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Twenty-one novel microsatellite DNA loci isolated from the Puget Sound white-crowned sparrow, *Zonotrichia leucophrys pugetensis*

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Abstract

The white-crowned sparrow, *Zonotrichia leucophrys*, has served as a model species for studies of song and reproductive physiology. Here, we describe primers for 21 novel microsatellite loci isolated from the Puget Sound subspecies, *Zonotrichia leucophrys pugetensis*, which will be useful for parentage and population genetic analyses. Based on genotypes from seven to 22 adult birds from one population, the average number of alleles per locus was 10.9 (four to 21 alleles) and observed heterozygosity varied from 0.50 to 1.00. All loci also amplified products in at least one of three other passerine species tested.

Keywords: microsatellite DNA loci, parentage analysis, Passeridae, *Zonotrichia leucophrys*

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The white-crowned sparrow, *Zonotrichia leucophrys*, is a small passerine bird with five currently recognized subspecies (*Zonotrichia leucophrys pugetensis*, *Z. l. gambelii*, *Z. l. nuttalli*, *Z. l. oriantha* and *Z. l. leucophrys*) varying in migratory behaviour and breeding biology (Chilton *et al*. 1995). Within some subspecies, geographically distinct song dialects occur (Baker 1975; Nelson *et al*. 2004) and some populations defined by dialect are genetically distinct from each other (e.g. *Z. l. oriantha*; MacDougall-Shackleton & MacDougall-Shackleton 2001) whereas others are not (e.g. *Z. l. pugetensis*; Soha *et al*. 2004). Here, we describe the identification of 21 novel highly polymorphic microsatellite loci and their cross-amplification in three other passerine species. We collected blood from white-crowned sparrows, *Z. l. pugetensis*, at Bullard's Beach State Park in Oregon, USA. Genomic DNA from one female Puget Sound white-crowned sparrow was extracted using phenol–chloroform, resuspended in TE (10 mM Tris pH 7.5, 1 mM EDTA) and subjected to the microsatellite enrichment protocol of Glenn & Schable (2007). Briefly, *Rsa*I restriction fragments were enriched for microsatellite repeats [Mix2 = $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$; Mix3 = $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $ACTC)_6$, $ACTG)_6$; Mix 4 = $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$] and ligated into a pCR 2.1-TOPO vector. Bidirectional sequence was determined for 78 inserts from the primary enrichment.

Candidate loci were selected by identifying sequences with more than six uninterrupted repeats and flanking regions with nonrepetitive sequences. Primer pairs were designed using MSATCOMMANDER (Faircloth 2008). To facilitate screening primers for polymorphic products, we attached one of

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Table 1 Characteristics of 21 microsatellite loci in *Zonotrichia leucophrys pugetensis* based on seven to 22 individuals from Bullard's Beach State Park, Oregon. We show information on the base repeat based on the sequenced clone, primer sequences, $MgCl_2$ concentration and annealing temperature (T_A) for optimized amplification, number of tested individuals (N), number of alleles (N_A), size range (bp) of alleles, observed (H_O) and expected (H_E) heterozygosities and difference from H_E with * indicating a significant difference based on the exact Hardy–Weinberg test in GENEPOP version 3.4 (http://genepop.curtin.edu.au/genepop_op1.html)

Locus	Accession no.	Repeat motif based on sequenced clone	Primer sequences (5′–3′)	$MgCl_2$, T_A	N	N_A	Size range (bp)	H_O	H_E	H_O-H_E
Zole_A01	EU410382	$GAAA_{13}$	F: TATCGAGCATTGCCCTCCC R: GCAGAGTATGAGGTTTTCCTTCC	2.5 mM, 59	12	21	328–437	0.833	0.989	–0.156*
Zole_A02	EU410383	$(CAGA)_4(GATA)_6(AGAT)_{11}$	F: GCAGCCATTTTGCTGTCATTC R: CCATCTGTCTGTCTTTCTGTCTG	3.5 mM, 59	13	9	170–196	0.923	0.772	0.151*
Zole_A08	EU410384	$ATCT_{13}$	F: ACCCAAAGTGCAAATCCCATC R: ACAAAGTCCCGTTTTCCTTGC	3.5 mM, 59	10	7	249–277	1.000	0.895	0.105
Zole_B01	EU410385	$ATCT_{17}$	F: GGACTGTGTTTCACTTCCTATC R: ACAGATGTTGCATTGCGG	2.5 mM, 59	11	16	231–308	0.909	0.970	–0.061
Zole_B03	EU410386	$AGAT_{14}$	F: GCCAAACTCAGTGACCTGC R: AGTTCCTGCACGGTTCTTC	2.5 mM, 59	11	7	235–259	0.818	0.797	0.021
Zole_B04	EU410387	AC_8	F: CCCATCCTTGCTGAAGTTCTC R: GACTCTGGTGTGGTAATGAAGG	2.5 mM, 59	12	4	208–214	0.500	0.486	0.014
Zole_C02	EU410388	$ATCC_{10}$	F: TCTGATATTTCAACAGCATGCAC R: GCGCAACATTTCCATGCAC	1.5 mM, 59	12	18	256–318	0.917	0.964	–0.047
Zole_C03	EU410389	$AGAT_{19}$	F: CCTTAGGTAATTGAAAATGTGTGC R: CTGTCAGTGTAAAATCAAAACACTCTG	3.5 mM, 55–45	7	11	194–264	1.000	0.956	0.044
Zole_C06	EU410390	$GGAT_{13}$	F: CCAGCCTGATTTCCCATGC R: TGTTGAGCATCTCTGGAGG	2.5 mM, 59	12	9	200–240	0.750	0.880	–0.130
Zole_C07	EU410391	$GGAT_{12}$, $AGAT_{11}$	F: TGCCAGCAACTCTGCCTC R: TGAGCTTCCAGCCCTTCAG	2.5 mM, 59	13	16	194–270	0.769	0.942	–0.173*
Zole_C11	EU410392	$ATCT_{14}$	F: TCCATGCTTCTGAACTGCC R: ACACCTGCTTTTCCTGACTG	3.5 mM, 59	15	11	168–200	1.000	0.869	0.131
Zole_C12	EU410393	AG_{13}	F: TAGGCAGGGACAGCAAGAC R: ACTACCAGAACCAACTAGGGG	3.5 mM, 59	10	9	223–247	0.900	0.874	0.026
Zole_E02	EU410394	GT_{10}, GT_6	F: GAGAACAGTCAAAGCGCCC R: GGCATCCTTCCCCTCAGTC	2.5 mM, 59	11	10	238–268	0.909	0.857	0.052
Zole_E11	EU410395	$ATCT_{13}$	F: AGAATGCTCTGGAACCGGC R: AGGACCTGTGTGCCAATTAAG	3.5 mM, 59	13	14	175–241	0.923	0.935	–0.012
Zole_F09	EU410396	$ATCT_{13}$	F: CAGCCTGTTCCATGCATCC R: GCTCGGTTCTTGCTCACAG	2.5 mM, 65–55	22	8	200–224	0.682	0.811	–0.129
Zole_F11	EU410397	$ATCC_{10}$	F: AACCAAGCCACCACAATGC R: GACAGGCACTAGGATGGGAG	3.5 mM, 59	12	7	230–270	0.833	0.848	–0.015
Zole_G03	EU410398	$AGAT_{12}$	F: GCCACGTTACACATCCTGC20 R: CTGGCATTCCAAAGCTGGG	2.5 mM, 65–55	12	16	214–271	0.917	0.960	–0.043
Zole_G10	EU410399	AG_{12}	F: TCAGAGTCCCATGTCTCACAG R: ACTCTTCCATAAGGGTTGAAATGG	2.5 mM, 59	12	4	201–213	0.917	0.598	0.319*
Zole_H02	EU410400	$GAGT_{14}$, $GACT_7$	F: ACTGTTCTTTTCTCCACCCAC R: GGTTGAATCCCAGGTGGAAAC	2.5 mM, 6 5–55	12	11	176–206	0.833	0.924	–0.091*
Zole_H05	EU410401	AC_9	F: ACTAGACAGAACAACATCATTGC R: AGTAGCAGACAGTAAACTTAGAATCC	3.5 mM, 59	8	3	254–266	0.500	0.464	0.036
Zole_H11	EU410402	CT_{11}	F: ACTGTAGCCATCTCATTAGCC R: GAGAGAAAGCAAGAAAGCAAGC	2.5 mM, 59	10	15	233–375	0.800	0.968	–0.168*

two possible engineered sequence tags (5′-CAGTCGGGC-GTCATCA or M13: 5′-CGCCAGGGTTTTCCCAGTCAC-GAC; see Glenn 2006) 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 (FAM, HEX or NED).

In total, primers were designed for 36 potential loci (25 loci with tetranucleotide repeats, two trinucleotide repeats and nine dinucleotide repeats) and locus-specific variation was assessed using DNA from seven to 22 (mean 11.6) unrelated adult birds. Primer sequences for 21 loci which successfully amplified using the conditions described below are shown in Table 1.

Table 2 Cross-amplification of 21 microsatellite loci from *Zonotrichia leucophrys* in three passerine species. *N* indicates the number of individual birds where amplification was attempted; each entry indicates number of samples amplifying and number of alleles found

		Locus										
Species	*N*	Zole_A01	Zole_A02	Zole_A08	Zole_B01	Zole_B03	Zole_B04	Zole_C02	Zole_C03	Zole_C06	Zole_C07	Zole_C11
Ammodramus savannarum	4	4/1	2/3	0/0	0/0	4/4	4/1	3/4	0/0	3/3	3/4	4/1
Spizella passerina	3	2/3	2/3	2/2	2/2	3/4	3/3	3/6	0/0	3/5	2/4	0/0
Catharus ustulatus	4	0/0	0/0	0/0	4/3	0/0	0/0	0/0	0/0	0/0	0/0	0/0

		Locus									
Species	*N*	Zole_C12	Zole_E02	Zole_E11	Zole_F09	Zole_F11	Zole_G03	Zole_G10	Zole_H02	Zole_H05	Zole_H11
Ammodramus savannarum	4	3/4	4/4	4/5	2/4	3/5	3/6	0/0	4/7	4/2	0/0
Spizella passerina	3	2/3	2/4	3/5	3/5	3/4	3/4	0/0	3/4	3/1	0/0
Catharus ustulatus	4	0/0	0/0	0/0	0/0	3/3	0/0	4/2	4/5	0/0	4/1

We set up 10-μL reactions containing 20 ng template DNA, 10× polymerase chain reaction (PCR) buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4), 0.2 mM each dNTP, between 1.5 and 3.5 mM $MgCl_2$, 0.05 U Platinum *Taq* polymerase (Invitrogen, Life Technologies) and 0.25 mM short and universal fluorescent primer and 0.025 mM tagged primer. Thermal cycling was carried out in an MJ PTC-200 thermal cycler (Bio-Rad); conditions included an initial hot start at 94 °C for 3 min, a cycle of 94 °C for 30 s, annealing temperature for 40 s, and extension at 72 °C for 40 s repeated 33 times or alternatively a touchdown programme combined with 15 cycles at the lowest annealing temperature, and a final extension step at 72 °C for 5 min (Table 1).

Products from primer pairs with different fluorescent labels were pooled and 1.5 μL of this mix was electrophoresed on an ABI PRISM 3100 genetic analyser. Fragments were sized using GENEMAPPER version 3.7 software.

We calculated observed and expected heterozygosities and null allele frequency with CERVUS 3.0 (Marshall *et al*. 1998; Kalinowksi *et al*. 2007) and tested whether loci were in Hardy–Weinberg equilibrium with GENEPOP version 3.4 (Raymond & Rousset 1995; see Table 1). Twenty-one primer pairs had null allele frequencies estimated at less than 0.15.

In parentage analyses, the 21 markers without null alleles will exclude the alleged parent with a probability of > 0.999 when the second parent is either known or not known.

We tested for linkage disequilibrium using GENEPOP version 3.4 (Fisher's method) and found no significant disequilibria between any loci after adjusting the critical *P* value for multiple tests.

DNA sequences of the 21 polymorphic loci were compared to already published sequences in GenBank with the online program BLASTN version 2.2.4 (Altschul *et al*. 1997). No significant matches were found confirming the uniqueness of the sequences.

We tested all 21 loci on three other passerines: the closely related grasshopper sparrow, *Ammodramus savannarum* and chipping sparrow, *Spizella passerina,* and the more distantly related Swainson's thrush, *Catharus ustulatus*. We completed one PCR attempt per sample using a touchdown programme from 55 to 45 °C (Table 2). Results show that 77–82% of the primer pairs amplified products in closely related sparrows, while only 23% of loci were amplified in the thrush.

The novel polymorphic microsatellites described here will be useful for detailed paternity and population genetic analyses in white-crowned sparrows and closely related birds.

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Isolation and characterization of microsatellite loci in the whale shark (*Rhincodon typus*)

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Abstract

In preparation for a study on population structure of the whale shark (*Rhincodon typus*), nine species-specific polymorphic microsatellite DNA markers were developed. An initial screening of 50 individuals from Holbox Island, Mexico found all nine loci to be polymorphic, with two to 17 alleles observed per locus. Observed and expected heterozygosity per locus ranged from 0.200 to 0.826 and from 0.213 to 0.857, respectively. Neither statistically significant deviations from Hardy–Weinberg expectations nor statistically significant linkage disequilibrium between loci were observed. These microsatellite loci appear suitable for examining population structure, kinship assessment and other applications.

Keywords: genomic library, microsatellites, *Rhincodon typus*, Rhincodontidae, whale shark

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The whale shark (*Rhincodon typus*) has a circumtropical distribution (Compagno 1984) and a K-selected life history characterized by slow growth, late maturation, long interpregnancy intervals and reduced population sizes (Colman 1997) making them susceptible to overexploitation (CITES 2002). Due to declining populations, they are listed as Vulnerable on the World Conservation Union red list, are included on México's red list of endangered species, and are listed in Appendix II of the Convention on International Trade in Endangered Species (CITES, Norman 2000; CITES 2002; DOF 2002). As with all widely distributed at-risk species, information on population structure and genetic diversity is needed to develop scientifically informed recovery and conservation strategies. A variety of genetic markers are available to examine genetic structure of populations; however, microsatellites have proven to be especially useful in studying population level genetic variability in managed organisms (e.g. Heist & Gold 1999).

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A suite of nine microsatellite DNA markers were developed using Glenn & Schable's (2005) protocol. Total genomic DNA was isolated from 20–50 mg of ethanol-preserved skin tissue collected in a minimally invasive manner from three individuals from Bahía de los Ángeles, Gulf of California, México in 2003 (Ramírez-Macías *et al*. 2007). A microsatellite-enriched partial DNA library was developed using the following mix of biotinylated oligos (Sigma-Genosys): AC_{12}, AG_{12}, AAT_8, ATG_8, $GGAT_6$, $ATCC_6$, $AACC_6$ and