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Authors: Latvís, Maribeth, Mortimer, Sebastian M. E., Morales-Briones, Diego F., Torpey, Samuel, Uribe-Convers, Simon, et al.

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PRIMERS FOR *CASTILLEJA* AND THEIR UTILITY ACROSS OROBANCHACEAE: I. CHLOROPLAST PRIMERS¹

MARIBETH LATVIS^{2,8}, SEBASTIAN M. E. MORTIMER^{3,4}, DIEGO F. MORALES-BRIONES^{3,4,5},
SAMUEL TORPEY^{3†}, SIMON URIBE-CONVERS⁶, SARAH J. JACOBS^{3,4,5}, SARAH MATHEWS⁷,
AND DAVID C. TANK^{3,4,5}

²Department of Natural Resource Management, South Dakota State University, 1390 College Avenue, Brookings, South Dakota 57007 USA; ³Department of Biological Sciences, University of Idaho, 875 Perimeter Drive, MS 3051, Moscow, Idaho 83844-3051 USA; ⁴Stillinger Herbarium, University of Idaho, 875 Perimeter Drive, MS 1133, Moscow, Idaho 83844-1133 USA; ⁵Institute for Bioinformatics and Evolutionary Studies (IBEST), University of Idaho, 875 Perimeter Drive, MS 3051, Moscow, Idaho 83844-3051 USA; ⁶Department of Ecology and Evolutionary Biology, University of Michigan, 830 North University Avenue, Ann Arbor, Michigan 48109 USA; and ⁷Australian National Herbarium, CSIRO National Research Collections, Canberra, Australia

- **Premise of the study:** Chloroplast primers were developed from genomic data for the taxonomically challenging genus *Castilleja*. We further tested the broader utility of these primers across Orobanchaceae, identifying a core set of chloroplast primers amplifying across the clade.
- **Methods and Results:** Using a combination of three low-coverage *Castilleja* genomes and sequence data from 12 *Castilleja* plastomes, 76 primer combinations were specifically designed and tested for *Castilleja*. The primers targeted the most variable portions of the plastome and were validated for their applicability across the clade. Of these, 38 primer combinations were subsequently evaluated in silico and then validated across other major clades in Orobanchaceae.
- **Conclusions:** These results demonstrate the utility of these primers, not only across *Castilleja*, but for other clades in Orobanchaceae—particularly hemiparasitic lineages—and will contribute to future phylogenetic studies of this important clade of parasitic plants.

Key words: *Castilleja*; chloroplast; hemiparasite; high-throughput sequencing; microfluidic PCR; Orobanchaceae.

The plastome is heavily relied upon in plant systematics, owing to its conserved nature and orthology, particularly for the study of deeper evolutionary divergences. Moreover, discordance between the uniparentally inherited plastome and the biparentally inherited nuclear genome may provide insights into introgression events and their direction (Twyford and Ennos, 2012). However, the low rate of molecular evolution in the plastome can become a hindrance when reconstructing relationships between closely related taxa, requiring large amounts of data to resolve these relationships (Uribe-Convers et al., 2016). In an attempt to alleviate this problem, several recent studies have leveraged available high-throughput sequencing data for the development of variable taxon-specific plastid (and nuclear) regions (e.g., Uribe-Convers et al., 2016).

Castilleja L. (Orobanchaceae; “the paintbrushes”) is a taxonomically challenging clade that includes ~200 hemiparasitic species, many of which have a complicated history of polyploidy and/or hybridization (Heckard and Chuang, 1977). Microsatellite markers have been developed in *Castilleja* for population genetic studies (Fant et al., 2013), and broader, genus-wide phylogenetic reconstructions within *Castilleja* used two chloroplast regions (*trnL-F* and the *rps16* intron), nuclear ribosomal spacers (ITS and ETS), and a low-copy nuclear gene (*waxy*) (Tank and Olmstead, 2008, 2009). However, species-level relationships lacked resolution in Tank and Olmstead (2008, 2009), limiting conclusions regarding diversification and hybridization. Here, we follow Uribe-Convers et al. (2016) for primer design and validation of the most highly variable chloroplast regions in *Castilleja*. Because these primers were designed for the Fluidigm Access Array microfluidic PCR system (Fluidigm, South San Francisco, California, USA), annealing temperature specifications are consistent across all primer combinations; this allows for parallelization of PCR and is ideal for high-throughput sequencing platforms (see Uribe-Convers et al., 2016 for application of this approach). Although our initial focus was the development of *Castilleja*-specific primers, we evaluated their utility in silico in three other lineages of Orobanchaceae to obtain a subset of “core” chloroplast primers with the potential to amplify across the clade. Once identified, we surveyed this set of core primers to assess their

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⁸Author for correspondence: Maribeth.Latvis@sdstate.edu

[†]Deceased 21 May 2017.

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TABLE 1. All primer pair sequences designed for *Castilleja* (names 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). Combinations are listed from most variable to least variable, according to our prioritization scheme (see text). 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 I: <i>Lindenbergia</i> sp. ^c	Clade II: <i>Schwalbea</i> <i>americana</i> ^c	Clade III: <i>Orobancha</i> <i>californica</i> ^c	Clade IV: <i>Lamoureauxia</i> <i>virgate</i> ^c	Clade V: <i>Rhinanthus</i> <i>electrolophilus</i> ^c	Clade VI: <i>Harveya</i> <i>purpurea</i> ^c	Clade VI: <i>Physocdix</i> <i>major</i> ^c	Paulowniaceae: <i>Paulownia</i> <i>elongata</i> ^c (outgroup)
Cas_120561_F_ Cas_121371_R (<i>ndh1-ndh1c</i>)	F: GTCCAAACGATCCCATACCA R: TTTAGTCGGTTACCGGTGT	810				X				
Cas_111970_F_ Cas_112789_R (<i>ndh1F-ycf1</i>)	F: GGTGGAAAGTGAGGAAGAAAGA R: TCAAGAGGAACAGGTTTGA	819				X				
Cas_129331_F_ Cas_130126_R (<i>ycf1</i>)	F: TGAGTTTAAATCAACCCGGAGA R: GACCCCTTTCCTGAACAAATCA	795				X				
Cas_112854_F_ Cas_113746_R (<i>ndh1F</i>)	F: ACATAGTATGTCTCGATTCATAAGGA R: GGAGGAGCCACTCTCTATT	892				X				
Cas_126859_F_ Cas_127713_R (<i>ycf1</i>)	F: GAACGGATCCAAAGATCTCTCTC R: GCGAAATGCGCTTTCATA	854				X				
Cas_59866_F_ Cas_60624_R (<i>accD</i>)	F: TTCCCGTCAAAGACATTCG R: GCGTGTGGAACAGCCCTCAG	758	X			X		X	X	X
Cas_127891_F_ Cas_128420_R (<i>ycf1</i>)	F: GGATCTCTTGATAGTGAAGAACAGA R: GAAGATCTGGACGATCGAA	529				X				
Cas_130168_F_ Cas_130760_R (<i>ndh1F-ycf1</i>)	F: ACAACCGAGTCTTGTTCATA R: GGTGGAAAGTGAGGAAGAAAGA	592				X				
Cas_126110_F_ Cas_126868_R (<i>ycf1</i>)	F: TCTAATCGATAATTAGGCCAAAGA R: GGATCGTCTTATCACAACA	758				X				
Cas_32159_F_ Cas_32745_R (<i>psbM-rn1E</i>)	F: AATCGGATCAATATCATGATATACAA R: ATTCCCAATCTACACGAG	586	X	X	X	X	X	X	X	X
Cas_77140_F_ Cas_78034_R (<i>psbH-psbE</i>)	F: TGTCTGCAATGGCTCTATTG R: TCATCTCTGTACAGCTCAAGCA	894				X				
Cas_10778_F_ Cas_11525_R (<i>psb16-rn1G</i>)	F: TCAGTTTGATGATCCTTTGATGA R: CATTGGCTCTTTATGGAA	747				X				
Cas_46472_F_ Cas_47162_R (<i>ycf3-rps4</i>)	F: GGAACTATTCGATTTTCATTG R: CTAACTGTGGAAATAAGTCTCC	690				X				
Cas_47758_F_ Cas_48638_R (<i>rps4-rn1L</i>)	F: CGCGATACGGACGTATTTA R: GAATTGTGATCAAGAAATCGAAATTA	880				X				
Cas_17609_F_ Cas_18412_R (<i>rps2-rpsC2</i>)	F: AACTCTCGCAGACCGGTAT R: CCACGATAGACCAAGCAATCA	803				X				
Cas_33546_F_ Cas_34499_R (<i>rnd-psbD</i>)	F: GACTCGTTTGGGAATTAATCAA R: CTTCAACCATTTCCGAGCAC	953				X				
Cas_67504_F_ Cas_68343_R (<i>psbE-psbL</i>)	F: TTGTACCGGAGGCATCTTTAG R: AACCGAAATAACTCGTTATAGTAAGCA	839				X				
Cas_72399_F_ Cas_73245_R (<i>rps12-clpP</i>)	F: GGGCTGGTTTAGATTGATCCT R: TTTCATGGATTATGATCGAGAG	846				X				
Cas_62840_F_ Cas_63772_R (<i>ycf4-cemA</i>)	F: AATCCGTTGACCCGCTACTGA R: AAGAGAGAAATCCACCAAGGTAA	932	X			X				X

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</i> <i>americana</i> ^d	Clade III: <i>Orobancha</i> <i>californica</i> ^e	Clade IV: <i>Castilleja</i> <i>lineariloba</i> , <i>C. pumila</i> , <i>C. lemmonii</i> ^f	Clade IV: <i>Lamoureauxia</i> <i>virgate</i> ^g	Clade IV: <i>Pedicularis</i> sp. ^c	Clade V: <i>Neobartisia</i> <i>filiformis</i> ^h	Clade V: <i>Rhinanthus</i> <i>electrololophus</i> ⁱ	Clade VI: <i>Harveya</i> <i>purpurea</i> ^j	Clade VI: <i>Physoclyx</i> <i>major</i> ^k	Paulowniaceae: <i>Paulownia</i> <i>elongata</i> ^l (outgroup)
Cas_65707_F_	F: CTGGGAAATCCCTGTGAC	927											
Cas_66634_R	R: TCCGGATTAGGTTATCCCTA					X	X						
Cas_69456_F	F: CAACCTAAGCGACCCCTAAATACA	718				X							
Cas_70174_R	R: TACTCGGCATCTTTCTCT												
Cas_73947_F	F: GGTTCCTTGACCAACACACAG	782				X							
Cas_5319_R	R: ATCCCAACAACACGACTTC												
Cas_48611_F	F: TGTAATTCGATTTCTTGATCAAT	909				X			X				
Cas_49520_R	R: CAGATACAGATTTGGCCATC												
Cas_29425_F	F: TTGAAGCGAAGTAGATAATTGA	866				X							
Cas_30291_R	R: TCTACACAGAGGCTACAACTGA												
Cas_125001_F	F: AATAATCCCAACGCTTACA	858				X							
Cas_125859_R	R: AATGTTTCAATTAGCTCTGAAATG												
Cas_21290_F	F: TGTTCTGATTTCTACAVATGATCGTTT	746	X		X	X			X	X	X		X
Cas_22036_R	R: CGTGAAGGCTTTCTTTAACA												
Cas_20851_F	F: CTGGTAGAGAGTGGTCGGATCT	456				X							
Cas_21307_R	R: GGTGAAATTGGGAGAAGCTG												
Cas_11589_F	F: AGCTTCCAAAGCTAAACGATG	872				X			X				
Cas_12461_R	R: CTGGAATCAGACCGCTATT												
Cas_47139_F	F: GGAGACCTTTATTCACCAGTTAG	550				X							
Cas_47689_R	R: TTTTCGATTGGGTATGGCTTC												
Cas_14073_F	F: TTCGATTCAATTTGGCTCTCA	651	X	X	X	X		X		X	X	X	X
Cas_14724_R	R: TGGAAAGGAGTGTGTGTGA												
Cas_122476_F	F: ACGGCTCTCATAGGTCA	797				X							
Cas_123331_R	R: TGCCTTTAAAGAAATTCATCTCA												
Cas_73947_F	F: TCTTGTCTCTGAATGGGCTC	551				X							
Cas_74498_R	R: GTTACGTTTCCACATCAAGTGA												
Cas_123306_F	F: AATGAGATGAATTCCTTTAACAAG	798	X			X							
Cas_124104_R	R: TGAATTTGGCTGATATTATGACG										X		X
Cas_24256_F	F: ATAAACCGGTAATCGCAAG	781				X							
Cas_25037_R	R: TGTTCATCCAGTCAATCCAA												
Cas_85769_F	F: CATCAGGATATACCATAGTTGCTTT	648				X							
Cas_86417_R	R: CCATAGGATGCCGTTCA												
Cas_36699_F	F: GGGTCCCGAGAAATATATGA	745				X							
Cas_37444_R	R: TTATTTCAAAATGGGAATCCTG												
Cas_61880_F	F: GCAATGGCTCTTTATTTCTTCA	951	X		X	X				X			X
Cas_62831_R	R: GGCCTCGGATGTCCTATTA												
Cas_71554_F	F: TCAATGGCTTCGGCTACTA	877				X							
Cas_72431_R	R: AATCATCCGGTTAGGATCAATC												
Cas_5508_F	F: CCGATTCAATTCCTTTAATTCG	722				X							
Cas_6230_R	R: TTAGCTCAACAGTTTGATAGCTTG												

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 amercanae</i>	Clade III: <i>Orobancha californica</i>	Clade IV: <i>Castilleja lineariloba</i> , <i>C. pumila</i> , <i>C. lemmonii</i> ^d	Clade IV: <i>Lamoureauxia virgate</i> ^e	Clade IV: <i>Pedicularis</i> sp. ^c	Clade V: <i>Neobarista filiformis</i> ^e	Clade V: <i>Rhinanthus alectorolophus</i> ^e	Clade VI: <i>Harveya purpurea</i> ^e	Clade VI: <i>Physoclyx major</i> ^e	Paulowniaceae: <i>Paulownia elongata</i> ^e (outgroup)
Cas_13394_F Cas_14062_R (<i>atpA-atpF</i>)	F: CGAGCAATACCATCGCTAC R: TTGGTTTCGGGAAGGATTAT	668	X	X		X	X	X		X	X	X	X
Cas_19198_F Cas_19976_R (<i>rpoC2</i>)	F: TCTCTGGAGTGGCCAAATAAG R: CTTTGTGTAATAAAGGCCAAA	778	X	X		X	X		X	X			X
Cas_124082_F Cas_124968_R (<i>ndhH</i>)	F: CGTCATAATATCAGCCAAATTCA R: ATGGACCCGAACGACTAGG	886				X							
Cas_52327_F Cas_52920_R (<i>ndhC-trnV</i>)	F: TCGATAAATACAGATACACCCCAATACA R: GCAAGAAATCCTAGGCGAAGA	593				X	X						
Cas_50548_F Cas_51414_R (<i>rnfF-ndhI</i>)	F: TCGGTTTCAGATACAAATAAATCCA R: AGGGTCATTTGTCTGCTTGG	866				X			X				
Cas_27800_F Cas_28512_R (<i>rpoB</i>)	F: CGGCTACAGAACGAATACGC R: GTATCGCGGGGATTAATTGG	712				X						X	
Cas_20009_F Cas_20813_R (<i>rpoC2</i>)	F: TTGTCTTGTGCCAATTCATATAC R: TCATCATTCCTCAATCG	804		X		X	X		X	X		X	X
Cas_14705_F Cas_15624_R (<i>atpH</i>)	F: TCACACACACTCCCTTTTCCA R: GATATCGAAGTAGTTGGATTAGTCA	919	X	X			X					X	
Cas_25017_F Cas_25720_R (<i>rpoC1-rpoB</i>)	F: TTGGATTGACTGGGANGACA R: TATTAGAGCGGCCAAGAAG	703	X	X			X	X	X	X	X	X	X
Cas_40118_F Cas_40881_R (<i>psaB</i>)	F: TCCTCGGATATATTGGTGATGA R: CAATTGGTTTACGCATTAATGAA	763		X			X			X	X	X	X
Cas_44810_F Cas_45699_R (<i>ycf3</i>)	F: ACACCGCTGCTCAAGACTTT R: CCATCGAAGTTTGTGAAGTG	889	X	X			X		X	X	X	X	X
Cas_121734_F Cas_122486_R (<i>ndhA-ndhI</i>)	F: CTGCGAGCTCGTATTGTTT R: TGAGGAGCGGTATGAGGTAAA	752	X				X				X	X	X
Cas_93851_F Cas_94660_R (<i>ycf2</i>)	F: CGGAGCTGGAAGTCTTAACCT R: GTCCGGGTAGAGACCAAGA	749	X		X		X		X	X	X	X	X
Cas_54595_F Cas_55457_R (<i>rnlM-atpB</i>)	F: GGGAGTCATTTGGTTCAAATCC R: GCGTTTCTTTATCACACCCCTTT	862	X	X	X		X		X	X	X	X	X
Cas_94709_F Cas_95300_R (<i>ycf2</i>)	F: CGGATCTAGTTCATGGGCTATT R: TCTGCAATAAATCTCGATGTGA	591	X	X	X		X	X	X	X	X	X	X
Cas_70574_F Cas_71412_R (<i>rps18-rp120</i>)	F: GGATCGAATTGATTATAGAAACATGA R: AGCTCGGAGCGTAGAACAACAA	838	X			X		X	X	X	X	X	
Cas_78484_F Cas_79253_R (<i>petB-petD</i>)	F: CTTTACCTCGGGACCAATC R: CAATGCAAGGAATGAATGC	769	X			X			X	X	X	X	
Cas_80388_F Cas_81242_R (<i>rpoA</i>)	F: TTTCTAGACTGCCCAATATCTGTTT R: AAGCCGACACAATAGGCATT	854							X	X	X	X	X
Cas_81995_F Cas_82887_R (<i>rpl36-rps8</i>)	F: CGCTACAGAACGAATACGC R: GTATCGCGGGATTAAATTG	892	X						X	X	X	X	X
Cas_85146_F Cas_85791_R (<i>rps3-rp122</i>)	F: TCGGAACGTATAGGAACAATAATCA R: GGCAACTATGGTATATCCTGATGTG	645		X	X		X			X	X	X	X

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</i> <i>americana</i> ^d	Clade III: <i>Orobancha</i> <i>californica</i> ^d	Clade IV: <i>Castilleja</i> <i>lineariloba</i> , <i>C. pumila</i> , <i>C. lemmonii</i> ^d	Clade IV: <i>Lamoureauxia</i> <i>virgata</i> ^e	Clade IV: <i>Pedicularis</i> sp. ^e	Clade V: <i>Neobartisia</i> <i>filiformis</i> ^e	Clade V: <i>Rhinanthus</i> <i>alecatorolophus</i> ^e	Clade VI: <i>Harveya</i> <i>purpurea</i> ^e	Clade VI: <i>Physoclyx</i> <i>major</i> ^e	Paulowniaceae: <i>Paulownia</i> <i>elongata</i> ^e (outgroup)
Cas_96241_F_	F: TCCGAGATCTCTTATGGAATTGC	789	X	X		X	X	X		X	X	X	X
Cas_97030_R_	R: TTCCATCGAATTGAGTATGATGTTG												
(<i>ycf1-ndhB</i>)													
Cas_38180_F_	F: CGCCCAAGATCAAGATATA	769				X			X		X	X	
Cas_38949_R_	R: AACTGAACCATATATGCAAGA												
(<i>trnG-rps14</i>)													
Cas_21932_F_	F: CGCGTCAGATATCCAGCAT	803	X	X		X	X	X	X	X	X	X	X
Cas_22735_R_	R: TCTCAGGCGCTGTCATATGTT												
(<i>rpoC2-rpoC1</i>)													
Cas_12567_F_	F: AGCGGTGATATCTTCCTTCA	832	X	X	X	X	X	X	X	X	X	X	X
Cas_13399_R_	R: TGCTCGTATTCACGGCTTG												
(<i>atpA</i>)													
Cas_25855_F_	F: GCATATGTCCACTGGAACG	802	X	X		X	X	X	X	X	X	X	X
Cas_26657_R_	R: AAGGCCCTGAAAGGATCACTA												
(<i>rpoB</i>)													
Cas_64793_F_	F: TAAGCCGCTGGATATTTGAGG	939				X							
Cas_65732_R_	R: AAATGCGGTACAGGGATTTTC												
(<i>petA-psbI</i>)													
Cas_23417_F_	F: TTCCTGAAGTATTTCCATCAATC	778	X	X		X	X	X	X	X	X	X	X
Cas_24195_R_	R: CGATACATTTCCCAATCGAG												
(<i>rpoC1</i>)													
Cas_66623_F_	F: ACCTAATCCGGAATATGAACCA	902				X							
Cas_67525_R_	R: TCTAAGATGCGCTCGGTACA												
(<i>psbI-petL</i>)													
Cas_90084_F_	F: AGAATCAGACCTATTCCGAAA	801	X	X		X	X	X	X	X	X	X	X
Cas_90885_R_	R: TGCTCCATTTATGTTGTGC												
(<i>ycf2</i>)													
Cas_18394_F_	F: TTGTTCTGCTATCTGTTGAAA	792	X	X		X	X	X	X	X	X	X	X
Cas_19186_R_	R: TGCCCATTTAGGAATCC												
(<i>rpoC2</i>)													
Cas_42062_F_	F: GGCCTAAAGCTGGGTATTT	835	X	X		X	X	X	X	X	X	X	X
Cas_42897_R_	R: TCAGGTGCATGTATCTTTACCG												
(<i>psaA</i>)													
Cas_92095_F_	F: CCAATGCATAGATCCATTC	840	X	X	X	X	X	X	X	X	X	X	X
Cas_92935_R_	R: TCGGTGCAGATGTAGGATACC												
(<i>ycf2</i>)													
Cas_87589_F_	F: TTGCTGCCGTTACTCTTCAG	923	X	X	X	X	X	X	X	X	X	X	X
Cas_88512_R_	R: AGAATCGGTGTGTGTATATTTCA												
(<i>rpl2-rpl23</i>)													
Cas_26951_F_	F: CTCCTATCCCTGAGACAAGG	757	X	X	X	X	X	X	X	X	X	X	
Cas_27708_R_	R: CGACTCTCAGAAATTTGGT												
(<i>rpoB</i>)													
Cas_91056_F_	F: AATGAAATATACGATCAACCAATT	839	X	X		X	X	X	X	X	X	X	X
Cas_91895_R_	R: TCATAATTATTGATACGGGCTTT												
(<i>ycf2</i>)													
Cas_104111_F_	F: TTGGTTTGACACTGCTCTTCA	834	X	X	X	X	X	X	X	X	X	X	X
Cas_104945_R_	R: ATTTACGCTCTTCTCTTTCG												
(<i>trnL-trnI6</i>)													
Cas_34914_F_	F: GAGCTTGCTCGATCTGTCTA	815	X	X	X	X	X	X	X	X	X	X	X
Cas_35729_R_	R: ATTGCTCCAGCCAGATAC												
(<i>psbD-psbC</i>)													

^aPrimer 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.

^bAmplicon length (bp) estimated from *Castilleja* plastome 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 both were PCR validated and had primer combinations evaluated in silico against their respective plastome assemblies (raw read files available in the NCBI Sequence Read Archive submission SRP100222).

performance using additional sampling across Orobanchaceae. Orobanchaceae represents the largest parasitic clade of angiosperms and has well-documented modifications to the plastome, such as reduction and accelerated rates of molecular evolution; however, the most comprehensive phylogenetic investigation to date was based on only five gene regions (McNeal et al., 2013). Thus, an expanded molecular toolkit will be of great benefit for future investigations in the clade.

METHODS AND RESULTS

Three species of *Castilleja* were selected for genome skimming (*C. cusickii* Greenm., *C. foliolosa* Hook. & Arn., *C. tenuis* (A. Heller) T. I. Chuang & Heckard; Appendix 1), with taxa chosen to include both annual and perennial lineages (National Center for Biotechnology Information [NCBI] Sequence Read Archive [SRA] accession SRP100222). DNA extraction, purification, Illumina library construction, and subsequent cleaning of reads followed Uribe-Convers et al. (2016). Samples were sequenced as 100-bp single-end reads on an Illumina HiSeq 2000 (Illumina, San Diego, California, USA) at the University of Oregon, and cleaned reads were assembled against a reference genome (*Sesamum indicum* L. JN637766) using the Alignreads pipeline version 2.25 (Straub et al., 2011). In addition to these three low-coverage genomes, we also used existing data for 12 *Castilleja* plastomes generated by Uribe-Convers et al. (2014) using a long-PCR approach. Fifteen plastomes in total were aligned using MAFFT version 7.017b under the default settings (Katoh and Standley, 2013).

We used a custom R script (Uribe-Convers et al., 2016) to identify the most variable regions of the alignment spanning 400–1000 bp that were flanked by conserved regions, enabling prioritization based on predicted amplicon size and variability. Regions containing ambiguous bases were discarded, and those missing from one or more taxa in the alignment, particularly in the plastomes generated through the long-PCR method, were given lesser priority. We used Primer3 (Untergasser et al., 2012) to design primer pairs for the selected regions with an annealing temperature of 60°C (±1°C), and allowing no more than three continuous nucleotides of the same base, following the specifications of the Fluidigm Access Array System protocol.

We validated each primer combination using PCR with three high-quality *Castilleja* DNA isolations chosen to represent major lineages, sensu Tank and Olmstead (2008) (*C. lineariloba* (Benth.) T. I. Chuang & Heckard, *C. lemmonii* A. Gray, and *C. pumila* Wedd.; Appendix 1), but different than those selected for genome skimming and primer design, and a negative control. Because we followed the approach of Uribe-Convers et al. (2016), it was necessary for our validation conditions to simulate the four-primer reaction of the Fluidigm microfluidic PCR using a standard thermocycler. Therefore, our target-specific primers include a 5' conserved sequence (CS) tag, obtained from the Fluidigm Access Array System protocol, which provides an annealing site for Illumina sequencing adapters and sample-specific barcodes. PCR amplification followed Uribe-Convers et al. (2016), and amplicons were visualized on a standard agarose gel. In total, 76 primer combinations were successfully designed and validated (Table 1).

To test the broader utility of our *Castilleja*-specific primers, we searched for matches in two published plastome assemblies for *Lamourouxia virgata* Kunth (Pedicularideae, Clade IV; Fig. 1) and *Neobartsia stricta* (Kunth) Uribe-Convers & Tank (Rhinanthaeae, Clade V) (NCBI SRA accessions SRR1023133 and SRR1023130, respectively; Uribe-Convers et al., 2014). We assembled the plastome for a third taxon, *Physocalyx major* Mart. (Buchneraeae, Clade VI; NCBI SRA accession SRP100222), to include in our comparison. *Physocalyx major* was sequenced on an Illumina HiSeq 2000 at the University of Oregon as 100-bp paired-end reads. Cleaned reads for *P. major* were mapped to three reference plastomes with one copy of the inverted repeat region removed (*Sesamum indicum* JN637766, *Neobartsia inaequalis* (Benth.) Uribe-Convers & Tank KF922718, *Castilleja paramensis* F. González & Pabón-Mora KT959111) using Bowtie2 (Langmead and Salzberg, 2012). Consensus sequences of the resultant contigs were obtained and used as local references. Contigs were then imported into Geneious R7 version 7.0.6 (Kearse et al., 2012), and a consensus sequence was obtained by calling regions with less than 5× coverage as “N” and using the “Highest Quality” as a threshold.

Separate BLAST databases were created for *Lamourouxia* Kunth, *Neobartsia* Uribe-Convers & Tank, and *Physocalyx* Pohl assemblies (–makeblastdb), and blastn_short was used to search for matching hits with the list of *Castilleja* chloroplast primers. Hits were further considered if both primer pairs (1) occurred on the same contig and (2) had predicted amplicon sizes between 350–1000 bp. Once we obtained a set of primer hits for the three taxa, they were validated with

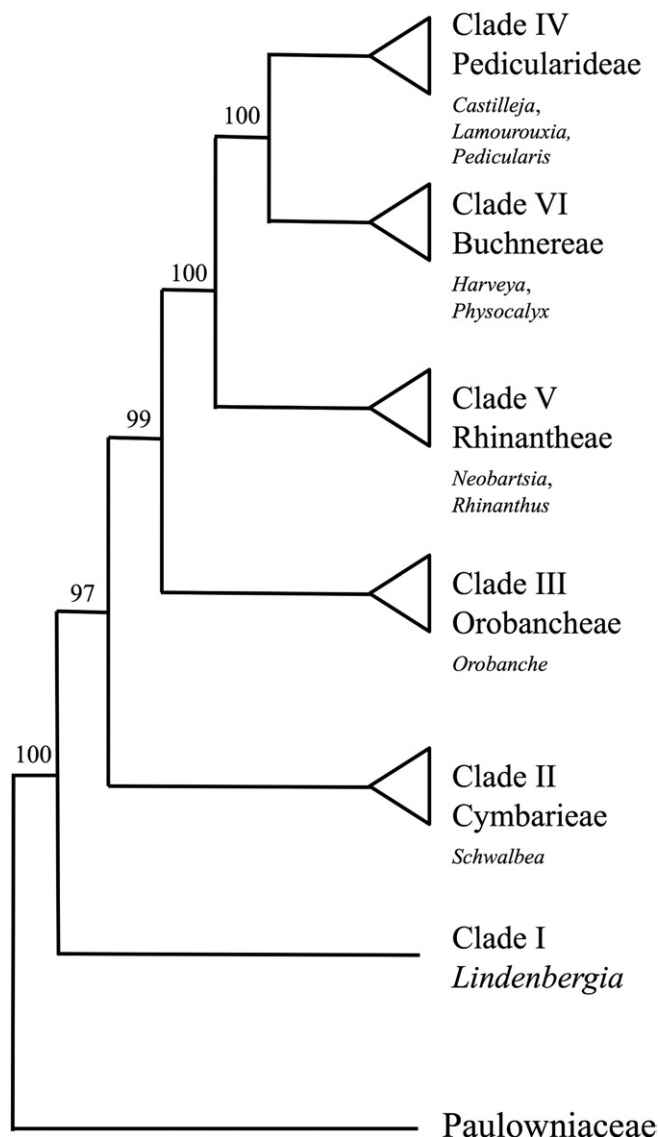


Fig. 1. Relationships among major clades within Orobanchaceae modified from McNeal et al. (2013), with taxa used for primer validation indicated (see text). Bootstrap support values for clades are indicated along the branches and follow McNeal et al. (2013).

PCR using *L. virgata*, *P. major*, and *Neobartsia filiformis* (Wedd.) Uribe-Convers & Tank (Appendix 1), as described above. Primer pairs with amplification in at least two out of three taxa above were chosen for another round of PCR validation with expanded taxon sampling that represented all major lineages of Orobanchaceae (sensu McNeal et al., 2013; Appendix 1): *Lindenbergia* sp. Lehm. (Clade I), *Schwalbea americana* L. (Cymbarieae, Clade II), *Orobanche californica* Cham. & Schltdl. (Orobancheae, Clade III), *Pedicularis* sp. L. (Pedicularideae, Clade IV), *Rhinanthus alectorolophus* (Scop.) Pollich (Rhinanthaeae, Clade V), *Harveya purpurea* Harv. (Buchneraeae, Clade VI), and *Paulownia* Siebold & Zucc. (Paulowniaceae; outgroup). As a positive control, we included CS-tagged “universal” primers for the *trnL-F* region (“trn-c” and “trn-f” of Taberlet et al., 1991, in Tank and Olmstead, 2008).

Out of the 76 primer pairs designed and validated for *Castilleja*, we identified 36 pairs with applicability across Orobanchaceae (referred to as core Orobanchaceae primers; these are boldfaced in Table 1). These were chosen based on amplification across a large phylogenetic breadth of the clade, but allowing for some failures. For example, *Orobanche*, a holoparasite, failed for most primer combinations, a result that is likely due to the reduction and modification of the plastome in this lineage (see Bennett and Mathews, 2006). Higher success rates were noted for hemiparasites.

CONCLUSIONS

We report 76 primer pairs designed to target the most variable regions of the chloroplast genome in *Castilleja*. We further demonstrate their utility across other major clades in Orobanchaceae, particularly with hemiparasitic taxa, and present a subset of 38 core Orobanchaceae primers. Although these primer combinations target similar highly variable plastid regions as in other angiosperm-wide studies (e.g., Ebert and Peakall, 2009), few of the primers reported here overlap directly with them. Two exceptions are Cas_11589 F (*trnG*) and Cas_61880 F (*psaI*) (Table 1), which were also developed by Ebert and Peakall (2009). Notably, our primer combinations were designed with the same annealing temperature to take advantage of the Fluidigm microfluidic PCR system and high-throughput sequencing platforms, but will also be useful for traditional PCR and Sanger sequencing.

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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-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>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 filiformis</i> (Wedd.) Uribe-Convers & Tank	Uribe-Convers 13-027 (ID)	La Paz, Bolivia	16.32796°S, 67.9457°W
<i>Orobanche californica</i> Cham. & Schltdl.	Bennett 72 (A)	Cultivated	Cultivated
<i>Paulownia elongata</i> Siebold & Zucc.	s.n. (A)	Cultivated	Cultivated (https://sheffields.com)
<i>Pedicularis</i> sp. L.	Krajsek and Bennett s.n. (A)	NA	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/>).