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Original article

Morphological, molecular identification and evaluation of antioxidant activity of seahorses from the Moroccan coasts

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ABSTRACT

Seahorses, part of the small marine teleost fish family *Syngnathidae*, are increasingly under threat due to habitat degradation and overfishing. Notably used in traditional Chinese medicine, these fish have demonstrated significant pharmacological and cosmetic properties. In Morocco, however, seahorses are minimally exploited. This study aims to explore the biodiversity of Moroccan seahorses, focusing on identifying species from the Atlantic and Mediterranean coasts both morphologically and molecularly, and evaluating their antioxidant activity.

The research involved collecting 62 dried seahorses from local fishermen. These specimens were subjected to detailed morphological and molecular identification through the DNA barcoding method, concentrating on the mitochondrial marker Cytochrome Oxidase I (COI) gene. Following DNA extraction and amplification, the sequences were analyzed for species identification and phylogenetic relationships. Additionally, the antioxidant activities of the seahorses were quantified using assays such as ABTS, reducing power, phosphomolybdenum, and β-carotene-linoleic acid.

The combined morphological and molecular analyses consistently identified all specimens as *Hippocampus hippocampus*, and phylogenetic trees suggested a close relation with European and Turkish counterparts. Furthermore, the antioxidant assays revealed significant activity, with the ABTS assay showing an IC50 of 14.571 mg/mL ± 0.334, and the β-carotene-linoleic acid assay showing an IC50 of 1.273 mg/mL ± 0.166. The reducing power and phosphomolybdenum assays recorded EC50 values of 1.868 mg/mL ± 0.033 and 1.156 mg/mL ± 0.112, respectively. These results confirm the high antioxidant potential of Moroccan seahorses, suggesting their therapeutic value and necessitating measures for their biodiversity preservation at a national level.

1. Introduction

Belonging to the genus *Hippocampus*, seahorses are diminutive marine fishes that reside in both tropical and temperate waters globally (Britz, 2017; Foster and Vincent, 2004). These species exhibit distinct sedentary behaviors, often occupying shallow water habitats and maintaining a reticent lifestyle. Noteworthy in the world of marine biology, their unique segmented anatomy features a pair of pectoral fins, a single dorsal fin, a modest anal fin, and a noticeable absence of a caudal fin. Moreover, they display unusual reproductive characteristics, notably male pregnancy (Foster and Vincent, 2004).

Discerning species within the *Hippocampus* genus can present difficulties when reliant on morphological attributes alone, considering the inherent diversity in size, color, and form. Therefore, the fusion of morphological and molecular techniques has emerged as the most effective strategy for examining the taxonomy and phylogeny of seahorses (Short et al., 2019; Silveira and Siccha-Ramirez, 2014). Of note, DNA barcoding has been recognized as a successful method for molecular identification (Dasmahapatra and Mallet, 2006; Guardone et al., 2017).

DNA barcoding, a prominent molecular identification technique, exploits genetic variances between species. Typically, mitochondrial DNA (mtDNA) is utilized due to its uniparental inheritance and high similarity within closely related species (Teske et al., 2003). The Cytochrome Oxidase subunit I (COI) gene, found in mtDNA, is frequently used as a DNA barcode due to its rapid mutation rate that induces

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sufficient genetic diversity for precise species identification, notably in marine taxa (Bucklin et al., 2021). Moreover, COI gene analysis offers valuable insights into intraspecific and interspecific relationships among diverse marine fishes and shellfish (Wanghe et al., 2022; Zhang and Hanner, 2012).

Hippocampus, or seahorses, are revered in Traditional Chinese Medicine (TCM) due to their antitumor, antioxidant, and antibacterial properties (Chen et al., 2015; Kumaravel et al., 2012). Seahorses are known to harbor bioactive antioxidant molecules that efficiently neutralize free radicals or active oxygen species (Kumaravel et al., 2012; Pan et al., 2007). As the demand for natural over synthetic antioxidants surges in the food and medical industries, research on these bioactive compounds escalates. Natural antioxidants, such as those found in seahorses, offer potential in combating free radical damage, preventing chronic diseases, and delaying lipid oxidation in food and medicine (Choi et al., 2007; Kang et al., 2008).

This study aims to investigate Moroccan seahorses along the Atlantic and Mediterranean coastlines, using morphological and molecular techniques for species identification and phylogenetic classification. It also evaluates their phenolic, flavonoid contents, and antioxidant properties, providing insights into their diversity and medicinal potential in Morocco.

2. Materials and methods

2.1. Sample collection

From January 2021 to January 2022, sixty-two dried adult seahorses were collected from traditional fishermen and purchased from sellers around seven locations in Morocco, covering both Mediterranean (Quaà Asserasse, Stehat, Chmaala, El Jebeha) and Atlantic (El Jadida, Agadir, Dakhla) coasts (Fig. 1). Samples were grouped based on geographical location and their sex. The distribution of collected samples, detailed in Table 1, includes 15 seahorses from Mediterranean locations and 47 from the Atlantic coast, with a composition of 31 males and 31 females. All samples were stored at −20 °C.

2.2. Identification of morphological features

The morphological characterization of the 62 dried seahorse samples was executed employing the guidelines set forth by Lourie et al. (2004). The processing methods used for drying seahorses inflicted some impairment on the morphological traits typically used in identification, including dorsal and pectoral fins. Consequently, this study focused on the examination of specific characteristics for seahorse identification: height, length of head and snout, number of trunk rings, tail rings, cheek spines, and eye spines. The metrics of these morphological characters for each group were precisely ascertained using a ruler. Additionally,

Table 1
Distribution of Collected Seahorse Samples by Sex and Location in Morocco.

	Location	Male	Female	Total number
Mediterranean	S1: Quaà Asserasse	2	3	5
	S2: Stehat	1	0	1
	S3: Chmaala	2	2	4
	S4: El Jebeha	2	3	5
Atlantic	S5: El Jadida	4	2	6
	S6: Agadir	17	19	36
	S7: Dakhla	3	2	5

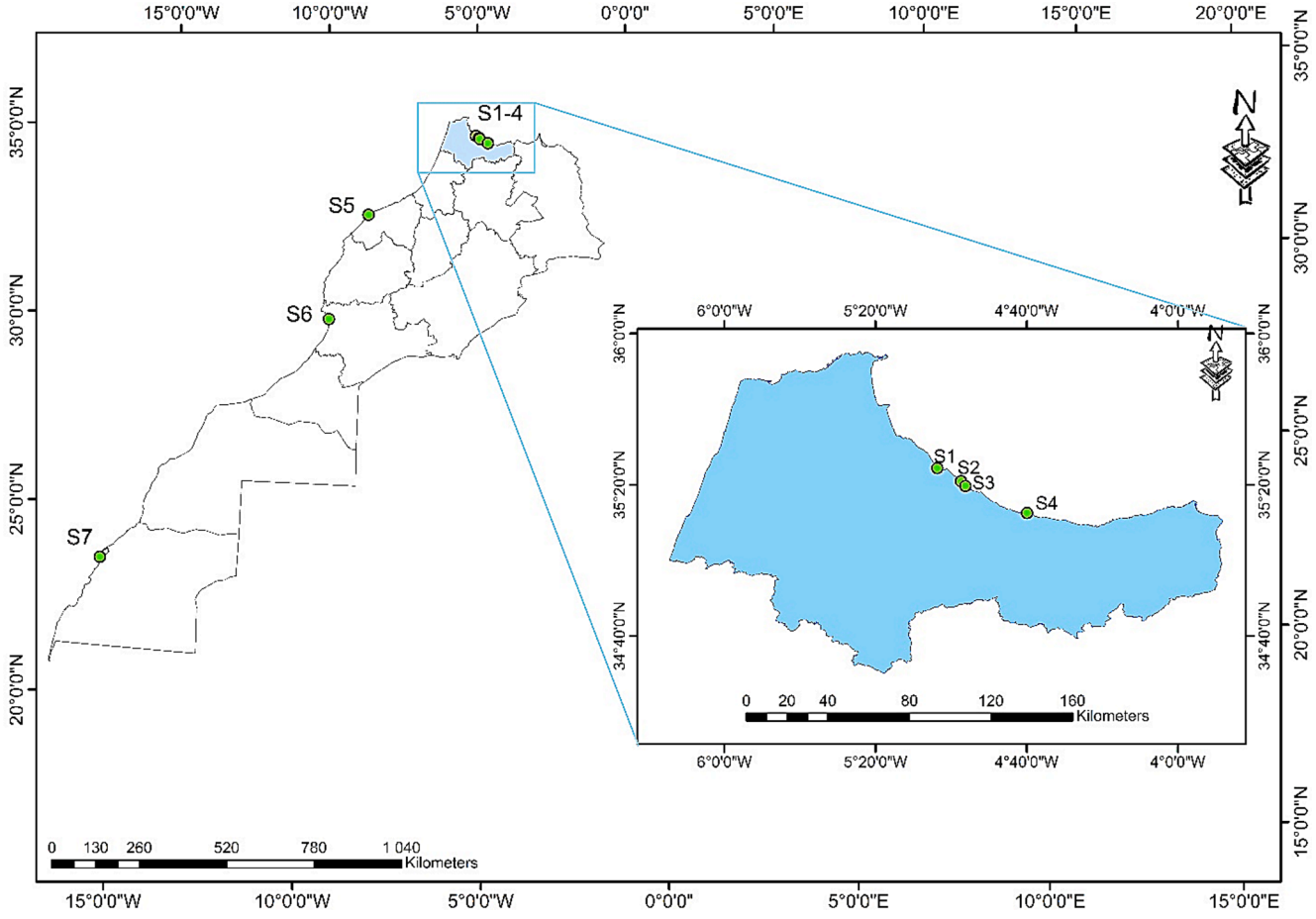

Fig. 1. Sampling sites of *Hippocampus* of the two Moroccan coasts. S1: Quaà Asserasse; S2: Stehat; S3: Chmaala; S4: El Jebeha; S5: El Jadida; S6: Agadir; S7: Dakhla.

meristic features for each group were counted manually.

2.3. Molecular identification

2.3.1. DNA extraction, amplification, control and sequencing

The standard phenol/chloroform extraction method was applied to extract genomic DNA from the tail muscle (Barnett and Larson, 2012). The extracted DNA was stored at −20 °C for subsequent use. The quantity of DNA from each sample was determined using a Qubit Fluorometer (Qubit 4 Fluorometer, Invitrogen ™, Thermo Fisher Scientific, Waltham, USA). To amplify the COI regions of the 62 seahorse samples, two pairs of universal primers were employed (Ward et al., 2005):

FishF1: 5′-TCAACCAACCACAAAGACATTGGCAC-3′;
FishR1: 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′.
and.
FishF2: 5′-TCGACTAATCATAAAGATATC GGCAC-3′;
FishR2: 5′-ACTTCAGGGTGACCGA AGAATCAGAA-3′.

The PCR reactions were performed in a 25 μL final volume, which consisted of 12.5 μL of 5X My Taq Reaction buffer, 0.5 μL of My Taq DNA Polymerase, 0.4 μM of each primer, 5 ng of genomic DNA and 10 μL of water. The reactions were run using a Biometra Thermocycler (Biometra, Goettingen, Germany) with the following conditions: a preheating step at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 60 s, primer annealing at 62 °C for 60 s, and extension at 72 °C for 60 s, with a final extension step at 72 °C for 10 min. The PCR products were visualized on a 1.5 % agarose gel to confirm the amplification success. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and then sent for sequencing to LGC Genomics GmbH using DNA sequencer ABI 3730 XL (Berlin, Germany).

2.3.2. Sequences alignment and phylogenetic tree

The sequences obtained from the amplicons of the COI gene were analyzed using the Basic Local Alignment Search Tool (BLASTn) on GenBank NCBI (https://blast.ncbi.nlm.nih.gov). This analysis was specifically conducted for the purpose of species identification. By aligning our sequences with those in the GenBank database. The aligned COI sequences of all *Hippocampus* were quantified by pairwise genetic divergence using the Kimura two-parameter (K2P) model. MEGA software (version 11.0) was used for sequence alignment, ensuring no gaps or stop codons were present, in addition due to low genetic distances (Nei and Kumar, 2000). An unrooted Neighbor-Joining (NJ) tree was built with GenBank sequences and our own dataset. In order to assess the robustness of the NJ tree, bootstrapping analysis with 1000 replicates using K2P distances was conducted (Felsenstein, 1985).

2.3.3. Genetic diversity

For each population, we calculated the following parameters using the DnaSP version 6.10 program (Librado and Rozas, 2009): the number of sequences (n), the number of haplotypes (h), the number of variable sites (ns), the haplotype diversity (hd), the nucleotide diversity (Pi), and the average number of nucleotide differences (k).

2.4. Phytochemical analysis

2.4.1. Preparation of extract

The process of extraction involved grinding different portions of the specimens to a fine powder and sieving the powder through a mesh with a size of 0.5 mm. 5 g of the resulting dry powder was then mixed with 100 mL of methanol (HPLC grade) and incubated for 24 h at 28 ± 2 °C at 100 rpm using a shaking incubator (“Witeg’’ Wise Cube WIS Precise) (Qian et al., 2008). The mixture was subjected to centrifugation at 3500 rpm for 10 min at 4 °C. The supernatant was filtered through Whatman No. 1 filter paper and the methanol was removed from the extract using rotary evaporation (D-LAB RE 100-Pro, USA) at 45 °C, and the extraction yield was expressed as a percentage. The dried extract was redissolved in methanol (HPLC grade, Sigma-Aldrich) to obtain a stock extract solution with a concentration of 25 mg/mL, which was used to evaluate various antioxidant activities.

2.4.2. Determination of total polyphenol content

The total phenolic (TP) content was determined using the Folin-Ciocalteau reagent with gallic acid as a reference, following the method described by Guenaou et al. (2021). To measure the absorbance at 765 nm, we employed a spectrophotometer (Jenway-6850, UK).

2.4.3. Determination of total flavonoid content

Total flavonoid (TF) content was quantified employing the aluminum chloride colorimetric method, utilizing quercetin as a standard, in accordance with the method delineated by Ahn et al. (2007).

2.5. Antioxidant activity

2.5.1. Reducing power assay

The reducing power assay, serving as an evaluation of the antioxidant potential of the sample solution, was executed. This assay measures the calorimetric reduction corresponding with the oxidation–reduction process of antioxidants in the sample, following the protocol prescribed by Oyaizu (1986).

2.5.2. Scavenging activity against ABTS free radical

The evaluation of the radical scavenging activity of ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) was performed according to the methodology described by Ezaouine et al. (2022).

2.5.3. Total antioxidant capacity: Phosphomolybdenum assay

The test was conducted using the method outlined by Trabelsi et al. (2012).

2.5.4. β-carotene blanching inhibition assay

The antioxidant activity was also evaluated using the bleaching-carotene linoleic acid method described by Nait Irahal et al. (2020).

2.5.5. Statistical analyses

The seahorse extract samples were analyzed in triplicate. The results were expressed as mean values and standard deviations. The data were subjected to statistical evaluation using a one-way analysis of variance (ANOVA), followed by Tukey’s HSD test. The significance level was set at $p < 0.05$, and the analysis was conducted using Prism 8 software for Windows (Graph Pad Software Inc., San Diego, CA, USA).

3. Results

3.1. Morphological identification

A morphological identification analysis was conducted on 62 seahorse individuals. The results showed that all individuals exhibited

Table 2
The main identification characteristics of Seahorse.

	Location			
Description	S1-4	S5	S6	S7
Number of individuals	15	6	36	5
Average height (cm)	8–11	11–13	6–10.5	8–10
Average head length (HL) (mm)	20–23	22–24	20–23	21–23
Average of snout length (SnL) (mm)	7–8	7–8	6–8	7–8
HL/SnL	2.85–2.87	3–3.14	2.85–3.33	2.87–3
Trunk rings (unit)	11	11	11	11
Tail rings (unit)	36–38	37–38	36–37	37
Cheek spines (unit)	2	2	2	2
Eye spine (unit)	2	2	2	2
Nose spine (unit)	1	1	1	1

similar characteristics for all criteria studied, as presented in Table 2. Seahorses from Mediterranean locations (S1-4) generally exhibited slightly larger sizes, with average heights ranging from 8 to 11 cm and head lengths of 20–23 mm. In contrast, seahorses from the Atlantic locations (S6-7) showed a broader size range of 6 to 10.5 cm, with similar head length ranges. However, an exception was observed in seahorses from location S5, which were longer than those from other locations, with lengths ranging from 11 to 13 cm and head lengths of 22–24 mm. The ratio of head length to snout length (HL/SnL) was found to be between 2.85 and 2.87 for the Mediterranean seahorses and ranged from 2.85 to 3.33 for the Atlantic seahorses. The color of the seahorses was observed to be either fully dark brown (in 34 individuals) or light brown (in 22 individuals), while six individuals were yellow as illustrated in Fig. 2. Additionally, the number of tail rings in all seahorses, whether from Atlantic or Mediterranean locations, was determined to be between 36 and 38. Each individual also exhibited 11 trunk rings, two cheek spines, two eye spines, and one nose spine. These morphological characteristics serve to confirm that all of the analyzed seahorses correspond to the species *Hippocampus hippocampus*.

3.2. Molecular identification

Amplification of the COI gene barcode sequence was performed on all samples, yielding a band of 650 bp in only 42 samples. The amplified products were sequenced, and the resulting sequences were aligned to a consensus length of 650 bp. Thirty-four of these sequences were deposited in the NCBI GenBank database, with accession numbers ranging from **ON823138**-**ON823152**/ **ON823154**-**ON823172**.

BLASTn analysis performed on the NCBI database confirmed that all specimens tested from the Atlantic and Mediterranean regions were identified as *Hippocampus hippocampus*, with identity values ranging from 99 to 100 %, as shown in Table 3.

3.3. Genetic diversity and phylogeny tree

A phylogenetic analysis of all COI sequences obtained in this study was conducted using the NJ method and *Solegnathus hardwickii* (GenBank accession no. **MH999466**) as an outgroup. The genetic distance was used to determine the degree of kinship between intraspecies and interspecies. The results of the genetic distance matrix analysis of the COI gene fragment in *H. hippocampus*, *Hippocampus* spp., and *Solegnathus* are presented in Table 4.

In examining genetic distances between the genus *Hippocampus* and *Solegnathus*, a range of 0.187 to 0.211 was identified, with the maximum genetic distance of 0.211 noted between H. *hippocampus* (**ON823143**) and *S. hardwickii*. The genetic distances between the species *H. hippocampus* and other seahorses of the genus *Hippocampus* were found to range from 0.034 to 0.143. *H. hippocampus* specimens collected from Moroccan coastal waters had a genetic distance of 0.000 to 0.011 with *H. hippocampus* (**KY176504**) from Turkey and *H. hippocampus* (**KC851882**) from Europe, meaning Moroccan seahorses are closely related to seahorses from Europe and Turkey.

To further illustrate the relationships between the species, the genetic distance values were utilized to construct a phylogenetic tree, depicted in Fig. 3. The tree shows that the two main clusters separated the genera *Hippocampus* and *Solegnathus*. The species of *H. hippocampus* we identified were grouped together in a subcluster that was distinct from other *Hippocampus* species. Additionally, the analysis revealed higher haplotype diversity (hd = 0.93939), nucleotide diversity (pi = 0.00904), and mean number of nucleotide differences (k = 3.45455) in the Mediterranean *H. hippocampus* population compared to the Atlantic population of *H. hippocampus*, as shown in Table 5.

3.4. Phytochemical analysis

The extraction yield of the sample was determined to be 5.2 % (w/w). The TP content of *H. hippocampus* was determined using a regression equation obtained from the calibration curve ($y = 0.007x$; $R^2 = 0.983$). The result showed a TP content of 11.87 ± 0.75 mg GAE/g. Additionally, the TF content was determined using a calibration curve equation ($y = 0.035x$; $R^2 = 0.999$). The analysis revealed a TF content of 5.467 ± 0.54 mg QE/g.

3.5. Antioxidant activities of H. hippocampus

In this study, the antioxidant activity of seahorse extract was evaluated through in vitro methods, including the ABTS cation radical scavenging ability assay, the reducing power test, the phosphomolybdate assay, and the β-carotene-linoleic acid assay. The results were compared to the standard antioxidant ascorbic acid (Fig. 4). The ABTS binding capacity of ascorbic acid and seahorse extract had an IC50 of 0.238 mg/mL ± 0.002 and 14.571 mg/mL ± 0.334, respectively. The

Fig. 2. Illustrative Example of Seahorses Harvested from the Moroccan Coastline.

Table 3
Sampling locations and results of species identification based on each specimen's barcode sequence using the BLASTN search tool from GenBank database.

Location	COI GenBank accession no.	Molecular identification	Identity (%)	Max Score
Quaa Asserasse	**ON823138**	*Hippocampus hippocampus*	99.68 %	1149
Quaa Asserasse	**ON823154**	*Hippocampus hippocampus*	99.84 %	1140
Quaa Asserasse	**ON823155**	*Hippocampus hippocampus*	99.84 %	1140
Quaa Asserasse	**ON823164**	*Hippocampus hippocampus*	99.35 %	1123
Stehat	**ON823139**	*Hippocampus hippocampus*	99.52 %	1127
El Jebha	**ON823142**	*Hippocampus hippocampus*	99.84 %	1133
El Jebha	**ON823143**	*Hippocampus hippocampus*	99.29 %	1022
El Jebha	**ON823158**	*Hippocampus hippocampus*	99.17 %	1081
El Jebha	**ON823165**	*Hippocampus hippocampus*	100 %	1140
El Jebha	**ON823166**	*Hippocampus hippocampus*	100 %	1146
Chmaala	**ON823159**	*Hippocampus hippocampus*	99.84 %	1140
Chmaala	**ON823167**	*Hippocampus hippocampus*	99.84 %	1140
El Jadida	**ON823152**	*Hippocampus hippocampus*	100 %	1144
Agadir	**ON823140**	*Hippocampus hippocampus*	99.84 %	1138
Agadir	**ON823141**	*Hippocampus hippocampus*	99.49 %	1077
Agadir	**ON823144**	*Hippocampus hippocampus*	99.68 %	1133
Agadir	**ON823145**	*Hippocampus hippocampus*	100 %	1144
Agadir	**ON823146**	*Hippocampus hippocampus*	99.84 %	1138
Agadir	**ON823147**	*Hippocampus hippocampus*	99.84 %	1127
Agadir	**ON823148**	*Hippocampus hippocampus*	99.68 %	1133
Agadir	**ON823149**	*Hippocampus hippocampus*	100 %	1133
Agadir	**ON823150**	*Hippocampus hippocampus*	100 %	1138
Agadir	**ON823156**	*Hippocampus hippocampus*	99.84 %	1175
Agadir	**ON823157**	*Hippocampus hippocampus*	100 %	1170
Agadir	**ON823160**	*Hippocampus hippocampus*	99.84 %	1125
Agadir	**ON823161**	*Hippocampus hippocampus*	100 %	1142
Agadir	**ON823168**	*Hippocampus hippocampus*	99.84 %	1144
Agadir	**ON823169**	*Hippocampus hippocampus*	100 %	1129
Agadir	**ON823170**	*Hippocampus hippocampus*	100 %	1140
Agadir	**ON823171**	*Hippocampus hippocampus*	100 %	1146
Agadir	**ON823172**	*Hippocampus hippocampus*	99.84 %	1140
Dakhla	**ON823151**	*Hippocampus hippocampus*	99.84 %	1133
Dakhla	**ON823162**	*Hippocampus hippocampus*	100 %	1146
Dakhla	**ON823163**	*Hippocampus hippocampus*	100 %	1142

Table 4
Genetic Distance Based on COI Gene Among Selected Species of *Hippocampus* and *Solegnathus*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1-*S.hardwickii* (**MH999466**)																										
2-*H.jayakari* (**MT558646**)	0,187																									
3-H.comes (**GQ502134**)	0,178	0,106																								
4-*H.kuda* (**FJ583553**)	0,192	0,122	0,118																							
5-*H.spinosissimus* (**MT558604**)	0,194	0,104	0,114	0,091																						
6-*H.patagonicus* (**KC851885**)	0,192	0,127	0,121	0,126	0,111																					
7-*H.erectus* (**KC851881**)	0,189	0,137	0,135	0,124	0,110	0,064																				
8-*H.hippocampus* (**KC851882**)	0,199	0,139	0,139	0,130	0,121	0,119	0,064	0,037																		
9-*H.hippocampus* (**KY176504**)	0,201	0,139	0,133	0,124	0,118	0,064	0,036	0,002																		
10-**ON823138**	0,201	0,138	0,134	0,125	0,119	0,067	0,038	0,005	0,003																	
11-**ON823154**	0,208	0,139	0,141	0,131	0,125	0,070	0,040	0,006	0,005	0,008																
12-**ON823164**	0,206	0,139	0,139	0,125	0,122	0,068	0,037	0,010	0,008	0,016	0,014															
13-**ON823139**	0,205	0,138	0,138	0,123	0,121	0,067	0,034	0,006	0,005	0,008	0,006	0,002														
14-**ON823165**	0,204	0,135	0,135	0,125	0,119	0,065	0,036	0,002	0,000	0,008	0,006	0,014	0,005													
15-**ON823166**	0,206	0,138	0,138	0,128	0,122	0,066	0,037	0,003	0,002	0,011	0,008	0,016	0,005	0,002												
16-**ON823143**	0,211	0,143	0,143	0,135	0,130	0,067	0,039	0,011	0,009	0,020	0,019	0,022	0,014	0,015	0,017											
17-**ON823159**	0,208	0,139	0,141	0,131	0,125	0,070	0,040	0,006	0,005	0,012	0,005	0,014	0,006	0,011	0,012	0,014										
18-**ON823167**	0,206	0,138	0,139	0,130	0,124	0,068	0,039	0,005	0,003	0,008	0,002	0,008	0,006	0,005	0,006	0,012	0,002									
19-**ON823152**	0,205	0,137	0,136	0,127	0,121	0,065	0,036	0,002	0,000	0,008	0,006	0,012	0,005	0,009	0,011	0,012	0,002	0,003								
20-**ON823156**	0,202	0,139	0,135	0,125	0,119	0,064	0,039	0,005	0,003	0,009	0,011	0,017	0,006	0,002	0,005	0,014	0,016	0,008	0,011							
21-**ON823140**	0,209	0,143	0,141	0,132	0,129	0,073	0,039	0,007	0,005	0,011	0,010	0,015	0,012	0,007	0,007	0,010	0,010	0,010	0,008	0,008						
22-**ON823168**	0,210	0,142	0,142	0,133	0,125	0,070	0,045	0,011	0,010	0,020	0,016	0,019	0,009	0,009	0,009	0,017	0,017	0,014	0,012	0,015	0,011					
23-**ON823150**	0,205	0,135	0,136	0,127	0,119	0,063	0,037	0,003	0,002	0,014	0,012	0,019	0,009	0,009	0,008	0,019	0,016	0,013	0,014	0,008	0,011	0,000				
24-**ON823151**	0,204	0,136	0,136	0,126	0,120	0,067	0,038	0,005	0,003	0,016	0,017	0,017	0,008	0,011	0,012	0,014	0,009	0,008	0,008	0,011	0,010	0,014	0,016			
25-**ON823162**	0,208	0,139	0,139	0,129	0,123	0,068	0,039	0,005	0,003	0,008	0,003	0,008	0,005	0,003	0,005	0,014	0,006	0,002	0,005	0,008	0,008	0,014	0,011	0,009		
26-**ON823163**	0,204	0,135	0,135	0,125	0,119	0,065	0,036	0,002	0,000	0,005	0,003	0,008	0,005	0,006	0,008	0,015	0,005	0,003	0,003	0,008	0,008	0,011	0,012	0,011	0,003	

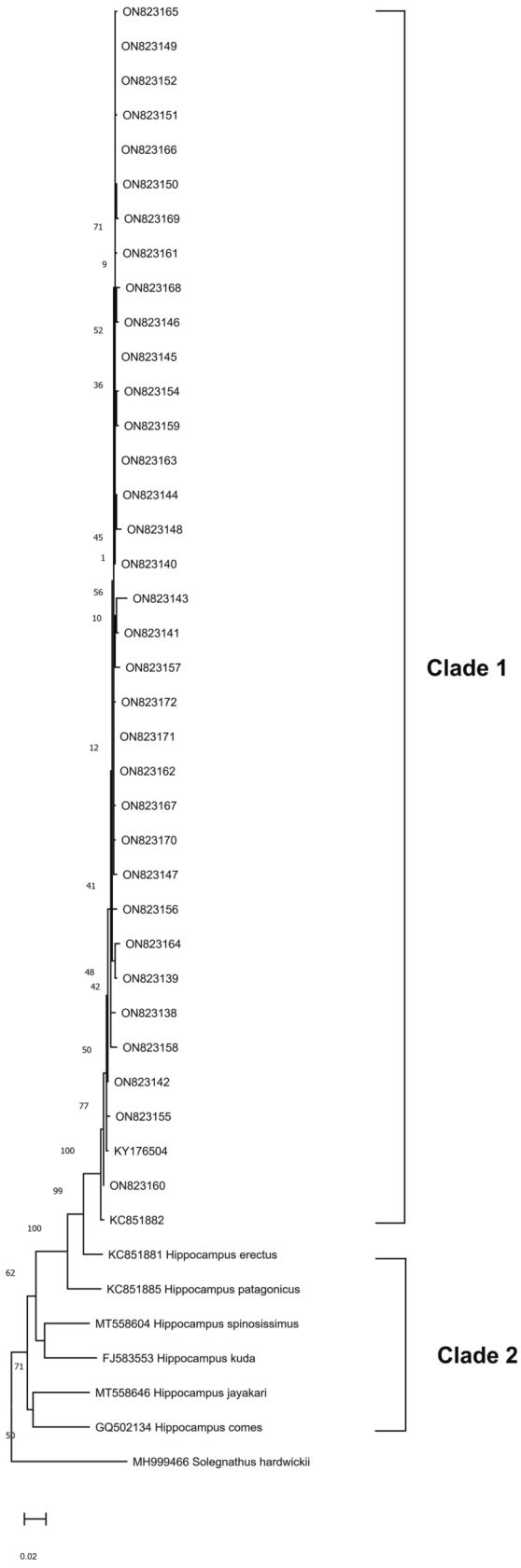

Fig. 3. NJ tree of seahorses inferred from COI sequences with 1000 bootstrap replicates.

reducing potential of seahorse extract was 1.868 mg/mL ± 0.033, while that of ascorbic acid was 0.070 mg/mL ± 0.002. Moreover, through the phosphomolybdate assay, it was demonstrated that the total antioxidant capacity of ascorbic acid surpasses that of the seahorse, with respective EC50 values of 0.095 mg/mL ± 0.008 and 1.156 mg/mL ± 0.112. Finally, the β-carotene-linoleic acid test showed the IC50 value of seahorse extract to be 1.273 mg/mL ± 0.166 and 0.015 mg/mL ± 0.300 for ascorbic acid.

4. Discussion

The morphological analysis of seahorse individuals sampled on the Atlantic and Mediterranean coasts of Morocco, according to the identification guide (Lourie et al., 2004), revealed that these samples correspond to the species *Hippocampus hippocampus*. This analysis can be difficult due to alterations in certain morphological characteristics (number of dorsal and pectoral fins) linked to abiotic factors (Wang et al., 2020). Molecular identification is an invaluable tool to discriminate between species in addition to morphological identification. The DNA barcode approach made it possible to validate, at a 99 % similarity threshold, the belonging of the individuals studied to the species *H. hippocampus* (Table 3). The phylogenetic tree shows that the individuals sampled on the Atlantic and Mediterranean coasts of Morocco belong to the same clade, indicating their close genetic relationship, in accordance with the findings from Li et al. (2000). This suggests that all these individuals belong to the species *H. hippocampus*.

COI gene analysis showed a genetic distance of 0.034–0.143 between the *H. hippocampus* species studied and other seahorses belonging to the genus *Hippocampus*. This genetic distance greater than 0.03 (3 %) is considered sufficient to establish species segregation (Hebert et al., 2003). However, the genetic distance between the H. *hippocampus* collected from Moroccan coastal waters and H. *hippocampus* (**KY176504**) from Turkey and H. *hippocampus* from Europe (**KC851882**) ranged between 0.000 and 0.011. Additionally, COI genes with a genetic distance of less than 0.02 (2 %) indicate that one species is closely related to another (Ratnasingham and Hebert, 2013). Thus, Moroccan seahorses are closely related to European and Turkish seahorses, this close genetic relationship suggests a shared evolutionary history and possible gene flow among the seahorse populations across these regions. The genetic similarity may result from historical migration patterns facilitated by ocean currents or other environmental factors that allowed for intermingling of these populations.

The haplotype diversity quantifies the probability that two randomly chosen alleles have different characteristics. Meanwhile, nucleotide diversity is expressed as the average number of nucleotide differences per site between compared DNA sequences in a pairwise manner (Nei, 1987). The haplotype diversity ranges from 0.80 to 0.94. In comparison with many other species, this is a high rate. However, nucleotide diversity varies between 0.00634 and 0.00904. Even though haplotype diversity is high, nucleotide diversity is low, indicating only minimal differences among haplotypes. This is further supported by the minimal spanning haplotype network, indicating the majority of differences between haplotypes are at single nucleotides (Nei, 1987). The difference between the levels of haplotype and nucleotide diversity suggests that the Atlantic and Mediterranean *H. hippocampus* populations have experienced a long period of stability in population evolution. This phenomenon is typical of marine fishes, as evidenced by high haplotype diversity in species such as *H. ingens* (Saarman et al., 2010), *H. trimaculatus* (Goswami et al., 2009; Lourie and Vincent, 2004), and *Sardina pilchardus* (Tinti et al., 2002).

The planktonic period of most seahorse species, go through between two and six weeks, is likely to result in widespread gene flow across geographically separated populations, creating genetic homogeneity (Foster and Vincent, 2004; Scales, 2010). Furthermore, the Mediterranean Sea is connected to the Atlantic Ocean via the Strait of Gibraltar. Geographic barriers did not appear to exist between the two populations

Table 5
Genetic diversity for *H. hippocampus*.

Population	Number of sequences (n)	Number of variable sites(s)	Number of haplotypes (h)	Haplotype diversity (hd)	Nucleotide diversity (Pi)	Average number of nucleotide differences (k)
Atlantic	29	23	10	0,80296	0,00634	2,42365
Mediterranean	12	16	9	0,93939	0,00904	3,45455

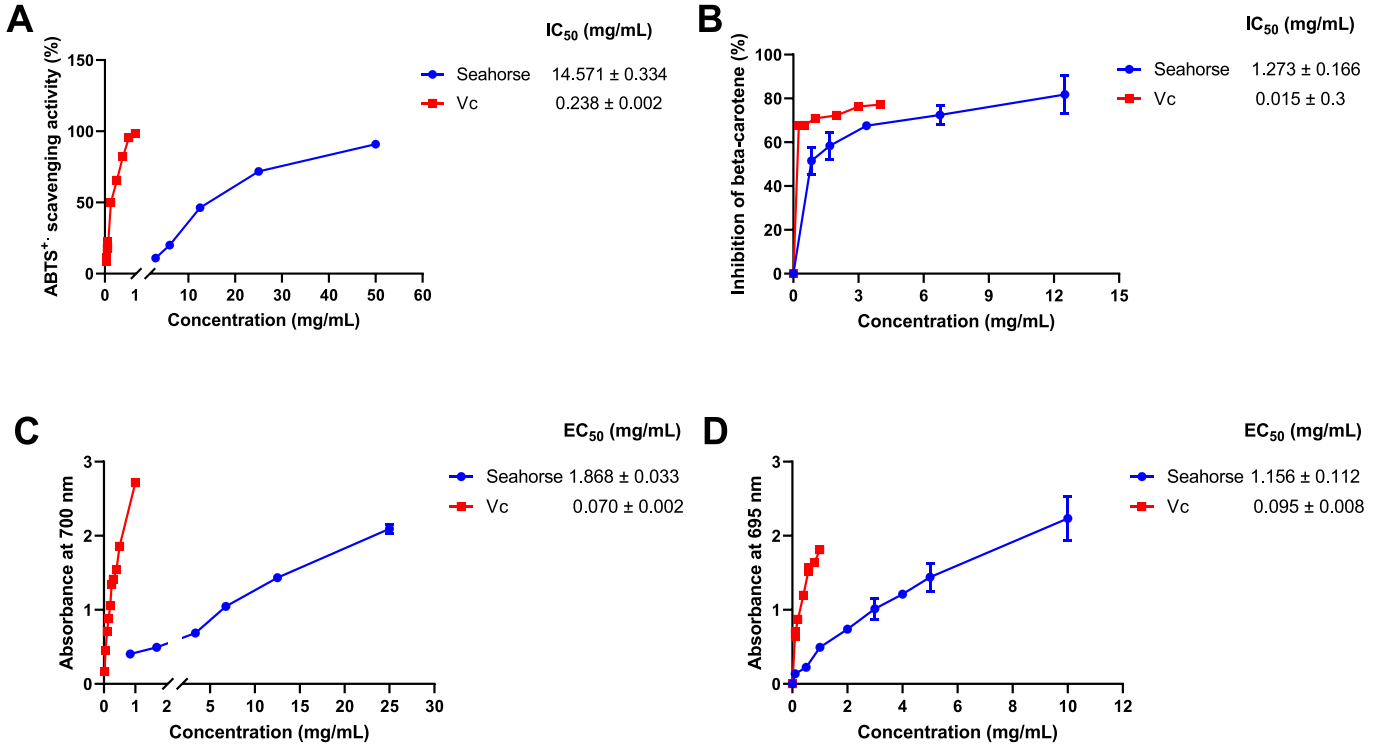

Fig. 4. Antioxidant activity of seahorse. A: ABTS; B: β-Carotene blanching inhibition; C: reducing power; D: total antioxidant capacity. All the data were expressed in mean ± standard deviation (SD) for three independent experiments.

of *H. hippocampus*, and the ocean currents between the Moroccan Atlantic coast and the Mediterranean coast could have contributed to increased gene exchange.

The mean TP content of *H. hippocampus* was determined to be 11.87 ± 0.75 mg GAE/g, which is lower compared to the TP content of H. kuda, which was 17.43 ± 1.30 mg/g (Qian et al., 2008). This discrepancy in TP content could be due to the lower extraction yield (5.2 %) observed in the current study compared to the yield of 6.9 % reported by Qian et al. (2008) for the methanol extract.

The antioxidant potential of the seahorse extract was evaluated using four assays, which yielded varied IC50 and EC50 values (Fig. 4). In the ABTS test, the IC50 value was 14.57 mg/mL ± 0.334, which was significantly lower than the antioxidant value of the positive control (ascorbic acid) (0.238 mg/mL ± 0.002). The results indicated a correlation between increasing polyphenol content and an increase in inhibition rate. This demonstrates the suitability of the ABTS assay for the evaluation of antioxidant activity in both lipophilic and hydrophilic antioxidants. A study performed on *H. japonicus* Kaup showed that this species has inhibitory properties related to the scavenging activity of the ABTS radical (Chen et al., 2010). The EC50 value, expressed as the power reduction result, was 1.868 ± 0.033 mg/mL. In comparison with the positive control (ascorbic acid), the reduction in the potency of the extracts varied significantly ($p < 0.05$) in accordance with other researchers, the ability to reduce free radicals depends on the structural conformation of phenolic compounds (Han et al., 2017). Although, the phosphomolybdenum assay demonstrated higher antioxidant activity of the seahorse extract, with an EC50 of 1.156 mg/mL ± 0.112. The hydrogen from the hydroxyl group can scavenge peroxide radicals and prevent other compounds from being oxidized. In the β-carotene-linoleic acid test, an IC50 value of 1.273 mg/mL ± 0.166 was obtained. Highly unsaturated β-carotene molecules rapidly discolor due to linoleate free radicals produced by the oxidation of linoleic acid (Yakubu et al., 2019). The addition of antioxidants can minimize the oxidation of β-carotenes by neutralizing free radicals, thus preventing the bleaching of β-carotenes (Singh et al., 2002).

5. Conclusion

The seahorses of the Moroccan Atlantic and Mediterranean coasts were determined to be *Hippocampus hippocampus* through combined morphological and molecular analysis using the COI sequence. *Hippocampus* specimens collected seem to belong to the same clade. Also, *Hippocampus hippocampus* possesses interesting antioxidant activities. This activity can be attributed to the presence of various compounds, including polyphenols. They have been shown to scavenge free radicals and protect against oxidative damage. Further research is needed to fully understand the mechanisms behind these effects and to determine the most effective ways to use the *hippocampus* as a supplement or therapeutic agent. Hence the need to better manage the biodiversity of this species.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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