Journal for Nature Conservation 62 (2021) 126030

Contents lists available at ScienceDirect

Journal for Nature Conservation

journal homepage: www.elsevier.com/locate/jnc

Molecular tools against the illegal exploitation of the threatened Pacific seahorse *Hippocampus ingens* Girard, 1858

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ARTICLE INFO

Keywords:
Seahorse
Mitochondrial
Poaching
Wildlife
Peru
Traditional Chinese medicine

ABSTRACT

The high demand for syngnathid species in the traditional Chinese medicine (TCM) trade is drastically affecting seahorse populations worldwide. Powdered seahorse not only represents the main material for the preparation of remedies in either TCM or folk medicine but is also being used to facilitate smuggling and avoid customs authorities in different countries. Here, we present the first primer set and standardized real time quantitative PCR (qPCR) protocol designed for a fast, accurate, sensitive, and reliable identification of one of the most threatened fish species in the world, the Pacific seahorse *Hippocampus ingens*, using DNA traces from powdered material. Our protocol efficiently detected quantities as low as 0.85 pg of seahorse DNA, showing a high specificity even in the presence of DNA from other non-target fish species. The qPCR protocol reported herein may be utilized as a powerful tool to monitor the illegal domestic trade and international trafficking of *H. ingens*, not only in poaching hotspot countries such as Ecuador and Peru but also in transit and final market destinations such as Hong Kong and China. In addition, we show photographic evidence of seahorse extraction and trade in northern Peru and southern Ecuador, highlighting the need for increased efforts to protect the Pacific seahorse.

1. Introduction

Wildlife trafficking is threatening some of the world's most iconic and beloved species, driving them to the brink of extinction (Scheffers, Oliveira, Lamb, & Edwards, 2019). Species including pangolin, rhino, saiga, tigers, sharks, and seahorses are among some of the most threatened by the trade and with elevated demand in traditional Chinese medicine (TCM), which is now practiced in more than 180 countries with an industry valued at US$60 billion (Fine, 2018; Reuters, 2019). The illegal trade of wildlife is one of the most profitable crimes, which is estimated to be worth up to US$23 billion annually (Nellemann et al., 2016), encouraging traffickers to adopt new strategies for transporting their hauls (BBC News, 2015). Powdered forms of endangered fauna (*e.g.* rhino horns, turtle shells, seahorses) can be easily disguised or declared as permitted items (*e.g.* of vegetal origin) to get past customs authorities and supply ready-made products to consumers. For instance, New Zealand wildlife authorities seized 25 kg of seahorse powder in 2007 (illegally exported from Australia but originated in China), which was declared as being a plant extract (Manifest: Journal of the Australian Customs Service, 2007). The same smuggling method was reported in Mexico and India, where seahorse exportation is banned, as to make them indistinguishable during inspection, they are dried and powdered to be illegally exported to Southeast Asia (Actualidad, 2021; Roundglass Sustain, 2018).

Seahorses (genus *Hippocampus* Rafinesque, 1810) are marine fishes of the family Syngnathidae, that inhabit shallow coastal and estuarine systems worldwide (Claassens, 2016; Foster & Vincent, 2004). According to the World Register of Marine Species (available at http://marinespecies.org; accessed in May 2021), 48 extant species belonging to the genus *Hippocampus* are currently recognized as valid (Froese & Pauly, 2021). They are characterized by sparse distribution, low mobility, small home-ranges and brood sizes, low fecundity and

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https://doi.org/10.1016/j.jnc.2021.126030
Received 19 February 2021; Received in revised form 28 May 2021; Accepted 9 June 2021
Available online 12 June 2021

lengthy parental care, which make their populations susceptible to anthropogenic disturbance such as habitat destruction and overexploitation (Claassens, 2016; Foster & Vincent, 2004; Koldewey & Martin-Smith, 2010). Furthermore, they are considered among the most charismatic marine animals and flagship species for marine conservation (Faleiro et al., 2015). In spite of that, it is estimated that about 24 million seahorses across 23 species are caught and traded every year in 75 countries (Actiman, 2016; Foster & Vincent, 2019), primarily for use in traditional Chinese medicine (TCM), and to a lesser extent as curios and live for ornamental display (Vincent et al., 2011Vincent, Giles, Czembor, & Foster, 2011). This unsustainable global exploitation is leading to a rapid decline in seahorse populations around the world (Stocks, Foster, Bat, & Vincent, 2017; Vincent et al., 2011). Concerns over the increasing overexploitation and global trade of different seahorse species led to the entire *Hippocampus* genus being listed on Appendix II of the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) in November 2002 with implementation in May 2004 (Koldewey & Martin-Smith, 2010).

South America harbors a wide range of aquatic ecosystems supporting rich species diversity (Miloslavich et al., 2011; Reis et al., 2016), which also makes this region susceptible to environmental crime such as illegal poaching and trade of protected species (Duri, 2020). In this region, two closely related seahorse species are the most commonly used for both domestic and non-domestic trade: the Pacific seahorse (*Hippocampus ingens*) and the longsnout seahorse (*H. reidi*) (Baum & Vincent, 2011; da Hora et al., 2016). The former represents the sole seahorse species in the eastern Pacific occurring from California in the United States to southern Peru, while the latter is distributed in the western Atlantic from North Carolina in the United States to southern Brazil (Lourie, Pollom, & Foster, 2016). Both species emerged as two of the six most impacted seahorses when the genus was proposed for inclusion in Appendix II of CITES (CITES, 2002; Saarman, Louie, & Hamilton, 2010).

Peru is not only one of the largest exporters of the Pacific seahorse *H. ingens*, but also has the largest known domestic seahorse trade in South America (Baum & Vincent, 2005). Baum and Vincent (2005) conducted an early survey of the Pacific seahorse trade in different cities along the Peruvian coast during the year 2000. The authors reported that seahorses were targeted by artisanal and commercial fishers in Tumbes and Piura, and divers in Pisco, for the curio, aquarium, and TCM, and were also landed as bycatch of purse seine and shrimp trawl fisheries. In 2004, the Peruvian Government banned the extraction and commercialization of seahorses imposing a permanent closed season over concerns of overfishing (RM Nº 306-2004-PRODUCE) (PRODUCE, 2004). However, despite the ban, a later study by Quiñe and Romero (2007) reported that the poaching of *H. ingens* for the domestic and international trade was still happening in several locations from northern Peru (*i.e.* Piura and Tumbes).

Hippocampus ingens is highly valued in the TCM due to its large size and smooth texture (Baum & Vincent, 2011), which is evidenced by several confiscation cases involving vast amounts of dried specimens by authorities of different nations including country of origin: Ecuador (El Universo, 2008), Mexico (Milenio, 2016), Peru (Actiman, 2016; GESTIÓN, 2019); transit countries: Germany (TRAFFIC, 2013), Panama (RPP, 2011); and destination countries: China (UCN, 2019), Hong Kong (ADMCF, 2019), Vietnam (VN Express International, 2017). Global records of medicinal uses of seahorses have been found in at least 33 countries, including Brazil, Ecuador, Mexico, and Peru (Baum & Vincent, 2011; Rosa, Defavari, Alves, & Oliveira, 2013). Powdered seahorse is the main form used for the preparation of remedies in either TCM or folk medicine, which can be consumed as "tea" or in combination with other products (Baum & Vincent, 2005, 2011; Rosa et al., 2013). In Peru, seahorses were found to be sold commercially for their use in TCM in Lima's Chinatown (Baum & Vincent, 2005); whereas in Brazil the use of seahorses for folk medicine is widespread, being used to treat asthma and bronchitis by mixing seahorse powder with water, soup or juice (Baum & Vincent, 2011; Rosa et al., 2013). In this respect, a molecular protocol for rapid and accurate identification of processed seahorse products (*i.e.* dry powdered) would become an invaluable tool for law enforcement, fighting not only illegal international trafficking but also the domestic trade for folk remedies.

Molecular forensic techniques have become a valuable and frequently used tool by custom officials to ensure the accurate identification of CITES-listed species, especially when dealing with juveniles or morphologically similar species (Cardeñosa, Gollock, & Chapman, 2019), and processed forms such as shark fins (Cardeñosa, Quinlan, Shea, & Chapman, 2018) and rhino horn (Ewart et al., 2018). Mitochondrial markers (*e.g.* COI, cytb, and 16S rRNA genes) have been successfully applied for the identification of dried seahorse and pipefish samples (Chang, Jang-Liaw, Lin, Fang, & Shao, 2013; Hou, Wen, Peng, & Guo, 2018; Sanders et al., 2008; Zeng et al., 2019). However, those studies aimed to identify several seahorse species using universal primers with additional post PCR steps such as DNA sequencing and bioinformatics analysis, which are time-consuming and comparatively expensive. The use of one-step real time quantitative PCR (qPCR) including group or species-specific primers achieves faster species identification results with higher discriminative power compared to conventional PCR; in addition to an extremely low risk for cross-contamination as reaction tubes remain closed during the whole analysis (Junior et al., 2013).

Despite the increasing trend in illegal international seahorse trade, to the best of our knowledge, there are no studies describing single-step qPCR assays for the identification of seahorse species in powdered material used in folk medicine or smuggling operations. This study aimed to evaluate the performance of a novel primer set targeting a partial fragment of the mitochondrial 16S rRNA gene through a rapid and sensitive qPCR protocol to identify the overexploited Pacific seahorse, *H. ingens*, using DNA samples from powdered material. As the powdered form is commercialized and used to facilitate illegal shipment in different countries (see above), this processed form is similarly expected to facilitate domestic transportation and exportation in Peru. Additionally, in order to obtain graphic evidence of the illegal seahorse extraction and trade, we visited fish landing sites, outdoor markets, handicraft fairs, and aquarium shops located in different coastal cities along the north-central Peruvian coast and southern Ecuador.

Table 1
Seahorse samples used in this study. Sample size, locality, sampling date, condition, and origin are shown.

Size (n)	Locality	Sampling date	Condition	Origin
1	Lima (Peru)	December 2009	Dried	Curio
40	Artisanal fish landing Culebras (Huarmey, Ancash, Peru)	January 29, 2016	Fresh	Confiscation by Peruvian authorities
1	La Gramita (Casma, Ancash, Peru)	October 10, 2018	Dried	Fisherman (bycatch of local artisanal fishery[a])
2	Parachique (Sechura, Piura, Peru)	March 5, 2019	Dried	Fisherman (catch zone Parachique)
2	Machala (El Oro, Ecuador)	June 20, 2019	Dried	Handicraft shop (local shrimp trawling bycatch[a])

[a] Information provided by seller or fisherman.

2. Material and methods

2.1. Validation of a qPCR assay for the identification of powdered seahorse samples

2.1.1. Sample collection and preparation of dried powdered samples

A total of 46 seahorse individuals were analyzed in this study (Table 1). Most of our samples (n = 44, 95.7 %) came from donations from Peruvian authorities, fishermen, and colleagues, in most cases information of date and capture locality were available. In 2016, Peruvian authorities seized 25 kg of fresh seahorses in Culebras fish landing site (Huarmey, Ancash, Peru, see Fig. 1) (GESTIÓN, 2016). Those confiscated seahorses were donated to the School of Biology of the National University of Santa (Ancash, Peru). The present study used 40 individuals from this donation. Four dried specimens collected in Lima (n = 1), Ancash (n = 1), and Piura (n = 2) (Fig. 1) were donated by colleagues, while two additional dried seahorse individuals were obtained from a handicraft fair during our fieldwork in Machala City (Ecuador) (Fig. 1). The six dried seahorse individuals were grounded to powder using new sterilized stainless-steel fine nutmeg graters (one for each specimen). To validate the novel seahorse primer set, 23 different non-target fish species covering a wide range of fish taxa (Table 2) were collected from local markets, landing sites, or donated by colleagues. A DNA barcoding approach (Ward, Zemlak, Innes, Last, & Hebert, 2005) was performed to ensure the accurate species-level identity of all non-target fish species.

2.1.2. DNA extraction and quantification

Genomic DNA from 40 fresh seahorse individuals and 23 non-target fish species was isolated using tail tissues and fin clips respectively, following the standard phenol-chloroform protocol (Sambrook & Russell, 2001). Genomic DNA extraction from each powdered seahorse sample was isolated from 30 mg of powder with the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). For the sensitivity test, an equi-mass mixture of seahorse powder prepared from the 6 dried collected individuals was performed using 8 mg from each individual and subjected to DNA isolation using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). Nucleic acid concentration and purity were calculated using an Epoch spectrophotometer (BioTek Instruments, Winooski, VT, USA).

2.1.3. Primer design

In order to design specific seahorse primers, all available complete mitochondrial 16S rRNA gene sequences from 29 fish species including 15 seahorses, 7 pipefish, seadragon (*Phycodurus eques*), bigeye tuna (*Thunnus obesus*), Japanese anchovy (*Engraulis japonicus*), dolphinfish (*Coryphaena hippurus*), smooth hammerhead shark (*Sphyrna zygaena*), and giant devil ray (*Mobula mobular*) were retrieved from their complete mitogenomes deposited in GenBank database (Table S1). All complete 16S rRNA gene sequences were multialigned using ClustalW (Thompson, Higgins, & Gibson, 1994) as implemented in MEGA 7 (Kumar, Stecher, & Tamura, 2016). Seahorse primers were manually designed targeting highly conserved nucleotide positions from both *H. ingens* (GenBank accession NC_024530) and *H. reidi* (GenBank accession NC_027931) sequences (Fig. 2). To ensure only target species amplification, mismatches at the 3′ end of the primer in non-target organisms (pipefish, seadragon, tuna, anchovy, dolphinfish, smooth hammerhead shark, and giant devil ray) were included. All primer sets were designed and tested *in silico* for secondary structure formation (hairpins, homo and hetero-dimers) using the IDT OligoAnalyzer tool (available at <http://www.idtdna.com>) and The Sequence Manipulation Suite (Stothard, 2000) (available at <http://www.bioinformatics.org/sms2/index.html>). All primers were designed to meet standard qPCR oligo characteristics: identical or similar melting temperatures (above or around 60°C under IDT OligoAnalyzer qPCR parameter sets), to render PCR products no longer than 140 bp in length, and with estimated delta G values for secondary structures higher (less negative) than −4.5 kcal/mol and −1 kcal/mol for dimers and hairpin formation respectively. To promote binding, at least one (but not more than three) cytosine/guanine residue was included in the last 5 bases at the 3′ of the primer ending ("GC clamp"). All designed primers were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, USA).

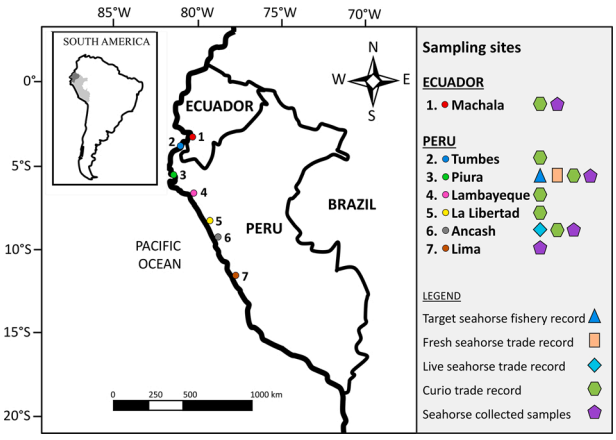

Fig. 1. Seahorse trade records and sampling localities from Peru and Ecuador. Target fishery, fresh, live, curio trade photographic records and collected seahorse samples are indicated by blue triangle, orange rectangle, sky blue diamond, green hexagon, and violet pentagon respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.1.4. Primer specificity

Confirmation of primer specificity by endpoint PCR and qPCR was determined with agarose electrophoresis resulting in specific bands of the expected size (136 bp) in all analyzed seahorse individuals (n = 46) with no cross-amplification in any of the 23 non-target fish species, and by melting curve analysis showing single sharp peaks with no visualization of primer dimer formation. Moreover, the identity of the yielded amplicons was further confirmed by DNA sequence analysis on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Hitachi, Foster City, CA, USA).

2.1.5. Endpoint PCR for primer validation

To verify the successful PCR amplification in all collected *H. ingens* specimens, the novel seahorse primers were subjected to endpoint PCR in a Veriti 96 Well thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR amplification conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s, and a final extension step at 72 °C for 7 min. PCR reactions consisted of 5 μl of Maximo *Taq* DNA Polymerase 2X-preMix (0.1 U/μl) (GeneOn, GmbH, Nurnberg, Germany), 0.2 μl each primer (10 μM), 1 μl of template DNA (10–20 ng/μl), 3.6 μl ultrapure H_2O. A negative amplification control reaction was included by adding 1 μl ultrapure H_2O instead of template DNA. Amplification was verified by 1.5 % agarose gel (EMD Millipore, Billerica, MA, USA) electrophoresis, amplicons were stained with GelRed Nucleic Acid Gel Stain (BIOTREND Chemikalien GmbH, Köln, Germany), visualized in a myECL Imager UV transilluminator (Thermo Scientific, Whaltham, MA, USA). A 50 bp DNA ladder GeneRuler (Fermentas Life Sciences Inc., Glen Burnie, Maryland, USA) was used to assess the molecular weight.

2.1.6. Duplex endpoint PCR for discrimination against non-target species

To confirm seahorse primer specificity without any cross-amplification in non-target species, a duplex PCR reaction was conducted using 23 non-target fish species template DNAs. An endogenous positive control was amplified in all analyzed species by using the fish universal primer set MiFish-U-F/R (designed for metabarcoding

Table 2
List of non-target species used to validate the specificity of the seahorse primer set HIP136 reported in this study. GenBank accession numbers and taxonomic classification are shown for all the non-target species.

GenBank accession	Common name	Species name	Family	Order	Class
MN839511/MN839512	Pipefish	*Leptonotus* sp.	Syngnathidae	Syngnathiformes	Actinopterygii
MN839513	Peruvian silverside	*Odontesthes regia*	Atherinopsidae	Atheriniformes	
MN839514	Pacific thread herring	*Opisthonema medirastre*	Clupeidae	Clupeiformes	
MN839515	Lumpfish searobin	*Prionotus stephanophrys*	Triglidae	Scorpaeniformes	
MN839516	Peruvian grunt	*Anisotremus scapularis*	Haemulidae	Perciformes	
MN839517	Burrito grunt	*A. interruptus*			
MN839518	Grey grunt	*Haemulon scudderii*			
MN839519	Arnillo drum	*Cheilotrema fasciatum*	Sciaenidae		
MN839520	Grape-eye seabass	*Hemilutjanus macrophthalmos*	Serranidae		
MN839521	Rooster hind	*Hyporthodus acanthistius*			
MN839522	Olive grouper	*Epinephelus cifuentesi*			
MN839523	Broomtail grouper	*Mycteroperca xenarcha*			
MN839524	Brick seabass	*Acanthistius pictus*			
MN839525	Southern rock bass	*Paralabrax callaensis*			
MN839526	Peruvian rock seabass	*Paralabrax humeralis*			
MH194449	Dolphinfish	*Coryphaena hippurus*	Coryphaenidae	Perciformes	
MH194486	Striped marlin	*Kajikia audax*	Istiophoridae		
MN839527	Pacific tripletail	*Lobotes pacificus*	Lobotidae		
MN839528	Cobia	*Rachycentron canadum*	Rachycentridae		
MN839529	Mocosa ruff	*Schedophilus haedrichi*	Centrolophidae		
MH194466	Swordfish	*Xiphias gladius*	Xiphiidae		
MN839530	Fourspot flounder	*Hippoglossina tetrophthalma*	Paralichthyidae	Pleuronectiformes	
MH194472	Smooth hammerhead	*Sphyrna zygaena*	Sphyrnidae	Carcharhiniformes	Chondrichthyes
MN839531	Pelagic thresher shark	*Alopias pelagicus*	Alopiidae	Lamniformes	

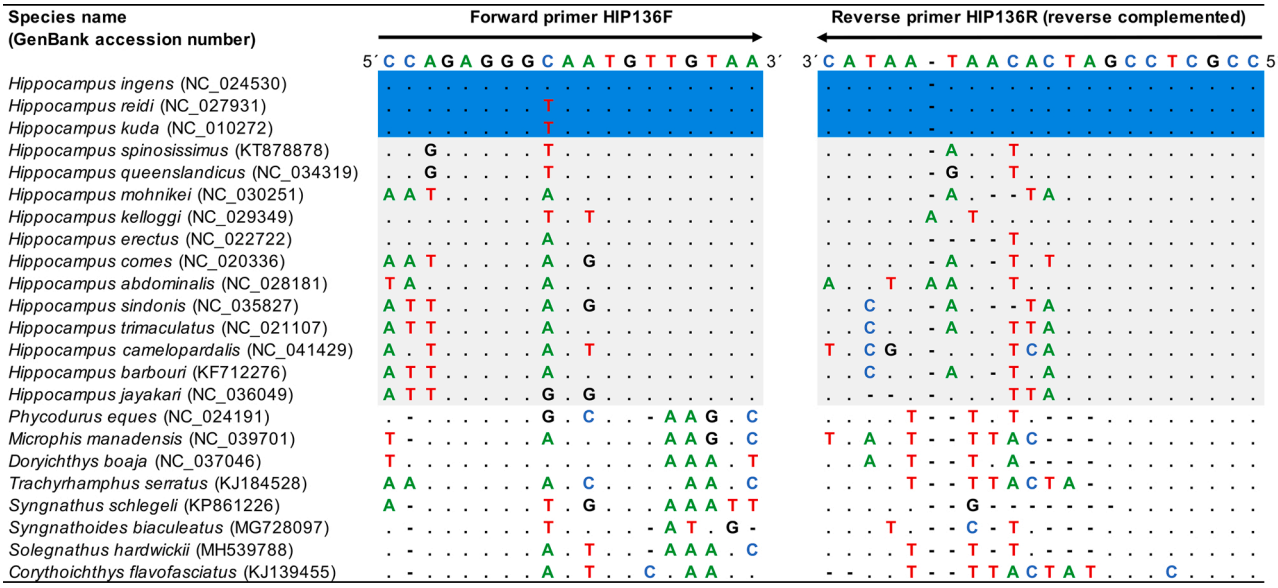

Fig. 2. Multialigned matrix of a partial fragment of the mitochondrial 16S rRNA gene indicating primer sequence binding sites in seahorse, pipefish, and seadragon species used for primer design. GenBank accession numbers are indicated in each species. Primer binding sequences highlighted in blue denote target seahorse species of the *H. kuda* complex. Primer binding sequences highlighted in grey denote non-target seahorse species. For the sake of clarity, sequences from other non-target fish species used for primer design (dolphinfish, Japanese anchovy, tuna, smooth hammerhead, and giant devil ray) were removed from this matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3
List of primer sets used in this work.

Primer name	Direction	Sequence (5′-3′)	Ta (°C)	Target species	Amplicon size (bp)	Target gene	Utility	Source
HIP136-F	Forward	CCAGAGGGCAATGTTGTAA	60	*Hippocampus ingens*	136	16S rRNA	Species ID by end-point and qPCR/ Sequencing	This study
HIP136-R	Reverse	GGCGAGGCTAGTGTTATTATG						
HIPCURV-F	Forward	TTAGTGGGCCTGAAAGCAG	60	*Hippocampus ingens*	416	16S rRNA	Standard curve construction	This study
HIPCURV-R	Reverse	CAGGCGGGACCTCTTAT						

environmental DNA) (Table 3) targeting a small fragment (220 bp approx.) of the mitochondrial 12S rRNA gene (Miya et al., 2015). Hence, the duplex PCR reaction included (in a single tube) our novel seahorse primer set HIP136 F/R (16S rRNA gene locus) and the fish universal primer set MiFish-U-F/R (12S rRNA gene locus). We expected to obtain two PCR products only in DNA samples of *H. ingens* (as a result of the co-amplification of both the 16S rRNA and 12S rRNA gene loci), while in each of the 23 non-target fish species a single PCR product (12S rRNA gene locus) was expected. The final optimized duplex PCR master mix consisted of 5 µl Maximo *Taq* DNA Polymerase 2X-preMix (0.1 U/µl) (GeneOn, GmbH, Nurnberg, Germany), 0.14 µl each seahorse primer HIP136 F/R (10 µM), 0.08 µl each universal primer MiFish-U-F/R (10 µM), 0.5 µl of template DNA (10–20 ng/µl), and 4.06 µl ultrapure H_2O, in a total volume of 10 µl. A negative amplification control reaction was included by adding 0.5 µl ultrapure H_2O instead of template DNA. The PCR amplification conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 32 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 15 s, and a final extension step at 72 °C for 5 min. Amplification products were visualized by agarose electrophoresis using the above-described methods.

2.1.7. Standard curve construction

For the standard curve construction, a fragment of 416 bp from the mitochondrial 16S rRNA gene (encompassing the smaller fragment of 136 bp amplified with the primer set HIP136) was PCR amplified using *H. ingens* genomic DNA with the novel primers HIPCURV-F and HIPCURV-R (Table 3) in a Veriti 96 Well thermal cycler (Applied Biosystems, Foster City, CA, USA), using the abovementioned thermal cycling profile (subsection endpoint PCR for primer validation). PCR master mix was prepared using 0.1 µl of Maximo *Taq* DNA Polymerase (5 U/µl) (GeneOn GmbH, Nurnberg, Germany), 0.2 µl each primer (10 µM), 1 µl of dNTP mix (2.5 mM each), 0.38 µl $MgCl_2$ (100 mM), 2 µl 10X buffer, 1 µl of template DNA, 15.12 µl ultrapure H_2O, in a total volume of 20 µl. The amplicon was purified using Exo Sap-IT (USB Corporation, Cleveland, OH, USA) following the instructions detailed in the product's manual. DNA quantification was estimated using an Epoch spectrophotometer (BioTek Instruments, Winooski, VT, USA). The purified amplicon was 10-fold serially diluted from 40 ng to 0.4 fg and subjected to qPCR to construct the standard curves, using 2 µl of each standard and three technical replicates for each dilution.

2.1.8. Sensitivity test

Limit of detection (LOD) of the qPCR was determined in triplicate using tenfold serial dilutions of *H. ingens* genomic DNA extracted from a powder mixture of 6 dried individuals, starting from 42.5 ng to a lower limit of 42.5 fg. DNA quantification was performed using an Epoch spectrophotometer (BioTek Instruments, Winooski, VT, USA). Real-time quantitative PCR conditions were the same as described below. To evaluate the qPCR amplification efficiency, a standard curve was created by plotting the Ct values of the dilution series against the logarithm of the DNA concentration.

2.1.9. Real-time quantitative PCR

The qPCR assay was performed on a LightCycler 480 II (Roche Diagnostics GmbH, Penzberg, Germany) and run in triplicate on 96-well reaction plates (Roche Diagnostics GmbH, Mannheim, Germany). Reactions were prepared in a total volume of 20 µl containing: 10 µl 2X SYBR Green I Master (Roche Diagnostics, Mannheim, Germany), 0.2 µl each primer (10 µM), 2 µl of template DNA (10 ng/µl), and 7.6 µl ultrapure H_2O. A negative amplification control reaction was included in each qPCR run by adding 2 µl ultrapure H_2O instead of template DNA. The amplification conditions used were an initial denaturation at 95 °C for 5 min, followed by 40 amplification cycles of 95 °C for 15 s and 60 °C for 1 min. A melting curve analysis was conducted from 60 °C for 1 min with a rate of 2.2 °C per second up to 95 °C with a continuous acquisition. All reactions were performed using a designated set of micropipettes for qPCR use only. A no-template control (NTC) and positive control (10 ng of *H. ingens* genomic DNA) were included in each replicate. Primer specificity was also checked by qPCR amplification and melting curves analyses against the 23 non-target fish species.

2.1.10. Mixed template analysis

To further test the amplification performance of our novel seahorse primers in powdered material containing target and non-target species DNA, a binary mixture of seahorse/pelagic thresher shark (*H. ingens* / *Alopias pelagicus*) of different known proportions (0.01 %, 0.1 %, 10 %, and 50 % w/w) was included in our qPCR assay. A dried shark filet sample (later authenticated by DNA analysis as the pelagic thresher shark *Alopias pelagicus*) was purchased from a local fish market (Chimbote, Ancash) and ground to powder using the same method described above. A total mass of 60 g was prepared for each mixed sample. Powder samples were weighed using a digital precision balance ED224S Extend ED (Sartorius China, Beijing, China). Mixed samples were subjected to DNA isolation using the standard phenol-chloroform protocol (Sambrook & Russell, 2001).

2.1.11. Direct qPCR assays

To improve the robustness of our protocol and to test its performance as a potential field-deployable tool, we performed direct qPCR amplification experiments (without DNA extraction step) using standard phosphate buffered saline (PBS 1X, pH 7.4, Sigma St Louis, MO, USA) as dilution buffer. Two types of nonextraction methods were performed: 1) Equipment-needed protocol: seahorse powder (15 and 30 mg) was mixed with PBS 1X (100 µl, 300 µl, 600 µl) by vortexing, heated in a hotplate at 55 °C for 20 min, vortexed, and quickly spun down by centrifuging (CSL-multiFUGE, Cleaver Scientific Ltd., Warwickshire, UK) at 5500 rpm for 5 s; and 2) Equipment-free protocol: seahorse powder (15 and 30 mg) was mixed in 300 µl PBS 1X by fingering and incubated at room temperature for 0, 5, 10, and 20 min. To test the effect of the sample volume on qPCR performance, the direct qPCR amplification was run in triplicates using 2, 3, and 4 µl of the supernatant as DNA template, using the same amplification protocol described in section "Real-time quantitative PCR".

2.1.12. Data analysis

The amplification efficiency and quantification cycles (Ct values) were calculated using the LightCycler 480 II Software (version 1.5.1.62) under the "Abs Quant/2nd Derivative Max" analyses. Mean values and standard deviations were calculated with Excel 2016 for macOS. Standard curve figures and R^2 values were obtained with GraphPad Prism7.

2.1.13. DNA sequence analysis

To confirm the positive amplification of the target gene region and species identity, qPCR products were sequenced in both directions using the same primer set (HIP136) on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Hitachi, Foster City, CA, USA) using the BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, Massachusetts, USA) at the Laboratory of Genetics, Physiology, and Reproduction (National University of Santa, Peru) and at Macrogen Inc. sequencing facilities (Korea). Consensus sequences were obtained from both the sense and antisense strands using MEGA 7 (Kumar et al., 2016) and BLAST compared against the public GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI, http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). Equally, non-target species used to validate the specificity of our primers were identified at species level using the primer sets FishF1 (5′-TCAACCAACCACAAAGACATTGGCAC-3′) and FishR1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) (Ward et al., 2005) or MiFish-U-F (5′-GTCGGTAAAACTCGTGCCAGC-3′) and MiFish-U-R (5′-CATAGTGGGGTATCTAATCCCAGTTTG-3′) (Miya et al., 2015), with previously described conditions (Marín et al., 2018). All sequences have been deposited in GenBank/DDBJ/EMBL DNA

databases with accession numbers from MN839505 to MN839531.

2.2. Collection of evidence for seahorse trafficking

Aiming to gather photographic evidence of the illegal trade of *H. ingens* in Peru and Ecuador, we visited main ports, local handicraft markets, pet shops and aquariums, and a Chinatown (from November 2018 to November 2019), all of which are located in 7 different cities along the north-central Peruvian coast to southern Ecuador (Fig. 1). These sites were selected on the basis of trade reports of *H. ingens* from a previous study (Baum & Vincent, 2005). Each of the localities included in the study was visited only once, except for the Chinatown from Lima which was visited twice. For the case of curios, we registered trading prices at point of sales. Most photographs were taken by the authors, and those kindly donated by colleagues are acknowledged in figure legends.

3. Results and discussion

3.1. Genetic assay

3.1.1. Specificity and primer performance

The specificity of the primer set HIP136 was evaluated by endpoint PCR and qPCR using 46 individuals of *H. ingens* and 23 non-target fish species covering a wide range of fish taxa including 21 genera, 15 families, and 7 orders. Endpoint PCR results showed specific target amplification of the expected size (136 bp) without any nonspecific amplicons or primer dimers in the 46 analyzed seahorse samples (Fig. 3). Melting curve analysis of qPCR products showed a single sharp melt peak at 78.09 °C ± 0.12 in all analyzed samples (frozen and powdered) (Fig. 4) confirming the absence of primer dimers or non-specific products. No cross-amplification was detected when the primer set HIP136 was tested against the 23 non-target fish DNA templates by the duplex endpoint PCR and qPCR assays. In the duplex PCR assay, DNA of all non-target fish species DNA yielded only the endogenous positive control locus (Fig. 5), whereas in the qPCR assay no amplification curve formation was observed during the 40 cycles of amplification (Fig. 6). Overall, qPCR efficiency values fit within the acceptable range of 90–110 % as described in the MIQE Guidelines (The Minimum Information for Publication of Quantitative Real-Time PCR Experiments) (Bustin et al., 2009), validating the accurate performance of the primers and robustness of our protocol. The BLAST analysis of sequences obtained from all powdered samples subjected to qPCR showed 100 % identity to *H. ingens* (GenBank accession numbers from MN839505 to MN839510). Due to the highly conserved regions present in seahorse species belonging to the *H. kuda* complex (in which our primers were designed, Fig. 2), further tests are needed to assess the performance of our novel primer set in the longsnout seahorse *H. reidi*, which is heavily exploited in Brazil (da Hora et al., 2016).

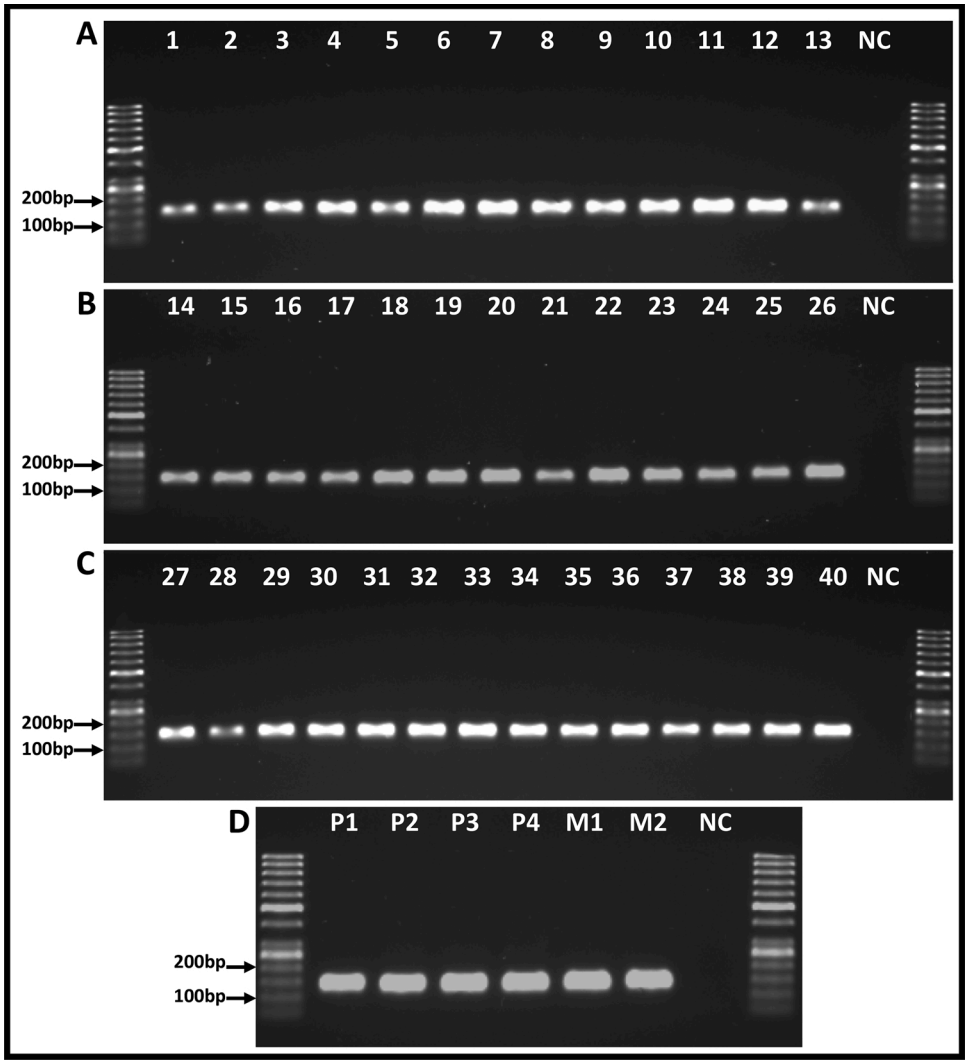

Fig. 3. Agarose gel electrophoresis showing the validation of the seahorse primer set HIP136 by conventional PCR in 46 individuals of *Hippocampus ingens*. Gel A, B, and C (wells 1-40): correspond to fresh-collected seahorse samples seized in Ancash (Peru) and donated to the School of Biology of the National University of Santa (Ancash, Peru). Gel D (wells P1, P2, P3, P4, M1, and M2): correspond to dried seahorse samples collected as curios in Peru (P1, P2, P3, and P4) and Ecuador (M1 and M2). NC: negative control.

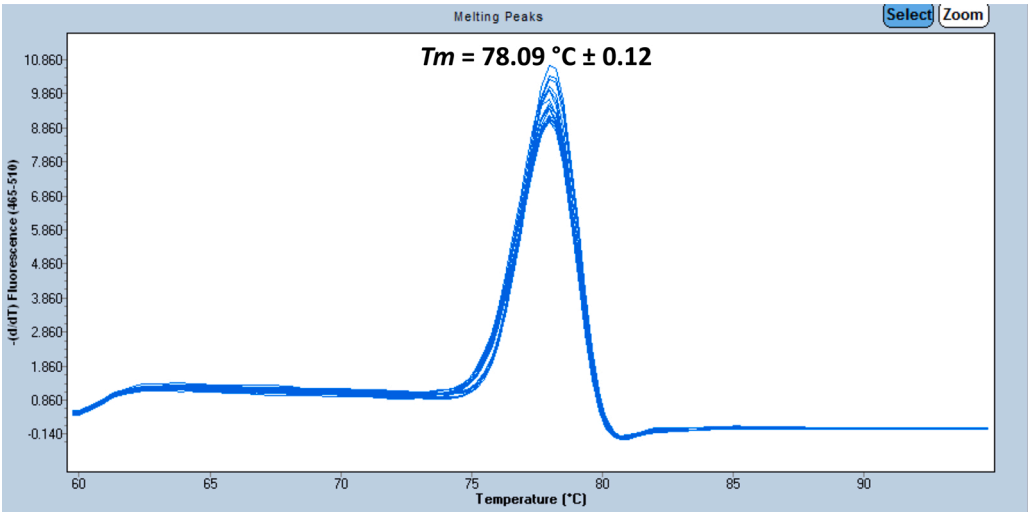

Fig. 4. Amplification specificity of a partial fragment (136 bp) of the mitochondrial 16S rRNA gene was confirmed by average melting peaks (*Tm* 78.09) obtained by qPCR using the seahorse primer set HIP136 in *Hippocampus ingens* DNA templates.

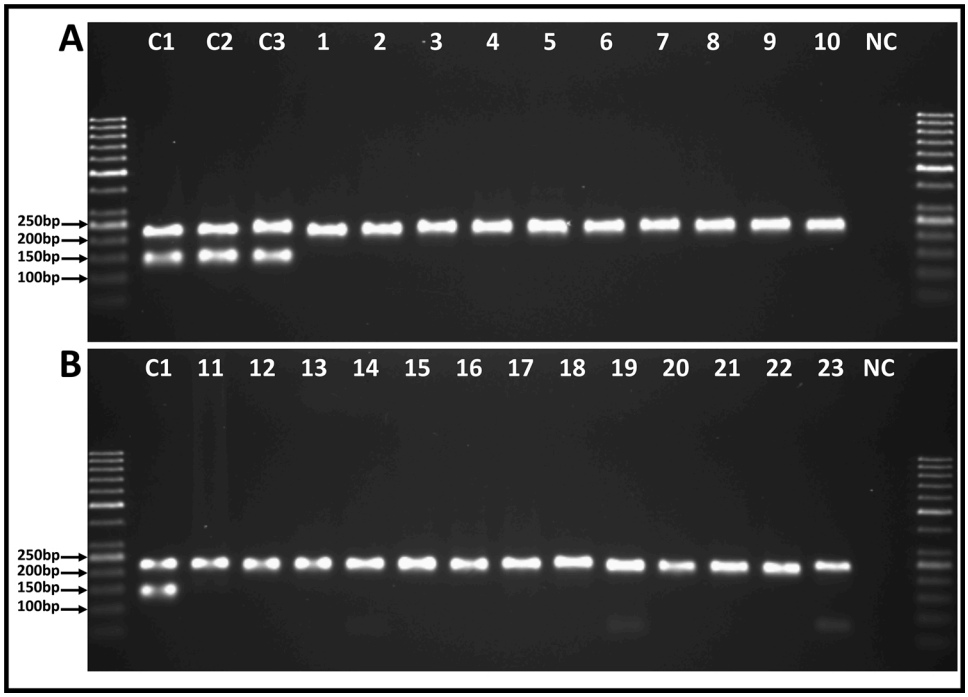

Fig. 5. Agarose gel electrophoresis validation results of a duplex PCR using the seahorse primers HIP136 F/R (16S rRNA locus, 136 bp) and universal fish primers (12S rRNA locus, about 220 bp) in target (*Hippocampus ingens*) and 23 non-target fish species. Gel A, well C1: powder seahorse, well C2: mixed powder from 6 seahorse individuals, well C3: mixed powder seahorse/pelagic thresher shark, well 1: Pipefish *Leptonotus* sp., well 2: Peruvian silverside *Odontesthes regia*, well 3: Pacific thread herring *Opisthonema medirastre*, well 4: Lumpfish searobin *Prionotus stephanophrys*, well 5: Peruvian grunt *Anisotremus scapularis*, well 6: Burrito grunt *A. interruptus*, well 7: Grey grunt *Haemulon scudderii*, well 8: Arnillo drum *Cheilotrema fasciatum*, well 9: Grape-eye seabass *Hemilutjanus macrophthalmos*, and well 10: Rooster hind *Hyporthodus acanthistius*, NC: negative control. Gel B, well 11: Olive grouper *Epinephelus cifuentesi*, well 12: Broomtail grouper *Mycteroperca xenarcha*, well 13: Brick seabass *Acanthistius pictus*, well 14: Southern rock bass *Paralabrax callaensis*, well 15: Peruvian rock seabass *P. humeralis*, well 16: Dolphinfish *Coryphaena hippurus*, well 17: Striped marlin *Kajikia audax*, well 18: Pacific tripletail *Lobotes pacificus*, well 19: Cobia *Rachycentron canadum*, well 20: Mocosa ruff *Schedophilus haedrichi*, well 21: Swordfish *Xiphias gladius*, well 22: Fourspot flounder *Hippoglossina tetrophthalma*, well 23: Smooth hammerhead *Sphyrna zygaena*. NC: negative control.

3.1.2. *Limit of detection test*

According to the MIQE guidelines (Bustin et al., 2009), the LOD is defined as the lowest amount of target DNA in a sample that can be reliably detected with > 95 % amplification success. The results of this study showed that the lower LOD was estimated to be 0.85 pg showing a mean Ct 32.87 ± 0.25 (Table 4 and Fig. 7). Based on the standard curve method, the calculated efficiency of the qPCR reaction was 97.71 % (R^2 = 0.9991, slope = −3.378) (Fig. 7). The Ct values were in the range of 16.18 ± 0.13 and 32.87 ± 0.25 (Table 4), confirming the DNA quality and absence of inhibitors in the powder mixture of 6 dried individuals used to make the serial dilutions used in the LOD test. The detection assay showed a high degree of reproducibility with low variation in Ct values across the technical replicates (Ct standard deviations ranging from 0.03 to 0.25, Table 4). No amplification was observed in NTC reactions. Overall, the LOD results demonstrated that our qPCR protocol provides a sensitive tool to monitor the presence of low amounts of template DNA from *H. ingens* in highly processed material such as dry powdered form.

3.1.3. *qPCR of powdered material and mixed template analysis*

The amplification efficiency of highly efficient primers should be between 90 % and 110 % (Bustin et al., 2009; Taylor et al., 2019). Based

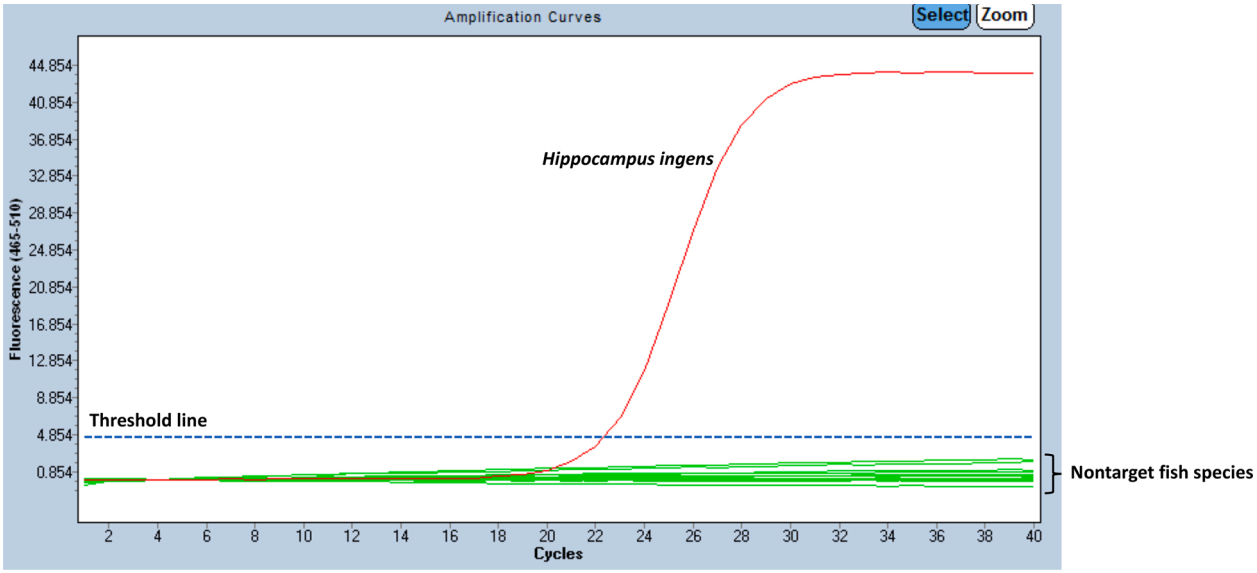

Fig. 6. qPCR amplification curve (SYBR Green fluorescence history vs. cycle numbers) using the seahorse primer set HIP136 in *Hippocampus ingens* (positive control, Ct = 22.18) *versus* 23 non-target fish species.

Table 4
Information on *Hippocampus ingens* standards used to determine the limit of detection (LOD) assay and cycle threshold (Ct) values. DNA in PCR (ng) was calculated by multiplying the 2 μl used in each reaction by the estimated concentration in each standard.

Sample	DNA in standard (ng/μl)	DNA in qPCR (ng)	Ct value			Mean Ct	Std Ct
			R1	R2	R3		
Standard 1	42.5	85	16.33	16.12	16.09	16.18	0.13
Standard 2	4.25	8.5	19.19	19.11	19.09	19.13	0.05
Standard 3	0.425	0.85	22.73	22.59	22.56	22.63	0.09
Standard 4	0.0425	0.085	26.05	25.96	25.98	26.00	0.05
Standard 5	0.00425	0.0085	29.60	29.56	29.62	29.59	0.03
Standard 6	0.000425	0.00085	32.61	32.92	33.09	32.87	0.25
Standard 7	0.0000425	0.000085	–	–	–	–	–

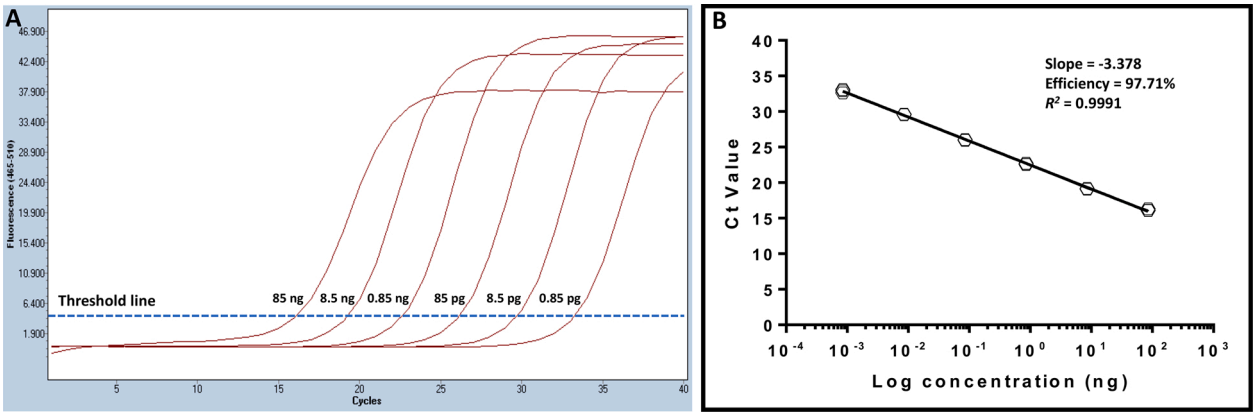

Fig. 7. Limit of detection (LOD) assay results. A) qPCR amplification curves (SYBR Green fluorescence history vs. cycle numbers). The primer set HIP136 was used to amplify tenfold serially diluted samples (ranging from 42.5 ng to 42.5 fg) of *Hippocampus ingens* genomic DNA from powdered form. The LOD was estimated to be 0.85 pg showing a mean Ct 32.87 ± 0.25. B) standard curve of the qPCR assay showing the $\log_{10}$ DNA concentration (ng) plotted against cycle threshold (Ct). The standard curve slope is -3.378, equivalent to an efficiency of 97.71 %.

on the standard curve method, the PCR efficiency of the assay, calculated from the slope of the linear regression curve using the equation $E = 10^{(-1/\text{slope})}$, was 1.93 or 92.6 % ($R^2$ =0.9997, slope = −3.513) (Table S2, Fig. 8). These results suggest a good correlation between Ct values and DNA concentration. All nine standard dilution samples presented a strong linear correlation (R^2>0.99). Based on qPCR results, Ct values for the six powdered seahorse samples and the mixed sample were determined (Table 5), with mean Ct values ranging from 14.93 ± 0.04 to 22.31 ± 0.16. To further ensure the primer efficiency and specificity of our assay in presence of genomic DNA from other seafood species, mixed seahorse and shark powder samples ranging from 0.01 to 50 % seahorse/pelagic thresher shark by mass (0.01 %, 0.1 %, 10 %, and 50 % w/w) were included in the qPCR reaction. The sensitivity of our assay was not affected by the presence of DNA from a different fish

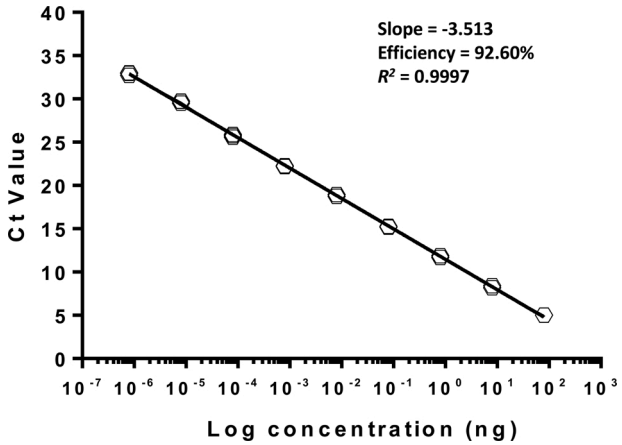

Fig. 8. Standard curve of the qPCR assay showing the $\log_{10}$ DNA concentration (ng) plotted against cycle threshold (Ct). A PCR product of 416 bp (comprising the smaller fragment of 136 bp amplified with the primer set HIP136) were used to create a standard curve with 9-point serial dilutions ranging from 40 ng to 0.4 fg. The standard curve slope is -3.513, equivalent to an efficiency of 92.60 %.

species. Fig. 5A shows seahorse locus amplification in presence of non-target pelagic thresher shark DNA (well in agarose gel labeled as "C3"), whereas in the qPCR assay all mixed samples were amplified with Ct values ranging from 22.92 to 35 (Table S3). Together, these results mean that the qPCR assay described here can be used to effectively detect small traces of *H. ingens* DNA, even in samples contaminated with DNA from other fish species.

3.1.4. Direct qPCR assay

In our direct qPCR assay, we tested two types of nonextraction methods: 1) Equipment-needed protocol and 2) Equipment-free protocol. Similar results were obtained with both methods, therefore the second method, which operates with no specialized equipment (no vortexer, heatblock, or centrifuge required), was selected for further analyses. The best results in terms of robust amplification and best Ct values were obtained using 30 mg of seahorse powder mixed in 300 µl of PBS 1X (without incubation at room temperature), using only 2 µl of the supernatant, which was applied directly to a qPCR mastermix. By using the direct qPCR assay, we were able to amplify all powder samples (success rate 100 %). Mean Ct values for the six powdered seahorse samples and the mixed sample were determined, ranging from 23.49 ± 0.14 to 32.17 ± 0.06 (Table 5). When we compare the results obtained by qPCR using isolated genomic DNA (mean Ct values ranged from 14.93 ± 0.04 to 22.31 ± 0.16), a shift in Ct values was found, possibly due to the presence of inhibitors in the direct qPCR template. However, the presence of inhibitors didn't hamper the qPCR detection. The direct qPCR trials proved to be able to detect all seahorse powder samples, which were run in triplicate. Overall, our direct qPCR method proved to be a quick and effective method for the detection of seahorse DNA in powdered samples. Besides, PBS helps to maintain the correct pH of the reaction (Thongjued, Chotigeat, Bumrungsri, Thanakiatkrai, & Kitpipit, 2019), and the mixed seahorse powder/PBS can be store at −15 °C, in cases where repeated qPCR tests are required.

3.2. Domestic and non-domestic trade of H. ingens

The illegal seahorse supply chain starts with direct poaching or extraction from incidental bycatch in fishing operations. Fig. 9A shows how artisanal divers fish for the targeted seahorse species in Sechura Bay (Piura, northern Peru). These illegally caught seahorses are then sold to mid-chain players at fish landing sites in Sechura Bay, as shown in Fig. 9B. Peru has the largest known domestic seahorse trade in South America, either for curios or for its use in TCM (Baum & Vincent, 2005). Baum and Vincent (2005) reported the domestic trade of dried seahorses during May and June in 2000 in different Peruvian (Ica, Lambayeque, Lima, Piura, and Tumbes) and Ecuadorian locations (Guayaquil, Manabi, Manta, and Quito). Twenty years later, our fieldwork found that dried seahorses sold as curios are still abundant along different cities in the north-central Peruvian coast including Ancash (Fig. 9G), La Libertad (Fig. 9H), Lambayeque (Fig. 9I), Tumbes (Fig. 9J), and Piura (Fig. 9K), and southern Ecuador (Puerto Bolívar, Machala City; Fig. 9L). Baum and Vincent (2005) also found seahorses sold in soups at the Chinese district (Chinatown) in Lima, priced at US$10 per bowl. In our study, we did not find seahorse trade in Lima's Chinatown (visited twice during August and October 2019). However, we cannot rule out the possibility that seahorse products are still being traded at Lima's Chinatown only to regular customers. We also provide evidence of live seahorse trade, which was found in an aquarium shop from Ancash (Fig. 9C).

In spite of the 2004 trade ban (PRODUCE, 2004), recent confiscation cases involving huge amounts of dried individuals of *H. ingens* from Peru are still making headlines, highlighting the major role that illegal Peruvian exports play in the international seahorse trade. For instance, the largest seahorse haul in Peru took place in October 2019, at the Port

Table 5
Quantitative detection of mitochondrial 16S rRNA gene fragment in seven samples of powdered seahorse *Hippocampus ingens* amplified with the primer set HIP136. Last column shows the mean Ct values obtained in the direct qPCR assay.

Sample	Replicate 1		Replicate 2		Replicate 3		Mean Ct	Std Ct	Mean concentration (ng)	Std concentration (ng)	Direct qPCR
	Ct value	Concentration (ng)	Ct value	Concentration (ng)	Ct value	Concentration (ng)					Mean Ct value (Std)
Powder 1	19.78	0.00383	19.64	0.0042	19.62	0.00427	19.68	0.09	0.0041	0.000238	32.17 (±0.06)
Powder 2	14.81	0.105	15.61	0.0614	14.76	0.109	15.06	0.48	0.0918	0.0264	25.51 (±0.70)
Powder 3	14.97	0.0942	14.89	0.0993	14.92	0.0978	14.93	0.04	0.0971	0.00262	25.89 (±0.39)
Powder 4	17.50	0.0175	17.45	0.0180	17.49	0.0175	17.48	0.03	0.0177	0.000304	23.49 (±0.14)
Powder 5	22.49	0.000645	22.19	0.000786	22.24	0.000759	22.31	0.16	0.00073	0.0000752	25.54 (±0.97)
Powder 6	15.80	0.0542	15.68	0.0588	15.71	0.0574	15.73	0.06	0.0568	0.00235	24.82 (±0.28)
Mixed Powder	17.47	0.0178	17.33	0.0195	17.42	0.0185	17.41	0.07	0.0186	0.000868	26.33 (±1.23)
Positive control	27.42	0.0000276	27.30	0.0000296	27.22	0.0000313	27.31	0.10	0.0000295	0.00000185	23.94 (±0.22)

Fig. 9. Seahorse fishery, bycatch, and trade in Peru and Ecuador. A: target seahorse fishery in Sechura Bay (Piura), the buyer offered US$1 and US$3 for small and large specimens respectively; B: fresh seahorse trade in Sechura Bay (Piura); C: live seahorse trade in an aquarium shop from Chimbote (Ancash), one adult individual was priced at US$15; D: seahorse bycatch in Peruvian scallop culture from Sechura Bay (Piura), E and F: seahorse and cornetfish bycatch detected in shrimp trawl fishery from Piura (photo credit: Iván Gómez, Project DYNAMICOPERU); G to L: curio seahorse trade (prices ranged from US$1 to US$8 for each specimen) G: Chimbote (Ancash); H: Huanchaco (La Libertad); I: Pimentel (Lambayeque); J: Tumbes (Tumbes); K: Catacaos (Piura, photo credit: Carolina Olano); and L: Puerto Bolívar (Ecuador).

of Callao near Lima, where 12.3 million specimens (weighing over 1 ton and valued at US$6 million in the black market) were discovered by government authorities (GESTIÓN, 2019). Also, in March 2017, Vietnamese customs officers at the Hai Phong Port (Hai Phong city, Vietnam) seized more than one ton of dried seahorses smuggled from Peru in disguise of cattle skin (VN Express International, 2017). Large seahorse hauls require big space for storage and transportation, sometimes concealed with other dried seafood or animal parts (*e.g.* cattle skin), and even chili peppers have been used to mask the odor of two tons of dried seahorses (Rosen & Smith, 2010). Powdered seahorses may represent an easier way to transport and store the haul, as well as avoid detection by customs officers. As abovementioned in Introduction section, there are confirmed incidents of seahorse powder trafficking in different countries (*i.e.*, India, Mexico, and New Zealand). Thus, it is very likely that those cases are not isolated events but part of a new tactic to transport the illegal goods. Powdered forms of endangered fauna not only facilitate smuggling efforts by evading detection at airports but also supply ready-to-use goods to consumers in Asia. If similar smuggling methods become more widespread, it will pose new challenges to customs officials and enforcement agencies. Thus, authorities must be prepared to effectively face those cases using rapid yet robust and sensitive molecular protocols for the identification of heavily used species in the TCM trade and/or CITES listed.

4. Conservation concerns

The International Union for Conservation of Nature (IUCN 2020, available at <https://www.iucnredlist.org>; accessed in December 2020) includes 51 seahorse species of which only 14 are listed as Threatened (two as Endangered and 12 as Vulnerable), 4 as Near Threatened, 13 as Least Concern, and 20 as Data Deficient. Substantial gaps in our knowledge even for overexploited seahorses is reflected in the large number of seahorse species listed as Data Deficient (Foster & Vincent, 2004). The Pacific seahorse, *H. ingens*, is currently undergoing a serious population decline throughout its entire range, which is mainly driven by overexploitation and trade (Pollom, 2017). Consequently, its current population trend is classified as "decreasing" by the IUCN. The creation of marine protected areas (MPAs) would benefit seahorse conservation through the protection of essential habitats and removing fishing pressures (Harasti, Martin-Smith, & Gladstone, 2014). In this regard, government approval of the proposal for the creation of the *Reserva Nacional Mar Tropical de Grau*, which includes four spots of rich marine diversity from northern Peru, would be of vital importance for the conservation of local seahorse populations, which are being drastically affected by illegal fishing gears like trawl and purse seine fisheries (Hooker & Ubillús, 2011).

Shrimp trawl bycatch is considered one of the major global threats to seahorses (Vincent, Sadovy de Mitcheson, Fowler, & Lieberman, 2014). Lawson, Foster, and Vincent (2017) reported that annual bycatch scales up to an estimate of 37 million seahorse individuals per year for the 21 countries included in their analysis. In Peru, incidental catches of *H. ingens* are caused by a variety of fishing gear including gill nets, purse seine, trap nets, and trawl fisheries (Lawson et al., 2017). A research project focused on protecting bycatch biodiversity found in shrimp trawl fishery from northern Peru (Project DYNAMICOPERU) has identified different fish species caught as bycatch, including those of the order Syngnathiformes such as seahorses and cornetfishes (Jaime Mendo personal communication, see Fig. 9E and F). We detected another

anthropogenic threat to seahorses, which occurs during farming activities of the Peruvian scallop *Argopecten purpuratus*. In Sechura Bay (a major scallop culture area in northern Peru) seahorses frequently get anchored to the nets used in lanterns (suspended systems) and sea ranching (bottom culture) (Fig. 9D). During scallop harvesting or net cleaning activities, divers may collect the incidentally caught seahorses to commercially sell them.

5. Conclusions and recommendations

Molecular approaches including qPCR play a significant role in wildlife conservation law enforcement. However, there is still a lack of effective protocols that allow for a rapid, sensitive, and efficient species identification of threatened seahorses from powdered material. This processed form, widely used in TCM, is also being used to facilitate smuggling efforts of different species including rhino horns, elephant ivory, pangolin scales, and seahorses. Our molecular detection assay demonstrated the specific qPCR amplification of the mitochondrial 16S rRNA locus in trace amounts of genomic DNA from powdered *H. ingens*. The newly reported primer set showed great sensitivity and accuracy, demonstrating that our protocol can be used to reliably identify *H. ingens* in dry powdered material. Frontline Customs officers have very little time to detain a suspicious package and determine whether it contains a protected species. Thus, quick species identification assays are crucial to facilitate rapid decision-making on the ground (Tempier, 2014). In our direct qPCR assay, which is performed directly on the seahorse powder without the need for DNA extraction (reducing the time, cost, and risk of cross-contamination), all samples were correctly identified in less than 80 min. The analysis time of our direct qPCR protocol can be shortened using a fast qPCR kit in a portable qPCR device like Biomeme's Franklin, which detects target DNA in just 30−60 min (biomeme.com). Recent advances in emerging biosensing technologies have been fueled by the advent of CRISPR/Cas systems and their power as nucleic acid biosensor, resulting in the development of ultrafast, non-invasive, equipment free, extraction free, amplification free protocols (*e.g.* Baerwald, Goodbla, & Nagarajan, 2020; Hajian et al., 2019; Williams et al., 2019). In the near future, wildlife forensics and customs agents could benefit from highly sensitive and rapid species identification using field-deployable CRISPR/Cas based techniques at border crossings (Baerwald et al., 2020).

Seahorses are one of the most illegally caught and trafficked fish in the world, regardless of the bans imposed by some countries including Peru. We showed that illegal target fishery and domestic trade of live, fresh, and dried seahorses are still happening in several locations along the north-central Peruvian coast. In light of the overwhelming evidence presented in this work, we urge the relevant authorities, scientific community, and NGOs to address this matter. Urgent action is needed to raise awareness among authorities, fishermen, and the general public about the massive depletion of Peruvian seahorse populations. Periodic monitoring programs of poaching and seahorse trade should be conducted by the authorities, and more severe sanctions against its illegal commerce should be imposed. There is still a huge gap in knowledge about the biology and exploitation of *H. ingens* populations from Peru. Further studies must be conducted to better understand the current population status of the Pacific seahorse, a harmless iconic species that deserve greater protection.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

A.M., R.A. and E.Z.M. designed the study and planned the fieldwork. A.M., R.A., C.V.LL., C.R., L.R.F., C.I., C.Y.B., L.C. and E.Z.M. performed fieldwork and sample collection. A.M. performed primer design and wrote the manuscript. A.M., L.R.F., I.A.J., and C.I. performed molecular work. A.M., C.V.LL., L.R.F., C.I., C.Y.B and L.C. contributed to data analyses and interpretation. All authors contributed to the final version of the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

We wish to thank Peter Teske (University of Johannesburg) for his useful comments on *H. kuda* complex and Yuri Hooker (Universidad Peruana Cayetano Heredia) for his valuable help during the identification of pipefish samples. The authors also wish to thank Jaime Mendo (Universidad Nacional Agraria La Molina) for his helpful comments on bycatch of Syngnathiformes from northern Peru (data taken from the Project DYNAMICOPERU: Developing of a dynamic co-management bycatch risk assessment to protect biodiversity in an artisanal shrimp trawl fishery in Peru). We also thank Ivan Gómez (Universidad Nacional Agraria La Molina, Project DYNAMICOPERU), Renato Gozzer (Redes-SP), Percy Ormeño (Universidad Nacional del Santa), Carolina Olano (Universidad Nacional de Trujillo), Mervin Guevara (IMARPE Tumbes), and Elizabeth Cardoza (Instituto Ricardo Ramos Plata, Piura) for providing fish samples, photographs of seahorse trade and bycatch, and for their kind support during the development of this study.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jnc.2021.126030.

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