The Bioscan 9(3):931-936, 2014
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

MITOCHONDRIAL DNA PHYLOGENY OF INDIAN SEAHORSE (*HIPPOCAMPUS KUDA*) BASED ON CYTOCHROME B GENE SEQUENCES

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KEYWORDS
Hippocampus
Seahorse
mtDNA
Cytochrome b
Phylogeny

Received on :
08.01.2014

Accepted on :
10.06.2014

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ABSTRACT
Syngnathidae family consists of 52 genera and over 215 species including *Hippocampus kuda*, popularly known as seahorse, which is one of the economically important species being utilized for Traditional Chinese Medicines. International Union for Nature and Natural Resources (IUCN) categorized this species as an endangered. We have carried out the genetic study on two populations (Tuticorin and Ratnagiri) of Indian seahorses using mtDNA cytochrome *b* sequence to reveal the genetic population structure and phylogenetic analysis of Indian seahorses. Our study revealed highly significant divergence in both Tuticorin (0.6429) and Ratnagiri (0.6667) seahorses Further, construction of NJ tree using mtDNA cytochrome *b* sequence revealed three lineages among *H. kuda*. One lineage consists of Tuticorin population, other lineage consists of Ratnagiri and the third lineage consists of samples from rest of the world. TMRCA (Time Most Recent Common Ancestors) value for kuda species of different regions was found to be 53,166 years ago. Thus suggests that the fall in the population size of *H. kuda* is due to overexploitation, but not because of any genetic bottleneck. The findings of the present study have important implications for in-situ conservation and stock management of hippocampus species along the Indian coasts.

INTRODUCTION

Syngnathidae family consists of fifty-two genera and over two hundred and fifteen species including *Hippocampus kuda*, popularly known as seahorse as described by Nelson (1994). *H. kuda* is a shallow-water species and generally found in seagrass/mangrove/estuarine/muddy areas less than 10 m deep as reported by Lourie *et al*. (2004). Recently, Thangaraj and Lipton (2007) had reported the occurrence of the *H. kuda* as are the most commonly available and economically important species in Indian waters. *H. kuda* is a species complex that can be divided into two major lineages, the first one from the Indian Ocean and the second one in the Western Pacific as reported by Teske *et al*. (2005). *H. kuda* is one of the most economically important species of the family Syngnathidae, being utilized for Traditional Chinese Medicines as reported by Lourie *et al*. (1999a). The estimated global trade of dried seahorses was over 20 million individuals seahorses (exceeding 50 metric tonnes) in 2000 for the traditional Chinese medicine market alone as reported by Salin *et al*. (2005); Lourie *et al*. (2004); Vincent, (1995a, b and c) and (1996a &b). 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 Rupees 2.673 million (US$ 70,000), with Chennai being the major port of trading activities as reported by MPEDA (2003). The commercial exploitation was carried out mainly at the Palk Bay and Gulf of Mannar areas in the south-east coast of India as reported by Salin *et al*. (2005). Overexploitation of seahorses from India had resulted in the decline of their population up to 60-70% and to stop this, Government of India took steps 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 (Madhabchandra, 2012). This decision thus impacted the trade of seahorses from India. International Union for Nature and Natural Resources (IUCN) categorized this species as an endangered. Lourie and Vincent (2004) sequenced a 696 bp fragment of cytochrome b (cyt b) gene of H. kuda from covering southern coasts of India, and suggested that the Indian populations were genetically similar to the populations from West of Wallace Line. Teske *et al*. (2005) based on the sequence information of mitochondrial control region (mtDNACR) analysed the colonization pattern of seahorses from Indian waters and reported distinct differences in genetic diversity between the south eastern (Tamil Nadu) and western Indian (Goa and Ratnagiri) populations of *H. kuda*. However, these studies suggested out the need for more comprehensive genetic data in order to get a clear picture of the population structure of the commercially important seahorse species from India. Attempts have been taken by Indian researchers to breed and rear seahorses in captivity for conservation have been successful in India in the recent past

for sea ranching in Indian coast as reported by Lipton and Thangaraj (2005). In order to plan an adequate conservation and management strategies for an endangered species, it is imperative to investigate its population history, geographical partitioning throughout its natural distributional range, and distribution of genetic diversity as reported by Lakra and Ayyappan, (2003). Genetic methods have great potential to distinguish distinct populations or stocks of fish species that cannot be identified by morphological and meristic characters (Teske *et al*. 2005 and Choudhary *et al*. 2012). Mitochondrial DNA (mtDNA) has been widely used as a molecular tool to identify both population structure and genetic variability because of its rapid evolutionary rate and almost complete maternal inheritance as reported by Kocher *et al*. (1989) and Irwin *et al*. (1991). Within mtDNA, the cyt b 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 to the population and recent divergence levels as reported by Chenoweth *et al*. (2002). In the present study, partial sequence information of the mtDNA cyt b gene was used to investigate the genetic population structure of two sea horse populations of *H. kuda* from Indian waters. The work was taken up to support the breeding and restocking programme of these seahorse species in Indian sea for in situ conservation purpose. The data are used to discuss the implications for conservation and management. Therefore, we attempted to study the mitochondrial (mtDNA) cytochrome b sequence of Indian seahorse (*H. kuda*) to assess the genetic population structure and phylogenetic relationship.

MATERIALS AND METHODS

Sample Collection and DNA isolation

A total of 50 live seahorses were collected from two different region of India, one at Tuticorin (east coast) and another at Ratnagiri (west coast). Of the 50 samples, 28 were collected from east coast, at Latitude of 8º48′ N and Longitude of 78º13′ E at the distance of 1 miles from the coast and at 5 metre depth using offshore trawlers and 22 were collected from west coast at Latitude of 16º55′ N and Longitude of 73º19′ E by hand nets and handpicking in shallow waters. Fin-clips were used whenever possible and captured seahorses were photographed and subsequently released into sea waters. Seahorse received appropriate anesthetics, to minimize pain and discomfort during preoperative, operative and postoperative procedures. All necessary approvals were obtained for experiments using seahorse fishes. Total DNA was extracted from the tissue samples according to the protocol modified and described by Thangaraj *et al*. (2000). Seahorses were dissected out and the gills were collected for DNA isolation. Gills were cut out into small pieces, ground with liquid nitrogen and suspended in reagent A (10mM Tris pH8.0, 320mM sucrose, 5mM $MgCl_2$, 1% triton X-100) and centrifuged at 3000rpm for 10 min. Supernatant was discarded and pellet was resuspended in 2mL of reagent B, (400mM Tris, 60mM EDTA, 150mMNaCl and 1%SDS) to which 40 μL of proteinase K (20 mg/mL) was added, mixed well and incubated at 50^{0}C in shaking waterbath for overnight. To the digest, 1mL of reagent C (5M sodium percholate) was added and shaken thoroughly. DNA was precipitated with ethanol after organic extraction with phenol and chloroform. DNA was washed with 70% ethanol and dissolved in TE buffer of pH 8.0 as reported by Thangaraj *et al*. (2003).

Table 1: Accession numbers of cytochrome b sequences belonging different genera and species of family Syngnathidae

Species	NCBI Accession no.
1. *Hippocampus zosterae*	AF356071
2. *Hippocampus sp*	AF356054
3.*Hippocampus abdominalis*	AF356065
4.*Hippocampus erectus*	AF356057
5. *Hippocampus comes*	AF356049
6. *Hippocampus barbori*	AF356048
7. *Hippocampus zosterae*	AF192706
8. *Hippocampus whitel*	AF192705
9. *Hippocampus whitel*	AF192704
10. *Hippocampus trimaculatus*	AF192703
11*Hippocampus trimaculatus*	AF192702
12. *Hippocampus trimaculatus*	AF192701
13. *Hippocampus trimaculatus*	AF192700
14.*Hippocampus trimaculatus*	AF192699
15.*Hippocampus trimaculatus*	AF192698
16. *Hippocampus subelongatus*	AF192697
17. *Hippocampus spinosissimus*	AF192696
18. *Hippocampus spinosissimus*	AF192695
19. *Hippocampus reidi*	AF192694
20. *Hippocampus reidi*	AF192693
21. *Hippocampus reidi*	AF192692
22. *Hippocampus mohnikei*	AF192689
23. *Hippocampus kuda*	AF356063
24. *Hippocampus kuda*	AF192687
25.*Hippocampus kuda*	AF192686
26. *Hippocampus kuda*	AF192685
27. *Hippocampus kuda*	AF192684
28. *Hippocampus kuda*	AF192683
29. *Hippocampus kuda*	AF192682
30. *Hippocampus kuda*	AF192681
31.*Hippocampus kuda*	AF192680
32. *Hippocampus kuda*	AF192679
33. *Hippocampus kelloggi*	AF192678
34. *Hippocampus kelloggi*	AF192677
35. *Hippocampus kelloggi*	AF192676
36.*Hippocampus breviceps*	AF192647
37. *Hippocampus camelopardalis*	AF192649
38.*Hippocampus capensis*	AF192652
39. *Hippocampus coronatus*	AF192659
40. *Hippocampus guttutatus*	AF192664
41. *Hippocampus hippocampus*	AF192666
42. *Hippocampus histrix*	AF192671
43. *Hippocampus ingens*	AF192674
44. *Syngnathus acus*	AF356073
45.*Syngnathus schlegeli*	AF356051
46.*Aulostomus strigosus*	AF327457
47.*Aulostomus maculatus*	AF327456
48 *Aulostomus chinensis*	AF327455
49.*Phyllopteryx taeniolatus*	AF356077
50 *Phyllopteryx taeniolatus*	AF356076
51.*Corythoichthys intestinalis*	AF356055
52.*Doryrhamphus dactyliphorous*	AF356047
53.*Microphis brachyurus*	AF356046
54.*Nerophis ophidion*	AF356043
55.Indostomus paradoxus	AP004438

PCR amplification and Sequencing:

The partial 315 bp seahorse cytochrome b gene was partially

Table 2: Different haplotypes of *H. kuda* from India and rest of the world.

Nt Position	498	501	518	523	526	555	564	565	569	573	583	594	606	612	620	657	658	669	678
Ref.	A	G	C	C	A	T	T	G	C	C	A	C	A	A	A	G	T	C	T
H1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H2	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
H4	.	.	.	.	C	C	G	.	G	G	.	.	.	.	C	.	G	.	.
H5	G	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H6	G	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H7	.	.	.	.	.	.	.	.	.	.	G	A	G	.	.	A	.	.	.
H8	.	A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
H9	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.
H10	.	A	.	.	.	.	.	.	.	.	.	A	G	.	.	.	.	T	.

Table 2: Cont.....

Nt Position	693	700	711	768	Tuticorin	Ratnagiri	India	Pakistan	Sri Lanka	Indo-Pacific	Thailand	Taiwan	Philippines	Japan	Hawain Island	Vietnam	Total haplotypes
Ref.	A	G	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-
H1	.	.	.	.	5		1		1	1							8
H2	.	.	.	.	1												1
H3		.	.	.	1												1
H4	.	.	.	.	1												1
H5	.	A	.	.		2		1									3
H6	.	A	.	.		1											1
H7	G	.	.	C			1										1
H8	.	.	.	.						1							1
H9	.	.	.	.						1					1	1	3
H10	.	.	C	.							1	1	1	1			4

amplified using universal primers (Verma and Singh 2003). Primer sequences were as follows*:*

forward (mcb398):5′ TACCATGAGGACAAATATCATTCTG3′

reverse (mcb869): 5′ CCTCCTAGTTTGTTAGGGA TGATCG 3′.

Primers were synthesized in an ABI392 oligosynthesiser (Perkin Elmer) at CCMB, Hyderabad and were used. Amplification was carried out in 10μL reaction volume containing 5.0ng of DNA, 10 pM of each primer, 200 pM of dNTPs, 1X PCR buffer containing 2.0 mM $MgCl_2$ and 2U of AmpliTaq Gold (Perkin Elmer). Amplification was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer) employing the conditions: 94ºC for 10 min; 30 cycles at 94ºC for 1 min, 55ºC for 1 min, 72ºC for 2 min and final extension at 72ºC for 5 min as per the protocol suggested by Verma and Singh., 2003. Amplified products were electrophorsed in 2% agarose gel. PCR products were directly sequenced as per the protocols modified and used by Thangaraj *et al*. (2003), using 50.0 ng (2.0µL) of PCR product, 4 pM (1.0µL) of primer, 4 µL of BigDye Terminator ready reaction mix (Perkin Elmer) and 3.0 µL of double distilled water to adjust the volume to 10.0 µl. Cycle sequencing was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer) employing the conditions: 30 cycles at 96ºC for 10 sec, 50ºC for 5 sec and 60º for 4 min. Extended products were purified by alcohol precipitation followed by washing with 70% alcohol. Purified samples were dissolved in 10 µl of 50% Hi-Di formamide and analysed in ABI 3700 automated DNA Analyzer (Perkin Elmer).

Data analysis

Partial sequence of 315 bp mtDNA cytochrome *b* sequence of the Indian seahorse of the present study were compared with the sequence from 33 different species of Hippocampus and 12 different genera of Syngnathidae family (downloaded from NCBI) and aligned using CLUSTAL X package as reported by Thompson *et al*. (1994). Accession number of sequences, which were downloaded, is given in Table 1. Sequence data generated from this article have been deposited with NCBI GenBank under accession numbers: (AY278753 - AY278763). The GENEDOC package (www.psc.edu/biomed/genedoc/gdpaf.html) was used for formatting the sequences to make them compatible with the desired software. Median joining network as described by Bandelt *et al*. (1999) was drawn using the Network program 3.1.1.1 (www.fluxus-engineering.com) with a weight of 10 and threshold 1. NJ/UPGMA, parsimony, and maximum likelihood trees were reconstructed using the PHYLIP Package Ver3.6 as reported by Felsenstein, (1993). Population pairwise $\ddot{o}_{ST}$ values, Nei's diversity value and Tau (t) values were computed using ARLEQUIN ver2.001 (http://anthropologie. unige/arlequin) as described by Excoffier *et al*. (1992). Mutation rate was calculated with formulae $\tau = 2\mu t$ as described by Luikart *et al*. (2001) using evolutionary time as 35 million years ago as suggested by Carrol *et al*. (1998).

RESULTS

Genetic diversity

Of the 315 bp sequence analysed, 13 substitutions were found among the Indian H. kuda. Of which 10 were be synonymous

Figure1: India map showing sampled area

and rest were nonsynonymous. Transition Vs transversion ratio of Tuticorin and Ratnagiri were 1:2 and 0:1, respectively. The Nie's diversity of the Tuticorin population was 0.6429 and Ratnagiri population was 0.6667. The mean number of pairwise differences of Tuticorn was found to be 2.25 + 1.39 and of Ratnagiri was 1.66 + 0.314. The genetic distance (Fst value) observed between Tuticorin and Ratnagiri population was 0.59741 (p = 0.005). Out of 28 and 22 samples analysed from Tuticorin and Ratnagiri, respectively, four haplotypes were observed in Tuticorin and two haplotypes in Ratnagiri samples (Table 2). Interestingly, there was no common haplotype found between Tuticorin and Ratnagiri samples. Median joining Network was constructed using Indian H. kuda along with H. kuda sequences from different parts of the world. The central node (Haplotype H1) of the network was shared by 5 samples from Tuticorin and one samples each from Sri Lanka, Indopacific and India (reported earlier). There were two haplotypes (H2 and H3) of Tuticorin, each one differ by single mutation from the central node. Another Tuticorin haplotypes H4 separated from the central node by 7 mutations (A498G, A583G, G657A, A693G, T768C, C594A, and A606G). Surprisingly, this is the only node that had 7 mutations (Fig. 2). There were two haplotypes separated from central node (H1) with two mutations. One of which was H. kuda isakuda (H8), a subspecies from Indopacific region (A612G, and G501A) and other haplotype (H8) consist of one seahorse each from Vietnam, Hawaiian and Indopacific (A606G and C594A). Haplotypes H7 and H10 differ from H9 by four mutations (A583G, G657A, A693G and T768C) and three mutations (T711C, C669T and G510A), respectively. Haplotype H5, consist of 2 Ratnagiri and 1 Pakistan seahorse were separated by three mutations (A498G, C523T, G700A). Haplotype H6, another Ratnagiri sample differ from H5 at one mutation (C518A).

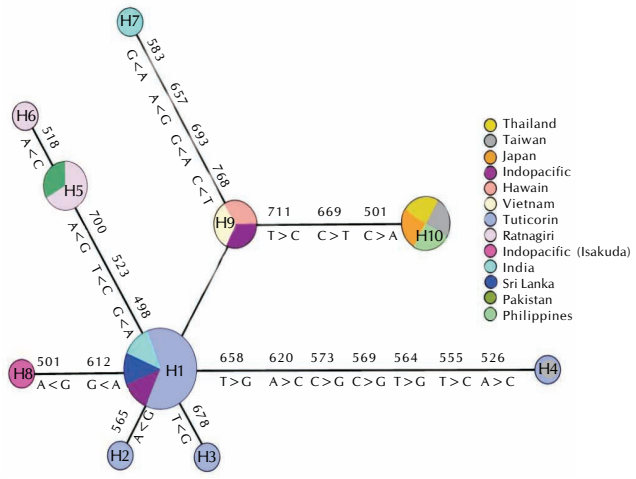

Figure 2: Median joining Network of *Hippocampus kuda*. Circles represent haplotypes and their size proportional to frequency

Phylogenetic Analysis

NJ tree (Fig. 3) reconstructed from sequences of different genera of Syngnathidae family showed star like phylogeny. Tree depectied three main clusters, one consists of pipefishes, which are ancestral to Hippocampus, second cluster consist of various species of Hippocampus, excluding kuda and third cluster consist of only H. kuda. Interestingly there were three lineages within H. kuda, one lineage contain H. kuda from Tuticorin, Sri Lanka, India (reported earlier) and Indopacific samples, Second lineage consist of Ratnagiri and one Pakistan sample and the third lineage consist of H. kuda from rest of the world (Fig. 3). Tuticorin and Ratnagiri clusters separated monophyletically with high bootstrap of 96/100. Low bootstrap values obtained within both Tuticorin and Ratnagiri lineages support their weak sub-branch.

Molecular dating

We calculated Time Most Recent Common Ancestor (TMRCA) values from available knowledge of divergence period of different genera of Syngnathidae family. Tau (τ) value resulted from Hippocampus species was found to be 49.083. Mutation rate was estimated to 0.022/site/million years i.e., 2200 years for each mutation. This mutation rate was fed into time estimate package of median joining network and TMRCA was calculated. TMRCA value for kuda species of different regions was found to be 53,166 years ago.

DISCUSSION

We have analysed the mtDNA cytochrome b sequences of H. kuda from east (28 samples) and west coast (22 samples) of India, which revealed 4 and 2 haplotypes, respectively. Intrestingly there were no common haplotypes between these two populations, though both the population inhabited in the same subcontinent. However, when samples from other geographical region such as Thailand, Taiwan, Phillipines, Indopacific, Hawaiian Islands, Sri Lanka, Pakistan, and Japan were compared, we found one haplotype being shared between 2 Ratnagiri and one Pakistan samples, similarly one haplotype was shared between 5 Tuticorin and one sample each from Sri Lankan, Indopacific, and Indian seahorse as

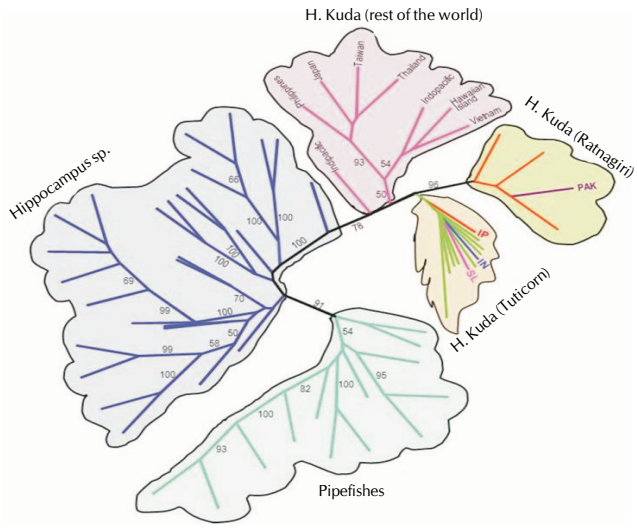

Figure 3: Neighbor-joining tree of Syngnathidae family. Tree shows three major clusters consisting of pipefishes, different species of Hippocampus and *Hippocampus kuda* of different regions. Cluster containing Hippocampus kuda shows three lineages. Bootstrap value above 50 is given

observed by Lourie and Vincent (1999b). We found significant diversity within and between Tuticorin and Ratnagiri samples. Computation of Nei's diversity showed high degree of diversity value in both of Tuticorin (0.6429) and Ratnagiri (0.6667) samples, despite very small sample size (Nei, 1987). Similar results have been reported by Song et al. (2008) in other teleosts and by Lourie and Vincent (2004) in seahorses. One of the objectives of this study was also to know whether the reduction in the sample size is due to high degree of homozygosity leading to bottleneck or due to over exploitation. Since both the populations showed significant diversity, reduction in the population size might be due to overexploitation this result is in concurrence with earlier reported work by Song et al. (2008). NJ tree constructed with 100 bootstraps as described by Hillis, (1993) revealed 3 major clusters, one consisting of pipefishes, ancestor to Hippocampus, another cluster consisted different Hippocampus species and the third cluster made of H. kuda species. It was interesting to observe three lineages within H. kuda species, one lineage consists of Ratnagiri and Pakistan, other lineage consists Tuticorin, Sri Lanka, Indopacific and Indian sample as in concurrence with Lourie and Vincent (2004) and Song *et al*. (2008), and third lineage consists of samples from rest of the world. Thus, we propose 3 different lineages within H. kuda species (Fig 3). Although both Tuticorin and Ratnagiri populations are from Indian subcontinent, both the populations are of different lineage. Probable reason for two lineages within the subcontinent could be because the Tuticorin is in east coast and the Ratnagiri in west coast. The findings of this study are of considerable importance in conjunction with the breeding and conservation programmes of H. kuda. The observed high values of genetic heterogeneity between the east and west coast populations of these seahorses suggest that they belong to different populations. The results are also indicative of low dispersal ability of seahorses as reported in other studies by Teske et al. (2005) and highlighted the role of life-history and dispersal strategies in gene-flow among populations. Wilson, et al. (2011) also suggested about estimation of TMRCA value which was also estimated that these three lineages might have separated about 53,000 years ago as per the TMRCA value. Our estimation is based on the information that the Syngnathidae family diverged into different genera and species before 35 million years ago as reported by Kimura (1980) and Carroll, (1988). From a genetics resource conservation and management perspective, the conclusion of the present study is expected to give precious demographic information to define the various breeding strategies of H. kuda for restocking. Genetic variation is pivotal for populations to adapt to changing environmental or demographic events. The efficacy of a restocking programme is influenced by the genetic variation of the Broodstock and associated propagation practices as reported by Lipton and Thangaraj (2005). Based on the present results, it is revealed that the broostock and wild populations of the species under study from the east and west coasts of India should be managed separately finds concurrence with Dhivya et al. (2011). It is suggested to utilize the progeny of broodstock from the same population/coast for restocking purposes to maintain genetic integrity. These strategies would also ensure that the original stock will not be contaminated from genetic materials elsewhere, thereby avoiding any possibilities of hybridization and dilution of gene pool, which could possibly lead to extinction of the native stock as reported by Hughes *et al*. (1999) and (2002). Hence, we suggest to ecologists and biologists and institutions to come forward to save seahorse dwindling populations from extinction by launching the restocking and sea ranching programme.

ACKNOWLEDGEMENT

We acknowledge Mr. Sunil Kumar Verma, Scientist, CCMB, Dr. K. Thangaraj, Scientist E, CCMB, for providing universal primers and deep insight for carrying out the molecular experiments at CCMB, Hyderabad.

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