Chapter 4

Cytogenetic Tools to Study the Biodiversity of Neotropical Fish: From the Classic to the Advent of Cell Culture

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Abstract

Neotropical Ichthyofauna is considered the richest and most diverse in the world. All this biodiversity has attracted attention from researchers from different areas of study, including the cytogenetics. Many cytogenetics studies have search to understand the evolution of macro and micro karyotype structure of these different groups of fish, and classical and molecular cytogenetics techniques have contributed significantly for all knowledge of this karyotypic diversity. Recently, the use of cell cultures as an alternative to obtaining mitotic chromosomes opening up new opportunities to study groups that have not been explored or have not yet been cytogenetically investigated. In this work, we take a chronological overview of the advances of different cytogenetic techniques ("*in vivo*" and "*in vitro*" methods to obtain the chromosome, C-banding, the detection of nucleolar organizer regions (Ag-RON), fluorescent *in situ* hybridization (FISH) with several repetitive probes and paint chromosome) over the decades and how these techniques helped elucidate questions of the organization and function of the fish genome.

Keywords: chromosome, karyotype evolution, molecular cytogenetics, fibroblast cells

1. Introduction

The Neotropical region includes the area between the north of Mexico and the south of South America. This is the richest and more diversity freshwater fish fauna in the world with approximately 5160 freshwater fish species, distributed in 739 genera, 69 families and 20 orders, which

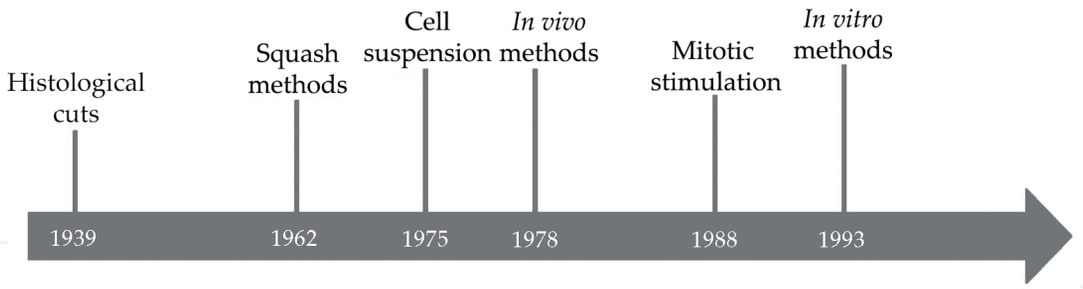

Figure 1. Timeline showing the major technical innovations that contribute to the development of fish cytogenetic.

represents one-third of all fishes on the planet [1]. A larger part of this diversity is grouped in Characiformes and Siluriformes, but there are still gaps in information in many groups [2].

All this diversity has been studied in different areas, including Cytogenetics. The refinement of cytogenetic techniques (**Figure 1**) provided the obtain of quality chromosome preparations that significantly increased the number of species studied and the description of their chromosomal characteristics, which contributed significantly to a better understanding of the genetic structures, evolution and systematic of the fishes [3–7].

2. Classical methods of cytogenetics to obtain metaphase chromosomes and their adaptations

Obtaining metaphase chromosomes is the most important point for cytogenetic studies, since any study to understand evolution and structure of the karyotypes of the species depends on this initial stage. It is known that many adjustments were made to improve the different techniques which it have arisen over the years in research within the fish cytogenetics.

The first studies with fish cytogenetics used fragments of testis previously fixed, included in paraffin and then submitted to cuts, like the experiments of Makino [8]. This methodology generated a certain doubt in its results, due to the uncertainty of the exact diploid number of each cell and was not employed with a significant number of species.

Subsequently, studies where the obtainment of chromosome depended on the squash technique were developed. In this case, a small fragment of tissue was directly crushed on a glass slide and fixed with acetic acid [9]. This technique often produced overlapping chromosomes, making it difficult to visualize the morphology and diploid number. Anyway, researches using this methodology continued and resulted in the creation of the "crushing machine" invented by Orlando Moreira Filho to minimize the injuries in the researcher's fingers [10].

The use of tissues to obtain metaphase chromosomes was not considered easily applied, because it was not easy to develop studies in the field [11]. Another relevant point is that it was not possible to regulate the rate of mitotic division and the condensation of the chromosomal arms. However, if it was known about the high hematopoietic activity of the anterior kidney in fishes [12] and from this organ, it was possible to obtain good metaphases, especially when subcutaneous or intraperitoneal stimulation of a mitogenic agent was performed [13, 14].

In 1971, Cole and Leavens [15] were the first to suggest the use of yeast as mitotic stimulating agents in hematopoietic tissues of reptiles and amphibians, but Lee and Elder [16] adapted this protocol for small mammals using a suspension of bread yeasts injected into the animal, observing that the chromosomes spread better and responded more effectively to banding treatments. For fishes, this methodology was adapted by Oliveira et al. [17], and it has been widely used over the years [18–20]. Other mitogenic agents were also employed in work with freshwater fish, such as phytohemagglutinin [21, 22], horse serum [23], parasitic infection as *Ichthyophthirius multifiliis*, or pharmaceutical agents [24, 25]. However, the use of enriched glucose solution of *Saccharomyces cerevisiae* (yeast activated suspension) is still the most used by its efficiency and low cost.

Since 1956, the works of Tjio, Levan, Ford, and Hamerton [26, 27] have reported about treating cytogenetic preparations with colchicine and hypotonic solutions, and the chromosomes have shown morphologically well-defined and that spread easily on the glass slide. Only in 1975, with the publication of a paper by McPhail and Jones, the chromosome preparations for cytogenetic studies in fish began to use this methodology [10].

The advances of chromosomal preparations in fishes have been boosted from the "air-drying" technique developed for mammals and later adapted for fish in "*in vivo*" [28] and *"in vitro"* [29] protocols. Both methods involved pre-treatment with colchicine. The use of this drug has enabled a direct control of chromosome condensation, which favored a more detailed study of the morphology of the chromosomes.

Another aspect that contributed to improving the quality of chromosome preparations was hypotonization process. Substances such as sodium citrate, distilled water and potassium chloride are used in the hypotonic treatment of the material; however, the potassium chloride is the most used in fish. In addition, the incubation temperature and hypotonization time should be adjusted according to the organism (e.g., in freshwater fish is common the hypotonization time of 21 min, while for marine fish is used from 30–35 min). After hypotonization, the cells are fixed in Carnoy's solution [21] and the cell suspension obtained is dropped into a glass slide for the rupture of the nuclear envelope [11] and thereby spreading the chromosomes for visualization of the diploid number and morphology.

Alternative methodologies have been published to improve chromosomal preparations in fish. Such methodologies, as proposed by Netto et al. [30], describe new proposed methodologies based on previously published protocols that allow cytogenetic analysis in individuals after death or that described by Blanco et al. [31], who proposed a protocol to be conducting in the field, where it eliminates the need for transportation of the specimens to the laboratory, but it is still not as common as the methodologies of Bertollo et al. [28] and Foresti et al. [29].

3. Chromosomal banding techniques and their contributions to the understanding of karyotypic macrostructure in several fish groups

Major breakthroughs in cytogenetic fish were possible with the development of differential staining techniques in the early 1970s that made it possible to understand the evolutionary

relationships in many fish groups. These methods allowed a better characterization of the chromosomal structure of the fish with appearance of markings along the chromosomes that before these techniques were based only in description of the number and chromosomal morphology. The main techniques used for chromosomal characterization in fish include the C and G banding techniques (not so usual due to compartmentalization of genomics) and silver nitrate staining.

The C-banding technique described by Summer [32] shows the patterns of the constitutive heterochromatin, and it has been widely used in cytogenetic studies of fish for characterization of similar karyotypes, especially to identify variations among species or populations of the same species [19, 33–36]. It was applied for first time in salmonid species [37, 38]. In fishes from the Neotropical region, the first studies were conducted in *Prochilodus* [39], *Eigenmannia* [40], and *Leporinus* [41], and since then, several studies have reported C bands in different fish species.

Most of this heterochromatin has been reported in centromeric and terminal regions of the chromosomes of most Neotropical fish species [34, 36, 42], while in some Loricariidae species it is possible to observe many heterochromatin blocks in the interstitial region [35], which appear to be a common feature for this group. In some species, heterochromatin can be more abundant [35, 43], whereas in other species these heterochromatin blocks are reduced [42]. Other studies have emphasized the importance of heterochromatin as a major source of karyotype diversification within and among some fish groups (e.g. 19). In some groups, it is possible to observe trends in relation to the behavior of heterochromatin, for example in Hypostominae, in which there is a relationship between the amount of heterochromatin and chromosome number of the species of this subfamily [44].

Not only did the C-banding technique provided a better characterization of the karyotypes but also the use of the silver nitrate staining technique that identifies the nucleolar organizing regions (NORs) became routine since the 1980s [45]. The NORs are chromosomal regions where the ribosomal RNA genes (45S = 18S + 5.8S + 28S) are located [46]. The first works using the technique in Neotropical fishes were in the species of Gymnotiformes [40, 47].

In general, two distribution patterns of NORs can be observed in fish, the first being the occurrence of only a single chromosome pair with NORs [33, 48, 49], while in other groups of fish more sites with NORs distributed in different chromosomes of the karyotype [36, 50, 51]. In fact, a single pair of NOR has been arbitrarily considered a plesiomorphic condition in fish [52]. Although this technique has been widely used, for the cost and ease, only 1.3% of the fish species had their NORs distribution investigated [46]. In some fish groups, it has been considered an excellent cytotaxonomic marker, as in *Apareidon* and *Paradon* of the Parondontidae family [53, 54]. In addition, polymorphism NORs have been evidenced with variation and size differences between homologous NORs [47].

4. How fluorescent *in situ* hybridization (FISH) and its variations have helped in the understanding of the evolution and organization of the fish genome?

The technique of fluorescence *in situ* hybridization (FISH) made it possible to physically map specific nucleotide sequences in the chromosomes of the species or group in study [55]. It was

first used by Buongiorno-Nardeli and Amaldi [56] in histological cuts and by Gall and Pardue [57] in chromosomes, but the adjustments to the protocols used to this day for fish studies are basically small changes from the original protocol proposed by Pinkel et al. [58]. This technique provided better results to investigate how chromosome diversity and organization of genomic segments occurred in fish chromosomes [59].

For example, cichlids are an interesting group of fish to be studied to explore different ecological niches and to report varied life strategies, morphology and behavior [60, 61], besides species important for fishing and aquarism [62]. Thus, many studies have search to understand more about the karyotypic macrostructure of this group of fish [66, 67], and the physical mapping of repetitive sequences has showing that such portions of the DNA may be involved in several chromosomal rearrangements in Cichlinae [63–67].

4.1. Ribosomal genes

In the genome of the eukaryotes, ribosomal genes are organized into two multigenic families, the 45S rDNA responsible for encoding the 18S, 5.8S and 26S/28S rRNAs and the other 5S rDNA, which encodes the 5S rRNA [59]. They are repeating sequences in *tandem*, and these genes are easily identified by FISH [68]. Several studies have searched to understand a little of the evolutionary dynamics of these repetitive sequences in the fish genome [5, 64, 67].

The 5S rRNA gene has been described in many fish groups, and it is located mainly in the interstitial region of the chromosome [59, 69–72], which may not only be a coincidence, but rather that this ribosomal minor distribution brings some advantage to the carrier genome [73]. It is known that the 5S rRNA is composed of a conserved region of 120 base pairs, separated from each other by the NTSs (not transcribed portions, which may vary in size or sequence). These variations have become important markers for specific species or specific populations.

Some studies with physical mapping of 5S rRNA in Anostomidae species have shown that the sites marked by the smaller ribosomal have been conserved during the karyotype evolution of the fish of this family [59, 70, 71, 74]. In *Brycon*, the physical mapping of 5S rRNA sequences was considered an important cytogenetic marker in the evolution of this group [75]. There is a variation in the number of chromosomes marked with the 5S rRNA in the genus *Astyanax*, with species with 1 pair [76, 77], species with 2 pairs [35, 76], until populations with 4 pairs, as in *A. scabripinnis* [78], and the distribution of these repetitive clusters seems to have been conserved in the group [76, 79, 80]. *Characidium* also have differences in relation to the number and location of the 5S rRNA clusters [81–83], and these variations are probably a reflection of the allopatric speciation occurred in populations of this genus.

In some fish species, more than one class of 5S rRNA gene has been identified, as reported in *Leporinus* [59]. This variation was due to differences in the sequences of portions not transcribed, and also it was reported in *Oreochromis niloticus* [84]. These sequences were found in pseudogenes and the 5S rRNA gene inverted; but in both works, the technique of FISH was contributed to identify the chromosomal location of the two classes of 5S rRNA. In the species, *Gymnotus sylvius* and *G. inaequilabiatus* were also detected two smaller classes, and with FISH, it was possible to observe that the two clusters of rRNA 5S are co-located in a chromosome pair, while the second class showed too marked in distinct chromosomes [85].

Many species of fish have the 18S rRNA gene co-located with the 5S rRNA gene [76, 86–89]; however, from the functional point of view, it would be more advantageous for two ribosomal classes to be on separate chromosomes since the transcription of them is made by distinct RNA polymerases, and the non-synteny is a way of ensuring that the 5S rRNA is not translocated to the rRNA 45S [70, 71], and allows the independent evolution of these genes [71].

Almeida-Toledo et al. [76] found that the genes 5S and 18S rRNA are co-located in five species of *Astyanax*, and such sequence was considered important markers for studying the evolutionary history of the group, including *A. altiparanae* and *A. lacustris*. This fact can be a sign of the recent separation of species, which previously belonged to a taxonomic unit of *A. bimaculatus* [90].

In the family Loricariidae, the FISH showed that most species have ribosomal sites in distinct chromosomes [91–95]. However, in the subfamily Neoplecostominae and Hypoptopomatinae [95], Hypostominae [92] and Loricariinae [91], these genes are in synteny condition, which is considered a primitive condition for the family, since it was found in the outgroup Trichomycteridae [19, 95]. According to Oliveira [19], the co-localization of 5S rDNA and 18S sites in Trichomycterus species is considered a plesiomorphic condition of the group, however the smaller ribosomal is more variable, since more labeled chromosomal pairs were observed, whereas the larger ribosomal was kept in only a couple, which according to the authors are homeologous.

Investigations using the genes rRNA 5S and 18S rRNA by Scacchetti et al. [83] showed that these genes are present in the sex chromosomes of some species of *Characidium*, indicating that the ribosomal can also participate in the differentiation process by chromosomes linked to sex in this group of fishes. In some fish, genome sequences of 18S rRNA 28S associated to heterochromatin have also been reported [69, 86], which seems to indicate that the constitutive heterochromatin may be involved in both the structural maintenance of the nucleolus and integrity of repetitions of ribosomal DNA [96].

4.2. Histones

The histone genes are composed of a genetic complex of a multigenic family (H2A, H2B, H3 and H4), which can vary in number of copies and organization genome [97]. In addition, they may be configured by H1 histone or spread throughout the genome [98]. In fishes, there are still a few studies that investigated the location and organization of these sequences, but in some of these studies histones are associated with ribosomal genes [85, 98, 99], and the genes H1, H3 and H4 are grouped in species of *Astyanax* [100, 101], as well as in the case of *Synbranchus*, where H3 and H4 are associated and spread throughout the genomes, likely to transposable elements [102]. This conformation was also observed in *Orestias ascotanensis* [103], where these sequences are organized into small copies. In *Characidium alipioi* [104], the H3 and H4 genes were mapped in a single chromosomal pair, which seems to be a conservative characteristic of the group [105].

4.3. snRNA

SnRNA genes are characterized in five RNA types (U1, U2, U4, U5, U6), non-coding, that are part of a large RNA-protein complex known as spliceosome machinery [106, 107]. The U2

gene is highly conserved in the genome of eukaryotes; however, the number of sites of these sequences may be different among species. This is because multigenic families may adopt different conservation strategies for their sequences [108].

Merlo et al. [109] and Úbeda-Manzanaro et al. [110] investigated the location of rRNA sequences U2 in species of the families Batrachoididae and Moronidae, while Manchado et al. [111] described U1 sites linked to smaller ribosomal in the genome of *Solea senegalensis*. However, few studies have been performed to map these sequences in Neotropical fishes. Study conducted by Cabral-de-Melo et al. [112] showed that the U1 snRNA gene in cichlids is found in just one chromosome pair, probably being a conserved feature in this group since the fragmentation of Gondwana [113]. On the other hand, the technique of FISH showed that the position of the snRNA U1 clusters can vary between distant species, and this is due to chromosomal rearrangements such as inversions and transpositions that modify and restructure the karyotypes of cichlids. The snRNA U1 sites were more variable between South American Cichlids than among the African species [112].

In *Gymnotus*, physical mapping of U2 snRNA sequences showed differences in the distribution of this gene, which can be clustered in homologous chromosomes as in most species or spread in several sites as in *G. pantanal*, an apomorphic condition [102]. In addition, the technique of FISH showed the U2 snRNA marked in a chromosome linked to sex in the species *G. pantanal* [102]. In other Neotropical fishes, these two configurations of the location of U2 snRNA gene can be found [83, 102, 103, 113, 114].

4.4. Telomeric probes

The telomere portions of the chromosomes are composed of repetitive sequences in tandem, which in vertebrates have been reported by sequence (TTAGGG)n [115]. In fish, these sites have already been mapped occupying regions of the telomeres [116, 117] and non-telomeric chromosome portions [118]. These interstitial marks contribute to studies about organization and macrostructural evolution of karyotypes, since they may answer some questions as fusion or inversions that modify the chromosomal structure of some species [117, 119]. Sometimes, these interstitial sites are the result of fusions but are not easily mapped because the karyotype in study may evolve and the telomeric sequences lose its function [55]. Another relevant point investigated in fish with a FISH technique using telomeric sequences is associated with satellite DNA [120, 121], which would be a response to the spreading of these regions in the interstitial regions. Scacchetti et al. [121] made it through the physical mapping of telomeric sequences in *Characidium* species, find interstitial markings in the chromosomes of some populations and, from there, carried out analyses that allowed establishing monophyletic group conditions. In Cioffi and Bertollo [122], telomeric interstitial markings were also observed in the neo-Y genome chromosome *Hoplias malabaricus*, which contributed to answer questions about the origins of the sexual system in this group of fishes.

4.5. Satellite DNA

Satellite DNA is composed of repetitive sequences that tend to accumulate in the chromosomes, especially in heterochromatic regions [123]. They are not protein coding and can form clusters on

the chromosome arms [123], which facilitate their physical mapping in the karyotype of interest. In the 1980s, satellite DNA families were first described in fish, and many works showed that they accumulate in the centromeric portion of the chromosomes and they may be related to the structural and functional roles of the centromere [124–126]. Some events such as unequal crossing over, transpositions and duplications may contribute to repetitive sequences including satellite DNA accumulating in heterochromatic regions, where they undergo less selective pressures and may thus evolve in the genome [127]. Some studies have used different satellite DNA probes to investigate the composition of supernumerary chromosomes in some species of fish [128–131].

4.6. Sex chromosome

Several studies attempted to understand the origin, evolution and maintenance of the sex-linked chromosomes [103], and fishes have become excellent models of studies because they have a wide and varied sexual system [122]. The sex chromosomes have been described in more than 7% of the fish karyotypes [132], and with the FISH technique, many satellite DNA sequences have been isolated and mapped in different species [133–137]. In some species of fishes, FISH technique has contributed to map sequences that characterize sex chromosomes undifferentiated by morphology or conventional staining, as is the case with guppy, within the family Poeciliidae [133, 138, 139]. And in other cases, the mappings of satellites sequences were important in work with morphologically differentiated sex chromosomes [135, 140]. Chromosome painting using W-chromosome-specific probe helped to answer about the common origin of this chromosome linked to sex in *Characidium* species [83, 141].

4.7. B chromosome

Many studies search to understand more about the origin, function and evolution of B chromosomes in fishes, since these are considered expendable parasites to supernumerary genome [142]. With the technique of FISH and advances in chromosomal painting, studies using themselves as probes it was possible to examine if there is homology of these extra chromosomes with the normal chromosomes of the karyotype, and from this understand possible answers about the origin and evolution of these chromosomes [51, 104, 130, 143, 144].

4.8. Fiber-FISH

The Fiber-FISH technique contributed greatly to the investigation of specific sites in the genome of Neotropical fish, since it allowed to determine the position of the genes in the chromatin fiber and to verify the organization of the gene sequences [145].

5. Culture of cells in fish: alternative tools for obtaining metaphase chromosomes

Cell culture is an *in vitro* technique widely used to isolate and maintain cells outside their original environment [146]. Briefly, a tissue fragment is aseptically removed from the individual and then mechanically and enzymatically dissociated or both. The isolated cells are

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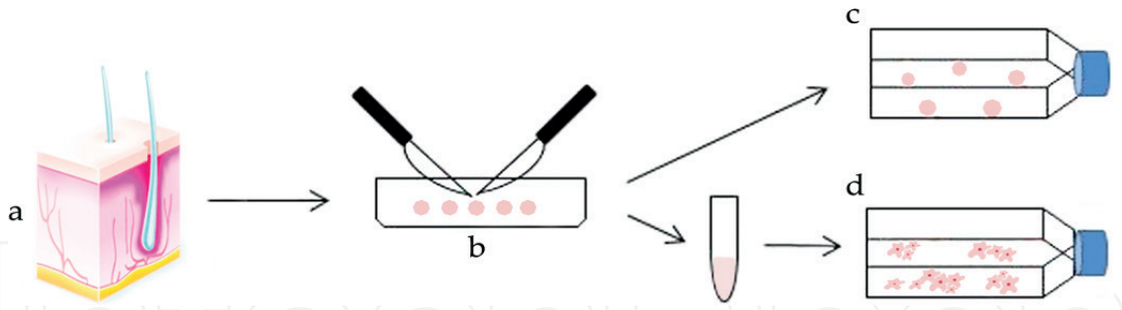

Figure 2. Scheme of obtaining cell culture. (a) Tissue; (b) disaggregation of the tissue mechanically; (c) tissue fragments (explant) grown in flasks with medium culture and (d) cells cultured in flasks after enzymatic disaggregation.

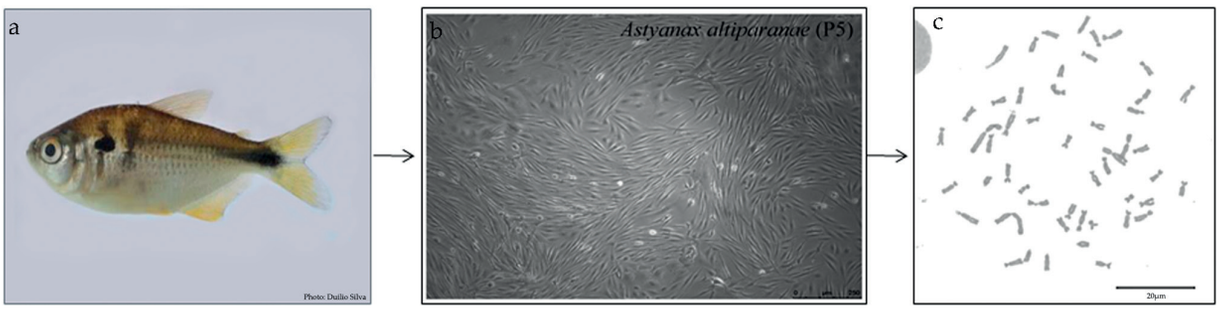

Figure 3. (a) *Astyanax altiparanae* (Characidae); (b) fibroblast cell line of *A. altiparanae* in the fifth passages and (c) mitotic chromosome of *A. altiparanae* obtain a cell line with diploid number of 2n = 50 chromosomes.

cultured in flasks with suitable medium with adjusted pH, antibiotic/antimycotic agents and fetal bovine serum (**Figure 2**). Cell culture is maintained at the appropriate temperature to the species under study and monitored daily for cell growth and possible contamination. When these cells cover the entire bottom of the flask (cell confluence), these cells are trypsinized and cultured in new vials (a process known as subculture or passage). These cells are treated with colchicine and after detached from the bottom of the flask are hypotonized, fixed with Carnoy's solution and then dropped onto slides (**Figure 3**) [147, 148].

Cell culture is still little used as a tool in fish cytogenetic studies [149–151], mainly by the difficulty of standardization of the technique of isolation and maintenance of cell cultures. Nevertheless, this technology is an excellent alternative to obtain good quality chromosome preparations, since it can be applied in cytogenetic studies of small and large species, in which it is difficult to work with direct methods of chromosome preparation or also in species used in aquaculture or endangered, when there is no possibility of sacrifice of animals [149]. Another advantage is that the methodology can provide the establishment of cell bank available at any time, so, in case of repetition of cytogenetic methodologies, it is not necessary to go back to the field for new individuals.

6. Conclusion

The advances of cytogenetic techniques have contributed directly in studies that search to investigate and understand the macro and micro karyotype structure of the most diverse

groups of Neotropical fish, and many questions have been answered with the use of these technologies, as well as new problems have arisen that it was not possible to investigate because of the difficulties of the techniques. It is known that there are still many gaps to be filled, but cytogenetics has grown a lot in recent years and morphological and /or phytogenetic tools have played an important role in cytogenetic advances.

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