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SHORT COMMUNICATION

Mitochondrial DNA extraction and sequencing of formalin-fixed archival snake tissue

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Abstract
Formalinized specimens used in mitochondrial DNA (mtDNA) studies have shown shortcomings with respect to efficacy of DNA isolation and subsequent PCR amplification. In order to pinpoint the source of some of the problems with formalinized tissues in reptiles, we compared the efficacy of isolation and amplification of mtDNA from two different snake species *Micrurus fulvius* and *Lampropeltis triangulum*—that differ in composition of tissues typically sampled (intercostal muscle tissue) by researchers performing mtDNA analyses in snakes and other reptiles. This study shows clearly that the more muscle-fiber rich *L. triangulum* tissues yield higher quality mtDNA that is easier to manipulate with PCR than the myocyte depauperate *Micrurus* tissues. We suggest that curatorial practice in making available formalin preserved specimens in reptiles should focus on tissue type, most appropriately on muscle-rich tissues. We reiterate previous caution about minimizing amplicon size and maximizing controls for contamination when working with formalin-preserved reptile tissues.

Keywords: *Mitochondrial DNA, Micrurus fulvius, Lampropeltis triangulum, Formalin*

Introduction

With many organisms, large numbers of specimens that can yield high-quality DNA for research are simply unavailable. Formalin-fixed tissues found in museum collections represent a potentially bountiful source of specimens. However, practice and the literature suggest that formalin-preserved specimens pose a serious methodological challenge and may not be useful at all for DNA sequence analysis. A recent meeting convened at the National Academy of Sciences discussed in detail these problems with formalin-preserved specimens in the context of DNA barcoding (Tang 2006).

DNA isolation from most formalinized tissues is problematic because formalin cross-links DNA and cellular proteins, and unbuffered formalin solutions may also degrade DNA (Gilbert et al. 2007). Such effects of formalin can impede the PCR and can either cause failure in the PCR or reduce the available sequence length of amplified products. Several methods have been used to extract formalin-fixed DNA, with varying degrees of success (Schander and Halanych 2003; Tang 2006; Gilbert et al. 2007). The National Academy of Sciences study recommended a more detailed examination of the factors involved in success and failure of DNA isolation procedures.

Consequently, we report here the impact of formalin preservation on tissue type in two species of snake—*Micrurus fulvius* and *Lampropeltis triangulum elapsoides*. These two species have different behavioral ecologies and anatomies. *Micrurus* belongs to the family Elapidae, and relies on highly toxic venom to subdue its prey and protect itself. *Lampropeltis* is a member of the Colubridae and is a powerful constrictor. Intercostal musculature is employed by *Lampropeltis* to kill its prey. On the other hand, *Micrurus* neither utilizes these muscles for predation

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nor for rapid motion. Differential muscular development should entail differential mitochondrial development and abundance, and perhaps result in differing degrees of DNA isolation efficacy in formalin-preserved specimens.

The current study used substantial numbers of organisms from sympatric populations of these snakes.

Materials and methods

Specimens

Out of a total of 138 specimens of *L. triangulum elapsoides* and 112 specimens of *M. fulvius* that were sampled for this study, 95 *Lampropeltis* and 81 *Micrurus* were obtained on loan or as tissue samples from various institutions and listed as formalin preserved (see Table I). Specimens were obtained in the field and from individual and institutional sources. Table I provides a further breakdown of sources of formalin-fixed tissues for each species. Institutional samples consisted of integumentary or organ tissue samples or loans of intact specimens, all of which are currently preserved in ethanol. Approximately 5 cm sections of intercostal muscle tissue were excised from each of the intact specimens. All tissue samples were stored in 95% ethanol at 4°C.

DNA extraction

The present study examines the efficacy of a method derived from Shedlock et al. (1997) (devised by Cathy Dayton of the US Fish and Wildlife Service: www.dmacc.edu/instructors/cmeckerman/formalin/dayton_protocol.doc). The Dayton and Shedlock approaches are members of the family of "soak methods" that rely on prolonged soaking in buffers, reportedly to disassociate formalin from tissues (Eckerman and Walsh 1997).

Following the Dayton protocol, 20 mg tissue was blotted dry and cross-linked in a Stratolinker (Stratagene Cloning Systems, La Jolla, CA, USA) for 15 s, after which the samples were suspended in 10 ml glycine–Tris–ethylenediamine tetraacetic acid buffer, consisting of 100 mM glycine, 10 mM Tris–HCl (pH 8.0) and 1 mM ethylenediamine tetraacetic acid. The buffer solution was prepared in 1000 ml aliquots. Tissue samples were soaked in the glycine–Tris–ethylenediamine tetraacetic acid buffer solution for a total of 72 h, during which time the solution was entirely replaced three times, after which there was no further agitation. The supernatant was then drained and the tissue was dried at 55°C until all of the visible liquid had evaporated, and was then cut into smaller pieces with a sterile razor blade and placed in 1.5 ml microcentrifuge tubes. Subsequent tissue processing employed the Gentra (now QIAGEN) Puregene Tissue Kit (QIAGEN, Valencia, CA, USA), which is essentially an alcohol/salt extraction method employing glycogen to coalesce the precipitated DNA. All DNA extracts were stored at −4°C.

Polymerase chain reaction

The nicotinamide adenine dinucleotide dehydrogenase subunit 4 (ND4) mitochondrial gene region was chosen for the phylogenetic component of this study because it reliably reflects evolutionary history (Russo et al. 1996) and it rapidly evolves, making it suitable for resolving relationships at the population-species boundary (Cracraft and Helm-Bychowski 1991). In particular, an 891 bp fragment of mitochondrial DNA (mtDNA) was used, including a 697 bp portion of the 3′ end of the ND4 gene, and a 194 bp section of three transfer ribonucleic acid (tRNA) genes (tRNA-His, tRNASer, tRNALeu) using primers known as ND4 (CACCTATGACTACCAAAAGCTCATGTAGAAGC) and Leu (TGGTGCAAATCCAAGTAAAAGTAATG) (Rodriguez-Robles and De Jesus-Escobar 1999). These primers have a proven track record with all squamates and, in fact, were initially designed for *Xenopus laevis*, so they were employed for amplification in both *Lampropeltis* and *Micrurus*.

Table I. Sources of formalin-fixed tissues.

Source	*Lampropeltis triangulum*	*Micrurus fulvius*	Tissue storage
Field collection	12	1	Cryo, buffer
Individual donation	14	15	Desiccation, EtOH, buffer
Institution			
Auburn University Museum	19	20	Formalin, EtOH
California Academy of Science	2	4	SED buffer, EtOH
Field Museum of Natural History	9	8	Formalin, EtOH
University of Georgia Museum of Natural History	10	5	Formalin, EtOH
Illinois Natural History Survey	11	4	Formalin, EtOH
Harvard Museum of Comparative Zoology	7	12	Formalin, EtOH
University of Florida	8	9	SED buffer, EtOH
University of Michigan Museum of Zoology	11	5	Formalin, EtOH
Smithsonian Institution	20	23	Formalin, EtOH
Carnegie Museum of Natural History	19	5	Formalin, EtOH
Total	142	111	

Table II. Primers used for amplification of formalin-fixed tissues.

Primer designation	Nucleotide	G–C%	T_m (°C)	Annealing temperature (°C)
F8_ND4_MDF	GCAGGCTCCATAGTACTAGC	55.0	54.6	50.4
R215_ND4_MDF	ATTGCGGCAATTACTARGCC	47.5	54.7	
F140_ND4_MDF	AACCTGCCTCCAACAAACG	52.6	61.1	56.0
R308_ND4_MDF	GGCTAAACAGAATAGTGCAGAGG	47.8	59.5	
F239_ND4_MDF	AAGTTTATCAGGAGCCATAGCC	45.5	58.8	55.0
R395_ND4_MDF	CCACCAAGTTGTGAGTATTGG	47.6	58.0	
F342_ND4_MDF	ATTGCCCACGGATTTACTTC	45.0	58.9	56.6
R476_ND4_MDF	CATCAGTTGAATAATGAGGATGC	39.1	58.6	
F361_ND4_MDF	CCTTACACGAGGATTCCACAA	47.6	60.0	55.0
R519_ND4_MDF	TGCTGTAATGAGTATTGATAGTCCAA	34.6	59.2	

In addition, due to the degraded nature of the DNA found in formalin-fixed tissues, sets of primers were designed bracketing overlapping 150–250 bp segments within a 561 bp section of the ND4–tRNA gene region, using the software package AMPLIFY 3.1 (http://www.engels.genetics.wisc.edu/amplify). These primer sets are presented in Table II. Designations refer to the starting position of the primer on a *Pituophis melanoleucus* (Genbank accession number AF141117) reference sequence. The same primer pairs were optimized and used for both *M. fulvius* and *L. triangulum*.

During all phases of the experiments reported on in this paper, we enforced common laboratory practices to either prevent or detect contamination. These practices include invalidation of an experiment if negative (water) controls showed positive PCR products, invalidation of an experiment where positive controls did not show amplification, and standard PCR approaches to avoid contamination. From those valid PCR reactions that were sequenced, we also rejected any sequences that when BLASTed to the NCBI database gave hits to species other than *L. triangulum elapsoides* or *M. fulvius*.

As a first step, optimum PCR annealing temperatures were determined for each primer pair. An Eppendorff Mastercycler ep (Eppendorf North America, Westbury, NY, USA) gradient thermocycler was used for this purpose. A ±0.5°C gradient was applied to the melting temperature (T_m) for each primer pair across six samples of each of six specimens (non-formalin-fixed) that had previously been successfully amplified. Optimum annealing temperatures are also listed for each primer pair in Table II. A second preliminary step was to test the primer pairs with formalin-fixed specimens. For this purpose, the AMNH Herpetology Collection provided five specimens of *L. triangulum triangulum* (accession numbers AMNH 91963, 16427, 93665, 3730, and 62832) with which three primer pairs were initially tested.

All PCRs were carried out with Eppendorff gradient thermocyclers. PuReTaq Ready-To-Go PCR beads (Amersham Biosciences, Amersham, UK) were used to amplify all formalin-fixed samples. The beads were placed in 21 μl water, to which 1 μl each of the primers were added, together with 2 μl template. The PCR cycle was ramped up at 96°C for 2 min, followed by 35–40 cycles of denaturation at 94°C for 30 s, annealing at the appropriate temperature for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 7 min, after which the reaction was held at 4°C. To give a very rough idea of the relative success of amplification with the selected primer pairs, electrophoresis of the PCR product yielded 32 bands out of 113 samples for primer pair 8/215, 31 bands out of 174 samples for 140/308, 43 bands out of 174 samples for 239/395, 99 bands out of 133 samples for 342/476, and 116 bands out of 168 samples for 361/519.

Sequencing

The PCR product was initially purified using the ethanol–isopropanol method. In later runs, a Biomek FXP Laboratory Automation Workstation robot (Beckman-Coulter, Inc., Fullerton, CA, USA) was used for PCR product purification. Cycle sequencing was carried out on the Eppendorff thermocycler, with a 94°C ramp-up for 5 min, followed by 40 cycles of melting at 94°C for 15 s, annealing at 50°C for 15 s and elongation at 60°C for 4 min. The reaction mixture consisted of 1 μl extender buffer, 1.0 μl primer, 0.5 μl Big Dye (Applied Biosystems, Inc., Foster City, CA, USA) and from 1 to 5 μl cleaned PCR product, depending on the intensity of the band it generated. The 5 μl reaction mixture was completed with distilled water. Cycle sequencing clean-up was carried out using the alcohol precipitation method (Lehn 2005). Sequencing was performed on an Applied Biosystems 3730/3730x DNA Analyzer.

Data analysis

Sequences were initially aligned and edited using SEQUENCHER 4.6 for Mac (Gene Codes Corporation, Ann Arbor, MI, USA). Additional alignment and editing was carried out with MacClade 4.08 for OSX (Sinauer Associates, Sunderland, MA, USA).

Results

Out of approximately 2000 sequencing reactions from all tissue sources, successful amplification from 97 *Lampropeltis* and 27 *Micrurus* sequences were obtained. Another 64 *Micrurus* sequences were obtained from formalin-fixed tissues, but, when submitted to BLAST searches, these latter *Micrurus* sequences aligned to other snake species, including *Lampropeltis triangulum*. These positive *Micrurus* amplifications were deemed the product of contamination. Table III presents the number and percentage of successful reactions by PCR primer pair or ND4 gene fragment. The *L. triangulum* DNA specimens, with success rates varying from 13 to 74%, clearly are more efficient at producing positive PCR products for all possible PCR fragments than the *Micrurus* samples, whose success rates ranged from 0 to 12%. Table IV presents the number and percentage of successfully sequenced *Micrurus* and *Lampropeltis* specimens by institution. There is no apparent relationship between institution and success rate.

One of the PCR fragments showed an unusually low success rate for both species (fragment 140–308). Given that this primer pair should produce an intermediate-length product, the length of the product most probably is not the cause of the low success rate of this primer pair. The low success rate of this primer pair may be the result of the primers not being as efficient as others we compared. The other four fragments were amplified at a relatively high rate for the *L. triangulum* DNA samples and at low rates for most of the *Micrurus* DNA samples. One primer pair, in particular, performed slightly better for *Micrurus* than the other primer pairs for this species (fragment 342–476). This PCR fragment is the smallest expected product at 136 bp and suggests that design of PCR primers to produce smaller fragments might allow for more of the *Micrurus* DNA samples to be successfully amplified. The success rate of the *L. triangulum* DNA samples is about 20-fold higher than in the *Micrurus* samples.

Table III. Successfully sequenced DNA fragments from formalin-fixed tissues.

	Number of sequences	
DNA fragment	*Lampropeltis triangulum*	*Micrurus fulvius*
8–215	47	1
140–308	12	0
239–395	50	2
342–476	70	10
361–519	63	0
Total	242	13

Discussion

We suggest that the great disparity between successful amplifications obtained for formalin-preserved *Micrurus* and *Lampropeltis* specimens is most probably due to the inherent differences in tissue makeup of the musculature of these two species. When dissecting out the intercostal muscle tissue from the two species, the tissue in the venomous *M. fulvius* was noticeably more diffuse than the denser musculature apparent in the constrictor *L. triangulum*. The implication is that the denser muscle tissue of the constricting colubrid may contain a greater number (or virtual number) of mitochondria—and more mtDNA—than the more diffuse tissue in the intercostal region of the venomous elapid. Although there have apparently been no relevant comparative studies on these organisms, the amount of both mitochondria and mtDNA is known to vary between cell types and within cell types at varying metabolic levels (Kazakova and Markosian 1966; Behrens and Himms-Hagen 1977; Robin and Wong 1988).

We can rule out primer specificity as a source of the disparity of amplification quality. Attempts were made to design *Micrurus*-specific primers, but these were discarded when they failed to produce amplification or sequences of reasonable length and quality PCR products. On the other hand, the ND4 primers easily amplified (non-formalin-fixed) *Micrurus* template DNA, although the proportion of sequences amplified was somewhat lower (data

Table IV. Successfully sequenced formalin-fixed specimens by institution of origin.

	Lampropeltis triangulum			*Micrurus fulvius*		
Institution	Total	Sequenced	Success rate (%)	Total	Sequenced	Success rate (%)
Auburn University Museum	19	14	74	20	1	5
Field Museum of Natural History	9	7	78	8	1	13
University of Georgia Museum of Natural History	10	6	60	5	1	20
Illinois Natural History Survey	4	0	0	4	0	0
Harvard Museum of Comparative Zoology	7	6	86	12	3	25
University of Michigan Museum of Zoology	9	4	44	4	1	25
Smithsonian Institution	20	10	50	23	1	4
Carnegie Museum of Natural History	19	14	74	5	0	0
Totals	95	61	64	81	8	10

not shown). We can also rule out differential post-formalin treatment as a factor in the disparity of success of amplification of the two species in this study, because specimens were shipped together in alcohol-saturated gauze and, in some cases, stored together in 90% ethanol.

In summary, the Dayton method appears capable of producing robust but differential results with formalin-fixed tissues. We show that careful consideration should be given to the type of tissue to be sampled in light of the biology of the organisms when formalin-preserved tissues are used. In the current study, tissue from dense muscle tissue preserved in formalin performed almost 20 times better than tissues from muscle depauperate tissues. We also reiterate recommendations with respect to formalin-preserved specimens made in previous studies: the amplification of short sequences is recommended for situations in which DNA degradation is likely, as is the case for formalin-fixed, archival tissues; and serious precautions must be taken to avoid contamination of samples at every level of processing, as is true with any archival tissue, including formalin-preserved tissues.

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