

South Dakota State University

## Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange

---

Natural Resource Management Faculty Publications

Department of Natural Resource Management

---

2017

### Primers for Castilleja and their Utility Across Orobanchaceae: II. Single-copy nuclear loci

Maribeth Latvis

*South Dakota State University*

Sarah J. Jacobs

*University of Idaho*

Sebastian M. E. Mortimer

*University of Idaho*

Melissa Richards

*University of Idaho*

Paul D. Blischak

*The Ohio State University*

*See next page for additional authors*

Follow this and additional works at: [https://openprairie.sdstate.edu/nrm\\_pubs](https://openprairie.sdstate.edu/nrm_pubs)

---

#### Recommended Citation

Latvis, Maribeth; Jacobs, Sarah J.; Mortimer, Sebastian M. E.; Richards, Melissa; Blischak, Paul D.; Mathews, Sarah; and Tank, David C., "Primers for Castilleja and their Utility Across Orobanchaceae: II. Single-copy nuclear loci" (2017). *Natural Resource Management Faculty Publications*. 295.  
[https://openprairie.sdstate.edu/nrm\\_pubs/295](https://openprairie.sdstate.edu/nrm_pubs/295)

This Article is brought to you for free and open access by the Department of Natural Resource Management at Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange. It has been accepted for inclusion in Natural Resource Management Faculty Publications by an authorized administrator of Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange. For more information, please contact [michael.biondo@sdstate.edu](mailto:michael.biondo@sdstate.edu).

---

**Authors**

Maribeth Latvis, Sarah J. Jacobs, Sebastian M. E. Mortimer, Melissa Richards, Paul D. Blischak, Sarah Mathews, and David C. Tank

## PRIMERS FOR *CASTILLEJA* AND THEIR UTILITY ACROSS OROBANCHACEAE: II. SINGLE-COPY NUCLEAR LOCI<sup>1</sup>

MARIBETH LATVIS<sup>2,8</sup>, SARAH J. JACOBS<sup>3,4,5</sup>, SEBASTIAN M. E. MORTIMER<sup>3,4</sup>,  
MELISSA RICHARDS<sup>3</sup>, PAUL D. BLISCHAK<sup>6</sup>, SARAH MATHEWS<sup>7</sup>, AND DAVID C. TANK<sup>3,4,5</sup>

<sup>2</sup>Department of Natural Resource Management, South Dakota State University, 1390 College Avenue, Brookings, South Dakota 57007 USA; <sup>3</sup>Department of Biological Sciences, University of Idaho, 875 Perimeter Drive, MS 3051, Moscow, Idaho 83844-3051 USA; <sup>4</sup>Stillinger Herbarium, University of Idaho, 875 Perimeter Drive, MS 1133, Moscow, Idaho 83844-1133 USA; <sup>5</sup>Institute for Bioinformatics and Evolutionary Studies (IBEST), University of Idaho, 875 Perimeter Drive, MS 3051, Moscow, Idaho 83844-3051 USA; <sup>6</sup>The Ohio State University, Department of Evolution, Ecology, and Organismal Biology, 318 W. 12th Avenue, Columbus, Ohio 43210 USA; and <sup>7</sup>Australian National Herbarium, CSIRO National Research Collections, Canberra, Australia

- *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 ribosomal ITS and ETS regions, the low-copy nuclear gene *waxy*, and the plastid *trnL-F* and *rps16* intron regions hinted at cytonuclear 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:

<sup>1</sup>Manuscript received 15 April 2017; revision accepted 20 July 2017.

This research was supported by resources at the Institute for Bioinformatics and Evolutionary Studies (IBEST; NIH/NCRR P20RR16448 and P20RR016454) and by the following awards from the National Science Foundation: DEB-1253463 (awarded to D.C.T.), DEB-1502061 (awarded to D.C.T. for S.J.J.), and DEB-1455399 (support for P.D.B.).

<sup>8</sup>Author for correspondence: Maribeth.Latvis@sdstate.edu

TABLE 1. Nuclear primer pairs designed for *Castilleja* (locus and region amplified), amplicon lengths, and validation results for Orobanchaceae and outgroup taxon *Paulownia*. All pairs were designed for an annealing temperature of 60°C (±1°C). Boldfaced rows correspond to core Orobanchaceae primers, defined by successful amplification in two or more major clades in Orobanchaceae (see Fig. 1).

| Locus (Region)          | Primer sequences (5'–3') <sup>a</sup>                       | Amplicon length (bp) <sup>b</sup> | Clade IV:<br><i>Castilleja</i>                   |  |   |   |  |  |  |   |   |   |   | Paulowniaceae:<br><i>Paulownia fortunei</i> (outgroup) |
|-------------------------|---|-----------------------------------|--|--|---|---|--|--|--|---|---|---|---|--|
|                         |   |                                   | Clade I:<br><i>Lindenbergia</i> sp. <sup>c</sup> | Clade II:<br><i>Schwalbea americana</i> <sup>c</sup> | Clade III:<br><i>Orobancha californica</i> <sup>c</sup> | Clade IV:<br><i>Lamourotaxia virgate</i> <sup>c</sup> | Clade IV:<br><i>Pedicularis</i> sp. <sup>c</sup> | Clade V:<br><i>Neobartsia peruviana</i> <sup>c</sup> | Clade V:<br><i>Rhinanthus electrolophilus</i> <sup>c</sup> | Clade VI:<br><i>Harveya purpurea</i> <sup>c</sup> | Clade VI:<br><i>Physocalyx major</i> <sup>c</sup> |   |   |  |
| A12g34560_688F          | F: CTFTTGTGGCAGCAAGTACC<br>R: AAAGCTGTGCACTGAAGC            | 498                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A12g34560_1186R (COSII) | F: TGACAAAACAGTCCAAATGCA<br>R: TTGGCAGGACGGGATTTAAGT        | 499                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A15g46630_851F          | F: TGAGTGGAAAGTTGAATGGC<br>R: AAGGCTGAAGGCATAGGAC           | 500                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A13g04260_165F          | F: ATGCACTGTGAGCCCTTT<br>R: GAGTGGCTGACTGAAGCT              | 511                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A15g12370_3513F         | F: TGCAATGCAAGTATCAGTCTTG<br>R: GACATGGGATTTGATCTTGGCAG     | 499                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A12g28390_1096F         | F: TTGCAAGATCCCAAGCTTTCTGC<br>R: GCGCCAGTTGTGTTTGG          | 496                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A12g26430_2136F         | F: CTGAACTCCCAAGATTTAGCAATAGTAC<br>R: TCAGGCAATGGACAGATTAGT | 499                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A14g24190_1278F         | F: CCGCAATACTTTATCAACTTCCGC<br>R: CTTGGGAAAGGATTTGAAACAG    | 799                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A15g27620_538F          | F: CAACACTCTTTGCTCTCAGG<br>R: GCTTCAGATGTTGATTCATCCCA       | 499                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A15g62010_1226F         | F: GTCACAGGAATAACTTTCATCTCC<br>R: GCGATGAGGCAATATTTGTGGTTG  | 500                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A15g48300_1121F         | F: GATAGCCTATAGCAATGCAACACC<br>R: AGCAATCTGGTGTAGGATG       | 501                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A13g62010_1425F         | F: AAACAACAACCTCGCAGCC<br>R: AAGTGTCTCTGGCTTACAGT           | 499                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A14g24830_948F          | F: TCCTAGAAGTGGTCAAGGAGT<br>R: CTGGTATAGCAGCCGGAAGT         | 499                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A13g04260_147F          | F: CGAATCCTAAACTTGCATGAAAGA<br>R: CGATGCTTCAAAATTTCCACGT    | 500                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A15g27620_1180F         | F: GGCAATGGTTGGTCTCACC<br>R: TGTFTGGACTGTGAAGAGTGT          | 499                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A15g26360_1811R (COSII) | F: TGAGACCATGGATGCAAAGC<br>R: GCTGTGCACTCAGAAAGCAG          | 500                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A12g29210_514F          | F: AGCCAAGCACTTACTCATCCT<br>R: CAGATTAAGGAAAGGCTGTAG        | 500                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A13g62010_2_1674F       | F: AGCCBAGCAGTACTCATCCT<br>R: ATATCTGTGTATAGCATGGCAACT      | 616                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A13g62010_1674F         | F: GTGCTATTAATGCACTGCACTTGT<br>R: CAGCATGAGCAGCAACATCTG     | 500                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A12g38020_1797F         | F: TTCTAACACGCCCAAGCCCA<br>R: CFTAGCTCTTCCCTGGGCA           | 499                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A12g38770_387F          | F: TCCAGCAATAGCACTATGCC<br>R: AATPATTGCCCTCAATGCTTGGC       | 490                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A14g11120_1226F         | F: GCATGTAGCCAGCAGCTTAA<br>R: CCGCTCGGAAGGGTTTATA           | 512                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A18g0150_1016F          | F: GGCBAACTGTAGAGAGCCCA<br>R: TGTAGAGAAAATAACATTCACAGCA     | 487                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A11g80150_1528R (PPR)   | F: CGAAATCTGTTCCGAGAGCG<br>R: CCACCTCACCTCTAGCTGG           | 467                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A11g04200_1908F         | F: CATCCTCATCGACTCTGAGG<br>R: TGCACGGAGTTAAATAATCATCG       | 500                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A12g07050_1067F         | F: ACFTCCCAATGAAAGCCCTT<br>R: GTCGATATCGGAATGATGCC          | 499                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |
| A14g02720_1652F         |   | 543                               | X  | X  | X   | X   | X  | X  | X  | X   | X   | X | X | X  |

TABLE I. Continued.

| Locus (Region)          | Primer sequences (5'-3') <sup>a</sup>                             | Amplicon length (bp) <sup>b</sup> | Clade I:<br><i>Lindenbergia</i> sp. <sup>c</sup> | Clade II:<br><i>Schwalbea americana</i> <sup>c</sup> | Clade III:<br><i>Orobancha californica</i> <sup>c</sup> | Clade IV:<br><i>Castilleja lineariloba</i> ,<br><i>C. pumila</i> ,<br><i>C. lemmonii</i> <sup>d</sup> | Clade IV:<br><i>Lamourossia virgate</i> <sup>c</sup> | Clade V:<br><i>Rhinanthus albertopolifus</i> <sup>c</sup> | Clade VI:<br><i>Harveya purpurea</i> <sup>c</sup> | Clade VI:<br><i>Physocalyx major</i> <sup>c</sup> | Paulowniaceae:<br><i>Paulownia fortunei</i> <sup>c</sup><br>(outgroup) |
|-------------------------|---|-----------------------------------|--|--|---|---|--|---|---|---|--|
| A15g26360_322F          | F: <b>TTCA</b> CGGTA <b>TCC</b> CA <b>TCTATGC</b>                 | 496                               | X  |  |   | X   |  |   |   | X   |  |
| A15g26360_818R (COSII)  | R: <b>ATG</b> TA <b>TG</b> T <b>CTG</b> T <b>GG</b> CA <b>CGA</b> |                                   |  |  |   |   |  |   |   |   |  |
| A15g49970_503F          | F: AGCAGCTACAAGACATCGC  | 495                               | X  |  |   | X   |  |   |   |   |  |
| A15g49970_998R (COSII)  | R: AGCTGGGAATGATGAAATCC   |                                   |  |  |   |   |  |   |   |   |  |
| A15g52210_456F          | F: ATCCAGCACAACCTCAAC   | 499                               | X  |  |   | X   |  |   |   |   |  |
| A15g52210_955R (COSII)  | R: GGATTTGCAAGAGCTCCAC  |                                   |  |  |   |   |  |   |   |   |  |
| A1g09620_1187F          | F: GFTCTACAGGAGCTGAAA   | 501                               | X  |  |   | X   |  |   |   |   |  |
| A1g09620_1688R (COSII)  | R: ACCGGACACCTCAGATCAA  |                                   |  |  |   |   |  |   |   |   |  |
| A1g09620_1490F          | F: AGCTCATATGTTGGAGGAGG   | 499                               | X  |  |   | X   |  |   |   |   |  |
| A1g09620_1989R (COSII)  | R: CCTCTCCATCCATGCAATCT   |                                   |  |  |   |   |  |   |   |   |  |
| A1g09620_1483F          | F: GTTCCAGGTCAACCCG   | 500                               | X  |  |   | X   |  |   |   |   |  |
| A1g28190_983R (COSII)   | R: TGCAACTGTTCTGAAATGG  |                                   |  |  |   |   |  |   |   |   |  |
| A2g38020_892F           | F: TTTCACATTTCTGTGCTTACCA   | 499                               | X  |  |   | X   |  |   |   |   |  |
| A2g38020_1391R (COSII)  | R: CGTACAGCAITTAAGACCGC   |                                   |  |  |   |   |  |   |   |   |  |
| A4g22670_93F            | F: GAATTTGGTTGGCATACAGC   | 496                               | X  |  |   | X   |  |   |   |   |  |
| A4g22670_589R (COSII)   | R: AGTCTAGCTTTCAGCCACA  |                                   |  |  |   |   |  |   |   |   |  |
| A15g26360_1322F         | F: TGFTTCTACCTTCTTGTACTCA   | 499                               | X  |  |   | X   |  |   |   |   |  |
| A15g26360_1821R (COSII) | R: GGTCCGATTAATGGACTTG  |                                   |  |  |   |   |  |   |   |   |  |
| A2g29210_902F           | F: TTGAATGCAGCAGTAAATGGAA   | 499                               | X  |  |   | X   |  |   |   |   |  |
| A2g29210_1401R (COSII)  | R: ACGCCCTACAGCAGTTTG   |                                   |  |  |   |   |  |   |   |   |  |
| A1g01940_828F           | F: TCATAACTATGTAAGCCAAACCATGAT                                    | 399                               | X  |  |   | X   |  |   |   |   |  |
| A1g01940_1227R (COSII)  | R: TATAAAGGTTTTCATGATACAAGTTGG                                    |                                   |  |  |   |   |  |   |   |   |  |
| A4g24620_1780F          | F: CTGTTCCCTGTATACCAAGATTTG                                       | 443                               | X  |  |   | X   |  |   |   |   |  |
| A4g24620_2223R (COSII)  | R: CAGTTATATTAACAGCTCAGATGG                                       |                                   |  |  |   |   |  |   |   |   |  |
| A15g62530_439F          | F: TGGAACAAATGCTTCGATTACTCC                                       | 499                               | X  |  |   | X   |  |   |   |   |  |
| A15g62530_938R (COSII)  | R: TGGCGGTGAGAGAGTCAATG   |                                   |  |  |   |   |  |   |   |   |  |
| A15g62010_1468F         | F: ACAATAGATATGACCTGCGCGA   | 501                               | X  |  |   | X   |  |   |   |   |  |
| A15g62010_1969R (COSII) | R: GCACATGGAGCAGGTTTG   |                                   |  |  |   |   |  |   |   |   |  |
| A4g11120_1399F          | F: CCCATGTGAAGTGGGATA   | 460                               | X  |  |   | X   |  |   |   |   |  |
| A4g11120_1859R (COSII)  | R: GCTTGTGAGCCATCCAG  |                                   |  |  |   |   |  |   |   |   |  |
| A2g41680_402F           | F: TGATCCGGATGTTGCTGCT  | 500                               | X  |  |   | X   |  |   |   |   |  |
| A2g41680_902R (COSII)   | R: GGATCCGGATGTTGCTGCT  |                                   |  |  |   |   |  |   |   |   |  |
| A15g22830_933F          | F: AAAGCCTAATGCACTATTGTAAC  | 499                               | X  |  |   | X   |  |   |   |   |  |
| A15g22830_1432R (COSII) | R: ACCAGTCTGTTTGTAAATACGA   |                                   |  |  |   |   |  |   |   |   |  |
| A15g26730_1284F         | F: AGATAACAATCTATCTGATCCATGGA                                     | 486                               | X  |  |   | X   |  |   |   |   |  |
| A15g26730_1770R (COSII) | R: TGTTTCAGGACAAGTGGCTCAG   |                                   |  |  |   |   |  |   |   |   |  |
| A2g17670_501F           | F: ATCCAATAGCCCGCCTTAC  | 497                               | X  |  |   | X   |  |   |   |   |  |
| A2g17670_998R (PPR)     | R: AAGGATTCGGTATAAAGCCTGAT  |                                   |  |  |   |   |  |   |   |   |  |
| A4g37170_962F           | F: CAACCTACCGGGTTTCTTCCAC   | 504                               | X  |  |   | X   |  |   |   |   |  |
| A4g37170_1466R (PPR)    | R: ATGGGTAATGCTCAGAATGGTCCAG                                      |                                   |  |  |   |   |  |   |   |   |  |
| A4g30825_1603F          | F: GGTGACATGTTGATCATCGGT  | 499                               | X  |  |   | X   |  |   |   |   |  |
| A4g30825_2102R (PPR)    | R: TTCAAAGCCCGACTTGACA  |                                   |  |  |   |   |  |   |   |   |  |
| A13g14730_878F          | F: ACGGGTGTAGCTAACAGG   | 500                               | X  |  |   | X   |  |   |   |   |  |
| A13g14730_1378R (PPR)   | R: AACGGCTTCTCATGCTC  |                                   |  |  |   |   |  |   |   |   |  |
| A15g18475_417F          | F: ACGGATGAAGCTATCGAGATGC   | 498                               | X  |  |   | X   |  |   |   |   |  |
| A15g18475_913R (PPR)    | R: AACATGACACACAATAATGCT  |                                   |  |  |   |   |  |   |   |   |  |
| A1g13040_1457F          | F: TGTAGCTCACATGTCAGGC  | 501                               | X  |  |   | X   |  |   |   |   |  |
| A1g13040_1958R (PPR)    | R: AGTGGAGCTCAAATGCAATGTT   |                                   |  |  |   |   |  |   |   |   |  |
| A1g16890_816F           | F: AAACCCATCGATCGTCAATTAATTTG                                     | 499                               | X  |  |   | X   |  |   |   |   |  |
| A1g16890_1315R (PPR)    | R: TCCACCTCCATTTGATGAAACTTTG                                      |                                   |  |  |   |   |  |   |   |   |  |
| A1g20300_782F           | F: GTGATCGAGCTTTGTGCC   | 504                               | X  |  |   | X   |  |   |   |   |  |
| A1g20300_1286R (PPR)    | R: TCGGCTCCACCTCATCTCA  |                                   |  |  |   |   |  |   |   |   |  |
| A1g07230_362F           | F: GGACCTCATCAACGGTTCC  | 508                               | X  |  |   | X   |  |   |   |   |  |
| A1g07230_870R (COSII)   | R: GTCAATCCACGGCGAAATCAA  |                                   |  |  |   |   |  |   |   |   |  |
| A1g17760_1370F          | F: TTGCTTACATCCCACTTCGG   | 497                               | X  |  |   | X   |  |   |   |   |  |
| A1g17760_1867R (COSII)  | R: AACATCAATTCAGTATTGAAACCCA                                      |                                   |  |  |   |   |  |   |   |   |  |
| A1g26640_1171F          | F: TGCAAGGCCCTGCAAAATTC   | 499                               | X  |  |   | X   |  |   |   |   |  |
| A1g26640_1670R (COSII)  | R: TGGGTCTCTCAGAAATTCATCTG  |                                   |  |  |   |   |  |   |   |   |  |

TABLE I. Continued.

| Locus (Region)         | Primer sequences (5'-3') <sup>a</sup> | Amplicon length (bp) <sup>b</sup> | Clade I:<br><i>Lindenbergia</i> sp. <sup>c</sup> | Clade II:<br><i>Schwalbea americana</i> sp. <sup>c</sup> | Clade III:<br><i>Orobanchae californica</i> | Clade IV:<br><i>Castilleja lineariloba</i> ,<br><i>C. pumila</i> ,<br><i>C. lemmonii</i> | Clade IV:<br><i>Lamourossia virgata</i> sp. <sup>c</sup> | Clade V:<br><i>Neobanksia peruviana</i> | Clade V:<br><i>Rhinanthus alectorolophus purpureae</i> | Clade VI:<br><i>Harveya Physocalyx major</i> | Paulowniaceae:<br><i>Paulownia fortunei</i> (outgroup) |
|------------------------|---------------------------------------|-----------------------------------|--|--|---|--|--|---|--|--|--|
| A1g30580_2218F         | F: TGAAAGGCTGTGAAGTTGA                | 500                               | X  |  |   |  |  |   |  |  |  |
| A1g30580_2718R (COSII) | R: GGATGGATCTTTGGGAGAAA               |                                   |  |  |   |  |  |   |  |  |  |
| A1g43860_2011F         | F: AGCATACAATTTCCAGGAGTGCATC          | 504                               | X  |  |   |  |  |   |  |  |  |
| A1g43860_2515R (COSII) | R: CTGGGGCCATCACAC                    |                                   |  |  |   |  |  |   |  |  |  |
| A1g59600_576F          | F: GGAGACCTGAAACTAGTATCC              | 499                               | X  |  |   |  |  |   |  |  |  |
| A1g59600_1075R (COSII) | R: AAGGTGTTGCTGAGCCTC                 |                                   |  |  |   |  |  |   |  |  |  |
| A1g69220_607F          | F: AGCCTCGTCCGTAACATCA                | 467                               | X  |  |   |  |  |   |  |  |  |
| A1g69220_1075R (COSII) | R: GGCACTGTATAAGCTCGAGA               |                                   |  |  |   |  |  |   |  |  |  |
| A2g03120_495F          | F: TGGGATCTGTGTAGTTTATCCAGA           | 500                               | X  |  |   |  |  |   |  |  |  |
| A2g03120_925R (COSII)  | R: ACGAAGAGCAGCAATCCCT                |                                   |  |  |   |  |  |   |  |  |  |
| A2g27170_2888F         | F: CCATCTTCATCCCTCCTCG                | 475                               | X  |  |   |  |  |   |  |  |  |
| A2g27170_3366R (COSII) | R: TTGACAGTCAATGGCTCAGCC              |                                   |  |  |   |  |  |   |  |  |  |
| A2g42490_4066F         | F: ACTTAGCCTTTGGACCAAGC               | 475                               | X  |  |   |  |  |   |  |  |  |
| A2g42490_4564R (COSII) | R: GCTTGGAGAAACGACAACT                |                                   |  |  |   |  |  |   |  |  |  |
| A3g03600_524F          | F: AGGCTCGAACTGTGTTGTC                | 499                               | X  |  |   |  |  |   |  |  |  |
| A3g03600_1023R (COSII) | R: GCCCTAAAACCTGCAACTC                |                                   |  |  |   |  |  |   |  |  |  |
| A3g04870_957F          | F: TCTTCCCATCTTCCCAAGGTC              | 455                               | X  |  |   |  |  |   |  |  |  |
| A3g04870_1412R (COSII) | R: ACTCAAAACAGGCTCAACTCA              |                                   |  |  |   |  |  |   |  |  |  |
| A3g05000_1412F         | F: ACCAACTAAGACAGCCTCAATCA            | 500                               | X  |  |   |  |  |   |  |  |  |
| A3g05000_1912R (COSII) | R: TTGCACTTTTACTTCCCTGTGG             |                                   |  |  |   |  |  |   |  |  |  |
| A3g09090_1427F         | F: TTTCTGTTTGTGATAGTTTACAGT           | 485                               | X  |  |   |  |  |   |  |  |  |
| A3g09090_1912R (COSII) | R: ATGGCCGACAAATGATCCA                |                                   |  |  |   |  |  |   |  |  |  |
| A3g13235_2063F         | F: GGCAACATGATCCGGCCTA                | 482                               | X  |  |   |  |  |   |  |  |  |
| A3g13235_2545R (COSII) | R: TTGGGAAACTAACCACACTCTGC            |                                   |  |  |   |  |  |   |  |  |  |
| A3g16150_956F          | F: ACGCAAGTTCAAGCATCAATGG             | 404                               | X  |  |   |  |  |   |  |  |  |
| A3g16150_1360R (COSII) | R: CCTTATTGCTCGGAATGCTCG              |                                   |  |  |   |  |  |   |  |  |  |
| A3g17205_926F          | F: TCAAGTCGAAACCACGATCTGA             | 499                               | X  |  |   |  |  |   |  |  |  |
| A3g17205_1425R (COSII) | R: GGACTGCTGGTAATGATCTG               |                                   |  |  |   |  |  |   |  |  |  |
| A3g55560_121F          | F: CAGAACTCCGGAGACTTCTCAC             | 499                               | X  |  |   |  |  |   |  |  |  |
| A3g55560_620R (COSII)  | R: GCCCACCGTCTTCAATACA                |                                   |  |  |   |  |  |   |  |  |  |
| A3g62580_1318F         | F: CATAATCGTCTGGGATTAACCG             | 499                               | X  |  |   |  |  |   |  |  |  |
| A3g62580_1817R (COSII) | R: GCAATCTGTTATCTTTACGCC              |                                   |  |  |   |  |  |   |  |  |  |
| A4g00560_2034F         | F: AAGCTGCCATGCTTTAAATGTC             | 494                               | X  |  |   |  |  |   |  |  |  |
| A4g00560_2528R (COSII) | R: GGAAACAGTCAATCCTCTCTCTG            |                                   |  |  |   |  |  |   |  |  |  |
| A4g12230_1167F         | F: TGTAGATTTGATCAAGCAATGG             | 417                               | X  |  |   |  |  |   |  |  |  |
| A4g12230_1584R (COSII) | R: TCAATGCTTGAATCCACTCATGG            |                                   |  |  |   |  |  |   |  |  |  |
| A4g23100_808F          | F: ATCACTCAAGAGAGAGCGG                | 499                               | X  |  |   |  |  |   |  |  |  |
| A4g23100_1307R (COSII) | R: CCTTCATATCGTATTTTACCACGG           |                                   |  |  |   |  |  |   |  |  |  |
| A4g29490_2406F         | F: CCAACCAACATGCAATGTCAGC             | 499                               | X  |  |   |  |  |   |  |  |  |
| A4g29490_2905R (COSII) | R: CCTAGCTACCTCAATGGCAG               |                                   |  |  |   |  |  |   |  |  |  |
| A4g32280_1754F         | F: CCGATGGCTAATCACCGCC                | 500                               | X  |  |   |  |  |   |  |  |  |
| A4g32280_2254R (COSII) | R: ATACGTTGACAGACTCGACA               |                                   |  |  |   |  |  |   |  |  |  |
| A4g33625_852F          | F: AGTAGGGCTCATTTAGAAACC              | 499                               | X  |  |   |  |  |   |  |  |  |
| A4g33625_1351R (COSII) | R: CATCTCTGTCGAAAGATTGG               |                                   |  |  |   |  |  |   |  |  |  |
| A4g38630_507F          | F: GGTACCTGTTCTAGGACGTGC              | 498                               | X  |  |   |  |  |   |  |  |  |
| A4g38630_1005R (COSII) | R: ATCCAAAGTTGGATCCACACC              |                                   |  |  |   |  |  |   |  |  |  |
| A5g06830_1899F         | F: GGCCCAACCCATTGCCATTA               | 499                               | X  |  |   |  |  |   |  |  |  |
| A5g06830_2398R (COSII) | R: ACTAGACTCTGGACTTTGACAG             |                                   |  |  |   |  |  |   |  |  |  |
| A5g07270_1779F         | F: CACATCCTTGCAACCTCAAGC              | 499                               | X  |  |   |  |  |   |  |  |  |
| A5g07270_2278R (COSII) | R: ACTGCTTCTAATGCTGCT                 |                                   |  |  |   |  |  |   |  |  |  |
| A5g12200_438F          | F: TCGGTTCTACTAGCTGCAAC               | 499                               | X  |  |   |  |  |   |  |  |  |
| A5g12200_937R (COSII)  | R: ATCAGAGTTAGGATGCTGTGT              |                                   |  |  |   |  |  |   |  |  |  |
| A5g13640_387F          | F: GGAACCTGATTTACAGGACTC              | 498                               | X  |  |   |  |  |   |  |  |  |
| A5g13640_885R (COSII)  | R: CTAGCTACGAGCAATGGC                 |                                   |  |  |   |  |  |   |  |  |  |
| A5g18580_933F          | F: GCTGCTTTATTCACGGTGT                | 499                               | X  |  |   |  |  |   |  |  |  |
| A5g18580_1432R (COSII) | R: ACAGTTACTCTGGTGAAGAG               |                                   |  |  |   |  |  |   |  |  |  |

TABLE 1. Continued.

| Locus (Region)           | Primer sequences (5'–3') <sup>a</sup> | Amplicon length (bp) <sup>b</sup> | Clade I:<br><i>Lindenbergia</i> sp. <sup>c</sup> | Clade II:<br><i>Schwalbea americanae</i> <sup>c</sup> | Clade III:<br><i>Orobanchae californicae</i> <sup>c</sup> | Clade IV:<br><i>Castilleja lineariloba</i> ,<br><i>C. pumila</i> ,<br><i>C. lemmonii</i> <sup>d</sup> | Clade IV:<br><i>Lamourouxia virgatae</i> <sup>e</sup> | Clade IV:<br><i>Pedicularis</i> sp. <sup>c</sup> | Clade V:<br><i>Neobartsia peruviana</i> <sup>c</sup> | Clade V:<br><i>Rhinanthus alectorolophus</i> <sup>c</sup> | Clade VI:<br><i>Harveya purpurea</i> <sup>c</sup> | Clade VI:<br><i>Physocalyx major</i> <sup>c</sup> | Paulowniaceae:<br><i>Paulownia fortunei</i> <sup>c</sup><br>(outgroup) |
|--------------------------|---------------------------------------|-----------------------------------|--|---|---|---|---|--|--|---|---|---|--|
| A15g20080_0925F          | F: ATACGAGATAGCATTTGGFACTCGA          | 499                               |  |   |   | X   |   |  |  |   |   |   |  |
| A15g20080_L1424R (COSII) | R: AGATGCCATCCTTAAGAACCCCTG           |                                   |  |   |   |   |   |  |  |   |   |   |  |
| A15g23540_1611F          | F: AATCCTCCATGAGTGAACCCCA             | 491                               |  |   |   | X   |   |  |  |   |   |   |  |
| A15g23540_2102R (COSII)  | R: ATTACTCTCAACATGGATGATAGTTGG        |                                   |  |   |   |   |   |  |  |   |   |   |  |
| A15g58490_254F           | F: GTATCAITCCCGCCCTAATTG              | 410                               |  |   |   | X   |   |  |  |   |   |   |  |
| A15g58490_664R (COSII)   | R: TTGCAAACTGTAAATATAGGCCCAA          |                                   |  |   |   |   |   |  |  |   |   |   |  |

<sup>a</sup>Primer sequence for the “*Castilleja*-specific primer.” To make the target-specific primer for subsequent microfluidic PCR, conserved sequence tags CSI (5'-ACACTGACGACATGGTTCTACA) and CS2 (5'-TACGGTAGCAGACTGGTCT) were added to each forward and reverse primer, respectively.

<sup>b</sup>Amplicon length (bp) estimated from BLAST hit alignments.

<sup>c</sup>PCR validations using DNAs from Bennett and Mathews (2006).

<sup>d</sup>PCR validations were considered successful for *Castilleja* when amplification occurred for all three taxa, representing one annual lineage (*C. lineariloba*) and two perennial lineages (*C. pumila* and *C. lemmonii*).

<sup>e</sup>Taxa that were both PCR validated and had primer combinations evaluated in silico against their respective assemblies (raw read files available in the NCBI Sequence Read Archive submission SRP100222).

Li et al., 2008; Yuan et al., 2009, 2010). We also test these primers for their broader applicability across Orobanchaceae following the approach outlined in Latvis et al. (2017a) with the goal of finding a subset of nuclear gene regions that would amplify across this more inclusive clade. Previous phylogenetic studies within Orobanchaceae employed the nuclear phytochrome genes *PHYA* and *PHYB*, the nuclear ribosomal ITS region, and the plastid *matK* and *rps2* genes. Orobanchaceae is the largest clade of parasitic angiosperms, and plastome reduction and accelerated rates of molecular evolution in retained plastid genes have been documented (see discussion in Bennett and Mathews, 2006). Additionally, phytochrome genes regulate responses to light and can be significantly modified in parasites (Bennett and Mathews, 2006). Therefore, the development of additional single-copy nuclear regions would provide a much-needed source of phylogenetic information in Orobanchaceae, and may provide a more reliable estimate of branch lengths for further studies of diversification and character evolution.

## 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 ~0.8x. 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 (Hellsten 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 *Lamourouxia multifida* Kunth using BLAST (Altschul et al., 1990). *Lamourouxia 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

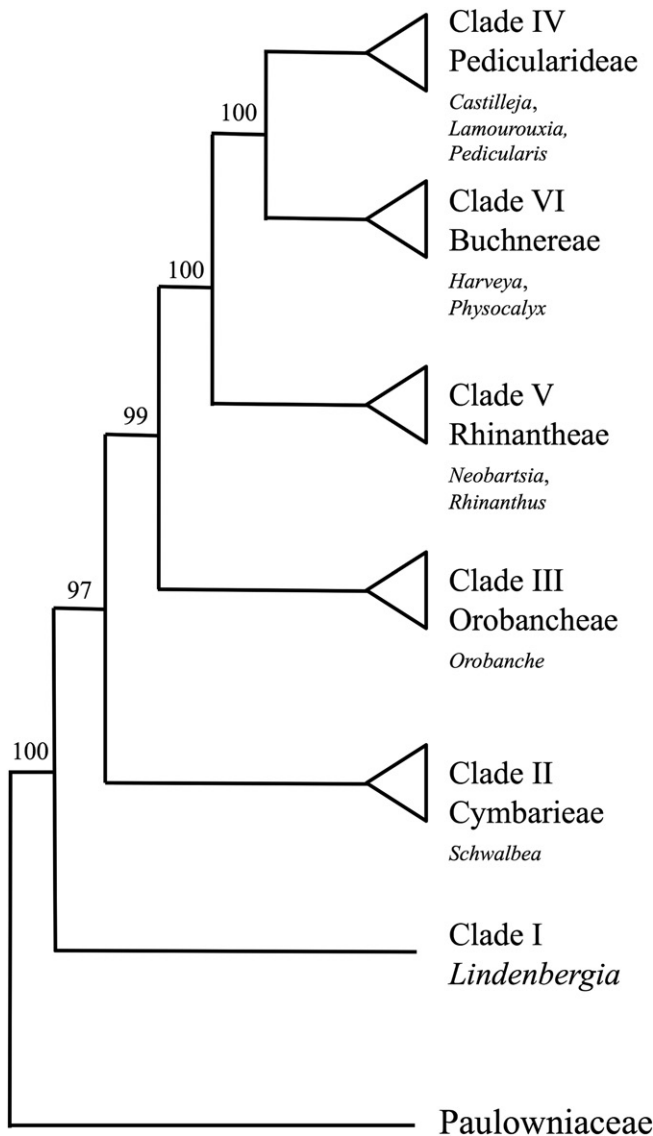


Fig. 1. Relationships among major clades within Orobanchaceae modified from McNeal et al. (2013) with taxa used for primer validation indicated (see text). Bootstrap support values for clades are indicated along the branches and follow McNeal et al. (2013). Reproduced from Latvis et al. (2017a).

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 Pedicularideae (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.

## LITERATURE CITED

- ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MEYER, AND D. J. LIPMAN. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215: 403–410.
- BANKEVICH, A., S. NURK, D. ANTIPOV, A. GUREVICH, M. DVORKIN, A. S. KULIKOV, V. LESIN, ET AL. 2012. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology* 19: 455–477.
- BENNETT, J. R., AND S. MATHEWS. 2006. Phylogeny of the parasitic plant family Orobanchaceae inferred from phytochrome A. *American Journal of Botany* 93: 1039–1051.
- BLISCHAK, P. D., A. J. WENZEL, AND A. D. WOLFE. 2014. Gene prediction and annotation in *Penstemon* (Plantaginaceae): A workflow for marker development from extremely low-coverage genome sequencing. *Applications in Plant Sciences* 2: 1400044.
- FANT, J. B., H. WOLF-WEINBERG, D. C. TANK, AND K. A. SKOGEN. 2013. Characterization of microsatellite loci in *Castilleja sessiliflora* and transferability to 24 *Castilleja* species (Orobanchaceae). *Applications in Plant Sciences* 1: 1200564.
- GODDEN, G. T., I. E. JORDAN-THADEN, S. CHAMALA, A. A. CROWL, N. GARCÍA, C. C. GERMAIN-AUBREY, J. M. HEANEY, ET AL. 2012. Making next-generation sequencing work for you: Approaches and practical considerations for marker development and phylogenetics. *Plant Ecology and Diversity* 5: 427–450.
- HELLSTEN, U., K. M. WRIGHT, J. JENKINS, S. SHU, Y. YUAN, S. R. WESSLER, J. SCHMUTZ, ET AL. 2013. Fine-scale variation in meiotic recombination in *Mimulus* inferred from population shotgun sequencing. *Proceedings of the National Academy of Sciences USA*.
- HUANG, X., AND A. MADAN. 1999. CAP3: A DNA sequence assembly program. *Genome Research* 9: 868–877.
- KATOH, K., AND D. M. STANDLEY. 2013. MAFFT Multiple Sequence Alignment Software version 7: Improvements in performance and usability. *Molecular Biology and Evolution* 30: 772–780.
- KEARSE, M., R. MOIR, A. WILSON, S. STONES-HAVAS, M. CHEUNG, S. STURROCK, S. BUXTON, ET AL. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics (Oxford, England)* 28: 1647–1649.
- LATVIS, M., S. M. E. MORTIMER, D. F. MORALES-BRIONES, S. TORPEY, S. URIBE-CONVERS, S. J. JACOBS, S. MATHEWS, AND D. C. TANK. 2017a. Primers for *Castilleja* and their utility across Orobanchaceae: I. Chloroplast primers. *Applications in Plant Sciences* 5: 1700020.
- LATVIS, M., S. J. JACOBS, S. M. E. MORTIMER, M. RICHARDS, P. D. BLISCHAK, S. MATHEWS, AND D. C. TANK. 2017b. Data from: Primers for *Castilleja* and their utility across Orobanchaceae. II. Single-copy nuclear loci. Dryad Digital Repository. <http://doi.org/10.5061/dryad.52v62>.
- LI, M., J. WUNDER, G. BISSOLI, E. SCARPONI, S. GAZZANI, E. BARBARO, H. SAEDLER, AND C. VAROTTO. 2008. Development of COS genes as universally applicable markers for phylogenetic reconstructions of closely related plant species. *Cladistics* 24: 727–745.



- MCNEAL, J. R., J. R. BENNETT, A. D. WOLFE, AND S. MATHEWS. 2013. Phylogeny and origins of holoparasitism in Orobanchaceae. *American Journal of Botany* 100: 971–983.
- TANK, D. C., AND R. G. OLMSTEAD. 2008. From annuals to perennials: Phylogeny of subtribe *Castillejinae* (Orobanchaceae). *American Journal of Botany* 95: 608–625.
- TANK, D. C., AND R. G. OLMSTEAD. 2009. The evolutionary origin of a second radiation of annual *Castilleja* (Orobanchaceae) species in South America: The role of long distance dispersal and allopolyploidy. *American Journal of Botany* 96: 1907–1921.
- TWYFORD, A. D., AND R. A. ENNOS. 2012. Next-generation hybridization and introgression. *Heredity* 108: 179–189.
- UNTERGASSER, A., I. CUTCUTACHE, T. KORESSAAR, J. YE, B. C. FAIRCLOTH, M. REMM, AND S. G. ROZEN. 2012. Primer3—New capabilities and interfaces. *Nucleic Acids Research* 40: e115.
- URIBE-CONVERS, S., J. R. DUKE, M. J. MOORE, AND D. C. TANK. 2014. A long-PCR based method for chloroplast genome enrichment and phylogenomics in angiosperms. *Applications in Plant Sciences* 2: 1300063.
- URIBE-CONVERS, S., M. L. SETTLES, AND D. C. TANK. 2016. A phylogenomic approach based on PCR target enrichment and high throughput sequencing: Resolving the diversity within the South American species of *Bartsia* L. (Orobanchaceae). *PLoS ONE* 11: e0148203.
- WU, F., L. A. MUELLER, D. CROUZILLAT, V. PÉTIARD, AND S. D. TANKSLEY. 2006. Combining bioinformatics and phylogenetics to identify large sets of single-copy orthologous genes (COSII) for comparative, evolutionary and systematic studies: A test case in the Euasterid plant clade. *Genetics* 174: 1407–1420.
- YUAN, Y.-W., C. LIU, H. E. MARX, AND R. G. OLMSTEAD. 2009. The pentatricopeptide repeat (PPR) gene family, a tremendous resource for plant phylogenetic studies. *New Phytologist* 182: 272–283.
- YUAN, Y., C. LIU, H. E. MARX, AND R. G. OLMSTEAD. 2010. An empirical demonstration of using pentatricopeptide repeat (PPR) genes as plant phylogenetic tools: Phylogeny of Verbenaceae and the *Verbena* complex. *Molecular Phylogenetics and Evolution* 54: 23–35.

APPENDIX 1. Voucher information for species used in this study.

| Species   | Voucher accession no. (Herbarium) <sup>a</sup> | Collection locality  | Geographic coordinates   |
|---|--|----------------------|--|
| <i>Castilleja cusickii</i> Greenm.                            | Tank 2009-01 (ID)                              | Idaho, USA           | 45.884241°N, 116.230195°W  |
| <i>Castilleja foliolosa</i> Hook. & Arn.                      | A. Colwell 03-09 (YM)                          | California, USA      | 35.3926°N, 120.3522°W  |
| <i>Castilleja lemmonii</i> A. Gray                            | Jacobs 2015-088 (ID)                           | California, USA      | 37.907982°N, 119.258583°W  |
| <i>Castilleja lineariloba</i> (Benth.) T. I. Chuang & Heckard | Tank 2002-04 (WTU)                             | California, USA      | 37.41387°N, 120.10833°W  |
| <i>Castilleja pumila</i> Wedd.                                | Uribe-Converts 2011-120 (ID)                   | La Libertad, Peru    | 7.99506°S, 78.44197°W  |
| <i>Castilleja tenuis</i> (A. Heller) T. I. Chuang & Heckard   | Tank 2001-13 (WTU)                             | Washington, USA      | 46.118133°N, 121.5158°W  |
| <i>Harveya purpurea</i> Harv.                                 | Randle 79 (OS)                                 | NA                   | NA   |
| <i>Lamourouxia multifida</i> Kunth                            | Mejia 695 (CAS)                                | Chiapas, Mexico      | NA   |
| <i>Lamourouxia virgata</i> Kunth                              | Mejia 581 (CAS)                                | Chiapas, Mexico      | 16.713611°N, 92.614722°W   |
| <i>Lindenbergia</i> sp. Kunth                                 | Armstrong 1163 (ISU)                           | NA                   | NA   |
| <i>Neobartsia peruviana</i> (Walp.) Uribe-Converts & Tank     | Uribe-Converts 13-011 (ID)                     | NA                   | NA   |
| <i>Orobanche californica</i> Cham. & Schltld.                 | Bennett 72 (A)                                 | Cultivated           | Cultivated   |
| <i>Paulownia fortunei</i> (Seem.) Hemsl.                      | s.n. (A)                                       | Cultivated           | Cultivated ( <a href="https://sheffields.com">https://sheffields.com</a> ) |
| <i>Pedicularis</i> sp. L.                                     | Krasek and Bennett s.n. (A)                    | Slovenia             | NA   |
| <i>Physocalyx major</i> Mart.                                 | G. O. Romão 2528 (ESA)                         | Minas Gerais, Brazil | 19.2635°S, 43.5508°W   |
| <i>Rhinanthus alectorolophus</i> (Scop.) Pollich              | Bennett 85 (A)                                 | NA                   | NA   |
| <i>Schwalbea americana</i> L.                                 | Kirkman s.n. (PAC)                             | NA                   | NA   |

Note: NA = not available.

<sup>a</sup>Herbarium acronyms are per Index Herbariorum (<http://sweetgum.nybg.org/science/ih/>).