UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS − RIO CLARO

ESTUDOS EVOLUTIVOS NO GÊNERO *Astyanax* (CHARACIFORMES, CHARACIDAE): ANÁLISES COMPARATIVAS BASEADAS NOS DIFERENTES NÚMEROS DIPLOIDES DESCRITOS PARA O GÊNERO

DIOVANI PISCOR

RIO CLARO − SP
Maio/2016

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Orientadora: Profª. Drª. Patricia Pasquali Parise-Maltempi

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RESUMO

O gênero *Astyanax* atualmente é visto como um grupo com incertezas filogenéticas, configurando-se até o presente momento como polifilético dentro da família Characidae. Atualmente, são descritas aproximadamente 150 espécies no gênero, e este se distribui sobre a região Neotropical com ocorrências desde o sul dos Estados Unidos até a região da Patagônica na Argentina. Dentro da região de ocorrência o gênero apresenta espécies com 2n = 36 cromossomos apenas para *Astyanax schubarti* e *Astyanax correntinus*, 2n = 46 para, por exemplo, *Astyanax fasciatus*, 2n = 48 para *Astyanax marionae*, 2n = 50 para *Astyanax altiparanae*, e 2n = 52 para *Astyanax* sp. Visto que o grupo apresenta variações significantes no número diploide, este trabalho teve como principal objetivo estudar o mapeamento cromossômico de DNAs repetitivos numa abordagem evolutiva. Nove espécies de *Astyanax* foram analisadas: *A. altiparanae* e *A. schubarti* provenientes da bacia do rio Piracicaba (SP), *A. abramis*, *A. asuncionensis* e *A. marionae* provenientes da bacia do rio Paraguai (MT), *A. bockmanni* proveniente da bacia do rio Iguatemi (MS), *A. eigenmanniorum* e *A. mexicanus* provenientes de loja de aquário, e diferentes populações de *A. fasciatus* provenientes da bacia do rio Corumbataí (SP). Para tanto, foram mapeados os genes de RNAr (18S e 5S), histona H3, RNAsn U2, microssatélites e DNA telomérico. Ainda, foram realizados estudos envolvendo técnicas clássicas de citogenética (Ag-NOR, banda-C e coloração com CMA_3/DAPI) e estudos do relógio molecular utilizando o gene mitocondrial citocromo *c* oxidase subunidade I (COI). São discutidos, ainda, parâmetros da evolução cromossômica e hipóteses sobre eventos particulares (cromossômicos e evolutivos) no grupo *Astyanax*.

Palavras-chave: DNA repetitivo, evolução cariotípica, FISH, relógio molecular, citocromo *c* oxidase I.

ABSTRACT

The genus *Astyanax* is currently seen as a group with phylogenetic confusion, setting up to date as polyphyletic within the family Characidae. Currently, there are about 150 described species in the genus, and this is distributed on the Neotropical region from the southern United States to the region of Patagonia in Argentina. Within the region of occurrence of the genus shows species with 2n = 36 chromosomes only *Astyanax schubarti* and *Astyanax correntinus*, 2n = 46, for example, to *Astyanax fasciatus*, 2n = 48 to *Astyanax marionae*, 2n = 50 to *Astyanax altiparanae*, and 2n = 52 to *Astyanax* sp. Since the group presents significant variations in the diploid number, this work focused primarily on the study of chromosomal mapping of repetitive DNAs with an evolutionary approach. Nine species of *Astyanax* were examined: *A. altiparanae* and *A*. *schubarti* from the Piracicaba River basin (SP), *A. abramis*, *A. asuncionensis* and *A. marionae* from the Paraguay River basin (MT), *A. bockmanni* from the Iguatemi River basin (MS), *A. eigenmanniorum* and *A. mexicanus* from the aquarium store, and *A. fasciatus* from the Corumbataí River basin (SP). Therefore, the genes mapped were rRNA (18S and 5S), histone H3, U2 snRNA, microsatellites and telomeric DNA. Further, studies were conducted involving classical cytogenetic techniques (Ag-NOR, C-band and staining with CMA_3/DAPI) and the molecular clock using the mitochondrial gene cytochrome *c* oxidase subunit I (COI). Are also discussed, parameters of chromosomal evolution and hypothesis about particular events (chromosomal and evolutionary) in the group *Astyanax*.

Keywords: repetitive DNA, karyotype evolution, FISH, molecular clock, cytochrome *c* oxidase I.

INTRODUÇÃO

1. INTRODUÇÃO

1.1. Considerações filogenéticas e taxonômicas do grupo *incertae sedis* em Characidae

Dentro da ordem Characiformes a família Characidae é o grupo com maior número de espécies. De acordo com Eschmeyer e Fong (2016) são 12 subfamílias e mais três clados (*Astyanax*, *Jupiaba* e *Nematobrycon*) e 1350 espécies, sendo destas 1103 consideradas válidas. Seus representantes estão presentes em diversos ambientes de água doce e distribuem-se no continente Americano desde o Texas e o Novo México nos Estados Unidos até o norte da Patagônia na Argentina (PAGE, BURR, 1991; ALMIRÓN et al., 1997).

A família Characidae apresenta peixes de pequeno e médio portes, como por exemplo exemplares do gênero *Astyanax*. Ao longo dos anos, muitos integrantes dessa família sofreram modificações na sua classificação. Sabe-se que as relações filogenéticas entre vários membros do grupo são incertas, no aspecto de representarem um grupo polifilético, o que é muito discutido até o presente momento.

A subfamília Tetragonopterinae, antes considerada como a subfamília com o maior número de espécies em Characidae (GÉRY, 1977), foi considerada como um grupo polifilético por Lima et al. (2003). A partir de então, somente o gênero *Tetragonopterus* foi mantido na subfamília e todos os demais gêneros de Tetragonopterinae foram alocados em *incertae sedis* dentro de Characidae (LIMA et al., 2003).

Entre os gêneros alocados em *incertae sedis* estão alguns dos grupos com maior número de espécies e taxonomicamente confusos de Characidae, como *Hyphessobrycon* (aproximadamente 170 espécies), *Astyanax* (aproximadamente 150 espécies), *Moenkhausia* (aproximadamente 90 espécies), *Bryconamericus* (aproximadamente 75 espécies), *Hemigrammus* (aproximadamente 70 espécies), entre outros (ESCHMEYER et al., 2016). Por outro lado, estudos recentes propõem hipóteses que levam à incerteza da manutenção de alguns gêneros em *incertae sedis*, como é o caso do gênero *Bryconamericus*, que foi alocado

em *incertae sedis* por Lima et al. (2003), mas que em estudos envolvendo dados moleculares, foi considerado polifilético, porém pertencente à subfamília Stevardiinae em Characidae (OLIVEIRA et al., 2011; THOMAZ et al., 2015).

Por outro lado, estudos com análises de sequências de DNA reforçam a ideia de que *Astyanax* não é um grupo natural dentro de Characidae (OLIVEIRA et al., 2011). Outros estudos, com base em análise de caracteres morfológicos, também têm apontado para o fato de que *Astyanax* seja um grupo polifilético em Characidae (MIRANDE, 2009; 2010; JAVONILLO et al., 2010).

1.2. História evolutiva e biogeográfica do gênero *Astyanax*

O gênero *Astyanax* é um bom modelo para investigar a importância relativa de padrões biogeográficos de vicariância e dispersão (ORNELAS-GARCÍA et al., 2008). Isto pode ser facilmente entendido, pois o gênero é amplamente distribuído na região Neotropical (MARINHO, LIMA, 2009), apresentando alta plasticidade fenotípica e capacidade de adaptação a diversos habitats (LOZANO-VILANO, CONTRERAS-BALDERAS, 1990; JEFFERY, 2001; DOWLING et al., 2002; STRECKER et al., 2003 *apud* ORNELAS-GARCÍA et al., 2008).

A região Neotropical, que compreende desde o México até o sul da América do Sul, é uma região com grande potencial e constantes avanços em estudos envolvendo a biogeografia histórica, visto que esta se configura como um território com grande biodiversidade (GOLDANI, 2012).

Dentro da região Neotropical, a Mesoamérica é uma das mais complexas áreas biogeográficas no mundo (CONTRERAS-BALDERAS, LOZANO-VILANO, 1996; MORRONE, 2002; ZALDIVAR-RIVERON et al., 2004; DOMÍNGUEZ-DOMÍNGUEZ et al., 2006; HUIDOBRO et al., 2006 *apud* ORNELAS-GARCÍA et al., 2008). Colonizações mais recentes da Mesoamérica por peixes primários de água doce (peixes que não toleram a salinidade da

água marinha) através do Estreito do Panamá foram evidenciadas por estudos filogeográficos de caracídeos (por exemplo, *Brycon*, *Bryconamericus*, *Eretmobrycon* e *Cyphocharax*). Esses estudos indicam várias ondas de expansão rápida da América do Sul durante o Plioceno (aproximadamente 3,3 milhões de anos atrás – Ma) (REEVES, BERMINGHAM, 2006 *apud* ORNELAS-GARCÍA et al., 2008).

O encerramento do Estreito do Panamá, no Plioceno (~3,3 Ma), têm sido postulado como uma das causas mais importantes do intercâmbio da fauna entre as regiões Neártica e Neotropical (BUSSING, 1985 *apud* ORNELAS-GARCÍA et al., 2008). As mudanças climáticas também são colocadas em pauta para a explicação da distribuição da fauna de peixes da Mesoamérica (REEVES, BERMINGHAM, 2006 *apud* ORNELAS-GARCÍA et al., 2008). Segundo Reeves e Bermingham (2006) e Strecker et al. (2004) *apud* Ornelas-García et al. (2008), o fechamento do Estreito do Panamá, aproximadamente 3,3 Ma, forneceu a primeira oportunidade para colonização da América Central por peixes da América do Sul.

De acordo com Strecker et al. (2004) a ictiofauna primária de água doce da América Central é pobre, provavelmente, por causa da origem geológica tardia da maioria das partes desta região geográfica e sua longa separação da América do Sul. Segundo os autores, os únicos grupos de peixes neotropicais a chegarem à América do Norte são representados por espécies do gênero *Rhamdia* e espécies do "complexo *Astyanax fasciatus*".

Um padrão filogeográfico de estruturação norte-sul da região da Mesoamérica foi proposto por Ornelas-García et al. (2008). Segundo os autores, os grupos filogenéticos de *Astyanax* (Mesoamérica), geralmente, não são sobrepostos, com exceção dos grupos I (México e América Central superior) e II (centro da América Central), que se sobrepõem na parte superior da bacia do Polochic da Guatemala, e grupos II e III (baixa América Central – inclui populações de *A. fasciatus* das bacias Ciruelas e Chires sobre a inclinação do Pacífico da Costa Rica), que se sobrepõem na bacia do Ciruelas da Costa Rica (ver mapa da Figura 1). Entretanto, Ornelas-García et al. (2008) concordam com a hipótese amplamente aceita de uma origem sul-americana para as espécies de *Astyanax* e outros caracídeos da América Central (GAYET et al., 2001; CALCAGNOTTO et al., 2005; REEVES, BERMINGHAM, 2006).

Poucos estudos neste sentido são explorados para o gênero *Astyanax*, porém sabe-se que eventos geológicos e climáticos contribuíram muito para a colonização de espécies deste gênero da América do Sul para a América Central. Em suma, é aceitável que, como dito anteriormente por alguns autores, o gênero *Astyanax*, assim como outros membros de Characidae, tiveram sua origem na América do Sul, porém não é muito discutido na literatura a biogeografia e a história evolutiva de *Astyanax* na América do Sul.

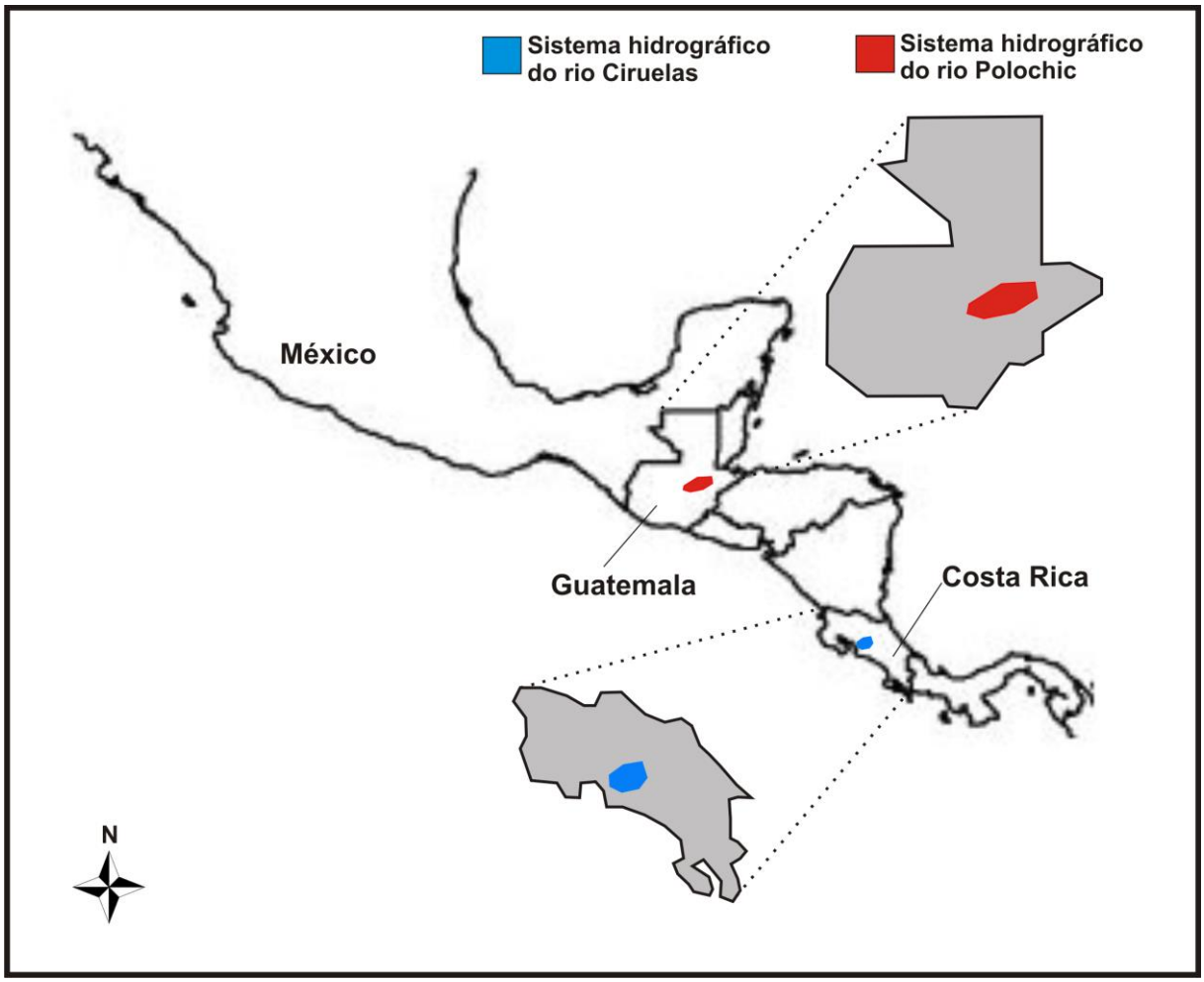

Figura 1. Mapa das localizações hidrográficas Mesoamericanas das sobreposições dos grupos filogenéticos de *Astyanax* (Grupos I, II e III) propostos por Ornelas-García et al. (2008).

1.3. O primeiro par metacêntrico como marcador cromossômico

Uma característica marcante compartilhada por muitas espécies de Characidae é o primeiro par cromossômico metacêntrico maior do que os demais cromossomos do complemento A (SCHEEL, 1973). A partir desse trabalho, a constatação foi corroborada por diferentes autores, tais como Salvador e Moreira-Filho (1992), Maistro et al. (1992), Margarido e Galetti (1996), Vicente et al. (1996), sendo este conhecimento, hoje, amplamente difundido e observado em Characidae.

Dentro de *incertae sedis* o primeiro par grande é compartilhado também entre a maioria das espécies, com algumas exceções. Por exemplo, em *Hyphessobrycon* as espécies com 2n = 50 cromossomos possuem o primeiro par metacêntrico grande, como observado para as espécies *Hyphessobrycon anisitsi*, *Hyphessobrycon leutkenii* e *Hyphessobrycon reticulatus* (MENDES et al., 2011; CARVALHO et al., 2002). Porém, a espécie *Hyphessobrycon eques* com 2n = 52 cromossomos, por mais que apresente o par metacêntrico maior que os demais cromossomos, este par não é tão notável como o par marcador de *H. anisitsi*, *H. leutkenii* e *H. reticulatus*, pelo menos considerando o tamanho do primeiro par em relação aos demais cromossomos.

Nos gêneros *Moenkausia* e *Hemigrammus* (também *incertae sedis*) a característica marcante do primeiro par metacêntrico relativamente maior que os demais cromossomos também é aplicada aos cromossomos das poucas espécies analisadas citogeneticamente descritas na literatura, tais como *Moenkhausia sanctaefilomenae* (PORTELA-CASTRO et al., 2001), *Moenkhausia oligolepis* (SANTOS, 2010), e *Hemigrammus marginatus* (GUIDINI, 2007).

Outro gênero de *incertae sedis* que apresenta algumas peculiaridades no que diz respeito ao par marcador metacêntrico de Characidae, é *Astyanax*. Neste gênero o número diploide varia de 2n = 36 a 2n = 52 cromossomos, sendo o mais frequente o número diploide 2n = 50 cromossomos. Nas espécies deste gênero o par marcador de Characidae é compartilhado por todas as espécies analisadas do ponto de vista citogenético, independente

da variação numérica cromossômica, ressalvando que em *Astyanax schubarti* e *Astyanax correntinus* (únicas espécies de *Astyanax* com número diploide 2n = 36), o par marcador de Characidae é o segundo par metacêntrico grande do cariótipo, considerando que *A. schubarti* possui um primeiro par metacêntrico ainda maior do que o primeiro par marcador de Characidae (DANIEL-SILVA; ALMEIDA-TOLEDO, 2001) e *A. correntinus* possui um par submetacêntrico maior do que todos os demais cromossomos (PAIZ et al., 2015).

Os cromossomos de *A. schubarti* e *A. altiparanae* foram analisados por Daniel-Silva e Almeida-Toledo (2005) após a incorporação do análogo de base 5-Bromodeoxiuridina (5-BrdU) que constataram que alguns cromossomos apresentavam homeologias. Um exemplo é o cromossomo 1 de *A. schubarti* que corresponde aos cromossomos 3 e 14 de *A. altiparanae* (o cromossomo 14 de *A. altiparanae* corresponde ao braço curto do cromossomo 1 de *A. schubarti*, e o cromossomo 3 de *A. altiparanae* corresponde ao braço longo do cromossomo 1 de *A. schubarti*). Outro exemplo é o cromossomo 2 de *A. schubarti* que corresponde, inteiramente, ao cromossomo 1 de *A. atiparanae*.

Esta característica cromossômica (primeiro par cromossômico metacêntico grande) tão pouco explorada em *Astyanax* desde os primeiros estudos citogenéticos entre as décadas de 1970 e 1980, reforça a necessidade de estudos mais aprofundados sobre a evolução dos cromossomos no grupo.

1.4. Considerações sobre as variações do número diploide e alguns exemplos de estudos cromossômicos revelados por técnicas clássicas no gênero *Astyanax*

Estudos citogenéticos sobre o gênero *Astyanax* têm mostrado considerável variação do número diploide, desde 2n = 36 cromossomos, por exemplo, para *A. schubarti* (Morelli et al., 1983), 2n = 46 para *Astyanax fasciatus* (FERREIRA-NETO et al., 2012), 2n = 48 para *Astyanax scabripinnis* (FERNANDES, MARTINS-SANTOS, 2003), 2n = 50 para a maioria das espécies, como para *A. altiparanae* e *Astyanax bockmanni* (FERNANDES, MARTINS-

SANTOS, 2004; KAVALCO et al., 2009), e recentemente foi descrito um cariótipo com 2n = 52 no gênero para *Astyanax* sp. (TENÓRIO et al., 2013).

Além da variação do número diploide, várias fórmulas cariotípicas são descritas para as diferentes espécies e populações de uma mesma espécie do gênero. Como exemplo tem-se o estudo de Medrado et al. (2008), no qual três populações de *A*. *fasciatus* pertencentes a diferentes bacias hidrográficas no estado da Bahia apresentaram número diploide de 2n = 48 cromossomos e três fórmulas cariotípicas diferentes, sugerindo a ocorrência de um complexo de espécies.

Complexos de espécies são comuns em *Astyanax*, por exemplo o "complexo *Astyanax scabripinnis*" sugerido por Moreira-Filho e Bertollo (1991) no qual o número diploide varia em 2n=46, 2n=48 e 2n=50 cromossomos, e o "complexo *Astyanax fasciatus*" onde número pode variar desde 2n=45 a 2n=50 cromossomos (CENTOFANTE et al., 2003), podendo estes apresentarem ou não cromossomos supranumerários (NÉO et al., 2000a,b ; FERREIRA-NETO et al., 2012).

A distribuição da macroestrutura da heterocromatina é bem estudada no gênero *Astyanax*. Por exemplo, Fernandes e Martins-Santos (2003) analisaram duas populações de *A. scabripinnis* da bacia do rio Ivaí (estado do Paraná, Brasil) e observaram um padrão similar de distribuição da heterocromatina constitutiva entre as duas populações, porém foi notada uma variação inter e intraindividual de blocos heterocromáticos. Segundo os autores, blocos grandes e fortemente corados foram observados nas regiões teloméricas em cromossomos subtelocêntricos e acrocêntricos. Mantovani et al. (2000) também relataram um similar polimorfismo interindividual em populações de *A. scabripinnis* provenientes dos córregos Centenário e Marrecas (bacia do rio Paranapanema). Outras populações também apresentaram variação inter e intraindividual de blocos de heterocromatina assim como diferentes números de cromossomos Ag-positivos (por exemplo, MANTOVANI et al., 2004; FERNANDES, MARTINS-SANTOS, 2006; MEDRADO et al., 2008)

As regiões organizadoras de nucléolo (RONs) são estudadas amplamente no gênero, apresentando variações de números e posições. Medrado et al. (2008), por exemplo,

relataram para três populações de *A. fasciatus*, pertencentes a rios de bacias da região nordeste do Brasil, três padrões de marcações Ag-RONs. Os autores descrevem que a população do rio Contas apresentou marcação na posição terminal do braço longo de um par subtelocêntrico e outro submetacêntrico, além de uma marcação na posição terminal do braço curto de um par metacêntrico. No entanto, as outras duas populações do rio Preto da Costa e córrego Mineiro apresentaram um par subtelocêntrico impregnado pelo íon prata, sendo que o mesmo parece ser o par Ag-RON principal (MEDRADO et al., 2008). Outros autores, relataram variações em número inter e intraindividual de cromossomos Ag-RONs marcados nas posições teloméricas para *A. paranae* e *A. scabripinnis* (VICARI et al., 2008a).

Regiões ricas em bases GC, as quais geralmente estão localizadas em segmentos heterocromáticos e/ou marcam regiões Ag-RONs, também são estudadas para espécies de *Astyanax* através da coloração por cromomicina A_3 (CMA_3). De acordo com Fernandes e Martins-Santos (2004), duas populações de *A. altiparanae* (uma dos rio Paraná e outra do rio dos Índios) apresentaram dois padrões de localização de regiões GC-ricas: a população do rio dos Índios mostraram que a maioria dos cromossomos das Ag-RONs eram ricos em bases GC (dos 10 cromossomos com marcações Ag-RONs, sete cromossomos mostraram marcações GC-ricas), enquanto que a população do rio Paraná mostrou que além dos cromossomos Ag-RONs, outros cromossomos apresentaram regiões ricas em bases GC (o braço curto do par 20, o qual é Ag-RON e heterocromático, e mais três cromossomos que não são Ag-RONs e nem heterocromáticos). Esses são alguns exemplos de variações de localização e posição da macroestrutura da heterocromatina, das RONs e de sítios CMA_3, porém, vários outros trabalhos demonstram que condições cromossômicas polimórficas são comuns para as espécies de *Astyanax*.

1.5. Cromossomos supranumerários e suas características em *Astyanax*

Cromossomos atípicos que não fazem parte do complemento A são denominados cromossomos Bs, supernumerários, supranumerários ou cromossomos extras. A ocorrência

desse tipo de cromossomo entre os indivíduos de uma população pode ser esporádica ou ser comumente encontrada para muitos representantes, podendo mostrar uma alta frequência entre os integrantes da população. É possível encontrar também variações em relação à morfologia, tamanho e número desse tipo de cromossomos (PAULS e BERTOLLO, 1983, 1990; VENERE et al., 1999; CAVALLARO et al., 2000; FERNANDES e MARTINS-SANTOS 2005; ARTONI et al., 2006).

Com relação à morfologia, os cromossomos supranumerários podem diferir entre os indivíduos de uma mesma espécie. Fernandes e Martins-Santos (2005) ao analisarem citogeneticamente exemplares de *A. scabripinnis* do córrego Tatupeba, bacia do rio Ivaí, encontraram três citótipos distintos referentes à presença de cromossomos supranumerários. No primeiro citótipo os indivíduos exibiram um macrocromossomo B do tipo metacêntrico totalmente heterocromático, no segundo citótipo os indivíduos portavam macrocromossomos B dos tipos metacêntrico, subtelocêntrico e acrocêntrico, parcialmente heterocromáticos, e no terceiro citótipo os exemplares apresentaram macrocromossomos B dos tipos metacêntrico e acrocêntrico, também parcialmente heterocromáticos.

Relacionado ao tamanho, os cromossomos supranumerários exibem notável variação no gênero *Astyanax*, apresentando macromossomos B (MAISTRO et al., 1992; VICENTE et al., 1996; MOREIRA-FILHO et al., 2001; FERRO et al., 2003) bem como microcromossomos B (STANGE; ALMEIDA-TOLEDO, 1993; KAVALCO; ALMEIDA-TOLEDO, 2007; HASHIMOTO et al., 2008).

Os cromossomos B também podem variar em número nas espécies do gênero *Astyanax*. Inicialmente, os cromossomos B foram estudados em *A. scabripinnis* a qual apresentou populações com zero, um ou dois cromossomos extras (ROCON-STANGE; ALMEIDA-TOLEDO, 1993; MIZOGUCHI; MARTINS-SANTOS, 1997; NÉO et al., 2000a, b; FERNANDES; MARTINS-SANTOS, 2005).

Apesar do aumento das informações disponíveis sobre a morfologia, herança, estrutura e outros aspectos relacionados aos cromossomos B em peixes, a exata origem e significado funcional desses elementos genômicos ainda permanece desconhecida

(SALVADOR; MOREIRA-FILHO, 1992; PORTO-FORESTI et al., 1997; JESUS et al., 2003; ARTONI et al., 2006), portanto hipóteses têm sido propostas para explicação de tal elemento no genoma. Uma delas foi proposta por Salvador e Moreira-Filho (1992), os quais realizaram o estudo da origem dos cromossomos supranumerários em uma população de *A. scabripinnis*, proveniente do município de Campos do Jordão e, segundo os autores, o elemento B nessa população poderia estar associado a não-disjunção mitótica do primeiro par metacêntrico durante a divisão celular, seguida por um processo de heterocromatização total ou parcial desse cromossomo extra.

Outros autores têm proposto a origem de cromossomos B a partir da formação de isocromossomos. Ao estudarem a espécie *A. scabripinnis*, Mestriner et al. (2000) detectaram a presença de sequências repetitivas (*As51*), também detectadas sobre cromossomos do complemento A, em ambos os braços do cromossomo B, reforçando, dessa forma, argumentos favoráveis sobre a hipótese de origem desses macrocromossomos extras pela formação de isocromossomos.

Apesar da tendência de citar a espécie *A. scabripinnis* como um modelo de estudo para cromossomos B em peixes, provavelmente devido ao número de estudos descritos disponíveis sobre a mesma, outras espécies do gênero *Astyanax* também apresentam cromossomos B em seus complementos. Por exemplo, Torres-Mariano e Morelli (2008) relataram a ocorrência de cromossomos supernumerários em *Astyanax eigenmanniorum*, Hashimoto et al. (2008) relataram a presença de cromossomos extras nos complementos de *A. altiparanae*, Daniel et al. (2012) em *A. bockmanni* e Silva et al. (2014) em *Astyanax paranae*.

Em um estudo recente, realizado em *A. paranae* por Silva et al. (2014), foi discutido que a hipótese para origem dos cromossomos Bs (um cromossomo grande metacêntrico e um cromossomo grande submetacêntrico) seria através de isocromossomos, uma vez que os autores verificaram que os dois braços cromossômicos tiveram marcações com sondas obtidas por microdissecção cromossômica de um único braço do cromossomo B metacêntrico grande. Segundo os autores foram identificados ainda, através do mapeamento de DNA

repetitivo (DNAr 18S e genes de Histonas), os prováveis pares do complemento A que originaram estes cromossomos Bs (pares 2 e 23).

Por fim, grandes esforços têm sido realizados na tentativa de entender a origem dos cromossomos B e explicar possíveis funções no genoma, porém, é notável que estes elementos não possuem uma origem ou função comum para os diferentes organismos.

1.6. Localização cromossômica de sequências repetitivas no táxon

O gênero *Astyanax* é o grupo mais estudado citogeneticamente entre os *incertae sedis*. Dentre as espécies do grupo extensivamente estudadas estão *A. altiparanae*, *A. scabripinnis* e *A. fasciatus* e uma das sequências repetitivas mais estudadas nesse grupo é a do DNA ribossomal (DNAr) 18S, parte da família multigênica do DNAr 45S.

A sequência do rDNA 18S apresenta variedade de localizações cromossômicas em *Astyanax*, principalmente entre as populações de uma mesma espécie. Por exemplo, Fernandes e Martins-Santos (2006) relataram para quatro populações de *A. altiparanae* provenientes da bacia do rio Paraná, quatro sítios de DNAr 18S para as populações do rio Paraná, córrego Tatupeba e córrego Maringá, e sete sítios de DNAr 18S para a população do córrego Keçaba. Por outro lado, a população de *A. altiparanae* do córrego Monjolinho (bacia do Alto Paraná, São Carlos, SP) apresentou apenas dois sítios de DNAr 18S (PERES et al., 2008). Além desses trabalhos, há outros relatando diferentes números e posições de sítios de DNAr 18S para as espécies de *Astyanax* (por exemplo, FERNANDES; MARTINS-SANTOS, 2004, 2005, 2006; KAVALCO; ALMEIDA-TOLEDO, 2007; HASHIMOTO et al., 2008; PERES et al., 2008; FERREIRA-NETO et al. 2012; PISCOR et al., 2015).

Outra sequência repetitiva extensivamente estudada no gênero *Astyanax* é a do DNAr 5S, que mostra padrões conservados para algumas espécies do gênero. Um desses padrões é compartilhado entre *A. fasciatus*, *A. scabripinnis*, *Astyanax parahybae*, *Astyanax* sp. B, *A. paranae*, *A. bockmanni*, *A. eigenmanniorum* e *Astyanax abramis*, as quais apresentaram um par metacêntrico marcado na porção pericentromérica e um par acrocêntrico ou

subtelocêntrico marcado na porção subterminal (ALMEIDA-TOLEDO et al. 2002; KAVALCO et al., 2004; MANTOVANI et al., 2005; VICARI et al., 2008b; KANTEK et al., 2008; KAVALCO et al, 2009; PISCOR et al., 2015). Outro padrão notável, de localização cromossômica de DNAr 5S, que pode ser observado em *A. altiparanae*, em *Astyanax bimaculatus* e em *Astyanax lacustris* é a presença de um par submetacêntrico com marcações pericentroméricas (ALMEIDA-TOLEDO et al., 2002; FERNANDES; MARTINS-SANTOS, 2006; DOMINGUES et al., 2007; FERREIRA-NETO et al., 2009; KAVALCO et al., 2011). Um terceiro padrão compartilhado entre *Astyanax janeiroensis*, *Astyanax* sp. C e *Astyanax* sp. D consiste na presença de um par acrocêntrico na posição pericentromérica marcado pelo DNAr 5S (KANTEK et al., 2008; VICARI et al., 2008b).

Além das sequências repetitivas mais comumente estudadas, como o DNAr 18S e 5S, outras sequências repetitivas têm sido analisadas, como por exemplo o DNA satélite *As51*, que foi caracterizado nos cromossomos de algumas espécies de *Astyanax*. De acordo com Mantovani et al. (2004) duas populações de *A. scabripinnis* com os números diploides 2n = 48 e 2n = 50 cromossomos mostraram que, através da técnica de hibridação *in situ* fluorescente (FISH), regiões de DNA satélite *As51* estão sobre regiões similares de heterocromatina revelada pela técnica de banda-C. Os autores verificaram que os grandes blocos heterocromáticos estavam localizados nas posições terminais dos braços longos, principalmente em cromossomos subtelocêntricos e acrocêntricos. Também foi notada variação de número e quantidade de heterocromatina distal e de marcações *As51* em ambas as populações (MANTOVANI et al., 2004).

O padrão de distinção da heterocromatina é um dos melhores marcadores cromossômicos para a separação das populações de *A. scabripinnis* (MOREIRA-FILHO; BERTOLLO, 1991; MAISTRO et al., 1998) e *A. fasciatus* (ARTONI et al., 2006). Do mesmo modo, estudos com DNA satélite *As51* demonstraram que grandes blocos de heterocromatina em *A. fasciatus* e *A. scabripinnis* apresentaram similaridade com esse tipo de satélite, indicando que o processo de diferenciação da heterocromatina-*As51* pode estar envolvido na evolução cromossômica dessas duas espécies (KANTEK et al., 2009). A sequência *As51*

também foi observada em outras espécies, tais com *A. paranae*, *Astyanax janeiroensis*, *A. scabripinnis*, *Astyanax* sp. D, *A. fasciatus* e *Astyanax hastatus* (KANTEK et al., 2009; KAVALCO et al., 2009). Kavalco et al. (2009) estudaram quatro populações de *A. hastatus* da bacia do rio Guapimirim (RJ) e verificaram três citótipos distintos, os quais não apresentaram nenhum sinal positivo através da técnica de FISH com sonda de DNA satélite *As51*.

Além dessas sequências repetitivas, outra sequência estudada em *Astyanax* é a do gene de histona H1. Hashimoto et al. (2011) estudaram a localização cromossômica da histona H1 em três espécies de *Astyanax*, mostrando que as espécies apresentaram dois pares cromossômicos marcados e, que um destes apresentava sintenia dos genes de DNAr 5S e da histona H1 em um par metacêntrico em *A. bockmanni* (par 2) e *A. fasciatus* (par 3), e um par submetacêntrico em *A. altiparanae* (par 12). Os autores verificaram ainda que o padrão de localização cromossômica dos genes de histona H1-DNAr 5S (sintenia), juntamente com a análise baseada nas sequências de histona H1, demonstraram que *A. fasciatus* e *A. bockmanni* estão intimamente relacionadas.

Outros genes, recentemente mapeados nos cromossomos de espécies do gênero *Astyanax*, como *A. paranae*, *A. fasciatus*, *A. bockmanni*, *A. altiparanae* e *A. jordani*, são aqueles envolvidos no processo de *splicing*, os genes da família DNAsn U (SILVA et al., 2015). Nesse estudo foi mostrado que os genes de RNAsn U2 são organizados em dois pares cromossômicos, exceto em *A. jordani* que apresentou sinais fluorescentes em apenas um par. Os autores também mostraram que os genes RNAsn U1 e U2 exibiram forte conservação em número de sítios cromossômicos por genoma, mas estes genes estão organizados em diferentes pares (SILVA et al., 2015).

Elementos transponíveis também têm sido mapeados nos cromossomos de algumas espécies do gênero. Pessenda (2012) estudou a localização cromossômica do retrotransposon *Rex*1 em *A. altiparanae*, *A. asuncionensis*, *A. eigenmannirorum* e *A. fasciatus*, e verificou que estas espécies apresentaram um padrão disperso para esta sequência. Segundo Silva et al. 2013, nas duas populações de *A. bockmanni* por eles analisadas, os

elementos *Rex3* estão compartimentalizados nas regiões de heterocromatina telomérica, assim como regiões eucromáticas e em sintenia com o DNAr 18S.

Por fim, estudos envolvendo diversas sequências repetitivas apresentados por diferentes autores para o gênero *Astyanax*, vêm confirmando ser este um grupo bastante interessante para estudos desse tipo, e que a localização cromossômica de sequências repetitivas em peixes pode ser de grande importância em comparações genômicas e evolutivas neste grupo.

HIPÓTESES

2. HIPÓTESES

1) O mapeamento cromossômico de sequências repetitivas aliado a dados moleculares poderia auxiliar no entendimento das relações evolutivas entre as espécies do gênero *Astyanax*?

2) Seria, o gênero *Astyanax*, um grupo monofilético dentro de Characidae?

JUSTIFICATIVA E OBJETIVOS

3. JUSTIFICATIVA E OBJETIVOS

Astyanax é um grupo interessante do ponto de vista citogenético e evolutivo porque apresenta espécies com, principalmente, 2n = 36, 46, 48, 50 e 52 cromossomos, sendo 2n = 50 o mais frequente no gênero. Com o intuito de obter informações que possam contribuir com o entendimento da origem e evolução dos cromossomos em espécies com diferentes números diploides ocorrentes no gênero, o principal foco deste trabalho foi estudar diversos marcadores cromossômicos. Para tanto, os principais objetivos foram:

1) Verificar a organização genômica de elementos primariamente repetitivos em espécies com diferentes números diploides, com o uso das técnicas de Ag-NOR, banda-C e dupla coloração por CMA_3/DAPI.

2) Caracterizar sequências repetitivas nos cromossomos de diferentes espécies de *Astyanax* com distintos números diploides para identificar panoramas gerais de organização entre as mesmas.

3) Associar dados citogenéticos com marcadores moleculares para identificar relações evolutivas associadas a datação da idade cronológica das unidades taxonômicas ou de grupos específicos dentro de *Astyanax*.

MATERIAIS E MÉTODOS

4. MATERIAIS

As coletas foram realizadas com autorização do Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) através da aquisição do documento para atividades com finalidade científica: SISBIO - Sistema de Autorização e Informação em Biodiversidade (Anexo I).

Os animais capturados foram levados ao laboratório de Citogenética Animal e colocados em aquários aerados até serem utilizados nos experimentos propostos no presente trabalho. Foram seguidos todos os cuidados recomendados pela Comissão de Ética no Uso de Animal (CEUA) do Instituto de Biociências da Universidade Estadual Paulista – UNESP, Campus de Rio Claro (Anexo II).

Os exemplares de *Astyanax altiparanae* (Garutti e Britski, 2000) e *Astyanax schubarti* (Britski, 1964) foram coletados no rio Piracicaba no estado de São Paulo (SP), Brasil; exemplares de *Astyanax fasciatus* (Cuvier, 1819) (2n = 46, 48 e 50 cromossomos – ver Figura 1), e também *A. altiparanae* foram coletados em afluentes da bacia do rio Corumbataí – SP, Brasil. Os exemplares de *Astyanax bockmanni* (Vari e Castro, 2007) foram coletados no córrego Guaçu no estado de Mato Grosso do Sul (MS), Brasil. Foram também analisados exemplares de *Astyanax abramis* (Jenyns, 1842) e *Astyanax asuncionensis* (Géry, 1972) pertencentes ao córrego Bento Gomes do estado de Mato Grosso (MT), Brasil e exemplares de *Astyanax marionae* (Eigenmann, 1911) pertencentes ao córrego Rio Claro – MT, Brasil. Foram também obtidos exemplares de *Astyanax mexicanus* (peixe de caverna albino sem olhos desenvolvidos) (De Filippi, 1853), e *Astyanax eigenmanniorum* (Cope, 1894) em loja de aquário. As espécies analisadas estão organizadas na Tabela 1 (ver também Figura 1).

Para as coletas foram utilizados aparelhos de pesca variados como redes de arrasto, covos, peneiras e equipamento de pesca elétrica. Os exemplares coletados foram colocados em aquários no Laboratório de Citogenética Animal da Universidade Estadual Paulista “Júlio de Mesquita Filho” (UNESP), Câmpus de Rio Claro – SP. Para cada exemplar foi fornecido um número de identificação e foi obtido o sexo, o qual foi identificado com o auxílio de microscópio de luz. Os exemplares foram narcotizados e utilizados para preparações

citogenéticas, fixados em etanol a 70%, e enviados para o Departamento de Zoologia da UNESP de São José do Rio Preto – SP para identificação pelo Prof. Dr. Francisco Langeani Neto, para o Departamento de Morfologia da UNESP de Botucatu – SP para identificação pelo Dr. Ricardo Britske, e para Unidade Universitária de Mundo Novo – MS (UEMS) para identificação pelo Prof. Dr. Carlos Alexandre Fernandes. Após identificação, os exemplares foram depositados na coleção Ictiológica do Departamento de Biologia da UNESP de Rio Claro − SP.

Tabela 1. Relação das espécies coletadas, número de indivíduos e procedências.

Espécies	N° de indivíduos	Estado	Bacias hidrográficas
Astyanax abramis	03	MT	Bacia do rio Paraguai (1)
Astyanax altiparanae[1]	05	SP	Bacia do rio Paraná (2)
Astyanax altiparanae[2]	05	SP	Bacia do rio Paraná (2)
Astyanax asuncionensis	04	MT	Bacia do rio Paraguai (1)
Astyanax bockmanni	03	MS	Bacia do rio Paraná (2)
Astyanax eigenmanniorum	03	Aquarismo	-
Astyanax fasciatus[3]	03	SP	Bacia do rio Paraná (2)
Astyanax fasciatus[4]	02	SP	Bacia do rio Paraná (2)
Astyanax fasciatus[5]	03	SP	Bacia do rio Paraná (2)
Astyanax fasciatus[1]	05	SP	Bacia do rio Paraná (2)
Astyanax marionae	06	MT	Bacia do rio Paraguai (1)
Astyanax mexicanus	09	Aquarismo	-
Astyanax schubarti	01	SP	Bacia do rio Paraná (2)

[1] Ribeirão Claro – SP (ponto 1); [2] Rio Piracicaba em Santa Maria da Serra – SP; [3] Afluente do rio Corumbataí – SP; [4] Afluente do rio Cabeça – SP; [5] Ribeirão Claro – SP (ponto 2); 1 e 2: Ver figura 1 (próxima página) para visualização das bacias hidrográficas.

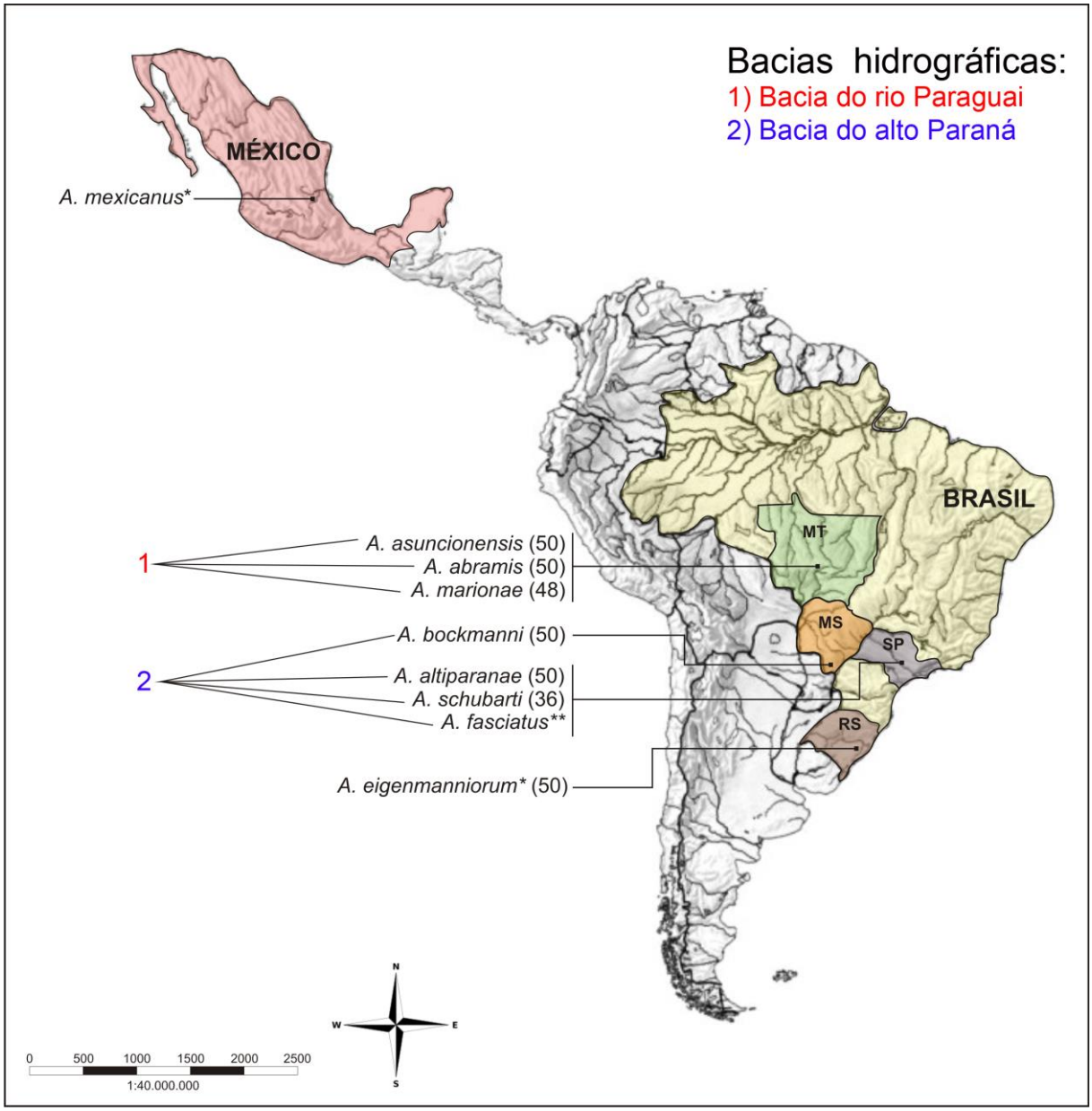

Figura 1. Mapa das localizações das espécies analisadas no presente trabalho. Observe que os números entre os parênteses são referentes aos números diploides das respectivas espécies; *Espécies provenientes de lojas de aquário no Brasil e suas respectivas localidades de origem; **Espécie com diferentes números diploides (2n = 46, 48 e 50).

5. MÉTODOS

5.1. Estimulação de células mitóticas

Para aumentar a quantidade de células mitóticas nas preparações, foi utilizada a técnica de injeção prévia de uma solução de fermento biológico nos animais, descrita inicialmente por Cole e Levans (1971) para répteis e anfíbios e adaptada por Oliveira et al. (1988) para peixes. O protocolo foi o seguinte:

1) Preparar uma solução de fermento biológico na seguinte proporção: 0,5g de fermento, 0,5g de açúcar e 7 mL de água destilada. Incubar em estufa por aproximadamente 20 minutos;

2) Injetar esta solução na região dorso-lateral do animal, na proporção de 1 mL para 100g de peso vivo. Manter o animal em um aquário bem aerado e com a temperatura em torno de 26°C por um período de aproximadamente 48 horas.

5.2. Obtenção de cromossomos mitóticos

Os cromossomos mitóticos foram obtidos de células extraídas das porções anterior e posterior do rim, segundo a metodologia de Foresti et al. (1981), com algumas modificações. O procedimento consiste em:

1) Injetar, na região intra-abdominal, solução aquosa de colchicina (0,025%) na proporção de 1 mL para cada 100g de peso do animal;

2) Deixar o peixe em aquário bem aerado, por 50 minutos;

3) Efetuar uma excisão abdominal, utilizando uma tesoura, partindo do ânus do animal até a região anterior. Logo após, realizar um corte lateral e paralelo às brânquias, e por último retirar a porção anterior e posterior do rim, e quando necessário as brânquias;

4) Colocar os tecidos retirados em placa de Petri contendo 7 mL de solução hipotônica de cloreto de potássio (KCl) a 0,075 M;

5) Fragmentar bem o material com o auxílio de pinças de dissecação e completar esse processo com uma seringa hipodérmica desprovida de agulha para se obter uma suspensão celular homogênea;

6) Colocar a suspensão obtida a 37°C, em estufa, durante 21 minutos;

7) Retirar da estufa, ressuspender cuidadosamente o material com o auxílio de pipeta Pasteur, e transferir a solução para um tubo de centrífuga. Adicionar 7 gotas de fixador gelado (Metanol e Ácido Acético na proporção de 3:1, respectivamente); agitar levemente a mistura com uma pipeta Pasteur e deixar repousar por 5 minutos a temperatura ambiente;

8) Adicionar cerca de 6 mL de fixador gelado e novamente agitar a mistura; levar à centrífuga durante 9 minutos a 1.000 rpm; descartar o sobrenadante com pipeta Pasteur;

9) Ressuspender o precipitado em 7 mL de fixador gelado e centrifugar por 9 minutos a 1000 rpm;

10) Repetir o item 9 por duas ou três vezes;

11) Após a última centrifugação, retirar o sobrenadante e adicionar 1,5 mL de fixador, transferindo a solução para um tubo do tipo Eppendorf de 1,5 mL.

12) Pingar o material em lâmina e deixar secar ao ar;

13) Corar com corante Giemsa a 10% diluída em solução tampão com pH ajustado em 6,8 por 10 minutos.

14) Lavar em água corrente e deixar secar ao ar, analisar a lâmina em microscópio óptico.

5.3. Análise morfométrica dos cromossomos

Os cromossomos foram mensurados com auxílio de um paquímetro e classificados de acordo com procedimento frequentemente utilizado para análise dos cromossomos de peixes no Brasil. Para tanto, a morfologia dos cromossomos foi determinada com base na razão de braços (RB), onde o valor da medida do braço longo (q) é dividido matematicamente pelo valor da medida do braço curto (p). Os cromossomos com dois braços foram classificados como metacêntricos (*m*), submetacêntricos (*sm*) e subtelocêntricos (*st*), com as razões compreendidas entre 1,0 a 1,70 para *m*, 1,71 a 3,0 para *sm* e 3,01 a 7,0 para *st*. Os

cromossomos com um braço foram considerados como acrocêntricos (*a*) com a razão acima de 7,0. Para o cálculo do número fundamental (NF) os cromossomos homólogos *m*, *sm* e *st* foram considerados como cromossomos de dois braços e os *a* com um braço.

5.4. Montagem dos cariótipos

Após as análises morfométricas dos cromossomos, os cariótipos foram montados com o auxílio do programa Adobe Photoshop CS4. Os cromossomos foram organizados em sequência do maior para o menor, seguindo a classificação (*m*, *sm*, *st* e *a*) de cima para baixo. As pranchas foram organizadas utilizando o programa CorelDRAW versão X4 (Corel® PHOTO-PAINT™ X4).

5.5. Montagem de idiogramas e cromossomos específicos

A criação de idiogramas e cromossomos específicos foi realizada através do auxílio de ferramentas especializadas do programa CorelDRAW versão X4 (Corel® PHOTO-PAINT™ X4).

5.6. Localização da heterocromatina

Para o estudo da heterocromatina foi utilizada a técnica de Summer (1972), seguindo os procedimentos abaixo:

1) Tratar a lâmina, já contendo as gotas do material para análise, com HCl a 0,2 N em temperatura ambiente, por 15 minutos;

2) Lavar a lâmina em água corrente e secar ao ar.

3) Incubar em solução salina de 2xSSC, a 60°C em banho-maria por 15 minutos.

4) Lavar em água corrente e secar ao ar;

5) Incubar a lâmina por 30 segundos em solução de hidróxido de bário [$Ba(OH)_2$] a 5%, sendo recém preparada e filtrada, em banho-maria a 42°C;

6) Lavar a lâmina rapidamente em solução de HCl 0,2 N, e depois em água destilada, deixar secar ao ar;

7) Incubar a lâmina em solução salina de 2xSSC a 60°C, por 1 hora;

8) Lavar em água destilada e secar ao ar;

9) Corar com Giemsa em solução tampão (pH 6,8) a 5% durante 5-10 minutos;

10) Lavar com água destilada e secar ao ar, analisar a lâmina em microscópio óptico.

5.7. Localização das regiões organizadoras de nucléolo pelo nitrato de prata

A metodologia para detecção das regiões organizadoras de nucléolo com uso de nitrato de prata ($AgNO_3$), foi realizada de acordo com a técnica de Howell e Black (1980) abaixo descrita:

1) Hidrolisar o material contido na lâmina por 3 minutos em HCl 1N a 60°C, em estufa;

2) Lavar em água corrente e secar ao ar;

3) Pingar uma gota de solução aquosa de gelatina e uma dota de água deionizada sobre a lâmina;

4) Adicionar sobre as gotas anteriores, duas gotas de solução de nitrato de prata ($AgNO_3$), e cobrir a lâmina com lamínula;

5) Incubar em câmara úmida a 60°C por um período de 3-5 minutos, ou até que a solução adquira uma coloração caramelada;

6) Lavar em água corrente e secar ao ar;

7) Corar com Giemsa em solução tampão (pH 6,8) a 2% durante 20-30 segundos e analisar em microscópio óptico.

5.8. Dupla coloração CMA_3/DAPI com cromossomos desnaturados

As regiões cromossômicas ricas em bases GC e AT foram detectadas utilizando a metodologia frequentemente utilizada no Laboratório de Citogenética da UNESP-Rio Claro, descrita abaixo:

1) Colocar as lâminas em solução de formamida 70% em 2xSSC a 70°C por 2 minutos;
2) Passar as lâminas por duas cubas com 2xSSC gelado por 2 minutos cada;
3) Passar as lâminas em série de etanol gelado a 70, 90 e 100%, 2 minutos cada. Deixar as lâminas secarem bem;
4) Montar as lâminas com 30 µL de cromomicina A_3 (CMA_3), deixar atuar por 1 hora em câmara escura na geladeira;
5) Passar as lâminas por três banhos de PBS 1x por 2 minutos cada;
6) Não deixar secar, montar as lâminas com 30 µL de Vectashield com DAPI (VECTOR), deixar atuar por 30 minutos;
7) Retirar o excesso de DAPI, e analisar em microscópio de fluorescência.

5.9. Extração de DNA genômico

A metodologia de extração de DNA genômico de tecidos sólidos foi empregada de acordo com a técnica de Fenol-Clorofórmio de Sambrook e Russel (2001):

1) Macerar o tecido (nadadeira, músculo ou fígado) dentro de um tubo do tipo Eppendorf de 1,5 mL com auxílio de um macerador ou tesoura;
2) Adicionar 430 µL de tampão de digestão, como descrito abaixo:

Reagentes	**Concentração**	**Volume (µL)**
NaCl	5 M	10
Tris-HCl (pH 8,0)	1 M	5
EDTA (pH 8,0)	0,5 M	25
SDS	10%	25
Proteinase K	10 mg/mL	5
H_2O_d q.s.p.*	-	430

3) Incubar em banho Maria a 40-50°C por cerca de 1 hora e 30 minutos (homogeneizar periodicamente), esse tempo pode ser maior ou menor dependendo do tecido utilizado;
4) Adicionar 500 μL de Fenol:Clorofórmio (1:1) e homogeneizar com movimentos rotatórios durante 15 minutos;
5) Centrifugar a 15.000 rpm durante 15 minutos a 4°C;
6) Transferir a camada superior (mais clara) para outro tubo do tipo Eppendorf de 1,5 mL;
7) Adicionar 0,2x o volume de NaCl 1 M e 2x o volume de etanol 100% gelado, homogeneizar suavemente para precipitar o DNA;
8) Centrifugar a 15.000 rpm durante 15 minutos a 4°C;
9) Descartar o sobrenadante e acrescentar cuidadosamente 375 μL de etanol 70%, sem agitar;
10) Centrifugar a 15.000 rpm durante 15 minutos a 4°C;
11) Descartar o sobrenadante e secar o *pellet* em estufa a 37°C;
12) Ressuspender com água mili-Q durante algumas horas
13) Verificar a quantidade do DNA em gel de Agarose (0,8%).

5.10. Obtenção de sondas de DNA repetitivos por PCR

1) A sonda de DNAr 18S foi amplificada através da técnica de PCR (Reação em Cadeia da Polimerase) usando DNA genômico de espécies de *Astyanax* e *primers* específicos (NS1 = 5'-GTA GTC ATA TGC TTG TCT C; NS8 = 5'-TCC GCA GGT TCA CCT ACG GA) descritos por White et al. (1990), de acordo com a seguinte tabela de especificação dos volumes e concentrações dos reagentes utilizados na reação:

Reagentes	Volume (µL)	Concentração
DNA	1	50 a 100 ng/µL
PCR *buffer* (Tampão)	5	10x
$MgCl_2$	4	50 Mm
Primer 1	1	0,6 ng/µL
Primer 2	1	0,6 ng/µL
dNTPs	1 (cada)	2 mM
Taq Polimerase	0,5	5 U/µL
Água	33,5	-
Volume final	**50 µL**	

O programa do termociclador para a amplificação do DNAr 18S foi:

1- 94°C − 5 min

2- 95°C − 1 min
3- 60°C − 1 min
4- 72°C − 1 min e 30 seg
} 35 ciclos

5- 72°C − 7 min
6- 4°C − ∞

2) A sonda de DNAr 5S foi amplificada através da técnica de PCR usando DNA genômico de *Astyanax* com os *primers* (A= 5'-TAC GCC CGA TCT CGT CCG ATC; B= 5'-CAG GCT GGT ATG GCC GTA AGC) descritos por Pendás et al. (1994) e Martins e Galetti (1999), de acordo com a reação descrita acima na tabela de especificação dos volumes e concentrações dos reagentes. O programa do termociclador para a amplificação do DNAr 5S foi:

1- 94°C − 5 min

2- 95°C – 30 seg
3- 45°C − 30 seg
4- 72°C − 30 seg
} 30 ciclos

5- 72°C − 7 min
6- 4°C − ∞

3) A sonda de histona H3 foi amplificada através da técnica de PCR usando DNA genômico de espécies de *Astyanax* com os *primers* (ScaH3F = 5’ GGC NMG NAC NAA RCA RAC; ScaH3R = 5’ TGD ATR TCY TTN GGC ATD AT) descritos por Cabral-de-Mello (2010), de acordo com a reação descrita acima na tabela de especificação dos volumes e concentrações dos reagentes. O programa do termociclador para a amplificação do gene de histona H3 foi:

1- 95°C − 5 min

2- 95°C – 40 seg
3- 55°C – 40 seg
4- 72°C – 2 min
} 34 ciclos

5- 72°C − 5 min
6- 12°C − ∞

4) A sonda de DNAsn U2 foi amplificada através da técnica de PCR usando DNA genômico de *Astyanax* com os *primers* (U2F = 5’ ATC GCT TCT CGG CCT TAT G; U2R = 5’ TCC CGG CGG TAC TGC AAT A) descritos por Bueno et al. (2013), de acordo com a reação descrita acima na tabela de especificação dos volumes e concentrações dos reagentes. O programa do termociclador para a amplificação do DNAsn U2 foi:

1- 94°C − 5 min

2- 95°C – 30 seg
3- 65°C – 30 seg } 35 ciclos
4- 72°C − 30 seg

5- 72°C − 5 min
6- 4°C − ∞

5.11. Marcação das sondas via PCR

As sondas foram marcadas com Biotina-11-dUTP (Roche) ou Digoxigenina-11-dUTP (Roche) por PCR, de acordo com a seguinte reação:

Reagentes	**Volume (µL)**	**Concentração**
Produto de PCR	1	~100 ng/µL
PCR *buffer* (Tampão)	5	10x
$MgCl_2$	4	50 mM
Primer 1	1	0,6 ng/µL
Primer 2	1	0,6 ng/µL
dATP	1	2 mM
dCTP	1	2 mM
dGTP	1	2 mM
dTTP	0,5	2 mM
D* ou B**	0,75	50 nmol
Taq Polimerase	0,5	5 U/µL
Água	33,25	-
Volume final	**50 µL**	

*D = Digoxigenina-11-dUTP; **B = Biotina-11-dUTP

Observação: Os programas utilizados para a marcação das sondas foram os mesmos utilizados para a amplificação.

5.12. Marcação das sondas via *nick translation*

Algumas sondas foram marcadas com Biotina-11-dUTP (Roche) ou Digoxigenina-11-dUTP (Roche) por *nick translation*, de acordo com a seguinte reação:

Reagentes	**Volume (µL)**	**Concentração**
Produto de PCR	5	~100 ng/µL
NT *buffer* (Tampão)	2	10x
dATP	2	0,4 mM
dCTP	2	0,4 mM
dGTP	2	0,4 mM
dTTP	0,7	0,4 mM
D* ou B**	0,52	50 nmol
DNA Polimerase I/DNase I	3	5 U/µL
Água	2,78	-
Volume final	**20 µL**	

*D = Digoxigenina-11-dUTP; **B = Biotina-11-dUTP

O programa do termociclador para a marcação via *nick translation* foi:

1- 16°C − 90 min
2- 65°C − 10 min
3- 10°C − ∞

5.13. Obtenção das sondas de Microssatélites

As sondas de microssatélites específicos foram cedidas pelo Prof. Dr. Diogo Cavalcanti Cabral-de-Melo (UNESP – Rio Claro). A marcação com biotina dos microssatélites específicos foi realizada durante a síntese dos mesmos na extremidade 5' (Sigma, St. Louis, MO, USA) como descrito por Milani e Cabral-de-Melo (2014). Os microssatélites utilizados foram os seguintes di- e tetra-nucleotídeos:

Microssatélites	Repetições
(CA)	15
(GA)	15
(CG)	15
(GACA)	4
(GATA)	8

5.14. Obtenção da sonda de DNA telomérico e FISH específico

A sonda de DNA telomérico foi adquirida pelo kit Telomere PNA FISH Kit/FITC (Dako Cytomation Denmark A/S Kit) e usada para hibridação de acordo com as recomendações específicas do protocolo do fabricante.

5.15. Hibridação *in situ* fluorescente (FISH) – técnica 1

A técnica de FISH foi realizada de acordo com Pinkel et al. (1986) com modificações feitas no Laboratório de Citogenética Animal da UNESP – Rio Claro descritas por Cabral-de-Mello et al. (2010):

PRIMEIRO DIA – tratamento das lâminas e desnaturação dos cromossomos

1) Deixar as lâminas com a suspensão de células em estufa a 37°C por pelo menos 4 horas antes de iniciar a técnica;

Limpeza com Pepsina

2) Incubar as lâminas em solução de Pepsina 0,005% a 37°C por aproximadamente 10 minutos (este tempo é variável de acordo com o material citogenético);
3) Lavar duas vezes por 2 minutos em 2xSSC;
4) Desidratar cada lâmina em série de etanol a 70, 90 e 100% por 5 minutos e secar;

Limpeza com RNase

5) Incubar as lâminas em 100 µL de RNase a 20 mM (concentração final de 0,4% em 2xSSC) a 37°C por 1 hora em câmara úmida;
6) Lavar três vezes por 5 minutos em 2xSSC;
7) Lavar uma vez durante 5 minutos em Triton 1x (1% de Triton-100x para 99% de 2xSSC);
8) Desidratar cada lâmina em série de etanol a 70, 90 e 100% por 5 minutos e secar;

Desnaturação dos cromossomos

9) Desnaturar o cromossomos com formamida 70% em 2xSSC a 70°C por aproximadamente 2 minutos (este tempo é variável de acordo com o material citogenético);
10) Desidratar cada lâmina em série de etanol gelado (-20°C) a 70, 90 e 100% por 5 minutos e secar;
11) Enquanto as lâminas são passadas pela série de etanol deve-se colocar a solução de hibridação contendo a sonda (especificação abaixo) para desnaturar a 95°C por 10 minutos;
12) Solução de hibridação: Preparar o tampão de hibridação com 15 µL de formamida (*CF=50%), 6 µL de sulfato de dextrano 50% (*CF=10%) e 3 µL de 20xSSC (*CF=2xSSC). Em um tubo do tipo Eppendorf, adicionar 6 µL da sonda marcada e 24 µL do tampão de hibridação. Após, armazenar a solução no gelo até o uso. *CF=Concentração Final;
13) Montar cada lâmina com 30 µL de solução de hibridação, cobrir com lamínula e colocar em câmara úmida a 37°C *overnight*.

SEGUNDO DIA – lavagem e detecção

Lavagem

14) Retirar as lamínulas cuidadosamente;
15) Lavar em 2xSSC a 72°C, sem agitação, por 5 minutos.
16) Transferir para a solução de 1xPBD (40 mL de 20xSSC + 2 mL de Triton-100x + 2 gramas de leite em pó, e completar para 200 mL com água mili-Q) a temperatura ambiente para proceder com a detecção (mínimo 10 e máximo 15 minutos);

Detecção

17) Aplicar 4 µL de anti-digoxigenina-rodamina (ROCHE) em 26 µL de 1xPBD se usar digoxigenina para marcar a sonda, ou 1 µL de estreptavidina conjugada a Alexa Fluor® 488 (INVITROGEN) em 100 µL de 1xPBD se usar biotina para a marcação da sonda;
18) Cobrir com lamínula e incubar por 1 hora em câmara úmida a 37°C;
19) Remover a lamínula e lavar três vezes em 1xPBD a 45°C por 5 minutos cada;
20) Montar a lâmina com 15 µL de Vectashield com DAPI (VECTOR) e armazenar em câmara escura na geladeira.

5.16. Hibridação *in situ* fluorescente (FISH) – técnica 2

A técnica de FISH foi realizada de acordo com Pinkel et al. (1986) com modificações feitas no Laboratório de Citogenética Animal da UNESP-Rio Claro descritas por Piscor et al. (2013):

PRIMEIRO DIA – tratamento das lâminas e desnaturação dos cromossomos

Obs.: Mesmos procedimentos do primeiro dia da técnica 1.

SEGUNDO DIA – lavagem e detecção

Lavagem

21) Retirar as lamínulas cuidadosamente;
22) Lavar 2x por 5 minutos com solução de estringência (50 % de formamida e 50 % de 1xSSC) a 45°C;
23) Lavar 2x por 5 minutos com 1xSSC a 45°C;
24) Lavar 1x por 5 minutos com 4xTriton (para cada 500 mL: 100 mL de 20xSSC + 250 µL de Triton 100x + 400 mL de água destilada) a temperatura ambiente.
25) Colocar 30 µL de tampão de bloqueio (misturar 1000 µL de 2xSSC + 10 µL de Triton 100x + 0,05 g de leite em pó desnatado, e centrifugar por 5 minutos a 10000 rpm) sobre cada lâmina, cobrir com lamínula por 5 minutos a temperatura ambiente.

Detecção

26) Aplicar 4 µL de anti-digoxigenina-rodamina (ROCHE) em 26 µL de tampão de bloqueio se usar digoxigenina para marcar a sonda, ou 1 µL de estreptavidina conjugada a Alexa Fluor® 488 (INVITROGEN) em 100 µL de tampão de bloqueio se usar biotina para a marcação da sonda;
27) Cobrir com lamínula e incubar por 1 hora em câmara úmida a 37°C;
28) Remover a lamínula e lavar rapidamente com 2xSSC em temperatura ambiente;
29) Lavar 2x por 5 minutos com 4xTriton (mesma preparação citada acima anteriormente) a 45°C;
30) Lavar rapidamente com 2xSSC em temperatura ambiente;
31) Montar a lâmina com 15 µL de Vectashield com DAPI (VECTOR) e armazenar em câmara escura na geladeira.

5.17. Amplificação do gene mitocondrial citocromo *c* oxidase I (COI)

O gene mitocondrial COI foi amplificado utilizando os *primers* descritos por Ward et al. (2005) através de reações preparadas utilizando o MIX PCR (QIAGEN) contendo: 5U de *Taq*

polimerase, tampão de enzima 10x, $MgCl_2$ a 1,5 mM e dNTPs a 200 µM, água Mili Q autoclavada, *primer foward* (F) 10 µM, *primer reverse* (R) 10 µM e DNA genômico. O programa de PCR utilizado foi:

1- 94°C − 2 min

2- 94°C – 30 seg
3- 50°C – 30 seg
4- 60°C − 4 min
} 30 ciclos

5- 72°C − 5 min
6- 12°C − ∞

5.18. Sequenciamento de DNA

As sequências amplificadas foram purificadas através do tratamento com a enzima EXOSAP (GE Healthcare), de acordo com o protocolo abaixo:

1) Limpar 10 µL do produto de PCR, adicionando 2 µL de EXOSAP e 2 µL de água Milli Q autoclavada;
2) Levar os tubos recém preparados ao termociclador em um ciclo de 1 hora a 37ºC e 15 minutos a 80ºC;
3) Retirar os tubos do termociclador e fazer 2 novos tubos, aliquotando em um tubo 5 µL do produto já limpo com 2,5 µL de *primer* F e em outro tubo *primer* R, seguindo os mesmos volumes do tubo um.

O DNA purificado foi enviado para o sequenciamento na Macrogen na Korea, onde foram realizadas a técnica de PCR de sequenciamento, a limpeza deste PCR e o sequenciamento num sequenciador automático de DNA modelo MEGABACE 1.000 (GE HealthCareT) com o kit *DYEnamic ET terminator reagent premix* para MEGABACE (Amersham Biociences).

5.19. Relógio molecular utilizando o gene mitocondrial COI

Foram utilizados os programas BEAUti v1.7.2 e BEAST v1.7.2 (Drummond et. al., 2012) para estimar os tempos de divergência entre as sequências e gerar uma árvore datando os momentos de separação das mesmas. O modelo "*Lognormal relaxed clock* (*uncorrelated*)" foi escolhido para o relógio molecular e o processo de especiação "*Yule*" para o parâmetro "*Tree Prior*". Eventos geológicos e fósseis calibraram as análises, o momento de colonização da América Central (que possibilitou a origem de *A. mexicanus*) foi datado no intervalo entre o soerguimento do Istmo do Panamá (há aproximadamente 3 milhões de anos – Ma) e o próprio evento de colonização da região pelo gênero *Astyanax* datado por Ornelas-García et. al. (2008) (há aproximadamente 8 Ma), também foi utilizado um fóssil de *Colossoma macropomum*, datado com pelo menos 15 Ma.

As análises de MCMC (Markov Chain Monte Carlo) foram rodadas com 10.000.000 gerações e parâmetros amostrados a cada 1.000 passos, as 10.000 amostras inicias foram descartadas (*burn-in* de 10%), e as demais foram sumarizadas e as topologias das árvores foram acessadas utilizando o programa TREEANOTATOR v1.7.2 (Drummond et. al., 2012) e visualizadas no programa FigTree v1.3.1 (Rambaut, 2008).

REFERÊNCIAS

REFERÊNCIAS BIBLIOGRÁFICAS

ALMEIDA-TOLEDO, L. F.; OZOUF-COSTAZ, C.; FORESTI, F.; BONILLO, C.; PORTO-FORESTI, F.; DANIEL-SILVA, M. F. Z. Conservation of the 5S-bearing chromosome pair and co-localization with major rDNA clusters in five species of *Astyanax* (Pisces, Characidae). **Cytogenetic and Genome Research**. v. 97, p. 229–233, 2002.

ALMIRÓN, A.; AZPELICUETA, M.; CASCIOTTA J.; LÓPEZ CAZORLA, A. Ichthyogeographic boundary between the Brazilian and Austral subregions in South America, Argentina. **Biogeographica**. v. 73, n. 1, p. 23–30, 1997.

ARTONI, R. F.; VICARI, M. R.; ENDLER, A. L.; CAVALLARO, Z. I.; JESUS, C. M.; ALMEIDA, M. C.; MOREIRA-FILHO, O.; BERTOLLO, L. A. C. Evolution of B chromosomes in the Prochilodontidae fish, *Prochilodus lineatus.* **Genetica**. v. 127, p. 277–284, 2006.

BUENO, D.; PALACIOS-GIMENEZ, O. M.; CABRAL-DE-MELLO, D. C. Chromosomal mapping of repetitive DNAs in *Abracris flavolineata* reveal possible ancestry for the B chromosome and surprisingly H3 histone spreading. **PLoS ONE**. v. 8, p. e66532, 2013.

BUSSING, W. A. Patterns of distribution of the Central American ichthyofauna. In: STEHLI, F. G.; WEBB, S. D., (Eds.). **The Great American Biotic Interchange**. New York, Plenum Press New York. 1985. p. 453−473.

CABRAL-DE-MELLO, D. C.; MOURA, R. C.; MARTINS, C. Chromosomal mapping of repetitive DNAs in the beetle *Dichotomius geminatus* provides the first evidence for an association of 5S rRNA and histone H3 genes in insects, and repetitive DNA similarity between the B chromosome and A complement. **Heredity**. v. 104, p. 393–400, 2010.

CALCAGNOTTO, D.; SCHAEFER, S. A.; DESALLE, R. Relationships among characiform fishes inferred from analysis of nuclear and mitochondrial gene sequences. **Molecular Phylogenetics and Evolution**. v. 36, n.1, p. 135–153, 2005.

CARVALHO, M. L.; OLIVEIRA, C.; FORESTI, F. Cytogenetics analysis of five species of the subfamily Tetragonopterinae (Teleostei, Characiformes, Characidae). **Caryologia**. v. 55, p. 181–188, 2002.

CAVALLARO, Z. I.; BERTOLLO, L. A. C.; PERFECTTI, F; CAMACHO, J. P. M. Frequency increase and mitotic stabilization of a B chromosome in fish *Prochilodus lineatus*. **Chromosome Research**. v. 8, p. 627–634, 2000.

CENTOFANTE, L.; BERTOLLO, L. A. C.; BUCKUP, P. A.; MOREIRA-FILHO, O. Chromosomal divergence and maintenance of sympatric *Characidium* fish species (Crenuchidae, Characidiinae). **Hereditas**. v. 138, n. 3, p. 213–218, 2003.

COLE, C. J.; LEVANS, C. R. Chromosome preparations of amphibians and reptiles: improved technique. **Herpetological Review**. v. 49, p.102, 1971.

CONTRERAS-BALDERAS, S. H. O.; LOZANO-VILANO, M. L. Punta de Morro, an interesting barrier for distributional patterns of Continental fishes in North and Central Veracruz, Mexico. **Pública Biologia Faculdad de Ciencias Biológicas Universidad Autónoma de Nuevo Léon México**. v. 16, p. 37−42, 1996.

DANIEL-SILVA, M. F. Z.; ALMEIDA-TOLEDO, L. F. Chromosome R-banding pattern and conservation of a marker chromosome in four species, genus *Astyanax* (Characidae, Tetragonopterinae). **Caryologia**. v. 54, p. 209–215, 2001.

DANIEL-SILVA, M. F. Z.; ALMEIDA-TOLEDO, L. F. Chromosome evolution in fish: BrdU replication patterns demonstrate chromosome homeologies in two species of the genus *Astyanax*. **Cytogenetic and Genome Research**. v. 109, p. 497–501, 2005.

DANIEL, S. N.; HASHIMOTO, D. T.; PANSONATO-ALVES, J. C.; FORESTI, F.; PORTO-FORESTI, F. Cytogenetic characterization of distinct B chromosomes in a population of the fish *Astyanax bockmanni* (Teleostei, Characiformes). **Caryologia**. v. 65, n. 3, p. 229–233, 2012.

DOMINGUES, M. S.; VICARI, M. R.; ABILHOA, V.; WANSER, J. P.; CESTARI, M. M.; BERTOLLO, L. A. C.; ALMEIDA, M. C.; ARTONI, R. F. Cytogenetic and comparative morphology of two allopatric populations of *Astyanax altiparanae* Garutti & Britski, 2000 (Teleostei: Characidae) from upper rio Paraná basin. **Neotropical Ichthyology**. v. 51, n. 1, p. 37–44, 2007.

DOMÍNGUEZ-DOMÍNGUEZ, O.; DOADRIO, I.; PÉREZ-PONCE-DE-LEÓN, G. Historical biogeography of some river basins in Central Mexico evidenced by their goodeine freshwater fishes: a preliminary hypothesis using secondary Brooks Parsimony Analysis (BPA). **Journal of Biogeography**. v. 33, p. 1437−1447, 2006.

DOWLING, T. E.; MARTASIAN, D. P.; JEFFERY, W. R. Evidence for multiple genetic forms with similar eyeless phenotypes in the blind cavefish, *Astyanax mexicanus*. **Molecular Biology and Evolution**. v. 19, n. 4, p. 446−455, 2002.

DRUMMOND, A. J.; SUCHARD, M. A.; XIE, D.; RAMBAUT, A. Bayesian phylogenetics with BEAUti and the BEAST 1.7. **Molecular Biology and Evolution**. v. 29, p. 1969-1973, 2012.

ESCHMEYER, W. N.; FONG, J. D. Catalog of Fishes. http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp. Acesso em 4 de abril de 2016.

ESCHMEYER, W. N.; FONG, J. D.; VAN-DER-LAAN R. Catalog of Fishes. http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp. Acesso em 4 de abril de 2016.

FERNANDES, C. A.; MARTINS-SANTOS, I. C. Cytogenetic characterization of two populations of *Astyanax scabripinnis* (Pisces, Characiformes) of the Ivaí basin PR Brazil. **Cytologia**. v. 68, p. 289–293, 2003.

FERNANDES, C. A.; MARTINS-SANTOS, I. C. Cytogenetic studies in two populations of the *Astyanax altiparanae* (Pisces, Characiformes). **Hereditas**. v. 141, p. 328–332, 2004.

FERNANDES, C. A.; MARTINS-SANTOS, I. C. Sympatric occurrence of three cytotypes and four morphological types of B chromosomes of *Astyanax scabripinnis* (Pisces, Characiformes) in the River Ivaí Basin, state of Paraná, Brazil. **Genetica**. v. 124, p. 301–306, 2005.

FERNANDES, C. A.; MARTINS-SANTOS, I. C. Mapping of the 18S and 5S ribosomal RNA genes in *Astyanax altiparanae* Garutti & Britski, 2000 (Teleostei, Characidae) from the upper Paraná river basin, Brazil. **Genetics and Molecular Biology**. v. 29, p. 464–468, 2006.

FERRO, D. A. M.; MOREIRA-FILHO, O.; BERTOLLO, L. A. C. B chromosome polymorphism in the fish, *Astyanax scabripinnis*. **Genetica**. v. 119, p. 147–153, 2003.

FERREIRA-NETO, M. F.; VICARI, M. R.; CAMARGO, E. F.; ARTONI, R. F.; MOREIRA-FILHO, O. Comparative cytogenetics among populations of *Astyanax altiparanae* (Characiformes, Characidae, *Incertae sedis*). **Genetics and Molecular Biology**. v. 32, n. 4, p. 792–796, 2009.

FERREIRA-NETO, M.; ARTONI, R. F.; VICARI, M. R.; MOREIRA-FILHO, O.; CAMACHO, J. P. M.; BAKKALI, M.; OLIVEIRA, C.; FORESTI, F. Cytogenetics three sympatric karyomorphs in the fish *Astyanax fasciatus* (Teleostei, Characidae) do not seem to hybridize in natural populations. **Comparative Cytogentics**. v. 6, n. 1, p. 29–40, 2012.

FORESTI, F.; ALMEIDA-TOLEDO, L. F., TOLEDO-FILHO, S. A. Polymorfic nature of nucleolus organizer regions in fishes. **Cytogenetics Cell Genetics**. v. 31, p. 137-144, 1981.

GAYET, M.; MARSHALL, L. G.; SEMPERE, T.; MEUNIER, F. J.; CAPPETTA, H.; RAGE, J. C. Middle Maastrichtian vertebrates (fishes, amphibians, dinosaurs and other reptiles, mammals) from Pajcha Pata (Bolivia). Biostratigraphic, palaeoecologic and palaeobiogeographic implications. **Palaeogeography Palaeoclimatology Palaeoecology**. v. 169, n. 1–2, p. 39–68, 2001.

GÉRY, J. **Characoids of the world**. Neptune City, T.F.H. Publications. 1977. 672 p.

GOLDANI A. Biogeografia histórica da região Neotropical: Análise de parcimônia de endemismo com dados distribucionais de peixes. **Revista Eletrônica de Biologia**. v. 5, n. 3, p. 12-41, 2012.

GUIDINI, L. C. F. **Estudos citogenéticos em espécies dos gêneros "*Hemigrammus*", "*Hyphessobrycon*" e "*Bryconamericus*" (Characidae) da planície de inundação do alto rio Paraná (PR)**. Dissertação de Mestrado. Universidade Estadual de Maringá, Maringá, PR. 2007. 57p.

HASHIMOTO, D. T.; GONÇALVES, V. R.; BORTOLOZZI, J.; FORESTI, F.; PORTO-FORESTI, F. First report of a B chromosome in a natural population of *Astyanax altiparanae* (Characiformes, Characidae). **Genetics and Molecular Biology**. v. 31, p. 275–278, 2008.

HASHIMOTO, D. T.; FERGUSON-SMITH, M. A.; RENS, W.; FORESTI, F.; Oliveira C, Foresti F. Chromosome mapping of H1 histone and 5S rRNA genes clusters in three species of *Astyanax* (Teleostei, Characiformes). **Cytogenetic and Genome Research**. v. 134, p. 64–71, 2011.

HOWELL, W. M.; BLACK, D. A. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. **Experientia**. v. 36, p. 1014-1015, 1980.

HUIDOBRO, L.; MORRONE, J. J.; VILLALOBOS, J. L.; ALVAREZ, F. Distributional patterns of freshwater taxa (fishes, crustaceans and plants) from the Mexican Transition Zone. **Journal of Biogeography**. v. 33, n. 4, p. 731−741, 2006.

JAVONILLO, R.; MALABARBA, L. R.; WEITZMAN, S. H.; BURNS, J. R. Relationships among major lineages of characid fishes (Teleostei: Ostariophysi: Characiformes), based on molecular sequence data. **Molecular Phylogenetics and Evolution**. v. 54, p. 498–511, 2010.

JEFFERY, W. R. Cave fish as a model system in evolutionary developmental biology. **Developmental Biology**. v. 231, p. 1−12, 2001.

JESUS, C. M.; GALETTI Jr., P. M.; VALENTINI, S. R.; MOREIRA-FILHO, O. Molecular characterization and chromosomal localization of two families of satellite DNA in *Prochilodus lineatus* (Pisces, Prochilodontidae), a species with B chromosomes. **Genetica**. v. 118, p. 25–32, 2003.

KANTEK, D. L. Z.; NOLETO, R. B.; MAURUTTO, F. A. M.; BERTOLLO, L. A. C.; MOREIRA-FILHO, O.; CESTARI, M. M. Cytotaxonomy of *Astyanax* (Characiformes, Characidae) from the Upper Iguaçu River Basin: confirmation of the occurrence of distinct evolutionary units. **Journal of Fish Biology**. v. 73, 2012–2020, 2008.

KANTEK, D. L. Z.; VICARI, M. R.; PERES, W. A. M.; CESTARI, M. M.; ARTONI, R. F.; BERTOLLO, L. A. C.; MOREIRA-FILHO, O. Chromosomal location and distribution of *As51* satellite DNA in five species of the genus *Astyanax* (Teleostei, Characidae, *Incertae sedis*). **Journal of Fish Biology**. v. 75, p. 408–421, 2009.

KAVALCO, K. F.; PAZZA, R.; BERTOLLO, L. A. C.; MOREIRA-FILHO O. Gene mapping of 5S rDNA sites in eight fish species from the Paraíba do Sul river basin, Brazil. **Cytogenetic and Genome Research**. v. 106, p. 107–110, 2004.

KAVALCO, K. F.; ALMEIDA-TOLEDO, L. F. Molecular cytogenetics of blind mexican tetra and comments on the karyotypic characteristics of genus *Astyanax* (Teleostei, Characidae). **Zebrafish**. v. 4, n. 2, p.103–111, 2007.

KAVALCO, K. F.; PAZZA R.; ALMEIDA-TOLEDO, L. F. *Astyanax bockmanni* Vari and Castro, 2007: an ambiguous karyotype in the *Astyanax* genus. **Genetica**. v. 136, n. 1, p. 135–139, 2009.

LIMA, F. C. T.; MALABARBA, L. R.; BUCKUP, P. A.; DA SILVA, J. F. P.; VARI, R. P.; HAROLD, A. et al. Genera *incertae sedis* in Characidae. In: REIS, R. E.; KULLANDER, S. E.; FERRARIS Jr., C. J., (Eds.). **Check List of the Freshwater Fishes of South and Central America**. Porto Alegre: Edipucrs. 2003. p. 106−169.

LOZANO-VILANO, M. L.; CONTRERAS-BALDERAS, S. *Astyanax armandoi*, n. sp. from Chiapas, Mexico (Pisces, Ostariophysi: Characidae) with a comparison to the nominal species *A. aeneus* and *A. mexicanus*. **Universidad y Ciencia**. v. 7, p. 95−107, 1990.

MAISTRO, E. L.; FORESTI, F.; OLIVEIRA, C.; ALMEIDA-TOLEDO, L. F. Occurrence of macro B chromosomes in *Astyanax scabripinnis paranae* (Pisces, Characiformes, Characidae). **Genetica**. v. 87, p. 101–106, 1992.

MAISTRO, E. L.; FORESTI, F.; OLIVEIRA, C. Comparative cytogenetic and morphological analysis of *Astyanax scabripinnis paranae* (Pisces, Characidae, Tetragonopterinae). **Genetics and Molecular Biology**. v. 21, p. 201–206, 1998.

MANTOVANI, M.; ABEL, L. D. S.; MESTRINER, C. A.; MOREIRA-FILHO, O. Accentuated polymorphism of heterochromatin and nucleolar organizer regions in *Astyanax scabripinnis* (Pisces, Characidae): tools for understanding karyotypic evolution. **Genetica**. v. 109, p. 161–168, 2000.

MANTOVANI, M.; ABEL, L. D. S.; MESTRINER, C. A.; MOREIRA-FILHO, O. Evidence of the differentiated structural arrangement of constitutive heterochromatin between two populations of *Astyanax scabripinnis* (Pisces, Characidae). **Genetics and Molecular Biology**. v. 27, n. 4, p. 536–542, 2004.

MANTOVANI, M.; ABEL L. D. S.; MOREIRA-FILHO, O. Conserved 5S and variable 45S rDNA chromosomal localization revealed by FISH in *Astyanax scabripinnis* (Pisces, Characidae). Genetica. v. 123, p. 211–216, 2005.

MARGARIDO, V. P.; GALETTI Jr., P. M. Chromosome studies in fish of the genus *Brycon* (Characiformes, Characidae, Bryconinae). **Cytobios**. v. 85, p. 219–228, 1996.

MARTINS, C.; GALETTI Jr., P. M. Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). **Chromosome Research**. v. 7, p. 363-367, 1999.

MARINHO, M. M. F.; LIMA F. C. T. *Astyanax ajuricaba*: a new species from the Amazon basin in Brazil (Characiformes:Characidae). **Neotropical Ichthyology**. v. 7, p. 169-174, 2009.

MESTRINER, C. A.; GALETTI Jr., P. M.; VALENTINI, S. R.; RUIZ, I. R. G.; ABEL, L. D. S.; MOREIRA-FILHO, O.; CAMACHO, R. P. M. Structural and functional evidence that a B chromosome in the characid *Astyanax scabripinnis* is an isochromosome. **Heredity**. v. 85, p. 1–9, 2000.

MEDRADO, A. S.; FIGUEIREDO, A. V. A.; WALDSCHMIDT, A. M.; AFFONSO, P. R. A. M.; CARNEIRO, P. L. S. Cytogenetic and morphological diversity in populations of *Astyanax fasciatus* (Teleostei, Characidae) from Brazilian northeastern river basins. **Genetics and Molecular Biology**. v. 31, p. 208–214, 2008.

MENDES, M. M.; ROSA, R.; GIULIANO-CAETANO, L.; DIAS, A. L. Karyotype diversity of four species of the *incertae sedis* group (Characidae) from different hydrographic basins: analysis of Ag-NORs, CMA_3 and 18S rDNA. **Genetics and Molecular Research**. v. 10, n. 4, p. 3596–3608, 2011.

MIZOGUCHI, S. M. H. N.; MARTINS-SANTOS. I. C. Macro- and microchromosomes B in females of *Astyanax scabripinnis* (Pisces, Characidae). **Hereditas**. v. 127, p. 249–253, 1997.

MILANI, D.; CABRAL-DE-MELLO, D. C. Microsatellite organization in the grasshopper *Abracris flavolineata* (Orthoptera: Acrididae) revealed by FISH mapping: remarkable spreading in the A and B chromosomes. **PLoS One**. v. 9, p. e97956, 2014.

MIRANDE, J. M. Weighted parsimony phylogeny of the family Characidae (Teleostei: Characiformes). **Cladistics**. v. 25, p. 574–613, 2009.

MIRANDE, J. M. Phylogeny of the family Characidae (Teleostei: Characiformes): from characters to taxonomy. **Neotropical Ichthyology**. v. 8, p. 385–568, 2010.

MOREIRA-FILHO, O.; BERTOLLO, L. A. C. *Astyanax scabripinnis* (Pisces, Characidae): a species complex. **Revista Brasileira de Genética**. v. 14, p. 331–357, 1991.

MOREIRA-FILHO, O.; FENOCCHIO, A. S.; PASTORI, M. C.; BERTOLLO, L. A. C. Occurrence of a metacentric macrochromosome B in different species of the genus *Astyanax* (Pisces, Characidae, Tetragonopterinae). **Cytologia**. v. 66, p. 59–64, 2001.

MORELLI, S.; BERTOLLO, L. A. C.; FORESTI, F.; MOREIRA–FILHO, O.; TOLEDO-FILHO, S. A. Cytogenetic considerations on the genus *Astyanax* (Pisces, Characidae). I Karyotypic variability. **Caryologia**. v. 36, p. 235–244, 1983.

MORRONE, J. J. Biogeographical regions under track and cladistics scrutiny. **Journal of Biogeography**. v. 29, n. 2, p. 149−152, 2002.

NÉO, D. M.; BERTOLLO, L. A. C.; MOREIRA-FILHO, O. Morphological differentiation and possible origin of B chromosomes in natural Brazilian population of *Astyanax scabripinnis* (Pisces, Characidae). **Genetica**. v. 108, p. 211–215, 2000a.

NÉO, D. M.; MOREIRA-FILHO, O.; CAMACHO, J. P. M. Altitudinal variation for B chromosome frequency in the characid fish *Astyanax scabripinnis*. **Heredity**. v. 85, p. 136–141, 2000b.

OLIVEIRA, C.; ALMEIDA-TOLEDO, L. F.; FORESTI, F.; BRITSKI, H. A.; TOLEDO-FILHO, S. A. Chromosome formulae of Neotropical freshwater fishes. **Revista Brasileira de Genética**. v.11, p. 577-624, 1988.

OLIVEIRA, C.; AVELINO, G. S.; ABE, K. T.; MARIGUELA, T. C.; BENINE, R. C.; ORTÍ, G.; VARI, R. P.; CASTRO, R. M. C. Phylogenetic relationships within the speciose family Characidae (Teleostei: Ostariophysi: Characiformes) based on multilocus analysis and extensive ingroup sampling. **BMC Evolutionary Biology**. v. 11, p. 275, 2011.

ORNELAS-GARCÍA, C. P.; DOMÍNGUEZ-DOMÍNGUEZ, O.; DOADRIO I. Evolutionary history of the fish genus *Astyanax* Baird & Girard (1854) (Actinopterygii, Characidae) in Mesoamerica reveals multiple morphological homoplasies. **BMC Evolutionary Biology**. v. 8, p. 340, 2008.

PAGE, L. M.; BURR B. M. **A field guide to freshwater fishes of North America north of Mexico**. Houghton Mifflin Company, Boston. 1991. p. 432.

PAIZ, L. M.; BAUMGÄRTNER, L.; Da GRAÇA, W. J.; MARGARIDO, V. P. Basic cytogenetics and physical mapping of ribosomal genes in four *Astyanax* species (Characiformes, Characidae) collected in Middle Paraná River, Iguassu National Park: considerations on taxonomy and systematics of the genus. **Comparative Cytogenetics**. v. 9, n. 1, p. 51–65, 2015.

PAULS, E.; BERTOLLO, L. A. C. Evidence for a system of a supernumerary chromosomes in *Prochilodus scrofa* Steindachner, 1881 (Pisces, Prochilodontidae). **Caryologia**. v. 36, p. 207–314, 1983.

PAULS, E.; BERTOLLO, L. A. C. Distribution of a supernumerary chromosome system and aspects of karyotypic evolution in the genus *Prochilodus* (Pisces, Prochilodontidae). **Genetica**. v. 81; p. 117–123, 1990.

PENDÁS, A. M.; MORÁN, P.; FREIJE, J. P.; GARCIA-VÁSQUEZ, E. Chromosomal location and nucleotide sequence of two tandem repeats of the Atlantic salmon 5S rDNA. **Cytogenetics and Cell Genetics**. v. 67, p. 31-36, 1994.

PERES, W. A. M.; BERTOLLO, L. A. C.; MOREIRA-FILHO, O. Physical mapping of the 18S and 5S ribosomal genes in nine Characidae species (Teleostei, Characiformes). **Genetics and Molecular Biology**. v. 31, p. 222–226, 2008.

PESSENDA, G. **Estudo da organização e mapeamento cromossômico do elemento transponível *Rex*1 em espécies do gênero *Astyanax* Baird & Girard, 1854 (Chacaciformes, Characidae) provenientes de diferentes bacias hidrográficas**. Trabalho de Conclusão de Curso. 2012. 42p.

PINKEL, D.; STRUME, T.; GRAY, J. W. Cytogenetic analysis using quantitative, highsensitivity, fluorescence hibridization. **Proceedings of the National Academy Sciences**. v. 83, p. 2934-2938, 1986.

PISCOR, D.; RIBACINKO-PISCOR, D. B.; FERNANDES, C. A.; PARISE-MALTEMPI P. P. Cytogenetic analysis in three *Bryconamericus* species (Characiformes, Characidae): first description of the 5S rDNA-bearing chromosome pairs in the genus. **Molecular Cytogenetics**. v. 6, p. 13, 2013.

PISCOR, D.; ALVES, A. L.; PARISE-MALTEMPI, P. P. Chromosomal microstructure diversity in three *Astyanax* (Characiformes, Characidae) species: comparative analysis of the chromosomal locations of the 18S and 5S rDNAs. **Zebrafish**. v. 12, n. 1, p. 81–90, 2015.

PORTELA-CASTRO, A. L. B. S.; JÚLIO, H. F.; NISHIYAMA, P. B. New occurrence of microchromosomes B in *Moenkhausia sanctaefilomenae* (Pisces, Characidae) from the Paraná River of Brazil: analysis of the synaptonemal complex. **Genetica**. v. 110, p. 277−283, 2001.

PORTO-FORESTI, F.; OLIVEIRA, C.; MAISTRO, E. L.; FORESTI, F. Estimated frequency of B-chromosomes and population density of *Astyanax scabripinnis paranae* in a small stream. **Brazilian Journal of Genetics**. v. 20, p. 377–380, 1997.

RAMBAUT, A. FigTREE. http://tree.bio. ed.ac.uk/software/figtree/. Acesso em março de 2008.

REEVES, R. G.; BERMINGHAM, E. Colonization, population expansion, and lineage turnover: phylogeography of Mesoamerican characiform fish. **Biological Journal of the Linnean Society**. v. 88, n.2, p. 235−255, 2006.

ROCON-STANGE, E. R.; ALMEIDA-TOLEDO, L. F. Supernumerary B chromosomes restricted to males in *Astyanax scabripinnis* (Pisces, Characidae). **Revista Brasileira de Genética**. v. 16, p. 601–615, 1993.

SALVADOR, L. B.; MOREIRA-FILHO, O. B chromosomes in *Astyanax scabripinnis* (Pisces, Characidae). **Heredity**. v. 69, p. 50–56, 1992.

SAMBROOK, J.; RUSSELL, D. W. **Molecular Cloning: a Laboratory Manual**. vol. 3. Set. Cold Spring Harbor Laboratory Publisher, 2001.

SANTOS, L. P. **Análise citogenética de peixes dos gêneros: *Astyanax*, *Moenkhausia*, e *Bryconops* provenientes das regiões de Uberlândia-MG e Monte do Carmo-TO**. Dissertação de Mestrado. 2010. 60p.

SCHEEL, J. J. **Fish chromosome and their evolution**. In: Internal Report of Danmartes Akvarvum. Charlotherlund, Denmark. 1973. 22p.

SILVA, D. M. Z. A.; PANSONATO-ALVES, J. C.; UTSUNOMIA, R.; DANIEL, S. N.; HASHIMOTO, D. T.; OLIVEIRA, C.; PORTO-FORESTI, F.; FORESTI, F. Chromosomal organization of repetitive DNA sequences in *Astyanax bockmanni* (Teleostei, Characiformes): Dispersive location, association and co-localization in the genome. **Genetica**. v. 141, p. 329–336, 2013.

SILVA, D. M. Z. A.; PANSONATO-ALVES, J. C.; UTSUNOMIA, R.; ARAYA-JAIME, C.; RUIZ-RUANO, F. J.; DANIEL, S. N.; HASHIMOTO, D. T.; OLIVEIRA, C.; CAMACHO, J. P. M.; PORTO-FORESTI, F.; FORESTI, F. Delimiting the origin of a B chromosome by FISH mapping, chromosome painting and DNA sequence analysis in *Astyanax paranae* (Teleostei, Characiformes). **PLoS ONE**. v. 9, n. 4, p. e94896, 2014.

SILVA, D. M. Z. A.; UTSUNOMIA, R.; PANSONATO-ALVES, J. C.; OLIVEIRA, C.; FORESTI, F. Chromosomal mapping of repetitive DNA sequences in five species of *Astyanax* (Characiformes, Characidae) reveals independent location of U1 and U2 snRNA sites and association of U1 snRNA and 5S rDNA. **Cytogenetic and Genome Research**. v. 146, p. 144–152, 2015.

STRECKER, U.; BERNATCHEZ, L.; WILKENS, H. Genetic divergence between cave and surface populations of *Astyanax* in Mexico (Characidae, Teleostei). **Molecular Ecology**. v. 12, n. 3, p. 699−710, 2003.

STRECKER, U.; FAUNDEZ, V. H.; WILKENS, H. Phylogeography of surface and cave *Astyanax* (Teleostei) from Central and North America based on cytochrome b sequence data. **Molecular Phylogenetics and Evolution**. v. 33, n. 2, p. 469-481, 2004.

SUMMER, A. T. A simple techine for demonstrating centromeric heterochromation. **Experimental Cell Research**. v. 75, p. 304−306, 1972.

TENÓRIO, R. C. C. O.; VITORINO, C. A.; SOUZA, I. L.; OLIVEIRA, C.; VENERE, P. C. Comparative cytogenetics in *Astyanax* (Characiformes: Characidae) with focus on the cytotaxonomy of the group. **Neotropical Ichthyology**. v. 11, n. 3, p. 553–564, 2013.

THOMAZ, A. T.; ARCILA, D.; ORTÍ, G.; MALABARBA L. R. Molecular phylogeny of the subfamily Stevardiinae Gill, 1858 (Characiformes: Characidae): classification and the evolution of reproductive traits. **BMC Evolutionary Biology**. v. 15, p.146, 2015.

TORRES-MARIANO, A. R.; MORELLI, S. B chromosomes in a population of *Astyanax eigenmanniorum* (Characiformes, Characidae) from the Araguari River Basin (Uberlândia, MG, Brazil). **Genetics and Molecular Biology**. v. 31, p. 246–249, 2008.

VENERE, P. C.; MYIAZAWA, C. S.; GALETTI Jr., P. M. New cases of supernumerary chromosomes in characiform fishes. **Genetic and Molecular Research**. v. 22, p. 345–349, 1999.

VICARI, M. R.; NOLETO, R. B.; ARTONI, R. F.; MOREIRA-FILHO, O.; BERTOLLO, L. A. C. Comparative cytogenetics among species of the *Astyanax scabripinnis* complex. Evolutionary and biogeographical inferences. **Genetics and Molecular Biology**. v. 31, p. 173–179, 2008a.

VICARI, M. R.; ARTONI, R. F.; MOREIRA-FILHO, O.; BERTOLLO, L. A. C. Co-localization of repetitive DNAs and silencing of major rDNA genes. A case report in the fish, *Astyanax janeiroensis*. **Cytogenetic and Genome Research**. v. 122, p. 67–72, 2008b.

VICENTE, V, E.; MOREIRA-FILHO, O.; CAMACHO, J. P. M. Sex-ratio distortion associated with the presence of a B chromosome in *Astyanax scabripinnis* (Teleostei, Characidae). **Cytogenetics and Cell Genetics**. v. 74, p. 70–75, 1996.

ZALDIVAR-RIVERON, A.; LEON-REGAGNON, V.; OCA, A. N. M. Phylogeny of the Mexican coastal leopard frogs of the *Rana berlandieri* group based on mtDNA sequences. **Molecular Phylogenetics and Evolution**. v. 30, n.1, p. 38−49, 2004.

WARD, R. D.; ZEMLAK, T. S.; INNES, B. H.; LAST, P. R.; HEBERT, P. D. DNA barcoding Australia's fish species. **Philosophical Transactions of the Royal Society B: Biological Sciences**. v. 360, p. 1847–1857, 2005.

WHITE, T. J.; BRUNS, T.; LEE, S.; TAYLOR, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: INNIS, M. A.; GELFAND, D. H.; SNINSKY, J. J.; WHITE, T. J. (Eds.). **PCR Protocols: a guide to methods and applications**. Academic Press, New York, USA. 1990. 315−322p.

RESULTADOS E DISCUSSÕES

6.1. CAPÍTULO I

CHROMOSOMAL MICROSTRUCTURE DIVERSITY IN THREE *Astyanax* (CHARACIFORMES, CHARACIDAE) SPECIES: COMPARATIVE ANALYSIS OF THE CHROMOSOMAL LOCATIONS OF THE 18S AND 5S rDNAs

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Chromosomal Microstructure Diversity in Three *Astyanax* (Characiformes, Characidae) Species: Comparative Analysis of the Chromosomal Locations of the 18S and 5S rDNAs

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Abstract

The species of genus *Astyanax* is widely distributed in freshwater neotropical zones. *Astyanax* is considered to be taxonomically confused, similar to other genera placed *incertae sedis* in Characidae. The cytogenetics of this genus is well characterized; species vary widely in diploid number, from 2n = 36 chromosomes in *Astyanax schubarti* to 2n = 50 for most species studied. The size, number, and position of different cytological markers vary among species and populations of *Astyanax*. We analyzed the karyotypes of individuals from three *Astyanax* species (*Astyanax abramis*, *Astyanax altiparanae*, and *Astyanax eigenmanniorum*) from populations not previously analyzed. We describe variations in several cytogenetic markers and the karyotypic relationships between them, specifically focusing on the characteristics of the conserved and divergent locations of the ribosomal genes. Our data are useful for establishing relationships between species and for investigating the karyotype evolution within the genus.

Introduction

The genus *Astyanax* (Baird and Girard, 1854) contains ~140 species[1] and is one of the most speciose within the Characidae family. Lima *et al*.[2] suggested that *Astyanax*, which belongs to the subfamily Tetragonopterinae,[3] should be placed (along with other genera) *incertae sedis* within Characidae, due to the lack of diagnostic characteristics that support the group as a monophyletic unit. A phylogenetic hypothesis based on DNA sequences reinforces the idea that *Astyanax* is not a natural group within Characidae.[4]

In *Astyanax*, cytogenetic studies have revealed wide variations in diploid number, from 2n = 36 in *Astyanax schubarti*[5] to 2n = 50 chromosomes for most species (including *Astyanax altiparanae*, *Astyanax bockmanni*, and others[6,7]), making this genus an interesting model for evolutionary studies. Different karyotype formulae and chromosomal marker features are observed among *Astyanax* species and among populations of the same species. Domingues *et al*.[8] showed that two populations of *A. altiparanae*, one from the upper Tibagi river and the other from the upper Iguaçu river, have different karyotype formulae and fundamental numbers (FNs), in addition to differences in chromosomal markers such as C-bands, argyrophylic nucleolar organizer regions (Ag-NORs), and chromomycin A_3 (CMA_3) staining.

Ribosomal DNA (rDNA) probes have been used widely as markers in cytogenetic studies; these markers have provided valuable information regarding the dynamics and location of ribosomal clusters on chromosomes. In eukaryotes, rDNAs are organized into two families, the 45S and the 5S rDNAs, both of which are repeated in tandem. Among *Astyanax* species, and among populations of the same species, these markers have both conserved and divergent features.[9–12]

In this study, we used various cytogenetic markers to obtain new chromosomal data for *A. altiparanae* and *Astyanax eigenmanniorum*, and to describe, for the first time, the karyotype of *Astyanax abramis*. Our findings contribute to the understanding of chromosomal evolution in the genus *Astyanax*.

Materials and Methods

Specimens and classical cytogenetics

In this study, we examined three *A. abramis* (not sexed) specimens from the Bento Gomes river in Mato Grosso state (MT), Brazil; five *A. altiparanae* (three females and two

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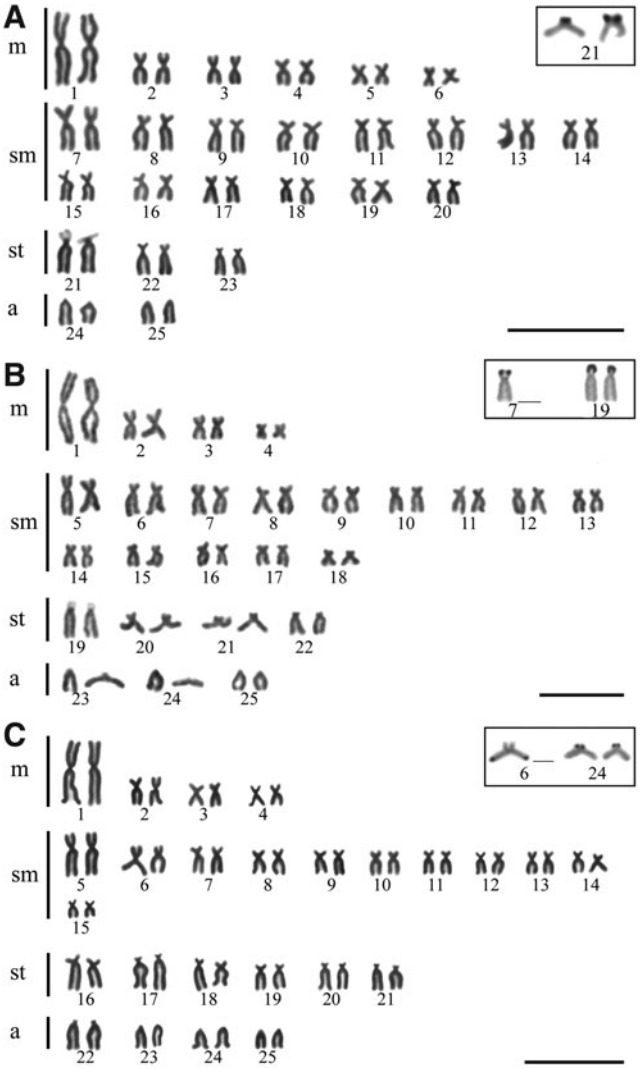

FIG. 1. Giemsa-stained chromosomes of *Astyanax*. **(A)** *Astyanax abramis.* **(B)** *Astyanax altiparanae*. **(C)** *Astyanax eigenmanniorum*. *Insets* show argyrophylic nucleolar organizer region (Ag-NOR)-bearing chromosome pairs. Scale bar = 10 μm.

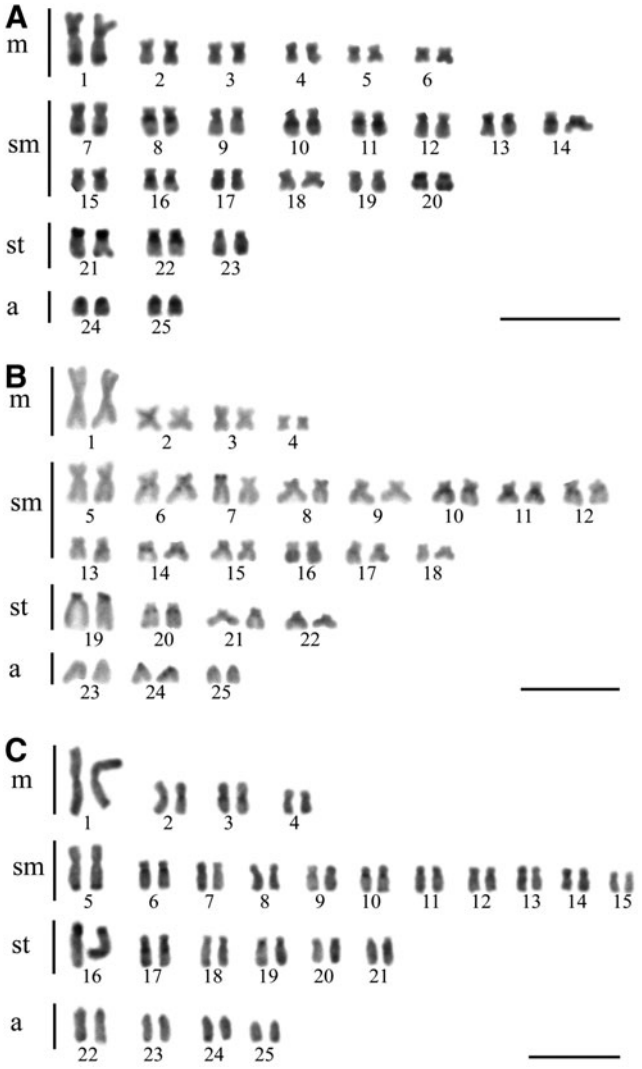

FIG. 2. Chromosomes showing the distribution of constitutive heterochromatin revealed by C-banding. **(A)** *A. abramis*. **(B)** *A. altiparanae*. **(C)** *A. eigenmanniorum*. Scale bar = 10 μm.

males) specimens from the Ribeirão Claro river in São Paulo state (SP), Brazil; and three *A. eigenmanniorum* (one female and two males) specimens from aquariophiles in Brazil. Chromosomes were obtained according to the methodology proposed by Foresti *et al.*[13] Based on the most common classification system used for fish chromosomes, the morphologies were determined according to the arms ratio: the length of the long arm (q) was divided by the length of the short arm (p). Chromosomes with two arms were classified as metacentric (m) (an arm ratio between 1 and 1.7), submetacentric (sm) (between 1.71 and 3), and subtelocentric (st) (between 3.01 and 7). Chromosomes with a single arm (arm ratio above 7) were considered acrocentric (a).

NORs were detected using silver ion impregnation as described by Howell and Black.[14] Heterochromatin was observed using the C-band technique proposed by Sumner.[15] CG- and AT-rich regions were identified by double-color CMA_3/4′,6-diamidino-2-phenylindole (DAPI) on denatured chromosomes, according to a technique commonly used in our laboratory. Briefly, our technique for CMA_3/DAPI staining is as follows: chromosomes were denatured by incubating slides in 70% formamide in 2× saline sodium citrate (SSC) at 70°C for 2 min, after the slides were immediately washed twice in cold 2× SSC for 2 min each, and then passed through a cold ethanol series (70%, 90%, and 100% for 2 min each). After drying, 30 μL of CMA_3 was added to each slide, and the slides were incubated at 4°C for 1 h in the dark. Next, slides were washed three times in 1× phosphate buffered saline for 2 min each, mounted in 30 μL of DAPI + antifade reagent, and incubated at 4°C for 1 h in the dark. Chromosomes were visualized with an Olympus BX51 microscope coupled to a digital camera (Olympus model D71), and images were captured using the DP Controller software.

DNA extraction and probe synthesis

Genomic DNA was extracted from fin samples as described by Sambrook and Russell.[16] The 18S rDNA probe

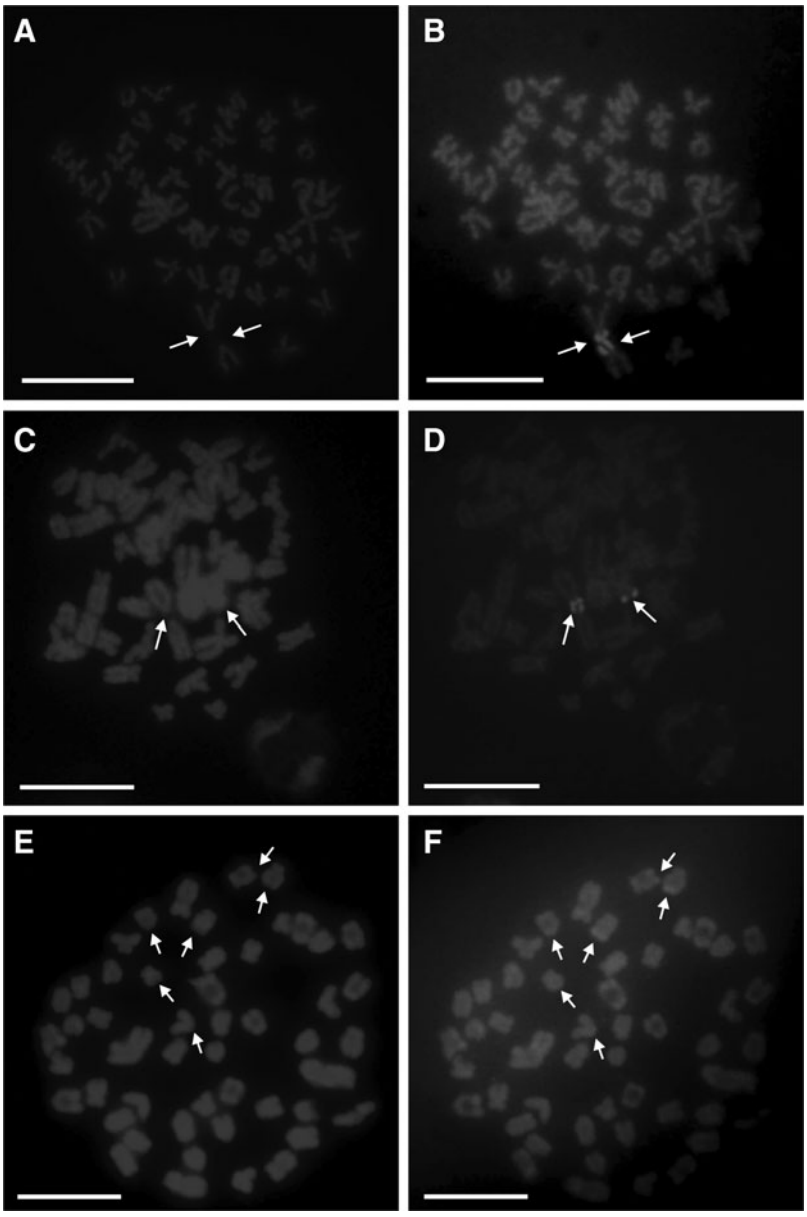

FIG. 3. Sequential chromomycin A_3/4′,6-diamidino-2-phenylindole (CMA_3/DAPI) staining. **(A, B)** *A. abramis.* **(C, D)** *A. altiparanae*. **(E, F)** *A. eigenmanniorum*. The *arrows* in **A, C** and **E** indicate DAPI and in **B, D** and **F** CMA3. Scale bar = 10 μm.

was obtained by polymerase chain reaction (PCR) using the primers NS1, 5′-GTAGTCATATGCTTGTCTC-3′, and NS8, 5′-TCCGCAGGTTCACCTACGGA-3′, as described by White *et al*.[17] The 5S rDNA probe was obtained by PCR using the primers A, 5′-TACGCCCGATCTCGTCCGATC-3′, and B, 5′-CAGGCTGGTATGGCCGTAAGC-3′, as described by Pendás *et al*.[18] as well as Martins and Galetti.[19]

Fluorescent in situ *hybridization*

The fluorescent *in situ* hybridization (FISH) technique used 18S and 5S rDNA probes tagged with digoxigenin-11-dUTP (Roche) and biotin-16-dUTP (Roche), according to the method of Pinkel *et al*.,[20] with modifications described by Marreta *et al*.[21] Slides prepared with metaphase chromosomes were incubated with RNase (20 ng/μL) for 1 h at 37°C and dehydrated using an ethanol series (70%, 90%, and 100%). Chromosomal DNA was then denatured for 2–3 min in 70% formamide in 2× SSC at 70°C, and then dehydrated immediately using an ethanol series (70%, 90%, and 100%). Hybridization solution (15 μL of formamide, 3 μL of 20× SSC, 6 μL of dextran sulfate, and 6 μL of rDNA probe) was incubated for 10 min at 95°C, and then applied to each slide. After overnight hybridization at 37°C, the slides were washed twice for 5 min each with 50% formamide in 2× SSC (pH 7.0) at 45°C, twice for 5 min each with 1× SSC at 45°C, and once for 5 min with 4× Triton (100 mL of 20× SSC, 400 mL of H_2O, and 250 μL of Triton X-100). Signals were detected using anti-digoxigenin-rhodamine (Roche) for digoxigenin and avidin-FITC (Sigma) for biotin. Chromosomes were counter-stained with DAPI, and slides were mounted using the Vectashield Mounting Medium.

Results

The *A. abramis* specimens had 2n = 50 chromosomes, and the karyotype contained 12 metacentric, 28 submetacentric, 6 subtelocentric, and 4 acrocentric chromosomes (12m + 28sm +

6st + 4a), yielding a FN of 96. A region of secondary constriction was evident in pair 21 (Fig. 1A). Heterochromatic regions were observed mainly in pericentromeric regions (pairs 1, 8, 10, 11, 13–16, 22, 24, and 25), and it was possible to see terminal blocks (pairs 1, 7, 20, and 23) (Fig. 2A). We also observed a large block of heterochromatin in the region, corresponding to the secondary constriction of pair 21 (Fig. 2A). The Ag-NORs, GC-rich regions, and 18S rDNA sites were also observed in the secondary constriction of pair 21 (Figs. 1A, 3B and 4A, respectively). The 5S rDNA sequences were located in the pericentromeric region on the long arms of pairs 2 and 23 (Fig. 4B).

The *A. altiparanae* specimens had 2n = 50 chromosomes, and the karyotype contained 8m + 28sm + 8st + 6a (FN = 94). A region of secondary constriction was evident in pair 19 (Fig. 1B). Three chromosomes contained Ag-NORs: pair 19 and one homolog of pair 7 (Fig. 1B). Blocks of heterochromatin were present in the pericentromeric regions of seven pairs (6, 10–12, 14, 20, and 24) and on the short arms of the same three Ag-NOR-bearing chromosomes (pair 19 and one homolog of pair 7) (Fig. 2B). GC-rich regions were also present in the same places as the Ag-NORs (Fig. 3D; note that the both 19 homologous chromosomes are connected by the terminal regions). The 18S rDNA probe labeled only the terminal regions of pair 19, not pair 7 (Fig. 4C). The 5S rDNA probe labeled the pericentromeric region of pair 5 (Fig. 4D).

A. eigenmanniorum had 2n = 50 chromosomes, and the karyotype contained 8m + 22sm + 12st + 8a (FN = 92) (Fig. 1C). Ag-NORs were observed in the terminal regions of the short arms of pair 24 and on the long arm of one homolog of pair 6 (Fig. 1C). Heterochromatin was evident in centromeric (pairs 2 and 19), pericentromeric (pairs 3, 6, 10, and 17), and terminal (pairs 5 and 16) regions (Fig. 2C), and GC-rich regions were detected in six chromosomes (pairs 6, 14, and 24) (Fig. 3F). The 18S rDNA sites occurred on the short arms of pairs 1, 14, and 24, and on the long arms of pairs 5 and 6, always in the terminal region (Fig. 4E). The 5S rDNA sites were located near the centromeres of pairs 2 and 22 (Fig. 4F).

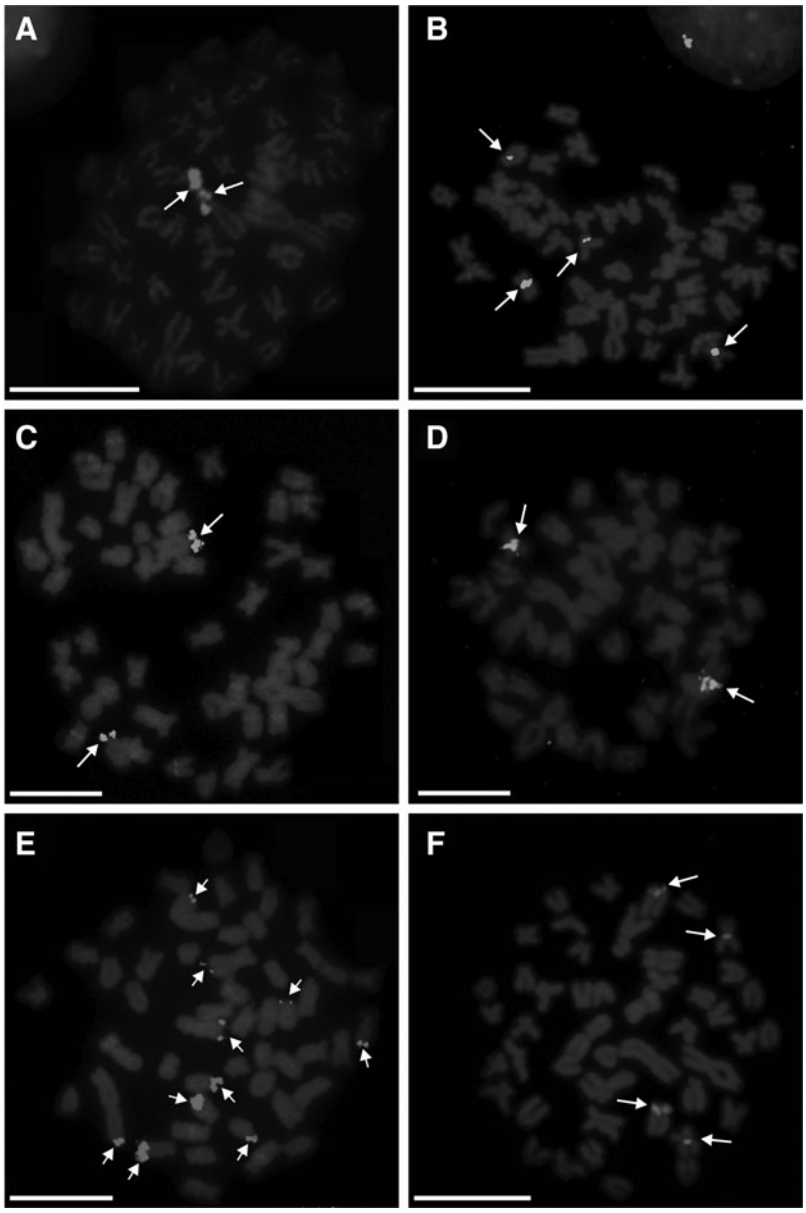

FIG. 4. Chromosomes of *Astyanax* species after fluorescent *in situ* hybridization to detect 18S ribosomal DNA (rDNA) and 5S rDNA. **(A, B)** *A. abramis.* **(C, D)** *A. altiparanae*. **(E, F)** *A. eigenmanniorum*. **(A, C,** and **E)** 18S rDNA probe. **(B, D,** and **F)** 5S rDNA probe. The *arrows* indicate the marked chromosomes. Scale bar = 10 μm.

In Figure 5, data on the chromosomal location of 5S rDNA in the three *Astyanax* species are summarized and compared with data from the literature on the chromosomal locations of this gene in other species of this genus.

Discussion

We observed variation in the Ag-NOR number, indicating that these regions are constantly in change of activity. Other authors described different numbers of chromosomes bearing NORs in the genus *Astyanax*.[6,10,22–25] According to Tenório *et al*.,[11] six *Astyanax* species had different Ag-NOR numbers. One pair was observed in *Astyanax argyrimarginatus*, *Astyanax* aff. *bimaculatus*, *Astyanax Elachylepis*, and *Astyanax* sp., whereas four pairs were observed in *Astyanax xavante* and two pairs in *A. altiparanae*. This variation in Ag-NOR number was also observed in *Astyanax scabripinnis* and *Astyanax fasciatus*.[24,26] According to Ferro *et al*.,[24] multiple Ag-NORs occur in *A. scabripinnis*, and this condition may be typical of *Astyanax* species (see e.g., Refs.[27–29]).

Several authors have pointed out that silver impregnation may or may not stain regions of heterochromatin, and that heterochromatin can also be stained with GC-specific fluorochromes.[30–34] According to Vitturi *et al*.,[35] regions marked by silver impregnation react positively to C-banding and fluoresce brightly after CMA_3 treatment, indicating that (in the scarab beetle *Thorectes intermedius* [Coleoptera]) the silver impregnates all heterochromatin and that heterochromatin consists of a highly repetitive DNA, GC-rich. In our study, one homolog of pair 7 in *A. altiparanae* was silver, CMA_3, and C-band positive (like pair 19), but was not marked by the 18S rDNA probe in FISH experiments, unlike pair 19. This suggests that this region in the homologous of pair 7 contains repetitive DNA that is very GC-rich, possibly representing a specific class of repetitive DNA.

In *Astyanax*, previous studies have found that the chromosomal locations of 18S rDNA are highly diverse among populations of the same species, as we also observed in this study (Table 1). Mantovani *et al*.[9] observed variations in the numbers of 18S rDNA sites in four *A. scabripinnis* populations. Two populations from the Paranapanema river basin (SP) had 4–16 sites, and two populations from the São Francisco river (Minas Gerais state) had 2–11 sites. The authors reported that the location of the 18S rDNA was variable in the *scabripinnis* complex, whereas the location of 5S rDNA was conserved; thus, multiple evolutionary trends for ribosomal gene placement may exist in the same genome.[9]

The 5S rDNA location on the chromosomes of some *Astyanax* species is conserved and this location has three configurations (Fig. 5). One configuration has 5S rDNA sites on both long arms near the centromeres of a metacentric chromosome pair and an acrocentric or subtelocentric pair—a configuration found in *A. fasciatus*, *A. scabripinnis*, *Astyanax parahybae*, *Astyanax* sp. B, *Astyanax paranae*, and *A. bockmanni*, as well as *A. eigenmanniorum* and *A. abramis* (described in this study).[7,36–39] Another configuration of 5S rDNA location is a pericentromeric site on a submetacentric pair, as seen in different *A. altiparanae* populations, including the population described in this article, as well as for *A. bimaculatus* and *Astyanax lacustris*.[8,10,36,40] A third configuration, consisting of

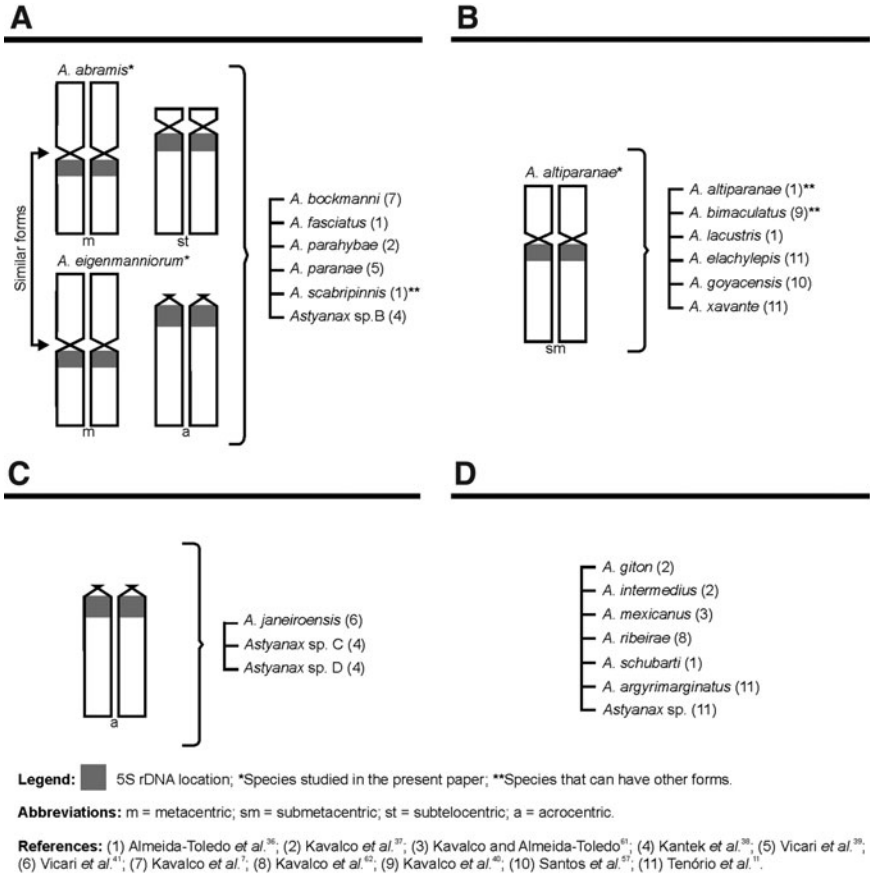

FIG. 5. Scheme showing the 5S rDNA location in *Astyanax* chromosomes. **(A)** First form. **(B)** Second form. **(C)** Third form. **(D)** Variant forms.

TABLE 1. LITERATURE REVIEW OF THE NUMBER OF CHROMOSOMES BEARING RIBOSOMAL DNA IN *ASTYANAX* SPECIES

Species	*Locality/city*	*State*	*5S*[a]	*18S*[b]	*References*
Astyanax altiparanae	Mogi-Guaçu river	SP	2	4	Almeida-Toledo *et al.*[36]
	Tibagi river	PR	2	7	Domingues *et al.*[8]
	Iguaçu river	PR	2	2	Domingues *et al.*[8]
	Keçaba stream	PR	2	7	Fernandes and Martins-Santos[10]
	Paraná river	PR	2	4	Fernandes and Martins-Santos[10]
	Tatupeba stream	PR	2	4	Fernandes and Martins-Santos[10]
	Maringá stream	PR	2	4	Fernandes and Martins-Santos[10]
	Monjolinho stream	SP	2	2	Peres *et al.*[46]
	Pântano stream	SP	2	2	Ferreira-Neto *et al.*[47]
	Feijão stream	SP	2	4	Ferreira-Neto *et al.*[47]
	Jordão river	PR	2	6	Ferreira-Neto *et al.*[47]
	Paraitinga river	SP	2	3	Kavalco *et al.*[40]
	Paranapanema river	SP	2	4	Kavalco *et al.*[40]
	Batalha river	SP	2	—	Hashimoto *et al.*[48]
	Água dos Patos stream	SP	2	4	Pacheco *et al.*[28]
	Igapó lake	PR	2	2	Pacheco *et al.*[28]
	Monjolinho stream	—	4	2	Tenório *et al.*[11]
Astyanax argyrimarginatus	Jaraguá stream	—	4	2	Tenório *et al.*[11]
Astyanax aff. *bimaculatus*	Ribeira de Iguape river	SP	2	3	Kavalco *et al.*[40]
	Guapimirim river	RJ	2	2	Kavalco *et al.*[40]
[c]	São Francisco river	—	2	2	Peres *et al.*[49]
[c]	Grande river	—	2	2	Peres *et al.*[49]
[c]	Piumhi river	—	2	2	Peres *et al.*[49]
	Dois de Agosto stream	—	4	2	Tenório *et al.*[11]
Astyanax bockmanni	Paranapanema river basin	SP	4	8	Kavalco *et al.*[7]
	Alambari river	SP	4	—	Hashimoto *et al.*[48]
	Alambari river	SP	—	7	Daniel *et al.*[50]
Astyanax eigenmanniorum	Eldorado do Sul	RS	—	3-4[d]	Mendes *et al.*[51]
Astyanax elachylepis	Taquaralzinho stream	—	2	2	Tenório *et al.*[11]
Astyanax fasciatus	Mogi-Guaçu river	SP	4	6	Almeida-Toledo *et al.*[36]
	Mogi-Guaçu river	MG	4	2	Pazza *et al.*[52]
	Mogi-Guaçu river	SP	4	2	Pazza *et al.*[52]
	Mogi-Guaçu river	SP	4	2	Pazza *et al.*[52]
	Mogi-Guaçu river basin	SP	4	6	Pazza *et al.*[53]
	Paranapanema river basin	SP	4	4	Pazza *et al.*[53]
	Grande river	—	4	3	Peres *et al.*[54]
	Piumhi river	—	4	6	Peres *et al.*[54]
	São Francisco river	—	6	2	Peres *et al.*[54]
	Rosas stream	PR	4	8	Fernandes *et al.*[55]
	Campo Novo river	SP	4	—	Hashimoto *et al.*[48]
	Água da Madalena stream	SP	4	10-10+B[e]	Ferreira-Neto *et al.*[56]
Astyanax giton	Paraitinga river	SP	—	7	Kavalco and Moreira-Filho[25]
	Paraitinga river	SP	10	—	Kavalco *et al.*[37]
Astyanax goyacensis	Taboquinha stream	TO	2	4	Santos *et al.*[57]
Astyanax hastatus	Guapimim river basin	RJ	—	6	Kavalco *et al.*[58]
Astyanax intermedius	Paraitinga river	SP	—	10	Kavalco and Moreira-Filho[25]
	Paraitinga river	SP	10	—	Kavalco *et al.*[37]
Astyanax janeiroensis	Sacovão and Açungui rivers	PR	2	20	Vicari *et al.*[41]
Astyanax jacuhiensis	Guaíba lake and tributaries	RS	—	2	Pacheco *et al.*[59]
Astyanax lacustris	São Francisco river basin	MG	2	4	Almeida-Toledo *et al.*[36]
	São Francisco river	MG	2	2	Peres *et al.*[46]
Astyanax laticeps	Forquetinha river	RS	—	3	Rosa *et al.*[60]
Astyanax mexicanus	Aquarium	—	6	8	Kavalco and Almeida-Toledo[61]
Astyanax paranae	Verde river	PR	4	13	Vicari *et al.*[39]
	Açungui river	PR	4	13	Vicari *et al.*[39]

(*continued*)

TABLE 1. (CONTINUED)

Species	*Locality/city*	*State*	*5S*[a]	*18S*[b]	*References*
Astyanax parahybae	Paraitinga river	SP	—	7	Kavalco and Moreira-Filho[25]
	Paraitinga river	SP	4	—	Kavalco *et al.*[37]
Astyanax ribeirae	Ribeira de Iguape river basin	SP	6	4	Kavalco *et al.*[62]
Astyanax scabripinnis	Lavrinha farm stream	SP	8	6	Ferro *et al.*[24]
	Fojo stream	SP	8	9	Ferro *et al.*[24]
	Capivari lagoon	SP	8	4	Ferro *et al.*[24]
	Carpas lake	SP	8	7	Ferro *et al.*[24]
	Canta Galo brook	SP	—	6-8[e]	Souza *et al.*[63]
	Tietê river	SP	4	4	Almeida-Toledo *et al.*[36]
	Macacos river	SP	—	6	Kavalco and Moreira-Filho[25]
	Macacos river	SP	6	—	Kavalco *et al.*[37]
	Centenário stream	PR	4	6-15-16[e]	Mantovani *et al.*[9]
	Marrecas stream	PR	6	4-12-16[e]	Mantovani *et al.*[9]
	Viveiro de Mudas stream	MG	4	4-7-8-11[e]	Mantovani *et al.*[9]
	Curral das Éguas stream	MG	4	2-3-4[e]	Mantovani *et al.*[9]
	Tatupeba stream	PR	4	14-16[e]	Fernandes and Martins-Santos[29]
	São Francisco river	MG	4	5	Peres *et al.*[46]
[f]	Santo Antônio river	PR	4	4-8[e]	Vicari *et al.*[39]
[f]	Jaguariaíva river	PR	4	8	Vicari *et al.*[39]
	Pedras stream	SP	6	5	Vicari *et al.*[64]
	Cascatinha stream	—	2	4	Santos *et al.*[12]
	Funari stream	—	2	6	Santos *et al.*[12]
	Barbosa waterfall	—	2	6	Santos *et al.*[12]
	Campão bonito stream	—	2	6	Santos *et al.*[12]
Astyanax schubarti	Mogi-Guaçu river	SP	4	4	Almeida-Toledo *et al.*[36]
Astyanax sp.	Grande stream	—	4	2	Tenório *et al.*[11]
Astyanax sp. B	Curitiba	PR	4	2	Kantek *et al.*[38]
Astyanax sp. C	Curitiba	PR	2	2	Kantek *et al.*[38]
Astyanax sp. D	Bicudo river	PR	2	15-17[e]	Kantek *et al.*[38]
Astyanax xavante	Avoadeira stream	—	2	6	Tenório *et al.*[11]

[a]5S rDNA.
[b]18S rDNA.
[c]*Astyanax* of the *bimaculatus* group.
[d]Interindividual variation.
[e]Intrapopulation variation.
[f]Unidentified species of the *Astyanax scabripinnis* species complex.
MT, Mato Grosso state; SP, São Paulo state; PR, Paraná state; RJ, Rio de Janeiro state; RS, Rio Grande do Sul state; MG, Minas Gerais state; TO, Tocantins state; rDNA, ribosomal DNA.

an acrocentric pair containing 5S rDNA in the pericentromeric position, is shared among *Astyanax janeiroensis*, *Astyanax* sp. C, and *Astyanax* sp. D.[38,41] According to Vicari *et al.*,[39] *Astyanax* species that have one pair of chromosomes bearing 5S rDNA sites exhibit a probable synapomorphic feature; similarly, species that have two pairs of chromosomes bearing 5S rDNA sites may also share a feature.

Telomeric regions favor the exchange of genetic material due to their proximity in the interphase nucleus, promoted by the ordering of chromosomes based on Rabl's model.[42] Consequently, because most 45S rDNA sequences are located in the terminal regions of many *Astyanax* chromosomes, this proximity could facilitate the transfer of these sequences to other regions. This could explain the large intra- and interspecific divergence of 18S rDNA locations in this group of fish and may explain why 5S rDNA configurations are conserved in some species: these species do not have telomeric 5S DNA sites, but instead have one or two sites near the centromere.

The action of transposable elements might also explain the divergence in the locations of 18S rDNA sequences among *Astyanax* species. The presence of different pairs containing 45S rDNA sites in *Astyanax* species may have arisen as a result of transposable elements inserting at these loci and transferring these sequences to other chromosomes, resulting in the reorganization of these on different chromosome pairs. Transposable elements have been proposed as one of the mechanisms responsible for the mobility of rDNA sequences to new sites.[43,44] According to Nakajima *et al*.,[45] the terminal regions of chromosomes can promote transposition events, leading to the dispersal of DNA segments.

Therefore, the conservation of 5S rDNA and the variability of 18S rDNA sites in different species, as demonstrated in this study, indicate that independent changes occur in the genome for these sequences, which can be caused by their location in different compartments of the interphase nucleus. Thus, these sequences may be subject to dynamics that can either maintain the conservation of their

location or contribute to their divergence by dispersing them to other regions of the genome.

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References

1. Eschmeyer WN, Fricke R. Catalog of fishes electronic version. 2014. Available at http://research.calacademy.org/research/ichthyology/catalog/fishcatmain.asp. (Accessed 09 April 2014).
2. Lima FCT, Malabarba LR, Buckup PA, Pezzi-da-Silva JF, Vari RP, Harold A, *et al.*: Genera *Incertae sedis* in Characidae. In: Check List of the Freshwater Fishes of South and Central America. Reis RE, Kullander SE, and Ferraris CJ Jr. (eds), pp. 106–169, Edipucrs, Porto Alegre, 2003.
3. Géry J: Characoids of the World. T.F.H. Publications, Inc., Neptune City, NJ, 1977.
4. Oliveira C, Avelino GS, Abe KT, Mariguela TC, Benine RC, Ortí G, *et al.* Phylogenetic relationships within the speciose family Characidae (Teleostei: Ostariophysi: Characiformes) based on multilocus analysis and extensive ingroup sampling. BMC Evol Biol 2011;11:1–25.
5. Morelli S, Bertollo LAC, Foresti F, Moreira-Filho O, Almeida-Toledo-Filho S. Cytogenetic considerations on the genus *Astyanax* (Pisces, Characidae). I. Karyotypic variability. Caryologia 1983;36:235–244.
6. Fernandes CA, Martins-Santos IC. Cytogenetics studies in two populations of *Astyanax altiparanae* (Pisces, Characiformes). Hereditas 2004;141:328–332.
7. Kavalco KF, Pazza R, Almeida-Toledo LF. *Astyanax bockmanni* Vari and Castro, 2007: an ambiguous karyotype in the *Astyanax* genus. Genetica 2009;136:135–139.
8. Domingues MS, Vicari MR, Abilhoa V, Wamser JP, Cestari MM, Bertollo LAC, *et al*. Cytogenetic and comparative morphology of two allopatric populations of *Astyanax altiparanae* Garutti & Britski, 2000 (Teleostei: Characidae) from upper rio Paraná basin. Neotrop Ichthyol 2007;5:37–44.
9. Mantovani M, Abel LDS, Moreira-Filho O. Conserved 5S and variable 45S rDNA chromosomal localisation revealed by FISH in *Astyanax scabripinnis* (Pisces, Characidae). Genetica 2005;123:211–216.
10. Fernandes CA, Martins-Santos IC. Mapping of the 18S and 5S ribosomal RNA genes in *Astyanax altiparanae* Garutti & Britski, 2000 (Teleostei, Characidae) from the upper Paraná river basin, Brazil. Genet Mol Biol 2006;29:464–468.
11. Tenório RCCO, Vitorino CA, Souza IL, Oliveira C, Venere PC. Comparative cytogenetics in *Astyanax* (Characiformes: Characidae) with focus on the cytotaxonomy of the group. Neotrop Ichthyol 2013;11:553–564.
12. Santos NM, Ferreira-Neto M, Artoni RF, Vicari MR, Bakkali M, Oliveira C, *et al.* A comparative structural cytogenetic study in three allopatric populations of *Astyanax scabripinnis* (Teleostei: Characidae). Zoologia 2012;29:159–166.
13. Foresti F, Almeida-Toledo LF, Toledo-Filho SA. Polymorphic nature of nucleous organizer regions in fishes. Cytogenet Cell Genet 1981;31:137–144.
14. Howell WM, Black DA. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 1980;36:1014–1015.
15. Sumner AT. A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 1972;75:304–306.
16. Sambrook J, Russell DW: Molecular Cloning: A Laboratory Manual. 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001.
17. White TJ, Bruns T, Lee S, Taylor J: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols—A Guide to Methods and Applications. Innis MA, Gelfand DH, Sninsky JJ, and White TJ (eds), pp. 315–322, Academic Press, NY, 1990.
18. Pendás AM, Morán P, Freije JP, Garcia-Vásquez E. Chromosomal location and nucleotide sequence of two tandem repeats of the Atlantic salmon 5S rDNA. Cytogenet Cell Genet 1994;67:31–36.
19. Martins C, Galetti PM Jr. Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chromosome Res 1999;7:363–367.
20. Pinkel D, Straume T, Gray JW. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci U S A 1986;83:2934–2938.
21. Marreta ME, Faldoni FLC, Parise-Maltempi PP. Cytogenetic mapping of the W chromosome in the genus *Leporinus* (Teleostei, Anastomidae) using a highly repetitive DNA sequence. J Fish Biol 2012;80:630–637.
22. Maistro EL, Foresti F, Oliveira C. Comparative cytogenetic and morphological analysis of *Astyanax scabripinnis paranae* (Pisces, Characidae, Tetragonopterinae). Genet Mol Biol 1998;21:201–206.
23. Maistro EL, Oliveira C, Foresti F. Sympatric occurrence of two cytotypes of *Astyanax scabripinnis* (Characiformes, Characidae). Genet Mol Biol 2000;23:365–369.
24. Ferro DAM, Néo DM, Moreira-Filho O, Bertollo LAC. Nucleolar organizing regions, 18S and 5S rDNA in *Astyanax scabripinnis* (Pisces, Characidae): populations distribution and functional diversity. Genetica 2001;110:55–62.
25. Kavalco KF, Moreira-Filho O. Cytogenetical analyses in four species of the genus *Astyanax* (Pisces, Characidae) from Paraíba do Sul river basin. Caryologia 2003;56:453–461.
26. Medrado AS, Figueiredo AVA, Waldschmidt AM, Affonso PRAM, Carneiro PLS. Cytogenetic and morphological diversity in populations of *Astyanax fasciatus* (Teleostei, Characidae) from Brazilian northeastem river basins. Genet Mol Biol 2008;31:208–214.
27. Yano CF, Moreira-Filho O, Margarido VP. Interpopulational comparative cytogenetics analysis among three *Astyanax* (Characiformes*: Incertae sedis*) species of two streams of upper Paraná river basin, Brazil. Biologia 2014;69:790–798.
28. Pacheco RB, Rosa R, Giuliano-Caetano L, Júlio HF Jr., Dias AL. Cytogenetic comparison between two allopatric populations of *Astyanax altiparanae* Garutti et Britski, 2000 (Teleostei, Characidae), with emphasis on the localization of 18S and 5S rDNA. Comp Cytogenet 2011;5:237–246.
29. Fernandes CA, Martins-Santos IC. Chromosomal location of 5S and 18S rRNA genes in three sympatric cytotypes of *Astyanax scabripinnis* (Characiformes, Characidae) from the Ivaí river basin, state of Paraná, Brazil. Caryologia 2006;59:253–259.
30. Gornung E, Gabrielli I, Cataudella S, Sola L. CMA_3-banding pattern and fluorescent *in situ* hybridization with ribosomal genes in zebrafish chromosomes. Chromosome Res 1997;5:40–46.

31. Rossi AR, Gornung E, Crosetti D. Cytogenetic analysis of *Liza ramada* (Pisces, Perciformes) by different staining techniques and fluorescent *in situ* hybridization. Heredity 1997;79:83–87.
32. Artoni RF, Molina WF, Bertollo LAC, Galetti PM Jr. Heterochromatin analysis in the fish species *Liposarcus anisitsi* (Siluriformes) and *Leporinus elongates* (Characiformes). Genet Mol Biol 1999;22:39–44.
33. Margarido VP, Galetti PM Jr. Amplification of a GC-rich heterochromatin in the freshwater fish *Leporinus desmotes* (Characiformes, Anostomidae). Genet Mol Biol 2000;23: 569–573.
34. Artoni RF, Bertollo LAC. Evolutionary aspects of the ZZ/ZW sex chromosome system in the Characidae fish, genus *Triportheus*. A monophyletic state and NOR location on the W chromosome. Heredity 2002;89:15–19.
35. Vitturi R, Colomba MS, Barbieri R, Zunino M. Ribosomal DNA location in the scarab beetle *Thorectes intermedius* (Costa) (Coleoptera: Geotrupidae) using banding and fluorescent *in-situ* hybridization. Chromosome Res 1999;7: 255–260.
36. Almeida-Toledo LF, Ozouf-Costaz C, Foresti F, Bonillo C, Porto-Foresti F, Daniel-Silva MFZ. Conservation of the 5S-bearing chromosome pair and co-localization with major rDNA clusters in five species of *Astyanax* (Pisces, Characidae). Cytogenet Genome Res 2002;97:229–233.
37. Kavalco KF, Pazza R, Bertollo LAC, Moreira-Filho O. Gene mapping of 5S rDNA sites in eight fish species from the Paraíba do Sul river basin, Brazil. Cytogenet Genome Res 2004;106:107–110.
38. Kantek DLZ, Noleto RB, Maurutto FAM, Bertollo LAC, Moreira-Filho O, Cestari MM. Cytotaxonomy of *Astyanax* (Characiformes, Characidae) from the Upper Iguaçu river basin: confirmation of the occurrence of distinct evolutionary units. J Fish Biol 2008;73:2012–2020.
39. Vicari MR, Noleto RB, Artoni RF, Moreira-Filho O, Bertollo LAC. Comparative cytogenetics among species of the *Astyanax scabripinnis* complex. Evolutionary and biogeographical inferences. Genet Mol Biol 2008;31:173–179.
40. Kavalco KF, Pazza R, Brandão KO, Garcia C, Almeida-Toledo LF. Comparative cytogenetics and molecular phylogeography in the group *Astyanax altiparanae-Astyanax* aff. *bimaculatus* (Teleostei, Characidae). Cytogenet Genome Res 2011;134:108–119.
41. Vicari MR, Artoni RF, Moreira-Filho O, Bertollo LAC. Colocalization of repetitive DNAs and silencing of major rRNA genes. A case report of the fish *Astyanax janeiroensis*. Cytogenet Genome Res 2008;122:67–72.
42. Schweizer D, Loidl J. A model for heterochromatin dispersion and the evolution of C-band patterns. Chromosome Today 1987;9:61–74.
43. Raskina O, Belyayev A, Nevo E. Activity of the *En*/*Spm*-like transposons in meiosis as a base for chromosome repatterning in a small, isolated, peripheral population of *Aegilops speltoides* Tausch. Chromosome Res 2004;12:153–161.
44. Raskina O, Barber JC, Nevo E, Belyayev A. Repetitive DNA and chromosomal rearrangements: speciation-related events in plant genomes. Cytogenet Genome Res 2008; 120:351–357.
45. Nakajima RT, Cabral-de-Mello DC, Valente GT, Venere PC, Martins C. Evolutionary dynamics of rRNA gene clusters in cichlid fish. BMC Evol Biol 2012;12:198.
46. Peres WAM, Bertollo LAC, Moreira-Filho O. Physical mapping of the 18S and 5S ribosomal genes in nine Characidae species (Teleostei, Characiformes). Genet Mol Biol 2008;31:222–226.
47. Ferreira-Neto M, Vicari MR, Camargo EF, Artoni RF, Moreira-Filho O. Comparative cytogenetics among populations of *Astyanax altiparanae* (Characiformes, Characidae, *Incertae sedis*). Genet Mol Biol 2009;32792–796.
48. Hashimoto DT, Ferguson-Smith MA, Rens W, Foresti F, Porto-Foresti F. Chromosome mapping of H1 histone and 5S rRNA gene clusters in three species of *Astyanax* (Teleostei, Characiformes). Cytogenet Genome Res 2011; 134:64–71.
49. Peres WAM, Bertollo LAC, Buckup PA, Blanco DR, Kantek DLZ, Moreira-Filho O. Invasion, dispersion and hybridization of fish associated to river transposition: karyotypic evidence in *Astyanax* ''*bimaculatus* group'' (Characiformes: Characidae). Rev Fish Biol Fish 2012;22:519–526.
50. Daniel SN, Hashimoto DT, Pansonato-Alves JC, Foresti F, Porto-Foresti F. Cytogenetic characterization of distinct B chromosomes in a population of the fish *Astyanax bockmanni* (Teleostei, Characiformes). Caryologia 2012;65:229–233.
51. Mendes MM, Rosa R, Giuliano-Caetano L, Dias AL. Karyotype diversity of four species of the *incertae sedis* group (Characidae) from different hydrographic basins: analysis of AgNORs, CMA3 and 18S rDNA. Genet Mol Res 2011;10:3596–3608.
52. Pazza R, Kavalco KF, Bertollo LAC. Chromosome polymorphism in *Astyanax fasciatus* (Teleostei, Characidae). 1. Karyotype analysis, Ag-NORs and mapping of the 18S and 5S ribosomal genes in sympatric karyotypes and their possible hybrid forms. Cytogenet Genome Res 2006;112: 313–319.
53. Pazza R, Kavalco SAF, Penteado PR, Kavalco KF, Almeida-Toledo LF. The species complex *Astyanax fasciatus* Cuvier (Teleostei, Characiformes)—a multidisciplinary approach. J Fish Biol 2008;72:2002–2010.
54. Peres WAM, Buckup PA, Kantek DLZ, Bertollo LAC, Moreira-Filho O. Chromosomal evidence of downstream dispersal of *Astyanax fasciatus* (Characiformes, Characidae) associated with river shed interconnection. Genetica 2009;137:305–311.
55. Fernandes CA, Martins-Santos IC, Bailly D. Karyotype analysis and mapping of the 18S and 5S ribosomal genes in *Astyanax fasciatus* (Teleostei, Characiformes) from Paranapanema river basin. Cytologia 2009;74:295–300.
56. Ferreira-Neto M, Artoni RF, Vicari MR, Moreira-Filho O, Camacho JPM, Bakkali M, *et al.* Three sympatric karyomorphs in the fish *Astyanax fasciatus* (Teleostei, Characidae) do not seem to hybridize in natural populations. Comp Cytogenet 2012;6:29–40.
57. Santos LP, Castro JP, Francisco CM, Vicari MR, Almeida MC, Goll LG, *et al.* Cytogenetic analysis in the neotropical fish *Astyanax goyacensis* Eigenmann, 1908 (Characidae, *Incertae sedis*): karyotype description and occurrence of B microchromosomes. Mol Cytogenet 2013;6:48.
58. Kavalco KF, Brandão KO, Pazza R, Almeida-Toledo LF. *Astyanax hastatus* Myers, 1928 (Teleostei, Characidae): a new species complex within the genus *Astyanax*? Genet Mol Biol 2009;32:477–483.
59. Pacheco RB, Giuliano-Caetano L, Júlio HF Jr., Dias AL. Cytogenetic data on *Astyanax jacuhiensis* (Characidae) in the lago Guaíba and tributaries, Brazil. Neotrop Ichthyol 2010;8:667–671.
60. Rosa R, Rubert M, Malabarba LR, Martins-Santos IC, Giuliano-Caetano L. Cytogenetic analysis of *Astyanax laticeps*

(Cope, 1894) (Ostariophysi: Characidae) from the laguna dos Patos system. Neotrop Ichthyol 2009;7:601–605.
61. Kavalco KF, Almeida-Toledo LF. Molecular cytogenetics of blind Mexican Tetra and comments on the karyotypic characteristics of genus *Astyanax* (Teleostei, Characidae). Zebrafish 2007;4:103–111.
62. Kavalco KF, Pazza R, Almeida-Toledo LF. Molecular cytogenetics of *Astyanax ribeirae* (Teleostei, Characidae), an endemic characin of the Atlantic rainforest. Nucleus 2010; 53:51–54.
63. Souza IL, Galián J, De-La-Rúa P, Bertollo LAC, Moreira-Filho O. Non-random Distribution of the GC-rich heterochromatin and nucleolar rDNA sites on *Astyanax scabripinnis* chromosomes. Cytologia 2001;66:85–91.
64. Vicari MR, Pistune HFM, Castro JP, Almeida MC, Bertollo LAC, Moreira-Filho O, *et al.* New insights on the origin of B chromosomes in *Astyanax scabripinnis* obtained by chromosome painting and FISH. Genetica 2011; 139:1073–1081.

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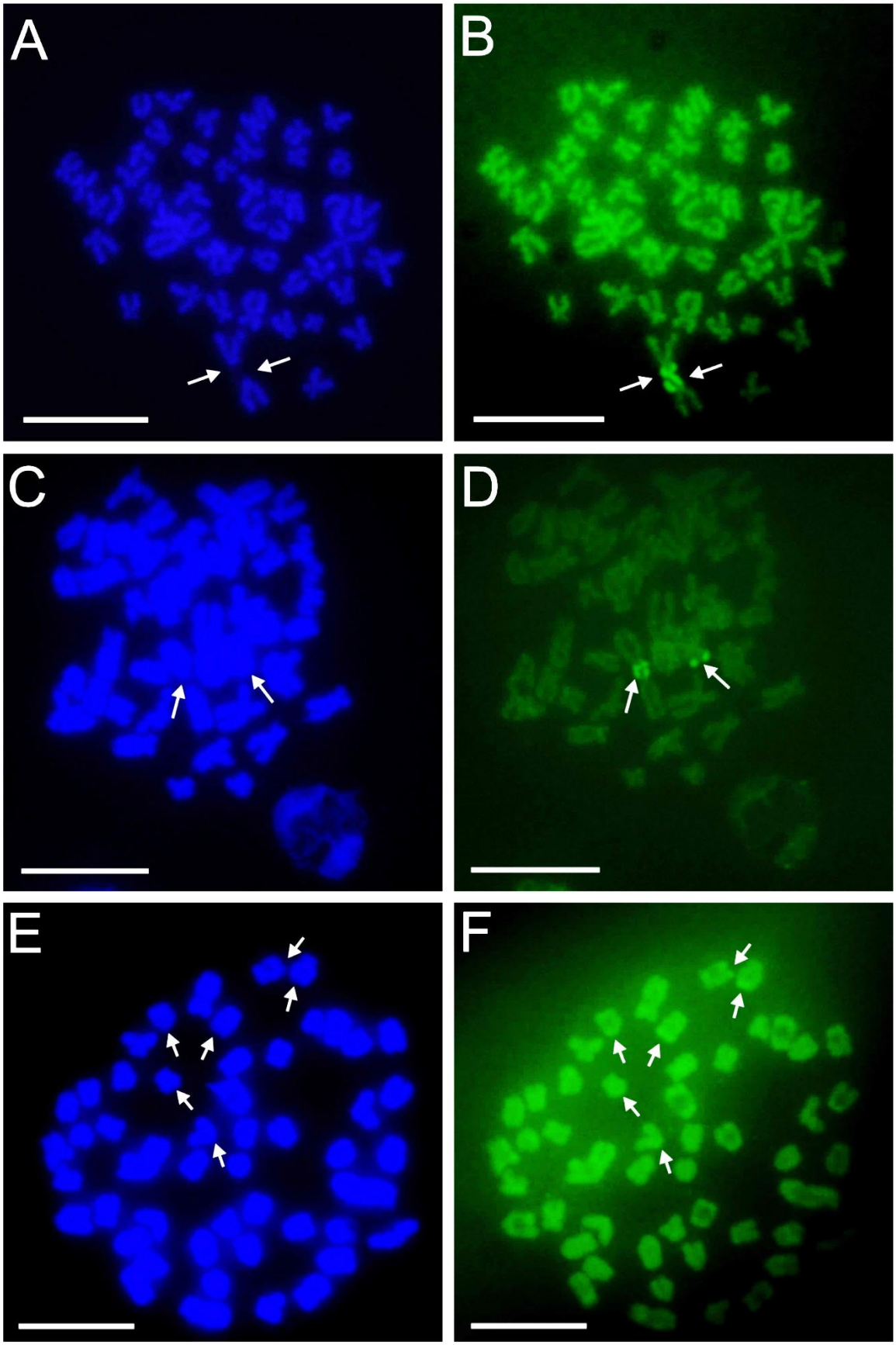
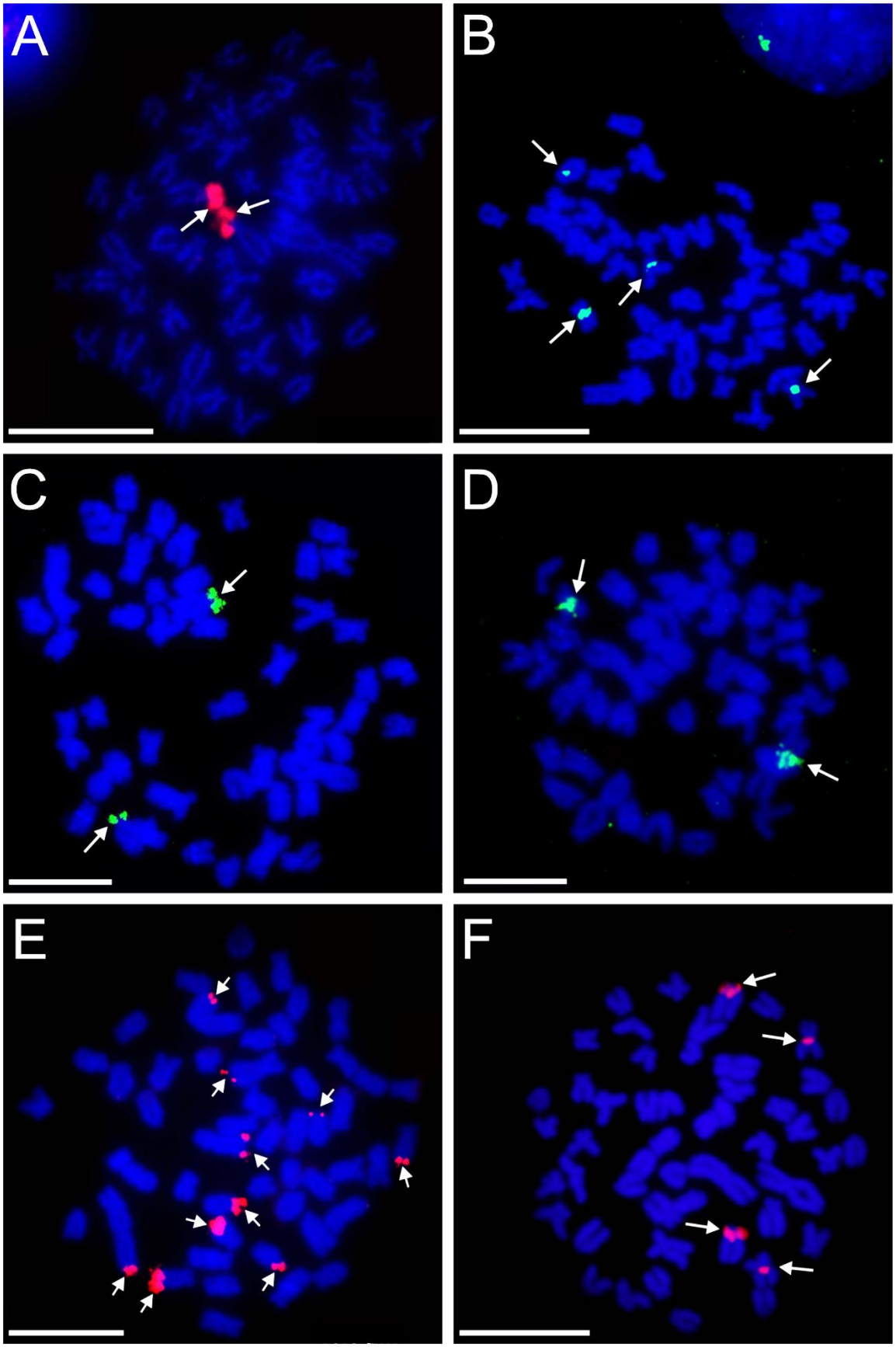


Color Figure 3. Sequential CMA_3/DAPI staining. **A–B.** *A. abramis.* **C–D.** *A. altiparanae*. **E–F.** *A. eigenmanniorum*. DAPI is shown in blue and CMA_3 in green. Bar = 10 µm.



Color Figure 4. Chromosomes of *Astyanax* species after fluorescence *in situ* hybridization to detect 18S rDNA and 5S rDNA. **A–B.** *A. abramis.* **C–D.** *A. altiparanae*. **E–F.** *A. eigenmanniorum*. **A, C,** and **E.** 18S rDNA probe. **B, D,** and **F.** 5S rDNA probe. Bar= 10 µm.

6.2. CAPÍTULO II

CHROMOSOMAL MAPPING OF H3 HISTONE AND 5S rRNA GENES IN EIGHT *Astyanax* (PISCES, CHARACIFORMES) SPECIES WITH DIFFERENT DIPLOID NUMBERS: SYNTENIC CONSERVATION OF REPETITIVE GENES

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ARTICLE

Chromosomal mapping of H3 histone and 5S rRNA genes in eight species of *Astyanax* (Pisces, Characiformes) with different diploid numbers: syntenic conservation of repetitive genes

Diovani Piscor and Patricia Pasquali Parise-Maltempi

Abstract: The genus *Astyanax* is widely distributed from the southern United States to northern Patagonia, Argentina. While cytogenetic studies have been performed for this genus, little is known about the histone gene families. The aim of this study was to examine the chromosomal relationships among the different species of *Astyanax*. The chromosomal locations of the 5S rRNA and H3 histone genes were determined in *A. abramis*, *A. asuncionensis*, *A. altiparanae*, *A. bockmanni*, *A. eigenmanniorum*, *A. mexicanus* (all $2n = 50$), *A. fasciatus* ($2n = 46$), and *A. schubarti* ($2n = 36$). All eight species exhibited H3 histone clusters on two chromosome pairs. In six species (*A. abramis*, *A. asuncionensis*, *A. altiparanae*, *A. bockmanni*, *A. eigenmanniorum*, and *A. fasciatus*), syntenic clusters of H3 histone and 5S rDNA were observed on metacentric (m) or submetacentric (sm) chromosomes. In seven species, clusters of 5S rDNA sequences were located on one or two chromosome pairs. In *A. mexicanus*, 5S rDNA clusters were located on four chromosome pairs. This study demonstrates that H3 histone clusters are conserved on two chromosome pairs in the genus *Astyanax*, and specific chromosomal features may contribute to the genomic organization of the H3 histone and 5S rRNA genes.

Key words: repetitive DNAs, karyotype evolution, FISH, Mexican blind cavefish, *Astyanax schubarti*.

Résumé : Le genre *Astyanax* présente une large aire de distribution allant du sud des Etats-Unis au nord de la Patagonie en Argentine. Bien que des études cytogénétiques aient été réalisées chez ce genre, peu de choses sont connues au sujet des familles de gènes codant pour les histones. Le but de cette étude était d'examiner les relations chromosomiques au sein des différentes espèces d'*Astyanax*. Les positions chromosomiques des gènes codant pour les ARNr 5S et les histones H3 ont été déterminées chez *A. abramis*, *A. asuncionensis*, *A. altiparanae*, *A. bockmanni*, *A. eigenmanniorum*, *A. mexicanus* (toutes à $2n = 50$), *A. fasciatus* ($2n = 46$) et *A. schubarti* ($2n = 36$). Chez les huit espèces, les gènes d'histones H3 étaient groupés sur deux paires de chromosomes. Chez six espèces (*A. abramis*, *A. asuncionensis*, *A. altiparanae*, *A. bockmanni*, *A. eigenmanniorum*, et *A. fasciatus*), les amas de gènes codant pour les histones H3 et l'ADNr 5S ont été observés sur des chromosomes métacentriques (m) ou submétacentriques (sm). Chez sept espèces, les amas de séquences d'ADNr 5S étaient situés sur une ou deux paires de chromosomes. Chez l'*A. mexicanus*, les ADNr 5S étaient répartis sur quatre paires de chromosomes. Cette étude montre que les amas de gènes codant pour les histones H3 sont conservés sur deux paires de chromosomes au sein du genre *Astyanax* et que des caractéristiques chromosomiques spécifiques pourraient contribuer à l'organisation génomique des gènes codant pour les histones H3 et les ARNr 5S. [Traduit par la Rédaction]

Mots-clés : ADN répétés, évolution caryotypique, FISH, tétra aveugle, *Astyanax schubarti*.

Introduction

Ribosomal genes, which constitute one of the major multigene families, are studied extensively in fish. These genes can characterize groups being located in one chromosome pair as in the genus *Apareiodon* (Parodontidae) (Vicari et al. 2006; Rosa et al. 2006; Bellafronte et al. 2009, 2011), or distributed in one or more pairs, ranging numerically depending on the population studied, as shown in *Astyanax scabripinnis* (Characidae) (Ferro et al. 2001; Almeida-Toledo et al. 2002; Santos et al. 2012). The chromosomal locations of 5S ribosomal DNA (rDNA) sequences are well conserved in species of the genus *Astyanax*; this contrasts with 45S rDNA locations, which are remarkably variable (Piscor et al. 2015).

Unlike the rDNA sequences, histone genes are generally found on one or two chromosome pairs in vertebrates, including mammals (Graves et al. 1985; Tripputi et al. 1986), amphibian (Turner et al. 1988), and fish (Pendás et al. 1994*a*; Hashimoto et al. 2011, 2013; Silva et al. 2014; Costa et al. 2014). Few investigations of histone

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gene locations in fish have been described in the literature. The research presented in this paper comprises the most comprehensive study to date of the relationships between H3 histone and 5S rRNA genes in the genus *Astyanax*.

Chromosomal mapping of histone genes in fish was first described by Pendás et al. (1994*a*) in a study of gene distribution in three species (brown trout, Atlantic salmon, and rainbow trout). In the genus *Astyanax*, Hashimoto et al. (2011) mapped the locations of the H1 histone and 5S rRNA genes in the chromosomes of three species (*A. altiparanae*, *A. fasciatus*, and *A. bockmanni*). Analysis of the gene sequences and their chromosomal locations in these species (two chromosome pairs with H1 histone cluster and one chromosome pair with 5S rDNA synteny in all three species) suggested that *A. fasciatus* and *A. bockmanni* were closely related. In the fish *Rachycentron canadum* (Perciformes), H3 histone genes mapped to terminal regions on the short and long arms of all chromosomes, and genes co-located with rDNAs on chromosome pair 2 (18S rDNA) and pairs 3 and 13 (5S rDNA) (Costa et al. 2014).

This paper aims to compare the degree of conservation of the locations of repetitive sequences (mainly H3 histone genes) among species of *Astyanax*, enhance our understanding of the chromosomal organization of the H3 histone and 5S rRNA genes, and determine the parameters underlying chromosome evolution in the *Astyanax* group.

Materials and methods

Ethical standards

The international guidelines for the care and use of laboratory animals follows the Guide to the Care and Use of Experimental Animals (Vol. 1, 2nd ed., 1993, and Vol. 2, 1984). The animals were captured with permission from Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio (number 43497-1), and the use for laboratory experiments was approved by the Animal Experimental Ethics Committee from Universidade Estadual Paulista – UNESP (protocol number: 2335).

Sampling and cytogenetic preparations

Twenty-seven specimens of *Astyanax* were obtained from locations in Brazil as follows: three *A. abramis* and four *A. asuncionensis* specimens from the Bento Gomes River in Mato Grosso state (MT); five *A. altiparanae* specimens and one *A. schubarti* specimen from the Piracicaba River in São Paulo state (SP); three *A. bockmanni* specimens from the Iguatemi River in Mato Grosso do Sul (MS); five *A. fasciatus* specimens from the Ribeirão Claro River in São Paulo state (SP); and three *A. mexicanus* and three *A. eigenmanniorum* specimens from aquariophiles in Brazil. Chromosomes were obtained as described by Foresti et al. (1981). Chromosome morphologies were determined according to the ratio of the arms (the most frequently used classification system for fish chromosomes). Briefly, the length of the long arm (q) was divided by the length of the short arm (p). Chromosomes with two arms and an arm ratio of 1–1.7 were classified as metacentric (m), those with two arms and an arm ratio of 1.71–3 were classified as submetacentric (sm), and those with two arms and an arm ratio of 3.01–7 were classified as subtelocentric (st). Chromosomes with a single arm (arm ratio > 7) were considered to be acrocentric (a).

DNA extraction, production of probes, and fluorescent in situ hybridization

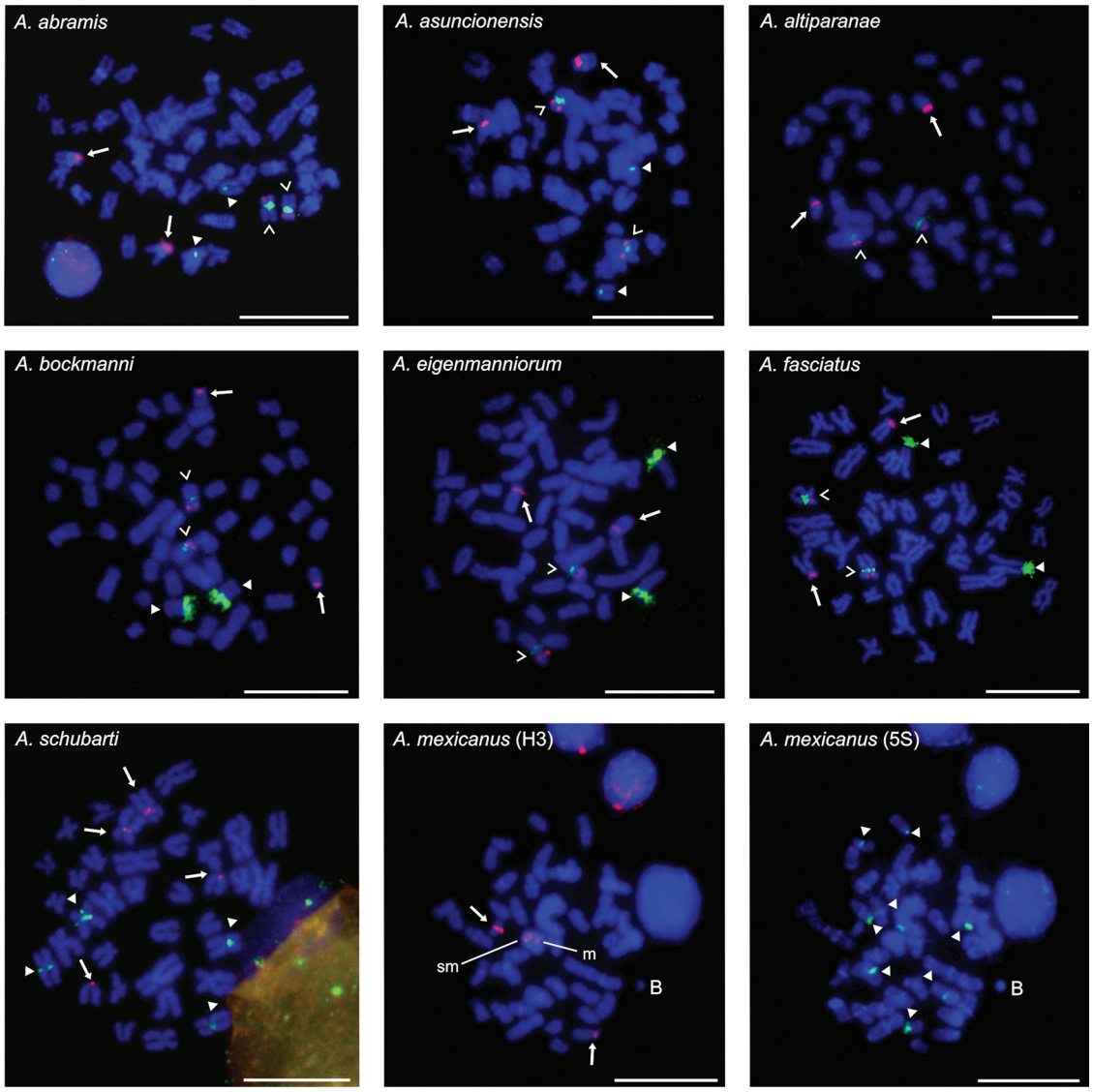
Genomic DNA was extracted from fin samples as described in Sambrook and Russell (2001). The 5S rDNA probe was prepared using polymerase chain reaction (PCR) with primers described by Pendás et al. (1994*b*) and Martins and Galetti (1999) (A, 5′-TACGCCCGATCTCGTCCGATC-3′, and B, 5′-CAGGCTGGTATGGCCGTAAGC-3′). The H3 histone gene probe was prepared using PCR with primers described by Cabral-de-Mello et al. (2010) (A, 5′-GGCNMGNACNAARCARAC-3′, and B, 5′-TGDATRTCYTTNGGCATDAT-3′). The 5S rDNA probe was labeled by PCR with biotin-14-dATP (Invitrogen, San Diego, Calif., USA) and digoxigenin-11-dUTP (Roche, Mannheim, Germany), and the H3 histone gene probe was labeled by PCR with digoxigenin-11-dUTP (Roche, Mannheim, Germany). Single, double, and sequential (on the same metaphase plate) fluorescent in situ hybridization (FISH) experiments were performed according to Pinkel et al. (1986) with modifications as described by Piscor et al. (2013). Chromosomes were counterstained with DAPI (4′,6-diamidino-2-phenylindole) and mounted using Vectashield Mounting Medium (Vector, Burlingame, Calif., USA). Chromosomes and fluorescent signals were visualized with an Olympus BX51 microscope coupled to a digital camera (Olympus model D71). Images were captured using the DP Controller software.

Results

The diploid number for six of the eight species of *Astyanax* was $2n = 50$ chromosomes (*A. abramis*, *A. asuncionensis*, *A. altiparanae*, *A. bockmanni*, *A. eigenmanniorum*, and *A. mexicanus*) (Fig. 1). All cells analyzed of *A. mexicanus* contained one acrocentric B microchromosome. *Astyanax fasciatus* had $2n = 46$ and *A. schubarti* had $2n = 36$ chromosomes (Fig. 1).

In six species of *Astyanax* (*A. abramis*, *A. asuncionensis*, *A. bockmanni*, *A. eigenmanniorum*, *A. fasciatus*, and *A. mexicanus*), the 5S rDNA was located on a metacentric pair (Figs. 1, 2A, 2B, 2D–2G). In *A. altiparanae*, the 5S rDNA was located on a submetacentric pair in the pericentromeric region (Figs. 1, 2C). Species with 5S rDNA on a metacentric pair also exhibited fluorescent signals in the pericentromeric regions on other chromosomes. Fluorescent 5S rDNA signal was noted on a subtelocentric pair in *A. abramis* (Figs. 1, 2A), an acrocentric pair in *A. asuncionensis* (Figs. 1, 2B), in *A. bockmanni* (Figs. 1, 2D), in *A. eigenmanniorum* (Figs. 1, 2E), in *A. fasciatus* (Figs. 1, 2F), and three pairs (one submetacentric and two acrocentrics) in *A. mexicanus* (Figs. 1, 2G). The species with lower diploid numbers,

Fig. 1. Location of 5S rDNA and H3 histone clusters on chromosomes of species of the genus *Astyanax*. The arrows indicate the H3 histone clusters, the arrowheads indicate the 5S rDNA clusters, and the slim arrowheads indicate chromosomes with 5S-H3 synteny. Note that in *A. mexicanus*, single FISH experiments (using 5S rDNA and H3 histone probes) were performed on the same metaphase plate. Scale bar = 10 μm.



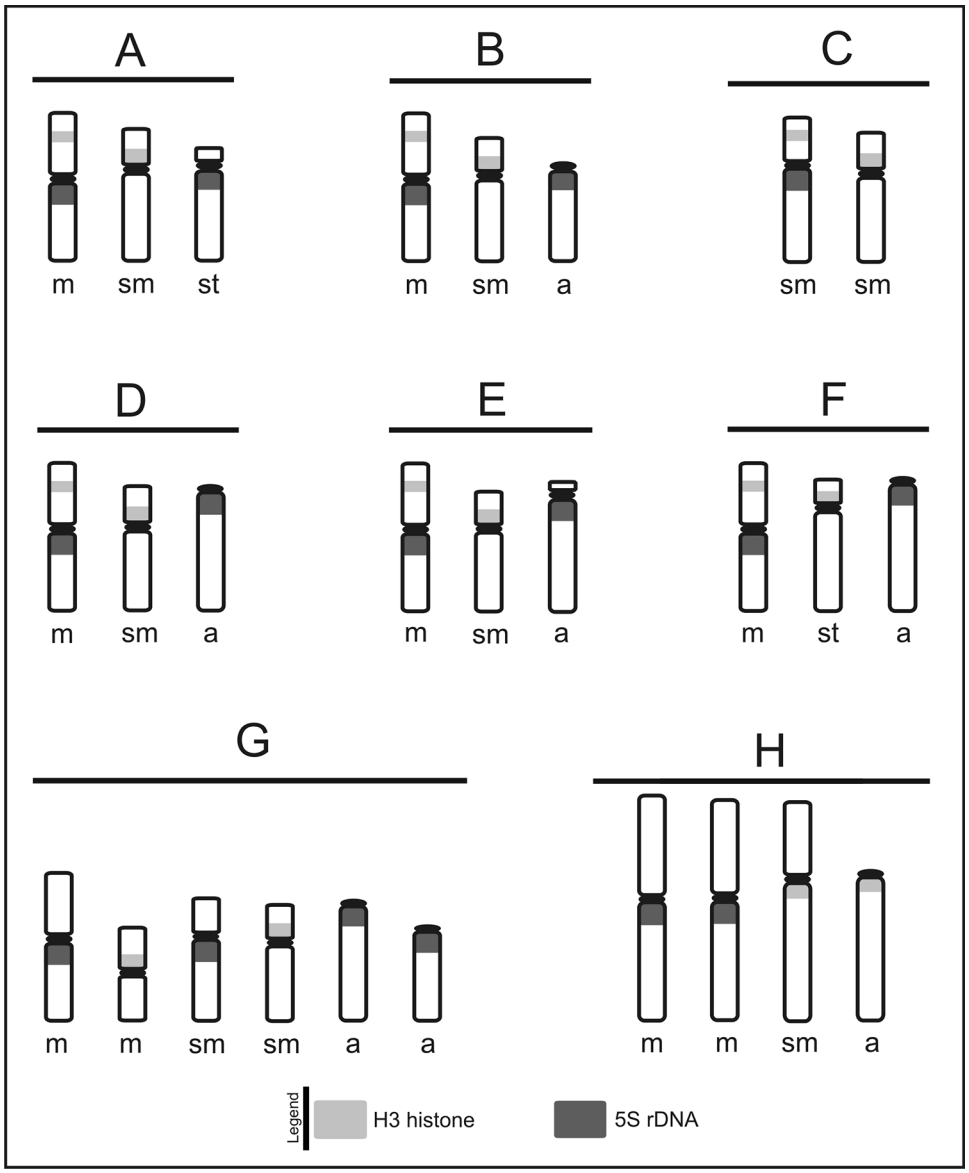
A. schubarti (2*n* = 36), harbored 5S rDNA clusters at pericentromeric regions on two metacentric pairs (Figs. 1, 2H).

H3 histone clusters were observed on two chromosome pairs in all eight examined species of *Astyanax* (Figs. 1, 2). One cluster was located in the interstitial region of the short arm on a metacentric pair in *A. abramis*, *A. asuncionensis*, *A. bockmanni*, *A. eigenmanniorum*, and *A. fasciatus* (Figs. 1, 2A, 2B, 2D, 2E, 2F, respectively) and submetacentric pair in *A. altiparanae* (Figs. 1, 2C). These same chromosome pairs exhibited synteny with the 5S rDNA sequences (Fig. 1: note the slim arrowheads). Additional H3 histone clusters were observed in the pericentromeric regions on a submetacentric pair in *A. abramis* (Figs. 1, 2A), *A. asuncionensis* (Figs. 1, 2B), *A. altiparanae* (Figs. 1, 2C), *A. bockmanni* (Figs. 1, 2D), *A. eigenmanniorum* (Figs. 1, 2E), and subtelocentric pair in *A. fasciatus* (Fig. 1, 2F). *Astyanax mexicanus* and *A. schubarti* did not display synteny between H3 histone and 5S rDNA clusters. In these species, H3 histone clusters were found in the pericentromeric regions of the short arm on a metacentric and submetacentric pairs (*A. mexicanus*; Figs. 1, 2G), and in pericentromeric regions of the long arm on a submetacentric and acrocentric pairs (*A. schubarti*; Figs. 1, 2H).

Discussion

The genus *Astyanax* displays variability among species with respect to chromosome structure, number, and morphology (see, for example, Morelli et al. 1983; Daniel-Silva and Almeida-Toledo 2001, 2005; Tenório et al. 2013). However, the current study showed that the H3 histone gene locations were conserved among species, particularly with

Fig. 2. Diagram showing chromosome pairs carrying 5S rDNA and H3 histone clusters in the eight species of *Astyanax* studied. (A) *A. abramis*, (B) *A. asuncionensis*, (C) *A. altiparanae*, (D) *A. bockmanni*, (E) *A. eigenmanniorum*, (F) *A. fasciatus*, (G) *A. mexicanus*, and (H) *A. schubarti*. Chromosome morphologies: m, metacentric; sm, submetacentric; st, subtelocentric; a, acrocentric.



respect to the pair numbers and morphologies of the chromosomes carrying the genes. The H3 histone genes were located on only two chromosome pairs in the eight species of *Astyanax* examined in this study. This corresponds with data from other populations of *Astyanax* as described by Hashimoto et al. (2011).

The similarities in the chromosomal locations make the H3 histone one of the most highly conserved gene location found in *Astyanax*. When compared with rDNA repetitive clusters, the histone genes exhibit the highest level of conservation, followed by the 5S rDNA, and the least conserved gene is the 18S rRNA (for additional review see, for example, Mantovani et al. 2005; Fernandes and Martins-Santos 2006; Piscor et al. 2015).

The chromosomal locations housing the different repetitive sequences may explain why some sequences of *Astyanax* are found at divergent sites, as for 45S rDNA, and other sequences show conserved locations, as for 5S rDNA. According to Nakajima et al. (2012), interstitial or proximal chromosomal regions tend to be more stable than the terminal sections. Thus, the chromosome location of 18S rDNA in terminal regions, like in *A. scabripinnis* (Mantovani et al. 2005), could be subject to transposition events or chromosome rearrangements leading the dispersion of these sequences throughout the genome. The stability of proximal regions, as seen on the pair 5 in *A. altiparanae* in the present paper, may be responsible for the conserved locations of 5S rRNA genes exhibited by several populations of this species (see also Fernandes and Martins-Santos 2006; Domingues et al. 2007; Piscor et al. 2015). This would also apply to the conserved locations of the histone genes in only two chromosome pairs,

since these sequences occupy most regions "protected" in the chromosomes of *Astyanax*, as pericentromeric and interstitial regions.

Synteny of H3 histone and 5S rDNA clusters was prevalent in the studied species of *Astyanax*. Although the clusters were distributed non-syntenically in *A. mexicanus* and *A. schubarti*, synteny was found in one chromosome pair (submetacentric or metacentric) for the other examined species. Hashimoto et al. (2011) examined three species and also observed synteny of histone (H1 histone) and 5S rRNA genes. Syntenic features such as adjacent location or co-location of sequences from other multigene families have been observed in fish but at lower frequencies than for the H3-5S genes. For example, synteny between two classes of ribosomal DNA (5S rDNA and 45S rDNA) was reported in a population of *A. scabripinnis*, and one of the chromosomes carrying 5S rDNA also harbored nucleolar organizer regions (NORs) (Mantovani et al. 2005). Synteny was also observed in other groups, including *Bryconamericus* aff. *iheringii* (Characidae), in which 18S and 5S ribosomal genes were adjacent to one another (Piscor et al. 2013), and *Triportheus nematurus* (Characidae), in which 5S rDNA clusters were adjacent to NORs (Diniz et al. 2009).

Another feature that supported our conservation hypothesis for the H3 histone genes, in the genus *Astyanax*, was the presence of clusters on one m chromosome pair and one sm or st chromosome pair in all species, except *A. schubarti* and *A. mexicanus*. In *A. schubarti*, H3 histone genes were located on two chromosome pairs (sm and a); however, the sm chromosome was larger than the sm/st chromosomes of the other species in this study (Fig. 2). In *A. mexicanus*, H3 histone genes were located on one small m chromosome pair and one sm chromosome pair; here, the m chromosome was smaller than the m chromosomes of the other species. On the other hand, the size and morphologies of the sm/st pairs (st in *A. fasciatus* and sm in the other species) were similar, and it is therefore possible that these chromosomes may be homeologous. However, we cannot exclude the possibility that microrearrangements might have stimulated subtle morphological differences in the submetacentric and subtelocentric pairs in these species of *Astyanax*.

Our data suggest that, due to variation of the number of chromosomes presented to the genus *Astyanax* studied here (modal number of $2n = 36$, $2n = 46$, and $2n = 50$ chromosomes), only two chromosomes carrying the genes of H3 histone seem to be preserved for the genus as a whole. However, two notable features of H3 histone chromosomal distribution attracted our attention. First, the chromosomes carrying the H3 histone genes in *A. schubarti*, which has the lowest diploid number ($2n = 36$), were particularly large. Second, no H3-5S synteny was observed in *A. schubarti* and *A. mexicanus* (Mexican blind cavefish). Observations by Morelli et al. (1983) suggested that the lower diploid number ($2n = 36$) in *A. schubarti* might be of recent origin. The lack of synteny in this species may be related to the chromosomal rearrangements involved in the reduction of the diploid number. Lack of synteny in *A. mexicanus* may be due to the ancient separation of this particular species, millions of years ago, from the species from South American river basins.

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References

Almeida-Toledo, L.F., Ozouf-Costaz, C., Foresti, F., Bonillo, C., Porto-Foresti, F., and Daniel-Silva, M.F.Z. 2002. Conservation of the 5S-bearing chromosome pair and co-localization with major rDNA clusters in five species of *Astyanax* (Pisces, Characidae). Cytogenet. Genome Res. **97**: 229–233. doi:10.1159/000066609. PMID:12438717.

Bellafronte, E., Vicari, M.R., Artoni, R.F., Margarido, V.P., and Moreira-Filho, O. 2009. Differentiated ZZ/ZW sex chromosomes in *Apareiodon ibitiensis* (Teleostei, Parodontidae): cytotaxonomy and biogeography. J. Fish Biol. **75**: 2313–2325. doi:10.1111/j.1095-8649.2009.02488.x. PMID:20738689.

Bellafronte, E., Schemberger, M.O., Moreira-Filho, O., Almeida, M.C., Artoni, R.F., Margarido, V.P., et al. 2011. Chromosomal markers in Parodontidae: an analysis of new and reviewed data with phylogenetic inferences. Rev. Fish Biol. Fish, **21**: 559–570. doi:10.1007/s11160-010-9177-3.

Cabral-de-Mello, D.C., Moura, R.C., and Martins, C. 2010. Chromosomal mapping of repetitive DNAs in the beetle *Dichotomius geminatus* provides the first evidence for an association of 5S rRNA and histone H3 genes in insects, and repetitive DNA similarity between the B chromosome and A complement. Heredity, **104**: 393–400. doi:10.1038/hdy.2009.126. PMID:19756039.

Costa, G.W.W.F., Cioffi, M.B., Bertollo, L.A.C., and Molina, W.F. 2014. Unusual dispersion of histone repeats on the whole chromosomal complement and their colocalization with ribosomal genes in *Rachycentron canadum* (Rachycentridae, Perciformes). Cytogenet. Genome Res. **144**(1): 62–67. doi:10.1159/000366301. PMID:25341625.

Daniel-Silva, M.F.Z., and Almeida-Toledo, L.F. 2001. Chromosome R-banding pattern and conservation of a marker chromosome in four species, genus *Astyanax* (Characidae, Tetragonopterinae). Caryologia, **54**: 209–215. doi:10.1080/00087114.2001.10589228.

Daniel-Silva, M.F.Z., and Almeida-Toledo, L.F. 2005. Chromosome evolution in fish: BrdU replication patterns demonstrate chromosome homeologies in two species of the genus *Astyanax*. Cytogenet. Genome Res. **109**: 497–501. doi:10.1159/000084209. PMID:15905644.

Diniz, D., Laudicina, A., and Bertollo, L.A.C. 2009. Chromosomal location of 18S and 5S rDNA sites in *Triportheus* fish species (Characiformes, Characidae). Genet. Mol. Biol. **32**: 37–41. doi:10.1590/S1415-47572009005000017. PMID:21637644.

Domingues, M.S., Vicari, M.R., Abilhoa, V., Wamser, J.P., Cestari, M.M., Bertollo, L.A.C., et al. 2007. Cytogenetic and comparative morphology of two allopatric populations of *Astyanax altiparanae* Garutti & Britski, 2000 (Teleostei: Characidae) from upper rio Paraná basin. Neotrop. Ichthyol. **5**(1): 37–44. doi:10.1590/S1679-62252007000100005.

Fernandes, C.A., and Martins-Santos, I.C. 2006. Mapping of the 18S and 5S ribosomal RNA genes in *Astyanax altiparanae* Garutti & Britski, 2000 (Teleostei, Characidae) from the upper Paraná river basin, Brazil. Genet. Mol. Biol. **29**(3): 464–468. doi:10.1590/S1415-47572006000300011.

Ferro, D.A.M., Néo, D.M., Moreira-Filho, O., and Bertollo, L.A.C. 2001. Nucleolar organizing regions, 18S and 5S rDNA in *Astyanax scabripinnis* (Pisces, Characidae): populations distribution and functional diversity. Genetica, **110**: 55–62. doi:10.1023/A:1017963217795. PMID:11519875.

Foresti, F., Almeida-Toledo, L.F., and Toledo-Filho, S.A. 1981. Polymorphic nature of nucleolus organizer regions in fishes. Cytogenet. Cell Genet. **31**: 134–141. doi:10.1159/000131639.

Graves, R.A., Marzluff, W.F., Giebelhaus, D.H., and Schultz, G.A. 1985. Quantitative and qualitative changes in histone gene expression during early mouse embryo development. Proc. Natl. Acad. Sci. U.S.A. **82**: 5685–5689. doi:10.1073/pnas.82.17.5685. PMID:3862090.

Hashimoto, D.T., Ferguson-Smith, M.A., Rens, W., Foresti, F., and Porto-Foresti, F. 2011. Chromosome mapping of H1 histone and 5S rRNA gene clusters in three species of *Astyanax* (Teleostei, Characiformes). Cytogenet. Genome Res. **134**: 64–71. doi:10.1159/000323512. PMID:21252491.

Hashimoto, D.T., Ferguson-Smith, M.A., Rens, W., Prado, F.D., Foresti, F., and Porto-Foresti, F. 2013. Cytogenetic mapping of H1 histone and ribosomal RNA genes in hybrids between catfish species *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum*. Cytogenet. Genome Res. **139**: 102–106. doi:10.1159/000345299. PMID:23208250.

Mantovani, M., Abel, L.D.S., and Moreira-Filho, O. 2005. Conserved 5S and variable 45S rDNA chromosomal localization revealed by FISH in *Astyanax scabripinnis* (Pisces, Characidae). Genetica, **123**: 211–216. doi:10.1007/s10709-004-2281-3. PMID:15954491.

Martins, C., and Galetti, P.M., Jr. 1999. Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chromosome Res. **7**: 363–367. doi:10.1023/A:1009216030316. PMID:10515211.

Morelli, S., Bertollo, L.A.C., Foresti, F., Moreira-Filho, O., and Toledo-Filho, S.A. 1983. Cytogenetic considerations on the genus *Astyanax* (Pisces, Characidae). I. Karyotypic variability. Caryologia **36**: 235–244. doi:10.1080/00087114.1983.10797664.

Nakajima, R.T., Cabral-de-Mello, D.C., Valente, G.T., Venere, P.C., and Martins, C. 2012. Evolutionary dynamics of rRNA gene clusters in cichlid fish. BMC Evol. Biol. **12**: 198. doi:10.1186/1471-2148-12-198. PMID:23035959.

Pendás, A.M., Morán, P., and García-Vázquez, E. 1994*a*. Organization and chromosomal location of the major histone cluster in brown trout, Atlantic salmon and rainbow trout. Chromosoma, **103**: 147–152. doi:10.1007/BF00352324. PMID:8055712.

Pendás, A.M., Morán, P., Freije, J.P., and García-Vázquez, E. 1994*b*. Chromosomal mapping and nucleotide sequence of two tandem repeats of Atlantic salmon 5S rDNA. Cytogenet Cell Genet. **67**: 31–36. doi:10.1159/000133792. PMID:8187548.

Pinkel, D., Straume, T., and Gray, J.W. 1986. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc. Natl. Acad. Sci. U.S.A. **83**: 2934–2938. doi:10.1073/pnas.83.9.2934. PMID:3458254.

Piscor, D., Ribacinko-Piscor, D.B., Fernandes, C.A., and Parise-Maltempi, P.P. 2013. Cytogenetic analysis in three *Bryconamericus* species (Characiformes, Characidae): first description of the 5S rDNA-bearing chromosome pairs in the genus. Mol. Cytogenet. **6**: 13. doi:10.1186/1755-8166-6-13. PMID:23547656.

Piscor, D., Alves, A.L., and Parise-Maltempi, P.P. 2015. Chromosomal microstructure diversity in three *Astyanax* (Characiformes, Characidae) species: comparative analysis of the chromosomal locations of the 18S and 5S rDNAs. Zebrafish, **12**(1): 81–90. doi:10.1089/zeb.2014.1036. PMID:25549064.

Rosa, R., Bellafronte, E., Moreira-Filho, O., and Margarido, V.P. 2006. Constitutive heterochromatin, 5S and 18S rDNA genes in *Apareiodon* sp. (Characiformes, Parodontidae) with a ZZ/ZW sex chromosome system. Genetica, **128**: 159–166. doi:10.1007/s10709-005-5700-1.

Sambrook, J., and Russell, D.W. 2001. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Santos, N.M., Ferreira-Neto, M., Artoni, R.F., Vicari, M.R., Bakkali, M., Oliveira, C., et al. 2012. A comparative structural cytogenetic study in three allopatric populations of *Astyanax scabripinnis* (Teleostei: Characidae). Zoologia, **29**(2): 159–166. doi:10.1590/S1984-46702012000200008.

Silva, D.M.Z.A., Pansonato-Alves, J.C., Utsunomia, R., Araya-Jaime, C., Ruiz-Ruano, F.J., Daniel, S.N., et al. 2014. Delimiting the origin of a B chromosome by FISH mapping, chromosome painting and DNA sequence analysis in *Astyanax paranae* (Teleostei, Characiformes). PLoS ONE, **9**(4): e94896. doi:10.1371/journal.pone.0094896. PMID:24736529.

Tenório, R.C.C.O., Vitorino, C.A., Souza, I.L., Oliveira, C., and Venere, P.C. 2013. Comparative cytogenetics in *Astyanax* (Characiformes: Characidae) with focus on the cytotaxonomy of the group. Neotrop. Ichthyol. **11**(3): 553–564. doi:10.1590/S1679-62252013000300008.

Tripputi, P., Emanuel, B.S., Croce, C.M., Green, L.G., Stein, G.S., and Stein, J.L. 1986. Human histone genes map to multiple chromosomes. Proc. Natl. Acad. Sci. U.S.A. **83**: 3185–3188. doi:10.1073/pnas.83.10.3185. PMID:3458175.

Turner, P.C., Bagenal, E.B.D., Vlad, M.T., and Woodland, H.R. 1988. The organization and expression of histone genes from *Xenopus borealis*. Nucl. Acids Res. **16**: 3471–3485. doi:10.1093/nar/16.8.3471. PMID:3375060.

Vicari, M.R., Moreira-Filho, O., Artoni, R.F., and Bertollo, L.A.C. 2006. ZZ/ZW sex chromosome system in an undescribed species of the genus *Apareiodon* (Characiformes, Parodontidae). Cytogenet Genome Res. **114**: 163–168. doi:10.1159/000093333. PMID:16825769.

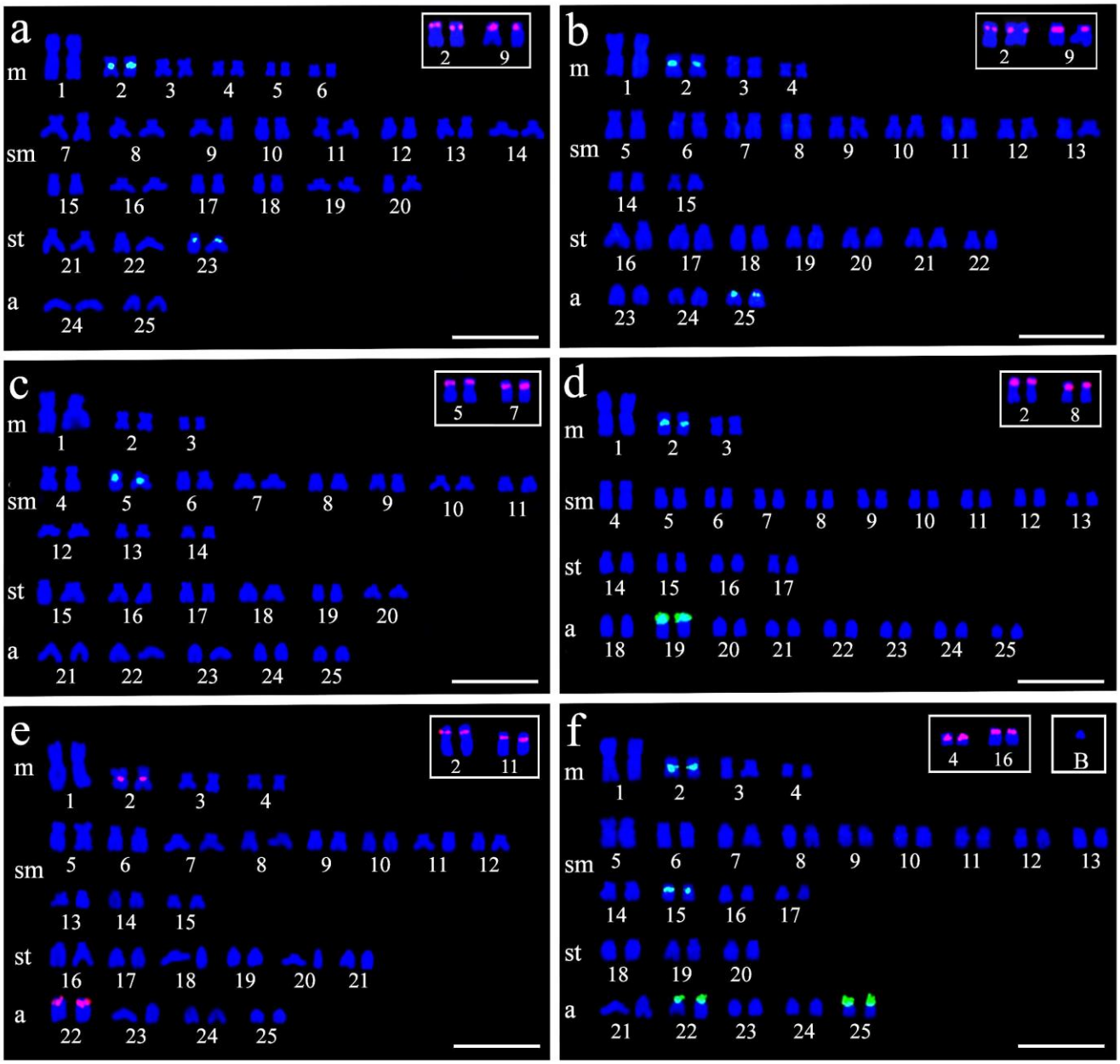

Figure 1. (Supplementary material). Location of 5S rDNA and H3 histone clusters on chromosomes of *Astyanax* species with 2n = 50 chromosomes. (**a**) *A. abramis*, (**b**) *A. asuncionensis*, (**c**) *A. altiparanae*, (**d**) *A. bockmanni*, (**e**) *A. eigenmanniorum*, and (**f**) *A. mexicanus*. Karyotypes show pairs with labeled 5S rDNA clusters and boxes illustrate pairs with H3 histone clusters.

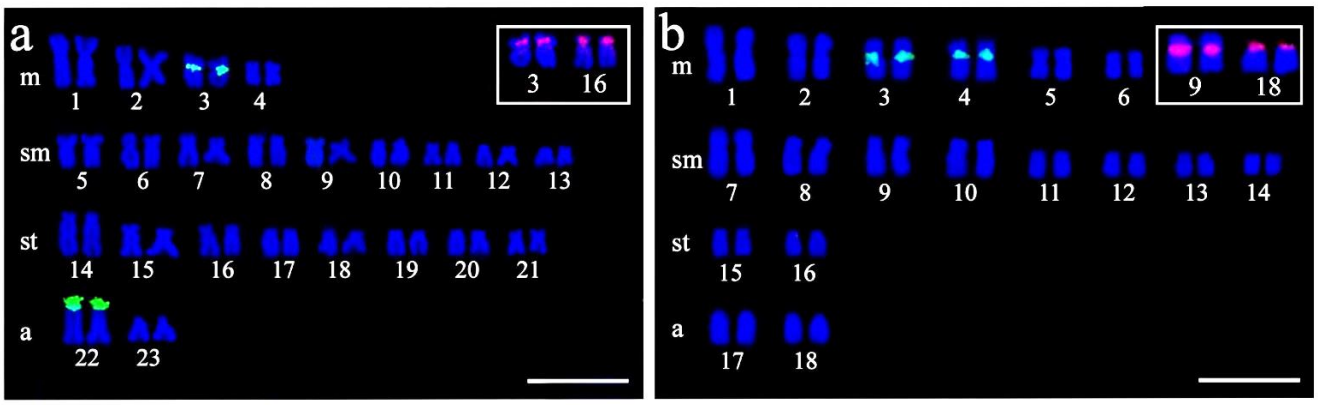

Figure 2. (Supplementary material). Location of 5S rDNA and H3 histone clusters on chromosomes of *A. fasciatus* (2n = 46) and *A. schubarti* (2n = 36). (**a**) *A. fasciatus,* and (**b**) *A. schubarti*. Karyotypes show pairs with labeled 5S rDNA clusters and boxes illustrate pairs with H3 histone clusters.

6.3. CAPÍTULO III

DISTINCT CLASSICAL AND MOLECULAR CYTOGENETICS BETWEEN *Astyanax marionae* AND *Astyanax fasciatus* (CHARACIFORMES: CHARACIDAE): A COMPARATIVE ORGANIZATION OF THE HETEROCHROMATIN AND REPETITIVE GENES

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Abstract

The *Astyanax* genus is well distributed in Neotropical freshwater and is considered taxonomically uncertain, even as other genera also allocated to the group *incertae sedis* in Characidae. This study aimed to analyze the karyotype of different populations of *A. fasciatus* (Corumbataí river basin – São Paulo state, Brazil) by Giemsa staining, band-C technique, and fluorescence *in situ* hybridization (FISH) using H3 histone and 5S rRNA genes and describing for the first time the chromosomal organization of these genes in *A. marionae* (Chapada dos Guimarães – Mato Grosso state, Brazil). The chromosomes of the three *A. fasciatus* populations were analyzed (two with 2n = 50 and one with 2n = 48), and the heterochromatin showed two organization forms (discrete and conspicuous blocks). The H3 histone and 5S rRNA genes were observed in the three populations of *A. fasciatus* on two chromosome pairs (one metacentric chromosome showed H3 histone and 5S rRNA gene clusters). In *A. marionae* (2n = 48), the H3 histone and 5S rRNA genes were observed in one acrocentric chromosome pair (distinct pairs). Here, the differences between karyotypes and heterochromatin were discussed and the chromosomal organization of H3 histone and 5S rRNA genes in *Astyanax* species focusing on the chromosome evolution in the group.

Keywords: H3 histone gene, rDNA, C-band, chromosomes.

INTRODUCTION

The *Astyanax* is one of the genera with the largest number of species in Characidae, containing approximately 140 valid species (Eschmeyer and Fong 2015). Their wide distribution from the South of the United States to Central Argentina (Lima et al. 2003), occupying a variety of habitats in rivers and streams, making this group one of the more complex genera among freshwater fishes. Lima et al. (2003) have allocated many genera into *incertae sedis* in Characidae, as e.g., *Hemigrammus*, *Hyphessobrycon*, *Moenkausia*, and *Astyanax*. More recently, others authors have also showed that *Astyanax* does not represent a monolyphyletic group (see Mirande 2010, Javonillo et al. 2010, Oliveira et al. 2011).

Cytogenetic studies of the *Astyanax* genus have revealed wide variation in the diploid number, ranging from 2n = 36 in *A. schubarti* (Morelli et al. 1983) and *A. correntinus* (Paiz et al. 2015) to 2n = 50 chromosomes for most species, e.g., *Astyanax altiparanae* and *Astyanax bockmanni* (Fernandes and Martins-Santos, 2004; Kavalco et al., 2009, respectively). Furthermore, some species of *Astyanax* may have more than one diploid number, as e.g., "*fasciatus* complex" which may present species with 2n = 45 to 2n = 50 chromosomes (Centofante et al. 2003).

Therefore, given the karyotype complexity found in the *Astyanax* genus, and considering that *A. marionae* and *A. fasciatus* are two species with similar morphology traits, these, would have similar cytogenetic characteristics? Thus, this study aimed to compare the chromosomes of *A. marionae* with different populations of *A. fasciatus*, and understand the chromosomal organization of the H3 histone and 5S rRNA genes and of the heterochromatin revealed by C-band.

MATERIAL AND METHODS

Sampling and classical cytogenetics

Three populations of *A. fasciatus* were studied: a population from the Cabeça river tributary, a population from the Ribeirão Claro river tributary, and a population from the Corumbataí river tributary (Corumbataí river basin, São Paulo state, Brazil). The individuals of *A. marionae* were captured in the Rio Claro stream (Paraguay river basin, Mato Grosso state – MT, Brazil). The metaphasic chromosomes were obtained by the methodology of Foresti et al. (1981) and stained with Giemsa (10% in phosphate buffer). The morphologies of chromosomes were determined according to the arms ratio, based on the more common classification system used for fish chromosomes in Brazil: the chromosomes with two arms and arm ratio (AR) of 1–1.7 of were classified as metacentric (m), those with AR of 1.71–3 as submetacentric (sm), and those with AR of 3.01–7 as subtelocentric (st). Chromosomes with a single arm (AR>7) were considered as acrocentric (a). Heterochromatin was observed using the C-band technique as proposed by Sumner (1972).

DNA extraction, production of probes, and fluorescence *in situ* hybridization

Genomic DNA was extracted, from fin samples of *Astyanax*, as described in Sambrook and Russell (2001). The 5S rDNA probe was prepared using polymerase chain reaction (PCR) with primers described by Pendás et al. (1994) and Martins and Galetti (1999) (A, 5′-TAC GCC CGA TCT CGT CCG ATC-3′, and B, 5′-CAG GCT GGT ATG GCC GTA AGC-3′). The H3 histone probe was prepared using PCR with primers described by Cabral-de-Mello et al. (2010) (A, 5′-GGC NMG NAC NAA RCA RAC, and B, 5′-TGD ATR TCY TTN GGC ATD AT). The 5S rDNA probe was labeled

by PCR with biotin-14-dATP (Invitrogen, San Diego, CA, USA), and the H3 histone probe was labeled by PCR with digoxigenin-11-dUTP (Roche, Mannheim, Germany). The Fluorescence *in situ* hybridization (FISH) experiments were performed according to Pinkel et al. (1986) with modifications as described by Piscor et al. (2013). Chromosomes were counterstained with Vectashield Mounting Medium (Vector, Burlingame, CA, USA) containing DAPI (4', 6-diamidino-2'-phenylindole). Chromosomes and fluorescent signals were visualized with an Olympus BX51 microscope coupled to a digital camera (Olympus model D71), and the images were captured using the DP Controller software.

RESULTS

The karyotype of *A. marionae* showed 8m, 24sm, 10st, 6a and Fundamental Number (FN) of 90 (Figure 1A). The heterochromatin C-banded was observed mainly on the centromeric and proximal regions (Figure 1B). Clusters of 5S rRNA and H3 histone genes were observed on pairs 22 (a) and 24 (a), respectively (Figure 1C).

The populations from the tributaries of Cabeça and Corumbataí rivers showed a diploid number of 2n = 50 chromosomes, and the Ribeirão Claro river population showed 2n = 48 chromosomes. The karyotypic formulae for the populations were: 16m, 12sm, 6st, 16a and FN = 84 for the Cabeça river tributary (Figure 2A), 10m, 20sm, 8st, 10a and FN = 86 for the Ribeirão Claro river tributary (Figure 2B), and 8m, 26sm, 6st, 10a and FN = 90 for the Corumbataí river tributary (Figura 2C). Heterochromatic regions were observed in two organization forms. The first form was noted for the Cabeça and Corumbataí populations in which showed more discrete

blocks (Figure 3A, C), and the second form was evidenced for the Ribeirão Claro population in which showed more conspicuous blocks (Figure 3B).

The H3 histone gene clusters were observed on two chromosome pairs in three populations examined of *A. fasciatus* (Figure 3D–F). The *A. fasciatus* population from Cabeça river tributary showed interstitial fluorescent signals on the pair 3 (m), and pericentromeric signals on the pair 15 (st) (Figure 3D). The *A. fasciatus* population from Corumbataí river tributary showed interstitial fluorescent signals on the pair 3 (m), and pericentromeric signals on the pair 18 (st) (Figure 3F). The *A. fasciatus* population from Ribeirão Claro river tributary showed interstitial fluorescent signals on the pair 2 (m), and pericentromeric signals on the pair 16 (st) (Figure 3E).

The 5S rDNA clusters were located on two chromosome pairs (Figure 3D–F). The *A. fasciatus* from Cabeça showed pericentromeric fluorescent signals on the pair 3 (m – same chromosome pair that bearing H3 histone genes), and on the pair 18 (a) (Figure 3D). The *A. fasciatus* from Corumbataí showed pericentromeric fluorescent signals on the pair 3 (m – same chromosome pair that bears H3 histone genes), and on the pair 21 (a) (Figure 3F). The *A. fasciatus* from Ribeirão Claro showed pericentromeric fluorescent signals on the pair 2 (m – same chromosome pair that bears H3 histone genes), and on the pair 20 (a) (Figure 3E).

A map with the hydrographic basins and the distributions of *A. marionae* and *A. fasciatus*, and representative chromosome pairs bearing H3 histone and 5S rDNA clusters are shown in Figure 4.

DISCUSSION

Astyanax fasciatus populations usually differ in the diploid number. According to Ferreira-Neto et al. (2012) this species can present diploid numbers 2n = 46, 48 and 50 chromosomes (the most common number is 2n = 48). However, previous results have shown karyomorphs with 2n = 45, 47, and 49 chromosomes, possibly result of hybridizations (see Pazza et al. 2006; Artoni et al. 2006). Furthermore, chromosome variations are evident among different populations of a species, primarily relating to the number and size of heterochromatin blocks (see, for example, Fernandes and Martins-Santos 2003; Fernandes and Martins-Santos 2004).

Conspicuous blocks of heterochromatin have been found for others *A. fasciatus* populations. Pazza et al. (2008) analyzed populations of *A. fasciatus* from three distinct sites along the Mogi-Guaçu River (Ouro Fino, Minas Gerais State – MG; Cachoeira de Emas, Pirassununga, São Paulo State – SP; Barrinha – SP, Brazil) and observed a similar pattern of constitutive heterochromatin organization on the 2n = 46 and 48 forms (telomeric region on long arms of submetacentric, subtelocentric and acrocentric chromosomes, and in the telomeric region on short arms of a submetacentric pair). Similar results were observed for the population from Ribeirão Claro River (2n = 48) studied here.

Moreover, the two heterochromatic organizations found for the three *A. fasciatus* populations, analyzed in this paper, we make us think that distinct evolutionary events may have influenced the organization of the heterochromatic segments. According to Peres et al. (2009) three *A. fasciatus* populations from the São Francisco River basin (MG) showed 2n = 48 chromosomes, two of which have shown remarkable heterochromatic blocks. The authors suggest that this characteristic may be due to the endemism of the population that has more discrete

heterochromatin blocks, different from that for the present work, where the three populations analyzed are part of the Corumbataí River basin and can maintain contact between them.

Astyanax marionae of the present paper presented heterochromatic blocks especially on the centromeric and pericentromeric regions in almost all of chromosomes. Krinski and Miyazawa (2014) also evidenced similar heterochromatin location in *A. marionae*. Moreover, Krinski and Miyazawa (2014) also discussed similar morphological characteristics between *A. marionae* and *A. fasciatus*, which have shown karyotypes with many similarities.

On the other hand, molecular cytogenetic data studied here exhibited two distinct chromosomal organization of repetitive sequences: (1) in *A. fasciatus* one pair metacentric bearing H3 histone cluster in interstitial region on the short arm, and 5S rDNA cluster in proximal region on the long arm, as one subtelocentric pair with proximal signal for the H3 histone and one acrocentric pair with proximal signal for the 5S rDNA are evident for the three populations; (2) in *A. marionae* the two genes (H3 histone and 5S rRNA) are organized on the proximal regions of two different acrocentric pairs.

Therefore, sequences of H3 histone may be located in homeologous pairs in the two *A. fasciatus* karyomorphs (2n = 48 and 2n = 50) observed in this work, and in karyomorph with 2n = 46 evidenced by other authors (Hashimoto et al. 2011, Pansonato-Alves et al. 2013, Silva et al. 2015, Piscor and Parise-Maltempi 2016). In *A. marionae* the location of H3-5S clusters is first described here, showing a particular form of organization. Peculiar forms have also been observed for *A. jordani* by Silva et al. (2015), *Astyanax schubarti* and *A. mexicanus* by Piscor and Parise-Maltempi (2016).

According to Piscor and Parise-Maltempi (2016) *A. fasciatus* and others species (*Astyanax altiparanae*, *Astyanax abramis*, *Astyanax asuncionensis*, *Astyanax bockmanni*, and *Astyanax eigenmanniorum*) present H3 histone clusters on the two chromosome pairs (one m/sm and other sm/st) with similar morphologies, except to *A. schubarti*, and *A. mexicanus* that showed different forms. In contrast, *A. marionae* is the species with the least derived system of H3 histone organization to genus *Astyanax* studied so far.

In this context, *A. marionae* and *A. fasciatus* although present similar morphological traits and chromosomal macrostructure features as discussed by Krinski and Miyazawa (2014), but differences on the organization of repetitive sequences are remarkable. About this, we suggest that the distinct organization forms of 5S rDNA and H3 histone clusters among these species do not exclude the possibility of closeness between them.

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References

Artoni RF, Shibatta OA, Gross MC, Schneider CH, Almeida MC, Vicari MR and Bertollo LAC (2006) *Astyanax* aff. *fasciatus* Cuvier, 1819 (Teleostei; Characidae): evidences of a species complex in the upper rio Tibagi basin (Paraná, Brazil). Neotrop Ichthyol 4(2)197-202.

Cabral-de-Mello DC, Moura RC and Martins C (2010) Chromosomal mapping of repetitive DNAs in the beetle *Dichotomius geminatus* provides the first evidence for an association of 5S rRNA and histone H3 genes in insects, and repetitive DNA similarity between the B chromosome and A complement. Heredity 104:393–400.

Centofante L, Bertollo LAC, Justi AJ and Moreira-Filho O (2003) Correlation of chromosomal and morphologic characters in two *Astyanax* species (Teleostei: Characidae). Ichthyol Explor Freswaters 14:361–368.

Eschmeyer WN and Fong JD. Catalog of Fishes. http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp (June 2, 2015).

Fernandes CA and Martins-Santos IC (2003) Cytogenetic characterization of two populations of *Astyanax scabripinnis* (Pisces, Characiformes) of the Ivaí basin PR Brazil. Cytologia 68:289-293.

Fernandes CA and Martins-Santos IC (2004) Cytogenetic studies in two populations of the *Astyanax altiparanae* (Pisces, Characiformes). Hereditas 141:328–332.

Ferreira-Neto M, Artoni RF, Vicari MR, Moreira-Filho O, Camacho JPM, Bakkali M, Oliveira C and Foresti F (2012) Cytogenetics three sympatric karyomorphs in the fish *Astyanax fasciatus* (Teleostei, Characidae) do not seem to hybridize in natural populations. Comp Cytogent 6(1):29–40.

Foresti F, Almeida-Toledo LF and Toledo-Filho SA (1981) Polymorphic nature of nucleous organizer regions in fishes. Cytogenet Cell Genet 31:134–141.

Hashimoto DT, Ferguson-Smith MA, Rens W, Foresti F and Porto-Foresti F (2011) Chromosome mapping of H1 histone and 5S rRNA gene clusters in three species of *Astyanax* (Teleostei, Characiformes). Cytogenet Genome Res 134:64–71.

Javonillo R, Malabarba LR, Weitzman SH and Burns JR (2010) Relationships among major lineages of characid fishes (Teleostei: Ostariophysi: Characiformes), based on molecular sequence data. Mol Phylogenet Evol 54:498–511.

Kavalco KF, Pazza R and Almeida-Toledo LF (2009) *Astyanax bockmanni* Vari and Castro, 2007: an ambiguous karyotype in the *Astyanax* genus. Genetica 136:135–139.

Krinski D and Miyazawa CS (2014) Cytogenetic analysis of three species of *Astyanax* genus (Pisces, Characidae, *Incertae sedis*) from freshwaters of upper Paraguay basin, Mato Grosso state, Brazil. J Life Science 8(1):51–57.

Lima FCT, Malabarba LR, Buckup PA, Silva JFP, Vari RP, Harold A, Benine R, Oyakawa OT, Pavanelli CS, Menezes NA, Lucena CA, Malabarba MCSL, Lucena ZMS, Reis RE, Langeani F, Casatti L, Bertaco VA, Moreira C and Lucinda PHF (2003) Genera *Incertae Sedis* in Characidae. In: Reis R, Kullander ESO and Ferraris Jr C (eds). Check List of the Freshwater Fishes of South and Central America. X edition. Edipucrs, Porto Alegre, pp 106–169.

Martins C and Galetti Jr PM (1999) Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chromosome Res 7:363–367.

Mirande JM (2010) Phylogeny of the family Characidae (Teleostei: Characiformes): from characters to taxonomy. Neotrop Ichthyol 8:385–568.

Morelli S, Bertollo LAC, Foresti F, Moreira-Filho O and Toledo-Filho SA(1983) Cytogenetic considerations on the genus *Astyanax* (Pisces, Characidae). I. Karyotypic variability. Caryologia 36:235–244.

Oliveira C, Avelino GS, Abe KT, Mariguela TC, Benine RC, Ortí G, Vari RP and Castro RMC (2011) Phylogenetic relationships within the speciose family Characidae (Teleostei, Ostariophysi, Characiformes) based on multilocus analysis and extensive ingroup sampling. BMC Evol Biol. 11:275.

Paiz LM, Baumgärtner L, da Graça WJ and Margarido VP (2015) Basic cytogenetics and physical mapping of ribosomal genes in four *Astyanax* species (Characiformes, Characidae) collected in Middle Paraná River, Iguassu National Park: considerations on taxonomy and systematics of the genus. Comp Cytogenet 9(1):51–65.

Pansonato-Alves JC, Hilsdorf AWS, Utsunomia R, Silva DMZA, Oliveira C and Foresti F (2013) Chromosomal mapping of repetitive DNA and cytochrome C oxidase I sequence analysis reveal differentiation among sympatric samples of *Astyanax fasciatus* (Characiformes, Characidae). Cytogenet Genome Res 141:133–142.

Pazza R, Kavalco KF and Bertollo LAC (2006) Chromosome polymorphism in *Astyanax fasciatus* (Teleostei, Characidae). 1 – Karyotypic analysis, Ag-NORs and mapping of the 18S and 5S ribosomal genes in sympatric karyotypes and their possible hybrid forms. Cytogenet Genome Res 112:313–319.

Pazza R, Kavalco KF and Bertollo LAC (2008) Chromosome polymorphism in *Astyanax fasciatus* (Teleostei, Characidae). 2 – Chromosomal location of a satellite DNA. Cytogenet Genome Res 122:61-66.

Pendás AM, Morán P, Freije JP and García-Vázquez E (1994) Chromosomal location and nucleotide sequence of two tandem repeats of the Atlantic salmon 5S rDNA. Cytogenet Cell Genet 67:31–36.

Peres WAM, Buckup PA, Kantek DLZ, Bertollo LAC and Moreira-Filho O (2009) Chromosomal evidence of downstream dispersal of *Astyanax fasciatus* (Characifiomes, Characidae) associated with river shed interconnection. Genetica 137:305–311.

Pinkel D, Straume T and Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 83:2934–2938.

Piscor D and Parise-Maltempi PP (2016) Chromosomal mapping of H3 histone and 5S rRNA genes in eight species of *Astyanax* (Pisces, Characiformes) with different diploid numbers: syntenic conservation of repetitive genes. Genome 59(3):167–172.

Piscor D, Ribacinko-Piscor DB, Fernandes CA and Parise-Maltempi PP (2013) Cytogenetic analysis in three *Bryconamericus* species (Characiformes, Characidae): first description of the 5S rDNA-bearing chromosome pairs in the genus. Mol Cytogenet 6:13.

Sambrook J and Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Silva DMZdA, Utsunomia R, Pansonato-Alves JC, Oliveira C and Foresti F (2015) Chromosomal mapping of repetitive DNA sequences in five species of *Astyanax* (Characiformes, Characidae) reveals independent location of U1 and U2 snRNA sites and association of U1 snRNA and 5S rDNA. Cytogenet Genome Res146:144–152.

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75:304–306.

334 **Table 1.** Cytogenetic data of the different populations of *Astyanax fasciatus* from tributaries of
335 Corumbataí river basin (São Paulo state, Brazil), and *A. marionae* from Chapada dos Guimarães
336 (Mato Grosso state, Brazil) studied in the present paper.

Populations	Diploid number	FN	Karyotype formulae	Localities/Basins
A. aff. *fasciatus*	2n = 50	84	16m, 12sm, 6st, 16a	Cabeça river tributary (SP)/ Corumbataí river basin
A. aff. *fasciatus*	2n = 50	90	8m, 26sm, 6st, 10a	Corumbataí river tributary (SP)/ Corumbataí river basin
A. fasciatus	2n = 48	86	10m, 20sm, 8st, 10a	Ribeirão Claro tributary (SP) Corumbataí river basin
A. marionae	2n = 48	90	8m, 24sm, 10st, 6a	Rio Claro stream (MT)/ Paraguay river basin

337 FN = fundamental number; SP = São Paulo state; MT = Mato Grosso state; m = metacentric; sm =
338 submetacentric; st = subtelocentric; a = acrocentric.

339

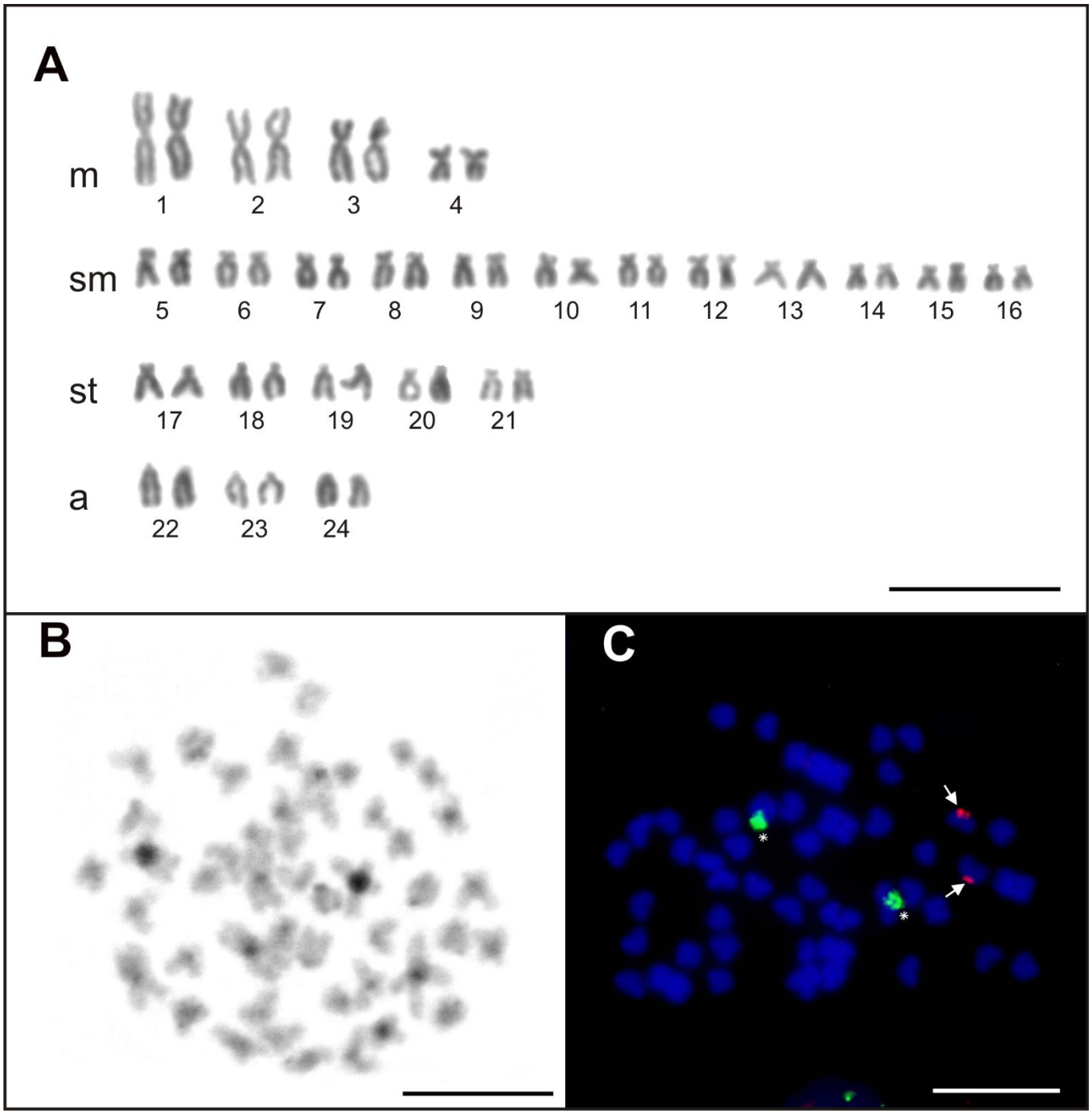

Figure 1. Cytogenetic data of *A. marionae*. **A.** Karyotype. **B.** Metaphase C-banded. **C.** Chromosomal location of H3 histone and 5S rDNA clusters. Arrowhead indicates the H3 histone cluster and asterisk indicates the 5S rDNA cluster. Bar = 10 µm.

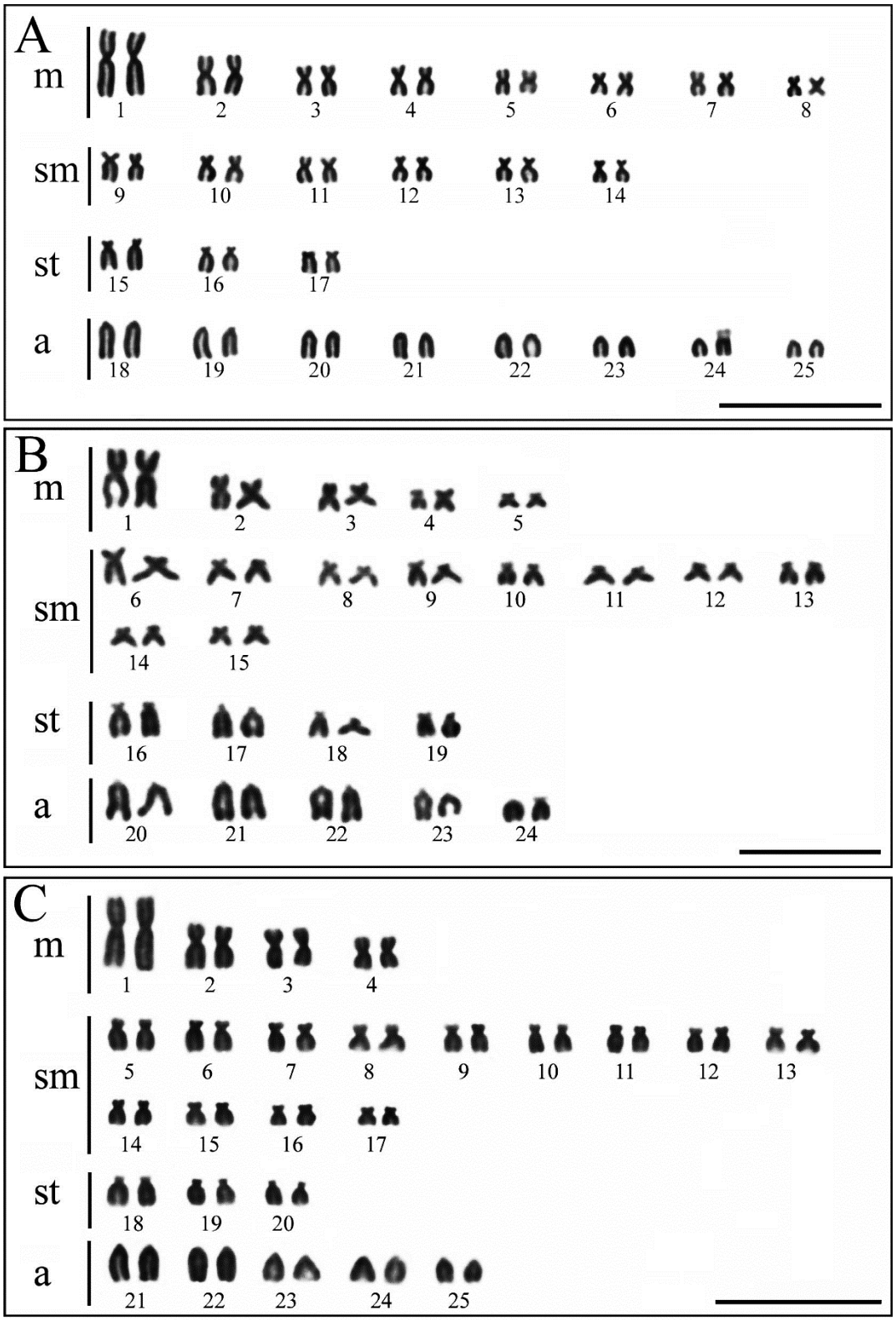

Figure 2. Chromosomes stained with Giemsa. **A.** Karyotype of *A. fasciatus* from the Cabeça river tributary (2n = 50). **B.** Karyotype of *A. fasciatus* from the Ribeirão Claro river tributary (2n = 48). **C.** Karyotype of *A. fasciatus* from the Corumbataí river tributary (2n = 50). Bar = 10 μm.

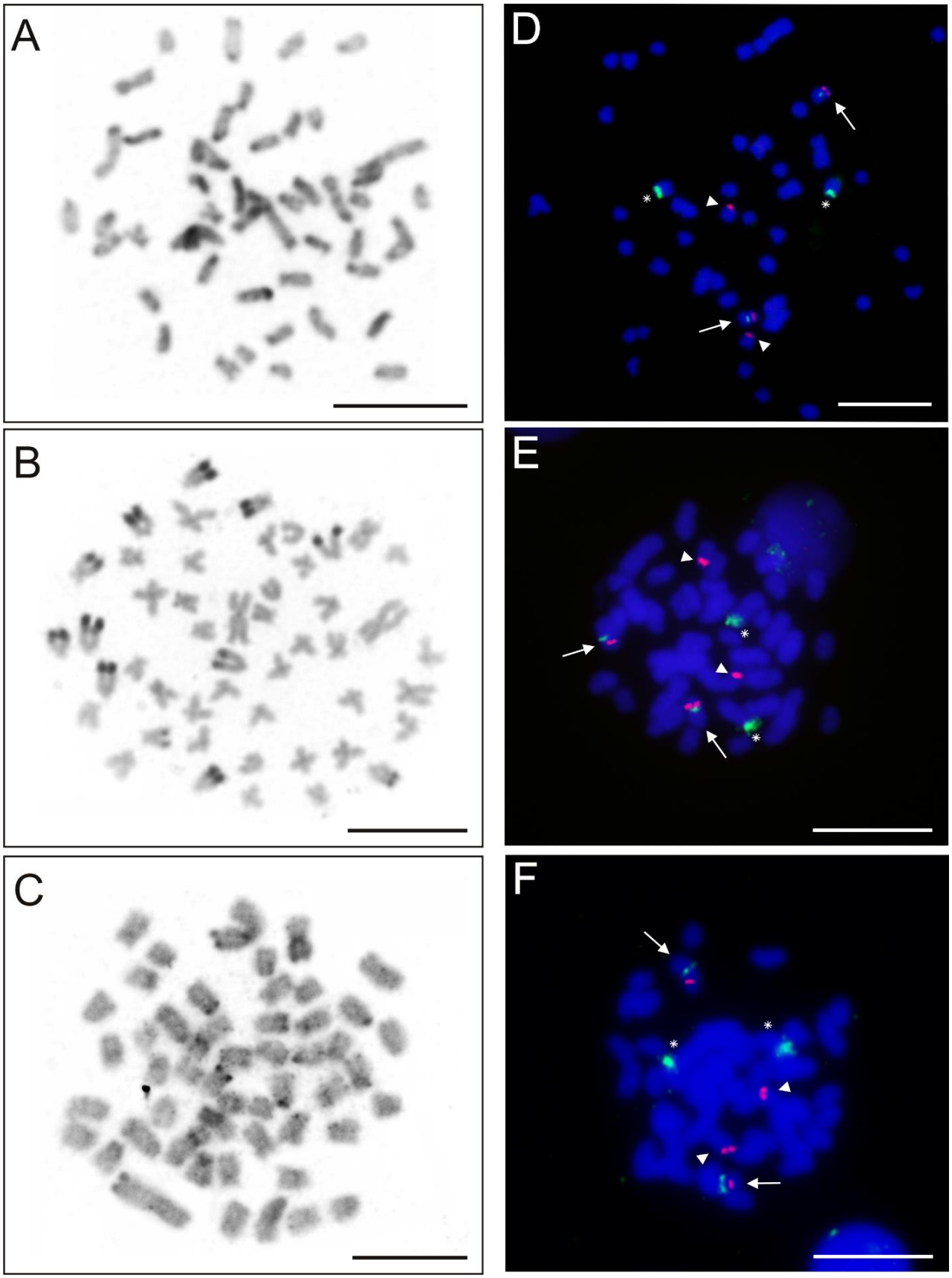

Figure 3. C-banding, H3 histone and 5S rRNA gene clusters location in karyomorphs of *A. fasciatus*. **A.** Metaphase C-banded of *A. fasciatus* from the Cabeça river tributary (2n = 50). **B.** Metaphase C-banded of *A. fasciatus* from the Ribeirão Claro river tributary (2n = 48). **C.** Metaphase C-banded of *A. fasciatus* from the Corumbataí river tributary (2n = 50). **D.** H3 histone and 5S rDNA clusters of *A. fasciatus* from the Corumbataí river tributary. **E.** H3 histone and 5S rDNA clusters of *A. fasciatus* from the Ribeirão Claro river tributary. **F.** H3 histone and 5S rDNA clusters of *A. fasciatus* from the Cabeça river tributary. The arrow indicates the metacentric chromosome with syntenic clusters (H3-5S), the arrowhead indicates the H3 histone cluster, and asterisk indicates the 5S rDNA cluster. Bar = 10 μm.

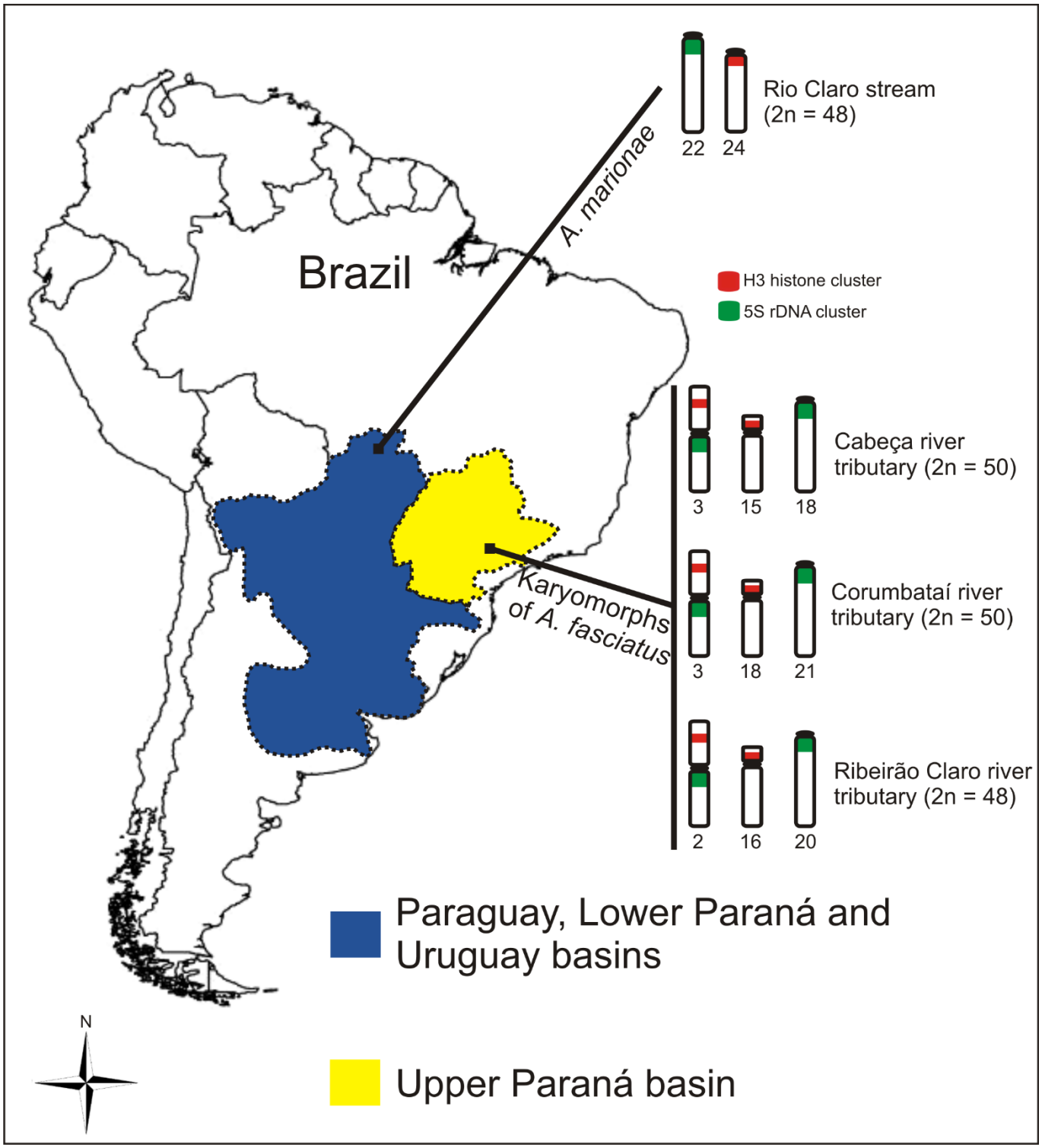

Figure 4. Map of the hydrographic basins and repetitive location on the chromosomes of *A. marionae* and *A. fasciatus*.

6.4. CAPÍTULO IV

HIGHLY SIMILAR MORPHOLOGIES BETWEEN CHROMOSOMES BEARING U2 snRNA GENE CLUSTERS IN THE GROUP *Astyanax* (CHARACIFORMES, CHARACIDAE): AN EVOLUTIONARY APPROACH IN SPECIES WITH 2N = 36, 46, 48, AND 50

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Abstract

Repetitive sequences and their chromosomal locations have been widely studied in species of the *Astyanax* genus. However, the chromosomal organization of U2 snDNA remains largely unknown. The aims of this study were to examine the chromosomal contexts of U2 snRNA and 5S rRNA genes in *Astyanax* species and determine the degree of chromosome morphological similarity between species with different diploid numbers. Clusters of U2 snDNA and 5S rDNA were determined in nine species of *Astyanax*, including two karyomorphs of *Astyanax fasciatus* Cuvier, 1819. All species exhibited U2 snDNA clusters on two chromosome pairs, except *Astyanax mexicanus* De Filippi, 1853 (one pair). The 5S rDNA clusters were located on one chromosome pair in *Astyanax altiparanae* Garutti and Britski, 2000 and *Astyanax marionae* Eigenmann, 1911, on two pairs in *Astyanax abramis* Jenyns, 1842, *Astyanax asuncionensis* Géry, 1972, *Astyanax bockmanni* Vari and Castro, 2007, *Astyanax eigenmanniorum* Cope, 1894, *A. fasciatus* (karyomorphs I and II) and *Astyanax schubarti* Britski, 1964, and on four pairs in *A. mexicanus*. The relationships between the repetitive sequences in different species suggest that *A. schubarti* and *A. mexicanus* exhibit an unusual U2 snDNA chromosomal format as a result of events occurring in the evolutionary history of the *Astyanax* group.

Keywords: Karyotype evolution, Repetitive DNA, Mexican blind cavefish, FISH, 5S rDNA.

Introduction

The *Astyanax* genus contains numerous species (about 140 species),[1] many of which have not been examined cytogenetically. Diploid number ranges from 2n = 36 chromosomes (e.g. *A. schubarti*)[2] to 2n = 50 chromosomes (most species), as in *A. altiparanae*[3] and *A. bockmanni*.[4] Intermediate diploid numbers are also observed, in species such as *A. fasciatus* (2n = 46)[5] and *Astyanax scabripinnis* Jenyns, 1842 (2n = 48).[6] However, the latter two species are considered to be species complexes that present variable diploid numbers (karyomorphs). For example, different karyomorphs of *A. fasciatus* were found under sympatric conditions.[7] *Astyanax* is therefore a promising model for studies of karyotype evolution.

The chromosomal locations of many repetitive sequences are well characterized in *Astyanax* and shown variations, as the locations of 45 ribosomal DNA (45 rDNA) sequences. Similarly, Fernandes and Martins-Santos[8] described four and seven sites of 18S rDNA in different *A. altiparanae* populations from the Paraná river basin (Paraná state – PR, Brazil), whereas Peres et al.[9] described only a single 18S rDNA site in a population of *A. altiparanae* from the upper Paraná river basin (São Paulo state – SP, Brazil). Conversely, fluorescent labeling showed that 5S rDNA location was conserved to the same chromosome pair in these *A. altiparanae* populations.

A general introduction to repetitive sequences was made above. For example, the chromosomal locations of histone and U small nuclear RNA (U snRNA) sequences are conserved.[10,11,12,13] More recently, Piscor and Parise-Maltempi[14] studied eight species of *Astyanax* and demonstrated that the chromosomal locations of H3 histone gene clusters were highly conserved in *A. abramis*, *A. asuncionensis*, *A. altiparanae*, *A. bockmanni*, *A. eigenmanniorum,* and *A. fasciatus*. Silva *et al.*[13] observed that the U1 and U2 snDNA clusters were located at different chromosomal sites in different *Astyanax* species, but exhibited strong conservation in the number of sites per genome.

Considering *Astyanax* a group with wide distribution and distinct cytogenetic features, the aims of this paper were to compare the chromosomal organization of U2 snDNA and 5S

rDNA in nine species, including those with different diploid numbers, and determine parameters underlying the chromosome evolution of U2 snRNA genes in the *Astyanax* genus.

Materials and methods

Sampling and classical cytogenetics

Astyanax specimens were obtained from locations in Brazil as follows: three *A. abramis* and four *A. asuncionensis* specimens from the Bento Gomes river in Mato Grosso state (MT); six *A. marionae* specimens from the Rio Claro stream in Mato Grosso state (MT); five *A. altiparanae* specimens and one *A. schubarti* specimen from the Piracicaba river in São Paulo state (SP); three *A. bockmanni* specimens from the Iguatemi river in Mato Grosso do Sul state (MS); two *A.* aff. *fasciatus* specimens (karyomorph I) from the Corumbataí river tributary (SP); five *A. fasciatus* specimens (karyomorph II) from the Ribeirão Claro river (SP); and three *A. mexicanus* and three *A. eigenmanniorum* specimens from aquariophiles in Brazil. Chromosomes were obtained as described by Foresti *et al.*[15] and chromosome morphologies were determined according to the arm ratios (the most frequently used classification system for fish chromosomes in Brazil), as cited by Piscor *et al.*[16]

Isolation of repetitive DNA probes and Fluorescence *in situ* Hybridization

Genomic DNA was extracted from fin samples as described in Sambrook and Russell.[17] The 5S rDNA probe was prepared using polymerase chain reaction (PCR) with primers described by Pendás *et al.*[18] and Martins and Galetti[19] (A, 5′-TAC GCC CGA TCT CGT CCG ATC-3′; and B, 5′-CAG GCT GGT ATG GCC GTA AGC-3′). The U2 snDNA probe was prepared using PCR with primers described by Bueno *et al.*[20] (U2F, 5′-ATC GCT TCT CGG CCT TAT G–3′; and U2R, 5′-TCC CGG CGG TAC TGC AAT A-3′). The 5S rDNA probe was labeled by PCR with biotin-14-dATP (Invitrogen, San Diego, CA, USA), and the U2 snDNA probe was labeled by PCR with digoxigenin-11-dUTP (Roche, Mannheim, Germany). Probes labeled with digoxigenin-11-dUTP were detected using anti-digoxigenin-rhodamine (Roche,

Mannheim, Germany), and probes labeled with biotin-14-dATP were detected using Alexa Fluor 488 conjugated streptavidin (Invitrogen, San Diego, CA, USA). Single and two-color fluorescence *in situ* hybridization (FISH) was performed using mitotic metaphasic chromosomes, according to Pinkel *et al.*[21] and with modifications as described by Cabral-de-Mello *et al.*[22] Chromosomes were counterstained with Vectashield Mounting Medium (Vector, Burlingame, CA, USA) containing DAPI (4',6-diamidino-2-phenylindole). Chromosomes and fluorescent signals were visualized with an Olympus BX51 microscope coupled to a digital camera (Olympus model D71). Images were captured using DP Controller software.

Results

Species with 2n = 50 chromosomes were *A. abramis*, *A. asuncionensis*, *A. altiparanae*, *A. bockmanni*, *A. eigenmanniorum*, *A. mexicanus* (Figs. 1A–F; Table 1), and *A.* aff. *fasciatus* (karyomorph I; the first described karyomorph for this population) (Fig. 2A; Table 1). All the examined *A. mexicanus* cells contained one acrocentric B chromosome (Fig. 1F, box). Species with smaller diploid numbers were *A. marionae* (2n = 48 chromosomes), *A. fasciatus* (karyomorph II; 2n = 46), and *A. schubarti* (2n = 36) (Figs. 2B–D, respectively; Table 1).

The U2 snDNA clusters were observed on two chromosome pairs in eight of the nine *Astyanax* species (including the two *A. fasciatus* karyomorphs) (Figs. 1, 2; Table 1). In *A. mexicanus,* U2 snDNA was observed on only one chromosome pair (Fig. 1F; Table 1). The U2 snDNA clusters were located on chromosome pairs 17 and 20 (sm) in *A. abramis* (Fig. 1A), pairs 11 and 13 (sm) in *A. asuncionensis* (Fig. 1B), pairs 10 and 13 (sm) in *A. altiparanae* (Fig. 1C), pairs 5 and 11 (sm) in *A. bockmanni* (Fig. 1D), pairs 15 (sm) and 16 (st) in *A. eigenmanniorum* (Fig. 1E), pair 8 (sm) in *A. mexicanus* (Fig. 1F), pairs 13 (sm) and 15 (st) in *A.* aff. *fasciatus* (karyomorph I) (Fig. 2A), pairs 5 and 9 (sm) in *A. marionae* (Fig. 2B), pairs 7 and 10 (sm) in *A. fasciatus* (karyomorph II) (Fig. 2C), and pairs 7 and 14 (sm) in *A. schubarti* (Fig. 2D). All fluorescent signals were located in the pericentromeric regions, with the exception of an interstitial signal observed on pair 7 in *A. schubarti* (Fig. 2D).

In five of the seven species with 2n = 50 chromosomes (*A. abramis*, *A. asuncionensis*, *A. bockmanni*, *A. eigenmanniorum*, and *A. mexicanus*) the 5S rDNA was located on pair 2 (m) (Figs. 1A–B, D–F, respectively). In *A. altiparanae,* the 5S rDNA was located on pair 5 (sm) (Fig. 1C), and in *A.* aff. *fasciatus* (karyomorph I) the 5S rDNA was observed on pairs 3 (m) and 21 (a) (Fig. 2A). Species with 5S rDNA on pair 2 (m) also exhibited signals in pericentromeric regions on other chromosomes. Pericentromeric fluorescent signals were noted on pair 23 (st) in *A. abramis* (Fig. 1A), pair 25 (a) in *A. asuncionensis* (Fig. 1B), pair 19 (a) in *A. bockmanni* (Fig. 1D), pair 22 (a) in *A. eigenmanniorum* (Fig. 1E), and on pairs 15 (sm), 22 (a), and 25 (a) in *A. mexicanus* (Fig. 1F). The three species with smaller diploid numbers, *A. marionae* (2n = 48), *A. fasciatus* karyomorph II (2n = 46) and *A. schubarti* (2n = 36), harbored 5S rDNA clusters at pericentromeric regions on pair 22 (a) (*A. marionae*; Fig. 2B), pairs 3 (m) and 22 (a) (*A. fasciatus* karyomorph II; Fig. 2C), and on the 3 and 4 (m) (*A. schubarti*; Fig. 2D).

A summary diagram indicating the chromosomal locations of the U2 snDNA clusters in the nine *Astyanax* species is shown in Figure 3. Note that three groups were formed: first group with two chromosome pairs bearing U2 snDNA clusters shared by several species (Figure 3A), second group with only one pair (Figure 3B), and third group with two pairs but the first pair with interstitial clusters (Figure 3C).

Discussion

As demonstrated by previous cytogenetic observations, the most common diploid number in the *Astyanax* genus is 2n = 50 chromosomes. This is consistent with the majority of species in the family Characidae.[2] However, other diploid chromosome numbers are observed in the *Astyanax* genus, such as the species with 2n = 36, 46, and 48 examined in this study. Furthermore, species complexes with variable diploid number are found in *Astyanax*, such as the "*scabripinnis* complex" and the "*fasciatus* complex" (see, for example,[23,24]). Here, *A. fasciatus* karyomorphs from the same river system were examined that had two different

diploid numbers (2n = 46 and 2n = 50). *Astyanax fasciatus* is known to have several karyomorphs, mostly with 2n = 46, 48, and 50 chromosomes.[7]

The *Astyanax* genus is distinguished cytogenetically by diploid number variability, from 2n = 36 chromosomes for *A. schubarti*[2] and *Astyanax correntinus* Holmberg, 1891[25] to 2n = 50 chromosomes for most species, e.g., *A. altiparanae* and *A. bockmanni*.[3,4] Variations in karyotype formula and fundamental number (FN) are also widely observed even in populations of the same species. For example, Fernandes and Martins-Santos[3] reported two different karyotype formulae and FN in two *A. altiparanae* populations from the Índios and Paraná rivers (PR, Brazil), and these values differ from those of the Piracicaba river (SP, Brazil) studied here. On the other hand, FISH examination have identified that repetitive sequences showed similar chromosomal locations in *Astyanax* species.

Chromosomal locations of 5S rDNA are conserved in some *Astyanax* and generally exhibit three forms.[26] The form found in most species, including the *A. fasciatus* karyomorphs analyzed here, exhibits one metacentric pair and one acrocentric or subtelocentric pair with 5S rDNA sites located on the long arm, both near the centromere.[26] According to Vicari *et al.*,[27] *Astyanax* species with two chromosome pairs bearing 5S rDNA sites exhibit probable synapomorphic features.

In fish, 5S rDNA and other repetitive DNA clusters may be located on the same chromosome pair. For example, while 5S rRNA and histone genes can occur on the same chromosome in *Astyanax* species,[10,14] the 5S rDNA is close to 18S rDNA clusters in *Bryconamericus* aff. *iheringii* Boulenger, 1887,[16] another characid fish . The chromosomal locations of the 5S rDNA and U2 snDNA clusters were not consistently linked in the *Astyanax* species examined here, because that these represent two distinct classes of repetitive DNA with completely different functions. Supiwong *et al.*[28] found that U2 snDNA and 5S rDNA sequences were also carried on different chromosome pairs in the naked catfish *Mystus bocourti* Bleeker, 1864 (Siluriformes). This spatial separation of 5S rDNA and U2 snDNA appears to be a common feature in fish (see, for example,[29,30]).

All the species examined here had clusters of U2 snDNA on two chromosome pairs, with the exception of *A. mexicanus* (one pair) (see the figure 3). Therefore, our results suggest that the two pairs with U2 snDNA clusters may represent a similar chromosome pairs shared by species of the first group (Figure 3A), and the only one chromosome pair of *A. mexicanus* (Figure 3B) and two pairs of *A. schubarti* (Figure 3C) represent different forms of genomic organization of U2 snRNA genes. These two different forms may be explained due to probable reduction of the diploid number (2n = 36) of *A. schubarti* and ancient separation of *A. mexicanus* from the South America as previously proposed by Piscor and Parise-Maltempi.[14]

Recently, Silva *et al.*[13] found U2 snDNA clusters on two chromosome pairs in different *Astyanax* species, except in *Astyanax jordani* Hubbs and Innes, 1936 (one pair). Silva *et al.*[13] also showed that, while U1 and U2 snRNA genes were located on different chromosome pairs in different species, the numbers of U1 and U2 sites per genome were strongly conserved. Martins and Galetti[19] proposed that 5S rDNA on a single pair of chromosomes probably represented a more ancient genomic condition in *Leporinus* Spix, 1829 (Anostomidae).

The eyed epigean form (surface fish) of *A. mexicanus* is widely distributed in northeastern Mexico and southern Texas, and the eyeless hypogean forms (cavefish) live in some caves inside this extension.[31] Therefore, an ancestral link is possible between the single pair of chromosomes carrying U2 snDNA in *A. mexicanus* from Mexico and single U2 snDNA pair in other species of *Astyanax* from North and Central America, as for example, *A. jordani* studied by Silva *et al.*[13] that also showed one chromosome pair bearing U2 snDNA clusters.

In summary, the variability in diploid chromosome number in the *Astyanax* genus is not reflected in the chromosomal organization of the U2 snRNA genes. However, U2 snDNA sites appear to be located on two chromosome pairs with medium size and similar morphologies in almost all *Astyanax* species. The U2 snDNA cluster stability could be the result of an evolutionary advantage or association with specific DNA segments or particular regions of the genome, which may have facilitated the maintenance of U2 snDNA on two chromosome pairs in South American *Astyanax* species.

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References

1. Eschmeyer WN, Fong JD. Catalog of Fishes. 2015; online available at http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp

2. Morelli S, Bertollo LAC, Foresti F, Moreira-Filho O, Toledo-Filho SA. Cytogenetic considerations on the genus *Astyanax* (Pisces, Characidae). I. Karyotypic variability. Caryologia 1983;36:235–244.

3. Fernandes CA, Martins-Santos IC; Cytogenetic studies in two populations of the *Astyanax altiparanae* (Pisces, Characiformes). Hereditas 2004;141:328–332.

4. Kavalco KF, Pazza R, Almeida-Toledo LF. *Astyanax bockmanni* Vari and Castro, 2007: an ambiguous karyotype in the *Astyanax* genus. Genetica 2009;136:135–139.

5. Torres-Mariano AR, Morelli S. Chromosomal analysis of *Astyanax fasciatus* (Pisces, Characidae) from the Araguari river, Uberlândia, MG, Brazil. Braz J Biol 2006;66(1A):161–165.

6. Maistro EL, Oliveira C, Foresti F. Sympatric occurrence of two cytotypes of *Astyanax scabripinnis* (Characiformes, Characidae). Genet Mol Biol 2000;23(2):365–369.

7. Ferreira-Neto M, Artoni RF, Vicari MR, Moreira-Filho O, Camacho JPM, Bakkali M, *et al.* Cytogenetics three sympatric karyomorphs in the fish *Astyanax fasciatus* (Teleostei, Characidae) do not seem to hybridize in natural populations. Comp Cytogent 2012;6(1):29–40.

8. Fernandes CA, Martins-Santos IC. Mapping of the 18S and 5S ribosomal RNA genes in *Astyanax altiparanae* Garutti & Britski, 2000 (Teleostei, Characidae) from the upper Paraná river basin, Brazil. Genet Mol Biol 2006;29(3):464–468.

9. Peres WAM, Bertollo LAC, Moreira-Filho O. Physical mapping of the 18S and 5S ribosomal genes in nine Characidae species (Teleostei, Characiformes). Genet Mol Biol 2008;31:222–226.

10. Hashimoto DT, Ferguson-Smith MA, Rens W, Foresti F, Porto-Foresti F. Chromosome mapping of H1 histone and 5S rRNA gene clusters in three species of *Astyanax* (Teleostei, Characiformes). Cytogenet Genome Res 2011;134:64–71.

11. Silva DMZA, Pansonato-Alves JC, Utsunomia R, Daniel SN, Hashimoto DT, Oliveira C, *et al.* Chromosomal organization of repetitive DNA sequences in *Astyanax bockmanni* (Teleostei, Characiformes): dispersive location, association and co-localization in the genome. Genetica 2013;141(7):329–336.

12. Silva DMZdA, Pansonato-Alves JC, Utsunomia R, Araya-Jaime C, Ruiz-Ruano FJ, Daniel SN, *et al.* Delimiting the origin of a B chromosome by FISH mapping, chromosome painting and DNA sequence analysis in *Astyanax paranae* (Teleostei, Characiformes). PLoS ONE 2014;9(4):e94896.

13. Silva DMZdA, Utsunomia R, Pansonato-Alves JC, Oliveira C, Foresti F. Chromosomal mapping of repetitive DNA sequences in five species of *Astyanax* (Characiformes, Characidae) reveals independent location of U1 and U2 snRNA sites and association of U1 snRNA and 5S rDNA. Cytogenet Genome Res 2015;146:144–152.

14. Piscor D, Parise-Maltempi PP. Chromosomal mapping of H3 histone and 5S rRNA genes in eight species of *Astyanax* (Pisces, Characiformes) with different diploid numbers: syntenic conservation of repetitive genes. Genome 2016;59(3):167–172.

15. Foresti F, Almeida-Toledo LF, Toledo-Filho SA. Polymorphic nature of nucleous organizer regions in fishes. Cytogenet Cell Genet 1981;31:134–141.

16. Piscor D, Ribacinko-Piscor DB, Fernandes CA, Parise-Maltempi PP. Cytogenetic analysis in three *Bryconamericus* species (Characiformes, Characidae): first description of the 5S rDNA-bearing chromosome pairs in the genus. Mol Cytogenet 2013;6:13.

17. Sambrook J, Russell DW: Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2001.

18. Pendás AM, Morán P, Freije JP, García-Vázquez E. Chromosomal location and nucleotide sequence of two tandem repeats of the Atlantic salmon 5S rDNA. Cytogenet Cell Genet 1994;67:31–36.

19. Martins C, Galetti Jr PM. Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chromosome Res 1999;7:363–367.

20. Bueno D, Palacios-Gimenez OM, Cabral-de-Mello DC. Chromosomal mapping of repetitive DNAs in *Abracris flavolineata* reveal possible ancestry for the B chromosome and surprisingly H3 histone spreading. PLoS ONE 2013;8:e66532.

21. Pinkel D, Straume T, Gray JW. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 1986;83:2934–2938.

22. Cabral-de-Mello DC, Moura RC, Martins C. Chromosomal mapping of repetitive DNAs in the beetle *Dichotomius geminatus* provides the first evidence for an association of 5S rRNA and histone H3 genes in insects, and repetitive DNA similarity between the B chromosome and A complement. Heredity 2010;104:393–400.

23. Moreira-Filho O, Bertollo LAC. *Astyanax scabripinnis* (Pisces, Characidae): a species complex. Rev Bras Genet 1991;14:331–357.

24. Pazza R, Kavalco SAF, Bertollo LAC. Chromosome polymorfism in *Astyanax fasciatus* (Teleostei, Characidae) 1. Karyotype analysis, Ag-NORs and mapping of 18S and 5S ribosomal genes in sympatric karyotypes and their possible hybrid forms. Cytogenet Genome Res 2006;112:313–319.

25. Paiz LM, Baumgärtner L, da Graça WJ, Margarido VP. Basic cytogenetics and physical mapping of ribosomal genes in four *Astyanax* species (Characiformes, Characidae) collected in Middle Paraná River, Iguassu National Park: considerations on taxonomy and systematics of the genus. Comp Cytogenet 2015;9(1):51–65.

26. Piscor D, Alves AL, Parise-Maltempi PP. Chromosomal microstructure diversity in three *Astyanax* (Characiformes, Characidae) species: comparative analysis of the chromosomal locations of the 18S and 5S rDNAs. Zebrafish 2015;12(1):81–90.

27. Vicari MR, Noleto RB, Artoni RF, Moreira-Filho O, Bertollo LAC Comparative cytogenetics among species of the *Astyanax scabripinnis* complex. Evolutionary and biogeographical inferences. Genet Mol Biol 2008;31:173–179.

28. Supiwong W, Liehr T, Cioffi MB, Chaveerach A, Kosyakova N, Pinthong K, *et al.* Karyotype and cytogenetic mapping of 9 classes of repetitive DNAs in the genome of the naked catfish *Mystus bocourti* (Siluriformes, Bagridae). Mol Cytogenet 2013;6:51.

29. Merlo MA, Pacchiarini T, Portela-Bens S, Cross I, Manchado M, Rebordinos L. Genetic characterization of *Plectorhinchus mediterraneus* yields important clues about genome organization and evolution of multigene families. BMC Genet 2012;13:33.

30. Utsunomia R, Scacchetti PC, Pansonato-Alves JC, Oliveira C, Foresti F. Comparative chromosome mapping of U2 snRNA and 5S rRNA genes in *Gymnotus* species (Gymnotiformes, Gymnotidae): evolutionary dynamics and sex chromosome linkage in *G. pantanal*. Cytogenet Genome Res 2014;142:286–292.

31. Dowling, TE, Martasian DP, Jeffery WR. Evidence for multiple genetic forms with similar eyeless phenotypes in the blind cavefish, *Astyanax mexicanus*. Mol Biol Evol 2002;19(4):446–455.

382 **Table 1.** Chromosomal data in species of *Astyanax* with U2 snDNA clusters.

Species	2n	karyotype formulae	FN[a]	5S[b]	U2[c]	References
A. abramis	50	12m + 28sm + 6st + 4a	96	4	4	Present study
A. asuncionensis	50	8m + 22sm + 14st + 6a	94	4	4	Present study
A. altiparanae	50	6m + 22sm + 12st + 10a	90	2	4	Present study
A. altiparanae	50	10m + 16sm + 16st + 8a	-	2	4	Silva *et al.*[13]
A. bockmanni	50	6m + 20sm + 8st + 16a	84	4	4	Present study
A. bockmanni	50	8m + 14sm + 12st + 16a	-	4	4	Silva *et al.*[13]
A. eigenmanniorum	50	8m + 22sm + 12st + 8a	92	4	4	Present study
A. aff. *fasciatus* (kary I)	50	8m + 20sm + 12st + 10a	90	4	4	Present study
A. fasciatus (kary II)	46	8m + 18sm + 16st + 4a	88	4	4	Present study
A. fasciatus	46	10m + 14sm + 14st +8a	-	4	4	Silva *et al.*[13]
A. jordani	50+B[d]	8m + 18sm + 12st + 12a	-	10	2	Silva *et al.*[13]
A. marionae	48	8m + 24sm + 10st + 6a	90	2	4	Present study
A. mexicanus	50+B[d]	8m + 26sm + 6st + 10a	90	8	2	Pressent study
A. paranae Eigenmann, 1914	50+B[d]	8m + 22sm + 10st + 10a	-	4	4	Silva *et al.*[13]
A. schubarti	36	12m + 16sm + 4st + 4a	68	4	4	Present study

383 [a]Fundamental numbers; [b]Numbers of clusters (5S rDNA); [c]Numbers of clusters (U2 snDNA); [d]B chromosomes.
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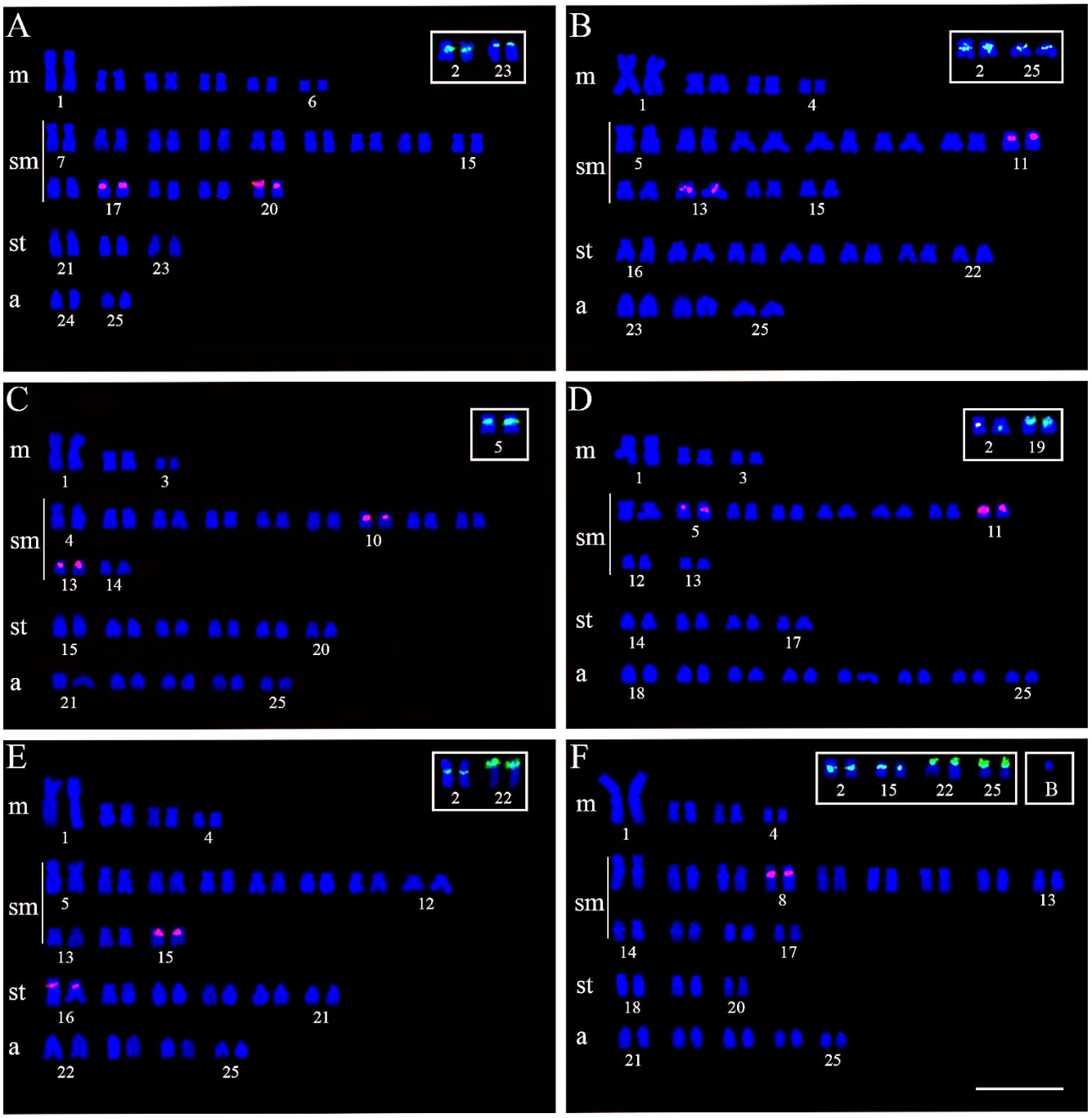

FIG. 1. Locations of U2 snDNA and 5S rDNA clusters on chromosomes of *Astyanax* species with 2n = 50 chromosomes. (**A**) *A. abramis*, (**B**) *A. asuncionensis*, (**C**) *A. altiparanae*, (**D**) *A. bockmanni*, (**E**) *A. eigenmanniorum*, and (**F**) *A. mexicanus*. Karyotypes indicate the chromosome pairs with U2 snDNA clusters, and boxes indicate pairs with 5S rDNA clusters. Bar = 10 µm.

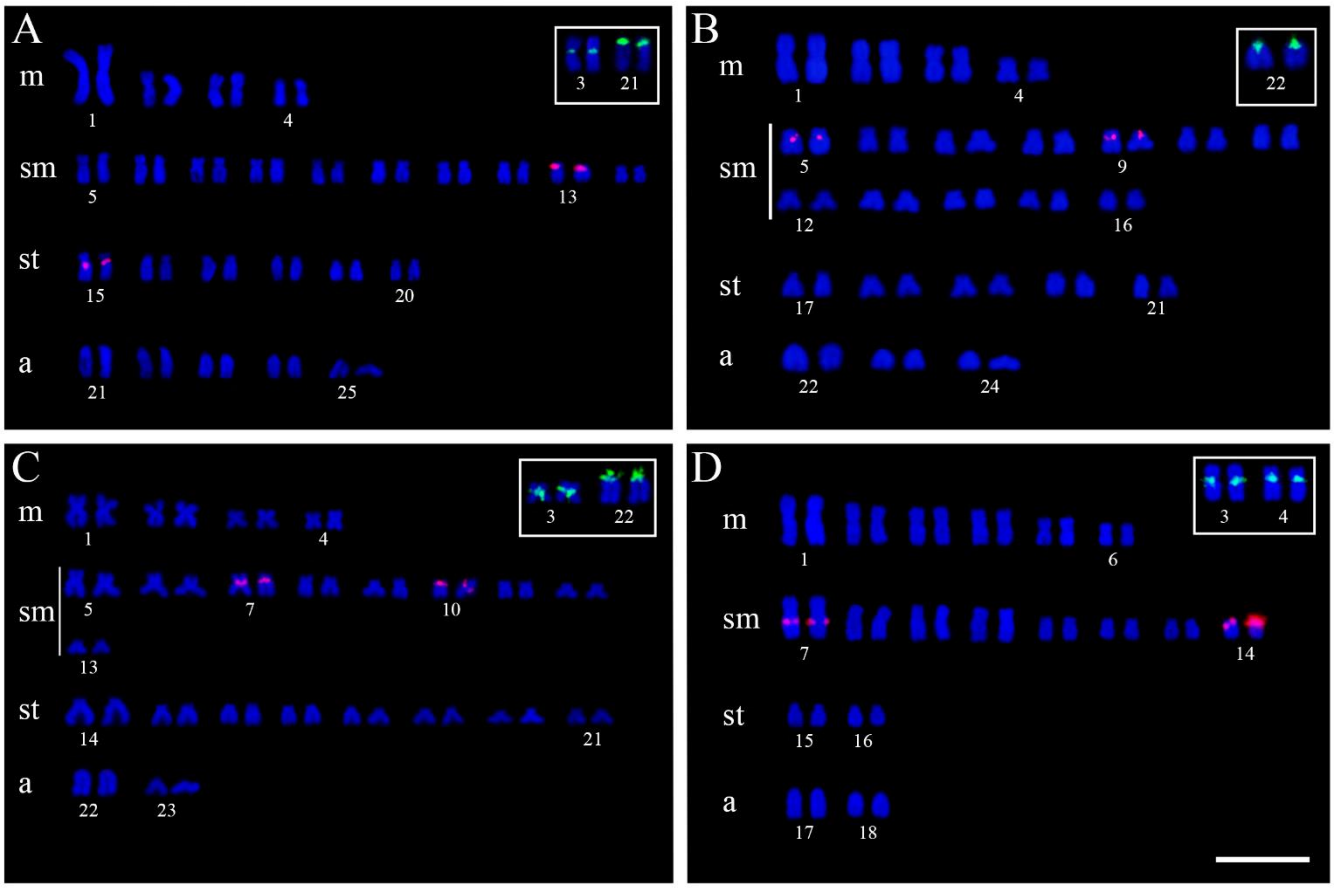

FIG. 2. Locations of U2 snDNA and 5S rDNA clusters on chromosomes of *A. marionae* (2n = 48), *A. schubarti* (2n = 36), and two *A. fasciatus* populations (karyomorph I, 2n = 50 and karyomorph II, 2n = 46). (**A**) *A.* aff. *fasciatus* (karyomorph I), (**B**) *A. marionae*, (**C**) *A. fasciatus* (karyomorph II), and (**D**) *A. schubarti*. Karyotypes indicate the chromosome pairs with U2 snDNA clusters, and boxes indicate pairs with 5S rDNA clusters. Bar = 10 µm.

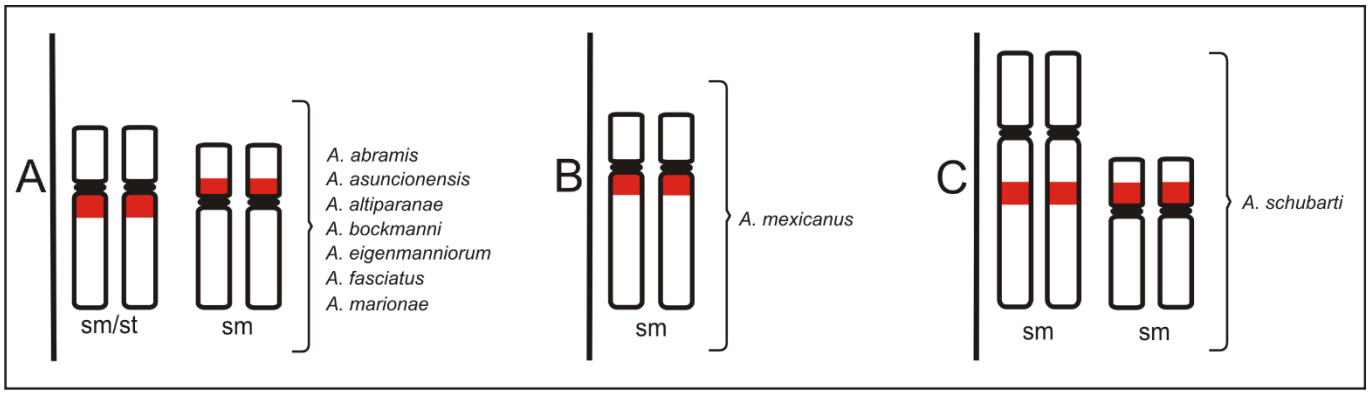

FIG. 3. Diagram indicating the chromosome pairs bearing U2 snDNA clusters in the nine *Astyanax* species. (**A**) Species with very similar chromosome pairs (pericentromeric regions), (**B**) *A. mexicanus,* with only one chromosome pair carrying U2 snDNA (pericentromeric region), and (**C**) *A. schubarti,* with two chromosome pairs carrying U2 snDNA (pair 7, interstitial location, and pair 14, pericentromeric location). U2 snDNA clusters are shown in red.

6.5. CAPÍTULO V

MICROSATELLITE ORGANIZATION IN THE B CHROMOSOME AND A CHROMOSOME COMPLEMENT IN *Astyanax* (CHARACIFORMES, CHARACIDAE) SPECIES

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Microsatellite Organization in the B Chromosome and A Chromosome Complement in *Astyanax* (Characiformes, Characidae) Species

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Key Words

Astyanax schubarti · Chromosome evolution · Heterochromatin · Mexican blind cavefish · Repetitive DNA

Abstract

The organization of microsatellites in B and sex chromosomes has been linked to chromosomal evolution in a number of animal groups. Here, the chromosomal organizations of $(CA)_{15}$, $(GA)_{15}$, $(CG)_{15}$, $(GACA)_4$, and $(GATA)_8$ microsatellites were examined in several *Astyanax* species with different diploid numbers: *Astyanax mexicanus* (2n = 50 + 1 B chromosome), *A. altiparanae* (2n = 50), *A. marionae* (2n = 48), *A. fasciatus* (2n = 46), and *A. schubarti* (2n = 36). The $(CA)_{15}$ and $(GA)_{15}$ microsatellites were dispersed across the chromosomes of *A. altiparanae* and *A. fasciatus* but were also observed as clusters (CA and GA for *A. altiparanae*, and CA for *A. fasciatus*). In *A. marionae* and *A. schubarti*, the $(CA)_{15}$ and $(GA)_{15}$ microsatellites were dispersed but were also observed as clustered signals and coincident with heterochromatic regions. In all 4 of these species, the $(CG)_{15}$ and $(GACA)_4$ microsatellites were dispersed across chromosomes, and the $(GATA)_8$ microsatellite was co-localized with 5S rDNA. In *A. mexicanus*, the $(CA)_{15}$, $(GA)_{15}$, $(CG)_{15}$, $(GATA)_8$, and $(GACA)_4$ microsatellites were weakly detected and dispersed across the chromosomes of the A complement. On the B chromosome, signals for the different microsatellites were weak, strong, absent, weak, and absent, respectively. The distribution of microsatellites and the locational relationship between microsatellites and 5S rDNA are discussed, and a possible evolutionary pathway is proposed for microsatellites in *Astyanax*.

Microsatellites are excellent markers for the use in studies of sex and B chromosomes [Pokorná et al., 2011; Milani and Cabral-de-Mello, 2014; Palacios-Gimenez and Cabral-de-Mello, 2015]. For example, abundant accumulation of microsatellites was detected in the W chromosome of the lacertid *Eremias velox* [Pokorná et al., 2011], and the authors suggested that accumulation of microsatellites in *E. velox* and other eukaryotic organisms might play an important role in sex chromosome dynamics [Pokorná et al., 2011].

Milani and Cabral-de-Mello [2014] found that, while the majority of examined microsatellites were scattered along the entire length of the B chromosome in *Abracris flavolineata* (grasshopper), conspicuous blocks were apparent and enriched for $(GA)_{15}$ and $(GAC)_{10}$ microsatellites. This suggested that microsatellites could play an important role in B chromosome evolution. In this context, the *Astyanax* genus can be regarded as a good model because of the variation in diploid numbers (e.g.

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Table 1. Microsatellite organization in the chromosomes of the A complement in *Astyanax* species

Species (diploid numbers)	N	Microsatellites					Localities
		$(CA)_{15}$	$(GA)_{15}$	$(CG)_{15}$	$(GACA)_4$	GATA/5S	
A. altiparanae (50)	5	C	C	S	S	P	SP, Brazil
A. fasciatus (46)	3	C	S	S	S	P	SP, Brazil
A. marionae (48)	6	Ccb	Ccb	S	S	P	MT, Brazil
A. schubarti (36)	1	Ccb	Ccb	S	S	P	SP, Brazil
A. mexicanus (50)	6	S	S	S	S	Ab	Mexico[a]

Ab = Absent but the GATA repeats showed spreading on the chromosomes of *A. mexicanus*; C = spread and with fluorescence signals clustered; Ccb = spread and with fluorescence signals clustered and coincident with C-band heterochromatin; P = present; S = fluorescence signals spread; SP = São Paulo state; MT = Mato Grosso state. [a] Mexican blind cavefish obtained from an aquarium store in Brazil.

A. schubarti with 2n = 36 and *A. altiparanae* with 2n = 50) and the presence of species possessing B chromosomes [Moreira-Filho et al., 2004].

Recent surveys on chromosomal microsatellite locations showed that organization in sex chromosomes differed between *Leporinus* and *Characidium* species [Poltronieri et al., 2014; Scacchetti et al., 2015]. For example, in W chromosomes of the *Leporinus* species, microsatellites accumulated primarily in heterochromatic regions of the long arms. However, microsatellite distributions differed between the 4 *Leporinus* species, despite the 4 species exhibiting similar patterns of heterochromatin distribution. This suggested that the heterochromatinization process is dynamic, free of selection, and subject to distinctive mechanisms after speciation [Poltronieri et al., 2014].

In this study the chromosomal organization of different microsatellite repeats in *Astyanax* species with different diploid numbers (2n = 36, 2n = 46, 2n = 48, and 2n = 50) was analyzed, and the microsatellite distribution in the B chromosome and A complement of *A. mexicanus* was examined. Possible scenarios for the evolution of microsatellite distribution and chromosomal clustering of GATA repeats and 5S rDNA are discussed.

Materials and Methods

Specimens and Classical Cytogenetics

The following animals were collected: *A. schubarti* and *A. altiparanae* from the Piracicaba river in São Paulo state, Brazil; *A. marionae* from the Rio Claro stream in Mato Grosso state, Brazil; *A. fasciatus* from the Ribeirão Claro river in São Paulo state, Brazil; and *A. mexicanus* (Mexican blind cavefish) from aquarists in Brazil (table 1). Mitotic metaphase chromosomes were prepared using the methods described by Foresti et al. [1981]. Heterochromatin was examined using the C-banding technique described by Sumner [1972].

DNA Extraction and Probe Synthesis

Genomic DNA was extracted from fin samples as described by Sambrook and Russell [2001]. PCR with the following primers was used to amplify the 5S rDNA probe: A (5′-TAC GCC CGA TCT CGT CCG ATC-3′) and B (5′-CAG GCT GGT ATG GCC GTA AGC-3′) as described by Pendás et al. [1994] and Martins and Galetti [1999]. The $(CA)_{15}$, $(GA)_{15}$, $(CG)_{15}$, $(GACA)_4$, and $(GATA)_8$ microsatellites were amplified and labeled with biotin during synthesis as described by Milani and Cabral-de-Mello [2014]. Microsatellites were donated by Prof. Dr. Diogo C. Cabral-de-Mello for use in laboratory experiments.

Fluorescence in situ Hybridization and Fiber-FISH

FISH experiments were performed according to Pinkel et al. [1986], with modifications as per Cabral-de-Mello et al. [2010]. Fiber-FISH was performed as described by Barros et al. [2011], and slides were used in double-FISH experiments with 5S rDNA and $(GATA)_8$ probes. Signals were detected using anti-digoxigenin-rhodamine (Roche, Mannheim, Germany) for digoxigenin and avidin Alexa Fluor 488 conjugate (Invitrogen, San Diego, Calif., USA) for biotin. Slides were mounted with Vectashield Mounting Medium (Vector, Burlingame, Calif., USA) containing DAPI (4′,6-diamidino-2-phenylindole) for chromosome counterstaining. Chromosome and fluorescence signals were visualized with an Olympus BX51 microscope coupled to a digital camera (Olympus model D71). Images were captured using DP Controller software.

Results

Diploid numbers of *A. altiparanae*, *A. marionae*, *A. fasciatus*, and *A. mexicanus* are given in table 1. In *A. mexicanus*, one small acrocentric B chromosome was ob-

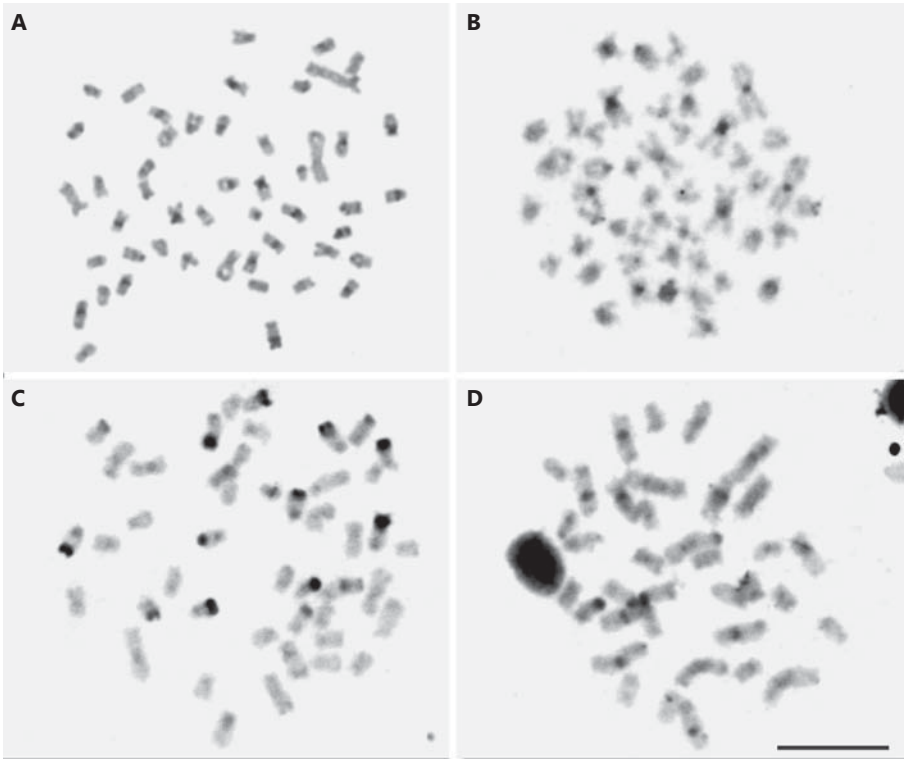

Fig. 1. C-banded metaphases of *A. altiparanae* (**A**), *A. marionae* (**B**), *A. fasciatus* (**C**), and *A. schubarti* (**D**). Bar = 10 μm.

served in all metaphases. Modest blocks of heterochromatin were observed, mainly in the pericentromeric and centromeric chromosomal regions in *A. altiparanae* (fig. 1A), pericentromeric and centromeric regions in *A. marionae* (fig. 1B), terminal regions in *A. fasciatus* (fig. 1C), and pericentromeric and centromeric regions in *A. schubarti* (fig. 1D). *A. mexicanus* chromosomes contained blocks of heterochromatin in the terminal and pericentromeric regions, and the B chromosome was C-band negative.

Microsatellites were distributed in 4 organizational formats:

1 Dispersed and with blocks of $(CA)_{15}$ and $(GA)_{15}$ microsatellites, corresponding to C-band heterochromatin in *A. marionae* and *A. schubarti* (fig. 2; note that the CA and GA repeats show more evident blocks in *A. schubarti*).
2 $(CA)_{15}$ and $(GA)_{15}$ microsatellites were spread across the chromosomes in *A. altiparanae* and *A. fasciatus* and were also found in clusters. However, the clusters did not correspond to C-band heterochromatic regions. CA and GA repeats formed clusters in *A. altiparanae*, but only CA repeats were clustered in *A. fasciatus* (fig. 2).
3 $(CG)_{15}$ and $(GACA)_4$ microsatellites were dispersed across the chromosomes in *A. altiparanae*, *A. marionae*, *A. fasciatus*, and *A. schubarti* (fig. 2).
4 The $(GATA)_8$ microsatellite was co-localized with 5S rDNA on pair 5 in *A. altiparanae*, pair 22 in *A. marionae*, pairs 3 and 22 in *A. fasciatus*, and pairs 3 and 4 in *A. schubarti* (figs. 3, 4; table 1).

In *A. mexicanus*, the $(CA)_{15}$, $(GA)_{15}$, $(CG)_{15}$, $(GACA)_4$, and $(GATA)_8$ microsatellites exhibited weak fluorescence signals on the chromosomes of the A complement (fig. 5; table 1). On the B chromosome, no signal was evident for $(CG)_{15}$ and $(GACA)_4$ microsatellites, $(CA)_{15}$ and $(GATA)_8$ microsatellites produced minimal signals, and $(GA)_{15}$ microsatellite exhibited stronger signals (fig. 5).

Discussion

Four microsatellite organizations were found in chromosomes of different *Astyanax* species with a range of diploid numbers: (1) clustered and coincident with C-band heterochromatin, (2) clustered and non-coincident with C-band heterochromatin, (3) dispersed across the chromosomes, and (4) co-localized with 5S rDNA. These

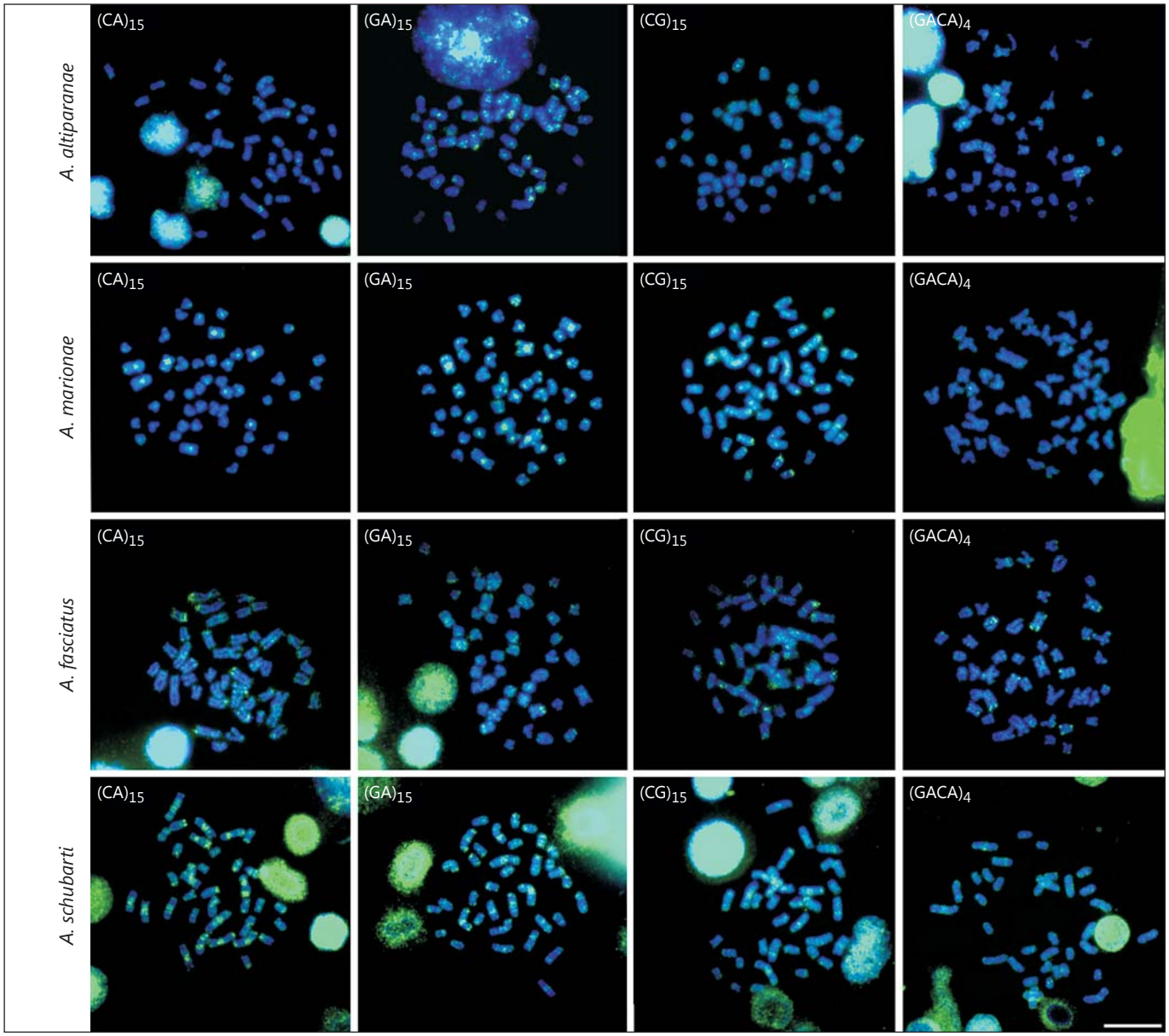

Fig. 2. Microsatellite distribution in chromosomes of *Astyanax* species. Bar = 10 μm.

results suggest that there may be 3 evolutionary pathways related to drive and/or genomic organization of microsatellites in *Astyanax* genomes: (1) the microsatellites can follow a free way to spreading and/or clustering, (2) the heterochromatin can play an important role in the genome organization of the microsatellites, and (3) the co-localization of 5S rDNA-GATA can stabilize DNA structures, acting as 'hot spots' for recombination, as discussed by Merlo et al. [2010].

B chromosomes carrying microsatellite DNA were described previously, for example, in the grasshopper *A. flavolineata* [Milani and Cabral-de-Mello, 2014]. For the authors, microsatellites located on the B chromosome may play an important role in the evolution of these elements. In the present study, *A. mexicanus* individuals contained 0 or 1 B chromosome. Thus, the low rate of recombination in the B chromosome of *A. mexicanus* may facilitate accumulation of microsatellites, as proposed by Charlesworth et al. [2005].

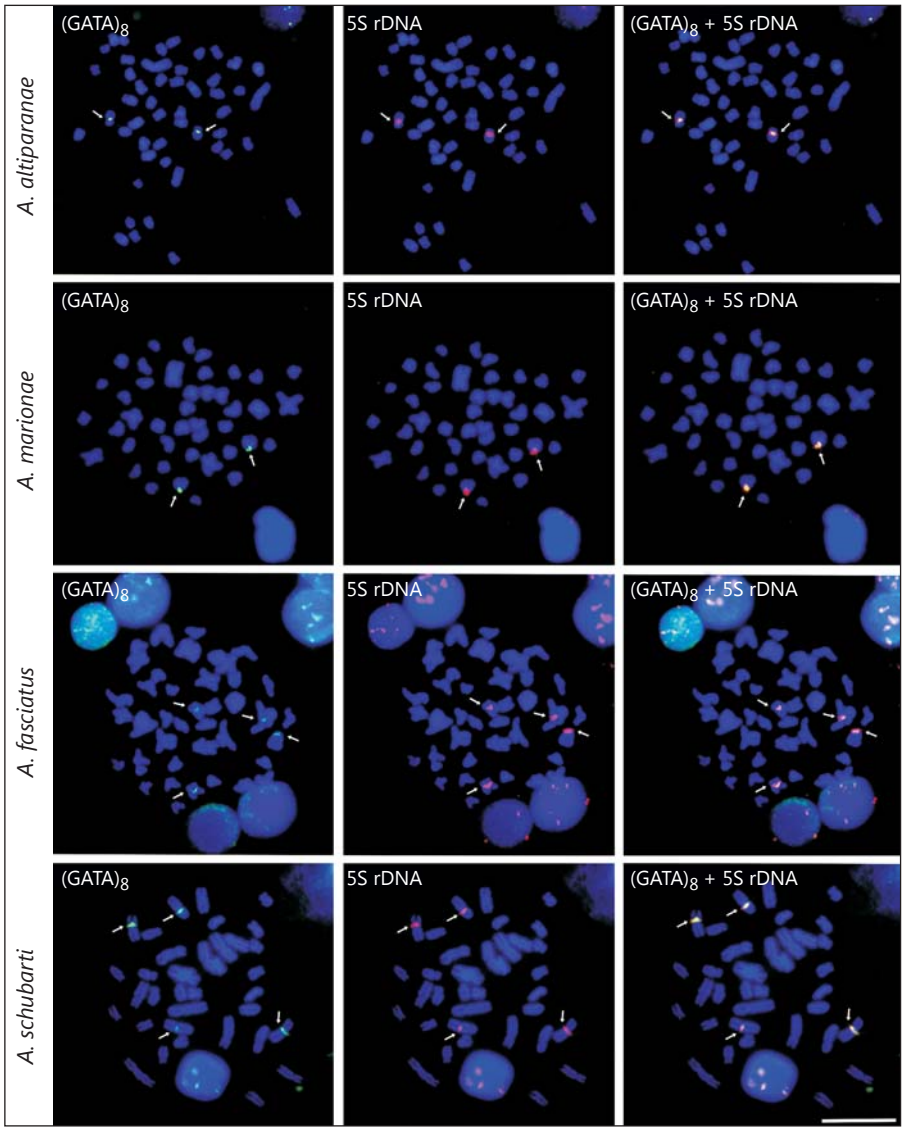

Fig. 3. Chromosomal location of 5S rDNA and GATA repeats in *Astyanax* species. Arrows indicate fluorescence signals. Bar = 10 μm.

Here, we observed chromosomal co-localization of 5S rDNA and the $(GATA)_8$ microsatellite. GATA repeats were found on chromosomes of other animal groups, for example, in the grasshopper *A. flavolineata* [Milani and Cabral-de-Mello, 2014], the fish *Halobatrachus didactylus* [Merlo et al., 2007], snakes *Liasis fuscus*, *Stegonotus cucullatus*, and *Notechis scutatus* [O'Meally et al., 2010], and lacertids *Coleonyx elegans*, *E. velox* [Pokorná et al., 2011], and *Aprasia parapulchella* [Matsubara et al., 2013]. However, in none of these species were GATA repeats co-localized with ribosomal DNA.

Using 2-color FISH, Úbeda-Manzanaro et al. [2010] examined $(GATA)_n$ and 5S rDNA in chromosomes from 4 species of fish of the Batrachoididae family *(Amphichthys cryptocentrus*, *Batrachoides manglae*, *Porichthys plectrodon*, and *Thalassophryne maculosa)*. GATA repeats were not co-localized with or adjacent to 5S rDNA in these species but were dispersed and abundant throughout all chromosomes. According to Úbeda-Manzanaro et al. [2010], GATA sequences cannot be used as chromosomal markers in these fish species. This is in contrast with the results from the present study, which indicate that GATA repeats can act as good chromosomal markers in South American *Astyanax* species.

Scrutiny of DNA sequences showed that microsatellites could be found within the non-transcribed spacers (NTSs) of 5S rDNA in fish [for examples, see Rocco et al., 2005; Pasolini et al., 2006; Pinhal et al., 2009; Merlo et al.,

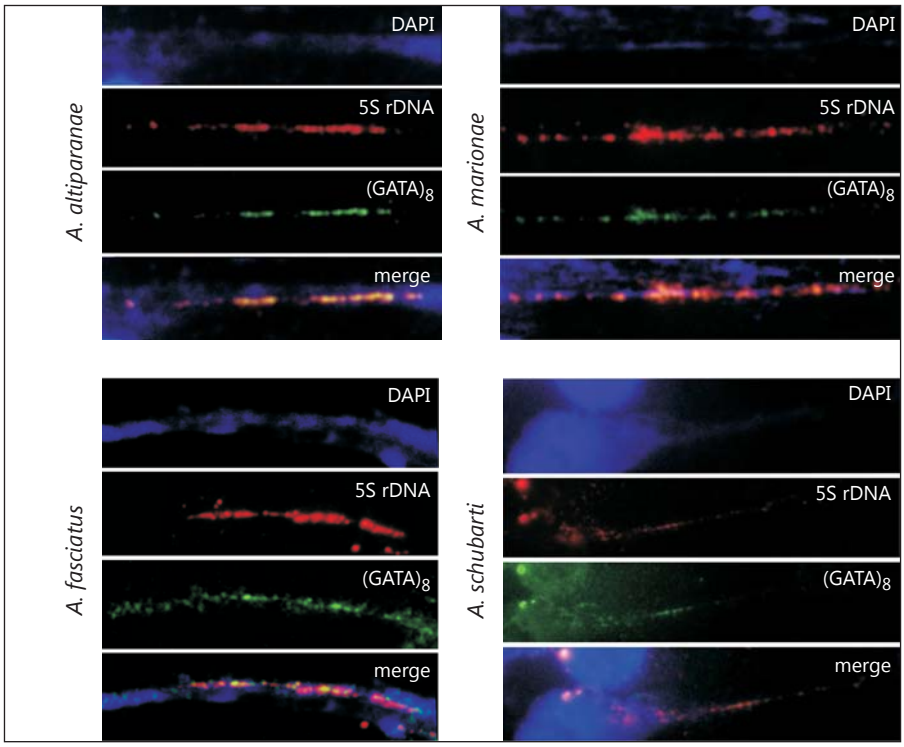

Fig. 4. Fiber-FISH of *Astyanax* chromosomes. The 5S rDNA and $(GATA)_8$ probes are co-localized.

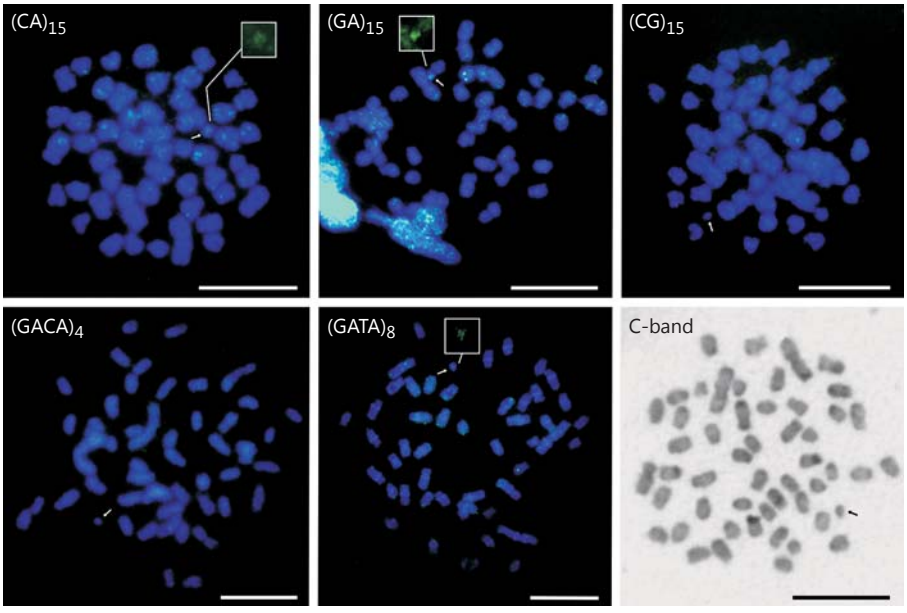

Fig. 5. Microsatellite distribution and constitutive heterochromatin revealed by C-banding in *A. mexicanus*. Arrows indicate the B chromosome, and **insets** show fluorescence signals on the B chromosome. Bars = 10 μm.

2010]. The microsatellite GTT was found within the NTS from 2 species *(Dicentrarchus labrax* and *D. punctatus)* of the Moronidae family [Merlo et al., 2010]. This suggested that the presence of microsatellite repeats favored the maintenance of tandem arrays of multigene families, as proposed by other authors [Liao and Weiner, 1995; Cross and Rebordinos, 2005].

Sequence analysis of 5S rDNA in 2 species of Batoidea, *Taeniura lymma* (Dasyatidae) and *Raja montagui* (Rajidae), revealed microsatellite repeats in the NTS regions of the 5S rDNA, and it was suggested that these repeats might exert an influence on gene regulation [Rocco et al., 2005].

The chromosomal co-location of GATA repeats and ribosomal DNA, which was maintained during evolution in different species with different diploid numbers, suggests an evolutionary advantage for the 5S rDNA-GATA combination in *Astyanax* chromosomes. Furthermore, the absence of 5S rDNA-GATA co-location in *A. mexicanus* indicates that the development of this feature may have occurred relatively recent in the evolutionary history of *Astyanax* species from South America.

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Statement of Ethics

All institutional guidelines for the care and use of laboratory animals were followed. Animals were captured with the permission of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio; No. 43497-1) and were used for laboratory experiments approved by the Animal Experimental Ethics Committee from Universidade Estadual Paulista (UNESP; protocol No. 2335).

Disclosure Statement

The authors have no conflicts of interest to declare.

References

Barros AV, Sczepanski TS, Cabrero J, Camacho JP, Vicari MR, Artoni RF: Fiber FISH reveals different patterns of high-resolution physical mapping for repetitive DNA in fish. Aquaculture 322:47–50 (2011).

Cabral-de-Mello DC, Moura RC, Martins C: Chromosomal mapping of repetitive DNAs in the beetle *Dichotomius geminatus* provides the first evidence for an association of 5S rRNA and histone H3 genes in insects, and repetitive DNA similarity between the B chromosome and A complement. Heredity 104: 393–400 (2010).

Charlesworth D, Charlesworth B, Marais G: Steps in the evolution of heteromorphic sex chromosomes. Heredity 95:118–128 (2005).

Cross I, Rebordinos L: 5S rDNA and U2 snRNA are linked in the genome of *Crassostrea angulata* and *Crassostrea gigas* oysters: does the (CT)n-(GA)n microsatellite stabilize this novel linkage of large tandem arrays? Genome 48:1116–1119 (2005).

Foresti F, Almeida-Toledo LF, Toledo-Filho SA: Polymorphic nature of nucleolus organizer regions in fishes. Cytogenet Cell Genet 31: 134–141 (1981).

Liao D, Weiner AM: Concerted evolution of the tandemly repeated genes encoding primate U2 small nuclear RNA (the *RNU2* locus) does not prevent rapid diversification of the (CT) n-(GA)n microsatellite embedded within the U2 repeat unit. Genomics 30:583–593 (1995).

Martins C, Galetti PM Jr: Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chromosome Res 7:363–367 (1999).

Matsubara K, Knopp T, Sarre SD, Georges A, Ezaz T: Karyotypic analysis and FISH mapping of microsatellite motifs reveal highly differentiated XX/XY sex chromosomes in the pink-tailed worm-lizard (*Aprasia parapulchella*, Pygopodidae, Squamata). Mol Cytogenet 6:60 (2013).

Merlo A, Cross I, Palazón JL, Sarasquete C, Rebordinos L: Chromosomal mapping of the major and minor ribosomal genes, $(GATA)_n$ and $(TTAGGG)_n$ by one-color and double-color FISH in the toadfish *Halobatrachus didactylus* (Teleostei: Batrachoididae). Genetica 131:195–200 (2007).

Merlo MA, Cross I, Chairi H, Manchado M, Rebordinos L: Analysis of three multigene families as useful tools in species characterization of two closely-related species, *Dicentrarchus labrax*, *Dicentrarchus punctatus* and their hybrids. Genes Genet Syst 85:341–349 (2010).

Milani D, Cabral-de-Mello DC: Microsatellite organization in the grasshopper *Abracris flavolineata* (Orthoptera: Acrididae) revealed by FISH mapping: remarkable spreading in the A and B chromosomes. PLoS One 9:e97956 (2014).

Moreira-Filho O, Galetti PM Jr, Bertollo LAC: B chromosomes in the fish *Astyanax scabripinnis* (Characidae, Tetragonopterinae): an overview in natural populations. Cytogenet Genome Res 106:230–234 (2004).

O'Meally D, Patel HR, Stiglec R, Sarre SD, Georges A, et al: Non-homologous sex chromosomes of birds and snakes share repetitive sequences. Chromosome Res 18:787–800 (2010).

Palacios-Gimenez OM, Cabral-de-Mello DC: Repetitive DNA chromosomal organization in the cricket *Cycloptiloides americanus*: a case of the unusual X_1X_20 sex chromosome system in Orthoptera. Mol Genet Genomics 290: 623–631 (2015).

Pasolini P, Costagliola D, Rocco L, Tinti F: Molecular organization of 5S rDNAs in Rajidae (Chondrichthyes): structural features and evolution of piscine 5S rRNA gene and non-transcribed intergenic spacers. J Mol Evol 62: 564–574 (2006).

Pendás AM, Morán P, Freije JP, García-Vázquez E: Chromosomal location and nucleotide sequence of two tandem repeats of the Atlantic salmon 5S rDNA. Cytogenet Cell Genet 67: 31–36 (1994).

Pinhal D, Araki CS, Godig OBF, Martins C: Molecular organization of 5S rDNA in sharks of the genus *Rhizoprionodon*: insights into the evolutionary dynamics of 5S rDNA in vertebrate genomes. Genet Res 91:61–72 (2009).

Pinkel D, Straume T, Gray JW: Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 83:2934–2938 (1986).

Pokorná M, Kratochvíl L, Kejnovský E: Microsatellite distribution on sex chromosomes at different stages of heteromorphism and heterochromatinization in two lizard species (Squamata: Eublepharidae: *Coleonyx elegans* and Lacertidae: *Eremias velox*). BMC Genet 12:90 (2011).

Poltronieri J, Marquioni V, Bertollo LA, Kejnovsky E, Molina WF, et al: Comparative chromosomal mapping of microsatellites in *Leporinus* species (Characiformes, Anostomidae): unequal accumulation on the W chromosomes. Cytogenet Genome Res 142: 40–45 (2014).

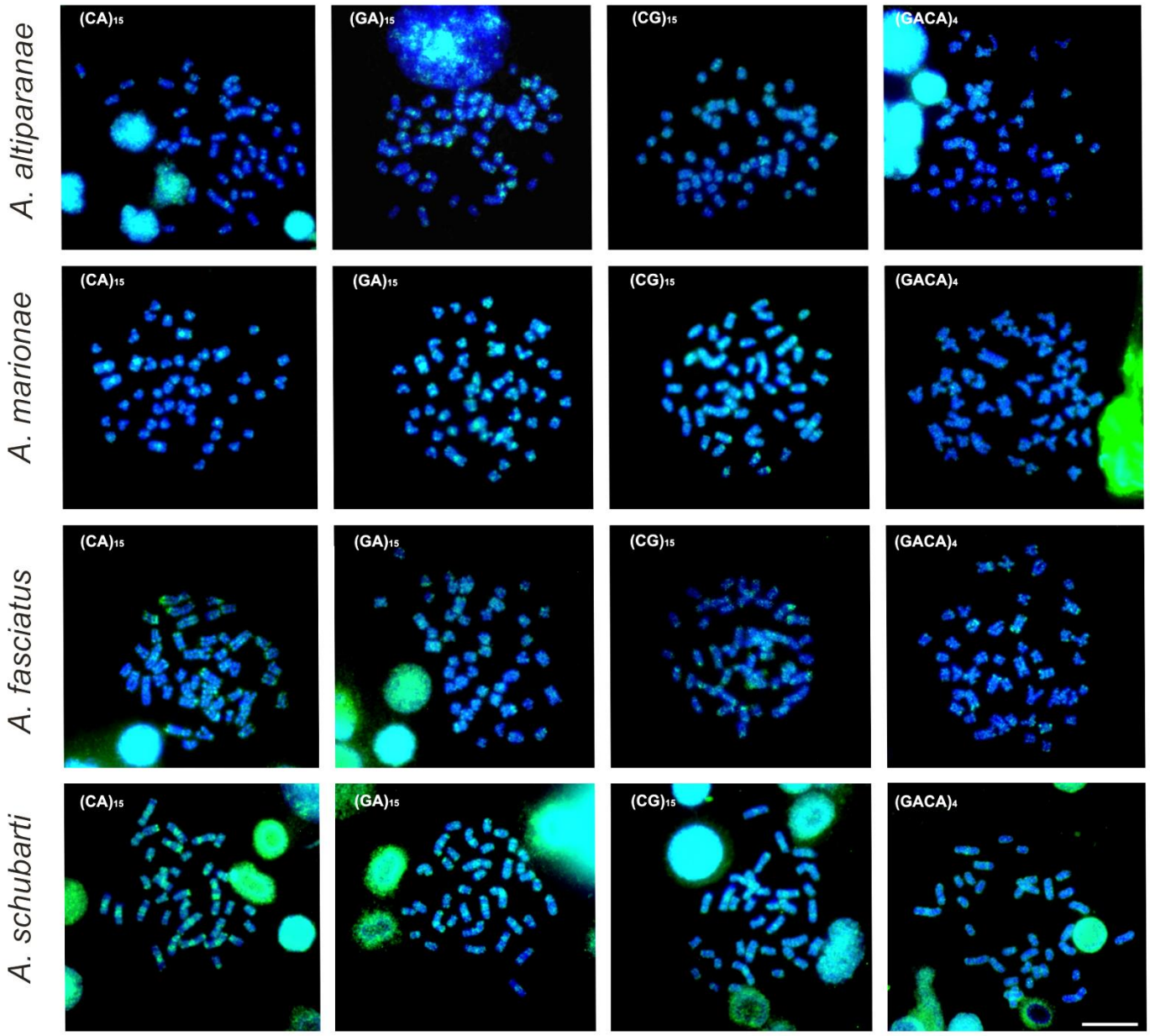
Rocco L, Costagliola D, Fiorillo M, Tinti F, Stingo V: Molecular and chromosomal analysis of ribosomal cistrons in two cartilaginous fish, *Taeniura lymma* and *Raja montagui* (Chondrichthyes, Batoidea). Genetica 123:245–253 (2005).

Sambrook J, Russell DW: Molecular Cloning: A Laboratory Manual, ed 3 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor 2001).

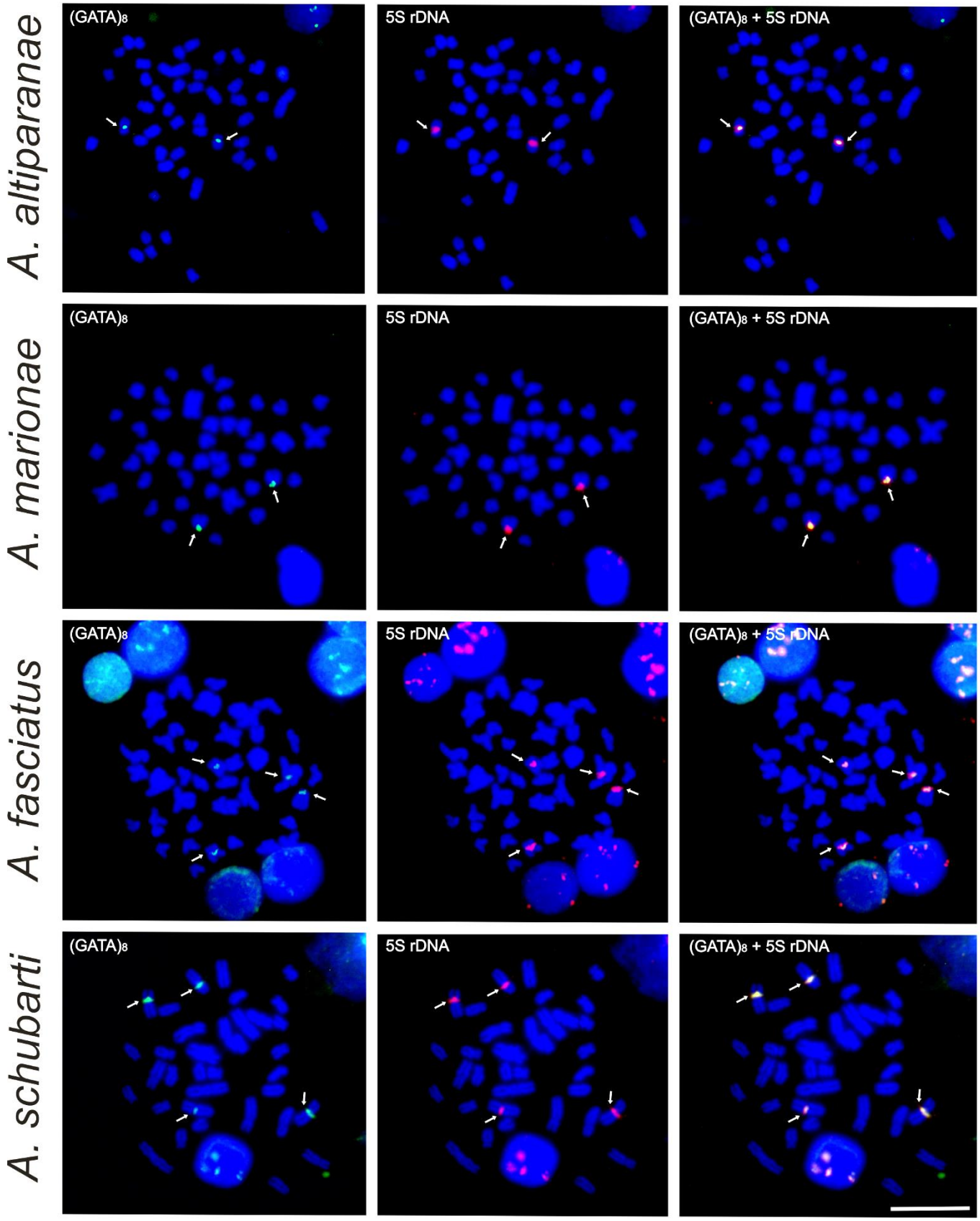
Scacchetti PC, Utsunomia R, Pansonato-Alves JC, Vicari MR, Artoni RF, et al: Chromosomal mapping of repetitive DNAs in *Characidium* (Teleostei, Characiformes): genomic organization and diversification of ZW sex chromosomes. Cytogenet Genome Res 146: 136–143 (2015).

Sumner AT: A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75:304–306 (1972).

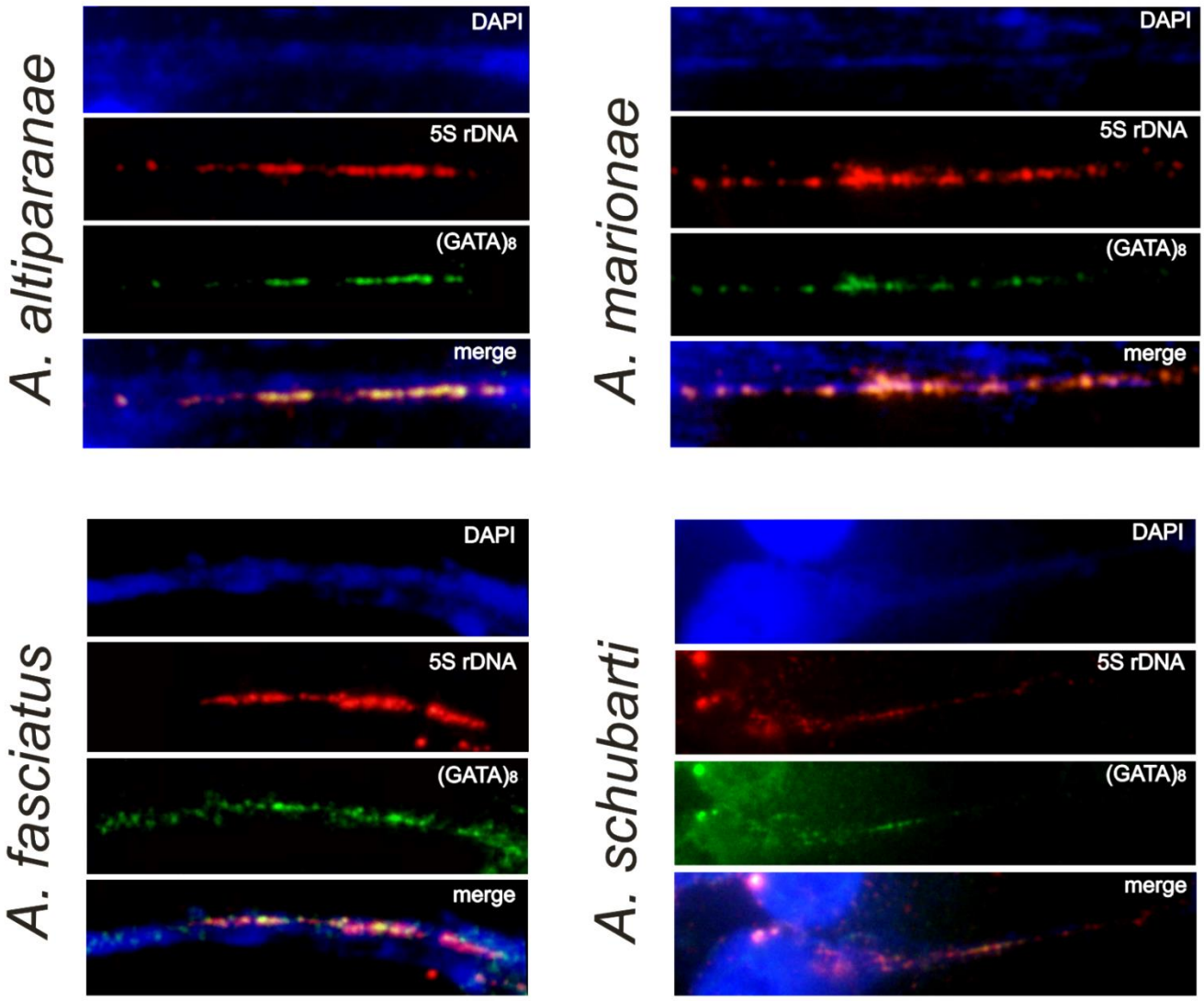
Úbeda-Manzanaro M, Merlo MA, Palazón JL, Cross I, Sarasquete C, Rebordinos L: Chromosomal mapping of the major and minor ribosomal genes, $(GATA)_n$ and U2 snRNA gene by double-colour FISH in species of the Batrachoididae family. Genetica 138:787–794 (2010).



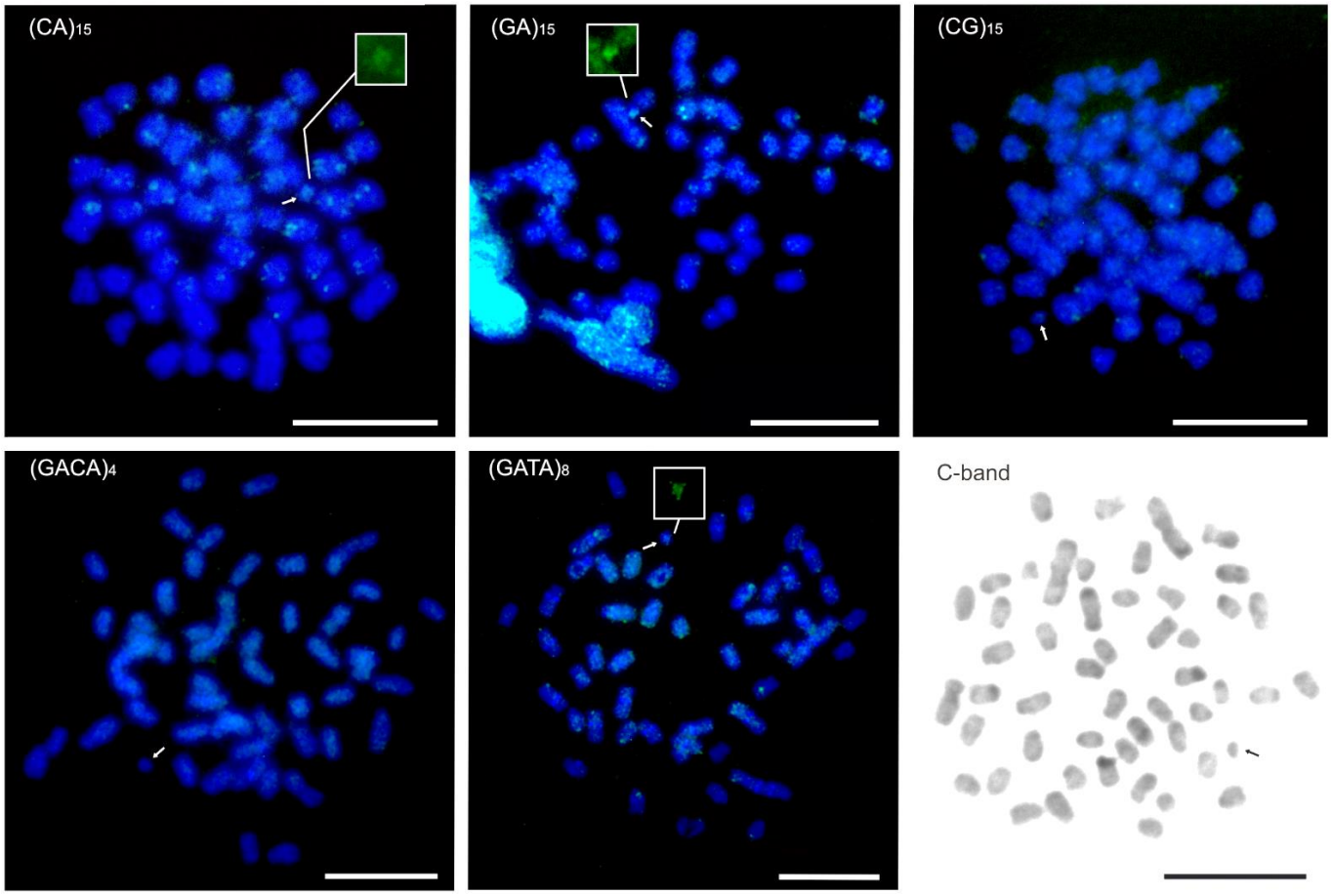
Color Figure 2. Microsatellite distribution on chromosomes of *Astyanax* species. Bar = 10 µm.



Color Figure 3. Chromosomal location of 5S rDNA and GATA repeats in *Astyanax* species. Arrows indicate fluorescent signals. Bar = 10 µm.



Color Figure 4. Fiber-FISH examination of *Astyanax* chromosomes. The 5S rDNA and $(GATA)_8$ probes are co-located.



Color Figure 5. Microsatellite distribution and constitutive heterochromatin revealed by the C-band technique in *A. mexicanus*. Arrows indicate the B chromosome, and boxes indicate fluorescent signals on the B chromosome. Bar = 10 µm.

6.6. CAPÍTULO VI

EVOLUTIONARY INFERENCES FOR THE ORIGIN OF CHROMOSOMAL CHARACTERISTICS IN THE GENUS *Astyanax* (CHARACIDAE, *Incertae sedis*): A CYTOGENETIC AND MOLECULAR APPROACH

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Abstract

Astyanax is a genus with wide distribution ranging South United States to North of Patagonia in Argentina and present species with 2n = 36, 46, 48 and 50 chromosomes. In this paper were analyzed cytogenetic and molecular data in species with different diploid numbers. In *Astyanax schubarti* (2n = 36) was observed two Ag-NOR bearing pairs, three pairs GC-rich and with 18S rDNA clusters, and C-band blocks were evident in pericentromeric and centromeric regions. *Astyanax fasciatus* (2n = 46) showed four Ag-NOR sites, four GC-rich sites, eight 18S rDNA clusters, and C-band blocks were evident in terminal regions. *Astyanax marionae* (2n = 48) presented one Ag-NOR bearing pair, four GC-rich sites, four 18S rDNA clusters, and C-band blocks were evident in pericentromeric and centromeric regions. In *Astyanax altiparanae* were observed six Ag-NOR sites, one pair GC-rich, one pair with 18S rDNA clusters, and band-C blocks were evident in pericentromeric and terminal regions. The fluorescent signals using telomeric probe were observed only on the telomeres in all the species studied here. Molecular clock showed three groups well-structured and an origin more ancient for *A. schubarti* about 2.31 Mya, and the separation of *A. mexicanus* nearly 4.4 Mya of the South America species. Here, will discuss the evolution of cytogenetic data using molecular data.

Keywords: Chromosomal evolution, Repetitive DNA, Mitochondrial DNA, Molecular clock.

Introduction

The Characidae family presents small and middle species of fish such as the *Astyanax*. Over the years, many members of this family has undergone changes in its classification. It is known that the phylogenetic relationships among the group members are doubtful, in the aspect of representing a polyphyletic group, which is much discussed until now (see discussion below).

The *Astyanax* genus has wide distribution, from Texas and New Mexico in the United States (Page and Burr 1991) to the North of Patagonia in Argentina (Almirón et al. 1997). This group is considered polyphyletic and was allocated in *incertae sedis* in Characidae amongst with other genera by Lima et al. (2003). From this, other studies have supported the hypothesis of polyphyletic *Astyanax* (see, Javonillo et al. 2010; Mirande 2010; Oliveira et al. 2011).

Since the revision considered by Lima et al. (2003), the number of valid *Astyanax* species almost doubled. According to Eschmeyer et al. (2016), more than 150 species are described for the genus, but its phylogenetic condition is still confused. Since this issue remains under discussion, other studies involving chromosomal and molecular analysis could contribute significantly to the evolutionary relationships among *Astyanax* species.

Cytogenetic has played an important role in recent decades on the study of the evolution of chromosomes in the different plant and animal groups (see, for example, Dobigny et al. 2004; Metcalfe et al. 2007; Landeen et al. 2013). From this, the use of chromosomal data combined with molecular analysis can contribute to the understanding of the evolutionary dynamics of chromosomes in the *Astyanax* group. Therefore, this study aimed to evaluate, cytogenetic and molecular data, *Astyanax* species with different diploid numbers through chromosomal techniques, analysis of mtDNA sequences and chromosomal data previously described in the literature, allowing a broad overview of chromosomal evolution in species from the Central and South America regions.

Materials and Methods

Sampling and classical cytogenetics

Were studied five individuals of *A. altiparanae* from the Piracicaba river in São Paulo state (SP), Brazil; six individuals of *A. marionae* from the Rio Claro stream in Mato Grosso state (MT), Brazil; three individuals of *A. fasciatus* from the Ribeirão Claro river (SP), Brazil; one individual of *A. schubarti* from the Piracicaba river (SP), Brazil; one individual of *Serrapinnus notomelas* from the Aquarium store in Rio Claro (SP), Brazil. Mitotic metaphase chromosomes were obtained according to the technique proposed by Foresti et al. (1981). The nucleolus organizer regions (NORs) were identified after impregnation by silver ion by technique of Howell and Black (1980) and the heterochromatin was observed using the C-band technique described by Sumner (1972). The double staining CMA_3/DAPI to identify regions GC/AT-rich was performed as described by Piscor et al. (2015).

DNA extraction and probe synthesis

The Genomic DNA was extracted of fins from the samples of *Astyanax* species as described by Sambrook and Russell (2001). The probes of 18S rDNA, 5S rDNA, and U2 snDNA were amplified by PCR (Polymerase chain reaction) using specific primers described by White et al. (1990), Pendás et al. (1994), and Bueno et al. (2013), respectively. The telomeric DNA probe was acquired by kit Telomere PNA FISH Kit/FITC (Dako Cytomation Denmark A/S Kit). The $(GATA)_8$ microsatellite was amplified and labeled with biotin during synthesis as described by Milani and Cabral-de-Mello (2014). Microsatellites were donated by Prof. Dr. Diogo C. Cabral-de-Mello (UNESP - Rio Claro) for use in laboratory experiments.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) experiments were performed according to Pinkel et al. (1986), with modifications described by Cabral-de-Mello et al. (2010) and Piscor

et al. (2013). Signals were detected using anti-digoxigenin-rhodamine (Roche, Mannheim, Germany) for digoxigenin and avidin Alexa Fluor 488 conjugate (Invitrogen, San Diego, CA, USA) for biotin. Slides were mounted with Vectashield Mounting Medium (Vector, Burlingame, CA, USA) containing DAPI (4', 6-diamidino-2-phenylindole). Chromosomes and fluorescent signals were visualized with an Olympus BX51 microscope coupled to a digital camera (Olympus model D71). Images were captured using DP Controller software.

Molecular clock using cytochrome *c* oxidase I (COI)

The sequences of COI were amplified and sequenced in *A. altiparanae*, *A. abramis*, *A. asuncionensis*, *A. bockmanni*, *A. eigenmanniorum*, *A. fasciatus*, *A. marionae*, and *A. schubati*. The COI for *A. mexicanus* and the outgroup (*S. notomelas*) were used from the *GenBanck* data (access numbers: GU702225.1 and HM126642.1, respectively).

Were used the programs BEAUti v1.7.2 and BEAST v1.7.2 (Drummond et. al. 2012) to estimate the times of divergence among the sequences and generate a tree dating moments of separation from them. The model "Lognormal relaxed clock (uncorrelated)" was chosen for the molecular clock and the process of speciation "Yule" for the parameter "Tree Prior". Geological and fossil events calibrate the analysis and the time of colonization of Central America (which enabled the origin of *A. mexicanus*) was dated in the interval between the uplift of the Isthmus of Panama (about 3 million years ago – Mya) and the event itself colonization of the region by genus *Astyanax* dated by Ornelas-García et. al. (2008) (eight Mya) it was also used a fossil of *Colossoma macropomum*, dated to at least 15 Mya.

The analyses of MCMC (Markov Chain Monte Carlo) were generated with 10.000.000 generations and parameters sampled to 1.000 steps. The 10.000 initial samples were discarded (burn-in of 10%), the others were summarized, and the topologies of trees were accessed using the program TREEANOTATOR v1.7.2 (Drummond et. al., 2012) and visualized by program FigTree v1.3.1 (Rambaut, 2008).

Results

Cytogenetic data in the genus *Astyanax*

The diploid number for *A. altiparanae* was 2n = 50 chromosomes, karyotype formula of 6m + 20sm + 8st + 16a, and fundamental number (FN) of 84. *Astyanax marionae* showed 2n = 48 chromosomes, karyotype formula of 8m + 24sm + 10st + 6a, and FN = 90. *Astyanax fasciatus* presented 2n = 46 chromosomes, karyotype formula of 8m + 18sm + 16st + 4a, and FN = 88. *Astyanax schubarti* showed 2n = 36 chromosomes, karyotype formula of 12m + 16sm + 4st + 4a, and FN = 68 (Figure 1a–d).

Ag-NORs sites were identified on pairs 21 and 24 and homologous of pairs 7 and 17 in *A. altiparanae*, on pair 17 in *A. marionae*, on pair 10 and homologous of pairs 7 and 15 in *A. fasciatus*, and on pairs 8 and 18 in *A. schubarti* (Figure 1, in box). The heterochromatin was observed mainly in the pericentromeric and centromeric regions of the chromosomes of *A. altiparanae* (Figure 2a), in pericentromeric and centromeric regions of *A. marionae* (Figure 2b), in terminal regions of *A. fasciatus* (Figure 2c), and in the pericentromeric and centromeric regions of *A. schubarti* (Figure 2d). GC-rich regions were observed on pair 21 in *A. altiparanae*, on pair 17 (one homologous with bitelomeric marking) and one homologous of pair acrocentric in *A. marionae*, on the same Ag-NOR regions in *A. fasciatus*, and on pairs 8, 17 and 18 in *A. schubarti* (Figure 3).

Fluorescent signals of 18S rDNA were observed on pair 21 in *A. altiparanae*, on pair 17 (one homologous with bitelomeric marking) and one homologous of pair acrocentric in *A. marionae*, on eight sites in *A. fasciatus*, and 8, 17 and 18 in *A. schubarti* (Figure 3). Signals of telomere probe does not observed on the centromeric, interstitial or pericentromeric regions of the chromosomes of *Astyanax* species (Figure 3).

Cytogenetic data of *S. notomelas*

Serrapinus notomelas was used in the molecular analysis as outgroup, it is a species that has no cytogenetic data with the U2 snDNA, and GATA repeats. Therefore, it showed 2n = 52 chromosomes and the repetitive sequences were located on two chromosome pairs

for U2 snDNA and one pair for 5S rDNA. The GATA repeats were clustered on three chromosome pairs and showed co-location with 5S rDNA sites (on one chromosome). The 5S rDNA also does not show clusters on the same chromosomes that presented U2 snDNA sites (Figure 4 – these data will be confirmed).

Molecular clock using COI

Molecular data presented accordance with the cladogram shown by Coutinho-Sanches and Dergam (2015), so we adapted the data for a better view of them (see Figure 5). The molecular clock showed clearly that three groups are well-structured: 1) humeral dark spot group (Hds); 2) diffuse humeral spot group (Dhs); and 3) Central and North America group (Cna) (Figure 6). This study also showed that the Hds and Cna groups diverged about 4.4 Mya (Millions of years ago), and *A. schubarti* is a species with ancient origin within of the Dhs group with a divergence of 2.31 Mya (Figure 7).

Discussion

Chromosome data and repetitive DNA locations in *Astyanax*

The *Astyanax* genus have five main diploid numbers, 2n = 36 chromosomes only in *A. schubarti* and *A. correntinus* (Morelli et al. 1983; Paiz et al. 2015), 2n = 46 e.g. to *A. fasciatus* (Morelli et al. 1983), 2n = 48 e.g. *A. scabripinnis* and *A. marionae* (Fernandes and Martins-Santos 2003; Krinski and Miyazawa 2014), and 2n = 50 for almost all others species e.g. *A. altiparanae*, *A. abramis*, and *A. bockmanni* (Piscor et al. 2015; Kavalco et al. 2009), as well as also observed for species studied in the present paper. More recently, 2n = 52 chromosomes are evaluated in the literature for the species *Astyanax* sp. (Tenório et al. 2013).

Some species of *Astyanax* may have wide geographical distribution and more of than one diploid number, as the case of species referred to as complex. It may be noted, e.g. the complex "*scabripinnis*" which presents 2n = 46, 2n = 48 and 2n = 50 chromosomes

(Moreira-Filho and Bertollo, 1991) and the complex "*fasciatus*" in which the diploid number ranges from 2n = 45 to 2n = 50 chromosomes (Centofante et al., 2003). On the other hand, chromosomal location analysis of repetitive sequences have significantly contributed to the understanding of chromosome evolution of these complexes of species, as well as all species studied cytogenetically within the *Astyanax* group.

Considering the studied of repetitive DNA for the *Astyanax* species, the 18S rRNA genes are more variants in chromosomal sites (see review Piscor et al. 2015), as observed in this study for *A. altiparanae*, *A. marionae*, *A. fasciatus* and *A. schubarti*. Specific chromosome forms of 5S rRNA gene clusters can be shared for some species and preserved groups begin to become evident as discussed by Piscor et al. (2015). These authors showed three preserved forms: the first was the presence of two chromosome pairs, one metacentric and one acrocentric pair with clusters on pericentromeric regions; the second form was the presence of one submetacentric pair with clusters on pericentromeric region; and the third form was the presence of one acrocentric pair with clusters on pericentromeric region.

Chromosomal organizations are best analysed when other repetitive are studied. In the case of histone genes, more conserved forms one or two pairs, in particular locations of 5S rDNA and histone genes on same chromosome pair (m or sm) are evidenced (see Hashimoto et al. 2011; Pansonato-Alves et al. 2013; Silva et al. 2014; Silva et al. 2015; Piscor and Parise-Maltempi 2016). According to Piscor and Parise-Maltempi (2016), two species showed different forms relationship to 5S-H3 chromosomal locations, *A. schubarti* (two chromosome pairs – sm) and *A. mexicanus* (two chromosome pairs – one m and one sm), both not showed 5S-H3 locations on the same pair. Similar to *A. mexicanus*, Silva et al. (2015) have observed a chromosomal organization pattern of H3 histone for *Astyanax jordani*.

U2 snRNA genes also showed high conservation of the number of clusters per genome (Silva et al 2015; Piscor et al. 2016). Piscor et al. (2016), for example, found forms preserved for seven species (*A. altiparanae*, *A. abramis*, *A. asuncionensis*, *A. bockmanni*,

A. eigenmanniorum, *A. fasciatus*, and *A. marionae*) and different forms to *A. schubarti* and *A. mexicanus*. These observations led the authors to believe that *A. schubarti* could represent a recent origin as proposed by Morelli et al. (1983), but discorded by the molecular clock data presented in this work, and *A. mexicanus* may represent a more recent origin for U2-chrosomosome location (because it has only one pair, as also *A. jordani* studied by Silva et al. 2015 – a closer species of *A. mexicanus*) once the outgroup *S. notomelas* exhibited two chromosome pairs.

Molecular analyses and evolution of cytogenetic features in the *Astyanax* genus

Our molecular results showed that of all the species studied here, *A. schubarti* (2n = 36), would be the type with an ancient origin with an approximate derivation of 2.31 Mya of the Hds group. Therefore, species which accumulate the derived systems of cytogenetic characteristics are *A. mexicanus* (Mexican blind cavefish – 2n=50), *A. schubarti* (2n=36) and *A. marionae* (2n=48). According to the molecular analyses presented by Coutinho-Sanches and Dergam (2015) *A. schubarti* form a well-structured group and close to *A. fasciatus*, *A. bockmanni*, and *A. eigenmanniorum*, *A. marionae* (see also the results of molecular clock), as well as *A. mexicanus* is closer to North and Central America species (see the figure 5 adapted from the Coutinho-Sanches and Dergam, 2015).

Based on morphological and molecular data, *Astyanax* have been considered a polyphyletic group by several authors (Calcagnotto et al. 2005; Mirande 2010; Javonillo et al. 2010; Oliveira et al. 2011). However, our data along with data showed by Coutinho-Sanches and Dergam (2015) have shown that for some species within of *Astyanax* represent well-structured groups, for example, Hds, Cna, and Dhs groups. On the other hand, we cannot fail to recognize that the genus when viewed in a general view is rather seen as a polyphyletic group.

However, the Cna group has diverged around 4.4 Mya of the Hds group, from analysis of the molecular clock in this paper. In this sense, several hypotheses are proposed

for colonization of Mesoamerica by freshwater fishes from South America, one of them, is the Cenozoic episode via the Antillean islands and/or a continental corridor (Rosen 1975; Rosen 1978; Bussing 1985 *apud* Ornelas-García et al. 2008). Molecular data suggested colonization of Mesoamerica by primary freshwater fishes about 4-7 Mya (Bermingham and Martin 1998; Perdices et al. 2002; Perdices et al. 2005; Concheiro-Perez et al. 2007 *apud* Ornelas-García et al. 2008), near the time of divergence between Hds and Cna groups shown in this study (~4.4 Mya).

Previous studies have indicate multiple waves of rapid expansion for fish characids from South America during the Pliocene ~3.3 Mya (Reeves and Bermingham, 2006 *apud* Ornelas-García et al. 2008). Our data reinforce the hypothesis already proposed on colonization *Astyanax* species from South America before the final uplift of the Isthmus of Panama ~3.3 Mya (see Coates and Oblando 1996; Iturralde-Vinent and MacPhee 1999; Bartoli et al., 2005 *apud* Ornelas-García et al. 2008). For example, Bermingham and Martin (1998) had proposed colonization of Mesoamerica between 4–7 Mya, prior to the final closure of the Panama Isthmus (~3.3 Mya).

Thus, our cytogenetic data rely on the evidence and indicate two conclusions:

1) *Astyanax mexicanus* represent a group within *Astyanax* that diverged a long time ago and so has distinct chromosomal characteristics compared to South American species;

2) Contrary to what was proposed by Morelli et al. 1983, *A. schubarti*, readily diverged earlier in evolutionary history, and may be have a particular chromosome system compared to other species of the genus.

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References

ALMIRÓN, A.; AZPELICUETA, M.; CASCIOTTA J.; LÓPEZ CAZORLA, A. Ichthyogeographic boundary between the Brazilian and Austral subregions in South America, Argentina. **Biogeographica**. v. 73, n. 1, p. 23–30, 1997.

BARTOLI, G.; SARNTHEIN, M.; WEINELT, M.; ERLENKEUSER, H.; GARBE-SCHONBERG, D.; LEA, D. W. Final closure of Panama and the onset of northern hemisphere glaciation. **Earth and Planetary Science Letters**. v. 237, n.1–2, p. 33–44, 2005.

BERMINGHAM, E.; MARTIN, A. P: Comparative mtDNA phylogeography of neotropical freshwater fishes: testing shared history to infer the evolutionary landscape of lower Central America. **Molecular Ecology**. v. 7, n.4, p. 499–517, 1998.

BUENO, D.; PALACIOS-GIMENEZ, O. M.; CABRAL-DE-MELLO, D. C. Chromosomal mapping of repetitive DNAs in *Abracris flavolineata* reveal possible ancestry for the B chromosome and surprisingly H3 histone spreading. **PLoS ONE**. v. 8, p. e66532, 2013.

CABRAL-DE-MELLO, D. C.; MOURA, R. C.; MARTINS, C. Chromosomal mapping of repetitive DNAs in the beetle *Dichotomius geminatus* provides the first evidence for an association of 5S rRNA and histone H3 genes in insects, and repetitive DNA similarity between the B chromosome and A complement. **Heredity**. v. 104, p. 393–400, 2010.

CALCAGNOTTO, D.; SCHAEFER, S. A.; DESALLE, R. Relationships among characiform fishes inferred from analysis of nuclear and mitochondrial gene sequences. **Molecular Phylogenetics and Evolution**. v. 36, n.1, p. 135–153, 2005.

CENTOFANTE, L.; BERTOLLO, L. A. C.; BUCKUP, P. A.; MOREIRA-FILHO, O. Chromosomal divergence and maintenance of sympatric *Characidium* fish species (Crenuchidae, Characidiinae). **Hereditas**. v. 138, n. 3, p. 213–218, 2003.

COATES, A.; OBLANDO, J. A. **The geologic evolution of the Central America Isthmus.** In *Evolution and Environmental in Tropical America* Edited by: Jackson JBC, Budd AF, Coates AG. Chicago: Chicago University Press, Chicago; 1996:21–56.

CONCHEIRO-PEREZ, G. A.; RICAN, O.; ORTI, G.; BERMINGHAM, E.; DOADRIO, I.; ZARDOYA, R. Phylogeny and biogeography of 91 species of heroine cichlids (Teleostei: Cichlidae) based on sequences of the cytochrome *b* gene. **Molecular Phylogenetics and Evolution**. v. 43, n. 1, p. 91–110, 2007.

COUTINHO-SANCHES, N.; DERGAM, J. A. Cytogenetic and molecular data suggest *Deuterodon pedri* Eigenmann, 1907 (Teleostei: Characidae) is a member of an ancient coastal group. **Zebrafish**. v. 12, n. 5, p. 357–365, 2015.

DOBIGNY, G.; DUCROZ, J. F.; ROBINSON, T. J.; VOLOBOUEV, V. Cytogenetics and cladistics. **Systematic Biology**. v. 53, p. 470–484, 2004.

ESCHMEYER, W. N.; FONG, J. D.; VAN-DER-LAAN R. Catalog of Fishes. http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp (acesso em 4/4/2016).

FERNANDES, C. A.; MARTINS-SANTOS, I. C. Cytogenetic characterization of two populations of *Astyanax scabripinnis* (Pisces, Characiformes) of the Ivaí basin PR Brazil. **Cytologia**. v. 68, p. 289–293, 2003.

FORESTI, F.; ALMEIDA-TOLEDO, L. F., TOLEDO-FILHO, S. A. Polymorfic nature of nucleous organizer regions in fishes. **Cytogenetics Cell Genetics**. v. 31, p. 137-144, 1981.

HASHIMOTO, D. T.; FERGUSON-SMITH, M. A.; RENS, W.; FORESTI, F.; Oliveira C, Foresti F. Chromosome mapping of H1 histone and 5S rRNA genes clusters in three species of *Astyanax* (Teleostei, Characiformes). **Cytogenetic and Genome Research**. v. 134, p. 64–71, 2011.

HOWELL, W. M.; BLACK, D. A. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. **Experientia**. v. 36, p. 1014-1015, 1980.

ITURRALDE-VINENT, M. A.; MACPHEE, R. D. E. Paleogeography of the Caribbean region: Implications for cenozoic biogeography. **Bulletin of the American Museum of Natural History**. p. 1–95. 1999.

JAVONILLO, R.; MALABARBA, L. R.; WEITZMAN, S. H.; BURNS, J. R. Relationships among major lineages of characid fishes (Teleostei: Ostariophysi: Characiformes), based on molecular sequence data. **Molecular Phylogenetics and Evolution**. v. 54, p. 498–511, 2010.

KAVALCO, K. F.; PAZZA R.; ALMEIDA-TOLEDO, L. F. *Astyanax bockmanni* Vari and Castro, 2007: an ambiguous karyotype in the *Astyanax* genus. **Genetica**. v. 136, n. 1, p. 135–139, 2009.

KRINSKI, D.; MIYAZAWA, C. S. Cytogenetic analysis of three species of *Astyanax* genus (Pisces, Characidae, *Incertae sedis*) from freshwaters of upper Paraguay basin, Mato Grosso state, Brazil. **Journal of Life Science**. v. 8, n. 1, p. 51–57, 2014.

LANDEEN, E. L.; PRESGRAVES, D. C. Evolution: from autosomes to sex chromosomes – and back. **Current Biology**. v. 23, p. R848-R850, 2013.

LIMA, F. C. T.; MALABARBA, L. R.; BUCKUP, P. A.; DA SILVA, J. F. P.; VARI, R. P.; HAROLD, A. et al. Genera *incertae sedis* in Characidae. In: REIS, R. E.; KULLANDER, S. E.; FERRARIS Jr., C. J., (Eds.). **Check List of the Freshwater Fishes of South and Central America**. Porto Alegre: Edipucrs. 2003. p. 106−169.

METCALFE, C. J.; BULAZEL, K.; FERRERI, G. C.; SCHROEDER-REITER, E.; WARNER, G.; RENS W.; OBERGFELL, C.; ELDRIDGE, M. D. B; O'NEILL, R. J. Genomic instability within centromeres of interspecific marsupial hybrids. **Genetics**. v. 177, p. 2507–2517, 2007.

MILANI, D.; CABRAL-DE-MELLO, D. C. Microsatellite organization in the grasshopper *Abracris flavolineata* (Orthoptera: Acrididae) revealed by FISH mapping: remarkable spreading in the A and B chromosomes. **PLoS One**. v. 9, p. e97956, 2014.

MIRANDE, J. M. Phylogeny of the family Characidae (Teleostei: Characiformes): from characters to taxonomy. **Neotropical Ichthyology**. v. 8, p. 385–568, 2010.

MOREIRA-FILHO, O.; BERTOLLO, L. A. C. *Astyanax scabripinnis* (Pisces, Characidae): a species complex. **Revista Brasileira de Genética**. v. 14, p. 331–357, 1991.

MORELLI, S.; BERTOLLO, L. A. C.; FORESTI, F.; MOREIRA–FILHO, O.; TOLEDO-FILHO, S. A. Cytogenetic considerations on the genus *Astyanax* (Pisces, Characidae). I Karyotypic variability. **Caryologia**. v. 36, p. 235–244, 1983.

OLIVEIRA, C.; AVELINO, G. S.; ABE, K. T.; MARIGUELA, T. C.; BENINE, R. C.; ORTÍ, G.; VARI, R. P.; CASTRO, R. M. C. Phylogenetic relationships within the speciose family Characidae (Teleostei: Ostariophysi: Characiformes) based on multilocus analysis and extensive ingroup sampling. **BMC Evolutionary Biology**. v. 11, p. 275, 2011.

ORNELAS-GARCÍA, C. P.; DOMÍNGUEZ-DOMÍNGUEZ, O.; DOADRIO I. Evolutionary history of the fish genus *Astyanax* Baird & Girard (1854) (Actinopterygii, Characidae) in Mesoamerica reveals multiple morphological homoplasies. **BMC Evolutionary Biology**. v. 8, p. 340, 2008.

PAGE, L. M.; BURR B. M. **A field guide to freshwater fishes of North America north of Mexico**. Houghton Mifflin Company, Boston. 1991. p. 432.

PANSONATO-ALVES, J. C.; HILSDORF, A. W. S., UTSUNOMIA, R.; SILVA, D. M. Z. A.; OLIVEIRA, C.; FORESTI, F. Chromosomal mapping of repetitive DNA and cytochrome *c* oxidase I sequence analysis reveal differentiation among sympatric samples of *Astyanax fasciatus* (Characiformes, Characidae). **Cytogenetic and Genome Research**. v. 141, p. 133–142, 2013.

PENDÁS, A. M.; MORÁN, P.; FREIJE, J. P.; GARCIA-VÁSQUEZ, E. Chromosomal location and nucleotide sequence of two tandem repeats of the Atlantic salmon 5S rDNA. **Cytogenetics and Cell Genetics**. v. 67, p. 31–36, 1994.

PERDICES, A.; BERMINGHAM, E. A. M.; DOADRIO, I. Evolutionary history of the genus *Rhamdia* (Teleostei: Pimelodidae) in Central America. **Molecular Phylogenetics and Evolution**. v. 25, p. 172–189, 2002.

PERDICES, A.; DOADRIO, I.; BERMINGHAM, E. A. M. Evolutionary history of the synbranchid eels (Teleostei: Synbranchidae) in Central America and the Caribbean islands inferred from their molecular phylogeny. **Molecular Phylogenetics and Evolution**. v. 37, n. 2, p. 460–473, 2005.

PINKEL, D.; STRUME, T.; GRAY, J. W. Cytogenetic analysis using quantitative, high sensitivity, fluorescence hibridization. **Proceedings of the National Academy Sciences**. v. 83, p. 2934–2938, 1986.

PISCOR, D.; RIBACINKO-PISCOR, D. B.; FERNANDES, C. A.; PARISE-MALTEMPI P. P. Cytogenetic analysis in three *Bryconamericus* species (Characiformes, Characidae): first description of the 5S rDNA-bearing chromosome pairs in the genus. **Molecular Cytogenetics**. v. 6, p. 13, 2013.

PISCOR, D.; ALVES, A. L.; PARISE-MALTEMPI, P. P. Chromosomal microstructure diversity in three *Astyanax* (Characiformes, Characidae) species: comparative analysis of the chromosomal locations of the 18S and 5S rDNAs. **Zebrafish**. v. 12, n. 1, p. 81–90, 2015.

PISCOR, D.; PARISE-MALTEMPI, P. P. Chromosomal mapping of H3 histone and 5S rRNA genes in eight species of *Astyanax* (Pisces, Characiformes) with different diploid numbers: syntenic conservation of repetitive genes. **Genome**. v. 59, n. 3, p. 167–172, 2016.

REEVES, R. G.; BERMINGHAM, E. Colonization, population expansion, and lineage turnover: phylogeography of Mesoamerican characiform fish. **Biological Journal of the Linnean Society**. v. 88, n.2, p. 235−255, 2006.

ROSEN, D. E. Vicariance Model of Caribbean Biogeography. **Systematic Zoology**. v. 24, n. 4, p. 431–464, 1975.

ROSEN, D. E. Vicariant patterns and historical explanation in biogeography. **Systematic Zoology**. v. 27, p. 159–188, 1978.

SAMBROOK, J.; RUSSELL, D. W. **Molecular Cloning: a Laboratory Manual**. vol. 3. Set. Cold Spring Harbor Laboratory Publisher, 2001.

SILVA, D. M. Z. A.; PANSONATO-ALVES, J. C.; UTSUNOMIA, R.; ARAYA-JAIME, C.; RUIZ-RUANO, F. J.; DANIEL, S. N.; HASHIMOTO, D. T.; OLIVEIRA, C.; CAMACHO, J. P. M.; PORTO-FORESTI, F.; FORESTI, F. Delimiting the origin of a B chromosome by FISH mapping, chromosome painting and DNA sequence analysis in *Astyanax paranae* (Teleostei, Characiformes). **PLoS ONE**. v. 9, n. 4, p. e94896, 2014.

SILVA, D. M. Z. A.; UTSUNOMIA, R.; PANSONATO-ALVES, J. C.; OLIVEIRA, C.; FORESTI, F. Chromosomal mapping of repetitive DNA sequences in five species of *Astyanax* (Characiformes, Characidae) reveals independent location of U1 and U2 snRNA sites and association of U1 snRNA and 5S rDNA. **Cytogenetic and Genome Research**. v. 146, p. 144–152, 2015.

SUMMER, A. T. A simple techine for demonstrating centromeric heterochromation. **Experimental Cell Research**. v. 75, p. 304−306, 1972.

TENÓRIO, R. C. C. O.; VITORINO, C. A.; SOUZA, I. L.; OLIVEIRA, C.; VENERE, P. C. Comparative cytogenetics in *Astyanax* (Characiformes: Characidae) with focus on the cytotaxonomy of the group. **Neotropical Ichthyology**. v. 11, n. 3, p. 553–564, 2013.

WHITE, T. J.; BRUNS, T.; LEE, S.; TAYLOR, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: INNIS, M. A.; GELFAND, D. H.; SNINSKY, J. J.; WHITE, T. J. (Eds.). **PCR Protocols: a guide to methods and applications**. Academic Press, New York, USA. 1990. p. 315−322.

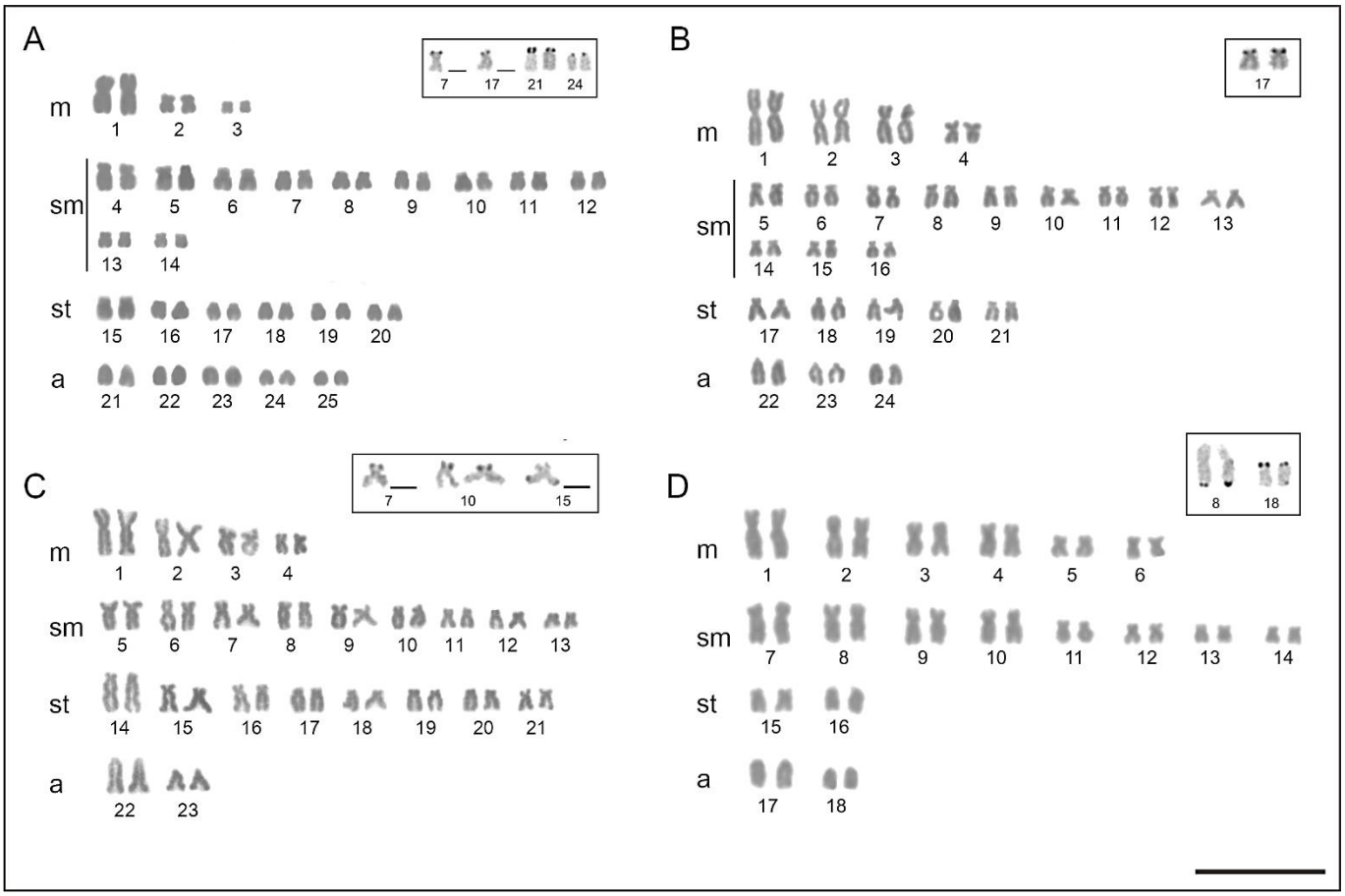

Figure 1. Karyotypes: (**A**) *A. altiparanae*; (**B**) *A. marionae*; (**C**) *A. fasciatus*; (**D**) *A. schubarti*. The Chromosomes Ag-NORs are indicate in the boxes. Bar = 10 µm.

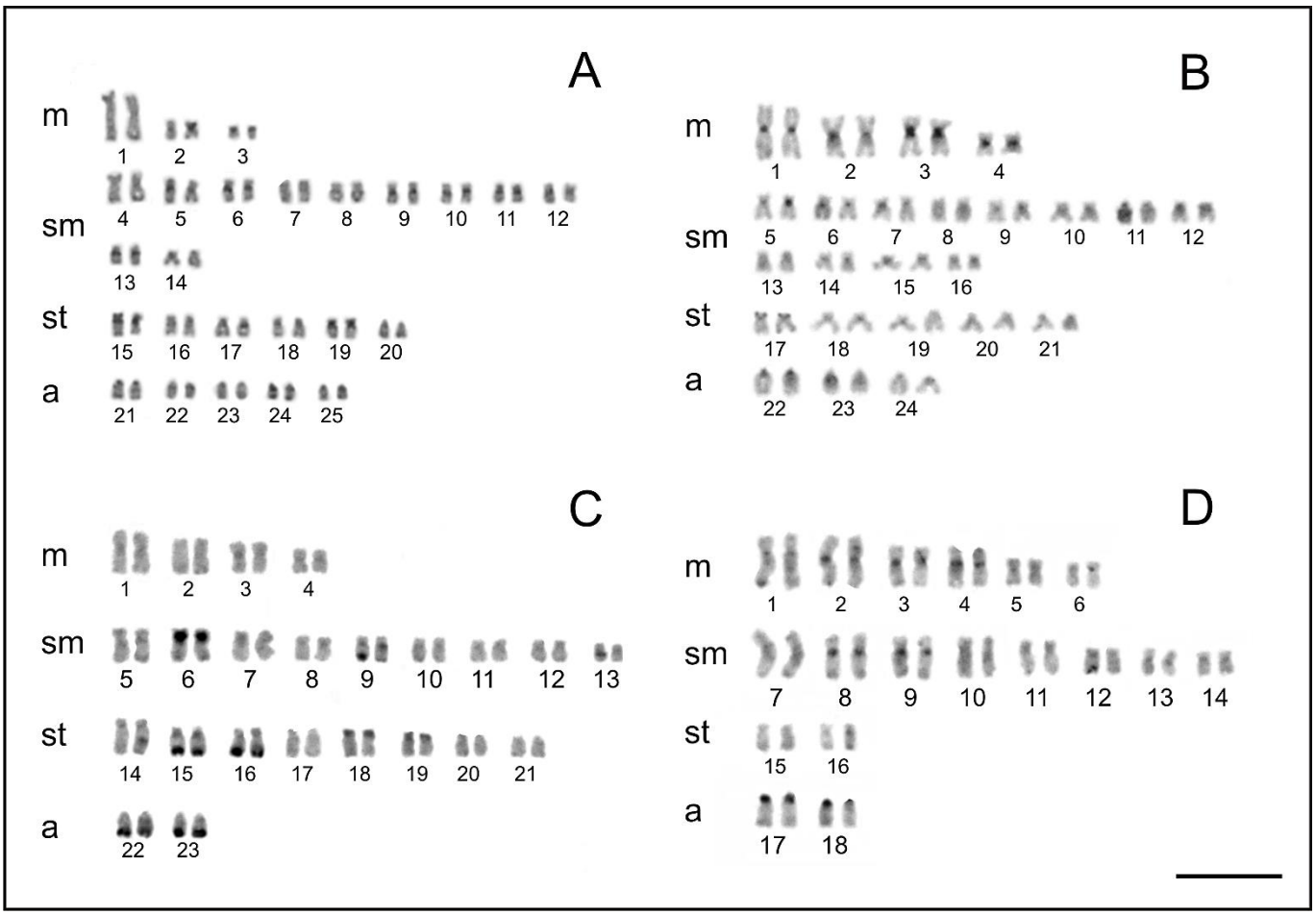

Figure 2. Chromosomes C-banded: (**A**) *A. altiparanae*; (**B**) *A. marionae*; (**C**) *A. fasciatus*; (**D**) *A. schubarti*.

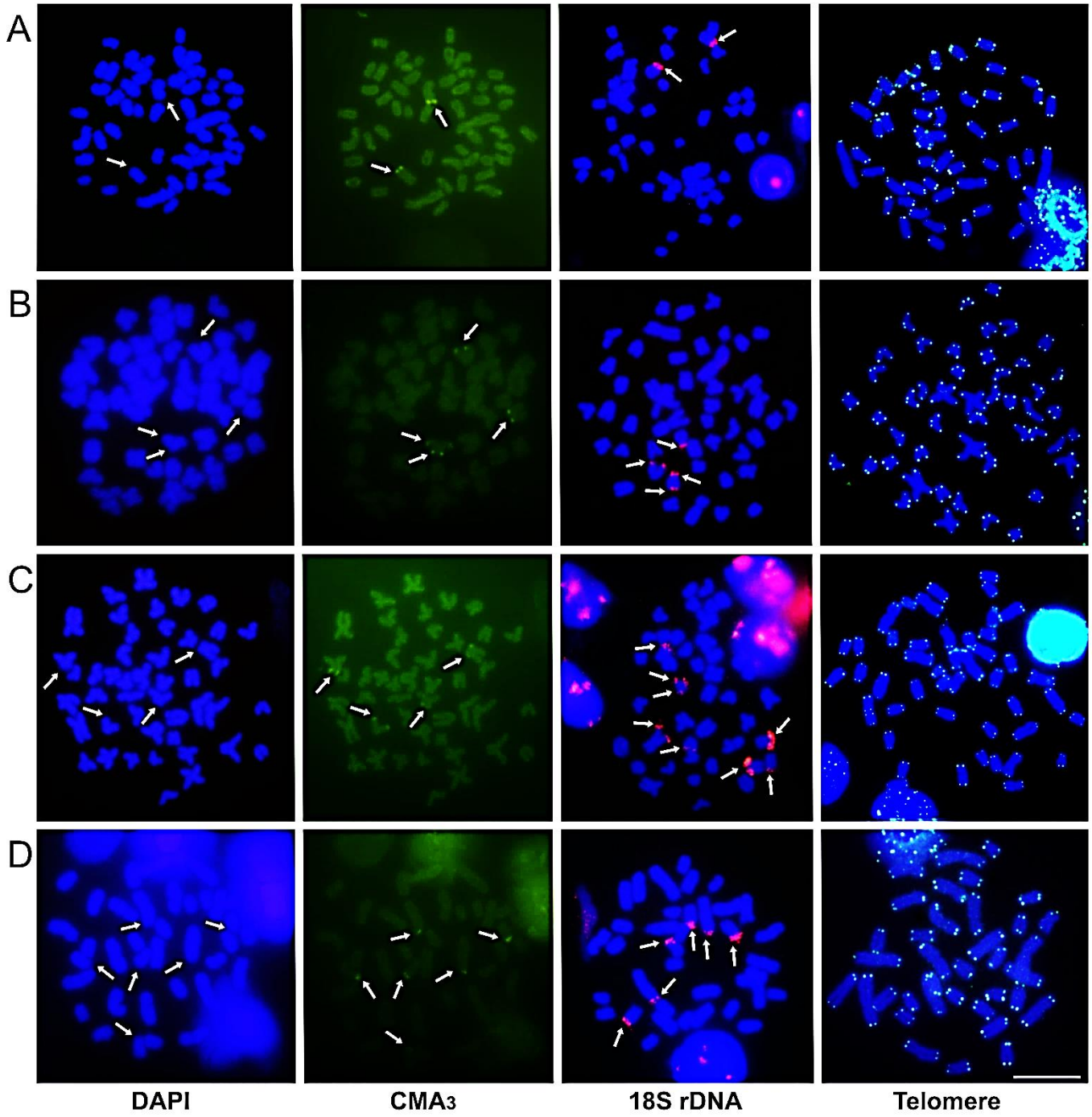

Figure 3. Chromosomes with CG-rich regions and fluorescent signals of repetitive sequences. (**A**) *A. altiparanae*; (**B**) *A. marionae*; (**C**) *A. fasciatus*; (**D**) *A. schubarti*. Bar = 10 µm

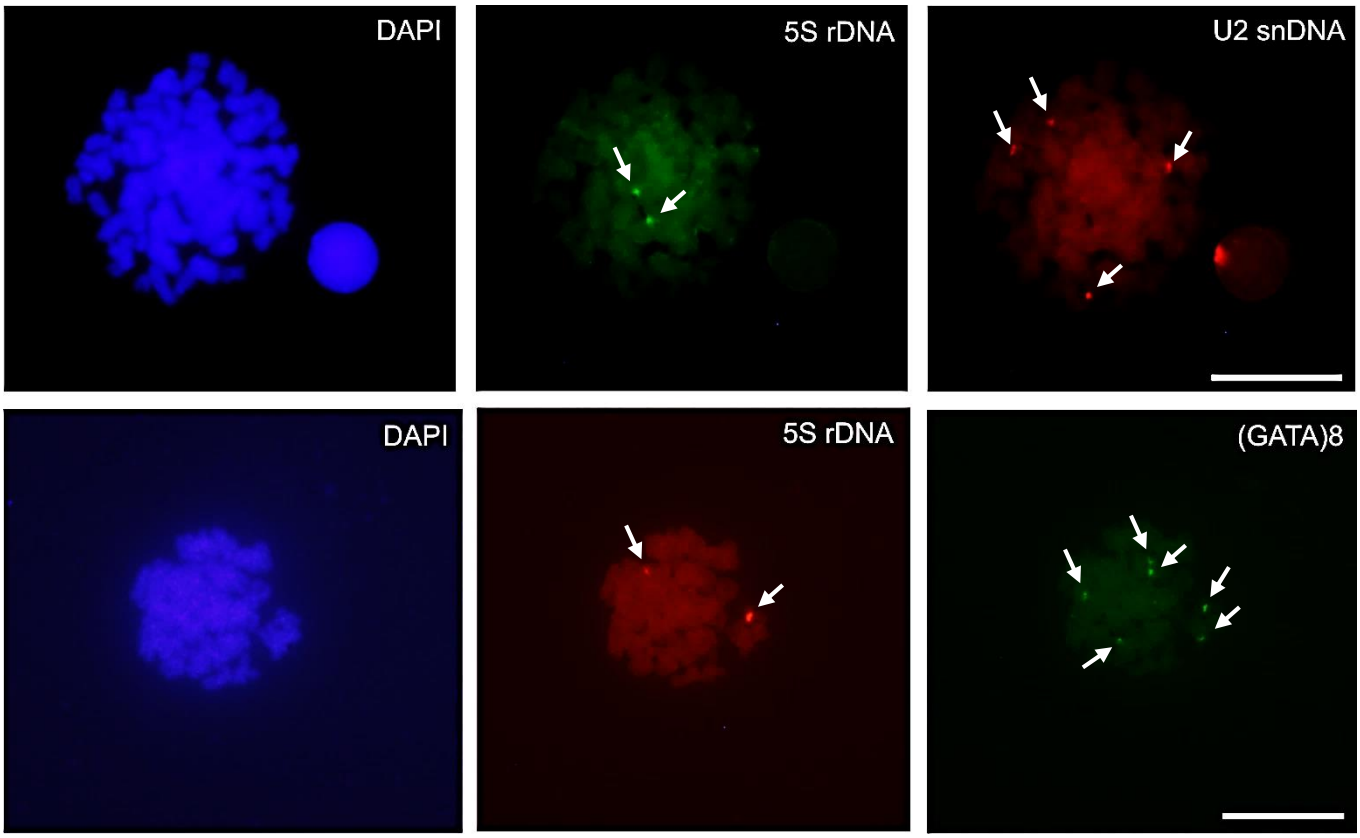

Figure 4. DAPI and fluorescent signals of repetitive sequences in *S. notomelas*. The arrows indicate the repetitive clusters. Bar = 10 µm.

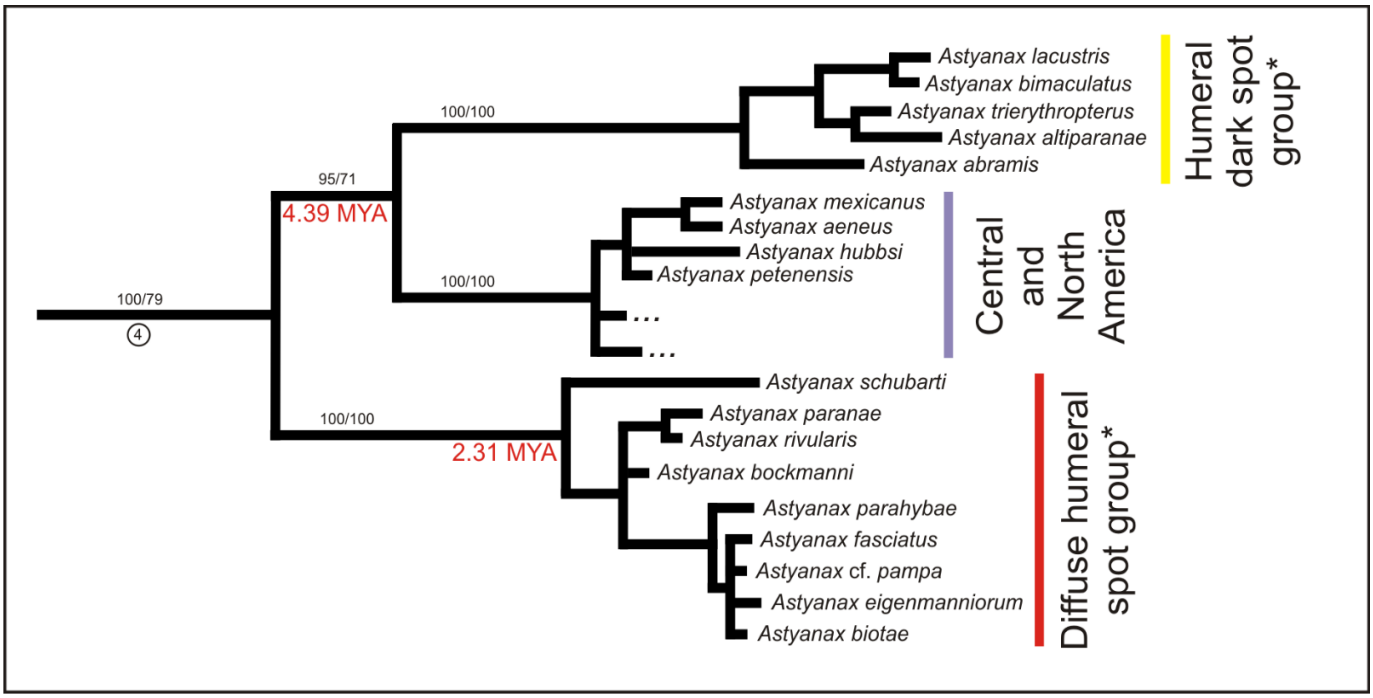

Figure 5. Cladogram adapted from Coutinho-Sanches and Dergam (2015). Note that three well-structured groups are formed. Red numbers indicate the separation in Millions of year ago (MYA) and the asterisks indicate the South American groups.

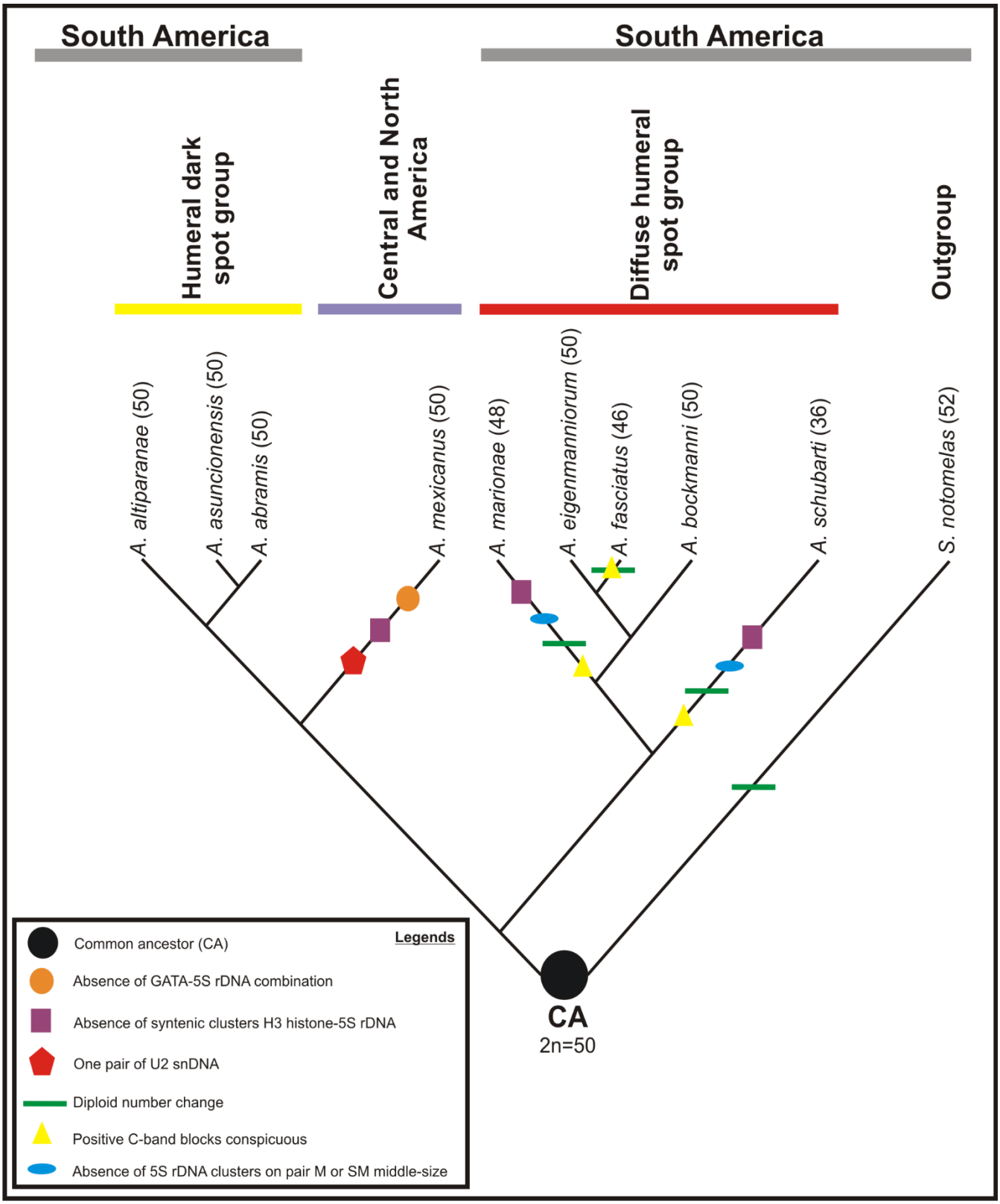

Figure 6. Schematic cladogram mounted from the molecular data of Coutinho-Sanches and Dergam (2015). Note that cytogenetic data were plotted in scheme.

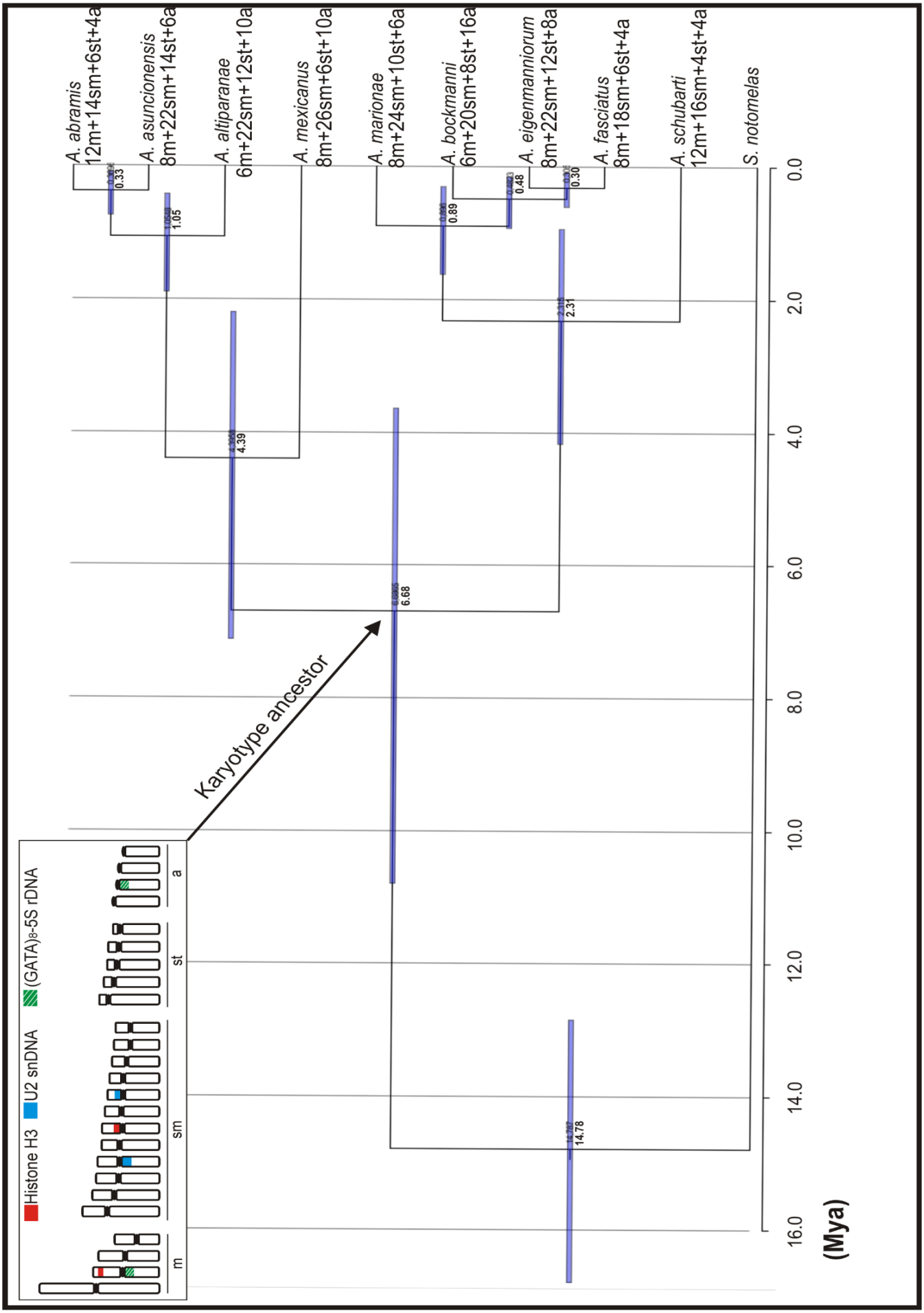

Figure 7. Molecular clock using COI gene. Note that the larger numbers represent the time in Millions of years.

CONCLUSÕES

7. CONCLUSÕES

Considerando os dados obtidos e as análises compreendidas nesta tese, é possível concluir que:

1) Entre as espécies estudadas, as que apresentaram características cromossômicas mais distintas foram *A. schubarti* (2n = 36) restrita a rios paulistas, *A. marionae* (2n = 48) da bacia do rio Paraguai e *A. mexicanus* (2n = 50 +1B) com distribuição entre sul da América do Norte e a América Central.

2) A localização do DNAr 5S parece ser conservada para alguns grupos dentro de *Astyanax*, diferentemente do DNAr 18S, o qual apresenta enorme variabilidade em termos de localização cromossômica e número de sítios.

3) Os genes de histona H3 apresentam localizações em cromossomos com morfologias similares para as espécies da América do Sul, com exceção de *A. schubarti* (2n = 36) e *A. marionae* (2n = 48).

4) Os cromossomos portadores dos genes de RNAsn U2 mostraram o maior grau de similaridade morfológica no grupo *Astyanax*. Mesmo em espécies com traços morfológicos muito similares, como por exemplo, *A. marionae* e *A. fasciatus*, e localizações cromossômicas dos genes H3-5S tão distintas, os genes U2 apresentaram localizações muito similares entre as duas espécies e as demais da América do Sul, com exceção de *A. schubarti*.

5) O estudo dos microssatélites foi de fundamental importância nesse trabalho, pois os resultados permitiram constatar a proximidade de grupos com características bastante peculiares dentro de *Astyanax*. Por exemplo, por mais que em *A. marionae* e *A. schubarti* as localizações cromossômicas dos genes de histona

H3 foram distintas, estas duas espécies compartilham uma característica em comum (co-localização 5S-GATA).

6) Espécies da América do Sul apresentaram-se mais próximas entre si do que com *A. mexicanus* (peixe cego de caverna do México).

7) Estudos mais conclusivos foram possíveis, e capazes de responder questões fundamentais relacionadas a origem e evolução dos cromossomos do grupo *Astyanax*, a partir de análises mais completas, como exemplificado neste trabalho com uso de dados de natureza citogenética e molecular.

8) *Astyanax* se configura, atualmente, como um gênero muito complexo filogeneticamente, e uma revisão taxonômica deveria ser avaliada para o grupo, levando em consideração não apenas caracteres morfológicos ou dados moleculares, mas diversos aspectos como, por exemplo, ecologia, comportamento, ciclo de vida e também dados citogenéticos, entre outros.

BIOGRAFIA

8. BIOGRAFIA

Diovani Piscor concluiu os ensinos Fundamental e Médio em escolas da rede pública em 2001 e 2004, respectivamente. Em 2003, na instituição CEFAM (Centro de Formação e Aperfeiçoamento para o Magistério) iniciou o curso de formação de professores (curso Normal) juntamente com o ensino Médio concluindo-o em dezembro de 2005. No ano seguinte, iniciou o curso de licenciatura em Ciências Biológicas pela Universidade Estadual de Mato Grosso do Sul (UEMS – Mundo Novo), e em fevereiro de 2010 recebeu o grau de licenciatura na área. Nesta mesma instituição sob a orientação do Prof. Dr. Carlos Alexandre Fernandes iniciou seus estudos em citogenética de peixes (gêneros *Bryconamericus* e *Astyanax*). Em fevereiro de 2010 foi aprovado no processo seletivo de mestrado em Ciências Biológicas (Biologia Celular e Molecular) da UNESP de Rio Claro – SP, e sob a orientação da Profa. Dra. Patricia Pasquali Parise Maltempi desenvolveu o projeto de pesquisa em citogenética, recebendo o título de mestre, na área citada anteriormente, em março de 2012. Em maio do mesmo ano ingressou no Doutorado (Biologia Celular e Molecular) pela mesma instituição (UNESP – Rio Claro), também sob a orientação da Profa. Dra. Patricia Pasquali Parise Maltempi, onde estudou e trabalhou durante quatro anos. Durante sua trajetória na pós-graduação sua pesquisa foi focada nos cromossomos de espécies dos gêneros *Bryconamericus* (Mestrado) e *Astyanax* (Doutorado). Ainda, durante o Doutorado realizou trabalhos paralelos com microdissecção cromossômica de roedores e peixes dos gêneros *Astyanax* e *Leporinus*. Desde a graduação seus esforços culminaram em um total de, atualmente, oito artigos publicados em periódicos internacionais da área.

ANEXOS

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 43497-1	Data da Emissão: 26/11/2014 15:18	Data para Revalidação*: 26/12/2015

* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Diovani Piscor	CPF: 345.026.938-04
Título do Projeto: Estudos evolutivos no gênero Astyanax (CHARACIFORMES, CHARACIDAE): análises comparativas baseadas nos diferentes números diploides descritos para o gênero	
Nome da Instituição : UNIVERSIDADE ESTADUAL PAULISTA	CNPJ: 48.031.918/0018-72

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coletas de animais em campo	03/2014	05/2016

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
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4	A autorização para envio ao exterior de material biológico não consignado deverá ser requerida por meio do endereço eletrônico www.ibama.gov.br (Serviços on-line - Licença para importação ou exportação de flora e fauna - CITES e não CITES).
5	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
6	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio e o material biológico coletado apreendido nos termos da legislação brasileira em vigor.
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8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1	SANTA MARIA DA SERRA	SP	Rio Piracicaba, rio Tietê.	Fora de UC Federal

Atividades X Táxons

#	Atividade	Táxons
1	Coleta/transporte de amostras biológicas in situ	Astyanax
2	Coleta/transporte de espécimes da fauna silvestre in situ	Astyanax schubarti (*Qtde: 30), Astyanax (*Qtde: 30)

* Quantidade de indivíduos por espécie, por localidade ou unidade de conservação, a serem coletados durante um ano.

Material e métodos

1	Amostras biológicas (Peixes)	Outras amostras biológicas(Pequenos pedaÃ§os de nadadeira)

Este documento (Autorização para atividades com finalidade científica) foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

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Dados do titular

Nome: Diovani Piscor	CPF: 345.026.938-04
Título do Projeto: Estudos evolutivos no gênero Astyanax (CHARACIFORMES, CHARACIDAE): análises comparativas baseadas nos diferentes números diploides descritos para o gênero	
Nome da Instituição : UNIVERSIDADE ESTADUAL PAULISTA	CNPJ: 48.031.918/0018-72

2	Método de captura/coleta (Peixes)	Outros petrechos(Covo), Rede de arrasto de fundo (tração motorizada): tangones, portas ou parelha, Tarrafa, Peneira, Coleta manual

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	UNIVERSIDADE ESTADUAL PAULISTA	

Código de autenticação: 48845224

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
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Nome da Instituição : UNIVERSIDADE ESTADUAL PAULISTA	CNPJ: 48.031.918/0018-72

Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº 03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

Este documento (Autorização para atividades com finalidade científica) foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

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Nome da Instituição : UNIVERSIDADE ESTADUAL PAULISTA	CNPJ: 48.031.918/0018-72

* Identificar o espécime no nível taxonômico possível.

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DECISÃO CEUA Nº 018/2012

Instituição: **UNESP – IB – CRC**	Departamento: Biologia
Protocolo nº: 2335	Data de Registro CEUA: 02.04.2012
Projeto de Pesquisa: "Estudos evolutivos no Gênero Astyanax (Characiformes, Characidae): análises comparativas baseadas nos diferentes números diplóides descritos para o gênero"	

Pesquisador Responsável: PATRÍCIA PASQUALI PARISE MALTEMPI.

Orientando(a): Diovani Piscor.

Colaborador(a): Carlos Alexandre Fernandes, Daniela Bocagini R. Piscor, Tiago Gazoni, Kenny U. Sato.

Objetivo Acadêmico:	( ) TCC ( ) Mestrado (X) Doutorado ( ) Outros – (especificar)

A Comissão de Ética no Uso de Animal - CEUA do Instituto de Biociências da UNESP – Campus de Rio Claro, em sua 12ª reunião ordinária, realizada em 30.05.2012.

(X)	**Aprovou** o Projeto de Pesquisa acima citado, ratificando o parecer emitido pelo relator.
( )	**Desde** que atendidas as **pendências** apontadas na reunião (vide anexo), **aprova** o Projeto de Pesquisa acima citado (prazo máximo de 30 dias).
( )	**Referendou** o Projeto de Pesquisa acima citado, ratificando o parecer emitido pelo relator.
( )	Aprovou **retornar** ao interessado para atendimento das pendências encontradas (prazo máximo de 30 dias).
( )	**Não** Aprovou.
( )	**Retirou**, devido à permanência das pendências.

Rio Claro, 30 de maio de 2012.

Prof. Dr. Mário Sérgio Palma
Coordenador da CEUA