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CELLULAR & MOLECULAR BIOLOGY LETTERS
Volume 11, (2006) pp 155 – 160
http://www.cmbl.org.pl

DOI: 10.2478/s11658-006-0013-7

Received: 21 December 2005
Revised form accepted: 21 February 2006

Short communication

THE ASSESSMENT OF DNA FROM MARINE ORGANISMS VIA A MODIFIED SALTING-OUT PROTOCOL

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Abstract: We developed a rapid, practical and non-toxic salting-out method for the extraction of DNA from marine organisms, and tested it on two representative species of Porifera and Cnidaria, both living in association with symbiotic zooxanthellae. We tested the efficiency of the protocol by comparing the output of the method for fresh tissue, frozen tissue and tissue stored in ethanol. It proved to be effective for extracting DNA in the case of the methods of preservation considered here, and for obtaining quantities of DNA comparable to those obtained via the traditional approach. The DNA from both species was of good quality. The DNA obtained was amplified by PCR using specific primers for the large ribosomal subunit, allowing the identification of the presence of both the host and symbiont genomes.

Key words: *Symbiodinium*, Porifera, Cnidaria, DNA, PCR

INTRODUCTION

Over the past three decades, an increase in the frequency and intensity of mass mortality events of benthic organisms in tropical marine areas was recorded [1, 2]. These events mainly affect organisms that are symbiotic with zooxanthellae: stony corals, forams, sponges, molluscs and tunicates.

The association between these symbiotic dinoflagellates and various marine invertebrates is important because they play an important role in the equilibrium

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of coral reef ecosystems [3, 4]. Therefore, the characterization of symbiotic zooxanthellae and the determination of their distribution could be a strong step forward in understanding how these associations react to climate change [5, 6] and evaluating the evolution of coral reefs from a new perspective [7-9]. The zooxanthellae belong to the genera *Symbiodinium* and *Gymniodinium*, although their taxonomy is still a matter of debate. The application of molecular techniques permitted remarkable progress in the study of symbiotic relationships, yielding the possibility to identify and discriminate between species with strictly correlated genotypes. To date, marine invertebrate DNA extraction has always been performed via the traditional method with phenol-chloroform [10-13], or using particular extraction kits [14-16]. The aim of this study was to individuate a rapid, practical and non-toxic method for the extraction of DNA from marine invertebrates. We therefore developed a modified version of the salting-out method [17], which had proved efficacious not only in human research [18]. This method allowed us to effectively extract DNA from marine organisms in a simple and rapid way, without using toxic substances or expensive kits. Since our scientific interests are focused on Porifera and Cnidaria, for each group, we chose a representative species living in association with symbiotic zooxanthellae.

MATERIALS AND METHODS

Specimens of *Cliona viridis* (Schmidt, 1862) (Porifera, Demospongiae) and *Eunicella singularis* (Esper, 1791) (Cnidaria, Octocorallia), were collected by scuba diving in the Marine Protected Area of Portofino (Ligurian Sea, Italy). All the specimens were transported in sea water to the laboratory for DNA extraction, and divided into three groups. One group was processed fresh, one was fixed in 70% ethanol, and the third was frozen at -20ºC. Tissue from specimens of *C. viridis* was sampled below the ectosomal surface to avoid epibionts. All the samples were weighed, cut into small fragments and processed for DNA extraction by the following modified salting-out method. 200 mg of each sample was put into 3 ml of Buffer A solution (SDS 20%, EDTA 5 mM, Tris-HCl 10 mM) with 0.3 µl of proteinase K (10 µg/ml) and incubated for three hours at 56ºC to lyse the host and symbiont cells. Proteins were precipitated in saturated CH_3COONa 6.1 M, with 1 ml added for each 4 ml of lysate. Due to the small quantity of DNA expected, we used CH_3COONa instead of NaCl to effectively precipitate the proteins. The samples were centrifuged for 20 min at 3500 rpm (10,000 g). Two volumes of 99.5% cold ethanol were added to each supernatant sample to precipitate the total genomic DNA. Cold ethanol, also precipitating DNA, was used instead of isopropanol to avoid the use of toxic compounds and to facilitate salt eliminations that do not precipitate with DNA. The samples were stored at -20 ± 1ºC for 30 min and then centrifuged for 30 minutes, 4500 rpm (12,000 g), at 4 ± 1ºC. The DNA pellets were dried for 10 min, resuspended in 250-500 µl of sterile distilled water, and then

spectrophotometrically assessed. The DNA quality was checked on a 1.5% agarose gel in TAE electrophoresis buffer 2x.

Statistical analyses were performed on the quantity of DNA extracted. The return data was log transformed to make the assumptions of analysis of variance, and was analysed with ANOVA. Due to the heterogeneity of variance, $P<0.01$ was choosen as the basal level of significance, as per Underwood [19]. Moreover, we performed the Tukey Test to make a multicomparison analysis on the return of the method with regard to the different storage methods.

To identify and characterize the host and symbiont genomes, the large ribosomal subunit (LSU rDNA) was amplified using the following specific primers: forward LS-1.3 5'-CGCTGAAATTAAGCATATAAGTAAG-3' and reverse LS-1.5 5'-AACGATTTGCACGTCAGTATC-3' (Wilcox, 1998). PCR reactions were carried out in a final volume of 25 µl including 50 ng DNA, 1X PCR Buffer II (Applied Biosystems), $MgCl_2$ (2mM) (Applied Biosystems), DMSO, dNTPs (1.2 mM) (Boehringer Mannheim), LS 1.3 and LS 1.5 (6 picoMoles each), and 1 U Ampli Taq DNA Polymerase (Applied Biosystems). The PCR conditions were as follows: initial denaturation for 2 min at 94ºC, 35 cycles consisting of 92ºC for 45 s, 55ºC for 1 min, 72ºC for 1.30 min, 72ºC for 7 min, and a final extension at 4ºC for 10 min. To selectively amplify only the symbiont's LS, we further developed a specific PCR reaction carried out in a volume of 25 µl using the same above conditions, but with an annealing temperature of 58ºC for 30 s. The results of PCR were visualised on 1.5% (w/v) agarose gel in TBE electrophoresis buffer 2X.

The two bands obtained from the LS amplification were separated and purified from the gel using a Microcon YM-50 filter (Millipore, Amicon) following the manufacturer's instructions. Sequencing reactions were performed in both directions using LS-PCR primers and an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) under the following conditions: primer 3 pM, Millipore-mix, Millipore-Buffer; initial denaturing period of 96ºC for 1 min, 25 cycles consisting of 96ºC for 10 s, 60ºC for 4 min, and a final extension period at 4ºC. The sequences were purified with 2 µl EDTA 125 mM and 2 µl sodium acetate 3 M, and transferred into 100 µl 100% ethanol. The samples were vortexed and stored for 20 minutes at -20ºC, and then the sequences were centrifuged at 13000 rpm, for 30 min at 4ºC. 14 µl formamide was added to the sequences before transferral onto plates for sequencing. The sequence identities were analysed using BLAST software in the Genebank (www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

The characterization of symbiotic zooxanthellae, which are associated with different marine invertebrates, could highlight how this association reacts to environmental modifications. Applying molecular techniques greatly facilitated the study of the symbiotic relationships, yielding the possibility to identify and

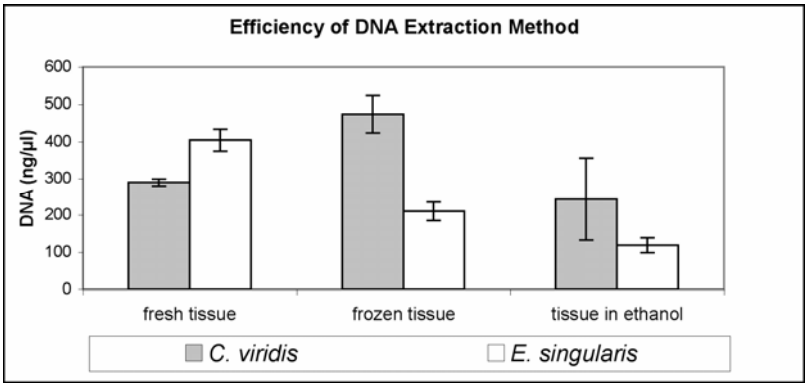

Fig. 1. The efficiency of the Salting Out Method.

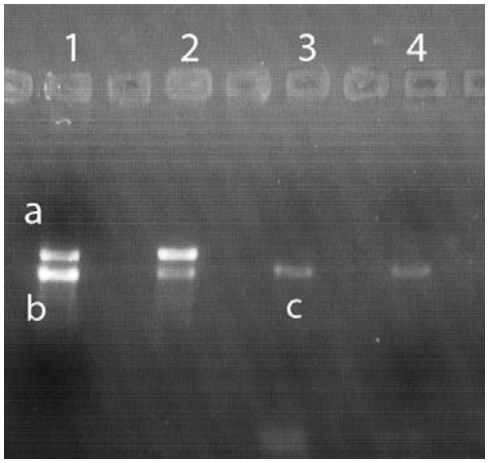

Fig. 2. Gel electrophoresis of LS PCR products. 1-3. *E. singularis* samples. 2-4. *C. viridis* samples; a- host's ribosomal large subunit, b - symbiont's ribosomal large subunit, c - symbiont's ribosomal large subunit obtained by selective PCR

discriminate between species with strictly correlated genotypes. The modified salting-out method of extraction allowed us to obtain a good concentration of genomic DNA from all the samples analysed, whether fresh, frozen or stored in ethanol, with a good ratio of DNA to proteins. Quantitatively, we extracted 289.3 ng/µl from fresh samples of *C. viridis* and 404 ng/µl from fresh samples of *E. singularis*; 473.6 ng/µl from frozen samples of *C. viridis* and 210.35 ng/µl from frozen samples of *E. singularis*; and 243.85 ng/µl from samples of *C. viridis* stored in ethanol and 118.33 ng/µl from samples of *E. singularis* stored in ethanol (mean values) (Fig. 1). By using this method, it was possible to get DNA from both of the hosts and their symbionts from all the samples. The DNA obtained by this method was efficiently obtained in quantities comparable

to those obtained with the traditional method, and the 260/280 ratios were 1.8-2.0, indicating the good quality of the DNA extracted. Such a method gives rise to only small variances in DNA quantity, mainly depending on the species (ANOVA $P<0.01$). Considering the same weight of tissue, both the sponge and the gorgonian showed a high return of DNA extracted, even if the latter always yielded lower values, probably due to the different tissue to skeleton ratio of the two species, which is lower in *E. singularis* owing to its heavy proteinaceous scleraxis. Occasionally, some differences were found depending on the method of tissue storage (ANOVA $P<0.01$): fresh and frozen samples gave higher levels of DNA than the samples stored in ethanol (Tukey Test $P<0.05$). Although sponge and gorgonian tissue are structurally different, the method proved to be effective for both host groups in the case of all the methods of storage used. Therefore, it can be considered very appropriate for the extraction of DNA from marine invertebrates. Moreover, unlike traditional phenol-chloroform extraction, the developed protocol permitted the practical extraction of DNA not with toxic reagents but only with salting solutions, and was very cheap compared to the commonly used and expensive extraction kits. The DNA obtained always proved to be amplifiable by PCR, and using LS-specific primers, we identified the presence of both host and symbiont genomes. PCR amplifications of LS allowed us to obtain two distinct bands of different molecular weight that were sequenced and blasted. The high band always identified the host (Fig. 2a), *C. viridis* or *E. singularis*, as per the original samples, and the low band (Fig. 2b) always identified the symbiont, belonging to *Symbiodinium microadriaticum* or *Symbiodinium* sp. Type A.

A modified amplification of the symbiont's large ribosomal subunit allowed us to obtain a single band (Fig. 2c) of the same molecular weight as the low band obtained with the previous PCR amplification (see Fig 2b), which was always identified after sequencing as *Symbiodinium microadriaticum* Freudenthal or *Symbiodinium* Type A.

REFERENCES

1. Glynn, P.W. Coral reef bleaching in the 1980s and possible connections with global warming. **Trends Ecol. Evol.** 6 (1991) 175-179.
2. Hayes, M.L., Bonaventura, J., Mitchell, T.P., Prosper, J.M., Shinn, E.A., Van Dolah, F. and Barber, R.T. How are climate and marine biological outbreaks functionally linked? **Hydrobiologia** 460 (2001) 213-220.
3. Muller-Parker, G. and D'Elia, C.F. Interactions between corals and their symbiotic algae. In: **Life and Death of Coral Reefs** (Birkeland, C. Ed.) Chapman & Hall, New York., 1997, 96-113.
4. Wakefield, T.S., Farmer, M.A. and Kempf, S.C. Revised description of the fine structure of in situ "zooxanthellae" genus *Symbiodinium*. **Biol. Bull.** 199 (2000) 76-84.

5. Goulet, T.L. and Coffroth, M.A. Stability of an octocoral-algal symbiosis over time and space. **Mar. Ecol. Prog. Ser.** 250 (2003) 117-124.
6. Rowan, R., Knowlton, N., Baker, A.C. and Jara, J. Landscape ecology of algal symbiont communities explains variation in episodes of coral bleaching. **Nature** 388 (1997) 265-269.
7. Cowen, R. The role of algal Symbiosis in reefs through time. **Palaios** 3 (1998) 221-227.
8. Hoegh-Guldberg, O. Climate change, coral bleaching and the future of the world's coral reefs**. Mar. Freshw. Res.** 50 (1999) 839-866.
9. Muscatine, L. and Porter, J.W. Reef corals – mutualistic symbioses adapted to nutrient-poor environments. **BioScience** 27 (1997) 454-460.
10. Junghans, H. and Metzlaff, M. A simple and rapid method for preparation of total plant DNA. **Biotechnique** 8 (1990) 176.
11. Coffroth, M.A., Lasker, H.R., Diamond, M.E., Bruenn, J.A. and Bermingham, E. DNA fingerprints of a gorgonian coral: a method for detecting clonal structure in a vegetative species. **Mar. Biol.** 114 (1992) 317-325.
12. Sinha, R.P, Dautz, M. and Hader, D.P. A simple and Efficient Method for the Quantitative Analysis of Thymine Cyanobacteria, Phytoplancton and Macroalgae. **Acta Protozool.** 40 (2001) 187-195.
13. Chen, C.A. and Yu, J.K. Universal primers for amplification of mithochondrial small subunit ribosomal RNA-encoding gene in scleractinian corals. **Mar. Biotechnol.** 2 (2000) 146-153.
14. LaJeunesse, T.C. Investigating the biodiversity, ecology and phylogeny of endosymbiotic dinoflagellates in the genus *Symbiodinium* using ITS region. In: Search of a "specie" level marker. **J. Phycol.** 37 (2001) 866-880.
15. Santos, S.R., Gutierrez-Riodriguez, C., Lasker, H.R. and Coffroth, M.A. *Symbiodinium* sp. Association in the gorgonian *Pseudopterogorgia elisabethae* in the Bahamas: high level of genetic variability and population structure in symbiotic dinoflagellates. **Mar. Biol.** 143 (2003) 111-120.
16. Crabbe, M.J. A novel method for the transport and analysis of genetic material from polyps and zooxanthellae of scleractinians corals. **J. Biochem. Biophys. Methods** 57 (2003) 171-176.
17. Miller, S.A., Dykes, D.D. and Polesky, H.F. A simple salting out procedure for extracting DNA from human nucleated cells. **Nucleic Acid Res.** 16 (1988), 1215.
18. Rawlings, L.H. and Donellan, S.C. Phylogeographic analysis of the green phyton, *Morelia viridis,* reveals criptic diversity. **Mol. Phylogen. Evol.** 27 (2003) 36-44.
19. Underwood, A.J. Techniques of analysis of variance in experimental marine biology and ecology. **Annu. Rev. Ocean. Mar. Biol.** 19 (1981) 513-605.