISSR technique, the genetic differentiation (G_{STI}) among *R. moulmainsense* populations was estimated to be 0.1290 and the number of migrants (Nm_I) was estimated as 3.3774 individuals per generation among populations. Genetic distance (D_I) between Tuyen Lam and Hon Nga populations was 0.0191, between Tuyen Lam and Bidoup populations were 0.0444 and between Hon Nga and Bidoup populations was 0.0490. The genetic relationship among three investigated populations based on ISSR markers is presented in Figure 1.

AMOVA analysis revealed that 16% of total genetic variability was among populations and the remaining 84% portioned within populations.

SCoT technique-induced data for genetic diversity and genetic variation among and within investigated populations

The SCoT techniques also yielded 66 bands with an average of 7.3 bands per primer. Genetic diversity parameters based on SCoT markers are shown in Table 4.

Table 3. Genetic diversity parameters based on ISSR markers in the three populations

Parameter	Tuyen Lam	Hon Nga	Bidoup	Species-level
H_{eI}	0.1259	0.1293	0.1708	0.1630
I_I	0.1885	0.1947	0.2512	0.2561
PPB_I (%)	36.36	36.36	43.94	57.58

Table 4. Genetic diversity parameters based on SCoT markers in the three populations

Parameter	Tuyen Lam	Hon Nga	Bidoup	Species-level
H_{eS}	0.0823	0.1574	0.2006	0.1864
I_S	0.1220	0.2316	0.2907	0.2951
PPB_S (%)	21.21	39.39	48.48	66.67

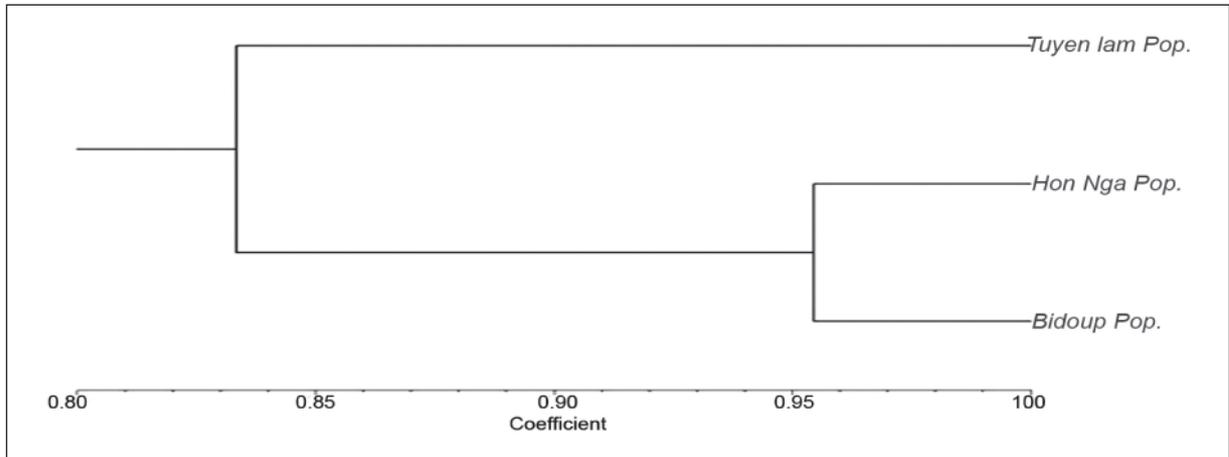


Fig. 1. Dendrogram expressing the genetic relationship among three investigated populations based on ISSR markers.

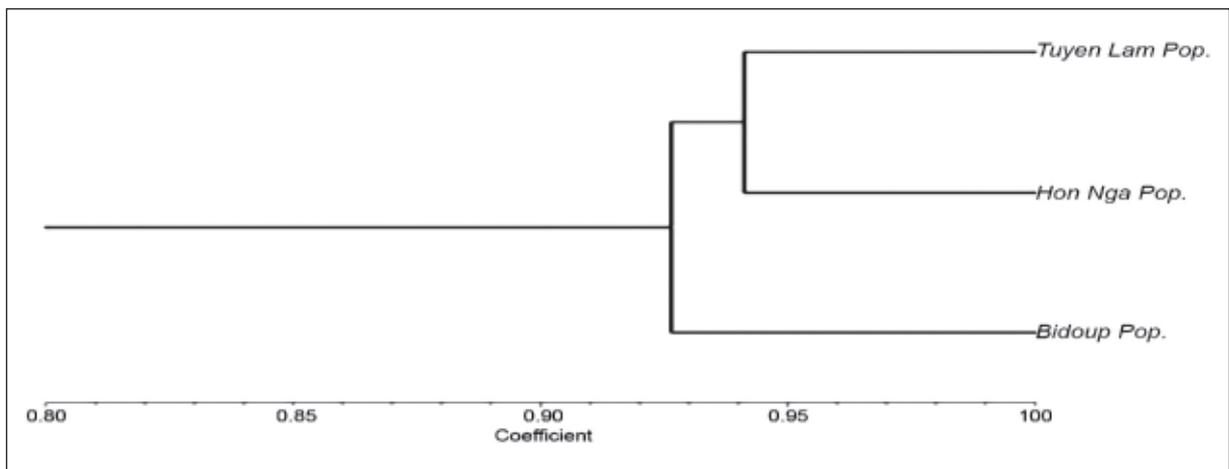


Fig. 2. Dendrogram representing the genetic relationship among three investigated populations based on SCoT markers.

The pairwise genetic similarity coefficients among investigated individuals of the Tuyen Lam population were between 0.864 and 1.000 with an average of $AGSC_S = 0.951$. Those for Hon Nga were between 0.758 and 1.000 with an average of $AGSC_S = 0.916$ and those for Bidoup were between 0.652 and 1.000 with an average of $AGSC_S = 0.895$. At the species level, the pairwise genetic similarity coefficients of 60 studied *R. moulmainense* individuals investigated in this study were between 0.561 and 1.000 with an average of $AGSC_S = 0.908$. Also, 16 pairs of individuals could not be distinguished.

Based on the SCoT technique, the genetic differentiation among *R. moulmainense* populations (G_{STS}) was estimated to be 0.2124 and the number of migrants (Nm_S) was estimated as 1.8539 individuals per generation among populations. Genetic distance (D_S) between Tuyen Lam population and Hon Nga population was 0.0606, between Tuyen Lam population and Bidoup population was 0.0796 and between Hon Nga population and Bidoup population was 0.0703. The genetic relationship among three investigated populations based on SCoT markers is presented in Figure 2.

AMOVA analysis revealed remarkable genetic differences among all three *R. moulmainense* populations, with 19% of total genetic variability portioned among populations and the remaining 81% portioned within populations.

Comparison of usability between the ISSR and SCoT techniques in studied populations

At the population level, ISSR and SCoT markers reflected the same general trend of H_e and I in three investigated populations: Tuyen Lam population < Hon Nga population < Tuyen Lam population in genetic diversity, but this trend was not clear based on values for percentage of polymorphic bands. The combined data also reflected the above trend for all of I , H_e , and PPB . Depended on each of the studied populations, I , H_e , and PPB values were higher either when using the ISSR technique or the SCoT technique, but based on all data sets the Bidoup population had the highest genetic diversity. DNA fingerprinting ability and characteristics of induced data of ISSR and SCoT techniques in total 60 investigated individuals are shown in Table 5.

Table 5 shows that the DNA fingerprinting ability including the rate of the chosen primers/initially tested primers and several revealed loci was similar for the ISSR technique and SCoT techniques. Generally, SCoT technique-induced data showed higher values of genetic diversity and population gene differentiation parameters than ISSR technique-induced data. Thus, ISSR technique-induced data inevitably showed higher gene differentiation among investigated individuals in studied populations. These comparative results again could be recognized through the average values of Polymorphism Information Content (PIC), Effective Multiplex Ratio (EMR), Marker Index (MI), and Resolving Power (Rp) achieved from ISSR and SCoT techniques in Table 6.

Data in Table 6 shows that in this study, the average of PIC, MI, and Rp using the ISSR primers was significantly higher than using the SCoT primers. This indicated that discriminatory ability,

efficiency, and ability of the primer combination when using the ISSR technique were better than using the SCoT technique. The average effective multiplex ratio (EMR) achieved from the SCoT technique was higher than from the ISSR showed that the system of the SCoT primers was more efficient than the system of the ISSR primers.

In general, in assessing the effectiveness of marker systems or DNA fingerprinting techniques in assessing population diversity and genetic variation, PIC reflects the discriminatory ability of the marker. The higher MI means the better the method and Rp is a parameter used to characterize the ability of the primer/marker combination to detect the differences between investigated genotypes. The higher the value of EMR means the more efficient the “primer-marker system” (Chesnokov & Artemyeva, 2015).

Genetic diversity and genetic variation of investigated *R. moulmainense* populations based on combined data

In previous research on population genetic diversity and genetic variation, the result generally recognized that increasing the number of investigated loci gives more reliable results (Carling & Brumfield, 2007). There have been various studies using a combination of two or more DNA fingerprinting techniques to study population genetics (Kumar *et al.*, 2014; Xu *et al.*, 2017a; Agarwal *et al.*, 2018; Talebi *et al.*, 2018). To best exploit all the data generated in this study, we combined the ISSR and SCoT data sets. Both marker types were dominant, making combination feasible. Genetic diversity parameters of the three populations and at the species level for the combined data set are shown in Table 7. Bidoup population was the highest genetically diverse, Tuyen Lam population was the lowest genetically diverse and Hon Nga population was intermediate.

These results may be explained by the history of the three populations over recent decades. While the habitat of the Bidoup population has been protected by Bidoup Nui Ba National Park. Hon

Table 5. DNA fingerprinting ability and genetic diversity/variation revealing feature of the ISSR and the SCoT techniques in studied populations

Criterion	ISSR technique	SCoT technique
Rate of the chosen primers/ initially tested primers	9/20	9/20
Average number of revealed loci per chosen primer	7.3	7.3
Percentage of polymorphic bands (PPB)	57.58%	66.67%
Expected heterozygosity (H_e)	0.1630	0.1864
Population gene differentiation (G_{ST})	0.1290	0.2124
Genetic variability among populations	16%	84%
Genetic variability within individuals of populations	19%	81%
Average pairwise genetic distance of populations	0.0375	0.0711
Average of pairwise genetic similarity coefficient ($AGSC$)	0.880	0.908

Table 6. Effect of used primers and techniques in genetic diversity estimation with studied population

No.	Primer code	Polymorphism Information Content (PIC)	Effective Multiplex Ratio (EMR)	Marker Index (MI)	Resolving Power (Rp)
1	UBC 17899	0.1941	6.8333	0.0049	2.0000
2	HB 8	0.2387	7.2500	0.0042	1.7667
3	UBC 807	0.2598	7.7667	0.0034	0.7333
4	HB12	0.2366	4.8000	0.0043	1.1333
5	UBC 808	0.2454	4.1167	0.0040	0.5667
6	ISSR 814	0.2674	5.3167	0.0030	1.0333
7	UBC 842	0.2773	9.2167	0.0022	1.3000
8	UBC856	0.2861	4.8500	0.0009	0.3000
9	UBC873	0.2083	4.3500	0.0048	1.5000
	Average of ISSR technique	0.2460	6.0556	0.0035	1.1481
10	SCoT 1	0.1091	9.8833	0.0004	0.2333
11	SCoT 9	0.1053	6.6667	0.0014	0.6667
12	SCoT 12	0.0932	6.3000	0.0027	1.4000
13	SCoT 13	0.0963	5.4667	0.0025	1.0667
14	SCoT 18	0.1089	6.8833	0.0005	0.2333
15	SCoT 19	0.1039	5.6667	0.0017	0.6667
16	SCoT 22	0.1049	6.6500	0.0015	0.7000
17	SCoT 29	0.0952	6.3500	0.0025	1.2333
18	SCoT 30	0.0991	8.3000	0.0022	1.4000
	Average of SCoT technique	0.1018	6.9074	0.0017	0.8444

Table 7. Genetic diversity parameters based on combined data in the three populations

Parameter	Tuyen Lam	Hon Nga	Bidoup	Species-level
H_{eS}	0.1041	0.1434	0.1857	0.1747
IS	0.1552	0.2131	0.2709	0.2756
PPB_S (%)	28.79	37.88	46.21	62.12

Nga population has been surrounded by rapidly extending agricultural regions and the Tuyen Lam habitat has declined in the area, extent, and quality due to the expansion of nearby agriculture and tourism (Figure 1).

There are several reviews of population genetics in a wide range of plants that possess similar life-history traits to *R. moulmainsense*, i.e. dicotyledons, long-lived perennial, regional distribution, a breeding system of outcrossing, and with seed dispersal by gravity. Studies by Hamrick and Godt (1996) using allozyme markers which showed $H_e = 0.128 - 0.190$, while Nybom and Bartish (2000) used RAPD markers which showed $H_e = 0.191 - 0.260$. Comparing to these previous studies, the genetic diversity of the currently studied *R. moulmainsense* population was much lower.

Comparing with other studies within the genus *Rhododendron*, including seven deciduous *Azalea* species (*Rhododendron* spp. section *Pentanthera*) native to the Eastern United States investigated by Chappell *et al.* (2008) using AFLP technique; six

R. trifolium populations sampled from Tibetan assessed by Xu *et al.* (2017a) using ISSR and AFLP techniques; four *R. aureum* Georgian populations in Changbai Mountain, China using ISSR and RAPD techniques by Liu *et al.* (2012); *R. arboreum* in temperate forest and the tropical forests of India populations using RAPD technique by Kuttapetty *et al.* (2014), the genetic diversity parameters values obtained in this study for each investigated population and species level were lower. Genetic diversity was similar when comparing the ISSR technique-induced genetic diversity parameters obtained in this study with the corresponding parameters based on ISSR techniques in studies of Xu *et al.* (2017a) and Liu *et al.* (2012). However, the H_e values obtained for three populations in the current study were medium to high compared to six invasive populations in Ireland and twelve native populations in Georgia and Spain of *R. ponticum* species in a study using AFLP markers conducted by Erfmeier and Bruehlheide (2011).

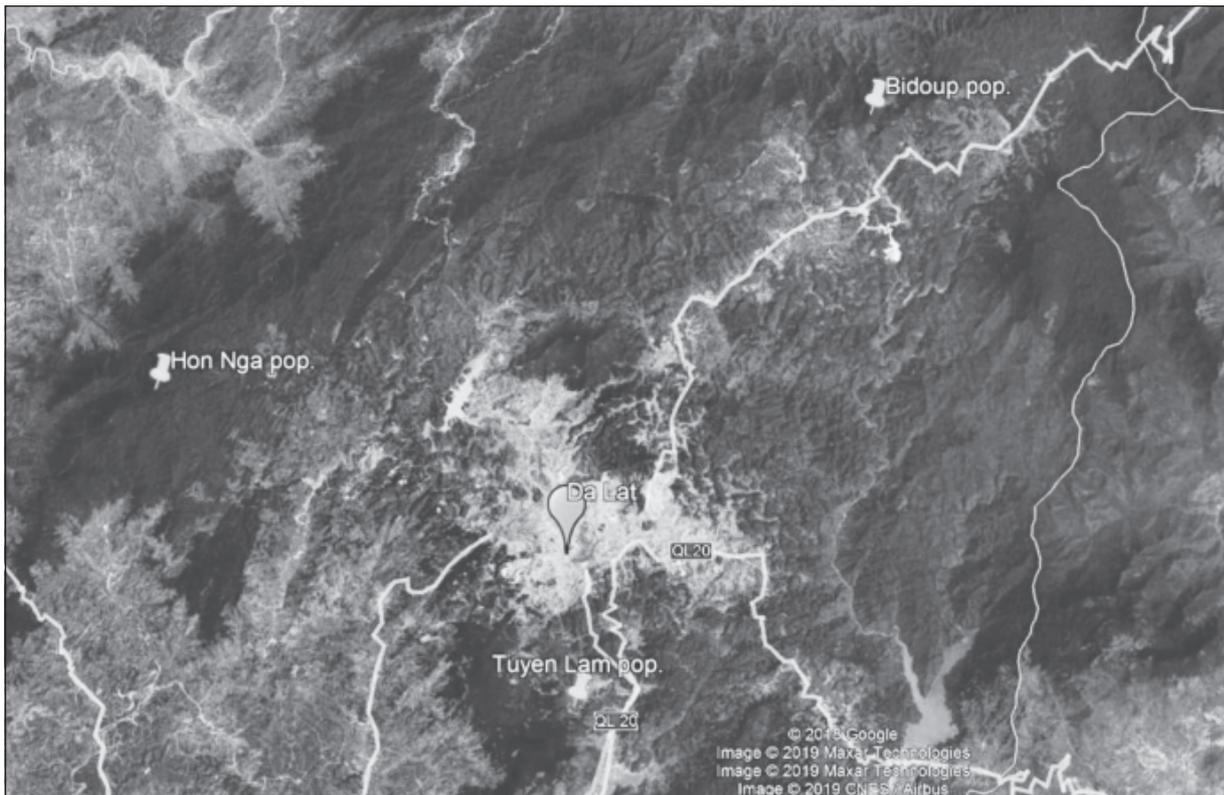


Fig. 3. Distribution of three investigated *R. moulmainsense* populations.

Based on combined data, the pairwise genetic similarity coefficients ($GSCs$) among investigated individuals of the Tuyen Lam population were between 0.841 and 0.985 with an average ($AGSC$) of 0.973. Pairs of RhC6 with RhC8, RhC16, RhC17, RhC18 were the most genetically dissimilar while pairs of RhC14-RhC18 and RhC19-RhC20 were the most genetically similar. In the Hon Nga population, $GSCs$ values were between 0.727 and 1.000 with an $AGSC$ of 0.911. The pair of RhB4-RhB18 was the most genetically different and the pair of RhB12-RhB13 was genetically similar in the Hon Nga population. Genetic similarity coefficients ($GSCs$) among investigated individuals of the Bidoup population were between 0.705 and 0.985 with an average ($AGSC$) of 0.895; pairs of RhA1-RhA2 and RhA4-RhA5 were the highest genetically similar, and pairs of RhA6-RhA4; RhA6-RhA5; RhA6-RhA5 were the lowest genetically similar. At the species level, the pairwise genetic similarity coefficients of 60 *R. moulmainsense* individuals investigated in this study were between 0.644 and 1.000 with an average of $AGSC = 0.894$, and the pair of RhA6-RhC15 was the most genetically different and the pair of RhB12-RhB13 was genetically identical. Thus, when combining data from both used techniques, the only pair of RhB12-RhB13 individuals could not be distinguished.

Based on these obtained genetic similarity coefficients, the UPGMA dendrogram for the genetic relationship among individuals investigated in this study is shown in Figure 4.

Using combined data, the genetic differentiation among *R. moulmainsense* populations was estimated to be $G_{STS} = 0.1735$ and the number of migrants was estimated as $Nm = 2.3824$ individuals per generation among these populations. According to the point of view of the systematics of genetic differentiation, $G_{STS} = 0.1735$ showed the large genetic differentiation of the investigated populations (De Vicente, 2003), and this differentiation was significantly low in comparison to the same genus natural populations including *R. trifolium* and *R. aureum* in the previous studies of Xu *et al.* (2017a) and Liu *et al.* (2012), but at a moderate level compared to seven deciduous *Azalea* species (*Rhododendron* spp. section *Pentanthera*) in the research of Chappell *et al.* (2008), and higher than two *R. protistum* var. *giganteum* populations in Gaoligong Mountain National Nature Reserve, China calculated based on AFLP markers (Wu *et al.*, 2015) and six *R. brachycarpum* populations in Korea that were examined using allozyme markers (Lee *et al.*, 2002).

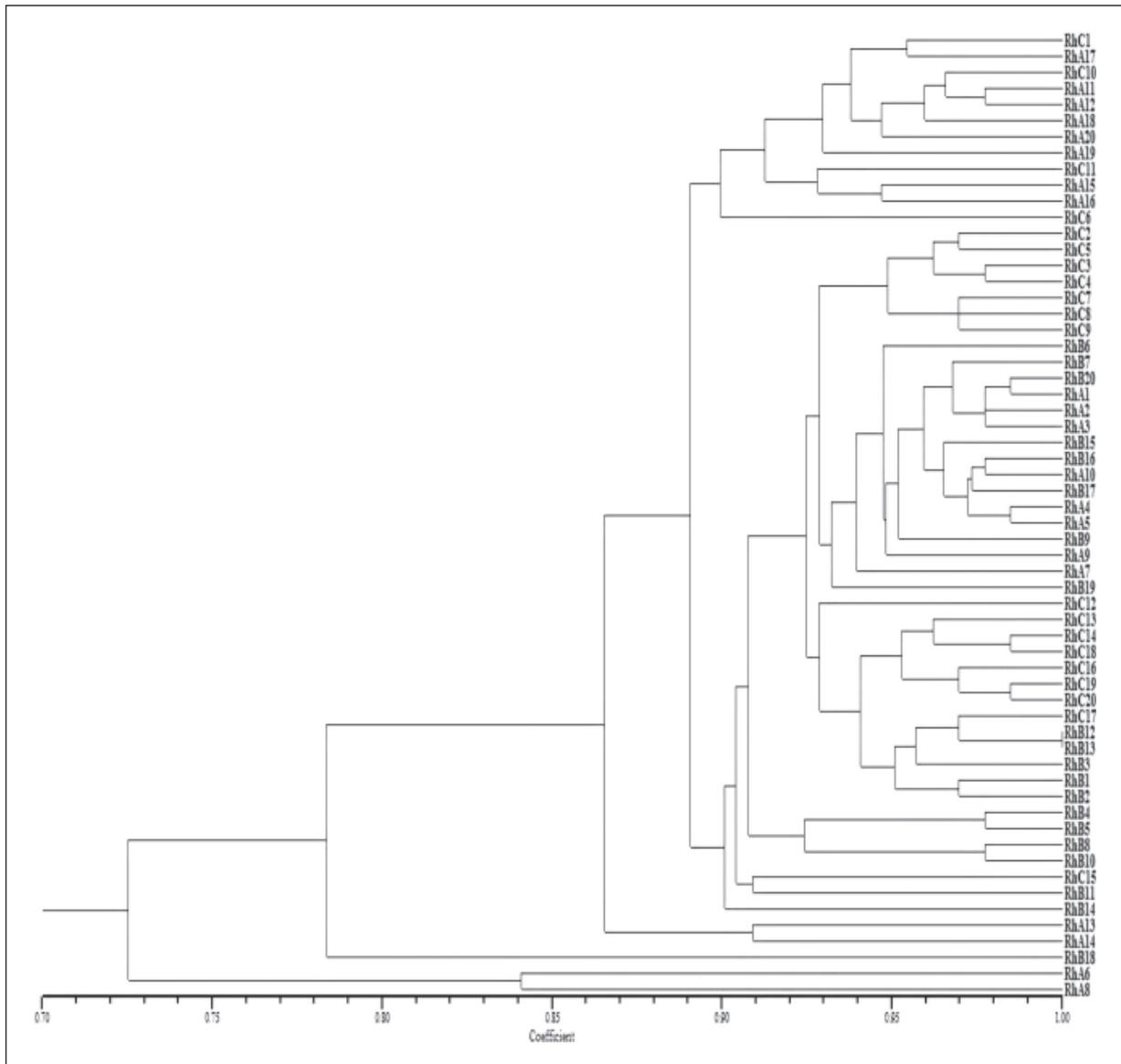


Fig. 4. Dendrogram for the genetic relationship among *R. moulmainsense* individuals investigated in this study at species level based on combined data.

The large genetic differentiation of *R. moulmainsense* populations in Lam Vien plateau lead to a genetic distance (D) between Tuyen Lam and Hon Nga populations of 0.0399, between Tuyen Lam and Bidoup of 0.0621, and between Hon Nga Bidoup of 0.0607. The geographic distance between Tuyen Lam population and Hon Nga population was about 27 km; between Tuyen Lam population and Bidoup population was about 35 km and between Bidoup population and Hon Nga population was about 40 km. The distance in a straight line from Bidoup population to Hon Nga population is further than to Tuyen Lam population, but the genetic distance between Bidoup and Hon Nga population was less than that of between Bidoup population and Tuyen Lam population (Figure 1). This can be explained

by the existence of a densely settled urban area, DaLat City, between Bidoup and Tuyen Lam. The genetic relationship among the three investigated populations based on combined data is presented in Figure 5.

The genetic relationship among the three populations based on combined data was more similar to that based on ISSR than to the relationship based on SCoT. Based on combined data, AMOVA analysis revealed genetic differences among all three *R. moulmainsense* populations, with 18% of total genetic variability portioned among populations and the remaining 82% portioned within populations. The proportion of genetic variance occurring within populations based on combined data was slightly higher than based on ISSR and substantially higher

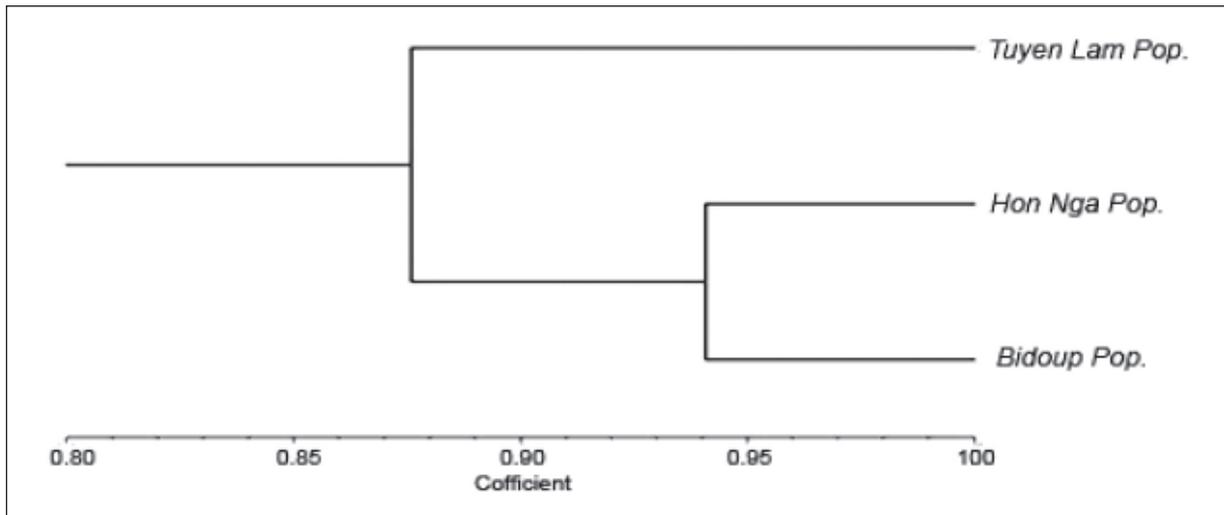


Fig. 5. Dendrogram representing the genetic relationship among three investigated populations based on combined data.

than that based on SCoT. But for all three types of data, the proportions of genetic variance among populations obtained in this study were higher than most previous studies on *Rhododendron* populations in the world including *R. trifolium* populations in Tibet (Xu *et al.*, 2017a), *R. aureum* populations in China (Liu *et al.*, 2012), *R. protistum* var. *giganteum* populations in China (Wu *et al.*, 2015), *R. nivale* populations in Tibetan (Xu *et al.*, 2017b), *R. ferrugineum* populations in Northern Apennines (Bruni *et al.*, 2012).

CONCLUSIONS

The combination of data obtained from the two techniques helped to distinguish the individuals more clearly. The genetic diversity of the investigated *R. moulmainsense* populations was lower in comparison to plant populations with similar life-history traits and many phylogenetically related populations, however, it was similar to or slightly higher compared to several other studies on other *Rhododendron* species. Genetic differentiation of the investigated populations was large according to the point of view of the systematics of genetic differentiation but may be larger, similar, or smaller comparing to other *Rhododendron* species. The proportion of genetic variance occurring within *R. moulmainsense* populations was high compared with other *Rhododendron* populations worldwide. Genetic diversity, genetic differentiation, and genetic distance of investigated *R. moulmainsense* populations may depend on human impacts on the habitats of this species.

The genetic diversity parameters based on combined data in this study can be applied for *in-situ* conservation of the *R. moulmainsense*

species in the Lam Vien plateau of Vietnam. For investigated populations, the ISSR technique was better than the SCoT technique in individual discriminatory ability, efficiency, and ability of the primer combination, but the SCoT primers were more efficient in reflecting genetic diversity of populations and differentiation among them.

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