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Molecular characterization of *Anthurium* genotypes by using DNA fingerprinting and SPAR markers

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ABSTRACT. We characterized single primer amplification reaction (SPAR) molecular markers from 20 genotypes of *Anthurium andraeanum* Lind., including 3 from commercial varieties and 17 from 2 communities in the State of Espírito Santo, Brazil. Twenty-four SPAR,

consisting of 7 random amplified polymorphic DNA and 17 inter-simple sequence repeat markers were used to estimate the genetic diversity of 20 *Anthurium* accessions. The set of SPAR markers generated 288 bands and showed an average polymorphism percentage of 93.39%, ranging from 71.43 to 100%. The polymorphism information content (PIC) of the random amplified polymorphic DNA primers averaged 0.364 and ranged from 0.258 to 0.490. Primer OPF 06 showed the lowest PIC, while OPAM 14 was the highest. The average PIC of the inter-simple sequence repeat primers was 0.299, with values ranging from 0.196 to 0.401. Primer UBC 845 had the lowest PIC (0.196), while primer UCB 810 had the highest (0.401). By using the complement of Jaccard's similarity index and unweighted pair group method with arithmetic mean clustering, 5 clusters were formed with a cophenetic correlation coefficient of 0.8093, indicating an acceptable clustering consistency. However, no genotype clustering patterns agreed with the morphological data. The *Anthurium* genotypes investigated in this study are a germplasm source for conservational research and may be used in improvement programs for this species.

Key words: Araceae; Breeding; DNA markers; Genetic diversity; Germplasm; Ornamentals

INTRODUCTION

Anthurium, which includes more than 600 species, is the most diverse genus in the family Araceae. *Anthurium andraeanum* Lind. is the second most widely traded tropical flower worldwide, second only to orchids (Castro et al., 2004). In Brazil, the total value of *Anthurium* traded in 2007 was estimated to be BRL 1 billion. The State of São Paulo, particularly Holambra, Atibaia and the Ribeira Valley areas, is the largest center of production and trade of *Anthurium* and is responsible for approximately 70% of the *Anthurium* market share (Kiyuna et al., 2007). However, production has increased in the states of Bahia, Ceará, and Pernambuco (Leme and Honório, 2004; Caldari Junior, 2004; de Assis et al., 2011). *Anthurium* are favored because of the size, color, and longevity of their inflorescence (da Silva et al., 2008; Nomura et al., 2011).

A demanding market requires uniform plants with high-quality flowers and a high yield (Stancato and Tucci, 2010). To meet these demands, knowledge regarding the genetic diversity among local cultivars is needed to support plant improvement programs and allow for exploitation of genetic variability in the production of new hybrids (Cabral et al., 2010).

Molecular markers are valuable tools for estimating genetic diversity (Vieira et al., 2007; Gonçalves et al., 2009; Oliveira et al., 2010; Cabral et al., 2011). These markers can be used to directly measure genetic diversity at the DNA level, eliminating the influence of environmental variations. Molecular markers also exhibit a large number of polymorphisms and are free from pleiotropy (Ferreira and Grattapaglia, 1998; Leal et al., 2010).

Recently, polymerase chain reaction (PCR)-based molecular markers, such as those based on a single primer amplification reaction (SPAR), have increasingly become efficacious tools for studying genetic diversity in plants (Ranade et al., 2009; Elmeer and Almalki, 2011;

Mani et al., 2011; Sharma et al., 2010, 2011), particularly for those species that have not been well-studied or those for which specific molecular markers had not been developed. Sharma et al. (2011) reported the efficiency of reactions involving 1 primer, such as the random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) methods, for analyzing genetic diversity in higher plants.

RAPD markers use 1 short 10-nucleotide primer that binds to an arbitrary sequence of the genome, eliminating the need for determining the sequence before amplification (Borém and Caixeta, 2009). Because RAPD primers randomly bind to several different complementary regions of the genome, this technique reveals high levels of polymorphism. ISSRs are based on microsatellite sequences and amplify genomic regions that are flanked by primer annealing sites (Wolfe, 2005). A combination of these types of markers has been used widely and successfully to estimate genetic diversity in plants (Pharmawati et al., 2004; Muthusamy et al., 2008; Kayis et al., 2010; Saleh, 2011; Shafie et al., 2011).

Although knowledge of the genetic variability among *Anthurium* accessions is crucial for developing new hybrids, few genetic studies examining *Anthurium* have been conducted (Nowbuth et al., 2005). Therefore, in this study, we characterized SPAR molecular markers from 20 genotypes of *A. andraeanum* Lind., including 3 from commercial varieties and 17 from 2 communities in the State of Espírito Santo.

MATERIAL AND METHODS

Genetic material

Twenty *Anthurium* genotypes were used: 12 were obtained from Nuvem Azul (NA) plant nursery located in the municipality of Venda Nova do Imigrante, ES, 5 were from Santo Antonio (SA) farm located in the district of Piaçu, municipality of Muniz Freire, ES, and 3 were commercially available genotypes (Table 1). The plants were grown in pots containing haplustox and supplemented with pieces of coconut shells and tree leaves to contribute to the drainage of excess of water and under mild weather conditions with low and indirect lighting in a greenhouse at the Centro de Ciências Agrárias da Universidade Federal do Espírito Santo.

DNA extraction

DNA was extracted from leaves following the protocol described by Doyle and Doyle (1990) and modified by Abdelnoor et al. (1995).

Approximately 200-300 mg leaves were ground in liquid nitrogen; the powdered leaves were transferred to labeled microcentrifuge tubes. Extraction buffer [700 µL: 2% cetyl trimethyl ammonium bromide (v/v), 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Tris-HCl, pH 8, 2% solid polyvinylpyrrolidone (w/v), and 0.2% β-mercaptoethanol (v/v)] was added, and the tubes were homogenized using a vortex mixer for approximately 20 s followed by incubation in a water bath at 65°C for 40 min. The samples were centrifuged (Heittich Zentrifugen MIKRO 200) for 5 min at 14,000 rpm, and the supernatants were transferred to new tubes. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the supernatant. The tubes were gently inverted and further centrifuged for 5 min

Table 1. Descriptions of each genotype analyzed, including location of origin, spathe color (SC), spathe shape (SS), young leaf color (YLC), lobe type (LT), and spadix color (SxC).

Genotypes	Origin	SC	SS	YLC	LT	SxC
SA 01	MF	PK	CO	LGr	CL	LP
SA 02	MF	PK	CO	LGr	CL	LP
SA 04	MF	RD	CO	LRd	OP	RD
SA 05	MF	PK	RO	LGr	SO	PK
NA 08	VNI	WH	RO	LGr	SO	LP
NA 09	VNI	LP	RO	PGr	OP	YW
SA 14	MF	WH	FU	LGr	SO	DP
NA 22	VNI	OR	FU	LGr	OP	RD
NA 24	VNI	WH	CO	LGr	OP	LP
NA 25*	VNI	-	-	DGr	OP	-
NA 26	VNI	WH	RO	LGr	SO	LP
NA 27	VNI	RD	RO	LGr	SO	YW
NA 28	VNI	RD	CO	DGr	SO	YW
NA 29	VNI	RD	FU	DRd	OP	RD
NA 30	VNI	LP	CO	LGr	OP	LP
NA 31	VNI	WH	CO	DGr	SO	DP
NA 33*	VNI	-	-	LGr	SO	-
Commercial samples						
Mississippi	Terra Viva	OR	CO	RGr	OP	WH
Sierra	Sitio Kolibri	RD	CO	LGr	OP	YW
Pink Champion	Sitio Kolibri	DP	CO	RGr	OP	DP

MF = Muniz Freire, ES; VNI = Venda Nova do Imigrante, ES. SC: PK = pink; RD = red; WH = white; LP = light pink; OR = orange; DP = dark pink. SS: CO = cordiform; RO = rounded; FU = fusiform. YLC: LGr = light green; LRd = light red; DRd = dark red; PGr = pinkish green; DGr = dark green; RGr = red-green. LT: CL = closed; OP = open; SO = slightly open. SxC: YW = yellow. *Did not exhibit floral structure.

at 14,000 rpm. The upper phases of the mixtures were transferred to new tubes, and ice-cold isopropanol (in a 1:1 ratio of isopropanol to supernatant) was added. The tubes were shaken gently and incubated at -20°C overnight.

Next, the samples were centrifuged for 10 min at 14,000 rpm. The supernatant was discarded and the precipitate was washed twice with 70% ethanol, once with 95% ethanol, and dried at room temperature. The precipitate was then resuspended in 0.2 mL 10 mM Tris-HCl and 1 mM EDTA, pH 8.0, and distilled water added to a final volume of 1 L containing RNase at a final concentration of 40 µg/mL and incubated for 1.5 h at 37°C.

DNA was visualized on 0.8% agarose gels and concentrations were estimated by comparison with molecular weight markers at concentrations of 25, 50, and 100 ng/µL. The DNA samples were diluted to 10 ng/µL.

Analysis of RAPD

To study RAPD, 7 primers from Operon Technologies (Alameda, CA, USA) were used (Table 2). PCR was performed in a thermocycler (TC Thechne 412) using 25-µL samples consisting of Tris-KCl, pH 8.3 (10 mM/50 mM), 2.8 mM $MgCl_2$, 0.6 mM dNTPs, 0.4 µM primer, 25 ng DNA, and 1 U *Taq* polymerase. The following amplification program was used: 40 cycles of denaturation for 2 min 15 s at 94°C, annealing for 30 s at 35°C, and elongation for 1 min at 72°C; and a final extension step for 7 min at 72°C. The reaction products were separated using electrophoresis on 1.2% agarose gel immersed in 10 mM sodium hydroxide and 40.43 mM boric acid under 100 V constant voltage for 4 h.

Table 2. Estimation of the genetic diversity among 20 genotypes of *Anthurium*.

RAPD	Primer	(5'-3') sequence	TAB	TPB	TMB	%P	PIC
1	OPF 06	GGGAATTCGG	7	7	0	100.00	0.258
2	OPG 18	GGCTCATGTG	2	2	0	100.00	0.444
3	OPG 14	GGATGAGACC	3	3	0	100.00	0.393
4	OPAI 08	AAGCCCCCCA	8	8	0	100.00	0.328
5	OPAI 13	ACGCTGCGAC	5	5	0	100.00	0.332
6	OPAM 14	TGGTTGCGGA	2	2	0	100.00	0.490
7	OPAI 05	GTCGTAGCGG	4	4	0	100.00	0.305
Total			31	31	0	-	-
Average			4.43	4.43	0	100.00	0.364
ISSR	Primer	(5'-3') sequence	TAB	TPB	TMB	%P	PIC
8	UBC 857	$(AC)_8YG$	16	15	1	93.75	0.342
9	UBC 810	$(GA)_8T$	18	18	0	100.00	0.401
10	UBC 880	$(GGAGA)_3$	10	10	0	100.00	0.280
11	UBC 808	$(AG)_8C$	11	9	2	81.82	0.307
12	UBC 862	$(AGC)_6$	6	5	1	83.33	0.234
13	UBC 855	$(AC)_8YT$	13	12	1	92.31	0.310
14	UBC 841	$(GA)_8YC$	24	22	2	91.67	0.266
15	UBC 849	$(GT)_8YA$	9	8	1	88.89	0.294
16	UBC 843	$(CT)_8RA$	12	9	3	75.00	0.235
17	UBC 834	$(AG)_8YT$	17	15	2	88.24	0.244
18	UBC 864	$(ATG)_6$	14	12	2	85.71	0.317
19	UBC 854	$(TC)_8RG$	14	14	0	100.00	0.338
20	UBC 890	$VHV(GT)_7$	18	17	1	94.44	0.331
21	UBC 859	$(TG)_8RG$	14	14	0	100.00	0.347
22	UBC 819	$(GT)_8A$	19	18	1	94.74	0.272
23	UBC 845	$(CT)_8RG$	21	15	6	71.43	0.196
24	UBC 827	$(AC)_8G$	21	21	0	100.00	0.364
Total			257	234	23	-	-
Average			15.12	13.76	1.35	91.05	0.299
SPARs total			288	265	23	93.39	0.318

SPAR = listed are the single primer amplification reaction; TAB = total amplified bands; TPB = total polymorphic bands; TMB = total monomorphic bands; %P = percentage of polymorphisms; PIC = polymorphism information content.

Analysis of ISSRs

ISSR experiments were performed using 17 primers (Table 2). Amplification reactions were performed in a thermocycler (ThechneTC 412) with 25-µL samples containing 2.4 mM $MgCl_2$, 0.25 mM Tris-KCl, pH 8.3, 0.25 mM of each dNTP, 0.2 µM primer, 1 U *Taq* DNA polymerase, and 30 ng DNA.

The following program was used for amplification: initial denaturation for 15 min at 94°C; 35 cycles for 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C; and a final extension step for 7 min at 72°C. For primers UBC 819, 845, and 827, the following program was used: 4 min at 94°C; 35 cycles for 1 min at 94°C, 1 min at 52°C, 2 min at 72°C; and a final extension step for 7 min at 72°C.

The reaction products were separated by electrophoresis on 2.5% agarose gel immersed in 1X TBE buffer (89 mM Tris base, 89 mM boric acid, 2.23 mM EDTA, distilled water to a final volume of 1 L) under constant voltage (110 V) for 4 h. A 100-base pair marker (Fermentas, Vilnius, Lithuania) was used to distinguish and estimate the molecular mass of the DNA fragments.

Statistical analysis

Molecular data were recorded based on the bands detected in the cultivars. A matrix of binary values for both markers was generated. A 1 or 0 denoted the presence or absence of bands, respectively, for each locus according to the gel results.

Genetic dissimilarity was estimated based on the complement of Jaccard's index using the GENES software (Cruz, 2013). The generated dissimilarity matrix was used to calculate the frequency of dissimilarity. The polymorphic information content (PIC) was calculated according to Roldán-Ruiz et al. (2000). The mean connection between groups was determined using unweighted pair group method with arithmetic mean for cluster formation. The cophenetic correlation coefficient (CCC) between the matrix of genetic dissimilarity and the matrix of cophenetic values was calculated using the R software to verify the clustering consistency.

RESULTS

Twenty-four SPAR primers were used to estimate genetic diversity among the 20 investigated *Anthurium* accessions (Table 1). A total of 288 bands were produced; of these, 265 polymorphic bands were present, corresponding to an average of 11.04 amplified polymorphic bands per primer (Table 2). The percentage of polymorphism varied from 71.43 to 100%, with an average of 93.39%.

In this study, the high percentage of polymorphisms identified in the *Anthurium* genotypes was caused by selection among different morphological traits. The results showed that *Anthurium* accessions exhibit wide genetic divergence, as detected using the SPAR markers.

The 7 RAPD primers generated 31 bands, all of which were polymorphic (100% polymorphism). The average number of bands generated per primer was 4.43 and ranged from 2 to 8. The lowest number of bands was generated by the primers OPAM 14 and OPG 18, and the highest by OPAI 08 (Table 2). The PIC values revealed the discriminatory power of the primers. For dominant markers such as RAPD and ISSRs, PIC should vary from 0 to 0.5: as it approaches 0.5, the discriminatory power of the primers increases. The RAPD primers used in this study consistently showed high discriminatory power.

The 17 ISSR primers used generated 257 total bands, of which 234 were polymorphic, yielding 91.05% polymorphism. The percentage of polymorphism varied from 71.43% with primer UBC 845 to 100% with primers UBC 810, UBC 880, UBC 854, UBC 859, and UBC 827. The highest number of bands, 24, was observed with primer UBC 841, while the lowest, 6, with primer UBC 862 (Table 2). The PIC of ISSR primers varied from 0.196 to 0.401 and averaged 0.229 (Table 2). The lowest PIC found for this marker was with primer UBC 845 (0.196), while the highest with UBC 810 (0.401).

A comparison of the 2 types of investigated markers showed that the average polymorphism per primer was higher for ISSRs per primer (13.76) than for RAPD primers (4.43). However, RAPD exhibited a higher average percentage of polymorphism (100%) compared to the ISSR primers (91.05%). This wide variation in the number of bands may be attributed to differences in the binding sites of both RAPD and ISSRs along the genome of the investigated genotypes. On average, RAPD markers showed a higher PIC (0.364) than ISSR primers (0.299) (Table 2), indicating greater information content in the RAPD primers.

The frequencies of dissimilarity for the 190 paired combinations of the 20 investigated genotypes by using SPAR markers (Figure 1) showed a non-uniform distribution. Most of the data points fell between 0.5 and 1, which included 99.5% of the total dissimilarity observed. The class with values from 0.70 to 0.79 exhibited the highest frequency of dissimilarity, comprising 48.42% of the total.

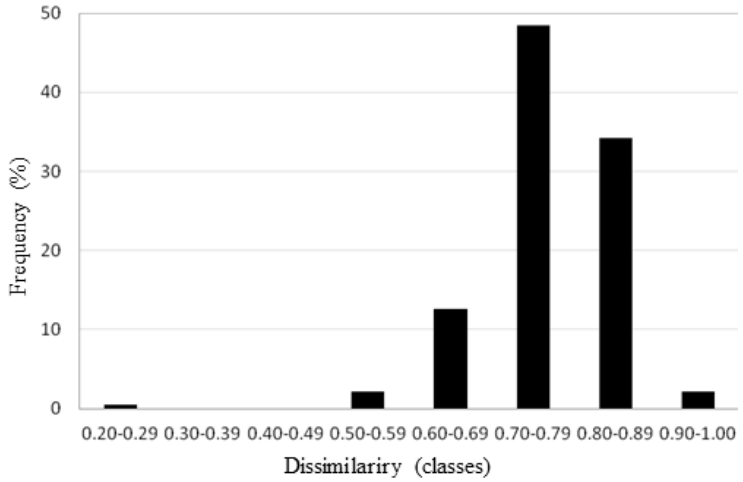

Figure 1. Distribution of the frequencies of dissimilarity among 20 genotypes of *Anthurium* obtained using single primer amplification reaction markers.

In the dendrogram of the 265 polymorphic bands for the 24 SPAR primer combinations, the shortest genetic distance between the individuals was 0.2727, the longest was 0.914, and the average was 0.7691. Genotypes SA 01 and SA 02 were the most closely related, while SA 01 and NA 33 were the least correlated. SA 01 and SA 02 shared common characteristics such as their origin, spathe color (SC), spathe shape (SS), young leaf color (YLC), lobe type (LT), and spadix color (SxC) (Table 1), confirming the low dissimilarity shown in the dendrogram (Figure 2). In contrast, genotypes SA 01 and NA 33 only shared one characteristic, YLC, explaining their greater dissimilarity.

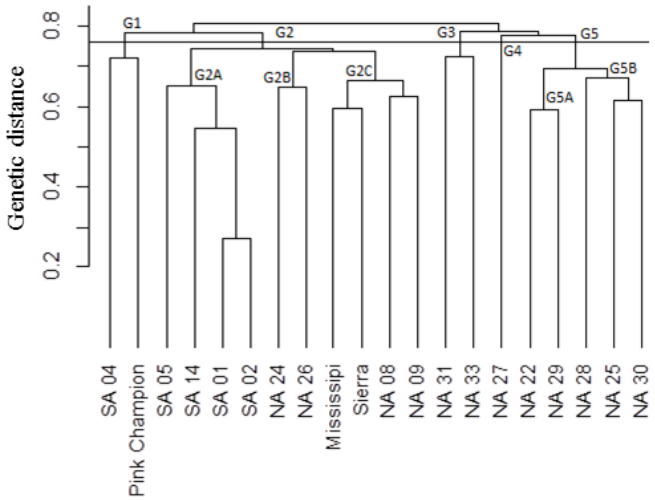

Figure 2. Dendrogram obtained using single primer amplification reaction markers and complemented by Jaccard's similarity index as generated using the unweighted pair group method with arithmetic mean (UPGMA) clustering method.

A cutoff point of 0.76 was used for dissimilarity in the dendrogram (Figure 2), resulting in the generation of 5 groups: G1-G5. Group G1 clustered genotypes SA 04 and the commercial cultivar Pink Champion, which share SS and LT characteristics (Table 1).

Group G2 was further divided into 3 subgroups, G2A, G2B, and G2C. Subgroup G2A clustered genotypes SA 05, SA 14, SA 01, and SA 02, which all share the same origin, and YLC. Subgroup G2B clustered genotypes NA 24 and NA 26, which share the same origin, as well as SC, YLC, and SxC. Subgroup G2C clustered 2 commercial cultivars, Mississippi and Sierra, and genotypes NA 08 and NA 09. These 4 genotypes have no common characteristics; however, upon comparison of 3 of the 4, genotypes NA 08, NA 09, and Sierra share YLC, while genotypes NA 09, Sierra, and Mississippi exhibit the same LT.

Group G3 clustered the genotypes NA 31 and NA 33, which share the same origin, YLC, and LT (Table 1). Group G4 contained only genotype, NA 27. Group G5 was further divided into 2 subgroups, G5A and G5B. Subgroup G5A clustered genotypes NA 22 and NA 29, which share the same origin, as well as SS, LT, and SxC. Subgroup G5B clustered genotypes NA 28, NA 25, and NA 30, which all share the same origin.

Among the 3 commercial cultivars, the lowest dissimilarity (0.596) was found between Mississippi and Sierra, which share SS and LT. Cultivars Mississippi and Pink Champion exhibited the greatest dissimilarity (0.835), but had the same SS, YLC, and LT (Table 1).

No pattern of genotype clustering agreed with the morphological data described in Table 1. This may be because the SPAR primers randomly annealed to the target genome. Therefore, the genetic relationship between the primers used and the morphological data is likely insignificant.

The calculated CCC was 0.8093, which is a good fit between the original data matrix and the matrix resulting from the dendrogram. Therefore, the clustering was acceptably consistent.

The CCC is the Pearson's linear correlation coefficient between the matrix of distances among cultivars resulting from the original data and the matrix of distances resulting from the dendrogram.

DISCUSSION

Sharma et al. (2011) estimated diversity within and between species of orchids using SPAR markers and found averages of 8.6 polymorphic bands per primer and 96.6% polymorphism. This high percentage of polymorphism may have resulted from using different species of orchids, leading to greater genetic distances between the investigated genotypes shown by the markers.

Similarly, RAPD results were reported by Kayis et al. (2010), who found an average of 5.59 polymorphic bands per RAPD primer. Pharmawati et al. (2004) evaluated the diversity among 16 genotypes of *Grevillea* using RAPD markers and found an average of 17.34 polymorphic bands per primer. Elmeer and Almalki (2011) assessed the genetic diversity of *Prosopis cineraria* and *P. juliflora* using RAPD markers and found 100% polymorphism. In this study, the PIC of the RAPD primers varied from 0.257 to 0.490, with an average of 0.364. Primer OPF 06 produced the lowest PIC, while OPAM 14 was the highest (Table 2). Saleh (2011) reported a similar average PIC of 0.377 in their analysis of the genetic diversity of *Arthrocnemum macrostachyum* using RAPD primers.

Similar to the results of ISSR evaluation, in a study on the genetic diversity of *A. macrostachyum*, Saleh (2011) found 90.91% polymorphism using ISSR primers. The average

number of amplifications per primer for the ISSR markers was 15.12. Muthusamy et al. (2008) assessed the genetic diversity of *Vigna umbellata* by using ISSR markers and found an average of 12.94 bands per primer.

Similar PIC results were reported by Muthusamy et al. (2008), who found average PICs of 0.243 and 0.203, for RAPD and ISSR primers, respectively. Kayis et al. (2010) found average PICs of 0.250 for ISSR.

According to Kayis et al. (2010), although some features indicate that using RAPD is more efficient than using ISSR markers, ISSRs may be more useful because of their greater repeatability, particularly in organisms such as *Anthurium* that have inadequate genomic information available for locus-specific genotyping. Several authors have reported the efficiency of using both types of markers (Ranade et al., 2009; Elmeer and Almalki, 2011; Mani et al., 2011; Sharma et al., 2010, 2011).

The frequencies of dissimilarity indicate a wide genetic variability among the genotypes under study. According to Loarce et al. (1996), improvement programs for breeding and selection of new cultivars are based on the exploitation of genetic variability within a species.

According to Cargnelutti Filho et al. (2010), as the magnitude of a CCC value approaches 1, the consistency of the clustering pattern is greater. The CCC calculated in this study showed good fit between the original data matrix and the matrix resulting from the dendrogram. Therefore, molecular characterization using SPAR markers is a very useful and effective method for estimating genetic diversity in *Anthurium* genotypes. In addition, the *Anthurium* genotypes investigated in this study consist of germplasm that may be promising for conservational research and improvement programs because of their wide genetic variability.

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