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Source: Applications in Plant Sciences, 1(4)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1200457

Applications in Plant Sciences 2013 1(4): 1200457

PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE MARKERS FOR *ALPINIA OXYPHYLLA* (ZINGIBERACEAE)[1]

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- *Premise of the study:* Microsatellite loci were isolated and characterized for population genetic studies of *Alpinia oxyphylla* (Zingiberaceae), a perennial rhizomatous herbaceous plant often used medicinally in China.
- *Methods and Results:* A total of 85 loci were identified using a magnetic bead enrichment method, of which 23 were polymorphic. The level of polymorphism was characterized in 32 individuals from two populations; the number of alleles per locus ranged from 1 to 13; and observed heterozygosity and expected heterozygosity varied from 0 to 1 (mean: 0.6441) and 0 to 0.887 (mean: 0.6241), respectively.
- *Conclusions:* The polymorphic microsatellite markers generated from this study will be useful for genetic diversity and structure analysis of *A. oxyphylla*.

Key words: *Alpinia oxyphylla*; genetic diversity; microsatellite; Zingiberaceae.

Alpinia oxyphylla Miq. belongs to the genus *Alpinia* Roxb. (Zingiberaceae), which comprises 250 species and is widely distributed in tropical and subtropical areas. Approximately 51 species are found in southern China (Wu and Larsen, 2000). *Alpinia oxyphylla* is endemic to southwestern China, and its fruits are used in traditional Chinese medicine for the treatment of intestinal disorders, urosis, and diuresis. *Alpinia oxyphylla* is also coded in the Chinese Pharmacopeia as an aromatic stomachic. Evidence suggests that the extract from the fruits of *A. oxyphylla* has significant neuroprotective activity (Yu et al., 2003; Wong et al., 2004).

A first step to better utilizing this medicinal plant is to understand more about the genetic diversity and population structure of the species. The limited research on characterization at the DNA level has hindered the improvement of *A. oxyphylla*, and little DNA variation has been detected by inter-simple sequence repeat (ISSR) markers (Wang et al., 2012). Here we present a set of polymorphic microsatellite markers that can provide an important tool for understanding genetic diversity and genetic structure of *A. oxyphylla*. Moreover, such information can serve as a guide to help characterize the chemical composition of this medicinal plant.

[1]Manuscript received 29 August 2012; revision accepted 3 October 2012.

The authors thank Dr. Zheng Feng Wang (South China Botanical Garden, Chinese Academy of Sciences) for his help in the experiments, and Dr. Kyle Dexter and Dr. Markus Ruhsam (Royal Botanic Garden Edinburgh) for their help in revising the manuscript. This work was supported by the Knowledge Innovation Program of the Chinese Academy of Sciences (grant KSCX2-YW-Z-0919), the National Natural Science Foundation of China (grants 30900089, 31200246, 31200176, 30870173), and the South China Botanical Garden–Shanghai Institute of Plant Physiology and Ecology Joint Fund.

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doi:10.3732/apps.1200457

METHODS AND RESULTS

Genomic DNA was extracted from dried leaves of *A. oxyphylla* individuals using the cetyltrimethylammonium bromide (CTAB) method (Doyle, 1990), with slight modifications for using sufficient β-mercaptoethanol as an antioxidant followed by purification using phenol-chloroform. Microsatellites were developed following the methods of Glenn and Schable (2005). Approximately 5 μg of DNA were digested with the restriction enzyme *Mse*I (New England BioLabs, Beverly, Massachusetts, USA). The restricted fragments were ligated with *Mse*I adapters (5′-TACTCAGGACTCAT-3′ and 5′-GACGATGAGTCCTGAG-3′) with T4 ligase (New England BioLabs) overnight at 16°C and then amplified using the *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′). PCR products were hybridized to a 5′ biotin-labeled oligonucleotide probe: $(GA)_{15}$, $(AC)_{15}$. Magnetic beads coated with streptavidin (Promega Corporation, Madison, Wisconsin, USA) were used to capture GA and AC repeats. The enriched fragments were amplified by PCR using *Mse*I-N as the primer, ligated into plasmids, cloned into the plasmid vector pGEM-T Easy Vector (TaKaRa Biotechnology Co., Dalian, Liaoning, China), and then transformed into *Escherichia coli* DH5α competent cells (TaKaRa Biotechnology Co.). The recombinant positive clones were identified by colony PCR using the universal M13R or M13F as primers (TaKaRa Biotechnology Co.). Approximately 200 positive colonies were sequenced (Beijing Genomics Institute, Beijing, China). Sequencing output showed that 85 sequences contained perfect microsatellites with sufficient flanking regions, and these were selected to design primers with the software Primer3 (Rozen and Skaletsky, 2000). Eight individuals were selected for assessment of polymorphisms and the optimal annealing temperature of microsatellite loci. PCR amplification was performed in 10-μL reaction mixtures, consisting of approximately 5 ng of template DNA, 1.5 μL 10× buffer, 5 pmol of each primer, 0.2 mmol of each dNTP, and 1 U of *Taq* DNA polymerase (TaKaRa Biotechnology Co.). The PCR program included 4 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature (48–64°C), 1 min at 72°C, and a final extension at 72°C for 10 min. Amplified products were subjected to electrophoresis in 6% denatured polyacrylamide gels using silver staining.

Of the 85 primer sets, 58 were capable of amplifying products from *A. oxyphylla* genomic DNA, of which 35 were monomorphic and 23 were polymorphic (Table 1 and Table 2). Only polymorphic primer pairs were further characterized. Specifically, the forward primer of each polymorphic primer pair was labeled with a fluorescent dye (6-FAM, HEX, or TAMRA). Thirty-two individuals in two geographically distinct populations from Guangdong and Hainan provinces in China (Appendix 1) were chosen to more thoroughly assay

TABLE 1. Characteristics of 23 polymorphic microsatellite primers developed for *Alpinia oxyphylla*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Size range (bp)	GenBank accession no.
YZ1	F: GGTAGAAGGATGTGCCCAAA	$(CT)_{12}$	50	180–203	JX422048
	R: ATCCACGTTTAGGTGCGAAG				
YZ2	F: AAGAAGCGTTGGTGAGAGGA	$(CT)_{20}$	52	182–203	JX422049
	R: GGGAGCCAACTCATCTCTGA				
YZ5	F: GAGGAGGACGAGGAGAGGAC	$(AGC)_7$	46	201–221	JX422050
	R: CCAACAGCCCTTCTTTTTGA				
YZ7	F: ACCGAGACTCCACTGCGTAT	$(AG)_8$	50	176–181	JX422051
	R: TCCTCGTTACTGTGCAATCG				
YZ10	F: CCATGGTTGTCGAGAAAGGT	$(GA)_{17}$	50	187–202	JX422052
	R: CACATTGCCTTCCTCTGTGA				
YZ12	F: GCTGAAGGGAAAAGATTTCG	$(CT)_{12}$	50	185–215	JX422053
	R: TGGAAAAGTTTATGGGTGTGC				
YZ16	F: ATGTGGAGTCATCGGTAGCC	$(GA)_8$	52	153–248	JX422054
	R: TATGTCACCTGCGACGTCAT				
YZ21	F: CGCAATTTGAGACCATCTGA	$(AG)_9(AG)_{22}$	60	203–230	JX422055
	R: GCATGTTTTCGGAGGAAGAG				
YZ22	F: CCAAACCCTAGCACGACAAT	$(GA)_{19}$	50	170–197	JX422056
	R: GACGTCACGAATGTTGGTTG				
YZ23	F: GCAGTGTGGTAAGCAGTCCA	$(GA)_{19}$	52	214–262	JX422057
	R: GGGCCAAAATTCAGTGCTAA				
YZ24	F: CATTCGATCTCCTTCGGTTC	$(AT)_8$	60	160–248	JX422058
	R: CTCATGAATGCTCACGGATG				
YZ27	F: CAAGCTATTGCCACGTGATG	$(AG)_2(GT)_9$	50	160–243	JX422059
	R: GGACCCAAGAAGATCGAACA				
YZ31	F: CTAAAGCGCCATCTCCAGTC	$(TG)_8$	52	223–245	JX422060
	R: GGCCATCGTGTAGAGGAACA				
YZ44	F: CAGGAAGATTGCGATGAAGC	$(CT)_8$	64	147–167	JX422061
	R: ACCCTACTCCTCCCATCTGC				
YZ45	F: TTGAAATTTGAAAAGCAACATCA	$(AG)_{11}$	52	142–153	JX422062
	R: CGAATTGGAAAGTAATTATATGACC				
YZ46	F: GCTAACTTGTCTTTCCTATTTCTCC	$(CT)_{13}$	52	150–177	JX422063
	R: CCTCGCCATCAAAATCATCT				
YZ53	F: TTCGGTGGAAGACAGAGACA	$(AG)_{11}$	49	169–186	JX422064
	R: TTGATTCTGCCTCCCATTTC				
YZ60	F: TCGACATGAAATCCCTACGA	$AGAC(AG)_{15}ACAG$	52	193–236	JX422065
	R: GTGAAGTGAAAGGGCGAAAG				
YZ61	F: TGACTCCAAACTTGCAGGAG	$(TC)_8$	55	160–192	JX422066
	R: AGCAGATCAATGCACGTGAG				
YZ65	F: GCGAAACCCTCTCATCCTTA	$(AG)_{14}$	48	174–203	JX422067
	R: CCATCTCCTCGTCCTTTTCA				
YZ66	F: ACCTGATGAGTTCCTTGCATC	$(AAG)_2(AG)_6$	48	148–176	JX422068
	R: AGCCAAATGAACGGACAGAT				
YZ67	F: TTGACTTGGGTATGGCAAAA	$(AG)_{13}$	48	226–232	JX422069
	R: AAGGTCGAGCAGGAGTAGCA				
YZ77	F: TCTGAACCAGGGAATCCAAA	$(CT)_9$	48	156–163	JX422070
	R: AACTCACTTGGAGGCCAACTT				

Note: T_a = annealing temperature.

polymorphism levels for the 23 primer pairs. PCR amplifications were carried out as described above. Equal volumes of PCR products amplified with two or three different dye-labeled primers were mixed together and used for genotyping. The PCR products were genotyped using an ABI Prism 3730 DNA Sequencer (Applied Biosystems).

Our results (Tables 1 and Table 2) indicate that these microsatellite markers are highly polymorphic. The number of alleles per locus ranged from one to 13, and observed heterozygosity and expected heterozygosity varied from 0 to 1 (mean: 0.6441) and 0 to 0.887 (mean: 0.6241; values obtained using POPGENE version 1.32; Yeh et al., 2000).

CONCLUSIONS

We developed and evaluated 23 polymorphic microsatellite markers from *A. oxyphylla*. These polymorphic microsatellite markers will be useful for investigating the genetic diversity and population structure of *A. oxyphylla*. The results should be helpful in efforts to exploit the medicinal value of the species.

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TABLE 2. Results of polymorphic primer screening in populations of *Alpinia oxyphylla*.[a]

	SM population				LC population			
Locus	*N*	*A*	H_o	H_e	*N*	*A*	H_o	H_e
YZ1	16	8	0.813	0.768	14	4	0.857	0.579
YZ2	16	8	0.813	0.754	15	5	0.800	0.582
YZ5	16	8	0.813	0.836	15	4	0.867	0.624
YZ7	16	3	0.188	0.275	16	4	0.438	0.678
YZ10	16	8	0.750	0.771	13	3	0.500	0.541
YZ12	16	8	0.938	0.795	16	4	0.875	0.369
YZ16	16	13	0.813	0.887	16	5	0.625	0.768
YZ21	13	8	0.875	0.836	15	8	0.933	0.827
YZ22	16	10	0.750	0.842	16	5	0.563	0.531
YZ23	16	13	0.750	0.869	16	6	1.000	0.684
YZ24	16	11	0.563	0.848	16	5	0.125	0.695
YZ27	16	1	0.000	0.000	13	4	0.375	0.326
YZ31	16	6	0.375	0.602	16	4	0.625	0.668
YZ44	13	4	0.538	0.615	16	4	1.000	0.643
YZ45	16	5	0.875	0.783	16	5	0.750	0.701
YZ46	16	10	0.688	0.777	16	5	0.625	0.492
YZ53	16	8	0.938	0.801	16	4	0.313	0.400
YZ60	16	9	1.000	0.814	16	7	1.000	0.840
YZ61	16	10	0.688	0.760	16	5	0.750	0.566
YZ65	16	4	0.313	0.451	16	4	0.375	0.475
YZ66	14	12	0.625	0.840	10	6	1.000	0.756
YZ67	16	2	0.188	0.170	16	2	0.313	0.342
YZ77	16	3	0.438	0.354	16	3	0.188	0.174

Note: A = number of alleles; H_e = mean expected heterozygosity; H_o = mean observed heterozygosity; N = sample size.

[a] See Appendix 1 for population and voucher information.

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APPENDIX 1. List of vouchers of *Alpinia oxyphylla* used in this study. All vouchers are deposited at the South China Botanical Garden Herbarium (IBSC).

Code	Location	Altitude (m)	Latitude	Longitude	Voucher no.
SM	Sanguan Mountain, Guangdong Province	670	21°56′42.68″N	111°07′1042″E	2012030901
LC	Li County, Hainan Province	303	18°22′41.15″N	109°19′44.55″E	2012032701