Advances in Biological Research 4 (1): 37-41, 2010
ISSN 1992-0067

Genetic Identity of Three Indian Populations of Three Spotted Seahorse, *Hippocampus trimaculatus*

[1]M. Thangaraj and [2]A.P. Lipton

[1]Centre for Advanced Study in Marine Biology,
Annamalai University, Parangipettai- 608 502, Tamil Nadu, India
[2]Vizhinjam Research Centre of Central Marine Fisheries Research Institute,
Vizhinjam, Thiruvananthapuram, Kerala- 695 521, India

Abstract: The genetic identity of three populations of *Hippocampus trimaculatus* was estimated using a molecular marker (Cyt b). About 620 bp of *Cyt b* gene sequence was compared and analysed. The genetic divergence between the Mullimunai and Tuticorin populations was 0.0016; between Mullimunai and Vizhinjam was 0.0016 and between Tuticorin and Vizhinjam the genetic distance was 0.0032. The nucleotide diversity was 0.00161 ± 0.0023 in all the three populations respectively. AMOVA result showed that the Φ_{ST} and the F_{ST} was 0.000 between the populations. But within populations, Φ_{ST} was 1.000 and F_{ST} was also 1.000 indicating no differences in populations.

Key words: Seahorse · *Hippocampus trimaculatus* · Genetic identity · MtDNA

INTRODUCTION

DNA markers are useful in fisheries for stock structure analysis, mixed stock analysis, species and hybrid identification, hatchery and transplanted stock monitoring, conservation and for mapping quantitative trait loci [1]. MtDNA seems to accumulate mutations more rapidly than do single copy nuclear genes. The rapid divergence between the mtDNAs of isolated populations often magnifies the detectable genetic distance between closely related taxa. Because of the haploid and maternal inheritance of mtDNA, a reduction in mtDNA cloned diversity is more likely than a reduction in nuclear heterozygosity in the event of population bottlenecks [1].

The cytochrome b gene (*Cyt b*) is one of the most important protein encoding genes on the heavy strand of mtDNA molecule and has been utilized in the studies of molecular evolution and classification of species [1, 2]. Cytochrome b sequences have been utilized widely as a 'Molecular clock' to estimate the chronology of speciation in several taxa [3, 4]. *Cyt b* gene contains both slowly and rapidly evolving codon positions, as well as more conservative and more variable regions or domains overall. Therefore, this gene has been used for a diversity of systematic questions, from "deep" phylogeny [6]. *Cyt b* gene has been used as a phylogenetic marker to resolve the relationships among cichlid fishes [2]. *Cyt b* divergence has also been studied in annual killifishes of the genus *Cynolebias* [7]. The evolutionary divergence among the lineages of ocean sunfish family Molidae has been revealed using the D-loop and *Cyt b* mitochondrial genes [8].

The evolutionary history of the endangered Knysna seahorse, *H. capensis* and the extent of geneflow among its three known populations were investigated by Teske *et al*. [9] using mitochondrial DNA sequence. Phylogenetic relationship among 93 specimens of 22 species of seahorses from the Atlantic and Indo-Pacific oceans was analysed using cytochrome b gene of mtDNA. Teske *et al*. [10] used mitochondrial control region (mtDNA CR) sequence to determine weather there was any support for the hypothesis that seahorses were able to colonise remote areas by means of rafting. Lourie *et al*. [11] studied the dispersal, habitat differences and comparative phylogeography of Southeast Asian seahorses using mitochondrial *Cyt b* gene sequence.

Seahorses command good market value as ornamental species and an ingredient in TCM outside India [12]. Species such as *H. kuda* and *H. trimaculatus* are exported in large numbers as dried products.

Corresponding Address: M. Thangaraj, Centre for Advanced Study in Marine Biology,
Annamalai University, Parangipettai- 608 502, Tamil Nadu, India

Unfortunately due to overexploitation and habitat destruction species such as *H. trimaculatus* are included in the IUCN Red Data Book of Threatened Animals [13]. However, no attempts have been made to study the stock structure and basic genetic profile of this species that are essential for fishery management, conservation and rehabilitation of this species in Indian waters.

In this article we discussed about the genetic similarity studies using the sequence data of mitochondrial *Cyt b* gene in three populations of *H. trimaculatus*.

MATERIALS AND METHODS

Hippocampus trimaculatus samples (n=10 per population) were collected from Mullimunai in the Palk Bay, Tuticorin in the Gulf of Mannar and Vizhinjam in south Malabar coast using small trawlnets and other conventional methods. *Hippocampus kuda* (n=2 each) and *Syngnathoides bioculeatus* (n=2) were collected from Tuticorin site for out grouping. The study area mape is given in Fig. 1. The tissue samples (finclips) for DNA extraction were collected from the live fish using non-invasive methods immediately after capture. The tissue samples were stored in sterile eppendorf tubes containing 95% ethanol and sealed with parafilm and kept at room temperature until further analysis. Total genomic DNA was isolated by a modified method of Sambrook *et al.* [14].

The Cytochrome-b gene amplifications were carried out in a gradient thermal cycler (M. J. Research, USA) employing seahorse specific *Cyt b* primers Shf1 (5'-CTACCTGCACCATCAAATATTTC-3') and Shr2 (5'-GAAGGTGAGTCCTCGT TG-3'). The polymerase chain reaction was performed in 25 µl reactions containing 1x assay buffer (100mM Tris, 500mM KCl, 0.1% gelatin, pH 9.0) with 1.5mM $MgCl_2$, 10 p moles/µL of primer mix, 10 mM dNTPs, 1.5 U Taq DNA polymerase and 20 ng of template DNA. To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. The reaction mixture was initially denatured at 95° C for 5 minutes followed by 29 cycles (94°C for 45 seconds, 54°C for 30 seconds and 72°C for 45 seconds, final extension 72° C for 5 minutes). The reaction was then subjected to a final extension at 72°C for 5 minutes.

3 µl PCR product along with marker (100bp DNA ladder) was run on 1% agarose gel with 1x TBE buffer for 30 minutes and stained with ethidium bromide. The gel was visualized under UV transilluminator and documented using Image Master VDS (Pharmacia Biotech, USA). The *Cyt b gene* was sequenced by a sequencing facility (Genei, India). The raw *Cyt b* gene sequences were

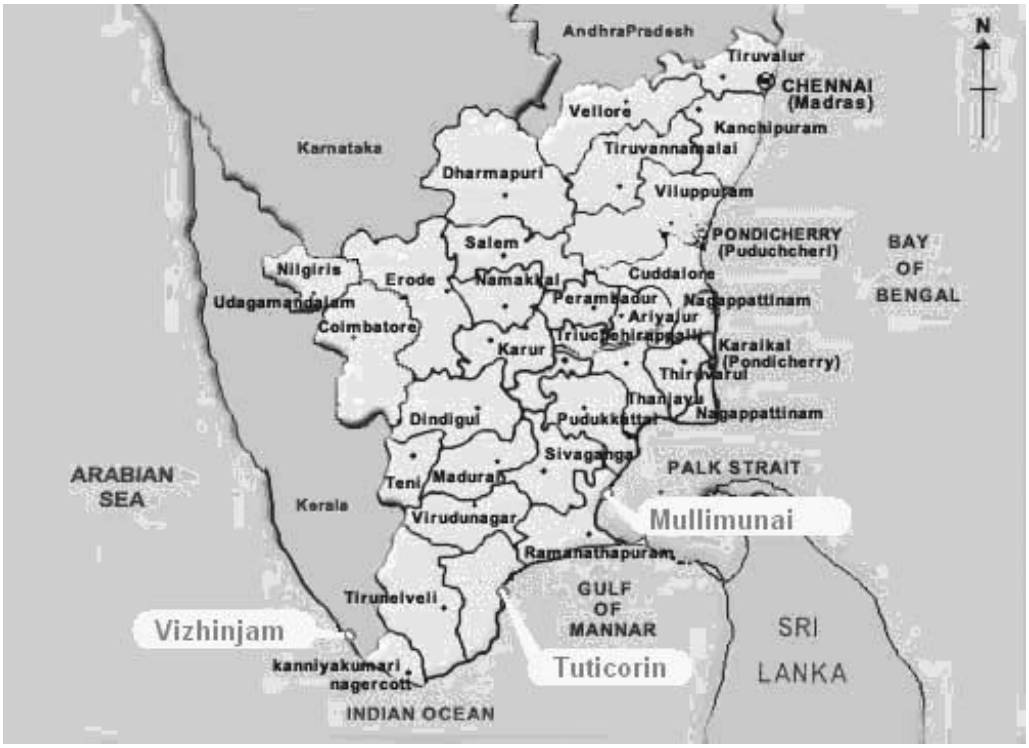

Fig. 1: Study area map

aligned and edited using BioEdit sequence alignment editor version 7.0.5.2 [15]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [16]. Haplotype and nucleotide diversity indices were calculated using ARLEQUIN version 2.0 [17] and MEGA ver. 3.1 [16].

To study the genetic divergence among and within populations, analysis of molecular variance (AMOVA) was used. AMOVAs were performed using haplotype frequency data alone (F_{ST}) and incorporating sequence divergence using pair-wise differences (ϕ_{ST}).

RESULTS

The banding pattern of amplified *Cyt b* gene of seahorses (*H. trimaculatus* and *H. kuda*) and pipefish (*Syngnathoides biaculeatus*) is presented in Fig.2. A total of 620 bases of *Cyt b* gene sequences of *H. trimaculatus* (3 populations) and two outgroup (*H. kuda* and *Syngnathoides biaculeatus*)) were unambiguously edited using BioEdit Sequence alignment editor version 7.0.5.2 [15] and aligned using CLUSTAL-W in BioEdit and checked manually. Polymorphic sites were rechecked with original sequence trace files. Identical sequences were assigned in the same haplotype identity and only a single example of each was used in the phylogenetic divergences assuming that identical haplotypes shared the same evolutionary origin. Haplotype definitions have been submitted to the NCBI GenBank (Accession numbers EF 189158 to EF 189167). The genetic divergence values with *Cyt b* sequence were estimated by BioEdit among three populations of *H. trimaculatus*. The genetic divergence value between the Mullimunai and Tuticorin populations was 0.0016; between Mullimunai and Vizhinjam the genetic distance was 0.0016 and between

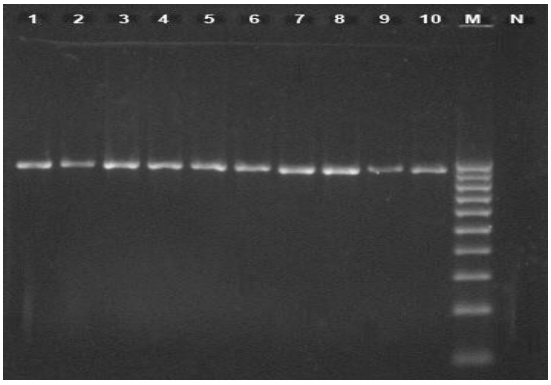

Fig. 2: Amplified Cyt b gene of seahorses (*H. kuda, H. trimaculatus*) and pipefish (*Syngnathoides biaculeatus*) (lanes 1-6 *H. trimaculatus*; 7-8 H. *kuda*; 9-10 S. *biaculeatus*; M-Moleculat weight marker 100 bp lader)

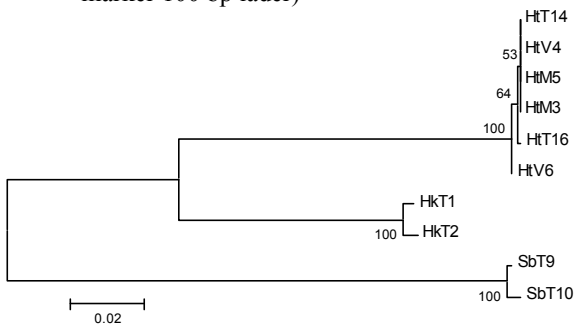

Fig. 3: Neighbour joining tree (1000 replications, K2P distance correlation) generated from *Cyt* b sequence data of *H. trimaculatus* (Htm3, Htm5= Mullimunai population; HtT14, HtT16= Tuticorin population; HtV4, HtV6= Vizhinjam population). Scale indicates genetic distance. The tree rooted with two out groups, *H. kuda* (HkT1, HkT2) and *S. biaculeatus* (SbT9, SbT10)

Table 1: Summary of molecular diversity analysis for *H. trimaculatus* from three locations in India

	Mullimunai	Tuticorin	Vizhinjam
K	1	2	2
S	1	1	1
h	0.0	0.5	0.5
$\pi \pm$ SD	0.00161 ± 0.0023	0.00162 ± 0.0023	0.00162 ± 0.0023

K = Number of Haplotypes; S = Number of Polymorphic Sites; h = Haplotype diversity; π = Nucleotide (sequence) diversity

Table 2: Results of AMOVA based on Cytochrome- *b* Sequence (620bp) data showing variation (1) for all collection sites and (2) within the individual collection sites

		All Populations	
		ϕ_{ST}	F_{ST}
1.	All Collection Sites		
	Among all sites	0.000	0.000
2.	Within individual collection sites		
	Mullimunai	1.000	1.000
	Tuticorin	1.000	1.000
	Vizhinjam	1.000	1.000

($P > 0.001$)

Tuticorin and Vizhinjam the genetic distance was 0.0016 and 0.0032 (two haplotypes). One haplotype was common to all the three populations.

The molecular diversity of three populations of *H. trimaculatus* is given in Table 1. There were two haplotypes in each population and each had only one polymorphic site. The nucleotide diversity was 0.00161 ± 0.0023 in all the three populations respectively. The AMOVA results are given in Table 2. This result showed that the Φ_{ST} and the F_{ST} was 0.000 between the populations. But within populations, Φ_{ST} was 1.000 and F_{ST} was also 1.000 indicating no differences in populations using *Cyt b* sequence analysis. The neighbor joining (NJ) analysis using Kimura 2 parameter yielded tree with high bootstrap support values as given in Fig. 3.

DISCUSSION

In our study all the populations contained two haplotypes and the haplotypes are same in their properties. Earlier report from South Africa by Teskei *et al.* [9] showed that seven haplotypes shared three populations of *H. capensis*. From the present study it can be understood that the populations in the three regions may be evolving relatively trough the same stochastic processes. Based on this sequence data, there is no evidence of population substructure among the Mullimunai, Tuticorin and Vizhinjam populations.

The sequence diversity in *H. trimaculatus* using Kimura 2 parameter model for *Cyt b* was very low (0.00161) in this present study compared to the same (9.1% to 37%) in many species in many genera of fishes [2]. Earlier reports by Lourie and Vincent [18] showed that the Indian Ocean population of *H. trimaculatus* had high haplotype diversity (h) and low nucleotide diversity whereas the Pacific Ocean population had low haplotype diversity and high nucleotide diversity based on *Cyt b* gene. But in the present study, low haplotype diversity and low nucleotide diversity in all the three populations suggests that the populations are getting mixed up as a result of successful dispersal events. Lourie *et al*. [11] reported that among the different seahorses, *H. trimaculatus* has the most widespread haplotypes (average clad distances of non-singleton haplotypes = 1169 km) indicating potentially high dispersal capabilities. The maximum distance between the most separated collection sites (Mullimunai and Vizhinjam) being only 500 km, possibilities of mixing up of populations without any restrictions can not be ruled out. The results support the earlier assumptions based on mark recapture investigation and stock assessment studies of many marine species by CMFRI [19, 20] that east and west coast populations of several crustacean and teleosts are homogeneous. Given the evidence for relatively rapid dispersal to South Malabar (Vizhinjam) from Palk bay (Mullimunai) through Tuticorin (Gulf of Mannar), the low nuecleotide diversity (π) values in *H. trimaculatus* among the three populations is not surprising. Ocean currents in these regions generally flow from east to west (from Palk Bay to south Malabar sea) during the northeast monsoon season (November-February) and in the reverse direction during the southwest monsoon season (May-September) apparently favouring the movement of haplotypes of eastern region to the west and vice-versa. The AMOVA results in the present study (F_{ST} = 0.000 and Φ_{ST} = 0.000) also support the 'homogeneous stock' hypothesis. One haplotype (HtM3, HtT14, HtT16, HtM5 and HtV4) is widespread in all the three populations, while haplotypes HtV6 and HtT6 are restricted to south Malabar and the Gulf of Mannar. It is possible that these patterns reflect the isolation of these smaller sea basins as observed in seahorses by Lourie and Vincent [18]. Further data are needed to critically assess this hypothesis.

Subsequent studies using more microsatellite markers and mtDNA sequence data (such as ATP synthase 6 or D loop/ control region along with *Cyt b*) will prove if the stocks of *H. trimaculatus* along the Indian coasts are homogeneous or not.

AKNOWLEGEDEMENT

The authors would like to thank The Director of CMFRI and Dr. A. Goplakrishnan, Principal Scientist, NBFGR, The Director, CAS MB, Annamalai University for encouragement and facilities. They also thankful to The Ministry of Environment and Forests, Govt. of India, New Delhi, for funding.

REFERENCES

1. Brahmane, M.P. and V.V. Sugunan, 2003. Role of molecular markers in fishery and aquaculture. Fishing Chimes 23(3): 11-14.
2. Farias, I.P., G. Orti, I. Sampaio, H. Schneider and A. Meyer, 2001. The cytochrome *b* gene as a phylogenetic marker: the limits of resolution for analyzing relationship among Cichlid fishes. J. Mol. Evol., 53: 89-103.

3. Sakai, T., Y. Kikkawa, K. Tsuchiya, M. Harada, M. Kanoe and M. Yoshiyuki, 2003. Molecular Phylogeny of Japanese rhinolophidae based on variation in the complete sequence of the mitochondrial Cytochrome-*b* gene. Genes. Genet. Syst., 78: 179-189.
4. Irwin, D.M., T.H. Kocher and A.C. Wilson, 1991. Evolution of the cyt *b* gene of mammals. J. Mol. Evol., 32: 138-144.
5. Smith, M.F. and J.L. Patton, 1993. The diversification of South American murid rodents: Evidence from mitochondrial DNA sequence data for the akodontine tribe. Biol. J. Linn. Soc., 50: 149-177.
6. Kumazawa, Y. and M. Nishida, 2000. Molecular phylogeny of Osteoglossoids: a new model for Gondwanian origin and plate tectonic transportation of the Asean Arowana. Mol. Biol. Evol., 17(2): 1869-1878.
7. Garcia, G., G. Wlasiuk and E.P. Lessa, 2000. High levels of mitochondrial Cyt *b* divergence in annual killifishes of the genus cynolebias (Cyprinodontiformes, Rivulidae). Zoo. J. Linn. Soc., 129: 93-110.
8. Bass, A.L., H. Dewar, T. Thys, J.T. Streelman and S.A. Karl, 2005. Evolutionary divergence among lineages of the Ocean sunfish family, Molidae (Tetraodontiformes). Mar. Biol., 137: 1077-1090.
9. Teske, P.R., M.I. Cherry and C.A. Matthee, 2003. Population genetics of the endangered Knysna seahorse, *Hippocampus capensis*. Mol. Ecol., 12: 1703-1715.
10. Teske, P.R., M.I. Cherry and C.A. Matthee, 2004. The evolutionary history of seahorses (Syngnathidae: *Hippocampus*): molecular data suggest a West Pacific and two invasions of the Atlantic Ocean. Mol. Phy. Evol., 30(2): 273-286.
11. Lourie, S.A., D.M. Green and A.C.J. Vincent, 2005. Dispersal, habitat differences and comparative phylogeography of South East Asian seahorses (Syngnathidae: *Hippocampus*). Mol. Ecol., 14: 1073-1094.
12. Lipton, A.P. and M. Thangaraj, 2002. Present status of seahorse fishing along the Palk Bay coast of Tamilnadu. Mar. Fish. Info. Serv. Tech. Exten. Seri., 174: 5-8.
13. Baillie, J. and B. Groombridge, 1996. IUCN red list of threatened animals. Gland, Switzelland: IUCN (World Conservation Union).
14. Sambrook, J., E.F. Fritsch and T. Maniatis, 1998. Molecular cloning: a laboratory manual.2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
15. 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: 95-8.
16. Kumar, S., K. Tamura and M. Nei, 2004. MEGA 3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinfo., 5: 150-163.
17. Schneider, S., D. Roessli and L. Excoffier, 2000. Arlequin: A software for population genetics data analysis. Ver 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.
18. Lourie, S.A. and A.C.J. Vincent, 2004. A marine fish follows Wallace's line: the phylogeography of the three spot seahorse (*Hippocampus trimaculatus*, Syngnathidae, Teleostei) in Southeast Asia. J. Biogeography, 31: 1975-1985.
19. Maniseri, M.K. And C. Manimaran, 1981. On the fishery of the Indian white prawn *Penaeus indicus* H.M. Edwards along the Tirunelveli Coast, Tamilnadu. Ind. J. Fish., 28: 208-216.
20. Rao, G.S., 1993. Stock assessment of *Penaeus* spp. off the east coast of India. Ind. J. Fish, 40(1): 1-19.