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A comparison of plucked feathers versus blood samples as DNA sources for molecular sexing

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ABSTRACT. Feathers are increasingly collected as a nondestructive source of DNA for avian genetic research. Although feather samples are not optimal in some important ways than more robust blood or tissue samples, feather sampling requires less training for field workers, results in shorter handling times for the organism, generates no hazardous wastes, and requires simpler storage procedures. Along with these largely positive attributes comes a set of challenges, particularly the relatively low copy number of DNA present in feather samples. We compared the utility and reliability of feathers to the more traditional blood samples as sources of DNA for polymerase chain reaction (PCR)-based molecular sexing of Black-capped Chickadees (*Poecile atricapilla*). DNA from 102 individuals was extracted separately from both single rectrices and from blood samples, and the sex of each bird was then determined using standard PCR-based methods. We found complete agreement between sex determinations based on feather versus blood DNA extractions. Slight variations in lab protocols were necessary to obtain consistent results from these two DNA sources; and we briefly discuss other sources of error that could occur in feather-based molecular sexing studies. This controlled comparison of feather versus blood samples demonstrates that plucked rectrices provide a highly reliable source of DNA for molecular sexing of wild birds.

SINOPSIS. **Una comparación entre el uso de plumas versus muestras de sangre como fuentes de ADN para estudios moleculares de determinación de sexo**

Se está incrementando la modalidad de usar plumas como una fuente no destructiva de ADN para llevar a cabo investigación genética en aves. Aunque las muestras tomadas de plumas, en algunas instancias no son óptimas que muestras más robustas como sangre y tejido, el uso de estas no requiere adiestramiento especial, toman menos tiempo en la manipulación del ave, no generan desperdicios peligrosos y requieren un almacenaje sencillo. Aunque hay atributos positivos en el uso de plumas, también existe el problema de obtener una muestra baja de ADN. Examinamos la utilidad y confiabilidad de muestras de plumas en comparación con técnicas más tradicionales como obtención de muestras de sangre como una fuente de ADN para el sexado de *Poecile atricapilla* utilizando PCR. A tales efectos se extrajo ADN, de rectrices, de 102 individuos y un número similar de muestras de sangre para determinar el sexo usando PCR. Encontramos resultados similares con ambos métodos. Sin embargo, fueron necesarios pequeñas variaciones en el protocolo de laboratorio para obtener resultados consistentes, de ambas fuentes de ADN. Discutimos algunas fuentes de error que pueden ocurrir cuando se utilizan plumas para la determinación del sexo utilizando técnicas moleculares. Este estudio demuestra que las rectrices son una fuente confiable para obtener buenas muestras de ADN y poder determinar el sexo en aves silvestres.

Key words: DNA, feather, molecular sexing, *Poecile atricapilla*, sex ratio

Recent reviews have highlighted the potentially broad utility of feathers as sources of DNA for molecular marker-based studies of avian behavior, ecology, and evolution (Taberlet and Bouvet 1991, Smith et al. 2003). With the now-common use of lab methods based on the polymerase chain reaction (PCR), DNA contained in feathers can be amplified and assayed using a variety of informative molecular techniques; and feather samples have been used successfully in molecular studies of sex ratios (Questiau et al. 2000), phylogeographic variation (Kimura et al. 2002), and paternity (Questiau et al. 1999). Along with avian feces (Idaghdour et al. 2003), nest materials (Pearce et al. 1996), and museum specimens (Mundy et al. 1997), feathers from live birds provide a simple and nonlethal means of obtaining genetic material when more destructive sampling would conflict with research goals.

Molecular sexing is one of the simplest and most widely used DNA-based assays applied to wild birds. To date, the most common sampling method for obtaining genetic material for sexing has involved acquiring blood samples during banding or from nestlings, because blood is relatively easy to obtain from captured individuals,

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and because the nucleated erythrocytes of birds make avian blood an unusually rich source of nuclear DNA. Feather samples have been used less often for molecular sexing, but they too represent an appealing tissue type, because plucking a single feather is one of the least invasive means of acquiring a genetic sample. This may be particularly important when permission to collect blood or tissue samples is difficult to obtain. For example, the present comparison is part of a larger study of chickadee overwinter survival that involves many nonprofessional citizen scientists, and we have found that participating landowners are much more likely to approve feather sampling than blood sampling from the birds visiting their backyard feeding stations. When compared to blood sampling, feather sampling requires less training for banders, results in shorter handling times (and hence less stress on the birds), generates no hazardous waste (e.g., sharps and exposure to blood), and the storage requirements for samples are simpler and less expensive.

As with other nondestructive sampling methods, feathers also present challenges as sources of DNA. First, feather samples are generally not associated with traditional museum-specimen vouchers, an important caveat particularly for studies with a phylogenetic component (Smith et al. 2003). Lethal vouchering, however, is often not feasible in studies addressing topics such as sex ratios or parentage when it is necessary to minimize disturbance to the focal population. Second, DNA in feather samples is typically present in much lower copy number than DNA from blood or tissue samples, and DNA from feathers may also be somewhat degraded if it originated from cells that died as the feather matured (such as pulp cells within the rachis or feather clots within the shaft; Leeton et al. 1993, Horvath et al. 2005). DNA yields from extractions of feather material are therefore correspondingly low and, in some cases, DNA derived from feathers has been reported to be a less reliable source of PCR templates than DNA from richer tissue types (Segelbacher 2002, Jensen et al. 2003). However, there have apparently been no controlled tests of PCR reliability that directly compare feather- and blood-derived DNA extracts.

The protocol now employed to sex birds in many studies targets an intron in the sex-linked CHD gene. In most bird species, the W- and Z-chromosome copies of this intron differ in length, rendering it straightforward to determine sex based on the presence of PCR products of one (in males) or two (in females) lengths (Griffiths et al. 1998). Here, we evaluate the reliability of using DNA obtained from feathers for the PCR-based molecular sexing of chickadees. By comparing molecular sex determinations from feather DNA directly with the results of molecular sexing tests from blood samples taken from the same individuals, we test whether feather DNA has a higher PCR failure rate, or a higher scoring error rate, compared to blood samples that typically give a 100% PCR success rate.

METHODS

Sample collection. Using mist-nets, we captured Black-capped Chickadees (*Poecile atricapilla*) at five feeding stations within 20 km of Ithaca, New York, USA, between 29 September and 6 December 2004. This species is sexually monomorphic (Pyle 1997), and so we were unable to determine the sex of individuals in the field. From each individual, we collected approximately 80 μl (equivalent to 1–2 drops) of blood in heparinized capillary tubes via brachial venipuncture with a sterile 27-gauge hypodermic needle. Blood samples were immediately transferred to tubes containing 0.5 ml of blood lysis buffer (100 mM Tris–HCl, pH 8; 100 mM Na_2 EDTA, 10 mM NaCl, 0.5% SDS; White and Densmore 1992). Buffer-preserved blood samples were kept at ambient temperatures for up to several days before being returned to the lab and stored at 4°C. Feather samples were obtained from the same individuals by pulling one outer rectrix from each bird. Feather samples were then kept at room temperature in individual glassine envelopes.

Laboratory methods. DNA extractions and PCR amplifications were conducted in a laboratory where pre- and post-PCRs are spatially separated, and assembled using dedicated pre-PCR equipment and aerosol-resistant barrier pipette tips. DNA was extracted from the blood samples using a Perfect gDNA Blood mini kit (Eppendorf, Westbury, NY, USA) with slight modifications of the manufacturer's protocol. Feather DNA was extracted from pulp cells inside the outer rachis using a DNeasy kit (QIAGEN Inc., Valencia, CA, USA) following the manufacturer's protocols, and taking

precautions to prevent cross-contamination (Ellegren 1992).

We amplified diagnostic sex-linked alleles using the P8/P2 primer set (Griffiths et al. 1998) that has previously been used successfully to sex Black-capped Chickadees (Ramsay et al. 2003) and other avian taxa. Amplification volumes totaled 10 μl and included either 1–10 ng (feathers) or 20–100 ng (blood) genomic DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.3 μM each primer, 200 μM dNTPs (Invitrogen Corp., Carlsbad, CA, USA), and 0.25 U of Jumpstart™ Taq DNA polymerase (Sigma-Aldrich Corp., St. Louis, MO, USA). Reactions were denatured for 1 min at 94°C followed by 30 (blood) or 40 (feathers and blood) cycles of 94°C denaturing for 30 s, 48°C annealing for 45 s, and 72°C extension for 45 s. A final 5-min extension at 72°C completed the PCR profile. We visualized 10 μl of each PCR sample on a 2% agarose gel containing 0.025 μg ethidium bromide per ml. Gels were run in tris-acetate-EDTA buffer (Sambrook and Russel 2001) at 80–96 V for 50–70 min. A 5-μg aliquot of a 1-Kb Plus™ DNA ladder (Invitrogen Corp., Carlsbad, CA, USA) was run with each row of samples as an unambiguous size standard. We photographed gels under UV light using a Gel Logic 100 Imaging System with 1D Image Analysis Software (version 3.6; Eastman Kodak Co., Rochester, NY, USA) and assigned sex by counting the number of visible bands in each lane and estimating fragment lengths relative to the adjacent DNA size standards. Blood and feather samples were run independently, and determination of sex for each set of reactions was conducted blind to the determinations made using the alternative DNA source.

DNA sequencing and allele identification. In a small number of feather and blood samples, an additional band was visible after electrophoresis. This band was slightly shorter than the consistently amplified products from the W- and Z-linked CHD alleles, and hence in these individuals males had two PCR bands and females three bands. The co-amplification of this seemingly spurious product in a sexing test that relies on the number of PCR bands is a concern. Attempts to eliminate the extra band by optimizing annealing temperatures and reagent concentrations were unsuccessful. To determine whether the extra band resulted from nonspecific amplification or from (for example) a duplication of the CHD locus, we gel-purified and sequenced all three bands from several female chickadees. Bands were excised from agarose gels and purified using a MinElute gel extraction kit (QIAGEN Inc., Valencia, CA, USA). These samples were sequenced using the amplification primers, following standard protocols (e.g., Lovette 2004). Locus identity was determined by comparing the resulting sequences to the GenBank database using BLAST searches.

Quantification of DNA yields. One feather and one blood sample from each of 19 individuals was tested to quantify DNA yield. Extraction product was diluted with water 1:50 or 1:100 (blood) and 1:1 or not at all (feathers). These diluted samples were then analyzed for absorbance at 260 and 280 nm using a SmartSpec 3000 Spectrophotometer with quartz micro spectrophotometer cell (Bio-Rad Labs., Hercules, CA, USA), and concentrations were calculated. A two-sample unequal variances *t*-test was performed on the results to determine the difference. Means are presented as ±1 SD.

RESULTS

We extracted DNA from both feather and blood samples from 102 Black-capped Chickadees of unknown sex. As described above, preliminary tests showed that both types of samples provided adequate DNA templates for the molecular sexing reactions, but that more PCR cycles were needed to achieve reliable results from the feather extracts. This difference is almost certainly due to the lower concentration of initial template in the lower-yield feather extracts.

As expected, the shorter Z-chromosome PCR band was clearly visible in all feather- and blood-derived reactions, and the presence/absence of the female-specific W-chromosome was also readily scored by visual inspection in all samples. Feather- versus blood-based sex determinations were consistent for all 102 individuals. Based on these sexing reactions, our sample included 49 female and 53 male chickadees.

Further results of some preliminary PCR tests are noteworthy, because they emphasize the importance of appropriate reaction optimization. PCR annealing temperatures greater than 53°C for females and 58°C for males yielded no product from the Z-chromosome locus. In

these cases, samples from females would display only a single band (from the W-chromosome) that could result in them being scored as males, whereas samples from males would display no band. We also noted an additional band that appeared in 15 feather-based and 3 blood-based reactions. Although the band was clearly of a different size than either of the target alleles, the presence of this spurious band could have caused these males to be scored as females in the absence of comparisons against true females. Sequences of the Z (283 nucleotides, not including primers) and W (340 nucleotides) fragments were similar to previously reported avian CHD loci. The shorter spurious band of 233 nucleotides was not homologous to any sequences in the GenBank database, suggesting that it resulted from co-amplification from an unknown locus unrelated to CHD. Representative sequences from each of these three loci (Z, W, and unidentified) have been archived in GenBank (accession numbers DQ068390, DQ068391, and DQ068392, respectively).

Optical spectrophotometry results indicated that DNA concentrations were more than an order of magnitude higher in blood (30.95 ± 18.79 μg/ml, $N = 19$) than in feathers (1.16 ± 0.72 μg/ml, $N = 19$), a significant difference in concentration ($t = 6.91$, $P < 0.0001$).

DISCUSSION

A potential concern with feather-based DNA sampling is that the small number of cells present on or within the feather rachis could result in inadequate DNA yields for molecular analysis (Taberlet et al. 1999). Our study provides evidence that feathers can provide sufficient DNA for molecular sexing reactions. Our direct comparison of feather- versus blood-derived DNA showed complete consistency in determining the sex of more than one hundred chickadees, suggesting that feathers are not associated with substantially higher PCR failure or scoring error rates.

Previous studies have shown that blood sampling does not alter the subsequent behavior or survival of free-living or captive birds (Stangel 1986, Hoysak and Weatherhead 1991). We know of no experimental tests of the fitness consequences of feather sampling, but we suspect that the effect sizes (if any) of the typical feather sampling protocols are equivalently low, suggesting that any such study would require very large sample sizes.

Blood samples unquestionably yield a greater amount of DNA than feathers, an advantage both for molecular sexing and for archiving this more concentrated and abundant product for other uses. The decision to sample blood versus feathers in a particular study should involve an informed assessment of the tradeoffs between these methods. Feather sampling provides lower DNA yields, but is faster and easier than blood sampling, and the reduced handling time may reduce stress on the birds during capture. Feather sampling may be a particularly attractive alternative at migration monitoring stations where high capture rates may preclude the additional handling time required to obtain blood samples. Feathers may also have other uses, such as age determination based on rectrix shape (Pyle 1997) or for analyses of isotopes and trace elements (Smith et al. 2003).

Recommendations. Because molecular sexing is often undertaken by field workers who have not previously conducted DNA-based analyses, we make the following general recommendations to new users of these techniques. Careful design of field sampling and sample labeling protocols is important.

As in all cases where samples are taken from wild birds, the collection, transport, and storage of feather samples are typically tightly regulated by federal, state, and local permits as applicable within a given country, and compliance with these regulations is essential. When possible, we recommend collecting two feathers from each sampled individual, as these replicates allow repeated, independent DNA extractions for later error checking, or allow for the use of DNA for multiple purposes (such as both molecular sexing and microsatellite genotyping).

Feather samples are best preserved frozen over the long term. We suggest placing feathers in individual glassine envelopes (labeled with collecting date, location, and band or other sample number using indelible ink) and sealing these envelopes in a plastic freezer bag to prevent icing. Samples should be archived in a non-frost-free freezer, as standard frost-free freezers cycle above freezing to remove ice, and this temperature cycling leads to DNA degradation.

PCR-based sexing is one of the simplest and most reliable molecular marker assays applied to birds, but nonetheless, laboratory protocols often require optimization for particular species.

Sexing primers sometimes fail to provide strong PCR amplifications in particular taxa, or the resulting bands may be so similar in size that they are difficult to resolve without specialized equipment for high-resolution electrophoresis. In such cases, we note that there are several alternative pairs of PCR primers that have been used broadly to sex wild birds (e.g., Griffiths et al. 1998, Kahn et al. 1998, Fridolfsson and Ellegren 1999). Preliminary tests using these alternative primer sets on a panel of known sex individuals is a strategic way to begin a molecular sexing project on a new taxon. All sets of sexing PCRs should include negative controls (with no template DNA added) to help identify contamination. Situations in which the female-specific, W-chromosome PCR band is consistently weaker than the Z-chromosome band require special scrutiny, as in this case, the weaker amplification of the diagnostic band could result in females being scored as males.

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