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Field preservation of marine invertebrate tissue for DNA analyses

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Abstract

Successful preservation of tissue samples is a prerequisite for long field studies in remote areas. However, there is little published information concerning field preservation of marine invertebrate tissues for DNA analyses. This omission is significant because marine biodiversity is centered in the Indo-Pacific, where immediate DNA analysis is often impossible. Consequently, we used an assay based on polymerase chain reaction (PCR) to examine the effect of five storage solutions and three temperature regimens on the degradation of DNA from four common classes of marine invertebrates (Anthozoa, Gastropoda, Polychaeta, and Scyphozoa). Control samples were cryopreserved. Storage solution and the type of tissue preserved were the best predictors of preservation success. The length of time in storage and the storage temperature also affected the preservation of DNA. A field test demonstrates that a solution of dimethylsulfoxide and sodium chloride (DMSO-NaCl) preserves a wide range of tissues for DNA analyses and is very simple to use in remote field locations.

Introduction

Plans to investigate scyphozoan phylogenies in the western equatorial Pacific Ocean required the preservation of tissue samples for long periods of time in a hot and humid climate. A survey of the literature revealed that, although many studies have identified methods suitable for preserving plant and animal tissues (Table 1), there were no recommendations on how best to preserve marine invertebrate tissues for DNA analyses. This as a significant omission for several reasons. First, DNA analyses are invaluable in studies of the evolution, systematics, and population genetics of marine invertebrates (e.g., see McMillan et al., 1991; Avise, 1994, p. 154; Burton and Lee, 1994; Palumbi, 1994; France et al., 1996). Second, marine invertebrates are becoming increasingly important to the pharmaceutical industry (Colin and Arneson, 1995). Finally, the marine environment harbors the greatest diversity of invertebrates (Brusca and Brusca, 1990, p. 5), and this diversity is highest in the Indo-Pacific (Colin and Arneson, 1995), where immediate analysis, or cryopreservation, of DNA is often impossible.

DNA is particularly susceptible to degradation by hydrolytic and oxidative endogenous nucleases (Dessauer et al., 1995), which, if not countered, break down highly informative long strands of DNA into small fragments of greatly reduced use for many analyses (Seutin et al., 1991). Enzyme activity, and consequently DNA degradation, may be limited by adjusting the ambient pH, salt concentration, or temperature (Dixon and Webb, 1979). Samples may be successfully preserved by a number of chemical or physical treatments (Table 1). Cryopreservation is the preferred method of DNA protection (Chase and Hills, 1991; Seutin et al., 1991; Rogstad, 1992; Post et al., 1993; Reiss et al., 1995), and may be accomplished by freezing samples over dry ice (−78°C) or in liquid nitrogen (−196°C). However, deep freezing is not always feasible. Both dry ice and liquid nitrogen are difficult to use in the field because they require careful handling and special equipment, and furthermore, strict regulations limit their transport by air (Liston and Rieseberg, 1990; Chase and Hills, 1991; Seutin et al., 1991; Dessauer et al., 1995).

This study was undertaken to identify an alternative to cryopreservation, suitable for the long-term storage of marine invertebrate tissues for DNA analyses, and appropriate for use at remote field sites. After reviewing the published literature, we

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Table 1. A synopsis of DNA-preservation methods.*

Sample type	Storage treatment											
	−80°C	−20°C	4°C	EtOH	CTAB	DMSO	DNAB	Urea	Queen's	Chem.†	Dried	Ref.‡
Plant leaf	√	√	√	*$^{95+}$	√					x^{a}	√	1
Fly tissue	√			√$^{80+}$						√^{b}	√	2
Wasp tissue	√			√^{100}						x^{f}, √^{g}	√	3
Beetle tissue	√			√^{95}			√			x^{c}		4
Ant tissue										√^{d}		5
Fish tissue						√		√		√^{d}		6
Bird tissue	√			x^{70}		√						7
Bird blood	√	√						√	√		√	7
Human blood										√^{e}		8
Foraminifera	√	√		x$^{\leq 100}$						x^{f}	*	9

* In the body of the table, a check mark (√) indicates successful preservation of DNA; x, degradation of DNA; *, conflicting results depending on precise protocol; blank cell, no information available; superscript numbers, % ethanol (EtOH); DNAB, DNA isolation buffer; superscript letters, other chemical treatments.

† Chemical treatments: [a]formalin, glutaraldehyde, EDTA, trichloroacetic acid, clorox, 25% NaCl, methanol/chloroform/proprionic acid, perfix; [b]propanol, methanol, Carnoy fixative, formal saline; [c]ethyl acetate, Carnoy fixative; [d]chelating ion-exchange resin; [e]phenol; [f]formalin; [g]ethylene glycerol.

‡ Reference numbers: [1]Pyle and Adams (1989), Chase and Hills (1991), Rogstad (1992), Nickrent (1994), Flournoy et al. (1996); [2]Post et al. (1993); [3]Dillon et al. (1996); [4]Reiss et al. (1995); [5]Altschmied et al. (1997); [6]Proebstel et al. (1993), Asahida et al. (1996), Altschmied et al. (1997); [7]Seutin et al. (1991); [8]Albariño and Romankowski (1989); [9]Holzmann and Pawlowski (1996).

chose to investigate the effects of five buffer solutions (70% ethanol, ''Queen's'' lysis buffer—see Experimental Procedures, DMSO-NaCl solution, hexadecyl trimethyl ammonium bromide [CTAB]–NaCl solution, and a urea extraction buffer) and storage at three temperatures (frozen, refrigerated, and ambient) on the long-term preservation of tissue from four marine invertebrate species. Desiccation was not included in this investigation because the high water content of many marine invertebrates (e.g., Scyphozoa, Polychaeta) would not be compatible with the requirement that samples be dried within 12 h to prevent degradation of DNA (Chase and Hills, 1991). The species were chosen to represent four classes of common marine invertebrates: gastropod mollusks (*Astraea undosa*), polychaete worms (*Phragmatopoma californica*), and two cnidarians *Anthopleura xanthogrammica* (anemone) and *Aurelia* sp. (scyphozoan jellyfish). Tissue from all species was stored in each solution and placed in all temperatures for up to 28 months prior to analyses. Control samples were cryopreserved in the absence of storage solutions. From the results of the experimental study, we decided to undertake a field test of DMSO-NaCl.

Results

The preservative used and the type of tissue preserved were the two principal factors influencing the degradation of sample quality. Lesser effects were attributable to the temperature and duration of storage (Figure 1A).

Visible physical degradation was negligible in all samples stored at −80°C. The physical appearance of tissues degraded in almost all other treatments (Figure 1A). Of these treatments, physical structure was preserved best by DMSO-NaCl and 70% ethanol; almost without exception structure could be easily identified after 28 months of storage in these solutions. In contrast, storage in urea resulted in complete dissolution of most samples during 28 months, the principal exceptions were those samples that were frozen. The Queen's and CTAB-NaCl methods were intermediate in performance—neither satisfactorily preserved the physical structure of samples. Typically, and regardless of preservative, degrading *Phragmatopoma* appeared as skeleton-like structures that would break up if disturbed. Degrading tissues of *Aurelia* became a slurry in the bottom of all tubes. Degradation of *Astraea* and *Anthopleura*, when not dissolved by urea, was commonly apparent as a loss of opacity of the tissues.

The physical condition of a sample was a poor indicator of the quality of DNA in that sample. High molecular weight DNA (~20 kb) was extracted from most samples stored for up to 28 months in all solutions or at −80°C, although considerable degradation of DNA also occurred in some of these samples

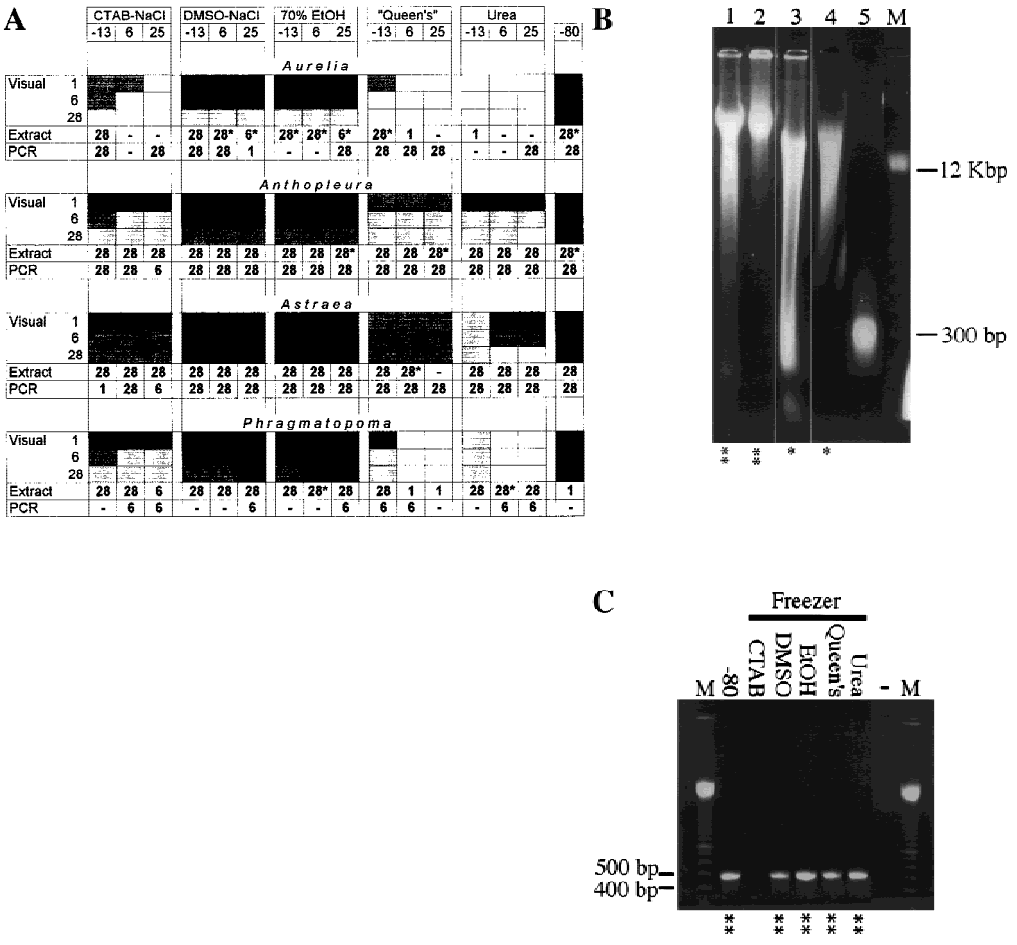

Figure 1A. Effect of storage treatments on the preservation of tissue samples for DNA analyses. Each column represents a combination of one storage solution and one storage temperature (−13°C, 6°C, 25°C), excepting ''−80,'' which indicates storage at −80°C alone. Within these columns, each cell indicates the effect of that treatment on the preservation of one of four tissue types, stored for 1, 6, or 28 months, assessed by three criteria: (1) preservation of the visual appearance of tissues, assessed on a 4-point scale from black (well preserved) through to white (poorly preserved); (2) the latest stage (months) at which high molecular weight DNA was successfully extracted from the sample; (3) the latest stage at which DNA extracts were successfully amplified using PCR. Amplifications using DNA from *Phragmatopoma* stored for 1 month were all unsuccessful. This is attributed to degradation of the DNA after extraction because fragments were successfully amplified from *Phragmatopoma* samples stored for 6 months. Asterisks indicate significant degradation of DNA although some high molecular weight DNA is still present.

Figure 1B. Examples of successful and unsuccessful extractions of high molecular weight DNA. **Successful: lane 1 (*Anthopleura,* stored in CTAB-NaCl at 6°C for 28 mo) and lane 2 (*Anthopleura,* DMSO-NaCl, 6°C, 28 mo). *Successful with degradation: lane 3 (*Anthopleura,* 70% ethanol, 25°C, 28 mo) and lane 4 (*Astraea,* Queen's, 6°C, 28 mo). Unsuccessful: lane 5 (*Astraea,* Queen's, 25°C, 28 mo). M indicates 100-bp ladder.

Figure 1C. Examples of successful (**) and unsuccessful amplification of the ITS-1 region by PCR. All products were amplified from *Astraea* stored at −13°C for 28 months; ''—'' denotes a negative control.

(Figures 1A, 1B). The failure of almost all treatments to prevent considerable degradation of DNA in samples of *Aurelia* is conspicuous.

The success of PCR amplification primarily reflected effects attributable to tissue type (Figures 1A, 1C). With few exceptions, DNA extracted from *Astraea* and *Anthopleura* was successfully amplified at each stage of the 28-month investigation. Amplifications of *Aurelia* DNA showed far greater variability in success and little pattern with regard to preservation method. Attempts to amplify ITS-1 from *Phragmatopoma* DNA, however, were more consistently unsuccessful. DNAs extracted from *Phragmatopoma* after 1 month and 28 months in

storage all failed to amplify; DNA extracted after 6 months in storage did yield amplification product but not in a predictable manner from any particular treatment. Of those samples stored at −80°C, only *Phragmatopoma* DNA failed to amplify after 28 months in storage.

These results show that the principal factors affecting sample preservation were tissue type and storage buffer. If tissue was not susceptible to degradation (*Astraea* and *Anthopleura*), then the storage solution had little effect on the preservation of DNA. Conversely, if samples were susceptible to degradation (*Aurelia* and *Phragmatopoma*), then the storage solution used had considerable influence on the success of preservation (Figure 1A). In these cases, preservation using CTAB-NaCl, Queen's, or urea was noticeably less successful than treatments using DMSO-NaCl or 70% ethanol.

Two other factors, duration and temperature of storage, also influenced the success of sample preservation. First, visual inspection of samples stored for 1, 6, and 28 months suggests that the greater the duration of storage prior to analysis, the greater the degradation of the sample. This pattern was most obvious from the visual assessments of physical quality, but was also reflected in increasing fragmentation of high molecular weight DNA with time. Second, in the few cases in which preservation success varies according to the storage temperature, it seems that greater degradation occurs at the higher storage temperature (e.g., see *Aurelia*), although this effect is neither consistent nor pervasive (and is arguably reversed in *Phragmatopoma*). Notably, temperature affected the appearance of some storage solutions. Refrigeration and freezing caused both CTAB-NaCl and urea solutions to separate into two layers (one white and one clear), while Queen's solution froze when kept at −13°C. The physical appearances of DMSO-NaCl and 70% ethanol were not affected by storage temperature.

Samples of marine invertebrates and fish collected in Palau and stored under refrigeration in DMSO-NaCl for between 6 and 18 months were in good physical condition. Most provided high molecular weight DNA, and all but one were amplified by the PCR using either cytochrome c oxidase subunit I or ''D-Loop'' primers (Figure 2).

Discussion

Many factors affect the preservation of DNA, including the type of tissue, the chemical and physical environment in which that tissue is stored, and the duration of storage. However, the interactions of these factors and their resultant effects on DNA preservation are difficult to predict a priori. Consequently, one might wish to test several alternative methods prior to field collections, or to use more than one preservation method once in the field (Chase and Hills, 1991; Rogstad, 1992). However, in the absence of such contingency plans, this research suggests that storage in DMSO-NaCl is the method most likely to result in successful preservation of tissue samples. Added precautions include storing the preserved samples at reduced temperature and performing DNA extractions at the earliest opportunity.

The most striking result of this study is the effect of different types of tissue on the success of preservation. These differences may be due to several factors. For example, samples of the two species that were most successfully preserved, *Astraea* and *Anthopleura,* were taken from muscular tissue, which is physically more robust than either the soft body of *Phragmatopoma* or the very delicate gonadal and gastric tissues of *Aurelia.* Second, a greater concentration of catalytic enzymes was most likely present in samples of *Phragmatopoma* and *Aurelia* than in samples of *Astraea* and *Anthopleura* as the former samples both included gastric material. Finally, some constituent of *Phragmatopoma* was responsible for making the successfully preserved and extracted high molecular weight DNA unavailable for amplification. Tissue type has also been found to affect the success of preservation and DNA analyses of samples from plants (Pyle and Adams, 1989; Chase & Hills, 1991; Rogstad, 1992) and birds (Seutin et al., 1991), and Altschmied et al. (1997) reported the abdomen of some ants must be discarded prior to preservation else the formic acid therein will depurinate the DNA. Other chemical interactions affecting the recovery of high molecular weight DNA have been noted by Flournoy et al. (1996).

In addition to tissue type, the choice of storage solution exerted an important influence on the rate of degradation of samples. In accord with Seutin et al. (1991), we found that DMSO-NaCl was the best solution in which to store tissues. This was the only solution that preserved recognizable tissue and amplifiable high molecular weight DNA from *Aurelia.* DMSO-NaCl may protect DNA in several ways. DMSO is a cryoprotectant (Dessauer et al., 1995) and thus prevents freeze-thaw damage of samples, although damage incurred in this way may not be a significant concern (Seutin et al.,

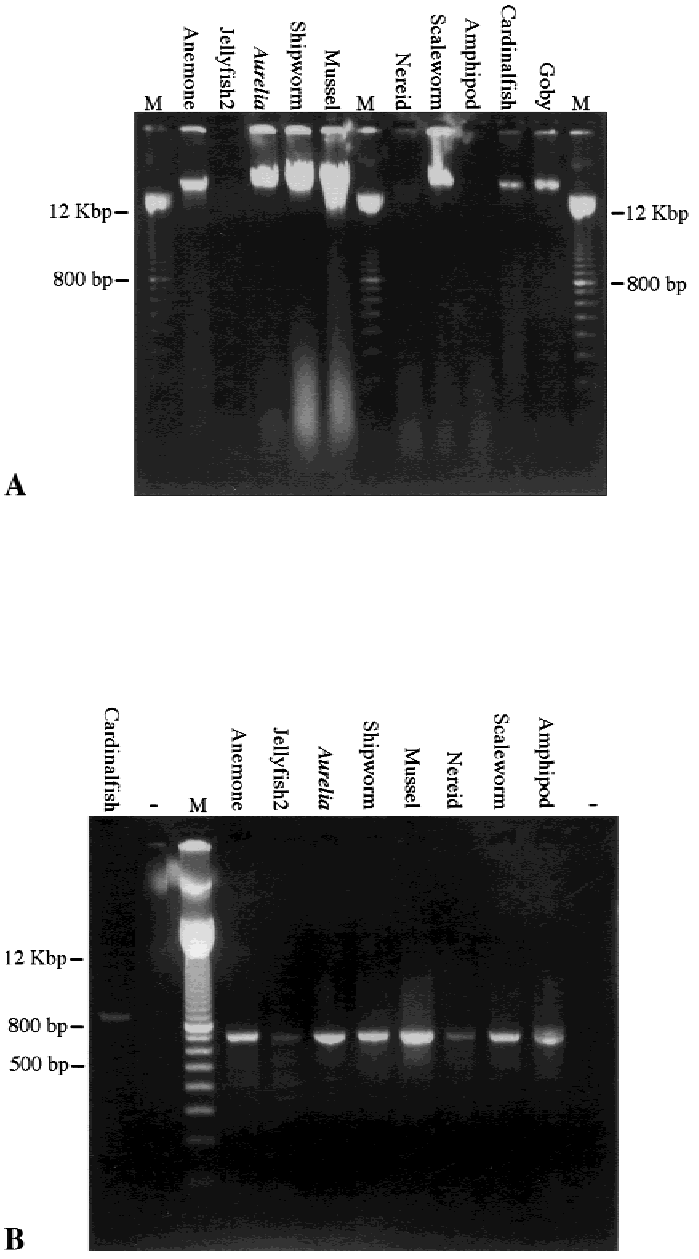

Figure 2. Results from a field test of the DMSO-NaCl preservation method. (A) DNA extracts from tissues of 9 taxa stored in DMSO-NaCl at 5°C for 6–18 months. (B) PCR products amplified from these DNA extracts.

1991). Also, by perturbing the structure of membrane-bound proteins, DMSO enhances the absorption into cells of materials, such as EDTA and NaCl, that inhibit nucleases (Seutin et al., 1991). The utility of high-salt solutions in preserving DNA was also demonstrated by the CTAB-NaCl treatment, although this method was less successful than preservation in 70% ethanol. Storage in ethanol dehydrates the sample and results in the denaturation and precipitation of proteins, including catabolic enzymes (Flournoy et al., 1996).

This study did produce unexpected results. First, the urea-based solution was suitable for long-term storage of DNA despite the assertion of Seutin et al. (1989) that, within 6 months, urea transforms into ammonia resulting in an elevated pH in which DNA is denatured. Asahida et al. (1996) have also found a urea-based preservative suitable for long-

term storage of tissues for DNA analyses. Second, preservation of DNA in lysis solutions (urea and Queen's) was arguably unaffected, or even improved, by lower storage temperatures although such buffers were designed for use at ambient temperature (Seutin et al., 1991). Clearly, any decline in the efficacy of these preservatives is, at least, offset by benefits of storage at lower temperatures. Third, these ''lysis'' solutions preserved DNA effectively within the stored tissue even though their intention is to lyse cells and release DNA into the surrounding solution (Seutin et al., 1991); cell lysis is not necessarily synonymous with movement of DNA from that cell into solution, particularly if the lysed cell is in the midst of a largely intact tissue sample.

Compared with tissue type and storage solution, the duration and temperature of storage had minor effects on the degradation of samples over the time course of this study. However, these minor effects agree with previous investigations that reported the quantity and quality of DNA recovered from samples progressively declines as the duration of storage increases (Post et al., 1993; Reiss et al., 1995), and that DNA degrades less rapidly in colder environments (Post et al., 1993; Poinar et al., 1996).

Preservation of tissue may be facilitated by finely dicing tissue to increase permeation of the storage solution into the sample (Seutin et al., 1991; Reiss et al., 1995). Dessauer et al. (1995) suggest that tissue should be minced into pieces no larger than 1 mm.[3] However, none of our samples that yielded high molecular weight DNA contained pieces less than 1 mm^3, with the exception of gastric filaments and gonads of *Aurelia*. Further, the preservation of intact physical structure is often desirable because samples may then be used for morphologic or parasitologic analyses as well as DNA analyses (Post et al., 1993; Reiss et al., 1995). Both physical structure and DNA were preserved well by DMSO-NaCl and 70% ethanol.

Although reproducing results has been a problem in previous studies (see Post et al., 1993), we experienced few discrepancies between independent tests, run concurrently by two of the authors. Consequently, the general patterns apparent in this study are expected to be robust to further investigation. The discrepancies that did occur were predominantly in species and storage groups that exhibited degradation in at least several treatments. In contrast, there were no contradictory results for any sample stored in DMSO-NaCl. This implies that, of the treatments investigated, storage in DMSO-NaCl is least sensitive to small inconsistencies (e.g., precise source and volume of tissue) that may occur during the preservation of samples.

Field testing of the DMSO-NaCl method supported the experimental results and also showed that this method is appropriate for a wide range of organisms and is extremely simple to use in the field. The DMSO-NaCl solution may be made and aliquoted into microcentrifuge tubes prior to departure. These tubes may be kept at any moderate temperature, before and after they are used to preserve samples, although chilling is preferable. The few items that need to be taken on each field excursion are the appropriate number of prepared microcentrifuge tubes, a small dissection kit with which to collect the sample, and materials such as sterile water, ethanol, and a cigarette lighter with which to clean samples and sterilize equipment. This small volume and mass of equipment is appropriate for lengthy collecting trips to remote sites.

Experimental Procedures

Collections were made on the coast of California between June 19 and 26, 1995. Tissues from *Aurelia* sp. were extracted at the field site and placed into storage solutions in temporary temperature conditions for the duration of sampling and transport to UCLA (<10 h). During sampling and transportation, deep-frozen, frozen, and refrigerated samples were kept on ice, while room-temperature samples were kept at ambient temperature. *Astraea undosa*, *Phragmatopoma californica*, and *Anthopleura xanthogrammica* were transported live to UCLA where they were kept in a seawater aquarium until tissues were dissected out.

Tissue samples, approximately 0.2 cm^3, were taken from *Phragmatopoma californica* (a single whole worm), *Astraea undosa* (foot muscle), *Anthopleura xanthogrammica* (body wall), and *Aurelia* sp. (gonad and stomach). Each sample was washed with 0.22 µm filtered seawater and then deposited into 500 µl of each of five storage solutions. The saturated NaCl-CTAB solution described by Rogstad (1992) was modified to consist of 0.1 M Tris (pH 8.0), 0.02 M EDTA (pH 8.0), 0.02% (wt/vol) CTAB and saturated with NaCl. This solution was autoclaved and cooled prior to the addition of β-mercaptoethanol to 0.002%. DMSO-NaCl solution is 20% DMSO, 0.25 M disodium-EDTA, and NaCl to saturation, pH 7.5 (Seutin et al., 1991); the pH of this solution must be above 8 for the EDTA to dissolve, and warming promotes dissolution of

NaCl. Queen's lysis buffer contains 0.01 M Tris, 0.01 M NaCl, 0.01 M disodium-EDTA, and 1.0% *n*-lauroylsarcosine, pH 8.0 (Seutin et al., 1991). Both DMSO-NaCl and Queen's solutions were sterilized by autoclaving prior to use. Urea buffer (168 g of urea, 25 ml of 5 M NaCl, 20 ml of 1 M Tris, 16 ml of 0.5 M EDTA, 40 ml of 10% sodium dodecyl sulfate (SDS), plus 170 ml of distilled water, pH 8.0) was filter-sterilized under vacuum through a 0.2-µm millipore filter; 100% ethanol was diluted to 70% using 4.0-µm millipore filtered seawater and then autoclaved.

Replicate samples of each of the 20 possible combinations of species tissue and storage solution were kept under three temperature regimens (mean ± 2.5°C): frozen (−13°C), refrigerated (6°C), and ambient (25°C). In addition to these experimental conditions, control samples of each tissue, with no added storage solution, were cryopreserved at −80°C. All samples were stored in opaque containers to prevent damage of samples by UV irradiation (Dessauer et al., 1995).

After 1 and 6 months in storage, two of the authors (K.A.R. and M.ND.) independently assessed the state of tissues stored in each of these conditions. The analyses at 28 months were performed solely by M.ND. First, the physical condition of tissues was visually assessed according to a 4-point scale, by comparing the appearance of stored tissue relative to the appearance of fresh material. Second, each treatment was scored for the presence or absence of high molecular weight DNA. Amplification of a PCR product from the extracted DNA was the third indicator of a successful preservation method.

In general, tissue pelleted out of the storage solution by centrifugation for 5 min at 14,000 rpm was used for the DNA extraction. The supernatant was pipetted off (see below*), and the pelleted tissue was resuspended in 600 µl CTAB (0.1 M Tris [pH 8.0], 0.02 M EDTA [ph 8.0], 0.02% [wt/vol] CTAB, 0.8 M NaCl, 0.002% β-mercaptoethanol) with 6 µl proteinase K (20 mg ml^{-1}) and digested at 42°C for 16 h. This proteinase K digestion was omitted for samples from *Aurelia*. DNA is liberated from these tissues by grinding in a dounce. Digested or ground samples were centrifuged for 5 min at 14,000 rpm before 300 µl of the supernatant was taken for DNA extraction. If samples were stored in urea or Queen's, however, only 150 µl of this supernatant was used in the DNA extraction; the other 150 µl was pipetted from the original preservative (see above*). When no tissue was preserved by urea or Queen's, 300 µl of the original preservative was used in the DNA extraction. The DNA extraction consisted of a single extraction with chloroform followed by repeated extractions with phenol:chloroform:isoamyl alcohol (25:24:1) until the interface between aqueous and organic phases was clear. A single chloroform:isoamyl alcohol (24:1) extraction was then completed before precipitating the DNA at −20°C for 1 h with 3 M sodium acetate and 100% ethanol. Purified DNA was resuspended in 30 µl of sterile water after the precipitate had been centrifuged for 28 min at 13,000 rpm, 6°C, and the resulting pellet was washed in 75% ethanol and dried at 25°C. The quality of DNA extract was determined by electrophoresing an 8-µm aliquot of each DNA sample across a 1.4% agarose minigel (1% TBE) containing ethidium bromide and scoring for either presence or absence of high molecular weight DNA. The ITS-1 region was amplified using the primers

5′GCGTTCGAARTGCGATGATCAA

and

5′-GTAGGTGAACCTGCAGAAGG

(Vogler and DeSalle, 1994). Approximately 1 ng of sample was added to each 25 µl total volume for PCR, set up according to the guidelines issued with *Taq* polymerase (Perkin Elmer). A ''hotstart'' was included before entering a 28-cycle PCR. Each cycle on the MJ Research MiniCycler comprised 45 s at 94°C, 45 s at 50°C, and 60 s at 72°C. PCR product was visualized as above, and scored for either presence or absence of a single strong band within the size range of previously sequenced ITS-1 regions (Vogler and DeSalle, 1994).

Based on preliminary (1-month) results, tissues from 10 additional taxa were sampled and stored for 6 to 18 months in DMSO-NaCl (~5°C) during field trips to the Republic of Palau, Micronesia, in 1996/1997 (6 mo, mussel, nereid polychaete; 9 mo, gammarid amphipod, goby, cardinalfish; 10 mo, anemone, *Aurelia*, shipworm, scaleworm; 18 mo, mastigiid jellyfish). All steps of the subsequent DNA extraction, PCR, and visualization of products were completed as above. At this time, other primers were used to amplify the mitochondrial control-region

(5′-TTCCACCTCTAACTCCCAAAGCTAG

and

5′-TATGCTTTAGTTAAGGCTACG,

Lee et al., 1995), or cytochrome c oxidase subunit I

(5′-GGTCAACAAATCATAAAGATATTGG

and

5′-TAAACTTCAGGGTGACCAAAAAATCA,
Folmer et al., 1994) from these samples.

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