ISSN 1817-3721, E-ISSN 1818-8745

Plant Tissue Cult. & Biotech. **28**(2): 171-182, 2018 (December)

PTC&B

Comparison of Genetic Variation of Anthurium (*Anthurium andraeanum*) Cultivars Using SCoT, CDDP and RAPD Markers

Abbas Saidi*, Zahra Daneshvar and Zohreh Hajibarat

Department of Plant Sciences and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, G.C., Tehran, Iran

Key words: Anthurium, Genetic diversity, CDDP, SCoT, RAPD

Abstract

To evaluate the genetic diversity among 10 cultivars of anthurium were performed using three molecular markers such as Start Codon Targeted (SCoT) and Conserved DNA-derived Polymorphism (CDDP), and Random Amplification of Polymorphic DNA (RAPD). Polymorphism index content (PIC) was calculated 0.39, 0.42 and 0.37 for RAPD, SCoT and CDDP, respectively. This result showed all the three molecular markers had almost an identical potential in estimating genetic diversity. Cluster analysis using SCoT, CDDP and RAPD divided the cultivars to three distinct clusters. The similarity matrix obtained through SCoT and CDDP was positively significantly correlated ($r = 0.76$, $p < 0.01$). This is the first report in which the efficiency of two targeted DNA region molecular markers (SCoT and CDDP) together with RAPD technique have been compared with each other in a set of anthurium cultivras. Results suggested that SCOT, CDDP and RAPD fingerprinting techniques are of sufficient ability to detect polymorphism in anthurium cultivars.

Introduction

Anthurium is the most diverse genus in Araceae. As the second most widely traded flower after orchids, this plant is of high value in trade and economy in tropical regions (Castro et al. 2004). Genetic diversity assistant in the efficient management of anthurium cultivras by breeders. Consequently, evaluation of the variation is a prerequisite tool to breed a given population. It also allows for exploitation of genetic variability in the production of new hybrids (Cabral et al.

*Author for correspondence: <abbas.saidi@gmail.com>

DOI: https://doi.org/10.3329/ptcb.v28i2.39676

2010). Anthurium (*Anthurium andraeanum* Lind.) is a slow-growing perennial plant that prefers shady and humid environment. Uniformity of flowers along with proper performance and high quality are of the most important traits that ensure the success of this plant in the market (Stancato and Tucci 2010). Therefore, in response to market demand (uniformity of flowers along with impressive colors), breeding program and genetic manipulation is needed to produce new hybrids. Traditionally, *Anthurium* species are often crossed with *A. andraeanum* in order to obtain hybrids with a wide color range. However, such crossing scheme has resulted in new cultivars with similar morphologies leading to difficulties in identifying their correct pedigree. Therefore, such conventional breeding methods are time-consuming and does not meet the stringent demands of production. The advent of PCR technology made a new set of markers available to scientists interested in comparing organisms at the molecular level. Today, the use of molecular markers at the DNA level has become popular because, such markers can be used to directly measure genetic diversity without the influence of environmental effects (Ranade et al. 2001). Some of different molecular marker systems used to evaluate genetic relationships among anthurium cultivars are including: RAPD (Collin et al. 2001, Debener et al. 2001), SPAR (Neto et al. 2104) and SSR (Wang and Chuang 2013). In recent years, more studies were attracted to gene-targeted markers (GTMs) such as start codon targeted polymorphism (SCoT (Collard and Mackill 2009a), conserved DNA-derived polymorphism (CDDP) (Collard and Mackill 2009a) in germplasm diversity assessment, targeted QTL mapping and marker-assisted breeding (Andersen and Lübberstedt 2003, Saidi et al.,2017). Due to longer primer chain and higher annealing temperature which are thought to improve reproducibility, SCoT and CDDP markers are more reliable and reproducible than arbitrary markers such as RAPDs or ISSR (Collard and Mackill 2009b). Like RAPD, SCoT and CDDP markers are used as single primer in a PCR amplification reaction. The SCoT and CDDP markers have been used previously in many crop plant species such as rice (Collard and Mackill 2009b), chickpea (Hajibarat et al. 2015), and wheat (Hamidi et al. 2014). The use of CDDP and SCoT markers for studying genetic diversity is reported here for the first time in anthurium cultivars. The objective of the present study was to investigate capabilities and potential of SCoT, CDDP and RAPD markers in determining genetic diversity among anthurium cultivars.

Materials and Methods

Ten cultivars of anthurium (Table 1) provided by the National Institute of Ornamental Plants (NIOP), Mahallat, Iran were studied. Morphological specifica-

tions of these cultivars are summarized in Table 1. DNA was extracted from young leaves of plants according to the modified CTAB method as described by Lassner et al. (1989). The purified total DNA was quantified by agarose gel electrophoresis using a known concentration of uncut λ DNA as a standard.

Table 1. Details of ten anthurium cultivars tested and their morphologic specifications.

Entry no.	Cultivar name	Flower shape	Main color
1	Fire	Standard	Red
2	Carnaval	Standard	White
3	Tropic Night	Brown	Brown
4	Baron	Obake	Salmon
5	Essencia	Obake	Green
6	Green	Standard	Pistach
7	Purple	Lancet	Previa
8	White	Standard	Presence
9	Orange	Standard	Sunglow
10	Pink	Cupped	Xavia

A set of 40 RAPD primers were procured of which 22 gave clear and polymorphic patterns. The polymorphic primers were then used for further analysis of 10 cultivars (Table 2). PCR amplifications were performed in 25 µl reactions including 30 ng sample DNA, 2.5 µM primer, 200 µM of each dNTP, 1.5 - 2.5 mM $MgCl_2$ and 1.5 unit of Taq DNA polymerase (Cinnagene, Iran). Thermal cycling included 3 min at 94°C followed by 35 cycles of denaturation at 93°C for 45 sec, annealing at optimum temperature for 45 sec, extension at 72°C for 90 sec, and a final extension cycle at 72°C for 10 min. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and scored for the presence or absence of bands.

Seven SCoT and 13 CDDP primers were selected for final amplification (Table 3). The amplification was performed in a thermal cycler (Eppendorf, Germany) with the following conditions: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 48°C (for SCoT analysis) and 50°C (for CDDP analysis) for 1 min each, extension at 72°C for 2 min and final extension at 72°C for 7 min. The PCR products obtained were separated on 2% agarose gel using 1 × TBE buffer at a constant voltage of 100 V for one hour. The gel stained with ethidium bromide and visualized using a gel documentation.

The amplified RAPD, SCoT and CDDP fragments obtained were scored for presence (1) or absence (0) of bands. Tree construction following an NJ tree using

a similarity matrix was performed through Splits Tree. The dissimilarity matrix thus obtained was subjected to cluster analysis using the un-weighted neighbor-joining analyses (UNJ) (Gascuel 1997), followed by bootstrap analysis with 1,000 permutations to obtain a dendrogram for all the 10 cultivars (Perrier et al. 2003). Mantel statistic was used to compare the similarity matrices as well as the dendrograms produced by the SCoT, CDDP and RAPD techniques. All these procedures were performed by appropriate routines in NTSYSpc version 2.0 (Rohlf 1997). Polymorphic information content (PIC) values were calculated for each SCoT, CDDP and RAPD primers according to the formula: PIC = 1 – ∑(Pij)2; where Pij is the frequency of the ith pattern revealed by the jth primer summed across all patterns revealed by the primers (Botstein et al. 1980). The Mantel test of significance (Mantel 1967) was also used to compare each pair of similarity matrices produced.

Table 2. Details about RAPD primers used to assess genetic diversity in anthurium.

No. primer ⟶	´35 ´sequence	Polymorphic band	Total	PIC	MI
OPQ-11	TCTCCGCAAC	7	7	0.39	2.8
OPAC-07	GTGGCCGATG	11	11	0.44	4.9
OPR-10	CCATTCCCCA	9	9	0.42	3.8
OPT -07	GGCAGGCTGT	6	6	0.41	2.5
OPK -07	AGCGAGCAAG	6	6	0.38	2.32
OS -09	TCCTGGTCCC	7	7	0.45	5.37
OPZ-17	CCTTCCCACT	12	12	0.41	2.9
OPAH -16	CAAGGTGGGT	8	8	0.42	3.4
OPAG-13	GGCTTGGCGA	6	6	0.41	2.5
OPC -20	ACTTCGCCAC	6	6	0.41	2.4
OPAF-10	GGTTGGAGAC	5	5	0.37	1.9
OPAD -06	AAGTGCACGG	5	5	0.4	2
OPAA -18	TGGTCCAGCC	8	8	0.41	3.3
OPH-12	ACGCGCATGT	4	4	0.33	1.3
OPA -02	TGCCGAGCTG	4	4	0.33	1.3
OPAAB-12	CCTGTACCGA	5	5	0.37	1.9
OPJ- 14	CACCCGGATG	3	3	0.3	0.9
OPD -06	AAGTGCACGG	5	5	0.39	1.9
OPI-16	TCTCCGCCCT	4	4	0.35	1.4
OPAA-04	AGGACTGCTC	6	6	0.4	2.4
OPP-13	CACCAGGTGA	11	11	0.45	4.9
OPE-10	GGAGTGCCTC	6	6	0.41	2.5

GC**%** = Primer G-C content, PIC = Polymorphic information content, MI = Marker index.

Results and Discussion

A total of 40 RAPD primers were utilized of which 22 revealed reliable banding patterns with high polymorphism and clear bands. The primers produced 144 fragments that all of which were polymorphic. The number of fragments generated by these RAPD primers was found to range from 3 to 12 bands. OPZ-17 primer produced the maximum number of polymorphic bands and OPJ-14 primer generated the minimum number of polymorphic bands. The polymorphism information content ranged from a high of 0.45 (OS -09 and OPP -13) to a low of 0.3 (OPJ-14) with an average of 0.439, indicating hyper-variability among the individuals studied. The marker index (MI) for RAPD was highest for the primer OS -09 (5.37) and lowest for the primer OPJ-14 (0.9) (Table 2). An average MI of 0.39 per primer was observed. Cluster analysis grouped anthurium cultivars into three distinct clusters (Fig. 1). The CDDP pattern obtained with OS-09 primer is shown in Fig. 1.

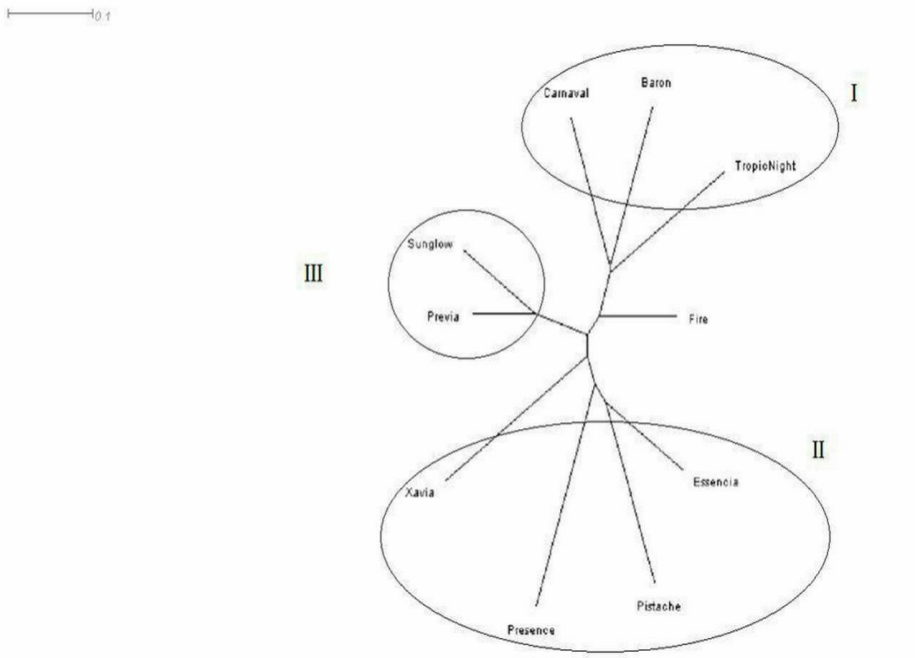

Fig. 1. Dendrogram of the 10 anthurium cultivars based on the genetic distance matrix developed using 22 RAPD markers.

A total of 74 polymorphic bands were detected amongst 10 anthurium cultivars using six CDDP markers. The number of products generated per primer was found to range from 2 (ABP1-3) to 8 (ABP1-1) bands with an average of 5.7 bands per primer.

Polymorphism information content (PIC) values ranged from 0.16 to 0.43, with an average value of 0.37 per primer (Table 3). Cluster analysis grouped anthurium cultivars into three distinct clusters. The first cluster had four cultivars. Clusters II and III contained 3 and 3 cultivars, respectively. The marker index was found to be highest for primer ABP1-3 (0.32) and lowest for the primer ABP1-1 (3.47) with an average MI of 2.26 per primer (Fig. 2).

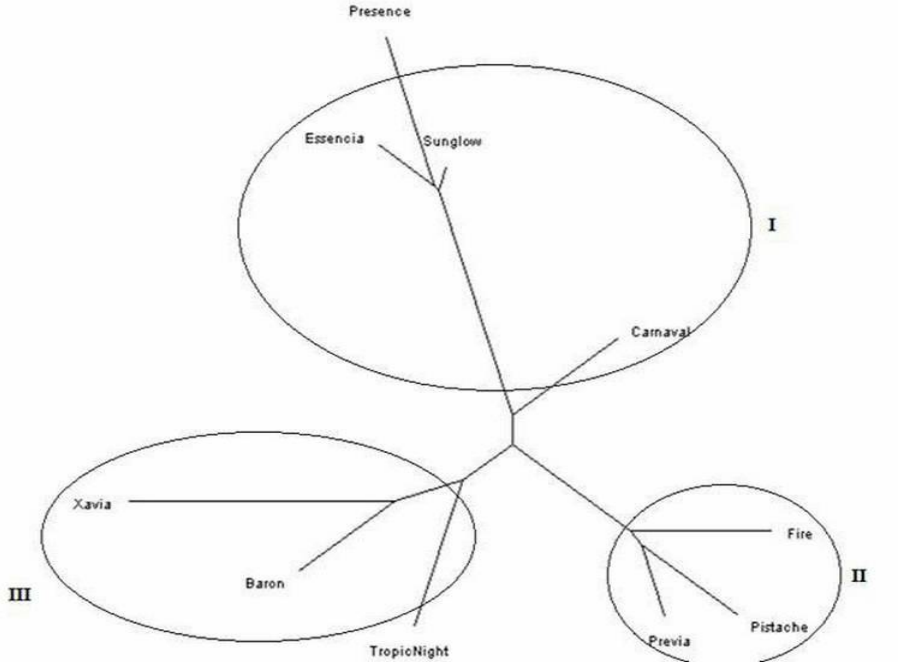

Fig. 2. Dendrogram of the 10 anthurium cultivars based on the genetic distances matrix developed using 13 CDDP markers.

Seven primers generated a total of 61 bands were polymorphic. The number of amplified products generated by SCoT primers were in the range of 7-11 bands. Primers SCoT-1 and SCoT-2 generated the most (7 bands) and primer SCoT-22 generated the least (11 bands) number of amplicons. On average, the number of polymorphic band was 8.7 per primer. PIC values ranged from 0.4 to 0.43, with an average value of 0.42 per primer (Table 4). Based on un-weighted neighbour-joining method, all 10 anthurium varieties were grouped into three distinct clusters. The estimates of MI were found to be highest for primer SCoT-22 (4.9) and the lowest with the primer SCoT-1 (2.81) with an average MI of 3.72 per primer. Clusters I, II and III included five, three and two cultivars, respectively (Fig. 3).

Table 3. Details of CDDP primers used to evaluate genetic diversity in anthurium.

Gene	Gene function	Sequence (5′ to 3′)	Primer name	Polymorphism band	Total band	GC (%)	PIC	MI
ABP1	Auxin-binding protein	Acsccsatcccaccgc	ABP1-1	8	8	73	0.433	3.47
		Cacgaggacctscagg	ABP1-3	2	2	69	0.16	0.32
ERF	Transcription factor involved in plant disease resistance pathway	Cactaccccggsctscg	ERF1	5	7	77	0.4	2
		Gcsgagatccgsgaccc	ERF2	7	7	77	0.42	2.9
Knox	Homeobox genes that function as transcription factors with a unique homeodomain	aagggsaagctsccsaag	Knox1	6	6	61	0.4	2.5
		cactggtgggagctscac	Knox2	5	7	67	0.42	2.1
		Aagcgscactggaagcc	Knox3	7	7	65	0.4	2.9
MYB	Unknown (implicated in secondary metabolism, abiotic and biotic stresses, cellular morphogenesis)	Ggcaaggctgccgg	MYB2	7	7	80	0.4	2.9
WRKY	Transcription factor for developmental and physiological roles	Gtggttgtgcttgcc	WRKY-R1	7	7	60	0.4	2.9
		gcctcgtasgtsgt	WRKY-R2	7	7	67	0.4	2.8
		gcasgtgtgctcgcc	WRKY-R3	6	6	73	0.4	2.3
		tggcgsaagtacggccag	WRKY-F1	4	4	67	0.33	1.33
MADS	Involved in controlling floral organ initiation and development	atgggccgsggcaaggtgc	MADS1	3	3	74	0.3	0.93

GC% = Primer G-C content, PIC = Polymorphic information content, MI = Marker index.

Table 4. Details about SCoT primers used to evaluate genetic diversity in anthurium.

A	Markers	Polymorphic band	Total	sequence ´3´ ⟶ 5	%GC	PIC	MI
1	SCoT1	7	7	CAACAATGGCTACCACCA	50	0.4	2.81
2	SCoT2	7	7	CAACAATGGCTACCACCC	55	0.41	2.88
3	SCoT13	9	9	ACGACATGGCGACCATCG	61	0.43	3.93
4	SCoT22	11	11	AACCATGGCTACCACCAC	55	0.44	4.91
5	SCoT28	9	9	CCATGGCTACCACCGCCA	66	0.43	3.85
6	SCoT35	10	10	CATGGCTACCACCGGCCC	72	0.43	4.36
7	SCoT36	8	8	GCAACAATGGCTACCACC	55	0.42	3.35

GC**%** = primer G-C content, PIC = Polymorphic Information Content, MI = Marker Index.

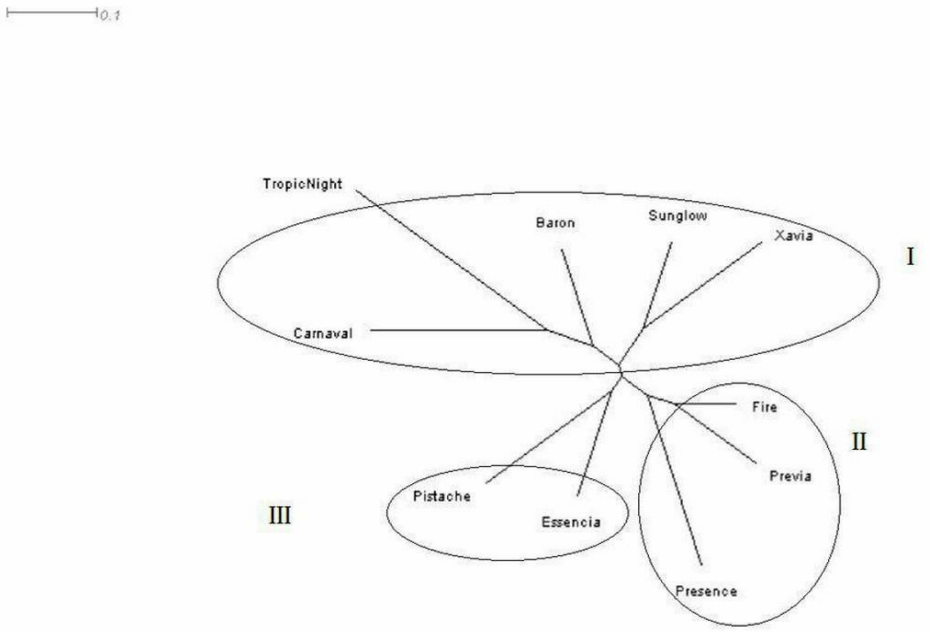

Fig. 3. Unweighted neighbor-joining method of arithmetic dendrogram of anthurium cultivars based on genetic distances computed from 7 SCoT molecular markers.

Mantel coefficient correlation test showed higher positive correlation between SCoT and RAPD matrices, indicating a consistent relationship between genetic distances from both marker systems. The correlation coefficient (r) was 0.77 between SCoT and RAPD, 0.76 between SCoT and CDDP, and 0.7 (significant $p > 0.01$) between RAPD and CDDP. All three molecular marker types showed positive and significant correlation with each other (Table 5). In order to estimate the genetic distance among genotypes, the dissimilarity matrix

was computed. The mean value of genetic distance obtained by SCoT, RAPD and CDDP markers were 0.27, 0.19 and 0.37, respectively.

Table 5. Mantel test correlation coefficients among similarity matrices obtained using CDDP, SCoT and RAPD markers.

Number	RAPD	SCoT	CDDP
RAPD	1		
SCoT	0.77**	1	
CDDP	0.70**	0.76**	1

Diversity in the population under study is one of the necessary tools for plant breeding. From this perspective, evaluation of diversity is prerequisite to modify any plant. Amongst various methods of diversity evaluation, molecular markers have been proved to be a fundamental and reliable tool for fingerprinting of varieties, establishing the fidelity of progenies, etc. (Khan and Pankajaksan 2010). The genetic background of most ornamentals is highly heterozygous and include many groups of species (Debener 2012). Genetic diversity has been estimated only on a small number of such flowers (Watanabe and Komamine 2000). Using three markers, our study suggested the presence of a significant polymorphism and revealed high level of variability in surveyed anthurium cultivars which is in agreement with those reported by Saeed et al. 2016 and Prajapati (2014). In this research three different molecular markers, RAPD, SCoT and CDDP were utilized to examine the extent of genetic variability among cultivars of anthurium. Use of SCoT and CDDP are the first marker report on anthruium. Our results demonstrated that CDDP and SCoT markers can be used as reliable techniques for detecting levels of DNA polymorphism and genetic relationship in anthurium. Conserved DNA regions sharing the same priming site, but differing in their genomic distribution may yield a large number of easily detectable length polymorphisms. Similarly, our results indicated that RAPD could be a useful marker for assessment of genetic diversity in anthurium as well. However, this type of marker system has its own limitations. For example, problems of reliability and transferability of RAPD data among laboratories, its dominant nature and low reproducibility in amplification of RAPD markers (Camlin 2000). According to our results, SCoT was also a suitable marker in studying genetic diversity with regards to its high polymorphic percentage, PIC (0.42) and MI (3.72) values. In total, SCoT marker had higher values than CDDP and RAPD markers in terms of all genetic parameters studied. Cluster analysis using delineated the cultivars in three major clusters by using data obtained from SCoT and CDDP marker techniques

because both techniques work based on single primer designed to link to the flanking regions of the ATG initiation codon on both DNA strands. In addition, these markers are usually reproducible and simple to use. Functional genes or gene families present in multiple copies in the plant genome can be amplified by short primers (Collard and Mackill 2009). In agreement with our study suggested the presence of a considerable polymorphism in the studied anthurium cultivars showing a high level of diversity (Naghavi et al. 2002, Tabaei-Aghdaei et al. 2006). In contrast, the study reported low level of genetic diversity within anthurium cultivars (Ghaffari et al. 2014). However, it is difficult to compare the polymorphism rate of SCoT and CDDP markers used in this study with other studied dominant markers (such as RAPD or ISSR) because of the difference in the number of primers and varieties used (Kobeissi et al. 2018). Investigation of the relationships between the studied markers through Mantel test revealed that all the three studied types of markers were significantly positively correlated with each other (Table 5). Compared with other correlations, correlation between SCoT and RAPD was higher indicating a consistent relationship between genetic distances from both marker systems. Although, the development of diversity for the three marker techniques was roughly equal, we anticipate that the source of detected diversity is different, as each technique targets different regions of the genome. Therefore, it is better to use more than one marker (as what we examined) to assess genetic diversity or diagnostic fingerprinting in anthurium. Since, SCoT markers exhibited a relatively higher PIC and MI values, we recommend the use of SCoT marker for estimating the genetic diversity among anthurium cultivars.

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