Characterization of 31 Microsatellite Markers for *Sinocalycanthus chinensis* (Calycanthaceae), an Endemic Endangered Species

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Primer Note

Characterization of 31 microsatellite markers for *Sinocalycanthus chinensis* (Calycanthaceae), an endemic endangered species[1]

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- *Premise of the study:* Thirty-one microsatellite markers were developed for *Sinocalycanthus chinensis* (Calycanthaceae), an endemic endangered species in China.
- *Methods and Results:* Twenty-one polymorphic and 10 monomorphic microsatellite markers of *S. chinensis* were developed using methods of biotin-streptavidin capture and capillary electrophoresis. The number of alleles per locus was one to 20 with an average of 4.677 in 90 individuals taken from two populations in Zhejiang Province and one population in Anhui Province in China. Mean observed and expected heterozygosity across all three populations were 0.403 ± 0.061 (0.033–1.000 per locus) and 0.510 ± 0.043 (0.032–0.797 per locus), respectively. Of these 31 loci, 29 were successfully amplified in *Calycanthus floridus*.
- *Conclusions:* These microsatellite markers will be useful for studies of population genetic diversity and phylogeny of *S. chinensis* and *C. floridus*.

Key words: Calycanthaceae; genetic diversity; microsatellite; polymorphic; *Sinocalycanthus chinensis*.

The monotypic genus *Sinocalycanthus chinensis* W. C. Cheng & S. Y. Chang within the family Calycanthaceae is an endemic, endangered plant species in China. *Sinocalycanthus chinensis* is a diploid ($2n = 22$; Jin et al., 2010), deciduous shrub characterized by large, individual flowers with a diameter of 4.5–7 cm (Cheng and Chang, 1964). Its high ornamental and medicinal value results in overharvesting and a highly restricted geographic distribution (Li and Jin, 2006). Some studies have focused on the genetic diversity and phylogeny of *S. chinensis* using random-amplified polymorphic DNA (RAPD) (Li and Jin, 2006), inter-simple sequence repeat (ISSR) (Ye et al., 2006; Jin and Li, 2007), amplified fragment length polymorphism (AFLP) (Zhao et al., 2014), and chloroplast simple sequence repeat (cpSSR) (Li et al., 2012) markers, but with limited resolution, low reproducibility, and/or low stability. In this study, microsatellites, a more powerful and effective marker due to their codominance, were developed for use in genetic investigation of three populations of *S. chinensis*.

METHODS AND RESULTS

Leaves of *S. chinensis* were collected from three populations (30 individuals in each population) distributed across three locations in China: Daleishan (DLS) (28.988717°N, 120.811367°E) in Tiantai County, Damingshan (DMS) (30.039817°N, 118.972933°E) in Lin'an city in Zhejiang Province, and Longxushan (LXS) (30.069167°N, 118.700167°E) in Jixi County in Anhui Province (Appendix 1). Leaves of *Calycanthus floridus* L. were collected from Zhenru Garden (31.253708°N, 121.398147°E) in Shanghai and Hangzhou Botanic Garden (30.255113°N, 121.116163°E) in Zhejiang Province in China (Appendix 1). Total genomic DNA was extracted from silica-dried leaves using the Plant Genomic DNA Kit (Tiangen, Beijing, China). A microsatellite-enriched library of *S. chinensis* was constructed using the biotin-streptavidin capture method (Zane et al., 2002). Genomic DNA was digested using *Mse*I (New England Biolabs, Beverly, Massachusetts, USA) at 37°C for 3 h, followed by 80°C for 20 min. After visualization by agarose gel electrophoresis, the DNA fragments (200–800 bp after digestion) were ligated to a *Mse*I-adapter pair (F: 5′-TACTCAGGACTCAT-3′, R: 5′-GACGATGAGTCCTGAG-3′) at 37°C for 2 h and then 65°C for 10 min. The ligation products were amplified as follows: 95°C for 3 min, followed by 20 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min. The PCR products were hybridized with a 5′ biotinylated probe $(AG)_{15}$ and captured with streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA). The enriched fragments were amplified as follows: 95°C for 3 min; 30 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min; and 72°C for 8 min. After separation by agarose gel electrophoresis, the PCR products were purified using the Multifunctional DNA Purification Kit (BioTeke, Beijing, China). The purified PCR products were ligated to pMD 19-T vector (TaKaRa Biotechnology Co., Dalian, China) at 72°C for 1 h, and then transformed into strain JM109 of *Escherichia coli* by transient thermal stimulation (ice bath for 30 min, 42°C water bath for 90 s, followed by ice bath for 2 min).

A total of 716 positive clones were chosen and tested by PCR using primers of $(AG)_{10}$ and M13F/M13R, respectively. One hundred and twenty-seven screened clones contained potential microsatellite motifs and were sequenced using an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA). A total of 107 (75 in the initial sequencing and 32 in the second sequencing) primer pairs were designed by the program Primer Premier 5 (PREMIER Biosoft International, Palo Alto, California, USA). These primers were tested for polymorphism in 90 *S. chinensis* individuals within the DLS, DMS, and LXS populations. PCR amplification was performed in a 10-µL reaction: 20 ng of genomic DNA template, 1.0 µL of 10× PCR buffer (with Mg^{2+}), 0.15 mM of each dNTPs, 0.05 µM of each primer, and 0.5 units of DNA *Taq* polymerase (TaKaRa Biotechnology Co.).

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Table 1. Characteristics of 31 microsatellite loci developed from *Sinocalycanthus chinensis*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
SC020*	F: GAATAAGGGGAGTGGACG R: GAGAAAGGAAGGAAATAAAA	$(TC)_8$	142	57	KY560159
SC056	F: ATAGAAAGCCTTGGTTG R: AGGGAAAACTCAAAAGA	$(GA)_9$	220–226	54	KY560160
SC061	F: CACTAAATGCTACCAAACG R: GAAAACATACCAACCAAAA	$(CT)_{16}$	205–223	54	KY560161
SC078	F: GAACCCTACAGAAACTTGAC R: GTGTTGTTAGATTGGGTGGT	$(GT)_{10}(GA)_{13}$	174–186	56	KY560162
SC093	F: TTCCGAGAACGAGAT R: TTTAGTCATGCCAATG	$(CT)_{24}$	94–112	48	KY560163
SC096*	F: AAACTCCTATTTCCTCCC R: TTTCAAACACCCTTCACA	$(AG)_{15}$	104	47	KY560164
SC098	F: CTGGTAGGTTTTGCTGCTTTT R: CGGATCTCCTTTCTTTCTTCA	$(AG)_{14}$	150–184	55	KY560165
SC107-2	F: ACCATCAAATAGAAACC R: GAGTCCTGAGAATAAGA	$(GA)_{10}$	90–106	57	KY560166
SC124	F: TACGGCGGTAATACAAGGG R: CTGAAACGCCATCCGACTC	$(AG)_8...(GA)_9$	220–246	60	KY560167
SC136*	F: GACAGGTTTTGGAGATG R: GGAGTGATTCCTTTGG	$(AG)_7$	124	50	KY560168
SC151	F: CCACAAAAGGTCAATGAG R: TCTGGATGGGTTGGACTA	$(GA)_{25}$	150–180	48	KY560169
SC197*	F: AAAACCAAACCAAGAGGAAGA R: GCCAACGTCAACATAAGTAGC	$(CT)_{16}$	183	52	KY560170
SC220	F: ATGACAATGCCAGGAGAT R: TCACGCTCCTCTGTTTCT	$(GA)_{15}$	203–213	49	KY560171
SC245*	F: GGGTTACTGGTTTGGTT R: GGGTCGGACAGTGAGTA	$(CT)_{15}$	188	50	KY560172
SC257*	F: GAGATAAGGAGATGGAT R: AAGTTGGACAGTGATGG	$(AG)_{12}$	199	45	KY560173
SC264	F: TGGGTTATTTGGTTTCA R: GTCGCAGTCACCTTCTC	$(GA)_9$	154–166	54	KY560174
SC280	F: GATTACCCTTCTTAGCAC R: CAGGTCCAGACTGATGAC	$(CT)_8(CA)_{12}$	308–322	52	KY560175
SC296	F: AAAAGAAGGACCATCAGTAT R: GTTGTATTGCATTCAAAGTT	$(TC)_{15}$	94–98	52	KY560176
SC301	F: TGTTTACATCATGCCAGT R: GCTCTACTCCCTGATTTT	$(CT)_9$	124–128	50	KY560177
SC318*	F: TGAGACTCGAAATCACCACT R: GGAGACAGAAGCAGCAGAAT	$(TC)_7$	199	50	KY560178
SC367	F: GAACAATGAAACCGAAGG R: TAGTTCAAATAAGAAGCAGAG	$(CT)_7$	170–184	54	KY560179
SC375	F: AAGTGTAAATATGCGGTGGA R: GCTGCCTCGAACAAGTCT	$(GA)_7$	113–123	50	KY560180
SC388*	F: CCATGATCCCAAGGTAAG R: AAGACAGAATGCCCCAAT	$(CT)_{11}$	255	56	KY560181
SC424	F: AGAAAGTAGGGGAGGGAAGC R: CACCCTTCAGTCGTGGAGCC	$(GA)_7$	222–246	57	KY560182
SC440*	F: ATGAAGATGTGATTTT R: CATTTGATTGAGATAA	$(TC)_{12}$	127	42	KY560183
SC472*	F: AGAAACCCAACAATAGTAGAAG R: ACAAGCACCCACCATACA	$(AG)_5(GA)_6$	159	55	KY560184
SC492	F: TACAAGGCTTACCGCACA R: GAGGATTTGAAAAGAACTGTTT	$(CT)_{14}$	163–215	46	KY560185
SC512-2	F: GGCACTTGGTGGTAG R: ATGGTCCTCACATCAG	$(AG)_{21}$	91–101	46	KY560186
SC537	F: ATTCCACAAACAATAATCTC R: TCTCCTTTCAAGCAACC	$(AG)_{17}$	160–168	49	KY560187
SC556-2	F: ACTATTCACCCTAGTTCTC R: CCATTTGACCCACTTA	$(TC)_{16}$	109–117	47	KY560188
SC673-2	F: TGACTCCCAATAAACAC R: TTCGAGCATCCAATAGC	$(GA)_8$	114–120	53	KY560189

Note: T_a = annealing temperature.
* Monomorphic microsatellite loci.

Microsatellite loci were amplified under the following conditions: 94°C for 3 min; 30 cycles of 94°C for 30 s, 41–60°C (annealing temperature) for 30 s, 72°C for 30 s; and extension at 72°C for 5 min. PCR products were visualized on 1.5% agarose gels and then resolved on a Fragment Analyzer automated capillary electrophoresis system (Advanced Analytical Technologies, Ankeny, Iowa, USA; kit DNF-900-K0500).

Table 2. Genetic diversity of 21 polymorphic microsatellite markers in three *Sinocalycanthus chinensis* populations.[a]

	Damingshan (N = 30)				Daleishan (N = 30)				Longxushan (N = 30)				Total (N = 90)			
Locus	n	A	H_o	H_e	n	A	H_o	H_e	n	A	H_o	H_e	n	A	H_o	H_e
SC056	30	2	0.000*	0.444	30	3	0.033*	0.609	30	3	0.000*	0.371	90	4	0.033	0.475
SC061	30	6	0.733	0.776	30	6	0.667	0.727	30	3	0.133*	0.598	90	10	0.511	0.700
SC078	30	4	0.300	0.579	30	7	0.567	0.736	30	5	0.533	0.626	90	7	0.467	0.647
SC093	30	6	0.567	0.767	29	7	0.517*	0.804	30	6	0.733	0.760	89	7	0.606	0.777
SC098	30	2	0.033	0.033	30	6	0.633	0.719	30	6	0.367*	0.617	90	9	0.344	0.456
SC107-2	30	6	0.500	0.661	30	6	0.467*	0.756	27	2	0.296	0.444	87	7	0.421	0.620
SC124	30	10	0.933*	0.811	30	8	0.500*	0.746	30	8	0.800*	0.835	90	14	0.933	0.797
SC151	30	6	1.000	0.752	30	6	0.567	0.779	30	5	0.600	0.562	90	8	0.722	0.698
SC220	30	2	0.267	0.231	30	7	0.567*	0.766	30	2	0.633	0.433	90	7	0.489	0.477
SC264	30	2	0.067*	0.180	30	2	0.133*	0.444	30	1	0.000	0.000	90	2	0.067	0.208
SC280	30	1	0.000	0.000	30	2	0.033	0.033	30	2	0.067	0.064	90	2	0.033	0.032
SC296	30	2	0.000*	0.124	30	3	0.267	0.527	30	2	0.000*	0.491	90	3	0.267	0.381
SC301	30	2	0.033	0.033	30	3	0.667	0.491	30	3	0.267	0.238	90	3	0.322	0.254
SC367	29	3	0.000*	0.585	30	5	0.100*	0.502	29	3	0.069*	0.447	88	5	0.069	0.511
SC375	30	2	1.000*	0.500	30	2	1.000*	0.500	30	2	1.000*	0.500	90	2	1.000	0.500
SC424	30	2	0.000*	0.124	29	9	0.241*	0.795	30	5	0.033*	0.578	89	9	0.137	0.499
SC492	30	8	0.267*	0.642	30	11	0.600*	0.854	30	10	0.833*	0.839	90	20	0.550	0.778
SC512-2	29	2	0.000*	0.408	29	4	0.000*	0.302	27	2	0.556	0.497	85	4	0.556	0.402
SC537	30	4	0.000*	0.611	30	5	0.133*	0.563	30	4	0.033*	0.517	90	5	0.083	0.564
SC556-2	30	4	0.267	0.317	30	4	0.567	0.668	30	4	0.433*	0.686	90	5	0.422	0.557
SC673-2	30	2	0.567	0.455	30	2	0.300	0.339	28	2	0.393	0.316	88	2	0.420	0.370

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals sampled; n = number of individuals successfully amplified.

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

* Significant deviation from Hardy–Weinberg equilibrium expectations after Bonferroni correction ($P < 0.001$).

The number of alleles, observed heterozygosity, expected heterozygosity, and linkage disequilibrium were estimated with the software FSTAT 2.9.3.2 (Goudet, 2001), and Hardy–Weinberg equilibrium was assessed using GenAlEx 6.3 (Peakall and Smouse, 2006). Of the 31 loci, 21 loci were polymorphic in at least two of the three tested populations, and the remaining 10 loci were monomorphic (Table 1). The number of alleles per locus ranged from one to 20, with an average of 4.677. In the 21 polymorphic markers, the average observed and expected heterozygosity in all three populations were 0.403 ± 0.061 (mean ± SEM [standard error of the mean]) (0.033–1.000 per locus) and 0.510 ± 0.043 (0.032–0.797 per locus), respectively (Table 2). Seven loci (SC056, SC124, SC367, SC375, SC424, SC492, SC537) significantly deviated from Hardy–Weinberg equilibrium in all three tested populations after Bonferroni correction ($P < 0.001$) (Table 2). Of these 31 loci, 29 were successfully amplified in *C. floridus* and also revealed high levels of polymorphism (Table 3).

CONCLUSIONS

In this study, 31 microsatellite markers were developed from the Chinese endemic endangered plant species *S. chinensis*. Twenty-one loci were polymorphic in three tested populations. The high transferability of these markers will provide a more effective method to research the population genetics and phylogeography of *S. chinensis* and the closely related species *C. floridus*.

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Table 3. Characterization of 31 microsatellite loci developed from *Sinocalycanthus chinensis* in two populations of *Calycanthus floridus*.[a]

	Shanghai Zhenru Park ($N = 7$)			Hangzhou Botanic Garden ($N = 2$)		
Locus	A	H_o	H_e	A	H_o	H_e
SC020	1	0.000	0.000	1	0.000	0.000
SC056	4	0.857	0.786	4	1.000	0.7500
SC061	5	0.714	0.726	4	1.000	0.7500
SC078	4	0.714	0.786	1	0.000	0.000
SC093	3	0.571	0.667	—	—	—
SC096	2	0.714	0.524	2	1.000	0.500
SC098	6	1.000	0.875	2	1.000	0.500
SC107-2	4	0.286	0.786	1	0.000	0.000
SC124	7	0.857	0.905	4	1.000	0.500
SC136	—	—	—	—	—	—
SC151	5	0.286	0.845	2	1.000	0.500
SC197	—	—	—	—	—	—
SC220	7	1.000	0.893	2	0.000	0.500
SC245	4	0.429	0.738	1	0.000	0.000
SC257	2	0.286	0.452	1	0.000	0.000
SC264	4	0.429	0.667	2	1.000	0.500
SC280	3	0.571	0.619	2	1.000	0.500
SC296	4	0.857	0.702	1	0.000	0.000
SC301	4	0.714	0.619	2	1.000	0.500
SC318	1	0.000	0.000	1	0.000	0.000
SC367	2	0.500	0.417	2	1.000	0.500
SC375	3	1.000	0.643	2	1.000	0.500
SC388	5	0.167	0.800	2	0.500	0.375
SC424	4	0.167	0.800	3	1.000	0.625
SC440	3	0.857	0.643	1	0.000	0.000
SC472	2	1.000	0.500	2	1.000	0.500
SC492	3	0.714	0.690	2	1.000	0.500
SC512-2	1	0.000	0.000	1	0.000	0.000
SC537	6	0.857	0.881	3	1.000	0.625
SC556-2	1	0.000	0.000	1	0.000	0.000
SC673-2	3	0.286	0.643	1	0.000	0.000

Note: — = no PCR products; 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.

Appendix 1. Locality information for the *Sinocalycanthus chinensis* and *Calycanthus floridus* samples used in this study.[a]

Species	Population ID	Collection locality	Geographic coordinates	Collector	Collection no.	N
Sinocalycanthus chinensis W. C. Cheng & S. Y. Chang	DMS	Damingshan, Zhejiang, China	30.039817°N, 118.972933°E	Xiao-Yan Wang	DLS1-30	30
Sinocalycanthus chinensis	DLS	Daleishan, Zhejiang, China	28.988717°N, 120.811367°E	Xiao-Yan Wang	DMS1-30	30
Sinocalycanthus chinensis	LXS	Longxushan, Anhui, China	30.069167°N, 118.700167°E	Jing-Jing Gu	AHJX1-30	30
Calycanthus floridus L.		Zhenru Garden, Shanghai, China	31.253708°N, 121.398147°E	Yong-Bin Shi	ZRCF1-6	7
Calycanthus floridus		Hangzhou Botanic Garden, Hangzhou, Zhejiang, China	30.255113°N, 121.116163°E	Chuan Chen	HZCF1-2	2

Note: N = number of individuals.
[a]All voucher specimens were deposited in Taizhou University, Taizhou, China.