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TECHNICAL NOTE

Development of twelve polymorphic microsatellite loci in polyploid endangered *Omphalogramma vincaeflora* Franch. (Primulaceae)

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Abstract A total of twelve polymorphic microsatellite loci were developed from polyploid endangered species, *Omphalogramma vincaeflora* (Primulaceae). These loci were screened for variability among 45 individuals from three populations in China. The primers amplified loci with allele number ranging from 3 to 9, with an average of 4.25 per locus. Polymorphism information content ranged from 0.23 to 0.86. Nei's genetic diversity ranged from 0.34 to 0.86. These primers provide an opportunity to use polymorphic DNA markers to study the population genetic structure and its breeding system in this species.

Keywords Microsatellite · *Omphalogramma vincaeflora* · Polyploidy · Genetic structure · Breeding system

Introduction

Omphalogramma vincaeflora Franch. is an endangered, perennial herbaceous plant and locally endemic to the alpine area in SW China. It grows in alpine meadows and forest margins at altitudes of 2200–4600 m (Hu and Kelso 1996). Currently, destruction and losing of habitat result in a rapid decline of population size and local population extinction. This species is in endangered status because of the small population size and restricted geographic distribution. Information of the population genetic structure is important to conserve and manage the existing populations for this endangered species. Moreover, all species of *Omphalogramma* are polyploidy, with chromosomes counts ranging from 48 to 96 in the genus (Bruun 1932; Huang 2004). *O. vincaeflora* is hexaploid with a chromosome number 2n = 6x = 72. Populations of *O. vincaeflora* show less well-known floral design, stigma-height dimorphism, and it involves discrete style-length morphs and shares some similarities with distyly (Lloyd and Webb 1992a). *O. vincaeflora* provides an excellent opportunity to investigate the breeding system of stigma-height dimorphism and the evolution of heterostyly. We report on the characterization of 12 polymorphic microsatellite loci to investigate genetic structure among populations for further conservation genetics studies. These markers also will be used for analyzing selfing rates and characterizing gene flow within *O. vincaeflora* populations to further clarify its breeding system.

Genomic DNAs were extracted using a Plant Genomic DNA Kit (TIANGEN, Beijing) from dry leaf tissue. The isolation of microsatellite loci from an enriched $(AG)_n$ library was performed according to the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol proposed by Zane et al. (2002). This method began with DNA digested with 2.5 U of *Mse*I restriction enzyme (NEB). The digested DNA fragments were ligated to *Mse*I adaptor pair (5′-TAC TCA GGA CTC AT-3′/5′-GAC GAT GAG TCC TGA G-3′). The digestion-ligation fragments were diluted 1:10 and 5 μl was amplified with adaptor-specific primers (5′-GAT GAG TCC TGA GTA AN-3′,

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hereafter refereed to as *Mse*I-N). The PCR products were hybridized with 0.15 μM of 5′-biotinylated $(AG)_{15}$ oligonucleotide probes in a total volume of 250 μl of SSC 4.2X and SDS 0.07% at 48°C for 2 h. Then, the DNA hybridized to biotinylated probes were selectively separated and captured by Streptavidin MagneSphere® Paramagnetic Particles (Promega) followed by two steps according to the procedure of Zane et al. (2002). Recovered DNA fragments were amplified with *Mse*I-N primers. The PCR products with purification using E.Z.N.A.® Gel Extraction Kit (Omega Biotek), were ligated into pMD18-T vector (Takara) according to the manufacturer's instructions, then transformed into *Escherichia coli* strain JM 109 competent cells (Takara). Transformants were plated, then insert-containing clones were selected by blue-white screening. The positive clones were tested using $(AG)_8$ and $M13^+$/$M13^-$ as primers, respectively (Li et al. 2007). In positive clones, PCR electrophoresis would show two DNA fragments, whereas, only the whole inserted fragment can be amplified in negative clone. One hundred forty-seven positive clones were chosen and sequenced on an ABI3700 DNA sequencer.

A total of 73 clones were found to contain a repeat region and 48 clones proved suitable for primer designing using Primer 5.0 (Clarke and Gorley 2001). These primers were tested for polymorphism in *O. vincaeflora* population. PCRs were performed in a 20-μl volume containing 50 ng of genomic DNA, 0.5 μM of each primer, 0.2 mM of dNTPs, 1× *Taq* buffer (100 mM Tris–HCl, pH8.8, 2.0 mM $MgCl_2$, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20) and 1 U of Takara *Taq* polymerase using a PTC200 thermocycler. PCR took place as follows: 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 50–60°C (optimized for each locus, Table 1) for 30 s, 72°C for 30 s, and a final extension step at 72°C for 8 min. Amplicons were denatured at 95°C for 7 min, then separated on 6% denaturing polyacrylamide gels and visualized by silver staining. Allele sizes were estimated using a 10-bp ladder molecular size standard (Invitrogen). The allele number, size range, number of bands per individual, and polymorphism information content (PIC) values were

Table 1 Characteristics of twelve microsatellite markers for *O. vincaeflora*

Locus	Primer sequence (5′ to 3′)	Repeat motif	N_A	Allele size (bp)	T_a (°C)	PIC	Nei's genetic diversity	GenBank accession no.
OvH011	F: GCTGGGAATTGGTCACTGAA R: AAAGATGCAACCAGACGAGG	$(GA)_7(GA)_4$	3	125	54	0.55	0.34	EU429540
OvH023	F:TCTTTGAAGCGGTTACGGTA R:AAACTTGGAGGACTTGGAGG	$(TC)_5(TC)_{12}(CT)_6$	3	291	54	0.55	0.67	EU429542
OvH102	F: TTCCCATGCACCATTATTTC R: ACTTCCTTCCTTCCCGTCTT	$(AG)_8(GA)_9$	5	191	54	0.76	0.67	EU429541
OvH153	F:AGAATAAGAGGAGCAGAAGT R:CAGAAGTAAGAGCGTAAGAG	$(CTT)_4(CTT)_4(CT)_{12}$	4	239	58	0.63	0.70	EU429543
OvH161	F: ACTCAATTGCATGAGACCAA R: CAGATAATGGCTGCTTTGAT	$(AG)_{17}$	3	225	56	0.53	0.75	EU429544
OvH165	F: TGAGGACCAAATGCGTTAGC R: GTTTTCACCCGTTTCGTCTC	$(AG)_9(AG)_4(GA)_5$	5	246	50	0.76	0.67	EU429545
OvH169	F: TAAAGTGGTCGTGTAGCGGA R: ATTTCGGCTGCCCTATTCTA	$(CTTC)_3(CT)_{15}$	3	282	52	0.38	0.67	EU429546
OvH175	F: GTCAAATCATAGGTCAAACG R: TAGGAGGACCTTGAGTGTAA	$(TC)_4(TC)_9$	3	236	54	0.23	0.37	EU429547
OvH182	F: GGCTGCCATACCTTTTAGAG R: GGAGATGAAAACCCCTTACC	$(AG)_3(GA)_4(GA)_{11}(GA)_3(GGAA)_3$	4	243	60	0.59	0.67	EU429548
OvH184	F: ATGGTATGGCAGGTGTTTTC R:ACACCAAAGATCGAAATGGA	$(TTC)_{11}(CGA)_3(CT)_{14}$	9	183	58	0.86	0.86	EU429549
OvH185	F: AAACCCTTTCATTTCTCCAC R: CTCCCATTTTCACCATCTTC	$(GA)_3(GA)_{10}(AAG)_3(AGA)_3$	6	183	52	0.77	0.67	EU429550
OvH186	F: CCTTCCTTTCCGTTTTCACC R: ATTTTCGGCAGCCCACATAG	$(TC)_4(TC)_{12}$	3	147	60	0.56	0.79	EU429551

N_A, number of observed alleles; T_a, PCR annealing temperature; PIC, polymorphism information content

calculated for each of the loci following the method of Baruah et al. (2003) using a web-based calculator (http://www.agri.huji.ac.il/-weller/hayim/parent/PIC.htm). Nei's genetic diversity (H_E) was estimated using GENOTYPE AND GENODIVE software allowing analysis of polyploidy data (Meirmans and Van Tienderen 2004).

A total of 45 individuals from three populations from Yunnan and Sichuan were used for genotyping. Twenty-five of the 48 primer pairs tested successfully amplified the target fragments, and 12 loci showed polymorphism (Table 1). The number of observed alleles ranged from 3 to 9 with an average of 4.25. PIC values ranged from 0.23 to 0.86, with ten of the twelve loci exhibiting PIC values greater than 0.5. Nei's genetic diversity ranged from 0.34 to 0.86. Up to 4 alleles per individual indicated the polyploidy nature of *O. vincaeflora* as we previously described as hexaploid. Because *O. vincaeflora* is polyploidy and the exact copy number for each locus is currently unknown, traditional measures of genetic variability, standard tests for deviations from Hardy–Weinberg equilibrium and linkage disequilibrium could not be determined. The microsatellite loci described here will be used to investigate the population genetic structure. Furthermore, these markers are intended to examine gene flow patterns and breeding system of this species.

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