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Chapter 8

Microsatellites for the Amazonian Fish *Hypophthalmus marginatus*

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Abstract

We isolated 41 and characterized 17 microsatellite loci for evaluating the genetic structure of the Amazonian fish *Hypophthalmus marginatus*, from the Tocantins and Araguaia River in the Eastern Amazonia. Of the 17 selected microsatellite sequences, 15 were dinucleotide repeats, 9 of which were perfect (5–31 repetitions) and 6 were composite motifs. Among these 17 microsatellites, only two were polymorphic. The average number of alleles (Na) observed in the five examined populations ranged from 3.5 to 4.5, while the average observed heterozygosity (Ho) ranged from 0.3 to 0.6. The allelic frequency was less homogeneous at the locus Hm 5 than that for the Hm 13. Genetic diversity was measured in three upstream and two downstream populations under the influence of the Tucuruí Hydroelectric Dam. Our findings provide evidence for low levels of genetic diversity in *H. marginatus* of the Tocantis basin possibility related to the Dam construction. The F_{st} and R_{st} analysis fits well with migratory characteristics of *H. marginatus*, suggesting the existence of a gene flow mainly in the upstream or downstream directions. To test the hypothesis that the Dam was responsible for the detected reduction on this species genetic diversity, a large number of genetic markers are recommended, covering geographic distribution range of the fish species.

Keywords: hydroelectric dam influence, migratory fishes, population genetic structure

1. Introduction

Migratory freshwater fishes are vulnerable to a variety of anthropogenic impacts, including harvesting, pollution, and other types of habitat disturbance. The impact of dams is not only limited to the transformation of habitats from lentic to lotic but they also isolate the population from their places of spawning to feeding grounds [1–3]. Therefore, a survival and reproduction

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of migratory fish can be directly affected by changing thermal and hydrodynamic conditions in their habitat [4]. In the Brazilian Amazonia, over 10 million hectares (ha) of forests are expected to become permanently flooded after the construction of new-planned dams [5]. Long-term monitoring of fish populations is available only for Tucuruí Dam in the Tocantins River [6–10], where alterations following impoundment reduced the fish diversity on the reservoir resulting in the increase of predators such as *Cichla* spp. Schneider 1801, and *Serrasalmus* spp. Lacepède 1803 [11]. Furthermore, a drastic reduction in fish production has been observed downstream of the dam, probably due to the low oxygen content of water that runs through the turbines and the blocking of fish migration [6, 12]. Harvesting of freshwater shrimp downstream the dam has dropped from 179 tons in 1981, before the dam construction, to only 62 tons in 1988 three years after dam construction, while fish landings declined from 4726 to 831 tons (the dam was built between 1984 and 1985) [13]. Catches in the reservoir increased to pre-flooding levels by the early 1990s [8], although nowadays migratory species such as *Hypophthalmus marginatus* are still not such abundant as before. Therefore, due to the great economic importance to local fisheries, the current genetic structure and species/biodiversity conservation of *H. marginatus* stocks in the low to medium Tocantins River is a matter of concern and was investigated here. Besides, helping to understand the impact of the construction of the Tucuruí Dam on the genetic variability, our results should contribute to the eventual development of population management strategies for the studied species and will hopefully arise concerns about the building of future dams in the Amazon.

2. Materials and methods

The Tocantins is largely a plateau river, flowing for most of its length within an enclosed valley, draining an area of 343,000 km^2. Over the past three decades, its basin has suffered from huge anthropogenic pressures, including widespread deforestation, mining, and the construction of the Tucuruí between 1984 and 1985. This dam is one of the world's largest hydroelectric dams, which has a reservoir of 2840 km^2, most of which was originally covered with primary *terra firme* forest [9, 10].

2.1. Samples

Eighty-two samples (14–19 per site) obtained from muscle or liver tissue of *H. marginatus* were stored in absolute ethanol and frozen at -20°C for future genomic DNA extraction, which was performed using Sambrook standard protocol [14]. The specimens were preserved in 4% formaldehyde and deposited in the ichthyological collection of the Museu Paraense Emílio Goeldí (MPEG 13375, MPEG 17486, MPEG 17499 and MPEG 17578). Microsatellite loci were characterized in *H. marginatus* individuals from four different points of the Tocantins River: Itupiranga (05°06'51.5"S, 49°21'34.9"W), Tucuruí (04°18'43.06"S, 49°19'58.1"W), Cametá (02° 03'27.5"S, 49°20'31.9"W), and Abaetetuba (01°40'42.6"S, 49°00'16.6"W). Additionally, samples from one point of Araguaia River (Conceição do Araguaia, 07°58'10.8" S, 49°11'0.6" W) were included in the analysis (**Figure 1**).

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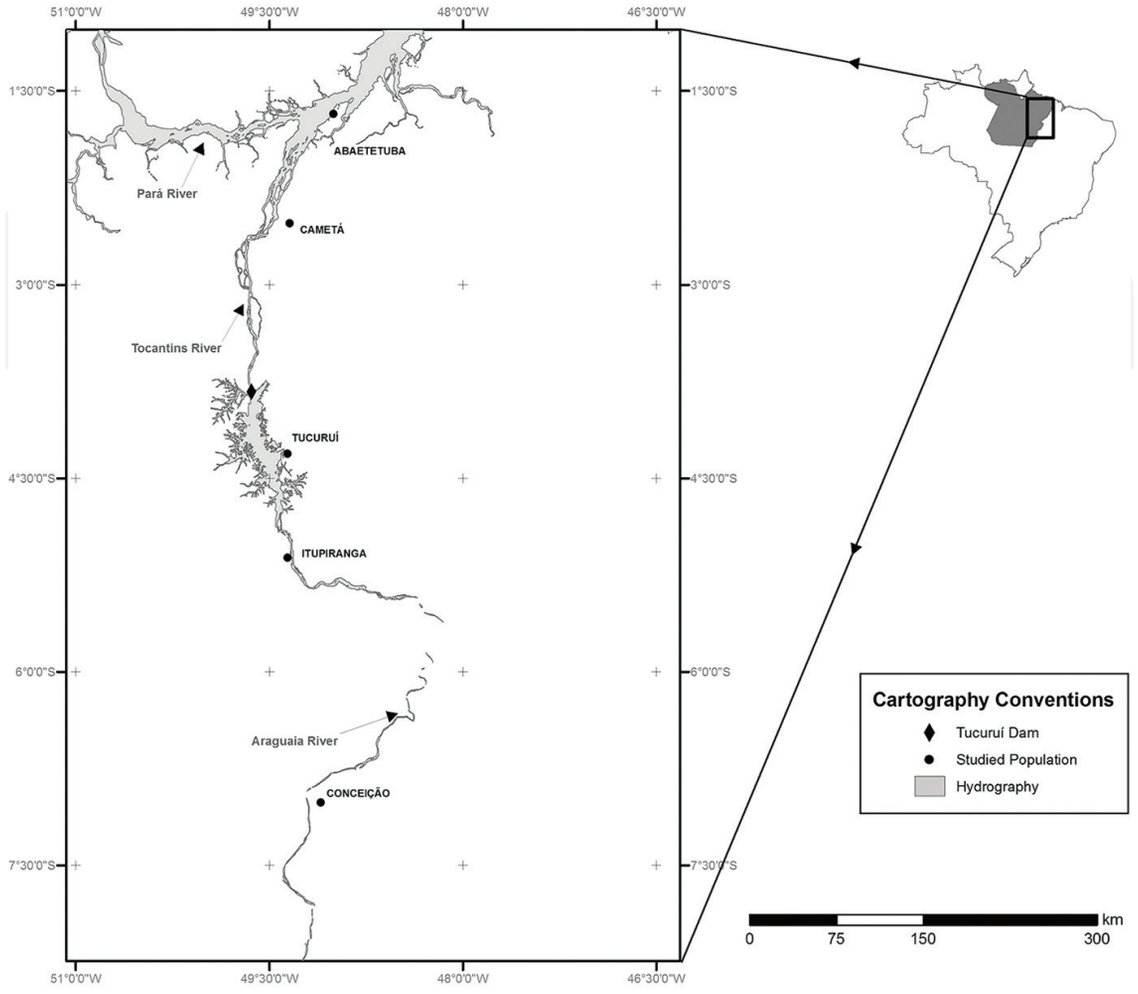

Figure 1. Distribution of the five stocks of *Hypophthalmus marginatus* sampled on the Tocantins and Araguaia Rivers in eastern Amazonia.

2.2. Microsatellite development

Molecular tools such as microsatellites markers can give us a picture of the distribution of the genetic variability of the natural populations of Amazonian fish [15, 16]. To assess the genetic parameters of the *Hypophthalmus* in the Amazon basin, we developed a partial genomic library of these fishes enriched for microsatellites following the method of selective hybridization with biotinylated probe types $(CT)_8$, conjugated to streptavidin-coated magnetic beads [17]. After hybridization, the microsatellite-enriched sequences were amplified by polymerase chain reaction (PCR), ligated into the pGEM-T Easy Vector (Promega Corp., Madison, USA) and transformed into *Escherichia coli* TOP 10 electroporated-competent bacteria. The transformed bacteria were plated on solid medium containing LB-ampicillin (100 mg/ml) + X-gal (2%), and after growth, the white colonies containing inserts were transferred and grown in 96-well plates in a liquid Tartoff-Hobbs Broth/ampicillin medium. A total of 96 positive clones were sequenced in both directions using the BigDye® Terminator v3.1 Cycle Sequencing Kit,

according to the manufacturer's instructions (Applied Biosystems, Carlsbad, USA). The sequences were edited and aligned in the software BioEdit [18].

A total of 96 clones were sequenced, and 17 of these were selected for primer design using the software Primer3 [19]. In all primer pairs, the forward primers had an M13(-21) tail added to its 5' end [20]. An optimal annealing temperature was inferred using a gradient PCR with temperatures set between 52.3 and 70.5°C (**Table 1**).

Genotyping reactions were carried out in the Biocycler Thermal Cycler MJ96+/MJ96G (Applied Biosystems), in a final volume of 10 μL. Each reaction contained 3.8 μL of MilliQ water, 1.2 μL of 50 mM $MgCl_2$, 1.0 μL of 10 mM dNTPs, 1.0 μL of PCR buffer (100 mM Tris-HCl, pH 8.5, 500 mM KCl), 0.4 μL of 2 μM tailed forward primer, 0.4 μL of 2 μM fluorescently labeled primer, 0.8 μL of 2 μM reverse primer, 0.3 μL of 2.5 U Taq DNA polymerase, and 1 μL of DNA (50–100 ng/μL). Reactions were submitted to the following cycling profile: hot start at 94°C for 60 s followed by 25 cycles of denaturing at 94°C for 30 s, annealing for 30 s at locus specific temperatures (**Table 1**), and extension at 68°C for 30 s; labeling step consisted of 20 cycles of denaturing at 94°C for 20 s, annealing at 52°C for 30 s, and extension at 72°C for 60 s; final extension was performed at 72°C for 30 min. The fragments present in 1 μL of PCR product were separated in 8% polyacrylamide denaturing gel in an ALFexpressTM II (Amersham Biosciences, Freiburg, Germany) automatic DNA sequencer. Amplicon size was estimated by the Allele Locator 1.03 software (Amersham Biosciences, India), based on the internal and external standards provided by the manufacturer.

Using the program Micro-Checker v2.2.3 [21], we verified that null alleles were not present in the data set. Genetic parameters were obtained with the program Arlequin 3.5 [22].

2.3. Data analysis

To measure the genetic variability within each population, the number of alleles per locus (A), effective number of alleles per locus (Ae), the observed heterozygosity (Ho), and expected (He) under Hardy-Weinberg equilibrium (HWE) for each locus and their averages were calculated using Popgene 32 software [23].

The Genepop 3.1b program [24] was used to test whether the data obtained are significant deviations from Hardy-Weinberg equilibrium and the occurrence of connection imbalance between the loci analyzed. This program uses a method of Markov chain to get an unbiased estimate of Fisher's exact test to detect a significant deficiency or excess of heterozygotes [25].

The differentiation between the populations was evaluated by comparing peer-to-peer two ways: 1. Estimates F_{ST} [26], using the Arlequin 3.5 program [22] and 2. Estimates R_{ST} [27], by program RSTCalc [28].

R_{ST} is an analog of Wright F_{ST}, which is based on the stepwise mutation model (SMM) for microsatellite loci and has the particularity of not displaying associated bias to differences in the size of the alleles in the samples of populations and / or differences the variance between

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Locus	Primer sequences (5'–3')	Repeat motif (5'–3')	T (°C)	Size range
Hm 2	GTTACTCGGGCTCATGGGTA	(GT)8A(TG)21	66.5	171
	TAGGGCTGAAGGTGAGTGCT			
Hm 4	CGTGCATCACTGGAGTCTTC	(TAAAAA)3	64.5	204
	ATGAAGGATGTCTGCGCTTT			
Hm 5*	GCAGCTACAGGGCATACTCC	$(AC)_9TG(CA)_5$	66	183
	CTCCCTGTCTCTGCACTTCC			
Hm 6	ACCACCATGCTTTAGCCAAG	(TG)5	64.5	178
	CTCTTGAGCCAGGAAACAGG			
Hm 7	GCCCCACGGCTATACATACA	(CA)23	56.4	222
	CTCTTGCTTACGCGTGGACT			
Hm 9	CCCCTTCTCATCGGAGAGTT	(TG)5	64.5	298
	CACAGACTGCATGCCACAC			
Hm 10	CCCGAGGCACTGTAGGTTAG	(GA)9	66	239
	ATGTGGGAATCCTGGTTCAG			
Hm 11	ATCAGTGCACCAGCATCAAG	(TG)5CA(TG)7	64.5	285
	CATCCTTGTGGGGATTTTTG			
Hm 12	CACCAGCACAGCTGATGATTA	(GA)12GC(GA)11	63.5	127
	GAGGCCCCTACAGTCACATT			
Hm 13*	GGACAAGGTTGTGTGGGTAAG	$(TG)_8$	66	162
	GGAGTAGTGACCCGTCTTCG			
Hm 14	TGGTGAAACATACCCTGTCG	(TG)31	68.1	125
	GAGGACACGAGAGAGTCACTGATA			
Hm 15	GAGTCTCCACACCACCTGCT	(CA)13CG(CA)5	58.8	125
	GAGCCTTTGTTATCTGGCTCA			
Hm 16	GTGAATTGGTGTTTCTAAAGTGG	(TG)15	60.8	100
	CCCTAGACAGGGTGCTACTCC			
Hm 17	GTTTCTAAAGTGCCCCTTAGTGTG	(TG)19A(GT)5	70.5	113
	GGCGCCACTCCATCGTAG			
Hm EH1	GTCTCCTCCCACAGTCCAAA	(TG)18	53.2	152
	GGGCAGGAACAACCCTAGAC			
Hm EH2	CTGCCCTGCTCTCGTGTTAT	(TG)21	53.2	257
	AATTCATTAAAAATCCTCAGCGTA			
Hm EH3	GTTTCCTCCCACAGTCCAAA	(TGGA)13	53.2	300
	AAAAATCGAAGGACAGGTAAAA			

*Polymorphic.

Table 1. Characteristics of microsatellite loci isolated from *Hypophthalmus marginatus*.

loci. Some authors argue that the estimates of FST show lower when compared to RST estimates in same analysis [29].

The existence of linkage disequilibrium between the loci was evaluated by Genepop 3.4 program [24].

3. Results

Among a total of 98 sequenced recombinant clones, 61 clones presented microsatellites. After sequence analysis, it was found that 41 clones showed more than four repeats of microsatellites and we have designed and purchased primers for 17 of SSRs.

Among the 17 selected microsatellite sequences were a hexanucleotide, a tetranucleotide, and 15 dinucleotide repeats. Of 15 dinucleotide repeats, nine were perfect (5–31 repetitions) and six were composite dinucleotide repeat type (**Table 1**). Of the 17 loci, only two (Hm 5 and Hm 13) were polymorphic among studied samples.

The average number of alleles (A) observed in five populations examined ranged from 3.5 to 4.5, while the average observed heterozygosity (Ho) ranged from 0.3 to 0.6 (**Table 2**). The allelic frequency was less homogeneous to the Hm 5 locus than for the Hm 13; its most frequent allele was the same for all populations (**Table 3**).

Significant values of F_{st} were observed in all comparisons including Abaetetuba and the comparison of Tucuruí × Conceição (**Table 4**) gave negative R_{st} values representing interactions where the variance within a population exceeds the variance between populations.

4. Discussions

Although tested just by two polymorphic SSRs, genetic variability of *H. marginatus* population is under the influence of the Tucuruí Dam on the Tocantis River and as measured by heterozygosity test it is relatively low, regarding the number of alleles (absolute and effective). Few studies use less than six microsatellite markers in population studies [30]. Although we would like to stress that the results presented here are preliminary, they are consistent with results obtained with other molecular markers. The importance of our work is that it described a library that can be used to test for the polymorphism kind of economic importance to the Amazon.

Some populations of *H. marginatus*, such as those of Abaetetuba and Conceição, had remarkable low values. We could indicate that the heterozygosity was similar to that shown by other Siluriformes [31].

A comparison made elsewhere of *H. marginatus* cytochrome b gene with the same genes in other freshwater fishes [32, 33] indicates very low genetic diversity levels in *H. marginatus*, possibly reflecting a low mutation rate in this species [34] or a characteristic of Pimelodidae, as found in other studies [35].

The heterozygosity values provided by two microsatellite loci in *H. marginatus* in five populations analyzed did not differ between populations upstream and downstream of

Locus											
Hm 5						**Hm 13**					
	Relative frequencies						**Relative frequencies**				
Size (bps)	**Conceição**	**Abaetetuba**	**Itupiranga**	**Cametá**	**Tucuruí**	**Size (bps)**	**Conceição**	**Abaetetuba**	**Itupiranga**	**Cametá**	**Tucuruí**
175			0.1786	0.026		146	0.0667	0.0714			
177		0.786				148	0.1000	0.0714		0.132	0.079
181	0.1000	0.107	0.3571	0.421	0.526	150	0.6333	0.4286	0.6429	0.684	0.632
183					0.053	152	0.200	0.4286	0.3571	0.184	0.289
185	0.133		0.1071	0.132	0.158						
187	0.233		0.1429	0.079	0.158						
189	0.500	0.0714	0.2143	0.316	0.105						
191		0.0357		0.026							
195	0.033										

Table 2. Average allelic frequencies for two microsatellite loci in five populations of *Hypophthalmus marginatus*.

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Population (N)	*Locus*	A	Ae	Ho	He	P – EHW
Abaetetuba (14)	Hm 5	4.0	4.0	0.3	0.4	0.3
	Hm 13	4.0	4.0	0.4	0.6	0.1
	Mean (SD)	**4.0 (0.0)**	**4.0 (0.0)**	**0.3 (0.1)**	**0.5 (0.2)**	**0.2 (0.1)**
Cametá (19)	Hm 5	6.0	5.5	0.6	0.7	0.2
	Hm 13	3.0	3.0	0.4	0.5	0.2
	Mean (SD)	**4.5 (2.1)**	**4.2 (1.8)**	**0.5 (0.1)**	**0.6 (0.1)**	**0.2 (0.0)**
Itupiranga (15)	Hm 5	5.0	5.0	0.7	0.8	0.1
	Hm 13	2.0	2.9	0.4	0.5	1.0
	Mean (SD)	**3.5 (2.1)**	**4.0 (1.5)**	**0.6 (0.2)**	**0.6 (0.2)**	**0.5 (0.6)**
Tucuruí (19)	Hm 5	5.0	4.9	0.6	0.7	0.4
	Hm 13	3.0	3.0	0.4	0.5	0.3
	Mean (SD)	**4.0 (1.4)**	**3.9 (1.3)**	**0.5 (0.1)**	**0.6 (0.1)**	**0.3 (0.1)**
Conceição (15)	Hm 5	5.0	4.9	0.5	0.7	0.4
	Hm 13	4.0	4.0	0.5	0.6	0.9
	Mean (SD)	**4.5 (0.7)**	**4.4 (0.6)**	**0.5 (0.0)**	**0.6 (0.1)**	**0.6 (0.3)**

N = sample size; A = number of alleles; Ae = allelic richness; Ho = observed heterozygosity; He = expected heterozygosity second Nei (1973); SD = Standard deviation.

Table 3. Variability intrapopulation in five populations of *Hypophthalmus marginatus*.

Population	*Conceição*	*Abaetetuba*	*Itupiranga*	*Cametá*	*Tucuruí*
Conceição	****	0.28 (0.000)	0.05 (0.05)	0.04 (0.09)	0.10 (0.001)
Abaetetuba	0.71 (0.0000)	****	0.24 (0.00)	0.28 (0.00)	0.27 (0.000)
Itupiranga	0.35 (0.0006)	0.26 (0.0061)	****	0.005 (0.41)	0.006 (0.350)
Cametá	0.20 (0.0100)	0.47 (0.0002)	0.04 (0.19)	****	0.008 (0.350)
Tucuruí	0.41 (0.0000)	0.42 (0.0000)	-0.02 (0.63)	0.02 (0.223)	****

Table 4. Comparisons of peer to peer between the populations of *H. marginatus* analyzed, with F_{ST} values (above the diagonal) calculated [21]according to Weir and Cockerham (1984), with their respective *P*-values (in quotes) and values of R_{st} (below the diagonal) calculated according to Michalakis and Excoffier (1996) with their respective *P*-values (in quotes).

Tucuruí Dam. In these populations, the observed heterozygosity was lower than expected heterozygosity for all populations, thus indicating that there is no evidence of population bottleneck. On the other hand, the distribution of heterozygosity did not vary much in comparison with other species of commercial fish such as arowana (*Osteoglossum bicirrhosum* (Vandelli 1829)) [15] or pirarucu (*Arapaima gigas*) [16].

There is no much information on populations of *H. marginatus*, or other *Hypophthalmus*, as this knowledge would have been instrumental in understanding the effects of the Tucuruí Dam on the populations of *H. marginatus* Tocantins and Araguaia. The values of F_{st} and R_{st} showed little differentiation among populations of the same side of the current course of the Tocantins River, for example, populations of Conceição and Itupiranga upstream of Tucuruí Dam or

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Abaetetuba and Cametá downstream of the same dam gave values of F_{st} low, as between populations separated by the dam as Abaetetuba and Conceição, accented F_{st} values indicating greater differentiation among populations, probably generated by low levels of gene flow.

Laroche and Durand [36] studied the genetic structure of the Percidae *Zinger asper* populations, an endangered endemic species, affected by the construction of a dam built on the River Rhone in France, and found significant genetic differences between upstream and downstream populations.

5. Preliminary conclusions

If low differentiation is prevalent, the geographic distribution range of large samples would be recommended for genetic analyses. It would also be useful to assess the levels of genetic variability within and among populations from different basins for a better understanding of population dynamics of these species. Although our conclusions were made by using only two microsatellite loci analyses, results are consistent with data from mitochondrial markers.

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