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Successful extraction of DNA from archived alcohol-fixed white-eye fish specimens using an ancient DNA protocol

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A protocol used routinely for rapid ancient DNA extraction was applied to fish tissue archived over 80 years ago. The method proved successful, whereas other extraction protocols failed. Researchers working on DNA from older archived fish samples are encouraged to continue to concentrate their efforts on 'white-eye' specimens, which indicate an alcohol-based fixative and are thus likely to yield viable DNA.

Key words: archived tissue; chloroform; formalin; museum specimen; PCR; phenol.

Natural history museums and various other research institutions worldwide house extensive collections of archived marine and freshwater fishes. These collections potentially can provide critical data for many biodiversity and conservation research questions, such as understanding how the distribution of populations (phylogeography; Avise, 2000) has been altered in the recent past by climate change (Leonard, 2008). There is a clear need for access to the historical information stored within the genomes of these archived museum specimens. To date, much effort to extract DNA from archived specimens has focused naturally on formalin-fixed specimens (Chakraborty *et al*., 2006; Tang, 2006), which form the bulk of those in museum collections. Results have been variable and, in some cases, unreliable, depending on the study organism and its method of fixation, which is often unknown. These extraction protocols are generally hit-and-miss and require considerable time, expertise and financial investment.

Early naturalists used available spirits (*e.g.* rum and brandy) or other alcohol-based preservatives (Fortey, 2008), yielding fixed specimens which could reveal viable DNA sequences. Formalin fixation became routine for fish specimens starting in the late 1800s. Once fixed, most specimens were transferred to ethanol or isopropanol for long-term storage. Embedded within research collections are some fish specimens that were fixed in alcohol and never exposed to formalin, probably

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because it was unavailable, even after formalin fixation became the standard protocol. Alcohol-fixed fish specimens may be identified by their distinctive white eyes: the central, spherical lens is white and cloudy rather than clear. They are considered inferior to formalin-fixed specimens for many special preparations, such as clearing and staining (Dingerkus & Uhler, 1977), as well as for long-term storage. Targeting these archived specimens, when available, may significantly increase successful DNA extractions and could substantially reduce the time and cost associated with attempts to extract DNA from formalin-fixed tissues of the same taxa, which often produces little or no result. Here, a protocol used routinely for ancient DNA (aDNA) extraction (Yang *et al*., 1998) was applied to white-eye specimens of the blue panchax *Aplocheilus panchax* (Hamilton 1822), collected in 1924 and 1930 and archived in the Smithsonian's National Museum of Natural History (USNM), and on an additional five white-eye specimens of the pygmy halfbeak *Dermogenys pusilla* Kuhl & van Hasselt 1823 collected in 1963. Non-white-eye samples, *i.e.* samples preserved in formalin, were included as controls. For comparison, three traditional and widely used DNA extraction methods were used: a phenol:chloroform (Sambrook *et al*., 1989) and a high-salt (Aljanabi & Martinez, 1997) procedure, and lastly a QIAamp DNA micro kit (www.qiagen.com), specially designed to recover low quantity DNA.

Various archived fish collections were surveyed to access *A. panchax* tissue. Three samples, collected on 5 April 1924 (USNM 109791, one specimen) and 10 March 1930 (USNM 109792, two specimens), by H. M. Smith from the Gulf of Koh Chang, Thailand, were chosen because their white eyes indicated alcohol-based fixation. Freshly caught fish display this characteristic within minutes of immersion in ethanol. Five non-white-eye archived *A. panchax* samples, *i.e.* preserved in formalin, of a similar age were included as controls [Raffles Museum of Biodiversity Research, Zoological Reference Collection (ZRC) 1082, Singapore: Serangoon, M. Tweedie, February 1938]. The protocol was also tested on an additional five white-eye specimens *D. pusilla* (ZRC 1233, Singapore: Sungei Kangkar, M. Dali, 14 March 1963).

Two DNA extraction techniques that are widely applied in molecular fish studies were trialled. The phenol–chloroform method (Sambrook *et al*., 1989) and a high-salt extraction method (Aljanabi & Martinez, 1997) were each replicated four times on the specimens. A kit for recovering small quantities of DNA was also used as above (QIAamp DNA micro kit). Subsequently, after negative results, an ancient DNA protocol that utilizes a QIAgen kit-based approach to recover minute amounts of DNA was also trialled. This method has been used successfully to amplify DNA from ancient southern elephant seal *Mirounga leonina* material, dating to *c*. 7000 years in age (de Bruyn *et al*., 2009). All aDNA work was conducted under strict aDNA procedures in a dedicated aDNA laboratory to avoid contamination (Gilbert *et al*., 2005).

For the aDNA extraction method, finclips were digested in 1 ml of extraction buffer, comprising 0·5% sodium dodecyl sulphate (SDS), 0·1 M EDTA (pH 8), 10 mM Tris–HCL and 50 µl proteinase K, at 50° C overnight. Digests were centrifuged at 13 000 *g* for 1 min, the aqueous phase was removed and added to five volumes of PB Buffer (Qiagen) and vortexed. This solution was passed through QIAquick spin filter columns (Qiagen) in 500 µl volumes, centrifuged at 13 000 *g* for 1 min, and the flow-through discarded. DNA was then twice washed with 700 µl of buffer PE (Qiagen), centrifuged as before, and the flow-through discarded. The filter column was placed in a sterile 1·5 ml tube, and DNA was eluted by applying

35 µl of buffer EB (Qiagen) to the column, and then centrifuged at 13 000 *g* for 1 min. DNA samples were stored at −20° C until required.

PCR (25 µl volumes) contained: 2 µl extract, 1 U *Taq*, 1× buffer, 1·5 mM $MgCl_2$ (Invitrogen Ltd; www.invitrogen.com), 0·2 µM each primer and 200 µM each dNTP. Control region primers used were: H16498 (5′-CCTGAAGTAGGAACCAGATG-3′) and L15995 (5′-CTCCACTATCAGCACCCAAAG-3′) (Meyer *et al*., 1990). PCR thermal cycles were set at: 95° C for 2 min, 40 cycles of 94° C for 45 s, 52° C for 45 s and 72° C for 45 s, followed by a final extension at 68° C for 10 min. Multiple negative controls (no DNA) were used for all PCR runs. PCR were purified using a Qiagen purification kit according to manufacturer's instructions. These purified PCR products were sequenced at Macrogen (www.macrogen.com). The sequence data generated have been deposited in GenBank (accession numbers: GQ377753–GQ377755).

Both traditional extraction methods (four replicates of each) and the QIAamp DNA micro kit yielded little trace of usable DNA, based initially on NanoDrop spectrophotometer (www.nanodrop.com) readings (Table I). In contrast, the aDNA method yielded a standard positive DNA reading after the first extraction attempt (22–35 ng μl^{-1}) on the alcohol-fixed (white-eye) samples, but did not work on the formalin-fixed samples. PCR supported these results, utilizing universal fish control-region mitochondrial DNA (mtDNA) primers (Meyer *et al*., 1990), targeting an *c*. 400 bp fragment (Fig. 1). No PCR bands were evident on the agarose gels from each of four replicates of the two traditional extraction methods, and the QIAamp DNA micro kit protocol (Fig. 1). Adding more DNA extract to the PCRs (5 and 10 µl) did not help. These PCR products did not sequence well, resulting in low-quality short sequences, after two sequencing attempts. Positive PCR amplicons of the correct size, and of high-quality yield, were identified on agarose gels from the aDNA extraction method from the alcohol-fixed specimens and yielded high-quality sequence data. A basic local alignment search tool (BLAST) search (through the National Center for Biotechnology Information website; www.ncbi.nlm.nih.gov) identified the sequences as *A. panchax*. This protocol was also trialled successfully on five archived white-eye specimens of *D. pusilla* after traditional extraction methods were unsuccessful. Subsequent trials of three sets of universal fish nuclear primers (Mlc-3, Act2, Cam-3; Atarhouch *et al*., 2003) on the aDNA extracts from alcohol-fixed specimens (white eyes) did not succeed (although these primers did work on DNA from contemporary samples). This probably results from the low copy number of nuclear (*i.e.* 2) *v*. mtDNA (*i.e.* 1000s) in tissue cells. This result is often found in ancient DNA studies where remains have not been well preserved. It is probable that the alcohol used to preserve (white-eye) samples in the 1930s was not of premium quality; nonetheless, mtDNA remains viable.

The ability to extract DNA from archived fish tissue is of great interest, as these molecular data may provide important historical information to address a multitude of biodiversity and conservation research questions. Careful examination of archived research collections can identify fish specimens with white eyes that were most probably fixed in alcohol, and thus are likely to yield usable DNA. The protocol outlined here proved successful in extracting usable mtDNA from such specimens for PCR and sequencing, yielding high-quality DNA sequences for subsequent analyses. The success of the method probably results from increased separation of DNA from PCR inhibitors compared with the other extraction procedures (Yang *et al*., 1998). It is unclear why the QIAamp DNA micro kit, which also utilizes silica-based spin

TABLE I. Quantity of archived white-eye *v*. non-white-eye fish DNA extracts measured on a NanoDrop spectrophotometer. The 260:280 absorbance ratio is given in parentheses. Letters after sample name (a to e) indicate unique individuals

Sample	Storage medium and year of collection	Phenol:chloroform method (ng l^{-1})	High salt method (ng μl^{-1})	QIAamp DNA micro kit (ng μl^{-1})	aDNA method (ng μl^{-1})
USNM 109791	Alcohol, white eyes (1924)	−3 (0)	1 (0·06)	2 (0·09)	35 (1·63)
USNM 109792a	Alcohol, white eyes (1930)	−2 (0)	0 (0)	1 (0·06)	26 (1·24)
USNM 109792b	Alcohol, white eyes (1930)	−5 (0)	3 (0·08)	3 (0·10)	28 (1·54)
ZRC 1233a	Alcohol, white eyes (1963)	−3 (0)	0 (0)	2 (0·09)	29 (1·60)
ZRC 1233b	Alcohol, white eyes (1963)	−1 (0)	2 (0·03)	3 (0·11)	31 (1·71)
ZRC 1233c	Alcohol, white eyes (1963)	−2 (0)	1 (0·05)	1 (0·03)	22 (1·34)
ZRC 1233d	Alcohol, white eyes (1963)	0 (0)	2 (0·06)	3 (0·12)	33 (1·45)
ZRC 1233e	Alcohol, white eyes (1963)	−1 (0)	1 (0·03)	1 (0·06)	28 (1·22)
ZRC 1082a	Formalin, non-white eyes (1938)	−2 (0)	−3 (0)	−5 (0)	−2 (0)
ZRC 1082b	Formalin, non-white eyes (1938)	−1 (0)	−1 (0)	−2 (0)	−1 (0)
ZRC 1082c	Formalin, non-white eyes (1938)	−4 (0)	−2 (0)	−1 (0)	−5 (0)
ZRC 1082d	Formalin, non-white eyes (1938)	−2 (0)	0 (0)	−2 (0)	−1 (0)
ZRC 1082e	Formalin, non-white eyes (1938)	−4 (0)	−2 (0)	−1 (0)	−0 (0)

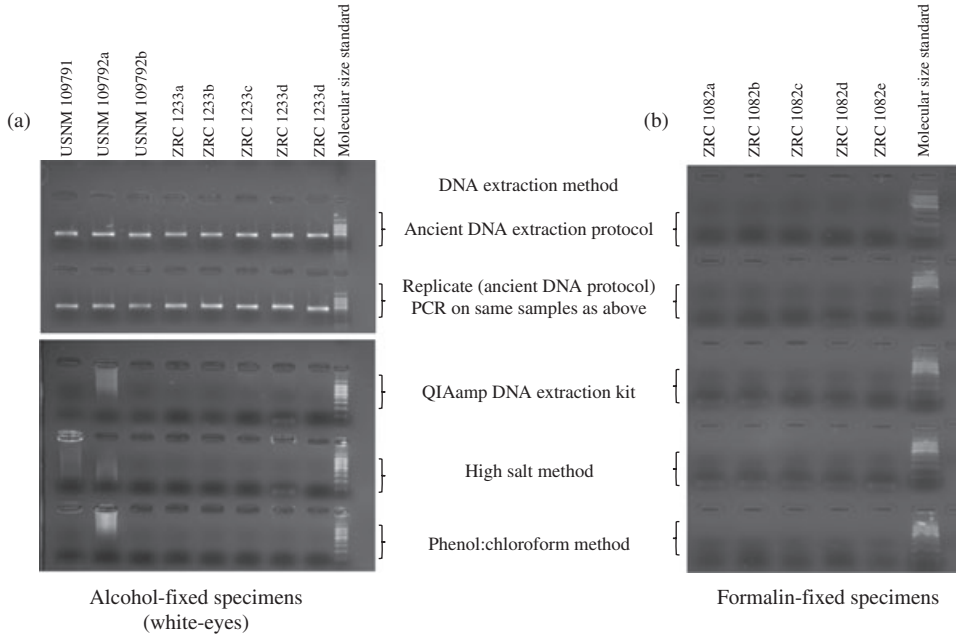

FIG. 1. Agarose gels (1·5%) displaying mitochondrial control region PCR products from (a) white-eye archived fish specimens and (b) formalin-fixed fish specimens, extracted using traditional methods and an ancient DNA protocol. Molecular size standard ladder is in 100 bp increments, bright lower band = 500 bp.

columns, did not work as well. This protocol should prove useful for accessing the vast historical record stored in the genomes of archived fishes.

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