The Chloroplast psbK-psbI Intergenic Region, a Potential Genetic Marker for Broad Sectional Relationships in Anthurium

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Abstract. Nuclear and chloroplast genetic markers have been extensively used for plant identification and molecular taxonomy studies. The efficacy of genetic markers to be used as DNA barcodes is under constant evaluation and improvement with identification of new barcodes that provide greater resolution and efficiency of amplification for specific species groups as well as distantly related plants. In this study, chloroplast DNA genetic markers for Anthurium, the largest genus in the Araceae family, were adapted from chloroplast markers previously designed for Lemma minor, a member of the same plant family. Primers for chloroplast region trnH-psbA, previously used for molecular systematic studies in Anthurium, as well as primers for the rpoB, rpoC1, psbK-psbI, matK, rbcL, and atpF-atpH regions, all located within the large single copy sequence in the chloroplast genome, were evaluated and found to efficiently amplify target sequences when using DNA of varied quality and concentration extracted from silica-dried leaves of selected accessioned species of Anthurium. The trnH-psbA, psbK-psbI, and atpF-atpH intergenic region primers were further evaluated using Anthurium species spanning different subgeneric groups. Of the intergeneric region primers tested, psbK-psbI primers were the most robust, yielding well-defined amplicons across Anthurium species that were consistent, with exceptions, within sectional groupings. Application of the psbK-psbI region amplicon as a visual marker for surveying sectional relationships in Anthurium is novel and serves as a model for the development of a diagnostic method for genotyping plants and testing for sample integrity from among species or germplasm collections. This work further demonstrates the use of dried plant tissue banks as a genetic reference and information resource to support basic research as well as ornamental plant characterization and improvement.

Research at the USDA ARS Pacific Basin Agricultural Research Center (PBARC) Tropical Plant Genetic Resources and Disease Research unit in Hilo, HI, includes support for the improvement of tropical plant ornamental and fruit crops, including cut and potted Anthurium for the Hawaiian floral industry. Anthurium are the top cut flower in Hawaii with sales in 2012 of 348,000 dozen flower stems valued at $3.34 million and 35,000 potted Anthurium were sold for a value of $267,000 (USDA, National Agriculture Statistics Service and Hawaii Department of Agriculture Agricultural Development Division, 2013). The genus Anthurium, the largest of the Araceae family, is comprised of ≈905 described species that range from southern Mexico to northern Argentina (Carlson, 2011; Govaerts et al., 2014), but based on the number of estimated new species to be described, this figure may increase to 1500 taxa (T.B. Croat, unpublished data; Boyce and Croat, 2012). Many modern commercial Anthurium cultivars are derived from interfertile hybrids, mainly from species within section Calomystrium, including A. andraeanum, A. amnicola, A. antioquiense, A. antrophyoides, A. armeniense, A. formosum, A. hoehmannii, A. kamemotoanum, and A. nymphaeifolium, as well as hybrids between species within the sections Porphyrochitonium and Cardiolonchium, and intersectional hybrids between Calomystrium and Cardiolonchium, among others (Kamimoto and Kuchhele, 1996). One of the ongoing research goals at PBARC is the development of molecular resources to identify origins of ornamental and horticultural diversity at the genome and molecular biological levels (Bliss and Suzuki, 2012) to support improvement of cultivars through breeding and biotechnological applications (Matsumoto et al., 2013).

In tropical ornamentals, including Anthurium, very few tools, simple or complex, for molecular evaluation of germplasm are available. Chloroplast gene markers have been used extensively for molecular identification in plants, although their use for species-level resolution is dependent on the genes used and the particular family of plants being studied (Shaw et al., 2005, 2007). Sequence analysis of the nuclear CHS gene intron and the chloroplast trnG intron, trnH-psbA, and trnC-ycf6 intergenic spacers in Anthurium has recently resulted in the reclassification and improved understanding of the various subgeneric sections and species relationships in this genus (Carlsen, 2011; Carlsen and Croat, 2013; Croat and Carlsen, 2013). This is the most comprehensive molecular phylogeny of Anthurium to date, providing a contemporary framework for identifying species, their origins, interrelationships, evolution, and diversification. Lemmaeae-specific chloroplast DNA markers for the rpoB, rpoC1, matK, and rbcL gene regions, and the trnH-psbA, atpF-atpH, and psbK-psbI intergenic regions were recently developed (Wang et al., 2010) based on a recommendation by the Consortium for the Barcode of Life (CBOL) (CBOL Plant Working Group, 2009), which allowed evaluation of DNA barcoding markers for species identification in this family.

In this study, we further explored the marker genes used in Lemma to supplement those presently used for molecular systematic studies in Anthurium as a result of their relatively close phylogenetic relationship (Rothwell et al., 2004). Although we did not have access to a large collection of live accessioned species of Anthurium, dried leaf material of species from previously identified collections was obtained from various sources and stored on silica. The goal of this study was to determine the appropriateness of using dried leaf material as the source of amplifiable DNA and for further development of diagnostic gene markers. Approaches for applying known phylogenetic information for practical diagnostic genotyping of Anthurium species or germplasm in collections were also investigated.

Materials and Methods

DNA extraction from Anthurium plant leaves. Anthurium leaves from various sources that had been stored in silica at ambient temperature were used as initial material for DNA extractions. Approximately 20 mg of leaf dry weight was placed into Lysing Matrix D tubes (MP Biomedicals) and homogenized for 20 s in MP Fast Prep-24 Buffer PL1. RNaseA obtained from NucleoSpin® Plant II kit (Macherey Nagel USA, Bethlehem, PA) was then added to each tube following recommended volumes and the mixture was homogenized for an additional 20 s. The contents of each tube were then centrifuged and transferred to a NucleoSpin® filter placed in a collection tube and purified using NucleoSpin® Plant II following the manufacturer’s recommendations. DNA yields were 5.3 to 347 ng·μL⁻¹ in a total volume of ≈50 μL.

Primer design. Anthurium chloroplast barcode gene marker primers were adapted from the Lemma minor chloroplast DNA barcode primer sequence (Wang et al., 2010) and target Anthurium polymerase chain reaction (PCR) product lengths estimated using sequences obtained from high-throughput sequencing of chloroplast-enriched DNA from...
A. andraeana Hort. ‘New Pahoa Red’ (Suzuki, unpublished data). Primers were obtained from Eurofins, MWG/Operon (Huntsville, AL) with primer Tm values based on the formula \[ T_m = 81.5 + 16.6 \log(0.1) + 41 \times \frac{G+C}{length} - 500/length \] provided by the manufacturer. Of the various target genes, rpoB, rpoCl, rbcL, and matK are coding genes, encoding the beta and N-terminus of the beta’s subunit, respectively, of the plastid RNA polymerase, the large subunit RuBisCO and matK, respectively. The remaining marker gene sites trnH-psbA, atpF-atpH, and psbK-psbl are intervening sequences between the transfer RNA H and photosystem II, D1 protein gene, adenosine-5’-triphosphate synthase subunit genes, and photosystem II reaction center protein genes, respectively.

Polymerase chain reaction amplification of DNA marker regions for Anthurium species. PCR reactions consisted of 25 μL 1× GoTaq Hot Start Master mix (Promega Corp., Madison, WI), 1 μM each of forward and reverse gene primers, and 1 μL of extracted DNA. Reactions were carried out by incubation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s, and a final extension of 72 °C for 5 min. PCR products were run in 2.0% agarose-1× Tris Borate EDTA gels containing 1× GelRed Nucleic Acid Stain (Phenix Research Products, Candler, NC). TrackIt™ 1.0Kb Plus DNA ladder (Life Technologies, Carlsbad, CA) was used as a molecular weight marker.

Results and Discussion

Polymerase chain reaction (PCR) primers specific to Anthurium were obtained by comparing primers previously designed for Lemma with a corresponding DNA sequence of the chloroplast genome of Anthurium andraeana Hort. ‘New Pahoa Red’, a popular red-spouted Hawaiian cut flower. Anthurium ‘New Pahoa Red’ chloroplast DNA sequence obtained from high-throughput sequencing of chloroplast-enriched DNA from this cultivar enabled the design of Anthurium-specific primers and prediction of target amplicons sizes (Table 1). PCR primer sequences for rpoB and atpF-atpH were unchanged between Lemma and Anthurium, whereas sequence differences were found between target L. minor and A. andraeana. Primer Tm was based on a general formula provided by the manufacturer (see “Materials and Methods”).

DNA was extracted from dried leaves of species representing 14 sections and two clades of the currently recognized 18 subgeneric sections of Anthurium (Croat and Sheffer, 1983) and additional newly recognized clades (Carlsen, 2011; Carlsen and Croat, 2013) (Table 2). Although Anthurium leaves were stored for greater than 2 years on silica at ambient temperature, with a few exceptions, relatively pure DNA as judged by spectrophotometric values (OD260/280 ~1.8) was obtained using a commercial plant DNA isolation kit. However, DNA yield did vary considerably (5 to 347 ng·μL−1), which we attribute to differences in the physical properties of the leaves that might have affected proper tissue preservation at the initial stages of the silica drying process and/or parameters affecting DNA extraction and quality such as

Table 1. Anthurium species chloroplast gene marker primers adapted from Lemma minor chloroplast DNA barcode primer sequences (Wang et al., 2010) based on A. andraeana Hort. ‘New Pahoa Red’ chloroplast DNA sequences. *

| Target gene regions | Lemma minor amplicon (bp) | Anthurium andraeana amplicon (bp) | Primers | Primer sequence | Primer length | Primer Tm |
|---------------------|---------------------------|----------------------------------|---------|-----------------|---------------|----------|
| trnH-psbA           | 300                       |                                  | *Aa.trnH-psbA.F | 5’-GTGATGCACTAGAAGCTGTC-3’ | 22            | 60.8     |
|                     |                           |                                  | Lm.trnH-psbA.F  | 5’-GTATGCAAGGAATGTTGTC-3’  | 23            | 68.1     |
|                     |                           |                                  | LmAa.trnH-psbA.R| 5’-GGGGGTTGTTGATCTTAC-3’  | 25            | 67.9     |
|                     |                           |                                  | LmAa.rpoB.F     | 5’-CTGGATGTTGAAAGAGATA-3’  | 21            | 54.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-GGCGAAGAGGGGAAGATCCG-3’ | 20            | 62.4     |
| rpoB                | 406                       | 426                              | *Aa.rpoC1.F     | 5’-GGCGAAGAGGGGAAGATCCG-3’ | 22            | 57.1     |
|                     |                           |                                  | LmAa.rpoB.F     | 5’-GGGAAAGGAAAGAGGCC-3’    | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 51.5     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 20            | 60.4     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 26            | 61.4     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 21            | 64.5     |
| psbK-psbl           | 544                       | 541                              | *Aa.psbK-psbL.F | 5’-GGCGAAGAGGGGAAGATCCG-3’ | 23            | 55.6     |
|                     |                           |                                  | LmAa.psbK-psbL.R| 5’-GGCGAAGAGGGGAAGATCCG-3’ | 23            | 55.6     |
|                     |                           |                                  | LmAa.psbK-psbL.R| 5’-GGCGAAGAGGGGAAGATCCG-3’ | 23            | 55.6     |
|                     |                           |                                  | LmAa.psbK-psbL.R| 5’-GGCGAAGAGGGGAAGATCCG-3’ | 23            | 55.6     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 20            | 62.4     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 21            | 64.5     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 22            | 57.1     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 20            | 62.4     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 21            | 64.5     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 22            | 57.1     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |
|                     |                           |                                  | LmAa.rpoB.R     | 5’-AAATGTTGAGAAAGATCCG-3’  | 19            | 55.8     |

* DNA bases shown in bold denote base pairs that differ between Anthurium (*Aa.xx*) and L. minor (*Lm.xx*) primers; LmAa.xx denotes primer sequences identical at the corresponding target gene regions of L. minor and A. andraeana. Primer Tm was based on a general formula provided by the manufacturer (see “Materials and Methods”).
| Subgeneric section | Sample no. | Species | Reference | Accession |
|--------------------|------------|---------|-----------|-----------|
| Calomystrium       | Calo 1     | Anthurium amnicola | Carlsen and Croat, 2013; | ABG 1991 1372 |
|                    | Calo 2     | Anthurium amnicola | Carlsen and Croat, 2013; | MSBG 1976-0053-002A |
|                    | Calo 3     | Anthurium amnicola | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 4     | Anthurium andraeanum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 5     | Anthurium andraeanum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 6     | Anthurium antioquiense | Carlsen and Croat, 2013; | HAIA |
|                    | Calo 7     | Anthurium antioquiense | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 8     | Anthurium antioquiense | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 9     | Anthurium armeniense | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 10    | Anthurium armeniense | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 11    | Anthurium emeraldense | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 12    | Anthurium formosum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 13    | Anthurium hoffmannii | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 14    | Anthurium hoffmannii | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 15    | Anthurium ochranthum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 16    | Anthurium ochranthum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 17    | Anthurium ochranthum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Calo 18    | Anthurium ochranthum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Card 1     | Anthurium cerrocampanense | MSBG 1976-0053-002A | MBG 84952 |
|                    | Card 2     | Anthurium cerrocampanense | MSBG 1976-0053-002A | MBG 84952 |
|                    | Card 3     | Anthurium magnificum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Card 4     | Anthurium ochranthum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Card 5     | Anthurium ochranthum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Card 6     | Anthurium ravenii | MSBG 1976-0053-002A | MBG 84952 |
|                    | Card 7     | Anthurium ravenii | MSBG 1976-0053-002A | MBG 84952 |
|                    | Card 8     | Anthurium warocqueanum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Card 9     | Anthurium warocqueanum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Card 10    | Anthurium warocqueanum | MSBG 1976-0053-002A | MBG 84952 |
|                    | Dac 1      | Anthurium clavigerum | Carlsen and Croat, 2013; | ABG 1991 1372 |
|                    | Dac 2      | Anthurium clavigerum | Carlsen and Croat, 2013; | MSBG 1991-0174A |
|                    | Dac 4      | Anthurium clavigerum | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Dac 5      | Anthurium kunthii | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Dec 1      | Anthurium pittieri | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Dig 1      | Anthurium lentii | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Gym 1      | Anthurium gymnopus | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Lep 1      | Anthurium gracile | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Lep 2      | Anthurium gracile | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Lep 3      | Anthurium gracile | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 1      | Anthurium guatemalense | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 2      | Anthurium llewelynii | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 3      | Anthurium salvini | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 4      | Anthurium schlechtendali | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 5      | Anthurium schlechtendali | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 6      | Anthurium schlechtendali | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 7      | Anthurium schlechtendali | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 8      | Anthurium solitarium | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 9      | Anthurium solitarium | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 10     | Anthurium sparreorum | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 11     | Anthurium upalaense | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 12     | Anthurium watermaliense | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 13     | Anthurium watermaliense | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Pac 14     | Anthurium watermaliense | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Poly 1     | Anthurium clemensioides | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Poly 2     | Anthurium clemensioides | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Poly 3     | Anthurium flexile | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
|                    | Por 1      | Anthurium bakeri | Carlsen and Croat, 2013; | MIA 36496, USDA ARS |
tissue maceration efficiency and presence of secondary compounds.

Extracted DNA of species from the section *Calomystrium* was chosen as a small sample set to test for PCR template quality and the efficiency of the various primers to amplify the target gene fragments studied here. These species were chosen for initial tests, because a large majority of the commercial hybrids of *Anthurium* derive from crosses between species in section *Calomystrium*, and we have ongoing interest in developing approaches for delineating their similarities and differences at the molecular level. Moreover, it would be predicted that the target chloroplast sequences of species sampled in this section would likely be conserved with corresponding sequences of *A. andraeanum* Hort. ‘New Pahoa Red’, a member species of section *Calomystrium*.

Previously, the *trnH-psbA* region was used for molecular systematic studies across a large number of widely related *Anthurium* species to reconstruct the molecular phylogeny of the genus (Carlsen, 2011; Carlsen and Croat, 2013) and the PCR primers designed in our study for this marker gene also resulted in efficient amplification from both the New Pahoa Red cultivar as well as other species in section *Calomystrium* (Table 2; Fig. 1A). In general, other PCR primer pairs tested were efficient in amplifying amplicons of expected size across species tested in this subgeneric *Anthurium* group (Fig. 1A–G) with amplicons close to the predicted marker gene amplicon size for ‘New Pahoa Red’ chloroplast DNA sequence (Table 1) based on migration in agarose gels relative to DNA molecular weight marker bands: *trnH-psbA* (300 to 400 bp), *rpoB* (400 to 500 bp), *rpoC1* (500 to 650 bp), *psbK-psbI* (500 to 650 bp), *rbcL* (500 to 650 bp), *atpF-atpH* (650 to 850 bp), and *matK* (850 to 1000 bp) (Fig. 1A–G). Amplicons were not visible in PCR reactions containing the samples ‘Calo 8, 9, and 15’ using primers representing *A. antioquiense*, *A. armeniaca*, and *A. nymphaefolium*; primers for *atpF-atpH* and ‘Calo 9 and 15’ using primers for *matK*, not readily visible for samples ‘Calo 2’ and ‘Calo 7’, primers for *atpF-atpH* representing one of three accession of *A. annicola*, and a second of three accessions of *A. antioquiense*. For samples ‘Calo 9’ and ‘Calo 15’ with DNAs OD$_{260/280}$ ≥ 1.45 or less and calculated DNA concentrations of 41.9 and 35.3 ng·µL$^{-1}$, respectively, low or no amplification was likely as a result of suboptimal DNA template quality because similar lower amplification efficiency was observed for these samples using other primer sets. These results demonstrate that the PCR primers were effective in amplifying the target gene amplicons from DNA of different *Anthurium* species and, in addition, the tissue storage and DNA extraction methods used were in general effective methods to obtain amplifiable DNA for this application.

According to Wang and collaborators (Wang et al., 2010), PCR products from the coding genes *rpoB*, *rpoC1*, *rbcL*, and *matK* were identical in product length across the 31 species tested from the subfamily Lemnaceae, whereas the intergenic sites *trnH-psbA*, *psbK-psbI*, and *atpF-atpH* varied considerably in amplicon size as a result of insertions/deletions. The *psbK-psbI* primers exhibited the highest amplification rate among different *Lemna* species and the most variability between species. Although intraspecies variability was high using *psbK-psbI* primers, it was less than that of *trnH-psbA*, a region that is often variable in length among different species or samples owing to its position at the junction of the chloroplast large single copy sequence and the inverted repeat (Wang et al., 2008). Based on these observations and our goal of identifying visible species markers, we tested primers for intergeneric sites *trnH-psbA*, *psbK-psbI*, and *atpF-atpH* with our collection of DNA extracted from various *Anthurium* species (Table 2). Figure 2A shows *trnH-psbA* amplicons from different species loaded in agarose gels according to subgeneric groupings. Among the different subgeneric groups, amplicons showed significant polymorphism compared with 335 bp predicted for *A. andraeanum* Hort. ‘New Pahoa Red’, migrating between the 300- and 500-bp DNA molecular weight markers,
Intraspecies variation was apparent between accessions of Cardiolonchium species *A. ravenii* (Card 6, 7), *Pachyneurium* species *A. schlechtendalii* (Pac 4, 5, 6, 7), and Mexican punctate species *A. lucens* (MP 1, 2, 3). Amplicons corresponding to *psbK-psbl* migrated in agarose gels between two main size classes, one consistent with the amplicon predicted for *A. andraeanum* Hort. (541 bp; Table 1) migrating in agarose gels above the 500-bp marker and a second size class below the 500-bp molecular weight marker (Fig. 2B). Single sample exceptions in amplicon size were observed for *A. roseospadix* (Calo 17) and *A. coriaceum* (Uro 2) of sections *Calomystrium* and *Urosadix*, respectively, whereas greater variation in incidence as well as amplicon size was apparent among species in section *Pachyneurium*. Intraspecies *psbK-psbl* amplicon variation was observed for *Pachyneurium* species, *A. schlechtendalii* (Pac 4, 5, 6, 7), *A. solitarium* (Pac 8, 9), and *Urosadix* section, *A. coriaceum* (Uro 1, 2). The *atpF-atpH* primer pair amplicon(s) migrated as three or four molecular weight species in agarose gels between 650 to 850 bp with the largest, major band consistent with 750 bp predicted for the amplicon from *A. andraeanum* Hort. ‘New Pahoa Red’ (Fig. 2C). In preliminary experiments, increasing the stringency of PCR annealing temperatures of up to 10°C did not substantially alter amplicon patterns for any of the primer pairs tested, suggesting that the smaller extraneous PCR products may result from subsegments of the target sequences. Size variation in the *atpF-atpH* amplicon(s) were apparent in section *Porphyrochitonium* between *A. bakeri* (Por 1, 2, 3) and other species of the section and possible differences between *A. leucocerum* and *A. pedatoradiatum* of the Mexican punctate clade. Intraspecies differences between *atpF-atpH* amplicon(s) were apparent between accessions of *A. solitarium* (Pac 8, 9) and *A. schlechtendalii* (Pac 4, 5, 6, 7) of section *Pachyneurium* and accessions of *A. gracile* (Lep 1, 2, 3) of section *Leptanthurium* with little if any other intersectional variation detected at this resolution.

Positive amplification was obtained for 96.4% of the *Anthurium* species DNA samples using *trnH-psbA* or *psbK-psbl* primer pairs and 91.6% of the *Anthurium* species DNA samples using *atpF-atpH* primer pairs. Three samples, ‘Card 1’ (*A. cerrocampanense*), ‘Por 5’ (*A. fragrantissimum*) and ‘Tet 2’ (*A. obtusum*), template DNAs had OD$_{260/280}$ 1.54 or less and lacked visible PCR amplification for samples ‘Calo 9’ and ‘Calo 15’ (*A. armeniense* and *A. nymphaeifolium*, respectively) using *atpF-atpH* (Fig. 2C) and other intergenic site primers (Fig. 1A–G). In the case of ‘Calo 8’ and ‘Card 4’ (*A. anti-ochraeae* and *A. ochraeae*), respectively with an OD$_{260/280}$ = 1.73 and a concentration
greater than 22 ng·µL⁻¹, low or no PCR product obtained with the atpF-atpH primer might also be attributable in part to primer design because similar results were observed in at least one other accession of *A. antioquiense* (‘Calo 7’) and *A. ochranthum* (‘Card 5’) using the same primers.

Of the three chloroplast intergenic sites examined, *psbK-psbl* varied sufficiently to differentiate distinct species on agarose gels compared with *atpF-atpH* and appeared to exhibit less intraspecific and intrasectional differences as compared with *trnH-psbA*. Of the two major *psbK-psbl* amplicon size classes observed, sections heavily represented by the larger *psbK-psbl* amplicon size class were *Cardiolonchium*, *Calomystrium*, and the Mexican epunctate clade represented by the species *A. leuconeurum* and *A. pedatoradatum* as well as *Urospadix* and the earliest diverging lineage represented by the section *Polyphyllium*, according to the molecular phylogenies by Carlsen (Carlsen, 2011; Carlsen and Croat, 2013) (Fig. 2B). By contrast, sections heavily represented by species with the smaller *psbK-psbl* amplicon included *Porphyrochitonium*, *Tetraspermium*, *Pachyneurium*, *Leptanthurium*, and *Dactylophyllum*, and the Mexican punctate clade represented by *A. lucens*, sections that appear to also cluster closer together phylogenetically (Carlsen, 2011; Carlsen and Croat, 2013). Despite heterogeneity in *psbK-psbl* amplicon size in certain tested *Anthurium* sections, the intraspecies variation observed in *Lemna* (Wang et al., 2010) and in *Anthurium* (this study), in addition to the monophyly of certain sections still being questioned and re-evaluated (Carlsen, 2011), this study shows the potential use of the *psbK-psbl* intergenic region for genotyping broad sectional relationships among species within *Anthurium*. Increasing the number of species and accessions sampled from the various *Anthurium* sections along with continued refinement of molecular phylogenetic classifications should enable further assessment of *psbK-psbl* amplicon size patterns among sectional groups. Increased sampling as well as re-verification of source material should in addition aid in vetting errors in taxonomic identification of samples that might be the origin of peculiar results found in this and future studies.

In this study, we examined a new set of gene markers that were previously used successfully in *Lemna* to resolve interspecies relationships based on DNA sequence comparisons. The *psbK-psbl* chloroplast gene region DNA sequence has also been used for species identification of various plants that are more distantly related to *Anthurium* (Lahaye et al., 2008), but to our knowledge has not been systematically examined at any level, DNA sequence, or amplicon size among species in this genus.

According to Carlsen (Carlsen, 2011; Carlsen and Croat, 2013), there is low-sequence divergence among *Anthurium* species for the chloroplast markers they used; she found that inclusion of the CHS nuclear gene intron in her molecular phylogenetic analyses better resolved monophyletic groups among *Anthurium* species as compared with the use of chloroplast gene markers alone (Carlsen, 2011; Carlsen and Croat, 2013). Nevertheless, chloroplast marker genes have been used to trace lineage in orchids (Khew and Chia, 2011; Tsai et al., 2012) and species or lineage-specific regions of the chloroplast genome have been identified from comparative analyses of related orchid genomes (Jheng
et al., 2012; Pan et al., 2012; Wu et al., 2010; Yang et al., 2013) to support breeding and ornamental improvement. In the study by Jheng and collaborators, gene markers were developed from the variable regions revealed by comparisons of whole genomes that could identify individual species by differences in PCR amplicon size (Jheng et al., 2012). This tool would be similarly useful in Anthurium, for example, in the evaluation of Anthurium pedigrees derived from interspecific crosses.

Rapid advances in chloroplast sequencing methodology (Atherton et al., 2010; Cronn et al., 2008, 2012; Moore et al., 2006; Nock et al., 2011; Parks et al., 2009; Stull et al., 2013) should increase the number of whole chloroplast genomes available for Anthurium thereby increasing chloroplast genome information available for Araceae (Ahmed et al., 2012; Mardanov et al., 2008; Wang and Messing, 2011). Sequencing of chloroplast genomes should allow identification of sites that provide higher phylogenetic resolution at lower taxonomic levels in Anthurium as well as aid in identification of species-specific markers most appropriate for diagnostic genotyping of germplasm in this genus. Like in the case of Lemna, gene markers useful for barcoding and species identification (Wang et al., 2010) need not necessarily be the same genes useful for resolving molecular phylogenetic relationships (Les et al., 2002). Improvements to the resolving power of the genotyping method could be made by multiplexing marker genes and the use of separation systems such as those used for amplified fragment length polymorphism analyses (Karudapuram and Larson, 2005) that enable high amplicon size resolution of large amplicon populations.

Development of simple genotyping markers or its approaches should complement more complex techniques developed to identify or characterize different cultivars or closely related species comprising commercial Anthurium germplasm (Bliss and Suzuki, 2012; Buldewo et al., 2012; Collette et al., 2004; Ge et al., 2012; Khan and Pankajaksan, 2010; Nowbuth et al., 2005; Ranamukhaarachchi et al., 2001; Wang and Chuang, 2013; Wang et al., 2013; Yu et al., 2012) to support improvement of ornamental and horticultural traits of Anthurium.

Finally, as demonstrated in this study, the availability of a tissue bank was invaluable as a genetic reference resource for testing hypotheses and for a comparative analysis of genes across Anthurium species. Dried leaf collections can be kept for relatively long periods without expensive facilities and although Anthurium is known to contain polysaccharides that inhibit PCR (Buldewo and Jaufeerally-Fakim, 2002), the commercially available plant column purification procedure used in this study yielded in most cases DNA template of sufficient quality and quantity.

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