http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 12 (3): 281-285, 2009
ISSN 1028-8880
© 2009 Asian Network for Scientific Information

Identification of *Panulirus homarus* Puerulus Larvae by Restriction Fragment Length Polymorphism of Mitochondrial Cytochrome Oxidase I Gene

[1]G. Dharani, [2]G.A. Maitrayee, [1]S. Karthikayalu, [1]T.S. Kumar, [1]M. Anbarasu and [1]M. Vijayakumaran
[1]Ocean Science and Technology for Islands, National Institute of Ocean Technology,
Pallikarani, Chennai-600 100, India
[2]School of Biotechnology, Vellore Institute of Technology, Vellore-632 014, India

Abstract: Molecular identification of puerulus larvae of *Panulirus homarus* of the genus *Panulirus* from Indian coast was studied by employing Polymerase Chain Reaction, Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of the mitochondrial DNA (mtDNA) Cytochrome Oxidase I (COI) gene by agarose gel electrophoresis and Denaturing Gradient Gel Electrophoresis (DGGE). The size of amplified fragment of COI gene was estimated to be approximately 1300 bp. Single fragment amplification was recorded during different stages of the life cycle. The RFLP digestion was carried out using five different restriction enzymes (BsplI, HhaI, RsaI, TaqI and AluI). The RFLP profile of the different endonucleases, varied between 1-5 restriction types. RFLP analysis using endonuclease TaqI enabled identification of *P. homarus* during different stages of its life history.

Key words: Spiny Lobster, Puerulus, PCR-RFLP, mtDNA COI gene

INTRODUCTION

Life cycle of spiny lobsters is complex, involving oceanic larval phase that extends over several months. Spiny lobsters brood their eggs in pleopods or swimmerets and release planktonic phyllosoma larvae (1-2 mm long) that are carried offshore by prevailing waves, wind and water currents. After a series of molts, increasing in size each time, the phyllosoma ultimately metamorphoses into puerulus, the lobster post larva. While the early phyllosoma larvae are carried away offshore, the late stages are brought back near shore by counter currents in the sea. Nearing the continental shelf, the final phyllosoma instar metamorphoses into the puerulus and starts its directional journey to its ultimate benthic habitat. Puerulus, which is glassy transparent, is a non feeding instar capable of swimming a long distance and moults into a post puerulus stage once it settles in its habitat (Booth and Phillips, 1994).

Due to the heavy market demand and high price for spiny lobsters in South East Asia, many countries such as Australia, New Zealand and India are exploring the possibility of growing wild collected spiny lobster puerulii in sea cages. Interestingly, Vietnam has taken big leap in spiny lobster farming by collection of puerulii and early juveniles with a production of over 1500 metric tones in about 30000 sea cages (Tuan and Mao, 2004). In India, the National Institute of Ocean Technology (NIOT), Chennai introduced farming and fattening of spiny lobsters in sea cages in the Southeast coast of Tamil Nadu. Large number of puerulii are being collected from these cages as well as from seaweed (*Kappaphycus alwarazi*) culture rafts in the nearby areas and used for stocking the cages (ijayakumaran *et al*., 2007). While life histories of many sub tropical spiny lobsters have been completed, the production of puerulii for aquaculture is still a distant dream due to low survival and extended larval period. The advantage of using wild caught juveniles for aquaculture are, low costs for obtaining animals for stocking as compared with hatchery production, availability of individuals fit for growout in sea cages, no risks of genetic pollution from deliberate or accidental release, reduced likelihood of transferring diseases and border range of economic benefits, including opportunities for coastal dwellers in developing countries to sell stock to larger enterprises (Hair *et al*., 2003). In addition, responsible capture and culture of wild juveniles can improve overall fisheries productivity for target species by circumventing the high rates of natural mortality associated with settlement of post larvae (Bell, 2004).

Though the wild collected puerulii from sea cages in India are predominantly of single species (*Panulirus homarus*), they are often mixed with other species like *Panulirus ornatus* and *Panulirus polyphagus*. Culture of

Corresponding Author: Gopal Dharani, Ocean Science and Technology for Islands, National Institute of Ocean Technology, Pallikarani, Chennai-600 100, India

mixed species of puerulus together is not advisable due to the variation in growth rate and productivity (NIOT, unpublished report). Hence, identification of puerulii is important in the context of aquaculture. The transparent puerulii larvae are delicate in nature and are difficult to identify before they complete initial moults. Identification of phyllosoma larvae of tropical Palinurids from plankton collections is still more complicated as no distinct differences could be observed in hatchery reared phyllosoma larvae of four species (Radhakrishnan and Vijayakumaran, 1995). Molecular identification of lobster species using Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) or nucleotide sequencing analysis in temperate species has been well documented (Silberman *et al*., 1994; Sarver *et al*., 1998; Sarver *et al*., 2000; Ptacek *et al*., 2001; Stamatis *et al*., 2004; Cannas *et al*., 2006; Rodriguez and Enriquez, 2006; Chow *et al*., 2006a, b), however such analysis for tropical spiny lobsters, particularly in Indian waters are lacking.

The present study is aimed to identify the puerulus larva of *Panulirus homarus* by employing polymerase chain reaction-restriction fragment length polymorphism of the Cytochrome Oxidase I (COI) gene.

MATERIALS AND METHODS

Adult *Panulirus homarus* (10 Nos.), berried *P. homarus* (2 Nos.) and puerulii (15 Nos.) were collected from the lobster culture cages in Tharuvaikulam, (8° 53' N: 78° 10' E), Tamil Nadu. Samples of eggs, abdominal muscle of puerulus and adult was collected carefully and preserved in 95% ethanol. The berried lobsters were transported and maintained at the seafront laboratory of NIOT at Neelankarai, Chennai and phyllosoma larvae, were collected and preserved upon hatching.

Genomic DNA was extracted from the egg, whole phyllosoma larvae, abdominal muscle of puerulii and adults with DNA extraction kit (Qiagen Pvt. Ltd., Germany). A fragment of mitochondrial Cytochrome Oxidase I (COI) gene, was amplified by Polymerase Chain Reaction (PCR) using gene specific primers. Four sets of primers described by Chow *et al*. (2006a), were modified, by inserting wobbles bases, into single primer: COI65F-5´-GGAGCTTGAGCT GGAATAGT- 3 (20 bp, 6254.12 mol. wt., 61.42 Tm), COI1342R-5´-GTGTAD GCRTCTGG RTA RTC- 3´ (20 bp, 6182.44 mol. wt., 60.06 Tm). The PCR amplification for all specimens was carried out in a 50 µL reaction mixture (PCR master mix 25 µL, forward primer 1 µL, reveres primer 1 µL, template 2 µL, nuclease free water 21 µL) as recommended by the manufacturer (Qiagen Pvt. Ltd., Germany). The reaction mixture was preheated at 94°C for 5 min followed by 35 cycle of amplification (at 94°C for 20 sec, 50-55°C for 30 sec and 72°C for 30 sec) with a final extension at 72°C for 5 min.

The amplified COI gene segment was eletrophoresied in agarose gel (0.8%) was eluted using gel cleanup kit (Perfectprep® Eppendorf) and the recovered DNA was used for restriction digestion. Restriction was performed with Alu 1, Taq 1, Bsp1I, RsaI and HhaI as 20 µL reaction mixture (nuclease free water 7 µL, buffer 2 µL, amplified DNA 10 µL, enzyme 1µL) in a thermal cycler (Eppendorf Germany) under conditions suggested by the manufacturer (Fermentas Life sciences, Canada) and eletrophoresied on agarose gel (0.8%) and Denaturing Gradient Gel Electrophoresis (DGGE). The DGGE was run with 8% stacking gel and resolving gel using urea and formamide gradient in resolving gel. The RFLP profiles of the individuals were documented and analyzed in gel documentation system.

RESULTS

The puerulii of *P. homarus* is transparent (Fig. 1), the total body length varying from 18.0 to 23.0 mm with a mean of 20.83±1.77 mm. The carapace length ranged from 5.8 to 9.4 mm with a mean of 7.78±1.16 mm, carapace width ranged from 3.2 to 5.4 mm with a mean of 4.49±0.57 mm, the total body weight ranged from 0.1 to 0.29 g with a mean of 0.20±0.06 g. The antenna were 2.1 times longer than the total body length (Fig. 1), the number of feeding, walking and swimming appendages were similar to that of the adult.

In *P. homarus*, a portion of mitochondrial gene COI could be amplified without much standardization, using the modified primer, however the amplifications were feeble, hence gradient PCR (50-60°C) was used and an optimum amplification was obtained in 52.5°C. Amplification of egg DNA was feeble and this problem was overcome using a blocking agent (bovine serum

Fig. 1: Photograph of *Panulirus homarus* puerulus

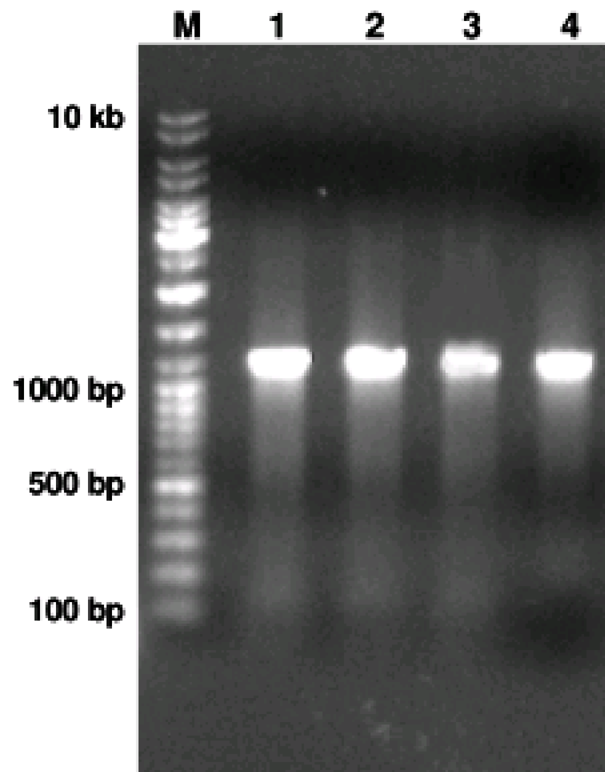

Fig. 2: Agarose gel electrophoresis, M-100 bp-10 kb DNA ladder, 1-4 PCR products of COI of adult *P. homarus*, puerulus, phyllosoma and egg

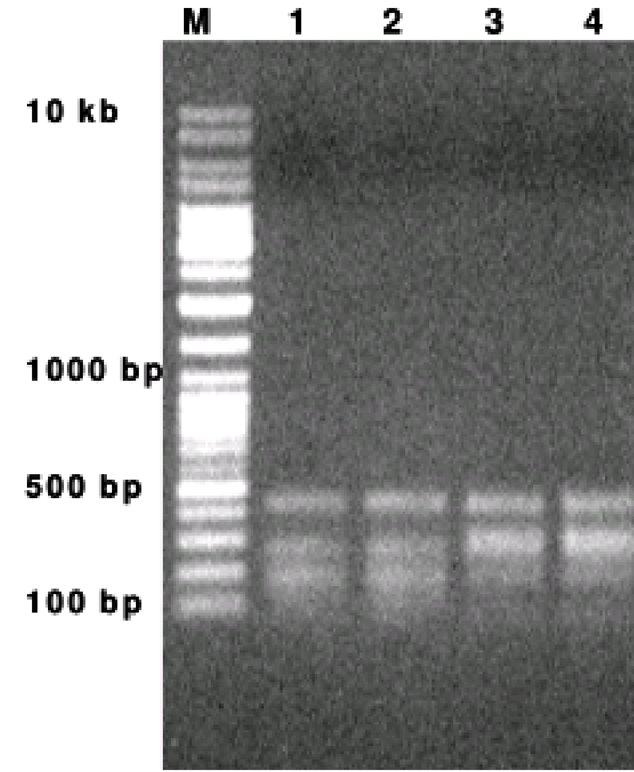

Fig. 4: Agarose gel electrophoresis, M-100 bp-10 kb DNA ladder, 1-4 Taq I RFLP of COI of adult *P. homarus*, puerulus, phyllosoma and egg

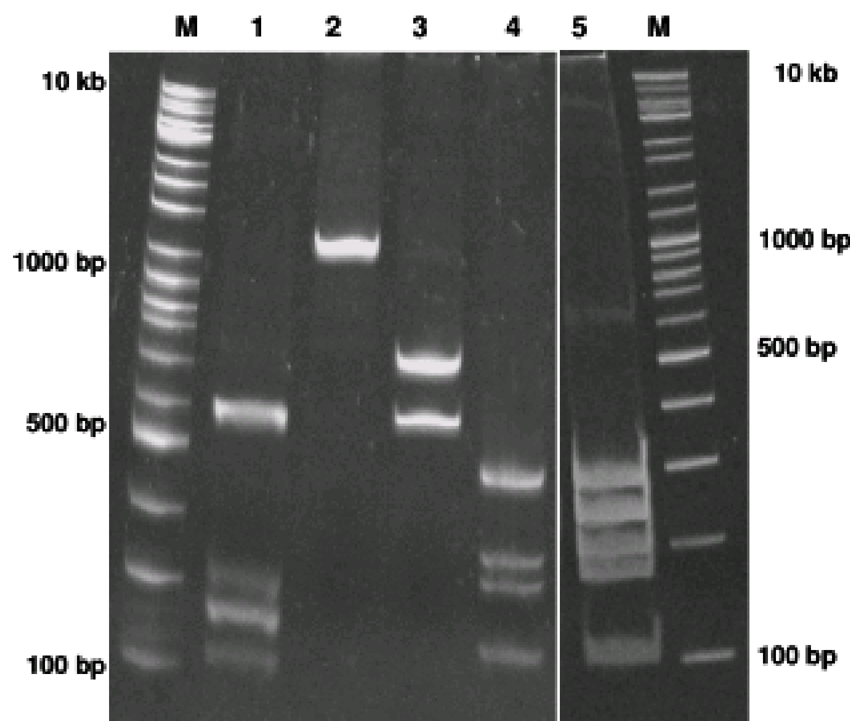

Fig. 3: DGGE of COI, M-100 bp-10 kb DNA ladder, Lanes 1- 5 PCR-RFLP of COI of adult *P. homarus* digested with BspLI, HhaI, RsaI, TaqI and AluI

albumin, 0.1 mg mL^{-1}) during PCR. The size of amplified fragment of COI gene was estimated to be approximately 1300 bp (Fig. 2). Out of the five restriction endonucleases used (AluI, TaqI, BspLI, RsaI and HhaI), four were found to have recognition site. TaqI, BspLI and AluI appear to have maximum number of restriction site in amplified COI fragment. The resolution of restricted products on the agarose gel was poor and good resolution was obtained in DGGE. The restriction endonucleases TaqI and BspLI, had three restriction site and yielded a fragment size of approximately, 150, 300, 360, 490 and 120, 260, 320, 600 bp, respectively. AluI had four restriction sites and yielded five fragments of approximately 100, 180, 200, 250 and 300 bp. RsaI has single restriction site and yielded two fragments of approximate 600 and 700 bp and Hha1 has no restriction site (Fig. 3). TaqI restriction profile of the egg, phyllosoma and peurulus larvae were similar to that of adult *P. homarus* (Fig. 4).

DISCUSSION

Puerulus settlement was recorded in the east coast of India throughout the year with a peak settlement during the month of March to July (Vijayakumaran *et al*., 2007). It is reported that the recruitment of puerulus larvae sometimes exceed the carrying capacity of the reef and approximately 20% for the recruit only survive and contribute to fisheries (Booth and Phillips, 1994). In lack of distinct morphological identification character for *P. homarus puerulus,* mitochondrial gene Cytochrome c Oxidase subunit 1 was taken for the species identification, because, COI has been used extensively in determining relationships among arthropods and establishing the phylogeny of the species, shrimp (Maggioni *et al*., 2001; Lavery *et al*., 2004), crab (arrison, 2004) and spiny lobster (Ptacek *et al*., 2001; Chow *et al*., 2006a, b). Exhibit enough sequence diversity to enable discrimination at the species level and broad-range primers are available for amplification of COI from diverse invertebrate and vertebrate phyla (Folmer *et al*., 1994).

The important outcome of the present investigation is the fact that TaqI restriction profile of the egg, phyllosoma and peurulus larvae were similar to that of adult *P. homarus*. No polymorphisms were recorded within the individuals of adult *P. homarus* studied and

distinct restriction patterns were recorded in the all life stages of *P. homarus*. Mitochondrial DNA sequence is considered to be the most powerful approach to resolve taxonomic uncertainties. Particularly, RFLP based methods have become conventional practice for identifying fish and crustacean species at all stages of the life cycle (Chow *et al*., 2006a, 1993). The results of the present study clearly indicate that restriction enzyme TaqI, can be used to identify and distinguish *P. homarus* puerulii and phyllosoma larvae from those of other lobster species. Further investigation on samples from wide geographical areas and sequence alignment will provide insight on the genetic structure of Indian spiny lobster *P. homarus.*

ACKNOWLEDGMENTS

The researchers are thankful to the Director NIOT for providing necessary facilities. We also thank our group head for his support and encouragement.

REFERENCES

Bell, J.D., 2004. Key issues for sustaining aquaculture production of the spiny lobster *Panulirus ornatus*, in Vietnam. Proceedings of the Workshop Spiny Lobster Ecology and Exploitation in the South Shina Sea Region, July 2004, Australian Centre for International Agricultural Research, Canberra, pp: 59-62.

Booth, J.D. and B.F. Phillips, 1994. Early life history of spiny lobster. Crustaceana, 66: 271-294.

Cannas, R., A. Cau, A.M. Deiana, S. Salvadori and J. Tagliavini, 2006. Discrimination between the Mediterranean Spiny lobster *Panulirus elephas* and *Panulirus mauritanicus* (Crustacea: Decapoda) by mitochondrial sequence analysis. Hydrobiologia, 557: 1-4.

Chow, S., M.E. Clarke and P.J. Walsh, 1993. PCR-RFLP analysis on thirteen Western Atlantic snappers (Subfamily Lutjaninae): A simple method for species and stock identification. Fish Bull., 91: 619-627.

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

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

Folmer, O., M. Black, W. Hoeh, R. Lutz and R. Vriejenhoek, 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol., 3: 294-299.

Hair, C., J. Bell and P. Doherty, 2003. The use of Wild Caught Juveniles in Coastal Aquaculture and its Application to Coral Reef Fishes. In: Responsible Marine Aquaculture, Stickney, R.R. and J.P. McVed (Eds.) CAB International, New York, ISBN 0851996043, pp: 327-353.

Harrison, J.S., 2004. Evolution, biogeography and the utility of mitochondrial 16S and COI genes in phylogenetic analysis of the crab genus Austinixa (Decapoda: Pinnotheridae). Mol. Phylogenet. Evol., 30: 743-754.

Lavery, S., T.Y. Chan, Y.K. Tam and K.H. Chu, 2004. Phylogenetic relationships and evolutionary history of the shrimp genus *Penaeus s.I* derived from mitochondrial DNA. Mol. Phylogenet. Evol., 31: 39-49.

Maggioni, R., A.D. Rogers, N. Maclean and F. D'Incao, 2001. Molecular phylogeny of Western Atlantic Farfantepenaeus and Litopenaeus shrimp based on mitochondrial 16S partia l sequences. Mol. Phylogenet. Evol., 18: 66-73.

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

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

Rodriguez, F.J.G. and R.P. Enriquez, 2006. Genetic differentiation of the California spiny lobster *Panulirus interruptus* (Randall, 1840) along the West coast of the Baja California Peninsula, Mexico. Mar. Biol., 148: 621-629.

Sarver, S.K., J.D. Silberman and P.J. Walsh, 1998. Mitochondrial DNA sequence evidence supporting the recognition of two subspecies or species of the Florida spiny lobster *Panulirus argus*. J. Crustacean Biol., 18: 177-186.

Sarver, S.K., D.W. Freshwater and P.J. Walsh, 2000. The occurrence of the provisional Brazilian subspecies of spiny lobster (*Panulirus argus westonii*) in Florida waters. Fishery Bull., 98: 870-873.

Silberman, J.D., S.K. Sarver and P.J. Walsh, 1994. Mitochondrial DNA variation and population structure in the spiny lobster *Panulirus argus*. Mar. Biol., 120: 601-608.

Stamatis, C., A. Triantafyllidis, K.A. Moutou and Z. Mamuris, 2004. Mitochondrial DNA variation in northeast Atlantic and Mediterranean populations of Norway lobster, *Nephrops norvegicus*. Mol. Ecol., 13: 1377-1390.

Tuan, L.A. and N.D. Mao, 2004. Present status of lobster cage culture in Vietnam spiny lobster ecology and exploitation in the South China Sea region. Proceedings of a Workshop Institute of Oceanography, Nha Trang, Vietnam, July 2004, CSIRO, Canberra, pp: 21-25.

Vijayakumaran, M., R. Venkatesan, T.S. Murugan, T.S. Kumar and D.K. Jha *et al*., 2007. Farming of spiny lobsters in sea cages in India. Proceedings of the 8th International Conference and Workshop on Lobster Biology and Management, Sept. 23-28, Charlottetown, Prince Edward Island , Canada, pp: 23-28.