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Morphological description and molecular characterisation of green aggregating sea anemone, *Anthopleura elegantissima* (Brandt, 1835) (Cnidaria: Anthozoa) from southwest coast of India

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Original Article

Abstract

Green aggregating sea anemone, *Anthopleura elegantissima* (Brandt 1835) is found in the intertidal region of Thirumullavaram (Lat. 8°89'53"N, Long. 76°55.61"E), southwest coast of India. During the survey, individuals of *A. elegantissima* were found to inhabit the crevices of intertidal rocky habitat. There are some studies on the distribution of *A. elegantissima* from Indian waters. However, little work has been done on the molecular description and characterisation of *A. elegantissima* in India. This study represents the first morphological description and molecular characterization (based on mitochondrial COI gene) of *A. elegantissima* from southwest coast of India. Molecular study based on COI sequences confirmed the speciation viz. phylogenetic tree (based on NJ analysis) and genetic distance data (intraspecific divergence up to 0.60% within the generated sequences).

Keywords: *Sea anemone, Anthopleura elegantissima, morphology, CO1 gene*

Introduction

Members of the sea anemone genus *Anthopleura* are inhabitants of rocky intertidal areas (Daly *et al*., 2017). *Anthopleura elegantissima* was first described as *Actinia elegantissima* (Brandt, 1835) in North America. This species occurs as colonial and solitary forms in the intertidal and shallow subtidal regions. Rocky intertidal zones tend to be more species-rich, in terms of number of species, than sandy intertidal zones (Archambault and Bourget, 1996) due to available space (Dayton, 1971) for attachment and settlement. Single clones were observed to occupy the tidal pools and channels over a year (Francis, 1979; Sebens, 1983). The genus *Anthopleura* is distinguished from other members in Actiniidae by its specific feature, acrorhagi and its adhesive verrucae (Stephenson, 1935; Daly and Fautin, 2020, Carlgren, 1949; Daly and den Hartog, 2004; Daly *et al.,* 2017). Colour of the anemone is commonly green with pinkish tentacle tips. White, yellow and grey coloured tips of tentacles were also seen (Shah *et al*., 2017). Green colour is due to the symbiotic association of zooxanthellae and zoochlorellae. In high light intensity, weight loss occurs in aposymbiotic hosts. During this condition, nutrition is imparted by algae present in the anemones (Muscatine, 1961; Tsuchida and Potts, 1994). Temperature has immense effect on the symbiotic algae (Saunders, 1997). Effects of illumination, feeding regime and

endosymbiotic algae significantly affect the growth of these intertidal anemones (Tsuchida and Potts, 1994). Biologically active agents present in these organisms have potential activity against pathogenic microorganisms (John *et al.*, 2015). Size of *A. elegantissima* fluctuated seasonally (Sebens, 1982). The organism is dioecious but exhibit sexual and asexual modes of reproduction. Reproduction occur in the winter, spring and summer, followed by spawning in late summer and autumn (Sebens, 1981). Longitudinal fission takes place in *A. elegantissima* (Mathew, 1979; Sebens, 1983) and results in cloning and aggression (Sebens, 1983) in the rocky shores.

In India, studies on anemone taxonomy and diversity are comparatively less. *A. elegantissima* was first documented from Saurashtra coast, Gujarat (Shah *et al.*, 2017) and then from Kanyakumari, southeast coast of India (John, 2019). There are some studies on the genus *Anthopleura* based on morphological characters. However, little work has been carried out on the molecular characterisation of *A. elegantissima* in Indian region. This is the first study which aims at the morphological description and molecular characterization (based on mitochondrial COI) of *A. elegantissima* from southwest coast of India.

Material and methods

Sampling

A. elegantissima is found in rocky habitat of Thirumullavaram area (Fig.1) of southwest coast (Lat. 8°89'53"N, Long. 76°55'61"E). Six individuals of this species were found solitary in the tidal rocks. Tidal height was noted and the specimens were collected during low tide (0.53 m). Specimens of *A. elegantissima* (Fig. 2) were taken from the intertidal rock using chisel and hammer and transported to the laboratory in live condition. Six live specimens were examined under a stereo-zoom microscope and were subject to morphological identification following the taxonomic keys provided by

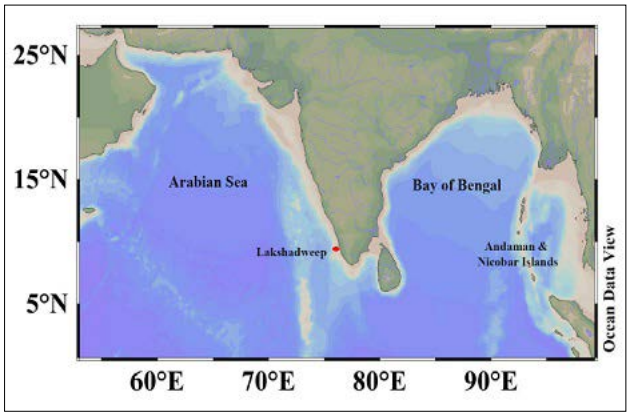

Fig. 1. Sampling site at Thirumullavaram (●) region of southwest coast of India

Fig. 2. Sea anemone, *A. elegantissima* in the rocky habitat at Thirumullavaram (southwest coast of India)

Carlgren (1949) and Shah *et al.* (2017). *A. elegantissima* were separated from the rock and were allowed to expand and anesthetized with menthol crystals. Photographs were taken with and without separating the pebble. Tissue from the tentacle, acrorhagi, filament, verrucae and basal disc were macerated and kept in slide for the identification of cnidae under 40X and 100X interference microscope. Length and width of undischarged cnidae were examined in Tcapture software. Identification of Cnidae was based on the details given by Mariscal (1974) and Ostman (2000). Tissue from the basal disc is used for the molecular studies. Molecular analyses were carried out to generate COI sequences and the data were submitted to NCBI database.

Molecular Analysis

Total genomic DNA was extracted from tissues of collected specimens following spin column method proposed in DNeasy Blood and Tissue Kit (Qiagen). Quality and quantity of extracted DNA samples were estimated using UV spectrophotometer (Nanodrop, 2000) at a wavelength range of 260/280 nm. Optical density (OD) of DNA extracts were measured and samples showing OD value between 1.7 and 2.0 were selected for PCR amplification.

50μl PCR reaction mix was prepared using 25μl Sigma Aldrich ReadyMix™ Taq PCR Reaction Mix with $MgCl^2$ 8 μl template (extracted genomic DNA), forward and reverse primers (1 μl each) and 15 μl demineralized water. Partial mitochondrial COI gene sequences from total genomic DNA was amplified using primer pair LCO1490–5′ GGTCAACAAATCATAAAGATATTGG 3′ and HCO2198–5′ TAAACTTCAGGGTGACCAAAAAATCA 3′ (Folmer *et al.*, 1994). Amplification was performed in a Corbett gradient thermal cycler following the temperature profile according to Bijukumar *et al.* (2015). Thermal profile consisted of an initial denaturation at 94°C for 1 min followed by 5 cycles of denaturation at 94°C for 30 s, 45°C (annealing

temperature) for 40s, extension at 72°C for 1 min, 35 cycles of 94°C for 30 s, 51°C for 40 s and a final extension at 72°C for 10 min. PCR amplicons were visualized on 1.2% agarose gel after electrophoresis and samples exhibiting intense bands were outsourced for purification and sequencing.

Editing of COI sequences were done manually using BioEdit 7.0.9 (Hall, 1999). Initial screening of nucleotide sequences was done using Basic Local Alignment Search Tool (BLASTn) for identifying homologous sequences in public database (Table 1) such as NCBI (Deepak and Harikrishnan, 2016). Sequences having more than 97% identity score towards their homologous sequences in public database were selected for molecular analyses (Shen *et al.,* 2016). Alignment and compilation of COI sequences were done using Clustal X (Thompson *et al.,* 1997). Genetic diversity indices of aligned sequences of *A. elegantissima* were analyzed using DnaSP 5.10 (Librado and Rozas, 2009) and Arlequin 3.1 (Excoffier *et al.,* 2005). All these sequences were submitted in National Center for Biotechnology Information (NCBI) database. In addition to six COI sequences generated, homologous mtCOI sequence of *A. elegantissima* and *A. buddemeieri* (outgroup) from NCBI database were also acquired (Table 1) and utilized for phylogenetic reconstruction and estimation of genetic distance. Phylogenetic tree based on Neighbour Joining analyses with 1000 bootstraps (differentiating haplotypes) and pair wise sequence distance data based on Kimura 2 Parameter model were generated using MEGA 5 (Tamura *et al.,* 2011).

Results

Systematics

Suborder : Enthemonae Rodriguez and Daly in Rodriguez *et al.*, 2014
Super family : Actinioidea Rafinesque, 1815
Family : Actiniidae Rafinesque, 1815
Genus : *Anthopleura* Duchassaing de Fonbressin & Michelotti, 1860
Anthopleura elegantissima (Brandt, 1835)

Synonymised names:

Actinia (Taractostephanus) elegantissima Brandt, 1835
Actinia elegantissima Brandt, 1835 (original binomen)
Anthopleura elegantissima
Anthopleura elegantissima
Bunodactis elegantissima (Brandt, 1835)
Cribrina elegantissima (Brandt, 1835)

Morphological description

The main morphological features of the specimens include oral disc, column and basal disc. Oral disc carries tentacles, acrorhagi and mouth. The specimen has the height of about 2 cm. Diameter of the oral disc (Fig. 3) is about 4.02 mm. Oral disc is wider than column. Actinopharynx contain two siphonoglyph. Expanded oral disc not much broader than

Table 1. Nucleotide sequences utilized for molecular analysis

Sl. No.	Species	Status	Accession number	Sampling location
1		Developed for this study	MG637430-35	South west coast, India
		Acquired from NCBI	GU443182	Oregon, USA
		Acquired from NCBI	AF480931	California, Monterey Bay USA
		Acquired from NCBI	GU443180	Monterey, USA
		Acquired from NCBI	MN448227	Washington, USA
		Acquired from NCBI	KM611880	British Columbia, Pacific Rim National Park, Canada
		Acquired from NCBI	KM611939	British Columbia, Pacific Rim National Park, Canada
	A. elegantissima	Acquired from NCBI	MF544697	British Columbia, Pacific Rim National Park, Canada
		Acquired from NCBI	MF545135	British Columbia, Pacific Rim National Park, Canada
		Acquired from NCBI	MF545142	British Columbia, Pacific Rim National Park, Canada
		Acquired from NCBI	MG421778	British Columbia, Bamfield Canada
		Acquired from NCBI	MG422391	British Columbia, Nanaimo, Piper's Lagoon Canada
		Acquired from NCBI	MG423411	British Columbia, Bamfield Canada
		Acquired from NCBI	MG421476	British Columbia, Bamfield Canada
2	*A. buddemeieri* (Outgroup)	Acquired from NCBI	KM259983	-

pedal disc. Oral disc is surrounded with 92 tentacles. Oral disc is green in colour with white radial lines at the base of the tentacle. Tentacles are long and slender, tapering to the end with white tip and are arranged in 4 cycles. Tentacles are of two types, one with green tip and the other with a pink. One is encircled by white. Inner row of tentacles is long and held upward and outer ones are shorter and extended horizontally. White flecks can be seen at the base of tentacles. There are two morphotypes of *A. elegantissima* in the collection; one with greenish column (Fig. 4) and other having brown colour with red verrucae. Length of the column is about 13.77 mm and has longitudinal rows of adhesive verrucae. Acrorhagi and adhesive verrucae are present at the distal end of the column, which are fully covered with particles surrounding the environment. Ten verrucae are arranged longitudinally and some are aggregate at the edge of the column. The diameter of verrucae is about 1.61 mm and is devoid of nematocyst.

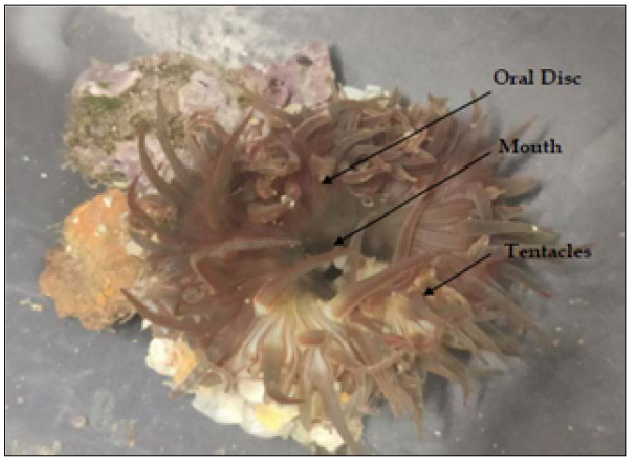

Fig. 3. Oral view of *A. elegantissima*

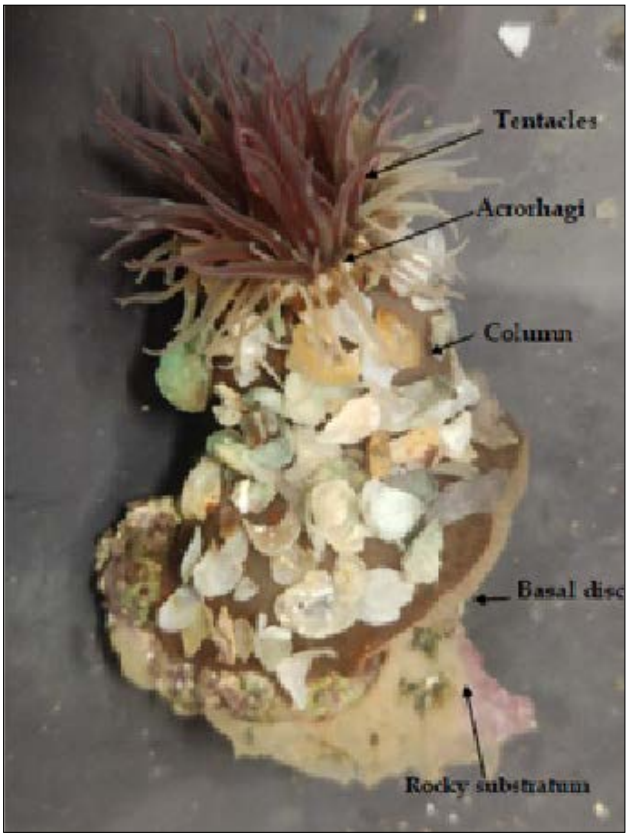

Fig. 4. Column view of *A. elegantissima* displaying acrorhagi

Mesentrial filaments with microbasic p-mastigophore present. Retractor muscle perfect. Sphincter more or less circumscribed. Basal disc is transparent with light orange or pale yellowish in colour. Well developed basal disc, which is broader than oral disc. Mesenterial insertions can be seen on the base of the basal disc. Diameter of basal disc is about 9.27 mm. No acontia present. Basitrich, spirocyst and microbasic p-mastigophore (Fig. 5) were present as cnidae in these anemones. When water level decreased during low tide, it retrieves its tentacles and form as a ball, which is to retain their water content.

Molecular analysis

COI sequences of *A. elegantissima* were devoid of insertions, deletions and stop codons. Genetic parameters for generated COI sequences are detailed in Table 3. Neighbour Joining tree and genetic distance data (with 1000 bootstraps) based on Kimura 2 Parameter (Fig. 6) were developed using generated and acquired COI sequences.

Fig. 5. (a) Spirocyst in tentacle (b) Spirocyst in column (c) Basitrich in column and (d) Microbasic p-mastigophore in the mesentrial filaments of *A. elegantissima*

Table 2. Genetic distance data based on COI sequences

MG637430							
A. elegantissima _AE1*							
MG637433							
A. elegantissima _AE4*	0.004						
MG637431							
A. elegantissima _AE2*	0.000	0.004					
MG637432							
A. elegantissima _AE3*	0.000	0.004	0.000				
MG637435							
A. elegantissima _AE6*	0.002	0.006	0.002	0.002			
MG637434							
A. elegantissima _AE5*	0.000	0.004	0.000	0.000	0.002		
KM612198							
A. elegantissima	0.023	0.027	0.023	0.023	0.025	0.023	
KM259983							
*A. buddemeieri*_Outgroup	0.091	0.095	0.091	0.091	0.093	0.091	0.072

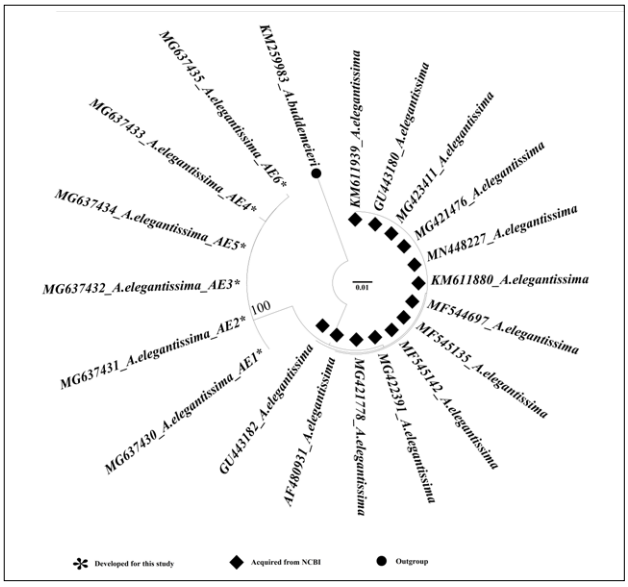

Fig. 6. Neighbour joining tree based on COI sequences with 1000 bootstrap replications

Table 3. Details of genetic parameters inferred for *A. elegantissima* COI sequences

Number of Sequences	6
Alignment length	528
Number of monomorphic sites	525
Number of polymorphic sites	3
Number of haplotypes	3
Haplotype diversity (Hd)	0.6000 +/- 0.2152
Nucleotide diversity (ϖ)	0.001894 +/- 0.001694
Mean number of pair wise difference	1.000000 +/-0.774597
Base frequency (%)	
A	23.26%
C	20.61%
G	21.78%
T	34.34%

Phylogenetic tree (NJ tree) confirmed the genetic congruency of COI sequences generated for this study with high bootstrap support (100%). COI sequences of *A. elegantissima* acquired from NCBI showed bootstrap support less than 70, which was unexpected. As expected, the selected outgroup *A. buddemeieri* represented the most diverse individuals within the tree (Fig. 6). In order to scrutinize the authority of the phylogenetic tree, distance matrix data (Table 2) was developed and analysed.

In genetic distance data, sequences of *A. elegantissima* showed intra-specific divergence between 0.20 to 2.7%. Intra-specific divergence within the developed sequences reached up to 0.60% only while the same for sequence acquired from NCBI reached up to 2.70%. The selected outgroup *A. buddemeieri* exhibited maximum genetic distance (inter-specific divergence) up to 9.5%, as expected.

Distribution

The occurrence of *A. elegantissima* was reported by Shah *et al.* (2017) from the mid-littoral zone of Sourashtra coast, Gujarat. For the present study, the samples were taken from the intertidal rocky habitat of Thirumullavaram, south west coast of India. This is the first report from this region.

Discussion

*A. elegantissima wa*s first reported in the Pacific coast of North America (Carlgren, 1952). Later, it was morphologically described (Brandt, 1835) as *A. elegantissima* and also found in Vancouver Island (Francis, 1979), British Island, Tatoosh Island, Washington, San Fransisco Bay, California (Hossfeld *et al.*, 2020), Costa Rica (Acuna *et al*., 2013), North America (Carlgren, 1952), Alaska and California (Acuna *et al.*, 2013). A massive settlement of *A. elegantissima* was recorded in Tatoosh Island, Washington (1972-1973) and in the mussel beds, Sanjuan Islands in 1974 and 1975 (Sebens, 1982).

The genus *Anthopleura* Duchassing de Fonbressin & Michelotti 1860, includes 46 species worldwide (Fautin, 2007). About 12 species were discovered under the genus *Anthopleura* from India. They were *Anthopleura panikkari* (Parulekar, 1968) from Mumbai, *A. nigrescens* (Mathew, 1979) from Kochi, *A. anneae* (Carlgren, 1940), *A. michaelseni* (Pax, 1920) long tentacled anemone, *A. nigrescens* (Verrill, 1928), dusky anemone *A. thallia* (Gosse, 1854), *A. waridi* (Carlgren, 1900), *A. anneae* (Carlgren, 1940) from Gulf of Mannar (Gopalakrishnan *et al.,* 2012), *A. anjunae* (den Hartog and Vennam, 1993) from Goa and *A. handi* in Andaman and Nicobar Islands (Raghunathan *et al*., 2014), *A. buddiemieri* in the western Indian Ocean (Bijukumar *et al*., 2015), *A. sola* and *A. dixoniana* (Shah *et al*., 2017) from Saurashtra, Gujarat. *A. elegantissima* was first discovered in

Saurashtra coast, Gujarat (Shah *et al*., 2017) and Kanyakumari (John, 2019), southeast coast of India. The present study is the first report on the occurrence of *A. elegantissima* along the coast of Kerala and is based on the molecular and morphological characteristics. *A. elegantissima* is confused with *A. buddiemieri* though *Anthopleura* is a controversial genus. A specialized character of *A. elegantissima* is the green oral disc with white and pink tips whereas *A. buddiemieri* has pale yellow column with red spots. *A. elegantissima* is also confused with other species such as *A. xanthogrammica* due to its greenish colour.

Accounting the limitations of morphology based conventional taxonomy, DNA-based diagnostic techniques using molecular markers have been utilized for resolving complications regarding species identification, systematics, phylogeny, population analysis and evolutionary history (Deepak and Harikrishnan, 2016, 2019; Lakra *et al*., 2013). Among the preferred molecular markers, mitochondrial markers are considered as a remedy for the above mentioned problems (Deepak and Harikrishnan, 2016). Among mitochondrial markers, COI is widely used in resolving the ambiguities in eukaryotes (Hebert *et al*., 2003; Deepak *et al*., 2015; Deepak and Harikrishnan, 2016, 2018, 2019; Sameera *et al.,* 2019; Balasubramanian *et al.,* 2016).

For this study, genotyping of *A. elegantissima* has been attempted for the first-time to develop mitochondrial marker COI and to estimate the molecular variance at intra-specific and inter-specific level. The nucleotide sequences developed for the present study constituted a single genetic entity with minimal diversity indices. Three polymorphic sites present within the generated sequences contributed three haplotypes within this species with moderate haplotype and lower nucleotide diversities. However, the intra-specific divergence among the acquired sequences was very high (up to 2.7%), but overall genetic distance contained under the threshold value of 3% as proposed by Hebert *et al.* (2003). Deepak *et al.* (2021) reported similar kind of results among *Carinotetraodon travancoricus* populations and hence there should be possibilities for population difference among generated and acquired sequences of *A. elegantissima* as they were inhabitants of different geographical areas.

Genetic diversity is influenced by many factors such as environmental factors, anthropogenic activities, habitation as well as low rate of mitochondrial evolution (Avise, 2004; Grant *et al.,* 2006). Genetic diversity at intra-population level could be well estimated based on nucleotide diversity with high haplotype diversity and low nucleotide diversity as indicators of population which had undergone rapid expansion (Nei and Li, 1979; Grant and Bowen, 1998). Results obtained for this study based on molecular marker sequences corroborates with this statement and suggest the possibilities for population expansion within *A. elegantissima.* It is also suggested to conduct studies over multiple populations of this species inhabiting diverse geographical realms using different molecular markers to study the mode of population expansion within this species.

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