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TECHNICAL NOTE

# Tetranucleotide microsatellite loci from eastern bluebirds *Sialia sialis*

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## Abstract

**We describe primers and polymerase chain reaction (PCR) conditions to amplify 18 tetranucleotide microsatellite DNA loci in eastern bluebirds (*Sialia sialis*). The primers were tested using individuals from two study sites in Georgia and South Carolina. Among individuals from the Georgia population ($n$ = 23), the primer pairs developed in this study yielded an average of 6.6 alleles per locus (range 2–12), an average observed heterozygosity of 0.56 (range 0.24–0.96) and an average polymorphic information content of 0.65 (range 0.3–0.86). Among individuals from the South Carolina population ($n$ = 19), the primer pairs yielded an average of 5.8 alleles per locus (range 2–9), an average observed heterozygosity of 0.56 (range 0.05–0.86) and an average polymorphic information content of 0.63 (range 0.29–0.83).**

*Keywords*: eastern bluebirds, microsatellites, primers, *Sialia sialis*, SSRs, tetranucleotide repeats

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Eastern bluebirds, *Sialia sialis*, are small, sexually dichromatic thrushes, in the family Muscicapidae that live in eastern North America and are among the most frequently studied and well known of North American passerines (Gowaty 1980; Gowaty & Plissner 1998). They are largely socially monogamous, yet social polygyny, social polyandry and helping behaviour are infrequent, but commonly reported social alternatives (Gowaty & Plissner 1998). Extrapair paternity (inferred from allozyme variation) and intraspecific nest parasitism are common in South Carolina populations (Gowaty & Karlin 1984; Gowaty & Bridges 1991a,b) and in Ontario (Meek *et al*. 1994).

Needed data include assignments of paternity when more than one male sires broods, maternal assignment when conspecific nest parasitism is detected, the genetic relatedness of adults in neighbourhoods and data on widespread dispersal, all of which can be inferred using microsatellite DNA. Using genetic parentage assignments, we can test hypotheses of the adaptive significance of mate-guarding, intrasexual aggression, male parental care, in addition to the effects upon extrapair paternity of female breeding synchrony, the density of breeders and the variation among females in their need for male parental care (Gowaty 1996a,b, 2006). Below, we describe the development of a panel of species-specific, tetranucleotide, microsatellite markers that will aid the collection of needed data and allow genetic parentage assignments.

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We extracted DNA from blood obtained from a female eastern bluebird using the GenomicPrep Blood DNA Isolation Kit (GE Healthcare). We digested DNA with *Rsa*I and *Bst*UI (New England Biolabs), ligated to double-stranded SuperSNX24 linkers and double enriched for $(AAAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(AATC)_6$, $(ACAG)_6$, $(ACTG)_6$, $(AAAC)_6$, $(AATG)_6$, $(AGAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(AAAT)_8$ and $(ACAT)_8$ (Glenn & Schable 2005). Product from polymerase chain reactions (PCRs) for each enrichment mix was pooled, ligated into PCR 2.1-TOPO vector and used to transform OneShot Top 10 chemically competent *Escherichia coli* cells (TOPO TA cloning kit, Invitrogen). We screened colonies for inserts using the β-galactosidase gene. Positive (white) colony PCR products of 500–1100 bp were sequenced using BigDye (version 3.1, PE Applied Biosystems) chemistry and ABI 3700 or ABI 3730xl sequencer. Sequences were assembled and edited in SEQUENCHER 4.2 (Gene Codes Corp.) and exported to EPHEMERIS 1.0 (available at www.uga.edu/

**Table 1** Characterization of 18 primer pairs amplifying tetranucleotide microsatellite loci from eastern bluebirds (*Sialia sialis*) collected in Athens-Clarke County, GA, USA

| Locus | Primer sequence (5′–3′) | GenBank Accession no. | Dye | Annealing start temp. (°C) | Repeats in cloned allele | Clone size (bp) | *N* | *A* | Size range (bp) | $H_O$ | $H_E$ | $P_{HW}$ | PIC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sialia2 U | ***CAGTCGGGCGTCATC***AGCAAAACCAGCAAACAA | DQ279128 | HEX | 65 | $(AATC)_5$ | 229 | 22 | 3 | 235–243 | 0.50 | 0.56 | 0.8 | 0.46 |
| Sialia2 L | GTTTGCTGGAAATCTCAACTCTAAT | | | | | | | | | | | | |
| Sialia6 U | ***CAGTCGGGCGTCATCA***TTCTCTTGGTGGTTTAGTG | DQ279141 | HEX | 65 | $(AAAC)_7$ | 265 | 22 | 5 | 270–290 | 0.68 | 0.77 | 0.08 | 0.71 |
| Sialia6 L | GTTTGCTTAGTGTTCTTTCACA | | | | | | | | | | | | |
| Sialia8 U | ***CAGTCGGGCGTCATCA***TGGGGAAGTGGAGTGAGT | DQ279145 | HEX | 65 | $(AAAC)_5$ | 344 | 20 | 6 | 354–366 | 0.50 | 0.73 | 0.06 | 0.68 |
| Sialia8 L | GTTTAAGGTGAGAATAATGTTGAC | | | | | | | | | | | | |
| Sialia11 U | GTTTGGCTTTCCCTTTATTTAT | DQ279154 | HEX | 65 | $(AAAC)_6$ | 244 | 17 | 9 | 245–269 | 0.77 | 0.83 | 0.5 | 0.78 |
| Sialia11 L | ***CAGTCGGGCGTCATC***CAGCTCCTACTGTTCTACTT | | | | | | | | | | | | |
| Sialia14 U | ***CAGTCGGGCGTCATCA***TTCCTAGTTGTATCCACTGA | DQ279162 | HEX | 60 | $(AAAC)_4$ | 383 | 21 | 2 | 381–385 | 0.24 | 0.44 | 0.04 | 0.34 |
| Sialia14 L | GTTTAGCTTACTTTCCAGATGTGT | | | | | | | | | | | | |
| Sialia15 U | GTTTAAGTAGCAAGGGTCAATGTG | DQ279163 | HEX | 65 | $(AAAC)_4$ | 238 | 23 | 4 | 236–244 | 0.17 | 0.31 | 0.01 | 0.29 |
| Sialia15 L | ***CAGTCGGGCGTCATC***AGAGGCAGAGAAGCAGTCAG | | | | | | | | | | | | |
| Sialia18 U | ***CAGTCGGGCGTCATC***AAGATCACATTTAGCAAGTCA | DQ279179 | HEX | 65 | $(AGAT)_6$ | 267 | 23 | 5 | 260–280 | 0.52 | 0.59 | 0.5 | 0.53 |
| Sialia18 L | GTTTACTAAGGCCACAAGAATC | | | | | | | | | | | | |
| Sialia20 U | ***CAGTCGGGCGTCATCA***TGTTCCACTGTTATCACG | DQ279185 | HEX | 65 | $(AGAT)_{11}$ | 312 | 21 | 10 | 286–326 | 0.29 | 0.83 | N/A | 0.79 |
| Sialia20 L | GTTTATAAAAGACTTAGCAGACATA | | | | | | | | | | | | |
| Sialia22 U | GTTTAGGAGGACTGATTTTCTACT | DQ279187 | FAM | 65 | $(AGAT)_{13}$ … $(CA)_6$ | 301 | 20 | 7 | 284–320 | 0.85 | 0.75 | 0.6 | 0.69 |
| Sialia22 L | ***CAGTCGGGCGTCATCA***TTACTGAGCACTTTTGACAC | | | | | | | | | | | | |
| Sialia23 U | GGAAACAGCTATGACCATGTCTCTCCAGTTGTTCTGA | DQ279190 | FAM | 65 | $(AGAT)_6$ | 240 | 18 | 5 | 238–262 | 0.39 | 0.76 | 0.01 | 0.70 |
| Sialia23 L | GTTTCTGATTGGGGCACTGAC | | | | | | | | | | | | |
| Sialia24 U | GTTTGCTCTGCTTTTTGTAT | DQ279191 | FAM | 65 | $(ATCT)_4$ … $(ATCT)_8$ | 330 | 17 | 12 | 295–339 | 0.59 | 0.90 | 0.002 | 0.86 |
| Sialia24 L | ***CAGTCGGGCGTCATCA***TCTACTTTTCTTTCCACTGTC | | | | | | | | | | | | |
| Sialia27 U | ***GGAAACAGCTATGACCAT***GCCTATCACCACCTTCT | DQ279197 | FAM | 65 | $(AGAT)_{17}$ | 377 | 23 | 7 | 330–362 | 0.78 | 0.79 | 0.7 | 0.74 |
| Sialia27 L | GTTTGACTTCCAGATGTAAACTT | | | | | | | | | | | | |
| Sialia28 U | ***CAGTCGGGCGTCATCA***TGGTTGCTTTGGTTATGTTG | DQ279203 | FAM | 65 | $(AGAT)_6$ | 258 | 22 | 6 | 246–274 | 0.96 | 0.80 | 0.8 | 0.75 |
| Sialia28 L | GTTTCTGGAAATGCTTGACTGAAT | | | | | | | | | | | | |
| Sialia30 U | ***GGAAACAGCTATGACCAT***CAAATGCCTGCCTCTCT | DQ279206 | FAM | 65 | $(AGAT)_5$ | 377 | 21 | 4 | 374–382 | 0.38 | 0.51 | 0.10 | 0.44 |
| Sialia30 L | GTTTCTTATTATGTGGTTTTCTCAC | | | | | | | | | | | | |
| Sialia34 U | ***CAGTCGGGCGTCATCA***GTGTGGAATGGAGGAGTAT | DQ279216 | FAM | 65 | $(ATCT)_5(AT)_7$ | 205 | 19 | 6 | 199–211 | 0.37 | 0.71 | 0.0001 | 0.65 |
| Sialia34 L | GTTTGAAAAAGGTGGAGGTTGAC | | | | | | | | | | | | |
| Sialia36 U | GTTTCTGTGAATTGCCTGAAA | DQ279218 | FAM | 60 | $(AGAT)_{13}$ | 335 | 23 | 10 | 319–363 | 0.78 | 0.88 | 0.02 | 0.84 |
| Sialia36 L | ***CAGTCGGGCGTCATCA***GGGAGGTTGTGAGTTGATA | | | | | | | | | | | | |
| Sialia37 U | ***CAGTCGGGCGTCATC***AGTTCATTAGCAAAACAAG | DQ279189 | FAM | 60 | $(AGAT)_6$ | 280 | 21 | 9 | 273–309 | 0.81 | 0.82 | 0.9 | 0.77 |
| Sialia37 L | GTTTGATGGTTATCTCAGTCACAG | | | | | | | | | | | | |
| Sialia38 U | GTTTGTGACTAGAGGTGGAGAA | DQ279193 | FAM | 60 | $(AT)_6$…$(AC)_7(AGAT)_8$ | 283 | 20 | 8 | 265–289 | 0.50 | 0.62 | 0.02 | 0.58 |
| Sialia38 L | ***CAGTCGGGCGTCATCA***CTCTCTCAGAGATACAAAGTG | | | | | | | | | | | | |

Sequences used to introduce sites for the universal primer are in bold italics.

Underlined bases indicate sharing of nucleotides between CAG (5′-CAGTCGGGCGTCATCA-3′) tag, M13R (5′-GGAAACAGCTATGACCAT-3′) tag, or GTTT 'pigtail' and the locus-specific primer binding site.

Clone size refers to the predicted size of the PCR product amplified from the clone used to develop each locus; *N*, number of individuals genotyped at each locus; *A*, number of alleles; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; $P_{HW}$, probability that genotype proportions conform to HWE; and PIC, polymorphic information content.

srel/DNA_Lab/dnacomputer_programs.htm) to search for microsatellite repeats. Basic local alignment search technique (BLAST) (Altschul *et al*. 1997) was used to identify duplicate sequences that were subsequently removed from consideration for primer development. We developed primers and added an M13-reverse or CAG tag to the 5′ end of one of each primer pair using OLIGO 4.0 (Molecular Biology Insights) to facilitate fluorescent size detection using ABI sequencers (Boutin-Ganache *et al*. 2001; Schable *et al*. 2002). Either a CAG or an M13-reverse tag was chosen for either the upper or the lower primer on the basis of minimizing self-complementarity, pair complementarity and secondary structure of each primer or primer pair. We added GTTT 'pigtails' to the 5′ end of primers lacking either CAG or M13-reverse tag to facilitate the nontemplated addition of adenosine by *Taq* polymerase (Brownstein *et al*. 1996).

We optimized primer pairs using six bluebird DNA samples obtained from wild individuals living on study sites on Clemson University (CU) farms in Anderson, Oconee and Pickens counties, South Carolina, and on University of Georgia (UGA) farms in Athens-Clarke and Oglethorpe Counties, Georgia. We extracted DNA from samples using the DNeasy Tissue kit (QIAGEN). PCR amplifications were performed in 10-μL volumes using MyCycler thermal cyclers (Bio-Rad). Final concentrations for optimizing reactions were 10 mM Tris pH 8.4; 50 mM KCl, 0.5 μM 'pigtailed', untagged primer; 0.05 μM CAG or M13-reverse tagged primer (CAG or M13-reverse + primer); 0.45 μM dye-labelled tag (HEX or FAM + CAG or M13-reverse); 1.5 mM $MgCl_2$; 0.5 mM dNTPs; 0.25 U JumpStart *Taq* DNA polymerase (Sigma-Aldrich) and 50 ng DNA. Reactions were optimized using two touchdown thermal cycling programs (Don *et al*. 1991), each encompassing a 10.5 °C span of annealing temperatures (ranges: 60–49.5 °C, 65.0–54.5 °C). Cycling parameters were 21 cycles at 95 °C for 20 s; highest annealing temperature for 20 s minus 0.5 °C per annealing cycle; and 72 °C for 90 s followed by 8 cycles at 95 °C for 20 s; 49.5 or 54.5 °C, respectively, for 30 s; 72 °C for 90 s; and a final extension period of 10 min at 72 °C. We checked products from PCR optimization for amplification and scored amplifying fragments using an ABI PRISM 377-96 sequencer with GeneScan ROX500 fluorescent size standard (PE Applied Biosystems). Results were analysed using GENESCAN software (PE Applied Biosystems), and we identified the optimal touchdown cycling scheme for each primer.

Following optimization, 23 individuals from UGA and 19 individuals from CU were genotyped using the reaction mix detailed previously and the optimum cycling profile for each primer. Results were scored using an ABI 3730xl sequencer (PE Applied Biosystems) with ROX500 fluorescent size standard. Fragments were sized and binned using GENEMAPPER version 3.7 software (PE Applied Biosystems). We calculated observed and expected heterozygosities and polymorphic information content for each locus using CERVUS 2.0 (Marshall *et al*. 1998). GENEPOP 3.4 (Raymond & Rousset 1995) was used to test for Hardy–Weinberg equilibrium (HWE) and genotypic linkage disequilibrium (LD), and we conducted a posteriori sequential Bonferroni correction (Rice 1989).

Table 1 summarizes the characteristics of 18 primer pairs developed from Eastern Bluebirds and tested using individuals from UGA. The number of alleles per locus ranges from two to 12, averaging 6.6. Total exclusionary power with both parents unknown is > 0.999. Following Bonferroni correction, LD was detected between Sialia11 and Sialia36 ($P = 0.00003$). No LD was detected between other loci following Bonferroni correction. Two loci (Sialia20, Sialia34) deviated from HWE ($P_{\text{Bonferroni}} < 0.01$) after Bonferroni correction. One additional locus (BP24) has a $P$ value < 0.01 but does not deviate from HWE following correction. Table 2 summarizes the characteristics of the primer pairs tested using individuals from CU. The number of alleles per locus ranges from two to nine, averaging 5.8. Total exclusionary power with both parents unknown is > 0.999. No LD was detected between loci following Bonferroni correction. Two loci (Sialia14, Sialia34) deviated from HWE ($P_{\text{Bonferroni}} < 0.01$) after Bonferroni correction. One additional locus (BP23) has a $P$ value < 0.01 but does not deviate from HWE following correction. In both populations, the

**Table 2** Characteristics of 18 primer pairs tested with eastern bluebirds (*Sialia sialis*) collected in Anderson, Oconee and Pickens counties, SC, USA

| Locus | $N$ | $A$ | Size range (bp) | $H_O$ | $H_E$ | $P_{HW}$ | PIC |
|---|---|---|---|---|---|---|---|
| Sialia2 | 16 | 2 | 235–243 | 0.50 | 0.52 | 1.0 | 0.38 |
| Sialia6 | 15 | 4 | 270–290 | 0.73 | 0.76 | 0.2 | 0.68 |
| Sialia8 | 17 | 7 | 354–366 | 0.71 | 0.83 | 0.3 | 0.78 |
| Sialia11 | 14 | 9 | 245–269 | 0.86 | 0.77 | 0.9 | 0.72 |
| Sialia14 | 19 | 2 | 381–385 | 0.05 | 0.51 | 0.0001 | 0.37 |
| Sialia15 | 19 | 3 | 236–244 | 0.32 | 0.32 | 0.5 | 0.29 |
| Sialia18 | 19 | 3 | 260–280 | 0.53 | 0.49 | 0.6 | 0.43 |
| Sialia20 | 18 | 6 | 286–326 | 0.50 | 0.81 | 0.02 | 0.75 |
| Sialia22 | 18 | 6 | 284–320 | 0.72 | 0.76 | 0.3 | 0.69 |
| Sialia23 | 14 | 6 | 238–262 | 0.29 | 0.66 | 0.002 | 0.61 |
| Sialia24 | 12 | 9 | 295–339 | 0.67 | 0.88 | 0.03 | 0.83 |
| Sialia27 | 18 | 9 | 330–362 | 0.56 | 0.83 | 0.02 | 0.78 |
| Sialia28 | 19 | 7 | 246–274 | 1.00 | 0.86 | 0.8 | 0.81 |
| Sialia30 | 16 | 2 | 374–382 | 0.25 | 0.39 | 0.2 | 0.31 |
| Sialia34 | 15 | 5 | 199–211 | 0.20 | 0.72 | N/A | 0.65 |
| Sialia36 | 19 | 9 | 319–363 | 0.84 | 0.85 | 0.3 | 0.81 |
| Sialia37 | 18 | 9 | 273–309 | 0.83 | 0.84 | 0.8 | 0.79 |
| Sialia38 | 18 | 6 | 265–289 | 0.44 | 0.66 | 0.03 | 0.60 |

$N$, number of individuals genotyped at each locus; $A$, number of alleles; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; $P_{HW}$, probability that genotype proportions conform to HWE; PIC, polymorphic information content.

observed deviation from HWE is probably the result of nonrandom selection of individuals that were genotyped and small sample size. However, this deviation could also indicate the presence of null alleles. Deviation from HWE at Sialia34 in both populations suggests the results at this locus may be more than just sampling artifact.

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