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TECHNICAL NOTE

Isolation and characterization of polymorphic microsatellite markers for the brown-headed nuthatch (*Sitta pusilla*)

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Abstract Populations of brown-headed nuthatches (*Sitta pusilla*) are declining throughout the species range. Here we characterize twelve polymorphic microsatellite loci for this species. Analysis of 32 presumably unrelated individuals from a single population revealed an average of 14.9 alleles per locus (range 4–25), an average observed heterozygosity of 0.74 (range 0.52–0.94) and an average polymorphic information content of 0.80 (range 0.57–0.95). We anticipate that these microsatellite markers will be useful for population genetic and behavioral studies on the brown-headed nuthatch and closely related species.

Keywords Brown-headed nuthatch · Microsatellites · Passerine · Primers · Sittidae

Introduction

The brown-headed nuthatch (*Sitta pusilla*) is a small passerine endemic to pine forests of the southeastern United States. This species has undergone substantial population declines due to habitat loss, degradation, and fragmentation (Withgott and Smith 1998). Their cooperative-breeding mating system, restricted dispersal, natal philopatry, and ecological specialization likely increases their susceptibility to habitat alteration, as seen in many other cooperative-breeding birds (Walters et al. 2004). We developed these microsatellite loci to analyze the population genetic structure and genetic mating system of this species.

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Methods

We constructed an enriched $(CA)_n$ microsatellite library using protocols from the University of Florida Interdisciplinary Center for Biotechnology Research Molecular Markers Workshop (Brazeau and Clark 2005), which were modified from Kandpal et al. (1994). Genomic DNA was isolated using the PUREGENE® DNA Purification Kit (Biozym, Hess.) from two individuals sampled at Tall Timbers Research Station (TTRS) in Leon County, Florida. Approximately 5 μg of genomic DNA from each individual was combined and digested, and fragments greater than 400 bp were selected using Chroma Spin® + TE 400 columns (Clonetech Laboratories). Fractionated genomic DNA was ligated to *Sau*3AI linkers, and recombinant fragments were amplified by PCR using the free linker oligonucleotide. $(CA)_n$ enrichment was completed by hybridizing to a biotinylated $(CA)_{15}$TATAAGATA probe and binding to an Avidin matrix (VECTREX® Avidin D, Vector Laboratories). The enriched pool was further amplified by PCR and these products ligated to pCR®2.1-TOPO® (Invitrogen) or pGEM®-T (Promega). Colonies were either screened by hybridization to a $(CA)_n$ probe followed by detection using the Phototope®-Star Chemiluminescent Detection Kit (New England Biolabs) or sequenced directly. Sequencing was performed on an ABI PRISM 377 or ABI PRISM 3100-Avant genetic analyzer

Table 1 Characterization of 12 brown-headed nuthatch (*Sitta pusilla*) microsatellite loci from individuals sampled in Leon County, Florida

Locus	Primer sequence (5′–3′)[a]	GenBank accession	*T*m	Final Mg^{2+}	Repeat motif	Size range (bp)	*n*	*k*	H_O	H_E	PIC
*Spu*L5-6	F: $_{HEX}$CTTCCTTGTGCATGGTTGAA	EF474467	60°	2.25	$(GT)_{27}$	233–295	29	17	0.93	0.93	0.905
	R: TCTACTGTCCCACGGGTAAAA										
*Spu*A6	F: $_{FAM}$ACCTCTAGCCTTGCTTGCAG	EF474468	64.5°	1.5	$(AC)_{11}$	191–231	31	12	0.52	0.71	0.671
	R: GCGAAGAATAGCAGGTTTGG										
*Spu*E19	F: $_{HEX}$TCCTGTGAGAGCAGCAAGAA	EF474469	66°	2.25	$GTGA(GT)_{10}$	235–325	29	10	0.59	0.72	0.684
	R: CTGGCATCAAAGGAAAGCAT										
*Spu*L4-31[b]	F: $_{FAM}$CCCCAAACCCAACTCTGTTA	EF474470	66°	2.25	$(GT)_{15}$	269–295	30	9	0.53	0.85	0.817
	R: TGCATTGGTTCATTACTAGATGCT										
*Spu*L4-3	F: $_{HEX}$AGTCAGCACATGGAACCACA	EF474472	66°	3.0[c]	$(GT)_{27}$	356–402	30	15	0.70	0.83	0.806
	R: **GTTT**AAACCCAGCAAACATTCCAC										
*Spu*L6-16	F: $_{FAM}$AGGCTCCCTGTGTAGGTGTG	EF474473	60°	2.0[c]	$(GT)_{27}$	302–356	31	21	0.94	0.91	0.891
	R: **GTTT**ATCCTTCAGGTGGGTGACTG										
*Spu*L4-30	F: $_{FAM}$ATGCACTGGGTTCCTGTGTT	EF474475	58°	2.25[c]	$(GT)_{29}$	264–312	29	21	0.83	0.92	0.901
	R: **GTTT**GTTCACATTGCTGGAAAGG										
*Spu*L6-2	F: **GTTT**AGTGTCCAACTATCCTCTGGGTGAA	EU935864	70°	3.0	$(GT)_{26}$	337–407	29	25	0.86	0.97	0.946
	R: $_{FAM}$CGAAGCCTTATGCCAAGACCAGA										
*Spu*L5-22	F: AAGAAGGGGCTCTGCTTTATG	EU935863	52°	2.25	$(GT)_{14}$	144–180	26	7	0.62	0.66	0.607
	R: $_{HEX}$CCATTGGCAAACAAATTCATCCA										
*Spu*L6-26[c]	F: $_{HEX}$CTGAGTGCGCTACAAAGCCATCAT	EU935865	65°	2.25	$(AC)_{32}$	294–370	32	24	0.88	0.94	0.923
	R: **GTTT**CTGTCTCCATCTGAAATGCCACA		60°								
*Spu*4-C6	F: TGGGATCAGGTAACAAGCTAGAA	EU935866	66°	3.0	$AT(GT)_4$	217–223	30	4	0.63	0.64	0.57
	R: $_{FAM}$ACTAAGCACAACCCTATGAGCAG										
*Spu*4-E7	F: GCTTGGGATCCACAGTTAGTATG	EU935867	63°	2.25	$(CA)_{13}(GA)_2CA$	369–423	32	14	0.88	0.90	0.876
	R: $_{HEX}$CCTAACTGACCCTCTCTGGATTT										

(T_m), annealing temperature; (*n*), number of individuals genotyped at each locus; (*k*), number of alleles found at each locus; (H_O), observed heterozygosity; (H_E), expected heterozygosity; (PIC), polymorphic information content

[a] Subscript denotes dye-label (HEX or FAM) at 5′ end. Bolded sequences were nucleotides added as part of a "pigtail"

[b] Locus out of Hardy–Weinberg Equilibrium after a sequential Bonferroni correction

[c] For annealing temperature, a touchdown cycle was used that involved 3 cycles at 65°, 3 at 64°, 3 at 63°, 3 at 62°, and then 23 at 60°

(Applied Biosystems). Primer pairs complementary to the microsatellite-flanking sequences were designed using primer 3 (Rozen and Skaletsky 2000). We optimized primer pairs and tested for polymorphism using 10 individuals from TTRS. Optimized PCR conditions consisted of 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 2 mM of each dNTP, 0.2 U *Taq* polymerase (New England BioLabs), 0.2 μM of the forward and reverse primer, and 8 ng of genomic DNA in a 10 μL reaction. Locus-specific optimized PCR conditions for can be found in Table 1. All PCRs began with 95°C (5 min); 35 cycles of 95° (60 s), primer-specific annealing and elongation conditions (Table 1); and a final extension at 72°C (30 min). We added a GTTT sequence to the 5′ end of five primers to facilitate the non-templated addition of adenosine by *Taq* polymerase, commonly referred to as "pigtailing" (Brownstein et al. 1996). Microsatellites were run on the ABI 3100-Avant and alleles were sized using GeneMarker® v. 1.5 (SoftGenetics LLC).

We genotyped 26–32 presumably unrelated individuals from TTRS for each polymorphic microsatellite locus. Characteristics of each primer pair are presented in Table 1. Total exclusion probabilities for the first and second parent, expected and observed heterozygosity, polymorphic information content, and null allele frequency estimates were calculated using CERVUS 3.0 (Kalinowski et al. 2007). Deviations from Hardy–Weinberg Equilibrium (HWE) and tests for linkage disequilibrium were tested using a Markov chain method provided in GENEPOP version 4.0.7 (Rousset 2008).

Results and discussion

These variable microsatellites, with an average number of alleles of 14.9 (range 4–25), give a high combined exclusion probability for the first and second parent (0.99996315 and 0.99999979, respectively). One locus (Table 1) significantly deviated from HWE following a sequential Bonferroni correction (Rice 1989). Departures from HWE for this locus may indicate heterozygote deficit consistent with the presence of null alleles, or a slight tendency towards inbreeding due to limited dispersal. No evidence for linkage disequilibrium ($P < 0.01$) was found between loci. Overall, these microsatellite loci are highly variable and should be valuable tools for studying many biological aspects of the brown-headed nuthatch.

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