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Investigating larval variability: Morphological and genetic analysis of Stage-I phyllosoma in five species of spiny lobsters (Palinuridae) from the southeast coast of India

N. S. Jeena, M. Anbarasu, Summaya Rahuman, Tinto Thomas, Wilson Sebastian and A. K. Abdul Nazar

[1]ICAR-Central Marine Fisheries Research Institute, Kochi- 682 018, Kerala, India.

*Correspondence e-mail: jeenacmfri@gmail.com
ORCiD: https://orcid.org/0000-0002-8142-0474

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Original Article

Abstract

Species identification of the planktonic phyllosoma larvae of spiny lobsters is a matter of conjecture due to their virtually uniform appearance, shared distribution ranges, and diminutive size. This study compares the morphological characteristics of the Stage-1 phyllosoma of five species of spiny lobsters, namely *Panulirus homarus, P. versicolor, P. ornatus, P. polyphagus,* and *P. longipes longipes*, which were allowed to spawn under captivity at CMFRI-Mandapam Regional Centre, Tamil Nadu, India. Additionally, the effectiveness of a novel primer pair that explicitly targets the hypervariable D-loop or control region (CR) of mitochondrial DNA, resulting in improved resolution of phyllosoma identification, is described. Closely related morphological characters, barring a few, were observed amongst the larvae. The primer pair was tested on larvae and adults of all species, sequence variation in their CR was examined, and evolutionary relationships were compared with the 16S rRNA (16S) and Cytochrome c oxidase subunit 1 (COI) sequences from previous studies. The lowest K2P genetic distance value based on the partial sequences of CR was found between *P. homarus* and *P. ornatus* (29.41%), while the highest was between *P. longipes longipes* and *P. polyphagus* (54.46 %). The results demonstrate the potential utility of the control region as an additional molecular marker that can be considered alongside protein-coding genes to provide more consistent phylogenetic signals. This region is also favoured for studying population structure and phylogeography in Palinuridae, and the new primers are expected to complement these studies.

Keywords: *Larval identification, Phyllosoma, molecular marker, mitochondrial, control region, spiny lobsters*

Introduction

Lobsters distributed along the coastline of India, with major landings from the northwest, southwest, and southeast coasts, constitute an esteemed fishery resource under extreme fishing pressure (Radhakrishnan *et al.,* 2019). Along the East coast of India, maximum fishing activity of rock lobsters was observed during the monsoon and post-monsoon periods, and large-size lobsters are usually noticed during the post-monsoon period spanning from November to January (Raju and Babu, 2015). *Panulirus homarus* (Linnaeus, 1758)*, P. versicolor* (Latreille, 1804)*, P. ornatus* (Fabricius, 1798)*, P. polyphagus* (Herbst, 1793), and two relatively rare species *P. longipes longipes* (A. Milne Edwards, 1868) and *P. penicillatus* (Olivier, 1791) are some spiny lobster species of the genus *Panulirus* White, 1847 that are reported to contribute to the fishery along the eastern coast (Nair *et al.,* 1973; Raju and Babu, 2015).

Spiny lobsters belong to the infra-order Achelata (Scholtz and Richter, 1995), with members characterized by a particular planktonic larval stage called phyllosoma. These larvae are known to be remarkable in having thin, flat, and transparent bodies and a long planktonic life, passing through different stages before metamorphosing into the subsequent phases ('Puerulus' and 'Nisto'). Plankton samples from the southeast coast of India revealed the presence of Phyllosoma decades in advance (Alikunhi, 1948; Prasad and Tampi, 1957; Tampi, 1973). Due to the close resemblance between the early stages, it is difficult to accurately identify phyllosoma on a species-specific basis or to determine the exact developmental stage (Chow *et al.,* 2006a). Furthermore, the peak breeding seasons

of different species overlap along the east coast, which can result in mixed species assemblages of larvae (Tampi, 1973).

In spiny lobsters, it is difficult to recognize morphological or morphometric elements that allow differentiation of the larvae of different species. Hence, rearing becomes a prerequisite for accurately identifying their larvae and post-larvae (Lucas and Ito, 1990). Species and developmental stages can be most reliably co-ordinated with phyllosoma hatched from berried females (Wakabayashi *et al.,* 2017). The history of successful attempts to correlate phyllosoma positively with respective species through captive breeding was initially attempted by Prasad and Tampi (1957, 1959) in *Panulirus burgeri* De Haan, 1841 (= *Panulirus homarus*), followed by *P. ornatus*. Later captive breeding attempts were carried out on *P. versicolor* (Deshmukh, 1968) and *P. polyphagus* (Kagwade and Kabli, 1991). Vijayakumaran *et al.* (2014) also tried some of the species listed above. These descriptions do not include the early phyllosoma of *P. longipes longipes* and *P. penicillatus*, which occur in India.

Molecular techniques such as PCR-RFLP of some mitochondrial genes were successfully attempted earlier to identify Phyllosoma of Indo-Pacific spiny lobsters of the genus *Panulirus* (Chow, 2006a; Shirai *et al.,* 2006). Nucleotide sequence analysis of COI (Chow *et al.,* 2006b), as well as a combination of mitochondrial COI, 16S rRNA, and nuclear gene regions (Tourinho *et al.*, 2012), were also reported to be useful in lobsters. Although COI is considered a powerful marker with versatile applications in Achelata (Singh *et al.,* 2017; Naro-Maciel *et al.,* 2011), there are studies indicating that DNA barcoding (COI) alone may sometimes fail in species discrimination (Bingpeng *et al.,* 2018). The likelihood of pseudogenes is also higher in decapods (Venera-Pontón *et al.,* 2020). Some research suggests that phyllosoma have a lower COI gene amplification success rate than adults (Chow *et al.*, 2006a). Therefore, an alternative marker would also benefit species identification of phyllosoma.

The mitochondrial Control Region (mtDNA-CR) or D-loop, a highly polymorphic and rapidly evolving DNA fragment, is an effective barcoding target in a variety of taxa (*eg.*, Sindičić *et al.*, 2011; McKenzie and Bremer, 2017). It has also been used successfully in population structure analysis (Dao *et al.,* 2015), and phylogeography (Diniz *et al.,* 2005) in lobsters. Bronstein *et al.* (2018) suggested that a non-coding region such as CR can infer the phylogeny of closely related species because it has a higher substitution rate and mitigates bias due to selection. A primer set that can amplify this region across different *Panulirus* spp., would be an effective tool for molecular tagging of phyllosoma that can be used with other mitochondrial genes for enhanced phylogenetic signals.

We aimed to develop a novel primer pair that can be used to amplify the mt-DNA CR across spiny lobster spp. at all life stages, so their larvae can be positively correlated with exact species. We conducted captive spawning of the female berried lobsters of five species of spiny lobsters collected from the southeast coast of India to compare their phyllosoma morphology. Also, the efficacy of CR data generated here was contrasted in phylogenetic analysis with the widely used COI and 16S rRNA markers.

Material and methods

Captive spawning

Ovigerous females of five spiny lobster species *viz. P. homarus*, *P. versicolor*, *P. ornatus, P. polyphagus,* and *P. longipes longipes* with fertilized eggs were collected from live lobster holding centres of the Gulf of Mannar (9°17'N Lat.; 79°8' E Lon.), between October 2015 and February 2016. Since good-quality brooders were not available for *P. penicillatus*, mature mating pairs of the species were kept in captivity for natural fertilization and breeding (Fig. 1). The carapace length (mm) and weight (g) of the ovigerous females used in this study were recorded using a vernier calliper and an electronic weight balance, respectively. They were maintained in separate 2.5 tonnes (T) fibre-reinforced plastic (FRP) tanks with 2 T of filtered seawater at the marine hatchery complex of the ICAR-Central Marine Fisheries Research Institute (CMFRI) Mandapam Regional Centre, Tamil Nadu. Species identification was

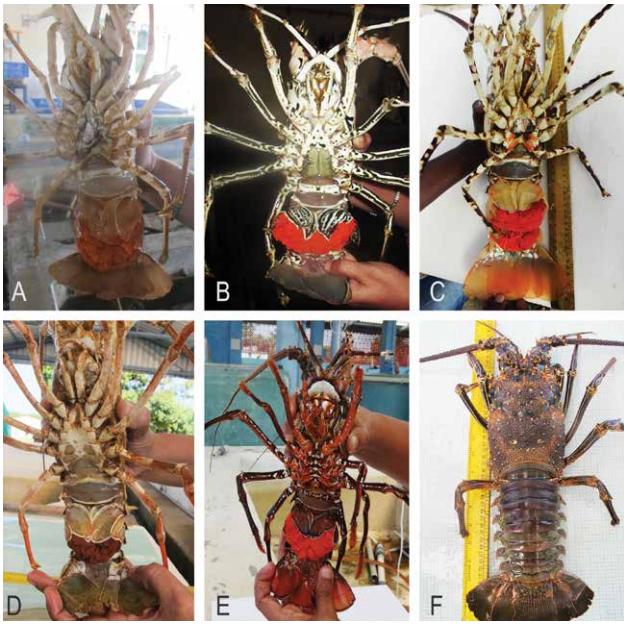

Fig. 1. Berried females of A) *Panulirus homarus*, B) *P. versicolor*, C) *P. ornatus*, D) *P. polyphagus* E) *P. longipes longipe*s used in this study. 'F' represents the mature female of *P. penicillatus*

done using the keys of the FAO Lobster Identification Sheet (Holthuis, 1991). The water was moderately aerated and the lobsters were fed with *Donax* sp. and squid meat once a day. A 100% water exchange was carried out daily in the morning hours. Water quality and hatching were monitored regularly. The larvae hatched after 1–2 weeks of incubation at a water temperature of 23.5–26.0 °C and a salinity of 33.5–35.0 ppt. After hatching, the female was removed from the breeding tank, and the antennal tip was preserved in ethanol for molecular studies. The healthy, newly hatched phyllosoma larvae were collected and preserved in 95% ethanol and 5% buffered formalin for molecular and morphometric analysis.

Larval morphometry, morphology and statistical analyses

Various body dimensions of Stage-1 phyllosoma larvae of five spiny lobster species were measured as described by Vijayakumaran *et al.* (2014), Radhakrishnan and Vijayakumaran (1995), Kagwade and Kabli (1991), and Deshmukh (1968). This was done using a Leica stereo zoom microscope (model S8APO) equipped with a Leica camera (model DFC280) and the Leica Application Suite V3 (LAS V3.8). Descriptive statistical analyses were performed using MS Excel to calculate the average, minimum, maximum, and standard deviation for the recorded morphometry data of stage I phyllosoma across five spiny lobster species. The measurements (Total length, Lengths and widths of carapace, abdomen, and thorax) and morphological features were compared with the literature cited above.

PCR amplification, sequencing and analysis

DNA extraction from the antennal tip of adult specimens and larvae was performed using the phenol-chloroform method (Sambrook and Russell, 2001). PCR primers were designed using Primer3 (Untergasser *et al.,* 2012) to amplify the mt-DNA CR (800 bp) based on the published reference genomes of the genus from NCBI GenBank (accession numbers: NC_016015, NC_014854, NC_028627, NC_014339, NC_004251, NC_028024). The forward primer LOB-CR-F was rooted in the small subunit ribosomal RNA gene (SSU rRNA- 12S), and the reverse primer LOB-CR-R was rooted in the Glutamyl-tRNA gene (tRNA-Gln). The PCR was carried out using the newly developed LOB-CR-F (5′-AGRWWATAGCAAGAATCAAACTATA-3′) and LOB-CR-R (5′-TGGTGTRAGTCCATTAYTYGT-′3) primer pair in the BIORAD S1000™ thermal cycler (Biorad, USA). The reactions were performed in 25 µl containing 2.5 µl 10x assay buffer, 1.5 µl MgCl2 (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 1 µl of 50-100 ng template DNA. The PCR cycle profiles were as follows. There was an initial denaturation of 4 min at 94 °C, 35 cycles of denaturation for 30 sec at 94 °C, 30 sec of annealing at 56 °C, 90 sec 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 bi-directionally. Forward and reverse sequences were aligned and edited in BIOEDIT (Hall, 1999). The CR sequences were compared to the nucleotide database using the NCBI BLAST server with default parameters. Query sequences were assigned as the species associated with sequences having more than 80% coverage and 86% similarity. For CR sequences, a lower similarity value was set as it is a highly variable domain that can be utilized even to detect stock-specific differences in lobsters (Abdullah *et al*., 2014). The phylogenetic analysis was carried out using MEGA X (Kumar *et al*., 2018). The evolutionary divergence between sequences was calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980), and the evolutionary history was inferred using the Maximum Likelihood (ML) method in the above software. For comparison with the outgroup *Homarus gammarus*, a final data set of 683 bp, including all gaps, was considered. Partial COI and 16S sequences of all species (including *P. penicillatus*) attempted in this study were also downloaded from GenBank and analyzed. For phylogenetic reconstruction, we selected the nucleotide substitution models HKY+G (Hasegawa *et al.,* 1985), TN92+G+I (Tamura, 1992), and T92+G for CR, COI, and 16S respectively. The genetic distances and the tree topologies were compared for the CR (505 bp), COI (642 bp), and 16S (451 bp) after the complete deletion of missing data.

Results and discussion

Larval morphometry and morphology

The carapace length and weight of ovigerous females of the five species used in this study were:

i) *P. homarus* – 65 mm, 300 g ii) *P. versicolour* – 130 mm, 550 g iii) *P. ornatus* – 150 mm, 2.2 kg iv) *P. polyphagus*- 97 mm, 400 g, and v). *P. longipes longipes* – 97 mm, 350 g respectively. All of them spawned successfully. Hatching occurred late at night or early in the morning for all species. The larvae were collected and observed under the microscope (NIKON-SMZ1500), and photographed (Fig. 2). Fully developed first phyllosoma larvae were released in all hatching events, and no naupliosoma or pre-phyllosoma stage larvae were observed. The schematic illustration of stage-I phyllosoma is given in Fig. 3 and important morphometric measurements are listed in Table 1. Successful fertilization could not be achieved with the *P. penicillatus* mating pair.

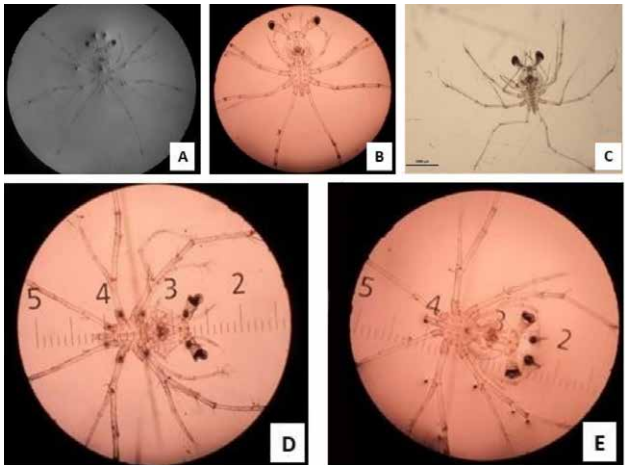

Fig. 2. Images of stage-I phyllosoma of five *Panulirus* spp. A) *Panulirus homarus* on the third day after hatching, B) *P. longipes longipes* on day 1, C) *P. ornatus* on day 2, D) *P. versicolor* on day 4, E) *P. polyphagus* on day 2. In D and E, the eyepiece scale is positioned over the organism

The present study attempts to compare the larval comparison of five species that have not yet been fully described in the region. The following characteristics were similar to the first-stage phyllosoma of all five species examined in this study. They are a) unsegmented eye complex, b) absence of an eyestalk c) uniramous and unsegmented antennule, with three long sensory setae d) uniramous and unsegmented antenna that is slightly shorter than the antennule e) Biramous anterior part of the first maxilla; two serrated terminal long setae and strong spines on the coxal and basal endite f) second maxilla with two segments, the larger basal and the smaller distal segments with four long plumose setae g) uniramous second maxilliped with five segments and without exopod h) biramous third maxilliped with an exopod consisting of three pairs of natatory setae i) the first and second pereiopods with five pairs of natatory setae on exopods, while the third pereiopod with small bud-shaped exopods without natatory setae j) pleon longer than coxa of the third pereiopod, with a postero-lateral spine and two to three short basal setae on each side, and k) absence of pleopod, uropod, and telson. Some morphological characteristics, such as the presence of the first maxilliped bud, the sub-exopodal spine in the 1st to 3rd pereiopods, the ventral coxal spine in the third maxilliped, and the 1st to 3rd pereiopods varied between the first phyllosoma of lobster species (Fig. 3 A-F). The key identification characteristics were compared with the published literature and presented in Table 2.

Closely related features could be observed in the phyllosoma larvae of different species. Phyllosoma are quite conservative in morphology and have not yet been characterized for all extant taxa (Govender *et al.*, 2019). Most known larvae of related lobster species showed many similarities, especially in the early stages, making their identification difficult. This

Table 1. Morphometric measurements (mm) of stage-I phyllosoma of the spiny lobsters in the present study

Species	Statistical measure	Total Length	Carapace Length	Carapace Width	Abdomen Length	Abdomen Width	Thorax Length	Thorax Width
P. homarus	Average	1.424	0.734	0.677	0.227	0.092	0.482	0.454
	*s.d	0.04	0.03	0.03	0.02	0.01	0.03	0.03
	Maximum	1.484	0.772	0.738	0.277	0.109	0.554	0.528
	Minimum	1.326	0.668	0.596	0.197	0.080	0.436	0.386
P. versicolour	Average	1.521	0.803	0.753	0.259	0.098	0.527	0.489
	*s.d	0.02	0.03	0.04	0.01	0.00	0.04	0.03
	Maximum	1.558	0.851	0.817	0.281	0.105	0.595	0.521
	Minimum	1.469	0.743	0.717	0.233	0.093	0.438	0.443
P. ornatus	Average	1.496	0.763	0.725	0.256	0.099	0.476	0.465
	*s.d	0.08	0.06	0.03	0.02	0.01	0.05	0.05
	Maximum	1.586	0.833	0.777	0.293	0.111	0.590	0.542
	Minimum	1.360	0.659	0.677	0.223	0.092	0.415	0.385
P. polyphagus	Average	1.468	0.771	0.692	0.235	0.093	0.526	0.466
	*s.d	0.04	0.03	0.02	0.01	0.01	0.05	0.03
	Maximum	1.524	0.824	0.736	0.251	0.105	0.581	0.510
	Minimum	1.362	0.705	0.652	0.216	0.081	0.427	0.425
P. longipes longipes	Average	1.566	0.867	0.720	0.241	0.095	0.576	0.509
	*s.d	0.09	0.03	0.03	0.02	0.00	0.04	0.01
	Maximum	1.656	0.910	0.766	0.267	0.097	0.633	0.517
	Minimum	1.377	0.815	0.663	0.212	0.091	0.537	0.491

*s.d, standard deviation.

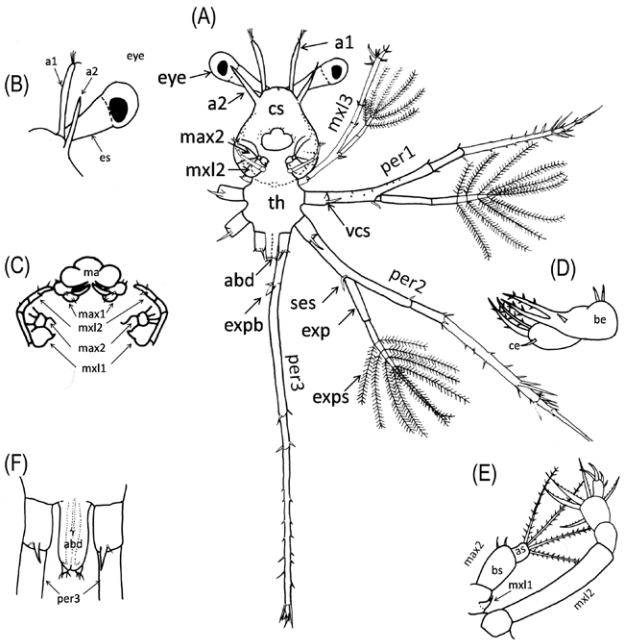

Fig. 3. A schematic illustration of stage-I *Palinurid phyllosoma* A) ventral view: a1 (antennule); a2 (antenna); eye, cs (cephalic shield); max2 (2nd maxilla); mxl2-3 (maxilliped2-3); th (thorax); Per1-3 (Pereiopod1-3); vcs (ventral coxal spine); abd (abdomen) ses (sub exopodal spine); exp (exopod); expb (exopod bud); exps (exopod setae); B) Ocular peduncle ventral view: eye; es (eyestalk); a1 (antennule); a2 (antenna); C) mouth parts: ma (mandible); max1-2 (maxilla1-2); mxl1-2 (maxilliped1-2); (D) coxal endite (ce) and basal endite (be) of left 1st maxilla, ventral view; E) 2nd maxilla (max2), bs (basal segment); as (apical segment); 1st maxilliped (mxl1) and 2nd maxilliped (mxl2), ventral view; F) 3rd pereiopod (per3) and abdomen (abd) ventral view

fact was pointed out earlier by Prasad and Tampi (1959), Berry (1974), and Prasad *et al.* (1975). A pear-shaped cephalic shield and large antennae are conserved for phyllosoma I in spiny lobsters. This trait is reported to have significance in the diversification of Achelata (Landeira *et al.,* 2023). It is reported that wild phyllosoma of *P. inflatus* and *P. penicillatus* can be determined only from larval stage IV (Muñoz-García *et al.*, 2014). In contrast to previous observations, a ventral coxal spine was present in the third maxilla of *P. polyphagus* (Table 2). A larval description of this species from the east coast of India is not available to compare current results and hence requires future studies. It is worth noting that there are morphological differences between mid to late-stage phyllosoma larvae of genetically isolated populations of *P. penicillatus* (Matsuda *et al.*, 2019), and this possibility needs to be investigated in *P. polyphagus* with further sampling from locations on the east coast. Although Deshmukh (1968) reported a short-lived (only a few hours) identical pre-phyllosoma in three of his experimental species, this stage could not be observed in *P. polyphagus* by Kagwade and Kabli (1991). The latter suggested that pre-phyllosoma could probably be a few larvae hitched before time into the artificial condition of aquaria. Our study was conducted in larger tanks that simulate more natural conditions, and the pre-phyllosoma stage could not be observed in any species.

Molecular analysis

The CR primer worked in all species at both larval and adult stages. The sequences from this study were submitted to GenBank under accession nos. MH817840–MH817845. The data obtained from the analysis of homology using BLAST revealed their species identity. CR sequences could be amplified only from the adult specimens of *P. penicillatus* which was included in the comparative analysis. The evolutionary divergence values obtained for the mitochondrial CR, COI, and 16S genes for all species are shown in Tables 3 and 4. The divergence values (in %) based on the partial sequences ranged from 29.41 (*P. homarus-P. ornatus*) to 54.46 (*P. longipes longipes- P. polyphagus*) for CR, 14.66 (*P. homarus -P. ornatus*) to 27.3 (*P. penicillatus-P. polyphagus*) for COI and 5.18 (*P. homarus- P. ornatus*)

Table 2. Comparison of morphological features of stage-I phyllosoma of five spiny lobster species in the present and previous studies. *Indicates differences in comparison

	Spiny lobster species				
Morphological features	*P. homarus*	*P. versicolour*	*P. ornatus*	*P. polyphagus*	*P. longipes longipes*
First maxilliped bud	Absent[1,2,5,6]	Present[1,2,5]	Absent[3,4], Present[1,2,5]	Present[1,2,7,8]	Present[1,2,9]
Ventral coxal spine in third maxilliped	Present[1,2,5,6]	Present[1,2,5]	Present[1,2,3,4,5,10]	Absent[2,7,8], *Present[1]	Present[1,2,9]
Ventral coxal spine in 1st to 3rdpereiopod	Present[1,2,5,6]	Present[1,2,5]	Present[1,2,3,4,5,10]	Present[1,2,7,8]	Present[1,2,9]
Sub-exopodal spine in first pereiopod	Present[1,2,5,6]	Present[1,2,5]	Absent[1,2,10], Present[3,4,5]	Absent[2], Present[1,7,8]	Absent[1,2,9]
Sub-exopodal spine in second pereiopod	Present[1,2,5,6]	Present[1,2,5]	Present[1,2,3,4,5,10]	Absent[2], Present[1,7,8]	Absent[1,2,9]
Sub-exopodal spine in third pereiopod	Present[1,2,5,6]	Present[1,2,5]	Present[1,2,3,4,5,10]	Absent[2], Present[1,7,8]	Absent[1,2,9]

[1]Present study; [2] Prasad *et al.,* 1975; [3] Duggan and Mc Kinnon, 2003; [4] Smith *et.al.*, 2009; [5] Vijayakumaran *et al.,* 2014; [6] Radhakrishnan and Vijayakumaran, 1995; [7] Deshmukh, 1968; [8] Kagwade and Kabli, 1991; [9] Matsuda and Yamakawa, 2000; [10] Prasad and Tampi, 1957

Table 3. Evolutionary divergence (K2P distance in %) over sequence pairs between groups for mtDNA- CR.

No.	Species	1	2	3	4	5	6
1	*Panulirus homarus*	———-					
2	*P. ornatus*	29.41	———-				
3	*P. polyphagus*	34.26	35.84	———-			
4	*P. versicolor*	39.60	38.71	37.52	———-		
5	*P. longipes longipes*	50.69	52.67	54.46	53.91	———-	
6	*P. penicillatus*	46.34	48.32	50.50	48.22	49.80	——-

Table 4. Evolutionary divergence (K2P distance in %) over sequence pairs between groups for mtDNA-COI (below the diagonal) and 16S rRNA (above the diagonal). The GenBank accession numbers are indicated next to the species in Fig. 4 B, C

No.	Species	1	2	3	4	5	6
1	*Panulirus homarus*	———-	5.18	8.63	8.16	19.25	21.83
2	*P. ornatus*	14.66	———-	8.34	7.15	20.24	21.84
3	*P. polyphagus*	17.27	16.88	———-	10.7	19.29	21.66
4	*P. versicolor*	17.25	18.04	17.06	———-	20.01	19.81
5	*P. longipes longipes*	21.96	26.08	24.59	25.95	———-	13.52
6	*P. penicillatus*	25.45	26.52	27.3	25.01	20.43	———-

to 21.84 (*P. penicillatus- P. ornatus*) for 16S regions. The phylogenetic trees reconstructed using the sequences are shown in Fig. 4 (A, B, and C). The cladograms showed a similar topology for all genes.

Molecular methods have opened new vistas for studying the biology of planktonic achelate larvae by facilitating their precise and rapid detection (Palero *et al.,* 2016, Konishi *et al.,* 2019, Ueda *et al.,* 2021, Konishi *et al.*, 2022, Genis-Armero *et al.*, 2023). The primer set developed in this study can be considered a primer of choice for the genetic identification of phyllosoma in panulirid lobsters, similar to studies on crab larvae from plankton samples (Ströher *et al.,* 2011). Previously, Diniz *et al.* (2005) developed primers to amplify CR in *Panulirus argus* and other closely related species. This primer proved to be useful for genetic studies in *P. argus* from the Atlantic and *P.homarus* from the Indo-West Pacific (Farhadi *et al.,* 2013; Lavery *et al.,* 2014). Species-specific primers for CR have been developed and used in population genetic studies of different spiny lobsters (Gopal *et al.,* 2006; García- Rodríguez and Perez-Enriquez, 2008; Abdullah *et al.,* 2014; Dao *et al.,* 2015). The control region primers developed in this study, which amplify a more extended region across all species, are expected to have applications in this regard. Analysis of evolutionary divergence showed that the genetic distance was much larger with CR compared to the protein-coding COI fragment. This is probably because CR is the longest non-coding and most polymorphic region in the mitochondrial genome (Bronstein *et al.,* 2018). The region is subject to

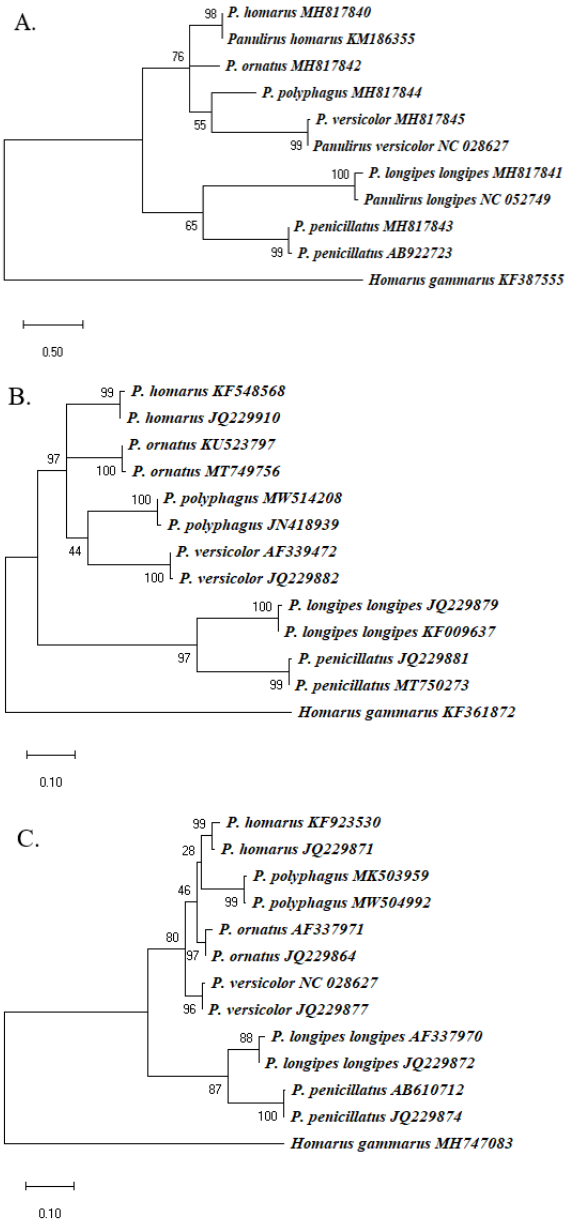

Fig. 4. Comparisons of Maximum likelihood trees based on partial nucleotide sequences of mitochondrial A) Control Region B) COI gene and C) 16S rRNA. Bootstrap values are listed at the nodes

fewer functional and structural constraints (Saccone *et al.,* 1987). This study appeared to have phylogenetic suitability similar to COI and 16S, which were used in previous studies (Ptacek *et al.,* 2001; Jeena *et al.,* 2015). The suitability of CR sequences for phylogenetic inference has been previously demonstrated in echinoids (Bronstein *et al.,* 2018) and fishes (Turanov *et al.,* 2019). Although some restrictive factors have been pointed out of CR in phylogenetic studies, positive features like high topological resolution and lack of substitution saturation make it a powerful marker (Bronstein *et al.,* 2018). The effectiveness of CR was compared with COI in *Nephrops*

norvegicus from the Atlantic and Mediterranean regions, revealing significant genetic differentiation between the regions (Gallagher *et al.,* 2018).

A successful combination of molecular and morphological methods can solve the mystery of cryptic species in plankton (Mcmanus and Katz, 2009). To effectively correlate phyllosoma with species and with molecular markers, captive breeding of all available species was required in the present study. The morphological information, along with the specific sequences generated by the new primer developed in this study, will find application in the identification of planktonic phyllosoma of the genus *Panulirus*. However, the intraspecific variation limit of CR needs to be standardized for each species due to its high degree of polymorphism. The above primer, which amplifies across species can have the potential use to generate longer sequences useful to analyze the genetic structure in spiny lobsters for their sustainable management.

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Author contributions

Conceptualization: NSJ; Methodology: NSJ, MA, WS; Data Collection and Analysis: NSJ, MA, SR, TT; Writing original draft: NSJ, MA; Supervision: AKAN

Data availability

The data supporting this study are publicly available at the NCBI repository under accession numbers MH817840–MH817845

Conflict of interest

The authors declare that they have no conflict of financial or non-financial interests that could have influenced the outcome or interpretation of the results.

Ethical statement

No ethical approval is required as the study does not include activities that require ethical approval or involve protected organisms/ human subjects/ collection of sensitive samples/ protected environments.

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References

Abdullah, M. F., S. Chow, M. Sakai, J. H. Cheng and H. Imai. 2014. Genetic diversity and population structure of pronghorn spiny lobster *Panulirus penicillatus* in the Pacific region. *Pac. Sci.,* 68 (2): 197-211.

Alikunhi, K. H. 1948. Note on the metamorphosis of phyllosoma larvae from Madras plankton. *Proc. 35th Indian Sci. Congr.*, Pt. III. Abstracts: 193 pp.

Berry, P. F. 1974. Palinurid and scyllarid lobster larvae of the Natal coast, South Africa. Oceanographic Research Institute, *Investigational report*, 34: 1-20.

Bingpeng, X., L. Heshan, Z. Zhilan, W. Chunguang, W. Yanguo and W. Jianjun. 2018. DNA barcoding for identification of fish species in the Taiwan Strait. *PLoS ONE,* 13 (6): e0198109.

Bronstein, O., A. Kroh and E. Haring. 2018. Mind the gap! The mitochondrial control region and its power as a phylogenetic marker in echinoids. *BMC Evol. Biol.*, 18 (1): 80.

Chow, S., N. Suzuki, N. Imai and T. Yoshimura. 2006a. Molecular species identification of spiny lobster phyllosoma larvae of the genus *Panulirus* from the northwestern Pacific. *Mar. Biotechnol.*, 8: 260-267.

Chow, S., H. Yamada and N. Suzuki. 2006b. Identification of mid to final stage lobster phyllosoma larvae of the genus *Panulirus* White, 1847 collected in the Ryukyu Archipelago. *Crustaceana.*, 79: 745-764.

Dao, H. T., C. Smith-Keune, E. Wolanski, C. M. Jones and D. R. Jerry. 2015. Oceanographic currents and local ecological knowledge indicate, and genetics does not refute, a contemporary pattern of larval dispersal for the ornate spiny lobster, *Panulirus ornatus* in the south-east Asian archipelago. *PLoS ONE,* 10 (5): e0124568.

Deshmukh, S. 1968. On the first phyllosomae of the Bombay spiny lobsters *(Panulirus)* with a note on the unidentified first *Panulirus* phyllosomae from India (Palinuridae). *Crustaceana Supplement*, II: 47-58.

Diniz, F. M., N. Maclean, M. Ogawa, I. H. Cintra and P. Bentzen. 2005. The hypervariable domain of the mitochondrial control region in Atlantic spiny lobsters and its potential as a marker for investigating phylogeographic structuring. *Mar. Biotechnol.*, 7 (5): 462-473.

Duggan, S. and A. D. Mckinnon. 2003. The early larval developmental stages of the spiny lobster *Panulirus ornatus* (Fabricius, 1798) cultured under laboratory conditions. *Crustaceana*, 76: 313-332.

Farhadi, A., H. Farhamand, M. A. Nematollahi, A. Jeffs and S. D. Lavery. 2013. Mitochondrial DNA population structure of the scalloped lobster *Panulirus homarus* (Linnaeus 1758) from the West Indian Ocean. *ICES J. Mar. Sci.*, 70 (7): 1491-1498.

Gallagher, J., J. A. Finarelli, J. P. Jonasson and J. Carlsson. 2018. Mitochondrial D-loop DNA analyses of Norway Lobster (*Nephrops norvegicus*) reveals genetic isolation between Atlantic and Mediterranean populations. *BioRxiv*: 258392.

García-Rodríguez, F. J. and R. Perez-Enriquez. 2008. Lack of genetic differentiation of blue spiny lobster *Panulirus inflatus* along the Pacific coast of Mexico inferred from mtDNA sequences. *Mar. Ecol. Prog. Ser.,* 361: 203-212.

Genis-Armero, R., J. Groeneveld, S. Singh, P. F. Clark, L. Corbari and F. Palero. 2023. Identification of phyllosoma larvae of the lobsters *Acantharctus* and *Biarctus* (Crustacea: Scyllaridae) from the SW Indian Ocean. *Zool. Stud.,* 62 pp.

Gopal, K., K. A. Tolley, J. C. Groeneveld and C. A. Matthee. 2006. Mitochondrial DNA variation in spiny lobster *Palinurus delagoae* suggests genetically structured populations in the southwestern Indian Ocean. *Mar. Ecol. Prog. Ser.,* 319: 191-198.

Govender, A., J. Groeneveld, S. Singh and S. Willows-Munro. 2019. The design and testing of mini-barcode markers in marine lobsters. *PLoS One*, 14 (1): e0210492.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41 (4): 95-98.

Hasegawa, M., H. Kishino and T. Yano. 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. *J. Mol. Evol.,* 22: 160-174.

Holthuis, L. B.1991. FAO species catalogue, vol. 13: Marine Lobsters of the World. An Annotated and Illustrated Catalogue of Species of Interest to Fisheries Known to Date. *FAO Fisheries Synopsis*, 125: 1-292.

Jeena, N. S., A. Gopalakrishnan, E. V. Radhakrishnan, J. K. Kizhakudan, V. S. Basheer, P. K. Asokan and J. K. Jena. 2015. Molecular phylogeny of commercially important lobster species from Indian coast inferred from mitochondrial and nuclear DNA sequences. *Mitochondrial DNA*, 27 (4):1-10.

Kagwade, P. V. and L. M. Kabli. 1991. Embryonic development of larvae on the pleopods of the spiny lobster *Panulirus polyphagus* (Herbst) and the sand lobster *Thenus orientalis* (Lund) from Bombay waters. *Indian J. Fish.*, 38 (2): 73-82.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.,* 16 (2): 111-120.

Konishi, K., T. Yanagimoto and S. Chow. 2019. Mid- to late stage phyllosoma larvae of *Panulirus brunneiflagellum* Sekiguchi and George, 2005 collected south of the Ogasawara Islands, Japan. Aquatic Animals, AA2019:1-7.

Kumar, S., G. Stecher and K. Tamura. 2018. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.*, 33: 1870-1874.

Landeira, J. M., D. Deville, E. Fatira, Z. Zhang, S. Thatje, Q. Lin, H. L. Santiago and K. Wakabayashi. 2023. Diversification of cephalic shield shape and antenna in phyllosoma I of slipper and spiny lobsters (Decapoda: Achelata). *Front. Mar. Sci.,* 10: 1070296.

Lavery, S. D., A. Farhadi, H. Farahmand, T. Y. Chan, A. Azhdehakoshpour and V. Thakur. 2014. Evolutionary Divergence of Geographic Subspecies within the Scalloped Spiny Lobster *Panulirus homarus* (Linnaeus 1758). *PLoS ONE,* 9 (6): e97247.

Lucas, J. S. and M. Ito. 1990. The complete larval development of the Scyllarid lobster, *Scyllarus demani* Holthuis, 1946 (Decapoda, Scyllaridae), in the laboratory. *Crustaceana.*, 58 (2): 144-167.

Matsuda, H. and T. Yamakawa. 2000. The complete development and morphological

changes of larval *Panulirus longipes* (Decapoda, Palinuridae) under laboratory conditions. *Fish. Sci.,* 66: 278-293.

Matsuda, H., M. Sakai, T. Yanagimoto and S. Chow. 2019. Morphological differences between phyllosoma larvae from the western–central and eastern Pacific populations of the pronghorn spiny lobster *Panulirus penicillatus*. *bioRxiv*, 667469.

Mckenzie, J. L. and J. R. Alvarado Bremer. 2017. Genetic identification of istiophorid larvae from the Gulf of Mexico based on the analysis of mitochondrial DNA control region sequences. *J. Fish. Biol.*, 90 (3): 1070-1079.

Muñoz-García, I., F. J. García-Rodríguez, R. González-Armas, R. Perez-Enriquez and M. Ayón-Parente. 2014. Taxonomy of the phyllosoma of *Panulirus inflatus* (Bouvier, 1895) and *P. gracilis* Streets, 1871, based on morphometry and molecular analysis. *Nauplius*, 22: 41-51.

Mcmanus, G. B. and L. A. Katz. 2009. Molecular and morphological methods for identifying plankton: what makes a successful marriage? *J. Plankton. Res.*, 31 (10): 1119-1129.

Nair, R. V., R. Soundararajan and K. Dorairaj. 1973. On the occurrence of *Panulirus longipes*, *Panulirus penicillatus* and *Panulirus polyphagus* in the Gulf of Mannar with notes on lobster fishery around Mandapam. *Indian J. Fish.*, 20: 333-350.

Naro-Maciel, E., B. Reid, K. E. Holmes, D. R. Brumbaugh, M. Martin and R. DeSalle. 2011. Mitochondrial DNA sequence variation in spiny lobsters: Population expansion, panmixia, and divergence. *Mar. Biol.,* ***158*** (9): 2027-2041.

Palero, F., R. Genis, M. Hall and C. Paul. 2016. DNA barcoding the phyllosoma of *Scyllarides squammosus* (H. Milne Edwards, 1837) (Decapoda: Achelata: Scyllaridae). *Front. Mar. Sci.,* 3. 10.3389/conf.FMARS.2016.05.00131.

Prasad, R. R. and P. R. S. Tampi. 1957. On the phyllosoma of Mandapam. *Proc. Natl. Acad. Sci. India,* 23B: 48–67.

Prasad, R. R and P. R. S. Tampi. 1959. A note on the first phyllosoma of *Panulirus burgeri* (de Haan). *Proc. Indian Acad. Sci.*, 49B: 397-401.

Prasad, R. R., P. R. S. Tampi and M. J. George. 1975. Phyllosoma larvae from the Indian Ocean collected by the Dana expedition 1928-1930. *J. Mar. Biol. Assoc. India.*, 17: 56-107.

Ptacek, M. B., S. K. Sarver, M. J. Childress and W. F. Hernkind. 2001. Molecular phylogeny of the spiny lobster genus *Panulirus* (Decapoda: Palinuridae). *Mar Freshwater Res.*,52 (8): 1037-1047.

Radhakrishnan, E. V. and M. Vijayakumaran. 1995. Early larval development of the spiny lobster *Panulirus homarus* (Linnaeus, 1758) reared in the laboratory. *Crustaceana*, 68 (2): 151-159.

Radhakrishnan, E. V., J. K. Kizhakudan, A. Saleela, A. P. Dineshbabu and S. Lakshmi Pillai. 2019. Lobster fisheries and management in India and Indian Ocean Rim countries. In: E. V. Radhakrishnan, B.F. Phillips, G. Achamveetil (Eds.). Lobsters: Biology, Fisheries and Aquaculture. *Springer.*, p. 219-350.

Raju, M. R. and D. E. Babu. 2015. Rock Lobster Fishery from East Coast of India. *Asian J. Exp. Sci.*, 29: 1-6.

Saccone, C., M. Attimonelli and E. Sbisa. 1987. Structural elements highly preserved during the evolution of the D-loop-containing region in vertebrate mitochondrial DNA. *J. Mol. Evol.,* 26: 205-211.

Sambrook, J. and D. W. Russell. 2001. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Scholtz, G. and S. Richter. 1995. Phylogenetic systematics of the reptantian Decapoda (Crustacea, Malacostraca). *Zool. Journ. Linn. Soc.*, 113: 289-328.

Shirai, S. M., T. Yoshimura, K. Konishi and T. Kobayashi. 2006. Identification of phyllosoma larvae: a molecular approach for Japanese *Panulirus* lobsters (Crustacea: Decapoda: Palinuridae) using mitochondrial rDNA region. *Species Diversity.*, 11 (4): 307-325.

Sindičić, M., T. Gomerčić, A. Galov, H. Arbanasić, J. Kusak, A. Slavica and D. Huber. 2011. Mitochondrial DNA control region as a tool for species identification and distinction between wolves and dogs from Croatia. *Veterinarski arhiv,* 81 (2): 249.

Singh, S. P., J. C. Groeneveld, A. Al-Marzouqi and S. Willows-Munro. 2017. A molecular phylogeny of the spiny lobster *Panulirus homarus* highlights a separately evolving lineage from the Southwest Indian Ocean. *Peer. J.*, 5: e3356.

Smith, G., M. Salmon, M. Kenway and M. Hall. 2009. Description of the larval morphology of captive reared *Panulirus ornatus* spiny lobsters, benchmarked against wild-caught specimens. *Aquaculture*, 295: 76-88.

Ströher, P. R., C. R. Firkowski, A. S. Freire and M. R. Pie. 2011. A molecular method for the detection of sally light foot crab larvae (*Grapsus grapsus*, Brachyura, Grapsidae) in plankton samples. *Genet. Mol. Biol.,* 34 (3): 529-531.

Tamura, K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Mol. Biol. Evol.,* 9 (4): 678–87.

Turanov, S. V., Y. H. Lee and Y. P. Kartavtsev. 2019. Structure, evolution and phylogenetic informativeness of eelpouts (Cottoidei: Zoarcales) mitochondrial control region sequences. *Mitochondrial DNA Part A.*, 30 (2): 264-272.

Thampi, P. R. S. 1973. The *phyllosoma* larvae of the *Indian*Ocean. *J. Mar. Biol. Assoc. India.,* 15 (2): 560-565.

Tourinho J. L., A. M. Solé-Cava and C. Lazoski. 2012. Cryptic species within the commercially most important lobster in the tropical Atlantic, the spiny lobster *Panulirus argus*, *Mar. Biol.*, 159: 1897-1906.

Ueda, K., T. Yanagimoto, S. Chow, M. Kuroki and T. Yamakawa. 2021. Molecular identification of mid to final stage slipper lobster phyllosoma larvae of the genus *Chelarctus* (Crustacea: Decapoda: Scyllaridae) collected in the Pacific with descriptions of their larval morphology. *Zool. Stud.,* 60 pp.

Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B.C. Faircloth, M. Remm and S. G. Rozen. 2012. Primer 3- new capabilities and interfaces. *Nucleic. Acids. Res.*, 40 (15): e115.

Vijayakumaran. M., A. Maharajan, S. Rajalakshmi, P. Jayagopal and M.C. Remani. 2014. Early larval stages of the spiny lobsters, *Panulirus homarus, Panulirus versicolor* and *Panulirus ornatus* cultured under laboratory conditions. *Int. J. Dev. Res.*, 4: 377-383.

Venera-Pontón, D. E., A. C. Driskell, S. De Grave, D. L. Felder, J. A. Scioli and R. Collin. 2020. Documenting decapod biodiversity in the Caribbean from DNA barcodes generated during field training in taxonomy. *Biodivers. Data J.,* 8: e47333.

Wakabayashi, K., C. H. Yang, J. Y. Shy, C. H. He and T. Y. Chan. 2017. Correct identification and redescription of the larval stages and early juveniles of the slipper lobster *Eduarctus martensii* (Pfeffer, 1881) (Decapoda: Scyllaridae). *J. Crustacean. Biol.*, 37: 204–219.