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Full Length Research Paper

Assessing genetic diversity of some *Anthurium andraeanum* Hort. cut-flower cultivars using RAPD Markers

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Randomly amplified polymorphic DNA (RAPD) markers fingerprinting were used to assess the level of genetic variations among 24 cut-flower *Anthurium andraeanum* Hort. cultivars. Eight decamer primers produced a total of 98 reproducible PCR bands that were used to calculate the Nei and Li's genetic distance (GD_{NL}) coefficients amongst the cultivars. GD_{NL} values ranged from 0.018 to 0.163 with an average of 0.09 (representing an average genetic similarity of 91.34%). This significantly low average genetic distance among the various cultivars indicated that genetic variation among the cultivars was low. A dendrogram, produced using unweighted pair group method using arithmetic averages (UPGMA), grouped the cultivars into four main clusters. Cultivar 'Antartica' was genetically distinct from all the others. 'Midori' and 'Bourgogne' together formed a cluster whereas the remaining 21 cultivars grouped into two clusters and were closely related to each other. Clusters did not relate to cultivar provenance or origin and were independent of floral colour and spathe category. Finding correlations between these morphological traits to RAPD markers would necessitate extensive primer screening. Nevertheless, RAPD markers fingerprinting allowed a rapid assessment of the level of genetic variation that would otherwise be difficult to evaluate using the limited number of morphological markers present among these closely related anthurium cultivars.

Key words: *Anthurium andraeanum* Hort., genetic variation, genetic distance, RAPD, fingerprinting, UPGMA, dendrogram, spathe**.**

INTRODUCTION

Anthurium is possibly one of the most complex genera in the Araceae family and is reported to encompass approximately 1000 species (Croat, 1992; Matsumoto and Kuehnle, 1997). Numerous *Anthurium* species are produced and traded internationally as cut-flowers, flowering potted plants and landscape plants. Most of the cut-flower anthuriums are believed to be hybrids of *Anthurium andraeanum* Linden ex André with several closely related species in the section *Calomystrium* (Croat and Sheffer, 1983) and have been referred to as *Anthurium andraeanum* Hort. (Kamemoto and Kuehnle, 1996).

Mauritius is the third largest cut-flower anthurium producing nation in the world and supplies around 10.2 million blooms annually. Numerous anthurium cultivars exhibiting an array of spathe colours (ranging from red, orange, pink, coral and white) and belonging to the three spathe categories namely standard (single- coloured heart- shaped), obaki (bicolours of green with another major anthocyanin colour) and tulip-type are produced in order to satisfy the various market preferences in colours, shades, floral sizes and shapes. Due to the increasing demand for cut flowers in the world market, large

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numbers of novel anthurium cultivars are continually being imported from the Netherlands and Hawaii for commercial cultivation. Identification of all these different cultivars has become a major issue due to their similar plant morphologies. So far, cultivars have mainly been identified based on spathe colour (Kobayashi et al., 1987), which is seldom available at the juvenile stage of plant development.

Variation in isozymes banding patterns was found to be low among some cut flower anthurium cultivars indicating the limited utility of such protein markers for cultivar identification purposes (Kobayashi et al., 1987). Karyotypic studies were reported to be of little use in delineating cut-flower anthurium cultivars and hybrids (Marutani et al., 1993) . Morphological and protein markers in anthuriums can easily be affected by environmental factors and plant maturity (Kamemoto and Kuehnle, 1996). Moreover, such markers are known to be often similar in closely related ornamental cultivars and not effective for identification purposes (Jan De Riek, 2001).

RAPD fingerprinting techniques have been used for the identification of horticultural crop varieties, description of cultivar genotypes and for protecting breeder's rights (Williams et al., 1990; Camlin, 2001; Debener, 2001b).Using this technique, an unlimited number of polymorphic bands can be produced with relative ease from minute amounts of genomic DNA (Welsh and McClelland, 1990) enabling assay to be performed at any stage of plant development. RAPD markers have been extensively used to distinguish intraspecific genetic variation in ornamental crops and detection of hybrids and clones (Collins et al., 2003; Arus, 2000; Debener, 2001a). Ranamukhaarachchi et al. (2001) showed that RAPD markers had the ability to identify pot-plant anthurium cultivars.

Identification of locally developed anthurium cultivars has become imperative since it is required for the provision of plant breeders' rights and is presently high on the local government's agenda. Furthermore, an understanding of the level of genetic variation among anthurium cultivars is crucial to local breeders involved in hybridisation programs for varietal development but is currently lacking. In this study, RAPD markers were used to evaluate the extent of genetic variation among some anthurium cut flower cultivars.

MATERIALS AND METHODS

Plant material

Twenty-four *A. andraeanum* Hort. cut-flower cultivars were used in this study. Spathe colour and origin of the cultivar are indicated in brackets: 'Paradisio'(greenish pink, Holland), 'Midori'(glossy green, Holland), 'Mauna kea'(white obake, Hawaii), 'AC10'(orange, Hawaii), 'Fleur des îles'(salmon green, Mauritius), 'Bianca'(white, Mauritius), 'José' (red, Mauritius), ' Chloë' (pale pink, Mauritius), 'KF1'(red obake, France), 'Mickey mouse'(dark red obake, Hawaii), 'Rose' (pink, Mauritius), 'UH' (dark pink, Hawaii), 'Ozaki' (bright red, Hawaii), 'La coquille'(pale pink, Mauritius), 'Marcovie'(rose coral, Mauritius), 'Breton'(rose, Mauritius), 'Michelle'(salmon, Mauritius), 'Fantasia'(cream, Holland), 'Salsa'(light red, Holland), 'Marian Seefurth'(pink, Hawaii), 'Tango'(reddish pink, Holland), 'Antartica'(white, Mauritius), 'Nitta'(orange, Hawaii) and 'Bourgogne'(wine red, Mauritius). *Spathiphyllum* sp., a member of the Araceae family, was collected to serve as out-group. The youngest, not fully expanded leaf was collected for DNA extraction.

DNA extraction

About two grams of leaf tissue were ground into a fine powder using liquid nitrogen in a mortar. Total genomic DNA was extracted using the DNeasy® Plant Mini kit (QIAGEN, GmbH, Hilden, Germany) following the manufacturer's protocol. DNA quality was evaluated on a 1.0% agarose gel stained with ethidium bromide (Sambrook et al., 1998). Quantity was measured using GeneQuant *pro* RNA/DNA Calculator (Biochrom Ltd, Cambridge, England).

DNA amplification

Optimisation of PCR conditions and thermal cycling parameters were performed in preliminary experiments. Optimised PCR amplification mixtures contained 2.5 mM Mg^{2+}, 200 µM of each dNTP (Roche Diagnostics GmbH, Mannheim, Germany), 2.5 pmol decamer primer (Operon Technologies, Alameda, California, USA), 0.5 units HotstarTaq™ DNA polymerase (QIAGEN GmbH, Germany), 50 ng of template DNA, 1x PCR buffer, 250 ng BSA(Life Technologies, UK) and 1x Q solution all in a final volume of 25 µl. RAPD reactions were performed in a PTC 220 DNA Engine Dyad (MJ Research. Inc.,Waltham , MA, USA). The thermal cycling program used was: 94°C for 15 min; 3 cycles of 94°C for25 s 35°C for 25 s and 72°C for 2 min followed by 40 cycles of 94°C for 25 s, 37°C for 25 s and 72°C for 2 min. Following a final extension of 72°C for 7 min, reactions were ended with an indefinite hold at 4°C. Negative control containing the amplification mixture without template DNA was also included to check the reproducibility and accuracy of PCR reactions. A 100 bp ladder (DNA molecular weight marker XIV, Roche Diagnostics, Germany) was loaded on each side of the gel for calculations of fragment size. Amplified DNA fragments were mixed with 10x loading buffer (20% w/v Ficoll 400, 0.1M EDTA, 1.0% SDS, 0.25% w/v Bromophenol blue) and separated on 1.8% (w/v) agarose gels (Agarose MP, Roche Diagnostics GmbH, Germany) in 1x TAE buffer. Gels were run for 2 h at 100 V, stained with ethidium bromide (0.4 µg ml^{-1}) for 45 min, washed in distilled water for 1.0 h and photographed on a UV transilluminator.

Primer screening

Initially, a total of fifty-two RAPD primers (Operon Technology Inc.; OPAJ 01-20, B 01-20 and OPAK 01-12) was screened with DNA from a subset of five arbitrarily chosen anthurium cultivars (Bourgogne, Antartica, Midori, Michelle and Nitta). Primers producing a fair number of normally distributed and well separated bands and giving the largest number of polymorphism were selected and used to amplify all the cultivars. Out of the 52 primers, eight primers that produced the most polymorphic bands were selected to fingerprint all the cultivars. Amplifications were repeated and consistent markers were included in the analysis.

Data analysis

Sizes of bands were estimated by comparison with the 100 bp DNA ladder. Fragments of identical size amplified with the same primer

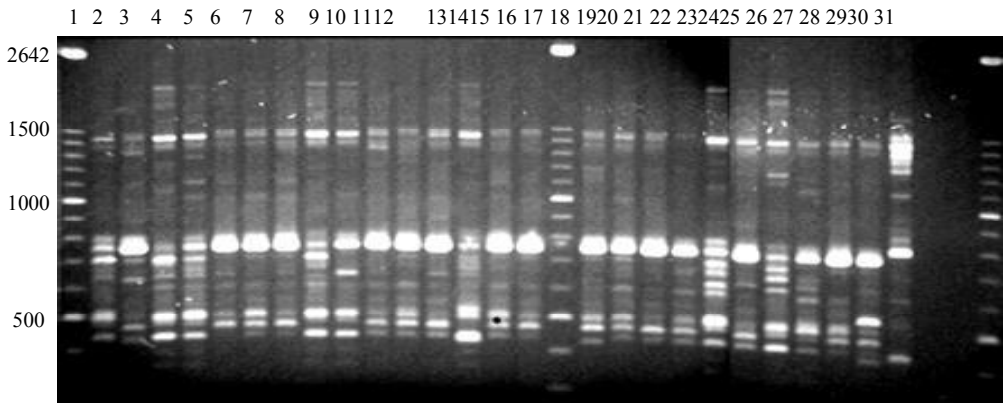

Figure 1. RAPD profiles obtained by amplifying 24 cultivars of *A. andraeanum* cultivars and *Spathiphyllum* species with primer OPAK 11. Lanes 1 to 31 are as follows: 100 bp DNA weight ladder (lane 1), *Paradiso* (lane 2), *Midori* (lane 3), *Mauna Kea* (lane 4), *AC 10* (lane 5), *Fleur des Iles* (lane 6), *Bianca* (lane 7) , *José* (lane 8), *Chloé* (lane 9), *KF1* (lane 10), *Mickey Mouse* (lane 11), *Rose* (lane 12), *UH* (lane 13), *Ozaki* (lane 14), *La Coquille* (lane 15), *Marcovie* (lane 16), 100bp DNA weight ladder (lane 17), *Breton* (lane 18) , *Michelle* (lane 19), *Fontasia* (lane 20), *Meringue* (lane 21) , *Salsa* (lane 22), *Marian Seefurth* (lane 23), *Tango* (lane 24), *Antartica* (lane 25), *Nitta* (lane 26), *Bourgogne* (lane 27), *Spathiphyllum* (lane 28) negative control with no DNA template (lane 29), negative control with no primer (lane 30)and 100 bp DNA weight ladder (lane 31).

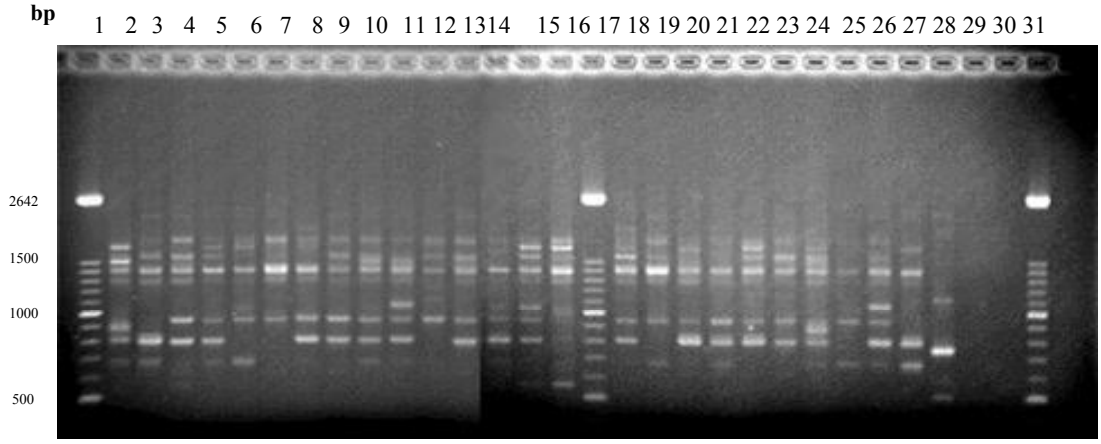

Figure 2. RAPD profiles generated by amplification of 25 cultivars of *A. andraeanum* and one *Spathiphyllum* species with primer BO2. Lanes 1 to 31 are as follows: 100 bp DNA weight ladder (lane 1), *Paradiso* (lane 2), *Midori* (lane 3), *Mauna Kea* (lane 4), *AC 10* (lane 5), *Fleur des Iles* (lane 6), *Bianca* (lane 7), *José* (lane 8), *Chloé* (lane 9), *KF1* (lane 10), *Mickey Mouse* (lane 11), *Rose* (lane 12), *UH* (lane 13), *Ozaki* (lane 14) , *La Coquille* (lane 15), *Marcovie* (lane 16) , 100 bp DNA weight ladder (lane 17), *Breton* (lane 18), *Michelle* (lane 19), *Fontasia* (lane 20), *Meringue* (lane 21), *Salsa* (lane 22), *Marian Seefurth* (lane 23), *Tango* (lane 24), *Antartica* (lane 25), *Nitta* (lane 26), *Bourgogne* (lane 27), *Spathiphyllum* (lane 28), negative control with no DNA template (lane 29), negative control with no primer (lane 30) and 100 bp DNA weight ladder (lane 31).

were considered to be the same DNA marker corresponding to one locus. For dominant markers like RAPDs, only two states (1 for present and 0 for absent) were distinguished at each band position. Scores for all cultivars were assembled into a binary matrix for total RAPD bands.

Nei and Li (1979) genetic distance coefficients (GD_{NL}) for all pairwise combinations of cultivars were generated from the binary matrix using PAUP software, version 4.0 (Swofford, 2000), and a genetic distance matrix was assembled. GD_{NL} measures the proportion of bands shared as the result of being inherited from a common ancestor (Mohammadi et al., 2003). A dendrogram showing the patterns of genetic relationship among the cultivars was constructed from the GD_{NL} coefficients using an unweighted paired group method using arithmetic averages (UPGMA) clustering option in PAUP 4.0b. Genetic similarities among cultivars were computed as [1-genetic distance] x 100% (Nei, 1987).

RESULTS AND DISCUSSION

The largest fragment amplified was in the range of 1500 to 2600 bp while the smallest but easily recognisable fragment was approximately of 300 bp. Most bands were concentrated between 500 to 1500 bp. Operon primers OPAK 11 (Figure 1) and BO2 (Figure 2) generated the largest number of bands compared to the others. The

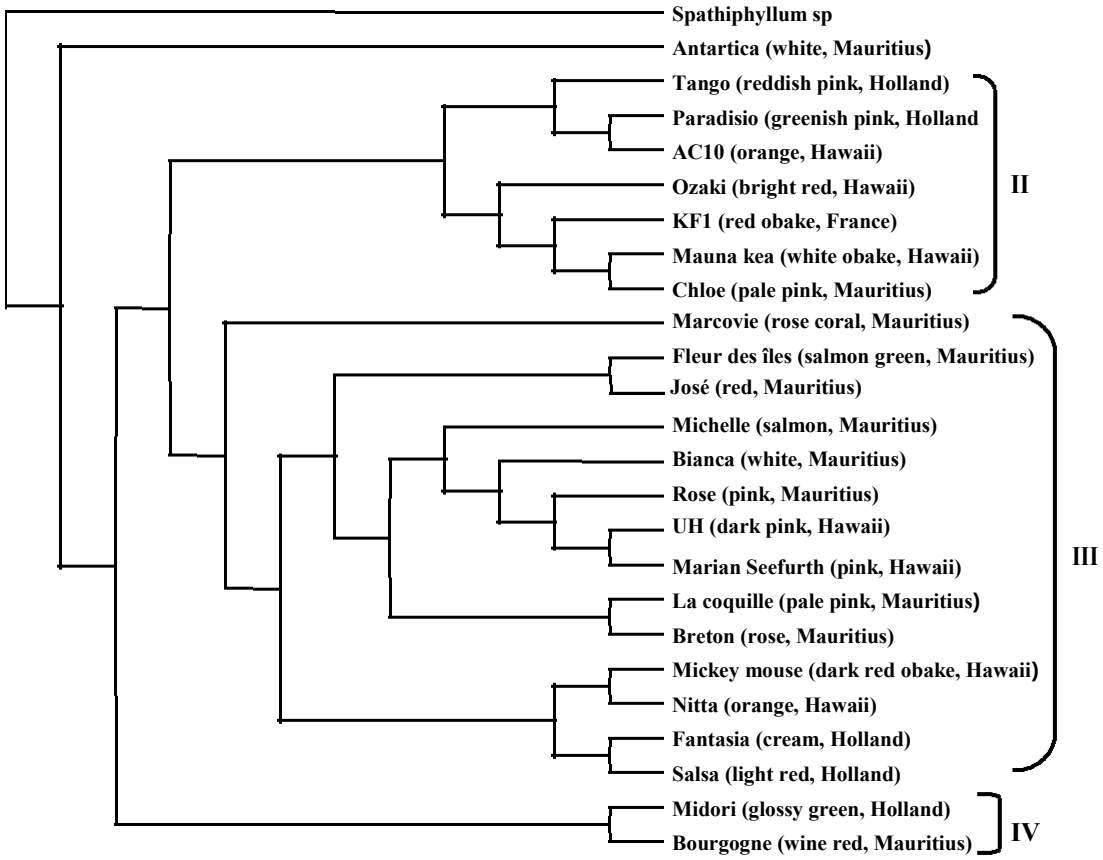

Figure 3. Dendrogram showing the relationships among the 24 *A. andraeanum* Hort. cultivars. UPGMA cluster analysis based on Nei and Li's genetic distance coefficients in PAUP* software was used to generate the dendrogram. *Spathiphyllum* sp. was used as an outgroup. Roman numerals indicate clusters. Spathe colours and country of provenance are indicated in bracket next to the respective cultivar.

number of bands scored for each primer varied from 4 to 16. The mean number and size of bands produced per primer for cut flower anthurium cultivars were comparable to those obtained by Ranamukhaarachchi et al (2001) with pot plant anthurium species. RAPD fingerprint patterns among cut-flower cultivars were similar indicating a high degree of genetic similarity.

Analysis of Genetic distance

Nei and Li genetic distance (GD_{NL}) coefficient values ranged from 0.018 to 0.163, with a mean value of 0.09. The low GD_{NL} values indicated that these anthurium cultivars were closely related to each other and resulted in their close clustering in the dendrogram (Figure 3). Two University of Hawaii releases namely 'UH' and 'Marian Seefurth' were separated by a GD_{NL} value of 0.018 (98.2 %genetic similarity).

The 24 *Anthurium* cut-flower cultivars grouped into four clusters in Figure 3. Locally developed cultivar 'Antartica' with white standard type spathes, resolved separately from the remaining anthurium cultivars. Cluster II grouped seven cultivars namely 'Tango', 'Paradisio', 'AC10', 'Ozaki', 'KF1', 'Mauna Kea' and 'Chloë'. Fourteen cultivars including 'Marcovie', 'Fleur des Iles', 'José', 'Michelle', 'Bianca', 'Rose', 'UH', 'Marian Seefurth', 'La coquille', 'Breton', 'Fantasia', 'Salsa', 'Mickey mouse' and 'Nitta' formed cluster III whereas 'Midori' and 'Bourgogne' formed the last cluster.

In general, cultivars related by pedigree were expected to cluster together in the dendrogram and share higher levels of varietal relationship. Pedigree information of many local varieties, however, was either untraceable or unavailable. Groupings in the dendrogram were found to be independent of cultivar provenance since Hawaiian varieties were interspersed with Dutch and Mauritian cultivars. Moreover, cultivars did not cluster together based on floral colour and spathe category. Cultivars with

standard spathe (i.e. with single-coloured spathe like 'Ozaki', 'Nitta' and 'Marian Seefurth') clustered with obakis varieties (bi-colours of green with another major spathe colour like 'Mickey mouse' and 'Mauna kea'). Finding correlations between agronomic characters like flower colour and spathe category with RAPD groupings at this stage were expected to be difficult since phenol-typic traits sample a very small region of the genome whereas RAPD markers are samples of DNA often originating from non-coding sequences that are randomly distributed throughout the genome (Dahlberg et al., 2002). Using a larger number of RAPD primers would increase genome coverage thus increasing the probability of identifying such phenotypic markers.

Numerous studies have supported the presence of a significantly high level of similarity among anthurium species and cultivars. Meiotic analyses (Sheffer and Croat, 1983; Marutani et al., 1993) indicated that most cut flower anthurium cultivars exhibited a high percentage of normal tetrads. Moreover, remarkably similar karyotypes of some representative cut flower anthurium hybrids and species indicated them to be very closely related (Marutani et al., 1988, 1993). Cross compatibility studies between *A. andraeanum* Hort. cultivars with numerous *Anthurium* species of the *Calomystrium* section further indicated their very close relationship (Marutani et al., 1988; Kamemoto and Kuehnle, 1996). Ranamukhaarachchi et al. (2001) showed the presence of a low level of genetic variability among flowering potted plant anthurium species using RAPD molecular fingerprinting.

Over the years, an intensive drive towards protection of new plant varieties has been noted. Although RAPD markers are known to be generally more discriminating than morphological traits, more powerful and reliable markers are required for accurate identification of anthurium cultivars to detect infringements and prevent frauds. With a higher multiplex ratio (the number of information points analysed per experiment) than RAPD, amplified fragment length polymorphism (AFLP) technique (Vos et al., 1995) has proven to be extremely effective in unequivocally distinguishing closely related vegetatively propagated horticultural genotypes and thus found suitable for assisting in protection of plant breeders rights (Xiang et al., 2003; Debener et al., 2004).

The genetic variability accessible in a gene pool is normally considered as being the major resource available to breeders (Ramanatha Rao and Hodgkin, 2002). The significantly low level of genetic variability detected among these cut flower anthurium cultivars highlighted the limited potential for cultivar improvement. Rapid replacement and elimination of numerous locally developed hybrids with commercially successful varieties have resulted in a gradual genetic erosion of the local anthurium gene-pool. Efficient handling of this low variability has thus become crucial for the success of the local anthurium industry. It is therefore imperative to collect and conserve older obsolete varieties which can eventually be accessed and used in future hybridisation events. Broadening the genetic base of local anthurium gene pool through the adoption of the introduced varieties in on-going hybridisation programs may represent a way to increase genetic variability.

In conclusion, RAPD markers have successfully been used to fingerprint and assess the extent of genetic variation among anthurium cultivars. Cultivars were found to be very closely related to each other. Knowledge on genetic diversity will help in the efficient management of anthurium germplasm by breeders.

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