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SPECIAL SECTION

Native Lampreys: Research and Conservation of Ancient Fishes

Unlocking the genomes of formalin-fixed freshwater fish specimens: An assessment of factors influencing DNA extraction quantity and quality

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Abstract

Objective: Recent technological developments may facilitate the description of evolutionary relationships and population genetic structure as well as other information relevant to fisheries management by using readily available natural history collections. Contemporary sequence capture and short-read sequencing methods offer opportunities to analyze highly fragmented DNA from formalin-fixed specimens so long as enough DNA of sufficient quality is recovered.

Methods: We compared two protocols developed to extract DNA from formalin-fixed tissues using specimens of three freshwater fishes: the Southern Brook Lamprey *Ichthyomyzon gagei*, Slimy Sculpin *Cottus cognatus*, and Brown Trout *Salmo trutta*. Extractions were attempted using hot alkali digestion with and without buffer wash pretreatments to compare the DNA concentration, purity, and fragment length of DNA recovered between extraction protocols, tissue types (muscle and caudal fin tissue for Brown Trout and Slimy Sculpin), and preservation periods (5 or 7 years for Southern Brook Lamprey).

Result: Likelihood models generally did not detect DNA quantity differences between extraction protocols nor tissue types; however, 6.0–8.7× more DNA was recovered from Slimy Sculpin caudal fins than from muscle tissue. Extraction protocol had mixed effects on DNA purity; the wash protocol outperformed the no-wash protocol for Slimy Sculpin and Brown Trout, but the reverse was true for the lamprey. Purer DNA was recovered from the caudal fins; however, fragment lengths were generally greater from muscle tissue for both ray-finned species.

Conclusion: Our results suggest that the best tissue for sampling may depend on the quality metric considered most important for a study's objectives and that omitting time-consuming tissue wash steps can yield DNA of quantity and quality comparable to DNA from more complex methods. Regardless of species, the DNA extracted from most samples using both protocols met quantity and quality thresholds that are likely to result in short-read sequencing success. These results provide optimism for unlocking the wealth of genetic information in natural history collections for use in fisheries management and conservation genomics.

KEYWORDS

fisheries, genetics, taxonomy and systematics

INTRODUCTION AND BACKGROUND

Natural history collections maintain abundant records of biodiversity (Soulé 1985; Burrell et al. 2015; Hykin et al. 2015; Wolf et al. 2015) and provide specimens for morphological and ecological studies of diverse taxa (Suarez and Tsutsui 2004; Winker 2004; Watson and Werb 2013; Hykin et al. 2015; Holmes et al. 2016). These collections can be used to resolve taxonomic questions, monitor species that are relevant to public health and safety concerns, track the history and progress of biological invasions, and measure organismal responses to global change (Suarez and Tsutsui 2004; Winker 2004; Watson and Werb 2013; Holmes et al. 2016). Additionally, the worldwide acceleration of extinction events, particularly in freshwater ecosystems (Jelks et al. 2008; Burkhead 2012; Closs et al. 2016), has further enhanced the value of phylogenetic and population genetic research using preserved specimens of rare or declining species (Burrell et al. 2015; Splendiani et al. 2017; Gaither and DiBattista 2022; Scarsbrook et al. 2023). In combination with the monitoring of extant populations, the analysis—including genetic examinations—of specimens housed in natural history collections can play an important role in conservation status assessments and informing management actions (Shaffer et al. 1998; Winker 2004; Sutherland et al. 2009; Lister et al. 2011; Watson and Werb 2013; Holmes et al. 2016; Hahn et al. 2021).

Until recently, common specimen preservation protocols have obstructed efforts to obtain suitable DNA from natural history collections for genetic studies. Fish specimens are typically fixed in a 10% formalin solution prior to long-term storage in 70% ethanol (Sidlauskas and Konstantinidis 2022). Formalin is inexpensive and effective at preserving soft tissues, including external characteristics and organs that are important for morphological and life history studies (Watson and Werb 2013; Holmes et al. 2016; Sotola et al. 2019; Vaz et al. 2022), but it forms DNA–DNA and DNA–protein cross-linkages and promotes DNA fragmentation and base modification (Simmons 1999; Hykin et al. 2015). These effects can inhibit the success of Sanger sequencing, the traditional technology used for DNA bar coding and sequencing of targeted loci, which relies upon DNA with low fragmentation and high integrity (Chase et al. 1998; Bucklin and Allen 2004; Campos and Gilbert 2012; Hykin et al. 2015). Recently developed methods for high-throughput short-read sequencing and DNA sequence capture can partially circumvent the effects of base modification and DNA fragmentation if enough DNA of sufficient purity is extracted. These methods act through their capacity to (1) generate sufficient sequence coverage for base modifications to stand out as lower-frequency variants and (2) obtain partial sequences of specific genomic regions from fragments that are too short to be recoverable using traditional polymerase chain reaction (PCR)-based approaches (Suchan et al. 2016; Murray et al. 2017; Feigin et al. 2018; Hahn et al. 2021). Using these methods, researchers have demonstrated some success in extracting enough DNA of sufficient quality for sequencing from diverse taxa, including invertebrates (Bucklin and Allen 2004), reptiles (Hekkala et al. 2011a, 2011b; Hykin et al. 2015; Straube et al. 2021), amphibians (Chatigny 2000; Hahn et al. 2021), and some fishes (Klanten et al. 2003; Diaz-Viloria et al. 2005; Splendiani et al. 2017; de Santana et al. 2021).

Although high-throughput short-read sequencing has the potential to circumvent several of the problems posed by formalin fixation, several criteria must still be met by the DNA extracted from specimen tissues: (1) a sufficient concentration of DNA is required for construction of sequencing libraries; (2) DNA must be sufficiently free of contaminants that inhibit the enzymatic reactions used in library preparation and sequencing; and (3) DNA fragments must be of sufficient length for the specific technology used (e.g., 150-cycle Illumina sequencing of DNA fragments <150 base pairs [bp] in length incorporates adapter sequences, causing data contamination and the inefficient use of sequencing reagents; Hykin et al. 2015; Hahn et al. 2021). The use of a heated alkali treatment on formalin-preserved tissues can break cross-links that formed during preservation, and the addition of prescribed tissue washes can remove excess formalin from the sample (Hykin et al. 2015; Splendiani et al. 2017; Billerman and Walsh 2019; Rocha et al. 2022). These methods have been successful in recovering DNA from specimens of several marine fish species (Zhang 2010; Hagedorn et al. 2018; Burrows et al. 2019; Jalenques et al. 2021; Agne et al. 2022; Scarsbrook et al. 2023) but have yet to be applied to many groups of freshwater fishes, including lampreys (order

Impact statement

Contemporary sequence capture and short-read sequencing methods may facilitate inference of evolutionary relationships, population genetic structure, and other information relevant to fisheries management from formalin-fixed natural history collections as long as enough DNA of sufficient quality is recovered. We assessed extraction protocols for two ray-finned fishes and one lamprey, and determined factors likely to result in sequencing success.

Petromyzontiformes). The added tissue wash protocol can reduce contaminants and yield purer DNA, but the protocol (1) requires several incubation steps that add nearly 1 day to the extraction process, (2) involves liquid transfers that can result in DNA loss, and (3) requires additional tubes and pipette tips that add cost and generate additional plastic waste.

Past research using formalin-fixed specimens has extracted DNA from bone, fin structures, dorsal muscle, and liver tissue, with varying success (Kearney and Stuart 2004; Raja et al. 2011; Hykin et al. 2015; Appleyard et al. 2021). Different types of tissue may vary in their inherent DNA concentration (i.e., cell density, ploidy, etc.) and the rate at which they uptake formalin when fixed (e.g., due to tissue depth or material composition), leading to differences in resulting quantities and quality during extraction (Hykin et al. 2015; Appleyard et al. 2021; Hahn et al. 2021). Because specimens in natural history collections are used for numerous morphological and genetic inquiries, less-destructive tissue sampling (i.e., muscle, fin) is typically preferred over liver or bone sampling that results in more damage to individual specimens (Shiozawa et al. 1992; Appleyard et al. 2021).

Prolonged exposure to formaldehyde or its derivatives (e.g., formic acid) results in extreme DNA degradation to specimens (Appleyard et al. 2021; Hahn et al. 2021). Appleyard et al. (2021) found that exposure to formalin for greater than 8 weeks severely limited the PCR amplification success of mini-barcode loci. Fortunately, many fish collections were fixed in formalin for shorter time periods and stored in ethanol for long-term preservation, and successful short-read sequencing has been achieved for preserved samples over 100 years old (Ruane and Austin 2017; Straube et al. 2021). A comparison of two formalin-fixed, ethanol-preserved collections recovered more DNA of higher quality from material that was exposed to ethanol for a shorter period (Hykin et al. 2015), but the degree to which subsequent storage time in ethanol damaged the DNA is unclear since the compared collections were not necessarily fixed in formalin for the same time period and under the same conditions.

In the present study, we compared the efficacy of an alkali treatment with and without tissue washes (i.e., standard protocol involving three sequential washes) for extracting DNA suitable for short-read sequencing from formalin-fixed specimens of three freshwater fish species. We examined the effect of these procedures on the quantity, purity, and fragment length of DNA that could be recovered from recently preserved specimens of Brown Trout *Salmo trutta* and Slimy Sculpin *Cottus cognatus* (14 days in formalin and no exposure to ethanol) and museum specimens of Southern Brook Lamprey *Ichthyomyzon gagei* (fixed in formalin, followed by 5 or 7 years of storage in 70% ethanol). Additionally, we examined the degree of association between these quantity and quality metrics and either tissue type (caudal fin versus dorsal muscle) for Slimy Sculpin and Brown Trout or preservation period (5 versus 7 years) for Southern Brook Lamprey. Our results provide several insights that could prove useful for researchers hoping to unlock the value of natural history collections for genetic research of taxonomically diverse freshwater fishes.

METHODS

Specimen collection

Eight Brown Trout (80–200 mm) and eight Slimy Sculpin (45–100 mm) were collected in February 2023 from Berge Coulee Creek near Coon Valley, Wisconsin. Each individual specimen was preserved whole, without incisions to enhance formalin uptake, was placed in a 10% formalin solution for 14 days, and then was rinsed with water prior to tissue sampling (Simmons 1999; Sidlauskas and Konstantinidis 2022). Additionally, 12 Southern Brook Lamprey specimens (55–151 mm) were acquired on loan from the Mississippi Museum of Natural Science. The lamprey specimens were collected from the Mobile and Pearl River basins (one locality and five localities, respectively); they were initially preserved using similar fixation techniques but were maintained in 70% ethanol for permanent storage for either 5 or 7 years. The 5- and 7-year preserved specimens were fixed using an identical protocol, allowing the storage time effect to be compared without the confounding effect of differences in fixation method.

Tissue collection

Two tissue types (i.e., dorsal muscle and caudal fin; 2.0 cm^2) were excised from each Brown Trout and Slimy Sculpin specimen. The samples were divided equally in half to provide paired samples for comparing the two DNA extraction protocols.

Tissue samples were collected from the gill region on the right side of each Southern Brook Lamprey specimen (1.5 cm^2; M. Wagner, U.S. Fish and Wildlife Service, personal communication) and were divided equally in half to provide paired samples from each fish (i.e., one-half per extraction protocol). We used only one tissue type for the lamprey samples in order to minimize damage to the museum specimens and maintain their integrity for future use. Insufficient tissue material was retrieved from four lamprey specimens that had been preserved for 7 years, so DNA was extracted using only the wash protocol for those specimens.

Extraction of DNA without tissue washes

The first protocol that was tested used a heated alkali pretreatment prior to extraction (Campos and Gilbert 2012). Tissue samples were stored in 2-mL screwcap O-ring tubes with an alkali digestion buffer (0.1-M NaOH; 1% sodium dodecyl sulfate solution) and heated in an autoclave for 20 min at 120°C, with a 5-min vapor removal period. After the sample was cooled for an additional 5 min, a 25:24:1 phenol : chloroform : isoamyl alcohol solution was added and the sample was mixed then centrifuged for 5 min at 17,000 *g* (Campos and Gilbert 2012). The upper aqueous layer was removed, added to a new tube with 1.0 volume isopropanol and 0.1 volume 3 M sodium acetate, and centrifuged at 17,000 *g* for 30 min. The resulting DNA pellet was rinsed with 85% ethanol and centrifuged once more before the ethanol was decanted, the samples were dried by evaporation, and the dried pellet was resuspended in tris–EDTA (TE) buffer (10-mM tris-HCl, 1-mM EDTA; pH 8.0). Resuspended DNA was stored at −20°C.

Extraction of DNA with tissue washes

Preceding the heated alkali treatment with a series of glycine–tris–EDTA (GTE) buffer washes may more efficiently cleave cross-linkage complexes and bind excess formalin to improve extract quality (Splendiani et al. 2017; Billerman and Walsh 2019; Rocha et al. 2022; Scarsbrook et al. 2023). A standard protocol was used for tissue washes that requires three sequential buffer washing steps following Hykin et al. (2015). First, the tissue samples were soaked in GTE buffer (100-mM glycine; 10-mM tris-HCl, pH 8.0; 1-mM EDTA), shaken at 100 rpm and 37°C for 2 h, and then rinsed with 100% ethanol for 1 min (Hykin et al. 2015). The 100% ethanol was removed, replaced with 70% ethanol for 5 min, and then replaced by sterile water for 10 min (Hykin et al. 2015). The second buffer wash cycle repeated the same process as the first. For the final wash cycle, tissues were soaked in GTE buffer and shaken at 100 rpm and 37°C for 12 h, followed by the same rinsing procedure as the previous two steps (Hykin et al. 2015). After the final tissue wash, the protocol that was used to extract DNA was the same as described for the nonwash protocol.

Quantifying the DNA concentration

The concentration of extracted DNA (ng/μL) was measured using a Qubit 3.0 fluorometer with the Qubit dsDNA (double-stranded DNA) HS (high-sensitivity) assay kit (Thermo Fisher Scientific), which uses a fluorescent dsDNA-binding dye to estimate DNA concentrations based on known standards (Simbolo et al. 2013; Psifidi et al. 2015). Samples with values of at least 0.02 ng/μL are detectable using the Qubit dsDNA HS kit (at 5 μL of sample added), and values of 1 ng/μL or greater are acceptable for low-input Illumina library preparation and eventual sequencing (UC Davis Genome Center 2023).

Quality of DNA using A260/A280 and A260/A230 ratios

A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) was used to assess DNA purity based on two absorbance ratios: A260/A280 and A260/A230. The A260/A280 ratio is diminished by contaminants absorbing at approximately 280 nm, including protein and phenol, and the A260/A230 ratio is diminished by contaminants absorbing at about 230 nm, including phenol, carbohydrates, proteins, guanidine, and magnetic beads (Thermo Fisher Scientific 2010). An A260/A280 ratio of 1.8 and an A260/A230 ratio of 2.0 are considered to represent "pure" DNA (i.e., free of contaminants; Thermo Fisher Scientific 2010). For analyses of DNA purity, deviations from 1.8 for the A260/A280 ratio and from 2.0 for the A260/A230 ratio were used as the response variables such that larger deviation values corresponded to less-pure DNA.

Lengths of DNA fragments

An approximate distribution of DNA fragment lengths was obtained by electrophoresing the DNA extracts on a 1.2% agarose gel at 80 V for 90 min. A Fisher BioReagents exACTGene low-scale DNA ladder (Thermo Fisher Scientific) was included in each row for fragment size comparison. The proportion of the gel smear corresponding to fragments having a length of at least 300 bp—a suitable minimum insert size for Illumina library preparation—was measured using ImageJ (Rasband 1997) following the method of Mulcahy et al. (2016).

Statistical analysis

Linear mixed-effects models fitted using maximum likelihood were used to describe the fixed effects of extraction protocol and tissue type or preservation time on each response variable with distributions that approximated normal. In these analyses, Wald chi-square tests were used to estimate *p*-values for each additive fixed effect. For response variables that did not meet the assumption of normality, a penalized quasi-likelihood model capable of handling non-normal and unbalanced designs with crossed random effects

was used. In these models, we used a Gaussian distribution with a log-link function. The crossed random effect in all mixed-effects models was the individual fish from which tissue samples were collected and DNA was extracted. Statistical significance was ascribed based on an α value of 0.05, and all analyses were completed using RStudio version 2022.12.0 Build 353 (https://posit.co).

RESULTS

Concentration of DNA

Nearly all Brown Trout (~81%), Slimy Sculpin (~91%), and Southern Brook Lamprey (95%) samples provided quantifiable concentrations of DNA (>0.02 ng/μL), regardless of the protocol, tissue type, or preservation period examined. The concentration of DNA recovered varied little between the two extraction protocols for any fish species evaluated or between the preservation periods for Southern Brook Lamprey (5 versus 7 years); however, the tissue type (caudal fin or muscle) was associated with significant differences in DNA concentration values for both Slimy Sculpin and Brown Trout. For Slimy Sculpin, caudal fin tissue yielded a significantly higher DNA concentration than muscle tissue, regardless of the extraction protocol (Table 1). Although a higher mean DNA concentration was recovered from Brown Trout fin tissue using the wash protocol and a higher mean concentration was recovered from muscle tissue using the nonwash protocol (Figure 1; Table 1), these differences between extraction protocols ($t_{22}=0.50$, $p=0.62$) and tissue types ($t_{22}=0.92$, $p=0.37$) were not statistically significant (Figure 1). Similarly, no significant difference in DNA quantity was observed between the two extraction protocols for Slimy Sculpin (Figure 2; $t_{22}=1.61$, $p=0.12$). Regardless of the protocol used, the amount of DNA recovered from Slimy Sculpin fin tissue greatly exceeded the quantities recovered from muscle tissue (Figure 2; Table 1; $t_{22}=2.70$, $p=0.01$). The observed differences in mean DNA concentrations recovered from Southern Brook Lamprey using the different extraction protocols and preservation periods were not statistically significant. Only small, nonsignificant differences in DNA concentration between the washed and nonwashed tissues ($t_7=0.80$, $p=0.45$) or between preservation durations ($t_{10}=0.30$, $p=0.77$) were observed (Table 1; Figure 3).

Quality of DNA from A260/A280 and A260/A230 ratios

No significant differences in the A260/A280 ratio were detected between the two extraction protocols for Brown

TABLE 1 Mean (standard error in parentheses) Brown Trout, Slimy Sculpin, and Southern Brook Lamprey specimen DNA extraction quantities and quality values for two extraction protocols (wash or no wash) and two tissue types (caudal fin or dorsal muscle; Brown Trout and Slimy Sculpin) or two preservation periods (5 or 7 years; Southern Brook Lamprey). The quality of DNA is described using DNA purity based on divergence from "ideal" A260/A280 and A260/A230 ratios (see Methods) and the percentage of DNA fragment lengths greater than 300 base pairs (bp).

Variable	Brown Trout ($n=8$ each)				Slimy Sculpin ($n=8$ each)				Southern Brook Lamprey			
	No wash; fin	No wash; muscle	Wash; fin	Wash; muscle	No wash; fin	No wash; muscle	Wash; fin	Wash; muscle	No wash; 5 years ($n=6$)	No wash; 7 years ($n=2$)	Wash; 5 years ($n=6$)	Wash; 7 years ($n=6$)
DNA quantity (ng/μL)	2.81 (1.20)	4.52 (1.54)	6.08 (2.08)	1.96 (0.59)	5.04 (1.55)	0.84 (0.32)	6.25 (2.59)	0.72 (0.25)	2.54 (1.01)	5.43 (3.54)	2.77 (1.33)	2.24 (0.65)
DNA purity (A260/A280)	0.07 (0.03)	0.23 (0.02)	0.11 (0.03)	0.32 (0.05)	0.17 (0.04)	0.24 (0.02)	0.20 (0.04)	0.29 (0.04)	0.28 (0.01)	0.29 (0.07)	0.32 (0.04)	0.43 (0.05)
DNA purity (A260/A230)	0.35 (0.12)	1.11 (0.12)	0.23 (0.11)	0.71 (0.19)	0.41 (0.11)	1.19 (0.09)	0.42 (0.18)	0.74 (0.11)	0.91 (0.18)	0.88 (0.38)	1.07 (0.20)	1.08 (0.26)
Percentage of DNA fragment lengths above 300 bp	27.88 (3.44)	32.18 (4.01)	27.41 (4.41)	34.71 (7.38)	37.03 (5.52)	40.38 (5.81)	31.35 (5.85)	37.70 (5.64)	21.32 (5.17)	21.65 (2.52)	25.53 (10.27)	23.66 (5.03)

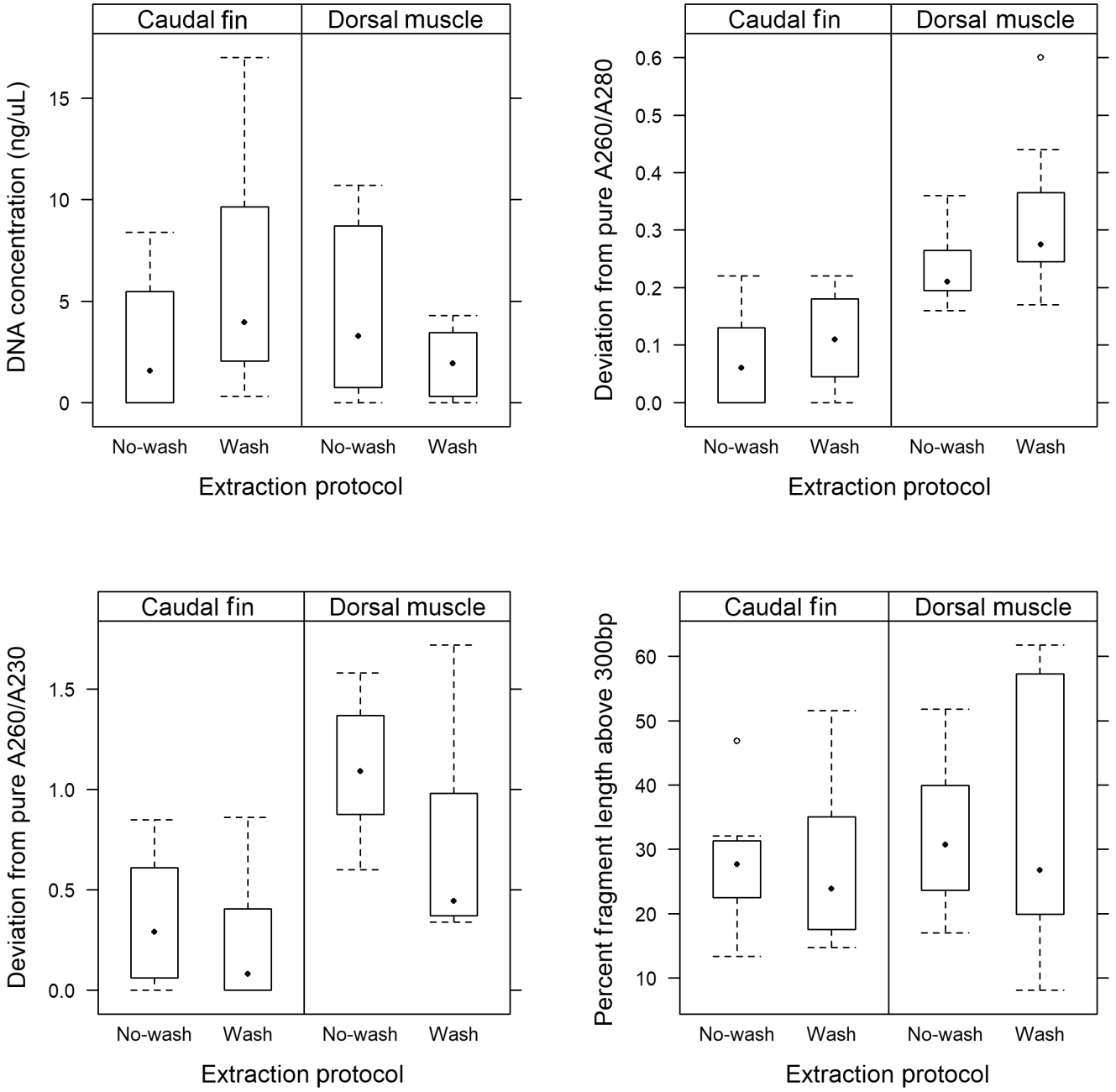

FIGURE 1 Comparison of DNA concentration, purity of DNA using A260/A280 and A260/A230 ratio differences, and the percentage of DNA fragment lengths greater than 300 base pairs (bp) among two DNA extraction protocols and two tissue types (caudal fin and dorsal muscle) for formalin-fixed Brown Trout. The two protocols involved the tissue being either washed with a buffered solution (wash) or not washed (no-wash) prior to a hot alkaline digestion and phenol : chloroform extraction. In each box plot, the box denotes the interquartile range (IQR), the closed black diamond within the box denotes the median, the whiskers denote the minimum value within Q1 − (1.5 × IQR) and maximum value within Q3 + (1.5 × IQR), and the open circles beyond the whiskers denote outlier values.

Trout (Figure 1; $t_{22}=1.92$, $p=0.06$) or Slimy Sculpin (Figure 2; $t_{22}=1.11$, $p=0.28$). However, significant differences in the A260/A230 ratio were detected between the two extraction protocols for both species (Brown Trout: $t_{22}=2.19$, $p=0.04$; Slimy Sculpin: $\chi^2=4.01$, df = 1, $p=0.04$). In both cases, the wash protocol outperformed the no-wash protocol in providing purer DNA (Figures 1 and 2). However, for Southern Brook Lamprey, we observed the opposite effect, with the no-wash protocol recovering purer DNA as measured by both the A260/A280 ratio (Figure 3; $\chi^2=6.19$, df = 1, $p=0.01$) and the A260/A230 ratio (Figure 3; $t_7=2.34$, $p=0.05$). Recovered caudal fin DNA exhibited significantly higher purity than muscle tissue for both Brown Trout (Table 1; Figure 1; A260/A280: $t_{22}=5.65$, $p<0.01$; A260/A230: $t_{22}=4.96$, $p<0.01$) and Slimy Sculpin (Table 1; Figure 2; A260/A280: $t_{22}=2.43$, $p=0.02$; A260/A230: $\chi^2=25.73$, df = 1, $p<0.01$) as measured by A260/A280 and A260/A230 ratios. The purity of DNA extracted

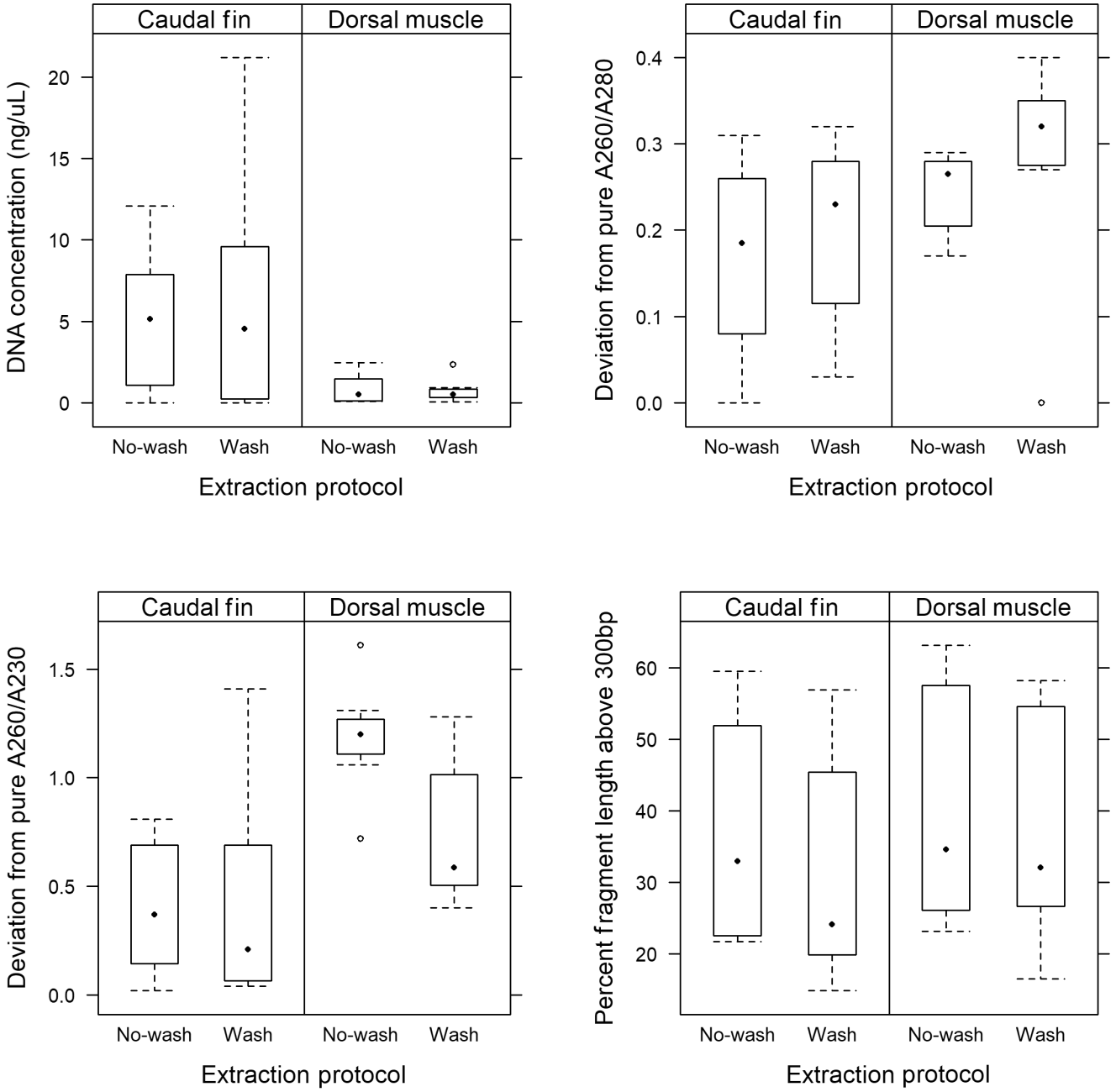

FIGURE 2 Comparison of DNA concentration, purity of DNA using A260/A280 and A260/A230 ratio differences, and the percentage of DNA fragment lengths greater than 300 base pairs (bp) among two DNA extraction protocols and two tissue types (caudal fin and dorsal muscle) for formalin-fixed Slimy Sculpin. The two protocols involved the tissue being either washed with a buffered solution (wash) or not washed (no-wash) prior to a hot alkaline digestion and phenol : chloroform extraction. In each box plot, the box denotes the interquartile range (IQR), the closed black diamond within the box denotes the median, the whiskers denote the minimum value within Q1 − (1.5 × IQR) and maximum value within Q3 + (1.5 × IQR), and the open circles beyond the whiskers denote outlier values.

from the caudal fins of these species was about 1.4–3.3× purer compared to muscle tissue for both A260/A280 and A260/A230 ratios (Table 1; Figures 1 and 2). No significant differences in DNA purity were observed between the two preservation periods evaluated for Southern Brook Lamprey specimens as measured by the A260/A280 ratio (Table 1; Figure 3; $\chi^2 = 3.77$, df = 1, $p = 0.05$) or the A260/A230 ratio (Table 1; Figure 3; $t_{10} = 0.04$, $p = 0.97$).

Lengths of DNA fragments

No significant differences in the percentage of fragment lengths greater than 300 bp were observed between the two extraction protocols for any of the species (Table 1; Brown Trout: $t_{22} = 1.16$, $p = 0.26$; Slimy Sculpin: $t_{22} = 1.75$, $p = 0.09$; Southern Brook Lamprey: $\chi^2 = 1.20$, df = 1, $p = 0.27$). However, dorsal muscle tissue provided a

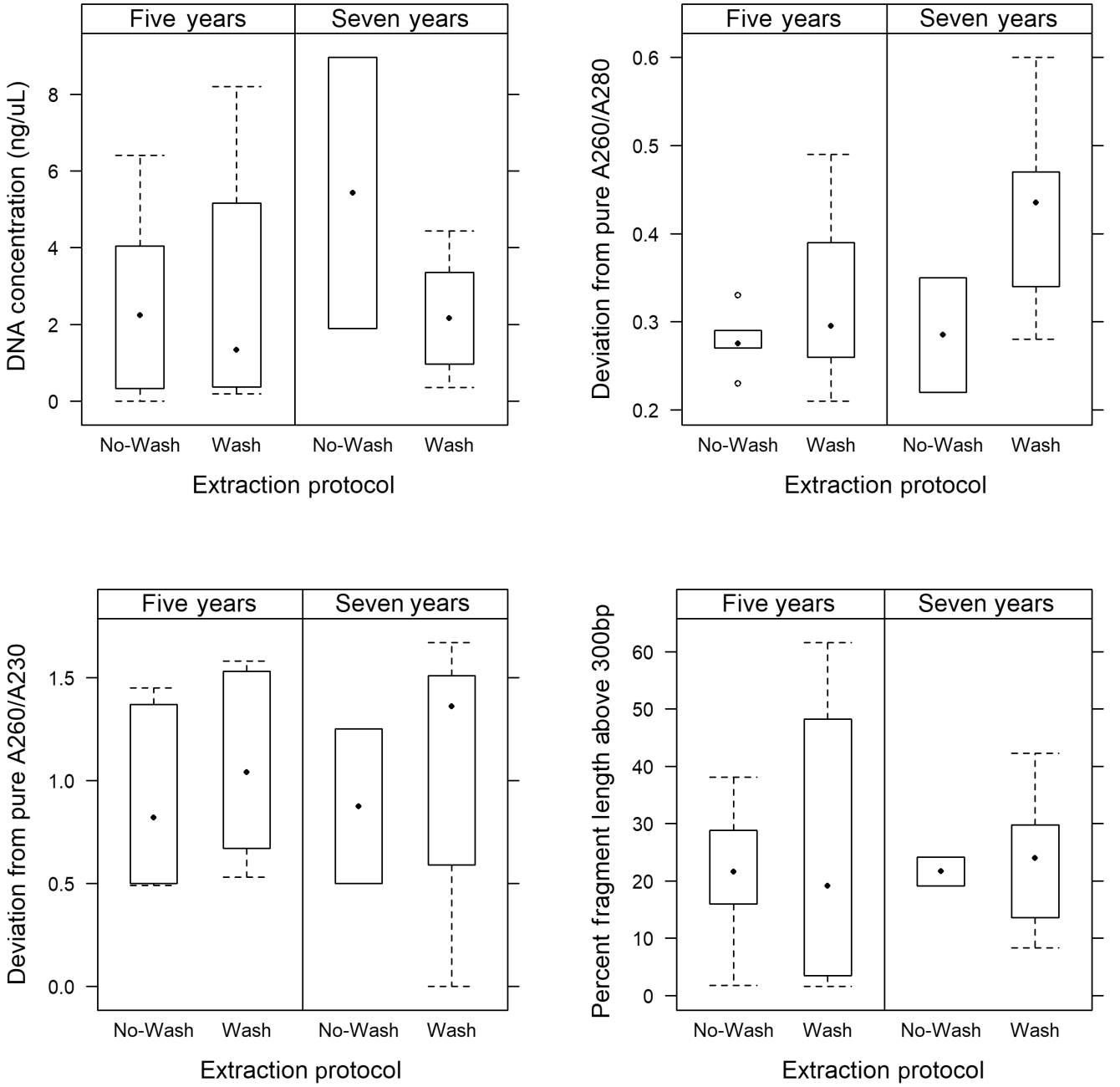

FIGURE 3 Comparison of DNA concentration, purity of DNA using A260/A280 and A260/A230 ratio differences, and the percentage of DNA fragment lengths greater than 300 base pairs (bp) among two DNA extraction protocols and two preservation times (5 and 7 years) for formalin-fixed Southern Brook Lamprey. The two protocols involved the tissue being either washed with a buffered solution (wash) or not washed (no-wash) prior to a hot alkaline digestion and phenol : chloroform extraction. In each box plot, the box denotes the interquartile range (IQR), the closed black diamond within the box denotes the median, the whiskers denote the minimum value within Q1 − (1.5 × IQR) and maximum value within Q3 + (1.5 × IQR), and the open circles beyond the whiskers denote outlier values.

significantly higher proportion of DNA fragments exceeding 300 bp than the caudal fin tissue for both Brown Trout (Table 1; Figure 1; $t_{22} = 2.53$, $p = 0.02$) and Slimy Sculpin (Table 1; Figure 2; $t_{22} = 2.09$, $p = 0.04$). Preservation period did not correspond to significant differences in the percentage of DNA fragments greater than 300 bp recovered from Southern Brook Lamprey specimens (Table 1; Figure 3; $\chi^2 = 0.15$, df = 1, $p = 0.70$).

DISCUSSION

Although DNA degradation from the formalin fixation of fish specimens has historically been an obstacle for genomic sequencing (Raja et al. 2011; Oosting et al. 2020; Appleyard et al. 2021), recent advances in DNA extraction techniques and sequencing technology may now enable the broad use of natural history collections. Such

inquiries can inform fisheries management decision making by describing the geographic representation of species and populations, clarifying taxonomic uncertainty, and assessing the results of past management actions. However, doing so requires DNA extraction protocols that can recover sufficient concentrations of DNA to be usable in low-input library preparation protocols, with sufficient purity to avoid inhibiting downstream enzymatic reactions and with sufficient fragment length to be usable for short-read sequencing. A number of methods may recover DNA that meets these quantity and quality criteria (Campos and Gilbert 2012; Hykin et al. 2015; Rocha et al. 2022; Scarsbrook et al. 2023), but the methods differ significantly in the amount of time, effort, and laboratory consumables required. In this study, we assessed two variations on a heated alkaline lysis protocol, with and without a series of three tissue washes, to determine whether the additional wash steps significantly improved the quantity and/or quality of DNA recovered from specimens of three freshwater fish species. In addition, we tested two other factors that may affect extraction efficacy: the tissue type sampled and the amount of preservation time in ethanol postfixation. Our results suggest that a variety of approaches can obtain DNA from diverse fishes that is suitable for high-throughput short-read sequencing, but careful selection of tissue type and laboratory protocols can help to attain better DNA quality or quantity based on species and/or preservation time. By including newly fixed specimens without subsequent ethanol preservation in our study design, we were also able to isolate the effects of fixation on DNA extraction success—a factor that is usually confounded with preservation in other studies of extraction from museum specimens. To our knowledge, a direct comparison between these protocols examining the quantity and quality of extracted DNA from formalin-fixed freshwater fish specimens has not been recorded. Our results provide further evidence that these methods can successfully recover DNA from formalin-fixed specimens in natural history collections, but the results also highlight important differences between species and between tissue types.

Protocols for DNA extraction

Although previous studies have demonstrated the value of including tissue washes prior to alkali treatment (Hykin et al. 2015; Splendiani et al. 2017; Billerman and Walsh 2019; Raxworthy and Smith 2021), our results were less conclusive regarding the value of the three washing steps for these three fishes. The wash protocol did not result in significantly higher DNA concentrations in any of the species, tissues, or preservation periods tested. Concentrations exhibited high variance for both protocols, and the direction of the mean concentration difference (wash > no-wash, or vice versa) differed between comparisons (Table 1). Similarly, the wash protocol did not result in a higher proportion of DNA fragments over 300 bp in length compared to the no-wash protocol. The DNA purity metrics exhibited a more complex pattern, with the wash protocol yielding DNA of higher purity in Brown Trout and Slimy Sculpin as measured by the A260/A230 ratio but not the A260/A280 ratio. Conversely, the no-wash protocol yielded purer DNA in Southern Brook Lamprey as measured by both the A260/A230 and A260/A280 ratios. Although the wash protocol may remove more formalin from the sample, it is also possible that buffer or ethanol residue or artifacts resulting from the washes can affect spectrophotometric readings. These factors appear to differ depending on the species and tissue sampled and perhaps based on the subsequent storage conditions of these specimens. It has not been established whether the differences in purity observed here are sufficient to result in different success rates in subsequent short-read sequencing, but preparation of reduced-representation sequencing libraries is currently underway for these specimens.

Tissue types

Natural history collections enable researchers to use specimens for diverse morphological and ecological studies (Suarez and Tsutsui 2004; Winker 2004; Watson and Werb 2013; Hykin et al. 2015; Holmes et al. 2016); therefore, it is important to target sampling to those tissues that are most likely to yield usable DNA while maintaining the physical and morphological integrity of the collection for future research (Holmes et al. 2016; Muhammad et al. 2016). Past research efforts have successfully extracted DNA from bone (Kearney and Stuart 2004; Stuart et al. 2006; Hykin et al. 2015), liver (Kearney and Stuart 2004; Raja et al. 2011; Hykin et al. 2015; Hahn et al. 2021; Scarsbrook et al. 2023), muscle (Kearney and Stuart 2004; Raja et al. 2011; Hahn et al. 2021), and different fins (Hykin et al. 2015; Appleyard et al. 2021) from formalin-fixed specimens. We found that caudal fin tissue yielded a higher DNA concentration in Slimy Sculpin compared to same-sized pieces of dorsal muscle tissue, but the two tissues did not yield significantly different concentrations of DNA in Brown Trout. Additionally, caudal fin tissue yielded purer DNA as measured by both the A260/A280 and A260/A230 ratios in both species. However, the dorsal muscle tissue yielded a higher proportion of DNA fragments greater than 300 bp in length for both species.

These results, which suggest a trade-off between the different methods by which DNA quantity and quality criteria are measured, are consistent with results of previous studies. Previous researchers have recovered greater amounts of DNA from fin tissue than from muscle tissue (Shiozawa et al. 1992; Appleyard et al. 2021), although the success of PCR amplification and DNA sequencing was increased by 2× when DNA was extracted from muscle tissue compared to fin tissue (Appleyard et al. 2021). This result appears consistent with our observation that less-fragmented DNA was obtained from muscle tissue. Our results suggest that although caudal fin tissue generally yielded more concentrated DNA and may be more easily washed of contaminants, deeper muscle tissue may suffer less DNA fragmentation damage, as has been similarly suggested for large, ossified structures (Sidlauskas and Konstantinidis 2022). Researchers may have to manage these trade-offs to obtain the DNA amount, purity, and structural integrity that are necessary for specific downstream applications while also considering the degree of destruction caused to the source collections (Muhammad et al. 2016).

Preservation period

Previous research has shown a significant association between length of exposure to formalin or formalin derivatives and decreased DNA quantity and/or quality (Raja et al. 2011). However, the effect of storage time is less clear for specimens that have been briefly fixed in formalin and subsequently stored in ethanol, since comparisons between differently aged specimens can be confounded by differences between fixation protocols and the fixation duration. Our study specifically compared specimens that were fixed using the same protocol, thus allowing the effect of storage time in ethanol to be directly compared. Our results largely failed to demonstrate significant differences in DNA quantity or quality between the two preservation periods tested for the Southern Brook Lamprey samples, although it should be noted that both periods are relatively short (i.e., 5 and 7 years). By examining samples that were fixed in formalin for 14 days but not subsequently preserved in ethanol, we were also able to directly examine the effects of fixation separate from those of ethanol storage. These samples exhibited low concentrations (means < 6.25 ng/μL), decreased purity, and high fragmentation, with levels similar to those in the Southern Brook Lamprey specimens that were preserved for 5–7 years. Although longer storage periods could exacerbate these effects, our results suggest that much of the DNA damage occurs relatively quickly after initial formalin exposure (i.e., within 2 weeks in this study). Therefore, as suggested by Hykin et al. (2015), researchers should (where possible) collect separate fresh tissue samples for DNA studies prior to preserving the rest of the specimen.

General conclusions

Recent advances in laboratory techniques and sequencing technology show great promise in unlocking the wealth of information that is stored in formalin-fixed museum specimens. However, challenges to using these preserved materials still exist, and it remains necessary to determine the best performing and least destructive tissue sampling and extraction protocols for many taxonomic groups of fishes. Our results suggest that tissue choice may be a more important consideration than the use of buffer tissue washes in terms of DNA concentration, purity, and fragment length. However, the optimal tissue choice may depend on which of these metrics is most important in a specific study. In our tests of Brown Trout and Slimy Sculpin, fin tissue yielded statistically similar or greater DNA concentrations and purer DNA compared to dorsal muscle tissue, but muscle tissue yielded a significantly higher proportion of fragments over 300 bp, suggesting an important trade-off between DNA quantity/purity and fragment integrity.

In all but one case (Slimy Sculpin muscle tissue), our extracts exhibited a mean concentration that was sufficient for low-scale Illumina sequencing (≥1 ng/μL). Higher DNA concentrations can produce higher read coverage and better overall sequencing results. However, since the amount of tissue sample can often be increased to achieve higher concentrations, the more pressing question for further examination becomes whether the purity and fragmentation levels are sufficient in real-world applications. Although contaminants can inhibit enzymatic reactions, including sequencing adapter ligation and PCR (Illumina 2023), past work has successfully sequenced samples despite contamination in DNA extractions from formalin-fixed maize (Abdel-Latif and Osman 2017), fish (Shiozawa et al. 1992; Scarsbrook et al. 2023), reptiles (Hykin et al. 2015), and amphibians (Pyron et al. 2022). If purity is the main consideration in a particular study, then our results suggest that caudal fin tissue may be superior to dorsal muscle. Our results are more ambiguous as to the benefit of the tissue washes on DNA purity. The protocol without the tissue washes generated better A260/A280 and A260/A230 ratios for Southern Brook Lamprey, but the protocol with the washes yielded better A260/A230 ratios for Brown Trout and Slimy Sculpin, suggesting that the efficiency of removing contaminants, either from the tissue or from the wash protocol itself, differs. However,

our results cannot distinguish whether this difference was due to the structure of the tissues (perhaps related to physiological or anatomical differences among species), ethanol storage of the specimen, or both. These effects could be tested with additional samples collected by using a study design that controls for other confounding factors.

The degree of DNA fragmentation is an important factor influencing sequencing success. Typically, a higher proportion of DNA fragments above 300 bp increases the chances of amplifying targeted DNA and sequencing (Abdel-Latif and Osman 2017; Garg et al. 2018; Scarsbrook et al. 2023), although PCR fragment amplification and sequencing have been successfully performed on even shorter lengths (~200 bp; Shiozawa et al. 1992; Hykin et al. 2015). In the present study, muscle tissue yielded a significantly greater proportion of fragments over 300 bp in length for both Brown Trout and Slimy Sculpin specimens. Although all samples exhibited evidence of fragments in the approximately 150–600-bp range that is typical of formalin-fixed samples (Sharpe et al. 2020) and suitable for short-read sequencing, the lower level of fragmentation exhibited by the dorsal muscle samples could significantly improve DNA sequencing success. Acquisition of larger fragments was not influenced in any of our tests by using tissue washes.

Another important question is the degree of base modification present in the different tissues and preparations and the level of sequencing depth required to identify these artifacts. To address this question, we are currently testing the suitability of the extracted DNA from Southern Brook Lamprey in this study for capture-assisted reduced-representation genome sequencing using double-digestion restriction site-associated DNA sequencing (Hoffberg et al. 2016; Suchan et al. 2016).

Management implications

Decreased financial support for natural history collections has resulted in the recent closures of some museums, losses of critical historical data, and a reduced number of new specimens added to collections (Suarez and Tsutsui 2004; Watson and Werb 2013; Dorfman 2018; Rohwer et al. 2022). Despite these challenges, the value of natural history collections continues to grow as new methods become available to obtain new data that are critical for scientific understanding and management (Chakraborty et al. 2006; Rohwer et al. 2022). Our research further contributes to the growing body of evidence that formalin-preserved fish specimens maintained by natural history collections can be suitable for genetic inquiries. Such research can provide guidance in conservation status assessments and can inform the management of economically valuable or at-risk fishes. These methods can be used to describe historical geographic ranges and range expansions or contractions through time, clarify phylogenetic and taxonomic questions that could affect determinations of rarity, track invasions, evaluate stocking histories and sources, assess population genetic structure and infer events such as bottlenecks or migrations, and identify representation of at-risk species (Shaffer et al. 1998; Winker 2004; Wandeler et al. 2007; Sutherland et al. 2009; Lister et al. 2011; Watson and Werb 2013; Holmes et al. 2016; Nualart et al. 2017; Hahn et al. 2021; Rohwer et al. 2022). The evolutionary relationships among the approximately 40 described species of lamprey and the population genetic structure of many species are largely unknown, resulting in uncertainties that now may be addressed using formalin-fixed museum specimens (Leidy and Moyle 1997; Potter et al. 2015). For example, the identity of geographically isolated populations of Southern Brook Lamprey *Ichthyomyzon* cf. *gagei* in Minnesota and Wisconsin that are either derived from southern populations or represent an autonomous divergence is currently being ascertained using these methods (Cochran 1987; Lyons et al. 1997). Despite the need for additional research to control potential confounding factors demonstrated in this study and to test these protocols across more taxonomic groups, our results provide evidence that these methods could unlock the wealth of information housed in natural history collections for use in fish management and conservation genomics of diverse lineages of freshwater fishes.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest declared in this article.

DATA AVAILABILITY STATEMENT

DNA quantity and quality data collected in this study are available through the Zenodo data repository (https://zenodo.org/record/8260472).

ETHICS STATEMENT

All research described in this study was conducted with the approval of the University of Wisconsin–La Crosse Institutional Animal Care and Use Committee (protocol #4–22).

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