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Comments by Eric Wong on the manuscript as well as on interpretation of the data are gratefully acknowledged. The NHGRI and USDA-SCD programs provided support for our work. Address correspondence to Dr. Edward J. Smith, 3130 Litton Reaves Hall, Virginia Tech, Blacksburg, VA 24061, USA. e-mail: esmith@vt.edu

Received 29 August 2000; accepted 2 January 2001.

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Small Bones from Dried Mammal Museum Specimens as a Reliable Source of DNA

BioTechniques 30:732-736 (April 2001)

Although many museums now routinely archive animal tissue for molecular systematic studies, the vast majority of museum specimens predate the advent of molecular techniques and consist of dried skins, skeletons, and formalin-fixed, ethanol-preserved tissues. The difficulties of obtaining usable DNA

from such specimens are well known (1,3,4,6,8), yet such specimens form a potentially invaluable source of data, especially for populations of animals that are extinct or locally extirpated. Engstrom et al. (3) pointed to the fact that bone may be a better source of DNA, particularly to obtain long fragments of DNA. However, the amount of material suggested by the authors from voucher specimens for PCR may be on the range of gram-quantities. During our studies of mitochondrial variation of fruit bats of the genus *Sturnira*, we were unable to amplify cytochrome *b* from standard phenol-chloroform extractions of museum specimens. Consequently, we refined extraction protocols using only milligram-quantities that have routinely yielded amplifiable DNA from up to 66-year-old museum skins, bones, and formalin-fixed, ethanol-preserved tissues from bats of the genus *Sturnira*. Our techniques are novel improvements of existing DNeasy® Tissue Extraction protocols (Qiagen, Valencia, CA, USA), apply to very tiny bones, and could potentially be used with other mammals and small vertebrates. These modifications are (i) repeated washing and rehydration of tissues; (ii) prolonged (up to 72 h) digestion and repeated additions of proteinase K; (iii) checking and adjusting the pH of the extracted DNA solution before adsorption on the Qiagen column; (iv) reducing the elution volume; and (v) amplification of short overlapping segments utilizing internal primers.

Samples consisted of museum specimens of *Sturnira*, frugivorous bats averaging about 20 g. Samples from dried specimens included bones (ribs 5–9 mm in length or 2–3 mm of the diaphysis of a wing bone) or 3 × 2 mm of dry skin with hair. Formalin-fixed, ethanol-preserved tissue samples included 3 × 2 mm pieces of liver or skin with muscle or 1–2 ribs. Liquid-preserved tissue was chopped into small (< 0.5 mm) pieces; dry bone was crushed within a folded piece of a 10 × 10 cm weighing paper (Fisher Scientific, Pittsburgh, PA, USA) with a pair of needle-nose pliers. Chopped or crushed samples were placed in 1.5-mL tubes and washed 3–5 times in 250 µL PBS (9) for 10 min at 55°C with occasional vortex mixing. The sample was spun briefly,

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Table 1. Comparison of Different Tissue Sources and Preservation Methods on Successful PCR Amplification from Museum Specimens of Bats

	Tissue Source	<i>n</i>	Successful PCR Amplifications	% Success
wet	liver	14	6	43
	bone	4	4	100
	skin	15	11	73
	wet subtotal	33	21	63
dry	bone	13	12	92
	skin	11	7	63
	dry subtotal	24	19	79
	overall total	57	40	70

"Wet" = formalin-fixed tissue stored in 70% ethanol.

and the wash solution was decanted. We assumed that the repeated washes rehydrate the tissues and might remove PCR inhibitors and residual fixatives.

Digestion and extraction was performed with the DNeasy tissue extrac-

tion kit (Qiagen), with modifications. These modifications consisted of (i) prolonged (up to 72 h) digestion with proteinase K; (ii) checking and adjusting the pH of the extracted DNA solution before adsorption on the Qiagen

column; and (iii) reducing the elution volume. To each tissue sample, 180 μ L buffer ATL (tissue lysis buffer; Qiagen) and 20 μ L proteinase K (20 mg/mL; Qiagen) were added; the sample was incubated at 55°C with occasional vortex mixing or gentle shaking. Digestion continued for 24–72 h until the tissues were completely lysed. We considered the lysis of the sample complete when no more solid materials (including bone) were observable with the naked eye. During the digestion, fresh 20- μ L aliquots of proteinase K were added every 12 h until lysis was complete. When digestion was complete, 200 μ L buffer AL (lysis buffer; Qiagen) were added, the mixture was vortex mixed, and incubated at 70°C for 10 min. Two hundred microliters of 100% ethanol were added, the sample was vortex mixed, and the pH was checked using color **pHast**[®] indicator strips (EM Science, Gibbstown, NJ, USA). The pH was adjusted to 7.0–6.5 with 0.25 M HCl; a pH greater than 7.0 will prevent adsorption of DNA onto the silica membrane of the Qiagen column. The pH-adjusted sample was passed through the Qiagen column; the column was washed with 500 μ L buffer AW1 (wash 1 buffer; Qiagen), 500 μ L buffer AW2 (wash 2 buffer; Qiagen), and then spun dry. DNA was eluted twice from the column in 40–50 μ L buffer AE (elution buffer; Qiagen) (10 mM Tris, pH 8.0); column and buffer were incubated for 5 min at 65°C before centrifugation to increase the yield of DNA from the membrane. Aliquots of the resulting total DNA were usually too weak to be visualized on an agarose gel.

We sequenced a portion of cytochrome *b* (approximately 850 bp total length) by amplifying four overlapping regions of approximately 250 bp each. The outermost primers were those of Pääbo et al. (7) and Edwards et al. (2), called here Cytb8F and Cytb7R, respectively. Some highly degraded templates could not be amplified in one piece; consequently, internal primer pairs were designed from consensus sequences of *Sturnira* obtained from fresh tissue samples of six different species. Those primers were named bat1F (5'-AGC-CACCGCATTCAACTCHG-3'), bat1R (5'-CCGTAGTTTACATCTCGGCAR-3'), bat2F (5'-TTCGCCGTCATAGCC-

Figure 1. Ease of amplification of cytochrome *b* from an 8-year-old bat museum specimen (wet material) and from a 19-year-old bat (dry material). Each sample was amplified using a forward primer (cyb 8) and one of four reverse primers, each yielding a product of successively greater length. Reverse primers and approximate length of product (when combined with forward primer cyb 8) are: 1R (279 bp); 2R (494 bp); 3R (698 bp); and cyb 7 (879 bp). Variables examined are tissue source, duration of rehydration (washed vs. unwashed), and duration of digestion (3 h vs. 72 h). The best amplifications and longest PCR products were obtained from dry bones digested for 72 h. Washing and rehydration before extraction appeared to have little or no effect. Amplifications from skin samples were inferior to those obtained from bone. Results from dry skin are not shown; results are similar to wet skin. Positive control template is bat DNA from saturated EDTA-DMSO saline (SED) buffer-preserved fresh kidney.

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ACA-3'), bat2R (5'-TG GTGATGAC-TGTTGCTCCYC-3'), bat3F (5'-CCT-MCTTCCCTTTATCGTAG-3'), and bat3R (5'-CYGGGTCTGATGGGAT-YCC-3'). These primers produced four overlapping products (each approximately 200 bases) that covered almost the entire cytochrome *b* region. Hot-start reactions (50 μ L) contained 5 μ L 10 $\times$ PCR buffer (Sigma, St. Louis, MO, USA) (without $MgCl_2$, 100 mM Tris-HCl, pH 8.3, 500 mM KCl), 7 μ L 25 mM $MgCl_2$, 1 μ L 10 mM dNTPs, 1 μ L each primer (10 pmol), 15 μ L of a saturated aqueous betaine solution, 15 μ L water, 5 μ L template, and 1 U *Taq* DNA polymerase (Sigma). The thermocycler program consisted of an initial denaturation at 93°C for 3 min, followed by 39 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, ending with a final elongation at 72°C for 3 min. Products were visualized on a 1% agarose gel stained with ethidium bromide and cleaned using QIAquick™ columns (Qiagen). Products were cycle sequenced using BigDye™ terminator mixture (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. Both strands were sequenced to assure accuracy in base calling. Controls consisted of template-free reactions (negative) and high-quality *Sturnira* DNA from fresh tissues (positive).

The overlapping sequences were assembled and analyzed cladistically with other *Sturnira* sequences. All sequences obtained from old museum specimens (up to 66 years old) fell into expected clades and aligned well with sequences from DNA isolated from fresh tissue samples. There was no clear relationship between the age of the sample and the amplification success.

Consistently, all attempts of extractions on wet or old dry material with standard phenol-chloroform methodology (5) were unsuccessful. Our modified Qiagen extraction protocol yielded amplifiable DNA on 70% of the samples (Table 1). To investigate the relative quality of template DNA from various tissue sources, we tried primer pairs of increasing successive length on each template and compared the results (Figure 1). Contrary to our expectations, small rib bones from dried skeletons and liquid-preserved samples proved to be the best source of DNA.

Among dry samples, small ear pieces (with skin, hairs, and muscles), skin, and bones provided DNA with an overall yield of 63%. Formalin-fixed, ethanol-preserved pieces produced DNA on 44% (liver) or 73% (skin) of the cases. As noted by Su et al. (10), "the success of DNA recovery depends largely on the original condition of the specimen". Many specimens "may not contain recoverable DNA due to overtreatment [or undertreatment] for antiseptic purposes or overdegradation [before or] in storage" (10).

In summary, old dried skins, skeletons, and formalin-fixed, ethanol-preserved mammal specimens represent a potentially valuable source of data. By applying these modifications (repeated washing and rehydration of tissues; repeated additions of proteinase K to facilitate complete digestion; amplification of short overlapping segments utilizing internal primers; and reduced elution volume), we obtained discrete amounts of amplifiable DNA from bats of the genus *Sturnira*. Although our study was limited to bats, we expect that these modified techniques should be applicable to the majority of vertebrate species. Our finding that small bones, only a few mm in length (less than 0.001 g), are an excellent source of relatively undegraded DNA increases the value of museum specimens to systematic biologists.

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We are grateful to the many curators that trusted their specimens to us for DNA extraction. Sequencing was done at the DNA Sequencing Core at the University of Florida. We thank Ginger Clark for critically reading the manuscript. This publication represents Florida Museum of Natural History Contributions to Mammalogy Number 2. Address correspondence to Carlos Alberto Iudica, Mammalogy, Florida Museum of Natural History, Dickinson Hall, P.O. Box 117800, University of Florida, Gainesville, FL 32611-7800, USA. e-mail: casaiud@flmnh.ufl.edu

Received 24 February 2000; accepted 27 November 2000.

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