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PRIMER NOTE

PRIMERS FOR LOW-COPY NUCLEAR GENES IN *METROSIDEROS* AND CROSS-AMPLIFICATION IN MYRTACEAE[1]

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- *Premise of the study:* Primers were developed to amplify low-copy nuclear genes in Hawaiian *Metrosideros* (Myrtaceae).
- *Methods and Results:* Data from a pooled 454 Titanium run of the partial transcriptomes of four *Metrosideros* taxa were used to identify the loci of interest. Ten exon-primed intron-crossing (EPIC) markers were amplified and sequenced directly with success in *Metrosideros*, as well as in a representative selection of Myrtaceae, including *Syzygium*, *Psidium*, and *Melaleuca* for most of the markers. The loci amplified ranged between 500 and 1100 bp, and up to 117 polymorphic sites were observed within an individual gene alignment. Two introns contained microsatellites in some of the species.
- *Conclusions:* These novel primer pairs should be useful for phylogenetic analysis and population genetics of a broad range of Myrtaceae, particularly the diverse fleshy-fruited tribes Syzygieae and Myrteae.

Key words: Hawai'i; *Metrosideros*; Myrtaceae; next-generation sequencing; phylogeny; single nuclear genes.

The genus *Metrosideros* Banks ex Gaertn. (Myrtaceae) is an emblematic genus of trees of the Pacific islands, being present on most high Pacific islands where it is particularly abundant in mountain and ridge vegetation. In Hawai'i, 'ōhi'a lehua (*M. polymorpha* Gaudich.) is an ecologically dominant tree with high intraspecific diversity (eight varieties) and four satellite species that is emerging as a model for studies of speciation in trees (Stacy et al., 2014). Low levels of variation in plastid (Percy et al., 2008) and ribosomal (Wright et al., 2000) DNA sequences and the occurrence of homoplasy in nuclear microsatellites (Harbaugh et al., 2009) have hampered understanding of *Metrosideros*' evolutionary history. Single-copy nuclear DNA sequences are likely to provide the necessary intermediate level of variation to improve understanding of the evolution of *Metrosideros*.

Metrosideros is part of a larger clade (BKMMST; Biffin et al., 2010) that comprises Backhousieae, Kanieae, Myrteae, Metrosidereae, Syzygieae, Tristanieae, and *Cloezia* Brongn. & Gris. This clade encompasses considerable morphologic diversity within the Myrtaceae family, including the two fleshy-fruited groups. The first group is the tribe Syzygieae, chiefly the genus *Syzygium* P. Browne ex Gaertn. (ca. 1000 species) including clove (*S. aromaticum* (L.) Merr. & L. M. Perry) and mountain apple (*S. malaccense* (L.) Merr. & L. M. Perry). The second group, Myrteae, comprises ca. 47 genera, including the large *Eugenia* L. (ca. 1100 species) and guava (*Psidium guajava* L.). Whether these two tribes are sister groups, and therefore whether fleshy fruits evolved once or twice in Myrtaceae, is still unclear. Therefore, phylogenetically useful markers are desirable for this entire group to answer such questions and to resolve systematic issues.

METHODS AND RESULTS

We obtained a pooled, partial transcriptome library from leaf and floral buds (fixed in the field in RNA*later* [QIAGEN, Germantown, Maryland, USA] and stored at −80°C) of four Hawaiian *Metrosideros* taxa: *M. rugosa* A. Gray (O'ahu), *M. tremuloides* (A. Heller) P. Knuth (O'ahu), *M. polymorpha* var. *incana* (H. Lév.) H. St. John (Big Island of Hawai'i), and *M. polymorpha* var. *newellii* (Rock) H. St. John (Big Island). Total RNA was isolated with the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), and mRNA were poly A selected using the Dynabeads mRNA Purification Kit (Life Technologies, Carlsbad, California, USA). cDNA libraries were prepared and normalized following the Roche-FLX Titanium cDNA Rapid Library Preparation Method Manual (Roche, Basel, Switzerland). RNA isolation, cDNA synthesis, and 454 sequencing were done at the University of Arizona Genetics Core Laboratory. The 454 run provided 715,065 reads with an average length of 401 bp. Adapters, ribosomal RNA, and low-quality and low-complexity sequences were removed/trimmed using SeqClean (http://sourceforge.net/projects/seqclean/), and each taxon was assembled separately by the TGI Clustering tools (TGICL; Pertea et al., 2003) using default settings. We searched for single-copy genes reported by Duarte et al. (2010) in the *Metrosideros* transcriptome through BLAST searches. We designed primers flanking each side of one intron for about 80 genes with Primer3 (Rozen and Skaletsky, 2000), setting the optimal annealing temperature to 60°C (minimum 59°C, maximum 61°C) and Max Poly X to 3, and using default settings for other parameters. We report here loci

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TABLE 1. Characterization of 10 single-copy nuclear markers designed to amplify in *Metrosideros* and fleshy-fruited Myrtaceae. Statistics on polymorphism are given for markers successfully sequenced in a sampling including all four of *Metrosideros polymorpha* var. *glaberrima*, *Syzygium sandwicense*, *Psidium guajava*, and *Melaleuca quinquenervia*.

Locus	Primer sequence (5′–3′)	Total/intron length, bp (+range)	Total no. of polymorphic sites (no. within exons)	Nucleotide diversity	Putative *Arabidopsis* homolog	Putative product
MeNu5	F: ATTCCAGCATCTTTGGGTGT R: CATCCGGATAACCAGTTGCT	913/726 (+7)	failed in *Psidium* and *Syzygium*		At5g52560	protein with UTP: sugar 1-phosphate uridylyltransferase activity
MeNu13	F: CAGCCAGTCGATCGAAGATT R: GGATCTCATCGCGAATCAAC	587/516 (+5)	failed in *Psidium* and *Syzygium*		At1g15220	protein with oxidoreductase activity present in the inner membrane of mitochondria
MeNu18	F: TAGTGTAGATGCGGCGACAA R: GCTGCTGCTTGTCCTTGAGT	521/408 (+118)	95 (2)	0.13148	At1g75200	flavodoxin family protein / radical SAM domain-containing protein
MeNu21	F: GGTCAGAGATGAATGGATGGA R: GCATCAGGAACTCCTGGGTA	794/716 (+39)	failed in *Psidium*		At2g45990	unknown protein
MeNu39	F: AGGCCCTTATATGGGTGGAG R: GGCTCAGTTCAAGGCATAGG	557/383 (+25)	82 (11)	0.06847	At5g57300	*S*-adenosyl-L-methionine-dependent methyltransferases superfamily protein
MeNu47	F: GAGGGTTGGGACTGGATTG R: CATCCCATATGCCAGTATTACG	801/746 (+316)	112 (0)	0.07340	At1g09010	glycoside hydrolase family 2 protein
MeNu61	F: GGGCCTCTCCCTATTGTGTT R: ACTGGATTGCCTTCTCGTTG	682/546 (+65)	heterozygote in *Melaleuca*		At1g74640	alpha/beta-Hydrolases superfamily protein
MeNu62	F: TAGAGGCACCCTCGAAGAAG R: ATAAGCCACTCCATCCATGC	606/447 (+410)	97 (15)	0.09018	At1g77930	Chaperone DnaJ-domain superfamily protein
MeNu79	F: GGGACCAATATGCTTTCACG R: CCACTTTCCGCAGCATTTAT	746/633 (+378)	failed in *Melaleuca*		At2g36810	ARM repeat superfamily protein
MeNu92	F: TTGGTGTCGCTCAAGGAAAT R: GGGCGAACAAATTCATCGTA	930/831 (+338)	117 (2)	0.06883	At3g15080	Polynucleotidyl transferase, ribonuclease H-like superfamily protein

that should be of potential use for phylogenetic studies in Myrtaceae, i.e., loci with relatively long amplicons (>500 bp) and successful amplifications across a range of species. We tested the primers on a single accession of *M. polymorpha* var. *glaberrima* (H. Lèv.) H. St. John, *P. guajava*, *Syzygium sandwicense* (A. Gray) Müll. Berol., and *Melaleuca quinquenervia* (Cav.) S. T. Blake. Three tribes of the BKMMST clade were thus represented, including the two fleshy-fruited tribes, and also the more distantly related tribe Melaleuceae.

Fresh or frozen leaves were homogenized with Lysing Matrix A tubes in the FastPrep-24 instrument for 40 s at 4.0 m/s (MP Biomedicals, Santa Ana, California, USA). DNA was extracted from the lysate using a NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany). Extracted DNA was precipitated with 100% ethanol and 3 M sodium acetate (pH 5.2), vacuum dried, washed with 70% ethanol, and resuspended in elution buffer (Macherey-Nagel). The nuclear regions were amplified using the following mix: 11.3 µL of H_2O, 4 µL of GoTaq 5× Buffer (Promega Corporation, Madison, Wisconsin, USA), 2 µL of $MgCl_2$ 25 mM, 1 µL of bovine serum albumin (BSA) 10 mg/mL, 0.4 µL of dNTP 1.25 mM, 0.2 µL of each primer 10 µM, 0.1 µL of GoTaq Flexi DNA polymerase 5 U/µL (Promega), and 0.8 µL of DNA template. The following amplification program was used: 2 min at 94°C, 38 cycles of 1 min at 94°C, 1 min at 63°C, 1 min at 72°C (1 min 30 s when the fragment exceeded 1000 bp), and a final extension of 5 min at 72°C. PCR products were sequenced directly at the Core Genetics Laboratory at the University of Hawai'i Hilo. DNA sequences were aligned with MUSCLE within MEGA 6.06 (Tamura et al., 2013).

We identified 10 genes that were successfully amplified in *Metrosideros* as well as *Syzygium*, *Psidium*, or *Melaleuca*; six worked in all four genera tested (Table 1). Direct sequencing was straightforward with a single exception. For one locus, MeNu61, the accession of *Melaleuca* appears to be heterozygous with the presence of two alleles differing by at least one indel; we could not obtain a clear direct sequence and did not attempt cloning. Alignment was straightforward in most genes, but somewhat difficult for MeNu18 and partly ambiguous for *Psidium* in MeNu47 because of a large indel. The cumulative number of polymorphic sites per gene across the four genera was high (>80) for most genes. We also identified microsatellites, e.g., $(CT)_{13}$ and $(CT)_{19}$ in MeNu18 for *M. polymorpha* and *S. sandwicense*, respectively, and $(TAAA)_{11}$ in MeNu79 for *P. guajava*. The levels of variation and usefulness of these microsatellites remain to be tested.

CONCLUSIONS

The 10 markers described here should be useful for investigations of the relationships within the BKMMST clade of Myrtaceae that includes all of the fleshy-fruited Myrtaceae. The level of polymorphism is promising for population genetic studies as well as for the resolution of phylogenetic relationships among genera of the tribe Myrteae and among species of large genera such as *Syzygium* or *Psidium*. They may also be useful in more distantly related Myrtaceae as indicated by their successful amplification in *Melaleuca*. Their usefulness at broader scales (e.g., across the entire family) may be limited by difficulty in alignment and homoplasy. Finally, they may facilitate discovery of microsatellites for population genetic studies in Myrtaceae.

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Appendix 1. Locality, voucher information, and GenBank accession numbers for MeNu5, MeNu13, MeNu18, MeNu21, MeNu39, MeNu47, MeNu61, MeNu62, MeNu79, and MeNu92. Voucher specimens are deposited at the National Tropical Botanical Garden, Kalaheo, Kauai, Hawai'i (PTBG).

Taxon	Locality	Voucher no.	GenBank accession no.
Metrosideros polymorpha var. *glaberrima* (H. Lèv.) H. St. John	Hawai'i, Kukuiopae	*Johansen et al. 55*	KJ940982, KJ940984, KJ883158, KJ940986, KJ883162, KJ883166, KJ883172, KJ883173, KJ940989, KJ883177
Psidium guajava L.	Hawai'i (introduced)	*Pillon 1432*	–, –, KJ883159, –, KJ883164, KJ883168, KJ883171, KJ883175, KJ940991, KJ883180
Syzygium sandwicense (A. Gray) Müll. Berol.	O'ahu, Kuliouou	*Johansen 60*	–, –, KJ883161, KJ940987, KJ883165, KJ883167, KJ883170, KJ883174, KJ940990, KJ883179
Melaleuca quinquenervia (Cav.) S. T. Blake	Hawai'i (introduced)	*Pillon 1430*	KJ940983, KJ940985, KJ883160, KJ940988, KJ883163, KJ883169, –, KJ883176, –, KJ883178