

SHORT COMMUNICATIONS

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AN IMPROVED EXTRACTION METHOD TO INCREASE DNA YIELD FROM MOLTED FEATHERS

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Abstract. To assess the value of molted feathers as a noninvasive source of DNA for genetic studies of Northern Goshawks (*Accipiter gentilis*), we isolated and quantified DNA from molted feathers and compared yields across five feather types. We also compared PCR success across the same five feather types using five microsatellite genetic markers of varying size. In addition, we compared DNA yields from a commonly used extraction method versus one we modified to increase DNA yield. Results indicated molted feathers provided on average 24 ng μl^{-1} of DNA, which is a relatively high DNA yield compared to other noninvasive tissue sources. Tail feathers yielded significantly more DNA than primary, secondary, and smaller feathers, yet all feather sizes produced equally high rates of PCR success. Although our modified extraction method increased the time required for processing feathers, it resulted in significantly higher yields of DNA as compared to the unmodified protocol.

Keywords: *Accipiter gentilis*, DNA extraction, DNA yield, molted feathers, noninvasive genetic sampling, Northern Goshawk.

Un Método Mejorado para Incrementar la Cantidad de ADN Obtenido de Plumas Mudadas

Resumen. Con el objetivo de determinar el valor de las plumas mudadas como método no invasivo para obtener ADN en estudios de *Accipiter gentilis*, aislamos y cuantificamos ADN de plumas mudadas y comparamos la utilidad de cinco tipos de plumas. También comparamos la amplificación de ADN por PCR entre los cinco tipos de plumas usando como marcadores genéticos cinco micro-satélites de diferentes tamaños. Además, comparamos la cantidad de ADN obtenida con un método de extracción comúnmente usado con la incorporación de una modificación que proponemos para incrementar la concentración de ADN obtenida de las muestras. Nuestros resultados sugieren que

las plumas mudadas proveen en promedio 24 ng μl^{-1} de ADN, lo cual es relativamente alto comparado con el uso de otros tejidos como fuente de obtención no invasivos. Significativamente mayor cantidad de ADN fue extraído de las plumas de la cola que de las remeras primarias, secundarias y plumas más pequeñas. Sin embargo, todos los tipos de pluma resultaron en altos niveles de amplificación por PCR. A pesar de que el método de extracción que proponemos incrementa el tiempo necesario para procesar las plumas, resulta en un aumento significativo de la cantidad de ADN extraído, comparado con el método tradicional.

The use of noninvasive techniques to sample genetic material from natural populations has greatly increased over the past decade, especially as advancement of molecular methods facilitates the use of tissues collected noninvasively. Most studies employing these techniques have focused on sampling mammals such as bears (Schwartz et al. 2006), wolves (Hausknecht et al. 2007), primates (Morin et al. 2001, McGrew et al. 2004), and elephants (Eggert et al. 2003), which are often difficult to capture. DNA has been sampled from hair (Goossens et al. 1998), feces (Tikel et al. 1996), urine (Nota and Takenaka 1999, Hausknecht et al. 2007), fish scales (Lucentini et al. 2006), shed snake skin (Bricker et al. 1996, Eguchi and Eguchi 2000), egg shells (Schmaltz et al. 2006), and blood spots left on snow from injured or proestrus female wolves (Scandura 2005). Likewise, mammal bones from owl pellets (Taberlet and Fumagalli 1996) have been used to detect prey species, identify trends in abundance, and to determine phylogeography (Miller et al. 2006).

Feathers molted annually also provide DNA, and several studies have used them successfully to identify species (Rudnick et al. 2007), determine sex (Griffiths and Tiwari 1995), identify parentage and individual turnover at nests (Rudnick et al. 2005), estimate abundance in nonbreeding populations (Rudnick et al. 2008), and assess population genetic structure (Segelbacher et al. 2003), phylogeography (Bayard de Volo 2008), and interspecific phylogeny (Seki 2006). Molted feathers are potentially a good source of DNA because feather shafts provide a microenvironment in which DNA molecules are protected from degrading conditions (e.g., solar radiation, hydrolysis, repeated freezing

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and thawing, and microorganisms). As feathers grow, they are supplied with blood containing nucleated red blood cells, and although the blood supply stops when feather growth is complete, residual cells remain inside the feather shafts and as part of the superior umbilicus (Horvath et al. 2005). Skin cells on the outside of the feather shaft can also supply DNA, although these cells are less protected from DNA degradation.

Our goals were to determine how much DNA can be recovered from feathers, with the following objectives: (1) optimize a DNA extraction method for molted feathers, and (2) test the effects of feather type (size) on DNA yield and success of PCR amplification of genetic markers. Using noninvasive techniques for genetic studies of Northern Goshawks (*Accipiter gentilis*) is necessary because of the difficulty and expense associated with studying these territorial but secretive long-lived forest predators (Bayard de Volo et al. 2005, Bayard de Volo 2008).

METHODS

SAMPLE COLLECTION AND PRESERVATION

We sampled molted feathers from free-ranging goshawks from across the North Kaibab Plateau in northern Arizona. Molted feathers were collected weekly from active nest sites throughout the breeding season (May–August, 1991–2000) by searching under nest trees and other large trees, downed logs, and stumps within nest sites. All feathers at each nest site were collected during weekly visits, and when new nests were located, we used only feathers that were freshly molted, avoiding those that were dirty, dry, and brittle, as these were likely molted in years previous to collection. Feathers were stored in envelopes according to year and territory in temperate dry, dark storage.

MOLTED FEATHER DNA EXTRACTION

We extracted DNA from 100 molted feathers randomly selected from our feather archive (10 per year, 1991–2000). Feather samples included primary, secondary, tail, alular, and wing- and body-covert feathers. We reduced the risk of sample contamination from DNA extraction or PCR amplification by performing all feather extractions in a separate lab from where blood samples were processed. Additionally, amplified DNA never entered the room where feather DNA extractions took place, and we always used aerosol-resistant tips on pipettes, which were dedicated to extraction of DNA from feathers. We routinely wiped all surfaces with 10% bleach (sodium hypochlorite; Prince and Andrus 1992), and all scissors, blades, and forceps were sterilized by soaking in a 50% bleach solution and then rinsed in sterile double-distilled water (ddH₂O).

The entire calamus tip was separated from the rest of each feather and soaked in 70% ethanol (EtOH) for 30 min, rinsed once in ddH₂O, and soaked again in ddH₂O for another 30 min. We produced replicates of each feather by splitting the calamus tip down its entire length (from the tip to where feather vanes start), cutting the shaft horizontally into small pieces (~0.3 cm, placed individually in sterile Petri dishes), which were then evenly distributed between two tubes. We ensured that each tube had one of the two proximate calamus tip halves, as this part of the feather likely contains much of the DNA.

Standard DNA Extraction. We isolated DNA from one set of replicates (hereafter replicates-1; $n = 100$) using a standard digestion of feather material, followed by an ammonium acetate separation of proteins and a cold isopropanol precipitation of nucleic acids (hereafter standard extraction). Specifically, the standard extraction digestion mix contained: 600 μ l TNE (Tris-NaCl-EDTA,

pH 7.5), 60 μ l 1 M Tris-HCl (pH 8.0), 25 μ l Proteinase K (25 mg ml⁻¹), 10 μ l 25% SDS (sodium dodecyl sulfate), and 5 μ l 1 M DTT (dithiothreitol; Appendix B). We prepared the DTT just prior to use because of the reagent's tendency to quickly lose activity upon going into solution (M. Sorenson, Boston University, pers. comm.). Feathers were digested for 24 hr in a water bath at 55°C and pulse-vortexed twice during that period. Following digestion, feather keratin was precipitated away from DNA by adding 233 μ l of 7.5 M ammonium acetate to each extract, vortexing for 20 sec, placing in an ice bath for 5 min, then centrifuging at 16 000 rpm for 10 min. The resultant supernatant was transferred to new tubes containing 600 μ l 100% isopropanol, after which 1 μ l glycogen (DNA carrier) was added to each extract. Extracts were then mixed by inverting tubes 50 times and were then placed in a freezer (–20°C) for 1 hr. Extracts were then centrifuged at 16 000 rpm for 10 min to pellet DNA, after which the isopropanol was poured off. To wash the pellet, 600 μ l 70% EtOH was added to each tube, which was then centrifuged at 16 000 rpm for 1 min, after which the EtOH was poured off. Pelleted DNA was air dried and then resuspended in 20 μ l TE (10 mM Tris, pH 8.0 + 1 M EDTA, pH 8.0). All centrifugation steps were performed in a cold centrifuge (4°C).

Modifying a DNA extraction method. To modify the standard extraction protocol, we extended the digestion time from 24 hr to 5–7 days, increased freezer time for the isopropanol precipitation from 1 hr to 12 hr, and increased cold centrifugation from 10 min to 30 min (S. Talbot, U.S. Geologic Survey, pers. comm.). We also increased Proteinase K from 25 μ l to 45 μ l and DTT from 5 μ l to 30 μ l, quantities more similar to those in protocols cited by Glenn (1996). These protocol changes were applied to only a subset of remaining feather replicates (hereafter replicates-2; $n = 53$). We then determined whether these changes increased DNA yield.

Quantifying DNA Yield. Following extractions, we quantified DNA yield (ng μ l⁻¹) for all replicates using a fluorometer (DyNAQuant 200, Hoefer Inc., Holliston, Massachusetts) and a Fluorescent DNA Quantitation Kit (Hoechst 33258 Dye; Bio-Rad Laboratories, Inc., Hercules, California). Instrument accuracy was checked every fifth sample using a concentration standard (calf thymus DNA prepared at 100 ng μ l⁻¹). Although, the fluorometer reports yields <10 ng μ l⁻¹, the quantitation kit's lower limit of accuracy is 10 ng μ l⁻¹. Nevertheless, we recorded all quantities including those <10 ng μ l⁻¹.

Effects of feather type. We determined the effects of feather type (size) on DNA yield and PCR success using 100 feather replicates (replicates-1), all subjected to the same standard extraction method. Feather age (time in archive) ranged 2–11 years.

GENETIC MARKERS AND PCR

Previously, we assessed genetic diversity for Kaibab goshawks using five microsatellite loci (Bayard de Volo et al. 2005) and used the same five genetic markers in this study. To prevent amplification of contaminating DNA, amplification reactions from feathers were prepared in an enclosed box containing two germicidal ultraviolet (UV) light bulbs (240 nm spectrum, 15 watt, model #G15T8, UV Process Supply Inc., Chicago, Illinois) to degrade contaminating DNA on PCR racks, pipettes and tubes. We used the UV lights for 20–30 min prior to setting up reactions.

Microsatellite markers were amplified as described in Bayard de Volo et al. (2005) with the following exceptions: (1) we added an additional 3 mM Mg₂Cl₃ to the PCR buffer; (2) we used positive controls (blood-derived DNA) to determine whether nonamplification was due to procedural errors or degraded feather DNA; (3) the number of PCR cycles was increased to 40 for most microsatellite loci (56 cycles for locus AGE-4; Bayard de Volo et al. 2005).

STATISTICAL ANALYSIS

To determine the efficacy of the modified extraction method, we used ANOVA to test two simple linear models where variation in DNA yield was explained by year of feather collection, extraction method (standard vs. modified), and sample within year (treated as a random variable). Likewise, variation in the number of loci amplified was explained by the same set of variables. We tested both models using PROC GLM and PROC MIXED in SAS (2001).

To assess the effects of feather type, we also used ANOVA (PROC GLM; SAS 2001) to test two simple linear models examining the effect of feather type on DNA yield, and feather type and DNA yield on number of loci amplified while controlling for year in both models. Lastly, we used Student's *t*-tests (two-way) to test the null hypothesis of no difference among least square means for DNA yields from different feather types. All statistical tests were assessed using $P = 0.05$ as a threshold for significance, and *t*-test multiple comparison *P*-values were Bonferroni adjusted.

RESULTS

We determined that two feather samples were statistical outliers, and thus removed them from all analyses. One feather had dried blood in the calamus that resulted in each of its replicates yielding $>1500 \text{ ng } \mu\text{l}^{-1}$, and another sample was identified as originating from a Cooper's hawk (*A. cooperi*).

MODIFYING THE EXTRACTION METHOD

Modifying the standard extraction method resulted in significantly higher DNA yields ($F_{1,50} = 19.7$, $P < 0.0001$), where mean yields were $\bar{X} = 15.2 \text{ ng } \mu\text{l}^{-1}$ (range: 1–81) and $\bar{X} = 27.3 \text{ ng } \mu\text{l}^{-1}$ (range: 0–99) for standard and modified protocols respectively (Fig. 1). The number of loci amplified, however, was not affected by extraction method ($F_{1,49} = 1.25$, $P = 0.26$).

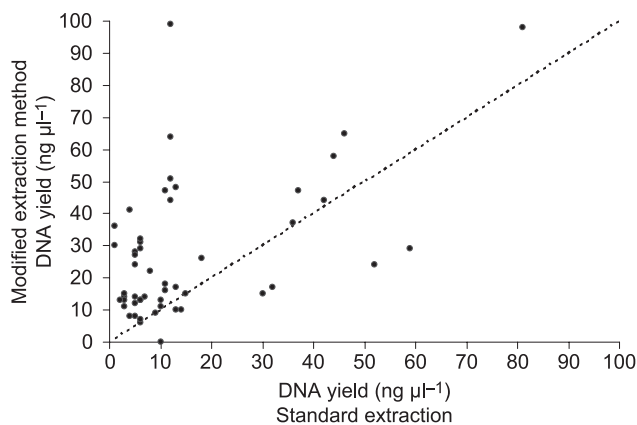


FIGURE 1. Pairwise relationship for replicates of feather samples undergoing either the standard or modified DNA extraction method. Points represent paired replicates ($n = 51$), and those falling on or near the 1:1 line indicate similar DNA yields. In some cases, multiple paired replicates had the same values and are therefore represented by a single point. Modifying the standard extraction method increased DNA yield, which is indicated by the number of points that lie above the line. Two replicates (feathers) were determined to be outliers and thus removed from analysis.

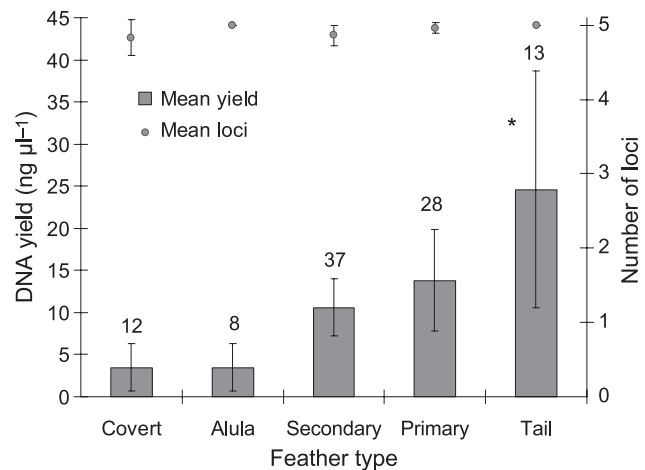


FIGURE 2. Means and 95% CI for DNA yield (bars) and success of microsatellite amplification (points) based on feather type (n appears above bars). DNA yields were for half-feather samples undergoing the standard extraction protocol. Feather type affected DNA yield, yet only remiges (tail feathers) yielded significantly more DNA ($P > 0.01$ for all pairwise comparisons). Feather type, however, did not greatly influence success of microsatellite amplification, where all but six feathers amplified all five loci. Two replicates (feathers) were determined to be outliers and thus removed from analysis.

EFFECTS OF FEATHER TYPE

Feather type significantly influenced DNA yield ($F_{4,84} = 6.7$, $P < 0.0001$), but neither DNA yield nor feather type influenced success of PCR amplification (Fig. 2). Interestingly, only tail feathers yielded significantly more DNA ($\bar{X} = 24.6 \text{ ng } \mu\text{l}^{-1}$, range: 1–81, $n = 13$) when compared to primary (13.8 ± 2.9 , $t_{40} = 2.4$, $P = 0.01$), secondary (10.6 ± 1.7 , $t_{49} = 3.2$, $P < 0.001$), alula (3.5 ± 1.2 , $t_{20} = 3.5$, $P = 0.001$), and covert (4.3 ± 1.1 , $t_{24} = 3.8$, $P < 0.001$) feathers (Fig. 2).

DISCUSSION

An important finding from this study was that DNA recovery from molted feathers was high relative to other noninvasive tissue sources. For example, Morin et al. (2001) reported fecal and hair yields from chimpanzees as $192 \text{ pg } \mu\text{l}^{-1}$ (range: 0–2550 $\text{pg } \mu\text{l}^{-1}$) and $38.4 \text{ pg } \mu\text{l}^{-1}$ (range: 0–228 $\text{pg } \mu\text{l}^{-1}$, single hair), respectively. DNA yields from free-floating feces of whales were 25 ng mg^{-1} dry weight, but only 1% of recovered the DNA was actually from whale (Gillett et al. 2008).

We also found that larger feathers (remiges and rectrices) provided higher yields of DNA relative to smaller feathers. Amplification success for DNA from smaller feathers, however, was just as high as that of larger feathers. Our results compare with one other study (Horvath et al. 2005) that measured DNA yields from feathers, but the authors did not report yields $<10 \text{ ng } \mu\text{l}^{-1}$, which precluded comparison with our results. Nevertheless, as was the case for our small feathers, all eight of their samples exhibited high amplification success despite low DNA yields. The high amplification success observed in this study was likely influenced by the fact that our sample was mostly comprised of feathers in good condition (i.e., recently molted without signs of degradation). Hogan et al. (2008) showed that feather condition significantly influenced amplification success of mtDNA and nuclear markers. Feather type, however, had varied effects on amplification success depending on the marker amplified and the condition of the feather (Hogan et al. 2008).

Another important finding was that we were able to improve DNA yields using several simple protocol changes. We found increasing digestion time and reagent concentrations facilitated increased breakdown of feather keratin. Likewise, increasing time in the freezer and for cold centrifugation helped increase precipitation of DNA molecules. Both steps increased processing time however, and compared with other commonly used methods (i.e., silica-filter methods and other extraction kits), ours takes longer to process samples. Worth noting is that our results are based on half-feather samples. Using the whole calamus tip, as well as the superior umbilicus (Horvath et al. 2005), will yield even more DNA, allowing final template volumes to be higher (30–50 μ l instead of the 20 μ l obtained in this study) while maintaining similar concentrations. However, for smaller feathers, which yielded on average <10 ng μ l⁻¹, using final template volumes of 20–30 μ l would allow for higher DNA concentrations. Appendices A and B provide details of the modified extraction protocol.

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APPENDIX A. Instructions for preparing molted feathers for DNA extraction, and a step-by-step detail of the modified DNA extraction protocol.

FEATHER PREPARATION

- Wipe all surfaces with 10% bleach. Run UV lights (if available) for 20 min. Heat water bath to 55°C.
 - Make sure there are plenty of sterile scissors, forceps, and plastic Petri dishes before you start. We have about 25 each sterile scissors and forceps ready for use to process ~20 samples at a time.
 - Use of nitrile gloves helps to reduce static electricity that causes difficulty in handling feather material.
1. Set out 1.5 ml centrifuge tubes.
 - a. Separate calamus tip from rest of feather and place in a tube. Make sure to cut above the superior umbilicus, so you can include it later.
 - b. Put feather back into envelope and write extraction date on envelope.
 2. Wash tip: fill tube with 70% Ethanol (EtOH) and soak for 30 min.
 3. Set out more 1.5 ml centrifuge tubes and fill with double-distilled water (ddH₂O); soak feathers for 30 min.
 4. Label 1.5 ml centrifuge tubes. These will be your digestion tubes, so label with sample numbers and date.
 - a. Use sterile Petri dish as a catching surface, and sterile scissors and sterile forceps for each sample.
 - b. Cut feather rachis longitudinally along its length. Stop before superior umbilicus.
 - c. Then cut horizontally ~5cm of the feather tip into 2–3 mm pieces and place into tube.
 - d. Cut out the superior umbilicus and place in tube. Cut it out as a small square around the “blood-dot.”
 - e. Pull out the papery material from inside the calamus and place in tube.

DNA EXTRACTION

1. Mix up digestion mix, using newly mixed DTT (Appendix B).
2. Pipette 700 µl digestion mix to each tube.
3. Incubate at 55°C until most of the material dissolves, overnight to one week, usually 3–4 days. In some cases, we add more Proteinase K (20 µl of 25 mg µl⁻¹) if material is not completely digested in two days.

Following digestion

4. Cool to room temperature.
5. Pipette 233 µl protein precipitation mix to each tube.
6. Pulse-vortex to mix.
7. Place in freezer (–20°C) for 30 min.
8. Centrifuge (4°C) at 13 000–16 000 rpm for 30 min.
 - a. Debris should be at the bottom of tube.
 - b. If some debris is still floating, recentrifuge for 10 min.
9. Label new 1.5 ml tubes; fill with 600 µl 100% isopropanol (DNA grade).
10. Pour supernatant into new tube (step 9) and leave debris behind.
11. Add 1 µl glycogen (DNA carrier) to each tube.
12. Mix samples by inverting tubes 50 times.
13. Place in freezer (–20°C) overnight.

Next day

14. Centrifuge (4°C) at 13 000–16 000 rpm for 30 min. Look for pellet at bottom of tube.
15. Pour off supernatant and drain tube on clean, absorbent paper.
16. Add 600 µl 70% EtOH (DNA grade).
17. Centrifuge (4°C) at 13 000–16 000 rpm for 2 min.
18. Carefully pour off EtOH.
19. Air-dry tubes to remove *all* excess EtOH. It may be necessary to leave overnight to dry.

When dried

20. Add 20–50 µl TE (Appendix B) to rehydrate pellet, flicking tube to mix. Can let rehydrate overnight before use, or warm (35°C) in a heating block to facilitate resuspension.
- Optional: quantify to establish DNA concentration. Typically, we use 1–2 µl for PCR.

APPENDIX B. Reagents and reagent mixes used in the modified DNA extraction protocol.

“Digestion Mix”: reagents used for feather digestion	
Reagents	Volume per extraction (µl)
1X TNE	600
1 M Tris-HCl	60
Proteinase K (20 mg ml ⁻¹)	45
25% SDS (weight/volume)	10
1 M DTT (newly mixed)	80
Total	795
Amount	
1X TNE	
100 mM NaCl	5.844 g
50 mM Tris	6.055 g
25 mM EDTA	9.306 g
Bring solution to pH 7.5 with HCl	
Bring to 1 L volume with ddH ₂ O	
Autoclave	
DTT	
Dithiothreitol	Add 1ml sterile H ₂ O to a tube containing a premeasured solid (0.1542 g) to make 1M solution
“Protein precipitation” reagent	
7.5 M Ammonium acetate	233
“Resuspending DNA pellet”	
TE	Volume per extraction (µl)
10 mM Tris pH 8.0	20–50
1 M EDTA pH 8.0	