Development and Characterization of Microsatellite Loci in the Pantropical Fern Hypolepis punctata (Dennstaedtiaceae)

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE LOCI IN THE PANTROPICAL FERN *HYPOLEPIS PUNCTATA* (DENNSTAEDTIACEAE)[1]

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- *Premise of the study:* Microsatellite loci were isolated in *Hypolepis punctata* (Dennstaedtiaceae) to further study the reproductive ecology of this species.
- *Methods and Results:* We developed 16 microsatellite loci from one sample of *H. punctata* using an enriched genomic library. These loci were characterized in 28 individuals. The number of alleles per locus ranged from two to 10, and the expected heterozygosity ranged from 0.036 to 0.845.
- *Conclusions:* The results indicate that the microsatellite markers can facilitate further studies on inferring the phylogeography and population genetics of *H. punctata* and related species.

Key words: Dennstaedtiaceae; *Hypolepis punctata*; microsatellite; phylogeography; population genetics.

Hypolepis punctata (Thunb.) Mett. ex Kuhn (Dennstaedtiaceae), i.e., downy ground fern, is a green, densely hairy, and glandulous fern that is widely distributed in tropical and subtropical regions in Asia and the Pacific (Brownsey, 1987). This plant is used in Chinese traditional medicine and contains pterosin, which has a cytotoxic effect on cancer cells (Lai, 2003). However, this species is often confused with *H. polypodioides* (Blume) Hook. and *H. resistens* (Kunze) Hook. (Xing and Wang, 2013), and using chloroplast markers (*rbcL*, *matK*, *trnL-F*, and *psbA-trnH*) is ineffective in improving identification accuracy (Shang et al., unpublished data). Moreover, *H. punctata* is an ideal species for studying mating system and sexual resource allocation because it exhibits high spore production and cloning habit. In addition, the wide distribution of this species may provide insight into the long-distance dispersal of homosporous ferns. Nuclear microsatellite markers are known as versatile molecular tools for ferns to solve the problem of inferring phylogeography or population genetics (Jiménez et al., 2008). In this study, we report on the development of 16 microsatellite markers for *H. punctata* to contribute to reproductive ecology and species differentiation research in the genus *Hypolepis* Bernh.

METHODS AND RESULTS

Total genomic DNA was extracted from the silica gel–dried leaves of an individual *H. punctata* specimen (voucher no.: JSL-WLSQ522; Appendix 1) collected from Wuling Mountain in Sangzhi County, Hunan Province, China, using a Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China).

A microsatellite-enriched library was built following the method presented by Glenn and Schable (2005) with slight modifications. Genomic DNA was digested with *Rsa*I and *Xmn*I (New England Biolabs, Ipswich, Massachusetts, USA) at 37°C overnight and subsequently ligated to the double-stranded adapter (forward 5′-GTTTAAGGCCTAGCTAGCAGAATC-3′, reverse 5′-pGATTCTGCTAGCTAGGCCTTAAACAAA-3′). The ligated DNA was randomly linked to one of the two single-stranded biotinylated microsatellite probes (5′-(CA) 15-Biotin, 5′-(GA) 15-Biotin). The hybridized DNA was then captured by streptavidin-coated paramagnetic beads (Dynabeads M-280 Streptavidin; Dynal Biotech, Oslo, Norway) and gathered using a magnetic particle-collecting unit (DynaMag-2 Magnet 12321D; Invitrogen, Waltham, Massachusetts, USA). The enriched DNA was amplified using the forward adapter as the primer. The product was then purified, ligated into the pGEM-T Easy Vector System (Promega Corporation, Madison, Wisconsin, USA), and cloned in Chemically Competent TOP10 *E. coli* cells (Tiangen Biotech). A total of 135 clones were selected and sequenced, in which 107 (~80%) contained simple sequence repeats. Among these, 83 had suitable lengths for primer design using Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). PCR amplifications were performed in 15-μL total volume with ~70 ng of genomic DNA, 10 μM of each primer, and 1× PCR mix (Tiangen Biotech). The PCR program consisted of 5 min of initial denaturation at 95°C, followed by 10 cycles of pre-PCR processing that involved 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 30 s of primer extension reaction at 72°C. The annealing temperature was reduced by 1°C per cycle. PCR amplification was continued for 25 more cycles at a constant annealing temperature of 50°C, and a final extension was performed at 72°C for 10 min. Finally, 16 pairs of primers (Table 1) were selected because they showed the clear bands of a single locus after agarose gel electrophoresis. The forward primer was labeled using one of the fluorescent dyes (FAM, TAMRA, or HEX) to detect polymorphism on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, California, USA).

To test marker efficiency, we used 28 individuals of *H. punctata* from three different populations (five individuals from Wuling Mountain [voucher: JSL-WLSQ522]; 16 from Nanling Mountain [voucher: YYH13169]; and seven from Bawangling Mountain, Hainan Island, China [voucher: SG2984]; Appendix 1). Samples were collected from different individuals, with a minimum interval of 100 m between them, to avoid sampling the same clone. The numbers of alleles per locus, observed heterozygosity, and expected heterozygosity were estimated using CERVUS 3.0 (Kalinowski et al., 2007). In addition, cross-amplifications

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TABLE 1. Characteristics of 16 microsatellite loci developed in *Hypolepis punctata*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	GenBank accession no.
SHH02	F: GTTGTCGTAATCCGCAAAGTGG	$(TC)_{19}$	292–294	KR270806
	R: CAGATATGAGCGTCATTATCTCGGT			
SHH13	F: CATGGATTTGTTCTCCCTATCTGC	$(GA)_{39}$	362–378	KR270807
	R: TGGCCTTTGGGGAACCTTAGTA			
SHH17	F: CAGCAGCAGAGGAACCTGACA	$(CT)_{15}$	445–447	KR270808
	R: ATTGCGAACCACCCATTGAC			
SHH19	F: TTGATGCCTCCATGACTATGCT	$(CA)_{24}$	264–288	KR270809
	R: TCACCTGTCCTCCCTAACTTCT			
SHH23	F: CGGAGCGGAAAGGTAGAACA	$(AC)_6AT(AC)_3$	235–245	KR270810
	R: TTTTGCCACTATTGCTGATGAA			
SHH33	F: TCTCCCTCCCTCGATCTCCTT	$(CT)_{17}$	246–258	KR270811
	R: ATGTGGTGCTTCTAGCTGCTGAC			
SHH34	F: AACCGTAACAGACGTGCAAACC	$(CT)_{20}(CA)_9$	441–453	KR270812
	R: TGTGAGAAGCAGCAAGTCCAAA			
SHH44	F: TGGTATCATAGGCCATTTTGTCC	$(CT)_{17}(CA)_{13}$	172–196	KR270813
	R: TAGAGGAGGGAGATGCATTGAGA			
SHH46	F: GGAATAAACCATGTAGGCAAGAGC	$(GA)_{13}$	121–123	KR270814
	R: CCAACGAGCCATGTGGACAA			
SHH51	F: TAGCAGTAAATAGTTTGTTACGTGCCC	$(CA)_6AA(CA)_3$	246–249	KR270815
	R: CCATCCGTTGTTGCCCCAT			
SHH55	F: GGAATCGCCAAGGAGATAATAA	$(AG)_{12}$	413–422	KR270816
	R: CCCTCTTTTCTCAATCTATGTCCC			
SHH56	F: AGAAGATGCTTGTCATAAGTAGGG	$(CT)_{20}$	421–438	KR270817
	R: AATGCTCAAGTCAAAAGTGCC			
SHH65	F: TCGATAGTGTTCGCGGGTAA	$(CT)_{23}(CA)_{11}$	271–283	KR270818
	R: GGGCATGGTGGTGACAAAGT			
SHH71	F: TTTCGTCTAAATCATGCTCTTTCC	$(CT)_{16}$	293–301	KR270819
	R: GCCTGTCTCGCTACCCGTAT			
SHH77	F: GATGAATAAAAGAACTTAAACCAAC	$(CA)_{10}$	439–451	KR270820
	R: AGCAAGAAAGGGAGAACGAG			
SHH78	F: ACAGTGATGGAAGGCTGAAAGTC	$(CT)_{10}$	238–242	KR270821
	R: GAGATGGCGTACCTATGGATGG			

were performed to test the transferability of the marker to five other *Hypolepis* species (two individuals of *H. polypodioides* [vouchers: SG765, SG767], one individual of *H. resistens* [voucher: SG2900], one individual of *H. tenuifolia* (G. Forst.) Bernh. [voucher: HN31], two individuals of *H. pallida* (Blume) Hook. [vouchers: YYH11628, YYH11629], and one individual of *H. brooksiae* Alderw. [voucher: SIWS19]; Appendix 1).

The number of alleles per locus ranged from two to 10, with an average of 4.75 (Table 2). Meanwhile, 14 of the loci presented a significant bias between the observed and expected heterozygosities, which might indicate selfing in these populations (Table 2). Furthermore, at least six loci were interspecifically amplifiable in each of the other five species. In particular, all 16 loci were amplifiable for *H. polypodioides* (Table 3).

CONCLUSIONS

A total of 16 polymorphic microsatellite loci were newly developed and characterized for *H. punctata*. These polymorphic microsatellite loci may provide good references for analyzing

TABLE 2. Genetic properties of the 16 newly developed microsatellites of *Hypolepis punctata*.

	Total (n = 28)			Hainan (n = 7)			Wuling (n = 5)			Nanling (n = 16)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
SHH02	2	0.036	0.036	1	0.000	0.000	1	0.000	0.000	2	0.063	0.063
SHH13	10	0.143	0.843	3	0.143	0.385	3	0.200	0.644	9	0.125	0.815
SHH17	3	0.071	0.491	2	0.000	0.264	2	0.200	0.556	3	0.063	0.179
SHH19	6	0.143	0.805	3	0.143	0.648	3	0.200	0.733	4	0.125	0.667
SHH23	3	0.143	0.137	1	0.000	0.000	2	0.400	0.356	3	0.125	0.123
SHH33	7	0.179	0.760	4	0.286	0.396	2	0.000	0.533	4	0.188	0.692
SHH34	6	0.143	0.732	2	0.000	0.440	3	0.400	0.644	3	0.125	0.573
SHH44	5	0.143	0.759	2	0.143	0.495	2	0.200	0.556	4	0.125	0.718
SHH46	2	0.143	0.468	2	0.143	0.143	2	0.200	0.556	3	0.188	0.534
SHH51	3	0.071	0.390	2	0.143	0.143	1	0.000	0.000	2	0.063	0.063
SHH55	5	0.143	0.606	4	0.143	0.495	2	0.200	0.200	2	0.125	0.444
SHH56	8	0.143	0.845	6	0.000	0.879	4	0.400	0.711	5	0.125	0.810
SHH65	6	0.143	0.779	2	0.143	0.143	2	0.200	0.200	4	0.125	0.756
SHH71	4	0.071	0.538	3	0.143	0.275	2	0.200	0.200	3	0.000	0.331
SHH77	4	0.071	0.610	1	0.000	0.000	1	0.000	0.000	3	0.125	0.486
SHH78	2	0.036	0.363	2	0.143	0.143	1	0.000	0.000	1	0.000	0.000

Note: A = number of sampled alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.

TABLE 3. Cross-amplification length (in base pairs) of 16 microsatellite loci from *Hypolepis punctata* in other *Hypolepis* species.

Locus	*H. polypodioides* (*n* = 2)	*H. resistens* (*n* = 1)	*H. tenuifolia* (*n* = 1)	*H. pallida* (*n* = 2)	*H. brooksiae* (*n* = 1)
SHH02	292	292	278–292	—	292
SHH13	366–368	382	382	372	—
SHH17	443	—	—	485	447–449
SHH19	264–272	290	—	—	—
SHH23	235–243	233–235	225	227–239	239
SHH33	248	276	—	—	—
SHH34	449	457	—	—	—
SHH44	150	—	140	152	—
SHH46	121	111–113	149	115	121–123
SHH51	246	287	—	—	—
SHH55	416	—	—	—	421–423
SHH56	431–433	—	425	417	429–431
SHH65	273–281	—	—	277	281–283
SHH71	293	—	—	—	293
SHH77	443	449	—	—	445
SHH78	238	—	—	—	240

Note: — = failed amplification; *n* = number of individuals sampled.

mating systems and population structures, identifying clones, estimating gene flow, and identifying related species. This research will considerably improve knowledge on the life history of ferns. In addition, the high transferability of these loci to other species from the genus *Hypolepis* is essential for future research on hybridization or speciation.

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APPENDIX 1. Voucher and locality information of all *Hypolepis* samples used in this study.[a]

Species	Voucher no.	Locality	Geographic coordinates
H. punctata (Thunb.) Mett. ex Kuhn	JSL-WLSQ522	Wuling Mountain, Hunan, China	29°18′31″N, 110°6′52″E
	YYH13169	Nanling Mountain, Guangdong, China	24°43′24″N, 114°15′46″E
	SG2984	Bawangling Mountain, Hainan, China	19°5′49″N, 109°13′32″E
H. polypodioides (Blume) Hook.	SG765, SG767	Fanjingshan Mountain, Guizhou, China	27°55′44″N, 108°41′17″E
H. resistens (Kunze) Hook.	SG2900	Bawangling Mountain, Hainan, China	19°5′28″N, 109°10′59″E
H. tenuifolia (G. Forst.) Bernh.	HN31	Wuzhishan Mountain, Hainan, China	18°55′1″N, 109°42′13″E
H. pallida (Blume) Hook.	YYH11628, YYH11629	Nantou County, Taiwan, China	NA
H. brooksiae Alderw.	SIWS19	Celebes Island, Indonesia	NA

Note: NA = not available.

[a] Specimens are deposited at the Shanghai Chenshan Botanical Garden Herbarium (CSH), except for voucher SIWS19, which is deposited at the Chinese National Herbarium, Institute of Botany, Chinese Academy of Sciences (PE).