Targeted enrichment methods allow for the extraction of hundreds of low-copy loci, which is useful for phylogenomic inference under the multispecies coalescent model (Lemmon et al., 2012; Heyduk et al., 2016). However, the bait sets required for target enrichment can be costly to produce and require genomic resources including reference genomes or transcriptome sequences (Hale et al., 2020). Universal bait sets, such as Angiosperms353 (Johnson et al., 2019), can ameliorate these challenges by enabling the generation of target gene sequences across a broad phylogenetic scale. Although this is an attractive option for some phylogenetic investigations, a “one-size-fits-all” approach may not be sufficient for disentangling phylogenetic relationships at lower taxonomic levels in many groups. Rapid species radiations, low sequence divergence, and gene and genome duplications (all rampant within the angiosperm clade) are likely to reduce the efficacy of using a universal bait set for species-level phylogeny reconstruction. Rather, it is likely that lineage-specific bait sets will be necessary to obtain sufficient variation to address questions at lower phylogenetic scales.

In this study, we address this issue by generating a single-copy gene bait set for the hyperdiverse Orchidaceae, the second-largest
family of flowering plants (behind Asteraeae) with over 27,000 recognized species (The Plant List, 2013). Numerous studies over the years have attempted to reconstruct relationships among species and lineages within the Orchidaceae, many with conflicting results (e.g., Freudenstein et al., 2004; Neubig et al., 2009; Chase et al., 2015). Phylogenetic support at lower taxonomic levels has been poor, primarily because of limited variation in genes commonly used for phylogenetic inference, e.g., ITS, rbcL, matK, chloroplast intergenic spacers (Freudenstein et al., 2004; Neubig et al., 2009). Hybrid enrichment has been used in orchids applying 517 nuclear genes (Granados Mendoza et al., 2020) and 446 nuclear, chloroplast, and mitochondrial genes (Bogarin et al., 2018), which have shown promise in resolving relationships in orchids of the genera Epidendrum L. and Lepanthes Sw., respectively.

In this study, we have generated an Orchidaceae-specific bait set including exons of 963 putatively single-copy nuclear genes (Unruh et al., 2018; Johnson et al., 2019), which we hypothesize will allow for more robust estimates of relationships with greater support. This bait set includes 754 genes from a previously generated data set (Unruh et al., 2018). Additionally, we identified 254 of the 353 Angiosperms353 genes (45 genes overlapping with the set of 754 genes) to be single copy in the Phalaenopsis equestris (Schauer) Rchb. f. genome (Cai et al., 2015; Johnson et al., 2019). This set, with overlap removed, was incorporated into the final Orchidaceae963 set. This allows for continuity among studies using either the Orchidaceae963 or Angiosperms353 bait sets. We tested the utility of the bait set in reconstructing relationships at the tribal level using a diverse sampling of orchid species from the three most speciose orchid subfamilies, Orchidoideae, Cypripedioideae, and Epidendroideae. Furthermore, we used the genus Acineta Lindl. as a test case for resolving species-level relationships. Species-level taxonomy in Acineta is currently based on floral morphology and has been controversial, with different authorities recognizing different numbers of species (Gerlach, 1999; Christenson, 2006), and no hypothesis of species relationships using molecular phylogenetics exists to date. This represents a general challenge in the Orchidaceae. Of the vast number of species resulting from a rapid radiation, many orchid clades have gone understudied, and the genes typically used for phylogenetic reconstruction in other plant groups do not contain sufficient variation to resolve species-level relationships in orchids (Neubig et al., 2009). Lineage-specific bait sets such as the Orchidaceae963 have the potential to advance our ability to resolve relationships at both deep and shallow scales and will facilitate species delimitation in the speciose orchid family.

METHODS

Probe design

Starting from an existing set of 775 genes that were found to be single copy across orchids (Unruh et al., 2018), we retrieved exon sequences for each gene from the Phalaenopsis equestris whole genome assembly (Cai et al., 2015). We removed any sequences that were less than 50 bp in length. For sequences ranging from 50–99 bp, we added flanking intron sequences on both sides to reach a minimum length of 100 bp needed for bait construction, called “padded” exons. After this filtering step, we were left with 5940 exon sequences for 754 genes. To identify additional single-copy loci, we performed a local BLAST search (Altschup et al., 1990) to find hits of the Angiosperms353 targets to the P. equestris genome (Cai et al., 2015). For each Angiosperms353 target, we filtered out any loci mapping to multiple contigs and loci with hit alignments with high-scoring segment pairs spanning a distance of more than 250 kbp on a single contig to account for potential duplicates. With the remaining hits, we used the P. equestris genomic coordinates, filtered and padded exons as previously described, and used the BEDTools getfasta function (Quinlan and Hall, 2010) to extract the sequences. This resulted in 763 exons for 254 genes. To compare the overlap between the set of 754 genes and the 254 single-copy Angiosperms353 targets identified in P. equestris, we ran a local BLAST search (Altschup et al., 1990). This resulted in a 45-gene overlap between both sets. We combined both exon sets and dropped the overlap from the set of 254 genes from the Angiosperms353 set. Finally, this resulted in a total of 6005 padded exons across 963 genes. This exon set was sent to Arbor Biosciences for synthesis of 100-bp tiled bait sets. The Orchidaceae963 bait set is available from Arbor Biosciences (Ann Arbor, Michigan, USA), and the exons used to generate the bait set are provided as Appendix S1.

Library preparation and sequencing

Utilizing an existing Orchidaceae taxonomy (Pridgeon et al., 1999, 2001, 2003, 2005, 2009, 2014), we sampled 28 species, including three species within the genus Acineta, to test the efficacy of this bait set at resolving species-level relationships (Appendix 1). Fresh leaf material was collected from plants in the Atlanta Botanical Garden Living Collection and dried on silica desiccant. Dried leaf tissue was ground in a FastPrep-24 (MP Biomedicals, Santa Ana, California, USA), and DNA was isolated using the sorbitol cetyltrimethylammonium bromide (CTAB) method (Doye, 1987; Storchova et al., 2000). DNA fragment size distribution was visually assessed on an agarose gel, and DNA concentration was quantified with the Qubit 3.0 fluorometer broad-range assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Total genomic DNA was sheared to an average fragment size of 550 bp using a Covaris sonicator (Covaris, Woburn, Massachusetts, USA) at either the Georgia Genomics and Bioinformatics Core at the University of Georgia (Athens, Georgia, USA; https://dna.uga.edu/) or the Emory Integrated Genomics Core (Atlanta, Georgia, USA). Dual-indexed libraries were generated using the KAPA LTP Library Preparation kit for Illumina (Roche, Basel, Switzerland) using dual-index primers from the EHS DNA Laboratory at the University of Georgia (Athens, Georgia, USA; http://www.baddna.uga.edu). Genomic libraries were amplified using 12 cycles and were quantified using the Qubit fluorometer high-sensitivity assay (Thermo Fisher Scientific) as well as using real-time quantitative PCR (qPCR). Average fragment size of the genomic DNA libraries was assessed using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Prior to hybridization, eight dual-indexed genomic DNA libraries were pooled in equal concentrations to include 100 ng of each library for a final 800-ng total pool. The pooled library was then used in the hybridization reaction with the custom orchid-specific bait set (Arbor Biosciences myBaits Target Capture Kit). Hybridization reactions were performed at 65°C for 24 h. Enriched libraries were amplified for 10 cycles, and PCR products were cleaned using Sera-Mag SpeedBeads (GE Healthcare Life Sciences, Pittsburgh, Pennsylvania, USA). Final hybridized libraries were quality checked on the Agilent Bioanalyzer, and concentration was measured with qPCR and the Qubit fluorometer. Multiple enriched
pooled libraries were sequenced on an Illumina NextSeq (300 cycles) PE150 High Output flow cell at the Georgia Genomics and Bioinformatics Core.

**Data analysis**

Gene assembly used Perl scripts in the Reads2Trees pipeline (Heyduk, 2014), except SPAdes was used in place of Trinity for contig assembly. Trimmmomatic was used to trim adapter sequences, remove reads with a quality score below 10, and remove reads less than 40 bp long (Bolger et al., 2014). Only reads where both members of the pair passed this quality filtering were used in downstream analysis. Genes were assembled de novo using SPAdes version 3.13.0 (Nurk et al., 2013), and contigs with greater than 98% similarity were merged using Bowtie2 (Langmead and Salzberg, 2012), and coverage was calculated using the “genomeCoverageBed” program in BEDTools (Quinlan and Hall, 2010). Genes were aligned using PRANK (Löytynoja and Goldman, 2005, 2008; Löytynoja, 2014). Multiple sequence alignments were cleaned with Gblocks using the default settings (Castresana, 2000; Talavera and Castresana, 2007). Maximum likelihood phylogenetic analysis was performed using RAxML on each Gblocks-filtered multiple sequence alignment. Clade support was assessed from 100 bootstrap replicates using the rapid bootstrapping algorithm in RAxML (Stamatakis, 2014). A species tree was estimated in ASTRAL-III (Zhang et al., 2018) to account for possible incomplete lineage sorting.

**Comparison of the Angiosperms353 and orchid-specific bait sets**

To compare the utility of the general angiosperm-wide (Angiosperms353) bait set with the Orchidaceae lineage-specific bait set, we generated phylogenetic trees using the 254 genes from the Angiosperms353 bait set that were included in the Orchidaceae963 bait set to compare to the species tree generated from the total set of 963 genes. Species trees were generated, and multi-locus bootstrapping and polytomy tests were run on the species tree as implemented in ASTRAL-III, which tests whether a node differs significantly from the null hypothesis of a polytomy (Sayaari and Mirarab, 2018; Zhang et al., 2018).

**Assessing the Orchidaceae963 bait set on species-level relationships**

To test the utility of the Orchidaceae bait set on resolving species relationships, we sequenced genes from three species in the genus Acineta,

**TABLE 1.** Taxonomy, read counts, and target gene and exon recovery information for species included in this study.

| Subfamily | Tribe | Subtribe | Species | Authority | Sequenced reads | Target exons recovered | Genes recovered |
|-----------|-------|----------|---------|-----------|----------------|-----------------------|-----------------|
| Cypripedioideae | Cranchideae | Godderyinae | Paphiopedilum exul | (Ridl) Rolfe | 2,753,445 | 653 | 430 |
| Cypripedioideae | Cranchideae | Spiranthinae | Phragmipedium | (Warsz. & Rchb. f.) Rolfe | 87,946,590 | 890 | 572 |
| Orchidoideae | Orchideae | Orchidinae | Anoectochilus chapaensis | Gagnep. | 72,034,725 | 988 | 559 |
| Epidendroideae | Arthureae | Arthureae | Coelogyne trinervis | (L.) Sw. | 4,809,455 | 2339 | 875 |
| Epidendroideae | Colliaeae | Colliaeae | Droseridium cladoniaeforme | (Rchb. f.) Schltr. | 26,652,164 | 2305 | 884 |
| Epidendroideae | Cymbideae | Catasetinae | Cymbidium ensifolium | (L.) Sw. | 2,447,334 | 2145 | 836 |
| Epidendroideae | Cymbideae | Cymbidinae | Coelogyne | Lindl. | 2,368,908 | 2033 | 825 |
| Epidendroideae | Cymbideae | Cymbidinae | Maxillaria | Rin. | 3,225,178 | 2081 | 847 |
| Epidendroideae | Cymbideae | Maxillariae | Maxillaria | Rin. | 4,572,350 | 2057 | 827 |
| Epidendroideae | Cymbideae | Oncidinae | Oncidium | (Nash ex Britton & Millsp.) Braem | 6,407,944 | 1724 | 776 |
| Epidendroideae | Stanhopeineae | Acineta | Acineta miyakei | G. Gerlach & M. H. Weber | 34,362,615 | 1961 | 841 |
| Epidendroideae | Stanhopeineae | Acineta | Acineta serra-turcica | Rchb. f. | 1,832,152 | 2095 | 834 |
| Epidendroideae | Stanhopeineae | Acineta | Acineta superba | (Kunth) Rchb. f. | 22,716,176 | 1980 | 837 |
| Epidendroideae | Stanhopeineae | Acineta | Acineta superba | (Kunth) Rchb. f. | 46,435,181 | 2005 | 836 |
| Epidendroideae | Stanhopeineae | Stanhopea | Stanhopea nicaraguensis | G. Gerlach | 1,518,041 | 2081 | 841 |
| Epidendroideae | Zygopetalinae | Pescatoria | Pescatoria lehmannii | Rchb. f. | 30,265,873 | 1923 | 807 |
| Epidendroideae | Zygopetalinae | Coelinae | Coelina bella | (Lem.) Rchb. f. | 53,909,915 | 2302 | 895 |
| Epidendroideae | Zygopetalinae | Encyclia | Encyclia polybulbon | (Sw.) Dressler | 33,176,407 | 1985 | 849 |
| Epidendroideae | Zygopetalinae | Lycomormium | Lycomormium squalidum | Hook. | 5,454,392 | 1948 | 812 |
| Epidendroideae | Zygopetalinae | Catasetinae | Catasetum integrum | Hook. | 20,258,165 | 1767 | 781 |
| Epidendroideae | Zygopetalinae | Lycomormium | Lycomormium squalidum | (Poepp. & Endl.) Rchb. f. | 2,368,908 | 2033 | 825 |
| Epidendroideae | Zygopetalinae | Catasetum | Catasetum | Lindl. | 3,225,178 | 2081 | 847 |
| Epidendroideae | Zygopetalinae | Maxillariae | Maxillaria | Lindl. | 4,572,350 | 2057 | 827 |
including two individuals of the species *A. superba* (Kunth) Rchb. f. The related genus *Stanhopea* Frost ex Hook. was used as an outgroup; the leaf sample was taken from a division of the plant used for the type specimen of *S. nicaraguensis* G. Gerlach (Table 1). Separate alignments of these five taxa were made in PRANK, and alignments were cleaned using Gblocks (see above). Cleared alignments of these five taxa were concatenated using concatenation scripts (https://github.com/ODiogoSilva/ElConcatenero) to assess the number of variable and parsimony informative sites across all genes in PAUP (Swofford, 1993). Individual gene trees were generated in RAxML, and clade support was assessed from 100 bootstrap replicates using the rapid bootstrapping algorithm in RAxML (Stamatakis, 2014). A species tree was reconstructed in ASTRAL-III (Zhang et al., 2018).

### RESULTS

Between 1,518,041 and 87,946,590 paired-end 150-base reads were generated for target-enriched genomic libraries (Table 1). The number of genes recovered from each species ranged from 430 in *Paphiopedilum exile* (Ridl.) Rolfe to 926 in *Phalaenopsis aphrodite* Rchb. f., and recovery was much higher in Epidendroideae taxa than other orchid subfamilies tested (Fig. 1). Most genes had relatively high occupancy; 826 genes were recovered for half or more taxa. However, 137 genes had fairly low capture success, capturing fewer than half of the tested taxa (Fig. 1). Additionally, of the 254 genes included from the Angiosperms353 bait set, we were able to recover between 18 genes in *Spiranthes* and 186 genes in *Phalaenopsis aphrodite* (Fig. 2). The mean total sequence assembly length (including targeted exons and adjacent non-coding sequence) ranged from 583 in *Paphiopedilum exile* to 1914 in *Coelia bella* (Lem.) Rchb. f., with a mean exon sequence length of 311 bp and a mean intron length of 907 bp (Fig. 3). Unlike capture success, variation in sequence assembly lengths did not correspond to taxon relationships (Fig. 3).

Species trees generated using the Angiosperms353 genes and the Orchidaceae963 gene set exhibited consistent subfamily and tribal relationships with one exception (Fig. 4). The Angiosperms353 tree exhibited support for a clade including the tribes Cymbideae and Vandeae that was sister to Epidendreae. However, for the node uniting the Cymbideae and Vandeae tribes, we were not able to reject the null hypothesis that the node is a polytomy. In contrast, we were able to reject a polytomy for the three tribes in the species tree generated using all of the Orchidaceae963 targets, and the species tree analysis yielded strong support for a clade uniting the tribes Cymbideae and Epidendreae, which was placed sister to the tribe Vandeae.

For the test on the three species in the genus *Acineta* and related *Stanhopea nicaraguensis*, we recovered more than 800 genes for four of the five taxa, including the outgroup *S. nicaraguensis*. The Gblocks-filtered concatenated alignment for these five taxa consisted of 2,090,511 sites, 69,675 variable sites, and 5934 parsimony informative sites. The ASTRAL-III species tree for *Acineta* + *Stanhopea nicaraguensis* showed 100% multi locus bootstrap support for all nodes, and polytomy tests rejected polytomies in the tree (Fig. 5).

### DISCUSSION

In this study, we designed and tested an Orchidaceae-specific bait set that has the potential to provide more resolution in the case of rapid radiations compared to the “one-size-fits-all” approach taken by the angiosperm-wide bait set (Johnson et al., 2019). The lower number of genes recovered from the Cypripedioideae and Orchidoideae clades and the 137 genes with low capture rates across the Orchidaceae can potentially be addressed with future reference-genome sequences for these subfamilies and improvements to target capture bioinformatic pipelines. For example, capture rates can be improved by using subfamily-specific exon files for read mapping or clustering methods to select multiple sequences per loci representative of sequence diversity across the family (Johnson et al., 2019; McClay et al., 2021).

Previous analyses of tribal relationships within the Epidendroideae subfamily have found a polytomy among the Cymbideae, Vandeae, and Epidendreae tribes (Freudenstein et al., 2004; Neubig et al., 2009). Other studies that are able to resolve the polytomy show three alternative results: (1) the Vandeae and Epidendreae tribes are closely related and together are sister to the Cymbideae (Cameron et al., 1999; Chase et al., 2015), (2) the Cymbideae and Epidendreae tribes are most closely related and together are sister to the Vandeae (Freudenstein and Chase, 2015), and (3) the Cymbideae and

---

**FIGURE 1.** Presence and absence of target genes in 28 orchid species. For each row, a colored vertical line indicates presence of the gene, and a white vertical line represents absence. Genes were ordered from left to right by the number of taxa with a gene present. The capture rate is high among most genes, and a low number on the right indicates a very low capture rate. Rows are grouped and colored by Orchidaceae subfamily, with Orchidoideae species in yellow, Cypripedioideae in green, and Epidendroideae in blue.
Vandeae tribes are most closely related and together are sister to the Epidendreae (Givnish et al., 2015). Interestingly, the relationship shown using the 963 genes from the Orchidaceae-specific bait set show the same relationships between the Vandeae, Epidendreae, and Cymbideae tribes as an analysis of seven nuclear, chloroplast, and mitochondrial genes for 312 taxa (Freudenstein and Chase, 2015). Despite nearly equal quartet frequencies for the three alternative tribal relationships (36%: [(C,E),V]; 35%: [(C,V),E]; 29%: [(E,V),C]), we were able to reject the null hypothesis of an ancestral polytomy giving rise to Vandeae, Epidendreae, and Cymbideae lineages using the ASTRAL-III polytomy test. The relationships shown in the Angiosperms353 tree (Cymbideae + Vandeae clade) are consistent with the topology recovered using whole chloroplast genome sequences (Givnish et al., 2015). The plastid genome is typically inherited uniparentally without recombination and, therefore, not subject to incomplete lineage sorting. Our species tree analyses of both the Angiosperms353 and Orchidaceae963 targeted gene sets indicate extensive incomplete lineage sorting of nuclear genes and rapid radiation of the Vandeae, Epidendreae, and Cymbideae tribal lineages. The sequences recovered with the Orchidaceae963 bait set provided sufficient statistical power for resolving relationships among the three tribes. The analysis presented here using the 963 genes from the Orchidaceae-specific bait set allows us to reconstruct a topology with greater support and resolves a polytomy in the case of a recent and rapid species radiation (Sayyari and Mirarab, 2018). In the future, examining which genes contribute to this resolution could be informative (Pease et al., 2016).

Additionally, we have demonstrated that the Orchidaceae963 bait set shows promise for characterizing species-level relationships, especially in epidendroid orchids. The genus Acineta consists of 20 species and is distributed in the Neotropics (Christenson, 2006). These species are pollinated by orchid bees in the tribe Euglossini (Dressler, 1968a, b, 1982). Species boundaries have been difficult to characterize (Christenson, 2006), and no phylogenetic reconstruction of species-level relationships has been established. Furthermore, studies of related taxa using a smaller number of genes have shown low resolution of species relationships (Whitten et al., 2000, 2014). Therefore, genomic resources such as the Orchidaceae963 bait set will provide the tools necessary to understand the complex relationships among orchid species and their pollinators in the face of recent and rapid diversification.

Lineage-specific bait sets, such as the Orchidaceae963 set described here, provide the genomic resources to sequence hundreds of single-copy nuclear genes for phylogenomics and population genomics while maintaining an overlapping set of genes with the broader-scale Angiosperms353 probe set to allow for comparison across studies. We have demonstrated that the Orchidaceae963 bait set provides sufficient variation to resolve recent and rapid radiations in the backbone of the orchid tree of life and at shallow phylogenetic scales with an example from the genus Acineta. There was a degree of phylogenetic bias in gene recovery, where we recovered hundreds more genes from species in

FIGURE 2. The number of genes captured for each taxon in the test set, showing both the total number captured (blue) and the number that are part of the Angiosperms353 universal probe set (yellow). Species in the Epidendroideae tended to have a greater number of genes captured than species in the subfamilies Cypripedioideae and Orchidoideae. Species are grouped by Orchidoideae subfamily.

FIGURE 3. Mean sequence length of exons and introns for each taxon. Mean exon length for a sample is shown in blue, and mean intron length is indicated in yellow. Species are grouped by Orchidoideae subfamily.
the Epidendroideae compared to species in the Orchidoideae and Cypripedioideae; this is likely because the baits were designed using sequences from the Phalaenopsis equestris genome, which is a member of the Epidendroideae. However, the number of genes in these two subfamilies was still very high, and utilizing a target file with diverse representative sequences during the assembly process may allow for greater gene recovery (McLay et al., 2021). Target sequence capture approaches show promise for population and conservation genomics (Villaverde et al., 2018; Hale et al., 2020), and lineage-specific bait sets such as the Orchidaceae963 will provide sufficient variation to estimate population genetic diversity and structure. Finally, the potential to derive DNA barcoding markers that exhibit greater diversity than typical rbcL or matK markers can be useful in combating the illegal orchid trade, which has enormous negative impacts on orchid populations worldwide (de Boer et al., 2017; Hinsley et al., 2017).

ACKNOWLEDGMENTS

The authors thank Amanda Cummings (University of Georgia) for performing hybridizations, and Becky Brinkman (manager of the Atlanta Botanical Garden Fuqua Orchid Center) for assistance sampling in the orchid collection. We thank the Georgia Genomics and Bioinformatics Core facility for generating high-quality sequence data. Funding was provided by the National Science Foundation (grant 1442199) and a private donation to the Atlanta Botanical Garden. We are grateful to Dr. Michael Fay and one anonymous reviewer for providing helpful feedback on this manuscript.

AUTHOR CONTRIBUTIONS

S.K.T. and J.H.L.M. designed the probe set. L.A.E. and E.E.D.C. designed the sampling strategy for testing the bait set. L.A.E. generated genomic DNA libraries. L.A.E. and S.K.T. analyzed data and wrote the manuscript. All authors approved the final version.

DATA AVAILABILITY STATEMENT

Raw sequence reads are available in the National Center for Biotechnology Information Sequence Read Archive (BioProject PRJNA690101). Multiple sequence alignments are available at https://github.com/laeserman/Orchidaceae963.

[Correction added on 01 June 2021, after first online publication: the hyperlink has been corrected in this paragraph.]

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. The exons used to generate the Orchidaceae963 bait set.

LITERATURE CITED

Altschup, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic Local Alignment Search Tool. Journal of Molecular Biology 215: 403–410.

Bogarin, D., O. A. Pérez-Escobar, D. Groenenberg, S. D. Holland, A. P. Karremans, E. M. Lemmon, A. R. Lemmon, et al. 2018. Anchored hybrid enrichment generated nuclear, plastid and mitochondrial markers resolve the Lepanthes...
Gerlach, G. 1999. 80. Subtribus: Stanhopeinae.

R. Schlechter, Die Orchideen

Dressler, R. L. 1982. Biology of the orchid bees (Euglossini). Evolution 22: 202–210.

de Boer, H. J., A. Ghorbani, V. Manzanilla, A.-C. Raclariu, A. Kreziou, S. Ounjai, Christenson, E. 2006. The genus Acineta

Chase, M. W., K. M. Cameron, J. V. Freudenstein, A. M. Pridgeon, G. Salazar, C. van den Berg, and A. Schuiteman. 2015. An updated classification of Orchidaceae. Botanical Journal of the Linnean Society 177: 151–174.

Christenson, E. 2006. The genus Acinetea: A review of this increasingly popular genus of stately Neotropical orchids. Orchid Digest 70: 232–251.

de Boer, H. J., A. Ghorbani, V. Manzanilla, A.-C. Raclariu, A. Kreziou, S. Ounjai, M. Osathanunkul, and B. Gravendeel. 2017. DNA metabarcoding of orchid-derived products reveals widespread illegal orchid trade. Proceedings of the Royal Society B: Biological Sciences 284: 20171182.

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

Dressler, R. L. 1968a. Observations on orchids and euglossine bees in Panama and Costa Rica. Revista de Biología Tropical 15: 143–183.

Dressler, R. L. 1968b. Pollination by euglossine bees. Evolution 22: 202–210.

Dressler, R. L. 1982. Biology of the orchid bees (Euglossini). Annual Review of Ecology and Systematics 13: 373–394.

Freudenstein, J. V., and M. W. Chase. 2015. Phylogenetic relationships in Epidendroideae (Orchidaceae), one of the great flowering plant radiations: Progressive specialization and diversification. Annals of Botany 115: 665–681.

Freudenstein, J. V., C. van den Berg, D. H. Goldman, P. J. Kores, M. Molvray, and M. W. Chase. 2004. An expanded plastid DNA phylogeny of Orchidaceae and analysis of jackknife branch support strategy. American Journal of Botany 91: 149–157.

Gerlach, G. 1999. 80. Subtribus: Stanhopeinae. In R. Schlechter, Die Orchideen III/3A, 2315–2435. Berlin, Germany.

Givnish, T. J., D. Spalink, M. Ames, S. P. Lyon, S. J. Hunter, A. Zuluaga, W. J. D. Iles, et al. 2015. Orchid phylogenomics and multiple drivers of their extraordinary diversification. Proceedings of the Royal Society B: Biological Sciences 282: 20151553.

Granados Mendoza, C., M. Jost, E. Hägsater, S. Magallón, C. van den Berg, E. M. Lemmon, A. R. Lemmon, et al. 2020. Target nuclear and off-target plastid hybrid enrichment data inform a range of evolutionary depths in the orchid genus Epidendrum. Frontiers in Plant Science 10: 1761.

Hale, H., E. M. Gardner, J. Viruel, L. Pokorny, and M. G. Johnson. 2020. Strategies for reducing per-sample costs in target capture sequencing for phylogenomics and population genomics in plants. Applications in Plant Sciences 8: e11337.

Heyduk, K. 2014. Reads2Trees Pipeline for analysis of sequence capture. Github repository https://github.com/kheyduk/reads2trees [accessed 16 February 2021].

Heyduk, K., J. D. Stephens, B. C. Faircloth, and T. C. Glenn. 2016. Targeted DNA region re-sequencing. In A. M. Arasney and J. L. Lavin Trueba [eds.], Field guidelines for genetic experimental designs in high-throughput sequencing, 43–68. Springer International Publishing, Cham, Switzerland.

Hinsley, A., H. J. de Boer, M. F. Fay, S. W. Gale, L. M. Gardiner, R. S. Gunasekara, P. Kumar, et al. 2017. A review of the trade in orchids and its implications for conservation. Botanical Journal of the Linnean Society 186: 435–455.

Huang, X., and A. Madan. 1999. CAP3: A DNA sequence assembly program. Genome Research 9: 868–877.

Johnson, M. G., L. Pokorny, S. Dodsworth, L. R. Botigué, R. S. Cowan, A. Devault, W. L. Eisenhardt, et al. 2019. A universal probe set for targeted sequencing of 353 nuclear genes from any flowering plant designed using k-medoids clustering. Systematic Biology 68: 594–606.

Langmead, B., and S. L. Salzberg. 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods 9: 357–359.

Lemmon, A. R., S. A. Emme, and E. M. Lemmon. 2012. Anchored hybrid enrichment for massively high-throughput phylogenomics. Systematic Biology 61: 727–744.

Löytynoja, A. 2014. Phylogeny-aware alignment with PRANK. In D. J. Russell [ed.], Multiple sequence alignment methods, 155–170. Humana Press, Totowa, New Jersey, USA.

Löytynoja, A., and N. Goldman. 2005. An algorithm for progressive multiple alignment of sequences with insertions. Proceedings of the National Academy of Sciences, USA 102: 10557–10562.

Löytynoja, A., and N. Goldman. 2008. Phylogeny-aware gap placement prevents errors in sequence alignment and evolutionary analysis. Science 320: 1632–1635.

Mclay, T. G. B., J. L. Birch, B. F. Gunn, W. Ning, J. A. Tate, L. Nauheimer, E. M. Joyce, et al. 2021. New targets acquired: Improving locus recovery from the Angiosperms353 probe set. Applications in Plant Sciences 9: e11420.

Neubah, K. M., W. M. Whitten, B. S. Carlsward, M. A. Blanco, L. Endara, N. H. Williams, and M. Moore. 2009. Phylogenetic utility of ycf1 in orchids: A plastid gene more variable than matK. Plant Systematics and Evolution 277: 75–84.

Nurk, S., A. Bankevich, D. Antipov, A. Gurevich, A. Korobeynikov, A. Lapidus, A. Priibelsky, et al. 2013. Assembling genomes and mini-metagenomes from highly chimeric reads. In M. Deng, R. Jiang, F. Sun, and X. Zhang [eds.], Research in computational molecular biology, 158–180. Springer, Berlin, Germany.

Pease, J. B., D. C. Haak, M. W. Hahn, and L. C. Moyle. 2016. Phylogenomics reveals three sources of adaptive variation during a rapid radiation. PLoS Biology 14: e1002379. https://doi.org/10.1371/journal.pbio.1002379.

Pridgeon, A. M., P. J. Cribb, M. W. Chase, and F. N. Rasmussen. 1999. Genera Orchidacearum, Volume 1: General Introduction, Apostasioideae, Cypripedioideae. Oxford University Press, Oxford, United Kingdom.

Pridgeon, A. M., P. J. Cribb, M. W. Chase, and F. N. Rasmussen. 2001. Genera Orchidacearum. Volume 2: Orchidoideae (Part One). Oxford University Press, Oxford, United Kingdom.

Pridgeon, A. M., P. J. Cribb, M. W. Chase, and F. N. Rasmussen. 2003. Genera Orchidacearum. Volume 3: Orchidoideae (Part Two), Vanilloideae. Oxford University Press, Oxford, United Kingdom.

Pridgeon, A. M., P. J. Cribb, M. W. Chase, and F. N. Rasmussen. 2005. Genera Orchidacearum. Volume 4: Epidendroideae (Part One). Oxford University Press, Oxford, United Kingdom.

Pridgeon, A. M., P. J. Cribb, M. W. Chase, and F. N. Rasmussen. 2009. Genera Orchidacearum. Volume 5: Epidendroideae (Part Two). Oxford University Press, Oxford, United Kingdom.

Pridgeon, A. M., P. J. Cribb, M. W. Chase, and F. N. Rasmussen. 2014. Genera Orchidacearum. Volume 6: Epidendroideae (Part Three). Oxford University Press, Oxford, United Kingdom.

Quinlan, A. R., and I. M. Hall. 2010. BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics 26: 841–842.

Sayyari, E., and S. Mirarab. 2018. Testing for polytomies in phylogenetic species trees using quartet frequencies. Genes 9: 132.

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

Storchova, H., R. Hrdlickova, J. Chrtek, M. Tetera, D. Fitze, and J. Fehrer. 2000. An improved method of DNA isolation from plants collected in the field and conserved in saturated NaCl/CTAB Solution. Taxon 49: 79–84.

Swoford, D. L. 1993. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods). Mac Version 3.1.1. Sinauer Associates, Sunderland, Massachusetts, USA.

Talavera, G., and J. Castresana. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Systematic Biology 56: 564–577.

The Plant List. 2013. Version 1.1. Available at http://www.thecanadianlist.org/ [accessed October 2020].

Unruh, S. A., M. R. McKain, Y.-I. Lee, T. Yukawa, M. K. McCormick, R. P. Shefferson, A. Smithson, et al. 2018. Phylotranscriptomic analysis and
APPENDIX 1. Provenance information for species used in this study.

| Species                        | Authority                  | Provenance                                         | ABG Living Collection no. | ABG Biorepository no. |
|-------------------------------|-----------------------------|----------------------------------------------------|---------------------------|-----------------------|
| Paphiopedilum exul            | (Ridl.) Rolfe               | United States Fish and Wildlife Service            | 20150810                  | 164                   |
| Phragmipedium longifolium     | (Warsz. & Rchb. f.) Rolfe   | Banos, Ecuador; Coll. Calaway H. Dodson            | 20111585                  | 163                   |
| Anoectochilus chapaensis      | Gagnep.                    | Coll. Douglas Goldman                                | 20140840                  | 185                   |
| Spiranthus sp.                |                             | Calhoun Co., Georgia; Coll. Ron Determann          | 20120193                  | 161                   |
| Platanthera blepharaglottis   | (Wild.) Lindl.              | Coll. Jeff Talbert; Deer Lake State Park, Florida  | L-20170054                | 158                   |
| Calapogon barbatus            | (Walter) Ames               | Coll. Jeff Talbert; Deer Lake State Park, Florida  | L-20160109                | 159                   |
| Coelogyne tri nervis          | Lindl.                     | Marie Selby Botanical Gardens                       | 20063209                  | 165                   |
| Gastrichis humboldti          | (Rchb. f.) Schltr.          | Louisiana Orchid Connection                         | 20163714                  | 166                   |
| Catasetum integerrimum         | Hook                       | Rudolph Jenny; F1218                                | 20031131                  | 167                   |
| Lycomormium squilulatum       | (Posep. & Endl.) Rchb. f.   | Carchi, Ecuador; Coll. Mark Whitten                 | 20111551                  | 168                   |
| Cymbidium ensifolium          | (L.) Sw.                    | United States Fish and Wildlife; 12580              | 20172133                  | 169                   |
| Eulaphia streptopetala       | Lindl.                     | Orchid Seedbank Project                             | 20172106                  | 170                   |
| Anguloa uniflora              | Ruiz & Pav.                | Cordillera Azul, Peru; Coll. Mark Whitten, F-1708  | 20111461                  | 171                   |
| Maxillaria teneru folia       | Lindl.                     | Cheekwood Botanical Garden                          | 20041060                  | 186                   |
| Tolumnia bahamensis           | (Nash ex Britton & Millsp.) Braem | Coll. Rob Rossmananith; Jonathan Dickinson State Park, Florida | L-20160078                | 157                   |
| Acineta mireyae               | G. Gerlach & M. H. Weber    | Bill Goldner                                        | 20021863                  | 96                    |
| Acineta sello-turcica         | Rchb. f.                   | Tropical Orchid Farm, TOF2291                       | 20071149                  | 77                    |
| Acineta superba               | (Kunth) Rchb. f.            | El Oro, Ecuador; Coll. Mark Whitten                 | 19940903                  | 98                    |
| Acineta superba               | (Kunth) Rchb. f.            | Paccha, Ecuador; Coll. F. L. Stevenson              | 19960940                  | 99                    |
| Stanhopea nicaraguaensis      | G. Gerlach                 | Jinotega, Ecuador; Coll. John Atwood                | 19901127                  | 106                   |
| Pescatoria lehmannii          | Rchb. f.                   | JEM Orchids                                         | 19940657                  | 172                   |
| Coelia bella                  | (Lem.) Rchb. f.             | Monrovia de Chiriqui, Costa Rica; Robert Dressler  | 20111479                  | 175                   |
| Encyclia polybulbon           | (Sw.) Dressler             | Cheekwood Botanical Garden, 1982-0089               | 20041097                  | 177                   |
| Liparis thombae               | J. J. Sm.                  | Ecuagenera Nursery                                  | 20150624                  | 179                   |
| Eria ornata                   | Lindl.                     | Clyde Bramblett                                     | 20050306                  | 180                   |
| Sobralia citrea               | Dressler                   | Cerro Jefe, Panama; Coll. Mark Whitten              | 20111610                  | 181                   |
| Phalaenopsis aphrodite        | Rchb. f.                   | Louisiana Orchid Connection                         | 20161292                  | 182                   |
| Angraecum breve               | Schltr.                    | Carter & Holmes Orchids                             | 20031832                  | 183                   |
| Polystachya concreta          | (Jacq.) Garay & H. R. Sweet| Puerto Rico, Coll. Mark Laroque                     | 20100385                  | 184                   |

Note: ABG = Atlanta Botanical Garden.