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Isolation and Characterization of 13 Microsatellite Loci from *Pedicularis rex* (lousewort)

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***Abstract. Pedicularis rex* C. B. Clarke ex Maxim., an endemic species with potential horticultural traits from Himalaya, has a unique cup-like petiole structure and highly infraspecific floral variation among members of the lousewort genus (Orobanchaceae). We developed 13 microsatellite markers from three microsatellite-enriched libraries (AG, AC, and AAG) of *P. rex* with a modified biotin–streptavidin capture technique. Polymorphism of each locus was assessed in 22 individuals with representation of five populations of *P. rex*. Number of alleles per locus (A) ranged from two to seven with an average of 4.38. The observed and expected heterozygosities varied from 0.03 to 0.86 and 0.45 to 0.84, respectively. Additionally, among the 13 identified microsatellite markers, 11 of them were successfully amplified in species *P. thamnophila*, and five of them showed polymorphisms. This study may provide important information for further investigation on the population genetics, introduction, and acclimatization of *P. rex* and its congeners.**

Pedicularis Linn. (Lousewort) in the family Orobanchaceae is one of the largest genera in angiosperms with ≈600 species primarily occurring in the mountains and alpine zones of the Northern Hemisphere (Li, 1948). The Himalaya Mountains represent a major diverse center and endemism of *Pedicularis* (Yang et al., 1998). Taxa in this region have long been of great biological interest, particularly pollination biology, speciation, and domestication (Li et al., 2007; Macior and Tang, 1997). As a result of the high diversity in vegetative and floral morphology (e.g., corolla type and color) and long flowering period, *Pedicularis* in the Himalayan area has great potential for being domesticated as popular ornamental plants (Li and Zhou, 2005). However, introduction and cultivation of *Pedicularis* from this area have not been well achieved so far, to a large extent as a result of the poorly known genetic mechanisms of these taxa.

Pedicularis rex C. B. Clarke ex Maxim is the most widely distributed species in *Pedicularis* from the Himalayas, both geographically and elevationally (Yu and Wang, 2008). It is characterized by a unique cup-like structure formed by fused petiole bases and highly floral variation in the genus. High genetic diversity in this species was revealed based on preliminary random amplified polymorphic DNA analysis (Li et al., 2005). Therefore, *P. rex* can be available as a model taxon for the study on evaluation and use of horticultural genetic resources of the Himalayan taxa of *Pedicularis*. Seed germinations of this species by controlling moist-chilling and gibberellic acid applications have been carried out for domestication. However, successful cultivation in the common landscape was still unresolved (Li et al., 2007; Ren and Guan, 2008).

The identification of molecular markers linked to important morphological traits will greatly facilitate maker-assisted breeding aimed at cultivar improvement (Yu et al., 2000). Microsatellite or simple sequence repeat (SSR) markers have been successfully used for genomic mapping, DNA fingerprinting, and maker-assisted selection in many plant species because of their reproducibility, nature codominant inheritance, and multiallelic form (Powell et al., 1996). In this study, we developed new microsatellite markers for further estimating the genetic diversity and population genetic structure of *P. rex*.

Twenty-two individuals of *P. rex* collected from five geographical locations in China were studied (Table 1). The primer pairs revealing polymorphism in *P. rex* were further investigated for cross-species amplification in its sister species *P. thamnophila* (Hand.-Mazz.) H.L.Li (Ree, 2005; Yang et al., 1998), which is also endemic to the southwest of China (Table 1). Specimen vouchers were deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

Genomic DNA of *P. rex* and *P. thamnophila* was extracted from silica gel-dried leaves using a CTAB methodology (Doyle and Doyle, 1987). A microsatellite genomic library was constructed and screened using a fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO) protocol with some modifications (Zane et al., 2002). First, total genomic DNA of *P. rex* (≈1000 ng) was completely digested with 2.5U of *Mse*I restriction enzyme (New England Biolabs) and then was ligated to a specific *Mse*I AFLP adaptor (5′-TAC TCA GGA CTC AT-3′/5′-GAC GAT GAG TCC TGA G-3′) using T4 DNA ligase (New England Biolabs). The digested-ligated fragments were diluted in a ratio of 1:10, and 5 μL of them were used for amplification reaction with an adaptor-specific primer (5′-GAT GAG TCC TGA GTA AN-3′). The amplified DNA fragments with a size range of 200 to 800 bp were hybridized to a 5-biotinylated [$(AG)_{15}$, $(AAG)_{10}$, or $(AC)_{15}$] probe and then selectively separated and captured by streptavidin-coated magnetic beads (Promega) (Zane et al., 2002). The recovered DNA fragments were re-amplified with the previously mentioned adaptor-specific primer. The purified polymerase chain reaction (PCR) products using a EZNA Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China) were then ligated into PGEM-T vector (Promega) and transformed into DH5α competent cells (Tiangen Biotech Co., Ltd., Beijing, China). The positive clones were tested using vector primers T3/T7 and specific primer $(AC)_{10}/(AG)_{10}/(AAG)_7$. In total, 367 positive clones were sequenced with an ABI PRISM 3730XL DNA sequencer (Applied Biosystems Inc.), and 220 (60%) clones contained microsatellites. Finally, a total of 95 pairs of primers were designed by Primer 5.0 (Clarke and Gorley, 2001).

Polymorphisms of all 95 microsatellite loci were assessed in the 22 individuals of *P. rex*. The PCR reactions were performed in 20 μL of reaction volume containing 30 to 80 ng genomic DNA, 0.6 μM of each primer, 10 μL 2 × Taq PCR MasterMix (Tiangen Biotech Co., Ltd., Beijing, China), 0.1 U Taq polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$. PCR amplifications were performed under the

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Table 1. Individuals information we sampled to evaluate the 13 microsatellite loci in *Pedicularis rex* (n = 22) and *P. thamnophila* (n = 4).

Taxon	Population	Number of individuals in one population	Voucher	Collection date	Altitude (m)	Locality
P. rex	Panxian, Guizhou	6	W.-B. Yu et al., LIDZ-0962	7 July 2008	2300	26°07′58.4″ N, 104°36′6.2 ″ E
	Muli, Sichuan	4	W.-B. Yu et al., LIDZ-0998	8 Aug. 2008	3165	28°06′7.2″ N, 101°9′0.0″ E
	Yajiang, Sichuan	3	W.-B. Yu et al., LIDZ-1169	18 Aug. 2005	3600	28°46′22.92″ N, 100°24′36.56″ E
	Kunming, Yunnan	3	P.-H. Huang, 08-H-01	6 Aug. 2002	2200	25°11′19.84″ N, 102°44′25.18″ E
	Zhongdian, Yunnan	6	W.-B. Yu et al., 057	16 Aug. 2005	3900	27°51′8.77″ N, 98°44′10.28″ E
P. thamnophila	Muli, Sichuan	2	W.-B. Yu et al., LIDZ-1015	9 Aug. 2008	2337	28°07′48.9″ N, 100°55′22.9″ E
	Daocheng, Sichuan	2	W.-B. Yu et al., LIDZ-1243	18 Aug. 2005	3600	29°00′35.93″ N, 100°29′53.47″ E

n = number of individuals used to evaluate the 13 microsatellite loci.

Table 2. Characteristics of 13 microsatellite loci developed for *Pedicularis rex* and cross-species amplification in *P. thamnophila*.

Locus	GenBank accession no.	Primer sequence (5′-3′)	Repeat motif	Size range (bp)	A	Ta (°C)	H_O	H_E	HWE *P* value	CA
PR1	GU441683	F: TTCTTCCCCTCTCACCTTCT R: CGGCATTACACATTTCAAAC	$(TC)_{17}(T)_7(CTT)_5$	90–160	7	58	0.47	0.77	0.0761	W
PR2	GU441684	F: GAGATAGAATTGGTGGTC R: AGTGCGATAAAGTGGTAG	$(A)_5(CA)_5T(AC)_4$	272–292	4	61	0.44	0.66	0.3042	P(2)[z]
PR3	GU441685	F: CAAGACTCCTCCTCCATC R: GAGAACAGAACCCAAACC	$(CA)_6$	94–104	4	60	0.06	0.48	0.0000	M
PR4	GU441686	F: GCTTGTCCGTATGCTTGA R: GCTTTTGTAGAGGCTTTGAT	$(A)_5(CA)_{22}$	192–224	7	57	0.86	0.84	0.5051	P(3)
PR5	GU441687	F: TTGCTACAAGAAACATACGC R: CTCAAGTGAAATCAACCCA	$(GA)_6$	221–263	5	56	0.36	0.73	0.0299	NA
PR6	GU441688	F: AAACCAGCCCAACAAATA R: TTCACTTCACTCCGCAAA	$(A)_6(CA)_9$	285–305	4	60	0.22	0.63	0.0009	P(3)
PR7	GU441689	F: TGCCTATTTGTGGAGACC R: TTGGCGTAGATTGAAGAC	$(AC)_{17}$	124–132	4	60	0.75	0.73	0.0000	P(3)
PR8	GU441690	F: CAGTGATTGATACAGGCTTATTGAG R: GCTGAAGAGAGACACGTATATGAAG	$(TG)_{16}$	98–146	6	61	0.47	0.79	0.1077	P(4)
PR9	GU441691	F: GTAAATCCCCGACATCCT R: TCCCTCCTCACCCTATCT	$(GA)_{13}$	181–187	3	60	0.11	0.45	0.0003	M
PR10	GU441692	F: CGCAGTTGGTAGAAGGTTC R: ATCAGCAAAAGGATAAGCAC	$(AC)_{17}$	183–197	3	60	0.44	0.62	0.1540	M
PR11	GU441693	F: CGAAGACGCCCAAAGATAG R: GCCATAGCATTTCCAAGAGT	$(CT)_{22}$	180–204	3	60	0.56	0.55	0.2970	M
PR12	GU441694	F: GGTGGCGTTGAAGAAGGT R: GCGAAGGGACGATGTTATT	$(GA)_{27}$	172–176	2	62	0.69	0.51	0.1905	M
PR13	GU441695	F: ACGGTTACATAGAGGCAAGG R: TAGTGGGGAAATAGCGGA	$(CTT)_5$	174–198	5	57	0.03	0.53	0.0000	NA

[z]The number in parentheses in the CA column means the observed alleles in *P. thamnophila*.
A = number of observed alleles; *Ta* = polymerase chain reaction annealing temperature; H_O = observed heterozygosity; H_E = expected heterozygosity; CA = cross-species amplification; W = weak amplification; P = polymorphic amplification; M = monomorphic amplification; NA = no amplification.

following conditions: 94 °C for 3 min followed by 32 to 35 cycles at 94 °C for 30 s at the annealing temperature for each specific primer (optimized for each locus; Table 2) for 30 s, 72 °C for 45 s, and a final extension step at 72 °C for 5 min. PCR products were separated on 8% polyacrylamide denaturing gel and visualized by silver staining (Creste et al., 2001). Standard genetic diversity parameters, departure from Hardy-Weinberg equilibrium (HWE), and linkage disequilibrium (LD) between pairs of loci were estimated using GENEPOP 3.4 (Raymond and Rousset, 1995; http://genepop.curtin.edu.au/). Tests for the presence of null alleles were performed in MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004).

PCR products with 13 of 95 pairs of primers displayed polymorphisms among different populations of *P. rex*. The number of alleles per locus (A) was two to seven with an average of 4.38. Observed (H_O) and expected (H_E) heterozygosities ranged from 0.03 to 0.86 and 0.45 to 0.84, respectively (Table 2). Five (PR3, PR6, PR7, PR9, and PR13) of the 13 microsatellite loci showed significant deviation from HWE ($P < 0.01$), and four of them (PR3, PR6, PR9, and PR13) could be attributed to the presence of null alleles. Among the five loci, locus PR7 had a very close value for H_O and H_E (H_O = 0.750; H_E = 0.730). The deviation from HWE could be attributed to the low genotypic diversity caused by self-pollinated breeding system (Li et al., 2005) or limited sampling of *P. rex* (only 22 individuals evaluated). Huang et al. (2008) developed 11 microsatellites for *P. verticillata* L. with a PIMA method and tested its application in the related species *P. ikomai* Sasaki. All loci were found to be significantly deviated from HWE that indicated a dramatic loss of genetic polymorphisms by genetic drift in fragmented populations of *P. verticillata*. In the GENEPOP analysis of LD, no significant LD was detected for any pair of loci (data not shown). A cross-species amplification test indicated that 11 of 13 primer pairs (except for loci PR5 and PR13) from *P. rex* successfully amplified in *P. thamnophila* (n = 4; Table 1). Of the 11 tested loci, five showed polymorphisms. These new microsatellite markers developed in this study may provide useful information for further investigation on the application of SSR assay in the horticultural domestication and breeding program of *P. rex* and its related Sino-Himalayan taxa.

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