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A comparison of sample types varying in invasiveness for use in DNA sex determination in an endangered population of greater Sage-Grouse (*Centrocercus uropihasianus*)

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Obtaining blood and muscle samples is often not feasible in genetic studies on endangered species, therefore researchers must adopt sampling techniques that are less invasive, but yield poorer quality DNA (Taberlet et al. 1999). Noninvasive sampling has increased in avian genetics over the last decade (Pearce et al. 1997; Segelbacher and Steinbrück 2001; Segelbacher 2002), but there has been no thorough comparison of the quality and quantity of DNA produced among sample types. Use of moderately invasive samples, such as mouth swabs (Poschadel and Möller 2004) and plucked feathers (Taberlet and Bouvet 1991), has also increased, but such samples have not been compared to other types.

The purpose of our study was to determine (1) whether DNA could be successfully extracted and amplified from a variety of noninvasive and moderately invasive sample types and (2) which sample types yield the best quality DNA for use in avian DNA sexing based on amplification of the chromo helicase DNA-binding (CHD) gene (Z fragment = 224 bp and W fragment = 252 bp). We evaluated three under utilized sample types (avian saliva, chick down, and predated eggshell membranes) along with common sample types for use in sex determination.

Greater Sage-Grouse (*Centrocercus urophasianus*) are endangered in Canada and the southern Alberta population has declined by 66–92% since the 1970s (Aldridge and Brigham 2003). We collected samples from the Alberta population from 1998–2004. Sample collection protocols and modifications to published DNA extraction protocols using Qiagen DNeasy® Tissue Kits and QIAamp® DNA Micro Kits (Qiagen, California, USA) are outlined in Table 1. Qiagen DNeasy® Tissue Kits were used for all samples with the exception of mouth swabs because low concentration DNA was produced. QIAamp® DNA Micro kits were used for mouth swabs and yielded high quality DNA (Table 1).

Samples were DNA sexed using the 1237L/1272H primer set and PCR cycling conditions from Kahn et al. (1998). Fifteen microlitre PCRs are described in Table 1. PCR products were separated on 3% agarose gels stained with ethidium bromide and visually scored. Low voltage (100 mV) was used for maximum separation and definition of sex-specific bands. All samples were sexed three times to ensure consistent results. Three separate PCRs (modified multiple-tubes approach; Segelbacher and Steinbrück (2001)) opposed to eight separate PCRs (multiple tubes approach; Taberlet et al. (1999)) were performed for each sample to avoid false typing of individuals with low DNA concentrations. Samples that did not have easily discernible sex-specific bands for all three tests were classified as unsuccessful. Negative controls in the form of tubes without samples were employed at

Table 1. Collection methods, DNA extraction, and PCR protocols for each sample type

Sample type	Sample collection method	DNA Extraction Kit[a]	Modification from published protocol	15 μl PCR
(1) Whole blood	Blood samples were taken from adults by clipping one toenail and dripping blood into a microcentrifuge tube	DNeasy® Tissue Kit	Modification to the whole nucleated blood protocol: (1) Step 3 – Add 190 μl of PBS to blood instead of 220 μl	7.96 μl millipore water, 1.0 μl 25 mM $MgCl_2$, 1.5 μl 10× Buffer[b], 1.2 μl 2 mM dNTP mix, 0.12 μl 20 mM forward primer, 0.12 μl 20 mM reverse primer, 0.1 μl 5 unit *Taq* polymerase, and 3 μl extracted DNA (PCR 1)
(2) Liver from dead birds	Liver samples were collected from carcasses	DNeasy® Tissue Kit	No modifications to the animal tissue protocol	(PCR 1)
(3) Autolytic or decomposing tissue	Abandoned unhatched eggs were collected and frozen at −20°C until embryos could be removed	DNeasy® Tissue Kit	Modification to the animal tissue protocol: (1) Step 2 – lyse finely chopped tissue for 24–36 h instead of 12–24 h at 50°C	(PCR 1)
(4) Mouth swabs	Q-Tip™ cotton swabs were rubbed along the sides of the oral cavity and the tongue to collect sloughed cells. Cotton tips were removed & placed in 2 ml microcentrifuge tubes and frozen at −20°C	QIAamp® DNA Micro Kit	Modification to the Omni Swab protocol: (1) Collect avian saliva sample with a cotton Q-Tip™ swab	(PCR 1)
(5) Plucked body contour feathers	Feathers were plucked from under the wing of each adult. Feathers were put in plastic bags with desiccant & stored at room temperature	DNeasy® Tissue Kit	Modifications to the animal tissue protocol: (1) Starting material =2–3 feather tips from plucked contour feathers	(PCR 2)
(6) Plucked chick down	Several abdominal feathers were removed. Feathers were put in plastic bags with desiccant & stored at room temperature	DNeasy® Tissue Kit	Modification to the tissue sample protocol: (1) Starting material =4–6 chick down feathers	(PCR 2)
(7) Hatched eggshell membrane	Shells and eggshell membranes were stored individually in plastic bags with desiccant at room temperature	DNeasy® Tissue Kit	Modifications to the animal tissue protocol: (1) Step 1 – use 400 μl of Buffer ATL instead of 180 μl; (2) Step 2 – use 40 μl of proteinase K instead of 20 μl	(PCR 1)
(8) Molted contour (body) feathers	Molted feathers were collected from leks (breeding grounds) and stored individually in plastic bags with desiccant & stored at room temperature	DNeasy® Tissue Kit	Modification to the tissue sample protocol: (1) Starting material = 1 feather tip from molted feathers	(PCR 2)

(9) Predated eggshell membrane	Each egg and/or egg fragment were stored individually in plastic bags with desiccant at room temperature	DNeasy® Tissue Kit	Modifications to the animal tissue protocol: (1) Step 1 – use 400 μl of Buffer ATL instead of 180 μl; (2) Step 2 – use 40 μl of proteinase K instead of 20 μl; (3) Lyse samples for 36–48 h instead of 12–24 h at 50°C	0.96 μl millipore water, 1.0 μl 25 mM $MgCl_2$, 1.5 μl 10× Buffer, 1.2 μl 2 mM dNTP mix, 0.12 μl 20 mM forward primer, 0.12 μl 20 mM reverse primer, 0.1 μl 5 unit *Taq* polymerase, and 10 μl extracted DNA (PCR 2)
(10) Molted flight feathers (wing) & tail feathers	Feathers were collected from leks (breeding grounds) and placed individually in plastic bags with desiccant & stored at room temperature	DNeasy® Tissue Kit	Modification to the tissue sample protocol: (1) Starting material = 1 feather tip from molted feathers	(PCR 2)

Modifications to DNA extraction protocols for each sample type using Qiagen DNeasy® Tissue Kits and QIAamp® DNA Micro Kits are listed. Full protocols including sample preparation can be found at: http://www.compusmart.ab.ca/kbush/DNAextraction.htm[a]Qiagen DNeasy® Tissue Kits were used for all sample types with the exception of mouth swabs. The DNeasy Tissue kit produced very low quality DNA from mouth swabs (10–15 ng/μl) so we used the QIAamp® DNA Micro kit and a slight modification to the Omni protocol to increase the concentration of the extracted DNA. The QIAamp® DNA Micro kit also works on all feathers samples and predated eggshell membranes and yields similar DNA concentrations to the DNeasy® Tissue Kit.[b]The 10× PCR buffer consisted of: 1 M KCl, 2 M Tris (pH 8.8), Triton X-100, Bovine Serum Albumin (BSA; 100 mg/ml), and millipore water.

Table 2. Success rate of DNA extracted from 10 avian sample types based on amplification of the CHD gene and DNA quantification

Sample type	Invasiveness category	DNA concentration (ng/μl)[a]	DNA extractions		
			Successful	Unsuccessful	Percent successfully sexed (%)
(1) Whole blood ($n = 216$)	Invasive/nondestructive	119	216	0	100
(2) Liver from dead birds ($n = 25$)	Noninvasive	98	25	0	100
(3) Autolytic or decomposing tissue ($n = 34$)	Noninvasive	57	34	0	100
(4) Mouth swabs ($n = 15$)	Moderately invasive/nondestructive	41	15	0	100
(5) Plucked contour (body) feathers ($n = 15$)	Moderately invasive/nondestructive	40	15	0	100
(6) Plucked chick down ($n = 74$)	Moderately invasive/nondestructive	12	71	3	95.9
(7) Hatched eggshell membrane ($n = 247$)	Noninvasive	39	236	11	95.5
(8) Molted body contour feathers ($n = 15$)	Noninvasive	13	9	6	60
(9) Predated eggshell membrane ($n = 439$)	Noninvasive	16	244	195	55.6
(10) Molted flight feathers (wing & tail feathers) ($n = 15$)	Noninvasive	0	0	15	0

[a]DNA concentration was calculated using the following formula: A_{260}nm×extinction coefficient×dilution factor (Sambrooke and Russell 2001). Since Qiagen uses an AE elution buffer, the extinction co-efficient for TE was used to estimate DNA concentration (44.6 μg/ml).

both the DNA extraction and PCR steps to detect contamination with exogenous DNA. DNA extractions were conducted in a separate room from post-PCR amplifications to minimize possible cross contamination and aerosol resistant filter tips were used at all steps.

DNA samples were quantified three times with UV absorbance at 260 nm using a 96-well SpectraMax 190® spectrophotometer plate reader and Softmax PRO® software.

All sample types yielded reproducible DNA sexing results with the exception of molted tail and flight feathers (Table 2). Plucked body contour feathers, mouth swabs, and autolytic tissue were highly reliable for correctly determining sex in samples of known sex and successful amplification of the CHD gene in samples of unknown sex (Table 2). Plucked chick down had the lowest DNA concentration, but had a 95.9% success rate (Table 2). Hatched eggshell membranes were a better source of DNA than predated eggshell membranes (Table 2). Molted contour feathers had a lower success rate (60%) for amplification of the CHD gene, but produced similar results to previous studies (Segelbacher 2002).

All samples of known sex ($n = 246$) were DNA sexed correctly and allelic dropout (allele amplification failure) was not observed. However, allelic dropout was observed in 25 eggshell membranes samples because of inconsistent amplification of the W-specific CHD band. These samples were excluded from further analysis and were considered unsuccessful.

We demonstrate that both noninvasive and moderately invasive samples provide useful alternatives to invasive sampling in sex determination studies of endangered avian populations. With further improvements to extraction methods, PCR protocols, and genotyping techniques, noninvasive samples may eventually produce consistently reliable results, eliminating the need for invasive sampling.

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