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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF 25 MICROSATELLITE PRIMERS FOR *ILEX CHINENSIS* (AQUIFOLIACEAE)[1]

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- *Premise of the study:* To evaluate genetic variation and structure of *Ilex chinensis* (Aquifoliaceae), a dioecious evergreen tree, we developed 25 microsatellite markers from its nuclear genome.
- *Methods and Results:* Based on the biotin-streptavidin capture method, 10 polymorphic and 15 monomorphic microsatellite markers were developed. Ten polymorphic loci were characterized by 87 individuals sampled from three populations located in Zhejiang Province and Shanghai, China. The number of alleles per locus varied from two to 12. The observed and expected heterozygosities were 0.0435–0.9032 and 0.3121–0.8343, respectively.
- *Conclusions:* These microsatellite markers can be useful for further genetic studies of *I. chinensis* populations, and so contribute to forest restoration and management.

Key words: Aquifoliaceae; evergreen broadleaved forests; genetic diversity; genetic structure; *Ilex chinensis*; simple sequence repeat (SSR).

Evergreen broadleaved forests (EBLFs) are zonal vegetation found in subtropical China. They support hyperdiverse species but have suffered from dramatic declines due to anthropogenic habitat loss and fragmentation. Less than 5% of old-growth EBLFs remain in subtropical China (Song and Chen, 2007). Thus, protecting and recovering EBLFs are crucial to sustainable ecosystem management. It has been suggested that one of the native dominant species in EBLFs, *Ilex chinensis* Sims (Aquifoliaceae), may be suitable for restoration of EBLFs. It is a dioecious evergreen tree, bearing small unisexual flowers and red globose drupes. Genetic variation provides important information for efficient management of fragmented forests and ecological restoration (Thomas et al., 2014). For this reason, it is necessary to delineate the genetic background of *I. chinensis*. In this study, 10 polymorphic microsatellites for *I. chinensis* were isolated and characterized. These can be used to evaluate the genetic diversity, genetic structure, and gene flow of this species.

METHODS AND RESULTS

Microsatellite loci were developed using the biotin-streptavidin capture method following the protocol reported by Liu et al. (2009) and Tong et al. (2012). Total genomic DNA was extracted from silica gel–dried leaf tissues of one individual of *I. chinensis* collected from Tiantong, Zhejiang Province, China, using a Plant Genomic DNA Extraction Kit (Tiangen, Beijing, China); GPS coordinates and voucher information are provided in Appendix 1. Approximately 250 ng of DNA was digested with the restriction enzyme *Mse*I (New England Biolabs, Beverly, Massachusetts, USA) and then linked with an *Mse*I-adapter pair (forward: 5′-TACTCAGGACTCAT-3′, reverse: 5′-GACGATGAGTCCTGAG-3′). The diluted products were used as templates for PCR with *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′) under the following 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 hybridized with 5′-biotinylated oligonucleotide probe $(AG)_{15}$ in a 250-µL hybridization solution at 48°C for 2 h. The products were captured by streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA). Then the diluted DNA fragments were amplified again by PCR for 30 cycles using the *Mse*I-N primer. After the PCR products were purified with a multifunctional DNA Extraction Kit (BioTeke, Beijing, China), they were ligated to pMD 19-T vector (TaKaRa Biotechnology Co., Dalian, China) followed by transformation into *Escherichia coli* strain JM109 by transient thermal stimulation.

A total of 456 clones were chosen and screened using $(AG)_{10}$ and $M13^+/M13^-$ as primers, respectively, producing 108 positive clones. They were sequenced on an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA), and 29 sequences were selected to design primers using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). Performance and polymorphism were tested for each locus using 24 *I. chinensis* individuals randomly selected from three populations located in Chun'an and Yuwang (Zhejiang Province, China) and Shanghai Botanical Garden (Shanghai, China), following the fluorescent labeling protocol of Schuelke (2000). Reactions were performed separately for each primer pair in 20-µL total volume containing 40 ng of template DNA, 1× PCR buffer, 2 mM Mg^{2+}, 0.2 mM of each dNTP, 0.1 µM M13(–21) sequencing primer (5′-TGTAAAACGACGGCCAGT-3′) labeled with HEX, ROX, or 6-FAM (Sangon Biotech, Shanghai, China), 0.025 µM forward primer with M13(–21) tail at its 5′ end, 0.1 µM reverse primer, and 2 units of *Taq* DNA polymerase (Sangon Biotech). Conditions of the PCR amplification were as follows: denaturation at 94°C for 5 min; then 30 cycles of 30 s at 94°C, 45 s at 54–65°C (depending on the specific locus) (Table 1), and 45 s at 72°C; followed by eight cycles of 30 s at 94°C, 45 s at 53°C, and 45 s at 72°C; and a final extension at 72°C for 10 min. The amplification products were visualized on an ABI 3730 automated sequencer, and alleles were called and binned using GeneMapper 4.0 software (Applied Biosystems). Finally, we obtained 10 polymorphic and 15 monomorphic loci (Table 1).

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TABLE 1. Characterization of 10 polymorphic and 15 monomorphic microsatellite loci developed in *Ilex chinensis*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_{as} (°C)	T_{af} (°C)	Fluorescent dye[b]	GenBank accession no.
DQ9	F: ACTTACGTCCACTCTTCG	$(GA)_9$	156	55	—	ROX	KT006006
	R: AGAAAGCGTGAGTTGTGA						
DQ20	F: AGCAAGCGTAAGTTGTGAG	$(TC)_8$	179	64	—	ROX	KT006007
	R: TTATAGTGGTTCGGTCTCG						
DQ27*	F: TAGTGGTTCGATCTGGTTG	$(GA)_{10}$	234–254	58	55	HEX	KP325082
	R: GCTCAAGTCATCGTCTCAA						
DQ39	F: TCCTCTACAGATGCAACCTC	$(GA)_7$	170	58	—	6-FAM	KT006008
	R: TCTCCATCAGTATTCCCCTC						
DQ41	F: CATAGAAACGCAAACAC	$(CA)_6…(GA)_6$	171	55	—	6-FAM	KT006009
	R: TTGGCAGATGTAAAGAC						
DQ43	F: TTCCTTCCGTTTTCTGGT	$(CT)_7$	323	57	—	HEX	KT006010
	R: TGCTAATTTCCGTGTTGC						
DQ56*	F: ATGCTCGTCATCTTCTTGG	$(TC)_{12}$	97–109	58	56	ROX	KP325075
	R: GAAATGATAGTGAGCGTGT						
DQ80*	F: GTTTAGTATCTCCACCTCC	$(AG)_9$	249–261	55	51	HEX	KP325076
	R: AATTCGATCACCCTCAT						
DQ111	F: ATTGACCCAACACGAACC	$(TC)_5…(CA)_5$	120	64	—	6-FAM	KT006011
	R: TAGAAGACGACGGAAAGC						
DQ137*	F: CTGTTCGGCTCATCA	$(TC)_5$	109–125	58	58.7	ROX	KP325083
	R: AACTCAAGACGCTGC						
DQ140	F: GGATATTATGCATTTGGGTC	$(CT)_9$	257	57	—	HEX	KT006012
	R: GGCTTGGATAATGGATTGGA						
DQ141	F: TGGTGGTTAGTGAGCAAT	$(GA)_8$	247	61	—	ROX	KT006013
	R: TAGAAAGCGTGAGTTGTG						
DQ146*	F: CCGACATATCAACCATC	$(AG)_9$	111–121	58	58.7	6-FAM	KP325074
	R: GTAAATAACGGCTCCAT						
DQ147	F: TTCTGTAACTCTTCCTCCAT	$(AG)_{11}$	218	54	—	ROX	KT006014
	R: CCCAACCCTAAATACCAT						
DQ158	F: CAAATCAGCAAATGAGCCT	$(TC)_5$	227	60.4	—	ROX	KT006015
	R: CCGAAAACCAACGAAATAG						
DQ159	F: GTGGCAATCGAATCATCTAG	$(GA)_6$	173	58	—	ROX	KT006016
	R: TTCTCACCTCTGTCCCTGTA						
DQ164	F: GTTTGTGGGGATCTTGCTC	$(CT)_{11}$	165	58.7	—	6-FAM	KT006017
	R: TGCCTCCACTTGACTCTGC						
DQ165	F: GAATACGCACGAGACAAG	$(AG)_{11}…(AG)_5$	118	58	—	6-FAM	KT006018
	R: CTAACTCCGGTAAAGGTC						
DQ168	F: TTATAGTGGTTCGGTCTCGG	$(GA)_{10}$	183	63	—	ROX	KT006019
	R: GATGGAAAGCGTAAGTTGTG						
DQ169*	F: ATTACCCTGAACACTCGTC	$(TC)_8$	210–236	60	58	HEX	KP325077
	R: CTTCAAAGCCTACAACCAC						
DQ175	F: GGCTTCATCTAACATTG	$(CT)_7$	169	57.5	—	6-FAM	KT006020
	R: TAATCACCTTGAACTCC						
DQ184*	F: GCGCATATCACAGGTAGGGT	$(TC)_{11}$	111–143	58.7	58.7	6-FAM	KP325079
	R: CTTCAGAGGTGATTGGGCAT						
DQ185*	F: CCAAATTGAGTTCCGTAG	$(TC)_7$	147–171	58.5	60	HEX	KP325080
	R: AGTTCCACAAACCCTTCT						
DQ188*	F: CCTAACTAACCACGACAC	$(CT)_{20}$	186–224	57	56	ROX	KP325078
	R: ATCTCCCATCCTACAATC						
DQ198*	F: AGTTCGGACGGCCTCCAATG	$(GA)_8$	178–182	65	65	6-FAM	KP325081
	R: TCTTTCACGTCACCGCCTCA						

Note: T_{af} = annealing temperature with fluorescent dyes labeling the forward primers; T_{as} = annealing temperature using the genotyping protocol of Schuelke (2000).

[a]All values are based on the samples representing three populations located in Chun'an and Yuwang in Zhejiang Province and Shanghai Botanical Garden in Shanghai, China (see Appendix 1).

[b]Fluorescent dyes (i.e., HEX, ROX, and 6-FAM) used for fragment analysis.

*Polymorphic microsatellite loci.

Ten polymorphic loci were further characterized in 87 *I. chinensis* individuals sampled from the three populations mentioned above (Appendix 1). Forward primers were labeled with one of the following fluorescent dyes: HEX, ROX, or 6-FAM (Sangon Biotech) (Table 1). PCR amplifications were performed separately for each locus in a 15-μL reaction volume containing 40 ng of template DNA, 1× PCR buffer, 1.5 mM Mg^{2+}, 0.2 mM of each dNTP, 0.1 μM of each primer, and 1 unit of *Taq* DNA polymerase (Sangon Biotech). PCR was performed under the following conditions: denaturation at 94°C for 5 min; followed by 30 cycles of 30 s at 94°C, 45 s at 51–65°C (Table 1), and 45 s at 72°C; and a final extension at 72°C for 8 min. The annealing temperatures of PCRs were different from those amplified using the Schuelke (2000) protocol, probably due to the fluorescent dye labeling the forward primers in the former. The amplification products were scanned on an ABI 3730 automated sequencer, and the alleles were called and binned using GeneMapper 4.0 software (Applied Biosystems).

All 10 polymorphic primer pairs amplifying high-quality PCR products showed moderate to high levels of polymorphism across the three populations. Using the software GENEPOP v4.0 (Rousset, 2008), results showed the number of alleles per locus to vary from two to 12 with an average of 4.8. The observed and expected heterozygosities ranged from 0.0435 to 0.9032 and 0.3121 to 0.8343, respectively (Table 2). Deviations from Hardy–Weinberg

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TABLE 2. Genetic properties of the 10 newly developed polymorphic microsatellites of *Ilex chinensis*.[a]

	Chun'an				Yuwang				Shanghai Botanical Garden			
Locus	*n*	*A*	H_o	H_e	*n*	*A*	H_o	H_e	*n*	*A*	H_o	H_e
DQ27	24	4	0.4167*	0.6933	29	6	0.4828	0.6146	23	4	0.2174	0.3121
DQ56	32	5	0.7500	0.7004	32	5	0.5625	0.6563	23	3	0.6522	0.6570
DQ80	32	5	0.5625*	0.7242	32	4	0.8125	0.6235	23	5	0.4348	0.6077
DQ137	27	2	0.3333	0.5094	28	2	0.5357	0.5084	23	4	0.1304*	0.4280
DQ146	32	3	0.6250	0.6171	32	3	0.5625	0.5298	23	3	0.6522	0.6773
DQ169	31	6	0.4194*	0.7356	31	6	0.3871	0.5198	23	4	0.3913*	0.5990
DQ184	31	7	0.4839*	0.6483	32	9	0.8438	0.8244	23	6	0.3478	0.3469
DQ185	32	3	0.1250*	0.4266	32	2	0.4063	0.4955	23	3	0.0435*	0.3295
DQ188	31	12	0.9032*	0.8017	32	12	0.7813	0.8343	23	8	0.7826	0.6986
DQ198	27	3	0.2593*	0.6115	31	3	0.2258	0.3527	22	3	0.2273*	0.6554

Note: *A* = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals genotyped.
[a] Locality and voucher information for the populations is available in Appendix 1.
* Indicates significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

equilibrium (HWE) and from linkage equilibrium were tested using GENEPOP v4.0 (Rousset, 2008) with sequential Bonferroni adjustment (Rice, 1989). No significant linkage disequilibrium ($P > 0.05$) was observed for each pair of loci. No locus showed significant departure from HWE in the Yuwang population. However, seven (DQ27, DQ80, DQ169, DQ184, DQ185, DQ188, and DQ198) and four loci (DQ137, DQ169, DQ185, and DQ198) significantly deviated from HWE ($P < 0.05$) in the Chun'an and Shanghai populations, respectively (Table 2).

CONCLUSIONS

The 25 microsatellites reported here for *I. chinensis* are appropriate for studies of the population's genetic structure. These analyses, in turn, can shed light on evolutionary forces such as the balance of mutation, gene flow, and genetic drift. Moreover, it can be expected that the genetic information of this dominant species based on these microsatellite loci may make a substantial contribution to the efficient conservation and management of EBLFs.

APPENDIX 1. Voucher and locality information of *Ilex chinensis* samples used in this study. Voucher specimens deposited at East China Normal University.

Voucher specimen ID	Collection locality	Geographic coordinates
T19200059	Tiantong, Zhejiang, China	29°48′56″N, 121°47′11″E
Chun'an	Zhejiang, China	29°30′29″N, 118°49′24″E
Yuwang	Zhejiang, China	29°51′04″N, 121°44′16″E
Shanghai Botanical Garden	Shanghai, China	31°08′48″N, 121°26′53″E

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