Development of EST-SSR Markers for Primula ovalifolia (Primulaceae) by Transcriptome Sequencing

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PRIMER NOTE

DEVELOPMENT OF EST-SSR MARKERS FOR *PRIMULA OVALIFOLIA* (PRIMULACEAE) BY TRANSCRIPTOME SEQUENCING[1]

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- *Premise of the study:* Microsatellite primers were developed for *Primula ovalifolia*, a member of *Primula* section *Petiolares* (Primulaceae), to study the population genetics and species delimitation in this section.
- *Methods and Results:* A total of 4753 markers were successfully designated from 5139 putative simple sequence repeat loci. We isolated 38 expressed sequence tag–simple sequence repeat markers from 220 selected marker sites and tested polymorphism in three populations of *P. ovalifolia*, one of *P. tardiflora*, and one of *P. epilosa*. The number of alleles per locus ranged from one to 19, and the observed and expected levels of heterozygosity varied from 0 to 0.938 and 0 to 0.915, respectively. Most of the loci could be successfully cross-amplified in the two congeneric species.
- *Conclusions:* These markers will be useful for further population genetic analysis and gene flow estimation of *P. ovalifolia* and its relatives.

Key words: EST-SSR marker; interspecific transferability; *Primula ovalifolia*; Primulaceae; transcriptome.

Primula L. section *Petiolares* Pax (Primulaceae) is mainly distributed in the Hengduanshan–Himalaya Mountains with only a few members occurring in Kashmir, central China, and other regions (Hu and Kelso, 1996). *Primula ovalifolia* Franch. and *P. tardiflora* (C. M. Hu) C. M. Hu are two closely related species in the section. *Primula ovalifolia* is widely distributed in southwestern and adjacent central China, around the Sichuan Basin, mainly growing in shaded habitats in broad-leaved forests and ravines, with altitudes ranging from 600 to 2500 m. *Primula tardiflora* is morphologically similar to *P. ovalifolia* but with a more limited distribution, known only from a single locality in E'mei Mountain. It was first considered as a subspecies of *P. ovalifolia*, and later was regarded as distinct from *P. ovalifolia* by its higher altitude habitat, later floral phenology, and some neglected vegetative traits (Hu and Kelso, 1996). A phylogeographic study based on chloroplast DNA data suggested that *P. tardiflora* is genetically close to the E'mei Mountain population of *P. ovalifolia* (Xie et al., 2012). Development of highly polymorphic nuclear markers (e.g., simple sequence repeats [SSRs]) will help to delineate between the two species. In *Primula*, only a few genomic SSR markers have been developed for several species thus far, such as *P. vulgaris* Huds. (Van et al., 2006), *P. obconica* Hance (Yan et al., 2010), *P. sieboldii* E. Morren (Ueno et al., 2011), *P. veris* L. (Bickler et al., 2013), *P. poissonii* Franch., and *P. wilsonii* Dunn (Zhang et al., 2013). Considering that these markers demonstrate low polymorphism and limited transferability, and that species of section *Petiolares* are phylogenetically distant from the above-named species, expressed sequence tag (EST)-SSRs specific to section *Petiolares* could provide useful tools for evolutionary and ecological studies in this section. In this study, we first obtained transcriptome data for *P. ovalifolia* using the Illumina platform and then designed marker pairs based on SSR loci. A subset of the markers was selected to investigate their polymorphism and transferability in congeneric species of *Primula*.

METHODS AND RESULTS

***Transcriptome sequencing*—**Plants of *P. ovalifolia* were collected from Mount E'mei, Sichuan Province, China. Leaves from one individual were sampled, frozen immediately in liquid nitrogen, and stored at −80°C for RNA extraction and transcriptome sequencing. RNA was extracted using TRIzol Reagent (QIAGEN, Dusseldorf, Germany) and delivered to Genepioneer Technologies Corporation (Nanjing, China) for construction of cDNA libraries and sequencing. The cDNA libraries were sequenced on the Illumina HiSeq 2500 platform (Illumina, San Diego, California, USA) according to the manufacturer's recommendations. A total of 49,094,910 raw reads were obtained and deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive under BioProject ID PRJNA379052 (accession no. SRP102475). Raw reads were first cleaned by trimming adapters and removing ambiguous reads ('N' > 10%) and low-quality reads (Phred score < 30). Clean reads were assembled into 142,468 transcripts using Trinity tools with default parameters (Haas et al., 2013) and were then clustered into 67,577 unigenes with TGICL version 2.1 (Pertea et al., 2003).

***Development of EST-SSR markers*—**The EST-SSR loci were identified from unigenes longer than 1 kb using MIcroSAtellite identification tool

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TABLE 1. Characteristics of 38 microsatellite loci identified in *Primula ovalifolia*.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent dye	GenBank accession no.
C11339	F: CGACTTCACTCCCACTGTTG	$(CT)_8$	278–310	HEX	MF716482
	R: GGTCAAAATCACCGGAAAGA				
C13959	F: TGGACGCCAATCTTTCTCAT	$(AG)_8$	266–282	FAM	MF716483
	R: TTGCATATCCCCTCCCAATA				
C14309	F: ATTGCAAGTGTTCTTTCGGC	$(TC)_{10}$	116–132	ROX	MF716484
	R: TCCCTTTGCTAAAAAGAAGGC				
C14710	F: TACACCGGTCGGAAGAAGTC	$(AG)_8$	258–276	TAMRA	MF716485
	R: GGTGTTCCTCCTAAATCCCC				
C15228	F: TTTCCAATCCATGTCGTTCA	$(AG)_8$	268–276	HEX	MF716486
	R: CAATTTGCACCCAACAAACA				
C21614	F: GCGCCGTGACATAAATCATA	$(CT)_9$	180–184	HEX	MF716487
	R: GGTGGAGGTGTTCGTAAGGA				
C23409	F: CAGTCAAATCACCAAGGGCT	$(CT)_8$	171–203	FAM	MF716488
	R: CAGCTTGTTCGATTGTTGGA				
C23644	F: GCGTAAGTAGTAGCGGTGGC	$(CT)_8$	238–268	TAMRA	MF716489
	R: CGCCCAATAACAAAACCAG				
C23746	F: CCACTGCCTCCATTACCATT	$(AG)_9$	112–114	TAMRA	MF716490
	R: AACGTTCCATTTTCAGGTGC				
C24233	F: CTGCAAAAACATGCTCTGGA	$(CT)_{10}$	284–298	HEX	MF716491
	R: GGGCAGTTTTGTGTCCATTT				
C24268	F: ATGGCAAATTCGGATTCAAG	$(AT)_8$	221–249	ROX	MF716492
	R: ACACGCACGTCTCCTCTTCT				
C24676	F: CCTGCAAACAGTTAGGCACA	$(GA)_{10}$	206–224	FAM	MF716493
	R: TTTCGCTATTTATCACCGCC				
C40984	F: AGGAGTGAGAGGGGTTTGGT	$(CT)_8$	145–153	TAMRA	MF716494
	R: CACAACAATTAAGCAGACAAAAA				
C45417	F: GGGGGAGCAGGAGTAATAGG	$(AT)_{10}$	166–252	ROX	MF716495
	R: CTTGAAAGTGGCAAGGCAAT				
C46430	F: CCAAGCCACACCACACAT	$(CT)_8$	285–305	ROX	MF716496
	R: GAGGACGGAGAGTACGCAAG				
C47095	F: GTCTGATCATGGCAGTGGTG	$(GA)_8$	212	HEX	MF716497
	R: GATCGGACGGTGGAGAATAA				
C48258	F: GGTGAATCATCACCCAATCC	$(GA)_9$	176–212	TAMRA	MF716498
	R: TGCCCAAACATATGCCTTCT				
C48475	F: GGCCCAAAGGAAAAGGATAA	$(AG)_8$	139–173	FAM	MF716499
	R: TGTGAGTGGAATTGGGAACA				
C50127	F: CCAGCGAGATTTGTGATTGA	$(TC)_8$	172–184	TAMRA	MF716500
	R: CAGATGAACATGTACACACCTGC				
C51170	F: GTACTCATCCGGCACCACTT	$(CT)_{10}$	294–322	ROX	MF716501
	R: AAAGCCGCAAGACCAGTAAA				
C53509	F: ACCATCCCAATTCCCTTCTC	$(TC)_8$	204–236	TAMRA	MF716502
	R: GCAGCAGTGACGACTGGTAA				
C53824	F: CTCGATCTCCAAGGGCTAAA	$(GT)_8$	248–254	TAMRA	MF716503
	R: CCCCTCTCTCTGTCATGGAA				
C53825	F: CAACAACAGGTTTTGGAGCA	$(AG)_9$	282–320	HEX	MF716504
	R: CCCTTGGGATCTCATCTTCA				
C53843	F: GGCAATAGTAGCCCCAAACA	$(GA)_8$	444–470	ROX	MF716505
	R: ATAACGCAACACCATCCCAG				
C53920	F: AACATGAGATGCCTGCACAA	$(CT)_9$	184–192	TAMRA	MF716506
	R: TGGGTCTGCATGTGAAAGAA				
C53962	F: GGTACAAAAGAAAAACGAGCTGA	$(CA)_8$	128–160	FAM	MF716507
	R: GGTAGGCGCAGCACTATGTT				
C55683	F: TGATGAAAAGTTGGGCATGA	$(AT)_9$	156–182	HEX	MF716508
	R: CACCGTATCGTGTGGAGATG				
C55722	F: CCATCGGCCTCATAAGAAAA	$(TA)_8$	206–220	TAMRA	MF716509
	R: GTATGCTCTCCCAGCTCCAC				
C57707	F: AGCAGCAAGAGCATTGGAGT	$(TC)_8$	225–239	ROX	MF716510
	R: TCATTGTTTCCAACTCTCACAAA				
C58437	F: ACCGGTCTACACCATGACCT	$(GA)_8$	252–256	ROX	MF716511
	R: CACACAAGGCTTCTTTGCAG				
C58140	F: AACACAATCTCGTATACTATCCATCA	$(AG)_6…(GA)_9$	280–304	HEX	MF716512
	R: GTAAATCTCGGCGTCGGTAA				
C59012	F: ATCGTCAACATCGTCGTCAG	$(AG)_8$	213–243	FAM	MF716513
	R: AGAGCGAGAAACCTCTTCCC				
C59078	F: CCGGCATTAAACACACTCAC	$(TA)_9$	166–180	FAM	MF716514
	R: CTACTGCTGCCGTGCATCTA				
C59155	F: TGCTTGCTTATTACCCTGCC	$(AG)_8$	145–151	HEX	MF716515
	R: AATTGTTGGCGTTGGAAGAC				

Table 1. Continued.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent dye	GenBank accession no.
C59702	F: TTAATCGTGACAGCCAGCAG	$(CT)_8$	182–200	TAMRA	MF716516
	R: GAGTCATCAATGCGAGGTGA				
C63472	F: ACGTGAAGCATGGTGCAATA	$(AT)_9$	206–234	FAM	MF716517
	R: CGGAAGCTTCTACTCGCCTA				
C65324	F: GCTCACCCTACCAACGATGT	$(GA)_9$	278–286	ROX	MF716518
	R: TCTCCACCGTCAAACCTACC				
C66329	F: CAAGGACCCGAATACTCCAA	$(AT)_9$	152–170	HEX	MF716519
	R: GATGGAATGGAAAAGGCAGA				

[a]A touchdown PCR program with annealing temperature of 60–50°C was used for all loci.

(MISA) based on the Perl language (Thiel et al., 2003). We searched for SSRs with motifs ranging from mono- to hexanucleotides in size, and 4753 primer pairs were designated from 5139 putative loci using Primer3 web version 0.4.0 (Rozen and Skaletsky, 1999). A total of 220 markers comprising two nucleotides with at least eight contiguous repeat units were chosen for screening, among which 102 primers produced clear bands with suitable fragment lengths (<500 bp) during the preliminary test with four individuals of *P. ovalifolia*.

These 102 loci were further tested with eight individuals of *P. ovalifolia*. PCR reactions were performed with three primers: a sequence-specific forward

Table 2. Results of initial primer screening in populations of *Primula* species.[a]

	P. ovalifolia									*P. tardiflora*			*P. epilosa*		
	OVA_EMS (n = 24)			OVA_HZG (n = 24)			OVA_BSH (n = 20)			TAR_EMS (n = 24)			EPI_PZ (n = 24)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
C11339	6	0.632	0.757	12	0.667	0.893	7	0.900	0.813*	3	0.136	0.210	1	0.000	0.000
C13959	4	0.313	0.619	3	0.250	0.507	3	0.056	0.517*	2	0.708	0.510	3	0.375	0.423
C14309	5	0.333	0.660	6	0.375	0.532	2	0.529	0.389	2	0.053	0.149	4	0.125	0.363*
C14710	6	0.833	0.779	7	0.708	0.764	8	0.857	0.847	3	0.813	0.679	3	0.565	0.569
C15228	5	0.579	0.731	5	0.300	0.278	3	0.300	0.296	—	—	—	1	0.000	0.000
C21614	3	0.231	0.495*	3	0.261	0.241	7	0.455	0.802	1	0.000	0.000	1	0.000	0.000
C23409	1	0.000	0.000	2	0.050	0.050	4	0.500	0.668	2	0.200	0.287	1	0.000	0.000
C23644	5	0.286	0.741*	12	0.739	0.887	4	0.842	0.698	3	0.667	0.519	2	0.083	0.082
C23746	1	0.000	0.000	2	0.125	0.120	2	0.000	0.278*	2	0.125	0.120	1	0.000	0.000
C24233	2	0.000	0.359	9	0.500	0.825*	3	0.049	0.050	2	0.375	0.311	2	0.261	0.232
C24268	10	0.500	0.870*	6	0.522	0.739	5	0.368	0.639*	6	0.429	0.606	1	0.000	0.000
C24676	3	0.087	0.405*	4	0.177	0.668*	4	0.938	0.666*	2	0.208	0.191	1	0.000	0.000
C40984	5	0.217	0.648*	2	0.895	0.508*	7	0.579	0.812*	6	0.333	0.559*	4	0.458	0.415
C45417	11	0.435	0.825	19	0.417	0.915	4	0.400	0.415*	6	0.250	0.509*	5	0.417	0.395
C46430	6	0.600	0.717	5	0.579	0.576	8	0.850	0.825	3	0.059	0.269	4	0.563	0.538
C47095	1	0.000	0.000	1	0.000	0.000	—	—	—	1	0.000	0.000	1	0.000	0.000
C48258	2	0.050	0.142	4	0.750	0.645	4	0.632	0.683*	1	0.000	0.000	4	0.652	0.654
C48475	5	0.278	0.819*	6	0.565	0.503	—	—	—	2	0.042	0.042	4	0.542	0.657
C50127	7	0.381	0.630	—	—	—	3	0.000	0.460*	3	0.750	0.573	4	0.318	0.698
C51170	6	0.750	0.761	11	0.542	0.872*	2	0.000	0.142*	3	0.476	0.441	—	—	—
C53509	11	0.632	0.871	6	0.263	0.713*	5	0.600	0.654	4	0.188	0.688*	—	—	—
C53824	7	0.778	0.764	13	0.667	0.884	—	—	—	5	0.304	0.442*	2	0.000	0.089*
C53825	1	0.000	0.000	2	0.526	0.398	3	0.368	0.547	—	—	—	2	0.375	0.311
C53843	8	0.765	0.743	10	0.870	0.876	5	0.800	0.643	3	0.435	0.445	5	0.539	0.634
C53920	3	0.044	0.086*	2	0.117	0.156	1	0.000	0.000	3	0.435	0.530	2	0.042	0.042
C53962	5	0.273	0.459	4	0.368	0.647	1	0.000	0.000	3	0.167	0.519*	2	0.046	0.206*
C55683	2	0.000	0.502	9	0.478	0.857*	3	0.063	0.576*	6	0.350	0.782*	4	0.154	0.665*
C55722	2	0.235	0.371	4	0.364	0.444	4	0.200	0.595*	1	0.000	0.000	6	0.667	0.660
C57707	8	0.810	0.829	8	0.609	0.837	3	0.083	0.344*	2	0.143	0.143	3	0.208	0.196
C58140	10	0.500	0.808*	9	0.500	0.825*	2	0.050	0.049	3	0.792	0.543*	—	—	—
C58437	3	0.684	0.522	2	0.067	0.067	2	0.600	0.495	2	0.478	0.476	—	—	—
C59012	2	0.053	0.053	3	0.191	0.180	3	0.200	0.184	3	0.762	0.638	1	0.000	0.000
C59078	2	0.263	0.309	—	—	—	1	0.000	0.000	3	0.385	0.631	2	0.048	0.048
C59155	3	0.046	0.09*	4	0.235	0.713	4	0.200	0.499*	1	0.000	0.000	1	0.000	0.000
C59702	6	0.563	0.794	6	0.409	0.538	3	0.111	0.106	4	0.200	0.407	2	0.208	0.311
C63472	4	0.154	0.542*	4	0.188	0.667*	5	0.375	0.734*	2	0.300	0.262	2	0.208	0.191
C65324	4	0.250	0.673*	—	—	—	3	0.789	0.517	4	0.292	0.645*	5	0.478	0.728*
C66329	1	0.000	0.000	3	0.042	0.451	1	0.000	0.000	6	0.524	0.436	1	0.000	0.000
Mean[b]	5.2	0.389	0.587	6.1	0.421	0.582	4.0	0.400	0.510	3.3	0.367	0.437	3.2	0.306	0.379

Note: — = unsuccessful amplification; A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.

[a]Locality and voucher information are provided in Appendix 1.

[b]Monomorphic loci are excluded.

* Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

primer with an M13(–21) tail at its 5′ end, a sequence-specific reverse primer, and the universal fluorescent-labeled M13(–21) primer (FAM, ROX, HEX, or TAMRA; Invitrogen, Guangzhou, Guangdong, China) (Schuelke, 2000). Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle, 1991). The amplified 10-μL mixture for SSRs included 5 μL of Master Mix (Generay Biotech, Guangzhou, China), 0.4 mM of each primer pair, 3.2 μL of deionized water, and 30–50 ng of genomic DNA. PCRs were run following a touchdown procedure with initial denaturation for 4 min at 94°C; followed by 10 cycles of 94°C for 35 s, 35 s at 60°C with an increment of –1°C per cycle, 45 s at 72°C; followed by 28 cycles of 94°C for 35 s, 35 s at 50°C, 45 s at 72°C; ending with an extra extension of 10 min at 72°C. PCR products were scanned by an ABI PRISM 3100 Genetic Analyzer using GeneScan 500 LIZ internal size standard (Invitrogen). Allele binning and calling were done using GeneMarker version 2.4.0 (SoftGenetics, State College, Pennsylvania, USA), and 38 primer pairs were selected for further polymorphism and transferability detection (Table 1). All of these SSR sequences have been deposited in GenBank (Table 1).

***Polymorphism and transferability assessment*—**To assess the polymorphism level of these 38 loci, we genotyped 20–24 individuals in each of five populations from three species (Appendix 1). DNA extraction, PCR amplification, and length assessment of PCR products were performed following the procedures described above. Linkage disequilibrium among loci per population and deviation from Hardy–Weinberg equilibrium were tested using FSTAT version 2.9.3 (Goudet, 2001). We used GenAlEx 6.5 (Peakall and Smouse, 2012) to calculate the number of observed alleles per locus (A), expected heterozygosity (H_e), and observed heterozygosity (H_o).

No significant linkage disequilibrium was detected among loci after Bonferroni correction at $\alpha = 0.05$ confidence level, and some loci showed significant deviations from Hardy–Weinberg equilibrium (Table 2). The 38 EST-SSRs displayed varied genetic diversity in three populations of *P. ovalifolia* (Table 2). A, H_o, and H_e for each locus ranged from one to 19, 0 to 0.938, and 0 to 0.915, respectively (Table 2). Excluding monomorphic loci, the polymorphic EST-SSR markers showed an average A of 5.2, 6.1, and 4.0; H_e of 0.587, 0.582, and 0.51; and H_o of 0.389, 0.421, and 0.40, in each population, respectively (Table 2). Out of the 38 SSR markers, 36 loci were successfully amplified in *P. tardiflora* and 31 loci showed polymorphism, with A ranging from two to six (Table 2). Similarly, 34 loci were successfully amplified in *P. epilosa* Craib, among which 23 loci showed polymorphism, with A ranging from two to six (Table 2). Overall, most of the EST-SSR markers developed for *P. ovalifolia* could be successfully cross-amplified, leading to a high transferability in the two congeneric species.

CONCLUSIONS

We developed and characterized 38 EST-SSR markers based on transcriptome sequencing of *P. ovalifolia*, a widely distributed species of *Primula* section *Petiolares*. These markers demonstrated high polymorphism in *P. ovalifolia*, with A ranging from one to 19, H_o from 0.000 to 0.938, and H_e from 0.000 to 0.915. Most of the markers could be successfully cross-amplified in congeneric species. These SSR makers are found to be useful tools for investigation of genetic structure and interspecific gene flow in this section.

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Appendix 1. Locality and voucher information for populations of *Primula ovalifolia*, *P. tardiflora*, and *P. epilosa* used in this study. Voucher specimens are deposited at the herbarium of South China Botanical Garden (IBSC), Guangzhou, Guangdong, China.

Species	Population code	Voucher no.	Location	Geographic coordinates	Altitude (m)	*n*
Primula ovalifolia Franch.	OVA_EMS	YS214	E'mei, China	29°32′55″N, 103°21′31″E	1702	24
	OVA_HZG	YS524	E'bian, China	29°02′42″N, 103°00′29″E	1793	24
	OVA_BSH	YS436	Pengzhou, China	31°14′09″N, 103°50′20″E	1900	20
Primula tardiflora (C. M. Hu) C. M. Hu	TAR_EMS	YS440	E'mei, China	29°32′49″N, 103°20′24″E	2448	24
Primula epilosa Craib	EPI_PZ	YS504	Pengzhou, China	31°11′32″N, 103°54′45″E	1300	24

Note: *n* = number of individuals sampled.