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Estudo da organização estrutural de elementos repetitivos isolados do genoma de *Leporinus elongatus* em diferentes espécies da família Anostomidae (Teleostei, Characiformes)

Edson Lourenço da Silva

Tese de Doutorado apresentada ao Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Rio Claro, para a obtenção do título de Doutor em Ciências Biológicas (Biologia Celular e Molecular)

Rio Claro
Estado de São Paulo – Brasil
Novembro de 2012

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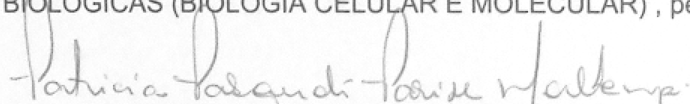

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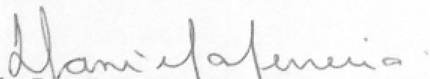

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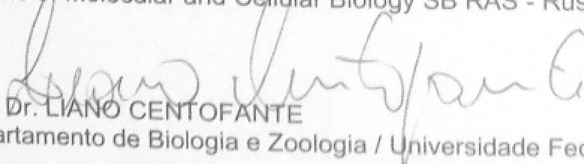

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RESUMO

A família Anostomidae compreende um grupo com 12 gêneros que apresenta ampla distribuição pela América do Sul e Central. Estudos citogenéticos de várias espécies da família mostram uma estrutura cariotípica bastante conservada, com o número diplóide igual a 54 cromossomos, dos tipos meta e submetacêntricos e apenas um par de cromossomos portador de regiões organizadoras de nucléolos (NOR) localizadas em posições e pares distintos nas diferentes espécies do grupo. Através de estudos envolvendo técnicas de citogenética molecular têm se conseguido uma caracterização mais resolutiva dos cromossomos de algumas espécies da família. Estas metodologias forneceram evidências de polimorfismos de NOR, diferentes padrões de distribuição da heterocromatina constitutiva, presença de seqüências repetitivas sexo-específicas e novos sistemas de cromossomos sexuais heteromórficos do tipo ZZ/ZW bem como a diferenciação de híbridos interespecíficos. Dessa forma, o presente trabalho teve como objetivo acrescentar novos dados que auxiliem no estabelecimento de padrões para esclarecer a história evolutiva dos cromossomos sexuais em espécies de Anostomidae, baseado no estudo da organização de seqüências repetitivas isoladas do genoma de *Leporinus elongatus* em diferentes espécies de Anostomidae. Para tanto, novos elementos repetitivos do genoma de *Leporinus elongatus* foram isolados através de restrição enzimática e usados em experimentos de hibridação fluorescente *in situ* (FISH) tanto em *L. elongatus* quanto nas espécies *L. macrocephalus*, *L. obtusidens*, *L. friderici*, *L. lacustris*, *L. striatus*, *Schizodon borellii*, *S. isognathus* e *Abramites hypselonotus*. Além disso, sondas de cromossomos inteiros obtidos por microdissecção também foram usadas nesses experimentos, a fim de buscar identificar homologias entre os cromossomos destas mesmas espécies, bem como a presença de cromossomos sexuais ainda em estágios de diferenciação naquelas espécies que não os apresentam na forma distinguível. O elemento repetitivo Le*Sma*I possui 378 pares de bases e mostrou-se exclusivo da espécie *L. elongatus* tanto machos quanto fêmeas. Trata-se de um satélite localizado próximo a NOR numa região altamente heterocromática. A presença deste DNA satélite reforça a idéia de diversidade de seqüências repetitivas no genoma de espécies de *L. elongatus* apesar da aparente estabilidade cromossômica reportada para as espécies de Anostomidae. Sondas de

Le*Sma*I e rDNA 18S foram usadas no mapeamento cromossômico das demais espécies da família deste estudo, contudo, não foi observado sinais de hibridação, mesmo em condições de baixa estringência. Os sítios de rDNA 18S mostram-se conservados, com relação ao número, mas apresentam um padrão diferenciado de localização, resultados de rearranjos que mantiveram tanto o número quanto a fórmula cariotípica de Anostomidae. Diferente do padrão observado em Le*Sma*I o elemento repetitivo Le*Spe*II, mostrou-se disperso no genoma de *L. elongatus* com uma distribuição semelhante a dos elementos LINES. Com 628 pares de bases, o elemento repetitivo Le*Spe*II não possui evidências de padrão sexo-específico, localizando-se mais comumente próximos as regiões terminais da maioria dos cromossomos de todas as espécies analisadas. Contudo não está presente nos cromossomos que compõem o sistema sexual múltiplo de *L. elongatus*, possivelmente, estando relacionado com o processo de origem dos mesmos. O elemento Le*Spe*I, já descrito em *L. elongatus* e caracterizado em *L. macrocephalus* e *L. obtusidens* foi hibridado nas demais espécies deste estudo, porém sem resultados positivos, reforçando sua ligação com os cromossomos sexuais das espécies de *Leporinus*. Os resultados dos experimentos de *crossfish* com sondas de cromossomos sexuais inteiros de *L. elongatus*, *L. macrocephalus* e *L. obtusidens* produziram um padrão de hibridação que permite sugerir uma origem comum para o sistema sexual diferenciado de *Leporinus.* Naquelas espécies sem cromossomos sexuais (*L. friderici*, *L. lacustris*, *L. striatus*, *S. borellii*, *S. isognathus* e *A. hypselonotus)*, não foram observados sinais de hibridação, o que indica a ausência de cromossomos sexuais crípticos compartilhando essas seqüências. Os resultados observados reforçam a estabilidade macro cariotípica das espécies de Anostomidae, e mostram a diversidade de elementos repetitivos presente no genoma destas espécies, bem como seu importante papel na diversificação dos mesmos.

Palavras-chave – banda C; FISH; NOR; piau; restrição enzimática;

ABSTRACT

The Anostomidae family comprises a group with 12 genera widely distributed throughout Central and South American Rivers. Cytogenetic studies carried out in several anostomids shows a karyotypic structure very conserved, with a diploid number equals to 54 chromosomes, metacentric and submetacentric, and only one chromosome pair carrier of nucleolar organizing regions (NOR), localized at distinct chromosome positions in the species of the family. The studies using molecular cytogenetic techniques have been providing a more accurate characterization of some species. These methods highlighted NOR polymorphisms, differential pattern of heterochromatin distribution, the presence of sex specific repetitive sequences, as well as new heteromorphic sex chromosomes and interspecific hybrids differentiation. Thus, the present study aimed to add new data to assist in to establish patterns to clarify the evolutionary history of sex chromosomes in Anostomidae species, based on the study of the organization of repetitive sequences isolated from *Leporinus elongatus* genome in different species of Anostomidae. For this, new repetitive elements were isolated from *L. elongatus* and used in Fluorescent in situ hybridisations experiments (FISH) in *L. elongatus* as well in the species *L. macrocephalus*, *L. obtusidens*, *L. friderici*, *L. lacustris*, *L. striatus*, *Schizodon borellii*, *S. isognathus* e *Abramites hypselonotus*. Indeed, probes of whole sex chromosomes obtained through microdissection were also used in order to identify similarities among the anostomids chromosomes and the presence of sex chromosomes in differentiation stages. The Le*Sma*I repetitive element has 378 bp and is exclusive to *L. elongatus* individuals. This is a satellite DNA localized near to the NOR in a highly heterochromatic region. The presence of this satellite DNA reinforces the idea of a diversity of repetitive sequences in the *L. elongatus* genome despite the chromosome stability reported to the group. Probes of Le*Sma*I element and 18S rDNA were used in a chromosome mapping in other anostomids species, however, no hybridisations signal were observed even after low stringency wash conditions. The 18S rDNA sites are conserved regarding the number, but are localized at distinct positions due the rearrangements that maintained both the number and karyotypic formulae of the Anostomidae. Different that observed in the Le*Sma*I distribution, the repetitive element Le*Spe*II is dispersed throughout the genome seeming with the LINES

elements. With 628 bp, this repetitive element not presents a sex specific pattern, being commonly near to the telomeres of all analyzed species. However, this element is absent in the multiple sex chromosomes of *L. elongatus* species, probable being related with the process of origin of this system. The Le*Spe*I element already described in *L. elongatus* and mapped in *L. macrocephalus* and *L. obtusidens* was hybridized against the chromosomes of the other anostomids, but without positive results, reinforcing the idea of relation with the sex chromosomes of *Leporinus*. The results of cross-fish with whole chromosome probes of *L. elongatus*, *L. macrocephalus* and *L. obtusidens* produced a hybridisation pattern that suggests a common origin for the ZZ/ZW sex system of *Leporinus*. In those species without differentiated sex chromosomes the signals were absents, indicating also the absence of cryptic sex chromosomes sharing this sequences. The results obtained here reinforce the stability of the karyotypic macro structure of the Anostomidae species, and shows the diversity of repetitive elements present in the genome of some species, as well as the role of these sequences in karyotypic diversification of anostomids.

Keywords C band; Enzymatic restriction; FISH; NOR.

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1 INTRODUÇÃO

1.1 Mecanismos de determinação sexual

Os mecanismos de determinação sexual nos organismos são variados, e mostram-se evolutivamente dinâmicos, provavelmente tendo se originado de forma independente nos principais grupos, mostrando uma considerável plasticidade evolutiva (CHARLESWORTH, 1991; MARÍN; BAKER, 1998; VALLENDER; LAHN, 2006) e aparentemente não seguindo nenhum padrão filogenético ou taxonômico (SWAIN, 2002).

Especialmente nos vertebrados, fica evidente a labilidade evolutiva da determinação sexual, uma vez que alterações naturais ou experimentais podem influenciar na determinação de machos e fêmeas de maneira diferenciada em alguns grupos (SEGAL, 1985). Sob condições ambientais o sexo é definido por um fator não genético vivenciado em um curto período após a concepção tais como temperatura, densidade, fatores sociais, e pH (BAROILLER; COTTA, 2001). Em alguns répteis, por exemplo, a temperatura define o sexo já nas primeiras fases do desenvolvimento embrionário (JANZEN; PHILIPS, 2006; VALLENDER; LAHN, 2006). Em alguns grupos de peixes como a tilápia do Nilo (*Oreochromis. niloticus*) tem se observado que a alta temperatura exerce alguma influência na atividade da enzima aromatase e na baixa síntese do hormônio estradiol, levando ao desenvolvimento de machos (DEVLIN; NAGAHAMA, 2002).

Em contraste, a determinação genotípica pode envolver sistemas monogênicos e poligênicos localizados em autossomos ou em cromossomos sexuais (DEVLIN; NAGAHAMA, 2002, JANZEN; PHILIPS, 2006). Os cenários genéticos relativos à determinação do sexo são extraordinariamente diversos e desafiam perguntas sobre a evolução e manutenção do sexo. Assim, segundo Schartl (2004a), por que um fenômeno biológico simples e altamente conservado como a existência de machos e fêmeas é controlado por mecanismos tão diversos, como por exemplo, a razão de cromossomos X para autossomos em drosófilas, ou a presença de um cromossomo Y em mamíferos machos? (ver SCHARTL, 2004a).

Em nível molecular, a determinação do sexo é ainda mais complexa (SCHARTL, 2004a) podendo envolver desde a ação de genes principais até a sua interação com múltiplos genes autossômicos (DEVLIN; NAGAHAMA, 2002). Além

disso, o mecanismo de atuação destes múltiplos genes pode funcionar como um modelo de cascata gênica na qual, aqueles que atuam no início desta cascata, desencadeiam todo o processo de determinação do sexo. No que diz respeito aos aspectos evolutivos, é sabido que os genes que atuam no topo da cascata são mais diversos ou variáveis que aqueles que atuam em posições abaixo da cadeia regulatória (MARIN; BAKER 1998; SCHARTL, 2004b).

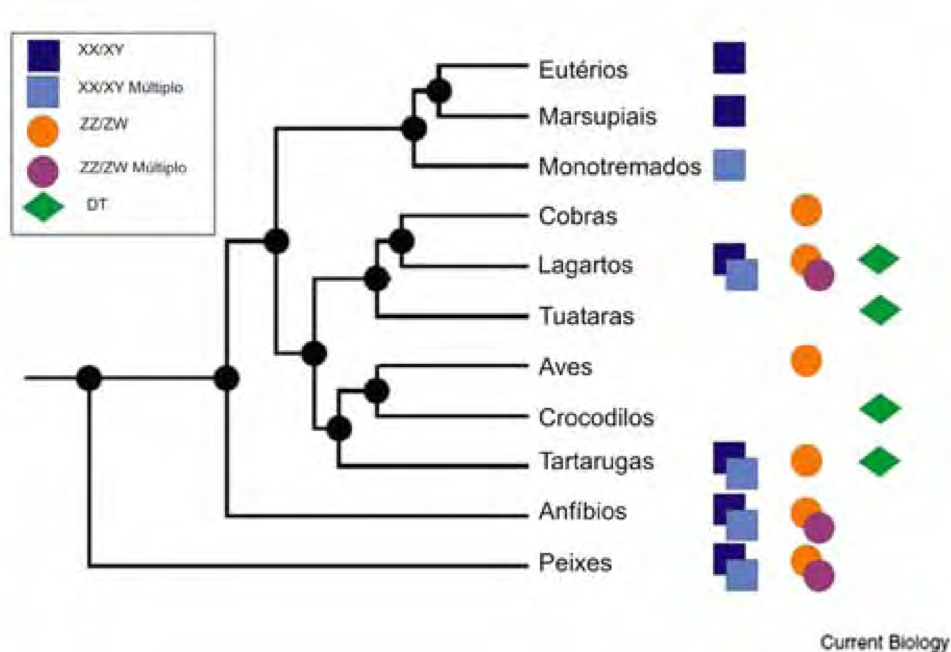

Figura 1. Filogenia dos maiores clados de vertebrados mostrando os sistemas de determinação do sexo encontrados em seus membros. DT= Dependente de temperatura. Adaptado de Ezaz et al. (2006).

O caso mais bem estudado de determinação genética é aquele presente nos mamíferos. Neste grupo, o gene de determinação principal é o *SRY*, que age diretamente na formação dos testículos atuando no topo de uma cascata que inclui outros genes como o *SOX9* e o fator de transcrição *DMRT1* (SCHARTL, 2004a). Ainda não está claro qual gene é responsável pela determinação do sexo em aves, anfíbios, répteis e peixes apesar de haver um consenso sobre quais são os melhores candidatos, principalmente no grupo das aves e répteis (SCHARTL, 2004a).

1.2 Origem e diferenciação dos cromossomos sexuais

A existência de um mecanismo genético de determinação do sexo não implica necessariamente na presença de cromossomos sexuais distinguíveis, embora usualmente isso ocorra (WHITE, 1977). Tipicamente nestes casos, um sexo heterogamético apresenta um par de cromossomos morfologicamente diferente, enquanto que o outro sexo, o homogamético, possui um par de cromossomos idênticos (BACHTROG, 2006). As diferenças morfológicas não estão somente relacionadas às diferenças de tamanho, mas também conteúdo gênico e quantidade de seqüências heterocromáticas no par de cromossomos (CHARLESWORTH et al., 2005).

Os cromossomos sexuais provavelmente derivam de um par de autossomos ancestral (OHNO, 1967); conseqüentemente no início do processo evolutivo, ambos os cromossomos ancestrais eram como seus progenitores, similares em forma e somente depois de certo tempo, adquiriram diferenças morfológicas suficientes para se tornaram heteromórficos (KONDO et al., 2006). Essas diferenças podem ter origem a partir da ausência de recombinação entre os homólogos, principalmente pela presença de inversões (DOBZHANSKY; EPLING, 1948) e acúmulo de heterocromatina. Como conseqüência da inibição da recombinação, ocorre a ampliação da região não recombinante e perda de conteúdo gênico no cromossomo sexo-específico com sua posterior degeneração (CHARLESWORTH; CHARLESWORTH, 2000). Atualmente é aceito que os sistemas de determinação sexual cromossômica evoluíram múltiplas vezes nos principais grupos, inclusive em grupos de espécies próximas (CHARLESWORTH, 1991; MATSUBARA et al., 2006; VALLENDER; LAHN, 2006; VOLFF; SCHARTL, 2001; TAKEHANA et al., 2007)

Nos animais que apresentam cromossomos diferenciados, a determinação genética do sexo segue dois tipos principais de heterogametia cromossômica: a feminina, com o sistema ZW (onde estas possuem exclusivamente o cromossomo W) e a masculina, com o sistema XY (onde o Y é exclusivo do macho) (WHITE, 1977; ALMEIDA-TOLEDO; FORESTI, 2001; OGATA et al., 2008) (Figura 1).

Além disso, existem as variantes múltiplas destes dois modelos, nestes casos entende-se por sistemas simples quando existem dois cromossomos distintos dos tipos XY ou ZW no sexo heterogamético, e dois idênticos, XX ou ZZ, no sexo homogamético. Nos sistemas múltiplos, tem-se no cariótipo mais de um

cromossomo X ou Z, ou mais de um Y ou W, como resultado de rearranjos estruturais, partindo-se de sistemas simples de cromossomos sexuais

Sistemas heterogaméticos em machos têm sido exaustivamente estudados em mamíferos, enquanto que a heterogametia em fêmeas é amplamente observada em aves e répteis (TAKEHANA et al., 2007; EZAZ et al., 2006). Nestes vertebrados, os cromossomos sexuais são altamente diferenciados sendo os cromossomos X e Z grandes e ricos em genes, comparando-se com o Y e W, que geralmente são menores, altamente heterocromáticos e contêm poucos genes (KONDO et al., 2006; TAKEHANA et al., 2007).

De maneira geral, dentro dos sistemas XY ou ZW, variações na morfologia dos cromossomos são prontamente explicadas por adição/deleção de heterocromatina ou fissões e rearranjos Robertsonianos ou ainda fusão *in tandem* produzindo sistemas múltiplos (ALMEIDA-TOLEDO; FORESTI, 2001). Mais interessante ainda é que alguns sistemas recentes têm evoluído por duplicação ou retrotransposição gênica ou ainda por variação e deleção de seqüências gênicas (EZAZ et al., 2006).

No que diz respeito aos aspectos evolutivos destes sistemas, em um mapeamento gênico comparativo em aves, répteis e humanos, Matsubara et al. (2006) não observaram homologia entre os cromossomos sexuais destes grupos, sugerindo que os mesmos foram derivados de diferentes pares de autossomos de um ancestral comum.

1.3 Peixes Neotropicais como modelo para estudos de evolução de sistemas de cromossomos sexuais: a importância dos elementos repetitivos no processo de diferenciação

Os DNAs repetitivos são constituídos por seqüências de diferentes comprimentos e composições que aparecem muitas vezes no genoma. Estão incluídos neste grupo os elementos arranjados em cadeia, compostos pelo DNA satélite (DNA altamente repetitivo), as seqüências de mini e microssatélites (DNA moderadamente repetitivo) e as seqüências repetitivas dispersas no genoma compostas por transposons e retrotransposons (DNA moderadamente repetitivo) (CHARSLESWORTH et al., 1994). DNAs satélites são seqüências não codificadoras

arranjadas em cadeia que geralmente estão localizadas próximas a regiões heterocromáticas dos cromossomos, nos centrômeros e telômeros (CHARLESWORTH et al. 1994).

Os transposons são classificados de acordo com seu modo de transposição. Assim, há transposons que se movimentam diretamente através de um mecanismo de “cut and paste” ou como retroelementos que se movimentam através de um RNA intermediário (CHARLESWORTH et al., 1994). Há dois tipos de retroelementos encontrados comumente no genoma de eucariontes: os SINEs (*Short Interspersed Nucleotide Elements*) e os LINEs (*Long Interspersed Nucleotide Elements*), que se distinguem um do outro pelo seus comprimento e estrutura molecular (SINGER, 1982; MARTIN, 1991; CHARLESWORTH et al, 1994; OKADA et al., 1997).

Seqüências repetitivas, de uma forma geral, estão altamente associadas com regiões heterocromáticas e por certo período de tempo foram consideradas como DNA lixo devido a sua função desconhecida no genoma (LIM; SIMMONS, 1994; DIMITRI et al., 1997; SUMNER, 2003). Entretanto, atualmente algumas repetições in tandem têm tido suas funções bem definidas no núcleo que variam desde cistrons ribossomais (LONG; DAWID, 1980; HESLOP-HARRISON, 1991), seqüências centroméricas (SUMNER, 2003) até genes que codificam para a síntese de histonas (MAXON et al., 1983).

Alem disso, as seqüências de DNA repetitivos têm sido estudadas em quase todos os grupos de vertebrados. Apesar de grande parte das informações disponíveis referirem-se aos mamíferos, sabe-se, que os peixes apresentam diversos tipos de DNAs repetitivos (VALENTE et al, 2010) que variam desde genes ribossomais, a elementos transponíveis das mais diferentes famílias (OZOUF-COASTAZ et al., 2004; MAZZUCHELLI; MARTINS, 2009; FERREIRA et al. 2010) bem como DNA satélites (NAKAYAMA et al., 1994; KOEHLER et al., 1997; PARISE-MALTEMPI et al., 2007, SILVA et al. 2012).

Esta classe constitui um grupo de animais extremamente diversificado que não constituem uma linhagem monofilética onde se incluem os agnatos (lampreias ou peixes bruxas), peixes com esqueleto ósseo, bem como os peixes cartilaginosos (tubarões e raias). Nelson (2006) sugere a presença de pelo menos 34.500 espécies de peixes em todo o mundo, e somente no ambiente de água doce são estimadas 15.000 espécies. Desse montante, cerca de 6.000 espécies são encontradas na

região Neotropical (REIS et al., 2003). Considerando o fato do genoma dos peixes evoluir de forma extremamente rápida (OSOUF-COSTAZ et al., 2004) e de sua posição filogenética basal entre os vertebrados, o estudo das particularidades de determinadas classes de DNA repetitivos tem se tornado cada vez mais importante, pois auxilia no entendimento das questões evolutivas da própria classe, como também dos demais vertebrados.

Em peixes, Os DNAs repetitivos têm sido amplamente estudados devido a sua ligação com os cromossomos sexuais, indicando que a diferenciação destes cromossomos esta freqüentemente relacionada ao acúmulo de seqüências de DNA repetitivos associados às regiões heterocromáticas (ver CIOFFI et al. 2011). Entretanto, se o ganho de segmentos heterocromáticos representa a causa ou conseqüência da redução da recombinação, é uma questão que permanece não resolvida (CHARLESWORTH et al., 1994).

No que diz respeito aos estudos dos sistemas de cromossomos sexuais os peixes Neotropicais constituem um grupo particularmente interessante visto que até o momento admite-se que somente uma minoria das espécies citogeneticamente estudadas apresentam sistemas de cromossomos sexuais heteromórficos, no entanto, sistemas simples e múltiplos já foram identificados em diferentes grupos (Tabela I) (ver ALMEIDA-TOLEDO; FORESTI, 2001; DEVLIN; NAGAHAMA, 2002; OLIVEIRA et al., 2009).

A heterogametia masculina está presente em um grande grupo de peixes, inclusive em famílias que apresentam cromossomos heteromórficos do tipo ZW (tabela 1). Nas espécies em que este sistema ocorre observa-se tanto sistemas simples quanto múltiplos, e sua origem tem sido atribuída a diferentes fatores, muitas vezes, evoluído mais de uma vez em grupos de espécies próximas.

Tabela 1. Sistemas de cromosssomos sexuais heteromórficos em famílias de peixes neotropicais envolvendo machos e fêmeas heterogaméticas (Adaptado de OLIVEIRA et al., 2009).

Família	Sistema sexual	
	Heterogametia feminina	**Heterogametia masculina**
Anostomidae	ZW; $Z_1W_1Z_2W_2$	
Characidae	ZW	XY
Cichlidae		XY
Crenuchidae	ZW	
Doradidae	ZW	
Erythrinidae		XY; X_1X_2Y; XY_1Y_2
Gasteropelecidae	ZW	
Gobiidae		XY; X_1X_2Y
Gymnotidae		X_1X_2Y
Heptapteridae	ZW	XY
Hypopomidae		X_1X_2Y
Loricariidae	ZW	XY; XO; XY_1Y_2
Parodontidae	ZW; ZW_1W_2	
Pimelodidadae		XY
Poeciliidae	ZW	XY
Prochilodontidae	ZW	
Rivulidae		XY
Sternopygidae	ZW	XY; X_1X_2Y

Os peixes da família Erythrinidae (Characiformes), por exemplo, constituem-se um bom modelo para estudos sobre a origem e evolução dos sistemas do tipo XX/XY. Os eritrinídeos possuem em geral uma grande variabilidade cariotípica (BERTOLLO et al., 2000; GIULIANO-CAETANO et al., 2001; DINIZ; BERTOLLO, 2003) sendo que só em *Erythrinus erythrinus* foram descritos ao menos quatro cariomorfos (A-D) nos quais foram observados desde cariomorfos sem cromossomos sexuais até sistemas simples e múltiplos (BERTOLLO et al., 2004). Através de técnicas de bandamentos diferenciados a origem de seus sistemas sexuais foi relacionada a eventos de translocação entre dois cromossomos de dois braços. Estudos adicionais comparando-se os diferentes cariomorfos através do uso de sondas de DNA repetitivos como rDNA, DNA satélites, seqüências teloméricas,

microsatélites e diferentes classes de elementos transponíveis demonstram que rearranjos cromossômicos e modificações genômicas foram significativos durante o curso da evolução cariotípica deste grupo (CIOFFI et al., 2012).

Na ordem Gymnotiformes, família Sternopygidae, um sistema semelhante pode ter se originado por eventos de fusão cêntrica entre cromossomos acrocêntricos em *Eigenmannia* sp. (SILVA; MARGARIDO, 2005). Experimentos de hibridação usando sondas de cromossomos sexuais inteiros extraídos de *Eigenmannia virescens* (citótipo XY) em *Eigenmannia* sp. mostraram a inexistência de homologia entre os cromossomos sexuais das duas espécies; e análises utilizando sequencias de DNA permitiram sugerir que ambas tiveram uma origem independente e relativamente recente (HENNING et al., 2008; HENNING et al., 2011)

A heterogametia feminina é o sistema de determinação mais freqüentemente encontrado entre os peixes (CENTOFANTE et al., 2002). Dependendo do grupo, a diferenciação entre os cromossomos Z e W ocorre de diversas formas: diferenças em tamanho dos cromossomos, como observado em espécies de *Triportheus* (ARTONI et al., 2001) e *Characidium* (Characiformes, Crenuchidae) (NOLETO et al., 2009); distinta posição do centrômero como ocorre em *Dormitator maculatus* (Perciformes, Eleotridae) (OLIVEIRA; ALMEIDA-TOLEDO, 2006); diferente morfologia observada em algumas espécies do gênero *Leporinus* (Characiformes, Anostomidae) (GALETTI; FORESTI, 1986; 1987; VENERE et al., 2004; PARISE-MALTEMPI et al., 2007); além dos distintos padrões de bandas G, R e C (VENERE et al., 2004, CARVALHO et al., 2002). Contudo, a maioria distingue-se pelo padrão de distribuição de heterocromatina constitutiva entre eles (ROSA et al., 2006).

Nos peixes neotropicais são vários os processos que deram origem aos cromossomos sexuais diferenciados do tipo ZZ/ZW. Em algumas espécies estão envolvidos acúmulos de segmentos heterocromáticos seguidos pelo aumento do cromossomo W em relação ao Z (CENTOFANTE et al., 2001) e acúmulos de heterocromatina seguido de perdas seqüencial de material genético (BERTOLLO; CAVALLARO, 1992; CARVALHO et al., 2002). Já em outras, substituições de segmentos heterocromáticos (CENTOFANTE et al., 2003) e rearranjos seguidos ou não pela perda de material heterocromático (ARTONI et al., 1998; ALMEIDA-TOLEDO et al., 2000; ROSA et al., 2006 para maior revisão).

A subfamília Ancistrinae (Siluriformes) é outro grupo interessante porque seus integrantes apresentam desde cromossomos sexuais indiferenciados até sistemas de heterogametia masculina e feminina. Cromossomos sexuais na tribo Ancistrini foram relatados pela primeira vez por Mariotto et al. (2004) e Mariotto e Miyazawa (2006), que verificaram a existência de dois sistemas de cromossomos sexuais simples, ZZ/ZW e XX/XY, para o complexo de espécies *Ancistrus* cf. *dubius* da bacia do Paraguai. Posteriormente, De Oliveira (2006) e De Oliveira et al. (2008) descreveram a ocorrência de cromossomos sexuais do tipo ZZ/ZW na espécie *Hemiancistrus spilomma* de um rio da bacia Araguaia-Tocantins e XX/XY em espécies do gênero *Ancistrus* da bacia Amazônica. Em *A.* cf. *dubius* o processo de diferenciação sexual cromossômica ocorreu pela adição de segmentos heterocromáticos do cromossomo X enquanto o cromossomo Y, totalmente eucromático, provavelmente mantém a estrutura cromossômica ancestral (MARIOTTO; MIYAZAWA, 2006).

As pesquisas citogenéticas em espécies da família Anostomidae (Teleostei, Characiformes) tiveram seu inicio ainda nos anos 80, e desde então, têm se observado uma considerável estabilidade cromossômica no que se diz respeito à organização da macroestrutura cariotípica das mais diferentes espécies estudadas: um número diplóide constante, um único par cromossômico portador da região organizadora de nucléolos (NOR), bem como a presença quase que exclusiva de cromossomos metacêntricos e submetacêntricos. Com relação aos cromossomos sexuais, os dados têm sido reportados somente para a existência de heterogametia em fêmeas, restrita até o momento a espécies do gênero *Leporinus*.

O gênero *Leporinus* é um modelo de estudo interessante porque apresenta cerca de 40 espécies e destas, uma minoria (*L. conirostris*, *L. elongatus*, *L.* aff. *elongatus*, *L. macrocephal*us, *L. obtusidens*, *L. reinhardti*, *L. trifasciatus* e *Leporinus* sp2.), apresenta sistema de cromossomos sexuais diferenciados, simples e múltiplos. No sistema ZW simples as fêmeas apresentam o cromossomo W grande, submetacêntrico e totalmente heterocromático. Em contraste, no cromossomo Z, presente em ambos os sexos, somente a terça parte distal do braço longo é heterocromática. Em *L. elongatus* no sistema sexual múltiplo, existe a presença de um grande cromossomo W_1 que possui o braço longo quase que completamente

heterocromático, e outros três cromossomos (Z_1, Z_2 e W_2) submetacêntricos com bandas de seqüências de DNA repetitivo (PARISE-MALTEMPI et al., 2007).

A similaridade morfológica entre os cromossomos sexuais das espécies do gênero *Leporinus* levou Galetti et al. (1995) a sugerirem que estes têm uma origem comum, onde um acúmulo de segmentos heterocromáticos inicial poderia ter sido o primeiro passo na diferenciação destes cromossomos sexuais. Contudo, na espécie *Leporinus* sp2, não há distinção morfológica entre os cromossomos Z e W, sendo diferenciados apenas pelo padrão de distribuição da heterocromatina constitutiva entre cromossomos homólogos do tipo metacêntrico (VÊNERE et al., 2004). A partir destes resultados pode se inferir que o sistema de cromossomos sexuais em *Leporinus* ainda está em processo de evolução (PARISE-MALTEMPI et al., 2007).

Em virtude dessas características e, com o acesso mais facilitado às técnicas refinadas de estudos dos cromossomos, grandes esforços têm sido aplicados no sentido de estudar a origem e evolução do sistema sexual que é tão peculiar entre as espécies de *Leporinus* (NAKAYAMA et al., 1994; KOEHLER et al., 1997; VENERE et al., 2004; PARISE-MALTEMPI et al., 2007, MARRETA et al. 2012; SILVA et al. 2012). Estes estudos baseiam-se no isolamento e análise de seqüências de DNAs repetitivos, bem como sua localização nos cromossomos através de experimentos de hibridação *in situ*. O mapeamento cromossômico dessas frações do genoma, principalmente aquelas provenientes de espécies como *L. elongatus* e *L. obtusidens,* têm mostrado uma íntima relação com os cromossomos sexuais, ricos em bandas heterocromáticas.

Nesse sentido, torna-se desafiadora a idéia de se buscar maiores informações que possam explicar esses padrões observados em Anostomidae. Os peixes que compõem esta família possuem pelo menos quatro particularidades que os fazem um modelo interessante de estudo: (I) apresentam espécies com ampla distribuição geográfica, que permite maiores comparações com trabalhos já realizados em outras regiões; (II) apresentam sistema de cromossomos sexuais heteromórficos que envolvem algumas espécies da família, permitindo inferências sobre a evolução destes sistemas através de comparações com as espécies que não os apresentam; (III) a existência de sondas de segmentos de DNAs repetitivos já isolados, que permitem estudos comparativos; (IV) os peixes da família Anostomidae são importantes na pesca de subsistência, esportiva e comercial. Assim sendo, o

acúmulo de informações sobre os aspectos biológicos destes animais enriquece ainda mais as ações de preservação e manejo racional dos estoques deste grupo de peixes.

Dessa forma, o presente trabalho visa acrescentar novos dados que permitam estabelecer padrões para esclarecer a história evolutiva dos cromossomos sexuais em espécies de Anostomidae.

2. OBJETIVOS ESPECÍFICOS

- Isolar, caracterizar e mapear elementos repetitivos de indivíduos de *L. elongatus* buscando suas relações com a presença dos cromossomos sexuais da espécie.

- Verificar a relação presença/ausência das seqüências obtidas e sua organização em espécies de diferentes gêneros da família Anostomidae, com e sem cromossomos sexuais diferenciados.

- Verificar a presença e a localização cromossômica da seqüência repetitiva Le*Spe*I já isolada de indivíduos de *L. elongatus* em diferentes espécies da família Anostomidae com e sem cromossomos sexuais diferenciados.

- Estudar a distribuição dos cromossomos sexuais em Anostomidae, buscando informações para explicar padrão de distribuição deste sistema na família.

- Agrupar as informações citogenéticas disponíveis para Anostomidae, buscando evidenciar os principais marcadores cromossômicos para a família.

3. MATERIAL E MÉTODOS

3.1 Procedimentos de campo

Foram realizadas expedições de coletas nos meses de dezembro/2009 e maio de 2010 em diversos rios que compõem a Bacia do Rio Paraguai, estado de Mato Grosso. Os pontos foram previamente determinados em função da facilidade de acesso e a possibilidade de se encontrar os animais (Figura 2). Para a captura foram utilizados diversos petrechos de pesca como anzol de diferentes medidas, tarrafas e redes de arrasto. Os exemplares foram levados ainda vivos para o Laboratório de Citogenética Animal do Departamento de Biologia e Zoologia da Universidade Federal de Mato Grosso- UFMT, onde foram feitas as preparações citogenéticas e a retirada de amostras de tecido para extração de DNA. Os exemplares de *L. elongatus* foram coletados em estações de piscicultura na região de Leme-SP (-22.130062,-47.451036). Os indivíduos foram catalogados e organizados na coleção ictiológica do Laboratório de Citogenética, Departamento de Biologia, Universidade Estadual Paulista "Julio de Mesquita Filho" campus de Rio Claro.

Figura 2. Locais de coleta das espécies de anostomídeos na bacia do Rio Paraguai.

3.2 Procedimentos em laboratório

3.2.1 Preparação de cromossomos mitóticos

As preparações cromossômicas foram realizadas a partir da porção anterior do rim dos animais seguindo a metodologia descrita por Foresti et al. (1981). O procedimento consistiu em injetar intra-abdominalmente, entre as nadadeiras peitorais e ventrais, solução aquosa de colchicina (0,025%) na proporção de 1 mL/ 100 g de peso do animal e deixar os peixes em aquário bem aerado por 50 minutos. Após este período, sacrificaram-se os animais para a retirada do rim que foi dissociado em solução de KCl (0,075M) com o auxílio de pinças de dissecção e pipeta Pasteur, até a obtenção de uma solução homogênea. A solução foi então transferida para um tubo de centrífuga e deixada por 21 minutos em uma estufa a 37ºC. Após este tempo, o material foi pré-fixado com 7 gotas de fixador gelado (metanol/ácido acético na proporção 3:1, respectivamente) por cinco minutos a temperatura ambiente e, seqüencialmente, foram adicionados 6 mL de fixador, com agitação da mistura e o tubo levado à centrífuga na velocidade de 900 rpm por 10 minutos.

Em seguida, o sobrenadante foi retirado e o precipitado ressuspendido em 7 mL de fixador, sendo a mistura centrifugada por 10 minutos a 900 -1000 rpm. Este passo foi repetido por duas vezes para que houvesse uma completa fixação e lavagem das células em suspensão. O material foi então armazenado em freezer a -20°C.

Para a análise cromossômica, algumas gotas da preparação foram pingadas sobre lâminas de microscopia previamente lavadas e aquecidas a 60ºC. Após a secagem total das lâminas, estas foram coradas com solução de Giemsa 5% por 10 minutos, as quais foram lavadas e secas ao ar para análise do material.

3.2.2 Detecção da Heterocromatina Constitutiva

Para a detecção da heterocromatina constitutiva empregou-se a técnica de bandamento C, descrita por Sumner (1972), com algumas adaptações. As Lâminas com os cromossomos mitóticos, foram tratadas com uma solução de ácido clorídrico 0,2N à temperatura ambiente durante 10 minutos. Após este tempo as lâminas foram

lavadas com destilada e deixar secar ao ar. As lâminas foram colocadas em solução salina de 2x SSC por 15 minutos, lavadas em água destilada e secas ao ar. Após a secagem, as lâminas foram incubadas em solução de Hidróxido de Bário a 1%, recém preparada e filtrada, em temperatura ambiente, durante 1 minutos e 15 segundos. A ação do hidróxido de bário foi interrompida submergindo as lâminas rapidamente na solução de HCl 0,2N, As lâminas foram incubadas em solução salina de 2xSSC, a 60ºC durante 45 minutos, lavadas em água destilada e secas ao ar. Para a análise, as preparações das lâminas foram coradas com solução de Giemsa a 3% durante 10 minutos, lavadas com água destilada e secas ao ar.

3.2.3 Impregnação por nitrato de prata coloidal

Para a visualização das regiões organizadoras de nucléolos (NOR), foi empregada a técnica de impregnação pelo nitrato de prata coloidal, descrita por Howell; Black (1980), com algumas adaptações. Foram colocadas duas gotas de solução de gelatina à 2% (acrescida de ácido fórmico na proporção de 1 mL para cada 100 mL de solução) em duas regiões diferentes da lâmina com preparações cromossomos. Em seguida foi acrescentada uma gota de solução aquosa de nitrato de prata 50% sobre cada gota de gelatina. Na seqüência, a lâmina foi coberta com uma lamínula e incubada em câmara úmida a 60ºC até que os cromossomos adquirissem uma coloração amarelada e os nucléolos uma coloração quase preta; em geral este tempo variou de 3 a 5 minutos. Decorrido este tempo, removeu-se a lamínula com jatos de água destilada. As lâminas foram secas ao ar.

3.2.4 Extração de DNA genômico

O DNA total foi obtido utilizando-se amostras de músculo e/ou fígado e empregando a técnica de fenol-clorofórmio-álcool isoamílico (Sambrook et al. 1989). Para isso, as amostras de tecidos foram colocadas em tubos eppendorf juntamente com a solução de lise (composta por 300 µL de TNE 1X; 30 µL de Tris-HCl 1M pH = 8,0; 20 µL de SDS 10% e 25 µL de Proteinase K/ 20mg/mL) recém preparada. Os tubos foram submetidos à brusca agitação (*vórtex*) por 15 segundos cada.

Após estes procedimentos, os tubos foram mantidos em estufa a 37ºC *overnight*, em seguida receberam 8 µL de solução aquosa de RNase na

concentração de 10 mg/mL e permaneceram em estufa na mesma temperatura por mais 1 hora, sendo agitados a cada 30 minutos. Transcorrido esse tempo, foram adicionados aos tubos com os tecidos completamente digeridos, 400 µL de uma mistura de fenol: clorofórmio: álcool isoamílico (25: 24: 1), utilizando-se a capela.

Mais uma vez o material foi submetido a uma brusca agitação por 30 segundos e, em seguida, centrifugado a 14.000 rpm por 10 minutos. Os tubos foram retirados da centrífuga com cuidado para não misturar as fases e o sobrenadante (fase alcoólica contendo DNA) foi transferido para um novo tubo devidamente identificado. Então, 60 µL de acetato de sódio 3M (pH= 5,3) foram adicionados e o tubo foi invertido lentamente para a completa mistura do sal com a solução.

Posteriormente, mais 600 µL de etanol absoluto gelado foram acrescentados aos tubos, os quais foram invertidos como no passo anterior. Os tubos foram mantidos no freezer por 30 minutos e centrifugados a 14.000 rpm por 30 minutos para a completa retirada da mistura etanol e acetato de sódio, em seguida o sobrenadante foi descartado, certificando-se que o precipitado de DNA continuou aderido à parede do tubo.

Os tubos receberam 150 µL de solução de etanol 70% a temperatura ambiente e foram novamente levados à centrífuga para agitação a 14.000 rpm por 30 minutos. O sobrenadante foi descartado cuidadosamente como na etapa anterior para que o precipitado de DNA continuasse aderido à parede do tubo. Esse passo foi repetido mais uma vez para garantir a retirada de todo o sal adicionado ao DNA.

A partir daí, os tubos foram colocados abertos sobre folhas de papel absorventes em estufa a 37ºC por 30 minutos para secagem do DNA. Após esse período, o DNA foi ressuspendido com 100 µL de água ultrapura autoclavada, por meio de agitação delicada do tubo para desprendimento total do DNA aderido à parede do tudo. O material foi levado para a estufa a 37ºC por 30 minutos e, posteriormente, transferido para a geladeira (4ºC) *overnight*. O armazenamento do mesmo foi feito em freezer (-20ºC) para conservação por longo prazo.

A integridade do DNA extraído foi aferida baseando-se no padrão eletroforético gerado após corrida em gel de agarose na concentração de 1%, corados com 2 µL SYBR Safe (10.000X) (Invitrogen®), em 1 µL tampão de corrida *blue juice* (10X) e visualizados em um transiluminador de luz ultravioleta.

3.2.5 Digestão enzimática para busca de seqüências repetitivas no genoma de espécies de Anostomidae

Foram realizados diversos ensaios para obtenção de fragmentos de restrição no genoma de indivíduos da família Anostomidae que pudessem ser utilizados como marcadores para as espécies analisadas. Foram seguidas as particularidades de cada enzima, de acordo com as especificações do fabricante, contudo o protocolo básico consistiu do uso de 30 µL de DNA (100 ng/µL), 57 µL de água ultrapura autoclavada, 10 µL tampão de enzima e 3 µL de enzima.

Os tubos foram incubados a 37ºC *overnight* e em seguida o DNA foi precipitado em 2 µL NaCl 5M e 204 µL de etanol 100% gelado. Esta preparação foi levada ao freezer -80ºC por duas horas. A mistura foi então centrifugada a 14.000 rpm por 10 minutos. O sobrenadante foi descartado e o material novamente centrifugado em 300 µL de etanol 70% gelado. O material foi seco em estufa e posteriormente ressuspendido em 10 µL de água autoclavada.

O material foi corrido em gel de agarose a 1,5% corado com SYBR safe (10.000X) (Invitrogen®), em 1µL tampão de corrida *blue juice* (10X) e visualizado em transiluminador de luz ultravioleta. As bandas visíveis foram cortadas e purificadas utilizando o kit Band purification (GE Healthcare) seguindo as especificações do fabricante. Os produtos foram estocados em freezer a -20ºC para posterior clonagem.

3.2.6 Clonagem dos produtos de restrição

Os produtos de restrição foram clonados com o *Kit pMos Blue* (GE Healthecare)

3.2.6.1 Ligação com vetor

Para cada fragmento a ser clonado foi preparado uma reação pK que consistia em colocar em um microtubo 2,5 µL de água ultrapura mais 1 µL do tampão pK 10x, 0,5 µL de DTT 100mM e 5 µL do produto a ser clonado. Essa

preparação foi incubada a 22ºC por 40 minutos e, transcorrido esse tempo, foi aquecida a 75ºC por 10 minutos e em seguida, colocada no gelo por 2 minutos.

Após a preparação da reação pK, foi preparada a ligação, a qual era constituída de 10 μL do produto obtido da reação de pK, 1 μL do vetor pBluescript e 1 μL de DNA ligase. A mistura foi incubada por um intervalo de 2 a 16 horas a 22ºC.

3.2.6.2 Transformação

Nessa etapa foram pipetados em um microtubo 20 μL de *Escherichia coli* linhagem dH5α competentes (Invitrogen) mais 4 μL do produto obtido na ligação. Essa mistura foi agitada suavemente. As células foram incubadas no gelo por 30 minutos e submetidas a um choque térmico por exatos 40 segundos no banho-maria a 42ºC e colocadas no gelo por 2 minutos.

A partir deste momento, foram adicionados 80 μL de meio SOC em cada tubo. Este foi levado ao *shaker* a 225 rpm por uma hora. Neste intervalo placas de cultivo foram preparadas adicionando-se ampicilina (100 mg/mL) à 30 μL de X-Gal. As células transformadas foram plaqueadas no meio seletivo e incubadas a 37ºC por 12-16h.

3.2.6.3 Manuseio dos clones recombinantes

As colônias de bactérias que receberam o inserto (colônias brancas) foram identificadas e selecionadas para uma nova cultura. Para isso, o meio L.B. líquido foi preparado (2 g Peptona, 2 g NaCl, 1 g Extrato de levedura, 200 mL de água, pH= 7,5) e distribuídos em alíquotas de 5 mL em tubos autoclavados e identificados e 2 μL de ampicilina (100 mg/mL) por mililitro de meio.

As colônias recombinantes foram retiradas da placa com ponteiras estéreis e inoculadas no novo meio e incubadas a 37ºC *overnight* sob agitação de 220 rpm. Após incubação, foram adicionados 300 μL de glicerol 100% em tubos criogênicos e 500 μL do meio cultivado. Os tubos foram identificados e conservados em *freezer* - 80ºC.

3.2.7 Ensaios para amplificação de seqüências repetitivas específicas a partir da Reação em Cadeia da Polimerase – PCR

Foram realizados diversos ensaios de PCR com o intuito de obter material para mapeamento dos elementos repetitivos nas espécies de Anostomidae, objeto deste estudo. Para tanto foram utilizados conjuntos de *primers* que flanqueiam regiões específicas dos vetores de clonagem, bem como *primers* de rDNA 18S.

A tabela 1 traz as particularidades dos conjunto de *primers,* bem como os protocolos iniciais de amplificação.

Tabela 2. *Primers* e protocolo inicial das reações para obtenção de seqüências especificas no genoma de diferentes espécies de Anostomidae.

Nome	*Primers*	Volume inicial	Programa inicial
18S rDNA	F- GTAGTCATATGCTTGTCTC R - GGCTGCTGGCACCAGACTTGC	-6,25 µL mix PCR*; -5,25 µL água; -0,5 µL *Primer* F; -0,5 µL *Primer* R; -0,5 µL DNA	1- 95℃ → 2' 2- 94℃→ 45" 3- 52℃→ 45" 4- 72℃→ 1' 30" (passos 2-4: 35X) 5- 72℃→ 5"
M13	F- GTA AAA CGA CGG CCA G R- CAG GAA ACA GCT ATG AC	-6,25 µL mix PCR; -5,25 µL água; -0,5 µL *Primer* F; -0,5 µL *Primer* R; -0,5 µL DNA	1- 95℃ → 2' 2- 94℃→ 45" 3- 50℃→ 45" 4- 72℃→ 1' 30" (passos 2-4: 35X) 5- 72℃→ 5"

*Na solução de MIX PCR Qiagen estão contidas os reagentes Taq DNA polimerase 5U, tampão da enzima 10X, $MgCl_2$ 1,5mM e dNTP (deoxinucleotídeo trifosfato) 200µM.

Os resultados das reações em cadeia da polimerase foram todos visualizados em gel de agarose a 1,5% corados com SYBR safe (10.000X) (Invitrogen®), em 1 µL tampão de corrida *blue juice* (10X), visualizados em transiluminador de luz ultravioleta. Os produtos foram estocados em freezer a -20ºC.

3.2.8 Seqüenciamentos das amostras

Para o seqüenciamento, foram utilizadas as amostras que possuíam por volta de 50 ng de DNA/µL. Estas amostras passaram pelo tratamento com a enzima ExoSAP-IT (GE Healthcare) que consistia em adicionar em um microtubo 10 µL de produto de PCR, 2 µL da enzima ExoSAP e 2 µL de água ultrapura autoclavada. Os tubos foram então levados ao termociclador em um ciclo de 1 hora a 37ºC e 15 minutos a 80ºC. As amostras foram mantidas no freezer (-20ºC) e o DNA purificado foi enviado para seqüenciamento na empresa MacroGen Inc. em Seul- Coréia.

3.2.9 Mapeamento cromossômico de seqüências específicas em espécies de Anostomidae

A técnica de hibridação *in situ* fluorescente (FISH) foi conduzida utilizando sondas de seqüências repetitivas já isoladas e cromossomos totais obtidos através de microdissecção. As sondas foram marcadas por PCR e/ou *nick translation*, utilizando os compostos biotina-14-dATP (Invitrogen) e/ou digoxigenina-11-dUTP (Roche). Os experimentos foram realizados segundo a metodologia de Pinkel et al. (1986) com algumas modificações nos tempos de desnaturação e lavagens pós hibridação. As sondas de cromossomos inteiros, obtidos por meio de microdissecção, foram fornecidas pela Profa. Dra. Patrícia Pasquali Parise Maltempi e encontram-se armazenadas, em *freezer* (-20° C), no Laboratório de Citogenética da Universidade Estadual Paulista "Júlio de Mesquita Filho" UNESP Campus de Rio Claro-SP. Excepcionalmente nos experimentos nos quais foram utilizadas sondas de cromossomos inteiros, os protocolos de hibridação foram feitos de acordo com Rens et al. 1999 e 2006.

3.2.9.1. Tratamento das lâminas para FISH

As preparações cromossômicas foram colocadas sobre lâminas previamente limpas, as quais foram mantidas em estufa a 37ºC por pelo menos 4 horas para total fixação do material. Para dar procedimento às técnicas de hibridação *in situ* fluorescente as lâminas foram tratadas com solução de pepsina e RNAse com o intuito de proporcionar um meio adequado para a hibridação.

Para o tratamento com pepsina incubou-se as lâminas por 10 minutos em uma solução contendo 99 mL de água destilada, 1 mL de HCl 1M e 50 µl de pepsina 10%. Em seguida, as lâminas foram lavadas duas vezes, por 2 minutos cada lavagem, em 2xSSC e incubadas em 100 µl de RNAse (5 µl de solução aquosa de RNAse 10 mg/mL mais 995 mL de 2xSSC) a 37ºC por 1 hora em câmara úmida. Após incubação, outras três lavagens, de 5 minutos cada, em 2xSSC foram efetuadas. Um último ciclo de lavagem foi realizado em Triton por 5 minutos, uma única vez. As lâminas foram desidratadas em série etílica 70, 90 e 100% por 5 minutos.

3.2.9.2 Desnaturação dos cromossomos

As lâminas previamente tratadas foram submetidas ao processo de desnaturação dos cromossomos que consistiu em adição de formamida 70% a 70ºC (70 mL de formamida PA em 30mL de 2xSSC). As lâminas foram mantidas nessa solução por 1 minuto e 45 segundos, variando de acordo com a qualidade das preparações citogenéticas. Em seguida, o material foi desidratado em série etílica (gelada a -20ºC) 70, 90 e 100% por cinco minutos cada concentração. Após esse procedimento o material foi mantido em temperatura ambiente para secagem. Enquanto as lâminas eram submetidas à serie etílica, as sondas foram desnaturadas.

3.2.9.3 Preparação das sondas

Os procedimentos de hibridação foram conduzidos tanto com sondas únicas quanto com múltiplas sondas. Para a preparação das sondas únicas, 2 µL da sonda marcada foram misturados 10 µL do tampão de hibridação (formamida 50% em 2× SSC e sulfato dextrano 10%) por lâmina em um tubo. A solução de hibridação foi desnaturada em termociclador a 95ºC por 10 minutos. Ao final da desnaturação, os tubos foram colocados imediatamente em gelo enquanto as lâminas secavam. A solução de hibridação de cada tubo foi colocada sobre a lâmina com os cromossomos desnaturados, coberta com lamínula e mantida *overnight* a 37ºC em câmara úmida.

Já a solução de hibridação com duas sondas foi preparada adicionando-se 8 µL da sonda A e 8 µL da sonda B em tubo pequeno para PCR. Na sequência, as sondas foram precipitadas em 50 µL de etanol 100% e 5 µL de acetato de sódio 3M por 1 hora em freezer a -80ºC.

Em seguida, a mistura foi centrifugada por 14 minutos a 14.000 rpm com o descarte do sobrenadante e secagem do precipitado em estufa a 37ºC. Depois de seco foi adicionado ao precipitado 10 µL de tampão de hibridação, o qual foi levado ao termociclador a 95ºC por 10 minutos para desnaturação. As lâminas foram montadas conforme procedimento já descrito para sondas únicas.

3.2.9.4 Lavagens e detecção

Após o tempo de hibridação, as lâminas montadas com a solução de hibridação e mantidas úmidas à temperatura constante foram submetidas aos processos de lavagem e detecção dos sinais de hibridação utilizando anticorpos e haptenos específicos. Para isso as lamínulas foram retiradas com cuidado e o material lavado duas vezes, cinco minutos cada, com solução de estringência (50 mL de formamida PA em 50 mL de 2xSSC) a 45ºC. Em seguida as lâminas foram lavadas duas vezes com 1xSSC a 45ºC por também cinco minutos cada. Outra lavagem de cinco minutos foi realizada com 4xT (20 mL de 20xSSC, 5 µL de Triton e 75 mL de água) em temperatura ambiente.

Após as lavagens, 30 µL de tampão de bloqueio (1mL de 2xSSC, 10 µL de Triton e 0,05 g de leite em pó desnatado) foram adicionados sobre cada lâmina que foram cobertas com lamínula ou filme plastico por 5 minutos a temperatura ambiente.

Para detecção de sondas marcadas com digoxigenina foram adicionados às lâminas 30 µL do anticorpo anti digoxigenina rodamina (4 µL de anti digoxigenina rodamina e 26 µL de tampão de bloqueio) e encubadas durante 30 minutos em câmara escura a 37ºC. Duas lavagens com 4xT a 45ºC, cinco minutos cada, foram realizadas para retirada do excesso de anti digoxigenina, seguidas de um rápido enxague com 2xSSC temperatura ambiente.

Para a montagem das lâminas, aproximadamente 15 µL de solução de DAPI+antifading foram adicionados sobre cada lâmina, as quais foram cobertas com lamínula e guardadas, no escuro, em geladeira.

Para detecção e amplificação do sinal de sondas marcadas com biotina cada lâmina contendo 6 µL de Avidina-FITC (1:100) e 24 µL de tampão de bloqueio foi mantida em estufa a 37ºC por 30 minutos. As lâminas foram lavadas por 2 minutos com 2xSSC a 45ºC sob agitação (*shaker*). Duas outras lavagens de 2 minutos cada com 4xT a 45ºC também sob agitação (*shaker*) foram realizadas.

Após esses procedimentos, cada lâmina foi incubada com 2 µL de Anti-avidina biotinilada e 38µL de tampão de bloqueio por 30 minutos a 37ºC. O processo de lavagem nesta etapa foi o mesmo descrito acima.

Mais um ciclo de amplificação do sinal com Avidina-FITC foi realizado seguido pelos mesmos procedimentos de lavagem. Ao final, as lâminas foram também montadas com 15 µL de solução de DAPI+antifading, cobertas com lamínula e guardadas, no escuro, em geladeira.

Para detecção dos sinais de mais de uma sonda, os procedimentos de lavagens e detecção foram os mesmos contudo, detectou-se primeiro os sinais marcados com digoxigenina e depois as sondas marcadas com biotina.

3.2.10 Hibridação em membrana

Clones das seqüências repetitivas foram utilizados para a análise da organização genômica destas seqüências em diferentes espécies de Anostomidae. Para tanto, o DNA genômico foi digerido com diferentes enzimas de restrição seguindo as especificações de cada fabricante. O DNA digerido foi submetido a técnica de eletroforese em gel de agarose (1,5%) por aproximadamente 6 horas. Em seguida o gel foi tratado e a transferência do DNA para a membrana foi realizada segundo Southern (1975) com modificações. Para a hibridação foi utilizado o Kit ECL *direct nucleic acid labelling and detection system* (Amersham Biosciences) seguindo as especificações do fabricante.

4. RESULTADOS

Os resultados obtidos com o desenvolvimento desta tese permitiram a elaboração de quatro artigos, dos quais três já foram submetidos a revistas especializadas e um está em fase de finalização. Os mesmos artigos estão formatados conforme as normas das respectivas revistas.

Artigo 1- Silva EL, Busso AF, Parise-Maltempi. PP (2012) Characterization and genome organization of a repetitive element associated with the nucleolus organizer region in *Leporinus elongatus* (Anostomidae: Characiformes). Accepted for publication in Cytogenetic and Genome Research.

Artigo 2- Silva EL, Borba RS, Parise-Maltempi. PP (2012) Chromosome mapping of repetitive sequences in Anostomidae species: implications for genomic and sex chromosome evolution. Accepted for publication in Molecular Cytogenetics.

Artigo 3- Silva EL, Rens W, Lowel F, Trifonov V, Ferguson-Smith M, Parise-Maltempi (2012) Comparative analysis of the sex chromosomes of the *Leporinus* fish (Teleostei, Characiformes) through cross painting of whole chromosome probes. Still in preparation.

Artigo 4- Silva EL, Borba RS, Parise-Maltempi. PP (2012) Comparative aspects of chromosome composition in Anostomidae species: a review of cytogenetic markers. Submitted to Reviews in fish biology and fisheries

ARTIGO 1

Characterization and genome organization of a repetitive element associated with the nucleolus organizer region in *Leporinus elongatus* (Anostomidae: Characiformes)

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Abstract

Chromosome mapping and studies of the genomic organization of repetitive DNA sequences provide valuable insights that enhance our evolutionary and structural understanding of these sequences, as well as identifying chromosomal rearrangements and sex determination. This study investigated the occurrence and organization of repetitive DNA sequences in *Leporinus elongatus* using restriction enzyme digestion and the mapping of sequences by chromosomal fluorescence in situ hybridisation (FISH). A 378-bp fragment with a 54.2% GC content was isolated after digestion with the *Sma*I restriction enzyme. BLASTN search found no similarity with previously described sequences, so this repetitive sequence was named Le*Sma*I. FISH experiments were conducted using *L. elongatus* and other Anostomidae species, i.e. *L. macrocephalus, L. obtusidens, L. striatus, L. lacustris, L. friderici, Schizodon borellii, S. isognathus,* and *Abramites hypselonotus* which detected signals that were unique to male and female *L. elongatus* individuals. Double-FISH using Le*Sma*I and 18S rDNA showed that Le*Sma*I was located in a nucleolus organizer region (NOR) in the male and female metaphases of *L. elongatus*. This report also discusses the role of repetitive DNA associated with NORs in the diversification of Anostomidae species karyotypes.

Key words FISH, Le*Sma*I, Neotropical fish, NOR, Repetitive DNA

Introduction

An interesting feature of the eukaryotic genome is the presence of large tracts of repetitive DNA sequences containing noncoding regions, including satellite, minisatellite and microsatellite DNA, as well as transposable elements [Sumner, 2003; Jurka et al., 2005; Plohl et al., 2008; Mazzuchelli and Martins, 2009] and high copy number coding multigene families such as ribosomal cistrons and histone genes [Long and Dawid, 1980; Maxson et al., 1983]. Tandem repeats, typically with monomer sizes ranging from 150 to 360 (bp), comprise most of the repetitive sequences found in the eukaryotic genome, which are arranged in arrays of thousands of adjacent monomers that can achieve megabase size in the genome [Goff et al., 2002].

Repetitive sequences are closely associated with heterochromatic regions in chromosomes and they were previously considered junk due to their unknown function in the genome [e.g. Lim and Simmons, 1994; Dimitri et al., 1997; Sumner, 2003]. However, some tandem repeats have been shown to have a defined functional role in the nucleus [Ceccarelli et al., 2010] as ribosomal cistrons [Long and Dawid, 1980; Heslop-Harrison, 1991], telomeric DNA [Zakian, 1995], centromeric DNA [Sumner, 2003; Rattner, 1991], and histone genes [Maxson et al., 1983]. An interesting feature of repetitive sequences is that some are highly conserved, whereas others evolve quickly with a high degree of divergence, so there are often few similarities among species [Hall et al., 2003].

Chromosome-specific satellite DNA can be a useful cytogenetic marker in teleost fish and it is sometimes used to understand phylogenetic relationships among different taxa, as well as for clarifying the cytotaxonomy of species complexes, to determine the likely origins of supernumerary chromosomes or characterizing sex chromosomes [see the review of Vicari et al., 2010]. The fish species *Leporinus elongatus* Valenciennes 1849 (Characiformes: Anostomidae) has been studied in terms of the occurrence and probable function of repetitive sequences in its genome [Nakayama et al., 1994; Parise-Maltempi et al., 2007; Marreta et al., 2012]. In this species, the sexual system is characterized by female heterogamety, where the W chromosome is larger than others, subtelocentric and almost totally heterochromatic. The repetitive DNA previously described in this species is distributed mainly in these

conspicuous sex chromosomes and appears to be related to the differentiation among homologous chromosomes [Parise-Maltempi et al., 2007]. The repetitive DNA known as Le*Spe*I is distributed differently in male and female individuals. In females, it is located in 3 submetacentric chromosomes, one of which corresponds to the W chromosome, whereas the other 2 medium-sized submetacentric chromosomes correspond to the nucleolus organizer region (NOR) carriers. The signals in the NOR chromosomes were observed in different locations: 2 signals in the long arm and one in the short arm of 1 of the homologs, and, in another homolog, only 1 cluster in the short arm. Due to this distribution pattern observed in *L. elongatu*s specimens the authors suggested a multiple $Z_1Z_1Z_2Z_2/Z_1W_1Z_2W_2$ sex chromosome system. In males, Le*Spe*I is restricted to the pericentromeric region of the Z.

Nakayama et al. [1994] also isolated 2 sex-specific DNA satellites from *L. elongatus*, which were cloned and used to investigate the structure and variability of the sex chromosomes in this species. The authors identified 3 atypical W chromosomes and proposed that they had undergone recombination with Z during female meiosis, thereby producing 3 new W chromosomes (W_1, W_2, and W_3) and 3 new Z chromosomes (Z_1, Z_2, and Z_3). These chromosomes were associated with normal male Z chromosomes during reproduction, resulting in 6 new genotypes (ZW_1, ZW_2, ZW_3, ZZ_1, ZZ_2, and ZZ_3).

The high heterochromatin content of *L. elongatus* chromosomes highlights the likely presence of a high number of repetitive sequences. Furthermore, the repetitive elements that are known to be linked to important genome regions such as sex chromosomes make the anostomids a useful model for studying the evolutionary dynamics of this interesting subset of the fish genome. The current study aimed to investigate the occurrence and organization of other repetitive DNA sequences in the *L. elongatus* genome using restriction enzyme digestion to inform a discussion of the role of repetitive DNA during the diversification of karyotypes in anostomid species.

Materials and Methods

Ten *L. elongatus* specimens (5 males and 5 females) were obtained from the Mogi-Guaçú River near the municipality of Pirassununga, São Paulo, Brazil, and used for the isolation of repetitive DNA. To verify the presence of repetitive elements

isolated from *L. elongatus* in other species of the Anostomidae family, additional studies were carried out with the species *L. macrocephalus* Garavello and Britiski 1988, *L. obtusidens*, *L. striatus* Kner 1858, *L. lacustris* Amaral Campos 1945, *L. friderici* Bloch 1794, *Schizodon borellii* Boulenger 1900, *S. isognathus* Kner 1858, and *Abramites hypselonotus* Günther 1868, which were collected in the Paraguay River basin, Mato Grosso State, Brazil.

Chromosomal preparations were obtained using kidney cells, as described by Foresti et al. [1981]. Genomic DNA was extracted from the liver and muscle according to standard protocols using proteinase K, followed by phenol/chloroform extraction [Sambrook et al., 1989]. Chromosomal nomenclature was based on the system of Levan et al. [1964] and the chromosomes were classified as metacentric submetacentric, subtelocentric, and acrocentric. Heterochromatin regions were visualized by C-banding [Sumner, 1972] and NORs were detected by silver nitrate (Ag-NORs) according to the procedure described by Howell and Black [1980].

Repeated DNA elements in *L. elongatus* were isolated by polymerase chain reaction (PCR) and restriction enzyme digestion of whole genomic DNA. PCR experiments were performed using the 18S rDNA-specific primer sets NS1 (5-GTAGTCATATGCTTGTCTC-3) and NS2 (5-GGCTGCTGGCACCAGACTTGC-3) [White et al., 1990].

Genomic DNA (3 μg) was digested using a set of different endonucleases. The digested products were submitted to electrophoresis on a 2% agarose gel and stained with ethidium bromide. Bands were excised from the gel, purified using GFX PCR DNA and a Gel Band Purification Kit (GE Healthcare), and cloned with a pMOS Kit (GE Healthcare), according to the manufacturer's instructions. Clones were sequenced with an ABI Prism 3130 (Applied Biosystems) automatic sequencer. Sequences were aligned using the Bioedit program and a BLASTN search was performed in GenBank to compare the cloned fragment with other repetitive sequences found in the database.

The genomic organization of isolated repetitive fragments was determined by Southern blot hybridisation using 10-ng aliquots of genomic DNA from males and females of all the studied species. Genomic DNA was digested using the restriction enzyme SmaI separated by electrophoresis on a 1% agarose gel, and the DNA

fragments were transferred onto a Hybond N+ nylon membrane by capillarity (GE Healthcare). Clones containing the repetitive fragment isolated from *L. elongatus* were used as probes and hybridized with an ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare), according to the manufacturer's instructions.

Fluorescence in situ hybridisation (FISH) analyses were performed according to the method of Pinkel et al. [1986] with some modifications, using 18S rDNA and the cloned sequence fragments as probes. The 18S rDNA was labeled with digoxigenin in a second cycle of reamplification using the PCR product as the template. For the chromosomal mapping of restriction fragments, the probes were labeled by nick translation with biotin-14-dATP (Invitrogen) according to the manufacturer's instructions.

Slides prepared with chromosomes were pretreated for 1 h with RNAse solution (5 µl of RNAse at 10 mg/ml in H2O and 995 µl of 2× SSC), following treatment with pepsin (50 µl of 10% pepsin in H2O, 1 ml of 1 M HCl and 99 ml of H2O). For each treatment, the slides were washed with 20 ml of 20× SSC, 50 µl of Triton, and 80 ml of H2O (at a final concentration of 0.5× SSC and 0.2% of Triton) at room temperature. The slides were submitted to an alcoholic dehydration series (70%, 90% and 100%) and the chromosomal DNA was denatured in formamide solution (70% in 2× SSC) for 2.5 min at 72°C, followed by dehydration with an alcoholic series (70%, 90% and 100%).

In the double-FISH experiment, 3 µl of each probe per slide was precipitated at –80°C for 1 h in a solution of 3 M sodium acetate solution and twice the volume of cold 100% ethanol. The probes were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was removed and the probes were centrifuged once more with 50 µl of 70% ethanol, dried, and then dissolved into 10 µl of hybridisation buffer (50% formamide in 2× SSC and 10% dextran sulphate). The probes were denatured at 95°C for 10 min and applied on the slide. The slides were covered with coverslips and incubated overnight at 37°C. Washing was performed at 45°C in a water bath. The slides were washed twice with the stringency solution (50% formamide and 2× SSC), followed by 2 washes with 1× SSC.

Hybridisation signals were detected using appropriate antibody sets composed of antidigoxigenin-rhodamine to detect the signals from the digoxigenin haptens and

anti-avidin, which was followed by the application of avidin-FITC to enhance the signals from biotin-labeled probes.

Chromosomes were counterstained with DAPI or propidium iodide, mounted with antifade solution, and observed using an Olympus BX51 microscope coupled to an Olympus digital camera (model D71). Chromosome images were captured using the DP Controller program.

Results

All individuals had the characteristic diploid chromosome number pattern observed in the Anostomidae (2n = 54 chromosomes). The heteromorphic sex chromosome system was present only in *L. elongatus, L. macrocephalus* and *L. obtusidens.*

Digestion of DNA from *L. elongatus* using the *Sma*I restriction enzyme visualized only 1 band of approximately 400 bp, which was referred to as Le*Sma*I. The fragment was successfully cloned; and the nucleotide content of 2 clones was 378 bp with 100% similarity and 54.2% GC nucleotide pairs (fig. 1). The BLASTN search for Le*Sma*I sequences did not find any significant similarity with the sequences available in GenBank. PCR with 18S rDNA primers successfully amplified the sequence and the fragment contained approximately 750 bp.

Enzyme digestion followed by Southern blotting hybridisation showed that Le*Sma*I was only present in the *L. elongatus* genome (fig. 2). In this species, the tandem repeat organization was visualized as a ladder pattern of bands, ranging from approximately 300 to over 3,000 bp in both sexes.

The chromosomal distributions identified by FISH experiments showed that the repetitive DNA Le*Sma*I and 18S rDNA sites were colocalized in *L. elongatus* (fig. 3), with positive hybridisation signals at the terminal region of the long arms of the second pair (Z_2W_2/Z_2Z_2) in females and males (fig. 3a, b). Silver nitrate impregnation confirmed the second pair as the Ag-NOR carrier, while C-band analysis indicated a high heterochromatin content in this chromosomal region (fig. 3f, g, respectively), in addition to another positioned at the terminal region of the p arm, which indicated the presence of at least 2 distinct heterochromatin blocks in this chromosome pair. All other species analyzed had no Le*Sma*I signals in the FISH experiments, even after

low stringency washing, whereas the simple cistrons of 18S rDNA signals were present in all the species analyzed in the experiment, but at different positions in the metacentric/submetacentric chromosomes (fig. 4).

Discussion

Previously analyzed Anostomidae species have a stable diploid number of 54 chromosomes and only 1 chromosome pair carrying the NOR [Galetti Jr et al., 1981, 1984; Molina and Galetti Jr, 2007; this study]. Despite this apparent stability, there are observable differences among karyotypes of these species, which have been associated with particular patterns of heterochromatin and NOR distribution [Silva EL, pers. commun.]. These variations in the heterochromatin distribution may have contributed to meiotic isolation between Z and W chromosomes [Galetti Jr and Foresti, 1986], dislocations of NOR-associated sites [Galetti Jr et al., 1991a] and chromosome reorganization due to quantitative and qualitative changes in heterochromatic segments [Galetti Jr et al., 1991b].

The presence of 2 different heterochromatic blocks in the second chromosome pair (Z_2W_2/Z_2Z_2) supported the diversity of heterochromatin distribution patterns in *L. elongatus*, which were detected by Koehler et al. [1997] using classical cytogenetic techniques. However, it was previously suggested that this feature was attributable to a population polymorphism, whereas the current study shows that this feature was due to 2 different repetitive DNA elements, i.e. the Le*Sma*I repetitive DNA described in this study and the Le*Spe*I repetitive DNA described by Parise-Maltempi et al. [2007], which were seen in all analyzed individuals. The molecular approach used here facilitated the analysis of the relevant genome section of *L. elongatus* and confirmed the report of Koehler et al. [1997], showing that these sequences were organized in distinct chromosomal positions, while the specific nucleotide content of each suggested that they were not related (fig. 3h).

In contrast to Le*Sma*I, the repetitive element Le*Spe*I was found in *L. elongatus* [Parise-Maltempi et al., 2007; Marreta et al., 2012], *L. macrocephalus*, and *L. obtusidens* [Marreta et al., 2012]. Fluorescent signals were observed in the long arm of the W chromosome in *L. elongatus* and in one of the homologs of the NOR-bearing chromosome pair. No fluorescent signals were detected in the Z or other

chromosomes and they were restricted to the W chromosomes in *L. macrocephalus* and *L. obtusidens*.

The genomic organization of the Le*Sma*I sequence comprised monomers ranging from 300 bp to over 3,000 bp suggesting a repetitive pattern without any indication of sex-specificity. Although repetitive, this sequence was unique to the regions adjacent to the NOR in *L. elongatus*, which formed an important species-specific marker.

The 18S rDNA FISH signals were restricted to the terminal position of only 1 chromosome pair and they varied among the chromosomes of different species. An exception was *L. striatus*, where the NOR was located in the interstitial region of the short arm of a metacentric chromosome. In this species, the NOR is strongly C-band positive, indicating a high proportion of heterochromatin [Silva EL, pers. commun.].

The occupation of interstitial positions by NORs in Anostomidae has also been reported for *L. octofasciatus* and *L. taeniatus* [Galetti Jr et al., 1981, 1991b]. Rearrangements involving inversions that maintained the original diploid number were probably responsible for the different NOR pattern phenotypes observed. Such rearrangements are also mainly responsible for the NOR phenotype differentiation in species from the Heptapteridae family [Borba et al., 2012].

The results of many studies using conventional cytogenetic techniques have shown that heterochromatin-NOR associations appear to be a common feature of anostomid species. Galetti Jr et al. [1991b] observed a high heterochromatin content around the centromere and NOR sites in *Leporellus vittatus*. The same pattern was also reported for *Schizodon nasutus, S. altoparanae, S. intermedius, S. knerii*, and *S. vittatus* [Martins and Galetti Jr, 1997].

The detailed cytogenetic analysis of *Leporinus* species by Koehler et al. [1997] showed that the NOR region was euchromatic, while neighboring heterochromatin regions had a strong C-banding mark in these species. Mithramycin treatment of the chromosomes of these species showed that NOR-bearing chromosomes were highly fluorescent in GC-ribosomal gene loci and interstitial C-bands [Koehler et al., 1997].

Thus, heterochromatin appears to be associated with the NOR in anostomid species, which may contribute to dislocations of cistrons and may be responsible for the karyotype differentiation among *Leporinus* species such as *L. piau, L. striatus, L.*

taeniatus, and *L. amblyrhynchus* [Galetti Jr et al., 1991a]. This type of association has also been reported in salmonid species, where ribosomal spacer units [Abuín et al., 1996], C-banding positive heterochromatin associated with rDNA sites [Fujiwara et al., 1998] and rDNA genes are interspersed with highly repeated DNA complexes [Pendás et al., 1993].

The genes that code for rDNA are highly conserved among unrelated groups, whereas the nontranscribed spacer has a highly variable sequence composition and size even among related species [Eickbush and Eickbush, 2007]. This characteristic may explain the absence of sequences similar to Le*Sma*I in the available databases probably because Le*Sma*I was localized in a nontranscribed region of rDNA.

Previous studies of the relationship of repetitive DNA and sex chromosomes in the Anostomidae suggested the unstable condition of these sequences in this family [see Parise-Maltempi et al., 2007; Marreta et al., 2012]. The current sequence characterization and organization of Le*Sma*I supports previous qualitative studies of the nucleotide content of heterochromatin blocks in the *L. elongatus* genome, despite the apparent chromosome macrostructure stability. Thus, the amount of diversified heterochromatic content observed and the peculiar species and sex-specific patterns may have been important for the diversification of the anostomids.

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Figure Legends

Fig. 1. Sequence alignment of Le*Sma*I clones. Dots indicate similar sequences.

Fig. 2. Southern Blot experiment of digested DNA with SmaI restriction enzyme using the Le*Sma*I sequence as probe in female (F) and male (M) of *Leporinus elongatus* individuals. In the experiments using genomic DNA of other Anostomidae species were not found hybridisation signals (data not shown).

Fig. 3. Fluorescence in situ hybridisation (FISH) using Le*Sma*I probe in individuals of *Leporinus elongatus*. A Le*Sma*I probe in female chromosome preparation. B Le*Sma*I in male chromosome preparation. C 18S rDNA probe. D Le*Sma*I probe. E 18S rDNA and Le*Sma*I probes combined. Chromosome pair 2 after F C-banding and G Ag-NOR techniques. E Idiogram showing the combination of 18S rDNA, Le*Sma*I and Le*Spe*I [Parise-Maltempi et al., 2007] repetitive sequences.

Fig. 4. Fluorescence in situ hybridisation (FISH) with 18S rDNA and Le*Sma*I probes in species of the Anostomidae family. A *Leporinus macrocephalus*. B *L. obtusidens*. C *L. friderici*. D *L. striatus*. E *L. lacustris*. F *Schizodon borellii*. G *S. isognathus*. H *Abramites hypselonotus*. Bar = 10µm.

Figure 1

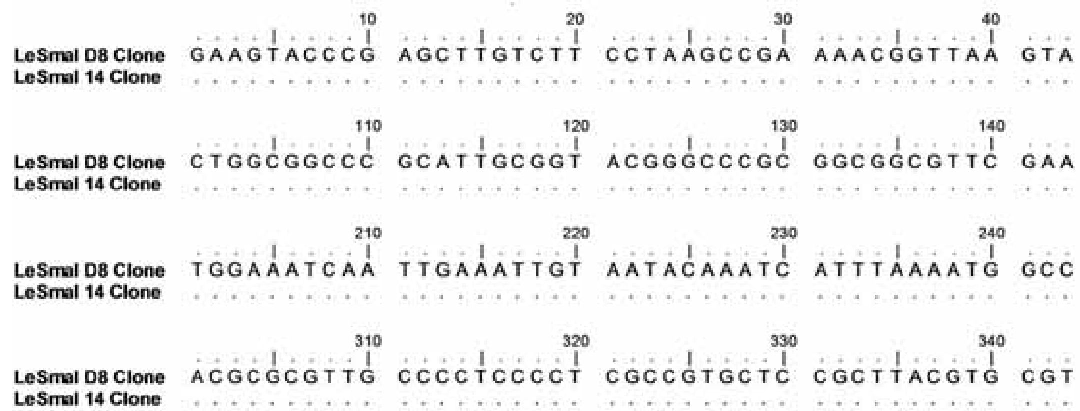


Figure 2

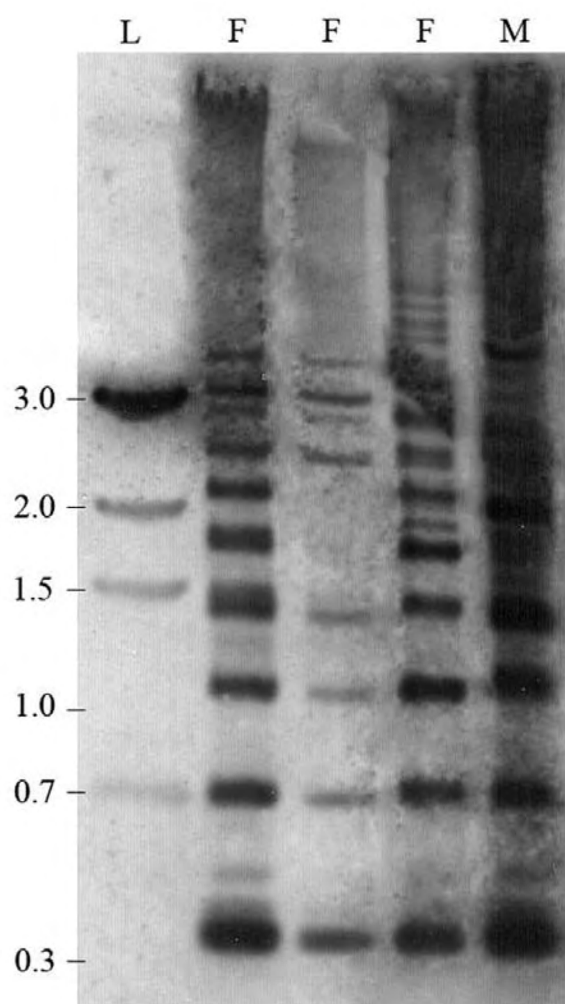


Figure 3

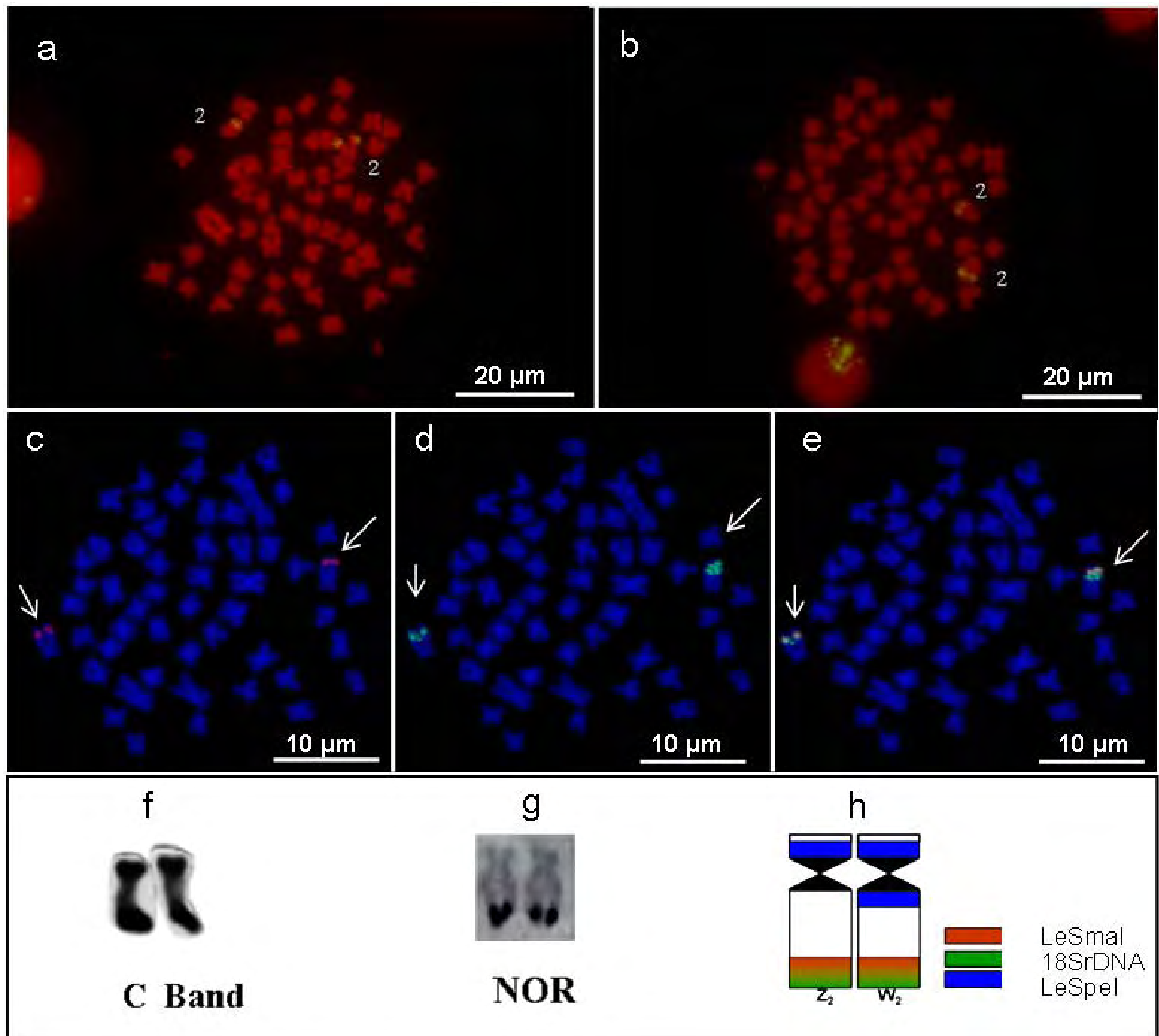
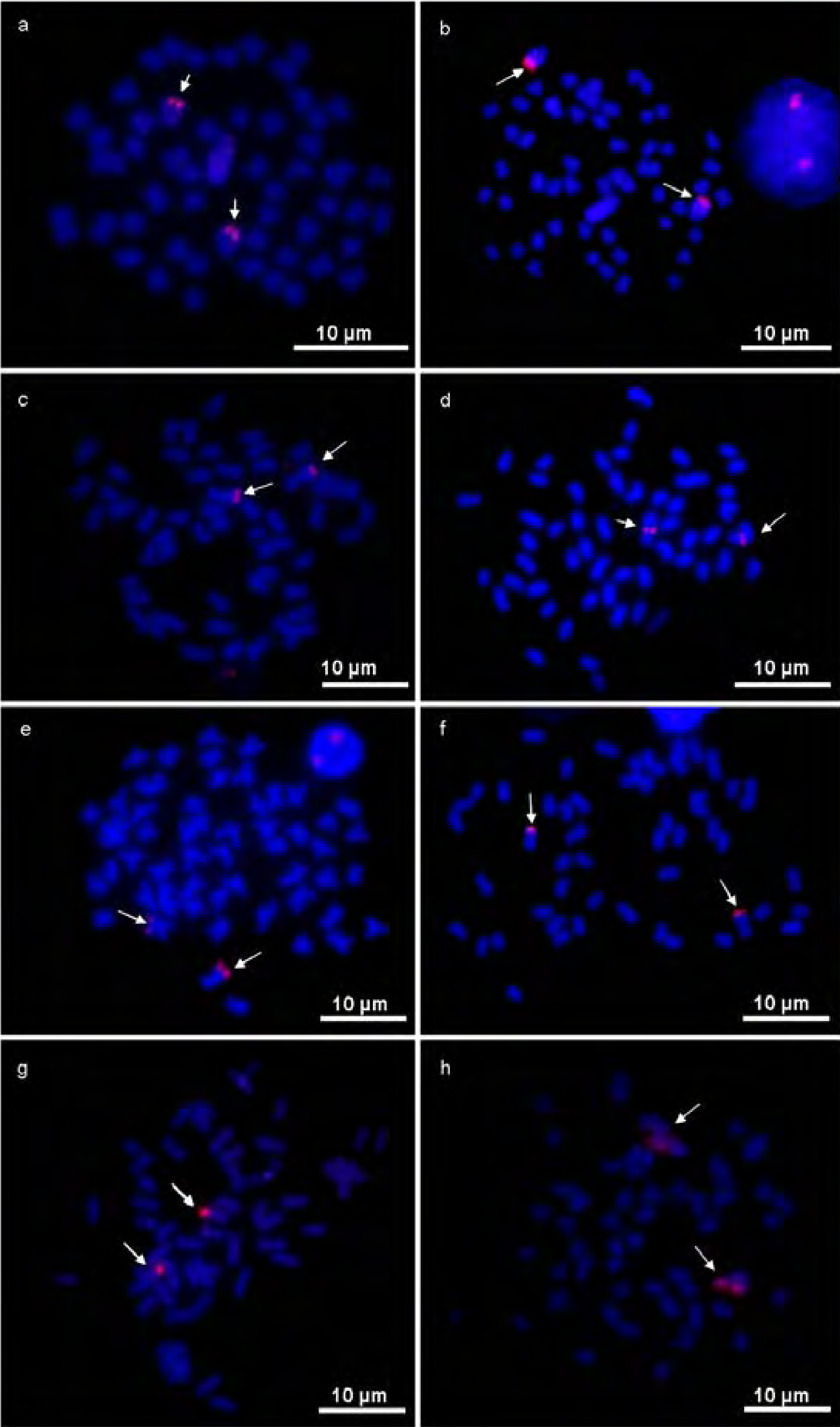


Figure 4



ARTIGO 2

Chromosome mapping of repetitive sequences in Anostomidae species: implications for genomic and sex chromosome evolution

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ABSTRACT

Background

The Anostomidae family is an interesting model system for the study of the influence of repetitive elements on genome composition, mainly due the presence of numerous heterochromatic segments and a peculiar system of female heterogamety restricted to a few species that comprise the *Leporinus* genus. The aim of this study was to isolate and identify important new repetitive DNA elements in Anostomidae through restriction enzyme digestion, followed by cloning, characterisation and chromosome mapping of this fragment. To identify repetitive elements in other *Leporinus* species and expand on studies of repetitive elements in Anostomidae, hybridisation experiments were also performed using probes of Le*Spe*I repetitive elements that were previously described.

Results

The 628-base pair (bp) Le*Spe*II fragment was hybridised to metaphase cells of *L. elongatus* individuals as well as those of *L. macrocephalus*, *L. obtusidens*, *L. striatus*, *L. lacustris*, *L. friderici*, *Schizodon borellii* and *S. isognathus*. In *L. elongatus*, both male and female cells contained small clusters of Le*Spe*II dispersed throughout all chromosomes, with enrichment near most of the terminal portions. In the sex chromosomes of *L. elongatus* (Z_1,Z_2/W_1W_2), however, this repeated element was absent. In the remaining species, a dispersed pattern of hybridisation was observed in all chromosomes without regard to sex. The repetitive element Le*Spe*I showed positive hybridisations signals only in *L. elongatus*, *L. macrocephalus* and *L. obtusidens*, i.e., species with differentiated sex chromosomes. In the remaining species, the Le*Spe*I element did not show hybridisation signals.

Conclusions

Results are discussed in terms of the effects of repetitive sequences on the differentiation of the Anostomidae genome with regards to sex chromosome evolution. Le*Spe*II showed hybridisation patterns typical of LINE elements. The differential distribution of this element may be linked to sex chromosome differentiation in *L. elongatus* species. The relationship between sex chromosome specificity and the Le*Spe*I element is confirmed.

Key words

Chromosomes, FISH, heterochromatin, Neotropical fish, sex chromosomes

BACKGROUND

Studies of Neotropical fishes indicate that almost none contain heteromorphic sex chromosomes. However, simple and multiple systems of heterogamety have been identified in various groups [1-3]. An interesting feature in the distribution of these systems is that for both male and female heterogamety, multiple and simple systems, as well the sporadic occurrence of heterogamety, can be found in related species and in different populations of the same species [1]. This diversity in the sex chromosome structure of fishes has been attributed in part to the dynamics of repetitive DNA [4-7].

In the eukaryotic genome, there are two classes of repetitive elements: sequences organised in tandem repeats such as satellites, minisatellites and microsatellites, and sequences dispersed in the genome as transposons or retrotransposons [8]. In eukaryotes, protein-coding sequences can constitute as little as 2–10% of the genome, with the remainder of the genome comprising introns, intragenic regions and a variety of repetitive sequences [9, 10].

The Anostomidae family is an interesting model system for the study of repetitive elements that influence the composition of the genome, particularly *Leporinus* species. This group is particularly attractive due to the presence of the peculiar sex chromosome system ZZ and ZW in some species. Some studies focusing on the isolation, characterisation and correlation of these repetitive elements with the presence and evolution of sex chromosomes have highlighted the great potential of studying this part of the genome in these Anostomidae species [4, 11-13].

The repetitive element Le*Spe*I, isolated from *L. elongatus* species [4], shows a sex-specific pattern that highlights the relationship of this element with the process of sex chromosome differentiation in *L. elongatus*, as well the congeneric species *L. macrocephalus* and *L. obtusidens* [12]. This sequence was not found in *L. friderici* (which lack differentiated sex chromosomes), reinforcing the notion that this element is linked to sex chromosome differentiation [12]. In addition, Le*Sma*I, another satellite DNA element isolated from *L. elongatus*, displays the species-specific characteristic

of localisation near the nucleolar organiser region in both males and females [13]. Interestingly, *L. elongatus* NOR-bearing chromosomes may comprise a multiple sex chromosome system (Z_2 and W_2).

Indeed, the participation of repetitive sequences in sex chromosome differentiation in Anostomidae is certain; however, the role of these sequences in this complex process is still unknown. In this study, we identified a new repetitive element from *L. elongatus*, Le*Spe*II. In addition, we investigated the localisation of the repetitive element Le*Spe*I in Anostomidae species to help elucidate the role of repetitive sequences in the diversification of the Anostomidae genome, particularly sex chromosome differentiation.

RESULTS

Chromosome number of anostomids and analysis of a repetitive sequence

Cytogenetic analysis of various anostomids revealed a diploid chromosome number of 54, with both metacentric- and submetacentric-type chromosomes present. The cloned fragments contained 628 bp, with high AT content (64.9%), with perfect sequence alignment among the clones (Figure 1). A BLASTN search did not reveal significant similarities between this sequence and previously identified sequences. This newly identified sequence was designated Le*Spe*II.

Genomic hybridisation of Le*Spe*II

The probe was labelled and hybridised to the digested genomic DNA of male and female individuals of *L. elongatus*, revealing many restriction fragments of various sizes, which indicates that Le*Spe*II is predominately dispersed and not tandemly arrayed (Figure 2).

Chromosomal distribution of repetitive DNA

The chromosomal distribution of the Le*Spe*II element was determined by Fluorescent *in situ* hybridisation (FISH) analysis using metaphase spreads of male and female *L. elongatus* individuals. Small clusters of signals were found to be

dispersed throughout all of the chromosomes, with enrichment near most terminal portions of the chromosomes (Figure 3 a). In the sex chromosomes of *L. elongatus*, however, this repeated element was absent, even after low stringency wash conditions were employed (Figure 3 a). In the remaining species, i.e., *L. macrocephalus*, *L. obtusidens L. friderici, L. striatus, L. lacustris*, *Schizodon borellii* and *S. isognathus*, a dispersed signal pattern was observed in all chromosomes, with no indication of sex specificity (Figure 3 b–h).

The distribution pattern of the Le*Spe*I element were similar to those described by Parise-Maltempi et al. [4] and Marreta et al. [12], who detected positive signals in the Z_2Z_2 chromosomes of males and the W_1Z_2 and W_2 chromosomes of female individuals of *L. elongatus* (Figure 4 a–h), while in *L. macrocephalus* and *L. obtusidens* females, positive signals were detected in the long arm of the W chromosome (Figure 4 b, c). *Leporinus friderici, L. striatus, L. lacustris*, *Schizodon borellii* and *S. isognathus* did not shows hybridisations signals of Le*Spe*I, even under low stringency conditions (Figure 4 d–h).

DISCUSSION

Several repetitive elements have been successfully isolated from *Leporinus* species. These elements exhibit wide diversity regarding chromosomal location and distribution [4,11-14]. The organisational patterns of these repetitive sequences demonstrate the dynamics of the fish genome, despite the apparent chromosomal stability that has been observed in the Anostomidae family since the first cytogenetic studies were performed in the 1980s (Silva, E.L. pers. commun.).

The characteristic predominantly dispersed distribution pattern of the Le*Spe*II element revealed by Southern blot analysis is consistent with the genomic organisation of most, if not all, LINEs that have already been analysed. Moreover, this characteristic distribution pattern differs from that of other repetitive elements of the anostomids that have been described. LINE elements comprise a class of Non-LTR retrotransposons representing an important group of repetitive DNA that has been widely studied in the genomes of many organisms [see 5]. The accumulation of Le*Spe*II near the telomeres of almost all chromosomes of the analysed anostomids is similar to the distribution pattern observed for the LINE CiLINE2 [15]. Higashiyama et

al. [16] suggested that because these elements display terminal distribution, they may play a stabilisation role in some circumstances, or they might form a structural cap to protect the terminal portion of the chromosome. In fact, the sex chromosome system of *L. elongatus* is considered to still be undergoing an evolutionary process [4]; thus, the absence of Le*Spe*II favours an environment of instability required for ongoing evolution.

In some groups of vertebrates, dispersed elements are arranged in clusters and blocks that can easily be visualised in [17, 18], but in some fish species, these elements have a widely scattered distribution pattern over all of the [19, 17]. These sequences have been associated with the evolution of the genome size of the host organism [20], and they have also been shown to elicit alterations in gene function by insertion, inducing chromosomal rearrangements and the production of coding and non-coding material, allowing the emergence of new genes or new regulatory sequences [21]. These elements are therefore potentially associated with speciation events [22].

A series of repetitive DNA families has been isolated from the *Leporinus* genus and used as probes in chromosome mapping. All of these families present different distribution patterns, adding features to some species, including sex-specific sequences and species-specific repeated elements. Nakayama et al. [11] described two different sequences, including one found in both Z and W chromosomes and the other representing a second family specific to the W chromosome. Le*Spe*I repetitive DNA was described as a sex-specific dispersed repetitive element showing distinct distribution patterns on two exclusive female chromosomes, named W_1 and W_2, by Parise-Maltempi et al. [4]. This sequence was used to probe chromosomal preparations from *L. macrocephalus, L. obtusidens* and *L. friderici*, which displayed positive signals only on the W chromosomes of *L. macrocephalus* and *L. obtusidens* [12]. Here, we examined the distribution patterns of Le*Spe*I in these species, as well as *L. striatus*, *L. lacustris*, *Schizodon borellii* and *S. isognathus*, which are all from different hydrographic basins, and we corroborated the sex specificities of this sequence. Besides the repetitive sequences associated with differentiated sex chromosomes, another satellite DNA, Le*Sma*I, has also been isolated from *L. elongatus* [13]. This satellite DNA, which is linked to nucleolar organiser regions

(classified as chromosomes Z_2 and W_2 by Parise-Maltempi et al. [4]), is exclusively found in *L. elongatus* and is not associated with sex.

The simple sex chromosome system originated from a pair of autosomes that, for some reason, stopped recombining and gradually diverged from each other [23]. Indeed, a series of heterochromatin acquisitions may have driven the morphological differentiation of simple sex chromosomes found in some *Leporinus* species to the multiple system observed in *L. elongatus*. Considering the direct relationship between the Le*Spe*I element and sex chromosome differentiation, at least in *L. elongatus*, *L. macrocephalus and L. obtusidens*, the presence of Le*Spe*II in all anostomids studied here, including those without differentiated sex chromosomes (such as *Leporinus friderici*, *L. striatus*, *L. lacustris*, *Schizodon borellii* and *S. isognathus*), may represent an ancient acquisition relative to that of the remaining repetitive elements already described in this family.

In the *L. elongatus* genome, the Le*Spe*II element is absent on the chromosomes that integrate the sex systems (W_1 Z_2 and W_2), but it is widely distributed in the remaining autosomes. The absence of this element on W_1 Z_2 and W_2 can be contributed to the fixation of the other repetitive elements. During another stage of differentiation, the accumulation of Le*Spe*I and Le*Sma*I sequences may have modified the accumulation of repetitive sequences in the *L. elongatus* genome [4, 13], and a remarkable event such as rearrangement may have promoted the formation of a multiple sex system in this species, since another heteromorphic pair was found only in this species [12].

In general, the acquisition of heterochromatin, which resulted in a series of important characteristics of anostomids, is the most important way that chromosomal differentiation can occur in the this family [24-26]. Our investigation of repetitive DNA sequences through FISH analysis revealed amazing new characteristics of this portion of the Anostomidae genome. Our results are useful for comparative genomics studies, and they provide new insights into the pathways of chromosomal diversification of the species in this family. The fishes, in general, comprise a basal group of vertebrates with a widely diverse distribution of sex chromosomes that originated from a series of events. Many of these events were related to changes or

multiplication of repetitive sequences. Therefore, investigating these events has enabled us to discover important details about vertebrate genome evolution.

MATERIALS AND METHODS

Chromosomal and genomic DNA preparation

Wild specimens of *Leporinus elongatus* (five males and eight females) were collected in the Mogi-Guaçú River, Pirassununga, São Paulo state, Brazil. Mitotic chromosome isolation and chromosome staining were performed according to Foresti et al. (1993) [27]. Additional cytogenetic studies were carried out with the species *L. macrocephalus* Garavello and Britiski 1988, *L. obtusidens*, *L. striatus* Kner 1858; *L. lacustris* Amaral Campos 1945, *L. friderici* (Bloch 1794), *Schizodon borellii* (Boulenger 1900) and *S. isognathus* Kner 1858, collected in the Paraguay River basin, Mato Grosso State, Brazil. Genomic DNA was extracted from liver and blood using standard methods [28].

The search for repetitive DNA was conducted using restriction enzyme digestions of genomic DNA of *L. elongatus* with different restriction endonucleases. The endonuclease *Spe*I produced a conspicuous band of approximately 650 bp. This DNA band was isolated from a gel, cloned into the pMOS Blue plasmid vector (Amersham Biosciences), and used for transformation in *E. coli* DH5α competent cells. Positive clones were identified and stored at -75°C for future analysis.

Sequencing and sequence analysis

Positives clones were isolated and sequenced using DYEnamic^{TM} ET Terminator Cycle Sequencing (Amersham Bioscience) and an ABI 377 automated DNA sequencer (Applied Biosystems). Nucleotide sequences were subjected to a [29] search at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast), and sequence alignment was performed using Clustal W [30] and manually checked.

Genomic organisation

The genomic organisation of the isolated repetitive fragment was determined by Southern blot hybridisation with 10 µg aliquots of genomic DNA from three *L. elongatus* males and three females. Genomic DNA was digested with the restriction enzyme *Spe*I, separated by electrophoresis in a 1.5% agarose gel and transferred onto a Hybond N+ nylon membrane (Amersham Bioscience) by capillary action. Clones bearing the isolated repetitive fragment were used as probes and hybridised under high stringency conditions using an ECL Direct Nucleic Acid Labelling and Detection Systems Kit (Amersham Biosciences), according to the manufacturer's instructions.

Chromosome mapping

Chromosome mapping of repetitive sequences was performed using the isolated fragment and probes of the Le*Spe*I repetitive element [4] through FISH, according to the method of Pinkel et al. [31] with modifications described in Silva et al. (2012) [13]. The probes were labelled with digoxigenin in a second cycle of reamplification using the clone as the template for PCR with an M13 primer set (F 5'-TGT AAA ACG ACG GCC AGT-3´; R 5'-CAG GAA ACA GCT ATG ACC-3'). Hybridisation signals were detected using appropriate antibody sets composed of antidigoxigenin-rhodamine to detect the signals from the hapten digoxigenin.

Chromosomes were counterstained with DAPI mounted with antifade solution and observed using an Olympus BX51 microscope coupled to an Olympus digital camera (model D71). Chromosome images were captured using the DP Controller program.

LIST OF ABBREVIATIONS

BLASTN - Basic Local Alignment Search Tool

DAPI - 4, 6 diamino-2-phenylindole

FISH – Fluorescent in situ hybridisation

LINEs - Long Interspersed Elements

LTR - Long Terminal Repeat

PCR -Polymerase Chain Reaction

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

ELS collected some of the animals, performed the cytogenetic studies and drafted the manuscript. RSB performed the cytogenetic studies and drafted the manuscript. PPPM collected some of the animals, supervised the cytogenetic studies, helped draft the manuscript and revised the final text. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1 Sequence of Le*Spe*II clone.

Figure 2 Southern blot of DNA digested with SpeI, using the Le*Spe*II sequence as probe in (F) female and (M) male *L. elongatus* individuals.

Figure 3 Mitotic metaphase chromosomes of Anostomidae representatives hybridised with Le*Spe*II repetitive element. a- *Leporinus elongatus* b- *L. macrocephalus* c- *L. obtusidens* d- *L. friderici* e- *L. striatus* f- *L. lacustris* G- *Schizodon borellii* H- *S isognathus*. Arrows indicate sex chromosomes in *L. elongatus.*

Figure 4 Mitotic metaphase chromosomes of Anostomidae representatives hybridised with Le*Spe*I repetitive element. a- *Leporinus elongatus* b- *L. macrocephalus* c- *L. obtusidens* d- *L. friderici* e- *L. striatus* f- *L. lacustris* G- *Schizodon borellii* H- *S isognathus*. Arrows indicate sex chromosomes in *L. elongatus, L. macrocephalus* and *L. obtusidens.* Arrows indicate sex chromosomes.

Figure 1

```
LeSpell    CTAGTTTCACACCTATTATTGGCATAAACAGGATCTACTGGGCTTATTATTTATTAACAC 60
           ............................................................ 60
LeSpell    TGCATACCATTCTCTAAGATACACCTGATATGTTTGTATATACAGTAATACAGTCCTGTG 120
           ............................................................ 120
LeSpell    CAGACGCTTTAGTCACTTGGAGACATTATTATATTTAAAATCTATTATTTCCAAACAGGA 180
           ............................................................ 180
LeSpell    AGCTTTTATTTACTCAGTAAACACTTTATTATCTTAATATTAAACAGTATTAATACACTA 240
           ............................................................ 240
LeSpell    AGTGTGTTTATTTAACCCTTTCCAAGCGTCTCCTTGTACCCAGTATTATTTACAGTTCTC 300
           ............................................................ 300
LeSpell    ATCTAATACCTGCTTTGACTGCTCAGTGCTTCATTAAGCTAAATCAAGATTGCTGCTGAA 360
           ............................................................ 360
LeSpell    GGAACTGAATACATTCATACAATGTCTGGGGCGTCCAGGAGAAATCTGGAANTACACTTT 420
           ............................................................ 420
LeSpell    GTTCTTCAAACTGGAGCCATTCAGTTCTACTTCTTTCACAATAAGATATTCATGTTACTT 480
           ............................................................ 480
LeSpell    TGTATTGTATGTATAATTATTTACATCTGTTTGAATCATATCTGCTGTTCTTACTGCTGA 540
           ............................................................ 540
LeSpell    ACANACAGTGTGGCGGTTGTGTTTCAAGGCCAACATCTTAGCTGCGGTAAGACTGGCTCA 600
           ............................................................ 600
LeSpell    TACAGTACGTCTCTATTACAGAGACTAG 628
           ............................ 628
```

Figure 2

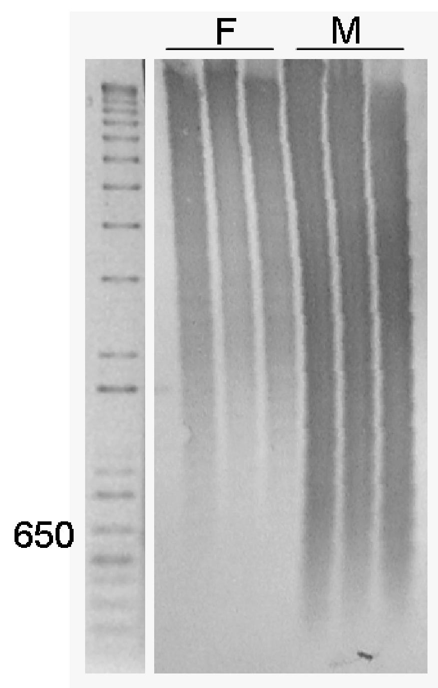


Figure 3

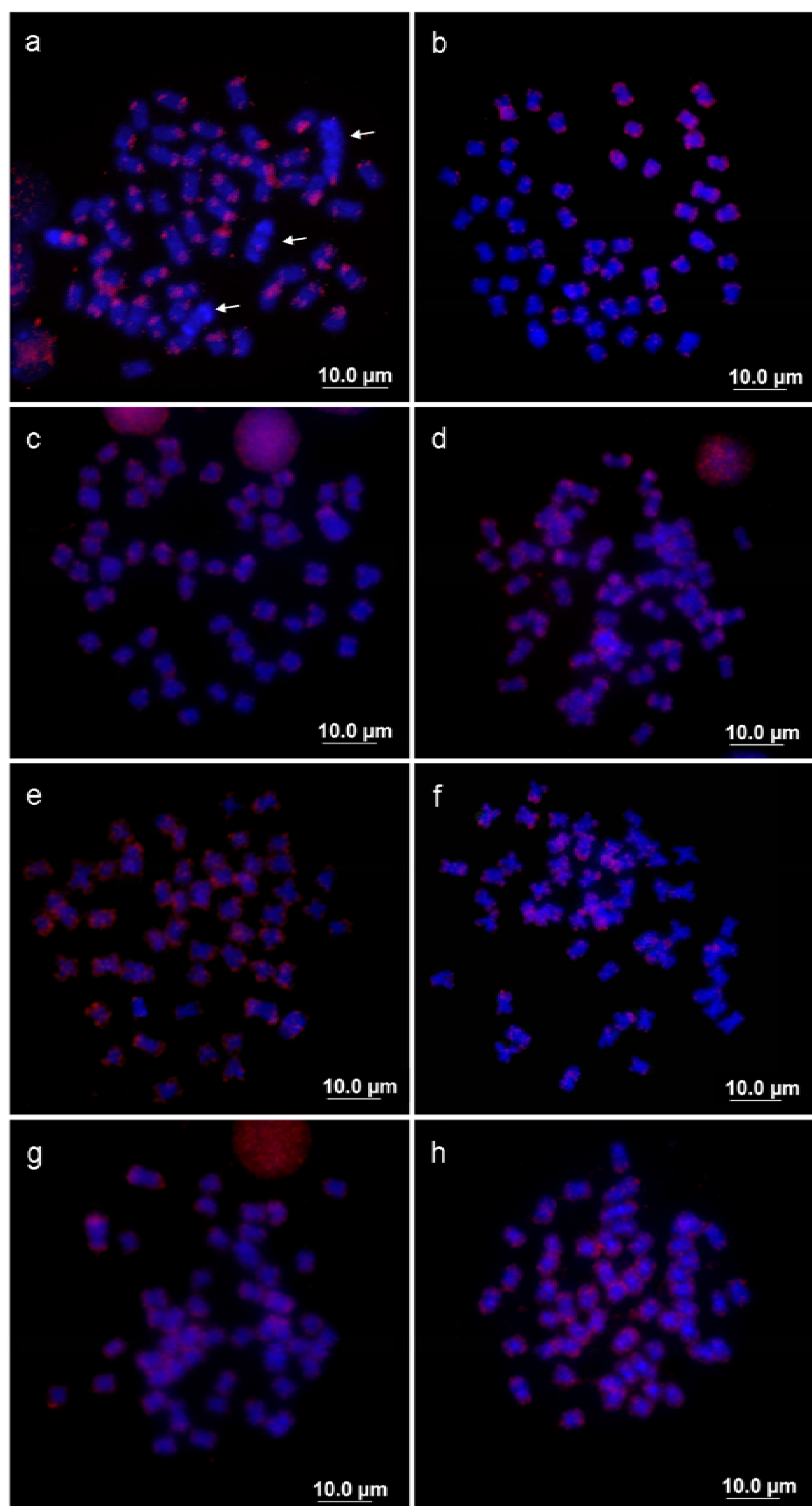
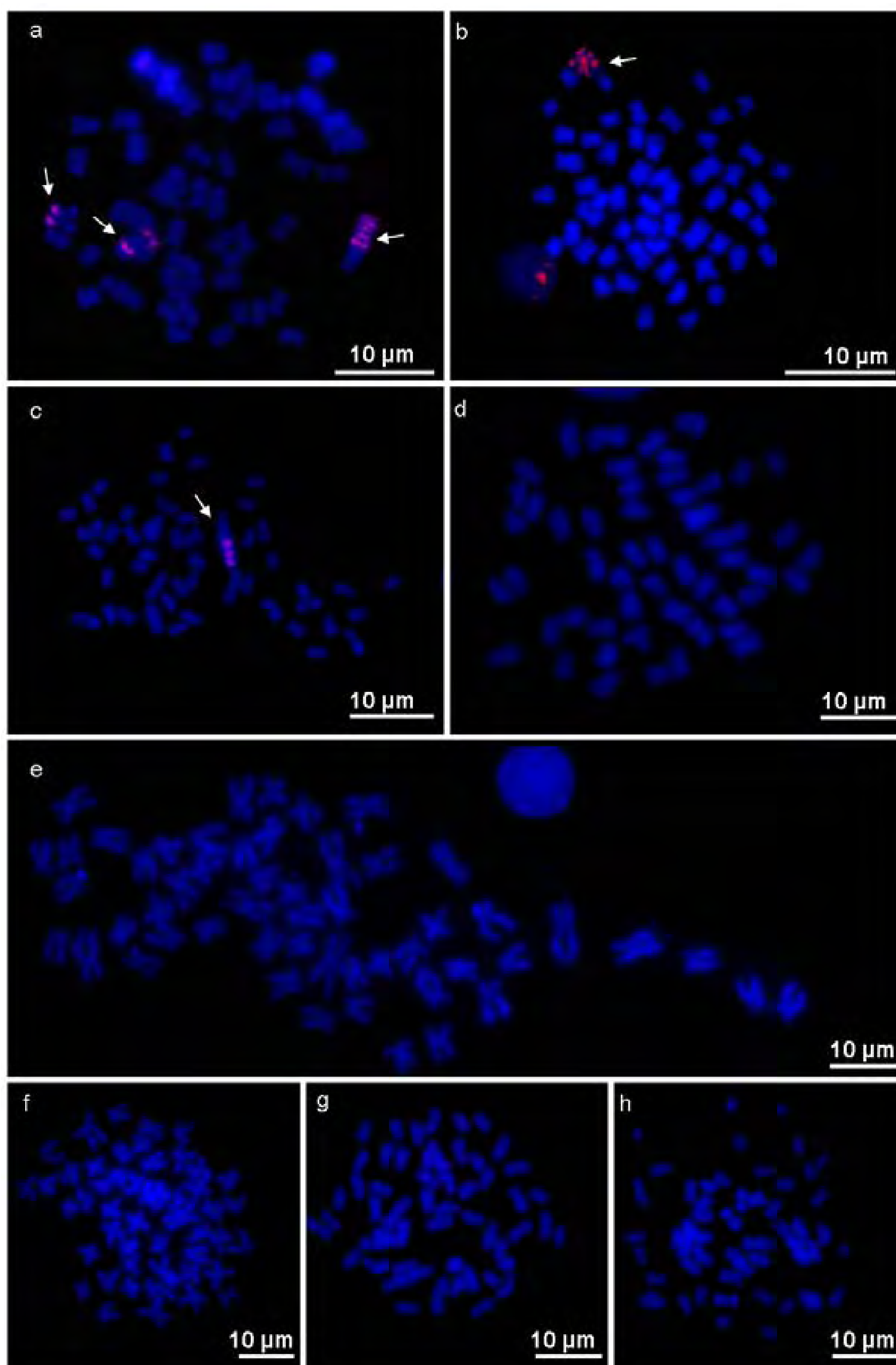


Figure 4



ARTIGO 3

Comparative analysis of the sex chromosomes of the *Leporinus* fish (Teleostei, Characiformes) through cross painting of whole chromosome probes

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ABSTRACT

The Anostomidae family is considered to be an interesting model for studies of sex chromosomes evolution particularly due to the unequal distribution of these systems restricted to some species of the genus *Leporinus*. An apparent chromosomal stability within the family, at least regarding the macrostructure, permits to make some hypothesis about the ways of sex chromosome diversification. In this study we used probes of W chromosomes in a series of cross species experiments trying to understand the major events regarding the sex chromosome evolution in this family. The WLe probes obtained from *Leporinus elongatus* and used against metaphases of this species showed signals in the entire W chromosome as well as on a portion of the Z. In female individuals of *L. macrocephalus* and *L. obtusidens*, the WLe probes painted the W chromosome as well as a part of the Z. The WLe probes in species without sex chromosomes (*L. friderici L. striatus*, *L. lacustris*, *Schizodon borelii* and *S. isognathus* did not paint anything. The WLm probes from *L. macrocephalus* shows the W chromosomes of *L. macrocephalus* entire painted and the Z almost fully hybridised. In the W1 chromosomes of *L. elongatus* and in the W chromosomes of *L. obtusidens*, the portion more intensely painted by WLm corresponding the long arms. In the male individuals, the experiments did not provided hybridisations signals. The probe WLo from W chromosomes of *L. obtusidens* show positive signals in all W chromosomes of *L. obtusidens*. Similarly strong signals were also observed restricted to the long arm of W1 chromosomes of *L. elongatus* and W of *L. macrocephalus*. Also as observed in WLm experiments, no signals were observed in male individuals of all analysed individuals. Our results highlight the common origin of sex chromosomes in *L. elongatus*, *L. macrocephalus* and *L. obtusidens*. The absence of signals in the species without differentiated sex chromosomes excludes the possibility of cryptic sex chromosomes that share Leporinus W sequences.

Key words: chromosome painting, FISH, Neotropical fishes, repetitive DNA, sex chromosomes

INTRODUCTION

The origin and evolution of sex chromosomes have interested evolutionary biologists for a long time. Although sex chromosomes evolve from an autosomes pair (Ohno 1967), over time they become different, in gene content and structure (Charlesworth et al. 2005). While sex chromosomes in most mammals are ancient, sex chromosomes in some fish, platypus, some insects and dioeciuos plants are evolutionarily young (Vyskot and Hobza 2004, Charlesworth et al. 2005).

Among vertebrates, fish present an enormous diversity of sex determination mechanisms, contrasting with the much more stable systems found in mammals and birds. The majority of teleost fish are gonochoristic, meaning that they exist as males and females, and the most gonochoristic species have genetic sex determination (GSD). Beside simultaneous and sequential hermaphroditism, gonochorism in fish is controlled by many forms of sex determination involving genetic and/or environmental factors, although temperature-dependent sex determination might be rarer than previously thought (Ospina-Alvarez and Piferrer 2008, Schultheis et al. 2009). Despite a large number of fish species described (around 34,500 according to Nelson, 2006), only a minor fraction has been thoroughly investigated and their sex determination mechanism unequivocally clarified (Devlin and Nagahama 2002,).

Within the vertebrate GSD species many variations have been found, ranging male or female heterogamety to polygenic sex determination. Multiple sex chromosomes or autosomal modifiers that enhance or antagonize the sex-determining genes on the gonosomes are quite frequent (Herpin and Schartl 2009). This, together with the fact that even between closely fish species the sex determination mechanisms can be different (Cioffi et al. 2011, Takehana et al. 2008, Tanaka et al. 2007, Ota et al. 2003, Devlin and Nagahama 2002, Volff and Schartl 2001), demonstrates the high evolutionary plasticity of this fundamental process (Herpin and Schartl 2009).

Sex chromosomes in *Leporinus* genus (Teleostei, Anostomidae) present some features that make it a very interesting group to study sex determination mechanisms and sex chromosome structure, composition and evolution, reflecting well the diversity of genetic sex determination observed at a broader scale on teleost fish.

The *Leporinus* group has around 40 species and eight of them show heteromorphic sex chromosome (*Leporinus conirostris* Steindachner 1875, *L. elongatus* Valenciennes 1850, *L.* aff. *elongatus*, *L. macrocephalus* Garavello and Britski, 1988, *L. obtusidens* (Valenciennes 1847), *L. reinhardti* Lütken, 1875, *L. trifasciatus* Steindachner, 1876 and *Leporinus* sp.). One feature of the sex chromosome of *Leporinus* species is their ability to add large blocks of heterochromatin. In fact, seven species of the genus (*Leporinus conirostris*, *L. elongatus*, *L.* aff. *elongatus*, *L. macrocephalus*, *L. obtusidens*, *L. reinhardti*, *L. trifasciatus*) present enlarged W chromosomes in relation with the Z, due the increase of heterochromatin, while one, *Leporinus* sp2 presents a karyotype with W chromosomes equals of its homologous Z and the remains autosomes. In this species the differentiation among W and others chromosomes is possible thought C band technique.

The hypothesis of repetitive elements accumulation as the major event in the differentiation of sex chromosome in Anostomidae is the most accepted (Nakayama et al. 2004, Parise-Maltempi et al. 2007). In fact repetitive elements also have been corroborated to discrimination among some species (Silva et al. 2012) and as putative responsible to the recent origin of a multiple $Z_1Z_1Z_2Z_2/Z_1W_1Z_2W_2$ sex chromosome in *Leporinus elongatus* (Parise-Maltempi et al. 2007). However whether the event of increase of heterochromatic segments and naturally repetitive DNA is cause or consequence yet remains undiscovered and the sex chromosome evolution of this species continues precisely unknown.

In this sense we got whole W chromosome probe of some species of *Leporinus* genus by manual microdissection in an attempt to provide sex-chromosome specific probes to get a view of the sex chromosome evolution in Anostomidae species through cross species hybridisation. Our results provide a new perspective in the Anostomidae genome study and have corroborating in the discussion about the origin of sex chromosomes.

MATERIALS AND METHODS

Cell culture and metaphase cell preparation

Leporinus elongatus chromosomes were obtained from culture cells. The fibroblast cells collected from fin tissue of *L. elongatus* cultures in DMEM medium supplemented with 10%FCS. The cells were maintained in 50% Amniomax C-100 (Invitrogen) serum at 37°C.

The cells were harvested after colcemid treatment (0.05ug/mL) for 2 hours, suspended in hypotonic solution (0.075M KCL) and then incubating at 37°C for 20 min. Cells were pelleted and resuspended three times in fresh ice cold fixative (3:1 methanol:acetic acid), then kept at -20°C until use.

For another Anostomidae species: *L. friderici, L. striatus*, *L. lacustris*, *Schizodon borelii* and *S. isognathus*, the chromosomes were obtained from direct cytological preparations produced from kidney samples according to the methods given by Foresti et al. (1981).

Chromosome microdissection and DNA amplification

Chromosome suspensions were dropped onto moist, 1% SDS- cleaned coverslips. The coverslips were washed in 1xPBS solution for 1 minute and after in a trypsin solution (35 mL 1xPBS and 5 mL trypsin) for 30 seconds. Were washed again in 1xPBS and stained with Giemsa 1% in PBS.

The microdissection was performed in an Eppendorf TransferMan NK2 (Eppendorf) coupled to a Zeiss Axiovert 40 CFL microscope, with glass needles made with a Nikon puller and sterilized by UV radiation. About 8 chromosomes were separately placed in 9uL DNAse-free ultrapure water and were then amplified by means of the GenomePlex Single Cell Whole Genome Amplification Kit (WGA4-Sigma).

Fluorescent *in situ* hybridisation (FISH)

The probes were labelled using the GenomePlex (WGA3 Reamplification KIT-Sigma) following the protocol, but changing the kit dNTP mix for a ½ T dNTP mix and also adding a biotin 16-dUTP in the reaction.

Fluorescent in situ hybridisation (FISH) was performed using the protocol described in Rens et al. (1999, 2006) with several modifications.

Slides were dehydrated through ethanol series; aged for 1 h at 60℃, denatured in 70% formamide/0.6× saline-sodium citrate (SSC) at 65º C for 2 minutes and dehydrated again. Three microliters of biotinylated probe were precipitated in ethanol using also *L. elongatus Cot* 1 DNA and resuspended in 13 µL of hybridisation buffer (40% deionized formamide (v/v), 10% dextran sulfate, 2×SSC, 0.05 M phosphate buffer, pH 7.3]. This mixture was denatured for 10 min at 65℃, pre-annealed at 37℃ for 1 hour and applied to each slide. Hybridisation was carried out at 37℃ for 12 hours for same species and three nights for cross species experiments. Post-hybridisation washes were performed in 40% formamide/1.8× SSC twice for 5 min each, followed by 2× SSC twice for 5 min each and 4× SSC with 0.05% Tween-20 (4xT) once for 4 min. washes were carried out at 42℃. Probe detection was carried out using 200 µL of diluted (1:500) Cy3-streptavidin antibody (Amersham) per slide at 37℃ for 30 min. After detection, slides were washed in 4xT three times for 3 min each at 42℃ and mounted in Vectashield mounting medium with 4′,6- diamidino-2-phenylindole (DAPI; Vector Laboratories).

Images were captured and processed using the CytoVision Genus system (Applied Imaging, USA) and a Cohu CCD camera mounted on an Olympus BX-60 microscope.

RESULTS

Hybridisations with WLe probes

The WLe probes used against chromosomes of *Leporinus elongatus* female showed complete hybridisations signals, painting the entire W chromosomes (Fig. 1 a). In female individuals of *L. macrocephalus*, the WLe probes painted one chromosome correspondent to the W chromosome that appears fully hybridised (Fig.

1 b) as well another chromosome almost totally, correspondent to the Z. In male individuals of *L. macrocephalus* the WLe probes painted de Z chromosomes in a position correspondent those observed in females (Fig. 1 d). The WLe probes painted completely the W chromosomes of female *L. obtusidens* individuals (Fig. 1 c). In the males, however, the signals were observed near to terminal portion of a pair of metacentric chromosomes correspondent to de Z (Fig. 1 e).

The fish experiments using WLe probes in individuals of *L. friderici, L. striatus*, *L. lacustris*, *Schizodon borelii* and *S. isognathus* did not produce any result even after low stringency wash conditions (data not show).

Hybridisations with WLm probes

The WLm probes show a high fidelity hybridisation pattern, being the W chromosomes of *L. macrocephalus* entire painted and other correspondent to the Z almost fully hybridised (Fig. 2 a). Indeed, many dots can be observed dispersed in other autosomes, indicating the sharing of others repetitive sequences. In the W1 chromosomes of *L. elongatus* and in the W chromosomes of *L. obtusidens*, the portion more intensely painted by WLm corresponding the long arms (Fig. 1 b, c). In the male individuals, the experiments did not provided hybridisations signals.

Hybridisations with WLo probes

Positive hybridisation signals of WLo probes were observed in all W chromosomes of *L. obtusidens* female individuals (Fig. 3 a). Similarly strong signals were also observed in the long arm of W1 chromosomes of *L. elongatus* and W of *L. macrocephalus*, but in the short arm, the signals were absent (Fig. 3 b, c). Also as observed in WLm experiments, no signals were observed in male individuals of all analysed individuals.

DISCUSSION

The W chromosome of *Leporinus* species is easily recognised by the higher size than autosomes and due the accumulation of heterochromatic blocks, allowing beyond recognition the micromanipulation procedure. While the sex chromosomes of the *Leporinus* species in general, are highly differentiated, the others chromosomes are included in the typical metacentric- submetacentric pattern, confirming the

proposal in which, 54 biarmed chromosomes a character ancient to the Anostomidae family (Galetti et al. 1981). The morphologic similarity among sex chromosomes found in the *Leporinus* species allowed the emergence of hypothesis about a common origin, in which an initial accumulation of heterochromatic segments may have been the first step in the differentiation of sex chromosomes (Galleti et al. 1995). An exception was identified in *Leporinus* sp2, in which the W chromosome have the same size of autosomes being in this species, the most evident difference among W and Z related to heterochromatic content predominantly mithramycin positive (Vênere et al. 2004). According these authors this system in *Leporinus* sp2 perhaps has originated independently from the ZW system previously described for other *Leporinus* species

Our hybridisation experiments confirmed that WLe is conserved as an entire unit only in *L. obtusidens*, while in *L. macrocephalus* the additional chromosome labelled indicates one evolutionary chromosome break that separates them. Indeed the WLm and WLo showed a similar model in the cross species experiments, being most intense those signals located on the heterochromatic regions of q arms. This pattern is possible due to the independent distribution of repetitive elements that compose these chromosome segments.

As already mentioned, the use of repetitive elements to map the dynamic of sex chromosomes in Anostomidae has been successfully employed. The repetitive element Le*Spe*I was described as a participant of the sex chromosome differentiation process in *L. elongatus* (Parise-Maltempi et al. 2007). This sequence was also mapped in *L macrocephalus* and *L. obtusidens*, evidencing some homologies and highlighting its importance on the differentiation (Marreta et al. 2012). However, trying to find some relation among the Le*Spe*I and putative cryptic sex differentiation process (Silva et al. 2012, in press) mapped this sequence in chromosomes of *Leporinus friderici L. striatus*, *L. lacustris*, *Schizodon borelii* and *S. isognathus,* but did not find positive hybridisation results.

The *Leporinus* genus has been hypothetically divided into two different groups according cytogenetic and molecular records (Galetti et al. 1995, Ferreira et al. 2007). Our data reinforces the idea of subdivision of *Leporinus* genus in at least two lineages: one comprised with species with differentiated sex chromosomes and

another with those that not have. The W specific probes retrieved from *L. elongatus*, *L. macrocephalus* and *L. obtusidens* systematically cross hybridised shows the synteny among theses sequences and based on this scenario, it is possible to hypothesize that the sex chromosomes of *L. elongatus*, *L. macrocephalus* and *L. obtusidens* had a common origin. According to our results, the hypothesis of cryptic sex chromosomes sharing *Leporinus* W sequences in remain anostomids (*Leporinus friderici L. striatus*, *L. lacustris*, *Schizodon borelii* and *S. isognathus*) can be discarded corroborating previous studies with repetitive sequences.

Cross-species chromosome painting is a powerful tool to delineate syntenic chromosome regions among closely and distantly related species and to reconstruct chromosomal phylogeny (Nanda et al. 2011). In fish specifically, studies have brought new insight into the evolution of sex chromosome systems, and shown that in many cases, very related group, has drawn quite different evolutionary paths (Phillips et al. 2001,Takehana et al. 2007a, Takehana et al. 2007b, Takehana et al. 2008, Henning et al. 2011, Cioffi et al. 2011). As in *Leporinus* species analysed, some other fish groups have a common origin of sex chromosomes (Ota et al. 2003, Diniz et al. 2008, Vicari et al. 2008, Pansonato-Alves et al. 2011) highlighting the wide diverse in the sex determining mechanisms fishes.

Our data indicate that although there are reported a high plasticity of sex determination mechanisms in fish, in the Anostomidae family, especially in some species of *Leporinus* genus the sex systems seems to be conserved and derived from a common ancestral pair. These results add new data to amount of studies using the *Leporinus* genus as a model, reinforcing the hypothesis that the ZW sex chromosome are present only in some related species, being absent, even in a non differentiated form in others anostomids. The data regarding fish sex determination available to comparison have been demonstrate a wide range of mechanisms in this Class, evidencing the proposal that it systems had evolved many times, differently even in related species.

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FIGURE LEGENDS

Fig.1 Cross fish with WLe probes. a- WLe X female *L. elongatus;* b- WLe X female *L. macrocephalus;* c- WLe x female *L. obtusidens;* d- WLe X male *L. macrocephalus;* e- WLe X male *L. obtusidens*; f- Scheme WLe probe hybridisation pattern.

Fig. 2 Cross fish with WLm probes. a- WLm X female *L. macrocephalus;* b- WLm X female *L. elongatus;* c- WLm x female *L. obtusidens*. Scheme WLm probe hybridisation pattern.

Fig. 3 Cross fish with WLo probes. a- WLo X female *L. obtusidens;* b- WLo X female *L. elongatus;* c- WLo x female *L. macrocephalus*; Scheme WLo probe hybridisation pattern.

Figure 1

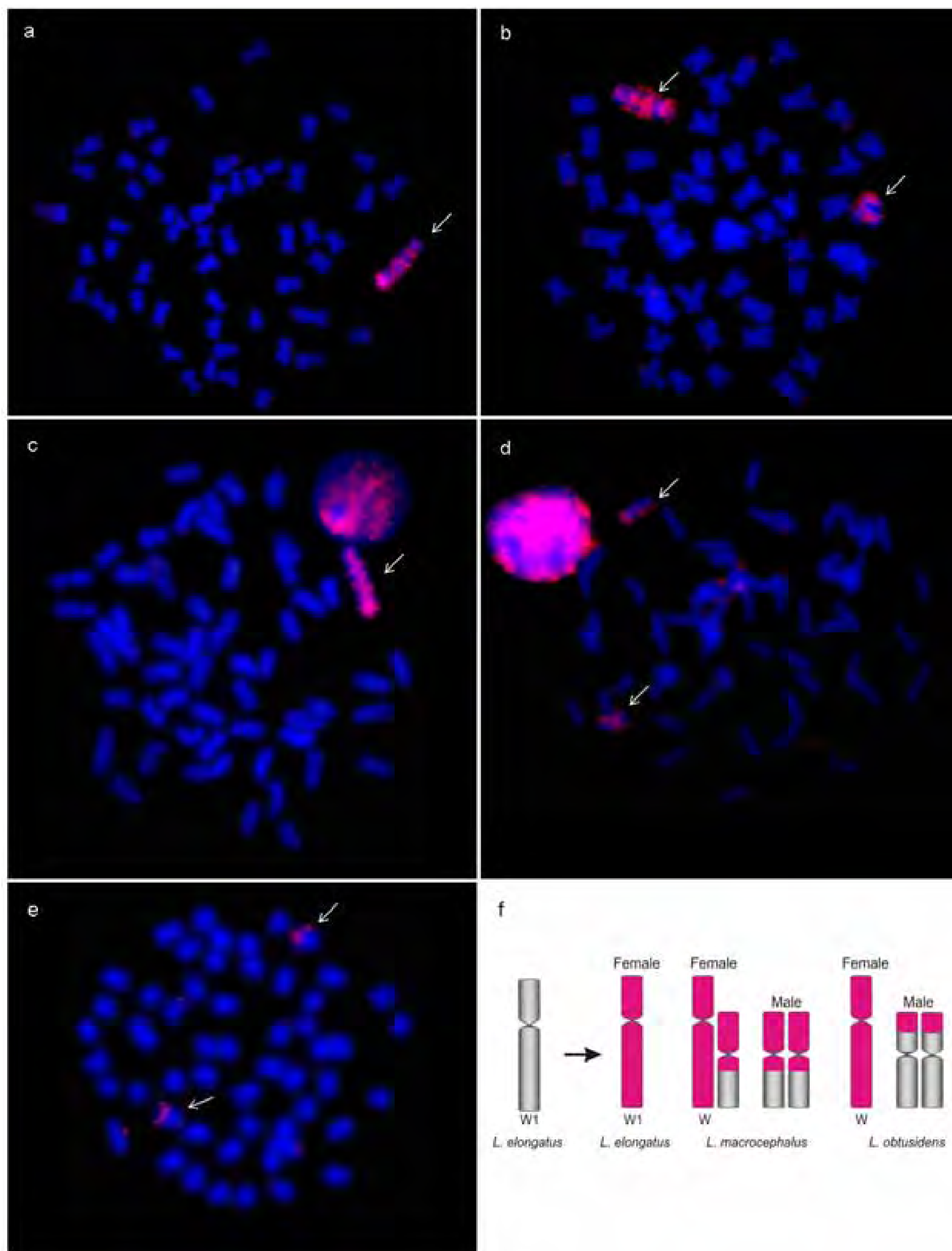


Figure 2

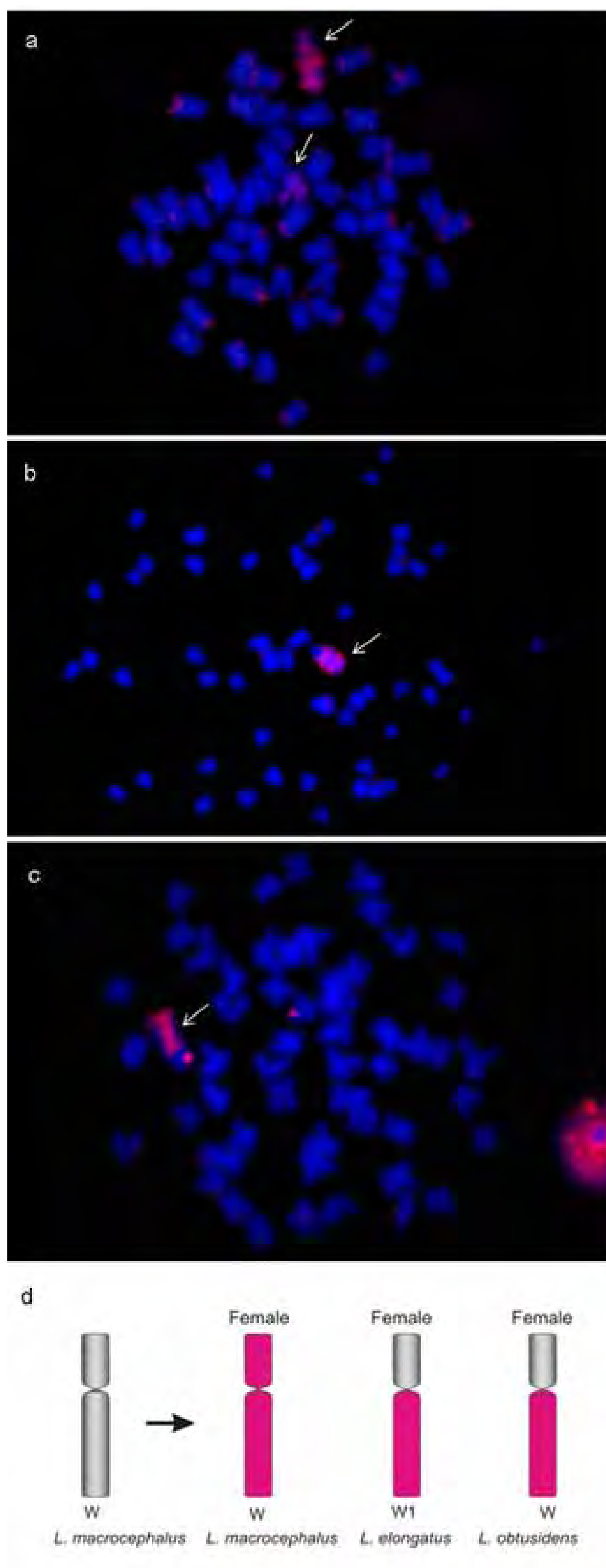
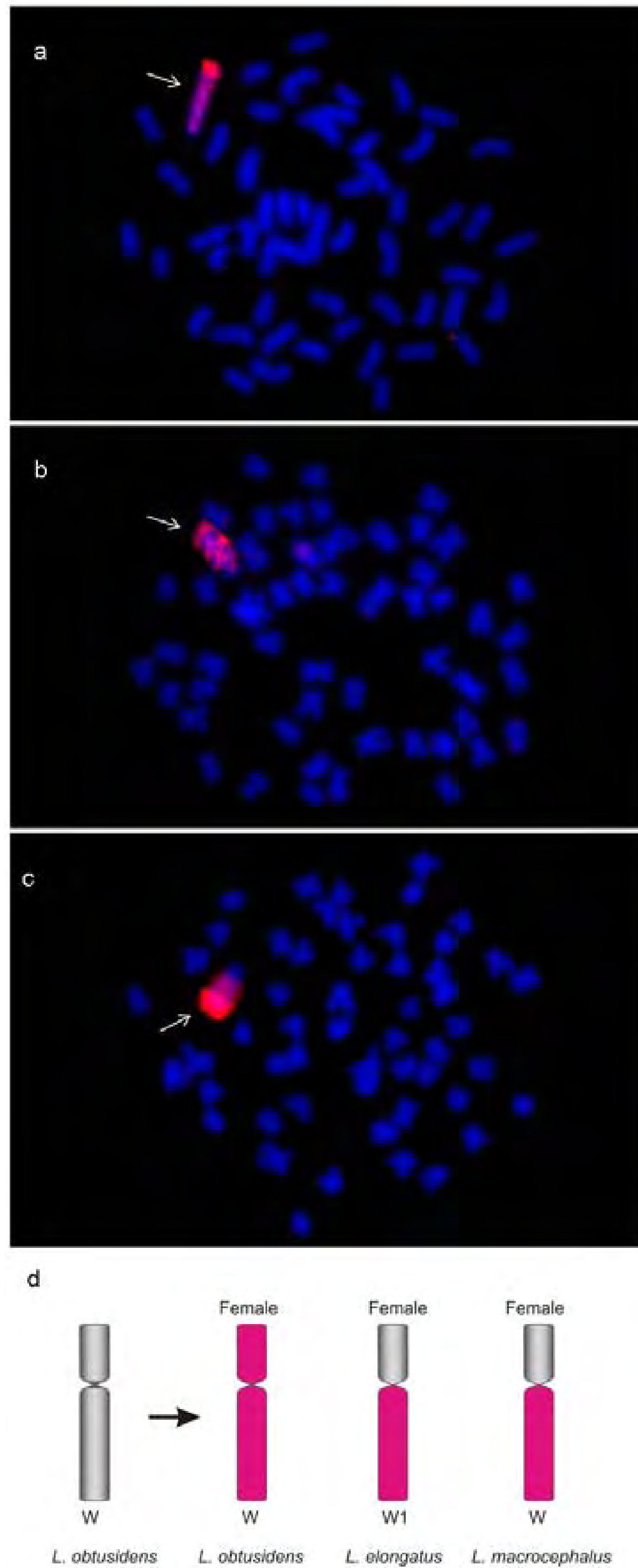


Figure 3



ARTIGO 4

Comparative aspects of chromosome composition in Anostomidae species: a review of cytogenetic markers

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ABSTRACT

The Anostomidae family, in the order Characiformes, comprises 12 genera and 138 described species. The anostomids occupy large river basins and tributaries and are broadly distributed in Central and South America. Despite numerous cytogenetic studies of anostomids, only few have focused on the comparative analysis of anostomid karyotype evolution. Therefore, this study was undertaken to compare the cytogenetics of most representative anostomid species, including *Abramites hypselonotus*, *Leporinus elongatus*, *L. friderici*, *L. lacustris*, *L. macrocephalus*, *L. obtusidens*, *L. striatus*, *Schizodon borellii*, and *S. isognathus*, using currently available data. These species possess quite a conserved karyotypic structure, with a diploid number of 54 metacentric and submetacentric chromosomes. Variations in chromosome number are related to the occurrence of B chromosomes in some species, which possibly originated from a recent event, without phylogenetic implications. All males and females contain a nucleolar organizer region (NOR) localized at different positions on metacentric/submetacentric chromosomes, as determined by fluorescent in situ hybridisation (FISH), using 18S rDNA probes. Rearrangements involving inversions that maintained the original diploid number were likely responsible for differences in NOR pattern phenotypes. Heteromorphic sex chromosomes are restricted to a few *Leporinus* species. Despite the apparent stability, differences among the karyotypes are associated with particular patterns of heterochromatin and NOR distribution. The increase in heterochromatic segments has probably contributed to the meiotic isolation of Z and W chromosomes, dislocations of NOR-associated sites and chromosome reorganization, based on changes in the quantity and/or quality of heterochromatic segments.

Keywords 18S rDNA, FISH, heterochromatin, repetitive DNA

INTRODUCTION

The Anostomidae family, in the order Characiformes, comprises 12 genera and 138 described species (Garavello and Britski 2003). The Anostomidae species are broadly distributed in Central and South America, occupying large river basins and tributaries, including the Magdalena and Atrato rivers in Colombia, the Orinoco River and Maracaibo Lake system in Venezuela and the Amazon, from inner drainages in Brazil to south of Buenos Aires, Argentina (Garavello and Britski 2003; Ringuelet et al. 1967). Most Anostomidae diversity occurs in the Amazon, Orinoco, Paraguay and Paraná rivers and their tributaries, with fewer species in isolated coastal rivers of the Guianas, the São Francisco basin, and the remaining short coastal rivers from north-eastern Brazil to Uruguay (Garavello and Britski 2003).

The anostomids can be distinguished from other Characiformes by their elongated bodies, short anal fins, and the absence of teeth in jaws and palates (Géry 1977). Regarding phylogeny, the family comprises a monophyletic group with the Chilodontidae, Prochilodontidae, and Curimatidae families (Vari 1983). According to Vari (1983), Anostomidae and Chilodontidae comprise one clade, while Prochilodontidae and Curimatidae comprise another. The relationship among the Anostomidae genera is strongly influenced by the series of synapomorphies that connect them (Vari 1983), but the monophyletic nature of the included genera has not been demonstrated (Mautari and Menezes 2006). The genera *Abramites*, *Anostomoides*, *Hypomasticus*, *Leporellus*, *Leporinus*, *Rhytiodus*, and *Schizodon*, include the majority of important species of this family (Sidlauskas and Vari 2008).

Cytogenetic studies in Anostomidae are relatively well documented. These studies have revealed quite a conserved karyotypic structure, with a diploid number of 54 metacentric and submetacentric chromosomes and a single chromosome pair carrier, the nucleolar organizer region (NOR) system, in distinct positions and pairs among the species, in addition to the heteromorphic sex chromosomes that are restricted to a few species of the *Leporinus* genus (Table 1).

Despite the apparent stability in karyotypic structure of the anostomids, slight differences among the karyotypes of many species were observed (see Table 1). Currently, the use of different chromosomal discrimination methods has enabled better characterization of some species, and the differences among these species

were shown to be more pronounced than previously thought. Among the major cytological markers, the polymorphism of the NOR, patterns of constitutive heterochromatin distribution (Galetti et al. 1984; Margarido and Galetti 2000), and the distinctive type ZZ/ZW heteromorphic sexual chromosome system, are most notable (Galetti and Foresti 1986; 1987; Venere et al. 2004; Parise-Maltempi et al. 2007; Marreta et al. 2012), as well as the different distribution patterns of 5S cistrons ribosomal DNA (rDNA) among the species (Martins and Galetti 2000). Heterochromatin plays an interesting role in karyotype differentiation among species (Galetti and Foresti 1986; Galetti and Foresti 1986; Galleti et al. 1991a; Galleti et al. 1991b; Nakayama et al. 1994; Khoeler et al. 1997; Parise-Maltempi et al. 2007; Marreta et al. 2012).

Classic and molecular cytogenetic studies have been indispensable for clarifying the karyotypic relationship and chromosome diversity among species of the Anostomidae family. The numerous karyotypic features of Anostomidae have undoubtedly been important for the formation of hypotheses concerning the genome organization of the group, but many details of this process remain to be understood. This study was undertaken to both review this topic and to offer new information concerning chromosome diversity in anostomids, by comparing the available chromosome data for Anostomidae genera and species and suggesting major chromosome markers for this family.

MATERIAL AND METHODS

Chromosomal and genomic DNA preparation

This study was carried out with the species *Leporinus elongatus* (Valenciennes 1849), *L. friderici* (Bloch 1794), *L. lacustris* (Amaral Campos 1945), *L. macrocephalus* (Garavello and Britiski 1988), *L. obtusidens* (Valenciennes, 1837), *L. striatus* (Kner 1858); *Schizodon borellii* (Boulenger 1900), *S. Isognathus* (Kner 1858) and *Abramites hypselonotus* (Günther 1868). All species were collected in the Paraguay River basin, Mato Grosso State, Brazil, with the exception of *Leporinus elongatus* and *L. obtusidens*, which were collected from a fish farm in Leme municipality, São Paulo state.

Direct cytological preparations were produced from kidney samples according to the methods of Foresti et al. (1993). Genomic DNA was extracted from livers and muscles with proteinase K digestion, followed by phenol/chloroform extraction, using standard protocols (Sambrook and Russel 2001).

Conventional cytogenetic analysis

Karyotype analysis was conducted after conventional staining with Giemsa. Chromosomes were morphologically classified using the modified nomenclature for fish of Levan et al. (1964), including the following: metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (t). The C bands were visualised after treatment following the protocols of Sumner et al. (1972)

Fluorescent in situ hybridisation (FISH)

The 18S rDNA probes were obtained by PCR using the primer sets NS1 5'-GTAGTCATATGCTTGTCTC-3' and NS2 5'-GGCTGCTGGCACCAGACTTGC-3' (White et al. 1990). The 18S rDNA was labelled with digoxigenin in a second cycle of reamplification using the PCR product as a template. FISH analyses were performed according to the methods of Pinkel et al. (1986) with the modifications employed by Silva et al. (2012). Chromosomes were counterstained with DAPI, mounted in antifading solution and observed using an Olympus BX51 microscope coupled to an Olympus digital camera (model D71). Chromosome images were captured using DP Controller software.

RESULTS

For the first time, the chromosomes of *Abramites hypselonotus* were analyzed. These chromosomes were found to be similar to those observed in other Anostomidae genera, and differentiated sex chromosomes were not observed (Fig. 1). *Leporinus elongatus, L. friderici, L. lacustris, L. macrocephalus, L. obtusidens, L. striatus, Schizodon borellii, S. isognathus*, have been well studied, and

the karyotypes found here for all males and females exhibited a diploid number of 54 chromosomes distributed into metacentric and submetacentric types as observed in the figure 2. Sex system chromosomes ZZ/ZW were observed in *Leporinus elongatus, L. macrocephalus* and *L. obtusidens*, according to previously published data.

All analyzed male and female Anostomidae individuals presented a simple NOR localized at different positions on metacentric/submetacentric chromosomes, as revealed by FISH experiments carried out with an 18S rDNA probe (Fig. 2).

DISCUSSION

Chromosome number

Currently, a total of 25 species of Anostomidae, from several South American river basins, have been cytogenetically studied. All of these species have biarmed chromosomes with a fundamental number (NF) of 108 (Table 1). The Anostomidae family exhibits an evolutionarily conserved and genetically stable chromosome set (Koehler et al. 1997; Galetti et al. 1991a; Galetti et al. 1991b; Galetti et al. 1995). The few variations in chromosome number that were previously observed could be attributed to the presence of supranumerary chromosomes in some specimens of *Schizodon nasutus*, *Leporinus friderici*, and *Leporinus* sp. (Pastori et al. 1997; Venere et al. 1989).

The origin of supranumerary chromosomes in anostomids is still unresolved. The apparent rare occurrence of such chromosomes in *Leporinus* species may indicate a recent occurrence of events, such as those observed in *Schizodon nasutus* (Pastori et al. 1997). However, the morphological similarities among supranumerary chromosomes of different Anostomidae species, suggest that these chromosomes may share an earlier, unique origin (Venere et al. 1989).

The evolutionarily conserved and genetically stable nature of the anostomid karyotype suggests that the diploid number and karyotypic formulae are ancient characteristics of the family (Galetti et al. 1981a). In fact, this condition is considered to represent synapomorphy within the families Chilodontidae, Prochilodontidae, and Curimatidae (Venere et al. 1989; Feldberg et al. 1992; Galetti et al. 1994; Venere et

al. 2008). This hypothesis is strongly supported by results of previous phylogenetic analyses of morphological traits (Vari 1983).

Chromosome localization of rDNA and NOR sites

In higher eukaryotes, the tandem arrays of rDNA genes are organized into two distinct multigene families. These multigene families are composed of hundreds to thousands of copies of genes organized into two distinct repeat classes, i.e., 45S rDNA and 5S rDNA (see Martins and Wasko 2004). The former class contains the genes that code for 18S, 5.8S and 26S–28S rRNAs alternating with non-transcribed regions called spacers (Long and David 1980), while 5S rDNA is the largest component of prokaryotic and eukaryotic ribosomes and is essential for protein synthesis (Long and Dawid, 1980; Vittorazzi et al. 2011).

Probes of 18S rDNA have been used to complement the data obtained from silver nitrate impregnation experiments to reveal the NOR. In Neotropical fishes, the presence of NORs appears to be a heterogenic characteristic, occurring as only one block on a chromosomal pair in some groups and as multiple blocks on others (Bertollo 1996). The analysis of NORs in various Anostomidae species revealed that independent of the homogeneity of karyotypic macrostructure, these regions are informative in the characterization of some species (Galetti et al. 1991a; Galetti et al. 1984; Koehler et al. 1997).

Among the species already analyzed, the phenotypic pattern of the NOR is relatively similar; this region is generally localized at terminal portions of chromosomes, except in some species of the *Leporinus* genus, although the chromosomes that contain this structure can vary. In *L. octofasciatus,* the NOR occupies the interstitial position on the q arm of chromosome 2, while in *L. striatus*, the NOR shares the same position, but it is located on the p arm. In *L. taeniatus,* NORs are located on the interstitial position of the p arm of the 23rd chromosome pair.

Although the presence of a NOR in the interstitial chromosome position is not common for the majority of Anostomidae species, the presence of this phenotype in a small group of species does not indicate that these species are more phylogenically related than other species. As phylogenetic analysis using sequences of nuclear and

mitochondrial DNA indicates, these species occupy different clades, which makes them relatively distant (Santos 2007). Thus, this difference in NOR pattern can be attributed to the occurrence of rearrangements that involved fissions and inversions, while still maintaining the original diploid number. Rearrangements such as these were responsible for most of the NOR phenotypic differentiation observed in species from the Heptapteridae family (Borba et al. 2011).

FISH using a 5S rDNA probe in *Leporinus elongatus*, *L. friderici*, *L. obtusidens*, *Schizodon vittatus*, *S. altoparanae*, *S. knerii*, *S. borellii*, *S. isognathus*, and *S. nasutus*, revealed that these cistrons are not related to nucleolar sites, but they are instead associated with other chromosome pairs (Martins and Galetti 1999; Martins and Galetti 2000). In the *Leporinus* and *Schizodon* genera, these cistrons are highly conserved regarding chromosomal localization. However, when sequences of 5S rDNA plus the non-transcribed spacer (NTS) were compared among seven species of the *Leporinus* genus (*Leporinus elongatus*, *L.* aff. *elongatus*, *L. friderici*, *L. macrocephalus*, *L. obtusidens*, *L. octofasciatus*, and *Leporinus* sp.), two monophyletic groups were revealed, one comprising species containing sex chromosomes and the other lacking sex chromosomes (Ferreira et al. 2007). The 5S rDNA probe also exhibited different distribution patterns in chromosome pairs among populations of *Leporellus vittatus* from three isolated hydrographic basins (Aguilar and Galleti 2008).

Sex chromosomes in Anostomidae: the role of Heterochromatin and Repetitive DNA

The existence of a genetic mechanism of sex determination does not necessarily imply the presence of distinguishable sex chromosomes, although such chromosome is usually present (White 1977). Most Neotropical fishes lack sex chromosomes, but simple and multiples systems had already been identified in different groups (Almeida-Toledo and Foresti 2001; Devlin and Nagahama 2002). An interesting feature of the distribution of these systems in fishes is that for both male and female heterogamety, simple and multiple systems, and sporadic occurrence, can be found in kindred species and in different populations within the same species (Almeida-Toledo and Foresti 2001).

Changes in the heterochromatic content of most anostomids are believed to be the major events contributing to important changes in the genome of the species. The *Leporinus* genus, for example, contains more than 40 species, only eight of which have differentiated sex chromosomes (Table 1). In all cases, the heterochromatin represents an effective way to produce meiotic isolation of the Z and W chromosomes, not only as a result of quantitative differences in heterochromatic content between the two chromosomes, but also qualitative differences, as was observed in snakes (Galetti and Foresti 1986).

In the simple female ZW system, the W chromosome is huge, submetacentric and fully heterochromatic. By contrast, in the Z chromosome, present in both sexes, the third distal part is heterochromatic. Species with multiple sex systems have a large W1 chromosome that has a long, completely heterochromatic arm and three others submetacentric chromosomes related to the sex system (Parise-Maltempi et al. 2007). The morphologic similarity among all sex chromosomes found in the *Leporinus* species led Galleti et al. (1995) to suggest that these chromosomes have a common origin, in which an initial accumulation of heterochromatic segments may have been the first step in the differentiation of sex chromosomes.

More recently, Venere et al. (2004) identified a new sex chromosome system in females of *Leporinus* sp2. Unlike most species with heterogametic sex, these females have a heterochromatic block in the W chromosome that is absent in the Z chromosome, but these chromosomes are the same size. These authors suggest that in *Leporinus* sp2, this system originated independently of those already attributed to this family.

For some time, it was thought that an XY-type sexual system existed in *Leporinus lacustris*. Galetti et al. (1981) suggested that this new sex chromosome system originated from the loss of the essential genetic material of a homolog pair, leading to the conversion of an X chromosome in the pair to a smaller Y chromosome. However, results from a previous study involving chromosome banding and the analysis of the synaptonemal complex by Mestriner et al. (1995) did not support this hypothesis.

Some repetitive sequences localized in heterochromatic regions isolated from *Leporinus* species evidenced the highly dynamic nature of this portion of the fish

genome. Parise-Maltempi et al. (2007) isolated repetitive DNA that was restricted to the conspicuous sexual system of *L. elongatus* (LeSpeI). This repetitive element is distributed differently in male and female individuals and seems to be related to the diversification of a multiple sex chromosome system in this species. This sequence, which was previously used to map the W chromosomes of other species of the genus, appears to have been involved in the differentiation of sex chromosomes in this genus, as it is only present in species with differentiated sex chromosomes (Marreta et al. 2012). The repetitive element Le*Spe*I was again used in hybridisation experiments to reveal cryptic sex chromosomes in *Leporinus lacustris*, *L. striatus*, *Schizodon borellii*, *S. isognathus*, and *Abramites hypselonotus*, but no indication of the presence of this sequence was observed (Silva et al. 2012, in press). Moreover, this repetitive element was successfully employed as a sex-specific probe to distinguish among parental contributions from female *L. macrocephalus* (Piauçu) and male *L. elongatus* (Piapara) in interspecific hybrids (Hashimoto et al. 2009).

The use of the Le*Spe*I repetitive sequence of *L. elongatus* confirmed the distinct heterochromatic C-banding patterns in chromosomes of the NOR pair in *L. elongatus* (Parise-Maltempi et al. 2007). These different C-banding patterns had previously been detected and are considered to represent a heterochromatin-related polymorphism between homologs of the NOR-bearing chromosome pair (Koehler et al. 1997; Molina et al. 1998; 2007). Recently, another satellite DNA, identified after restriction enzyme digestions, showed hybridisation signals that were restricted to the NOR region of *L. elongatus* chromosomes, reinforcing the presence of at least two different classes of heterochromatin in this pair (Silva et al. 2012).

Another sequence, a LINE element named Le*Spe*II also isolated from *L. elongatus*, was used against different species of Anostomidae in FISH experiments (Silva et al. 2012, in press). The results revealed a wide distribution pattern in all chromosomes of anostomids comparable with the patterns observed for previously described transposon elements. This result suggests that this sequence may be related to the evolution of different components of the genome.

Nakayama et al. (1994) also isolated two sex-specific satellite DNAs of *L. elongatus* and identified three atypical W chromosomes, suggesting that recombination events occurred between W and Z during female meiosis, resulting in

three new W chromosomes (W_1, W_2 and W_3) and three new Z chromosomes (Z_1, Z_2 and Z_3). These chromosomes associated with normal Z male chromosomes during reproduction, resulting in six new genotypes (ZW_1, ZW_2, ZW_3, ZZ_1, ZZ_2 and ZZ_3).

Cross-FISH using probes of whole W chromosomes of *L. macrocephalus* and *L. obtusidens* and W1 of *L. elongatus* produced a hybridisation pattern that suggests a common origin of the differentiated chromosomes of these species. However, in fish without sex chromosomes, this was not observed, indicating the absence of cryptic sex chromosomes sharing these sequences (Silva et al. 2012, unpublished data).

Another satellite, 483 bp in length, was isolated from *L. obtusidens* and hybridized to chromosomes from various species of the same genus. However, this satellite was only observed in *L. elongatus*, where it was present in the pericentromeric region of only one chromosome pair. This study showed that this satellite is an important cytogenetic marker for this species (Koehler et al. 1997).

The heterochromatin that appears to be associated with the NOR may also contribute to the dislocations at this sites. This event seems to be responsible for the differentiation between some species of *Leporinus piau*, *L. striatus*, *L. taeniatus*, and *L. amblyrhyncus* (Galleti et al. 1991a). Heterochromatic segments linked to the NOR were also observed in other Anostomidae species, including *L. elongatus*, *L. friderici*, *L. obtusidens*, *Leporellus vittatus*, *Schizodon altoparanae*, *S. fasciatus*, *S. intermedius*, *S. knerii*, *S. nasutus*, and *S. vittatus*. These segments have played an important role in the reorganization of chromosomes, through changes in quantity and/or quality (Galleti et al. 1991b; Koehler et al. 1997; Martins and Galleti 1997).

CONCLUSIONS

Despite the apparent karyotypic stability of anostomids, a variety of species-specific and sex-specific sequences are available, making it possible to establish specific chromosomal markers for the family. Also, considering the wealth of information about chromosome organization of Anostomidae species, these fish have great potential as a model system for the study of chromosome evolution and the origin of sex chromosomes.

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Legends caption

FIG. 1 Karyotype of *Abramites hypselonotus* showing the typical diploid number of Anostomidae (A). C-banded metaphase of *Abramites hypselonotus* Bar =10 µm.

FIG. 2 Idiogram of representative species of Anostomidae showing the major cytogenetic markers available.

Figure 1

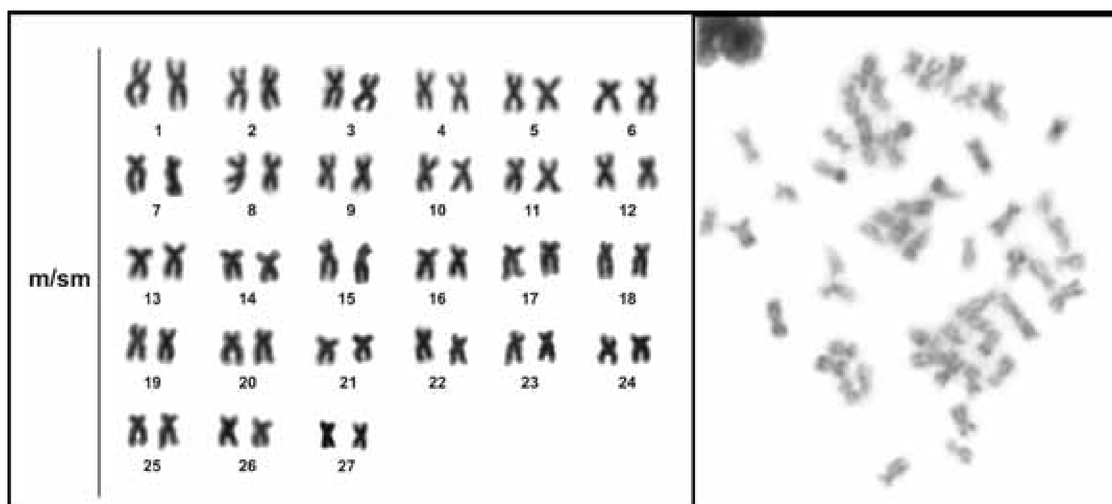

Figure 2

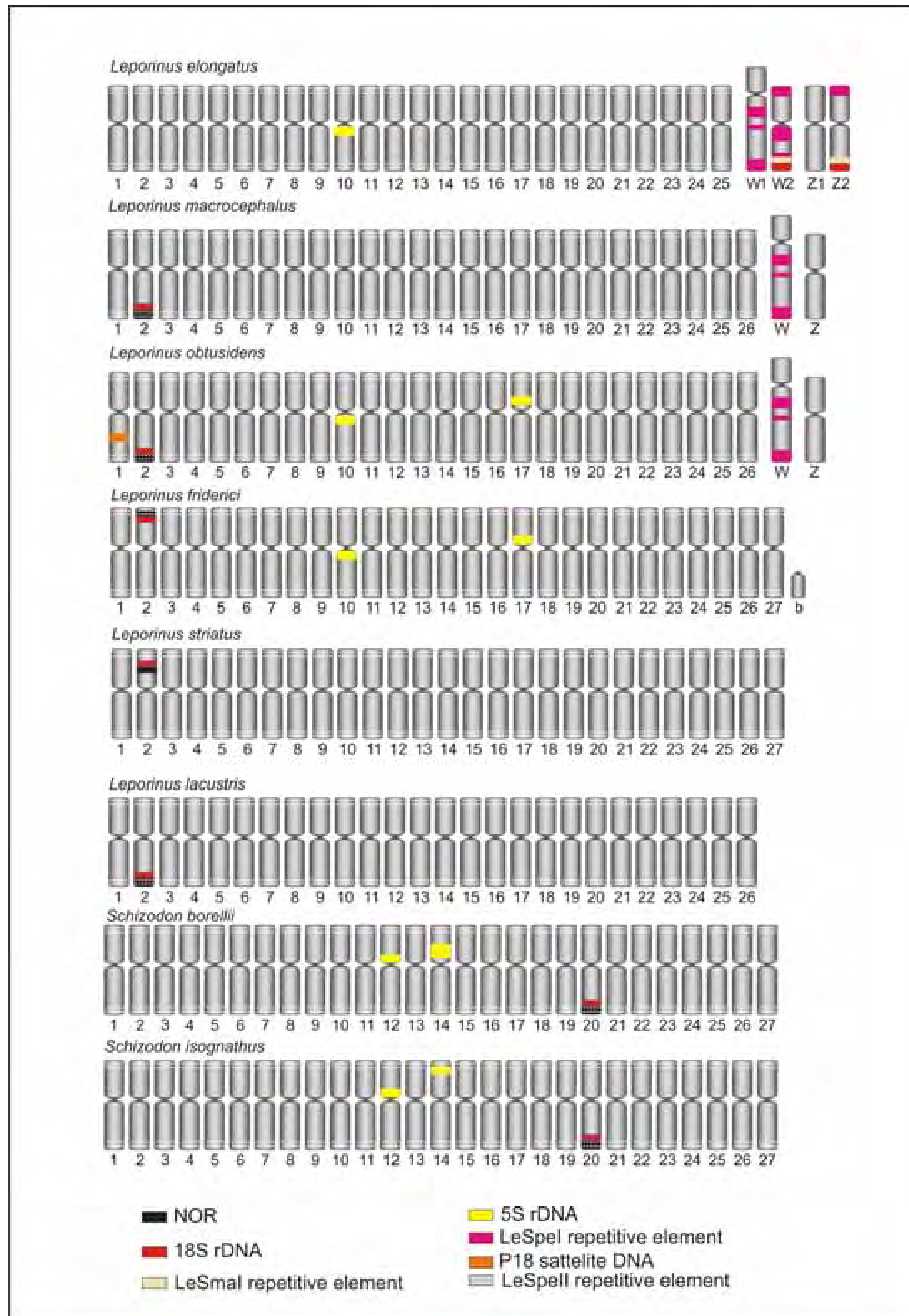

Table 1 The diploid number (2n), karyotypic formulae, number of the nucleolar organising regions (NOR), and banding types available data of species from Anostomidae family

CMA3= Chromomycin A3 satin; C= C band; D= Dystamycin stain; MM= Mytramycin stain; HSS= banding by hot saline solution; p18= repetitive DNA; RGB= Incorporation of 5'bromodeoxyuridine; LeSpeI= repetitive sequence; 45S, 5S and 18S= ribosomal sequences

Genus/specie	River	Drainage	2n	Sex chromosome	Karyotype	NOR	Band	B	References
Abramites									
A. hypselonotus	-	Paraguay River	54	-	M/SM	1	18S	-	Present paper
A. solarii		Amazon Basin	54		M/SM	1	C		Martins et al. 2000
Anostomus									
Anostomus ternetzi		Amazon Basin	54		M/SM	1	C		Martins et al. 2000
Leporellus	-	-	54	-	-		-		
L. vittatus	-	-	54	-	-	1	-	-	Galetti et al. 1984
L. vittatus	Mogi-Guaçú	Paraná River	54	-	-	-	-	-	Galetti et al. 1981a
L. vittatus	Mogi-Guaçú	Paraná River	54	-	M/SM	1	CMA_3, D, 45S, 5S	-	Aguilar; Galletti 2008
L. vittatus	São Francisco	São Francisco	54	-	M/SM	1	CMA_3, D, 45S, 5S	-	Aguilar; Galletti 2008
L. vittatus	Araguaia	Araguaia-Tocantins	54	-	M/SM	1	CMA_3, D, 45S, 5S	-	Aguilar; Galletti 2008
L. vittatus	Mogi-Guaçú	Paraná River	54	-	M/SM	1	C	-	Galetti et al. 1991a
L. vittatus	São Francisco	São Francisco		-	M/SM	1	C	-	Galetti et al. 1991a
Leporinus	-	-	54		-		-		
Leporinus sp.	Rio das mortes	Araguaia-Tocantins	54	-	-	-	C, CMA_3	1	Venere et al. 1999
Leporinus sp.	Miranda	Paraguay	-	ZZ ZW	-	-	C	-	Galetti; Foresti 1986
Leporinus sp.	Araguaia	Araguaia-Tocantins	54	-	-	-	5S	-	Ferreira et al. 2007
Leporinus sp2.	Araguaia	Araguaia-Tocantins	-	-	26m 28sm	1	C, MM	-	Venere et al. 2004
L. aff. elongatus	São Francisco	São Francisco	54	-	-	-	5S	-	Ferreira et al. (2007)

Table 1 cont.

	River	Drainage	2n	Sex chromosome	Karyotype	NOR	Band	B	References
L. amblyrhyncus	São Francisco	São Francisco	-	-	M/SM	1	C	-	Galetti et al. (1991b)
L. cf. elongatus	São Francisco	São Francisco	54	-	-	-	5S	-	Martins; Galetti (2001)
L. desmotes	Tocantins	Tocantins	54	-	M/SM	1	C, MM	-	Margarido; Galetti (2000)
L. elongatus	Mogi-Guaçú	Paraná River	54	ZZ ZW	-	1	5S	-	Martins; Galetti (1999)
L. desmotes	Tocantins	Tocantins	54	-	M/SM	1	C, MM	-	Margarido; Galetti (2000)
L. elongatus	Mogi-Guaçú	Paraná River	54	ZZ ZW	-	1	5S	-	Martins; Galetti (1999)
L. desmotes	Tocantins	Tocantins	54	-	M/SM	1	C, MM	-	Margarido; Galetti (2000)
L. elongatus	Mogi-Guaçú	Paraná River	54	ZZ ZW	-	1	5S	-	Martins; Galetti (1999)
L. elongatus	Mogi-Guaçú	Paraná River	-	ZZ ZW	-	1	C, MM, HSS	-	Artoni et al. (1999)
L. elongatus	-	-	54	-	-	-	P18	-	Koehler et al. 1997 a
L. elongatus	Mogi-Guaçú	Paraná River	54	ZZ ZW	M/SM	1	C, MM	-	Koehler et al. (1997 b)
L. elongatus	Mogi-Guaçú	Paraná River	54	ZZ ZW	-	-	C	-	Galetti; Foresti (1986)
L. elongatus	-	-	54	-	-	1	-	-	Galetti et al. (1984)
L. elongatus	-	-	54	ZZ ZW	M/SM	1	C	-	Porto-Foresti et al. (2008)
L. elongatus	Mogi-Guaçú	Paraná River	54	ZZ ZW	M/SM	1	C D	-	Molina et al. (2008)
L. elongatus	Paranapanema	Paraná River	54	ZZ ZW	M/SM	1	C D	-	Molina et al. (2008)
L. elongatus	Paranapanema	Paraná River	54	ZZ ZW	M/SM	1	C, RGB	-	Molina; Galetti (2007)
L. elongatus	Mogi-Guaçú	Paraná River	54	ZZ ZW	M/SM	1	C, LeSpeI	-	Parise-Maltempi et al. (2007)
L. elongatus	São Francisco	São Francisco	3n 81	ZZ ZW	M/SM	-	MM	-	Molina et al. (2007)

Table 1 cont.

	River	Drainage	2n	Sex chromosome	Karyotype	NOR	Band	B	References
L. elongatus	Mogi-Guaçú	Paraná River	54	-	-	-	5S	-	Martins; Galetti (2001)
L. elongatus	Mogi-Guaçú	Mogi-Guaçú	54	ZZ ZW	M/SM	1	Le*Sma*I	-	Silva et al. (2012)
L. elongatus	-	-		ZZ ZW	M/SM	1	18S	-	Present paper
L. friderici	Mogi-Guaçú	Paraná River	54	-	-	1	5S	-	Martins; Galetti (1999)
L. friderici	-	-	54	-	-	-	C, CMA_3	1	Venere et al. (1999)
L. friderici	Araguaia	Araguaia-Tocantins	54	-	-	-	C, CMA_3	1	Venere et al. (1999)
L. friderici	Mogi-Guaçú	Paraná River	54	-	M/SM	1	C, MM	-	Koehler et al. (1997b)
L. friderici	Madeira	Amazon	54	-	M/SM	1	C	-	Galetti et al. (1991a)
L. friderici	-	-	54	-	-	1	-	-	Galetti et al. (1984)
L. friderici	Mogi-Guaçú	Paraná River	-	-	-	-	-	-	Galetti et al. (1981a)
L. friderici	Mogi-Guaçú	Paraná River	-	-	-	-	5S	-	Ferreira et al. (2007)
L. friderici	Mogi-Guaçú	Paraná River	54	-	-	-	5S	-	Martins; Galetti (2001)
L. friderici	-	Paraguay River	54	-	M/SM	1	18S	-	Present paper
L. lacustris	Mogi-Guaçú	Paraná River	54	XX XY	-	-	-	-	Galetti et al. (1981b)
L. lacustris	-	-	-	-	-	1	-	-	Galetti et al. (1984)
L. lacustris	-	Paraguay River		-	M/SM	1	18S	-	Present paper
L. macrocephalus	Paraguay	Paraguay River	54	-	-	-	5S	-	Ferreira et al. (2007)
L. macrocephalus	-	-	54	ZZ ZW	M/SM	1	C	-	Porto-Foresti et al. (2008)
L. macrocephalus	-	Paraguay River	54	ZZ ZW	M/SM	1	18S	-	Present paper
L. obtusidens	-	-	54	-	-	1	-	-	Galetti et al. 1984

Table 1 cont.

	River	Drainage	2n	Sex chromosome	Karyotype	NOR	Band	B	References
L. obtusidens	Mogi-Guaçú	Paraná River	-	ZZ ZW	-	1	5S	-	Martins and Galetti (1999)
L. obtusidens	-	-	54	-	-	-	P18	-	Koehler et al. (1997)
L. friderici	Mogi-Guaçú	Paraná River	-	-	-	-	-	-	Galetti et al. (1981a)
L. friderici	Mogi-Guaçú	Paraná River	-	-	-	-	5S	-	Ferreira et al. (2007)
L. friderici	Mogi-Guaçú	Paraná River	54	-	-	-	5S	-	Martins; Galetti (2001)
L. friderici	-	Paraguay River	54	-	M/SM	1	18S	-	Present paper
L. lacustris	Mogi-Guaçú	Paraná River	54	XX XY	-	-	-	-	Galetti et al. (1981b)
L. lacustris	-	-	-	-	-	1	-	-	Galetti et al. (1984)
L. obtusidens	Mogi-Guaçú	Paraná River	54	ZZ ZW	M/SM	1	C, MM	-	Koehler et al. (1997b)
L. obtusidens	Mogi-Guaçú	Paraná River	54	ZZ ZW	-	-	-	-	Galetti et al. (1981b)
L. obtusidens	Paranapanema	Paraná River	-	ZZ ZW	M/SM	1	C, RGB	-	Molina; Galetti (2007)
L. obtusidens	Mogi-Guaçú	Paraná River	-	-	-	-	5S	-	Ferreira et al. (2007)
L. obtusidens	Mogi-Guaçú	Paraná River	-	-	-	-	5S	-	Martins; Galetti (2001)
L. obtusidens	-	-	54	ZZ ZW	M/SM	1	18S	-	Present paper
L. octofasciatus	Paranapanema	Paraná River	54	-	-	-	5S	-	Ferreira et al. (2007)
L. octofasciatus	-	-	54	-	-	1	-	-	Galetti et al. (1984)
L. octofasciatus	-	-	54	-	-	-	-	-	Galetti et al. (1981a)
L. piau	São Francisco	São Francisco	54	-		1	C		Galetti et al. (1991a)
L. reinhardti	São Francisco	São Francisco	54	ZZ ZW	-	-	C	-	Galetti; Foresti (1986)
L. reinhardti	São Francisco	São Francisco	54	ZZ ZW	M/SM	1	C, RGB	-	Molina; Galetti (2007)
L. silvestrii	Mogi-Guaçú	Paraná River	54	ZZ ZW	-	-	-	-	Galetti et al. (1981b)
L. striatus	Mogi-Guaçú	Paraná River	54	-	M/SM	1	C	-	Galetti et al. (1991b)
L. striatus	-	-	54	-	-	1	-	-	Galetti et al. (1984)

Table 1 cont.	River	Drainage	2n	Sex chromosome	Karyotype	NOR	Band	B	References
L. striatus	Mogi-Guaçú	Paraná River	54	-	-	-	-	-	Galetti et al. (1981a)
L. striatus	-	Paraguay River	54	-	M/SM	1	18S	-	Present paper
L. taeniatus	São Francisco	São Francisco	54	-	M/SM	1	C	-	Galetti et al. (1991b)
L. trifasciatus	Araguaia	Araguaia-Tocantins	54	ZZ ZW	22m 30sm	1	C, MM	-	Venere et al. (2004)
Piaupara (hybrid)	-	-		ZZ ZW	M/SM	1 ht	C	-	Porto-Foresti et al. (2008)
Pseudanos									
P. trimaculatus		Amazon basin	54		M/SM	1	C	-	Martins et al. (2000)
Schizodon									
S. altoparanae	Paranapanema	Paraná River	54	-	-	1	5S	-	Martins; Galetti (2000)
S. altoparanae	Paranapanema	Paraná River	54	-	M/SM	1	C, MM	-	Martins; Galetti (1997)
S. borellii	Cuiabá	Paraguay River	54	-	-	1	5S	-	Martins; Galetti (2000)
S. borellii	Miranda	Paraguay River	54	-	-	1	5S	-	Martins; Galetti (2000)
Schizodon									
S. altoparanae	Paranapanema	Paraná River	54	-	-	1	5S	-	Martins; Galetti (2000)
S. altoparanae	Paranapanema	Paraná River	54	-	M/SM	1	C, MM	-	Martins; Galetti (1997)
S. borellii	Cuiabá	Paraguay River	54	-	-	1	5S	-	Martins; Galetti (2000)
S. borellii	Miranda	Paraguay River	54	-	-	1	5S	-	Martins; Galetti (2000)
S. borellii	Vermelho	Paraguay River	54	-	-	1	5S	-	Martins; Galetti (2000)
S. borellii	-	Paraguay River	54	-	M/SM	1	18S	-	Present paper
S. fasciatus	Madeira	Amazon	54	-	M/SM	1	C	-	Galetti et al. (1991a)
S. intermedius	Águas S. Pedro	Paraná River	54	-	M/SM	1	C, MM	-	Martins; Galetti (1997)
S. isognathus	Vermelho	Paraguay River	54	-	-	1	5S	-	Martins; Galetti (2000)
S. isognathus	-	Paraguay River	54	-	M/SM	1	18S	-	Present paper

Table 1 cont.	River	Drainage	2n	Sex chromosome	Karyotype	NOR	Band	B	References
S. knerii	São Francisco	São Francisco	54	-	-	1	5S	-	Martins; Galetti (2000)
S. knerii	São Francisco	São Francisco	54	-	M/SM	1	C, MM	-	Martins; Galetti (1997)
S. nasutus	Mogi-Guaçú	Paraná River	54	-	-	1	5S	-	Martins; Galetti (2000)
S. nasutus	Paraná	Paraná River	54	-	-	1	5S	-	Martins; Galetti (2000)
S. nasutus	Mogi-Guaçú	Paraná River	54	-	M/SM	1	C, MM	-	Martins; Galetti (1997)
S. nasutus	Paraná	Paraná River	54	-	M/SM	1	C, MM	-	Martins; Galetti (1997)
S. nasutus	Paraná	Paraná River	54	-	32m 22sm	1	C	1	Pastori et al. (1997)
S. nasutus	Mogi-Guaçú	Paraná River	54	-	M/SM	1	C	-	Galetti et al. (1991a)
S. nasutus	-	-	54	-	-	1	-	-	Galetti et al. (1984)
S. nasutus	Mogi	Paraná River	54	-	-	-	-	-	Galetti et al. (1981a)
S. vittatus	Amazon	Amazon	54	-	M/SM	1	C, MM	-	Martins; Galetti (1997)
S. vittatus	Araguaia	Araguaia- Tocantins	-	-	M/SM	1	C, MM	-	Martins; Galetti (1997)

5. CONSIDERAÇÕES FINAIS

Os resultados do presente trabalho permitiram uma visão mais abrangente sobre a diversidade dos elementos repetitivos no genoma de espécies da família Anostomidae. O mapeamento destas seqüências permitiu uma série de observações que devem ser ressaltadas, como por exemplo, que:

- Os elementos repetitivos presentes no genoma de espécies da família Anostomidae tiveram importante papel na diversificação do cariótipo, apesar da aparente estabilidade cromossômica há tempos relatada para a família.

- O elemento repetitivo Le*Sma*I constitui uma fração do segmento heterocromático de *L. elongatus* e de alguma forma está relacionado com a diferenciação do sistema múltiplo de cromossomos sexuais da espécie. Constituem-se também interessantes marcadores espécie-específicos.

- O elemento repetitivo Le*Spe*II apresenta a particularidade de estar disperso no genoma de todas as espécies analisadas. Sua presença predominantemente nos autossomos, pode ter favorecido o surgimento e fixação de outros elementos repetitivos como Le*Spe*I e Le*Sma*I nos cromossomos sexuais de *L. elongatus*.

- Os resultados obtidos através da hibridação com sondas dos cromossomos W de *L. macrocephalus* e *L. obtusidens* e o cromossomo W_1 provenientes de *L. elongatus* indicam que os cromossomos sexuais nestas espécies possuem uma origem comum. As espécies *L. friderici*, *L. striatus*, *L. lacustris, S. borellii* e *S. isognathus* não compartilham seqüências desses cromossomos.

6. FINAL CONSIDERATIONS

The results presented here allowed a comprehensive vision about the diversity of repetitive elements in anostomids genomes. The chromosome mapping of these sequences also permits a series of considerations that can be highlighted:

- The repetitive elements present in the anostomids genome have had an important role in the karyotype diversification, despite the apparent chromosomal stability for many times reported to the family.

- The repetitive element Le*Sma*I constitutes a fraction of heterochromatic segments of *L. elongatus* and somehow is related to differentiation of the multiple sex chromosome system of this species. It is also an interesting species-specific marker.

- The repetitive element Le*Spe*II has the particularity of being dispersed in the genome of all species analyzed. Their presence predominantly in autosomes, may have favoured the emergence and establishment of other repetitive elements as Le*Spe*I Le*Sma*I and the sex chromosomes of *L. elongatus*.

- The results obtained through hybridisation with probes of W chromosomes from *L. macrocephalus* and *L. obtusidens* and W_1 chromosome from *L. elongatus* indicate the sex chromosomes in these species have a common origin.

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