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Comparative study on various Methods for Field Preservation and DNA Extraction of Zoanthids

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Abstract

Zoanthids, the benthic Anthozoans are found in nearly all coastal marine environments. Zoanthids have become recent interests in the field of scientific study because of the challenge they offer in their taxonomic discernment due to the morphological variability within a species. In these study different methods has been carried out for sample preservation and DNA extraction from Zoanthids. Sample collection and preservation in different fixatives is influential on the quality of DNA during the extraction procedure. The present study is an effort to examine six field fixatives and tries to establish an effective DNA extraction protocol for Anthozoans. Zoanthids were collected and preserved in fixatives like: 1) absolute ethanol, 2) 70% ethanol, 3) lyses buffer with CTAB, 4) Phosphate Buffer Saline with SDS 5) Liquid Nitrogen and 6) Dry Ice. The genomic DNA extraction was carried out using four different protocols, viz. Lyses buffer with proteinase K (LBWPK) 2) Lyses buffer with cetyl trimethyl ammonium bromide (LBWCTAB) 3) Lyses buffer with guanidine Isothiocyanate (LBWGIT) 4) TriZol. The most effective preservative and DNA extraction method, both in term of quality and quantity were confirmed by Gel Analysis and purity was checked by spectrophotometric method.

Keywords: Zoanthids, anthozoans, saurashtra coast, tissue fixation, dna extraction.

Introduction

The encrusting anemone, Zoanthid is distributed worldwide in shallow Sub-tropical and tropical waters, and is commonly found on rocks and coral reef edges exposed to waves and/or currents. Despite their relative abundance, Zoanthids have been overlooked by scholars, because of the intrinsic difficulty in establishing a sound taxonomy based on external morphological criteria and internal morphological examinations due to the presence of sand and detritus in their body. DNA bar-coding is a new technique[1], which reassesses conservation priorities[2] to help increase taxonomic knowledge. Molecular approaches using allozymes and DNA marker, DNA sequencing and phylogenetic analysis[3-5] have begun to reassess the diversity. For all molecular techniques DNA extraction from the animals is a very important step. Biologists have been extracting DNA from various materials using different protocols and have been improving these protocols continuously for better extraction of DNA[6-7]. In spite of this, the quality and quantity of the extracted DNA varies according to the organism, preservation conditions, storage duration and the DNA extraction protocol[8]. It is generally seen that the highest quality of DNA is extracted from live specimens[9], live specimens frozen at -80˚C[8], or live specimens quick frozen in liquid nitrogen[10]. However, these methods are not always practical for field biologists. Reports on comparison of preservation methods have been published on marine invertebrates since 1996[11-15], and a recent study was carried out on Poriferans[16].

With the aim of finding out the best protocol for preservation and DNA extraction of Zoanthids, four protocols were used. Thus, varied protocols were compared for the evaluation of the quality and quantity of Genomic DNA extracted and its suitability for PCR amplification.

Material and Methods

Collection, Preservation and Storage: Zoanthids were collected from the Sutrapada of Saurashtra coast of Gujarat (Latitude 20°5'N; Longitude: 70°29'E). The samples were collected and washed for the removal of symbionts, debris etc. Each sample was preserved in six different preservatives, viz. i. absolute ethanol, ii. 70% ethanol, iii. lyses buffer with CTAB, iv. Phosphate Buffer Saline with 30% glycerol v. Liquid Nitrogen and vi. Dry Ice. Samples for each fixative were taken in triplicates and stored in them for three to four days at room temperature.

DNA Extraction: Zoanthid collection was done and all specimens were divided in fragments of same size and were weighed. They were frozen in dry-ice after removal from sea water. Samples were then subjected to four protocols of genomic DNA extraction i.e.: 1. Lyses buffer with proteinase K (LBWPK)[17] Lyses buffer with cetyl trimethyl ammonium bromide (LBWCTAB) Lyses buffer with guanidine Isothiocyanate (LBWGIT).

Determination of quality and quantity of DNA: The DNA integrity then validated in 0.8% agarose gels run in TAE 1X (40 mM Tris acetate, 1 mM EDTA). For the standardization of the

quantification of method, each sample applied on a gel had its volume adjusted according to the initial wet weight. In order to evaluate the quality of the genomic DNA, gel analysis and Spectrophotometric method was carried out, $A_{260/280}$ was measured and concentration of DNA was calculated using formula:

Concentration of DNA (ng/µl): O.D (260 nm) x 50 x Dilution Factor

PCR Amplification test: The cytochrome oxidase subunit I (COI) gene was amplified by using LCO1490-f (5' GGTCAACAAATCATAAAGATATTGG) and HCO2198-r (5'-TAAACTTCAGGGTGACCAAAAAATCA). Each 20 µl PCR amplification reaction mixture contained 1 µl DNA template (50ng/ µl), 1 µl forward primer, 1 µl reverse primer, 1.6 µl Tween 20, 5.4 µl milli Q water and 10 µl AmpliTaq Gold® 360 master mix. PCR amplification was carried out in a Veriti 96 well thermal cycler. An initial denaturation step of 1 min at 95ºC was followed by 35 cycles of 1 min at 94˚C, 1 min at 40˚C and 1.5 min at 72˚C, with an additional final step of 7 min at 72ºC for final expansion. The amplified bands were separated by electrophoresis on 2% agarose gels in 1 X TAE. The size of the amplified fragments was estimated by comparison with standard DNA ladders.

Results and Discussion

DNA extraction was carried out from all the samples that were preserved in six different fixatives. There were great dissimilarity in DNA quantity and quality observed in all the procedures used here. It was observed that lysis buffer with proteinase K (figure-1d) was improved protocol than LBWCTAB (figure-1a), LBWGIT (figure-1b) and TriZol (figure-1c) as it extracted good quality and quantity of DNA for all the preservatives. Figure-2 and figure-3 shows average $A_{260/280}$ and DNA concentration from the samples preserved in six different fixatives. Sample preserved in absolute alcohol obtained good $A_{260/280}$ ratio that is 1.84 and concentration of DNA was 260 ng/µl. Pinto et al. used LBWPK for DNA extraction from sea anemone and Sinniger et al., used LBWGIT and Mythili Krishna and Gophane used LBWCTAB for DNA extraction. However, Salgado et. al., mentioned Lyses buffer with guanidine hydrochloride is best procedure for DNA extraction from the porifera.

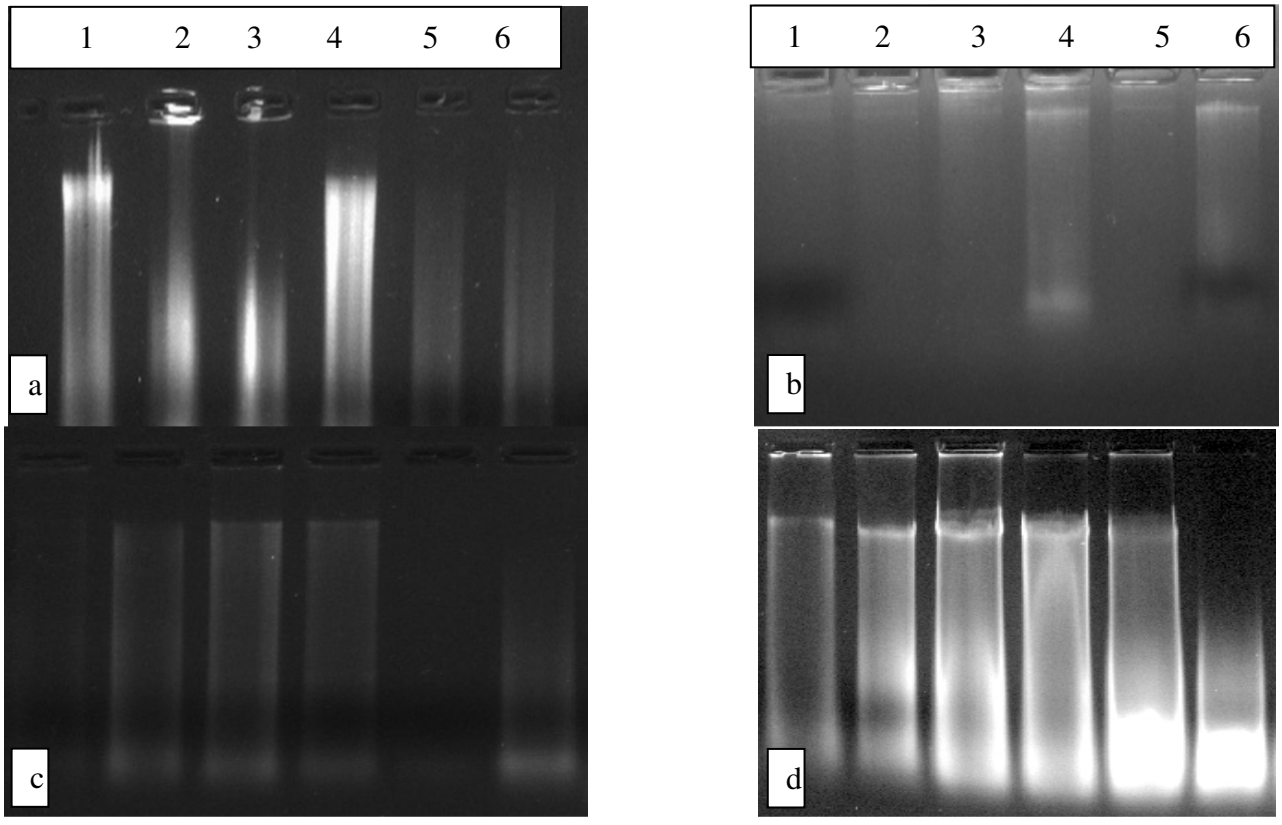

Figure-1

a) DNA extraction with LBWCTAB, b) DNA extraction with LBWGIT, c) DNA extraction with Trizol d) DNA extraction with LBWPK. Well 1- Sample preserved in Phosphate Buffer Saline with 30% Glycerol, Well 2- Sample preserved in lysis buffer with CTAB, Well 3- Sample preserved in Dry Ice, Well 4- Sample preserved in Absolute Ethanol, Well 5- Sample preserved in 70% Ethanol, Well 6- Sample preserved in Liquid Nitrogen

Sample preserved in absolute alcohol has better DNA than sample preserved in 70% alcohol. Ethanol (EtOH) is also known to be killing agent as well as a preservative, with a critical percentage level. Ethanol dries out water from tissue and cells, dehydrating the tissue and thus preserving DNA. Absolute Ethanol (99.5%-99.9%) when ideally combined with cold temperature is best for optimum preserving conditions for keeping DNA integrated for over a hundred years[18]. 70% ethanol is also used as a preservative but it caused degradation of DNA as it contain 30% of other material (mostly water), which causes degradation of DNA. DNA is better preserved using dry (pinned) specimens, than with material stored at 70% ethanol. Krishna and Gophane[19] and Reimer et al.[20] has used absolute alcohol and 99% ethanol respectively, for preservation of Zoanthids for molecular analysis while Reimer and Fujii, used 75% alcohol for preservation of zoanthids. Framptom et. al., mentioned 95% ethanol as a best preservative for bees. Sample preserved in PBS with 30% glycerol and dry ice has also given a good result with CTAB method, while sample preserved in lysis buffer with CTAB has been degraded and that has affected the quality and quantity of DNA. Sample preserved in liquid nitrogen has difficulty in homogenization due to presence of ice crystals of sea water and that has affected DNA due to mechanical sheared. However it has been observed that Protenase K is better for *Palythoa* species while Guanidium isothiocynate has shown better extraction of DNA from *Zoanthus* species. Despite the variation in DNA quantity and quality, all extraction procedures resulted into DNA that rendered single PCR products for COI gene that is of 648 bp fragment (figure-4).

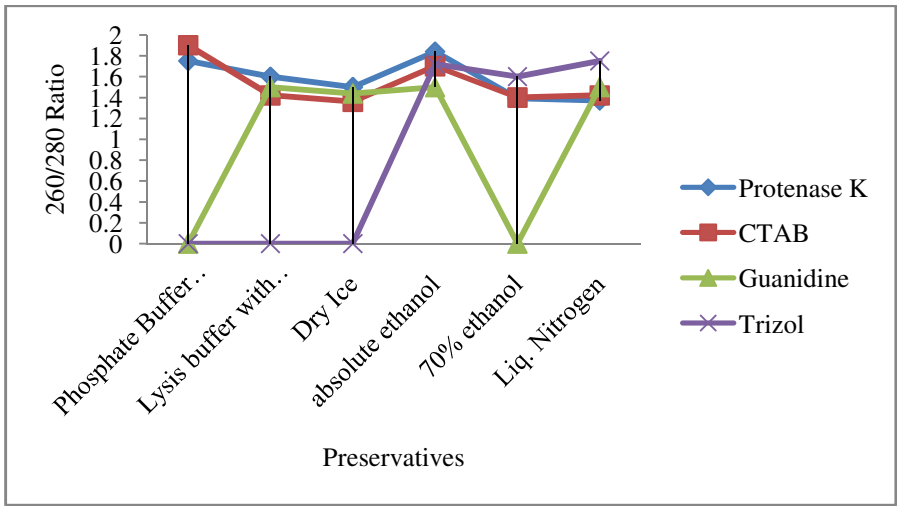

Figure-2
$A_{260/280}$ of preserved sample

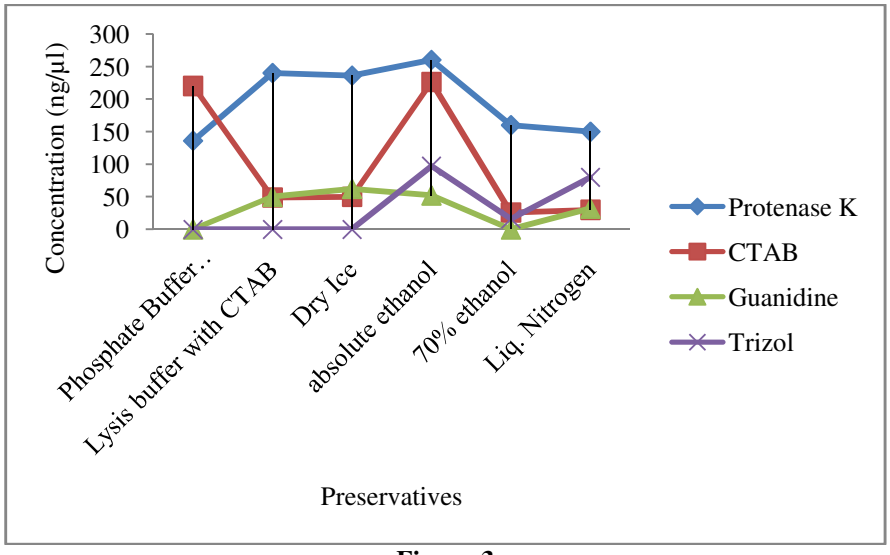

Figure-3
DNA concentration of preserved sample

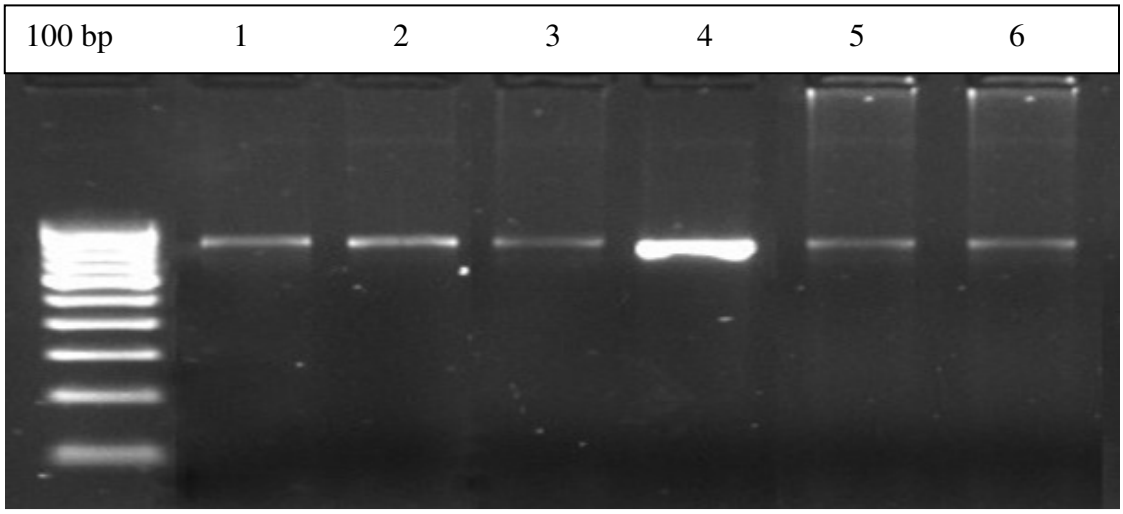

Figure-4

Agarose gel (2%) electrophoresis showing COI PCR amplification from one Zoanthids individual preserved in six different fixatives., Well 1- Sample preserved in Phosphate Buffer Saline with 30% Glycerol, Well 2- Sample preserved in lysis buffer with CTAB, Well 3- Sample preserved in Dry Ice, Well 4- Sample preserved in Absolute Ethanol, Well 5- Sample preserved in 70% Ethanol, Well 6- Sample preserved in Liquid Nitrogen

Conclusion

Our results represents that sample can be preserved in all preservatives that were tested here. Based on gel analysis, most effective preservation methods, both in quality and quantity, were Absolute alcohol and PBS with 30% glycerol. Regarding the DNA extraction procedures LBWPK, LBWCTAB, LBWGH and the Trizol®; LBWPK is most effective among all the method tested here as it produced high quality genomic DNA. DNA extracted by any of the methods followed here yielded potential material for PCR products for COI gene.

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