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CONTRIBUTIONS OF CONVENTIONAL AND MOLECULAR CYTOGENETICS TO TAXONOMIC AND EVOLUTIONARY STUDIES OF NEOTROPICAL FISHES

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Abstract: The present work aimed, through the method of bibliographic review, to show that conventional and molecular cytogenetics is one of the tools for taxonomic and evolutionary studies of neotropical fishes. Based on the methodology used, which was divided into two stages, the first being booking and data collect and the second the analysis and discussion of the results, we got three studies that used cytogenetics in fishes and whose results (indications that chromosome dynamics may be involved in evolution and speciation) contributed to what we intended to show in this work: the assertion that conventional and molecular methods of cytogenetics in the study of fishes have much to add to the scientific knowledge of evolutionary biology and taxonomy of this group of vertebrates.

Keywords: Conventional cytogenetics, Molecular cytogenetics, Evolutionary Studies, Taxonomic studies, Neotropical fishes.

INTRODUCTION

The fish group is a vast group of aquatic vertebrates, whose fundamental characteristics for definition are the presence of fins and gill respiration. This taxon comprises the oldest and most diverse within the Vertebrata clade, constituting five of the nine current vertebrate classes and about half of the approximately 58,000 vertebrate species (HICKMAN et al., 2016).

Regarding the geographic distribution of fishes, it can be said that ichthyofauna covers numerous regions and, among these, the Neotropical. Neotropical waters, which comprise the continental environments of the extreme south of North America, all Central and South America, have the most diverse freshwater ichthyofauna on the planet, with more than 7,000 fishes' species, but estimates are based on around a much larger number. In the Amazon basin, for example, the number of described species is 2,266, that is, 15% of the total known to science and the greatest diversity of freshwater fishes in the world (ALBERT e REIS, 2011; GOLDANI, 2012; AMAZON FISH PROJECT, 2021).

Despite valid numbers and estimates, many ichthyofauna individuals are not known or are poorly studied from a cytogenetic point of view. However, the fish group is one of the most interesting for studies of genetic variability and evolution among vertebrates, because, in addition to the unique aspects of each species, the most of species are karyotypically unique. "This diversity is, in part, what makes understanding their evolutionary history and establishing a classification so challenging and, at the same time, fascinating" (WHITE, 1978; NELSON et al., 2016; p. 1).

In this scenario, cytogenetics, which studies chromosomes, proves to be an excellent tool that can assist in taxonomic and evolutionary studies of this important group of vertebrates, since in taxonomy there are still many unresolved issues regarding the systematization/classification of these animals (WHITE, 1978 apud ZIERMNICZAK, 2011).

On cytogenetic techniques for the study of fishes, there are the conventional ones, being the C-banding, for the differential staining of Constitutive Heterochromatin (CH) – repetitive DNA sequences with little or no transcriptional activity; and impregnation by silver nitrate (Ag-NOR), for the detection of Nucleolar Organizer Regions (NORs) – chromatin associated with the nucleolus, representing an important cytogenetic marker in fishes, as they correspond to the location of ribosomal genes 45S that were active during the last interphase, the most known; in addition to the use of base-specific fluorochromes (Chromomycin A_3 – $CMA_{3,}$ Mithramycin e 4',6-diamidino-2- phenylindole – DAPI) for the detection of regions rich in the nitrogenous bases Adenine-Thymine or Guanine-Cytosine

(SUMNER, 1972; HOWELL e BLACK, 1980; ARTONI, 2000).

As for molecular techniques, the detection of specific DNA sequences using molecular markers such as ribosomal DNA probes (rDNA) telomeric sequences, etc., extracted from other organisms, has been highlighted in recent decades, thus giving rise to Molecular Cytogenetics, in which Fluorescent *in situ* Hybridization (FISH) is the most used method in fishes. In this method, a fluorochrome-labeled DNA sequence (probe) pairs with the target complementary DNA to be analyzed. The use of FISH in chromosomal mapping of repetitive DNAs has allowed a more resolute analysis of the structure, behavior, and evolution of fish chromosomes (DA SILVA et al., 2021; DE SOUZA et al., 2021).

In general, conventional, and molecular cytogenetics have a lot to add to scientific knowledge regarding the systematics and evolutionary study of fishes. In the context of conventional cytogenetics, C-banding, for example, can show chromosomal differences between related species and populations of the same species based on their distribution pattern in the genome, proving to be an important marker for comparative cytogenetic analysis. Molecular cytogenetics, in turn, may suggest the occurrence of chromosomal rearrangements related to speciation. As an example, one can mention the genomic mapping in a cryptic species, *Gymnotus carapo*, which present great karyotypic diversity and that such diversity is much greater than what conventional cytogenetics supposes, that is, repetitive sequences in DNA, in general, are useful as markers for the study of genomic and chromosomal evolution, since they can be correlated with processes of reproductive isolation and speciation, resulting from the constant reorganization (MARTINS e GALETTI, 2001; SYMONOVÁ et al., 2003; NAGAMACHI et al., 2010; DA SILVA et al., 2021).

From this theoretical basis, the present work aimed to address the importance of cytogenetics as a tool to the taxonomic and evolutionary studies of fishes, emphasizing Amazonian species, based on the literature review of some published scientific articles, whose themes are related to cytogenetic analyzes of species that occur in rivers of the Amazon basin, as well as their phylogenetic implications.

METHODOLOGY

This work was performed using the bibliographic research method which, according to Gil (2008), consists of research that is developed from material already prepared, consisting mainly of books and scientific articles. Thus, the research was divided into two stages: booking and data collect; and analysis and discussion of the results:

BOOKING AND DATA COLLECT

A search for keywords such as “fishes cytogenetics”; “conventional cytogenetics”; “molecular cytogenetics”; “ribosomal DNA probes”; “fish systematic”; “neotropical fishes” etc. was performed on Google Scholar. Then, there was an exploratory reading of all the material acquired, aiming to verify if the consulted works were of interest for the present work. The articles acquired were selected based on two criteria: the specimens that were the object of research are neotropical; the techniques used in the study are conventional and molecular cytogenetics.

ANALYSIS AND DISCUSSION OF RESULTS

In the analysis of the results, there was a reading to order and summarize the information present in the authors’ productions and extract the most relevant ones, so that they made it possible to obtain content about the theme proposed in this work: contributions of conventional and molecular cytogenetics to taxonomic and evolutionary studies of neotropical fishes. Finally, the discussion of the results consisted of categories, which emerge from the previous moment (analysis of the results), analyzed, and discussed from a theoretical framework related to the theme of the work (GIL, 2008).

RESULTS AND DISCUSSION

From the bibliographic research, 10 articles were obtained, of which 3 were interesting for this work. Below are the titles and information of the chosen research – which will later be mentioned as the name of the respective author:

I. Chromosomal characterization of two species of the genus *Steatogenys* (Gymnotiformes: Rhamphichthyoidea: Steatogenini) from the Amazon basin: sex chromosomes and correlations with Gymnotiformes phylogeny, by Cardoso et al. (2011), published in Reviews in Fish Biology and Fisheries, DOI: 10.1007/s11160-010-9196-0;

II. Comparative cytogenetics of two species of the genus *Scobrinancristrus* (Siluriformes, Loricariidae, Ancristrini) from the Xingu River, Brazil, by Cardoso et al. (2013), published in Comparative Cytogenetics, DOI: 10.3897/CompCytogen.v7i1.4128;

III. *In situ* localization of ribosomal sites in *Peckoltia* and *Ancistomus* (Loricariidae: Hypostominae) from the Amazon basin, by Pety et al. (2018), published in Zebrafish, DOI: 10.1089/zeb.2017.1523.

Regarding the methods used in the three surveys, table 1 lists each author with their methods.

As for the findings and reflections of the research, the first, by Cardoso et al. (2011) brought information about chromosomal diversity within the genus *Steatogenys*, as well as the mechanisms that lead to chromosomal differentiation, from analyzes of *Steatogenys elegans* and *Steatogenys duidae* populations from the Amazon basin. The authors reported 2n = 50 for both species and different karyotypic formula, showing that the karyotypes remain conserved. There was heteromorphism in the karyotype of females with the ZZ/ZW

	Cardoso et al. (2011)	Cardoso et al. (2013)	Pety et al. (2018)
Conventional Method			
C-banding	X	X	
Ag-NOR	X	X	X
CMA_3 or DAPI	X	X	
Molecular Method			
FISH with 5S rDNA			X
FISH with 18S rDNA	X	X	X
FISH with telomeric sequences		X	

Table 1 – Authors and their research method

Source: Prepared by the author (2021).

sex chromosome system only in *S. elegans*, in which the presence of two heterochromatic blocks between a euchromatic is an indication that the W chromosome differed compared to the Z chromosome, leading to the hypothesis of evolution of this system: the ancestor heterochromatic block has been subdivided into two independent blocks through a paracentric inversion. About chromosomes and phylogenetic relationships in Steatogenini – a monophyletic tribe (comprises three genera: *Steatogenys*, *Hypopygus*, and *Stegostenopos*), whose position within the superfamily Rhamphichthyoidea (Rhamphichthyidae + Hypopomidae) is not very well defined currently, two hypotheses were considered by Cardoso et al. (2011), the first being by Albert (2001), who states that the tribe Steatogenini belongs to the family Hypopomidae (figure 1-a). The data obtained by Cardoso et al. (2011), when compared with a species of the family Hypopomidae (*Hypopygus lepturus*), show that 2n = 50 remained, but other species of this family would have a reduction in this number. The second hypothesis, in which Alves-Gomes et al. (1995) and Sullivan (1997) state that Steatogenini belongs to family Rhamphichthyidae (figure 1-b), fits better with the cytogenetic data obtained by Cardoso et al. (2011), as the karyotypes of Steatogenini species are more like *Rhamphichthys*, and the diploid number 2n = 50 would be synapomorphic (CARDOSO et al., 2011).

The second research, by Cardoso et al. (2013), brought the first karyotypic characterizations of sympatric species from the Xingu River, *Scobinancistrus aureatus* – endemic to the Xingu River, and *Scobinancistrus pariolispos*, of the genus *Scobinancistrus* – tribe Acistrini, subfamily Hypostominae and family Loricariidae. The authors reported that these species have the same diploid number – 2n = 52; differences in the karyotypic formula; constitutive heterochromatin distribution; Nucleolar Organizer Regions (NORs) only in pair 3 of each species, in the interstitial region of the long arms in *S. aureatus* and in the distal region of the short arms in *S. pariolispos*, both adjacent to the constitutive heterochromatin blocks. Based on these results, Cardoso et al. (2013) were able to verify that the same diploid number with different karyotypic formula is consistent with other species of Ancistrini. This can be explained by the occurrence of chromosomal inversions that modify the structure of chromosomes without changing their number, but 2n = 54 is a basal number

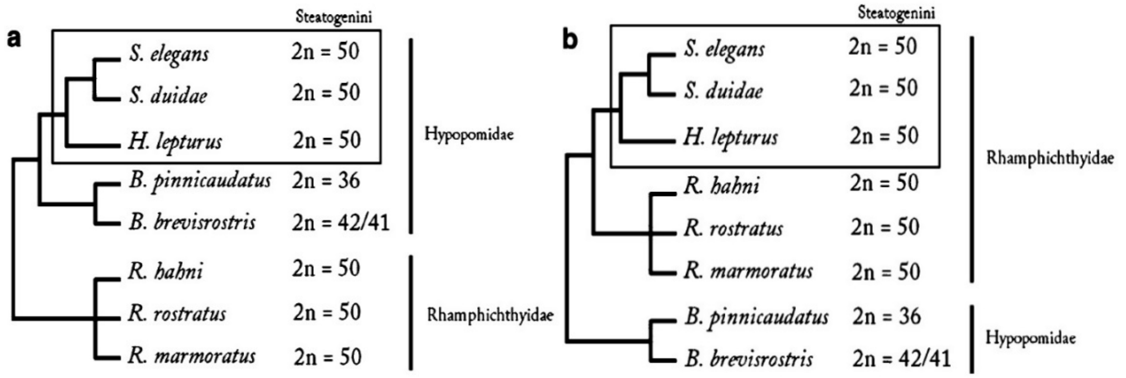

Figure 1 – Gymnotiformes phylogeny. a- Phylogenetic tree according to Albert (2001); b- Phylogenetic tree based on Alves-Gomes et al. (1995) and Sullivan (1997)

Source: Cardoso et al. (2011).

for Loricariidae, therefore, it is believed that the reduction to 2n = 52 for most Ancistrini species must be a result of fusion events. FISH with telomeric probes hybridized at the ends of all chromosomes, without interstitial telomeric labeling. Regarding the NORs, their location on the same pair of chromosomes in the two species is a condition shared by most species of the tribe Ancistrini, however, in the two species studied, the fact that they are in different regions of the pair indicates that the chromosomes carrying NOR are not the same in *S. aureatus* and *S. pariolispos*, which suggests the occurrence of events that changed the position of NOR within the karyotype. Furthermore, NORs were positive for CMA3 and negative for DAPI, indicating that ribosomal DNA is rich in Guanine-Cytosine, an event frequently described in fishes. Heterochromatin was positive for DAPI, that is, rich in Adenine-Thymine, but the pattern differed between the species, indicating that processes related to DNA dynamics may be involved in the karyotypic differentiation of these two species, which can be used in taxonomic identification. Furthermore, the sympatric occurrence of these two species suggests that the karyotypic differences identified may have been a post-zygotic reproductive isolation mechanism during the speciation process (CARDOSO et al., 2013).

The third and final study, by Pety et al. (2018), mapped the major and minor rDNA sites in the karyotypes of species of the subfamily Hypostominae, being five species of the genus *Peckoltia* (*Peckoltia vittata*, *Peckoltia cavatica*, *Peckoltia multispinis*, *Peckoltia oligospila* and *Peckoltia sabaji*) and one of the genus *Ancistomus* (*Ancistomus feldbergae*), from the Amazon basin, through FISH using 5S rDNA and 18S rDNA, to discuss the mechanisms of organization and diversification of these clusters. All the specimens presented 2n = 52, which is supposed to be a synapomorphy of the tribe Ancistrini – subfamily Hypostominae; divergences in chromosomal morphologies, which must result from inversions, mainly pericentric, identified as the main source of karyotype evolution in Ancistrini. Pety et al. (2018) also reported the presence of a single NOR on chromosome 20 of *A. feldbergae*, 9 of *P. cavatica*, 5 of *P. multispinis,* and 10 of *P. vittata;* and several NORs on chromosomes 10 and 20 of *P. oligospila* and 7 and 20 of *P. sabaji*. As for the location of the 18S and 5S rDNA sites, it was divergent in all six species, there were cases wherein minor (5S), and major (18S) rDNA sites were in different chromosome pairs and cases wherein in these two classes of rDNA were syntenic and even colocalized. It is noteworthy that the presence of a 5S rDNA site in the interstitial region on the same chromosome with the only NOR may be a primitive remnant condition for the *Peckoltia* clade. Furthermore, Pety et al. (2018) emphasized that these dynamics found in rDNA clusters indicate that other types of chromosomal rearrangements, in addition to inversions, should have occurred during the karyotype divergence of these species and that transpositions and duplications seem to be important evolutionary events, involved at least in the dissemination of rDNA clusters in this group. The authors conclude by saying that the data generated can provide taxonomic markers and, consequently, be of great use in the identification of *Peckoltia* species, in addition to showing evidence of a post-zygotic reproductive isolation mechanism among the sympatric species of the Xingu River.

Based on data presented in studies by Cardoso et al. (2011), Cardoso et al. (2013) and Pety et al. (2018), whose methods were conventional and molecular cytogenetics and where it was found that in the first article, the authors observed chromosomal rearrangements, as well as a cytogenetic perspective for the taxonomy of the species studied; in the second, karyotypic differences were also observed between the objects of study, which led to the idea of a post-zygotic reproductive isolation mechanism during the speciation process; and the third, which showed that the positions of rDNA sites in the genome can be involved in the dynamics of chromosomal evolution, making these marker sites useful for identification and evolutionary biology, one can affirm the relevance of conventional and molecular cytogenetics as an interesting approach and tool for taxonomic and evolutionary studies of Neotropical fishes, since it is from cytogenetic analysis that it is possible to observe phenomena of diversification in the genome and, consequently, speciation and/or evolution, such as those observed by the authors of the three articles, which converges with the ideas of Bertollo et al. (1986) and Moreira-Filho and Bertollo (1991), who state that the greater applicability of fishes cytogenetics refers to evolutionary studies. These are associated with taxonomy and thus, cytotaxonomy, which in turn is one of the goals of the cytogenetics of neotropical fishes, even more in taxonomically poorly resolved groups.

Corroborating this:

> The cytogenetics of neotropical fishes can be considered an excellent tool to be used in association with data from morphology, biogeography, behavior, and molecular genetics, to get closer to a real evolutionary history of organisms (ARTONI, 2000, p. 53).

In recent years, fish cytogenetics has been accumulating data that allow establishing evolutionary trends between, for example, the Characiformes and Siluriformes orders, the most representative groups in number of species in the Neotropical region. And, speaking of evolutionary issues, according to the fundamental theorem of natural selection, evolutionary changes are determined by the diversity present in the genetic material. About cryptic species – morphologically identical with independent evolutionary units, cytogenetic analysis is fundamental for the correct identification, and systematization. Furthermore, it is the genetic makeup of a species that provides its adaptive and evolutionary potential (ARTONI, 2000; ARTONI e MATTIELO, 2003; GALETTI et al., 2008).

In Brazil, since the 70s, when three research groups from the Paulista State University (UNESP, Botucatu, SP); Federal University of São Carlos (UFSCar, São Carlos, SP); and University of São Paulo (USP, São Paulo, SP) started the cytogenetics of fishes, so far, having expanded to more than twenty research groups, located in different Brazilian regions and also in Argentina, many significant evolutionary and cytotaxonomic contributions were achieved, improving knowledge about biodiversity within the rich Neotropical ichthyofauna (BERTOLLO et al., 2017).

CONCLUSION

The results of the research mentioned here indicate that conventional and molecular cytogenetic methods allow the observation of numerous events that occur in the genome and, consequently, such events may be related to the chromosomal evolution of fishes, contributing to the study of evolutionary biology and/or providing information to assist in taxonomic organization. From this perspective, the objective of the present work was achieved, since it brought evidence that cytogenetics can bring contributions, adding to the content of the scientific collection of taxonomy and the study of the evolution of Neotropical fish species.

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