Development of Microsatellite Loci for Cyclocarya paliurus (Juglandaceae), A Monotypic Species in Subtropical China

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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE LOCI FOR *CYCLOCARYA PALIURUS* (JUGLANDACEAE), A MONOTYPIC SPECIES IN SUBTROPICAL CHINA[1]

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- *Premise of the study:* Microsatellite loci were developed for a monotypic species endemic to subtropical China, *Cyclocarya paliurus*, to help infer the evolutionary histories of ancient monotypic genera in subtropical China.
- *Methods and Results:* Using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol, 28 primer sets were identified in two wild populations. All loci were polymorphic, with the number of alleles per locus ranging from two to eight. The expected and observed heterozygosities ranged from 0.153 to 0.802 and from 0 to 0.750, respectively. The transferability of the 28 primer pairs was tested on *Juglans regia*, *Pterocarya stenoptera*, and *Platycarya strobilacea*. Twenty-one (75.0%), 22 (78.6%), and 15 (53.6%) markers were successfully amplified in *J. regia*, *P. stenoptera*, and *P. strobilacea*, respectively.
- *Conclusions:* These loci will be useful for in-depth analysis of genetic diversity and phylogeographical variation throughout the distribution range of *C. paliurus.*

Key words: *Cyclocarya paliurus*; Juglandaceae; microsatellite marker; population genetics.

Subtropical China includes a host of taxa that are presumed to be phylogenetically primitive, with many occurring as monotypic taxa. *Cyclocarya paliurus* (Batalin) Iljinsk. is a medium-sized (up to 30 m) deciduous tree growing in montane forests (ca. 400–2500 m a.s.l.) (Lu et al., 1999). This species has a scattered distribution in subtropical China and is the only representative in the genus *Cyclocarya* Iljinsk., often known as a "living fossil" (Ying et al., 1993; Wu et al., 2003). Therefore, it is an ideal candidate for inferring the evolutionary histories of ancient monotypic genera in subtropical China, e.g., inferring refugial locations and the predominant pattern of migration that has led to their present geographical range. Moreover, *C. paliurus* has multiple commercial uses and is widely exploited. The leaves taste sweet and are used as an ingredient in functional foods or beverages in China. *Cyclocarya paliurus* has preventive effects against hypolipidemia and diabetes mellitus and improves mental efficiency, antihypertensive action, and immunomodulation (Kurihara et al., 2003; Jiang et al., 2006; Xie et al., 2006). The increasing demand for *C. paliurus* in tea production and medical uses has already resulted in a rapid decline of population size and local extinctions at many natural places (D.-M. Fan, personal observation). Consequently, it is necessary to quantify patterns of genetic diversity and gene flow to develop adequate management strategies for the long-term conservation of this species and to ensure the rational use of wild genetic resources. In this study, we isolate and characterize 28 novel microsatellite loci for *C. paliurus*, which is the first step toward investigating the genetic diversity and spatial genetic structure of this species.

METHODS AND RESULTS

We sampled 24 *C. paliurus* trees in two natural populations (Jinggangshan, Jiangxi: 26.51707°N, 114.09920°E, n = 12; Yuyao, Fujian: 29.75192°N, 121.08393°E, n = 12). Voucher specimens for each population were deposited in the Jiangxi Agricultural University herbarium (accession no.: JXAU35129 and JXAU35158). Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987), and microsatellites were isolated using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol (Zane et al., 2002). A single individual from the Jinggangshan population was used to prepare the microsatellite-enriched library. Total genomic DNA (ca. 250–500 ng) was completely digested with 2.5 U of *Mse*I restriction enzyme and then ligated to an *Mse*I AFLP adapter (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) using T4 DNA ligase (MBI, Fermentas, Vilnius, Lithuania). The digested-ligated fragments were diluted in a ratio of 1 : 10, and 5 μL of them were used for amplification reactions with adapter-specific primers (5′-GATGAGTCCTGAGTAAN-3′/5′-TTACTCAGGACTCATCN-3′). The amplified DNA fragments (200–800 bp) were enriched for simple sequence repeats by magnetic bead selection with a 5′-biotinylated probe [$(AG)_{15}$ or $(AC)_{15}$, respectively]. Enriched DNA fragments were reamplified with *Mse*I-N primers. The PCR products were purified using SanPrep Column DNA Gel Extraction Kit (Sangon Bio-Tech, Shanghai, China). Purified DNA fragments were ligated into pGEM-T Easy Vector (Promega Corporation, Madison, Wisconsin, USA), and then transformed into DH5α competent cells (Tiangen Biotech, Beijing, China). The positive clones were tested by PCR using vector primers T7/Sp6 and primers $(AC)_{10}$/$(AG)_{10}$. In total, 337 clones with positive inserts were sequenced with an ABI PRISM 3730xl DNA sequencer (Applied Biosystems, Carlsbad, California, USA).

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TABLE 1. Characteristics of 28 microsatellite primers developed in *Cyclocarya paliurus*.

Locus	T_a (°C)	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	Transferability[a]	GenBank accession no.
CYC007	58	F: ATTGGATGAGGTCTTGTC R: TTCTACTCGCTTAGTCCC	$(CT)_9$	149	0, 0, 0	JX575769
CYC010	56	F: TAACAAAGCGAGTATAAG R: AAAGGCTAACATAGAAC	$(AG)_{10}$	203	1, 0, 0	JX575770
CYC015	62	F: ACCCCTCAA GTCCCACCA R: CCAGATACACATGCACAC	$(CT)_{11}$	178	2, 1, 2	JX575771
CYC019	61	F: ATTCCCCACCCCCATCTC R: CTCCTCCAGCGCACATAA	$(CT)_8$	201	0, 1, 1	JX575772
CYC029	56	F: CCTAATCTTCTCCCCTCG R: GAGAATAAGAGCACCACC	$(CT)_8$	122	0, 0, 0	JX575773
CYC036	57	F: ATCGTCCTGGTG ATGTTG R: AGGTCCTCCTTCCTTTGG	$(AC)_7$	167	2, 1, 0	JX575774
CYC045	49	F: GCAAAACATTCTTAGG R: ACTTGGTCA AATAGTC	$(GT)_6$	143	2, 1, 1	JX575775
CYC049	59	F: TGCCTCAATCCCAAAGAC R: AATTACGCCGAAGGGGTC	$(TG)_7(AG)_7$	208	2, 1, 0	JX575776
CYC052	60	F: CCACTTCGTGATCTGCCG R: AGGGAAAGAAAGCGAGAT	$(CT)_9$	125	1, 3, 0	JX575777
CYC053	59	F: AGATGGCTTTTCAGATTTG R: CGGAAACTTGAATCAGAG	$(CT)_{12}$	105	1, 2, 1	JX575778
CYC055	58	F: CTGGCACGCACAATACAC R: CCA AAAAGGGTTGAGCTT	$(CT)_{10}$	109	0, 0, 0	JX575779
CYC059	60	F: GCTGATGGTAATGGTTTTTAG R: ACA AAACCGACTGACAACAA	$(CT)_{10}(CTCTGT)_5$	175	1, 1, 1	JX575780
CYC060	62	F: CCTCAAGTCTGTGGCTCT R: GAACCGAAGCCAGTAAAG	$(CT)_7$	133	1, 1, 0	JX575781
CYC062	51	F: GTGAGAGAATGAAATGAA R: TATGTGATTAGATTGAGC	$(CT)_{12}$	131	1, 1, 1	JX575782
CYC074	59	F: TAACGGAGACGGATAAGG R: ACGCCCTAA AATGTGACT	$(AG)_6$	136	0, 0, 0	JX575783
CYC078	57	F: TGAGGGAGAGCGAGGAT R: CGTTACTGTAGCGGTTTG	$(AG)_5(AG)_5$	145	1, 2, 1	JX575784
CYC083	54	F: ACCCAA AAGAAAAGCA R: CGGTGAAATCTACTCCAA	$(AG)_6AA(AG)_7$	103	0, 0, 0	JX575785
CYC092	53	F: AAGGAGAAAGAGAAAAC R: AGAACTGGAGAGGCGAAC	$(AG)_7$	129	2, 1, 2	JX575786
CYC095	62	F: TGGAACTGGGAACGGTG R: ACCCCCTTCGTCGTCTTT	$(GA)_8$	151	2, 1, 1	JX575787
CYC099	60	F: TGGAGGTGAGACTTGCC R: AGGGTCACCATTAGATCATT	$(AG)_{10}$	209	1, 1, 0	JX575788
CYC106	60	F: GAGAGAGAGAGAGAGAGTG R: CCAGGACAGGGGTAGACA	$(AG)_{13}(GT)_5$	143	1, 1, 1	JX575789
CYC114	53	F: AAACTCTCTCTTTCTCAC R: CGTTGGTATTTAGGTCTA	$(AC)_{11}AT(AC)_{17}$	155	1, 2, 1	JX575790
CYC125	53	F: CGTAATGGAATTTAGTCC R: GAATACCACCAATCCTTT	$(CT)_{19}$	189	0, 2, 0	JX575791
CYC129	57	F: GTTATGCTAAAGCCTCGC R: GAGGAAAGAAAGAGTTGG	$(CT)_{11}$	136	1, 1, 2	JX575792
CYC130	57	F: TGCCAAGAGTGACAGATT R: GTTGATGATAGTTTGTAGAG	$(CT)_{13}(AC)_{11}$	223	1, 1, 2	JX575793
CYC132	65	F: AGCCACCGCTAGGAAGCA R: GGGCGTTACAGTGGGAGA	$(CT)_{12}$	122	1, 1, 1	JX575794
CYC148	59	F: TCCTCCACTTCCAATGAT R: AGAGGAGCAAACAAACAT	$(CT)_{17}$	196	1, 1, 1	JX575795
CYC150	60	F: AGAGATTAGCTCGGGTCT R: GATCCA AAACTGAAGGGA	$(TG)_{13}(AG)_{15}$	126	4, 2, 1	JX575796

Note: T_a = annealing temperature when run individually.

[a]Number of alleles detected in cross-amplification of *Juglans regia*, *Pterocarya stenoptera*, and *Platycarya strobilacea*.

A total of 153 sequences contained microsatellite repeats, and 137 with relatively long flanking regions were used to design primers using OLIGO 7.0 software (Rychlik, 2007).

Polymorphism of all loci with newly designed primer pairs was assessed with all 24 individuals sampled. The PCR reactions were performed in a 20-μL reaction volume containing 50–100 ng of genomic DNA, 0.5 μM of each primer, and 10 μL 2× *Taq* PCR MasterMix (0.1 U *Taq* polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl [pH 8.3], 100 mM KCl, and 3 mM $MgCl_2$; Tiangen Biotech, Beijing, China). PCR amplifications were conducted under the following conditions: 95°C for 3 min followed by 32–35 cycles at 94°C for 45 s, at the annealing temperature for each specific primer (optimized for each locus, Table 1) for 45 s, 72°C for 45 s; and a final extension step at 72°C for 5 min. PCR products were separated by 8% nondenaturing PAGE gel and stained with a silver-staining method. A portion of PCR products were checked using QIAxcel for capillary gel electrophoresis (QIAGEN, Düsseldorf, Germany).

TABLE 2. Results of initial primer screening in populations of *Cyclocarya paliurus*.

	Jinggangshan population (N = 12)			Yuyao population (N = 12)		
Locus	A	H_e	H_o	A	H_e	H_o
CYC007	3	0.538	0.333	2	0.463	0.000
CYC010	3	0.403	0.167	5	0.417	0.250
CYC015	4	0.580	0.083	2	0.278	0.000
CYC019	2	0.153	0.000	2	0.180	0.000
CYC029	4	0.413	0.167	2	0.219	0.000
CYC036	2	0.444	0.000	2	0.486	0.167
CYC045	3	0.628	0.182	3	0.486	0.000
CYC049	2	0.153	0.000	4	0.462	0.083
CYC052	2	0.153	0.000	4	0.608	0.083
CYC053	2	0.153	0.167	2	0.375	0.500
CYC055	5	0.722	0.333	4	0.733	0.250
CYC059	4	0.618	0.333	4	0.573	0.167
CYC060	3	0.594	0.250	4	0.663	0.083
CYC062	2	0.500	0.667	2	0.486	0.333
CYC074	2	0.434	0.455	2	0.469	0.750
CYC078	4	0.583	0.000	3	0.569	0.167
CYC083	4	0.358	0.417	8	0.740	0.250
CYC092	2	0.483	0.091	2	0.375	0.333
CYC095	2	0.219	0.250	3	0.403	0.500
CYC099	2	0.413	0.250	2	0.153	0.000
CYC106	2	0.278	0.167	3	0.594	0.083
CYC114	2	0.219	0.250	2	0.165	0.182
CYC125	4	0.681	0.000	6	0.750	0.167
CYC129	3	0.611	0.250	3	0.517	0.083
CYC130	4	0.413	0.417	7	0.802	0.417
CYC132	4	0.635	0.083	5	0.764	0.417
CYC148	3	0.426	0.333	7	0.778	0.750
CYC150	4	0.622	0.083	4	0.726	0.250

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

Among the 137 primer pairs, 28 successfully amplified in all samples. All 28 primer pairs displayed polymorphism. Standard genetic diversity parameters, e.g., the number of alleles per locus, expected heterozygosity, and observed heterozygosity, were calculated using the package GENEPOP (version 4.0; Raymond and Rousset, 1995). The number of alleles per locus ranged from two to eight, with a mean of 3.3. The expected and observed heterozygosities ranged from 0.153 to 0.802 and from 0 to 0.750, respectively (Table 2). The marker transferability of the polymorphic primer pairs was tested on three closely related species, *Juglans regia* L., *Pterocarya stenoptera* C. DC., and *Platycarya strobilacea* Siebold & Zucc. (three individuals for each species), using the same PCR conditions as previously described. Leaf samples of these three species were collected from cultivated trees in Jiangxi Agricultural University, Nanchang, China (28.76073°N, 115.82740°E; voucher no. JXAU35160–JXAU35162). Twenty-one markers (75.0%) were successfully amplified in *J. regia*, 22 (78.6%) in *P. stenoptera*, and 15 (53.6%) in *P. strobilacea* (Table 1).

CONCLUSIONS

The 28 microsatellite markers developed in this study will be useful for detection of genetic diversity in *C. paliurus* populations and elucidation of population dynamics. These markers will also help to develop viable strategies for the conservation and management of this monotypic genus. In addition, more than half have been successfully amplified in three closely related species; thus, these markers may represent a set of useful molecular tools for population genetic studies in other species of Juglandaceae.

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