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The genetic linkage maps of Anthurium species based on RAPD, ISSR and SRAP markers

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ABSTRACT

We report the construction of genetic linkage maps of two species *Anthurium ornatum* (*A. ornatum*) and *Anthurium andreanum* (*A. andreanum*) based on random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) and sequence related amplified polymorphism (SRAP) markers using a double-pseudo test cross mapping strategy. Mapping population of 43 F1 individuals was developed using a natural mutant IIHR selection A1 (*A. ornatum* or *Anthurium nymphaefolium*) and a variety called Eternity (*A. andreanum*). A total of 228 markers, including 99 RAPDs, 21 ISSRs and 108 SRAP combinations were used for the linkage group analysis. The linkage map of *A. ornatum* consisted of 10 linkage groups and a doublet covering 1233.5 cM , with a total of 85 markers, whereas the linkage map of *A. andreanum* consisted of 12 linkage groups covering a distance of 1023.5 cM with 78 markers. The constructed linkage map covered a total genome length of 77% and 73% of *A. ornatum* and *A. Andreanum*, respectively. This study provides an important basis for identification and mapping of horticultural traits, which would be exploited in Marker—Assisted Selection in Anthurium breeding programs.

1. Introduction

Anthurium, one of the leading cut flowers in the world, is known for its attractive foliage. It is the largest genus of the Araceae family. The genus *Anthurium* encompasses over 1500 species, of which around 600 species originated from Tropical America; the best known species among them is *Anthurium andreanum* (*A. andreanum*). The aroid varieties currently available in the market are the result of hybridization occurred between species *A. andreanum*, *Anthurium antioquiense* and *Anthurium ornatum*. *A. andreanum* and *A. ornatum* are diploid species $2n = 30$ (Sheffer et al., 1983; Cotias-de-Oliveira et al., 1999) with moderate genome size (Bliss and Suzuki, 2012).

In the recent years, DNA markers including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), inter specific simple repeats (ISSR) and sequence related amplified polymorphism (SRAP) markers, have undergone rapid development. These markers have been used for identification of germplasm, genetic diversity and quantitative trait loci (QTLs); linkage map would be a stepping stone towards the identification of QTLs related to economic traits.

Similar to many trees, fruits and nut crops, Anthurium is also an out-breeding heterozygous crop with long generation time and inbreeding depression, limiting its ability to produce populations for mapping studies. Therefore, in order to generate linkage maps, F1 progeny (obtained from crossing two highly heterozygous parent clones) and the double pseudo-test cross strategy are used. This method two independent maps are constructed for each parent (Weeden, 1994; Atienza et al., 2002; LaRosa et al., 2003). A number of genetic linkage maps have been constructed for high commercial ornamental crops such as Dendrobium (Xue et al., 2010; Lu et al., 2012), Chrysanthemum (Zhang et al., 2010), lily (Abe et al., 2002), rose (Dugo et al., 2005; Oyant et al., 2008). Linkage map would further improvise the breeding program of Anthurium through marker assisted selection (MAS).

There have been relatively few reports regarding the use of molecular markers within Anthurium cultivars. Isozymes were the first molecular marker used for genotypic characterization in Anthurium (Kobayashi et al., 1987), yet those markers are very limited and display low polymorphism. Since then, genomic markers based on DNA polymorphism, RAPD and ISSR molecular

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Fig. 1. Morphological features of IIHR selection A1 (*Anthurium ornatum*)—(a) and variety Eternity (*Anthurium andreanum*)—(b) which were used for deriving F1 progeny.

markers, have been used for genetic diversity analyses (Nowbuth et al., 2005; Khan et al., 2010; Buldewo et al., 2012). Bliss and Suzuki (2012) estimated the genome size and chromosomal number of 77 accessions representing 34 species and 9 cultivars of Anthurium. Wang et al. (2013) developed and characterized novel microsatellite markers using Araceae expressed sequence tags (ESTs). Recently, Cui et al. (2012) have constructed genetic linkage map of *A. andreanum* based sequence-related amplified polymorphism (SRAP). Cheng-dan et al. (2013) have used SRAP marker to analyze the genetic relationship of 33 *A. andreanum* cultivars.

A previous program on breeding novel characters in Anthurium conducted at the Indian Institute of Horticultural Research (IIHR) Bangalore has lead to a natural mutant 'IIHR selection A1′ in *A. ornatum*. We chose this mutant as female parent in this study. The variety, 'Eternity' belongs to *A. andreanum* and is grown commercially. The hybrid progenies selected from these species have the potential to breed a novel Anthurium cultivar with fragrance, resistance to bacterial blight along with good economical characters derived from cv. Eternity. To achieve this goal, we attempted to construct a genetic linkage map for *A. ornatum* and *A. andreanum* to identify important traits to improve Anthurium breeding program.

2. Materials and methods

2.1. Plant material

IIHR selection A1 (Fig. 1a) and Eternity (Fig. 1b) are two genetically distinct varieties with different morphological traits (Patil, 2008) such as spathe length (IIHR selection A1: 14.25 cm vs Eternity: 10.45 cm). However, both of them share a similar white spathe color. In terms of spadix length and diameter IIHR selection A1 is featured with short and thick spadix (4.78 cm in length and 1.6 cm in diameter). Eternity is marked with long and thin spadix (6.43 cm length with 0.98 cm diameter). IIHR selection A1 is highly resistant to bacterial blight and emits minty fragrance during sigma receptivity that is absent in Eternity. The IIHR selection A1 has also been registered as a novel line at National Bureau of Plant Genetic Resources (NBPGR) with registration no "INGR 08047" (Aswath and Devender, 2005). The dichogamy type of progeny was determined in the spring, generally in the morning between 8am and 9am. Pistil receptivity was assessed by first dusting pollen onto the stigma, followed by blowing off the excess pollen. A receptive stigma has a sticky surface to which a large number of pollen grains adhere. The seed set on the spadix were further germinated for developing F1 progenies. Since it is an interspecific cross seed setting is difficult. Only 100 seeds out of total of 175 seeds collected initially for germination showed efficient germination. A total of 43 progenies which had good growth and expressed high heterozygosity for morphological characters, were selected as F1 progenies. Genomic DNA was extracted from F1 progenies and parents using cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) with some minor modifications. The DNA concentration was determined using GeneQuant and further confirmed on 0.8% agarose gels based on the intensity of fluorescence stained with ethidium bromide.

2.2. Molecular markers analysis

In order to determine clear polymorphism and reproducibility a total of 410 RAPD primer, 20 ISSR primers and 208 SRAP primer combinations including 13 forward and 16 reverse were screened on the parental samples (IIHR selection A1 and Eternity). The primers showing polymorphism were genotyped for 43 F1 progenies.

The polymerase chain reaction (PCR) reactions for RAPD and ISSR were carried out in 25 μl volume containing 50 ng of template DNA, 1.8 mM $MgCl_2$, 1X PCR reaction buffer, 200 μM of dNTPs (deoxynucleotide triphosphate), 0.25 μM of each primer and 1 U Taq DNA polymerase. The PCR reaction solution for SRAP markers was prepared for 25 μl containing 50 ng of template DNA, 2.5 mM $MgCl_2$, 320 μM dNTPs, 0.3 μM of each primer and 1.5 U *Taq* DNA polymerase. All the reactions were performed in Technie thermal cycler (Bibby Scientific Asia Limited).

A touch down PCR program was used for RAPD amplification with conditions: 94 °C for 15 min, followed by 3 cycles of 94 °C for 25 s, 35 °C for 25 s, 72 °C for 2 min and 40 cycles of 94 °C for 25 s, 37 °C for 25 s and 72 °C for 2 min along wing a final extension of 72 °C for 7 min.

Amplification of ISSR primers was programmed for 94 °C for 4 min, followed by 35 cycles of 94 °C for 45 s, specific annealing temperature (53 °C to 60 °C) for 45 s, 72 °C for 1 min and final extension for 7 min.

PCR program was used for SRAP primer amplification was 94 °C for 7 min followed by five cycles of 94 °C for 60 s, 35 °C for 60 s, 72 °C for 60 s, 35 cycles of 94 °C for 60 s, 50 °C for 60 s, 72 °C for 60 s with a final extension at 72 °C for 7 min.

The amplified product of RAPD and ISSR primers was detected by 1.6% agarose gels. The SRAP fragments were separated on 8% polyacrylamide gels. The electrophoretic patterns of the PCR products were recorded digitally using a UVIpro Platinum Gel-Doc image analysis system. The DNA bands were scored for the presence and absence.

2.3. Marker nomenclature

The nomenclature followed for scoring the data in polymorphic fragments is important to identify the markers used in the analysis. RAPD markers are named with arbitrary sequence alphabet followed by the primer number in the sequence and the polymorphic fragment (either alphabet or number). In case of ISSR markers the name of the markers begins with 'I' followed by the primer number and number of the polymorphic fragment. Whereas in SRAP, the name start with 'M' followed by the number of forward and reserve primers, and ends with the polymorphic fragment numbered using alphabet.

2.4. Mapping and linkage analysis

RAPD, ISSR and SRAP primers were first assayed for both parents (IIHR selection A1 and Eternity). Each PCR reaction and gel running of PCR products were repeated twice and only reliable markers were considered for further analysis. Reproducible and clear fragments showing polymorphism between parents were further processed in the segregating progeny. Markers were scored in the mapping population of 43 F1 individuals for their presence or absence in two different data sets according to their parental origin. Linkage maps were constructed separately for each parent using the double pseudo-testcross mapping method (Weeden, 1994), utilizing the segregation data for polymorphic markers heterozygous in one or both parents with particularly application in mapping dominant markers like RAPDs (Xue et al., 2010).

The markers were separated into three types: (a) maternal testcross markers that were heterozygous (band present) IIHR selection A1, (b) paternal testcross markers that segregated only within Eternity, and (c) intercross markers that segregated within both parents. All markers were tested for a Mendelian segregation ratio of 1:1 or 3:1 using Chi-square analysis ($P < 0.05$). Those markers that segregated in a Mendelian fashion were used for linkage map construction in both the parents. Thus, two separate data sets were prepared and analyzed using MapMaker/EXP version 3.0 (Lincoln et al., 1992). Markers were grouped by two point linkage using LOD 5. Distances between adjacent marker loci were computed using Kosambi's mapping function, while the linkage maps were drawn using Mapdraw 2.1 software (Liu and Meng, 2003).

2.5. Estimation of genome length

Genome length was estimated calculating the average framework marker spacing (s) i.e., by dividing the summed length of all linkage groups by the number of intervals (number of markers minus number of linkage groups). The total length of the genomes (L) was calculated by simply adding $2s$ to the length of each linkage group to account for the chromosome that ends beyond the terminal markers (Fishman et al., 2001).

Table 1
Markers scored in the Anthurium using RAPD, ISSR and SRAP analysis.

	RAPD markers	ISSR marker	SRAP marker
Number of primers screened	410	20	208
Number of polymorphic primers	42	8	30
Number of polymorphic fragments	99	21	108
Number of testcross markers in IIHR selection A1	49	12	55
Number of testcross markers in Eternity	47	7	40
Number of intercross marker	3	2	3
Total number of markers produced	99	21	108

3. Results

3.1. Molecular markers

Only 42 (10.2%) primers, out of a total 410 RAPD primers initially screened between the parents, showed 99 polymorphic markers ranging from 200 to 2000 bp (Table 1). Each RAPD primer amplified one to three polymorphic bands with an average of 2.8 markers per loci. Among the 99 markers, 96 were testcross markers while the other three markers were intercross marker. Out of these, 96 markers the observed heterozygosities were 51% (49 markers) and 48% (47 markers) in *A. ornatum* and *A. andrearum*, respectively.

In the case of ISSR primers, out of 20 primers screened, only eight were informative for map construction which produced 21 loci. Each primer produced one to two polymorphic markers with an average of 1.2 marker per loci. Among the 21 markers, 19 markers were testcross markers and while two markers were intercross. Of the 19 test cross markers, 12 (63%) were from *A. ornatum* while 7 (36%) were from *A. andrearum*.

Among the 208 SRAP primer combinations screened between the parents, 30 primer combinations generated 108 polymorphic markers. Each primer produced three to four polymorphic markers with an average of 3.6 markers per loci per combination. Out of the 108 markers, 95 markers were testcross markers while 13 were intercross markers. Among the 95 test cross markers, 55 markers (57%) and 40 markers (42%) were from *A. ornatum* and *A. andrearum*, respectively.

3.2. Genetic linkage map construction

Overall 228 markers, including both testcross and intercross markers, from RAPD, ISSR and SRAP were considered for linkage map construction. Other markers that did not follow Mendelian segregation were not included. The 228 markers were subjected to two point linkage analysis with a LOD of 5. Linkage distances were computed using the Kosambi mapping function As per the double pseudo test cross, the linkage map was constructed separately for each parent.

For *A. ornatum*, a total of 123 marker were taken for analysis, of which 85 markers were linked to 10 major groups and a doublet (Fig. 2), while the rest 38 markers were left unlinked. The length of

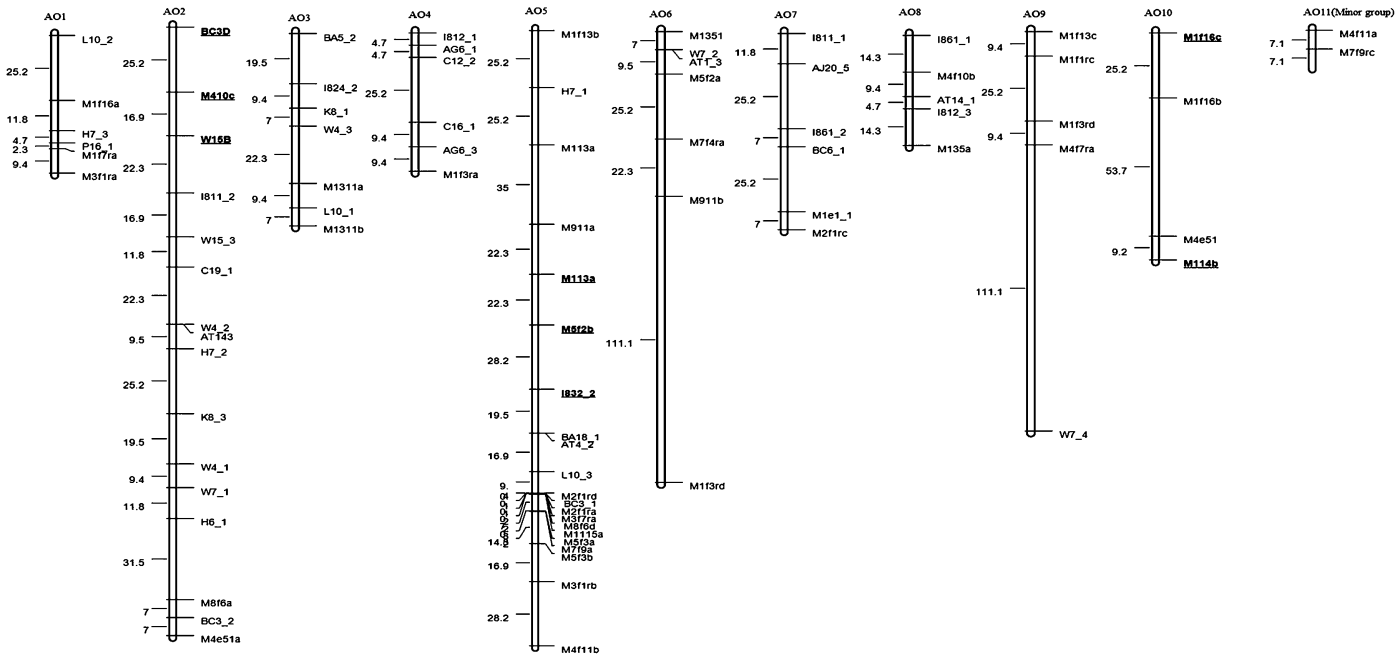

Fig. 2. Genetic linkage map of *A. ornatum* (IIHR selection A1) consisting of RAPD, ISSR and SRAP markers. Distances in centiMorgans are indicated to the left and marker names to the right of each linkage group. Doublets are shown as 'minor group'.

Table 2
Shared markers placed on different group on both *A. ornatum* and *A. Andreanum*.

Intercross markers	Marker type	In *A. ornatum*	In *A. andreanum*
BC3D	RAPD	AO2	AA5
W15B	RAPD	AO2	AA5
I832_2	ISSR	AO5	AA5
M410c	SRAP	AO2′	AA5
M1f16c	SRAP	AO10	AA12
M5f2b	SRAP	AO5	AA3
M114b	SRAP	AO10	AA12
M113a	SRAP	AO5	AA11

the linkage group (LG) varied from 2.4 cM to 111.1cM with mean LG of 13.49 cm. Each group comprised of 3 to 22 markers with mean of 8.6 loci per LG covering a total distance of 1233.5 cM.

In the framework of *A. andreanum*, a total of 105 markers were used for analysis. Of which 75 markers were linked into 12 group (Fig. 3) leaving 30 markers unlinked. Here, each group consisted of 4 to 11 markers. This map outlined a total distance of 1023.5 cM with a mean of 13.3 loci per LG.

Intercross markers underlined markers (Fig. 2 and Fig. 3) were used to find homologous LGs among the parents. Both *A. ornatum* and *A. andreanum* shared eight (47%) of the 17 intercross markers. Three linkage groups of *A. ornatum* could be paired with four linkage groups of *A. andreanum* (Table 2). Total genome size of *A. ornatum* was estimated to be 1,585.5 cM while that of *A. andreanum* was estimated to be 1395.5 cM. Consequently, the linkage groups with DNA markers covered 77% and 73% of the *A. ornatum* and *A. andrearum* genomes, respectively.

4. Discussion

This study presents the construction of linkage maps of two species of Anthurium namely *A. ornatum* and *A. andreanum* using a double pseudo test cross strategy. This strategy was first put in practice in forest tree by Grattapaglia and Sederoff, 1994 who constructed a linkage map of two parents of interspecific full-sib cross of Eucalyptus. The use of dominant markers in these crosses resulted in three type of segregating markers, (a) a test (1:1 segregating) marker inherited by the pollen parent. (b) a test (1:1 segregating) marker inherited by the seed parent and (c) intercross (3:1 segregating) marker inherited from both the parents. For an out-crossing species such as Anthurium, the double pseudo-testcross mapping strategy is well suited for use with dominant markers. Both *A. ornatum* and *A. andreanum* displayed high heterozygosity (Patil et al., 2009). RAPD and ISSR are both PCR based dominant markers. They share a lot of advantages, like relatively little template DNA, the ability to be resolved without the use of radioactivity and good levels of polymorphism (Sudheer et al., 2005). The most important feature of these markers is that they can be used without prior knowledge of the target template DNA sequence. Other markers such as RFLP and SSRs, require extensive sequencing before marker development.

Compared to RAPD and ISSR, the SRAP marker is a relatively new type of molecular marker that is more suitable for practical applications because of its features such as simplicity, low-cost test, secure and rich in polymorphism (Li and Quiros, 2001). In SRAP, there is a difference of GC content between gene coding sequences and other sequences in plant genomes. This difference was used to design two sets of SRAP primers. The forward primers contained a GGCC cassette closing the 3′ end of SRAP primers that might preferentially anneal to the GC-rich regions while the reverse SRAP primer set was incorporated with an AATT cassette that would preferentially anneal SRAP primers to introns. SRAP could preferentially amplify gene-rich regions in a genome. The authors found that a certain percentage of RAPD and SRAP markers were co-dominant (Li and Quiros, 2001). However, we did not find co-dominant RAPD and SRAP markers in the current study on Anthurium.

Intercross markers are less informative than test cross markers because the dominant phenotype comprises three indistinguishable genotypes (++,+−,−+) (Crespel et al., 2002). However, these markers are of interest because of their use as "locus bridges" to align homologous linkage groups between the two maps. If enough markers are in common between maps, the maps can be combined into a single integrated linkage map. In the present study, the

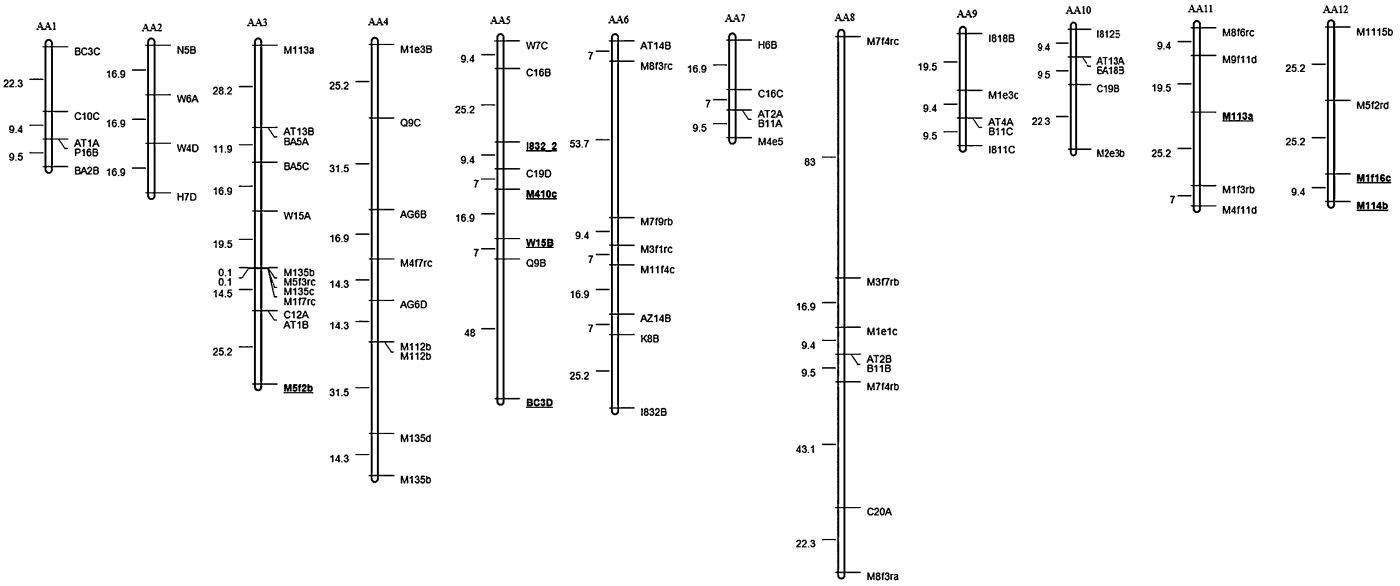

Fig. 3. Genetic linkage map of *A. andreanum* (Eternity) consisting of RAPD, ISSR and SRAP markers. Distances in centiMorgans are indicated to the left and marker names to the right of each linkage group.

percentage of intercross markers RAPD (7%), ISSR (10%) and SRAP (6%) were less thus, cannot be compared with intraspecific crosses. Although 17 intercross markers were identified only seven markers were grouped in both linkage maps (Table 2). In order to align and compare the homology of both maps effectively, either more intercross markers need to be generated and mapped on a larger progeny set or preferably, more informative co-dominant marker types, such as SSRs need to be placed on the maps (Sudheer et al., 2005). We could not locate phenotypic traits on these maps due to fact that the progenies are still under evaluation.

A LOD score greater than 3.0 is considered as the evidence for linkage; higher LOD score could be used for better linkage maps. In this study, we have used a LOD score of 5, slightly higher than the recommended mapping studies. *A. ornatum* had 11 linkage groups and *A. andreanum* had 12 linkage groups. According to the linkage mapping theory, the number of linkage groups should be in par with number of haploid chromosomes. The linkage groups are lower than the haploid number of chromosomes in Anthurium ($n = 15$) indicating that the maps are not saturated. To saturate the map additional markers of different type and larger population size are required. In this study, less population probably could be the cause for large markers being unlinked thereby accounting for less saturation of the linkage map. A similar study was carried out in Ananas species initially were a small population of 46 F1 were used to construct linkage map (Carlier et al., 2004). This linkage map is also a step forward for improvising and developing a dense map. We have tried our best to cover the genome length of 1233.5 cM and 1023.5 cM in *A. ornatum* and *A. andreanum*, respectively, covering 77% and 73% of the total length of the two species, respectively. In *A. ornatum*, linkage groups AO-2 and AO-5 and in *A. andreanum* linkage group AA-3 showed more markers linked. This is due to the occurrence of fewer recombination in the adjacent markers. Absence of recombination between the close markers, results in these markers being assigned in the same map position.

To the best our knowledge, this is the first report of the individual linkage maps for both *A. ornatum* and *A. andreanum*. Apart from our study, there is only a single report of genetic linkage map of *A. andreanum* using SRAP marker system (Cui et al., 2012). Most of the economic as well as disease resistant traits in Anthurium appear to have complex mode of inheritance. The current study is probably a great stepping stone in identifying the chromosomal loci that control important traits such as stalk length, stalk diameter, spathe length and diameter, spadix length and diameter, fragrance and resistance to diseases like bacterial blight. With further saturation of this map will lead to identification of all important horticultural traits thereby aiding in marker assisted selection in future.

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