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Authors: Wang, Chunbo, Guo, Zhiyou, Huang, Xilian, and Huang, Lu

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF 15 MICROSATELLITE MARKERS FOR *CEPHALOTAXUS FORTUNEI* (CEPHALOTAXACEAE)[1]

CHUNBO WANG[2,4], ZHIYOU GUO[2], XILIAN HUANG[2], AND LU HUANG[3]

[2]Department of Life Sciences, Qiannan Normal College for Nationalities, Duyun 558000, People's Republic of China; and [3]State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, People's Republic of China

- *Premise of the study:* To survey population variation and the adaptive evolution of *Cephalotaxus fortunei* (Cephalotaxaceae), an endemic and endangered conifer in China, microsatellite markers were developed and characterized for this species.
- *Methods and Results:* Based on the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol, 15 microsatellite markers were developed for *C. fortunei*, 13 of which were polymorphic within a sample of 75 individuals representing five natural populations. The number of alleles per locus ranged from one to seven. The expected and observed heterozygosities were 0.108–0.738 and 0.000–1.000, respectively. Ten polymorphic loci were also successfully amplified in *C. oliveri*.
- *Conclusions:* These polymorphic loci provide a valuable tool for population genetic analysis of *C. fortunei*, which will contribute to its management and conservation.

Key words: Cephalotaxaceae; *Cephalotaxus fortunei*; cross-amplification; FIASCO; genetic analysis; microsatellite primers.

Cephalotaxus fortunei Hook. is a perennial, coniferous shrub or small tree belonging to the family Cephalotaxaceae. Endemic to China, *C. fortunei* is mainly distributed from the subtropical regions up to the northernmost Qinling Mountains and Huai River in central China, occurring in locations with an elevation between 200 and 3700 m (Zhou et al., 1997). Because it contains the anticancer alkaloid harringtonine, *C. fortunei* is important for medicinal use in treating leucocythemia (Shi et al., 2010). Its natural populations in China face threats of deforestation, other human-induced disturbances, and overexploitation. At present, *C. fortunei* is listed as a Category V threatened plant by the international Conifer Specialist Group (He et al., 1996). Thus, a deeper understanding of genetic variation and population structure of this species using polymorphic DNA markers will provide valuable information for developing conservation strategies.

In this study, we developed 15 microsatellite loci for *C. fortunei* using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) approach (Zane et al., 2002), and we also examined their ability to be cross-amplified in *C. oliveri* Mast.

METHODS AND RESULTS

Seventy-five individuals of *C. fortunei* from five populations were collected in its natural distribution area from 2014 to 2015, and voucher specimens were deposited at the herbarium of Qiannan Normal College for Nationalities (Appendix 1). Young and healthy leaves were preserved in silica gel. All samples were stored at −20°C until processed. Total genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol with −20°C propanone pretreatment to eliminate polysaccharides (Su et al., 1998).

The FIASCO method was used to develop microsatellite loci using one individual of *C. fortunei* from the Guizhou population (voucher: *CB Wang 201406, JP3* [QNCN]). Approximately 3 µg of DNA was digested with *Mse*I (New England Biolabs, Ipswich, Massachusetts, USA). The DNA digestion fragments were linked to an *Mse*I adapter pair (F: 5′-TACTCAGGACTCAT-3′, R: 5′-GACGATGAGTCCTGAG-3′) with T4 ligase at 4°C overnight (Pan et al., 2011). A diluted digestion-ligation mixture (1 : 10) was directly amplified using the following program: 95°C for 30 s, 60°C for 20 min, and 72°C for 1.5 min for 23 cycles with *Mse*I-N primers (5′-GATGAGTCCTGAGTAAN-3′). Then, we used 5′-biotinylated $(AC)_{15}$ and streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA) to hybridize and capture the PCR product (Miao et al., 2012). Enriched fragments were recovered with PCR amplification as described above, using *Mse*I-N as the primers. Purifying with a multifunctional DNA Extraction Kit (OMEGA Bio-Tek, Norcross, Georgia, USA), the PCR products were then ligated into pTA2 vector (Toyobo, Osaka, Japan) and transformed into *E. coli* DH5α competent cells. A total of 80 clones were selected by blue-white screening and tested by PCR using $M13^+/M13^-$ as primers. Seventy-six positive clones were chosen to be sequenced on an ABI Prism 3730 automated DNA sequencer (Invitrogen, Guangzhou, China). Out of the 76 clones, 27 clones contained simple sequence repeats, of which 12 were discarded because they were unsuitable for designing primers. Primers for the remaining 15 sequences were designed using Primer Premier 5.0 (Clark and Gorley, 2001).

We used 75 individuals from five populations to test the polymorphism of the newly developed primer pairs (Table 1). The PCR amplifications were performed in a 20-µL reaction containing 1 µL of genomic DNA, 2 µL of PCR buffer, 0.5 µL 10 mM each primer, 1 µL 10 mM dNTP mixture, and 1 unit *Taq* DNA polymerase (TaKaRa Biotechnology Co., Dalian, China). PCR profiles were as follows: an initial denaturation at 94°C for 5 min; followed by 35 cycles at 94°C for 45 s, annealing temperature for 30 s, extension at 72°C for 1 min; and final extension at 72°C for 10 min (Table 1). PCR products were electrophoresed on 6% polyacrylamide denaturing gels by silver staining using a 50-bp ladder. Thirteen of the 15 loci were found to be polymorphic. The sizes of all amplification products matched the expected lengths.

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[4]Author for correspondence: wchunb@mail2.sysu.edu.cn

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TABLE 1. Characteristics of 15 microsatellite loci for *Cephalotaxus fortunei*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
CF1	F: GCCCTAAACGCTTCTCAA R: CGGTACGGGATAGCAAGA	$(AC)_{17}$	129	54	KT832555
CF2	F: ACGATTCCCGAGATTCAT R: ACGGTCAGAGTTGTAGCG	$(TG)_{12}$	139	57	KT832556
CF3	F: CGGGTATTCCAGGGCTAA R: TCCGCGTTACGTCAGGTT	$(AC)_{13}TGC(TC)_{18}$	207	54	KT832557
CF4	F: CCGCGTGGGACATTCTAG R: CCATGGACTTGGGCAACA	$(GT)_{15}$	122	53.5	KT832558
CF5	F: GTAGAAAACTTCACAGGGAC R: ACACGCGATGTGCTAAAC	$(CA)_{19}$	114	56	KT832559
CF6	F: CTCAGGCACTGGGCAATC R: CGCTGTAGGCGTCGATTT	$(TG)_{26}$	204	54.5	KT832560
CF7	F: ATTCCCGAACTTCCCAGG R: CTCACAGTAAACGGCGTC	$(AC)_{25}$	122	57	KT832561
CF8	F: GGCAATCCCTTGGGTTAG R: CTAAAGCCTCTGGGACGC	$(AC)_{29}$	105	53	KT832562
CF9	F: CTAAGCACGACTGGACAAAG R: GGCGCTGAATCCGACACT	$(CA)_{11}$	102	55	KT832563
CF10	F: AGCGCCCATTTGAAAGTA R: TGCCGATTAGTGGAAGTGTA	$(TC)_9AA(CA)_{12}$	258	58	KT832564
CF11	F: CGTAGGCAACCCGCTTTC R: GGCGATCCGATTGACACC	$(TG)_{23}$	124	55	KT832565
CF12	F: CCCGTAAGTGACTGTCCG R: TTAGCCGTTGAAATGTGC	$(AC)_{21}$	106	56	KT832566
CF13	F: ATCCGATTTCGCCGTGTT R: CTTGACGGTGCCATTGTG	$(GT)_{17}$	125	57	KT832567
CF14*	F: CTTACCCAGGCAAATGTG R: GTATCGGCCCTTTGGTAG	$(GT)_8CTA(CA)_7$	103	54	KT832568
CF15*	F: TACCTCGGGAGACATCAT R: CTCGTTAGTAGCCCGTTGG	$(TG)_{16}$	141	56	KT832569

Note: T_a = annealing temperature.
* Monomorphic loci.

The effective number of alleles (A_e), observed heterozygosity (H_o), expected heterozygosity (H_e), and departure from Hardy–Weinberg equilibrium (HWE) were estimated by GenAlEx version 6.4 (Peakall and Smouse, 2006). Linkage disequilibrium (LD) across all populations was tested using GENEPOP version 4.0.10 (Rousset, 2008). The occurrence of null alleles was investigated using MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004). The number of alleles per locus varied from one to seven, with a total of 247 alleles scored across the 75 individuals. H_e and H_o ranged from 0.108 to 0.738 and from 0.000 to 1.000, respectively. All loci were found to be in HWE. No null alleles were detected, and no significant LD ($P > 0.05$) was detected (Table 2). Furthermore, all 15 loci were successfully amplified in 75 individuals of *C. oliveri* from five populations (Appendix 1). Of these, 10 loci (CF1–CF10) were polymorphic (Table 3).

CONCLUSIONS

In this study, we developed 15 microsatellite loci for *C. fortunei*, 13 of which were polymorphic. The genetic information based

TABLE 2. Genetic diversity of 13 polymorphic microsatellite loci in *Cephalotaxus fortunei* populations.[a]

	Enshi population ($N = 15$)				Suining population ($N = 15$)				Jinping population ($N = 15$)				Jinggangshan population ($N = 15$)				Shiping population ($N = 15$)			
Locus	A	A_e	H_o	H_e	A	A_e	H_o	H_e	A	A_e	H_o	H_e	A	A_e	H_o	H_e	A	A_e	H_o	H_e
CF1	4	3.437	0.323	0.235	5	4.274	0.6020	0.474	4	3.7540	0.383	0.277	2	1.372	0.247	0.209	2	1.842	0.488	0.421
CF2	5	3.573	0.466	0.401	4	3.527	0.386	0.305	2	1.463	0.707	0.635	3	2.889	0.600	0.478	6	4.791	0.873	0.598
CF3	2	1.331	0.197	0.114	2	1.510	0.208	0.173	3	2.582	0.538	0.281	5	3.996	0.584	0.501	4	3.588	0.731	0.627
CF4	7	5.716	1.000	0.738	6	5.0282	0.877	0.539	3	2.375	0.436	0.374	3	2.037	0.373	0.286	3	2.736	0.217	0.190
CF5	3	2.554	0.252	0.243	2	1.742	0.273	0.209	3	2.486	0.211	0.207	2	1.814	0.137	0.126	3	1.477	0.562	0.392
CF6	3	2.764	0.485	0.386	5	4.337	0.409	0.317	3	2.371	0.319	0.188	2	1.753	0.281	0.194	4	3.522	0.613	0.485
CF7	2	1.724	0.536	0.524	3	2.646	0.434	0.282	5	4.877	0.485	0.429	3	1.344	0.813	0.528	5	3.985	0.741	0.677
CF8	4	3.015	0.632	0.206	3	2.127	0.211	0.193	6	4.835	0.947	0.487	1	1.000	0.000	0.108	2	1.371	0.318	0.251
CF9	3	2.544	0.530	0.381	4	3.486	0.785	0.274	4	2.742	0.374	0.218	3	2.378	0.713	0.454	2	1.319	0.301	0.217
CF10	3	2.322	0.274	0.197	3	2.712	0.277	0.176	3	2.615	0.598	0.472	3	2.086	0.251	0.136	2	1.436	0.462	0.366
CF11	4	3.706	0.522	0.209	3	2.854	0.306	0.298	1	1.000	0.000	0.108	4	2.792	0.815	0.630	4	3.091	0.750	0.519
CF12	4	3.418	0.387	0.328	3	2.371	0.299	0.187	2	1.784	0.193	0.180	3	2.514	0.277	0.217	4	3.802	0.800	0.718
CF13	4	3.273	0.623	0.544	3	2.668	0.275	0.204	5	4.613	0.828	0.382	5	4.281	0.626	0.464	4	3.517	0.732	0.635

Note: A = actual number of alleles; A_e = effective number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size for each population.
[a] Locality and voucher information is available in Appendix 1.

TABLE 3. Genetic diversity in five *Cephalotaxus oliveri* populations using 10 polymorphic microsatellite loci originally developed in *C. fortunei*.[a]

	Changyang population (N = 15)				Hupingshan population (N = 15)				Fanjingshan population (N = 15)				Anfu population (N = 15)				Daweishan population (N = 15)			
Locus	A	A_e	H_o	H_e	A	A_e	H_o	H_e	A	A_e	H_o	H_e	A	A_e	H_o	H_e	A	A_e	H_o	H_e
CF1	3	2.766	0.439	0.382	5	3.638	0.2040	0.126	3	2.5270	0.205	0.193	2	1.771	0.385	0.218	2	1.426	0.536	0.412
CF2	5	3.432	0.628	0.571	3	2.757	0.527	0.483	2	1.343	0.284	0.205	6	4.648	1.000	0.831	3	2.825	0.429	0.375
CF3	4	2.488	0.482	0.276	2	1.436	0.266	0.214	5	3.719	0.799	0.510	3	2.731	0.426	0.304	4	3.463	0.671	0.482
CF4	5	3.653	0.927	0.803	4	2.7882	0.726	0.548	3	2.380	0.372	0.274	5	3.547	0.418	0.373	3	2.319	0.317	0.202
CF5	3	2.072	0.372	0.210	3	2.382	0.353	0.213	2	1.826	0.179	0.137	2	1.380	0.173	0.137	4	2.331	0.828	0.746
CF6	3	1.364	0.518	0.454	4	3.738	0.536	0.437	3	2.737	0.257	0.208	4	3.283	0.218	0.148	3	2.2523	0.602	0.560
CF7	4	3.632	0.7398	0.571	3	2.442	0.267	0.189	5	4.566	0.703	0.542	2	1.739	0.852	0.727	4	3.092	0.718	0.592
CF8	4	3.281	0.835	0.706	1	1.000	0.000	0.116	5	4.201	0.808	0.746	2	1.514	0.177	0.119	4	3.304	0.892	0.539
CF9	3	2.737	0.243	0.217	5	4.237	0.845	0.677	4	3.782	0.527	0.327	3	2.747	0.727	0.542	2	1.109	0.283	0.203
CF10	3	2.455	0.306	0.258	2	1.536	0.252	0.165	4	3.091	0.361	0.296	3	2.806	0.317	0.231	3	2.377	0.737	0.586

Note: A = actual number of alleles; A_e = effective number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size for each population.

[a] Locality and voucher information is available in Appendix 1.

on these newly developed microsatellite loci will contribute to the management and conservation of *C. fortunei*. In addition, the successful cross-species amplification of the loci in *C. oliveri* implies that they will provide an opportunity to further investigate the adaptive evolution of *Cephalotaxus* species.

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APPENDIX 1. Geographic location and voucher information of each population for *Cephalotaxus fortunei* and *C. oliveri* in this study. All voucher specimens were deposited at the herbarium of Qiannan Normal College for Nationalities (QNCN).

Species	Population	Geographic coordinates	Voucher specimens
Cephalotaxus fortunei	Enshi, Hubei Province	30°17′N, 109°23′E	*CB Wang 201406*, *ES1*
	Suining, Hunan Province	26°30′N, 109°30′E	*CB Wang 201406*, *SN2*
	Jinping, Guizhou Province	26°41′N, 109°11′E	*CB Wang 201406*, *JP3*
	Jinggangshan, Jiangxi Province	26°35′N, 114°08′E	*CB Wang 201406*, *JGS4*
	Shiping, Yunnan Province	23°43′N, 102°25′E	*CB Wang 201406*, *SP5*
Cephalotaxus oliveri	Changyang, Hubei Province	30°17′N, 109°23′E	*ZY Guo 201311*, *CY1*
	Hupingshan, Hunan Province	26°30′N, 109°30′E	*ZY Guo 201311*, *HPS2*
	Fanjingshan, Guizhou Province	26°41′N, 109°11′E	*ZY Guo 201311*, *FJS3*
	Anfu, Jiangxi Province	26°35′N, 114°08′E	*ZY Guo 201311*, *AF4*
	Daweishan, Yunnan Province	23°43′N, 102°25 ′E	*ZY Guo 201311*, *DWS5*