difference may be due to the smaller size of the angiosperm pollen and the inability of the 1-mm (diameter) glass beads to efficiently rupture the pollen cell wall. Smaller beads may be appropriate for small diameter pollen. Total DNA concentration per mg of pollen varied with species, ranging from 3.9 µg per 50 mg of yellow poplar pollen to 109 µg per 50 mg of ponderosa pine pollen.

Based on the above results, the procedure in Table 1 is recommended. The procedure is rapid, requires very few solutions and is effective in isolating genomic DNA from non-germinated pollen grains. Genomic DNA from ponderosa pine, Virginia pine, loblolly pine, red spruce, Fraser fir, yellow poplar, eastern cottonwood and European black cottonwood has been successfully isolated by using the above procedure, with an average yield of 40 µg of DNA from 50 mg of pollen across all samples and all species tested. This procedure provides additional advantages over traditional plant DNA isolation methods. The first and most beneficial is that the DNA is not degraded as has been seen with other techniques where physical maceration has been used. The second is that the procedure provides high yields of DNA from a small amount of pollen. Although this procedure has at present been tested on a limited number of species, it could be of great help to others working with pollen. The DNA isolated by this procedure has been effectively used in randomly amplified polymorphic DNA polymerase chain reaction (i.e., amplification using four decamer primers yielded 6–10 bands per primer), alkaline gel electrophoresis and alkaline unwinding assays for quantification of genetic damage induced by UV-B radiation.

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Enhanced DNA Extraction and PCR Amplification of Mitochondrial Genes from Formalin-Fixed Museum Specimens

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Enzymatic methods to clone and manipulate DNA using the polymerase chain reaction (PCR) (9,13,19) in conjunction with rapidly advancing DNA sequencing technologies (1) are allowing an increasing number of investigators to work directly with DNA from a wide variety of organisms and tissue samples. Detailed genetic studies are now becoming feasible using fossil amber, bone, leaves, mummies (4,6,8,11), single hair follicles (17), dried blood stains (22), dung (5) and a myriad of archival museum specimens including...
bird and mammal skins, formalin-fixed fishes and copepods (3,16,18,21). Archival specimens are of profound importance to comparative biology because of the enormous wealth of biological information potentially available in systematic collections of plants and animals preserved around the world in museums, universities and private facilities. Most museum specimens are comprised of either dried tissue (such as skins, leaves and skeletal preparations) or fluid-preserved tissue stored in either formalin or alcohol. DNA purification and subsequent PCR amplification from some formalin-fixed tissues has been demonstrated by several investigators (7,10,12,16,21) but has remained problematic with many samples.

In this report, we outline a method of DNA extraction and PCR amplification of relatively large target fragments from formalin-fixed, fluid-preserved tissues. The approach was refined and tested with widely used PCR primers and formalin-fixed museum specimens of various condition and with taxa for which authentic DNA sequence data from fresh or frozen samples was already available. Standard methods were not successful with these archival samples, and the modifications used in this report form a sound basis for further investigations aimed at optimizing the purification and sequencing of DNA from fish, amphibian, reptile and invertebrate museum specimens fixed with formalin.

With formalin-fixed, fluid-preserved tissue, conventional nucleic acid extraction protocols typically produce low-yield and low-quality DNA, largely unsuitable for PCR amplification and subsequent nucleic acid sequencing, or else limit work to small fragments of less than a few hundred base pairs. Although a number of different models have been proposed, the exact mechanism as to how formaldehyde insolubilizes proteins is not known. Since it is a monofunctional aldehyde, it presumably cannot form protein crosslinks directly as has been suggested by some investigators. Formaldehyde does not directly attack nucleic acids but does react with thiols, free amino groups and phenolic groups in amino acids and proteins (2,15). The fact that in situ hybridization is successfully used to detect RNA expression in formalin-fixed samples suggests that nucleic acids are not destroyed by formalin, but rather are made difficult to extract. Paraffin-embedded material, widely used in clinical applications, may be less refractory because processes associated with fixation cease to occur after embedding, while these reactions are likely to continue to a greater degree in aqueous alcohol.

By comparing standard extraction methods (enzymatic proteolysis followed by phenol/chloroform extraction) using ethidium bromide (EtBr) gel electrophoresis and Hoechst dye fluorescence at each step, we found that: (i) formalin-fixed tissue homogenates initially contain much more nucleic acid than alcohol-fixed ones; (ii) proteolysis is greatly inhibited in formalin-fixed samples, presumably due to damage to the protease and/or refractory nature of formalin-fixed proteins; and (iii) most of the DNA is lost upon phenol/chloroform extraction of formalin-fixed samples (but not alcohol-fixed ones), indicating that DNA remains associated with protein complexes, resulting in the typical low yields. To flush excess formalin from the system and hence protect the effectiveness of proteolysis, tissues of museum specimens were successively incubated in large excess volumes of buffer before digestion. Controlled extractions with a short dialysis of homogenized, formalin-fixed tissue showed a modest improvement in DNA yield with the addition of glycine to the pre-digestion buffer (data not shown). It is probable that addition of other primary amino groups could have a similar effect.

The protocol outlined in detail below has been used to successfully extract genomic DNA and amplify relatively large target fragments from archived specimens encompassing a wide range of preservation histories. The present report includes data from rare deep-sea fishes (suborder Ceroidei) one specimen of which was fixed more than 85 years ago (Figure 1, Hg1 ZMUC J.78). In general, we have found that material fixed with formalin and subsequently stored in alcohol by standard museum procedures within the last quarter century typically yields more high-molecular-weight DNA than does much older material, although this general pattern will be clearly influenced by the condition and curatorial history unique to each specimen. The size of the frequently studied mitochondrial gene fragments presented in this report (16S rRNA = 570 bp, cytochrome b = 470 bp; for details of primer annealing sites, see References 14 and 19) is much larger than the low-molecular-weight fragments (ca. 100–200 bp) one is typically limited to amplifying when using degraded DNA templates conventionally extracted from formalin-fixed tissue. For 12 different museum specimens of variable condition, the modified procedure below yielded 28 positive PCR products out of 34 total reactions conducted in four distinct experimental series that included both positive (fresh tissue) and negative controls. To confirm the absence of contamination or PCR artifacts, the nucleotide sequences of cloned products from formalin-fixed specimens were verified by direct DNA sequencing and comparison to authentic sequences available from fresh material and published data (see Figure 2).

For modified DNA extraction: (i) small pieces of formalin-fixed mussel tissue (ca. 0.5 cm²) were dissected with sterile razors, traces of integument Figure 1. PCR products of templates extracted from formalin-fixed museum specimens. Lanes designated M are 1-kb ladders; lanes containing mitochondrial 570-bp 16S rRNA and 470-bp cytochrome b products are indicated; and lane N is a negative control. Lanes correspond to archived specimens as follows: Gv Giganactus vanhoef- feni (UW221178, collected 1991); Oe Oneirodes eschrichti (UW22372, collected 1990); Ch Cera- tias hoeltalli (UW22322, collected 1991); Hg1 Himantolophus groenlandicus (ZMUC J.78, col- lected 1909); Ls Lasiorhagias beebei (ISH5542- 79, collected 1979); Pw Pterichthys wedli (ZMUC 922153, collected 1982); Cj Caulo- phryne jordani (ZMUC WH4, collected 1985); Oa Oneirodes acanthius (UW21263, collected 1974); and Hg2 Himantolophus groenlandicus (UW22179, collected 1991).
were completely removed, and then the pieces were washed for three successive 24-hour periods (rotary shaker on low rpm; room temperature) in fresh solutions of 10 mL of 1× GTE (100 mM glycine, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to act as a binding agent for excess formalin; (ii) tissues were air-dried and completely digested in 50 µL of extraction buffer (1% sodium dodecyl sulfate [SDS], 25 mM Tris-HCl, pH 7.5, 100 mM EDTA) at 65°C for 24 h. Twenty microliters of 1 M dithiothreitol and 100 µL of proteinase K (10 mg/mL) were added at the beginning of digestion, and an additional 50 µL of proteinase K (10 mg/mL) and 10 µL of DNase-free RNase (10 mg/mL) were added after the first 10 h of digestion; (iii) Avoiding vortex mixing and disturbances that can shear DNA, modified phenol/chloroform extractions were performed as follows: (a) completed digestions were extracted in 500 µL of equilibrated phenol. Supernatants were saved and then extracted two more times in 500 µL of equilibrated phenol; (b) supernatants were extracted twice with 500 µL of 25:24:1 solution of phenol:chloroform:isooamyl alcohol; and (c) supernatants were extracted twice with 500 µL of 24:1 solution of chloroform:isooamyl alcohol; (iv) DNA in supernatants was precipitated by adding 2.5 vol cold absolute ethanol stored at -80°C, and samples were immediately placed at -20°C for 24 h; (v) DNA precipitates were spun by adding 2.5 vol cold absolute ethanol stored at -80°C, and samples were immediately placed at -20°C for 24 h; (vi) DNA precipitates were spun for 30 min in a microcentrifuge at 10,000 x g. (vii) absolute ethanol was removed. Pellets were rinsed twice with 50 µL of 70% ethanol and thoroughly air-dried; and (viii) purified DNA was resuspended in 40 µL of 1× TE (pH 8.0) for quantitation.

For PCR amplification: (i) 250 ng of total cellular DNA from each sample were used as a template in 25 µL of buffer containing 67 mM Tris-HCl (pH 9.0), 16.6 mM ammonium sulfate, 6.7 mM MgCl₂, 10 mM mercaptoethanol, 400 µM each dATP, dCTP, dGTP and dTTP, 12.5 pmol of each oligonucleotide primer and 0.15 U of Taq DNA polymerase. The reactions were loaded into the hot 96°C block of the thermal cycler (GeneAmp® PCR System 9600; Perkin-Elmer, Norwalk, CT, USA) and subjected to the following thermal cycles: 96°C for 10 s; 3 cycles at 94°C for 10 s denaturing, 45°C for 15 s annealing, 72°C for 30 s extension; 22 cycles at 94°C for 10 s denaturing, 56°C for 15 s annealing and 72°C for 30 s extension; and (ii) Secondary amplification reactions containing identical reagents and volumes as those listed above, but using 2 µL of primary reaction product as DNA template, were loaded into the hot 96°C block and subjected to the following thermal cycles: 96°C for 10 s; 3 cycles at 94°C for 10 s denaturing, 50°C for 15 s annealing, 72°C for 30 s extension; 12 cycles at 94°C for 10 s denaturing, 56°C for 15 s annealing and

Figure 2. DNA sequence comparison of formalin-fixed museum specimens and fresh material. Illustrative sequence alignments are shown above corresponding aligned chromatographs for: (A) formalin-fixed Ceratias hoelbolli (UW22322), cytochrome b; (B) fresh C. hoelbolli, cytochrome b; (C) Gigantactis vanhoeffeni (UW221178), 16S rRNA; and (D) fresh G. vanhoeffeni 16S rRNA. All sequences align easily with published comparative data for these gene fragments.
72°C for 30 s extension.

For product purification and DNA sequencing: (i) reaction products were subjected to electrophoresis on 1% wt/vol agarose gels, stained with EtBr and visualized by ultraviolet transillumination; (ii) product reactions were cleaned directly through QIAquick™ Spin PCR Purification Preparations (Qiagen, Chatsworth, CA, USA) per manufacturer’s recommendations; (iii) cleaned templates were dried in a vacuum centrifuge (Model RC10.10; Jouan, Winchester, VA, USA) and rehydrated in 20 µL of sterile water; (iv) sequencing reactions were prepared with a Taq DyeDeoxy™ Termination Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA, USA) using 9.5 µL of purified product template per reaction (100–500 ng). Reaction products were cleaned through Centri-Sep™ Spin Columns (Princeton Separations, Adelphi, NJ, USA) and run on an Applied Biosystems Model 373A Automated DNA Sequencer, according to manufacturer’s recommendations.

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