ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2016; 2(12): 18-22
www.allresearchjournal.com
Received: 04-10-2016
Accepted: 05-11-2016

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Conventional and molecular taxonomy of Chiton species (*Chiton tuticorinensis*) collected from Tiruchendur coastal water, Southeast coast of India

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Abstract
In this present study, chiton species were identified both from morphological as well as molecular level. Therefore, conventional and molecular method (DNA barcoding) are moving parallel to each other that's why we attain the targets like biodiversity conservation, palaeo-biology and evolution, census of life of earth, monitoring health and remediation of the environment and so on.

Keywords: *Chiton tuticorinensis,* taxonomy, SEM, DNA barcoding

1. Introduction
Chitons (Mollusca: Polyplacophora) include approximately 600 marine species. They live primarily in rocky, intertidal habitats, although some have been found at depths up to 7000 m (Campbell and Fautin, 2001) [4]. These organisms are relatively sedentary and are generally more abundant on exposed rocks, living in crevices or adhering to the underside of rocks using pressure and they exert on their ventral foot and belt. They prefer to live among algae in intertidal and submarine zones (Cruz and Sotela, 1984; Piercy, 1987) [6, 17]. They are one of the most important factors in controlling algae growth, in the intertidal zone. Chitons are especially important in ecosystems where they are the dominant grazer, and have been found to increase the biomass of its prey through grazing.
Molecular techniques have the potential to provide definite species, thereby overcoming the taxonomic uncertainty and also to construct the phylogenetic tree of the evolutionary patterns. The phylogenetic relationships among species of tropical marine taxa that occur in multiple bio-geographic regions illuminate the importance of these phenomena in determining broad-scale historic patterns of divergence. Each and every organism has unique DNA sequences that can be used as a biomarker for detection and identification. Molecular techniques are now more frequently employed in taxonomic analysis and revealing numerous cases of cryptic species in all groups, including molluscs (Williams and Reid, 2004; Collin, 2005; Duda *et al*., 2000; Malaquias and Reid, 2008) [29, 5, 9, 13]. Molecular data are now routinely used in combination with shell and anatomical characteristics for identification and sometimes become the ultimate proof of the existence of separate and new species. Although molecular markers have started to play an important part (Blastex, 2003 and Herbert *et al*., 2003) [3, 10]. The mitochondrial gene cytochrome c oxidase I (COI) was chosen as the standard gene for DNA bar-coding because it shows a conserved region sequence that facilitates the design of universal primer applicable to a diverse group of organisms; COI also apparently functions well to discriminate species (Herbert *et al*., 2003, 2004; Ward *et al*., 2005 and Hajibabaei *et al*., 2006) [10, 11, 28].
DNA barcoding is a DNA-based species identification method in which molecular biology and Bioinformatics are combined. PCR and sequencing techniques coupled with IT technology have provided a new method of classification, termed DNA taxonomy. Hebert *et al*. (2003) [10] suggested a section of the mitochondrial DNA gene cytochrome-c oxidase subunit I (COI). Once sequenced, this gene fragment could be used as a "barcode" to distinguish between species. COI is the best candidate for this taxonomic tool, as it has a high degree of conservation and insertions which is rare in deletions (Moritz and Cicero, 2004) [14]. Compared to the nuclear genome, the mitochondrial genome lacks introns, which

have restricted exposure to recombination, and has a haploid mode of inheritance (Saccone *et al*., 1999) [21].

The lack of information on class Polyplacophora of the entire coast of India prompted the present investigation. The earlier knowledge on the chitons was limited in India to short term studies, thus the present study was concentrated to observe the species distribution remarks of chitons along the Tamilnadu coastline.

2. Materials and Methods

2.1 Description of study area

Tiruchendur is located at the latitude and longitude of 8.4833° N, 78.1167° E extending over a distance of 32.78 km of the Southeastern Coastline borders of the Bay of Bengal. It has sandy beaches and some areas are rocky in nature.

2.2 Sample Collection

The samples of Chitons were collected from rocky shores of Tiruchendur, Tamil Nadu from the southeast coast of India. Live species of chitons were dissected and foot was clipped from anesthetized specimens and preserved in 95% ethanol and sequenced for a 658-bp fragment of the Barcoding COI gene, while shells were kept intact for morphological analyses. Live specimens were fixed in 10% formalin for further identification.

2.3 Sample identification

The samples were identified and the morphological features were coded according to Sigwart (2009) [24], including 69 characters for shell, girdle, radula, and gill arrangement. These keys are used primarily macroscopic, external characters: shell features, general girdle features, and gills. In many cases, definite identification may be dependent on more detailed features, especially the radula, aesthete arrangement and microscopic characters of the gill armature. Characters formulated from shell morphology are used to code chiton taxa in the phylogenetic analysis.

Preparation of objects for Scanning Electronic Microscopy (SEM) was described in SchWaBe & Ruthen Steiner (2001) [22]. Micrographs were taken using a Hitachi S 2400 SEM. Chitons were cleaned and kept under the microscope and their visuals were captured by camera lucida and drawn the external structure for identification key character.

2.4 For molecular Identification

For molecular identification DNA barcoding were adopted and the methodology are given below:

2.5 Genomic DNA Isolation

Genomic DNA was isolated from the tissues using the NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer instructions.

Tissues were placed in a 1.5 ml Microcentrifuge tube. 180 µl of T1 buffer and 25 µl of proteinase K was added and incubated at 56 ºC in a water bath until the tissue was completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. 200 µl of B3 buffer was added and incubated at 70ºC for 10 minutes. 210 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and the DNA was eluted out using 50 µl of BE buffer.

2.6 Agarose Gel Electrophoresis for DNA Quality check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed in 0.5X TBE as electrophoresis buffer at 75 V until the bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using a Gel documentation system (Bio-Rad).

2.7 PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume, which contained 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers.

2.6 Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
COX1	LCO	Forward	GGTCAACAAATCA TAAAGATATTGG
	HCO	Reverse	TAAACTTCAGGGT GACCAAAAAATCA

The PCR amplification was carried out in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems).

2.7 Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml Ethidium Bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as an electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV Trans illuminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

2.8 Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al*., 2010) [7].

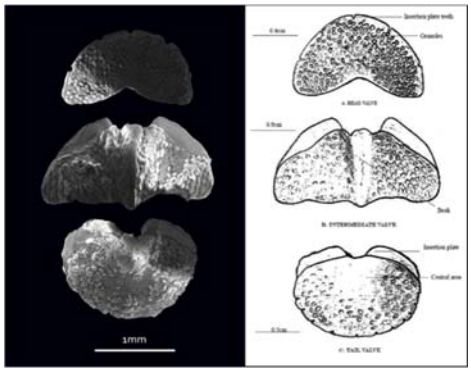
2.9 BLAST
BLAST analysis was performed to obtain the highly similar sequences from NCBI database. Based on the percentage similarity of other COI sequences, chiton species were selected to reveal the evolutionary relationship among the species.

2.10 Phylogenetic analysis
The DNA sequences were aligned using ClustalX (ver. 2.0), (Larkin *et al*., 2007) [12]. The phylogenetic tree constructed using MEGA 7 (Molecular Evolutionary Genetic Analysis) software. MEGA is an integrated tool for conducting automatic and manual sequence alignment, inferring Phylogenetic trees, mining the interest based online DNA databases, estimating the rates of Molecular evolution, and testing evolutionary hypothesis.

2.11 Gene Bank Submission
All the sequences were submitted to the NCBI gene bank through their BANKIT program according to NCBI's procedure.

3. Results
3.1 Morphological identification
Kingdom Animalia
Phylum Mollusca
Class Polyplacophora
Order Neoloricata
Family Chitonidae
Genus *Chiton*
Species *C. tuticorinensis*

Fig 1- (A)



(B) (C)

Fig 1: Showing (A) Whole animal (B) SEM images of *Chiton tuticorinensis* head valve, intermediate valve and tail valve (C) Outline of shell valves of head, intermediate and tail.

3.2 Synonyms
Chiton tuticorinensis H.C. Ray & P. Roychoudhury, 1968

3.3 Materials Examined
This chiton occurs in low intertidal zones attached to rocky substrate at the depth of 0.5m.

3.4 Distribution
The specimens described here where collected from rocky surface of Tiruchendur station.

3.5 Remarks
C. tuticorinensis was first described by Ray & in Tuticorin.

3.6 Molecular identification
The sequence of cytochrome oxidase subunit I (COI) genes of *C. tuticorinensis* from Tiruchendur coast of India are given below.

3.7 Sequence of Cytochrome Oxidase subunit I gene (PRNCAS6) of *Chiton tuticorinensis* from Southeast coast of India. (Accession No: KY235767).
AACTTTATATATCTTATTTGGAATTTGATCAGGCTT
AGTTGGAACAGCCTTAAGACTTTTAATCCGAGCTG
AATTAGGTCAACCAGGCGCATTGTTAGGAGATGAT
CAACTTTATAATGTAATTGTTACAGCACATGCTTTT
GTAATAATTTTTTTCTTAGTAATACCTATAATAATT
GGAGGATTTGGAAATTGATTAGTCCCACTTATACTT
GGCGCACCTGACATAGCCTTTCCACGATTAAATAA
TATAAGATTTTGGCTTTTGCCTCCAGCTCTTTGTCTC
CTCTTAGCATCAGCAGCTGTAGAAAGAGGAGTAGG
TACAGGATGAACTGTATACCCCCCTCTAGCTAGAA
ATATTGCCCACGCCGGAGGTTCAGTAGACCTGGCC
ATCTTTTCACTACATCTTGCCGGAGTTTCTTCTATTC
TAGGAGCAGTAAACTTCATTACAACCGTTTTTAATA
TACGATGAAAAGGAATACAATTAGAACGTCTCCCA
CTTTTCGTTTGATCTGTAAAAATTACAGCAATCCTT
TTACTTCTATCCCTTCCAGTTTTAGCTGGAGGTATT
ACTATACTTTTAACTGATCGAAATTTTAACACTGCC
TTTTTTGACCCAGCAGGAGGAGGAGATCCTATTTTA
TACCAACACCTCTTC

Several other cytochrome oxidase subunit I sequence of the *C. tuticorinensis* species were downloaded from NCBI database via basic local alignment search tools (BLAST) http://blast.ncbi.nlm.nih.gov/Blast.cgi for constructing phylogenetic tree. The phylogenetic tree was constructed using shuffled input and the genetic distance was calculated using pairwise distance analysis and maximum livelihood method. The phylogenetic tree was constructed to test the efficacy of COI gene was delineate *C. tuticorinensis* to its species level. The barcode sequence (COI) was used as an out group in the phylogenetic tree.

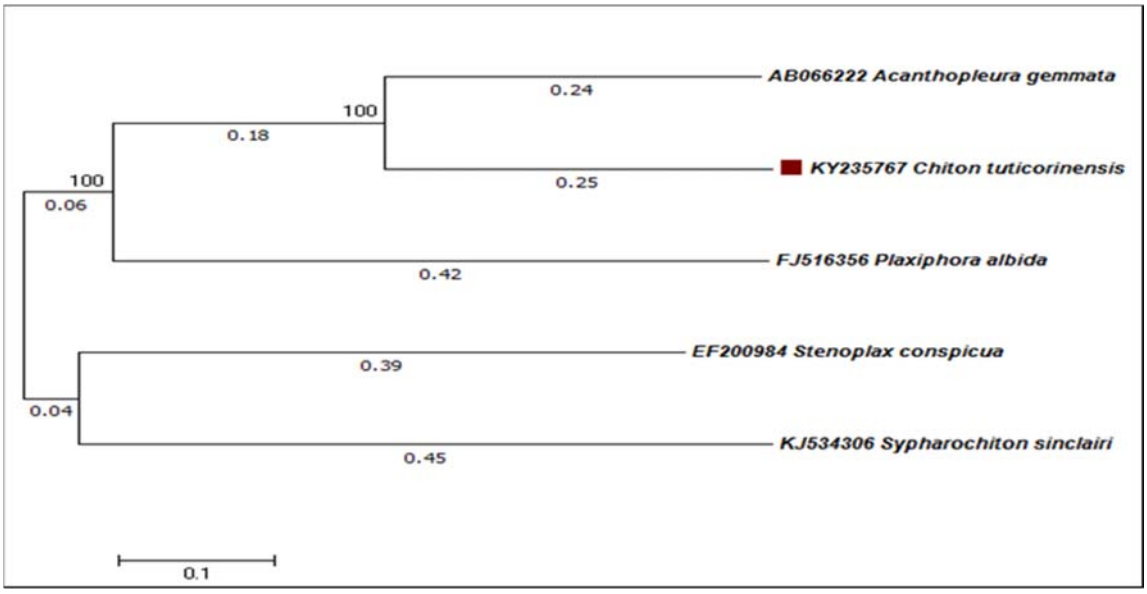


Fig 2: Phylogenetic tree analysis of the COI sequence of *C. tuticorinensis*

Table 1: The sequence similarity of *C. tuticorinensis* with the obtained sequences through NCBI database based on COI gene

S. No	Species Name	Accession No (NCBI)	Sequence Similarity (%)
1	*Plaxiphora albida*	FJ516356	85%
2	*Sypharochiton sinclairi*	KJ534306	82%
3	*Stenoplax conspicua*	EF200984	82%
4	*Acanthopleura gemmata*	AB066222	82%

The sequence similarity of COI gene of *C. tuticorinensis* during the BLAST showed maximum similarity (85%) in *P. albida* and the minimum similarity (82%) in *Acanthopleura gemmata.*

4. Discussion

Chitons (Mollusca) are locally abundant elements of the fauna collected from rocky shore and found distributed evenly throughout the study areas. Several species are described by were known only from their type material, and the present study expands the range and the known populations considerably. However, the studies on biodiversity often suffer from a lack of reliable baseline data with which to compare our current findings. Additional work by many ecologists indicates that near shore fauna, including molluscs, are highly sensitive to changes in their marine environments (Southward, 1980) [26].

Schwabe (2006) [23] collected two species of polyplacophora from Andaman Sea. One of them *Leptochiton vietnamensis* is recorded for the first time from the Indian Ocean. The second species is *Craspedochiton tetrica* (Carpenter in Pilsbry, 1893) [18], a species formerly known as synonym of *Craspedochiton laqueatus* (Sowerby, 1842) [27]. Sigwart (2007) [25] recorded a new species of fossil polyplacophoran *Leptochiton faksensis* from the Danian (Lower Paleocene) of Denmark is described over 450 individual disarticulated plates.

Molecular phylogenetic study including members of this fauna found preliminary evidence for cryptic species in some, but not all, morpho species of these chitons. A combination of molecular and anatomical study will shed more light on these questions in future. But currently, DNA barcoding is a widely accepted molecular taxonomic method which uses a short genetic marker to facilitate identification of a particular species even by non-specialist in molecular biology. DNA barcoding can reliably be assigned unknown specimens to known species, also flagging potential cryptic species and genetically distant populations (Radulovici *et al*., 2010) [20]. The popularity of COI DNA 'barcoding' is increasing rapidly, with mass amounts of invertebrates and vertebrates collected in the field inevitably becoming a mass of data to be added. With so much data needing to be processed, 'taxonomic implement' exist just as much for molecular data as it does for traditional collections.

Okusu *et al*. (2003) [15] published the first phylogenetic analysis of the molluscan class Polyplacophora using DNA sequence data. The clustering pattern of COI barcodes flagged misidentifications, guided, taxonomic decisions and facilitated the detection of diversity overlooked by the current taxonomic system. It could be understood that there are no records apart from the present study in the NCBI database on the barcode of Class Polyplacophora from Indian coastal waters especially along Tamil Nadu coastline, whereas several authors in molecular taxonomy have DNA barcoded on various flora and fauna from Indian coastal waters (Persis *et al*., 2009; Ajmal Khan *et al*., 2010; 2011, Prasannakumar *et al*., 2011) [16, 2, 1, 19].

The present study reveals that, the study areas sustain more species of chiton which is due to the natural rocks are found in these regions. Most of the species were found in intertidal region during the study period. To gain focus on chitons along southeast coast of India. In the present study the class Polyplacophora species collected were identified by using COI gene sequences and submitted to the NCBI data base.

5. Conclusion

This study merits as first report on the distribution, barcode of chiton and taxonomy of chitons from Tamil Nadu. Phylogenetic studies of Polyplacophora based on morphology are scarce. Hence the addition of an independent source of evidence such as molecular data is important. While this analysis points towards interesting issues in chiton systematics, it mostly serves as a first step towards further analyses of morphological and molecular attributes.

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