The presence of the seahorse *Hippocampus patagonicus* in the Argentine Sea based on the cytochrome b sequence of mitochondrial DNA

by

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ABSTRACT. - This study aimed at sequencing the mitochondrial cytochrome b gene (cyt b) of seahorses morphologically identified as *Hippocampus patagonicus* Piacentino & Luzzatto, 2004 from the two known localities where the species occur in the Argentine Sea (San Antonio Oeste and Mar del Plata). Based on the lack of substitutions within cyt b found between the individuals of both localities, the geographical range of *H. patagonicus* was extended up to the Northern limit of the Argentine Sea. A phylogenetic analysis comparing the cyt b sequences of *Hippocampus* species revealed that *H. patagonicus* together with *H. hippocampus* and *H. erectus* belong to a monophyletic group of species. The comparison of the cyt b sequence of *Hippocampus cf. erectus* from Southern Brazil with that one of *H. patagonicus* resulted in scarce differences. This could suggest the presence of *H. patagonicus* in southern Brazil, although further investigation is needed to resolve the taxonomic status of *H. erectus* in the South Western Atlantic.

RÉSUMÉ. - Présence de l'hippocampe *Hippocampus patagonicus* en mer d'Argentine démontrée par séquençage du cytochrome b.

La présente étude a pour but de déterminer la séquence du cytochrome *b* (cyt b) d'hippocampes échantillonnés dans la mer d'Argentine (à San Antonio Oeste et Mar del Plata) et préalablement identifiés sur des bases morphologiques comme appartenant à l'espèce *Hippocampus patagonicus* Piacentino & Luzzatto, 2004. L'absence de substitutions observée entre les séquences de cyt b provenant d'individus des deux localités échantillonnées permet d'élargir l'aire de répartition de l'espèce *H. patagonicus* depuis le sud de la Patagonie jusqu'à la limite nord de la mer d'Argentine. Une analyse phylogénétique comparant les séquences de cyt b issues de différentes espèces du genre *Hippocampus* démontre que *H. patagonicus*, *H. hippocampus* et *H. erectus* (prélevé au large du Brésil) appartiennent à un groupe monophylétique. En outre, le peu de différences relevées entre les séquences de cyt b de *H. erectus* (individu brésilien) et de *H. patagonicus* indiquent que la résolution du statut taxinomique de *H. erectus* présent dans l'Atlantique sud-ouest nécessite de nouvelles investigations.

Key words. - Syngnathidae - *Hippocampus patagonicus* - South Western Atlantic - cyt b - Record.

The genus *Hippocampus* Rafinesque, 1810 is distributed in temperate and tropical coastal waters between 50° N and 50° S (Lourie *et al*., 2004). The populations of many species within the genus *Hippocampus* have declined due to overexploitation and degradation of their natural habitats. The entire genus was included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) appendix II due to seahorse traffic mainly for the production of traditional medicines. In addition, almost all the species (37) have been listed by the International Union for Conservation of Nature (IUCN). Seven of them have been listed as Vulnerable, one as Endangered, one as Least Concern and 28 as Data Deficient. In order to contribute to the conservation and management of wild populations of *Hippocampus* many scientists have focused their efforts on understanding its natural history (Foster and Vincent, 2004) and identifying species (Lourie *et al*., 2004).

The genus has been poorly recorded in certain areas as is the case of the Argentine Sea. The first recorded seahorse in Argentina was identified as *Hippocampus guttulatus* Cuvier, 1829 (Berg, 1895). Ringuelet and Aramburu (1960), Menni *et al*. (1984), Cousseau and Denegri (1995) listed the presence of *Hippocampus punctulatus* Guichenot, 1853, which was considered a synonym of *Hippocampus erectus* Perry,

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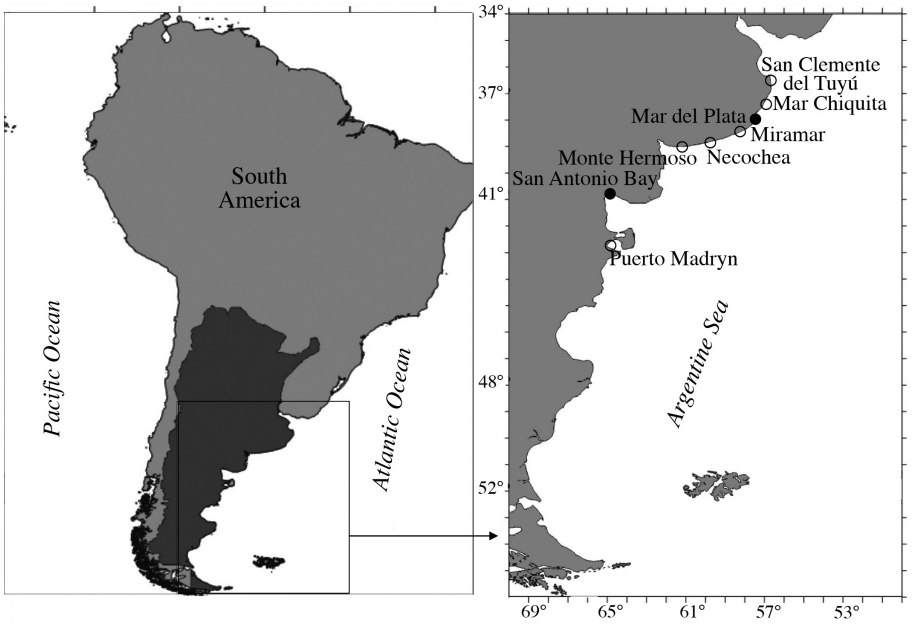

Figure 1. - Dots indicate the sampling localities of *Hippocampus patagonicus* for DNA extraction, indicating permanent seahorse populations. Open dots indicate localities showing sporadically occurrence records of seahorses.

1810 (Vari, 1982). *Hippocampus patagonicus* (Piacentino & Luzzatto, 2004) was recently described as a new species for Argentinean waters and the reported geographical range included the northern Patagonia area (Puerto Madryn-San Antonio Bay) (Fig. 1). Piacentino (2008) expanded its range southwards to Puerto Deseado in south Patagonia and fixed its northern limit to the Rio Negro estuary (49º S to 40ºS, respectively).

The southernmost distribution of *H. erectus* is considered doubtful. The species has been reported to be occurring as far south as Brazil (Lourie *et al*., 1999), and Uruguay (Teixeira and Musick, 2001). Baum *et al*. (2003) expanded the geographical range of the species southwards to Argentina. Lourie *et al*. (2004) clarified that the latter is a suspected distribution. On the other hand, based on cyt b sequence, Casey *et al*. (2004) reported that there is molecular evidence to consider the existence of more than a single taxon within *H. erectus*. Given that classical morphological measurements (Lourie *et al*., 2004) could not be sufficient to separate related species of seahorses (Teske *et al*., 2007a) the name *H. patagonicus* should be validated.

Table I. - Morphological measurements of the individuals sampled.

	San Antonio Bay (N = 4)	Mar del Plata (N = 4)
Height (mm)	132 ± 13	103 ± 12
Trunk rings (N°)	11	11
Tail rings (N°)	38 (38-39)	38 (37-38)
Dorsal fin rays (N°)	17 (16-18)	17 (17-18)
Anal fin rays (N°)	4	4
Pectoral fin rays (N°)	13 (13-14)	13

In this work cyt b of seahorses of geographically distant localities of Argentina was sequenced and compared to the cyt b sequences previously obtained from *H. erectus* (Casey *et al*., 2004). The results contribute to the understanding of the phylogeny of *H. erectus* complex and of the geographical range and taxonomic status of *H. patagonicus*.

MATERIAL STUDIED, AREA DESCRIPTION, METHODS AND TECHNIQUES

Sample acquisition and DNA extraction

Individuals morphologically identified as *H. patagonicus* from two different populations in the Argentine Sea were sampled (Tab. I, Fig. 1). Individuals were collected from San Antonio Bay (40°45'S; 64°54'W), the type locality of *H. patagonicus*. In this locality, seahorses were found to be associated to sessile invertebrates (tube polychaetes and sponges) and algae on a shallow sandy bottom. Also, individuals were collected in Mar del Plata dock (38°02'S; 57°32'W), a recently created artificial environment with a shallow mud/sandy bottom. These two permanent populations (the one present in San Antonio Bay and the other in Mar del Plata dock) are, so far, the only known distribution localities along the marine coast of Argentina. Seahorses were obtained by scuba diving at depths between 1 and 6 m at both localities and kept in aquaria devices for aquaculture purposes. After their natural death, seahorses were preserved in 96% ethanol.

Four individuals from each locality were used for DNA extraction. Tissue samples of approximately 50 mg were removed and DNA was extracted as described previously (Kocher *et al*., 1989). Briefly, tissue samples were digested

in lysis buffer [100 mM Tris-HCl pH 7.6, 10 mM EDTA, 100 mM NaCl, 0.1% SDS, 50 mM DTT, 0.5 μg/ml Proteinase K] with gentle agitation at 37°C for 2 hours. Samples were extracted with chlorophorm:isoamyl alcohol (24:1), ethanol precipitated, washed with 70% ethanol and resuspended in water.

Primer design, PCR and sequence alignment

Primers CytbUp (5'-CCGGAATTCTAACCAGGACTAATGGCTTG-3') and CytbDown (5'-CCGGAATTCGATTTTGTTTTCCATTCAAC-3') were designed according to highly conserved regions of cyt b of different fish species. For cloning purposes, primers were designed as 20 base pair (bp)-long oligonucleotides specific for cyt b, with additional 9 bp-tails that contain sites for the EcoRI restriction enzyme. A polymerase chain reaction (PCR) was carried out using *Taq* polymerase (Invitrogen), primers CytbUp and CytbDown, and seahorse DNA as template. The PCR consisted of 5 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, and 1:20 minutes of extension at 72°C; followed by 30 additional cycles with 30 seconds of annealing at 64°C. The PCR product was cloned into pGEM-T Easy (Promega) and sequenced in both directions using primers T7 and SP6, which flank the cloning region in the pGEM-T Easy vector. Sequence analyses and alignments were performed using EditSeq and MegAlign (DNASTAR).

Phylogenetic analysis

The cyt b sequences of *Hippocampus* species published in the GenBank have been compared with the sequences obtained in the present study. The comparisons were made with the Molecular Phylogenetic Analysis by Maximum Likelihood method with Mega5 package (Tamura *et al*., 2011). The bootstrap consensus tree was inferred after 500 replicates. The information used to perform the analysis were the GenBank accession code, the taxonomic identity given by the authors and the published cyt b sequences.

Museum material

In order to preserve the samples for future studies, four seahorses from Mar del Plata (Accession numbers MMPEAA S/001, S/002, S/003 and S/004) and four seahorses from the San Antonio Bay (Accession numbers MMPEAA S/011, S/012, S/026 and S/027) have been deposited in the Syngnathid Collection of Museo Municipal de Ciencias Naturales "Lorenzo Scaglia". The samples have been preserved in ethanol 96º.

RESULTS

The obtained 1144-base pairs (bp) PCR product corresponds to positions 14331-15472 of the complete sequence of mitochondrial DNA of *Hippocampus kuda* Bleeker, 1852 (GenBank accession number AP005985), which includes a 36-bp upstream non-coding region and 1108 bp of cyt b. Comparison of the obtained sequences showed no difference among individuals obtained from Mar del Plata or San Antonio Bay (GenBank accession no. EU871944 for Mar del Plata and EU871945 for San Antonio Bay). Thus, specimens of both localities resulted in one haplotype.

The cyt b sequences obtained for *H. patagonicus* in Mar del Plata and San Antonio Bay resulted in minor differences compared to the previously obtained by Casey *et al*. (2004) for the Brazilian *H.* cf. *erectus*. The consensus sequence of *H. patagonicus* diverged from the Brazilian seahorse sequence (GeneBank accession number AF192660) in 6 nucleotide substitutions (390: G-A, 612: A-G, 909: C-T, 979: A-C, 1072: G-T and 1075: C-T), indicating a 0.53% difference at the nucleotide level. The first three substitutions are synonymous, whereas the last three caused the substitutions M329L, D358Y and L359F, respectively.

The phylogenetic analysis showed that *H. hippocampus*, *H. erectus* and *H. patagonicus* constituted a group of species separated from other seahorse complexes/species (Fig. 2, Casey *et al*., 2004). A single seahorse identified as *H. erectus* is grouped in the *H. patagonicus* branch. This is the Brazilian *H. erectus* analysed above.

DISCUSSION

The lack of substitutions among the cyt b sequence of all specimens analysed indicates that there is not taxonomic divergence between the populations of San Antonio Bay and the recently created environment of Mar del Plata. Therefore, genes/sequences with a higher degree of sequence variability should be analysed to assess possible variability between these two populations.

The geographical range of *H. patagonicus* in the Argentine Sea considered in the present work includes the Río de la Plata River (San Clemente del Tuyú) in the north to the locality of Puerto Madryn in the south.

Previous studies (Piacentino and Luzzatto, 2004; Piacentino, 2008) reported occasional records of seahorses (as by-catch, found dried on the beach or as deposited Museum material) between the localities of Mar del Plata and San Antonio Bay (e.g. Miramar, Necochea, Monte Hermoso) (Fig. 1). Sporadic individuals have also been reported to the north of Mar del Plata and to the south of San Antonio Bay (e.g. in San Clemente del Tuyú and Puerto Madryn, respectively) (Fig. 1). All these records corresponded to isolated individuals findings, rather than the presence of a stable population. The recorded individuals to the south of Puerto Madryn (Piacentino, 2008) could not be confirmed by the present work. The Museum records provided by Piacentino

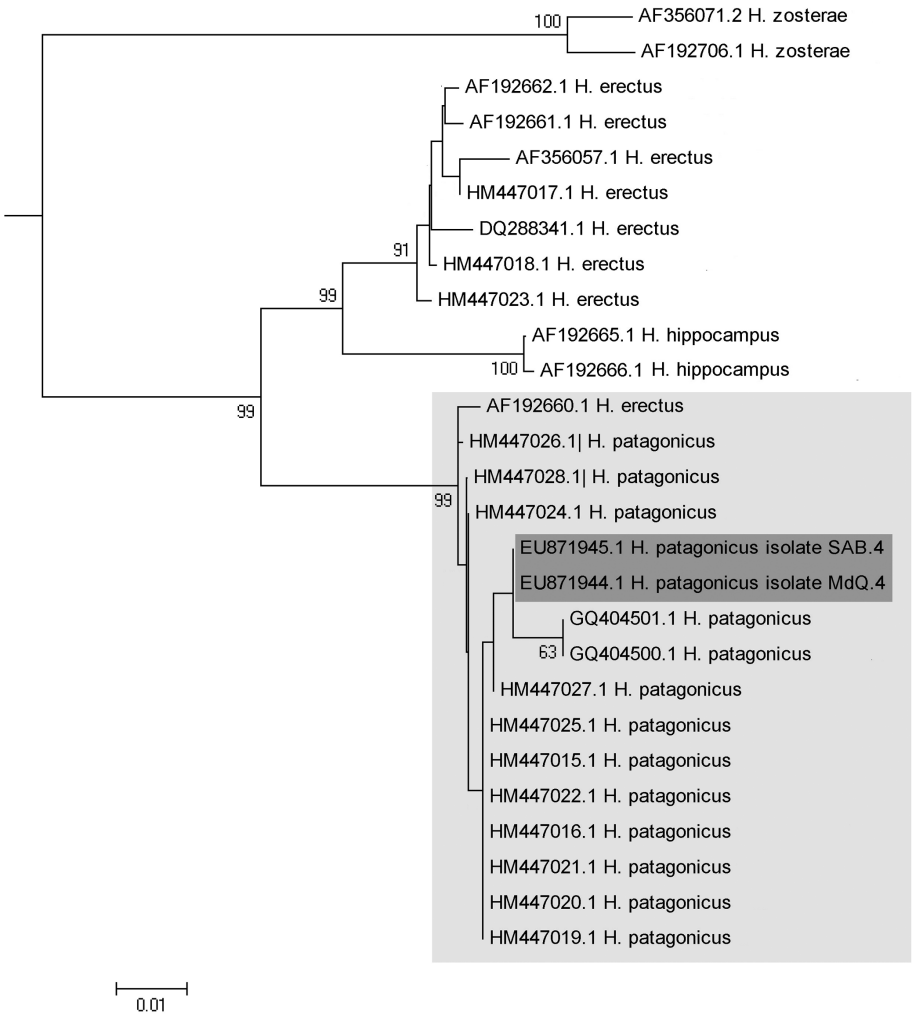

Figure 2. - Molecular Phylogenetic Analysis by Maximum Likelihood method of *Hippocampus***.** The GenBank accession code and the taxonomic identity given by the authors are indicated in the tree. The branch containing the *H. patagonicus* group and the sequences informed in the present work are highlighted. This is a Casey *et al*., 2004 complementary *Hippocampus* phylogenetic analysis showing only the branches analysed in the present work.

(2008) neither included seahorses in Southern Patagonia nor specified how the reports were obtained. Piacentino (2008) also found seahorses to the north of Rio Negro Estuary and set the northernmost limit of *H. patagonicus* up to this point, saying nothing about the taxonomic status of the seahorses found in Buenos Aires Province. The cyt b sequence analysed in the present work strongly revealed the identity of *H. patagonicus* at this locality.

Rafts have been proposed as a mechanism of dispersion and speciation within the genus (Teske *et al*., 2005, 2007b). So far, the populations inhabiting San Antonio Bay and Mar del Plata are the only known *H. patagonicus* permanent populations in the Argentine Sea. The occasionally reported specimens could be the consequence of the settlement of juveniles in inadequate places for their reproduction after being dispersed by rafts. Juvenile individuals from San Antonio bay have been found using rafts as a way of dispersion. Furthermore, aquaria experiments suggested that *H. patagonicus* from 20 days to two months old prefer floating rather than bottom-attached substrates (unpubl. data). These observations could support the hypotheses that *H. patagonicus* settled down in Mar del Plata recently (decades ago), after the construction of the dock. This dock had implied a radical modification of the shoreline creating protected environment along an exposed coast.

The genus could have experienced a recent expansion into the Argentine Sea based on the minor substitutions observed in the cyt b between the sequence provided by Casey *et al*. (2004) for the Brazilian *H.* cf. *erectus* and the sequence presented herein (0.53%). Casey *et al*. (2004) also reported wider sequence differences (5.9%) between *H.* cf. *erectus* of Brazil and *H. erectus* of two localities in The United States, setting uncertainty on the taxonomic uniqueness of the species. Moreover, the low difference found in the cyt b sequence between *H.* cf. *erectus* and *H. patagonicus* suggest that they are the same species, *H. patagonicus*. A more detailed analysis, including molecular and morphological measurements, should be performed to clarify the taxonomic

status of the populations assigned to the species *H. erectus* along the Southwestern Atlantic Ocean.

In summary, the results presented here allow to expand the geographical range of *H. patagonicus* up to the northern limit of the Argentine Sea and to suspect its presence at lower latitudes in the Southwestern Atlantic Ocean. Piacentino (2008) identified the species in South Patagonia but the data should be confirmed.

Conservation concerns

The species of *Hippocampus* suffer a severe decline due to overexploitation and habitat degradation worldwide (Foster and Vincent, 2004). So far, the seahorses of the South Atlantic Ocean are not considered as a conservation issue mainly for its recent description and the lack of knowledge about their biological characteristics. *H. patagonicus* is not listed in the IUCN red list but *H. erectus* is regarded as Vulnerable. *H.* cf. *erectus* has been reported as a highly traded seahorse in southern Brazil (Rosa *et al*., 2011). In Argentina, Piacentino (2008) reported that seahorses are traded alive and dry. It is necessary to establish the identity of the seahorses assigned to *H.* cf. *erectus* and to set the magnitude of the threats that Argentinean populations are facing. It is also needed to clearly establish the geographical range of the species in order to set their conservation status.

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