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Isolation and characterization of 50 nuclear microsatellite markers for *Cathaya argyrophylla*, a Chinese endemic conifer[1]

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- *Premise of the study*: Microsatellite primers were developed for the endangered *Cathaya argyrophylla* (Pinaceae) to investigate its genetic diversity and population genetic structure, as well as its evolutionary history.
- *Methods and Results*: Fifty dinucleotide microsatellite loci were identified in two populations. The number of alleles per locus ranged from 1 to 6, with a mean of 2.84. The observed and expected heterozygosities ranged from 0 to 0.889 and from 0 to 0.779, respectively.
- *Conclusions*: These markers will facilitate further studies on the population genetics and evolutionary history of *Cathaya argyrophylla*.

Key words: *Cathaya argyrophylla*; microsatellites; Pinaceae; population genetics; SSR.

Cathaya argyrophylla Chun & Kuang is an endangered conifer, with a total number of mature individuals less than 2 000 (Sun et al., 1994). Fossil evidence indicates that this species was once widely distributed in the Northern Hemisphere and has disappeared from North America, Europe, and most of Asia because of Late Tertiary climatic oscillations and Quaternary glaciation (Wang, 1990; Liu and Basinger, 2000). Currently, *C. argyrophylla* occurs only in several widely separated subtropical areas in southern China and has not undergone population expansion like most other conifers since the Quaternary glaciation (Wang, 1990; Wang and Ge, 2006). Notably, almost all extant *C. argyrophylla* populations face serious threats and a high risk of extinction because of habitat deterioration and loss (Xie and Chen, 1999; Wang and Ge, 2006). It is of great importance, therefore, to understand the evolutionary history and population genetics of this endangered species and to take concrete measures to conserve and recover its populations. To this end, efficient and highly resolved molecular markers are needed. Here, we report a set of novel polymorphic microsatellites for *Cathaya argyrophylla*, which provides useful markers for studying population genetics and evolutionary history as well as developing a conservation strategy for this species.

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METHODS AND RESULTS

Total DNA was digested from silica-gel-dried leaves using the CTAB method (Doyle and Doyle, 1987) and the construction of a microsatellite-enriched library followed Glenn and Schable (2005) with some modifications. Total DNA was digested with *Rsa* I and *Xmn* I (New England Biolabs, Ipswich, MA) and then ligated to the double-strand Super SNX-24 linker (forward 5′-GTT TAA GGC CTA GCTAGC AGA ATC-3′, reverse 5′-pGAT TCT GCT AGC TAG GCC TTA AAC AAA-3′). The ligated DNA was randomly linked to one of three single-strand bio-tinylated microsatellite probes (5′-$(CA)_{15}$-Biotin, 5′-$(GA)_{15}$-Biotin, 5′-$(AAT)_{15}$-Biotin). Hybridized DNA was captured by streptavidin-coated paramagnetic beads (Dynal Biotech Dynabeads M-280 Streptavidin, Oslo, Norway) and gathered with a magnetic particle-collecting unit (MPC, Dynal Biotech Dynal MPC-S, Dynal, Oslo, Norway). The enriched DNA was amplified using superSNX-24 linker-forward as a primer, and the product was purified, ligated into pGEM-T easy vector (Promega Corp., Madison, Wisconsin, USA) and cloned in Top 10 competent cells of *E. coli* (TransGen Biotech, Beijing, China). Three-hundred-four positive clones were selected and sequenced, and 208 (68%) contained SSRs. Of them, 146 sequences were selected for primer design with Primer premier 5.0 (Premier Biosoft International, Silicon Valley, California, USA) and finally 50 pairs of primers (Table 1) were chosen because they showed single and clear bands. The forward primer was labeled with one of the fluorescent dyes (FAM, TAMRA, or HEX) for polymorphism detection on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, California, USA).

Forty-nine individuals were sampled from two of the four separate glacial refugia (Wang and Ge, 2006): 31 trees from population WHD in the Dayao Mountains and 18 trees from population LLD in the Bamian Mountains. The number of alleles per locus (A), the observed and expected heterozygosity (H_O *and* H_E), and the deviations from Hardy–Weinberg equilibrium (HWE) were analyzed using POPGENE software (Yeh et al., 1997). The pairwise linkage disequilibrium (LD) was evaluated by the software FSTAT (Goudet, 1995).

The number of alleles per locus ranged from 1 to 6, with a mean of 2.84. Five loci in WHD and nine in LLD are monomorphic. However, only three loci were monomorphic across two populations, indicating a high level of polymorphism at the species level (Table 2). The observed and expected heterozygosity per locus ranged from 0 to 0.8889 (an average of 0.3714) and from 0 to 0.7794 (an average of 0.4102), respectively (Table 2). Nine loci (WZS04, WZS08, WZS12, WZS30, WZS34, WZS35, WZS36, WZS43, WZS48) in WHD and four loci (WZS15,

TABLE 1. Characteristics of 50 polymorphic microsatellite markers in *Cathaya argyrophylla.*

Locus	Primer sequence (5′–3′)	Repeat motif	Size range (bp)	Ta (°C)	GenBank Accession No.
WZS01	F:TTCAGAACCACAAGGACAAAT R:TGGACAAAACATAATAAAGGA	$(TC)_{17}ACTC(GT)_{19}$	313–339	48	GU140043
WZS02	F:TACTTTTCCCGTAGAACTCA R:CTCACATTGTAGCTTGGATT	$(AC)_{18}$	191–207	48	GU140044
WZS03	F:AATCTTGTTTGGCATTTCTC R:TGGGGTTTTAAGGTTGTGAC	$(CT)_{23}$	223–246	53	GU140045
WZS04	F:ATTTGATAGTTTCTTTCCATTG R:GTTGTTCGTTTTCTTATTTTGT	$(AC)_{17}$	192–198	48	GU140046
WZS05	F:ATCCATACCCTTTCTATCACCC R:GCTATAACTAAACTAATTTCCACCC	$(AC)_{25}$	163–189	53	GU140047
WZS06	F:TGAACAAATCCATAAAGACAT R:GATGAATCTGTCAATCTCGTC	$(AC)_{10}AA(AC)_{13}$	291–331	56	GU140048
WZS07	F:CACGAAGTGGGATTTGAA R:CGAATTTGCATCTTTATGTCTAT	$(CA_{)19}$	224–228	56	GU140049
WZS08	F:TGTCAAGGTCGTATGTTCA R:AATGGGGAAGTAGGTGTAT	$(TG)_{18}$	161–177	56	GU140050
WZS09	F:TGCACAAAATTAAAACAC R:CTAGAAAGCATAGCTCACT	$(AC)_{17}$	156–178	48	GU140051
WZS10	F:CCCTTTAAGTTTGGAACAA R:AGGATGGTGGGATGAATAG	$(AC)_{12}(AAC)_7$	180–183	53	GU140052
WZS11	F:AAAGTCATACACCACCACA R:GGTCTGACACTATTTCCCT	$(TC)_{18}CC(TC)_8$	200–218	56	GU140053
WZS12	F:CACATTCCATCCACATTTT R:AGAGCCTTCCCCTACTTTT	$(AG)_{12}$	180–184	56	GU140054
WZS13	F:AAGCCCAATGTTTAGACCA R:GCCCCTCCTAGATTAGTCC	$(AC)_{30}$	138–146	56	GU140055
WZS14	F:TTGGATACAAACCCATCTCACCTA R:GGAATGAACTGAAAATGTCACGAA	$(CA)_{11}(AT)_6$	159–169	57	GU140056
WZS15	F:AAAATTAGCTCATTCTTTACATAG R:GTTATACATATTGAAATCTAGGTG	$(AC)_{17}$	139–166	57	GU140057
WZS16	F:GAATTATCCATTTCTCCTCGTG R:CCTATGAATCTCCCAAAACTAC	$(CA)_{13}$	255–257	59	GU140058
WZS17	F:ATAGTCCTTGTCCCTTCTTCT R:CATAACTCACCAAATAGTTTCT	$(TA)_5(TG)_{13}$	246–250	57	GU140059
WZS18	F:CAGAGCCACTCTAAACCA R:GTAAACACTGCCTCCAAA	$(CA)_{19}$	242–266	50	GU140060
WZS19	F:ACAAATCTTCAACATAGTCCATCC R:CTCTTGCTAATTCGATGCCATAT	$(GC)_5(AC)_8$	405–409	59	GU140061
WZS20	F:CTAAATCAAACCCACTCCTCCA R:AACTCATTCCACCATTCCTCTA	$(CT)_{17}(GT)_{23}$	418–456	53	GU140062
WZS21	F:AAGTATAAGCATGAGCCCTCT R:GGTGTTCCAAAGTTAATCCA	$(GAT)_8$	313–319	50	GU140063
WZS22	F:TTTTGGTAGGGCTAGTTTTC R:ACTTGGGTTTCCATTTGATC	$(TG)_{18}$	300–314	53	GU140064
WZS23	F:AACTCCCTCACCACAATC R:GGAAATATCACCGAGACAC	$(CA)_8$	212–217	60	HM751233
WZS24	F:CAACCCTATTTTCCACTAA R:TCTATCTTGACAATCTACCCT	$(TG)_8C(GT)_4$	404–419	50	GU140066
WZS25	F:GATTCATTATTGGTTCTC R:ATCATCCTACATAGTCACAT	$(TG)_{14}$	263–290	46	GU140067
WZS26	F:GTAGGGTTCAGCTATTCAGG R:TTACTTGGTTTAGGGTTTCC	$(GT)_8(GA)_{13}$	248–254	60	HM751234
WZS27	F:TCTTGCTCCACAAATGTTG R:GAAGTTTAGGACCCGAAAA	$(CA)_{18}$	223–247	58	HM751235
WZS28	F:TGTAACTGGAGTATGTTCGTTT R:TGATATGATTCTTATGTGGTGAC	$(AG)_{11}$	361–367	60	HM751236
WZS29	F:GACCCACCTCCAACAGTTATTT R:TCTTGTCGTTCTCCCACTTTCT	$(TG)_{18}$	438–450	48	GU140071
WZS30	F:TTCTACTGGAAACCATCGC R:GCATAATAACAATCAAGGAAG	$(TG)_{25}$	147–189	53	HM751212
WZS31	F:ATTTGCTCAATGAACCCACCA R:CCGAGGCTTGGACTATGGTAA	$(CA)_8A(AC)_8$	130–153	55	HM751213
WZS32	F:TACAAGGAACTGTTGAAATGC R:GAAGAGTGGTGCTGGGGATAG	$(TA)_5(TG)_{10}$	424–426	50	HM751214
WZS33	F:GCCACAACATAGACAATCAACA R:TGCTAGTCAGGGGTTTTATCA	$(CA)_{19}$	246–263	58	HM751215
WZS34	F:TATAGGGGCGAGTCATTTA R:GGAAGCAGATAGTGAGAAGTA	$(TC)_{16}$	440–461	48	HM751216

TABLE 1. Continued.

Locus	Primer sequence (5′–3′)	Repeat motif	Size range (bp)	Ta (°C)	GenBank Accession No.
WZS35	F:GGTGATAAGTAAGAGGCTTGGGAGA R:TGGAAGAAGAAAAGAATGAGAAACG	$(GA)_{15}$	349–368	63	HM751217
WZS36	F:TCAAGGTCGTATGTTCAAAAC R:CAAATGGGGAAGTAGGTGTAT	$(TC)_{18}$	157–173	55	HM751218
WZS37	F:TATTTTGGGATTTTCCTTGG R:TTTATGCCTCCTTTATGGTC	$(CA)_{25}$	408–444	54	HM751219
WZS38	F:GTAAATGGGATTGGTGTA R:ACATAATGATAGAGTTTGGA	$(TG)_8$	360–368	50	HM751221
WZS39	F:GAGCACCAACCATAGGAAGA R:TTTAACCAAGGGCACATACC	$(GA)_{17}$	160–178	58	HM751222
WZS40	F:AATCTCACCTTAATTTCCTT R:GACTTACAAACACCTCGTT	$(CA)_{15}(TA)_5(GA)_8$	420–429	55	HM751223
WZS41	F:TCACTAAGAGGGAATGTTGAGAAT R:AGAACTGTGCGTAGCCTTGGAAAT	$(AG)_{15}$	351–353	61	HM751224
WZS42	F:GTGTAATTCCTTTCATGTTC R:TCTCCACCTCTTATTCCTA	$(TC)_{15}GCTCT(CA)_{13}$	434–452	52	HM751225
WZS43	F:CGCACAGTGTATCGTCTTGG R:TGGGGTTTCTGTCTTTTGATT	$(CA)_{10}(TA)_6$	104–108	53	HM751226
WZS44	F:GCAAGACTGCTCATAAAATA R:GAGGAGGTGAAGGATACAAA	$(CA)_{19}$	212–252	58	HM751227
WZS45	F:GTTTTCTTCGTTTCAGTTGTTCG R:CTAAGGGAAGGTTGTTGGGTTT	$(CT)_7$	440	60	HM751228
WZS46	F:ACTGCTCAATCAACGAAAT R:AAAGATAGGTCAAGATAAAGG	$(CT)_{14}$	299–334	50	HM751229
WZS47	ATAGAAGATGAAGACCAAAT CACCATAATGTCAATATGTAT	$(CA)_{11}$	144–152	48	HM751230
WZS48	F:GTTGGGTGAAATGGATGTTA R:TTTTGTAGTGGGATGACTCT	$(TG)_{12}$	250–258	51	HM751231
WZS49	F:CTGGCGGGTGATGGTT R:ACAGGGAAAAGGTTTAGATGA	$(AC)_{21}$	365	50	HM751220
WZS50	AGTAAATGAGTTTGCAGGTT CATCTAGCACAAGTAAGGGA	$(TG)_8$	361	51	HM751232

Ta, annealing temperature.

TABLE 2. Results of initial primer screening in *Cathaya argyrophylla.*

	WHD (N = 31)			LLD (N = 18)				WHD (N = 31)			LLD (N = 18)		
Locus	A	H_O	H_E	A	H_O	H_E	Locus	A	H_O	H_E	A	H_O	H_E
WZS1	6	0.7742	0.6822	5	0.7222	0.7778	WZS26	3	0.5	0.6198	1	0	0
WZS2	2	0.0714	0.0701	2	0.5000	0.5127	WZS27	3	0.4828	0.5245	2	0.3889	0.3222
WZS3	6	0.7419	0.7684	3	0.6250	0.621	WZS28	2	0.2414	0.3128	2	0.3333	0.2857
WZS4	2	0.0323	0.4045	2	0.1875	0.2722	WZS29	3	0.6129	0.6753	2	0.2941	0.2585
WZS5	6	0.5484	0.6177	1	0	0	WZS30	5	0.5667	0.6107	2	0.2353	0.5134
WZS6	4	0.3846	0.3341	3	0.7778	0.681	WZS31	2	0.5161	0.4654	5	0.5882	0.6488
WZS7	2	0.4516	0.4188	2	0.1667	0.1571	WZS32	2	0.2857	0.2987	2	0.6667	0.5079
WZS8	3	0.1724	0.4156	3	0.5000	0.4012	WZS33	2	0.5333	0.5085	3	0.6471	0.5508
WZS9	4	0.1613	0.2094	2	0.5000	0.5127	WZS34	3	0.0667	0.2418	5	0.3889	0.7540
WZS10	3	0.5333	0.5356	2	0.4444	0.4571	WZS35	6	0.6000	0.7085	3	0.6471	0.5704
WZS11	6	0.5484	0.5727	4	0.6111	0.7286	WZS36	3	0.1613	0.4426	3	0.3889	0.3984
WZS12	3	0.2000	0.4654	2	0.5556	0.5143	WZS37	5	0.7000	0.7333	2	0.5556	0.4127
WZS13	2	0.4516	0.4654	3	0.3889	0.3381	WZS38	2	0.5000	0.4627	1	0	0
WZS14	2	0.3462	0.3824	2	0.3889	0.3857	WZS39	3	0.1613	0.2565	2	0.3529	0.2995
WZS15	5	0.5667	0.6638	5	0.3889	0.7095	WZS40	3	0.5862	0.6515	3	0.6111	0.6079
WZS16	2	0.0345	0.0345	1	0	0	WZS41	2	0.1333	0.1266	2	0.1250	0.1210
WZS17	3	0.7222	0.6651	1	0	0	WZS42	5	0.4516	0.6145	2	0.5294	0.508
WZS18	3	0.1000	0.0977	3	0.5556	0.5	WZS43	2	0.0667	0.3638	2	0.4444	0.4127
WZS19	1	0	0	3	0.1667	0.5	WZS44	6	0.6000	0.7531	3	0.6667	0.5889
WZS20	5	0.7333	0.6932	5	0.8889	0.7794	WZS45	1	0	0	1	0	0
WZS21	2	0.2333	0.2096	1	0	0	WZS46	4	0.5000	0.6791	3	0.6111	0.627
WZS22	6	0.6552	0.7816	2	0.2222	0.2032	WZS47	4	0.5862	0.6582	4	0.125	0.6351
WZS23	1	0	0	2	0.6111	0.4365	WZS48	2	0	0.0655	5	0.4706	0.7005
WZS24	2	0.2258	0.2967	2	0.5294	0.5080	WZS49	1	0	0	1	0	0
WZS25	3	0.5172	0.4985	2	0.2778	0.2460	WZS50	1	0	0	1	0	0
							Mean	3.18	0.36116	0.4211	2.5	0.3816	0.39936

A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

WZS19, WZS34, WZS47) in LLD showed significant deviation from HWE ($P < 0.01$) as a result of heterozygote deficiency. No significant linkage disequilibrium was detected among pairs of loci in each population, suggesting that these microsatellites are independent markers and suitable for genetic studies on *C. argyrophylla* populations.

CONCLUSIONS

We present 50 nuclear microsatellite markers developed specifically from *C. argyrophylla*. Although previous studies detected general patterns of population genetics and potential refugia of this species (Ge et al., 1998; Wang and Ge, 2006), in-depth investigation of population history and dynamics has been restricted due to lack of efficient and highly resolved molecular markers. This set of novel markers represents a set of highly polymorphic, selectively neutral and codominant markers, and would be useful in our further studies on the mating system, gene flow, and the underlying mechanism of the endangerment of *C. argyrophylla*. Such information would help in developing plans for the recovery and management of this endangered species.

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