

NICHOLAS JACOB WALKER

**CYTOGENETIC AND MOLECULAR CHARACTERIZATION OF TWO
POPULATIONS OF MINNOWS *ASTYANAX GITON* EIGENMANN 1908
AND *ASTYANAX* AFF. *FASCIATUS* (CHARACIFORMES: CHARACIDAE)
IN THE SÃO BARTOLOMEU CREEK, MINAS GERAIS STATE, BRAZIL**

Dissertação apresentada à
Universidade Federal de Viçosa,
como parte das exigências do
Programa de Pós-Graduação em
Biologia Animal, para obtenção do
título de *Magister Scientiae*.

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APROVADA: 7 de março de 2013.

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RESUMO

WALKER, Nicholas Jacob., M.Sc., Universidade Federal de Viçosa, Março de 2013. **Citogenética e caracterização molecular de duas populações de peixes *Astyanax giton* Eigenmann 1908 e *Astyanax* aff. *fasciatus* (Characiformes: Characidae) na ribeira São Bartolomeu, Minas Gerais, Brasil.** Orientador: Jorge Abdala Dergam Dos Santos.

Os peixes neotropicais de água doce são os mais abundantes em termos de espécies, e os peixes da família Characidae têm a maior diversidade entre eles. O gênero *Astyanax* contém 130 espécies, que se encontram em condição de *incertae sedis* em termos sistemáticos. Porém, as espécies podem ser diferenciadas com estudos de padrões morfológicos, citogenéticos e moleculares. As populações do gênero *Astyanax* do ribeirão São Bartolomeu de Minas Gerais, Brasil, nunca foram identificadas. Neste estudo, peixes do gênero *Astyanax* de duas represas da Universidade Federal de Viçosa foram analisados usando técnicas citogenéticas, incluindo coloração convencional (Giemsa), padrões de heterocromatina (banda-C), regiões de organizadores nucleolares (NOR) e hibridização fluorescente *in situ*. Os dados cromossômicos foram comparados com os caracteres morfológicos de cada espécime (número de raios da nadadeira anal, número de escamas na linha lateral, disposição das escamas e padrões dentários). Os cariótipos de 4m+18sm+22st+6t e 10m+20sm+12st+6t para *A. giton* e *A. fasciatus* não têm sido registrados anteriormente, e para *A. giton*, isto é o segundo cariótipo para esta bacia. As sequências de mtDNA de COI indicam que *A. giton* é próxima de *A. giton* da mesma bacia e mais distante de *A. scabripinnis* e *A. intermedius*, e *Astyanax* aff. *fasciatus* é no mesmo clado de outros *A. fasciatus* e *A. parahybae*.

ABSTRACT

WALKER, Nicholas Jacob., M.Sc., Universidade Federal de Viçosa, March 2013. **Cytogenetic and molecular characterization of two populations of minnows *Astyanax giton* Eigenmann 1908 and *Astyanax* aff. *fasciatus* (Characiformes: Characidae) in the São Bartolomeu Creek, Minas Gerais State, Brazil.** Adviser: Jorge Abdala Dergam Dos Santos.

Neotropical freshwater fish are the most abundant vertebrate species, with Characidae fish representing the greatest diversity among them. The genus *Astyanax* contains approximately 130 species, all of which are currently considered *incertae sedis* in systematic terms. Because of vague original descriptions, many local populations are difficult to identify. In these cases, the combined analysis of morphological, cytogenetic and molecular data may provide useful information for determining their taxonomic status. We applied this approach on two populations of *Astyanax* occurring in the São Bartolomeu Creek of Minas Gerais State, Brazil, a tributary of the Doce River Basin. Morphological characters included the analysis of lateral line scales, teeth, anal fin rays. Cytogenetic techniques included conventional coloration (Giemsa), heterochromatin patterns (C-Banding), nucleolar organizer regions (NORs) and fluorescent *in situ* hybridization (FISH). Molecular techniques were performed using a 634 bp cytochrome oxidase subunit I fragment of mitochondrial DNA. All characters indicate that both species are *Astyanax giton* and *Astyanax fasciatus* respectively. The karyotypes of 4m+18sm+22st+6t for 10m+20sm+12st+6t and *A. giton* and *Astyanax* aff. *fasciatus* respectively had not been previously reported, and for *A. giton*, this is the second karyotype in the Doce River Basin and the first incidence of B chromosomes. The mtDNA sequence COI indicate that *A. giton* is closely associated to other *A. giton* from the same basin and more distantly related to *Astyanax scabripinnis* and *Astyanax intermedius*, whereas *Astyanax*

aff.*fasciatus* forms a strongly supported clade with *A. fasciatus* from other basins and *A. parahybae*.

LITERATURE REVIEW

Few published works exist regarding *Astyanax giton*. According to Fishbase, *A. giton* is found in the Paraíba do Sul River Basin and coastal streams of Espírito Santo and Rio de Janeiro States, Brazil [1]. Recently, *A. giton* has been found in the Sossego Creek in Viçosa, Minas Gerais State, Brazil [2].

Eigenmann [3] defined *A. giton* by the following characteristics: Snout more than half the length of the eye; two or three teeth in the front row of the premaxillary; a caudal band, depth 2.5 to 2.6; eye 2.5; anal fin with 23-24 rays; 34 scales on the lateral line with 5 above and four below as well as a diffuse (sometimes absent) vertically elongate humeral spot crossing the third scale of the lateral line. Eigenmann based these characters on two specimens from the Rio Paraíba in Eastern Brazil.

Astyanax giton from the Paraíba do Sul River Basin, Serra da Bocaina, São Paulo State, Brazil presented a diploid number of $2n=50$ and a karyotype of $6m+8sm+8st+28t$, FN=72 [4]. Both *A. giton* and *A. intermedius* from this river showed five chromosome pairs bearing 5SrDNA sites, all telocentrics except for one subtelocentric in *A. giton*. The occurrence of the same number of 5S rDNA sites in similar locations supports the proximity of these species [4,5].

Populations of *A. giton* from the Paraitinga River and Jacuí Stream presented the same diploid number and karyotype as those from the Paraíba do Sul River Basin [4,6]. In the Paraitinga River and Jacuí Stream, C-banding revealed heterochromatin in the interstitial regions of chromosomes 4, 5, 6 and 9 as well as in the pericentromeric regions of many chromosomes. Silver nitrate staining in the nucleolar organizer regions (Ag-NOR) from two sites on the Paraitinga River showed multiple NORs, up to six markings, and fluorescent in situ hybridization with the 18S rDNA probe revealed up to ten markings, many of them very reduced [5].

The karyotype and heterochromatin of *A. giton* from the Paraitinga was similar to *A. intermedius* in this river, with the latter species having a karyotypic

formula of $6m+8sm+4st+32t$ and heterochromatin in many pericentromeric regions. No GC or AT positive heterochromatin was found in either species and the heterochromatin did not hybridize with the As51 satellite DNA probes, a rarity in fish. These data indicate that *A. giton* and *A. intermedius* belong to a different clade than *A. scabripinnis* and *A. parahybae*, which is supported by a 5S metacentric marker pair observed in the latter two species but not in the former [4,6,7].

Astyanax fasciatus was described by Cuvier in 1819 with Brazil as its type locality. This species is widely distributed, ranging from the Rio Negro in Patagonia to Mexico and southern Texas [3,8,9]. Different localities have more or less distinct varieties of *A. fasciatus*. These varieties are often only differentiated statistically; i.e., individuals are impossible to distinguish but a large number of specimens may be distinguished from those of another locality. *Astyanax fasciatus* is absent from some rivers where it has probably taken on distinct forms, such as in the Paraíba do Sul River. In this river it is replaced by the similar *A. parahybae*, which Eigenmann considered a distinct form of *A. fasciatus* formed by river isolation. This is the result of species isolation in rivers independently emptying into the Atlantic in eastern and southeastern Brazil, Central America and Mexico.

Astyanax fasciatus is characterized morphologically by a depth of caudal peduncle more than half the length of the head. It usually has a single tooth in the maxillary. The depth is 2.3 to 3. Scales below the lateral line are in parallel series. The caudal band is simple and median [3]. In the Três Marias River, *A. fasciatus* present an anal fin with 22 to 32 rays, a lateral line with 37 to 41 scales, with seven or eight above and 5.5 to 6.5 below, a length of 2.6 to 3.2 times its height, and one maxillary tooth [10].

Astyanax fasciatus is considered a species complex based on morphological differentiation among basins and karyotypical diversity [11-16]. Several chromosome polymorphisms have been observed in *A. fasciatus*, with fundamental numbers ranging from 66 to 90 [13,17]. Multiple cytotypes have been observed in the same location, as in the Mogi-Guaçu River, which contains

three variants in sympatry, providing further evidence for considering *A. fasciatus* as a species complex [13]. Chromosomal inversions favor speciation, especially over sites of geographical co-occurrence [9].

Diverse cytotypes, including $2n=45$, $2n=46$, $2n=47$, $2n=48$ and $2n=50$, have been found in sympatry [15]. In a study of *A. fasciatus* in the Água da Madalena stream, Botucatu, São Paulo State, Brazil, three karyomorphs ($2n=46$, $2n=48$ and $2n=50$) were found in sympatry. Based on the absence of intermediate chromosome numbers, the restriction of B chromosomes to the $2n=46$ karyomorph and the absence of heteromorphism for the 18S rDNA sites, these karyomorphs are unlikely to hybridize and are considered three separate species [15]. Diploid numbers for *A. fasciatus* are highly variable across streams and basins.

Astyanax fasciatus has diploid numbers of $2n=45$, 46, 47 or 48, sometimes in sympatry, in the Rio Grande do Sul Basin [13,16]. However, populations of this fish in the Upper Tibagi River may present karyotypes of $6m+18sm+14s+10t$ ($2n=48$) or $8+18sm+14st+10t$ ($2n=50$), as well as heterochromative variations within the same karyotype [14]. Cytotypes without gene flow can be considered distinct taxonomic units, even in sympatry.

The $2n=46$ cytotype is present in other river systems as well, including the Araguari River in Uberlândia, Minas Gerais State, Brazil. Here, specimens of *A. fasciatus* presented a karyotype of $14m+16sm+10st+6t$ (FN=86) with Ag-NORs in the terminal region of the short arm in the first submetacentric chromosome pair and in the short arms of the third metacentric pair [18].

Populations of *A. fasciatus* of the Tibagi River showed variable Ag-NOR sites, with one cytotype having 2-3 and the other 1-3. The Ag-NOR sites show evidence of transposition, fission, deletion, translocation and inactivation, and the presence of multiple NORs could indicate a plesiomorphy within this genus. Differences in the karyotypes, constitutional heterogeneity, and 18S/5S sites do not seem to represent fitness or selective adaptation in *Astyanax* [19].

Histone genes constitute a complex multigene family with a variable number of copies in one or more chromosome regions [11,20]. The H1 linker

histone genes located in the pericentromeric regions of two chromosomes in *A. fasciatus* were identical to those in *A. altiparanae* and *A. bockmanni*, indicating these are a conserved feature of this genus. *Astyanax fasciatus* also presented clusters of 5S rDNA on one telocentric pair, a trait also found in *A. bockmanni* but not *A. altiparanae*. The 5S and H1 genes co-localized on chromosome 3 and 5S hybridization revealed clusters in the pericentromeric regions of chromosome 23 [11].

Populations of *Astyanax fasciatus* in the Upper Paraná, Araguaia-Tocantins, Contas and São Francisco basins present multiple karyotypes in sympatry, reviewed in [15]. In the Água da Madalana Stream, Botucatu, São Paulo Brazil, a population of *A. fasciatus* included a $2n=46$ cytotype (FN=84, $5m+8sm+6st+4a$), a $2n=48$ cytotype (FN=86, $5m+8sm+6st+4a$) and a $2n=50$ cytotype (FN=84, $5m+8sm+4st+8a$). All contain similar constitutive heterochromatin and occur in sympatry, with no differences between males and females.

The cytotypes may be differentiated by morphology using canonical variate analysis, indicating the variants may be the result of gene flow or recent isolation. Karyotype morphology of the $2n=48$ cytotype was different at Furnas 2 of the Vila Velha State Park in Paraná State, which is separated from other bodies of water and prohibits gene flow. The $2n=48$ and $2n=50$ cytotypes contain heterochromatin mainly distributed as very conspicuous blocks in the telomeric regions of the long arms of the telocentric chromosomes. One $2n=49$ individual was found, with seven metacentric chromosomes and heterochromatin equal to the 48 and 50 cytotypes -- possibly a rare hybrid. A distinct $2n=50$ cytotype in this region presented only a few chromosomes with heterochromatin, almost always located interstitially [14].

In *A. fasciatus* from the Upper Paraná, NORs are present in the distal portions of the short arms of two submetacentric chromosomes. 5S markers were present in the pericentric region of two metacentric and two telocentric chromosomes, with 28S markers present in the distal portion of the short arm of

two submetacentric chromosomes, colocalized with 5S in the two metacentric and telocentric chromosomes [7].

In the Mogi-Guaçu river, the headwaters (at Ouro-Fino) contain *A. fasciatus* with diploid numbers of $2n=48$ and $2n=47$; in the middle region (Cachoeira de Emas, Pirassununga) they have diploid numbers of $2n=48$, $2n=46$, $2n=45$, $2n=47$ and $2n=46$ but with different chromosome morphology; and near the confluence of the Mogi-Guaçu with the Pardo River (Barrisha), $2n=46$ and $2n=48$ [13]. NOR, 18S and 5S markers were conserved within all cytotypes. The $2n=45$ cytotype is likely the result of centric fusion among telocentric chromosomes as no $2n=44$ cytotype has been found. At Emas Falls, in the middle of the Mogi-Guaçu River (where fish cannot go upstream), most *A. fasciatus* are $2n=46$ with few $2n=48$ and chromosomal variants [9].

This scenario is reversed at localities upstream (Ouro Fino, all $2n=48$) and downstream (Barrinha, most $2n=48$). Some specimens from the Tietê River may have invaded the Mogi-Guaçu at a certain point between Emas falls and Barrinha, possibly through floods that temporarily connected tributaries from each main river, or by human activities. An occasional hybrid of $2n=48$ and $2n=50$ is found in the Tibagi River, a tributary of Paranapanema [9].

NORs are variable between cytotypes in the Mogi-Guaçu, ranging from two (most common) to eight with FISH 18S markers located in the NORs. Other fluorescent markings were smaller, always telomeric and located in subtelocentric and telocentric chromosomes, although more difficult to see and not detectable in all specimens. All cytotypes have a ribosomal site on the subterminal region of the short arm of one submetacentric pair and 5S markings in the pericentromeric region of the short arms of one telocentric pair [13]. These markings indicate the proximity of the cytotypes and a recent divergence [13]. The $2n=46$ cytotype has six variations, likely the result of random interbreeding between cytotypes, i.e. karyotype plasticity. Although differences in karyotype may cause reproductive isolation, this is not the case in this river [21].

These chromosome combinations are not enough to adversely affect fitness, as evidenced by marker chromosomes from several $2n=48$ variants. It is

possible that Cachoeira de Emas contains populations that converged during migrations. $2n=46$ could have come from the Paraná River, through either the Tietê or Grande rivers, or small floods in areas where the Tietê and Mogi-Guaçu rivers are close [13].

If $2n=46$ is an invading karyotype, the ability to interbreed may be helping it succeed [13]. Shared features of the karyotypes suggest recent divergence, suggesting two populations or two species [19]. Chromosomal speciation may cause reproductive isolation because hybrids may be infertile, although that does not appear to be the case in this river [13]. The $2n=46$ invader is likely able to interbreed with $2n=48$, as indicated by the frequency and location of $2n=46$ cytotypes in nearby Paraná Basin collection sites and constitutive heterochromatin in terminal regions on long arms of various chromosomes and in the terminal regions of the short arms of submetacentric chromosomes in both cytotypes, with these regions being GC-rich and containing NORs [16].

Banding patterns differ among *Astyanax*. C-banding is highly variable, with polymorphisms occurring even within populations. GC-rich heterochromatic regions are often associated with NORs [9]. Fifty nine percent AT satellite DNA was identified in a portion of the *A. scabripinnis* genome [22] containing monomeric units of 51 bp, known as As51 [23]. This sequence has also been identified in *A. fasciatus*, *A. janae* and *A. paranae*. Although satellites are likely targets for breaks and fusions, As51 has not been associated with modifications to the diploid number in *A. fasciatus* or *A. scabripinnis* [23].

Nevertheless, hybrid forms have not been directly detected in the Mogi-Guaçu Basin, although molecular markers show some gene flow among cytotypes [19]. As51 satellite sites have been found in the terminal regions of the long arms of one telocentric, four subtelocentric and three submetacentric pairs in the $2n=48$ cytotype, and in one telocentric, three subtelocentric and four submetacentric pairs in the $2n=46$ cytotype, with additional As51 sites in the variant pairs. CMA3 staining showed GC-rich heterochromatin exclusively related to the NOR sites [16].

Several species of *Astyanax* including *A. altiparanae*, *A. lacustris*, *A. fasciatus*, *A. schubarti* and *A. scabripinnis* show conserved localization of 5S/28S markers between species, indicating a close phylogenetic relationship [7], with 5S visible in more than one pair [24]. NORs are almost always fewer than regions detected by FISH. These 5S locations are absent in *A. giton* and *A. intermedius*, suggesting polyphyly or divergence within this genus [13]. R-banding patterns have been observed in four species (*A. altiparanae*, *A. fasciatus*, *A. schubarti* and *A. scabripinnis*) in one large submetacentric chromosome and one small pair of metacentric chromosomes, colocalized with C-band negative late replicating regions [7].

In *Astyanax*, heterochromatin may be balanced between AT and GC content [6] or 59% AT [22]. *Astyanax fasciatus* and *A. paraguayae* have As51 DNA, while *A. giton* and *A. intermedius* do not. Both the 2n=46 and 2n=58 forms of *A. fasciatus* have 8 pairs of As51, which is similar to that of *A. scabripinnis* from the Piracicaba River [12,16]. It is possible that As51 was the ancestral condition and that the pairs have increased with time as a result of Robertsonian and non-Robertsonian rearrangements that could duplicate or disperse satellite DNA. Most As51 sites are homologous, but slight variations exist. Variations between karyotypes could be the result of intrachromosomal rearrangements involving telocentric chromosomes with terminal As51 sites on the long arms, which were absent in the 2n=45 forms [16].

Not all regions where 28S rDNA colocalized with 5S rDNA were Ag+, possibly because the 5S genes were associated with rDNA genes that did not form a nucleolus because the gene sequences were too small to be detected [7]. In *A. fasciatus* from the Upper Paraná, co-localization was observed in one of the telocentric pairs [24]. 5S rDNA is composed of tandemly repeated units of approx 120 bp, intercalated by a non-transcribed spacer (nts). The 120 bp repeated units are stable, while the nts changes more rapidly [4]. Several species of *Astyanax*, including *A. fasciatus*, have a 5S rDNA cluster in the distal region of a metacentric chromosome [4,7].

B-chromosomes are dispensable supernumerary chromosomes characterized by their lack of homology with regular ones [20] and do not obey Mendelian laws of inheritance [25]. B chromosomes are mostly composed of repetitive DNA sequences, especially ribosomal DNA, satellite DNA and in rare cases, protein coding genes [11]. It is estimated B chromosomes are present in 10-15% of eukaryotes [25] but this is impossible to determine with certainty as by definition they are not found in all individuals and are likely to be missed if the karyotype is based on only one individual [26].

B chromosomes can vary substantially in their size, frequency and other characteristics. They may be macro, intermediate or micro and most are heterochromatic. Many Bs contain rRNA genes detectable by both NOR and FISH [25]. B chromosomes may originate in one of two ways. They can be intraspecific, from the host genome (most common) or interspecific, from another species, .e.g. the result of hybridization [27]. As B chromosomes do not synapse or recombine, they evolve independently of the A set. Therefore, regardless of origin, the evolutionary pathways of B chromosomes are the same in either case and B chromosome morphology is not necessarily reflective of origin.

There are several ways to test B chromosome origins. Banding patterns and repetitive sequences may be compared between the B and A sets. If no matches are found, the B chromosome can be compared to the a set of related species. Chromosomal fragments may also be visible in interspecific crosses, and phylogenetic analyses may be done on the transposable elements of both the B and A sets [27].

The simplest method for B chromosomes to originate is for them to come from the A set via duplication or fragments after fusions or amplification of a paracentromeric region. Fragments of paternal chromosomes sometimes survive even in cytoplasmic incompatible crosses [27]. That similar B chromosomes are found in similar species suggests they are long lived and may even survive speciation events -- or they could be the result of more recent hybridization events [25,27].

B chromosomes in *Astyanax* can vary within species (by size or morphology). They may also vary by sex or altitude, meaning may be an adaptive trait [9]. There is no evidence on the precise role of B chromosomes on individual fitness in *Astyanax*[9]. Species of *Astyanax*, such as *A. fasciatus*, *A. scabripinnis* and *A. schubarti* present B-chromosomes [28,29]. The origin of supernumerary chromosomes is unknown, but it is accepted that they result from amplification of other parts of the genome [30] and A-chromosomes follow their own evolutionary pathway (Mestriner, 2000). The metacentric macro B chromosome is the most common in *Astyanax*[11].

In *A. fasciatus*, B chromosomes are reported for the $2n=46$ karyomorph in the São Francisco Basin [15,31,32] and a $2n=49$ karyomorph has been observed in the Tibagi River, in the Upper Paraná River Basin, Ponta Grossa, Paraná, Brazil [14].

There are two groups of B chromosomes in *Astyanax*. The first is composed of species with large metacentric Bs, including *A. fasciatus*, *A. scabripinnis*[32-36], *A. eigenmanniorum*, *A. schubarti*, and *A. bockmanni*[32], suggesting a common origin. This hypothesis could be tested by comparing DNA sequences of B chromosomes. The second is in the *A. scabripinnis* group, where the presence of distinct types of supernumerary chromosomes has been commonly reported in several populations ranging from large metacentric/submetacentric elements to small metacentric or even microchromosomes [9].

B chromosomes may result from interspecific hybridization, supported by the presence of DNA sequences in the B chromosome matching those of the A chromosome of related species, as seen in *A. fasciatus*[37]. However, an ancestral B preceding speciation events is supported by As51 satellite DNA found in B chromosomes of *A. scabripinnis* populations. [9]. The B chromosome may originate from a nondisjunction of the first pair of chromosomes during meiosis, with subsequent heterochromatinization [29]. Another hypothesis is that shared DNA sequences on A and B chromosomes evidence that the B chromosomes are isochromes from the A set [22], which was confirmed in *A.*

scabripinnis by finding that B chromosomes resulted from tandem duplications of heterochromatin segments in acrocentric pair 24, as well as sharing some repetitive DNA sequences [36,37]. This is based on the presence of an interstitial heterochromatic band in the long arm of this pair, which is believed to cause interstitial As51 sites at symmetric distances from the centromere in both arms of the B chromosome. B chromosomes may also vary by gender, with individual females or some populations containing an additional B [22]. A large metacentric B chromosome was found in *A. scabripinnis*, *A. eigenmanniorum*, *A. schubarti* and *A. fasciatus*[32,37]. This suggests that this B originated prior to species differentiation, but molecular analysis is needed to confirm. B chromosomes have not been documented in *A. giton*. No phenotypic effects resulting from B chromosomes have been observed in *Astyanax* [37].

As in the second hypothesis, rapid heterochromatization may occur by genetic imprinting to deactivate B chromosomes, which explains the presence of heterochromatin in many Bs. Sometimes the B forms anew in each generation due to non-disjunction [27]. Rapid heterochromatization of B chromosomes often occurs (as seen by C-Banding) and may be necessary for stability and positive selection. B chromosomes may also be a precursor to sex chromosomes (especially the y chromosomes, which also move to opposite poles during spermatogenesis) which are also high in repeats [25,27]. B chromosomes may originate from either autosomes or sex chromosomes, although the latter are not found in any populations of *A. fasciatus* or *A. giton*.

B chromosomes often contain rDNA to encode ribosomal RNAs, typically visualized as NORs (secondary constrictions). NORs may also be suspect to chromosome breakage, which could create Bs that could then lose rDNA segments, affecting their size. Bs may also accumulate fragments from various sources [27].

Bs may have no phenotypic effects, or they may affect vigor, fertility or fecundity. For example, B chromosomes result in color changes in some plants [25]. Bs may also exert influence simply by their presence, e.g. by containing NORs. [27]. One negative effect of Bs is that having more chromosomes means

more DNA to replicate, meaning a longer cell cycle and larger cells. This slows development in some species. Bs may be more common in species with smaller genomes and fewer chromosomes [25].

B tolerance is dependent on the effects of supernumerary chromosomes. If fitness decreases with Bs, complete intolerance results. If fitness is unaffected with some B chromosomes, but is reduced when more are present, there will be incomplete tolerance (the most common case). If fitness is independent of Bs, the A set will be tolerant. Overtolerance occurs when the B increases host fitness. This last case is most likely when the B arises from hybridization as it includes new DNA. As the effects of B chromosomes are usually negative, they may be more common in favorable environments with less selective pressure [25].

Methylation has been proposed for deactivating sex chromosomes and may also function with late-replicating B chromosomes to make them genetically inert [27]. B chromosomes that are transcriptionally active with no apparent phenotypic effects could have their transcripts nullified by transposon insertion.

Meiosis is always asymmetric, especially in females with polar bodies and oocytes [25]. Since Bs are not paired during meiosis, they can go to their preferred meiotic pole, possibly increasing their transmission rate. Bs may also result from nondisjunction during meiosis anaphase. Bs are often unstable during meiosis, with a transmission rate of less than 0.5. A rate of greater than 0.5 indicates accumulation, or drive. B drive is a disregard for Mendelian transmission (also known as an unfair transmission pattern) and is necessary for their survival. B chromosomes must have accumulation methods, or they would only make sense as being beneficial; they likely begin by being selfish even if they become inactive later [25]. Due to meiosis, it is possible for the B to always originate from one sex. They can occur during female meiosis by migrating from one oocyte to another, as well as in males by avoiding heterochromatin [27].

Bs may be considered parasitic if they have driven or damage the host (virulence). In species in which the B chromosomes do not drive, they could be suppressed by the A genome. The host (A set) may evolve resistance, but the B

may evolve another form, continuing the cycle. For example, in species of mealy bugs the B tries to decondense and segregate with the sperm, but drive suppressor genes in the A set prevent this from occurring. The selection to eliminate B chromosomes, however, is usually weak. Genes for their removal are only helpful to individuals with them, and in most cases the B has very small effects. On the other hand, the B is selecting for its very survival [25].

Bs are selected at the level of the chromosome and impact higher levels of organization [25]. They can affect gene expression of the A set, NOR expression and the level of recombination in the A set (chiasma effect). The latter is hypothesized to occur for one of four reasons:

- 1) The adaptation hypothesis: B chromosomes enable higher levels of genetic variation, leading to faster population evolution. This is criticized because it suggests parasites are selected for positive host effects.
- 2) Passive effects: Chiasma effects are the result of extra DNA, but this cannot explain contradictory effects.
- 3) The increased chiasma frequency is the result of parasitic Bs, because some recombinant progeny will be resistant to B chromosomes and individuals with Bs will have higher rates of recombination. (This is a variation of Matt Ridley's Red Queen hypothesis [38], that recombination increases when conditions are poor.)
- 4) Changes in chiasma frequency serve the selfish purposes of the B chromosome, i.e. Bs pair with Bs to reduce meiotic loss with the side effect of more B chiasma, or the formation of bivalent Bs is blocked, or Chiasma results from restricted A-B pairing.

When chiasma increases in populations with driving Bs, the third explanation is most likely. The response is proportional to the intensity of B parasitic Bs, and contradictory results could indicate the B is not driving (near-neutral). Where chiasma decreases, it could be that Bs are heterotic or beneficial. In populations with even numbers of B chromosomes, it may be because the B chromosomes are able to pair (hypothesis 4). If odd and even

numbers of Bs are present in different ratios, one may have more drive than the other [25].

Many B chromosomes are stable, in which the drive mechanism and selection are balanced. In these cases, the B becomes more dependent on drift and population size, unless it can acquire an even number of Bs and regular meiotic behavior to incorporate itself into the A set [25]. B equilibrium (stability) results from two opposing forces: first, the accumulation of Bs by either the selfish chromosome theory or a high transmission ratio; and a change in individual fitness. As the survival of B chromosomes is ultimately linked to their hosts, they may become less harmful over time (which would reduce the probability of phenotypic effects) [25]. Newly formed Bs would therefore be expected to be the most harmful.

B chromosomes have three options -- they can remain as a parasite, they can acquire a function and integrate themselves into the host (the selfish gene, working horizontally; although this is rare and only possible when already common) or go extinct. They may change from parasitic to neutral and back many times. The A set may completely suppress drive, or do nothing. But the B has a secret weapon -- because it is genetically indispensable, it can support a high mutation rate without serious consequences, which explains the large number of B variants in many species. Even a neutralized B can still last more than 10,000 generations, plenty of time to regain drive/parasitism [25]. According to Muller's ratchet, deleterious mutations build up in asexual species, so sexual reproduction is needed to eliminate them. As B chromosomes are not subject to recombination, they may also be subject to these mutations [27].

B chromosomes may in some cases be considered genomic parasites [26], as in the selfish gene theory [39]. This has been theorized for populations of *A. scabripinnis* with B chromosomes at high altitude but not at lower elevation [40]. Under this explanation, more B chromosomes would be expected in areas where environmental conditions are more favorable for this species -- e.g. headwater streams, higher altitude, etc., where decreased predation and interspecific competition allow higher tolerance of parasitic B chromosomes [37].

In populations of *A. scabripinnis*, populations at the highest altitudes were the most likely to have large metacentric B chromosomes [24], but they had fewer NORs than low altitude populations [37].

C-Banding has shown that B chromosomes can be totally or partially heterochromatic [28]. Some *Astyanax* populations show faint paracentromeric C-bands in all chromosomes, being more conspicuous distally on the long arms of telocentric A chromosomes [22]. Additionally, C-band locations may coincide with those of NOR, and interstitial C-bands are sometimes present on the arms of B chromosomes.

Astyanax fasciatus from the Campo Novo River, Paraná Basin, São Paulo State, Brazil (22°23'8.35"S, 49°0'55.63"W) presented a diploid number of $2n=46$, karyotype of $10m+14st+20st+2t$ and $FN=90$ [11]. These authors suggest that the $2n=46$ *A. fasciatus* are probably the result of fusions from the ancestral state of $2n=50$. Two out of 25 individuals had medium-sized telocentric B chromosomes of similar size and morphology to pair 23, but it is unlikely that they originated from the A set because they do not have similar rDNA or hisDNA.

Astyanaxbimaculatus, *A. fasciatus*, *A. schubarti*, and *A. scabripinnis* subjected to R-Banding showed one large submetacentric and two small submetacentric markers, indicating a common origin among them [7,8]. R- and G-banding of a population of *A. scabripinnis paranae* (now known as *A. paranae*, separate from the *A. scabripinnis* complex [41]) from Cascatina stream showed a pattern of longitudinal differentiation in B chromosomes [29].

Silver nitrate (Ag-NOR banding) can identify the 45S rDNA located in the NORs, however the 5S rDNA sites require more specific techniques, e.g. fluorescent in situ hybridization (FISH) [24]. Intrapopulation NOR site variation may represent genomic variations in populations with stable karyotypes [24].

Astyanax spp. are found in two reservoirs of the São Bartolomeu Creek at the Federal University of Viçosa in Minas Gerais State, Brazil, and have never been formally identified. We hypothesize that they are *A. giton* and *A. fasciatus*, known respectively as the silver minnow and red-tailed minnow. These species

show cytogenetic variance across basins and each may constitute a separate species complex. This hypothesis may be tested by cytogenetic characters and morphology.

Dam construction, flow regulation, mining, deforestation and industrial and agricultural pollution increasingly affect major river basins by modifying fish habitats and altering fish life-cycles. However, compared to mammals, birds and amphibians, relatively few South American freshwater fish have been scientifically assessed as endangered species by IUCN or other criteria, even [42].

Cytogenetic studies, combined with mitochondrial DNA and morphology, will facilitate characterization of these species within *Astyanax*. Cytogenetic studies of chromosomal data, including karyotyping, C-Banding, Ag-NOR and the FISH technique will be performed. Cytogenetic studies can show if these individuals represent populations of *A. giton* and *A. fasciatus*, and can be used for interpopulational cytogenetic comparisons to provide information on evolutionary structure of this genus.

1. INTRODUCTION

Neotropical freshwater fish comprise approximately 6,000 of the 13,000 species worldwide, in 25 orders and 71 families [9]. These fishes represent the most diversified vertebrate group and are adequate for studies of genetic diversity and evolution [43].

Neotropical fishes present high karyotypic variability, with changes in the diploid number, sex chromosome systems, supernumerary chromosomes, natural triploidy, and diversity of constitutive heterochromatin and nucleolar organizer regions (NORs) [44]. Some families of Neotropical fish are characterized by stable diploid numbers, such as Anostomidae (including the genera *Abramites*, *Anostomus*, *Laemolyta*, *Leporellus*, *Leporinus*, *Pseudanos*, *Rhytiodus* and *Schizodon*), Prochilodontidae, (including *Prochilodus* and *Semaprochilodus*), Parodontidae (including *Apareiodon* and *Parodon*), Curimatidae (including *Curimata*, *Curimatella*, *Curimatopsis* and *Cyphocharax*), Cynodontidae (including *Cynodon*, *Hydrolycus* and *Rhaphydon*), Hemiodontidae (including *Anodus*, *Argonectes*, *Bivibranchia* and *Hemiodus*) and Pseudopimelodidae (including *Lophiosilurus*, *Microglanis* and *Pseudopimelodus*), all of which present $2n=54$ (excluding rare instances of B chromosomes) [45].

In other cases, the diploid numbers may be more variable. In the family Erythrinidae, *Hoplias lacerdae* presents a conservation of the diploid number $2n=50$ in the Usina Limoeira (São Jose do Rio Pardo) and CA UNESP (both in São Paulo State), in the Rio São Francisco and Uberlândia (Minas Gerais State) and the Rio Pitinga (Amazonas). This diploid number is also found in *H. aff. lacerdae* in the Rio Grande (Sacramento, Minas Gerais). On the other hand, *H. malabaricus* is variable, presenting $2n=40$ (males 39) in specimens from the Monjolinho, Piracicaba, Três Pontes, Passa-Cinco, Luiz Antônio, Tietê and Lobo rivers, (São Paulo); Rio das Pedras (Minas Gerais) and $2n=40$ (both sexes) in the Rio São Francisco (Minas Gerais), Manaus (Amazonas), Tucurul (Pará), Porto Velho (Rondônia), Cuiaba Pontal do Araguaia Rio Sepotuba (Mato

Grosso); Goiás Velho (Goiás), Corumba and Miranda (Mato Grosso do Sul), Paramaribo (Suriname) and the Rio Aguapey in Argentina. This last location also includes $2n=42$ cytotypes, as do the Doce River, Lake Carioca (of the Doce River), Lake Patos, Rio Grande, Rio Piranga and Rio Sapucal (all located in Minas Gerais); Rio Juquiá, Rio Jurumirim and Lake Guaporé (São Paulo); Rio Vorá, São Mateus do Sul, Curitiba, Rio Jurumirim, Rio Iguazu and Rio Tibagi (Paraná) and Araguaiana (Mato Grosso) [45]. In total, seven cytotypes have been found for *H. malabaricus* [46].

The Characiformes are one of the main orders of Ostariophysan fishes, including approximately 1,900 valid species belonging to over 270 genera, currently arranged in 18 families in Africa, Central America and South America [47,48]. It is one of the most numerous freshwater fish fauna in the Neotropical region, second only to catfish [49]. Phylogenetic relationships within Characiformes are not clear [50].

Characidae is the most diverse family and comprises 58% of this order, with approximately 250 South American genera and 1,100 species, [8,37,48,51,52]. It comprises the majority of freshwater fish in Brazil, with 20 subfamilies and approximately 400 species [10,37]. Phylogenetic relationships of Characidae, however, are not known (but see [21]). Genera of this family have variable diploid numbers, with $2n=52$ in *Bryconamericus*, *Cheirodon*, *Exodon*, *Inpaichthys*, *Markiana*, *Piabina*, *Triportheus*, *Charax*, *Cynopotamus*, *Galeocharax*, *Roebooides*, *Odontostilbe*, *Serrapinus* and *Mimagoniates*; 48 in *Bryconops*; 50 in *Ctenobrycon*, *Hasemania*, *Hollandichthys*, *Moenkhausia*, *Nematobrycon*, *Pselogrammus*, *Salminus*, *Aphyocharacinae*, *Brycon*, *Deuterodon* and *Oligosarcus*, the latter two closely related to *Astyanax*. Diploid numbers of 60 or greater are found in *Pristobrycon*, *Pygocentrus*, *Serrasalmus* and *Metynnis*, while lower numbers are found in *Phenacogaster* (46) or *Paracheirodon*, which presents 32, 36, 50 or 52 chromosomes [45].

More specific trends are also evident in genera such as *Astyanax*, which can vary in diploid numbers, chromosome morphology, banding patterns and the presence of B chromosomes, especially across species or basins. A diploid

number of $2n=50$ is considered basal for the genus [53] as found for *A. bimaculatus*, with most *Astyanax* containing between $2n=46$ and $2n=50$ chromosomes [7,15] except for *A. schubarti* which has $2n=36$ [7,54].

The genus *Astyanax* was described in 1854[55] based on samples collected from Baird and Girard in Texas and Mexico. The original description lists the presence of an adipose fin, large scales, dorsal fin above the ventrals a non-serrated abdominal line and a double row of teeth on both the upper and lower jaw, flattened with several conical spines or processes upon their edge, lacking canine and palative teeth. The double row of mandibular teeth is not reported in later descriptions of this genus.

Astyanax was described again by Carl Eigenmann in 1917, with samples from South America[3]. Eigenmann characterized *Astyanax* as small compressed fishes, more or less elongate, reaching a length of 150 mm and usually much smaller (with the exception being the larger *Astyanax maximus*). Fishes of this genus have a complete lateral line, pre-maxillary teeth in two series (internal series with four or usually five teeth), a mandible with strong frontal teeth and minute conical teeth on the sides. The crowns of the premaxillary and mandibular are usually ridged and denticulate, with few or no teeth in the maxillary. Maxillary bone is short, with its anterior edge forming a more or less continuous curve from end to end. The lateral line rarely has more than 39 scales although it can be as high as 41 in some specimens of *A. fasciatus*, *A. taeniatus* and *A. scabripinnis*. The lateral line curves slightly in the front, and runs along the fourth longitudinal series of scales, above the ventral fin or higher. The scales are of normal size and cover the base of the rays of the tail, although the rest of the tail is naked. Regular and complete series of scales are found between the occipital process and the dorsal. The gill rakers are setiform and no predorsal spine is present. Body length is about three times body height [3,10].

Some geographically isolated fish assemblages are restricted to headwaters, separated by physical, chemical or biotic barriers, resulting in distinct evolutionary pathways within a single hydrographic system. This may be

the reason *Astyanax* is one of the most speciose genera in the Neotropics, including several species complexes [9]. *Astyanax* is regarded as *incertae sedis* because of its likely polyphyletic origin [9] and considered a recent group with chromosome polymorphism in several species [21]. *Astyanax* has extensive morphological diversification and highly complex taxonomy with a number of species complexes [15,37,52].

Although doubts remain on the monophyly of *Astyanax*[3,4,7,8,56], this genus is an excellent model for all kinds of studies of evolutionary mechanisms [41]. It is one of the best documented Neotropical fish genera from a cytogenetic standpoint, with the species complexes *Astyanax scabripinnis*, *A. fasciatus* and *A. altiparanae* (the latter is sometimes referred to as *A. bimaculatus*) being the most studied [41]. Species complexes are characterized by wide cytogenetic variation, mainly regarding distinct diploid numbers. Karyotypes, however, could also differ because of non-Robertsonian rearrangements. Whether or not individual cytotypes are considered distinct species depends on whether or not they can hybridize (karyotype plasticity) [41]. Some examples of karyotypes are as follows:

Astyanax scabripinnis from Rio Agua do Rancho, Paraná present $2n=50$, $6m+28sm+16t$, 4 NORs, C-Bands and 0-2 micro B chromosomes; *A. bimaculatus* from the Rio Paraguai, Mato Grosso do Sul present $2n=50$, $6m+26sm+12st+6t$, 1 NOR, C-, Q- and Mytramycin Bands and no Bs; *A. altiparanae* from Fundo Altenas, Minas Gerais are $2n=50$, $8m+28sm+6st+8t$, 2 NORs, C-Banding, Chromomycin A3 and no Bs; *A. aff. abramis* from the Rio Bento Gomes, Mato Grosso are $2n=50$, $22m+10sm+6st+12t$, 1 NOR, C-Banding, Chromomycin A3 and no Bs; *A. eigenmanniorum* from the Marrecas, Paraná are $2n=50$, $6m+20sm+8st+16t$, 5 NORs, C-Bands, Chromomycin A3 and no Bs; *A. schubarti* from the Rio Mogi-Guaçu, São Paulo are $2n=36$, $12m+16sm+4st+4t$, 1 NOR, C-Bands and no Bs; and *A. fasciatus* from the Rio São Francisco, Minas Gerais are $2n=46$, $6m+24sm+8st+8a$, 4 NORs, C-Bands, and 0-1 large metacentric Bs [45].

Astyanax fasciatus has highly variable karyotype morphology [41], with two standard diploid numbers of $2n=46$ and $2n=48$ and some $2n=50$ specimens with affinities to the *A. fasciatus* group in the Tibagi River Basin [9]. Several cytotypes may occur in sympatry, as in the Mogi-Guaçu River. The karyotype of *Astyanax giton* is also geographically variable although a diploid number of $2n=50$ is conserved. A comparison of *Astyanax* spp. cytotypes across basins is shown (Table 1).

Table 1. Comparison of diploid numbers and cytotypes of *Astyanax fasciatus*, *A. parahybae* and *A. giton* across several basins in Brazil

| Species | Location | Dipl oid | Bs | m | s m | st | t | |
|--|--|-------------|----|--------|--------|--------|--------|---|
| <i>A. fasciatus</i> | Meia Ponte River, Goiânia, GO, Paranáiiba River | 46 | | 2 8 | | 1 8 | | |
| | Mogi-Guaçu River, Pirassununga, SP, Upper Paraná River | 46 | | 1 4 | 2 0 | 1 0 | 2 | |
| | Juquiá River, Registro, SP, Upper Paraná River | 48 | | 1 0 | 2 4 | 1 2 | 2 | |
| | Mogi-Guaçu River, Upper Paraná River | 46 | | 3 2 | | 1 4 | | |
| | Paiol Grande Stream, São Bento do Sapacuaí, SP, Grande River | 48 | | 1 2 | 2 0 | 1 2 | 4 | |
| | | | 45 | | 1 2 | 2 0 | 1 0 | 3 |
| | | | | | 1 2 | 2 0 | 1 0 | 4 |
| | | | 46 | | 2 1 | 0 2 | 0 1 | 4 |
| | | | | | 1 2 | 1 9 | 1 0 | 6 |
| | Mogi-Guaçu River, Pirassununga, SP, Upper Paraná River | | | | 1 2 | 2 1 | 1 0 | 4 |
| | | | 47 | | 1 2 | 2 0 | 1 0 | 5 |
| | | | | | 1 2 | 2 0 | 1 0 | 4 |
| | | | 48 | | 8 | 2 2 | 1 2 | 6 |
| | Mogi-Guaçu River, Ouro Fino, MG, Upper Paraná River | | 47 | | 1 2 | 2 0 | 1 0 | 5 |
| | | | | | 8 | 2 2 | 1 2 | 6 |
| Mogi-Guaçu River, Barrinha, SP, Upper Paraná River | | 46 | | 1 2 | 2 0 | 1 0 | 4 | |
| | | | | 8 | 2 2 | 1 2 | 6 | |
| Piracicaba River, Piracicaba, SP, Tietê | | 46 | | 1 | 2 | 1 | 4 | |

| | | | | | | | |
|--------------------------------|---|-----------|------------|----------------|----------------|----------------|----------|
| | River | | | 2 | 0 | 0 | |
| | Araguari River, Uberlândia, MG, Paranaíba River | 46 | | 1 4 | 1 6 | 1 0 | 6 |
| | São Francisco River, MG | 46 | 0-1 | 1 2 | 1 6 | 4 4 | 4 |
| <i>Astyanax aff. fasciatus</i> | São Bartolomeu Creek, Viçosa, MG, Doce River | 48 | 0-2 | 1 0 | 2 0 | 1 2 | 6 |
| <i>A. parahybae</i> | Paraíbuna River, Paraíba do Sul River | 48 | | 8 | 1 8 | 1 2 | 1 0 |
| | Paraitinga River, Paraíba do Sul River | 48 | | 8 | 1 8 | 1 2 | 1 0 |
| <i>A. giton</i> | Paraitinga River, Cunha, SP, Paraíba do Sul River | 50 | | 6 | 8 | 8 | 2 8 |
| | Jacuí Stream, Cunha, SP, Paraíba do Sul River | 50 | | 6 | 8 | 8 | 2 8 |
| | Sossego Dam, Viçosa, MG, Doce River | 50 | | 6 | 8 | 2 4 | 1 2 |
| | São Bartolomeu Creek, Viçosa, MG, Doce River | 50 | 0-1 | 4 | 1 8 | 2 2 | 6 |

Brazilian states abbreviated as follows: GO = Goiás, MG = Minas Gerais, SP = São Paulo. Table adapted from data reviewed by Pazza & Kavalco [9]. Data for *Astyanax giton* from Sossego from Aguiar [2], *A. fasciatus* from São Francisco from (UNESP) [45] and *Astyanax aff. fasciatus* and *A. giton* from São Bartolomeu (bold) from this work.

In terms of morphology, Eigenmann [3] points out that "the true *A. scabripinnis* shades through [*A. intermedius*] into *A. taeniatus*.... I think it would be possible to arrange specimens of *A. scabripinnis*, *A. intermedius* and *A. taeniatus* from the Paraíba into series in respect to any one character and get complete integrations." In *Astyanax taeniatus*, the teeth on the sides of the lower jaw tend to become graduate, a condition leading to the distinguishing character of *Deuterodon*[3]. *Astyanax taeniatus* usually has three teeth in the front row of the premaxillary, 19 to 24 rays in the anal fin, two maxillary teeth, 32 to 39 scales on the lateral line with 5 to 7 above and 4 or 5 below. The teeth of *A. obscurus*, *A. taeniatus*, *A. giton* and *A. intermedius* all share the common trait of gradually decreasing, unlike most other *Astyanax* (including *A. fasciatus*) which exhibit an abrupt reduction in size between the anterior and posterior dentary teeth [57].

Astyanax altiparanae is considered a species complex because of significant inter-populational karyotypic variation (similar to *A. fasciatus* and *A.*

scabripinnis). karyotypic variation may be caused by structural rearrangements, e.g. pericentric inversions. This is observed in *A. giton* and *A. intermedius*, which are mainly differentiated cytogenetically by a paracentric inversion in the chromosomal pair bearing 5S rDNA [9].

Astyanax giton is found in the Paraíba do Sul River Basin and coastal streams of Espírito Santo and Rio de Janeiro States [1] and in the Doce River Basin in Minas Gerais State, Brazil [2]. *Astyanax giton* is characterized morphologically by two or three teeth in the front row of the premaxillary, an anal fin with 23-24 rays, 34 scales on the lateral line with 5 above and four below as well as a diffuse (sometimes absent) vertically elongate humeral spot crossing the third scale of the lateral line [3].

Populations morphologically similar to *Astyanax fasciatus* are distributed from the Rio Negro in Patagonia to Mexico and southern Texas [3,8]. *Astyanax fasciatus* is characterized morphologically by a single maxillary tooth, an anal fin with 22 to 32 rays, a lateral line with 37 to 41 scales, with seven or eight above and 5.5 to 6.5 below, a length of 2.6 to 3.2 times its height, and one maxillary tooth [10]. *Astyanax fasciatus* is considered a species complex, based on morphological differentiation among basins and karyotypical diversity [11-16]. Diploid numbers for *A. fasciatus* are highly variable across streams and basins. *Astyanax fasciatus* is absent from the Paraíba do Sul River in which it is replaced by *A. parahybae*, which may be a distinct form of *A. fasciatus* resulting from rivers independently emptying into the Atlantic in eastern and southeastern Brazil, Central America and Mexico [3,58].

Although the southeastern region of Brazil is known for its high levels of endemism (these rivers are considered a biogeographical unit [59], with 95% of species being considered endemic [58]), few species of *Astyanax* have been karyotyped in this area. Samples of *Astyanax* were captured in two reservoirs of the São Bartolomeu Creek, at the Federal University of Viçosa in Minas Gerais State, Brazil. Cytogenetic characterization of these fishes was done using karyotypes with Giemsa, Ag-NOR banding, C-Banding, and the FISH technique. These data were complemented with morphological and molecular analyses.

2. MATERIALS AND METHODS

2.1 COLLECTION AREA

Ninety-one individuals (32 *A. giton* and 59 *A. fasciatus*) were captured from two reservoirs of the São Bartolomeu Creek at the Federal University of Viçosa, in Viçosa, Minas Gerais State, Brazil from October 2011 to July 2012. The São Bartolomeu creek is part of the Doce River Basin, in southeast Brazil, (18° 45' and 21° 15' S and 39°55'and 43° 45'W). Fish were captured with rods from two reservoirs, one near the Quatro Pilastras (20°45'32.81"S 45°52'22.54"W) hereafter referred to as reservoir 1, and a second upstream dam (20°46'06.31"S 42°52'17.27"W), hereafter referred to as reservoir 2 (Figure 1). These reservoirs were selected because they are somewhat isolated and have never been studied cytogenetically. They are located within the Atlantic Rainforest, an area considered a biodiversity hotspot, with only 8% of original forest remaining [60].

Although fish can swim from Reservoir 2 to Reservoir 1 via a waterfall, they cannot go in the opposite direction, suggesting the possibility of differences in species composition between these reservoirs. Fifty fish (23 *A. giton* and 27 *A. fasciatus*) were captured from Reservoir 1 and 41 fish (9 *A. giton* and 32 *A. fasciatus*) from Reservoir 2. One male *A. giton* was captured from Reservoir 1; the other 31 were female. Thirteen male *A. fasciatus* were captured from Reservoir 1 along with 14 females, and six males and 26 female *A. fasciatus* were captured from Reservoir 2. *Astyanax giton* were more difficult to capture in both reservoirs and circumstantial evidence indicates that this species actively avoids gillnets.

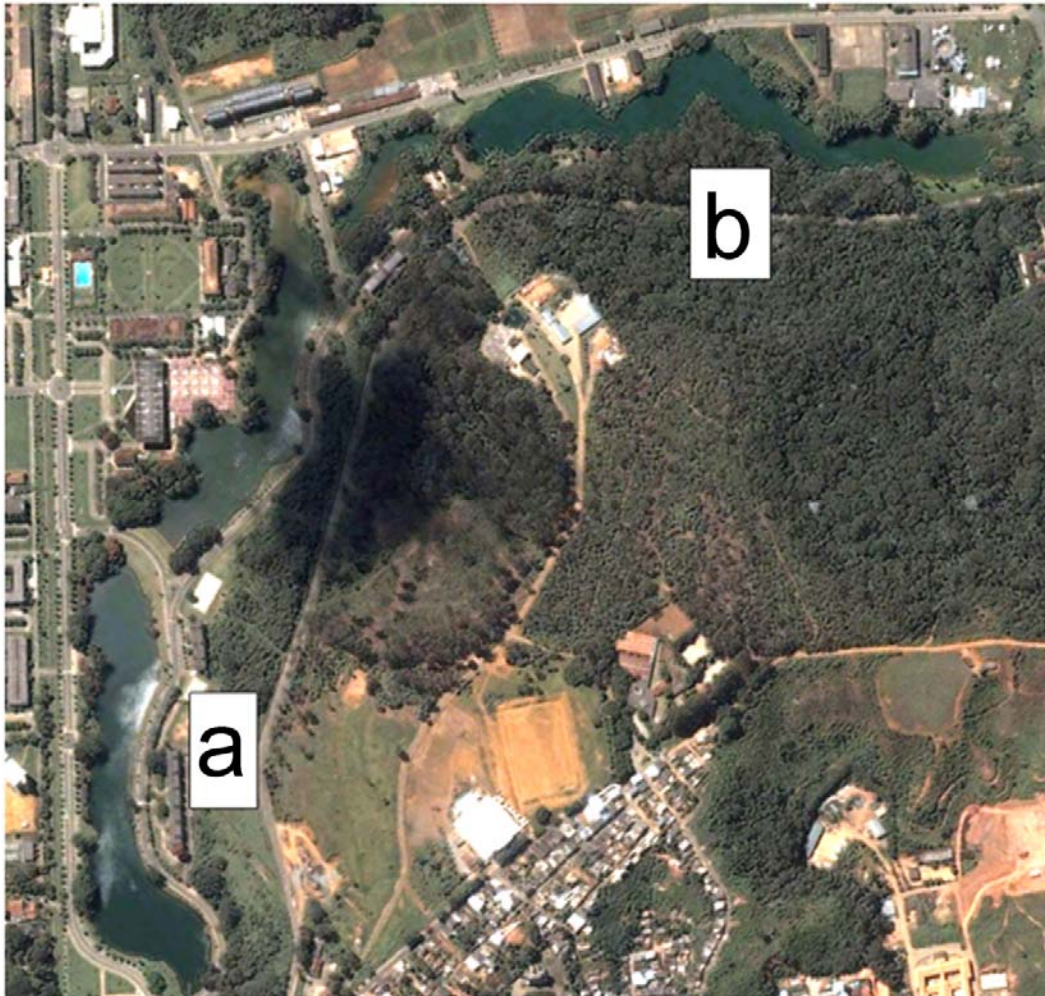


Figure 1. Collection areas within the Federal University of Viçosa (UFV) in Viçosa, Minas Gerais State, Brazil. a) Reservoir 1; b) Reservoir 2. Map adapted from Google Earth imagery.

Fish were kept in aquariums for cytogenetic analysis, fixed in 10% formalin for one week, transferred to 70% ethanol, deposited in the ichthyological collection at the João Moojen Zoological Museum for morphological studies or sent to Dr. Vinicius A. Bertaco at the University of Rio Grande Do Sul for taxonomic confirmation.

2.2 MORPHOLOGICAL PROTOCOLS

The sex of each fish was determined by external sexual dimorphism, based on presence (males) or absence (females) of anal fin hooks and later

confirmed by type of gonad. Morphometric data were analyzed [3,10,56] including the length and height of the fish, the aspect ratio (length/height), the number of rays in the anal fin, the number of scales on, above and below the lateral line, and the number of teeth in the dentary, maxillary and pre-maxillary. Data were analyzed using the software program PAST v. 2.17b [61]. Data were transformed using the option to remove size from distances in PAST with isometric Burnaby then plotted with principal component analysis (PCA) with a correlation matrix and disregarding groups.

For PCA, fish were separated by species and the following thirteen morphological characters were used: 1) Total length, 2) Body height, 3) Aspect ratio (length/height), 4) Scales above lateral line, 5) Scales below lateral line, 6) Scales on lateral line, 7) Number of rays on anal fin, 8) Dentary teeth (center), 9) Dentary teeth (side), 10) Maxillary teeth, 11) Number of teeth in the first premaxillary row, 12) Number of teeth in the second premaxillary row, and 13) Number of teeth in the third premaxillary row. This last attribute was observed only in a single female *Astyanax aff.fasciatus* from reservoir 1 containing a single tooth in the third row; the rest of the fish had only two rows as expected for this genus.

2.3 CYTOGENETIC PROTOCOL

Cytogenetic analyses were carried out on 60 fish with specimens from both reservoirs. Mitotic chromosomes were obtained from cell suspensions of the anterior kidney, using the conventional air-drying method [62] after fish were anaesthetized with clove oil [63]. In addition to the standard Giemsa method, chromosomes were analyzed using silver nitrate staining [64] to visualize the nucleolar organizing regions (Ag-NOR), C-banding was used to detect C-positive heterochromatin [65] and fluorescent in situ hybridization (FISH) was performed [66] using 28S and 5S DNA sequence probes.

About 30 metaphase spreads were analyzed per specimen (total 1700) to determine the diploid chromosome number and karyotypic structure. Chromosomes were classified as metacentric (m), submetacentric (sm),

subtelocentric (st) or telocentric (t) according to centromeric index values [67] using the program ImageJ 1.46.

2.4 MOLECULAR DNA

DNA extraction followed [68] on six specimens. Fragments of cytochrome oxidase subunit I (COI) were amplified using primers L8524 (5'-AA Y CCT GAR ACT GAC CAT G-3') and H9236 (5'-GTT AGT GGT CAK GGG CTT GGR TC-3') [69]. Double-stranded DNA was synthesized in 50 μ l reactions containing 10 μ l dNTPs (1 mM), 5 μ l reaction buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 μ l MgCl₂ (50 mM), 2 μ l of each primer (10 mM), 0.5 μ l (2.5 U) Taq DNA polymerase (Phoentria), 2 μ l template DNA (100 ng/ μ l) and 26.5 μ l H₂O. PCR conditions were as follows: 94°C (2 min), five cycles of 94°C (45 s), 54°C (45 s) and 72°C (1.5 min) and 29 cycles of 94°C (45 s), 58°C (45 s) and 72°C (1.5 min). PCR products were purified using Qiaquick (Qiagen; www.qiagen.com): 5 μ l of the purified PCR product were used in a 10 μ l cycle sequencing reaction using a rhodamine terminator cycle sequencing kit (PE Applied Biosystems; www.appliedbiosystems.com). Sequences were aligned using MEGA 5.0 [70]. The model of molecular evolution that best fitted the data was GTR+I+G chosen using MrModeltest 2.3 [71] and haplotype divergence was estimated within and between haplogroups using this model. Phylogenetic trees were constructed with Bayesian inference (MB) using MrBayes v. 3.2.1.x64 [72] with ten million Markov-Chain Monte-Carlo (MCMC) steps [73] with 25% burn-in to produce posterior probabilities of nodes in the tree. *Oligosarcus argenteus* was used as an outgroup but removed from the final trees. Sequences were deposited in GenBank (accession numbers XXXXXXXX). Tree diagrams were produced using FigTree 1.4.0.

Table 2. Samples used for mtDNA phylogenetic analysis of *Astyanax* aff. *fasciatus* and *A. giton* compared with *A. intermedius*, *A. fasciatus*, *A. scabripinnis* and *A. parahybae* from various basins

| Genbank | Species | Basin | | Location (GPS) | Reference |
|------------|--------------------------------|---------------|----|----------------|-------------------------------|
| GU702127.1 | <i>A. intermedius</i> | | | | |
| GU702128.1 | <i>A. intermedius</i> | Paraíba | do | 23.374 S | [74] |
| GU702129.1 | <i>A. intermedius</i> | Sul | | 46.053 W | |
| GU702130.1 | <i>A. intermedius</i> | | | | |
| | <i>A. intermedius</i> | Paraíba | do | 19°51'10.97", | Unpublished research (Beagle) |
| | <i>A. intermedius</i> | Sul | | 42°48'0.73" | |
| | <i>A. intermedius</i> | | | | |
| | <i>A. intermedius</i> | | | | |
| | <i>A. intermedius</i> | Doce | | 20°29'02.31" S | [2] |
| | <i>A. intermedius</i> | | | 42°49'58.16" S | |
| | <i>A. intermedius</i> | | | | |
| | <i>A. intermedius</i> | | | | |
| GU702120.1 | <i>A. scabripinnis</i> | Paraíba | do | 22.60 S | [74] |
| GU702225.1 | <i>A. scabripinnis</i> | Sul | | 45.161 W | |
| GU702190.1 | <i>A. scabripinnis</i> | | | | |
| | <i>A. giton</i> | Doce | | 20°29'02.31" S | [2] |
| | <i>A. giton</i> | | | 42°49'58.16" S | |
| | <i>A. giton</i> | | | | |
| | <i>A. giton</i> | Doce | | 20°45'32.81" S | Present work |
| | <i>A. giton</i> | | | 45°52'22.54" W | |
| | <i>A. giton</i> | | | | |
| | <i>Astyanax fasciatus</i> aff. | Doce | | 20°46'06.31" S | |
| | <i>Astyanax fasciatus</i> aff. | | | 42°52'17.27" W | |
| | <i>Astyanax fasciatus</i> aff. | | | | |
| GU702088.1 | <i>A. parahybae</i> | | | | |
| HM064982.1 | <i>A. parahybae</i> | Paraíba | do | 23.369 S | [74] |
| HM064976.1 | <i>A. parahybae</i> | Sul | | 46.024 W | |
| HM064977.1 | <i>A. parahybae</i> | | | | |
| JQ353530.1 | <i>A. fasciatus</i> | | | 23.5238 S | [75] |
| | | | | 45.8896 W | |
| JQ353557.1 | <i>A. fasciatus</i> | Upper Paraná | | 25.3779 S | |
| | | | | 49.8054 W | |
| JQ353550.1 | <i>A. fasciatus</i> | | | 21.5748 S | |
| | | | | 46.3236 W | |
| JQ353545.1 | <i>A. fasciatus</i> | São Francisco | | 18.4003 S | |
| JQ353546.1 | <i>A. fasciatus</i> | | | 45.471 W | |
| JQ353589.1 | <i>A. fasciatus</i> | Upper Paraná | | 15.6673 S | |
| | | | | 47.9524 W | |
| | <i>A. bimaculatus</i> | Doce | | 20°12'26.49"S | Unpublished research (Beagle) |
| | <i>A. bimaculatus</i> | | | 42°52'37.80"W | |

3. RESULTS

3.1 MORPHOLOGY

Astyanax giton was characterized by a total length 67.96 to 92.45 mm, height 20.05 to 30 mm, 5 to 6.5 (one with 7) scales above the lateral line, 4 to 6 (one with 6.5) scales below the lateral line, 31 to 39 (most 33 to 38) scales on the lateral line, 16 to 21 (most 18 to 21) rays on the anal fin, 4 (one with 3) to 5 central dentary teeth, 0 to 3 lateral dentary teeth, 0 to 2 teeth in the maxillary, 2 to 4 (one with 5) teeth in the first row of the premaxillary and 3 to 5 teeth in the second row of the premaxillary and a black vertically elongated humeral spot.

Astyanax aff. *fasciatus* was characterized by a length 70.7 to 116.9 mm, height 19 to 34.1 mm, 6 to 7.5 (one with 8) scales above the lateral line, 5 to 6.5 scales below the lateral line, 35 to 40 scales on the lateral line, 16 to 24 (most 18 to 24) rays on the anal fin, 3 to 5 central dentary teeth, 0 to 5 lateral dentary teeth, 0 to 2 teeth in the maxillary, 3 to 4 (one with 5) teeth in the first row of the premaxillary and 3 to 5 teeth in the second row of the premaxillary. A single specimen appeared to have one tooth in a third premaxillary row. Most specimens of *A. aff.fasciatus* presented red coloring on the tail fin, which is common for this species. Photos of *A. aff.fasciatus* and *A. giton* showing morphology are presented (Figure 2).

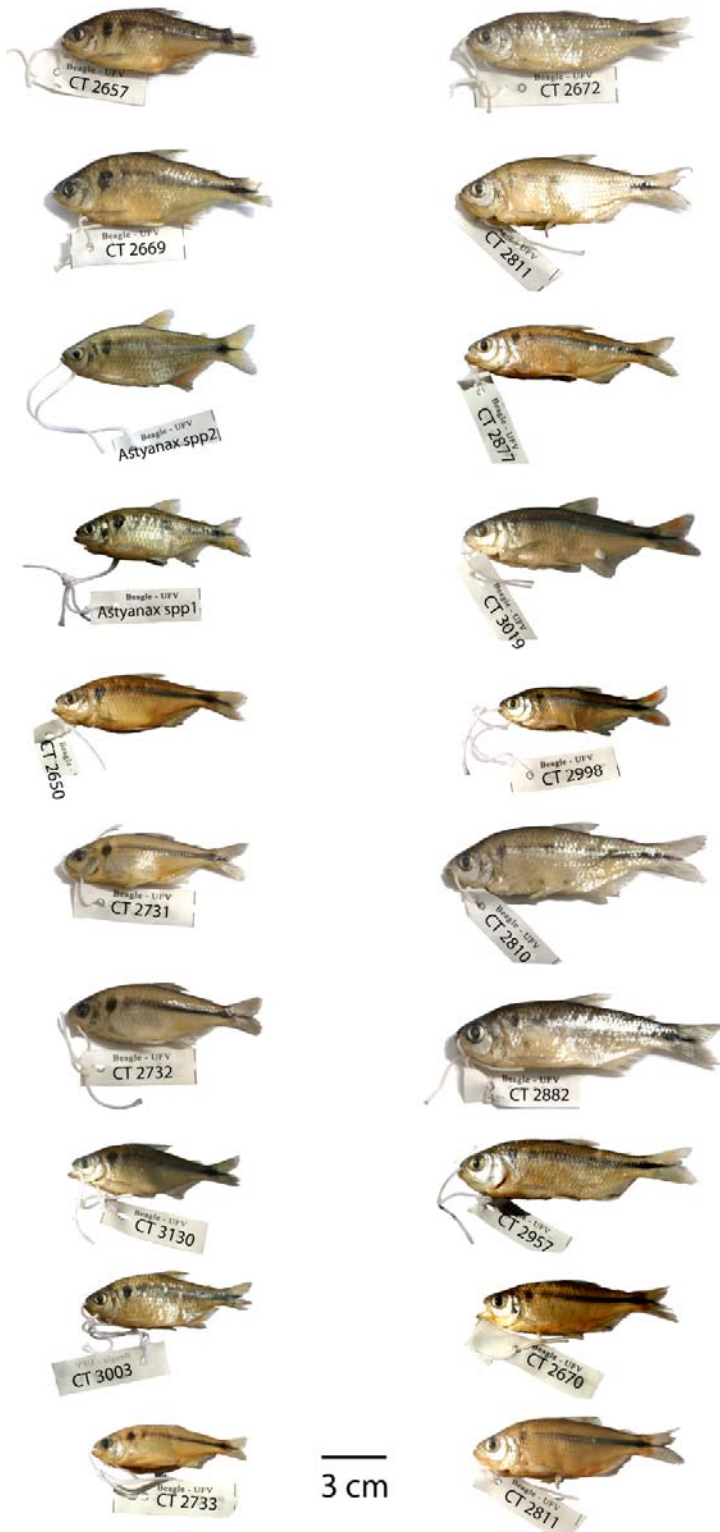


Figure 2. Specimens of *Astyanax giton* (left) and *Astyanax aff. fasciatus* (right) from the São Bartolomeu Creek, a tributary of the Doce River Basin.

3.1.1 PRINCIPAL COMPONENT ANALYSIS

There were no significant differences in morphology between fish of the same species from each reservoir. Fish of the same species from different reservoirs could not be differentiated visually. PCA placed each species slightly differently, although overlapped (Figure 3).

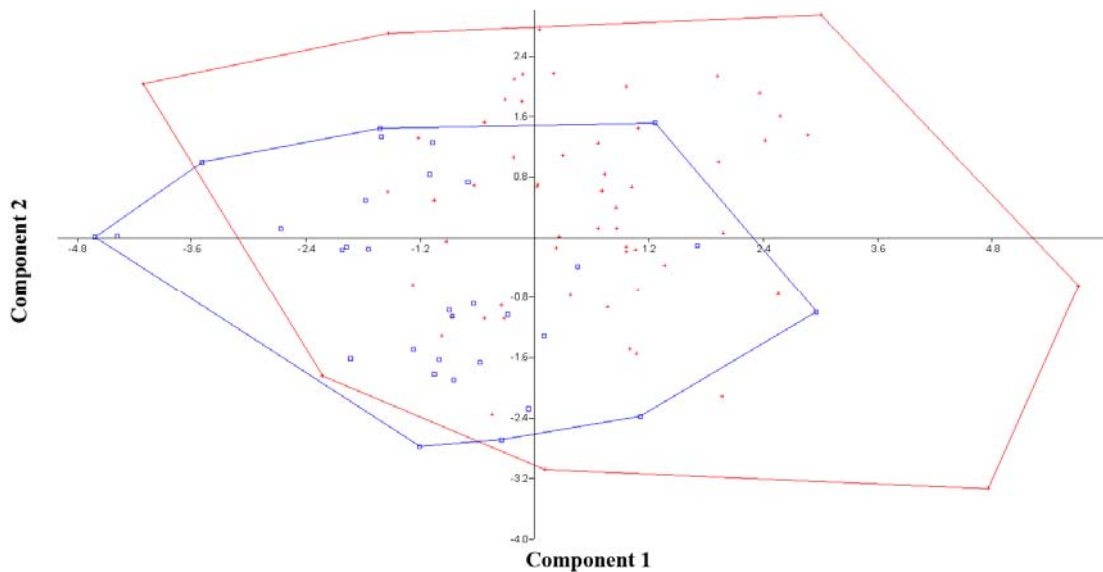


Figure 3. Principal component analysis (PCA) for *Astyanax giton* (blue) and *Astyanax aff. fasciatus* (red), combining data from both reservoirs. Most important coefficients were length (0.4595), height (-0.56) and aspect ratio (0.5554) for component 1 (y-axis) and length (-0.4028), scales below lateral line (0.4133), scales on lateral line (0.4919) and anal fin rays (0.456).

3.2 CYTOGENETICS

Astyanax giton presented a karyotypic formula of $4m+18sm+22st+6t$ (FN=94) (Figure 4). There were no differences between karyotypes of males and females.

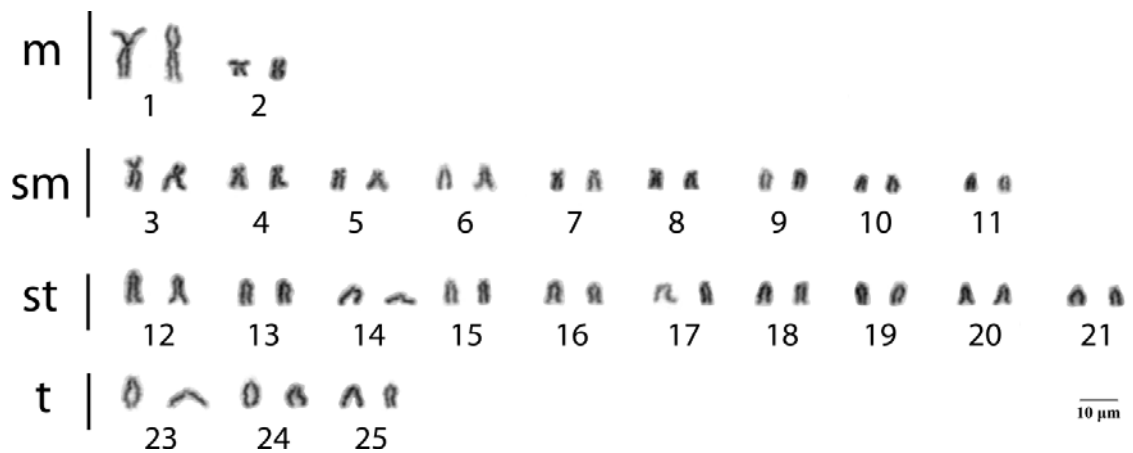


Figure 4. Karyotype of *Astyanax giton* from the São Bertolomeu Creek, a tributary of the Doce River Basin. Karyotypic formula: 4m+18sm+22st+6t.

Astyanax giton presented a secondary constriction in the first subtelocentric pair (not visible in all metaphases). NORs were visible in the short arms of one submetacentric pair and one subtelocentric pair (Figure 5). C-banding revealed the presence of heterochromatin in all centromeres and in the telomeric regions of the first metacentric pair, two submetacentric pairs and two subtelocentric pairs and one telocentric pair. Interstitial markings were visible in the long arms of one submetacentric pair (Figure 6).

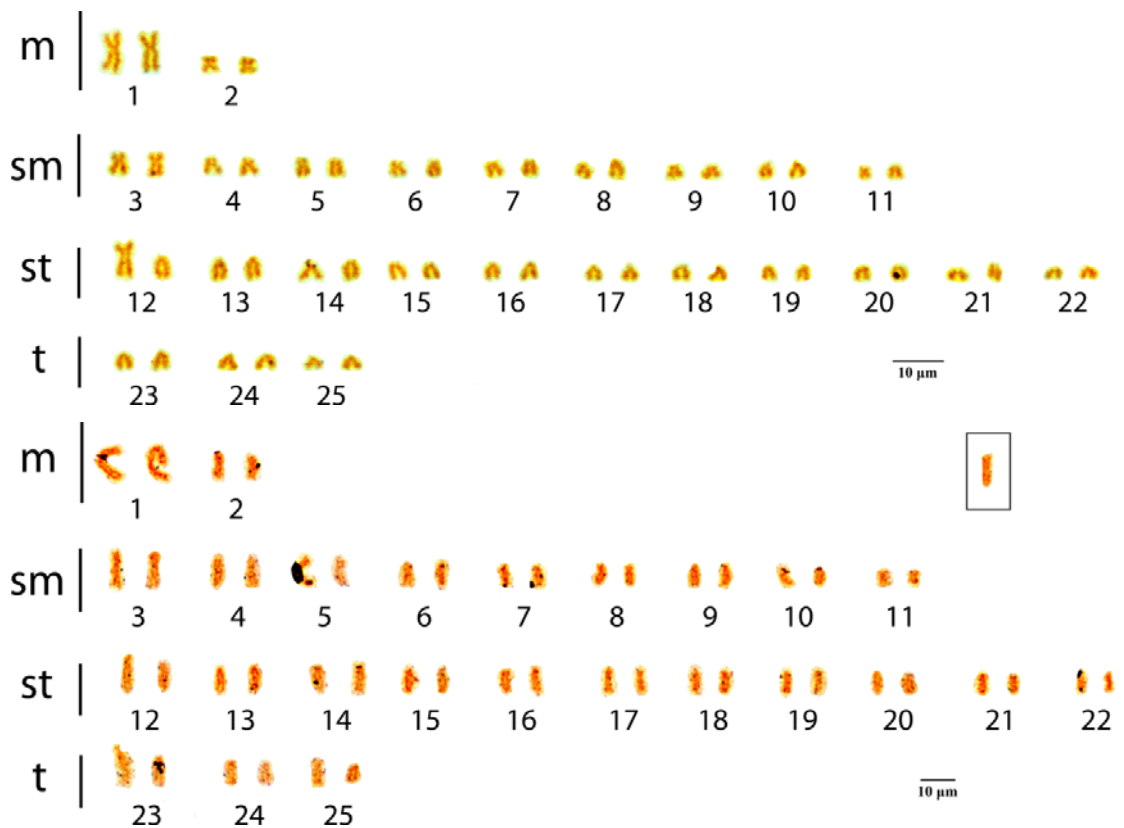


Figure 5. NOR sites in *Astyanax giton* from the São Bartolomeu Creek, a tributary of the Doce River Basin. The top photo shows one NOR in the short arm of one submetacentric chromosome in the 14th pair; the bottom photo shows NOR sites in the short arms of submetacentric pair 10 and subtelocentric pair 14. A B chromosome is shown in the box.

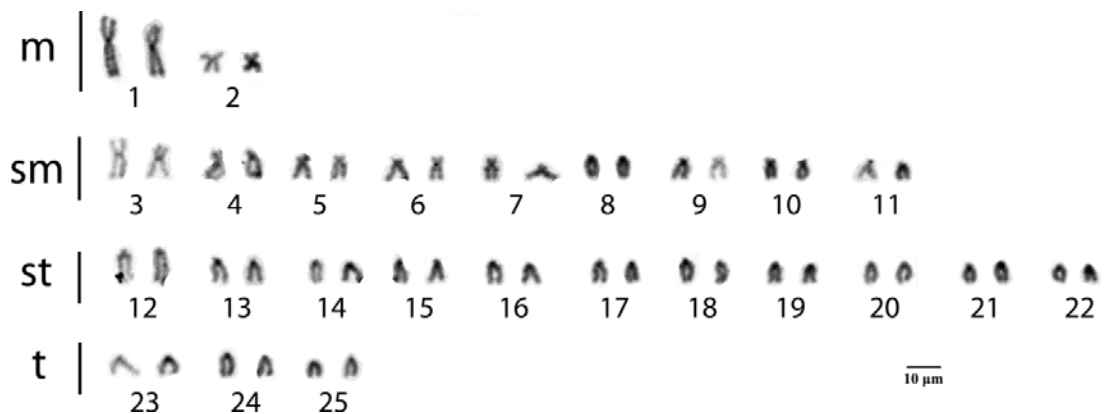


Figure 6. C-banding in *Astyanax giton* from the São Bertolomeu Creek, a tributary of the Doce River Basin. Heterochromatin is seen in the centromeres of all chromosomes, in the telomeric regions of the long arms of pairs 1, 4, 8, 18, 21 and 24, and interstitially in the long arms of the 7th pair.

Astyanax aff. fasciatus presented a karyotypic formula of 10m+20sm+12st+6t (FN=90) (Figure 7). There were no differences between karyotypes of males and females.

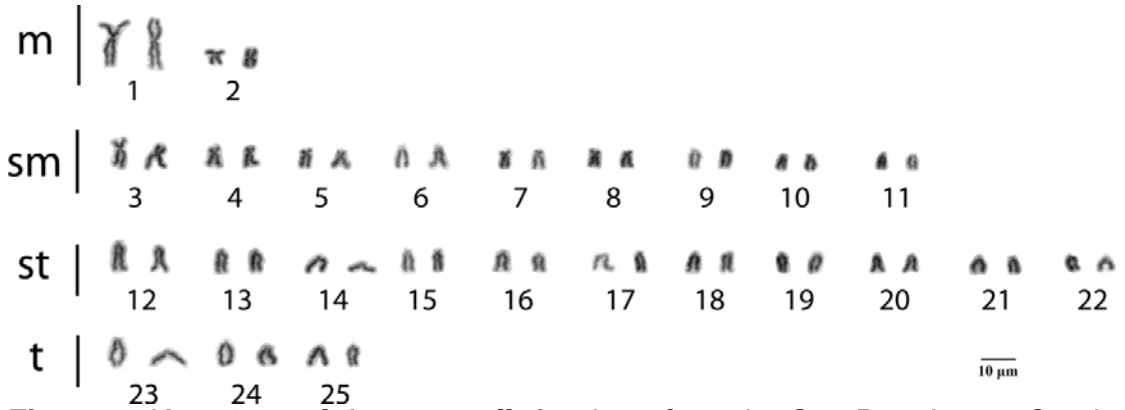


Figure 7. Karyotype of *Astyanax aff. fasciatus* from the São Bartolomeu Creek, a tributary of the Doce River Basin. Karyotypic formula: 10m+20sm+12st+6t.

This species had secondary constrictions in the long arms of one subtelocentric pair and one telocentric pair. Both secondary constrictions coincided with NOR sites and three additional NORs were present in the short arms of three submetacentric chromosomes (Figure 8).

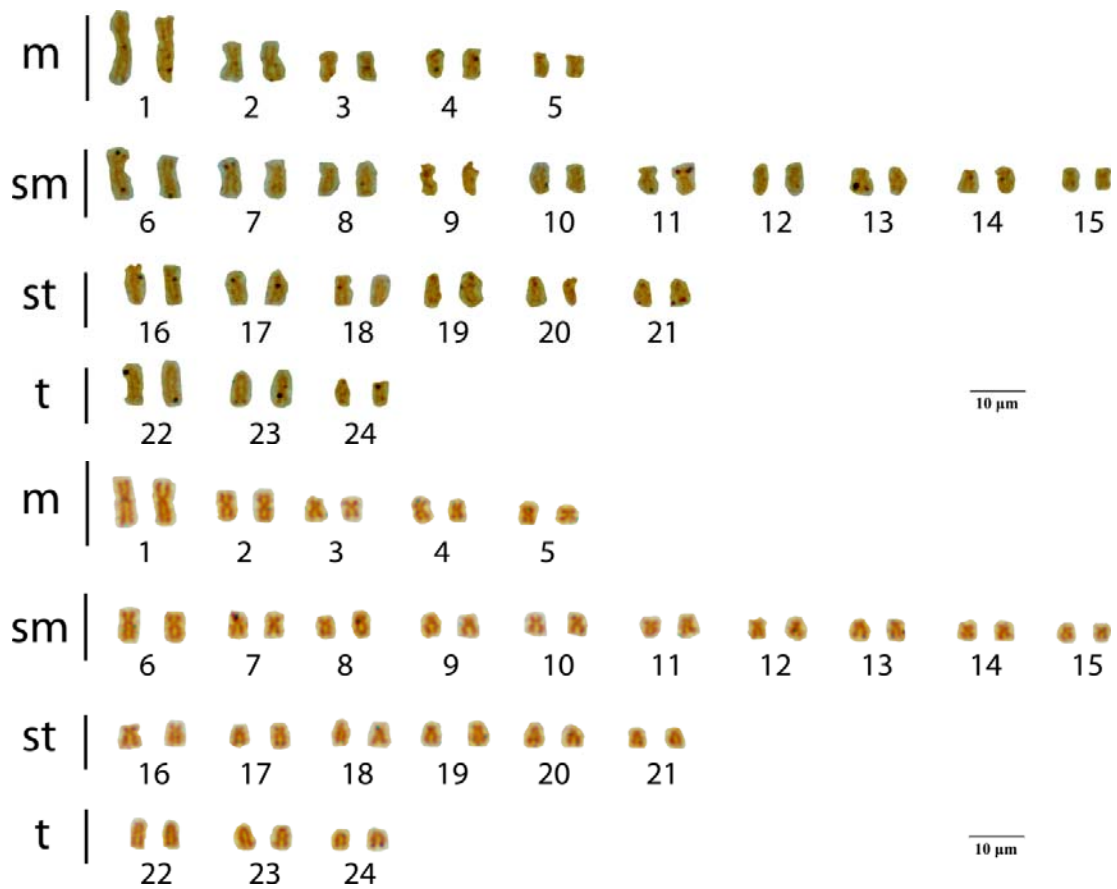
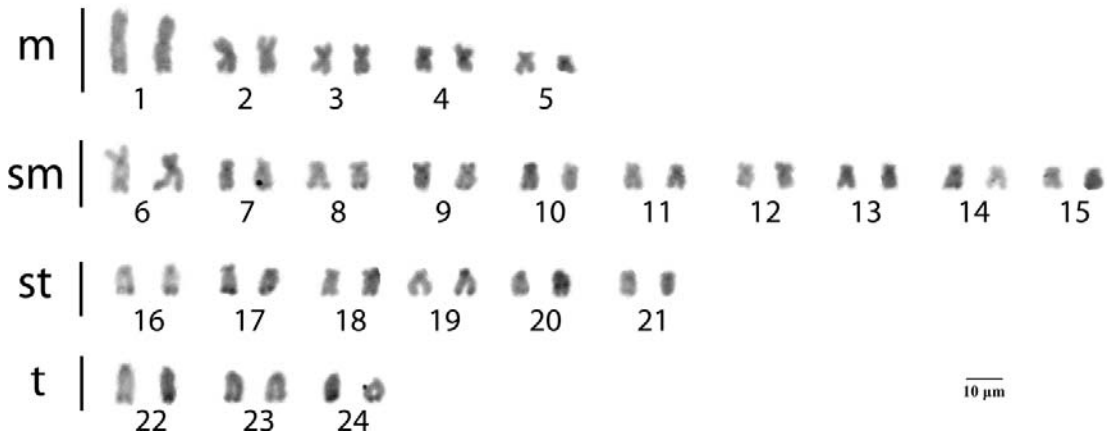


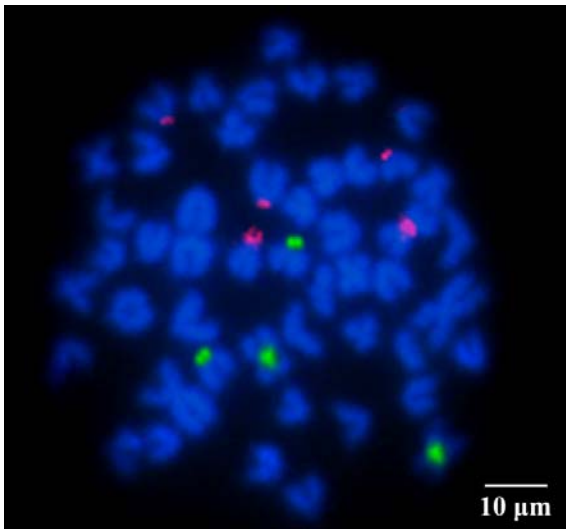
Figure 8. NOR sites in *Astyanax aff. fasciatus* from the São Bartolomeu Creek, a tributary of the Doce River Basin. In the top photo, NORs are visible in the short arms of three submetacentric chromosomes in pairs 6, 11 and 13, in the bottom photo they are visible in the long arms of one subtelo-centric chromosome (pair 17) and one telocentric chromosome (pair 24).

C-banding revealed regions of heterochromatin in nearly all centromeres, as well as in the telomeric regions of the long arms of the first metacentric pair, four submetacentric pairs (plus one in pair 14), two subtelo-centric pairs and all three telocentric pairs. Interstitial markings were visible in the long arms of one subtelo-centric chromosome of the 20th pair (Figure 9a). A distinctive interstitial heterochromatin band is visible in one subtelo-centric chromosome. Fluorescence in situ hybridization (FISH) showed hybridization of the 28S probes (marked in red) in the short arms of two subtelo-centric/telocentric pairs and in the centromeric region of one metacentric pair. 5S probes (green) were

visible in the short arms of one telocentric pair and the centromeres of one metacentric pair.



(a)



(b)

Figure 9. a) C-Banding in *Astyanax* aff. *fasciatus* from the São Bartolomeu Creek, a tributary of the Doce River Basin. Heterochromatin visible in nearly all centromeres, in the distal portions of the long arms of the first metacentric pair, four submetacentric pairs (plus one in pair 14), two subtelocentric pairs and all three telocentric pairs. Interstitial markings visible in the long arms of one subtelocentric chromosome of the 20th pair. b) Fluorescence in situ hybridization (FISH): 28S probes are shown in red and visible in the short arms of two subtelocentric/telocentric pairs and in the centromeric region of one metacentric pair. 5S probes are shown in green, visible in the short arms of one telocentric pair and the centromeres of one metacentric pair.

3.3B CHROMOSOMES

Supernumerary (b) chromosomes were found in five fish, one *Astyanax* aff. *fasciatus* and four *A. giton*, including the only male of this species, all from Reservoir 1 (Table 3). All B chromosomes were micro except in one *A. giton* that had a macro B chromosome resembling the first metacentric pair. The *Astyanax* aff. *fasciatus* had one metaphase with a single B chromosome and a second with two B chromosomes and was the only specimen to present more than one supernumerary chromosome.

Table 3. Presence of B chromosomes

| Accession number | Species | Sex | Metaphases containing B chromosomes |
|------------------|---------------------------------------|-----|-------------------------------------|
| CT 2651 | <i>A. giton</i> | M | 1/18 (5.6%) |
| CT 2664 | <i>Astyanax</i> aff. <i>fasciatus</i> | M | 2/13 (15.4%) |
| CT 3003 | <i>A. giton</i> | F | 2/2 (100%) |
| CT 3005 | <i>A. giton</i> | F | 12/26 (46.2%) |
| CT 3130 | <i>A. giton</i> | F | 3/41 (7.3%) |

Four out of 30 *A. giton* (13.3%) and one out of 30 *Astyanax* aff. *fasciatus* contained B chromosomes (3.3%) (Figures 10 and 11, respectively). Morphologically, CT 3003 and CT 3005 were unique among *A. giton* in presenting a red anal fin and were the only female specimens of this species to have a micro B chromosome.

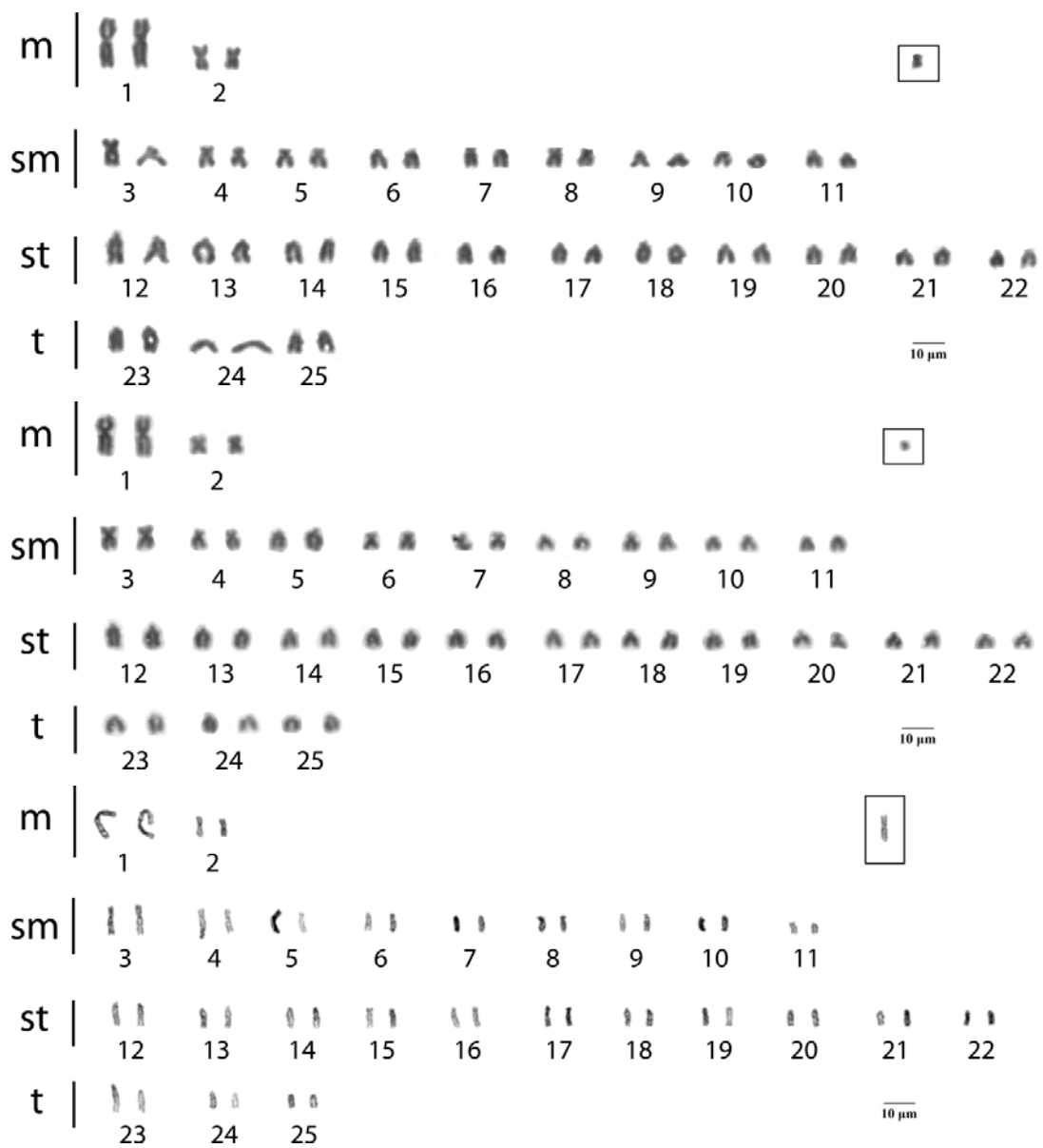


Figure 10. B chromosomes in *Astyanax giton*. Top two karyotypes show micro B chromosomes in male and female *A. giton* respectively, with the third karyotype showing a macro metacentric B chromosome.

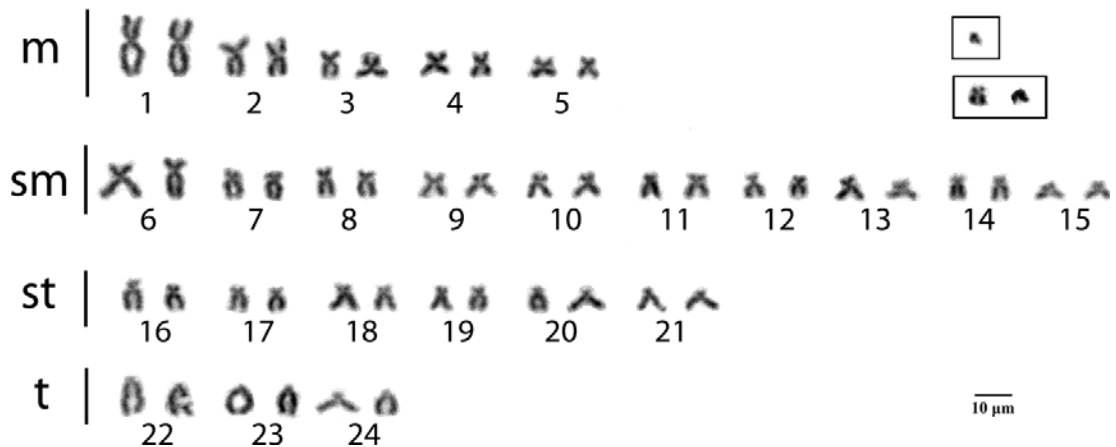


Figure 11. Karyotype of *Astyanax* aff. *fasciatus* CT 2664. B chromosomes from two separate metaphases are shown in boxes.

3.4 MOLECULAR DNA

Based on mitochondrial data, the species morphologically similar to *A.giton* grouped with *A. giton* from the nearby Sossego Dam (Viçosa, Minas Gerais State, Brazil) while those resembling *Astyanax* aff.*fasciatus* grouped with *A. fasciatus* and *A. parahybae*, a species similar to *A. fasciatus* but found only in the Rio Paraíba do Sul, where *A. fasciatus* is absent (Figure 12).

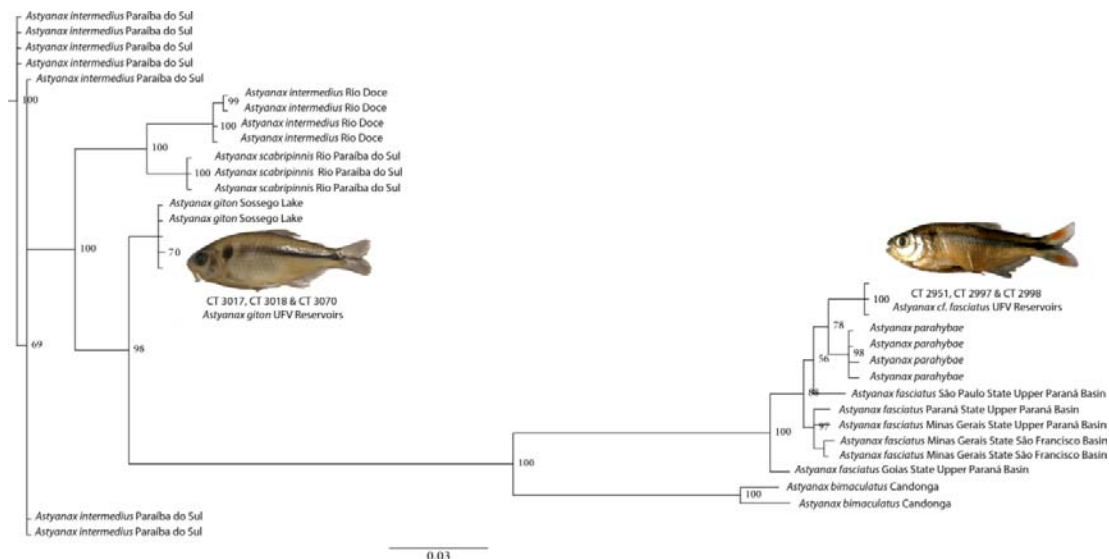


Figure 12. Phylogenetic analysis based on mtDNA trees using the gene COI with 34 fish samples from various basins. *Astyanax giton* from the UFV reservoirs are in the same clade as *A. giton* from Sossego Dam. *Astyanax* aff. *fasciatus* forms a very supported clade with *A. parahybae* and *A. fasciatus*.

4. DISCUSSION

The Neotropical freshwater fish fauna has long been considered the richest in the world [59], the result of a long evolutionary history of isolation and specialization [46]. Freshwater fishes are highly suitable for the recovery of past biogeographic processes and indicators of past paleohydrology [59], due to their obligatory relationship with water [46], as related forms can only occur in isolated basins if the water bodies were connected in the past.

Coastal and continental drainages may share such a past connection, based on cytogenetic evidence from *H. malabaricus*, which presents seven similar cytotypes between the Ivaí, Iguaçu, Tibagi and Ribeira do Iguape rivers. Stocks of *Astyanax aff. fasciatus* from the Furnas region might also represent evidence of prior connection between basins [9].

The geologic evolution of the chain of high mountains associated with the eastern limits of the Brazilian Shield allow vicariance and isolation of fish species [58]. The Atlantic drainages (Doce, Paraíba do Sul and Ribeira) have existed since the break-up of Gondwanaland [46]. They include (from North to South) the Paraguaçu, Contas, Pardo and Doce rivers. These rivers have a large branching system of headwaters adjacent to the eastern headwaters of the São Francisco. Among these drainages are numerous smaller drainages that have no contact with the São Francisco Basin [58].

During periods of low sea level, the coastal plane and associated river basins were more extensive, with coastal fish species able to disperse and occupy extensive areas. Populations occupying different coastal subbasins became isolated due to repeated episodes of sea level rise, resulting in speciation and population differentiation [58]. Studies of fish fauna from the Doce river show phylogenetically related fauna in the coastal and continental basins, although the exact causes of these relationships are not always clear [46].

Compared to Amazonian Rivers, eastern coastal drainages are characterized by relatively low diversity but considered a biogeographical unit [59] due to high levels of endemism, with 95% of species considered endemic [58]. Most species associated with the eastern margin of the Brazilian shield are

endemics of either coastal or inland basins, a few species occurring on both sides of the watershed divide. species occurring on both sides of the main watershed divide (e.g. *Astyanax fasciatus*) are said to have a "pattern C" type of geographic distribution [58], which includes stream piracy between coastal and crystalline Brazilian shield rivers [76]*Astyanax taeniatus*, a coastal freshwater fish, reaches the northern limit of its distribution range in the Mucuri and Jucuruçu rivers [59]

Most species inhabiting the high mountain stream Guaratuba do not occur elsewhere, but one exception is *A. paranae*, which is common in the upper headwaters of the Tietê. This could be due to either erosion or tectonic fault reactivation [58]. *Astyanax altiparanae* is present in the coastal upper Rio Itatinga as well as the upper Tietê, presumably the result of stream capture by erosion. This species is often cited as being endemic to the upper Paraná, but has a vicariant form *A. lacustris* that inhabits the São Francisco basin. Morphological characters of both species are variable and overlapping [58].

The area north of the mouth of the Doce River is composed of beach ridges, deltas, sea cliffs and sandstone reefs, geologic structures that could divide fish populations [59]. Bathymetric surveys of this area show that various channels converge on the Abrolhos depression. The Pleistocene lagoons are delimited to the south by the mouth of the Doce River and to the north by the Abrolhos Formation. Along with *A. fasciatus*, the trahira, *Hoplias malabaricus* has one of the largest distributional ranges, occurring from Panamá to the Buenos Aires Province in Argentina [46].

In the case of *Hoplias malabaricus*, closely related haplotypes were found in the Doce and São Mateus rivers, the latter located in Espírito Santo State, Brazil. Samples from coastal basins presented a sister group relationship with haplotypes from these two rivers, but not with the Rio Grande or São Francisco [46]. Haplotypes of *H. malabaricus* from the Itanhem and Jucurussu rivers show a close phylogenetic relationship, supporting a more recent common palaeohydrological history involving basins within this region [59]. Molecular

data indicate no evidence of genetic exchange between the Doce and Paraíba do Sul river populations of *H. malabaricus* during glacial periods [59]

The lack of data on geographic distributions and phylogeny of Neotropical freshwater fishes make it difficult to formulate hypotheses of biogeographic histories [56]. One possibility is that species of widespread distribution are relatively ancient, with spatial continuity broken by recent events of marine transgression (explaining the slight differences in *Astyanax* across basins). Species of restricted distribution may be also be related to these events.

4.1 MORPHOLOGY

The systematics of *Astyanax* are poorly understood, with the Atlantic Rainforest region containing several undescribed species [77]. Some "species" of this genus are widespread but vary geographically. Phylogenetic and taxonomic relationships among these fishes, as well as those of limited distribution, are not well known. Previous studies of *Astyanax* relied on morphological characters such as dentition, lateral line scales, tail fin and osteology [56]. As the species of *Astyanax* are morphologically similar, however, it has been difficult to separate them based on this criteria.

Based on morphological characters, Professor Vinicius de Araújo Bertaco at the Universidade Federal de Rio Grande do Sul confirmed our identification of *A. giton*, based on the key of Melo [56] that describes this species as follows: Dentary teeth gradually decrease in size from the symphysis, differentiating this species from *A. scabripinnis*. with the base of the teeth in the dentary and internal series of the premaxillar being thin. The central cuspid is approximately the same size as the side cuspids, with the maximum number of cuspids exceeding 8. The interopercular does not expand further and does not cover a portion of the sub-opercular. Body height is 28.9 to 37.4% of the pattern length, with 34 to 39 scales on the lateral line, with a rounded naked area between the second and third infra-orbital.

Eigenmann differentiated *A. fasciatus parahybae* from *A. fasciatus* by the presence of a series of scales below the lateral line deflected toward the anal fin

by the interpolation of supplementary series. *Astyanax fasciatus parahybae* also has a head of 4.25 to 4.44; depth of 2.5 to 2.66; 27 to 34 (usually 31 or 32) rays in the anal fin, a lateral line with 37 to 41 (usually 39 or 40) rays. an eye equal to the interorbital; one maxillary tooth; 4 to 5 teeth in the outer row of the premaxillary in a 2:1 ratio[3]. Melo also identified *A. parahybae* as having irregularly placed scales above the anal fin and the anal fin having 29 or more rays, both of which differentiate this species from *A. fasciatus* and definitively separate it from the *A. fasciatus* complex[56].

According to Eigenmann, when *A. fasciatus*, *A. scabripinnis* and *A. taeniatus* are found together in the same river it is possible to differentiate one from another, but across different rivers and basins each undergoes so many modifications it is impossible to clearly define them morphologically [3]. *Astyanax taeniatus* in one river could be confused for *A. scabripinnis*, *A. fasciatus*, *A. giton* or *A. intermedius* in another. Lütken described both *A. taeniatus* and *A. scabripinnis* as *A. scabripinnis rivularis*. Jenyns recognized *A. taeniatus* as an intermediate form of *A. scabripinnis*. Bertaco recognized *A. fasciatus* as *A. intermedius*. Eigenmann described *A. intermedius* as the center of a triangle formed by *A. scabripinnis*, *A. fasciatus* and *A. taeniatus*.

Species assigned to *Astyanax* are not a monophyletic entity [8,78]. Current definition relies on morphological characters such as two rows or teeth in the premaxillary, a complete lateral line and the presence of a caudal fin, which are widespread in Characidae [3]. *Astyanax intermedius* has been included in the *Astyanax scabripinnis* species complex [78]. *Astyanax intermedius* has a humeral spot similar in form to *A. microschemos*; however *A. intermedius* is distinguished from this species by having a larger interorbital width (29.7-34.1% vs. 32.5-47.8%)

Astyanax giton is most easily distinguished from *A. fasciatus* by the presence of a vertically elongate black humeral spot. *Astyanax fasciatus* presents a less conspicuous humeral spot, with the differences being most pronounced after the fish have been fixed in alcohol. Body shape also differs between these fish, with *A. giton* on average having a deeper body while *A.*

fasciatus is more elongate. The median aspect ratios (length/height) for *A. giton* and *A. fasciatus* were 3.46 and 3.80. Both species were differentiated by PCA, concordant with Eigenmann's statement that fish of *Astyanax* can often be differentiated from one another only in larger samples [3]. The sex ratio for *A. giton* from the UFV reservoirs (1 out of 32) was consistent with data from Sossego Lake, where 8 males and 171 females were captured [2].

4.2 CYTOGENETICS

The cytogenetics of *A. giton* from the UFV Reservoirs and the Sossego Dam differ. Although both present a diploid number of $2n=50$, the karyotype of *A. giton* from Sossego was $6m+8sm+24st+12t$ and $FN=88$ [2]. This population presented multiple heterochromatic markings with C-banding and DAPI, mostly on the small arms of the submetacentric and subtelocentric chromosomes. A faint marking also visible on the centromeric region of the largest metacentric chromosome. C-banding showed heterochromatin in four chromosome pairs, including a subtelocentric chromosome with bitelomeric markings. Giemsa-stained C-banding matched 18S rDNA fluorescence patterns, with this probe also showing markings in the telomeric portions of the largest metacentric pair. The NORs were present in two subtelocentric chromosomes that appear similar to the two NOR-bearing chromosomes of the *A. giton* studied here. Only one pair of chromosomes was marked with the 5S rDNA probe in the Sossego Dam [2].

Astyanax giton from the two reservoirs present 22 subtelocentric and 12 telocentric chromosomes, differing slightly from the Sossego population which had 24 subtelocentrics and 12 telocentrics. These populations contrast with the many (28-32) telocentric chromosomes in populations from the Paraíba do Sul River Basin [4], similar to *A. intermedius*, *A. ribeirae* and populations of *A. hastatus* and *A. scabripinnis* from coastal rivers, all of which have 34 and 38 subtelocentric and telocentric chromosomes [60]. Although both populations of *A. giton* from the Doce River present many more subtelocentrics than those from the Paraíba do Sul, the combined totals are still within the range of combined subtelocentric and telocentric chromosomes.

However, *A. giton* from both the Paraíba do Sul River and Sossego Dam both present one pair of NORs and 10 chromosomes bearing 18S rDNA clusters[5], although the *A. giton* from Paraíba do Sul also contain several chromosomes bearing 5S clusters [2]. Additionally, the Sossego Dam contains triploid *A. giton* ($3n=75$) that were not observed in the UFV Reservoirs. There are at least six species of *Astyanax* with triploid individuals: *A. eigenmanniorum*, *A. scabripinnis*, *A. schubarti*, *Astyanax* aff. *fasciatus* (from the Tibagi River in Vila Velha, Paraná State, Brazil), *Astyanax* sp. D [9] and *A. giton* (from the Sossego Dam in the Doce River Basin, Minas Gerais State, Brazil) [2]. Some triploid *A. scabripinnis* also carry B chromosomes [9].

To the best of our knowledge, this is the first reporting of this macro metacentric B in *A. giton*. As the size and morphology of the macro B are similar to those of supernumerary chromosomes in *A. fasciatus*, *A. scabripinnis*, *A. eigenmanniorum*, *A. schubarti* and *A. bockmanni*, it is possible that this B chromosome predates these speciation events [32-36]; however, the morphology of a supernumerary chromosome is not necessarily indicative of its origin [25,27].

As there was no evidence of hybridization between the species, the B chromosomes could have arisen from the A set or an ancient event of hybrid introgression. B chromosomes were rare in the *Astyanax* studied from these reservoirs, with only 20 out of 1,700 (1.2%) metaphases containing any supernumerary chromosomes. Because of their rarity, the B chromosomes could not be tested for banding or heterochromatin, nor linked to males or females in particular. The *A. giton* with the metacentric macro B was stained for NOR, but the B did not present markings. Attempts to stain slides containing male gonads with Giemsa were inconclusive as chromosome morphology could not be determined.

The chromosomal change rate in *Astyanax* is calculated at $e = 3.582$. Using mtDNA, this genus has a divergence time of about 19 My, with remarkable radiation during the last 5My, especially within *Astyanax fasciatus*, which presents the shortest divergence time. The rate of karyotypic evolution is

higher than other Characiformes, with speciation events resulting from pericentric inversions, heterochromatin redistribution and Robertsonian rearrangements [79].

Astyanax fasciatus from Araras, São Paulo State, Brazil, in the Mogi-Guaçu River Basin (part of the Paraná River System) were studied cytogenetically [80]. This population presented a diploid number of $2n=48$ and a karyotype of $8m+22sm+12st+6t$, FN=90 -- the closest published karyotype to *A. aff. fasciatus* of the Doce River ($10sm+20sm+12st+6A$, FN=90).

NORs are highly variable among *Astyanax fasciatus*, with cytotypes in the Mogi-Guaçu River ranging from two to eight NOR sites [13]. In *A. aff. fasciatus* from the reservoirs, NORs were found in the terminal regions of the short arms of three submetacentric chromosomes, as well as in the terminal regions of the long arms of one subtelocentric and one telocentric chromosome, the latter two coincident with secondary constrictions. NORs in the terminal regions of the short arms of two submetacentric chromosomes (with size polymorphism) were observed in the Araguari River, a population with a diploid number of $2n=46$ and a karyotype of $14m+16sm+10st+6t$ (FN=86) [18]. These NOR sites were also observed in *A. fasciatus* from the Upper Paraná River [7]. The Araras population had two NORs, in the distal region of the long arms of a pair of telocentric chromosomes [80], similar to those found in the subtelocentric and telocentric chromosomes from *A. aff. fasciatus* in the Doce River.

C-Banding in *A. aff. fasciatus* presented heterochromatin in nearly all centromeres, telomeric regions of long arms of 1st metacentric pair, four submetacentric pairs (plus one in pair 14), two subtelocentric pairs and all three telocentric pairs. Interstitial heterochromatin bandings were visible in long arms of one subtelocentric chromosome of the 20th pair. The Araras population was similar constitutive heterochromatin (C-Banding) in the distal regions of long arms of submetacentric, subtelocentric and telocentric chromosomes, and short arms of some other chromosomes.

Astyanax fasciatus from the Mogi-Guaçu river presented constitutive heterochromatin in terminal regions on long arms of sm, st, t and terminal

regions of short arms of sm chromosomes in both 48/46, which are GC-rich and contain NORs [16]. The regions from the long arms of the sm, st and t terminal regions were coincident with data from *A. aff. fasciatus*. Similar markings among the Mogi-Guaçu cytotypes indicate the proximity of these lineages and a recent divergence. Different cytotypes may cause reproductive isolation, although this does not appear to be the case given the presence of $2n=47$ and $2n=45$ specimens [13].

FISH in *A. aff. fasciatus* from the Doce River presented 5S markings in the short arms of one telocentric pair and the centromeres of one metacentric pair, and 28S markings in the short arms of two subtelocentric/telocentric pairs and the centromere of one metacentric chromosome. 28S and 18S probes hybridize at the same sites and may therefore be compared across populations and species.

Specimens of *A. fasciatus* from the Upper Paraná present 28S markers in the distal portion of the short arms of two submetacentric chromosomes and 5S markers in the pericentric region of two metacentric (similar to *A. aff. fasciatus* from the Doce River) and in two telocentric chromosomes, colocalized with 28S in the two metacentric and telocentric chromosomes [7].

Several species of *Astyanax* including *A. altiparanae*, *A. lacustris*, *A. fasciatus*, *A. schubarti* and *A. scabripinnis* show conserved localization of 5S/28S markers between species, indicating a close phylogenetic relationship [7] and 5S can be seen in more than one pair [24]. NORs are almost always fewer than regions detected by FISH, because some 5S genes are associated with rDNA genes that do not form a nucleolus because the gene sequences were too small to be detected [7]. These 5S locations are absent in *A. giton* and *A. intermedius*, suggesting polyphyly or divergence within this genus [13]. *Astyanax fasciatus* from the Paraná and Paraíba do Sul basins have a 5S rDNA cluster in the distal region of a metacentric chromosome [4,7].

In *A. fasciatus* from the Mogi-Guaçu River, FISH 18S markers were located in the NORs [13]. Other fluorescent markings were smaller, always telomeric and located in subtelocentric and telocentric chromosomes, consistent

with some of the markings observed for *A. aff.fasciatus*. All cytotypes from the Mogi-Guaçu had a ribosomal site on the subterminal region of the short arm of one submetacentric pair and 5S markings in the pericentromeric region of the short arms of one telocentric pair [13], also observed in *A. aff.fasciatus* from the Doce River.

In the Araras population of *A. fasciatus*, FISH revealed two chromosomes pairs with 5S, one at the pericentromeric region of one metacentric chromosome and the other at the proximal region of a telocentric chromosome, exactly like *A. aff.fasciatus* from the Doce River. Also in Araras, three submetacentric chromosomes presented 18S markings, two in the terminal region of the short arm and one in the terminal region of the long arm.

The first two 18S sites coincide with Ag-NOR in *A. aff.fasciatus* from the Doce River, but 28S/18S probes did not hybridize at these sites. Although homology with the original gene sequence of *A. fasciatus* from Araras should be sufficient to hybridize with the rDNA probe, in *A. aff. fasciatus* they may no longer represent an active gene or functional gene product and therefore no longer present an Ag-NOR [9]. This may occur if mutations have resulted in pseudogenes or functionless sequences (as observed in *H. malabaricus*). Although the 28S sites in *A. aff.fasciatus* did not coincide with those of Ag-Nor, one on the metacentric chromosome did correspond to C-banding.

A single chromosomal pair bearing 5S DNA, often interstitially (known as marker chromosomes) is observed in several *Astyanax* and probably ancestral for this group. A second pair is also found in *A. scabripinnis*, *A. parahybae*, *A. schubarti* and *A. fasciatus*[9]. Other *Astyanax*, however, present several chromosomes bearing 5S but lack the marker pair, as is the case of *A. giton*, *A. intermedius*, *A. janeiroensis*, *A. ribeirae* and *A. fasciatus* from the Doce River, indicating these may have been lost in coastal populations [9,60].

CT 3005 and CT 3003 were the only female *A. giton* to have both a red anal fin and a micro B chromosome. Although it is possible these traits may be

correlated, a much larger sample size and sequencing of the B chromosomes would be necessary to draw such a conclusion and determine their origin.

Astyanax fasciatus is known to occupy main river channels and form large shoals with migratory habits; therefore it is less susceptible to barriers to gene flow than other species of this genus [31,32]. Therefore it may have more opportunities to hybridize and pick up B chromosomes from interspecific crosses. Metaphases containing B chromosomes are shown (Figures 8 and 9). *Astyanax altiparanae* is one of the most widely distributed groups of this genus, closely related to *A. aff. bimaculatus*. These species are morphologically similar because of the elliptic humeral spot and yellow tail [21]. *Astyanax altiparanae* from the Tietê and Paranapanema rivers have diploid numbers of $2n=50$. Karyotypes differ, with multiple cytotypes occurring in sympatry, indicating *A. altiparanae* is a species complex. Hybridization has not been reported and distinct cytotypes may represent operational taxonomic units, or cryptic species.

However, molecular data indicate they are a monophyletic group despite population-specific haplotypes, with the populations being neither very diverse nor distant genetically [21]. All populations have one pair of NORs, in the terminal portions of either the long or short arms of subtelocentric or telocentric pairs, depending on the population, similar to *A. bimaculatus*[21]. C-Banding was present in the terminal and interstitial regions of several chromosomes. 18S rDNA was present in multiple sites (two to four), located in the terminal portions of subtelocentric and telocentric chromosomes, except for one site in the terminal portion of the short arms of a single metacentric chromosome of the first pair from the Pilar do Sul River in São Paulo State, Brazil[21]. FISH with 5S revealed two interstitial sites on metacentric or submetacentric chromosomes. These two sites are common to *A. bockmanni*, *A. fasciatus* [60], *A. parahybae*, *A. scabripinnis* and *A. schubarti* [21]. With the addition of *A. eigenmanniorum*, these are also the same species that feature the large metacentric B chromosome found in *A. giton* from these reservoirs.

4.3 MOLECULAR DNA

Based on mitochondrial DNA, the *A. giton* from the two reservoirs are identical to those of Sossego Dam. One difference between these populations, however, is that the *A. giton* from Sossego are hybridizing with *Oligosarcus argenteus*, but no hybrids were found at either reservoir despite the presence this genus. If hybridization were advantageous it would presumably occur in both lakes; if disadvantageous, *Astyanax* and *Oligosarcus* should be good species and not interbreed. It may be that the species have adapted different strategies based on conditions unique to each area, as the size of the water system may drive evolution through physical, chemical and biotic populational barriers [37]. It is also possible that the B chromosomes found in *A. giton* are the result of a past hybridization event, either with *Oligosarcus argenteus* or another species. As B chromosomes are heteropycnotic (i.e., they do not pair with the a set) interspecific interactions may be sufficient that chromosomes that cannot pair will evolve as Bs [27].

Molecular data place *A. aff.fasciatus* completely in the same clade as *A. fasciatus* and *A. parahybae*. As stated by Eigenmann, *A. parahybae* may be a distinct form of *A. fasciatus* in the Paraíba do Sul River, where *A. fasciatus* is absent, the result of lineages being isolated in rivers on the eastern coastal basins [3,58], although it is currently defined as a separate species [9,56].

When populations present similar morphology and molecular data but differ by cytotype, the question arises whether or not to treat them as distinct species. In a study of four populations of *A. hastatus* from the Guapimirim River Basin (Rio de Janeiro State, Brazil, three cyotypes were found, all $2n=50$ [41]. These were considered a group of cryptic species, not species complexes, because each cytotype could be precisely identified and biological boundaries among analyzed specimens were supported by cytogenetic markers, revealing a lack of interbreeding and hybrid forms. The cyotypes could not be differentiated through morphometric analysis [41]. According to the Darwinian species concept (species recognized as morphological and genotypic cluster) these cannot be considered individual species. According to biological concept of Mayr, however,

they should correspond to real species since distinct karyotypes found with limited gene flow but no evidence of hybridization. This type of analysis could be applied to *A. fasciatus* and *A. giton*.

5. CONCLUSION

Two species of *Astyanax* from the Doce River were analyzed using morphological, cytogenetic and molecular techniques. All characters indicate these fish are *A. giton* and *Astyanax aff.fasciatus*. Morphological characters overlap but may be differentiated using statistical analysis. Diploid numbers were consistent with those previously documented for these species but chromosome morphology differ. *Astyanax giton* may be a species complex based on a new karyotype occurring in the Doce River basin and the presence of B chromosomes. The mtDNA fragment COI indicates these fish are in separate clades. Follow-up studies could include a comparison of *Astyanax aff.fasciatus* with populations of *A. fasciatus* and *A. paraguayana*, testing the idea of species complexes versus cryptic species and sequencing the supernumerary B chromosomes of both species.

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