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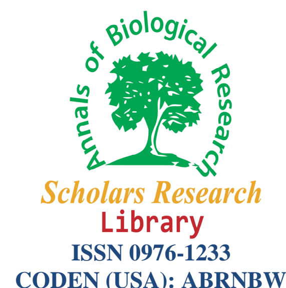

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First Molecular Report and Phylogenetic Analysis of Crinoidea from Rameswaram Island, South East Coast of India

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ABSTRACT

Crinoidea is a class of echinoderms that degenerate very easily due to the presence of very temperature sensitive proteins, hence the samples obtained could not be identified easily, resulting in the identification of the samples at a molecular level by sequencing the 650 –bp region from the 5' end of the mitochondrial CO1 region. The sequenced genes were identified as three different species of Crinoids; Cenometra sp, Tropiometra sp and Comatella sp submitted to the National center of bioinformatics (NCBI) which provided the three finds with accession numbers. The sequences were compared with other closely related sequences and analyzed using the CLUSTAL X software to attain a multiple sequence alignment and Mega (Molecular Evolutionary Genetic Analysis) to construct a Phylogenic tree through which the evolutionary relationships of the three samples analyzed could be observed in the study.

Key words: Crinoidea, Echinoderm, Phylogeny, *Cenometra sp*, *Tropiometra sp, Comatella sp,* Molecular taxonomy.

INTRODUCTION

Sustainable conservation of species requires, among other things, appropriate knowledge about the diversity of life at different hierarchical levels, including physiological, ecological, biogeographical, and systematic information [1,2], taxonomists [3,4,5], accurate species identification remains an imperative condition to investigate on biodiversity and conservation. To date, traditional taxonomy relies mostly on diagnostic morphological characters, requiring expert knowledge to identify specimens. In this regard, DNA barcoding for the molecular taxonomy studies has proved to be a useful alternative method for rapid global biodiversity assessment, providing an accurate identification system for living organisms [6,7,8,9]. DNA barcoding translates expert taxonomic knowledge into a widely accessible format, DNA sequences, allowing a much broader range of scientists to identify specimens [10]. This method of species identification is based on detecting sequence diversity in a single standardized DNA fragment, namely, mitochondrial Cytochrome c Oxidase Subunit I (COI) [6]. Examination of nucleotide sequence diversity of this gene allows the grouping of unknown specimens with a prior defined taxonomic species [11,12] based on the assumption that intraspecific genetic divergence is lower than the interspecific one [6,13,14]. This method has provided a high degree of taxonomic resolution (> 94%) for most of the species examined across several animal groups [6,14,15]. In marine organisms, such as invertebrates and macroalgae, species identification using standard taxonomic analyses can be notoriously difficult [16]. This is because these taxa often show morphologic convergence and phenotypic plasticity, resulting in taxonomic lumping or splitting of species [17,18]. In particular, echinoderms constitute a large and diverse group of invertebrates highly diverse in terms of their morphologies and

ecologies. However, the identification of species based on morphological traits is complex due to the high levels of homoplasy [19,20,21]. In this regard, the use of complementary techniques such as DNA sequences may enhance taxonomic and systematic studies. [13] have indicated that molecular taxonomy (DNA Barcode) holds promise for identification in taxonomically well-understood and thoroughly sampled clades. In this respect, the molecular taxonomy of echinoderms of the Rameshwaram Island in the south east coast of India received a particular attention in the present study. These studies represent an important improvement of species identification of a particularly rich and complex fauna.

DNA barcoding (molecular taxonomy) has been proposed as a means of identifying animal species. The method is based on sequencing an approximately 650-bp region from 5' end of the mitochondrial cytochrome oxidase 1 (CO1) gene [6]. A referenced library of barcodes is established based on sequences from verified specimens, backed up wherever possible by voucher specimens retention, and unknown specimens identified by matching their barcodes to the references library [22]. The Echinodermata is an exclusively marine phylum comprising about 7000 extant species. Adults are largely bottom dwellers from inter tidal zone to deep sea trenches, and often constitute a major proportion of marine biomass. If DNA barcoding could be shown to be effective for Echinodermata it would enable ready identification of all their life-history stages, from larvae to adults. Echinoderm larvae are usually pelagic and are extremely difficult to distinguish. Asteroid larvae, for example, can rarely be identified morphologically to family, let alone genus of species [23]. Adults too can be hard to discriminate, being morphologically plastic within species and sometimes of variable colour. It should also flag taxa that are in fact species complexes; many echinoderm species remain undescribed. Exacerbating the identification problem is paucity of well trained taxonomic specialists. Consequently, DNA barcoding (molecular taxonomy) of echinoderms could be a major boon to marine ecosystem studies [24,25].

DNA sequencing has been used in an attempt to match asteroid larvae with known sequences from the adults of 44 species, in a study utilizing five tRNA mitochondrial genes [23]. Wild caught cloning asteroid larvae fell into four distinct genetic groups, but none perfectly matched any of the known reference sequences. This failure presumably reflects an inadequate reference database, but larval placement to likely genera or families was achieved. DNA barcoding might be a potentially valuable method for identifying echinoderm species, making it available to a much broader range of researchers and particularly to non-specialists. The development of DNA barcoding for echinoderms is still scarce, so the present study is especially DNA sequence analysis was processed for Crinoidea class only.

MATERIALS AND METHODS

1. Wet Lab Method:

Effective DNA barcoding depends on the quality of the biological materials.

(i) Sample collection and documentation:

The individual specimens were freezed in plastic bags and labeled to record vessel, expedition name, code, locality and station number, latitude and longitude, date spaces name and collectors name. The labeled specimens were stored in freezer. Tissues samples for DNA extraction were preserved in fresh 95% ethanol and stored in a freezer.

(ii) DNA extraction:

The tissue was placed in 1.5 ml eppendorf tube and 500 µl of solution I (50mM Tris-HCl pH 8, 20mM EDTA pH8 and 2% SDS) was added. The tissue was homogenized with sterile homogenizer and 5 µl of Proteinase K (20mg/ml) was added and quick vortexed. The sample was incubated at 55ºC in water bath for 2 hours with occasional mixing. Following incubation the sample was chilled over ice for 10 minutes and 250 µl of solution II (6M NaCl) was added and inverted several times for thorough mixing. The tube was chilled on ice for 5 minutes and centrifuged at 8000rpm for 15 minutes. About 500 µl of supernatant was carefully collected in to new-labeled 1.5 ml tube and twice the volume (*i.e.* 1ml) of 100% AR grade Ethanol was added to precipitate the DNA. The precipitate was pellet down at 8000rpm for 5 minutes and the supernatant was removed without touching the pellet. The DNA pellet was rinsed with 500 µl of cold Ethanol and centrifuged at 11000 rpm for 5 minutes. The supernatant was carefully removed and the excess liquid was drained using pipette. The pellet was partially dried (devoid of Ethanol) with lid off at 55ºC on heating block. The pellet was re-suspended with 50-200 µl of fresh sterile H_2O depending on size of pellet (100 µl average) by gently pipetting sample with wide-bore filter tip until dissolved. This dissolved DNA acted as a template for Polymerase Chain Reaction (PCR).

Determination of Quality of isolated DNA:
The gel casting unit was prepared according to manufactures instruction. !% Agarose gel was prepared by weighing 1g of Agarose in 1X TBE buffer. The Agarose solution was heated to dissolve Agarose and 1.5µl of Ethidium Bromide was added before cooling. After cooling it to 50ºC, they were poured into gel casting plate with already adjusted gel comb. After solidification the comb was removed and the plate was loaded in gel casting unit loaded with 1X TBE running buffer. 2µl of DNA solution was added to the wells and the runned at 70 V for 20 minutes. Then the gels are observed in UV trans-illuminator with protective sheilds.

(iii) PCR and DNA sequencing:
A standard protocol for CO1 amplification in fish was followed approximately 658 bp can be amplified from the region of CO1 gene from mitochondrial DNA. Different combinations of newly designed primer were used. Folmer's primers, LCO/HCO primers are use to amplify barcodes of Crinoidea.

LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'
HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'

The PCR condition includes, hot start with 94˚C for 1min, 5 cycles of 94˚C for 30 sec, annealing at 45 - 50˚C for 40 sec, and extension at 72˚C for 1min, 30-35 cycles of 94˚C for 30 sec, 51 -54˚C for 40 sec, and final extension at 72˚C for 10 min. The amplicons were gel checked and processed for DNA sequencing. The amplified PCR products were sequenced with automated DNA sequence machine (MegaBace), Bioserve Biotechnologies, Hyderabad.

2. Dry Lab Method:
(i) Retrieving sequences from NCBI:
Three much related Genus of *Cenometra, Tropiometra, and Comatella* COX I DNA sequences were collected from National Center for Biotechnology Information (NCBI) database. These sequences were collected in the fasta format and were saved in notepad. There were nine barcodes available for genus *Cenometra, Tropiometra, Comatella.* The accession numbers of these barcodes were given in Table 1.

(ii) Multiple Alignment- Clustal X:
Clustal X is a new windows interface for the ClustalW multiple sequence alignment program. It provides an integrated environment for performing multiple sequence and profile alignments and analyzing the results. One can cut and paste sequences to change the order of the alignment; you can select a subset of sequences to be aligned; you can select a sub-range of the alignment to be realigned and inserted back into the original alignment. Alignment quality analysis can be performed and low-scoring segments or exceptional residues can be highlighted. Multiple alignments were prepared for the cytochrome c oxidase subunit1 (COX I) using ClustalX with default setting.

In the file menu of ClustalX, load sequences option was clicked. FASTA formated COX1 DNA sequences were loaded on the ClustalX window. Then Multiple Alignment Mode was selected. A single sequence data area was then displayed. Multiple alignments were carried out in 3 stages: *Viz.,*

1) All sequences are compared to each other (pair wise alignments);
2) A dendrogram (like a phylogenetic tree) is constructed, describing the approximate groupings of the sequences by similarity (stored in a file).
3) The final multiple alignment is carried out, using the dendrogram as a guide.

The 3 stages are carried out automatically by the Do Complete Alignment option. In the Alignment menu, Do complete alignment option was clicked. The alignment was displayed on the screen with the sequence names on the left hand side. The sequence alignment was for display only; it cannot be edited here (except for changing the sequence order by cutting-and-pasting on the sequence names). A ruler was displayed below the sequences, starting at 1 for the first residue position (residue numbers in the sequence input file are ignored). The line above the ruler was used to mark strongly conserved positions. '*' indicates positions which have a single, fully conserved residue. After alignment of COXI DNA sequences multiple alignment sequences were loaded into MEGA ver.4.1 software.

(iii) MEGA (Molecular Evolutionary Genetic Analysis) ver.4.1:
The objective of the MEGA software is to provide tools for exploring, discovering, and analyzing DNA and protein sequences from an evolutionary perspective**.** MEGA contains an array of input data and multiple results explorers

for visual representation; the handling and editing of sequence data, sequence alignments, inferred phylogenetic trees; and estimated evolutionary distances. The results explorers allow users to browse, edit, summarize, export, and generate publication-quality captions for their results. MEGA 4 also includes distance matrix and phylogeny explorers as well as advanced graphical modules for the visual representation of input data and output results. MEGA 4 is distinct from previous versions; we have made a special effort to retain the user-friendly interface that researchers have come to identify with *MEGA*.

MEGA 4 software can be used to constructing the phylogenetic tree of the multiple alignment sequences from the Clustal X. The Neighbor-Joining tree was built based on the Kimura 2 Parametric distances for COXI DNA sequences. The tree was constructed based on the COXI sequences fragment showing phylogenetic relationship among 4 species of class Crinoidea from *Cenometra, Tropiometra, and Comatella genus*.

RESULTS

In the present study 4 Crinoidea sample were analysed for DNA sequences. The combined effect of PCR primers and conditions yielded favorable quality and quantity of amplicon for sequencing. The chromatograms generated from the sequencer were double checked manually for miscals and base spacing. After cropping out noises at initial and fag end of chromatogram, about 642 to 645 nucleotides could be revered and subjected to further analysis along with the sequences retrieved from NCBI (Box 1)(Table: 1) .

Table: 1. Most relevant species DAN sequences from the NCBI

S.no	Species name	Acc.no
1.	*Cenometra bella*	GU327851
2.	*Himerometra magnipinn*	GQ913326
3.	*Himerometra robustipi*	GQ913316
4.	*Ptilometra macronema*	GU327866
5.	*Tropiometra afra*	GU327867
6.	*Tropiometra carinata*	GU480555
7.	*Oxycomanthus japonicu*	GU327860
8.	*Comanthina schlegelii*	GQ913317
9.	*Comatella nigra*	GU327858

Box 1: The barcode sequence generated from this work

>*Comatella*_ sp_ KC815535
ttatattttc tttttggggc ttgggcggga atggggggaa cggctttaag aattataatt
cgagcagaat tggcccaacc gggttctttt ttgggggacg accaaattta taaggtaatt
gtaacttccc acgctttggt aatgattttt tttatggtaa tgcctgtaat gattgggggt
tttggaaatt gattaattcc acttatgatc ggggccccgg atttggcttt tccccgggta
aaaaaaatga gtttttggct tttaccccct tcttttttgt tgttgttggc ttcggcgggg
gtggaaaggg gggtgggaac gggttgaaca atttaccccc ctttatctag aaaaatcgcg
catgctggag ggtctgttga tcttgcaatt ttttctttac atatagctgg tgcttcttca
ataattgctt ctatcaaatt tattactact ataataaaaa tgcgttctcc tggtattact
tttgatcgtt tatctctttt tgtttggtct atttttatta ccacttttct tcttttgtta
tctctaccgg ttttggcagg ggcaataaca atgcttttaa cggaccgaaa tataaataca
acttttttg acccggcggg gggaggtgat cctatattgt tt

> *Tropiometra*_sp_ KC763371
atgccaataa tgataggggg ttttggaaat tggttaatcc ctttaatgat aggttccccg
gatttgtctt ttcctcgtgt aaaaaaaatg aggttttgac ttcttcctcc ttcttttctt
cttttattag cttctgctgg tgtggaaagg ggggtgggta ctggttgaac aatatatcct
cctttgtcta ggggtttggc tcatgcaggg ggttcggtgg acctggcaat tttttctttg
cacattgcgg gggcttcttc aataatagct tcaataaaat ttattactac tataataaaa
atgcgctctc ctggtgttac ttttgatcgt ttgcctcttt ttgtttgatc tgtttttata
acggcttttc tccttttgtt atctttaccg gttttagcgg gggcaataac aatgctccta
actgatcgta aagttaaaac tacttttttt gacccagctg gggggaggtga tcctattttg
tttcagcatt tgttttggtt ttttggtcat cctgaggttt atattcttat tttacctggt
tttggtatga tttctcatgt tgttgctcat tattctggta agagagaacc ttttggttat
ttgggaatgg tttatgctat ggttgctata gggatattag gtttt

> *Cenometra*_sp_ KC815536
tttttgtttg gggcttgggc gggtatgatt ggaacggctt tgagaataat aatccgtact
gagttggccc agcctggttc ttttttgggg gatgaccaaa tttataaagt aatagtaact

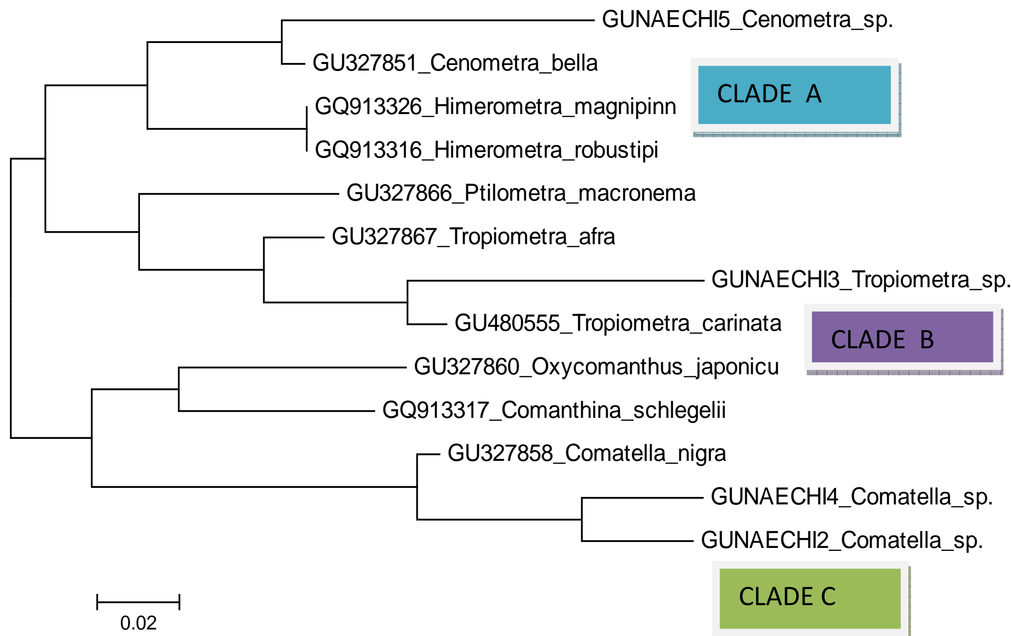
tctcatgctt tgataatgat tttttttatg gtaatgccaa taatgatggg gggttttgga
aattggttaa ttcctttaat gatgggggcc ccggatttgg cttttccccg ggtaaaaaaa
atgagttttt ggcttttgcc tccttctttt attttgttac tggcttcaac gggggtggaa
agtggagttg gtactggttg aactatttat cctcctttgt caaggggttt agcccatgcg
gggggttcgg tggacctggc tattttttct ttacatattg ctggtgcttc ttctatagtt
gcttctataa attttataac tactataata aaaatgcgtt ctcctggtgt tacttttgat
cgtttacctc tttttgtttg atctgctttt ataactgctt ttcttctgtt attatctctt
ccagttttag cgggggcaat aacaatgctc ctaacggacc gaaatgtaaa tacaactttt
tttgacccgg caggtggtgg ggacccaatt ttatttcaac attt

> >*Comatella_* sp_ KC763370
ttggcccaac cgggttcttt tttgggggat gatcaaatat ataaagttat tgtgacttct
catgctttgg taatgatttt ttttatggta atgcctgtaa tgattggggg ttttggaaat
tgattaattc cacttatgat tggtgccccg gatttggctt ttccccgggt aaaaaaaatg
agtttttgac ttttacctcc ttcttttttg ttattattgg cttcggcggg ggtggaaagg
ggggtgggaa caggttgaac aatttatcct cctttatcta gaaaaattgc ccatgcgggg
gggtctgttg atcttgcaat tttttcttta catatagctg gtgcttcttc aataattgct
tctatcaaat ttataacaac aataataaaa atgcgttctc ctggtattac ttttgatcgt
ttatcccttt ttgtttggtc tatttttatt acaacttttc tccttttgtt atccctcccg
gttttggcag gggcaataac aatgctttta acggaccgaa atataaatac aacttttttt
gaccctgccg gtggaggtga cccaattttg tttcagcatt tattttggtt ttttggtcac
ccggaggttt atattctaat attacctggt tttggaatga tttt

Table: 2. Results of barcodes generated, its length and corresponding accession number in NCBI was given.

S.No.	Animal list	GenBank Accession number	Nucleotide length
1	*Comatella sp.*	KC815535	642
2	*Cenometra sp.*	KC815536	644
3	*Tropiometra sp.*	KC763371	645
4	*Comatella sp.*	KC763370	644

Fig 1. Neighbor-joining tree drawn by Kimura2-parametric distance using COI barcodes of Crinoidea. Segregation in separate clade confirms the barcoding signals in the COI gene.



Phylogenetic analysis

The barcode sequences generated through this study was submitted at NCBI and accession numbers assigned were tabulated (Table 2).

The phylogenetic tree construction was performed through MEGA ver. 4. 0 using Neighborhood joining method. The sister taxa *Cenometra, Tropiometra* and, Comatella *used* as out group has been clearly segregated in the Phylogram (Fig. 1).

This proves that COI could delineate related species with high efficacy. All the echinoderms of *Crinoidea* got aggregated in three clades in the same branch of the tree. This variation may be due to differences in their phylogeography.

DISCUSSION

[6] suggested that DNA-based identification founded on the mitochondrial Cytochrome c Oxidase Subunit I would serve as the core of a global bio-identification system for animal life. The idea of using nucleotide sequences as barcodes for species identification has stirred up debates in the community of taxonomists and systematists. DNA barcoding has met with spirited reaction from scientists, especially systematists, ranging from enthusiastic endorsement to vociferous opposition. For example, many stress the fact that DNA barcoding does not provide reliable information above the species level, while others indicate that it is inapplicable at the species level, but may still have merit for higher-level groups. Others resent what they see as a gross oversimplification of the science of taxonomy. And, more practically, some suggest that recently diverged species might not be distinguishable on the basis of their COI sequences. Due to various phenomena, [13] found that some 23% of animal species are polyphyletic if their mtDNA data are accurate, indicating that using an mtDNA barcode to assign a species name to an animal will be ambiguous or erroneous some 23% of the time .

The DNA barcoding debate resembles the phenetics debate of decades gone by. It remains to be seen whether what is now touted as a revolution in taxonomy will eventually go the same way as phenetic approaches, of which was claimed exactly the same decades ago, but which were all but rejected when they failed to live up to overblown expectations. Controversy surrounding DNA barcoding stems not so much from the method itself, but rather from extravagant claims that it will supersede or radically transform traditional taxonomy. Other critics fear a "big science" initiative like barcoding will make funding even more scarce for already underfunded disciplines like taxonomy, but barcoders respond that they compete for funding not with fields like taxonomy, but instead with other big science fields, such as medicine and genomics.

This study has thrown light upon the genetic diversity of genera *Cenometra, Tropiometra* and, *Comatella sp.* The study included 4 barcodes belonging to 3 Genus belonging to *Cenometra sp, Tropiometra sp* and *Comatella* sp. About 642-645 nucleotides of barcode region from class Crinoidea had been generated from this work. Before initiating analysis, NCBI database was scanned for number of barcodes available for members of class Crinoidea and the most relevant data were used for phylogeny tree construction. The barcode of class Crinoidea generated through this work is the first sequence submitted in NCBI. And this will act as a reference sequence for identification of the study animal around the world.

The phylogram drawn using COI distinguished the members in to three separate clades, Clade A is populated with *Cenometra sp.* , Clade B is populated with *Tropiometra* sp and clade C populated with *Comatella* sp.

Despite its methodological shortcomings and limitations, DNA barcoding studies have reinvigorated the development of systematic studies and taxonomic inventories around the world. The increased development of molecular systematic and taxonomy provide exciting opportunities to enrich our understanding of ancient and widespread taxa such as marine echinoderms, and support the initiatives for conserving global biodiversity. Creating and organizing a DNA database for precise identification of Echinoderms would be the next target in continuum of this effort.

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