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PERSPECTIVE

Extraction of nuclear DNA from bone of skeletonized and fluid-preserved museum specimens

Abstract Obtaining DNA sequences, particularly nuclear DNA, from museum specimens is challenging. We sequenced nuclear DNA from small bone fragments of skeletonized and fluid-fixed museum specimens of squamate reptiles by using a forensic protocol developed for isolating DNA from human bones. The method yielded high quality nuclear DNA sequences from bones taken from 11 of 21 (52.4%) skeletonized or desiccated specimens, the oldest of which dated back to 1938, and 1 of 9 (11.1%) fluid-preserved specimens, which was collected in 1957.

Key words DNA extraction, bone, formalin, museum specimens, nuclear DNA

Scientists have been collecting and preserving animals for centuries and this record of biodiversity is currently held in natural history museums around the world. Most of the preserved vertebrates in natural history museum collections do not have associated tissue samples available for DNA study because they were collected prior to the molecular revolution in systematic biology during the late 20th century, or the collectors chose not to preserve tissue samples when the voucher specimens were prepared. Molecular systematists have been attempting to recover usable DNA from preserved museum specimens, particularly for evolutionary studies on extinct or rare species (Cooper, 1994; Parham *et al*., 2004), on individuals from extinct or politically inaccessible populations (Wirgin *et al*., 1997; Barnes *et al*., 2002), or to document changes in genetic diversity over time (Bouzat *et al*., 1998; Pergams *et al*., 2003). Consequently, a number of protocols have been developed and used in recent years for extracting and amplifying DNA from formalin-fixed museum specimens, skeletonized museum specimens, and field-collected bone samples (e.g. Taberlet & Fumagalli, 1996; Shedlock *et al*., 1997; Wirgin *et al*., 1997; Chatigny, 2000; Iudica *et al*., 2001; Austin & Arnold, 2002; Barnes *et al*., 2002; Fang *et al*., 2002; Lambert *et al*., 2002; Schander & Halanych, 2003; Austin *et al*., 2004; Rohland *et al*., 2004; Wisely *et al*., 2004). Most protocols for extracting and amplifying DNA from ancient, degraded, or formalin-fixed samples have been used to obtain mitochondrial rather than nuclear DNA, probably because mitochondrial DNA occurs in higher copy number in the cell and is more likely to be retrieved (Hofreiter *et al*., 2001; Huynen *et al*., 2003; Isenberg, 2005). However, some workers may want to obtain nuclear rather than mitochondrial DNA sequences from ancient, degraded, or formalin-fixed samples owing to a need for an additional data set that is independent of the mitochondrial genome, an interest in paternal inheritance (Tosi *et al*., 2000; Scribner *et al*., 2001), problems with mitochondrial pseudogenes (Zhang & Hewitt, 1996) or introgression (Wilson & Bernatchez, 1998), or a need for molecular markers that are phylogenetically informative at deep divergences (Graybeal, 1994).

The largely separate scientific discipline of forensic molecular genetics has a related interest in obtaining DNA evidence from trace and degraded samples of body fluids, hair, bones, and teeth (e.g. Hochmeister *et al*., 1991; Fisher *et al*., 1993; Hoss & Paabo, 1993; Cattaneo *et al*., 1997; Prado *et al*., 1997). While the forensic and molecular systematic applications may differ once the DNA has been extracted, the primary goal of obtaining high quality DNA with greater yields is shared by both scientific groups.

We were presented with the challenge of obtaining nuclear DNA sequences from museum specimens of amphisbaenians ("worm-lizards"), a poorly known group of small, fossorial, squamate reptiles for which few fresh tissues are available. We obtained high quality nuclear DNA sequences from very small bone fragments of skeletonized and fluid-fixed museum specimens of squamate reptiles by modifying a simple molecular forensic protocol that was developed for recovering DNA from human bones (Isenberg, 2005). The sequences were used to obtain the first molecular based phylogeny for

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Taxon	Source	Tissue type	Weight (g)	Year collected	Sequencing success
Agamodon anguliceps	CG 2440	Dry bone; 7 ribs	–	1962	*c-mos* and RAG-1
Amphisbaena alba	CG 1216	Dry bone; 2 vertebrae, 3 ribs	–	1958	*c-mos* and RAG-1
Amphisbaena alba	FMNH 17803	Wet bone; 1 vertebrae	0.42	1932	No
Ancylocranium ionidesi	CG 1129	Dry bone; 5 vertebrae + associated ribs	–	1959	No
Anops kingii	CG 2776	Dry bone; 6 vertebrae + associated ribs	–	1963	*c-mos* and RAG-1
Ancylocranium sp.	CG 1131	Dry bone	–	1959	No
Aulura anomala	CG 2766	Dry bone; 3 vertebrae, 6 ribs	–	unknown	*c-mos* only; RAG-1 failed
Baikia africana	BM 1964.253	Desiccated specimen	–	1964 or earlier	No
Chirindia swynnertoni	CG 4037	Dry bone; ribs	–	1969	*c-mos* and RAG-1
Chirindia swynnertoni	CG 4032	Desiccated specimen	–	1969	No
Cynisca leucura	CG 3759	Dry bone; 3 vertebrae + associated ribs	–	1969	*c-mos* and RAG-1
Cynisca sp.	FMNH 224277	Wet bone	–	unknown	No
Dalophia ellenbergeri	FMNH 134554	Wet bone; vertebrae	0.02	unknown	No
Dalophia ellenbergeri	FMNH 142694	Wet bone	–	unknown	No
Dalophia longicauda	CG 5318	Dry bone	–	unknown	No
Dalophia pistillum	CG 13528	Dry bone; 2 vertebrae + associated ribs	–	unknown	No
Leposternon microcephalum	CG no number	Dry bone; vertebrae + associated ribs	–	unknown	No
Leposternon sp.	CG no number	Dry bone; 3 vertebrae, 6 ribs	–	unknown	*c-mos* and RAG-1
Loveridgea ionidesi	FMNH 129609	Wet bone	–	1958	No
Monopeltis capensis	FMNH 80030	Wet bone; 3 vertebrae	0.19	1957	*c-mos* and RAG-1
Rhineura floridana	FMNH 43394	Wet bone; 4 vertebrae	0.05	1939	No
Rhineura floridana	FMNH 211863	Wet bone	–	1980	No
Zygaspis quadrifrons	FMNH 82549	Wet bone	–	1958	No
Zygaspis quadrifrons	CG 4829	Dry bone; vertebrae and ribs	–	1969	No
Tupinambis teguixin	FMNH 22393	Dry bleached bone	0.15	1939	No
Hemidactylus garnotii	FMNH 206754	Dry bone; 7 ribs	–	1976	No
Mabuya multifasciata	FMNH 229939	Dry bone; 3 ribs	–	unknown	No
Iguana iguana	FMNH 211878	Dry bone	0.12	prior to 1971	*c-mos* only; RAG-1 not attempted
Iguana iguana	FMNH 22476	Dry bone	0.12	1938	*c-mos* only; RAG-1 not attempted
Acrochordus granulatus	FMNH 221398	Dry bone; 3 ribs	–	unknown	*c-mos* only; RAG-1 not attempted

Table 1 Bone samples of squamate reptiles extracted using the protocol reported in the text, and results of attempts to sequence fragments of the nuclear *c-mos* and RAG-1 genes. Dry bone was removed from skeletonized museum specimens. Wet bone was removed from fluid-preserved museum specimens stored in 70% ethanol but probably initially fixed in formalin. FMNH refers to catalogued specimens, and CG and BM to uncatalogued specimens, at the Field Museum of Natural History, Chicago. Sequencing attempts were considered successful only if high quality, complete fragments of *c-mos* (357 bp) and RAG-1 (459 bp) were obtained.

worm-lizards (Kearney & Stuart, 2004). Here we summarize our successes and failures using this protocol on museum specimens of squamate reptiles of differing types and ages.

The preservation histories of the squamate specimens that we used are incompletely known. Fluid-preserved museum specimens were whole specimens stored in 70% ethanol, but these were probably initially fixed in formalin (for at least one day, but often many years) based on standard collecting practices during the mid-20th century and the hardened condition of the specimens. Skeletonized and desiccated specimens were probably initially treated as wet specimens, but skeletonized specimens were later purposely prepared as skeletons and desiccated specimens were ones that had accidentally dried out. To minimize the loss of morphological information, we used

either vertebrae or ribs because there are numerous samples of each per individual. Bones dissected from fluid-preserved specimens had some soft tissue attached, and this was included with the bone in the extraction process. Bone may act as a barrier against autolytic, oxidative, and hydrolytic damage of DNA, including in museum specimens that have been fluid-preserved (Cooper, 1994), and this may explain some of our success with bone.

The extraction and amplification protocols are reported in detail in Kearney & Stuart (2004). Briefly, precaution was taken against contamination by using UV-sterilized supplies inside a Purifier PCR Enclosure (Labconco) in a separate room from where fresh squamate tissues were extracted. Bones were washed in GTE Buffer to bind any residual formalin (Shedlock *et al*., 1997), decalcified in EDTA, and extracted for several days in TNES Buffer (which contained both SDS and DTT) and daily additions of a large quantity (300 μg) of proteinase-K. DNA was purified using silica-based columns provided in the Dneasy Tissue Kit (Qiagen). The amplification protocol utilized bovine serum albumin (BSA; New England BioLabs) to prevent polymerase chain reaction (PCR) inhibitors, a relatively large amount of DNA template (4 μl DNA template per 25 μl PCR reaction) to overcome low extraction yield, the high-quality Taq polymerase AmpliTaq Gold (Roche), and extra cycles (40 total) of the PCR reaction.

Two fragments of 185 and 267 bp of the nuclear oocyte maturation factor Mos (*c-mos*) and three fragments of 218, 192–225 and 249 bp of the nuclear recombination activating protein 1 (RAG-1) gene were amplified using primers reported in Kearney & Stuart (2004). The fragments overlapped in variable regions by 22–70 bp after primer sequences were trimmed to avoid generating chimeric sequences during analysis (Olson & Hassanin, 2003), yielding a total alignment of 357 bp of *c-mos* and 459 bp of RAG-1. Sequences were compared with those of other squamates generated in our laboratory to verify authenticity, and submitted to GenBank (accession numbers AY444009-AY444030, AY444035, AY569648-AY569650, AY444036-AY444056, AY444061-AY444062).

The electropherograms of the nuclear DNA sequences had strong signal with very little or no background noise. High quality nuclear DNA sequences were obtained from bones sampled from 11 of 21 (52.4%) skeletonized or desiccated specimens, and 1 of 9 (11.1%) fluid-preserved specimens. Sequences were obtained from skeletonized specimens collected since 1938 and from a fluid-preserved specimen collected in 1957 (Table 1). We believe it is likely that a higher number of successes would have been achieved if we had attempted to amplify and sequence mitochondrial DNA rather than nuclear DNA, owing to the characteristics of these genomes, but this remains to be tested.

Although we did not exhaustively test alternative methods, we were able to obtain enough nuclear DNA to perform a phylogenetic analysis of worm-lizards with sufficient taxonomic sampling (Kearney & Stuart, 2004). We hope this extension of a recent forensic science protocol for extraction of DNA from skeletal remains to museum specimens will contribute toward improved methods for obtaining DNA from the large collection of organic diversity housed in the world's natural history museums.

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