

Isolation and characterization of 11 microsatellite loci from *Carex macrocephala* (Cyperaceae)

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Abstract *Carex macrocephala* (Cyperaceae) is a species of great conservation concern along the northwest coast of North America. Using a biotinylated-streptavidin bead protocol, we successfully isolated and characterized 11 microsatellite loci from a *C. macrocephala* genomic library. Although these loci are polymorphic, the scored populations exhibit a significant deficiency in heterozygosity. *Carex macrocephala* is a self-pollinating rhizomatously spreading sedge, consequently this may contribute to the observed patterns.

Keywords Sedge · Clonal population genetics · Coastal organism · Genetic diversity

Introduction

Carex macrocephala (Cyperaceae), the large-headed sedge is one of two species within the *Carex macrocephala* species complex along with *C. kobomugi*. *Carex macrocephala* is a perennial that spreads rhizomatously across moderately disturbed areas on sandy beaches and sand dunes along the north Pacific coast. Its range extends from

mid-Oregon north to Alaska and the Aleutian Islands to the Kamchatka Peninsula, around the Sea of Okhotsk, and south to Hokkaido Island of Japan. It has been listed as a species of concern in numerous counties in Oregon and Washington states, USA, and has been officially red-listed as a threatened/endangered species along the coast of British Columbia, Canada (Harper et al. 1994). Previous studies have identified 14 polymorphic microsatellite loci within closely related *C. kobomugi* (Ohsako and Yamane 2007), and we have developed a unique set of microsatellite loci from *C. macrocephala*. With these loci we will be able to test the relationships among the lineages of the *C. macrocephala* species complex, provide a means to identify clonal levels within populations of *C. macrocephala* along the northwest coast of North America; and obtain population genetic indices from populations along the northwest coast of North America to determine levels of gene flow and population structure.

A size fractionated genomic library was constructed from a single individual from *C. macrocephala* (King voucher specimen 36). Total genomic DNA was isolated from 5 g of fresh leaf tissue using a modified 2× CTAB protocol (Roalson et al. 2001). A total of 50 µg of extracted DNA was digested with 3 units of the *Sau3aI* restriction enzyme for 30 min. DNA fragments ranging from 300 to 1,000 bp in length were size selected with a 3% agarose gel. Fragments imbedded in the agarose gel were extracted with a Wizard SV Gel Clean-Up System (Promega Corp.). The SAULB (5'-GATCCCAAGCTTC CCGGGTACCGC-3') and SAULA (3'-GGTTCGAAGGG CCCATGGCG-5') linkers were added to the genomic fragments by first denaturing the linkers at 95°C and then annealing them together at 60°C for 10 min in a 250 mM NaCl solution. These annealed linkers were then ligated onto the genomic fragments using 2 units of T4 DNA

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Table 1 Characterization of polymorphic microsatellite loci from *Carex macrocephala*

Locus	GenBank accession no.	Primers	T _a (°C)	Repeat	Dye	Clone allele size	A _{total}	VI		QCI			
								A _{VI}	H _o	H _e	A _{QCI}	H _o	H _e
CM01	EU525889	f-CAAAGGAGAGAAATTCTCTG r-GATCAGGTCATACCAAGTAT	58	(CT) ₁₄	6FAM	220	5	4	0.2	0.58*	5	0.167	0.428*
CM07	EU525890	f-ATATCATGGGCACCTGGTCTTC r-ATCATCTAGTATTCCACAGAG	50	(CT) ₁₇	NED	144	6	6	0.167	0.56*	6	0.139	0.689*
CM12	EU525886	f-TCGCAAACGCCCTTCAAATAAC r-GACGTGCCCTTCCGCCAGTAG	46	(GT) ₃₉	VIC	231	5	5	0.067	0.744*	4	0.056	0.42*
CM13	EU525887	f-CGCAGCACCCAAACAGTTTA r-ACACGTGCACACACGTACCC	46	(GT) ₄₇	PET	217	6	6	0.133	0.801*	6	0.111	0.782*
CM16	EU525888	f-GGCACATGTACGACATGAGA r-ATCACGACAGCATGGTGTAC	46	(GCT) ₂₂ (GTT) ₂₅ (GAT) ₁₂ (GCT) ₃₆	PET	528	5	4	0	0.581*	5	0	0.701*
CM25	EU525883	f-CGGTGTGGTGGGCTGATA r-CGCTTCTCCGTTTCTTTTGT	58	(CT) ₉	6FAM	380	2	2	0	0.183*	2	0	0.127*
CM27	EU525884	f-GATTTAGTACAGCCACACAG r-ACCAACCAGTCAGCCTCTCA	55	(CT) ₆	VIC	435	9	8	0.133	0.743*	8	0.111	0.795*
CM28	EU525885	f-GACCCGGAAACCAGCTGAAC r-GTACCCCACTCCGACGCATAA	55	(GT) ₄₅	NED	175	5	5	0	0.601*	5	0	0.75*
CM35	EU525880	f-AGGAGGAGGAGGAGCAA r-GCGGAGGAGGGCAGAGATA	57	(GCT) ₆	VIC	200	4	3	0	0.242*	4	0	0.35*
CM36	EU525881	f-TGGCGGAGAAGGGGAGGTTA r-CGTTTCAGGGGATACAAAGTT	57	(CT) ₁₂	PET	305	5	5	0.033	0.655*	5	0.028	0.674*
CM39	EU525882	f-ATCCACCGTTGCCACCTATT r-ACCGTTGTGATGCTTAGT	55	(CAG) ₁₉ (CAA) ₅ (CAG) ₁₁ (CAA) ₁₈	6FAM	304	4	2	0.067	0.066	4	0.056	0.218*

VI is the Vancouver Island population and QCI is the Queen Charlotte Island population; T_a = annealing temperature; A = number of alleles; H_o = observed heterozygote frequency; H_e = expected heterozygote frequency; Significant deviation from Hardy–Weinberg equilibrium (*) $P < 0.01$

ligase at 16°C for 12 h, then heated to 70°C for 15 min to deactivate the enzyme.

The prepared genomic fragments were PCR amplified using the SAULA protocol 95°C for 1 min, 72°C for 1 min, 67°C for 1 min, and 72°C for 2 min repeated 30 times, followed by a 30 min 72°C extension. The amplified products were enriched for (GT)₂₅, (CT)₂₅, (CCT)₁₇, and (CAG)₁₇ repeats using the biotinylated-streptavidin bead method developed by Hamilton et al. (1999). All hybridizations took place at 50°C for 12 h followed by washes of 55°C for (GT)₂₅ and (CT)₂₅ and 65°C for (CCT)₁₇ and (CAG)₁₇. These hybridizations and washes were carried out twice, each followed by a PCR amplification (Hamilton et al. 1999). After the third PCR amplification the enriched fragments were cloned using the pGEM T-Easy Vector System (Promega). Cloned fragments were sequenced using Big Dye Terminators 3.1 on an Applied Biosystems (City, State) 3730 automated DNA sequencer. Sequences were screened and edited using Sequencher 4.5 (GeneCodes Corporation, City State). Approximately 42% of the sequences contained simple sequence repeats (SSRs).

Thirty-six of the cloned sequences were used to design locus specific primers to test for amplification efficiency and polymorphism. Priming sites were chosen manually. After PCR optimization twenty-five of the thirty-six loci amplified successfully, and of these, 11 exhibited polymorphism using agarose gel electrophoresis.

To test for levels of polymorphism we selected two populations of *C. macrocephala* from the coast of North America, a population from Vancouver Island (VI), and a population from the Queen Charlotte Islands (QCI). Each population had a sample size of 30 individuals. All loci were amplified using a PCR cycling protocol of 95°C for 3 min, followed by 30 repeated steps of 95°C for 30 s, the appropriate annealing temperature, T_a, for 30 s, 72°C for 15 s, with a final extension step at 72°C for 30 min. PCR reactions were comprised of 1 µL of 10× buffer with 1.5 mM of MgCl₂, 1 µL of 4 µM dNTPs, 1 µL of 5 µM 5' and 3' primers, 1 unit of TAQ DNA Polymerase, and 5.9 µL of H₂O. Amplified fragments were run on an Applied Biosystems 3730 and scored using GENEMAPPER 3.7 (Applied Biosystems). Tests for Hardy–Weinberg Equilibrium (HWE) were carried out using GENEPOP (Raymond and Rousset 1995) and summarized in Table 1. Tests for linkage disequilibrium were run in FSTAT (Goudet 1995). Significance levels were adjusted using sequential Bonferroni corrections (Rice 1989).

Our data set shows a low level of heterozygosity with observed values, H_o, ranging from 0 to 0.2. To assess whether null alleles may be causing the pattern of heterozygote deficiency we used the program MICROCHECKER (Van Oosterhout et al. 2004) to estimate null allele values at each locus. This did not significantly change our observed deficiency of heterozygotes, and as such did not alter the significance of the deviation from HWE. No loci showed significant linkage disequilibrium after Bonferroni correction. These loci were also successfully amplified in *C. kobomugi*, and exhibited polymorphisms between *C. macrocephala*, in agarose gels, but were not further tested. Intraspecific polymorphism levels were not tested within *C. kobomugi*. The levels of variation observed within these microsatellite loci will be sufficient to test our population genetic hypotheses along the North American coast.

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