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Hereditary multiplicity in (*Anthurium andraeanum)* cultivars in Mauritius

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Anthurium andraeanum is an important ornamental in the flower industry in Mauritius. Classical phenotype methods of identification, although still very useful, are difficult to use between very closely related Anthurium cultivars. The objectives of this study were to assess the genetic diversity of 12 *A. andraeanum* cultivars in Mauritius using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers and identify cultivar specific markers for the genetic profiling of *A. andraeanum* cultivars. Polymorphism among the 12 cultivars was assessed using RAPD and ISSR primers. Reproducible results were used for statistical analysis. The presence and absence of bands were scored as 1 and 0, respectively to form a matrix from which a dendrogram was obtained using NTSYS. Dendrograms were obtained from matrices derived from RAPD and ISSR analyses to give an estimate of the genetic distance between the tested cultivars. Both ISSR and RAPD were found to be useful tools in differentiating locally grown *A. andraeanum.*

Key words: *Anthurium andraeanum*, RAPD, ISSR, genetic diversity, cluster analysis.

INTRODUCTION

Anthurium andraeanum (family Araceae) is a perennial, herbaceous, ornamental plant known for its highly attractive and long lasting spathes. The plant has good market value worldwide and the main producing countries are the Netherlands and Hawaii (Kuenhle et al., 2001). *Anthuriums* were introduced to Mauritius in the early twentieth century and have undergone about a hundred years of random cultivation and hybridization in search of plants with desirable characteristics (Reetoo, 1989).

Since the 1960's, floriculture in Mauritius has been dominated by *A. andraeanum* and it has become a well-established export industry.

Mauritius exports about 10.2 million stems per year and is the third largest exporter of *A. andraeanum* on the world market (Nowbuth et al., 2005). Although the local growers are currently able to meet export demands,

Mauritian growers are experiencing fierce competition from Hawaii and Holland leading to a decline in exports over the past few years. Market demands make it imperative that local producers diversify the cultivars. This can be achieved by breeding new cultivars, importing exotic cultivars from Holland and Hawaii or reaching for new markets. Some growers have started breeding programs. Selection is based principally on visual screening for useful traits such as flower colour, spadix orientation, spathe shape and yield.

Although some breeders have varieties obtained from complex hybridization schemes, no proper records of crossing schemes and parent selection are available. These breeders are hesitant to bring the novel Anthuriums to the market for fear of losing their germplasm to other producers since the material can be easily propagated by tissue culture or other vegetative means. Some of the locally bred Anthuriums cultivated commercially by growers include 'Anouchka', 'Michele', 'Mado', 'Francesca', 'Monica' and 'Bourgogne'.

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Table 1. Main characteristics of *Anthurium* accessions used in this study.

Name	Primary Phenotype- Flower colour	Secondary Phenotype- Plant Height	Tertiary Phenotype- Leaf shape
Acropolis	White	Average	Flat + heart shape + mickey-mouse lobes
Carre	Red (heart-shape spathe)	Average	Large, long, flat and heart shape
Mado	Salmon Pink	Short	Roundish, flat, large and pointed end
Marian Seefurth	Pink	Average	Flat, large heart shape with pointed ends
Midori	Green	Average	Flat, large heart shape with pointed ends
Nitta	Orange	Average	Oblong, flat heart shape with pointed ends
Norflora	Brick red	Average	Oblong, heart shape, pointed ends and overlapping lobes
Ozaki	Orange red	Average	Oblong, long heart shape with pointed ends
Paradiso	Obake	Tall	Large, oblong, heart shape with mickey-mouse lobes
Scorpio	Light Orange red	Average	Oblong, heart shape with pointed ends
Tropical	Red (round spathe)	Average	Oblong + heart-shape
UH69	Pink	Average	Flat, large heart shape with pointed ends

Tall, 150 cm above ground level; average: 50 to 100 cm above ground level; short, up to 50 cm above ground level; large, the width of the leaf is more than one third of the length; pointed ends, leaf ends with a pointed tip.

Most of the commercial cultivars in Mauritius originated from Hawaii and Holland. Therefore, commercialization of these Anthurium cultivars involves the payment of royalties. Classical phenotypic methods of identification, although still very useful, are difficult to use between very closely related Anthurium cultivars. The accuracy of phenotypic selection is not only impeded by external factors such as genotype x environment interactions or developmental stage of the plant but is also subject to human interpretations.

A large number of molecular techniques are now available for assessing the genetic diversity of plants. These molecular approaches are especially informative because the markers are phenotypically neutral and are not subject to environmental effects (Ranade et al., 2001).

The development of a DNA typing tool for *A. andraeanum* should encourage growers to breed for novel traits and release already developed potential commercial cultivars. RAPD markers have been used to assess genetic relationships in a wide range of plant species including *Anthurium* (Ranamukhaarachchi et al., 2001) examined the genetic relationships of nine morphologically similar pot plant cultivars of *A. andraeanum* and concluded that there was a close genetic relationship among the cultivars. ISSR and RAPD markers were found to be useful in current breeding programs of *A. andraeanum,* for estimating the genetic similarity among genotypes and for the identification of cultivars (Jau-Yueh et al., 2001).

Nowbuth et al. (2005) reported low genetic variability in Anthurium but found that RAPD was useful in differentiating locally bred varieties of in Mauritius. A high degree of polymorphism was reported in a study of 12 Anthurium varieties in India assessed with RAPDs (Khan

and Pankajaksan, 2010). The objectives of this study were to (i) assess the genetic diversity of 12 *A. andraeanum* cultivars in Mauritius using RAPD and ISSR markers and (2) identify cultivar specific markers for the genetic profiling of *A. andraeanum* cultivars*.*

MATERIALS AND METHODS

The plant material used in this study was obtained from two large-scale anonymous producers in Mauritius. The leaf and spathe of the cultivars used for DNA extraction (Table 1) were collected in the morning. Some of the main morphological features of these cultivars are summarized in Table 1. The cultivars were selected on the basis of their different morphological features.

DNA extraction and RAPD analysis

DNA was extracted according to the method described in Buldewo and Jaufeerally-Fakim (2002). A total of 58 random primers purchased from Operon Technologies (Alameda, Calif, USA) were used as single primers for RAPD analysis. In addition, four primers that showed high diversity in characterizing the molecular diversity of taro (Irwin et al., 1998) were used in this study. The reaction mix for a 30 µl reaction contained: 25 mM $MgCl_2$, 200 µM dNTP, 10 pmoles of primer, 1X reaction buffer, 1 unit *Taq* Polymerase (Boehringer Mannheim, Germany) and 50 ng DNA templates. Amplification was carried out in a Techne thermal cycler (Bibby Scientific Ltd., Staffordshire, UK) programmed for one initial denaturation cycle at 94°C for 2 min followed by 40 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 1 min followed by one final extension cycle of 72°C for 10 min.

Optimization of the RAPD reactions was carried out by varying the concentrations of template, primer, dNTP, $MgCl_2$ and by using different amplification conditions such as annealing temperatures and the number of cycles. Finally, two operon primers and four primers used by Irwin et al. (1998) were selected for this study (Table 2).

The amplified fragments were separated on 1.5% agarose gels,

Table 2. List of selected primers and annealing temperatures used for RAPD analysis.

Primer	Primer sequence	Annealing temperature (°C)
OPB20	GGA CCC TTA C	37
OPA18	AGG TGA CCG T	37
AM7	TTG TTG CTG TGG GTA TAG CAT CAT	60
AN 1	ACT TCA TGC TAT GTG GCG ACT	60
B2	GGC GTC GGT TTC CAT TAT	58
B11	TGT GCC GAC GAT GTT GAT GCA AT	67

Table 3. Matrix of genetic distances obtained from RAPD data among Anthurium cultivars.

Paradiso	1.000											
Norflora	0.412	1.000										
Mado	0.286	0.404	1.000									
Midori	0.273	0.174	0.224	1.000								
Tropical	0.316	0.378	0.360	0.244	1.000							
Scorpio	0.200	0.227	0.200	0.143	0.167	1.000						
Ozaki	0.128	0.191	0.216	0.220	0.184	0.111	1.000					
Nitta	0.146	0.180	0.250	0.178	0.196	0.178	0.146	1.000				
UH69	0.206	0.262	0.255	0.205	0.250	0.175	0.140	0.130	1.000			
Marian Seefurth	0.147	0.214	0.188	0.154	0.128	0.125	0.270	0.163	0.222	1.000		
Carre	0.200	0.277	0.269	0.174	0.240	0.227	0.244	0.180	0.293	0.186	1.000	
Acropolis	0.188	0.282	0.244	0.158	0.268	0.158	0.150	0.140	0.194	0.171	0.282	1.000

stained with ethidium bromide and photographed under UV light. Two molecular weight markers, 123 bp from Gibco (Carlsbad, CA, USA) and molecular marker VI (Boehringer Mannheim) were used to estimate DNA fragment sizes. The RAPD reactions were repeated to verify the reproducibility of the banding patterns.

Amplification with ISSR primers

20 microsatellite motifs were screened to assess their potential in detecting polymorphism among the *Anthurium* cultivars. Amplification with ISSR primers were carried out as described by Guo et al. (2006). The amplicons were resolved in 2.5% agarose gels and stained with ethidium bromide. Four primers with motifs $(CCA)_5$, $(CTC)_5$, $(GTT)_5$ and $(CTGA)_4$ were selected for this study since they showed the highest diversity and uniqueness of some cultivars. Molecular weight marker VI (Boehringer Mannheim) was used to estimate the fragment sizes.

Data analysis

Clear and reproducible bands were scored as present (1) or absent (0). The data were statistically analyzed using the NTsys software package (Rohlf, 1997). Pairwise distance matrices were computed based on the Jaccard's coefficient of similarity (Sneath and Sokal, 1973). The distance matrices for the RAPD and ISSR data are shown in Tables 3 and 4, respectively. Separate dendrograms were created for the RAPD and ISSR data with the unweighted pair group method with arithmetic averaging (UPGMA) for cluster analysis (Rohlf, 1993).

RESULTS AND DISCUSSION

RAPD analysis

The genetic distance obtained from the RAPD data in this study ranged from 0.1 to 0.41 (Table 3). Previous studies showed that genetic distances among *Anthurium* cultivars ranged from 0.018 to 0.163 with a mean value of 0.09 (Nowbuth et al., 2005). Genetic distance values ranged from 0.74 to 0.91 among the 20 Anthurium cultivars in Brazil (Neto et al., 2008). Genetic distance co-efficient values for the 12 varieties of Anthurium ranged from 0.1851 to 0.7333 (Khan and Pankajaksan, 2010). The low genetic distance values obtained in this and other studies is ascribed to the close genetic relationship among *Anthurium* cultivars in Mauritius. It is known that most of the commercial *Anthurium* cultivars in Mauritius originated from only two countries, The Netherlands and Hawaii.

These plants were clonally propagated for many years preserving the crop's genetic identity. Low genetic distance values have also been recorded for other vegetatively propagated crops such as banana (Pillay et al., 2001), varieties of *Agave tequilana* (Gil-Vega et al., 2001; 2006) and sugarcane cultivars in India (Kawar et al., 2009).

Table 4. Matrix of genetic distances obtained from ISSR data among Anthurium cultivars.

|	Marian Seefurth	UH69	Carre	Acropolis	Paradiso	Midori	Nitta	Tropical	Norflora	Mado	Ozaki	Scorpio
Marian Seefurth	1.000											
UH69	0.556	1.000										
Carre	0.429	0.583	1.000									
Acropolis	0.519	0.444	0.480	1.000								
Paradiso	0.370	0.400	0.500	0.478	1.000							
Midori	0.571	0.615	0.481	0.519	0.370	1.000						
Nitta	0.654	0.783	0.444	0.429	0.440	0.593	1.000					
Tropical	0.444	0.609	0.458	0.440	0.455	0.560	0.652	1.000				
Norflora	0.577	0.560	0.480	0.520	0.478	0.640	0.600	0.565	1.000			
Mado	0.423	0.591	0.375	0.308	0.304	0.542	0.565	0.684	0.545	1.000		
Ozaki	0.536	0.708	0.500	0.429	0.440	0.593	0.615	0.583	0.481	0.565	1.000	
Scorpio	0.259	0.391	0.304	0.409	0.350	0.259	0.375	0.318	0.240	0.350	0.375	1.000
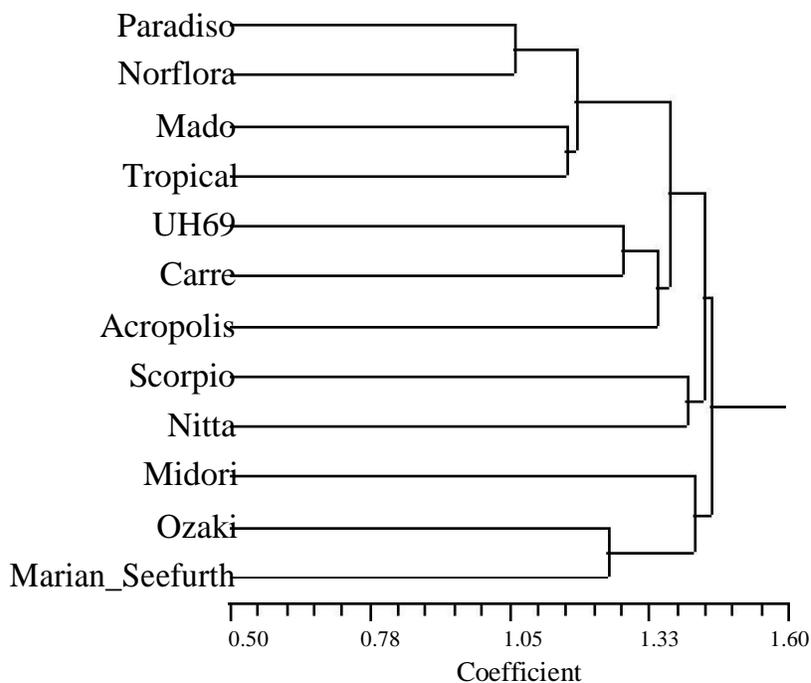
Figure 1. Dendrogram generated from RAPD data showing clustering of Anthurium cultivars.

The dendrogram derived from RAPD banding patterns (Figure 1) grouped the 12 cultivars into four clusters. The first cluster consisted of 'Paradiso', 'Norflora', 'Mado' and 'Tropical'. In this cluster, the two pairs of cultivars 'Paradiso' and 'Norflora', and 'Mado' and 'Tropical' shared a sister relationship. The second cluster consisted of 'UH69' and 'Carre' and 'Acropolis'; in this cluster 'UH69' and 'Carre' were closer than they were to 'Acropolis'. The third cluster consisted of the cultivars 'Scorpio' and 'Nitta' while the fourth cluster consisted of 'Midori', 'Ozaki' and 'Marian Seefurth'. In the latter cluster, Ozaki' and 'Marian Seefurth' were more closely related (Figure 1). The clustering of 'Midori' and 'Ozaki' is in agreement with the similarity of their morphological characteristics such as the spathe, spadix orientation, leaf venation as well as general leaf appearance. The only phenotypic character that separates the two cultivars is spathe colour. It appears that spathe color alone may not be a reliable character to group *Anthurium* cultivars since the dendrogram grouped cultivars with different spathe colors. For example, 'Marian Seefurth' (pink spathe) was found to be closer to 'Ozaki' (orange spathe) than to 'UH 69' (pink spathe) which in turn was found to be closer to 'Carre' (red spathe).
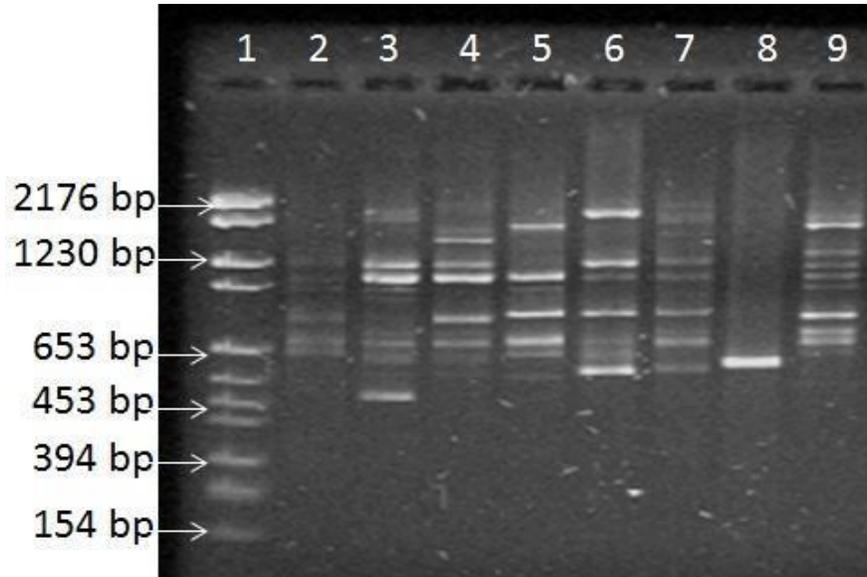
Figure 2. RAPD profiles obtained by amplifying nine cultivars of *A. andraeanum* with primer OPA 18. Cultivars and lanes numbers are represented as follows: molecular weight marker VI (1), UH 69 (2), Acropolis (3), Ozaki (4), Mado (5), Tropical (6), Carre (7), Scorpio (8), and Nitta (9).

Although the cultivars shared many common bands, some of the primers produced banding patterns that were cultivar specific. For example, the banding profiles produced with OPA 18 were able to differentiate a number of cultivars (Figure 2).

In Figure 2, 'Acropolis' (lane 3) could be distinguished from the rest of the cultivars by the presence of low molecular weight band of about 500 bp. This band was not present in any of the other cultivars and is diagnostic for 'Acropolis'. Similarly, the cultivar "Mado" (lane 5) did not possess the 1.53 kbp fragment that appeared in all the other cultivars except 'Nitta'. The cultivar 'Scorpio' (lane 8) produced a single diagnostic fragment of 520 bp and lacked the higher and lower molecular weight bands present in the other cultivars.

The four primers (AM7, B2, B11, and AN1) also produced unique fingerprints that were able to identify all the cultivars (data not shown). The amplification patterns obtained with primer B11 (Figure 2) produced unique DNA fingerprints for each cultivar (Table 4) and was able to differentiate the 12 cultivars (Figure 2). The four cultivars 'UH69', 'Marian Seefurth', 'Carre' and 'Acropolis' were distinctive in that only low molecular weight bands were observed. 'Acropolis' for example produced a typical three-band profile which was not seen in the others. This primer appeared to be most promising for cultivar identification in *Anthurium* (Figure 3).

ISSR

The genetic distances from the ISSR data ranged from

0.24 to 0.78 (Table 4). These values are much higher than those obtained with the RAPD data and may be due to the fewer primers used for the ISSR analysis.

The dendrogram obtained from the ISSR data produced three clusters. Cluster one was composed of two groups. One group consisted of the cultivars 'Marian Seefurth, 'Midori', and 'Norflora' while the second group consisted of the Hawaian cultivar 'UH 69', 'Nitta', 'Ozaki', 'Tropical' and 'Mado'. In group one 'Midori', and 'Norflora' formed a sister relationship while in group two 'UH69' and 'Nitta', and 'Tropical' and 'Mado' were sister groups. Cluster two comprised the cultivars 'Carre', 'Paradiso' that were more closely related and 'Acropolis'. A single cultivar 'Scorpio' made up the third cluster (Figure 4).

As expected, the cultivars shared a large number of alleles after amplification with the four ISSR primers used in this study. Despite this, the ISSR primers exhibited unique polymorphisms that were useful in distinguishing some of the cultivars readily. Our discussion will be based on one of the most useful microsatellite primer motifs $(CCA)_5$. Although both 'Marian Seefurth' and 'UH 69' are known to have identical morphological features (Table 1) with both cultivars having pink spathes, the two cultivars can be distinguished by their unique banding profiles as shown by the arrows in lanes 2 and 3 in Figure 5. For example, 'Marian Seefurth' (Figure 5, lane 2) can be distinguished from 'UH69' (Figure 5, lane 3) by the unique band at 896 bp. These bands are absent in 'Marian Seefurth'. In addition, one of the triple bands was present at the 1504 bp position in 'UH69' (Figure 5, lane 3 is absent in 'Marian Seefurth'. In summer, it is very difficult to differentiate between these two cultivars in
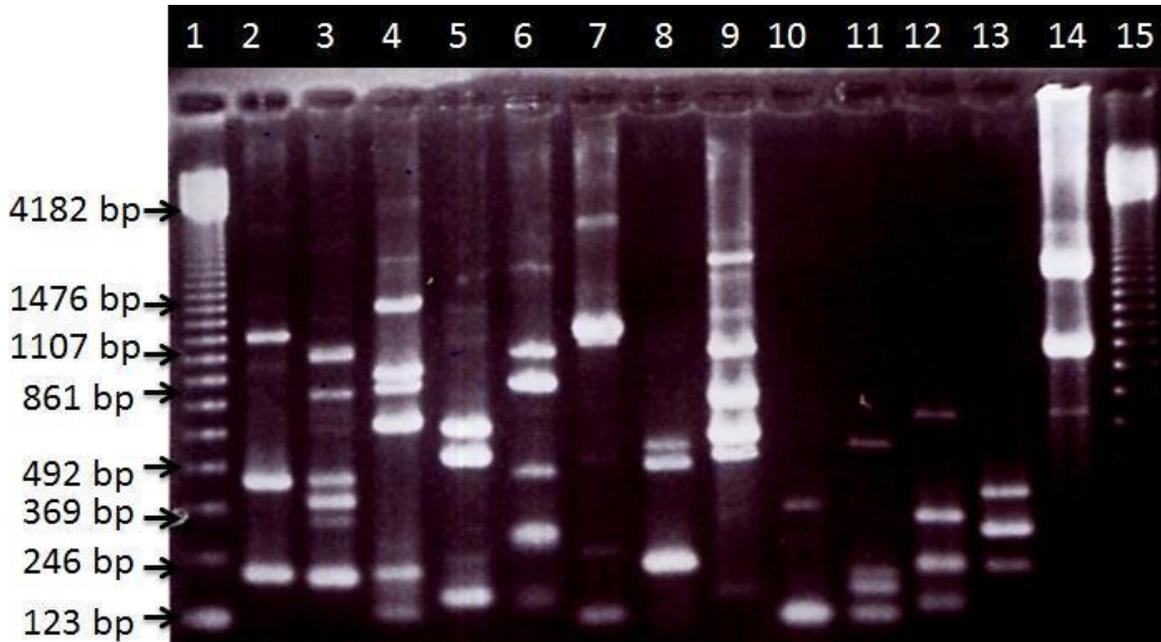
Figure 3. RAPD profiles obtained by amplifying 12 cultivars of *A. andraeanum* with primer B11. Cultivars and lanes numbers are represented as follows: 123 bp marker from Gibco (1, 15), Paradiso (2), Norflora (3), Mado (4), Midori (5), Tropical (6), Scorpio (7), Ozaki (8), Nitta (9), UH 69 (10), Marian Seefurth (11), Carre (12), Acropolis (13).
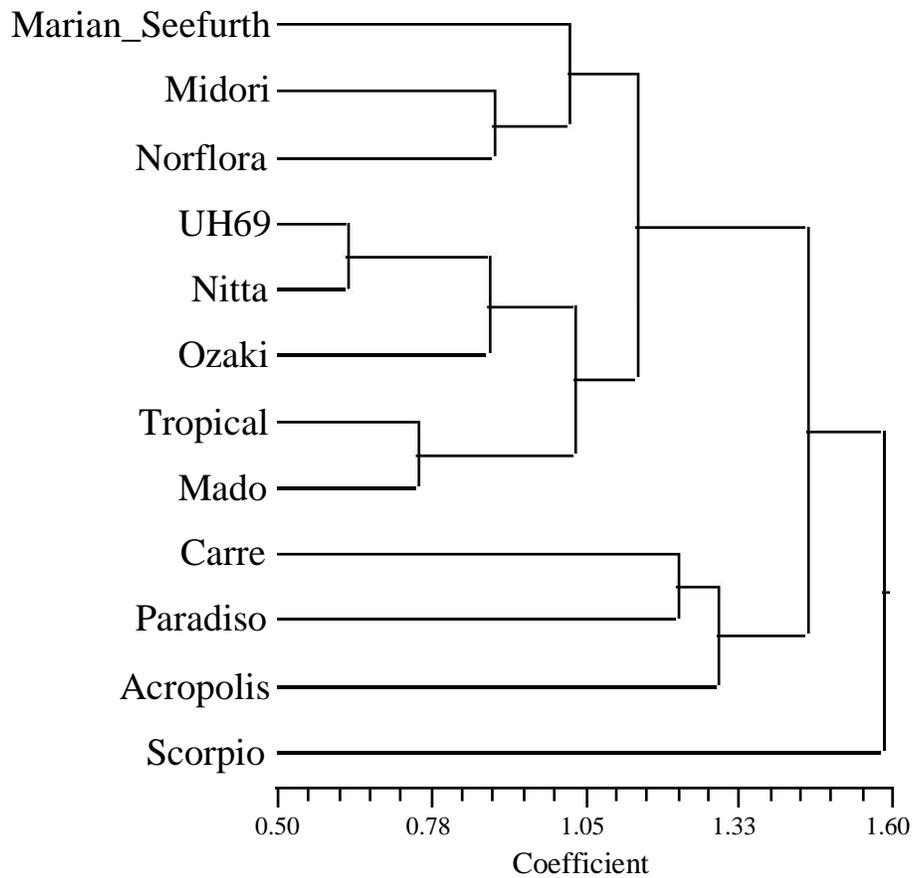

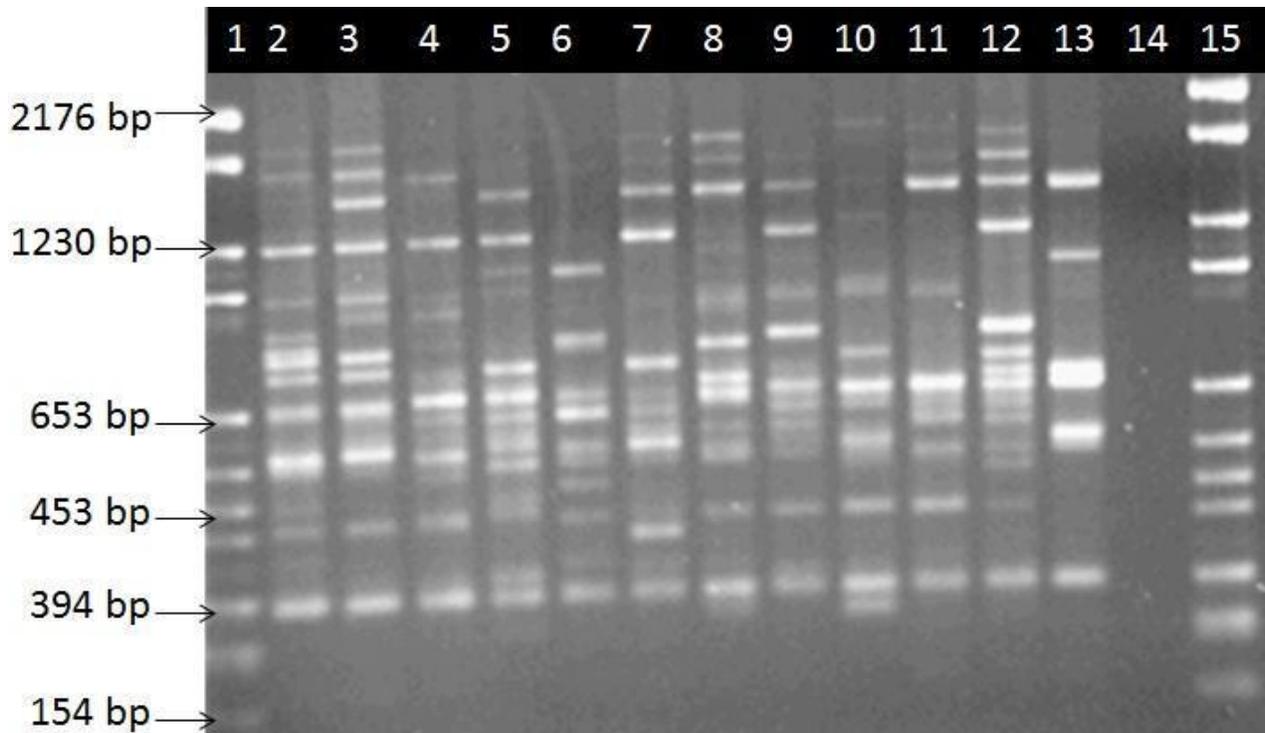**Figure 4.** Dendrogram generated from ISSR data.

Figure 5. Amplification patterns obtained with microsatellite motif (CCA)$_5$. The cultivars and lanes are represented by: marker VI (B.M.) (1, 15), Marian Seefurth (2), UH 69 (3), Carre (4), Acropolis (5), Paradiso (6), Midori (7), Nitta (8), Tropical (9), Norflora, (10), Mado (11), Ozaki (12), and Scorpio (13).

large plantations as 'Marian Seefurth' gets 'bleached' and appears similar in colour to 'UH 69'.

Similarly, the 'Carre' (Figure 5, lane 4) and 'Tropical' (Figure 5, lane 9) with red spathes had unique bands at 1700 bp and 896 bp, respectively that could different the cultivars at the molecular level. 'Norflora' (Figure 5, lane 10,) is the only cultivar that has a low molecular weight band of about 230 bp. The cultivars 'Nitta' (lane 8) and 'Ozaki '(lane 12) both with orange spathes could be distinguished from their unique banding patterns.

In Figure 5, 'Midori' could also be distinguished from 'Ozaki'. Producers of *A. andraeanum* indicated that differentiating between these two cultivars is problematic since they exhibit similar phenotypic characteristics such as spathe shape, spadix orientation and even leaf venation and were considered to be genetically close. However, the RAPD and ISSR-derived dendrograms (Figures 1 and 4) showed that the two cultivars are not genetically close.

Comparison between RAPD and ISSR dendrograms

One of the striking results obtained from this study is that the cultivars 'Mado' and 'Tropical' formed sister relationships with both the RAPD and ISSR data despite their morphological dissimilarities. The cultivar 'Tropical'

originated from The Netherlands while 'Mado' is regarded as a Mauritian cultivar. The close genetic relationship between the two cultivars and the absence of pedigree data suggests that 'Mado' was derived from 'Tropical' or *vice-versa*. While the RAPD data showed that 'Ozaki' and 'Marian Seefurth' were closely related, the ISSR data did not group these two cultivars closely. This anomaly needs further investigation. 'Scorpio' clustered with 'Nitta' with RAPD data, but remained as a single cultivar in a clade of its own with ISSR analysis.

To a certain extent, the clustering of the cultivars seemed to correspond with their geographic origin in both the RAPD and ISSR dendrograms (Figure 5, lane 9). For instance, the cultivars of Dutch origin 'Paradiso', 'Mado', and 'Tropical' appeared in the same cluster in the dendrogram (Figure 5) while 'Paradiso' and 'Acropolis', Tropical and 'Mado' appeared closer together in lane 9.

Similarly, Cultivars from Hawaii "Marian Seefurth' and 'Ozaki' were closely related in Figure 5 and while 'UH69', 'Nitta' and 'Ozaki' were grouped together in lane 9.

Conclusion

Phenotypic characterization of plants as used in describing *A. andraeanum* cultivars suffers from the influence of environmental factors on the morphological/

phenotypic descriptors. This problem is now being resolved by using molecular markers that are based on naturally occurring polymorphisms in DNA sequences. This study highlighted the importance of using RAPD and ISSR to distinguish cultivars of *A. andraeanum*.

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