Primers for Castilleja and their Utility Across Orobanchaceae: II. Single-Copy Nuclear Loci

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PRIMERS FOR CASTILLEJA AND THEIR UTILITY ACROSS OROBANCHACEAE: II. SINGLE-COPY NUCLEAR LOCI

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Premise of the study: We developed primers targeting nuclear loci in Castilleja with the goal of reconstructing the evolutionary history of this challenging clade. These primers were tested across other major clades in Orobanchaceae to assess their broader utility.

Methods and Results: We assembled low-coverage genomes for three taxa in Castilleja and developed primer combinations for the single-copy conserved ortholog set (COSII) and the pentatricopeptide repeat (PPR) gene family. These primer combinations were designed to take advantage of the Fluidigm microfluidic PCR platform and are well suited for high-throughput sequencing applications. Eighty-seven primers were designed for Castilleja, and 27 were found to have broader utility in Orobanchaceae.

Conclusions: These results demonstrate the utility of these primers, not only across Castilleja but for other lineages within Orobanchaceae as well. This expanded molecular toolkit will be an asset to future phylogenetic studies in Castilleja and throughout Orobanchaceae.

Key words: Castilleja; high-throughput sequencing; microfluidic PCR; Orobanchaceae; single-copy nuclear genes.

Although the plastome has long been considered the workhorse of phylogenetic inference in plants, reliance on chloroplast data alone may limit the ability to identify inheritance patterns of polyploids, as well as introgression and hybridization events (Godden et al., 2012; Twyford and Ennos, 2012). The reliable estimation of an underlying species tree also depends upon the acquisition of multiple, unlinked loci—especially for recent and rapid radiations—shifting the focus toward the development of single- or low-copy nuclear gene regions for phylogenetic analyses. The process of identifying and developing these nuclear gene regions using traditional methods can be time consuming and costly, but the increasing availability of high-throughput sequencing data, as well as new bioinformatic approaches, allows for the efficient and cost-effective exploration of the nuclear genome.

Here we focus on developing a suite of putatively single-copy nuclear gene regions in Castilleja L. (Orobanchaceae; “the paintbrushes”), a clade rich with polyploid and hybrid taxa, and the product of an ongoing rapid radiation (Tank and Olmstead, 2008, 2009). Previous studies using the nuclear rDNA ITS and ETS regions, the low-copy nuclear gene waxy, and the plastid trnL-F and rps16 intron regions hinted at cyto-nuclear discordance in some taxa, and most relationships among closely related taxa were unresolved (Tank and Olmstead, 2008, 2009). We recently developed primer combinations targeting the most variable regions of the plastome in Castilleja (Latvis et al., 2017a), and now present a companion set of nuclear primers with the goal of obtaining a resolved species tree for this challenging clade, as well as to aid in the detection of introgression and hybrid speciation. Primers for microsatellite markers have also been developed by Fant et al. (2013) for population-level investigations. In part, we follow the approach outlined by Blischak et al. (2014) to develop nuclear primer combinations from genome-skimming data, while following specifications for the Fluidigm Access Array microfluidic PCR system (Fluidigm, South San Francisco, California, USA) (see Latvis et al., 2017a). Thus, all primer combinations use the same annealing temperature of 60°C and may be amplified in parallel prior to high-throughput sequencing or traditional Sanger sequencing. We specifically target putatively single-copy genes from the conserved ortholog set (COSII) and pentatricopeptide repeat (PPR) domains, both of which have been highlighted for their phylogenetic utility in plants (COSII: Wu et al., 2006; PPR domain: Olmstead, 2008, 2009). Previous studies using the nuclear ribosomal ITS and ETS regions, the low-copy nuclear gene waxy, and the plastid trnL-F and rps16 intron regions hinted at cyto-nuclear discordance in some taxa, and most relationships among closely related taxa were unresolved (Tank and Olmstead, 2008, 2009). We recently developed primer combinations targeting the most variable regions of the plastome in Castilleja (Latvis et al., 2017a), and now present a companion set of nuclear primers with the goal of obtaining a resolved species tree for this challenging clade, as well as to aid in the detection of introgression and hybrid speciation. Primers for microsatellite markers have also been developed by Fant et al. (2013) for population-level investigations. In part, we follow the approach outlined by Blischak et al. (2014) to develop nuclear primer combinations from genome-skimming data, while following specifications for the Fluidigm Access Array microfluidic PCR system (Fluidigm, South San Francisco, California, USA) (see Latvis et al., 2017a). Thus, all primer combinations use the same annealing temperature of 60°C and may be amplified in parallel prior to high-throughput sequencing or traditional Sanger sequencing. We specifically target putatively single-copy genes from the conserved ortholog set (COSII) and pentatricopeptide repeat (PPR) domains, both of which have been highlighted for their phylogenetic utility in plants (COSII: Wu et al., 2006; PPR domain: Olmstead, 2008, 2009).
### Table 1. Nuclear primer pairs designed for *Castilleja* (locus and region amplified), amplicon lengths, and validation results for Orobancheaceae and outgroup taxon *Paulownia*. All pairs were designed for an annealing temperature of 60°C (±1°C). Boldfaced rows correspond to core Orobancheaceae primers, defined by successful amplification in two or more major clades in Orobancheaceae (see Fig. 1).

| Locus (Region) | Primer sequences (5′-3′)` | Amplicon length (bp) | Clade I: Lindbergia sp. | Clade II: Schistospora americana | Clade III: *Orobanche californica* | Clade IV: *Castilleja lutea* | Clade V: *Lamourousia virga* | Clade IV: *Pediculata* | Clade V: *Neoepibolus peruvianus* | Clade V: *Rhobanthus alectorolophus* | Clade VI: *Harveya porphyra* | Clade VI: *Physocalyx major* | Paulownia (outgroup) |
|----------------|-----------------------------|----------------------|------------------------|-------------------------------|-------------------------------|-----------------------------|-----------------------------|------------------------|-----------------------------|-------------------------------|------------------------|------------------------|----------------------|
| At1g34560_688F | F: CTGTGGTGGCAGCAATCAGC | 498 | X | X | X | X | X | X | X | X | X |  | | |
| At2g54560_1185F (COSII) |  |  |  |  |  |  |  |  |  |  |  |  |  | |
| At5g44630_1330F | F: TGCAACCCAGTCCCAAATGCA | 499 | X | X | X | X | X | X | X | X |  |  |  | |
| At5g44630_1330F (COSII) | R: TGGACAGCGGCGAATTGAGT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At3g04260_665F | R: AAGGCTTCAATCAGATGGAAC |  |  |  |  |  |  |  |  |  |  |  |  | |
| At4g28390_1994F | F: CATGCTGTGGAAGCTTGGC | 511 | X | X | X | X | X | X | X | X |  |  |  | |
| At2g3930_1959F | R: GAGATCTGAGTCAATGTG |  |  |  |  |  |  |  |  |  |  |  |  | |
| At3g26430_2136F | F: TCTGACAGTCCACCTGTTG | 496 | X | X | X | X | X | X | X | X |  |  |  | |
| At4g21430_1278F | R: CTTAGGTCAGGTAATGTAATAC | 499 | X | X | X | X | X | X | X | X |  |  |  | |
| At4g21490_1777F | F: TGACGATTTCCATCAATGTTGCC | 799 | X | X | X | X | X | X | X | X |  |  |  | |
| At5g27620_535F | R: CCTGGAGAAGGATGTGAGCG |  |  |  |  |  |  |  |  |  |  |  |  | |
| At5g27620_1335F (COSII) | R: TCCGTGAAAGCAGGAGTG | 499 | X | X | X | X | X | X | X | X |  |  |  | |
| At3g62010_1226F | F: CAAAAGCTTCGGTCTCAGG | 499 | X | X | X | X | X | X | X | X |  |  |  | |
| At3g62010_1725F | R: GCTTCAAGTGTTGAGGTTCCACCA | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At3g62010_1725F (COSII) |  |  |  |  |  |  |  |  |  |  |  |  |  | |
| At4g48800_1121F | F: GCATCGGATATCTCCATTCTCC | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At4g48800_1121F (COSII) | R: GGATAGAAGATAATGGTGGT |  |  |  |  |  |  |  |  |  |  |  |  | |
| At3g62010_1245F | F: GATGACCTAGATGGTAAAGGC | 501 | X | X | X | X | X | X | X | X |  |  |  | |
| At3g62010_1926F | R: AAGCCAATGGTTGAGGATTG | 502 | X | X | X | X | X | X | X | X |  |  |  | |
| At4g24380_948F | R: AAGGCTTCAATCAGATGGAAC | 499 | X | X | X | X | X | X | X | X |  |  |  | |
| At4g28380_1447F | R: CTTAGGTCAGGTAATGTAATAC | 499 | X | X | X | X | X | X | X | X |  |  |  | |
| At3g62010_2467F | F: TCTGACAGTCCACCTGTTG | 499 | X | X | X | X | X | X | X | X |  |  |  | |
| At5g27620_11180F | R: CCAATCCATACCTCCTAGGAAAGAAGAAG | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At5g27620_1680R (COSII) | R: CAGTGCTTCACAAATTTGCTGATG | 499 | X | X | X | X | X | X | X | X |  |  |  | |
| At5g27620_1811R | F: GCCATGTTGTTGTTGCTTACCC | 499 | X | X | X | X | X | X | X | X |  |  |  | |
| At1g29210_118F | R: TGTGGGACTTGGTAGGATG | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At2g29210_1064F | R: CTTAGGTCAGGTAATGTAATAC | 499 | X | X | X | X | X | X | X | X |  |  |  | |
| At3g62010_2174F | F: ACSGACGACTTACTACCTTCTT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At1g20300_2174R (COSII) | R: CAGCCTTCAAGCGAGCGCTGGT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At1g20300_2174R | R: CAGCCTTCAAGCGAGCGCTGGT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At1g20300_2174R | R: CAGCCTTCAAGCGAGCGCTGGT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At1g20300_2174R | R: CAGCCTTCAAGCGAGCGCTGGT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At1g20300_2174R | R: CAGCCTTCAAGCGAGCGCTGGT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At1g20300_2174R | R: CAGCCTTCAAGCGAGCGCTGGT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At1g20300_2174R | R: CAGCCTTCAAGCGAGCGCTGGT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At1g20300_2174R | R: CAGCCTTCAAGCGAGCGCTGGT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At1g20300_2174R | R: CAGCCTTCAAGCGAGCGCTGGT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| At1g20300_2174R | R: CAGCCTTCAAGCGAGCGCTGGT | 500 | X | X | X | X | X | X | X | X |  |  |  | |
| Locus (Region) | Primer sequences (5'-3') | Amplicon length (bp) | Clade I: Lindernbergia sp. | Clade II: Schwallaea americana | Clade III: Orobanchaceae | Clade IV: Castilleja lineariloba, C. pumila, C. lemmonii | Clade IV: Lamprosia virgata | Clade IV: Pedicularis sp. | Clade V: Neobartsia peruviana | Clade V: Rhinanthus alectorolophus | Clade VI: Harveya purpurea | Clade VI: Physocalyx major | Paulowniaceae: Paulownia fortunei (outgroup) |
|---------------|--------------------------|----------------------|-------------------------|-----------------------------|------------------------|---------------------------------|-----------------|-----------------|------------------------|------------------------|-----------------|---------------------|------------------|
| At5g26360_322F | F: ATGGGAGCTATATGACGAGAGTTG | 496 | X | X | | | | | | | | | |
| At5g26360_818R | R: TGTCAAGACATGCAAATACGC | | | | | | | | | | | | |
| At5g26360_1322F | F: TTACCGCCTGACATAGATGC | 496 | X | X | | | | | | | | | |
| At5g26360_1821R | R: ACTGAGCAGAAGAGGCTGCG | | | | | | | | | | | | |
| Locus (Region) | Primer sequences (5′–3′) | Clade I: *Linaria annua* | Clade II: *Calystegia sepium* | Clade III: *Cupanopsis crispata* | Clade IV: *Cupanopsis gigantea* | Clade V: *Cupanopsis tomentosa* | Clade VI: *Physocalyx minor* |
|---------------|--------------------------|------------------------|----------------------------|----------------------------|----------------------------|----------------------------|---------------------------|
| At1g09000_1928F | F: AAACGTGCTGAGAAGAACC  
R: TTTATGCGGTAAGTCAGAG | X | X |  | X |  |  |
| At1g12550_1000F | F: AGCCTGGAGAACGCTGACTAC  
R: AAAAGACAGGTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: CCGACAGGAAGGCGAGGCG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACCAGTGAGGTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: AAAGCAGGACGGGGGTACG | X | X |  | X |  |  |
| At1g12550_1000R | F: AGCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: GGCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TTCACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: TACCGCTGGACTGACCGGCAG | X | X |  | X |  |  |
| At1g12550_1000R | F: ACTACGCTGGACTGACCGGCAG | X | X |  | X |  |  |
Table 1. Continued.

| Accession | Clade | Castilleja lutea | Castilleja lemmonii | Castilleja pusilla | Miscanthus × giganteus | Sporobolus paniculatus | Wild Oat | Other | Total contigs |
|-----------|-------|------------------|---------------------|-------------------|-----------------------|-----------------------|---------|-------|-------------|
| EJ211534  | Clade V | ● | ● | ● | ● | ● | ● | ● | ● |
| EJ211535  | Clade V | ● | ● | ● | ● | ● | ● | ● | ● |
| EJ211536  | Clade V | ● | ● | ● | ● | ● | ● | ● | ● |
| EJ211537  | Clade V | ● | ● | ● | ● | ● | ● | ● | ● |

a Primer sequence for the “Castilleja-specific primer.”

b Percentage of alignment length (bp) estimated from BLAST hit alignments.

c PCR validations were considered successful for Castilleja when amplification occurred for all three taxa, representing one annual lineage (C. fortunei) and two perennial lineages (C. pumila and C. cusickii).

d PCR validations using DNA from Bennett and Mathews (2006).

e PCR validations using DNAs from Bennett and Mathews (2006).

METHODS AND RESULTS

We assembled contigs from raw reads of three low-coverage Castilleja genomes, C. cusickii Greenm., C. foliolosa Hook. & Arn., and C. tenuis (A. Heller) T. I. Chuang & Heckard (Latvis et al., 2017a; National Center for Biotechnology Information [NCBI] Sequence Read Archive [SRA] accession SRP100222) using CAP3 (Huang and Madan, 1999) with the default settings. The accessions were sequenced as 100-bp single-end reads on an Illumina HiSeq 2000 (Illumina, San Diego, California, USA), yielding ~12.5 million reads per taxon (Uribe-Convers et al., 2014) and an average depth of coverage of ~0.8×. These taxa include both annual and perennial lineages of Castilleja and span the phylogenetic breadth of the clade (Tank and Olmstead, 2008, 2009). These assemblies were then culled to include only contigs of 1 Kb or larger using a custom R script. The culled assemblies and script are available from the Dryad Digital Repository (http://doi.org/10.5061/dryad.52v62; Latvis et al., 2017b).

To search for hits among our contigs, available COS sequences were obtained from Sol genomics (https://solgenomics.net), and PPR loci were mined from the *Mimulus* L. genome on Phytozome (Heller et al., 2013; https://phytozome.jgi.doe.gov) using the 127 PPR orthologs identified in Arabidopsis Heynh. by Yuan et al. (2009) as references. Both gene sets may be found in Uribe-Convers et al. (2016) and were used to construct local BLAST databases for the search (–makeblastdb). We used TBLASTX to search each *Castilleja* CAP3 assembly (with contigs of 1 KB or greater) against both the COS and PPR databases, indicating tab-delimited output (–outfmt 6). Output files were filtered for alignment length >200 and a maximum E-value of 1e-10, and were culled to include only unique hits.

Hits shared between *C. cusickii*, *C. foliolosa*, and *C. tenuis* were placed together into individual FASTA files (data available from the Dryad repository: http://doi.org/10.5061/dryad.52v62; Latvis et al., 2017b), imported into Geneious R7 version 7.0.6 (Kearse et al., 2012), and aligned with MAFFT version 7.017b under the default settings (Katoh and Standley, 2013). We designed primer pairs using Primer3 (Untergasser et al., 2012) using the same specifications for the Fluidigm Access Array system as Latvis et al. (2017a), but with a size range between 400–525 bp and an optimal size of 500 bp. We designed 10–30 primer pairs for each identified locus and prioritized them based on desired size and the presence of multiple G or C bases at the 3' end of the primers (GC clamp). This also allowed us to design overlapping sets of primers with the potential to combine them after sequencing to produce longer contigs for downstream phylogenetic analyses. Suitable primer pairs were validated for *Castilleja* with PCR following the same amplification protocol and using the same *Castilleja* accessions as in Latvis et al. (2017a) and visualized on an agarose gel. We present 87 nuclear primer combinations specifically designed and validated for *Castilleja* (Table 1).

To investigate whether any of these primer combinations would amplify successfully across Orobanchaceae, we searched for our selected *Castilleja* primers against an assembled low-coverage genome for *Lamourousia multifida* Kunth using BLAST (Altschul et al., 1990). *Lamourousia multifida* was sequenced on an Illumina HiSeq 2000 at the University of Oregon as 100-bp paired-end reads, and contigs were assembled using SPAdes (Bankevich et al., 2012) under the
default settings. BLAST search parameters, assessment of suitable hits, and subsequent PCR validation with Lamourouxia virgata Kunth, Physocalyx major Mart., and Neobartsia filiformis (Wedd.) Uribe-Convers & Tank are described in Latvis et al. (2017a). Primer combinations with successful amplification in Lamourouxia Kunth and at least one other taxon were selected for further PCR testing with other major lineages in Orobanchaceae (sensu McNeal et al., 2013; Fig. 1). This second round of PCR validation follows Latvis et al. (2017a), except that two of the accessions used for testing were changed. As in Latvis et al. (2017a), we also included a negative control and conserved sequence–tagged “universal” primers for the trnL-F region as a positive control for all primer pairs. We used Neobartsia peruviana (Walp.) Uribe-Convers & Tank instead of N. filiformis, and Paulownia fortunei (Seem.) Hemsl. instead of P. elongata Siebold & Zucc. (Appendix 1). Of the 87 nuclear primer combinations specifically designed for Castilleja, we identified 27 with broader applicability in Orobanchaceae, chosen if they successfully amplified in Physocalyx (Clade IV, including Castilleja, Lamourouxia, and Pedicularis L.) and at least one of the other major clades highlighted in Fig. 1. Validation results are presented in Table 1 with these “core Orobanchaceae” combinations boldfaced.

CONCLUSIONS

We present 87 nuclear primer pairs specifically designed for Castilleja that target COSII and PPR loci. Although we target the same putative single-copy nuclear domains as previous studies (Wu et al., 2006; Li et al., 2008; Yuan et al., 2010; Blischak et al., 2014; Uribe-Convers et al., 2016), we developed primers for different loci and present unique primer combinations in this study. As with our chloroplast primers (Latvis et al., 2017a), all combinations were designed with the Fluidigm microfluidic PCR system in mind, allowing for parallelization of amplification for downstream high-throughput sequencing platforms. Of these, we identify a set of 27 primer combinations with broader utility across Orobanchaceae. The development of primers for putatively single-copy nuclear loci will greatly enhance efforts to understand evolutionary history at multiple taxonomic scales, both for Castilleja and across Orobanchaceae.

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| Species                  | Voucher accession no. (Herbarium) | Collection locality | Geographic coordinates          |
|-------------------------|-----------------------------------|---------------------|---------------------------------|
| Castilleja cusickii     | Tank 2009-01 (ID)                  | Idaho, USA          | 45.884241°N, 116.230195°W       |
| Castilleja foliolarosa  | A. Colwell 03-09 (YM)              | California, USA     | 35.3926°N, 120.3522°W          |
| Castilleja lemmonii     | Jacobs 2015-088 (ID)               | California, USA     | 37.907982°N, 120.10833°W       |
| Castilleja lineariloba  | Tank 2002-04 (WTU)                 | California, USA     | 37.41387°N, 120.10833°W        |
| Castilleja pumila       | Uribe-Convers 2011-120 (ID)       | La Libertad, Peru   | 7.99506°S, 78.44197°W         |
| Castilleja tenax        | Tank 2003-13 (WTU)                 | Washington, USA     | 46.11813°N, 121.5158°W        |
| Harveya purpurea        | Randle 79 (OS)                     | NA                  | NA                             |
| Lamuroxoa multifida    | Mejia 695 (CAS)                   | Chiapas, Mexico     | NA                             |
| Lamuroxoa virgata      | Mejia 581 (CAS)                   | Chiapas, Mexico     | 16.713611°N, 92.614722°W       |
| Lindenbergia sp.       | Armstrong 1163 (ISU)              | NA                  | NA                             |
| Neobartsia peruviana    | Uribe-Convers 13-011 (ID)         | NA                  | NA                             |
| Orobanche californica   | Bennett 72 (A)                    | Cultivated          | Cultivated                     |
| Paeonia fortunei        | s.d. (A)                          | Cultivated          | (https://sheffield.com)         |
| Pedicularis sp.         | Krasek and Bennett s.n. (A)       | Slovenia            | NA                             |
| Physocalyx major        | G. O. Romão 2528 (ESA)            | Minas Gerais, Brazil| 19.2635°S, 43.5508°W          |
| Rhinanthis alectorophus | Bennett 85 (A)                    | NA                  | NA                             |
| Schwalbea americana    | Kirkman s.n. (PAC)                | NA                  | NA                             |

Note: NA = not available.

*Herbarium acronyms are per Index Herbariorum (http://sweetgum.nybg.org/science/ih/).