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Development of Polymorphic Tetranucleotide Microsatellite Markers for New World Warblers (Aves: Passeriformes: Parulidae) with Broad Cross-species Utility

Rauri C. K. Bowie,[1,2,5] Kevin A. Feldheim,[3] Zachary R. Hanna,[1,2] Anna B. Sellas,[1,4] and Carla Cicero[1]

ABSTRACT.—We describe the isolation of 10 microsatellite loci with tetranucleotide repeat motifs from the Orange-crowned Warbler (*Oreothlypis celata*) by using an enrichment protocol. All 10 loci were variable across two sampled populations (Alaska, California Channel Islands); the number of alleles ranged from 2–23, and observed heterozygosity ranged from 0.333–1.000. We tested the utility of these loci across an additional 13 parulid species, spanning the evolutionary and ecological diversity of the Parulidae. Nine of our 10 primer pairs were successfully PCR-amplified across at least 12 species, with the remaining locus being amplified across 9 parulid species. We expect the identified loci to be of particular use in studies of phylogeography, determination of cryptic species boundaries, hybrid zone dynamics, and paternity assignment across New World warblers. *Received 11 July 2016. Accepted 19 December 2016.*

Key words: *Basileuterus*, *Cardellina*, *Geothlypis*, *Mniotilta*, nuclear markers, Orange-crowned Warbler, *Oreothlypis*, *Parkesia*, Parulidae, *Seiurus*, *Setophaga*.

The New World warblers (Parulidae) represent a large and colorful radiation (119 species, 18 genera) of oscine songbirds (Gill and Donsker 2016). Most species occur in the tropics; however, almost half are long-distance migrants that breed in North America and move farther south to the tropics for winter. Many of the species are vulnerable to habitat fragmentation of both their breeding and winter grounds. Destruction of habitat threatens some 24 species (22%) of parulids, with two species (Bachman's Warbler [*Vermivora bachmanii*] and Semper's Warbler [*Leucopeza semperi*]) almost certainly having become extinct in the recent past (Winkler et al. 2015).

To better understand the potential impacts of habitat fragmentation on warbler population dynamics, there is a need to estimate the extent to which individuals move among populations. For the long-term persistence of small populations, gene flow among populations provides a buffer against stochastic population fluctuations and a means to reduce the loss of genetic diversity through genetic drift as well as to mitigate the potential effects of inbreeding depression (Hanski 1998, Frankham 2015). Microsatellite markers, because of their high mutation rates, have proven useful in assessing genetic connectivity and diversity among breeding populations of tropical species (e.g., Caro et al. 2013), as well as among wintering and breeding populations of migratory species (e.g., Bray and Hockey 2015).

The Orange-crowned Warbler (*Oreothlypis celata*, formerly *Vermivora celata,* Chesser et al. 2011) is a widespread summer resident across much of western and northern North America. This species winters in the southern United States, Mexico, and parts of Central America (Gilbert et al. 2010). Some populations of Orange-crowned Warblers from California, including those on the Channel Islands, are resident year-round. Of the four currently recognized subspecies (AOU 1957, Gilbert et al. 2010), three (*celata*, *orestera,* and *lutescens*) have geographically broad breeding distributions. The fourth subspecies, *sordida*, has a restricted distribution on the California Channel Islands, the Coronados, and the Todos Santos Islands off the coast of northwestern Baja, and is

[1] Museum of Vertebrate Zoology, 3101 Valley Life Science Building, University of California, Berkeley, CA 94720, USA.

[2] Department of Integrative Biology, Valley Life Science Building, University of California, Berkeley, CA 94720, USA.

[3] Pritzker Laboratory for Molecular Systematics and Evolution, The Field Museum, 1400 S. Lake Shore Drive, Chicago, IL 60605, USA.

[4] Center for Comparative Genomics, California Academy of Sciences, 55 Music Concourse Drive, San Francisco, CA 94118, USA.

[5] Corresponding author; e-mail: bowie@berkeley.edu

locally distributed on the mainland of coastal southern California. The extent of genetic isolation of island *sordida* populations is uncertain. Here, we describe the isolation and characterization of 10 microsatellite loci for the Orange-crowned Warbler and determine the utility of these loci across another 13 parulid species in eight genera. All 10 microsatellite loci are characterized by the presence of tetranucleotide repeat motifs, which are easier to score consistently than di- or trinucleotide repeats because of the reduction in the number of stutter bands.

METHODS

Laboratory Procedures

A microsatellite library was developed from one individual Orange-crowned Warbler (MVZ 179477) using a modification (see Sellas et al. 2008, de Ponte Machado et al. 2009) of the enrichment protocol described by Glenn and Schable (2005). Briefly, we extracted genomic DNA using the DNeasy Blood and Tissue kit (QIAGEN Inc., Germantown, MD, USA). DNA from one individual was digested with the restriction enzymes *RsaI* and *XmnI* (New England Biolabs, Ipswich, MA, USA) prior to ligation of SuperSNX24 linkers onto the fragments. We then hybridized the fragments to several biotin-labeled tetranucleotide probes (e.g., $[ACAG]_8$; $[AAGT]_8$; $[AGAT]_8$; $[ACAT]_8$) to form a dimer, which was attached to streptavidin-coated magnetic beads (Dynabeads M-270; Invitrogen Corp., Carlsbad, CA, USA) and washed twice with 2X SSC, 0.1% SDS and four times with 1X SSC, 0.1% SDS at 52 °C before ethanol precipitation. We removed these dimers from the beads, performed a 'recovery' PCR, and then cloned them using a TOPO-TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA) and sequenced them on the AB3730 platform (Applied Biosystems Inc., Foster City, CA, USA). A total of 192 colonies were sequenced, of which 37 contained repetitive elements. From these, we selected 15 loci containing tetranucleotide repeats for primer development. Primers were designed using PRIMER ver. 3 (Rozen and Skaletsky 2000), and the forward primer was 5′ tagged with one of four different flourophores (HEX, FAM, PET, NED). Ten of the primer pairs were identified as optimal based on peak morphology and variability (Table 1).

To test the efficacies of our 10 selected tetranucleotide loci, we screened 45 individuals of *O. celata*: 30 *O. c. sordida* from the California Channel Islands, and 15 *O. c. celata* from Fairbanks, Alaska. These two populations were selected because they represent the edge of the species' distribution, hence where we may expect heterozygosity and allelic diversity to be low. Thus, our results from scoring the 45 individuals from these two populations likely represents a conservative estimate of efficacy of the microsatellite markers reported here. These specimens are accessioned in the collections of the Museum of Vertebrate Zoology, University of California, Berkeley (Online Appendix). They were collected under animal ethical approval R118 granted by the UC Berkeley Institutional Animal Care and Use Committee.

We first ran a series of gradient PCRs with annealing temperatures ranging from 54–64 °C, and used this information to optimize the PCR thermal cycling conditions. PCR reactions (10 µl) consisted of 1X PCR Buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3; Invitrogen Corp., Carslbad, CA, USA), 0.6 µM of each primer, 200 µM of each dNTP, 0.6 Units *Taq* (Invitrogen Corp., Carslbad, CA, USA) and ~5–10 ng genomic DNA. Thermal cycling profiles were as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 sec, T_a (annealing temperature Table 1) for 15 sec, and an extension at 72 °C for 15 sec. The PCR products for six loci (Oce34, Oce50, Oce102, Oce103, Oce128, Oce179) were cleaned using a T4 DNA polymerase treatment (Ginot et al. 1996). All PCR products were run on an AB3730 automated sequencer together with the GSLIZ500 size standard (Applied Biosystems Inc., Foster City, CA, USA). Fragment analysis and genotyping were performed using GENEMAPPER ver. 4.0 (Applied Biosystems Inc., Foster City, CA, USA).

We subsequently tested cross-species PCR-amplification of the microsatellites isolated from *O. celata* on 13 parulid species representing eight genera (Table 2) to assess the broad utility of the markers. We generated genotypes using the same PCR-amplification conditions as for *O. celata*.

TABLE 1. Summary statistics of microsatellite loci isolated from the Orange-crowned Warbler (*Oreothlypis celata*). Ta is the optimized annealing temperature. *N* is the sample size. H_O and H_E represent the observed and expected heterozygosity, respectively.

				Channel Islands (*O. celata sordida*)					Alaska (*O. celata celata*)					
Locus	Primer sequence (5′–3′) and dye label	Repeat motif	T_a (°C)	*N*	No. of alleles	Allele size range (bp)	H_O	H_E	*N*	No. of alleles	Allele size range (bp)	H_O	H_E	GenBank Accession no.
Oce34	TAATGCAGGGGAACAAAACC (NED) ACATGTGTATGCTTGCTTTA	$(TAGA)_{14}$	50	30	9	133–165	0.700	0.779	15	6	149–165	0.333[a]	0.763	AA00000
Oce50	TCTCTAGTCCTGCACTGTGAC (FAM) AGGTAGGATGCAGTGAGGTG	$(TGGA)_{17}$	55	30	23	178–298	0.900	0.945	15	18	154–282	1.000	0.959	AA00000
Oce70	TCCAGCATCTGTTAGTTGAC (FAM) CAGAAAGCTGAGTTGGCATG	$(TACA)_6$	55	30	3	168–180	0.500	0.505	15	2	176–180	0.333	0.508	AA00000
Oce102	GCCTTAGCTTTTCATTTCTCCA (NED) TCTCTTTTCAGATCAAGTGTTTGC	$(TATC)_{11}$	55	30	7	185–213	0.800	0.802	15	8	165–213	0.733	0.839	AA00000
Oce103	AGCCTGTCATTAGCCTTCCA (HEX) CACCCATTGGAGAGTTTTCTTC	$(TATC)_4TACC(TATC)_4$	53	30	2	150–154	0.467	0.364	15	7	146–178	0.667	0.648	AA00000
Oce109	AGCTGGGAGTGTCAGGAGAA (PET) AAAGTTTTTGGACGCACACA	$(TATC)_9GATC(TATC)_7$	50	30	8	214–242	0.833	0.829	15	7	210–242	0.933	0.839	AA00000
Oce116	TCTTCCCCTGTCTGAGACTACTG (PET) CTCAAGGCTGAAGCAAGCTC	$(TAGA)_{12}$	55	30	8	157–185	0.700	0.788	15	6	161–181	0.733	0.749	AA00000
Oce128	CAGAGCCAGAAAAAGAAGCA (NED) TGTCATAGGTCTGGAAGCAAAA	$(TAGA)_{15}$	53	29	14	123–187	0.724	0.704	15	12	127–199	0.933	0.913	AA00000
Oce167	CCCAATGCTAAAGAATGGTGA (FAM) TGTGAGGCACCAGTTATCCA	$(TATC)_8TATT(TATC)_{12}$	55	29	15	146–234	0.655	0.902	15	14	150–262	0.667	0.880	AA00000
Oce179	CGGATGGTTTTTGGACAGAT (NED) CAGGAAACCCTTGCAAGAAA	$(TATC)_{11}$	55	30	7	215–239	0.833	0.775	15	6	219–239	0.867	0.770	AA00000

[a] Significant deviation from Hardy-Weinberg equilibrium, after Bonferroni correction ($P < 0.005$). No loci were linked in either population, after Bonferroni correction ($P > 0.001$).

TABLE 2. Cross-species PCR-amplification of microsatellite loci isolated from the Orange-crowned Warbler (*Oreothlypis celata*). Two individuals (four alleles) were screened per species unless otherwise noted.

Species		Locus									
		Oce34	Oce50	Oce70	Oce102	Oce103	Oce109	Oce116	Oce128	Oce167	Oce179
Basileuterus belli	$N_{alleles}$	—	3	2	3	4	—	4	4	—	4
	Size	—	172–272	147–171	161–173	175–219	—	157–179	134–150	—	199–217
Cardellina pusilla	$N_{alleles}$	3	2	1	4	3	4	-	3	3	4
	Size	131–163	131–135	164	161–193	147–171	298–374	-	146–162	143–155	218–234
Geothlypis trichas	$N_{alleles}$	2	3	1	4	3	4	3	4	2	4
	Size	157–165	169–209	176	185–213	158–214	290–338	169–181	147–163	142–146	239–255
Geothlypis tolmiei	$N_{alleles}$	3	4	3	4	3	4	4	2	4	3
	Size	140–156	169–205	172–180	181–229	178–190	228–278	169–175	143–151	132–156	207–239
Mniotilta varia	$N_{alleles}$	4	3	2	2	4	4	4	3	3	3
	Size	122–158	135–159	176–180	169–181	132–158	256–320	157–191	134–150	138–146	246–270
Oreothlypis ruficapilla	$N_{alleles}$	3	4	1	4	3	4	3	3	3	4
	Size	145–157	197–221	176	192–208	167–175	223–375	162–182	147–163	201–213	232–252
Oreothlypis virginiae	$N_{alleles}$	3	4	1	4	4	4	4	3	4	3
	Size	145–157	209–253	176	188–212	154–178	223–287	165–181	151–159	208–280	220–248
Parkesia noveboracensis[a]	$N_{alleles}$	2	2	2	2	2	—	2	1	1	2
	Size	136–148	209–257	180–192	166–182	169–173	—	161–169	164	151	231–235
Seiurus aurocapilla[a]	$N_{alleles}$	1	1	2	2	2	—	2	2	1	2
	Size	153	130	184–192	217–229	158–162	—	169–173	160–164	133	231–343
Setophaga americana	$N_{alleles}$	3	3	3	2	4	—	2	2	3	3
	Size	136–152	205–269	172–180	177–181	166–218	—	169–181	154–210	132–156	225–245
Setophaga coronata	$N_{alleles}$	3	2	3	3	4	4	3	3	3	3
	Size	137–153	153–161	176–196	149–173	136–164	262–284	144–182	139–151	133–141	228–276
Setophaga occidentalis	$N_{alleles}$	4	3	3	3	4	4	3	2[a]	3	4
	Size	114–150	153–197	171–187	168–176	154–222	241–429	124–186	135–139	138–146	218–236
Setophaga petechia	$N_{alleles}$	3	1	3	3	4	2[a]	2[a]	—	3	3
	Size	153–161	197	172–180	156–164	155–167	277–303	149–157	—	133–141	210–218

[a] Only one individual scored.

Statistical Analyses

For the data collected from each population of *O. celata* (Table 1), we assessed both the likelihood of scoring errors because of allele stutter and the probability of large allele dropout (null alleles) using MICROCHECKER ver. 2.2.3 (van Oosterhout et al. 2004). We calculated observed and expected heterozygosities using Arlequin ver. 3.1 (Excoffier et al. 2005), and we tested for linkage disequilibrium and Hardy-Weinberg equilibrium using GENEPOP ver. 4.0 (Raymond and Rousset 1995).

RESULTS

All 10 tetranucleotide microsatellite loci were polymorphic in both the Channel Island and Alaskan populations of *O. celata,* with the number of alleles ranging from 2–23 (Table 1). We did not detect null alleles. Observed heterozygosities ranged from 0.467–0.900 (expected heterozygosities 0.364–0.945) in the Channel Islands population and from 0.333–1.000 (expected heterozygosities 0.508–0.959) in the Alaskan population (Table 1). We found only one locus (Oce34) in the Alaska population to be out of Hardy-Weinberg equilibrium after applying Bonferroni correction for multiple comparisons (Rice 1989). No loci were linked in either population after Bonferroni correction.

The cross-species PCR-amplification for all 10 microsatellite loci was generally successful across diverse parulid species and genera (Table 2). Five loci were PCR-amplified in all 13 species we tested, four loci in 12 of the species tested, and one locus in nine parulid species.

DISCUSSION

The 10 tetranucleotide microsatellite loci isolated for the Orange-crowned Warbler were all variable across the two distinct populations we screened for variability: one population from the California Channel Islands (*O. c. sordida*), and the other, Fairbanks, Alaska (*O. c. celata*). The extent to which the populations of *sordida* from the Channel Islands are isolated from the mainland is uncertain. The most pertinent comparison is with the Horned Lark (*Eremophila alpestris*), where coalescent analyses of mitochondrial data indicate ongoing gene flow between mainland and island populations, with the island populations likely acting as a source of emigrants (Mason et al. 2014). However, this finding has been the exception with respect to studies of gene flow between bird populations from the mainland and Channel Islands. Phylogeographic studies of the Loggerhead Shrike (*Lanius ludovicianus*; Caballero and Ashley 2011), Song Sparrow (*Melospiza melodia*; Wilson et al. 2015), California Scrub-Jay ([*Aphelocoma californica*] and Island Scrub-Jay [*A. insularis*]; Delaney and Wayne 2005, Langin et al. 2015) and Spotted Towhee (*Pipilo maculatus*; Walsh 2015) all inferred cessation of gene flow and isolation between populations from the Channel Islands and mainland. These results are reflective of taxonomy, with the Channel Islands harboring endemic subspecies of Loggerhead Shrike (*L. l. anthonyi*), Song Sparrow (*M. m. graminea*), Spotted Towhee (*P. m. clementae*), and the Island Scrub-Jay being one of only two bird species endemic to California. The development of a panel of 10 variable nuclear DNA loci in this manuscript will enable study of the extent to which *O. c. sordida* populations are isolated on the Channel Islands. Results from such a study will likely have important conservation implications for this locally distributed taxon.

The microsatellite loci isolated from *O. celata* include some hypervariable loci (Oce 50, 23 alleles, Table 1) but on average are comparable in variability (10 loci, 9 alleles) to those isolated from other widespread oscine passerine bird species: *Henicorhina leucophyrys* (8 loci, 6–21 alleles; Bowie et al. 2012), *Batis crypta* (9 loci, 2–11 alleles; Wogan et al. 2016), *Zosterops virens* (10 loci, 2–13 alleles; Oatley et al. 2013), *Cossypha natalensis* (13 loci, 4–12 alleles; Wogan et al. 2015b), and *Sigelus silens* (13 loci, 2–11 alleles; Wogan et al. 2015a).

All but one of our 10 primer pairs worked to PCR-amplify microsatellite loci across 12 or 13 species of parulids, with the remaining locus readily amplifying nine species (Table 2). Although we did not test the primer pairs across many individuals per species, the 13 parulid species that we screened span the full evolutionary and ecological diversity of the Parulidae (Lovette et al. 2010). We expect these new loci to be of particular use in studies of phylogeography, determination of cryptic species boundaries, hy-

brid zone dynamics, and paternity assignment. In summary, these new loci will facilitate the documentation of spatial evolutionary patterns and life-history attributes of most parulid species, thereby providing a means for conservation managers to rapidly obtain necessary data for augmenting management plans of threatened taxa.

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Isolation and Characterization of Polymorphic Microsatellite Loci from the Rufous-throated Antbird *Gymnopithys rufigula* (Aves: Thamnophilidae)

Juliana Menger,[1,2,3,6] Michael Gerth,[4] Jasmin Unrein,[2] Klaus Henle,[1,5] and Martin Schlegel[2,5]

ABSTRACT.—Fourteen microsatellite loci were characterized for the Rufous-throated Antbird (Thamnophilidae: *Gymnopithys rufigula*), an Amazonian obligate ant-following bird. Eight novel polymorphic microsatellite loci were isolated using next-generation sequencing and six additional loci were cross-amplified using primers previously designed for other bird species. All 14 loci were screened using 40 samples from central Amazon. The number of alleles per locus ranged from 3 to 20, whilst the observed and expected heterozygosities varied from 0.200 to 0.975, and 0.186 to 0.928, respectively. No linkage disequilibrium was found among the loci. Only one locus deviated from Hardy-Weinberg equilibrium. Among the eight novel loci, six were cross-amplified in at least one additional Thamnophilidae species, whilst three loci cross-amplified in two other Dendrocolaptidae species. This set of markers will be useful for future population genetic structure and parentage analysis of Amazonian forest birds. *Received 27 April 2016. Accepted 6 October 2016.*

Key words: Amazonia, ant-following birds, cross-amplification, next-generation sequencing, nuclear markers.

Genetic and genomic markers are lacking for most Amazonian bird species, including the Rufous-throated Antbird (Thamnophilidae: *Gymnopithys rufigula*), an obligate army ant-follower that occurs exclusively in the Guiana Shield, northern Amazonia (Naka et al. 2012). The species inhabits the understory of lowland terra firme forests, where it joins multispecies flocks that feed on insects fleeing from army-ant swarms (Willis and Oniki 1978). Bird attendance at army ant swarms is one of the most impressive foraging strategies in tropical forests (Brumfield et al. 2007). Such specialization has evolved to effectively exploit army-ant swarms as a food resource and includes morphological and behavioral changes, such as loss of feeding territoriality (O'Donnell et al. 2012). As army ant-following birds tend to forage over large areas,

[1] UFZ-Helmholtz Centre for Environmental Research, Department of Conservation Biology, 04318 Leipzig, Germany.

[2] Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, 04103 Leipzig, Germany.

[3] Coordenação de Pesquisa em Biodiversidade, Instituto Nacional de Pesquisas da Amazônia - INPA, Manaus, Brazil.

[4] Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK.

[5] German Centre for Integrative Biodiversity Research (iDiv), Halle-Jena-Leipzig, Deutscher Platz 5e, D-04103 Leipzig, Germany.

[6] Corresponding author; e-mail: j.menger@ufz.de