International Journal of Zoology Studies
ISSN: 2455-7269
Impact Factor: RJIF 5.14
www.zoologyjournals.com
Volume 3; Issue 2; March 2018; Page No. 132-134

Comparative study on DNA extraction methods for PCR amplification of COI gene from ascidians of Indian coast

Kaleemullah Khan B, ML Mohammed Kaleem Arshan, A Soban Akram, H Abdul Jaffar Ali*
Department of Biotechnology, Islamiah College (Autonomous), Vaniyambadi, Tamil Nadu, India

Abstract
Ascidians (Tunicates/Urochordates) are excellent animal models for developmental, evolutionary and molecular biology studies. Isolation of DNA is the basic phase for most of the molecular biology studies including DNA barcoding. So far there is no definite method for the isolation of DNA from ascidians. In an effort to overwhelm this problem, three different methods such as Phenol-Chloroform, Salting out and Kit method were evaluated for DNA extraction. Results showed that among the three methods, kit method, after few modifications, yielded sufficient quantity and quality of DNA. The yield of DNA between the samples and methods was significant ($P<0.005$). This method is more pronounced for the PCR amplification of COI gene with tunicate primer.

Keywords: ascidian, COI gene, DNA extraction, PCR amplification, tunicate primer

1. Introduction
Ascidians otherwise called Tunicates are marine benthic sessile organisms. They rapidly grow on a variety of substrates. They share a major part in the aquatic biodiversity. Ascidians have been known for their multifarious functions in pharmacology such as antimicrobial, antioxidant, antitumor activities, etc. Ascidians are also used as model organisms to several chordates as the ascidian larvae possess a prototype of chordate body plan [1]. Recently, the ascidian *Ciona intestinalis* has been used to study a neurodegenerative disorder, Alzheimer's disease in human beings [2].

Towards biodiversity, morphology-based ascidian taxonomy is quite difficult and requires skilled taxonomists. Moreover, identification of species in its early embryonic and larval stages is still a challenge in the conventional taxonomy. This signalled the need of a molecular based identification method involving the sequencing of CO1 (cytochrome coxidase subunit 1) gene known as DNA barcoding.

DNA barcoding, neurodegenerative and other molecular biology studies involve isolation of genomic DNA, PCR amplification and sequencing of specific genetic markers. Till date there is no specific method for DNA isolation from ascidians. Moreover, the presence of pigments, keratin and spicules in marine invertebrates interfere in the extraction of DNA and affect the quantity and quality of the extracted nucleic acids [3]. Furthermore, a variety of marine organisms, including ascidians, possess polyphenolic compounds, dopa-containing proteins and a other pigments which interferes in the extraction of high molecular weight DNA and RNA [4, 5].

A typical DNA extraction protocol should be effective, swift, direct and safe with minimal risk for the user. It should result in high quality and quantity of DNA and less usage of hazardous chemicals. In the present study, three different methods such as Phenol-Chloroform method, Salting out method and Kit method were evaluated for the extraction of whole genomic DNA fromchosen ascidians.

2. Materials and method
Specimens of ascidians were collected during the low tide level in intertidal water from Thoothukudi harbour area (8.7507 N 78.2029 E), India. For morphological studies, the specimens were narcotized and preserved in 10% buffered formalin whereas for molecular studies, specimens were preserved in 95% ethanol at -20ºC.

Genomic DNA extraction
Three different methods were assessed to extract the whole genomic DNA from ascidian tissue. A muscle tissue from mantle body of solitary ascidian and several whole zooids of colonial ascidians were used for DNA extraction. Before homogenizing, ethanol was removed from the tissues to avoid destabilization of the extracted DNA and also to prevent the interference of ethanolduring PCR and DNA sequencing.

Procedure 1: The whole genomic DNA was extracted using the standard Phenol-Chloroform method [6]. 25 mg tissue of each sample was homogenized and transferred to the tubes with lysis buffer containing 10 mM TrisHCl (pH 8.0), 1 mM EDTA (pH 8.0), 5 M NaCl, 1% SDS and proteinase K (20mg/ml). RNase A (10 mg/ml) was added separately to the each tube and incubated at 55ºC for 3 hours. The lysate was centrifuged at 10,000 rpm for 10 minutes at 4ºC and washed with phenol: chloroform : isoamyl alcohol (25:24:1). DNA was extracted using 95% ethanol and the pellet was washed with 70% ethanol. The DNA pellet was dissolved in 100 µl TE buffer and stored at -20ºC.

Procedure 2: DNA was isolated using salting out protocol. Tissue of 25 mg from each sample was homogenized using mortar and pestle. 25 mM TrisHCl (pH 8.0), 20 mM EDTA (pH 8.0), 2% SDS and 20 mg/ml proteinase K was added to the homogenate and incubated at 55ºC for 2 hours. To the lysate saturated NaCl was added and centrifuged at 8000 rpm

for 15 minutes. DNA was precipitated using 100% ethanol and the pellet was dissolved in 100 µl TE buffer.

Procedure 3: The genomic DNA extraction was performed with kit (QIAamp® DNA mini kit, Qiagen, Germany) following the manufacturer instruction with few modifications such as doubling the volume of proteinase K, increase in elution time and reducing the volume of elution buffer for high yield of DNA. For this method 25 mg of each ascidian tissue was taken and the carried out the extraction process.
The quantification of extracted DNA samples were performed using Nano Drop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the DNA yield was calculated for all the three methods using the standard formula.

DNA yield (µg) = DNA concentration × total sample volume (ml)

Pcr Amplification
The genomic DNA isolated from three different methods were subjected to amplification of Barcode region i.e. CO1 gene using the tunicate primers Tun_F (5'-TCGACTAATCATAAAGATATTA-3') and Tun_R(5'-AACTTGTATTTAAATTACGATC-3') as described in Stefaniak *et al*. (2009) [7] with master mix (Qiagen, Germany). PCR amplifications were performed in 25 µl reaction volume containing 12.5 µl master mix, 1. 0 µM of both primers and 50-100 ng of template DNA with the following thermocyclic conditions: 94ºC, 3 minutes; 35 cycles of 94ºC, 30 sec; 39ºC, 30 sec; 72ºC, 30 sec; 72ºC, 10 minutes and a final hold at 4'C. The amplified products were purified using Qiagen®QIAquick PCR Purification Kit and the quality was analysed using Agarose Gel Electrophoresis.
The results are presented as means ±SD and are processed statistically by analysis of variance (ANOVA).

3. Results and Discussion
Three solitary and two colonial ascidians were sampled for DNA isolation in this study. Amonssg which, *Perophora multiclathrata* yielded high quantity of DNA in all the three methods. The concentration and yield of DNA obtained from ascidian samples are depicted in Table 1. The concentration range in the Phenol-Chloroform method was between 43.7and 140.3 µg/ml. In Salting out method the range was between 38.2 and 102.1 µg/ml. whereas, in the Qiagen kit method the concentration range was between 87.3 and 280.6 µg/ml. The yield of DNA between the samples and methods was significant (P<0.005).

Table 1. Mean values of DNA concentration and yield of ascidian tissues by three methods (3 replicates, mean ± SD)

Ascidian Species	DNA Concentration (µg/ml)		
	PC	SO	Kit
Phallusia nigra	98.4±09.84	88.6±7.97	196.8±21.65
Microcosmus exasperates	43.7±03.49	38.2±3.06	87.3±8.73
M. squamiger	52.8±04.75	60.3±6.03	105.7±12. 8
Perophora multiclathrata	140.3±15.43	102.1±11.23	280.6±22.45
Polyclinum indicum	44.7±03.58	48.7±5.84	89.3±8.03

Tissue lysis in all the three methods involved the use of SDS and Proteinase K, yet lysis was found effective in kit method resulting in a clear lysate whereas, in other two methods the lysis was not efficient resulting in indistinct solution with debris. Kit method yielded good quality DNA after adopting few modifications in the standard protocol. Doubling the volume of Proteinase K improved the lysis step and increased elution time and reduced volume of TE buffer ensured the quality DNA.
Among the three methods, kit method was less laborious and involved non-hazardous chemicals. Phenol-Chloroform and Salting out method involved the use of phenol which is highly corrosive in nature.
The nano spectrophotometry results showed that among the three methods, kit method yielded ample quantity of DNA (Fig 1). Moreover, PCR amplification using the PCR master mix resulted distinct bands for the amplified DNA extracted from the kit method (Fig 2). Whereas, PCR amplification showed no bands for DNA isolated using the other two methods.

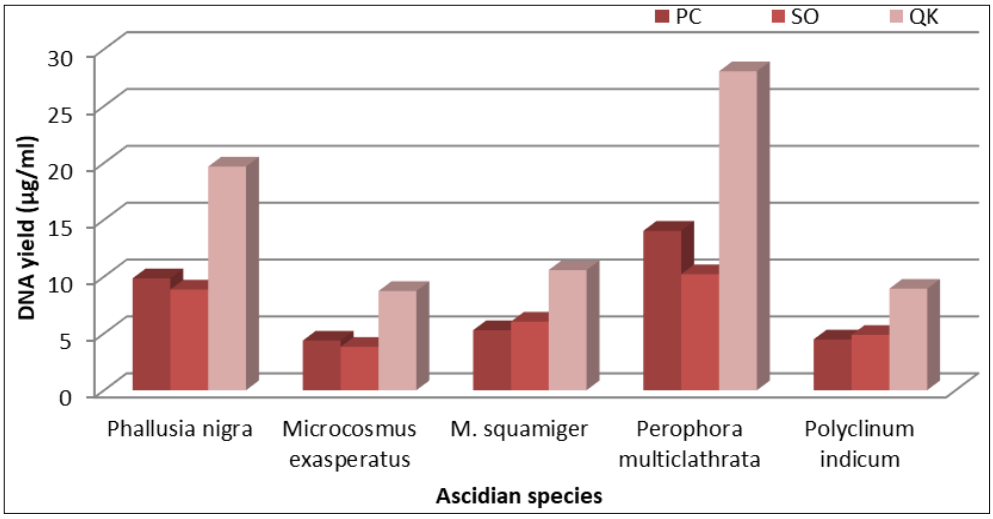

Fig 1: Yield of DNA from ascidian tissues in three different methods

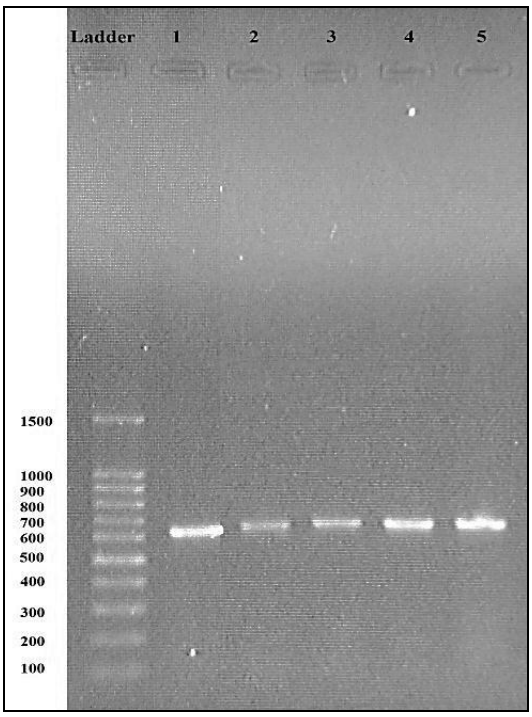

Fig 2: Gel image of amplified CO1 gene using Qiagen PCR master mix

The results revealed significant differences among the three procedures and only kit method yielded satisfactory outputs with good quality and quantity DNA suitable for sequencing of DNA barcode region (CO1 gene) as well as other molecular studies. This could be justified with the reports of many authors [8, 9, 10] who adopted kit method only rather than manual methods for ascidians.

4. Acknowledgement
Our deep sense of gratitude to Department of Biotechnology, Government of India for the financial support (BT/PR6801/AAQ/3/609/2012) and also to our College Secretary and Principal for their motivation.

5. References

1. Satoh N. Developmental Biology of Ascidians (Cambridge: Cambridge University Press), 1994.
2. Virata MJ. A novel invertebrate chordate model for Alzheimer's disease using the ascidian *Ciona intestinalis*. PhD Thesis, University of California, San Diego, USA, 2009.
3. Ribout C, Carpentieri C. Automated genomic DNA purification of marine organisms on the epMotion® 5075 VAC from Eppendorf. Application Note, Eppendorf, 2013.
4. Tamarin A, Lewis P, Askey J. The structure and formation of the byssus attachment plaque in Mytilus. Journal of Morphology.1976; 149(2):199-221.
5. Waite JH. Andersen SO. 3, 4-Dihydroxyphenylalanine sclerotization of periostracum in *Mytilus edulis* L. Biological Bulletin (Woodse Hole, Mass.). 1980; 158:164-173.
6. Sambrook J, Russell DW. Molecular cloning. A laboratory manual. 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 2001.
7. Stefaniak L, Lambert G, Gittenberger A, Zhang H, Lin S, Whitlatch RB. Genetic conspecificity of the worldwide populations of *Didemnum vexillum* Kott, 2002. Aquatic Invasion, 2009; 4:29-44.
8. Hess JE, Swalla BJ, Moran P. New molecular markers to genetically differentiate populations of *Didemnum vexillum* (Kott, 2002) – an invasive ascidian species. Aquatic Invasion, 2009; 4:299-310.
9. Smith K, Stefaniak L, Saito Y, Gemmill C, Cary C, Fidler A. Increased inter-colony fusion rates are associated with reduced COI Haplotype diversity in an invasive colonial ascidian *Didemnum vexillum*. PLoS ONE. 2012; 7(1):e30473.
10. Rocha RM, Kremer LP, Fehlauer-Ale KH. Lack of COI variation for *Clavelina oblonga* (Tunicata, Ascidiacea) in Brazil: Evidence for its human-mediated transportation. Aquatic Invasion. 2012; 7(3):419-424.