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Primer Note

Development and characterization of EST-SSR markers in the Chinese medicinal plant *Callerya speciosa* (Fabaceae)[1]

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- *Premise of the study:* The first microsatellite primers were developed for *Callerya speciosa*, an important traditional medicinal plant with island-mainland distributions in China, to further investigate its genetic variability and population structure.
- *Methods and Results:* The microsatellite-containing sequences were selected from a cDNA library of *C. speciosa*. In total, 58 primer pairs were designed, and 25 of the corresponding loci showed clear amplification. Polymorphisms were assessed in two different natural populations. The mean number of alleles per locus ranged from two to nine. Observed and expected heterozygosity per loci ranged from 0.067 to 0.938 and 0.064 to 0.836, respectively. One out of 25 loci showed departure from Hardy–Weinberg equilibrium expectations in both populations, and three pairs of loci showed significant linkage disequilibrium after Bonferroni correction.
- *Conclusions:* These microsatellite markers will be useful tools for genetic and conservation studies and to understand the evolutionary processes in *Callerya* species.

Key words: *Callerya speciosa*; conservation; EST-SSR; island-mainland distributions; Millettieae; population genetics.

Callerya speciosa (Champ. ex Benth.) Schot belongs to the tropical genus *Callerya* Endl. of the Fabaceae family, is native to Southeast Asia, and is widespread in tropical and subtropical forests of Hainan Island and southern mainland China. It is almost certain that Hainan Island has experienced repeated processes of connection and disconnection with the Chinese mainland through the Pleistocene epoch (Hope, 2005; Yan, 2006). The geological events combined with different environmental conditions and geographical isolation played an important role in determining the genetic structure and evolutionary process of *C. speciosa* on Hainan Island and adjacent areas on the Chinese mainland. *Callerya speciosa* is a well-known medicinal plant; the roots of this plant have been applied for centuries in traditional Chinese medicine for the treatment of rheumatoid arthritis (Zong et al., 2009). In folk remedies, the swollen roots are also used to make tonic soup and tonic wine. Unfortunately, the high demand for *C. speciosa* has caused a serious reduction in the number of roots available to harvest in the wild (Li et al., 2010). Our field surveys over the past several years have revealed that the current patchy distribution of this species is a remnant of a more extensive former distribution because of unsustainable exploitation and habitat deforestation. Therefore, an appropriate conservation program is urgently needed to prevent further loss of *C. speciosa*.

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Simple sequence repeat (SSR) markers are useful and popular tools for population genetic studies and conservation management of biological resources; they also have appeal to phylogeographers and landscape geneticists as a nuclear complement to chloroplast DNA (cpDNA). The aim of this study was to develop SSR markers derived from expressed sequence tags (ESTs) to analyze effects of historical events on genetic structure, population differentiation, and diversity of natural populations of *C. speciosa* and to provide useful information for design of conservation strategies in *Callerya* species.

METHODS AND RESULTS

Samples of *C. speciosa* collected from four sites (Nanfeng: 19.40431°N, 109.62747°E; Jiangbian: 18.82390°N, 109.33491°E; Dinghushan: 23.17085°N, 112.53962°E; Xuwen: 20.41562°N, 110.23157°E) were cultivated at the nursery of the Tropical Crops Genetic Resources Institute (TCGRI, Chinese Academy of Tropical Agricultural Science), Hainan, China. Voucher specimens of every sampled population were deposited in the herbarium of TCGRI (Appendix 1). Total RNA was extracted from the roots of one individual of *C. speciosa* from the Nanfeng population using the cetyltrimethylammonium bromide (CTAB) method (Le Provost et al., 2007) and further purified with *Oligotex-dT30* (Super) mRNA Purification Kit (TaKaRa Biotechnology Co., Dalian, Liaoning, China). Then a complementary DNA (cDNA) library was constructed using a cDNA Synthesis Kit (TaKaRa Biotechnology Co.) and sequenced using an ABI PRISM 3730xl DNA Analyzer with the ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) by TaKaRa Biotechnology Co. A total of 1573 EST sequences were obtained, ranging in size from 308 to 732 bp with an average length of 641 bp, and 1009 putative unigenes were constructed by CodonCode Aligner (http://www.codoncode.com/aligner/index.htm). To eliminate redundancy, all assembled sequences containing microsatellites were used for similarity search against the National Center for Biotechnology Information (NCBI) nonredundant (nr) database using the Basic Local Alignment Search Tool (BLASTX) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx) with an *E*-value cutoff of 1e-5

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Table 1. Characteristics of the 25 EST-SSR primers developed in *Callerya speciosa*.

Locus (GenBank accession no.)	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	BLAST top hit description [organism]	BLAST top hit accession no.	*E*-value
Ndl_001 (JX046819)	F: TCTGAAGCACCATCACCAAG R: TGAGGTACAAGGGTTCACGA	$(AG)_{13}AC(AG)_2$	213–236	—		
Ndl_002 (JX046820)	F: TATCTGCTGCCACATCTTCG R: AACCACCCACCTTGCATAAG	$(AT)_{10}(GT)_7$	268–292	Lectin-related polypeptide [*Robinia pseudoacacia*]	BAA36416.1	6e-74
Ndl_003 (JX046821)	F: ACCCTCTCCTTGCCCAGTAT R: CCCAGCTGAACAAGAGCTTC	$(AT)_8$	288–296	Predicted homeobox protein knotted-1-like 2-like isoform 2 [*Glycine max*]	XP_003522219.1	1e-28
Ndl_004 (JX046822)	F: TCCGACAATGTCAAGATCCA R: CCACGGGGTGGTTATAATTG	$(CCG)_6$	277–282	Ethylene-responsive transcription factor [*Medicago truncatula*]	XP_003593630.1	2e-40
Ndl_010 (JX046823)	F: TCTTGGAGGATGAAGGATGG R: GACTTCTAGTCCCCCGCTCT	$(TCT)_5$	289–298	Predicted hypothetical protein [*Glycine max*]	XP_003535809.1	1e-92
Ndl_011 (JX046824)	F: TTGTGGCAGATGGAACACTC R: TGACACTGTCCCTACCGTCA	$(GGT)_6$	262–275	Predicted zinc finger protein ZAT10-like [*Glycine max*]	XP_003525928.1	8e-107
Ndl_013 (JX046825)	F: AAGGGGATTAGGGTTTACGG R: GCTCTTCGGCTTCTTTGTTG	$(AAC)_6$	161–189	Cap-binding protein-like protein [*Phaseolus vulgaris*]	ABU54819.1	5e-133
Ndl_015 (JX046826)	F: AAGATCCAACAACTCAACTCTGG R: TCTACACCCAGAAAGAGAGAAGG	$(CT)_{11}$	226–245	Predicted hypothetical protein [*Glycine max*]	XP_003549641.1	4e-20
Ndl_017 (JX046827)	F: CGGAGCTACAAGGGTTCCTA R: TGCAGGGTTATGGTGAATGA	$(CAG)_{10}$	248–276	Predicted hypothetical protein [*Glycine max*]	XP_003535716.1	6e-88
Ndl_019 (JX046828)	F: CTGTGTGAACTTTCTTGTGTAACC R: GGTGACTCGTTGTGGTGTGT	$(AG)_{16}$	281–296	ARG10 [*Vigna radiata*]	BAA25187.1	4e-141
Ndl_020 (JX046829)	F: GACTTCTAGTCCCCCGCTCT R: TCTTGGAGGATGAAGGATGG	$(AAG)_5$	291–298	Predicted hypothetical protein [*Glycine max*]	XP_003535809.1	1e-92
Ndl_021 (JX046830)	F: GATCAGATGGCTCTGGAAGC R: GCTTGACGGTAGGGACAGTG	$(GCG)_6$	235–241	Predicted zinc finger protein ZAT10-like [*Glycine max*]	XP_003525928.1	8e-107
Ndl_022 (JX046831)	F: CACTGTCCCTACCGTCAAGC R: TTGTGGCAGATGGAACACTC	$(ACC)_6$	261–272	Predicted zinc finger protein ZAT10-like [*Glycine max*]	XP_003525928.1	8e-107
Ndl_028 (JX046832)	F: CTAGTGGCTCCAATGGTGGT R: AATTGCAGGGGTCATCAAAG	$(GCA)_6GAG(GCA)_2$	276–289	Predicted protein TIME FOR COFFEE-like [*Glycine max*]	XP_003556039.1	6e-75
Ndl_031 (JX046833)	F: TTCAATCCGGAGCTACAAGG R: TGCAGGGTTATGGTGAATGA	$(CAG)_{10}$	248–269	Predicted hypothetical protein [*Glycine max*]	XP_003535716.1	7e-53
Ndl_032 (JX046834)	F: GCTGTTAATTTGCATAAGGGTAAGC R: CAAGGAGATCGCGAATCAAT	$(TATT)_6$	263–286	—		
Ndl_033 (JX046835)	F: GGAGCACTCAAAACCCAAAA R: TACGTGCATGCTCGAAGAAC	$(AG)_{12}$	140–158	Hypothetical protein [*Glycine max*]	NP_001241027.1	3e-95
Ndl_038 (JX046836)	F: GTCTCCACCTTCCAACTCCA R: CACCTAATTGCTGCTGCTGA	$(CAG)_2CAA(CAG)_6$	181–193	Predicted hypothetical protein [*Glycine max*]	XP_003528481.1	4e-28
Ndl_042 (JX046837)	F: ATTCCATTCCCAATGGTACG R: TCTTCTCCGAAGCCTGTTGT	$(CT)_{10}$	162–178	60S ribosomal protein L11, putative [*Ricinus communis*]	XP_002522234.1	1e-98
Ndl_043 (JX046838)	F: GGAGTTTTCAGGAAGGCACA R: CCTTTCACTTGCTTTTGTCCA	$(AAC)_7$	242–266	Predicted probable WRKY transcription factor 33 [*Glycine max*]	XP_003544908.1	3e-93
Ndl_047 (JX046839)	F: GCCTGTGCCTTTTCTCTCTG R: CTCGAACTGGGTTTCCTCAA	$(GA)_{11}$	235–247	Putative transcription factor EREBP [*Trifolium pratense*]	BAE71206.1	3e-56
Ndl_049 (JX046840)	F: ACTGACTCCACACCACACCA R: TGGTACCCAGGTTCGATAGC	$(GAA)_7(GAAG)_5(AGG)_2$	182–196	Predicted F-box protein PP2-A15-like [*Glycine max*]	XP_003521431.1	9e-66
Ndl_050 (JX046841)	F: GTGGTGGTGTTCCTGCTTCT R: ACGGTGGGAACCCTCTTAAT	$(TGT)_{10}$	252–275	Hypothetical protein MTR_5g093390 [*Medicago truncatula*]	XP_003617600.1	1e-19
Ndl_051 (JX046842)	F: TGGACCTCAACATGATGCTC R: TTCCCTGCGGAGAAGAAGTA	$(CCA)_6$	192–212	Hypothetical protein MTR_2g101130 [*Medicago truncatula*]	XP_003597685.1	1e-77
Ndl_053 (JX046843)	F: CTTAGGCGGTGGTTGATGTT R: CCAGAAGAAGCAGAGGATGG	$(CTT)_{10}$	220–246	Hypothetical protein MTR_7g065150 [*Medicago truncatula*]	XP_003623160.1	2e-25

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Table 2. Genetic diversity of the 25 polymorphic EST-SSR markers in two natural populations of *Callerya speciosa.*

Locus	Nanfeng population (N = 25)				Dinghushan population (N = 25)			
	A	H_o	H_e	F_{IS}	A	H_o	H_e	F_{IS}
Ndl_001	6	0.692	0.651	−0.024	4	0.667	0.684	0.060
Ndl_002	5	0.250	0.777	0.695*	5	0.750	0.68	−0.071
Ndl_003	2	0.625	0.469	−0.304	3	0.125	0.119	−0.017
Ndl_004	2	0.188	0.498	0.643*	2	0.077	0.488	0.854*
Ndl_010	3	0.125	0.225	0.469	4	0.563	0.639	0.151
Ndl_011	3	0.625	0.643	0.060	3	0.571	0.426	−0.308
Ndl_013	4	0.750	0.668	−0.091	2	0.125	0.117	−0.034
Ndl_015	3	0.462	0.462	0.040	4	0.188	0.229	0.211
Ndl_017	8	0.938	0.813	−0.122	5	0.800	0.733	−0.057
Ndl_019	5	0.750	0.707	−0.029	4	0.563	0.725	0.254
Ndl_020	2	0.067	0.064	0.000	2	0.563	0.498	−0.098
Ndl_021	4	0.688	0.574	−0.166	3	0.875	0.586	−0.469
Ndl_022	3	0.533	0.598	0.142	3	0.438	0.354	−0.207
Ndl_028	5	0.750	0.686	−0.062	2	0.563	0.404	−0.364
Ndl_031	9	0.933	0.809	−0.120	5	0.786	0.717	−0.059
Ndl_032	5	0.467	0.742	0.401*	5	0.800	0.744	−0.040
Ndl_033	8	0.800	0.836	0.077	5	0.500	0.693	0.308
Ndl_038	3	0.286	0.253	−0.095	4	0.563	0.572	0.049
Ndl_042	7	0.867	0.702	−0.201	5	0.563	0.574	0.053
Ndl_043	2	0.438	0.498	0.153	5	0.800	0.722	−0.073
Ndl_047	5	0.733	0.736	0.038	3	0.267	0.24	−0.077
Ndl_049	4	0.438	0.611	0.314*	3	0.250	0.225	−0.081
Ndl_050	4	0.625	0.637	0.051	4	0.714	0.61	−0.135
Ndl_051	4	0.667	0.553	−0.172	3	0.200	0.184	−0.050
Ndl_053	5	0.667	0.709	0.094	4	0.467	0.464	0.030
Mean	4.4	0.575	0.597		3.6	0.511	0.497	

Note: A = number of alleles per locus; F_{IS} = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size.
* Significant departures from Hardy–Weinberg equilibrium at $P < 0.05$.

(results as shown in Table 1). In total, 58 sequences had a microsatellite insert with a dinucleotide of at least seven repeat units or a tetra- or trinucleotide of at least five repeat units, were chosen using the Simple Sequence Repeat Identification Tool (SSRIT; http://www.gramene.org/db/searches/ssrtool) (Temnykh et al., 2001), and the primer pairs were designed based on the flanking sequences of the microsatellite loci using Primer3 software (Rozen and Skaletsky, 2000).

The genomic DNA of all individuals of *C. speciosa* from every sampled population was extracted using a DNeasy plant DNA isolation kit (QIAGEN, Hilden, Germany). For each primer pair, two samples were amplified and their amplification products run on 2% agarose gels. PCR amplifications were performed in a 10-µL reaction containing 10 mM Tris-HCl (pH 8.4), 50 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM of each primer, 1.0 U of *Taq* polymerase (TaKaRa Biotechnology Co.), and 50 ng of genomic DNA. Amplifications were performed as follows: 94°C for 5 min, 35 cycles of denaturation for 50 s at 94°C, annealing for 50 s at 57°C, extension for 90 s at 72°C, and a final extension at 72°C for 10 min. For primers that showed single locus amplification patterns, polymorphisms were evaluated using DNA of four individuals from four different sampled populations of *C. speciosa*, by PCR carried out according to the protocol described above. The amplified products were screened on a 6% polyacrylamide denaturing gel and visualized by silver staining. A 10-bp DNA ladder (Promega Corporation, Madison, Wisconsin, USA) was used to identify alleles. Results showed that 44 (excluding three with PCR product sizes considerably larger than expected) of the 58 primer pairs were amplified successfully. Among these loci, 25 were polymorphic with a clear fragment pattern, 11 had multibanding patterns, and the other eight were monomorphic. The 25 loci (Table 1) that yielded clear chromatograms and polymorphisms were further screened for their suitability using 50 individuals from Nanfeng (Hainan Island) and Dinghushan (Chinese mainland) (N = 25 for each population). The degree of polymorphism, including the number of alleles (A), observed heterozygosity (H_o), expected heterozygosity (H_e), and fixation index (F_{IS}), was calculated for each locus and population using GenAlEx version 6 (Peakall and Smouse, 2006). Tests for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) after Bonferroni correction were performed using GENEPOP version 4 (Rousset, 2008).

All of the polymorphism results are summarized in Table 2. The mean number of alleles per locus was 4.4 (range: 2–9) and 3.6 (range: 2–5) for the Nanfeng and Dinghushan populations, respectively. The observed heterozygosity ranged from 0.067 to 0.938 (average: 0.575) in the Nanfeng population and from 0.077 to 0.875 (average: 0.511) in the Dinghushan population. The expected heterozygosity ranged from 0.064 to 0.836 (average: 0.597) in the Nanfeng population and from 0.117 to 0.744 (average: 0.497) in the Dinghushan population. Only for one locus (Ndl_004), the observed proportions showed significant deviation from those expected under HWE ($P < 0.05$) in both populations. Significant linkage disequilibrium was found in three pairs of loci (Ndl_010 and Ndl_020, Ndl_011 and Ndl_022, and Ndl_017 and Ndl_031) across both populations after Bonferroni correction ($P < 0.0001$).

CONCLUSIONS

The 25 microsatellite loci presented here are the first set of SSR markers for the genus *Callerya*, and should provide a useful tool for genetic diversity studies and conservation of genetic resources. These EST-SSR markers may also be applied to taxonomy, phylogeography, cultivar identification, and molecular-assisted selection in breeding programs of *C. speciosa*.

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Appendix 1. Samples of *Callerya speciosa* used in this study.

Code	Accession no.[a]	Locality (Geographical coordinates)	Voucher no.[b]
NF	NF01, NF02, NF03, NF04, NF07, NF08, NF10, NF11, NF12, NF13, NF15, NF16, NF17, NF18, NF19, NF21, NF22, NF23, NF25, NF26	Nanfeng, Danzhou city, Hainan Province, China (19.40431°N, 109.62747°E)	NDL0160
DHS	DHS01, DHS02, DHS03, DHS04, DHS05, DHS06, DHS07, DHS08, DHS10, DHS13, DHS14, DHS15, DHS17, DHS18, DHS19, DHS20, DHS21, DHS22, DHS23, DHS24	Dinghushan, Guangzhou city, Guangdong Province, China (23.17085°N, 112.53962°E)	NDL0581
JB	DF08	Jiangbian, Dongfang city, Hainan Province, China (18.82390°N, 109.33491°E)	NDL0050
XW	XW25	Xuwen, Zhanjiang city, Guangdong Province, China (20.41562°N, 110.23157°E)	NDL0213

[a] Samples are cultivated at the nursery of the Tropical Crops Genetic Resources Institute (TCGRI, Chinese Academy of Tropical Agricultural Science), Hainan, China.
[b] Voucher specimens are deposited at TCGRI herbarium.

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