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TECHNICAL NOTE

Isolation and characterization of microsatellite markers for *Ligularia hodgsonii* Hook. (Asteraceae)

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Abstract *Ligularia hodgsonii* is one of widely distributed *Ligularia* species in south China, central China, Far East area of Russia and Japan. In this study, we described the development of 14 microsatellite markers from the genome of *L. hodgsonii* using the FIASCO protocol. Polymorphism of each locus was assessed in 30 adult individuals of the *Ligularia*. The average allele number of the microsatellites was 3.0 per locus, ranging from 2 to 5. The observed (H_O) and expected (H_E) heterozygosities varied from 0.2000 to 0.7333 and from 0.3910 to 0.7598. The marker transferability of the 14 primer pairs was tested on other two congeneric species that also occur in China.

Keywords *Ligularia hodgsonii* · FIASCO · Microsatellite markers · Polymorphism

Ligularia Cass. (Asteraceae) in the Hengduan Mountains area provides interesting samples for the study of plant diversity, since the genus is highly diversified and their genetic differentiation is considered to be still continuing in the area. The chemical and genetic diversity of some *Ligularia* species has been investigated by chemical constituents and DNA sequences for exploring its speciation and evolution (Nagano et al. 2007, 2006; Kuroda et al. 2007; Torihata et al. 2007; Hanai et al. 2005). In order to devise adequate genetic diversification, conservation, and management strategies for *Ligularia* species, we developed microsatellite markers (SSR) from *Ligularia hodgsonii* using the Fast Isolation by AFLP of Sequences Containing repeats protocol (FIASCO) (Zane et al. 2002); at the same time, no nuclear microsatellite primers have been reported for *Ligularia*. Compared to the ISSR fingerprinting, microsatellites show numerous advantages since they are locus-specific, codominant, highly reproducible and usually highly polymorphic (Powell et al. 1996).

Ligularia hodgsonii Hook, a perennation herbage plant, is distributed in south China, central China, Far East area of Russia and Japan. Its genomic DNAs were extracted from dry leaf tissue, which was ground in liquid nitrogen, using a CTAB methodology (Doyle and Doyle 1987). Total genomic DNAs were completely digested with MseI and then ligated to an MseI AFLP adaptor. A diluted digestion–ligation mixture (1:10) was amplified with adaptor-specific primers (5′-GAT GAG TCC TGA GTA AN-3′). Amplified DNA fragments, with a size range of 200–800 bp, were enriched for repeats by magnetic bead selection with a 5′-biotinylated $(AC)_{15}$, $(AG)_{15}$ and $(AAG)_7$ probe, respectively. Enriched fragments were amplified again with adaptor-specific primers. PCR products were purified using an EZNA® Gel Extraction Kit (Omega Bio-Tek). Purified DNA fragments were ligated into the pGEM-T vector (Promega), and transformed into DH5αcells. Positive clones were tested by PCR using (AC) 10/(AG) 10/(AAG) 7 and T7/Sp6 as primers, respectively. In total, 124 clones with positive inserts were sequenced with an ABI PRISM 3730XL DNA sequencer. A total of 92 (74.2%) sequences were found to contain microsatellite repeats, and 40 of them were suitable for designing locus-specific primers, using the PRIMER 5.0 program (Clarke and Gorley 2001).

Polymorphisms of these loci were assessed in 30 individuals collected from six populations. All microsatellites were amplified in a total volume of 15 μl which contained 50 ng template DNA, 0.6 μM of each primer, 7.5 μl 2× Taq

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Table 1 Specific primer sequences and characterization for 14 microsatellite loci isolated from *Ligularia hodgsonii*

Locus	Repeat motif	Primer sequences (5′–3′)	T_a(°C)	Allele size(bp)	*N*	*A*	H_O	H_E
Lho12	$(GT)_2TC(GT)_6$	F: CTCCTTTCTACTCCTCTATG	54	276–295	30	3	0.6667	0.4949
		R: CAAGAATACGAAGATTTACC						
Lho17	$(GT)_5$	F: TTGCCTCAAAGGTCTCTT	54	132–142	30	3	0.2667	0.4637*
		R: CCCTAACACCACTCAATG						
Lho29	$(TC)_5…(AC)_7$	F: TCCACTCACTAAGGGGAACA	58	143–153	30	2	0.0667	0.5695*
		R: ACGGATCGTTAGGGTTCA						
Lho35	$(TC)_9$	F: AACCATCGCTGCACATTC	57	170–210	30	5	0.4000	0.7070*
		R: GCAACACCACCACTGACG						
Lho36	(CT)5…(AC)5…(AC)5	F: ACCTTCGAATTATTCTTTCGC	57	229–250	30	4	0.3000	0.4092
		R: TCTCAGAGCTTTTCAGTGTCTAT						
Lho37	$(GA)_4T(AG)_3…(AG)_6$	F: ACTCCCAAAATACTTTCAAAC	56	300–325	30	3	0.4667	0.3910
		R: CATGTCCTGCAAATGGGT						
Lho38	$(AC)_5…(AC)_4…(AC)_7$	F: CTTACACCTCCGAACTGATC	57	272–290	30	3	0.6000	0.5667
		R: TCTAAAAGGGAAATGGAAACA						
Lho40	$(CT)_6…(TG)_4$	F: ATCATACCTTGCCTCAAAGT	57	192–202	30	2	0.2000	0.4881*
		R: CAATAGTTCCGAACACCCT						
Lho41	$(AG)_4AT(AG)_8…(AG)_9$	F: ACGAGTAGACACCCAACGTC	57	180–205	30	5	0.7333	0.7508
		R: CCTTTCTTCCCCAACACAA						
Lho64	$(ATG)_{6…}$(AG)rich	F: CGAATGACATGAACACCAC	57	160–195	30	4	0.3333	0.4933*
		R: CCTTCCTCCTTGAGCCTAT						
Lho75	$(CT)_{18}$	F: CCACCATCATTTTCTGTAG	54	185–220	30	5	0.4000	0.6085*
		R: GTATGAGACACCACCGAAT						
Lho77	$(TG)_7$	F: AGTTTTGTAGTAAAACGGAGTT	55	178–198	30	3	0.7333	0.6729
		R: CGCATAAATAATGTAAGCA						
Lho114	$(GA)_6$	F: AGTTCGGTTTGCTGCTAT	55	220–235	30	3	0.5000	0.5082
		R: TGGGCTTATGGACTTGAT						
Lho154	$(GT)_6…(GT)_7T(GT)_6$	F: CACCTTCTCCTCCTACACG	58	175–195	30	4	0.4667	0.5622*
		R: CCTAGATCTTCATCTCTTTTCT						

* Indicates the observed heterozygosity is significantly different from the expected heterozygosity under Hardy–Weinberg equilibrium ($P < 0.01$); T_a, PCR annealing temperature; *N*, number of individuals; *A*, number of alleles revealed; H_O, observed heterozygosity; H_E, expected heterozygosity

PCR MasterMix (Tiangen; 0.1 U Taq Polymerase/μl, 0.5 mM dNTP each, 20 mM Tris–HCl (PH8.3), 100 mM KCl, 3 mM MgCl2). PCR amplifications were conducted on an MJ PTC-200 Thermal Cycler using the following condition: initial denaturing at 97°C for 4 min; 32 cycles of 1 min at 94°C, 1 min at the annealing temperature for each designed specific primer, 40 s at 72°C; and finally 8 min at 72°C. PCR products were then electrophorzed on 6% denaturing polyacrylamide gel and visualized using silver staining. Allele sizes were estimated using a 10 or 20 bp ladder molecular size standard (Invitrogen).

Thirty-nine of the forty primer pairs successfully amplified the target regions. Of them, fourteen displayed polymorphisms (Table 1). Standard genetic diversity parameters, departure from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between pairs of loci

Table 2 Cross-species amplification of microsatellites cloned from *Ligularia hodgsonii* in other *Ligularia* species, *L. tongolensis* and *L. pleurocaulis*

Species	Locus													
	Lho12	Lho17	Lho29	Lho35	Lho36	Lho37	Lho38	Lho40	Lho41	Lho64	Lho75	Lho77	Lho114	Lho154
L. tongolensis	P	P	M	P	P	P	M	P	–	M	–	P	P	P
L. pleurocaulis	P	M	–	P	P	M	P	P	P	P	M	M	M	P

M Monomorphic, *P* polymorphic (number of alleles), – no specific product

were estimated in POPGENE version 3.4 (Raymond and Rousset 1995). Over all, the number of alleles per locus ranged from 2 to 5, with an average of 3.0. The observed and expected heterozygosities, H_O and H_E, ranged from 0.2000 to 0.7333 and from 0.3910 to 0.7598, respectively. The locus Lho17, Lho29, Lho35, Lho40, Lho64, Lho75, and Lho154 were deviated significantly from Hardy–Weinberg equilibrium (HWE) ($P < 0.01$) due to excessive homozygosity.

For cross-species application, these 14 primer pairs were tested in other two *Ligularia* species, *L. tongolensis* and *L. pleuroca*ulis (Table 2), which also occur in China. Eleven of them were successfully amplified in all species, whereas Lho41 and Lho75 failed amplification in *L. tongolensis*, Lho29 failed amplification in *L. pleurocaulis*. The polymorphic microsatellite loci presented here would be useful for assessing the population genetic structure of *L. hodgsonii* and its sisters.

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