Molecular Ecology Notes (2005) **5**, 39–41 doi: 10.1111/j.1471-8286.2004.00823.x

PRIMER NOTE

Tetranucleotide microsatellites for *aquila* and *haliaeetus* eagles

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Abstract

A unique community of four syntopic eagle species exists in north-central Kazakhstan. Questions about behaviour and genetics in these four species would benefit from the development of microsatellite markers. We isolated eight polymorphic microsatellite repeats $(AAAG)_n$ from the eastern imperial eagle (*Aquila heliaca*) genome using a hybridization enrichment technique. These loci revealed moderate diversity in a local population of eastern imperial eagles (observed heterozygosity 0.26–0.78), and were also polymorphic in steppe eagles (*A. nipalensis*) and white-tailed sea eagles (*Haliaeetus albicilla*). These primers may be polymorphic in other species of *Aquila* and *Haliaeetus* eagles.

Keywords: *Aquila*, eagles, enrichment, *Haliaeetus*, tetranucleotide microsatellites

Received 2 June 2004; revision accepted 10 September 2004

The *Zapovedniks* (national nature reserves) of the former Soviet Union are important habitats for northern hemisphere raptors. In north-central Kazakhstan, these reserves protect critical habitat for a unique community of four similar eagle species: eastern imperial; steppe; golden (*A. chryseatos*); and white-tailed sea eagles (Bragin 1989; Katzner *et al*. 2003). Addressing ecological and genetic questions such as paternity, territoriality, philopatry, and gene flow in these four populations requires the development of useful genetic marker systems. While dinucleotide microsatellite markers are available for eagles (Nesje & Roed 2000; Martinez-Cruz *et al*. 2002), we chose to isolate tetranucleotide repeats in order to minimize polymerase slippage and simplify allele scoring.

Field collections were made in 1998–2000 at the Naurzum Zapovednik reserve near Karamendy (formerly Dokuchaevka), Kazakhstan (51 N, 64 E). Quill bases from adult feathers were gathered at nest and roost sites. Nestling samples consisted of a single growing feather plucked from a chick's back or 100–500 μL of blood collected from a toenail clip. Samples were preserved in lysis buffer (100 mM Tris-HCl pH 8.4, 50 mM EDTA, 100 mM NaCl, 2% SDS) at the field site until processing. This buffer provided excellent DNA protection despite nonfrozen storage at fluctuating temperatures for up to four months. DNA extractions from blood were performed using a Puregene™ kit (Gentra Systems).

We isolated AAAG repeats using an enrichment technique designed by Armour *et al*. (1994) with changes by Li *et al*. (1997). We made a number of further modifications, starting with the addition of 25 μg of DNA (originally 10 μg) to the *Sau*I digest and the harvest of a larger size fraction (600–1500 bp, originally 300–1000 bp) from low melting-point agarose (Fisher, LMP Agarose). We chose larger product sizes to counteract the tendency of small templates to dominate downstream PCRs. The amount of PCR product used in each bead selection was increased to 1 μg (originally 0.2 μg), and an $(AAAG)_6$ probe was hybridized at 55 °C. After transformation, inserts from clones grown in broth culture were amplified using universal primers M13F and M13R. Ninety-six amplicons larger than 300 bp were sequenced using an ABI377 (Applied Biosystems). Twenty-nine of these (30.2%) yielded AAAG repeats from 12 unique loci. Primers were designed for each unique sequence using OLIGO 6.53 (MBI). All forward and reverse primers were given a nontemplate

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Table 1 Primer sequence, repeat motif, and polymorphism data for eight eastern imperial eagle microsatellite markers

GenBank Acc. #	Locus	Primer sequences 5′–3′*	repeat motif	*A. heliaca* (n = 23)	*A. nipalensis* (n = 4)¶	*Haliaeetus albicilla* (n = 13)
				Allele sizes†‡ and H_O (H_E)§		
AY631063	IEAAAG04	F: GCATGTAACAAGTTTAATGTTGATGG	$(AAAG)_6(AAAC)_4(AAAG)_6$	235, 239, **243**, 259	231, 235, **239**, 243	199, 207, **211**, 215, 219
		R: GTTGGAAACAGGACATGTTAAGC		0.26 (0.24)	1.00 (0.75)	0.77 (0.73)
AY631064	IEAAAG05	F: GTCTGAATCCAGCACAGGGAC	$(AAAG)_7$	**109**	101, **109**	127, 135, 147, **149**, 155, 163,
		R: GGCAGAAAGCTCATGTTTGC		0.00 (0.00)	0.00 (0.43)	0.83 (0.70)
AY631065	IEAAAG09	F: GAACAATTTCAGGTAGATATTACCTGCAATA	$(RAAG)_{18}$	482, **486**, 490, 494	482, 486, 490, **494**	**465**
		R: GTATTGACTGCACATCATTGTGG		0.52 (0.59)	0.75 (0.82)	0.00 (0.00)
AY631066	IEAAAG11	F: GGCTGACTGAGCTCCAGAGC	$(AAAG)_{26}$	332, **336**, 340, 344	**336**, 340, 352, 356	342, **346**, 348
		R: GTGTTTGCTTCATCGAAACAGC		0.70 (0.68)	0.75 (0.75)	0.31 (0.41)
AY631067	IEAAAG12	F: GCTGCTGCTAAGAATCACTCATGTAC	$(AAAG)_{10}(GAAG)_3(AAAG)_5$	**132**, **136**	123, **131**, 135	102, 110, **114**
		R: GTGGGAAGGTGGGTTGTCAG		0.78 (0.50)	0.25 (0.61)	0.85 (0.59)
AY631068	IEAAAG13	F: GAATACCACAATAAGAGGCAGAGTG	$(AAAG)_3(RAAG)_{13}(AAAG)_{16}$	**192**, 246, 250, 254, 258	192, 250, **254**	**217**
		R: GTCTAAAATGAAGTGAATCTGTAAGACAG		0.70 (0.60)	0.75 (0.75)	0.00 (0.00)
AY631069	IEAAAG14	F: GTCCAGATTCCCTGCTAGAAAGC	$(AAAG)_{18}$	**200**, **204**	196, **200**	**176**, 180, 184, 188
		R: GTTGGAGAGTCTAAGCACTGAATCAG		0.43 (0.50)	0.50 (0.43)	0.92 (0.64)
AY631070	IEAAAG15	F: GAGAATAATTTTTGAAAAGCAGTAGG	$(AAAG)_7$	111, **115**, 119, 123	114, 118, **122**, 130	123, **131**
		R: GCTTAGTTGTTCAGAGGACGGTAAG		0.48 (0.47)	0.75 (0.89)	0.69 (0.45)

*Note the addition of a G to the 5′ end of each primer to promote adenylation by *Taq* DNA Polymerase.
†Apparent size on ABI377 (typically 2–3 bases larger than actual sequence with these loci).
‡Bold denotes most common allele.
§H_O, observed heterozygosity (direct count); H_E, expected heterozygosity under Hardy–Weinberg equilibrium. H_O and H_E were calculated in TFPGA (Miller 1997).
¶Nei's unbiased heterozygosity was used to calculate H_E in steppe eagles, since n was low (Nei 1978).

5′-guanine to increase the adenylation efficiency of *Taq* DNA polymerase (Brownstein *et al*. 1996). Enhanced adenylation markedly decreases the number of confounding split peaks observed on polyacrylamide gels.

The 12 unique microsatellite loci were tested against a panel of 23 eastern imperial eagles. Each PCR was performed in a final volume of 25 μL and contained 50 ng genomic DNA, 1.0 unit Platinum® *Taq* DNA Polymerase (Gibco BRL), 1× PCR buffer (20 mM Tris pH 8.4 and 50 mM KCl), 3.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM forward and reverse primers, and 0.5–2.0 μM fluorescent dUTP (Applied Biosystems dR110, dR6G, or dTAMRA). Reactions were heated to 94 °C for 5 min to release the Platinum® *Taq* antibody, then subjected to 30 cycles of 94 °C for 20 s, 65 °C for 30 s, and 72 °C for 30 s. Products from individual PCRs were pooled and electrophoresed through 5.0% Long-Ranger (FMC) polyacrylamide gels on an ABI377 (Applied Biosystems).

Four of the 12 primer pairs generated nonspecific products that could not be optimized. A fifth amplified cleanly but was monomorphic in eastern imperial eagles (IEAAAG05). The remaining seven loci were polymorphic, with an average of 3.25 alleles per locus and observed heterozygosities (H_O) of 0.26–0.78 (Table 1). Observed and expected heterozygosities in *A. heliaca* were similar, suggesting that null alleles (if present) are not segregating at high frequencies, and thus the markers should be useful for population genetic studies. The mean H_O of this marker set (0.48) was comparable to that from 18 dinucleotide markers tested against 38 Spanish imperial eagles (0.45, Martinez-Cruz *et al*. 2002). In all loci, polymerase slippage and nontarget amplification were inconsequential, demonstrating the benefit of tetranucleotide repeats. Allele sizes were easily assigned when post-amplification products were pooled into the following two mixes: mix 1: IEAAAG04, IEAAAG05, IEAAAG09, and IEAAAG12; mix 2: IEAAAG11, IEAAAG13, IEAAAG14, IEAAAG15.

Cross-species amplification in four steppe eagles and 13 white-tailed sea eagles was robust with all eight primers. Observed heterozygosity and the number of alleles were quite different in these two species (Table 1). Surprisingly, IEAAAG05 was highly polymorphic in white-tailed sea eagles (six alleles, $H_O = 0.83$), despite its monomorphism in eastern imperial eagles. This locus may be polymorphic in a wider geographical sample of eastern imperials, since the 23 individuals we tested were all collected from one Zapovednik reserve near Karamendy. In contrast, markers IEAAAG09 and IEAAAG13 were monomorphic in white-tailed sea eagles. Their allele sizes were different from the two *Aquila* species we tested therefore it is possible they will be useful for assigning genus of anonymous adult feathers. Our eight markers are robust in two genera of eagles, which is consistent with reports for other raptor microsatellites (Nesje & Roed 2000; Martinez-Cruz *et al*. 2002; D. Dawson *pers. com*.). It is likely that these tetranucleotide microsatellites will be useful in a range of *Aquila* and *Haliaeetus* species in addition to those we tested.

Acknowledgements

Tatyana Bragina, Anatoly Taran, Jaraslaw Matusiak Taran, Seth Layman, Heather Triplett, Rashid Shigapov, Fergus Crystal, Alexander Popkov, Alexander Sagalov, and Douglas Grier provided critical field support and assisted in field collections. We thank Dr Shou-Hsien Li for advice about this protocol. Financial support for this project was provided to TEK by the US-EPA STAR Graduate Research Fellowship Program. We acknowledge assistance from the Naurzum Zapovednik and the government of Kazakhstan. Collections were gathered and imported under the appropriate Russian, United States and Kazakhstan CITES and MBO permits; details of these permits are available upon request.

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