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Territorial male red-winged blackbirds from five locations in the United States and Canada were genotyped using a suite of six microsatellite loci. Each population possessed unique alleles, but numbers of alleles per locus (range = 7.3–8.8) and expected multilocus heterozygosities (range = 0.76–0.80) were similar in all populations. Significant overall allele frequency differences were detected between some population pairs, and some pairwise F_{st} values were significant (but small). However, F_{st} among populations, although significant, was also small (0.009). Despite revealing low levels of population structure, the high multilocus polymorphism indicates these loci will be valuable in the genetic analysis of behavior and reproductive strategies in this species.

KEY WORDS: red-winged blackbird; microsatellite; *Agelaius phoeniceus.*

INTRODUCTION

Red-winged blackbirds (*Agelaius phoeniceus*) are abundant across North America and capable of long-distance movements. However, they also exhibit breeding site fidelity (Dolbeer, 1978), and show morphological (James *et al.*, 1984; Linz *et al.*, 1993) and vocalization differences (Kroodsma and James, 1994) on a regional geographical scale. Allozymes have revealed high levels of variation within red-winged blackbird populations and little genetic differentiation among them except

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for large genetic differences between populations in California and those across the rest of the United States (Gavin *et al*., 1991). Ball *et al.* (1988) detected "mild" mitochondrial DNA structure among red-winged blackbird populations from 14 U.S. states, as well as one Canadian province and two Mexican states.

Given the greater rate of evolution at microsatellite loci, they may be informative enough to detect population structure in species that lack allozyme structure. Furthermore, male red-winged blackbirds are territorial and exhibit site fidelity (reviewed in James *et al.*, 1984), so biparentally inherited markers may reveal population structure not detected using maternally inherited mitochondrial markers. In many other species, microsatellite data are useful for estimating population differentiation, gene flow, and kinship. If sufficient differences are detected in microsatellite allele frequencies, unknown individuals can be assigned to most likely source populations.

Red-winged blackbirds can cause locally severe damage to agricultural crops (Linz *et al.*, 2000), with estimates of sunflower damage in the northern Great Plains of North America exceeding U.S. $2,000,000 annually (Peer *et al.*, 2003). Genetic markers to study relationships among regional or finer scale populations would be useful for developing management tools for this species. Our goal was to identify polymorphic microsatellite loci in red-winged blackbirds, and determine levels of variation within and among five populations sampled across North America. To our knowledge this is the first report of microsatellite variation among red-winged blackbird populations.

MATERIALS AND METHODS

We identified 10 potentially useful microsatellite loci from the literature: 7 that had been developed in great-tailed grackles (*Quiscalus mexicanus*—Hughes *et al.*, 1998) and 3 in brown-headed cowbirds (*Molothrus ater*—Gibbs *et al.*, 1997). Muscle or blood was taken from 95 territorial male red-winged blackbirds collected in two Canadian provinces (Alberta and Manitoba), and three states in the United States (California, Louisiana, and Minnesota) in June and July 2001 (Table I).

Table I. Red-Winged Blackbird Samples Used in This Study

Population	Collection dates	*n*	Subspecies
California	July 2001	19	*A. p. phoeniceus*
Louisiana	July 5, 2001	20	*A. p. littoralis*
Alberta	June 29, 2001	23	*A. p. phoeniceus*
Minnesota	June 6–11, 2001	16	*A. p. phoeniceus*
Manitoba	June 19, 2001	16	*A. p. phoeniceus*

DNA was isolated from muscle by using a Qiagen tissue kit (Valencia, CA) and the manufacturer's protocol. A drop of blood was placed in lysis buffer (Longmire *et al.*, 1988), which was stored and shipped at room temperature, then frozen at -20°C. An aliquot was digested with Proteinase K at 55°C for 3 h. Proteins were precipitated by addition of NaCl and removed by centrifugation. DNA was precipitated with ethanol, collected by centrifugation, rehydrated in TE, reprecipitated, and quantified by fluorimetry.

All 10 loci were screened in red-winged blackbirds. Ten-microliter screening amplifications included 200 μM dNTPs (Promega, Madison, WI), 0.5 μM primers, 0.5 U Taq polymerase (Promega, Madison, WI), 1$\times$ buffer, 100 ng template DNA, 1.5 mM $MgCl_2$, as well as 0.1 μL of fluorescent dNTPs (Applied Biosystems, Foster City, CA). Locus-specific annealing temperatures ranged from 50 to 58°C and were determined empirically. Cycling parameters were 94°C for 2 min, then 35 cycles of 94°C for 30 s; locus-specific annealing temperature for 30 s, 72°C for 30 s, and a final extension step of 72°C for 2 min. Amplification products were held at 4°C or -20°C until they were electrophoresed on 6% Long Ranger gels (BMA, Rockland, ME) using a fluorescent internal size standard (400HD; Applied Biosystems, Foster City, CA) on an ABI Prism 377 (Applied Biosystems, Foster City, CA). Six microsatellite loci (Mau10, Mau20, Mau23, Qm10, Qm21, and Qm37) that yielded unambiguous amplification products were selected for further analyses. Forward primers labeled with FAM, HEX, or TET (ABI, Foster City, CA; MWG Biotech, High Point, NC) were then used to collect genotypes for 95 individuals from five locations (Table I). DNA from individuals was amplified and electrophoresed as described for fluorescent dNTP amplifications, with only slight modifications to some annealing temperatures. Genotypes were determined using Genescan (ver. 3.2.1) and Genotyper (ver. 2.5) software (Applied Biosystems, Foster City, CA).

Departures from Hardy–Weinberg proportions and linkage disequilibrium between pairs of loci were tested using Fisher's exact test in the Genetic Data Analysis program (GDA; Lewis and Zaykin, 1999). Average numbers of alleles per locus, unique alleles, expected (H_e) and observed (H_o) heterozygosities, genetic distances (Nei, 1978), and F statistics (significance of which was based on 95% confidence intervals determined by bootstrapping across loci; Weir and Cockerham 1984) were also determined using GDA. Allele frequencies for each locus and population were determined in Genepop (v3.2a; Raymond and Rousset, 1995) and allele frequency differences between population pairs were tested using Fisher's exact test. Significance of multiple pairwise comparisons was corrected using a sequential Bonferroni adjustment (Rice, 1989). Probabilities of exclusion and polymorphism information content (PIC) values were determined using Cervus (Marshall *et al.*, 1998), although our sample sizes were insufficient for analysis of null alleles with that software.

RESULTS

Six of 10 loci were useful, including 1 (Qm37) that had been reported previously as not amplifying red-winged blackbird DNA (Hughes *et al.*, 1998). Over all samples, the number of alleles per locus ranged from 7 (Qm21) to 19 (Mau20). The average number of alleles was 8.2 and the average expected heterozygosity (H_e) was 0.774. Significant departures from Hardy–Weinberg proportions were detected only at Mau20 in birds from Alberta, Manitoba, and Minnesota. No overall evidence for genetic linkage of loci was detected. Allele frequencies by collection site, average number of alleles per locus, multilocus heterozygosity, and probability of exclusion are provided in Tables II and III. Unique alleles were detected at low (<0.10) frequencies in all populations and a single population (Manitoba) possessed a unique allele at a frequency of 0.12 (Table II). Multilocus probabilities of exclusion for the first parent ranged from 0.944 to 0.977 (Table III), and indicate these loci would be useful in establishing familial relationships. Multilocus PIC values assessed over all populations ranged from 0.703 to 0.748. Qm21 was consistently the least informative locus and Mau20 was the most informative.

Nei's (1978) genetic distances between population pairs were small (Table IV). Pairwise comparisons revealed significant overall allele frequency differences between California and Louisiana, Louisiana and Minnesota, and Alberta and both Louisiana and California. However, single locus F_{st} values among populations were all less than 0.02 and the multilocus F_{st} among populations, although significant, was also small (0.009), indicating that little of the genetic variation was partitioned among populations. Only two pairwise multilocus F_{st} values (between Minnesota and both California and Louisiana) were significant, but they too were small (Table IV). Although larger overall heterozygote deficiencies correspond to birds collected over larger geographic areas (Alberta, $F_{is} = 0.13$, Minnesota, $F_{is} = 0.10$; Manitoba, $F_{is} = 0.07$) than those from much smaller geographic areas (California, $F_{is} = 0.02$; Louisiana, $F_{is} = 0.03$), the lack of deviations from Hardy–Weinberg expectations indicated that none of the samples were from admixed populations. Overall, slight heterozygote deficiencies were observed within and among populations ($F_{is} = 0.07$, $F_{it} = 0.08$), but they were not significant. Finally, although more significant pairwise allele frequency differences were detected between subspecies than within one subspecies, pairwise F_{st} values between subspecies were no greater than those within subspecies.

DISCUSSION

We identified six microsatellite loci that are polymorphic in red-winged blackbirds, and we present allele size ranges and estimates of allele frequencies in putative populations from across North America. Low levels of genetic structuring among populations in this initial microsatellite survey may reflect either relatively recent

Table II. Microsatellite Allele Frequencies for Red-Winged Blackbird Populations[a]

	Population						Population				
	CA	LA	AB	MN	MB		CA	LA	AB	MN	MB
Qm10						161	0.08	0.28	0.15	0.30	0.11
215	0.03	0.03	0.03	0.00	0.04	163	0.38	0.20	0.18	0.17	0.21
218	0.05	0.00	0.03	0.04	0.00	165	0.03	0.10	0.05	0.03	0.07
221	0.00	0.07	0.08	0.04	0.11	167	0.03	0.03	0.03	0.00	0.21
224	0.18	0.17	0.03	0.19	0.21	169	0.05	0.03	0.15	0.03	0.00
227	0.13	0.17	0.08	0.04	0.04	171	0.00	0.05	0.03	0.00	0.04
230	0.13	0.23	0.20	0.04	0.14	177	0.03	0.00	0.00	0.00	0.04
233	0.24	0.20	0.25	0.35	0.25	Mau20					
236	0.05	0.00	0.15	0.19	0.04	99	0.13	0.05	0.05	0.04	0.07
239	0.03	0.00	0.10	0.08	0.07	103	0.00	0.00	0.00	0.07*	0.00
242	0.16	0.10	0.03	0.00	0.07	107	0.00	0.08	0.03	0.00	0.04
245	0.00	0.03	0.03	0.04	0.04	109	0.13	0.03	0.10	0.11	0.04
248	0.00	0.00	0.03*	0.00	0.00	111	0.00	0.13	0.20	0.00	0.07
Qm21						113	0.00	0.00	0.00	0.04*	0.00
137	0.03	0.06	0.00	0.07	0.00	116	0.03	0.00	0.00	0.00	0.11
140	0.08	0.03	0.00	0.00	0.03	118	0.05	0.05	0.05	0.11	0.00
143	0.73	0.74	0.71	0.61	0.66	120	0.05	0.03	0.03	0.11	0.04
146	0.08	0.12	0.19	0.14	0.22	122	0.13	0.00	0.03	0.00	0.11
149	0.10	0.00	0.07	0.14	0.09	124	0.18	0.18	0.13	0.07	0.11
152	0.00	0.06	0.00	0.04	0.00	126	0.08	0.13	0.10	0.11	0.11
155	0.00	0.00	0.02*	0.00	0.00	128	0.08	0.13	0.05	0.21	0.07
Qm37						130	0.00	0.08	0.08	0.04	0.04
114	0.00	0.00	0.05*	0.00	0.00	132	0.03	0.11	0.08	0.00	0.07
117	0.15	0.21	0.24	0.29	0.10	134	0.15	0.00	0.03	0.07	0.11
120	0.15	0.05	0.10	0.25	0.03	136	0.00	0.00	0.05	0.00	0.04
123	0.10	0.00	0.10	0.07	0.10	143	0.00	0.00	0.03*	0.00	0.00
126	0.25	0.55	0.31	0.36	0.40	149	0.00	0.00	0.00	0.04*	0.00
129	0.08	0.03	0.12	0.00	0.13	Mau23					
132	0.08	0.11	0.02	0.00	0.10	151	0.00	0.00	0.00	0.00	0.04*
135	0.13	0.05	0.05	0.04	0.10	153	0.00	0.00	0.00	0.00	0.12*
140	0.00	0.00	0.02*	0.00	0.00	157	0.03	0.03	0.00	0.04	0.00
153	0.03	0.00	0.00	0.00	0.03	159	0.13	0.14	0.13	0.14	0.08
156	0.03*	0.00	0.00	0.00	0.00	161	0.15	0.06	0.34	0.29	0.23
159	0.03*	0.00	0.00	0.00	0.00	163	0.33	0.25	0.38	0.43	0.35
Mau10						165	0.08	0.19	0.13	0.11	0.15
153	0.03	0.05	0.05	0.00	0.04	167	0.23	0.19	0.03	0.00	0.00
155	0.00	0.00	0.00	0.03*	0.00	169	0.08	0.08	0.00	0.00	0.00
157	0.05	0.00	0.00	0.03	0.00	171	0.00	0.06*	0.00	0.00	0.00
158	0.00	0.03	0.00	0.03	0.00	173	0.00	0.00	0.00	0.00	0.04*
159	0.35	0.25	0.38	0.37	0.29						

[a]CA – California; LA – Louisiana; AB – Alberta; MB – Manitoba; MN – Minnesota. Allele sizes in base pairs are under locus names. Unique alleles are indicated with an asterisk.

Table III. Measures of Genetic Variability for Red-Winged Blackbirds

Population	Average number of alleles per locus	Multilocus heterozygosity		Probability of exclusion
		Expected	Observed	
California	8.5	0.78	0.77	0.97
Louisiana	7.8	0.76	0.74	0.96
Alberta	8.8	0.77	0.67	0.97
Minnesota	7.3	0.77	0.69	0.94
Manitoba	8.5	0.80	0.75	0.98

range expansion from a common source, current levels of gene flow sufficiently high to limit genetic differentiation among populations, or both. Indeed, although most red-winged blackbirds exhibit nest site fidelity, those that do not may disperse tremendous distances (Dolbeer, 1978).

This suite of microsatellite loci will be of use to researchers examining relatedness among individual red-winged blackbirds, and could provide a valuable tool for studies examining reproductive behavior in this polygynous species. However, in an analysis using larger sample sizes, a high null allele frequency was detected at locus Mau20 in all five locations (0.09–0.28; not shown), indicating this locus is not suitable for analyses of relatedness in this species. Given that we detected some overall significant allele frequency differences between population pairs, larger sample sizes, and/or more loci may improve regional population resolution. We are currently analyzing much larger breeding season samples to better investigate the potential utility of these microsatellite loci. We acknowledge that detection of fine-scale local differentiation is unlikely, given that the allele frequency differences occur only between populations separated by vast distances. The high levels of variation (numbers of alleles/locus and heterozygosity) detected indicate red-winged-blackbird-specific loci are not likely to reveal greater levels of differentiation among breeding populations.

Table IV. Pairwise Genetic Distances and F_{st} Values Between Red-Winged Blackbird Populations[a]

	California	Louisiana	Alberta	Manitoba	Minnesota
California	—	0.016	0.011	0.002	0.015*
Louisiana	0.016	—	0.015	0.006	0.023*
Alberta	0.011	0.015	—	−0.005	−0.002
Manitoba	0.002	0.007	−0.005	—	0.006
Minnesota	0.015	0.023	−0.002	0.006	—

[a] Genetic distances (Nei, 1978) are below the diagonal. F_{st} values are above the diagonal. Significant values are indicated with an asterisk. Negative values are not different from 0.

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