HILTON JEFERSON ALVES CARDOSO DE AGUIAR

FIRST REPORT ON SPONTANEOUS HYBRIDIZATION BETWEEN *Astyanax giton* Baird & Girard 1854 and *Oligosarcus argenteus* Günther 1864 (PISCES : CHARACIDAE): ECOLOGICAL AND PHYLOGENETIC INFERENCES

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

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APROVADA: 14 de fevereiro de 2011.

Prof. Rubens Pazza
(Co-orientador)

Profª.Karine Frehner Kavalco

Prof. Jorge Abdala Dergam dos Santos
(Orientador)

To Luísa, my darling, who since the first moment impelled to me keep studying genetics, even when there was no family around and available helping hands.

"For nothing can seem foul to those that win"

Henry IV. Part I; Act V; Scene I

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Resumo

AGUIAR, Hilton Jeferson Alves Cardoso. M. Sc., Universidade Federal de Viçosa, fevereiro de 2011. **Primeiro relato de hibridização espontânea entre *Astyanax giton* Baird & Girard 1854 e *Oligosarcus argenteus* Günther 1864 (Pisces : Characidae): inferências ecológicas e filogenéticas**. Orientador: Jorge Abdala Dergam. Co-orientadores: Silvia das Graças Pompolo e Rubens Pazza.

A complexa família Characidae é parte da fauna ictiológica neotropical e conta com várias espécies e gêneros em condição de *Incertae Sedis*. Os gêneros *Astyanax* e *Oligosarcus*, considerados muito aparentados, estão incluídos nesta família e abrangem espécies de pequeno tamanho e expressiva abundância em muitos rios e córregos da América do sul. Na bacia do rio Doce, no sudeste brasileiro, uma análise morfológica preliminar permitiu a identificação de cinco peixes "semelhantes à *Astyanax*" que continham no seu osso maxilar de 8 a 13 dentes. Esses cinco peixes, chamados no presente trabalho de dentuços, foram coletados em simpatria com as espécies *Astyanax bimaculatus* (Linneaus, 1758), *Astyanax giton* (Eigenmann, 1908) e *Oligosarcus argenteus* Günther, 1864. De modo a determinar a natureza biológica dos dentuços, foi realizado um estudo multidisciplinar envolvendo dados morfológicos citogenéticos e moleculares. Os dentuços apresentaram configurações intermediárias entre as espécies *A. giton* e *O. argenteus* no que diz respeito ao número de escamas na linha lateral e ao número de dentes no osso maxilar. As análises citogenéticas, feitas através das técnicas de Giemsa, bandeamento NOR, bandeamento-C, fluorocromos, e FISH, indicaram que todas as espécies de caracídeos contaram com número diploide 2n=50 cromossomos diferindo, porém, em vários caracteres de sua morfologia cromossômica. Os dentuços caracterizaram-se por apresentar altos índices de variação cromossômica tanto intra- como inter-individual. Além do mais, eles contaram com vários cromossomos não pareáveis assim como cromossomos de tamanho diminuto que não são observados em nenhuma daquelas espécies simpátricas de Characidae. Três espécimes dos dentuços apresentaram seu fragmento de DNA do gene mitocondrial citocromo b (475 pb) idêntico aquele de *O. argenteus* e, contudo, todos os dentuços compartilhavam mais alelos ISSR com os espécimes de *A. giton* do que com os espécimes de *O. argenteus*. Por outro lado, uma espécie de dentuço conta com o gene *cyt. b* idêntico aquele das

espécies de *A. giton* estudadas. A análise de fragmentos ITS-1 (1123 pb) mostrou que os dentuços têm sequencias mais relacionadas à *Oligosarcus argenteus*. Os dados, desse modo, sugeriram que os dentuços são híbridos entre as espécies *A. giton* e *O. argenteus* representando assim o primeiro caso de hibridismo espontâneo entre dois gêneros de peixes neotropicais. A relevância de tal descoberta para a biologia da conservação e para as investigações filogenéticas foram discutidas.

Abstract

AGUIAR, Hilton Jeferson Alves Cardoso. M. Sc., Universidade Federal de Viçosa, February, 2011. **First report on spontaneous hybridization between *Astyanax giton* Baird & Girard 1854 and *Oligosarcus argenteus* Günther 1864 (Pisces : Characidae): Ecological and Phylogenetic inferences**. Advisor: Jorge Abdala Dergam. Co-advisors: Silvia das Graças Pompolo and Rubens Pazza**.**

Within the Neotropical fish fauna, the taxonomically complex family Characidae has many species and genera in *Incertae Sedis* condition. Within this family, the closely related genera *Astyanax* and *Oligosarcus* are represented by small sized fishes that are an expressive proportion of the freshwater biodiversity in many rivers of South America. In the Doce River Basin, Southeastern Brazil, a preliminary morphologic analysis indicated the presence of 5 *Astyanax*-like fish with unusually high numbers (8-13) of maxillary teeth which are referred as "toothed morphs". These fishes were collected in sympatry with *Astyanax bimaculatus* (Linneaus, 1758), *Astyanax giton* (Eigenmann, 1908), and *Oligosarcus argenteus* Günther, 1864. To determine the biological status of them a comparative multidisciplinary approach involving morphologic, cytogenetic and molecular data was conducted, with the toothed morphs and their sympatric species. The toothed morphs showed an intermediate position in lateral line scale numbers and maxillary teeth number between *A. giton* and *O. argenteus*. Cytogenetic analyses (Giemsa, NOR, C-banding, fluorochromes and FISH) indicated that all sympatric characids were 2n=50, although they differed from each other in many other karyotypic characters. The toothed morphs were characterized by high levels of intra and inter-individual chromosomal variation including several unpairable chromosomes and tiny chromosomes that were not observed in either of the other sympatric species. Three toothed morphs specimens had their cytochrome b DNA fragment (475 bp) identical to *O. argenteus*, with one exception which its *cyt. b* DNA sequence was identical to *A. giton*. Moreover, all toothed morphs ITS-1 DNA sequences were characterized by their similitude to those sequences of *Oligosarcus argenteus*. On the other hand, all toothed morphs shared more ISSR alleles with *A. giton.* The data suggested that the toothed morphs were hybrids between *A. giton* and *O. argenteus*, representing the first evidence for spontaneous hybridization between two Neotropical

fish genera. The relevance of such findings in conservation biology and phylogeny assessment were discussed

Chapter I

Overview

“ …And yet these Rarities might be allow'd,
To Man, that sov'raign thing and proud;
Had he not dealt between the Bark and Tree,
***Forbidden mixtures** there to see.*
No Plant now knew the Stock from which it came;
He grafts upon the Wild the Tame…”

The Mower against Gardens
Andrew Marvell (1621-1678)

The Characiformes and the Characidae problem

Among bony fish, the order Characifomes encompasses the most morphologically diverse ordinal assemblage of the Ostariophysan fishes (Vari 1998). Paralleling the morphological diversity of characiforms is their notable species-level diversity (Nelson 2006). Such morphological traits have yielded, and will continue to yield a trove of valuable information pertinent to phylogenetic questions at all levels within the order (Vari 1998). The Characins belong to a large family of freshwater tropical fishes with roughly eight hundred species recognizable by their adipose fin and distinctive teeth in their jaws (Moyle 1995). However, the recent research throws doubts about the monophyly of the taxon (Weitzman and Malabarba 1998). The conflicts between cladistics and traditional or phenetic “placement” of various taxa in the Characidae demonstrate that at this time we know little about the relationships of putative characid subgroups with one another or with other characiform groups. In consequence, eighty eight Characiformes genera were listed as *Incertae Sedis* including 399 Characidae specimens taxonomically poorly known and possibly belonging to genera not considered monophyletic (Lima et al. 2003). These mostly small fishes are abundant in rivers and other aquatic habitats throughout the Neotropical region including the highly speciose *Astyanax* Baird & Girard (1854) and *Oligosarcus* Günther (1864) genera.

Genera *Astyanax* and *Oligosarcus*

Astyanax and *Oligosarcus* species inhabit heterogeneous environments, as a result of the highly complex Neotropical hydrographic history, which favoured several tectonic processes that resulted in vicariant and dispersal events (Lundberg et al. 1998; Ribeiro 2003). Some lineages could be split, undergo the process of speciation and differentiation between them and later, through random vicariant events, they could be brought together again, in secondary contact.

As a dominant freshwater fish genus of South America, *Astyanax* is found from Patagonia to the United States, and on both slopes of the Cordilleras. Most of its species are popularly known as "lambaris" or, in the aquarium trade, as "tetras". This genus was described by Baird and Girard (1854), in Baird (1859), honouring Astyanax, the son of Hector and considered *Astyanax argentatus* from riverine streams of Texas as species type. Currently, *A. argentatus* is considered senior synonym to *Astyanax mexicanus* (De Filippi, 1853). This genus of tetras is also one of the most studied among Characidae, with at least 86 known species (Lima et al. 2003). Recently, more than 20 new species have been described (*e.g.* Bertaco and Garutti 2007; Bertaco and Lucena 2006; Garavello and Sampaio 2010; Haluch and Abilhoa 2005; Melo and Buckup 2006 among others). The complete diagnosis of the genus *Astyanax* proposed by Baird and Girard (1854) and followed by Eigenmann (1921) takes into account a combination of morphological characters, where the most important are: small size, compressed body more or less elongate, rarely reaching a length of 150 mm without ectopterygoid and canine teeth, two rows of pre-maxillary teeth, five teeth in the inner pre-maxillary series, complete lateral line, presence of adipose fin and naked caudal fin. Unfortunately those characters are not diagnostic for *Astyanax* but widespread among all Characidae genera, suggesting the polyphyletic condition of this genus (Weitzman and Malabarba 1998). The complex evolutionary history of *Astyanax* is closely linked to its headwater behaviour. Some geographically isolated fish populations restricted to headwaters of some tributaries have been identified all over the large tropical riverine systems and remain as separated assemblages by physical, chemical or biotic barriers which may lead to several evolutionary pathways within the same hydrographic system (Lowe-McConnell 1969). Doubtfully, all these specimens had the same evolutionary pathways to the common ancestry. It seems more plausible instead, that their body shape is be the result of convergent evolution, or in other words, the body shape similarity is a homoplasy.

Another important Characidae genus is *Oligosarcus* which is much less specious, with only 19 valid species (Menezes 1987; Menezes and Ribeiro 2010; Ribeiro 2007; Ribeiro et al. 2007).Their South American endemic species are found in the Parana-Paraguay basin and in coastal streams of eastern and southern Brazil, Uruguay and north-eastern Argentina (Lima et al. 2003). The *Oligosarcus* species are popularly known in Portuguese South America as "bocarras" or "cachorras" and in

Spanish South America as “dientudos”. Günther (1864) considered *Oligosarcus argenteus* from the Velhas River Basin as type species for the genus, which was diagnosed, among other characters by having: head and body compressed of moderate size, oblong and covered with scales; lateral line complete; teeth conical, in a single series in the intermaxillary (pre-maxillary), maxillary, mandible (dentary) and on the palatine bones; presence of canine teeth in the premaxillary and mandible. The phylogenetic condition of *Oligosarcus* inside Characifomes has been subject to debate since its description. In a first moment *Oligosarcus, Paroligosaracus* and *Acestrorhynchus* genera were clustered together inside the tribe Acestrorhynchini, outside the family Characidae but in the family Acestrorhynchidae (Menezes 1969). *Oligosarcus* was placed as the sister taxon of *Paroligosarcus pintoi* which is characterized by distinct teeth morphology with tri- and penta-cuspid teeth on the anterior portion of the dentary. Such condition was considered plesiomorphic since all *Oligosarcus* and *Acestrorhynchus* species have conical or canine teeth at the anterior dentary series. However, subsequent osteological data changed *Paroligosarcus* genus status and placed *P. pintoi* inside O*ligosarcus*, now recognized as *O. pintoi* (Menezes and Géry 1983).

The osteological review made by Menezes and Géry (1983) also revealed that Acestrorhynchini could not be considered a monophyletic taxa because both *Acestrorhyncus* and *Oligosarcus* have several particular derived characters. A subsequent revision analysis made by Buckup (1991) proposed the separation of the genus *Oligosarcus* from Acestrorhinchini and its inclusion in the subfamily Tetragonopterinae (Characidae) because of the absence of the supraorbital bone. Inside Tetragonopterinae, *Oligosarcus* should also be closely related to *Astyanax*. The genus *Oligosarcus* has strong evidences suggesting monophyletic condition, such as the presence of tricuspid teeth along most of the ectopterygoid length (Ribeiro et al. 2007). Even so, *Oligosarcus* was also included in *Incertae Sedis* (Lima et al. 2003) due to its unknown phylogenetic relationships with the other Characidae.

Despite the high morphological difference between those two genera, recent phylogenetic studies place them together as sister groups (Ortí and Meyer 1997; Castro et al. 2004). Moreover, morphological analysis (*e.g.* Lucena 1993), and molecular analysis (Calcagnotto et al. 2005; Javonillo et al. 2010) suggest that *Astyanax* is

paraphyletic. Javonillo et al. (2010) also include *Oligosarcus* within "*Astyanax*". The relationship of both genera with the other groups in Characidae remains unclear (Hattori et al. 2007). These studies do not include all possible mechanisms that could be responsible for this confusing relationship between the two genera.

Fish Cytogenetics and Related Concepts

Cytogenetics can be defined as the study of the normal or abnormal chromosome set and their genetic properties (Griffiths et al. 2004). However the comprehension of the mechanisms of genetic changes between chromosomes can never be understood without the cytological point of view of the chromosome set. Chromosomes are part of living cells and therefore, part of their mechanical basic principles of growing, replicating and dying. As genetic structures each chromosome contains a fragment of the code of life and is a keystone on the heritage process. As cytological structures they obey to all cell rules of mitosis and the even more dramatic meiosis.

Usually, each animal and plant species accounts with its specific chromosome set – the karyotype. However a single karyotype, as the number of chromosomes and their specific morphology, may be apparently present in another species. Therefore, objective karyological characterization of each species requires several specific chromosome markers obtained by banding patterns and *in situ* hybridization. There are also cases in which a species or a population cannot be characterized by one single karyotype but several variations, called cytotypes. When those variations are numerical, they are result of mechanical complications during cell divisions or due to the presence of supernumerary chromosomes, also called B chromosomes. When the variation is related to the morphology of distinct chromosomes, they could be the result of specific chromosomal rearrangements. All new rearrangements require the specimens undergo a heterozygous condition, which generates cytological polymorphisms. There is a strong evolutionary importance on those polymorphisms. Commonly they are related to reproductive disorders resultant of meiotic disturbances.

The chromosomal heterozygozity condition and its role in the speciation process have been largely discussed in the cytogenetics literature. Ernst Mayr considered such rearrangements as simply a post-speciation afterthought (Kearney and Hewitt 2009). White (1978), however argued that if this were so, we would expect to frequently encounter the situation where a 'young' species or semi-species had different karyotypes in different parts of its range, without being associated with any genetic isolation (and this is definitely not the case). Its stasipatric model also proposes that the heterozygote condition is so deleterious that different mechanisms developed to balance the polymorphisms may lead to speciation (Spirito 1998). King (1993) suggests also that this homozygous advantage is considered unimportant since several species developed non-Mendelian modes of segregate two chromosome types in the heterokaryotype. At present times, several studies suggests that those heterokaryotypes may be naturally fixed or not (Barton 2001; Navarro and Barton 2003). What will decide their fate inside a natural population, the balance between native chromosomes and rearranged chromosomes, shall be their selective value to the natural selection (Kawakami et al. 2011).

Because of their sheer numbers and old age as a group, fish are particularly informative for genetic, cytogenetic and evolutionary studies. The large amount of cytogenetic data already obtained for fishes has been useful for improving the phylogenetic comprehension of the taxon. Such cytogenetic data pointed the existence of large variability on fish diploid number, ranging from a low extreme value of 2n=16 in the cyprinodontid, *Nothobranchius rachovii,* to a high of 2n=168 in the petromyzontiform lamprey, *Petromyzon marinus* (Hoar 1979). Interestingly, the diploid number 2n=48 chromosomes can be found throughout several Teleostei (Denton 1973) leading to the suggestion that this karyotype configuration may represent the ancestral chromosome complement of all vertebrates (Ohno et al. 1968). In Characiformes the diploid numbers vary from 2n = 22 to 2n = 102 (Porto et al. 1992) but the great majority, however has chromosome numbers between 2n=40 and 2n=60 (Klinkhardt et al. 1995). The genus *Astyanax* is characterized by remarkable karyotypic diversity (Pazza and Kavalco 2007), including the occurrence of different diploid numbers among individuals from allopatric populations pertaining to the same nominal species (Moreira-Filho and Bertollo 1991). *Astyanax* species also have their diploid numbers, ranging from 2n=36 in *A. schubarti* Britski (1964) to 2n=50 chromosomes (Arefjev

1990; Moreira-Filho and Bertollo 1991; Morelli et al. 1983; Pazza and Kavalco 2007; Oliveira et al 1988; Portela et al. 1988 and others). However, all *Oligosarcus* species studied cytogenetically have 2n=50 chromosomes (Kavalco et al. 2005) differing only on the morphology of their chromosomes.

The ancestral karyotype within the genus *Astyanax* was likely composed of 2n=50 chromosomes (Pazza and Kavalco 2007) with a large metacentric chromosome pair in the complement (Scheel 1972). However, the huge chromosome variability, both inter and intra-populational, among *Astyanax* species suggests the existence for at least three groups, also called “species complexes” (Moreira-Filho and Bertollo 1991; Fernandes and Martins-Santos 2004). This expression “species-complex” refers to cases where two or more species are difficult to define because of their complex patterns of variation (Nelson 2006). When levels of variation are studied using cytogenetic tools, it is common to observe cryptic species instead of species complexes in Neotropical, fishes, since remarkable differences in chromosomes usually imply unviable breeding (Kavalco et al. 2009).

Generally, fish chromosomes are smaller in size than chromosomes in most vertebrates. The length of the average fish chromosome is between 2 and 5 µm. Many species possess numerous small chromosomes of 2µm or less, but which are nonetheless easily seen using the light microscope. Very large chromosomes of 15-30µm in length, such as those in the lungfish, *Lepidosiren paradoxa* (Ohno et al. 1968) are rare. On the other hand, extremely small chromosomes, the so called microchromosomes, were also reported in primitive species (Ohno et al. 1969). These authors also suggest that although microchromosomes could have arisen independently at the reptilian stage of vertebrate evolution, their presence in these relict fishes imply that microchromosomes were already present in ancestral fish.

Each pair of homologous chromosome is assumed to differ genetically from all other chromosome pairs in the same cell. Manifestations of some of these differences comprise the morphological “phenotype” or karyotype and include differences between chromosome pairs in relative size, shape, and centromere position. Sturtevant wrote in 1925 that for a geneticist, many of the comparisons between karyotypes from different species would seem of little significance, because to him (geneticist) it is not the shape and size of chromosomes which are important, but the genes contained in them. Such

“gene master importance” view was later abandoned when a better comprehension of the nature of chromosomes became available. One of these findings was the discovery of the position effect, when the same gene may have different functions or activities when placed in different places of the same chromosome. Another relevant aspect was the existence of the crossing over mechanism in spreading genetic variability produced by each individual throughout the population. As a result of a long history, karyotypic differences among species or taxa are routinely used to determine phenetic similarities and to infer phylogenetic relationships (Gold 1979).

Sporadic events of polyploidy in natural populations resulting in natural triploidy have been detected in a considerable number of specimens from unrelated species in genus *Astyanax*. Out of nine cases of natural triploidy reported for tropical fish, five occurred in species of this genus (Almeida-Toledo et al. 2000). The higher incidence of triploidy in *Astyanax* may be due to the diversified environments in which these fish live, with greater exposure to abrupt changes in temperature (Fauaz et al. 1994). These recurrent triploids are probably derived from the fertilization of unreduced egg cells, possibly originating from thermal shocks that may have occurred during fertilization thus disturbing the second meiotic division (Almeida-Toledo et al. 2000).

In fish cytogenetics the chromosomes usually are arranged following the nomenclature proposed by Levan et al. (1964). This nomenclature is based on the position of the centromere on the chromosome and four morphological chromosome categories have been erected: metacentric (m), submetacentric (sm), subtelocentric (st) and telocentric (t). There are another two categories almost never used on cytogenetic studies: metacentric (M), when the centromere is positioned exactly on the middle of the chromosome suggesting symmetry; and telocentric (T), when the chromosome has only one arm. There is a great discussion about the true nature of the so called telocentric (T) and its arm absence. White (1954), affirms that telocentric chromosomes with a truly terminal centromere do not occur naturally, they could be the result of poorly fixed materials that lead the small arm to merge with the larger one. In consequence, he coined the term “acrocentric” to avoid confusion with the term “telocentric” where acrocentric chromosomes should be those chromosomes with the terminal centromere followed by a small arm. In other words, acrocentric chromosomes are the same of the telocentric (t) chromosomes from Levan et al. (1964). However,

Levan et al. (1964) proposed this classification "based on morphologic observations rather than theoretical hypothesis" and so, "chromosomes in which no second arm can be seen should be designed telocentric, until evidence to the contrary may come forward". The current refinement of cytogenetic techniques and the use of high quality equipment for obtaining chromosome pictures have made it possible to see small arms that where invisible in the past. Thus, the correct classification of one species' chromosomes should incorporate the cytogenetic theory of the chromosome structure: all stable eukaryote chromosomes have at least, two telomeres and one centromere.

The importance of the chromosomal telomeric region is paramount. Since the decade of 30's, Muller stated that the loss of this region would lead to the fusion of the broken chromatid to another (Muller 1932). Furtheremore, he suggested that chromosomes with natural ends are capable of indefinite survival in the course of successive cell divisions, while the ones with broken ends are not (Muller and Herskowitz 1954). To Muller, the ends of the natural chromosome ends (which he called *telomeres*) are self-perpetuating structures that are unable to persist on an interstitial position in the chromosome.

Another fundamental chromosome structure, the centromere, is a region that enables the accurate partition of newly replicated sister chromatids between daughter cells during mitosis and meiosis. Although the functional role of the centromeres is most evident at metaphase and anaphase they are permanent as autonomous segments of chromosomes which can be seen at other stages of the cycle in suitable material (White 1954). The centromere holds the sister chromatids together and through its centromere DNA-protein complex, known as the kinetochore, binds to spindle microtubules bringing accurate chromosome movements (Dobie et al. 1999). In addition, centromeres regulate the progression of cell cycle and are critical in sensing completion of metaphase. They also trigger the onset of anaphase (Nasmyth 2002). The satellite DNA that surrounds the centromere structure is usually A-T rich but adenines or timines are not randomly distributed within the sequence. The periodic distribution of A-T tracts normally induces curvature of the DNA helix axis and the formation of tertiary structures to form a super helix (Fitzgerald et al. 1994). Such a structure is thought to be important for the tight packing of DNA and proteins in heterochromatin (Ugarkovic 2009).

The remarkable importance of both centromere and telomeres on chromosome structure and dynamics is clear, and seems completely unlikely that they can occupy the very same place on the chromatin. The idea of having telocentric chromosomes (T) where the centromere is placed at the telomeric end of the chromosome cannot prevail. With few exceptions, all chromosomes have a centromere and two arms with telomeres on their edges. This basic concept has a key role on fish phylogeny by means of cytogenetic data since different karyotypes are compared taking into account their Fundamental Number (FN). Matthey (1945) used the term *nombre fondamental* (N.F.) to indicate the number of major chromosome arms in a species, counting each metacentric as two arms and each telocentric as one. This concept must be carefully used as a proportion of the telocentric chromosomes among the other chromosomes of the karyotype, where each telocentric has the value (1) and the other kinds of chromosomes has the value (2). Several citations mentioning “bi-armed” and “one-armed” chromosomes can be found on fish cytogenetical literature and they should be avoided. It must be stated that, in natural conditions, all chromosomes are “bi-armed”.

Phenotypic Plasticity and Heterochrony

The heterogeneous environments inhabited by *Astyanax* and *Oligosarcus* specimens may be closely connected to the huge morphological variation found among different specimens of each genus. High degrees of speciation itself may be an important consequence to these peculiar Neotropical fresh waters. Phenotypic plasticity is rarely mentioned in the literature even though it plays a major role in the diversity of the morphs (Romero and Green 2005). The array of phenotypes developed by a genotype over an array of environments is genetically variable and its capacity for plasticity is therefore subject to natural selection (Pigliucci 2001). Those individuals with a heritable higher capacity to express specific adaptive traits under appropriate conditions can be expected to be favoured by natural selection. Phenotypic plasticity provides reproductive advantage over genetically fixed phenotypes because environmentally induced phenotypes have higher probabilities of fitting to prevailing environmental conditions than genetically fixed ones (Whiteman 1994). Perhaps the

most common “mechanism” for developmental plasticity is heterochrony. The term was coined by Haeckel in 1875 and indicates a variety of alterations in the rate or timing of developmental events, resulting in an array of peramorphic (overdeveloped) or paedomorphic (juvenilized) forms of descendants (Gould 1977). There is still some arguing on the heterochrony definition and West-Eberhard (2003) suggested that it is not a “mechanism” of evolution and like duplication, deletion and reversion; it should describe a *kind* of variation that has become established during a cross-generational transition, a change in the phenotypic composition of a population or species. In other words, heterochrony should not be interpreted as a cause of such changes. Notwithstanding, the genetic variation responsible for these “shifts” is poorly understood yet, the heterochronic shift concept has been useful in trying to understand how morphological differences between homogeneous taxa could have originated (Schichnes and Freeling 1998). Goldschmidt (1938; 1952) proposed that all mutations produce a change in some developmental step which occurs at a definite time and stage of development as well as in a definite region of the embryo. This author also suggested that chromosomal rearrangements remain as an important process able to generate hypothetical macromutations that may cause large-scale evolutionary changes in a “saltational” way. Later, Fink (1982) emphasized the necessity of phylogenies to polarize directions of heterochronic change between species. There are currently three characteristics of the development of organs and organisms which are subject to heterochronic change: onset, rate and/or offset, and they embrace the essential components of any change in development: growth, allometry, scaling and functional adaptation. It is important to state that when invoking heterochrony it is critical to demonstrate that one or other of onset, rate and offset has been altered in a descendent relative to the condition in an ancestor (Hall 1999). Heterochrony is a valuable concept because it links development and evolution in fairly understandable ways and therefore, its application as the main explanation for so much evolutionary change must be made carefully since it seems unlikely that this process is a predominant mode of evolutionary change (Thomson 1988). The genetic mechanisms of heterochrony are not well understood yet however, it seems that they are more connected to genetic expression than to the presence or absence of specific genes. An increasing number of papers have shown how shifts in the relative timing of onset or offset of particular genes – genetic heterochrony - may produce significant phenotypic change (*e.g.* Smith 2003). This link

of molecular genetics with the classical heterochrony mystery is providing a clear bound between microevolutionary processes and macroevolutionary changes. The paedomorphic features in fish are commonly referred to as reductive and considered to be due to the loss of terminal stages in the developmental sequence (Weitzman and Vari 1988). Miniaturization of the body is commonly reported among different vertebrate groups. The phenomenon of miniaturization is a phylogenetic statement, implying that the group under consideration evolved from a larger ancestor. The body size is among the most important determinants of species function and ecological role. However, the broad significance of miniaturization for organismal biology and evolution, including its high frequency among major groups of animals, has been largely unappreciated (Hanken and Wake 1993). Miniaturization involves not only small body *per se*, but also the consequent and often dramatic effects of extreme size reduction on anatomy, physiology, ecology, life history and behaviour (Peters 1986). There are some costs and also some compensation on size decrease. In freshwater fishes, a standard length of 25-26 mm has been used as the maximum size for miniature species (Weitzman and Vari 1988) and this represents one-fourth to one-fifth the average size of living teleosts (Helfman 2009). At least 85 species of South American freshwater fishes are regarded as miniature, representing 5 orders, 11 families and 40 genera (Weitzman and Vari 1988). The reduced and simplified adult morphology that characterizes many miniaturized taxa often bears a strong resemblance to sub-adult or even embryonic stages of larger close relatives. This resemblance typically is the primary evidence used to define the morphology of many miniaturized species as paedomorphic and as having evolved via precocious truncation of the ancestral developmental program (Gould 1977). The rates of morphological variability are also connected to the body size decrease and novel morphological features are readily tied to it since the novelty may lie, in part, on the effect of size reduction in a morphogenetic way (Hanken 1985). Novelty associated with miniaturization may be responsible for distinctiveness in newly established clades and can be reinforced by the parallel or convergent evolution of similar, if not identical, modifications in disparate lineages that have evolved small size (Hanken and Wake, 1993). The genus *Astyanax* is characterized by high phenotypic plasticity and a capacity to adapt to diverse habitats (Strecker et al. 2003). There are clear evidences of relatively fast adaptations of these fish to new habitats and environments, with ecological specialization and morphological differentiation. The

commonest adaptation is related to the body shape and have been demonstrated that populations inhabiting fast-water streams usually have elongated body shapes (Haluch and Abilhoa 2005). Considerable attention has been given to the evolution of developmental mechanisms and adaptation to cave environments (Protas et al. 2007). Cahn (1958) carried out the first detailed comparative analysis of eye development in *Astyanax* surface fish and cavefish showing, by means of histological data, that embryos of both populations actually develop a primordial eye, which gradually disappear during larval stage. Her work has been confirmed several times (Behrens et al. 1997; Jeffery et al. 2003). The cavefish eye actually develops normally for a considerable length of time before finally disappearing due to failure to keep up with the overall body growth (Jeffery 2005) what indicates a clear ontogenetic misfit between the eye and the body development. The epigean and the hypogean species also differ on their feeding apparatus: the hypogean species has larger jaws with 3-4 teeth in the maxillary bone whereas the epigean one has only 1-2 teeth on that bone. Jaw modifications are accompanied by larger maxillary bones which ossify earlier during cavefish development, an example of heterochrony. These modifications have converged in different cavefish populations and are likely to be under the influence of strong selective pressure (Jeffery 2001).

In fact heterochrony and homoplasy interconnect in the case of paedomorphic parallelism, in which related clades in essence back down (in a phylogenetic sense). Therefore, the morphological similitude between different phylogenetic groups of Neotropical Characidae should be analysed carefully by means of plasticity and heterochrony morphological concepts since these fish are grouped by their morphological plesiomorphies and questioned by their molecular divergences.

Hybridism

The mechanisms responsible for generating the enormous biological diversity have received paramount attention from naturalists. Among them, the natural process of hybridization has been studied by men since earlier ages. Aristotle himself studied cases of hybridism among dogs and foxes and partridge and common chicken. He had

difficult to explain, however, the sterility of the mule. For him, hybrids do not necessarily truly breed – after several generations of interbreeding, they supposed to eventually revert to the female appearance, just as seeds of plants come to vary according to the soil on which they grow (Anton and Preus 1992). Hybridism is a major asset to all works that intend to understand what a species is, because a single hybrid specimen represents the living proof of the breakage of main barriers that keep different species apart. Owing to the gradualness of the speciation process one should find in nature, populations that are on the way to becoming separate species, but have not yet quite completed the process. Such "semi-species" are indeed found (Mayr 1999). The "idea of species" is a major reference framework for virtually all subfields of biology sciences, mainly due to its importance in systematics (Queiroz 2005). Related to that relevance, hybridism occupies a central position to those who try to unveil the nature of the species concept. As a consequence, the definition of hybridization is as difficult as the definition of species. One of the striking phenomena of recently formed hybrid populations is their tremendous variation. Both parental types, as well as all the possible recombinations of the parental characters, may be found in the same effective breeding population (Mayr 1999). The difficulty of delimiting the term "hybridization" against the various forms of intraspecific interbreeding have been warned by Mayr (1999) who suggested that the use of this term is undoubtedly justified only if individuals of different families, genera or "good species" interbreed. Hybrids differ from individuals of the parental species not only in morphology but usually also in fertility and viability (heterosis). Zoologists have tended to see hybridization as a problem for taxonomy: should populations that remain distinct despite genetic exchange be considered members of the same species (Barton 2001)? Even those who developed the biological species concept (Dobzhansky 1953; Mayr 1966) regarded gene flow through animal hybrid populations as ineffective; hybrid zones were seen as being either transient, with natural selection reinforcing mating preferences to give fully isolated species, or if stable, merely as obstacles to further divergence. Most hybrids present problems to produce the normal number of viable gametes, a biological response to hybridism called "hybrid sterility". Sterility may range from slight to complete and it is not necessarily correlated with a reduction in viability (Mayr 1966). The complex problem that Aristotle firstly found with the mule sterility raised several concerns about the main causative reasons for hybrid sterility. A fundamental discovery concerning hybrid

sterility was made by Federly (1913) who suggested that the chromosomes usually fail to form bivalents at meiosis in sterile and semi-sterile hybrids in consequence of several abnormalities observed on their spermatocytes. This link between abnormalities in spermatocytes and hybrid sterility led Dobzhansky and Beadle (1936) perform an elegant experiment transplanting larvae testes between hybrids of *Drosophila* and pure species. They confirmed that the sterility is connected to the hybrid spermatogenesis since the gonads of the pure species develop and work normally on hybrid specimens. The key role of hybrid sterility led Theodosius Dobzhansky (1953) to pioneer the genetic study of speciation, by mapping its main responsible genetic factors. He also created the view that speciation represents the transformation of within-population variation into between-taxa differences through the evolution of inherent reproductive isolating barriers. Indeed, chromosomal incompatibilities are strongly connected to hybrid sterility however, Bateson (1909), Dobzhansky (1936) and Muller (1940; 1942) independently discovered that the main reasons for those incompatibilities, even among species with similar karyotypes, are the epistatic deleterious interactions between different sets of complementary genes. Hybridization, therefore, indicates relationship, but it should be stated emphatically, that the degree of sterility observed in the hybrids does not indicate the degree of relationship (Mayr 1999).

Charles Darwin himself recognized that cross-fertilization results, in many situations, in higher biological vigour than crosses between inbred specimens within the same species (Gowen 1952). The term heterosis, or hybrid vigour, is used to characterize the increased function of any biological quality in a hybrid offspring. In other words, it is the occurrence of a genetically superior offspring from the combination of distinct parental genes. The heterosis phenomenon is also the opposite of the inbreeding depression caused by endogamy (Falconer 1987). Actually, the only frame of reference which makes the concept of heterosis meaningful is the greater fitness or adaptive value of the progeny compared with that of the parents (Dobzhansky 1949). However, there is an open question about how this combination can actually produce an increase in the fitness of the heterozygote, as compared to both homozygotes, since the characters favoured by natural selection are the result of several genes in heterozygous condition (Buzzati-Traverso 1952). The genetic mechanisms which produce heterosis are different in each case. Since most mutations arising in any given species are deleterious, and since in outbred species, deleterious dominant

mutants are eliminated by selection more rapidly than recessive ones; the populations accumulate stores of concealed variability which consist mainly of deleterious recessive variants (Dobzhansky 1949). Taking this idea into account two theories, which are competing but not mutually exclusive, try to explain heterosis: the dominance hypothesis suggests that the hybrid vigour is a consequence of interactions between dominant favourable genes. Therefore, the heterozygosis condition is not essential for heterosis (Cruz 2005; Davenport 1908). On the other hand, the overdominance hypothesis suggests that the vigour originates from certain combinations of alleles, attributing heterosis to the heterozygosis (Shull 1908). It seems that to the comprehension of species evolution and speciation, the overdominance hypothesis remains more plausible. Dobzhansky (1949) also suggests that heterosis is caused by the specific interaction effects of gene complexes which have emerged in the process of evolution under a controlling influence of natural selection. Therefore, the likely alternative to explain the increased vigour of hybrids between natural populations is due to certain gene loci, where the heterozygote is superior to either homozygote, and due to gene interactions (Crow 1948). Among natural populations, fitness varies smoothly with genotype because of the interaction of many genes with large numbers of alleles with small effect (Barton 2001). The vast genetic database obtained from *Drosophila* strains also seems to corroborate the overdominance hypothesis (Dobzhansky 1950; Muller and Falk 1961). Studying natural populations of *Drosophila* in Central America, Dobzhansky and Pavlovsky (1955) found in Honduras a *D. tropicalis* population with high proportions of heterozygotes for a specific inversion in the second chromosome that lead the homozygous progeny to death in egg or larval stages on every generation. However, even sacrificing about one-half of its zygotes in every generation, the fitness of the heterotic heterozygotes is so high that the population is considered by the authors a “flourishing one”. Heterosis is in short, a form of evolutionary adaptation, characteristic of sexually reproducing and cross-fertilizing species: heterosis is, therefore, a result of natural selection (Dobzhansky 1949; 1953).

At least four kinds of factors (ecological, ethological, mechanical and genetic) participate in preventing the production of hybrid individuals between sympatric species. The mere fact that such hybrids occur indicates that some of the isolating mechanisms may broke down (Mayr 1999). An important outcome of hybridization is the introgression of the gene pool of one taxon (*eg.* species) into another taxon. When

the hybridization involves a rare taxon, Arnold (1997) suggests three important results: increase of its fitness; addition of genetic variation; and the construction of a reservoir of parental-like genotypes/phenotypes. Barton (2001) suggests that even if those barriers get broken the hybrid fitness frequently drops when compared to their parents'. However, the increase in variability still remains the key. Actually few hybrid genotypes might be fitter than the parental ones because only the hybrid specimens have some degree of gene interaction. It is unlikely that the fittest possible gene combination would occur in the pure lineage of either parental species (Turelli et al. 2001). If hybrids are capable to survive the competition with their parents, with all their problems, they must be able to hit new adaptive combinations of genes (Mallet 2007). The introgression event is considered a spontaneous consequence of hybridization by Mallet (2005) who suggests that the first (F_1) hybrid should be the most difficult to produce because they are result of a singular event: closely related and diverging species living in sympatry; once they are produced the backcrossing to one of the parental lineage is much more straightforward. Barton (2001) also shares this point of view and says that if fertile F_1 hybrids are produced these will almost certainly backcross to one or other parental genotype because of their variability. The possibility of introgression, or not, were taken into account by Allendorf et al. (2001) who categorized hybridization in six distinct types whereas the last three types are the result of anthropogenic work: 1) natural hybrid taxon; 2) natural introgression; 3) natural hybrid zone; 4) hybridization without introgression (F_1 sterile); 5) widespread introgression (recent hybridization geographically restricted); 6) complete admixture (when conservation actions fail and all populations become hybrid swarms).

It is sometimes argued that intraspecific hybridization should not be a concern because populations of the same species generally share alleles so that new genetic types that might cause outbreeding depression will not be created by introgression (Keenan 2000). Moreover, it was suggested that the anthropogenic introduction of new genetic variation into a population, by a process called supplementation, could generally be beneficial and provide genetic variation so that natural selection can increase the fitness of populations (Cuenco et al. 1993). This seems absurd since each population has its own and characteristic gene pool. By introducing alien genes in a threatened population the true result shall be the complete extinction of the original population and the rise of a new population but with different gene pool. The impact of introduced

species or lineages is widely known by their negative effects on the natural species by competing with or preying on them or destroying their habitat (Gurevitch & Padilla 2004). However the extinction by hybridization and introgression with the introduced species on native fauna is a serious menace too (Allendorf et al. 2001; Orr 1998; Rhymer and Simberloff 1996). Since a hybrid has unusual gene combinations its adaptive value, if compared to its parents, is unpredictable. By means of introgression and gene exchange two well adapted and natural populations could drastically change their gene pools with severe consequences to their fitness (Dowling and Hoeh 1991; Dowling et al. 1989), and usually, the introgressant genes predominantly flow from the common to rarer species (Allendorf et al. 2001). Introgressive hybridization may have been a persistent feature of the adaptive radiation through most of natural history, facilitating evolutionary diversification and occasionally affecting both the speed and direction of evolution (Grant et al. 2005). However the predatory behaviour of human kind makes almost impossible to consider, nowadays, hybridization and introgression as pure natural processes. Therefore, it is complicated to distinguish natural and anthropogenic hybridization (Allendorf et al. 2001; Dowling and Secor 1997), and this is central feature for conservation biology. When this distinction is not achievable, the alternative is to either not allow protection of natural hybrids or to protect anthropogenic hybrids that could contribute to extinction of parental species and waste limited resources available for conservation. The most widely known case relating hybridism and environmental biology is that of the problematic red wolf (named *Canis rufus)* which wasted great amounts of preservation resources when, actually, it was not a natural species but a hybrid of the grey wolf (*Canis lupus*) and the coyote (*Canis latrans*) (Wayne & Gittleman 1995). This hybridization event was strongly associated with the extensive agricultural cultivation of the southern United States by European settlers beginning around 250 years ago (Reich et al. 1999). The hybridism problem is so serious among conservationists that several pejorative connotations for introgression were published as: “contamination”, “infection”, “genetic deterioration”, “genetic pollution”, “genetic aggression” and others (Rhymer and Simberloff 1996).

Natural hybridization is believed to be more common in fishes than on other group of vertebrates (Mayr 1966), and moreover, non-native fish species have been introduced extensively worldwide (Smith 1992).The largest fish hybrid database reports the existence of at least 3.759 natural and artificial cases of hybridization (Schwartz

1972; 1981). This amount, however, should be currently much larger. Several groups of fishes from western United States exhibit evidence of past introgression, implicating a general causative factor. Ecosystems of this region have gone through dramatic change, including considerable tectonic activity and progressive aridification. This process had severe impact on aquatic ecosystems, likely producing cycles of isolation and sympatry (Dowling and Secor 1997). Man-caused habitat changes in North America have also been correlated with hybridization between both previously allopatric and sympatric pairs of species and sympatric species that rarely or never hybridize in nature and hybridize freely in laboratory conditions (Hubbs 1955). Several characteristics of fish species may account for their ease to hybridize: external fertilization, weak ethological isolating mechanisms, unequal abundance of the two parental species, competition for limited spawning habitat, and susceptibility to secondary contact between recently evolved forms (Campton, 1988). The data of Neotropical hybrid fish species is scarce and only four cases are known for the family Characidae. The first one is related to the blind tetra *Astyanax jordani* and *A. mexicanus* (Kullander 1999). Two other cases involve fish species from *Serrasalmus* genus, also called piranhas. In one case the cytogenetic data suggested the hybridism between two isolated populations of *Serrasalmus spilopleura* (Cestari and Galetti Jr. 1992). Hybridism in piranhas has also been studied by Hubert et al. (2007; 2008) between *Serrasalmus compressus* and *S. hollandi* in a sympatric population of Madeira River. The fourth case of hybridism was documented for two different sympatric populations of *Astyanax fasciatus* with distinct chromosome configuration (Pazza et al. 2006). All four documented cases of hybridism among Characidae species involve crossing between close species or different populations from a same species and therefore a hybridism between different genera or higher taxa have never been discussed. However, it must be stated that hybridization may be strongly underestimated when we consider that many pairs of species are only weakly diagnosable using morphology (Mallet 2008). The major importance of hybridism events to Characidae fish is that the instable phylogenetic situation of the species belonging to the *Incertae Sedis* group may be the result of several horizontal transferences among different genera that once lived in sympatry. As the phylogenetic investigations of this group suggest absence of monophyly for distinct groups, the event of introgression should be taken into account as an explanation when phylogenetic trees inferred from different genes are in conflict (Baak and Rieseberg 2007).

Final Aspects

The relationship between the Characidae genus *Astyanax* and *Oligosarcus* is complex under the scope of phylogenetics and when morphologically intermediary specimens are discovered several hypothesis must be raised for the correct understanding of the biological processes that made those specimens rise in sympatry. Such intermediary characteristics may be the result of random heterochronic variations on *A. giton* or *O. argenteus* leading to morphologic changes. However, they also may be resultant of hybridism between those two sympatric species. Each hypothesis has distinct biological implications and phylogenetic impact for the Characidae fish and therefore, the conclusion must be carefully taken. The more tools available to unveil this mystery, the best explanation for this biological incident is possible to reach. When morphology, cytogenetics and molecular biology techniques are combined to detect the variability of these morphologically distinct specimens and used to contrast these morphological variants with the other sympatric species, the results suggests that the hybridism hypothesis is more plausible. However, all knowledge about both hypotheses is always necessary for conclusive affirmations about this case that may be able to answer several questions about the relationship of the different Characidae groups. The results of these techniques and their main conclusions are discussed on the following chapter.

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Chapter II

Spontaneous hybridization between *Oligosarcus argenteus* Günther 1864 and *Astyanax giton* Baird & Girard 1854 (Pisces: Characidae)

“The view generally entertained by naturalists is that species, when intercrossed, have been specially endowed with the quality of sterility, in order to prevent the confusion of all organic forms”

Charles Darwin, 1859

Spontaneous hybridization between *Oligosarcus argenteus* Günther 1864 and *Astyanax giton* Baird & Girard 1854 (Pisces: Characidae)

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Keywords: Hybrid, Chromosome Polymorphism, Characiformes, *Incertae Sedis*

Abstract

Within the Neotropical fish fauna, the taxonomically complex family Characidae has many species and genera in *Incertae Sedis* condition. Within this family, the closely related genera *Astyanax* and *Oligosarcus* are represented by small sized fishes that are an expressive proportion of the freshwater biodiversity in many rivers of South America. In the Doce River Basin, South-eastern Brazil, 5 characid specimens “toothed morphs” with unusual morphology were analysed with morphological, cytogenetic and molecular techniques and compared to the sympatric characids *Astyanax bimaculatus*, *Astyanax giton* and *Oligosarcus argenteus*. The toothed morphs showed an intermediate position in lateral line scale numbers and maxillary teeth number between *A. giton* and *O. argenteus*. Cytogenetic analyses (Giemsa, NOR, C-banding, fluorochromes and FISH) indicated that all sympatric characids were 2n=50, although they differed from each other in many other karyotypic characters. The toothed morphs were characterized by high levels of intra and inter-individual chromosomal variation including several unpairable chromosomes and tiny chromosomes that were not observed either in *A. giton* or *O. argenteus.* Two toothed morphs had their cytochrome b DNA fragment (475 bp) identical to *O. argenteus* but one toothed morph had its sequence compatible to the *A. giton* species. However a different picture can be seen at the nuclear DNA where all toothed morphs had their ITS1 DNA sequence (1123 bp) similar to *O. argenteus* including the specimen with distinct mtDNA. Moreover, all toothed morphs shared more ISSR alleles with *A. giton,* than to *O. argenteus.* The data suggested that the toothed morphs were hybrids between *A. giton* and *O. argenteus*, representing the first evidence for spontaneous hybridization between two Neotropical fish genera. The relevance of such findings in conservation biology and phylogeny assessment was discussed.

Introduction

The Neotropical freshwater ichthyofauna is remarkably rich and diverse (Schaefer 1998). The order Characiformes has more than 1600 species in 16 families (Reis et al. 2003) including the family Characidae, which include the popular tetras. The Characidae are widespread throughout American continent, from south Texas to Patagonia (Eigenmann 1921), and are very common both in lotic or lentic waters. This family has not been diagnosed as a monophyletic group (Weitzman and Malabarba 1998). The conflicts between cladistic and traditional phenetic approaches in Characidae demonstrate that at this time we know little about the relationships of putative characid subgroups with one another or with other characiform groups (Weitzman and Malabarba 1998). Such uncertainty led to the inclusion of a great number of genera from the Characidae family to the *Incertae Sedis* condition (Lima et al. 2003) including the genera *Astyanax* and *Oligosarcus*.

Macroevolutionary processes may be the historic outcome of microevolutionary dynamics (Ridley 2004), a level that is gauged by the patterns of variation in natural populations. At the population level, processes such as migration and gene flow (or even hybridization) may superimpose to genetic drift and divergence. Horizontal transfer has been frequently reported among North American and Old World freshwater fishes (Schwartz 1972), but there is little information on spontaneous hybridization between Neotropical species. A survey in a headwater dam in the Latão Creek, a tributary of the Doce River Basin, yielded 5 *Astyanax*-like fish with numerous maxillary teeth (ca. 8-13) which are hereafter referred as the toothed morphs. Lima (1998) collected one single specimen with few similar characteristics at Paraiba do Sul river basin. Unfortunately the author only commented the similitude of this specimen to *Astyanax* and *Oligosarcus* without any discussion of its origin and suggested the creation of a new genus for this single specimen. In Sossego's dam, these toothed morphs are sympatric with *Astyanax bimaculatus* (Linneaus, 1758); *Astyanax giton,* Eigenmann, 1908 and *Oligosarcus argenteus,* Günther, 1864. These three species are easily distinguished morphologically. To determine the biological nature of these "toothed morph" fishes, it was conducted a comparative multidisciplinary approach including morphologic, cytogenetic, and molecular data, on the toothed morphs and on the other sympatric species. In the last decades, the combined application of these independent character sets has allowed to

address whether populations or individuals belong to different species or not. Morphological characters allow determining the possible uniqueness of the toothed morphs, their similarity to any of their sympatric species or, in the case of hybrids, an intermediate condition between their putative parental species (Hubbs 1955; Welsh and Cincotta 2004). Cytogenetic data have also been quite instrumental to characterize the biological nature of problematic specimens, because it is well known that related species have chromosomal differences (King 1993; White 1978). Finally, uniparental loci, such as mitochondrial DNA are excellent markers for characterizing new taxa (reciprocal monophyly) or for detecting genetic horizontal transfer events (hybridization). However, under such circunstances nuclear genetic markers such as the ITS regions may be more suitable for this kind of investigation since they evolve fast but with the effects of the concerted evolution what minimizes the degree of intraspecific variation, and make the ITS regions suitable for phylogenetic comparisons among closely related taxa (Chow et al. 2006; Presa et al. 2002). Additionaly, rDNA genes are recombining, biparental markers, which can reveal recent gene flow and hybridization events (Mayer and Soltis 1999, Sang et al. 1995). Moreover, multilocus markers, as ISSR, are efficient to determine the similarity of genotypes between species. They are also good markers for identifying different sympatric groups characterized by chromosome polymorphisms in a given Characidae population (Pazza et al. 2007). The possibility of hybridism and introgression between Characidae species may help the phylogenetic understanding of this unsolved group of South American freshwater fishes.

Material and Methods

Characterization of the study area and overall proceedings

Specimens were collected at a dam in the headwaters of Latão Creek, in the Municipality of Coimbra (20°51'24" S, 42°48'10" W) in the state of Minas Gerais (Fig. 1). The Latão Creek is a tributary of the Piranga River, within the Doce River Basin. The dam was built in 1978 and has approximately 2 ha. A multiple (morphologic, cytogenetic, and molecular) approach was used to compare the toothed morph (Fig. 2) to the other sympatric species (Table 1): *Astyanax bimaculatus* (Linnaeus 1758)*, Astyanax giton* Eigenmann, 1908 and *Oligosarcus argenteus* Günther, 1864. After the cytogenetic and molecular protocols, all specimens were fixed in 10% formalin, transferred to 75% alcohol, and deposited in the ichthyological collections of Zoology Museum of the Universidade Federal de Viçosa (Minas Gearis, Brazil) and PUC-RS (Rio Grande do Sul, Brazil).

Morphometric protocols. Morphometric measurements were carried out using a manual 0.05 mm precision calliper, and characters followed Eigenmann (1921). Sixteen traits were taken from each specimen: standard length/head (**HEAD)**, standard length/maximum body depth (**DEPTH**), head/eye (**EYE**), head/interorbital distance (**IO**), head/snout (**SNOUT**), free margin of maxillary bone/anterior-posterior length of the pre-maxillary (**FMM)**, head without operculum/free maxillary border (**PMMB**), total dorsal fin rays (**D**), total anal fin rays (**A**), number of scales between dorsal fin and lateral line (**D-LL**), number of scales on lateral line (**LL**), number of scales between pelvic fin and lateral line (**P-LL**), teeth on the front pre-maxillary row (**TFR**), teeth on the inner pre-maxillary row (**TIR**), teeth on the maxillary (**TM**) and teeth on the dentary bone (**DENTARY**). Morphometric and meristic data were subject to principal component analysis (PCA) by correlation (normalized var-covar) data matrix since the variables are measured in different units (morphometrical and meristical). The analysis were carried out using the software PAST v. 2.01 (Hammer et al. 2010). The jaw bones from *A. bimaculatus*, *A. giton*, *O. argenteus* and the toothed morph were also subject to Scanning Electron Microscopy (SEM) using a LEO SEM model 1430VP.

Cytogenetic protocols. Cell division was stimulated *in vivo* with 2 daily applications of Imunovac®, a commercially available solution of different kinds of antigens following (Molina 2001; Molina et al. 2010). Fish were previously anesthetized with clove oil according to Henyey et al. (2002) and somatic metaphases were obtained from kidney cells by the "air drying" method of Egozcue (1971) and modified by Bertollo et al. (1978). Techniques included Giemsa staining, C-banding according to Sumner (1972) and combined with DAPI staining (Swarça et al. 2003); sequential fluorochrome with CMA_3/DAPI followed Schweizer (1976) and Sola et al. (1992). The Nuclear Organizer Regions (NORs) were detected by silver staining method (Howell and Black 1980). Fluorescent *in situ* hybridization (FISH) followed Pinkel et al. (1986) with 18S and 5S probes from *Hoplias malabaricus* (Cioffi et al. 2009) and *Leporinus elongatus* (Martins and Galetti 1999) respectively. Chromosomes were classified in metacentrics (m), submetacentrics (sm), subtelocentrics (st) and telocentrics (t) following Levan et al. (1964). Digital images were captured on Olympus BX 60 microscope using Q color 3® Olympus software and the chromosomes were measured using Image Pro Plus®. The chromosomes were assembled in karyotypes using Corel Photopaint X4™ and Adobe Photoshop CS2®.

Molecular protocols The DNA extraction followed Boyce et al. (1989) and was conducted on 28 specimens: 10 specimens of *Oligosarcus argenteus,* 1 specimen of *Oligosarcus paranensis* Menezes and Géry, 1983, 10 specimens of *Astyanax giton,* 1 specimen of *Astyanax bimaculatus* from the Sossego Dam, 1 specimen of *Astyanax bimaculatus* from Tiririca Lake (19°45'S; 42°27'W), and the 5 toothed morphs. For mtDNA analysis, a 475 bp fragment of cytochrome b was sequenced, using primers GLU-5 (Table 2) (Hillis et al. 1996). Double stranded DNA was synthesized in 50 µL reactions containing 10 µL dNTPs (1 mM), 5 µL reaction buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 µL $MgCl_2$ (50 mM), 2 µL of each primer (10 mM), 0.5 µL (2.5 U) of Taq DNA polymerase (Phoneutria), 2 µL of template DNA (100 ng/µL) and 26.5 µL of H_2O. PCR conditions were as follows: 94 °C (2 min), 5 cycles of 94 °C (45 s), 54 °C (45 s), 72 °C (1,5 min), 29 cycles of 94 °C (45 sec), 58 °C (45 s), 72 °C (1,5 min). PCR products were purified using Qiaquick (Qiagen) and were sequenced in Macrogen Corp., South Korea. The sequences were aligned using CLUSTAL W as implemented in MEGA 4.0 (Tamura et al. 2007) and the analysis was performed using Neighbour Joining (NJ) (Saitou and Nei 1987).

Internal Transcribed Spacers (ITS). Nucleotide sequences of a conserved primer pair (ITS1 and 5.8S) used to amplify the ITS1 region were obtained from Duke University web site (http://www.biology.duke.edu/fungi/mycolab/primers.htm). The forward primer (ITS1) was designed to anneal near the 3' end of 18S rDNA, and the reverse primer (5.8S), to anneal near the 5' end of 5.8S rDNA (Table 2). The ITS1 sequence was amplified using Taq polymerase Phoneutria® with GC buffer which considerably improved amplification over standard Taq protocols. Initial denaturation at 96 ºC for 2 min, was followed by 25 cycles of amplification (denaturation at 96 ºC for 0-5 min, annealing at 58 ºC for 0-5 min and extension at 74 ºC for 1 min) with a final extension at 74 ºC for 10 min. Six specimens of *Oligosarcus argenteus*, five specimens of *Astyanax giton* and four toothed morphs were subjected to the previous protocol. *A. bimaculatus* was discarded as a possible parental.

Cloning ITS fragments. The amplification products were purified directly from a 1,5% agarose gel by using Kit Wizard ® SV Gel and PCR Clean-Up System (PROMEGA) and bounded to Vector pGEM®-T Easy Vector System (PROMEGA) following the manufactory's suggestions. The vectors were applied to transform ultracompetent DH5a *E. coli* cells. The transformant clones were selected by alpha-complementation, and the recombinant transformant by plasmid extraction through fast colony lysis. The transformant clones were selected, multiplied and the recombinant plasmids were extracted and purified by the usage of the S.N.A.P. Miniprep Kit from INVITROGEN ®, as suggested by the manufactory. The purified plasmid was submitted to sequencing by automatic sequencer model MegaBACE (GE-Healthcare) with M13 oligonucleotides specific to the utilized cloning vector. The partial amplification products were purified with the Enzyme mix Exo-Sap-IT® (USB corporation) and directly sequenced with the same oligonucleotides used in the amplification process.

Inter Simple Sequence Repeat (ISSR) protocols. After *A. bimaculatus* was discarded as a putative parental, ISSR amplification was used in the 2 remaining species and the toothed morphs, to estimate the nuclear DNA similarity of the toothed morphs. Twelve ISSR primers (UBC primer set # 9, Biotechnology Laboratory, University of British Columbia, Canada) were assayed in a pilot test on randomly chosen individuals of *A. giton and O. argenteus* and the toothed morphs. Different concentrations of several reaction components and annealing temperatures were tested. Three primers produced strong bands and were evaluated further for ISSR polymorphism (Table 2). PCR amplifications were carried out in a

total volume of 20 µL comprising 20 ng template DNA, 2.0 µL 10X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 2% formamide, 0,2 µM primer, 0,75 U Taq DNA polymerase (Phoneutria) and ultrapure water. PCR amplification was programmed on MJ Research PT 100 as follows: a denaturation step at 94 ºC, 45s annealing at 45 ºC -53 ºC, 90s extension at 72 ºC followed by 7 min extension at 72º C. Amplification products were separated via electrophoresis on 6% (w/v) polyacrilamyde gels with 1X TBE buffer at constant voltage (100v) for approximately 4-6 hours. Amplified fragments were identified using a 1 kb DNA Ladder (Invitrogen). Bands were visualized with silver nitrate. ISSR bands were treated as dominant genetic markers and scored as 1 (present) or 0 (absent) binary characters. Only polymorphic bands that could be unambiguously scored across all the surveyed individuals were considered for further analysis. The resulting data matrix was analysed using TFPGA v. 1.3 (Miller 1997) for UPGMA, by means of Nei`s (1972) original distance. The analysis was used to construct a phenogram representing the genetic distances among each sample.

Results

Morphological data. Most of the morphological patterns of variation placed the toothed morphs in an intermediate position between *A.giton* and *O. argenteus*. The method of principal components (PCA) is a procedure for finding hypothetical variables (components) which account for as much of the variance in multidimensional data as possible (Harper 1999). These new variables are linear combinations of the original variables. This method appears to have useful application to analysis of fish hybrids, partly because it is free of the disadvantages of the hybrid indices and discriminant functions (Smith 1973). *Astyanax bimaculatus*, *Astyanax giton* and *Oligosarcus argenteus* were characterized by diagnostic morphology and meristic patterns (Table 3; Fig. 3). The toothed morphs and the two species of *Astyanax* lacked the ectopterygoid teeth series that characterize *O. argenteus*. The maxillary teeth number and morphology characterized each of the species at the Sossego's Dam: *A. bimaculatus* lacked such teeth, *A. giton* were characterized by low numbers (0-3) and hexacuspid and pentacuspid teeth, *O. argenteus* had the highest number (15-26) of tricuspid and conic maxillary teeth; and toothed morphs had high numbers (8-13) of pentacuspid and tricuspid teeth (Table 4; Fig. 4). Toothed morphs also differed in the number of lateral line scales and the dentary tooth number from the other two species (Table 3).

Cytogenetic data. All three species and the toothed morphs shared the same diploid number of 2n=50 chromosomes (Fig. 5). The chromosome morphology, however, was characteristic for each species (Table 5). The four toothed morphs showed high levels of chromosome variation both within and among individuals (Fig. 6). Unique chromosome morphologies were observed, including a large submetacentric and tiny acrocentrics or metacentrics. Most of these chromosomes lacked homologues and were placed in a special group (Fig. 6; Table 6); a trend that was particularly evident to the toothed morph CT 2214, which showed 2n=51 in more than half of the analysed metaphases, suggesting the modal diploid number for this specimen was 2n=51 (Table 6). The most stable karyotypic formula was observed in the individual CT 1945 and therefore the cytogenetic results of the remaining toothed morphs were referred to it (Table 7).

Among the three species and the toothed morph, *Astyanax bimaculatus* showed the highest level of karyotypic divergence. Its C-banding/DAPI pattern showed pericentromeric heterochromatin in sm and st chromosomes (Fig. 7a), while Giemsa-stained C-banding showed heterochromatic blocks at the pericentromeric region of only one pair of submetacentric chromosomes (Fig. 7b). Two non-homologous chromosomes (st and t) showed silver stained blocks (Fig. 7c-d), and the location of some heterochromatic blocks matched the silver-stained regions. These chromosomes also presented CMA_3 fluorescence, but the subtelocentric chromosome had a marking at its short arm instead of the telomeric region of its long arm (Fig. 8a-b). The 5S rDNA FISH probe also showed a mark on the telomeric region of the long arm of a telocentric chromosome and also on the telomeric region of the small arm of a subtelocentric chromosome. (Fig. 8c). The 18S FISH probe hybridized with 10 m, sm and st chromosomes (Fig. 8c). The few pericentromeric marks seemed equivalent to the heterochromatin blocks identified using the C-banding technique.

In *A. giton*, the C-banding/DAPI showed multiple heterochromatic markings, most of them on the small arms of submetacentric and subtelocentric chromosomes (Fig 7e). A faint marking was also found on the centromeric region of the largest metacentric chromosome. The C-banding technique showed heterochromatin only on four chromosome pairs including a large subtelocentric chromosome showing bitelomeric markings (Fig. 7f). All Giemsa-stained C-banding matched 18S rDNA fluorescence patterns, although this probe also showed additional markings at telomeric portions of the largest metacentric pair (Fig. 8f). The Ag-stained metaphases showed two different small chromosomes of subtelocentric morphologies as NOR bearers (Fig. 7g-h). Also, only one pair of chromosomes was marked with the 5S rDNA probe (Fig. 8f). The small difference between the two chromosomes marked with silver was not evident with the CMA_3 fluorochrome staining (Fig. 8d-e).

The karyotype of *O. argenteus* was also characterized by high numbers of telocentric chromosomes. The technique of both C-banding and C-banding/DAPI sequential yielded similar patterns (Fig. 7i-j). A pair of small subtelocentric chromosomes seemed to carry a heterochromatic block on their small arms, and only one chromosome from the largest metacentric pair presented heterochromatin block on the distal region of their long arms. The nucleolar organizer region was located in the small arm of a pair of medium sized subtelocentric chromosomes (Fig. 7k-l). The 5S rDNA cluster was also located on the small arm of a pair of subtelocentric chromosomes. (Fig. 8i). All 18S rDNA clusters (Fig. 8i) were

associated to CMA_3 marks (Fig. 8g-h), except for the heterochromatic block at the distal long arm of the larger metacentric chromosome. Also, a pair of large subtelocentric chromosomes carried two 18S rDNA clusters in both telomeres, a bitelomeric marking that was not shown by CMA_3 staining, restricted however to the long arm of one of its homologues and to the short arm of its counterpart.

Finally, for the toothed morphs, the C-banding (Giemsa and DAPI sequential stain) showed only faint markings (Fig. 7m-n) on telomeric region of a submetacentric chromosome small arm and, also on the interstitial region of the long arm of a single telocentric chromosome. Two NOR sites were located on a pair of subtelocentrics (Fig. 7o-p). Only one chromosome pair carries the 5S rDNA region as confirmed by the FISH technique (Fig. 8l). CMA_3 and 18S rDNA FISH coincided for same regions in 4 chromosome pairs (Fig. 8j-k). The only exception was the telomeric 18S rDNA cluster on the larger metacentric chromosome, which was not evidenced with CMA_3. This toothed morph was characterized by relatively few subtelocentric chromosomes, a condition shared with *O. argenteus* but its karyotype also included a unique feature: the presence of a large pair of telocentrics, its 19th chromosome pair that represented roughly two thirds of the first chromosome pair. None of other studied species had such a large telocentric chromosome pair. The toothed morphs were cytogenetically unique since their karyotypes showed diverse variations (Fig. 6). There were also remarkable differences between the banding patterns of these sympatric species and the toothed morph specimen (Table 7).

Molecular data. The cytochrome b mtDNA fragment was identical in three of the toothed morphs and the sympatric *O. argenteus* and thus, the Neighbour Joining analysis clustered the toothed morph specimens as closely related to the genus *Oligosarcus*. However, the toothed morph CT 2213 clustered with the *Astyanax giton* branch (Fig. 9). The ITS-1 fragments showed similar results of the mitochondrial sequence, though the toothed morphs, including CT 2213, clustered with the *Oligosarcus* instead of the *Astyanax* branch (Fig. 10). More than one sequence was obtained for the toothed morph CT 2213 and *Astyanax giton* CT 2093 as a result of the multiple genomic ITS sequences extracted from nuclear DNA. In the ISSR analysis, ten marker loci were obtained distinguishing *A. giton* and *O. argenteus* where the toothed morphs remained as mosaics between these two species. The UPGMA

phenogram of the ISSR matrix clustered *O. argenteus* and *A. giton* in two different groups (Fig. 11), while the toothed morphs formed a distinct group within the *A. giton* group.

The relationship between the haplotypes of *A. giton*, *O. argenteus* and the toothed morphs may be related to introgressive hybridization and also to ancestral polymorphism (Dobzhansky 1953; Wu et al. 2011). If the observed degree of mtDNA sequence variation found among these three Sossego's groups can also be found among other species from both *Astyanax* and *Oligosarcus* genera, the pattern of variation should be related to ancestral polymorphisms. In order to determine which kind of relationship can be related to the mtDNA variation obtained in the sympatric *A, giton* and *O. argenteus* specimens, a mtDNA comparison including *Astyanax bimaculatus* and *Oligosarcus paranensis* species was made (Fig. 12). The analysis showed that the toothed morphs clustered with *Oligosarcus solitarius* and *Oligosarcus paranensis* in a distinct branch of that one of *Astyanax giton* and *Astyanx bimaculatus*. Therefore, the mtDNA relatedness found among *A. giton*, *O. argenteus* and the toothed morph should not be associated with commom ancestral polymorphisms.

Discussion

The PCA pattern indicates the intermediate character of the toothed morphs between *A. giton* and *O. argenteus*. Besides these general morphological aspects, the morphology of the maxillary teeth was also informative. Cusp morphology in toothed morphs included tricuspid teeth (typical of *O. argenteus*) and pentacuspid teeth (characteristic of *A. giton*). Moreover, the toothed morphs showed two rows of pre-maxillary teeth – a trait not shared with any known *Oligosarcus* specimen. Many studies indicate that hybrid fish are morphologically intermediate between the parental morphotypes (Hubbs 1955). However, in other cases many hybrid characters show a range of patterns of nonadditive inheritance (Smith 1992). With respect to some traits, a hybrid may fall well outside the range of parental morphological variation. Such traits are said to be heterotic and may result in cases where the hybrids exceeds the range of variation exhibited by its parents (positive heterosis) or when hybrids fall below the range of parental variation (negative heterosis) (McCarthy 2006). Within the hybrid genome, different alleles can interact following two main hypotheses: the dominant hypothesis, which has enjoyed long theoretical support, posits that deleterious recessive alleles are complemented in hybrids by fitter alleles from the alternate parent, generating a possible increase in vigour. In contrast, the overdominance hypothesis attributes hybrid vigour to the synergistic interactions of alleles at a heterozygous locus (Baak and Rieseberg 2007). Here, success depends on the fixation of favourable new gene combinations from the two parental species (Barton 2001). There is no doubt that the combination of two different genotypes may result on unique phenotypes not present on the parental species. The Bateson-Dobzhansky-Muller theory states that the sterility of hybrids is related to the epistatic interactions between gene clusters of each parent (Bateson 1909; Dobzhansky 1936; 1953; and Muller 1940; 1942) and remains as a classic example of how singular combinations can be found only in hybrid specimens but not on the parental lineage. Through dominance effects, some traits were expressed more similar to *A. giton* and others to *O. argenteus* (such as absence of palatine teeth). Some traits, on the other hand, showed intermediate condition between both putative parental, such as the maxillary teeth. These toothed morphs are therefore distinct from the specimen described by Lima (1998) at the Paraiba do Sul River basin mainly due to the absence of ectopterygoid teeth and the presence of pentacuspid teeth at the maxillary.

The cytogenetic data suggested a mixed suite of *A. giton* and *O. argenteus* characters in the toothed morphs. The toothed morph CT 1945, characterized by a more conservative karyotype, had an unusual banding pattern. All banding techniques when combined provided the identity of each group of Sossego's dam and therefore represent good markers. The species *A. bimaculatus* possesses the most distinctive pattern characterized by two non-homologous chromosomes bearing the Ag-NOR clusters. The C-banding/DAPI shows ten chromosome pairs with heterochromatin blocks, several of them located at the pericentromeric region. However, the most informative data is the location of 5S-rDNA clusters. Only *A. bimaculatus* has two chromosomes pairs bearing this kind of rDNA. The species *A. giton* similarly to *A. bimaculatus* has several chromosomes carrying heterochromatin blocks and only two chromosomes marked with CMA_3. However differentiates of *A. bimaculatus* by having a single pair of chromosomes carrying the 5S-rDNA clusters, instead of two. *Oligosarcus argenteus* possesses a clear divergence of that pattern shown by these two *Astyanax* species since he presented only few chromosomes marked by C-Banding/DAPI technique and 6 chromosomes marked by the CMA_3 technique. The main distinctive character is the presence of only 8 chromosomes carrying 18S rDNA clusters, instead of 10 chromosomes as seen in these sympatric *Astynax* species. The toothed morph presented a mixed suit of markers and therefore unique. The C-banding (both Giemsa and DAPI stained), NOR banding and CMA_3 banding patterns were closer to *O. argenteus* than to *A. giton*. However, the 18S FISH pattern was very similar to the pattern seen in *A. giton*. On the other hand, 5S FISH markings were conserved, exactly as in *A. giton* and *O. argenteus*.

The karyotype of *A. giton* from Sossego's dam was also different from that already described for a population in Paraiba do Sul River basin, distinguishing only by the difference between the proportion of subtelocentric and telocentric chromosomes, where the *A. giton* population from Sossego has 12 subtelocentric chromosomes pairs and the population from Paraiba do Sul River basin has only 4 subtelocentric chromosomes. Moreover, both populations presented, in the majority of their individuals, a single pair of chromosomes Ag-NOR marked and 10 chromosomes baring the 18S rDNA clusters (Kavalco and Moreira-Filho 2003). Unfortunately the authors did not pointed the location of such 18S clusters in the karyotype. The illustration, however, show that the main difference between

these two populations is the lack of the chromosome pair which bears the 18S rDNA on both telomeric regions.

The lack of cytogenetic data about *O. argenteus* complicate any comparison between different populations of this species. Paiva (2008) described the karyotype of *O. argenteus* from São Bartolomeu River (also a tributary of Turvo River) which differed from the Sossego population only in the number of metacentric and subtelocentric chromosomes, but they share the same Fundamental Number. The *O. argenteus* population of São Bartolomeu River presented 4m+16sm+14st+16t and the population from Sossego was characterized by 6m+16sm+12st+16st. Moreover, the São Bartolomeu population showed several heterochromatic blocks at the telomeric regions of different chromosomes, where one pair presented the bitelomeric mark. The karyotype of *O. argenteus* from Sossego was poorer on such heterochromatic blocks, but it counts with the bitelomeric mark on a large submetacentric chromosome. Also, it was found by Paiva (2008) that the specimens from São Bartolomeu River have two chromosome pairs bearing the Ag-NORs, contrasting with the single chromosome pair bearing the Ag-NOR for specimens from Sossego's dam. The pattern of *O. argenteus* from another geographically close riverine system, the Casca River, was also informative. The individuals from that locality presented 10 chromosomes carrying the 18S rDNA clusters (including a chromosome with 18S rDNA clusters on both telomeric regions) (Barros, unpublished data). This is a remarkable difference between these two close populations of *O. argenteus*. Since in Sossego *Oligosarcus argenteus* is the only species with 8 chromosomes bearing the 18S rDNA clusters, the rDNA configuration of that closest riverine population may suggest the uniqueness of the *Oligosarcus* from Sossego.

A population of *Oligosarcus macrolepis* (Steindachner 1877), studied by Falcão and Bertollo (1985) collected from the Turvo River (Rio Doce basin) had similar karyotypes of that described in the present work except for differences between submetacentric and subtelocentric chromosomes. The karyotype described by Falcão and Bertollo (1985) was characterized by 6m+22sm+6st+16t.

In lower vertebrates, NORs can be evidenced not only by silver staining but also by GC-specific fluorochromes such as chromomycin A_3 and mithramycin. The great advantage of using fluorochromes is that it is possible to identify the 45S clusters of the NOR, independent of their activity (Schmid 1980; Schmid and Guttenbach 1988). Ag-NORs in the

genus *Oligosarcus* present an intra-and interspecific variation in number and a remarkable size heteromorphism in some populations (Kavalco et al. 2005). However, when the number of 18S rDNA markers are the same, the disturbances obtained through the ag-NOR technique should be linked to variations on the expression of the rDNA and the specific proteins connected to these active regions (Miller et al. 1976).

The 5S rDNA multigene family consists of a highly conserved coding sequence of 120bp forming arrays of several tandem copies, which are kept separated from each other by variable non-transcribed spacers (NTS) (Danna et al. 1996). This kind of rDNA gene is a smaller DNA sequence that does not participate in the formation of the nucleolus (Peres et al. 2008). In several organisms, the 5S rDNA genes appear strongly constrained to only one chromosome pair, while NORs are often present in multiple chromosomes (Suzuki et al. 1996, Mäkinem et al. 1997). The 5S clusters may also occur in more than a single chromosome pair (Martins and Galleti-Jr 1999) as exemplified by a population of *A. giton* with several chromosomes bearing the 5S clusters (Kavalco et al. 2004). The 5S rDNA clusters presents a different pattern of variation among fish species. Since the sequences of the 45S and 5S rDNA genes remained greatly conserved during the evolution of fish (Fujiwara et al. 1998; Martins and Galleti-Jr 2001), these sites may constitute important cytotaxonomical markers in this group (Garcia and Moreira-Filho 2008). To the present data, unfortunately, the 5S rDNA cluster represented a good marker only to identify *A. bimaculatus* since all other sympatric characids presented a single pair of chromosomes bearing those ribosomal genes. On the other hand *A. giton* from Sossego and Paraíba do Sul River basin populations are highly different because the first population has a single pair of chromosomes bearing the 5S clusters and the second population counts with 10 chromosomes carrying such genes (Kavalco et al. 2004). It seems highly improbable that all these divergence is connected only to Robertsonian chromosomal rearrangements. Despite the number of 5S rDNA clusters remains as good markers to distinguish these two populations, they should not be used to characterize *A. giton*.

The morphological aspects of the chromosomal structure among the four karyotyped toothed morphs revealed the wide range of variation characteristic of some fish hybrids (LeGrande et al. 1984). All toothed morphs presented several degrees of chromosomal abnormalities by possessing numerous chromosomes unable to be paired including tiny chromosomes, of metacentric and telocentric morphology. The toothed morph CT 2214,

presented an additional small chromosome in more than a half of its cells. Chromosome variability with mitotic origin is not a novelty, and several variations (mainly in heterochromatic blocks) were documented in many vertebrates, invertebrates and plant species (Kurnit 1979). The chromosome heterozigosity among kangaroo hybrid species suggested to O'Neill et al. (2001) that the chromosome rearrangements found in such hybrids do not occur randomly. Such variability may be explained by the concept of specific chromosomal territories inside the nucleus as proposed by Boveri (1909). It is currently accepted that the chromosomes are not randomly displayed inside the nucleus, but organized in specific chromosome territories that are essential components of the higher-order chromatin architecture of the vertebrate cell nucleus (Tanabe et al. 2002; Tashiro et al. 2007). Such arrangements have several implications on gene expression and chromosome evolution. It has been argued that heterochromatin blocks may play a role in the evolving nuclear architecture (Dechat et al. 2008; Manuelidis 1990), such as position of genes relative to the heterochromatin, which may strongly affect their transcription (Baxter et al. 2002; Lanctôt et al. 2007). The CT-IC cytogenetic model states that the positioning of genes into proper nuclear compartments is an essential part of gene-activation and gene silencing mechanisms (Cremer and Cremer 2001). Chromosome translocations that join heterochromatin segments with gene-dense chromatin segments may therefore lead to radial chromatin shifts depending on the size and composition of the respective segments (Tanabe et al. 2002). Not surprisingly, every subtle change on the position of some chromosome fragments (*e.g.* Position Effect) may have several and unpredictable phenotypic results. Hybridization can result in genomic changes including alterations to gene expression, chromosomal structure, and genome size (Baak and Rieseberg 2007). In the toothed morphs, hybrid somatic cells containing sets of chromosomes belonging to each parental may face a complex condition for each chromosome position inside the nucleus, which may result in non-random interaction between chromosomes inside the interphasic nucleus. According to John (1980), the occurrence of rearrangements involving homolog chromosomes is a rare event. Baak and Rieseberg (2007) suggest that in hybrid cells the karyotypic differences may arise through the sorting of chromosomal rearrangements that distinguish the parental species or may arise *de novo*. Chromosome fission or Robertsonian translocations may be responsible for the additional chromosome that characterized toothed morph CT 2214. The presence of high chromosome mosaicism on this specimen suggests that the rearrangements involved occurred late in the embryonic development (O'Neill 2001). Such chromosome instability in somatic cells may

bear phenotypic implications. Gene incompatibility of the parental species may lead to severe or lethal physiological disturbances, as reported for hybrids of crosses between the close swordfish species *Xiphophorus maculatus* and *Xiphophorus helleri.* These hybrids frequently develop melanotic tumours (Mayr 1966). Similar incompatibilities of the parental genomes have been described for species crosses in a number of animal and plant genera (Ghadially and Gordon 1957; Mayr 1966) and may lead to reductions on fertility capacity (Dobzhansky 1953; Garagna et al. 2002). However, this concept of hybridization as an evolutionary dead-end in animals is being challenged by reports of frequent hybridization between closely related species (Avise 1994; Barton and Hewitt 1989). These chromosomal rearrangements, mainly inversions, in hybrid swarms have their remarkable importance when carrying advantageous traits, which may lead to quickly fixation in the population (Stemshorn et al. 2011)

Mitochondrial DNA has been widely used in evolutionary studies because of its uniparental mode of inheritance, high rate of evolution, and ease of isolation and characterization (Moritz et al. 1987) and some mitochondrial genes could be used as phylogenetic markers to resolve relationships among taxa that diverged as long as 65 to 300 million years ago (*e.g.*, Ortí and Meyer 1997). On the other hand, the biparental way of inheritance of ITS sequences is advantageous when compared to the uniparental inheritance of organellar DNAs (Feliner and Roselló 2007) since the concerted evolution of tandem repetitive families, such as rDNA, make them useful in phylogenetic studies as a result of the homogenization of the variation within species and populations, whereas divergence is stressed between them (Hillis and Dixon 1991; Nei and Rooney 2005; Separack et al. 1988). The fast evolution of such sequences generates great amount of variability and when concerted evolution is not fully operating, duplicated ribosomal loci do not necessarily remain functional and some arrays may degenerate into pseudogenes (Feliner and Roselló 2007). The inter-simple-sequence-repeats (ISSR) have applications in studies of population structure, parentage, individual identification, and may be used for studies of hybridization (Scribner et al. 2001). The three molecular approaches indicated different aspects of the toothed morphs genetic background, suggesting a matrilineal *O. argenteus* origin and a closer nuclear allelic identity to *A. giton*. The specimen CT 2213 represents the main clue that the toothed morphs are indeed hybrids since its matrilineal origin lies on *Astyanax giton* and its nuclear ITS-1 DNA is similar to those of *Oligosarcus argenteus* specimens. In the ten ISSR

loci that were considered good markers for *A. giton* or *O. argenteus,* the toothed morphs presented intermediary characters for nine loci. The degree of variance found among the paternal *A. giton* contribution is surprising, considering the rarity of male specimens of *A. giton* at Sossego's Dam (Table 1).

Hybrid occurrence is a common phenomenon among fishes, especially in fresh water ones (Hubbs 1955) and Schwartz (1972; 1981) reviewed 3,759 references related to fish hybridization. Several factors have been proposed as possible causes for the high incidence of hybridization among closely related fish taxa: external fertilization; weak behavioural isolating mechanisms; unequal abundance of the two parental species; competition for limited spawning habitat; and susceptibility to secondary contact between recently evolved forms (Hubbs 1955; Scribner et al. 2001); anthropogenic habitat alterations and introduction of exotic species may also be current relevant factors (*e.g.*, Welsh and Cincotta 2004). Smith's (1992) revision indicates three main assumptions on fish hybridization literature: 1) the likelihood of hybridization and the fertility of hybrids are proportional to genetic similarity (Hubbs 1955), which is condemned by Mayr (1999). Genetic compatibility between species can be considered a plesiomorphic trait that may or may not be blocked by subsequent evolution; 2) hybridization and introgression are promoted mainly by anthropogenic disturbance of habitat integrity, "hybridization of the habitat" (Miller et al.1989). Fossil and molecular evidences suggest, however, that hybridization has occurred in ancient as well as in modern times (Smith 1992). Anthropogenic disturbances are recent additions to normal geological, climatic and ecological modifications of habitats; 3) some assumptions on fish hybridism suggest they are "intermediate" between the parental morphotypes (Hubbs 1955). However some observations and experiments indicate, to the contrary, that diverse characters show a range of patterns of nonadditive inheritance as commented above.

Four cases of spontaneous hybridism have been reported for Characidae fish, but they were either inferred and/or are restricted to the same nominal species. One of them is the blind troglodyte *Astyanax jordani* and the epigean species *Astyanax mexicanus*, as a result of sympatry when specimens of the latter species become trapped in caves with *A. jordani* (Kullander 1999). Pazza et al. (2006) reported two sympatric strains of *A. fasciatus* from Mogi-Guaçu River, with different chromosome configurations that suggest hybridization events. The other two cases are related to piranhas *Serrasalmus*. Based on chromosome morphology, Cestari and Galetti Jr. (1992) inferred the existence of hybridization between

two different and isolated populations of *S. spilopleura*: from the Upper Paraná and the Paraguay River basins. Hubert et al. (2007; 2008) studied 3 closely related species from Madeira River (*Serrasalmus compressus*, *Serrasalmus hollandi* and *Serrasalmus* sp.) by means of mitochondrial and nuclear DNA data. They indicated that recent isolation and ancestral polymorphism alone are unlikely to produce the observed high levels of haplotype sharing and the authors propose mtDNA introgression through hybridization as the most likely cause. Currently, genetic similarity of sympatric populations can be interpreted as evidence of a sympatric origin of species, evidence of introgressive hybridization or both (Grant et al. 2005).

Introgressive hybridization is a common natural phenomenon and many examples in animals, and mainly in plants, have been reported (Anderson 1949). The remarkable importance of hybridism zones as "natural laboratories for evolutionary process" (Hewitt 1988, 2011) must not mask its ecological impact. There has been considerable debate in the ecological and evolutionary literature over the relative importance and rate by which microevolutionary processes, operating at the population level, result in the separation and differentiation of lineages, populations and ultimately in speciation. As a microevolutionary process, hybridism influences the trajectory of around 10% of animal species, particularly in young, recently diverged species (Arnold 1997). Hybridization is usually infrequent in nature between sympatric species (Mallet 2008); when it occurs, however, it may have vast consequences. Scribner et al. (2001) propose that over evolutionary time scales, hybridization processes can be an important source of diversification in fish species, as exemplified by the hybrid origin of the *Poecilia* complex. Horizontal gene flow among different groups of Characidae may likewise result in deep alterations in morphological and molecular data at many levels of the systematic hierarchy. Gene transfer may explain the paraphyletic condition of the genus *Astyanax* as reported by Lucena (1993), Calcagnotto et al. (2005), and Javonillo et al. (2010). Although the Sossego's Dam is a clear example of an anthropogenic habitat, past geologic events may have mimic small environments that favoured gene transfer among different lineages; these scenarios are not strange to the Neotropical hydrographical history (Lundberg et al. 1998; Ribeiro 2003). The ancient role of natural hybridization process may bear consequences at high evolutionary levels because several populations in a particular geographic context may be result of hybridization between two different taxa (Grant and Grant 1997). To better understand the relationship among the different Characidae

taxa, mainly those considered *Incertae Sedis*, it is essential to take into account the possibility of hybridization events, both anthropogenic and natural.

The relevance of hybrids has been increasing in conservation biology. Hybridization may lead to the formation of new species, to extinction of native species or both (Moyle and Cech 2004) by swamping and replacing endemic genic pools with exotic genes from invasive species (Epifanio and Nielsen 2001; Tanaka 2007). Rates of hybridization and introgression have increased dramatically worldwide because of widespread intentional and incidental transpositions of organisms and habitat modifications by humans (Allendorf et al. 2010) and the harmful effects of hybridization, with or without introgression, have led to the extinction of many populations and species in many plant and animal taxa (Allendorf et al. 2001; Gurevitch and Padilla 2004; Rhymer and Simberloff 1996). Although there are no records of fish introductions for the Sossego's Dam, *A. giton* is known only from this locality and therefore, it is also the only known locale where this species is sympatric with *O. argenteus*.

To determine whether toothed morphs are ecological dead-ends or are able to reproduce with each other or introgress to their parental lineages, analysis of many functional aspects are still needed (*e.g.,* gonadal and gametic development, and reproductive behavior). There is a common belief that hybridization almost never results in gene flow and introgression as a consequence of several post-zygotic natural barriers (Mayr 1966). However, Mallet (2005) suggested that the F_1 hybrids should be the most difficult to achieve because they would be the result of special events. Barton (2001) also asserted that if fertile F_1 hybrids are produced, they will almost certainly backcross to one or other parental genotype. Although the toothed morphs seemed to be the F_1 hybrids, they were represented by both sexes and their gonads were normally developed.

Most hybrid zones reflect a balance between migration and selection against hybrids (Barton and Hewitt 1989). However, it must be stressed that at the Sossego dam the fish population is small and isolated so, there is no possible migration and genetic exchange. Moreover, the strong sexual disequilibrium may reduce the effective size of that population allowing it to the dispersive effect of the genetic drift. As pointed out by Payseur (2010) the inheritance process can contribute additional inter-locus heterogeneity to patterns of introgression which are different between nuclear and mitochondrial DNA since variation in effective population size leads to disparate rates of genetic drift and also, genomic

compartments are expected to show discordant levels of introgression in species with sex-biased dispersal. Some studies on the radiation of several small fish species found that sympatric pair of species appeared monophyletic in their mitochondrial but poly- or paraphyletic in their nuclear genes, suggesting hybridization of colonizing species, followed by the fixation of one parental mtDNA (Seehausen 2004). These new recombinants might aid the fixation of novel genotypes that are well suited to fill vacant niches in the novel environments in which adaptive radiations occur (Schilthuizen et al. 2004). These radiations generates a great amount of variation subject to strong selection purging incompatibility alleles and other alleles linked to them (Seehausen 2004). However, alleles there were rare in either parent population might them hitchhike to high frequencies in the hybrid population (Schilthuizen et al. 1999; 2001) (the "rare allele effect") influencing, therefore, several morphological traits. This kind of radiation influenced by hybridization events and also a balance between drift and selection against hybrids may be a good model for understanding some of the variation among the Neotropical Characidae fish.

The genetic barriers involving chromosomal rearrangements are much stronger than single-locus barriers and so, they are far more likely to trigger a snowball accumulation of post-zygotic incompatibilities that can lead to reproductive isolation of the species (Navarro and Barton 2003) or their eventual hybrids. Therefore, the relationship between the toothed morphs and their parental species will be at least partially limited by their chromosomal incompatibilities. Overall karyotypic similarity between the parental species allowed the production of the toothed morphs. More critically, it is unknown whether other mechanisms acting on the reproductive tissues may counteract the unbalanced karyotypes seen in the renal tissue. Some species of the livebearer *Poeciliopsis* are known to have hybrid origin (Schultz 1961) and developed different mechanisms to avoid chromosomal incompatibilities on heterozygotes. The "hybridogenesis" allow this species to transmit only the maternal chromosomes of each generation to the eggs, with the consequent meiotic loss of the male chromosomes (Schultz 1969).

Conclusions

The toothed morph specimens were considered hybrids between *Astyanax giton* and *Oligosarcus argenteus*. Hybridization bears potentially high impact on the phylogeny of Characidae fish since the horizontal gene flow may character combinations that might preclude the establishment of monophyletic characters for involved taxa. Although the high levels of chromosomal variation and instability found in the toothed morphs strongly suggest their hybrid nature, the absence of meiotic data did not allow determining the effects of the hybrid karyotype on fertility. Therefore, the introgression of *Oligosarcus argenteus* genes inside *Astyanax giton* specimens demands more information about the genetic structure on both species. The existence of spontaneous intergeneric hybrids in an anthropogenic environment underlines still another threat to the Neotropical fish biodiversity.

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Figures

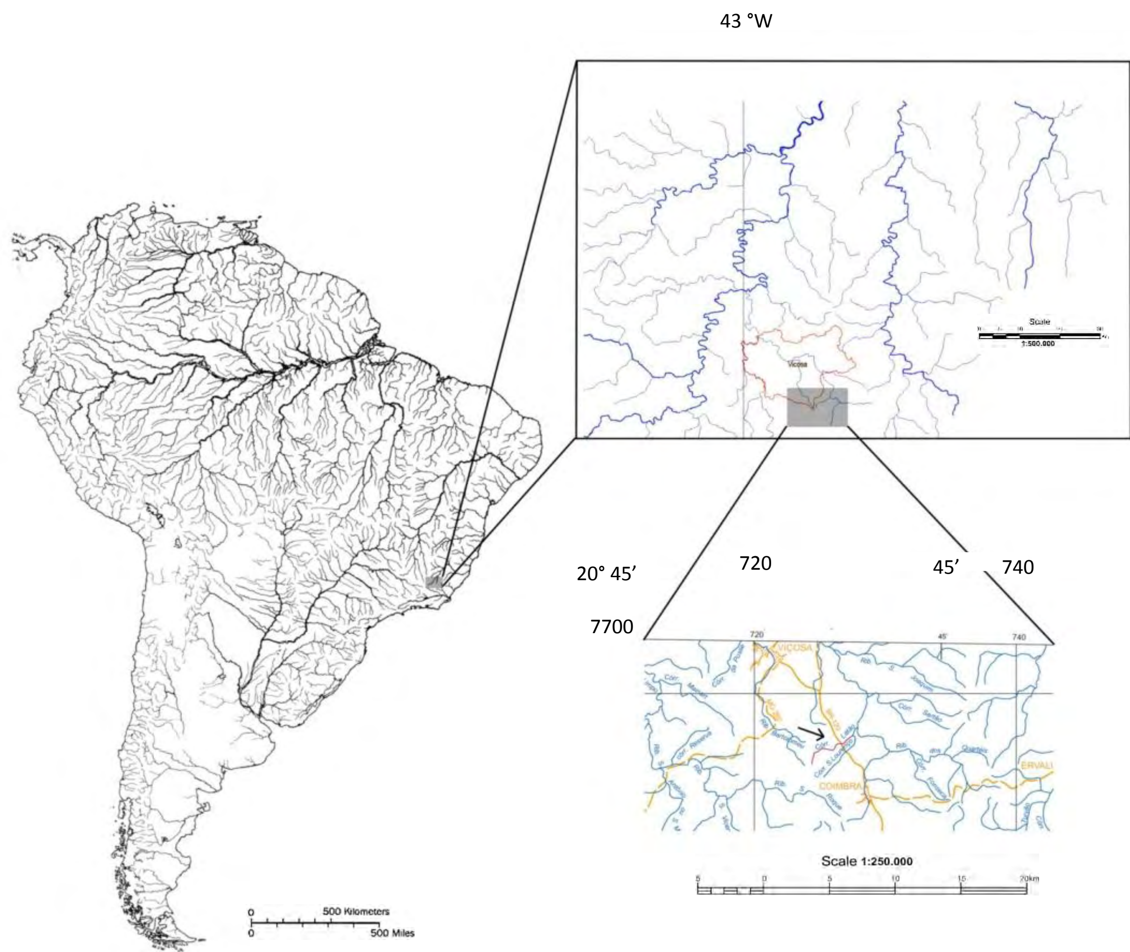


Figure 1. Location of Latão stream (arrow) in south-eastern Brazil.

Figure 2. Toothed morph found at Sossego's Dam.

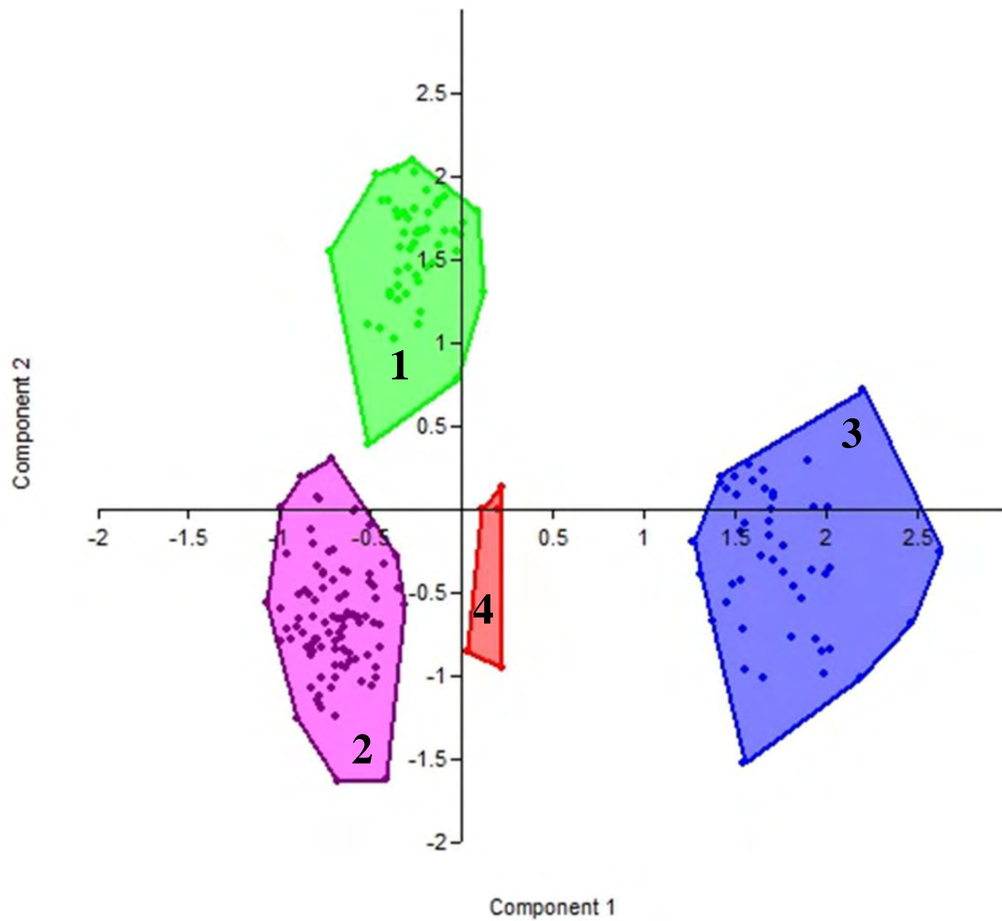


Figure 3. Principal Component Analysis (PCA) of a morphological correlation data matrix including: **(1)** *A. bimaculatus*; **(2)** *A. giton*; **(3)** *O. argenteus* and **(4)** the toothed morphs.

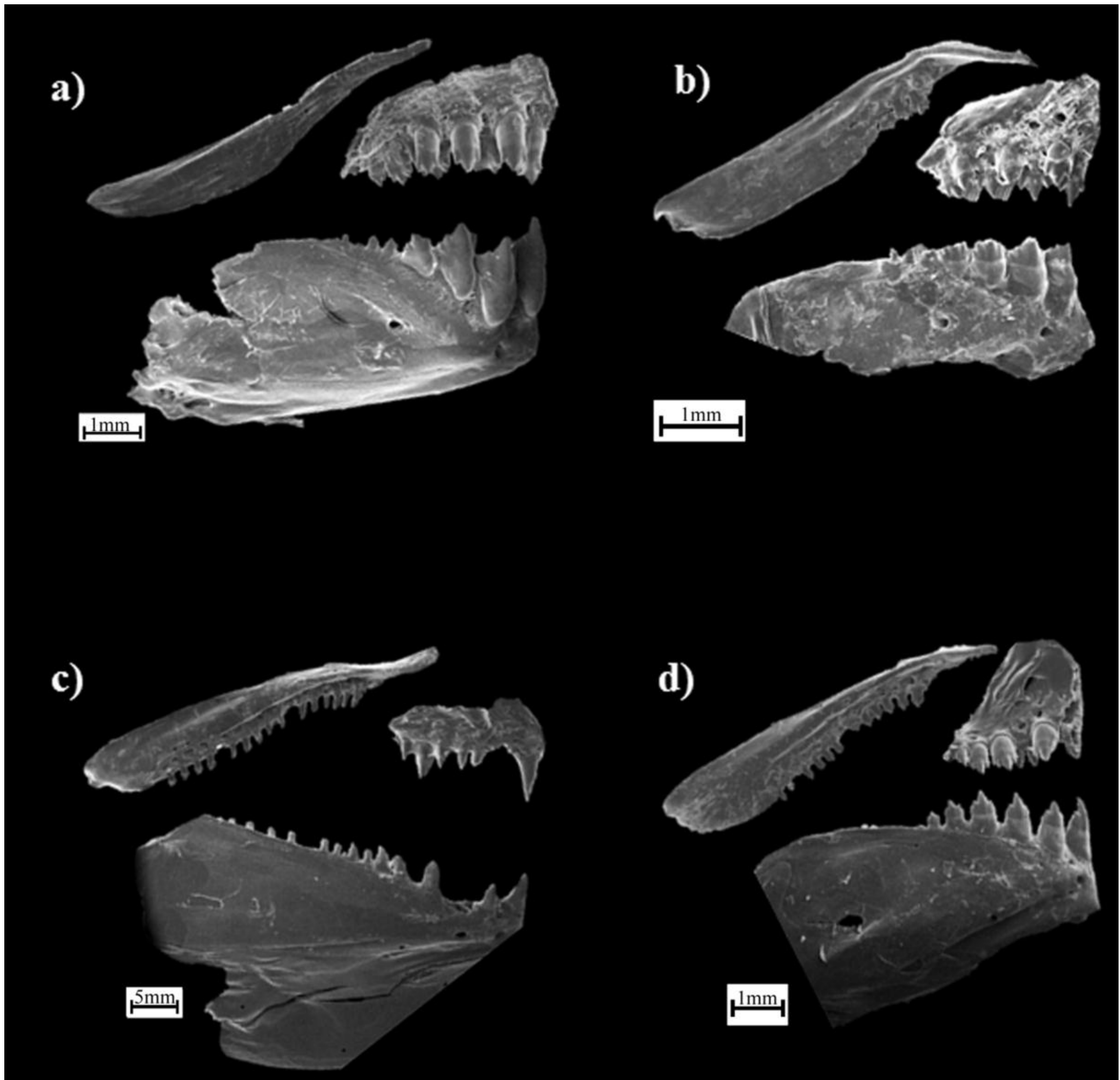


Figure 4. Scanning Electronic Microscopy image of the four simpatric Characidae species' jaw from Sossego. a) *Astyanax bimaculatus*; b) *Astyanax giton;* c) *Oligosarcus argenteus* and d) toothed morph.

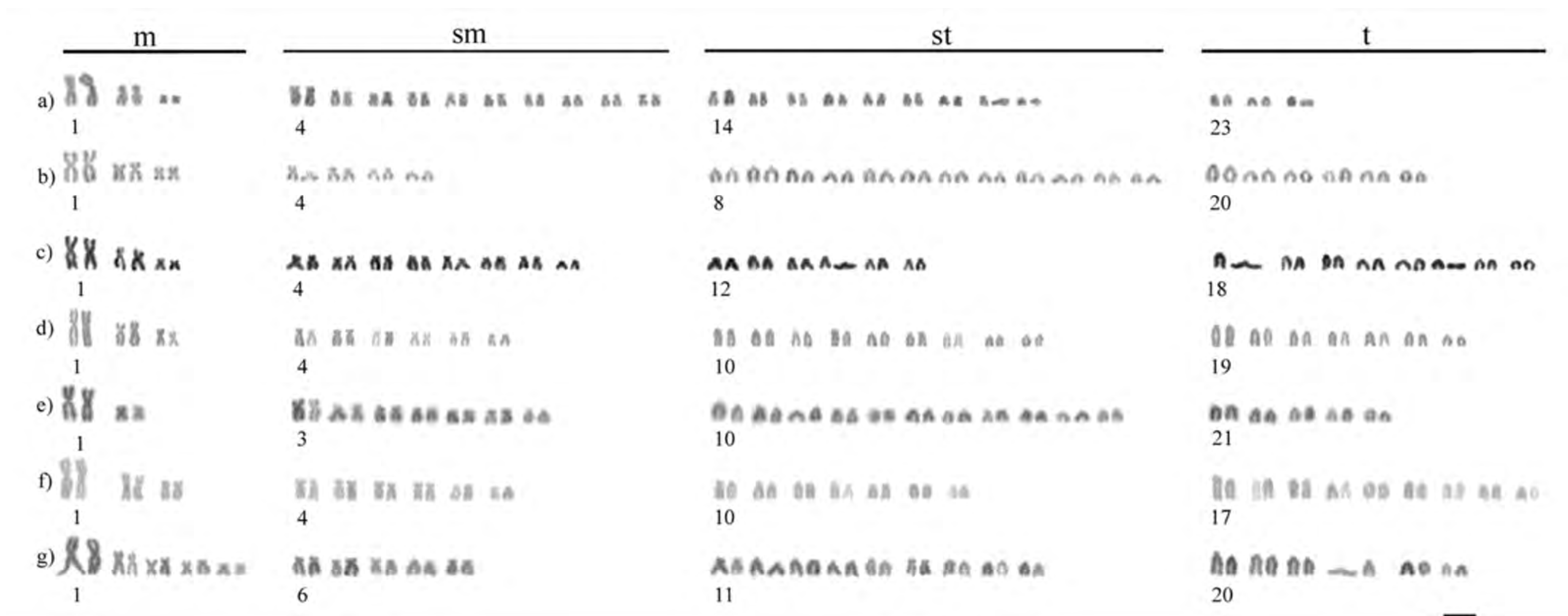


Figure 5. Giemsa-stained karyotypes of the Characidae fish species that are sympatric in the Sossego Dam. **a)** *Astyanax bimaculatus*; **b)** *Astyanax giton*; **c)** *Oligosarcus argenteus;* Some toothed morph karyotypes: **d)** CT 1945; **e)** CT 2064; **e)** CT 2138; **f)** CT 2213; **g)** CT 2214. Bar = 5µm.

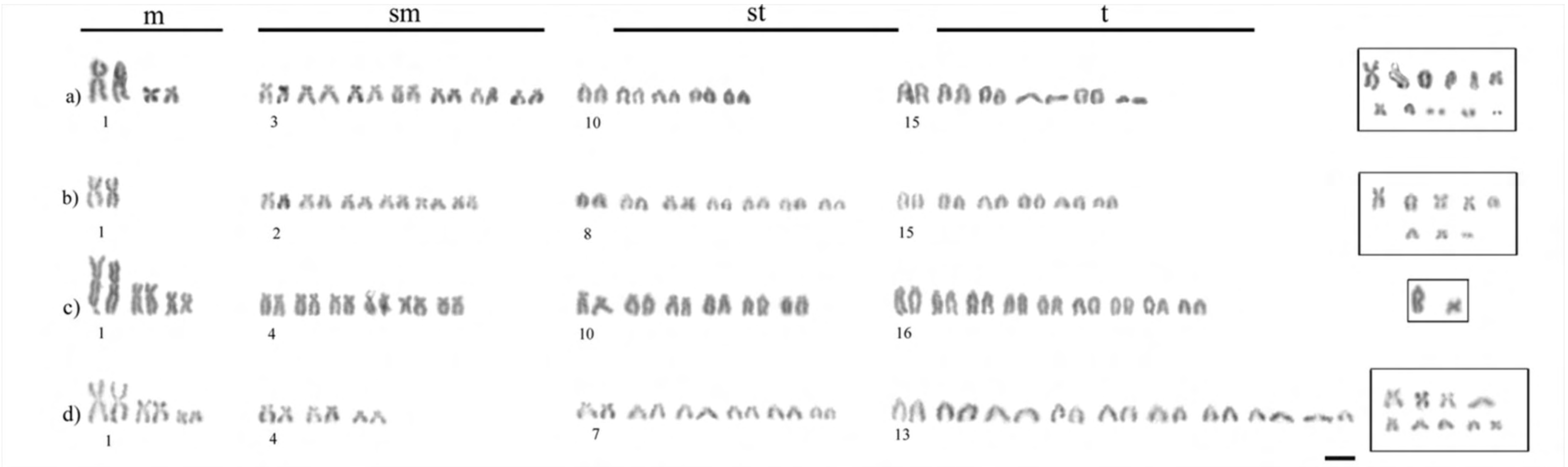


Figure 6. Some variant karyotypes observed in the toothed morph specimens. **a)** CT 1945 with 3 dot-like chromosomes; **b)** CT 2138 with one dot-like chromosome; **c)** CT 2213 with one unpairable small metacentric chromosome; d) CT 2214 with 51 chromosomes. The inset showing unpairable chromosomes. Bar = 5µm.

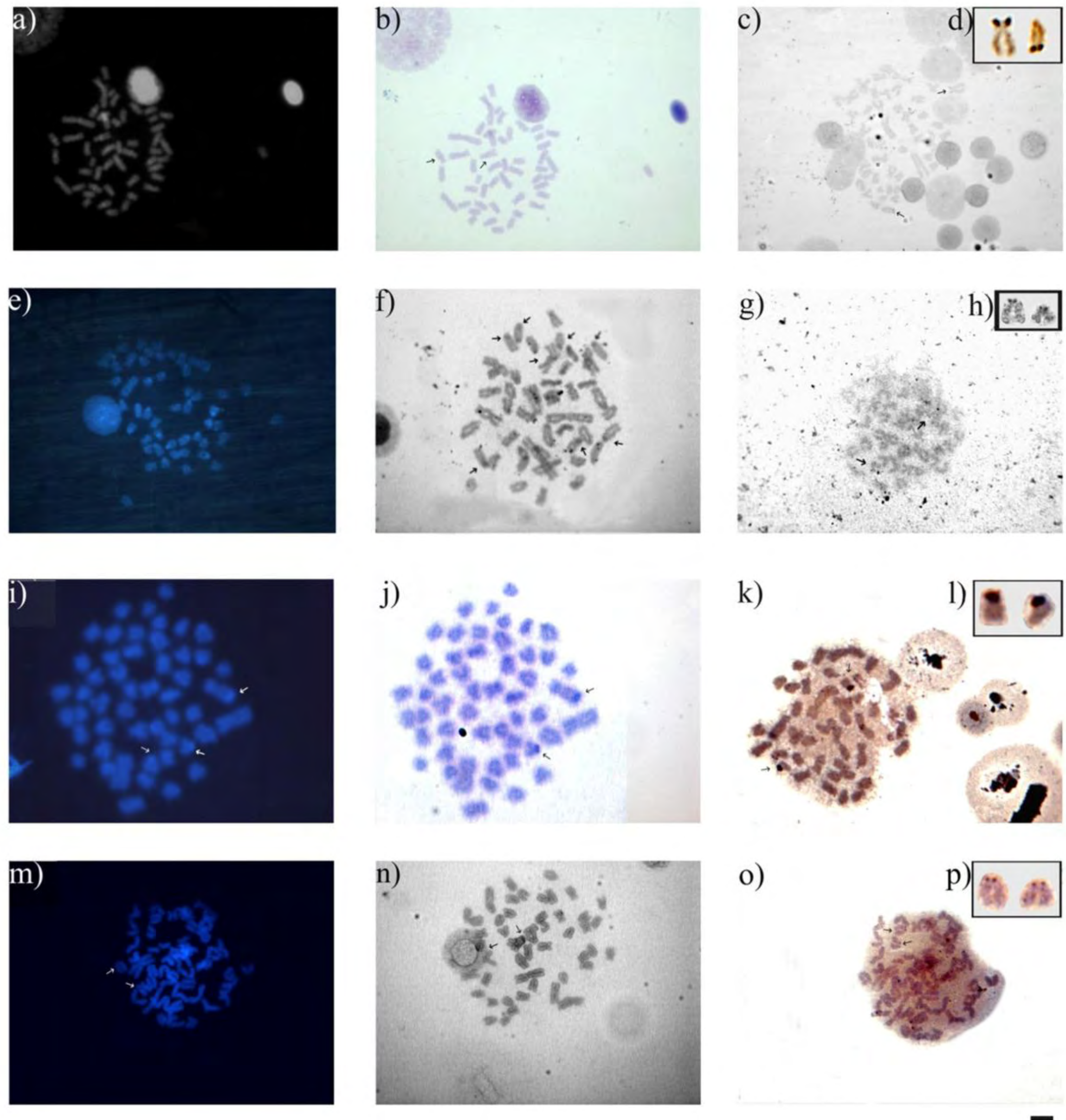


Figure 7. Chromosome banding patterns. *Astyanax bimaculatus* (a-d), *Astyanax giton* (e-h), *Oligosarcus argenteus* (i-l) and Toothed morph (m-p): DAPI-stained C-banding (**a, e, i** and **m**), arrows indicating heterochromatin blocks; Giemsa-stained C-banding (**b, f, j** and **n**), arrows indicating heterochromatin blocks; NOR-banding (**c**,**g**,**k** and **o**), Arrows indicate NOR clusters. Insets showing NOR-bearing chromosomes (**d, h, l** and **p**). Bar = 5 µm

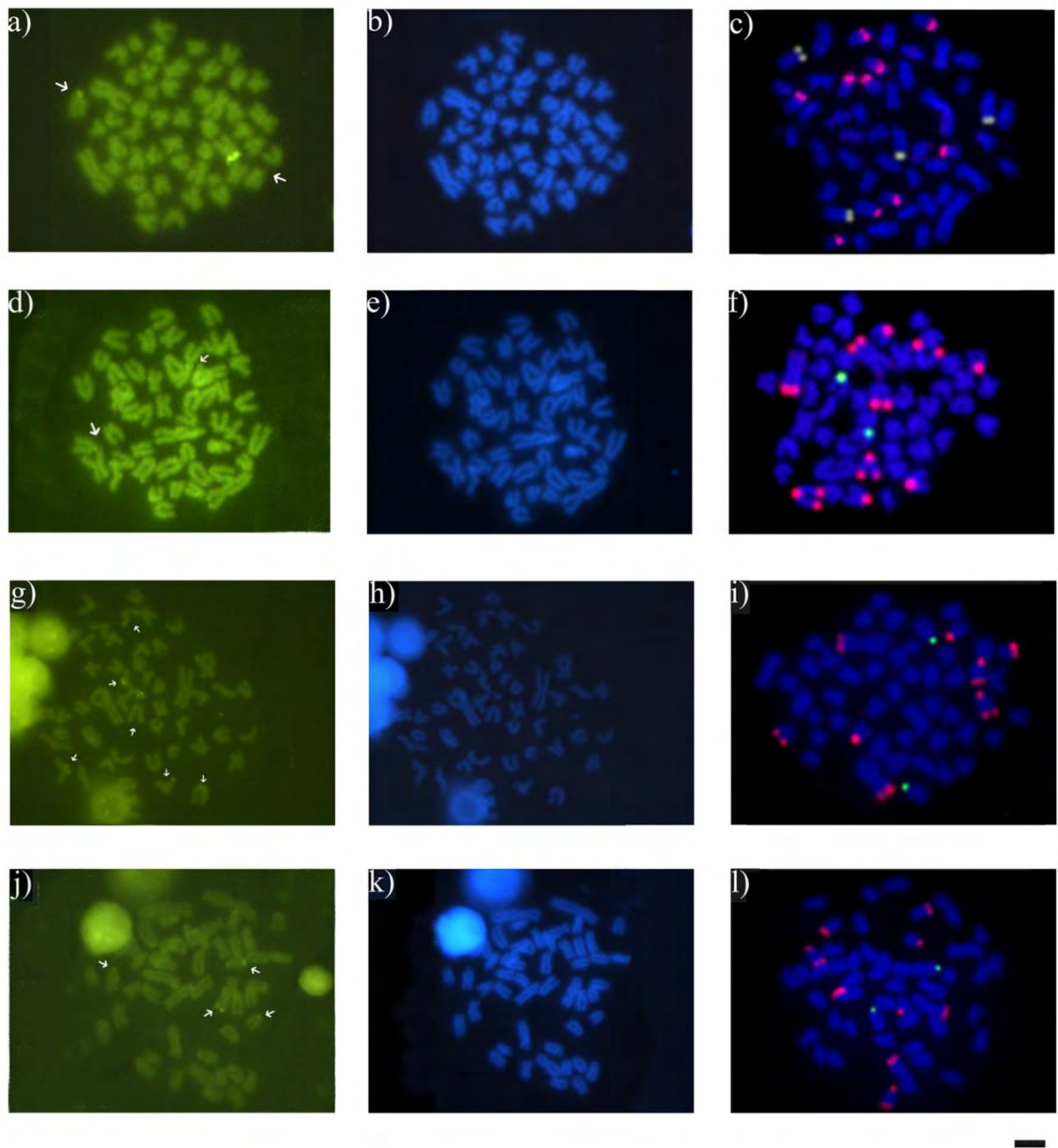


Figure 8. Fluorescent *In Situ* Hybridization (FISH) and fluorochrome-stained chromosomes. *Astyanax bimaculatus* (a-c), *Astyanax giton* (d-f), *Oligosarcus argenteus* (g-i) and Toothed morph (j-l): CMA_3 stained (**a, d, g** and **j**), arrows indicate GC-rich DNA; DAPI stained (**b, c, h** and **k**); FISH-treated chromosomes (c, **f**, **i** and **l**); green and red marks indicate 5S NOR clusters and 18S NOR clusters respectively. Bar = 5 µm

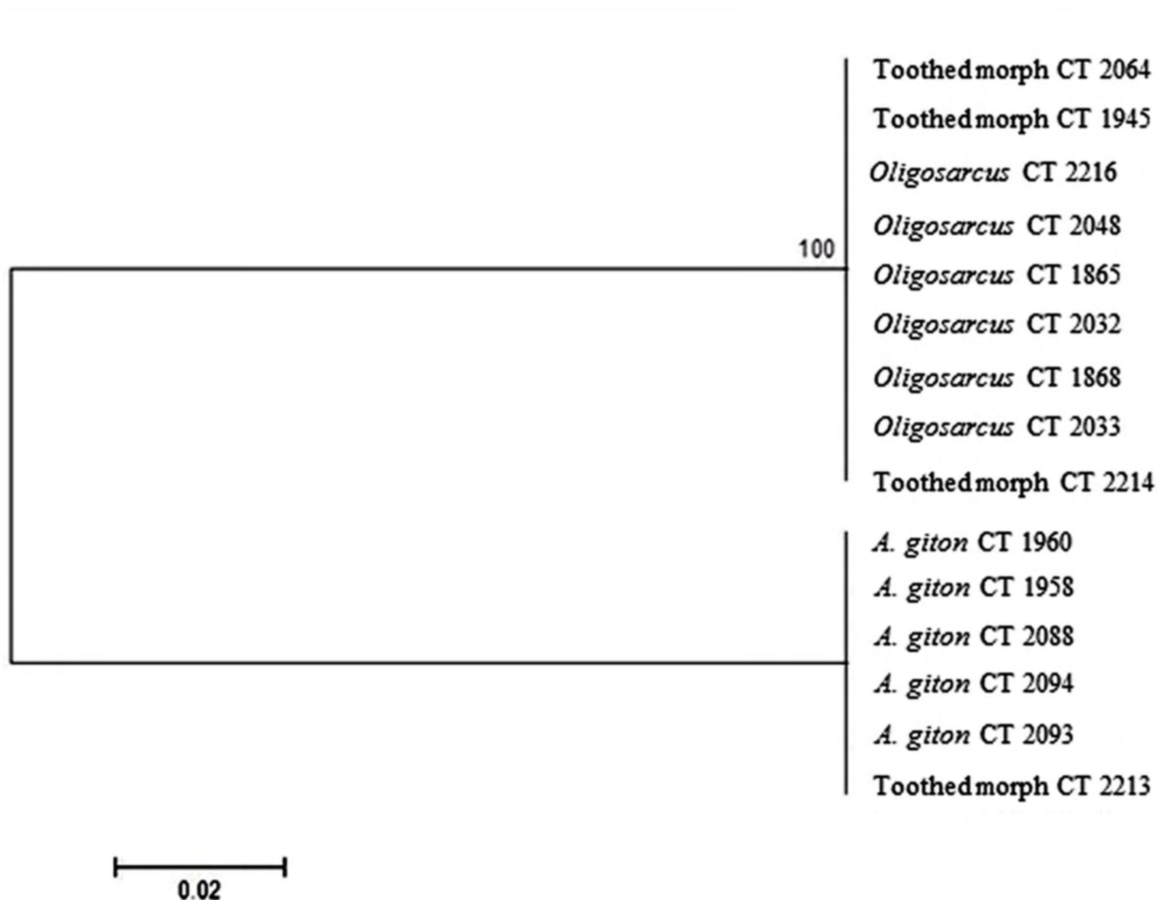


Figure 9. Neighbour Joining analysis based on a 475 bp mtDNA of the cytb fragment of *Astyanax giton*, *Oligosarcus argenteus* and 4 toothed morphs, obtained with the Glu-5 primer. The DNA sequence of the toothed morphs are identical to the one of *O. argenteus* except by the toothed morph CT 2213 which has the *A. giton* DNA sequence. Bootstrap = 1200

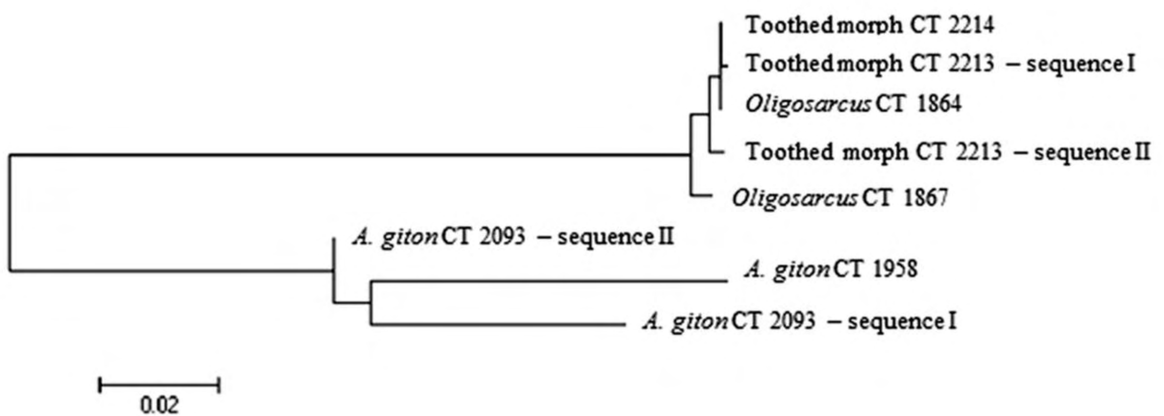


Figure 10. Neighbour Joining analysis based on a 1123 bp ITS-1 sequence of nuclear DNA fragment of *Astyanax giton*, *Oligosarcus argenteus* and 3 toothed morphs, showing that the DNA sequence of all the toothed morphs are identical to the one of *O. argenteus* including the CT 2213 which has *A. giton* mitochondrial DNA. Bootstrap = 1200

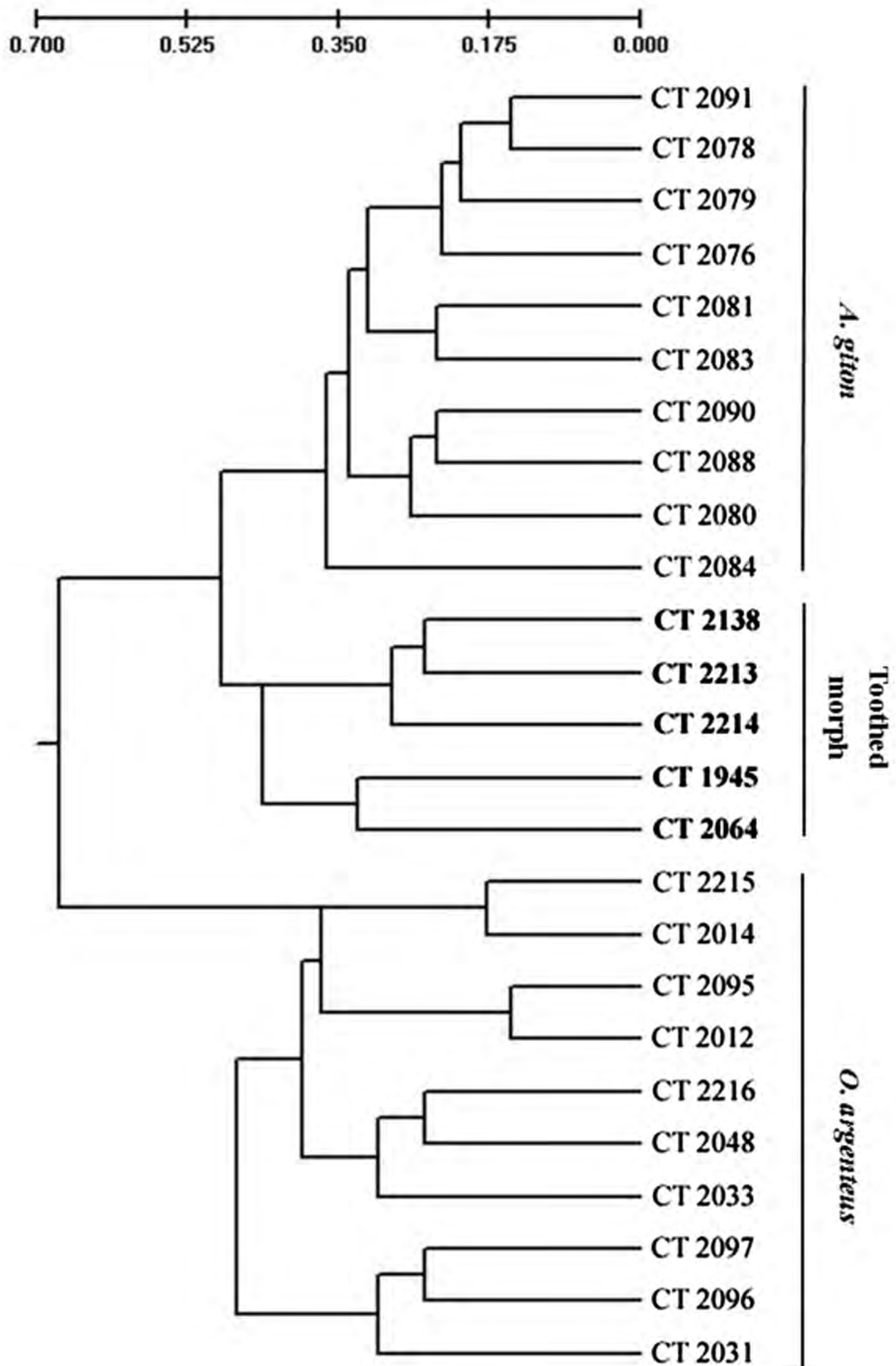


Figure 11. UPGMA cluster analysis based in ISSR loci in *A. giton*, *O. argenteus* and the toothed morph specimens. Bootstrap = 1200.

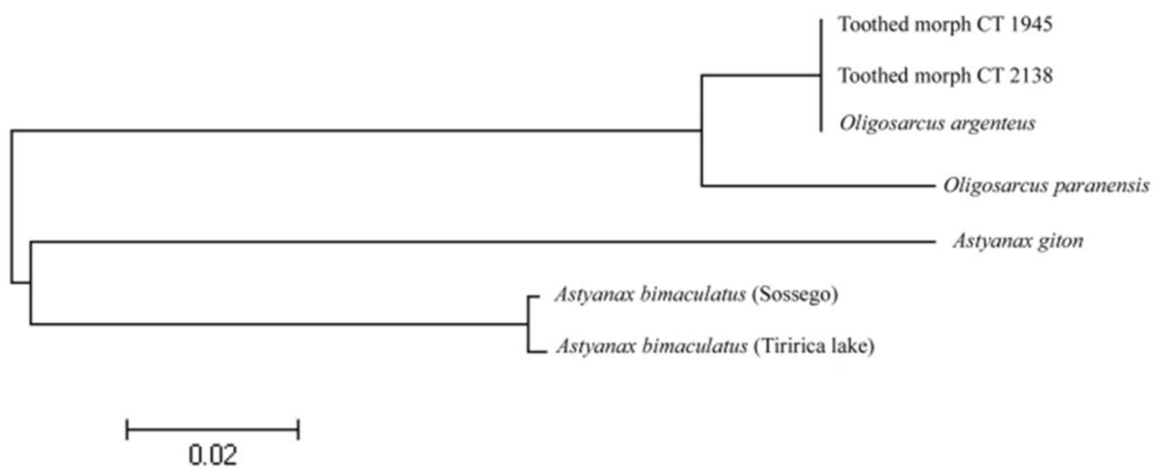


Figure 12. Neighbour Joining analysis based on a 407 bp cytb mitochondrial DNA fragment of *Astyanax bimaculatus*, *Astyanax giton*, *Oligosarcus argenteus*, *Oligosarcus paranensis*, and 2 toothed morphs, showing that the DNA sequence of the latter was identical to the one of *O. argenteus*. Bootstrap = 1200

Tables

Table 1 Collected specimens at Sossego`s Dam and gender proportion

Gender	**Sympatric groups**				**Total**
	Astyanax bimaculatus	*Astyanax giton*	*Oligosarcus argenteus*	Toothed morph	
Male	7	8	9	3	27
Female	45	171	38	2	256
Total	52	179	47	5	**283**

Table 2 Selected *cyt. b*, ISSR and ITS primers for analysis. Their sequences and *Tm*.
R = (A or G); Y = (C or T)

	Primer	Sequence	*Tm*	number of polymorphic loci
cyt b	Glu-5	TGACATGAAAAAYCAYCGTTG	54 °C	
ISSR	Terry	GTG GTG GTG GTG RC	53 ºC	38
	UBC861	ACC ACC ACC ACC ACC ACC	45 ºC	25
	UBC835	AGA GAG AGA GAG AGA GYC	53 ºC	23
ITS	Forward	TCC GTA GGT CCT GCG G	58 ºC	
	Reverse	CGC TGC GTT CTT CAT CG'	58 ºC	

Table 3 Comparison of morphometrical and meristical data of the sympatric *A. bimaculatus, A. giton, O. argenteus* and the Toothed morphs

	Astyanax bimaculatus		*Astyanax giton*		*Oligosarcus argenteus*		Toothed morph	
Character	Average /(mode)	Std. error	Average /(mode)	Std. error	Average /(mode)	Std. error	Average /(mode)	Std. error
Head	4.17	0,04	4,16	0,03	3,55	0,02	3,92	0,07
Depth	2,5	0,02	3,00	0,02	3,43	0,03	3,18	0,07
D.	(11)	0,04	(11)	0,04	(11)	0,04	(11)	0,32
A.	(29)	0,17	(24)	0,15	(26)	0,31	(25)	0,74
D-LL	(8)	0,08	(5)	0,06	(8)	0,10	(6)	0,24
LL *	(37)	0,12	(33)	0,26	(44)	0,52	(38)	1,24
P-LL	(7)	0,09	(4)	0,06	(6)	0,12	(6)	0,37
Eye	3,02	0,03	2,43	0,02	3,68	0,07	2,68	0,16
IO	1,69	0,02	2,09	0,02	2,36	0,05	2,19	0,05
Snout	4,67	0,08	4,34	0,06	4,05	0,08	4,25	0,18
FMM	2,10	0,06	2,23	0,04	2,89	0,10	2,33	0,06
PMMB	0,55	0,02	0,5	0	0,73	0,04	0,61	0,01
TFR	(4)	0,02	(3)	0,06	(7)	0,09	(4)	0,51
TIR	(5)	0,02	(5)	0,02	(0)	0	(5)	0,2
TM *	(0)	0	(2)	0,05	(18)	0,37	(11)	0,84
Dentary *	(8)	0,17	(9)	0,12	(16)	0,41	(13)	1,0

* most informative morphological traits.

Table 4 Morphology of the maxillary teeth of the sympatric species and the toothed morph from Sossego Dam.

Species	Number of cusps			
	conical	tricuspid	pentacuspid	hexacuspid
A. bimaculatus	No	No	No	No
A. giton	No	No	**Yes**	**Yes**
O. argenteus	**Yes**	**Yes**	No	No
Toothed morph	No	**Yes**	**Yes**	No

Table 5 Chromosomal formulae of sympatric species of Characidae and the toothed morphs in the Sossego Dam.

	Number of chromosomes (2n)				
Species	**metacentric (m)**	**submetacentric (sm)**	**subtelocentric (st)**	**telocentric (t)**	**Total**
A. bimaculatus	6	20	18	6	50
A. giton	6	8	24	12	50
O. argenteus	6	16	12	16	50
Toothed morph	variable	variable	variable	variable	48-51

Table 6 Karyotype configurations found in the four toothed morph specimens.

Karyotype (2n)	Toothed morph specimens				Total
	CT 1945	CT 2138	CT 2213	CT 2214	
Others	34	18	35	21	108
48 Chromosomes	17	8	17	8	50
49 Chromosomes	17	28	30	12	87
50 Chromosomes	52	87	167	29	335
Total	120	141	249	70	580
Others + 1 small	0	2	0	0	2
48 Chromosomes + 1 small	0	8	5	1	8
49 Chromosomes + 1 small	3	10	30	10	53
50 Chromosomes + 1 small	2	4	2	82	90
Total	5	24	37	93	153
Final Total	125	159	286	163	733

Table 7 Cytogenetic techniques results for the three species and the toothed morph from Sossego's dam.

Group	**FN**	Number of chromosomes marked					
		C Banding	NOR Banding	C-Banding/ DAPI	CMA_3	18S FISH	5S FISH
Astyanax bimaculatus	94	2	2 (non-homologous)	20	2	10	4
Astyanax giton	88	8	2	28	2	10	2
Oligosarcus argenteus	84	2 (non-homologous)	2	3	6	8	2
Toothed morph	Variable	2	2	2	8	10	2

Appendix

Figures

APPENDIX A. Overview of the Sossego Dam at Latão Stream

APPENDIX B. Formalin fixed toothed morph specimens from Sossego's Dam. **a)** CT 1945; **b)** CT 2064

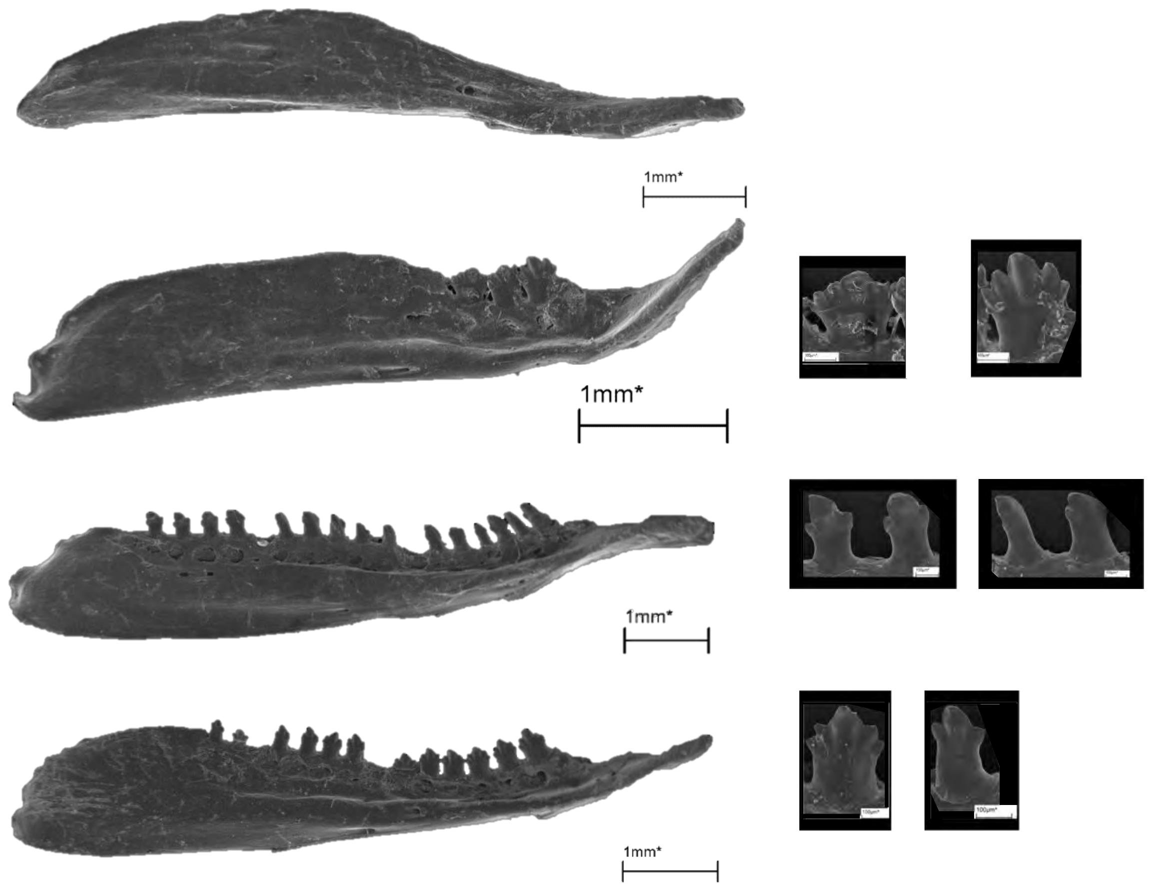
a)

b)

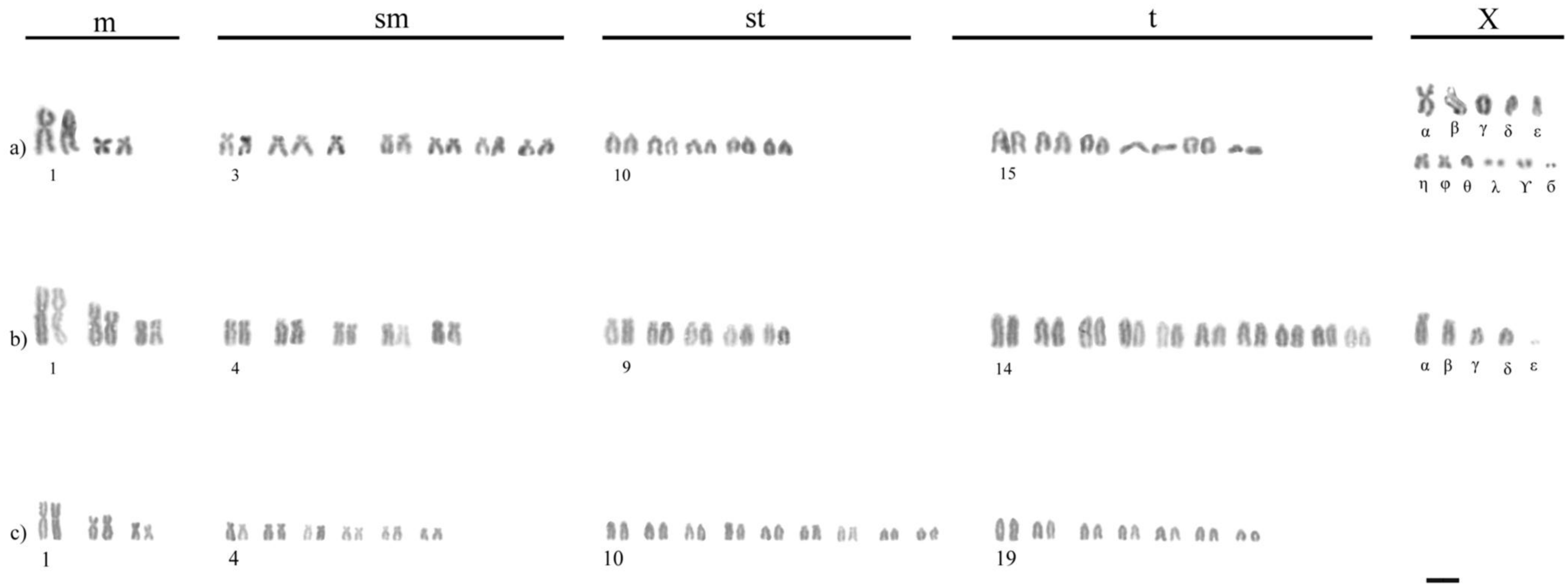
c)

APPENDIX C. Three sympatric Characidae species found at Sossego's dam. **a)** *Astyanax bimaculatus*; **b)** *Astyanax giton*; **c)** *Oligosarcus argenteus*.

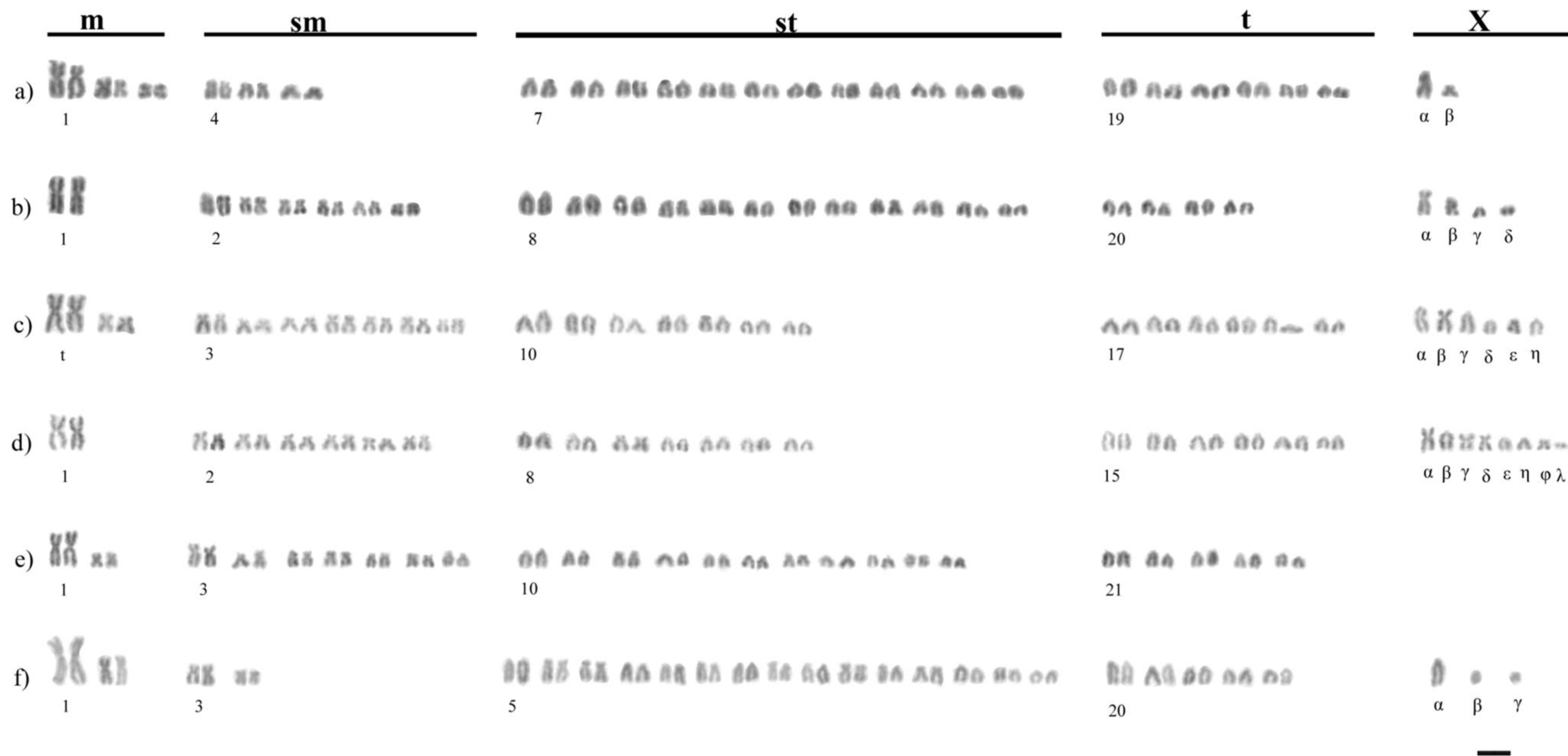
APPENDIX D. Comparative picture of *Astyanax giton* (bottom) and *Oligosarcus argenteus* (middle and top). The *A. giton* species seems more similar to young individuals of *O. argenteus* in spite of the larger specimens suggesting heterochronic variations.



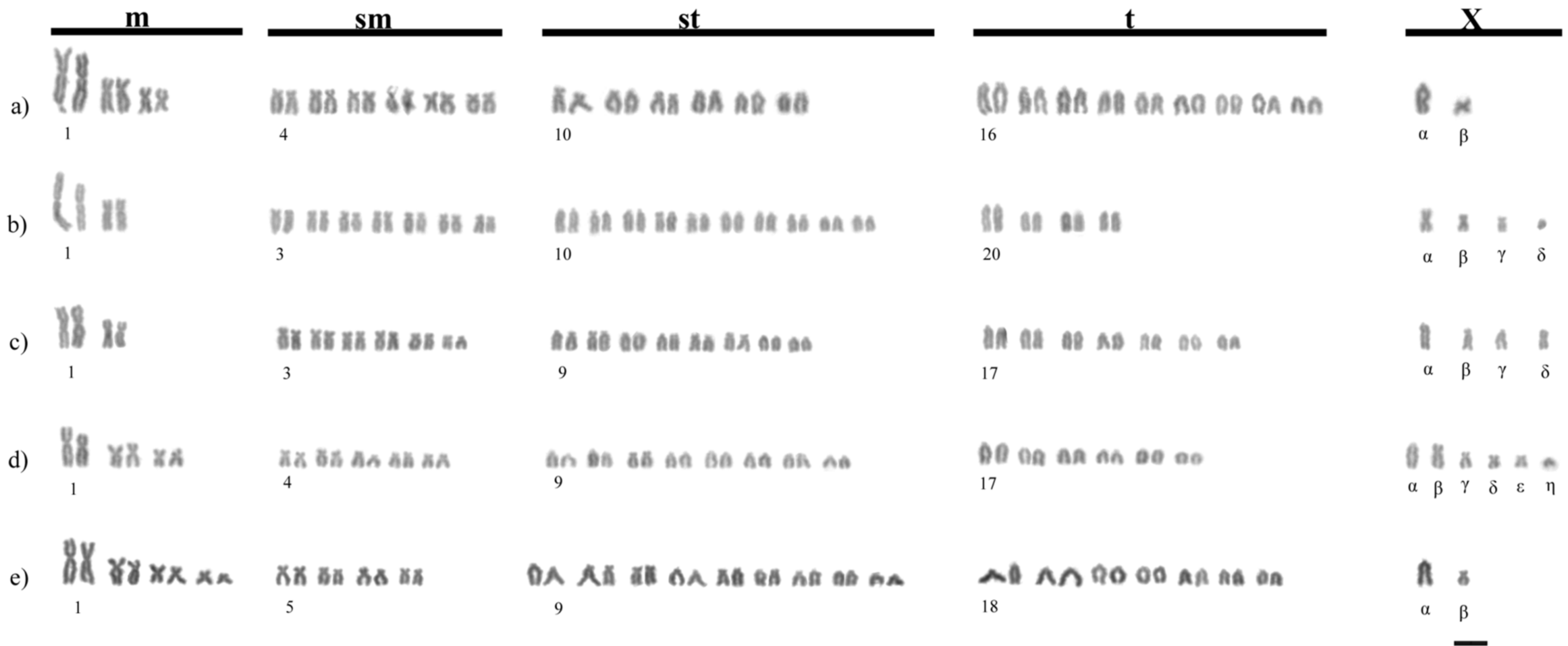
APPENDIX E. Scanning Electronic Microscopy image of the left maxillary bone of: a) *Astyanax bimaculatus*; b) *Astyanax giton*; c) *Oligosarcus argenteus*; and d) Toothed morph. Cusp details of the teeth inside the boxes



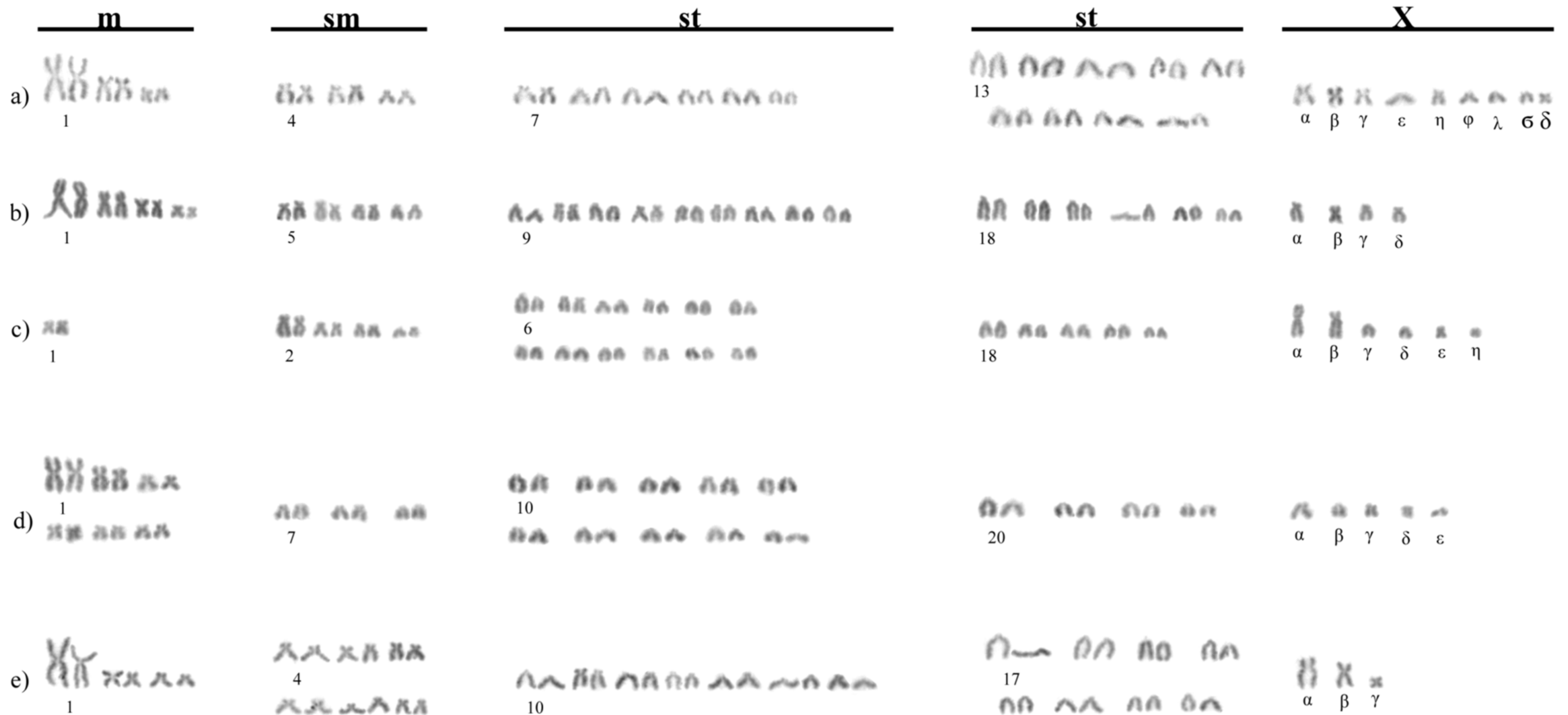
APPENDIX F. Karyotypes from the toothed morph specimens. Board showing the three karyotypes found for the specimen CT 1945. **a)** Karyotype with 3 pairs of dot like chromosomes; **b)** Karyotype with one pair of dot like chromosome; **c)** Karyotype with a large telocentric pair of chromosomes. The “X” group contains chromosomes that do not pair with other chromosomes. Bar = 5µm.



APPENDIX F (cont.). Karyotypes from the toothed morph specimens. Board showing the six karyotypes found for the specimen CT 2138. **a)** Karyotype with a tiny metacentric; **b)** Karyotype with a pair of dot like chromosomes; **c)** Karyotype with large and not able to pair submetacentric chromosomes; **d)** Karyotype 2n = 48 with a pair of dot like chromosomes; **e)** Karyotype apparently normal; **f)** Karyotype with a large and a tiny telocentric and a tiny metacentric without any pair. The "X" group contains chromosomes that do not pair with other chromosomes. Bar = 5µm

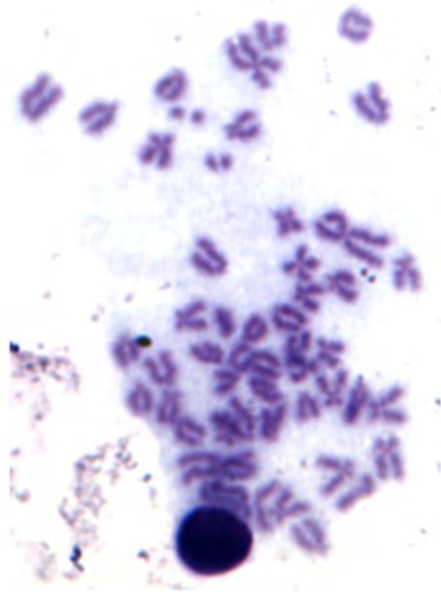


APPENDIX F (cont.). Karyotypes from the toothed morph specimens. Board showing the five karyotypes found for the specimen CT 2213. **a)** Karyotype with a tiny metacentric; **b)** Karyotype with a tiny telocentric without any pair; **c)** Karyotype with large telocentric chromosomes; **d)** Karyotype with a large submetacentric and a tiny telocentric, both without any pair; **e)** Karyotype with a large telocentric without any pair; The "X" group contains chromosomes that do not pair with another chromosomes. Bar = 5μm.

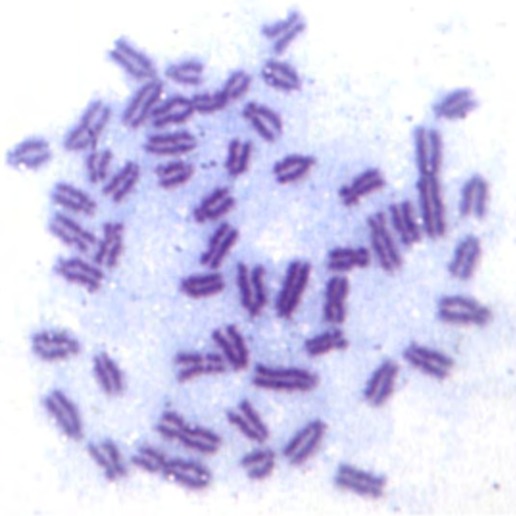


APPENDIX F (cont.). Karyotypes from the toothed morph specimens. Board showing the five karyotypes found for the specimen CT 2214. **a)** Karyotype with a tiny metacentric and telocentric without any pair; **b)** Karyotype with a small submetacentric without any pair; **c)** Karyotype with a tiny telocentric without any pair; **d)** Karyotype with small metacentric and telocentric, both without any pair; **e)** Karyotype with a small telocentric without any pair. The "X" group contains chromosomes that do not pair with other chromosomes. Bar = 5µm.

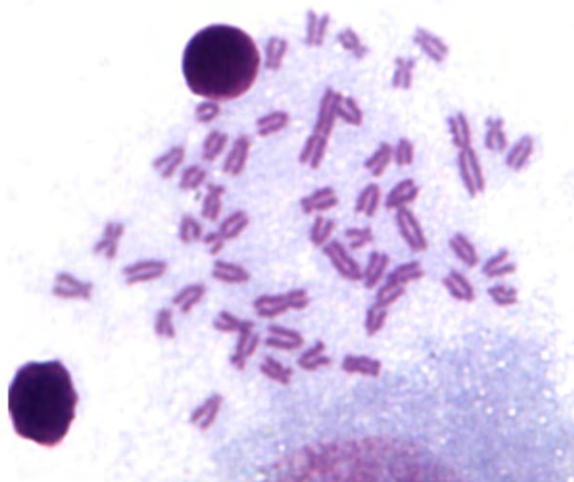
a)



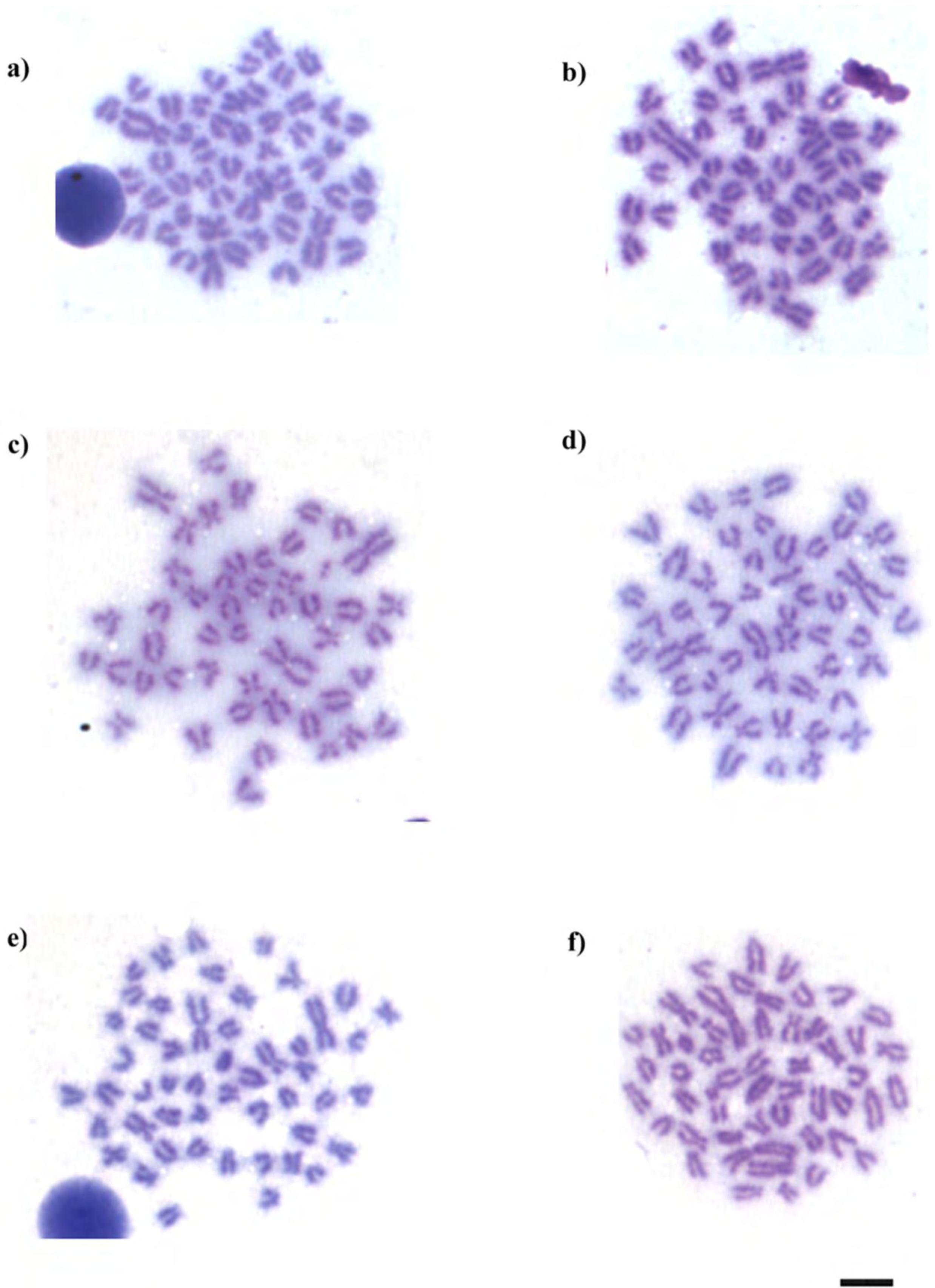
b)



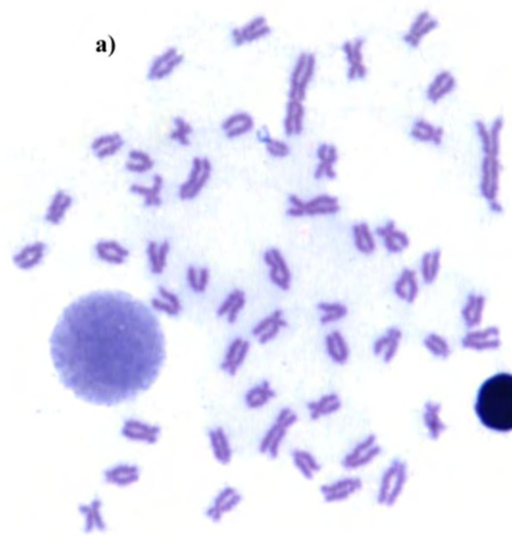
c)

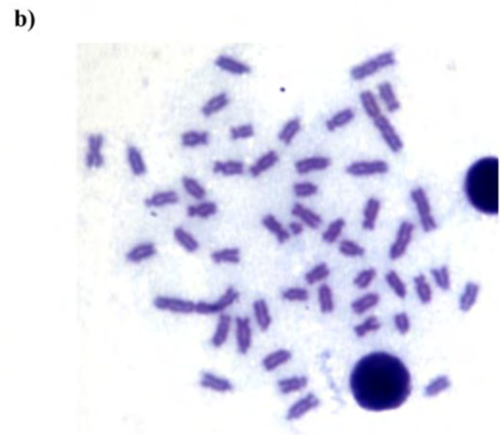


APPENDIX G. Metaphasis from the toothed morph specimens. The three metaphasis used to construct the karyotype board of the specimen CT 1945 shown in **APPENDIX F.** Bar = 5μm.



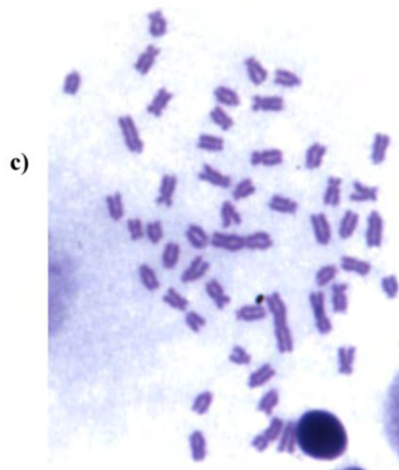
APPENDIX G (cont.). Metaphasis from the toothed morph specimens. The six metaphasis used to construct the karyotype board of the specimen CT 2138 shown in **APPENDIX F**. Bar = 5µm.



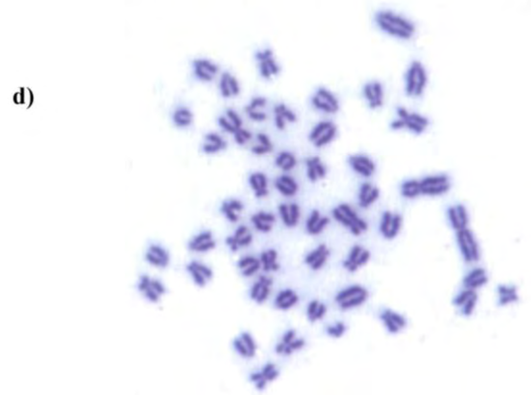


b)

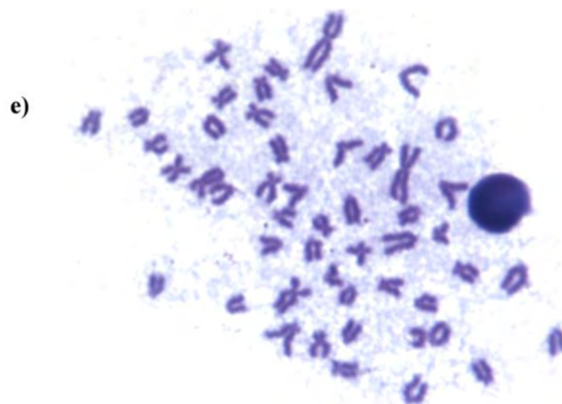
c)



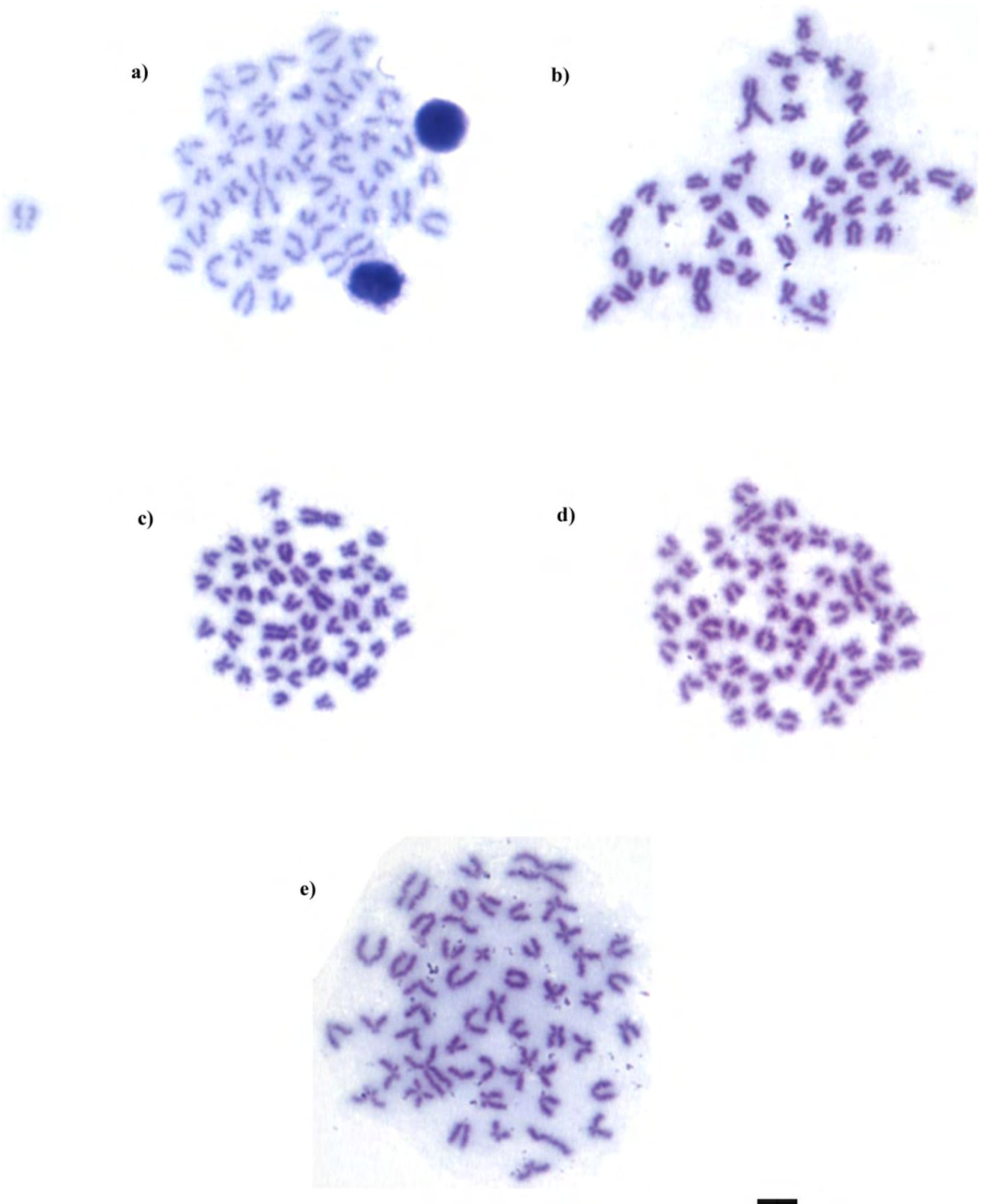
d)



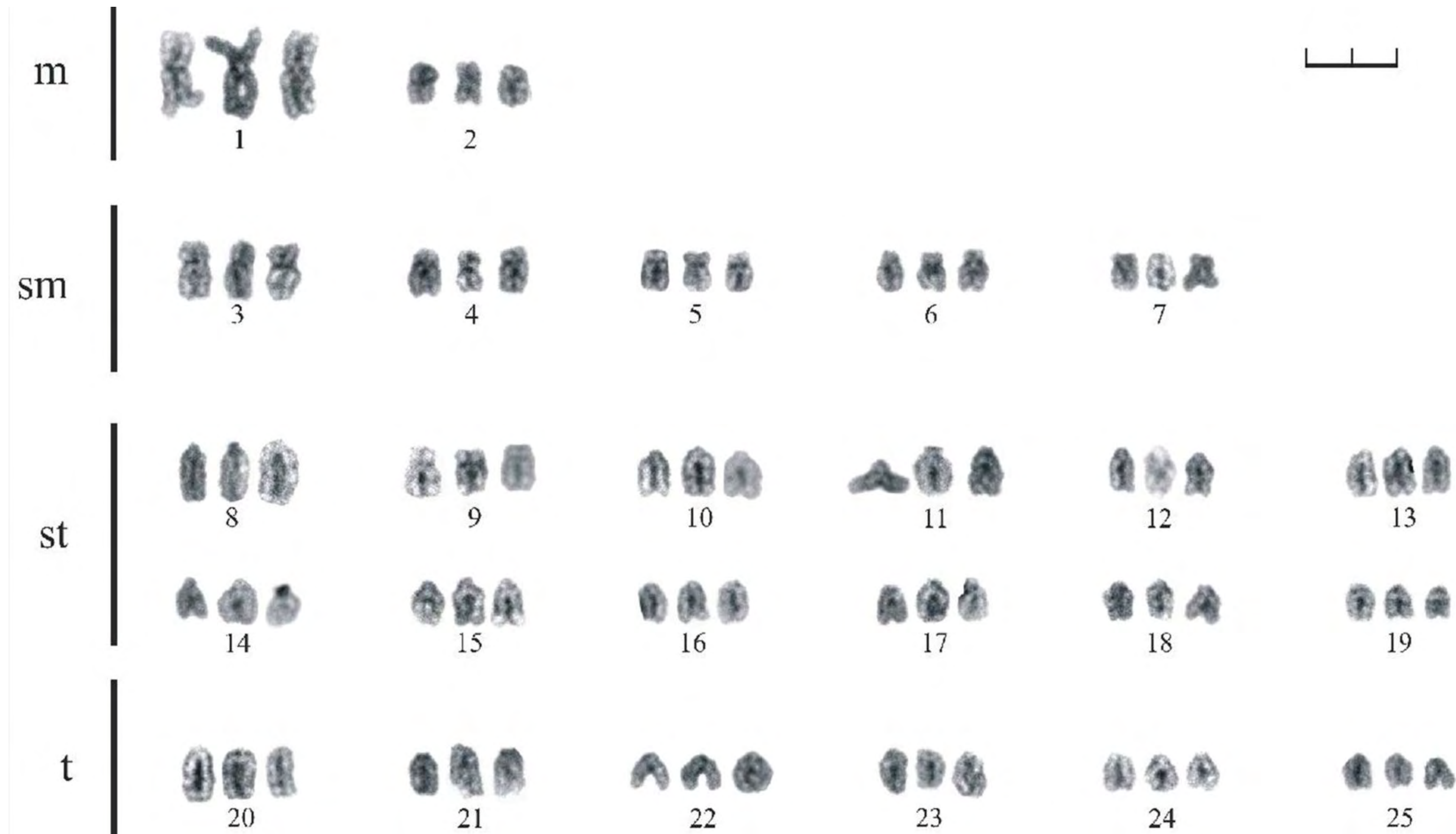
e)



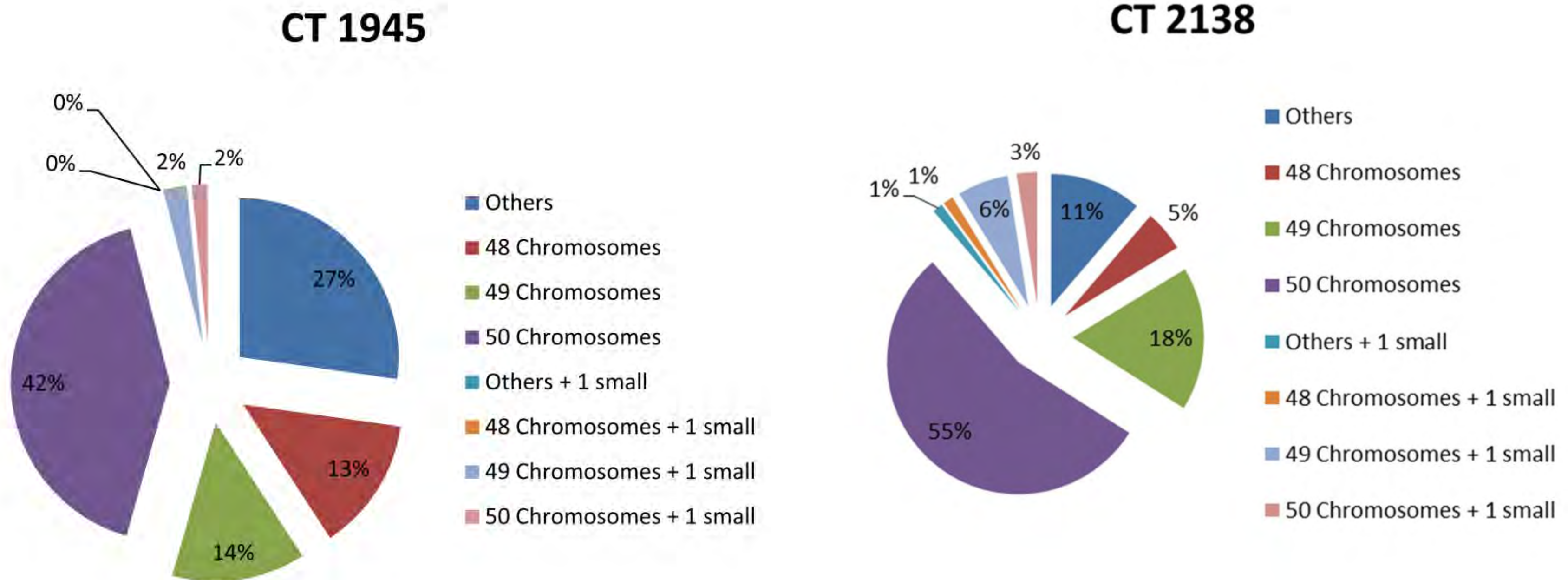
APPENDIX G (cont.). Metaphasis from the toothed morph specimens. The five metaphasis used to construct the karyotype board of the specimen CT 2213 shown in **APPENDIX F**. Bar = 5μm.



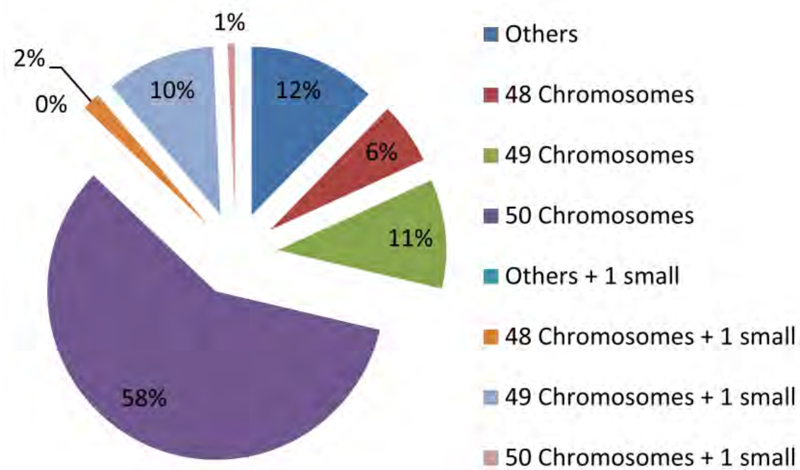
APPENDIX G (cont.). Metaphasis from the toothed morph specimens. The five metaphasis used to construct the karyotype of the specimen CT 2214 shown in **APPENDIX F**. Bar = 5µm.

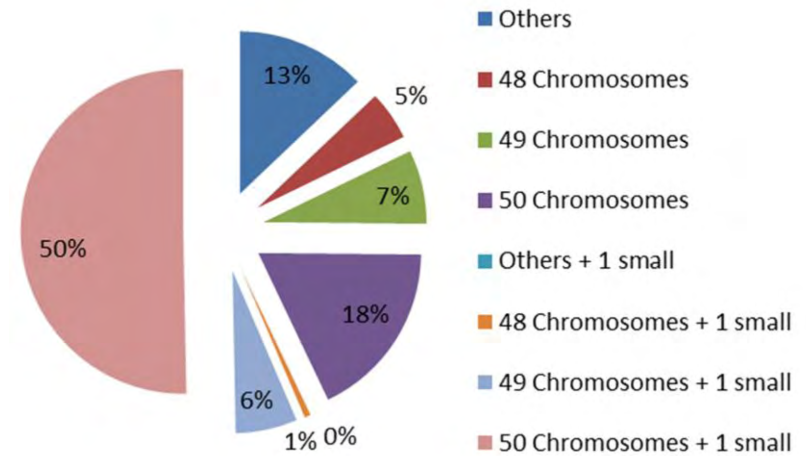


APPENDIX H. Karyotype from a triploid specimen identified as *Astyanax giton,* suggesting meiotic disorders. Bar = 5µm.

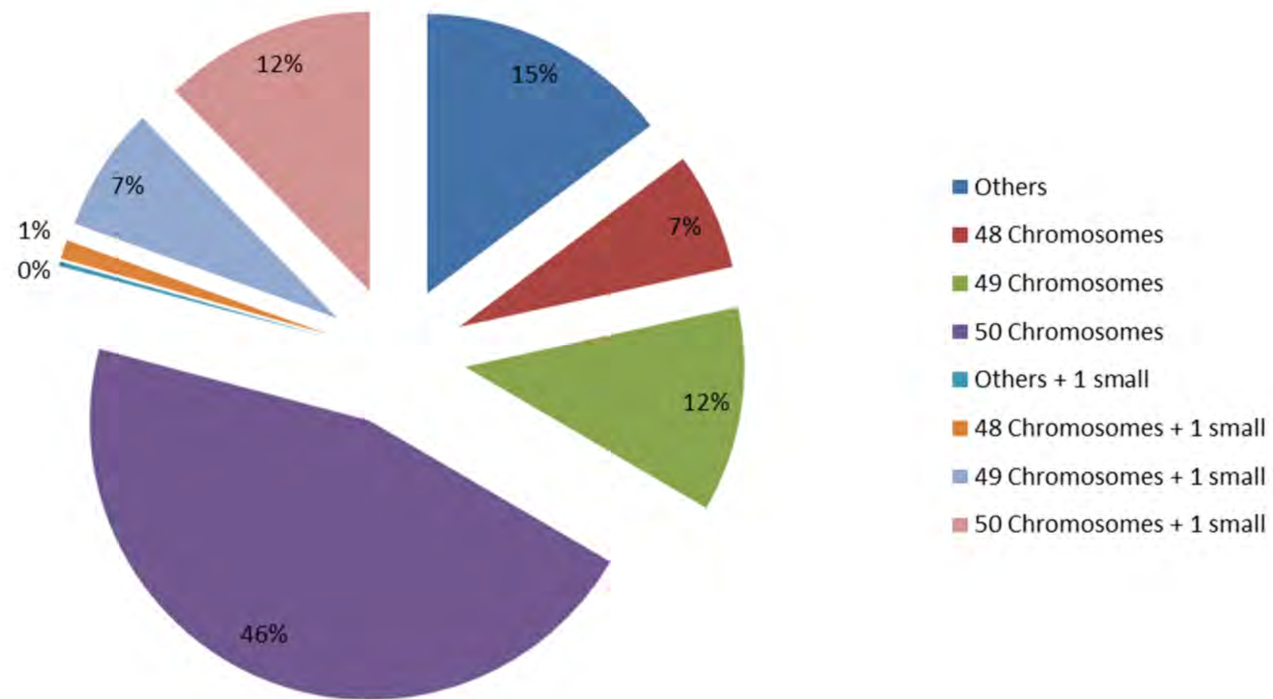


APPENDIX I. The proportions of all karyotypes found in toothed morphs. Specimens CT 1945 (left) and CT 2138 (right).

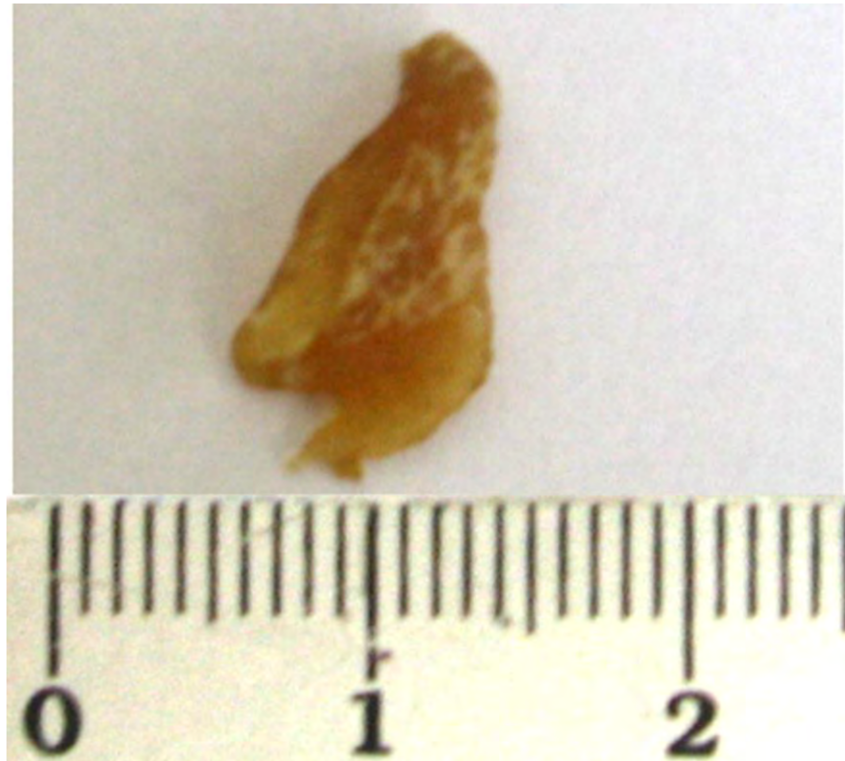




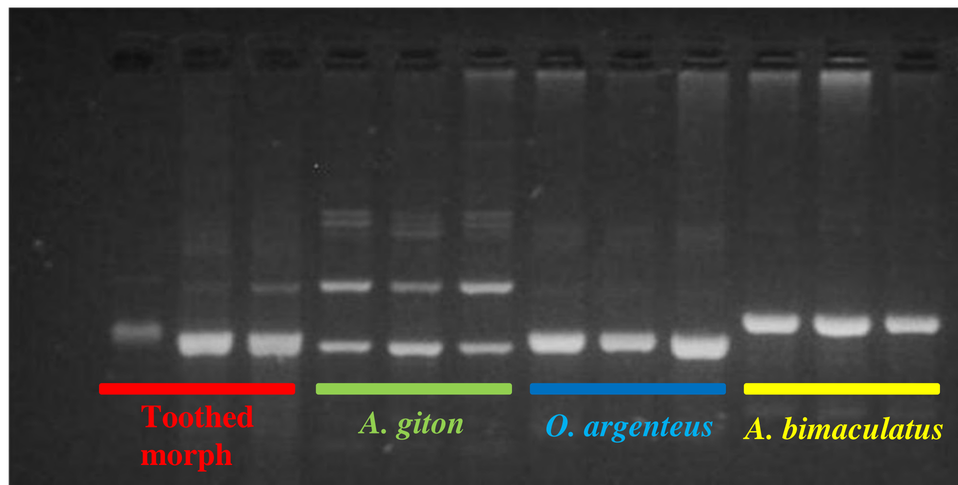
APPENDIX I (cont.). The proportions of all karyotypes found in toothed morphs. Specimens CT 2213 (left) and CT 2214 (right).



APPENDIX I (cont.). The proportions of all karyotypes found in toothed morphs. All four toothed morphs cytogenetically studied put together



APPENDIX J. A solid tumour extracted from the viscerae of an *A. giton* specimen, suggesting serious mitotic disorders. It occupies 200mm^3 of volume and 0,19g of dry weight.



APPENDIX K. 3% agarose gel of DNA fragments from the four groups of Sossego's dam amplified by a universal ITS-1 primer. More than one single fragment can be seen for the toothed morph and *A. giton* studied specimens. It may suggest strongest relationship between the Toothed morph and *A. giton* in despite of *O. argenteus*. However these extra bands may also represent paralog bands

Tables

APPENDIX L. Comparison of morphometrical and meristical data of *A. bimaculatus*, *A. giton, O. argenteus* and the toothed morphs. Maximum and minimum of each analyzed character and its standard deviation.

	Astyanax bimaculatus		*Astyanax giton*		*Oligosarcus argenteus*		Toothed morph	
Character	Max-Min	Std. deviation	Max-Min	Std. deviation	Max-Min	Std. deviation	Max-Min	Std. deviation
Head	5,38 – 3,75	0,26	5,16 – 3,56	0,26	4,0 – 3,22	0,15	4,07 – 3,72	0,15
Depth	2,89 – 2,28	0,12	4,65 – 2,48	0,24	3,88 – 3,06	0,2	3,42 – 3,05	0,15
D.	11 – 9	0,31	11 – 9	0,37	12 – 10	0,25	12 – 10	0,71
A.	31 – 24	1,23	31 – 19	1,5	38 – 22	2,12	28 – 24	1,64
D-LL	8 – 6	0,55	6 – 3	0,56	9 – 6	0,7	7 – 6	0,55
LL *	39 – 35	0,85	39 – 24	2,63	49 – 30	3,56	39 – 32	2,77
P-LL	7 – 4	0,64	6 – 3	0,59	8 – 5	0,72	6 – 4	0,84
Eye	3,46 – 2,23	0,22	3,6 – 1,42	0,21	4,93 – 1,82	0,49	3,02 – 2,1	0,35
IO	1,94 – 1,43	0,11	2,6 – 1,68	0,21	3,16 – 1,85	0,33	2,34 – 2,04	0,12
Snout	6,01 – 3,31	0,57	7,25 – 2,32	0,64	5,25 – 2,33	0,52	4,74 – 3,79	0,41
FMM	4,45 – 1,67	0,46	4,46 – 1,38	0,45	6,35 – 1,23	0,68	2,48 – 2,11	0,14
PMMB	1,28 – 0,47	0,11	0,64 – 0,35	0,05	1,91 – 0,27	0,28	0,65 – 0,58	0,03
TFR	5 – 4	0,14	7 – 2	0,65	8 – 6	0,64	5 – 2	1,14
TIR	5 – 4	0,14	6 – 4	0,17	0 – 0	0	5 – 4	0,45
TM*	0 – 0	0	3 – 0	0,53	26 – 15	2,55	13 – 8	1,87
Dentary *	11 – 5	1,25	11 – 6	1,06	24 – 12	2,81	16 – 10	2,30

* most informative morphological traits.