Morphological and molecular evidence for range extension and first occurrence of the Japanese seahorse, *Hippocampus mohnikei* (Bleeker 1853) in a bay-estuarine system of Goa, central west coast of India

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Abstract

Accurate information of taxonomy and distribution range of seahorse species (genus *Hippocampus*) is the first step in preparing threat assessments and designing effective conservation measures. Here, we report the range expansion and first occurrence of the Japanese seahorse, *Hippocampus mohnikei* (Bleeker, 1853) from the Mandovi estuarine ecosystem of Goa, central west coast of India based on morpho-molecular analyses. The morphometric and meristic traits particularly, short snout length, double cheek spine, low coronet and long tail observed in all four collected specimens matched with the reported diagnostic morphological criteria of *H. mohnikei*. The seahorse mitochondrial cytochrome oxidase subunit I (COI, 609 bp) and cytochrome *b* (Cyt *b*, 639 bp) genes were partially sequenced for conclusive genetic identification of the species under study. Using the maximum similarity with GenBank database, maximum likelihood network and subsequent morphological analysis, the identity of the collected seahorse species was reconfirmed as *H. mohnikei*. With this new report, the distributional range of *H. mohnikei* extended significantly to the westward side from its hitherto known range of Japan and Vietnam.The possible role of prevailing oceanic circulation in the Indo-Pacific region in facilitating dispersal and range extension of *H. mohnikei* has been highlighted. Comparison of the pair-wise genetic distances (Kimura 2-parameter) based on COI and Cyt *b* sequences revealed that the conspecificity of Goa specimens with populations of *H. mohnikei* from Vietnam and Thailand rather than Japan. To test the hypothesis whether *H. mohnikei* populations are vagrants or residents of a bay-estuarine system, long-term inter-annual sampling and analyses are warranted.

Introduction

Seahorses (Syngnathiformes; Syngnathidae) belonging to the single genus, *Hippocampus* (Rafinesque, 1810) are a fascinating and remarkable group of fishes with their unusual body

shape and their biology, with males incubating the fertilized eggs in a brood pouch [1]. They are small, cryptic, and sedentary marine fishes that occur worldwide in shallow temperate to tropical waters in a wide variety of habitats, including seagrass beds, estuaries, coral and rocky reefs and mangroves [2−4]. Their remarkable ability to camouflage with structurally complex habitats further reduces the risk of predation [5], while providing stealth for prey capture [6]. However, such biological traits often also make them unnoticeable from coastal habitat, particularly in those regions where seahorse trade is absent. Often overlooked by most fishermen, cryptic nature, ability to camouflage and the sparse distribution restrict biologists to identify their presence in thick coastal marine habitats.

Out of 41 valid seahorse species (Genus *Hippocampus*) throughout the world [7], *Hippocampus kelloggi*, *H. kuda*, *H. spinosissimus* and *H. trimaculatus* are most common in Indian coastal marine waters [8,9]. In addition, sightings based on morphological identification of single specimens, *H. mohnikei* from the Palk Bay, southeastern India [10], *H. borboniensis* [11] and *H. montebelloensis* [12] from Gulf of Mannar, southeast coast of India and *H. camelopardalis* from Gulf of Kachchh, northwest coast of India [13] have been reported. Another seahorse species, *H. histrix* is also suspected to occur in Indian coastal waters [14].

The utility of morphological traits commonly used in diagnosis of *Hippocampus* spp. is quite challenging as they lack certain key physical features (e.g. pelvic and caudal fins), high variability and overlapping of body proportions, colour (camouflage) and skin filaments within and between species [3,15]. On the other hand, molecular methods have proved helpful in resolving morphologically challenging seahorse taxonomy [7,16], phylogenetic relationship within genus, *Hippocampus* [17,18], natural species boundaries [19−21] and genetic variability [20,22] of many seahorse species. An integrated approach of combination of morphological and genetic analyses [23] would aid in the management of demographically

separate populations as independent units and allow international legal mechanisms and international agreements such as the convention on international trade in endangered species (CITES) to work effectively [24].

Generally, seahorses are sedentary fish species with small home range and patchy distribution in sheltered areas such as seagrasses and seaweeds [2,25,26]. However, instances of small migration of seahorses in search of proper habitat, food or holdfasts have been reported [26,27]. Evidence of long-distance dispersal of seahorse species via rafting (floating debris and seaweeds) [2,28,9] and subsequent colonisation [20] have been documented. The role of oceanic currents facilitating as stepping-stones in long-distance dispersal and range expansion of many seahorse species has also been highlighted [29−34].

The Japanese seahorse or Lemur-tail seahorse, *Hippocampus mohnikei* (Bleeker, 1853) is a small coastal seahorse species (5–8 cm) inhabiting seagrasses, mangroves, oyster beds and mussel farms along the coasts of continental Asia from southeastern India to Korea and Japan [35]. Previous reports [15,36−38] indicated that the distribution of *H. mohnikei* is restricted or native to the coastal waters around Japan and Vietnam. As a consequence of recent exploitations [35,39−41], the status of *H. mohnikei* in the IUCN Red List of Threatened Species has been listed as 'vulnerable' [42].

A probable occurrence of *H. mohnikei* along the central west coast of India (CWCI) began in February 2017 when a fisherman posted a picture of seahorse incidentally caught in gill net operated in the Chapora estuary, Goa (India) (15.6120° N, 73.7506° E) on social media [S1 Fig.] which was suspected to be an adult male of *H. mohnikei*. Sustained follow-up surveys for its occurrence in the surrounding environs of the Chapora estuary did not yield in any further reporting. This contribution describes the range expansion and first occurrence of *H. mohnikei* from the Mandovi estuarine ecosystem, Goa, CWCI. To test further the hypothesis that the morphologically identified seahorse species is *H. mohnikei*, gene markers (COI and

Cyt *b* genes) were employed for reconfirmation. Potential role of ocean currents on the range expansion of *H. mohnikei* to north-western Asia have also been discussed. In the wake of the vulnerability of seahorse populations to the threats such as habitat alteration/destruction and fishing pressure globally, the present sighting of *H. mohnikei* in a bay-estuarine system is of considerable conservation and biogeographic significance.

Materials and Methods

Collection site and seahorses

The collection site of seahorses (Brittona, Goa, India, 15.3059°N, 73.5073° E, Fig 1) is a shallow water littoral environment which is in the proximity to the mangrove-dominated Chorao Island in the Mandovi estuary, CWCI. The currents are largely tide dominated, the tides being semi-diurnal with a mean amplitude of 1.3 m. Currents are also influenced by the large seasonal freshwater influx [43] during the summer monsoon season (June to September). Temporal variations in abiotic and biotic factors influenced by monsoonal precipitation and seasonal upwelling render the collection location as one of the most ecologically complex ecosystems [44,45]. Significant lowering of salinity occurs when the freshwater discharge is at its maximum during monsoon. During the post- and pre-monsoon periods, the salinity at the surface and bottom remains high (~25 ppt). The bottom topography at the collection site is characterized by muddy and comprises mainly of silt and clay with rich organic matter content [46]. The collection site is reported to harbour rich and diverse fishery resources and its importance in supporting the life history stages of several marine teleosts has also been well-documented [47]. Present report is based on four specimens of *H. mohnikei* (Fig. 2. and Fig. 3) that were incidentally caught in bag nets attached to the fixed stakes (total length, 25 m; mouth length, 5 m; cod end mesh size, 10 mm) operated at the water depth of ~6 m in the Mandovi estuary during July 2018. Freshly landed dead seahorses

as incidental catch in bag nets attached to the stakes operated by local fishermen in the estuarine system formed the study material. No live seahorses were specifically caught for the present study. Fin clips from these dead seahorses were used for molecular analysis. The present study did not involve any experimentation with live animals and hence ethics committee's approval for the usage of live animals for experiments is inconsequential.

Fig 1. Sampling localities of *H. mohnikei*. A map showing major rivers and estuaries of Goa (central west coast of India). Location of a suspected adult male *H. mohnikei* in Chapora estuary (●); collection site of confirmed report of *H. mohnikei* in the Mandovi estuary (★).

Fig 2. Collected seahorses from Goa. Specimens of *Hippocampus mohnikei* (MK1 and MK2 = females; MK3 and MK4 = males) collected from the Mandovi estuary, Goa, central west coast of India.

Fig 3. Close-up of *H. mohnikei*. A closer view of *Hippocampus mohnikei* (A-male; B-Female) collected from the Mandovi estuary, Goa, central west coast of India.

Morphological analysis

Before subjecting them to a detailed analysis of morphological characteristics, seahorses were carefully photographed (Nikon D7200, Japan) and sexed via observation of presence or absence of brood pouch. Morphometric measurements and meristic counts were carried out as per standardized protocols [14,15,48]. Morphometric characters such as height (Ht, linear distance from the top of the head to the tip of the stretched tail), standard length (SL), head length (HL), trunk length (TrL) and tail length (TaL) were measured. Measurements of snout length (SnL), ratio of head/snout lengths (HL/SnL), snout depth (SnD), head depth (HD),

151 pectoral fin base length (PL) and dorsal fin base length (DL) were also conducted. All
152 measurements were taken with the help of digital caliper to 0.1 mm and repeated to ensure
153 accuracy. Coronet height (CH), trunk depths on 4th trunk ring (TD4) and 9th trunk ring (TD9)
154 were measured. The presence/absence of spines and dermal appendages was also noted.
155 Meristic characters such as the number of trunk rings (TrR), tail rings (TaR), dorsal fin rays
156 (DF), pectoral fin rays (PF) and anal fin rays (AF) were counted under stereo zoom
157 microscope (Olympus SZX7, Japan) and the values were confirmed by three time counting.
158 Brood pouch dimensions were also taken in case of male specimens.

159 The salient morphometric and meristic characters of the collected seahorse specimens
160 were compared with the reported diagnostic features for different seahorse species [14,15,23].
161 The voucher material is deposited in the national marine biodiversity repository at CSIR-
162 National Institute of Oceanography, Goa, India (deposition ID: NIO1015/19).

163

164 Statistical analysis

165 Morphological data of *H. mohnikei* and two other commonly occurring seahorse species (*H.*
166 *kuda* and *H. trimaculatus*) was evaluated by one-way analysis of variance (ANOVA)
167 followed by the Duncan's multiple-range tests [49]. The level of significance was tested at
168 5.0%, represented as $P < 0.05$. A comparison of morphological characters and meristic
169 features of *H. mohnikei* (n = 4) with *H. kuda* (n = 10) and *H. trimaculatus* (n = 10) was made
170 through principal component analysis (PCA). PCA was employed in which factor loadings
171 based on components were used to determine the morphometric factors using PRIMER
172 software (version 6) after square-root transformation.

173

174 Molecular analysis: DNA extraction, PCR amplification and sequencing

175 For gene-based phylogenetic analysis, fragments of dorsal fin from fresh seahorse were
176 removed and preserved in 95% ethanol at –20°C until subjected to DNA extraction. DNA
177 extraction was carried out as described [50] with a few modifications. In brief, lysis buffer
178 and sodium dodecyl sulfate were added to the fin tissue and vortexed. Further, RNase and
179 Proteinase K were added to the mixture and incubated for 15–20 minutes at room
180 temperature. After the incubation period, the DNA was extracted using
181 phenol:chloroform:isoamyl alcohol (25:24:1) mixture. Precipitated DNA was washed with
182 70% cold ethanol. The gel electrophoresis was performed to confirm the quality and integrity
183 of DNA.

184 Seahorse mitochondrial cytochrome oxidase subunit I (COI) gene was amplified using
185 primers, forward (5'TCAACTAATCACAAAGACATCGGCAC3′) and reverse
186 (5'ACTTCGGGGTGCCCAAAGAATC3′) [51]. Similarly, mitochondrial cytochrome *b* (Cyt
187 *b*) gene fragment was also amplified using PCR with seahorse-specific primers, forward
188 (5'AACYAGGACYAATGRCTTGA3') and reverse (5′GCASWAGGGAGGRKTTTAAC3′)
189 [40]. Reactions were carried out in a thermal cycler (Veriti® 96-well Thermal Cycler,
190 Applied Biosystems, CA, USA). The amplification of COI and Cyt *b* genes was carried out
191 by following cycling conditions as described [52] with few modifications; an initial
192 denaturation step of 95°C for 2 minutes followed by 39 cycles of 94°C for 30s, 55°C for 30s
193 72°C for 1 minute and a final extension at 72°C for 2 minutes. Amplified PCR products of
194 both COI and Cyt *b* genes were verified on agarose gel (1.5%) and purified for sequencing
195 using PCR purification kit (Sigma Aldrich, USA). Gene sequence of the PCR products was
196 determined using a taq dye deoxy terminator cycle sequencing kit (PerkinElmer, CA, USA)
197 following supplier's protocols. Sequencing reaction products were analyzed on 373
198 automated DNA Sequencer (Model 313 xl; Applied Biosystems, CA, USA). Amplification of

199 the mitochondrial COI and Cyt *b* genes resulted in fragments of 609 bp and 639 bp,
200 respectively.
201

202 Phylogenetic analysis

203 Sequences of Goa specimens (GenBank accession numbers MK330041.1 and MK112274.2
204 for COI and Cyt *b* genes, respectively) were compared using the basic local alignment search
205 tool (BLAST) of NCBI [53] (http://www.ncbi.nih.gov/BLAST). Sequences of Goa specimens
206 were aligned with those of morphologically similar seahorse species (*H. coronatus* and *H.*
207 *mohnikei*) and the most commonly occurring species in the Indo-Pacific region (*H.*
208 *camelopardalis*, *H. comes, H. histrix, H. kelloggi, H. kuda, H. sindonis, H. spinosissimus* and
209 *H. trimaculatus*) retrieved from the GenBank database. The mitochondrial COI and Cyt *b*
210 gene sequences were aligned using the ClustalW algorithm implemented in MEGA 7 [54]. To
211 ascertain stable and reliable relationships among the seahorse species, phylogenetic trees
212 were constructed through maximum likelihood (ML) method. The analyses were performed
213 in MEGA 7, and confidence levels were assessed using 1000 bootstrap replications. The
214 alligator pipefish, *Syngnathoides biaculeatus* was used as an outgroup species for
215 phylogenetic analysis to differentiate between different seahorse species. The genetic
216 distances of *H. mohnikei* from different geographical locations were calculated using Kimura
217 2-parameter (K2P) distance model [55] implemented in MEGA 7 [54]. Codon positions
218 included were 1st+2nd+3rd+noncoding. All ambiguous positions with alignment gaps and
219 missing data were removed from the sequences (pair-wise deletion option).
220

221 Prevailing ocean circulation

222 In order to understand the basin-wide circulation in the Indo-Pacific region (encompassing
223 South China Sea, East China Sea, and southeastern Bay of Bengal), monthly mean

climatology was prepared based on daily data on U (zonal) and V (meridional) components of geostrophic current velocities derived from Aviso merged product for the years, 2007–2017. Altimeter products by Ssalto/Duacs and distributed by Aviso+, with support from Cnes (https://www.aviso.altimetry.fr) were used.

Results

Material examined

Colour of the head and body of freshly collected seahorse specimens was almost dark brown to dark black. The entire body was covered with low spines and the coronet was observed to be short with five tiny projections. Two prominent cheek spines and two small rounded spines below the eye were discernible in all four specimens. (Fig 2. and Fig 3.).

Morphological taxonomy

Data on detailed morphometric measurements and meristic counts recorded on four seahorse specimens from Goa are presented in Table 1. Measured height (Ht) and standard length (SL) of specimens varied between 39.2 and 54.2 mm (48.2 ± 6.5 mm) and 47.3 and 60.1 mm (54.95 ± 5.48 mm), respectively. Trunk lengths (TrL) and tail lengths (TaL) varied between 15.1 and 19.4 mm (16.45 ± 2.02 mm) and 24.2 and 33.1 mm (30.15 ± 4.04 mm), respectively. TaL was observed to be relatively long compared to the TrL of the body. The ratio of the head length (HL) to snout length (SnL) of all specimens varied between 3.2 and 3.5.

Number of trunk rings (TrR) in all specimens were 11 whereas, the number of tail rings (TaR) varied narrowly between 37 and 39. The number of TrR supporting dorsal fin was two and the number of TaR supporting dorsal fin was counted to be one in all specimens. Number of dorsal fin rays, pectoral fin rays and anal fin rays recorded were 15–16, 12–14 and 4, respectively. Slight enlargements, respectively of 1st, 4th, 7th, and $10/11^{th}$TrR and 4th, $7/8^{th}$, $9/10^{th}$, $13/14^{th}$TaR were prominently discernible. The measured morphometric and meristic

249 characters of Goa specimens are consistent with the morphological descriptions for *H.*
250 *mohnikei* reported by Lourie et al. [14,15,23] for specimens collected from Vietnam and
251 Japan.
252
253 **Table 1. Detailed morphology of *H. mohnikei*.** Morphological characteristics of four
254 specimens of *Hippocampus mohnikei* collected from the Mandovi estuary, Goa, central west
255 coast of India (MK1 and MK2 = Female; MK3 and MK4 = Male).

	MK1	**MK2**	**MK3**	**MK4**
Morphometric characters (mm)				
Height (Ht)	39.2	54.2	48.1	51.3
Standard length (SL)	47.3	60.1	55.2	57.2
Trunk length (TrL)	16.1	19.4	15.2	15.1
Tail length (TaL)	24.2	32.1	31.2	33.1
Head length (HL)	8.5	9.1	9.2	8.7
Snout length (SnL)	2.5	3.1	3.3	2.5
HL/SnL ratio	3.4	3.2	3.3	3.5
Snout depth (SnD)	1.5	2.1	2.1	2.1
Head depth (HD)	5.5	7.1	6.9	7.2
Eye orbit	1.5	2.1	2.2	2.1
Trunk depth between 4th and 5th trunk ring (TD4)	3.1	4.2	3.9	4.1
Trunk depth between 9th and 10th trunk ring (TD9)	4.7	6.1	5.8	5.9
Pectoral fin base length	2.1	2.1	2.1	2.2
Dorsal fin base length	3.5	4.1	4.1	3.9

Anal fin base length	0.5	0.5	0.5	0.5
Pouch length	-	-	6.2	6.6
Pouch depth	-	-	3.1	2.5
Pouch width	-	-	3.2	3.3
Meristic features (numbers)				
Trunk Rings (TrR)	11	11	11	11
Tail Rings (TaR)	37	38	37	39
TrR supporting dorsal fin	2	2	2	2
TaR supporting dorsal fin	1	1	1	1
Cheek spines	2	2	2	2
Eye spines	2	2	2	2
Enlargement of trunk Rings	1, 4, 7, 11	1, 7, 10	1, 4, 7, 11	1, 4, 7, 10
Enlargement of tail Rings	4, 7, 10, 13	4, 8, 10, 13	4, 7, 9, 13	4, 7, 10, 14
Dorsal fin rays	15	16	16	16
Pectoral fin rays	12	14	14	14
Anal fin rays	4	4	4	4

Comparative morphology

A comparative morphological features of *H. mohnikei* and other two commonly occurring seahorse species (*H. kuda* and *H. trimaculatus*) is presented in Table 2. Fresh specimens of *H. kuda* was orange to dark brown with reddish spots over the body with single eye and cheek spine, rounded and backward curled coronet and deeper head and rounded or less developed body spines [14,15]. *H. trimaculatus* has three prominent black spots on the dorso-lateral surface of the first, fourth and seventh trunk rings [14,15] which are clearly absent in *H. mohnikei*. Single eye and hooked like cheek spine also observed in *H. trimaculatus*. Snout

265 length (SnL) of *H. mohnikei* was shorter compared to the *H. kuda* and *H. trimaculatus*. These
266 morphological characters clearly distinguishes *H. mohnikei* from *H. kuda* and *H.*
267 *trimaculatus*.

268 PCA used to elucidate the factors contributing to morphological differences in *H.*
269 *mohnikei, H. kuda* and *H. trimaculatus* showed that many morphometric and meristic
270 characters play an important role in differentiating the seahorse species. Results of PCA
271 analysis revealed that 11 morphometric and meristic characters integrated into two principal
272 components (PC1 and PC2) which cumulatively contributed to 97% of total variance (Table
273 3). The PC1 which explained 81% of total variation consisted of morphometric characters
274 such as SL, TaL, TD4, HL, TD9, TrL, HD, SnL and SnD while, the PC2 which explained
275 16% of total variation consisted of the TaR and DF. The derived ordination diagram after
276 plugging standardized morphological data of all individuals of three seahorse species into
277 PCA is depicted in Fig. 4. PCA ordination diagram showed that three seahorse species were
278 clustered out distinctly in orientation space. Therefore, based on comparison of
279 morphological traits [14,15,23] and the results of PCA, the identity of seahorse specimens
280 collected from Goa was confirmed as *H. mohnikei*.

281

282

283 **Table 2. Morphological comparison of seahorses.** Comparison of morphological characteristics of *H.*
284 *mohnikei* with other two seahorse species, (*H. kuda* and *H. trimaculatus)*. Sample size for each species are
285 shown in parentheses, Mean±SD denoted with different superscripts in same row significantly differ from
286 each other (P < 0.05).

Morphometrics (mm)	***H. mohnikei* (*n* = 4)**	***H. kuda* (*n* = 10)**	***H. trimaculatus* (*n* = 10)**
Height (Ht)	48.2 ± 6.50[a]	157.73 ± 19.24[b]	93.06 ± 14.71[c]
Standard Length (SL)	54.95 ± 5.48[a]	182.65 ± 11.64[b]	109.87 ± 6.45[c]
Trunk Length (TrL)	16.45 ± 2.02[a]	47.83 ± 2.54[b]	26.40 ± 2.12[c]
Tail Length (TaL)	30.15 ± 4.04[a]	104.43 ± 9.07[b]	61.70 ± 3.40[c]
Head Length (HL)	8.83 ± 0.41[a]	30.39 ± 1.88[b]	21.70 ± 1.77[c]
Snout Length (SnL)	2.67 ± 0.21[a]	12.06 ± 1.04[b]	8.90 ± 0.99[c]
HL/SnL ratio	3.33 ± 0.14[a]	2.54 ± 0.16[b]	2.45 ± 0.19[bc]
Snout Depth	1.95 ± 0.30[a]	4.25 ± 0.41[b]	3.45 ± 0.16[c]
Head Depth	6.68 ± 0.79[a]	14.80 ± 1.84[b]	9.50 ± 0.53[c]
TD4	3.83 ± 0.50[a]	12.16 ± 1.19[b]	6.75± 0.86[c]
TD9	5.63 ± 0.63[a]	18.68 ± 1.51[b]	9.55 ± 0.90[c]
Pectoral fin base length	2.03 ± 0.1[a]	7.07 ± 1.2[b]	4.75 ± 0.6[c]
Dorsal fin base length	3.88 ± 0.3[a]	15.73 ± 1.8[b]	9.80 ± 0.8[c]
Meristic features (numbers)			
Trunk Rings (TrR)	11 ± 0.00	11 ± 0.00	11 ± 0.00
Tail Rings (TaR)	38 ± 1.00[a]	36 ± 1.00[b]	41 ± 1.00[c]
TrR supporting Dorsal Fin	2 ± 0.00	2 ± 0.00	2 ± 0.00
TaR supporting Dorsal Fin	1 ± 0.00	1 ± 0.00	1 ± 0.00
Cheek Spines	2 ± 0.00[a]	1 ± 0.00[b]	1 ± 0.00[c]
Eye spines	2 ± 0.00[a]	1 ± 0.00[b]	1 ± 0.00[c]
Dorsal fin rays	16 ± 1.00[a]	18 ± 1.00[b]	20 ± 1.00[c]
Pectoral fin rays	14 ± 1.00[a]	16 ± 1.00[b]	17 ± 1.00[c]

Table 3. PCA analysis of seahorse species. Loadings of components matrix and contributions of each axis to total variance ranked as per their coefficients (eigen values > ± 0.30 are included; values in parentheses indicate variance explained by each principal component).

Morphological characters	**Coefficients**
Principal component 1 (81%)	
SL	0.334
TaL	0.333
TD4	0.329
TD9	0.327
HL	0.327
TrL	0.325
HD	0.324
SnL	0.318
SnD	0.314
Principal component 2 (16%)	
TaR	0.663
DF	0.655

Fig 4. PCA analysis scores. Scores of factor loadings plot of principal component analysis of three species, *H. mohnikei*, *H. kuda* and *H. trimaculatus* based on morphological data.

313 Phylogenetic analysis and genetic distance

314 PCR for the mitochondrial COI and Cyt *b* genes resulted in 609 bp and 639 bp amplicons,
315 respectively. Phylogenetic trees constructed with sequences of COI and Cyt *b* genes of Goa
316 specimens with those of morphologically similar and most abundant seahorse species in the
317 Indo-Pacific region are depicted in Fig. 5 and Fig. 6, respectively. Sequences of COI
318 (GenBank accession number MK330041.1) and Cyt *b* (GenBank accession number
319 MK112274.2) genes of Goa specimens showed similarities of 100% with *H. mohnikei*
320 (GenBank accession number: GQ502157.1) and 93% with *H. mohnikei* (GenBank accession
321 number: EU179924.1), respectively. Based on maximum similarities, the genetic identity of
322 Goa specimens was confirmed as *H. mohnikei*. Genetic distance describes the kinship
323 distance between intraspecies and interspecies. Calculated pair-wise genetic distance (K2P)
324 values based on COI and Cyt *b* sequences of *H. mohnikei* from Goa and other localities
325 (Japan, Korea, China, Vietnam and Thailand) are presented in Table 4 and Table 5,
326 respectively. Relatively, *H. mohnikei* from Goa showed the least genetic distances of 6.5%
327 (COI) and 5.4% (Cyt *b*) with populations of *H. mohnikei* from Vietnam (COI) and Thailand
328 (Cyt *b*), respectively.

329

330 **Fig 5. Phylogenetic relationships of Goa specimen with other seahorses species based on**
331 **COI gene sequences.** Phylogenetic relationships of Goa specimen (MK330041.1) with
332 closely related species based on cytochrome oxidase sub-unit I gene (COI) constructed
333 through maximum likelihood method with bootstrap values (1000 replicates) as percentage at
334 the nodes. The alligator pipefish, *Syngnathoides biaculeatus* (KY066152.1) served as an
335 outgroup species. Scale bar = genetic distance of 0.05.

336

Fig. 6. Phylogenetic relationships of Goa specimen with other seahorses species based on Cyt *b* gene sequences. Phylogenetic relationships of Goa specimen (MK112274.2) and closely related seahorse species based on cytochrome *b* gene (Cyt *b*) constructed through maximum likelihood method with bootstrap values (1000 replicates) as percentage at the nodes. The alligator pipefish, *Syngnathoides biaculeatus* (EF189167.1) served as an out-group species. Scale bar = genetic distance of 0.05.

Table 4. Pair-wise genetic distance based on COI gene sequences. Pair-wise genetic distances (Kimura 2-parameter model) based on COI gene sequences of *Hippocampus mohnikei* populations between Goa and the Indo-Pacific region. Values in parentheses indicate percentage.

Localities of *H. mohnikei* with GenBank Accession number		1	2	3	4
1	Goa, India (MK330041.1)	0.000			
2	Japan (GQ502158.1)	0.085 (8.5)	0.000		
3	China (KX689046.1)	0.100 (10.0)	0.032 (3.2)	0.000	
4	Vietnam (GQ502157.1)	0.065 (6.5)	0.092 (9.2)	0.104 (10.4)	0.000

354 **Table 5. Pair-wise genetic distance based on Cyt *b* gene sequences.** Pair-wise genetic
355 distances (Kimura 2-parameter model) based on Cyt *b* gene sequences of *Hippocampus*
356 *mohnikei* populations between Goa and the Indo-Pacific region. Values in parentheses
357 indicate percentage.

Localities of *H. mohnikei* with GenBank Accession number		1	2	3	4	5
1	Goa, India (MK112274.2)	0.000				
2	South Korea (KT731897.1)	0.126 (12.6)	0.000			
3	China (KC527556.1)	0.128 (12.8)	0.006 (0.6)	0.000		
4	Japan (AF192689.1)	0.134 (13.4)	0.050 (5.0)	0.047 (4.70)	0.000	
5	Thailand (EU179924.1)	0.054 (5.4)	0.132 (13.2)	0.128 (12.8)	0.140 (14.0)	0.000

358

359 ## Ocean circulation

360 The climatological currents generated for the winter season (December to February) from
361 2007 to 2017 for the Indo-Pacific region are depicted in Fig. 7. As seen from the figure that
362 the circulation shows a large-scale flow moving from the East China Sea to the South China
363 Sea. From the South China Sea, the major part of the flow turns southeastward entering into
364 the Java Sea and a small part turns northwestward to eastern Bay of Bengal (BoB) and the
365 Andaman Sea through Malacca Strait. From eastern BoB, the surface waters flow westward
366 as a part of the north equatorial current (NEC). On encountering the coast of Sri Lanka, a part
367 of the NEC move northward into the northern BoB, while the rest moves westward and into
368 the southern Arabian Sea, south of Sri Lanka. The NEC feeds into the wintertime west India
369 coastal current (WICC) and moves waters from NEC towards north into the Arabian Sea
370 along its eastern boundary.

Fig. 7. Oceanic circulation in Indo-Pacific region. Climatological surface currents during winter season (December–February) for the Indo-Pacific region. Red dots indicates the geographic locations of distribution of *Hippocampus mohnikei.* (WICC= West Indian Coastal Current, EICC= East India Coastal Current, NEC= North Equatorial Current and SCSWC= South China Sea Warm Current).

Discussion

The distributional range of *Hippocampus mohnikei* is still unresolved as [14,15] confirmed the nativity of this species to Japan and Vietnam and likely extending throughout the South China Sea and the Gulf of Thailand [14,15,23]. Recent reports on the occurrence of *H. mohnikei* from many other non-native locations in north- and south-eastern coastal waters of Asia [10,35,41,51,56,57] suggest its significantly extended distribution in the Indo-Pacific region (Fig. 8). The sighting of *H. mohnikei* in the Palk Bay, southeast coast of India [10] is till date the known range limit towards northwestern Asian coastal waters. In southeastern Asian coastal waters, *H. mohnikei* has been reported from Singapore [57] as well as from Cambodia, Malaysia, Thailand and Vietnam [35]. Towards northeast Asian coastal waters [56,58] reported *H. mohnikei* in southern Korea, whereas, [41,51] reported *H. mohnikei* from China.

Fig. 8. Reported sightings of *H. mohnikei* in Indo-Pacific region. Map showing native range, recent sightings and all reported sightings for *Hippocampus mohnikei* in the Indo-Pacific region. Sightings are classified as new (this study, ★), recently published (2004–2016, (▲) and original records [14] (*). Map was modified from Aylesworth et al. [35] and re-created in the World Geodetic System (WGS) 84 datum.

396 The present report on the occurrence of *H. mohnikei* in a bay-estuarine system of Goa,
397 CWCI as confirmed by both morpho-molecular analysis is the most recent represents a
398 significant westward expansion compared to the previously known distributional range of this
399 species [35]. Since the reporting of *H. mohnikei* solely based on morphological characters
400 from the Palk Bay, southeast coast of India [10], no further sightings of this species have
401 been reported from coastal marine waters of India. Therefore, a comparison of genetically
402 distinct populations of *H. mohnikei* inhabiting native (Japan and Vietnam) and extended
403 ranges in the Indo-Pacific region (Fig. 8) with Goa specimens gains significance. Lourie et al.
404 [7,23] reported that a Vietnamese specimen of *H. mohnikei* was genetically distinct from *H.*
405 *mohnikei* from Japan. Results of phylogenetic constructed of sequences of COI (GenBank
406 accession number, MK330041.1) and Cyt *b* (GenBank accession number MK112274.2)
407 genes of Goa specimens showed maximum similarities of 100% with (GenBank accession
408 number GQ502157.1) and 93% (GenBank accession number EU179924.1) with *H. mohnikei*
409 from Vietnam and Thailand, respectively. Furthermore, a comparison of pair-wise genetic
410 distance values (K2P) based on sequences COI (Table 4) and Cyt *b* (Table 5) genes revealed
411 the conspecificity of Goa specimens with populations of *H. mohnikei* from Vietnam (K2P
412 value, 6.5%) and Thailand (K2P value, 5.4%) rather than Japan (K2P values, 8.5% and
413 13.4% for COI and Cyt *b* sequences, respectively). In contrast, high pair-wise K2P values of
414 sequences of Cyt *b* between *H. mohnikei* populations from the Gulf of Thailand and those
415 from China (13.54−14.46%) and Japan (14.42−14.79%) have been recently reported [59]. A
416 comparative assessment of K2P values of the present study with those from the Gulf of
417 Thailand [59] suggest that *H. mohnikei* populations from Goa and the Gulf of Thailand might
418 be genetically distinct from China and Japan. Also, a possible genetic differentiation in
419 populations of *H. mohnikei* inhabiting coastal marine waters of northeastern Asia (Japan,
420 Korea and China) and southeastern Asia (Vietnam, Thailand, Cambodia, Malaysia, Singapore

421 and India) may not be ruled out. Furthermore, least K2P values between disjunct populations
422 of *H. mohnikei* (Goa vs Vietnam, COI gene) and (Goa vs. Thailand, Cyt *b* gene) may reflect
423 on the possibility of cryptic species within *H. mohnikei* populations [7,23,59] which needs
424 further confirmation.

425 Recent studies have advanced our understanding on habitat associations of *H. mohnikei*
426 compared to what has been described in the IUCN Red List Assessment [42]. Previously, *H.*
427 *mohnikei* was reported only in association with seagrass beds in Japan and in an estuary in
428 Vietnam [15,23]. Association of *H. mohnikei* populations with seagrass beds (*Zostera* spp.) in
429 Japan [58,60], Korea [56] and China [41] has been reported. Whereas the specimen reported
430 from the Palk Bay [10] was associated with two other additional genera of seagrass
431 (*Halophila* and *Thalassia*). Recently, published report on the habitat preferences of *H.*
432 *mohnikei* from Southeast Asia [35] indicate its association with seagrass beds of *Cymodocea*
433 spp., *Enhalus* spp., *Halodule* spp., *Halophila* spp., *Syringodium* spp. and *Thalassia* spp.
434 Furthermore, these authors also report the association of *H. mohnikei* with mangroves, oyster
435 beds, mussel farms and sandy beaches, whereas, [57] has reported the its occurrence in
436 association with seaweeds in Singapore. From these studies, it is evident that *H. mohnikei*
437 occupies a wide variety of habitats.

438 All previous reports on the habitat descriptions of *H. mohnikei* were located in the coastal
439 waters and bay-estuarine systems in the depth range of 0–10 m [10,15,23,35,41,56].
440 Interestingly, the occurrence of small juveniles of *H. mohnikei* in association with highly
441 dense live prey organisms in the offshore waters of Japan has also been reported [37]. The
442 present report of *H. mohnikei* is based on specimens caught as an incidental catch in bag nets
443 fixed to the stakes operated in the Mandovi estuary, Goa. Although precise information on
444 the habitat of *H. mohnikei*, could not be ascertained, it is worth describing the surrounding
445 environs at the site of collection. Predominantly, the estuarine stretches of the River Mandovi

446 have dense mangrove coverage and extensive oyster beds [61] and patches of seagrass,
447 *Halophila ovalis* and *H. beccarii* [62]. The channel network of Mandovi estuary is
448 characterised by islands (Chorao, Divar and Vanxim) and many sheltered areas interspersed
449 with dense mangrove ecosystem. The present occurrence of *H. mohnikei* in a bay-estuarine
450 system of Goa is not quite surprising as these are the typical habitats where this species has
451 been previously reported. For ascertaining habitat shift by disjunct populations of *H.*
452 *mohnikei.* further intense studies on habitat specificities of different life stages therefore, are
453 warranted.

454 Food availability has been considered as one of the factors influencing the abundance and
455 distribution of seahorses [19]. Zooplankton such as *Oithona davisae*, Calanoida and *Penilia*
456 *avirostris* have been identified as the main prey organisms for juvenile and young *H.*
457 *mohnikei* in Tokyo Bay [37], whereas the adults have been reported to prey upon mainly
458 larger organisms such as gammarid and caprellid amphipods [58]. Nutrient-rich waters of the
459 Mandovi estuarine system sustain high primary productivity and abundance of zooplanktonic
460 prey organisms such as copepods [63−65] which are essential for the survival and
461 establishment of seahorse populations. Considering the prevailing environmental conditions,
462 mangrove shelter and abundance of prey organisms, the likelihood of establishment and
463 colonization of seahorse populations in these sheltered areas is quite high.

464 Factors such as habitat, diet, and even anthropogenic activities are the main reasons cited
465 for the migration of seahorses [66]. Furthermore, instances of seahorses for long distance
466 dispersal by means of rafting along with seaweeds or any other suitable holdfast under the
467 influence of ocean currents during juvenile stages have been documented [2,20]. Southward
468 migration of *H. histrix* ~1800 km under the influence of East Australian Current in the Great
469 Barrier Reef has been reported [31]. Furthermore, ~1100 km migration from the south to the
470 northeast coast of India by *H. fuscus* under the influence of East Indian Coastal Current has

471 been also reported [33]. Direct evidence for passive long-distance migration of *H.*
472 *trimaculatus* at Malacca Strait has been documented recently [34].

473 Newborn juveniles of many seahorse species undergo a pelagic phase (planktonic)
474 ranging between two and six weeks depending on the species before they attain a settlement
475 phase (benthic) [2,67]. Consequently, planktonic phase juveniles may facilitate in widespread
476 gene flow across geographically disjunct populations and resulting in genetic homogeneity
477 [68]. The occurrence of small juveniles of *H. mohnikei* (15.1−45.5 mm) in association with
478 highly dense live prey organisms in offshore waters of Japan [37] suggests that *H. mohnikei*
479 might undergo a prolonged planktonic phase. Furthermore, a seasonal (warm and cold
480 season) inshore-offshore migration by *H. mohnikei* populations inhabiting Chinese coast has
481 been confirmed [51]. Higher dispersal capabilities of seahorse juveniles with floating debris,
482 prey swirls and with seaweed rafts than adults has been reported [2,28,34]. It has been
483 postulated that marine species with greater longevity, free swimming and feeding
484 (planktotrophic) larval phases have relatively a high dispersal abilities and thus promoting
485 genetic exchange between populations [69]. A relatively prolonged planktonic phase as in
486 case of *H. mohnikei* may facilitate the high degree of dispersal under the influence of oceanic
487 circulation.

488 The confirmed occurrence of four sub-adult specimens (Mandovi estuary) and suspected
489 occurrence of an adult specimen of *H. mohnikei* in the proximately adjacent bay-estuarine
490 ecosystem (Chapora estuary) of Goa, CWCI could be as a consequence of either long-
491 distance dispersal from Southeast Asian countries or stepping-stone mediated dispersal as
492 revealed by oceanic currents circulation in the Indo-Pacific region. It is clearly seen from Fig.
493 7 that water mass from southeastern Asian coasts and east coast of India enters into the
494 Arabian Sea as a result of winter circulation [70]. In the absence of geographical barriers
495 between *H. mohnikei* populations of Goa and Southeast Asian countries, ocean currents

496 flowing from regions where sightings of *H. mohnikei* may contribute to wide dispersal.
497 Therefore, it is plausible that oceanic currents may be aiding long-distance or stepping stone
498 dispersal of *H. mohnikei* populations from different geographical locations such as
499 Cambodia, Malaysia, Thailand and Vietnam [14,15,35,59], Singapore [57] and the Palk Bay,
500 India [10] to the west coast of India.

501 Conversation with local fishermen who operate stake nets at the collection site revealed
502 that sightings of seahorses are relatively more common during summer monsoon season than
503 in non-monsoonal months. During the summer monsoon period, a number of rivulets and
504 rivers connected to the Mandovi estuarine network discharge large amounts of freshwater
505 [71]. Consequently, it may be possible that the rainwater discharge from sheltered areas (the
506 preferred seahorse habitats) may entail seahorses to flow downstream along with currents due
507 to their poor swimming abilities. It is well known that the west coast of India comes under the
508 influence of substantial coastal cold-water upwelling event during the summer monsoon
509 season [45,71]. It has been reported that the survival and dispersal of tropical marine species
510 are known to be impacted by a drastic decrease in water temperature associated with
511 upwelling [72−74]. Consequently, seahorse populations in western coastal India might
512 undergo seasonal fluctuations [20]. A significant decrement in abundance and diversity of
513 demersal fishes during the summer monsoon in a bay-estuarine system of the Mandovi river
514 has been documented [47]. As a consequence of the reduction in salinity and drop in
515 temperature associated with monsoonal precipitation and upwelling, respectively the seahorse
516 populations might face limitations in growth and recruitment along the west coast of India.
517 Interestingly, unlike most subtropical seahorse species, the greater tolerance by native
518 populations of *H. mohnikei* to cold water inhabiting in the Western Pacific has been reported
519 [29]. However, further temperature stress simulation studies are required to ascertain whether
520 translocated populations of *H. mohnikei* also possess a similar trait or not.

Conclusions

Recognising the potential of seahorses as a popular flagship species in estuarine conservation and sustainable management [1,75,76], significant westward range expansion of *H. mohnikei* is of considerable biogeographic and conservation importance. Further intense sampling and analysis of *H. mohnikei* populations along the Indian coast are therefore required to confirm whether the present occurrence of *H. mohnikei* in a bay-estuarine system, CWCI represents (i) an isolated translocation or (ii) naturally occurring facilitated by oceanic circulation or (iii) the first sighting of an unreported of fairly established populations of this vulnerable seahorse species.

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Author Contributions

Conceived and designed the experiments: SVS RAS RDSK: Performed the experiments: SVS RAS RDSK MSS HTK. Analyzed the data: SVS RAS MSS RDSK JN. Contributed reagents/materials/analysis tools: JN HTK. Wrote the paper: RAS SVS RDSK.

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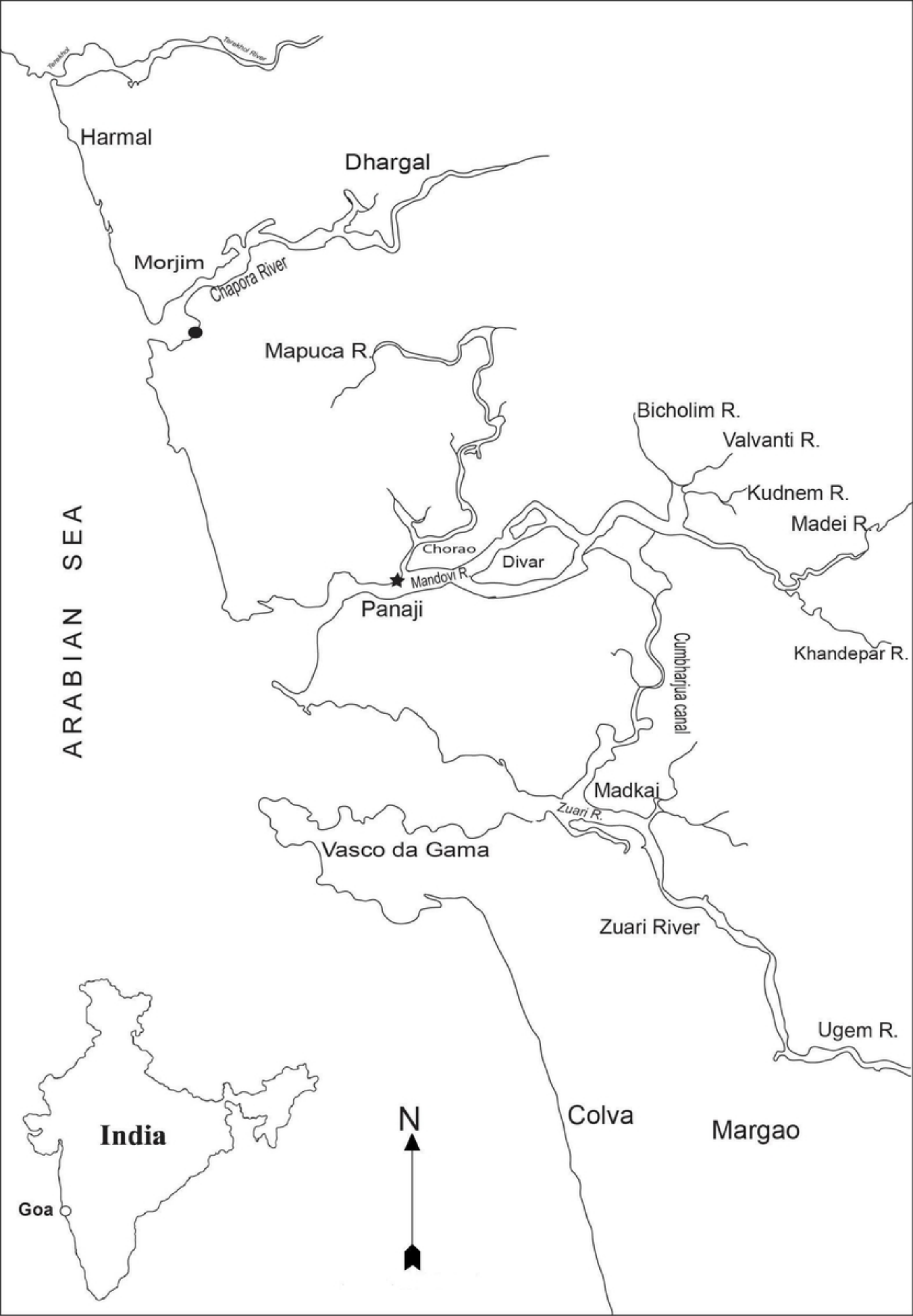
Supporting Information

S1 Fig. Picture of *H. mohnikei* posted on social media. Male specimen of *Hippocampus mohnikei* caught by gill net in the Chapora estuary, Goa, India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Terekhol River
Harmal
Dhargal
Morjim
Chapora River
Mapuca R.
Bicholim R.
Valvanti R.
Kudnem R.
Madei R.
ARABIAN SEA
Chorao
Divar
Mandovi R.
Panaji
Khandepar R.
Cumbharjua canal
Madkai
Zuari R.
Vasco da Gama
Zuari River
Ugem R.
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Goa
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Colva
Margao

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A)

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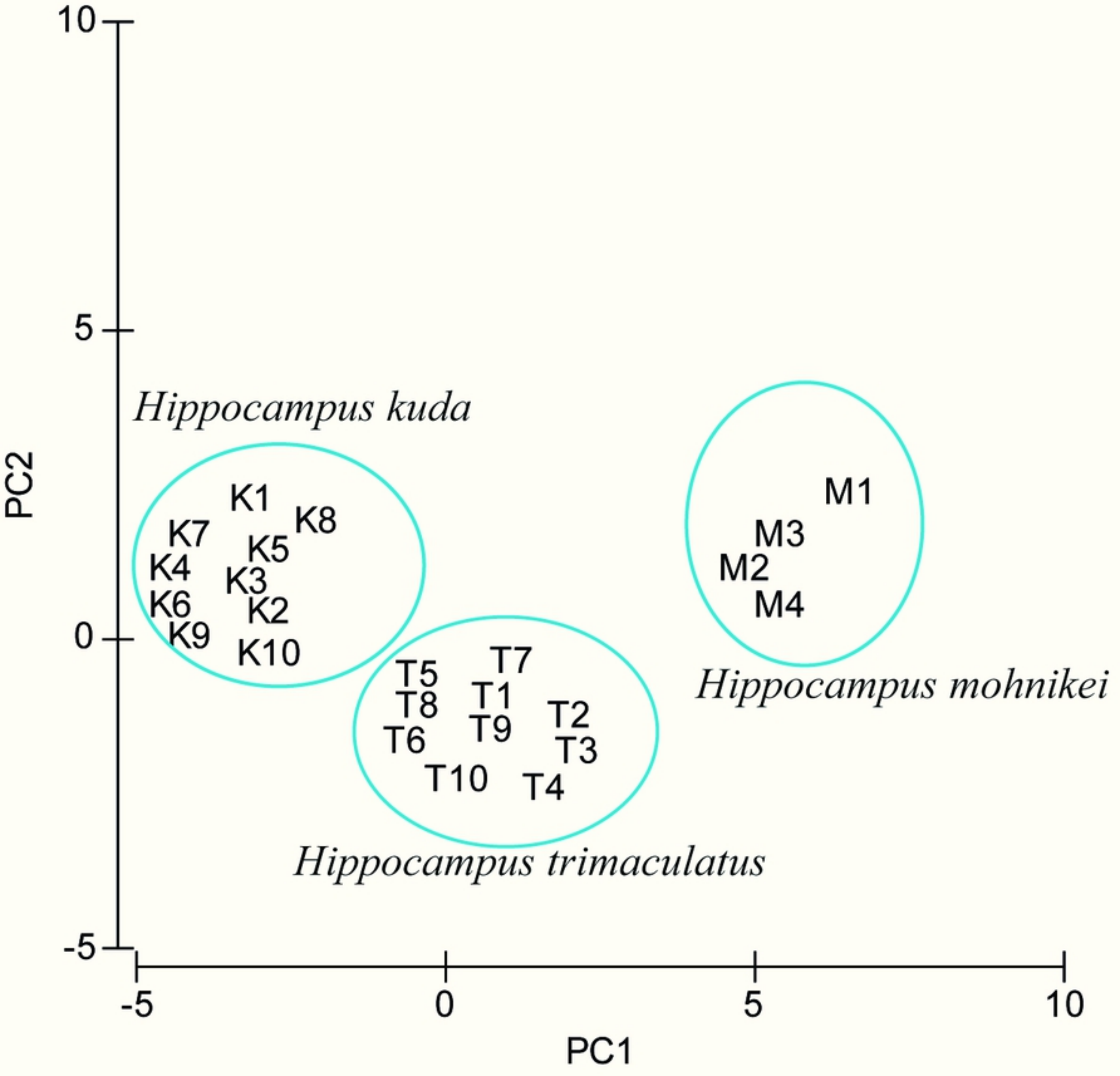
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K10
M1
M3
M2
M4
Hippocampus mohnikei
T7
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Hippocampus trimaculatus
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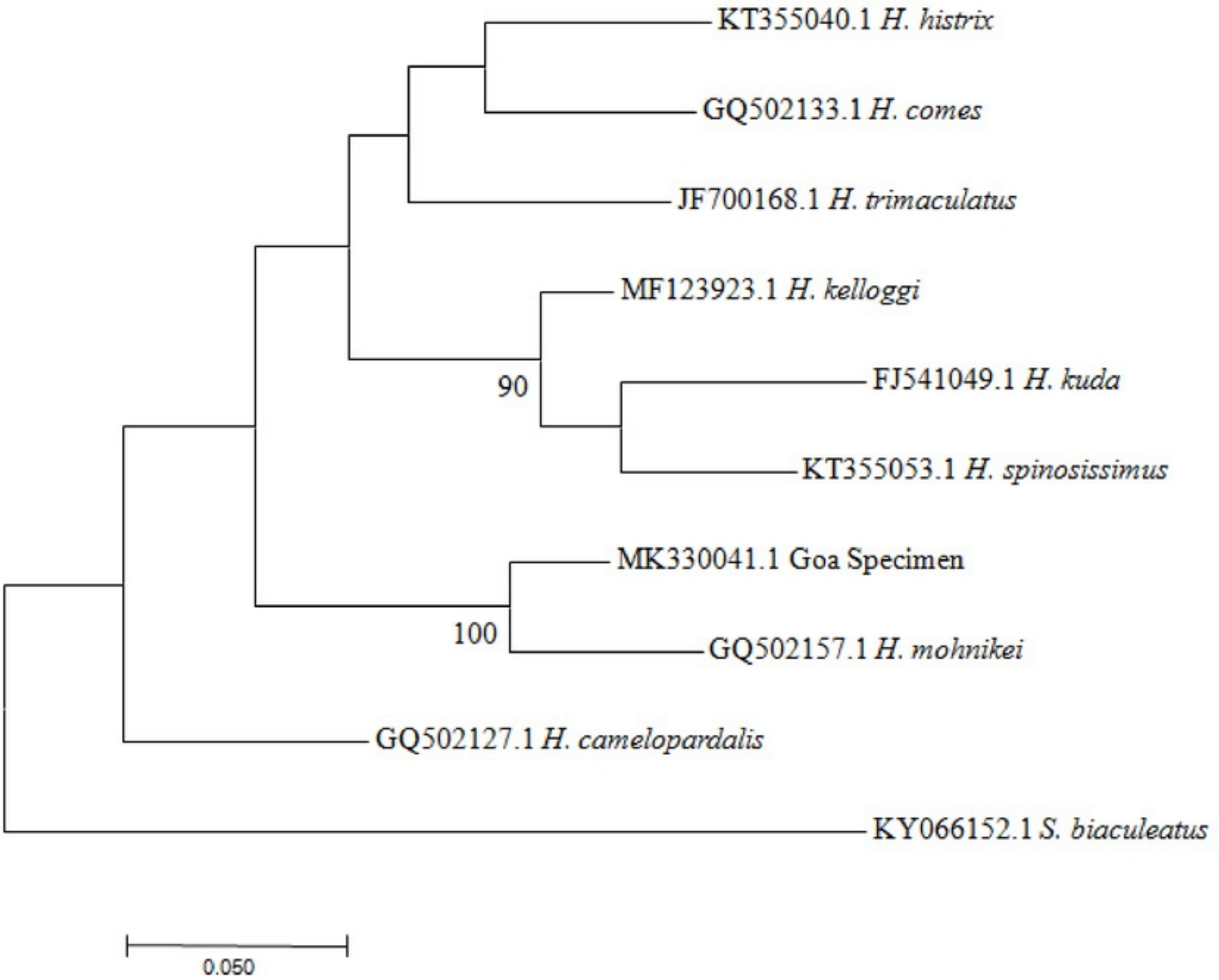
PC1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MF123923.1 H. kelloggi
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KT355053.1 H. spinosissimus
MK330041.1 Goa Specimen
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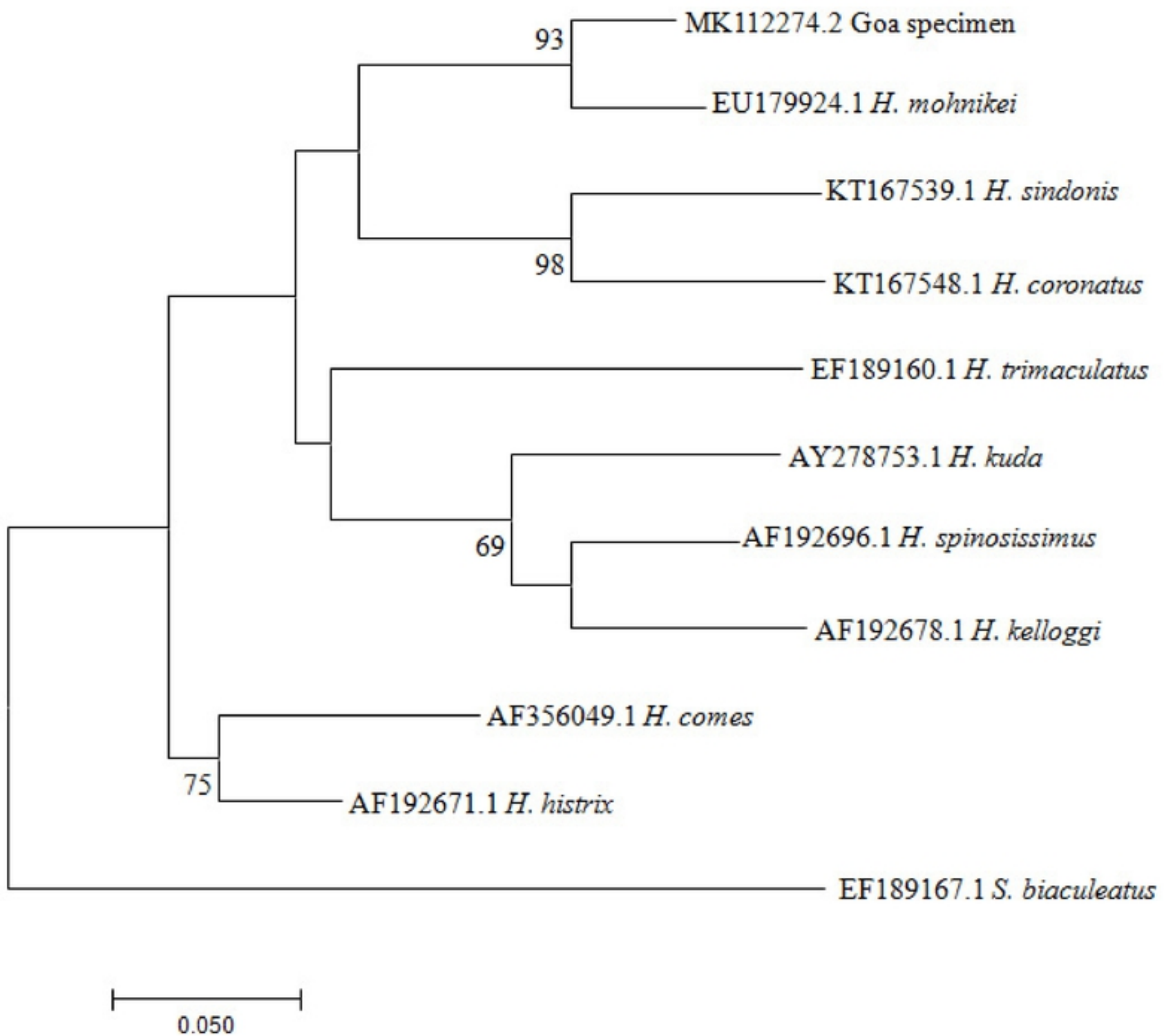
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EF189160.1 H. trimaculatus
AY278753.1 H. kuda
69
AF192696.1 H. spinosissimus
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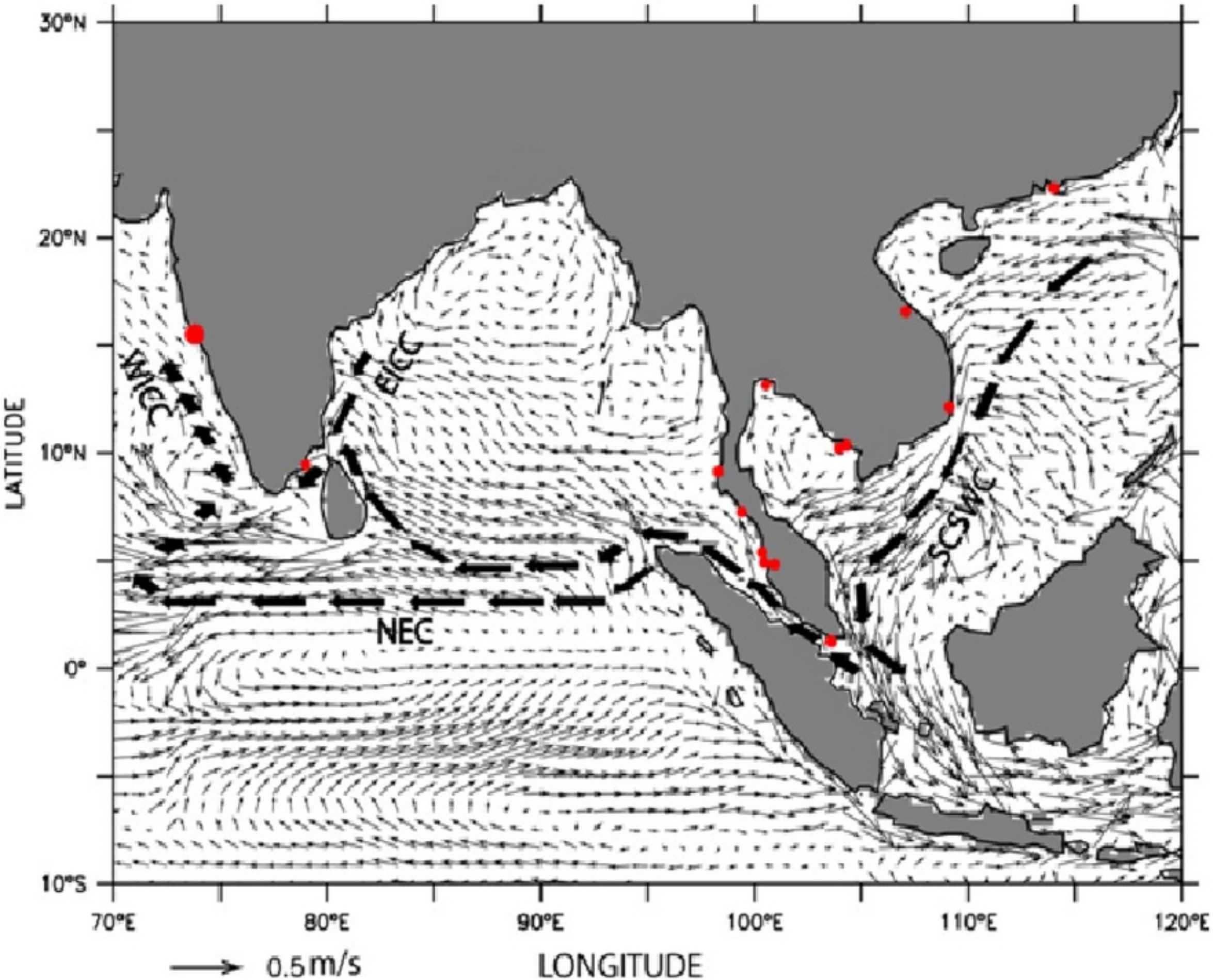
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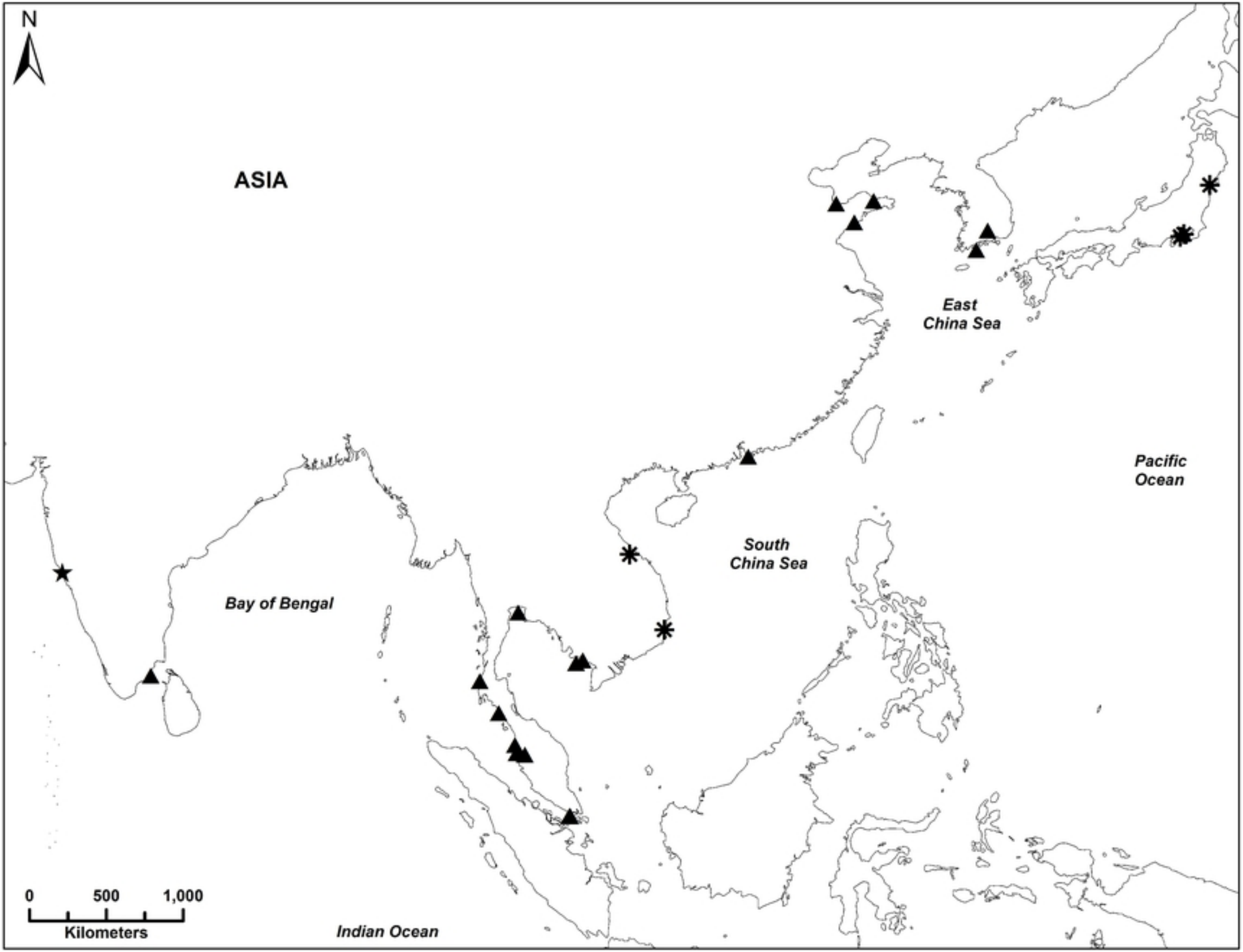
N
ASIA
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