Bald Eagle *(Haliaeetus leucocephalus)* Feathers as an Alternative to Blood for Microsatellite DNA Analysis: Toward a Non–invasive Technique for Conservation Genetics

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Abstract: The bald eagle *(Haliaeetus leucocephalus)* is currently classified as threatened in the lower 48 United States. In Massachusetts, only 12 active nesting sites presently exist, and the majority of breeding birds originated from a population of eaglets imported from Nova Scotia in the 1980s. Previous work using Random Amplified Polymorphic DNA (RAPD) technique demonstrated a genetic diversity among Massachusetts's eagles of only 22 percent. RAPD, while useful for genetic analysis of blood samples, proved to be inappropriate for analysis of feather samples from the same birds. To address this, our current work aimed at determining whether microsatellites would yield identical information for blood and feather samples from the same animal. Utilizing GenBank sequences, a total of 24 microsatellite primer sets representing 18 loci were designed and tested for allele polymorphism using DNA from eight blood samples and a single annealing temperature in the polymerase chain reaction (PCR). Thirteen microsatellites (54%) representing 11 loci were polymorphic, and three of these were selected to compare allele sizes in blood and feather DNA from the same eaglet. Preliminary results using microsatellite AJ620425 showed that 18 out of 44 blood/feather pairs amplified alleles of similar sizes. Feather DNA of the other 26 blood/feather pairs tested did not amplify any alleles. Data suggest that microsatellite alleles from blood and feather of the same bird may be consistent, and the microsatellite technique could be useful for non–invasive conservation genetics studies. However, multiple repetitions of the experiment are needed in order to determine optimum DNA concentration for feather samples. Additionally, refinements to the protocol will be necessary to obtain greater amplification of feather DNA which tended to give somewhat weak signals.

Keywords: conservation genetics, microsatellites, blood, feather, bald eagle, DNA, *Haliaeetus leucocephalus*

INTRODUCTION

The bald eagle (*Haliaeetus leucocephalus*), driven nearly to extinction in the lower 48 United States, has made an extraordinary recovery. Threatened first by direct hunting for the feather trade and by reductions in prey base through big game and waterfowl hunting, the eagles benefited from the 1940 passage of the Bald Eagle Protection Act, forbidding their capture or destruction. Despite modest population gains under that Act, post World War II usage of DDT and other organochlorine pesticides sent bald eagle numbers plummeting to only 417 nesting pairs throughout the U.S. in 1963, excluding Alaska (Federal Register 1999). A 1972 ban on DDT, passage of the Endangered Species Act in 1973, and subsequent aggressive management plans set the bald eagle on a path toward steady population increase. Downlisted to threatened in all the lower 48 states, the eagles continued reproductive success led the U.S. Fish and Wildlife Service (USFW) to seek to delist the species entirely in 1999. Their action failed mainly due to widespread fear that once authority for conservation were turned over to the individual states, the eagles would likely continue to thrive in some areas while suffering major declines in others primarily because of habitat loss and lack of federal funding for continued population studies (Booth 2000). Bald eagles are not divided into particular subspecies, but the concern over local effects of delisting is warranted given the site fidelity of the birds. Upon reaching maturity, bald eagles will return to their natal area to breed, and once established, the birds will return to the same nest year after year. They form close pair bonds as well, and these factors, when taken together, point to some degree of genetic isolation in local populations (Lesica and Allendorf 1994). Alpers et al (2004) suggests that conservation policy makers would do well to base management decisions on the underlying genetic structure of wildlife populations, rather than simple species or subspecies categorizations. Given its highly variable distribution in North America, the bald eagle seems particularly in need of this sort of scrutiny.

In the state of Massachusetts bald eagles were extirpated by 1950. Reintroduction of 42 nestlings

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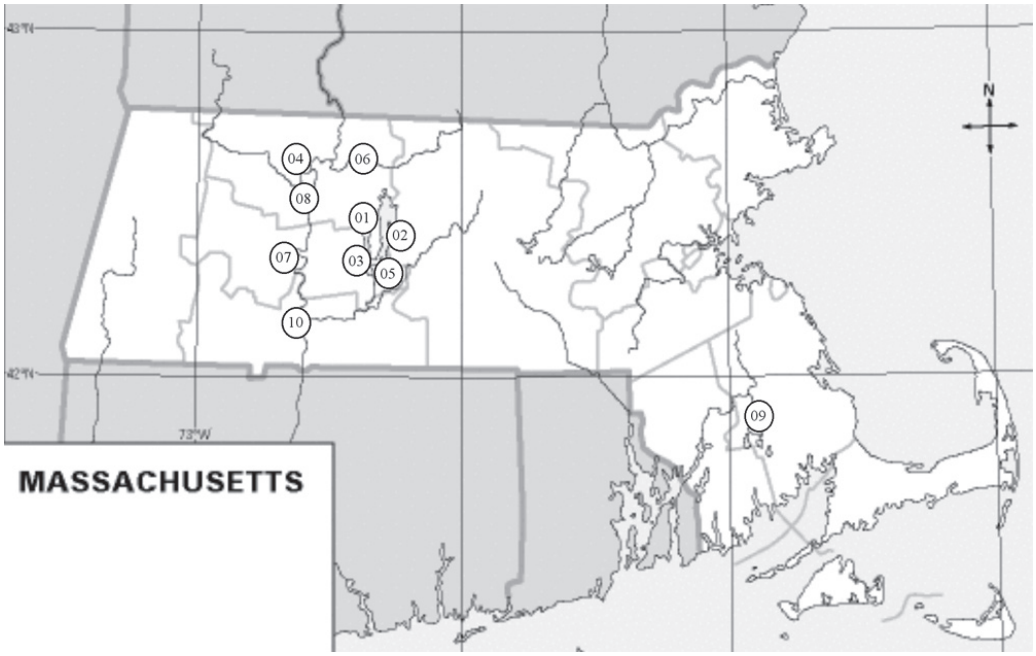

Figure 1. Geographic locations of bald eagle nests in Massachusetts. The territories are identified chronologically in the order in which they were discovered: 01=Russ Mountain, Quabbin, 02=Mount Pomeroy, Quabbin, 03=Quabbin Park, 04=Barton Island, Connecticut River, 05=Mt. Lizzie/ Little Quabbin, Quabbin, 06=Hamilton Island, Quabbin, 07=Oxbow–North Hampton, Connecticut River, 08=River Road Deerfield, Connecticut River, 09=Pocksha Pond, Middleboro, 10=Turnpike, Connecticut River, West Springfield.

from Nova Scotia between 1982 and 1986 formed the basis for the current population of 12 breeding pairs (DeGraaf and Yamasaki 2001). Given such a small group from which most Massachusetts bald eagles are descended, it was not surprising when previous work using RAPD analysis demonstrated a level of genetic diversity of only 22 percent among Massachusetts eaglets banded between 1994 and 1996 (Alcivar–Warren et al 2003; Mark et al 2003). That work also attempted to show that RAPD analysis of both blood and feather samples yield identical genetic information. This did not, however, prove true.

This study looks to microsatellites to determine if they generate identical information for blood and feather samples. Microsatellites, or simple sequence repeats (SSRs), are repetitive sequences in an organism's DNA, present in both the coding and non–coding regions (Li et al 2004). They are ideal markers for population genetics, parentage testing and gene mapping due to their variability and abundance in the genome, as well as their Mendelian pattern of inheritance and co–dominant expression (Meehan et al 2003 and references therein). Particularly when they fall within the unexpressed portion of the genome, these sequences can and do undergo high mutation rates. These high mutation rates result in high levels of polymorphism, without any deleterious effect on the organism's fitness. Microsatellites appear to be hypermutable even when found in coding regions suggesting their unusually mercurial nature (Bayliss et al 2004). For this reason, microsatellites are particularly well suited to genetic analysis of closely related individuals (Haymer 1994). A limited number of microsatellites have been isolated from the bald eagle genome with a total of 25 sequences currently available through the public GenBank database (Culver et al 2004). A genomic library for Massachusetts bald eagles has been cloned, however the recombinant clones are still awaiting sequencing.

The objectives in this study were to a) identify polymorphisms in microsatellite containing sequences in the bald eagle genome, and b) use three polymorphic markers to determine if microsatellite alleles of blood and feather from a given bird would yield identical information. Feathers have proved a reliable source of microsatellite DNA in other avian species (Segelbacher and Storch 2002). If the same holds true for the bald eagle, future population analysis and genomics of this species and many other endangered and threatened birds will not be hampered when blood samples are difficult or impossible to obtain.

Materials and Methods

Sample collection and DNA extraction. Both feather and, when feasible, blood samples from each eaglet banded at Massachusetts nest sites (Table 1 and Figure 1) from 1994 through 2000 were obtained through cooperation with Massachusetts Fish and Wildlife. Blood was collected from brachial veins and stored in EDTA and heparinized vacutainers. One to four breast feathers per bird were collected in glass tubes in the field and both blood and feather samples were kept at minus 80°C until processing. Catherine Mark, DVM, performed DNA extraction on all samples through 1996 in the summer of that year.

Blood nucleic acids were isolated using either a

guanidine (GT) based or SDS (Lysis) based protocol (Alcivar et al 1989). Feathers were processed using either the same Lysis protocol with some minor modifications or a Chelex based protocol (Mark et al 2004). Samples obtained after 1996 were all processed during the summer of 2004 using the Lysis protocol. DNA quality control was performed on all samples using agarose gel electrophoresis, and only samples that showed high molecular weight DNA were included in the microsatellite analysis.

Microsatellite loci amplification and scoring. Microsatellite containing sequences were obtained from GenBank, with one or more microsatellites identified from each of the following sequences: AJ620420, AJ620424, AJ620425, AJ620433, AJ620434, AJ620435 (Culver et al 2004). The Primer3 software program (Rozen and Skaletsky 1996, 1997) was used to design 24 oligonucleotide primer sets flanking one or more motifs within a given sequence. These primer sets, which represented 18 different genomic loci, were selected based on uniqueness of the primer sequence. A microsatellite was defined as any sequence containing three or more repeats of a di–, tri–, or tetranucletide (Xu et al 1999; Meehan et al 2003). Motifs of only three repeats were included because microsatellites are particularly rare in avian genomes (Khatib et al 1993; Longmire et al 1999); interestingly, their relative paucity in birds may be due to the weight constraints imposed by flight having ramifications all the way down to the molecular level (Primmer et al 1997).

All 24 reverse primers were fluorescently labeled with either 6–FAM, TET, or HEX dyes (Integrated DNA Technologies, Inc., Coralville, IA) and used to test for polymorphisms. Table 2 details the forward and reverse primer sequences, core motifs and expected allele sizes of tested microsatellites. Because the authors could not be certain of the results feather samples would yield with microsatellites, only blood samples were used for preliminary polymorphism testing. Eight animals were selected (numbers 11, 13, 16, 18, 30, 35, 50S, 57S in Table 1). The authors attempted to maximize the diversity among the eight by using animals from as many different geographic locations as possible, and by including fostered chicks raised in Massachusetts nests, but with biological parents from locations as diverse as Michigan and the Philadelphia Zoo. This strategy was intended to maximize the likelihood of finding polymorphic markers. Subsequently, 44 birds were selected from the total population in Table 1 for confirmation of microsatellite allele similarity between blood and feather. Any bird for which both blood and feather samples were available was included in the analysis (sample numbers in bold in Table 1).

Microsatellite genotyping was performed following the user's manual of ABI PRISM® 377 DNA sequencer (Applied Biosystems, Foster City, CA). PCR conditions followed lab protocols developed for shrimp with some modifications (Alcivar–Warren et al unpublished). The PCR reaction mixture consisted of 0.3 µM template DNA, 0.33 µM reverse primer (fluorescently labeled) and forward primer, 0.13 mM dNTPs, 0.04 U/µl Taq polymerase (Promega Corporation, Fitchburg Center, WI), 2.5 mM $MgCl_2$ and 1X buffer in a total of volume of 15 µl. The thermal cycler (PTC–100, MJ Research, Waltham, MA) profile was (1) 95°C for 12 minutes, (2) 94°C for one minute, (3) 52°C for one minute, (4) 72°C for two minutes, (5) repeat steps two thru four for 30 cycles, and (6) 72°C for 30 minutes (Alcivar–Warren et al unpublished). Amplified products of all three reactions were multiplexed into a mixture containing standard volumes as follows: 2uL for TET containing mixture, 3uL for 6–FAM containing mixture, and 4uL HEX containing mixture. The products were then electrophoresed in polyacrylamide gels following ABI PRISM® 377 manufacturer's procedures. Electrophoresis data were analyzed by GeneScan version 2.1 and Genotyper version 2.0 software. To avoid inaccuracy in scoring among different gels, a control DNA sample provided by ABI of known genotype was included in each set of samples for each gel. The allele sizes of amplified products were estimated by at least two different researchers (D. Meola and S. Courchesne) using an internal size standard, GeneScan 500 (Applied Biosystems). A microsatellite was regarded as polymorphic when the frequency of the most common allele was equal to or less than 0.99 (Nei 1987).

Results

Polymorphism analysis of 24 primer sets. Thirteen (five FAM–, four TET– and four HEX) of the 24 primers tested were polymorphic using a single annealing temperature (Table 3). Most of the samples genotyped with 6–FAM and TET labeled primers showed two different alleles, but samples genotyped with HEX labeled primers amplified a single allele of variable sizes (Table 3). This suggests that allele amplification conditions for the HEX primers may need to be optimized taking into account the possible presence of null alleles caused by mutations in primer sequences.

The observed heterozygosity for polymorphic 6–FAM and TET labeled primers (Table 3) ranged

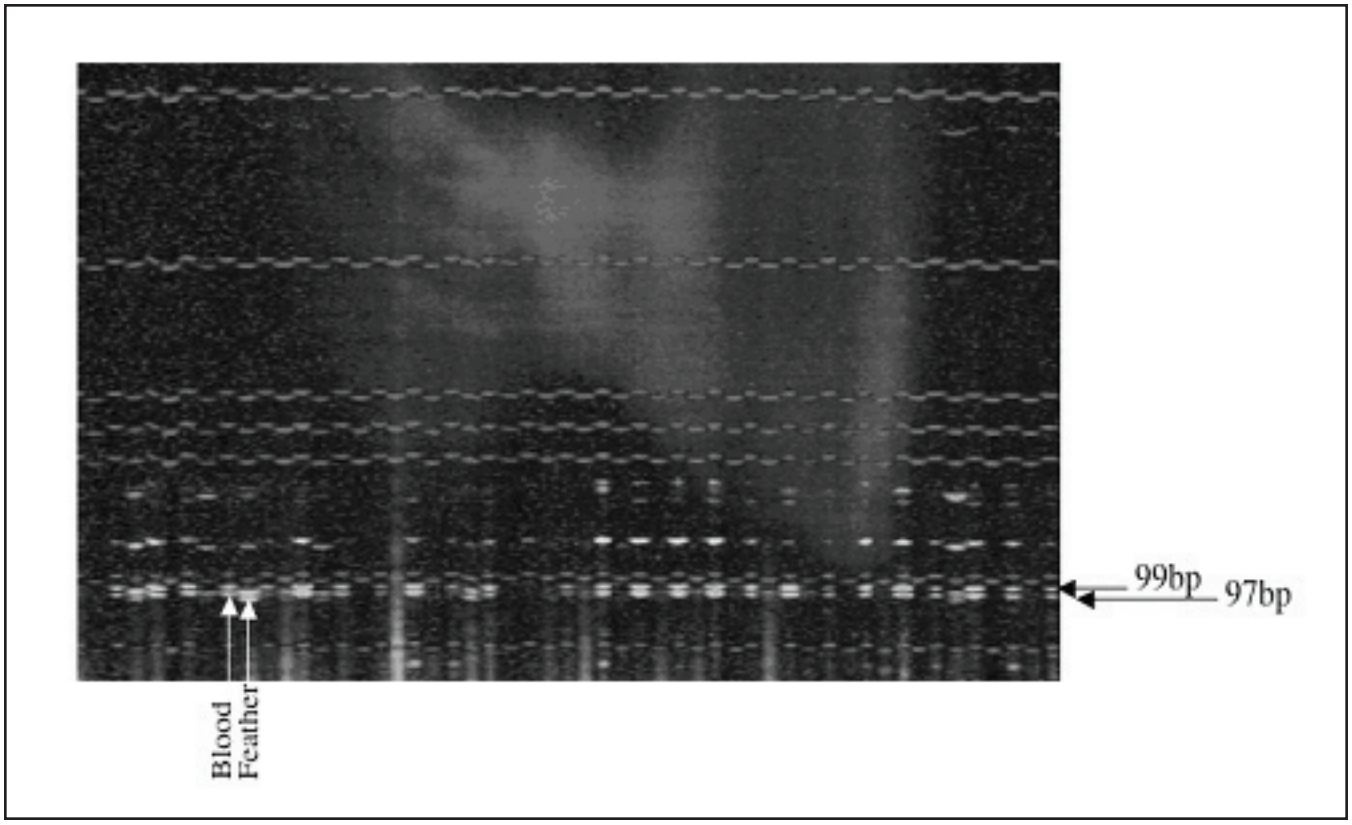

Figure 2. Microsatellite allele amplification of blood (b) and feather (f) of same eaglets using three fluorescently labeled primers in an ABI PRISM® 377 DNA Genotyper. Arrows denoting allele size are approximate and represent results from the TET primer.

from 25 to 100 percent and shows that these markers could be useful for genetic analysis. In this study, all samples with a single band are regarded as homozygous although they could be one amplified allele and one null allele (Pemberton et al 1995).

It appears that even short motifs of only four repeats can amplify polymorphic alleles. This is shown with the useful polymorphic primer sets of locus AJ620420b (6–FAM labeled) which flanked the short motif $(AT)_4$ whereas primer sets of AJ620420a (HEX labeled) which flanked the longer motif $(GT)_{14}$ need further optimization of allele amplification conditions.

The HEX and 6–FAM labeled primers gave weaker signals than have previously been seen with the shrimp work, but the signals were sufficient to determine polymorphism (Table 3).

Analysis of blood versus feather DNA from the same animal. The most polymorphic marker from each dye category was chosen to generate a microsatellite multiplexing assay (one polymorphic primer set each for 6–FAM, TET, and HEX dyes) so that each sample was electrophoresed with three markers simultaneously. These markers were AJ620420(b), AJ620425(d), and AJ620423 respectively. Because of the variability in signal intensity, the volumes of 6–FAM and HEX mixtures were increased by 1uL each in order to maximize the intensity of the bands.

Preliminary findings showed that the TET labeled primer (AJ620425d) gave the best results by far of the three primers with 18 (41%) out of 44 blood/feather pairs amplifying alleles of similar sizes. The feather DNA of the other 26 blood/feather pairs tested failed to amplify (bands labeled with arrows at approximately 99 to 101 base pairs in Figure 2). These results need to be repeated first with the same protocol used to detect polymorphism with the initial eight samples (without dye changes) before proceeding with further modifications. Also, the experiment must undergo multiple repetitions in order to rule out some human or technical error in the feather protocol. DNA from a single feather is found in far lower concentrations than blood and many other standard tissue types. Because of this, it is often difficult to determine an accurate DNA concentration. Further repetitions will aid in determining whether these difficulties are inherent in using feather as a substrate, or whether the error lies with the researchers. Once the requisite repeat experiments are complete, the protocol will be scrutinized. The 6–FAM and HEX primers would likely benefit from optimization of allele amplification conditions,

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Table 1. Bald Eagle samples collected from eaglets banded between 1994 and 2000.

Sample	Location	Study	Date	Blood	Feather	Parent (M)	Parent (F)	Siblings	Sex
1	Russ Mountain	W-60	6/8/95	yes	yes	Russ '82?	#4 '85?	W-61, 62	M
1A	Russ Mountain	W-60	6/8/95	no	yes	Russ '82?	#4 '85?	W-61,62	M
2	Russ Mountain	W-61	6/8/95	yes	yes	Russ '82?	#4 '85?	W-60, 62	F
2A	Russ Mountain	W-61	6/8/95	no	yes	Russ '82?	#4 '85?	W-60, 62	F
3	Russ Mountain	W-62	6/8/95	yes	yes	Russ '82?	#4 '85?	W-60, 61	F
3A	Russ Mountain	W-62	6/8/95	no	yes	Russ '82?	#4 '85?	W-60, 61	F
4	Conway	W-64	5/21/96	yes	yes	NC	Maine	n/a	F?
4A	Conway	W-64	5/21/96	no	yes	NC	Maine	n/a	F?
5	Barton Cove	W-65	5/21/96	yes	yes	known	known	W-66	F?
5A	Barton Cove	W-65	5/21/96	no	yes	known	known	W-66	F?
6	Barton Cove	W-66	5/21/96	yes	yes	known	known	W-65	M?
6A	Barton Cove	W-66	5/21/96	no	yes	known	known	W-65	M?
7	Middleboro	W-69	5/24/96	yes	yes	?	pres.,bnd.	W-70	F
8	Middleboro	W-70	5/24/96	yes	yes	?	pres.,bnd.	W-69	M
9	Hamilton Island	W-71	5/30/96	yes	yes	present	present	W-72	M
10	Hamilton Island	W-72	5/30/96	yes	yes	present	present	W-71	M
11	Russ Mountain	W-73	5/30/96	yes	yes	present	present	n/a	M
12	Little Quabbin	W-74	5/31/96	yes	yes	n/a	n/a	n/a	F
13	Brooksville	U	6/19/96	yes	yes	unknown	unknown	unknown	M
14	Petersham	W-46	5/31/95	yes	yes	'84 Mr.01+ '85 #3		found dead	F?
15	Hamilton Island	W-48	5/31/95	yes	no	Hamilton, >'86		W-49	F?
16	Hamilton Island	W-49	5/31/95	yes	yes	Hamilton, >'86		W-48	M?
17	Hampshire Co.	W-50	6/1/95	yes	yes	1986-1987		n/a	M
18	Little Quabbin	W-51	5/19/95	yes	yes	Michigan	Phil. Zoo	n/a	M?
19	Barton Cove	W-52	6/2/95	yes	yes	1986	1985	W-53	F?
20	Barton Cove	W-53	6/2/95	yes	yes	1986	1985	W-52	M

Table 1 continued.

Sample	Location	Study	Date	Blood	Feather	Parent (M)	Parent (F)	Siblings	Sex
21	Barton Cove	W-53	6/2/95	yes	yes	1986	1985	W-52	M
22	Conway	W-54	6/2/95	yes	yes	captive		n/a	F?
23	Hampshire Co.	W-55	6/2/95	yes	yes	unknown		W-56,57	M?
24	Hampshire Co.	W-56	6/2/95	yes	yes	unknown		W-55,57	M?
25	Hampshire Co.	W-57	6/2/95	yes	yes	unknown		W-55,56	F
26	Pocksha Pond	W-58	6/5/95	yes	yes	unknown		W-59	M?
27	Pocksha Pond	W-59	6/5/95	yes	yes	unknown		W-58	M
28	Barton Cove	W-37	5/26/94	yes	yes	1986	1985	n/a	M?
29	Hamilton Island	W-38	5/31/94	yes	yes	unknown		n/a	U
30	Pocksha Pond	W-39	6/6/94	yes	yes	unknown		n/a	F?
31	Hamilton Island	W-40	6/8/94	yes	yes	>86		W-41	F?
32	Hamilton Island	W-41	6/8/94	yes	yes	>86		W-40	M?
33	S. Penninsula	W-42	6/9/94	yes	yes	1984 + 85 #8			F?
34	Petersham	W-43	6/10/94	yes	yes	84 Mr. 01 + '85 #3		W-44	F?
35	Petersham	W-44	6/10/94	yes	yes	84 Mr. 01 + '85 #3		W-43	F?
38	W. Springfield	W-77	6/6/96	no	yes	2nd generation?		W-78	M
39	W. Springfield	W-78	6/6/96	no	yes	2nd generation?		W-77	F
40	Pocksha Pond	W-81	5/29/97	yes	yes	unknown		W-82	F?
41	Pocksha Pond	W-82	5/29/97	yes	yes	unknown		W-81	M?
42	Conway	W-83	5/30/97	yes	yes	Virginia	Patuxent	n/a	F
43	Little Quabbin	W-84	5/30/97	yes	yes	unknown		W-85	M
44	Little Quabbin	W-85	5/30/97	yes	yes	unknown		W-84	M
45	Russ Mountain	W-86	6/4/97	yes	yes	Russ '82?	#4 '85?	W-87,88	F
46	Russ Mountain	W-87	6/4/97	yes	yes	Russ '82?	#4 '85?	W-86,88	F
47	Russ Mountain	W-88	6/4/97	yes	yes	Russ '82?	#4 '85?	W-86,87	F
50	S. Penninsula	W-89	6/10/97	yes	yes	unknown		n/a	M

Table 1 continued.

Sample	Location	Study	Date	Blood	Feather	Parent (M)	Parent (F)	Siblings	Sex
50S	Wellfleet	WB-8	7/9/99	yes	yes	unknown-not found in nest		unknown	U
51S	U	WB-9	8/21/99	yes	no	Bullet(Alaska)	Maine	WC-0	M
52S	U	WC-0	8/21/99	yes	no	Bullet(Alaska)	Maine	WB-9	F
53S	U	WC-1	8/21/99	yes	no	Pair #2		n/a	F
54S	Barton Cove	W-92	5/21/98	yes	yes	New York	85 N.Scotia	n/a	U
55S	U	U	7/9/99	no	yes	unknown		U	U
56S	Hamilton Island	W-96	5/28/98	no	yes	unknown-new nest		W-95	M
57S	Mt. Pomeroy	W-90	5/28/98	yes	yes	Bullet(Alaska)	Maine	W-91	M
58S	Little Quabbin	W-91	5/28/98	yes	yes	Bullet(Alaska)	Maine	W-90	F
59S	Russ Mountain	W-98	5/28/98	yes	yes	unknown		W-97,99	M
60S	Russ Mountain	W-99	5/28/98	yes	yes	unknown		W-97,98	F
61S	Hamilton Island	W-95	5/28/98	yes	yes	unknown-new nest		W-96	M
62S	Poksha Pond	W-94	5/26/98	no	yes	unknown		W-93	U
63S	Poksha Pond	W-93	5/26/98	no	yes	unknown		W-94	U
64S	Mt. Pomeroy	WA-0	5/29/98	yes	yes	unknown		foster W90	F
65S	Little Quabbin	WO-0	5/29/98	no	yes	unknown		foster W91	F
66S	W. Springfield	WA-1	6/5/98	yes	yes	W18	W29	WA-2	F
67S	W. Springfield	WA-2	6/5/98	no	yes	W18	W29	WA-1	F
68S	Russ Mountain	W-97	5/28/98	yes	yes	unknown		W-98,99	F
69S	S. Penninsula	W-75	5/6/96	no	yes	Quabbin Park Pair		W-76	M
70S	S. Penninsula	W-76	5/6/96	no	yes	Quabbin Park Pair		W-75	F
71S	Little Quabbin	WD-0	6/8/00	yes	yes	unknown		WD-1	M
72S	Little Quabbin	WD-1	6/8/00	yes	yes	unknown		WD-0	F
73S	Mt. Pomeroy	WD-2	6/9/00	yes	no	unknown		U	F
74S	U	U	6/13/97	no	yes	unknown		U	U
75S	U	Bullet	3/18/97	no	yes	unknown		U	U
76S	U	U	6/1/98	yes	no	unknown		U	U

including primer sequences, annealing temperatures, etc. In order to repeat this experiment, future efforts toward isolating DNA from feathers to yield higher quality as well as quantity DNA need to be undertaken.

Results from the HEX primers were particularly intriguing; after polymorphism testing, all but one HEX labeled primer that amplified at all gave only a single peak. The appearance of a single allele typically brings to mind the possibility of a null allele, or a mutation in the primer sequence which causes the animal to appear to be a homozygote when, in fact, it is not. This eventuality was well elucidated by Pemberton et al (1994). In this experiment, however, only the HEX primers showed a single allele of variable size. It is assumed that those primers derive from randomly distributed loci in the eagle genome. This suggests that all eight animals selected for polymorphism testing were homozygotes, which is unlikely given their diverse parentage. Perhaps the problem lies either with effects of the HEX dye itself on DNA, or with PCR conditions set for the HEX primers. We look forward to repeating the experiment to see if the same single peak appears again for the HEX primer.

Discussion

Data suggest that microsatellite alleles from blood and feather may be consistent, and microsatellite technique could be useful for non–invasive conservation genetics studies. However, multiple repetitions of the experiment are needed in order to determine optimum DNA concentration for feather samples and before any generalizations can be made about the value of using feathers as a substrate for bald eagle population or conservation genetics.

Future work could focus both on testing the remaining primers deemed polymorphic in this study, and also on developing primers from sequences of recombinant plasmids obtained from a genomic library cloned using blood DNA of a Massachusetts bird (Alcivar–Warren et al 2003). The results from the TET labeled primer AJ620425d, while hardly definitive, are at least promising that with continued work we may indeed see strong evidence that microsatellites are a reliable source of genomic DNA in bald eagles. If this technique ultimately proves to have some utility, future population genetics and genomics research will have gained a valuable tool. The authors hope to apply this technique to the continued monitoring of not only Massachusetts eagles, but also populations tht were previously inaccessible due to the implausibility of obtaining blood samples. This technique will permit collaborators without the experience or ability to draw blood to contribute genetic data, and it will also reduce stress and risk of injury to the birds themselves. In the end, the authors hope to have contributed to the ongoing discussion of how best to aid the recovery and conservation efforts of a cherished national symbol and an indispensable link in the natural chain.

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