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Isolation and Characterization of Polymorphic Microsatellite Markers in *Calocedrus macrolepis* Kurz (Cupressaceae)

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Abstract. **The Chinese Incense-cedar (*Calocedrus macrolepis* Kruz), an important wood and ornamental tree, is native to southwest China and also in northern Vietnam, Laos, Thailand, and Myanmar. As a result of ecological degradation in these areas, Chinese Incense-cedar was considered a vulnerable species according to the criteria of the International Union for the Conservation of Nature and Natural Resources. In the current report, we developed and characterized 13 novel microsatellite markers for this species using the protocol of fast isolation by amplified fragment length polymorphism of sequences containing repeats. Polymorphism of each locus was assessed in 36 individuals from nine geographical populations. The number of alleles per locus ranged from two to nine with an average of 6.08. The observed and expected heterozygosities ranged from 0.0000 to 1.0000 and from 0.1549 to 0.8912 with averages of 0.6688 and 0.6815, respectively. Four of the 13 loci were significantly deviated from Hardy-Weinberg expectations. No significant linkage disequilibrium was detected. These polymorphic microsatellite markers would be useful tools for investigating genetic population structure and diversity to establish conservation strategy for this interesting and vulnerable species.**

The genus *Calocedrus* has only three members, California Incense-cedar (*C. decurrens* Florin), Chinese Incense-cedar (*C. macrolepis* Kruz), and Taiwan Incense-cedar [*C. macrolepis* var. *formosana* (Florin) W.C.Cheng & L.K.Fu] in the cypress family Cupressaceae. The Chinese Incense-cedar is native to southwest China and also in northern Vietnam, northern Laos, extreme northern Thailand, and northeastern Myanmar. It is a medium-sized tree to 25 to 35 m tall and trunk up to 2-m diameter with glaucous green leaves on the upper side of the shoots (Farjon, 2005; Fu et al., 1999). As a result of its glaucous green leaves and beautiful shape, Chinese Incense-cedar has been introduced into many gardens or parks worldwide as an ornamental tree. In past decades, Chinese Incense-cedar was overharvested for its valuable wood; moreover, its habitat was seriously degraded ecologically with economic development in southwestern China. The size of wild populations is dramatically shrinking, but there is an overall decline is continuing in whole distribution areas. Chinese Incense-cedar has been classified as a vulnerable species according to the criteria of the International Union for the Conservation of Nature and Natural Resources Red List in 2001 (Farjon, 2001) and has been listed as an endangered species in China (Fu, 1995) and Vietnam (World Conservation Monitoring Centre, 1997). To establish conservation strategies, it is important to characterize the genetic population structure and diversity for this species. Unfortunately, very few molecular markers are available to investigate the genetic diversity for Chinese Incense-cedar to date. In this report, we describe development of 13 polymorphic microsatellite loci for ongoing population genetic research in Chinese Incense-cedar using the protocol of the fast isolation by amplified fragment length polymorphism (AFLP) of sequences containing repeats (Zane et al., 2002).

Genomic DNA samples were extracted from dry leaf tissue, which was ground in liquid nitrogen using a CTAB methodology (Doyle and Doyle, 1987). Total genomic DNA (≈500 ng) derived were completely digested with *Mse*I and then ligated to an *Mse*I AFLP adaptor. A diluted digestion–ligation mixture (1:10) was amplified with adaptor-specific primers (5′-GATGAGTC CTGAGTAAN-3′). Amplified DNA fragments, with a size range of 200 to 800 bp, were enriched for repeats by magnetic bead selection with a 5′-biotinylated $(AC)_{15}$ and $(AG)_{15}$ and $(AAG)_{10}$ probe, respectively. Enriched fragments were amplified again with adaptor-specific primers. Polymerase chain reaction (PCR) products were purified using an EZNA Gel Extraction Kit (Omega Bio-Tek, USA). Purified DNA fragments were ligated into the pGEM-T vector (Promega, USA) and transformed into DH5αcells. Positive clones were tested by PCR using $(AC)_{10}$/$(AG)_{10}$/$(AAG)_7$ and T_7/Sp_6 as primers, respectively.

In total, 460 clones with positive inserts were chosen and sequenced with an ABI PRISM 3730XL DNA sequencer. A total of 167 (36%) sequences were found to contain microsatellite repeats, and 68 of them were suitable for designing locus-specific primers using PRIMER3 (http://www.broad.mit.edu/genome_software/). Polymorphisms of all 68 microsatellite loci were assessed in 36 samples of *C. macrolepis* from nine natural populations in China (seven from Yunnan province and two from Guizhou provience; Table 1). The PCR reactions were performed in 15 μL of reaction containing 30 to 50 ng genomic DNA, 0.6 μM of each primer, 7.5 μL 2 × Taq PCR MasterMix [Tiangen, China; 0.1 U Taq polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$)]. PCR amplifications were conducted under the following conditions: 97 °C for 3 min followed by 30 to 36 cycles at 94 °C for 30 s at the annealing temperature for each specific primer (optimized for each locus; Table 2) for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. PCR products were separated on 8% polyacrylamide denaturing gel using a 20-bp DNA ladder molecular size standard (TaKaRa, China) by silver staining. In all,

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13 primer pairs displayed polymorphisms (Fig. 1). Standard genetic diversity parameters, departure from Hardy-Weinberg equilibrium and linkage disequilibrium between pairs of loci, were performed using the software Genepop 4.0 (available at http://genepop.curtin.edu.au/). The number of alleles per locus (A) was two to nine with an average of 6.08; values for observed and expected heterozygosities ranged from 0.0000 to 1.0000 and from 0.1549 to 0.8912 with averages of 0.6688 and 0.6815, respectively (Table 2). Four of the 13 loci (Cm4, Cm7, Cm11, and Cm12) were significantly deviated from Hardy-Weinberg expectations ($P < 0.05$), which might be the result of small population size and isolated samples used in this study. No significant linkage disequilibrium was detected between the comparisons of these loci ($P < 0.001$) in our analysis.

Table 1. Populations of *Calocedrus macrolepis* for microsatellite marker development.

Population	Altitude (m)	Latitude (N)	Longitude (E)	Sample size
Yimen, Yunnan	1800	24°58′	102°16′	4
Changning, Yunnan	1700	24°53′	99°42′	4
Tengchong, Yunnan	1500	25°14′	98°37′	4
Lincang, Yunnan	1700	23°39′	100°5′	4
Mojiang, Yunnan	1500	23°32′	101°40′	4
Puer, Yunnan	1300	22°51′	100°56′	4
Yuanjiang, Yunnan	1500	23°25′	101°48′	4
Leishan, Guizhou	1200	26°31′	108°21′	4
Congjiang, Guizhou	1100	25°56′	108°12′	4

Table 2. Specific primer sequences and characterization for 13 microsatellite loci isolated from *Calocedrus macrolepis.*

Locus	Repeat motif	Primer sequences (5′–3′)	Ta (°C)	Allele size (bp)	A	H_O	H_E	GenBank accession no.
Cm1	$(AAG)_4$	F: TTTGGGTTTTCTTCGTTTTC R: ACATTCCAAAAGTTCCCTATCT	55	230–236	2	0.0000	0.1549	GU047325
Cm2	$(AC)_{28}$	F: AGACCTCCTACACATTATTCAA R: ACAAGAAAAAGAAGAAGTAGGTA	50	198–270	9	0.8056	0.6874	GU047326
Cm3	$(AG)_{23}$	F: TGGGTTGACCAGTGCTTCT R: ATGCCCAACACCTCATTAGA	55	156–200	7	0.8333	0.7786	GU047327
Cm4*	$(TG)_{17}$	F: AAAACTTCTAAGACCTCAACCT R: CAAGGAAGGTAGTTTGTAGAGA	53	180–212	4	1.0000	0.7109	GU047328
Cm5	$(TG)_{17}$	F: GAAGTTTACCATTTGTCGAA R: GTGTCTTCCAATATGAATCG	53	168–220	6	0.7778	0.8138	GU047329
Cm6	$(AC)_{17}$	F: AAAAGAATCCAAAACACACA R: CCCACCTGTACATTTCTCTA	53	158–178	5	0.1667	0.5708	GU047330
Cm7*	$(CA)_9$	F: CCTAACACAAACTCCAAGAAGA R: TTGGACACTCAAAAGCAATAAT	54	190–216	5	0.8889	0.5704	GU047331
Cm8	$(TC)_{18}$	F: CACCATTTTGATTTCTCATTTG R: AAACCACTCTAAAGGGAGAATAA	55	110–126	4	0.4722	0.5802	GU047332
Cm9	$(TC)_{16}(AC)_{19}$	F: ACCACTCTCTAACCCCACAAT R: GAGAGGGATAGAGGGTGAAAC	55	158–212	7	0.7222	0.6706	GU047333
Cm10	$(AC)_{11}(TC)_{15}$	F: GATAGGGAAAAACAACCTTACT R: TCATAAATCATTCACGGTTCTA	53	118–158	8	0.6389	0.8690	GU047334
Cm11*	$(AG)_{25}$	F: CAGCTCTAGGCGAGTATTTA R: CTTCCTCTCTCCTATGTTCC	53	171–213	5	0.8889	0.7254	GU047335
Cm12*	$(TG)_9$	F: TCCCTACTTTTTGGTTTTTTATT R: ACATTGGGCTCATTGATTTC	55	106–178	9	0.9722	0.8912	GU047336
Cm13	$(TG)_{10}(AG)_{31}$	F: CTACTTCATCACCCCTAAAATA R: TGACTACCATAAAAGCTCTCTC	53	110–174	8	0.5278	0.8369	GU047337

Ta = polymerase chain reaction annealing temperature; A = number of alleles revealed; H_O = observed heterozygosity; H_E = expected heterozygosity; F = forward; R = reverse.
*Significant departure from Hardy-Weinberg equilibrium ($P < 0.05$).

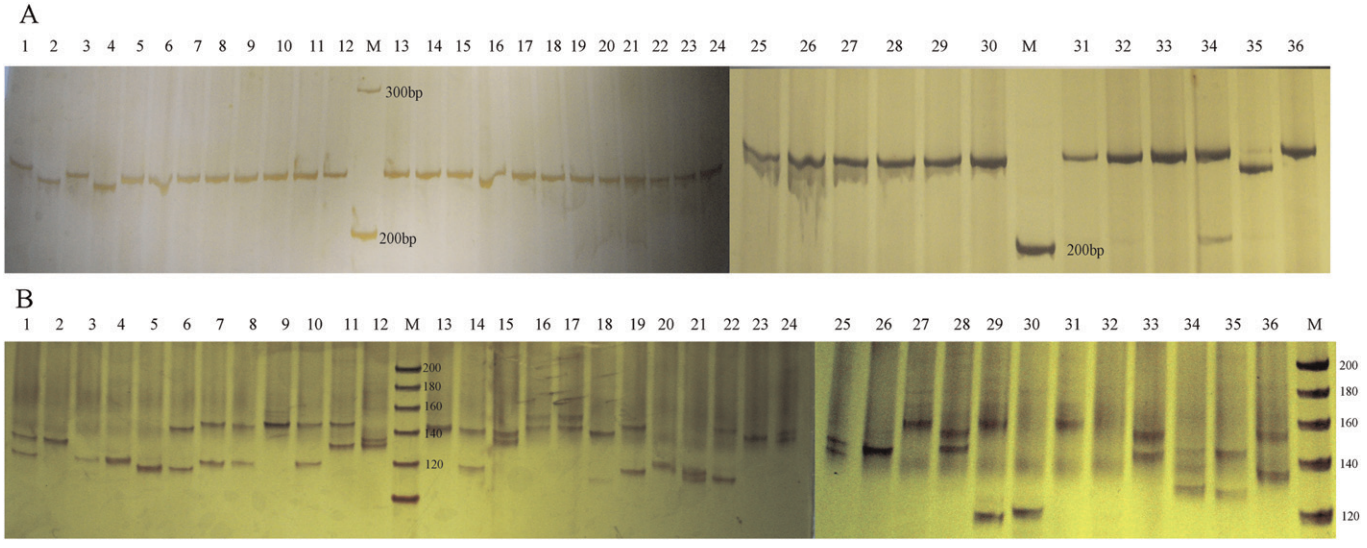

Fig. 1. Simple sequence repeat profile of two loci of *Calocedrus macrolepis*. (**A**) Cm1 locus; (**B**) Cm10 locus; Lanes 1–36, samples used for microsatellite loci assessment; Lane M, molecular marker (20-bp ladder).

These polymorphic microsatellite markers would be useful tools for investigating genetic population structure and diversity to establish conservation strategy for this interesting and vulnerable species.

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