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SHORT RESEARCH NOTE

Genetic heterogeneity in the Indian stocks of seahorse (*Hippocampus kuda* and *Hippocampus trimaculatus*) inferred from mtDNA cytochrome b gene

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Abstract Genetic stock structure analysis of two seahorse species from the south east and south west coasts of India (37 samples of *Hippocampus kuda* and 39 samples of *Hippocampus trimaculatus* from Kollam (Kerala) and Mandapam (Tamil Nadu)) was carried out through sequence variation analysis of a 350 bp cytochrome *b* fragment of mitochondrial DNA. This was taken up to support the breeding and restocking programme of these species in natural habitats for conservation purpose. The occurrence of strong genetic subdivision among the samples, detected by the analysis of molecular variance (AMOVA), and significant Φ_{ST} values indicated that stocks of both the species in the two Indian coasts are distinct. The findings of the present study have important implications for conservation and management of these two species and we recommend stock-specific, breeding assisted sea-ranching programme for *H. kuda* and *H. trimaculatus* along the Indian coasts.

Keywords *Hippocampus kuda* · *Hippocampus trimaculatus* · Seahorse · mtDNA · Cytochrome *b* · Population genetic analysis · Conservation

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The family Syngnathidae consists of about 33 species of seahorses worldwide (Lourie et al., 2004), and of these, five species viz., *Hippocampus fuscus*, *Hippocampus histrix*, *Hippocampus kelloggi*, *Hippocampus kuda*, and *Hippocampus trimaculatus* are reported from India (Lourie et al., 1999, 2004; Lipton & Thangaraj, 2002). Recently, Thangaraj & Lipton (2007) had reported the occurrence of the sixth species, the Japanese seahorse (*H. mohnikei*) also from Indian coast. *H. kuda* and *H. trimaculatus* are the most commonly available and economically important species in Indian waters. *H. kuda* is a shallow-water species and generally found in seagrass/mangrove/estuarine/muddy areas less than 10 m deep (Lourie et al., 2004), while *H. trimaculatus* is found at depths of at least 10–15 m, evidently on more open substrates such as sand or gravel and/or in association with octocorals or sponges (Lourie et al., 1999). *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 (Teske et al., 2005). The three-spot seahorse (*H. trimaculatus*) distributed in the Indo-Pacific region also contains two distant lineages: the Western (from India to Japan) and the Eastern (Philippines, Sabah and Eastern Indonesia) lineages (Lourie & Vincent, 2004).

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; Vincent, 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 Rupees 2.673 million (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). Overexploitation 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 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. This has impacted the trade of seahorses from India.

Although the seahorses have been used for various purposes, there is a dearth of information on the population structure of *H. kuda* and *H. trimaculatus* inhabiting different ecological regions along the Indian coast. Knowledge on seahorses from India is limited to the studies mainly on their taxonomy based on morphology, species diversity, and distribution (Salin et al., 2005). Lourie & Vincent (2004) sequenced a 696 bp fragment of cytochrome *b* (cyt *b*) gene of *H. trimaculatus* from Indo-Pacific region covering southern coasts of India, and concluded 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) studied the colonization pattern of seahorses from Indo-Pacific region including 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 pointed out the need for more comprehensive data in order to get a clear picture of the population structure of the two commercially important species from different regions including India.

Attempts to breed and rear seahorses in captivity for conservation have been successful in India in the recent past, and steps have been initiated to sea-ranch seahorses in south east and south west coasts of India by the research organizations funded by the Indian government (Lipton & Thangaraj, 2005). In order to devise adequate conservation and management strategies for an endangered species, it is important to investigate its population history, geographical partitioning throughout its natural distributional range, and distribution of genetic diversity (Lakra & 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). Mitochondrial DNA (mtDNA) has been widely used to identify both population structure and genetic variability because of its rapid evolutionary rate and almost complete maternal inheritance (Song et al., 2008). 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 (Chenoweth et al., 2002, Kotlik & Berrebi, 2001). In the present study, partial sequence information of the mtDNA cyt *b* gene was used to investigate the population structure of two sea horse species, *H. kuda* and *H. trimaculatus* from Indian waters. The work was taken up to support the breeding and restocking programme of these two species in Indian seas for conservation purpose. The data are used to discuss the implications for conservation and management of programmes of these two species in natural habitats.

About 37 samples of *H. kuda* and 39 samples of *H. trimaculatus* were collected from two different regions of India. Of the 37 samples of *H. kuda*, 20 were from Mandapam, Tamil Nadu (East coast of India), at latitude 9° 16′39″ and longitude 79° 07′27″ E; and 17 were from Kollam, Kerala (West coast of India), at latitude 8° 52′29″ N and longitude 76° 35′18″ E. Of the 39 samples of *H. trimaculatus*, 15 were from landings at Kollam and 24 were from Mandapam.

Fin-clips were used whenever possible and captured seahorses were photographed and subsequently released. Total DNA was extracted from the tissue samples according to the protocol described by Thangaraj et al. (2003). Cyt *b* gene (350 bp) of *H. kuda* and *H. trimaculatus* were amplified using universal primers—forward (mcb398): 5′TAC CATGAGGACAAATATCATTCTG3′ and reverse (mcb869): 5′CCTCCTAGTTTGTTAGGGATTGAT CG3′ (Verma & Singh, 2003). The reaction mixture (20 µl) contained 5 ng of DNA, 10 pM of each primer, 200 µM of dNTPs, 1X PCR buffer containing 2.0 mM $MgCl_2$ and 2 U of AmpliTaq Gold (Perkin Elmer). Amplification was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer) employing the conditions: 95°C for 10 min, 35 cycles of 95°C for 45 s, 51°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min. Amplified products were electrophoresed in 2% agarose gel. PCR products were sequenced using 50 ng (2 µl) of PCR product, 4 pM (1.0 µl) of primer, 4 µl of BigDye terminator ready reaction mix (Perkin Elmer, Foster City, USA) and 3.0 µl of double distilled water to adjust the volume to 10.00 µl (Thangaraj et al., 2003). Before cycle sequencing, 5 µl PCR products were treated with 2 µl of Exosap and incubated 37°C for 15 min followed by 80°C for 15 min. Cycle sequencing was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer) employing the conditions: 30 cycles at 95°C for 10 s, 50°C for 5 s and 60°C for 4 min. Extended products were purified by alcohol precipitation followed by washing with 80% alcohol. Purified products were dissolved in 10 µl of 50% Hi-Di formamide and analysed in ABI 3700 automated DNA Analyser (Perkin Elmer, Foster City, USA). The sequences were identified as mtDNA cyt *b* region through comparison with 11 other species of seahorses and other syngnathids based on BLAST in the NCBI and all the haplotypes were submitted to the GenBank under accession numbers EF641032-EF641107. All nucleotide sequences were aligned using CLUSTAL X 1.8 multiple alignment programme (Thompson et al., 1997) and refined manually. The GENEDOC package (www.psc.edu/biomed/genedoc/gdpf.html) was used for formatting the sequences to make them compatible with the desired software. Sequence polymorphism was analysed, genetic divergence values within and between populations were estimated and a neighbor-joining (NJ) tree was constructed for all the haplotypes according to Kimura 2-parameter (K2P) model using PHYLIP ver 3.6 (Felsenstein, 1993) in MEGA version 3.1 (Kumar et al., 2004). The NJ tree was rooted with the cyt *b* sequences of *H. kuda* and *H. trimaculatus* from the Pacific Ocean (NCBI GenBank Accession #s AF192682 and DQ912706, respectively). Nucleotide diversity (π) and haplotype diversity (h) were estimated using DnaSP 4.0 (Rozas et al., 2003). Population structure was evaluated using the analysis of molecular variance (AMOVA) model in the ARLEQUIN ver 3.0 software (Excoffier et al., 2005). Fixation indices (Φ_{ST}) analogous to F_{ST} (Hudson et al., 2002), based on the haplotype frequency distribution and levels of sequence divergence among haplotypes, 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.

A total of 350 bp of the cyt *b* gene fragment were successfully sequenced in 37 individuals of *H. kuda* and 39 individuals of *H. trimaculatus* from Mandapam and Kollam populations. In total, eight unique haplotypes with six variable sites (1.7%) were identified in 37 samples of *H. kuda* and nine unique haplotypes with six variable sites (1.7%) were identified in 39 samples of *H. trimaculatus* (Table 1). In both the species, among the polymorphic sites, most were transitions and only a few were transversions. As expected, most of the changes occurred at the 3rd position of the codon, resulting in always synonymous substitutions. The mean number of nucleotide composition in *H. kuda* was A = 27.80%, T = 28.50%, C = 29.50% and G = 14.20% and in *H. trimaculatus* was A = 26.80%, T = 29.10%, C = 29.20% and G = 14.90%.

In *H. kuda*, of the eight haplotypes, four were specific to Mandapam population and the remaining four were unique to Kollam. Similarly, out of the nine haplotypes in *H. trimaculatus*, five haplotypes were from Kollam population and the remaining four were found in the Mandapam population. Interestingly, these two populations did not share any cyt *b* haplotypes in either the species. Nucleotide and haplotype diversities for different populations are shown in

Table 1 Variable nucleotide positions of cytochrome *b* haplotypes, nucleotide and haplotype diversities in Mandapam and Kollam samples of *H. kuda* and *H. trimaculatus*

Haplotypes	Nucleotide position[#]						Haplotype and nucleotide diversities				
Hippocampus kuda							*Hippocampus kuda*				
	12	*24*	*36*	*48*	*72*	*344*	Population	Number of sequences	Number of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)
Mandapam HkM 1	T	C	T	A	C	A					
Mandapam HkM 2	·	T	C	·	·	·	Mandapam	20	4	0.52	0.004
Mandapam HkM 3	·	T	·	G	·	·					
Mandapam HkM 4	·	T	·	·	·	·					
Kollam HkK 1	·	T	·	·	·	G	Kollam	17	4	0.61	0.006
Kollam HkK 2	C	T	·	·	·	G					
Kollam HkK 3	·	T	·	·	T	G					
Kollam HkK 4	·	T	·	·	·	C	Overall	37	8	0.56	0.005
Hippocampus trimaculatus							*Hippocampus trimaculatus*				
	15	*18*	*30*	*38*	*48*	*109*					
Mandapam HtM1	A	C	G	T	A	C	Mandapam	24	4	0.47	0.007
Mandapam HtM2	G	·	·	·	·	·					
Mandapam HtM3	G	T	·	·	·	·					
Mandapam HtM4	G	·	·	C	·	·	Kollam	15	5	0.39	0.005
Kollam HtK1	G	·	·	·	·	G					
Kollam HtK2	G	·	A	·	·	G					
Kollam HtK3	G	·	·	·	T	·					
Kollam HtK4	G	·	·	·	T	G	Overall	39	9	0.43	0.006
Kollam HtK5	·	·	·	·	·	G					

[#] The vertical numbers indicate the position of variable nucleotides within the 350-bp cytochrome *b* sequence. Dots indicate that the same nucleotide is present as in haplotype 1

Table 1. Haplotype diversity was high, but nucleotide diversity was low for overall populations in both the species (for *H. kuda* 0.005 and for *H. trimaculatus* 0.006). The sequence data also revealed six polymorphic sites (substitutions) in both the populations of *H. kuda* and *H. trimaculatus* (two each in Kollam and four each in Mandapam). Transition to transversion ratio (overall populations) for *H. kuda* and *H. trimaculatus* were 2.0 and 1.3, respectively.

The mean genetic divergence value between the two populations of *H. kuda* was 0.00524 and of *H. trimaculatus* was 0.00525. Based on Kimura-2 Parameter, pair-wise genetic divergence among haplotypes of both the species is given in Table 2. Pair-wise Φ_{ST} values after sequential Bonferroni corrections indicated significant levels of genetic differentiation in both the species between Kollam and Mandapam (*H. kuda* $\Phi_{ST} = 0.6528$, $P < 0.001$; *H. trimaculatus* $\Phi_{ST} = 0.6337$, $P < 0.001$). The AMOVA was conducted to describe variance components of Kollam and Mandapam populations based on the haplotype frequency differences. Most of the molecular variance was observed to occur among populations (65.72% for *H. kuda* and 62.68% for *H. trimaculatus*). The values were significant ($P < 0.001$) and revealed the occurrence of genetic partitioning between Kollam and Mandapam populations in both the species (Table 3). A NJ tree separated the cyt *b* haplotypes of populations of both the species into two main clusters with high bootstrap support (92–100%) indicating the distinct genetic structuring between the east and west coast populations of sea horses along Indian coasts (Fig. 1).

Table 2 Pair-wise K2P genetic divergences values among haplotypes of *H. kuda* and *H. trimaculatus* respectively as observed in phylogenetic trees

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]
Hippocampus kuda									
[1]									
[2]	0.00287								
[3]	0.00287	0.00575							
[4]	0.00000	0.00287	0.00287						
[5]	0.00575	0.00865	0.00865	0.00575					
[6]	0.00575	0.00865	0.00865	0.00575	0.00575				
[7]	0.00575	0.00865	0.00865	0.00575	0.00575	0.00575			
[8]	0.00287	0.00575	0.00575	0.00287	0.00287	0.00287	0.00287		
[1]	*H. kuda* Kollam HkK1								
[2]	*H. kuda* Kollam HkK2								
[3]	*H. kuda* Kollam HkK3								
[4]	*H. kuda* Kollam HkK4								
[5]	*H. kuda* Mandapam HkM1								
[6]	*H. kuda* Mandapam HkM2								
[7]	*H. kuda* Mandapam HkM3								
[8]	*H. kuda* Mandapam HkM4								
Hippocampus trimaculatus									
[1]									
[2]	0.00286								
[3]	0.00573	0.00286							
[4]	0.00573	0.00286	0.00573						
[5]	0.00572	0.00286	0.00572	0.00572					
[6]	0.00860	0.00572	0.00860	0.00860	0.00286				
[7]	0.00860	0.00572	0.00860	0.00860	0.00286	0.00572			
[8]	0.00860	0.00572	0.00860	0.00860	0.00286	0.00572	0.00000		
[9]	0.00572	0.00286	0.00572	0.00572	0.00000	0.00286	0.00286	0.00286	
[1]	*H. trimaculatus* Mandapam HtM1								
[2]	*H. trimaculatus* Mandapam HtM2								
[3]	*H. trimaculatus* Mandapam HtM3								
[4]	*H. trimaculatus* Mandapam HtM4								
[5]	*H. trimaculatus* Kollam HtK1								
[6]	*H. trimaculatus* Kollam HtK2								
[7]	*H. trimaculatus* Kollam HtK3								
[8]	*H. trimaculatus* Kollam HtK4								
[9]	*H. trimaculatus* Kollam HtK5								

The aim of the study was to obtain a clear picture of the population genetic structure of the two seahorse species of high commercial importance *H. kuda* and *H. trimaculatus* from Indian waters, which will be helpful in their conservation and restocking programmes in natural habitats. Recent attempts to breed and rear seahorses in captivity have been successful in India (Lipton & Thangaraj, 2005). This success has opened an opportunity for mass production of these valued species for restocking in natural habitats for conservation purposes. Information on within species genetic diversity is highly essential for the management and protection of wild fish resources.

Table 3 AMOVA of mtDNA cytochrome *b* sequences for two populations of *H. kuda* and *H. trimaculatus*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indices	*P*-value
Hippocampus kuda						
Among populations	2	29.363	1.08919 Va	65.72	$\Phi_{ST} = 0.65719$**	$P < 0.001$
Within populations	40	17.846	0.56815 Vb	34.28		
Total	42	47.209	1.65734			
Hippocampus trimaculatus						
Among populations	2	15.089	0.50088 Va	62.68	$\Phi_{ST} = 0.62689$**	$P < 0.001$
Within populations	42	8.389	0.29810 Vb	37.32		
Total	44	23.478	0.79898			

** Significant test after 10,000 permutations

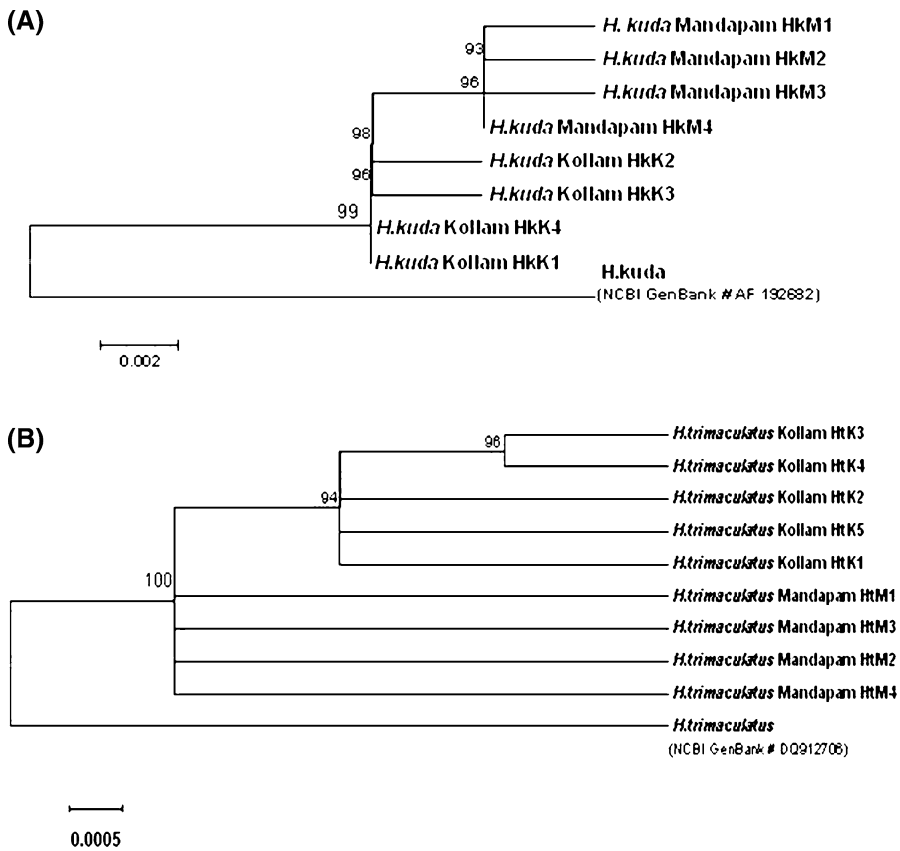

Fig. 1 Neighbor-joining tree of the mtDNA cyt *b* regions of *Hippocampus kuda* (**A**) and *Hippocampus trimaculatus* (**B**). The numbers at each node represent bootstrap proportions (%) based on 1,000 pseudoreplications

The mtDNA cyt gene partial sequences revealed eight haplotypes in *H. kuda* and nine haplotypes in *H. trimaculatus* based on the nucleotide variation. The overall haplotype (h) and nucleotide diversity (π) in *H. kuda* was 0.56 and 0.005, respectively, and in *H. trimaculatus* 0.43 and 0.006, respectively. The haplotype diversity was high compared to the nucleotide (sequence) diversity in both the species, primarily because the haplotypes were only separated by a maximum of five nucleotide differences. Similar results have been reported by Tinti et al. (2002) and Song et al. (2008) in other teleosts and by Lourie & Vincent (2004) in *H. trimaculatus* across huge geographical distances (>10,000 km from India to Japan)

on the continental shelf. Lourie & Vincent (2004) suggested that this phenomenon (low sequence diversity, yet high haplotype diversity) may be as a result of the more recent range expansion events that would have erased any previous phylogenetic structure in *H. trimaculatus* in the region.

Analyzing 100 samples of *H. trimaculatus* from the Indo-Pacific region (including two samples from Kerala (south-west Coast of India) and six samples from Tamil Nadu (south-east coast of India), Lourie & Vincent (2004) demonstrated a major east–west genetic split similar to the terrestrial Wallace Line. They also reported the distribution of the most abundant haplotype, A12 (696 bp cyt *b* gene) from India to Java (Indonesia). Interestingly, they observed the fixed haplotype difference in *H. trimaculatus* between the east and west coasts of India—the haplotypes A19 and A32 of lineage A were confined to the Kerala (south-west) coast while the haplotypes A01, A02, A12, and A28 restricted to the coasts of Tamil Nadu. Though the sample size was small from Indian waters, their study indicated little or no migration occurred between the Indian populations of *H. trimaculatus*. The present study was focussed entirely on Indian population(s) of *H. trimaculatus* and used a larger sample size (39). The significant pair-wise comparison of Φ_{ST} and the AMOVA values (350 bp cyt *b* gene) between Kollam (Kerala, 15 samples, collection site adjacent to Lourie & Vincent, 2004) and Mandapam (Tamil Nadu, 24 samples, collection site same as in Lourie & Vincent, 2004) and the absence of common haplotypes between the populations, all indicated the occurrence of distinct population structure in *H. trimaculatus* in Indian waters as observed by Lourie & Vincent (2004). 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. trimaculatus* in the region on a contemporary time-scale.

The large differences amongst Kollam (west coast, 17 samples) and Mandapam (east coast, 20 samples) populations of *H. kuda* observed in the present study (supported by significant Φ_{ST} and AMOVA and no sharing of haplotypes) were expected since Teske et al. (2005) using 380 bp sequence data of mtDNA control region (CR) reported striking differences in terms of genetic diversity between the Tamil Nadu (24 samples) and Ratnagiri/Goa (11 samples, west coast) lineages of *H. kuda*, even though the CR basal/root haplotype was shared by the east and west coast populations of *H. kuda.* However, in the present study, no cyt *b* haplotypes were shared by Kollam (Kerala) and Mandapam (Tamil Nadu) populations of this species. Kollam is approximately 1,360 km south of Ratnagiri and is closer to the east coast (Tamil Nadu). Within mtDNA, the non-coding CR evolves five times faster than the coding region and often has higher variability and greater efficiency to share intra-specific differences in teleosts (Song et al., 2008). Considering these two aspects, further deeper analyses with intensive sampling are warranted in order to examine the occurrence of more regional populations of *H. kuda* in Indian waters.

The findings of this study are of considerable importance in conjunction with the breeding and conservation programmes of *H. kuda* and *H. trimaculatus*. 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 (Lourie et al., 2005; Teske et al., 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). However, Morgan (2007) has suggested planktonic phases in *H. spinosissimus* (8–10 days) and *H. comes* (5–10 days).The results of the present study 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 *H. kuda* populations from Indian waters. From a resource conservation and management perspective, the conclusion of the present study is expected to give precious demographic information to define the breeding strategies of both *H. kuda* and *H. trimaculatus* 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 (Allendorf et al., 1987). Based on the present results, it seems intuitive that the broodstocks and wild populations of the two species under study from the east and west coasts of India should be managed separately. It is advisable to utilize the progeny of broodstock from the same population/coast for restocking purposes or that broodstocks are sourced from the respective population to be stocked to maintain genetic integrity. These strategies would ensure that the original stock will not be contaminated from genetic materials else where, thereby avoiding any possibilities of hybridization and dilution of gene pool, which could possibly lead to extinction of the native stock (Hughes et al., 1999, 2002).

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