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Development and characterization of polymorphic microsatellite markers in *Castanopsis sclerophylla* (Fagaceae)[1]

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- *Premise of the study:* We developed polymorphic microsatellite primers in *Castanopsis sclerophylla* (Lindley & Paxton) Schottky (Fagaceae), a dominant canopy tree, to provide markers for further studies on the genetic structure and mating system of this species.
- *Methods and Results:* Ten polymorphic microsatellite loci were isolated and successfully amplified in three *C. sclerophylla* populations (Huangshanjian, Shilin, and Guanmiao) from Chun'an, Zhejiang Province, China. The number of alleles per locus in these populations ranged from 3 to 17. The observed and expected heterozygosities were 0.100–0.977 and 0.294–0.916, respectively.
- *Conclusions:* These microsatellite loci displayed moderate or high levels of polymorphism within the examined populations, showing the utility of primers in studying the genetic variation, parentage, and mating system of *C. sclerophylla*.

Key words: *Castanopsis sclerophylla*; genetic diversity; microsatellites; polymorphism.

Castanopsis sclerophylla (Lindley & Paxton) Schottky (Fagaceae) is a monoecious, wind-pollinated, and evergreen broad-leaved canopy tree that is widely distributed in subtropical eastern Asia. This species is gravity-dispersed, and rodents may serve as a secondary disperser. As a dominant species in the zonal vegetation, evergreen broad-leaved forests of subtropical China, this species plays an important role in maintaining the function and stability of local ecosystems (Zhang et al., 2007). However, the subtropical areas of eastern China have long been affected by human activities, and *C. sclerophylla*–dominated forests have been extensively fragmented. Analysis of the genetic variation of dominant species will provide a sound basis for the management of fragmented forests and successful reforestation (Young et al., 1996; Chen, 2000). Owing to codominance and high polymorphism, microsatellite loci are a very useful tool for analyzing population genetic variation (Selkoe and Toonen, 2006). In this study, we developed primers for the polymorphic microsatellites of *C. sclerophylla* to enable further analysis of the genetic structure, parentage, and mating system of this species.

METHODS AND RESULTS

The biotin-streptavidin capture method was used to develop the microsatellite primers (Liu et al., 2009; Xu et al., 2010). We extracted total genomic DNA from silica-gel-dried seedling leaf tissue of an individual of *C. sclerophylla* using a Plant Genomic DNA Extraction Kit (Tiangen, Beijing, China). The DNA was digested with the restriction enzyme *Mse*I (New England Biolabs, Beverly, Massachusetts, USA) to obtain fragments of 200–1 000 bp. We then ligated these fragments to an *Mse*I-adaptor pair. Five-microliter volumes of the products were used as templates for PCR with *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′). PCR was carried out in a total reaction volume of 20 μl under the following reaction conditions: denaturation at 95°C for 3 min; followed by 17 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min. To isolate the fragments containing simple sequence repeats, the PCR products were denatured at 95°C for 5 min and then hybridized to a 5′-biotinylated oligonucleotide probe $(AG)_{15}$ in 250 μl hybridization solution at 48°C for 2 h. The products were captured using magnetic streptavidin-coated beads (Promega, Madison, Wisconsin, USA). After washing away the unhybridized DNA, we eluted the captured DNA fragments from the beads in 50 μl of 1×TE. We amplified the products by PCR for 30 cycles using the *Mse*I-N primer described previously. We then purified the PCR products using a multifunction DNA Extraction Kit (Bioteke, Beijing, China), ligated these into pMD 19-T vector (Takara, Dalian, China), and transformed into *E. coli* strain JM109 by electroporation. A total of 758 clones were screened using $(AG)_{10}$ and $M13^+$/$M13^-$ as primers. Using an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA), 131 positive clones were sequenced. Among these, 59 sequences were discarded because the flanking regions of the repeat sequences were not suitable for designing primers. The remaining 72 sequences were used to design primers using Primer Premier 5.0 software (http://www.premierbiosoft.com).

We examined the amplification of the 72 primer pairs using 21 *C. sclerophylla* individuals randomly selected from populations in Chun'an County, Zhejiang Province. PCR was performed under the following conditions: denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 52–62.5°C for 45 s (Table 1), and 72°C for 45 s; and then extension at 72°C for 10 min. The total volume of the reaction was 20 μl and contained the following reactants: 1× PCR buffer, 1.5 mM Mg^{2+}, 0.2 mM of each dNTP, 0.1 μM of each primer, 0.4U of *Taq* DNA polymerase (Sangon, Shanghai, China), and 50 ng sample DNA. Amplified products were electrophoresed on 8% denaturing polyacrylamide gels and visualized by silver staining using pUC19 DNA/*Msp*I (H*pa*II) (Fermentas, Vilnius, Lithuania) as the ladder. Ultimately, we acquired 10 polymorphic loci (Table 1).

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TABLE 1. Repeat motif, primer sequences (including fluorescent dye markers), annealing temperature (*Ta*), number of alleles (A), fragment size range, and GenBank accession number of the 10 isolated microsatellite loci.

Locus	Primer pair sequence (5′–3′)	Motif	*Ta* (°C)	A	Size range (bp)	GenBank Accession No.
CS43	F: <6-FAM>CTCTATCTCGCAAGCGTGTGA R: CCCCATTTTGTGGTTCTAAGGT	$(CT)_9$	61.5	6	92–106	HQ265409
CS92	F: <TAMRA>CAGAAACCAAAAAAGAACAG R: ACACACAAGAAAACAAAAGC	$(GA)_{12}…(AT)_3$	61	8	151–171	HQ265410
CS137	F: <ROX>GAACTCACAAGTCACAACCCT R: TTCCTCTCTTTCTTTGCCCAT	$(GA)_4C(AG)_6…(AG)_7$	57	7	135–157	HQ265408
CS561	F: <6-FAM>ATTCATCTGGTGGGGTTT R: GGAGTGGAGTTAGAAGACGAT	$(CT)_{20}$	55	17	316–352	HQ265411
CS576	F: <TAMRA>CAGGAACAAATATCAAACGG R: AAAGAGCCCTAATAGCAATG	$(AG)_6G(GA)_{13}$	62	6	166–180	HQ265412
CS620	F: <6-FAM>AGGTGTAGAAGGAAAAAAGC R: AGACTGACTCAAAACAATAAAG	$(CT)_{12}$	62.5	15	118–154	HQ265416
CS627	F: <6-FAM>CGCTACTGTAACTGGAATGG R: CTCAATGTTTGTGTTGGTGT	$(CT)_{16}$	62	14	190–232	HQ265417
CS687	F: <ROX>GTGTAAAGTTTGACCCATTG R: AGCAGCCACCATTTCAGTTC	$(CT)_{12}$	59	7	129–151	HQ265413
CS697	F: <6-FAM>TTTCCTTCACAAAAAGGTTG R: AGCAAAAAAATACTCCCAAA	$(CT)_9(CA)_8$	57.5	3	87–91	HQ265414
CS721	F: <HEX>AAGCAATGAGTAGAGATAATGATG R: GAGAGATGTAGAGAATAGATGGAA	$(CT)_{16}$	52	10	119–139	HQ265415

TABLE 2. Number of samples genotyped (N), number of alleles (*A*), and observed (H_O) and expected (H_E) heterozygosity of the 10 polymorphic microsatellite loci in three *Castanopsis sclerophylla* populations.

	Huangshanjian (29°34′10″N, 119°06′03″E)				Shilin (29°30′42″N, 119°04′47″E)				Guanmiao (29°32′15″N, 119°05′54″E)			
Locus	N	*A*	H_O	H_E	N	*A*	H_O	H_E	N	*A*	H_O	H_E
CS43	10	4	0.600	0.721	18	3	0.444	0.538	43	6	0.233	0.294
CS92	10	5	0.700	0.626	18	5	0.833	0.697	43	8	0.465	0.517
CS137	10	5	0.800	0.753	18	5	0.444	0.568	43	7	0.721	0.697
CS561	10	11	0.700	0.916	18	10	0.944	0.862	43	12	0.791	0.789
CS576	8	5	0.625	0.825	18	6	0.833	0.771	43	5	0.977	0.649
CS620	9	8	0.778	0.876	18	8	0.556	0.706	35	12	0.743	0.810
CS627	10	6	0.500	0.737	18	7	0.444	0.756	42	11	0.405	0.772
CS687	10	6	0.600	0.632	18	3	0.500	0.560	43	4	0.535	0.524
CS697	10	2	0.100	0.395	18	3	0.222	0.367	43	3	0.163	0.450
CS721	10	7	0.700	0.789	16	7	0.188	0.776	41	9	0.561	0.797
Mean	9.7	5.9	0.610	0.727	17.8	5.7	0.541	0.660	41.9	7.7	0.559	0.630

To further check the variation of the 10 loci, we extracted genomic DNA from 71 *C. sclerophylla* individuals sampled from Huangshanjian, Shilin, and Guanmiao in Chun'an County, Zhejiang Province (Appendix 1), and then performed PCR using the same thermocycle program mentioned earlier. The 10 polymorphic primers were labeled with one of the following fluorescent dyes: 5′HEX, 5′TAMRA, 5′ROX, or 5′6-FAM (Sangon, Shanghai, China). To get the best resolution, we performed the PCR amplifications separately in a total volume of 15 μl with the above reactions and scanned the products in two combinations in an ABI 3130 genetic analyzer using GS500(−250)LIZ as the internal lane standard. We used the software GENEMAPPER 4.0 (Applied Biosystems, Foster City, California, USA) to analyze the data and discriminate alleles.

All 10 primer pairs amplified high-quality PCR products showing moderate to high levels of polymorphism across the three populations. Using the software GENEPOP v4.0 (Rousset, 2008), we found that the number of alleles per locus ranged from 3 to 17, with an average of 9.3 (Table 1). At the population level, the number of alleles per locus was 2–12. The observed heterozygosity (H_O) ranged from 0.100 to 0.977, and the expected heterozygosity (H_E) ranged from 0.294 to 0.916 (Table 2).

CONCLUSIONS

The developed microsatellite loci showed high levels of polymorphism in *C. sclerophylla*. These markers will be useful tools for analyzing within- and among-population genetic variation, parentage, and mating system of this species. This information will be important for the management of fragmented forests and ecological restoration of zonal vegetation (Zhang et al., 2006; Jian et al., 2008; Liu et al., 2008).

LITERATURE CITED

CHEN, X. Y. 2000. Effects of habitat fragmentation on genetic structure of plant populations and implications for the biodiversity conservation. *Acta Ecologica Sinica* 20: 884–892.

JIAN, Y., L. SHI, D. LI, C. C. ZHANG, M. M. SHI, R. WANG, AND X. Y. CHEN. 2008. Effects of deforestation on the genetic structure of *Castanopsis sclerophylla. Acta Ecologica Sinica* 28: 6228–6234.

LIU, M., M. M. SHI, M. H. LIU, AND X. Y. CHEN. 2009. Isolation and characterization of microsatellite loci in *Fagus longipetiolata* Seem. (Fagaceae). *Conservation Genetics* 10: 1981–1983.

LIU, M. H., X. Y. CHEN, X. ZHANG, AND D. W. SHEN. 2008. A population genetic evaluation of ecological restoration with the case study on *Cyclobalanopsis myrsinaefolia* (Fagaceae). *Plant Ecology* 197: 31–41.

ROUSSET, F. 2008. GENEPOP'007: A complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

SELKOE, K. A., AND R. J. TOONEN. 2006. Microsatellites for ecologists: A practical guide to using and evaluating microsatellite markers. *Ecology Letters* 9: 615–629.

XU, N. N., S. YU, J. G. ZHANG, P. K. E. TSANG, AND X. Y. CHEN. 2010. Microsatellite primers for *Halophila ovalis* and cross-amplification in *H. minor* (Hydrocharitaceae). *American Journal of Botany* 97: e56–e57.

YOUNG, A., T. BOYLE, AND T. BROWN. 1996. The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology & Evolution* 11: 413–418.

APPENDIX 1. Information on voucher specimens deposited at East China Normal University.

Taxon—*Voucher specimens*, Locality in East China
Castanopsis sclerophylla—HSJ1, Chun'an, Zhejiang, China; *SL2*, Chun'an, Zhejiang, China; *GM3*, Chun'an, Zhejiang, China.

ZHANG, X., X. Y. CHEN, AND D. ZHANG. 2006. Effects of regeneration methods on RAPD-based genetic variation of *Cyclobalanopsis glauca* (Fagaceae). *New Forests* 32: 347–356.

ZHANG, X., G. F. XU, D. W. SHEN, Y. J. GU, H. GAO, X. H. LUO, AND X. Y. CHEN. 2007. Maintenance and natural regeneration of *Castanopsis sclerophylla* populations on islands of Qiandao Lake Region. *Acta Ecologica Sinica* 27: 424–431.