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Molecular Level Evolutionary Relationship of the Colonial Ascidian *Eudistoma Viride* From Southeast Coast of India

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Abstract

The colonial ascidian *Eudistoma viride* was collected from the under surface of calcrete rocks near Hare Island, Tuticorin coast and stored in 95% (v/v) ethanol at -20°C. The ascidian was sequenced at 608 bp region of the mitochondrial cytochrome oxidase subunit I gene (COI) for phylogenetic analysis. Few ascidian barcode sequences were extracted via FASTA format from NCBI to study phylogenetic tree and genetic distances within same genus and other genus ascidians sequences. In the Phylogenetic analysis the ascidian *E. viride* sequence shows 72% similarity with another genus ascidian species *Aplidium pseudolobatum* and 100% similarity with pervious submitted ascidian sequences *E. viride* GAPB10. In maximum likelihood analysis the ascidian *E. viride* sequence shows 99% similarity with pervious submitted ascidian sequences *E. viride* GAPB10. These molecular phylogenetic studies suggest that improved knowledge of ascidian diversity could lead to the recognition of more than one distinct genus of ascidian. Such identification of ascidian species through bioinformatics and molecular phylogeny is to value in biodiversity and conservation of marine species.

Keywords: ascidian, Tuticorin, mtDNA, NCBI, maximum likelihood, maximum parsimony

INTRODUCTION

Mitochondrial DNA (mtDNA) analysis has been employed in the evolutionary study of the animal species for more than 30 years (Avise and Walker 1999). Its higher mutational rate and lower effective population size than the nuclear DNA make mtDNA a powerful tool to probe for evolutionary studies. This fact provoked a proposal to standardize DNA-based species identification by analyzing a uniform segment of the mitochondrial genome. A library of sequences from taxonomically verified voucher specimens could be built with this approach which could serve as DNA identifiers for species, in short, DNA barcodes (Herbert et al. 2003). For animals, 648 bp segment of the mitochondrial gene cytochrome C oxidase I (COI), which can be readily recovered from diverse species with a limited set of primers, was declared as a DNA barcode (Kevin et al. 2007). In addition, by assigning specimens to known species, DNA barcoding can speed the discovery of new species, as large sequence differences in animal mtDNA generally signal species status. Since, marine animals have been the subject of intensive taxonomic analysis they provide an excellent opportunity to test the efficacy of barcode-based species delimitation. For this approach to be effective, it must be possible to distinguish between intraspecific and interspecific mtDNA variation. The simplest test is whether the genetic distance within the species is lesser than those between species.

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). Morphology-based tunicate taxonomy is a highly-specialized discipline but the misidentification of species is a frequent problem (Lamber 2006; Geller et al. 2010).

Ascidians have few external characteristics that are species specific and can easily be mistaken with similar looking ascidians. Many colonial ascidians exhibit remarkable variation in colony color and form, and colony appearance is sometimes substantially different within the same species and/or very similar among different species. Thus, to accurately identify the species, colonies must be dissected under a stereo microscope to examine their zooid morphology. Zooids are often simple in morphology and do not provide many features for taxonomic identification. However, the integration of molecular and morphological identification techniques can provide a stronger taxonomic confirmation of organisms. From Tuticorin coast of India ‘‘barcoding’’ of mitochondrial cytochrome oxidase I (COI) gene sequences of only two species of colonial ascidians of the genus *Didemnum* – *Didemnum granulatum* (JQ013198) and *D. psammathodes* (JN624758) has been attempted (Sri Kumaran et al. 2013). The genus *Eudistoma* exhibits considerable variation in external morphology, but there is an apparent lack of internal morphological variation and the zooids are difficult to distinguish among species, which can create difficulties for species identification, especially in preserved material (Kott 1990). So, this study was aimed at exploring the ‘‘barcoding’’ of mitochondrial cytochrome oxidase I (COI) gene sequences and phylogenetic status of the ascidian *E. viride* collected from the Tuticorin Coast of India.

MATERIALS AND METHODS

Sample collection

The colonial ascidian *E. viride* was collected from the under surface of calcrete rocks near Hare Island, Tuticorin coast (Lat. 8° 46' 20. 72" N and Long. 78° 11' 57. 91" E) of India (Fig. 1). Fig. 2 shows the colony of *E. viride*.

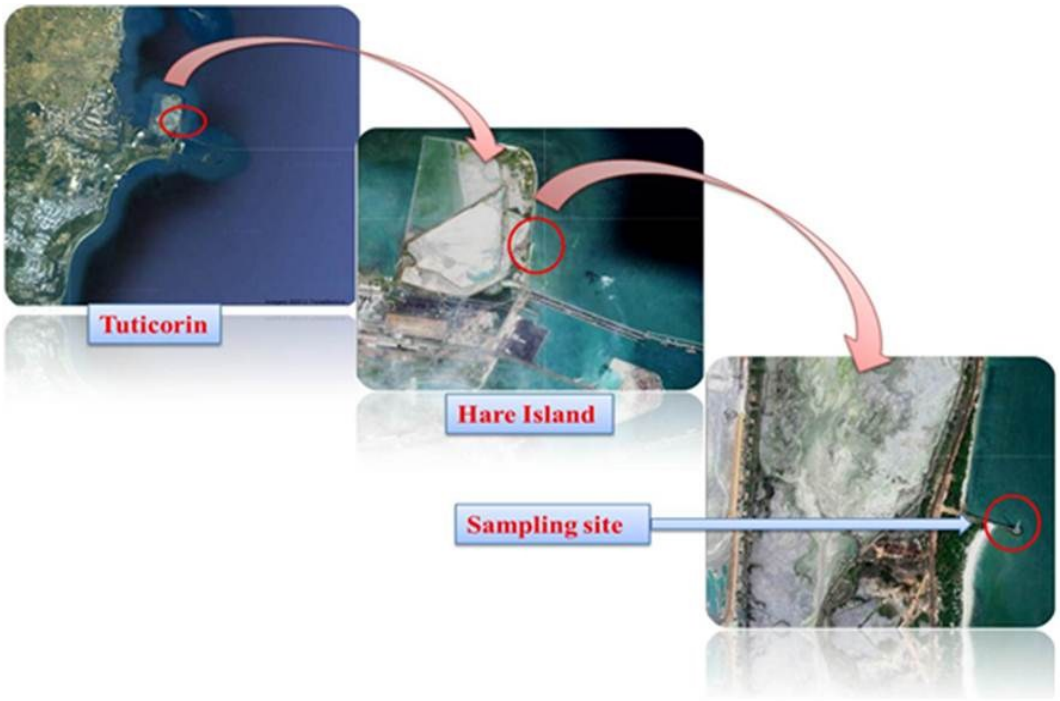

Fig. 1. Collection site

Fig. 2. Colonial ascidian *Eudistoma viride*

The sample was thoroughly washed with sea water to remove sand, mud and overgrowing organisms at the site of collection. 25 mg of ascidian tissue was removed from each lobe using sterile blade and stored in 95% (v/v) ethanol at -20°C. Salt out protocol was adopted for precise and quick DNA isolation. Standard literature of Monniot and Monniot (2001) was used for identification. Voucher specimen No AS 2234 have been deposited in the National Collection of ascidian in the Museum of the Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin-628 002.

DNA extraction and mitochondrial cytochrome oxidase I (COI) DNA sequencing

The DNA was extracted from ascidian tissues by the following method of Sri Kumaran (2013). LCO1490: 5'GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 :5'TAAACTTCAGGGTGACCAAAAAATCA 3' primers were employed for COI amplification (Folmer 1994). The sample was loaded onto Mega Bace sequencer (MB 1000) at Bioserve Biotechnologies, Pvt. Ltd. Hyderabad, India.

COI sequence analysis

The electropherenogram generated by automated DNA sequencer was read by Chromas Pro vl.42 (Technelysium Pvt. Ltd., Tewantin, Queensland, Australia) and the sequence was carefully checked for mis-calls and base spacing. Few ascidians sequences were extracted *via* FASTA format from NCBI. Clustal X 2.0.6 (www.clustal.org) was used to align the nucleotide sequence (Thomson, 1997). The nucleotide content of collected barcode was estimated by BioEdit version 7.0.9.0 sequence alignment editor (Hall, 1999). MEGA 6 was used to construct phylogenetic tree via the neighborhood joining method using Kimura 2 parameter and to calculate genetic distance of the given set of sequences (Tamura et al. 2011). The previously submitted ascidian sequences were selected for phylogenetic tree construction.

RESULTS

The mitochondrial Cytochrome oxidase I (COI) DNA sequence has been deposited in Genbank and accession number was obtained as JQ403421. The final length after alignment and trimming was of 608 base pairs (bp) and free of gaps. Various ascidian sequences were selected form NCBI for compare the evolutionary relationship with *E. viride* sequence at genus level. By using these various genus of ascidian sequences the phylogenetic tree of *E. viride* was constructed (Fig. 3).

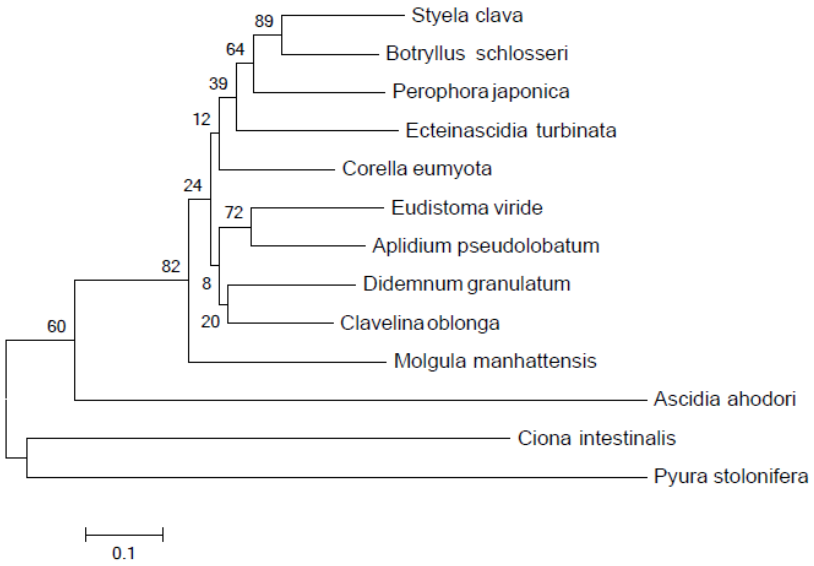

Fig. 3. Comparison of the evolutionary relationship with various genuses of ascidians

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 4.30352733 is shown. The percentage of replicate trees in which the associated ascidian sequence clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Tamura et al. 2007) and are in the units of the number of base substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+ Noncoding. All ambiguous positions were removed for each sequence pair. In the Phylogenetic analysis, the ascidian *Eudistroma viride* sequence shows 72% similarity with other genus ascidian species *Aplidium pseudolobatum*.

The evolutionary relationship between interspecies was inferred using the Neighbor-Joining method (Fig. 4) (Saitou and Nei 1987).

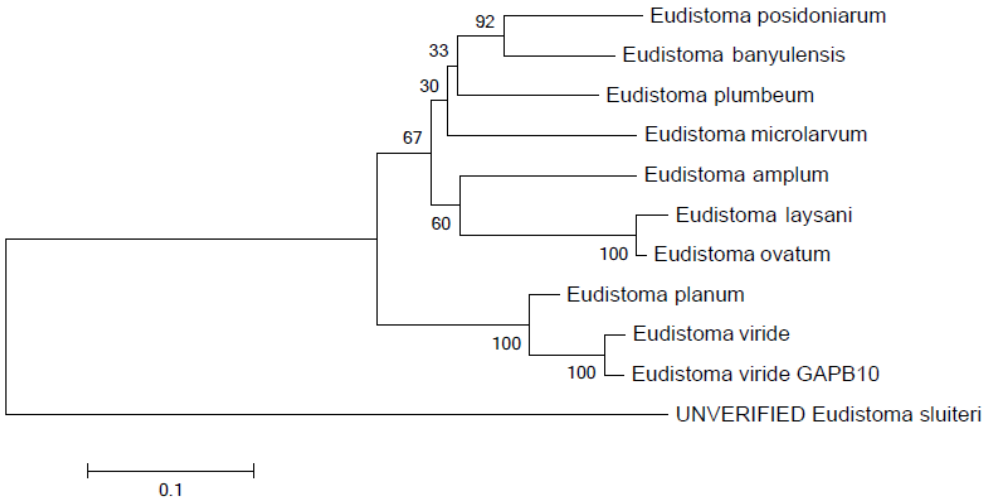

Fig. 4. Comparison of the evolutionary relationship within same species of ascidians sequences

The optimal tree with the sum of branch length = 1.48246815 is shown. The percentage of replicate trees in which the associated ascidian sequence clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Tamura et al. 2007) and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All ambiguous positions were removed for each sequence pair. There was a total of 513 positions in the final dataset. In the Phylogenetic analysis, the ascidian *Eudistroma viride* sequence shows 100% similarity with previously submitted ascidian *Eudistroma viride* GAPB10 sequence.

The maximum likelihood analysis also studied in interspecies of ascidian (Fig. 5). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood (-3145.2235) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 492 positions in the final dataset. In maximum likelihood analysis, the ascidian *Eudistroma viride* sequence shows 99% similarity with previously submitted ascidian *Eudistroma viride* GAPB10.

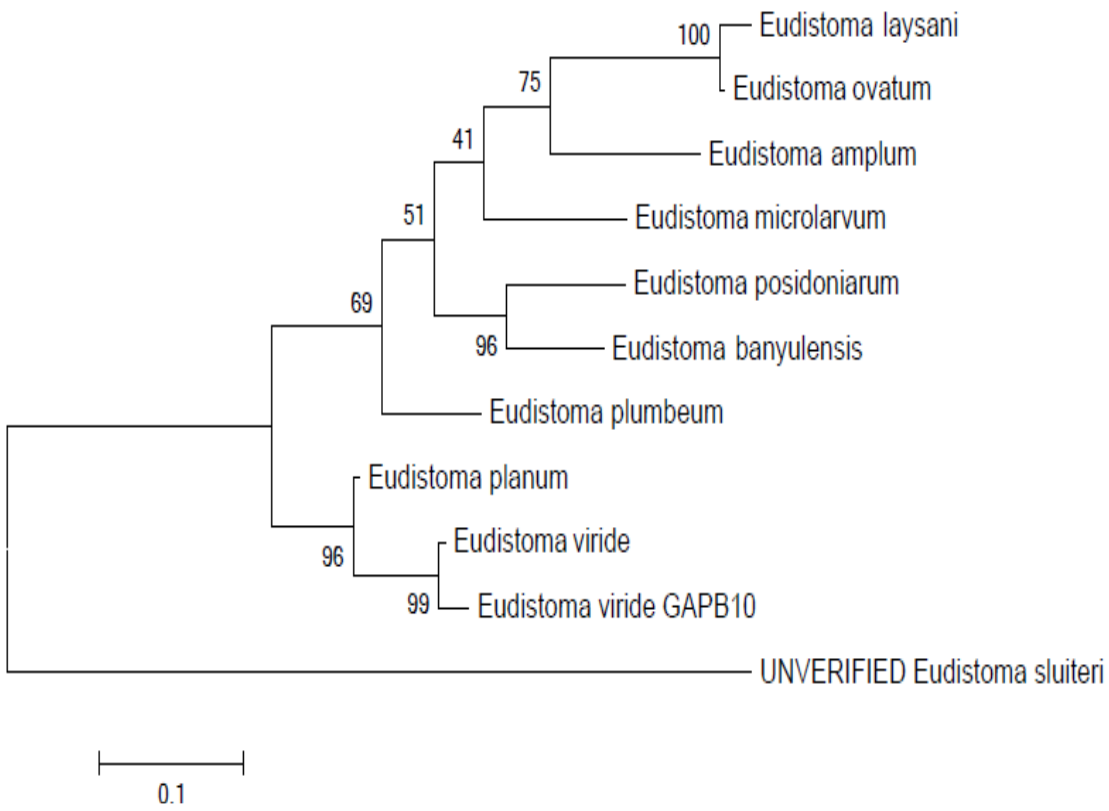

Fig. 5. Molecular Phylogenetic analysis of ascidian *Eudistoma viride* by Maximum Likelihood method

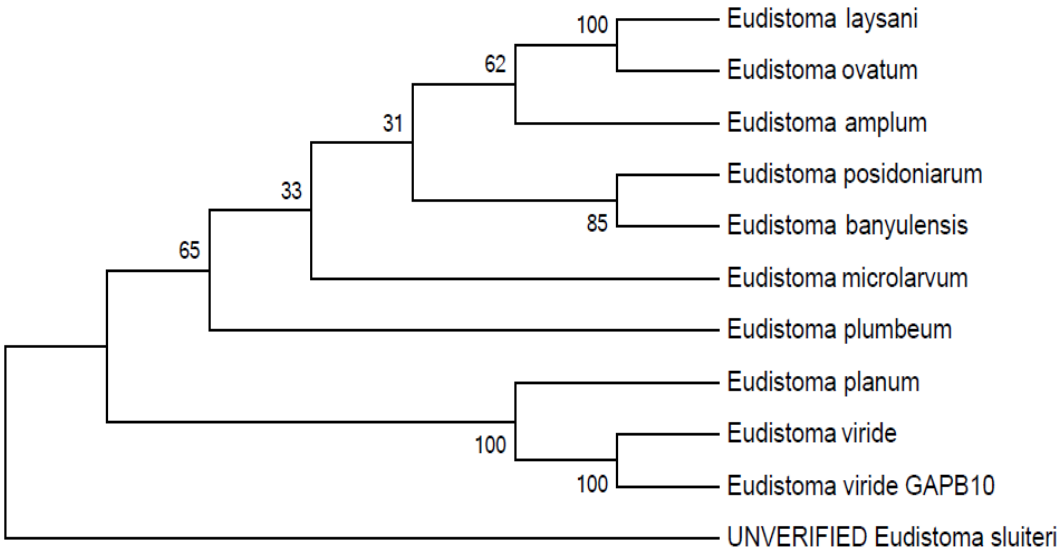

Fig. 6. Maximum Parsimony analysis of ascidian *Eudistoma viride*

Maximum Parsimony analysis of ascidian *Eudistoma viride* was shows in Fig .6. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 3 most parsimonious trees (length = 627) is shown. The consistency index is (0.614907), the retention index is (0.592105), and the composite index is 0.416457 (0.364090) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 492 positions in the final dataset.

The percentage of nucleotides A, C, G and T present in mitochondrial cytochrome oxidase I (COI) DNA sequence of ascidian *E. viride* were 22.86, 15.63, 19.57 and 41.94 respectively (Fig. 7.).

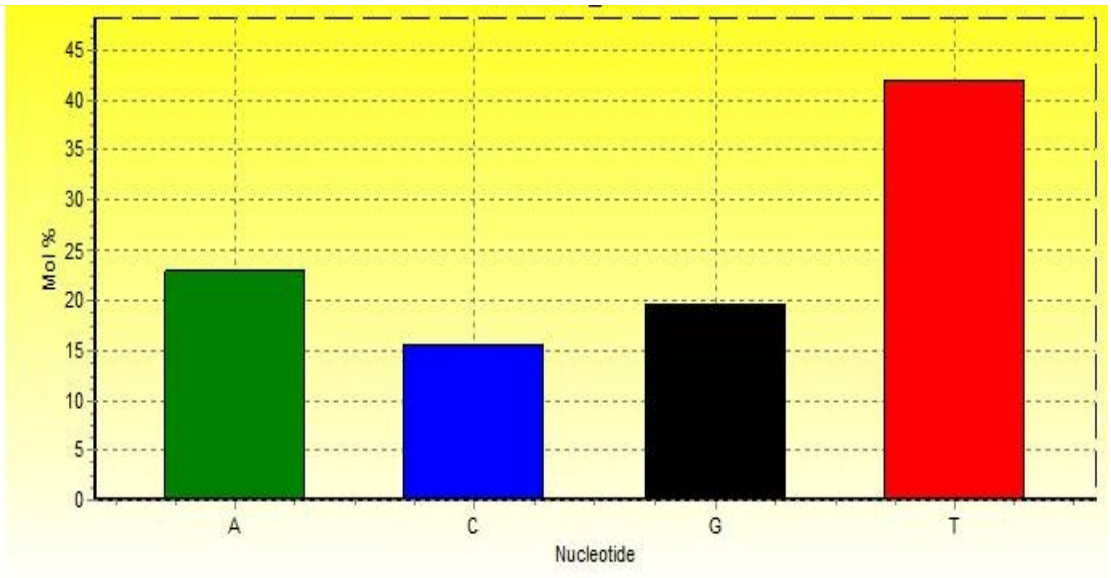

Fig. 7. Percentage of nucleotides presents in mitochondrial cytochrome oxidase I (COI) DNA sequence of ascidian *E. viride* (JQ403421)

The GC and AT content was 35.2 and 64.8%. Genetic distance (without parenthesis) with standard error (in parenthesis) is given in Table 1.

Table 1. The genetic distance of *Eudistroma viride* (JQ403421) with standard error between the selected ascidians.

	Eudistoma viride	***Eudistoma viride GAPB10***	***Eudistoma planum***	***Eudistoma laysani***	***Eudistoma ovatum***	***Eudistoma microlarvum***	***Eudistoma amplum***	***Eudistoma posidoniarum***	***Eudistoma banyulensis***	***Eudistoma plumbeum***	***UNVERIFIED Eudistoma sluiteri***
Eudistoma viride		(0.007)	(0.012)	(0.030)	(0.030)	(0.029)	(0.032)	(0.029)	(0.029)	(0.025)	(0.064)
Eudistoma viride GAPB10	0.024		(0.014)	(0.030)	(0.030)	(0.029)	(0.031)	(0.028)	(0.029)	(0.024)	(0.061)
Eudistoma planum	0.067	0.083		(0.028)	(0.027)	(0.026)	(0.028)	(0.027)	(0.026)	(0.022)	(0.060)
Eudistoma laysani	0.337	0.343	0.308		(0.007)	(0.025)	(0.024)	(0.025)	(0.024)	(0.025)	(0.066)
Eudistoma ovatum	0.335	0.341	0.297	0.024		(0.024)	(0.023)	(0.024)	(0.024)	(0.024)	(0.067)
Eudistoma microlarvum	0.298	0.300	0.256	0.263	0.247		(0.024)	(0.023)	(0.022)	(0.022)	(0.069)
Eudistoma amplum	0.329	0.323	0.282	0.235	0.216	0.234		(0.027)	(0.023)	(0.025)	(0.064)
Eudistoma posidoniarum	0.304	0.298	0.271	0.266	0.250	0.231	0.254		(0.019)	(0.020)	(0.068)
Eudistoma banyulensis	0.304	0.307	0.262	0.244	0.230	0.215	0.235	0.151		(0.021)	(0.063)
Eudistoma plumbeum	0.256	0.248	0.198	0.259	0.237	0.205	0.238	0.188	0.189		(0.065)
UNVERIFIED Eudistoma sluiteri	0.774	0.755	0.740	0.773	0.771	0.806	0.758	0.802	0.748	0.771	

The numbers of base substitutions per site from between sequences are shown Table 1. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates). Analyses were conducted using the Kimura 2-parameter model (Kimura 1980). The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There was a total of 513 positions in the final dataset.

DISCUSSION

The cytochrome oxidase I (COI) gene of mitochrondrial DNA (mtDNA) is a common gene useful for molecular identification of species and for uncovering patterns of diversity within and among populations and in communities (Muirhead et al. 2008). It is a logical first choice for species identification and may be useful for early detection of propagules for management purposes. A relatively short (~ 650 base) fragment of COI has been used in the identification of species which allows the acquisition of reliable sequences from single reads using standard cycle sequencing, and is generally highly conserved within species (Hajibabaei et al. 2007). However, COI might be expected to be useful at lower taxonomic levels (Stach et al. 2002), and has

subsequently been used in a phylogenetic analysis of species of ascidians (Turon and Lopez-Legentil 2004).

In the present study, mitochondrial cytochrome oxidase I (COI) DNA sequence of ascidian *E. viride* (JQ403421) has been deposited in NCBI databases and compared with other genus and interspecies of ascidian sequences. The BLAST results provided confirmation of our taxonomic identifications. This study compares the evolutionary relationships on various genus and same genus level of ascidian sequences. The newly determined mtDNAs of *Eudistoma* species and the comparative data reported here highlight the strong differences in mtDNA evolutionary dynamics between ascidians and remaining chordates. Comparative analysis of mitochondrial COI sequences of colonial ascidian, *E. viride* revealed contrasting patterns of genetic structure.

DNA sequence data are being used to assess the taxonomic status of colonial ascidians with variable morphologies (Lopez-Legentil and Turon 2005). Invasive species may show morphological similarities, or differences, to specimens from the source population, but would be expected to show low intra-specific DNA sequence divergence with the source population and high inter-specific divergence with congeners. However, as many invasive species actually maintain a low level of genetic diversity in introduced regions, the time since introduction is difficult to estimate from molecular datasets (Silva and Smith 2008).

Phylogeographic studies of taxa across biogeographical ranges can contribute to the elucidation of cryptic species was proposed by findings of Tarjuelo et al. (2001) which suggested two distinct clades due to lack of gene flow between the harbor and rock reef population by employing COI gene as a effective tool.

Knowledge of diversity of ascidians in the waters around India is very less. In particular, the scale and ecological significance of the establishment of non-native marine invertebrate species is poorly understood or even quantified. The Indian coast, being dotted with 12 major ports and a number of minor ports is susceptible for bioinvasions and hence warrants a close watch. Little information exists from marine ecosystems of India regarding the presence and distribution of alien and cryptogenic ascidians. A comparison of the pre-2000 ascidian survey data in Indian waters with that of the post-2000 period showed that more than 300 species of ascidians including more than 170 new species were belonging to 10 families and 38 genera reported so far from Indian waters comprising both colonial and solitary form, reported in the later period (Renganathan 1990, Meenakshi 2004, Meenakshi and Senthamarai 2006a,b,c; Ali and Sivakumar 2007) and the total number of ascidians increased to more than 450. This clearly indicates that taxonomical studies on ascidians in India have been expanded. However, distributional information of alien ascidians is lacking. Some information is available on the impact of ascidians as marine fouling species (Venkat et al. 1995) but these ascidians were not categorized into either alien or native species. It is reported that 6% of the total 205 non-indigenous taxa introduced into Indian seas in the post-1960 period were represented by ascidians (Rao, 2005). Bhavanarayana and Ganapati (1971) studied the ascidian species among pelagic tunicates from the inshore waters of Visakhapatnam. Recently few researchers

are contributing their research in molecular identification of ascidians. Sri Kumaran et al. (2014) studied mitochondrial cytochrome oxidase I (COI) DNA sequencing of the ascidians *Didemnum granulatum* (JQ013198) and *D. psammathodes* (JN624758), Selva Prabhu et al. (2012) studies Mitochondrial Cytochrome Oxidase I gene sequences of Ascidian *Polyclinum madrasensis* (Sebestian, 1952) from Gulf of Mannar, Southeast coast of India. Iyappan et al. (2015) studied molecular identification of four ascidians from the Palk Bay Region, southeast coast of India. Jaffar Ali et al. (2016) studied distribution and invasiveness of a colonial ascidian, *Didemnum psammathodes,* along the southern Indian coastal water. Mondal et al. (2016) studied 8 non-indigenous ascidians of Andaman and Nicobar Islands, India. Jaffar Ali et al. (2014) studied non- indigenous ascidians in V. O. Chidambaram port, Thoothukudi, India. Recently Jaffar Ali and Ahmed (2016). Worked on DNA barcoding of two solitary ascidians, *Herdmania momus* Savigny, 1816 and *Microcosmus squamiger* Michaelsen, 1927 from Tuticorin coast, India. As Tuticorin is one of the major ports in India, a thorough prolonged future investigation is needed to distinguish the harmful from the harmless invasive/alien species and to identify the impacts of the former on native biodiversity. This will also help in detecting new invasion of exotic species and documenting significant range and extensions of damage to the habitat at the regional level. Without a concerted effort to conserve the local native nothing can be achieved at a global level. This type of early warning at regional level will be helpful to restore the diversity of species in different habitats of the Tuticorin coast which is very close to the Gulf of Mannar National Marine Park, a well-preserved area for its rich marine diversity with rich corals. More data are still needed to identify the source and confirm mixing of port populations.

CONCLUSION

The COI sequence in the phylogram constructed clearly clustered the ascidian species in individual group proving the efficiency of COI gene in delineating the member of *Eudistoma* to the species level. Hence, we conclude that COI sequence could be potentially used to identify the individual of ascidians to species level.

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Declaration of interest

We declare that we do not have conflict of interest.

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