

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

Authors: Latvís, Maribeth, Jacobs, Sarah J., Mortimer, Sebastian M. E., Richards, Melissa, Blischak, Paul D., et al.

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## 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:

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<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')        | Amplicon length (bp) | Clade IV:                                        |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             | Paulowniaceae: |
|---------------------------|---------------------------------|----------------------|--------------------------------------------------|-------------------------------|-------------------------------------------------------------------|--------------------------------------------------------------------------------------------|-----------------------------------------------------------------|-----------------------------------------------------------|-----------------------------------------------------------------|--------------------------------------------------------------------|-------------------------------------------------------------|-------------------------------------------------------------|----------------|
|                           |                                 |                      | Clade I:<br><i>Lindenbergia</i> sp. <sup>c</sup> | Clade II:<br><i>Schwalbea</i> | Clade III:<br><i>Orobancha</i><br><i>californica</i> <sup>c</sup> | Clade IV:<br><i>lineariloba</i> ,<br><i>C. punila</i> ,<br><i>C. lemmonii</i> <sup>a</sup> | Clade IV:<br><i>Lamoureauxia</i><br><i>virgata</i> <sup>c</sup> | Clade V:<br><i>Pedicularis</i><br><i>sp.</i> <sup>c</sup> | Clade V:<br><i>Neobarbista</i><br><i>peruviana</i> <sup>c</sup> | Clade VI:<br><i>Rhinanthus</i><br><i>dictyonophus</i> <sup>c</sup> | Clade VI:<br><i>Harveya</i><br><i>purpurea</i> <sup>c</sup> | Clade VI:<br><i>Physocalyx</i><br><i>major</i> <sup>c</sup> |                |
| A12g34560_688F            | F: CTGTTTGTCGGACGACGTACC        | 498                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A12g34560_1186R (COSII)   | R: AAAGGTGTGTGCACTGAAGC         |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A15g46630_851F            | F: TGACAAACAGTTCACAAATGCA       | 499                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A15g46630_1350R (COSII)   | R: TTGGCAGGACGGGATTTAAGT        |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A13g04260_165F            | F: GGAGTGGGAAGTTGAATTGGC        | 500                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A13g04260_165R (COSII)    | R: AAGGCTGAAGGCATAGGAGC         |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A15g12370_3513F           | F: ATGCACTGCTGAGCCCTTT          | 511                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A15g12370_3513R (COSII)   | R: GAGATGCCCTGACTGAAGCT         |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A12g28390_1096F           | F: TGCAATGCAAGTTATCAGTCTTG      | 499                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A12g28390_1595R (COSII)   | R: GACATGGGATTTGATCTTGGCAG      |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A12g26430_2136F           | F: TTGCAAGATTCACAGCTTCTTGTG     | 496                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A12g26430_2136R (COSII)   | R: GCGCCGATTTGTTTGG             |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A14g24190_1278F           | F: CTGAACTCCAGATTAGCAATAGTAC    | 499                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A14g24190_1278R (COSII)   | R: TCAGGCATTTGGACAGATTAGGT      |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A15g27620_538F            | F: CCGCATATCTTATCACTTCGCG       | 799                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A15g27620_1337R (COSII)   | R: CTTGGGAAAGGATTTGAAACAG       |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A13g62010_1226F           | F: CACATCTGCTTTGCTCTCAGG        | 499                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A13g62010_1725R (COSII)   | R: GCTTCAGATTTGATTCATCCCA       |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A15g48300_1121F           | F: GCTCCAGGAATAACTTCACTTCC      | 500                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A15g48300_1621R (COSII)   | R: GCAGTAAGGCATATTTTGTGTTG      |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A13g62010_1425F           | F: GAGAGCCATAGCATAAGCAACCC      | 501                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A13g62010_1926R (COSII)   | R: AGGCAATCTGGTGGTAGGATG        |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A14g24830_948F            | F: AACCAACCACTCGCAGCC           | 499                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A14g24830_1447R (COSII)   | R: AAGTGTCTCTGCTCTACAGT         |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A13g04260_147F            | F: TCCTAGAAGATGGTCACAGGAGT      | 499                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A13g04260_646R (COSII)    | R: CTGCTATACGACCCGAGT           |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A15g27620_1180F           | F: CGAATCCTAACTTGCAATGAAAGA     | 500                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A15g27620_1680R (COSII)   | R: CGATGCTTCAAAATTTCCAGT        |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A15g26360_1312F           | F: GGCAATGGTTGGTTCTCACC         | 499                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A15g26360_1811R (COSII)   | R: TGTGTTGGACTTGTGAAGAGTGT      |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A12g29210_514F            | F: TGAGACCATGGATTGCAAAAGC       | 500                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A12g29210_1014R (COSII)   | R: GCTGTGCACTCAGAACGAG          |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A13g62010_2_1674F         | F: AGCCAAGCACTTACTCATCTCT       | 500                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A13g62010_2_2174R (COSII) | R: CAGATCAAGGAGAGGCTGTAG        |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A13g62010_1674F           | F: AGCCAAGCACTTACTCATCTCT       | 616                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A13g62010_2290R (COSII)   | R: ATATCTGTGTATGATGCAATGCAACT   |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A12g38020_1797F           | F: GTGCTATTAA TGCACTGACTTGT     | 500                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A12g38020_2297R (COSII)   | R: CAGCATGACGACACATCTG          |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A12g38770_387F            | F: TTCTTAACACGCCCAAGCCA         | 499                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A12g38770_886R (COSII)    | R: CTTAGTCTCTTCCCTGGGCA         |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A14g11120_1226F           | F: TCCGCAATACGACCTATGCC         | 490                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A14g11120_1716R (COSII)   | R: AATTTATGCTCAATTTGCTTGGC      |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A15g80150_1016F           | F: GCATGTAGGCGACGAGCTTA         | 512                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A11g80150_1528R (PPR)     | R: GCGTCTCGGAAGGGTTTATA         |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A11g04200_1908F           | F: GCAACTGTATGAGAGGCCCA         | 487                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A11g04200_2395R (COSII)   | R: GACTCTCTTCTCAATGCGAGTG       |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A12g07050_1067F           | F: GCAACTGTATGAGAGGCCCA         | 467                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A12g07050_1534R (COSII)   | R: TGTAGAGAGAGAGATTAACATTACAGCA |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A14g02720_1652F           | F: CGAAATCTGTTTTCGAGAGCG        | 500                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A14g02720_2152R (COSII)   | R: CCACCTCACCTCTACGTTGG         |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A13g09920_917F            | F: CATCTCTCATGACTCTGAGG         | 499                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A13g09920_1416R (COSII)   | R: TGCAACGAGTTAAAGAAATTCATCG    |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |
| A13g09920_1316F           | F: ACCTCCCAATGAA GAGCCTT        | 543                  | X                                                | X                             | X                                                                 | X                                                                                          | X                                                               | X                                                         | X                                                               | X                                                                  | X                                                           | X                                                           | X              |
| A13g09920_1859R (COSII)   | R: GTGATATATCGGAATGATGCC        |                      |                                                  |                               |                                                                   |                                                                                            |                                                                 |                                                           |                                                                 |                                                                    |                                                             |                                                             |                |

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</i><br><i>americana</i> <sup>c</sup> | Clade III:<br><i>Orobanchae</i><br><i>californica</i> <sup>c</sup> | Clade IV:<br><i>Castilleja</i><br><i>lineariloba</i> ,<br><i>C. pumila</i> ,<br><i>C. lemmonii</i> <sup>d</sup> | Clade IV:<br><i>Lamoureauxia</i><br><i>virgata</i> <sup>e</sup> | Clade IV:<br><i>Pedicularis</i><br><i>sp.</i> <sup>e</sup> | Clade V:<br><i>Neobanksia</i><br><i>peruviana</i> <sup>e</sup> | Clade V:<br><i>Rhinanthus</i><br><i>electrolophus</i> <sup>e</sup> | Clade VI:<br><i>Harveya</i><br><i>purpurea</i> <sup>e</sup> | Clade VI:<br><i>Physocalyx</i><br><i>major</i> <sup>e</sup> | Paulowniaceae:<br><i>Paulownia</i><br><i>fortunei</i> <sup>e</sup><br>(outgroup) |
|--------------------------------------------|------------------------------------------------------------------|-----------------------------------|--------------------------------------------------|----------------------------------------------------------------|--------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|------------------------------------------------------------|----------------------------------------------------------------|--------------------------------------------------------------------|-------------------------------------------------------------|-------------------------------------------------------------|----------------------------------------------------------------------------------|
| A15g26360_322F<br>A15g26360_818R (COSII)   | F: <b>TTACCGGTAATCCCATCTATGC</b><br>R: <b>ATGCTATGTGTGGCAGCA</b> | 496                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             | X                                                           |                                                                                  |
| A15g49970_503F<br>A15g49970_998R (COSII)   | F: AGCAGCTACAAGACATCGC<br>R: AGCTGGGAAATGATGAATCC                | 495                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A15g52210_456F<br>A15g52210_955R (COSII)   | F: ATCCAGCACAACCTCAAC<br>R: GGATTTGCAAGGAGCTCCAC                 | 499                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A1g09620_1187F<br>A1g09620_1688R (COSII)   | F: GGCTACACGAGGCTGAAA<br>R: ACCCGACACCTTCAGATCAA                 | 501                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A1g09620_1490F<br>A1g09620_1989R (COSII)   | F: AGCTCATATGTTGAGGAGG<br>R: CCTCTCCATCCATGCAATCT                | 499                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A1g28190_483F<br>A1g28190_983R (COSII)     | F: GTTCCACGGTCAACCGC<br>R: TGCAACCTGTTTCTGTAATGG                 | 500                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A2g38020_892F<br>A2g38020_1391R (COSII)    | F: TTTGACATTTCTGTTGCTTACCA<br>R: CGTACAGCATTTAAGACGCG            | 499                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A4g22670_93F<br>A4g22670_589R (COSII)      | F: GAATTTGGTTGGCATACAGC<br>R: AGTCTAGCTTTGAGCCACA                | 496                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A15g26360_1322F<br>A15g26360_1821R (COSII) | F: TGTTCTCACCTTTCTGTACTCA<br>R: GGTGCAATATGTTGGACTTG             | 499                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A2g29210_902F<br>A2g29210_1401R (COSII)    | F: TTGAATGCAAGCAGTTAATGAA<br>R: ACCGCCATACAGCAGTTTG              | 499                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A1g01940_828F<br>A1g01940_1227R (COSII)    | F: TCATAACTATGTAAGCCAAACATGAT<br>R: TATAAAGGTTTCATGATACAGGTGG    | 399                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A4g24620_1780F<br>A4g24620_2223R (COSII)   | F: CTGTTCCCTGTATACCAAGATTG<br>R: CAGTTATATCAACAGCTCAGATGG        | 443                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A15g62530_439F<br>A15g62530_938R (COSII)   | F: TGGAACAAATGCTTCGATTACTCC<br>R: TGCGGCTGAGAGAGTCATG            | 499                               |                                                  |                                                                |                                                                    | X                                                                                                               | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A13g62010_1468F<br>A13g62010_1969R (COSII) | F: ACATAATAGATGACCTGCGCA<br>R: GCACATGGAGCAGGTTTG                | 501                               |                                                  |                                                                |                                                                    |                                                                                                                 | X                                                               |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A4g11120_1399F<br>A4g11120_1859R (COSII)   | F: CCCATGTGAAGTGGGATA<br>R: GCTTGGCAGCCATTCAG                    | 460                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A2g41680_402F<br>A2g41680_902R (COSII)     | F: GTACACAAACAAGTCTCGGC<br>R: GGATCCGGATGTTTGCTGT                | 500                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A15g22830_933F<br>A15g22830_1432R (COSII)  | F: AAAGCCCTAATGCACTATTGTGAAC<br>R: ACCAGTCTCTGTTGCTAATACGA       | 499                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A13g26730_1284F<br>A13g26730_1770R (COSII) | F: AGATACTCTTATCTGATCATGA<br>R: TGTTTCAGGACAAGTGGCTCAG           | 486                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A2g17670_501F<br>A2g17670_998R (PPR)       | F: ATCCAATAGCCGCTTAC<br>R: AAGGAGTTCGGTATATAAGCCTGAT             | 497                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A4g37170_962F<br>A4g37170_1466R (PPR)      | F: CAACCTATACGGGTTTCTTCAC<br>R: ATGGGTATGCTCAGAATGGTCAG          | 504                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A4g30825_1603F<br>A4g30825_2102R (PPR)     | F: GTTGACATGGTGTATGATCGGT<br>R: TTCAAGACCCGACTTGACA              | 499                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A13g14730_878F<br>A13g14730_1378R (PPR)    | F: ACGGGTGTCTCTCAACAGG<br>R: AAGGGCTTCTCTCTATGCTC                | 500                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A15g18475_417F<br>A15g18475_915R (PPR)     | F: ACGGATGAAGCTATCGAGATGG<br>R: AACATGACACACATAATATGCT           | 498                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A1g13040_1457F<br>A1g13040_1958R (PPR)     | F: TGTAGCTCACCATGTCAGGC<br>R: AGTGGAGCTCAAAATGATAGGT             | 501                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A1g16890_816F<br>A1g16890_1315R (PPR)      | F: AAACCATCGATCGTCTATTACTTTG<br>R: TCCACCTCCATTTGATGAAACTTG      | 499                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A1g20300_782F<br>A1g20300_1286R (PPR)      | F: GTGATCGACGCTTTGTGCC<br>R: TCGGCTCCACATCATCTCA                 | 504                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A1g07230_362F<br>A1g07230_870R (COSII)     | F: GGACCTCATCAACGGTTC<br>R: GTCAATCCACGCCGAAATCAA                | 508                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A1g17760_1370F<br>A1g17760_1867R (COSII)   | F: CTTGCTCATTCCTCACTTCGG<br>R: AACATCTTCAGTATTGAAACCA            | 497                               |                                                  |                                                                |                                                                    | X                                                                                                               |                                                                 |                                                            |                                                                |                                                                    |                                                             |                                                             |                                                                                  |
| A1g26640_1171F<br>A1g26640_1670R (COSII)   | F: TGCAAGAGCCCTGACAATTC<br>R: TGGGTCCTCAGAAATTCATCTG             | 499                               |                                                  |                                                                |                                                                    | 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>Orobanchaceae</i> | Clade IV:<br><i>Castilleja lineariloba</i> ,<br><i>C. pumila</i> ,<br><i>C. lemmingii</i> <sup>d</sup> | Clade IV:<br><i>Lamoureauxia virgata</i> <sup>e</sup> | Clade IV:<br><i>Pedicularis</i> sp. <sup>e</sup> | Clade V:<br><i>Neobanksia peruviana</i> <sup>e</sup> | Clade V:<br><i>Rhinanthus alectonolophus</i> <sup>e</sup> | Clade VI:<br><i>Harveya purpurea</i> <sup>e</sup> | Clade VI:<br><i>Physocalyx major</i> <sup>e</sup> | Paulowniaceae:<br><i>Paulownia fortunei</i> <sup>e</sup><br>(outgroup) |
|-------------------------|---------------------------------------|-----------------------------------|--------------------------------------------------|------------------------------------------------------|------------------------------------|--------------------------------------------------------------------------------------------------------|-------------------------------------------------------|--------------------------------------------------|------------------------------------------------------|-----------------------------------------------------------|---------------------------------------------------|---------------------------------------------------|------------------------------------------------------------------------|
| At1g30580_2218F         | F: TGAAAGGCTGCTGAAGTTGA               | 500                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At1g30580_2718R (COSII) | R: GGATGATCTTTGGGAGAAAC               |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At1g43860_2011F         | F: AGCATACAATTCAGGAGTGCATC            | 504                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At1g43860_2515R (COSII) | R: CTCGGCGACCATCACAC                  |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At1g59600_576F          | F: GGAGACCTGAAACTAGGTCC               | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At1g59600_1075R (COSII) | R: AAGTGTGTTGCTGAGCTC                 |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At1g69220_607F          | F: AGCTCGTCGCTGAACATCA                | 467                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At1g69220_1075R (COSII) | R: GGCGATGTATAAAGCTCGAGA              |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At2g03120_425F          | F: TGGGATCTGTTGTAGTTTATCCAGA          | 500                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At2g03120_925R (COSII)  | R: ACGAAGACGACATCCCT                  |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At2g27170_2888F         | F: CCATCTTCATCCCTCCTGC                | 475                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At2g27170_3366R (COSII) | R: TTGACAGTCAATGGCTCAGCC              |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At2g42490_4066F         | F: ACTTAGCCCTTGGACCAACC               | 475                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At2g42490_4564R (COSII) | R: GCTGGAGAAACGACAACTC                |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g03600_524F          | F: AGGCTCGAACTGTGTTGTC                | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g03600_1023R (COSII) | R: GCCTTAAACCCCTCAACTC                |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g04870_957F          | F: TCTTCCCATCTTCCCAAGGTC              | 455                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g04870_1412R (COSII) | R: ACTCAAAACAGGCTCAACTCA              |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g05000_1412F         | F: ACCAACTAAACAGAGCCCTCAATCA          | 500                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g09090_1427F         | R: TTGCACTTTTACTTTCCCTGTGG            |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g09090_1912R (COSII) | F: TTTCTGTTTGTGATAGTTTAGCAGT          | 485                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g09090_1912R (COSII) | R: ATGCCCGCACAAATGATCCA               |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g13235_2063F         | F: GGCAACATGATCCGGCCTA                | 482                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g13235_2545R (COSII) | R: TTGGGAAACTAAACCAACTCTGC            |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g16150_956F          | F: ACAGCAAGTCAACGATATCAATGG           | 404                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g16150_956F          | R: CCTTATTTGCTCGAATGCTCG              |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g16150_1360R (COSII) | F: TCAAGTCGAAACCAAGCATCTGA            | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g17205_926F          | R: GGACTGCTGGTAATGCATCTG              |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g17205_1425R (COSII) | F: CAGAACTCCGAGACTTCTCAAC             | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g55560_121F          | R: GCCACCGTCCTTCAATFACA               |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g55360_620R (COSII)  | F: CATAATCTGCTTGGGATTAACCG            | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g62580_1318F         | R: GCAATCTGTTATCTTTACGCC              |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At3g62580_1817R (COSII) | F: AAGCTGCCATGCTTTAAATGTC             | 494                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g00560_2034F         | R: GGAAACAGCTCATCTCTCTCTTG            |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g12230_1167F         | F: TGTAGATTGGATCAAGCAATGGC            | 417                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g12230_1167F         | R: TCAATGCTTGAATCCACTCATGG            |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g12230_1584R (COSII) | F: ATCACTCAAGAGAGAGGCGG               | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g23100_808F          | R: CCTTCATATATCGTATTCTACACGG          |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g23100_1307R (COSII) | F: CCAACCAACTGCTATGTCAGC              | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g29490_2406F         | R: CCTAGCTCACCTCATGGCAG               |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g32280_1754F         | F: CCGATGGCTAATCACCGCC                | 500                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g32280_2254R (COSII) | R: ATACGTTGCACAGACTCGACA              |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g33625_852F          | F: AGGTAGGCTCATTTGTAACCC              | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g33625_1351R (COSII) | R: CATCTCTGCTCAAGAAATTGG              |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g38630_507F          | F: GTTACCTGTTCTAGGACGTGC              | 498                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At4g38630_1005R (COSII) | R: ATCAAGTTGGGATCCACCC                |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At5g06830_1899F         | F: GGCCCAACCCATTGCCATTA               | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At5g06830_2398R (COSII) | R: ACTAGACTCTGGACTTTGCAAG             |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At5g07270_1779F         | F: CACATCCTTGCAACCTCAAGC              | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At5g07270_2278R (COSII) | R: ACTGCTCTTCAATAGGCTGCT              |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At5g12200_438F          | F: TCGGTTCTTACTAGTGTCAAC              | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At5g12200_937R (COSII)  | R: ATCAGAGTTAGGGATGCTGTGT             |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At5g13640_387F          | F: GGGAACCTGATTACAGGGAATC             | 498                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At5g13640_885R (COSII)  | R: CTAGCTACGAGCAATGCG                 |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At5g18580_933F          | F: GCTGCTTTTATGACGGTGT                | 499                               |                                                  |                                                      |                                    | X                                                                                                      |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| At5g18580_1432R (COSII) | R: ACAGTTACCTGCGTGAAGAG               |                                   |                                                  |                                                      |                                    |                                                                                                        |                                                       |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |

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 americana</i> <sup>c</sup> | Clade III:<br><i>Orobanchaceae</i> | Clade IV:<br><i>Castilleja lineariloba</i> ,<br><i>C. pumila</i> ,<br><i>C. lemmonii</i> <sup>d</sup> | Clade IV:<br><i>Lamouroxia virgata</i> <sup>e</sup> | Clade IV:<br><i>Pedicularis</i> sp. <sup>e</sup> | Clade V:<br><i>Neobartsia peruviana</i> <sup>e</sup> | Clade V:<br><i>Rhinanthus alectonophilus</i> <sup>e</sup> | Clade VI:<br><i>Harveya purpurea</i> <sup>e</sup> | Clade VI:<br><i>Physocalyx major</i> <sup>e</sup> | Paulowniaceae:<br><i>Paulownia fortunei</i> <sup>e</sup><br>(outgroup) |
|--------------------------|---------------------------------------|-----------------------------------|--------------------------------------------------|------------------------------------------------------|------------------------------------|-------------------------------------------------------------------------------------------------------|-----------------------------------------------------|--------------------------------------------------|------------------------------------------------------|-----------------------------------------------------------|---------------------------------------------------|---------------------------------------------------|------------------------------------------------------------------------|
| A15g20080_925F           | F: ATACGAGATAGCATTTGGTACTCGA          | 499                               |                                                  |                                                      |                                    | X                                                                                                     |                                                     |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| A15g20080_L1424R (COSII) | R: AGATGCCATCCTTAAGAACCCCTG           |                                   |                                                  |                                                      |                                    |                                                                                                       |                                                     |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| A15g23540_L1611F         | F: AATCCTTCCATGAGTGAACCCCA            | 491                               |                                                  |                                                      |                                    | X                                                                                                     |                                                     |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| A15g23540_L102R (COSII)  | R: ATTACTCTCAACATGGATGATAGTTGG        |                                   |                                                  |                                                      |                                    |                                                                                                       |                                                     |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| A15g58490_254F           | F: GCTATCAATCCAGCCCTAATTG             | 410                               |                                                  |                                                      |                                    | X                                                                                                     |                                                     |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |
| A15g58490_664R (COSII)   | R: TTGCAATCTGTAAATTAGGCCCAA           |                                   |                                                  |                                                      |                                    |                                                                                                       |                                                     |                                                  |                                                      |                                                           |                                                   |                                                   |                                                                        |

<sup>a</sup>Primer sequence for the “*Castilleja*-specific primer.” To make the target-specific primer for subsequent microfluidic PCR, conserved sequence tags CS1 (5'-ACACTGACGACATGGTTCTTACA) and CS2 (5'-TACGGTAGCAGACTTGGTCT) 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 *Lamouroxia multifida* Kunth using BLAST (Altschul et al., 1990). *Lamouroxia 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

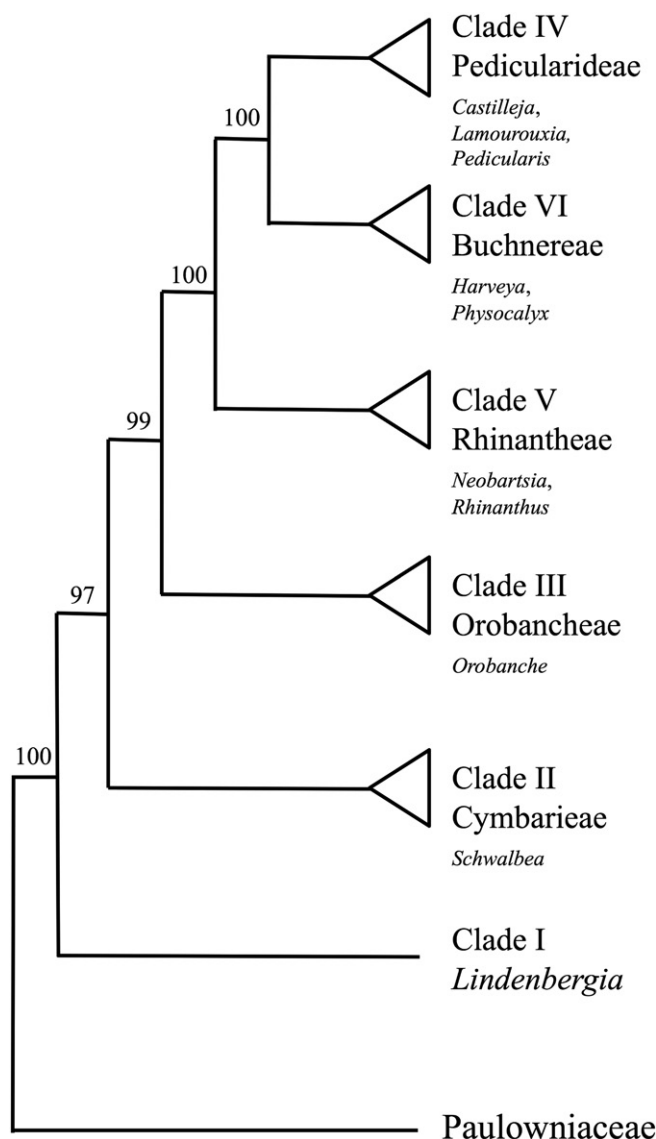


Fig. 1. Relationships among major clades within Orobanchaceae modified from McNeal et al. (2013) with taxa used for primer validation indicated (see text). Bootstrap support values for clades are indicated along the branches and follow McNeal et al. (2013). Reproduced from Latvis et al. (2017a).

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.

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# 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-Convers 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>Lamouroxia multifida</i> Kunth                             | Mejia 695 (CAS)                                | Chiapas, Mexico      | NA                                                                         |
| <i>Lamouroxia 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-Convers & Tank      | Uribe-Convers 13-011 (ID)                      | NA                   | NA                                                                         |
| <i>Orobanche californica</i> Cham. & Schltdl.                 | 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/>).