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Effectiveness of alternative organic solvents in field preservation of whole barnacles for PCR analyses

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There are few reports of non-cryogenic preservation methods for marine invertebrates, so potable alcohol and acetone-based nail varnish remover (NVR) are for the first time evaluated against absolute ethanol as short-term preservatives of whole barnacles. Performance of ethanol and NVR-preserved material was comparable, but potable alcohol was significantly worse. These results are of practical importance for fieldwork in remote areas where laboratory chemicals are unattainable but potable alcohol or NVR are locally available. Of these, acetone-based NVR would be the solvent of preference.

INTRODUCTION

The development of the polymerase chain reaction (PCR) has facilitated areas of research in marine biology involving the application of biomolecular techniques. There is therefore an increasing appreciation of the importance of archival collections of tissue samples for future use in molecular studies. A more immediate problem is to preserve samples in the field so that they will be in appropriate condition for such analyses.

Immediate freezing in liquid nitrogen or dry ice is the best method of field preserving samples for PCR-based analyses (Dessauer et al., 1996). The structural integrity of DNA can be maintained long-term for material kept frozen, particularly in ultracold freezers or in liquid nitrogen. If cryopreservation is not possible, then immersion in ethanol is usually recommended (e.g. Dessauer et al., 1996). Compared with the numerous reports for other (mainly terrestrial) organisms (for reviews and further references, see Dessauer et al., 1996 or Dawson et al., 1998), there are relatively few published tests on non-cryopreservation methods for marine invertebrates. Dawson et al. (1998) pointed out that this is unfortunate given that much marine biodiversity occurs in remote areas where access to laboratory materials and facilities is difficult. They tested 70% ethanol, lysis buffer, DMSO–NaCl solution, NaCl/CTAB solution, and a urea extraction solution for four classes of marine invertebrates: Anthozoa, Gastropoda, Polychaeta, and Scyphozoa. DMSO–NaCl was suggested as the most effective method. However, the short-comings are that, first, the solution requires careful handling because DMSO is a skin irritant and can transport toxic compounds into the body (Mason, 1971)—this may be a problem under field conditions where there may be a higher risk of accidental spillage or container failure. Second, chemical solutions necessitate prior preparation, thus ruling out opportunistic sampling in remote areas. Alternative preservatives are high concentration laboratory-grade ethanol (Dessauer et al., 1996) or acetone (Fukatsu, 1999). However, such materials are difficult to transport due to their flammable properties. Thus for sites without a local source, laboratory-grade organic solvents may not be easily transported for use.

Here, we present tests on two alternatives that are more readily obtained: potable alcohol and nail varnish remover (NVR). Easy availability enables unplanned sampling, or sampling by those who do not have easy access to laboratory chemicals (e.g. volunteers or scientists in under-developed regions). To our knowledge, empirical tests of these materials have not been previously published. Here, we evaluate them for preserving barnacles (Cirripedia), for which tests of non-cryopreservative methods are lacking. Molecular applications in barnacle studies have been increasing in importance, most notably in studies examining associations between population genetic structure, selection at specific loci, and environmental heterogeneity (e.g. Schmidt et al., 2000). Such molecular ecological applications, particularly for natural populations in remote locations, would be facilitated by improvement of non-cryopreservative methods.

RESULTS AND DISCUSSION

Laboratory-grade absolute ethanol was used as the control. One brand of gin (Tesco brand, 37.5% volume) and one brand of NVR (KK Classic, KK Toiletries, UK) were arbitrarily used, with the presumption that any other would perform similarly. Since some studies may require intact organisms, and dissection may be difficult in the field, whole barnacles were preserved. *Balanus perforatus* Bruguiere, 1789 collected from the Gower (South Wales, UK) were immersed in at least an equal volume of preservative (about 60 ml) in 'Leaktite' 120-ml sterile pots (Elkay). These remained at room temperature for a maximum of nine months—a realistic time period for the longest duration of most field studies. For each treatment, the DNA of each of 25 barnacles was extracted using the PUREGENE DNA isolation kit (Gentra Systems)

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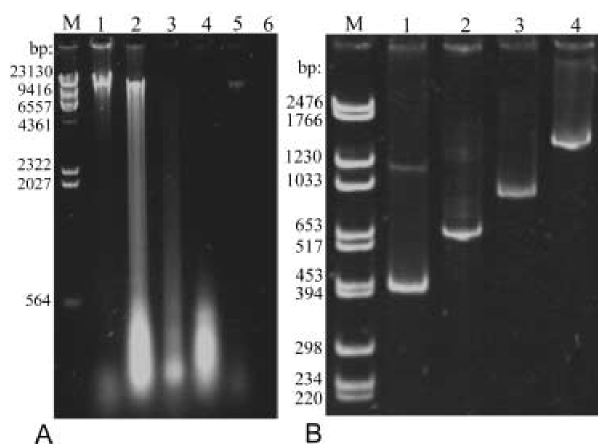

Figure 1. Examples of gel electrophoresis for whole genomic DNA and PCR products. (A) Six categories of sample quality ranked from best to worst (1 to 6). 1, high-molecular weight DNA; 2, some high-molecular weight DNA, but also some shearing; 3, most of the DNA is sheared; 4, only highly sheared DNA; 5, little DNA in the sample (sheared or not); 6, no DNA visible; M, molecular weight marker; (B) the PCR products amplified from *Balanus perforatus*. 1, 12S mtDNA fragment (409 bp); 2, 18S nuclear DNA fragment (619 bp); 3, COI/IIb mtDNA fragment (853 bp); 4, COI/IIa mtDNA fragment (1315 bp).

following the manufacturer's instructions, and re-hydrated in 40 μl of the kit's hydration solution.

Three microlitres of the DNA extract sample were electrophoresed through 1% agarose gel (Invitrogen), and the results documented with the Gel Doc 2000 system (Biorad) (Figure 1A). DNA quality was visually assessed and categorized as in Figure 1A. Stock DNA concentration (DNA yield) was assessed with a Genequant spectrophotometer (Pharmacia). The PCR efficiency was assessed with a panel of four primer sets targeting different DNA regions of various fragment sizes (Figure 1B). The COI/IIa fragment spans the cytochrome oxidase subunits I and II (COI and COII) of mitochondrial DNA (see Power et al., 1999 for primer sequences). For a shorter fragment (COI/COIIb), we designed a new primer targeting a section just within the COII region: (COIIb): 5′-AAGG(A/T)GA(G/A)GCTCTATCTTG. The 12S fragment is again shorter (Figure 1B) (primer sequences in Mokady et al., 2000). The final fragment is amplified from the nuclear 18S rDNA gene (primer sequences in Reischl et al., 1997). Ten samples for each treatment were tested. For all, 5 ng of extracted DNA was used in 25 μl reaction mixes. Mixes included 100 μM dNTPs, 4 mM $MgCl_2$, Buffer IV (ABGene: 750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20), 1 unit *Taq* DNA polymerase (ABGene). Reactions in a DNA Engine (MJ Research) thermocycler followed recommended cycling programmes (references are those as for primer sequences, the only modifications being reductions of magnesium chloride for 18S and 12S to 2mM and 1mM respectively). The protocol for COI/COIIb was: 94°C for 1 min; 94°C for 1 min; 48°C for 1 min; 72°C for 2 min—29 cycles; 72°C for 7 min. To assess PCR success, the number of successful reactions were tallied: 0 indicated failure for all four primer sets, a value of one indicated success for only one primer set, and so forth, for a maximum index value of four.

Average DNA yield of barnacles from each preservation treatment were similar and not significantly different (analysis of variance, F=2.622, df=3,18, P=0.056). In contrast, DNA quality was significantly different among treatments (Kruskal–Wallis test, df=2, P<0.001). The DNA of almost half the samples preserved in gin were either completely sheared or in very small amounts (categories 4 to 6) and were of significantly lower quality than ethanol—(Mann–Whitney test, Z=−3.905, P<0.001) or NVR-preserved samples (Mann–Whitney test, Z=−3.112, P=0.002). Samples preserved in NVR and ethanol were similar (Mann–Whitney test, Z=−0.705, P=0.481) and mainly of intermediate quality (categories 2 to 3; Figure 2A). There was a significant correlation between DNA quality and the degree of PCR success (Spearman rank correlation, P=0.002). Other studies have found similar associations (Dean & Ballard, 2001; Lee & Griffiths, 2003).

The method of preservation had a significant effect on PCR success (Kruskal–Wallis test, df=2, P=0.025). Whereas the average PCR success for NVR and ethanol

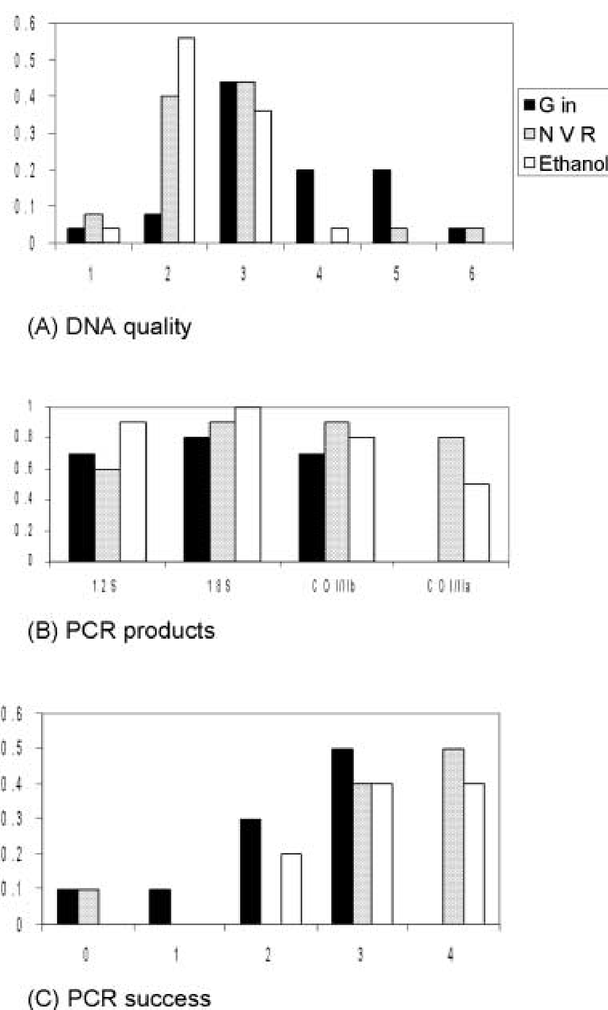

Figure 2. Sample frequencies in (A) categories of DNA quality (1 to 6; see Figure 1A); (B) successful PCR amplifications of target DNA fragments; (C) successful amplification for none, one, two, three or four PCR fragments.

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preserved samples were similar (3.20 ±SE 0.39 and 3.20 ±SE 0.25 respectively), that of the gin-preserved samples were lower (2.20 ±SE 0.33). The large COI/IIa fragment could not be amplified from any of the gin-preserved samples, although some success was achieved for other fragments (Figure 2B). In contrast, the COI/IIa fragment could be amplified for half or more of the samples preserved in either ethanol or NVR. For all data, the success of PCR amplification was significantly associated with the size of the DNA fragment (likelihood ratio test, df=3, P=0.001), but this was no longer significant when gin-preserved samples were excluded (likelihood ratio test, df=3, P=0.078), or when COI/IIa was excluded (likelihood ratio test, df=3, P=0.234).

In summary, the performance of NVR as a short-term preservative of whole barnacle specimens for PCR analyses was found to be as good as that of absolute ethanol. Samples preserved in gin yielded DNA of lesser quality with higher degrees of PCR failure. Nevertheless, for these samples, there was some reasonable success in the amplification of shorter PCR fragments. Given the similarilty in performance between NVR and absolute ethanol, and previous demonstration of the effectiveness of acetone for terrestrial invertebrates (Fukatsu, 1999), it is likely that NVR will also be effective for other marine invertebrates. Nevertheless, Dawson et al. (1998) recommended that the preservation of specific species of interest should be tested prior to actual fieldwork. The results of this study suggest that NVR or acetone could certainly be included in such experimental trials. Use of locally available NVR will negate the problem of transportation to the field site. The acetone-containing NVR is still a flammable substance, but once the sample has been fixed in it, excess solvent can be poured away just prior to transporting the samples back to the laboratory. Indeed, this is the standard practice for ethanol-preserved material. Thus, in situations where laboratory chemicals are not immediately available, NVR is a suitable material for the short-term preservation of samples intended for use in PCR analyses.

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