

# Chromosomes as Tools for Discovering Biodiversity – The Case of Erythrinidae Fish Family

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## 1. Introduction

Biodiversity or biological diversity is the diversity of life, extant or extinct. All of the biodiversity found on Earth today consists of many millions of distinct biological species and is the product of more than 4 billion years of evolution. Although the origin of life has not been correctly determined by science, some evidence suggests that life may already have been well-established only a few hundred million years after the formation of the Earth. Estimates of the number of extant global macroscopic species vary from 2 million to 100 million, with a best estimate of approximately 13–14 million, and the vast majority is represented by insects. However, biodiversity is not evenly distributed; rather, it varies greatly across the globe as well as within regions. A “biodiversity hotspot” can be defined as a region with a high level of endemic species, and while they can be found all over the world, the majority of them are forest areas, and most are located in the tropics (Myers, 1988).

In fact, it is not a coincidence that the world’s biodiversity hotspots are also the centers of evolutionary change for numerous species. Evolution produces biodiversity, and in turn, a more diverse biological environment creates more selective pressures, which drive evolution. The biodiversity of a specific region is often measured by determining the number of species found there. Although biodiversity concept with its complex mutual evolutionary interrelationships is not merely a species inventory, complete list of reliably identified species is a basic prerequisite for addressing various biodiversity issues. Therefore, for an accurate assessment of biodiversity, it is first necessary that a correct definition of the term species as well as the methods to differentiate between species be considered. However, to identify species requires i) appropriate species concept, ii) informative diagnostic characters that correctly separate species, i.e. diagnosable unit. The problem of species concepts in ichthyology has been extensively discussed (Nelson 1999, Ruffing et al., 2002, Mooi & Gil, 2010) and compete for acceptance. One of them, and also the one used in this book chapter, is the “evolutionary species concept” (Mayr 1942), which defines a species

not only according to appearance but as members of populations that actually or potentially interbreed in nature in such a way that they are necessarily reproductively isolated from others, has its own independent evolutionary fate and its own historical tendencies, thus representing separate evolutionary lineage.

However, species can be identified by means of diagnostic characters at different levels of organism/genome organization (Ráb et al., 2007). One of these levels, genetic diversity, is composed of the diversity in organisms and populations arising from genetic and genomic variants. Genetic variation is a fundamental characteristic of most biota. Recently, advances in genetic technologies have permitted deep investigations of the genetic variation among distinct living or even extinct groups. Such methodologies have enabled the exploration of various questions related to biodiversity and its conservation (i.e., determination of genetic variability, population delineation, characterization of the germplasm, genotoxicity, comparative genomic studies, forensic analysis, phylogeographic patterns and evolutionary inferences). Among these approaches, cytogenetic studies have proved to be a useful tool in several cases by identifying the chromosomal characteristics of the genomes of a species. Indeed, in some situations, changes in chromosome number and structure have been correlated with a number of novel morphological and environmental traits leading to habitat divergence and adaptation (Hoffmann & Rieseberg, 2008). In this chapter, we advocate the view that, as compared to various other genetic marks, cytogenetics can reveal set of characters, many times diagnostic ones, that are not accessible by other methods and thus explore another level of genetic component of biodiversity. We exemplify this view using the Erythrinidae fish family in which classic and molecular cytogenetic techniques were useful to compare the degree of chromosomal diversity over a species geographical range, providing important tools for evolutionary and taxonomic studies, besides improving the knowledge of the genome diversification and the biological biodiversity.

## 2. Cytogenetics and biological investigation

For many years, classical conventional karyotyping methods have been used to determine chromosome number and morphology as well as the presence of morphologically differentiated sex chromosome systems in many animal and plant species. This approach has contributed significantly to the present knowledge of chromosomal diversity and/or stability among many distinct taxa. The advent of chromosome banding techniques (i.e., C-, G-, R-, Q- and H-banding and AgNORs, DAPI and CMA<sub>3</sub> staining) allowed for the differentiation of specific regions along chromosomes, and many important karyotypic changes could be demonstrated with such methodologies. The recent combined use of cytogenetic, genetic and refined molecular studies has helped us to understand the connection between genomic organization and the functional biology of chromosomes, species adaptation and species survival, and this has begun a new era of evolutionary genomics and phylogenomics.

Major advances in cytogenetics arose in the last two decades with the application of *in situ* detection of DNA sequences on chromosomes, parts of chromosomes or even whole genomic DNA. The earliest *in situ* hybridizations, performed in the late 1960s, were not fluorescent but instead utilized probes labeled with radioisotopes (i.e., <sup>3</sup>H, <sup>35</sup>S, <sup>125</sup>I and <sup>32</sup>P). Since the beginning of the 1980s, probes started being labeled with non-radioactive

molecules. Although several methods based on enzymatic reactions using alkaline phosphatase, beta-galactosidase or horseradish peroxidase were available, the most commonly used method in the subsequent years was based on the utilization of fluorescent elements; therefore, the technique was named (Fluorescence *In Situ* Hybridization) (FISH) (Pinkel et al. 1986). In the FISH technique, probe detection and experimental results are based on the observation of fluorescent signals through an epifluorescence microscope. FISH emerged as a useful alternative to older hybridization methods because the fluorescent systems had more precise definition of the hybridization signals as compared to the radioactive or enzymatic methods (Gall & Pardue, 1969). Indeed, FISH technology, where a DNA probe is hybridized to its complementary sequences on previously fixed and denatured chromosomal DNA preparations, appeared to be superior to previous *in situ* technologies, as it allowed the simultaneous use of different fluorescence systems for multi-probe analysis and the detection of more than one target sequence simultaneously. The analysis of FISH results requires the use of an epifluorescence microscope coupled with digital cameras connected directly to a computer for the image acquisition. Additionally, nowadays some computer softwares can also be used for image manipulation, increasing the final quality of the figures.

One of the most important applications of the FISH technique has been its use in the physical mapping of DNA sequences on chromosomes. Its development has led to the advancement of chromosome studies not only for physical mapping and genome analyses but also as a tool for evolutionary and phylogenetic studies. With FISH, it is possible to map the location of DNA sequences across related species and genera to show not only their probable conservation but also their diversification throughout evolutionary processes. Thus, the advent of FISH allowed the transition from the “classical” (black-and-white) to the “molecular cytogenetic” (color) and combined with genomic data recently to phylogenomic era, which allowed the integration of molecular information of DNA sequences with its physical location along chromosomes and in genomes (Schwarzacher, 2003) (**Figure 1**).

The simple characterization of the karyotype in some species may be sufficient to identify intra- and inter-cytotype variants and to characterize species. However, in most cases, just the the karyotype descriptions appear to be inconclusive when not coupled with other methods capable of generating more accurate chromosomal markers for cytotaxonomy or phylogenetic applications. In part, this stems from the inability to discern either the mechanisms involved in karyotype evolution of some groups (homologies *versus* convergences) or the genesis of novel chromosomal structures. In some groups, such as Perciformes fishes, karyotypes and cytogenetic aspects associated with the chromosomal structure, identified by conventional cytogenetic techniques, show a vast number of species sharing the same karyotypic patterning, which restricts their use for taxonomic and phylogenetic inferences (Molina, 2007; Motta-Neto et al., 2011).

In contrast, the molecular organization and cytogenetic mapping of many genes might be a significant data set for the characterization of particular segments of biota, providing very important information for phylogenomics. Remarkably, a substantial fraction of any eukaryotic genome consists of repetitive DNA sequences including satellites, minisatellites, microsatellites and transposable elements (Jurka et al., 2005). These repetitive DNA sequences are thought to arise through many mechanisms, from direct sequence amplification by unequal recombination of homologous DNA regions to the reverse flow of

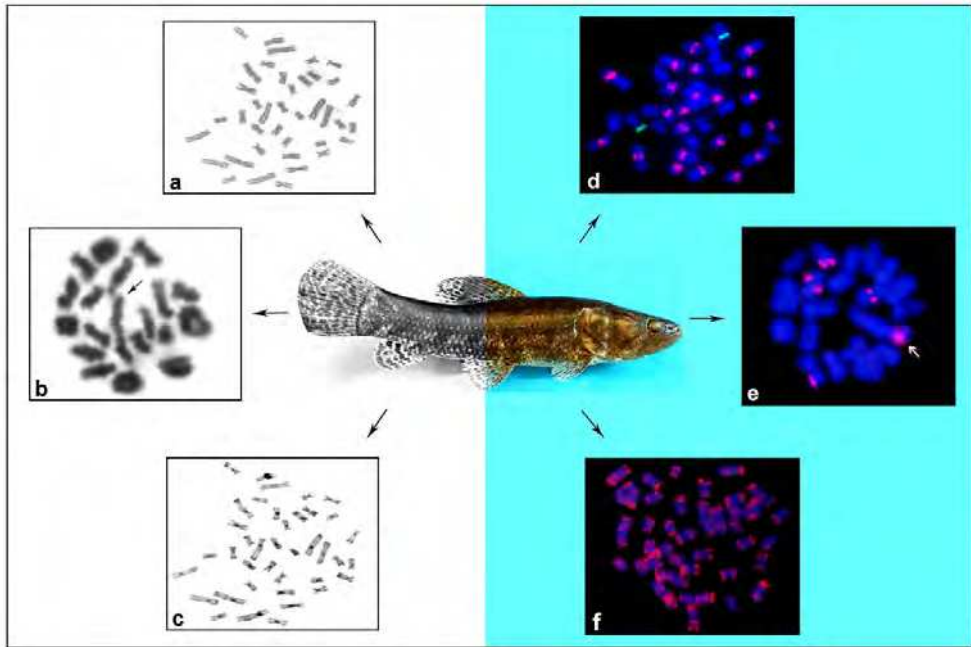


Fig. 1. Conventional (a, b and c) x molecular (d, e, and f) cytogenetic analyses in chromosomes of males of the karyomorph D of the fish *Hoplias malabaricus* ( $2n = 39, X_1X_2Y$  sex chromosomes) highlighting the transition from the "black-and-white" to the "color" era. Giemsa-stained mitotic chromosomes (a) and diakinesis/metaphase I meiotic cell (b); C-banded (c); 5S rDNA (green) and satellite 5SHindIII-DNA (red) hybridized to mitotic chromosomes (d); diakinesis/metaphase I meiotic cell displaying 18S rDNA sites (red) in the synapsed chromosomes (e); simple sequence repeat (GA)<sub>15</sub> (red) hybridized to mitotic chromosomes. The arrows indicate the sex trivalent.

genetic information using an intermediate RNA molecule. Due to the hypervariability of tandem repeats, such genomic segments are highly polymorphic and considered to be good molecular markers for genotyping individuals and populations (Jeffreys et al., 1985). The repetitive fraction of the genome was long considered to be "junk DNA" with no clear function, which was reinforced by indications that these sequences were not transcribed in eukaryotes (Doolittle & Sapienza, 1980). However, accumulated data from eukaryotic species of diverse taxonomic origins have challenged this view over the past few years (Bonaccorsi & Lohe, 1991), supporting a major role of repetitive DNA sequences in the structural and functional evolution of genes and genomes in a variety of organisms (Biémont & Vieira, 2006).

Today, many studies have been conducted using dispersed or *in tandem* repetitive DNA sequences as probes for FISH cytogenetic mapping in distinct living organisms; these sequences include simple sequence repeats (Figure 2a), satellite DNA (Figure 2b), BAC clones (Figure 2c, e), and rRNA genes (Figure 2d, f). In general, these probes provide highly visible signals due to their abundant repetition and distribution in the genome, and they

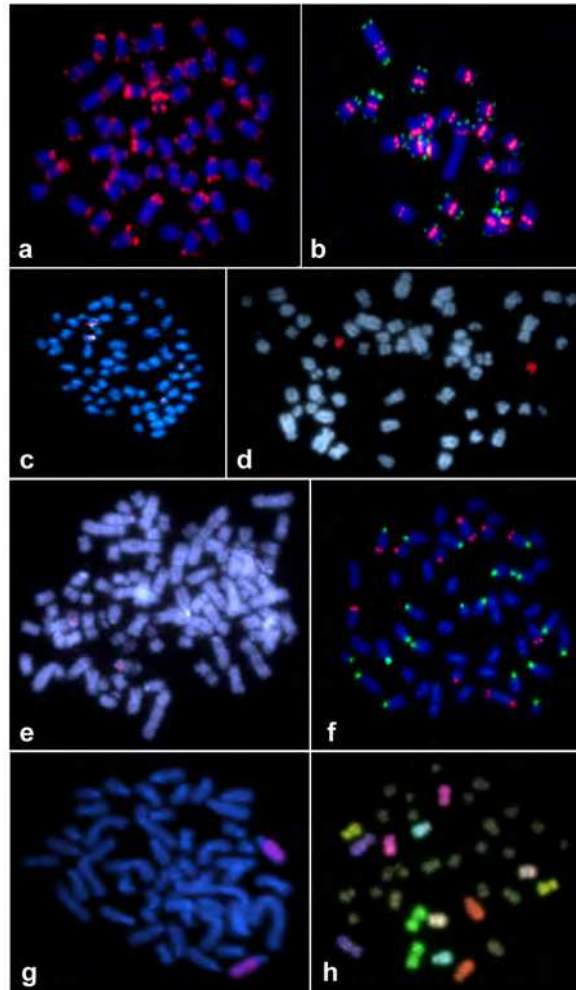


Fig. 2. Fluorescence *in situ* hybridization to metaphase chromosomes of distinct organisms using different probes. (a) simple sequence repeat (CA)<sub>15</sub> (red) in the fish *Leporinus elongatus*; (b) Centromeric STAR-C tandem repeat (red) and subtelomeric X43.1 tandem repeat (green) in the plant *Silene latifolia*; (c) BAC-FISH in the moth *Biston betularia* using BAC clones of sulfamidase (green) and *Irtp* (red); (d) 45 rDNA (red) in the eagle *Spizaetus tyrannus*; (e) BAC-FISH in the hawk *Leucopternis albicollis* using BAC clones derived from a *Gallus gallus* microchromosome; (f) 5S rDNA (green) and 18S rDNA (red) in the fish *Erythrinus erythrinus*; (g) chromosome paint probe of human chromosome no. 7 (red) hybridized to the chromosomes of the monkey *Alouatta fusca*; (h) multi-color FISH hybridization of cat painting probes hybridized to the chromosomes of the leopard *Panthera pardus*. The FISH image (b) is courtesy of Eduard Kejnovsky (Academic Science of Czech Republic, Czech Republic), (c) of František Marec (Biology Centre of the Academy of Sciences of the Czech Republic, Czech Republic), (d and e) of Edivaldo HC de Oliveira (Instituto Evandro Chagas, Brazil), and (h) of Vladimir Trifonov (Institute of Chemical Biology and Fundamental Medicine, Russia).

might even generate a unique FISH karyotype for each species (e.g., Badaeva et al., 2007), enabling an evolutionary and phylogenetic view of related species. In contrast to functional genes, repetitive DNA sequences are thought to have evolved under different conditions, escaping from the selective pressures that act on the non-repetitive segments (Charlesworth et al., 1994). In this sense, they represent good chromosomal markers to detect recent differentiation events.

In addition, Genome *In Situ* Hybridization (GISH) and chromosome painting are new and useful tools for investigating biodiversity. GISH allows for the comparison of genomes using the genomic DNA of one organism as a probe for the DNA of another organism. This method offers new perspectives in phylogenetic and systematic studies by determining and testing hypotheses of genomic relatedness between species. In turn, chromosome painting has also provided significant support to comparative cytogenetics by highlighting chromosomal changes that took place during the evolution of a species. DNA probes covering an entire chromosome can be developed using chromosome sorting with microdissection-based or flow-sorting methods. Such whole chromosome probes (wcp) allow the tracking of homologous and/or segments of chromosomes among related species and have been a powerful tool for evolutionary studies being used to identify homologous chromosome segments among different species, rearrangements and thereby karyotype differentiation. In recent years, complete karyotypes of many animal and plant species have been analyzed by chromosome painting, which have added to our understanding of genomic reorganization and chromosome evolution (Griffin et al., 2007, Ferguson-Smith & Trifonov 2007; Teruel et al., 2009; Yang & Graphodatsky, 2009; Cioffi et al., 2011a; Pokorná et al., 2011) (**Figure 2g, h**).

In summary, the development and improvement of cytogenetic FISH analyses have substantially expanded the methods of chromosome studies and have played an important role in the precise characterization of the structure of genomes. The current availability of an ever increasing number of completely sequenced eukaryotic genomes has opened new “avenues” for advancing cytogenetics. Coupled with the application of bioinformatics, the integration of chromosome analysis and genomic data represents promising tools for the future of cytogenetics. However, classical information regarding chromosome number and morphology and banding data is not outdated and therefore should still be useful to elucidate a range of both basic and applied aspects, ranging from cytotaxonomy to karyotype evolution.

In fact, a number of groups of organisms show a high diversity of species. However, this diversity is frequently distributed in a phylogenetically uneven way. Among fish taxa, for example, an order might contain anywhere from two to over ten thousand species (Nelson, 2006). In groups widely distributed and difficult to access, diversity is only estimated, and the number of species identified each year continues to dramatically increase. Many of these species are known to be endemic; thus, they have a crucial, pivotal role in biological conservation. Indeed, issues related to cryptic biodiversity and its correct identification continue to demand constant attention.

### **3. Neotropical fish and biodiversity**

#### **3.1 Fish: Diversity and functional role in evolutionary studies**

Fish exhibit the greatest biodiversity among the vertebrates, making this group extremely attractive to study a number of evolutionary questions. The term "fish" most precisely

describes any non-tetrapodal craniate that has gills throughout life and whose limbs, if any, are in the shape of fins. However, fish do not constitute a monophyletic group but are instead a paraphyletic collection of taxa including hagfish, lampreys, sharks, rays and the finned bony fish. The latter is by far the most diverse group and is well represented in freshwaters, while the others are predominantly marine groups. Nelson (2006) suggested the presence of almost 34,500 fish species out of the almost 55,000 recognized living vertebrate species.

Generally, each continent has a distinctive freshwater fish fauna, and the observed patterns of fish distribution are the result of physical barriers disrupting past fish dispersal and different temperature adaptations amongst the various groups. Most species occur in the tropical and subtropical regions, and there is an overall reduction in diversity in temperate and polar regions (Lévêque et al., 2007). Specific aspects of the spatial distribution of this group, such as subdivisions according to biogeographical barriers and biological aspects such as length of life, population size, degree of mobility, behavior patterns, and aspects of sex determination are reflected in their chromosomal patterning. The wide spectrum of mechanisms for reproduction, sex determination and sexual differentiation in fish species also illustrates the plasticity of their genomes, with many species exhibiting hermaphroditism and some even changing sex at a specific stage in their life cycle. Indeed, fish show a range of sex determination mechanisms, from male or female heterogametic sex determination to environmental sex determination (Devlin & Nakayama, 2002). It has been suggested that all this diversity might be related to the fact that fish genomes seem to undergo genetic changes more rapidly than in other vertebrate groups (Venkatesh, 2003).

Although fish have traditionally been the subject of comparative evolutionary studies, they have now drawn attention as models in genomics and molecular genetics research, and there are many ongoing or completed genome sequencing projects, including those for the catfish *Ictalurus punctatus*, the rainbow trout *Oncorhynchus mykiss*, the Atlantic salmon *Salmo salar*, the three-spined stickleback *Gasterosteus aculeatus*, the Nile tilapia *Oreochromis niloticus*, the two pufferfish *Takifugu rubripes* and *Tetraodon nigroviridis*, the platyfish *Xiphophorus maculatus*, the medaka *Oryzias latipes*, the spined loach *Cobitis taenia* and the popular zebrafish (*Danio rerio*), which is a commonly used model organism for studies of vertebrate development and gene function (Mayden et al., 2007).

### 3.2 The Neotropical region and freshwater fish biodiversity

Concerning all of the biodiversity on Earth, the Neotropical region has the largest repository of genetic information, and its biodiversity has an enormous economic importance in addition to its ecological relevance. The number of freshwater fish species in the world is estimated to be approximately 15,000. Although a substantial component of the Neotropical fish fauna is still unknown, approximately 6,000 freshwater fish species are found in this region (Reis et al., 2003), which corresponds to approximately 45% of all freshwater fish species in the world (Oliveira et al., 2007).

The hydrographic system that drains the Neotropical region is highly branched covering a large and ecologically diverse area and containing an extremely diverse fish fauna, one of the world's richest in number of species. Phylogenetic and biogeographic patterns

indicate that, in most groups of Neotropical fishes, diversification occurred incrementally over large spatial and temporal scales, with speciation occurring over much of the continental platform and requiring tens of millions of years. Complementary, vicariance and species dispersal processes have profoundly influenced the formation of new species and the taxonomic composition of regional biotas. Together, these processes interact in a complex duet of Earth history events and biological diversification (Albert & Reis, 2011). Therefore, the exploration of Neotropical fish evolution requires a multidisciplinary approach to gain a more complete understanding, and cytogenetics has contributed to it as an important tool to support/correct systematic studies and the corresponding taxonomical constructions.

### 3.2.1 Cytogenetic studies in Neotropical fishes

Among Neotropical fish, there are many nominal species (i.e. group of individuals appointed as taxonomically unique and but not necessarily validated by additional biological and genetic studies) with a large geographic distribution that are found in different river basins isolated by millions of years. In this region, small and widely distributed fish that inhabit small streams with limited opportunity to migrate tend to possess an increased rate of speciation and form a “species complex”.

In such ecological systems, cytogenetic studies have made important contributions toward a better understanding of Neotropical fish fauna, showing that many local populations have different chromosomal characteristics. However, most of these studies utilized classical techniques involving conventional staining and simple banding procedures such as C-banding, the detection of nucleolar organizer regions (AgNOR) and fluorochrome staining techniques. Despite this apparent limitation, the results have provided important data that have revealed interesting and significant components of this biodiversity, which have helped to elucidate the evolutionary pathways of distinct fish groups. A number of cases of species complexes, populational polytypy, the presence of B-chromosomes, diverse sex heteromorphic chromosome systems and spontaneous polyploidization have been reported in various Neotropical fish species.

Karyotype data have shown an extensive variability between different species and higher taxonomic categories. Chromosome numbers are known for 1,047 Neotropical freshwater species and 109 marine species, ranging from  $2n = 20$  in *Pterolebias longipinnis* to  $2n = 134$  in *Corydoras aeneus* (Oliveira et al., 2007). In general, most Neotropical fish families contain species for which some karyotypic data are available, which shows different evolutionary trends and patterns. When linked to biological features, evolutionary time and geomorphological history, the karyotypic variability can be better understood, allowing for inferences about their evolution and diversity (Oliveira et al., 2007).

In this context, some fish groups have certainly been used as models and are deeper studied. One of the more didactic models that illustrate the importance of cytogenetics in identifying cryptic components of biodiversity is undoubtedly the family Erythrinidae. In addition to conventional chromosome studies, molecular cytogenetic analyses were used to find new chromosomal characteristics for comparative genomics and to provide insights into karyotype differentiation inside the “species complex” of *H. malabaricus* and *E. erythrinus*, which are widespread in the continental waters of South America.



## 4. Erythrinidae - A fish family as an example for investigating biodiversity

### 4.1 The Erythrinidae family: General features

The characiform fish family Erythrinidae is a small group composed of three recognized genera, *Hoplias*, *Hoplerythrinus* and *Erythrinus*. It is widespread throughout South America, with a remarkable preference for a great variety of lentic environments such as small and large rivers and lagoons (Oyakawa, 2003) (Figure 3). They are typically carnivorous fish, and several species are broadly distributed throughout the main South America hydrographic basins. The family Erythrinidae has likely a close relationship to the families Lebiasinidae, Ctenoluciidae and the African Hepsetidae (Vari, 1995). Due to their sedentary habits, they are not able to overcome obstacles such as waterfalls and large rapids, which apparently contribute to a reduced gene flow between populations in the same hydrographic river basin.

The fish diversity found in the Neotropics hinders the real definition of many species. In fact, several species have the karyotype described, but have been identified only until the genus level (Oliveira et al., 2007). The taxonomy of the Erythrinidae fishes also reflects this trouble and is still to be better resolved. All the genera appear to have a number of not described species, nowadays included in a same nominal species. Despite the revision for some species of the *Hoplias lacerdae* group (Oyakawa & Mattox, 2009), no revision studies is available for the remaining *Hoplias* species, as well as for the *Hoplerythrinus*, and *Erythrinus* genera.

The erythrinids are fishes that, in general, possess large karyotypic variation (Bertollo et al., 2000; Giuliano-Caetano et al., 2001; Diniz & Bertollo, 2003) and represent excellent models for exploring biodiversity through cytogenetic investigations and for understanding the mechanisms of genomic diversity. The initial cytogenetic studies in this group, mainly based on Giemsa-stained chromosomes, showed the presence of intra-specific variations, with extensive karyotype diversity found among populations in terms of the diploid chromosome number (2n), karyotype composition and different sex chromosome systems. In addition to conventional chromosome studies, molecular cytogenetic analyses proved useful in identifying new cytogenetic characteristics for comparative genomics (Table 1).

### 4.2 Chromosomal and karyotype diversification among Erythrinidae fishes

The genus *Hoplerythrinus* contains three species, *H. cinereus*, *H. gronovii* and *H. unitaeniatus* (Oyakawa, 2003), however *H. unitaeniatus* has been cytogenetically analyzed only. A comparative cytogenetic analysis of populations from different Brazilian river basins showed karyotype diversity in this species. Both chromosome number and other karyotypic variations were found among populations, with 2n ranging from 2n = 48 to 2n = 52 and with variable numbers of acrocentric chromosomes (Giuliano-Caetano et al., 2001; Diniz & Bertollo, 2003). However, to date, no heteromorphic sex chromosomes was detected in this species. The available cytogenetic data suggest that *H. unitaeniatus* might include several distinct species and that these fishes require detailed taxonomic analysis to reveal their actual systematic diversity.

The genus *Hoplias* is composed of two large “species groups” (*H. malabaricus* and *H. lacerdae*). The *H. lacerdae* group includes six recently recognized species: *H. brasiliensis*, *H. aimara*,

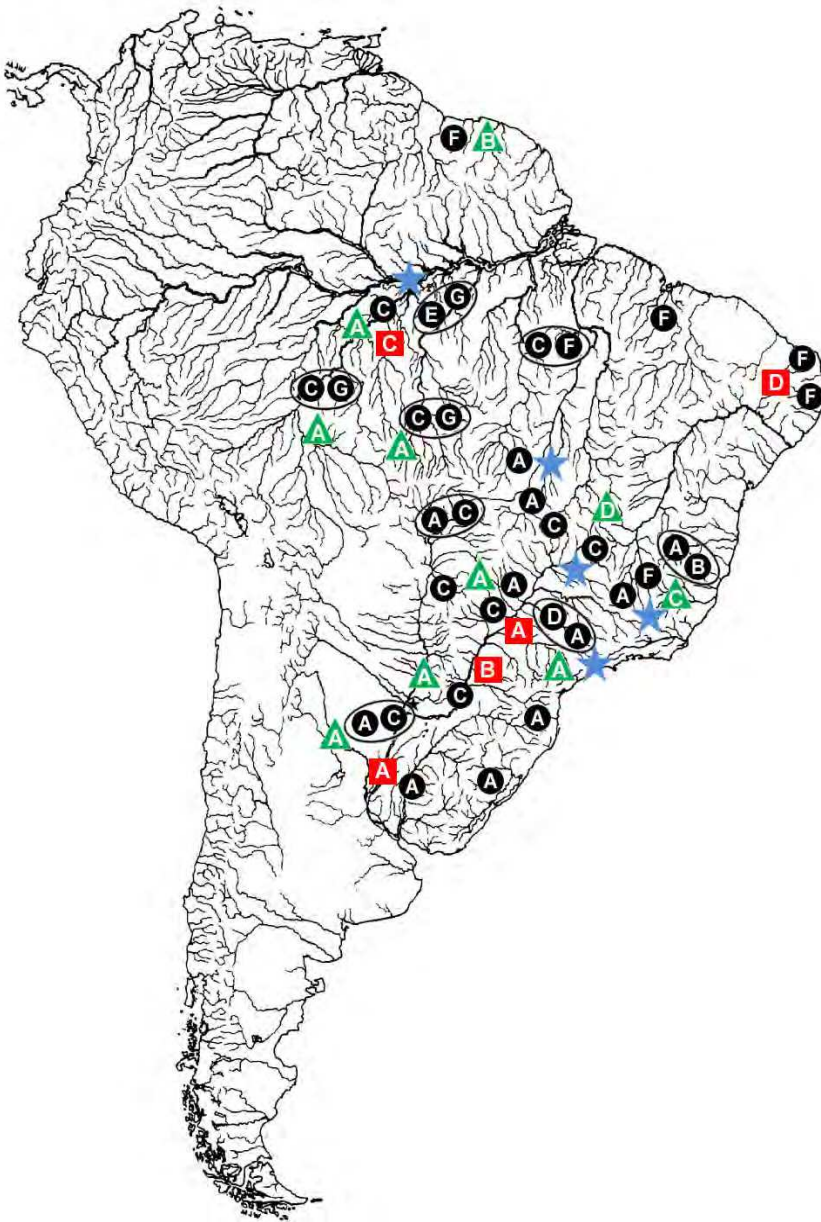


Fig. 3. Distribution of *Hoplias malabaricus* karyomorphs A-G (circles); *Hoplias lacerdae* species group (stars); *Hoplerythrinus unitaeniatus* karyomorphs A-D (triangles) and *Erythrinus erythrinus* karyomorphs A-D (squares) in the South America. The large open circles indicate some of the sympatric conditions already detected among distinct *H. malabaricus* karyomorphs.

Species/ Diploid number	Karyotype	Sex chromosomes
<i>Hoplias malabaricus</i>		
A 2n=42	♂ 42 m/sm	Not differentiated
B 2n=42	♀ 40 m/sm + 2 st	XX
	♂ 41 m/sm + 1 st	XY
C 2n=40	♀ 40 m/sm	XX
	♂ 40 m/sm	XY
D 2n=40/39	♀ 40 m/sm	X <sub>1</sub> X <sub>1</sub> X <sub>2</sub> X <sub>2</sub>
	♂ 39 m/sm	X <sub>1</sub> X <sub>2</sub> Y
E 2n=42	♂ 40 m/sm + 2a	Not differentiated
F 2n=40	♂ 40 m/sm	Not differentiated
G 2n=40/41	♀ 40 m/sm	XX
	♂ 40 m/sm	XY <sub>1</sub> Y <sub>2</sub>
<i>Erythrinus erythrinus</i>		
A 2n=54	♂ 46 a + 2 st + 6 m	Not differentiated
B 2n=54/53	♀ 46 a + 2 st + 6m	X <sub>1</sub> X <sub>1</sub> X <sub>2</sub> X <sub>2</sub>
	♂ 44 a + 2 st + 7m	X <sub>1</sub> X <sub>2</sub> Y
C 2n=52/51	♀ 38 a + 6 st + 8 m/sm	X <sub>1</sub> X <sub>1</sub> X <sub>2</sub> X <sub>2</sub>
	♂ 36 a + 6 st + 9 m/sm	X <sub>1</sub> X <sub>2</sub> Y
D 2n=52/51	♀ 44 a + 2 st + 6 m/sm	X <sub>1</sub> X <sub>1</sub> X <sub>2</sub> X <sub>2</sub>
	♂ 42 a + 2 st + 7 m/sm	X <sub>1</sub> X <sub>2</sub> Y
<i>Hoplerythrinus uniteniatus</i>		
A 2n=48	♂ 48 m/sm	Not differentiated
B 2n=48	♂ 46 m/sm + 2 a	Not differentiated
C 2n=52	♂ 46 m/sm + 6 a	Not differentiated
D 2n=52	♂ 44 m/sm + 2 st + 4 a	Not differentiated
<i>Hoplias lacerdae</i> group		
2n=50	♂ 50 m/sm	Not differentiated

Table 1. Karyotype data for the Erythrinidae fish family. m = metacentric; sm = submetacentric and a = acrocentric chromosomes

*H. currupira*, *H. intermedius*, *H. australis* and *H. lacerdae* (Oyakawa & Mattox, 2009). This group of species has a conserved karyotype with an invariable diploid chromosome number ( $2n = 50$ ) and a karyotype with m and sm chromosomes but no morphologically differentiated sex chromosomes (Bertollo et al., 1978; Morelli et al., 2007; Blanco et al., 2011). Therefore, it seems that speciation in the *lacerdae* group was not accompanied by observable changes at the chromosome level. Such conserved karyotypes and other chromosomal characteristics represent an exception for Erythrinidae fish as compared with the huge karyotypic diversity that has generally been observed in other species of this family.

*H. malabaricus* and *E. erythrinus* are the cytogenetically most studied fish in the Erythrinidae family. Both species have a large geographic distribution in different river basins isolated by millions of years. Conventional and molecular chromosomal markers have proved to be useful indicators for identifying cryptic species diversity. Previous extensive cytogenetic

comparative studies showed that many local populations have different karyotypes and other chromosomal characteristics, with a broad range of sex chromosome systems. The overall cytogenetic data clearly suggest that these are an assemblage of species with unresolved taxonomy (Bertollo, 2007) placed under one name that evidently represents a catch-all taxon. More recently, the chromosomal mapping of repetitive DNA families combined with chromosomal painting analysis has improved the understanding of the evolutionary mechanisms involved in the generation of the complex genomic variability in these fish.

#### 4.2.1 *Erythrinus erythrinus*

The genus *Erythrinus* contains only two species, *E. kesslei* and *E. erythrinus* (Oyakawa, 2003), and cytogenetic analyses are available for the later only.

Conventional cytogenetic analyses revealed the presence of extensive karyotype diversity among and within populations of the four currently identified karyomorphs (A to D) (Bertollo et al., 2004). Karyomorph A is composed of populations with  $2n = 54$ , which have very similar karyotypes composed of 6 metacentric (m), 2 subtelocentric (st) and 46 acrocentric (a) chromosomes and have an absence of differentiated sex chromosomes. Karyomorphs B, C and D share an  $X_1X_1X_2X_2/X_1X_2Y$  sex chromosome system, but they differ in their number of chromosomes and karyotype composition. While karyomorph B has  $2n = 54$  ( $6m + 2st + 46a$ ) chromosomes in females and  $2n = 53$  ( $7m + 2st + 44a$ ) in males, both karyomorphs C and D have  $2n = 52/51$  but differ in their karyotypes, with  $6m + 2sm + 6st + 38a$  in females and  $7m + 2sm + 6st + 36a$  in males of karyomorph C and  $4m + 2sm + 2st + 44a$  in females and  $5m + 2sm + 2st + 42a$  in males of karyomorph D (Figure 4). The prevalence of acrocentric chromosomes is a particular characteristic that differentiates karyotypes of *E. erythrinus* from those of other erythrinids (Bertollo et al., 2000; Giuliano-Caetano et al., 2001).

The most frequent chromosome number for characiform fish is  $2n = 54$ , and this number likely represents their ancestral diploid chromosome number (Oliveira et al., 2007). Karyomorph A of *E. erythrinus*, with a diploid chromosome number of  $2n = 54$  and a karyotype dominated by acrocentric chromosomes with undifferentiated sex chromosomes, may represent the most ancestral karyotype among representatives of Erythrinidae. In this view, the occurrence of a lower chromosome number, an increase in the proportion of biarmed chromosomes in the karyotype and the presence of differentiated sex chromosomes represent derived characteristics in the members of karyomorphs B-D. It was hypothesized that karyomorphs B-D resulted from centric fusions between two non-homologous acrocentric pairs producing the submetacentric chromosomes found in their karyotypes and from pericentric inversions, which together generated the observed karyotypic differentiation (Bertollo et al., 2004).

Additional comparative studies of karyomorphs A and D using cytogenetic mapping of repetitive DNA, such as rDNA repeats, satellite DNA, telomeric sequences and classes of TEs, demonstrated that chromosomal rearrangements and genomic modifications were significant events during the course of karyotypic differentiation of this fish. The presence of Interstitial Telomeric Sequences (ITS) in the centromeric region of the only submetacentric chromosome pair found in karyomorph D indicated that a centric fusion created this pair, which is not found in karyomorph A (Cioffi et al., 2010). The most remarkable difference

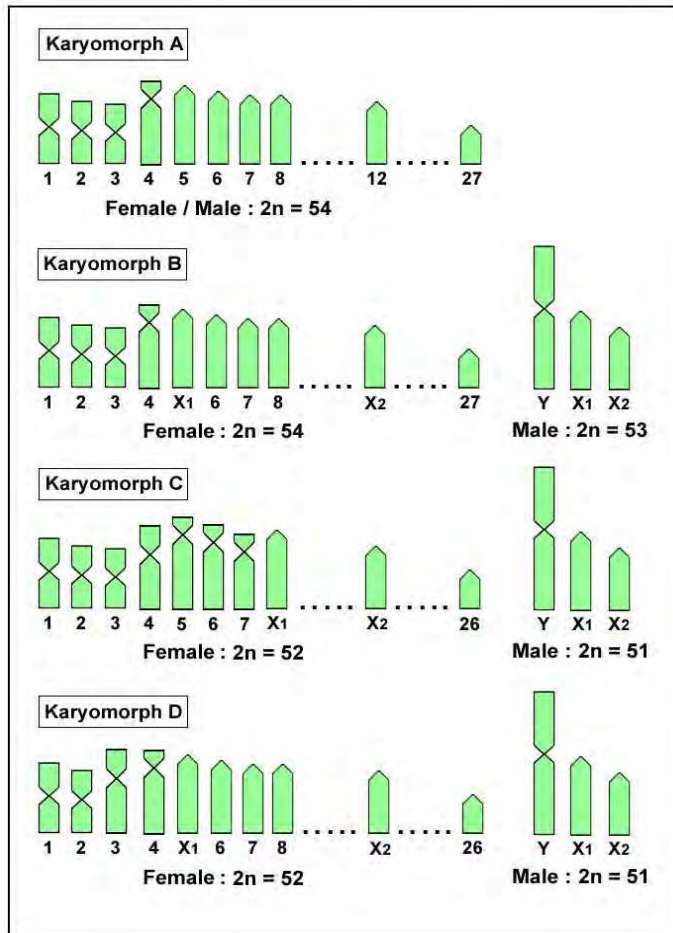


Fig. 4. Extensive chromosomal variability found in the fish *Erythrinus erythrinus*. Partial idiograms of karyomorphs A-D showing their well-defined differences regarding the diploid number, chromosomal morphology and sex chromosome systems.

between karyomorphs A and D was the distribution of 5S rDNA/*Rex3* sites. These sequences co-localized to the centromeric region of several chromosomes. However, while a single chromosome pair was found to bear these sites in karyomorph A, a surprisingly large number of these sequences were found in karyomorph D, with 22 sites in females and 21 in males. Thus, a huge dispersal of 5S rDNA/*Rex3* elements throughout the centromeric regions of the acrocentric chromosomes had occurred; the retroelement *Rex3* might have inserted into a 5S rDNA sequence, giving rise to a 5S rDNA-*Rex3* complex that then moved and dispersed the complex in the genome (Cioffi et al., 2010). Taking into account that karyomorph D represents a derived form as compared to karyomorph A, the chromosomes of this karyomorph may have undergone further rearrangements during the evolutionary process mediated by retrotransposon activity (Figure 5).

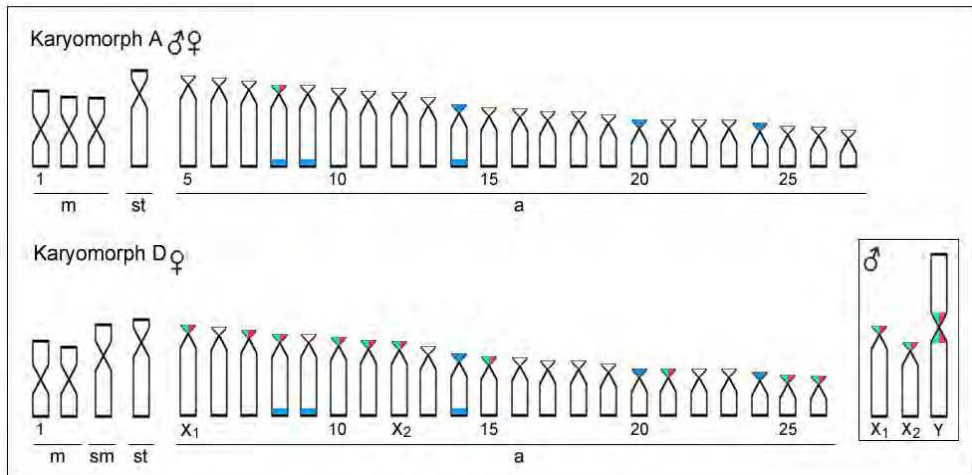


Fig. 5. Representative idiogram of *Erythrinus erythrinus* karyomorphs A and D highlighting the chromosomal distribution of the 18S rDNA (blue) and 5S rDNA/*Rex3* sequences (green/red) in karyomorph A and the expanded distribution of the latter sites in karyomorph D. The sex chromosomes are boxed.

Another remarkable characteristic of *E. erythrinus* karyotypes is the multiple  $X_1X_1X_2X_2/X_1X_2Y$  sex chromosome system shared by karyomorphs B–D. Bertollo et al. (2004) proposed that a centric fusion between two non-homologous acrocentric chromosomes might have created the large metacentric Y chromosome and, consequently, the unpaired  $X_1$  and  $X_2$  chromosomes in the male karyotypes, as this sex system appears to have originated before the divergence of these three karyomorphs.

A comparative analysis of male and female karyotypes clearly indicated that the large metacentric Y chromosome originated by a centric fusion harboring characteristic ITS in its centromeric region. Accordingly, the resulting non-homologous acrocentric chromosomes in the male karyotype correspond to the  $X_1$  and  $X_2$  chromosomes (Cioffi et al., 2011a). Chromosome painting also suggested that the  $X_1X_2Y$  sex system of *E. erythrinus* was derived from an XY sex pair still morphologically undifferentiated, as found in karyomorph A (Cioffi et al., 2011a), for which there seems to be no apparent specific markers (Cioffi et al., 2011b).

#### 4.2.2 *Hoplias malabaricus*

*H. malabaricus* cytogenetic analyses showed that several populations possess different karyotypes and other chromosomal characteristics (Bertollo et al., 2000). This species is well adapted to life in small populations with low vagility, which may facilitate the stochastic fixation of chromosomal rearrangements (Faria & Navarro, 2010). Currently, seven karyomorphs (A to G) were easily identified by their number of chromosomes, karyotypes and the presence or absence as well as the size of heteromorphic sex chromosomes (Bertollo et al., 2000).

Conventional cytogenetic analyses were able to distinguish two major karyotype groups in *H. malabaricus*, one consisting of karyomorphs A, B, C, and D (Group 1) and the other containing karyomorphs E, F, and G (Group 2) (Figure 6). Despite their differences in chromosome number, karyomorphs A-D have fairly similar karyotypes, which are different from those of karyomorphs E-G (Bertollo et al., 2000). To date, karyomorphs A-D are the best analyzed. Biogeographical data clearly showed that, while karyomorphs A and C have a wide distribution, karyomorphs B and D are endemic to particular regions (Bertollo et al., 2000; Cioffi et al., 2009). It is noteworthy that some karyomorphs, mainly those ones showing a wider geographical distribution, can be found in sympatric or even in syntopic situations in Brazilian and some other South American regions (Figure 3). In all these cases, no apparent hybrid forms were found suggesting the reproductive isolation between the karyomorphs and, in this way, reinforcing the occurrence of a species complex (Bertollo et al., 2000). Additional RAPD-PCR genomic markers also demonstrated the lack of genetic flow between karyomorph pairs A-C and A-D (Degam et al., 1990), which is compatible with the karyotypic data.

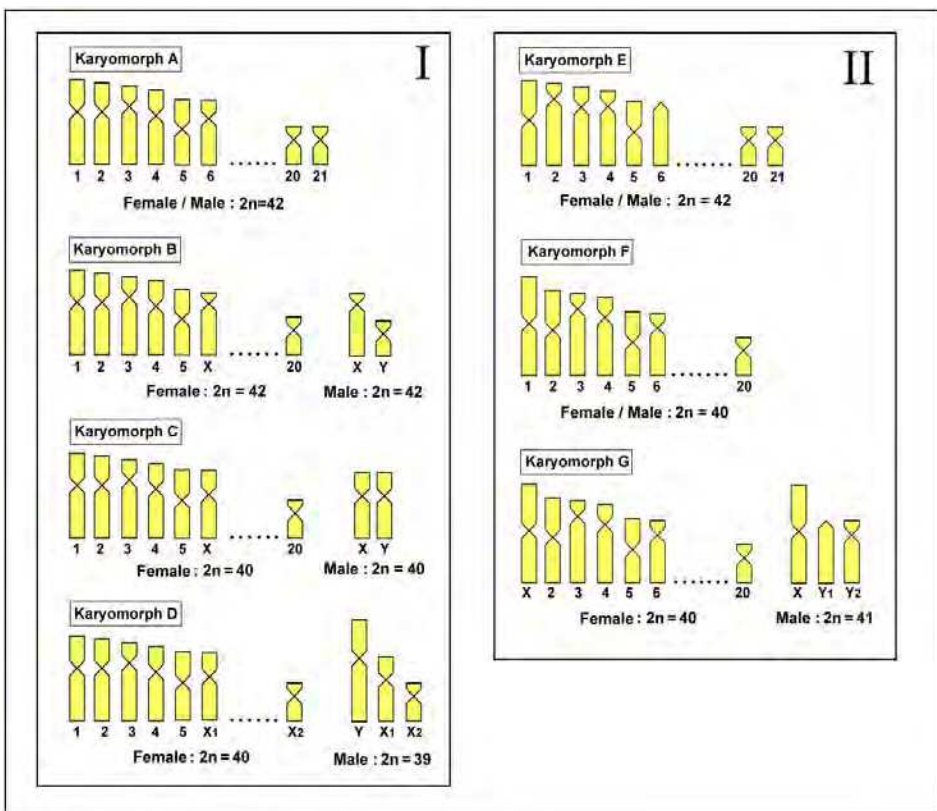


Fig. 6. Extensive chromosomal variability found in the species *Hoplias malabaricus*. Partial idiograms of the karyomorphs A-D (Group I) and E-G (Group II), showing their well-defined differences regarding diploid number, chromosomal morphology and sex chromosome systems.

The *in situ* investigation of repetitive DNA sequences added new informative characteristics that are useful for comparative genomics at the chromosomal level, providing insights into the cytogenetic relationships among *H. malabaricus* karyomorphs A-D. The cytogenetic mapping of repetitive DNA classes (rDNA repeats, satellite DNA, telomeric sequences, several TEs and microsatellite repeats) has provided useful chromosomal markers, highlighting the close relationship among these four karyomorphs and giving additional support to the proposition that they constitute a closely related evolutionary group within *H. malabaricus* (Figure 7). The use of repetitive DNA sequences as probes for FISH analyses

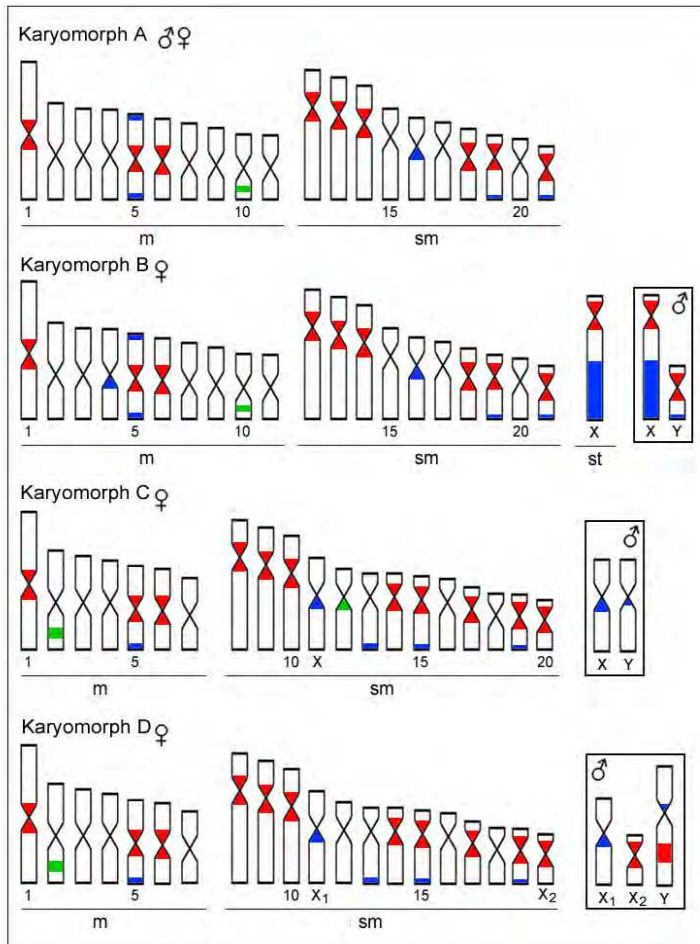


Fig. 7. Representative idiogram of *Hoplias malabaricus* karyomorphs A-D, highlighting the distribution of different classes of repetitive DNA. The locations of the satellite 5S *Hind*III-DNA, 18S rDNA and 5S rDNA sites on the chromosomes are indicated in red, blue and green, respectively. Note that several chromosomes bearing these repetitive DNA sequences were shared by all karyomorphs alongside some karyomorph-specific chromosomal markers. The sex chromosomes are boxed.



has greatly contributed to the study of fish karyotypic evolution, not only because this method provides additional information about the structure of their chromosomes but also because it allows the comparison of genomes of different species (Nanda et al., 2000). In fact, the repetitive DNA fraction of the genome was effective in identifying significant genomic changes that occurred during the differentiation of these karyomorphs of *H. malabaricus*.

The presence of different sex chromosome systems is also a significant characteristic of the *H. malabaricus* genome. Three karyomorphs (A, E and F) lack identifiable heteromorphic sex chromosomes, whereas three others (B, D and G) possess well-differentiated sex chromosome systems, an XX/XY system in karyomorph B, an  $X_1X_1X_2X_2/X_1X_2Y$  system in karyomorph D and an XX/ $XY_1Y_2$  system in karyomorph G (Bertollo et al., 2000), in addition to an early differentiated XX/XY sex chromosome system found in karyomorph C (Cioffi & Bertollo, 2010). Variation in the amount of several types of repetitive DNA has been shown to be associated with sex chromosome evolution in *H. malabaricus*. Remarkably, a clear tendency of sex chromosomes to accumulate repetitive DNA was demonstrated (Cioffi et al., 2009, 2010; Rosa et al., 2009). In general, karyomorphs that possess well-differentiated sex chromosomes (B, D and G) show a restricted geographical distribution, indicating their derived origin. Chromosome painting using whole sex chromosomes as probes has helped to determine the origin of the sex chromosomes in *H. malabaricus*. Homology was demonstrated between specific chromosomes of karyomorphs A and B as well as between specific chromosomes of karyomorphs C and D (Cioffi et al., 2011c), indicating that the sex systems evolved independently in the different karyomorphs of *H. malabaricus*. Undoubtedly, this is an important feature considering that the presence of distinct sex chromosome systems might represent a determining factor for the reproductive incompatibility between karyomorphs.

## 5. Conclusion

Cytogenetics as a whole, as well as fish cytogenetics, has experienced major methodological advances over the years. Much progress has been made in the chromosomal analysis of fish in general, and particularly from the pioneering studies of Neotropical fishes in the 70's. In fact, several improvements in cytogenetic methodologies, specially the advances in molecular cytogenetics, have added to our understanding of chromosomal evolution. Particularly, the mapping of specific DNA sequences on chromosomes by FISH and chromosome painting have proved to be powerful tools. The use of such methodologies has enhanced studies on the molecular composition of the chromosomes and the mechanisms that led to the significant karyotypic differentiation observed in fish. In this context, the Neotropical Erythrinidae family was chosen to illustrate how classical and molecular cytogenetic techniques were useful for comparing the degree of chromosomal diversity over the geographical range of a species, providing important tools for evolutionary and taxonomic studies and increasing knowledge of genomic diversification. The data obtained to date with the use of classical cytogenetic methods and the additional improvements provided by molecular cytogenetics highlighted the hidden biodiversity in distinct species of the Erythrinidae family. Thus, in at least three nominal species of this family, i.e., *Hoplias malabaricus*, *Erythrinus erythrinus* and *Hopletythrinus unitaeniatus*, the karyotype and chromosomal diversity points to the existence of a set of distinct species under evolutionary species concept rather than single biological entities, that are widely distributed throughout

the Neotropical region. However, the cases highlighted in the erythrinid fishes can also be found in many other fish species. Chromosomal studies with fishes from different regions of the world have provided reliable information on the inherent diversity of this group. Thus, cytogenetics revealed a powerful tool for discovering biodiversity, with useful applications in evolutionary, taxonomic, phylogenetic and conservation studies.

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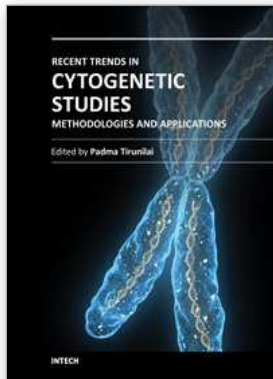
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## **Recent Trends in Cytogenetic Studies - Methodologies and Applications**

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Recent Trends in Cytogenetic Studies - Methodologies and Applications deals with recent trends in cytogenetics with minute details of methodologies that can be adopted in clinical laboratories. The chapters deal with basic methods of primary cultures, cell lines and their applications; microtechnologies and automations; array CGH for the diagnosis of fetal conditions; approaches to acute lymphoblastic and myeloblastic leukemias in patients and survivors of atomic bomb exposure; use of digital image technology and using chromosomes as tools to discover biodiversity. While concentrating on the advanced methodologies in cytogenetic studies and their applications, authors have pointed out the need to develop cytogenetic labs with modern tools to facilitate precise and effective diagnosis to benefit the patient population.

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