



The Past and Present of an Estuarine-Resident Fish, the “Four-Eyed Fish” *Anableps anableps* (Cyprinodontiformes, Anablepidae), Revealed by mtDNA Sequences

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Abstract

Historical events, such as changes in sea level during the Pleistocene glacial cycles, had a strong impact on coastal habitats, limiting connectivity and promoting the genetic divergence of various species. In this study, we evaluated the influence of climate oscillations and the possibility of estuary function as a barrier to gene flow among populations of the four-eyed fish, *Anableps anableps*. This species is fully estuarine-resident, has internal fertilization, is viviparous and does not migrate across long distances. These features make the four-eyed fish an excellent model for the study of evolutionary processes related to genetic differentiation of species and populations in estuaries. The evolutionary history of *A. anableps* was inferred from phylogeographic and population analyses using sequences of the mitochondrial DNA Control Region of 13 populations distributed in the Amazon and Northeast Coast of Brazil from Calcoene (Amapa) to Parnaíba (Piauí). The 83 retrieved haplotypes show a pattern of four distinct mitochondrial lineages, with up to 3.4% nucleotide divergence among them. The evolutionary reconstruction suggests that these lineages diverged recently in the late Pleistocene/early Holocene after the Atlantic Ocean reaching current levels. Analysis of variability, neutrality and the genetic expansion pattern revealed that the lineages have distinct characteristics, which were shaped by the different geomorphological features of coastal regions combined with sea level oscillations over a very long period of time. Only few neighboring populations show a discreet gene flow. This study may also be helpful for designing new experiments to better understand the geomorphological evolutionary history of the estuaries of the Amazon and the Northeast Coast of Brazil using estuarine-resident species as a model.

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Introduction

Organisms that inhabit coastal waters such as estuaries or lagoons may show more genetic differentiation than strictly marine species due to the discontinuity of these natural ecosystems and the relative isolation of the great ocean currents [1]. Estuaries are important transition zones between freshwater and marine environments [2] and are ephemeral in geological terms [3]; they have complex dynamics with daily and seasonal changes in salinity, turbidity and temperature. Furthermore, estuaries represent an environment of high productivity and are considered natural nurseries for many marine and estuarine species [4,5].

Historical events, such as changes in the sea level during the Pleistocene glacial cycles, may have exerted a strong impact on coastal habitats by limiting connectivity and promoting genetic divergence between species [6–9]. During the Pleistocene, there

was an alternation between glacial and interglacial periods [10]. In the last glacial period, the sea level dropped approximately 130 m below the current level, leaving the continental shelf completely exposed [11]. In the Holocene, the current interglacial period that began approximately 10,000 years ago, the sea level began to rise, creating the current estuaries approximately 5100 years ago [3].

The fluctuations in sea level in Brazil caused changes in coastal and estuarine regions, mainly in the Amazon coast. The Amazon River Delta is located in this region, which has the largest discharge of freshwater in the world [12] and could potentially serve as a barrier to the dispersal of species. The coastal regions of both Amapa and Para states have different geomorphological features [13–15]. The coast of Amapa is classified into two regions, Estuarine (nearest the mouth of the Amazon), and Oceanic (further north, encompassing the Amapa and Calcoene estuaries) [16]. For Para and Maranhão states, the authors have

described five different geomorphological sectors: the Para Platform, Caete Basin, Gurupi Bay, Turiçu Bay and Cumã Bay [17].

In the Para Platform (estuaries 4 to 7), great cliffs are maintained by sediment from the Pirabas/Barreiras formation, which acts as barriers to the external action of waves and tidal currents. In this area, the estuaries are narrow, with a length of only two kilometers. The estuarine channels may reach up to 60 km on the mainland and have no interconnections between adjacent estuaries [15]. Conversely, the Caete Basin (estuaries 8 to 11) is characterized by the presence of inactive cliffs with widely distributed estuaries reaching up to 30 km in length. The estuarine channels in this region may extend up to 100 km towards the continent and, unlike the Para Platform, the estuaries here are interconnected [15], which also appears to be the case for the other estuaries from Maranhao to the Parnaiba Delta.

Several studies indicate that marine environments can operate as barriers inhibiting gene flow between organisms that spend their entire life cycle in estuary ecosystems, as is the case for the four-eyed fish, *Anableps anableps* Linnaeus, 1758. This species belongs to the family Anablepidae and is considered an estuarine-resident species [18].

A. anableps has attracted the attention of many researchers because its retina is split horizontally, allowing for an aerial view and an aquatic view simultaneously [19,20]. It is an epipelagic fish that can adapt only to a narrow range of salinity (stenohaline), and it is viviparous with internal fertilization [21]. The distribution of *A. anableps* extends from the Gulf of Paria in Venezuela to the Parnaiba Delta in the state of Piauí, Brazil. Moreover, the *Anableps* genus has two other species: *A. microlepis* Müller & Troschel, 1844, which is considered the sister species of *A. anableps* with whom it lives in sympatry in South America, and *A. dowie* Gill, 1861, which occurs in the Pacific (Central America) and is considered the most primitive species of the genus [22].

The life history of *A. anableps* shows that this fish has a low potential for dispersal, mainly because it does not have a period of planktonic larval dispersal (PLD) and is stenohaline and resides in estuarine regions throughout its life cycle; therefore, phylogeographic studies are of great importance in uncovering the role of historical events in the diversification of this species. Nevertheless, *A. anableps* is an excellent target for phylogeographic studies due to its previously described biological and ecological characteristics. In this context, the phylogeographic study of *A. anableps* will be of great importance for understanding the patterns of genetic variability, gene flow and levels of connectivity among populations in the estuaries of the Amazon and Northeast Brazil. Coalescence analyses may also shed light on how the evolutionary history of the estuaries correlates with the sea level alterations that occurred during the Pleistocene and the Holocene.

Results

Genetic diversity

Comparison among the 393 sequences obtained in the present work showed 70 variable sites (52 parsimony informative and 18 singleton variable sites) that defined 83 haplotypes. Forty-two haplotypes (43%) are represented by a single sequence in the whole sample, which are mostly concentrated in the Para Platform. The haplotype diversity varied from 0 in the Parnaiba estuary to 0.933 in the Amazonas river estuary.

Nine haplotypes were detected for the two populations of the Amapa state coast: haplotype 1, which is the most frequent, is observed in the Calcoene and Amapa estuaries, which also share haplotype 3. Haplotype 2 was observed only in Calcoene estuary

while haplotypes 4 to 9 occur only in specimens of the Amapa river estuary. The populations from the Amazonas river mouth to the Maracana estuary (3 to 6) do not share haplotypes as shown in Table 1. Haplotype 43 is widely distributed, occurring in one population from the Para Platform (Urindeua), in all populations from the Caete Basin, and also in populations from Maranhao and Parnaiba Delta. Curiously, haplotype 43 is the only haplotype present in fish of the Parnaiba Delta (Table 1).

Population structure

Results from AMOVA show that the genetic variation among regions (54.07%) was higher than the variation among populations within regions (17.43%). However, the variation within populations (28.5%) was larger than the variation among populations within regions (17.43%) (Table S1).

Pairwise F_{st} values between populations were all significant except for the comparisons between the Calcoene (1) vs the Amapa (2), Urindeua (7) vs Japerica (8) vs, and Caete (10) vs Sao Marcos (12) (Table 2). The highest pairwise F_{st} value (0.99309) resulted from comparisons between Calcoene (1) and Sao Marcos (12), while the lowest value (0.01339) resulted from comparisons between Urindeua (7) and Japerica (8).

Haplotype network

The haplotype network (Fig. S1) clearly distinguishes four major divisions identified as **Lineage 1** (the Amapa region), **Lineage 2** (the Amazon river mouth estuary), **Lineage 3** (the Para Platform) and **Lineage 4** (from Japerica to Parnaiba). The haplotype network showed that the Amazon estuary lineage (in blue) was slightly differentiated from the Para Platform population, but they are derived from a common ancestor. The populations from the Para Platform clearly show some degree of sub-structuring as a consequence of the relative isolation of populations from this region, a pattern demonstrated by a high number of unshared haplotypes. Conversely, the populations from Japerica to Parnaiba show a large number of shared haplotypes and a star-like pattern indicative of substantial gene flow. However, a surprising result was a close phylogenetic relationship of some haplotypes from Amapa and Calcoene (haplotypes 2, 4 and 6) with some haplotypes from the Caete estuary (haplotypes 69, 70, 71 and 72). As these geographic areas are 500 km apart and no evidence of present day connectivity is observed between these two regions, this certainly suggests a historical relationship between these two populations.

Maximum likelihood analysis

Maximum likelihood analysis produced the unrooted phylogenetic tree depicted in Fig. 1, which is very similar to the pattern showed by the haplotype network. The results clearly display the same four lineages described above. Again, the four haplotypes from the Caete estuary are more closely related to the haplotypes from Amapa and Calcoene than to any haplotype from the Caete estuary and neighboring estuaries.

Demographic history

The results of F_u 's F_s and R_2 are presented in Table 3, including the significance of the simulated p-values. F_u 's F_s and R_2 were all negative but not significant for all populations from the Amapa coast (1 and 2) and Amazonas (3) as well as for three populations from the Para Platform (5–7), suggesting that these populations did not have an excess of rare nucleotide site variants compared to the expectation under a neutral model of evolution. Conversely, three populations in the Caete Basin (8, 9 and 11)

Table 1. Haplotype distribution by estuary.

Haplotypes	No.	Estuaries													
		1	2	3	4	5	6	7	8	9	10	11	12	13	
1	36	+	+												
2	1	+													
3	2	+	+												
4 to 9	12	+													
10 to 16	10		+												
17 to 19	17			+											
20 to 29	26				+										
30 to 32	35					+									
33	2					+									
34	1					+									
35 to 42	12						+								
43	65							+	+	+	+	+	+	+	+
44 to 45	3						+								
46	2														
47	2							+							
48	27								+	+		+			
49	6									+					
50	1														
51	4														
52 to 54	3														
55 to 57	4										+				
58 to 62	25														
63	10														
64-72	48														+
73 to 80	13													+	
81 to 83	26														+
	393														

The gray band indicates the Caete basin region plus Maranhao and Parnaiba estuaries; No = Number of haplotypes. Shared haplotypes were included in the rectangle.
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Table 2. Fst results for a pairwise population comparison (lower diagonal) and associated significance indications (upper diagonal).

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	0	ns	+	+	+	+	+	+	+	+	+	+	+
2	0.04195	0	+	+	+	+	+	+	+	+	+	+	+
3	0.97701	0.93216	0	+	+	+	+	+	+	+	+	+	+
4	0.96699	0.79903	0.8291	0	+	+	+	+	+	+	+	+	+
5	0.97337	0.91289	0.87763	0.63051	0	+	+	+	+	+	+	+	+
6	0.97686	0.84764	0.8767	0.64441	0.73499	0	+	+	+	+	+	+	+
7	0.96593	0.88517	0.96827	0.9510	0.96626	0.95477	0	ns	+	+	+	+	+
8	0.97646	0.88365	0.96847	0.95964	0.96901	0.96446	0.01339	0	+	+	+	+	+
9	0.21060	0.20455	0.78662	0.39189	0.68702	0.39965	0.39250	0.37186	0	+	+	+	+
10	0.97984	0.89587	0.97639	0.96801	0.97388	0.96957	0.18512	0.31842	0.33559	0	+	ns	+
11	0.97194	0.85115	0.96501	0.95626	0.96447	0.95962	0.38279	0.48772	0.19813	0.39686	0	+	+
12	0.99309	0.84830	0.96034	0.97215	0.96772	0.9769	0.10793	0.29739	0.24258	-0.02949	0.4189	0	+
13	0.75683	0.66764	0.77574	0.41010	0.68617	0.35274	0.61623	0.60177	0.22681	0.62504	0.51252	0.4936	0

Light and dark gray boxes indicate Fst values in the Para Platform and the Caete basin, respectively.
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showed negative and significant Fu's Fs or R2 estimates, indicating an excess of rare mutations when compared to the expected under the neutral model of evolution, which can be interpreted as a result of recent population expansion. Otherwise, these values can be explained by balancing selection on a nearby locus, although studies proving selection on the mitochondrial genome in natural populations are rare.

In perfect agreement with Fu's F and R2 results, the Bayesian Skyline Plot showed evidence of demographic expansion only in Lineage 4 (Japerica to Parnaiba), (see Fig. 2). However as divergence rates of control region are extremely variable in fishes the estimate obtained here (~18,000 years ago) should be taken with caution due to uncertainty of divergence rates [23,24]. Furthermore, there are evidences that estuaries were formed at about 5,000 years ago [3], therefore the inferred divergences should be much younger. Conversely, Lineages 1 (the Amapa coast) and 3 (the Para Platform) did not show any evidence of demographic expansion. Thus, it appears that these populations remained demographically stable during their entire existence. Lineage 2 (Amazon river mouth) was not tested because of its small size.

Bayesian Analysis of Population Structure (BAPS)

Bayesian analysis of genetic structure identified four clusters (Fig. 3): Cluster I, all haplotypes from Calcoene and Amapa, one haplotype from Amazonas and four haplotypes from the Caete estuary; Cluster II, all remaining haplotypes from Amazonas; Cluster III, Paracauari, Curuça, Maracana, and Urindeua; Cluster IV, a few haplotypes from Urindeua plus Japerica, Quatipuru, Caete, Gurupi, Sao Marcos and Parnaiba. The four clusters correspond exactly to the same four lineages detected by the ML tree (Fig. 1).

Discussion

Phylogeographic Pattern & Past scenarios

The genetic analyses using the mtDNA Control Region revealed a phylogeographic pattern with four distinct mitochondrial lineages in populations of *A. anableps* from the North and Northeast coasts of Brazil.

Many studies with estuarine fishes suggest Pleistocene glacial events as the main cause of the genetic structuring [7–9,25–27], and some studies with fresh water fishes [28], sponges [29], shrimps [30] and mollusks [31] ascribed the genetic structuring to Pleistocene vicariant events, with some in the beginning (1.806 million years before present) and others at the end of the Pleistocene (11,700 years before present). It is well known that, with the glacial advance during the Pleistocene, huge volumes of water were immobilized in continental ice sheets, resulting in short-term sea level drops of 100 meters or more over the entire surface of the Earth. At approximately 100,000 years ago, the sea level in the Amazon coast was approximately 130 m below the present day level [32]. The sea level oscillated during the last glaciation and reached its lowest level at the Last Glacial Maximum [33]. Under this circumstance, all the discharge of the Amazon river was most likely spread along the shelf, which means that the Amazon plume as it is today practically did not exist at that time (LGM). Moreover, the oceanic current regimes were quite different from what they are now. Therefore, it is quite possible that no physical (Amazon plume) or physicochemical (high salinity) barriers existed as they do now, allowing an ample dispersion of *Anableps* along the coast. Therefore, it is possible that allopatric refuges due to variations in sea level during the Pleistocene-Holocene associated with the biology of the species,

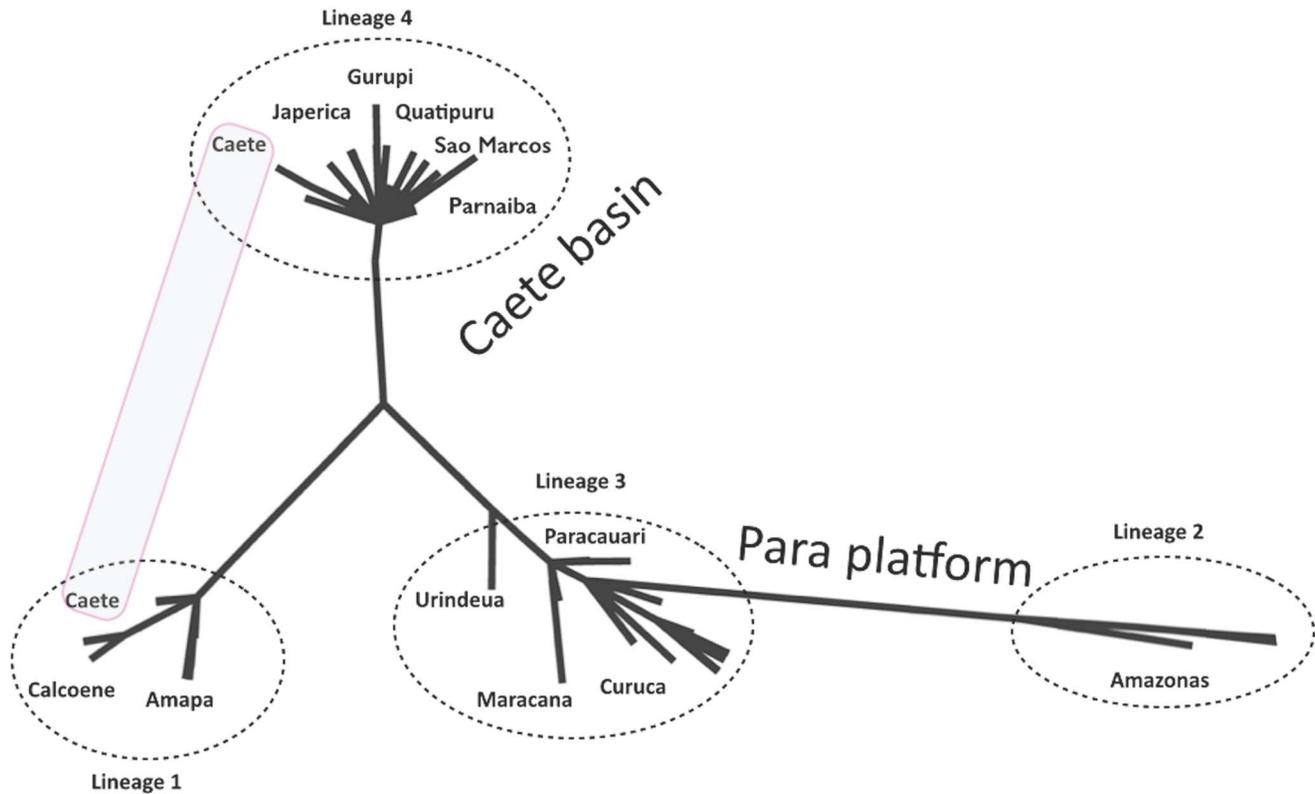


Figure 1. Maximum likelihood tree of the mitochondrial sequence of four-eyed fish from the North coast. This unrooted maximum-likelihood tree depicted the historical connection of haplotypes from the Caete and Amapa basins, which is indicated by a gray rectangle. The four lineages are delineated by interrupted line circles.
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such as viviparity and low potential for dispersal may have been the main force that promoted the recent divergence observed between populations and for the phylogeographic pattern observed for this species today. The divergence among lineages of the four-eyed-fish are comparable to those found in other species such as *Chelon haematocheilus*, a fish species from Asia [9], where three mitochondrial lineages were also detected that differentiated sometime between the middle and the end of the Pleistocene.

Isolation by Distance

The Mantel test showed some evidence of isolation by distance when all populations were analyzed together ($r = 0.45$; $P < 0.01$), but when each region was analyzed separately, no evidence of IBD was found. The structure observed in *A. anableps* can be explained by the geomorphological scenario of the study area, which was dramatically affected by sea level changes [3,34]. Lineage 1 was isolated in the middle to upper portion of the Amapa coast, but curiously, Lineage 2 (the Amazonas river mouth) is more closely related to Lineage 3 (the Para Platform) than to the Amapa Lineage 1, which is not consistent with the current flow of the Amazonas river to the north.

The geographic distribution of Lineages 2 (Amazonas), 3 (Para Platform) and 4 (Caete Basin to Parnaiba) matches the geomorphological landscape of the region. Therefore, it is highly probable that the wide distribution of tidal plains and the absence of interconnection between the estuaries have been essential for the isolation of Lineage 3 in the Para Platform. In contrast, there is an opposite pattern in the Caete Basin, which favored the

demographic and spatial expansion of Lineage 4. Importantly, in contrast to the proposal of at least five geomorphological sectors [17], the entire region from the Caete estuary to the Parnaiba Delta seems to be a unique biogeographical region for populations of *A. anableps*. Therefore, the genetic data obtained here clearly distinguish the Para Platform and the Caete Basin but do not show differences between populations of the Caete Basin and those of Sao Marcos and Parnaiba. Thus, the genetic pattern of *Anableps* mitochondrial lineages helps to distinguish four biogeographical regions in the whole area of study: Oceanic Amapa (Calcoene and Amapa), the Amazonas river Mouth (near Macapa), the Para Platform (Paracauari, Curuca, Maracana, and Urindeua) and Caete-Parnaiba (Japerica, Caete, Quatipuru, Gurupi, Sao Marcos and the Parnaiba estuary).

The unexpectedly high genetic similarity between the four haplotypes from the Caete estuary and the haplotypes from Amapa (Lineage 1) may represent historical signatures of colonization of the Amapa estuaries by ancestral haplotypes from Caete that are widely distributed or are at least distributed from Caete to Amapa thousands of years ago, when the sea level was 130 m below the present level. Extinction of intermediate populations due to geomorphological changes, gradual increases in the sea level, formation of the Amazon plume, and oceanic currents may also have contributed. Lineage 1 is practically restricted to the Amapa State coast with no evidence of significant gene flow to neighboring populations, which is corroborated by the Bayesian Skyline Plot, suggesting that these populations did not experience a significant demographic expansion since colonization.

Table 3. Indexes of genetic variability and neutrality tests estimated in populations of *A. anableps* from 13 estuaries.

Collecting Places	Code	N	Hap	h	π	R2	Prob	Fu's Fs	Prob
Calcoene river estuary	1	23	3	0.170	0.00057	0.1492	0.3547	-1.30	0.06
Anapa river estuary	2	28	8	0.696	0.00460	0.1126	0.3967	-1.18	0.28
Amazonas river estuary	3	10	7	0.933	0.01065	0.1480	0.2939	-0.78	0.31
Paracauari river estuary	4	17	3	0.588	0.00250	0.1931	0.7843	1.44	0.80
Curuca Bay	5	26	10	0.775	0.00434	0.0687	0.0208	-3.78	0.01
Maracana river estuary	6	30	5	0.361	0.00140	0.0597	0.0001	-1.78	0.06
Urindeua Bay	7	30	14	0.903	0.01239	0.1635	0.9052	-2.22	0.19
Japerica river estuary	8	28	9	0.791	0.00352	0.0890	0.1298	-3.21	0.02
Quatipuru river estuary	9	22	8	0.701	0.00259	0.0810	0.0117	-3.95	0.005
Caete river estuary	10	98	17	0.788	0.01458	0.1164	0.8030	1.12	0.70
Gurupi river estuary	11	38	9	0.565	0.00154	0.0495	0.0008	-6.60	0.00
Sao Marcos Bay	12	30	5	0.501	0.00177	0.1039	0.2669	-1.16	0.20
Parmaiba river estuary	13	13	1	ne	ne	ne	ne	ne	ne
Total		393	83	0.937					

N = number of individuals; Hap = number of haplotypes; h = nucleotide diversity; π = nucleotide diversity; Prob = probability.
doi:10.1371/journal.pone.0101727.t003

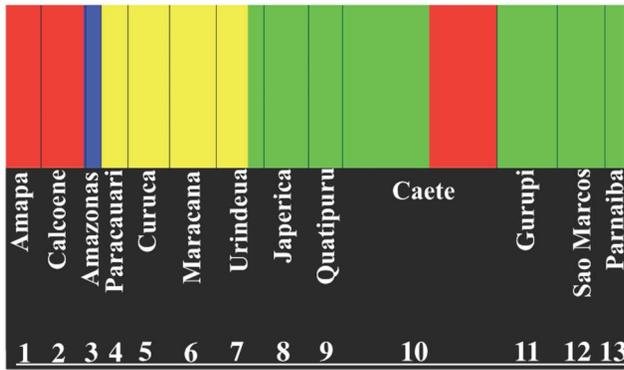


Figure 2. Bayesian analysis of population structure of the four-eyed fish from the North and Northeast coast. Locality and estuary names are shown. The clusters are indicated by colors: cluster 1 is red, cluster 2 is blue, cluster 3 is yellow, and cluster 4 is green. The haplotypes from Lineage 4, which are historically correlated with the Amapa basin, are represented in red inside cluster 4. doi:10.1371/journal.pone.0101727.g002

The genetic reconstruction performed here suggests that at least two distinct historical events helped shape the evolutionary history of *A. anableps* in northern Brazil, and both seem to be related to global events such as glaciations. The drop in the sea level during the Last Glacial period, which reached its lowest level (−130 meters) in Last Glacial Maximum (LGM; ~22,000 to 19,000 years ago [33]), and the gradual rise to the current sea level (last 5,000 years [35]) drove the distribution and structuring of the populations as we know it today. Therefore, the exposure of the Atlantic shelf at the LGM period may have presented to the ancestral four-eyed-fish the opportunity for dispersion along the coast through a coastal lagoon environment [33] or even an Amazon “fresh water sea”. Second, and more recently, the rise of the sea level, the establishment of the Amazon plume, the configuration of the Marajo Archipelago [36], and the Oceanic Currents shaped the current distribution and genetic structuring of the *Anableps* populations analyzed here. It is interesting to note that the formation of the Marajó Archipelago during the recent Holocene separated the population of *Anableps* of the Amazonas river mouth from that of the Para Platform, from which the former is genetically derived.

Because the migration of four-eyed fish appears to be highly restricted to environments of low salinity [37], this suggests that

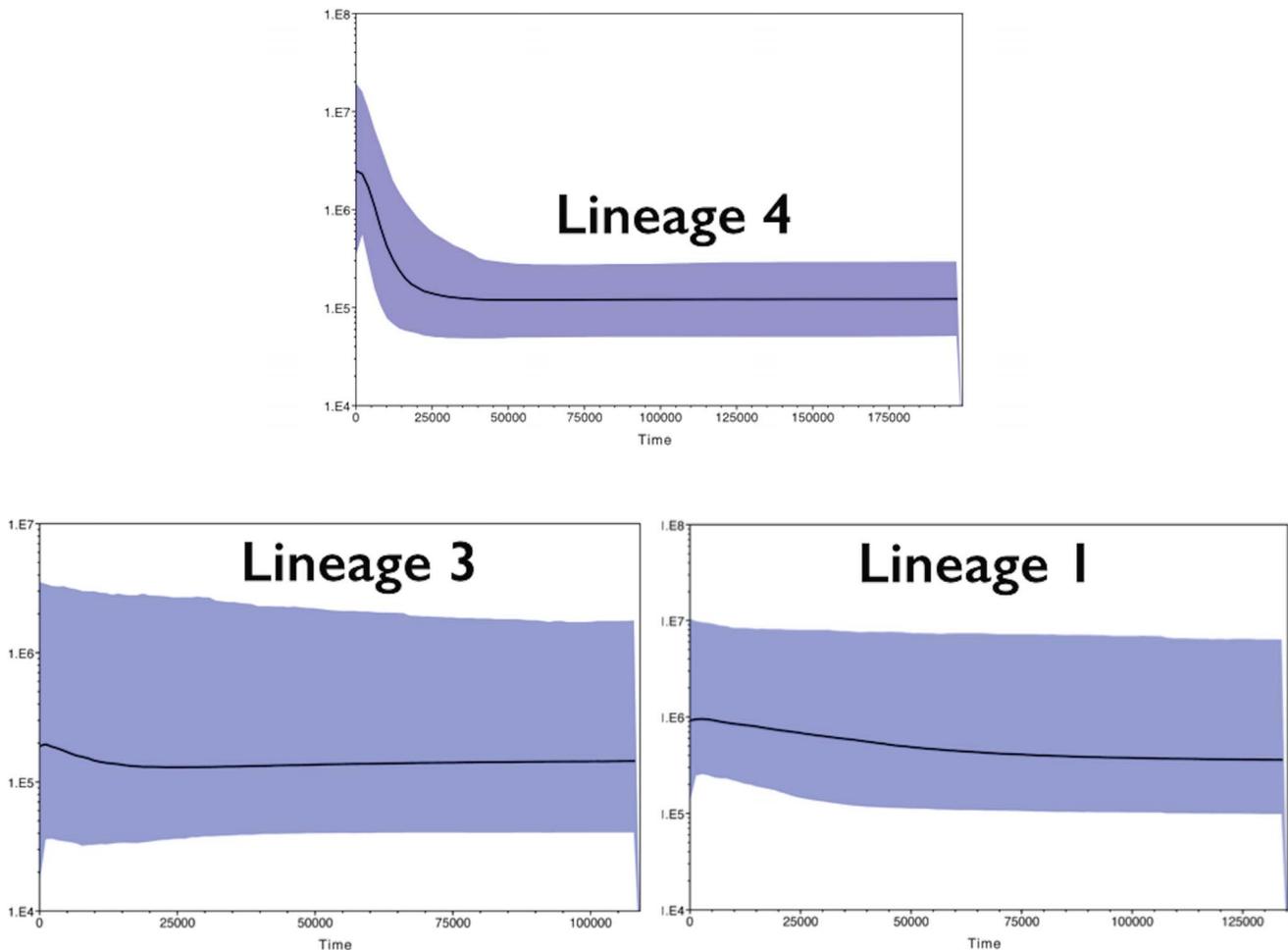


Figure 3. Bayesian Skyline Plot of three mitochondrial lineages of the four-eyed fish. Curves for each one of the three lineages are identified in the graph. The Y-axis indicates effective population size (N_e) x generation time, while the X-axis indicates mean time in thousands of years before present. The thick line represents the average, and the blue band represents the standard error. doi:10.1371/journal.pone.0101727.g003

the only possible connection between neighboring estuaries are by inland interconnections among the estuaries and not by the sea coast. Therefore, the absence of interconnection among the estuaries of the Para Platform provided the opportunity for the isolation of *Anableps* populations independent of distance. This isolation is clearly validated by the absence of shared haplotypes in this region and high values of pairwise *F_{st}*. With the exception of neighboring populations from the Maracana and Urindeua estuaries that share few haplotypes, the remaining populations from this sector show strong estuary fidelity. Bayesian Skyline Plot analysis (Fig. 2) also suggests that these populations did not experience a significant demographic expansion since colonization, possibly due to the relative isolation of each population due to their inaccessible estuaries. In contrast, for populations of the Caete Basin-Parnaiba, Bayesian Skyline plotting and the low *F_{st}* values confirm the significant gene flow among the populations in this sector and provide evidence for expansion and genetic structuring probable during late Pleistocene and early Holocene.

Conclusions

The present study revealed high genetic divergence among populations of *Anableps* and uncovered four distinct mitochondrial lineages for *A. anableps* on the North and Northeast Amazon coast of Brazil. The study also suggested that the genetic structuring of these populations is correlated to events of the Holocene (11,700 to present).

The discrepancy of the gene flow pattern observed along the coast of Para fits well with the geomorphological features that are known as the Para Platform and the Caete Basin. The former has no interconnections between adjacent estuaries, while in the latter, these connections are evident. This analysis identifies only four biogeographic regions (Amapa, the Amazonas river Mouth, the Para Platform and Caete-Parnaiba) instead of the several geomorphological sectors described by geologists. The divergence observed in populations of *A. anableps* confirms that estuarine-resident organisms exhibit a degree of differentiation that is higher than strictly marine species [7,8,27]. Therefore, the results of the present work can be useful for designing new experiments to better understand the evolutionary history of the estuaries on the Brazilian coast using organisms that are biologically similar to the four-eyed-fish *Anableps*.

Methods

Ethics statement

Animal samples were collected and manipulated (DNA extraction, capture, transport and handling) under a permit (N° 12773-1) provided by the Environment Ministry (MMA) that was given to Iracilda Sampaio. All work was performed in compliance with and approved by the Ethics Committee of the University Federal do Para.

Sampling

For the present study, samples of muscle tissue were obtained from a total of 393 specimens collected in 13 estuaries from four Brazilian states: Amapa, Para, Maranhão and Piauí, practically covering the entire range of the geographic distribution of the four-eyed fish in Brazil (Table 4; Fig. 4). Specimens were captured using fishnets and then euthanized by an overdose of tricaine methanesulphonate. After that a fragment of 5 to 10 mm of muscle tissue was collected from dorsal side of the fish. Muscle tissue samples were then fixed in absolute ethanol and later stored in a freezer at -20°C until DNA extraction.

DNA extraction, PCR amplification and DNA sequencing

Total DNA was isolated from muscle tissue by digestion with proteinase K using a modified protocol with phenol chloroform and precipitation with sodium acetate and isopropanol [38]. A fragment of 464 base pairs of the Control Region (CR) was amplified by polymerase chain reaction (PCR) using the following primers: D-Loop L1 (5'-CCTAACTCCCAAAGCTAGG-TATTC-3') and D-Loop H1(5'-TGTTTATCACTGCTGA-GAGTTCCCT-3') [39]. Each PCR reaction consisted of 4 μl dNTP (1.25 mM), 2.5 μl buffer (10X), 1 μl MgCl_2 (50 mM), 0.25 μl of each primer (100 ng/ μl), 1 μl DNA (200 ng/ μl), 0.2 μl Taq DNA polymerase (5 U/ μl) and distilled water to complete the final reaction volume of 25 μl . The amplification program consisted of an initial step of 94°C for 3 min (initial denaturation) followed by 35 cycles at 94°C for 30 seconds (denaturation), 57°C for 1 minute (annealing), and 72°C for 1 minute (extension). A final extension period of 7 minutes at 72°C was used. The amplified products were cleaned with EXOSAP-IT, and then, cycle sequencing was conducted using the Applied Biosystems Big Dye (ABI Prism™ Dye Terminator Cycle Sequencing Reading Reaction – PE Applied Biosystems – Carlsbad, CA-USA). The product was cleaned by precipitation and electrophoresed in an automatic capillary ABI 3130 (Applied Biosystems – Carlsbad, CA-USA) sequencer. The 393 DNA sequences were deposited in Genbank under the accession numbers from KJ639422 to KJ639814.

Population analyses

The sequences obtained were aligned in the program CLUSTAL W [40] implemented in BioEdit [41] and edited manually. All statistical parameters and tests were estimated using Arlequin v. 3.5 [42]. Genetic variability within populations was estimated by computing the haplotype (*h*) and nucleotide diversities (π). Partitioning of genetic variation within and among populations was calculated using analysis of molecular variance (AMOVA) [42] by computing the conventional **F**-statistics from haplotypes with 1000 permutations using Arlequin v. 3.5 [42].

The optimal model of sequence evolution was determined using the Akaike Information Criterion with Kakusan v.4 [43]. The resulting model was the Hasegawa-Kishino-Yano (HKY) with among-site rate variation assuming a gamma distribution. Using this model in a script, generated by Kakusan v.4, a maximum-likelihood phylogenetic tree was reconstructed with PhyML version 3.0 [44]. From this tree, a haplotype genealogy was generated using Haploviewer 1.0 [45].

To test for past population expansion, two statistical tests were used: *F_u*'s *F_s*, which uses the distribution of haplotypes (or alleles), and the *R₂* Statistic, which is based on the relationships between singletons and the average number of nucleotide differences. *F_u*'s *F_s* and *R₂* statistics [46] proved to be very strong for this purpose, especially when population sample sizes are large (>50 , *F_u*'s *F_s*) or when sample sizes are small (<10 , *R₂*). Furthermore, the power of the *R₂* statistic is relatively high when the number of segregating sites is low (e.g., <20) [46]. The significance of *F_u*'s *F_s* and *R₂* was obtained by examining the null distribution of 5,000 coalescent simulations of these statistics using DnaSP v.5 [47]. Significantly large negative *F_u*'s *F_s* values and significantly positive *R₂* values were taken as evidence of a population expansion.

The reconstruction of genealogy and demographic history was made in a Bayesian framework using the Bayesian Skyline Plot implemented in Beast v1.7.5 [48]. As there is no fossil record to calibrate the time, the mutation rate was used to inform the prior distribution of the rate in the Bayesian analysis. The averaged divergence rate for the fish mtDNA control region used in the

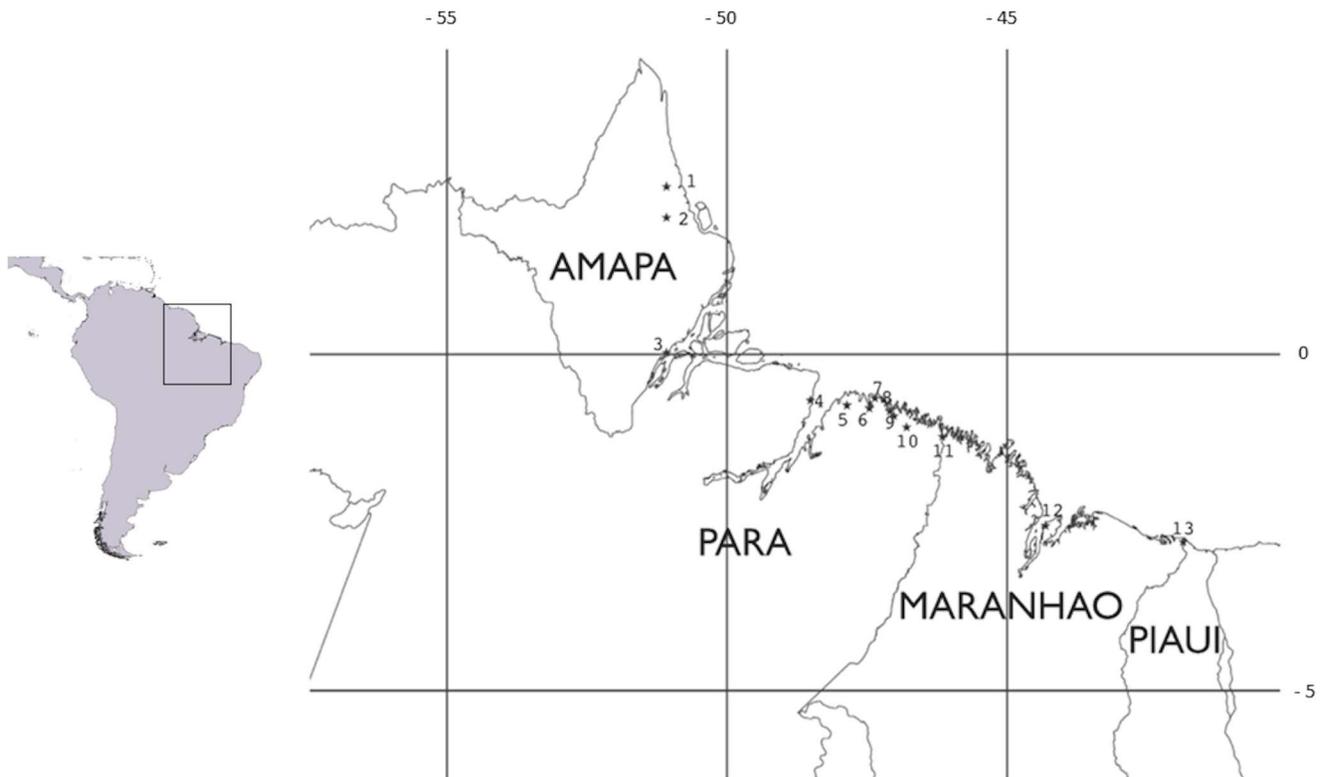


Figure 4. Map of the distribution of *Anableps anableps* populations sampled in the present study. Amapa, Para and Maranhao are states from North and Piaui from Northeast of Brazil. The estuaries are represented by the following numbers: 1 = Calcoene; 2 = Amapa; 3 = Amazonas; 4 = Paracauari; 5 = Curuca; 6 = Maracana; 7 = Urindeua; 8 = Japerica; 9 = Quatipuru; 10 = Caete; 11 = Gurupi; 12 = Sao Marcos; 13 = Parnaiba. doi:10.1371/journal.pone.0101727.g004

Table 4. Origin, number of *A. Anableps* samples collected and geographic coordinates of the estuaries.

Collecting Sites	Code	Number	Longitude	Latitude
Amapa-Curuca-Amazonas				
Calcoene	1	23	-50.825245	2.500976
Amapa	2	28	-50.794464	2.051082
Amazonas	3	10	-51.104504	0.000842
Para Platform				
Paracauari	4	17	-48.534042	-0.717226
Curuça	5	26	-47.851547	-0.738175
Maracana	6	30	-47.462582	-0.765942
Urindeua	7	30	-47.346126	-0.631339
Caete Basin				
Japerica	8	28	-47.089771	-0.8276
Quatipuru	9	22	-47.005656	-0.899611
Caete	10	98	-46.678575	-0.980565
Gurupi	11	38	-46.139187	-1.221839
Sao Marcos estuary - Parnaiba Delta				
Sao Marcos	12	30	-44.551371	-2.532688
Parnaiba	13	13	-41.814806	-2.908765
Total		393		

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present work was 3.6×10^{-8} [49], which has been extensively used in fishes when the fossil record is absent.

Effective population sizes (N_e) against time were drawn for each one of the following lineages: 1 (Amapa, 51 individuals), 3 (the Para Platform, 103 individuals), and 4 (the Caete Basin, 229 individuals). Lineage 2 was not tested because it has only 10 individuals. The analyses were run in Beast v1.7.5 for 100 million iterations using default parameters and priors (HKY substitution model without site heterogeneity; strict molecular clock model; tree prior: coalescent Bayesian skyline, with 10 groups and piecewise-constant skyline model; operators: auto-optimize; log parameters: every 10,000 iterations). The results of the analyses were visualized using Tracer v1.5 [50]. Convergence of the chains to the stationary distribution was systematically confirmed by visual inspection of plotted posterior estimates.

The Mantel test was performed in Arlequin v. 3.5 [42] to check if the patterns of genetic differentiation found in *A. anableps* correspond to a model of isolation by distance (IBD). Four options were used: 1) all 13 populations; 2) Caete Basin; (3) Para Platform; and (4) Amapa Basin. The matrices used were pairwise *Fst* and geographic distances among populations.

To infer the number of subpopulations and to assign individual samples to these groups, we employed the BAPS6 software [51] a program for Bayesian inference of genetic structure in a population. When testing for population clusters, 10 replicates for every level of *k* (*k* is the maximum number of clusters) up to

k = 13 were run. The number of reference individuals was set to 200, and admixture analyses were run 50 times per individual.

Supporting Information

Figure S1 Haplotype genealogy of the mitochondrial sequence based on a maximum-likelihood tree. Circles represent haplotypes; the size is proportional to the number of individuals, and small blue dots represent intermediate, unsampled haplotypes. The four lineages are identified by distinct colors. (TIFF)

Table S1
(DOC)

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Author Contributions

Conceived and designed the experiments: LAW HS IS. Performed the experiments: LAW JMC IS. Analyzed the data: HS NAN MV USP. Contributed reagents/materials/analysis tools: JMC LAW. Wrote the paper: LAW HS IS.

References

- Bilton DT, Paula J, Bishop JDD (2002) Dispersal, Genetic Differentiation and Speciation in Estuarine Organisms. *Estuarine, Coastal and Shelf Science* 55: 937–952.
- Miranda LB, Castro BM, Kjerfve B (2002) *Princípios de Oceanografia Física de estuários*. São Paulo: EDUSP.
- Cohen M, Filho P, Lara R, Behling H, Angulo R (2005) A Model of Holocene Mangrove Development and Relative Sea-level Changes on the Bragança Peninsula (Northern Brazil). *Wetlands Ecology and Management* 13: 433–443.
- Butler AJ (1999) *Seagrass in Australia: strategic review and development of an R & D plan*/Alan Butler and Peter Jernakoff; Jernakoff P, editor. Collingwood, Vic. CSIRO Publishing.
- Beck MW, Heck KL Jr, Able KW, Childers D, Eggleston D, et al. (2001) The identification, conservation, and management of estuarine and marine nurseries for fish and invertebrates. *Bioscience* 51: 633–641.
- Hewitt G (2000) The genetic legacy of the Quaternary ice ages. *Nature* 405: 907–913.
- Beheregaray LB, Sunnucks P (2001) Fine-scale genetic structure, estuarine colonization and incipient speciation in the marine silverside fish *Odontesthes argentinensis*. *Molecular Ecology* 10: 2849–2866.
- Chenoweth SF, Hughes JM (2003) Oceanic interchange and nonequilibrium population structure in the estuarine dependent Indo-Pacific tasselfish, *Polynemus sheridani*. *Molecular Ecology* 12: 2387–2397.
- Liu J-X, Gao T-X, Wu S-F, Zhang Y-P (2007) Pleistocene isolation in the Northwestern Pacific marginal seas and limited dispersal in a marine fish, *Chelon haematocheilus* (Temminck & Schlegel, 1845). *Molecular Ecology* 16: 275–288.
- Imbrie J, Boyle EA, Clemens SC, Duffy A, Howard WR, et al. (1992) On the Structure and Origin of Major Glaciation Cycles 1. Linear Responses to Milankovitch Forcing. *Paleoceanography* 7: 701–738.
- Lambeck K, Esat TM, Potter E-K (2002) Links between climate and sea levels for the past three million years. *Nature* 419: 199–206.
- Salati E, Vose R (1984) Amazon basin: a system in equilibrium. *Science* 225: 129–138.
- Franzini E (1992) Evolution of the geomorphology of the coast of the State of Para, Brazil. In: Prost MT, editor. *Evolution des littoraux de Guyane et de la Zone Caraïbe Meridionale pendant le Quaternaire*. Paris: ORSTOM. pp. 203–230.
- Souza-Filho PWM (2000) Tectonic control on the coastal zone geomorphology of the northeastern Para state. *Revista Brasileira de Geociências* 30: 527–530.
- Souza-Filho PWM (2005) Costa de manguezais de macromaré da Amazônia: cenários morfológicos, mapeamento e quantificação de áreas usando dados de sensores remotos. *Revista Brasileira de Geofísica* 23: 427–435.
- Torres AM, El-Robrini M (2006) Amapa. In: Muehe D, editor. *Erosão e progradação do litoral brasileiro*. Brasília: Ministério do Meio Ambiente. pp. 1–40.
- Souza-Filho PW, Lessa GC, Cohen MC, Costa FR, Lara RJ (2009) The subsiding macrotidal barrier estuarine system of the eastern Amazon coast, Northern Brazil. *Geology and Geomorphology of Holocene Coastal Barriers of Brazil*: Springer. pp. 347–375.
- Nelson JS (2006) *Fishes of the world*. New York: John Wiley and Sons, Inc. 601p.
- Sivak JG (1976) Optics of the eye of the "four-eyed fish" (*Anableps anableps*). *Vision Research* 16: 531–534, IN536.
- Schwab IR, Ho V, Roth A, Blankenship TN, Fitzgerald PG (2001) Evolutionary attempts at 4 eyes in vertebrates. *Transactions of the American Ophthalmological Society* 99: 145–157.
- Turner CL (1938) Adaptations for viviparity in embryos and ovary of *Anableps Anableps*. *Journal of Morphology* 62: 323–349.
- Ghedotti MJ (2003) Family Anablepidae (four-eyed fishes, one sided livebearers and the white eye). In: Reis RE, Kullander SO, Ferraris CJ, editors. *Checklist of the freshwater fishes of South and Central America*. Porto Alegre: EDIPUCRS. pp. 582–585.
- Waters JM, Rowe DL, Apte S, King TM, Wallis GP, et al. (2007) Geological Dates and Molecular Rates: Rapid Divergence of Rivers and Their Biotas. *Systematic Biology* 56: 271–282.
- Page T, Hughes J (2010) Comparing the performance of multiple mitochondrial genes in the analysis of Australian freshwater fishes. *Journal of Fish Biology* 77: 2093–2122.
- Domingues VS, Faria CL, Stefanni S, Santos RS, Brito A, et al. (2007) Genetic divergence in the Atlantic Mediterranean Montagu's blenny, *Coryphoblennius galerita* (Linnaeus 1758) revealed by molecular and morphological characters. *Molecular Ecology* 16: 3592–3605.
- Chenoweth SF, Hughes JM, Keenan CP, Lavery S (1998) When oceans meet: a teleost shows secondary intergradation at an Indian-Pacific interface. *Proceedings of the Royal Society of London Series B: Biological Sciences* 265: 415–420.
- Durand JD, Tine M, Panfili J, Thiaw OT, Lac R (2005) Impact of glaciations and geographic distance on the genetic structure of a tropical estuarine fish *Ethmalosa fimbriata* (Clupeidae S Bowdich 1825). *Molecular Phylogenetics and Evolution* 36: 277–287.
- Faulks L, Gilligan D, Beheregaray L (2010) Evolution and maintenance of divergent lineages in an endangered freshwater fish, *Macquaria australasica*. *Conservation Genetics* 11: 921–934.
- Wörheide G, Hooper JNA, Degnan BM (2002) Phylogeography of western Pacific *Leucetta 'chagosensis'* (Porifera: Calcarea) from ribosomal DNA sequences: implications for population history and conservation of the Great Barrier Reef World Heritage Area (Australia). *Molecular Ecology* 11: 1753–1768.
- Benzie JAH, Ballment E, Forbes AT, Demetriades NT, Sugama K, et al. (2002) Mitochondrial DNA variation in Indo-Pacific populations of the giant tiger prawn, *Penaeus monodon*. *Molecular Ecology* 11: 2553–2569.
- Imron, Jeffrey B, Hale P, Degnan BM, Degnan SM (2007) Pleistocene isolation and recent gene flow in *Haliois asinina*, an Indo-Pacific vetigastropod with limited dispersal capacity. *Molecular Ecology* 16: 289–304.

32. El-Robrini E, Souza Filho PWM, Silva CA, Silveira OFM, Faria L Jr, et al. Registro das oscilações do nível do mar na plataforma continental norte do Brasil; 1992; Manaus. pp. 73–75.
33. Yokoyama Y, Lambeck K, De Deckker P, Johnston P, Fifield LK (2000) Timing of the Last Glacial Maximum from observed sea-level minima. *Nature* 406: 713–716.
34. Haberle SG, Maslin MA (1999) Late Quaternary Vegetation and Climate Change in the Amazon Basin Based on a 50,000 Year Pollen Record from the Amazon Fan, ODP Site 932. *Quaternary Research* 51: 27–38.
35. Filho PS, Cohen M, Lara R, Lessa G, Koch B, et al. (2006) Holocene coastal evolution and facies model of the Bragança macrotidal flat on the Amazon Mangrove Coast, Northern Brazil. *Journal of Coastal Research*: 306–310.
36. Rossetti DF, Valeriano MM, Góes AM, Thales M (2008) Palaeodrainage on Marajó Island, northern Brazil, in relation to Holocene relative sea-level dynamics. *The Holocene* 18: 923–934.
37. Nascimento FL, Assunção MIS (2008) Ecologia reprodutiva dos tralhos *Anableps anableps* e *Anableps microlepis* (Pisces: Osteichthyes: Cyprinodontiformes: Anablepidae) no rio Paracatuari, ilha de Marajó, Pará, Brasil. *Boletim do Museu Paraense Emílio Goeldi* 3: 229–240.
38. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.
39. Santa Brígida EL, Cunha DB, Rego PS, Sampaio I, Schneider H, et al. (2007) Population analysis of *Scomberomorus cavalla* (Perciformes, Scombridae) from the Northern and Northeastern coast of Brazil. *Brazilian Journal of Biology* 67: 919–924.
40. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673–4680.
41. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.
42. Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564–567.
43. Tanabe AS (2007) kakusan: a computer program to automate the selection of a nucleotide substitution model and the configuration of a mixed model on multilocus data. *Molecular Ecology Notes* 7: 962–964.
44. Guindon Sp, Dufayard J-Fβ, Lefort V, Anisimova M, Hordijk W, et al. (2010) New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology* 59: 307–321.
45. Salzburger W, Ewing GB, Von Haeseler A (2011) The performance of phylogenetic algorithms in estimating haplotype genealogies with migration. *Molecular Ecology* 20: 1952–1963.
46. Ramos-Onsins SE, Rozas J (2002) Statistical Properties of New Neutrality Tests Against Population Growth. *Molecular Biology and Evolution* 19: 2092–2100.
47. Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25: 1451–1452.
48. Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian Phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* 29: 1969–1973.
49. Donaldson KA, Wilson RR (1999) Amphi-Panamaic geminates of snook (Percoidae: Centropomidae) provide a calibration of the divergence rates in the mitochondrial DNA control region of fishes. *Molecular Phylogenetics and Evolution* 13: 208–213.
50. Rambaut A, Drummond AJ (2009) *Tracer*. 1.5 ed.
51. Corander J, Waldmann P, Marttinen P, Sillanpää MJ (2004) BAPS 2: enhanced possibilities for the analysis of genetic population structure. *Bioinformatics* 20: 2363–2369.