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More than skin and bones: Comparing extraction methods and alternative sources of DNA from avian museum specimens

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Abstract

Next-generation sequencing has greatly expanded the utility and value of museum collections by revealing specimens as genomic resources. As the field of museum genomics grows, so does the need for extraction methods that maximize DNA yields. For avian museum specimens, the established method of extracting DNA from toe pads works well for most specimens. However, for some specimens, especially those of birds that are very small or very large, toe pads can be a poor source of DNA. In this study, we apply two DNA extraction methods (phenol–chloroform and silica column) to three different sources of DNA (toe pad, skin punch and bone) from 10 historical avian museum specimens. We show that a modified phenol–chloroform protocol yielded significantly more DNA than a silica column protocol (e.g., Qiagen DNeasy Blood & Tissue Kit) across all tissue types. However, extractions using the silica column protocol contained longer fragments on average than those using the phenol–chloroform protocol, probably as a result of loss of small fragments through the silica column. While toe pads yielded more DNA than skin punches and bone fragments, skin punches proved to be a reliable alternative source of DNA and might be especially appealing when toe pad extractions are impractical. Overall, we found that historical bird museum specimens contain substantial amounts of DNA for genomic studies under most extraction scenarios, but that a phenol–chloroform protocol consistently provides the high quantities of DNA required for most current genomic protocols.

KEYWORDS
DNA extraction, historical DNA, natural history collections, NGS

1 | INTRODUCTION

Over the last decade, next-generation sequencing (NGS) has transformed museum specimens into important sources of genomic material (Burrell, Disotell, & Bergey, 2015; Green et al., 2010; Hofreiter et al., 2015; Knapp & Hofreiter, 2010; McCormack et al., 2017; Rizzi, Lari, Gigli, De Bellis, & Caramelli, 2012). Many studies now regularly incorporate molecular data from museum specimens in order to fill in sampling gaps of rare or extinct species (Anmarkrud & Lifjeld, 2017; Besnard et al., 2016; Hofreiter et al., 2015; Thomas, Schaffner, Wilson, & Pääbo, 1989). DNA from historical specimens is also increasingly being used as a baseline for studying genetic change over time across historical or evolutionary timescales (Buerki et al., 2015; Habel, Husemann, Finger, Danley, & Zachos, 2014; Holmes et al., 2016). As the number of these genomic projects multiplies, so too will the need for DNA from museum specimens.

One of the major limitations in the burgeoning field of museum genomics is the finite nature of the specimen and our ability to extract DNA from it, which often places specimen preservation and specimen use at counter-purposes. DNA extracted from museum

specimens is invariably fragmented and damaged due to a number of factors including specimen age and preservation method (Axelsson, Willerslev, Gilbert, & Nielsen, 2008; Briggs et al., 2010; Dabney et al., 2013; Handt, Höss, Krings, & Pääbo, 1994; Hawkins et al., 2016; Molak & Ho, 2011; Pääbo, 1989; Stiller et al., 2006; Willerslev & Cooper, 2005). At least one study has suggested that it is our inefficient extraction protocols, and not any intrinsic lack of DNA in the specimen itself, that explains low yields from ancient and historical samples (Barta et al., 2014). However, recent successes in extracting and sequencing DNA from formerly intractable museum specimens collected in the past two centuries show promise for future studies (Cornetti, Girardi, Ghielmi, & Vernesi, 2018; Ruane & Austin, 2017; Smith, Von Bargen, DeHaan, Scheerer, & Meeuwig, 2019; Wood, González, Lloyd, Coddington, & Scharff, 2018). Regardless, until we see a major paradigm-shifting advance in how DNA is extracted from historical samples, improvements to DNA yields must come through modifications to existing protocols and available sources of DNA. Furthermore, as museum specimens are both invaluable and irreplaceable, the main focus for optimizing DNA extraction from them should be to increase DNA yields while minimizing destructive sampling.

Numerous studies have compared extraction protocols on a variety of ancient and historical tissues, including mammalian bones, teeth and skin, and insects, plants and reptiles (Bi et al., 2013; Gamba et al., 2016; Hofreiter, 2012; Liu et al., 2018; McGuire et al., 2018; Rohland, Glocke, Aximu-Petri, & Meyer, 2018; Rothe & Nagy, 2016; Singh & Bahuguna, 2014; Staats et al., 2013; Tin, Economo, & Mikheyev, 2014). One recent study of kit fox specimens showed that extractions of different sources of sample tissue yielded varying DNA amounts and quality, affecting downstream molecular processes (Lonsinger, Daniel, Adams, & Waits, 2019). For birds, DNA has been successfully extracted from museum skins, eggshells and feathers, but exploration and comparison of DNA from different tissue sources are limited (Grealy, Bunce, & Holleley, 2019; Oskam et al., 2010; Rawlence, Wood, Armstrong, & Cooper, 2009; Sefc, Payne, & Sorenson, 2003).

Bird museum study skins provide a valuable source of historical DNA due to their relatively benign method of preservation (natural drying) as compared to formalin preservation of herpetological and ichthyological specimens. Pieces of tissue cut from the feet of bird specimens (i.e., toe pads) are the most common source of DNA because tissue sampling is minimally destructive and has in the past provided sufficient DNA for most applications (Fulton, Wagner, & Shapiro, 2012; McCormack, Tsai, & Faircloth, 2016; Mundy, Unitt, & Woodruff, 1997). Toe pads from small birds such as hummingbirds and warblers, however, are difficult to cut and produce lower DNA yields. Conversely, toe pads from large birds, such as herons and parrots, are prone to rotting, which degrades DNA. To reduce rotting and damage to the specimen, the feet of large birds were sometimes injected with formalin, which also adversely affects DNA yields and downstream NGS processes (Simmons, 2014).

It is therefore worth considering other potential sources of DNA on a bird study skin besides toe pads. Most birds grow their feathers in tracts, with patches of featherless skin called apteria that could be sampled without leaving obvious damage to the specimen (Töpfer, Gamauf, & Haring, 2011). Hard tissues, like bone and teeth, have been shown in some cases to provide the most intact DNA (Wandeler, Hoeck, & Keller, 2007). Typical bird study skins contain some remaining bones inside them, such as most of the skull and leg bones, and the distal ends of wing bones. These bones, although difficult to access, could be especially rich in high-quality DNA, and are relatively hidden from the outside appearance of the specimen.

In addition to tissue type, extraction methodology could affect DNA yields, or interact with tissue type to affect yields. Silica column methodologies, such as the widely used Qiagen DNeasy Blood & Tissue Kit, in which DNA binds to a silica membrane and is washed of impurities, differ from classic phenol–chloroform methods, in which DNA is trapped and sequestered in an aqueous layer and then precipitated from solution. There have been some comparisons of silica column and phenol–chloroform protocols on lizard and mammal museum specimens, but no direct comparisons for avian museum specimens (Cornetti et al., 2018; Hykin, Bi, & McGuire, 2015; Nishiguchi et al., 2002; Rohland & Hofreiter, 2007; Singh & Bahuguna, 2014). Furthermore, extraction protocols and tissue types could differ in the amount and length of DNA fragments recovered, with implications for various downstream NGS uses, but this has not been investigated. Here, we compare two types of extraction protocols (a standard silica column kit and a phenol–chloroform method) on three tissue types (toe pads, skin and bones) from avian museum specimens to determine which method and tissue yields the most and highest quality DNA by looking at both DNA concentration and fragment lengths.

2 | METHODS

2.1 | Sampling

We sampled 10 bird specimens representing a range of bird species from the teaching collection of the Moore Laboratory of Zoology at Occidental College (Table 1). All specimens probably date to the early 20th century, based on tags and condition; however, only five of the specimens had an associated year of collection. From each specimen, we cut two samples of the three different tissue types while wearing gloves and using separate, sterile surgical blades and clean forceps (see Figure S1 for photographs of sampling locations and relative damage to specimens). For toe pads, we chose the toes with the largest toe pad and cut as close to the bone as possible. For skin (hereafter called a "skin punch"), we brushed aside the feathers to expose the pectoral apterium. We then used a scalpel to cut a 5 × 5-mm piece of skin. To obtain bone fragments, we cut the skin around the distal end of the tibiotarsus, just proximal to the joint between the tibiotarsus and tarsometatarsus, closest to the feather line, and removed the leg from the specimen. We cut a piece of bone off from the top of the exposed tibiotarsus. We weighed each sample in milligrams for normalization of DNA yield.

TABLE 1 Avian museum specimens and summary statistics for extracted DNA

MLZ teaching collection ID	Scientific name	Common name	Collection year	Toe pads				Skin punches				Bone fragments			
				Silica column		Phenol–chloroform		Silica column		Phenol–chloroform		Silica column		Phenol–chloroform	
				Yield (ng/mg)	Average size (bp)	Yield (ng/mg)	Average size (bp)	Yield (ng/mg)	Average size (bp)	Yield (ng/mg)	Average size (bp)	Yield (ng/mg)	Average size (bp)	Yield (ng/mg)	Average size (bp)
12	*Mergus merganser*	Common merganser	1927	58	104	431	99	1	64	17	40	24	136	32	86
18	*Callipepla gambeli*	Gambel's quail	1907	26	97	189	86	16	144	127	53	22	139	75	68
83	*Asio otus*	Long-eared owl	–	55	179	354	67	6	134	261	55	29	133	127	75
84	*Asio flammeus*	Short-eared owl	–	122	216	208	99	2	100	92	51	57	99	119	78
89	*Melanerpes formicivorus*	Acorn woodpecker	–	105	216	325	59	9	129	462	53	38	127	283	78
117	*Nucifraga columbiana*	Clark's nutcracker	1905	21	138	158	68	3	164	202	47	62	167	347	87
118	*Corvus brachyrhynchos*	American crow	–	38	171	114	71	5	143	106	61	6	137	37	65
120	*Cyanocitta stelleri*	Stellar's jay	–	79	115	528	60	59	174	645	55	23	158	225	96
138	*Cinclus mexicanus*	American dipper	1905	92	213	937	72	44	150	259	50	19	154	42	105
278	*Melozone crissalis*	California towhee	1908	7	–	737	61	9	132	680	47	48	119	114	120

2.2 | DNA extraction

We carried out all extractions in a sterile area with equipment designated for historical DNA and included a negative control in each batch. Even though we did not conduct PCR as part of this experiment, our protocol includes washing away potential inhibitors of downstream enzymatic reactions and contaminating bacteria, using 100% ethanol at room temperature for 5 min. Next, we rehydrated each sample for 5 min in 1× STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 0.1 mM EDTA) at room temperature. We digested all samples in 180 µl Buffer ATL (Qiagen) and 20 µl of Proteinase K at 56°C. If there was still a large piece of undissolved tissue after 2 hr of digestion, we mashed each sample with a sterile minipestle and continued incubating for an additional 2 hr. One hour prior to the completion of incubation, we added 25 µl of 1 M dithiothrietol to ensure complete digestion.

We extracted DNA from each sample using two methods. First, we followed a modified silica column protocol using the Qiagen DNeasy Blood & Tissue Kit (Fulton et al., 2012; Mundy et al., 1997). We modified the standard protocol by performing two additions of 50 µl of pre-warmed Buffer AE (56°C) to each Qiagen column and incubating for 5 min at room temperature before centrifuging for a total elution of 100 µl instead of a single addition of 200 µl at room temperature.

We also performed a phenol–chloroform extraction on each tissue type, modified from Green and Sambrook (2012). Modifications included transferring digested samples to Phase Lock Light Gel tubes (Quanta BioSciences) and adding equal parts of phenol, chloroform and isoamyl alcohol (ThermoFisher Scientific). We saved the upper aqueous layer, performed an ethanol precipitation and stored samples in 10 mM Tris-HCl pH 7.5. We performed a high ratio solid phase reversible immobilization (SPRI) cleanup using a home-made mix (Rohland & Reich, 2012) intended to retain all DNA, including small fragments, and remove salts and PCR inhibitors prior to downstream reactions. A detailed step-by-step protocol is available in Appendix S1 and from the Dryad Digital Repository: https://doi.org/10.5061/dryad.0nj71g0.

2.3 | DNA quantification and analysis

We measured DNA concentrations (ng/µl) using the dsDNA High Sensitivity kit on a Qubit Fluorometer 2.0 (ThermoFisher Scientific). To visualize DNA extractions, we performed gel electrophoresis on 2% agarose gels stained with SYBR Safe DNA Gel Stain (Invitrogen). Fragment size was calculated using the Agilent 2100 Expert Software. We quantified and analysed an initial small batch of samples from each protocol type before and after performing the SPRI cleanup. We did not find noteworthy differences in total DNA or fragment distribution after cleanup, confirming that our bead clean up was probably not a factor affecting the results. We thus report and analyse values based on post-cleanup quantification and analyses. We normalized the concentrations based on amount of starting material to control for the fact that tissue samples varied in size

and mass. We tested for significant DNA yield and fragment length differences between extraction protocols and among tissue types separately using a paired *t* test design in STATA version 14.2. To use all data in one analysis instead of partitioning it, we also used a multivariate two-way repeated-measures ANOVA in the CAR1 package in RSTUDIO version 3.4.3. To assess for an effect of year of collection on DNA yields, we used linear regression in STATA version 14.2.

3 | RESULTS

We successfully extracted DNA from all sample types using both protocols (Table 1). There was no significant correlation between time since collection and DNA yields for any extraction and tissue type combination for the five specimens that had a known year of collection, suggesting no important role of specimen age within the narrow range of specimen ages we investigated here (e.g., all specimens were ~100–120 years old). Phenol–chloroform extractions yielded more DNA per weight of dry tissue (ng/mg) than silica column extractions (Figure 1, Table 1). This effect was large, as much as 10-fold higher DNA yields in some specimens, and was significant across the three paired *t* test comparisons for toe pad ($t = -4.00$, $df = 9$, $p = .003$), skin punch ($t = -3.80$, $df = 9$, $p = .004$) and bone ($t = -3.41$, $df = 9$, $p = .008$).

For tissue type, toe pad samples yielded the most DNA regardless of extraction protocol. Skin punches yielded the least DNA for silica column extractions and bone yielded the least DNA for phenol–chloroform extractions (Figure 1). In paired *t* tests separated by extraction type, for phenol–chloroform extractions, the higher yields were not significant for toe pad versus skin punch ($t = 1.42$, $df = 9$, $p = .188$) and skin punch versus bone ($t = 2.14$, $df = 9$, $p = .061$), but the higher yield of toe pad versus bone was significant ($t = 2.59$, $df = 9$, $p = .029$). For silica column extractions, the higher yield of toe pad versus skin punch was significant ($t = 3.70$, $df = 9$, $p = .005$), but the higher yields of toe pad versus bone ($t = 2.08$, $df = 9$, $p = .067$) and skin punch versus bone were not significant ($t = -1.77$, $df = 9$, $p = .110$). Most of the nonsignificant results were close to the $p < .05$ threshold, and the effect sizes were large. Power analyses suggested that, across the four nonsignificant results above, there was a 30%–80% chance of failing to detect significance, given the effect sizes, standard deviations and sample sizes. A multivariate repeated-measures ANOVA, which uses all of the data, and controls for both extraction type and tissue type, also returned a *p*-value close to statistical significance for differences in DNA yield among tissue types ($df = 8$, $F = 3.35$, $p = .085$).

DNA was degraded across all protocols and sample types with an average fragment length of 106 bp with a range of averages among samples from 40 to 216 bp (Figure 2). Fragment length did not decrease with age across any protocol and tissue type combination. Silica column extractions produced significantly longer average fragment lengths for all tissue types: toe pad (average for silica column = 161 bp; average for phenol–chloroform = 76 bp; $df = 8$, $t = -4.74$, $p = .002$), skin punch (average for silica column = 133 bp; average for phenol–chloroform = 51 bp; $df = 9$, $t = 9.00$, $p < .001$), and bone (average for silica column = 137 bp; average for phenol–chloroform = 86 bp; $df = 9$, $t = 6.58$, $p < .001$). There were no significant differences in fragment length between any tissue types when testing within extraction protocol (e.g., testing between bone and skin punch within silica column extraction type), except that toe pads produced longer fragments than skin punches for phenol–chloroform extractions (average for toe pad = 74 bp; average for skin punch = 51 bp; $df = 9$, $t = 3.98$, $p = .003$).

4 | DISCUSSION

Our results show that a phenol–chloroform extraction protocol outperformed a silica column protocol in amount of DNA by a margin

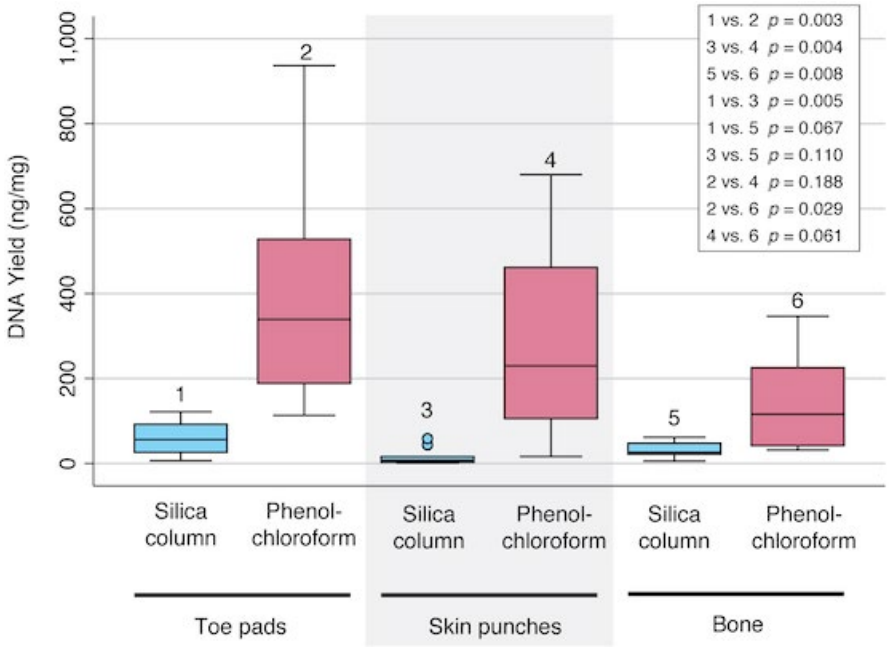

FIGURE 1 DNA yields from two different extraction protocols and three different DNA sources from bird museum skins. The box indicates the limits of the 25th (lower) and 75th (upper) percentile and the centre line represents the median. The whiskers represent the upper and lower adjacent values. Dots represent outliers from the adjacent values. Results of paired *t* tests are shown in the box with numbered comparisons corresponding to numbers above each treatment

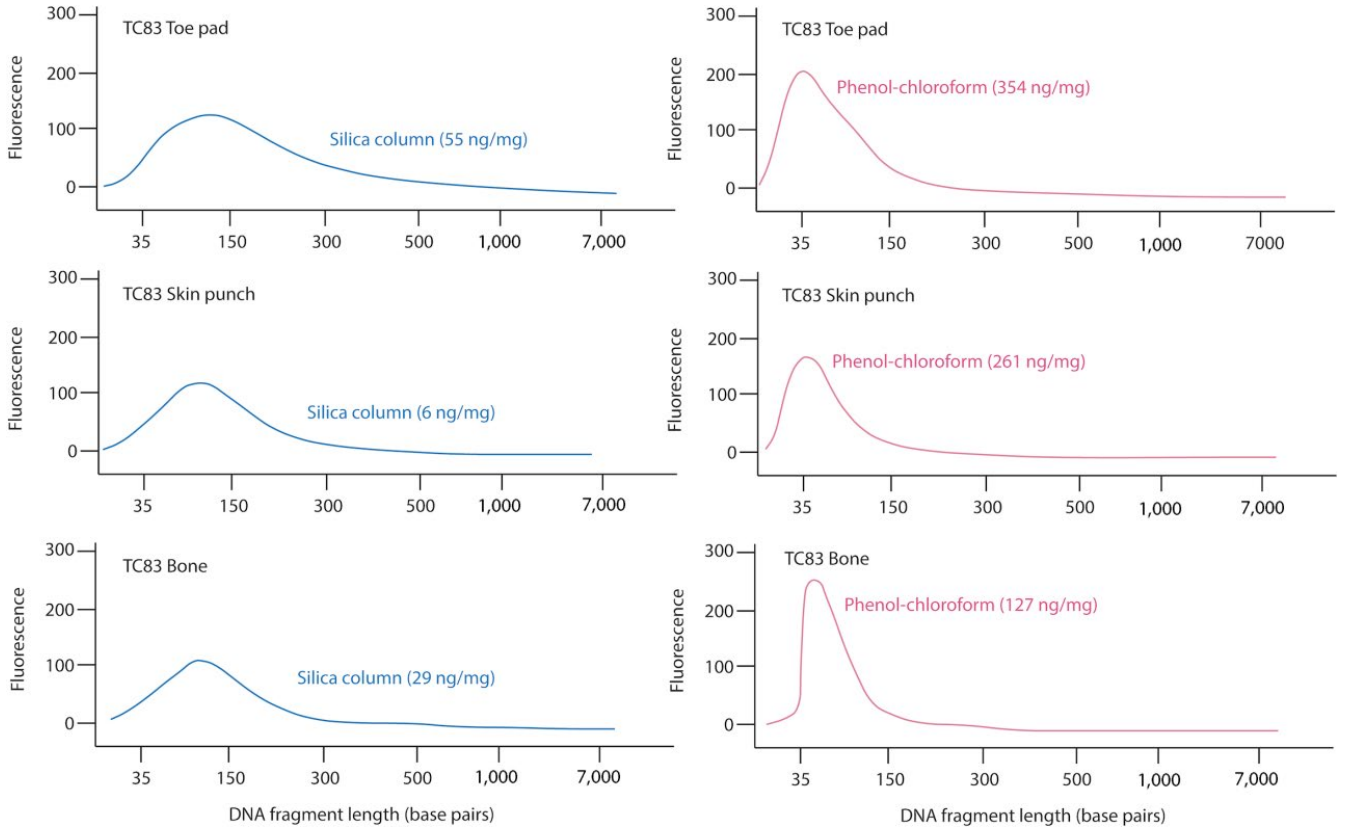

FIGURE 2 Fragment length profiles for each tissue and type of extraction for *Asio otus* (TC83) showing the higher number of short fragments obtained from the phenol–chloroform protocol. DNA yield is shown in parentheses for each sample. Note that the fluorescence peak and the area under the curve do not readily translate to DNA yield and cannot be compared among samples

that was both statistically significant and of large effect, yielding, in some cases, 10 times as much total DNA. Our results agree with at least two other studies comparing phenol–chloroform and silica-based extractions of DNA from museum specimens (Hykin et al., 2015; Nishiguchi et al., 2002). However, this result has not been universal. One study on snakes preserved in ethanol found little difference in yield between the two methods and opted for the simpler silica column extraction (Ruane & Austin, 2017). Another study on lizards preserved in ethanol found better yields from silica-based extractions (McGuire et al., 2018). Although neither of these studies compared extraction results quantitatively, the results highlight that there might be substantial differences in how extraction protocols work with museum specimens under different preservation conditions, something we did not test here. One important best practice is to save a portion of tissue in case multiple extractions are necessary (Cooper, 2000; Fulton, 2012).

Even though silica column DNA yields were far lower, they recovered longer average DNA fragment lengths than phenol–chloroform extractions. This could be explained by smaller fragments washing through the silica spin column, skewing average yields toward longer lengths, whereas with the phenol–chloroform protocol, more overall DNA was recovered but most of it was in a lower range of fragment lengths. In other words, it was not clear in our results whether the silica column protocol actually recovered more long DNA fragments or whether the difference in average length was solely due to differential loss of short fragments.

Investigating which protocols specifically maximize long fragments is an interesting avenue for future study, as several NGS protocols, such as restriction site-associated DNA sequencing (RADseq) and sequence capture of ultraconserved elements, perform better with longer input DNA fragments (Burrell et al., 2015; Gnirke et al., 2009; Hofreiter et al., 2015; Linck, Hanna, Sellas, & Dumbacher, 2017; Mamanova et al., 2010; McCormack et al., 2016; Toews et al., 2016). Few prior studies report fragment distribution from DNA extractions, and none has compared fragment lengths across extraction protocols. As the number of genomic applications for museum specimens grows, each with its own needs in terms of input DNA, fragment distribution will be an increasingly important factor in determining which extraction protocol is best suited to a particular NGS application.

Differences in DNA yields among toe pads, skin punches and bone were not as large as those between extraction protocols. Toe pads had the highest DNA yields from both extraction methods, and although the effect sizes were generally large, the results were not always significant. Skin punches performed particularly poorly with the silica column protocol, although it was not clear why, with eight of the 10 samples falling below the last quartile of the other tissue sources in terms of DNA yield. What these results suggest is that

although toe pads yield the most DNA, skin punches extracted with phenol–chloroform are an adequate alternative source of DNA when toe pads are small and difficult to cut or when initial extractions are unsuccessful. While it is noteworthy that bone inside specimens provides substantial quantities of DNA, bones are not easy to access within most bird specimens without causing noticeable damage to the specimen. Additionally, lower DNA yields from bone relative to other sources make them an unappealing option, and therefore they should only be utilized as a last resort and with the utmost care toward the preservation of the specimen.

To put our results in more practical terms for use with NGS studies, many library preparation kits tout a lower limit of only 1 ng of input DNA. Based on this, DNA yields from any of our protocols or tissues would not necessarily be limiting for library preparation-based applications. However, final constructed library output is only a fraction of total input DNA, thus using a starting DNA quantity at the upper suggested limit (e.g., 1 μg for a number of popular kits including Illumina TruSeq kits, Kapa Biosystems Hyper kits and NEBNext Ultra Kit) will maximize cost–benefit ratio, reagent efficiency and available DNA for downstream applications such as sequence capture. The silica column protocol produced values below this 1-μg threshold in all 30 cases except for three bone samples. In contrast, phenol–chloroform extractions on all tissue types produced values above this threshold in 25 of 30 cases (except two skin punches and three bone samples).

For some NGS techniques, both the quantity and the quality of input DNA matter. For example, double-digest RADseq (ddRADseq) requires a large quantity of starting DNA consisting of long fragments because the method works by selecting a small proportion of overall fragments that are homologous and contain two restriction-enzyme cut sites (Peterson, Weber, Kay, Fisher, & Hoekstra, 2012). Providers of ddRADseq service request up to 2 μg of starting DNA, which was only recovered from phenol–chloroform extractions, including all toe pad samples. While whole-genome amplification might offer a solution to low-quantity DNA samples (Turchinovich et al., 2014), the fragmented quality of historical DNA is a further impediment to achieving homologous fragments with ddRADseq (Burrell et al., 2015; Graham et al., 2015). For the moment, sequence capture or methods that combine RADseq and sequence capture—such as RADcap (Hoffberg et al., 2016) or hyRAD (Suchan et al., 2016)—are best suited to both the quality and the quantity of DNA achievable from historical samples.

As museum specimens are increasingly used as sources of DNA, the need for maximizing yields from finite materials will only grow. Until radically new approaches to DNA extraction are invented, improvements will come from modifying the protocols and sources of DNA we have at hand. We emphasize that there is not one single, universal extraction method that will work for the entire diversity of museum specimens as they differ widely in taxa, age and preservation method. We did not include a modern sample in this study, and did not seek to investigate changes in DNA over time, but previous work demonstrates the steady degradation in DNA fragment size that occurs over 140 years since the time of collection, which might also interact with extraction method (McCormack et al., 2017, 2016). Here, we demonstrate one method, phenol–chloroform extraction of toe pads, that works best for avian museum specimens that are around 100 years old. That said, skin punches are an adequate replacement, despite being virtually untapped as a DNA source. Future study of fragment length profiles from extraction methods and tissue sources could help to provide better matches between extraction protocols and downstream NGS applications.

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AUTHOR CONTRIBUTIONS

W.L.E.T., J.E.M. and J.M.M. designed the study. W.L.E.T. and M.E.S. collected and analysed the data. W.L.E.T. and J.E.M. wrote the manuscript with critical input from all authors.

DATA AVAILABILITY STATEMENT

A step-by-step phenol–chloroform protocol for DNA extraction from avian museum specimens is available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.0nj71g0.

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SUPPORTING INFORMATION

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