Technical Note

Title

Isolation and characterisation of microsatellite loci in *Calystegia soldanella* (Convolvulaceae), an endangered coastal plant isolated in Lake Biwa, Japan

Running title: Primers for microsatellite loci in *Calystegia*

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Abstract

Eight microsatellite loci of *Calystegia soldanella* useful for comparisons of the genetic structure of isolated populations in the ancient Lake Biwa and coastal populations in Japan were isolated and characterised. The number of alleles ranged from 2 to 5. The expected (H_E) and observed (H_O) heterozygosities were 0.097-0.583 and 0.000-0.380, respectively, from 100 individuals from Lake Biwa and coastal populations. Seven of the eight loci exhibited significantly fewer heterozygotes than expected based on the Hardy-Weinberg equilibrium (P<0.05). These primers amplifying microsatellites in *C. soldanella* may provide a population genetics tool useful in the establishment of a conservation strategy.

Keywords *Calystegia soldanella*, Conservation genetics, Heterozygosity, Lake Biwa, Microsatellite

Calystegia soldanella (L.) Roem. et Scult. is a perennial coastal herb of the family Convolvulaceae that is globally distributed across sandy seashores of temperate zones. This plant is self-incompatible and proliferates by sexual reproduction as well as clonal propagation through the elongation of rhizomes. In Japan, *C. soldanella* is the most common species of sandy coastal vegetation and also occurs on the sandy shores of Lake Biwa, an ancient lake that was established ca. 400 MYA ago and harbours coastal plant taxa. *C. soldanella, Vitex rotundifolia* L., *Lathyrus maritimus* (L.) Bigel. and *Pinus thunbergii* Parl. are assumed to have migrated from coastal populations in ancient times and have been isolated from them for a very long period of time (Kitamura 1968).

The lakeshore environment at Lake Biwa has been heavily disturbed by development, and *C. soldanella* has been protected as a threatened species in Shiga Prefecture. In the present study, we aimed to clarify the comparative genetic structure of *C. soldanella* between Lake Biwa and coastal populations using single-sequence repeat (SSR) markers, and here we report the development of microsatellite loci for this species.

Genomic DNA of *Calystegia soldanella* was extracted from fresh leaves collected from populations in Lake Biwa and the nearby coast (Ise Bay, Pacific side). Fresh leaf material was frozen in liquid nitrogen and then ground into a fine powder. After polysaccharides were removed from the powder using HEPES buffer (pH 8.0; Setoguchi and Ohba 1995), genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). The extracted DNA was dissolved in 100 μL of TE buffer.

Microsatellite loci were isolated using an improved technique for isolating codominant compound microsatellite markers (Lian and Hougetsu 2002, Lian et al.

2006). DNA was digested with the blunt-end restriction enzymes *Ssp*I, *Eco*RV and *Alu*I. The restricted fragments were then ligated with a specific blunt adaptor (consisting of the 48-mer

5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3'

and an 8-mer with the 3'-end capped with an amino residue: 5'-ACCAGCCC-NH_2-3') using a DNA ligation kit (Takara Bio). The ligated fragments were treated with ddGTP using AmpliTaq Gold (Applied Biosystems) to block polymerase-catalysed extension of the 8-mer adaptor strand. Fragments were amplified from the *Ssp*I, *Eco*RV and *Alu*I DNA libraries using one of the compound SSR primers $(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ and an adaptor primer (5'-CTATAGGGCACGCGTGGT-3'). After electrophoresis on a 1.5% agarose gel, the fragments (400–800 bp) were purified using the Geneclean II kit (Bio 101, Vista, CA, USA) and cloned using the Qiagen PCR Cloning plus Kit (Qiagen) in accordance with the manufacturer's instructions. Recombinant clones were identified using blue/white screening on LB agar plates containing ampicillin, X-gal and IPTG. Ninety-six insert-positive clones were amplified using forward and reverse primers and sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). For 48 fragments containing the $(AC)_6(AG)_n$ or $(TC)_6(AC)_n$ compound SSR sequence at one end, a specific primer was designed using Primer3 v. 0.4.0 (Rozen and Skaletsky 2000). PCR amplifications were performed following the standard protocol of the Qiagen Multiplex PCR Kit (Qiagen) in a final volume of 6 µl. Compound SSR primers [$(AC)_6(AG)_5$ or $(TC)_6(AG)_5$] were labelled with the fluorochromes 6-FAM or HEX (Applied Biosystems). The amplification profiles included initial denaturation at 95°C for 15 min; followed by 35 cycles of 30 s at 94°C, 1.5 min at 57°C (the annealing temperature of the primer pair) and 1 min at 72°C; and a final extension at 60°C for 30

min. The size of the PCR products was measured using the ABI PRISMTM 3100 Genetic Analyzer (Applied Biosystems) and GeneMapper^{TM} analysis software (Applied Biosystems).

Eight microsatellite loci that showed a clear and strong band on the electrophoretic gel were detected in the preliminary screening. For further characterisation, 100 individuals of *Calystegia soldanella* were genotyped using the procedure described above. These samples were collected from the Lake Biwa population (50 individuals) and the nearby coastal population in Ise Bay (50 individuals). Allelic diversity per locus ranged from 2 to 5, with an average of 3.125 (Table 1). The expected heterozygosity (H_E) ranged from 0.097 to 0.583, while observed (H_O) heterozygosities were 0.000-0.380. Deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium at each locus was calculated using FSTAT version 2.9.3.2 (Goudet 1995). Significant deviations ($P < 0.05$) from HWE were detected for seven of the eight loci, with the exception of TC10. The deviations from HWE would be attributable to very low genetic diversity ascribed to a bottleneck effect at the time of population establishment. Coastal plant populations in Japan tend to be damaged by the tidal waves of typhoons and become reestablished from a limited number of seeds that were buried and/or drifted ashore. In fact, most of the *Calystegia* populations harbour single cpDNA haplotypes, whereas we have found seven haplotypes from the coastal and Lake Biwa populations in Japan (Setoguchi et al. unpublished). The developed loci were independent based on 560 permutations among 28 possible pairwise locus comparisons for testing linkage disequilibrium, with the exception of two loci, AC2 and AC41.

The eight microsatellite loci presented here should be useful for measuring genetic diversity and gene flow, as well as for elucidating intra-population genetic structure in

isolated populations of *Calystegia soldanella* at ancient Lake Biwa.

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table

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Table 1 Characteristics of the eight nSSR loci isolated from *Calystegia soldanella*

Locus	Accession number	Primer Sequence (5' to 3')	Total					Lake Biwa			Ise Bay (coastal population)		
			Size range (bp)	No. of allels	H_O	H_E	*P value*	No. of allels	H_O	H_E	No. of allels	H_O	H_E
AC2	AB453809	R:TCATGACTCCTAAGTGGGAAAGA	91-93	2	0.008	0.505	0.006	2	0.040	0.153	2	0.120	0.182
		F:ACACACACACACAGAGAGAGAG											
AC5	AB453810	R:AAAACAAGAAATCTGAAAGCACAA	372-374	2	0.000	0.358	0.006	2	0.000	0.184	2	0.000	0.470
		F:ACACACACACACAGAGAGAGAGAG											
AC24	AB453811	R:AGGCCAGGAGATTCGGTAAT	142-154	4	0.008	0.352	0.006	2	0.040	0.040	4	0.120	0.556
		F:ACACACACACACAGAGAGAGAG											
AC30	AB453812	R:TCATGACTCCTAAGTGGGAAAGA	91-93	2	0.009	0.505	0.006	2	0.080	0.183	2	0.100	0.199
		F:ACACACACACACAGAGAGAGAG											
AC41	AB453813	R:TTAGGTTTGGCTCACAGATGG	396-402	4	0.009	0.532	0.006	3	0.100	0.298	4	0.080	0.236
		F:ACACACACACACAGAGAGAGAG											
TC4	AB453814	R:AAATGGACAGTGCCAGAAGG	353-357	3	0.006	0.097	0.019	1	NA	NA	3	0.120	0.188
		F:TCTCTCTCTCTCACACACACAC											
TC10	AB453815	R:AATCTTACCCCTCTAGGCCG	230-234	3	0.370	0.348	0.850	2	0.300	0.261	3	0.440	0.422
		F:TCTCTCTCTCTCACACACACAC											
TC11	AB453816	R:CAGTGAAGTAAACTTACAAAAATCATG	88-96	5	0.380	0.583	0.006	3	0.180	0.175	5	0.580	0.629
		F:TCTCTCTCTCTCACACACACAC											
Average				3.125	0.099	0.410	0.006	2.125	0.093	0.162	3.125	0.195	0.360

Abbreviation: NA, not applicable.