DALAT UNIVERSITY JOURNAL OF SCIENCE Volume 12, Issue 3, 2022 43-55

POPULATION GENETIC DIVERSITY OF *CAMELLIA DILINHENSIS* ON THE DI LINH PLATEAU OF VIETNAM REVEALED BY ISSR AND SCOT MARKERS

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Article history
Received: July 22nd, 2021
Received in revised form: February 10th, 2022 | Accepted: February 28th, 2022
Available online: March 31st, 2022

Abstract

Genetic diversity of naturally distributed Camellia dilinhensis populations on the Di Linh plateau of Vietnam was assessed by the inter-simple sequence repeat (ISSR) and start codon targeted (SCoT) techniques separately and then by combining data from both techniques for satisfactory results. The genetic diversity parameters and genetic distances among individuals found with the ISSR technique (H_{eI} = 0.1420, I_I = 0.2092, PPB_I = 35.22%, $GSCs_I$ = 0.765–0.988, and $AGSC_I$ = 0.915) are lower than those found with the SCoT technique (H_{eS} = 0.2100, I_S = 0.381, PPB_S = 52.27%, $GSCs_S$ = 0.644–0.985, and $AGSC_S$ = 0.866). Based on the combined data from both techniques, the level of genetic diversity of the investigated population is PPB = 43.77%, H_e = 0.1720, I = 0.2582, and the genetic similarities among individuals are GSCs = 0.764–0.973 with an average of AGSC = 0.894. The SCoT technique differentiated between individuals better and reflected a higher level of genetic diversity in the population than the ISSR technique, but the ISSR technique revealed more loci in Camellia dilinhensis plants than did the SCoT technique.

Keywords: *Camellia dilinhensis*; Genetic diversity; ISSR; Population; ScoT; Vietnam.

DOI: http://dx.doi.org/10.37569/DalatUniversity.12.3.954(2022)

Article type: (peer-reviewed) Full-length research article

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1. INTRODUCTION

Camellia is the largest genus of the Theaceae family, with more than 200 species (Vijayan et al., 2009). The distribution of *Camellia* species is concentrated in Southeast Asia and China, where 80% of the *Camellia* genus species in the world occur (Gao et al., 2005). *Camellia* species have been used as tea, in fatty/seed oil technology, in medicine, and as bonsai/ornamental plants.

To date, a group of golden camellias has been recorded in China and Vietnam consisting of 52 yellow flower *Camellia* species. These species contain many active ingredients in their leaves and flowers that make them valuable plants for medicine (Tran et al., 2019). *Camellia dilinhensis*, one member of the group's golden camellias, was recorded on the Di Linh plateau, Lam Dong Province of Vietnam as an endemic species by Tran Ninh and Luong Van Dung (Tran & Luong, 2013). *Camellia dilinhensis* is known from a single small location (estimated area of occupancy less than 500 km^2) with a small population and low natural regeneration. Agricultural expansion and illegal logging are causing a continuing decline in the extent and habitat quality of this species. Thus, *C. dilinhensis* has been listed as Endangered, i.e., En B2ab (iii, v) in accordance with IUCN standards (Beech et al., 2017). Activities for sustainable conservation and development of this species are necessary and urgent.

High genetic diversity helps plant populations adapt to climate change and maintain their existence through generations. Understanding population genetic diversity is required for plant resources conservation, development, and sustainable exploitation. Plant population genetic diversity parameters are used to establish and apply suitable strategies for *in situ* and *ex situ* conservation and development of plant populations (Hogbin & Peakall, 1999). This method has been applied in a large range of plant species, including several *Camellia* species such as *C. nitidissima* (Tang et al., 2006; Wei et al., 2008), *C. euphlebia* (Wei et al., 2005), *C. reticulate* (Wang & Ruan, 2012), and *C. spp.* (Dang et al., 2017). However, the population genetic diversity of *C. dilinhensis* in Vietnam has not been assessed. Therefore, a thorough understanding of the population genetic diversity of *C. dilinhensis* in Vietnam is desirable. According to Luu et al. (2020):

> With the rapid development of biotechnology, molecular markers have become powerful tools for analyzing genetic diversity (Ouborg et al., 1999). Various approaches are available for DNA fingerprinting, such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), inter-simple sequence repeat (ISSR) (Kumar et al., 2009), and start codon targeted (SCoT) (Collard & Mackill, 2009), etc. Each of these possesses its own advantages and disadvantages, but ISSR and SCoT have proved to be simple, low-cost, easy-to-implement, and worthy markers for assessing population genetic diversity. Both are even considered dominant techniques. While the ISSR technique targets DNA regions between the simple sequence repeats (microsatellites) that can belong to either the transcribed regions or the non-transcribed regions of the genome, the SCoT technique targets the start codon of functional genes. However, in principle, these techniques are similar

when using a single oligonucleotide as a forward and reverse primer in PCR (Collard & Mackill, 2009). The ISSR and SCoT techniques were proposed for use in conjunction with other DNA fingerprinting techniques for applications such as genetic analysis and quantitative trait loci mapping, especially by laboratories with a preference for agarose gel electrophoresis (Collard & Mackill 2009; Nagaoka & Ogihara, 1997).

The main purpose of this study is to assess the genetic diversity and similarity among individuals of the *C. dilinhensis* population on the Di Linh plateau of Vietnam, thereby acquiring the basic data needed for conservation and sustainable utilization of this medicinal/ornamental plant. In addition, comparisons between the ISSR and SCoT techniques of assessing genetic diversity of a population are also presented to technically contribute to the general use of DNA fingerprinting techniques.

2. MATERIALS AND METHODS

2.1. Materials

Twenty-five individuals were randomly chosen from their natural habitat on the Di Linh plateau (Lam Dong Province) as a representative sample of the endemic *C. dilinhensis* population to assess its genetic diversity. Samples from the chosen individuals were labeled CaD1 to CaD25 and brought to the Plant Breeding Technology Laboratory of Dalat University for study. Fresh leaves from each of the sampled individuals were used separately as material for DNA extraction.

2.2. DNA extraction

CTAB protocol I (Weising et al., 2005) with a modification of adding 10% SDS to the extraction buffer was used to extract total genomic DNA from fresh leaves. Three DNA samples were extracted from each field trip sample. Extracted DNA was dissolved in TE buffer. The DNA concentration and purity were identified by the spectrophotometry method (Weising et al., 2005) using a NanoScan2 system (Analytik Jena). The DNA samples with OD260/OD280 values between 1.7 and 2.0 (preferably closer to 1.8) were chosen to represent sampled individuals in the study. DNA samples were kept at -20 ºC for the subsequent PCRs.

2.3. DNA fingerprinting

DNA fingerprints of samples were generated by the ISSR and SCoT techniques. The primers used in this study were synthesized by PhuSa Biochem Ltd. (Vietnam). The ISSR primers were synthesized according to the primer set suggested by the University of British Columbia (Canada) and Zagazig University (Egypt) (Ahmed, 2005), and the SCoT primers were synthesized according to the primer set published by Collard and Mackill (2009). In both techniques, the screened primers that yielded bright clear bands and possessed at least one polymorphic band in five randomly tested *C. dilinhensis* samples were chosen for DNA fingerprinting. In all, 9/20 ISSR primers and 9/20 SCoT

primers satisfied these criteria and were chosen for DNA fingerprinting. Characteristics of the primers used in the study are shown in Table 1.

Table 1. Characteristics of the primers used in the study

No.	Primer code	Sequence 3' to 5'	T_a (ºC)	Chosen after screening	Number of induced bands	*PPB* (%)
1	UBC 17899	5'-$(CA)_6$ A/G G-3'	54	×	17	23.52
2	HB 12	5'-$(CAC)_3$ GC-3'	52	×	17	70.59
3	HB 9	5'-$(GT)_6$ GG-3'	52	×	16	31.25
4	HB 8	5'-$(GA)_6$ GG-3'	52	×	20	30.00
5	UBC 808	5' -$(AG)_8$ C-3'	52	×	19	36.84
6	ISSR 814	5' -$(CT)_8$ TG-3'	51.5	×	22	31.82
7	UBC 856	5'-$(AC)_8$ T/C A-3'	52	×	19	47.37
8	UBC 842	5'-$(GA)_8$ T/C G-3'	51.5	×	18	33.33
9	UBC 873	5'-$(GACA)_4$ -3'	52	×	18	12.22
10	HB 15	5'-$(GTG)_3$GC-3'	52			
11	UBC 807	5'-$(AG)_8$ T-3'	54			
12	UBC 844	5' -$(CT)_8$ AC-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				166	
	Average of ISSR technique based on selected primers				18.44	35.22
21	SCoT 1	CAACAATGGCTACCACCA	50	×	15	53.33
22	SCoT 12	ACGACATGGCGACCAACG	50	×	14	50.00
23	SCoT 18	ACCATGGCTACCACCGCC	50	×	17	41.17
24	SCoT 3	CAACAATGGCTACCACCG	50	×	14	35.71
25	SCoT 13	ACGACATGGCGACCATCG	50	×	17	52.94
26	SCoT 19	ACCATGGCTACCACCGGC	50	×	14	64.28
27	SCoT 29	CCATGGCTACCACCGGCC	50	×	15	60.00
28	SCoT 30	CCATGGCTACCACCGGCG	50	×	14	85.71

Table 1. Characteristics of the used primers in the study (cont.)

No.	Primer code	Sequence 3' to 5'	T_a (ºC)	Chosen after screening	Number of induced bands	*PPB* (%)
29	SCoT 22	AACCATGGCTACCACCAC	50	×	11	27.27
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				131	
	Average of SCoT technique based on selected primers				14.55	52.27

PCRs were performed in 50 µl reactions containing 25 µl MyTaq HS Red Mix (Bioline), 0.2 µM primer, and approximately 30 ng of DNA templates. The amplifications followed the following programs:

In the ISSR technique: initial denaturation at 94 ºC for 5 min, 10 cycles of 94 ºC for 15 s, annealing temperature +5 (Ta + 5) ºC (Table 3) for 15 s, decrements of 0.5 ºC /cycle, 72 ºC for 45 s, 30 cycles of 94 ºC for 15 s, annealing temperature for 15 s, 72 ºC for 45 s, and final extension at 72 ºC for 10 min.

In the SCoT technique: initial denaturation at 94 ºC for 5 min, 36 cycles of 94 ºC for 15 s, 50 ºC for 15 s, 72 ºC for 45 s, and final extension at 72 ºC for 10 min.

The PCR products were separated in 2.5% agarose gel using TBE buffer at 80 V for 2.5 hours, stained with ethidium bromide (0.5 µg/ml), and photographed under 254/312 nm wavelength light using a UVP GelStudio Plus System (Analytik Jena, Germany).

2.4. Data analysis

Since ISSR and SCoT markers are dominant, 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*), the heterozygosity/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 the UPGMA dendrogram for the genetic relationship among studied samples were calculated using NTSYSpc 2.1 (Numerical Taxonomy and Multivariate Analysis System) software (Rohlf, 2004). The average of these genetic similarity coefficients (*AGSC*) was also calculated.

Genetic diversity and similarity among individuals in the investigated population were evaluated based on data obtained separately with the ISSR technique (parameters denoted H_{eI}, I_I, PPB_I, $GSCs_I$, and $AGSC_I$) and the SCoT technique (parameters denoted H_{eS}, I_S, PPB_S, $GSCs_S$, and $AGSC_S$) for technical comparison. Based on the combined data from both techniques, the adequate level of population genetic diversity and genetic similarity among individuals was evaluated and denoted H_e, *I*, *PPB*, *GSC*s, and *AGSC*.

The criteria used to compare the usability of the techniques for the studied population are the ratio of the chosen primers (ones that responded to the criteria) to the initially tested primers, the number of revealed loci per primer, the level of population genetic diversity indicated by the *PPB*, H_e, and *I* parameters, and the level of genetic difference among samples, as shown by their pairwise genetic similarity coefficients. In addition, the discriminatory ability of each DNA fingerprinting technique was calculated as its polymorphism information content (PIC). The number of revealed loci was found from the efficiency of the primer-marker system, expressed as effective multiplex ratio (EMR). The efficiency of the method was measured by calculating the marker index (MI), and the ability of the primer combination to detect differences between investigated samples was expressed by resolving power (Rp). The PIC, EMR, MI, and Rp values were calculated from the ISSR and SCoT data separately using iMEC, an online marker efficiency calculator that is available at https://irscope.shinyapps.io/iMEC/ (Amiryousefi et al., 2018). The calculated values were then compared between the two techniques.

3. RESULTS AND DISCUSSION

3.1. Data generated by ISSR technique for genetic diversity and genetic relationship among individuals of the investigated population

Based on the ISSR technique, the percentage of polymorphic bands of the *C. dilinhensis* population in Vietnam was PPB_I = 35.22%. The expected heterozygosity of this population was H_{eI} = 0.1420, and its Shannon index was I_I = 0.2092.

Genetic similarity coefficients between pairs of individuals in the investigated population were in the range of $GSCs_I$ = 0.765–0.988 with an average value of $AGSC_I$ = 0.915.

3.2. Data generated by SCoT technique for genetic diversity and genetic relationship among individuals of the investigated population

Results shown in Table 1 indicate that, based on the SCoT technique, the percentage of polymorphic bands of the *C. dilinhensis* population was $PPB_S = 52.27\%$. The expected heterozygosity of this population was $H_{eS} = 0.2100$, and its Shannon index was $I_S = 0.3081$.

Genetic similarity coefficients between pairs of individuals in the investigated population were in the range of $GSCs_S = 0.644–0.985$ with an average value of $AGSC_S = 0.866$.

3.3. Genetic diversity of the investigated population based on pooled data

Genetic diversity assessments are better and more exact when using more data and investigating more loci in the genome (Carling & Brumfield, 2007). Thus, to exploit all data acquired in this study, the ISSR and SCoT data were combined to assess the genetic diversity of *C. dilinhensis* population. The combination of these data for population genetic diversity assessment is possible because both are dominant data.

Based on the pooled data, the percentage of polymorphic bands of the studied population was $PPB = 43.77\%$. The expected heterozygosity of this population was $H_e = 0.1720$, and the Shannon index was $I = 0.2528$.

There are several studies on a wide range of plants that possess the same life history traits as *C. dilinhensis*, i.e., dicotyledons, long-lived perennial, endemic distribution, outcrossing breeding system, and seed dispersal by gravity. These studies include Hamrick and Godt (1996), which found $H_e = 0.105–0.180$, and Nybom and Bartish (2000), which found $H_e = 0.191–0.260$. The more satisfactory comparison in this case is between our results and those of Nybom and Bartish (2000) because they assessed genetic diversity using RAPD markers – the dominant markers. Compared to the study of Nybom and Bartish (2000), the genetic diversity of the *C. dilinhensis* population was lower.

Compared to the golden flower camellia (*Camellia spp*.) accessions in Quang Ninh Province of Vietnam that were investigated using RAPD and ISSR markers by Dang et al. (2017), the genetic diversity based on the percentage of polymorphic bands of the *C. dilinhensis* population was also lower.

According to Tang et al. (2006), there are six populations of yellow camellias (*Camellia nitidissima*) from Guangxi in southern China. Genetic diversity assessment of these populations by RAPD and AFLP markers separately showed lower *PPB* and H_e values than did the *C. dilinhensis* population.

Using ISSR markers, Wei et al. (2005), Wei et al. (2008), and Wang and Ruan (2012) estimated the genetic diversity of four *C. euphlebia* populations in Guangxi, 13 *C. nitidissima* populations in Guangxi, and six C. *reticulata* populations in southwest China. Their values for the percentage of polymorphic bands (*PPB*) and the expected

heterozygosity (H_e) of the studied populations were lower than those of the *C. dilinhensis* population in the current study, as found from the pooled data. The parameters for *C. dilinhensis* calculated from the ISSR data were higher than those of the *C. nitidissima* populations in Guangxi (Wei et al., 2008), similar to the four *C. euphlebia* populations in Guangxi (Wei et al., 2005) and lower than the six *C. reticulata* populations in southwest China (Wang & Ruan, 2012).

3.4. Genetic relationship among individuals of the investigated population based on combined data

Genetic similarity coefficients between pairs of individuals in the investigated population were in the range of *GSC*s = 0.764–0.973, with an average value of *AGSC* = 0.894. The CaD6-CaD10 pair was the most genetically similar, while the greatest genetic distance was observed for the CaD5-CaD22 pair.

A UPGMA dendrogram for the genetic relationships among the studied individuals was constructed based on the genetic similarity coefficients and is shown in Figure 1.

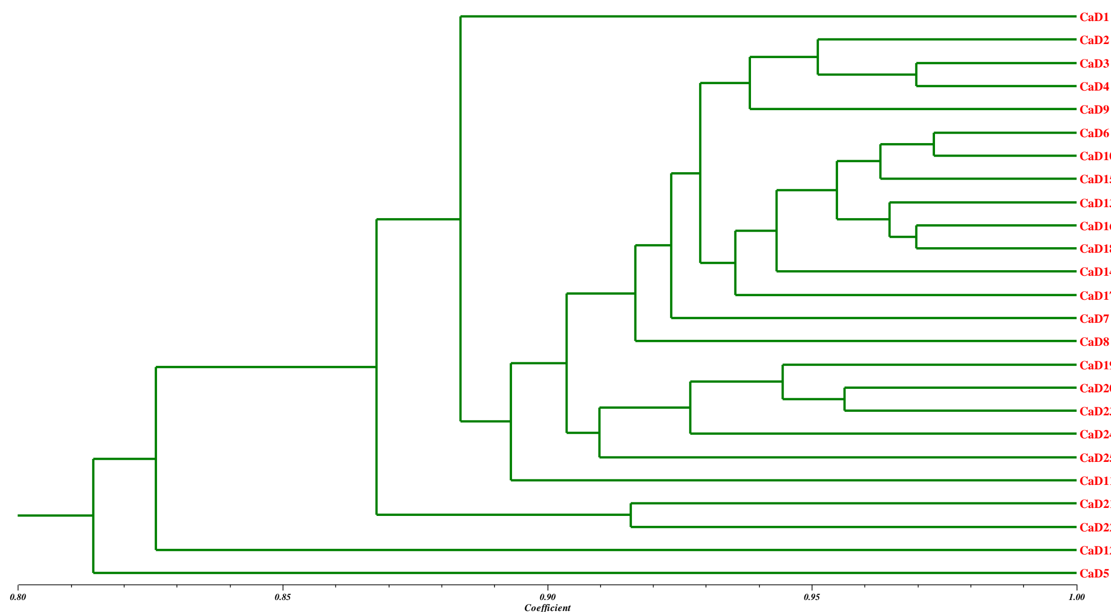

Figure 1. Combined data-based UPGMA dendrogram for the genetic relationships among individuals of the studied *C. dilinensis* population

Genetic similarity coefficients (based on pooled data) between pairs of individuals in the investigated *C. dilinhensis* population were higher, but in a narrower range, than in golden flower camellia (*Camellia spp.*) accessions from Quang Ninh Province, Vietnam, which were investigated using combined data from the RAPD and ISSR techniques by Dang et al. (2017).

The highest genetic similarity coefficient among individuals of the investigated *C. dilinhensis* population was 0.973, showing that all of the investigated individuals grew from seeds. In other words, the population has an outcrossing breeding system.

The individuals in the UPGMA dendrogram for the genetic relationships among the *C. dilinhensis* population (Figure 1) can be divided into six groups based on the combined data from both techniques. Group 1 includes only CaD1. Group 2 includes CaD2, CaD3, CaD4, CaD9, CaD6, CaD10, CaD15, CaD13, CaD16, CaD18, CaD14, CaD17, CaD7, CaD8, CaD19, CaD20, CaD23, CaD24, and CaD25. Group 3 includes only CaD11. Group 4 includes CaD21 and CaD22. Group 5 includes only CaD12, and Group 6 includes only CaD5. Most individuals belong to Group 2; the other groups included only one or two individuals. Because the investigated population has been found to be the only endemic population of *C. dilinhensis*, the individuals that do not belong to the main group cannot be the result of migration from other places. They may be the result of the reproduction of the harvested parents, and their contribution to the next generations were trivial.

3.5. Comparison of usability between the techniques used in the studied population

DNA fingerprinting ability and data characteristics of the ISSR and SCoT techniques are given in Table 2 for the studied population.

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

Criterion	ISSR technique	SCoT technique
Ratio of chosen primers/initially tested primers	9/20	9/20
Average number of revealed loci per chosen primer	16.60	14.65
Percentage of polymorphic bands (*PPB*)	35.22%	52.27%
Expected heterozygosity (H_e)	0.1420	0.2100
Shannon index (*I*)	0.2092	0.3081
Average of pairwise genetic similarity coefficient (*AGSC*)	0.915	0.866

Table 2 shows an equal ratio of chosen primers to initially tested primers for the ISSR and SCoT techniques. For the amount of acquired information, the average number of revealed loci per chosen primer was higher (1.14-fold) with the ISSR technique than the SCoT technique. This indicates that the ISSR technique reveals more loci in the plant genomes than the SCoT technique.

However, the ability of the SCoT technique to reflect the genetic diversity of the population was significantly higher than that of the ISSR technique. This can be recognized more clearly when observing the higher percentages of polymorphic bands (1.48-fold), expected heterozygosity (1.48-fold), and Shannon index (1.47-fold).

The average pairwise genetic similarity coefficient among the investigated individuals, as determined by the SCoT technique, was lower (0.95-fold) than by the ISSR

technique. This shows that the SCoT technique reflected the genetic difference among investigated samples better than the ISSR technique.

The results can be explained by the nature of the two techniques. The ISSR technique reveals the DNA regions between simple sequence repeats (DNA microsatellites) that can belong to either the transcribed or the non-transcribed regions of the genome, while the SCoT technique targets the start codon of functional genes, which exit in transcribed regions only. Another explanation may be that the genetic diversity of the DNA microsatellites in the investigated population was not high, but it was higher than the functional genes.

The above comparative results can be seen again in the average values of polymorphism information content (PIC), effective multiplex ratio (EMR), marker index (MI), and resolving power (Rp) that were achieved with the ISSR and SCoT techniques (Table 3).

Table 3. Effect of primers and techniques used to estimate genetic diversity in the studied population

No.	Primer code	Polymorphism information content (PIC)	Effective multiplex ratio (EMR)	Marker index (MI)	Resolving power (Rp)
1	UBC 17899	0.1263	16.7600	0.0011	0.4800
2	HB 12	0.0764	13.6400	0.0102	4.3200
3	HB 9	0.1259	15.6800	0.0015	0.6400
4	HB 8	0.1256	19.5200	0.0018	0.9600
5	UBC 808	0.0966	16.2800	0.0084	4.3200
6	ISSR 814	0.1220	20.8800	0.0037	2.2400
7	UBC 856	0.1209	17.9200	0.0040	1.2800
8	UBC 842	0.1240	17.3200	0.0028	1.3600
9	UBC 873	0.1179	16.7200	0.0049	2.5600
	Average of ISSR technique	0.1151	17.1911	0.0043	2.0178
10	SCoT 1	0.1868	12.8000	0.0085	2.6400
11	SCoT 12	0.1901	12.0800	0.0082	2.8000
12	SCoT 18	0.2036	15.4000	0.0062	2.0800
13	SCoT 3	0.2106	13.0800	0.0046	1.8400
14	SCoT 13	0.2148	16.2800	0.0031	1.4400
15	SCoT 19	0.2019	12.6000	0.0065	2.8000
16	SCoT 29	0.1923	13.0400	0.0079	2.4000
17	SCoT 30	0.1271	9.6800	0.0118	5.1200
18	SCoT 22	0.1970	9.7200	0.0073	0.7200
	Average of SCoT technique	0.1916	12.7422	0.0071	2.4267

The average values of PIC, MI, and Rp were significantly higher with the SCoT technique primers than with the ISSR technique primers. This indicates that the discriminatory ability and efficiency of the primer combination are better with the SCoT technique than with the ISSR technique. That the average effective multiplex ratio (EMR) achieved with the ISSR technique is higher than with the SCoT technique is supported by the above result that more loci are revealed with the ISSR technique than the SCoT technique. According to Chesnokov and Artemyeva (2015), PIC reflects the discriminatory ability of the marker, and the higher the value of EMR, the more efficient the “primer-marker system.” A higher MI means the method is better, and Rp is a parameter used to characterize the ability of the primer/marker combination to detect the differences between investigated genotypes.

4. CONCLUSION

The genetic diversity of the investigated *C. dilinhensis* population was slightly lower than for plant populations with the same life history traits, but in comparison with phylogenetically close populations, genetic diversity may be higher, similar, or lower depending on the investigated taxa. The genetic diversity parameters found in this study from the combined data of the ISSR and SCoT techniques should be considered for *in situ* conservation of the *C. dilinhensis* species on the Di Linh plateau of Vietnam. As for the technical aspect of the investigation, the ISSR technique revealed more loci in the plant genomes than the SCoT technique, but the SCoT technique differentiated the individuals better and revealed genetic diversity more efficiently than the ISSR technique.

ACKNOWLEDGMENTS

The authors would like to express their sincere thanks to Dr. Luong Van Dung (Dalat University) and Ms. Nguyen Thi Hanh (TTC School Dalat) for their help in investigating the natural distribution of the studied population and in collecting the samples.

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