




# Genetic diversity and populational structure of the seahorse *Hippocampus reidi* (Syngnathidae) in north-eastern Brazil: A conservationist approach

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## Abstract

1. Anthropogenic influence on the environment has caused extensive damage, with several species becoming vulnerable to extinction, such as *Hippocampus reidi*, the seahorse with the largest distribution area in Brazilian estuaries. For instance, in the Maracaípe River estuary, north-eastern Brazil, once a sanctuary for the species, this seahorse is now increasingly difficult to find.
2. Recovery strategies for species threatened with extinction include the introduction of individuals from a demographically healthy population, or the captive breeding and subsequent release of individuals from the population at risk. This study evaluated the evolutionary potential and the best way to manage the recovery of the Maracaípe seahorse population.
3. The genetic diversity of 30 adults and 99 juveniles of *H. reidi* collected in Maracaípe were compared with those of 29 adults from a nearby, demographically healthy population in Jericoacoara National Park, Ceará. Inter simple sequence repeat (ISSR) polymerase chain reaction (PCR) was used, amplifying 96 polymorphic fragments from the studied groups.
4. Adults and juveniles from Maracaípe had high diversity and low genetic differentiation. The neighbour-joining method, based on the number-of-differences model, revealed two clusters: one with adults and juveniles from Maracaípe; and the other with adults from Jericoacoara only. The most probable number of genetic groups ( $K = 2$ ) was also obtained using Evanno's test, which was confirmed by STRUCTURE. The analysis of molecular variance showed that more than 30% of the differences occur between these two groups (Maracaípe and Jericoacoara).
5. The population differentiation observed between Maracaípe and Jericoacoara suggests genetic incompatibility between the individuals in each area, and points to the risk of outbreeding depression. Therefore, a possible conservation strategy for the Maracaípe population is a native captive breeding programme and the release of offspring in this location, aiming at the demographic recovery of the population in the area.

## KEYWORDS

coastal, conservation evaluation, estuary, fish, genetics, recovery

## 1 | INTRODUCTION

Conservation issues continue to cause increasing concern worldwide. The loss of species takes place at such a fast pace that it has been estimated that the sixth mass extinction on the planet is imminent (Barnosky et al., 2011). In the marine environment, where several species play a central role in various biogeochemical processes that maintain the biosphere, approximately 1% of organisms is at some risk of extinction. For fish, this number rises to 4% (Vié, Hilton-Taylor, & Stuart, 2009). Moreover, these values also increase as a result of anthropic disturbances, such as habitat pollution and destruction, as well as the over-exploitation of numerous species (Reis et al., 2016; Worm et al., 2006).

All over the world, seahorses are threatened by anthropic pressures. Of the 41 species described to date (Lourie, Pollom, & Foster, 2016), 30 have been assessed by the International Union for Conservation of Nature (IUCN). Of these 30 species, only one was classified as being of 'Least Concern' (IUCN, 2017). Since 2002, all seahorse species have been included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). More specifically in Brazil, all three native species, namely *Hippocampus erectus* Perry, 1810, *Hippocampus patagonicus* Piacentino & Luzzatto, 2004, and *Hippocampus reidi* Ginsburg, 1933, are classified as being vulnerable by the Ministry for the Environment (Ministério do Meio Ambiente, 2014). The most abundant species in Brazilian estuaries, *H. reidi* (Silveira, 2011), has been recorded in Atlantic Ocean waters from Florida, USA, to Rio Grande do Sul, Brazil (Lourie, Foster, Cooper, & Vincent, 2004; Silveira, Siccha-Ramirez, Silva, & Oliveira, 2014).

Owing to its estuarine habits, *H. reidi* has become a tourist attraction in special events called 'seahorse tours', held in some locations in Brazil, where tourists interact directly with these animals in their natural environment (Silveira, 2005; Ternes, Gerhardinger, & Schiavetti, 2016). Two areas in Brazil stand out regarding these tours: Maracaípe River estuary and Jericoacoara National Park, in the states of Pernambuco and Ceará, respectively. Recent surveys have revealed that the population density of *H. reidi* in Jericoacoara is high, compared with the values observed in other areas of the Brazilian coast (Almeida, Silva, Santos, & Silveira, 2015; Lopes, Silva, & Silveira, 2015; Mai & Rosa, 2009; Silveira, Ramineli, Veja, Rosa, & Azevedo, 2011; Vidon & Silveira, 2015). Research shows that it has remained essentially constant in the past few years (Silveira, Ferreira, & Silva, 2015); however, such a favourable scenario is not observed in Maracaípe, where the *H. reidi* population has decreased considerably. For example, between 2001 and 2010, an 80% decrease was observed in the population of this species in the area (Furtado, 2010; Silveira et al., 2011).

One of the anthropic pressures responsible for the decline in *H. reidi* populations in Maracaípe is the 'seahorse tour' that, contrary to what happens in Jericoacoara, is carried out too intensively and without any regulation. Periodic surveys conducted in Maracaípe have shown that the demographic reduction in the *H. reidi* population between 2001 and 2011 coincided with the increase in the number of fishermen who organize 'seahorse tours', from seven to almost 40 in the region. During these tours, which take place several times a day, fishermen capture seahorses to show them to tourists, with no regard for the age and reproduction status of these animals (Silveira et al., 2011).

The first step in any conservation strategy to help the recovery of a threatened species is to identify the reasons for the decrease in the number of individuals, so that these causes can be offset or mitigated (IUCN Species Survival Commission (SSC), 2013). The intense anthropic pressures that have threatened the Maracaípe estuarine ecosystem prompted the legal authorities of the state of Pernambuco to request the establishment of a conservation unit in the region, which will regulate local tourist attractions, including the 'seahorse tours' (Ministério Público de Pernambuco (MPPE), 2017). In addition to this measure, assessing the genetic diversity of *H. reidi* in Maracaípe is important in the evaluation of the evolutionary potential of the species (Frankham, Ballou, & Briscoe, 2010).

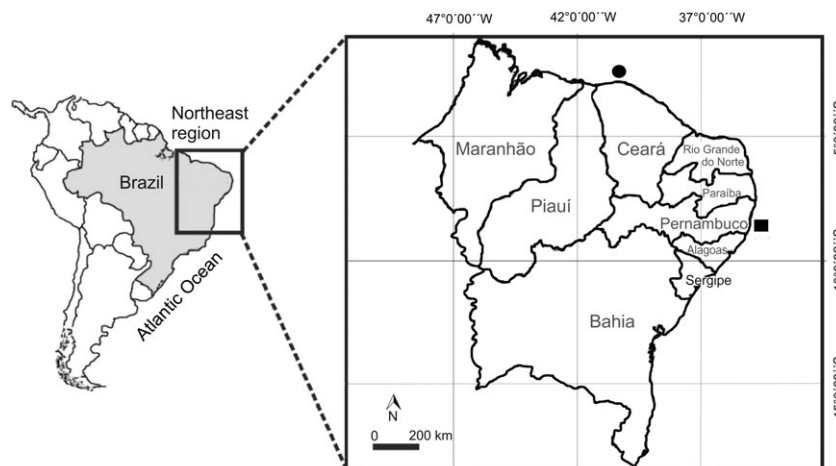
If populations do not recover naturally after the problems causing demographic decline are mitigated or solved, it will be necessary to adopt further appropriate management measures. Threatened populations may recover based on the introduction of demographically, epidemiologically, and genetically healthy individuals from other populations (Edmands, 2007). Another strategy adopted for this purpose is reproduction in captivity of individuals that belong to the population to be recovered, and the subsequent implementation of reinforcement strategies for the offspring spawned in nature (i.e. the introduction of captive-bred individuals; Williams & Hoffman, 2009). In both approaches it is necessary to evaluate the genetic diversity of the individuals that belong to the demographically weakened population (Edmands, 2002). Genetic diversity should also be investigated in migrant individuals retrieved from a technically suitable population (as in the first approach) or in the offspring spawned in captivity (as in the second approach) (Edmands, 2002). With the introduction of individuals from other locations, the genetic differentiation of the populations involved should be studied as a means to prevent outbreeding depression (Edmands & Deimler, 2004; Gharrett, Smoker, Reisenbichler, & Taylor, 1999). With reproduction in captivity, endogamy and loss of genetic diversity must be avoided. Endogamy may lead to the expression of recessive deleterious alleles in homozygotes, whereas a loss of genetic diversity could result in poor adaptation (Edmands, 2007; Galloway & Etterson, 2005).

The present study investigated the genetic diversity of adults of *H. reidi* from the Maracaípe River estuary and Jericoacoara National Park, both in north-east Brazil. Juveniles spawned from *H. reidi* adults captured in Maracaípe and kept in captivity were also analysed. The data obtained allowed for an assessment of the evolutionary potential of the Maracaípe population, and the most appropriate conservation strategy to help the recovery of this population: i.e. (i) the introduction of migrants from a demographically healthy population (Jericoacoara); or (ii) the reproduction in captivity of individuals from Maracaípe, and the subsequent use of the offspring in the reinforcement of the population in its natural environment.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection

Thirty adult *H. reidi* individuals collected in the Maracaípe River estuary and 29 animals from the Jericoacoara National Park were used in



**FIGURE 1** Map of South America (left), highlighting Brazil (grey). The rectangle indicates the north-east region of the country. The inset shows *Hippocampus reidi* collection sites: Jericoacoara National Park (circle) and Maracaípe River estuary (square)

the genetic analysis (Figure 1). Juveniles spawned from 10 couples from Maracaípe kept in captivity at the Marine Aquaculture Laboratory (Projeto *Hippocampus*) were also examined. Nine or 10 juveniles from each couple were included, representing roughly 2% of the offspring from each pregnancy (Silveira & Fontoura, 2010). Half of the juveniles analysed were from the first filial generation (F1) of individuals collected in nature, whereas the other half included F2 seahorses.

Adult individuals were manually captured by a free-diver. A small fragment (1 × 2 mm) of the dorsal fin was excised and stored in absolute ethanol for DNA extraction. The excision of a sample of the dorsal fin does not affect the growth and survival rates of seahorses (R. B. Silveira, pers. comm., November 2017). In juveniles, DNA was extracted using the whole specimen. This study was authorized by Chico Mendes Biodiversity Conservation Institute (ICMBio), permit number 40311-2, and by the Ethics Committee for the Use of Animals in Research (CEUA), permit number 068/2015.

## 2.2 | DNA extraction and amplification

The DNA samples were extracted according to the method developed by Medrano, Aasen, and Sharrow (1990), with modifications. Four primers, (CA)<sub>6</sub>AT, (CA)<sub>6</sub>GC, (CA)<sub>6</sub>AG, and (AGC)<sub>4</sub>T, were selected from a set of 24, to facilitate the production of a high number of easily identifiable polymorphic DNA fragments and the reproducibility of the results in the same individual.

The nuclear DNA was amplified by polymerase chain reaction (PCR) using the selected inter simple sequence repeat (ISSR) primers. The total volume of amplification reactions was 20 μL, containing 20 ng DNA template, 0.25 μM of each deoxynucleotide (dNTP), 200 nM primer, 2.5 μL PCR 10X buffer, ... 1 mM MgCl<sub>2</sub>, 1 Taq DNA polymerase unit (Invitrogen, Carlsbad, CA, USA), and distilled water. A negative control (without DNA) was used in each reaction to assess contamination. PCR parameters were: a 4-min denaturation step at 94°C, followed by 40 amplification cycles of 1 min at 94°C, 1 min annealing at 50°C, and 2 min at 72°C, followed by an extension cycle of 5 min at 72°C.

The amplified DNA fragments were stained with GelRed (Biotium, Hayward, CA, USA) and electrophoresed on 2.5% agarose gels in Tris-borate-EDTA (TBE) 1X buffer at 85 V, 95 mA, and 95 W, kept constant for 1 h. Next, gels were photographed under ultraviolet light.

The size of DNA fragments was estimated using a 100-bp ladder (Invitrogen). One-third of the samples were amplified in duplicates to ensure the reliability and reproducibility of the results. Only DNA fragments that were amplified consistently were included in the analysis.

## 2.3 | Data analysis

Using the data obtained from the ISSR, binary presence-absence tables were constructed. The results of this matrix were analysed in FAMM 1.3 (Schlüter & Harris, 2006) to estimate the number of polymorphic loci and the presence of exclusive DNA fragments.

Phylogenetic trees were constructed by the neighbour-joining method and the number-of-differences model using MEGA 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The same model was employed to calculate the inter- and intra-group genetic distances. STRUCTURE 2.3, which evaluates the genetic structure using Bayesian analysis, was used to estimate the most probable number of populations ( $K$ ; Pritchard, Stephens, & Donnelly, 2000). The software was run with five independent chains for each  $K$  value from 1 to 10, with 10<sup>6</sup> replications, and the first 10<sup>4</sup> generations were discarded as burn-in. The absence of mixture and independent allele frequency models were used in this analysis. The most probable number of populations was estimated by Evanno's test according to the model values ( $\Delta K$ ), based on a second-order rate of change for  $K$  using the likelihood function (Evanno, Regnaut, & Goudet, 2005). The analysis of molecular variance (AMOVA) was conducted in ARLEQUIN 3.5 (Excoffier & Lischer, 2010), generating inter- and intra-group distance matrices.

Tajima's  $D$  parameter (Tajima, 1989) was used to evaluate the presence of variation in population size with DNASP 5.1 (Rozas, Sánchez-Delbarrio, Messeguer, & Rozas, 2003). This program was also employed to estimate the pair-to-pair distribution of the expected and the observed differences (pairwise mismatch distributions).

## 3 | RESULTS

The combined results obtained with the four ISSR primers enabled the identification of 96 loci between 300 and 2000 bp, all of which were polymorphic in the 158 *H. reidi* individuals included in the analysis

**TABLE 1** Populations, number of individuals (*n*), and number of polymorphic DNA fragments with four ISSR primers for *Hippocampus reidi* from north-eastern Brazil. The juveniles were spawned from adults collected in the Maracaípe River estuary and kept in captivity

Population	<i>n</i>	Polymorphic DNA fragments
Adults from the Maracaípe River estuary, Pernambuco	30	91
Juveniles from the Maracaípe River estuary, Pernambuco	99	94
Adults from the Jericoacoara National Park, Ceará	29	89

**TABLE 2** Genetic distance in *Hippocampus reidi* from the north-eastern Brazil coast, measured using four ISSR primers. Values were calculated as means of the number of pairwise differences. Grey cells indicate the intrapopulation distance. White cells represent the interpopulation distance

	Jericoacoara (adults)	Maracaípe (adults)	Maracaípe (juveniles)
Jericoacoara (adults)	22.399		
Maracaípe (adults)	41.975	34.602	
Maracaípe (juveniles)	42.485	34.066	33.805

(Table 1). No identical combinations of ISSR fragments were observed in the animals. The genetic variability of individuals captured in Jericoacoara was low, being approximately 50% higher in the two groups of individuals from Maracaípe (adults and juveniles). The genetic distances for Maracaípe adults and juveniles were similar within and between groups (Table 2).

The similarity tree constructed employing the neighbour-joining method and the number-of-differences model had two main clusters: one included adults and juveniles collected in Maracaípe; the other was formed by adults from Jericoacoara (Figure 2).

The results obtained using STRUCTURE revealed that the most probable number of genetic populations, inferred through Evanno's test, was  $K = 2$  ( $\Delta K = 166.003$ ; Figure 3a). This analysis revealed the existence of one population formed by *H. reidi* juveniles and adults from Maracaípe, whereas the other included adults from Jericoacoara (Figure 3b). Yet,  $K = 3$  was poorly supported, according to Evanno's test ( $\Delta K = 0.091$ ).

The AMOVA was carried out considering two possibilities. The first was based on the three groups investigated, whereas the second was developed considering the results obtained with STRUCTURE, which divided the sample into two populations. The second option had the most robust structure for the species studied ( $F_{st} = 0.301$ ), discriminating Jericoacoara adults from the *H. reidi* adults and juveniles collected in Maracaípe (Table 3). The analysis including the three groups (Jericoacoara adults and Maracaípe adults and juveniles) had low population differentiation ( $F_{st} = 0.092$ ; Table 3). As this analysis took into consideration the pairwise differences between groups, this result reflects the poor genetic differentiation between adults and juveniles captured in Maracaípe, which formed one single population.

The analysis of the pairwise mismatch distribution showed that there is no population expansion (Figure S1). The same result was observed using Tajima's *D*-test (2.096;  $P = 0.927$ ).

## 4 | DISCUSSION

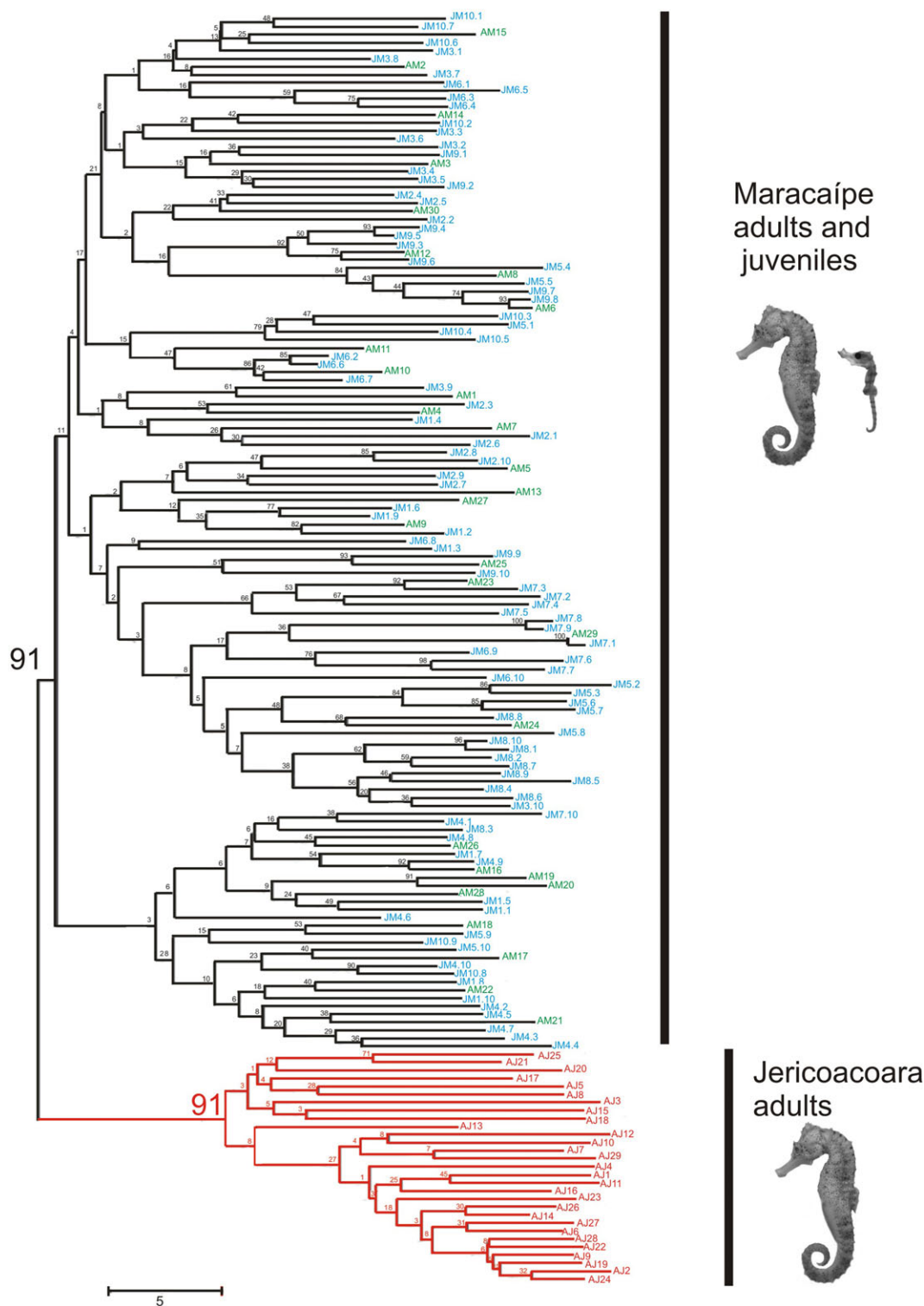
Any loss of genetic variability may limit the adaptive capacity of populations against environmental challenges, more intensely and

immediately if the population is formed by a small number of individuals (Frankham et al., 2010). Contrary to expectation, the *H. reidi* population from Maracaípe, which exhibited a marked decrease in the density of individuals, had higher genetic diversity compared with that from Jericoacoara, where recent surveys indicated the existence of a demographically large and stable population (Silveira et al., 2015). This result points to the evolutionary potential of the population from Maracaípe. This means that, if a conservation unit is established in the area (MPPE, 2017), then this population may recover naturally, producing offspring that are well adapted to this environment.

The genetic diversity of *H. reidi* has been analysed using mitochondrial and nuclear genes from several locations in the distribution area of this species (Casey, Hall, Stanley, & Vincent, 2004; Silveira et al., 2014; Teske, Hamilton, Matthee, & Barker, 2007). These studies investigated a small number of individuals and reported high genetic diversity. The same was observed in the present study, but using a larger sample size. Such results may be a useful tool in the development of successful conservation strategies for threatened *H. reidi* populations.

The genetic data obtained indicate, for the first time, the existence of a populational structure of *H. reidi*. This organization has been reported for other species of the genus. For example, research on mitochondrial (Choudhry, Lakra, & Choudhry, 2014; Goswami et al., 2009; Lourie, Green, & Vincent, 2005; Lourie & Vincent, 2004; Singh et al., 2011; Teske et al., 2005) and nuclear genes (Thangaraj, Lipton, John, & Gopalakrishnan, 2012) identified the genetic population structure of *Hippocampus barbouri*, *Hippocampus kuda*, *Hippocampus spinosissimus*, and *Hippocampus trimaculatus* in the Indian and Pacific Oceans. Additionally, in the northern Atlantic Ocean, investigations based on mitochondrial genes observed the genetic population structure of *Hippocampus hippocampus* (Woodall, Koldewey, & Shaw, 2011) and *Hippocampus zosterae* (Fedrizzi et al., 2015). Seahorses have biological characteristics that reduce the connectivity between populations, such as poor dispersion capacity, internal fecundation and pregnancy, absence of a larval stage, adult site fidelity, and small home range (Boisseau, 1967; Curtis & Vincent, 2005; Teske et al., 2005).

In the region adjacent to the area studied here, other fish species have also exhibited similar population structure. Such is the case of

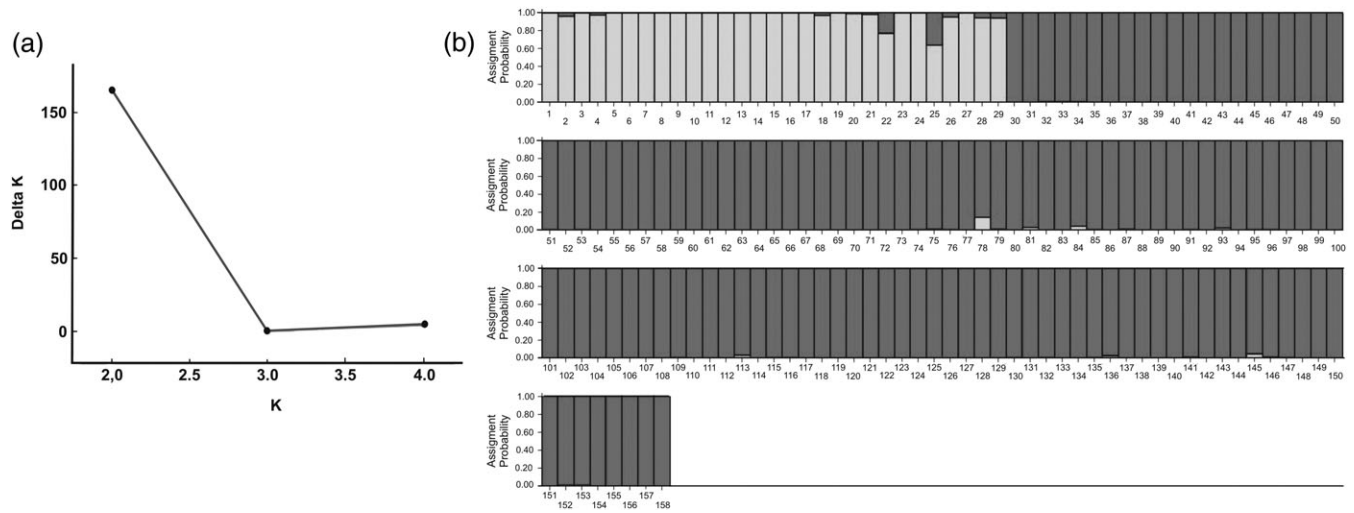


**FIGURE 2** Neighbour-joining tree for *Hippocampus reidi*, constructed using the number-of-differences model. Abbreviations: AJ, adults from Jericoacoara National Park, Ceará (red); AM, adults from Maracaípe River estuary, Pernambuco (green); JM, Juveniles spawned in captivity from Maracaípe individuals (blue)

*Epinephelus itajara*, *Holocanthus ciliaris*, and *Macrodon ancylodon*, which were evaluated using mitochondrial and nuclear genes (Affonso & Galetti Jr, 2007; Damasceno et al., 2015; Santos, Schneider, & Sampaio, 2003). Sea currents and environmental conditions may explain these findings (Boehm et al., 2013).

High genetic differentiation was observed between *H. reidi* individuals collected in Maracaípe and Jericoacoara. Owing to the

differentiation of these two populations, the resulting hybrids may present poor adaptation as a consequence of: (i) the interruption of the favourable interactions between genes and the environment (Templeton, 1986); (ii) the interaction between alleles that have never been tested together (Schierup & Christiansen, 1996); and (iii) the interruption of the epistatic interactions caused by recombination (Waser, 1993). Together, these three events promote a phenomenon called



**FIGURE 3** Evanno's test and Bayesian analysis carried out in STRUCTURE. (a)  $\Delta K$  values to determine the ideal number of population groups between samples of *Hippocampus reidi* formed by adult and juvenile individuals collected in Maracaípe River estuary and adult specimens from Jericoacoara National Park using four ISSR primers. (b) Bayesian analysis of 158 samples of *H. reidi* for the model with  $K = 2$ . Each vertical bar represents one individual. The length of the shaded segment indicates the proportion of an individual assigned to one genetic group: 1–29, adults captured in Jericoacoara; 30–50, adults collected in Maracaípe; and 60–158, juveniles spawned from individuals from the Maracaípe and kept in captivity

**TABLE 3** Molecular analysis of variance for 158 *Hippocampus reidi* individuals from populations living in the Maracaípe River estuary, Pernambuco (adults and juveniles), and Jericoacoara National Park, Ceará (adults), using four ISSR primers (df: degrees of freedom). Two groups: (adults from Jericoacoara) and (adults + juveniles from Maracaípe). Three groups: (adults from Jericoacoara), (adults from Maracaípe), and (juveniles from Maracaípe)

Cluster	Source of variation	df	Sum of squares	Variance component	Percentage variation
Two groups					
	Interpopulation	1	341.922	6.883	30.17
	Intrapopulation	157	2485.819	15.934	69.83
Three groups					
	Interpopulation	2	355.361	3.798	09.16
	Intrapopulation	156	2490.740	15.966	90.84

'outbreeding depression'. The negative effects of the crossing of genetically distinct populations have been observed for invertebrates (Armbruster, Bradshaw, Steiner, & Holzapfel, 1999; Aspi, 2000; Burton, 1990a, 1990b; Escobar, Nicot, & David, 2008; Peer & Taborsky, 2005), amphibians (Sagvik, Uller, & Olsson, 2005), birds (Marr, Keller, & Arcese, 2002), and fishes (Gharrett et al., 1999; Gharrett & Smoker, 1991). In view of this, the data obtained in the present study do not support the idea of introducing *H. reidi* individuals from Jericoacoara in Maracaípe.

The values of genetic diversity of juveniles and adults captured in Maracaípe were qualitatively and quantitatively similar, showing that there was no loss of variability in the formation of the breeding stock. The establishment of a conservation unit in Maracaípe will not stop the 'seahorse tour' from happening, which is a likely cause of the demographic decrease of *H. reidi* in the area; however, the impact of this tourist activity will be reduced by following a set of guidelines and reducing the frequency of tours (MPPE, 2017). In the event that these measures do not result in the efficient recovery of this

population within 5 years, then a captive breeding programme and the use of offspring for the reinforcement of this population may be an appropriate management strategy.

Some authors have highlighted the fact that differentiation and genetic erosion should be avoided in the establishment of breeding stocks in captivity (Ebenhard, 1995; Williams & Hoffman, 2009). The loss of genetic diversity during reproduction in captivity, compared with wild populations, has been reported in several investigations (Allard, 1988; Briscoe et al., 1992; Verspoor, 1988). The excessive number of generations kept in captivity was suggested to be the main reason behind the loss of genetic diversity and the risk of endogamy (Briscoe et al., 1992). In the present study, the juveniles analysed were the first or second generation held in captivity, which may explain the high genetic diversity and the fact that they are similar to the population from which they spawned.

Since 2001, the population density (the number of individuals per square metre) of *H. reidi* has been assessed periodically at several sites in Maracaípe (Silveira, 2005; Silveira et al., 2011; R. B. Silveira, pers. comm., November 2017). Studies on the genetic diversity of this population have also been conducted since 2012 (Neves et al., 2014; Neves et al., 2015; Neves, Cardoso, Garcia, Montes, & Silveira, 2017). These methods have been shown to be suitable in population surveys, and we suggest that they should be continued in order to assess the success and the number of required introductions, if this management strategy is adopted. Based on the *H. reidi* growth curve, established using data obtained in the natural environment of Maracaípe, we believe that the introduction of juveniles could be implemented at the age of 3 months (Silveira, 2005). The time for introduction would possibly be March, so that they may reach maturity during the forthcoming reproductive season (between May and November), when 50% or more of the *H. reidi* population is reproducing in Maracaípe (Silveira, 2005).

The noteworthy threat of extinction observed for several seahorse species across the globe underscores the importance of

studies to evaluate the aspects surrounding the conservation of these fishes. This work suggests an appropriate management approach to help the recovery of the *H. reidi* population in the Maracaípe River estuary, an area that has been considered a sanctuary for the species and that is seriously threatened today. The analysis of genetic diversity showed that this population has high variability and, consequently, presents evolutionary potential. If this population does not recover naturally with the establishment of a conservation unit, and the organization and regulation of the 'seahorse tours', reproduction in captivity and the reinforcement of the Maracaípe population with juvenile offspring would be an appropriate conservation strategy.

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