Molecular Ecology Resources (2008) **8**, 113–115 doi: 10.1111/j.1471-8286.2007.01885.x

PERMANENT GENETIC RESOURCES

Novel microsatellite markers for the saltmarsh sharp-tailed sparrow, *Ammodramus caudacutus* (Aves: Passeriformes)

CHRISTOPHER E. HILL,* SCOTT TOMKO,* CRIS HAGEN,† NANCY A. SCHABLE† and TRAVIS C. GLENN†

**Department of Biology, Coastal Carolina University, PO Box 261958, Conway, SC 29528, USA, †Savannah River Ecology Laboratory, University of Georgia, Drawer E, Aiken, SC 29802, USA*

Abstract

We have developed eight high-quality microsatellite DNA loci for the saltmarsh sharp-tailed sparrow and one additional locus with evidence of null alleles. In a sample of 250–350 individuals, the average number of alleles per locus was 14.7 and average observed heterozygosity was 0.80. These loci were tested in three additional species of emberizid sparrows, indicating that more than half of the loci could be useful in other sparrows.

Keywords: *Ammodramus*, birds, Emberizidae, microsatellites, PCR primers, SSR

Received 18 March 2007; revision accepted 10 May 2007

The saltmarsh sharp-tailed sparrow, *Ammodramus caudacutus* (hereafter: 'saltmarsh sparrow') is confined to tidal marshes, where it is a species of national conservation concern (USFWS 2002). This species and the others in the genus *Ammodramus* have been the subjects of studies of speciation (Greenlaw 1993; Rising & Avise 1993), hybrid introgression (Shriver *et al*. 2005), and of mating system (Greenlaw & Rising 1994 and references therein; Hill & Post 2005). The social mating system of the saltmarsh sparrow, scramble competition polygyny, is nearly unique among songbirds. To trace paternity of saltmarsh sparrow chicks, we developed a set of polymorphic microsatellite loci.

We used the microsatellite enrichment protocol of Glenn and Schable (Glenn & Schable 2005). Briefly, DNA was extracted from the blood (preserved in lysis buffer 0.1 M Tris, 0.1 M EDTA, 0.01 M NaCl, 0.5% SDS, pH 8.0) of a female saltmarsh sparrow. Genomic DNA was purified by phenol–chloroform extraction with an RNase step and precipitated in ethanol, then reconstituted in TE (10 mM Tris pH 8, 2 mM EDTA). The DNA was digested with *Rsa*I and simultaneously ligated to superSNX linkers with T4 ligase. The fragments with linkers were enriched for microsatellite repeats by hybridization to 3′ biotinylated oligonucleotides followed by capture on streptavidin-coated magnetic beads. The sequences of the oligos used for enrichments included $(TG)_{12}$ $(AG)_{12}$ $(AAG)_8$ $(ATC)_8$ $(AAC)_8$ $(AAT)_8$ $(ACT)_8$ $(AAAC)_6$ $(AAAG)_6$ $(AATC)_6$ $(AATG)_6$ $(ACCT)_6$ $(ACAG)_6$ $(ACTC)_6$ $(ACTG)_6$ $(AAAT)_8$ $(AACT)_8$ $(AAGT)_8$ $(ACAT)_8$ and $(AGAT)_8$.

The captured fragments were eluted and then recovered by polymerase chain reaction (PCR) with linker-specific primers, creating a primary-enriched library. The primary library was enriched again, producing a second, serially (double) enriched library. The recovery products from both libraries were ligated into pCR 2.1-TOPO vector, inserted into One Shot Top10 chemically competent *Escherichia coli* cells, and screened for successful insertion using the β-galactosidase gene with materials and protocols provided by the TOPO TA cloning kit (Invitrogen). A total of 384 colonies from the primary enrichment and 192 colonies from the double enrichment were checked for inserts between 500 bp and 1000 bp by PCR with plasmid primers.

Bidirectional sequence was determined for 92 inserts from the primary enrichment and 192 inserts from the double enrichment using ABI BigDye Terminator version 1.1 chemistry at 0.25×, Centrisep columns, and an ABI PRISM 310 genetic analyser. Resulting sequences were examined in SEQUENCING ANALYSIS software (Applied Biosystems), with the forward and reverse sequences assembled using CHROMAS PRO (Technelysium Ltd). Candidate loci were selected by examining sequences and choosing those with 10 perfect repeats and both flanking regions showing good quality, nonrepetitive sequence.

We designed primers for candidate loci using PRIMER 3 (Rozen & Skaletsky 2000). To facilitate screening primers

Correspondence: C. E. Hill, Fax: (843) 349 2201; E-mail: chill@coastal.edu

Table 1 Characteristics of nine new microsatellite loci from the saltmarsh sparrow, *Ammodramus caucadutus*. Size ranges, number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities from 250 to 350 individuals from Connecticut. 'Clone', size of PCR product in clone

Locus GenBank Accession no.	Primer sequences (5′–3′) 5′ fluorescent tag as indicated	Repeat motif	Clone (bp)	Range (bp)	N_A	H_O	H_E	Amplification conditions [($MgCl_2$), AT, #cycles]
Aca01 EF447093	6FAM-AGCCCACTAATGGGTTTTCC TGAGTGTTCAAAGTTGCCAGA	$(TCTA)_{14}(TCA)_2$-TCTATCA$(TCTA)_{13}$	229	165–229	15	0.867	0.877	2.5 mM, 56C, 32
Aca04† EF447094	6FAM-CAATGGTGACTGCAATCCTG CCTCAGCCATTTCTGTTGTCT	$(TCTA)_{11}(TC)_3$TATCC-$(TCTA)_{13}$	211	157–291	32	0.858	0.902	2.5 mM, 56C, 32
Aca05 EF447095	HEX-CCTGCTAGGCTGCATCTTCT GAGTGTCATCACATTTGTACTTTGG	$(TGTC)_2(TATG)_7(TATC)_{14}$	203	193–239	12	0.823	0.860	2.5 mM, 56C, 32
Aca08 EF447096	6FAM-TAGCCACAAGCAAGACCTGA CTGTGACAGGAAGGGCAGTT	$(AC)_{18}$	175	165–185	10	0.767	0.790	3.0 mM, 56C, 32
Aca10 EF447097	NED-GAAATGTGGGGAGATGCTGT TGCTATTAGGTCTTTTCATTGTCC	$(TATC)(TGTC)(TATC)_{11}$	190	158–194	10	0.795	0.775	2.5 mM, 56C, 32
Aca11 EF447098	NED-AGCTTCCCATACCTGAATGC GAAAGGCATGAGTTTACAGTGG	$(AGAT)_{12}$	142	130–166	14	0.789	0.832*	2.5 mM, 56C, 32
Aca12‡ EF447099	HEX-GCTTGTTTCCTGTTCCCAAA AATCGGATCCATAGACTTCAAA	$(GATA)_9G(GATA)_5(GACA)_2$-$(GACG)(GATA)_2GATG$	243	213–257	20	0.785	0.902	2.5 mM, 56C, 32
Aca17 EF447100	NED-GGAGCATGTGACAATGGAGT TCTGTGCTGTTCCAAGCAGA	$(TCTA)_{13}(TC)_9$	242	234–258	8	0.752	0.771	2.5 mM, 50C, 40
Aca21§ EF447101	NED-CCCTCTCCCTGGCTACTCTT CTGGTGCTTCTGGCTCAGT	$(ATCT)_{14}(TC)_8$	247	223–259	11	0.595	0.773*	2.5 mM, 50C, 40

*Significant departure from HWE; †although a tetranucleotide repeat, with typical 4-bp stutter, Aca04 has alleles spaced every 2 bp for much of its range; ‡although a tetranucleotide repeat, Aca12 has two series of alleles differing by a single base pair, so that spacing between allelic sizes in base pair goes 3,1,3,1, etc; §Aca21 has known, fairly common null alleles, with frequency estimated at 0.125.

for polymorphic products on a DNA sequencer, we attached an engineered sequence tag (5′-CAGTCGGGCGTCATCA) to the 5′ end of the shorter of the two locus-specific primers, and prepared a cocktail containing three primers: the tagged locus specific primer, the reverse locus specific primer, and a third primer with the engineered sequence and bearing a 5′ dye label (see www.uga.edu/srel/DNA_Lab/protocols.htm). After screening, we ordered 5′ dye-labelled locus-specific primers for loci that amplified and appeared polymorphic. Further amplifications were carried out with the locus specific primers in 9- L reactions containing 25 ng template DNA, 2 pmol each primer, 0.2 mM each dNTP, 0.3 U *Taq* DNA polymerase (Promega) in 1 × PCR buffer with 2.5 mM $MgCl_2$. Thermal cycling was carried out in a Thermo Hybaid PX2 cycler as follows: 32 cycles of (30 s at 94 C, 30 s at 56 C and 30 s at 72 C), followed by a single 40-min hold at 72 C (See Table 1 for exceptions).

PCR products were electrophoresed on an ABI PRISM 310 genetic analyser and sized and binned using GENESCAN and GENOTYPER software. We calculated heterozygosity and null allele frequency with CERVUS 2.0 (Marshall *et al*. 1998), and tested for genetic disequilibrium using GENEPOP 3.4 (Raymond & Rousset 1995).

Preliminary sequencing of 92 clones from the primary enrichment produced only three loci suitable for primer design. Of 109 doubly enriched sequences, however, 88 contained microsatellites of at least seven repeats. The trinucleotide repeat motifs AAG and AGG were observed in 10 clones, always with long stretches of other repeat motifs with varying combinations of A and G. We interpreted the AAG and AGG clones to be derived from highly repetitive elements within the saltmarsh sparrow genome. Of the 91 clones with microsatellites, we initially designed primers for 13. Of the 13 primer pairs, six produced a product that appeared to be polymorphic and seven failed to amplify reliably. Redesign of six of the nonworking primer pairs produced one more useful locus, and designing primers for a further 10 loci provided two more. At this stage, we discovered that one locus, Aca21, had a nonamplifying 'null' allele at frequency of about 12%, detectable by apparent homozygote excess and confirmed by comparing offspring with known mothers. Redesign of both forward and reverse primers for Aca21 always produced consistent amplification, but no primer combination eliminated the null allele.

The nine loci are described in Table 1. The average number of alleles found in a large sample of saltmarsh sparrows was 14.7. Average observed heterozygosity was 0.80 (excluding Aca21). Tests for linkage disequilibrium showed no significant linkage between pairs of loci after sequential Bonferroni correction.

Table 2 Cross amplification of nine microsatellite loci from the saltmarsh sparrow, *Ammodramus caucadutus*, in three other species of emberizid sparrows. Each entry indicates percentage of samples amplifying and number of alleles found. *N*, number of individual birds where amplification was attempted

		Locus								
Species	*N*	Aca01	Aca04	Aca05	Aca08	Aca10	Aca11	Aca12	Aca17	Aca21
Ammodramus nelsoni	8	75/5	88/8	68/5	100/5	100/5	88/7	88/5	68/6	57/2
Ammodramus maritima	48	55/5	0/0	100/13	100/11	73/9	86/11	0/0	98/12	25/4
Melospiza melodia	8	100/6	0/0	75/3	100/8	100/10	0/0	100/5	0/0	88/5

We tested these loci on three other emberizid sparrow species: sister species *Ammodramus nelsoni*, congener *Ammodramus maritimus*, and the more distantly related *Melospiza melodia*. Results of cross-amplification trials are shown in Table 2, indicating that these loci will be of use for a variety of sparrow species. In these trials, only one PCR attempt was made per sample, and the listed amplification rate may be conservative.

We will use these markers to investigate multiple mating by female saltmarsh sparrows, and to trace reproductive success of males who cannot otherwise be linked to their offspring. These markers could prove valuable in population genetic and paternity studies of many sparrow species.

Acknowledgements

Financial assistance was provided by the Franklin and Virginia Spivey Ornithology endowment, the Department of Biology at Coastal Carolina University and award DE-FC09-07SR22506 from the US Department of Energy to the University of Georgia Research Foundation.

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