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TECHNICAL NOTE

Isolation and characterization of 13 microsatellite loci from *Incarvillea mairei* (Bignoniaceae), an endemic species to the Himalaya-Hengduan mountains region

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Abstract *Incarvillea mairei* (H. Léveillé) Grierson (Bignoniaceae) is an endemic species to the Himalaya-Hengduan Mountains region. Here, we developed 13 microsatellite markers from *I. mairei* using a modified biotin-streptavidin capture method. Number of alleles per locus (NA) ranged from 3 to 7 with an average of 4.615. The observed (H_O) and expected (H_E) heterozygosities were from 0.050 to 0.800 and from 0.249 to 0.815, respectively. Additionally, among the 13 identified microsatellite markers, 12 of them were successfully amplified in other three congeneric species, and most of them showed polymorphic. Obtained evidences suggest that these markers provide a useful tool for further study of the population genetic structure and the breeding system in this species or/and infra-generic species.

Keywords *Incarvillea mairei* · Genetic structure · Breeding system · Microsatellites · Himalaya-Hengduan Mountains region

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Introduction

Incarvillea Juss is a notable genus with herbaceous and temperate distributed species in contrast with other woody and tropical distributed members in family Bignoniaceae. The diversification of some herbaceous species appears to have involved rapid migration, geographic isolation and reproductive isolation, and is considered to be related to the uplift of the Himalaya Mountains (Wu et al. 2003; Chen et al. 2005). *Incarvillea mairei* (H. Léveillé) Grierson, a perennial herb, is endemic to the Himalaya-Hengduan Mountains region and particularly occurs in rocky grass slopes and meadows at altitudes ranging from 2,500 to 4,500 m. It is very interesting that this early-flowering species bears specific floral structures, which are characterized by a large sensitive stigma composed of two lobes and the anther-appendage. Our primary study showed that a set of reproductive strategies of this species were able to ensure reproductive success in harsh alpine environment. In order to further investigate its genetic structure and the breeding system, here we developed 13 microsatellite markers from *I. mairei*, and tested their utility for other three congeneric species (i.e. *I. arguta*, *I. lutea* and *I. younghusbandii*).

Genomic DNA samples of *I. mairei* were extracted from silica-gel-dried leaves using a CTAB methodology (Doyle and Doyle 1987). A microsatellite enriched library was then conducted using a modified biotin-streptavidin capture method (Chen et al. 2008). In brief, total genomic DNA (approximate 500–1,000 ng) was completely digested with 2.5 U of *Mse*I restriction enzyme (NEB), and then was ligated to a specific *Mse*I AFLP adaptor 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 adaptor-specific

Table 1 Characteristics of 13 microsatellite loci developed for *Incarvillea mairei*

Locus	Primer sequence (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	NA	H_O	H_E
IM2	F AACCCGCTCAAAACCACT R TGTATGTCCTCCAATGCTAAAG	$(AG)_8$	126–145	60	4	0.550	0.670*
IM6	F TGTATGTAGCGGCGGTGTA R CCTTTCATTGCCATCCTTC	$(TC)_6TT(TC)_6$	170–195	58	5	0.675	0.526
IM7	F TGTGCCTATCTTCTTGTTCATC R TTCCACTCGTTTTCTCTCCA	$(TG)_8$	190–230	54	7	0.575	0.726
IM26	F GTACCCCTCATACCTTCAAG R TACGAATAGAGCGAACCC	$(AC)_7$–$(TC)_6$	112–135	55	3	0.350	0.534
IM28	F CATCATCCGCAGGAAAGT R GCATCAATACAGGGCAAT	$TT(CT)_{12}CC$	188–216	56	4	0.450	0.592
IM30	F AGCCAAATGGATTCAGGT R TCAGTGTAAACGGATGCC	$(CTT)_7$	155–182	60	6	0.375	0.716*
IM32	F TCCTTTCTTCCATCTTCATT R AGGCTGGGGTTTTGTTTG	$(TC)_{13}$	212–240	55	5	0.475	0.736*
IM33	F ACTAAAACTCGGCTCGGT R TCAACTATTCCCTCCTCGT	$(TTC)_6$	185–220	58	3	0.050	0.297*
IM48	F AGTGGTAGCATCACAGTT R AGATACGAAATCATAGCC	$(AT)_4$ $(AAG)_9$	102–145	52	7	0.550	0.815*
IM73	F CCACTTCTGCCACATTTC R ATTCTGTTTCTGTGCCTATCT	$(AT)_2(GTTT)_2(GT)_6$	181–225	58	5	0.750	0.756*
IM75	F CTTCTCCTTCTTCCCACG R AATATACGCTGAACTTCCATC	$(TTC)_5$	196–215	58	3	0.175	0.616*
IM76	F CCTCTTCCTTTCCTTCATCT R AAGTTGTAGCAGGCACCG	$(GT)_5(AG)_2(GT)_3$	200–226	58	3	0.225	0.249
IM78	F ATGGAACAATCACCTAAG R TATTCGTATTTACCAGACA	$(CCG)_7$–$(GT)_8$	205–238	58	5	0.800	0.669

NA, number of observed alleles; T_a, PCR annealing temperature; H_O, observed heterozygosity; H_E, excepted heterozygosity; Statistically significant deviation from Hardy–Weinberg expectation is indicated by *($P < 0.01$)

primers (5′-GATGAGTCCTGA GTA AN-3′). The amplified DNA fragments, with a size range of 200–800 bp, were hybridized to a 5-biotinylated [$(AG)_{15}$, $(AAG)_{10}$ or $(AC)_{15}$] probe, then selectively separated and captured by streptavidin-coated magnetic beads (Promega) (Zane et al. 2002). The recovered DNA fragments were re-amplified with *Mse*I-N primers. The purified PCR products using EZNA Gel Extraction Kit (Omega Bio-Tek), were then ligated into PGEM-T vector (Promega), and transformed into DH5α competent cells (Tiangen). The positive clones were tested using vector primers SP6/T7 and specific primer $(AC)_{10}$/$(AG)_{10}$/$(AAG)_7$, respectively. In total, 240 positive clones were sequenced with an ABI PRISM 3730XL DNA sequencer, and 130 clones contained microsatellites. Finally, a total of 80 primer pairs were predicted suitable for primer designing using Primer 5.0 (Clarke and Gorley 2001).

Polymorphisms of all 80 microsatellite loci were assessed in 40 individuals of *I. mairei* from six populations in the Himalaya-Hengduan Mountains region. The PCR reactions were performed in 15 μl of reaction volume containing 30–50 ng genomic DNA, 0.6 μM of each primer, 7.5 μl 2 × Taq PCR MasterMix (Tiangen; 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 conducted under the following conditions: 94°C for 3 min followed by 30–36 cycles at 94°C for 30 s, at the annealing temperature for each specific primer (optimized for each locus, Table 1) for 30 s, 72°C for 1 min, and a final extension step at 72°C for 8 min. PCR products were separated on 8% polyacrylamide denaturing gel using a 20-bp ladder molecular size standard by silver staining.

In all, PCR products with 13 primer pairs displayed polymorphisms among different populations. Standard genetic diversity parameters, departure from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium between pairs of loci were estimated in GENEPOP version 4.0 (Raymond and Rousset 1995). The number of alleles per locus (*NA*) was 3–7 with an average of 4.615, values for observed (H_O) and expected (H_E) heterozygosities

Table 2 Cross-species amplification of 13 polymorphic microsatellite loci islolated from *Incarvillea mairei* in *I. arguta*, *I. lutea* and *I. younghusbandii*

Locus	*I. arguta* ($n = 8$)	*I. lutea* ($n = 8$)	*I. younghusbandii* ($n = 8$)
IM2	W	M	P(2)
IM6	NA	P(3)	P(4)
IM7	W	P(3)	P(5)
IM26	W	P(3)	M
IM28	W	P(2)	P(4)
IM30	P(3)	P(2)	P(3)
IM32	W	P(3)	M
IM33	W	M	W
IM48	W	P(3)	P(3)
IM73	M	P(3)	P(4)
IM75	P(3)	P(2)	P(3)
IM76	P(2)	P(3)	P(3)
IM78	P(3)	P(2)	P(4)

NA no amplification, *W* weak amplifications, *M* monomorphic amplification, *P* polymorphic amplification

ranged from 0.050 to 0.800 and from 0.249 to 0.815, with averages of 0.462 and 0.608, respectively (Table 1). For all 13 microsatellite loci, except IM6, IM7, IM26, IM28, IM76 and IM78, the genotypic frequencies showed significant deviation from HWE ($P < 0.01$) indicating the possibility of null alleles, non-random mating, or the Wahlund effect. Significant LD was detected in four locus pairs: (IM6, IM7), (IM26, IM6) (IM73, IM7) and (IM73, IM6).

Cross-species amplification in other three species (*I. arguta*, *I. lutea* and *I. younghusbandii*) was tested using eight individuals each. Twelve of them were successfully amplified in all three congeneric species, whereas the primer IM6 failed to amplify in *I. arguta*, and most of them revealed polymorphism in various species (Table 2). Eleven microsatellite loci (84.62%) showed polymorphisms in *I. lutea*, ten loci (76.92%) in *I. younghusbandii* and four loci (30.77%) in *I. arguta*, respectively. The results indicated that there was a high potential for transferring microsatellite markers in the genus *Incarvillea*. Therefore, the 13 novel polymorphic microsatellite loci developed here will be useful for understanding the population genetic structure and further investigating the breeding system and gene flow patterns of *I. mairei* and its related species.

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