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PRIMERS FOR *CASTILLEJA* AND THEIR UTILITY ACROSS OROBANCHACEAE: II. SINGLE-COPY NUCLEAR LOCI¹

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- *Premise of the study:* We developed primers targeting nuclear loci in *Castilleja* with the goal of reconstructing the evolutionary history of this challenging clade. These primers were tested across other major clades in Orobanchaceae to assess their broader utility.
- *Methods and Results:* We assembled low-coverage genomes for three taxa in *Castilleja* and developed primer combinations for the single-copy conserved ortholog set (COSII) and the pentatricopeptide repeat (PPR) gene family. These primer combinations were designed to take advantage of the Fluidigm microfluidic PCR platform and are well suited for high-throughput sequencing applications. Eighty-seven primers were designed for *Castilleja*, and 27 were found to have broader utility in Orobanchaceae.
- *Conclusions:* These results demonstrate the utility of these primers, not only across *Castilleja*, but for other lineages within Orobanchaceae as well. This expanded molecular toolkit will be an asset to future phylogenetic studies in *Castilleja* and throughout Orobanchaceae.

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

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

Here we focus on developing a suite of putatively single-copy nuclear gene regions in *Castilleja* L. (Orobanchaceae);

“the paintbrushes”), a clade rich with polyploid and hybrid taxa, and the product of an ongoing rapid radiation (Tank and Olmstead, 2008, 2009). Previous studies using the nuclear 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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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') ^a	Amplicon length (bp) ^b	Clade IV: <i>Castilleja</i>										Paulowniaceae: <i>Paulownia fortunei</i> (outgroup)	
			Clade I: <i>Lindenbergia</i> sp. ^c	Clade II: <i>Schwalbea americana</i> ^c	Clade III: <i>Orobancha californica</i> ^c	Clade IV: <i>Lamourotaxia virgate</i> ^c	Clade IV: <i>Pedicularis</i> sp. ^c	Clade V: <i>Neobartsia peruviana</i> ^c	Clade V: <i>Rhinanthus electrolophilus</i> ^c	Clade VI: <i>Harveya purpurea</i> ^c	Clade VI: <i>Physocalyx major</i> ^c			
A12g34560_688F	F: CTGTTTGTGGCAGCAAGTACC R: AAAGGCTGTGCACTGAAGC	498	X	X	X	X	X	X	X	X	X	X	X	X
A12g34560_1186R (COSII)	F: TGACAAAACAGTCCAAATGCA R: TTGGCAGGACGGGATTTAAGT	499	X	X	X	X	X	X	X	X	X	X	X	X
A15g46630_851F	F: TGAGTGGAAAGTTGAATGGC R: AAGGCTGAAGGCATAGGAC	500	X	X	X	X	X	X	X	X	X	X	X	X
A13g04260_165F	F: ATGCACTGTAAGCCCTTT R: GAGTGGCTGACTGAAGCT	511	X	X	X	X	X	X	X	X	X	X	X	X
A15g12370_3513F	F: TGCAATGCAAGTATCAGTCTTG R: GACATGGATTTGATCTTGGCAG	499	X	X	X	X	X	X	X	X	X	X	X	X
A12g28390_1096F	F: TTGCAAGATTCGAAGCTTTGTC R: GCGCCAGTTGTGTTTG	496	X	X	X	X	X	X	X	X	X	X	X	X
A12g26430_2136F	F: CTGAACTCCAGATTTAGCAATAGTAC R: TCAGGCATTTGGACAGATTAGT	499	X	X	X	X	X	X	X	X	X	X	X	X
A14g24190_1278F	F: CCACAATCTTTATCAACTCCGC R: CTTGGGAAAGGATTTGAAACAG	799	X	X	X	X	X	X	X	X	X	X	X	X
A15g27620_538F	F: CAACAATCTTTGCTCTCAGG R: GFTTCAGATGTTGATTCATCCA	499	X	X	X	X	X	X	X	X	X	X	X	X
A13g62010_1226F	F: GTCACAGAAATCACTTCACTTCC R: GCAGTAAAGGCAATATTTGGTTG	500	X	X	X	X	X	X	X	X	X	X	X	X
A15g48300_1121F	F: GATAGCCTATGATTAAGCAACCC R: AGCAATCTGGTGTAGGATG	501	X	X	X	X	X	X	X	X	X	X	X	X
A13g62010_1425F	F: AAACAACACCTCGCAGCC R: AAGTGTCTCTGGCTTACAGT	499	X	X	X	X	X	X	X	X	X	X	X	X
A14g24830_948F	F: TCCTAGAAGTGGTCAAGGAGT R: CTGGTATGACGCCGGAAGT	499	X	X	X	X	X	X	X	X	X	X	X	X
A13g04260_147F	F: CGAATCTTAACTTGCATGAAAGA R: CGATGCTTCAAATTTCCACGT	500	X	X	X	X	X	X	X	X	X	X	X	X
A15g27620_1180F	F: GGCAATGGTTGGTCTCACC R: TGTFTGGACTGTGAAGAGTGT	499	X	X	X	X	X	X	X	X	X	X	X	X
A15g26360_1312F	F: TGAGACATGGATGCAAAGC R: GCTGTGCACTCAGAAGCAG	500	X	X	X	X	X	X	X	X	X	X	X	X
A12g29210_514F	F: AGCCAAGCACTTACTCACTCCT R: CAGATTAAGGAAGGCTGTAG	500	X	X	X	X	X	X	X	X	X	X	X	X
A13g62010_2_1674F	F: AGCCAAGCACTTACTCACTCCT R: CAGATTAAGGAAGGCTGTAG	500	X	X	X	X	X	X	X	X	X	X	X	X
A13g62010_2_2174R (COSII)	F: AGCCAAGCACTTACTCACTCCT R: CAGATTAAGGAAGGCTGTAG	500	X	X	X	X	X	X	X	X	X	X	X	X
A13g62010_1674F	F: AGCCAAGCACTTACTCACTCCT R: CAGATTAAGGAAGGCTGTAG	500	X	X	X	X	X	X	X	X	X	X	X	X
A13g62010_2290R (COSII)	F: AGCCAAGCACTTACTCACTCCT R: CAGATTAAGGAAGGCTGTAG	500	X	X	X	X	X	X	X	X	X	X	X	X
A12g38020_1797F	F: GTCGTATTAAATGCACTGACTTGT R: CAGCATGACGACCAACATCTG	500	X	X	X	X	X	X	X	X	X	X	X	X
A12g38020_2297R (COSII)	F: TTTCTAACACGCCCAAGCCA R: CFTAGCTCTTCCCTGGGCA	499	X	X	X	X	X	X	X	X	X	X	X	X
A12g38770_387F	F: TCCAGCAATACGACTATGCC R: AATFATTCCTCAATTCCTTGGC	490	X	X	X	X	X	X	X	X	X	X	X	X
A14g11120_1226F	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG	490	X	X	X	X	X	X	X	X	X	X	X	X
A14g11120_1716R (COSII)	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG	512	X	X	X	X	X	X	X	X	X	X	X	X
A11g80150_1016F	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG	487	X	X	X	X	X	X	X	X	X	X	X	X
A11g80150_1528R (PPR)	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG	467	X	X	X	X	X	X	X	X	X	X	X	X
A11g04200_1908F	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG	500	X	X	X	X	X	X	X	X	X	X	X	X
A12g07050_1067F	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG	499	X	X	X	X	X	X	X	X	X	X	X	X
A12g07050_1652F	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG	499	X	X	X	X	X	X	X	X	X	X	X	X
A14g02720_1652F	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG	499	X	X	X	X	X	X	X	X	X	X	X	X
A14g02720_2152R (COSII)	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG	543	X	X	X	X	X	X	X	X	X	X	X	X
A13g09920_917F	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG													
A13g09920_1416R (COSII)	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG													
A13g09920_1316F	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG													
A13g09920_1859R (COSII)	F: GATGAGAAAATAACATTCACAGCA R: GACTCTCTTCAATGCCAGTG													

TABLE I. Continued.

Locus (Region)	Primer sequences (5'-3') ^a	Amplicon length (bp) ^b	Clade I: <i>Lindenbergia</i> sp. ^c	Clade II: <i>Schwalbea americana</i> ^c	Clade III: <i>Orobancha</i>	Clade IV: <i>Castilleja lineariloba</i> , <i>C. pumila</i> , <i>C. lemmonii</i> ^d	Clade IV: <i>Lamourosaxia virgate</i> ^e	Clade IV: <i>Pedicularis</i> sp. ^c	Clade V: <i>Neobanksia peruviana</i> ^c	Clade V: <i>Rhinanthus alectorolophus</i> ^c	Clade VI: <i>Harveya purpurea</i> ^c	Clade VI: <i>Physocalyx major</i> ^c	Paulowniaceae: <i>Paulownia fortunei</i> ^c (outgroup)
A15g26360_322F A15g26360_818R (COSII)	F: TTCAACGGTATTCCTCATATGCG R: AGCAGCTACAAGACATCGC	496	X			X							
A15g49970_503F A15g49970_998R (COSII)	F: AGCAGCTACAAGACATCGC R: AGCTGGGAATGATGAAATCC	495	X			X							
A15g52210_456F A15g52210_955R (COSII)	F: ATCCAGCACAACCTCCAC R: GGATTCGAAGAGCTCCAC	499	X			X							
A1g09620_1187F A1g09620_1688R (COSII)	F: GFTCTACAGGAGCTGAAA R: ACCGACACCTCAGATCAA	501	X			X							
A1g09620_1490F A1g09620_1688R (COSII)	F: AGCTCATATGTTGGAGGAGG R: CCTCTCCATCCATGCAATCT	499	X			X							
A1g28190_483F A1g28190_983R (COSII)	F: GTTCCAGGTCACCCCG R: TGCAACTGTTCTGATGG	500	X			X							
A2g38020_892F A2g38020_1391R (COSII)	F: TTTCACATTTCTGTGCTTACCA R: CGTACAGCAITTAAGACCGC	499	X			X							
A4g22670_93F A4g22670_589R (COSII)	F: GAATTTGGTTGGCATACAGC R: AGTCTAGCTTTCAGCCACA	496	X			X							
A15g26360_1322F A15g26360_1821R (COSII)	F: TGGTCTCACCTTCTTGTACTCA R: GGTCCGATTAATGTTGGACTTG	499	X			X							
A2g29210_902F A2g29210_1401R (COSII)	F: TTGAATGACAGCAGTAAATGGAA R: ACCGCCATACAGCAGTTTG	499	X			X							
A1g01940_828F A1g01940_1227R (COSII)	F: TCATAACTATGTAAGCCAAACCATGAT R: TATAAAGGTTTTCATGATACAAGTTGG	399	X			X							
A4g24620_1780F A4g24620_2223R (COSII)	F: CTGTTCCCTGTATACCAAGATTTG R: CAGTTATATTAACAGCTCAGATGG	443	X			X							
A15g62530_439F A15g62530_938R (COSII)	F: TGGAAACAATGCTTCGATTACTCC R: TGCCGGTGAAGAGATCAG	499	X			X							
A3g62010_1468F A3g62010_1969R (COSII)	F: ACAATAGATATGACCTGCGCGA R: GCACATGGAGCAGGTTTG	501											
A4g11120_1399F A4g11120_1859R (COSII)	F: CCCATGTGAAGTGGGATTA R: GCTTGTGAGCCATCCAG	460	X			X							
A2g41680_402F A2g41680_902R (COSII)	F: GGATCCGGATGTTGCTGCT R: AAAGCCATAAGCATCTATTGTGAAC	500	X			X							
A15g22830_933F A15g22830_1432R (COSII)	F: AACAGTCTGTTTCTAATACGA R: ACCAGTCTGTTTCTAATACGA	499	X			X							
A15g26730_1284F A15g26730_1770R (COSII)	F: AGATAACATCTATCTGATCCATGGA R: TGTTTCAGGACAAGTGGCTCAG	486	X			X							
A2g17670_501F A2g17670_998R (PPR)	F: ATCCAAATAGCCGCTTAC R: AAGGATTCGGTATAAAGCCTGAT	497	X			X							
A4g37170_962F A4g37170_1466R (PPR)	F: CAACCTACCGGGTTTCTTCCAC R: ATGGGATAGCTCAGAATGGTCCAG	504	X			X							
A4g30825_1603F A4g30825_2102R (PPR)	F: GTTGACATGTTGATCATCGGT R: TTCAAAGCCGGACTTGACA	499	X			X							
A13g14730_878F A13g14730_1378R (PPR)	F: ACGGGTCTGACATAACAGG R: AAGGGCTTCTCATGCTC	500	X			X							
A15g18475_417F A15g18475_913R (PPR)	F: ACGGATGAAGCTATCGAGATGC R: AACCATGCACACAATAATGCT	498	X			X							
A1g13040_1457F A1g13040_1958R (PPR)	F: GTGAGCTCACATGTCAGGC R: AGTGGAGCTCAAATGCAATGTT	501	X			X							
A1g16890_816F A1g16890_1315R (PPR)	F: AAACCCATCGATCGTCAATTAATTTG R: TCCACCTCCATTTGATGAAACTTTG	499	X			X							
A1g20300_782F A1g20300_1286R (PPR)	F: GTGATCGAGCTTTGTGCC R: TCGGCTCCACCTCATCTCA	504	X			X							
A1g07230_362F A1g07230_870R (COSII)	F: GGACCTCATCAACGGTTCC R: GTCAATCCACGGCGAAATCAA	508	X			X							
A1g17760_1370F A1g17760_1867R (COSII)	F: TTGCTTACATCCCACTTCGG R: AACCATTTCCAGTATTGAAACCCA	497	X			X							
A1g26640_1171F A1g26640_1670R (COSII)	F: TCGAAGGCCGTGCAAAATTC R: TGGGTCTCTCAGAAATTCATCTG	499	X			X							

TABLE I. Continued.

Locus (Region)	Primer sequences (5'-3') ^a	Amplicon length (bp) ^b	Clade I: <i>Lindenbergia</i> sp. ^c	Clade II: <i>Schwalbea americana</i> sp. ^c	Clade III: <i>Orobanchae californica</i>	Clade IV: <i>Castilleja lineariloba</i> , <i>C. pumila</i> , <i>C. lemmonii</i>	Clade IV: <i>Lamourossia virgata</i> sp. ^c	Clade V: <i>Neobanksia peruviana</i>	Clade V: <i>Rhinanthus alectorolophus purpureae</i>	Clade VI: <i>Harveya Physocalyx major</i>	Paulowniaceae: <i>Paulownia fortunei</i> (outgroup)
A1g30580_2218F	F: TGAAAGGCTGTGAAGTTGA	500	X								
A1g30580_2718R (COSII)	R: GGATGGATCTTTGGGAGAAA										
A1g43860_2011F	F: AGCATACAATTTCCAGGAGTGCATC	504	X								
A1g43860_2515R (COSII)	R: CTGGGCGACATCACAC										
A1g59600_576F	F: GGAGACCTGAAACTAGTATCC	499	X								
A1g59600_1075R (COSII)	R: AAGGTGTTGCTGAGCCTC										
A1g69220_607F	F: AGCCTCGTCCGTAACATCA	467	X								
A1g69220_1075R (COSII)	R: GGCACTGTATAAGCTCGAGA										
A2g03120_495F	F: TGGGATCTGTGTAGTTTATCCAGA	500	X								
A2g03120_925R (COSII)	R: ACGAAGAGCAGCAATCCCT										
A2g27170_2888F	F: CCATCTTCATCCCTCCTCG	475	X								
A2g27170_3366R (COSII)	R: TTGACAGTCAATGGCTCAGCC										
A2g42490_4066F	F: ACTTAGCCTTTGGACCAAGC	475	X								
A2g42490_4564R (COSII)	R: GCTTGGAGAAACGACAACT										
A3g03600_524F	F: AGGCTCGAACTGTGTTGTC	499	X								
A3g03600_1023R (COSII)	R: GCCCTAAAACCTGCAACTC										
A3g04870_957F	F: TCTTCCCATCTTCCCAAGGTC	455	X								
A3g04870_1412R (COSII)	R: ACTCAAAACAGGCTCAACTCA										
A3g05000_1412F	F: ACCAACTAACAAGAGCCTCAATCA	500	X								
A3g05000_1912R (COSII)	R: TTGCACTTTTACTTCCCTGTGG										
A3g09090_1427F	F: TTTCTGTTTGTGCAATAGTTAGCAGT	485	X								
A3g09090_1912R (COSII)	R: ATGCCCGCACAAATGATCCA										
A3g13235_2063F	F: GGCAACATGATCCGGCCTA	482	X								
A3g13235_2545R (COSII)	R: TTGGGAAACTAACCACACTCTGC										
A3g16150_956F	F: ACGCAAGTCAACGATATCAATGG	404	X								
A3g16150_1360R (COSII)	R: CCTTATTGCTCGGAATGCTCG										
A3g17205_926F	F: TCAAGTCGAACCCAGCATCTGA	499	X								
A3g17205_1425R (COSII)	R: GGACTGCTGGTAATGATCTG										
A3g55560_121F	F: CAGAACTCCGGAGACTTCTCAC	499	X								
A3g55560_620R (COSII)	R: GCCCACCGTCTTCAATCA										
A3g62580_1318F	F: CATAATCGTCTGGGATTAACCG	499	X								
A3g62580_1817R (COSII)	R: GCAATCTGTTATCTTTACGCC										
A4g00560_2034F	F: AAGCTGCCATGCTTTAAATGTC	494	X								
A4g00560_2528R (COSII)	R: GAAACAGTCAATCCTCTCTCTTG										
A4g12230_1167F	F: TGTAGATTTGATCAAAAGCAATGG	417	X								
A4g12230_1584R (COSII)	R: TCAATGCTTGAATCCACTCATGG										
A4g23100_808F	F: ATCACTCAAAAGAGAGGGGG	499	X								
A4g23100_1307R (COSII)	R: CCTTCATATCGTATTTTACCACGG										
A4g29490_2406F	F: CCAACCAACATGCAATGTCAGC	499	X								
A4g29490_2905R (COSII)	R: CCTAGCTACCTCAATGGCAG										
A4g32280_1754F	F: CCGATGGCTAATCACCGCC	500	X								
A4g32280_2254R (COSII)	R: ATACGTTGCACAGACTCGACA										
A4g33625_852F	F: AGTAGGGCTCATTTAGAAACC	499	X								
A4g33625_1351R (COSII)	R: CATCTCTGTCGAAAGAAATGG										
A4g38630_507F	F: GGTACCTGTTCTAGGACGTGC	498	X								
A4g38630_1005R (COSII)	R: ATCCAAGTTGGATCCACACC										
A5g06830_1899F	F: GGCCCAACCCATTGCCATTA	499	X								
A5g06830_2398R (COSII)	R: ACTAGACTCTGGACTTTGCAGG										
A5g07270_1779F	F: CACATCCTTGCACCTCAAGC	499	X								
A5g07270_2278R (COSII)	R: ACTGCTTCTAATGTTGCT										
A5g12200_438F	F: TCGGTTCTTACTAGCTGCAAC	499	X								
A5g12200_937R (COSII)	R: ATCAGAGTTAGGGATGCTGTGT										
A5g13640_387F	F: GGAACCTGATTAACAGGACTC	498	X								
A5g13640_885R (COSII)	R: CTAGCTACGAGCAATGGC										
A5g18580_933F	F: GCTGCTTTATTCACGGTGT	499	X								
A5g18580_1432R (COSII)	R: ACAGTTACTCTGGTGAAGAG										

TABLE 1. Continued.

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

^aPrimer sequence for the “*Castilleja*-specific primer.” To make the target-specific primer for subsequent microfluidic PCR, conserved sequence tags CSI (5'-ACACTGACGACATGGTTCTACA) and CS2 (5'-TACGGTAGCAGACTTGGTCT) were added to each forward and reverse primer, respectively.

^bAmplicon length (bp) estimated from BLAST hit alignments.

^cPCR validations using DNAs from Bennett and Mathews (2006).

^dPCR 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*).

^eTaxa that were both PCR validated and had primer combinations evaluated in silico against their respective assemblies (raw read files available in the NCBI Sequence Read Archive submission SRP100222).

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

METHODS AND RESULTS

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

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

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

To investigate whether any of these primer combinations would amplify successfully across Orobanchaceae, we searched for our selected *Castilleja* primers against an assembled low-coverage genome for *Lamourouxia multifida* Kunth using BLAST (Altschul et al., 1990). *Lamourouxia multifida* was sequenced on an Illumina HiSeq 2000 at the University of Oregon as 100-bp paired-end reads, and contigs were assembled using SPAdes (Bankevich et al., 2012) under the

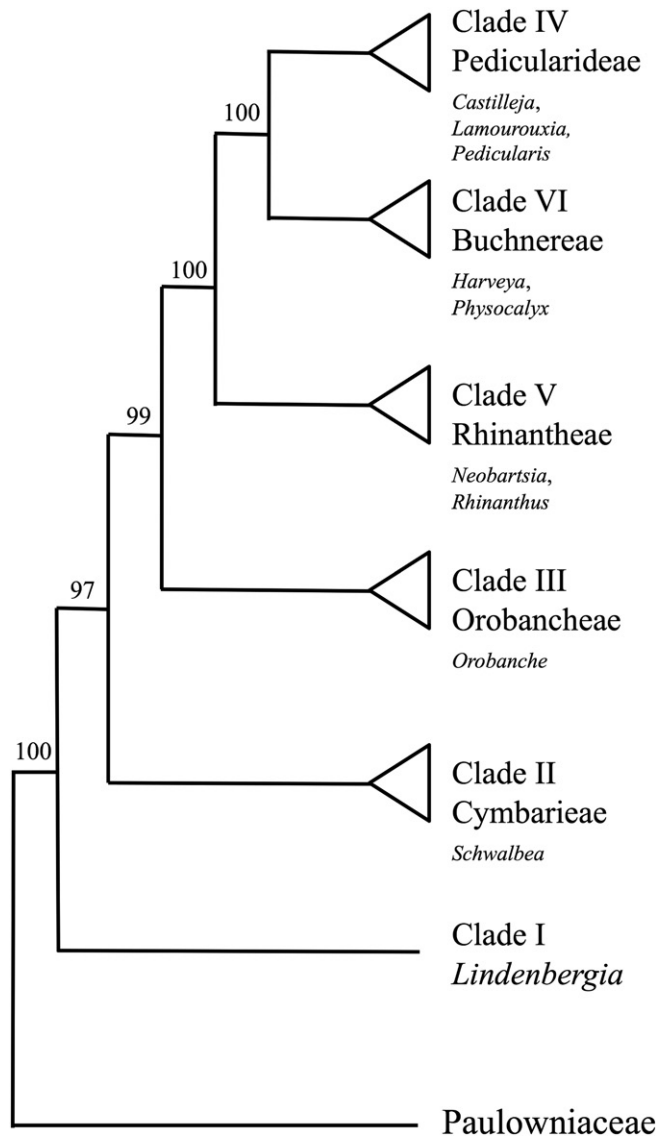


Fig. 1. Relationships among major clades within Orobanchaceae modified from McNeal et al. (2013) with taxa used for primer validation indicated (see text). Bootstrap support values for clades are indicated along the branches and follow McNeal et al. (2013). Reproduced from Latvis et al. (2017a).

default settings. BLAST search parameters, assessment of suitable hits, and subsequent PCR validation with *Lamourouxia virgata* Kunth, *Physocalyx major* Mart., and *Neobartsia filiformis* (Wedd.) Uribe-Convers & Tank are described in Latvis et al. (2017a). Primer combinations with successful amplification in *Lamourouxia* Kunth and at least one other taxon were selected for further PCR testing with other major lineages in Orobanchaceae (sensu McNeal et al., 2013; Fig. 1). This second round of PCR validation follows Latvis et al. (2017a), except that two of the accessions used for testing were changed. As in Latvis et al. (2017a), we also included a negative control and conserved sequence-tagged “universal” primers for the *trnL-F* region as a positive control for all primer pairs. We used *Neobartsia peruviana* (Walp.) Uribe-Convers & Tank instead of *N. filiformis*, and *Paulownia fortunei* (Seem.) Hemsl. instead of *P. elongata* Siebold & Zucc. (Appendix 1). Of the 87 nuclear primer combinations specifically designed for *Castilleja*, we identified 27 with broader applicability in Orobanchaceae, chosen if they successfully amplified in Pedicularideae (Clade IV; including *Castilleja*, *Lamourouxia*, and *Pedicularis* L.) and at least one of the other major clades highlighted in Fig. 1. Validation results are presented in Table 1 with these “core Orobanchaceae” combinations boldfaced.

CONCLUSIONS

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

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

Species	Voucher accession no. (Herbarium) ^a	Collection locality	Geographic coordinates
<i>Castilleja cusickii</i> Greenm.	Tank 2009-01 (ID)	Idaho, USA	45.884241°N, 116.230195°W
<i>Castilleja foliolosa</i> Hook. & Arn.	A. Colwell 03-09 (YM)	California, USA	35.3926°N, 120.3522°W
<i>Castilleja lemmonii</i> A. Gray	Jacobs 2015-088 (ID)	California, USA	37.907982°N, 119.258583°W
<i>Castilleja lineariloba</i> (Benth.) T. I. Chuang & Heckard	Tank 2002-04 (WTU)	California, USA	37.41387°N, 120.10833°W
<i>Castilleja pumila</i> Wedd.	Uribe-Converts 2011-120 (ID)	La Libertad, Peru	7.99506°S, 78.44197°W
<i>Castilleja tenuis</i> (A. Heller) T. I. Chuang & Heckard	Tank 2001-13 (WTU)	Washington, USA	46.118133°N, 121.5158°W
<i>Harveya purpurea</i> Harv.	Randle 79 (OS)	NA	NA
<i>Lamourouxia multifida</i> Kunth	Mejia 695 (CAS)	Chiapas, Mexico	NA
<i>Lamourouxia virgata</i> Kunth	Mejia 581 (CAS)	Chiapas, Mexico	16.713611°N, 92.614722°W
<i>Lindenbergia</i> sp. Kunth	Armstrong 1163 (ISU)	NA	NA
<i>Neobartsia peruviana</i> (Walp.) Uribe-Converts & Tank	Uribe-Converts 13-011 (ID)	NA	NA
<i>Orobanche californica</i> Cham. & Schltld.	Bennett 72 (A)	Cultivated	Cultivated
<i>Paulownia fortunei</i> (Seem.) Hemsl.	s.n. (A)	Cultivated	Cultivated (https://sheffields.com)
<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.

^aHerbarium acronyms are per Index Herbariorum (<http://sweetgum.nybg.org/science/ih/>).