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Biofouling and comparative phylogeographic status of the turtle barnacle *Chelonibia testudinaria* on various hosts of the Coromandel coast, India

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Biofouling by the coronuloid barnacle *Chelonibia patula* (synonimized as *C. testudinaria*) was observed during the collection and maintenance of live broodstock of a few species of commercially important crustaceans at Mandapam Regional Centre of ICAR-CMFRI located along the Coromandel coast of Tamil Nadu, India. We assessed the genetic identity of *C. patula* found on the various crustacean hosts (*Panulirus polyphagus, Portunus pelagicus* and *Charybdis natator*) and turtle, olive ridley (*Lepidochelys olivacea*) from the Gulf of Mannar Biosphere Reserve. The phylogeographic studies of turtle barnacles from all potential hosts, carried out across world oceans about their species paradox, do not represent collection record from India and hosts like lobsters and olive ridleys. The mitochondrial Cytochrome *c* oxidase subunit 1 (COI) analysis of this epibiont from various hosts indicated that species from the south-east coast of India belong to the Indian Ocean/Western Pacific clade of *C. testudinaria*. The very low K2P genetic distance (0.008) between *C. patula* and *C. testudinaria*, on comparison with existing GenBank records, supports this finding. The phylogeographic structuring and evolutionary divergence between the clades of *C. testudinaria* with COI sequence data indicated that the Indian Ocean/Western Pacific and Eastern Pacific clades are the most divergent.

[Keywords: Epibiont; *Chelonibia patula;* Crustacean hosts; Indian coast; Mitochondrial COI]

Introduction

The different species of barnacles in the genus *Chelonibia* were often identified by host type and described according to their variation in morphology[1]. The obligate commensal crustacean *Chelonibia patula* (Ranzani 1818) historically known as a crab barnacle was believed to be a generalist on various crustacean hosts while the turtle barnacle *Chelonibia testudinaria* (Linnaeus 1758) is supposed to have narrow host specialisation on different species of sea turtles[1,2]. *C. patula* has been recorded from various hosts like crabs[3,4], lobsters[5] and sea snakes[6] from Indian waters and different oceanic basins of the world[1,6,7]. Various phylogeographic studies using molecular markers indicated that out of the four established species of turtle barnacles, three species, *viz.*, *C. testudinaria/C. patula/C. manati* represent host morphotypes whose host specificity is a case of phenotypic plasticity to combat for divergent hosts inhabiting heterogenous environments, and hence they should be synonymized as a single species under the more senior Linnaean epithet *testudinaria*[1,6]. Hence, in this study, the name *C. patula*/*C. testudinaria* is used interchangeably for the crab barnacle, which were morphologically identified as *C. patula* from various hosts. Among the number of phylogeographic studies carried out across world oceans about the species paradox of turtle barnacles from all potential hosts, genetic information from Indian coast is meager and collection record of the same from lobster hosts and olive ridleys has not yet been represented. The genetic data from this study will supplement the limited genetic information in the database on the turtle barnacle species from Northern Indian Ocean.

Materials and Methods

Sample collection

Live broodstock collection of various crustaceans for experimental seed production activities was carried out at the Mandapam Regional Centre of ICAR-Central Marine Fisheries Research Institute in 2015-2017. During this, crabs like *Portunus pelagicus, Charybdis natator* and *Charybdis feriatus* infested with barnacles were observed randomly in commercial trawl landings at

Mandapam, Gulf of Mannar, south-east coast of India. Another species infested was the mud spiny lobster, *Panulirus polyphagus*. The infested specimens were collected and brought to the laboratory in live condition without any external damage for recording their morphometric measurements and fouling organisms were documented and preserved. Barnacles were collected from the olive ridley (*Lepidochelys olivacea*) with the assistance of local fishermen from the Islands in the Gulf of Mannar which were immediately released back to sea after removing barnacles. All the samples collected from commercial landings were neither under protection by law nor needed permission for collection. Samples of live barnacles were collected from the carapace of all the host species. They were preserved in 90% ethanol and 10% buffered formalin for DNA isolation and barnacle morphology studies, respectively.

Barnacle morphology and identification

The animal as a whole and the dissected parts of the shell were observed under stereo dissecting microscope (NIKON-SMZ1500) and photographed (EvolutionVF-SNQ15949). Barnacles were dissected and the shell parameters including shell length and orifice length along the rostral-carinal axis, maximum shell height, length of all the margins in the scutum and tergum were measured to the nearest mm using the software Image pro-6 Expresser. Barnacles were morphologically identified using the keys of Nilsson-Cantell (1938)[4], Wagh and Bal (1974)[5] and Fernando (2006)[8].

DNA isolation, polymerase chain reaction (PCR) and sequencing

DNA was isolated from the visceral tissue of each barnacle using phenol-chloroform method[9]. Amplification of partial sequences of Cytochrome *c* oxidase subunit 1 (COI) gene was carried out using the primer set LCO1490/ HCO2198[10]. PCR reactions were carried out in BIORAD T100^{TM} thermal cycler (Biorad, USA). The reactions were performed in 25 µl containing 2.5 µl 10x assay buffer, 1.5 µl $MgCl_2$ (1.5 µM), 0.5 µl of 10 µM of each primer, 0.5 µl of 10 µM dNTPs, 1 U Taq DNA polymerase (Sigma Aldrich, USA) and 1µl of 50-100 ng template DNA. The PCR cycling profiles were as follows: An initial denaturation of 4 min at 94 °C, 30 cycles of denaturation for 30 s at 94 °C, 30 s of annealing at 42 °C, 45 seconds of extension at 72 °C, and a final extension of 7 min at 72 °C. The PCR products were checked on 1.5% agarose gels and directly sequenced unidirectionally.

Evolutionary analyses

The sequences were compared to the GenBank database using the NCBI BLAST server. The software MEGA version 7[11] was employed for calculating the sequence divergence values and phylogenetic tree reconstruction of the 560 bp long final dataset. The GTR+I[12] model was selected as the best-fit model in maximum likelihood analysis. Relative support for tree topology was obtained by bootstrapping using 1000 iterations of the data matrix.

Results

The carapace width (CW) of commercially important crab species infested by barnacles ranged from 58-162 mm (*P. pelagicus*), 94-102 mm (*C. natator*), 110-115 mm (*C. feriatus*) and carapace length (CL) of lobster was 153 mm. The basal diameter of crab barnacles which were loosely cemented with the hosts during the present study ranged between 3-23 mm with its shell height between 1.5-11 mm. The number of barnacles fouled on single crab *P. pelagicus*, varied from one to 49. Dorsal side was abundantly fouled by barnacles in four different locations, *viz.*, carapace, cheliped, walking legs and swimming legs (fifth pereopods) in crabs, but found only on the carapace in lobster. Barnacles were placed mostly on the central region of the carapace of the hosts (Fig. 1).

Barnacles were identified as *Chelonibia patula*, from various crustacean hosts (Fig. 1), based on the shell morphology. It was the only ecosymbiont barnacle species attached to the crabs *P. pelagicus* and *C. feriatus* and lobster *P. polyphagus* examined during this period. *C. natator* specimen was infested by *C. patula* along with oyster spat and bryozoans. *C. patula* loosely cemented to different locations of the hosts, left no impressions when removed. The shell of *C. patula* is smooth, conical or oval in shape, thin and fragile, white or yellowish white in color, radii broad and smooth with oblique summits and consists of six plates (Fig. 1F). The shape of tubiferous calcareous base and the shell height varied in accordance with the place of attachment on the host. In a patchy attachment, it has irregular base with flattened shell. In the case of barnacle attached on the cheliped and coxa of crab, the base was compressed, has irregular shape and elongated shell. Their shell

height was more or less double when compared to those attached on the carapace.

The external shell of *C. patula* is conical and with smooth surface and has wedges with smooth edges at the sutures (Figs. 1E and G). Small-sized individuals attached randomly on the shell surface and orifice openings were identified as dwarf complemental males (Figs. 1 F and 1G). The large adults have an average of 17.6 mm ± 1.3 (SD) basal diameter and hosted 2-5 dwarf males with an average maximum basal diameter of 2.9 mm ± 0.7 (SD).

C. testudinaria collected from olive ridley had the typical stellate pattern on the shell. It had a lower aspect and a thicker shell compared to *C. patula*. The wedges at the sutures have indentations or teeth (Fig.1H) and dwarf males were seen located randomly along the sutures.

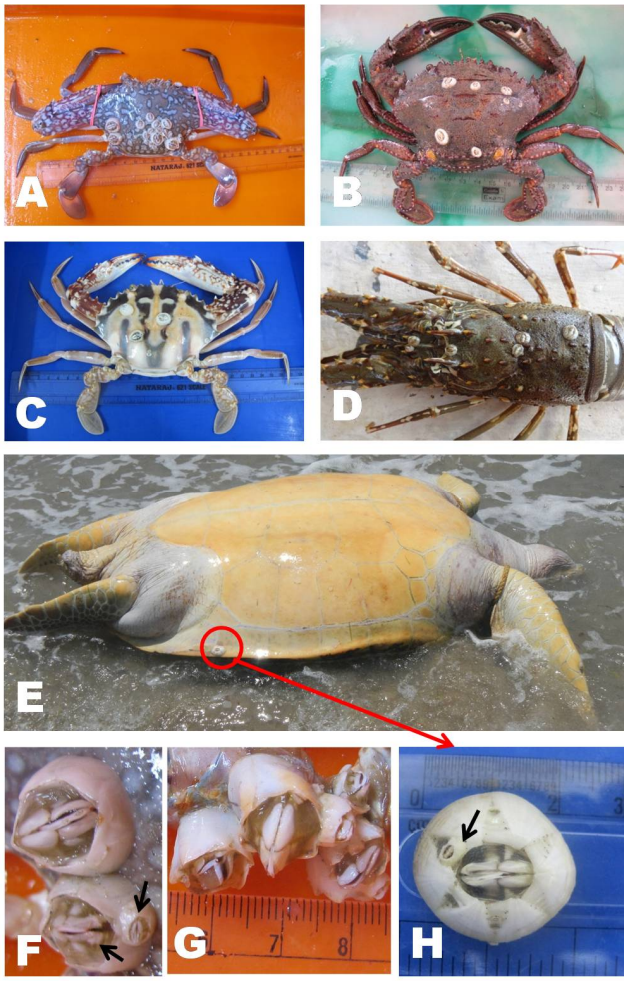

Fig. 1. — Biofuling of *Chelonibia testudinaria* on the crabs *Portunus pelagicus* (A), *Charybdis natator* (B) and *Charybdis feriatus* (C), lobster *Panulirus polyphagus* (D), sea turtle *Lepidochelys olivacea* (E), proximal view of *C. testudinaria* patchy attachment on the crab carapace (F), coxa of waking leg (G) and *C. testudinaria* collected from *Lepidochelys olivacea* (H). The small dwarf males (indicated by black arrows) settled randomly on shell surface and orifice opening.

Analyses of the mitochondrial COI gene fragments revealed that the barnacles fouled on all the crustaceans and turtle belonged to *C. patula/C. testudinaria* clade. The mitochondrial COI sequences from this study have been deposited under GenBank accessions KY273914 to KY273916. These were compared with representative sequences across world oceans which resulted in three distinct phylogeographic clades (Indian Ocean/W. Pacific, Atlantic/Mediterranean, Eastern Pacific). Populations of *C. testudinaria* in the Atlantic Ocean formed a sister clade to the western Pacific populations (Fig. 3). Barnacles from the present study, clustered together with *C. testudinaria*/*C.patula* from the Indian Ocean/Western Pacific upon COI analysis with a very low K2P genetic distance of 0.008 between *C. testudinaria* and *C. patula* taken together across oceans (Table 1). The K2P distances between these three clades ranged between 0.112 and 0.136 (Table 2).

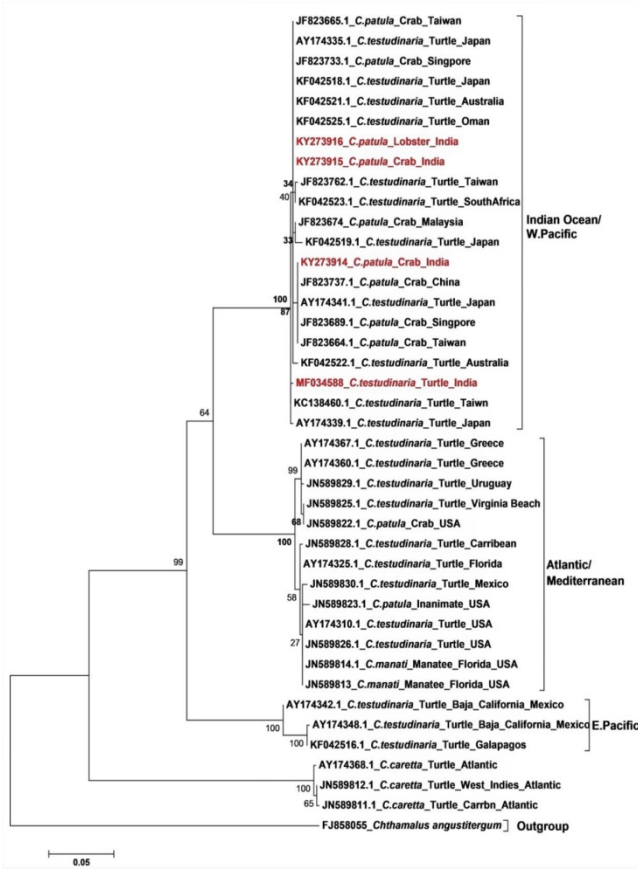

Fig. 2. — Molecular phylogenetic analysis by maximum likelihood method based on general time reversible model. Sequences are given along with species designation, host organism and geographic region.

Table 1 — Net evolutionary divergence between groups of sequences (Species-wise)

	1	2	3	4
C. patula	-----			
C. testudinaria	0.008	-----		
C. manati	0.082	0.037	-----	
C. caretta	0.224	0.199	0.236	-----

Table 2 — Net evolutionary divergence between groups of sequences (Clade-wise)

	1	2	3
Indian Ocean/W. Pacific	-----		
Atlantic/Mediterranean	0.112	-----	
E. Pacific	0.136	0.124	-----

Discussion

Decapod crustacean species like lobster and crab are important in the commercial fisheries and human food and its symbionts have received much attention. Lobster (*P. polyphagus*) and crabs were inhabited by barnacle *C. patula* having different densities and size frequencies. Average diameter of the barnacle, *C. patula* attached to the crabs ranged from 3 to 23 mm which is similar to the result reported by Afshin *et al*. (2012)[13] on *P. pelagicus* (0.7 to 2.1 cm). Though there is an early record of *C. patula* on *P. polyphagus* from India[5], this barnacle from lobster hosts has not been represented in any of the reported phylogenetic studies[1,7,14].

In the present investigation, it has been observed that the barnacles were most abundant in the central region on dorsal side of carapace of hosts than any other part examined. No fouling was observed in ventral side of the crustacean hosts. Tania (2010)[15] also reported that most of the barnacle larvae get settled on the dorsal side of the crabs, since it is exposed to more light and has a more attractive microbial layer. In case of ventral surface, attachment of barnacles was less because it might have experienced more abrasion and siltation when the crabs walk along the seafloor. An interesting observation in the entire study was *C. patula* attached on the swimming legs (fifth pereopods) particularly, the swimming paddles of *P. pelagicus* showed high rate of adhesion mechanism and strength. The barnacle is usually found attached in the middle of the carapace of crustacean hosts. Site selection and attachment by barnacles can be influenced by complex chemical and physical cues from the substratum[9,16,17], associated biofilms[18,19] and the nearby environment[20,21].

In this study, *C. patula* loosely cemented to four different locations of the crab hosts, leaving no impression when removed. The barnacles merely use their basibont for substratum and transport. Benefits to the barnacles include increased dispersal, access to consistent feeding currents, and perhaps most importantly escape from predators[22]. The barnacle is generally reported to have no adverse effect on the health of crustacean hosts[3,13,14], but their presence in high numbers or in unusual locations (e.g., attaching in swimming pedals and coxa of leg) certainly has adverse consequences for the host. In general, crabs infested by barnacle lose their market values because of aesthetic problem. The present data provides a base line to study the ectosymbiotism of barnacle and crab. It also directs the studies to be carried out in relation to the effect of barnacle fouling on health, growth, reproduction and ecological issues of commercially important crustaceans.

No consistent morphological differences could be determined between the specimens sampled from different hosts. But, the overall shape of the barnacles varied due to place of attachment on the host specially in crabs. It is widely acknowledged that the intertidal acorn barnacles experiencing variation in predation pressure and wave action develop different shell forms[23,24]. The observed morphological divergence may be driven by environmental response. ‘‘Common garden’’[25] or reciprocal transplant experiments[26] are needed to determine the actual environmental factors that shape the shell morphology of the turtle barnacles. Pilsbry (1916)[27] mentions phenotypic plasticity in *Chelonibia*, particularly in referring to *C. manati*-like forms removed from sea turtles, stating that these differences seem to correspond to host selection where *C. testudinaria* is “admirably adapted to the rough conditions of existence on the backs of sea turtles, the walls being enormously thickened and the stature low,” whereas the relatively fragile and lighter *C. patula* is specialized for living on motile marine-estuarine animals, particularly crabs. Moreover, Frick and Ross (2001)[28] reported that they do display morphological variation within species, and when shell morphologies are not entirely consistent or differ from established taxonomic characters, *Chelonibia* species are often identified by the host type.

Further, Zardus et al. (2014)[1] state that morphological variability is correlated with the host type and there is likely great functional significance

among the different forms of *C. testudinaria* related to host association. Host life styles (where hosts live and feed), their movements influencing water flow to the barnacles and the variation in efforts hosts make in removing barnacles, may be important factors influencing the variety of forms exhibited by this species. But in the present observation, differences in the *C. patula* tubiferous calcareous base shape and shell height noticed in the same host with different place of attachment, may be associated with their modes of attachment. In general, barnacles employ adhesion mechanism to attach on a surface; the shell wall of *C. patula* is thin and fragile which help in forming a broad base with a large surface (e.g., carapace) and irregular base with a small surface (e.g., cheliped and coxa region of walking and swimming leg), appropriate for attaching by adhesion. The high adaptation in morphology possibly improves fitness of the barnacle species towards optimal in heterogeneous environments and enables it to be a successful epibiotic biofouler[14]. Further extended investigations into larval ecology and developmental genetics of *C. patula* are needed to fully determine the forces governing morphological variation within this species.

Most of the barnacle species are hermaphroditic, especially the intertidal species which live in dense population. In some species where the mating group size is small and patchy, these species often have dwarf males attached on the large hermaphrodites or females to facilitate mating success[14]. Complemental males have previously been reported for *C. patula*[29] and *C. testudinaria*[30] as in the present observation.

Mitochondrial markers are useful tools to delineate barnacle species due to their fast evolving nature and are commonly applied in barnacle phylogenetic and population genetic studies[31,32,33]. Based on the molecular results, we concluded that the barnacles sampled from lobster and the three portunid crab hosts represent the same species, *viz*., *Chelonibia patula*. The very low genetic distance (K2P- 0.008) of *C. patula* and *C. testudinaria* from different hosts on comparison with GenBank records indicated that both species are the same. On phylogeographic analysis, barnacles from this study belonged to the Indian Ocean/Western Pacific clade of *C. patula*/*C.testudinaria* which formed a sister clade to *C. testudinaria* in the Atlantic Ocean. The eastern Pacific population was found to be the most divergent, congruent with previous findings[1]. The morphological plasticity of the species on various crustacean hosts and turtle observed in the present study along with genetic data and phylogeographic analysis support the finding that host-specificity of the species assemblage is a case of phenotypic plasticity[1]. The first genetic data of turtle barnacle from the Indian coast will supplement the phylogeographic information of the species across oceanic basins.

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