INTRODUCTION

Sea anemones (Anthozoa: Actiniaria) are simple askeletal polyp animals, and a very diverse, ecologically important group of organisms (Schick, 1991). In spite of the many techniques that have been developed, the isolation of template DNA for PCR (Saiki et al., 1988) amplified from individual, small organisms can be difficult. Recently, DNA-based techniques have been used to determine the relationship of the Cnidaria to other metazoa and to examine relationships within the Cnidaria (e.g., Hori and Satow, 1991; Christen et al., 1991; Bridge et al., 1992; 1995; Chen et al., 1995; Beagley et al., 1995; Veron et al., 1996; France et al., 1996; Odorico and Miller, 1997; Romano and Palumbi, 1997). Such approaches are elucidating long-standing controversies about relationships within the Cnidaria.

There are few techniques available for the extraction of DNA from sea anemone species (e.g., Wolstenholme, 1992; Pont-Kingdon et al., 1994; Finnerty and Martindale, 1997; Fautin and Smith, 1997) and these studies followed standard protocols previously described for other metazoan organisms (e.g., those of Wolstenholme and Fauron, 1976; Shure et al., 1983; Winnepenninckx et al., 1993; Folmer et al., 1994).

We have had success in obtaining good DNA templates using an optimization of the protocol described by Chen et al. (1995) to extract and subsequently amplify DNA from sea anemone specimens. The total DNA extracted was used as a template in polymerase chain reaction (PCR) experiments. The nuclear DNA was analyzed using the single primer amplification reaction technique (SPAR) (Gupta et al., 1994). These pilot experiments were carried out using the following species: Aiptasia pallida Verrill, 1864, Bellactis ilkalyseae Dube, 1983, Anthopleura krebsi Duchassaing & Michelotti, 1860, Carcinactis dolosa Riemann-Zünneck, 1975, Paratelmatectis sp. (Pinto, S.M., unpublished data) and one species of corallimorpharian, Discosoma carlgreni (Watzl, 1922).

This article reviews the published literature and suggests a feasible DNA extraction method to obtain large amounts of pure and integral DNA which can be used in different kinds of molecular analyses.

RESULTS

DNA extraction

We unsuccessfully attempted to extract DNA from sea anemones using previously published protocols. We then optimized the protocols by including some important additional steps involving careful adjustments in tissue maceration, followed by optimization of the concentrations of proteinase K and by modification of the standard phenol:chloroform extraction techniques.

Total genomic DNA was extracted from pieces of 100% ethanol-preserved sea anemones. For most specimens small pieces (approximately 0.5 to 1.0 cm) of tissue from the pedal disc were used, which avoided possible amplification of the zooxanthellae DNA (Fautin and Smith, 1997). In all steps, shaking was for 15 min and centrifugation for 4 min at 6500 rpm, except in the third step that centrifugation was at 13,000 rpm. Prior to extraction the tissue fragments were placed in an Eppendorf tube and incubated at 35°C for 2 h in order to withdraw excess etha-
In step one, small pieces of tissue were ground into powder under liquid nitrogen and then placed in a final volume of 400 µl of lysis buffer (10 mM Tris-HCl, pH 8.0, containing 0.25 M EDTA, pH 8.0, 2% (w/v) sodium dodecyl sulfate, SDS). In step two, RNAse was added to a final concentration of 0.6 mg/ml and the sample was incubated for 30 min at 37°C in order to eliminate any RNA molecules. After that, proteinase K was added to a final concentration of 1 mg/ml and the mixture incubated for 72 h at 37°C. After incubation an equal volume of phenol was added to the sample, which was then shaken and centrifuged. Approximately 360 µl of the supernatant solution was then transferred to a clean tube and an equal volume of phenol added. Shaking and centrifugation were performed using the same conditions as above and 340 µl of the supernatant was transferred to a new tube with an equal volume of chloroform:isoamylalcohol (24:1) and gently shaken before centrifugation. In step three, 300 µl of the supernatant was transferred to a new tube containing 30 µl of 6 M NaCl and gently stirred. The DNA was precipitated with 2.5 volumes of very cold absolute ethanol and the samples centrifuged for 4 min at 13,000 rpm. The supernatant was drained off and the pellet was washed with 70% ethanol and air-dried for 24 h with the tube inverted. The samples were resuspended in an appropriate volume of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and incubated at 37°C for at least 48 h, until completely soluble.

DNA was quantified in a spectrophotometer (Itachi model U-2000), the mean purity value was 1.8 ± 0.1 and extracted DNA concentration was 300-1,400 ng/µl. The integrity of the extracted DNA was checked using 2 µl of each sample on a 1.0% agarose gel stained with ethidium bromide. Figure 1 shows that the samples presented no signs of degradation. Figure 1 (upper half, lanes 6-9) also shows four unsuccessful DNA extractions, where samples were incubated (24 h at 37°C) in the presence of proteinase K.

DNA extracted from sea anemone samples was used as a template in PCR amplifications with the SPAR technique (Gupta et al., 1994). The tetranucleotide repeat primers used were 16 bases long. A variety of primers were assayed with the most informative outcomes from (GACA)₄. In a study of the population structure of Actiniaria species being undertaken by S.M. Pinto and her colleagues, (GACA)₄ is being used as a molecular marker under the name Micro 5.

**PCR reactions**

DNA (1 ng) was amplified in a total volume of 30 µl containing 10 mM Tris-HCl, pH 8.4, 0.5% nonidet P-40, 50 mM KCl, 5.0 mM MgCl₂, 100 µM each of dNTP (dGTP, dATP, dCTP and dTTP), 5 pmol (GACA)₄ primers and 1.25 units of TaqDNA polymerase (Life Technologies). Amplifications were performed in a Perkin Elmer TC1 thermocycler for 35 cycles. The first 5 cycles consisted of 45 s at 94°C, 60 s at 51°C, and 60 s at 72°C. The final 30 cycles consisted of 45 s at 92°C, 60 s at 48°C, and 60 s at 72°C. All products were visualized on a 1.4% agarose gel stained with ethidium bromide (Figure 2). Negative controls lacking sea anemone DNA were included in PCR assays to monitor any possible contamination (Palumbi, 1996).

**DISCUSSION**

The systematics of sea anemones is currently based on only a few morphological characters (see McCommas, 1991; Fautin and Smith, 1997), and may potentially improve the use of molecular characteristics for clarifying phylogenetic relationships among genera and species. The utility of DNA molecular markers for addressing evolutionary questions in sea anemones has already been demonstrated by Pont-Kingdon et al. (1994), Beagley et al. (1996), Finnerty and Martindale (1997), and Fautin and Smith (1997).

The results of our study show the striking effects of some details on the success of the DNA extraction protocol. The tissue type, maceration and digestion of tissue and amount of time allowed for proteinase K incubation were all essential for obtaining high-quality DNA.

![Figure 1](image-url) - Total DNA extracted from sea anemone tissues. Upper: M, molecular weight (digested with HindIII) and L, molecular weight 123 DNA ladder (GibcoBRL); lanes: 1 - Anthothoe chilensis, 2 - Bellactis ilkalyseae, 3 - Paratelmatactis sp., 4 - Anthopleura krebsi, 5 - Aiptasia pallida, 6-9 - negative results by extraction using 24-h incubation with proteinase K. Bottom: 1 - A. chilensis (positive control), 2 - B. ilkalyseae, 3 - Paratelmatactis sp., 4 - Discosoma carlgreni, 5 - A. krebsi, 6 - C. dolosa.
Tissue type has been found to affect the success of DNA analyses of samples from plants (Chase and Hillis, 1991; Rogstad, 1992), birds (Seutin et al., 1991) and insects (Altschmied et al., 1997). We concluded that the dehydration of tissue until there is no trace of ethanol was the best solution to avoid interference in the reactions occurring during the extraction. Given the hard consistency of the tissue the use of liquid nitrogen to homogenize the dehydrated tissue until there is no trace of ethanol was found to be the best solution to avoid interference in the reactions occurring during the extraction. Samples of pure DNA are invaluable in studies of genetic evolution and systematic phylogenetic approaches using molecular data. The integrity of DNA is especially important for the amplification of large fragments. Figure 1 shows no signs of DNA degradation in the samples so that they could be used to amplify large fragments. High concentrations of extracted DNA (300-1400 ng/µl) have allowed us to begin a genetic stock of several species of sea anemone. The method described in this paper is a feasible method which allows the isolation of good DNA from tissues of sea anemones, and the protocol may also be applicable to other fleshy marine cnidarians.

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