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# Development of polymorphic microsatellite markers for *Incarvillea sinensis* (Bignoniaceae)[1]

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- *Premise of the study*: Microsatellite markers were developed for *Incarvillea sinensis* var. *sinensis* (Bignoniaceae), an annual herb endemic to Inner Mongolia, to study the degree to which delayed self-fertilization is favored.
- *Methods and Results*: Eight polymorphic primer sets were isolated and characterized in two Inner Mongolia populations of *I. sinensis* var. *sinensis* with a relatively simple and fast subcloning method. Numbers of alleles per locus ranged from 2 to 7, with observed and expected heterozygosities ranging from 0 to 0.261 and from 0 to 0.778, respectively.
- *Conclusions*: These markers will be useful for future studies of self-fertilization adaptability in *I. sinensis* var. *sinensis.*


**Key words:** *Incarvillea sinensis;* microsatellite; polymorphism; self-fertilization; subcloning.

The evolution of self-fertilization and the conditions under which it is favored are major subjects in the evolution and ecology of reproduction (Stebbins, 1974; Barrett, 2003; Goodwillie et al., 2005). Lloyd and Schoen (1992) classified the autonomous self-pollination mode into three general categories, termed “prior,” “competing,” and “delayed,” according to whether self-pollination occurs before, during, or after opportunities for outcrossing in a flower. Of the three modes, delayed selfing is considered especially advantageous because it provides reproductive assurance when pollinator visitation is unpredictable within and among flowering seasons. However, the realized benefit of reproductive assurance will depend not only on the efficiency of autonomous selfing, but also on the magnitude of inbreeding depression (Husband and Schemske, 1996). We aim to study the adaptability of delayed self-fertilization in *Incarvillea sinensis* Lamarck var. *sinensis* (Bignoniaceae), an annual herb endemic to Inner Mongolia with wind-mediated delayed selfing (Qu et al., 2007). We plan to use nuclear microsatellites as markers for estimating selfing rate and inbreeding depression. However, microsatellites have not been previously reported from the genus *Incarvillea*. Because microsatellites are common in the genomes of most taxa, direct sequencing of whole-genome restriction digests can be used to identify repeat sequences. Here a relatively simple and fast subcloning method was used to develop microsatellite markers.

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## METHODS AND RESULTS

Total genomic DNA of leaves sampled from Mu Us Sand land in Inner Mongolia (39°29′37″N, 110°11′29″E), was extracted using a plant genomic DNA Kit (Tiangen, Beijing, China). The extracted DNA was digested to 500–2000 bp fragments by restriction enzyme Sau3AI (Takara, Tokyo, Japan). DNA fragments were cloned into pUC19/BamHI vectors (Fermentas MBI, Toronto, Canada), and then transformed into Trans1-T1 Phage Resistant Chemically competent cell (Transgen, Beijing, China). Plasmid DNA was extracted using the Wizard SV96 System (Promega, Biotech Co., Ltd., Beijing, China) and then fragments of 500–2000 bp were sequenced by using an ABI PRISM 3730 Genetic Analyzer with a BigDye Terminator Sequencing Kit (Applied Biosystems, Foster City, California, USA). Of the 501 plasmids sequenced, 11 were found to contain di-nucleotide, trinucleotide, and penta-nucleotide repeat sequences. A total of 50 primer pairs flanking microsatellite sequences were designed using Primer Premier 5.0 (Lalitha, 2000), and 10 individuals of *I. sinensis* var. *sinensis* were used for primer screening. Eight pairs amplified single products of the expected lengths when electrophoresed on agarose gels; these pairs were fluorescently labeled and further tested for polymorphism on an ABI PRISM 3730 Genetic Analyzer using GeneMapper 4.0 and LIZ 500 as an internal size standard (Applied Biosystems). PCR amplifications were performed in a 20-uL reaction mixture consisting of approximately 5.0 ng of template DNA, 10 mm Tris-HCl (pH 8.3), 50 mm KCl, 1.5 mm $MgCl_2$, 0.2 mm of each dNTP, 0.25 um of each primer [forward- labeled with FAM, HEX, or TAMRA], and 1 U Taq polymerase (Takara). Samples were incubated in a Bio-Rad DNA EngineR Peltier Thermal Cycler. The PCR conditions included an initial denaturation step of 5 min at 94°C, followed by 30 cycles of 45 s at 94°C, 30 s at an annealing temperature of 53–58°C and 45 s at 72°C, followed by a final extension step of 10 min at 72°C (Table 1). The microsatellite loci were characterized using 22–23 individuals per population of *I. sinensis* var. *sinensis* (POP-*EM*: 39°29′36″N, 110°11′28″E, and POP-*EN:* 39°29′25″N, 110°11′19″E). PCR amplification followed the same protocol and conditions described above. Cross-species amplification was performed on three individuals of five *Incarvillea* species (*I. lutea* Bur. et Franch., *I. delavayi* Bur. et Franch., *I. Arguta* (Royle) Royle, *I. Mairei* (H. Leveille) Grierson, *I. grandifloral* (Wehrhahn) Grierson) using the same amplification conditions for *I. sinensis* var. *sinensis.*

All the eight loci gave satisfactory results with polymorphic and single-locus amplification products. For these loci, the numbers of alleles per locus, observed and expected heterozygosities, genotypic linkage disequilibrium, and deviations from Hardy–Weinberg equilibrium (HWE) for each locus in each population were calculated using FSTAT version 2.9.3 (Gouldet, 2001). The number of alleles per locus ranged from two to seven, with an average of 5.50, and the expected and observed heterozygosities ranged from 0 to 0.261 and from 0 to 0.778, respectively. No linkage disequilibria were detected at these

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Table 1. Characteristics of eight microsatellite loci in *Incarvillea sinensis*: locus name, primer sequence, repeat motif and range of product size, annealing temperature ($T_a$) and GenBank accession number.

| Locus | Primers sequences (5′–3′) | Repeat motif | Size Range (bp) | *Ta* (°C) | GenBank Accession No. |
|---|---|---|---|---|---|
| In01 | F: TAMRA-TTATCAGAGCCTAAACGG<br>R: CGCAGCAGTAGCTGTAT | $(AT)_9$ | 233–247 | 53 | HQ848937 |
| In02 | F: HEX-TATTGTGATTCTTGGTTG<br>R: GTAGGGTTATTACAGCGT | $(ATAAT)_9$ | 175–225 | 53 | HQ848938 |
| In03 | F: TAMRA-ATGGTAGGGTTTATTTTG<br>R: TGTGATTTCTGGGTTAGA | $(TA)_{15}$ | 250–268 | 53 | HQ848939 |
| In05 | F: HEX-TTCACCGCTTACAAACTG<br>R: TAGGCAAACAACTTCAATA | $(TA)_{15}$ | 189–197 | 55 | HQ848940 |
| In08 | F: HEX-ATGACGATGATTATGATGATG<br>R: CGGTATTGTTTGGGCACTC | $(ATG)_{13}$ | 282–294 | 58 | HQ848941 |
| In831 | F: FAM-GGGAATTTCTCGATACGT<br>R: TAGACCAACCATCAGGCT | $(ATTTT)_9$ | 264–269 | 53 | HQ848944 |
| In821 | F: FAM-ATTTCGTTACAATCTAATCG<br>R: AGTTACAGCATTGCTGGT | $(AAT)_{18}$ | 215–230 | 53 | HQ848942 |
| In822 | F: FAM-AGAAGAAGTGGATGATGT<br>R: GAACCACAACTCTACCTC | $(ATT)_{19}$ | 235–275 | 53 | HQ848943 |

Table 2. Results of initial primer screening in two populations of *Incarvillea sinensis*. Shown for each primer pair are the number of alleles (*A*), average observed ($H_o$) and expected heterozygosity ($H_e$).

| | *POP-EM (N = 23)* | | | *POP-EN(N = 22)* | | |
|---|---|---|---|---|---|---|
| Locus | A | $H_o$ | $H_e$ | A | $H_o$ | $H_e$ |
| In01 | 5 | 0.217 | 0.668 | 4 | 0.045 | 0.411 |
| In02 | 2 | 0.000 | 0.169 | 3 | 0.045 | 0.132 |
| In03 | 7 | 0.261 | 0.758 | 4 | 0.227 | 0.585 |
| In05 | 5 | 0.261 | 0.778 | 6 | 0.182 | 0.610 |
| In08 | 4 | 0.261 | 0.729 | 3 | 0.182 | 0.317 |
| In831 | 2 | 0.174 | 0.502 | 1 | 0.000 | 0.000 |
| In821 | 6 | 0.217 | 0.765 | 6 | 0.136 | 0.570 |
| In822 | 4 | 0.000 | 0.339 | 2 | 0.091 | 0.089 |

loci at $P < 0.05$ significance levels. All loci showed a significant deficiency of heterozygotes, which may be the outcome of self-fertilization (Table 2). Only locus In08 showed high transferability among the five *Incarvillea* species, while amplification success was poor for other loci.

## CONCLUSIONS

The polymorphic markers identified at eight loci will provide sufficient power for accurate estimates of selfing rates and inbreeding depression in evaluation of *I. sinensis* var. *sinensis.* Compared with the Fast Isolation by AFLP of Sequences Containing (FIASCO) repeats protocol, the subcloning protocol is time- and cost-effective, but depends on, to some extent, the abundance of microsatellites in the genome.

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