Unlocking inaccessible historical genomes preserved in formalin

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Abstract

Background

Museum specimens represent an unparalleled record of historical genomic data. However, the wide-spread practice of formalin preservation has thus far impeded genomic analysis of a large proportion of specimens. Limited DNA sequencing from formalin-preserved specimens has yielded low genomic coverage with unpredictable success. We set out to refine sample processing methods and to identify specimen characteristics predictive of sequencing success. With a set of taxonomically diverse specimens collected between 1936 and 2015 and ranging in preservation quality, we compared the efficacy of several end-to-end whole genome sequencing workflows alongside a k-mer-based trimming-free read alignment approach to maximize mapping of endogenous sequence.

Results

We recovered complete mitochondrial genomes and up to 3X nuclear genome coverage from formalin-fixed tissues. Hot alkaline lysis coupled with phenol-chloroform extraction out-performed proteinase K digestion in recovering DNA, while library preparation method had little impact on sequencing success. The strongest predictor of DNA yield was overall specimen condition, which additively interacts with preservation conditions to accelerate DNA degradation.

Conclusions

We demonstrate a significant advance in capability beyond limited recovery of a small number of loci via PCR or target-capture sequencing. To facilitate strategic selection of suitable specimens for genomic sequencing, we present a decision-making framework that utilizes independent and non-destructive assessment criteria. Sequencing of formalin-fixed specimens will contribute to a greater understanding of temporal trends in genetic adaptation, including those associated with a changing climate. Our work enhances the value of museum collections

42 worldwide by unlocking genomes of specimens that have been disregarded as a valid molecular
43 resource.
44

45 **Keywords**: DNA, formaldehyde, formalin-fixed, genome, hot alkali, museum, museomics,
46 preservation media

Background

Natural history collections are a window into the recent past, offering a view of historical biodiversity that is unparalleled in its detail. Collected over the last 250 years, voucher specimens document a period of time over which humans have had a devastating impact on the natural world (1). The comprehensive metadata associated with each specimen (collection date, location, sex, weight, age, etc.), phenotypic data (e.g., color, size, gut contents) and genomic data can be used to monitor ecosystem health and study the mechanisms driving adaptation, evolution, speciation and extinction (2,3). The value of collections as sources of historical genetic material has been recognized for the past 30 years, with numerous pathways emerging to retrieve high-quality DNA from challenging archival vertebrate tissues such as skins (4), feathers (5,6), eggshells (7,8) and toe pads (9).

DNA degradation associated with preservation method and aging has limited most genetic studies of museum specimens to interrogation of relatively few loci via PCR amplification, often targeting the high copy mitochondrial genome. For phylogenetic studies where a survey of many-fold more loci improves understanding of species' evolutionary history (10–12), genome-wide analyses are increasingly becoming common place. With demand for historical genome-wide data on the rise, newly-developed target-capture approaches now facilitate broader genomic survey from degraded museum specimens (13–15). In some cases, recovery and assembly of whole historical genomes has been achieved (16,17), including, extinct from species (e.g., the Tasmanian tiger (18)). While technological advances are enabling recovery of genomic data from many museum specimens, genomic study of those preserved with 10% formalin (3.4% w/v formaldehyde) has thus far been very limited.

Formalin-fixation, followed by storage in ethanol, is a common curatorial method used to preserve soft tissue structure. Of the 1.9 million records of preserved chordates within the open-

71 access Atlas of Living Australia (ALA) specimen database (19), 33% are classified as "spirit-
72 preserved" (preserved in ethanol with or without prior formalin-fixation). A search for
73 "formalin" preparation within the ALA's chordate records indicates at least 4% of specimens
74 (N = 77,301) have been formalin-fixed. This is likely a severe underestimate because formalin-
75 fixation is not consistently recorded by all collections. Notably, for fish, reptiles and
76 amphibians, formalin-fixation has historically been the primary method used to preserve tissues
77 long-term while mammals and birds are commonly dry-preserved. Most collections now
78 archive frozen fresh tissue specifically as a genomic resource. However, prior to the 1980s,
79 spirit-preservation was the only method used to preserve soft tissue. Thus, spirit collections
80 offer the only opportunity to obtain genetic data from a large proportion of older specimens,
81 holotypes and some of the world's most biodiverse vertebrate taxonomic groups.

82 Genomic study of formalin-preserved museum specimens has lagged behind because DNA
83 extracted from such tissues is typically low-yield and highly fragmented. PCR amplification of
84 formalin-degraded DNA templates is generally restricted to few, short genomic loci, which
85 provide limited phylogenetic resolution (20). Formalin fixation presents further challenges by
86 inducing numerous molecular lesions, such as strand breaks, base misincorporation, and both
87 intra- and intermolecular cross-links (21–23). Formaldehyde damage to DNA templates can
88 result in sequencing artefacts that are difficult to differentiate from true genetic variants
89 (22,23). Because PCR amplification of damaged DNA is particularly prone to sequencing
90 artefacts, it is preferable to perform deep next-generation sequencing of amplicons (20) or to
91 avoid amplicon approaches altogether through whole genome sequencing (WGS) of degraded
92 templates (24). Coupled with library preparation methods optimized for low-input and
93 damaged DNA templates (25,26), high-throughput sequencing can generate enough coverage
94 to call genomic variants with high confidence (27). Thus, WGS and reduced representations of

genomes could provide a way to overcome the challenges associated with formalin damage and accurately reconstruct historical genetic variation from formalin-preserved tissues.

Promisingly, WGS of formalin-fixed paraffin-embedded (FFPE) archival tissues has become routine in clinical and medical contexts (28). However, museum specimens are often older, exposed to higher concentrations of formaldehyde, incubated in the fixative for longer (29) and in most cases have not been preserved in ideal conditions. Common museum practices, such as failure to rinse specimens prior to permanent storage in ethanol, result in prolonged formaldehyde exposure (30). Indeed, many specimens can be in contact with formaldehyde (or its derivatives, such as formic acid) for the entirety of their tenure in a collection. Prolonged formaldehyde exposure, especially under acidic conditions, is thought to result in more extreme DNA degradation (20,31). The damage resulting from the preservation process compounds with DNA damage due to natural decomposition, which can be extensive and often precedes any obvious visual indicators of decomposition (32). Unfortunately, the time between death and preservation (post-mortem interval) is highly variable and rarely recorded. In light of these additional challenges, WGS methods used with FFPE tissues are relevant but not directly transferable to formalin-fixed museum tissues.

Of the few genetic studies of formalin-fixed museum specimens, most have targeted nuclear (33–37) and high copy mitochondrial (20,38,39) loci via PCR amplification due to the difficulty and unpredictability of nuclear DNA extraction. There are few examples of broader-scale genomic sequencing of formalin-fixed museum specimens and none have recovered whole vertebrate genomes. Hot alkaline extraction followed by WGS of a single 30-year-old formalin-preserved *Anolis* lizard yielded sufficient coverage to reconstruct the entire mitochondrial genome (40). Using the same method, whole genomes were recovered for the bioluminescent bacterial symbionts contained within light organs of formalin-preserved cardinalfish (41). Using a proteinase K digestion method, sufficient gDNA was recovered for

120 capture and sequencing of ultra-conserved elements from formalin-preserved snakes (42).
121 Hybridization capture baits have also been used to recover the mitochondrial genome from a
122 120-year-old formalin-preserved Crimean green lizard (43). Highlighting the difficulty of
123 recovering gDNA from formalin-preserved specimens, numerous studies have reported failure
124 to extract and amplify gDNA from formalin-preserved museum tissues (20,44,45). In this
125 context, it is unfortunate yet wise to be hesitant to conduct destructive sampling of formalin-
126 preserved specimens for the purposes of costly WGS.

127 Recent reports of successful, albeit limited, genomic sequencing in formalin-preserved
128 specimens indicate WGS of higher quality specimens is possible. However, without a
129 framework to guide specimen selection, genomic work on formalin-preserved museum tissues
130 will remain infeasible. It is likely impossible to fully know the numerous and interdependent
131 factors driving sequencing success (e.g., age of the specimen (46,47), method of preservation
132 (48), post-mortem interval (32) and heat and light exposure during storage). However,
133 identification of metrics with which to pre-screen specimens for sequencing suitability will
134 improve yield of genomic data while reducing unnecessary destruction of specimens. With
135 screening criteria in hand, museum curators will be less reluctant to grant destructive sampling
136 (49) and researchers will be more inclined to include historical specimens in their analyses.

137 To facilitate informed-selection of formalin-preserved museum specimens for WGS, we set
138 out to further refine appropriate extraction and library preparation methods and to identify
139 specimen characteristics predictive of DNA extraction and sequencing success. First, we
140 investigated the relationship between residual formaldehyde concentration and pH in
141 preservation media through a survey of specimens in the Australian National Wildlife
142 Collection (ANWC; Crace, Australia). Next, in a phased approach, we compared DNA yield
143 achieved with three extraction methods - (1) hot alkaline lysis digestion followed by phenol-
144 chloroform extraction, (2) proteinase K digestion followed by phenol-chloroform extraction

and (3) proteinase K digestion followed by silica spin column purification. We then applied the best-performing DNA extraction method to terrestrial vertebrate specimens representing the broad range of tissue quality observed in museum specimens and tested performance of two library preparation methods – (1) single-stranded method v2.0 (ss2) (25) and (2) BEST double-stranded method (dsBEST) (26). Placing our results into context with a comprehensive and unbiased survey of collection-wide spirit preservation conditions, we present a decision-making framework to accelerate and facilitate genomic research using formalin-preserved specimens.

Results

Preservation media condition survey

Within 149 ANWC specimen jars surveyed (23 amphibian, 40 mammal, 40 reptile, and 46 avian), preservation media pH ranged from 4.8–8.4 with 70 (47%), 61 (41%) and 18 (12%) having neutral (6.5–7.5), low (< 6.5) and high (> 7.5) pH, respectively. Residual formaldehyde concentration ([F]) ranged from 0–40,000 mg/L. High [F] (> 1000 mg/L) was detected in 61% of low pH jars, 6% of neutral pH jars and 0% of high pH jars. We assumed specimens in jars yielding [F] = 0 (n = 82) were preserved with ethanol and without exposure to formaldehyde. Consistent with the practice of fixing specimens with unbuffered formalin combined with the gradual degeneration of formaldehyde to formic acid, the pH of the formalin-preserved samples (range 4.8–7.1; mean = 6.2) was significantly lower than for the ethanol-preserved samples (range 6.1–8.4; mean = 7.1) (T-test; $p < 0.0001$; Supplementary Figure 1A). The recorded collection date of the specimens ranged from 1936–2015. The time since collection (age) of the ethanol-preserved specimens (mean = 40.1 years) was not significantly different than the formalin-preserved specimens (mean = 36.1 years) (Supplementary Figure 1B). Among the

169 formalin-preserved samples, [F] and pH were negatively correlated ($R = -0.6$, $p < 0.001$; Figure
170 1). Age was not significantly correlated with either [F] or pH. Of the 12 specimens selected for
171 sequencing, collection date ranged from 1962–2006 and pH ranged from 4.9–8.2. Three
172 sequenced specimens were ethanol-preserved and nine sequenced specimens were formalin-
173 preserved with [F] ranging from 325–20,000 mg/L (Table 1).

174

175 **Table 1. Specimen metadata and independently assessed preservation quality metrics**
176 **for samples selected for sequencing**

177 Twelve specimens (three ethanol-preserved and nine formalin-preserved) from the ANWC
178 spirit vault were selected for DNA extraction and sequencing. Unique ANWC specimen IDs,
179 species names, common name, recorded year of collection, residual formaldehyde
180 concentration in the preservation media (mg/L), pH and tissue sampled for extraction are
181 given.

Preservation	Specimen ID	Species name	Common name	Collection year	[F] (mg/L)	pH	Tissue sampled
Ethanol	ANWC B30438	*Phalacrocorax carbo*	Great black cormorant	1977	0	8.2	Skin
	ANWC B00001	*Aquila audax*	Wedge-tailed eagle	1973	0	7.68	Liver
	ANWC M15492	*Phascolarctos cinereus*	Koala	1971	0	7	Muscle
Formalin	ANWC A02522	*Rhinella marina*	Cane toad	2002	2050	6.41	Liver
	ANWC M11465	*Macropus eugenii*	Tammar wallaby	1989	8000	5.26	Liver
	ANWC R03280	*Crocodylus porosus*	Saltwater crocodile	1973	4000	6.31	Liver
	ANWC B47838	*Melopsittacus undulatus*	Budgerigar	1996	5000	6.3	Liver
	ANWC R06312	*Pogona minima*	Dwarf dragon	1986	1800	7.04	Liver
	ANWC R01545	*Pogona vitticeps*	Central dragon	1971	325	6.24	Liver
	ANWC B40690	*Taeniopygia guttata*	Zebra finch	1986	20000	4.86	Muscle
	ANWC B34691	*Falco cenchroides*	Australian kestrel	2006	2000	5.45	Liver
	ANWC M03973	*Ornithorhynchus anatinus*	Platypus	1962	10000	5.79	Muscle

182

DNA quantification

We compared DNA yield from the hot alkaline lysis (HA), proteinase K plus phenol-chloroform (proK-PC) and proteinase K plus column (proK-col) extraction methods for the *Rhinella marina*, *Macropus eugenii* and *Crocodylus porosus* specimens and observed no significant differences between extraction methods (one-way ANOVA; Supplemental Figure 3A). However, the HA method produced more DNA from the two poor quality specimens (*M. eugenii* and *C. porosus*) compared to either of the proteinase K methods (Table 2). Thus, we predicted the HA method would perform better on specimens ranging broadly in preservation quality and we used this method to extract the remaining nine specimens. HA extraction yielded DNA detectable by high sensitivity Qubit for all twelve specimens. Two ethanol-preserved specimens (*Aquila audax* and *Phascolarctos cinereus*) and two formalin- preserved specimens (*R. marina* and *Melopsittacus undulatus*) yielded > 1,000 ng total DNA from 50 mg of tissue (Table 2). Three specimens, *Phalacrocorax carbo*, *Taeniopygia guttata* and *Ornithorhynchus anatinus*, yielded particularly low (< 100 ng) total DNA from 50 mg of tissue (Table 2). We observed no significant difference in DNA yield between ethanol and formalin-preserved specimens (T-test; Supplemental Figure 3B). However, mean DNA yield from ethanol-preserved specimens was more than double that from formalin-preserved specimens. Mean DNA yield from formalin-preserved specimens in preservation media with low pH (< 6) was not significantly different from those in media with neutral to high pH (> 6) (Supplemental Figure 3C). DNA yield was significantly higher from formalin-preserved liver tissue compared to non-liver tissue (T-test; $p < 0.05$; Supplemental Figure 3D). Both [F] and age showed a negative but non-significant correlation with DNA yield from formalin-preserved specimens (Supplemental Figures 3E and 3F).

Table 2. Sequencing and alignment statistics

For all specimens, DNA yield is given for the individual extractions of 50 mg of tissue. For the remaining metrics, the values shown were calculated having combined both the ss2 and dsBEST libraries. The number of raw reads is given as a sum of all single reads (R1 and R2) from the paired-end sequencing run. Reads aligned indicates the percent of raw reads aligned to reference genome after removal of PCR and optical duplicates. The mean aligned insert length is the mean length (in bp) of the aligned portion of the read. C_{nuc} is the coverage of the nuclear genome. C_{mt} is the proportion of mitochondrial genome with greater than 30X coverage. C_{pot} is the estimated potential genomic coverage if the full library had been sequenced, calculated from the estimated library complexity. MRM is the number of reads aligned to the mitochondrial genome per one million raw reads.

Preservation	Species	Extraction method	DNA yield from 50 mg (ng)	Raw reads (million)	Reads aligned (%)	Mean aligned insert length (bp)	C_{nuc}	C_{mt}	C_{pot}	MRM
Formalin	*Rhinella marina*	HA	1,860	434	21	65	2.2	0.78	6.2	52
		proK-col	666	77	40	81	1	0	6.2	14
		proK-PC	2,550	321	15	74	1.2	0.42	11.4	29
	Macropus eugenii	HA	271	306	8	56	0.5	0.59	2.7	50
		proK-col	4	17	1	67	0	0	0.1	3
		proK-PC	33	801	< 1	65	0	0	0.1	2
	Crocodylus porosus	HA	130	23	< 1	67	0	0	0	11
		proK-col	None detected	160	< 1	70	0	0	0.1	12
		proK-PC	79	294	< 1	62	0	0	0	2
	Melopsittacus undulatus	HA	2,400	318	20	60	3.1	0.94	23.6	201
	Pogona minima	HA	521	367	7	58	0.8	0.51	7.5	29
	Pogona vitticeps	HA	672	432	15	59	2.1	0.85	7.9	52
	Taeniopygia guttata	HA	15	62	< 1	66	0	0	0	1
	Falco cenchroides	HA	690	303	5	56	0.7	0.12	2.1	14
	Ornithorhynchus anatinus	HA	22	520	< 1	70	0	0.13	0.8	20
Ethanol	*Phalacrocorax carbo*	HA	57	292	< 1	69	0.10	0.90	0.60	50.00
	Aquila audax	HA	1,932	282	67	76	11.3	0.98	323	2515
	Phascolarctos cinereus	HA	1,254	423	60	76	5.4	0.94	93	2606

Pre-alignment library quality assessment

Prior to alignment, we used FastQC to assess the quality of paired-end reads from ss2 and dsBEST libraries. All libraries contained a high proportion of adapter content and low read quality score beginning at roughly 50 bp, consistent with highly fragmented input DNA. Focusing on the first 75 bp of the raw reads, mean sequence quality was slightly but significantly higher for read 2 (mean Phred score = 34.3) than for read 1 (mean Phred score = 33.7) across all libraries (paired T-test; $p < 0.001$). Likewise, the mean sequence quality was significantly higher in ss2 libraries compared to the corresponding dsBEST libraries for both read 1 (mean of the differences = 2.1; paired T-test; $p < 0.001$) and read 2 (mean of the differences = 0.79; paired T-test; $p < 0.01$). Mean sequence quality was not significantly different between reads derived from ethanol and formalin-preserved tissues, even when excluding libraries prepared from less than 200 ng of input DNA (paired T-test). We found evidence of cross-contamination in several libraries prepared from low DNA yield extractions. Compared to negative controls, both *O. anatinus* libraries and all but two *C. porosus* libraries showed a higher number of reads classified as genus *Mus* by Kraken2 (Supplementary Table 2). The *O. anatinus* libraries also contained a high percentage of reads classified as *Homo sapiens* (9.7% and 25%). The *O. anatinus* and *C. porosus* tissues were among those that yielded the least DNA. The *O. anatinus* HA extraction yielded just 22 ng. The *C. porosus* HA and proK-PC extractions yielded 130 and 79 ng, respectively, while the proK-col extraction yielded no detectable DNA. The only other specimens to yield less than 500 ng were the *P. carbo*, *T. guttata* and *M. eugenii*.

Relative alignment quality from three extraction methods

We used three indicators of alignment quality to compare the relative success of the three extraction methods on the *R. marina*, *M. eugenii* and *C. porosus* specimens: percent of raw reads aligned to the genome (% alignment), the number of reads aligned to the mitochondrial

242 genome per million raw reads (MRM) and the mean aligned insert length. Among these three
243 specimens, we observed no significant differences between library preparation methods in any
244 of the three alignment quality indicators (paired T-tests). Therefore, we took the mean of the
245 two library preparations to compare extraction methods across each alignment quality
246 indicator. Again, we observed no significant difference between the three extraction methods
247 applied to the *R. marina*, *M. eugenii* and *C. porosus* specimens in any of the three alignment
248 quality indicators (one-way ANOVA). All six *C. porosus* libraries yielded < 1% alignment
249 (Figure 2A and Table 2), indicating failure of all extraction and library preparation methods on
250 this specimen. Excluding the *C. porosus* libraries, we observed significant differences in MRM
251 between the extraction methods (one-way ANOVA; $p < 0.05$) with the HA method producing
252 significantly more MRM than both the proK-col and proK-PC methods (Tukey tests; $p < 0.05$).
253 We observed no significant difference in MRM between the proK-col and proK-PC methods
254 (Tukey tests) nor in % alignment or mean insert length between the three extraction methods
255 (one-way ANOVA).

256 **Effect of specimen quality on sequencing success**

257 The percentage of aligned reads removed by optical and PCR de-duplication varied between
258 8.8% and 99.5% across all libraries. Among the HA alignments, de-duplication reduced
259 significantly more mapped reads from dsBEST libraries than from ss2 libraries (paired T-test;
260 $p < 0.01$). Combining the ss2 and dsBEST libraries for each HA extraction, de-duplication
261 removed more than double the percentage of reads (69.8% versus 32.8%) from poor quality
262 specimens (those yielding < 1% reads aligned) compared to better quality specimens (those
263 yielding > 1% reads aligned). However, this difference was not significant (T-test). De-
264 duplication removed significantly more reads from the formalin-preserved specimens (mean =
265 54.6%) than from the ethanol-preserved specimens (mean = 16.7%) (T-test; $p < 0.01$).
266 Following de-duplication, the mean percent of mapped reads remaining was 44% and 59% for

the dsBEST and ss2 HA libraries, respectively. Across all specimens extracted using the HA method, we observed no significant differences between library preparation methods in any of the three alignment quality indicators (paired T-tests). Therefore, we conducted further comparison of the effect of specimen quality on alignment success taking the mean of each alignment quality indicator from the two HA library preps.

HA extraction of one of three ethanol-preserved specimens (*P. carbo*) and three of nine formalin-preserved specimens (*C. porosus*, *T. guttata* and *O. anatinus*) produced < 1% aligned reads (Table 2), indicating equal rates of very poor sequencing success with ethanol- and formalin-preserved tissues. Excluding the specimens with < 1% aligned reads, the ethanol-preserved specimens produced a significantly higher percentage of aligned reads (T-test; $p < 0.01$). Two of the three ethanol-preserved specimens (*A. audax* and *P. cinereus*) produced > 60% aligned reads while the remaining six formalin-preserved specimens (*R. marina*, *M. eugenii*, *M. undulatus*, *Pogona minima*, *Pogona vitticeps* and *Falco cenchroides*) produced between 5% and 21% aligned reads (Table 2). Excluding the specimens with < 1% aligned reads, the mean insert length was significantly longer for the ethanol-preserved specimens (mean = 76 bp) compared to the formalin-preserved specimens (mean = 59 bp) (T-test; $p < 0.0001$). MRM was also significantly higher for the ethanol-preserved specimens (mean = 2,560) compared to the formalin-preserved specimens (mean = 43) (T-test: $p < 0.01$).

The percentage of reads aligned increased with preservation media pH ($R = 0.44$; Figure 3A), decreased with preservation media [F] ($R = -0.53$; Figure 3B) and decreased with specimen age ($R = -0.46$; Figure 3C), although these correlations were not statistically significant. The percentage of aligned reads was significantly higher in specimens sampled with liver than those sampled with muscle and skin (T-test; $p < 0.05$; Figure 3D). Of the specimens yielding poor sequencing success (< 1% reads aligned), all but *C. porosus* were sampled with either muscle

or skin as liver was not present. The only specimen sampled with a tissue other than liver to yield a percent of reads aligned > 1% was the ethanol-preserved *P. cinereus*.

Genome sequencing coverage

Nuclear genome coverage (C_{nuc}) of the deduplicated alignments was < 1X for the majority of libraries. Since raw read yield was highly variable, C_{nuc} is not an appropriate measure with which to compare the extraction or library preparation methods. However, it is noteworthy that we achieved C_{nuc} > 1X for two of the ethanol-preserved specimens and three of formalin-preserved specimens. Combining all libraries for a given specimen, we achieved a total of 5.4X and 11.3X C_{nuc} for the ethanol-preserved *P. cinereus* and *A. audax* specimens, respectively (Table 2). Likewise, we achieved a total of 2.1X, 3.1X and 4.4X C_{nuc} for the formalin-preserved *P. vitticeps*, *M. undulatus* and *R. marina* specimens, respectively (Table 2). To estimate the potential for improving C_{nuc} through re-sequencing of the prepared libraries, we calculated potential genomic coverage (C_{pot}) (Table 2). Combining all libraries for a given specimen, C_{pot} exceeded 20X for the *R. marina* and *M. undulatus* and exceeded 75X for the *P. cinereus* and *A. audax*. Focussing on the mitochondrial genome, the proportion of sites with 30X or higher coverage (C_{mt}) was nearly complete (> 0.9) for all three ethanol-preserved specimens (Table 2). C_{mt} for the formalin-preserved *M. undulatus* (0.94) was comparable to that of the ethanol-preserved specimens. C_{mt} was moderate to high (> 0.5) for five of the formalin-preserved specimens (Table 2). Only the *C. porosus*, *T. guttata*, *F. cenchroides* and *O. anatinus* yielded very poor C_{mt} (< 0.15).

Read length periodicity

From the aligned insert lengths estimated with Picard, we plotted the frequency of reads between 50 and 100 bp (Figure 4). This plot revealed a pattern of read length periodicity in several specimens, notably those that resulted in higher mapping success. We observed prominent periodicity of approximately 10.1 bp in the *R. marina* specimen extracted with the

316 proK-PC method. While less pronounced, we observed read length periodicity of
317 approximately 10.8 bp in the HA extractions of *R. marina*, *P. vitticeps*, *P. minima*, *F.*
318 *cenchroides*, *A. audax* and *P. cinereus*. The pattern of periodicity was observed in both the
319 dsBEST and ss2 libraries, however, it was slightly more pronounced in the dsBEST libraries.

320 ## Discussion

321 In this study, we present evidence challenging the common perception that formalin-preserved
322 museum specimens are devoid of accessible DNA. Processed with a tailored molecular and
323 bioinformatic workflow, formalin-preserved specimens had an overall sequencing success rate
324 equivalent to ethanol-preserved specimens, albeit with recovery of a lower percentage of
325 sequence reads mapping to the reference genome. Contrary to popular belief, we found
326 genome-wide nuclear data is retrievable from some formalin-preserved museum specimens,
327 even with a moderate investment of sequencing effort (with 30% of formalin-preserved
328 specimens, we achieved > 2X nuclear genome coverage from 300-500 million raw reads). We
329 also show reconstruction of large sections of the mitochondrial genome is possible even in poor
330 quality specimens where limited nuclear data were recovered (with 55% of formalin-preserved
331 specimens, we achieved > 30X coverage of more than 50% of the mitochondrial genome).
332 Investigating specimens covering a range of preservation quality, we also developed a decision-
333 making framework to improve sequencing success rate and prioritize suitable specimens. Our
334 findings support a considered and targeted sequencing approach that transforms thousands of
335 spirit collection specimens into a new molecular resource. Improved access to genomic data
336 held in these specimens has the potential to inform research into the mechanisms driving
337 adaptation, evolution, speciation and extinction.

Hot alkaline lysis effectively recovers gDNA from formalin-preserved archival tissues suitable for next generation sequencing.

Originally developed for DNA extraction from FFPE sections, the HA method relies on high heat (120°C) under alkaline conditions (pH = 13) to break strong inter- and intramolecular cross links and utilizes organic extraction to maximize capture of fragmented gDNA from formalin-preserved tissues (50–52). This method has been applied to museum specimens to successfully recover sections of the mitochondrial genome in trout (53) and full mitochondrial genomes from lizards (40) and bacterial symbionts (41). Here we show the HA yields gDNA in adequate quantities for WGS from higher-quality formalin-preserved museum specimens. Coupled with library preparation methods designed to efficiently convert degraded DNA, we produced complex sequencing libraries with the potential to recover full vertebrate genomes when mapped using a strategy optimized to maximize recovery of endogenous sequence. Our results indicate that the HA method is appropriate for DNA extraction from a broad range of taxa preserved under various conditions, making it well-suited for application in both museum and pathological settings.

In a small-scale comparison to proK digestion with either phenol-chloroform extraction or column purification, the HA method performed superiorly for poor quality formalin-preserved specimens. We experienced equal success rates with the HA method in formalin and ethanol-preserved tissues. It is not standard practice to apply the HA method to ethanol-preserved specimens, which do not suffer from cross-linking, but we implemented it in this study to serve as a comparison to formalin-fixed tissues. Thus, while the HA method is likely unnecessarily harsh for recovery of DNA from tissues not crosslinked with formaldehyde, we propose this extraction method is suitable across a wide range of tissue qualities and preservation conditions observed in museum spirit collections. And, given that we achieved relatively high yield from the ethanol-preserved tissues, we propose that the HA method is appropriate in cases where

363 contact with formalin cannot be determined. We caution; however, the HA method's success
364 may be limited to DNA-rich tissues such as liver. Our HA extractions of formalin-preserved
365 muscle and ethanol-preserved skin tissue failed to yield adequate gDNA for sequencing, while
366 our HA extraction of ethanol-preserved muscle tissue was less successful than our extraction
367 of ethanol-preserved liver tissue. HA extraction has been previously observed to perform
368 poorly compared to cetyltrimethylam-monium bromide (CTAB) protocols on formalin-
369 preserved mammalian heart tissue (54). We also note that, preservation conditions being equal,
370 DNA yield may differ between taxonomic groups due to factors such blood cell nucleation.
371 Due to low sample size, we were not able to test if the lack of nucleated red blood cells in
372 mammal tissues impacted DNA yield.

373 **aDNA library preparation methods effectively capture DNA extracted from formalin-**
374 **preserved archival tissues**

375 DNA degradation in museum specimens is a significant challenge to genome sequencing. To
376 improve our conversion of degraded DNA from formalin-preserved tissues into high quality
377 library molecules, we utilized two library preparation methods developed specifically for
378 degraded aDNA templates. We tested the ss2 (46) and dsBEST (47) methods on DNA extracted
379 from both ethanol and formalin-preserved archival tissues. Sequence quality was significantly
380 higher for libraries prepared using the ss2 method compared to the dsBEST protocol. However,
381 this quality difference did not result in significantly lower rates of read alignment or reduced
382 mapped insert length for the dsBEST libraries. While we did not see differences in
383 contamination rates between the two methods, an advantage of the dsBEST method is its
384 reliance on fewer tube transfers and additions of solution, thus reducing opportunities to lose
385 DNA and introduce contaminants. The ss2 and dsBEST methods performed similarly on all
386 twelve of our archival templates, indicating both are well-suited to prepare libraries from DNA
387 extracted from ethanol and formalin-preserved tissues. Alternative library preparation methods

388 developed specifically for degraded DNA may prove equally effective. To maximize
389 conversion of fragmented archival DNA template, we advise using a library preparation
390 method designed to capture small fragments whilst minimising contamination risk. Overall, we
391 observed samples with very low DNA yield (< 200 ng from 50 mg of tissue) did not produce
392 libraries with high rates of mapping success. Thus, as a cost-saving measure, we advise
393 quantifying DNA templates prior to library preparation and focussing sequencing effort on
394 higher yielding samples.

395 **High alignment rates of fragmented DNA are achieved through exhaustive match**
396 **searching**

397 Removal of adapter sequence and low-quality bases via read-trimming is a standard pre-
398 processing procedure conducted on raw sequencing reads prior to mapping. In the context of
399 libraries prepared from highly degraded templates, filtering and trimming can reduce the
400 dataset substantially. For example, pre-processing of the library prepared from a formalin-
401 preserved *Anolis* lizard reduced the dataset to 13.5% of the raw data (40). Although filtering
402 and trimming are effective at removing PCR duplicates and erroneous bases introduced through
403 library preparation and sequencing, quality control parameters should be optimized to avoid
404 removing informative endogenous sequence, particularly with data derived from highly
405 fragmented low-input templates. Compared to DNA extractions from fresh tissue, our
406 extractions from formalin-preserved specimens were highly fragmented as is typical of aDNA
407 sources (55). We opted to trial a computationally efficient approach that eliminates loss of
408 endogenous sequence during pre-processing. The kalign function from the open source kit4b
409 toolkit performs alignments of raw reads by searching for the maximum length match within
410 the read to the reference sequence regardless of the match's position within the read. For each
411 raw read, kalign performs a rapid complete exhaustive match search across the indexed
412 reference genome. The match search is performed recursively through seed expansions

generated along the read length. The longest match to endogenous sequence is retrieved while satisfying the minimum length threshold of the match. Using this approach, we aligned up to 21% and 67% of raw reads from formalin and ethanol-preserved tissues, respectively. These alignment rates are consistent with the degree of degradation in the DNA we extracted from spirit-preserved museum specimens being intermediate between that of fresh and truly ancient tissues. A previous application of the ss2 method yielded a maximum of 11.3% mappable reads from libraries prepared from aDNA tissue sources (25). The same study yielded 60% and 68% mappable reads from libraries prepared from horse and pig liver stored in buffered formalin for 5 and 11 years, respectively (25). In comparison, our modest alignment rates may be the result of tissues of intermediate age and using a different metric of calculating the percent of mapped reads.

Sequencing success is strongly influenced by specimen integrity prior to fixation

To explore the effects of formalin-fixation on sequencing success, we selected three specimens preserved with ethanol only and nine specimens preserved with formalin. We found no significant difference in DNA yield between the ethanol and formalin-preserved specimens and the differences we observed in DNA fragment lengths were minimal. Furthermore, we observed equal rates of very poor sequencing success within ethanol and formalin-preserved specimens, indicating preservation method is not a strict determinant of sequencing success. Older, poor-quality ethanol-preserved specimens have previously been shown to be as problematic for genomic analyses as formalin-preserved specimens (42,56). This is not to say preservation method does not impact sequencing success. Two of our ethanol-preserved specimens (*P. cinereus* and *A. audax*) had much higher mapping rates (60% and 67% reads aligned, respectively) than even our most successful formalin-preserved specimens (*R. marina,* produced 21% reads aligned with the HA method). Our findings indicate WGS of formalin-preserved museum specimens is possible using HA extraction paired with a library preparation

optimized for conversion of degraded DNA. However, as with all potential DNA sources, the overall integrity of the tissue will ultimately determine sequencing success.

The specimens with poor sequencing success (< 1% reads aligned) were largely older, their preservation media had lower pH and higher [F] and they were sampled with a tissue other than liver. On the contrary, the specimens with better sequencing success were preserved more recently, their preservation media had neutral pH and lower [F] and the tissue sampled was liver. We calculated the correlation between specimen quality measures ([F], pH, age and tissue type) and both DNA yield and mapping success. Tissue type was the only quality measure significantly associated with lower DNA yield, with liver yielding significantly more DNA than either muscle or skin. Our higher success with liver is consistent with findings of a previous study comparing sequencing success from liver, muscle and tail-tip in a formalin-preserved *Anolis* lizard (40). However, in that study, the tissues were extracted using different methods and thus it could not be determined if success was driven by tissue type or extraction method.

Post-mortem DNA degradation occurs more rapidly in liver relative to other bodily tissues including skeletal muscle, heart and brain (57,58). In the museum curatorial setting, specimens undergo varying degrees of post-mortem decay prior to fixation. As is the case for most museum specimens, the length of the post-mortem interval (PMI) was not recorded for the specimens used in this study. Given expected rapid decay of the viscera, we used the visual appearance of the gut contents as a reasonable proxy for the length of the PMI. The four specimens used in this study that lacked liver tissue were visibly more degraded than those with intact liver tissue (Supplementary Figure 2). In the case of the *P. cinereus*, *P. carbo* and *O. anatinus*, the complete absence of viscera indicated the internal organs were likely well-degraded and discarded prior to fixation. For specimens preserved after a long PMI, DNA integrity throughout the carcass would be lower than in specimens preserved after a short PMI.

463 Therefore, we conclude that the higher yield from specimens sampled with liver is a reflection
464 of overall specimen quality and DNA damage occurring post-mortem but prior to fixation.

465 **Re-thinking formalin damage**

466 Formalin-preserved museum specimens have long been considered intractable sources of
467 gDNA. Encouragingly, we found specimen contact with formaldehyde does not prohibit DNA
468 sequencing if tissue decomposition occurring prior to fixation is minimized. With appropriate
469 sample vetting (Figure 5), HA extraction and DNA library preparation optimized for degraded
470 DNA, historical genomic data may be extracted from many formalin-preserved specimens.
471 These data will not be of similar quality to those recovered from fresh or ethanol-preserved
472 tissues. However, higher sequencing volume and borrowing of analytical methods from the
473 field of aDNA may facilitate reconstruction of historical genomes from formalin-preserved
474 tissues. We found evidence that DNA damage in formalin-preserved specimens shares
475 characteristics with that of aDNA. In addition to capturing shorter fragments with low mapping
476 rates, we observed a pattern of read length periodicity of approximately 10 bp. This is
477 consistent with observations in aDNA specimens (59) and is an interval that coincides with the
478 length of a turn of the DNA helix. Pederson et al (2014) attributed the 10 bp read periodicity
479 in specimens greater than 4,000 years old to protection of the DNA by nucleosomes
480 preferentially positioned at 10 bp intervals. We observed a striking periodicity pattern
481 averaging 10.8 bp in HA extracted samples and 10.1bp in the proK-PC samples. The shorter
482 periodicity in the proK treated samples may be due to reduced protection of the ends of DNA
483 fragments by digestion of the nucleosomes during extraction. We did not observe a signal of
484 nucleosome occupancy in read depth or in enrichment of fragments of nucleosome length (147
485 bp) as did Pederson et al., perhaps because we sequenced shorter fragments to comparatively
486 low depth. However, the appearance of 10 bp periodicity suggests it may be possible to infer

nucleosome occupancy from patterns of DNA degradation observed in formalin-preserved specimens if higher coverage is achieved.

Managing expectations

We have shown WGS of formalin-preserved museum specimens is feasible and success can be improved through specimen quality vetting. We stress; however, measures of specimen quality are imperfect and the key parameters may vary between and within museum collections. Modern collection institutions aim to limit light exposure and temperature variation within their spirit vaults. With older specimens, the likelihood they have been exposed to undocumented DNA-degrading conditions increases. We found the age of the specimen was not strongly predictive of sequencing success, however, we did not sample specimens collected prior to the 1960s. This warrants further investigation into the extent to which intact DNA can be extracted from much older formalin-preserved specimens.

While preservation media pH and [F] were not predictive of sequencing success in our specimens, we note these measures do not always accurately reflect preservation condition. Most institutions periodically top up the specimen jars in their spirit vaults to replace ethanol lost through evaporation. In some cases, the preservation media is replaced entirely. Thus, media pH and [F] values at the time of sampling for sequencing may not reflect preservation and long-term storage conditions. With additional sampling of older and more varied specimens, it may be possible to establish clear correlates of sequencing success associated with pH and [F].

Both researchers and museums would benefit from an improved set of guidelines for strategic decision making based on independent quality metrics rather than qualitative *ad hoc* assessments. This will empower researchers to most effectively deploy their sequencing budgets and support museums in deciding when to grant requests for destructive sampling. A

cost-benefit analysis should be conducted prior to genomic sequencing of museum specimens. From the perspective of the museum, destructive sampling should be avoided if the specimen is unlikely to yield sufficient DNA to achieve a project's aims. From the perspective of the researcher, sequencing of high-quality specimens should be prioritized to generate high-quality data. To assist in making these assessments, we provide a decision-making tree (Figure 5) for use by both curators and researchers to determine which specimens are likely to be appropriate for genomic analyses.

Ultimately, museum curators decide if the potential benefit of sequencing outweighs the damage to the specimen through destructive sampling. Once sampling and DNA extraction has been completed, the decision to proceed with library preparation and sequencing can be made on the basis of DNA yield. We found specimens with high DNA yield (> 1,500 ng/50 mg tissue) produced a high percentage (> 20%) of mappable reads while specimens with low DNA yield (< 200 ng/50 mg tissue) produced virtually no mappable reads. While specimens yielding between 200–1,500 ng of DNA per 50 mg tissue produced relatively low genomic coverage, they did produce high coverage of the mitochondrial genome. Thus, reconstruction of historical mitochondrial haplotypes may be possible from specimens yielding low quantities of DNA. When nuclear data is required, high-volume sequencing should be reserved for high-quality specimens. Generally speaking, most research projects aim to sequence a small number of museum specimens with which to provide a base-line for comparison to contemporary specimens. In light of the limited availability of historical specimens in collections, it is often reasonable and feasible to allocate a relatively large budget to conduct deep sequencing of a small number of specimens.

Conclusions

Our results demonstrate formalin-fixation is not a complete barrier to WGS in museum specimens. While success is not a guarantee, the use of HA lysis for DNA extraction followed by an appropriate sequencing library preparation optimized for degraded DNA can produce libraries of sufficient complexity for genomic analyses. When selecting specimens for sequencing, our results indicate those with poor gut integrity are least likely to yield sufficient DNA for sequencing.

Methods

Preservation media condition survey

We conducted an unbiased survey of the ANWC spirit vault to measure variation in preservation characteristics that can be sampled without disturbing the specimen. We randomly selected 149 specimen jars spanning a range of taxonomic groups and ages, and removed a 25 mL aliquot of preservation media. We measured pH using an Orion™ Versa Star Pro™ benchtop pH meter (*Thermo Scientific*) and [F] using MQuant® test strips (*Merck*). Where [F] was at the upper detection limit of the test strips, we diluted the aliquot 1:10 with ultrapure water and remeasured, extrapolating the neat concentration of the media by multiplying the measurement by the dilution factor.

Specimen selection

To select specimens for genomic sequencing, we first identified those with a publicly available whole-genome reference for the specimen species or closely related species. Of these specimens, we selected 12 representing a range of taxonomic groups, preservation conditions and ages and sampled 50 mg of tissue. We sampled liver tissue when it was available. Muscle was sampled from an ethanol-preserved *P. cinereus* specimen and from formalin-preserved *T. guttata* and *O. anatinus* specimens. Skin was sampled from an ethanol-preserved *P. carbo*. All

specimens sampled with liver were preserved as whole animals whereas substantial portions of the body were absent from those specimens sampled with muscle or skin (Supplementary Figure 2). From the nine formalin-preserved specimens, we selected three with which to test the relative success of three DNA extraction methods. To represent "good" quality formalin-preserved specimens, we selected a cane toad (*R. marina*) preserved in 2002. Visually, this specimen appeared minimally degraded and measurements of the storage media indicated low [F] and a neutral pH. To represent "poor" quality formalin-preserved specimens, we selected a tammar wallaby (*M. eugenii*) preserved in 1989 and a saltwater crocodile (*C. porosus*) preserved in 1973. Visually, these two "poor" specimens were reasonably well-preserved, however, measurements of the storage media indicated substantial [F] in both specimen jars and mildly acidic pH in that of the wallaby.

Tissue preparation

Prior to DNA extraction, we liquid nitrogen pulverized all dissected tissue into a fine powder using a cryoPREP® (*Covaris*) dry pulverizer (three impacts to a TT05 tissueTUBE^{TM} on intensity setting three; 10 sec in liquid nitrogen between impacts). We then stored the pulverized tissue powder in 70% ethanol at -80°C until further processing. We re-hydrated the pulverized tissue by stepping it into 50% ethanol, 30% ethanol then TE buffer with rocking for 10 min intervals. For the nine formalin-fixed tissues, we quenched excess formaldehyde by rocking for 2 hrs in 1 mL GTE buffer (100 mM glycine, 10 mM Tris-HCL, pH 8.0, 1 mM EDTA), followed by a further wash in fresh GTE for 2 hrs and a final fresh GTE wash overnight at room temperature. We removed the GTE buffer and washed with rocking in sterile water for 10 min.

Proteinase K DNA extraction

We conducted two variations on a standard proteinase K (proK) digestion. For each specimen, we digested two 50 mg (wet weight) aliquots of tissue overnight at 55°C with 30 µL of 20

582 mg/mL proteinase K in 970 µL lysis buffer (10 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM
583 EDTA, 1% SDS). We isolated DNA from the proK lysates with either (A) three extractions of
584 phenol-chloroform followed by ethanol precipitation (proK-PC), resuspending the DNA in 30
585 µL TE, or (B) a QIAquick PCR purification column (*Qiagen*) (proK-col), following the
586 manufacturer's instructions and eluting the DNA in 30 µL TE. Alongside the museum tissues,
587 we processed tissue-free controls. We quantified extracted dsDNA using a Qubit fluorometer
588 and high sensitivity (HS) DNA kit (*Invitrogen*).

589 **Hot alkaline lysis DNA extraction**

590 For the hot alkaline lysis (HA) extractions, we heated 50 mg (wet weight) tissue aliquots to
591 120°C for 25 min in 500 µL of alkali buffer (0.1 M NaOH with 1% SDS, pH 13) according to
592 methods described in (52). We purified DNA from the lysate with three phenol-chloroform
593 extractions followed by ethanol precipitation, resuspending the DNA in 30 µL TE. Alongside
594 the museum tissues, we processed tissue-free controls. We quantified extracted dsDNA using
595 a Qubit fluorometer and HS DNA kit.

596 **Library preparation methods**

597 To avoid cross-contamination, we prepared all sequencing libraries in the Ecogenomics and
598 Bioinformatics Laboratory trace facility at the Australian National University following
599 standard anti-contamination procedures. We prepared libraries from all DNA extracts and
600 tissue-free controls using two methods developed for high efficiency conversion of fragmented
601 aDNA; the single-stranded method v2.0 (ss2) (25) and the BEST double-stranded method
602 (dsBEST) (26). Concurrently, we prepared DNA-free control libraries. For sequencing of Read
603 1 in both library preparation methods, we used an adapter with the sequence 5`–
604 AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC–3`. For sequencing of Read 2, we
605 used adapters with the sequences 5`–GGAAGAGCGTCGTGTAGGGAAAGAGTGT–3` and
606 5`–AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT–3` for the ss2 and dsBEST

607 methods, respectively. We removed excess adapter and primer dimer by isolating fragments
608 between 160 bp and 400 bp from the resulting libraries using the PippinHT size-selection
609 system (*Sage Science*). We further purified the libraries with a MinElute PCR purification kit
610 (*Qiagen)* and quantitated the library concentrations using the LabChip GXII (*PerkinElmer*)
611 capillary electrophoresis system. We then pooled the libraries in approximately equimolar
612 concentrations and measured the concentration of the final pooled library using a Qubit
613 fluorometer and HS DNA kit. The Australian Genome Research Facility sequenced the pooled
614 library on a 150 bp paired-end S4 flow cell on the Illumina NovaSeq 6000 platform.

615 **Quality control of raw reads**

616 We computed quality control metrics for the raw reads using FastQC v.0.11.8 (60). Our adapter
617 content analysis included both default Illumina adapters and our custom library adapters. To
618 rapidly detect library contamination by non-target species' DNA, we classified the taxonomic
619 origin of reads using Kraken2 v.2.0.9b (61). We estimated the number of unique fragments
620 present in the raw sequence libraries with the EstimateLibraryComplexity function of PICARD
621 v.2.9.2 (62).

622 **Alignment**

623 We aligned reads to reference nuclear and mitochondrial genomes obtained from the DNA Zoo
624 Consortium (63,64) and GenBank (65) (Supplementary Table 1). Species-specific reference
625 genomes were not available for three of the specimens. For *A. audax*, *F. cenchroides* and *P.*
626 *minima,* we used the reference genomes of species in the same genera- *A. chrysaetos*, *F.*
627 *perigrinus* and *P. vitticeps*, respectively (Supplementary Table 1). We hard-masked the eleven
628 genomes with RepeatMasker v.4.1.0 (66) including our ss2 and dsBEST library adapters in the
629 repeat database and applying the -qq option allowing 10% less sensitivity while decreasing
630 processing time. We aligned raw reads with the kalign function of the ngskit4b tool suite
631 v.200218 (67) with options -c25 (--minchimeric=<int>; minimum chimeric length as a

632 percentage of probe length) -l25 (--minacceptreadlen=<int>; after any end trimming only
633 accept read for further processing if read is at least this length) -d50 (--pairminlen=<int>;
634 accept paired end alignments with observed insert sizes of at least this) -U4 (--pemode=<int>;
635 paired end processing mode: 4 - paired end no orphan recovery treating orphan ends as SE).
636 We removed PCR and optical duplicates from the alignments using the MarkDuplicates
637 function of PICARD enabling REMOVE_DUPLICATES=TRUE. For each de-duplicated
638 alignment, we computed a histogram of aligned insert lengths and calculated the mean aligned
639 insert length using the CollectInsertSizeMetrics function of PICARD.

640 **Genome coverage analyses**

641 We estimated nuclear genome coverage (C_{nuc}) as the number of unique aligned reads multiplied
642 by the mean insert length divided by unmasked genome size. To estimate how much genomic
643 coverage could be achieved by increasing sequencing depth, we calculated the sequenced
644 proportion of the prepared library as the number of read pairs examined divided by the
645 estimated library size. We estimated the number of possible reads represented in the prepared
646 library by dividing the number of actual reads aligned by the sequenced proportion of the
647 library. We then roughly estimated the potential genomic coverage represented in the full
648 prepared library (C_{pot}) as: $(\#\ possible\ reads \times mean\ insert\ length\ (bp)) \div$
649 $genome\ size\ (bp)$. To calculate the proportion of mitochondrial genome sites with 30X or
650 greater coverage (C_{mt}), we executed the Samtools *depth* function (68) on SAM files for the
651 mitochondrial contigs for each species combined across all libraries.

652 **Statistical analyses**

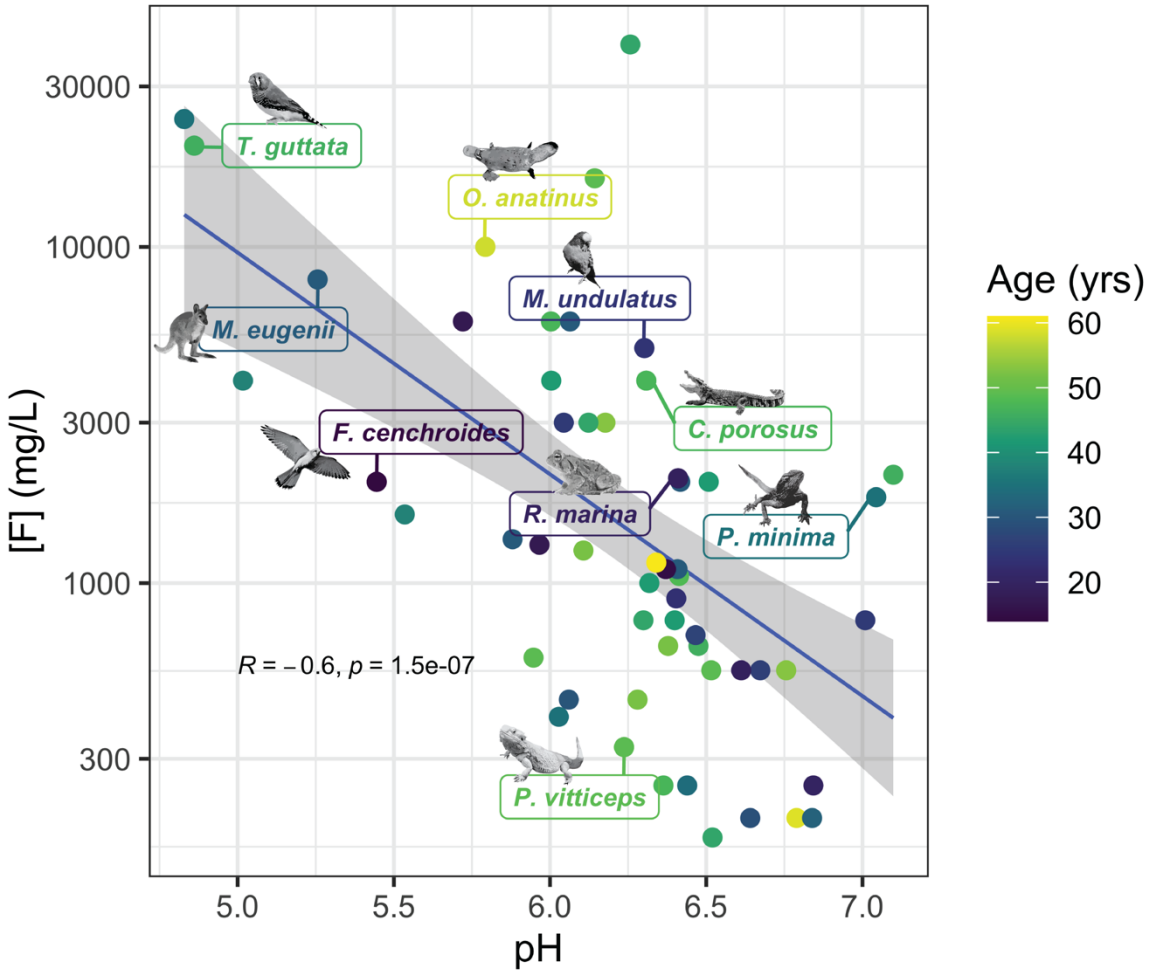
653 We performed statistical analyses in the R environment, v.4.0.2 (69) and produced figures
654 using the packages *ggplot2* (70) and *ggpubr* (71). To test if the residuals of data were normally
655 distributed, we ran Shapiro-Wilk tests with the function *shapiro.test*. We conducted T-tests
656 with the function *t.test*, analyses of variance (ANOVA) with the function *aov* and computed

confidence intervals using Tukey's Honest Significant Difference method (Tukey test) with the function *TukeyHSD* in the base package *stats*. We computed Pearson correlation coefficients with associated p-values with the *ggpubr* function *stat_cor*.

Figures

Figure 1. Preservation media survey results of formalin-fixed specimens in the Australian National Wildlife Collection

Residual formaldehyde concentration [F] (mg/L) is shown on a log-scale in relation to pH. Individual specimens (N = 65) are colored by the time since their collection (age) and the specimens selected for sequencing are indicated by species name. A linear model was used to fit a regression line and standard error is shown in grey; R = Pearson's correlation coefficient.



669 **Figure 2. Effectiveness of extraction and library preparation methods for *R. marina*, *C.***

670 ***porosus* and *M. eugenii* specimens.**

671 (A) Alignment to the whole genome expressed as the percentage of reads aligning (B)

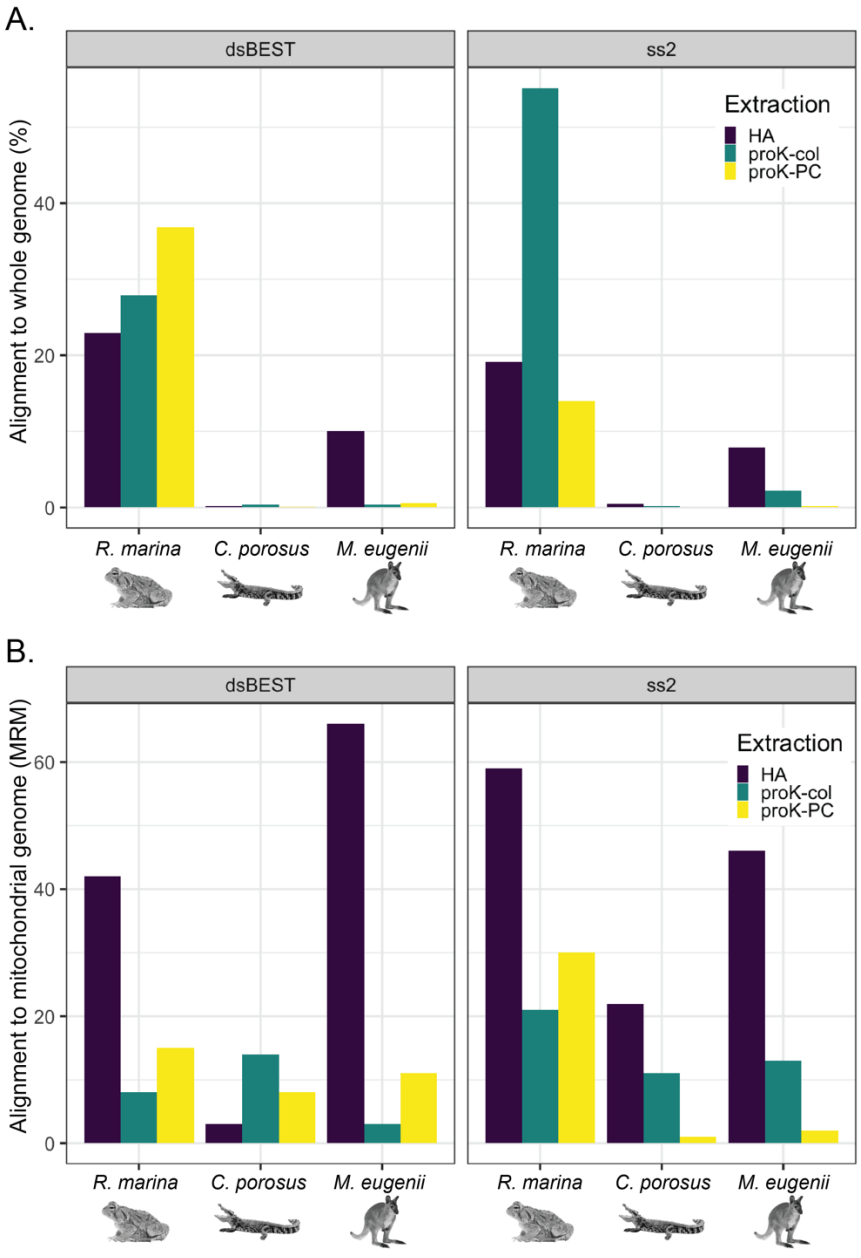
672 Alignment to the mitochondrial genome expressed as the number of reads aligned per million

673 raw reads (MRM). dsBEST = BEST double-stranded method (26); ss2 = single-stranded

674 method v2.0 (25); HA = hot alkaline lysis; proK-col = proteinase K digestion followed by

675 column purification; proK-PC = proteinase K digestion followed by phenol-chloroform

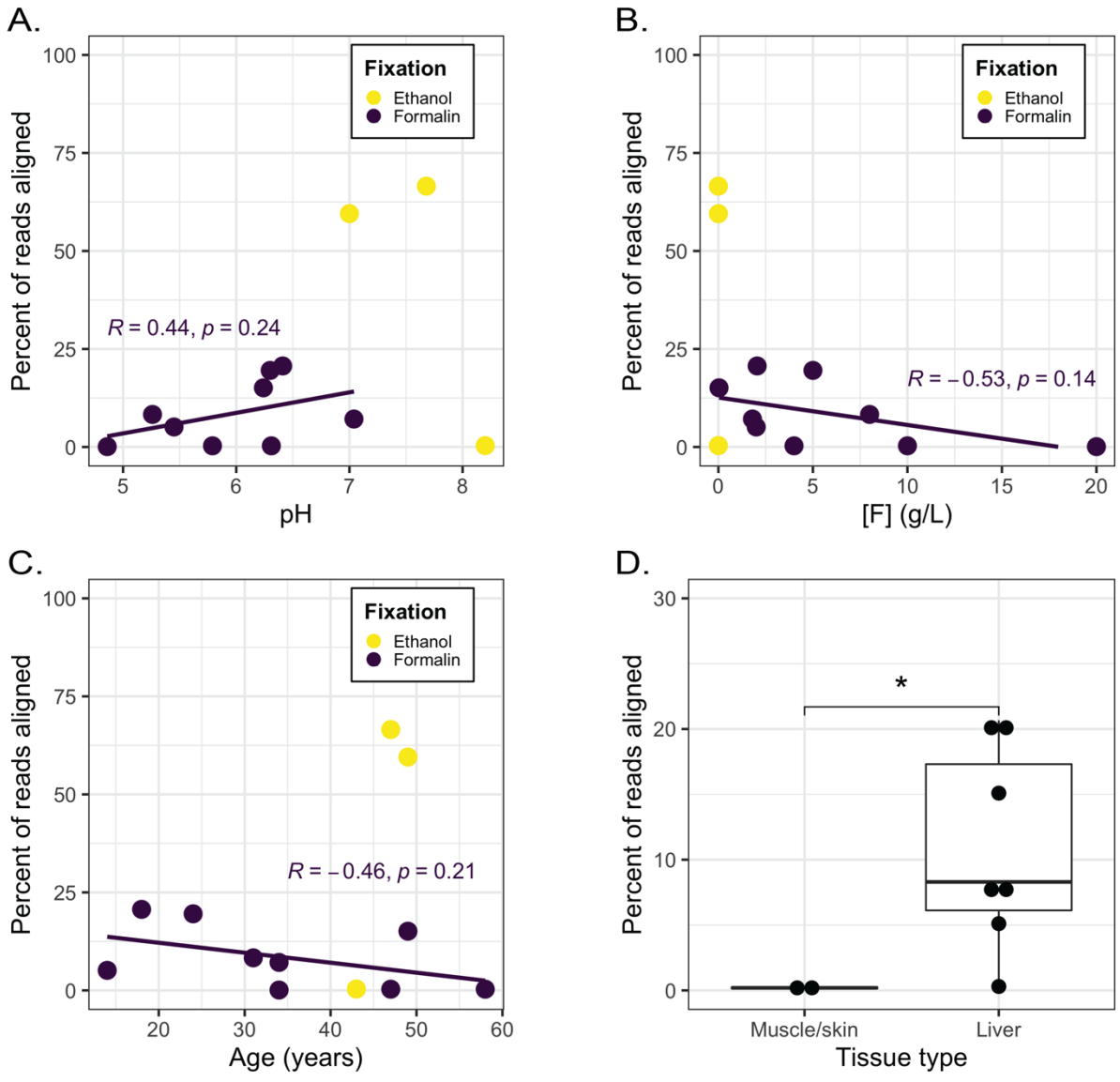
676 extraction.



677

678 **Figure 3. Alignment results for hot alkali extracted samples**

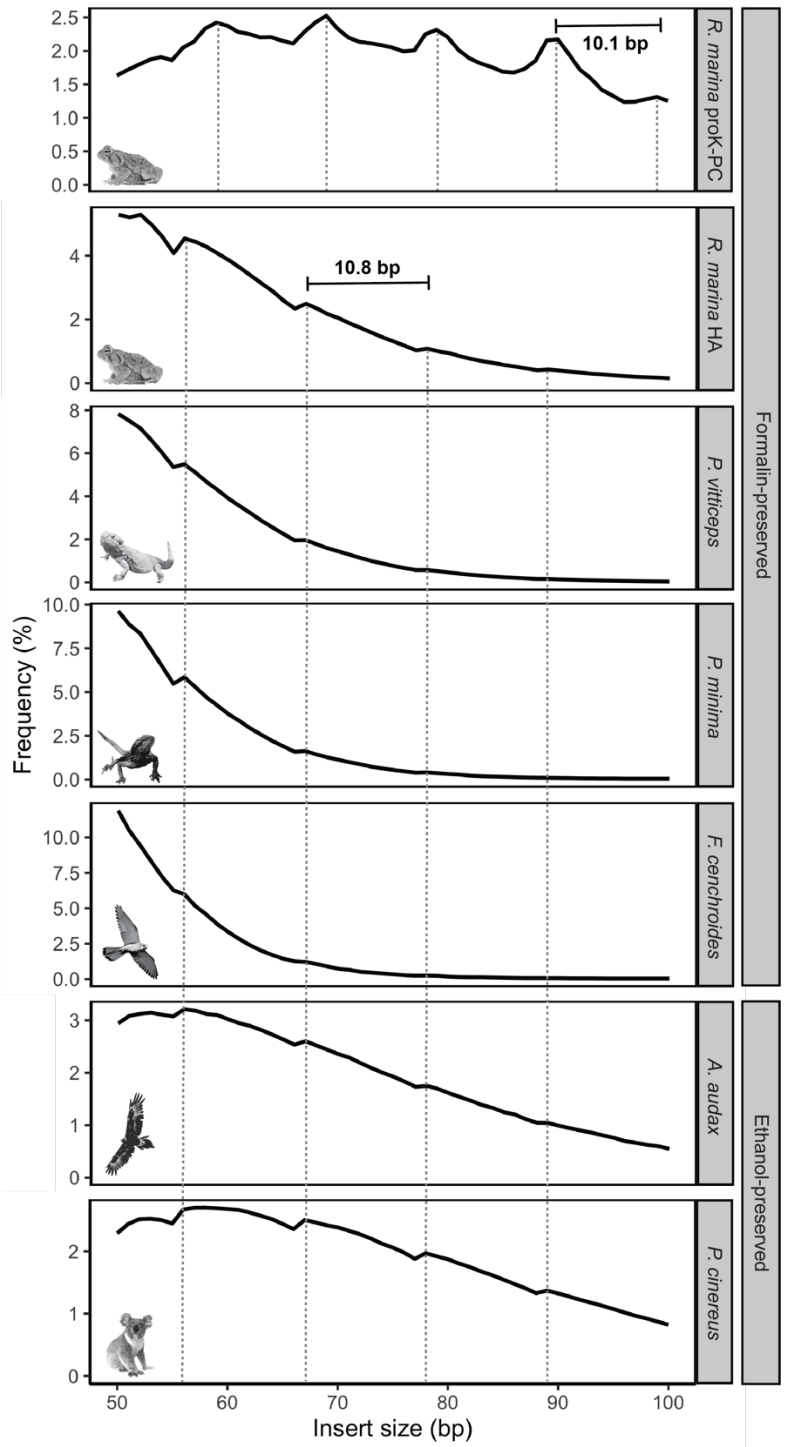
679 The correlation between the percentage of reads aligned to the whole genome (combining
680 both library preparations of the hot alkali extracted specimens) and (A) preservation media
681 pH, (B) preservation media formaldehyde concentration (g/L), (C) number of years in the
682 collection and (D) tissue sampled. In A-C, all specimens are shown colored by their fixation
683 type and R = Pearson's correlation coefficient for the formalin-fixed specimens. In D, only
684 the formalin-preserved specimens are plotted and individual specimens are shown with black
685 dots, * = $p < 0.05$.



686

687 **Figure 4. Libraries with read periodicity**

688 The frequency of insert lengths, in bp, estimated from the mapped dsBEST libraries is shown
689 for six preserved specimens. Read periodicity in the *R. marina* libraries from the proteinase K
690 with phenol-chloroform (proK-PC) extractions averages 10.1 bp while periodicity in libraries
691 from the hot alkali extractions of six specimens averages 10.8 bp.



692

693 **Figure 5. Decision-making tree for a priori estimation of likely sequencing success in**

694 **spirit-preserved museum specimens.**

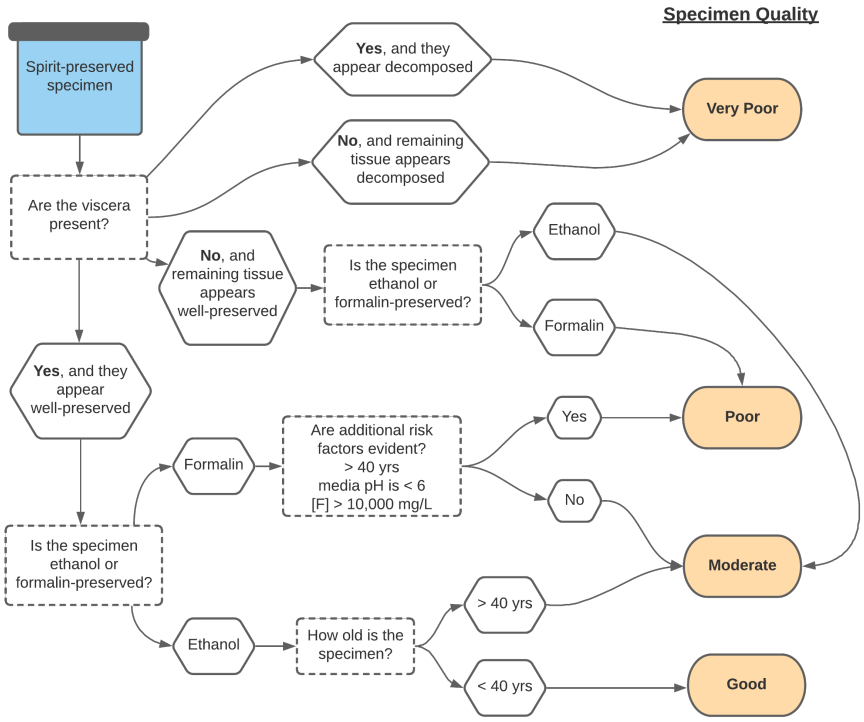
695 Green ticks indicate the specimen is well-suited the sequencing application and there is a high

696 likelihood of success. Black question marks indicate the specimen is marginal for the

697 sequencing application and there is high variation in the likelihood of success. Red crosses

698 indicate the specimen is not well-suited for the sequencing application and there is a low

699 likelihood of success.



700

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The sequencing data generated and analysed in this study are archived in the CSIRO Data Access Portal. Correspondence and requests for materials should be addressed to CEH (clare.holleley@csiro.au)

Competing interests

The authors declare they have no competing interests.

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Authors' contributions

This study was conceived by CEH. Experiments were designed by CEH, MRA and AG and conducted by MRA and AG. Data analysis was conducted by EEH, JS, MRA and AG and advised by DMG and CEH. All authors contributed to the writing and editing of the manuscript.

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