Global Advanced Research Journal of Biotechnology Vol. 1(2) pp. 012-016, September, 2012
Available online http://garj.org/garjb/index.htm

Full Length Research Paper

First Record of Mitochondrial Cytochrome Oxidase I gene sequences of Ascidian *Polyclinum madrasensis* (Sebestian, 1952) from Gulf of Mannar, Southeast coast of India

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Accepted 08 August, 2012

Recent studies have revealed that many marine invertebrates are closely associated with diverse microorganisms, frequently resulting in the production of compounds of biomedical interest. During the present investigation mitochondrial cytochrome oxidase I gene sequences was used to identify the tunicate as *Polyclinum madrasensis* (Sebestian, 1952) this is the first report of this species worldwide. Solitary ascidian *Polyclinum madrasensis* was collected from the Gulf of Mannar coast in April 2011. The amplified product was sequenced by a commercial lab. The size of the COI was 643 bp in length. The processed sequence was deposited in Gen Bank and got the accession number *Polyclinum* JN 107814. This study highlights the power of molecular method for species identification and India's need for an extensive, systematic molecular inventory of its existing marine invertebrate biodiversity.

Keywords: Biomedical; Ascidian; Molecular Identification; *Polyclinum madrasensis;*

INTRODUCTION

Ascidians, or sea squirts, are members of the class Ascidiacea, within Tunicata that exhibit diverse life history strategies (Satoh 1994; Burighel and Cloney 1997; Davidson *et.al* 2004). The ascidians settle on all kinds of surfaces; hard rocks, stones, hulls of ships, branches and roots of trees, algae, floating objects, sand and muddy surfaces. In soft substratum either the animals skin down keeping the siphons above, or the test sends projections like 'foot' or root like processes to fix into the sand. They are distributed in different places extending from the tropic to the Polar Regions. The great majority of forms occur in the littoral zone. They are the major components of fouling community occurring on the hulls of ships, piers, pilings, test panels, buoys, floats, cables and various other harbor installations. From the evolutionary points of view, ascidians occupy an interesting position between invertebrates and chordates.While the terrestrial ecological changes are relatively well documented, marine ecological changes are much less described. In particular, the scale and ecological significance of the establishment of non-native marine invertebrate species is poorly understood or even quantified. As recently as the last decade several invasive tunicate species have

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become established in New Zealand coastal waters including *Didemnum vexillum* Kott, 2002 (Coffey 2001), *Styela clava* Herdman, 1881 (Davis and Davis 2006) and *Eudistoma elongatum* (Herdman, 1886) (Smith et al. 2007). Morphology-based tunicate taxonomy is a highly specialized discipline and the misidentification of species is a frequent problem (Lambert 2009; Geller et al. 2010). However, recent genome analyses suggest that tunicates may be more closely related to vertebrates than cephalochordates (Blair and Hedges 2005; Philippe and others 2005; Delsuc *et.al* 2006). Also, tunicates possess neural crest cells (Jeffery *et.al* 2004) and placodes (Manni *et.al* 2004; Bassham and Postlethwait 2005; Mazet *et.al* 2005) that are lacking in cephalochordates. Tunicates typically have long branch-lengths, which confound phylogenetic analyses and create artifacts (Blair and Hedges 2005; Zeng and Swalla 2005; Delsuc *et.al*, 2006). In summary, the placement of the tunicates within deuterostomes has been problematic (Winchell *et.al* 2002; Blair and Hedges 2005; Zeng and Swalla 2005; Delsuc *et.al* 2006), even though studies have shown that tunicates are monophyletic (Swalla *et.al* 2000; Stach and Turbeville 2002; Winchell *et.al* 2002).Ascidian tadpoles have key chordate characteristics such as a notochord and a dorsal hollow nerve cord (Swalla 2004a, 2004b), but these traits are lost after metamorphosis. Adult ascidians may be solitary and sexual or colonial and alternating between sexual and asexual reproduction by budding (Berrill 1935, 1936; Nakauchi 1982; Burighel and Cloney 1997). Colonial ascidians tend to be ovoviviparous, producing large eggs and releasing adultated larvae that stay in the water column for only a short period of time before settling and initiating metamorphosis into the adult form (Berrill 1935, 1936; Jeffery and Swalla 1992; Burighel and Cloney 1997; Davidson and others 2004). Solitary either release large numbers of relatively small eggs into the water column, where fertilization and subsequent development into tadpole larvae takes place, or brood large, highly differentiated larvae (Berrill 1935).Ascidians were originally divided into colonial and solitary species by taxonomists, but in the early part of the 20th century classification based on branchial sac and gonad morphology became universally accepted (Van Name 1945; Berrill 1950; Nishikawa 1990; Kott 1998; Monniot F and Monniot C 2001; Monniot and others 2001; Lambert 2005). Recently, phylogenies based on DNA sequences have helped to clarify some evolutionary relationships among the tunicates, although most phylogenies are entirely consistent with the taxonomic relationships (Wada and others 1992; Hadfield and others 1995; Wada 1998; Cameron and others 2000; Swalla and others 2000; Stach and Turbeville 2002; Winchell and others 2002; Turon and Lo´ pez-Legentil 2004).The use of DNA sequence data to identify marine species is proving especially useful in situations where traditional morphology-based discrimination of taxa is very difficult and / or controversial (Darling and Blum 2007; Miura 2007; Geller et al. 2010). Indeed the successes of this approach have led to the development of internationally standardized molecular methodologies and associated public access databases explicitly for DNA sequence based species identification (Ratnasingham and Hebert, 2007). In this study, *P.madrasensis* were collected in Gulf of Mannar coastal region. Here we report the cytochrome oxidase I (COI) gene sequences as well as several morphological characters to identify *P. madrasensis* in Gulf of Mannar coastal waters.

Study Area

The Gulf of Mannar Biosphere Reserve (08º to 09 º; 78 º12' to 70 º 14'E) covers an area of 1,050,000 hectares on the Southeast coast of India. It is one of the world's richest regions from a marine biodiversity perspective. In the present study totally, 26 stations were selected from Rameswaram to Tuticorin for the ascidians diversity.

Taxonomy

The class includes the sessile tunicates either solitary or compound with an external covering the tunic, body may be undivided or divided into a thorax and abdomen and sometimes also into a post abdomen. There are two openings in the test, the oral and atrial apertures. The oral siphon leads into the enlarged pharynx or branchial sac. At the base of the oral siphon simple or branched branchial tentacles are present. The lateral and dorsal side of the branchial sac is surrounded by the atrium. Atrial siphon may open directly to the exterior or into a cloacal cavity in which case an atrial languest is present muscles on the body wall consisting of circular muscles around the siphons and longitudinal and circular muscles in the trunk region. The branchial sac is perforated by stigmata. On the roof of the pharynx, dorsal tubercle is present. A mid –ventral ciliated groove, the endostyle is present in the branchial sac. Branchial papillae at the junction of the internal longitudinal vessels with the transverse vessels may or may not be present; the pharynx leads into the oesophagus, stomach, intestine and anus. A circulatory system and a rather limited nervous system are present.

They are hermaphrodites and many have developed mechanism of sperm-ova incompatibility or the male and female gametes maturing at different times to avoid self fertilization. Development is indirect with a tadpole larva. Most compound and some simple ascidians are viviparous, retaining the fertilized eggs in the atrial chamber until hatching- larval period last only for a few hours. In compound ascidians, asexual reproduction by

budding transforms the initial zooid into a colony. It differs from other genera of Polyclinidae by the following characters. No longitudinal folds in stomach, branchial lobes six, ovary in post abdomen. Abdomen and post abdomen separated by constriction, gut loop twisted.

***P. madrasensis* (Sebestian, 1952)**

The test is usually soft in preservative. Colonies are cushions to about 6 cm in diameter and up to 1.5 cm thick. Test gelatinous, translucent internally. Colonies are black in preservative. No sands embedded in the surface of the test. Zooids are long. Atrial lips long originated from the body wall anterior to the atrial opening. There are 12-14 rows of up to 14 relatively short oval stigmata.

Species composition and distribution of ascidians along the gulf of mannar

Most of the marine organisms have two phases in their life cycle, the pelagic larval stage and benthic adult stage. The planktonic larval stage in their life cycle introduces the potential for considerable spatial and temporal variation. The spatial and temporal variability in patterns of settlement and recruitment of marine invertebrates can strongly influence the distribution and abundance of adult populations. It is noteworthy to mention here the ascidians were collected from 16 different habitats from trawl, intertidal, deep sea, hull of ships, barge, pipeline, pearl oyster farm, pearl oyster cage, peal oyster bed, seaweed raft,seaweed ropes, fishing harbour ,fish landing centre, cement block, rocks pillars and dead coral.

MATERIALS AND METHODS

DNA extraction, amplification, and purification

For collected ascidian sample, 3-5 mm^2 section of siphon tissue was cut and finely diced. The diced tissue was digested and purified following a modified lithium chloride/chloroform protocol (Gemmell & Akiyama, 1996).DNA pellets were suspended in 100µL TE8(10mM Tris- HCL,pH8.0,1mM EDTA) and stored at -20°C. DNA concentration was measured spectrophometrically using a Nanodrop (Nanodrop Technologies Ins.USA).The mitochondrial COI gene was amplified with primers adapted from Folmer et al. (1994):

BAS1_COI1F-CO9, 5'-GTACTGAGCTTTCACAAACTGGGCAAT-3',

BAS1_COI1R-DO9, 5'-TGAAAAAGAATAGGATCTCTCCTTCC-3'.

PCR amplification was performed in 20µL reaction volume, consisting of 1Xbuffer (50mM KCL, 10mM Tris HCL.pH.8.0), 1.5mM $MgCl_2$ 200µm dNTPs, 0.5µm each primer, 0.5U *Taq*(Invitrogen),12.9µL double-distilled, autoclaved water plus 2µL of template DNA. Thermal cycling parametersincluded an initial denaturation at 94°C for 2 minutes, followed by 48°C(COI)for 20 sec, and 72°C, for 30 sec, before a final 7 minute extension at 72°C.COI PCR products were not purified for sequencing reactions because there was no difference in the quality of purified and unpurified sequence products.

Primer Design

The yeast cytosolic NADP(+)-dependent isocitrate dehydrogenate gene (IDP2) was previously isolated and cloned (Loftus et al., 1994). We used the gene sequence published at that time to create primers which would amplify the 1.2 kb IDP2 gene sequence using PCR. Primers were engineered to amplify the open reading frame of the gene, placing a BamHI site directly before the start codon, and a HindIII site directly after the stop codon (Forward BAS1_COI1F-CO9, 5'-GTACTGAGCTTTCACAAACTGGGCAAT-3', Reverse BAS1_COI1R-DO9, 5'-TGAAAAAGAATAGGATCTCTCCTTCC-3').

Sequencing

For COI, labeled PCR primers BAS1_COI1F-CO9 and BAS1_COI1R-DO9 were used (mentioned above). COI sequencing reactions were performed using a Big Dye V3.1 sequencing kit (Applied Biosystems), per manufacturer's instructions. COI sequencing reactions were optimized by adding a heat-denature step for 5 min at 98°C before adding the dye-terminator mix to sequence through a difficult region after a 643bp poly-A tail (Kieleczawa, 2006).

RESULTS AND DISCUSSION

The amplified product was sequenced by a commercial lab. The size of the COI was 643 bp in length. The processed sequence was deposited in GenBank and got the accession number Polyclinum JN 107814.

The study is significant in the aspects of first report on partial sequencing of the *P.madrasensis*. Future research is focused on the *P.madrasensis* associated bacteria. The bacterial communities found on the tunic surfaces of the sedentary ascidians is said to be more diverse. The association of bacteria may be vertically transmitted and involved in the production of secondary metabolites that deter predators of the ascidians. Understanding the

phylogenetic relationships of the three major Urochordate groups within the deuterostomes is a major key factor to understand the evolution of the chordates. The associated bacteria might be vertically transmitted and involved in the production of secondary metabolites that deter predators of the ascidians. The mitochondrial gene sequences and morphological characters were used to identify the tunicates as *P.madrasensis*. We have prepared a detailed phylogenetic analysis of urochordates based on new urochordate mitochondral DNA sequences.

The possible ecological and economic implications of this introduced tunicate species are unknown but it might result in identification of new biologically active compounds. In addition, microbes associated with invertebrates might also play important functions in the ecosystem. Ascidians are prolific producers of secondary metabolites (e.g., Ireland et al., 1988; Rinehart, 2000). Although the precise role of these compounds in ascidian function is largely unclear, they might function as protection against predation or colonization by unwanted or pathogenic microorganisms (Pisut and Pawlik, 2002; Moss et al., 2003; Ramasamy and Murugan, 2003). Notwithstanding the widely recognized logistical and statistical challenges of taxonomic assignments based solely on sequence data, this study again highlights the power of molecular methods for species identification when such approaches are well-supported by classical morphology-based taxonomy (Ratnasingham and Hebert 2007; Borisenko et al. 2009; Radulovici et al. 2009). This study also underscores a need for extensive molecular inventories of the extant marine invertebrate biodiversity in those regions that wish to effectively monitor and / or control the ongoing anthropogenic spread of invasive marine species (Radulovici et al. 2009). We conclude that *P.madrasensis* is present in the gulf of mannar (South east coast of India) and, to the best of our knowledge, this is the first record of *P.madrasensis* in the Indian waters. The possible ecological and economic implications of this introduced tunicate species are unknown but it might result in biofouling issues.

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