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Indian Journal of Animal Sciences **81** (1): 97–101, January 2011

Molecular identification and phylogenetic relationship of seahorse, *Hippocampus kuda* (Bleeker 1852) using mitochondrial 16S rRNA and COI gene sequences from east and west coasts of India

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Received: 6 October 2009; Accepted: 22 October 2010

ABSTRACT

Traditionally, fish species identification is based on morphological characters, yet, in many cases it is difficult to establish identity as in the case of seahorses which lack key species-diagnostic morphological features. The spotted or yellow seahorse - *Hippocampus kuda* has a complex identity and the samples collected from the east and west coasts of India were analyzed for the species identification and phylogenetic relationship, based on partial sequence information of mitochondrial genes - 16S rRNA and Cytochrome Oxidase subunit I (COI). Estimates of genetic divergence with both 16S rRNA and COI genes, when compared with the sequence divergence values of *H. kuda* from other continents (as obtained from NCBI accessions) were sufficient enough to discriminate individuals of the same species from Indian waters. Pair-wise f_{ST} values using AMOVA indicated significant levels of genetic differentiation of *H. kuda* populations among east coast, Kerala and Konkan populations; however, no significant genetic partitioning was observed between the Palk Bay and Gulf of Mannar populations.

Key words: Cytochrome oxidase subunit I (COI), *Hippocampus kuda*, Mitochondrial DNA, Seahorse, 16S rRNA gene

The seahorses belong to family *Syngnathidae*, which also includes pipefishes, pipehorses and seadragons. There are about 50 species reported across the world and they have been found to inhabit coral reefs, seagrass beds and also coastal mangroves. Morphological revisions of seahorse have been carried out by Lourie *et al*. (1999a). Based on the morphometric characters, the species inhabiting Indian waters are *Hippocampus kuda* (Bleeker 1852), *H. fuscus* (Ruppell 1838), *H. trimaculatus* (Leach 1814), *H. kelloggi* (Jordon and Snyder 1901), and *H. histrix* (Kaup 1856). There are also reports of *H. spinosissimus* (Weber 1913) having suspected distribution (Lourie 2004) and recently *H. borboniensis* and *H. mohnikei* have also been reported (Thangaraj and Lipton 2007).

The global trade of dried seahorses was estimated to be over 20 million individuals (exceeding 50 metric tonnes) in 2000 for the traditional Chinese medicine market alone (Salin *et al*. 2005, Lourie *et al*. 2004 and Vincent 1994, 1995, 1996). India was one of the largest seahorse exporters until 2001–2002 and according to official estimates, about 4.34 tonnes of seahorses were exported from India mainly to Singapore, United Arab Emirates and Hong Kong during 2001–2002, earning a total of 2.673 million rupees (US$ 70,000), with Chennai being the major port of trading activities (Anon 2003). The commercial exploitation was carried out mainly at the Palk Bay and Gulf of Mannar areas in the South-east coast of India (Salin *et al*. 2005). Over-exploitation of both the species of seahorses from India had resulted in the decline of their population up to 70% (Salin *et al*. 2005) and to curb this, Government of India took steps to ban the fishing and trade by declaring all members of the family *Syngnathidae* from Indian waters as protected species under the Schedule I (Part 2A) of the Indian Wildlife (Protection) Act, 1972 through a Notification No. 1–4/95 WL1 dated 11 July, 2001. The IUCN Red List (2006) has listed 20 species of seahorse as 'vulnerable'; 11 as 'data deficient' and one *(H. capensis)* as 'endangered'. The national assessment reports seahorses as threatened in Australia (entire genus *Hippocampus*), France *(H. guttulatus*), China *(H. kelloggi*), Portugal *(H. hippocampus* and *H. ramulosus* = *H. guttulatus*), South Africa

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(H. capensis), Ukraine *(H. guttulatus microstephanus*), and Vietnam *(H. histrix, H. japonicus, H. kelloggi, H. kuda* and *H. trimaculatus*) (Lourie *et al.* 2004).

Identification of seahorse based on morphology alone is always doubtful, as the seahorses are conservative in morphology and lack certain key physical features (e g pelvic and caudal fins) which are often used in the morphometric analysis of several fish species (Knowlton 1993). Moreover, *H. kuda, H. fuscus, H. spinosissimus* and *H. borboiensis* species have been found to show overlapping morphometric features. Molecular markers especially those that uncover fixed allelic differences at diagnostic loci, are proving increasingly valuable in identifying species in recent years. In particular, non-recombining mitochondrial DNA (mtDNA) has received strong support for its use as a marker in conservation genetics, forensic, taxonomic, and ecological studies (Avise 1995, Moritz 1994). The well-characterized mitochondrial 16SrRNA and COI genes have proved to be a robust evolutionary marker for the analysis of intergeneric and interspecific relationships in many marine fish and shellfish (Lakra *et al*. 2008). We have used 592 base-pair (bp) 16S rRNA and 655 bp COI for identification and phylogenetic comparison of *H. kuda* from the Indian waters.

MATERIALS AND METHODS

Collection of samples

The seahorse samples were collected from the east and west coasts of India covering Thondi, Mullimunai, Pamban (Palk Bay, Tamil Nadu); Vellipatti, Thirespuram, Tiruchendur (Gulf of Mannar, Tamil Nadu); Vizhinjam, Kollam, Cochin (Kerala Coast), Karwar, Kumta, Panjim Estuary, Marmagao and Ratnagiri (Konkan Coast) (Table 1). For species identification and nomenclature, seahorse identification guide by Lourie *et al.* (2004) was followed. Fin samples were collected either as by-catch of fish trawlers or directly from fishermen and preserved in 95% ethanol. A non-invasive fin clip procedure was applied for tissue collection (Lourie 1999). All morphometric data were collected following the Lourie (1999, 2004). The measurements were taken using dial calipers to the nearest 0.1 mm on the right side of the seahorse and repeated to ensure accuracy and the mean value taken.

DNA extraction and PCR amplification

DNA was extracted using Phenol-chloroform method (Sambrook *et al.* 1989). The extracted DNA was stored at –20°C until used. The mitochondrial 16S rRNA gene was amplified in a 50 µl reaction volume with 5 µl of 10 × *Taq* polymerase buffer, 0.2 mM of each dNTP, 0.4µM of each primer, 2.5 U of *Taq* polymerase and 5 µl genomic DNA using the thermal cycler PTC 200. The primers used for the amplification of the partial 16S rRNA gene were 16SAR (5'-CG CCTGTTTATCAAAAACAT–3') and 16SBR (5'-CC GGTCTGAACTCAGATCACGT–3') (Palumbi *et al*. 1991). The thermal profile used was 36 repetitions of a three step cycle consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1.5 min including 4 min for initial denaturation at 94°C and 7 min for the final extension at 72°C. COI gene was also amplified in a final concentration of 50 µl volume with a final concentration of 5 µl of 10 × *Taq* polymerase buffer, 2 µl of $MgCl_2$ (50 mM), 0.25 µl of each dNTP (0.05 mM), 0.5 µl of each primer (0.01 mM), 0.6 U of *Taq* polymerase and 5µl of genomic DNA. The primers used for the amplification of the 655 bp COI gene were FishF1–5'-TCAACCAACCACAAAGACATTGGCAC–3' and Fish R1–5'-TAGACTTCTGGGTGGCCAAAGAATCA–3' (Ward *et al*. 2005). The thermal regime consisted of an initial step of 2 min at 95°C followed by 35 cycles of 40s at 94°C, 40s at 54°C and 1 min 10s at 72°C followed in turn by final extension of 10 min at 72°C. PCR products were visualized on 1.2% agarose gels and the most intense products were

Table 1. Collection of samples of *Hippocampus* spp.

Collection site	Coast/population	Latitude/longitude	Samplesize
Thondi, Mullimunai	Palk Bay (East coast)	9° 45′N 79° 04′E	22
Pamban,		9° 10′N 79° 28′E	32
Mandapam		9° 22′N 78° 52′E	35
Vellipatti, Tuticorin	Gulf of Mannar (East coast)	8° 48′N 78° 11′E	9
Thirespuram, Tuticorin		8° 48′N 78° 09′E	8
Tiruchendur, Tuticorin		8° 30′N 78° 11′E	15
Vizhinjam	Kerala coast	8° 29′N 76° 59′E	25
Kollam		9° 7′N 76° 29′E	18
Cochin		9° 58′N 76° 17′E	23
Karwar, Karnataka	Konkan coast	14° 48′N 74° 11′E	8
Kumta, Karnataka		14° 26′N 74° 27′E	9
Panjim Estuary		15° 3′N 73° 55′E	16
Marmagao		15° 25′N 73° 43′E	11
Ratnagiri,Maharastra		16°55′N 73° 19′E	30

selected for sequencing. Products were labeled using the Cycle sequencing Kit and sequenced bidirectionally using a capillary sequencer following manufacturer's instructions.

Sequence analysis

The sequences obtained were edited using the *EditSeq*, a sequence editor and import/export tool in Lasergene software and were then aligned in MegAlign. A general purpose multiple sequence alignment program Clustal W (Thompson *et al*. 1994) and BIOEDIT sequence alignment editor version 7.0.5.2 (Hall 1999) was also used. All the sequences were submitted to the GenBank (Accession numbers - FJ211362 - FJ211367, FJ211278 - FJ211285. FJ176578, FJ176581, FJ176586 - FJ176592, EU930325 - EU930330). Pair-wise evolutionary distance among haplotypes was determined by the Kimura 2–Parameter method using the software program MEGA 3.1 (Molecular Evolutionary Genetics Analysis) (Kumar *et al.* 2004). Gaps were considered as missing data on the phylogenetic reconstructions. Neighbour Joining (NJ) and Maximum Parsimony (MP) trees were constructed using MEGA 3 using *Fistularia petimba* from NCBI Gen Bank as an outgroup.To verify the robustness of the internal nodes of NJ and MP trees, bootstrap analysis was carried out using 1000 pseudoreplications. was evaluated using Analysis of molecular variance (ANOVA) model in the ARLEQUIN ver 3.0 software (Excoffier *et al*. 2005) was employed to study the population structure of *H. kuda* from all 4 coasts. Fixation indices ($\hat{O}_{ST}$) analogous to F_{ST} based on haplotype frequency distribution were also calculated to assess genetic divergence for overall and between population pairs. The statistical significance of the total and pair-wise fixation indices was estimated by comparing the observed distribution with a null distribution, generated by 10,000 permutations of the data matrix. Multiple tests of the same null hypothesis were subjected to table-wide sequential Bonferroni correction to avoid elevated Type I error rates.

RESULTS AND DISCUSSION

An average of 592 bp (range 581–605bp) of sequenced product was obtained from *H. kuda* samples using the 16S rRNA mtDNA primers. The nucleotide sequence comparison of PCR amplified 16S rRNA gene segment among the *H. kuda* samples collected from different locations of the east and west coasts of India showed, 5 unique haplotypes with 4 variable (0.68%), 2 parsimony informative and 2 singleton sites. Among the 4 polymorphic sites, 3 were transitions, and only 1 was transversion with an average transition/ transversion ratio as 2.22. The mean number of nucleotide composition in *H. kuda* was A = 32.2%, T = 24.4%, C = 22.8% and G = 20.5%. Of the 5 haplotypes of 16S rRNA, 2 (H1, H2) were specific to the east coast of India (Palk Bay and Gulf of Mannar) and the remaining 3 (H3, H4, H5) were unique to the west coast (Kerala and Konkan). Interestingly, the Kerala (H3, H4) and Konkan (H5) populations did not share any haplotypes. Haplotype diversity was high, but nucleotide diversity was low for overall populations. The overall mean genetic divergence value among the 4 populations with 16S rRNA was 0.0041 based on Kimura 2 parameter. Pair-wise genetic divergence among the haplotype is given in Table 2. Estimates of genetic divergence when compared with the sequence divergence values of *H. kuda* from other continents (as obtained from NCBI accessions; value varied from 0.032–0.7%; expected value within species for 16SrRNA in teleosts < 1.5%) were sufficient enough to discriminate individuals of the same species from Indian waters. Pair-wise Φ_{ST} values after sequential Bonferroni corrections indicated significant levels of genetic differentiation among east coast, Kerala and Konkan populations, however, no significant genetic partitioning was observed between the Palk Bay and Gulf of Mannar populations of *H. kuda*. [Between Palk Bay and Gulf of Mannar – 0.029 (NS); between East coast and Kerala 0.0581 ($P<0.001$); between East and Konkan Coasts 0.0892 ($P<0.001$) and between Konkan Coast and Kerala 0.0901 ($P<0.001$)]. The ANOVA analysis based on the haplotype frequency differences indicated that most of the molecular variance occurred among populations (76.1%). The values were significant ($P<0.001$) and revealed the occurrence of genetic partitioning among east coast, Kerala and Konkan populations. The NJ and MP trees revealed identical phylogenetic relationship among the samples collected from four coasts and separated the 16S r RNA haplotypes into 3 clusters with high bootstrap support (Fig. 1) in most of the nodes indicating the distinct genetic structure among the 3 populations of *H. kuda* from the Indian coast.

Table 2. Pair-wise K2P genetic divergence among 16S rRNA haplotypes of *Hippocampus kuda.*

Haplotypes	H1	H2	H3	H4	H5
H1	–				
H2	0.00171	–			
H3	0.00342	0.00413	–		
H4	0.00513	0.00543	0.00171	–	
H5	0.00571	0.00685	0.00297	0.00343	–

A total of 655 base pairs of COI gene fragments were successfully sequenced from *H. kuda*. The nucleotide sequence comparison showed 8 unique haplotypes with 25

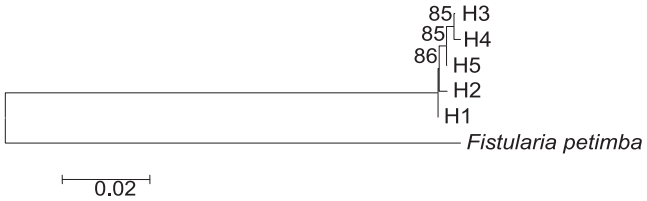

Fig 1. Evolutionary relationship of *Hippocampus kuda* populations based on 16S rRNA sequence data (Neighbour-Joining method).

Table 3. Pair-wise K2P genetic divergence among COI haplotypes of *Hippocampus kuda.*

Haplotypes	H1	H2	H3	H4	H5	H6	H7	H8
H1	–							
H2	0.00153	–						
H3	0.00306	0.00307	–					
H4	0.00217	0.00234	0.0014	–				
H5	0.00560	0.00587	0.00559	0.00568	–			
H6	0.00642	0.00666	0.00687	0.00649	0.00518	–		
H7	0.00669	0.00613	0.00650	0.00628	0.00779	0.00763	–	
H8	0.0189	0.00997	0.0160	0.00803	0.00594	0.0107	0.0046	–

variable, 13 parsimony informative and 12 singleton sites. Among the 25 polymorphic sites, 18 were transitions and only seven were transversion with an average transition/ transversion ratio as 2.57%. Majority of the changes occurred in the third codon position. The mean number of nucleotide position was A = 25.7% T= 33.7%, G= 17.6% and C = 22.9% of the 8 haplotypes, four (H1, H2, H3, H4) were specific to the east coast (Palk Bay and Gulf of Mannar), 2 to Kerala coast (H5, H6) and the remaining 2 (H7, H8) to Konkan Coast. The mean genetic divergence values among the 4 populations with COI was 0.0067 based on Kimura 2 parameter. Pair-wise genetic divergence among the haplotypes is given in Table 3. Estimates of genetic divergence when compared with the sequence divergence values of *H. kuda* from other continents (as obtained from NCBI accessions; value varied from 0.95–2.19%; expected value within species for COI in teleosts < 3.5% (Lakra *et al.* 2008)) were sufficient to discriminate individuals of the same species from Indian waters. Pair-wise Φ_{ST} values after sequential Bonferroni corrections indicated significant levels of genetic differentiation among east coast, Kerala and Konkan populations [between Palk Bay and Gulf of Mannar –0.008 (NS); between east coast and Kerala 0.0974 (P<0.0001); between east coast and Konkan region 0.109 (P<0.001) and between Konkan and Kerala 0.0795 (P<0.001)].As in 16S rRNA analysis, no significant genetic partitioning was observed between the Palk Bay and Gulf of Mannar Populations of *H. kuda* using the COI sequences. The AMOVA indicated that most of the molecular variance was occurred among populations (62.5%). The NJ and MP

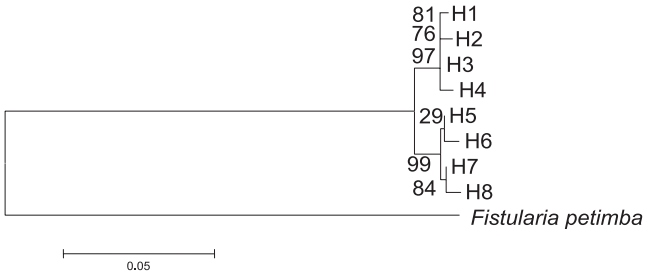

Fig. 2. Phylogenetic relationship of *Hippocampus kuda* populations based on COI partial sequence (Neighbor-Joining method)

trees revealed identical phylogenetic relationship among the samples collected from 4 coasts and separated the haplotypes of *H. kuda* population into 3 clusters with high bootstrap support (Fig. 2) in most of the nodes indicating the distinct genetic structure among the populations from the Indian coast.

The inability to identify individuals to species is one of the major factors limiting the questions that can be addressed in many ecological studies for conservation and rehabilitation of fishes. Morphological characters are sometimes of limited value for identification and differentiation purposes because they show a considerable intraspecific variation and differences among species are small. The conserved nature of 16S rRNA and COI mitochondrial gene segments enable them as diagnostic molecular markers in identification and resolution of taxonomic ambiguity. The technique of DNA barcoding has highlighted the expanding use of the COI, as a genetic marker for species identification (Hebert 2003). The present study on sequence analysis of *H. kuda* for 16S rRNA and COI gene segments yielded similar results. Estimates of genetic divergence with both 16S rRNA and COI genes when compared with the sequence divergence values of *H. kuda* from other continents (as obtained from NCBI accessions) were sufficient enough to discriminate individuals of the same species from Indian waters. These values and the levels of inter-specific variation correspond well with the reports in other teleosts (Lakra *et al.* 2008). Presence of common haplotypes and non-significant Φ_{ST} value between samples of Palk Strait and the adjacent Gulf of Mannar indicate the absence of distinct population structure in *H. kuda* along the east coast of India. But, significant pair-wise comparison of Φ_{ST} and the AMOVA values (with both 16S rRNA and COI) among east, Kerala and Konkan coasts and the absence of common haplotypes between the populations - all indicated the occurrence of 3 distinct population structure in *H. kuda* in Indian waters. The fact that no haplotypes was shared between the populations, suggest an interruption of gene flow for an efficient number of generations in Indian waters as observed by Lourie *et al.* (2004) in *H. trimaculatus*. The results are also indicative of low dispersal ability of seahorses as reported in other studies (Lourie *et al*. 2005, Teske *et al*. 2004 and 2005) and highlight

the role of life-history and dispersal strategies in gene-flow among populations. Seahorses lack larval stages with dispersal abilities, but colonization of distant habitats by small number of founding individuals by rafting (distribution by attaching to a raft such as floating seaweed) has been reported in many species, resulting in genetically isolated lineages (Teske *et al*. 2005). Our results may also reflect a genetic adaptation to specific climatic or environmental conditions, prolonged isolation of populations or possibly repeated extinction and recolonization events by small founding populations. Generally environmental conditions play a role in determining the dispersal and survival of tropical marine species (Maree *et al*. 2000, Bowen *et al*. 2001). Teske *et al*. (2005) attributed the strikingly different oceanographic conditions between east and west coasts of India as a major factor for the large differences in genetic diversity of seahorse populations from Indian waters. A finer-scale population genetics and phylogeography of the species with more intensive sampling at microgeographic level along the Indian coast line (possibly involving neighboring countries such as Sri Lanka and The Maldives) and using additional nuclear DNA markers (e.g., microsatellites or fast evolving single copy nuclear DNA) would be valuable in order to obtain a clear insight on the levels of gene flow between the populations of *H. kuda* in the region on a contemporary time-scale.

ACKNOWLEDGMENTS

The authors are grateful to Dr S Ayyappan, Director General, ICAR, for his support and encouragement.

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