

Tetranucleotide microsatellite markers for the Brown-headed Cowbird *Molothrus ater*

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The Brown-headed Cowbird *Molothrus ater* is the principal avian species in North America that displays obligate brood parasitism (Rothstein 1994, Johnsgard 1997). A cowbird reproduces by laying her eggs in the nests of other birds, and the host species incubates the eggs and raises the young cowbirds to fledging, often to the detriment of the host's own offspring (Ortega 1998, Morrison et al. 1999, Smith et al. in press). Because of this unusual mode of reproduction and the lack of association between the female cowbird and her young, it has long been difficult to collect information on even

basic aspects of cowbird reproduction. The major elusive questions include: average fecundity (the number of eggs laid by a single adult female cowbird in a season); relative host specificity (the number of different host nests and of different host species parasitized by a single female cowbird); individual laying strategy (whether female cowbirds preferentially parasitize particular host species in a given community); spatial extent of exploitation (how large a laying area is used by a single female); and nature of cowbird mating system (such as monogamy, polygyny or polyandry).

Molecular genetic techniques offered the first opportunity to address these questions in the past decade (Hahn and Fleischer 1995, Gibbs et al. 1997, Alderson et al. 1999, Hahn et al. 1999). Molecular genetic approaches that identify highly polymorphic regions in the cowbird genome are best suited to decipher genetic relationships such as maternity and paternity within a population of cowbirds given the dispersed laying pattern and lack of parental care.

Microsatellites are short, tandemly repeating DNA sequences containing six or fewer nucleotides per repeat unit. Microsatellites are currently considered to be the system of choice for performing genetic analysis because they are present in the genomes of all higher organisms, highly polymorphic, and easily analyzed in a locus-specific manner using the polymerase chain reaction, PCR (Tautz and Renz 1984, Weber and May 1989, Epplen 1991). Among the different microsatellite repeat motifs, tetranucleotide repeats (such as repeats of GATA) are highly desirable for developing locus-specific markers due to their highly polymorphic nature and characteristic lack of stutter bands following PCR amplification (Weber et al. 1993, White et al. 1999).

Two previous reports have described the development of seven microsatellite markers for the cowbird (Gibbs et al. 1997, Alderson et al. 1999). These markers were based on dinucleotide GT repeats and were used to study parentage in this species. In order to further facilitate genetic studies of cowbirds, we have developed markers for four new microsatellite loci in the cowbird. These new markers, which are described in this report, are highly

polymorphic and based on the tetranucleotide repeat GATA_n.

Materials and methods

Cowbird DNA was isolated from peripheral blood following the method described in Longmire et al. (1992). A multiple representation cosmid library was constructed from female cowbird DNA. As reported elsewhere, that library was used in a hybridization-based study to examine the representation of microsatellites within the cowbird genome (Longmire et al. 1999). Repeats that were examined included all possible mono-, di-, and trinucleotide repeat motifs and the tetranucleotide repeat GATA_n. Besides providing insights into the abundance of different microsatellite motifs within cowbird DNA, that study also served to identify cosmids that contained microsatellites that could be further processed to develop locus-specific markers. Cosmids found to contain GATA repeats were subcloned into plasmids using modifications to an approach originally described by Weissenbach et al. (1992). Briefly, the cosmids were digested with a mixture of *Hae*III, *Alu*I and *Rsa*I, dephosphorylated and then subcloned into the *Sma*I site of pUC19. Sequencing several of the GATA-positive subclones revealed four independent microsatellite loci for which primer pairs could be designed.

DNA was amplified in a Perkin Elmer thermal cycler (model 9600). Reaction mixtures contained approximately 100 ng of cowbird genomic DNA, 0.8 units of *Taq*

Table 1. Characteristics of cowbird GATA microsatellite loci. Data derived from analysis of 309 birds.

Locus	Sequenced allele	Primers	Number of alleles	Size range (bp)	Observed heterozygosity
CB.1	(GGTA) ₄ (GATA) ₁₀	F: 5'-ACT TGT CTG ATT TAC TGA TGG ATA AAG CCT-3' R: 5'-AAT CAA GTA TAA TGT TCT GCG TA-3'	20	198-277	84.1%
CB.12	(GATA) ₆ GAAGTTA (GATA) ₃ (GA) ₂ (GATA) ₁₀	F: 5'-AAC GCT AGA TAT TGG ACA GTC AGA CCT-3' R: 5'-GAG GGG AAA GCG CCC GAC CTT GTT-3'	31	272-353	92.9%
CB.15	(GATA) ₄ GGTA (GATA) ₁₂	F: 5'-GGC TGT TAT AAT ATT TAA AAT AGG ATT CAC T-3' R: 5'-TAA TTC ATC TAG CAT CTT TTG AAG TCA CTT-3'	23	233-318	84.1%
CB.16	(GGAT) ₄ (GATA) ₁₁ N ₁₆ (GATA) ₂ N ₂₅ (GATA) ₁₂	F: 5'-AAT TAT TGA AAT CTC ACT TAA ATC CAC ATG AA-3' R: 5'-CAG TTG GAG TAA GAA CCT GAG TCT GCA-3'	102	247-822	91.6%

Alleles larger than the largest size standard used (822 bp) were not scored at locus 16. These very large alleles were present in 20 birds.

DNA polymerase (Perkin Elmer), 0.1 units of *Pfu* I, 2 μ l of 10 \times PCR buffer with ficoll/tartazane and 20 mM MgCl₂ (Idaho Technology), 0.25 μ M of each primer, 250 μ M dNTPs (Perkin Elmer), and distilled water in a final reaction volume of 25 μ l. In addition, fluorescently labeled dNTPs (either dUTP[R110], dUTP[R6G], or dUTP[TAMRA]) were added for PCR reactions for loci CB.1, CB.12, CB.15 in concentrations recommended by the manufacturer (ABI). For locus CB.16, sharper peaks were obtained when one of the primers was fluorescently labeled with FAM than when labeled dNTPs were used. Thermal cycling conditions included an initial 2-min denaturation at 94°C, followed by 32 cycles at 94°C for 10 s, 5 s at 55°C, 30 s (45 s for locus CB.16) at 72°C. Following amplification, PCR fragments were analyzed on an ABI 373-stretch autosequencer. To improve the resolution of larger fragments at locus CB.16 we used Long Ranger sequencing gel solution (FMC BioProducts). Results were compiled using GeneScan version 2.1 and Genotyper version 1.1.1 software (ABI).

Results

Variability at the four GATA loci was examined in 309 cowbirds collected in Millbrook, NY. As summarized in Table 1, all four of the loci were highly polymorphic with at least 20 alleles being present at each locus within our study population. In each case, the level of heterozygosity was greater than 80%. These attributes indicate that the GATA markers described in this report will be valuable for population genetic studies. In addition to being useful for population genetic studies, locus 16 will also be interesting to study at the level of genomic organization due to the extreme number of alleles that are present at this locus.

Discussion

The abundance of microsatellites within bird genomes is approximately an order of magnitude lower than in humans and other mammals (Primmer et al. 1997, Longmire et al. 1999). This increases the value of the cowbird microsatellites described in this paper. In addition, these markers are the first tetranucleotide microsatellite markers described for cowbirds so far and have the advantage of being easier to score and more reproducible than more commonly used dinucleotide repeats. The use of these markers, together with those previously described by Gibbs et al. (1997) and by Alderson et al. (1999) will allow more accurate parentage studies to be conducted in cowbirds.

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