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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE LOCI FOR SMOOTH CORDGRASS, *SPARTINA ALTERNIFLORA* (POACEAE)[1]

WUXIA GUO[2], YELIN HUANG[2,3], ZIWEN HE[2], YUBIN YAN[2], RENCHAO ZHOU[2], AND SUHUA SHI[2,3]

[2]Guangdong Key Laboratory of Plant Resources and State Key Laboratory of Biocontrol, Sun Yat-Sen University,
Guangzhou 510275, People's Republic of China

- *Premise of the study: Spartina alterniflora* is one of the nine most notoriously invasive plants in China. Microsatellite markers were developed for this species to investigate its invasiveness and genetic diversity.
- *Methods and Results:* Fifteen polymorphic and seven monomorphic simple sequence repeat (SSR) markers derived from expressed sequence tags (ESTs) were identified and screened in 60 samples of *S. alterniflora*. The number of alleles per polymorphic locus ranged from two to eight, with an average of 3.8 alleles per polymorphic locus. The expected heterozygosity and observed heterozygosity based on seven disomic loci ranged from 0.27 to 0.46 and 0.21 to 0.51, respectively. The average Shannon index ranged from 0.26 to 0.94 in eight nondisomic loci.
- *Conclusions:* The SSR markers described here may be useful for further investigation of population genetics and invasion dynamics of *S. alterniflora*.

Key words: invasive species; microsatellite; Poaceae; *Spartina alterniflora*; transcriptome.

Spartina alterniflora Loisel. (Chloridoideae, Poaceae) is a perennial grass native to the Atlantic and Gulf coasts of North America, and has been used in coastal restoration programs in many countries (Daehler and Strong, 1996). However, *S. alterniflora* is highly invasive in many parts of the world where it is introduced. In China, the species has been listed as one of the nine most notoriously invasive plants (Zhi et al., 2007). Recently, we have sequenced the transcriptome of *S. alterniflora* using the next-generation sequencing platform Illumina Genome Analyzer II to understand its invasion in China (Guo et al., unpublished data). The transcriptome sequences contain abundant simple sequence repeat (SSR) markers, which should be very useful in population genetic studies. Here, for the first time, we identified several thousand expressed sequence tag (EST)–derived simple sequence repeat (ESSR) markers from the RNA-seq data of *S. alterniflora*. Compared to genomic SSR markers, ESSRs are easier and less expensive to develop, as well as more transferable across taxonomic boundaries (Ellis and Burke, 2007).

METHODS AND RESULTS

Transcriptome sequencing of *S. alterniflora* was conducted using the Illumina Genome Analyzer II system. In total, 14.55 million 90-nucleotide paired-end reads were obtained and assembled into 69 899 contigs with an average length of 503 nucleotides by using two short-read assemblers—Trinity and CAP3 (Huang and Madan, 1999; Grabherr et al., 2011). These unique sequences (i.e., ESTs) were further screened for the presence of microsatellites using MISA (http://pgrc.ipk-gatersleben.de/misa). A total of 3052 potential ESSRs were identified. The microsatellites were defined as di-, tri-, tetra-, penta-, and hexanucleotide SSRs with a minimum of four contiguous repeat units. The most abundant repeat type was trinucleotide (47.8%, 1460), followed by dinucleotide (38.7%, 1182), tetranucleotide (8.1%, 247), pentanucleotide (3.7%, 114), and hexanucleotide (1.6%, 49) repeat units. Primer3 software (Rozen and Skaletsky, 2000) was used to design 50 primer pairs with an expected product size ranging from 100 to 300 bp. Sixty individuals of *S. alterniflora* representing five populations in China (Appendix 1) were used to evaluate the polymorphisms of the microsatellite loci.

Genomic DNA from each individual was extracted from silica gel–dried leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle, 1991). PCR amplifications were performed in a final volume of 20 μL, containing 2 μL 10× PCR buffer, 2 μL of 2 mM each dNTPs, 1.2 μL 25 mM $MgCl_2$, 1 μL 10 pM forward primer, 1 μL 10 pM reverse primer, 2 U *Taq* DNA polymerase (Sangon, Shanghai, China), and 10 ng of genomic DNA. The PCR reactions were conducted with the following conditions in a thermocycler (Bio-Rad Laboratories, Hercules, California, USA): initial denaturation at 94°C for 5 min, followed by 34 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, with a final extension cycle at 72°C for 10 min. PCR products were electrophoresed on 8% polyacrylamide denaturing gel and visualized by silver staining. The band size was estimated by comparison with a 20-bp DNA ladder (Fermentas, Vilnius, Lithuania). Twenty-six (52%) of the primer pairs failed to amplify products, two (4.0%) generated complex band patterns that were difficult to genotype, seven (14%) were monomorphic, and 15 (30%) displayed clear polymorphisms (Table 1). To determine the function of polymorphic SSR-associated unigenes, ESSRs were evaluated for connections with genes of known functions; those 22 sequences (including seven monomorphic and 15 polymorphic loci, respectively) were blasted against the GenBank nonredundant database using BLASTX (Altschul et al., 1997) with an *E*-value of 10^{-10}.

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[3]Authors for correspondence: lsshyl@mail.sysu.edu.cn, lssssh@mail.sysu.edu.cn

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Table 1. Characteristics of 15 polymorphic and seven monomorphic ESSRs developed in *Spartina alterniflora*.

ESSR locus	Primer sequences (5′–3′)	Repeat motif	Size (bp)	T_a (°C)	Location	*A*	PIC	GenBank accession no.	Putative function
SaESP01	F: TATCCCCAGACACCCACAGT R: CATTCTCTGGGTCTGCAACA	$(\text{TC})_{10}$	268	60	3′ UTR	6	0.70	JU981477	grancalcin
SaESP02	F: GAAGAGACCGTTCAGGTTGG R: CCCGGCGACTAACTCTCAT	$(\text{TGA})_{6}$	214	60	5′ UTR	3	0.42	JU981475	cytochrome c
SaESP03	F: CGTGCCGACCAAGTAAAGTT R: ACCGACAGCGTGTTCCTC	$(\text{CTC})_{6}$	256	60	ORF	4	0.53	JU981468	AIR12 precursor
SaESP04	F: AGCGAAGGGAAGATCTCGAC R: GGAGGCCTTTTTAATAGCCG	$(\text{TC})_{9}$	155	60	5′ UTR	3	—	JW662119	acyl-desaturase
SaESP05	F: CTTGCAGCGGCTATCCTTAC R: ACGAGACCTTCGCTTTTGAA	$(\text{GCG})_{6}$	245	60	ORF	1	—	JW662116	DNA binding protein
SaESP06	F: AACCTGAAGTGCGTAAGCGT R: CTTCCCCCAACACTTCGATA	$(\text{AT})_{9}$	245	60	ORF	5	0.46	JU981466	large secreted protein, putative
SaESP07	F: CCCAGCACCTCTGATTTGAT R: ATCCACCTCTACCATGCGTC	$(\text{TTC})_{6}$	132	60	3′ UTR	3	0.58	JU981470	bZIP transcription factor
SaESP08	F: TGCTAAGATTGGAGCAGGGT R: GCTTACATTACCGCCAAAGC	$(\text{TAC})_{6}$	266	60	3′ UTR	1	—	JW662120	serine acetyltransferase 3
SaESP09	F: GACTTTACCGCGAAGAGCC R: AGGAAGCCCAAAACACACAC	$(\text{TTC})_{7}$	192	60	5′ UTR	3	0.43	JU981464	mitogen-activated protein kinase kinase kinase 2–like
SaESP10	F: CGAAAGGTTAAGCCAATCCA R: ACGAAAGTTGCGGGTACAAC	$(\text{CT})_{9}$	211	60	5′ UTR	8	0.82	JU981473	hypothetical protein
SaESP11	F: ACAAACTCGGCCTCCTCTTT R: ATAAGTACCCGCCCTTGTCC	$(\text{CT})_{8}$	171	60	5′ UTR	3	—	JW662118	CBL-interacting protein kinase 1
SaESP12	F: GGAGCAACAAAGACAGAGCC R: CGACTCGTGGTTGGTGAAG	$(\text{CAC})_{6}$	215	60	ORF	1	—	JW662115	hypothetical protein
SaESP13	F: CGATCCACTGGTACTGGGAC R: GGCTGCCATTATCGATTGTT	$(\text{TGCC})_{5}$	196	60	3′ UTR	2	0.37	JU981471	ribokinase
SaESP14	F: TCGTCACGTTGACTTGTGGT R: TGCTGCTTCCCTTTGATCTT	$(\text{GA})_{8}$	249	60	3′ UTR	4	0.62	JU981469	hypothetical protein
SaESP17	F: TGCTTCATGCGTTGATTAGC R: TGAGATGAAGCCTGTGGAGA	$(\text{CA})_{9}$	150	60	3′ UTR	5	0.58	JU981465	glycosyltransferase
SaESP18	F: GCCACAACAAGAGTTGGGTT R: GCTGGTCCAAAGAAATCAGA	$(\text{AAT})_{6}$	171	60	3′ UTR	4	0.45	JU981474	transcription factor
SaESP19	F: GCGCCATTACCACAGAGG R: ATACGATCTGCCCTGTTTCG	$(\text{CT})_{7}$	165	60	5′ UTR	5	0.63	JU981467	choline/ethanolamine kinase
SaESP20	F: TGTAGCTGTTAGCATTGGCG R: AGGACCAGCAGAGGACAGAG	$(\text{AG})_{10}$	170	60	3′ UTR	3	0.54	JU981476	transcription factor
SaESP21	F: ATACCGCAACGAAAGCAAAG R: CTACTGCACCGACAAGTGGA	$(\text{TTC})_{6}$	205	60	3′ UTR	1	—	JW662114	xyloglucan xyloglucosyl transferase
SaESP22	F: CCAGCGTCTCCTCTACAACC R: CAGGAAACAAACGGACATGA	$(\text{TTC})_{6}$	277	60	3′ UTR	1	—	JW662117	hypothetical protein
SaESP23	F: ATCCGTGCGTCTCTGTCTCT R: CCACCATGATGCATAACAGC	$(\text{GTCA})_{5}$	255	60	3′ UTR	2	0.24	JU981478	cyclophilin
SaESP24	F: ACCCTGCTAGATATGCACGC R: TTGTCGAAGGAGTAGGAGGC	$(\text{CCT})_{6}$	263	60	ORF	2	0.37	JU981472	hypothetical protein

Note: *A* = number of alleles detected; *PIC* = polymorphism information content; T_a = optimal annealing temperature.

All of the sequences showed significant similarities to known genes (Table 1). The allele number (*A*) and polymorphism information content (PIC) were calculated for each of the loci using a Web-based calculator (http://www.genomics.liv.ac.uk/animal/pic.html). The number of alleles per polymorphic locus ranged from two to eight, with an average of 3.80 alleles per polymorphic locus. The number of alleles per individual ranged from one to six, which was consistent with the hexaploidy of *S. alterniflora*. Seven (46.67%) of 15 polymorphic loci showed the disomic pattern with a maximum of two alleles per individual. These 15 polymorphic microsatellite loci are further characterized in Tables 2 and 3. The expected heterozygosity (H_e) and observed heterozygosity (H_o) for each disomic locus were calculated using POPGENE (version 1.32; Yeh and Boyle, 1997). The Shannon index for each nondisomic locus was calculated using POLYSAT (version 1.2-1; Clark and Jasieniuk, 2011). The H_e for those loci was also calculated using the Web-based calculator (http://www.genomics.liv.ac.uk/animal/pic.html), assuming Hardy–Weinberg equilibrium (HWE). In general, *S. alterniflora* showed a moderate level of genetic polymorphisms in China. The average H_o ranged from 0.21 to 0.51, and the H_e ranged from 0.27 to 0.46 based on seven disomic loci. The average Shannon index (*I*) ranged from 0.26 to 0.94 in eight nondisomic loci. Five disomic loci (including SaESP06 in all five populations; SaESP09 in the Fujian, Shanghai, and Jiangsu populations; SaESP13 in the Taiwan and Hong Kong populations; SaESP18 in the Taiwan population; and SaESP19 in the Fujian population) deviated significantly from HWE ($P < 0.05$). It is likely that the observed departures from HWE are due to the presence of null alleles or a result of mixed reproductive modes (selfing and outcrossing) in *S. alterniflora*. The monomorphism at some loci observed for some populations may also reflect nonequilibrium population dynamics resulting from its recent introduction and spread in China (Tables 2 and 3).

CONCLUSIONS

In this study, we report the development and characterization of a set of ESSRs, which was derived from a large-scale transcriptome sequencing of *S. alterniflora* using the Illumina Genome Analyzer II system. Fifteen polymorphic and seven

Table 2. Characterization of seven disomic polymorphic microsatellite loci in *Spartina alterniflora*.

	Fujian (N = 12)			Taiwan (N = 12)			Shanghai (N = 12)			Jiangsu (N = 12)			Hong Kong (N = 12)		
Disomic 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
SaESP06	4	1.0000^{+}	0.7536	2	1.0000^{+}	0.5217	4	1.0000^{+}	0.6014	2	1.0000^{+}	0.5217	2	1.0000^{+}	0.5217
SaESP09	2	0.1667*	0.5217	2	0.5000	0.3913	2	0.0000*	0.2899	3	0.1667*	0.4203	1	0.0000	0.0000
SaESP13	2	0.7500	0.5181	2	0.0000*	0.4638	2	0.3333	0.5072	2	0.6667	0.5072	1	0.1667*	0.4638
SaESP18	3	0.3333	0.3007	2	0.0000*	0.5072	2	0.0833	0.0833	2	0.2500	0.2283	1	0.4167	0.5181
SaESP19	3	0.0833*	0.3587	1	0.0000	0.0000	4	0.5833	0.4855	4	0.8333	0.7065	1	0.1667	0.1594
SaESP23	2	0.1667	0.1594	1	0.0000	0.0000	2	0.5833	0.4312	2	0.2500	0.4312	1	0.0000	0.0000
SaESP24	2	0.5000	0.3913	1	0.0000	0.0000	2	0.3636	0.4156	2	0.4167	0.4312	1	0.7273	0.5195

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

* Heterozygote deficiency ($P < 0.05$).

$^{+}$Heterozygote excess ($P < 0.05$).

Table 3. Characterization of eight nondisomic polymorphic microsatellite loci in *Spartina alterniflora*.

	Fujian (N = 12)			Taiwan (N = 12)			Shanghai (N = 12)			Jiangsu (N = 12)			Hong Kong (N = 12)		
Nondisomic locus	A	I	H_e	A	I	H_e	A	I	H_e	A	I	H_e	A	I	H_e
SaESP01	6	0.5868	0.7140	3	0.0000	0.0000	6	0.5623	0.7733	6	0.5661	0.7285	3	0.0000	0.0000
SaESP02	3	0.8676	0.5694	2	0.0000	0.0000	2	0.5623	0.0000	3	0.9596	0.5952	2	0.0000	0.0000
SaESP03	3	0.8240	0.6145	4	1.1187	0.6076	4	0.8370	0.5826	3	0.5661	0.4545	3	0.8240	0.5947
SaESP07	3	0.2868	0.6661	3	0.6365	0.6531	3	0.6931	0.6250	3	0.6792	0.6420	3	0.6792	0.6420
SaESP10	6	0.8676	0.6304	3	0.0000	0.0000	8	1.4735	0.8247	7	0.8877	0.8328	6	0.5623	0.3822
SaESP14	3	0.5623	0.0988	2	0.0000	0.0000	4	1.1988	0.6973	4	1.5171	0.6811	4	0.8676	0.6990
SaESP17	3	1.0114	0.0000	2	0.0000	0.0000	5	1.3144	0.6961	5	1.4241	0.7267	2	0.0000	0.0000
SaESP20	3	1.1269	0.6454	3	0.2868	0.5406	3	0.8877	0.5261	2	0.4506	0.0000	2	0.0000	0.0000

Note: A = number of alleles; H_e = expected heterozygosity; I = Shannon index.

monomorphic microsatellite markers were identified. Seven of those polymorphic loci display disomic inheritance. These newly developed ESSRs should be valuable for population genetic studies of this invasive species, and they can be used as new tools to trace its invasion history in China.

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Appendix 1. Voucher information for five populations of *S. alterniflora* used in this study. All vouchers are deposited at the Herbarium of National Sun Yat-Sen University.

Voucher no.	Collection locality	Geographic coordinates
Shi 091105	Fuzhou, Fujian, China	26°01′04″N, 119°37′19″E
Xia 110401	Mipu, Hong Kong, China	22°29′45″N, 114°02′47″E
Xia 110411	Nantong, Jiangsu, China	32°33′96″N, 121°01′67″E
Xia 110415	Chongming, Shanghai, China	31°36′21″N, 121°49′85″E
Shi 101015	Taibei, Taiwan, China	25°07′34″N, 121°27′30″E