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Comparative cytogenetic survey of the giant bonytongue *Arapaima* fish (Osteoglossiformes: Arapaimidae), across different Amazonian and Tocantins/Araguaia River basins

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The South American giant fishes of the genus *Arapaima*, commonly known as pirarucu, are one of the most iconic among Osteoglossiformes. Previously cytogenetic studies have identified their karyotype characteristics; however, characterization of cytotaxonomic differentiation across their distribution range remains unknown. In this study, we compared chromosomal characteristics using conventional and molecular cytogenetic protocols in pirarucu populations from the Amazon and Tocantins-Araguaia river basins to verify if there is differentiation among representatives of this genus. Our data revealed that individuals from all populations present the same diploid chromosome number 2n=56 and karyotype composed of 14 pairs of meta- to submetacentric and 14 pairs of subtelo- to acrocentric chromosomes. The minor and major rDNA sites are in separate chromosomal pairs, in which major rDNA sites corresponds to large heterochromatic blocks. Comparative genomic hybridizations (CGH) showed that the genome of these populations shared a great portion of repetitive elements, due to a lack of substantial specific signals. Our comparative cytogenetic data analysis of pirarucu suggested that, although significant genetic differences occur among populations, their general karyotype patterns remain conserved.

Keywords: CGH, Chromosome banding, Fish cytotaxonomy, Osteoglossomorpha, rDNA FISH.

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Os peixes gigantes da América do Sul do gênero *Arapaima*, comumente conhecidos como pirarucus, são um dos mais icônicos de Osteoglossiformes. Estudos citogenéticos prévios identificaram suas características cariotípicas, entretanto a caracterização da diferenciação citotaxonômica através de suas distribuições geográficas ainda são desconhecidas. Nesse estudo, nós comparamos características cromossômicas utilizando técnicas de citogenética clássica e molecular em populações das bacias dos rios Amazonas e Tocantins-Araguaia, a fim de verificar se há alguma diferenciação entre representantes desse gênero. Nossos dados revelaram que indivíduos de todas as populações apresentam número diploide de 2n=56 cromossomos e que seus cariótipos são compostos de 14 pares de cromossomos meta- e submetacêntricos e 14 pares de subtelo- e acrocêntricos. Os sítios maiores e menores de rDNA estão localizados em pares cromossômicos separados, onde os sítios maiores de rDNA correspondem a grandes blocos heterocromáticos. Hibridizações genômicas comparativas (CGH) mostraram que o genoma dos espécimes dessas populações é amplamente compartilhado, devido à falta de sinais substanciais específicos. Nossos dados de citogenética comparativa do pirarucu sugerem que embora diferenças genéticas significativas ocorram entre populações, os padrões cariotípicos gerais se mantêm conservados.

Palavras-chave: Bandamento cromossômico, CGH, Citotaxonomia, Osteoglossomorpha, DNAr FISH.

INTRODUCTION

Distributed throughout South America, Africa, Australia, and South Asia realms, the Osteoglossiformes represents one of the ancient teleost lineages containing several taxa important for fisheries and aquaculture (Castello *et al*., 2009; Medipally *et al*., 2016; Maldonado *et al*., 2017).

Fishes of the genus *Arapaima* Müller, 1843, commonly named as pirarucu, are among the largest freshwater fish species, reaching up to two and a half meters in length and 200 kg in weight (Nelson *et al*., 2016). Their natural distribution extends throughout the Brazilian basins of Tocantins-Araguaia, Amazon, and Essequibo (Guyana) rivers, usually in lakes and showing a preference for calm waters, known as "várzeas" (Lowe-McConnell, 1987; Queiroz, 2000; Castello, 2008; Reis *et al*., 2016).

Despite their high economic and conservation importance, as well as the voluminous literature about them, few information is available focusing on karyotype structure and mapping of chromosomal markers in pirarucu (Marques *et al*., 2006; Rosa *et al*., 2009; Oliveira *et al*., 2019). The first karyotype description of pirarucu, under the name *Arapaima gigas* (Schinz, 1822) (from Araguaia River, Araguaiana - MT), reported a diploid chromosome number 2n=56, karyotype containing 14 m-sm + 14 st-a pair of chromosomes and only one chromosome pair bearing Nuclear Organizer Regions (NORs) (Marques *et al*., 2006). These results were also observed in a recent reassessment (Oliveira *et al*., 2019), and it was observed that the karyotype of pirarucu remarkably differs from its sister taxon, the African *Heterotis niloticus* (Cuvier, 1829), the latter possessing 2n=40 with different content and distribution patterns of repetitive DNAs.

The genus *Arapaima* was considered monotypic since Günther (1867), who synonymized earlier described species with *A. gigas*. However, a recent taxonomic revision of species diversity in the genus indicated the validity of the species, namely *A. arapaima* (Valenciennes, 1847), *A. mapae* (Valenciennes, 1847), *A. agassizii* (Valenciennes, 1847) and the proposition of the new species *A. leptosoma* Stewart, 2013. But, recent publications analyzing the genetic diversity present in *Arapaima* considered it as a monotypic genus (Farias *et al*., 2019; Torati *et al*., 2019). In this study, although all analyzed specimens have been classified as *A. gigas* based on morphological characteristics during voucher deposit, we still accept the potential existence of other cryptic species as proposed by Stewart (2013a,b).

Cytogenetic techniques have been frequently used as helpful tools on fish species with complex taxonomy, as well as to characterize fish biodiversity (Artoni *et al*., 2006; Bertollo, 2007; Pansonato-Alves *et al*., 2013; Cioffi *et al*., 2018; Pazza *et al*., 2018). Furthermore, it is now possible to gain insights into evolutionary relationships using comparative genomic hybridization (CGH), a useful approach for fish genomes characterization (Symonová *et al*., 2015), as demonstrated for Lebiasinidae (Sassi *et al*., 2020), Notopteridae (Barby *et al*., 2019) and Osteoglossidae (Cioffi *et al*., 2019) fish families.

In this study, we analysed individuals of *Arapaima gigas* from regions covering part of its geographical distribution from the Amazon and Tocantins-Araguaia rivers basins. We applied conventional and molecular cytogenetic procedures, including chromosomal distributions of repetitive DNAs and CGH among populations from both river systems to assess potential divergences in their genomes. The main objective of our study was to investigate whether the cytogenetic data support different evolutionary patterns among populations of pirarucu fishes or if they represent a case of taxonomic diversity not associated with cytotaxonomic differentiation despite their wide geographical distribution.

MATERIAL AND METHODS

Material, conventional and molecular cytogenetic protocols. Samples of pirarucu from six localities (three from the Amazon river basin and three from the Tocantins-Araguaia river basin) were analyzed (Tab. 1, Fig. 1). Voucher specimens were deposited in the Museu de Zoologia da Universidade de São Paulo (MZUSP) (Tab. 1).

Metaphase chromosomes were obtained using the conventional air-drying method (Bertollo *et al*., 2015), with some adaptations given by Oliveira *et al.* (2019). Conventional staining was done using 5% Giemsa solution in phosphate buffer, pH 6.8, for 10 min. C-positive heterochromatin was detected following Sumner (1972). A total of 10 repetitive DNA sequences, namely three multigene families (U2 snDNA, 5S and 18S rDNAs) and seven microsatellite repeat motifs $(A)_{30}$, $(CA)_{15}$, $(GA)_{15}$, $(CAC)_{10}$, $(CGG)_{10}$, $(GAA)_{10}$ and $(GAG)_{10}$ were isolated and mapped by fluorescence *in situ* hybridization (FISH) procedures, following Yano *et al.* (2017). The microsatellite probes were directly labeled with Cy3 during synthesis according to Kubat *et al.* (2008). Both rDNA sequences (18S and 5S rDNAs) were produced following Cioffi *et al.* (2009) and Pendás *et al.* (1994), respectively. The U2 snDNA probe was produced according to

Silva *et al.* (2015). All these probes were directly labeled with Spectrum Orange-dUTP by nick translation, according to manufacturer's recommendations (Roche, Mannheim, Germany), with the exception of 5S rDNA, which was directly labeled with Spectrum Green-dUTP, also by nick translation (Roche, Mannheim, Germany).

TABLE 1 | Localities, numbers, sex and voucher codes of *Arapaima* individuals analyzed in this study. Voucher specimens were deposited in the Museu de Zoologia da Universidade de São Paulo (MZUSP).

Locality Description	N	Code	Basin	Geographical coordinates	Deposit number
Castanho Lake, Manaus, AM	04♂, 05♀	CAS	Am	3°42'48.4"S 60°31'11.6"W	123955
Panta Leão Lake, Mamirauá Reserve, Alvarães, AM	07♂, 08♀	MAM	Am	2°54'58.1"S 64°49'29.1"W	123953
Onças Lake, Codajás, AM	11♂, 06♀	COD	Am	3°53'17.1"S 62°07'36.2"W	123954
Marginal lake to the Santa Tereza River, tributary of the Tocantins River, Peixes, TO	06♂, 04♀	PEI	To-Ar	11°54'31.7"S 48°38'02.7"W	121642
Marginal lake to the Javaé River, tributary of the Araguaia River, Lagoa da Confusão, TO	07♂, 06♀	JAV	To-Ar	11°00'27.1"S 49°56'01.8"W	121639
Xavantinho River, tributary of the Araguaia River, São Félix do Araguaia, MT	09♂, 08♀	SFA	To-Ar	11°42'07.2"S 50°50'15.4"W	121643

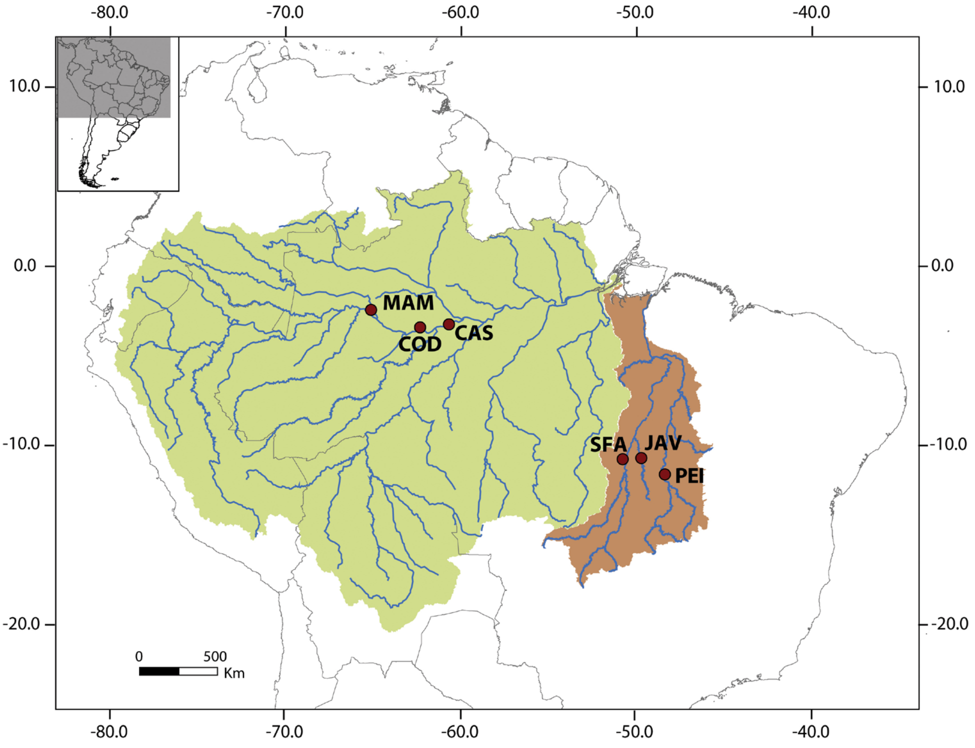

FIGURE 1 | Map of northern part of South America showing the sampling sites of *Arapaima* individuals analyzed in this study from Araguaia-Tocantins (brown) and Amazon (green) River basins, coded in the Tab. 1.

Comparative Genomic Hybridization (CGH). CGH experiments were performed to compare the genomic composition of individuals from populations located in different hydrographic basins. The gDNA of individuals from the JAV population (Tocantins-Araguaia basin) was compared with the gDNA of individuals from the MAM population (Amazon basin), by using probes of labeled gDNA isolated from individuals of each basin against metaphase chromosomes from samples of each basin. For this purpose, the gDNA of individuals from the MAM population was labeled with digoxigenin-11-dUTP using DIG-nick-translation Mix (Roche, Mannheim, Germany), while the gDNA of individuals from JAV population was labeled with biotin-16-dUTP using BIO-nick-translation Mix (Roche). For blocking the repetitive sequences, we used C_0t-1 DNA prepared according to Zwick *et al.* (1997), in all experiments. The final probe cocktail for each slide was composed by 500ng of gDNA of individuals from the MAM population + 500ng of gDNA of individuals from the JAV population + 15μg of derived C_0t-1 DNA of each specimen. The probes were ethanol-precipitated, and the dry pellets were suspended in hybridization buffer containing 50% formamide, 2×SSC, 10% SDS, 10% dextran sulfate and Denhardt's buffer, pH 7.0.

Microscopic Analysis and Image Processing. At least 30 metaphase spreads per individual were analyzed to confirm the 2n, karyotype structure, and results of FISH experiments. Images were captured using an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan) with Cool SNAP, and processed using Image-Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a) and according to Levan *et al.* (1964).

RESULTS

Karyotypes, chromosome banding, and rDNA FISH. Both males and females from all populations had conserved diploid chromosome number 2n=56, with karyotypes composed of 28 m-sm + 28 st-a chromosomes and a number of chromosomal arms (NF value) of 84 (Fig. 2). C-positive heterochromatic bands were distributed in the pericentromeric regions of all chromosomes, except for some additional conspicuous telomeric blocks in some chromosome pairs (Fig. 2). The 5S and 18S rDNA sites were interstitially located on the long (q) arms of the pair No. 1 and on the short (p) arms of the pair No. 2, respectively (Fig. 2).

Microsatellites and U2 snDNA distribution. The $(GC)_{15,}$ $(CAA)_{10,}$ $(CAC)_{10,}$ $(CAG)_{10}$, $(CAT)_{10,}$ $(CGG)_{10,}$ $(GAA)_{10}$ and $(GAG)_{10}$ repeats displayed scattered hybridization signals throughout the genome of all individuals from all populations (Figs. 3–4). Specifically, $(CGG)_{10}$ repeats displayed conspicuous signals corresponding to the NORs (Figs. 3–4). No significant inter/intrapopulation variation regarding the microsatellite distribution was observed. The U2 snDNA was mapped onto the interstitial position of one small acrocentric pair in individuals from all populations. To demonstrate these patterns, an individual from a representative population from each basin, *i.e.*, Javaé (To-Ar) and Mamirauá (MAM), were selected and here presented. The other results can be found in the supplementary figures **S1**, **S2**, **S3** and **S4**.

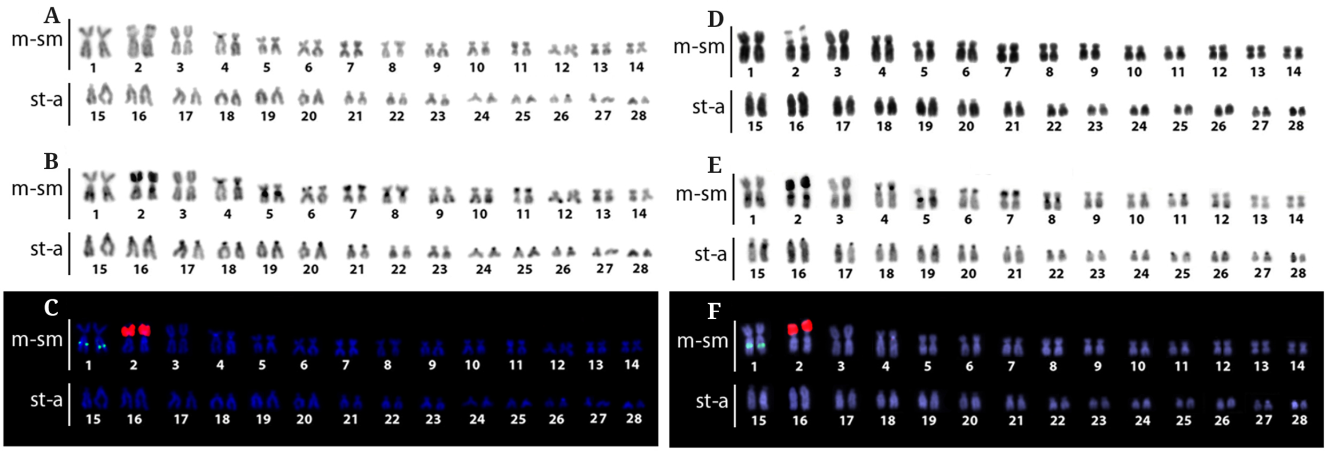

FIGURE 2 | Karyotypes of *Arapaima gigas* male (**A**–**C**) and female (**D**–**F**) from Javaé (JAV) population (Araguaia-Tocantins River basin), sequentially arranged from Giemsa-stained (**A** and **D**), C-banded (**B** and **E**), and double-FISH (**C** and **F**) with 5S rDNA (green) and 18S rDNA (red) labeled chromosomes. Bar=5 µm.

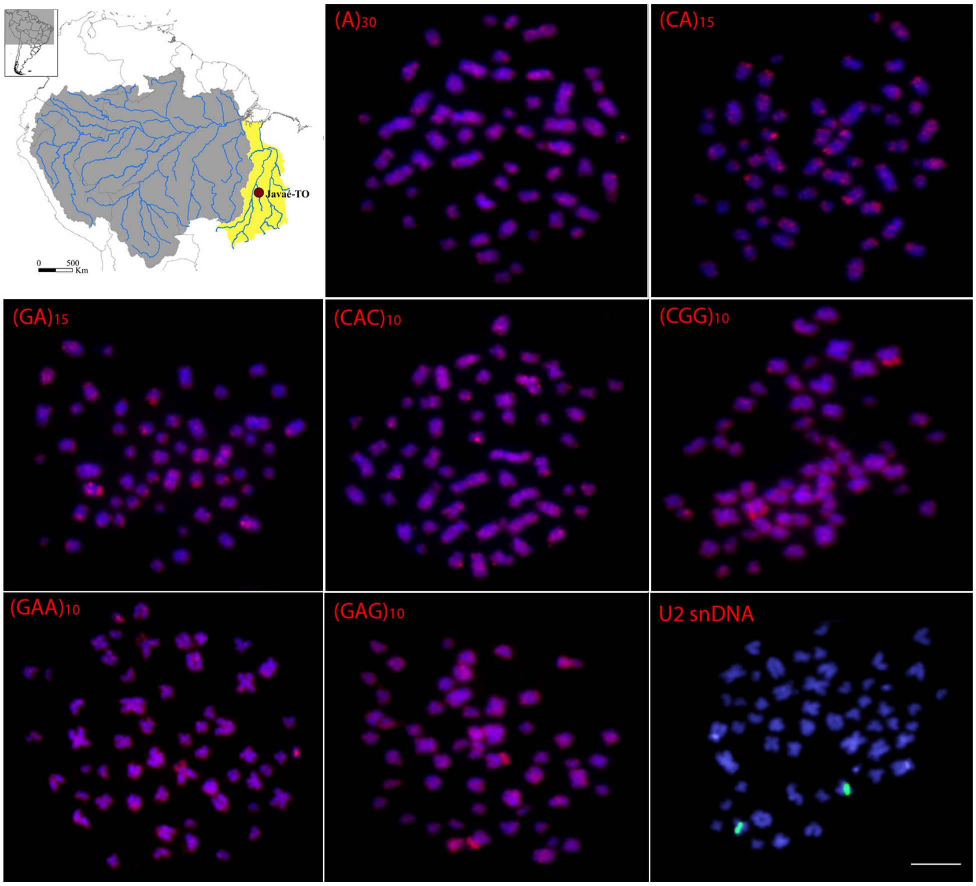

FIGURE 3 | Metaphase plates of *Arapaima gigas* from Javaé (JAV) population (Araguaia-Tocantins River basin) hybridized with repetitive DNA sequences, including mono-, di- and trinucleotide microsatellites and the multigene families U2 snDNA. Bar=5 µm.

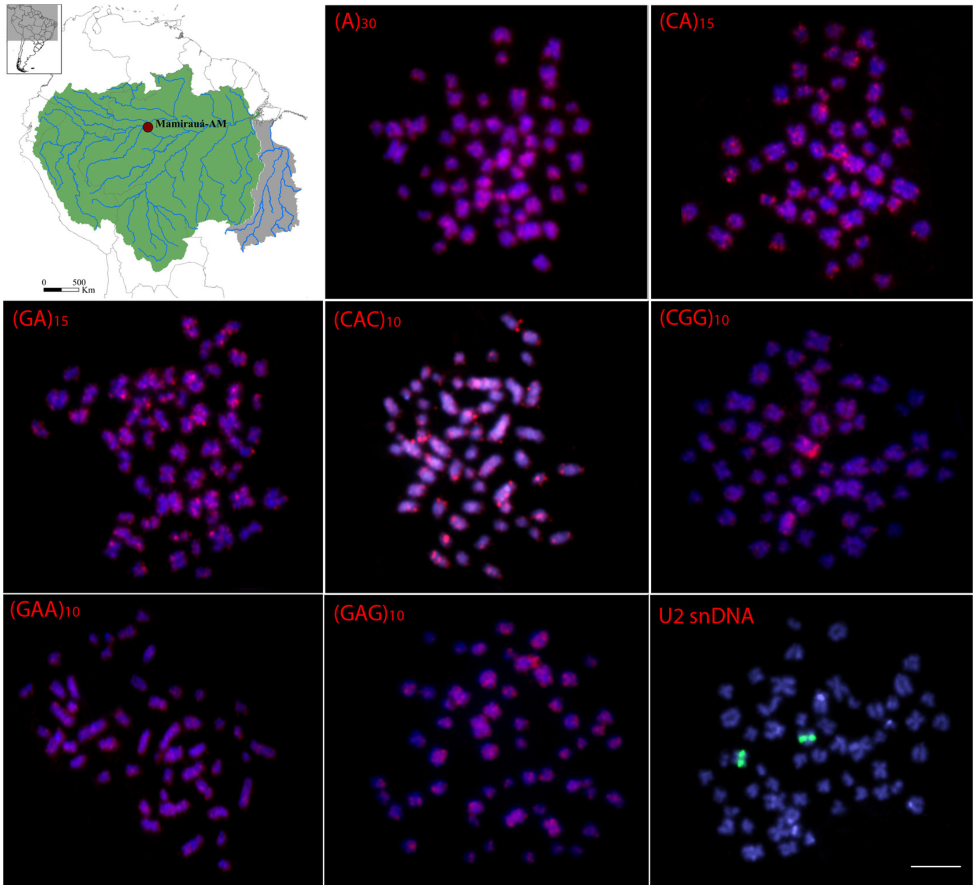

FIGURE 4 | Metaphase plates of *Arapaima gigas* from Mamirauá (MAM) population (Amazon River basin) hybridized with repetitive DNA sequences, including mono-, di- and trinucleotide microsatellites and the multigene families U2 snDNA. Bar=5 µm.

Comparative Genomic Hybridization. The comparative hybridization of gDNAs probes of individuals from JAV (To-Ar) and MAM (Am) populations produced essentially a large number of overlapping signals, highlighting the heterochromatic blocks that occurred in the centromeric and terminal regions of some chromosomes. Some of these signals were considerably stronger and linked to the major rDNA sites. However, the hybridization against metaphase chromosomes of individuals from the MAM population particularly highlighted some over-represented signals, probably linked to a higher copy number of repetitive sequences in some chromosomal regions (Fig. 5).

DISCUSSION

Cytogenetic and genomic characteristics among basins. The same cytogenetic characteristics, *i.e.*, the same karyotype structures and the same patterns of chromosomal mapping of repetitive DNAs, were found for both sexes from all the populations in both basins. Therefore, our results agree with the previous reports of Marques *et al.* (2006), Rosa *et al.* (2009) and Oliveira *et al.* (2019). The CGH comparison, an appropriate

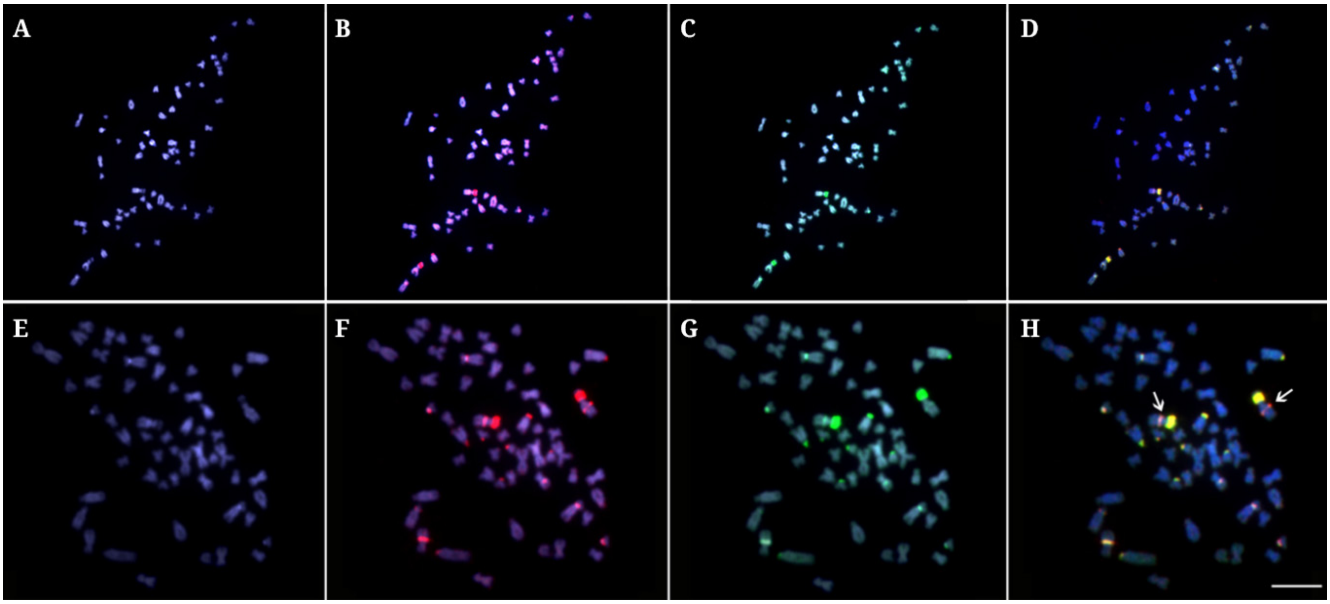

FIGURE 5 | Comparative genomic hybridization (CGH) experiments on metaphase chromosomes of *Arapaima* individuals from the Javaé (JAV) (**A**–**D**) and Mamirauá (MAM) populations (**E**–**H**). First column (**A** and **E**): DAPI images of chromosomes; Second column (**B** and **F**): Hybridization pattern using gDNA of *A. gigas* from MAM population (red): Third column (**C** and **G**): Hybridization pattern using the gDNA of *A. gigas* from JAV population (green); Fourth column (**D** and **H**) overlap of the images. The shared regions are highlighted in yellow. The arrows indicate the higher abundance of some repetitions in individuals from MAM population. Bar=5 µm.

approach for comparing the genome divergence between closely related species and/or populations, as well as hybrids (Symonová *et al*., 2015; Moraes *et al*., 2017; Sember *et al*., 2018; Sassi *et al*., 2019), showed a low genomic divergence between the populations from the To-Ar and Am basins.

Karyotypic and morphological patterns are often indicators of the lifestyle of a species (Pellestor *et al*., 2011; Rabosky *et al*., 2013). *Arapaima* fishes are considered sedentary animals having low migratory activities, with a preference for low-oxygenated lentic environments, specialized parental care and a high degree of endogamy (Hrbek *et al*., 2005). Fish species with such behavioral characteristics usually display a high level of karyotype diversity among populations, as observed in the genus *Channa* Scopoli, 1777 (Channidae) (Cioffi *et al*., 2015), in the Erythrinidae species *Hoplias malabaricus* (Bloch, 1794) and *Erythrinus erythrinus* (Bloch & Schneider, 1801) (reviewed in Bertollo, 2007 and Cioffi *et al*., 2012), and in the Synbranchidae species *Synbranchus marmoratus* Bloch, 1795 and *Monopterus albus* (Zuiew, 1793) (reviewed in Supiwong *et al*., 2019), among others. However, due their complicated and not fully solved taxonomy, *Arapaima* seems to represent another case of higher taxonomic diversity, which is not associated with differentiated cytogenetic parameters. Examples of these processes can be observed in the genus *Esox* Linnaeus, 1758 (Symonová *et al*., 2017), in Leuciscidae family (Ayata *et al*., 2019) and in many marine fish groups (reviewed in Molina, 2007).

However, it is noteworthy that this low karyotype chromosomal differentiation in both karyotype structure and repetitive DNA levels is not substantiated by single nucleotide polymorphism (SNPs) dataset analyses. Indeed, our previous studies demonstrated a low genetic diversity for *A. gigas* populations from the To-Ar basin, but not for those from the Am basin, where a higher diversity level comparable to the SNP diversity of other taxa from the same region was detected (Oliveira *et al*., 2020). This lower genetic diversity for the To-Ar populations, especially from those of the upper section of the basin, was also found by additional studies (Alencar-Leão, 2009; Vitorino *et al*., 2017; Torati *et al*., 2019).

These contrasting cytogenetic and sequence patterns presented by pirarucu populations may be associated with multiple factors. Among them, a population homogenizing effect over time can be attributed to the hydrological dynamics of the region where these fishes are currently found. Indeed, the Brazilian Amazon region shows long flooding duration and flow cycles allowing the fish dispersion for long periods, a process known as lateral migration (Castello, 2008). Besides, rivers from the To-Ar basin are situated in an active tectonic depression, which resulted in the development of extensive alluvial plains. In this scenario, there is evidence for ichthyofaunal exchanges among the headwaters of the Tocantins River and all other major drainages on its boundaries, including several ones from the Am basin (*e.g*., Xingu River) (Rossetti, Valeriano, 2007; Lima, Ribeiro, 2011). Besides that, along with several other fishes, *Arapaima* has shown a population decline due to the loss of natural habitats and commercial over-exploitation (Allan *et al*., 2005; Castello *et al*., 2011). In addition to this recent decline due to anthropogenic activities, paleogeographic reconstructions (Oliveira *et al*., 2019) indicated that during the last glacial period the pirarucu fishes distribution was constrained under severe climatic conditions, with scattered suitable habitats and restricted areas of refuge. Additionally, a deep leaning model analysis indicates that the Tocantins-Araguaia river basin was colonized by the population from the Amazon river basin (Oliveira *et al*., 2020).

Altogether, these features possibly played a major role in shaping modern genetic diversity and chromosomal conservatism among populations. In this sense, the pattern of genetic differentiation among distinct populations most likely arose due to natural and human actions reducing their size and distribution area providing differentiation over time. Under these size fluctuations, an accentuated genetic drift and bottleneck effects are not also ruled out (Wright, 1931; Lande, 1988). On the other hand, chromosomal differentiation usually requires more time and more specific conditions to be fixed, such as complete geographic isolation of small populations without gene flow among them. In this respect, the regional hydrological dynamism may act in the opposite direction preventing major changes in the karyotypes.

Despite the high economic importance of pirarucu fishes, research on their evolutionary, genetic and chromosomal characteristics deserves more in-depth studies. This study focused, for the first time, on a comparative cytogenetic survey of pirarucu populations from the Amazon and Tocantins-Araguaia rivers basins, in order to characterize possible karyotype differentiation among them. It was shown that those populations retain a remarkable karyotype and/or chromosomal stability, as revealed both by conventional and molecular cytogenetic markers.

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AUTHOR CONTRIBUTION

Ezequiel A. de Oliveira: Conceived and the study, Conducted the experiments, Analyzed the data, Wrote the manuscript, Read and approved the final version.

Francisco de M. C. Sassi: Wrote the manuscript, Read and approved the final version.

Manolo F. Perez: Conceived and the study, Conducted the experiments, Read and approved the final version.

Luiz A. C. Bertollo: Conceived and the study, Analyzed the data, Wrote the manuscript, Read and approved the final version.

Petr Ráb: Wrote the manuscript, Read and approved the final version.

Tariq Ezaz: Analyzed the data, Wrote the manuscript, Read and approved the final version.

Terumi Hatanaka: Conducted the experiments, Read and approved the final version.
Patrik F. Viana: Conducted the experiments, Read and approved the final version.
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Edivaldo H. C. de Oliveira: Wrote the manuscript, Read and approved the final version.
Marcelo de B. Cioffi: Conceived and the study, Conducted the experiments, Analyzed the data, Wrote the manuscript, Read and approved the final version.

ETHICAL STATEMENT

Individuals were captured using traps with the proper authorization of Brazilian Environmental Agencies (ICMBIO/ SISBIO - License No. 48290-1 and SISGEN (License No. A96FF09). Laboratory experiments followed ethical conduct and were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (UFSCar – Process number CEUA 9506260315).

COMPETING INTERESTS

The authors declare no competing interests.

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