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New Data from Old Specimens

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Museums provide a wealth of scientific information via preserved natural history specimens, including but not limited to dietary, morphological, and geographic distributions of organisms. In the modern molecular age, however, fluid-preserved museum collections have not always been at the frontline for generating useable data, despite the fact that for some species, only museum specimens are known, with no fresh genetic materials available. We are now at a major shift in our ability to use museum specimens for molecular phylogenetics, where modern subgenomic sequencing techniques better allow for successfully sequencing hundreds to thousands of phylogenetically informative loci for historical specimens, including formalin- and fluid-preserved amphibians and reptiles. Here, I review the current state of the field, with respect to studies which have successfully generated high-throughput molecular datasets using fluid-preserved specimens for herpetofauna, particularly for systematic studies. Although only six publications fitting the search criteria were found, these studies provide a wealth of knowledge on the uses of museum herpetological specimens for modern work and illustrate just how important historical specimens are for enhancing our current understanding of species genetic structure, phylogenetic placements, and for disentangling taxonomic conundrums. In an age where both museums and general collecting come under critique from the general public, this review emphasizes the continued importance of museum specimens across all subfields in the study of amphibians and reptiles.

THE advent of the polymerase chain reaction in the 1980s revolutionized the field of systematics, allowing for the collection of hundreds or even thousands of informative characters in the form of nucleotides. Compared to morphological datasets, these DNA sequence datasets were relatively easy and fast to generate; starting in the late 20th and into the 21st century, molecular phylogenetics went from novel to a standard data type for tree-building, and the subfield of phylogeography was simultaneously born (Avise et al., 1987; Avise, 1998, 2000, 2009; Hickerson et al., 2010). With this change in methods, a parallel shift in scientific collecting occurred, where tissues appropriate for future DNA extraction, often liver, muscle, or blood, were frequently taken prior to fixation of the specimen for museum collections. However, before the common use of DNA for phylogenetic work, entire specimens were fixed in chemicals that were generally unconducive to DNA sequencing, making them largely unsuitable for broad or large-scale molecular work. In particular, the fluid-fixation methods for amphibians and reptiles starting in the late 1800s are still used today and rely on formalin-fixation and ethanol fluid-preservation (Simmons, 2014). These methods make extracting DNA in sufficient quantities and qualities for DNA sequencing and phylogenetic analysis challenging and sometimes impossible, though not from a lack of effort.

As early as the 1990s and continuing to the present, attempts have been made to extract phylogenetically useable DNA from non-human, formalin-preserved specimens (e.g., fishes; Shiozawa et al., 1992; Shedlock et al., 1997; Wirgin et al., 1997), dried museum skins and toepads (e.g., mammals; Rowe et al., 2011; birds; Töpfer et al., 2011), and even including ancient and extinct animals (e.g., moas; Cooper et al., 2001; subfossil tortoises; Austin et al., 2002; cave bears; Rohland et al., 2010). In 2000, a DNA extraction protocol specifically aimed at formalin-fixed, fluid-preserved amphibians and reptiles was published (Chatigny, 2000), subsequently followed by a general review of methods for extraction of formalin-fixed tissues (Schander and Halanych, 2003). Multiple studies, especially those focused primarily on the amplification of mitochondrial genes, but some, including nuclear loci, had some level of success with fluid-preserved specimens, resulting in historical DNA sequences from snakes (Grazziotin et al., 2006; Friedman and DeSalle, 2008; Green et al., 2010), lizards (Austin et al., 2004; Austin and Melville, 2006; Stuart et al., 2006; Cornetti et al., 2018), turtles (Stuart and Fritz, 2008; Garofalo et al., 2011; Fritz et al., 2014), frogs (Austin and Melville, 2006; Chambers and Hebert, 2016), and salamanders (Yuan et al., 2018).

The aforementioned studies clearly show that obtaining viable DNA from fluid-preserved and formalin-fixed specimens is possible using approaches that included standard DNA extractions (either via silica spin-column kits or phenol-chloroform based extractions) and PCR, often via "primer-walking." However, these studies had somewhat mixed success (e.g., Chakraborty et al., 2006; Stuart et al., 2006), with a general commonality of short sequence lengths (<200 bp) and often limited to mitochondrial genes (e.g., Shedlock et al., 1997). In addition, the finer details in some of these studies are lacking with respect to which or how many of the targeted tissues were from formalinized specimens, the range of sequence lengths recovered among specimens, whether extractions were repeated multiple times, and other miscellaneous but important details, making it sometimes unclear just how successful the methods were in generating sequences that were ultimately useful for tree inference particularly in studies that did not include a phylogeny.

The advances of broadly accessible molecular technology since the late 2000s has resulted in researchers exploring modified DNA-extraction protocols (e.g., Gilbert et al., 2007; Hykin et al., 2015; McCormack et al., 2016; Yuan et al., 2018), using high-sensitivity methods to determine more

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precisely the quantity of DNA in resulting extracts (e.g., real-time PCR, Qubit® high-sensitivity assay kits) and harnessing high-throughput sequencing (HTS) options for degraded or ancient DNA (e.g., Carpenter et al., 2013; Staats et al., 2013; Hykin et al., 2015; Ruane and Austin, 2017). Combined, these methods are potentially less time consuming and more conducive to generating large numbers of loci that are useful in a phylogenetic framework when compared to earlier protocols that relied on Sanger sequencing. Subsequently, we have recently seen an increase in the number of studies using HTS methods for genomic or subgenomic sampling from formalin-fixed and/or fluid-preserved specimens. Here, I focus on and review the use of fluid-preserved museum amphibians and reptiles in high-throughput sequencing applications with respect to systematic inference over the past ten years.

MATERIALS AND METHODS

I performed a Google Scholar search in June of 2019, with the search set to return results from 2009–2019, using the following search terms: "molecular phylogenetics AND fluid-preserved" and "amphibian OR reptile OR frog OR toad OR snake OR lizard OR crocodile OR alligator OR turtle" and results were sorted by relevance. In order to gather the most papers possible using HTS for fluid-preserved reptile and amphibian specimens, an additional search was performed, examining all of the papers citing Hykin et al. (2015) and Ruane and Austin (2017), as both of these papers have been frequently used as references in recent studies where extraction of DNA from historical specimens is performed, especially for amphibians and reptiles.

RESULTS

The Google Scholar search returned 126 entries. Many of these papers did not sequence historical specimens for the publication, were not focused on amphibians or reptiles, or both. For the Hykin et al. (2015) paper, 43 citation entries were available, and for Ruane and Austin (2017), 47 citations entries were available; many of these overlapped. In total, six publications using fluid-preserved squamate specimens with HTS were identified, including Hykin et al. (2015) and Ruane and Austin (2017). These six studies are discussed in detail below. No amphibian studies were found, although molecular studies using fluid-preserved amphibians with Sanger sequencing are known, as cited above. It is worth noting that additional publications were found that used extraction protocols similar to the HTS papers to generate DNA sequences from fluid-preserved or historical specimens (e.g., Cornetti et al., 2018; Yuan et al., 2018). These studies were not using HTS, so are not discussed further.

DISCUSSION

Despite the demonstrated successes using fluid-preserved specimens in modern phylogenomic work, the application of these methods is still in its infancy. Although few in number, the studies thus far have greatly underscored the importance of herpetological museum specimens in phylogenomic work, as the many of the species sequenced have been the only known specimen for the species, focus on extinct animals, and have supported previously posited hypotheses based on morphology as well as novel but robust estimations for taxonomic placements for herpetofauna. The first herpetological paper to make use of HTS and fluid-preserved specimens is only from 2015 (Hykin et al., 2015), although the methods making this possible had been available since the earlier 2000s.

Hykin et al. (2015) is the pivotal herpetological study to use minimally destructive sampling of historical museum specimens for modern phylogenomics. Their goal was to determine if a whole genome shotgun sequencing approach could generate useable nucleotide sequences from preserved specimens of *Anolis carolinensis* (Green Anole) that could subsequently be included in a phylogeny with modern sequences of *Anolis*. The authors tried several protocol variations to determine the best practices for this type of work with respect to tissue type (liver vs. leg muscle vs. tail tip), age of the specimen (~100 years old vs. 30 years old), and type of extraction (phenol-chloroform vs. silica-based spin column kit). Their results indicated that the highest DNA quantities came from liver tissue using the phenol-chloroform protocol from the 30-year-old specimen. No useable sequence was ultimately obtained from the 100-year-old specimen. To determine the practical utility of their methods, the authors aligned the resulting HTS reads of the 30-year-old specimen to an *Anolis carolinensis* reference genome; although the entire mitogenome was present for the sample, they used only the mtDNA locus ND2, due to its availability from earlier work on *Anolis*, to build a phylogeny including multiple specimens of *A. carolinensis* as well as additional species of *Anolis*. The formalin-fixed specimen was accurately placed within the *A. carolinensis* clade as closely related to other samples from the same region, illustrating the phylogenetic informativeness of the 30-year-old formalinized ND2 sequence.

The historical specimen work on snakes from Ruane and Austin (2017) is a direct result of the suggestion in Hykin et al. (2015) that target-capture may be a highly suitable method for degraded, formalinized samples. Ruane and Austin first identified 21 snakes from museum collections (primarily the California Academy of Sciences and the Museum of Comparative Zoology at Harvard University), representing rare or unavailable species for modern tissue samples and, at the time, not previously included in any molecular phylogenies. These snakes included species known only as museum specimens and holotypes, and comprised a mixture of snakes preserved and stored in ethanol, as well as individuals that were formalin-fixed prior to ethanol preservation. Using a modified extraction protocol with the Qiagen® DNAeasy blood and tissue kit, based largely on longer incubation times, large amounts of liver tissue, and hotter temperatures for certain aspects of the standard protocol, Ruane and Austin extracted quantifiable DNA from more than half of the snakes as measured using a high-sensitivity Qubit® flourometer assay kit. They successfully sequenced 100s–1000s of ultraconserved elements (UCEs) using a target capture approach for ten of the snakes in total, albeit with significantly shorter average locus lengths when compared to UCEs from modern tissue samples. Part of this success may be due to having a smaller than typical number of individuals (13 total) on a single Illumina® sequencing lane, as many studies include 100–200 individuals per lane for similar work with modern samples; because of the lower quality and smaller quantity of DNA in historical fluid-specimen samples, using large numbers of samples and/or

combining the historical samples with modern samples in a sequencing lane could result in lower numbers of reads for historical samples (J. Enk, ArborBiosciences, pers. comm.). These snakes were incorporated into a phylogeny of modern tissue samples from Streicher and Wiens (2016), and the resulting phylogeny supported several previous taxonomic hypotheses for snakes based on morphology. A result of particular note was finding the fossorial Indian snake *Xylophis stenorhynchus* as the sister taxon to the Asian Pareidae, a family of primarily arboreal gastropod-feeding specialists. The genus *Xylophis* has a history of taxonomic instability with respect to its familial placement, with suggestions that it is a colubrid, natricid, or xenodermatid (Gower and Winkler, 2007) but no previous hints of affinity with Pareidae.

To determine the validity of the surprising result of *Xylophis* + Pareidae found in Ruane and Austin (2017), Deepak et al. (2018) leveraged one of the benefits of HTS, specifically target capture approaches. Rather than generate all new sequences to further examine the placement of *Xylophis*, the authors "pulled" the mtDNA bycatch from the Ruane and Austin (2017) dataset for *X. stenorhynchus*; a necessity for its inclusion, as this species of *Xylophis* is only available as museum specimens, with no fresh or new samples known. Although this did not result in a full *Xylophis* mitogenome, which can often be constructed from fresh-tissue target capture datasets due to the higher per cell copy number mitogenomes that get sequenced as molecular bycatch, enough mtDNA sequence was recoverable to combine the sample of *X. stenorhynchus* with newly generated Sanger-sequencing loci from the other two species of *Xylophis*. The resulting tree added to the evidence that *Xylophis* is the sister taxon to the pareids and was officially described as a subfamily within Pareidae (Deepak et al., 2018).

Allentoft et al. (2018), following the successes from Hykin et al. (2015) and Ruane and Austin (2017), conducted a phylogeographic study using ethanol-preserved soft tissues (muscle and skin) as well as tooth and bone fragments from three *Zamenis longissimus* (Aesculapian Snake) collected between 1810–1863. These individuals are from an extirpated Danish population of the snake, and the focus of the study was an examination of the past distribution and postglacial recolonization patterns for *Z. longissimus*. The extraction of the fluid-preserved snakes was based on Ruane and Austin (2017), and the authors used a shotgun approach for sequencing similar to Hykin et al. (2015). Two of the three samples resulted in enough reads to successfully use ~1,630 base pairs of mtDNA from each snake for phylogeographic analyses (Cyt *b* and COI). The authors concluded that the two successfully sequenced Danish *Z. longissimus* are part of an eastern lineage of Aesculapian Snakes, supporting a Balkan glacial refugium hypothesis for this species.

Perhaps one of the most important aspects of being able to sequence fluid-preserved herpetological specimens is accessing DNA from holotypes that were collected well before genetic sampling was standard. This is relevant when the holotype or type series are the only material for a species available and accessing the DNA of such specimens can also help to disentangle current taxonomic confusion, even when the species is currently being collected. An exemplar of this use is the work of McGuire et al. (2018) on the *Draco fimbriatus* group (Asian flying lizards). In this group of lizards, species identification is based primarily on coloration, which ultimately fades in preservation, thus making museum specimens difficult to identify with accuracy. Two species in particular cause a taxonomic conundrum: *D. cristatellus*, which was described with a single and already-preserved specimen, meaning coloration was not as in life; and *D. punctatus*, described after *D. cristatellus* as distinct, but with no clear indication in the description of how the two species differ phenotypically; a detailed review of the somewhat capricious taxonomy of these species is provided in McGuire et al. (2018). To determine whether these two lizards do in fact represent distinct species and to then better determine which recently collected *Draco* are from which species, McGuire et al. (2018) developed an extraction protocol specifically designed for ethanol-preserved specimens for use on the holotype of *D. cristatellus* using liver tissue. This extraction method is well detailed by the authors, but it is worth pointing out that the successful extraction included a specific step using a high-concentration salt buffer that aids in recovering short DNA fragments. The resulting *D. cristatellus* was sequenced on an Illumina® platform using a shotgun approach. The mtDNA locus ND2 was recoverable from the holotype, and the authors combined this with ND2 sequences from 65 additional *Draco* to determine the phylogenetic position of the holotype of *D. cristatellus*. As an aside, some additional ND2 sequences from *Draco* were generated from the mtDNA bycatch of a different HTS target capture study and used in this study. The results of this work showed that a more recently collected (from 1996) individual of *Draco* initially assigned to *D. cristatellus* was, in fact, most closely related to the holotype of *D. cristatellus* and that these two individuals are distinct from the other three species in the *D. fimbriatus* group, including *D. punctatus*. These results support the assertion that *D. cristatellus* is a valid species. The publicly available mtDNA sequences generated for the holotype of *D. cristellatus* as well as the now-confirmed more recently sampled individual should allow researchers to better identify new specimens of *Draco*, even when coloration confuses the issue.

Similar in scope, Mayer and Gamble (2019) conducted a pilot study to ultimately determine the phylogenetic placement of *Anolis roosevelti* (Culebra Giant Anole), a species known only from six fluid-preserved individuals last collected in the 1930s (at least four fixed in ethanol only) and for which taxonomic affinity is only surmised based on the morphology of the preserved specimens. Following the extraction protocol of Ruane and Austin (2017) and using a shotgun sequencing approach on the Illumina® platform, Mayer and Gamble initially extracted and sequenced historical specimens of the common and similar *A. cristatellus* (Crested Anole). This was done to determine that the minimal but still destructive sampling of the limited specimens of *A. roosevelti* for HTS would not be in vain. The 12 chosen specimens of *A. cristatellus* were also fixed in ethanol or isopropanol, encompassed similar collection ages as the specimens of *A. roosevelti*, and included one sample that was a modern specimen that was preserved with DNA sequencing in mind. The authors were able to assemble whole mitogenomes for half of the samples and partial mitogenomes for three of the samples. For the ten successfully sequenced individuals, a phylogenetic tree using the mtDNA ND2 gene was inferred including samples of *A. cristatellus* from previous work on the species (Reynolds et al.,

2017). The placement of all of the historical fluid-preserved samples was as expected with respect to geographic clustering in the tree, therefore showing that this method is appropriate and likely to work similarly for samples of *A. roosevelti* in the future.

***Conclusions.*—**To date, shotgun sequencing and target-capture approaches have been the successfully tested HTS methods for generating DNA from fluid-preserved museum specimens with a variety of modified DNA extraction protocols, with most using liver tissue and designed to increase the quantity of DNA recovered from such specimens. Although the focus here is on amphibians and reptiles with respect to methodological review, there has been success in other fluid-preserved taxa, including fishes, using restriction-site associated DNA sequencing methodologies (RADseq; Smith et al., 2019), a method not used in any of the herpetofaunal studies reviewed here, but certainly worth future exploration as RADseq is typically lower in cost when compared to the aforementioned methods. Indeed, during production of this paper, O'Connell et al. (2021, Authorea Preprints, DOI: 10.22541/au.161598002.27985989/v1) found some success using RADseq to generate SNPS from formalinized salamander specimens. When working with fluid-preserved specimens in a molecular phylogenetic context, investigators should thoroughly examine any and all pertinent literature to determine which methods or approaches may best serve their study systems, budgets, and research questions.

One general, but important, consideration for this type of work is for authors to make it as clear as possible which specimens included in studies are from fluid-preserved and/or formalin-fixed tissues, as this can greatly aid researchers when trying to decide their own approach to using similar specimens. Furthermore, specific details, even if not the main focus of the study, on how well the sequencing worked for such specimens, reported as number of sequence reads, lengths of loci, and similar is important information that should not be overlooked in its utility. These details allow one to understand better the benefits, limits, and what are reasonable expectations for including fluid-preserved and formalin-fixed individuals in modern phylogenomic studies.

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