Org. Divers. Evol. 3, 195–205 (2003)
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DNA, PCR and formalinized animal tissue – a short review and protocols

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Received 5 December 2002 · Accepted 24 March 2003

Abstract

Formaldehyde was first prepared in 1859, and since then has been in widespread use for fixing and preserving medical and biological specimens. The value of such archival material has increased considerably because several methods for extracting DNA from formaldehyde-fixed animal tissue have been developed. Most of these, however, either require large amounts of tissue (rarely available) or recover only short fragments of DNA. Here we summarize current knowledge of and experience with such published methods, look at some of the known problems, and develop an additional method based on embedding the tissue in agarose prior to treatment with proteinase-K and GeneReleaser™. With this method we have obtained mitochondrial DNA useful for PCR reactions from as little as 3 mg tissue of more than 30 years old formaldehyde-fixed aplacophoran molluscs. We examine the conditions under which obtaining relatively high-quality DNA from formaldehyde-fixed material is possible, making previously collected samples accessible for molecular studies in genetics, systematics and related fields. The purpose of this short review is to acquaint molecular systematists with some of the methodological advances and considerations in using formaldehyde-preserved material.

Key words: amplification, extraction, fixation, formalin, formaldehyde, PCR

Introduction

Obtaining material for studies of phylogeny, systematics and phylogeography is often a laborious and costly buisness, and key species are often excluded due to lack of material. In the course of the increasing destruction of habitats and the accompanying loss of biodiversity, specimens in museum collections often become irreplaceable when the populations and species they came from are extinguished. Such specimens may then represent the last and only sources for molecular data. Certain habitats also offer special problems. For example, obtaining quality DNA from deep-sea organisms is usually problematic because material is formaldehyde-fixed in bulk, rendering it less useful for molecular studies. For reasons of cost, oceanographic expeditions are rarely funded for the primary purpose of molecular (or morphological) evolutionary analyses, further limiting the availability of useful animal tissue. Ironically, a proper morphological and molecular-taxonomic framework is critical to many marine ecology studies. Given this situation, the need to access formaldehyde-fixed samples is all the more critical, making the use of archival collections increasingly important. Much of this material has been fixed in formaldehyde for other purposes limiting its utility for DNA studies. Initial attempts to use formaldehyde-fixed material for molecular studies were made in the medical field to study genetic diseases (e.g. Goelz et al. 1985). The yields of DNA in the early attempts were generally low, and the conclusion was that the results were largely dependent on fixation time and type of storage. Later protocols achieved greater success using prolonged extraction (6 hr–7 d) of DNA with proteinase-K (e.g. Dubeau et al. 1986, Bramwell & Burns 1988, Bunker & Locker 1989, Warford et al. 1988, Rogers et al. 1990, Wright & Manos 1990, Forsthoefel

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1439-6092/03/03/03-195 $ 15.00/0

et al. 1992, Shiozawa et al. 1992, O'Leary et al. 1994, France & Kocher 1996) and with the use of chemical agents to break protein cross linkages (Johnson et al. 1995, Chatigny 2000).

Several different attempts have been made to access various types of formalinized tissue. When we started working with such samples we had several questions. How exactly does formaldehyde interact with the DNA? What is it about the process of fixation that inhibits subsequent retrieval of DNA? Are there preferable conditions during preservation and/or extraction that will facilitate recovery of DNA? When is it not even worth trying? Although many of these issues were mentioned in various publications, we did not find a concise source that addressed the needs of molecular evolutionary biologists. Thus, in order to help provide further access to formalinized samples and to help other researchers with similar questions, we compiled a mini-review about DNA extraction from formalinized tissue. This review is not meant to be exhaustive, but instead to be practical and to provide summary answers to key questions. It is built largely on the literature and our combined experience with marine invertebrate specimens.

Review

History

Formaldehyde was first prepared by A. Butlerov in 1859, but he failed to characterize the substance. In 1868 A. W. von Hofmann prepared formaldehyde using a different method, and subsequently identified it. Commercial production was started on a limited scale in the United States in 1901 (Walker 1964). Since the late 1800s it has been a main ingredient in many fluids used for preserving biological and medical samples (Blum 1893, 1894; Jones 1976; Fox et al. 1985; Johnson et al. 1995). Museums and other institutions all over the world are holding large collections of preserved tissue samples as a service to the scientific community. These have been preserved for a number of purposes such as histology, gross anatomy, cytology, and taxonomy. Only in the past two decades a need has arisen for the use of archival collections for DNA-related purposes. A major part of the archival collections are specimens that were fixed in formaldehyde prior to storage in alcohol (Chatigny 2000). Such specimens are used for medical research (e.g. Forsthoefel et al. 1992, Savioz et al. 1997), or for biological studies of vertebrates (e.g. Shiozawa et al. 1992) as well as invertebrates (e.g. France & Kocher 1996, Chase et al. 1998).

Pure formaldehyde at room temperature is a flammable, colorless gas with the chemical formula CH_2O, which condenses on chilling to give a liquid that boils at −19 °C and freezes to a crystalline solid at −118 °C. Both the gas and the liquid polymerize readily and can therefore be kept in the pure monomeric state only for a limited time. For this reason, formaldehyde is sold and transported in solution or in the polymerized state. In its aqueous solutions, formaldehyde is almost completely hydrated. These hydrates have a relatively high degree of stability. Formaldehyde is marketed chiefly in the form of aqueous solutions containing about 35–50% by weight dissolved CH_2O, the standard being 37% containing 7–15% methanol to prevent precipitation of polymers. The standard 37% formaldehyde solution is also known under the trade names "Formalin" and "Formol". The pH usually ranges from 2.8–4.0. In aqueous solutions formaldehyde is present principally in the form of the monohydrate, methylene glycol $CH_2(OH)_2$, and a series of low molecular weight polymeric hydrates. The concentration of monomeric formaldehyde is well under 0.1%. Formaldehyde is also readily soluble in alcohols. In these solutions the dissolved aldehyde is in the form of simple hemiacetals having the type formula $ROCH_2OH$ that probably are in equilibrium with polyoxymethylene derivatives in more concentrated solutions. Solutions of formaldehyde in methanol, propanol, *n*-butanol and isobutanol are commercially available (Walker 1964, e.g. Sigma–Aldrich catalogue 2002–2003). Formaldehyde is considered a health hazard, and may have primary irritant and an immunogenic effect, as well as posing potential carcinogenic and mutagenic risks (Yodaiken 1981, Björkman & Christensen 1982, Ma & Harris 1988).

Formaldehyde effects on tissue

The initial chemistry of formaldehyde fixation is reviewed in French & Edsall (1945), and Walker (1964). Its primary mode of action in the fixation process is to combine with functional groups of certain amino acids, thereby denaturing proteins. In the primary reaction the oxygen atoms undergoes hydrogen bonding with primary amines to cross-link proteins. The reactions with proteins are numerous and complex, because it can combine with a number of different functional groups. Under favorable conditions formaldehyde forms methylene bridges between functional groups. The exact reactions are highly dependant on physical factors such as pH, whether or not the formaldehyde is buffered, the concentration, temperature, fixation time, etc. (Thompson 1966, Crisan & Mattson 1993, Hamazaki et al. 1993, Koshiba et al. 1993). Some of the reactions are rapid while others are slow, some are reversible and some are not (French & Edsall 1945, Freifelder & Davison 1963, Jackson 1978, Yu et al. 1980).

Although formaldehyde is known as a potent agent for cross-linking DNA, DNA to protein, and protein to

protein (Chaw et al. 1980, Ma & Harris 1988, Crisan & Mattson 1993, Chang & Loew 1994), the reactivity toward free double-stranded DNA is very low (Trifonov et al. 1967; McGhee & von Hippel 1975 a, b, 1976 a, b). Such cross-links can be partially broken, permitting limited success with PCR, electrophoresis and slot blot experiments (Jackson 1978, Jackson & Chalkley 1981, Solomon & Varshavsky 1985, Orlando & Pardo 1993). At a neutral pH, formaldehyde can react with three of the bases in DNA: cytosine, guanine and adenine (Fraenkel-Conrat 1954; McGhee & von Hippel 1975 a, b, 1976 a, b; Neubauer et al. 1992). This can create a reactive formaldehyde compound via the methylene group, that can hinder primer annealing, inhibit renaturation, and suppress the replication procedure in the PCR (Karlsen et al. 1994). Karlsen et al. (1994) noted that only 2.5% of DNA-protein cross-links need to remain to cause the polymerase enzyme to malfunction after 200 bp. AT-rich regions are thought to be more susceptible to reaction with formaldehyde than regions dominated by CG base pairs (Chang & Loew 1994).

Long exposure to formaldehyde leads to further reactions which have not been well characterized. For example, Rumph & Williams (1986) noted that less formaldehyde could be eluted from tissue stored for 100 days than from tissue stored for 50 or 75 days, suggesting that secondary reactions had occurred. The interaction with common impurities and substances formed by prolonged storage, such as formic acid, methanol, methylal, methyl formate and polymeres of various compositions, also remains largely uninvestigated.

Formaldehyde is not a good medium for preserving DNA, and today tissue for molecular studies is either frozen or fixed in alcohol or other media (e.g. Seutin et al. 1991, Shiozawa et al. 1992, Fukatsu 1999, Liu et al. 2001 – but see Pavelic et al. 1996). Nonetheless, the bulk of most wet collections are only available as formaldehyde-fixed material. Numerous attempts have been made to utilize this material for molecular studies, with mixed success. Below, we discuss specific problems concerning the use of material fixed in formaldehyde and in an Appendix compile published protocols for DNA extraction of formaldehyde-fixed animal tissue. The purpose of this paper is not to evaluate the merits of the various protocols, but to show the possibilities at hand, and also to point out some difficulties to be taken into consideration.

Fixation and storage

Formaldehyde fixation degrades DNA. Shibata (1994) concluded that because of this degradation PCR targets should be less than 400 bp. However, Goelz et al. (1985) found DNA fragments up to 10,000 bp from tissues fixed in 4% neutral-buffered formaldehyde, and Savioz et al. (1997) successfully amplified an 838 bp long fragment from 46 years old formaldehyde-fixed tissue.

Crisan & Mattson (1993) have reviewed the factors influencing the success of obtaining high-quality DNA from fixed (and embedded) tissue. They list the following as the most important: 1) The chemical composition of the fixative; 2) The duration of fixation; 3) The duration of tissue hypoxia (which is proportional to the amount of DNA degradation); 4) The size of the specimen and its permeability to the fixative; 5) The length of storage time.

A problem with most archival material, especially zoological material, is that although we know that it has been fixed in formaldehyde prior to storage in alcohol, the details of the fixation are usually not known. For example, the formaldehyde may or may not have been buffered, fixation time varies from a few hours to years, and fixation temperatures vary drastically. For some purposes even boiling formaldehyde solution has been used to achieve rapid fixation (Warén 1983). Even though formaldehyde is relatively inactive towards double-stranded DNA, the high temperature of boiling formaldehyde denatures DNA. Once single-stranded, DNA reacts rapidly with formaldehyde by hydroxymethylation, inhibiting future re-annealing of the strands. Koshiba et al. (1993) noted that higher fixation temperature resulted in higher DNA degradation. Fixation and storage at 4 °C greatly reduced degradation. O'Leary et al. (1994) found that fixation at 0 °C provided a DNA template that was unsuitable for PCR and concluded that this was probably due to supercoiling phenomena of the DNA double helix.

The time from collection to actual fixation may vary from sample to sample within a series, but this factor greatly influences the possibility of extracting useful DNA (R. J. Etter & J. Zardus, pers. comm. 2000; see also France & Kocher 1996 on "collecting effect".) Samples allowed to warm, for example in the sun for a couple of hours prior to fixation, may still be useful for identification and/or histological purposes but will be virtually useless for DNA extraction. With terrestrial gastropods it is common practice to relax the animals by drowning them in water before fixation, which causes hypoxia. This procedure seems to degrade the DNA (CS, unpubl. data).

Some samples believed to have been fixed in formaldehyde solution have actually been fixed in Bouin's fluid, Carnoy's solution, Zenker's formic solution, Zenker's acetic solution, or some other frequently used histological fixative. Such fixation may seriously hamper the possibility to extract any DNA from the samples. Tissue fixed in Carnoy's solution and AMeX fixation (Acetone – Methyl benzoate – Xylene) have been reported to give good yields of high-quality DNA (Gall et al. 1993, O'Leary et al. 1994, Pavelic et al. 1996). Fix-

ation in mercuric chloride based fixatives seems to prevent any possibilities of extracting DNA (e.g. O'Leary et al. 1994). Glutaraldehyde and acrolein are other commonly used fixatives, especially for ultrastructural work. Such material is commonly not placed in general collections and therefore little is known about the effect of these chemicals on DNA. France & Kocher (1996) attempted to extract DNA from deep-sea crustaceans fixed in glutaraldehyde, without any success. All published attempts to obtain DNA for PCR from tissue fixed in Bouin's fluid have given negative results (e.g. Gall et al. 1993, O'Leary et al. 1994, Pavelic et al. 1996).

Neutral-buffered formaldehyde is far better than any acidic fixative (e.g. Nuovo & Silverstein 1988, Hamazaki et al. 1993). Low-pH formaldehyde, or formaldehyde with a high formic acid content, causes a greater degradation of the DNA than neutral buffered (Koshiba et al. 1993). Prolonged fixation also seems to reduce DNA yield (e.g. Rogers et al. 1990, Greer et al. 1991, Forsthoefel et al. 1992, Hamazaki et al. 1993, Karlsen et al. 1994, Inoue et al. 1996), especially if non-buffered formaldehyde is used. The number of both direct and indirect reactions with the DNA will be greater when the time of exposure to the fixative is prolonged. Karlsen et al. (1994) found a correlation between concentration and purity of DNA isolated from tissues fixed for 8 and 48 hours respectively, but not for DNA fixed for 96 hours, which indicated that longer formaldehyde reaction time with DNA in solution changes the extraction capacity. Inoue et al. (1996) managed to extract DNA from tissue fixed in non-buffered formaldehyde for 1–6 days, but not for 7. Some institutions are known to store their biological samples in the actual fixation fluid indefinitely, which is bound to have negative effects on the possibility to obtain good quality DNA. This was also noted by France & Kocher (1996) who failed to extract DNA from material that had not been transferred out of the formaldehyde used for fixation.

Even if prolonged storage seems to decrease the amount of DNA obtained, useful DNA has been extracted from 10–85 years old fixed tissue (Shibata et al. 1988, Shiozawa et al. 1992, Gall et al. 1993, Wang et al. 1994, France & Kocher 1996, Pavelic et al. 1996, Savioz et al. 1997, Shedlock et al. 1997, Chase et al. 1998, Schander & Halanych 2000). Pavelic et al. (1996) found that strands >2000 bp were still intact even after 40 years of storage, and Savioz et al. (1997) reported that strands 20 kb long were present in fixed material 46 years old.

The concentration of the fixation media (i.e. formaldehyde) may have an effect, but this has not been well studied. For zoological purposes 1–10% formaldehyde solution is commonly used for fixation.

Archival zoological material is commonly stored in alcohol, a good medium for removing formaldehyde, for many years (decades) after fixation. Rumph & Williams (1986) investigated the efficiency of water, ethanol solutions and ethylene glycol for extracting formaldehyde from fixed muscle tissue. Their conclusion was that all these fluids are effective in extracting formaldehyde from fixed tissues but ethanol was slightly superior. Unfortunately they only tested a series of 20% to 40% ethanol solutions, whereas most archival samples are stored in 70–80% solutions. It seems certain, however, that only minute traces of formaldehyde (if any) are left in archival material stored in alcohol for a long time. Another substance useful for formaldehyde extraction is phenoxyethanol (Frølish et al. 1984), but the effect of this substance on the preservation of DNA has not been investigated.

De Giorgi et al. (1994) tested the PCR-inhibiting effects of formaldehyde and concluded that it is only slightly inhibiting in higher concentrations. It is therefore safe to conclude that residuals of formaldehyde in the tissue does not constitute a problem for PCR on archival material stored in alcohol. Other substances present (e.g, high concentrations of potatissum chloride, urea and porphyrins derived from haeme and drug metabolites) may inhibit the effect of PCR (Doyle & O'Leary 1992, O'Leary et al. 1994). Thus, the available evidence suggests that cross-linking and DNA damage caused by formaldehyde, and not the presence of formaldehyde, is the main factor in inhibiting PCR amplification from formaldehyde-fixed tissue.

Extraction

Although the fixation conditions clearly influence the quality of formaldehyde-fixed DNA, optimization of extraction procedures can be critical to obtaining usable DNA. Extraction methods can differ in performance with different species (e.g. Whittier et al. 1999). As more protocols become published, it will be possible to select a protocol optimal for the species studied. For example, SDS (sodium dodecyl sulphate), used in some extraction protocols, can inhibit *Tag*-polymerase, resulting in lower yields of PCR product (e.g. Jackson et al. 1990). It is nevertheless a part of some protocols for extracting DNA from archival animal tissues (e.g. Shiozawa et al. 1992, De Giorgi et al. 1994, France & Kocher 1996).

Proteinase-K is commonly used in DNA extractions from animal tissue. Numerous studies show that prolonged digestion with proteinase-K gives higher yields of DNA than other methods (e.g. Shiozawa et al. 1992, Crisan & Mattson 1993, France & Kocher 1996, Shedlock et al. 1997).

A procedure common to many of the protocols (including ours) is the use of phenol-chloroform extraction. Some studies have shown that this procedure might further damage, or at least remove, an unnecessarily large amount of fragile archival DNA (Wang et al. 1994,

Shedlock et al. 1997). Finding alternatives to this procedure therefore opens possibilities for further improvement of the protocols. Wang et al. (1994) obtained sufficient DNA using the chelex-100 method (see also, for example, Gill et al. 1992, Walsh et al. 1991).

The amount and type of tissue are important considerations. Archival tissue often originates from a single collection and is unique. Many protocols have therefore beeen developed for working with small metazoan animals (e.g. Schizas et al. 1997), fragments thereof (e.g. Schander & Halanych 2000), or skin scrapings (e.g. Chatigny 2000). For small metazoan animals, the entire specimen is often needed, but for larger animals the yield may vary with the tissue used. Shiozawa et al. (1992), working on salmonid fishes, found that muscle and liver yielded equal amounts of DNA, and gut tissue somewhat more.

Many medical studies have extracted DNA from histological material that was embedded in paraffin after fixation. Some studies (e.g. Goelz et al. 1985, Bunker & Locker 1989, Gall et al. 1993, Date et al. 1997, Cawkwell & Quirke 2000) indicate that there is less degradation is in such material, and Inoue et al. (1996) actually recommends that paraffin embedding should be performed after fixation on material to be used for PCR. Serial sectioning from paraffin-embedded material is a routine method in many zoological and medical disciplines, and both sections and embedded material could be a useful source of DNA for molecular studies. Commonly used histological stains do not seem to interfere with PCR (e.g. Pavelic et al. 1996), making paraffin-embedded specimens accessible to DNA-related techniques.

A few other approaches have been tried and deserve further study. Shedlock et al. (1997) noticed that the addition of glycine to the pre-digestion buffer led to an improvement in the DNA yield, and concluded that addition of other primary amino groups probably could have similar effects. This needs to be investigated further. Chemical means to break protein cross-linkages, for example DTT (dithiothereitol; see Johnson et al. 1995, Shedlock et al. 1997) and DTE (dithioerythritol; see Chatigny 2000) have been used in a few studies. There are additional substances capable of breaking protein cross-linkages, and their use in extractions of DNA from formaldehyde-fixed tissues may open new possibilities.

PCR

Weirich et al. (1997) found that PCR–products from extractions of formaldehyde-fixed tissue were more specific and reproducible using HPLC-purified primers compared to using non-purified primer based PCR products. These findings have not been tested in any animal protocol, but might be a way to further improve the results. For degraded DNA another possibility may be to HPLC-purify the PCR products before sequencing. DNA from formaldehyde-fixed tissue may be more or less degraded, and it is sometimes not possible to amplify the full fragment desired. However, a nested series of internal primers can be constructed (e.g. Chase et al. 1998) to overcome the problem of short fragments and minimize the risk of contamination by constructing more species-specific primers.

Additional considerations

A major problem using archival material is contamination. This problem is twofold. Firstly, archival DNA is more or less degraded, and therefore any contaminant constitutes a serious competitor for amplification (Cawkwell & Quirke 2000). Chase et al. (1998) found human contamination a serious problem in samples of minute deep-sea bivalves. We also noted (human) contaminations in some of our extractions, and strongly recommend performing a BLAST search with any sequence obtained from archival material. Secondly, there are indications that material handled during storage will be a poorer source of DNA than material that has not been handled. France & Kocher (1996) noted that samples that had been extensively manipulated (i.e. sorted under the microscope) amplified worse than unmanipulated samples. As recommended by those authors, all extractions should be carried out with replicates whenever possible.

Another problem is the possibility that formaldehyde fixation has induced apparent substitutions in nucleotides. Karlsen et al. (1994) note that although double-stranded DNA remains in formaldehyde-fixed tissue, there is a risk that it has been modified. A number of infidelities in a 634 bp long rDNA fragment amplified from fixed tissue of the nematode worm *Caenorhabditis elegans* where found when compared to sequences from unfixed material (De Giorgi et al. 1994). The artifacts consisted of single-site mutations where G and T were inserted in the sequence. Other errors involved multiple nucleotide deletions. These were sometimes so severe that the sequences were impossible to align. It was also noted that the extent of the difference can vary from sample to sample, and multiple extracts from the same formaldehyde-fixed specimens should be amplified and sequenced. France & Kocher (1996) also tested for any infidelities in obtained sequences, but found no obvious artifacts. Some variation was found, but only at sites known to be variable within species. They also found no sequence variation when independent extractions were made from the same specimen. This has also been the case in other published studies comparing sequences obtained from fresh and formaldehyde-fixed material (e.g. Shiozawa et al. 1992, Shedlock et al. 1997).

Compilation of protocols

In the work of most molecular systematists and molecular phylogeographers, the extraction of DNA from formalinized material is the most critical step for successful amplification. Fortunately, it is also the step over which the investigator has the most control. Presumably, if the investigator is working with formalinized material, the material is rare or hard to obtain and was probably fixed by someone else. Thus, the investigator had no control over fixation, making the extraction the next most important consideration. To this end, we summarize some of the different extraction approaches that have been used successfully (see Appendix). We did not explicitly test all of these protocols ourselves. However, based on the literature and experience, these protocols appear to be the most successful.

Acknowledgements

We thank John Zardus (University of Hawaii) and Malin Strand (University of Göteborg) for testing our protocol, and Amélie H. Scheltema for encouragement for the project and comments on the manuscript. Rüdiger Bieler (Field Museum of Natural History) also commented on the manuscript. Iben Heiner (University of Copenhagen), Mats Olsson, Thomas Dahlgren and Per Sundberg (all University of Göteborg) helped us with obtaining literature.

The project has been supported to various degrees by grants from Colliander Foundation, Carl Trygger Foundation, Schwartz Foundation, PADI Foundation, Wilhelm & Martina Lundgren Foundation, The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Contract number 22.5/2001-1029), by the Swedish Research Council (contract no. 621-2002-313; to CS), and U.S. National Science Foundation PEET (Partnerships for Enhancing Expertise in Taxonomy) grants DEB-9521930 (to A. H. Scheltema) and DEB-0075618 and EAR-0120646 (to KMH). This is contribution #10866 from the Woods Hole Oceanographic Institution, and a contribution from the Arctic Station of the University of Copenhagen.

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Appendix

The following protocols have been designed specifically for work with zoological material. Although we did not try all of them firsthand, they appear to be the most promising protocols based on the biology and chemistry of formaldehyde fixation. The listed protocols are summarized in Table 1.

Shiozawa et al. (1992)

Shiozawa et al. (1992) worked with formaldehyde-fixed museum specimens of fish, and the d-loop region of the mitochondrion was successfully amplified.

Approximately 1 cm² fin-tissue was placed in 20 volumes of TE9 buffer (500 mM Tris, 20 mM EDTA, 10 mM NaCl, pH 9.0; after Goelz et al. 1985). The buffer was changed twice over 24 hours. Tissue was minced with a clean razor blade and placed in 15 ml centrifuge tubes with 10 ml of TE9 and 0.1 g of SDS. Five µg of proteinase K were added to each sample, and the tubes were capped and incubated in a shaking water bath for 24 hours at 55 °C. An additional 5 mg of proteinase K and 0.1 mg SDS were added to each sample and the tubes returned to the shaking water bath for 50 hours at 55 °C to remove residual undigested tissue. The samples were transferred to 30 ml tubes, and an equal volume of phenol-chloroform was added to each. The tubes were inverted several times to mix and then centrifuged at 10,000 rpm for 10 minutes. The aqueous phase from each sample was removed with an inverted glass pipette and placed into a clean 30 ml tubes and the procedure repeated. A final extraction of the aqueous phase was made with one volume of chloroform and centrifuged at 10,000 rpm for 10 minutes. The aqueous phase from each sample was transferred to a new tube and 0.1 volume of 3 M sodium acetate solution was added. The mixtures were precipitated with one volume of 95% ethyl alcohol and stored overnight at –20 °C. Each sample was centrifuged at 10,000 rpm for 10 minutes and the supernatant carefully poured off, leaving a DNA pellet. The pellets were washed with 70% ethyl alcohol and centrifuged again for 10 minutes at 10,000 rpm. The alcohol was poured off and the samples allowed to air dry. The pellets were resuspended in 3 mM Tris, 0,2 mM EDTA solution (pH 7.2). RNAse was added to a final concentration of 20 µg/ml.

De Giorgi et al. (1994)

De Giorgi et al. (1994) studied a 643 bp long fragment of 26S rDNA from formaldehyde-fixed and fresh material of the nematodes *Xiphinema* sp. and *Caenorhabditis elegans*.

A single specimen of nematode was placed in a 1.5 ml tube containing distilled water. The sample was washed several times by centrifuging and discarding the supernatant. After the last centrifugation, 500 µl extraction buffer (10 mM Tris pH 8, 2 mM EDTA pH 8, 10 mM NaCl, 1% SDS, 8 mg/ml dithiothereitol, and 0.4 mg/ml proteinase K) were added and incubated at 37 °C for 1–3 h with occasional, gentle mixing. The lysate was extracted, once with equal volume of phenol and once with chloroform-isoamyl alcohol. Nucleic acids were precipitated by adding 1 µg of tRNA, 0.3 M (f.c) sodium aceatate and 2 volumes of 95% ethanol at –20 °C. After centrifugation at 12,000 rpm, the pellet was washed twice with 70% ethanol and dissolved in 20 µl of distilled sterile water.

France & Kocher (1996)

France & Kocher (1996), working on crustaceans, compared a number of protocols adapted mainly from medical, or vertebrate, uses, and got most consistent positive results by using variations of the Shiozawa et al. (1992) protocol. They were able to amplify 153–259 bp fragments of COI and up to 523 bp of 16S.

Prior to mincing, tissues were soaked in buffer (500 mM Tris pH 9.0; 20 mM EDTA; 10 mM NaCl) at room temperature for 24 hours, with one or two buffer changes. Tissues were then minced and added to 1 ml of buffer plus 50 µl of 20% SDS and 25 µl of 20 mg/ml proteinase K and incubated at 55 °C. After 24 hours, another 25 µl of 20% SDS and 25 µl of 29 mg/ml proteinase K were added, and incubation was continued for another 48 hours at 55 °C. A subsequent modification to the protocol involved adding half of the second aliquot of proteinase K after 24 hours and the reminder 24 hours later. The DNA was extracted twice with phenol:chloroform (1:1, v:v), and once with chloroform, followed by an overnight ethanol precipitation at –20 °C. The precipitate was vacuum-dried and resuspended in 50–100 µl of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA).

Shedlock et al. (1997)

Shedlock et al. (1997) worked on archival specimens of fish, amphibians, reptiles and invertebrates fixed in formaldehyde. 470 & 570 bp were amplified from tissue up to 85 years old.

Small pieces of formalin-fixed tissue (ca 0.5 cm^3) were dissected and traces of integument removed. The pieces were washed in fresh solutions of 10 ml of 1x GTE (100 mM glycine, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at room temperature for three successive 24 h periods (rotary shaker). The tissues were air-dried and completely digested in 500 µl of extraction buffer (1% SDS, 25 mM Tris-Hcl, pH 7.5, 100 mM EDTA) at 65 °C for 24 h. 20 µl of 1 M dithiothereitol and 100 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. Modified phenol/chloroform extractions were performed, avoiding vortex mixing and disturbances that can shear DNA. 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. Supernatants were extracted twice with 500 µl of 25:24:1 solution of phenol:chloroform:isoamyl alcohol, and supernatants were extracted twice with 500 µl of 24:1 solution of chloroform:isoamyl alcohol. DNA in supernatants was precipitated by adding 2.5 volumes cold absolute alcohol stored at –80 °C, and samples were immediately placed at –20 °C for 24 h. DNA precipitates were spun for 30 minutes in a microcentrifuge at 10,000 g. Absolute ethanol was removed and pellets were rinsed twice with 50 µl of 70% ethanol and thoroughly air-dried. Purified DNA was resuspended in 40 µl of 1 × TE (pH 8).

Chase et al. (1998)

Chase et al. (1998), working on molluscs, found the protocols by France & Kocher (1996) and Shedlock et al. (1997) cumbersome for small amounts of tissue. They instead used a modification of a commercially available DNA extraction kit.

The tissue was placed in microcentrifuge tubes with 200 µl of tissue lysis buffer ATL from the QIAamp® Tissue Extraction Kit (Qiagen, Chatsworth, CA, USA) and incubated for 24 h at 55 °C. Then 5 µl of a 50mg/ml solution of proteinase K and an additional 95 µl of lysis buffer were added and incubation continued at 55 °C for another 72 h. The extraction then followed the manufacturer's instructions with the exception that the buffer AL and ethanol were increased from 200 to 300 µl.

Whittier et al. (1999)

Whittier et al. (1999) extracted DNA for PCR amplification of COII from primate fecal samples fixed in formaldehyde. Two different protocols were used, the GT protocol and the Lysis protocol (Alcivar et al. 1989). Their conclusion was that the different protocols were optimal for different species.

In the GT protocol samples were homogenized in 5 ml of guanidine isothiocyanate buffer (GTB) and centrifuged at 2680 g for 10 minutes to remove debris. N-laurylsarcosine (2%) and cesium chloride (0.15 g/ml) were added to the supernatant before layering on 5.7 M caesium chloride cushion. Samples were centrifuged at 23 °C for 14–18 h at 140,000 × g, and the supernatant containing DNA was mixed with an equal volume of TE buffer (0.01 M Tris pH 7.5, 0.001 M EDTA pH 8.0) and 2.5 volumes of 100% ethanol and precipitated at –20 °C overnight. DNA was centrifuged at 5100 g for 30 min at 4 °C. The pellet was rinsed with 70% ETOH and then processed according to the lysis protocol.

The lysis protocol entailed homogenization of samples in 5 ml of lysis buffer, and incubation with 100 µg proteinase K ml^{-1} at 37 °C overnight before being transferred to 1 M NaCl. 1/10 volume of 10% cetyl trimethylammonium bromide (CTAB)/0.7 M NaCl solution was added, and the samples were incubated at 65 °C for 10 minutes. Samples were then extracted twice with an equal volume of chloroform and once with phenol:chloroform:isoamyl alcohol (25:24:1 by volume). DNA was precipitated with 2.5 volumes of 100% ethanol at –20 °C overnight, and the final pellet was resuspended in ultrapure distilled water.

Chatigny (2000)

Chatigny (2000) worked on formaldehyde-fixed museum samples of amphibians and reptiles. The average size of the amplified product was approximately 700 bp.

Tissues were ground to a fine powder in liquid nitrogen using a small mortar and pestle. Approximately 50 mg of ground tissue were placed in a 1.5 ml micro centrifuge tube along with 1 ml of STE buffer (0.1 M NaCl, 0.05 M Tris-HCl pH 7.5, 0.001 M EDTA), 25 µl of 20 mg/ml proteinase K, 50 µl of 20% SDS, and 2.5 µl of 20 mg/ml of dithioerythritol. The aggregate was gently mixed and then incubated in a gently shaking water bath for 24 h at 55 °C, An additional 25 µl of proteinase K, 25 µl SDS, and 2.5 µl of dithioerythritol were then added and the mixture was placed back into the water bath for 48 h at 55 °C. The mixture was then placed in a 15 ml centrifuge tube and subjected to two standard phenol:chloroform:isoamyl alcohol (25:24:1) extractions. The aqueous layer was placed in a clean tube and 1/10 volume of 2 M sodium chloride was added. The mixture was precipitated with 1 volume of cold (–20 °C) 95% ethanol and left at –20 °C overnight. Each tube was then centrifuged for 30 min at 7000 g and the supernatant removed using a micropipette. Since in most cases no pellet was visible, care was taken to avoid disturbing the liquid at the very bottom of the tube. The remaining liquid was evaporated in a convection oven for 2 h at 55 °C. The DNA was resuspended in 50 µl of TE buffer (0.001 M Tris-HCL pH 7.5, 0.0001 M EDTA).

Yue & Orban (2001)

This protocol was developed for the extraction of DNA from fish scales, both fresh and formaldehyde-fixed. Up to 600 bp long fragments were amplified.

The scales (1–4) were dried by wiping with a paper towel and then placed into a 1.5 ml Eppendorf tube containing 200 µl 5% Chelex 100 (Bio-Rad, Hercules, CA, USA) in sterile water. The tubes were boiled in a water bath for 10 minutes, and then allowed to cool at room temperature. 200 µg of proteinase K (Boehringer-Mannheim, Mannheim, Germany) was added to each tube, followed by incubation at 55 °C for 1 hour. The tubes were centrifuged at 10,000 g for 5 minutes and the supernatant was transferred to a new 1.5 ml tube. After adding 540 µl of 6 M NaI and 8 µl 100% (wt/vol) silica to each tube, they were vortexed for 5 seconds, followed by slight shaking for 2 minutes. The tubes were then briefly spun for 5 seconds, and the supernatant was removed using a pipette. One ml of wash solution (10 mM Tris pH 7.5, 1 mM EDTA pH 7.5, 100 mM NaCl, and 50% ethanol) was added to each tube, followed by vortexing for 10 seconds. Tubes were then centrifuged at 10,000 g for 30 seconds. The supernatant was removed, and the silica-bound genomic DNA was dried at 37 °C for 5 minutes. The DNA was then eluted by adding 40 µl of destilled water of 1x TE buffer and centrifugation at 10,000 g for 1 minute. The supernatant (approx. 40 µl) containing genomic DNA was transferred into new tubes.

Silica was obtained from Sigma (St. Louis, Mo., USA; Cat. No. S-5631) and prepared according to Bloom et al. (1990) with a slight modification. 10 g of silica were put into 100 ml of sterile destilled water, then shaken vigorously overnight. The silica was then allowed to settle for 10–12 hours, the supernatant was removed by pipetting or decanting, then the silica was resuspended in 10 ml of 6 M sodium iodide (approx. concentration 100% wt/vol). The treated silica could be stored in the dark at room temperature for at least three months.

No PCR was possible if the silica step was omitted from the protocol.

Schander & Halanych (new)

Our new protocol basically combines elements from the procedures of Schizas et al. (1997) and Savioz et al. (1997). The main components have been presented in an abstract from the 9th Deep-Sea Biology symposium (Schander & Halanych 2000). The protocol has been tested on small amounts of formaldehyde-fixed molluscan tissue, and on formaldehyde-fixed crustaceans.

3–78 mg of formalin-fixed tissue were cut into <1 mm^3 pieces and rinsed in 1x TE (10 mM TRIS, 1 mM EDTA, pH 8.0) twice overnight at 4 °C. The tissue was subsequently cut into smaller pieces, resuspended in 500 µl TE9 (500 mM TRIS, 20 mM EDTA, 10 mM NaCL, pH 9.0) and ground with a pestle. After incubation at 50 °C for 5 minutes, 100 µl of a 1% solution of low melting point agarose (SeaPlaque GTG Agarose, FMC) melted in TE9 were added directly into the 1.5 ml Eppendorf tubes and placed on ice. The tubes were then transferred into a –70 °C freezer for 30 minutes. This serves two purposes: it alters the structure of the agarose and makes the sample easier to handle when taken out of the tube. The agarose plugs were then removed by cutting the tubes open, and the plugs were subjected to proteinase-K treatment in new Eppendorf tubes. 4.0 mg proteinase K (Boehringer-Mannheim, 14–22 mg/ml) were used per 100 mg tissue in 1 ml of incubation solution (10 mM TRIS, 500mM EDTA, 1% N-lauroyl-sarcosine sodium salt, pH 8.4) and treated overnight in a water bath at 50 °C. They were subsequently denatured at 95 °C for 5 minutes. The samples were spun for 10 minutes at 14,000 rpm. The supernatant was mixed with an equal amount of phenol, mixed and again spun at 14,000 rpm for 10 minutes. The supernatant was mixed with 2 parts ice-cold 100% ethyl alcohol and 1/10 volume NaAc, and placed in a –20 °C-freezer overnight prior to centrifugation for 15 minutes at 14,000 rpm. The pellets were cleaned in 500 µl 70% ethyl alcohol and centrifuged for 10 minutes at 14.000 rpm. The pellets were dried using a Speed Vac (SAVANT speed vacuum dryer system) and resuspended in 60 µl PCR buffer (Promega). The buffer was mixed with an equal amount of GeneReleaser™ (BioVentures) and the protocol suggested by the manufacturer was followed. The tubes were then centrifuged for a short time and the supernatant (approximately 60 µl) was pipetted and placed in a new, labeled Eppendorf tube. 3 µl of the solution were then used in a PCR.

For comparison, QiaGen DNeasy and QiaAmp kits were used according to the manufacturer's recommendations. This procedure yielded too little DNA to allow PCR.

Table 1. Summary of DNA extraction protocols described in the Appendix.

Publication	Organism/tissue	Age	Amount	Fragment	Length (bp)	Proteinase K	Phenol	Chelex
Shiozawa et al. (1992)	fish	66 years	1 cm^2	mitochondrial d-loop	120	24 hours	Yes	No
De Giorgi et al. (1994)	nematodes	? (days)	single specimen	26S	643	1–3 hours	Yes	No
France & Kocher (1996)	amphipods	? (years)	single specimen	COI 16S	153–259 523	24+48 hours	Yes	No
Shedlock et al. (1997)	fish, amphibians, reptiles, invertebrates	85 years	0.5 cm^3	16S Cyt b	570 470	24 hours	Yes	No
Chase et al. (1998)	molluscs	30 years	1.0–5.0 mm	16S	330	72 hours	No	No
Whittier et al. (1999)	primate faeces	50 days	0.5–2.0 g	COII	418	overnight	Yes	No
Chatigny (2000)	amphibians, reptiles	?	50 mg	Cyt b	700	24 hours	Yes	No
Yue & Orban (2001)	fish scales	?	1–4 scales	tubulin	600	1 hour	No	Yes
Schander & Halanych (new)	molluscs, crustaceans	30 years	3–78 mg	16S COI ITS1–2	450 600 1000	overnight	Yes	No