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Research article

A simple molecular protocol for the identification of hybrid Western Atlantic seahorses, *Hippocampus erectus* × *H. reidi*, and potential consequences of hybrids for conservation

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Abstract
Seahorses hold an iconic status and are popular exhibits in zoos and public aquaria, where they are often on display in multi-species systems. Two of the more popularly kept species are the lined seahorse, *Hippocampus erectus*, and the longsnout seahorse, *H. reidi*. These two species are from different evolutionary subclades, but can produce viable hybrid F_1 offspring, therefore species segregation should be maintained for seahorse conservation breeding programmes. Hybrid *H. erectus* ♂ × *H. reidi* ♀ F_1 offspring exhibit higher median meristic counts for various traits, although large ranges in counts make it difficult to identify hybrids by meristics alone. A molecular protocol was developed to identify both the parent species and the reciprocal hybrids using polymerase chain reaction restriction-fragment length polymorphism (PCR-RFLP). The PCR-RFLP protocol employed the use of the *BsrBI* and *Ms1I* restriction enzymes at the *Tmo-4c4* and *S7* loci, respectively. The developed protocol was effective at discerning hybrids (F_1) from the parent species and identifying some post-F_1 *H. erectus* × *H. reidi* hybrids, but not the direction of the cross. Although captive-bred hybrids may be considered to pose a threat to wild populations if released, there are many benefits to producing captive hybrid seahorses, including improved aquaculture techniques that can curb the wild collection of seahorses.

Introduction

The intriguing nature of seahorses and anthropogenic threats to them provides a great platform to increase public awareness of marine conservation issues, and has made syngnathid exhibits quite popular in zoos and public aquaria (Koldewey 2005). A variety of factors such as specimen size, colour and ease of rearing offspring has made the lined seahorse (*Hippocampus erectus*), the longsnout seahorse (*H. reidi*) and the spotted seahorse (*H. kuda*) the three most popular species on display in public aquaria (Zimmerman 2011), where seahorses are often co-exhibited in a multi-species Syngnathiformes system that may include multiple seahorse species (Syngnathidae: Hippocampinae), pipefishes (Syngnathidae: Syngnathinae) and shrimpfishes (Syngnathidae: Centriscinae).

Zoos and aquaria can play an integral role in the conservation of threatened and endangered species, in both the wild setting and by safeguarding captive populations (Kleiman et al. 1986; Snyder and Snyder 2000). The Association of Zoos and Aquariums (AZA) implements and manages the Species Survival Plan® (SSP) programmes (Association of Zoos and Aquariums 2009). The mission of the SSP programmes is to cooperatively manage specific taxa of interest within a suite of AZA-accredited facilities and related participants (Association of Zoos and Aquariums 2009), for the purpose of maintaining a healthy, genetically diverse, and demographically varied AZA population and ultimately aid in conserving these species in the wild. An SSP programme for *H. erectus* has been implemented, and, like all SSPs, hinges on 'wild-type' species individuals (i.e. non-hybrid individuals).

Across a diverse range of taxa, including fishes, hybridisation occurs quite extensively in nature (Hubbs 1933, 1955; Scribner et al. 2001). Hybrid crosses can exhibit greater fitness than parent species, earlier less fit generations can contribute to gene introgression, or establish hybrid taxa (Arnold et al. 1999), and thus natural hybridisation can have a large influence on evolution (Rieseberg et al. 1990; Smith et al. 2003; Tiedemann et al. 2005). Wild interspecific matings in the family Syngnathidae

are rare (Wilson 2006; Otero-Ferrer et al. 2011). Two male bay pipefish (*Syngnathus leptorhynchus*) carried broods that were genetically confirmed to contain eggs from barred pipefish (*S. auliscus*), but no hybrid adults were found in the study population (Wilson 2006), while in seahorses, two male *H. algiricus* were recorded producing viable hybrid offspring with *H. hippocampus* (Otero-Ferrer et al. 2011).

In captive seahorses, a strain called the 'Chester strain' or *Hippocampus* '*chesteri*' was developed at the Chester Zoo in the United Kingdom, and is the most frequently kept seahorse in European exhibits (Bull and Mitchell 2002). This strain is allegedly a hybrid strain, but details are scant. Mitochondrial DNA analysis (cytochrome b and control region) has shown that the strain matrilineally aligns with *H. kuda* (Woodall et al. 2009), and is now referred to as *H. kuda* 'Chester'. However, mitochondrial DNA analysis does not preclude the possibility of hybrid origin; it only reveals the maternal identity. Hybridisation between *H. erectus* and *H. reidi* has been observed previously (Seahorse Source Inc., Fort Pierce, Florida USA). Meristic traits are known to be plastic in fishes (Hubbs 1922), can vary widely in seahorses (Lourie et al. 1999, 2004), and overlap between *H. erectus* and *H. reidi* (Table 1). Given that unrecognised captive hybridisations can cause serious problems for captive breeding programmes, molecular analysis can be an invaluable tool in identifying intraspecific hybrids (Tiedemann et al. 2005; do Prado et al. 2011).

In 2012, hybrid offspring between *H. erectus* ♂ × *H. reidi* ♀ were documented at the Florida Institute of Technology's Vero Beach Marine Laboratory (Vero Beach, Florida, USA). Owing to the lack of information on seahorse hybridization, the main purpose of this study was to develop a molecular protocol for the identification of the parental species, *H. erectus* and *H. reidi*, and their reciprocal interspecific hybrids using polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (RFLP) methods. Furthermore this study aimed to confirm the hybrid origin of various potential hybrid offspring through the protocols developed. The secondary goal of this study was to evaluate the meristic features of the hybrid *H. erectus* ♂ × *H. reidi* ♀ offspring in contrast to the parent species up to 30 days of age.

Methods

Progeny origin and hybridisation events

Hippocampus erectus

Reference specimens of *H. erectus* consist of nine wild-collected specimens from across Florida, USA, and one aquacultured *H. erectus* specimen (of direct wild descent). Either ethanol or frozen (-20 °C) preserved dorsal fin tissue or tail muscle tissue served as genetic reference materials for *H. erectus* (Table 2).

A brooding *H. erectus* (Here-015, Table 2) was collected from Sebastian Inlet, Florida, USA on 27 February 2013 and maintained at the Vero Beach Marine Laboratory (VBML, Vero Beach, Florida; http://research.fit.edu/vbml/). The brooding male released 660 viable offspring four days after acquisition, and the brood was labelled Here-Cohort1.

Hippocampus reidi

Genetic reference materials for *H. reidi* were acquired from either broodstock pairs of wild origin or filial one (F_1) progeny of wild broodstock that were unrelated to broodstock already sampled (Table 2).

Hippocampus reidi progeny were acquired from a broodstock pair at Seahorse Source Inc. (Fort Pierce, Florida, USA). A pair released 972 viable offspring in August 2013, and this cohort was labeled Hrei-Cohort1.

Hippocampus erectus ♂ × *H. reidi* ♀

In September 2012, one breeding pair of *H. reidi* (of which the female is Hrei-002, and male is Hrei-001, Table 2), wild caught from Fortaleza, Brazil and one breeding pair of *H. erectus*, F_1 captive raised (from wild broodstock collected in Florida, USA; of which the male is Here-GL, Table 2), were housed together in a 342-L bare bottom flow-through rectangular tank furnished with artificial coral at the VBML. By late September, the female *H. erectus* died, and courtship behaviors between the female *H. reidi* (Hrei-002, Table 2) and male *H. erectus* (Here-GL, Table 2) began despite the presence of the male *H. reidi* (Hrei-001, Table 2). On 22 October 2012, a copulation event between the *H. reidi* female and *H. erectus* male was noted and recorded with video equipment. After a gestation period of 20 days, 727 viable offspring were released. This cohort of *H. erectus* ♂ × *H. reidi* ♀ was labeled Hrxe-Cohort1. Three additional cohorts were produced by this interspecies pair: Hrxe-Cohort2, Hrxe-Cohort3, and Hrxe-Cohort4. A total of 737 neonates were released after a 15-day gestation for Hrxe-Cohort2, 668 neonates after 15 days for Hrxe-Cohort3, and 683 neonates after 17 days for Hrxe-Cohort4. All four cohorts were visually confirmed to have been produced from the mating (egg transfer) between Hrei-002 and Here-GL. To reduce the number of animals involved in the study and cost, we elected to analyse only the first and last cohort. A total of 10 specimens from Hrxe-Cohort1 and 10 from Hrxe-Cohort4 (five males and five females of those cohorts) were analysed genetically (Table 2).

Hippocampus erectus ♀ × *H. reidi* ♂

Table 1. Meristic characteristics (traits) of *Hippocampus erectus* and *H. reidi* (Lourie et al. 2004), and their hybrid. Numbers in parentheses indicate trait range. Mode values presented for Lourie et al. 2004 and median for this study. Modes for this study are presented in brackets if different from median.

	Lourie et al. 2004		This study*		
Trait	*Hippocampus erectus*	*Hippocampus reidi*	*Hippocampus erectus*	*Hippocampus reidi*	*H. erectus* ♂ × *H. reidi* ♀
Dorsal fin rays	18–19 (16 – 20)	17 (16 – 19)	17 (15 – 29)	17 (14 – 19)	18 (16 – 20)
Pectoral fin rays	15–16 (14 – 18)	16 (15 – 17)	13 [12] (11 – 15)	15 [16] (11 – 18)	12 (10 – 15)
Trunk rings	11	11	12 [11] (10 – 14)	11 (9 – 14)	14 (11 – 15)
Tail rings	36 (34 – 39)	35 (31 – 39)	33 (26 – 27)	30 (22 – 34)	36 (31 – 39)

*Specimens in this study ranged from 5 to 30 days post-release in age, and data presented are pooled across ages.

Table 2. Samples analysed in this study, including morphological species identification, cohort (for reared batches), genetic (PCR-RFLP) species determination, sex, and sampling location/specimen source.

No.	Specimen ID	Morphological species	Cohort	PCR-RFLP determined species	Sex	Source/collection location	
						Aquacultured/ captive raised	Wild
1	Here-GL[*†]	*H. erectus*		*H. erectus*	♂	Seahorse Source Inc.	
2	Here-001	*H. erectus*		*H. erectus*	♂	St. Lucie, Florida, USA	
3	Here-015[‡]	*H. erectus*		*H. erectus*	♂		Sebastian Inlet, Florida, USA
4	Here-016	*H. erectus*		*H. erectus*	J		Fort Pierce, Florida, USA
5	Here-017	*H. erectus*		*H. erectus*	♂		Goat Creek, Valkaria , Florida, USA
6	Here-022	*H. erectus*		*H. erectus*	♀		Mosquito Lagoon, Florida, USA
7	Here-029	*H. erectus*		*H. erectus*	♀		Sebastian Inlet, Florida, USA
8	Here-031	*H. erectus*		*H. erectus*	♂		Marathon Key, Florida, USA
9	Here-032	*H. erectus*		*H. erectus*	N		Banana River, Florida, USA
10	Here-035	*H. erectus*		*H. erectus*	♂		Gulf Coast Shores, Florida, USA
11	Here-119[§]	*H. erectus*	Here-Cohort1	*H. erectus*	♀	Captive raised progeny of Here-015	
12	Here-120[§]	*H. erectus*	Here-Cohort1	*H. erectus*	♀	Captive raised progeny of Here-015	
13	Hrei-001	*H. reidi*		*H. reidi*	♂		Fortaleza, Brazil
14	Hrei-002[†]	*H. reidi*		*H. reidi*	♀		Fortaleza, Brazil
15	Hrei-003	*H. reidi*		*H. reidi*	♀		Fortaleza, Brazil
16	Hrei-005[*]	*H. reidi*		*H. reidi*	♂	Seahorse Source Inc.	
17	Hrei-006[*]	*H. reidi*		*H. reidi*	♂	Seahorse Source Inc.	
18	Hrei-007[*]	*H. reidi*		*H. reidi*	♀	Southwatch Inc.	
19	Hrei-008[*]	*H. reidi*		*H. reidi*	♀	Southwatch Inc.	
20	Hrei-009[*]	*H. reidi*		*H. reidi*	♀	Southwatch Inc.	
21	Hrei-010[*]	*H. reidi*		*H. reidi*	J	Seahorse Source Inc.	
22	Hrei-013[*]	*H. reidi*		*H. reidi*	♀	Seahorse Source Inc.	
23	Hrei-014[§]	*H. reidi*	Hrei-Cohort1	*H. reidi*	J	Seahorse Source Inc.	
24	Hrei-015[§]	*H. reidi*	Hrei-Cohort1	*H. reidi*	J	Seahorse Source Inc.	
25 – 34	Hrxe-001 – Hrxe-010	*H. erectus* ♂ × *H. reidi* ♀	Hrxe-Cohort1	hybrid	5 ♂, 5 ♀	Vero Beach Marine Laboratory	
35 – 44	Hrxe-011 – Hrxe-020	*H. erectus* ♂ × *H. reidi* ♀	Hrxe-Cohort4	hybrid	5 ♂, 5 ♀	Vero Beach Marine Laboratory	
45 – 54	Hexr-001 – Hexr-010	*H. erectus* ♀ × *H. reidi* ♂	Hexr-Cohort1	hybrid	1 N, 5 ♂, 4 ♀	Seahorse Source Inc.	
55 – 64	MM-001 – MM-010	*H. erectus* (putative)		post-F_1-hybrid	2♂, 8 ♀	Mote Marine Laboratory	

[*]Reference specimens that were aquacultured, but no shared pedigree. [†]Sire and dam of the *H. erectus* ♂ × *H. reidi* ♀ hybrids. [‡]Sire of the *H. erectus* for meristic analysis. [§]Validation of species cohorts for meristic analysis. J = Juveniles, N = neonate (newborn). Post-F_1 refers to any progeny produced from crossing an F_1 with any other specimen, whether it be another F_1 (their progeny being F_2) or different specimens from different lineages. "PCR-RFLP Determined Species" refers to the species or hybrid designation of the specimens after the PCR-RFLP technique developed in this study has been applied.

Seahorse Source Inc. co-housed some of their *H. erectus* and *H. reidi* for a brief period in 2014. During that period a successful mating event occurred between a female *H. erectus* and male *H. reidi*., which resulted in approximately 450 hybrid offspring being released. The gestation period for this cohort is unknown. Ten specimens from this cohort (Hexr-Cohort1), were also analysed genetically (Table 2).

Suspected captive H. erectus × H. reidi

The Seahorse Conservation Laboratory at The Mote Marine Laboratory (SCL, Sarasota, Florida; http://mote.org/) runs a captive breeding programme that supplies captive-bred *H. erectus* to 20 AZA accredited zoos and aquariums in the USA. Up to 40% of *H. erectus* on display at those facilities were produced by the SCL. In the past, the SCL has co-housed (F_1 and F_2) *H. erectus*

reared at the SCL and post-F_1 (i.e. any progeny produced from crossing any F_1 with any other specimen) *H. reidi* reared at the Shedd Aquarium (Chicago, Illinois, http://www.sheddaquarium.org/) (SCL, pers. comm.). The SCL suspected possible hybrids at their facility, after learning about possible hybridisation between the two species at the 2013 Regional Aquatics Workshop (Pham et al. 2013). Fin clip samples from a total of 10 specimens exhibiting *H. erectus* morphological features were received from the SCL (Table 2). These putative '*H. erectus*' specimens were genetically analysed. These specimens were produced in April and June 2013, originated from the commingled species broodstock tank, and have undetermined parentage (SCL, pers. comm.).

Genetics

PCR and sequencing

Live specimen dorsal fin tissue was sampled with a non-invasive technique (Woodall et al. 2012), fin clips, or tail muscle tissue was sampled from dead specimens. A Qiagen DNeasy blood and tissue kit (Qiagen Inc., Valencia, California, USA) was used to extract and purify total genomic DNA from tissue samples. Polymerase chain reaction (PCR) with methods based on Kocher et al. (1989) were employed to amplify the partial sequences of first intron (RP1) of the nuclear S7-like ribosomal protein (*S7*) and the nuclear Tmo-4c4 gene (*Tmo-4c4*) with the primer pairs S7RPEX1F and S7RPEX2R (Chow and Hazama 1998), and Tmo4c4F and Tmo4c4R (Streelman and Karl 1997), respectively (Table 3). Amplification was conducted with Fisher BioReagents™ *ex*ACTG*ene*™ Core Reagents Set B (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) following the PCR and DNA purification techniques described by Ho et al. (2012). Purified products were sent to the University of Florida for sequencing in both the forward and reverse directions using an Applied Biosystems Model 3130 Genetic analyser. The expected amplicon sizes were ~500 basepairs (bp) for *Tmo-4c4* and ~650 for *S7*. The sequences were edited and assembled using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI USA; http://www.genecodes.com) and deposited in GenBank. Two hybrids were also sequenced (Hrxe-001 and Hrxe-011), but GenBank does not accept sequences of hybrid specimens.

PCR-RFLP

The polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis methods described by Prado et al. (2011) were employed with modification. Restriction maps for the sequenced *H. reidi* (*S7*: Hrei-001 = KJ741262, *Tmo-4c4*: Hrei-007 = KJ741264, Hrei-008 = KJ741265) and *H. erectus* (Here-GL for both *S7* = KJ741261 and *Tmo-4c4* = KJ741263) specimens were analysed using the software NEBCUTTER V2.0 (Vincze et al. 2003), along with reference sequences of *H. erectus* and *H. reidi* from GenBank (KC811841.1, KC811851.1, AY277339.1, DQ288386.1). Restriction digestion was conducted following the manufacturer's protocol, where PCR products were digested by restriction enzymes BsrBI and Ms1I (New England Biolabs Inc., Ipswich, Massachusetts, USA) in a final volume of 50 µL containing 44 µL of PCR products, 5 µL of 10X NEBuffer, and 1 µL of restriction enzyme. Reactions were incubated at 37 °C for 2 hours, and 5 µL of restriction product was visualised with a 1% agarose electrophoresis gel.

Meristic analysis

Rearing and husbandry protocols

For meristic analysis one batch of *H. erectus* (offspring of Here-015, Here-Cohort1), one batch of *H. reidi* (from Seahorse Source, Hrei-Cohort1), and two batches of *H. erectus* ♂ × *H. reidi* ♀ (Hrxe-Cohort1 and Hrxe-Cohort4) were used. Meristics analysis was fatal and the reciprocal hybrid was not investigated for meristics due to limited offspring produced and commercial limitations from Seahorse Source. To verify that *H. erectus* and *H. reidi* were genetically *H. erectus* and *H. reidi*, PCR-RFLP was conducted on two specimens from each batch (Here-119 and Here-120 from Here-Cohort1; Hrei-014 and Hrei-015 from Hrei-Cohort1).

Experimental setup and meristic counts

The neonates (offspring) were reared from 0 days post-release (DPR) to 30 DPR at the VBML using the protocol described by Pham and Lin (2013). Each cohort (batch: species or hybrid) was reared in triplicate (n = 3) and each tank stocked with 100 neonates. Due to timing of reproductive events, each cohort was reared independently (sequentially) in time. Each tank was considered the replicate unit for cultivation, and each tank was sampled for three specimens at 5, 10, 15, 20, and 30 DPR. Thus each species was samples for 9 specimens at each time period (across three tanks) for a total (*n*) of 45 seahorses for each cohort. Data was pooled for each species and the two hybrid cohorts were pooled, i.e. *n* = 45 for *H. erectus* and *H. reidi*, and *n* = 90 for *H. erectus* ♂× *H. reidi* ♀. Sampled specimens were preserved in 10% formalin. The following meristic traits were quantified following Lourie et al. (1999): number of dorsal fin rays, number of pectoral fin rays, number of trunk rings, and number of tail rings.

Statistics

Specimens were reared in triplicate, but meristic trait sets can be unique to individuals and as such analysis was performed on individuals instead of the tank as unit. To test the effect of species (hybrid included) on the combined set of meristic traits, a permutational multivariate analysis of co-variance (perMANCOVA) was employed with species (hybrid included) as a fixed factor and age as a covariate. Contingent on the results of the perMANCOVA, the effect of species (hybrid included) on each meristic trait individually was tested with a permutational

Table 3. Primers employed for the amplification of markers in this study.

Marker	Primer name	Sequence (5′ → 3′)	Annealing temperature employed	Reference
S7	S7RPEX1F	TGGCCTCTTCCTTGGCCGTC	55 °C*	Chow and Hazama 1998
	S7RPEX2R	AACTCGTCTGGCTTTTCGCC		
Tmo-4c4	Tmo4c4F	CCTCCGGCCTTCCTAAAACCTCTC	55 °C	Streelman and Karl 1997
	Tmo4c4R	CATCGTGCTCCTGGGTGACAAAGT		

*The suggested annealing temperature is 60° C; however, 55° C was used effectively to match *Tmo-4c4* protocol.

Table 4. Fixed polymorphisms between *Hippocampus erectus* and *H. reidi* at the *S7* and *Tmo-4c4* loci.

Locus	Fixed polymorphisms															
S7	Position	5′	85	88	100	211	218	220	269	280	285	288	328	368	378	426	3′
	H. erectus	…	T	T	T	G	R	C	G	C	A	G	C	A	G	A	…
	H. reidi	…	A	G	A	T	–	G	T	A	T	T	A	T	A	C	…
Tmo-4C4	Position	5′	430	437	471	475	488	489	499	539	546	572	573	574	575	3′	
	H. erectus	…	G	G	A	G	A	A	A	C	G	A	T	T	T	…	
	H. reidi	…	T	T	C	A	G	G	G	G	T	–	–	–	–	…	
	Position	5′	2	75	89	102	113	123	125	128	195	246	254	259	344	3′	
	H. erectus	…	C	C	G	G	T	A	C	C	A	C	A	T	A	…	
	H. reidi	…	A	T	A	A	G	G	T	A	G	G	G	G	T	…	

analysis of co-variance (perANCOVA) on each trait separately with species (hybrid included) as a fixed factor and age as a covariate. Significant trait perANCOVAs were contrasted with fixed pairwise perANCOVAs as a post-hoc with a Holm-Bonferroni multiple comparison correction. All meristic traits are presented as pooled and with median ± median absolute deviation (MAD).

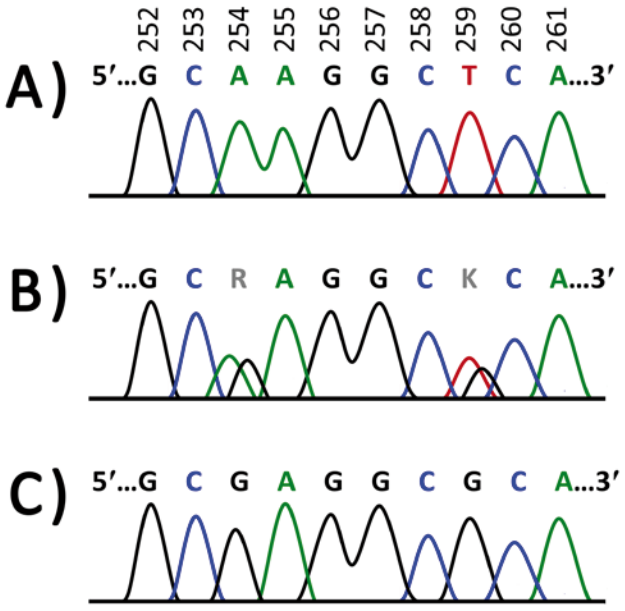

Figure 1. Reproduction of a 10 basepair section of the *Tmo-4c4* sequence chromatopherograms for A) *Hippocampus erectus*, B) *H. erectus* ♂ × *H. reidi* ♀, and C) *H. reidi* from position #252–261. Two fixed polymorphisms are present in the illustrated segments at positions #254 and #259. The hybrid specimen shows two half strength peaks at those positions, corresponding to the two parent species polymorphisms.

Results

Genetics

PCR and sequencing

For *S7*, a 625 bp fragment was amplified for *H. reidi*, and a 630 bp fragment for *H. erectus*. There were 22 polymorphic sites between the two species (Table 4), and a region of low quality read in *H. erectus* between bp 217–329 that resulted in numerous ambiguities. A single base pair indel (insertion/deletion) was detected at bp 218 and 4 bp (ATTT repeat) indel at bp 572 for *H. erectus*. For *Tmo-4c4*, a 501 bp fragment was amplified for both *H. erectus* and *H. reidi* and 13 polymorphic sites were identified between the two species (Table 4). For both *S7* and *Tmo4c4*, sequences of the hybrid specimens (Hrxe-001 and Hrxe-011) exhibited an ambiguous signal reflective of the parental sequences at the fixed polymorphic sites (Fig. 1). Sequences of the species specimens have been deposited in GenBank (KJ741261 – KJ741265). Hybrid sequences cannot be deposited in GenBank.

PCR-RFLP

NEBCUTTER (Vincze et al. 2003) identified five enzymes that had exclusive cleavage in *H. reidi* (*Ms1I*, *BstAPI*, *BtgI*, *BstZ17I*, and *HpyCH4III*), and eight enzymes in *H. erectus* (*BanII*, *N1aIV*, *NciI*, *BciVI*, *BmrI*, *AflII*, *NmeAIII*, and *Bs1I*) for the *S7* locus. For the *Tmo-4c4* locus four enzymes in *H. reidi* (*HpyCH4V*, *BsmAI*, *BcoDI*, and *HpyCH4IV*) and seven enzymes in *H. erectus* (*NgoMIV*, *BsrFI*, *HpaII*, *MspI*, *NaeI*, *BsrBI*, and *EarI*) were identified. Based on the fragment size yields, and quality of sequence reads, one enzyme was selected for each species.

The restriction enzyme *Ms1I* (New England Biolabs Inc., Ipswich, Massachusetts, USA) was identified to specifically cleave *H. reidi* at the *S7* locus, and predicted the 625 bp *S7* fragment would be cleaved to yield 84 and 541 bp fragments (104 and 561 bp fragments including the primers). Note that there is a 5 bp indel between the two species: *H. erectus* has a length of 630 bp for *S7*.

The restriction enzyme *BsrBI* (New England Biolabs Inc.) was identified to specifically cleave *H. erectus* at the *Tmo-4c4* locus,

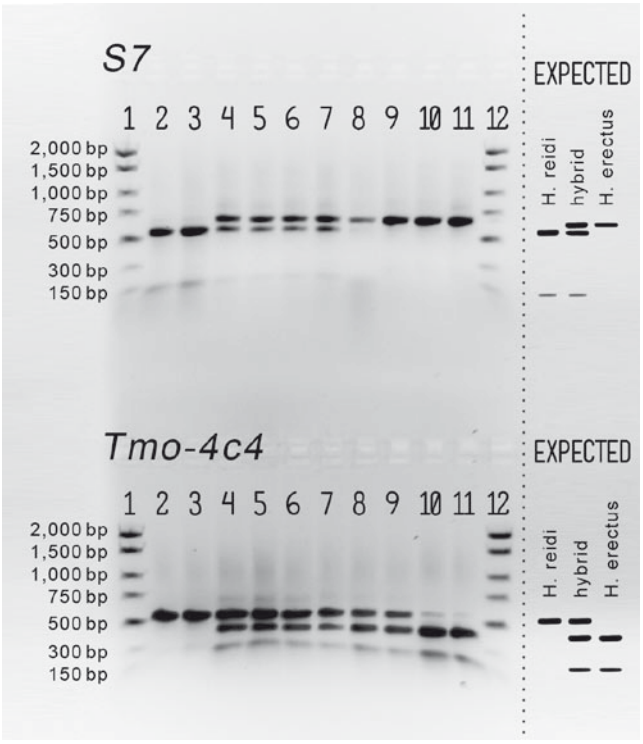

Figure 2. PCR-RFLP (inverted grayscale) patterns for *S7* nuclear gene (top) and *Tmo-4c4* nuclear gene (bottom). Lane contents are as follows: 1 & 12 = molecular weight marker, 2 & 3 = *Hippocampus reidi* (Hrei-01 & Hrei-02), 4 & 5 = *H. erectus* ♂ × *H. reidi* ♀ (Hrxe-01 & Hrxe-02), 6 & 7 = *H. erectus* ♀ × *H. reidi* ♂ (Hexr-01 & Hexr-02), 8 & 9 = putative *H. erectus* (MM-01 & MM-02), and 10 & 11 = *H. erectus* (Here-GL & Here-01). Note that faint bands under 300 bp, and some arching is present where outside lanes ran faster than inside lanes.

and predicted the 501 bp *Tmo-4c4* fragment would be cleaved to yield 104 and 397 bp fragments (128 and 421 bp fragments including the primers).

S7 locus

PCR products yielded *S7* fragments that were slightly larger than ~600 bp on the gel for all specimens (625 & 630 bp from sequencing). Enzymatic restriction with *Ms1I* for all *H. reidi* specimens revealed two bands, an intense and a faint band, that coincided with the predicted cleavage products of 561 bp and 104 bp fragments, respectively (Fig. 2, top, lane 2 & 3). Enzymatic restriction with *Ms1I* for all *H. erectus* specimens revealed a single intense band that was slightly larger than ~600 bp, coinciding with an undigested PCR product (Fig. 2, top, lane 10 & 11). Hybrid specimens of both directional crosses presented with three bands, two intense and one faint band. The bands coincided with undigested PCR product for the *H. erectus* allele, and of 561 bp and 104 bp fragments for the *H. reidi* allele (Fig. 2, top, lane 4 – 7). All Mote seahorse specimens (MM) exhibited one intense band that coincided with un-cleaved PCR product for *S7* (Fig. 2, top, lane 8 & 9).

Tmo-4c4 locus

PCR products yielded *Tmo-4c4* fragments that were slightly larger than ~500 bp on the gel for all specimens (501 bp from sequencing). Enzymatic restriction with *BsrBI* for all *H. reidi* specimens revealed a single intense band that was slightly larger than ~500 bp, coinciding with an undigested PCR product (Fig. 2, bottom, lanes 2 & 3). Enzymatic restriction with *BsrBI* for all *H. erectus* specimens revealed two bands, an intense and a faint band, that coincided with the predicted cleavage products of 421 bp and 128 bp fragments, respectively (Fig. 2, bottom, lanes 10 & 11). Some arching was present in the bottom gel, which makes the faint bands appear larger than they are. Hybrid specimens of both directional crosses presented three bands, two intense and one faint band. The bands coincided with undigested PCR product for the *H. reidi* allele, and of 421 bp and 128 bp fragments for the *H. erectus* allele (Fig. 2, bottom, lanes 4–7). All Mote specimens (MM) exhibited three bands for *Tmo-4c4*, a similar pattern to the hybrid crosses (Fig. 2, bottom, lanes 8 & 9).

Meristic analysis

The *H. erectus* and *H. reidi* batches used for meristic analysis were genetically confirmed to be *H. erectus* and *H. reidi* using PCR-RFLP. The perMANCOVA revealed a significant effect of species (hybrid included) and age as a covariate on the combined dependent variables of dorsal fin rays, pectoral fin rays, tail rings, and trunk rings (Table 5). Individual perANCOVAs on each response variable revealed a significant effect of species (hybrid included) and age as a covariate on pectoral fin rays and trunk rings (Table 5). Furthermore, a significant effect of species (hybrid included) on dorsal fin rays and tail rings was found, with no significant effect of age as a covariate (Table 5).

Table 5. Results of perMANCOVA and perANCOVA test on various meristic characteristics (traits). Significant values highlighted in italics.

Statistical model	Independent variable	Measure	Pseudo-*F*	d.f.	*P* (permutational)	R^2
perMANCOVA	Species (hybrid included)	Combined	47.799	2,131	<0.001	0.3825
	Age (covariate)	Combined	23.345	1,131	*<0.001*	0.0934
perANCOVA	Species (hybrid included)	Dorsal fin rays	16.457	2,131	*<0.001*	
	Age (covariate)	Dorsal fin rays	0.284	1,131	0.4013	
	Species (hybrid included)	Pectoral fin rays	48.262	2,131	*<0.001*	
	Age (covariate)	Pectoral fin rays	57.642	1,131	*<0.001*	
	Species (hybrid included)	Tail rings	65.353	2,131	*<0.001*	
	Age (covariate)	Tail rings	0.111	1,131	0.5811	
	Species (hybrid included)	Trunk rings	44.549	2,131	*<0.001*	
	Age (covariate)	Trunk rings	7.741	1,131	*0.0116*	

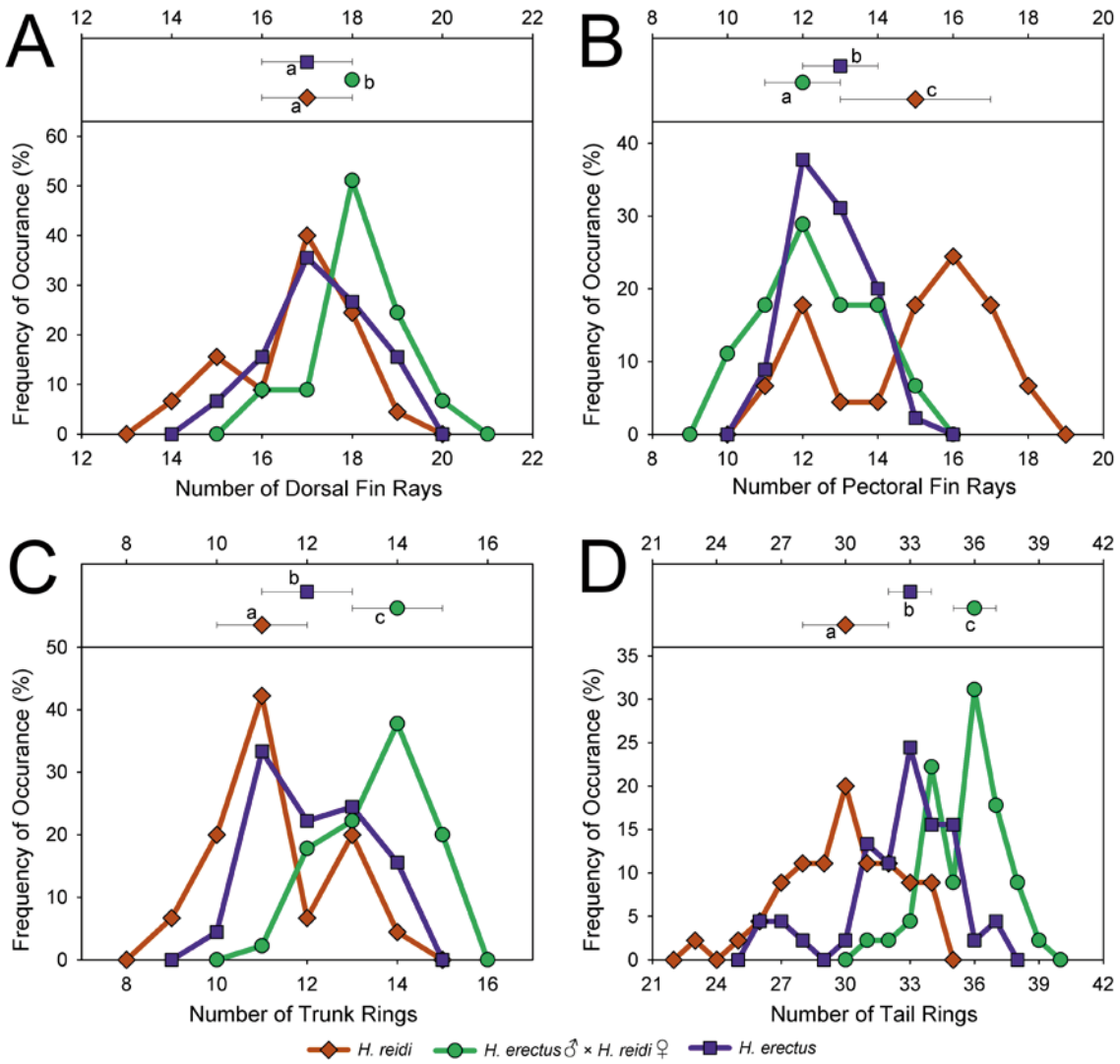

Figure 3. Median ± Median Absolute Deviation (MAD) [**A**] dorsal fin rays, [**B**] pectoral fin rays, [**C**] trunk rings, and [**D**] tail ring counts of *Hippocampus reidi*, *H. erectus*, and *H. erectus* ♂ × *H. reidi* ♀. Frequency distributions of each meristic trait presented in bottom section of each panel. Sample size (*n*) = 9 for each age period (5, 10, 15, 20, and 30 days post-release) for a total pooled sample size (*n*) = 45 for each species. Two hybrid cohorts were reared and pooled for a sample size (*n*) = 90 for hybrid. Potential differences in age were accounted for using age as a covariate, and data presented as pooled. Significant differences in median number of rays are indicated by different lower case letters.

Dorsal fin ray counts for *H. reidi* ranged from 14 to 19, while *H. erectus* ranged from 15 to 19 dorsal fin rays. Both species had a median of 17 dorsal fin rays (Fig. 3A). However the dorsal fin ray counts in the hybrids ranged from 16 to 20 rays and had median number of 18 rays, which was significantly higher than either parent species (Fig. 3A). Pectoral fin ray counts ranged from 11 to 18 for *H. reidi*, 11 to 15 in *H. erectus*, and 10 to 15 rays in the hybrids (Fig. 3B). All three groups had significantly different median numbers of pectoral fin rays, with the median number of rays in hybrids being less than both parent species. *Hippocampus reidi* trunk ring counts ranged from 9 to 14, *H. erectus* counts from 10 to 14, and hybrid counts from 11 to 15 (Fig. 3C). All three groups had significantly different median numbers of trunk rings, with the hybrids having a median of two and three more trunk rings than *H. erectus* and *H. reidi*, respectively (Fig. 3C). Tail ring counts had very broad ranges, with *H. reidi* ranging from 22 to 34 rings, *H. erectus* from 26 to 37, and the hybrids from 31 to 39 (Fig. 3D). All medians were significantly different, with *H. reidi* at 30, *H. erectus* at 33, and the hybrids with more rings than either parent species at 36 (Fig. 3D).

Discussion

Traditionally, morphological examination — including colour patterns and meristics — is used to identify seahorse species (Lourie et al. 2004). At young ages, colour patterns have not yet fully matured, and may not be useful for identification. Additionally, meristic traits can be plastic (Hubbs 1922) and encompass a wide range in counts within a species (Lourie et al. 1999). Meristic traits in hybrids tend to be intermediate between parent species (Hubbs 1955; Fleming et al. 2014); however of the four meristic traits investigated for the *H. erectus* ♂ × *H. reidi* ♀ hybrids, three exhibited significantly higher medians, and one significantly lower than either parent species. Similarly, Leary (1983) reported consistently high meristic counts in hybrid salmonids. Certain critical periods of development determine counts of meristic traits in fishes (Tåning 1950; Lindsey 1954; MacCrimmon and Kwain 1969; Lindsey and Harrington 1972; Ali and Lindsey 1974). Leary et al. (1985) proposed that differences in timing and duration of critical periods between parent species can lead to higher meristic counts than either parent species. Nevertheless, the medians for

the hybrids fall within the observed count distributions of one or both parent species. Coupled with the wide range in counts, it is unlikely that high confidence identifications of potential hybrids can be made from investigating meristics alone.

In cases of cryptic morphology, molecular markers have been applied successfully for the detection of hybrids and genetic introgression in other fishes (Perez et al. 1999; Congiu et al. 2001; Scribner et al. 2001; Park et al. 2003; Gante et al. 2004; Tiedemann et al. 2005; Gunnell et al. 2008; Aboim et al. 2010; do Prado et al. 2011). Here we were able to effectively detect interspecific *Hippocampus* hybridisation at the molecular level using PCR-RFLP and DNA sequencing. PCR-RFLP showed the parent species were homozygous for both *S7* and *Tmo-4c4* markers about the cleavage polymorphism. The F_1 progeny from the VBML and Seahorse Source were all heterozygous for both markers, consistent with F_1 interspecific hybrids (see Fig. 2), making the *BsrBI* and *Ms1I* enzymes effective for the discrimination of *H*. *erectus*, *H*. *reidi*, and their reciprocal F_1 interspecific crosses. However, the direction of the cross cannot be discerned with the current protocol. Specimens from SCL at Mote Marine Laboratory were determined to be heterozygous for the *Tmo-4c4* marker, but homozygous for the *H*. *erectus* allele for the *S7* marker. The partial heterozygosity of the Mote specimens indicates post-F_1 hybrids, and likely products of a back cross to *H*. *erectus*. Currently, it is unknown if F_1 hybrids can cross and produce F_2 progeny; however, F_1 hybrids have been backcrossed to *H*. *erectus* in the past (Seahorse Source Inc.).

Hippocampus erectus ranges from as far north as Nova Scotia (Canada) down to the Amazon River Barrier in Brazil (Lourie et al. 2004; Boehm et al. 2013), while *H*. *reidi* ranges from the southern United States of America down to the southernmost reaches of Brazil (Lourie et al. 2004; Boehm et al. 2013). This makes the entire Gulf of Mexico and the wider Caribbean a sympatric contact zone between *H*. *erectus* and *H*. *reidi*; however, the lack of wild hybrid documentation may be a result of a scarcity of studies employing combined informative nuclear and mitochondrial markers with such a purpose (Wilson 2006; López et al. 2010).

The evolutionary origin of seahorses — upright posture — dates back to the splitting off from the pygmy pipehorse lineage in the late Oligocene, 33.9–23 MYA (Teske and Beheregaray 2009). Four major *Hippocampus* clades, separated by millions of years of divergence, are recognised (Teske et al. 2004; Teske and Beheregaray 2009). One of the clades is subdivided into four subclades, two of which invaded the Atlantic on separate occasions (Teske et al. 2004). *Hippocampus erectus* is a descendent of the first invasion before the closure of the Tethyan seaway 14–6.7 MYA (Hsü and Bernoulli 1978; Sonnenfeld 1985; Vrielynk et al. 1997), while the clade containing *H*. *reidi* invaded after the closure (Teske et al. 2004). *Hippocampus erectus* and *H. reidi* produced viable hybrid offspring, even though they are separated by millions of years of divergence (Teske et al. 2004; Teske and Beheregaray 2009; Boehm et al. 2013). It appears that the degree of evolutionary separation between *Hippocampus* species has not led to any substantial initial postzygotic barriers, though the reproductive capabilities of the hybrids still need to be evaluated in detail. As wild seahorse hybridisation is scarce, prezygotic barriers such as allopatry, complex reproductive behaviours, and population densities in sympatric species can be important factors that curb hybridisation (Vincent 1994; Vincent and Sadler 1995; Masonjones and Lewis 1996; Jones et al. 1998; Jones and Avise 2001; Wilson et al. 2003; Foster and Vincent 2004; Otero-Ferrer et al. 2011).

Wild seahorse populations tend to be patchy and densities have been reported to be very low — typically less than 0.5 individuals/m^2 (Perante et al. 2002; Bell et al. 2003; Foster and Vincent 2004; Moreau and Vincent 2004; Freret-Meurer and Andreata 2008), but some patches have been recorded with densities as high as 10 individuals/m^2 (Foster and Vincent 2004). Display aquaria and other captive conditions such as aquaculture settings are known to house seahorses in extremely dense conditions. High density coupled with the lack of predators, an optimal and stable breeding environment, and plentiful access to a high quality diet, may cause the cross-species behavioural barriers to disintegrate and lead to hybridisation events in captivity, even in the presence of conspecifics.

The AZA has implemented an SSP programme for *Hippocampus* and these programmes hinge on stocks genetically reflective of the wild species (no hybridisation or gene introgression from other species). However, there are institutions that have in the past or currently co-house *H*. *erectus* and *H*. *reidi*. This in itself is harmless, unless progeny from such housings are reared and thus bring uncertainty into the progeny's parentage – especially since copulation events often go undocumented and parentage is paternally inferred, thus masking the potential for a heterospecific female. This is emphasised by the post-F_1 individuals from Mote, where backcrossing could have gone undetected. It is important to segregate the species in captive settings if the stocks are to be used in breeding programmes.

The production and keeping of hybrid seahorses has been intensely discouraged under any circumstances (Project Seahorse 2009) to, for example, safeguard captive breeding programmes and wild populations from potential hybrid releases. This rigid framework sits well within the guise of the SSPs and other conservation breeding efforts. However, since F_1 hybrids can easily go undetected, for facilities that need to maintain species-specific breeding programmes, it is advisable that incoming stocks are genetically screened prior to entering a breeding programme. The protocol described herein is effective for discriminating F_1 and some post-F_1 individuals. However, additional markers need to be assayed to be able to accurately detect the many possible post-F_1 hybrids and backcrosses. Furthermore, in facilities that currently co-house *H*. *erectus* and *H*. *reidi* — or have in the past — and have produced progeny from comingled broodstock, it would be advisable to genetically screen any current progeny for potential hybrids and genetic introgression to avoid threatening the viability of the breeding programme.

Although there may be a perceived risk to wild populations in producing hybrids, the use of hybrids in aquaculture is widespread and has many benefits, such as novel colour patterns (Baensch and Tamaru 2009), sensory repertoire expansion (Sandkam et al. 2013), increased growth rates (Tuncer et al. 1990; Gunther et al. 2005), manipulation of sex ratios (Wolters and DeMay 1996), production of sterile animals (Khan et al. 1990), and 'hybrid vigour' (Rahman et al. 1995). With exceptionally high demand for seahorses for the traditional Chinese medicine trade (Vincent 1995), the use of hybrid crosses can aid in the aquaculture production of seahorses for these markets. The increased efficiency of aquaculture production of seahorses can benefit wild populations, by helping to curb wild collections. It is feared that if hybrids become mainstream in aquaria (public or private), it can threaten the integrity of captive lines in breeding programmes due to the difficulty in identifying hybrids morphologically. Furthermore, if interspecific hybrid seahorses are to be employed, detailed records ought to be kept and line vigour and productivity traced, as post-F_1 hybrids may exhibit reduced vigour (Templeton 1986) and offspring viability. Furthermore, the development and employment of more fine-scale genetic markers, such as DNA microsatellites or single nucleotide polymorphism, can aid in the assessment of the degree of relatedness among individuals and facilitate the selection of optimal pairs for breeding to either curb or encourage hybridisation. Nonetheless, it is the responsibility of the host institution or breeder to verify stock origin and if necessary the genetic makeup (hybrid or introgressed) of any

animal they intend to bring into a breeding programme (which is already a goal of AZA's SSP programmes), whether hybrids exist or not. Thus, the dismissal of the production and use of hybrids can be very shortsighted, and robs us of a potentially viable, albeit nontraditional, avenue of seahorse conservation.

Conclusion

Two western Atlantic seahorse species from different evolutionary subclades — *Hippocampus erectus* and *H. reidi* — can reciprocally produce viable hybrid F_1 offspring. The hybrid F_1 exhibit higher median meristic counts for various traits, although large ranges in counts make it difficult to identify hybrids by meristics alone. Using PCR-RFLP, the use of both the *BsrBI* and *Ms1I* enzymes at the *Tmo-4c4* and *S7* loci, respectively, are effective at identifying F_1 and some post-F_1 hybrids. This study shows that under captive conditions *H. erectus* and *H. reidi* can produce viable offspring and that the offspring can be readily reared. This illustrates the importance of species segregation for breeding programmes. Hybrid seahorses bred in captivity are believed to pose a threat to wild populations if released. However, there may be many benefits to producing captive hybrid seahorses, including improved aquaculture techniques that can curb the wild collection of seahorses that merit further research.

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