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 *Lemna 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 intergenic 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 (Carlsen, 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 *Calomystrum*, including *A. andraeanum*,

A. amnicola, *A. antioquiense*, *A. antrophyoides*, *A. armenense*, *A. formosum*, *A. hoffmannii*, *A. kamemotoanum*, and *A. nymphaeifolium*, as well as hybrids between species within the sections *Porphyrochitonium* and *Cardiolorchium*, and intersectional hybrids between *Calomystrum* and *Cardiolorchium*, among others (Kamemoto and Kuehnle, 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. Lemnaceae-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 *Lemna* 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 gene marker primers were adapted from the *Lemna 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

Table 1. *Anthurium* species chloroplast gene marker primers adapted from *Lemna minor* chloroplast DNA barcode primer sequences (Wang et al., 2010) based on *A. andraeanum* Hort. 'New Paho Red' chloroplast DNA sequences.^z

Target gene regions	<i>Lemna minor</i> amplicon (bp)	<i>Anthurium andraeanum</i> amplicon (bp)	Primers	Primer sequence	Primer length	Primer T _m
<i>trnH-psbA</i>	300	335	*Aa.trnH-psbA.F Lm.trnH-psbA.F LmAa.trnH-psbA.R	5'-GTGATGCATGAACGTAATGCTC-3' 5'-GTTATGCACGAACGTAATGCTC-3' 5'-CGCGCGTGGTGGATTACAAATCC-3'	22	60.8
<i>rpoB</i>	406	426	LmAa.rpoB.F LmAa.rpoB.R	5'-ATGCAGCGTCAAGCAGTTCCGTTCC-3' 5'-TCGGATGTGAAAAGAAGTATA-3'	23 21	68.1 67.9
<i>rpoC1</i>	509	529	*Aa.rpoC1.F Lm.rpoC1.F LmAa.rpoC1.R	5'-GGCAAAGAGGGAAGATTCCG-3' 5'-GGAAAAGAGGGAAGATTCCG-3'; 5'-CAATTAGCATATCTTGAAGTTGG-3'	20 22	62.4 57.1
<i>psbK-psbI</i>	544	541	*Aa.psbK-psbI.F Lm.psbK-psbI.F LmAa.psbK-psbI.R	5'-TTAGCCTTTGTTTGGCAAG-3' 5'-TTAGCATTGTTTGGCAAG-3' 5'-AAAGTTTGGAGTAAGCAT-3'	19 19	55.8 51.5
<i>rbcL</i>	580	599	*Aa.rbcL.F Lm.rbcL.F *Aa.rbcL.R Lm.rbcL.R	5'-GTAAATCAAGTCCACCACG-3' 5'-GTAAATCAAGTCCACCACG-3'; 5'-ATGTCACCACAAACAGAACTAAAGC-3' 5'-ATGTCACCACAAACAGAGACTAAAGC-3'	20 26	60.4 61.4
<i>atpF-atpH</i>	675	750	LmAa.atpF-atpH.F LmAa.atpF-atpH.R	5'-ACTCGCACACTCCCTTTCC-3' 5'-GCTTTTATGGAAGCTTTAAACAAT-3'	21 23	64.5 55.6
<i>matK</i>	862	889	*Aa.matK.F Lm.matK.F *Aa.matK.R Lm.matK.R	5'-CGTACCGTACTTTTATGTTTACGAG-3' 5'-CGTACTGTACTTTTATGTTTACGAG-3'; 5'-ATCCCATCATCTGGAAATCTGGTTTC-3' 5'-ATCCGGTCCATCTAGAAATATTGGTTC-3'	25 27	61.3 66.1

^zDNA 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. andraeanum*. Primer T_m was based on a general formula provided by the manufacturer (see "Materials and Methods").

A. andraeanum Hort. 'New Paho Red' (Suzuki, unpublished data). Primers were obtained from Eurofins, MWG/Operon (Huntsville, AL) with primer T_m values based on the formula $[T_m = 81.5 + 16.6 \times \log(0.1) + 41 \times (G+C)/\text{length} - 500/\text{length}]$ provided by the manufacturer. Of the various target genes, *rpoB*, *rpoC1*, *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 maturase K, respectively. The remaining marker gene sites *trnH-psbA*, *atpF-atpH*, and *psbK-psbI* 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 *Lemna* with a corresponding DNA sequence of the chloroplast genome of *Anthurium andraeanum* Hort. 'New Paho Red', a popular red-spaced Hawaiian cut flower. *Anthurium* 'New Paho 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 amplicon

sizes (Table 1). PCR primer sequences for *rpoB* and *atpF-atpH* were unchanged between *Lemna* and *Anthurium*, whereas sequence differences were found between target *Lemna* and *Anthurium* that warranted base changes in at least one primer of the primer pairs for *rpoC1*, *trnH-psbA*, and *psbK-psbI*, and differences in both primers of a primer pair found for *rbcL* and *matK* (Table 1). This result suggests that primer target sites for *rpoB* and *atpF-atpH* are more conserved between *Lemna* and *Anthurium* compared with those of the other gene sequences examined. The *Lemna* and *Anthurium* *matK* primers contained the most differences from among the primer pairs studied here, a finding that is in line with the observation that *matK* is one of the most variable coding genes in the chloroplast of Angiosperms (Hilu et al., 2003). Predicted amplicon sizes of the *Anthurium* marker gene matched the relative size order of the corresponding target amplicons of *Lemna minor*.

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 ($OD_{260/280} \approx 1.8$) 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

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Table 2. *Anthurium* species used for chloroplast gene marker primer testing in this study.^z

Subgeneric section	Sample no.	Species	Reference	Accession
<i>Calomystrium</i>	Calo 1	<i>Anthurium amnicola</i>	Carlsen and Croat, 2013; Croat and Sheffer, 1983	ABG 1991 1372
	Calo 2	<i>Anthurium amnicola</i>		MSBG 1976-0053-002A
	Calo 3	<i>Anthurium amnicola</i>		MBG 84952
	Calo 4	<i>Anthurium andraeanum</i>	Croat and Sheffer, 1983	ABG 1991 1368
	Calo 5	<i>Anthurium andraeanum</i> Hort. 'New Paho Red'		HAIA
	Calo 6	<i>Anthurium antioquiense</i>	Carlsen and Croat, 2013	MBG 81407
	Calo 7	<i>Anthurium antioquiense</i>		MSBG 1996-0276A
	Calo 8	<i>Anthurium antioquiense</i>		NYBG 1383/78*
	Calo 9	<i>Anthurium armenense</i>	Croat, 1983	MSBG 1979-1055A
	Calo 10	<i>Anthurium armenense</i>		MBG 63434e
	Calo 11	<i>Anthurium esmeraldense</i>	N/A	ABG 2007 2399
	Calo 12	<i>Anthurium formosum</i>	Carlsen and Croat, 2013	MSBG 1991-0158A
	Calo 13	<i>Anthurium hoffmannii</i>	Carlsen and Croat, 2013	MSBG 1993-0176A
	Calo 14	<i>Anthurium hoffmannii</i>		MBG 66203
	Calo 15	<i>Anthurium nymphaeifolium</i>	Croat and Sheffer, 1983	MBG 55262
	Calo 16	<i>Anthurium nymphaeifolium</i>		NYBG 33/678A
	Calo 17	<i>Anthurium roseospadix</i>	Croat and Sheffer, 1983	MBG 74076
	Calo 18	<i>Anthurium sanctifidense</i>	Croat, 1986	MIA 36188, USDA ARS
<i>Cardiolonchium</i>	Card 1	<i>Anthurium cerrocampanense</i>	Croat, 1986	MSBG 1980-0429A
	Card 2	<i>Anthurium cerrocampanense</i>		MBG 76663b
	Card 3	<i>Anthurium magnificum</i>	Croat and Sheffer, 1983	MIA 36498, USDA ARS
	Card 4	<i>Anthurium ochranthum</i>	Croat (personal communication)	MBG 75190a
	Card 5	<i>Anthurium ochranthum</i>		MBG 69861a
	Card 6	<i>Anthurium ravenii</i>	Croat (personal communication)	MSBG 1980-0425A
	Card 7	<i>Anthurium ravenii</i>		MBG 74778a
	Card 8	<i>Anthurium warocqueanum</i>	Croat and Sheffer, 1983	ABG 1993 0478
	Card 9	<i>Anthurium warocqueanum</i>		MSBG 2006-0001A
<i>Dactylophyllum</i>	Dac 10	<i>Anthurium warocqueanum</i>		MBG 101538
	Dac 1	<i>Anthurium clavigerum</i>	Carlsen and Croat, 2013; Croat and Carlsen, 2013	ABG 2001 1433
	Dac 2	<i>Anthurium clavigerum</i>		MSBG 1991-0174A
	Dac 3	<i>Anthurium clavigerum</i>		USBG 77-0094
	Dac 4	<i>Anthurium clavigerum</i>		MIA 36159, USDA ARS
<i>Decurrentia</i>	Dac 5	<i>Anthurium kunthii</i>	Carlsen and Croat, 2013; Croat and Carlsen, 2013	MIA 36496, USDA ARS
	Dec 1	<i>Anthurium pittieri</i>	Croat et al., 2005	JBM 84-2010
<i>Digittinervium</i>	Dig 1	<i>Anthurium lentii</i>	Croat and Sheffer, 1983	MBG 35902b
<i>Gymnopodium</i>	Gym 1	<i>Anthurium gymnopus</i>	Croat and Sheffer, 1983; Engler, 1905	CJBN 2009.3.213
<i>Leptanthurium</i>	Lep 1	<i>Anthurium gracile</i>	Carlsen and Croat, 2013	ABG 1998 0680
	Lep 2	<i>Anthurium gracile</i>		MSBG 2001-0232A
	Lep 3	<i>Anthurium gracile</i>		MBG 95664
<i>Pachyneurium</i>	Pac 1	<i>Anthurium guatemalense</i>	N/A	MIA 36495, USDA ARS
	Pac 2	<i>Anthurium llewelynii</i>	Croat, 1991	MIA 36497, USDA ARS
	Pac 3	<i>Anthurium salvinii</i>	Croat, 1991	MIA 36500, USDA ARS
	Pac 4	<i>Anthurium schlechtendalii</i>	Croat, 1991	MSBG 1977-3108A
	Pac 5	<i>Anthurium schlechtendalii</i>		NYBG 993/79*A
	Pac 6	<i>Anthurium schlechtendalii</i>		NYBG 993/93*A
	Pac 7	<i>Anthurium schlechtendalii</i> ssp. <i>schlechtendalii</i>		MBG 78640
	Pac 8	<i>Anthurium solitarium</i>	Croat, 1991	JBM 2083-2000
	Pac 9	<i>Anthurium solitarium</i>		MBG 53699b
	Pac 10	<i>Anthurium sparreorum</i>	Croat, 1991	MIA 36501, USDA ARS
	Pac 11	<i>Anthurium upalaense</i>	Croat, 1991	MIA 36502, USDA ARS
	Pac 12	<i>Anthurium watermaliense</i>	Croat, 1991	MSBG 1977-2832A
	Pac 13	<i>Anthurium watermaliense</i>		MBG 78766
	Pac 14	<i>Anthurium watermaliense</i>		USBG 07-0534
<i>Polyphyllum</i>	Poly 1	<i>Anthurium clidemioides</i>	Carlsen and Croat, 2013	MBG 79567
	Poly 2	<i>Anthurium clidemioides</i>		JBVL 850002
	Poly 3	<i>Anthurium flexile</i> ssp. <i>muelleri</i>	Carlsen and Croat, 2013	MBG 100348b
<i>Porphyrochitonium</i>	Por 1	<i>Anthurium bakeri</i>	Croat and Sheffer, 1983	MBG 78747c

(Continued on next page)

Table 2. (Continued) *Anthurium* species used for chloroplast gene marker primer testing in this study.^z

Subgeneric section	Sample no.	Species	Reference	Accession
	Por 2	<i>Anthurium bakeri</i>		NYBG 897/63*
	Por 3	<i>Anthurium bakeri</i>		USBG 77-0090
	Por 4	<i>Anthurium durandii</i>	Croat, 1983	MIA 36182, USDA ARS
	Por 5	<i>Anthurium fragrantissimum</i>	Croat, 1986	MBG 76860
	Por 6	<i>Anthurium wendlingeri</i>	Croat and Sheffer, 1983	ABG 2007 2507
	Por 7	<i>Anthurium wendlingeri</i>		MSBG 1977-1989A
	Por 8	<i>Anthurium wendlingeri</i>		MBG 95418
	Por 9	<i>Anthurium wendlingeri</i>		USBG 01-1412
<i>Semaephyllum</i>	Sem 1	<i>Anthurium trilobum</i>	Carlsen and Croat, 2007	MIA 36184, USDA ARS
<i>Tetraspermium</i>	Tet 1	<i>Anthurium obtusum</i>	Carlsen and Croat, 2013	ABG 1997 0478
	Tet 2	<i>Anthurium obtusum</i>		MBG 82905
	Tet 3	<i>Anthurium scandens</i> ssp. <i>pusillum</i>		ABG 1998 0667
	Tet 4	<i>Anthurium scandens</i> ssp. <i>pusillum</i>		USBG 98-1900
	Tet 5	<i>Anthurium scandens</i> ssp. <i>scandens</i>	Carlsen and Croat, 2013	ABG 1991 1433
	Tet 6	<i>Anthurium scandens</i> ssp. <i>scandens</i>		MBG 47671
<i>Urospadix</i>	Uro 1	<i>Anthurium coriaceum</i>	Carlsen and Croat, 2013	NYBG 574/65*A
	Uro 2	<i>Anthurium coriaceum</i>		USBG 98-1747
	Uro 3	<i>Anthurium radicans</i>	Carlsen and Croat, 2013	ABG 1991 1495
	Uro 4	<i>Anthurium radicans</i>		MSBG 1975-0053-003A
	Uro 5	<i>Anthurium radicans</i>		USBG 98-2591
<i>Xialophyllum</i>	Xia 1	<i>Anthurium microspadix</i>	Croat and Sheffer, 1983	MBG 100186
Other classifications				
Mexican Epunctate clade	ME 1	<i>Anthurium leuconeurum</i>	Carlsen and Croat, 2013	MSBG 1980-1683B
Mexican Epunctate clade	ME 2	<i>Anthurium pedatoradiatum</i>	Carlsen and Croat, 2013	MIA 36499, USDA ARS
Mexican Punctate clade	MP 1	<i>Anthurium lucens</i>	Carlsen and Croat, 2013	MSBG 1980-1619A
Mexican Punctate clade	MP 2	<i>Anthurium lucens</i>		MBG 78702
Mexican Punctate clade	MP 3	<i>Anthurium lucens</i>		NYBG 103/81*

^zAccessioned dried leaf tissue samples were obtained from Atlanta Botanical Garden (ABG), Conservatoire et Jardins Botaniques de Nancy (CJBN), Hawaii Anthurium Industry Association (HAIA), Jardin Botanique de Montréal (JBM), Jardin Botanique de la Ville de Lyon (JBVL), Marie Selby Botanical Gardens (MSBG), Missouri Botanical Garden (MBG), New York Botanical Garden (NYBG), U.S. Botanic Garden (USBG), U.S. Department of Agriculture, Agriculture Research Service, Subtropical Horticulture Research Station, National Clonal Germplasm Repository (USDA ARS SHRS NCGR or USDA ARS).

N/A = original reference not available.

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 Paho 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 Paho 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 Paho 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' (accessions representing *A. antioquiense*, *A. armenianse*, and *A. nymphaeifolium*), 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 accessions of *A. amnicola*, 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 intraspecific 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 intergenic 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 Paho Red', migrating between the 300- and 500-bp DNA molecular weight markers,

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplifications of target chloroplast gene marker regions from *Anthurium* species belonging to subgeneric section *Calomystrum*. *Anthurium* species samples are labeled according to Table 2. *Anthurium* primers used in amplification reactions corresponded to target genes (A) *trnH-psbA*, (B) *rpoB*, (C) *rpoC1*, (D) *psbK-psbI*, (E) *rbcL*, (F) *atpF-atpH*, and (G) *matK*. DNA markers.

a size range where amplicon size differences are relatively easily resolved on agarose gels. Interestingly, species in the crown group *Polyphyllum* apparently have the largest *trnH-psbA* amplicons compared with species in other sections tested. Among sections excluding *Decurrentia*, *Digittinervium*,

Gymnopodium, *Leptanthurium* and *Semaephyllum*, *Xialophyllum*, and the Mexican Punctate clade, represented by two or more species, variation in *trnH-psbA* size was most apparent between species within sections *Cardiolonchium*, *Pachyneurium*, *Porphyrochitonium*, *Tetraspermium*, and *Urospadix*.

Intraspecies variation was apparent between accessions of *Cardiolonchium* species *A. ravenii* (Card 6, 7), *Pachyneurium* species *A. schlehtendalii* (Pac 4, 5, 6, 7), and Mexican punctate species *A. lucens* (MP 1, 2, 3). Amplicons corresponding to *psbK-psbI* 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 *Calomystrum* and *Urospadix*, respectively, whereas greater variation in incidence as well as amplicon size was apparent among species in section *Pachyneurium*. Intraspecies *psbK-psbI* amplicon variation was observed for *Pachyneurium* species, *A. schlehtendalii* (Pac 4, 5, 6, 7), *A. solitarium* (Pac 8, 9), and *Urospadix* 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° 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. leuconeurum* and *A. pedatoradiatum* of the Mexican epunctate clade. Intraspecies differences between *atpF-atpH* amplicon(s) were apparent between accessions of *A. solitarium* (Pac 8, 9) and *A. schlehtendalii* (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-psbI* primer pairs and 91.6% of the *Anthurium* species DNA samples using *atpF-atpH* primer pairs. Three samples, 'Card 1' (*A. cerrocampense*), 'Por 5' (*A. fragrantissimum*) and 'Tet 2' (*A. obtusum*), template DNAs had OD_{260/280} 1.54 or less and lacked visible PCR amplicons for all chloroplast *trnH-psbA*, *psbK-psbI*, and *atpF-atpH* intergenic site primers suggesting that the lack of amplification might be affected by DNA template quality and amount. This could also be the cause of similar, low or no amplicon amplification for samples 'Calo 9' and 'Calo 15' (*A. armenense* 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-quense* and *A. ochranthum*, respectively) with an OD_{260/280} = 1.73 and a concentration

Fig. 2. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplified target chloroplast sequence amplicons from *Anthurium* species loaded according to subgeneric grouping. (A) *trnH-psbA* amplicons, (B) *psbK-psbI* amplicons, (C) *atpF-atpH* amplicons. DNA markers (lane M) are labeled at relevant positions with corresponding molecular weight in base pairs.

greater than $22 \text{ ng} \cdot \mu\text{L}^{-1}$, 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-psbI* 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-psbI* amplicon size classes observed, sections heavily represented by the larger *psbK-psbI* amplicon size class were *Cardiolonchium*, *Calomys-trium*, and the Mexican epunctate clade represented by the species *A. leuconeurum* and *A. pedatoradiatum* 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 the smaller *psbK-psbI* amplicon included *Porphyrochitonium*, *Tetraspermium*, *Pachyneurium*, *Leptanthurium*, *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-psbI* amplicon size in certain tested *Anthurium* sections, the intraspecific 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-psbI* 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-psbI* amplicon size patterns among sectional groups. Increased sampling as well as reverification 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-psbI* 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

Fig. 2. (Continued)

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., 20008, 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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Fig. 2. (Continued)

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