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RESEARCH PAPER

Disentangling the controversial identity of the halfbeak stock (*Hemiramphus brasiliensis* and *H. balao*) from northeastern Brazil using multilocus DNA markers

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Abstract Reliable biological identification is essential for effective management programs for fishery resources. In many cases, individuals of species with overlapping features and/or subtle morphological differences can be misidentified by traditional taxonomic procedures. The conservation status (i.e., genetic diversity) of commercial fishery stocks of the Southern Atlantic are in general poorly understood. The halfbeak populations found off the coast of northeastern Brazil represent a suitable model for testing controversial identifications, given that the two local species (*Hemiramphus brasiliensis* and *H. balao*) not only present very subtle morphological differences, but are also harvested intensively. The present study examined the potential occurrence of the two species off the coast of the Brazilian state of Pernambuco using a multilocus DNA approach, which also provided insights into the conservation status of these stocks in relation to the genetic variation found in both mitochondrial and nuclear regions, analyzed by molecular systematics and population genetics parameters. The results indicated the presence of only one halfbeak species in the region, reinforced by phylogenetic relationships and population genetics in both mitochondrial and nuclear loci. The conservation status of the stocks in terms of their genetic diversity appears to be good despite the intense exploitation. The results of this study provide a new perspective for the conservation and management of this fishery resource, particularly given the fact that the intensive exploitation over the last five decades appears to have impacted a single species, rather than two.

Keywords Halfbeaks · ISSRs · DNA barcode · Cytochrome b · Fishery genetics

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Introduction

Many groups of plants and animals are still subject to controversial identifications, despite the development of modern techniques such as DNA sequencing and genomics (Packer et al. 2009), which have often proved more effective than traditional morphology-based approaches. The elimination of these uncertainties is important for successful conservation and management practices (Frankham et al. 2004). While there has been some criticism of the efficacy of DNA data for the assessment of species identification (Bridge et al. 2003; Song et al. 2008; Hazkani-Covo et al. 2010), the robustness of this approach has been well supported (Avise 2000, 2004; Frankham et al. 2004; Padial et al. 2010). In particular, this approach has been used successfully for a number of endangered animals (Bowen et al. 1992; Roberts et al. 2004; Vianna et al. 2006; Caragiulo et al. 2014).

A number of DNA-based studies have provided evidence on fish population structure and species diversity off the Atlantic coast of South America (Santos et al. 2003, 2006; Fraga et al. 2007; Prodocimo et al. 2008; Sodré et al. 2012; Mendonça et al. 2013). The data from these studies have contributed to the development of effective guidelines for the management of fishery stocks in Brazil, in particular populations with poorly-defined taxonomic status.

The exploitation of fishery resources is a traditional human activity that presents fundamental challenges for the sustainable exploitation of stocks. For example, groupers represent an important fishery resource, but populations are in decline worldwide, primarily because of the inadequate monitoring and management of stocks. Approximately one quarter of all grouper species are list by the IUCN (Mitcheson et al. 2013), and recent genetic analyses have provided important diagnostic insights into the species- and population-level diversity of these fishes (Craig et al. 2009; Almany et al. 2013; Portnoy et al. 2013; Seyoum et al. 2013; Silva-Oliveira et al. 2013; Torres et al. 2013). These observed genetic variations might indicates the need for more species- and population-level monitoring focused on improvements on the management of stocks.

The ballyhoo (*Hemiramphus brasiliensis*) and the balao (*H. balao*) halfbeaks, are found in northeastern Brazil (Lovejoy 2000). These species are an important fishery resource in the region (primarily in the states of Pernambuco, Alagoas and Rio Grande do Norte), accounting for approximately 90 % of the landings by weight from seine fishing in northeastern Brazil (Lessa et al. 2006). These halfbeaks are very similar morphologically, with only a few diagnostic features, such as the size of the pectoral fin and the color of the lobes of the caudal fin (Collette 2003). However, fin color can only be discerned reliably in live or fresh individuals, and is of little use for the diagnosis of fixed specimens. The two species are harvested together and are considered to be the same resource in Florida (USA) and Pernambuco (Berkeley 1975; Mcbride and Thurman 2003; Ibama 2005).

Considering the potential impact of controversial identifications on the management of fishery resources, the present study used a multilocus DNA approach to evaluate the presence of ballyhoo and balao halfbeaks as distinct species off the coast of the Brazilian state of Pernambuco. The methods used included in silico and in vitro procedures of Polymerase Chain Reactions-Restriction Fragment Length Polymorphisms at the Cytochrome Oxidase I gene (COI—PCR–RFLPs), Inter Simple Sequence Repeats (ISSRs) and sequences of the Cytochrome b gene. The data were also used to evaluate the conservation status (i.e. genetic diversity) of the local populations, and comments are provided on the potential use of these data for the sustainable exploitation of halfbeak stocks in the region.

Materials and methods

A total of 64 specimens (32 identified in the field as *H. brasiliensis* and 32 as *H. balao*; Fig. 1) were collected at sea with the aid of local fishermen, and identified in the field (freshly caught) by the authors based on the only valid halfbeak identification key provided by Collette (2003). Samples of muscle and fin tissue were preserved in 95–96 % ethanol (Merck) and stored at −20 °C.

Five specimens of each species were selected randomly for the in vitro PCR-RFLP procedure. The DNA was purified by the phenol–chloroform-isoamylic alcohol method (Sambrook and Russell 2001). The COI barcode fragments (~700 bps) were amplified by PCR using the primers FishF1 5′ TCAAC CAACCACAAAGACATTGGCAC 3′ and FishR1 5′ TAGACTTCTGGGTGGCCAAAGAATCA 3′ (Ward et al. 2005). The PCRs were prepared in a total volume

Fig. 1 Freshly caught specimens of **a** *H. brasiliensis* and **b** *H. balao*

of 25 µl. The reactions comprised 2.25 µl of 10× PCR buffer, 1.25 µl of $MgCl_2$ (50 mM), 0.25 µl of each primer (0.01 mM), 0.5 µl of the dNTP mix (0.05 mM), 0.625 U of Taq (New England Biolabs), 0.5–2.0 µl of template DNA, and 18.75 µl of ultrapure H_2O (Ward et al. 2005; Ivanova et al. 2007). The reactions consisted of an initial cycle of 2 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 54.8 °C, and 60 s at 72 °C, with a final extension step of 10 min at 72 °C.

The COI RFLP profiles were obtained from reactions in a final volume of 20 µL containing 5–10 µL of the PCR product, 20 µL of the 10× buffer of the respective enzyme (as indicated by the manufacturer), 10 units of the enzyme (Table 1: New England Biolabs), and ultrapure water to complete the final volume. When necessary, 0.2 µL of 100× bovine serum albumin was added. The enzymes were chosen taking into account the molecular features of each enzyme. Enzymes comprising 4 nucleotides are treated as frequent-site types, and those with more than 4 nucleotides are considered to be rare-site types. Therefore, our approach aimed to examine inter-species variation using two categories of restriction enzyme, as in other fish species (Torres et al. 2013). The RFLP reactions were run over 90 min at the temperatures recommended by the manufacturer of each enzyme. The products of these reactions were visualized by electrophoresis in 1.5 % agarose gel immersed in TBE buffer and stained with GelGreen™ (Biotium) for subsequent photography. The molecular weight of the fragments was estimated using a 100-bp molecular ladder (New England Biolabs). Different reagent brands were tested, but distinct RFLP profiles were not detected.

Table 1 The enzymes used and their restriction sites (*)

Enzyme	Restriction site
AluI	AG * CT
BamHI	G * GATCC
EcoRI	G * AATTC
HaeIII	GG * CC
HhaI	GC * GC
MboI	GATC *

In all experiments the temperature used was 37 °C for 1 h

Bioinformatics were used (pDRAW32 v.1.1.121 software; www.acaclone.com) for control RFLP experiments based on voucher COI sequences from the Bold system [Barcode of life system; Bold codes *H. balao* MFSP09409 (voucher LBPV35107—Laboratório de Biologia de Peixes, Unesp, Botucatu, Brazil) and MFSP09407 (voucher LBPV35110—Laboratório de Biologia de Peixes, Unesp, Botucatu, Brazil); Bold codes *H. brasiliensis* BZLWE007-08 (voucher BZLW8007 from Belize, USNM—Smithsonian Institution) and CURA119-09 (voucher CURA8119 from Curaçao, USNM—Smithsonian Institution)]. The in silico strategy aimed to test for the in vitro COI profiles observed in the study species. The experiments were conducted using the same in vitro conditions and enzymes as described above.

The ISSR markers were obtained by testing 17 primers for the di- and tri-nucleotide repeat motifs shown previously to provide consistent results for the assessment of a number of different taxa, including fishes (Li et al. 2013; Moysés et al. 2010; Liu et al. 2009; Pazza et al. 2007). The seven most polymorphic primers (Table 2) were selected for the present study. The PCR reactions comprised 0.2 U of Taq (New

England Biolabs), 2.5 μl of 10× buffer, 0.5 μl of each primer (50 μM), 0.2 μM of dNTP mix, and 20 ng of DNA. The reactions consisted of an initial step of 94 °C for 3 min, 39 cycles of 40 s at 94 °C, 40 s at the optimal temperature for each primer (Table 2), and 2 min at 72 °C, with a final extension of 7 min at 72 °C. The PCR products were visualized by electrophoresis in 1.8 % agarose gel in TBE buffer and stained with GelGreen™ (Biotium), and then photographed.

The ISSR profiles were transformed into a binary matrix where the presence of a band was coded as 1 and its absence as 0. In order to avoid the use of presumed markers in one or another sample, only clear and well-defined bands were identified as markers. The matrix was analyzed using two grouping methods: Neighbor-Joining (NJ; Saitou and Nei 1987) and Maximum Parsimony (MP; Fitch 1971). Both analyses used *Hyporhamphus unifasciatus* as the outgroup. All analyses were run in PAUP* v.4.0b10 (Swofford 2002) and visualized in its PaupUp v.1.0.3.1 graphic interface (Calendini and Martin 2005). The MP analysis was based on heuristic searches, with the characters designated as "not-ordered" with equal weights. The maximum number of trees analyzed was 100,000 with 5,000 replications based on the random addition of terminals and the tree-bisection-reconnection (TBR) algorithm for branch swapping. The strict consensus tree was computed and the robustness of the resulting topologies (NJ and MP) was assessed through bootstrap and jackknife analyses with 1,000 pseudo-replicates.

Population parameters (AMOVA, Φ_{ST}, G_{ST}, and Nm) were calculated in Arlequin v.3.11 (Excoffier et al. 2005) and Popgene v.1.3.1 (Yeh et al. 1999).

Table 2 List of the ISSR primers used with their respective motifs and annealing temperatures

Primer	Motif (5′-3′)	Annealing temperature (°C)
ISSR1	$(AG)_8$ + T	50.4
ISSR2	$(AG)_8$ + C	52.4
ISSR3	$(GA)_8$ + T	50.4
ISSR7	$(AG)_8$ + YA	54
ISSR11	$(CT)_8$ + RA	50
ISSR14	$(GGAC)_3$ + A	51
ISSR15	$(GGAC)_3$ + T	51

Genetic diversity was determined by estimating the number of polymorphic loci. The genetic structuring of the populations was also assessed using a Bayesian approach in the Structure 2.3.3 software (Falush et al. 2003, 2007; Hubisz et al. 2009; Pritchard et al. 2000). In order to determine the number of populations (K) within the complete data set, ten independent simulations for K = 1–10 with 100,000 burn-in interactions were computed. The analysis was performed using both the admixture model and allele frequencies correlated among populations. The number of genetically distinct populations was estimated using the protocol described by Evanno et al. (2005).

Sequences of the cytochrome b gene (cyt b) were obtained from 15 individuals of each species, selected randomly from the available specimens. The PCR used the primer set of L14725 (forward—5′CGAAACTA ATGACTTGAAAAACCACCGTTG3′) (apud Santos et al. 2003) and MVZ16 (reverse—5′AAATAGGAA RTATCAYTCTGGTTTRAT3′) (Smith and Patton 1993), following Santos et al. (2006). The PCR products were purified using EXOSAP-IT (Affimetrix) enzymes, according to the protocol provided by the manufacturer. The cyt b fragments obtained from this process were sequenced bi-directionally using the BigDye Terminator v.3.1 Cycle Sequencing kit (www.appliedbiosystems.com), following the manufacturer's instructions, and the products were analyzed in an automated DNA sequencer (ABI 3500, Applied Biosystems).

Sequences were then edited by eye and aligned with BioEdit v.5.0.9 (Hall 1999), using the ClustalW multiple alignment tool, with gap opening at 15 and gap extension penalties at 0.3 (Hall 2001). Additional voucher cyt b sequences from GenBank [Accession numbers AF243872.1—AF243873.1 (*H. balao* voucher University of Florida; UF 99879; Tortugas Bank, Florida Keys, Monroe County); AF243864.1—AF243865.1 (*H. brasiliensis* voucher Cornell University; CU 75111; Long Key, Florida and Tortugas Bank, Florida Keys, Monroe County)] were included in the dataset in order to enrich the analyses. Asymmetric extremities of the sequences were trimmed following alignment to avoid unexpected results. The alignment was exported as a nexus file and the evolutionary model that best explained the variance among the sites was identified with jModelTest, based on the Akaike information criterion, AICc (Darriba et al. 2012).

The resulting alignment was analyzed by NJ and Bayesian Inference (BI) methods using PAUP* v.4.0b10 (Swofford, 2002) with its PaupUp v.1.0.3.1 graphic interface (Calendini and Martin 2005) and MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003), respectively. *Hiporamphus unifasciatus* (AF231665.1) was used as outgroup for both analyses. The NJ analysis was conducted using the model parameters obtained by jModelTest and the robustness of the branches was measured by 1,000 bootstrap and jack-knife pseudo-replicates. The BI analysis had 3,031,000 generations with a burn-in period of 750. The resulting majority rule consensus topology was obtained from a total of 29,561 trees showing the posterior probabilities of each yielded branch.

An analysis of molecular variance (AMOVA) was used to test possible deviations from a uniform genetic structure between species for both ISSR and cyt b data (Excoffier et al.1992) by using Arlequin 3.5.1.2 (Excoffier and Lischer 2010), with Φ_{ST} values also being calculated. For the cyt b sequences, haplotype (H) and nucleotide diversity (π), tests for population expansion (Tajima's D and Fu's Fs), mismatch distributions, genetic differentiation (G_{ST}), genetic flow (Nm), and the Chi-square test for genetic differentiation between species were calculated in DnaSP v.5.10.00 (Librado and Rozas 2009) considering each species as distinct populations. The haplotype network was defined in Network v.4.516 (available at www.fluxus-engineering.com) using the median-joining criterion.

Results

The analysis of the in vitro PCR–RFLPs from the COI barcode region obtained fragments of between 350 and ~700 base pairs (bps) in length (Table 3). However, the exact same COI profiles were recorded for all the specimens analyzed, regardless of the enzyme used (Fig. 2a–f). The in silico control analyses found no differences in the COI profiles in the voucher sequences of *H. balao* and *H. braziliensis* from São Paulo (Brazil) and Curaçao. The AluI enzyme also revealed similar COI profiles between *H. balao* and *H. braziliensis* from São Paulo and Curaçao, although they were different from the profiles observed in the in vitro experiments. In addition, a different COI profile was observed in the voucher sequence of *H. braziliensis* from Belize (Fig. 3a–d).

Table 3 The fragment lengths of the COI PCR-RFLPs obtained from the *H. brasiliensis* and *H. balao* specimens collected from the coast of Pernambuco, Northeastern Brazil

Enzyme	Approximate fragment size (base pairs)	
	H. brasiliensis	*H. balao*
AluI	350	350
BamHI	525	525
EcoRI	600	600
HaeIII	500	500
HhaI	700	700
MboI	400	400

A total of 103 ISSR bands were obtained, with fragment lengths varying from 250 to 3,000 bps. The analysis indicated that 99.03 % of the variation is found within the population as a whole, with only 88.34 % being attributed to *Hemiramphus balao*, and 80.58 % to *H. brasiliensis*. The NJ and MP topologies revealed no genetic or evolutionary differentiation between the putative species (Figs. 4, 5). The Bayesian analysis indicated the presence of two genetic populations (K = 2), although they both included members of the two taxa (Fig. 6). The results of the AMOVA indicated that only 9.52 % of the genetic variation is found between the two species, with the remaining 90.48 % occurring within species. A Φ_{ST} value of only 0.09521 was found between species (Table 4; $p < 0.01$). The genetic differentiation (G_{ST}) and gene flow (Nm) observed between species were 0.0456 and 8.4991, respectively.

A total of 544 comparable sites were found in the final alignment of the cyt b sequences, with a total of 34 variable sites and 10 distinct haplotypes (Table 5). Based on the Akaike information criterion, jModeltest selected the TVM+G model ($-\ln L = 1{,}603.3230$; frqA = 0.2616; freqC = 0.2869; freqG = 0.1624; freqT = 0.2891; gamma shape = 0.7690) as the best fit for the variation among sites. The results of the AMOVA indicated that only 6.43 % of the genetic variation is found between species, with the remaining 93.57 % arising within species. The observed Φ_{ST} between species was 0.06428 (Table 6; $p < 0.01$).

The results of the NJ and BI analyses were exactly the same, and neither topology was consistent with any phylogenetic differentiation between species. A close

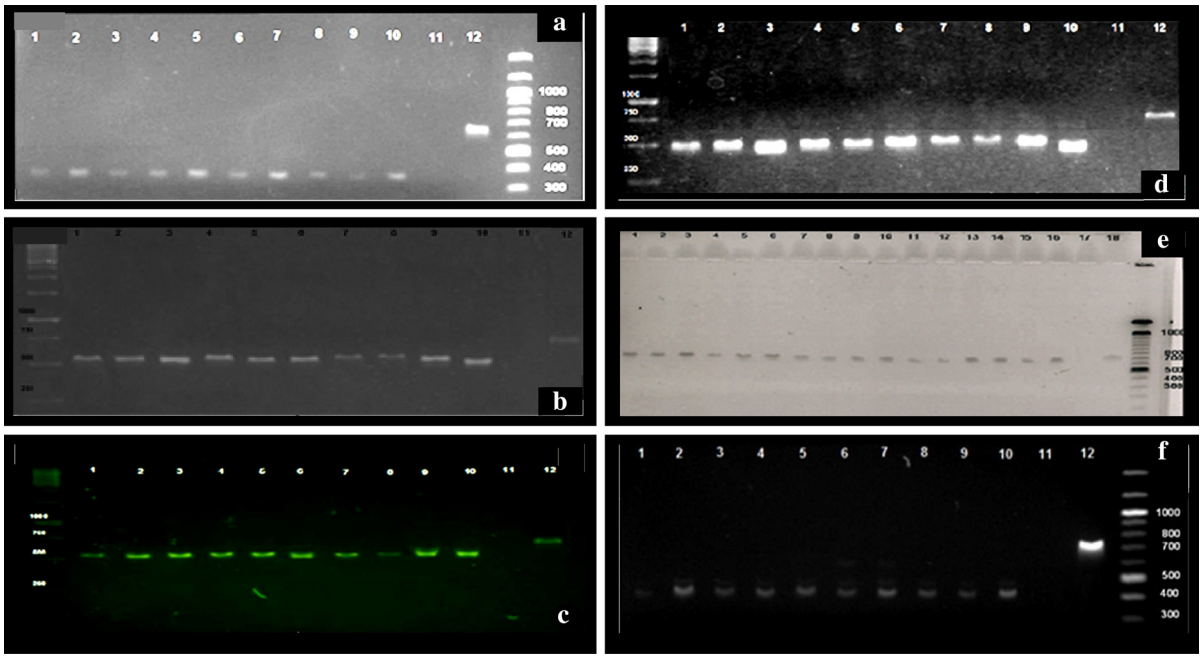

Fig. 2 Summary of the results obtained for the COI PCR-RFLPs. **a** AluI, **b** BamHI, **c** EcoRI, **d** HaeIII, **e** HhaI, and **f** MboI. *Numbers 1–5* = *H. brasiliensis* and *6–10* = *H. balao*. *Numbers 11* and *12* are, respectively, the negative control (all reagents except DNA) and an undigested COI fragment (positive control)

relationship was also found between the halfbeaks sampled in the present study and the voucher specimens of North American *H. brasiliensis*. The voucher cytb sequences of *H. balao* formed a distinct clade with strong branching support (Fig. 7). The genetic differentiation (G_{ST}) and gene flow (Nm) observed between the study species were 0.04196 and 5.71, respectively. The Chi-square test for genetic differentiation between species was not significant ($p = 0.1961$).

The haplotype network (Fig. 8a) revealed two haplogroups differentiated by 23 mutations. These mutations represented a total of three percent of divergence between the two haplogroups. One group is comprised of haplotypes from both species, one of which (H2) was the most common in both populations. The second group (H1 and H2) consisted exclusively of *H. balao* haplotypes. The demographic history delineated by the mismatch distribution indicated a bimodal pattern of population growth (Fig. 8b).

Discussion

In conservation science, the reliable identification of focal species is essential for the development of effective management proposals, given that each species or evolutionary significant unit will be adapted to specific environmental conditions (Frankham et al. 2004). Many fish taxa are poorly defined due to the limitations of morphological criteria for the identification of closely-related species, especially in juvenile specimens (Lleonart et al. 2006). The halfbeaks studied here are a good example of this problem, given that the morphological and anatomical features used to distinguish *H. balao* from *H. brasiliensis* (Collette 2003) are not only extremely subtle, but can only be observed in freshly-caught specimens. A reliable diagnosis is thus extremely difficult, and in many cases, the species is misidentified (Berkeley 1975; Ibama 2005). However the multilocus approach provided in this study clearly resolved this impediment, as shown below.

The results obtained in the present study by in vitro COI (barcode region) PCR-RFLPs indicate that only one genetic stock—that is, a single taxon—of halfbeaks is found in the Brazilian state of Pernambuco. This conclusion is supported by the complete absence of distinct COI profiles—which would be expected for different species (Carvalho et al. 2011; Hosseinali et al. 2011)—despite the use of six different enzymes.

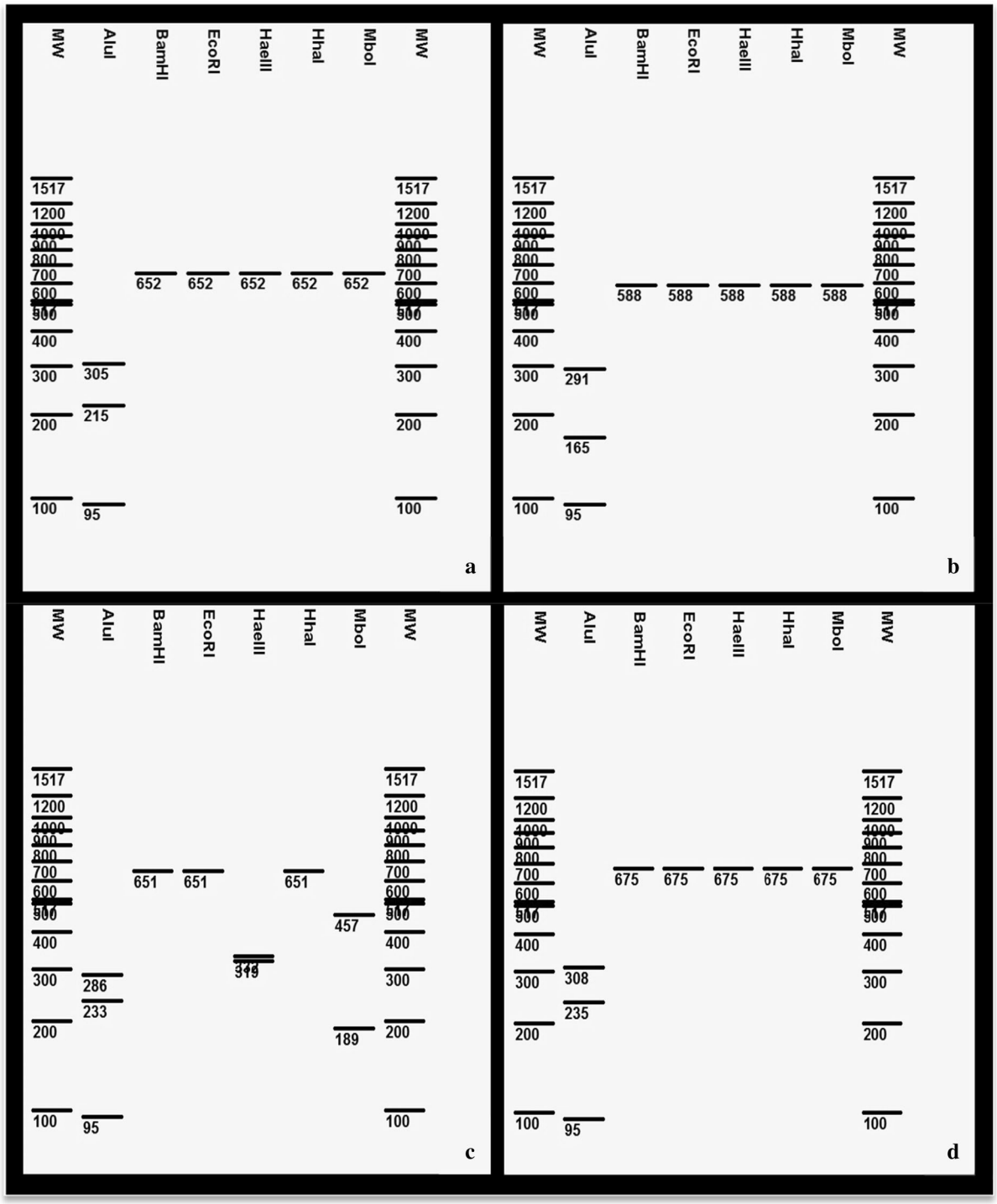

Fig. 3 In silico PCR-RFLPs results: **a** vouchered COI sequence—*H. balao*1 from the coast of the state of São Paulo, **b** vouchered COI sequence—*H. balao*2 from the coast of the state of São Paulo, **c** vouchered COI sequence—*H. brasiliensis* from Belize, and **d** vouchered COI sequence—*H. brasiliensis* from Curaçao. First and last columns = New England Biolabs 100 bp ladder. The remaining columns show the RFLP profiles obtained by each enzyme

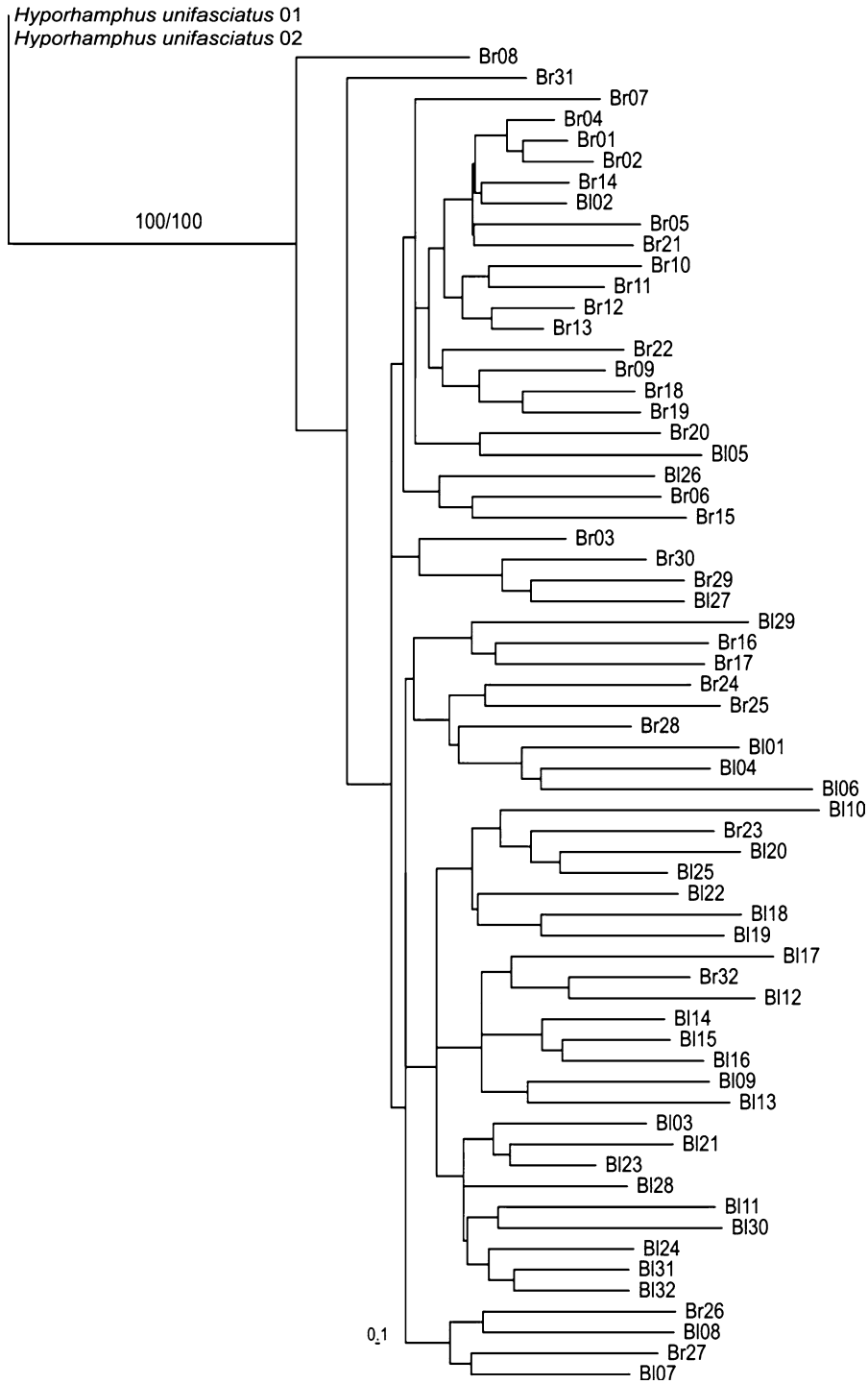

Fig. 4 The NJ topology based on the ISSR markers. *Numbers* above the branches are the bootstrap/jackknife support values. Br = *H. brasiliensis* and Bl = *H. balao*

Recent studies of the COI barcode region have revealed its potential not only for the validation of species, but also the characterization of population structuring within species (Keith and Hedin 2012; Carvalho et al. 2013a, b; Torres et al. 2013). However the evidence found in the present study indicated clearly that a single morphology-based polymorphic species is found off the coast of Pernambuco in

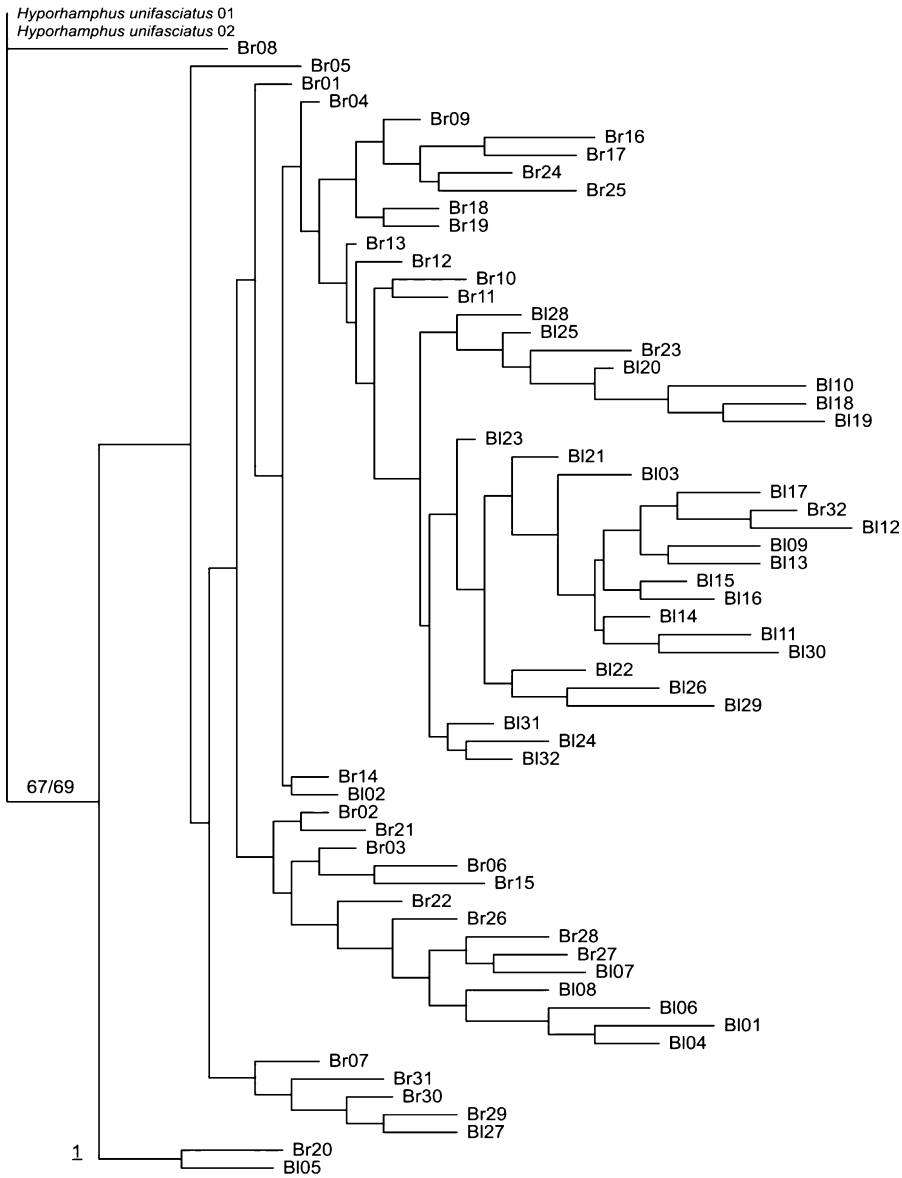

Fig. 5 The MP topology based on the ISSR markers. *Numbers* above the branches are the bootstrap/jackknife support values. Br = *H. brasiliensis* and Bl = *H. balao*

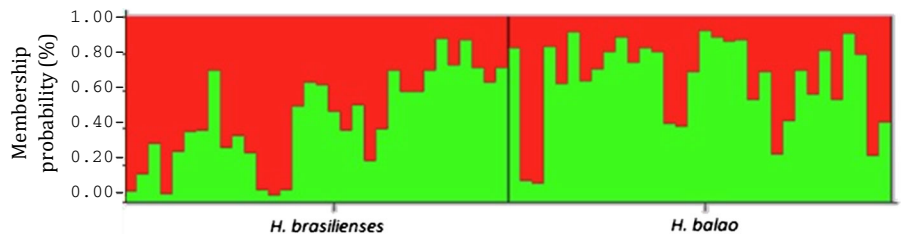

Fig. 6 Estimated Bayesian species assignments (*Structure*). The x axis represents the likelihood (*Q values*) of the genetic profile of each specimen (*vertical bars*). *Green* and *red* coloration represent the two (k = 2) genetic pools identified in the analysis. Note the genetic admixture between species

Table 4 Results of the AMOVA for the ISSR markers analyzed with the specimens of *H. brasiliensis* and *H. balao* being treated as distinct populations

Source of variation	Degrees of freedom	Sum of squares	Components of variance	% of variance
Among populations	1	49.016	1.18101 va	9.52
Within populations	62	695.844	11.22329 vb	90.48
Total	63	744.859	12.4043	100
Φ_{ST}	0.09521			

($p < 0.01$)

Table 5 Overall cyt b sequence statistical parameters for the *H. brasiliensis* and *H. balao* specimens analyzed: n = sample size, h = number of haplotypes, hd = haplotype diversity, k = mean number of nucleotide differences, and π = nucleotide diversity

Species	n	h	hd	k	π	Tajima's D	Fu and Li's F
H. brasiliensis	15	3	0.3619	0.95238	0.00128	−0.99157 ^{ns}	−1.14464^{ns}
H. balao	15	8	0.7908	9.38095	0.01263	−1.07923 ^{ns}	0.27005^{ns}
H. brasiliensis + *H. balao*	30	10	0.6023	5.34023	0.00719	−1.83318*	−0.45219^{ns}

Ns not significant

($p = 0.1/p > 0.1$); * $p < 0.05$

Table 6 Results of the AMOVA for the cyt b sequences obtained from the specimens analyzed, with *H. brasiliensis* and *H. balao* being treated as distinct populations

Source of variation	Df	Sum of squares	Components of variance	% of variance
Among populations	1	4.767	0.16127 va	9.52
Within populations	28	65.733	2.34763 vb	90.48
Total	30	70.5	2.50889	100
Φ_{ST}	0.06428			

Df degrees of freedom

($p < 0.01$)

northeastern Brazil. The results also reinforce the use of the PCR-RFLP approach as a rapid and cost-effective complementary molecular technique for taxonomic studies.

The in silico COI RFLP experiments reinforced the results obtained in the in vitro experiments, that is, a lack of differentiation in the *Hemiramphus* spp. specimens from São Paulo (Brazil) and Curaçao in relation to the specimens analyzed here. The different AluI profile observed here is potentially consistent with intraspecific-level variation, given the characteristic of this enzyme as a producer of very frequent site-based restrictions. Some very similar examples of intraspecific RFLP variation detected by COI are found in the literature on fishes and other groups of animals (Mariguela et al. 2011; Sinclair et al. 2011; Ribeiro et al. 2012; Keskin and Atar 2012; Keskin et al. 2013; Barry et al. 2013).

A distinct COI profile was observed in the voucher sequence from Belize (Caribbean Sea). The Greater Caribbean province has been identified as a marine biodiversity hotspot due to its high biological diversity (Floeter et al. 2008). A number of studies have also demonstrated profound genetic divisions between populations from the Caribbean and rest of the tropical Atlantic province (Vianna et al. 2006; Caballero et al. 2013) including those of fish species (Nirchio and Cequea 1998; Rocha et al. 2002; Nirchio et al. 2005;

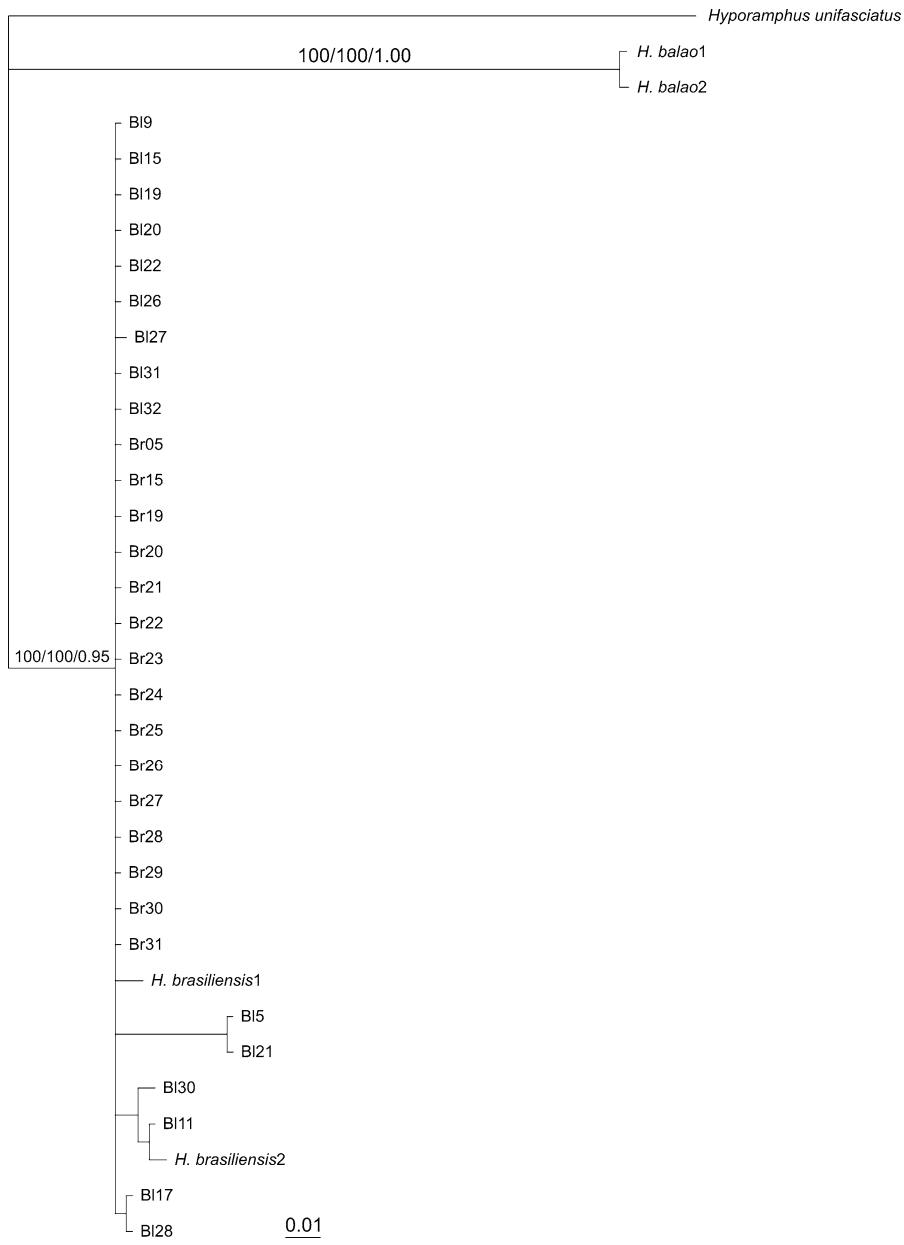

Fig. 7 Bayesian topology based on the cyt b sequences obtained in the present study. *Numbers* above the branches are the bootstrap/jackknife values and posterior probabilities. Br = *H. brasiliensis* and Bl = *H. balao* from Pernambuco, *H. brasiliensis* 1 and 2 and *H. balao* 1 and 2 are from Florida

Fraga et al. 2007), and the distinct COI profiles detected in the *H. brasiliensis* sequences from Belize reaffirm this pattern. These findings support the need for further genetic and phylogeographic research in the region, in particular to evaluate the potential role of the Caribbean as a reservoir of cryptic genetic lineages.

Nuclear markers such as ISSRs have been frequently used in studies of evolutionary history at the population and species levels (Casu et al. 2009; Moysés et al. 2010; Kumla et al. 2012; Li et al. 2013). In the present case, the data indicated high mean genetic variation (84 %), which contrasts considerably with the situation found in other fish species—both freshwater and marine—subject to intense harvesting (Tatarenkov et al. 2011; Pinsky and Palumbi 2014). Fishery records from Pernambuco indicated that halfbeaks have been exploited intensively over the past 45 years. In one coastal region alone, around 15 tons of halfbeak were landed annually in the mid-

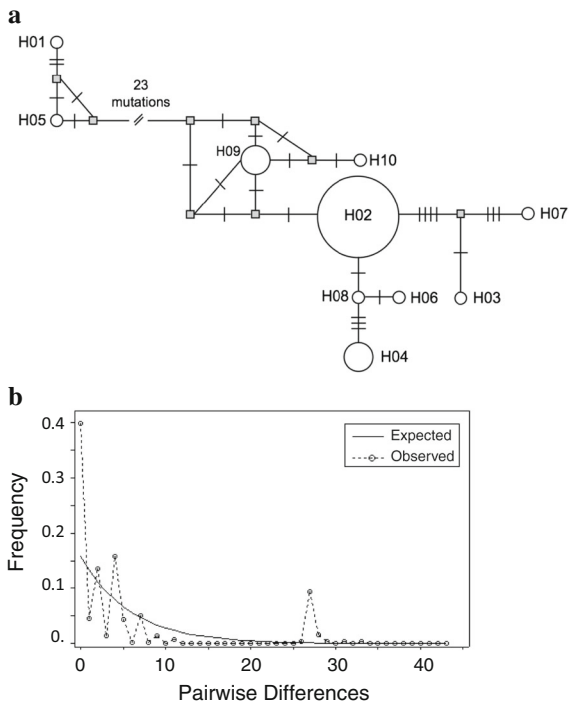

Fig. 8 **a** The haplotype network (median-joining) obtained for the cyt b lineages of *H. brasiliensis* and *H. balao* obtained in the present study. The *circles* labeled H1–H10 represent the ten haplotypes identified by the analyses. *The size of circles* represents the frequency of the haplotypes. The *perpendicular lines* on the branches represent the number of mutations between the haplotypes, and the *gray boxes* represent lost or unsampled haplotypes. **b** The mismatch distribution obtained for the cyt b sequences of *H. brasiliensis* and *H. balao* from Pernambuco, Brazil

1960s (Santos 1967). As a single specimen weighs approximately 100 g on average this represents the harvesting of more than 400 fishes per day. Despite this intensive exploitation of stocks, the genetic variability recorded in the present study indicates that the halfbeaks are still potentially well prepared to deal with changes in environmental conditions. This is further emphasized by the phenotypic plasticity of the species, which reflects marked genetic variability, rather than diagnostic differences between species, given that species with little genetic variation tend to have homogeneous phenotypes (Frankham et al. 2004).

The ISSR data demonstrated very similar genetic profiles, indicating a high degree of genomic cohesiveness in both species. The NJ topology failed to differentiate *H. balao* from *H. brasilienses*, reinforcing the lack of any genetic distinction between the populations of these two species in Pernambuco, and suggesting intense gene flow between both morphospecies. These conclusions were further confirmed by the MP topology, and the analysis of the ISSRs reconfirmed the lack of any species-level differentiation, as indicated by the DNA barcode data. In summary, there is no nuclear DNA evidence of the presence of distinct halfbeak species (ballyhoo and balao) off the coast of Pernambuco.

Wright's (1978) F_{ST} (Φ_{ST}) statistic is inversely related to gene flow, and it indicates that the within-species genetic variance might reflect population-level subdivisions. The value was low (0.09521; $p < 0.01$) and the majority of the variance (90.48 %) was found within populations. These results failed to confirm the genetic structuring expected for two well-defined species. According to Wright (1978) and Hartl and Clark (2010), F_{ST} (Φ_{ST}) values of between 0.05 and 0.15 indicate weak structuring, further reinforcing the conclusion that only a single species of *Hemiramphus* is found off the coast of Pernambuco.

The Bayesian structuring analysis indicated the presence of $K = 2$ well-admixed *Hemiramphus* populations, favoring the method's null hypothesis (Evanno et al. 2005) and reinforcing the lack of genetic or evolutionary distinctiveness. This approach is able to detect admixed genomes, such as those of *Psedoplatystoma* analyzed by Carvalho et al. (2013a, b), who showed that the valid species have quite distinct genomes from that of their hybrids. This situation is quite different from the one observed in the *Hemiraphus* genomes in the present study. These conclusions are further supported by the values of G_{ST} (0.0556) and Nm (8.4991), which indicate strong cohesion between halfbeak populations in Pernambuco [details on the interpretation of the G_{ST} and Nm values can be found in Wright 1978, Hartl and Clark 2010, Hedrick 1999, and Mills and Allendorf 1996].

The cytochrome b gene is one of the most amply studied mitochondrial regions and it has been used to assess species-level phylogenies in many organisms, especially fishes (Meyer 1994; Farias et al. 2001; Craig et al. 2009; Kullander et al. 2010; Boguski et al. 2012; DiBattista et al. 2012; Byrne et al. 2013). When treated as separate populations, both species presented high levels of haplotype (Hd) and nucleotide (π) diversity compared with other overexploited or protected marine fishes (Santos et al. 2006; Craig et al. 2009; Daly-Engel et al. 2012; Byrne et al. 2013).

These findings reinforce the favorable genetic potential of the halfbeak stock from northeastern Brazil and suggest a satisfactory to good conservation status of this fishery resource, in spite of the intense exploitation.

The NJ and BI topologies (Fig. 6) further reinforced the results of the COI RFLP experiments, and indicated no phylogenetic differentiation between the Pernambuco halfbeaks. The *H. balao* vouchered specimens from Florida (USA) formed a clearly distinct lineage, although the voucher *H. brasiliensis* from Florida grouped together with the specimens analyzed in the present study, indicating that this is the species found in Pernambuco.

The haplotype network indicated two haplogroups with 3 % of differentiation. Studies of other congeneric fish species have found differentiation in cyt b sequences at least two orders of magnitude higher than this (Craig et al. 2009; DiBattista et al. 2013; Byrne et al. 2013). The sum of the evidence from the present study thus fails to show that two halfbeak species are found in the study area, as previously supposed, but rather confirm that only a single species—*H. brasiliensis*—occurrs off the coast of Pernambuco.

The data provided in this study provide a new perspective for the conservation of the ballyhoo halfbeak stocks of the study region. Despite the good conservation status of these stocks, in terms of their genetic variation, further exploitation of this fishery resource requires careful consideration. While the intense exploitation of two distinct taxa may have minor depletive effects on their stocks, the results of the present study indicated that a single species is exploited, demanding caution in the use of genetic variation as a management parameter. Overlooking the genetic variation observed in the present study may accentuate the decline in stocks, and render harvesting unsustainable in the near future. Thus, management measures that guarantee the maintenance of the current degrees of genetic variation would be preferable in a scenario of the intense exploitation of existing stocks.

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References

Almany GR, Hamilton RJ, Bode M, Matawai M, Potuku T, Saenz-Agudelo P, Planes S, Berumen ML, Rhodes KL, Thorrold SR, Russ GR, Jones GP (2013) Dispersal of grouper larvae drives local resource sharing in a coral reef fishery. Curr Biol 23(7):626–630

Avise JC (2000) Phylogeography: The History and Formation of Species. Harvard University Press

Avise JC (2004) Molecular markers, natural history and evolution. Sinauer Associates, Sunderland

Barry PD, Tamone SL, Tallmon DA (2013) A complex pattern of population structure in the North Pacific giant octopus *Enteroctopus dofleini* (Wülker, 1910). J Mollus Stud 79(2):133–138

Berkeley SA (1975) Fishery and biology of balyhoo on the southeast Florida coast. University of Miami, Sea Grant Program, p 15

Boguski DA, Reid SB, Goodman DH, Docker MF (2012) Genetic diversity, endemism and phylogeny of lampreys within the genus *Lampetra* sensu stricto (Petromyzontiformes: Petromyzontidae) in western North America. J Fish Biol 81:1891–1914

Bowen BW, Meylan AB, Ross JP, Limpus CJ, Balazs GH, Avise JC (1992) Global population structure and natural history of the green turtle (*Chelonia mydas*) in terms of matriarchal phylogeny. Evolution 46(4):865–881

Bridge PD, Roberts PJ, Spooner BM, Panchal G (2003) On the unreliability of published DNA sequences. New Phytol 160:43–48

Byrne RJ, Bernardi G, Avise JC (2013) Spatiotemporal genetic structure in a protected marine fish, the california grunion (*Leuresthes tenuis*), and Relatedness in the genus *Leuresthes*. J Hered 104(4):521–531

Caballero S, Santos MCO, Sanches A, Mignucci-Giannoni AA (2013) Initial description of the phylogeography, population structure and genetic diversity of Atlantic spotted dolphins from Brazil and the Caribbean, inferred from analyses of mitochondrial and nuclear DNA. Biochem Syst Ecol 48:263–270

Calendini F, Martin JF (2005). PaupUP v1.0.3.1 A free graphical frontend for Paup* Dos software

Caragiulo A, Dias-Freedman I, Clark JA, Rabinowitz S, Amato G (2014) Mitochondrial DNA sequencevariation and phylogeography of Neotropic pumas (*Puma concolor*). Mitoch DNA 25(4):304–312

Carvalho DC, Oliveira D, Pompeu PS, Leal CG, Oliveira C, Hanner R (2011) Deep barcode divergence in Brazilian freshwater fishes: the case of the São Francisco River basin. Mitoch DNA 22(S1):80–86

Carvalho DC, Oliveira DAA, Beheregaray L, Torres RA (2013a) Hidden genetic diversity and distinct evolutionarily significant units in an commercially important Neotropical apex predator, the catfish *Pseudoplatystoma corruscans*. Conserv Genet 13(6):1671–1675

Carvalho DC, Seerig AS, Brasil BSAF, Crepaldi DV, Oliveira DAA (2013b) Molecular identification of the hybrid between the catfish species *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum* using a set of eight microsatellite markers. J Fish Biol 83(3):671–676

Casu M, Lai T, Curini-Galletti M, Ruiu A, Pais A (2009) Indentification of mediterranean *Diplodus* spp. and *Dentex dentex* (Sparidae) by means of DNA Inter-Simpre Sequence Repeat (ISSR) markers. J Exp Mar Biol Ecol 368(2):147–152

Collette BB (2003) Hemiramphidae. In: KE Carpenter (ed) FAO species identification guide for fishery purposes. The living marine resources of the Western Central Atlantic. Vol 2: Bony fishes part 1 (Acipenseridae to Grammatidae). Ref No [50879] Key No [966] pp 1135–1144

Craig MT, Graham RT, Torres RA, Hyde JR, Freitas MO, Ferreira BP, Hostim-Silva M, Gerhardinger LC, Bertoncini AA, Robertson DR (2009) How many species of goliath grouper are there? Cryptic genetic divergence in a threatened marine fish and the resurrection of a geopolitical species. Endang Spec Res 7:167–174

Daly-Engel TS, Randall JE, Bowen BW (2012) Is the great barracuda (*Sphyraena barracuda*) a reef fish or a pelagic fish? The phylogeographic perpective. Mar Biol 159(5): 975–985

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nat Methods 9(8):772

DiBattista JD, Berumen ML, Gaither MR, Rocha LA, Eble JA, Choat JH, Craih MT, Skilings DJ, Bowen BW (2013) After continents divide: comparative phylogeography of reef fishes from the Red Sea and Indian Ocean. J Biogeogr 40(6):1170–1181

DiBattista JD, Waldrop E, Bowen BW, Schultz JK, Gaither MR, Pyle RL, Rocha LA (2012) Twisted sister species of pygmy angelfishes: discordance between taxonomy, coloration, and phylogenetics. Coral Reefs 31:839–851

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure : a simulation study. Mol Ecol 14:2611–2620

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 10:564–567

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distance among DNA haplotypes: application to human mitochondrial DNA data. Genetics 131:479–491

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164(4):1567–1587

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Not 4(7):574–578

Farias I, Ortí G, Sampaio I, Schneider H, Meyer A (2001) The cytochrome b gene as a phylogenetic marker: the limits of resolution for analyzing relationships among cichlid fishes. J Mol Evol 53:89–103

Floeter SR, Rocha LA, Robertson DR, Joyeux JC, Smith-Vaniz WF, Wirtz P, Edwards AJ, Barreiros JP, Ferreira CEL, Gasparini JL, Brito A, Falcón JM, Bowen BW, Bernardi G (2008) Atlantic reef fish biogeography and evolution. J Biogeogr 35:22–47

Fraga E, Schneider H, Nirchio M, Santa-Brigida E, Rodrigues-Filho LF, Sampaio I (2007) Molecular phylogenetic analyses of mullets (Mugilidae, Mugiliformes) based on two mitochondrial genes. J Appl Ichthyol 23:598–604

Frankham R, Ballou J, Briscoe D, Mcinnes K (2004) A primer of conservation genetics. Cambridge University Press, Cambridge

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Hall BG (2001) Phylogenetics trees made easy. A how to manual for molecular biologists. Sinauer Associates. Inc, Sunderland

Hartl DL, Clark AG (2010) Princípios de Genética de Populações, 4th edn. ArtMed, Porto Alegre-RS

Hazkani-Covo E, Zeller RM, Martin W (2010) Molecular poltergeists: mitochondrial DNA copies (*numts*) in sequenced nuclear genomes. PLoS Genet 6(2):e1000834. doi:10.1371/journal.pgen.1000834

Hedrick PW (1999) Highly variable loci and their interpretation in evolution and conservation. Evolution 53(2):313–318

Hosseinali A, Homayoun HS, Aria AA, Shahrokh S (2011) Cytochrome c oxidase subunit 1 barcode data of fish of the Nayband National Park in the Persian Gulf and analysis using meta-data flag several cryptic species. Mol Ecol Res 11(3):461–472

Hubisz MJ, Falush D, Stephens M, Pritchard JK (2009) Inferring weak population structure with the assistance of sample group information. Mol Ecol Res 9:1322–1332

Huelsenbeck JP, Ronquist F (2001) MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17(8):754–755

Ibama (2005) Boletim Estatístico da Pesca marítima e Estuarina do Nordeste do Brasil—2004. Centro de Pesquisas e Gestão dos Recursos Pesqueiros do Litoral Nordeste. Tamandaré—PE

Ivanova NI, Zemlak TS, Hanner RH, Hebert PDN (2007) Universal primer cocktails for fish DNA barcoding. Mol Ecol Not 7(4):544–548

Keith R, Hedin M (2012) Extreme mitochondrial population subdivision in southern Appalachian paleoendemic spiders (Araneae: Hypochilidae: *Hypochilus*), with implications for species delimitation. J Arachnol 40(2):167–181

Keskin E, Atar HH (2012) Genetic structuring of European anchovy (*Engraulis encrasicolus*) populations through mitochondrial DNA sequences. Mitochon DNA 23(2):62–69

Keskin E, Ağdamar S, Tarkan AS (2013) DNA barcoding common non-native freshwater fish species in Turkey: low genetic diversity but high population structuring. Mitochon DNA 24(3):276–287

Kullander SO, Norén M, Friðriksson GB, Santos de Lucena CA (2010) Phylogenetic relationships of species of *Crenicichla* (Teleostei: Cichlidae) from southern South America based

on the mitochondrial cytochrome *b* gene. J Zool Syst Evol Res 48:248–258

Kumla S, Doolgindachbaporn S, Sudmoon R, Sattayasai N (2012) Genetic variation, population structure and identification of yellow catfish, *Mystus nemurus* (CandV) in Thailand using RAPD, ISSR and SCAR marker. Mol Biol Rep 39:5201–5210

Lessa R, Vieira ACS, Monteiro A, Santos JS, Lima MM, Cunha EJ, Souza-Jr JCA, Bezerra S, Travassos PEPF, Oliveira BABR (2006) Diagnóstico da pesca no litoral do estado de Pernambuco. In: Isaac VJ, Martins AS, Haimovici M, Andriguetto-Filho JM (eds) A pesca marinha e estuarina do Brasil no início do século XXI: recursos, tecnologias, aspectos socioeconômicos e institucionais. Editora Universitária UFPA, Bélem, pp 67–91

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452

Liu Y-G, Yu Z-G, Bao B-L, Sun X-Q, Shi Q-L, Liu L-X (2009) Population genetics studies of half-smooth tongue sole *Cynoglossus semilaevis* using ISSR markers. Biochem Syst Ecol 36:821–827

Li W, Sun W-X, Fan J, Zhang C-C (2013) Genetic diversity of wild and cultured swamp eel (*Monopterus albus*) populations from central China revealed by ISSR markers. Biol 68(4):727–732

Lleonart J, Taconet M, Lamboeuf M (2006) Integrating information on marine species identification for fishery purposes. Mar Ecol Prog Ser 316:231–238

Lovejoy NR (2000) Reinterpreting recapitulation: systematics of needlefishes and their allies (Teleostei: Beloniformes). Evolution 54:1349–1362

Mariguela TC, Paiva LRS, Foresti F, Oliveira C (2011) 5S rDNA chromosomal mapping and COI sequence analysis reveal differentiation among distinct populations of a characid fish *Serrapinnus notomelas*. Rev Fish Biol Fish 21(4):779–788

Mcbride RS, Thurman PE (2003) Reproductive biology of *Hemiramphus brasiliensis* and *H. balao* (Hemiramphidae): maturation, spawning, frequency, and fecundity. Biol Bull 204:57–67

Mendonça FF, Oliveira C, Gadig OBF, Foresti F (2013) Diversity and genetic population structure of the Brazilian sharpnose shark *Rhizoprionodon lalandii*. Aquat Conserv: Mar Freshw Ecosyst 23(6):850–857

Meyer A (1994) Shortcomings of the cytochrome b gene as a molecular marker. Trends Ecol Evol 9(8):278–280

Mills LS, Allendorf FW (1996) The one-migrant-per-generation rule in conservation and management. Conserv Biol 10(6):1509–1518

Mitcheson YS, Craig MT, Bertoncini AA, Carpenter KE, Cheung WWL, Choat JH, Cornish AS, Fennessy ST, Ferreira BP, Heemstra PC, Liu M, Myers RF, Pollard DA, Rhodes KL, Rocha LA, Russell BC, Samoilys MA, Sanciangco J (2013) Fishing groupers towards extinction: a global assessment of threats and extinction risks in a billion dollar fishery. Fish Fisher 14(2):119–136

Moysés CB, Daniel-Silva MFZ, Lopes CE, Almeida-Toledo LF (2010) Cytotype-specific ISSR profiles and karyotypes in the Neotropical genus *Eigenmannia* (Teleostei: Gymnotiformes). Genetica 138:179–189

Nirchio M, Cequea H (1998) Karyology of *Mugil liza* and *M. curema* from Venezuela. Bol Investig Mar Cost 27:45–50

Nirchio M, Cipriano R, Cestari M, Fenocchio AS (2005) Cytogenetical and morphological features reveal significant differences among Venezuelan and Brazilian samples of *Mugil curema* (Teleostei: Mugilidae). Neotrop Ichthyol 3(1):107–110

Packer L, Gibbs J, Sheffield C, Hanner R (2009) DNA barcoding and the mediocrity of morphology. Mol Ecol Res 9(Suppl 1):42–50

Padial JM, Miralles A, De la Riva I, Vences M (2010) The integrative future of taxonomy. Front Zool 7:16

Pazza R, Kavalco KF, Prioli SMAP, Prioli AJ, Bertollo AC (2007) Chromosome polymorphism in Astyanax fasciatus(Teleostei, Characidae), Part 3: analysis of the RAPD and ISSR molecular markers. Biochem Syst Ecol 35:843–851

Pinsky ML, Palumbi SR (2014) Meta-analysis reveals lower genetic diversity in overfished populations. Mol Ecol 23:29–39

Portnoy DS, Hollenbeck CM, Renshaw MA, Cummings NJ, Gold JR (2013) Does mating behaviour affect connectivity in marine fishes? Comparative population genetics of two protogynous groupers (Family Serranidae). Mol Ecol 22:301–313

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Prodocimo V, Tscha MK, Pie MR, Oliveira-Neto JF, Ostrensky A, Boeger WA (2008) Lack of genetic differentiation in the fat snook *Centropomus parallelus* (Teleostei: Centropomidae) along the Brazilian coast. J Fish Biol 73:2075–2082

Ribeiro AO, Caires RA, Mariguela TC, Pereira LHG, Hanner R, Oliveira C (2012) DNA barcodes identify marine fishes of São Paulo State, Brazil. Mol Ecol Res 12:1012–1020

Roberts MA, Schwartz TS, Karl SA (2004) Global population genetic structure and male-mediated gene glow in the green sea turtle (*Chelonia mydas*): analysis of microsatellite loci. Genetics 166:1857–1870

Rocha LA, Bass AL, Robertson DR, Bowen BW (2002) Adult habitat preferences; larval dispersal; and the comparative phylogeography of three Atlantic surgeonfishes (Teleostei: Acanthuridae). Mol Ecol 11:243–252

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12):1572–1574

Saitou N, Nei M (1987) The Neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406–425

Sambrook J, Russell DW (2001) Molecular cloning. A Laboratory Manual. I, II, III, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Santos SM (1967) Contribuição ao estudo da agulha-preta (*Hemiramphus brasiliensis*) (Pisces, Beloniformes, Hamiramphidae). Trab Oceanográf 9(11):285–304

Santos S, Schneider H, Sampaio I (2003) Genetic differentiation of *Macrodon ancylodon* (Sciaenidae, Perciformes) populations in Atlantic coastal waters of South America as revealed by mtDNA analysis. Genet Mol Biol 26(2): 151–161

Santos S, Hrbek T, Farias IP, Schneider H, Sampaio I (2006) Population genetic structuring of the king weakfish,

Macrodon ancylodon (Sciaenidae), in Atlantic coastal waters of South America: deep genetic divergence without morphological change. Mol Ecol 15:4361–4373

Seyoum S, Tringali MD, Barthel BL, Puchulutegui C, Davis MC, Collins AB, Craig MT (2013) Isolation and characterization of 29 polymorphic microsatellite markers for the endangered Atlantic goliath grouper (*Epinephelus itajara*), and the Pacific goliath grouper (*E. quinquefasciatus*). Conserv Genet Res 5:729–732

Silva-Oliveira GC, Silva ABC, Oliveira Y, Nunes ZP, Torres RA, Sampaio I, Vallinoto M (2013) New nuclear primers for molecular studies of Epinephelidae fishes. Conserv Genet Res 5(1):165–168

Sinclair W, Newman SJ, Vianna GMS, Williams S, Aspden WJ (2011) Spatial subdivision and genetic diversity in populations on the east and west coasts of Australia: the multifaceted case of *Nautilus pompilius* (Mollusca, Cephalopoda). Rev Fisher Sci 19(1):52–61

Smith MF, Patton JL (1993) The diversification of South American murid rodents: evidence from mitochondrial DNA sequence data for the Akodontine tribe. Biol J Linn Soc 50:149–177

Sodré D, Rodrigues-Filho LFS, Souza RFC, Rêgo PS, Schneider H, Sampaio I, Vallinoto M (2012) Inclusion of South American samples reveals new population structuringof the blacktip shark (*Carcharhinus limbatus*) in the western Atlantic. Genet Mol Biol 35(4):752–760

Song H, Buhay JE, Whiting MF, Crandall KA (2008) Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proc Natl Acad Sci 115(36):13486–13491

Swofford DL (2002) PAUP*. Phylogenetic analysis using parsimony (*And Other Methods). V.4.0b10. Sunderland, Massachucets. Sinauer

Tatarenkov A, Lima SMQ, Avise JC (2011) Extreme homogeneity and low genetic diversity in *Kryptolebias ocellatus* from south-eastern Brazil suggest a recent foundation for this androdioecious fish population. J Fish Biol 79:2095–2105

Torres RA, Feitosa RB, Carvalho DC, Freitas MO, Hostim-Silva M, Ferreira BP (2013) DNA barcoding approaches for fishing authentication of exploited grouper species including the endangered and legally protected goliath grouper *Epinephelus itajara*. Scient Mar 77:409–418

Vianna JA, Bonde RK, Caballero S, Giraldo JP, Lima RP, Clark A, Marmontel M, Morales-Vela B, Souza MJ, Parr L, Rodríguez-Lopez MA, Mignucci-Giannoni AA, Powell JA, Santos FR (2006) Phylogeography, phylogeny and hybridization in trichechid sirenians: implications for manatee conservation. Mol Ecol 15:433–447

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN (2005) DNA barcoding Australia's fish species. Phil Trans R Soc B 360:1847–1857

Wright S (1978) Evolution and the genetics of populations, vol 4. University of Chicago Press, Chicago, USA

Yeh FCT, Boyle ZYE, Xiyan JM (1999) PopGene. Version 1.31: Microsoft Windows-based freeware for population genetic analysis. University of Alberta and Center for International Forestry Research