

TECHNICAL ARTICLE

Species identification of birds through genetic analysis of naturally shed feathers

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Abstract

Genetic analysis of noninvasively collected bird feathers is of growing importance to avian ecology; however, most genetic studies that utilize feathers make no mention of the need to verify their species of origin. While plumage patterns and collection location often are indicative of species identity, broad-scale feather collections may require definitive species identification prior to analysis. Genetic species identification has been applied to noninvasively collected samples from a wide range of taxa but, to date, these techniques have not been widely used on bird feathers. Here, we develop and test a polymerase chain reaction (PCR)-based technique for identifying eastern imperial eagle (*Aquila heliaca*) samples among a vast number of noninvasively collected feathers. Species identification is accomplished by amplifying a fragment of the mitochondrial cytochrome *c* oxidase I gene, then digesting that fragment with a restriction enzyme. The resulting species-specific restriction fragment length polymorphisms (RFLPs) are easily visualized by gel electrophoresis. We tested this PCR-RFLP assay on over 300 individuals that had been genetically identified from noninvasively collected feathers and demonstrated that the assay is both reliable and robust for DNA of low quality and quantity. The genetic methods of species identification used to develop this assay can readily be applied to other bird assemblages, making them particularly relevant to a broad range of future avian research.

Keywords: cytochrome *c* oxidase I, eagle, feather, noninvasive, RFLP, DNA barcoding

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Introduction

A growing number of studies are utilizing noninvasively collected samples (feathers, hair, scat, etc.) to characterize ecological or demographic attributes of populations that are either difficult or impossible to investigate by more traditional means. Because the donor species of noninvasively collected samples must often be verified, genetic methods of species identification have become invaluable resources (e.g. Lucchini *et al.* 2002; Palomares *et al.* 2002; Kurose *et al.* 2005; Smith *et al.* 2006). Although naturally shed feathers have long been identified as

suitable sources of DNA for avian research (Pearce *et al.* 1997; Segelbacher 2002; Horvath *et al.* 2005), most genetic studies that exploit these types of samples make no mention of the need to verify the feathers' species of origin. In some cases, plumage patterns or site-specific data are diagnostic for species, but in many cases, they are not. In these situations, the species origin of noninvasively collected feathers must be verified if results are to be credible.

The species origin of noninvasively collected feathers must be carefully evaluated when a number of species with similar plumage patterns frequent areas where feathers have been collected. For example, consider feathers collected haphazardly from a productive wetland: if multiple species with similar plumage patterns are present, the feathers' donor species should be ascertained before any species-specific analyses can occur. Genetic species identification also can be useful in situations where

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feathers are collected by less traditional 'noninvasive' means, but for which species identity is nonetheless ambiguous. Predator dietary patterns often are characterized by examining faeces or regurgitated pellet castings (Korschgen 1980), but these studies rarely catalogue avian prey by species (e.g. Gilbert & Nancekivell 1982; Farrell *et al.* 2000). Genetic methods of species identification are capable of determining the species identity of ambiguous remains, which better characterizes a predator's impact on local avian communities (e.g. Scribner & Bowen 1998). Genetic species identification also can be helpful for studies that investigate bird-aircraft collisions. When a bird collides with an aircraft, a small number of feathers often are the only evidence remaining (e.g. Manville 1963; Laybourne 1974). When those feathers lack distinctive plumage patterns, genetic methods of species identification could assign species identity when more conventional, visual techniques of species identification prove inconclusive.

We have used naturally shed feathers to investigate several aspects of eastern imperial eagle (*Aquila heliaca*; EIE) biology in Central Asia. Our previous work focused on a breeding population of EIEs (Rudnick *et al.* 2005) and utilized feathers that were noninvasively collected from EIE nesting sites. Because breeding EIEs are highly territorial, there were few cases for which we had reason to doubt a feather's donor species. Our ongoing work, however, is focusing on nonbreeding EIEs present at our field site. To study this nonterritorial component of the EIE population, we have collected thousands of feathers from communal roosting areas. Many of the samples we collected were body feathers that lacked distinctive plumage patterns. To complicate matters, approximately 25 raptor species breed within our study site including three additional eagle species (Katzner *et al.* 2003), the feathers of which sometimes resemble those of EIEs: the steppe eagle (*Aquila nipalensis*; SE), the golden eagle (*Aquila chrysaetos*; GE) and the white-tailed eagle (*Haliaeetus albicilla*; WE). Thus, it was necessary for us to genetically verify the donor species of samples prior to intraspecific analyses of individuals.

We used a fragment of the mitochondrial cytochrome *c* oxidase I gene (COI) to develop a robust assay for genetically discriminating among the four eagle species present at our study site. We first used reference samples of known species origin to characterize COI sequences (haplotypes) both within and among these eagle species, then developed a restriction fragment length polymorphism (RFLP) assay to distinguish among each species' mitochondrial DNA (mtDNA) haplotypes. This RFLP assay was tested on several hundred noninvasively collected eagle feathers, and the mtDNA results were evaluated in light of microsatellite data on the same set of samples. Herein, we describe the molecular assay we used to identify EIEs and discuss how methods of genetic species identification can contribute to future avian research.

Methods

Reference samples

Two types of reference samples were used to design the species identification assay: developing blood feathers plucked directly from eagle chicks and toepad tissue taken from museum specimens. Developing blood feathers were used as reference samples for EIEs, WEs and SEs, while museum specimens were used as reference samples for GEs.

Developing blood feathers were collected over a 7-year period (1998–2004) from EIE, WE and SE chicks hatched at the Naurzum Zapovednik, a national nature reserve in north-central Kazakhstan. Samples were placed in a lysis buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA, 10 mM NaCl, 2% SDS) immediately upon collection, and ultimately stored at –80 °C. In general, eagles are socially monogamous and exhibit high nesting site fidelity both within and among breeding seasons (del Hoyo *et al.* 1994). Consequently, our collection of chick samples was replete with full-siblings. To minimize pseudoreplication among samples, we chose reference individuals for EIEs, SEs and WEs in the following manner. For each species, the breeding season with the greatest number of sampled nesting sites was identified. Then, one chick from each nest was randomly chosen for analysis. Because our previous work empirically supported the assumption that eagles exhibit high nesting site fidelity between breeding seasons (Rudnick *et al.* 2005), we also supplemented these samples with chicks from the year that directly pre- and proceeded the primary sampling year by the following means. We identified all nesting sites that were sampled in the supplementary years but not in the primary year, then added one chick from each of those nests to the analyses. In total, we obtained tissue samples from 30 EIE chicks, 9 WE chicks and 6 SE chicks for use as reference samples.

The Bald and Golden Eagle Protection Act prohibits known import of GE samples into the United States. For this reason, GE chicks were not directly sampled. Instead, we obtained samples of toepad tissue from 20 GE specimens located at the Field Museum of Natural History in Chicago, IL (USA). Specimens were originally collected between 1908 and 1941 throughout both the United States and Central Asia.

DNA methods

DNA was isolated from chick samples following the methods in Rudnick *et al.* (2005). DNA was similarly isolated from toepad tissue, with the exception that samples were first soaked in 100% ethanol for 24–48 h to remove polymerase chain reaction (PCR) inhibitors. A negative control containing no eagle tissue was included in each group of DNA extractions.

The mtDNA COI gene has previously been shown to exhibit adequate interspecific sequence divergence to discriminate among a wide variety of bird species that span at least 19 avian orders (Hebert *et al.* 2004). We initially screened the BirdF1/BirdR1 primers published by Hebert *et al.* (2004) and found them to robustly amplify a 746-bp fragment of COI in our EIE, WE and SE chick samples. However, because amplification proved inconsistent for noninvasively collected feathers that yield suboptimal DNA, we redesigned BirdR1 to amplify a shorter fragment of COI. Primers BirdF1 and EagleR2b (the redesigned BirdR1 primer; 5'-ATTGATRGCCTGTGATAAA-3') robustly amplify a 485-bp fragment of COI from both chick and noninvasively collected feather samples. PCRs for BirdF1/EagleR2b were performed in a final volume of 12 µL and contained 1× Thermopol PCR buffer, 0.2 mM each dNTP, 0.3 µM each primer, 1.0 U *Taq* DNA Polymerase (New England BioLabs), and 0.008 U *Vent* DNA polymerase (New England BioLabs) to reduce *Taq* error (Mattila *et al.* 1991). The thermal profile included an initial denaturation step of 94 °C for 2 min, followed by 32 cycles of 94 °C for 1 min, 59 °C for 30 s, and 72 °C for 1 min. A final extension step of 72 °C for 5 min concluded the profile.

DNA isolated from GE toepad tissue was highly degraded and COI failed to reliably amplify for these samples with BirdF1/EagleR2b. Consequently, we designed a series of primers to amplify the target fragment of COI in four short, overlapping amplicons (Table 1). PCR conditions and thermal profiles for all primer pairs were identical to those listed for BirdF1/EagleR2b, with the exception of the following annealing temperatures: 45 °C annealing temperature for primer pairs BirdF1/GE01 and GE02/GE03; 50 °C annealing temperature for primer pairs GE04/GE05 and GE06/GE07.

Negative control PCRs (those without template DNA) were routinely conducted to rule out sample contamination. All PCR products were cleaned with a sodium acetate-ethanol precipitation and sequenced in both directions with the amplification primers. Sequencing reactions utilized BigDye version 3.1 (Applied Biosystems), following the manufacturer's protocol modified to one-eighth reactions.

Developing a method for species identification

SEQUENCHER 4.6 (Genecodes Corporation) was used to align amplicons, ensure sequences contained open reading frames (to guard against amplification of nuclear pseudogenes), and generate consensus sequences for all individuals. MODELTEST 3.6 (Posada & Crandall 1998) was used to identify the most appropriate model of evolution for analysing the amplified region of COI, and that model was then used for all subsequent sequence analyses. PAUP* 4.0b10 (Swofford 2004) was used to calculate pairwise maximum-likelihood (ML) distances for all unique COI haplotypes. Mean intraspecific ML distances were calculated for each species, then these values were used to calculate a grand mean for intraspecific ML distance observed across all species. Mean interspecific ML distance was calculated from all possible interspecific pairs of COI haplotypes. PAUP* 4.0b10 also was used to generate an ML tree with 1000 bootstrap replicates, to provide a graphic representation of species divergence. A peregrine falcon (*Falco peregrinus*) COI sequence was downloaded from GenBank and used as an outgroup.

COI sequences from all four eagle species were compared with one another to identify a restriction enzyme that would produce species-specific RFLPs, taking into account the intraspecific variation observed in our reference samples. The restriction enzyme *Nla*III easily discriminated among the four species *in silico*, and we validated the use of this enzyme empirically. Restriction digests were performed in a final volume of 30 µL and contained 10 µL COI PCR product, 1× NEBuffer 4 (New England BioLabs), 3 µg BSA and 1.0 U *Nla*III. Digests were incubated at 37 °C for 1 h, then electrophoresed in 2% agarose gels (sodium boric acid buffer system; Brody & Kern 2004) and stained with ethidium bromide for visualization. Reference samples of known species origin were run on each gel for comparison.

Testing samples of unknown species origin

Naturally shed feathers were noninvasively collected from four communal raptor roosts at the Naurzum Zapovednik.

	Primer	Sequence (5'–3')	Fragment size (bp)
Amplicon 1	BirdF1	sequence from Hebert <i>et al.</i> (2004)	184
	GE01	CTATGAAGAAGATTATTACRAAAGCA	
Amplicon 2	GE02	TCYTAGGCGAYGACCAAATC	157
	GE03	TTGTTTATRCGTGGGAAGGCTA	
Amplicon 3	GE04	GGAAACTGACTTGTYYCCMCTC	218
	GE05	TGWAGGGARAAGATFRGCTAGGTC	
Amplicon 4	GE06	CCCAITAGCSGGYAAACATA	158
	GE07	GGGTGTYTGGTATTGGAGA	

Table 1 Primers designed to amplify the target fragment of COI in four short, overlapping amplicons. Fragment sizes are given in number of base pairs (bp)

Sampling locations were chosen because they were in areas where significant numbers of EIEs have been regularly observed. Only feathers that were likely to be EIE in origin were collected (i.e. feathers obviously not from EIEs were ignored), but many samples were body feathers that lacked distinctive plumage patterns. Sampled feathers were placed in paper envelopes and stored dry at room temperature. DNA was isolated from single feathers, and the species origin of all individuals was elucidated by the newly developed RFLP assay. One or two feathers were screened per individual, and any sample producing an RFLP profile that failed to match one of the known eagle profiles was subsequently sequenced. All samples in this study were also genotyped at a suite of seven microsatellite loci that were used to assign individual-specific DNA fingerprints, and a total of 314 individuals were identified among the noninvasively collected feathers (Rudnick *et al.* submitted).

Results

Initial characterization of COI

When the 485-bp fragment of COI was considered, four haplotypes were found in EIEs ($n = 30$), two haplotypes were found in SEs ($n = 6$), and all WEs shared a single haplotype ($n = 9$). Of the 20 GE samples obtained, only two produced sequences for the complete fragment of COI under consideration. These two samples share a single haplotype and represent individuals collected from the United States in 1935 (specimen 100792) and Afghanistan in 1939 (specimen 228823). Overall, no COI haplotypes were shared among eagle species. Sequences representing all unique haplotypes have been deposited into the GenBank database under the accession nos DQ834322–DQ834329.

Using the Akaike Information criterion, MODELTEST 3.6 picked GTR + I as the most appropriate model of evolution for the relevant region of COI. Using this model, pairwise maximum-likelihood (ML) distances were calculated among unique haplotypes. Mean ML distance among species was 10.84% and the grand mean of ML distances within species was 0.15%. ML trees exhibited strong bootstrap support (ranging from 86% to 99% across nodes) for intraspecific monophyly. The restriction enzyme *Nla*III produced diagnostic RFLPs for all four eagle species under consideration (Fig. 1). There was no intraspecific RFLP variation among COI haplotypes in any species (i.e. *Nla*III discriminates among species, not among haplotypes within a species).

Testing samples of unknown species origin

Our newly developed RFLP analysis was used to identify the species of 314 individuals identified from noninvasively

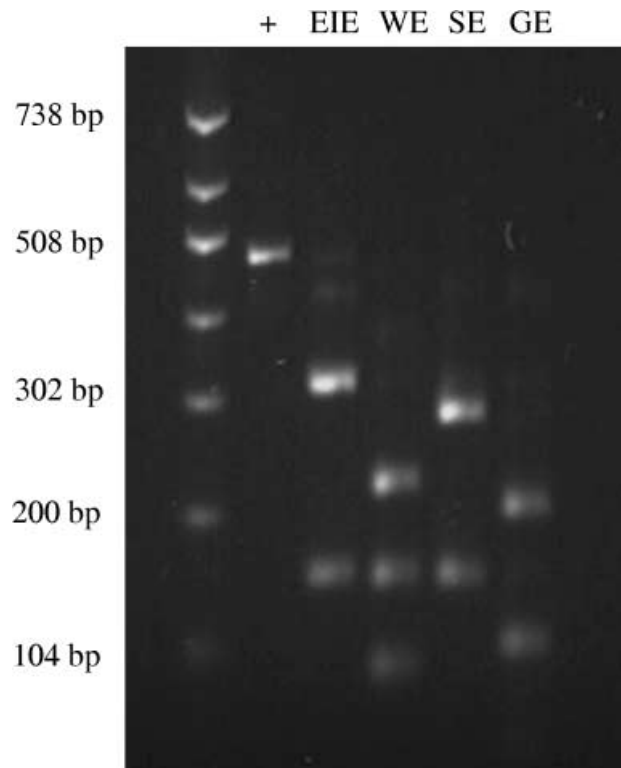


Fig. 1 A gel image of COI RFLP profiles for all eagle species under consideration. Uncut PCR product is indicated by a plus-sign. Restriction fragment sizes in base pairs (bp) are as follows: EIE 160/325; SE 30/160/295; WE 94/160/231; GE 50/110–116/209.

collected feathers. A total of 262 individuals were identified as EIEs, 13 individuals as WEs, and 1 individual as a GE. Six individuals exhibited a single RFLP profile that failed to match one of the expected eagle profiles. The species identity of the remaining 32 individuals (10%) could not be ascertained because of insufficient DNA quantities.

DNA sequences from the six birds exhibiting anomalous RFLP profiles revealed a unique COI haplotype shared among these individuals. This unique sequence was compared to all known eagle haplotypes, and found to differ from one of the EIE haplotypes by only a single nucleotide substitution that created an *Nla*III restriction site. Thus, these individuals were identified as EIEs that shared a previously unrecognized haplotype.

Discussion

Traditional methods of identifying a feather's species of origin involve matching species-specific feather characteristics to reference individuals (Chandler 1916). Feather characteristics include macroscopic attributes like plumage pattern, texture and size, but microscopic attributes of the plumulaceous barbs also can be utilized (e.g. Robertson *et al.* 1984; Dove

1997). Traditional methods of species identification are advantageous because they require little specialized equipment. However, a significant disadvantage of these methods is that accurate species identification can require extensive experience. Genetic methods of species identification are a valuable alternative to traditional techniques because they are less dependent on user interpretation and are more portable (i.e. there are only a few experts in feather microstructure, but there are many genetic laboratories where these types of assays are routine). Here, we demonstrate the use of genetic analyses to successfully identify 268 eastern imperial eagles from among thousands of noninvasively collected feathers.

Species identification by genetic means is usually accomplished through one of three techniques: phylogenetic reconstruction (Baker & Palumbi 1994; Malik *et al.* 1997; Alacs *et al.* 2003; Domingo-Roura *et al.* 2006), species-specific primer amplification (Shivji *et al.* 2002; Kurose *et al.* 2005), or PCR-RFLP analysis (Hold *et al.* 2001; Armstrong *et al.* 2003; Gomez-Moliner *et al.* 2004). We chose a PCR-RFLP approach for several reasons. Because of the large number of samples requiring analysis (~300 individuals), phylogenetic reconstruction would have been burdensome. With species-specific primer amplification, PCR primers are designed for all likely species of origin such that each primer pair produces a PCR product of unique size. Samples that fail to amplify are assumed to come from a donor species not included in the assay. Because DNA isolated from noninvasively collected samples is often of low quantity and quality, it was possible that the species-specific primer approach would have produced a high proportion of false negatives. Consequently, we felt a PCR-RFLP approach would provide a more robust assay.

We used reference samples to develop and phylogenetically groundtruth a PCR-RFLP assay for species identification. We initially screened a previously published primer set that amplified a 746-bp fragment of COI, but found this fragment did not reliably amplify in DNA of low quantities and qualities. We redesigned one of the primers to amplify a shorter fragment of COI (485 bp) and found this new primer pair to be both reliable and robust for even suboptimal DNA. When the PCR-RFLP assay was tested on a data set of 314 individuals, 282 were definitively identified as one of the four eagle species under consideration. Only six individuals exhibited anomalous RFLPs, and those samples were easily resolved with DNA sequencing. The species identity of the remaining 32 individuals could not be determined because of lack of DNA as opposed to a flaw in the species identification assay. It is worth noting that we successfully identified the donor species of ~90% of the noninvasive samples we assayed and furthermore, that our results were entirely consistent with species-specific differences observed in microsatellite data collected for these samples (Rudnick *et al.* submitted).

One important aspect of our approach to designing a PCR-RFLP species identification assay was the initial characterization of intraspecific variation. Multiple COI haplotypes were discovered in two out of the four eagle species under consideration, and we were careful to choose a restriction enzyme that would produce the same species-specific RFLP across the range of intraspecific haplotypes. Even so, when our RFLP assay was applied to a large data set, an uncharacterized EIE haplotype produced an RFLP profile unexpected for the species. Heist & Gold (1999) reported similar results when developing a genetic method of species identification for a suite of shark species; they found that spinner sharks from the North Atlantic produce different RFLPs than spinner sharks from the Pacific coast of Australia. In the ~400-bp fragment of mtDNA evaluated in their study, only two nucleotide differences were found between the two groups of spinner sharks. However, as with our work, one of these differences coincidentally resulted in a restriction site disparity. Collectively, these results highlight the importance of characterizing intraspecific variation prior to developing a PCR-RFLP assay for species identification.

As the number of studies that utilize naturally shed feathers continues to rise, genetic species identification will become increasingly important for avian research. The method we have described for developing a PCR-RFLP assay for species identification can be applied to any assemblage of bird species. The mtDNA fragment we employed was previously identified as an appropriate tool for genetic species identification in birds (Hebert *et al.* 2004), and our results enhance that claim. As more and more sequences are deposited in public databases (e.g. GenBank) and molecular assays for species identification become easier to develop, we envision that molecular techniques will supplement if not supplant other means of feather identification in situations where positive identification by visual means is cryptic.

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References

- Alacs E, Alpers D, de Tores PJ, Dillon M, Spencer PBS (2003) Identifying the presence of quokkas (*Setonix brachyurus*) and

- other macropods using cytochrome *b* analyses from faeces. *Wildlife Research*, **3**, 41–47.
- Armstrong KF, McHugh P, Chinn W, Frampton ER, Walsh PJ (2003) Tussock moth species arriving on imported used vehicles determined by DNA analysis. *Biosecurity*, **56**, 16–20.
- Baker CS, Palumbi SR (1994) Which whales are hunted? A molecular genetic approach to monitoring whaling. *Science*, **265**, 1538–1539.
- Brody JR, Kern SE (2004) Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. *BioTechniques*, **36**, 214–216.
- Chandler AC (1916) A study of feathers, with reference to their taxonomic significance. *University of California Publications in Zoology*, **13**, 243–446.
- Domingo-Roura X, Marmi J, Ferrando A *et al.* (2006) Badger hair in shaving brushes comes from protected Eurasian badgers. *Biological Conservation*, **128**, 425–430.
- Dove CJ (1997) Quantification of microscopic feather characters used in the identification of North American plovers. *Condor*, **99**, 47–57.
- Farrell LE, Roman J, Sunquist ME (2000) Dietary separation of sympatric carnivores identified by molecular analysis of scats. *Molecular Ecology*, **9**, 1583–1590.
- Gilbert FF, Nancekivell EG (1982) Food habits of mink (*Mustela vison*) and otter (*Lutra canadensis*) in northeastern Alberta. *Canadian Journal of Zoology*, **60**, 1282–1288.
- Gomez-Moliner BJ, Cabria MT, Rubines J *et al.* (2004) PCR-RFLP identification of mustelid species: European mink (*Mustela lutreola*), American mink (*M. vison*), and polecat (*M. putorius*) by analysis of excremental DNA. *Journal of Zoology*, **262**, 311–316.
- Hebert PDN, Stoeckle MY, Zemplak TS, Francis CM (2004) Identification of birds through DNA barcodes. *PLoS Biology*, **2**, 1657–1663.
- Heist EJ, Gold JR (1999) Genetic identification of sharks in the U.S. Atlantic large coastal shark fishery. *Fishery Bulletin*, **97**, 53–61.
- Hold GL, Russell VJ, Pryde SE *et al.* (2001) Validation of a PCR-RFLP based method for the identification of salmon species in food products. *European Food Research and Technology*, **212**, 385–389.
- Horvath MB, Martinez-Cruz B, Negro JJ, Kalmar L, Godoy JA (2005) An overlooked DNA source for non-invasive genetic analysis in birds. *Journal of Avian Biology*, **36**, 84–88.
- del Hoyo J, Elliott A, Sargatal J (eds) (1994) *Handbook of the Birds of the World, Vol. 2. New World Vultures to Guinea-fowl*. Lynx Edicions, Barcelona, Spain.
- Katzner TE, Bragin EA, Knick ST, Smith AT (2003) Coexistence in a multi-species assemblage of eagles in central Asia. *Condor*, **105**, 538–551.
- Korschgen LJ (1980) Procedures for food-habits analyses. In: *Wildlife Management Techniques Manual* (ed. Schemnitz SD), pp. 113–127. The Wildlife Society, Washington, D.C.
- Kurose N, Masuda R, Tataru M (2005) Fecal DNA analysis for identifying species and sex of sympatric carnivores: a noninvasive method for conservation on the Tsushima Islands, Japan. *Journal of Heredity*, **6**, 688–697.
- Labourne RC (1974) Collision between a vulture and an aircraft at an altitude of 37 000 ft. *Wilson Bulletin*, **86**, 461–462.
- Lucchini V, Fabbri E, Marucco F *et al.* (2002) Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the western Italian Alps. *Molecular Ecology*, **11**, 857–868.
- Malik S, Wilson PJ, Smith RJ, Lavigne DM, White BN (1997) Pinniped penises in trade: a molecular-genetic investigation. *Conservation Biology*, **11**, 1365–1374.
- Manville RH (1963) Altitude record for mallard. *Wilson Bulletin*, **75**, 92.
- Mattila P, Korpela J, Tenkanen T, Pitkanen K (1991) Fidelity of DNA synthesis by the *Thermococcus litoralis* DNA polymerase — an extremely heat stable enzyme with proofreading activity. *Nucleic Acids Research*, **19**, 4967–4973.
- Palomares F, Godoy JA, Piriz A, O'Brien SJ, Johnson WE (2002) Faecal genetic analysis to determine the presence and distribution of elusive carnivores: design and feasibility for the Iberian lynx. *Molecular Ecology*, **11**, 2171–2182.
- Pearce JM, Fields RL, Scribner KT (1997) Nest materials as a source of genetic data for avian ecological studies. *Journal of Field Ornithology*, **68**, 471–481.
- Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.
- Robertson J, Harkin C, Govan J (1984) The identification of bird feathers: scheme for feather examination. *Science and Justice*, **24**, 85–98.
- Rudnick JA, Katzner TE, Bragin EA, Rhodes OE Jr, DeWoody JA (2005) Using naturally shed feathers for individual identification, genetic parentage analyses, and population monitoring in an endangered Eastern imperial eagle (*Aquila heliaca*) population from Kazakhstan. *Molecular Ecology*, **14**, 2959–2967.
- Rudnick JA, Katzner TE, Bragin EA, DeWoody JA (submitted) A noninvasive Genetic Evaluation of Population Size and Natal Philopatry of Non-breeding Imperial Eagles (*Aquila Heliaca*) in Central Asia.
- Scribner KT, Bowen TD (1998) Microsatellites identify depredated waterfowl remains from glaucous gull stomachs. *Molecular Ecology*, **7**, 1401–1405.
- Segelbacher G (2002) Noninvasive genetic analysis in birds: testing reliability of feather samples. *Molecular Ecology Notes*, **2**, 367–369.
- Shivji M, Clarke S, Pank M *et al.* (2002) Genetic identification of pelagic shark body parts for conservation and trade monitoring. *Conservation Biology*, **16**, 1036–1047.
- Smith DA, Ralls K, Hurt A *et al.* (2006) Assessing reliability of microsatellite genotypes from kit fox faecal samples using genetic and GIS analyses. *Molecular Ecology*, **15**, 387–406.
- Swofford DL (2004) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Sinauer & Associates, Sunderland, Massachusetts.