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Short Communications

Assessment of genetic variation in closely related seahorse species (Genus: *Hippocampus*) using mtDNA marker

M Thangaraj* and A P Lipton

Marine Biotechnology Laboratory, Vizhinjam Research Centre of Central Marine Fisheries Research Institute, Vizhinjam, Thiruvananthapuram 695 521, India

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Phylogenetic relationships among two species of seahorses (*Hippocampus kuda* & *H. trimaculatus*) and one species of pipe fish (*Syngnathoides biaculeatus*) from Gulf of Mannar were analysed using cytochrome b gene (*cyt b*) sequence data (Acc. No. EF 189158 & EF 189167). The genetic distance value between *H. kuda* and *H. trimaculatus* was found to be 0.155; while between *H. kuda* and *S. biaculeatus*, it was 0.256, and between *H. trimaculatus* and *S. biaculeatus*, 0.290. The percentage of identical pairs between *H. kuda* and *H. trimaculatus* was 85.90, and between *H. kuda* and *S. biaculeatus*, 79.19. The percentage of transition and transversion between two seahorse species (*H. kuda* & *H. trimaculatus*) was lesser than between seahorse and pipe fish (*S. biaculeatus*). The results suggested that further phylogenetic studies are required to determine the genetic relationship in all Indian seahorse species.

Keywords: Cytochrome b, genetic distance, pipefish, seahorse

The mitochondrial genome is a single, small, double stranded, 16 to 20 kb circular molecule contained in multiple copies in mitochondria[1]. As there is no paternal contribution of mtDNA and no known recombination between mitochondrial genomes, the mtDNA is clonally inherited and is treated as a single locus in population studies[2]. 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[3,4]. Mitochondrial *cyt b* sequences have proven to contain phylogenetic signal at many different axonomic levels in numerous taxa, including fish[5,6]. *Cyt b* gene sequences have been utilized widely as a 'molecular clock' to estimate the chronology of speciation in several taxa[7,8]. Overall, *cyt b* contains both slowly and rapidly evolving codon positions, as well as more conservative and more variable regions or domains. Therefore, this gene has been used for diversity and phylogenetic determination studies[6,9]. The evolutionary history of the endangered Knysna seahorse, *Hippocampus capensis*, and the extent of geneflow among its three known populations were investigated by Teske *et al*[10] using 1038 bp of mtDNA control region sequence. The phylogenetic data suggests that high diversity in the Indo-Pacific resulted from speciation events, dating from the Pleistocene to the Miocene or earlier[11]. Teske *et al*[10] used 4 molecular markers, such as, nuclear RP1 and aldolase gene and mt 16S rRNA and *cyt b* genes to determine the phylogenetic relationship among 32 species of the genus *Hippocampus*.

Seahorse populations are declining year by year not only in India but throughout the world, because of over-fishing and increasing demand in Chinese market. To study the genetic structure and differences between and within species is essential to conserve these species effectively. So in this study, we have tried to find out the genetic differences between two commonly available seahorse and one pipefish (as out group) species in Gulf of Mannar using mitochondrial gene sequence.

Finclips of *H. trimaculatus, H. kuda* and *S. biaculeatus* (n=10 each) were collected from the live fish using non-invasive methods immediately after the capture. The tissue samples were stored in sterile eppendorf tubes containing 95% ethanol. Total genomic DNA was isolated by a modified method of Sambrook *et al*[13]. The mitochondrial *Cyt b* gene was amplified with the primers Shf1 (5'CTACCTGCACCATCAAATATTTC-3') and Shr2 (5'-CGGAAGGTGAGTCCTC GTTG -3'). The details of these primer sequences were taken from earlier report[11]. DNA amplifications were performed in 25 µL reactions containing 1X assay buffer (100 m*M* Tris, 500 m*M* KCl, 0.1% gelatin, *p*H 9.0) with 1.5 m*M* $MgCl_2$, 10 pmoles/µL of primer mix, 10 m*M* dNTPs, 1.5 U Taq DNA polymerase and 20 ng of template DNA. The amplification profile was 95°C for 5 min followed by 29 cycles of 94°C for

*Author for correspondence:
Tel: 91-4144-243223; Mobile: 91-9486133396
Email: coralholder@yahoo.com
Present Address: Centre for Advanced Study in Marine Biology, Annamalai University, Parangipettai 608 502, India

45 sec, 54°C for 30 sec and 72°C for 45 sec and a final extension 72°C for 5 min. The cleaned up PCR product was sequenced by a sequencing facility (Genei, Bangalore, India). The sequences were aligned by eye using the Bio-Edit program[14]. Nucleotide diversity, genetic variation substitutions rates between transition and transversion was performed by MEGA (Ver. 3.1)[15].

The banding pattern of mtDNA *Cyt b* gene of seahorses (*H. trimaculatus* & *H. kuda*) and pipefish (*Syngnathoides biaculeatus*) is presented in Fig. 1. A total of 620 bases of *Cyt b* gene partial sequences of *H. trimaculatus, H. kuda* and *S. biaculeatus* were unambiguously edited using BioEdit sequence alignment editor[14] 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 (Acc. No. EF 189158-EF 189167).

The genetic divergence values with *Cyt b* sequence were estimated by BioEdit among 3 species. The genetic distance value between *H. kuda* and *H. trimaculatus* was 0.155; while it was 0.256 between *H. kuda* and *S. biaculeatus*, and was 0.290 between *H. trimaculatus* and *S. biaculeatus*.

Of the 620 nucleotides of *Cyt b*, the percentage of identical pairs between *H. kuda* and *H. trimaculatus* was 85.90, and between *H. kuda* and *S. biaculeatus*, it was 79.19. The percentage of transition and transversion between two seahorse species (*H. kuda*, *H. trimaculatus*) was lesser than between seahorse and pipe fish (*S. biaculeatus*). The details are given in Table 1. The neighbor joining (NJ) analysis using Kimura 2 parameter yielded a tree with high bootstrap support values as given in Fig. 2. The aim of this study was to assess the genetic variation between the seahorse and pipe fish. Seahorses and its relatives have a good market value as ornamental species and an ingredient in TCM outside India[16]. Species such as *H. kuda* and *H. trimaculatus* are exported in large numbers as dried products. Unfortunately due to overexploitation and habitat destruction species such as *H. trimaculatus* are included in the IUCN Red Data Book of Threatened Animals[17]. The exact population status of a species must be established before adapting any management and conservation strategies.

Within the mitochondrial genome, the 16s ribosomal DNA gene, and the *CO1* gene are widely used owing to their slowest mutation and lower substitution

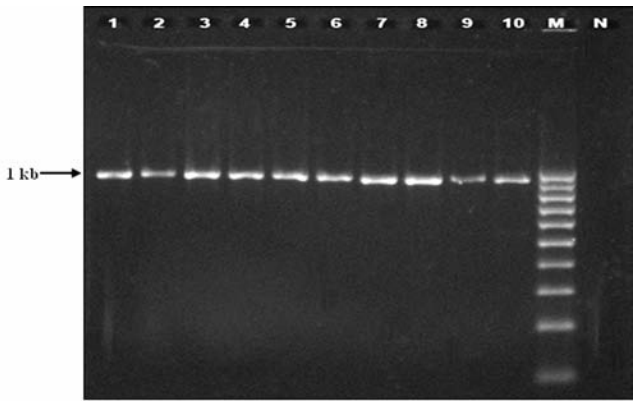

Fig. 1—mtDNA *Cyt b* gene banding pattern of seahorses: *H. kuda* (lanes 1-6), *H. trimaculatus* (lane 7 & 8), *S. biaculeatus* (lanes 9 & 10); Molecular weight marker, 100 bp lader (M).

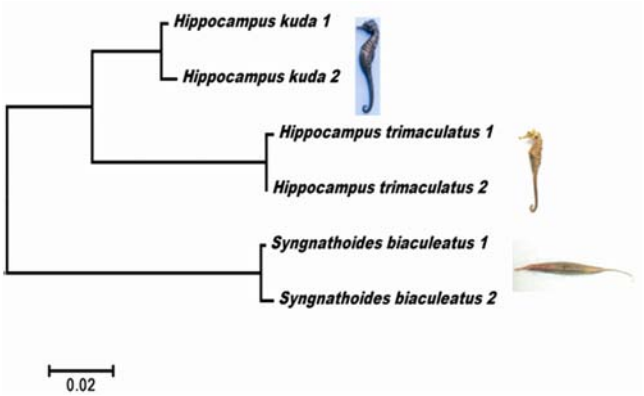

Fig. 2—NJ tree generated from *Cyt b* sequence data, scale indicates the genetic distance.

Table.1—The percentage of pair wise nucleotide variations above diagonal and genetic distance below diagonal

Species	*H. trimaculatus*			*H. kuda*			*S. biaculeatus*		
	I	Ts	Tv	I	Ts	Tv	I	Ts	Tv
H. trimaculatus	***	***	***	85.90	10.32	3.38	76.93	12.58	10.32
H. kuda		0.155		***	***	***	79.19	11.12	9.51
S. biaculeatus		0.290			0.256		***	***	***

I = Identical; Ts = Transition; Tv = Transversion

rates compared to other mtDNA genes. These genes have been reported to be useful when analyzing families and species[18]. *Cyt b* evolves relatively faster than 16S rRNA and this gene has been used to study stock specific differences in many fish species[19]. In the present study, the genetic distance between *H. kuda* and *H. trimaculatus* was 0.155. This result is comparable with the earlier study by Casey *et al*[11], where the genetic distance between *H. kuda* and *H. trimaculatus* was 0.178. In their study the *H. kuda* sample was collected from India and *H. trimaculatus* sample was collected from Philippines. In our study, both the samples were collected from Indian waters only. Because of more geographic distance the genetic distance between *H. kuda* and *H. trimaculatus* was varied. In a recent study using Type I marker (*Cyt b*), it has been shown that the genetic variation between various populations of *H. trimaculatus* was very less[20]. Though the *S. biaculeatus* specimen showed more genetic distance (<0.25), we consider *S. biaculeatus* as an out group in phylogenetic tree analysis (Fig. 2). Subsequent studies using more mtDNA sequence data (such as ATP synthase 6 or D loop/control region along with *Cyt b*) will strengthen the genetic distance between *H. kuda, H. trimaculatus* and also other Indian seahorse species.

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