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PRIMER NOTE

PRIMERS FOR LOW-COPY NUCLEAR GENES IN THE HAWAIIAN ENDEMIC *CLERMONTIA* (CAMPANULACEAE) AND CROSS-AMPLIFICATION IN LOBELIOIDEAE¹

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- *Premise of the study:* Primers were developed to amplify 12 intron-less, low-copy nuclear genes in the Hawaiian genus *Clermontia* (Campanulaceae), a suspected tetraploid.
- *Methods and Results:* Data from a pooled 454 titanium run of the partial transcriptomes of seven *Clermontia* species were used to identify the loci of interest. Most loci were amplified and sequenced directly with success in a representative selection of lobeliads even though several of these loci turned out to be duplicated. Levels of variation were comparable to those observed in commonly used plastid and ribosomal markers.
- *Conclusions:* We found evidence of a genome duplication that likely predates the diversification of the Hawaiian lobeliads. Some genes nevertheless appear to be single-copy and should be useful for phylogenetic studies of *Clermontia* or the entire Lobelioideae subfamily.

Key words: *Clermontia*; Hawai'i; Lobelioideae; low-copy nuclear genes; next-generation sequencing; polyploidy.

Although phylogenetic studies of plants rely heavily on plastid and nuclear ribosomal loci, the limitations of these loci are well known. Plastid loci are uniparentally inherited and susceptible to chloroplast capture (Rieseberg and Soltis, 1991). Furthermore, their low variation is particularly problematic for investigations of recent radiations such as those in the Hawaiian Islands, and indeed these markers have provided only limited resolution there. Nuclear ribosomal loci are subject to complex concerted evolution (Alvarez and Wendel, 2003), which can be incomplete so that multiple ancient alleles are maintained within a single individual, or rapid such that traces of hybridization are quickly erased. As a result, both plastid and ribosomal markers provide only incomplete information when hybridization is common.

The genus *Clermontia* Gaudich. (Campanulaceae, subfamily Lobelioideae) comprises 22 species endemic to Hawai'i, 13 of which are on the IUCN Red List of Threatened Species. Species identification in the field is often difficult, particularly in the absence of flowers, and apparent hybrids can be common. *Clermontia* and other Hawaiian lobeliads (six genera total) form a

monophyletic clade that represents the largest plant radiation in Hawai'i (Givnish et al., 2009). The members of this clade, as well as the members of clade 4 of Antonelli (2008), in which the Hawaiian clade is nested, are suspected paleotetraploids (Lammers, 1988). To find genetic markers that will be useful for phylogenetics and DNA barcoding within *Clermontia*, we used 454 data of partial cDNA libraries to design primers for single-exon nuclear loci in *Clermontia* and then tested their cross-amplification in Campanulaceae.

METHODS AND RESULTS

We obtained a pooled, partial transcriptome library from leaf and floral buds (fixed in the field in RNAlater [QIAGEN, Gaithersburg, Maryland, USA] and stored at -80°C) of seven taxa: *Clermontia arborescens* (H. Mann) Hillebr., *C. clermontoides* (Gaudich.) A. Heller, *C. fauriei* H. Lévl., *C. kakeana* Meyen, *C. kohalae* Rock, *C. parviflora* Gaudich. ex A. Gray, and *C. peleana* Rock. RNA isolation, cDNA synthesis, and 454 sequencing were done at the University of Arizona Genetics Core Laboratory. The 454 run provided 1.4 million reads with an average length of 395 bp. 454 adapters, ribosomal RNA, and low-quality and low-complexity sequences were removed/trimmed using SeqClean (<http://compbio.dfci.harvard.edu/tgi/software/>), and each taxon was assembled separately by the TGI Clustering tools (TGICL; Pertea et al., 2003), using default settings. We conducted BLAST searches of the 400 most highly expressed genes in *Arabidopsis* (C. Fizames, personal communication) against our data in CLC DNA Workbench (CLC bio, Aarhus, Denmark) to identify a set of genes with high coverage within each of all or most of the species. We selected loci (generally only a small portion of a gene) that comprised a single, long exon (200 bp) with matches in multiple species, and designed primers with FastPCR (PrimerDigital Ltd., Helsinki, Finland; <http://www.primerdigital.com/fastpcr.html>) for their amplification using default settings. The presence of introns was tested by comparison with genomic and cDNA sequences in the *Arabidopsis* Information Resource database (www.arabidopsis.org). Avoiding introns allowed the direct sequencing of accessions even in the case of gene duplications; introns often contained indels,

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TABLE 1. Identity of the 12 intron-less, low-copy nuclear genes identified in this study, with primer sequences, results from cross-amplification tests, and inference of putative gene duplication.^a

Gene	Putative <i>Arabidopsis</i> homolog	Putative product	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	Length (bp)	Failed sequences	Duplication in Hawai'i
<i>Clerm1</i>	At1g14320	RPL10 (ribosomal protein)	GACCTGCTAGGTGTTACCGT	ATCTTCTGACGACACAGGGA	484		yes
<i>Clerm2</i>	At1g61520	Lhca#3 (chlorophyll-binding protein)	TTGGATTTCGACCCCACTTGG	ACTTAAGGCTGGTCTACGAC	594	<i>Campanula</i>	no
<i>Clerm3</i>	At2g05070	Lhcb2.2 (chlorophyll-binding protein)	GGTGACTACGGATGGGACAC	CATCAGCCAGTCCAAGTGGGT	382		yes
<i>Clerm4</i>	At3g26520	TIP2 (tonoplast intrinsic protein)	GATGACATTCGGGCTGGTA	CAGTAGACCCAGTGGTGG	210	<i>Campanula</i>	no
<i>Clerm5</i>	At4g18100	RPL32e (ribosomal protein)	ATTGATTCGCCGGGTGAGAA	GTCACCAACATCAAGCT	248	<i>Campanula, Lobelia</i>	<i>Clermontia</i>
<i>Clerm6</i>	At3g13920	EIF4-1 (translation initiation factor)	TTTGAGAGGCCCTCTCTCAA	TTGCCCTACGCAACATGTCAA	351		yes
<i>Clerm7</i>	At4g14880	ATCYS3-A (acetylserine(thiol)lyase)	TCTTATCGAACCCAAAGTGG	TCGGATTGGCAGGATCTCTC	257		no
<i>Clerm8</i>	At5g05170	CESA3 (cellulose synthase)	AGGCCATCGCAATGGCTG	GCGAAATACCACTCTGGAGC	264		no
<i>Clerm9</i>	At3g23810	SAHH2 (S-adenosyl-L-homocysteine hydrolase)	CATGCTCTTAGCCGACTTCG	ACCATTAGCCTGCATTTGG	608		no
<i>Clerm10</i>	At1g79550	PGK (phosphoglycerate kinase)	GTCGAAGTGGCAATGATTGC	CCTTCAGTGGAAAGCATGAGC	212		yes
<i>Clerm11</i>	At1g66580	SAG24 (senescence-associated gene/ribosomal protein L10e)	GACCTGCTAGGTGTTACCGT	CCCTCATACCAAGTCTGGAGC	339		yes
<i>Clerm12</i>	At3g56940	Magnesium protoporphyrin IX monomethyl ester cyclase	TCTACACGACGGATTTCGAGG	TGAGGCGTCTTACCAAGCTC	264		yes

^a Missing sequences are due to failed amplification (*Clerm5*), weak amplification of a different gene (*Clerm2*).

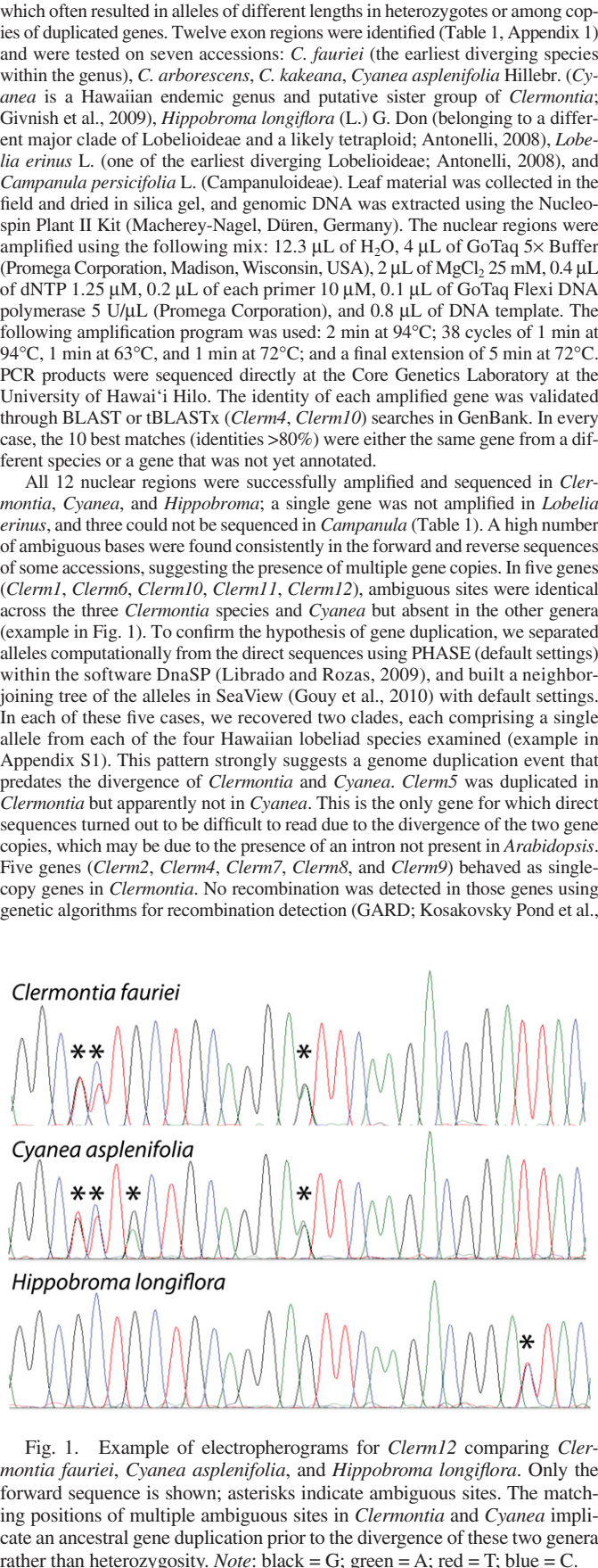


TABLE 2. Comparison of variation of the five apparently nonduplicated nuclear genes with three plastid and two nuclear ribosomal loci.^a

Characteristic	<i>Clerm2</i>	<i>Clerm4</i>	<i>Clerm7</i>	<i>Clerm8</i>	<i>Clerm9</i>	<i>rbcL</i>	<i>matK</i>	<i>psbA-trnH</i>	ITS	ETS
Length (bp)	594	210	257	264	608	599	889	460–462	874–877	594–600
No. of variable sites	12	5	4	5	5	10	16	9	17	12
Percent variable sites	2.0	2.4	1.6	1.9	0.8	1.7	1.8	1.9	1.9	2.0
1st position (nonsynonymous)	2 (2)	1 (1)	0	1 (1)	0	3 (1)	9 (8)	NA	NA	NA
2nd position (nonsynonymous)	2 (2)	0	0	1 (1)	0	0	3 (3)	NA	NA	NA
3rd position (nonsynonymous)	8 (2)	4 (0)	4 (0)	3 (0)	5 (0)	7 (3)	4 (1)	NA	NA	NA
Indels	0	0	0	0	0	0	0	2	1	2

Note: NA = not applicable.
^aFor each gene, variation was measured on a sample that included *Clermontia fauriei*, *C. kakeana*, *C. arborescens*, and *Cyanea asplenifolia*.

2006; <http://www.datamonkey.org/>). The percentage of variable sites within each of these genes was comparable to those of the plastid loci *rbcL*, *matK*, and *psbA-trnH* and the nuclear ribosomal ITS and ETS (Table 2). Genotyping of a broader taxonomic sample of *Clermontia* revealed a much greater number of variants at these newly described nuclear genes and a different pattern of evolution compared to plastid genes (Pillon et al., 2013).

CONCLUSIONS

The selection of intron-less regions proved successful for the amplification and direct sequencing of several nuclear loci and the detection of duplicated genes. The large number of gene duplications shared between *Clermontia* and *Cyanea* strongly supports the hypothesis of whole-genome duplication that predates the diversification of the lobeliads in Hawai‘i. Whole genome duplication has similarly been demonstrated in Hawaiian silverswords (Barrier et al., 1999). Despite the genome duplication, we nevertheless identified a number of apparently single-copy genes, whether due to the loss of one copy in each case or the selectivity of our primers for one copy. Geographic and taxonomic patterns of variations of two of these markers within *Clermontia* are examined in a study of their potential use as DNA barcodes (Pillon et al., 2013).

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APPENDIX 1. Location information, voucher specimens, and GenBank accession numbers for *Clerm1*, *Clerm2*, *Clerm3*, *Clerm4*, *Clerm5*, *Clerm6*, *Clerm7*, *Clerm8*, *Clerm9*, *Clerm10*, *Clerm11*, *Clerm12*, *ETS*, *ITS*, *matK*, *psbA-trnH*, and *rbcL*. For duplicated genes, only cDNA sequences were submitted, when available. Voucher specimens were deposited at the Herbarium of the University of Hawai‘i (HAW). The vouchers for *Clermontia arborescens* and *Campanula persicifolia* have been lost, and vouchers were not collected for *Cyanea asplenifolia* because it is an endangered plant. — signifies that no sequence is available for the particular locus for that accession.

<i>Clermontia arborescens</i> (H. Mann) Hillebr. subsp. waihae (Wawra) Lammers: Maui, Pu‘u Kukui, 20°55’26”N, 156°36’11”W, no voucher: JX500281, JX500282, JX500287, JX500291, JX500293, JX500298, JX500300, JX500305, JX500312, JX500318, JX500324, JX500327, —, JX500333, JX500337, JX500341, JX500345, JX500349.	<i>Cyanea asplenifolia</i> Hillebr.: Maui, no voucher (Volcano Rare Plant Facility): —, JX500289, —, JX500295, JX500299, —, JX500307, JX500314, JX500320, —, —, —, JX500335, JX500339, JX500343, JX500347, JX500351.
<i>Clermontia fauriei</i> H. Lév.: Kaua‘i, Alaka‘i swamp trail, 22°9’12”N, 159°37’52”W, <i>Johansen</i> 1: JX500283, JX500288, —, JX500294, —, JX500301, JX500306, JX500313, JX500319, —, —, JX500330, JX500334, JX500338, JX500342, JX500346, JX500349.	<i>Hippobroma longiflora</i> (L.) G. Don: Hawai‘i (introduced), <i>Pillon</i> 1426: —, —, —, JX500296, —, —, JX500308, JX500315, —, JX500324, —, —, —, —, —, —.
<i>Clermontia kakeana</i> Meyen: Maui, Pu‘u Kukui, 22°56’3”N, 156°36’53”W, <i>Johansen</i> 5: —, JX500286, —, JX500292, —, —, JX500304, JX500311, JX500317, —, —, —, JX500332, JX500336, JX500340, JX500344, JX500348.	<i>Lobelia erinus</i> L.: Hawai‘i (cultivated), <i>Pillon</i> 1427: JX500284, JX500290, —, JX500297, —, JX500302, JX500309, JX500316, JX500321, JX500325, JX500328, —, —, —, —, —, —.
	<i>Campanula persicifolia</i> L.: France (cultivated), no voucher: JX500285, —, —, —, —, JX500303, JX500310, —, JX500322, JX500326, JX500329, JX500331, —, —, —, —, —.