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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF EST-SSR MARKERS IN *OSTRYOPSIS* (BETULACEAE)[1]

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- *Premise of the study:* A set of expressed sequence tag (EST) microsatellite markers were developed and characterized using next-generation sequencing technology for the Chinese genus *Ostryopsis* (Betulaceae).
- *Methods and Results:* A total of 38 high-quality simple sequence repeat (SSR) primers were identified, of which 15 could be successfully amplified. Subsequently, we selected 80 individuals to represent the three species of the genus to evaluate the efficacy of these markers for examining genetic diversity of each species in the future. We found that the number of alleles per locus ranged from one to nine, with an average of 3.8. The expected heterozygosity and observed heterozygosity per locus varied from 0 to 0.829 and from 0 to 1, respectively, with their respective mean values as 0.483 and 0.416.
- *Conclusions:* These EST-SSR markers will be useful for evaluating the range-wide genetic diversity of each species and examining genetic divergence and gene flow between the three species.

Key words: Betulaceae; EST-SSR marker; next-generation sequencing technology; ortholog genes; *Ostryopsis*.

Ostryopsis Decne. (Betulaceae) is a small genus endemic to China, consisting of only three recognized species: *O. davidiana* Decne., *O. nobilis* Balf. f. & W. W. Sm., and *O. intermedia* B. Tian & J. Q. Liu (Tian et al., 2010). *Ostryopsis davidiana* is mainly distributed in northern China, while *O. nobilis* and *O. intermedia* are limited to southwestern China. The northern vs. southwestern distributions of the three species in this genus suggest that *Ostryopsis* is a good model system to explore species divergence of plants in response to both habitat and temperature change across China. Until now, a total of 10 simple sequence repeat (SSR) markers have been developed for *O. davidiana* (Qiu et al., 2009). However, some of these markers could not be successfully amplified with the other two species. Furthermore, the development of expressed sequence tag (EST)–SSR markers is particularly attractive because they represent coding regions of the genome. EST-derived SSRs developed from one species can be easily amplified and used in closely related species (Wünsch, 2009). Here, we report 15 new polymorphic EST-SSR loci for *Ostryopsis*, which will facilitate the characterization of genetic diversity of each species in the genus and examination of gene flow and genetic divergence between the three species.

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METHODS AND RESULTS

In this study, 20 or 30 individuals per species were collected for *O. davidiana* (Yijun, Shaanxi Province; Diebu, Gansu Province), *O. nobilis* (Tangdui, Yunnan Province; Jirenhe, Yunnan Province), and *O. intermedia* (Judian, Yunnan Province; Deqin, Yunnan Province) from their native distributions in China, and the voucher specimens were deposited in the herbarium of Lanzhou University (LZU), Lanzhou, China (Appendix 1). Genomic DNA was extracted using a cetyltrimethylammonium bromide (CTAB) procedure from leaves of each individual per species (Ghangal et al., 2009). The total RNA of *O. davidiana* and *O. nobilis* was also extracted using a CTAB procedure (Ghangal et al., 2009) and then the complementary DNA (cDNA) libraries were constructed and sequenced, respectively.

Approximately 4 μg of RNAs were purified using poly(dT)-conjugated beads (Life Technologies, Carlsbad, California, USA) to clear poly(A)-tagged mRNA. These RNAs were then broken into ~200-bp fragments under divalent cations at 75°C. We synthesized the first strand of cDNA by the reverse transcriptase with random hexmer primers, and the second strand of cDNA by RNase H (Invitrogen, Ghent, Belgium) and DNA polymerase I (New England BioLabs, Ipswich, Massachusetts, USA). We sequenced the transcriptome on an Illumina (Solexa) Genome Analyzer II (Illumina Inc., San Diego, California, USA). After removing adapter sequences, we filtered and assembled two data sets of raw reads from *O. davidiana* and *O. nobilis* as described in Qiu et al. (2011). To identify orthologous genes between two species, their reads were mapped back against the assembled unigenes using Bowtie 2 (Langmead and Salzberg, 2012). We recalled single-nucleotide polymorphisms (SNPs) and indel calling with SAMtools (Li et al., 2009). We identified EST-SSRs using MISA (http://pgrc.ipk-gatersleben.de/misa) based on the orthologous unigene sequences. Because the de novo assembly introduced multiple indels, we therefore removed the shared ones by two orthologous unigenes. We used only the indels with end-to-end alignment as candidate SSR regions for further primer design. The final analyses resulted in a total of 72 and 69 SSR indels according to the above reference unigenes from two species, respectively. We then designed the paired primers with Primer3 software (Rozen and Skaletsky, 2000). Primers were not retained when they targeted to the SNP region of the unigenes. In this way, we obtained a total of 38 primer pairs from both species.

Table 1. Characteristics of 15 polymorphic EST-SSRs developed in *Ostryopsis*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
Osd02	F: TCCCACTCTCCAGTGCATTAGTA	$(TCG)_6$	273	59	JZ078196
	R: GACAAAATCCCCGACAAGGA				
Osd09	F: GCTCCACCCTGTTCGTCTG	$(CCA)_5$	191	58	JZ078197
	R: AGCTGCTGGTTTATTATTGTTCC				
Osd10	F: AGCACCCTCTTGGTCAGTT	$(CAA)_6$	259	56	JZ078198
	R: TTGCATTTCTTGGCATTCTC				
Osd13	F: CTTCACAGTCACCCTCTTCAGC	$(CCT)_5$	354	59	JZ078199
	R: GGAGGGACTCGTGGGTTAGA				
Osd14	F: AAGAGGTGTCCCATTTGCT	$(AAC)_9$	288	55	JZ078200
	R: CATTGAAGAGCTTCGTCCAT				
Osd15	F: TGACTCGGAAGAACTGGCTG	$(GAA)_{11}$	262	58	JZ078201
	R: TTCGGCCTCATCATTCACAT				
Osd16	F: AAAAGAACCCAAGAACCG	$(AGC)_6$	371	53	JZ078202
	R: CATAGTAGTCGTAACTCCTCCC				
Osd17	F: GGAGACCCTTGTGGTTGCT	$(CTT)_6$	298	57	JZ078203
	R: TTAATGGGAGCGCTTTTCA				
Osd19	F: CCAATGGCTTGTCGTCCTG	$(TCT)_7$	204	58	JZ078204
	R: GGAGATGAGGTCAGTTTCGAAG				
Osd20	F: AGGGAAGAAGCAAACTGCTATT	$(GAA)_5$	283	57	JZ078205
	R: GCCTATTCGCTATAATACCTTGA				
Osd23	F: GGGGACCTGTACCAGACCATA	$(GAG)_6$	270	57	JZ078206
	R: TCTTCTTGCACTCCTCTACGG				
Osd27	F: GCGCACTCTAGCATCACTCC	$(TCT)_8$	231	58	JZ078207
	R: GGGTCGGTTGCTCCAGTT				
Osd30	F: TCTTCATCGTATAGCAGCCT	$(CAT)_{10}$	264	53	JZ078208
	R: CTCGCAGTTGACAACCAA				
Osd32	F: GATGGCAGTTGTGAGGACA	$(AGC)_6$	214	52	JZ078209
	R: TGTCGGCTTTAGGTTTAGAT				
Osd33	F: TGAATCCCAGAATTTCCAACAT	$(CAG)_9$	158	58	JZ078210
	R: GCTCCAACTTTACAGGTCCAA				

Note: T_a = optimal annealing temperature.

To evaluate polymorphisms of these primer pairs and their possible amplifications in the other species, we selected five individuals from each of the three species. PCR reactions were carried out in a 20-μL solution containing 20 ng of DNA template, 5 pmol of each primer, 100 μM each of dNTPs, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.2 U of *Taq* polymerase (Intron Biotechnology, Seongnam, South Korea). After a denaturing step of 5 min at 95°C, a touchdown amplification program was performed. This profile included a denaturing step of 45 s at 95°C and an extension step of 30 s at 72°C. The initial annealing step was 45 s at 60°C for one cycle, and subsequently the temperature was decreased by 0.7°C for every cycle to a final temperature of 53°C. This annealing temperature was employed for the last 30 cycles of the amplification, followed by one cycle of 72°C for 10 min. PCR products were assayed on 1.5% (w/v) agarose gels to test the utility of the primers. Finally, a total of 15 primer pairs were successfully amplified across all three species and all of them displayed clear polymorphisms (Table 1).

Subsequently, we selected a total of 80 individuals from the three species to evaluate the potential value of these markers for estimating genetic diversity of each species. Fluorescence-based SSR genotyping was performed using a

Table 2. Genetic diversity statistics for each sampled population of the three *Ostryopsis* species based on 15 pairs of EST-SSR primers.[a]

	O. davidiana						*O. nobilis*						*O. intermedia*					
	Yijun (N = 10)			Diebu (N = 10)			Tangdui (N = 15)			Jirenhe (N = 15)			Judian (N = 15)			Deqin (N = 15)		
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	A	H_o	H_e
Osd02	7	0.500	0.700	9	0.800	0.825	9	0.533	0.811	5	0.429	0.620	7	0.533	0.760	6	0.333	0.671
Osd09	5	0.500	0.735	4	0.300	0.610	4	0.200	0.606	5	0.357	0.620	2	0.400	0.320	5	0.133	0.647
Osd10	2	0.400	0.320	2	0.200	0.500	3	0.467	0.527	3	0.500	0.457	5	0.667	0.691	6	0.067	0.447
Osd13	2	1.000	0.500	3	0.500	0.580	5	0.267	0.558	7	0.357	0.518	2	0.000	0.444	1	0.000	0.000
Osd14	1	0.000	0.000	2	0.100	0.095	4	0.200	0.293	5	0.286	0.505	5	0.733	0.709	3	0.333	0.287
Osd15	2	1.000	0.500	4	0.300	0.595	3	0.333	0.371	3	0.429	0.513	3	0.200	0.384	4	0.333	0.429
Osd16	1	0.000	0.000	2	0.200	0.320	3	0.267	0.371	3	0.214	0.401	1	0.000	0.000	3	0.200	0.184
Osd17	3	0.500	0.535	3	0.600	0.515	5	0.667	0.656	3	0.571	0.574	4	0.267	0.589	2	0.067	0.180
Osd19	3	0.900	0.635	3	0.600	0.540	3	0.467	0.451	3	0.357	0.304	4	0.400	0.522	3	0.133	0.127
Osd20	2	0.100	0.095	2	0.100	0.095	2	0.267	0.320	4	0.429	0.610	3	0.333	0.331	4	0.200	0.296
Osd23	3	0.300	0.515	3	0.700	0.645	4	0.600	0.544	5	0.571	0.643	2	0.000	0.124	2	0.000	0.124
Osd27	2	0.100	0.095	4	0.500	0.665	4	0.400	0.389	5	0.643	0.676	2	0.067	0.064	3	0.067	0.127
Osd30	1	0.000	0.000	4	0.700	0.630	6	0.533	0.584	4	0.571	0.497	1	0.000	0.000	3	0.200	0.418
Osd32	1	0.000	0.000	2	0.100	0.095	1	0.000	0.000	3	0.071	0.304	2	0.000	0.124	2	0.000	0.124
Osd33	3	0.500	0.395	4	0.700	0.615	7	0.933	0.804	7	0.786	0.829	6	0.467	0.722	7	0.733	0.738

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.

[a]Locality and voucher information for the populations is provided in Appendix 1.

modified method of Hayden et al. (2008). Briefly, the forward primers of the 15 EST-SSRs were labeled with 6-FAM, VIC, or NED fluorescent tags (Applied Biosystems, Foster City, California, USA). The PCR reactions were carried out separately for each microsatellite as described above. Amplification products for which size and color did not overlap were pooled together for simultaneous detection of the amplified alleles.

To characterize each EST-SSR marker, we calculated three genetic diversity statistics using POPGENE version 1.31 (Yeh et al., 1999): number of alleles per locus, observed heterozygosity, and expected heterozygosity (Table 2). We found that the number of alleles ranged from one to nine, with an average of 3.8 alleles per locus. The expected heterozygosity and observed heterozygosity ranged from 0 to 0.829 and 0 to 1, respectively, with their respective mean values as 0.483 and 0.416 (Table 2).

CONCLUSIONS

We developed 15 polymorphic EST-SSR markers for *Ostryopsis* from two cDNA libraries. The polymorphisms of these markers were further evaluated with 80 individuals representing the three species. These newly developed EST-SSRs have a high degree of universality between species and will be useful for studying the genetic diversity of each species and genetic divergence between the three species.

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Appendix 1. Locality information for the sampled populations of the *Ostryopsis* species used in this study. All voucher specimens are deposited at the herbarium of Lanzhou University (LZU), Lanzhou, China.

Species	*N*	Population	Geographic coordinates	Altitude (m)	Voucher
O. davidiana	10	Yijun, Shaanxi	35°28′N, 109°09′E	1113	YJ01–YJ09, YJ11
	10	Diebu, Gansu	34°07′N, 103°10′E	2566	DB01DB10
O. nobilis	15	Tangdui, Yunnan	27°59′N, 99°34′E	2900	TD01–TD12, TD14, TD19, TD20
	15	Jirenhe, Yunnan	27°48′N, 99°28′E	1950	JR01–JR13, JR16, JR17
O. intermedia	15	Judian, Yunnan	27°17′N, 99°39′E	1950	JD01–JD15
	15	Deqin, Yunnan	28°22′N, 98°54′E	2870	DQ01–DQ15