Research Article

Diversification of Hawaiian *Cyrtandra* (Gesneriaceae) under the influence of incomplete lineage sorting and hybridization

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Abstract *Cyrtandra* (Gesneriaceae) is a genus of flowering plants with over 800 species distributed throughout Southeast Asia and the Pacific Islands. On the Hawaiian Islands, 60 named species and over 89 putative hybrids exist, most of which are identified on the basis of morphology. Despite many previous studies on the Hawaiian *Cyrtandra* lineage, questions regarding the reconciliation of morphology and genetics remain, many of which can be attributed to the relatively young age and evidence of hybridization between species. We utilized targeted enrichment, high-throughput sequencing, and modern phylogenomics tools to test 31 Hawaiian *Cyrtandra* samples (22 species, two putative hybrids, four species with two samples each, one species with four samples) and two outgroups for species relationships and hybridization in the presence of incomplete lineage sorting (ILS). Both concatenated and species-tree methods were used to reconstruct species relationships, and network analyses were conducted to test for hybridization. We expected to see high levels of ILS and putative hybrids intermediate to their parent species. Phylogenies reconstructed from the concatenated and species-tree methods were highly incongruent, most likely due to high levels of incomplete lineage sorting. Network analyses inferred gene flow within this lineage, but not always between taxa that we expected. Multiple hybridizations were inferred, but many were on deeper branches of the island lineages suggesting a long history of hybridization. We demonstrated the utility of high-throughput sequencing and a phylogenomic approach using 569 loci to understanding species relationships and gene flow in the presence of ILS.

Key words: *Cyrtandra*, gene flow, Gesneriaceae, Hawaiian Islands, hyb-seq, phylogenomics.

1 Introduction

Since Darwin's (1859) first observation of species diversity on the Galapagos Islands, oceanic island systems have played a key role in the study of evolutionary processes such as speciation and adaptive radiation (Carlquist, 1974; Schluter, 2000; Emerson, 2002). Although the underlying processes of diversification within species radiations remain poorly understood (Yoder et al., 2010), the natural isolation, young ages, and small areas of island systems make them ideal settings for studying the mechanisms of evolution (Funk & Wagner, 1995; Fleischer et al., 1998; Price & Clague, 2002; Price, 2004; Price & Wagner, 2004). The Hawaiian Islands in particular are an excellent site for biogeographic and phylogenetic studies due to its geological history, small total area, and highly isolated location nearly 4000 km from the nearest land mass. The Hawaiian Islands are a hotspot archipelago, a chain of age-sorted islands formed by the northwest movement of the Pacific plate over an area of active volcanism. Kaua'i, the oldest of the current six main high islands, was formed approximately 4.7 million years ago (Mya) and sits in the

northwest, while Hawai'i, the youngest and largest of the high islands, was formed approximately 0.5 Mya and sits in the southeast (Price & Clague, 2002). The three high islands of Moloka'i, Lana'i, and Maui are products of submergence and erosion of a larger prehistoric island called Maui Nui, which formed around 1.2 Mya (Carson & Clague, 1995; Price & Elliott-Fisk, 2004). Evidence has shown that most of the Hawaiian flora and fauna likely diversified on the current high islands over the most recent 5 My (Price & Clague, 2002). However, there are lineages, such as the Hawaiian lobeliads (Givnish et al., 2009), which are thought to have colonized the now eroded atolls when they were higher islands situated northwest of the main high islands, which are estimated to have formed between 7 and 28 Mya, prior to dispersing to the current high Hawaiian Islands (Price & Clague, 2002).

Due to its extreme isolation, the Hawaiian Islands have one of the highest rates of species endemism, with 89% of its flowering plant species restricted to these islands (Wagner et al., 1990; Carson & Clague, 1995; Price, 2004). Among angiosperms, approximately 259 natural introductions are

inferred to have given rise to over 1000 species, of which nearly 90% are endemic to the Hawaiian Islands and over 50% are endemic to a single island (Wagner et al., 1990; Price, 2004; Price & Wagner, 2018). Much of the diversity within each of the Hawaiian angiosperm lineages is likely to have resulted from a single colonization event to the archipelago followed by dispersal throughout the islands and includes lineages such as the Hawaiian silversword alliance (Baldwin & Sanderson, 1998), Hawaiian *Psychotria* L. (Nepokroeff et al., 2003), Hawaiian lobeliads (Givnish et al., 2009), *Schiedea* Cham. & Schltdl. (Willyard et al., 2011), Hawaiian Stachydae (Roy et al., 2013; Welch et al., 2016), Hawaiian *Silene* L. (Eggens et al., 2007), and *Hesperomannia* A.Gray (Kim et al., 1998; Morden & Harbin, 2013), though there are angiosperm groups such as the Hawaiian *Scaevola* L. (Howarth et al., 2003) and Hawaiian *Coprosma* J.R.Forst. & G.Forst. (Cantley et al., 2014), which are inferred to have colonized the islands more than once. The rapid radiation of the Hawaiian flora has resulted in an array of morphologically diverse lineages exhibiting low levels of genetic variation (Baldwin & Sanderson, 1998; Lindqvist & Albert, 2002; Clark et al., 2009; Givnish et al., 2009; Baldwin & Wagner, 2010), and many are thought to hybridize leading to further difficulty in understanding their evolutionary histories (Smith et al., 1996; Wagner et al., 1999; Pillon et al., 2013b; Roy et al., 2013; Cantley et al., 2014; Stacy et al., 2014). One such hybridization-prone lineage with high levels of phenotypic variation and low levels of genetic diversity is Hawaiian *Cyrtandra* J.R.Forst. & G.Forst. (Gesneriaceae).

Hybridization has long been known to play a role in the diversification and adaptation of plants (Zirkle, 1934; Anderson, 1953; Anderson & Stebbins, 1954; Stebbins, 1959; Mallet, 2005), and evidence for its importance continues to grow (Mallet, 2008; Soltis & Soltis, 2009; Abbott et al., 2013). It is estimated that approximately 25% of all plant species, especially younger lineages, are involved in hybridization and introgression with other plant species (Mallet, 2005), but it has been suggested this is a significant underestimation due to approaches used in developing classification and to lack of study in many areas, especially islands (Raven, 1976; Ellstrand et al., 1996). Hybridization studies prior to molecular sequence data relied on phenotypic observations and comparisons, which were largely insufficient for understanding the importance of introgression in plant evolution as the extent of gene flow could not be accurately measured (Anderson, 1948; Goulet et al., 2017). The advent of Sanger sequencing has made molecular phylogenetics possible, allowing for greater insight into the evolutionary histories of plant lineages and mechanisms of hybridization (Savolainen & Chase, 2003). Phylogenetic evidence suggests that introgression in the ancestry of many lineages is common, and that hybridization is a major factor in species evolution, especially among plants (McDade, 2000). However, testing for hybridization in the presence of incomplete lineage sorting (ILS) has been difficult without the use of genome or transcriptome data. Modern phylogenomic methods and current high-throughput sequencing (HTS) techniques of whole genomes and transcriptomes have furthered our understanding of evolutionary processes and have given us greater insight into hybridization in plants by allowing us to test for underlying genetic structure and relationships within and between lineages (Soltis & Soltis, 2009; Jackson et al., 2016; Solís-Lemus & Ané, 2016).

A multitude of different HTS techniques have been used to resolve difficult groups and understand how their evolutionary histories have been affected by hybridization and/or ILS. Restriction-site associated DNA sequencing (RAD-seq; Hipp et al., 2014; Vargas et al., 2017), transcriptomics (Yang & Smith, 2014; Roberts & Roalson, 2018), genome skimming (Straub et al., 2012; Bock et al., 2014; Weitemier et al., 2014), and targeted enrichment (Cronn et al., 2012; Stull et al., 2013; Weitemier et al., 2014; Folk et al., 2015, 2017), among other methods, have been used in phylogenomic studies. Targeted enrichment is among the leading strategies for obtaining large amounts of data for relatively low cost, which has yielded highly informative data for resolving difficult groups, particularly those under putative ILS and/or hybridization complexity (*Asclepias*, Weitemier et al., 2014; *Heuchera*, Folk et al., 2017). Furthermore, modern phylogenomic methods running on accessible and powerful computing clusters has allowed researchers to test for hybridization in the presence of ILS using large datasets in relatively short amounts of time (Than et al., 2008; Solís-Lemus & Ané, 2016; Blischak et al., 2018).

Cyrtandra is the most species-rich genus in the family Gesneriaceae, with over 800 species representing 15–20% of all species within the family (Wagner et al., 1999; Cronk et al., 2005). Found in the understory of many Old World tropical forests, the morphologically diverse species within this genus are often long-lived shrubs and small trees, though they are also sometimes herbs, lianas, and epiphytes (Wagner et al., 1990). This genus is likely to have evolved in the Indo-Malaysia region of Southeast Asia and later dispersed throughout the Pacific to become one of the most widely distributed plant genera in the region (Burtt, 2001; Cronk et al., 2005; Clark et al., 2008, 2009). Based on the highly similar habitats (tropical forest understory) across the broad geographic region occupied by the genus, *Cyrtandra* is likely to have diverged under a dispersal-mediated allopatry model rather than ecological pressures (Cronk et al., 2005; Clark et al., 2008). To date, all examined species of *Cyrtandra* are diploid (Kiehn, 2005) and there is no evidence of polyploidy in the group. Prior phylogenetic studies support monophyly of the Pacific clade, suggesting that the ~300 species within this clade diversified within the last 22 million years (Clark et al., 2009; Johnson et al., 2017). While species of *Cyrtandra* are morphologically quite diverse across their range, species of Pacific clade are much more homogeneous, as they are almost exclusively understory shrubs or small trees with white flowers and fleshy berries (Cronk et al., 2005). However, the Fijian and Hawaiian lineages of *Cyrtandra* exhibit marked morphological diversity in other characters (Gillett, 1967).

Hawaiian *Cyrtandra* are, most likely, of Fijian origin and colonized Kaua'i after its formation approximately 4.7 Mya (Clark et al., 2008, 2009; Johnson et al., 2017). To date, the most comprehensive study on morphological diversity was conducted on the Hawaiian species of *Cyrtandra* (Wagner et al., 1990), but many questions regarding their evolutionary histories remain. Currently 60 named species of Hawaiian *Cyrtandra* are recognized based on morphological (Wagner et al., 1990) and genetic (Clark et al., 2008, 2009; Pillon et al.,

2013a, 2013b) characters (Fig. 1). Interestingly, 51 of the 60 species are single-island endemics and the majority of species have a narrow distribution within islands, with up to eight species observed to inhabit a single community (Wagner et al., 1990). O'ahu has the most species diversity (24 species) followed by Kaua'i (14), Maui (12), Moloka'i and Hawai'i (10 each), and Lana'i (3; Wagner & Roalson, unpubl.). It should also be noted that six of the nine multi-island species are only found on multiple islands of the Maui Nui complex, and it is not clear whether these are derived from a

Fig. 1. **A,** *Cyrtandra wawrae*, Kaua'i, Wailua River, 2017, K. R. Wood (image only), showing the post-anthesis persistent calyx and peltate leaf. **B,** *C. cordifolia*, O'ahu, *Wagner 5691* (BISH), showing corolla and calyx shape. **C,** *C. longifolia*, Kaua'i, La'au Summit, 2009, K. R. Wood (image only), showing leaves and flowers. **D,** *C. paludosa* subsp. *microcarpa*, Kaua'i, Blue Hole, headwaters of N fork of Wailua River on 21 Feb 2017, showing young fruit after calyx drops. **E,** *C.* sp., Kaua'i, headwaters of Waikoko, *Wood 12775* (PTBG), showing unique leaf base. **F,** *C. hawaiensis*, Maui, East Maui, Pipiwai Stream, *Wagner et al. 5855* (BISH, US), showing calyx shape. **G,** *C. grayi*, Maui, West Maui, Wagner (image only), showing habit and leaves. **H,** *C. paludosa* subsp. *microcarpa*, Kaua'i, Blue Hole, headwaters of N fork of Wailua River on 21 Feb 2017, showing flower. **I,** *C. platyphylla*, Maui: East Maui, *Wagner* et al. *5920* (BISH), showing pubescence, leaf, and flowers.

broad distribution when Maui Nui was a single island or dispersal after submersion and separation of the upland areas into separate islands. Eighty-nine putative Hawaiian hybrid combinations have been listed to date (Wagner et al., 1990; Wagner & Roalson, unpubl.), primarily based on morphological intermediacy and sympatry of their putative parent species, though molecular evidence does exist for a few putative hybrids (Smith et al., 1996).

Species-diagnostic haplotypes have been found for species on Kaua'i but not for species on the younger islands of O'ahu or Hawai'i (Pillon et al., 2013a) and phylogenetic analyses using nuclear and chloroplast loci show that species of *Cyrtandra* from Kaua'i form a well-supported lineage in the Hawaiian clade while Hawai'i taxa do not (Clark et al., 2009; Johnson et al., 2015). This evidence suggests that the diversity of *Cyrtandra* across the Hawaiian Islands is the result of a classic progression rule pattern from the oldest to youngest as has been hypothesized for a number of other Hawaiian lineages such as *Metrosideros* (Percy et al., 2008) and *Psychotria* (Nepokroeff et al., 2003), though Johnson et al. (2017) present evidence that *Cyrtandra* is likely to have dispersed from both old to young and young to old islands in the Hawaiian Islands.

Hawaiian *Cyrtandra* poses a complex problem, where morphology and genetics have not been reconciled despite numerous attempts to do so (Smith et al., 1996; Cronk et al., 2005; Clark et al., 2008, 2009; Pillon et al., 2013a, 2013b; Johnson et al., 2017). The confusing array of morphological diversity has been attributed to hybridization (Smith et al., 1996; Pillon et al., 2013a, 2013b; Johnson et al., 2019) and convergence (Clark et al., 2008), though understanding the patterns of introgression or convergence has been difficult with the molecular data that has been generated for these organisms. Here, we use targeted enrichment to sequence 569 loci from 33 taxa including putative hybrids. We hypothesize that both incomplete lineage sorting and hybridization will be evident in the genetic data recovered for these taxa. Additionally, we hypothesize that genetic data will reflect the morphology of these species. While the stepping-stone model of dispersal is well accepted, we expect *Cyrtandra* to also exhibit evidence of back-dispersal on the basis on morphology and the results from Johnson et al. (2017). The advent of HTS and modern phylogenomic techniques has allowed us to further our understanding of Hawaiian *Cyrtandra*, and we hope the results from this research positively influence how other Hawaiian and island-dwelling flora are studied.

2 Materials and Methods

2.1 Sampling
We sampled 31 specimens of *Cyrtandra* from across the Hawaiian Islands, and two species of *Cyrtandra* from Fiji to use as outgroups based on prior studies of this genus (Table 1; Cronk et al., 2005; Clark et al., 2008; Johnson et al., 2017). We chose to sample across the islands rather than on a single island in order to look for biogeographical patterns that may inform the direction of future studies on this and other Hawaiian lineages. Many species of *Cyrtandra* are found only on a single island, but for those found across multiple islands, we attempted to sample multiple specimens. We sampled 22 species (four species with two samples, one with four samples) and two putative hybrids, including putative parent species in the sampling. These individuals were hypothesized to be hybrids on the basis of intermediate morphology in the field and include *C. hawaiensis* × *C. grayana* (Moloka'i) and *C. longifolia* × *C. kauaiensis* (Kaua'i; Table 1). Genomic DNA was extracted from silica-dried leaf material housed in the Roalson lab or supplied by the National Tropical Botanic Garden (NTBG) using a CTAB protocol (Doyle & Doyle, 1987).

2.2 Library preparation and sequencing
DNAs were checked for quality using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and poor-quality samples were cleaned using an ethanol precipitation step. DNAs were then quantified with a Qubit (dsDNA BR assay, Thermo Fisher Scientific). We prepared 33 genomic DNA libraries using a NEBNext Ultra II kit (New England Biolabs, Ipswich, MA, USA) starting with between 70 ng-1μg of input DNA sheared for 350 bp fragments with a Covaris sonicator using the manufacturer recommended settings (model M220; Covaris, Woburn, MA, USA). Ampure XP beads (Beckman Coulter, Brea, CA, USA) were used in bead-based size selection steps, using 0.25x volumes for the first step and 0.1x volumes for the second. PCR enrichment used between five and ten cycles depending on the amount of input DNA. Libraries were barcoded using NEBNext multiplex oligos (New England Biolabs) and quantified using qPCR (NEBNext quant kit, New England Biolabs). Probes for target enrichment of our libraries were designed with the Hyb-Seq protocol (Weitemier et al., 2014) using a *Boea hygrometrica* genome (Xiao et al., 2015) and *Primulina pteropoda* transcriptome (Ai et al., 2015) — two Old World species of Gesneriaceae. The Hyb-Seq protocol was meant for use with a transcriptome and genome from the same species, but because there are no transcriptomes or complete genomes available for *Cyrtandra*, we opted to use two available and related Old World gesneriad taxa. An unfiltered, biotinylated baitset was synthesized for us using 3x tiling and a bait length of 120 bp totaling 3420 unique baits by MYcroarray (Ann Arbor, MI; now Arbor Biosciences, USA). We followed the MyBaits v.3 protocol but using pools of six libraries and between 98–126 ng of input DNA (14–18 ng DNA per library) in 7 μL per enrichment reactions (six total reactions). Hybridization was performed at 65°C for 25.5 hours and NEB 2x HiFi MasterMix (New England Biolabs) was used in the final amplification step after removing the beads from the enriched library pools. The thermocycler profile used 15 μL of template, an annealing temperature of 60°C for 30 seconds, an extension temperature of 72°C for 35 seconds, and 18 total cycles. Enriched library pools were cleaned using a Monarch PCR & DNA cleanup kit (New England Biolabs), quantified using a Qubit (dsDNA HS assay; Thermo Fischer Scientific), and checked for quality and size distribution using a Fragment Analyzer (Advanced Analytical, Ankeny, IA, USA). Enriched pools were then combined in equimolar amounts to create a final pool of 36 samples, followed by a final clean-up step using 0.7x volumes of AxyPrep purification beads (Thomas Scientific, Swedesboro, NJ, USA). The final library was sequenced on an Illumina MiSeq (Clinical

Table 1 Sampled specimens, collectors, herbarium, and location. All specimens unless otherwise indicated are from the Hawaiian Islands so only island name is given.

Species (sample designation, when necessary)	Voucher[1]
Cyrtandra calpidicarpa (Rock) H.St.John & Storey	O'ahu, *J. R. Clark 571* (PTBG)
Cyrtandra cordifolia Gaudich.	O'ahu, *J. R. Clark 579* (PTBG)
Cyrtandra giffardii Rock	Hawai'i, *K. Wood 12258* (PTBG)
Cyrtandra grayana Hillebr.	Moloka'i, *K. Wood 11401* (PTBG)
Cyrtandra grandiflora Gaudich.	O'ahu, *J. R. Clark 577* (PTBG)
Cyrtandra grayi C.B.Clarke (-1)	Maui, *J. R. Clark 667* (PTBG)
(-2)	Moloka'i, *H. Oppenheimer H110624* (BISH)
Cyrtandra hawaiensis C.B.Clarke (-1)	Maui, *J. R. Clark 662* (PTBG)
(-2)	Moloka'i, *K. Wood 11385* (PTBG)
(-3)	O'ahu, *J. R. Clark 569* (PTBG)
(-4)	O'ahu, *J. R. Clark 567* (PTBG)
Cyrtandra hawaiensis × *C. grayana*	Moloka'i, *K. Wood 11392* (PTBG)
Cyrtandra kauaiensis Wawra	Kaua'i, *J. R. Clark 556* (PTBG)
Cyrtandra kaulantha Wawra	O'ahu, *J. R. Clark 572* (PTBG)
Cyrtandra longifolia (Wawra) Hillebr.	Kaua'i, *J. R. Clark 551* (PTBG)
Cyrtandra longifolia × *C. kauaiensis*	Kaua'i, *J. R. Clark 552* (PTBG)
Cyrtandra lysiosepala (A.Gray) C.B.Clarke	Hawai'i, *K. Wood 12418* (PTBG)
Cyrtandra macrocalyx Hillebr.	Moloka'i, *H. Oppenheimer H110622* (BISH)
Cyrtandra munroi C.N.Forbes (-1)	Lana'i, *H. Oppenheimer H120638* (BISH)
(-2)	Maui, *J. R. Clark 678* (PTBG)
Cyrtandra occulta A.C.Sm.	Fiji, *J. R. Clark 694* (PTBG)
Cyrtandra paludosa Gaudich.	Maui, *J. R. Clark 680* (PTBG)
Cyrtandra platyphylla C.B.Clarke	Maui, *H. Oppenheimer H100512* (BISH)
Cyrtandra sandwicensis (H. Lév) H.St.John & Storey (-1)	O'ahu, *J. R. Clark 576* (PTBG)
(-2)	O'ahu, *J. R. Clark 578* (PTBG)
Cyrtandra tintinnabula Rock	Hawai'i, *S. Perlman 17676* (PTBG)
Cyrtandra victoriae Gillespie	Fiji, *M. Johnson MJ049* (SUVA)
Cyrtandra wagneri Lorence & Perlman	Hawai'i, *S. Perlman 17675* (PTBG)
Cyrtandra wainihaensis H.Lév.	Kaua'i, *J. R. Clark 549* (PTBG)
Cyrtandra wawrae C.B.Clarke (-1)	Kaua'i, *J. R. Clark 550* (PTBG)
(-2)	Kaua'i, *K. Wood 17317* (PTBG)
Cyrtandra sp. nov. Kaua'i	Kaua'i, *K. Wood 12775* (PTBG)
Cyrtandra sp. nov. E. Maui	Maui, *K. Wood 6249* (PTBG)

[1]Herbaria abbreviations are as follows: BISH: Bishop Museum; PTBG: National Tropical Botanic Garden; SUVA: University of the South Pacific.

Genomics Center, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA) using paired-end 250 bp reads. Reads were demultiplexed through the MiSeq controller software.

2.3 Marker assembly and dataset creation
Raw reads were first assessed for quality using the tools implemented in FastQC (Simon, 2010). Trimmomatic v. 0.36 (Bolger et al., 2014) was then used to remove contaminating adapter sequences, trim the first 13 bases from each read, and filter low-quality paired-end reads with a sliding window of 5 bp and only accepting quality scores (phred33) of at least Q20 (options HEADCROP:13 LEADING:3 TRAILING:3 SLIDINGWINDOW5:20 MINLEN:36). For each sample, we used the default settings in Geneious 8 (Kearse et al., 2012) to merge paired-end reads and then map reads to the probe-set file as the reference (per suggestions by Weitemier et al., 2014). For mapping, a single reference sequence was constructed that contained each locus separated by 200 N's. This approach was used so that a single mapping run per species would be performed rather than separate mapping runs per locus per sample. Consensus sequences were extracted for each locus for each sample. "Splash-zone" regions, reads from the intron sequences flanking the exon sequences from the probe-set, were also extracted. Default settings allowed for ambiguity calls in cases of multiple alleles, and regions where coverage was not at least 5x were called as "?" and treated as missing data.

2.4 Gene tree estimation
Individual loci were aligned using MAFFT v. 7.2.71 (Katoh et al., 2009) using the automatic setting (--auto). For each locus, the alignments were trimmed using Phyutility v.2.7.1 (Smith & Dunn, 2008) for minimal column occupancy at a 0.5 cutoff values prior to reconstructing the final ortholog tree. Final gene tree estimation and bootstrapping (100 replicates per alignment) were performed using RAxML v. 8.2.9 (Stamatakis, 2014) using the GTRCAT model for all alignments at each column-occupancy cutoff. SumTrees (Sukumaran &

Holder, 2010) was used to summarize bootstrap results onto the best-scoring maximum likelihood gene trees and to collapse nodes with <30% bootstrap support in order to minimize potential impacts of gene-tree estimation error on species-tree reconstructions (Mirarab & Warnow, 2015; Sayyari & Mirarab, 2016).

2.5 Species tree estimation

For each locus at the 0.5 column occupancy cutoff, both gene alignments (for concatenation analyses, described below) and gene trees (for summary-coalescent analyses, described below) were used in species tree estimations. Because assumptions behind any one phylogenetic analysis are unlikely to apply for our dataset, we used both concatenation and summary-coalescent methods in a variety of analyses. A supermatrix was created for each dataset including all of our taxa by concatenating all loci with Phyutility. A likelihood analysis was performed in RAxML using the GTRGAMMA model and partitioning the alignment with loci treated as separate partitions. Two hundred rapid bootstrap replicates followed by an ML tree search (option -f a) from every fifth replicate were used for the concatenated alignments.

Under scenarios of high ILS, it has been demonstrated that concatenation has lower power to reconstruct phylogenetic relationships (Mirarab et al., 2014; Chou et al., 2015). Therefore, we addressed this possibility using the summary-coalescent method implemented in ASTRAL v. 5.6.1 (Mirarab & Warnow, 2015; Zhang et al., 2017) with default settings for summary-coalescent species tree estimation. Branch support was estimated with standard ASTRAL support values (i.e., local posterior probabilities; Sayyari & Mirarab, 2016) and multilocus bootstrapping (Seo, 2008), hereafter referred to as ASTRAL-pp and ASTRAL-mlbs, respectively. Additionally, quartet scores were calculated for the ASTRAL species trees to measure the amount of gene tree conflict around each branch.

2.6 Analyses of introgression on islands

Gene trees built from the alignments with 0.5 column occupancy cutoff were segregated into four exclusive pools prior to introgression analyses. Each pool corresponded the taxa present on each island group, as follows: 1) Kaua'i: *C. wainihaensis, C. kauaiensis, C. longifolia* × *C. kauaiensis, C. longifolia, C. wawrae 1, C. wawrae 2,* and C. sp. nov. Kaua'i; 2) O'ahu: *C. hawaiensis 3, C. grandiflora, C. kaulantha, C. hawaiensis 4, C. calpidicarpa, C. sandwicensis 1, C. sandwicensis 2,* and *C. cordifolia;* 3) Maui Nui: *C. hawaiensis 2, C. hawaiensis* × *C. grayana, C. grayi 2, C. macrocalyx, C. grayana 2, C. hawaiensis 1, C. munroi 1, C. paludosa, C.* sp. nov. E. Maui, *C. munroi 2, C. grayi 1,* and *C. platyphylla;* and 4) Hawai'i: *C. tintinabula, C. lysiosepala, C. giffardii,* and *C. wagneri.* Because Hawai'i was sampled for only 4 taxa, we did not include it for subsequent network estimations. Gene tree bootstrap cutoffs were kept at 30% as applied through SumTrees during gene tree estimation.

2.7 Phylogenetic network estimation

Each set of pruned gene trees was used in the Julia package in PHYLONETWORKS (Solís-Lemus & Ané, 2016; Solís-Lemus et al., 2017) to infer phylogenetic networks by island group in the presence of incomplete lineage sorting and hybridization.

We used SNaQ (Species Network applying Quartets; Solís-Lemus & Ané, 2016) to evaluate the most likely network and to calculate γ, the vector of inheritance probabilities describing the proportion of loci inherited by a hybrid node from each of its parents. We performed nested analyses that allowed from zero to four hybridization (h) events and compared the negative log psuedolikelihood scores. Optimization in each nested analysis was performed for 5 or 10 independent runs. A sharp improvement in negative log pseudolikelihood scores is expected for each nested analysis, with a slow, linear improvement after h reaches the optimal value. Finally, we selected the best network with h hybridization events when the pseudolikelihood values hit a plateau and the allowance of additional hybrid branches does not significantly improve the pseudolikelihood values. Branch and hybrid branch support was assessed on the best network using 100 bootstrap replicates.

For comparison with SNaQ and using the same set of gene trees, phylogenetic networks were additionally estimated for each island group using PhyloNet v. 3.6.4 (Than et al., 2008) using a maximum pseudolikelihood approach (Yu & Nakhlah, 2015). Nested searches were performed over 10 independent runs, allowing from zero to four hybridization events, and optimizing branch lengths and inheritance probabilities under full likelihood. For model selection we used three criteria: the Akaike Information Criterion (AIC; Akaike, 1973), the bias-corrected Akaike Information Criterion (AICc; Sugiura, 1978), and the Bayesian Information Criterion (BIC; Schwarz, 1978). The number of parameters equals the number of branch lengths being estimated, plus the number of hybridization probabilities being estimated, and the number of gene trees used to estimate the likelihood. We chose the best model with the lowest information criterion score, with preference given toward BIC. Tanglegrams were created in Dendroscope (Huson & Scornavacca, 2012) to compare network estimations from SNaQ and PhyloNet. Because the outputs for both programs are semi-directed, unrooted networks, we oriented the networks based on the ASTRAL topology.

2.8 *D* and $\hat{f}_d$ statistics

Incongruence among *Cyrtandra* species relationships was further tested using the *D*-statistic, also known as the ABBA-BABA test (Durand et al., 2011), and the $\hat{f}_d$ statistic (Martin et al., 2015), which estimates the fraction of the genome shared through introgression. For these analyses we used the 0.5 column occupancy concatenated alignment and removed all positions with gaps. The R package HYBRID-CHECK (Ward & Oosterhout, 2016) was used to calculate statistics and count the number of ABBA and BABA site patterns in four-population phylogenies. The *D* and $\hat{f}_d$ statistics were calculated across four-population phylogenies where network analyses indicated potential hybridization events. Specifically, when the network analyses indicated potential hybridization events between two populations to produce a third hybrid population, we calculated the *D* and $\hat{f}_d$ statistics for two tests of gene flow: (a) between the first population and the third, and (b) between the second population and the third (Fig. 2A). For example, on Maui-Nui a hybridization event was indicated between *C. hawaiensis* 2 and *C. paludosa* to produce *C. hawaiensis* 1 (Figs. 2B, 2C). We
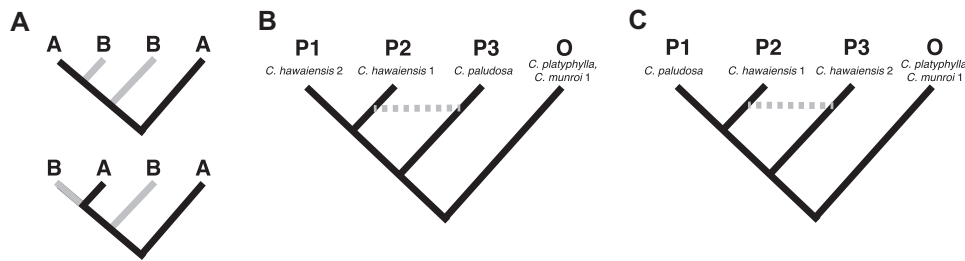
Fig. 2. Schematic representation of the ABBA-BABA tests for gene flow among Hawaiian *Cyrtandra*. **A,** The *D*-statistic measures the asymmetry in the occurrence of two incongruent allele patterns (ABBA and BABA) among four selected populations (Population 1 [P1], Population 2 [P2], Population 3 [P3], and an outgroup [O]). These frequencies should arise with equal frequency in the absence of introgression, but deviate if P3 exchanges genes with either P1 or P2. The *D*-statistic will be positive if ABBA site patterns are more prevalent than BABA site patterns. **B,** Test for hybridization between *C. paludosa* (P3) and *C. hawaiensis* 1 (P2). **C,** Test for hybridization between *C. hawaiensis* 2 (P3) and *C. hawaiensis* 1 (P2).

therefore calculated the *D* and $\hat{f}_d$ statistics in two separate analyses to test for gene flow between *C. hawaiensis* 2 and *C. hawaiensis* 1, and between *C. paludosa* and *C. hawaiensis* 1. We expect equal counts of the two site patterns (ABBA and BABA) when incomplete lineage sorting (ILS) causes discordance. On the other hand, if discordance is caused by gene flow, we expect the ABBA site patterns to be more prevalent than the other (i.e., *D* values will be positive). Although a full genome (or other linkage information) is not currently available for *Cyrtandra*, our loci likely represent a sampling of mostly unlinked markers from across the genome. Under these circumstances, a jackknife approach was used to test for genome-wide variation in incongruence (Meyer et al., 2012; Eaton & Ree, 2013).

3 Results

3.1 Hyb-seq and read mapping
Our probe-set design from the Hyb-Seq protocol (Weitemier et al., 2014) contained 570 sequences for enrichment totaling 180 784 bp, with an average length of 317.2 bp per probe. The largest and shortest probe sequences were 2 053 bp and 120 bp, respectively. Preliminary analyses on datasets with and without the intron regions flanking the targeted exons were conducted. Here, we focus on the dataset including the intron sequences flanking the exons as the data was more informative. After removing the two samples (*C. procera* and *C. procera* × *C. grayana*) with low percentages of mapped reads, the average sequencing coverage across samples was 226.3 × (Table 2). The least-covered sample had 58.0X average coverage and the most-covered sample had 593.3X average coverage. Only three samples had sequences for one of the loci, which was removed prior to analyses. Following individual alignment and filtering of 569 loci, each locus had an average length of 819.3 bp. The largest aligned locus was 2590 bp and the shortest was 233 bp. The aligned length of the concatenated 33-taxon, 569 locus supermatrix was 466 201 bp. 58 683 characters (12.6%) were parsimony informative and 8.31% of the matrix was missing data. Considering only the ingroup taxa, 52 029 characters (11.2%) were parsimony informative and 8.37% of the matrix was missing data.

3.2 Concatenated phylogenetic inference
An unpartitioned maximum likelihood analyses was conducted on the concatenated dataset using 2500 bootstrap replicates. The concatenated analysis yielded a phylogeny with bootstrap support (BS) ranging from BS = 4 to BS = 100 (Fig. 3A). Most Kaua'i taxa (*Cyrtandra wainihaensis, C. longifolia, C. wawrae,* and *C.* sp. nov. Kaua'i) formed a paraphyletic group in relation to the rest of the taxa with moderately strong support (BS > 89). Two Kaua'i samples, *C. kauaiensis* and *C. longifolia* × *C. kauaiensis* were sister to each other (BS = 100) and were in a clade with O'ahu and Maui Nui taxa. O'ahu, Maui Nui, and Hawai'i taxa were interspersed throughout the concatenated phylogeny. Bootstrap support for the clade containing all but the Kaua'i grade of taxa was strong (BS = 95), but support for the two clades nested in that grade were low (BS = 75 and BS = 74). The four sampled *C. hawaiensis* were in two separate clades. *Cyrtandra hawaiensis*-3 and -4, *C. grandiflora, C. hawaiensis* × *C. grayana,* and *C. kaulantha* formed a clade (BS = 10), whereas *C. hawaiensis*-1 and -2 form a clade sister to a clade with *C. calpidicarpa, C. munroi*-1, *C. paludosa, C. longifolia* × *C. kauaiensis,* and *C. kauaiensis* (BS = 75). *Cyrtandra longifolia* × *C. kauaiensis* was sister to *C. kauaiensis* (BS = 100). The rest of the sampling is distributed in three clades: one from O'ahu (*C. sandwicensis*-1 and -2 and *C. cordifolia;* BS = 100), one from Maui Nui (*C. grayi*-2, *C. munroi*-2, *C. macrocalyx,* and *C. grayana;* BS = 100), and a third from Maui Nui and Hawai'i (*C. giffardii, C. tintinnabula, C. grayi*-1, *C.* sp. nov. E. Maui, *C. platyphylla, C. lysiosepala,* and *C. wagneri;* BS = 100).

3.3 Coalescent phylogenetic inference
We generated maximum likelihood trees with 100 bootstrap replicates for each gene, and then used these gene trees to create species trees in ASTRAL. The ASTRAL analysis on our dataset for all taxa yielded a phylogeny with a different topology than that found for the concatenated dataset (Fig. 3B). The ASTRAL analysis reconstructed a species tree where the Kaua'i taxa formed a paraphyletic group in relation to the rest of our sampled taxa. The two sampled *Cyrtandra wawrae* and *C.* sp. nov. Kaua'i formed a clade (ASTRAL-mlbs = 79; ASTRAL-pp = 0.65) sister to all other Hawaiian taxa, while *C. wainihaensis, C. kauaiensis,*

Table 2 Summary of targeted enrichment sequencing and mapped reads.

Sample	Raw Reads	Trimmed Reads	Assembled Reads	Assembled Reads (%)	Unassembled Reads	Extracted Loci	Mean Coverage
C. calpidicarpa	699 974	686 958	562 663	0.82	92 721	569	286.30×
C. cordifolia	843 912	828 834	657 681	0.79	122 291	570	344.06×
C. giffardii	375 466	369 328	324 892	0.88	27 954	569	188.37×
C. grayana	736 298	723 810	605 696	0.84	74 834	569	328.00×
C. grandiflora	766 312	752 264	601 847	0.80	101 423	569	311.88×
C. grayi-1	288 814	284 660	247 223	0.87	26 679	569	141.35×
C. grayi-2	411 964	405 334	343 433	0.85	40 143	569	194.30×
C. hawaiensis-1	281 798	277 608	233 635	0.84	33 675	569	132.45×
C. hawaiensis-2	660 106	648 308	545 274	0.84	60 644	569	284.31×
C. hawaiensis-3	186 366	180 942	115 875	0.64	51 919	567	57.98×
C. hawaiensis-4	636 918	627 200	485 865	0.77	112 401	569	265.38×
C. hawaiensis × *C. grayana*	609 880	600 490	508 345	0.85	63 963	569	294.62×
C. kauaiensis	711 810	699 500	618 927	0.88	40 471	569	324.02×
C. kaulantha	1 018 210	1 000 560	792 993	0.79	147 151	570	423.28×
C. longifolia	582 082	573 036	505 417	0.88	42 693	569	288.90×
C. longifolia × *C. kauaiensis*	623 864	612 832	530 140	0.87	46 032	569	273.26×
C. lysiosepala	1 397 914	1 374 832	1 086 373	0.79	224 379	566	593.31×
C. macrocalyx	734 202	722 204	615 422	0.85	67 566	569	346.65×
C. munroi-1	248 414	243 204	181 760	0.75	43 932	568	83.82×
C. munroi-2	409 700	403 332	349 810	0.87	35 630	569	197.42×
C. occulta	560 408	548 246	339 712	0.62	188 270	569	169.97×
C. paludosa	179 246	176 166	151 344	0.86	15 606	569	82.85×
C. platyphylla	117 110	115 296	98 654	0.86	12 554	568	59.12×
C. sandwicensis-1	650 000	638 106	412 039	0.65	199 341	569	200.59×
C. sandwicensis-2	639 090	628 388	477 252	0.76	108 226	569	254.25×
C. tintinnabula	409 766	402 994	352 425	0.87	27 621	569	201.66×
C. victoriae	528 964	519 828	322 053	0.62	170 657	569	166.63×
C. wagneri	384 676	378 898	342 816	0.90	19 580	569	199.89×
C. wainihaensis	283 526	279 196	235 793	0.84	27 991	569	136.71×
C. wawrae-1	420 752	410 126	278 095	0.68	106 721	568	140.35×
C. wawrae-2	439 702	432 262	287 115	0.66	126 679	569	141.40×
C. sp. nov. Kaua'i	481 400	473 074	307 485	0.65	143 211	569	152.69×
C. sp. nov. E. Maui	423 958	416 928	338 505	0.81	63 267	569	200.74×

C. longifolia × *C. kauaiensis,* and *C. longifolia* formed a clade (ASTRAL-mlbs = 100; ASTRAL-pp = 0.94) sister to all non-Kaua'i taxa. *Cyrtandra grandiflora, C. hawaiensis* × *C. grayana, C. kaulantha, C. hawaiensis,* and *C. calpidicarpa* formed a clade (ASTRAL-mlbs = 65; ASTRAL-pp = 0.92) sister to the rest of Hawaiian *Cyrtandra. Cyrtandra munroi*-1 and *C. paludosa* var. *paludosa* formed a weak to moderately supported clade (ASTRAL-mlbs = 88; ASTRAL-pp = 0.42) sister to the remaining taxa. *Cyrtandra* sp. nov. E. Maui, *C. munroi*-2, *C. cordifolia,* and *C. sandwicensis* formed a clade (ASTRAL-mlbs = 67; ASTRAL-pp = 0.6) sister to a clade formed by the remaining Maui Nui and Hawai'i taxa (ASTRAL-mlbs = 73; ASTRAL-pp = 0.57). Quartet scores for ingroup branches of ASTRAL species trees found very low scores (<50; Fig. S1). A higher quartet score indicates that a larger proportion of the gene trees share the same topology as the species tree, and this pattern is considered to represent significant incomplete lineage sorting (Sayyari & Mirarab, 2016).

3.4 Evidence of gene flow in Hawaiian *Cyrtandra*

We analyzed three datasets for gene flow associated with three island sample sets: Kaua'i, O'ahu, and Maui Nui (Figs. 4–6; Table 3; Table S1). With only four samples, we could not test for hybridization in Hawai'i. SNaQ and Phylonet do not require a priori designation of hybrid branches to test, but ABBA-BABA does. We limited which branches to test with ABBA-BABA to those that were inferred to be hybrid branches in the SNaQ analyses. SNaQ, Phylonet, and ABBA-BABA tests for hybridization in Kaua'i agreed on one hybrid branch, *C. longifolia* × *C. kauaiensis,* which had been previously hypothesized based on morphology (Fig. 4; Table 3). Phylonet supported one hybrid branch as the best fit with all statistical measures applied (log likelihood, AIC, AICc, and BIC; Table S1). SNaQ vectors of inheritance probabilities (γ) from each parent suggest more gene copies have been contributed from *C. longiflora* (γ = 0.779) than *C. kauaiensis* (γ = 0.221).
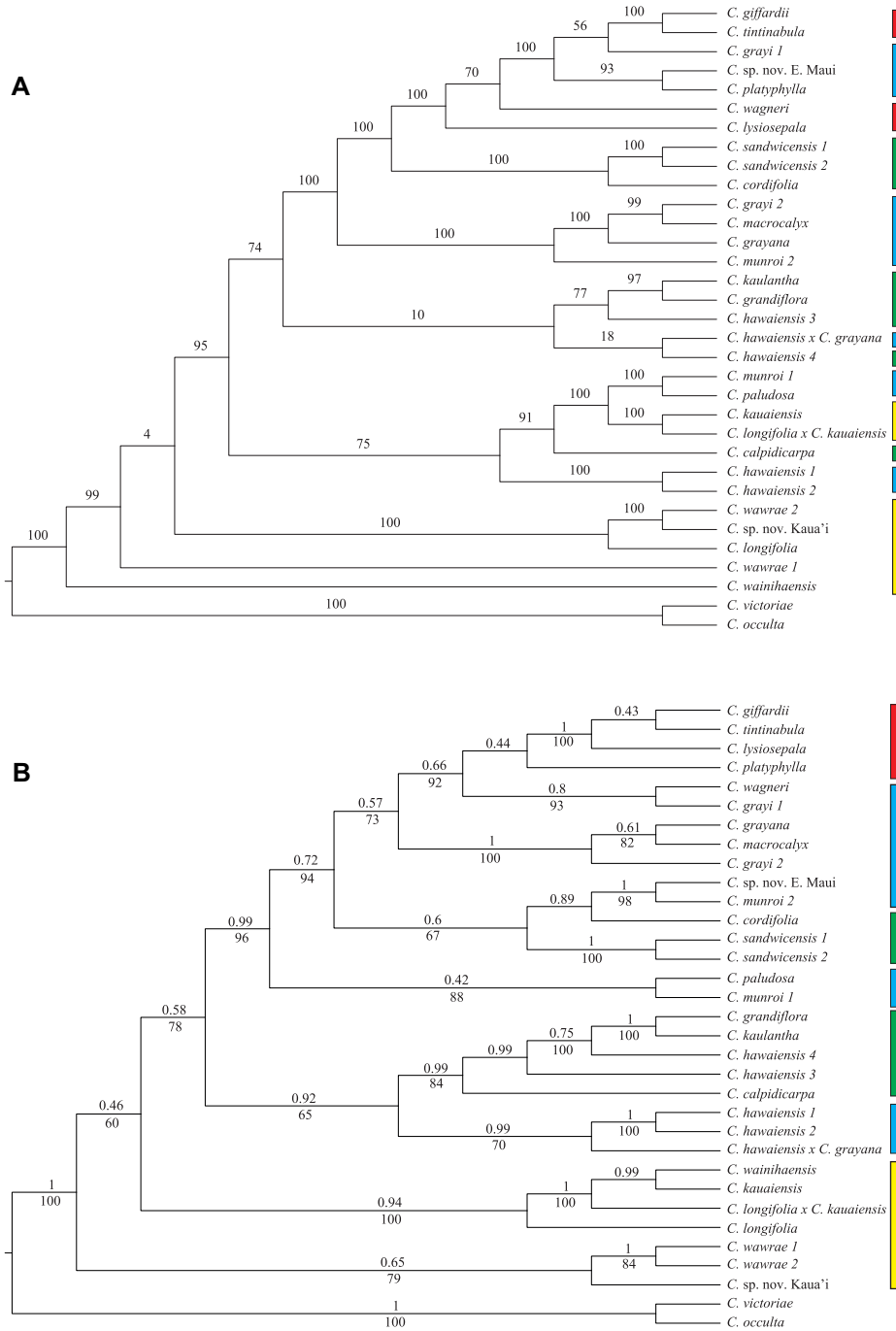
Fig. 3. Phylogenetic hypotheses of relationships in Hawaiian *Cyrtandra*. **A,** Concatenated ML tree inferred in RAxML. Numbers above branches are bootstrap values. **B,** ASTRAL phylogenetic hypothesis. Numbers above branches are local posterior probabilities (ASTRAL-pp) and those below branches are multilocus bootstrap values (ASTRAL-mlbs). For both phylogenetic hypotheses, color bars to the right of species names are island location as follows: yellow = Kaua'i, green = O'ahu, blue = Maui Nui, and red = Hawai'i.

Inference of hybridization in O'ahu *Cyrtandra* was more complex, with disagreement on the number of hybridization events depending on the method and statistical measure (Fig. 5; Table 3; Table S1). Phylonet inferred either zero (BIC; Fig. 5; Table S1) or three (log likelihood, AIC, AICc; Fig. 5; Table S1) hybridization events while SNaQ inferred two (Fig. 5;

Table S1). ABBA-BABA supported both SNaQ hybridization events as significant (Table 3). SNaQ suggests *C. kaulantha* to be a hybrid, which has been previously suggested (unpubl. data), with vectors of inheritance probabilities from *C. grandiflora* ($\gamma = 0.746$) and *C. hawaiensis*-3 ($\gamma = 0.254$). SNaQ also infers a deeper hybridization event between
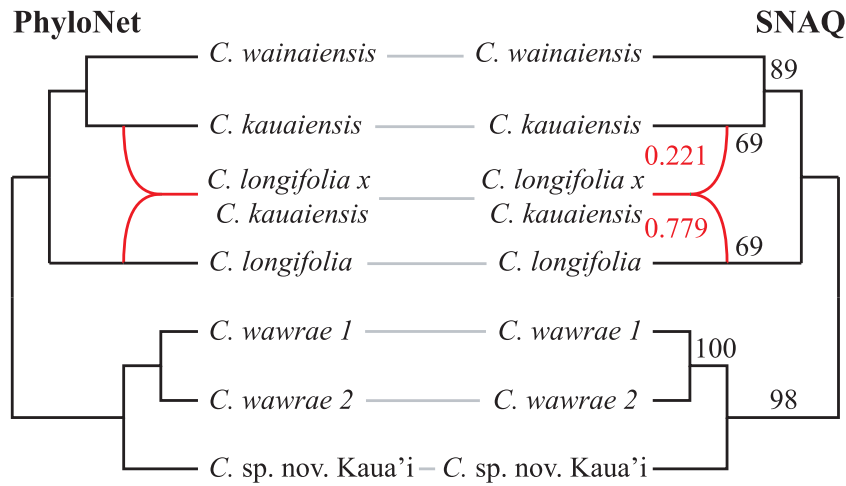
Fig. 4. Inference of hybridization on Kaua'i. Inference of hybrid branches using PhyloNet (left) and SNaQ (right). Hybrid branches are colored red and the SNaQ hypothesis includes bootstrap support above each branch (in black) and vectors of inheritance probabilities (γ) in red for each parental lineage.
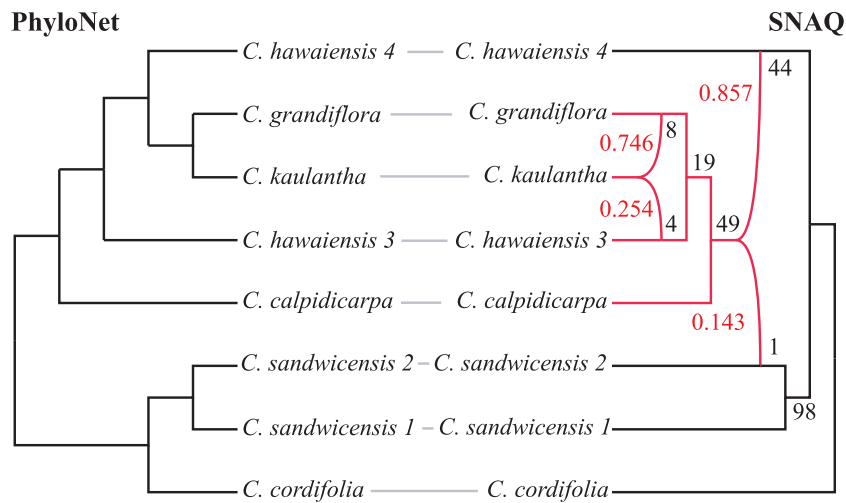

Fig. 5. Inference of hybridization on O'ahu. Inference of hybrid branches using PhyloNet (left) and SNaQ (right). Hybrid branches are colored red and the SNaQ hypothesis includes bootstrap support above each branch and vectors of inheritance probabilities (γ) in red for each parental lineage.
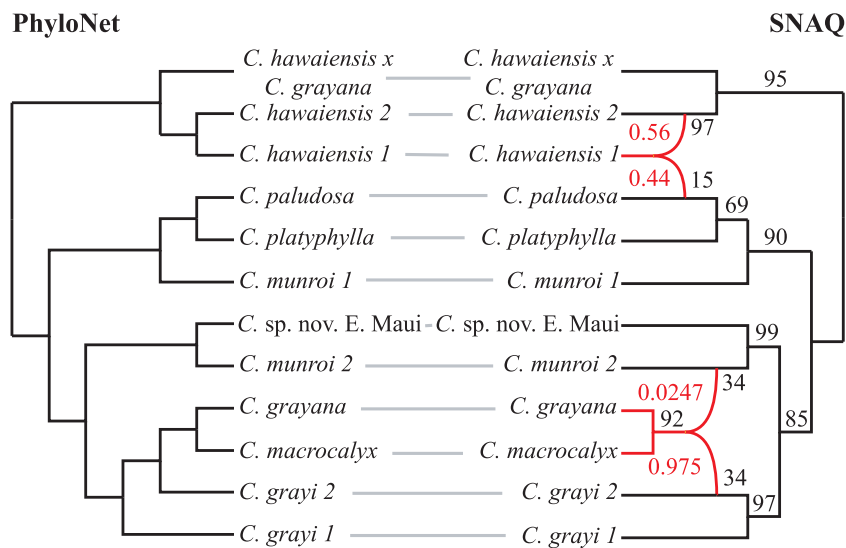

Fig. 6. Inference of hybridization on Maui Nui. Inference of hybrid branches using PhyloNet (left) and SNaQ (right). Hybrid branches are colored red and the SNaQ hypothesis includes bootstrap support above each branch and vectors of inheritance probabilities (γ) in red for each parental lineage.

Table 3 Summary of ABBA-BABA and $\hat{f}_d$ tests for introgression

P1	P2	P3	O	D	P-value	$\hat{f}_d$
Kaua'i						
C. kauaiensis	C. longifolia x kauaiensis	C. longifolia	C. wawrae 1, C. wawrae 2, C. sp. nov. Kaua'i	0.204	<0.001	0.026
C. longifolia	C. longifolia x kauaiensis	C. kauaiensis	C. wawrae 1, C. wawrae 2, C. sp. nov. Kaua'i	0.499	<0.001	0.099
Oahu						
C. hawaiensis 4	C. grandiflora, C. kaulantha, C. hawaiensis 3, C. calpidicarpa	C. sandwicensis 2	C. cordifolia	0.034	<0.001	0.009
C. sandwicensis 2	C. grandiflora, C. kaulantha, C. hawaiensis 3, C. calpidicarpa	C. hawaiensis 4	C. cordifolia	0.286	<0.001	0.085
C. hawaiensis 4	C. grandiflora, C. kaulantha, C. hawaiensis 3, C. calpidicarpa	C. sandwicensis 2	C. cordifolia, C. sandwicensis 1	-0.016	<0.001	0.002
C. sandwicensis 2	C. grandiflora, C. kaulantha, C. hawaiensis 3, C. calpidicarpa	C. hawaiensis 4	C. cordifolia, C. sandwicensis 1	0.435	<0.001	0.099
Maui Nui						
C. hawaiensis x grayana	C. hawaiensis 1	C. paludosa	C. platyphylla, C. munroi 1	0.039	<0.001	0.005
C. paludosa	C. hawaiensis 1	C. hawaiensis x grayana, C. hawaiensis 2	C. platyphylla, C. munroi 1	0.413	<0.001	0.132
C. hawaiensis x grayana, C. hawaiensis 2	C. hawaiensis 1	C. paludosa	C. grayi 1, C. grayi 2, C. grayana, C. macrocarlyx, C. sp. nov. E. Maui, C. munroi 2	0.103	<0.001	0.012
C. paludosa	C. hawaiensis 1	C. hawaiensis x grayana, C. hawaiensis 2	C. grayi 1, C. grayi 2, C. grayana, C. macrocalyx, C. sp. nov. E. Maui, C. munroi 2	0.332	<0.001	0.085
C. grayi 1, C. grayi 2	C. grayana, C. macrocalyx	C. munroi 2	C. hawaiensis x grayana, C. hawaiensis 1, C. hawaiensis 2	0.154	<0.001	0.032
C. munroi 2	C. grayana, C. macrocalyx	C. grayi 1, C. grayi 2	C. hawaiensis x grayana, C. hawaiensis 1, C. hawaiensis 2	0.080	<0.001	0.030
C. grayi 1, C. grayi 2	C. grayana, C. macrocalyx	C. munroi 2	C. paludosa, C. platyphylla, C. munroi 1	0.238	<0.001	0.051
C. munroi 2	C. grayana, C. macrocalyx	C. grayi 1, C. grayi 2	C. paludosa, C. platyphylla, C. munroi 1	0.145	<0.001	0.055
C. grayi 1, C. grayi 2	C. grayana, C. macrocalyx	C. munroi 2	C. sp. nov. E. Maui	0.204	<0.001	0.067
C. munroi 2	C. grayana, C. macrocalyx	C. grayi 1, C. grayi 2	C. sp. nov. E. Maui	0.316	<0.001	0.167

Abbreviations: P1, population 1; P2, population 2; P3, population 3; O, outgroup.

C. hawaiensis-4 ($\gamma = 0.857$) and *C. sandwicensis*-2 ($\gamma = 0.143$) leading to the clade of *C. calpidicarpa*, *C. grandiflora*, *C. hawaiensis*-3, and *C. kaulantha* (Fig. 5).

Maui Nui *Cyrtandra* was inferred to include either zero hybridization events (Phylonet AIC, AICc, BIC; Fig. 6; Table S1) or two hybridization events (Phylonet log likelihood, SNaQ, ABBA-BABA; Fig. 6; Table 3; Table S1). SNaQ-inferred hybrids include *C. hawaiensis*-1 with vectors of inheritance from *C. hawaiensis*-2 ($\gamma = 0.56$) and *C. paludosa* ($\gamma = 0.44$), and the clade of *C. grayana* + *C. macrocalyx* with vectors of inheritance from *C. grayi*-2 ($\gamma = 0.975$) and *C. munroi*-2 ($\gamma = 0.0247$).

4 Discussion

On island systems, the evolutionary forces of hybridization and gene flow have been thought to play important roles in the diversification of lineages, though the underlying processes are not well understood (Yoder et al., 2010; Ellstrand et al., 1996). HTS methods and modern genomic tools provide new avenues to test for the effects of hybridization in diversification (Gompert & Buerkle, 2016; Payseur & Rieseberg, 2016; Vallejo-Marín & Hiscock, 2016). Here, we use targeted enrichment strategies implemented through Hyb-Seq (Weitemier et al., 2014) to demonstrate its utility in understanding phylogenetic relationships of Hawaiian *Cyrtandra,* and to test morphologically-based hypotheses of gene flow in this lineage. Through species tree methods and analyses of gene discordance, we provide evidence of ILS and gene flow in Hawaiian *Cyrtandra.*

For our system, targeted enrichment via Hyb-Seq (Weitemier et al., 2014) was a cost-effective method of obtaining large amounts of data from multiple samples for phylogenetic analyses. A limitation of targeted enrichment is that a genome and transcriptome from the genus of interest are often required to identify low-copy regions for sequencing. However, as Weitemier et al. (2014) and we demonstrate, a genome and transcriptome from closely related taxa may be sufficient for identifying highly-conserved regions for sequencing. In our study, we utilized the genome of *Boea hygrometrica* (Xiao et al., 2015) and the transcriptome of *Primulina pteropoda* (Ai et al., 2015) as input for the Hyb-Seq protocol (Weitemier et al., 2014) because a complete genome and transcriptome were not available for *Cyrtandra.* Our probe-set thus targets less data than those of other studies that utilize a genome and transcriptome from the genus of interest (Straub et al., 2012; Weitemier et al., 2014; Folk et al., 2017). However, we obtained and aligned over 400 000 bp of data for each sample, which is significantly more than prior studies in *Cyrtandra* (Cronk et al., 2005; Clark et al., 2008, 2009; Pillon et al., 2013b; Johnson et al., 2017) and has allowed us to better hypothesize species relationships and instances of gene flow within this lineage.

4.1 Concatenated vs ASTRAL analyses
The concatenated and coalescent analyses conducted here yielded phylogenies with incongruent topologies (Fig. 3). Because concatenated analyses assume that all loci share the same evolutionary history, the phylogenetic tree estimated is identical to estimating a single gene tree. Long loci provide greater signal than short loci in concatenation, which can

result in a resolved tree with a biased topology. Furthermore, concatenation has been shown to output incorrect trees when ILS is widespread (Kubatko & Degnan, 2007). Coalescent methods on the other hand are able to produce accurate species trees in comparison to concatenation methods when ILS is present and there is good signal in the gene trees (Liu et al., 2008; Degnan & Rosenberg, 2009; Edwards, 2009). A number of recent phylogenomic studies using both concatenated and coalescent methods (Song et al., 2012; Stephens et al., 2015; Folk et al., 2017) resulted in phylogenies that were largely congruent, but it is clear in Hawaiian *Cyrtandra* that the high level of ILS has influenced the topology of the concatenated phylogeny. ILS is expected in Hawaiian *Cyrtandra* due to its young age and is evident through the quartet values generated in ASTRAL (Fig. S1). In lineages with low levels of ILS, the major quartet score (q1) at nodes is often high (> 60), while low scores (< 50) indicate high levels of ILS (Mirarab et al., 2014). In our ASTRAL species trees, we see that most nodes have almost equal proportions of quartet scores (q1, q2, q3 = ~33), indicating very high levels of ILS. We used the ASTRAL species trees in tests of gene flow and to compare our findings with those of prior studies, as we consider the ASTRAL phylogenetic hypothesis to be a more accurate depiction of relationships given the high levels of ILS.

4.2 Phylogenetic relationships among species of *Cyrtandra*
The phylogenetic hypotheses presented here largely agree with the previous phylogenetic studies on this group (Cronk et al., 2005; Clark et al., 2008, 2009; Johnson et al., 2017, 2019). Prior studies relied on relatively few loci to reconstruct hypotheses of species relationships in Hawaiian *Cyrtandra*. Cronk et al. (2005) utilized the nuclear ITS spacer, and Clark et al. (2008, 2009) utilized nuclear ITS, nuclear ETS, and plastid *psbA-trnH* spacer regions to estimate species relationships. Pillon et al. (2013b) showed how discordance between gene histories due to hybridization may skew hypotheses of species relationships, especially when few loci are used, and demonstrated the use of low-copy nuclear loci in testing for species relationships in this and other recent plant radiations (Pillon et al., 2013a). Johnson et al. (2017) utilized the nuclear low-copy *Cyrt1* and plastid *rpl32-trnL* regions in addition to loci from prior studies, and was able to reconstruct a more resolved picture of species relationships in Hawaiian *Cyrtandra*, further demonstrating the use of larger datasets including low-copy exon regions in young plant lineages. Johnson et al. (2019) used nine nuclear loci to reconstruct relationships in the Hawaiian Islands, but support for relationships was low and no attempt was made to distinguish between ILS and hybridization. Throughout our species tree, nodes are generally more strongly supported than the previous phylogenetic hypotheses, most likely due to the large number of loci used in comparison to prior studies (569 to ≤ five) and species-tree methods, which account for ILS. However, there are still unsupported nodes (BS < 50) indicating that: (1) gene flow and ILS are obscuring the relationships in Hawaiian *Cyrtandra* at these nodes, or (2) despite having a large amount of data, our datasets are not informative at these nodes given our sampling, or (3) these very short branches are real and represent very rapid divergence of those lineages. In this study, we used relatively few taxa (22 species and 2 putative hybrids), and only for five

species did we sample more than one individual. Wider sampling across species and hybrids of Hawaiian *Cyrtandra*, including more individuals per species, and considering allelic variation among those individuals is likely to yield a clearer picture of species relationships in this lineage.

From our ASTRAL species tree (Fig. 3B), we infer an initial colonization of Kaua'i (the oldest of the main islands) with subsequent dispersal to O'ahu, Maui Nui, and then Hawai'i (progression rule), which has been hypothesized in prior studies (Clark et al., 2008, 2009; Johnson et al., 2017). Furthermore, our phylogeny suggests that the back-dispersal hypotheses of Johnson et al. (2017) has likely occurred, although denser sampling is necessary to adequately test this.

4.3 Species boundaries, ILS, and hybridization in Hawaiian *Cyrtandra*

Species and putative hybrids in Hawaiian *Cyrtandra* are identified on the basis of morphology and/or geographical location. In our sampling, we included multiple samples of *C. wawrae, C. hawaiensis, C. sandwicensis, C. munroi,* and *C. grayi* in order to test if the morphological delimitation of these species is reflected in a molecular phylogeny. *Cyrtandra wawrae* and *C. sandwicensis* samples are each monophyletic (Fig. 3B), while samples of *C. hawaiensis, C. munroi,* and *C. grayi* are not in the phylogenetic hypotheses including all Hawaiian samples (Fig. 3B). We infer slightly different relationships when we include samples from a single island or island group (Figs. 4–6), and this is particularly true for those samples inferred to be involved in hybridization. Given the speciation process, at shallow phylogenetic scales we would not necessarily expect species to be reciprocally monophyletic (Rosenberg, 2003; Kizirian & Donnelly, 2004; Mehta & Rosenberg, 2018); however, where samples within species do not coalesce to the exclusion of other species, we have an opportunity to explore these relationships to test our morphological species concepts. The two samples of *C. grayi* and *C. munroi* are from different islands of Maui Nui: *C. grayi*-1 from Maui, *C. grayi*-2 from Moloka'i, *C. munroi*-1 from Lana'i, and *C. munroi*-2 from Maui. These patterns are suggestive that either samples from the same species from different islands are in the process of diverging from each other, samples from the same species from different islands have introgressed with different species leading to different phylogenetic placements, or that samples from different islands were placed within the same taxonomic species in error and the entities on these islands are not closely related. Studies on the influence of hybridization on species boundaries in other Hawaiian plants have predominantly taken a population genetic/fragment analysis approach (Smith et al., 1996; Friar et al., 2007), or gene incongruence approach among a relatively limited number of sequenced loci (Friar et al., 2008; Willyard et al., 2011; Knope et al., 2012; Roy et al., 2013), and most evidence regarding Hawaiian plant hybridization is morphological (Wagner et al., 1990, 1999). This study is the first for a Hawaiian plant lineage to explicitly test for the distinct influence of ILS and hybridization.

Cyrtandra grayi is distributed on Moloka'i and West Maui, and has been hypothesized to hybridize with four other species (Wagner et al., 1990; Wagner & Roalson, unpubl.). Of the two samples we included here, *C. grayi*-1 is placed in a clade with samples from the island of Hawai'i despite being from

West Maui, whereas *C. grayi*-2 is in a clade with samples of *C. grayana* and *C. macrocalyx,* all three of which were sampled from Moloka'i (Fig. 3B). These relationships are somewhat different when hybridization is considered, as SNaQ suggests a monophyletic *C. grayi* but with *C. grayi*-2 contributing to the ancestor of the *C. grayana + C. macrocalyx* clade (Fig. 6). The other putative parent, *C. munroi*-2 is not known from Moloka'i, and so its involvement in the formation of this hybrid lineage needs to be explored further. *Cyrtandra grayi* and *C. grayana* have been previously hypothesized to hybridize (Wagner et al., 1990; Wagner & Roalson, unpubl.), but neither of these species have been proposed to hybridize with *C. macrocalyx,* and only *C. grayana* has been proposed to hybridize with *C. munroi* on Lana'i (Wagner & Roalson, unpubl.). Morphologically, these four species are fairly easy to distinguish, particularly *C. macrocalyx* and *C. munroi,* but discrimination of *C. grayi* and *C. grayana* is somewhat more difficult given the (hypothesized) more frequent hybrid formation between those two species (Wagner & Roalson, unpubl.).

Cyrtandra munroi-1 from Lana'i is placed as the sister taxon to a sample of *C. paludosa* from Maui, whereas *C. munroi*-2 (West Maui) is placed sister to *C.* sp. nov. E. Maui and in a clade with two species from O'ahu (*C. cordifolia* and *C. sandwicensis;* Fig. 3B). *Cyrtandra paludosa* is known from all of the Hawaiian Islands except Lana'i, and *C. munroi* and *C. paludosa* are quite morphologically distinct, particularly based on calyx morphology (Wagner et al., 1990, 1999). When the hybridization analyses are considered, there is clear, strongly supported phylogenetic separation of the two *C. munroi* samples (Fig. 6). The type of *C. munroi* is from Lana'i, and previous work has suggested that the West Maui populations are somewhat different from the Lana'i populations based on leaf and pubescence morphology (Wagner & Roalson, unpubl.), but denser sampling will be needed to infer our species concepts in this group and whether the Lana'i and West Maui populations should be considered different species.

Species circumscription of *C. hawaiensis* s.l. is one of the most difficult in Hawaiian *Cyrtandra* (Wagner et al., 1990). As it is currently circumscribed, it is found on O'ahu, Moloka'i, Maui, and Hawai'i (Wagner & Roalson, unpubl.). Wagner et al. (1990) discuss that the distribution of morphological variation makes it very difficult to partition more finely, and we have here included four samples of *C. hawaiensis* s.l. from three islands to start to address the circumscription of this species. It is also one of the species most commonly inferred to be hybridizing with other species with nine different hybrid combinations (Wagner & Roalson, unpubl.). The ASTRAL phylogenetic hypothesis (Fig. 3B) supports a clade with all of the samples of *C. hawaiensis* along with morphologically similar species *C. calpidicarpa, C. grandiflora,* and *C. kaulantha. Cyrtandra hawaiensis*-1 (Maui), *C. hawaiensis*-2 (Moloka'i), and *C. hawaiensis × C. grayana* (Moloka'i) together form a clade sister to all of the *C. hawaiensis* and related species from O'ahu, which may suggest that either the O'ahu and Maui Nui lineages of *C. hawaiensis* might be separate lineages/species, and/or that islands clustering is being driven by hybridization among members of this clade when they come into contact.

Analyses to assess the influence of hybridization among *C. hawaiensis* and relatives (Figs. 5,6; Table 3; Table S1) either suggest no hybridization among these species (as measured by the current sampling), or a more complicated combination

of historical and more recent hybridization in the group. On Maui Nui, one hybridization event is inferred with SNaQ associated with *C. hawaiensis*: *C. hawaiensis*-1 is hypothesized to be the result of a hybridization between *C. hawaiensis*-2 ($\gamma = 0.56$) and *C. paludosa* ($\gamma = 0.44$), and these inheritance vectors are close to what would be expected for an F1 hybrid. Interestingly, a field-identified sample denoted as *C. hawaiensis* × *C. grayana* from Moloka'i does not show any signature of hybrid introgression among the genotypes sampled here (Fig. 6). Alternatively, on O'ahu, introgression between *C. hawaiensis*-4 and *C. sandwicensis*-2 genotypes/ genetic lineages contribute to a clade of four samples (Fig. 5), with further introgression within this clade between *C. grandiflora* ($\gamma = 0.746$) and *C. hawaiensis*-3 ($\gamma = 0.254$) leading to *C. kaulantha*. While more sampling will be necessary to tease apart these potentially complicated introgression patterns, these results suggest a number of possible insights. First, it has been previously proposed that *C. kaulantha* might be of hybrid origin (Wagner et al., 1990). Based on morphological features it was thought to have been the result of hybridization between *C. hawaiensis* and *C. laxiflora*, but we cannot directly test that parentage here as we have not sampled *C. laxiflora* in these analyses. However, *C. sandwicensis* is very closely related to *C. laxiflora* and the hybridization results that suggest *C. sandwicensis* contributed genetic material to *C. kaulantha* supports the general hypothesis that the *C. laxiflora*/*C. sandwicensis* morphological group contributed to the hybrid origin of *C. kaulantha*. These results would suggest *C. calpidicarpa* is not hybridizing with *C. kaulantha*, despite growing sympatrically with it in a number of localities. These results also suggest that neither of the two *C. hawaiensis* samples included from O'ahu are the direct product of hybridization and instead have either contributed in the distant past to a diversified clade (*C. hawaiensis*-4) or are contributing to more recent hybrid speciation (*C. hawaiensis*-3). In all, the relationships among *C. hawaiensis* s.l. populations and related species is quite complex, particularly on O'ahu. The data is suggestive that *C. hawaiensis* on O'ahu might be best considered as a separate lineage from *C. hawaiensis* on other islands, but additional sampling across O'ahu, adding as-yet-unsampled populations from Hawai'i, and morphological comparisons within *C. hawaiensis* s.l. across islands will be necessary to properly reassess these relationships.

Cyrtandra sp. nov. Kaua'i and *C.* sp. nov. E. Maui are included to test the relationships of these as yet undescribed entities. *Cyrtandra* sp. nov. Kaua'i is morphologically similar to *C. wawrae* from Kaua'i (Wagner & Wood, unpubl.), and these form a clade separate from the other Kaua'i taxa implying that this undescribed species is closely related to *C. wawrae* as expected (Fig. 3B). *Cyrtandra* sp. nov. E. Maui forms a clade with *C. munroi*-2 (W. Maui) in all analyses (Figs. 3B & 6). Given the uncertainty of the species status of *C. munroi* on Lana'i versus W. Maui, this needs further study, particularly in relation to this undescribed species.

5 Conclusion

Many hypotheses of species relationships in Hawaiian *Cyrtandra* have been proposed over the years on the basis of morphology and Sanger sequences (Gillett, 1967; Wagner

et al., 1990; Smith et al., 1996; Cronk et al., 2005; Clark et al., 2008; Johnson et al., 2017). However, these hypotheses struggled to overcome the influences of incomplete lineage sorting and hybridization evident in this lineage. Here we demonstrated the utility of HTS and a phylogenomic approach to understanding species relationships and gene flow in the presence of ILS. The application of this Hyb-Seq probe set coupled with explicit tests for ILS and hybridization provide a powerful approach to understanding lineage diversification in *Cyrtandra*. The limitations of concatenated analyses in lineages exhibiting high levels of ILS is demonstrated here through the incongruence of the concatenated phylogeny with the ASTRAL phylogenies, and their incongruence with other biological data available for *Cyrtandra*. Evident in our ASTRAL species trees is progression of *Cyrtandra* from the oldest island of Kaua'i to the youngest of Hawai'i, with possible back-dispersals from Maui Nui to O'ahu and Hawai'i to Maui Nui.

These results additionally have implications for the conservation of species. While some taxa are currently considered to be hybrids based on their morphological traits, some of these might not be of hybrid origin based on the results here and may require a reconsideration of the morphological variability within species. We acknowledge that wider sampling of both sympatric and allopatric species and populations across their ranges will allow us to better understand species relationships and the timing, direction, and magnitude of gene flow in this group, in addition to how many species of Hawaiian *Cyrtandra* may actually exist. Future studies incorporating wider sampling are certain to refine and solidify the hypotheses we have proposed here. This study contributes a new hypothesis framework for the diversification of *Cyrtandra* and other young lineages where hybrid lineage formation through time could be one of the major contributors to the diversification process.

Acknowledgements

Data Accessibility

Raw reads for the enriched libraries generated in this study are deposited in the NCBI Sequence Read Archive (BioProject ID: PRJNA531912). Assembled sequences, data files, alignments, and trees are available from the Dryad Digital Repository: doi:10.5061/dryad.s7h937n. Scripts and data for analyses are available from https://github.com/wrroberts/Cyrtandra-phylogenomics.

References

Abbott R, Albach D, Ansell S, Arntzen JW, Baird SJE, Bierne N, Boughman J, Brelsford A, Buerkle CA, Buggs R, Butlin RK,

Dieckmann U, Eroukhmanoff F, Grill A, Cahan SH, Hermansen JS, Hewitt G, Hudson AG, Jiggins C, Jones J, Keller B, Marczewski T, Mallet J, Martinez-Rodriguez P, Möst M, Mullen S, Nichols R, Nolte AW, Parisod C, Pfennig K, Rice AM, Ritchie MG, Seifert B, Smadja CM, Stelkens R, Szymura JM, Väinölä R, Wolf JBW, Zinner D. 2013. Hybridization and speciation. *Journal of Evolutionary Biology* 26: 229–246.

Ai B, Gao Y, Zhang X, Tao J, Kang M, Huang H. 2015. Comparative transcriptome resources of eleven *Primulina* species, a group of 'stone plants' from a biodiversity hot spot. *Molecular Ecology Resources* 15: 619–632.

Akaike H 1973. Information theory and an extension of the maximum likelihood principle. In: Petrov BN, Csáki F eds. *2nd International Symposium on Information Theory, Tsahkadsor, Armenia, USSR, September 2-8, 1971*. Budapest: Akadémiai Kiadó. 267–281.

Anderson E. 1948. Hybridization of the habitat. *Evolution* 2: 1–9.

Anderson E. 1953. Introgressive hybridization. *Biological Reviews* 28: 280–307.

Anderson E, Stebbins GL. 1954. Hybridization as an evolutionary stimulus. *Evolution* 8: 378–388.

Baldwin BG, Sanderson MJ. 1998. Age and rate of diversification of the Hawaiian silversword alliance (Compositae). *Proceedings of the National Academy of Sciences USA* 95: 9402–9406.

Baldwin BG, Wagner WL. 2010. Hawaiian angiosperm radiations of North American origin. *Annals of Botany* 105: 849–879.

Blischak PD, Chifman J, Wolfe AD, Kubatko LS. 2018. HyDe: a Python package for genome-scale hybridization detection. *Systematic Biology* 67: 821–829.

Bock DG, Kane NC, Ebert DP, Rieseberg LH. 2014. Genome skimming reveals the origin of the Jerusalem Artichoke tuber crop species: neither from Jerusalem nor an artichoke. *New Phytologist* 201: 1021–1030.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114–2120.

Burtt BL. 2001. A survey of the genus *Cyrtandra* (Gesneriaceae). *Phytomorphology* 51: 393–404.

Cantley JT, Swenson NG, Markey A, Keeley SC. 2014. Biogeographic insights on Pacific *Coprosma* (Rubiaceae) indicate two colonizations to the Hawaiian Islands. *Botanical Journal of the Linnean Society* 174: 412–424.

Carlquist S. 1974. *Island Biology*. New York: Columbia University Press.

Carson HL, Clague DA. 1995. Geology and biogeography of the Hawaiian Islands. In: Wagner WL, Funk VA eds.*Hawaiian biogeography: Evolution on a hot spot archipelago*. Washington: Smithsonian Institution Press. 14–29.

Chou J, Gupta A, Yaduvanshi S, Davidson R, Nute M, Mirarab S, Warnow T. 2015. A comparative study of SVDquartets and other coalescent-based species tree estimation methods. *BMC Genomics* 16: S2.

Clark JR, Ree RH, Alfaro M, King MG, Wagner WL, Roalson EH. 2008. A comparative study in ancestral range reconstruction methods: retracing the uncertain histories of insular lineages. *Systematic Biology* 57: 693–707.

Clark JR, Wagner WL, Roalson EH. 2009. Patterns of diversification and ancestral range reconstruction in the southeast Asian-Pacific angiosperm lineage *Cyrtandra* (Gesneriaceae). *Molecular Phylogenetics and Evolution* 53: 982–994.

Cronk QC, Kiehn M, Wagner WL, Smith JF. 2005. Evolution of *Cyrtandra* (Gesneriaceae) in the Pacific Ocean: the origin of a supertramp clade. *American Journal of Botany* 92: 1017–1024.

Cronn R, Knaus BJ, Liston A, Maughan PJ, Parks M, Syring JV, Udall J. 2012. Targeted enrichment strategies for next-generation plant biology. *American Journal of Botany* 99: 291–311.

Darwin C. 1859. *The Origin of Species*. New York: Oxford University Press.

Degnan JH, Rosenberg NA. 2009. Gene tree discordance, phylogenetic inference and the multispecies coalescent. *Trends in Ecology & Evolution* 24: 332–340.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Durand EY, Patterson N, Reich D, Slatkin M. 2011. Testing for ancient admixture between closely related populations. *Molecular Biology and Evolution* 28: 2239–2252.

Eaton DAR, Ree RH. 2013. Inferring Phylogeny and introgression using RADseq data: an example from flowering plants (*Pedicularis*: Orobanchaceae). *Systematic Biology* 62: 689–706.

Edwards SV. 2009. Is a new and general theory of molecular systematics emerging? *Evolution* 63: 1–19.

Eggens F, Popp M, Nepokroeff M, Wagner WL, Oxelman B. 2007. The origin and number of introductions of the Hawaiian endemic *Silene* species (Caryophyllaceae). *American Journal of Botany* 94: 210–218.

Ellstrand NC, Whitkus R, Rieseberg LH. 1996. Distribution of spontaneous plant hybrids. *Proceedings of the National Academy of Sciences USA* 93: 5090–5093.

Emerson BC. 2002. Evolution on oceanic islands: molecular phylogenetic approaches to understanding pattern and process. *Molecular Ecology* 11: 951–966.

Fleischer RC, Mcintosh CE, Tarr CL. 1998. Evolution on a volcanic conveyor belt: using phylogeographic reconstructions and K–Ar-based ages of the Hawaiian Islands to estimate molecular evolutionary rates. *Molecular Ecology* 7: 533–545.

Folk RA, Mandel JR, Freudenstein JV. 2015. A protocol for targeted enrichment of intron-containing sequence markers for recent radiations: a phylogenomic example from *Heuchera* (Saxifragaceae). *Applications in Plant Sciences* 3: 1500039.

Folk RA, Mandel JR, Freudenstein JV. 2017. Ancestral gene flow and parallel organellar genome capture result in extreme phylogenomic discord in a lineage of angiosperms. *Systematic Biology* 66: 320–337.

Friar EA, Cruse-Sanders JM, McGlaghlin ME. 2007. Gene flow in *Dubautia arborea* and *D. ciliolata*: the roles of ecology and isolation by distance in maintaining species boundaries despite ongoing hybridization. *Molecular Ecology* 16: 4028–4038.

Friar EA, Prince LM, Cruse-Sanders JM, McGlaughlin ME, Butterworth CA, Baldwin BG. 2008. Hybrid origin and genomic mosaicism of *Dubautia scabra* (Hawaiian Silversword Alliance; Asteraceae-Madiinae). *Systematic Botany* 33: 589–597.

Funk VA, Wagner WL. 1995. Biogeography of seven ancient Hawaiian plant lineages. In: Wagner WL, Funk VA eds. *Hawaiian biogeography: Evolution on a hot spot archipelago*. Washington DC: Smithsonian Institution Press. 160–194.

Gillett GW. 1967. The genus *Cyrtandra* in Fiji. *Contributions of the U.S. National Herbarium* 37: 107–159.

Givnish TJ, Millam KC, Mast AR, Paterson TB, Theim TJ, Hipp AL, Henss JM, Smith JF, Wood KR, Sytsma KJ. 2009. Origin, adaptive radiation and diversification of the Hawaiian lobeliads (Asterales: Campanulaceae). *Proceedings of the Royal Society B: Biological Sciences* 276: 407–416.

Gompert Z, Buerkle CA. 2016. What, if anything, are hybrids: enduring truths and challenges associated with population structure and gene flow. *Evolutionary Applications* 9: 909–923.

Goulet BE, Roda F, Hopkins R. 2017. Hybridization in plants: old ideas, new techniques. *Plant Physiology* 173: 65–78.

Hipp AL, Eaton DAR, Cavender-Bares J, Fitzek E, Nipper R, Manos PS. 2014. A framework phylogeny of the American Oak clade based on sequenced RAD data. *PLoS One* 9: e93975.

Howarth DG, Gustafsson MH, Baum DA, Motley TJ. 2003. Phylogenetics of the genus Scaevola (Goodeniaceae): implication for dispersal patterns across the Pacific Basin and colonization of the Hawaiian Islands. *American Journal of Botany* 90: 915–923.

Huson DH, Scornavacca C. 2012. Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. *Systematic Biology* 61: 1061–1067.

Jackson ND, Carstens BC, Morales AE, O'Meara BC. 2016. Species delimitation with gene flow. *Systematic Biology* 66: 799–812.

Johnson MA, Clark JR, Wagner WL, McDade LA. 2017. A molecular phylogeny of the Pacific clade of Cyrtandra (Gesneriaceae) reveals a Fijian origin, recent diversification, and the importance of founder events. *Molecular Phylogenetics and Evolution* 116: 30–48.

Johnson MA, Pillon Y, Sakishima T, Price DK, Stacy EA. 2019. Multiple colonizations, hybridization and uneven diversification in Cyrtandra (Gesneriaceae) lineages on Hawai'i Island. *Journal of Biogeography* 46: 1178–1196. https://doi.org/10.1111/jbi.13567, [accessed 20 April 2019].

Johnson MA, Price DK, Price JP, Stacy EA. 2015. Postzygotic barriers isolate sympatric species of Cyrtandra (Gesneriaceae) in Hawaiian montane forest understories. *American Journal of Botany* 102: 1870–1882.

Katoh K, Asimenos G, Toh H. 2009. Multiple alignment of DNA sequences with MAFFT. In: Posada D ed. *Bioinformatics for DNA sequence analysis*. Totowa, NJ: Humana Press. 39–64.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28: 1647–1649.

Kiehn M. 2005. Chromosome numbers of Hawaiian angiosperms: new records and comments. *Pacific Science* 59: 363–377.

Kim H-G, Keeley SC, Vroom PS, Jansen RK. 1998. Molecular evidence for an African origin of the Hawaiian endemic Hesperomannia (Asteraceae). *Proceedings of the National Academy of Sciences USA* 95: 15440–15445.

Kizirian D, Donnelly MA. 2004. The criterion of reciprocal monophyly and classification of nested diversity at the species level. *Molecular Phylogenetics and Evolution* 32: 1072–1076.

Knope ML, Morden CW, Funk VA, Fukami T. 2012. Area and the rapid radiation of Hawaiian Bidens (Asteraceae). *Journal of Biogeography* 39: 1206–1216.

Kubatko LS, Degnan JH. 2007. Inconsistency of phylogenetic estimates from concatenated data under coalescence. *Systematic Biology* 56: 17–24.

Lindqvist C, Albert VA. 2002. Origin of the Hawaiian endemic mints within North American Stachys (Lamiaceae). *American Journal of Botany* 89: 1709–1724.

Liu L, Pearl DK, Brumfield RT, Edwards SV. 2008. Estimating species trees using multiple-allele DNA sequence data. *Evolution* 62: 2080–2091.

Mallet J. 2005. Hybridization as an invasion of the genome. *Trends in Ecology & Evolution* 20: 229–237.

Mallet J. 2008. Hybridization, ecological races and the nature of species: empirical evidence for the ease of speciation. *Philosophical Transactions of the Royal Society B: Biological Sciences* 363: 2971–2986.

Martin SH, Davey JW, Jiggins CD. 2015. Evaluating the use of ABBA-BABA statistics to locate introgressed loci. *Molecular Biology and Evolution* 32: 244–257.

McDade LA. 2000. Hybridization and phylogenetics. In: Wiens JJ ed. *Phylogenetic analysis of morphological data*. Washington DC: Smithsonian Institution Press. 146–164.

Mehta RS, Rosenberg NA. 2018. The probability of reciprocal monophyly of gene lineages in three and four species. *Theoretical Population Biology* https://doi.org/10.1016/j.tpb.2018.04.004

Meyer M, Kircher M, Gansauge M-T, Li H, Racimo F, Mallick S, Schraiber JG, Jay F, Prüfer K, de Filippo C, Sudmant PH, Alkan C, Fu Q, Do R, Rohland N, Tandon A, Siebauer M, Green RE, Bryc K, Briggs AW, Stenzel U, Dabney J, Shendure J, Kitzman J, Hammer MF, Shunkov MV, Derevianko AP, Patterson N, Andrés AM, Eichler EE, Slatkin M, Reich D, Kelso J, Pääbo S. 2012. A high-coverage genome sequence from an archaic Denisovan individual. *Science* 338: 222–226.

Mirarab S, Reaz R, Bayzid MS, Zimmermann T, Swenson MS, Warnow T. 2014. ASTRAL: genome-scale coalescent-based species tree estimation. *Bioinformatics* 30: i541–i548.

Mirarab S, Warnow T. 2015. ASTRAL-II: coalescent-based species tree estimation with many hundreds of taxa and thousands of genes. *Bioinformatics* 31: i44–i52.

Morden CW, Harbin SC. 2013. Evolution and biogeographic origins of the endemic Hawaiian genus Hesperomannia (Asteraceae). *Pacific Science* 67: 219–235.

Nepokroeff M, Sytsma KJ, Wagner WL, Zimmer EA. 2003. Reconstructing ancestral patterns of colonization and dispersal in the Hawaiian understory tree genus Psychotria (Rubiaceae): a comparison of parsimony and likelihood approaches. *Systematic Biology* 52: 820–838.

Payseur BA, Rieseberg LH. 2016. A genomic perspective on hybridization and speciation. *Molecular Ecology* 25: 2337–2360.

Percy DM, Garver AM, Wagner WL, James HF, Cunningham CW, Miller SE, Fleischer RC. 2008. Progressive island colonization and ancient origin of Hawaiian Metrosideros (Myrtaceae). *Proceedings of the Royal Society B: Biological Sciences* 275: 1479–1490.

Pillon Y, Johansen J, Sakishima T, Chamala S, Barbazuk WB, Roalson EH, Price DK, Stacy EA. 2013a. Potential use of low-copy nuclear genes in DNA barcoding: a comparison with plastid genes in two Hawaiian plant radiations. *BMC Evolutionary Biology* 13: 1.

Pillon Y, Johansen JB, Sakishima T, Roalson EH, Price DK, Stacy EA. 2013b. Gene discordance in phylogenomics of recent plant radiations, an example from Hawaiian Cyrtandra (Gesneriaceae). *Molecular Phylogenetics and Evolution* 69: 293–298.

Price JP. 2004. Floristic biogeography of the Hawaiian Islands: influences of area, environment and paleogeography. *Journal of Biogeography* 31: 487–500.

Price JP, Clague DA. 2002. How old is the Hawaiian biota? Geology and phylogeny suggest recent divergence. *Proceedings of the Royal Society B: Biological Sciences* 269: 2429–2435.

Price JP, Elliott-Fisk D. 2004. Topographic history of the Maui Nui complex, Hawai'i, and its implications for biogeography. *Pacific Science* 58: 27–45.

Price JP, Wagner WL. 2004. Speciation in Hawaiian angiosperm lineages: cause, consequence, and mode. *Evolution* 58: 2185–2200.

Price JP, Wagner WL. 2018. Origins of the Hawaiian flora: Phylogenies and biogeography reveal patterns of long-distance dispersal. *Journal of Systematics and Evolution* 56: 600–620. https://doi.org/.org/10.1111/jse.12465

Raven PH. 1976. Systematics and plant population biology. *Systematic Botany* 1: 284–316.

Roberts WR, Roalson EH. 2018. Phylogenomic analyses reveal extensive gene flow within the magic flowers (Achimenes). *American Journal of Botany* 105: 726–740.

Rosenberg NA. 2003. The shapes of neutral gene geneologies in two species: probabilities of monophyly, paraphyly, and polyphyly in a coalescent model. *Evolution* 57: 1465–1477.

Roy T, Chang T-H, Lan T, Lindqvist C. 2013. Phylogeny and biogeography of New World Stachydeae (Lamiaceae) with emphasis on the origin and diversification of Hawaiian and South American taxa. *Molecular Phylogenetics and Evolution* 69: 218–238.

Savolainen V, Chase MW. 2003. A decade of progress in plant molecular phylogenetics. *Trends in Genetics* 19: 717–724.

Sayyari E, Mirarab S. 2016. Fast coalescent-based computation of local branch support from quartet frequencies. *Molecular Biology and Evolution* 33: 1654–1668.

Schluter D. 2000. *The Ecology of Adaptive Radiation.* Oxford: Oxford University Press.

Schwarz G. 1978. Estimating the dimension of a model. *Annals of Statistics* 6: 461–464.

Seo TK. 2008. Calculating bootstrap probabilities of phylogeny using multilocus sequence data. *Molecular Biology and Evolution* 25: 960–971.

Simon A 2010. FastQC: a quality control tool for high throughput sequence data [online]. Available from http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ [accessed 1 August 2017].

Smith JF, Burke CC, Wagner WL. 1996. Interspecific hybridization in natural populations of *Cyrtandra* (Gesneriaceae) on the Hawaiian Islands: evidence from RAPD markers. *Plant Systematics and Evolution* 200: 61–77.

Smith SA, Dunn CW. 2008. Phyutility: a phyloinformatics tool for trees, alignments and molecular data. *Bioinformatics* 24: 715–716.

Solís-Lemus C, Ané C. 2016. Inferring phylogenetic networks with maximum pseudolikelihood under incomplete lineage sorting. *PLoS Genetics* 12: e1005896.

Solís-Lemus C, Bastide P, Ané C. 2017. PhyloNetworks: a package for phylogenetic networks. *Molecular Biology and Evolution* 34: 3292–3298.

Soltis PS, Soltis DE. 2009. The role of hybridization in plant speciation. *Annual Review of Plant Biology* 60: 561–588.

Song S, Liu L, Edwards SV, Wu S. 2012. Resolving conflict in eutherian mammal phylogeny using phylogenomics and the multispecies coalescent model. *Proceedings of the National Academy of Sciences USA* 109: 14942–14947.

Stacy EA, Johansen JB, Sakishima T, Price DK, Pillon Y. 2014. Incipient radiation within the dominant Hawaiian tree *Metrosideros polymorpha* . *Heredity* 113: 334–342.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1312–1313.

Stebbins GL. 1959. The role of hybridization in evolution. *Proceedings of the American Philosophical Society* 103: 231–251.

Stephens JD, Rogers WL, Heyduk K, Cruse-Sanders JM, Determann RO, Glenn TC, Malmberg RL. 2015. Resolving phylogenetic relationships of the recently radiated carnivorous plant genus *Sarracenia* using target enrichment. *Molecular Phylogenetics and Evolution* 85: 76–87.

Straub SCK, Parks M, Weitemier K, Fishbein M, Cronn RC, Liston A. 2012. Navigating the tip of the genomic iceberg: Next-generation sequencing for plant systematics. *American Journal of Botany* 99: 349–364.

Stull GW, Moore MJ, Mandala VS, Douglas NA, Kates H-R, Qi X, Brockington SF, Soltis PS, Soltis DE, Gitzendanner MA. 2013. A targeted enrichment strategy for massively parallel sequencing of angiosperm plastid genomes. *Applications in Plant Sciences* 1: 1200497.

Sugiura N. 1978. Further analysts of the data by Akaike's information criterion and the finite corrections. *Communications in Statistics – Theory and Methods* 7: 13–26.

Sukumaran JA, Holder MT. 2010. DendroPy: a Python library for phylogenetic computing. *Bioinformatics* 26: 1569–1571.

Than C, Ruths D, Nakhleh L. 2008. PhyloNet: a software package for analyzing and reconstructing reticulate evolutionary relationships. *BMC Bioinformatics* 9: 322.

Vallejo-Marín M, Hiscock SJ. 2016. Hybridization and hybrid speciation under global change. *New Phytologist* 211: 1170–1187.

Vargas OM, Ortiz EM, Simpson BB. 2017. Conflicting phylogenomic signals reveal a pattern of reticulate evolution in a recent high-Andean diversification (Asteraceae: Astereae: *Diplostephium*). *New Phytologist* 214: 1736–1750.

Wagner WL, Herbst DR, Sohmer SH. 1990. *Manual of the Flowering Plants of Hawai'i, 2 volumes.* Honolulu: University of Hawaii Press, Bishop Museum Press.

Wagner WL, Herbst DR, Sohmer SH. 1999. *Manual of the Flowering Plants of Hawai'i, 2 volumes, revised edition with supplement by Wagner WL and Herbst DR.* Honolulu: University of Hawaii Press, Bishop Museum Press.

Ward BJ, Oosterhout C. 2016. HYBRIDCHECK: software for the rapid detection, visualization and dating of recombinant regions in genome sequence data. *Molecular Ecology Resources* 16: 534–539.

Weitemier K, Straub SCK, Cronn RC, Fishbein M, Schmickl R, McDonnell A, Liston A. 2014. Hyb-Seq: combining target enrichment and genome skimming for plant phylogenomics. *Applications in Plant Sciences* 2: 1400042.

Welch AJ, Collins K, Ratan A, Drautz-Moses DI, Schuster SC, Lindqvist C. 2016. The quest to resolve recent radiations: plastid phylogenomics of extinct and endangered Hawaiian endemic mints (Lamiaceae). *Molecular Phylogenetics and Evolution* 99: 16–33.

Willyard A, Wallace LE, Wagner WL, Weller SG, Sakai AK, Nepokroeff M. 2011. Estimating the species tree for Hawaiian Schiedea (Caryophyllaceae) from multiple loci in the presence of reticulate evolution. *Molecular Phylogenetics and Evolution* 60: 29–48.

Xiao L, Yang G, Zhang L, Yang X, Zhao S, Ji Z, Zhou Q, Hu M, Wang Y, Chen M, Xu Y, Jin H, Xiao X, Hu G, Bao F, Hu Y, Wan P, Li L, Deng X, Kuang T, Xiang C, Zhu J-K, Oliver MJ, He Y. 2015. The resurrection genome of *Boea hygrometrica*: a blueprint for survival of dehydration. *Proceedings of the National Academy of Sciences USA* 112: 5833–5837.

Yang Y, Smith SA. 2014. Orthology inference in nonmodel organisms using transcriptomes and low-coverage genomes: improving accuracy and matrix occupancy for phylogenomics. *Molecular Biology and Evolution* 31: 3081–3092.

Yoder JB, Clancey E, Des Roches S, Eastman JM, Gentry L, Godsoe W, Hagey TJ, Jochimsen D, Oswald BP, Robertson J, Sarver BAJ, Schenk JJ, Spear SF, Harmon LJ. 2010. Ecological opportunity and the origin

of adaptive radiations: ecological opportunity and origin of adaptive radiations. *Journal of Evolutionary Biology* 23: 1581–1596.

Yu Y, Nakhlah L. 2015. A maximum pseudo-likelihood approach for phylogenetic networks. *BMC Genomics* 16: S10.

Zhang C, Sayyari E, Mirarab S 2017. ASTRAL-III: increased scalability and impacts of contracting low support branches. In: Meidanis J, Nakhleh L eds. *Comparative genomics*. 15th International Workshop, RECOMB CG 2017, Barcelona, Spain, October 4–6, 2017, Proceedings. Springer International Publishing. 53–75.

Zirkle C. 1934. More records of plant hybridization before Koelreuter. *Journal of Heredity* 25: 3–18.

Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.12519/suppinfo:

Fig. S1. ASTRAL tree with quartet scores at each branch. The three scores (q1, q2, and q3) on each branch correspond to the species tree, first alternate, and second alternate.

Table S1. Model fit summaries. Estimates of the number of hybrid edges and model fit under each scenario for PhyloNet and SNaQ. Best fit models are shaded for each statistical measure.