Chromosomes in Focus: Basic Cytogenetics, Light Microscopy and the Case of Neotropical Fish

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Despite of all advances in cytogenetic techniques, basic information regarding chromosomal number and morphology coupled with simple banding procedures and light microscopy analysis are no longer obsolete. Neotropical fish constitute a good model to understand the importance of these studies. Cases of species complex, populational polymorphisms, presence of B-chromosomes and diverse sex chromosome systems, as well as natural polyplodization have been reported in distinct species and families of this extensive group, mostly derived from conventional Giemsa-staining. In the present work, we selected some representatives of the order Characiformes, a dominant freshwater group throughout neotropical region, to show the application of simple, fast and cost-saving cytogenetic studies on assessing both inter- and intra-specific genetic diversity. Specimens of *Astyanax* sp., *Astyanax aff. bimaculatus* (Characidae), *Hoplias malabaricus* (Erythrinidae) and *Acestrorhynchus lacustris* (Acestrorhynchidae) were collected along Contas river basin in the state of Bahia, northeastern Brazil. These results represent the first cytogenetic data of such species/populations and they can be used to compare the degree of chromosomal diversity over their geographical range, providing important tools for phylogeographic, evolutionary and taxonomic studies, besides helping further conservation programmes. Based on the available data, we encourage students and researchers all over the world to focus on basic cytogenetic studies in order to increase the information about chromosomal patterns of fish species. This approach seems to be particularly suitable to research centers in developing countries, usually surrounded by poorly known biodiversity hotspots and limited financial resources.

Keywords chromosomes, Characiformes, karyotype, NORs

1. Introduction

Cytogenetic studies allow analyzing the chromosomal behavior in the organization and transmission of genetic information, variability mechanisms and evolutionary pathways, besides contributing to the genetic improvement of domestic species [1]. Therefore, cytogenetics is mainly focused on structure and chemical/genetic organization of chromosomes, linking two formerly unrelated sciences, cytology and genetics [2]. Cytologic studies got started with the invention of the first microscope in the seventieth century. From 1850 to 1900, the identification of cell components had presented a fast development and the term "chromosome" was created by Waldeyer as a reference to small structures observed in the nucleus during certain stages of the cell cycle [3]. Later, after the re-discovery of Mendelian analysis and the formulation of chromosome theory of inheritance by Wilson, Sutton and Boven in 1902-1903, the so-called cytogenetic science was properly initiated [2].

Over the last decades, cytogenetic techniques have been constantly improved and several modern technologies are currently available. Nowadays, the processes of chromosomal pairing and alignment during meiosis, able to ensure such "elegant chromosome dance", are well known [4]. The combination of refined cytologic, genetic and molecular studies has launched a new era of evolutionary genomics, helping us understand how the genome organization translates into the functional biology of chromosomes, species adaptation and survival [5]. Molecular cytogenetics, mainly involving *in situ*

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hybridization, stands out as a good example of such advances, being able to locate precisely specific genes or DNA sequences on chromosomes of several species [6, 7]. However, basic information, regarding chromosomal number and morphology, coupled with simple banding techniques are no longer obsolete and they might be useful to elucidate an array of both basic and applied aspects, ranging from cytotaxonomy to karyotype evolution [8].

Neotropical fish constitute a good model to understand the importance of cytogenetics, leading to several inferences about genetic and evolutionary patterns of studied species. Fish species of the order Characiformes represent a dominant freshwater group throughout neotropical region [9] displaying a remarkable phenotypic and karyotypic variability [7]. For instance, the Bolivian pygmy blue characin, *Xenurobrycon polyancistrus*, presents a total length of 17 mm, while Amazonian tambaqui, *Colossoma macropomum*, can reach 1 m in length and 30 kg in weight [10] and the chromosomal diploid number can range from 2n=28 in *Hemigrammus* [11] up to 2n=102 in *Potamorhina altamazonica* [12]. After the separation of Africa and South America in the late Cretaceous, about 90 millions years ago, American characiform fishes have independently evolved different life habitats, body shapes, breeding strategies and feeding mechanisms and several phylogenetic lineages have arisen [10]. More than 1,400 species distributed in nearly 260 genera are recognized at present, and several recently described species are about to increase this number [13-16].

Most of cytogenetic studies in neotropical fish are based on basic techniques, involving conventional staining and simple banding procedures such as C-banding and detection of active nucleolar-(NOR) sites. Despite of this apparent limitation, the results provided by such reports have played a major role on the understanding of evolutionary pathways within distinct fish groups. Cases of species complex, populational polymorphisms, presence of B-chromosomes and diverse sex chromosome systems, as well as natural polyplodization have been reported in distinct neotropical species, mostly derived from conventional Giemsa-staining either associated or not with banding techniques [8].

In the present work, we selected some representatives from distinct families within the order Characiformes, common in a northeastern river basin in Brazil, to show the application of simple, fast and cost-saving cytogenetic studies on assessing both inter- and intra-specific genetic diversity. The present results, based only on light microscopy analysis, represent the first chromosomal information about these species/populations.

2. Material and Methods

2.1 Fish sampling sites

Specimens of *Astyanax* sp, *Astyanax aff. bimaculatus* (Characidae), *Hoplias malabaricus* (Erythrinidae), *Curimatella* sp. (Curimatidae) and *Acestrorhynchus lacustris* (Acestrorhynchidae) were collected using set gill and drift nets at different sites along Contas river basin (main river and tributaries), an important hydrographic system, comprising about 55,000 Km^2 in the state of Bahia, northeastern Brazil (Table 1).

Table 1 Collection sites and number of specimens analyzed.

Species	Collection site.	Sample number
Astyanax sp.	Contas river	43
	Preto do Costa river	40
Astyanax aff. bimaculatus	Contas river	15
	Preto do Costa river	2
	Oricó river	2
Hoplias malabaricus	Contas river (and marginal lakes)	11
	Preto do Costa river	9
	Bom-sem-farinha river	6
Curimatella sp.	Jibóia river	4
Acestrorhynchus lacustris	Oricó river	2

Fish samples were transported to the laboratory and kept in separated tanks prior cytogenetic analyses. The specimens were identified and deposited in the fish collection at UESB.

2.2 Chromosomal studies

Mitotic stimulation was performed on collected specimens 72h prior cytogenetic preparation by intra-muscular or intra-peritoneal inoculation of the pharmaceutical compound Munolan, Allergan Frumtost, (76mg/2.5ml) [17]. Metaphasic chromosomes were obtained from kidney cells according to the air-drying technique [18] and the slides were stained with Giemsa at 5% for conventional analyses. In order to show the feasibility and low costs of chromosomal obtaining in fish species, this procedure is described in details as follows:

a) After 72h of mitotic stimulation, apply 0.025% of colchicine solution (1ml/100mg) into the peritoneal cavity of live fish specimens (just behind the pectoral fin insertion). In case of very small individuals, we have obtained good results using 50µl of colchicine per animal. The animals are then kept in aquarium for 30 minutes;
b) Then, pieces of anterior kidney (and, alternatively, spleen) should be removed and cut into small tissue fragments in a small plate, containing 10ml of hypotonic solution (KCl 0.075M);
c) Mix the solution with a Pasteur pipette and keep it for 20 minutes at 37^{o} C;
d) Add five to 10 drops of fresh Carnoy's fixative (methanol: acetic acid 3:1 at 4^{o}C);
e) Centrifugate the material at 1,000 to 1,500 rpm for 10 minutes and discard the supernatant;
f) Add 6ml of fixative (recently prepared and kept in freezer) to each centrifuge tube;
g) Centrifugate the material at 1,000 to 1,500 rpm for 10 minutes and discard the supernatant;
h) Repeat (f) and (g) steps twice;
i) After the last centrifugation, discard the supernatant and add fixative at a proportion of 1:1 to pellet (usually about 1.5ml), mixing the solution until obtaining a homogeneous cell suspension, that can be stored in microcentrifuge tubes at -20^{o}C indefinitely;
j) Put three drops of the cell suspension onto a glass slide, covered with a thin water layer at 60^{o}C;
k) After air-drying, stain the material with 5% Giemsa solution (diluted in phosphate buffer pH 6.8) for 10 minutes.

The best metaphases were digitally photographed and printed in high-quality photographic paper for chromosomal measurements, karyotyping and NOR analysis. The chromosomal pairs were arranged in decreasing size order and classified as metacentric (M), submetacentric (SM), subtelocentric (ST) and acrocentric (A) according to arm ratio [19]. Active nucleolar organizer regions were detected by the one-step method of silver nitrate staining (Ag-NOR) [20].

3. Results

The specimens identified as *Astyanax* sp. presented 2n=48 chromosomes. However, differences in chromosomal morphology could be identified between samples from the main channel of Contas river and one of its tributaries, Preto do Costa river. While the former presented 8M+24SM+12ST+4A (fundamental arm number, FN=92), the latter was characterized by 8M+24SM+10ST+6A (FN=90). Active NORs were identified, mainly, on telomeres of a large subtelocentric pair in both populations and, sporadically, on SM and M chromosomes of some individuals from Contas river (Fig. 1).

In contrast, both diploid number and chromosomal morphology were similar among analyzed individuals of *Astyanax aff. bimaculatus*. All specimens presented 2n=50 chromosomes, composed of 6M+28SM+12ST+4A (FN=92). Silver nitrate staining revealed a remarkable inter- and intra-population variation, ranging from one to four chromosomes bearing telomeric NORs (Fig. 2).

All specimens and populations of *H. malabaricus* analyzed presented 2n=40 M-SM chromosomes (FN=80). Multiple Ag-NOR sites were detected at telomeric region on chromosomal pairs 6 and 8 in sampled populations (Fig. 3).

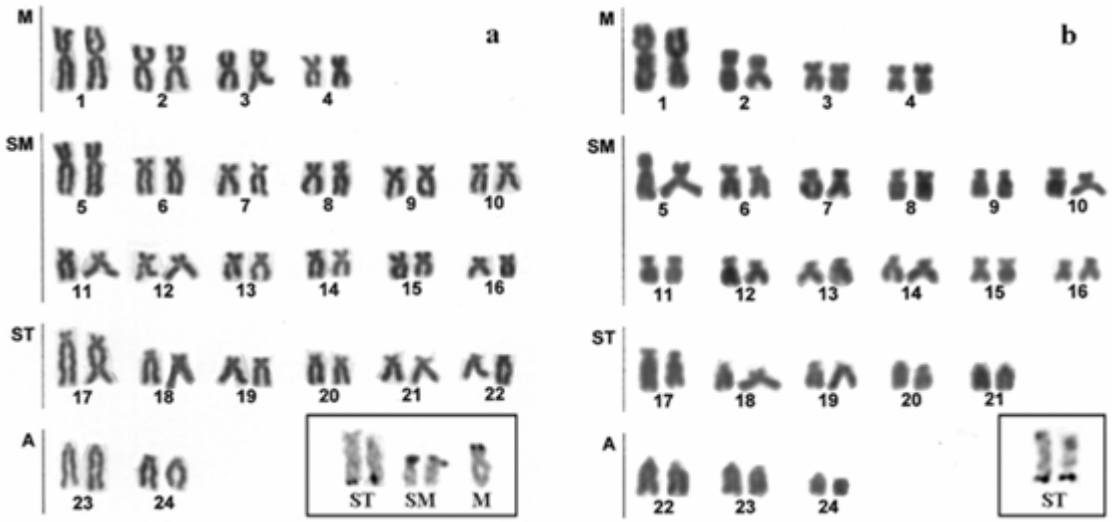

Fig. 1 Giemsa-stained karyotypes of *Astyanax* sp.; a) Contas river population (2n=48, FN=92), b) Preto do Costa river population (2n=48, FN=90). The NOR-bearing chromosomes after silver nitrate staining for each population are shown inbox.

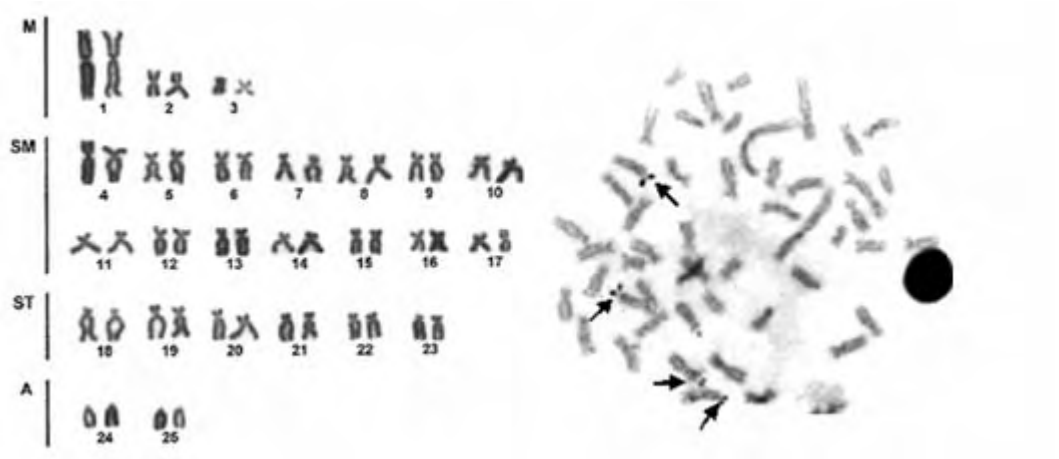

Fig. 2 Giemsa-stained karyotype of *Astyanax aff. bimaculatus* (2n=50, FN=92) and, on the right, a metaphase spread after silver nitrate staining showing up to four NOR-bearing chromosomes (indicated by arrows).

Fig. 3 Metaphase spread (on left) and karyotype (on right) of *Hoplias malabaricus* (2n=40, FN=80). The two NOR-bearing chromosomal pairs after silver nitrate staining are shown inbox.

Curimatella sp. was characterized by the presence of 54 chromosomes, all meta-submetacentric (FN=108). Single NORs were identified in this species by silver nitrate staining, with positive marks on short arms of a medium-sized SM chromosomal pair (Fig. 4). The NOR-bearing chromosomal pair could not be precisely identified since chromosomal size decreased subtly between pairs.

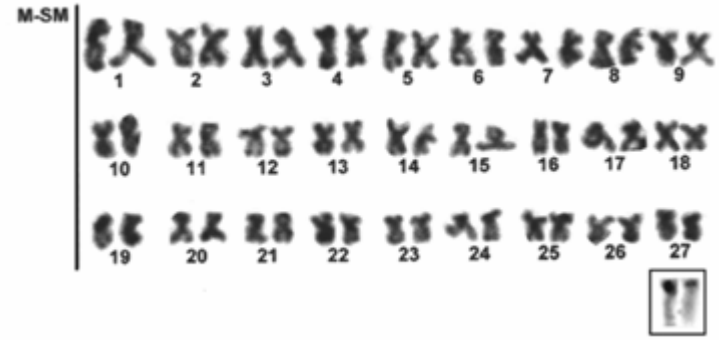

Fig. 4 Giemsa-stained karyotype of *Curimatella* sp. (2n=54, FN=108). The NOR-bearing chromosomes after silver nitrate staining are shown inbox

Preliminary results in *A. lacustris* from Contas river basin revealed a karyotype composed of 16M+30SM+2ST+2A (2n=50, FN=98). A single NOR system was also found in this species, comprising the subtelocentric chromosomal pair. Marks on short arms of the NOR-bearing pair were constantly heteromorphic and homologous chromosomes were often associated in metaphase spreads (Fig. 5).

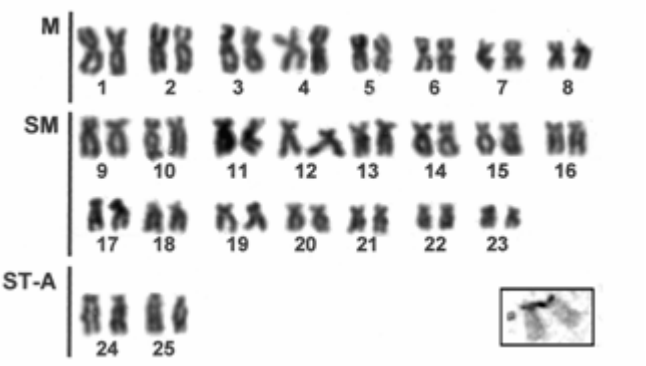

Fig. 5 Giemsa-stained karyotype of *Acestrorhynchus lacustris* (2n=50, FN=98). In detail, a partial metaphase showing the homologous chromosomes associated by NORs after silver nitrate staining.

Evidences of a sex chromosome system were absent in all species and populations herein analyzed.

4. Discussion

The present results, although preliminary, provided some interesting features about evolutionary, cytotaxonomic and conservation management issues of characiform fish from Contas river basin. However, the scope of the present study is to show the relevance and application of basic cytogenetics on assessing levels of biodiversity, using neotropical fish as a model. Therefore, the following topics related to discussion of each species are restricted to general considerations.

Cytogenetic studies have revealed two major evolutionary trends regarding chromosomal diversification within Characiformes. One group comprises families and species characterized by a remarkable karyotypic variability, including small characins such as *Astyanax* spp. and fishes from family Erythrinidae [7, 8]. Usually, taxonomy and phylogenetics of these groups are controversial and cytogenetic analyses can represent a useful tool. In several cases, chromosomal data have supported the existence of species complex or sibling species, such as in *Astyanax scabripinnis* [21, 22], *A. fasciatus* [23], *Hoplias malabaricus* [24] and *H. unitaeniatus* [25], among many others. On the other hand, families like Anostomidae, Curimatidae, Prochilodontidae, Chilodontidae, and Parodontidae present little numerical and structural chromosomal variation, representing a cytogenetically conserved group [7, 8, 26, 27]. Actually, these families seem to be monophyletic, comprising a group of migratory fish species that usually swim in large schools [28]. This behavior could favor gene flow among populations and restrain the fixation of chromosomal rearrangements. A similar statement is postulated to explain low levels of chromosomal diversity in highly connected populations, such as marine fishes [29, 30]. Nevertheless, some peculiar chromosomal features can be found even in these apparently conserved groups, such as the presence of supernumerary chromosomes in some Prochilodontidae, Curimatidae and Parodontidae species [31] and a ZZ/ZW sex chromosome system in several *Leporinus* representatives (Anostomidae) [32].

The karyotypes described for the species of the genus *Astyanax* in the present work corroborates the chromosomal plasticity of this group of small characins, widespread throughout the neotropical region. The species *Astyanax* sp. was formerly considered a synonym for *Astyanax fasciatus* [33]. This "species" is now considered a complex and may present a wide chromosomal diversity, with diploid numbers ranging from 2n=45 to 48 and several distinct chromosomal formulae [23]. The differentiated chromosomal pattern reported for rivers within a same hydrographic basin (Preto do Costa and Contas) supports the high chromosomal plasticity of this group and suggests that a further taxonomic review is required to define precisely both evolutionary units. In fact, cytogenetic studies coupled with morphometric analyses in populations of *A.* sp (formerly *A. fasciatus*) revealed striking differences among populations in Contas river basin and nearby areas [33].

The species *A. bimaculatus* also represents a widely distributed fish in Brazilian rivers and recent morphological studies have separated this species into other taxonomic units such as, for instance, *A. altiparanae* from South Brazil [34]. The karyotype of *A. aff. bimaculatus* populations from Contas river basin were identical and the diploid number found (2n=50) is the same described elsewhere in this group of species [21, 35, 36]. However, the present data reveal that populations from the state of Bahia present differentiated karyotypic formulae when compared to other Brazilian hydrographic basins, indicating a high structural chromosomal diversity in this group. The number and location of active nucleolar organizer regions in both *Astyanax* species herein analyzed have also presented a remarkable variability within and between populations, reinforcing their degree of cytogenetic variation. Additional studies using distinct chromosomal markers might, eventually, reveal other inter-population differences within each group.

Similarly, cytogenetic features in the species *Hoplias malabaricus* (Erythrinidae) reported in the present work can be useful to understand some biogeographic pattern and chromosomal evolutionary pathways in this group. Based on Giemsa-stained karyotypes, seven cytotypes were previously described for this species (often referred as a complex of sibling species) along South American river systems, involving different diploid numbers (2n=39 to 2n=42), chromosome morphology and presence or

absence of distinct sex chromosome systems [24, 37]. Few populations from northeast region of Brazil have been analyzed and the present results are the first data available for populations restricted to Contas river basin. The karyotype found in our samples (2n= 40 M/SM chromosomes) corresponds to the cytotype A, also found in a close hydrographic system, the São Francisco river basin. The presence of two NOR-bearing chromosomal pairs is another feature shared by populations from both basins [24, 38]. These data, combined with the similar geological history of both hydrographic systems suggest a common origin for these populations.

On the other hand, Curimatidae species have been regarded as a typical conservative characiform group under a cytogenetic focus. Most of species analyzed so far present a homogeneous karyotype composed of 54 bi-armed chromosomes [28, 39, 40] and the curimatid representative herein selected is no exception. Curiously, icthyological studies have reported the genus *Curimatella* in the São Francisco river basin (*C. lepidura*) but not in other eastern Brazilian river basins [9]. However, taxonomic analyses in the individuals sampled in this work identified them as a *Curimatella* species by the unique presence of scales in the caudal fins. The cytogenetic data have also revealed a karyotype very similar to that reported for *C. lepidura* [40], but considering the high degree of chromosomal similarity within this family, it is yet to be defined whether this species represents a new occurrence of *C. lepidura* or an undescribed new species. Chromosomal markers, such as the location of nucleolar organizer regions, might play a major role on distinguishing closely related species [26]. For instance, species of the family Anostomidae (a sister-group of Curimatidae) present similar karyotypes (2n=54, FN=108) but NOR-bearing chromosomes constitute species-specific markers able to precisely identify *Leporinus* species [41]. Further studies about NOR sites and other banding techniques in *Curimatella* sp. may eventually provide useful chromosomal features helpful to compare the cytogenetic patterns in curimatid species.

The family Acestrorhynchidae comprises a single genus; *Acestrorhynchus* [9]. Although widely distributed throughout South America, few species of this family have been cytogenetically studied, showing a constant presence of 2n=50 chromosomes [42]. Comparing the present results for *A. lacustris* in the Contas tiver basin (16M+30SM+2ST+2A) with previous reports in populations from Mogi-Guaçu river in Southeastern Brazil (12M+32SM+4ST+2A) [42], a structural inter-population difference in the chromosomal structure can be inferred, probably related to pericentric inversions. Besides, the first information about the location of active ribosomal sites is presented in this species.

Finally, it should be pointed out that the present analyses are dependent on few reagents and simple equipments (basically a centrifuge, a temperature-controlled chamber and a light microscope) when compared to other genetic methodologies, such as those involving direct DNA studies. Furthermore, digital photographic systems are now easily afforded and represent a major advance in cytogenetics. Traditionally, cytogenetic laboratories have been dependent on a photographic facility and researchers should be able (and skillful) to handle all the laborious steps involving film and photography developing. Nowadays, a high-resolution digital camera attached to a light microscope and a good printer turns the work easier and faster. Additionally, if one wants to go to the expenses, several imaging capture software systems are on market and they are very adequate for routine cytogenetic analyses.

Based on the available data, we encourage students and researchers all over the world to focus on basic cytogenetic studies in order to increase the information about chromosomal patterns of fish species. This dataset can be extremely useful to understand the evolutionary history of species, chromosomal rearrangements, and establish levels of inter- and intra-specific diversity, allowing phylogenetic and taxonomic inferences. Such approach seems to be particularly suitable to research centers in developing countries, usually surrounded by poorly known biodiversity hotspots and limited financial resources.

Acknowledgements The financial support by FAPESB, CNPq and UESB is gratefully acknowledged. The authors also thank Dr. Roberto E. Reis (PUC-RS) for the taxonomic identification of species.

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