Isolation and Characterization of Microsatellite Markers in the Lepisorus clathratus Complex (Polypodiaceae)

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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE MARKERS IN THE *LEPISORUS CLATHRATUS* COMPLEX (POLYPODIACEAE)[1]

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- *Premise of the study:* Microsatellites were designed and characterized in the Sino-Himalayan fern *Lepisorus clathratus* complex (Polypodiaceae) to further study the phylogeography and reproductive ecology of this species.
- *Methods and Results:* From a genomic library obtained by next-generation sequencing, 10 polymorphic and six monomorphic microsatellite loci were developed. In one population of *L. clathratus* from Taibaishan in central China, the number of alleles observed for these microsatellites ranged from seven to 29, and observed and expected heterozygosity ranged from 0.463 to 0.919 and from 0.797 to 0.947, respectively. Cross-amplification in other taxa within this complex was successful, but cross-amplification was poor for other congeneric species.
- *Conclusions:* This set of newly developed microsatellite markers will be useful for assessing genetic diversity, population structure, and mating system, and to infer polyploid origin in the *L. clathratus* complex.

Key words: ferns; *Lepisorus clathratus* complex; microsatellites; next-generation sequencing; polyploid origin; Polypodiaceae.

Lepisorus clathratus (C. B. Clarke) Ching (Polypodiaceae) is an alpine fern species complex, mainly distributed in the Hengduan Mountains, the Qinghai–Tibetan Plateau and adjacent areas, and in other mountain regions of high latitude in northern China, Russia, and Japan. The monophyly of the *L. clathratus* complex has been confirmed by phylogenetic study of the genus (Wang et al., 2010). However, morphological variation, especially of the two types of sporangia (dehiscent and indehiscent), has resulted in confusing taxonomic treatments. For example, in the *Flora of China*, six species of the *L. clathratus* complex are recorded: *L. clathratus*, *L. thaipaiensis* Ching & S. K. Wu, *L. crassipes* Ching & Y. X. Lin, *L. albertii* (Regel) Ching, *L. waltonii* (Ching) S. L. Yu, and *L. likiangensis* Ching & S. K. Wu (Qi et al., 2013). Moreover, the indehiscent sporangia may promote self-fertilization by reducing the ability of spores to spread out. The hypothesis that these two types of sporangia have different mating systems needs to be tested by suitable molecular tools, such as microsatellite markers. In addition, the phylogeography of the *L. clathratus* complex has been reconstructed with two chloroplast DNA regions (Wang et al., 2011), but these lack complementary nuclear data. Another recent study (Wang et al., 2012) preliminarily confirmed the occurrence of polyploidy and hybridization in the *L. clathratus* complex, but its polyploid origin has yet to be studied.

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Microsatellite markers are considered the most suitable for genetic studies because they are codominant, highly polymorphic, and have abundant, specific, and uniformly distributed loci in plant genomes (Mantello et al., 2012). Simple sequence repeat (SSR) markers are versatile molecular tools for ferns to solve the problem of inferring phylogeography or population genetics (Jiménez et al., 2008) and can be used to infer allopolyploid or autopolyploid origin (Palop-Esteban et al., 2012). However, to date, no microsatellite markers have been developed in the *L. clathratus* complex. The aim of the current study was to isolate a set of microsatellite markers in the *L. clathratus* complex to facilitate further study of its genetic structure, gene flow pattern, mating system, and polyploid origin.

METHODS AND RESULTS

Sampling these plants was difficult because populations of this species are usually very small, owing to the extremely alpine habitats. Therefore, we paid particular attention to large populations. Additionally, because polyploidy is widespread in this species, we focused on diploid individuals, for which genotyping was more easily accomplished. According to the requirements noted above, a population of 41 diploid individuals (determined by chromosome counting and flow cytometry, unpublished data) of *L. clathratus* (regular sporangia type) was collected at Taibaishan, Shaanxi, China, with a minimum interval of 50 m between individuals, to avoid sampling the same clone. Total DNA was extracted from silica gel–dried leaves using the Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China). One individual was used for next-generation sequencing (NGS). The NGS libraries were generated using aliquots of ca. 10 μg of genomic DNA and sequenced on the Roche 454 GS-FLX Titanium platform (454 Life Sciences, Branford, Connecticut, USA). The obtained sequence reads were assembled using Newbler 2.6 (Roche Diagnostics, Mannheim, Germany) with a 96% minimum overlap identity. In total, 440 Mbp of sequence reads were obtained. Dinucleotide and trinucleotide repeats of more than four iterations were searched using the Perl program "SSR_finder.pl" (Tóth et al., 2000; Yu et al.,

TABLE 1. Characteristics of 16 nuclear microsatellite loci isolated from the *Lepisorus clathratus* complex.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Fluorescent label	Allele size range (bp)	GenBank accession no.
LC017	F: CCAATATCAAAACTCATTGTGC	$(TG)_{36}$	FAM	123–295	KX171640
	R: GCTTACTTGCTACACCCAAGG				
LC019	F: TAGTAGCAGACGAGACAAGCAA	$(CA)_{12}$	HEX	110–174	KX171641
	R: GACAATCATTTGTTGTGCATTC				
LC038	F: TACTTGTGTAAATGACACGGGA	$(TG)_{28}$	TAMRA	213–285	KX171642
	R: AAATGCGAGTGAGAGAAAGAGT				
LC065	F: ATATAGCTTGCGTGAAACACCT	$(AG)_{19}$	HEX	114–202	KX171643
	R: CGTCATTTGAGTAAAGAGGGAG				
LC067	F: TTTTGCTTTCTGTTGGTTAGGT	$(AG)_{24}$	TAMRA	131–237	KX171644
	R: ATGAGCATCCCTCACATAGAAG				
LC068	F: GCATTAATGGGCTTTTCCTT	$(AG)_{21}$	HEX	125–181	KX171645
	R: AGTGTGGGATCTGTGGTAGG				
LC089	F: CATTATTTTGGCTACATGGCTT	$(AG)_{20}$	TAMRA	163–259	KX171646
	R: ACATTGTGTCCCCAATATCAAT				
LC090	F: GCATACGTAAGTGGGTGGAG	$(AG)_{21}$	FAM	107–207	KX171647
	R: TTTGCTTTGCCCTCTCTATG				
LC092	F: CTCCATGCTGACCCTTACAT	$(AG)_{24}$	HEX	230–330	KX171648
	R: AAAGAGGAGAGCGCTTCAAT				
LC105	F: AAACTGCATGGATGTGACAG	$(AG)_{27}$	TAMRA	179–229	KX171649
	R: GTAACGGGAGCTAGGGAAAG				
LC002	F: CCATGAAAAGGTATAGAATGGG	$(CA)_{25}T(CA)_2AT(CA)_{44}$	FAM	273	KX171650
	R: CCCAACAGATGTAATGGACTTT				
LC009	F: CCTTGAGGTGCTTGTAGGTAAG	$(TG)_{21}$	HEX	163	KX171651
	R: ACAAACTCAAAGCAGTGTAGCA				
LC029	F: AGATGGGTACGAAGGATTACCT	$(AG)_{22}$	TAMRA	169	KX171652
	R: TGAGCCACTCAACTAACACTTG				
LC043	F: AGTTTGTTGGTGGTTGTGCT	$(TG)_{14}$	FAM	238	KX171653
	R: TCTCCCAGGTGATGATGAGT				
LC073	F: GCATGAGCCATGTCGAGT	$(AG)_{25}$	HEX	160	KX171654
	R: ATCCATTCTCTGGCTCTCACT				
LC099	F: CGTCTATGTAGGAAATTGGAGC	$(AG)_{16}$	TAMRA	225	KX171655
	R: TTCTAGGTTCCTTTCTCCCTTC				

[a]Annealing temperature for all reactions was 58°C.

2011). A pair of primers flanking each repeat was designed to amplify the fragment containing repeats using Primer3 (Untergasser et al., 2012). The optimal primer size was set to a range of 18–26 bases and the optimal melting temperature to 58°C. The optimal product size was set to 100 to 400 bp, and the remaining parameters were left as the default settings. In 10 randomly chosen individuals, we tested 109 primer pairs for amplification using the PCR conditions described below, and 22 of them successfully amplified a single band. PCR reactions were performed in a total volume of 20 µL containing 10 µL of 2× *Taq* PCR MasterMix (500 µM dNTP, 3 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase, and 20 mM Tris-HCl; Tiangen Biotech), ca. 25 ng of DNA, and 0.2 µM each of forward primer and reverse primer. The PCR process consisted of the following steps: predenaturation (10 min at 94°C); 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 58°C), and extension (30 s at 72°C); and a final extension of 7 min at 72°C using a Veriti 96-Well Thermal Cycler (Applied Biosystems, Waltham, Massachusetts, USA). To confirm the sequence of each of the 22 primer pairs and their PCR products, we performed cloning with the pEASY-T3 Cloning Kit (Transgen Biotech, Beijing, China). For each individual, four clones were sequenced using primer M13F on an ABI3730 automatic sequencer (Applied Biosystems). Sixteen primer pairs containing real SSRs were confirmed.

The 16 primer pairs were further tested for genotyping all individuals using the 5′ fluorescence-labeled forward primers (FAM, HEX, or TAMRA). The PCR reagents used and thermal cycler program were the same as described above. PCR products were run on an ABI 3730XL sequencer with GeneScan 500 LIZ Size Standard (Applied Biosystems). Sizes were determined with GeneMarker version 2.2 (SoftGenetics, State College, Pennsylvania, USA). The number of alleles (A), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F), and departures from Hardy–Weinberg equilibrium (HWE) were calculated in GenAlEx 6.5 (Peakall and Smouse, 2012).

Ten primer pairs proved to be polymorphic while the remaining six were monomorphic (Table 1). Alleles per locus numbered seven to 29 (average 21.1); H_o and H_e ranged from 0.463 to 0.919 and from 0.797 to 0.947, respectively (Table 2). Five loci (LC019, LC068, LC089, LC090, LC105) departed from HWE ($P < 0.05$ or $P < 0.001$) (Table 2).

Additionally, we performed cross-species amplification to test the transferability of these markers to other members of the *L. clathratus* complex: *L. waltonii* (irregular sporangia type, diploid, determined by chromosome counting and flow cytometry, unpublished data; 8 individuals) and *L. likiangensis* (tetraploid, determined by chromosome counting and flow cytometry, unpublished data; 8 individuals), as well as two distantly related congeneric species, *L. scolopendrium* (Buch.-Ham. ex Ching) Mehra & Bir (8 individuals) and *L. morrisonensis* (Hayata) H. Itô (10 individuals) (Appendix 1).

Cross-amplification in *L. waltonii* was moderately successful (Table 3), suggesting a relatively recent divergence from *L. clathratus*. Low monomorphic

TABLE 2. Genetic properties of 10 polymorphic nuclear microsatellite loci developed in *Lepisorus clathratus* for 41 individuals sampled in central China.[a]

Locus	N	A	H_o	H_e	F[b]
LC017	39	21	0.795	0.872	0.089
LC019	41	7	0.463	0.797	0.418***
LC038	37	27	0.919	0.947	0.030
LC065	40	16	0.800	0.881	0.092
LC067	40	26	0.700	0.939	0.255
LC068	40	23	0.800	0.939	0.148*
LC089	36	29	0.833	0.947	0.120***
LC090	35	23	0.686	0.899	0.237***
LC092	39	18	0.821	0.883	0.071
LC105	39	21	0.487	0.921	0.471***

Note: A = number of alleles; F = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals genotyped.

[a]Voucher and locality information are provided in Appendix 1.

[b]Deviations from Hardy–Weinberg equilibrium: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

TABLE 3. Cross-amplification results and genetic properties of microsatellite loci developed for *Lepisorus clathratus* in *L. waltonii*, *L. likiangensis*, *L. scolopendrium*, and *L. morrisonensis*.[a]

	L. waltonii (n = 8)			*L. likiangensis* (n = 8)			*L. scolopendrium* (n = 8)			*L. morrisonensis* (n = 10)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
LC017	8	0.625	0.797	—	—	—	—	—	—	—	—	—
LC019	—	—	—	2	1.000	0.500	—	—	—	—	—	—
LC038	—	—	—	—	—	—	—	—	—	—	—	—
LC065	7	1	0.766	2	1.000	0.500	3	0.125	0.539	5	0.300	0.730
LC067	5	0.125	0.570	1	0.000	0.000	—	—	—	—	—	—
LC068	7	0.625	0.797	2	1.000	0.500	4	1.000	0.742	6	1	0.750
LC089	10	1	0.844	2	1.000	0.500	—	—	—	—	—	—
LC090	—	—	—	1	0.000	0.000	—	—	—	—	—	—
LC092	—	—	—	2	1.000	0.500	—	—	—	—	—	—
LC105	5	0.750	0.727	1	0.000	0.000	—	—	—	—	—	—

Note: — = failed amplification; A = number of alleles; F = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.

[a]Voucher and locality information are provided in Appendix 1.

pattern in the tetraploid *L. likiangensis* population revealed extremely low diversity (Table 3). The markers showed quite low transferability in *L. scolopendrium* and *L. morrisonensis*, with just two markers successfully amplifying (Table 3).

CONCLUSIONS

We successfully developed and amplified the first set of microsatellite markers for the Sino-Himalayan fern, *L. clathratus* complex. Among them, 10 microsatellite markers display a high level of polymorphism that will help to estimate more reliable genetic diversity parameters and to further reconstruct the population history of the *L. clathratus* complex. These markers may also be useful tools to study mating system and infer polyploid origin in the *L. clathratus* complex, and to explore other taxonomic problems.

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APPENDIX 1. Voucher and locality information of all *Lepisorus* samples used in this study. Vouchers are deposited at the Chinese National Herbarium of the Institute of Botany, Chinese Academy of Sciences (PE).

Species	Collection no.	Locality	Geographic coordinates
L. clathratus (C. B. Clarke) Ching	*Chun-Ce Guo & Chong-Wu 080619*	Taibaishan, Shaanxi, China	34.0390°N, 107.7202°E
L. waltonii (Ching) S. L. Yu	*Cun-Feng Zhao & Zhong-Shuai Zhang NR1-26*	Lhasa, Xizang, China	29.7130°N, 91.0994°E
L. likiangensis Ching & S. K. Wu	*Xian-Chun Zhang 7608*	Yulong Xueshan, Yunnan, China	27.0283°N, 100.2634°E
L. scolopendrium (Buch.-Ham. ex Ching) Mehra & Bir	*Yi-Zhen Shao 2622*	Xizang, China	NA
L. morrisonensis (Hayata) H. Itô	*Cun-Feng Zhao & Zhong-Shuai Zhang XZ-25*	Sejila, Xizang, China	29.5663°N, 94.5735°E

Note: NA = not available.