UNIVERSIDADE FEDERAL DE SÃO CARLOS

PÓS-GRADUAÇÃO EM GENÉTICA EVOLUTIVA

E BIOLOGIA MOLECULAR

Evolução cromossômica na família Lebiasinidae (Teleostei, Characiformes), com enfoque em espécies do gênero *Lebiasina*

Francisco de Menezes Cavalcante Sassi

São Carlos – SP, Brasil

2020

UNIVERSIDADE FEDERAL DE SÃO CARLOS

PÓS-GRADUAÇÃO EM GENÉTICA EVOLUTIVA E BIOLOGIA MOLECULAR

Francisco de Menezes Cavalcante Sassi

Evolução cromossômica na família Lebiasinidae (Teleostei, Characiformes), com enfoque em espécies do gênero *Lebiasina*

Dissertação de Mestrado apresentada ao programa de Pós-Graduação em Genética Evolutiva e Biologia Molecular do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de Mestre em Ciências (Ciências Biológicas).

Área de Concentração: Genética e Evolução.

Orientador: **Prof. Dr. Marcelo de Bello Cioffi**

São Carlos – SP, Brasil

2020

UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Genética Evolutiva e Biologia Molecular

Folha de Aprovação

Assinaturas dos membros da comissão examinadora que avaliou e aprovou a Defesa de Dissertação de Mestrado do candidato Francisco de Menezes Cavalcante Sassi, realizada em 06/02/2020:

Prof. Dr. Marcelo de Bello Cioffi
UFSCar

Profa. Dra. Karine Frehner Kavalco
UFV

Profa. Dra. Mara Cristina de Almeida
UEPG

Identificação Bibliográfica

Nome e sobrenome: **Francisco de Menezes Cavalcante Sassi**

Título da Dissertação: **Evolução cromossômica na família Lebiasinidae (Teleostei, Characiformes), com enfoque em espécies do gênero *Lebiasina***.

Título da Dissertação em Inglês: **Chromosomal Evolution in Lebiasinidae family (Teleostei, Characiformes), with emphasis on *Lebiasina* species**.

Programa de estudo: Pós-Graduação em Genética Evolutiva e Biologia Molecular

Orientador: Prof. Dr. Marcelo de Bello Cioffi

Ano de início: 2018

Ano da defesa: 2020

Palavras-chave: Peixes, Cromossomos, Evolução, DNAs Repetitivos

Keywords: Fishes, Chromosomes, Evolution, Repetitive DNAs

"Em nosso tempo, temos assistido à emergência de um modelo diferente de ciência, caracterizado pela dependência de grandes recursos financeiros, a privatização e o sigilo da pesquisa, a mercantilização dos resultados, a burocratização das instituições e a instrumentalização da ciência, submetendo-a a interesses extra-científicos (...) Se opor a esta tendência é necessário para rejuvenescer o ethos da ciência, retornando aos princípios mertonianos, mas ao mesmo tempo, reformulando-os, de modo mais adequado para as atuais estruturas institucionais da ciência pós-acadêmica."

Piotr Sztompka

AGRADECIMENTOS

- À minha mãe Marina e minha irmã Ana Clara, que me apoiaram e guiaram em todas as escolhas que tive que fazer para chegar até aqui, sempre com muito amor e carinho.

- Ao meu pai Roberto, que independentemente da situação, sempre acreditou no meu potencial;

- À minha vó Helena, meu avô Nick, meu tio Álvaro, minha madrinha Arlete, meu segundo pai Valdir, por todas as palavras de apoio e incentivo que com certeza me ajudaram e muito a chegar aqui;

- Ao Professor Doutor Luiz Antônio Carlos Bertollo, que é inspiração para todo cientista, e acreditou na missão de me orientar durante o mestrado;

- Ao Professor Doutor Orlando Moreira Filho, por todas as ideias e discussões científicas que tivemos, assim como de certa forma política, me fazendo enxergar um lado importante da profissão de biólogo;

- Ao Professor Doutor Marcelo de Bello Cioffi, que desde o início me orientou neste mestrado e me recebeu de portas abertas ao LCP.

- Aos Professores Doutores Rubens Pasa e Karine Frehner Kavalco, que me ensinaram muito ao longo dessa jornada, me introduzindo à citogenética e à divulgação científica, mostrando que o papel do cientista é muito maior do que só na ciência.

- À equipe do Laboratório de Citogenética de Peixes: Piau, Toninho, Carlinha, Caio, Ezek, Manolo, Renata, Terumi, Gustavo, Geize, Lari, Fernando, Pedro, Priscila, Juliana e Giovana, que tornaram a rotina algo prazeroso, também me ensinando muito sobre ciência e sobre a vida;

- Ao Luiz, Vinícius (Alemão), Mauro Yida e Matheus Lewi, aos quais agradeço todos os dias por terem aparecido na minha vida. Encontrar amigos como vocês não é fácil, obrigado por tudo;

- Aos meus amigos de república, que me aturaram durante esses anos de convivência. Sinto saudades de todos: Vitor Augusto, Rodrigo (Capela), Gabriel, Matheus Lunardi, Vinícius Barros e Renan Rodrigues;

- Aos amigos que fiz em São Carlos: Robson, Daniel (Foguete), Arthurzão, Luan (Atchim), Fernando, Bruno, Thiagão e 2K, por todas as risadas e momentos de descontração;

- A toda a equipe do LaGEEvo da UFV-CRP, que ajudaram, e muito, a me formar como pessoa e pesquisador, em especial à Snaydia, Rosana, Dinaíza, Winston, Marcos Silva, Igão, Rafaella, Gabi Leles, Larissa, Gabi Oliveira e Iuri Silva, torço muito por todos vocês;

- Aos meus amigos de Guarujá, em especial para o pessoal do Debate: Lelo, Rodrigo, Caio, Felipe, Enzo e Pedro, obrigado por todas as risadas que demos juntos;

- À toda equipe do Poeira Estelar, Próxima Parada: Cerrado, e Rock com Ciência, projetos de extensão que sinto orgulho de fazer parte.

- Ao Augusto Luiz Ferreira Jr., por todas as conversas que tivemos e ensinamentos transmitidos;

- Ao Professor Doutor Mauro Nirchio, que cedeu algumas das amostras analisadas nesse trabalho;

- À Doutora Manoela Maria Ferreira Marinho, pela identificação dos exemplares de Lebiasinidae utilizados neste e em outros trabalhos;

- À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, pela bolsa de mestrado concedida.

- À Alexandra Elbakyan, pelo SciHub e pela representação de resistência que ela é.

Agradeço a todos vocês. Sem vocês, esse trabalho não seria possível.

RESUMO

A família Lebiasinidae é composta por sete gêneros de pequenos peixes de água doce, sendo encontrados em pequenos riachos da América Central e do Sul. Os membros desta família apresentam uma grande diversidade de formas corpóreas e coloração, mas suas relações evolutivas se mantêm inexplicadas. Nosso objetivo é realizar as primeiras análises citogenéticas em duas espécies do gênero *Lebiasina*, permitindo investigações acerca das características cariotípicas e de sua evolução cromossômica. Para isso, *Lebiasina bimaculata* e *L. melanoguttata* apresentaram 2n = 36 cromossomos em seu cariótipo, exclusivamente composto por cromossomos meta/submetacêntricos. A heterocromatina C-positiva se localizou nas regiões centroméricas e teloméricas de diversos cromossomos em ambas as espécies. Em *L. melanoguttata*, uma série intersticial de heterocromatina C-positiva é obervada, sendo ausente em *L. bimaculata.* O rDNA 5S se encontra na região intersticial do braço q do primeiro par cromossômico em ambas as espécies, com um sítio adicional na região telomérica do cromossomo 13 em *L. melanoguttata*. O rDNA 18S está localizado na região telomérica do terceiro par em *L. bimaculata*, enquanto *L. melanoguttata* apresenta múltiplas marcações localizadas na região telomérica dos braços longos dos pares 1 e 3, na região telomérica do braço curto dos pares 7 e 9 e em ambos os telômeros do segundo par cromossômico. A pintura cromossômica total com sonda do primeiro par cromossômico revelou o compartilhamento total do conteúdo genômico deste par entre ambas as espécies de *Lebiasina* e *Boulengerella* (Ctenoluciidae), evidenciando o relacionamento próximo destas famílias. Adicionalmente, a hibridização genômica comparativa entre as duas espécies de *Lebiasina* revelou um alto grau de diferenciação genômica entre elas. Outro experimento de hibridização genômica comparativa revelou uma região telomérica sexo-específica no terceiro par cromossômico de fêmeas de *L. bimaculata*. Este resultado, assim como o padrão diferencial de bandas C-positivas, coloração diferencial CMA3+ e a hibridização fluorescente com o microssatélite

$(CGG)_n$ indica duas possibilidades i) a presença de uma variação de número de cópias, gerando diferenças entre os pares homólogos, ou ii) um sistema cromossômico sexual nascente do tipo ZZ/ZW. Além disso, é possível observar duas tendências na evolução cromossômica da família Lebiasinidae, sendo a primeira responsável por uma provável conservação do número diploide em Lebiasininae e, contrariamente, a tendência de acrocentrização em Pyrrhulininae.

ABSTRACT

The Lebiasinidae family is composed by seven genus of small freshwater fishes that can be found in small rivers of Central and South America. Members of this family presents a great diversity in form and body color, but their evolutionary relationships are still unclear. Our objective was to realize the first cytogenetical analysis in two species of *Lebiasina* genus, aiming to clarify their karyotypic characteristics and their chromosomal evolution. For this, *Lebiasina bimaculata* and *L. melanoguttata* presents 2n = 36 as their diploid number, exclusively composed by bi-armed chromosomes. The C-positive heterochromatin was located in centromeric and telomeric regions of several chromosomes in both species. But, in *L. melanoguttata* there is a remarkable interstitial series of c-positive heterochromatin, absent in *L. bimaculata*. The rDNA 5S is located in the interstitial region on q-arm of the first chromosomic pair in both species, with an additional site on telomeric region of 13 chromosome of *L. melanoguttata*. The 18S rDNA can be found in telomeric region of the third pair in *L. bimaculata*, while *L. melanoguttata* presents multiple marks located in telomeric region on long arm of pairs 1 and 3, in telomeric region on short arm of 7 and 9 pairs and in both regions of the second chromosomic pair. The whole chromosome painting with a probe from the first chromosomic pair showed a share of genomic content between the two *Lebiasina* species and *Boulengerella* (Ctenoluciidae), evidencing the close relationship between this two families. Additionally, the comparative genomic hybridization among the two *Lebiasina* species reveals a high level of genomic differentiation amid them. Another experiment of comparative genomic hybridization reveals a telomeric sex-specific region on the third chromosomic pair of *L. bimaculata* females. This result with the differential pattern of c-banding, CMA3+ differential stain and the fluorescent in situ hybridization with the microsatellite probe (CGG)n indicates two possibilities (i) the presence of a copy number variation process, generating differences between the homologous pair, or (ii) a nascent sex chromosome system of ZZ/ZW type. Besides

that, two chromosomal evolutionary pathways were recognized in Lebiasinidae family, being the first responsible for a probable diploid number conservation in Lebiasininae and, in contrast, an acrocentrization tendency in Pyrrhulininae.

LISTA DE FIGURAS

Material e Métodos

Capítulo 1

d). Genomic probes from *L. melanoguttata* and *N. trifasciatus* hybridized against *L. melanoguttata* chromosomes (**e – h**). Genomic probes from *L. melanoguttata* and *N. beckfordi* hybridized against *L. melanoguttata* chromosomes (**i – l**). Genomic probes from *L. melanoguttata* and *N. eques* hybridized against *L. melanoguttata* chromosomes (**m – p**). Genomic probes from *L. melanoguttata* and *N. marginatus* hybridized against *L. melanoguttata* chromosomes (**q** – **t**). First column (**a, e, i, m** and **q**): DAPI images (blue); second column (**b**, **f, j**, **n** and **r**): hybridization patterns using gDNA probe from *L. melanoguttata*; third column (**c, g, k, o** and **s**): hybridization patterns using gDNA probes from *N. unifasciatus*, *N. trifasciatus*, *N. beckfordi*, *N. eques* and *N. marginatus*, respectively; fourth column (**d**, **h, l, p** and **t**) merged images of both genomic probes and DAPI staining depicting the shared regions in yellow. Bar = 5 μm.

LISTA DE TABELAS

SUMÁRIO

I – INTRODUÇÃO

1.1 Peixes: diversidade e modelo para estudos evolutivos

Os peixes compreendem o grupo mais diverso dentre os vertebrados viventes, com mais de 30 mil espécies atualmente descritas (Nelson *et al*., 2016). No entanto, o grupo deve ser considerado parafilético, pois não inclui descendentes todos de um mesmo ancestral. Tal riqueza taxonômica encontra-se acompanhada de uma grande variedade de hábitos e habitats explorados por esse grupo. Essa diversidade de espécies fica ainda mais notável quando consideramos o ambiente de água doce, que abriga mais de 40% de toda as espécies de peixes (Nelson *et al*., 2016), sendo 25% destas na região Neotropical (Vari & Malabarba, 1998), estendendo-se do sul dos Estados Unidos até o sul da Argentina.

Os peixes de água doce constituem um modelo importante para estudos evolutivos e biogeográficos, pois sua evolução pode ser intimamente associada à evolução geológica da Terra (Lundberg, 1993). Consequentemente, cada continente apresenta uma fauna particular de peixes de água doce, onde os padrões distintos de distribuição são decorrentes de barreiras físicas que interromperam a dispersão de muitas espécies antepassadas. A maioria das espécies de peixes ocorre nas regiões tropicais e subtropicais, com uma redução global da diversidade em regiões temperadas e polares (Berra, 2001). A África tropical, o sudeste asiático e a bacia do rio Amazonas se destacam pela riqueza de espécies (Lévêque *et al*. 2008, Nelson *et al*. 2016), enquanto a região da América Central é relativamente pobre em diversidade em decorrência de sua história geológica.

Grande parte da diversidade de peixes de água doce está incluída na ordem Characiformes (Nelson *et al*., 2016), onde são encontrados grupos com baixa tolerância a água salgada e uma notável variação morfológica, o que os torna particularmente atraentes para estudos evolutivos. Essa ordem encontra-se dividida em duas subordens monofiléticas: Citharinoidei (peixes africanos, 110 espécies divididas em duas famílias) e Characoidei (mais

de 2 mil espécies distribuídas em 22 famílias, sendo apenas duas destas africanas) (Betancur-R *et al*., 2018). A grande diversificação dos Characoidei é datada de antes da fragmentação final da Gondwana, sugerindo um papel preponderante das rotas de dispersão pós-fragmentação entre a África e a América do Sul (Chen *et al*., 2013).

Tem sido sugerido que os peixes poderiam apresentar modificações genômicas mais rápidas do que aquelas que ocorrem em outros grupos de vertebrados (Venkatesh, 2003), o que poderia influenciar no grau de diversidade que apresentam. Além de poliploidizações, vários outros eventos, como duplicações gênicas independentes, ocorreram nesse grupo. De fato, há evidências substanciais de que um evento antigo de duplicação do genoma (tetraploidização) estaria relacionado com a diversificação de funções dos genes e especiação em várias linhagens (Zhang *et al*. 2002). O amplo espectro de sistemas de determinação do sexo encontrado nos peixes também ilustra a plasticidade de seus genomas, associado ao fato de que muitas espécies apresentam hermafroditismo, incluindo até mesmo mudança de sexo em um estágio específico de seu ciclo de vida (Ohno, 1967; Devlin & Nagahama, 2002).

Considerando que os peixes ocupam posição basal na filogenia dos vertebrados, estudos em espécies modelos contribuem para esclarecer diversas questões relacionadas com a organização do genoma e evolução dos vertebrados como um todo. No entanto, a enorme biodiversidade desse grupo ainda permanece em grande parte inexplorada. Neste cenário, os estudos citogenéticos vêm fornecendo contribuições importantes para o conhecimento do genoma dos peixes. Nas últimas duas décadas, uma quantidade significativa de dados tem sido gerada, incluindo o mapeamento de sequências de DNAs repetitivos nos cromossomos de diversas espécies (Cioffi & Bertollo, 2012). Tais sequências tem se revelado importantes na organização estrutural e funcional dos genomas (Schueler *et al*. 2001; Biémont & Vieira, 2006), além de proporcionar novos conhecimentos sobre processos evolutivos em muitas espécies de vertebrados.

1.2 Bandamentos e marcadores cromossômicos

A utilização de diversos marcadores cromossômicos tem se mostrado uma ferramenta essencial na caracterização da biodiversidade entre os peixes possibilitando, inclusive, a identificação de espécies crípticas (Moreira-Filho e Bertollo, 1991; Bertollo *et al*., 2000; Cioffi *et al*., 2012). A partir dos primeiros bandamentos cromossômicos permitindo a comparação de bandas para o pareamento correto dos cromossomos homólogos (Guerra, 1988), a citogenética molecular foi gradativamente aperfeiçoando suas metodologias propiciando atualmente diversos procedimentos para uma análise evolutiva mais elaborada. A seguir são consideradas algumas dessas possibilidades que vem sendo utilizadas entre os peixes.

1.2.1 Mapeamento de DNAs repetitivos e regiões heterocromáticas

O bandamento C surgiu no início dos anos 1970 objetivando a identificação das regiões ricas em heterocromatina constitutiva, sendo descrita pela primeira vez por Sumner (1972). Essas regiões são geralmente compostas por diversas sequências de DNAs repetitivos e apresentam-se principalmente localizadas nas regiões pericentroméricas e teloméricas dos cromossomos (Charlesworth, 1994; Plohl *et al*., 2008; López-Flores e Garrido-Ramos, 2012). A fração repetitiva do DNA compõe mais de 80% do genoma eucariótico, apresentando uma alta variabilidade (Gregory, 2005; Plohl *et al*., 2008). Em espécies de peixes dos gêneros *Brycon* e *Leporinus,* a distribuição da heterocromatina constitutiva é relevante na diferenciação das espécies, visto que poucas variações na macroestrutura cariotípica são observadas (Galetti Jr. *et al*, 1991; Margarido e Galleti Jr., 1999; Margarido e Galetti Jr., 2000). Além disso, esse tipo de bandamento possibilita a identificação de sistemas cromossômicos sexuais, como observado em *Hoplias malabaricus* (Bertollo *et al*., 1997) e *Hypostomus* aff. *ancistroides* (Rocha-Reis *et al*., 2018).

Adicionalmente, a localização cromossômica dos genes ribossomais tem também possibilitado elucidar diversos processos relacionados à evolução cromossômica entre os peixes (Gornung, 2013; Rebordinos *et al*., 2013). Os genes ribossomais dão origem ao RNA ribossômico, participando ativamente na síntese proteica, sendo estes expressos por duas famílias multigênicas: o rDNA 45S e o rDNA 5S. No segmento de rDNA 45S encontram-se codificados os rDNAs 28S, 5,8S e 18S, separados por duas regiões conhecidas como espaçadores internos transcritos (ITS1 e ITS2), além de dois espaçadores externos também transcritos (ETS1 e ETS2) e um espaçador não transcrito (NTS). Já o rDNA 5S encontra-se organizado por uma série de repetições, separadas por NTS, podendo ser também encontrado na forma de pseudogenes dispersos nos cromossomos (Lafontaine e Tollervey, 2001).

A detecção das chamadas regiões organizadoras de nucléolos (NORs) foi, primordialmente, realizada a partir da impregnação de nitrato de Prata nos cromossomos (Ag-NOR), conforme proposta de Howell e Black (1980). Dada que a região organizadora de nucléolos é composta por DNAs ribossomais (genes ribossomais 45S e proteínas acessórias) a técnica Ag-NOR foi utilizada, por muitos anos, como a principal forma de se detectar essa família de DNA ribossomal. No entanto, com o advento da citogenética molecular, particularmente a hibridização fluorescente *in situ* (FISH), a posição real dos genes ribossomais, assim como o seu número de cópias, puderam ser mais bem investigadas. A partir do surgimento da FISH e do desenvolvimento de sondas de rDNA, o mapeamento desses genes nos cromossomos tornou-se a primeira opção dos citogeneticistas para a caracterização da biodiversidade, sendo também utilizados em estudos de sistemática (Sochorová *et al*., 2018). Apesar da alta conservação das sequências ribossomais e de sua evolução em concerto (processo que mantém a funcionalidade e homogeneidade das sequências), os rDNAs tendem a mudar em número de cópias e em posição nos cromossomos (Zimmer *et al*., 1981; Dover, 1982; Schubert e Wobus, 1985; Dubcovsky e Dvorak, 1995; Averbeck e Eickbush, 2005; Roy

et al., 2005; McTaggart *et al*., 2007; Wang *et al*., 2017; Sochorová *et al*., 2018), o que os torna extremamente atraentes para estudos evolutivos.

Além das sequências ribossomais, os microssatélites também se destacam entre os DNAs repetitivos, sendo constituídos por pequenas sequências repetidas em tandem, com unidades de repetição de até seis nucleotídeos, encontrados em todos os eucariotos (López-Flores e Garrido Ramos, 2012; Cioffi e Bertollo, 2012). Este tipo de DNA pode se agrupar em longas repetições, constituídas por centenas a milhares de unidades, também se associando com a heterocromatina de diversas espécies (Martins, 2007; Cioffi *et al*., 2011). Em peixes, tais sequências podem ser localizadas comumente nas regiões teloméricas, centroméricas e nos cromossomos sexuais, agrupadas a outras sequências repetitivas (Cioffi e Bertollo, 2012).

Por fim, as sequências teloméricas também se encontram entre aquelas que provêm informações importantes sobre a evolução cromossômica. Tais sequências são ricas em guanina $(TTAGGG)_n$ e desempenham um papel fundamental na sobrevivência dos organismos, estabilizando os cromossomos e permitindo a replicação completa das suas regiões terminais (Blackburn, 1994). Nos eventos de fusão cromossômica restos de regiões teloméricas podem ser encontradas na região onde a fusão ocorreu, conhecidos como sítios teloméricos intersticiais (ITS) (Meyne *et al*., 1990). No entanto, nem sempre os ITS podem ser detectados a partir de um processo de fusão, provavelmente pela perda de tais regiões durante o rearranjo (Schubert *et al*., 1992).

1.2.2 Hibridização genômica comparativa (CGH) e Pintura cromossômica (WCP)

A hibridização genômica comparativa (CGH), utilizando sondas de genomas inteiros, possibilita a comparação de regiões compartilhadas ou que apresentam ganho ou perda de sequências de DNA entre as espécies analisadas, bem como a variação no número de cópias de uma sequência genômica específica. Também desenvolvida inicialmente com enfoque clínico

(Kallioniemi *et al*., 1992), a técnica CGH expandiu suas aplicações, tornando-se uma ferramenta valiosa nos estudos evolutivos, com particular interesse na detecção de poliploidias e hibridizações (Chester *et al*., 2010). Tornou-se também de utilidade nas pesquisas de cromossomos sexuais, permitindo a identificação de homologias entre sistemas, conforme demonstrado no gênero *Triportheus* (Yano *et al*., 2016). Entre os peixes tal procedimento vem sendo cada vez mais utilizado, conforme demonstrado pela quantidade de trabalhos publicados nos últimos anos utilizando tal abordagem (Oliveira e Sember *et al*., 2017; Freitas *et al*. 2017; Moraes *et al*., 2017; 2019; Toma *et al*., 2019; Oliveira *et al*., 2019), ressaltando a relevância de metodologias resolutivas para o avanço da ciência.

Apesar da possibilidade do mapeamento de diversos genes por FISH, o surgimento da pintura cromossômica total (WCP), no final da década de 1990, veio dar um impulso adicional nas abordagens citogenéticas. Esta metodologia surgiu com um enfoque predominantemente clínico, voltado para a detecção de rearranjos cromossômicos envolvidos principalmente com o câncer (Blough *et al*., 1998), utilizando sondas derivadas de cromossomos inteiros (whole chromosome painting) no processo de hibridização. Adicionalmente, as sondas para a pintura podem ser também construídas a partir de segmentos cromossômicos (PCP – partial chromosome paint) fazendo com que diversos loci genômicos possam ser hibridizados.

No entanto, a pintura cromossômica passou a ser também aplicada para estudos evolutivos comparando a hibridização de sondas cromossômicas entre espécies relacionadas, num processo conhecido como cross-FISH ou Zoo-FISH (Yang *et al*., 2009). A obtenção de cromossomos para a construção das sondas pode ser realizada por microdissecção ou por citometria de fluxo, seguido pela amplificação por primers degenerados (DOP-PCR) (Yang *et al*., 2009). Em peixes, tal técnica começou a ser amplamente utilizada a partir da década de 2010, permitindo estudos sobre origem e evolução de cromossomos sexuais e autossômicos em

diversas espécies (Nagamashi *et al.*, 2010; Cioffi *et al*., 2013, Yano *et al*., 2016; Freitas *et al.*, 2017; Oliveira e Sember, 2017; Moraes *et al*., 2017; Barby *et al*., 2019; Moraes *et al*., 2019).

1.3 A família Lebiasinidae, com enfoque no gênero *Lebiasina*

Endêmicos da região Neotropical, os peixes da família Lebiasinidae são encontrados em pequenos riachos da América Central (Costa Rica e Panamá) e América do Sul, exceto o Chile (Fricke *et al*., 2019). São englobados em duas subfamílias, Lebiasininae e Pyrrhulininae, apresentando grande variação de formatos corporais e coloração, o que os tornam particularmente atrativos para a aquariofilia (Weitzman & Weitzman, 2003). Diversos representantes dessa família apresentam-se miniaturizados (Weitzman e Vari, 1988), com um tamanho corpóreo bastante reduzido, como por exemplo *Nannostomus anduzei* com 1,6 cm de comprimento. Além das características de tamanho, grande parte dos Lebiasinidae apresenta dimorfismo sexual visível a olho nu como, por exemplo, a presença de tubérculos nupciais em *Lebiasina* (Netto-Ferreira, 2006).

A família Lebiasinidae foi previamente correlacionada com alguns outros grupos da ictiofauna Neotropical como, por exemplo, Erythrinidae, Ctenoluciidae e Hepsetidae, em decorrência de seus caracteres morfológicos (Oyakawa, 1997; Buckup, 1998). Posteriormente, análises filogenéticas propuseram Erythrinidae e Hepsetidae como grupos irmãos, assim como Lebiasinidae e Serrasalmidae (Orti & Meyer, 1997). Mais tarde, foi proposto que Erythrinidae e Crenuchidae seriam famílias relacionadas entre si, enquanto Ctenoluciidae seria mais relacionada com Lebiasinidae (Calcagnotto *et al*., 2005). Utilizando dados filogenéticos moleculares, Oliveira *et al*. (2011) sugeriram que muitas das sinapomorfias morfológicas entre Erythrinidae, Ctenoluciidae, Hepsetidae e Lebiasinidae poderiam corresponder apenas a convergências associadas a modificações para o estilo de vida predatório desses grupos, mas apoiando uma provável proximidade entre Ctenolucidae e Lebiasinidae. Concordantemente, tal

proximidade foi corroborada pelos estudos moleculares de Arcila *et al*. (2017) e Betancur-R *et al*. (2018).

O gênero *Lebiasina* apresenta 18 espécies válidas (Fricke *et al*., 2019) (**Tabela 1**), distribuídas pelo escudo Andino e Guianas, além de três espécies (*L. marilynae*, *L. melanoguttata* e *L. minuta*) que são encontradas na Serra do Cachimbo – PA, Brasil. Esse gênero é considerado o mais basal dentre os Lebiasinidae e pode ser diagnosticado pela ausência de nadadeira adiposa e a presença de alvéolos na porção anterior da câmara posterior da bexiga natatória (Netto-Ferreira, 2010). Espécies intermediárias entre *Lebiasina* e *Piabucina* podem ser encontradas como, por exemplo, *P. boruca* e *P. festae*, questionando assim a validade do gênero *Piabucina*. Neste sentido, tem sido proposta a sinonimização desses dois gêneros (Géry, 1977; Taphorn, 1992; Géry e Zarske, 2002; Netto-Ferreira, 2010; Netto-Ferreira, 2012). Contudo, apesar das divergências taxonômicas, o monofiletismo da subfamília Lebiasininae é corroborado por nove sinapormofias morfológicas (Netto-Ferreira, 2006).

Tabela 1. Espécies válidas do gênero *Lebiasina*, conforme Fricke *et al*. (2019).

Gênero *Lebiasina*
Lebiasina bimaculata Valenciennes, 1847
Lebiasina multimaculata Boulenger, 1911
Lebiasina intermedia Meinken, 1936
Lebiasina uruyensis Fernández-Yépez, 1967
Lebiasina floridablancaensis Ardila Rodríguez, 1994
Lebiasina provenzanoi Ardila Rodríguez, 1999
Lebiasina yuruaniensis Ardila Rodríguez, 2000
Lebiasina chucuriensis Ardila Rodríguez, 2001
Lebiasina narinensis Ardila Rodríguez, 2002
Lebiasina taphorni Ardila Rodríguez, 2004
Lebiasina colombia Ardila Rodríguez, 2008
Lebiasina ortegai Ardila Rodríguez, 2008
Lebiasina chocoensis Ardila Rodríguez, 2010
Lebiasina yepezi Netto-Ferreira, Oyakawa, Zuanon & Nolasco, 2011
Lebiasina marilynae Netto-Ferreira, 2012
Lebiasina melanoguttata Netto-Ferreira, 2012
Lebiasina minuta Netto-Ferreira, 2012
Lebiasina ardilai Netto-Ferreira, Lopez-Fernandez, Taphorn & Liverpool, 2013

1.4 Estudos cromossômicos na família Lebiasinidae

Estudos citogenéticos têm fornecido contribuições relevantes na caracterização da biodiversidade ictiofaunística (Cioffi *et al*., 2018). Enquanto muitas famílias evidenciam-se mais conservadas no tocante a organização de seus cariótipos, como por exemplo Parodontidae, Anostomidae e Prochilodontidae, outras se mostram altamente diversificadas, a exemplo de Erythrinidae e Characidae. Tal diversidade se manifesta pela ocorrência de diversos complexos de espécies onde distintos cariomorfos evidenciam ausência de fluxo gênico, mesmo quando em condições de simpatria e sintopia (Moreira-Filho & Bertollo, 1991; Bertollo *et al*., 2000; Bertollo, 2007; Cioffi *et al*, 2012; Bertollo *et al*., 2018).

Contudo, a família Lebiasinidae permanece muito pouco estudada do ponto de vista citogenético, sendo os dados existentes predominantemente restritos à caracterização do número diploide de algumas espécies (Scheel, 1973; Arai, 2011), com algumas poucas

investigações mais pormenorizadas (Arefjev, 1990; Oliveira *et al*., 1991; Moraes *et al*., 2017; Moraes *et al*., 2019; Toma *et al*., 2019; Sassi *et al*. 2019). Apesar dessa carência investigativa, os dados existentes apontam para uma diversidade de números cromossômicos entre as espécies, variando de 2n = 22 em *Nannostomus unifasciatus* a 2n = 46 em *N. trifasciatus* (Arai, 2011). Entretanto, é possível que também ocorram problemas de identificação para várias das espécies examinadas, considerando diferentes números cromossômicos relacionados a uma mesma espécie nominal (**Tabela 2**). Rearranjos Robertsonianos se apresentam como um dos prováveis fatores geradores dessa diferenciação cariotípica, como sugerido pelo número cromossômico mais reduzido e a ocorrência de grandes cromossomos metacêntricos em *N. unifasciatus* (Arefjev, 1990).

Recentemente, análises investigativas mais aprofundadas começaram a ser implementadas na família Lebiasinidae, abrindo novas perspectivas para a citogenética evolutiva desse grupo (Moraes *et al.*, 2017). Duas espécies de *Pyrrhulina* (*P. australis* e *P.* aff. *australis*) foram investigadas por procedimentos da citogenética convencional e molecular, incluindo o mapeamento de DNAs repetitivos, hibridização genômica comparativa (CGH) e pintura cromossômica total (WCP). As duas espécies mostraram possuir o mesmo número diploide (2n = 40), assim como a mesma fórmula cariotípica (4st + 36a), sem diferenciações entre os sexos. Contudo, ambas exibem diferenciações genômicas acentuadas, evidenciando a ocorrência de unidades evolutivas distintas (Moraes *et al*., 2017). Por sua vez, apesar das especificidades inerentes a cada grupo, algumas correlações quanto à estrutura cariotípica e distribuição de classes de DNAs repetitivos foram evidenciadas entre *Pyrrhulina* e *Erythrinus erythrinus* (Erythrinidae) (Moraes *et al*., 2017), apontando marcadores promissores para as análises de relacionamentos entre grupos da nossa ictiofauna.

Tabela 2. Dados citogenéticos de espécies da família Lebiasinidae. Tabela publicada em Sassi *et al*. (2019) e atualizada em Toma *et al*. (2019).

Espécies	2n (sexo)	Cariótipo	Referência
Copeina			
C. guttata	42 (?)	-	Scheell, 1973
C. guttata	42♂♀	2m+4sm+36st/a	Toma *et al*., 2019
Copella			
C. arnoldi	44 (?)	-	Scheell, 1973
C. nattereri	36 (?)	-	Scheell, 1973
Copella sp.	26 (?)	-	Scheell, 1973
Copella sp.	24 (?)	-	Scheell, 1973
Nannostomus			
N. beckfordi (A)	42 ♂	2m+40a	Arefjev, 1990
N. beckfordi (B)	44 (?)	-	Scheell, 1973
N. beckfordi (C)	36 (?)	-	Scheell, 1973
N. eques (A)	34 (?)	34a	Arefjev, 1990
N. eques (B)	36 (?)	-	Scheell, 1973
N. arrisoni	40 (?)	-	Scheell, 1973
N. marginatus	42 (?)	-	Scheell, 1973
N. trifasciatus (A)	46 (?)	-	Scheell, 1973
N. trifasciatus (B)	38 (?)	-	Scheell, 1973
N. trifasciatus (C)	30 (?)	-	Scheell, 1973
N. trifasciatus (D)	24 (?)	-	Scheell, 1973
N. unifasciatus	22 (?)	-	Scheell, 1973
Pyrrhulina			
Pyrrhulina cf. *australis*	40♂♀	6st+34a	Oliveira *et al*., 1991
Pyrrhulina sp.	42 (?)	2m+2sm+38st/a	Oliveira *et al*., 1992
P. australis	40♂♀	4st+36a	Moraes *et al*., 2017
Pyrrhulina cf. *australis*	40♂♀	4st+36a	Moraes *et al*., 2017
P. brevis	42♂♀	2sm + 4st + 36a	Moraes *et al*., 2019
P. semifasciata	41♂42♀	1m + 4st + 36a ♂ 4st + 38a ♀	Moraes *et al*., 2019

Adicionalmente, duas outras espécies de *Pyrrhulina*, *Pyrrhulina brevis* e *P. semifasciata*, foram também analisadas por diferentes procedimentos da citogenética clássica e molecular (Moraes *et al*., 2019). Neste caso, enquanto que *P. brevis* evidenciou 2n = 42 cromossomos (2sm + 4st + 36a) em ambos os sexos, *P. semifasciata* apresentou 2n = 42 cromossomos nas fêmeas (4st + 38a) e 2n=41 cromossomos nos machos (1m + 4st + 36a) em

virtude da ocorrência de um sistema sexual do tipo $X_1X_1X_2X_2/X_1X_2Y$. Até então esta é a primeira evidência de um sistema de cromossomos sexuais diferenciado para a família, originado pela fusão cêntrica entre dois cromossomos acrocêntricos não-homólogos nessa espécie (Moraes *et al*., 2019).

Além dessas abordagens no gênero *Pyrrhulina*, outros grupos representativos da família Lebiasinidae, como espécies de *Lebiasina* (presente estudo) e de *Copeina* (Toma *et al*., 2019), tem sido objeto de estudos citogenéticos. Em *Copeina guttata*, o número diploide encontrado foi de 42 cromossomos (2m + 4sm + 36 st-a), reforçando a tendência evolutiva divergente entre as espécies de Lebiasinidae. Além disso, os experimentos de hibridização genômica comparativa revelaram o compartilhamento moderado de algumas poucas regiões repetitivas entre *Copeina*, *Lebiasina* e *Pyrrhulina*, exceto para uma forte hibridização comum entre os sítios de rDNA 18S (Toma *et al*., 2019).

Assim sendo, apesar das dificuldades técnicas gradativamente superadas para a obtenção de boas preparações cromossômicas entre os Lebiasinidae, o uso de marcadores da citogenética clássica e molecular têm propiciado avanços recentes para a caracterização cariotípica e o entendimento da evolução cromossômica nessa família.

II - JUSTIFICATIVA E OBJETIVOS

Lebiasinidae situa-se entre as muitas famílias de peixes que ainda carecem de investigações citogenéticas resolutivas que possam evidenciar e esclarecer seus processos evolutivos, bem como inter-relações com outros grupos da ictiofauna. Vencidas as primeiras dificuldades em se obter boas preparações cromossômicas em espécies miniaturas, como é o caso de diversos representantes dessa família, análises recentes têm evidenciado que Lebiasinidae é um grupo extremamente promissor para investigações evolutivas intra- e inter-genéricas, assim como intra- e inter-familiares. De fato, as abordagens já disponíveis em relação à diversidade cariotípica numérica e estrutural, bem como genômica entre as espécies, suportam o interesse investigativo desse grupo. Embora suas relações com outros grupos de Characiformes sejam questões que ainda requerem esclarecimentos adicionais, filogenias moleculares recentemente obtidas apontam para sua proximidade com a família Ctenoluciidae. Esta família, investigada por Souza *et al*. (2017), evidencia o número diploide 2n = 36 (14m+16sm+6st) em todas as espécies analisadas (*Boulengerella cuvieri*, *B. lateristriga*, *B. lucius* e *B. maculata*), mas com padrão de distribuição da heterocromatina espécie-específico. Adicionalmente, outra espécie de Ctenoluciidae do gênero *Ctenolucius* também mantém o número diploide (2n = 36), mas com fórmula cariotípica diferenciada (26m/sm+10st/a) (Arefjev, 1990). Assim sendo, tais subsídios abrem perspectivas promissoras para as investigações cromossômicas em espécies de Lebiasinidae, considerando também os avanços metodológicos incorporados na citogenética de peixes propiciando importantes marcadores cromossômicos para estudos evolutivos em níveis intra- e inter-familiares.

Neste sentido, a presente pesquisa teve como enfoque os seguintes objetivos:

2.1 Objetivo geral

O presente estudo objetivou avançar no conhecimento dos processos evolutivos da família Lebiasinidae, tendo em vista suas relações cromossômicas em nível intra-genérico, intra-familiar e inter-familiares. Foram enfocadas espécies de *Lebiasina* desprovidas de informações cromossômicas, tendo em vista que esse gênero representa o grupo mais basal da família, possibilitando assim abordagens comparativas importantes do ponto de vista evolutivo.

2.2 Objetivos específicos

Tendo em vista atingir o objetivo geral proposto para este estudo, os seguintes objetivos específicos foram priorizados:

2.2.1 – Caracterizar o cariótipo de machos e fêmeas de espécies de *Lebiasina* quanto ao número e a morfologia dos cromossomos, assim como a distribuição da heterocromatina C-positiva.

2.2.2 – Investigar a constituição genômica das espécies pela distribuição de sequências de DNAs repetitivos nos cromossomos, mapeamento citogenético dos DNAs ribossomais 5S e 18S e de sequências microssatélites.

2.2.3 – Prospectar a ocorrência de cromossomos sexuais diferenciados nesse grupo, com base nos caracteres cromossômicos presentes em cada sexo e de marcadores específicos.

2.2.4 – Avaliar o compartilhamento de frações do genoma pela hibridização genômica comparativa (CGH) e experimentos de pintura cromossômica total (WCP) entre as espécies investigadas.

2.2.5 – Buscar subsídios, com bases nos marcadores obtidos, a respeito da proximidade evolutiva entre Lebiasinidae e Ctenoluciidae.

III – MATERIAL E MÉTODOS

3.1 – Material

No presente estudo, foi possível investigar duas espécies representativas do gênero *Lebiasina*, *L. melanoguttata* Netto-Ferreira, 2012 e *L. bimaculata* Valenciennes, 1847 (**Figura 1**). A espécie *L. melanoguttata* é encontrada na região da Serra do Cachimbo, ao sul do Pará e norte do Mato Grosso, na bacia do rio Xingu, onde foi originalmente descrita. A coleta contou com autorização da agência de controle ambiental ICMBIO/SISBIO (Licença Nº 48628-2) e SISGEN (A96FF09). Já a espécie *L. bimaculata* é encontrada em rios do Equador e Peru, a oeste dos Andes, e na bacia do rio Marañon (Fricke *et al*., 2019). Amostras dessa espécie, provenientes da bacia do rio Arenillas (Equador) (**Tabela 1** e **Figura 2**) foram gentilmente cedidas pelo Dr. Mauro Nirchio, docente na Universidad Técnica de Machal (Equador). Os animais foram depositados no Museu de Zoologia da Universidade de São Paulo (MZUSP), números de voucher 124457 e 124625.

Figura 1. Espécies utilizadas no presente trabalho. a) *Lebiasina bimaculata* (Figura retirada de Netto-Ferreira, 2010). b) *Lebiasina melanoguttata* (acervo pessoal).

Tabela 1. Coordenadas geográficas dos pontos de coleta das espécies de *Lebiasina* analisadas e número amostral (N).

Espécie	Local	N
Lebiasina bimaculata	Lagos do rio Arenillas – El Oro (Equador) (S03°30'57.204", O80°3'44.2656")	**04♂, 03♀**
Lebiasina melanoguttata	Altamira – PA (Brasil) (S08° 46' 59,4", O54°58'26,9")	**10♂, 04♀**
Lebiasina melanoguttata	Cachoeira da Serra – PA (Brasil) (S08°58'18,7", O54°58'18,7")	**04♂, 18♀**

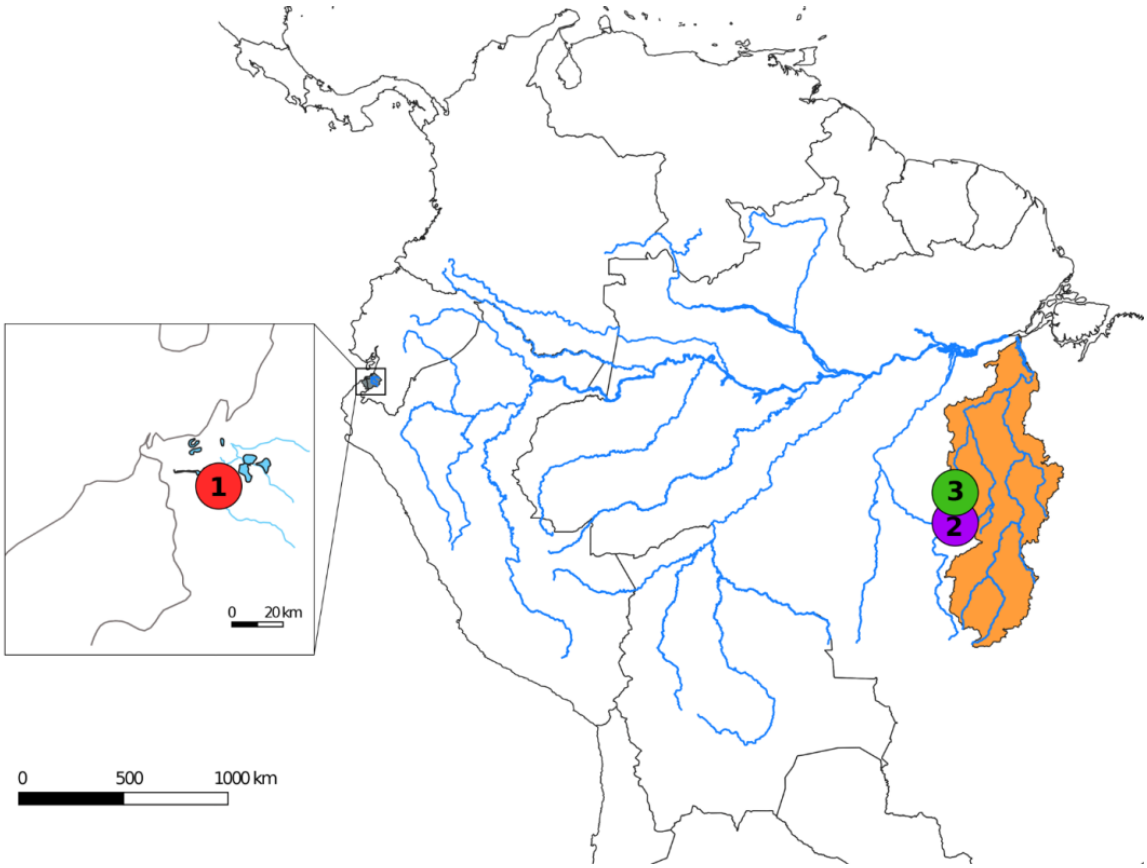


Figura 2. Mapa parcial da América do Sul, evidenciando os pontos de coleta de *Lebiasina bimaculata* (1 – círculo vermelho) e *L. melanoguttata* (2 e 3 – círculos roxo e verde), destacando a bacia do rio Xingu (em laranja). Figura organizada com o auxílio dos softwares QGIS Desktop 3.6.3, Inkscape 0.92 e Adobe Photoshop CC 2015.

3.2 Métodos

3.2.1 Preparação cromossômica, bandamento-C e coloração diferencial por Cromomicina A_3

Os cromossomos mitóticos foram obtidos de acordo com Bertollo *et al*. (2015). Os animais foram previamente submetidos ao tratamento com Colchicina (1 ml de solução a 0,005%/100 gramas de peso), por um período de 45 minutos. Após procedimentos anestésicos, foram extraídos fragmentos do rim anterior e posterior, procedendo-se a hipotonização celular em cloreto de Potássio (KCl) 0,075M durante 20 minutos a 36 °C, a fixação em álcool metílico (3 partes): ácido acético glacial (1 parte) e a preparação das lâminas, conforme a metodologia usual de "*air drying*". A seguir, os cromossomos foram corados com Giemsa 5% (pH 6.8) por 13 minutos. Todos os procedimentos seguiram as condutas éticas e de anestesia aprovadas pelo Comitê de Ética em Experimentação e Uso Animal da Universidade Federal de São Carlos (Processo CEUA 1853260315).

A identificação da heterocromatina C-positiva foi realizada pelo bandamento C, conforme Sumner (1972). O material foi tratado com ácido clorídrico (HCl) 0,2N a temperatura ambiente, por 15-20 minutos, seguindo-se a lavagem em água destilada e secagem ao ar. Posteriormente, as lâminas foram incubadas em uma solução de hidróxido de Bário (BaOH) 5%, a 60 °C, por um período de 30-60 segundos, seguido por rápida imersão em HCl 0,2N, lavagem em água destilada e secagem ao ar. Por fim, as lâminas foram tratadas com uma solução 2xSSC a 60 °C por 30-60 minutos, lavadas em água destilada e coradas com iodeto de Propídio a 50 μg/ml (Lui *et al*., 2012).

A coloração diferencial com Cromomicina A_3 (CMA_3) e 4',6'-diamidino-2-phenylindole (DAPI) foi utilizada para a detecção de regiões cromossômicas ricas em GC e AT, respectivamente, de acordo com Schmid (1980).

3.2.2 Hibridização fluorescente *in situ* (FISH) - mapeamento de DNAs repetitivos

Foram utilizadas sondas dos DNAs ribossomais 5S e 18S, isoladas do genoma de *Hoplias malabaricus* (Characiformes, Erythrinidae), clonadas em vetores plasmidiais e propagadas em células competentes de *Escherichia coli* DH5α (Invitrogen, San Diego, CA, USA). A sonda de rDNA 5S inclui 120 pares de base (pb) do gene transcriptor do rRNA 5S e 200pb do espaçador não-transcrito (NTS - *Nontriscribed spacer*) (Martins *et al*., 2006). A sonda de rDNA 18S corresponde a 1400pb do segmento desse gene obtido pela Reação de Polimerase em Cadeia (PCR –*Polymerase Chain Reaction*), a partir do DNA nuclear (Cioffi *et al*., 2009). A marcação das sondas foi realizada com kit de Nick-Translation mix (Roche, Manheim, Alemanha), sendo a sonda 5S marcada com Spectrum Green-dUTP e a sonda 18S marcada com Spectrum Orange-dUTP (Vysis, Downers Grove, IL, USA), conforme as instruções do fabricante.

Além das famílias multigênicas acima descritas, foram utilizadas sondas de sequências repetitivas de pequeno tamanho, correspondendo aos microssatélites $(CA)_{15}$, $(GA)_{15}$, $(CAT)_{10}$ e $(CGG)_{10}$. Essas sequências foram diretamente marcadas durante a síntese com Cy3, com exceção de $(GA)_{15}$, cuja marcação se deu com Fluoresceína modificada com isocianato (FITC), seguindo o protocolo descrito em Kubat *et al*. (2008). Adicionalmente, sondas de sequências teloméricas $(TTAGGG)_n$, foram também empregadas (sonda DAKO Telomere PNA FISH Kit/FITC (DAKO, Glostrup, Dinamarca).

O procedimento de hibridização seguiu o protocolo descrito em Yano *et al*. (2017). As lâminas foram tratadas por 1h com 70µl da solução de RNAse (1µl de RNAse (10µg/ml) + 1ml 2xSSC), em câmara úmida a 37 °C. Após este tempo, as lâminas foram lavadas em PBS 1x sob agitação por 5 min, seguindo-se tratamento com 50µl de solução de Pepsina (495µl de H_2O miliq autoclavada + 5µl HCl 1M + 1,5 µl Pepsina (20mg/µl)). Após nova lavagem das lâminas com PBS 1x, sob agitação por 5 minutos, procedeu-se rápida lavagem em série alcoólica, 70%,

85% e 100%, dois minutos em cada banho. Para a desnaturação dos cromossomos, as lâminas foram submetidas ao tratamento com Formamida a 72 °C por 3min e 15s, sucedendo-se lavagem em série alcoólica como no passo anterior, alterando apenas o álcool 70% que nesta etapa deve se encontrar gelado. O mix de hibridização conteve 2µl da sonda desejada mais 18µl de sulfato dextrano, sendo desnaturado em termociclador a 86 °C por 10min. Após, 20µl deste mix foi aplicado em cada lâmina, com incubação a 37 °C por aproximadamente 16h. Passado este tempo, as lâminas foram lavadas em 2xSSC e 1xSSC, ambos os procedimentos sob agitação por 5 min. Para as sondas com marcação indireta (rDNAs 5S e 18S) procedeu-se a detecção em solução contendo 995µl NFDM (*Non-Fat Dry Milk*) + 5µl de contracorante, sendo 20µl de tal solução aplicada nas lâminas, seguido de incubação em câmara úmida por 1h a 37 °C. As lâminas então foram lavadas em Tween, posteriormente em PBS 1x, desidratadas em série alcoólica e contra coradas com 15µl de DAPI + Antifading.

3.2.3 Hibridização genômica comparativa

Os DNAs genômicos (gDNAs) de *Lebiasina bimaculata*, *Lebiasina melanoguttata*, *Boulengerella lateristriga* (Ctenoluciidae), *Copella nattereri*, *Copeina guttata*, *Nannostomus beckfordi*, *N. eques*, *N. unifasciatus*, *N. marginatus*, *N. trifasciatus*, *Pyrrhulina australis*, *Pyrrhulina* aff. *australis*, *P. brevis* e *P. semifasciata* foram extraídos a partir do fígado, seguindo o método de fenol-clorofórmio-álcool isoamílico (Sambrook e Russell, 2001). Quatro ensaios experimentais foram considerados, com as hibridizações realizadas conforme o protocolo descrito em Symonová *et al* (2013). Os dois primeiros ensaios focaram em comparações intra-específicas entre machos e fêmeas de ambas as espécies de *Lebiasina*. Para isso, o gDNA de machos e fêmeas de *L. bimaculata* e *L. melanoguttata* foram marcados com Spectrum Orange-dUTP e Spectrum Green-dUTP, respectivamente, utilizando o Nick-Translation mix kit (Roche, Manheim, Alemanha) e hibridizados em cromossomos de machos

e fêmeas de cada espécie. Para o bloqueio de sequências repetitivas foi utilizado o C0t-1 DNA em todos os experimentos, o qual corresponde a fração de gDNA enriquecida para sequências alta e moderadamente repetitivas, preparado conforme Zwick *et al*. (1997). O mix final para cada lâmina foi composto de 500 ng de gDNA derivado de machos, 500 ng de gDNA obtido das fêmeas e 15µg de C0t-1 DNA. As sondas foram precipitadas com etanol, seguido da mistura dos pellets secos com um buffer de hibridização contendo formamida 50%, 2xSSC, dodecil sulfato de sódio 10%, sulfato dextrano 10% e buffer Denhardt's (1% Ficoll, 1% polivinilpirrolidona e 1% albumina do soro bovino) com pH corrigido para 7.0.

O terceiro ensaio focou em comparações interespecíficas e intergenéricas: *Lebiasina bimaculata* x *L. melanoguttata* e *L. melanoguttata* x *Boulengerella lateristriga*, *Copella nattereri*, *Copeina guttata*, *Nannostomus beckfordi*, *N. eques*, *N. unifasciatus*, *N. marginatus*, *N. trifasciatus*, *Pyrrhulina australis*, *P.* aff. *australis*, *P. brevis* e *P. semifasciata*. Para isso, o gDNA derivado de fêmeas de *L. melanoguttata* foi marcado diretamente com o Atto488 (verde) com auxílio do kit de marcação Nick-translation (Jena Bioscience, Jena, Germany), enquanto os gDNA de *C. guttata*, *C. nattereri*, *P. australis*, *Pyrrhulina* aff. *australis*, *P. brevis*, *P. semifasciata*, *N. eques*, *N. marginatus*, *N. trifasciatus* e *N. unifasciatus* foram diretamente marcados com Atto550 (vermelho) com o mesmo kit de marcação.

Por fim, o quarto ensaio objetivou a comparação genômica das famílias Lebiasinidae e Ctenoluciidae. Assim, sondas genômicas derivadas de fêmeas de *L. bimaculata* (Lebiasinidae) e *B. lateristriga* (Ctenoluciidae) foram hibridizadas nos cromossomos de fêmeas de *L. bimaculata*. Para isso, o gDNA de *L. bimaculata* e de *B. lateristriga* foram respectivamente marcados com Spectrum Green-dUTP e Spectrum Orange-dUTP, utilizando o Nick-Translation mix kit (Roche, Manheim, Alemanha). A mistura final consistiu em 500 ng de gDNA derivado de fêmea de *L. bimaculata*, 500 ng de gDNA derivado de fêmea de *B. lateristriga* e 15µg de

C0t-1 DNA derivado de fêmeas de cada espécie, diluído no buffer de hibridização acima descrito.

3.2.4 Pintura cromossômica total (WCP)

Um experimento de pintura cromossômica total foi realizado entre espécies (Zoo-FISH), seguindo o protocolo descrito em Yano *et al*. (2017). Para tanto foi selecionado o primeiro par cromossômico de *L. bimaculata* e *B. lateristriga*, visto que estes se destacam facilmente dos demais cromossomos do cariótipo em relação ao tamanho, permitindo assim sua identificação precisa após coloração com Giemsa. Dezesseis cópias do primeiro par cromossômico de ambas as espécies foram isoladas por microdissecção e amplificados seguindo o procedimento descrito em Yang *et al*. (2009). As sondas foram denominadas LEB-1 e BOU-1 e marcadas com Spectrum Orange-dUTP e Spectrum Green-dUTP (Vysis, Downers Grove, IL, USA), respectivamente, em uma Reação de Polimerase em Cadeia com primer degenerado de oligonucleotídeos (DOP-PCR: *Degenerated Oligonucleotide-Primed Polymerase Chain Reaction*), utilizando 1µl do produto primeiramente amplificado como DNA molde (Yang *et al*., 2009). As sondas foram hibridizadas em preparações cromossômicas de *L. bimaculata* e *L. melanoguttata*.

3.2.5 – Análises

Trinta metáfases por indivíduo foram analisadas para confirmar o número diploide 2n, a estrutura cariotípica e os resultados da FISH. As imagens foram analisadas e capturadas em microscópio Olympus BX50 (Olympus Corporation, Ishikawa, Japão) com CoolSNAP e processadas utilizando o software Image Pro Plus 4.1 (Media Cybernetics, Silver Spring, MD, USA). Os cromossomos foram classificados como metacêntricos (m) ou submetacêntricos (sm), seguindo a classificação proposta por Levan *et al*. (1964), a partir da razão de braços.

Além disso, a confecção de mapas foi realizada nos softwares QGIS Desktop 3.6.3, Inkscape 0.92 e Adobe Photoshop CC 2015. Este último software foi também utilizado para a montagem dos cariótipos, ideogramas e o tratamento das demais imagens.

IV - Resultados e Discussão

Os resultados e as discussões foram compilados na forma de artigos científicos, sendo o primeiro capítulo publicado na revista **"International Journal of Molecular Sciences"** (IF = 4.18 e Qualis A2). Já o segundo capítulo será submetido para a revista "**Genes**" (IF = 3.484 e Qualis A2), em sua edição especial intitulada "Chromosome-Centric View of the Genome Organization and Evolution".

Capítulo 1

Chromosomal Evolution and Evolutionary Relationships of *Lebiasina* Species (Characiformes, Lebiasinidae)

Francisco de Menezes Cavalcante Sassi, Ezequiel Aguiar de Oliveira, Luiz Antonio Carlos Bertollo, Mauro Nirchio, Terumi Hatanaka, Manoela Maria Ferreira Marinho, Orlando Moreira Filho, Rouben Aroutiounian, Thomas Liehr, Ahmed B.H. Al-Rikabi e Marcelo de Bello Cioffi.

International Journal of Molecular Sciences 20 (12): 2944 (2019)

Abstract

We present the first cytogenetic data for *Lebiasina bimaculata* and *L. melanoguttata* with the aim of (1) investigating evolutionary events within *Lebiasina* and their relationships with other Lebiasinidae genera and (2) checking the evolutionary relationships between Lebiasinidae and Ctenoluciidae. Both species have a diploid number 2n = 36 with similar karyotypes and microsatellite distribution patterns but present contrasting C-positive heterochromatin and CMA_3^+ banding patterns. The remarkable interstitial series of C-positive heterochromatin occurring in *L. melanoguttata* is absent in *L. bimaculata*. Accordingly, *L. bimaculata* shows the ribosomal DNA sites as the only GC-rich (CMA_3^+) regions, while *L. melanoguttata* shows evidence of a clear intercalated CMA_3^+ banding pattern. In addition, the multiple 5S and 18S rDNA sites in *L. melanogutatta* contrast with single sites present in *L. bimaculata*. Comparative genomic hybridization (CGH) experiments also revealed a high level of genomic differentiation between both species. A polymorphic state of a conspicuous C-positive, CMA_3^+, and (CGG)n band was found only to occur in *L. bimaculata* females, and its possible relationship with a nascent sex chromosome system is discussed. Whole chromosome painting (WCP) and CGH experiments indicate that the *Lebiasina* species examined and *Boulengerella maculata* share similar chromosomal sequences, thus supporting the relatedness between them and the evolutionary relationships between the Lebiasinidae and Ctenoluciidae families.

Introduction

Lebiasinidae (Characiformes) are small freshwater fishes comprising approximately 74 valid species widely distributed throughout South and Central America, from Costa Rica to Argentina (Weitzman and Weitzman, 2003; Fricke *et al*., 2019). Two subfamilies and seven genera are currently recognized: Lebiasininae (*Lebiasina*, *Piabucina*, and *Derhamia*) and Pyrrhulininae (*Pyrrhulina*, *Nannostomus*, *Copeina*, and *Copella*) (Fricke *et al*., 2019). Several lebiasinids experienced an evolutionary gradual body miniaturization, resulting in very small-sized taxa (Weitzman and Vari, 1988).

Several *Lebiasina* species need taxonomic revision to better elucidate their identities. Although an unpublished phylogenetic analysis considers this genus to be the most basal of Lebiasinidae (Netto-Ferreira, 2010), further studies are necessary to characterize the evolutionary relationships within the family. In addition, the phylogenetic position of Lebiasinidae with respect to other Characiformes groups is also not well defined. In this sense, it has been proposed as being closely related to different Characiformes families, such as Ctenoluciidae, Erythrinidae, and Hepsetidae (Oyakawa, 1998; Buckup, 1998; de Pinna *et al*., 2018). However, recent phylogenetic analyses based on molecular data have repeatedly considered Lebiasinidae as closely related to Ctenoluciidae (Oliveira *et al*., 2011; Arcila *et al*., 2017; Betancur-R *et al*., 2019).

In this context, methodological advances in cytogenetics have improved the knowledge of fish biodiversity by providing useful taxonomic and evolutionary data (Cioffi *et al*., 2018). Although a large number of neotropical fish species has been cytogenetically analyzed so far, lebiasinids remain poorly explored under this approach, with most of the available data limited to haploid (n) and/or diploid (2n) number descriptions (**Table 1**). This scarcity of data is probably linked with the small size of many species, which makes the obtaining good metaphase plates difficult, both in terms of quantity and quality. The available data points to

great variation in the chromosome numbers of some taxa such as *Nannostomus*, in which the chromosome number ranges from 2n = 22 in *Nannostomus unifasciatus* to 2n = 46 in *Nannostomus trifasciatus* (Arai, 2011). However, it is possible that misidentifications have led to different 2n numbers for the same nominal species (**Table 1**). In fact, many Lebiasinidae species are poorly diagnosed, mainly due to the fact that some present great variation in color pattern (which may be related to sexual dimorphism) alongside with destroyed type material, which constitutes a barrier for their proper identification (Netto-Ferreira *et al*., 2013; Marinho and Menezes, 2017).

Recently, some fine-scale molecular cytogenetic approaches, such as comparative genomic hybridization (CGH) and whole chromosome painting (WCP), have been applied in several fish groups, allowing a deeper understanding of their karyotypes and genomic evolution (Moraes *et al*., 2019; Barby *et al*., 2019; Carvalho *et al*., 2017; de Freitas *et al*., 2017; de Oliveira *et al*., 2019; Sember *et al*., 2018). In this context, *Pyrrhulina* represents the only Lebiasinidae genus where, besides conventional analysis, molecular cytogenetic approaches have also been performed (Moraes *et al*., 2017; 2019). CGH experiments were able to show evidence of a range of specific differentiations between two morphologically similar species, thus pointing to their particular evolutionary history and differential taxonomy (Moraes *et al.*, 2017). Moreover, Whole Chromosome Painting (WCP) experiments were useful for demonstrating the origin and evolution of a multiple X1X2Y sex chromosome system in *Pyrrhulina semifasciata* as well as the occurrence of putative undifferentiated sex chromosomes in the three other congeneric species (Moraes *et al*., 2019).

On the other hand, Ctenoluciidae is a small family of Neotropical fishes composed of the genera *Ctenolucius*, with two species, and *Boulengerella*, with five species (Vari and Malabarba, 1998; Nelson *et al*., 2016). Cytogenetic analyses conducted in four *Boulengerella* species demonstrated a conservative chromosomal pattern, with all species presenting 2n = 36

chromosomes and similar C-positive heterochromatin and ribosomal DNA (rDNA) distribution patterns (de Souza e Sousa, 2017). In addition, a male heteromorphic state regarding the Nucleolar Organizer Regions (NOR)-carrying chromosome pair was also observed in all species, thus suggesting a putative XX/XY sex chromosome system (de Souza e Sousa, 2017).

The present study represents part of a series focusing on the cytogenetics and cytogenomics of Lebiasinidae fishes. Here, we provide, for the first time, cytogenetic data for two *Lebiasina* species (*Lebiasina bimaculata* and *Lebiasina melanoguttata*) using multipronged cytogenetic approaches including C- and CMA3 banding, repetitive DNA mapping, CGH, and WCP experiments. *L. bimaculata* is known to be present in Ecuador and Peru in drainages west of Andes, and in the upper Marañon basin, while *L. melanoguttata* occurs in the tributaries of rio Curuá, rio Xingú basin, Serra do Cachimbo, and Pará, Brasil (Weitzman and Weitzman, 2003). We aimed to investigate chromosomal evolutionary processes within this genus and their relationships with other Lebiasinidae genera, as well as to provide additional evidence of the phylogenetic proximity between the Lebiasinidae and Ctenoluciidae families.

Table 1. Chromosomal data for the Lebiasinidae family. The symbol ♂ was used to represent the males and ♀ for the females. The question mark (?) was used when the sexes were not identifiable, The karyotype formula uses "m" as metacentric, "sm" as submetacentric, "st" as subtelocentric and "a" as acrocentric chromosomes

Species	2n (sex)	Karyotype	Reference
Copeina			
C. guttata	42 (?)	-	Scheell, 1973
Copella			
C. arnoldi	44 (?)	-	Scheell, 1973
C. nattereri	36 (?)	-	Scheell, 1973
Copella sp.	26 (?)	-	Scheell, 1973
Copella sp.	24 (?)	-	Scheell, 1973
Nannostomus			
N. beckfordi (A)	42 ♂	2m+40a	Arefjev, 1990
N. beckfordi (B)	44 (?)	-	Scheell, 1973
N. beckfordi (C)	36 (?)	-	Scheell, 1973
N. eques (A)	34 (?)	34a	Arefjev, 1990
N. eques (B)	36 (?)	-	Scheell, 1973
N. arrisoni	40 (?)	-	Scheell, 1973
N. marginatus	42 (?)	-	Scheell, 1973
N. trifasciatus (A)	46 (?)	-	Scheell, 1973
N. trifasciatus (B)	38 (?)	-	Scheell, 1973
N. trifasciatus (C)	30 (?)	-	Scheell, 1973
N. trifasciatus (D)	24 (?)	-	Scheell, 1973
N. unifasciatus	22 (?)	-	Scheell, 1973
Pyrrhulina			
Pyrrhulina cf. *australis*	40♂♀	6st+34a	Oliveira *et al*., 1991
Pyrrhulina sp.	42 (?)	2m+2sm+38st/a	Oliveira *et al*., 1992
P. australis	40♂♀	4st+36a	Moraes *et al*., 2017
Pyrrhulina cf. *australis*	40♂♀	4st+36a	Moraes *et al*., 2017
P. brevis	42♂♀	2sm + 4st + 36a	Moraes *et al*., 2019
P. semifasciata	41♂42♀	1m + 4st + 36a ♂ 4st + 38a ♀	Moraes *et al*., 2019

Results

Karyotypes and C-Banding

Both *Lebiasina* species showed the same chromosome number and karyotypes composed exclusively by m and sm chromosomes (2n = 36m/sm, FN = 72) (**Figure 1 a, d and Figure S1**). The C-positive heterochromatin was located in the centromeric and telomeric regions of several chromosomes in both species, but *L. melanoguttata* displayed an exclusive set of conspicuous interstitial C-bands (**Figure 1 b, e and Figure 2**). Besides, a female heteromorphism concerning an enlarged C-positive telomeric constriction was observed in only one homologue of pair 3 in *L. bimaculata* (**Figure 1, boxed and Figure 2**).

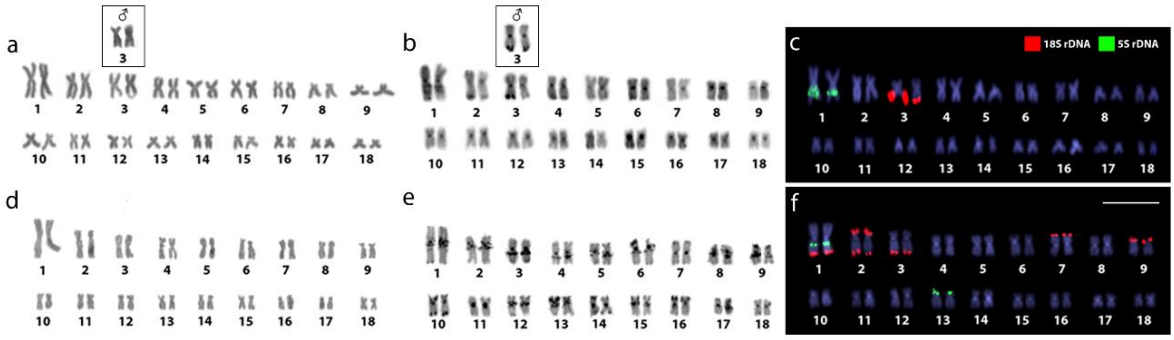


Figure 1. Female karyotypes of *Lebiasina bimaculata* (a, b, and c) and *Lebiasina melanoguttata* (d, e, and f) arranged after different cytogenetic procedures. Giemsa staining (a,d), C-banding (b,e), and dual-color fluorescence in situ hybridization (FISH) with 18S (red) and 5S (green) ribosomal DNA probes (c,f). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in blue. The inserts highlight the homomorphic condition related to pair 3 in the males of *L. bimaculata*. Scale bar = 5 μm

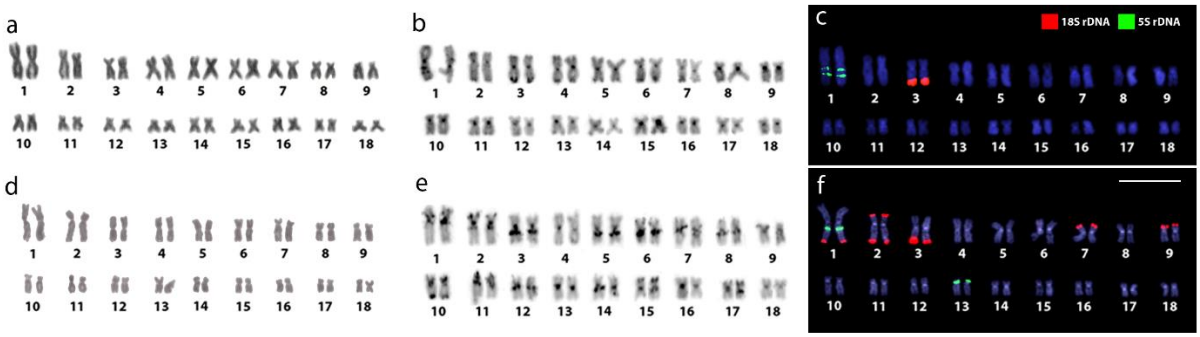
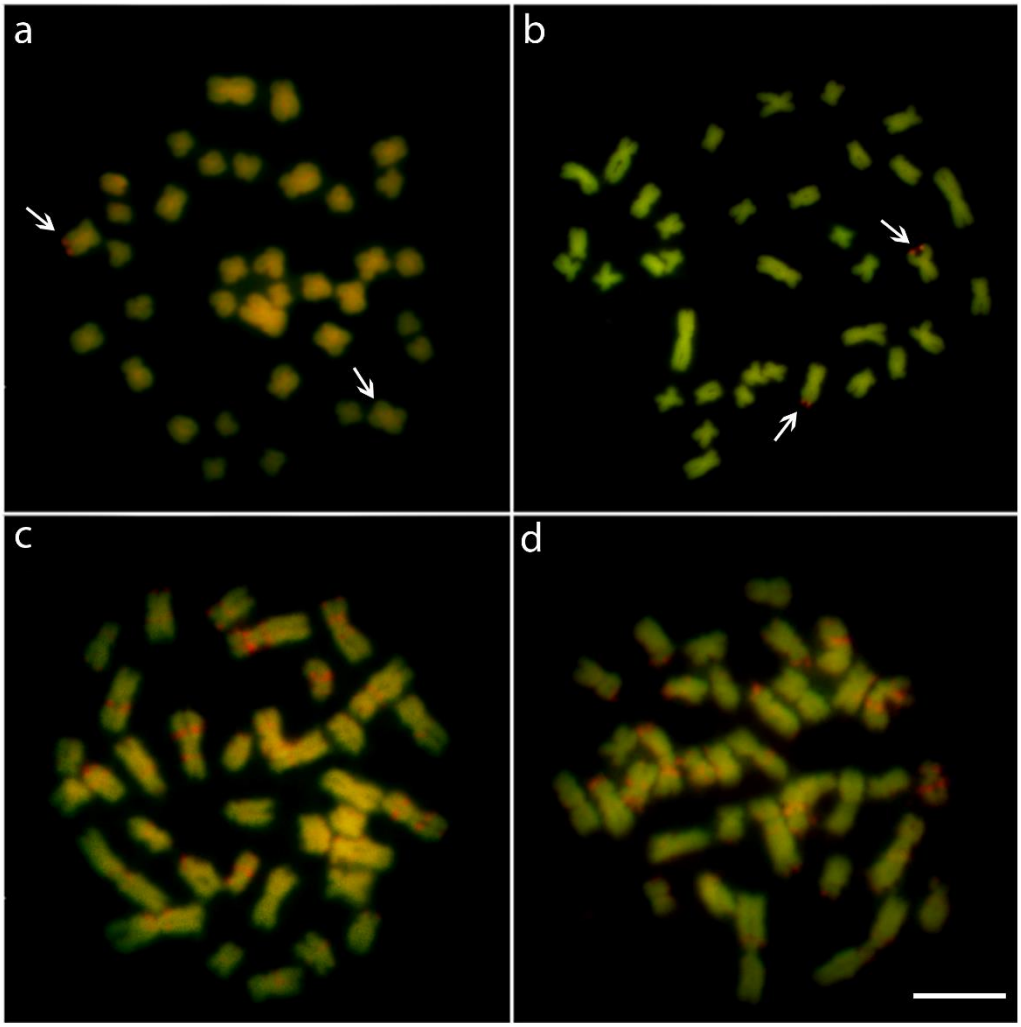


Figure 2. Male karyotypes of *Lebiasina bimaculata* (a, b and c) and *Lebiasina melanoguttata* (d, e and f) arranged after different cytogenetic procedures. Giemsa staining (a, d); C-banding (b, e) and dual-colour FISH with 18S (red) and 5S (green) rDNA probes (c, f). Chromosomes are counterstained with DAPI (blue). Bar = 5 μm

Chromosomal Mapping of Repetitive DNAs and CMA3 Banding

In both species, pair 1 bears interstitial 5S rDNA sequences on the long arms with an additional site on the short arms of pair 13 of *Lebiasina melanoguttata*. In this species, 12 telomeric 18S rDNA sites were observed, comprising five chromosomal pairs, including bi-telomeric sites in pair 2 and a syntenic condition with the 18S rDNA site in pair 1. On the contrary, *L. bimaculata* showed 18S rDNA sequences restricted only to the telomeric region of pair 3 (**Figure 1c, f**).

CMA3+ bands (GC-rich regions) in *L. bimaculata* were found to be exclusively co-located with the 18S rDNA sites. The same sex-associated polymorphic scenario related to C-banding was also highlighted by this fluorochrome staining. Thus, in contrast to males, only one Chromomycin A3 (CMA3)+ mark occurs in the female metaphases. On the other hand, besides the 18S rDNA regions, a clear set of CMA3+ bands and 4',6-diamidino-2-phenylindole (DAPI)+ (AT-rich) bands were highlighted on the chromosomes of *L. melanoguttata* (**Figure 3**).



685

686 **Figure 3**. Metaphase plates of female (a) and male (b) *Lebiasina bimaculata* and male
687 (c) and female (d) *Lebiasina melanoguttata* after DAPI-CMA3 staining. The arrows
688 indicate the unique CMA3+ site and its polymorphic state between male and females
689 of *L. bimaculata*. In *L. melanoguttata*, males and females display a set of CMA3+
690 (GC-rich) and DAPI+ (AT-rich) regions on the chromosomes. Scale bar = 5 µm.

691 Chromosomal mapping with the microsatellites probes (CA)n, (GA)n, (CGG)n, and
692 (CAT)n displayed a similar pattern for males and females of both species. The microsatellites
693 (CA)n and (GA)n exhibit conspicuous subtelomeric signals in almost all chromosomes.
694 (CGG)n motifs have a dispersed distribution throughout most of the chromosomes, along with
695 a conspicuous telomeric cluster in one chromosome pair. Notably, a polymorphic scenario
696 between males and females also occurs in *L. bimaculata*, as reported for the C-banding, 18S

rDNA, and CMA3 patterns. Here, only one (CGG)n telomeric cluster is present in female metaphases, in contrast to two found in males (**Figure 4, boxed**). The microsatellite (CAT)n presents a dispersed distribution, with accumulation in the telomeric regions of some chromosomes in *L. bimaculata*, but in several regions in *L. melanoguttata* (**Figure 4**).

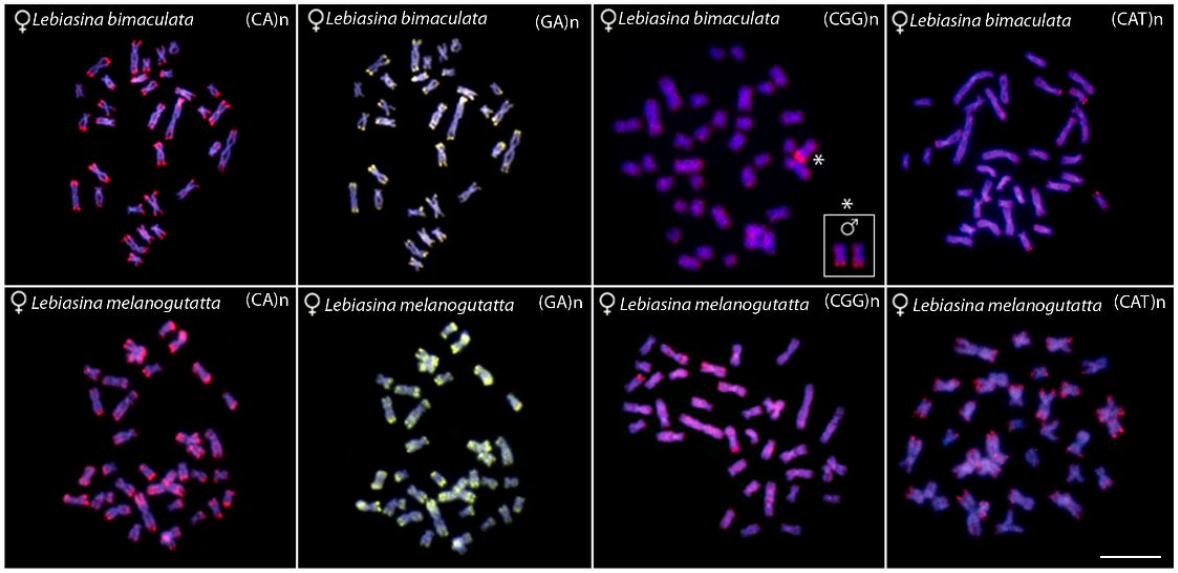


Figure 4. Metaphase plates of *Lebiasina bimaculata* (upper line) and *Lebiasina melanoguttata* hybridized with the microsatellite probes (CA)n, (GA)n, (CGG)n, and (CAT)n, respectively, showing the general distribution pattern of these repetitive DNAs in the chromosomes. Bar = 5 μm.

FISH with the (TTAGGG)n probe revealed hybridization signals only on the telomeric regions of all chromosomes, without interstitial telomeric sites (ITS), in both species (**Figure 5**).

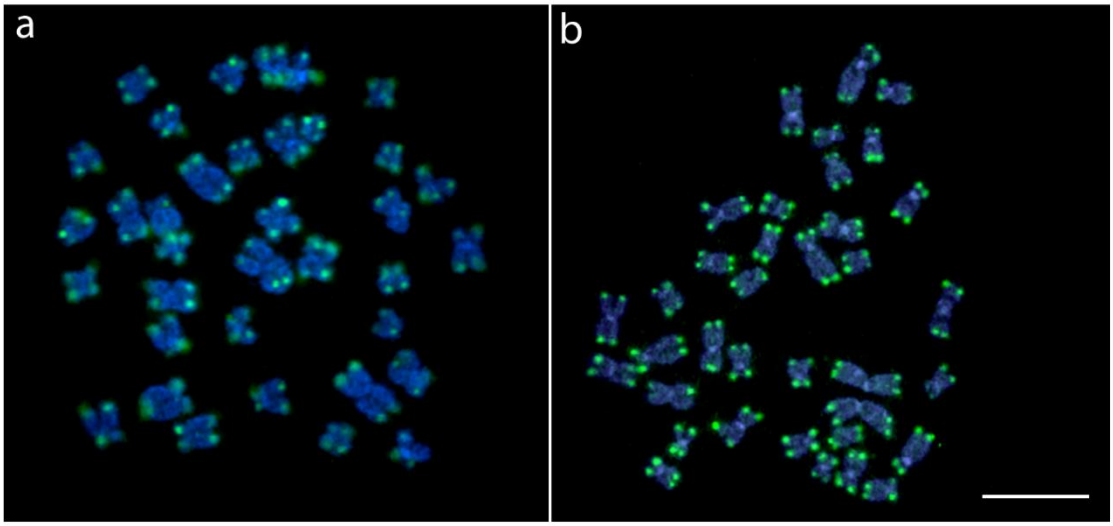


Figure 5. Female metaphase plate of *Lebiasina bimaculata* (a) and *Lebiasina melanoguttata* (b) showing the distribution of the telomeric (TTAGGG)n repeats. Bar = 5 µm.

Comparative Genomic Hybridization (CGH)

The genomic DNA (gDNA) comparison between *Lebiasina bimaculata* and *L. melanoguttata* revealed a high level of compartmentalization, with both species presenting a distinct composition of repetitive DNA sequences which vary both in quantity and distribution (**Figure 6i–l**). The CGH between males and females of *L. bimaculata* highlighted the presence of specific signals for females in the telomeric region of chromosome pair 3 (**Figure 6a–d**), the same polymorphic region identified by C-banding, 18S rDNA, (CGG)n, and CMA3+. However, no differences were observed between *L. melanoguttata* males and females (**Figure 6e–h**). The genomic comparison between *Lebiasina* and *Boulengerella* species showed that Lebiasinidae and Ctenoluciidae share several repetitive DNA segments (**Figure 6m–p**), especially in the telomeric regions.

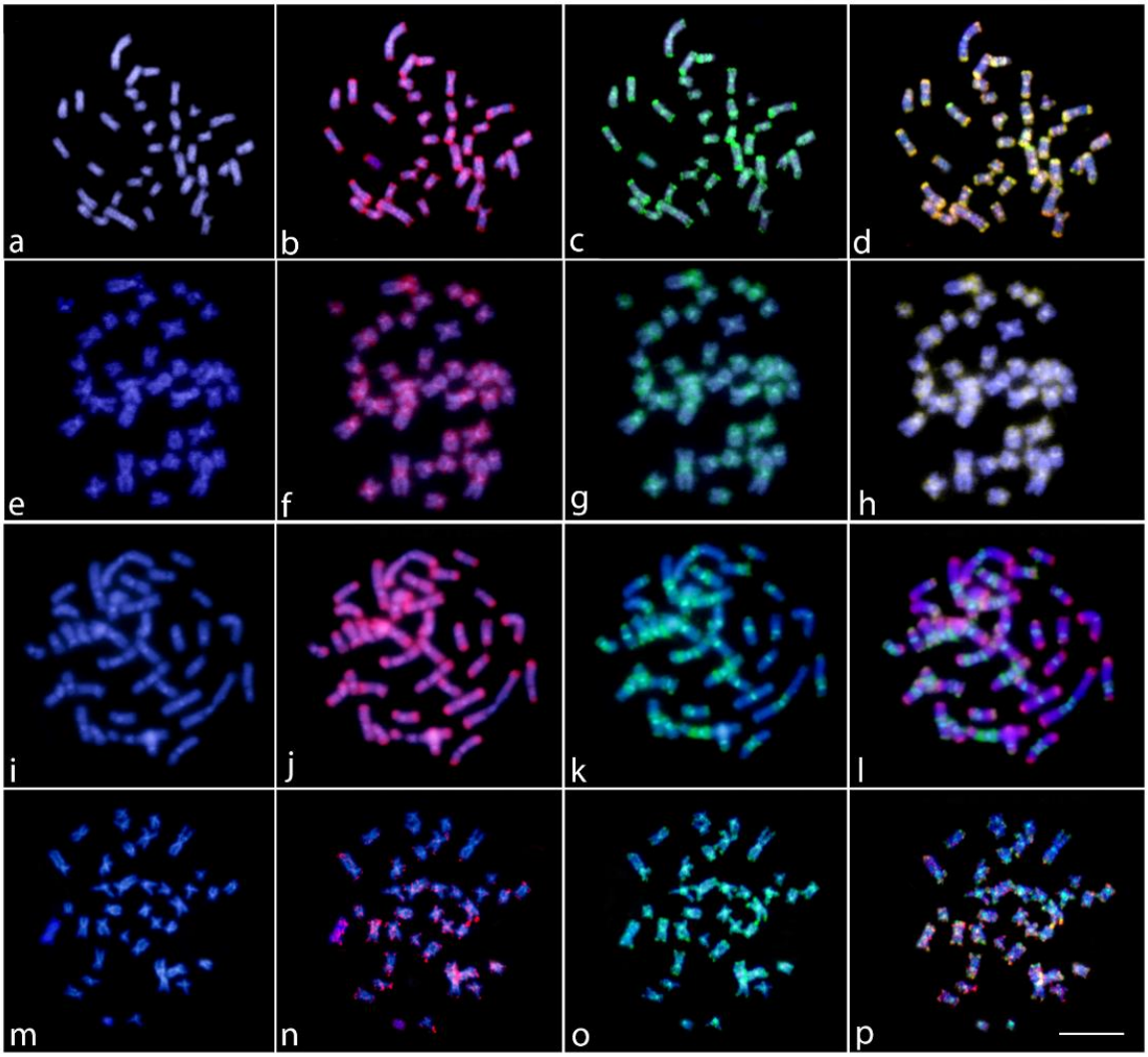


Figure 6. Comparative genomic hybridization (CGH) for intra- and interspecific comparison in the female metaphase plates of *Lebiasina bimaculata* (a–d and m–p) and *L. melanoguttata* (e–h and i–l). Male- and female-derived genomic probes from *L. bimaculata* mapped against female chromosomes of *L. bimaculata* (a–d); Male- and female-derived genomic probes from *L. melanoguttata* mapped against female chromosomes of *L. melanoguttata* (e–h); female-derived genomic probes from both *L. bimaculata* and *L. melanoguttata* hybridized together against female chromosomes of *L. melanoguttata* (i–l); and female-derived genomic probes from both *L. bimaculata* and *Boulengerella lateristriga* (Ctenolucidae) hybridized together against female chromosomes of *L. bimaculata* (m–p). First column (a, e, i, m): DAPI images (blue); second column (b, f, j, n): hybridization patterns using male gDNA of *L. bimaculata* (b), male gDNA of *L. melanoguttata* (f), female gDNA of *L. melanoguttata* (j), and female gDNA of *B. lateristriga* probes (red); third column (c, g, k, o): hybridization patterns using female

gDNA of *L. bimaculata* (c and o) and female gDNA of *L. melanoguttata* (g and k) probes (green); fourth column (d, h, l, p): merged images of both genomic probes and DAPI staining. The common genomic regions are depicted in yellow. Scale bar = 5 µm.

Whole Chromosome Painting (WCP)

The quality of the chromosome probes (LEB-1 and BOU-1) was validated by mapping them back onto the chromosomal background of *L. bimaculata* and *Boulengerella lateristriga* (data not shown), respectively, using species-specific Cot1-DNA as the suppressor. As expected, the first chromosome pair was completely painted in both species. Besides, both probes completely painted pair 1 of *L. bimaculata* and *L. melanoguttata*, indicating that the first chromosomal pair of these three species represents homologous ones, with a great conservation of their genomic content, size, and morphology (**Figure 7 and 8**).

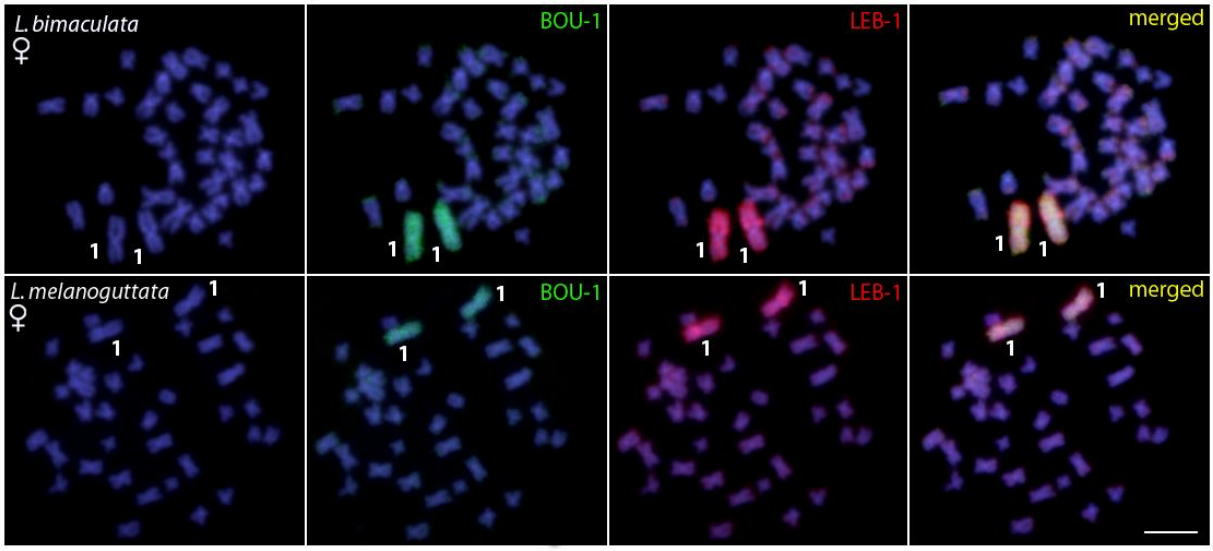


Figure 7. WCP with the LEB-1 (red) and BOU-1 (green) probes derived from pair 1 of *Lebiasina bimaculata* and *Boulengerella lateristriga*, respectively, hybridized against female metaphase chromosomes of *Lebiasina bimaculata* and *Lebiasina melanoguttata*. No differences between the sexes were observed. Bar = 5 µm.

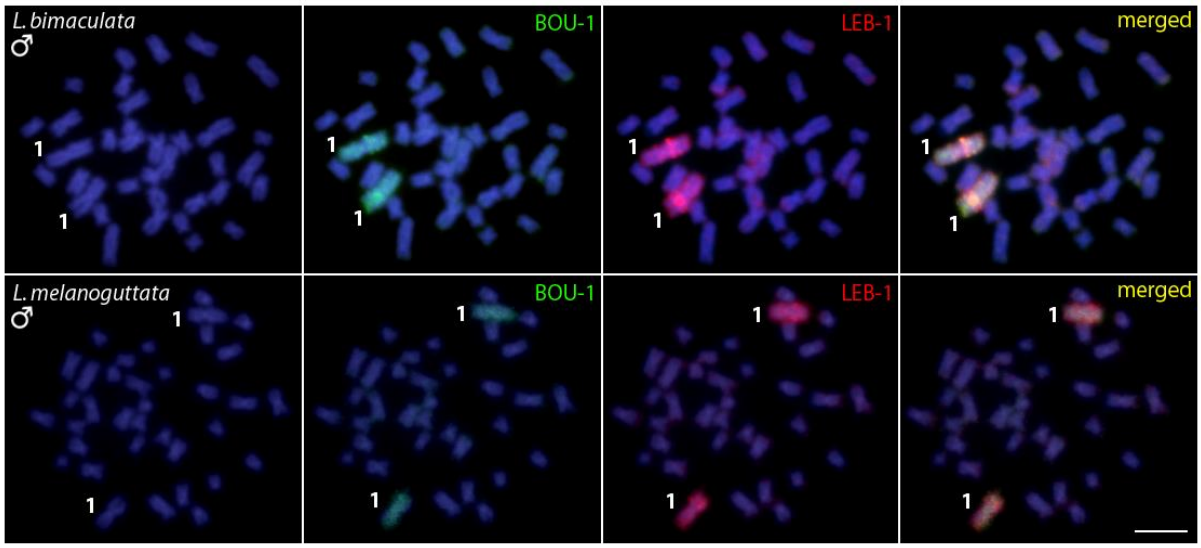


Figure 8. Zoo-FISH with the BOU-1 (green) and LEB-1 (red) probes derived from the pair 1 of *Lebiasina bimaculata* and *Boulengerella lateristriga*, respectively, hybridized against male metaphase plates of *Lebiasina bimaculata* and *Lebiasina melanoguttata*. Bar = 5 μm

Discussion

Chromosomal Features of Lebiasina Species

Both *Lebiasina* species presented the same diploid number (2n = 36), composed exclusively of bi-armed (m and sm) chromosomes. According to the data summarized in **Table 1**, this feature represents an exception among Lebiasinidae, since all of the other species analyzed harbor karyotypes dominated by mono-armed (st/a) chromosomes. The presence of karyotypes composed predominantly of mono-armed chromosomes seems to be a characteristic of most derived fish clades, where the basal ones display mainly biarmed ones (Nirchio *et al*., 2014). Beyond the differences found between basal and derived orders in fish phylogeny, the tendency towards chromosome acrocentrization seems to occur even within groups at the family level. For example, the ancestral karyotype reconstruction analysis performed in the family Carangidae has shown that although the diploid number 2n = 48 is conserved in the family, karyotypes with higher numbers of biarmed chromosomes (m/sm) are predominant in basal clades, whereas a higher proportion of acrocentric chromosomes with a decreasing tendency or complete elimination of biarmed chromosomes is observed in most derivative species (Jacobina *et al*., 2014). In the case of *Lebiasina*, which is considered basal in the family, the presence of biarmed chromosomes must represent a basal condition for the family, a fact that is also reinforced by the absence of any ITS signal on their chromosomes (**Figure 5**). In this sense, the high 2n variation present in other Lebiasinidae species suggests that multiple chromosomal rearrangements, including fission events, might have produced the huge chromosomal differentiations in number and morphology within this fish group.

It is known that chromosomal rearrangements can foster adaptation to heterogeneous environments by limiting genomic recombination, and thus, they may be directly linked to speciation processes (White, 1978; Lowry and Willis, 2010; Jay *et al*., 2018; Mérot *et al*., 2018;

Supiwong *et al*., 2019). Such rearrangements could be facilitated by common fragile sites that propitiate breaks and gaps that frequently occur at the heterochromatin–euchromatin borders (Arlt *et al*., 2006; Badaeeva *et al*., 2007). Of course, this evolutionary pathway, which appears to fit Lebiasinidae, should be highly corroborated as other genera and species are investigated by advanced chromosomal procedures, a type of study that is presently ongoing in our research group.

In turn, the remarkable series of interstitial C-positive heterochromatin in several chromosomes of *L. melanoguttata* — as also observed in other lebiasinid species such as *Pyrrhulina* aff. *australis* (Moraes *et al*., 2017) and *P. brevis* (Moraes *et al*., 2019) — is of particular relevance. An inherent feature of heterochromatin is its complex composition of tandem repeats of several repetitive DNA sequences (López-Flores and Garrido-Ramos, 2012), including some rDNA and microsatellite sequences, such as those mapped here. In *Lebiasina*, most of these sequences are species-specific, as demonstrated by the CGH experiments (**Figure 6**). Repetitive DNA also might form secondary chromosomal structures with the potential to induce replication fork stalling, leading to DNA breakage (Barra and Fachinetti, 2018). As the correlation between repetitive DNA sequences, fragile sites, and chromosomal rearrangements is widely known and documented (Raskina *et al*., 2008; Barros *et al*., 2017), our results point to a direct correlation between the content of the genomic repetitive elements and the karyotype divergence experienced by lebiasinid fishes. In fact, despite having the same 2n and karyotype structure, both *Lebiasina* species display divergent C-positive heterochromatin, CMA3+ banding, and rDNA distribution patterns, with the noteworthy interstitial series of C-positive heterochromatin being absent in *L. bimaculata*. Accordingly, the latter also shows the rDNA sites as the only GC-rich regions in the karyotype, in contrast with the rich CMA3+ banding pattern found in *L. melanoguttata*.

Lebiasina bimaculata presents single 5S and 18S rDNA sites, with the latter associated with GC-rich heterochromatin (**Figures 1, 2, and 3**). This pattern represents the most common scenario found in fish (Gornung, 2013; Sochorová *et al*., 2017), in contrast to warm-blooded vertebrates, which present genomic GC heterogeneity (Symonová *et al*., 2016). In turn, *L. melanogutatta* displays multiple rDNA sites and a clear set of intercalated CMA3+ and DAPI+ bands (**Figures 1, 2 and 3**). In addition, to clearly differentiate it from *L. bimaculata*, this banding pattern also represents a remarkable exception among fishes, since just few species have presented such a GC-compartmentalized genome thus far (Symonová *et al*., 2016). On the other hand, the diversity in the number of the rDNA loci, with the spreading of the 18S repeats to five chromosomal pairs, including bi-telomeric sites and 18S/5S rDNA synteny, has already been documented for other fish groups (Sochorová *et al*., 2017). It is pointed out that such divergences among closely related species may create sub-chromosomal background diversification that is directly linked with some speciation events (Symonová *et al*., 2016).

Although generally following the common pattern found among fishes (Cioffi and Bertollo, 2012), microsatellite mapping enabled some specificity to be shown between the *Lebiasina* species. A strong accumulation of the (GA)n and (CA)n repeats was found in the genomes of both species, especially in the subtelomeric regions, indicating the occurrence of very large perfect or degenerate arrays. Likewise, both species displayed a dispersed distribution of the (CGG)n repeats among all chromosomes. However, a remarkable sex-specific accumulation was observed in *L. bimaculata*.

In accordance with the above-mentioned features, the genomic comparison determined by CGH experiments also showed that both species differ in the composition and distribution of their repetitive sequences (**Figures 6 and 9**). A similar scenario has also been found in some other fish groups, such as in Notopteridae (Osteoglossiformes), where most species, although retaining a relatively conserved karyotype with a long evolutionary time (>120 Mya), show

significant genomic diversity highlighted by CGH and DArT-Seq analysis (Barby *et al*., 2019). In addition, it is noteworthy that *L. bimaculata* displays a particular telomeric female signals in chromosome pair 3, the same region that shows the differential (CGG)n+/CMA3+/18S rDNA+ constitution in this chromosome. This chromosomal scenario and its potential relationships with sex-specific regions/chromosomes are discussed in depth below.

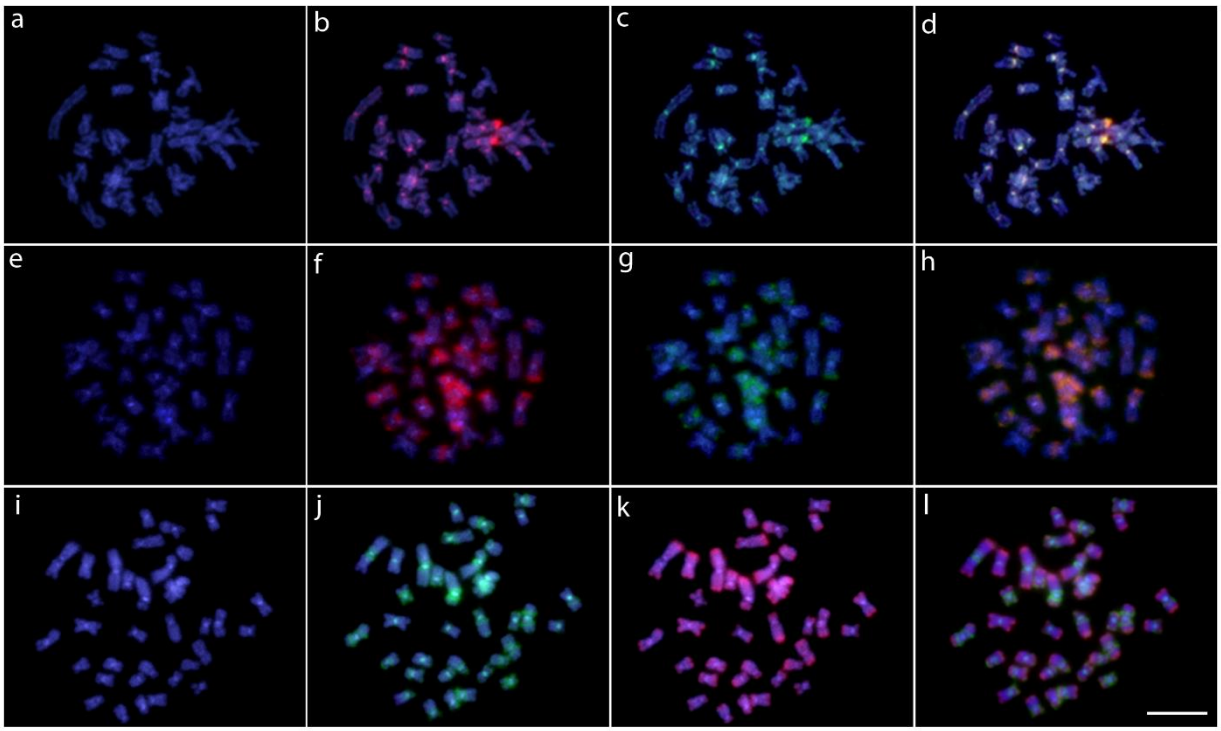


Figure 9. Comparative genomic hybridization (CGH) for intra- and interspecific comparison in male metaphase plates of *L. bimaculata* (a-d) and *L. melanoguttata* (e-h and i-l). Male and female-derived genomic probes from *L. bimaculata* mapped against male chromosomes of *L. bimaculata* (a-d); Male- and female-derived genomic probes from *L. melanoguttata* mapped against male chromosomes of *L. melanoguttata* (e-h); Male-derived genomic probes from *L. melanoguttata* (red) and *L. bimaculata* (green) hybridized together against male chromosomes of *L. melanoguttata* (i and l). First column (a-e-i): DAPI images (blue) of male *L. bimaculata* (a) and *L. melanoguttata* (e and i) metaphases; Second column (b-f-j): hybridization pattern using male gDNA of *L. bimaculata* (b – red; j - green) and male gDNA of *L. melanoguttata* (f - red); Third column (c-g-k): hybridization pattern using female gDNA of *L. bimaculata* (c - green) and female gDNA of *L. melanoguttata* (g - green and k - red); Fourth column (d-h-i): merged images of both genomic probes and DAPI staining. The common genomic regions are depicted in yellow. Bar = 5 µm.

Heterochromatin Polymorphism and CGH: Putative Sex Chromosomes in L. bimaculata?

Our results revealed that a differentiation between sexes occurs in the genome of *Lebiasina bimaculata*, where the females differ from males for a set of chromosomal markers. Giemsa staining, C-banding, 18S rDNA, CGG(n) and CMA3+ mapping showed characteristics for only one homologue of female pair 3. In addition, after intraspecific CGH experiments, females also showed conspicuous "specific" signals in both chromosomes of the third pair, whereas in the male genome, they appeared to be absent or perhaps with a very small and discrete size.

It is known that the rRNA gene amplification system is unique in maintaining a species-specific number of rDNA copies (Kobayashi, 2016). In addition, it is also possible that unequal sister chromatid recombination or retrotransposition lead to copy number variation of some rDNA (Symonová *et al*., 2016). The process that maintains the homogeneity and functionality of rDNA is concerted Evolution (Zimmer *et al*., 1980; Dover, 1982), probably mediated by homologous and non-homologous recombination, since it is observable that the copy number and position of rDNA on chromosomes (Sochorová *et al*., 2017; Roy *et al*., 2005; Wang *et al*., 2017). Three mechanisms can generate copy number variation in humans: two recombination-based (nonallelic homologous recombination and nonhomologous end-joining) methods and retrotransposition (Zhang *et al*., 2009). In this sense, a variation in the number of the 18S rDNA copies, associated with a set of other associated repetitive DNAs appears to be a possible explanation for the differentiation observed.

Despite the small sampling size, it is not clear why such features manifest only in females. In fact, if this situation represents a polymorphic autosomal condition, it would be expected to occur in both sexes. Could this female trait have some correlation with a possible sex determining system, despite the absence of a morphologically heteromorphic chromosome pair in the karyotype? Sex chromosome systems with heteromorphic chromosomes are present in about 5% of actinopterygian fish (Arai, 2011). Unfortunately, classical cytogenetic methods

have some limitations for highlighting sex chromosome systems, unless a distinct differentiation is already present in the sex pair, thus underestimating their real occurrence (Gamble *et al.*, 2015). The sex determination in fish depends on a complex series of interconnected biochemical processes that can be mono or polygenic, and cytogenetic differences between heteromorphic pairs may be too small to be observed by current techniques (Gold *et al*., 1980; Devlin and Nagahama, 2002). However, this scenario has changed in the last years with the advent and popularization of cytogenomics. As *L. bimaculata* shows a copy number variation only in females, it is not possible to disregard its probable significance. In this view, the emergence of a sex chromosomal system in a very early evolutionary stage, characterized by the remaining morphological similarity in the proto-sex pair but already with discrimination in its genomic content, also appears to be a possible explanation. If so, it is plausible that the third female chromosome bearing the differentiated genomic content will constitute the future W chromosome of the emerging ZZ/ZW sex system.

Although a definite conclusion is not possible at this time, our hypothesis seems to be very similar to what is found in *Boulengerella*, a representative genus of Ctenoluciidae. This taxon shares similar characteristics to *L. bimaculata*, also presenting different sizes for the distal rDNA 18S sites and the corresponding C-banded region of only one homologue of a chromosome pair in the karyotype. However, in this case, the male specimens are the differentiated sex, thus suggesting a probable XX/XY sex chromosome system for Boulengerella (de Souza e Sousa *et al*., 2017). In this sense, it is significant that Ctenoluciidae is thought to be related to Lebiasinidae (Arcila *et al*., 2017; Betancur-R *et al*., 2019). This scenario provides a unique opportunity for fine-scale analysis of a putative nascent sex chromosomes, and further analysis involving sequencing analysis will be performed to fully understand this scenario.

Relationships between Lebiasinidae and Ctenolucidae

Previous phylogenetic studies have suggested a close relationship between Lebiasinidae and Erythrinidae, Ctenoluciidae, and Hepsetidae (Oyakawa, 1998; Buckup, 1998; Calcagnotto *et al*., 2005), but with distinct arrangements within this group. Recently, the use of new sequencing technology, together with phylogenetic reconstructions, has provided evidence that Lebiasinidae and Ctenoluciidae are sister groups (Arcila *et al*., 2017; Betancur-R *et al*., 2019). In this way, we performed a comparative analysis between *Lebiasina* and *Boulengerella* species, representative taxa of the Ctenolucidae family, in order to investigate their relatedness at the chromosomal level.

Notably, our results highlighted several similarities between *Lebiasina* and *Boulengerella* species, here represented by *B. lateristriga*, both at the chromosomal and genomic levels. At the level of the karyotype macrostructure, they have the same diploid number (2n = 36) as well as both having exclusively bi-armed chromosomes. However, similarities between Lebiasinidae and Ctenoluciidae go beyond to the 2n number and karyotype macrostructure. Furthermore, the CGH and WCP experiments also indicated their evolutionary relatedness. The comparative analysis of the gDNA of *L. bimaculata* and *B. lateristriga* provided evidence of the co-localization of scattered hybridization signals in many chromosomes of *L. bimaculata*, thus revealing the shared repetitive content of these regions. As expected, a range of non-overlapping species-specific signals also occurs, as the result of their specific evolutionary history (**Figure 6**). Remarkably, the Zoo-FISH analyses using both BOU-1 and LEB-1 probes showed complete homology between the first chromosomal pair of *L. bimaculata* and *B. lateristriga* (**Figure 7**), and such homology also extends to other *Bourengella* and *Ctenolucius* species (de Souza e Sousa, 2019). Despite the fact that probes from just one chromosomal pair were applied, the conservation of these syntenic regions between Lebiasinidae and Ctenolucidae species introduces the expectation that several other regions may have been

remained conserved during the course of their genome differentiation, despite the spatio-temporal isolation.

Materials and Methods

Individuals

The collection sites, numbers, and genders of individuals investigated are presented in **Figure 9** and **Table 2**. Samples were collected with the authorization of the environmental agency ICMBIO/SISBIO (License number 48628-2) and SISGEN (A96FF09). The specimens were properly identified by evaluation of their meristic characteristics and deposited in the fish collection site of the Museu de Zoologia da Universidade de São Paulo (MZUSP) under the voucher numbers 124457 and 124625.

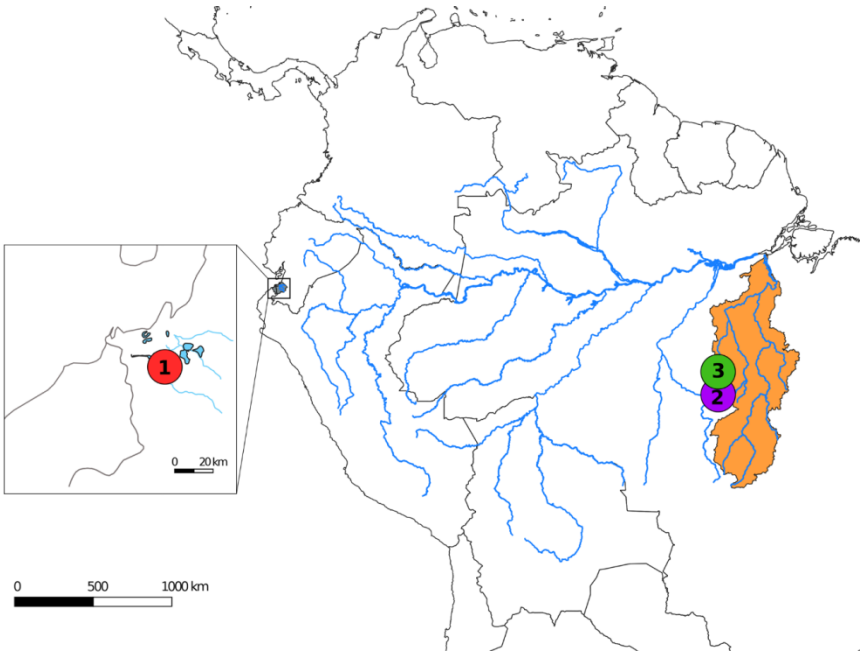


Figure 10. Map of South America highlighting the collection sites of Lebiasina bimaculata (1—red circle) and L. melanoguttata (2—purple and 3—green circles). The maps were created using the following software: QGis 3.4.3, Inkscape 0.92, and Photoshop 7.0.

Table 2. Collection sites of the Lebiasina species analyzed with the sample size (N).

Species	Locality	N
Lebiasina bimaculata	Arenillas river lakes—El Oro (Ecuador) (S03°30'57.204", W80°3'44.2656")	04♂, 03♀
Lebiasina melanoguttata	Altamira—PA (Brazil) (S08° 46' 59,4", W54°58'26,9")	10♂, 04♀
Lebiasina melanoguttata	Cachoeira da Serra—PA (Brazil) (S08°58'18,7", W54°58'18,7")	04♂, 18♀

Chromosome Preparations, C- and CMA3 Bandings

Mitotic chromosomes were obtained by the protocol described in Bertollo *et al*. (2015). The experiments followed ethical and anesthesia conducts and were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (Process number CEUA 1853260315). Chromomycin A3 and DAPI fluorescent staining was performed as described by Schmid (1980). The C-positive heterochromatin (C-banding) was identified according to Sumner (1972).

Fluorescence In Situ Hybridization (FISH) for Repetitive DNA Mapping

Two tandemly-arrayed DNA sequences isolated from the genome of an Erythrinidae species, *Hoplias malabaricus*, previously cloned into plasmid vectors and propagated in competent cells of *Escherichia coli* DH5α (Invitrogen, San Diego, CA, USA), were used. The first probe contained a 5S rDNA repeat copy and included 120 base pairs (bp) of the 5S rRNA transcribing gene and 200 bp of the nontranscribed spacer (NTS) (Martins *et al*., 2006). The second probe corresponded to a 1400 bp segment of the 18S rRNA gene obtained via PCR from nuclear DNA (Cioffi *et al*., 2009). These probes were directly labeled with the Nick-Translation mix kit (Roche, Manheim, Germany). The 5S rDNA was labeled with Spectrum Green-dUTP,

and the 18S rDNA was labeled with Spectrum Orange-dUTP (Vysis, Downers Grove, IL, USA), according to the manufacturer's manual. The small repetitive sequences (CA)15, (GA)15, (CAT)10, and (CGG)10 were directly labeled with Cy-3 (with the exception of (GA)15 which was direct labeled with FITC) during the synthesis, as described by [62]. Telomeric (TTAGGG)n sequences were also mapped using the DAKO Telomere PNA FISH Kit/FITC (DAKO, Glostrup, Denmark).

Comparative Genome Hybridization (CGH)

The gDNAs of *L. bimaculata*, *L. melanoguttata*, and *Boulengerella lateristriga* (Ctenolucidae, previously analyzed in (de Souza e Sousa *et al*., 2017)) were extracted from liver tissue by the standard phenol-chloroform-isoamylalkohol method (Sambrook and Russell, 2001). Four different experimental designs were used for this study. The first two assays were focused on intraspecific comparisons between males and females of both *Lebiasina* species. For this purpose, gDNA of males and females of *L. melanoguttata* and *L. bimaculata* was labelled with Spectrum Orange-dUTP and Spectrum Green-dUTP, respectively, using the Nick-Translation mix kit (Roche, Manheim, Germany), and hybridized against the male and female chromosome background of each species. For blocking the repetitive sequences in all experiments, we used C0t-1 DNA (i.e., a fraction of genomic DNA enriched for highly and moderately repetitive sequences) prepared according to (Zwick *et al*., 1997). The final probe mixture for each slide was composed of 500 ng of male-derived gDNA, 500 ng of female-derived DNA, and 15 μg of female-derived C0t-1 DNA. The probe was precipitated with ethanol and the dry pellets were mixed with a hybridization buffer containing 50% formamide, 2×SSC, 10% SDS, 10% dextran sulfate, and Denhardt's buffer at pH 7.0.

In the third set of the experiments, we focused on interspecific genomic comparisons between *Lebiasina* species. Male and female-derived genomic probes from *L. bimaculata* and

L. melanoguttata were hybridized together onto male and female chromosomal backgrounds of *L. bimaculata*. For this purpose, the gDNA of males and females of *L. melanoguttata* and *L. bimaculata* was labelled with Spectrum Green-dUTP and Spectrum Orange-dUTP, respectively, using the Nick-Translation kit (Roche, Manheim, Germany). The final probe cocktail was composed of 500 ng of male or female-derived gDNA of *L. melanoguttata*, 500 ng of male or female-derived DNA of *L. bimaculata*, and 15 μg of female-derived C0t-1 DNA from each species diluted in the hybridization buffer described above.

Finally, the fourth assay was focused on interfamily genomic comparisons. Female-derived genomic probes from both *L. bimaculata* and *B. lateristriga* (Ctenoluciidae) were hybridized together onto female chromosomes of *L. bimaculata*. For this purpose, female gDNA of *L. bimaculata* and *B. lateristriga* was labeled with Spectrum Green-dUTP and Spectrum Orange-dUTP, respectively, using the Nick-Translation mix kit (Roche, Manheim, Germany). The final probe cocktail was composed of 500 ng of female-derived gDNA of *L. bimaculata*, 500 ng of female-derived DNA of *B. lateristriga*, and 15 μg of female-derived C0t-1 DNA of each species, diluted in the hybridization buffer described above.

The hybridization experiments were performed according to (Symonová *et al*., 2013).

Whole Chromosome Painting (WCP)

For cross-species painting, we selected the first chromosome pair from the *L. bimaculata* and *B. lateristriga* complement, as they unambiguously represent the largest element in the karyotypes. This allowed us to precisely identify both homologues after Giemsa staining. Sixteen copies of the first chromosome pair (pair 1) of *B. lateristriga* and *L. bimaculata* were isolated by glass-based microdissection and amplified using the procedure described in (Yang *et al*., 2009). The probes were referred to as BOU-1 and LEB-1 and they were labeled with

Spectrum Green-dUTP and Spectrum-Orange-dUTP (Vysis, Downers Grove, IL, USA), respectively, in a secondary Degenarate Oligonucleotide-Primed Polymerase Chain Reaction (DOP PCR) using 1 µL of the primarily amplified product as template DNA (Yang *et al*., 2009). Chromosomal preparations from *L. bimaculata* and *L. melanoguttata* females were used for Zoo-FISH experiments and the following hybridization procedures (Yano *et al*., 2017).

Analyses

At least 30 metaphase spreads per individual were analyzed to confirm the 2n number, karyotype structure, and FISH results. Images were captured using an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan) with CoolSNAP and processed using Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA). Chromosomes were classified as metacentric (m) or submetacentric (sm), according to their arm ratios (Levan *et al*., 1964).

Conclusions

This study provides the first chromosomal data for *Lebiasina* species, allowing for the investigation of the karyoevolutionary process between two *Lebiasina* species and their relationships, as well as their relationship with other Lebiasinidae species and with other fish families. The particular chromosomal characteristics that differ in both *Lebiasina* species at the inner chromosomal organization level clearly show that similarities shared in their karyotype macrostructures were, in fact, followed by a remarkable intra-genomic variation during their evolutionary history. Furthermore, considering both the basal condition of *Lebiasina* and the overall chromosomal data for other Lebiasinidae genera, it is likely that huge chromosomal rearrangements, both in number as well as in morphology, have occurred during the diversification of this family. Furthermore, our results indicate a close evolutionary relationship between Lebiasinidae and Ctenoluciidae, as previously proposed by some molecular and morphological phylogenies. Particularly noteworthy is the heteromorphic condition presented by *L. bimaculata* females on the third chromosome pair of the karyotype, a feature that is similarly found among males of *Boulengerella lateristriga* (Characiformes, Ctenoluciidae). Such similarity suggests a copy number variation that could probably lead to evolutionary processes of sex chromosomes in both families, however, this deserves further investigation.

Capítulo 2

Understanding the chromosomal evolution of Lebiasinidae fishes (Teleostei, Characiformes)

Francisco de Menezes Cavalcante Sassi, Terumi Hatanaka, Renata Luiza Rosa de Moraes, Ezequiel Aguiar de Oliveira, Gustavo Akira Toma, Luiz Antonio Carlos Bertollo, Alexandr Sember, José Francisco de Sousa e Souza, Patrik F. Viana, Eliana Feldberg, Mauro Nirchio, Manoela Maria Ferreira Marinho, Orlando Moreira Filho, Marcelo de Bello Cioffi.

Revista a ser submetido: **Genes (MDPI)**

Abstract

We present comparative genomic hybridization experiments in several Lebiasinidae species and a revision of the distribution of ribosomal sequences among them. By that, eleven species of several Brazilian rivers were analyzed. The mitotic chromosomes were obtained by the classical air-drying technique. Schematic representations of the rDNA 18S and 5S loci were designed. Ten sets of experiments were developed in order to hybridize genomic DNA of *Copeina*, *Copella*, *Nannostomus* and *Pyrrhulina* species in metaphase plates of *Lebiasina melanoguttata*. Our experiments revealed two major pathways on chromosomal evolution of these species: i) conservation of 2n=36 bi-armed chromosomes in Lebiasininae, as a basal condition, and ii) high numeric and structural chromosomal rearrangements in Pyrrhulininae, with a notable tendency towards acrocentrization. The ribosomal DNA distribution also revealed a marked differentiation during the chromosomal evolution of Lebiasinidae, since both single and multiple sites, in addition to a wide range of chromosomal locations can be found. With some few exceptions, the terminal position of 18S rDNA on chromosomes appears as a common feature in Lebiasinidae analyzed species. Altogether with Ctenoluciidae, this pattern can be considered a symplesiomorphism for both families. In addition to the specific repetitive DNA content that characterizes the genome of each particular species, *Lebiasina* also keeps inter-specific repetitive sequences, thus reinforcing its proposed basal condition within Lebiasinidae.

Introduction

Advanced molecular approaches have been widely applied in cytogenetic studies of several animal groups, providing useful insights about their karyotype and genome evolution. However, although in fish such procedures have also improved investigations as a whole, chromosomal analysis of several taxa are still incipient. Obtaining good metaphases plates, both in quantity and quality, stands out as the reason for such deficit, mainly for small-sized fishes. Thus, dealing with chromosomes of miniature species, which reach a maximum length of 26 mm (Weitzman and Vari, 1988), is a challenge, but also a possible procedure (Moraes *et al*., 2017; Sousa e Souza *et al*., 2017; Moraes *et al*., 2019; Sassi *et al*., 2019; Toma *et al*., 2019).

Lebiasinidae is a freshwater fish family comprising about 75 valid species, distributed throughout Central and South America, except Chile, which experienced a body miniaturization along their evolution (Weitzman and Vari, 1988; Fricke *et al*., 2019). Netto-Ferreira (2010), based on morphological characters, recognized two distinguishable subfamilies: i) Lebiasininae comprising the *Lebiasina* and *Piabucina* genera and ii) Pyrrhulininae including the *Copeina*, *Copella*, *Derhamia*, *Nannostomus* and *Pyrrhulina* genera.

The phylogenetic position of Lebiasinidae in relation to other Characiformes groups have been quite discussed (Oyakawa, 1997; Orti & Meyer, 1997; Buckup, 1998; Calcagnotto *et al*., 2005), but without a definitive conclusion. Recent analyses based on molecular data show that Ctenoluciidae emerges as the sister group of Lebiasinidae (Oliveira *et al*., 2011; Arcila *et al*., 2017; Betancur-R *et al*., 2019). This proposal was also corroborated by cytogenetic approaches (Sassi *et al*., 2019), where whole chromosome painting (WCP) experiments with probes from the first chromosome pair of *Lebiasina bimaculata* (Lebiasinidae) and *Boulengerella lateristriga* (Ctenoluciidae) revealed a complete homology between them, a fact also extended to other Ctenoluciidae species. Additionally, the comparative genomic analysis (CGH) experiment showed co-localized scattered signals on *L. bimaculata* and *B. lateristriga*

chromosomes, indicating that shared syntenic regions remained conserved during the evolutionary process of these groups (Sassi *et al*., 2019).

Lebiasina (Lebiasininae) is one of the most unexplored Lebiasinidae genera, with no cytogenetic data until a report provided by Sassi *et al*. (2019). It is considered a basal group concerning the other lebiasinids, with morphological (Netto-Ferreira, 2010) and cytogenetic (Sassi *et al*., 2019) features corroborating such position. This makes *Lebiasina* an interesting group for evolutionary studies. In fact, basal clades are important links making possible useful comparisons at intra- and interfamilial levels. For such purposes, the CGH is a helpful methodology that have improved the evolutionary cytogenetic by comparing entire genomes. Although initially developed to suit clinical approaches (Kallioniemi *et al*., 1992), CGH is now successfully used to trace evolutionary trends among different biological groups. Among fishes, distinctive evolutionary processes (including the differentiation of sex chromosomes) have been highlighted among different species using this useful procedure (Yano *et al*., 2016; Oliveira and Sember *et al*., 2017; Moraes *et al*., 2019; Sassi *et al*., 2019).

This study is part of a series focusing in the chromosomal evolution of the Lebiasinidae family. Here, it was used CGH experiments for hybridizing genomes of several lebiasinid species, and revised the distribution of ribosomal sequences among them, thus providing additional insights on the chromosomal evolution of this family.

Material and Methods

Samples

Eleven Lebiasinidae species from several Brazilian rivers were analyzed (**Figure 1; Table 1**). Collection had authorization from Brazilian Environmental Agencies: ICMBIO/SISBIO (License number 48628-2) and SISGEN (A96FF09). Specimens were identified and deposited in the Museu de Zoologia da Universidade de São Paulo (MZUSP), under the voucher numbers 119077, 119079, 123073, 123077, 123080, 124457, 124625, 124915.

Chromosomes and Ideograms

Mitotic chromosomes were obtained by the classical air-drying technique (Bertollo *et al*., 2015) from kidney cells. All experiments followed the ethical/anesthesia conducts and were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (Process number CEUA 1853260315). Schematic representations, aiming an overview of chromosomal distribution of the 5S and 18S rDNA sequences in Ctenoluciidae and Lebiasinidae, were organized under the Adobe Photoshop CC 2015, according the data from Sousa e Souza *et al*. (2017), Moraes *et al*. (2017; 2019), Sassi *et al*. (2019), Toma *et al*. (2019) and Sember *et al*. (2019).

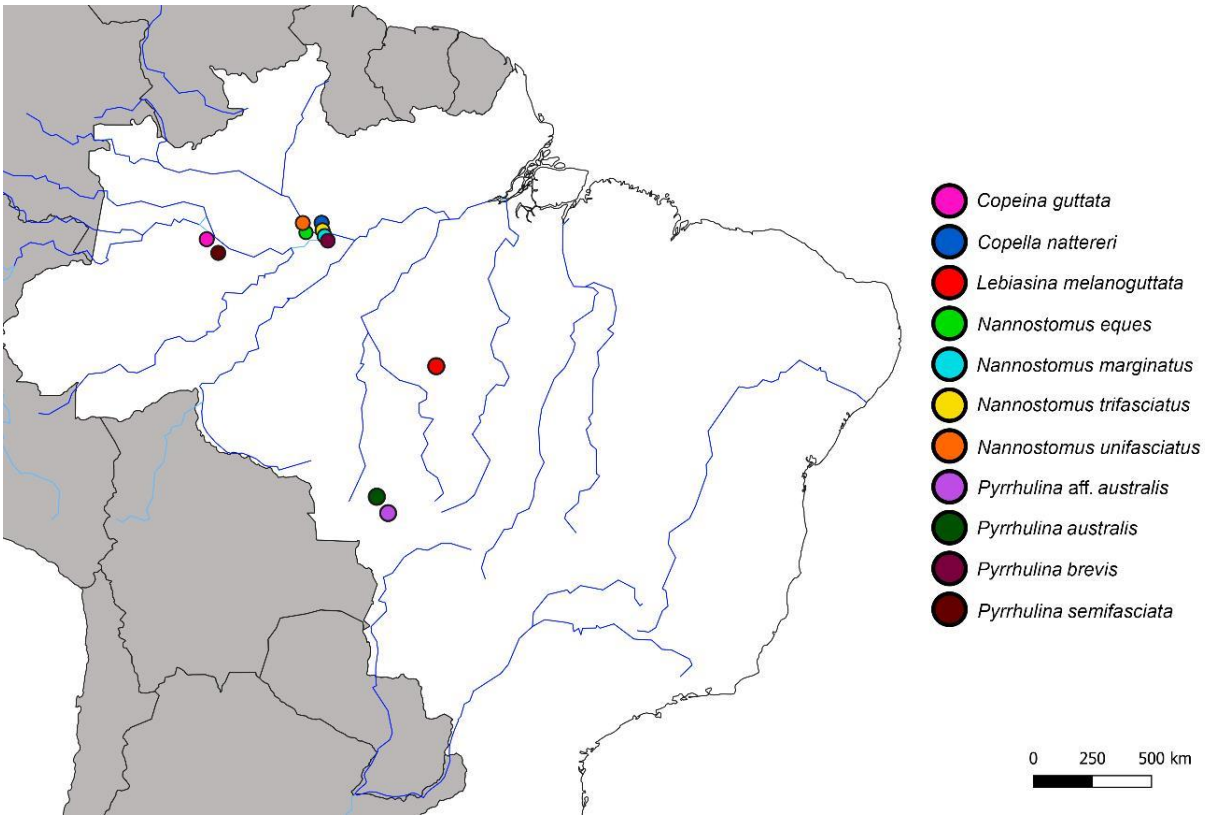


Figure 1. Partial South America map highlighting the Brazilian collection sites of *Copeina guttata*, *Copella nattereri*, *Lebiasina melanoguttata*, *Nannostomus eques*, *N. marginatus*, *N. trifasciatus*, *N. unifasciatus*, *Pyrrhulina australis*, *Pyrrhulina* aff. *australis*, *P. brevis* and *P. semifasciata*. The map was produced using the softwares QGis 3.4.4, Inkscape 0.92, and Photoshop CC 2015.

Table 1. Collection sites and sample sizes (*N*) of the analyzed species.

Species	Locality	N
Copeina guttata	Tefé – AM (Brazil) (S03°23'07.7", W64°46'43.7")	11♀; 06♂
Copella nattereri	Manaus – AM (Brazil) (S02°35'42.9", W60°02'23.8")	04♀; 06♂
Lebiasina melanoguttata	Cachoeira da Serra - PA (Brazil) (S08°58'18,7", W54°58'18,7")	22♀; 14♂
Nannostomus eques	Manaus – AM (Brazil) (S02°47'58.1", W60°29'19.8")	02♀; 02♂
N. marginatus	Manaus – AM (Brazil) (S02°55'53.9", W59°58'30.7")	03♀; 05♂
N. trifasciatus	Manaus – AM (Brazil) (S02°44'59.6", W60°01'37.9")	07♀; 12♂
N. unifasciatus	Manaus – AM (Brazil) (S02°47'58.1", W60°29'19.8")	05♀; 07♂
Pyrrhulina australis	Santo Afonso – MT (Brazil) (S14°27'25.2", W57°34'35.2")	30♀; 18♂
Pyrrhulina aff. *australis*	Barra do Bugres – MT (Brazil) (S15°04'27.5", W57°11'05.4")	22♀; 16♂
P. brevis	Manaus – AM (Brazil) (S02°55'53.9", W59°58'30.7")	13♀; 17♂
P. semifasciata	Tefé – AM (Brazil) (S3°39'45.8", W64°35'33.3")	07♀; 12♂

Probes for Comparative Genomic Hybridization (CGH)

Ten sets of experiments were developed in order to hybridize the genomic DNA (gDNA) of *Copeina*, *Copella*, *Nannostomus* and *Pyrrhulina* species in metaphase plates of *Lebiasina melanoguttata*. For this purpose, the female-derived gDNA of *L. melanoguttata*, *C. guttata*, *C. nattereri*, *P. australis*, *Pyrrhulina* aff. *australis*, *P. brevis*, *P. semifasciata*, *N. eques*, *N. marginatus*, *N. trifasciatus* and *N. unifasciatus* were extracted from liver tissues by a standard phenol-chloroform-isoamyl alcohol method (Sambrook and Russell, 2001). For all assays, the female-derived gDNA of *L. melanoguttata* was directly labeled with Atto488 (green fluorescence) using the Nick-translation labeling kit (Jena Bioscience, Jena, Germany), while the gDNA of *C. guttata*, *C. nattereri*, *P. australis*, *Pyrrhulina* aff. *australis*, *P. brevis*, *P. semifasciata*, *N. eques*, *N. marginatus*, *N. trifasciatus* and *N. unifasciatus* were direct labeled

with Atto550 (red fluorescence) also using the Nick-translation labeling kit (Jena Bioscience, Jena, Germany). The final hybridization mixtures contained 500 ng of *L. melanoguttata* gDNA plus 500 ng of gDNA from one of the above described species. In all experiments repetitive sequences were blocked using 25 μg of C_0t-1 DNA, prepared according to Zwick *et al*. (1997), and dissolved in 20 μl of the hybridization buffer (50% formamide, 2x SSC, 10% SDS, 10% dextran sulfate and Denhardt's buffer, pH 7.0).

Fluorescence *in situ* Hybridization (FISH) for CGH

CGH experiments were performed following the protocol of Symonová *et al*. (2015). Slides were first aged for 1 to 2 hours at 60 °C and then treated with RNase A (20 μg/ml; 90 min at 37 °C in a wet chamber), and pepsin (50 μg/ml; 3 min at 37 °C). Chromosomes were denatured in 75% formamide diluted in 2x SSC at 74 °C for 3 min. At the same time, the probes were also denatured at 86 °C for 10 min and chilled on ice for 10 min. Then, the hybridization mix was applied to the slides, followed by a three-days incubation in a wet chamber (37 °C). The non-specific hybridization was removed by a stringent washing at 44 °C, two washes in 50% formamide/2x SSC (10 min each), three washes in 1x SSC (7 min each), and a final wash in 2x SSC at room temperature. Chromosomes were counterstained with DAPI (1.2 μg/ml) and mounted in an antifade solution (Vector, Burlingame, CA, USA).

Results

Chromosomal distribution of the ribosomal sequences among Lebiasinidae and Ctenoluciidae species

Boulengerella (Ctenoluciidae) species (**Figure 2a**) have 5S rDNA sites located in the terminal and pericentromeric regions of the first and the tenth chromosome pairs, respectively. The only exception for this pattern occurs in *B. lucius*, which has the fourth chromosome pair, instead of the tenth one, bearing these sites. Concerning the 18S rDNA it is found only in the telomeric region of the 18th pair of all *Boulengerella* species (Souza e Sousa *et al*., 2017).

Nannostomus species (**Figure 2d)**, exhibit 5S rDNA sequences in only one chromosome pair, although with a marked variation in position, i.e. (1) telomeric region of the short arms of the pairs 03 of *N. eques* and 04 of *N. marginatus*, (2) proximal region of the long arms of the pair 07 of *N. unifasciatus*, and (3) telomeric region of the pair 18 of *N. beckfordi*. In its turn, the 18S rDNA sites have a more varied distribution both in number and location among species: (1) one signal in the telomeric region of the short arms of the 2nd chromosome pair of *N. beckfordi*, (2) two signals, both in the interstitial region of the long arms of the 2nd pair of *N. unifasciatus*, (3) one signal in the telomeric region of the short arms of the chromosomes 02 and 18 in *N. eques* and (4) one telomeric signal in the short arms of the pair 03 of *N. marginatus*, with an additional pericentromeric signal in the long arms of the pair 19 (Sember *et al.*, 2019).

Lebiasina species (**Figure 2c)** also have distinct patterns of ribosomal DNA distribution. The Ecuadorian species, *L. bimaculata*, presents 5S sites in the interstitial position of the first chromosome pair and 18S sites in the telomeric region of the pair 03. On the other hand, the Brazilian species, *L. melanoguttata*, presents multiple 18S sites, being 12 telomeric ones comprising the chromosome pairs 01, 02, 03, 07 and 09, but also including bi-telomeric sites in the pair 02. The 5S rDNA sequences occur in the proximal region of the long arms of the chromosome 01, thus showing a syntenic distribution with the 18S rDNA, and also in the short arms of the pair 13 (Sassi *et al.*, 2019).

Copeina guttata (**Figure 2b**), presents 5S rDNA marks in the proximal region of the long arms of the second chromosome pair, and also in the short arms of the 15th one. On other hand, the 18S rDNA has a single distribution, being located in the short arms of the pair 04 (Toma *et al*., 2019.)

Pyrrhulina species have a most diversified pattern of ribosomal distribution than the other Lebiasinidae, with multiple 5S and 18S rDNA sites **(Figure 2e).** In *P. semifasciata*, the short arms of the pairs 07, 08, 09, 15 and 21 possess 5S rDNA sequences, while the 18S rDNA ones are located in the chromosomes 01, 03, 06 and 11. Likewise, *P. brevis* also presents five chromosomes with 5S rDNA sequences in their short arms (pairs 03, 07, 08, 10 and 14). In the 7th pair, an additional interstitial signal occurs on the long arms, as well as in the chromosome 10 but in the proximal region. Synthenia occurs in both pairs 03 and 14, where 5S and 18S rDNA sequences are shared in the short arms, in addition to pair 11 bearing only 18S sites. In *P. australis*, 18S rDNA is found in the short arms of the pairs 01, 06, 11 and 19, in both telomeric regions of the pair 04, and also in this same region of the long arms of the pair 07. Colocalized 5S and 18S sequences occur in the short arms of the pair14, in addition to 5S sites in the short arms of the chromosomes 03, 07, 08, 09, 10, 15 and 16. *P.* aff. *australis* presents four chromosomes with 5S rDNA (pairs 03, 07, 15 and 16) in their short arms. 18S sequences also occur in the 7th pair, but in the telomeric region of the long arms, besides an additional site in the short arms of the pair 06 (Moraes *et al.*, 2017, 2019).

Comparative Genomic Hybridization (CGH)

CGH experiments revealed that a significant level of genomic divergence occurs among *L. melanoguttata* and the other lebiasinid species (**Figures 3-5)** In fact, a high level of species-specific genomic compartmentalization stood out, with distinct patterns of repetitive DNA

sequences both in amount and distribution in the chromosomes. In addition, some inter-specific segments of repetitive DNAs were also highlighted as shared among species.

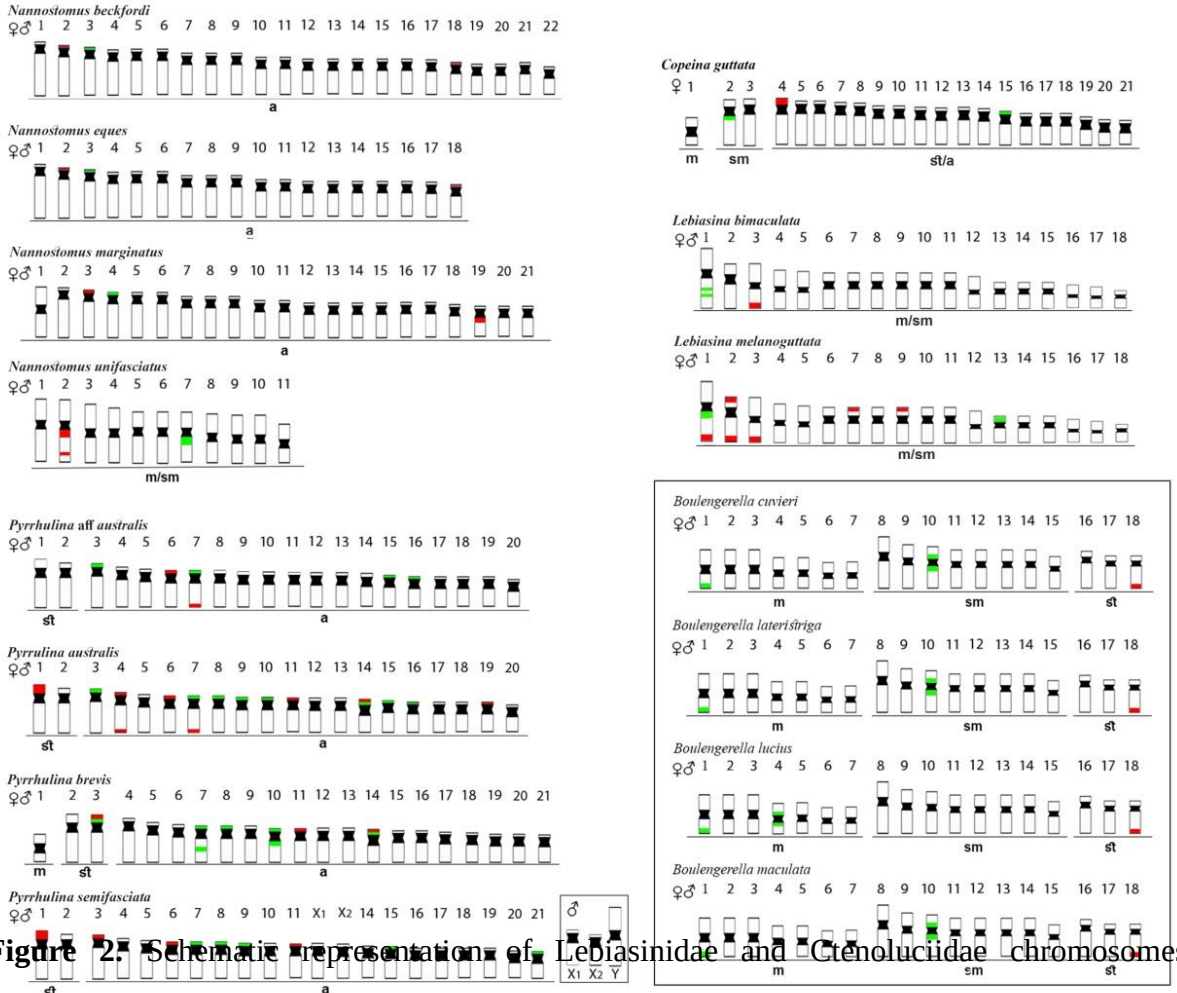


Figure 2. Schematic representation of Lebiasinidae and Ctenoluciidae chromosomes, highlighting the position of 5S rDNA (green) and 18S rDNA (red). The box highlights a sex chromosome system in *Pyrrhulina semifasciata*. FISH data were taken from de Sousa e Souza *et al*. (2017); Moraes *et al*. (2017; 2019), Sassi *et al*. (2019), Toma *et al*. (*in prep*) and Sember *et al*. (*in prep*).

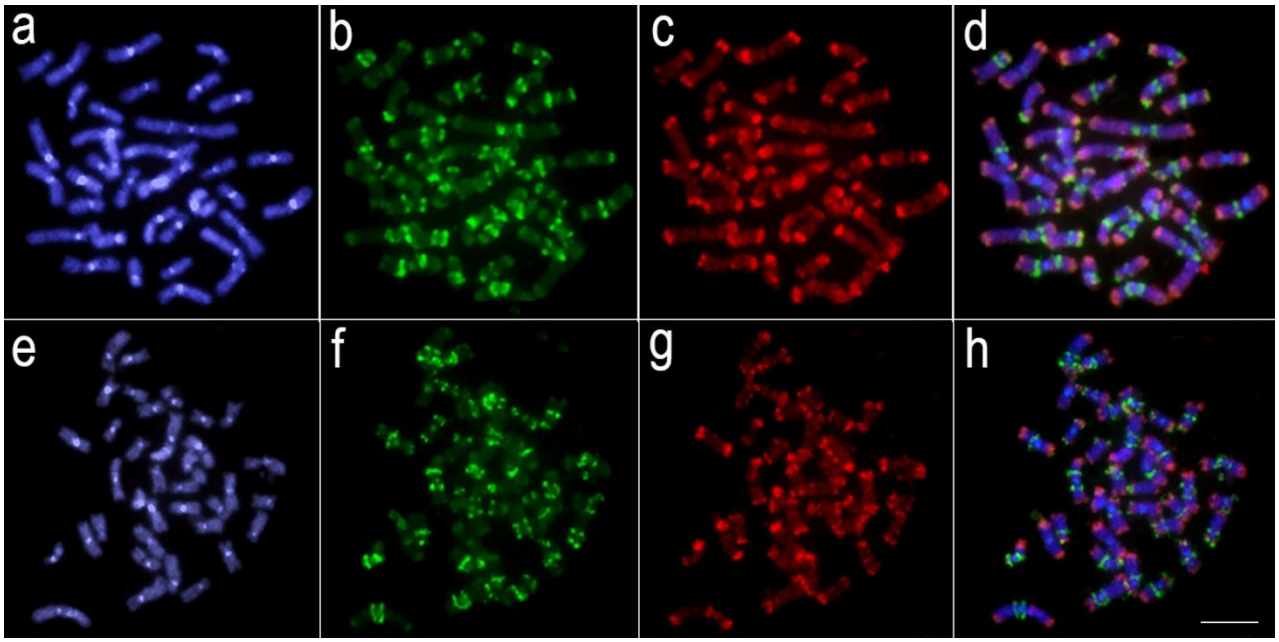


Figure 3. Comparative genomic hybridization using the gDNA of *Lebiasina melanoguttata*, *Copeina guttata* and *Copella nattereri* against chromosomal background of *Lebiasina melanoguttata* . Genomic probes from *L. melanoguttata* and *Copeina guttata* hybridized against *L. melanoguttata* chromosomes (**a – d**). Genomic probes from *L. melanoguttata* and *Copella nattereri* hybridized against *L. melanoguttata* chromosomes (**e – h**). First column (**a** and **e**): DAPI images (blue); second column (**b** and **f**): hybridization patterns using gDNA probe from *L. melanoguttata*; third column (**c** and **g**): hybridization patterns using gDNA probes from *Copeina guttata* and *Copella nattereri*, respectively; fourth column (**d** and **h**) merged images of both genomic probes and DAPI staining depicting the common regions in yellow. Bar = 5 µm.

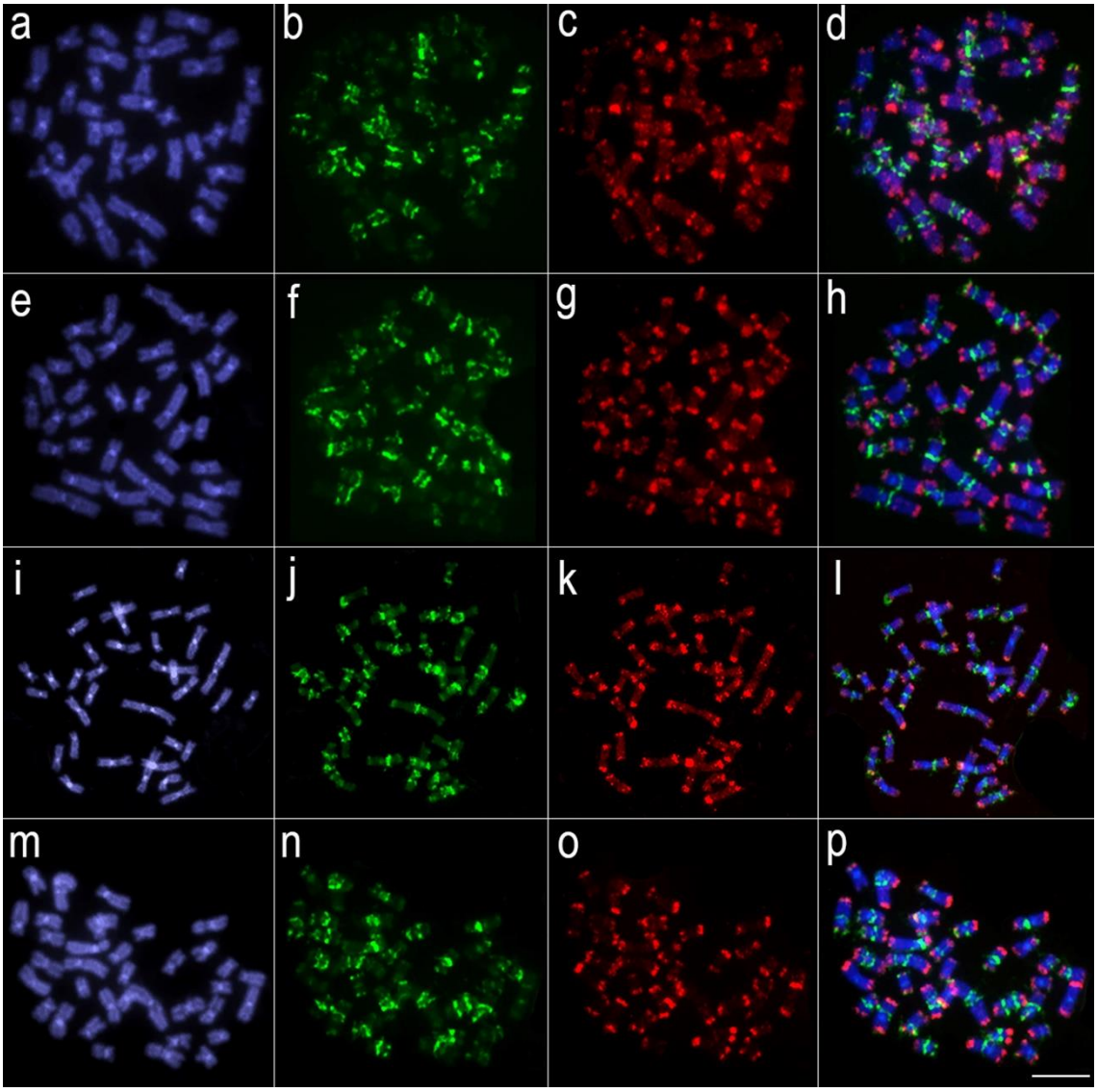


Figure 4. Comparative genomic hybridization using the gDNA of *Lebiasina melanoguttata* and *Pyrrhulina* species against chromosomal background of *Lebiasina melanoguttata*. Genomic probes from *L. melanoguttata* and *P. australis* hybridized against *L. melanoguttata* chromosomes (**a – d**). Genomic probes from *L. melanoguttata* and *Pyrrhulina* aff. *australis* hybridized against *L. melanoguttata* chromosomes (**e – h**). Genomic probes from *L. melanoguttata* and *P. brevis* hybridized against *L. melanoguttata* chromosomes (**i – l**). Genome from *L. melanoguttata* and *P. semifasciata* hybridized against *L. melanoguttata* chromosomes (**m – p**). First column (**a, e, i** and **m**): DAPI images (blue); second column (**b**, **f, j** and **n**): hybridization patterns using gDNA probe from *L. melanoguttata*; third column (**c, g, k** and **o**): hybridization patterns using gDNA probes from *P. australis*, *Pyrrhulina* aff. *australis*, *P. brevis* and *P. semifasciata*, respectively; fourth column (**d**, **h, l** and **p**) merged images of both genomic probes and DAPI staining depicting the shared regions in yellow. Bar = 5 µm.

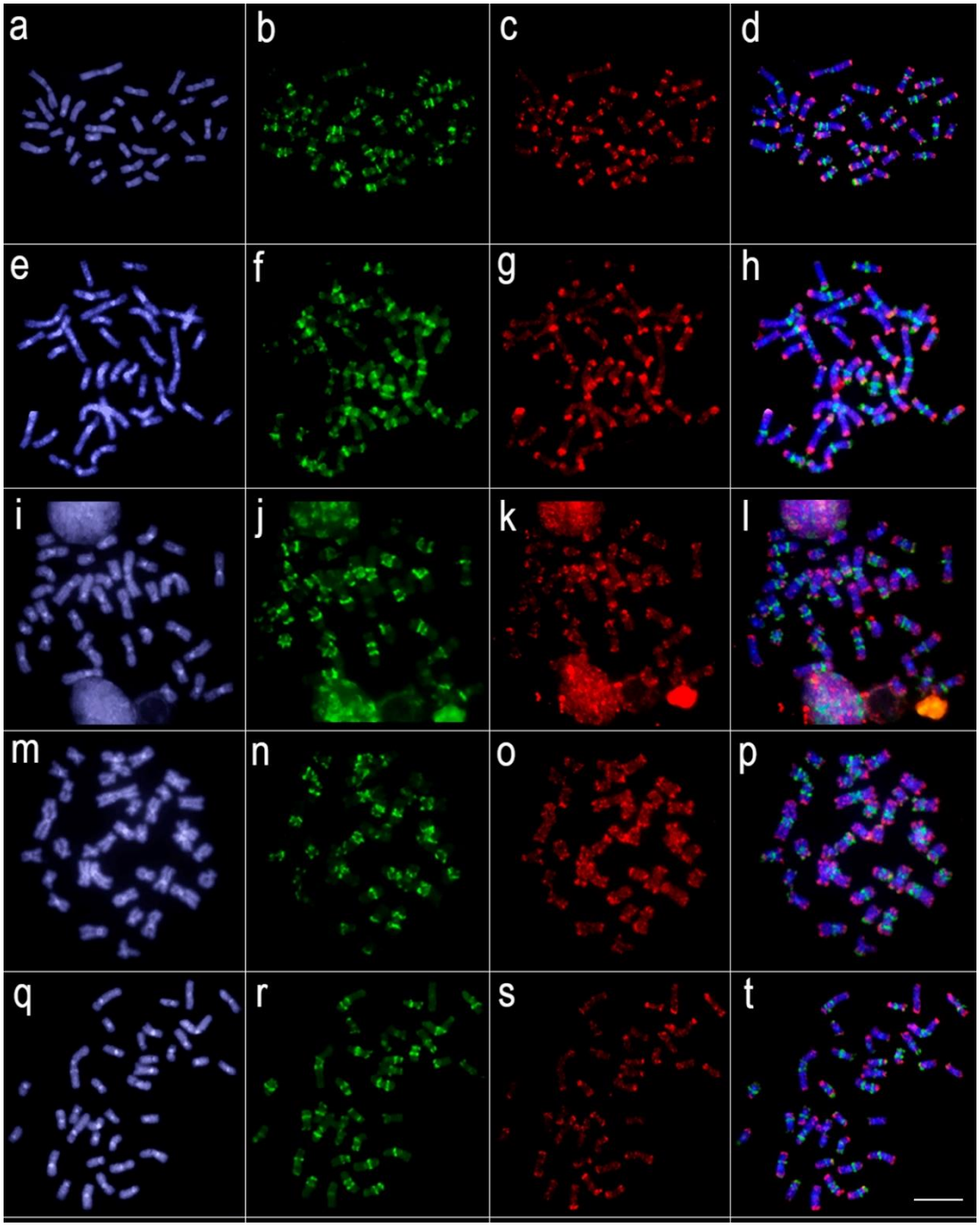


Figure 5. Comparative genomic hybridization among *Lebiasina melanoguttata* and *Nannostomus* species. Genomic probes from *L. melanoguttata* and *N. unifasciatus* hybridized against *L. melanoguttata* chromosomes (**a – d**). Genomic probes from *L. melanoguttata* and *N. trifasciatus* hybridized against *L. melanoguttata* chromosomes (**e – h**). Genomic probes from *L. melanoguttata* and *N. beckfordi* hybridized against *L. melanoguttata* chromosomes (**i – l**). Genomic probes from *L. melanoguttata* and *N. eques* hybridized against *L. melanoguttata* chromosomes (**m – p**). Genomic probes from *L. melanoguttata* and *N. marginatus* hybridized against *L. melanoguttata* chromosomes (**q** – **t**). First column (**a, e, i, m** and **q**): DAPI images (blue); second column (**b**, **f, j**, **n** and **r**): hybridization patterns using gDNA probe from *L. melanoguttata*; third column (**c, g, k, o** and **s**): hybridization patterns using gDNA probes from *N. unifasciatus, N. trifasciatus, N. beckfordi, N. eques* and *N. marginatus*, respectively; fourth column (**d**, **h, l, p** and **t**) merged images of both genomic probes and DAPI staining depicting the shared regions in yellow. Bar = 5 µm.

Discussion

Evolutionary trends in the Lebiasinidae family

Huge chromosomal rearrangements both in number and morphology, which may be probably linked to speciation processes, took place during the diversification of the Lebiasinidae family. Altogether, two major pathways can be recognized on chromosomal evolution of lebiasinids: i) conservation of 2n=36 bi-armed chromosomes in the Lebiasininae family as a basal condition, in accordance the scenario found in the putative sister family Ctenoluciidae; ii) high numeric and structural chromosomal rearrangements in the Pyrrhulininae subfamily, with a notable tendency towards acrocentrization (**Figure 2**). These findings fit with some proposals stating that several derived fish clades predominantly present mono-armed chromosomes while basal ones have more bi-armed chromosomes (Nirchio *et al*, 2014).

Teleost fishes display varied arrangements of chromosomal evolution. It is noteworthy, for example, that several Characiformes groups have a somewhat conserved karyotype organization pattern, maintaining the diploid number around 2n=54 and a relatively similar chromosomal morphology. This is the case for Anostomidae, Curimatidae, Prochilodontidae, Hemiodontidae and Chilodontidae (Arai, 2011), and also for some families belonging to Perciformes (Galetti *et al*., 2000). Such characteristics could be related to a karyotypic orthoselection (White, 1973), leading to a bi-armed chromosomes conservation. However, rapid and recent speciation events can also create conserved karyotypes (Sola *et al*., 1981), a fact that cannot be ruled out for the Lebiasinidae family, since it's only phylogenetic analysis does not make reference to divergence time (Netto-Ferreira, 2010). Indeed, the Lebiasininae subfamily presents a conserved karyotype macrostructure, although with interspecific genomic divergences (Sassi *et al*., 2019). In its turn, other fish groups show marked divergences in their karyotype structure as, for example, the Erythrinidae (Bertollo, 2007; Cioffi *et al*., 2012) and Characidae in the Characiformes (Arai, 2011), and the Loricariidae in Siluriformes (Artoni and

Bertollo, 1996; Giuliano-Caetano, 1998; Kavalco *et al*., 2005). Notably, both trends are present in the Lebiasinidae family, thus differentiating the evolutionary pathways followed by the Lebiasininae and Pyrrhulininae subfamilies.

The divergent evolutionary pathways between Lebiasininae and Pyrrhulininae are also pointed out by CGH experiments, where sequences of repetitive DNAs hybridize in different loci among species, thus pointing to a high degree of genomic divergence among them. It is noteworthy that divergent patterns of hybridization occur even among closely related species, such as between *L. bimaculata* and *L. melanoguttata* (Sassi *et al*., 2019), and *P. semifasciata* and *P. brevis* (Moraes *et al*., 2019), revealing species-specific CGH signals. Concerning *Lebiasina*, this is a somewhat expected feature, since *L. melanoguttata* is highly endemic in the Brazilian Serra do Cachimbo, remaining isolated from several other Lebiasinidae species for a distance of 1500 km (Goulding *et al*., 2003; Netto-Ferreira, 2012). The presence of two other *Lebiasina* species (*L. marilynae* and *L. minuta*) in this same isolated area suggests the occurrence allopatric speciation events (Netto-Ferreira, 2012), favoring the emergence of different patterns of genomic organization. However, in addition of such general genomic divergence, it is also evident that inter-specific hybridization of repetitive sequences still occurs in *Lebiasina* chromosomes, in this way supporting the proposal of its proposed basal position in the Lebiasinidae family (Arcila *et al*., 2017; Betancur-R *et al*., 2018).

The ribosomal DNA distribution is also a characteristic that experienced a marked differentiation during the chromosomal evolution of the Lebiasinidae. In fact, our review evidences that these sequences can be distributed since a single site in the karyotype (i.e. *Lebiasina bimaculata*) to multiple ones (i.e. *Pyrrhulina australis*), in addition to a wide range of chromosomal locations. The evolution of rDNA sequences follows the concept of concerted evolution, maintaining the functionality and homogeneity of these genes (Zimmer *et* al., 1980; Dover, 1982). However, since homologous and non-homologous recombination are processes

that mediates the concerted evolution, it is possible that unequal sister chromatid recombination or retrotransposition lead to favour a copy number variation of such sequences (Roy *et al*., 2005; Symonová *et al*., 2015; Sochorová *et al*., 2018; Wang *et al*., 2017). Indeed, this copy number variation can generate some non-transcribed rDNA copies, that have an extreme importance on genome integrity (Kobayashi, 2014). In fishes, copy number variation of ribosomal DNAs are highly reported, since their gene regulation processes seems to be more relaxed than in higher vertebrates (Symonová and Howell, 2018). In its turn, it is meaningful that Ctenoluciidae fishes present a conserved pattern of rDNA distribution. In this family a single site of 18S rDNA is found in all species, with a single exception for *B. lucius* (Souza e Sousa *et al*., 2018). Since the basal *Lebiasina* genus, shares this same single pattern, it is possible that this characteristic has arisen before the divergency of Lebiasinidae and Ctenoluciidae.

With some few exceptions, the chromosome terminal position of the 18S rDNA appears as a common feature for *Nannostomus*, *Pyrrhulina*, *Lebiasina* and *Copeina*. Altogether with Ctenoluciidae, this pattern can be considered a symplesiomorphism for both families. In fact, the terminal position of 45S rDNA is a common trait for several groups, including fish, in contrast to the 5S loci that appear to have a more frequent interstitial location along the chromosomes (Sochorová *et al*., 2018). However, this later condition does not apply to Lebiasinidae and even Ctenoluciidae, where both terminal and intersticial positions are highlighted, but with a preferential location at the ends in *Nannostomus* and *Pyrrhulina.*

It is also noteworthy that *N. unifasciatus* and *P. brevis* exhibit particular arrangements of ribosomal DNAs. To some extent, this is an expected trait for *N. unifasciatus*, since this species has the lowest diploid number among Lebiasinidae fishes, with 2n = 22 and the karyotype probably shaped by Robertsonian fusions (Arefjev, 1990). In its turn, peri- and/or paracentric inversions appear to have had important role on the karyotype differentiation of *P.*

brevis (Moraes *et al.*, 2019). In this sense, besides the action of possible transposable elements, it is possible that rDNA sequences have been shift by such rearrangements during the karyotype evolution. Furthermore, syntenic 5S and 18S sites occur in *L. melanoguttata* (pair 01), *P. australis* (pairs 07 and 14), *P.* aff. *australis* (pair 07) and *P. brevis* (pairs 03 and 14). The co-localization of both rDNA classes in the same chromosome may increase the recombination frequency between them (Sochorová *et al*., 2018), and, in association with heterochromatin, may act as recombination hotspots (Salvadori *et al*., 1995; Sola *et al*., 2003; Gornung, 2013).

Concluding remarks

The studies of Arcila *et al*. (2017), and Betancur-R *et al*. (2018), indicated the proximity to the Lebiasinidae and Ctenoluciidae families, besides corroborating the monophilia of the two lebiasinid subfamilies, Lebiasininae and Pyrrhulininae. Meaningly, conventional and molecular cytogenetic data that have been progressively improved for miniature fishes, actually corroborate and strengthen the proposed proximity relationship between Lebiasinidae and Ctenoluciidae. Additionally, it is also notorious the evolutionary divergence that appears to differentiate both Lebiasinidae subfamilies. In fact, the chromosomal diversity in Pyrrhulininae hugely contrasts with the apparent conservatism in the Lebiasininae subfamily. Furthermore, in addition to the specific repetitive DNA content that characterizes the genome of each particular species, *Lebiasina* also keeps inter-specific repetitive sequences, thus reinforcing its proposed basal condition within Lebiasinidae. Even keeping in mind that representative species of both subfamilies still require further investigations, the results now available already provide significant advances in understanding the chromosomal evolution of Lebiasinidae fish, a group of the Neotropical Ichthyofauna previously lacking resolute cytogenetic investigations.

V – CONSIDERAÇÕES FINAIS

A caracterização citogenética de espécies do gênero *Lebiasina*, ainda não estudadas, permitiu inferir que duas rotas evolutivas diferenciais provavelmente ocorrem na família Lebiasinidae, ou seja: i) conservação do número diploide 2n = 36 na subfamília Lebiasininae, com cromossomos metacêntricos/submetacêntricos, caracterizando a provável condição ancestral na família, em concordância com o que também ocorre na família Ctenoluciidae, grupo irmão de Lebiasinidae; ii) acentuada diversidade numérica e estrutural, com relevante acrocentrização na subfamília Pyrrhulininae. No que se refere especificamente às duas espécies de *Lebiasina*, apesar de conservarem o mesmo número diploide, *Lebiasina bimaculata* e *Lebiasina melanoguttata* mostram características extremamente divergentes quanto à composição genômica, conforme evidenciado pelos experimentos de hibridização genômica comparativa. Adicionalmente, destaca-se também a dispersão diferencial dos sítios de DNAs ribossomais 5S e 18S em *L. melanoguttata*, assim como a presença de uma inversão paracêntrica associada ao rDNA 5S, ressaltando as diferenciações ocorridas ao longo da história evolutiva dessas espécies. É possível que alguns fatores possam estar propiciando o espalhamento dessa classe de DNA ao longo do genoma como, por exemplo, a ocorrência de sítios frágeis e um acúmulo do número de cópias gerado por crossing-over desigual. Contudo, características cariotípicas diferenciais entre *L. melanoguttata* e *L. bimaculata* seriam mesmo esperadas em virtude do isolamento geográfico entre essas espécies, possibilitando assim uma evolução divergente entre elas pela ausência de fluxo gênico. Os dados ora obtidos, somado às demais informações cromossômicas já disponíveis para os peixes lebiasinídeos, evidenciam que esta família é marcada por intensos processos de rearranjos cromossômicos, o que pode ser um fator associado aos processos de especiação nesse grupo. Particularmente em *L. bimaculata* o padrão de heterocromatinização diferencial entre machos e fêmeas levanta a hipótese de um possível sistema sexual do tipo ZZ/ZW nesta espécie. Significativamente, tal padrão se

assemelha muito ao encontrado entre as espécies de *Boulengerella* (Characiformes, Ctenoluciidae), demonstrando que processos como a variação no número de cópias gênicas podem estar relacionados com eventos de diferenciação de cromossomos sexuais nestas famílias relacionadas. Por sua vez, o compartilhamento completo do genoma do primeiro par cromossômico entre espécies de Lebiasinidae e Ctenoluciidae corrobora a proximidade entre estas duas famílias, reforçando assim as análises filogenéticas apresentadas com base em dados moleculares.

VI – BIOGRAFIA

Filho de jornalistas, nasceu no litoral paulista (Guarujá – SP), onde viveu até os 17 anos. Ao longo desse tempo, trabalhou no Jornal do Guarujá, onde teve um contato mais próximo com o jornalismo. No entanto, devido à um estágio no Acqua Mundo, o aquário de Guarujá, descobriu sua grande paixão. No ano de 2014, ingressou no curso de bacharelado em Ciências Biológicas na Universidade Federal de Viçosa, *Campus* Rio Paranaíba (UFV-CRP). Neste mesmo ano, iniciou seus estudos e seu primeiro estágio, no Laboratório de Genética Ecológica e Evolutiva (LaGEEvo), onde foi apresentado pela Dr. Karine Kavalco e pelo Dr. Rubens Pasa à citogenética evolutiva, inicialmente auxiliando nos trabalhos de citogenética vegetal (*Brassica napus*). Ao longo dos quatro anos de graduação, desenvolveu projetos com diversos organismos (*Astyanax*, *Hoplias*, *Hypostomus*) na citogenética animal. Também atuou em projetos de divulgação científica já existentes no laboratório (Biologia na Web, Rock com Ciência, Folha Biológica), resgatando assim seu contato próximo com o jornalismo. Também na UFV-CRP, participou da fundação e como Diretor Administrativo Financeiro no Centro Acadêmico de Ciências Biológicas – CABio Colmeia (2017). Além disso, na mesma instituição, atuou na composição dos materiais didáticos da UFV-CRP, desenvolvendo atividades de taxidermia de pequenos e grandes vertebrados. Por fim, desenvolveu sua monografia com morfometria geométrica em espécies do gênero *Hypostomus*, avaliando a diversidade e configuração corporal desses animais na bacia do rio Paranaíba. Em 2018, graduou-se em Ciências Biológicas, com ênfase em Conservação da Biodiversidade e retornou ao estado de São Paulo, mais precisamente na cidade de São Carlos, onde ingressou no mestrado em Genética Evolutiva e Biologia Molecular pela Universidade de São Carlos, sob orientação do professor Dr. Luiz Antonio Carlos Bertollo. Ao longo do mestrado também desenvolveu atividades de divulgação científica, nos projetos Poeira Estelar, Próxima Parada: Cerrado e Rock com Ciência. Já no ano de 2019, passou a ser orientado pelo professor Dr. Marcelo de Bello Cioffi, onde conclui a primeira etapa da pós-graduação.

VII – REFERÊNCIAS BIBLIOGRÁFICAS

Almeida-Toledo, L.F.D.; Foresti, F.; Daniel, M.D.F.Z.; Toledo-Filho, S.D.A. Sex chromosome evolution in fish: the formation of the neo-Y chromosome in *Eigenmannia* (Gymnotiformes). *Chromosoma* **2000**, 109:197-200.

Altmanová, M.; Rovatsos, M.; Kratochvíl, L.; Johnson Pokorná, M. Minute Y chromosomes and karyotype evolution in Madagascan iguanas (Squamata: Iguania: Opluridae). *Biological Journal of the Linnean Society* **2016**, 118: 618-633.

Arai, R. *Fish Karyotypes: A Check List*. Springer Science & Business Media: Tokyo, Japan, 2011.

Arcila, D.; Petry, P.; Ortí, G. Phylogenetic relationships of the family Tarumaniidae (Characiformes) based on nuclear and mitochondrial data. *Neotropical Ichthyology*, **2018** 16 (3).

Arcila, D.; Ortí, G.; Vari, R.; Armbruster, J.W.; Stiassny, M.L.J.; Ko, K.D.; Sabaj, M.H.; Lundberg, J.; Revell, L.J.; Betancur, R.R. Genome-wide interrogation advances resolution of recalcitrant groups in the tree of life. *Nat. Ecol. Evol.* **2017**, 1: 1–10.

Ardila Rodríguez, C.A. *Lebiasina chucuriensis* una nueva especie de pez para Colombia (Teleostei: Characiformes: Lebiasinidae). *Revista Unimetro* **2001**, 13.27-28: 1-20.

Ardila-Rodríguez, C.A. *Lebiasina chocoensis*, una nueva especie de pez para Colombia (Teleostei: Characiformes: Lebiasinidae, Lebiasininae). *Peces del Departamento del Chocó* **2010**, 1: 1-20.

Ardila-Rodríguez, C.A. *Lebiasina colombia* (Characiformes: Lebiasinidae), nueva especie cuenca del río Sinú, Colombia. *Dahlia* **2008**, 10: 27-32.

Ardila-Rodríguez, C.A. *Lebiasina floridablancaensis*, una nueva especie de pez para Colombia (Teleostei: Characiformes: Lebiasinidae). *Revista Unimetro* **1994**, 10.19: 1-8.

Ardila-Rodríguez, C.A. *Lebiasina nariñensis*, una nueva especie de pez para Colombia (Teleostei: Characiformes, Lebiasinidae). *Dahlia* **2002**, 5: 11-18.

Ardila-Rodríguez, C.A. *Lebiasina ortegai* (Characiformes: Lebiasinidae), nueva especie, sistema del río Cauca, Colombia. *Dahlia* **2008**, 10: 17-25.

Ardila-Rodríguez, C.A. *Lebiasina provenzanoi*, una nueva especie de pez para Venezuela (Teleostei: Characiformes: Lebiasinidae). *Revista Unimetro* **1999,** 13.5: 1-10.

Ardila-Rodríguez, C.A. *Lebiasina taphorni* (Pisces: Characiformes, Lebiasinidae), una nueva especie. *Dahlia* **2004**, 7: 57-65.

Ardila-Rodríguez, C.A. *Lebiasina yuruaniensis*, una nueva especie de pez para Venezuela (Teleostei: Characiformes: Lebiasinidae). Revista Unimetro 2000, 13: 25-26.

Arefjev, V.A. Problems of karyotypic variability in the family Characidae (Pisces, Characiformes) with the description of somatic karyotypes for six species of tetras. *Caryologia* **1990**, 43: 305–319.

Arlt, M.F.; Durkin, S.G.; Ragland, R.L.; Glover, T.W. Common fragile sites as targets for chromosome rearrangements. *DNA Repair* **2006**, 5: 1126–1135.

Artoni, R.F.; Bertollo, L.A.C. Cytogenetic studies on Hypostominae (Pisces, Siluriformes, Loricariidae). Considerations on karyotype evolution in the genus *Hypostomus*. *Caryologia* **1996**, 49: 81-90.

Averbeck, K.T.; Eickbush, T.H. Monitoring the mode and tempo of concerted evolution in the Drosophila melanogaster rDNA locus. *Genetics* **2005**, 171.4: 1837-1846.

Badaeva, E.D.; Dedkova, O.S.; Gay, G.; Pukhalskyi, V.A.; Zelenin, A. V.; Bernard, S.; Bernard, M. Chromosomal rearrangements in wheat: their types and distribution. *Genome* **2007**, 50: 907–926.

Barby, F.F.; Bertollo, L.A.C.; de Oliveira, E.A.; Yano, C.F.; Hatanaka, T.; Ráb, P.; Sember, A.; Ezaz, T.; Artoni, R.F.; Liehr, T.; Al-Rikabi, A.H., Trifonov, V., De Oliveira, E.H.C., Molina, W.V., Jegede, O.I., Tanomtong, A., Cioffi, M.B. Emerging patterns of genome organization in Notopteridae species (Teleostei, Osteoglossiformes) as revealed by Zoo-FISH and Comparative Genomic Hybridization (CGH). *Sci. Rep.* **2019**, 9: 1112.

1683 Barra, V.; Fachinetti, D. The dark side of centromeres: Types, causes and consequences of
1684 structural abnormalities implicating centromeric DNA. *Nat. Commun.* **2018**, 9: 4340.

1685 Barros, A.V.; Wolski, M.A.V.; Nogaroto, V.; Almeida, M.C.; Moreira-Filho, O.; Vicari, M.R.
1686 Fragile sites, dysfunctional telomere and chromosome fusions: What is 5S rDNA role? *Gene*
1687 **2017**, 608: 20–27.

1688 Berra, T.M. *Freshwater fish distribution*. Academic Press, 2001.

1689 Bertollo, L.A.C. Chromosome evolution in the Neotropical Erythrinidae fish family: an
1690 overview. *Fish cytogenetics* **2007**, 195: 211.

1691 Bertollo, L.A.C.; Born, G.G.; Dergam, J.A.; Fenocchio, A.S.; Moreira-Filho, O. A biodiversity
1692 approach in the neotropical Erythrinidae fish, *Hoplias malabaricus*. Karyotypic survey,
1693 geographic distribution of cytotypes and cytotaxonomic considerations. *Chromosome*
1694 *Research* **2000**, 8.7: 603-613.

1695 Bertollo, L.A.C.; Cioffi, M.B.; Moreira-Filho, O. Direct chromosome preparation from
1696 freshwater teleost fishes. In *Fish cytogenetic techniques (Chondrichthyans and Teleosts)*,
1697 Ozouf-Costaz, C., Pisano, E., Foresti, F., Almeida Toledo, L.F., Eds.; CRC Press: Enfield, CT,
1698 USA. **2015**, pp. 21–26.

1699 Bertollo, L.A.C.; Fontes, M.S.; Fenocchio, A.S.; Cano, J. The X1X2Y sex chromosome system
1700 in the fish *Hoplias malabaricus*. I. G-, C-and chromosome replication banding. *Chromosome*
1701 *research* **1997**, 5.7: 493-499.

1702 Betancur-R., R.; Arcila, D.; Vari, R.P.; Hughes, L.C.; Oliveira, C.; Sabaj, M.H.; Ortí, G.
1703 Phylogenomic incongruence, hypothesis testing, and taxonomic sampling: The monophyly of
1704 characiform fishes. *Evolution* **2019**, 73: 329–345.

1705 Biémont, C.; Vieira, C. Junk DNA as an evolutionary force. *Nature* **2006**, 443.7111: 521.

1706 Blackburn, E.H. Telomeres: no end in sight. *Cell* **1994**, 77.5: 621-623.

1707 Blough, R.I.; Heerema, N.A.; Ulbright, T.M.; Smolarek, T.A.; Roth, L.M.; Einhorn, L.H.
1708 Interphase chromosome painting of paraffin-embedded tissue in the differential diagnosis of

possible germ cell tumors. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc* **1998**, 11.7: 634-641.

Boulenger, G.A. *Catalogue of the Freshwater Fishes of Africa in the British Museum (Natural History)*. London. *Vol*2, **1911**.

Buckup, P.A. Relationships of the Characidiinae and phylogeny of characiform fishes (Teleostei: Ostariophysi). In *Phylogeny and Classification of Neotropical Fishes*, Malabarba, L.R., Ed.; EdiPUCRS: Porto Alegre, Brasil, **1998**, pp. 123–144.

Calcagnotto, D.; Schaefer, S.A.; DeSalle, R. Relationships among characiform fishes inferred from analysis of nuclear and mitochondrial gene sequences. *Mol. Phylogenet. Evol.* **2005**, 36: 135–153.

Carvalho, P.C.; de Oliveira, E.A.; Bertollo, L.A.C.; Yano, C.F.; Oliveira, C.; Decru, E.; Jegede, O.I.; Hatanaka, T.; Liehr, T.; Al-Rikabi, A.B.H.; Cioffi, M.B. First chromosomal analysis in Hepsetidae (Actinopterygii, Characiformes): Insights into relationship between African and Neotropical fish groups. *Front. Genet.* **2017**, 8: 203.

Charlesworth, B. *Evolution in age-structured populations*. Vol. 2. Cambridge: Cambridge University Press, **1994**.

Chen, H.; Cooke, D.R.; Baker, M.J. Mesozoic iron oxide copper-gold mineralization in the central Andes and the Gondwana Supercontinent breakup. *Economic Geology* **2013**, 108.1: 37-44.

Chester, M.; Leitch, A.R.; Soltis, P.S.; Soltis, D.E. Review of the application of modern cytogenetic methods (FISH/GISH) to the study of reticulation (polyploidy/hybridization). *Genes* **2010**, 1.2: 166-192.

Cioffi, M.B.; Bertollo, L.A.C. Chromosomal distribution and evolution of repetitive DNAs in fish. In *Repetitive DNA*; Garrido-Ramos, M.A., Ed.; Karger: Basel, Switzerland, **2012**, pp. 197–221.

Cioffi, M.B.; Camacho, J.P.M.; Bertollo, L.A.C. Repetitive DNAs and differentiation of sex chromosomes in neotropical fishes. *Cytogenetic and Genome Research* **2011**, 132.3: 188-194.

Cioffi, M.B.; Liehr, T.; Trifonov, V.; Molina, W.F.; Bertollo, L.A.C. Independent sex chromsomome evolution in lower vertebrates. A molecular cytogenetic overwiew in the Erythrinidae fish family. *Cytogenet. Genome Res.***2013**, 141: 186-194.

Cioffi, M.B.; Martins, C.; Centofante, L.; Jacobina, U.; Bertollo, L.A.C. Chromosomal variability among allopatric populations of Erythrinidae fish *Hoplias malabaricus*: Mapping of three classes of repetitive DNAs. *Cytogenet. Genome Res.* **2009**, 125: 132–141.

Cioffi, M.B.; Moreira-Filho, O.; Almeida-Toledo, L.F.; Bertollo, L.A.C. The contrasting role of heterochromatin in the differentiation of sex chromosomes: an overview from Neotropical fishes. *Journal of Fish Biology* **2012**, 80.6: 2125-2139.

Cioffi, M.B.; Moreira-Filho, O.; Ráb, P.; Sember, A.; Molina, W.F.; Bertollo, L.A.C. Conventional cytogenetic cpproaches—Useful and indispensable tools in discovering fish biodiversity. *Curr. Genet. Med. Rep.* **2018**, 6: 176–186.

De Freitas, N.L.; Al-Rikabi, A.B.H.; Bertollo, L.A.C.; Ezaz, T.; Yano, C.F.; de Oliveira, E.A.; Hatanaka, T.; Cioffi, M.B. Early stages of XY sex chromosomes differentiation in the fish *Hoplias malabaricus* (Characiformes, Erythrinidae) revealed by DNA repeats accumulation. *Curr. Genomics* **2017**, 19: 216–226.

De Oliveira, E.A.; Bertollo, L.A.C.; Rab, P.; Ezaz, T.; Yano, C.F.; Hatanaka, T.; Jegede, O.I.; Tanomtong, A.; Liehr, T.; Sember, A.; Maruyama, S.R., Feldberg, E., Viana, P.F., Cioffi, M.B. Cytogenetics, genomics and biodiversity of the South American and African Arapaimidae fish family (Teleostei, Osteoglossiformes). *PLoS One* **2019**, 14: 1–19.

De Pinna, M.; Zuanon, J.; Rapp Py-Daniel, L.; Petry, P. A new family of neotropical freshwater fishes from deep fossorial amazonian habitat, with a reappraisal of morphological characiform phylogeny (Teleostei: Ostariophysi). *Zool. J. Linn. Soc.* **2018**, 182: 76–106.

De Souza E Sousa, J.F.; Viana, P.F.; Bertollo, L.A.C.; Cioffi, M.B.; Feldberg, E. Evolutionary relationships among *Boulengerella* species (Ctenoluciidae, Characiformes): Genomic organization of repetitive DNAs and highly conserved karyotypes. *Cytogenet. Genome Res.* **2017**, 152: 194–203.

Devlin, R.H.; Nagahama, Y. Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* **2002**, 208: 191–364.

Dion-Côté, A.M.; Symonová, R.; Ráb, P.; Bernatchez, L. Reproductive isolation in a nascent species pair is associated with aneuploidy in hybrid offspring. *Proceedings of the Royal* Society B: Biological Sciences 2015 282: 20142862

Doležálková, M.; Sember, A.; Marec, F.; Ráb, P.; Plötner, J.; Choleva, L. Is premeiotic genome elimination an exclusive mechanism for hemiclonal reproduction in hybrid males of the genus Pelophylax? *BMC genetics* **2016** 17: 100.

Dover, G.A. Molecular drive: A cohesive model of species evolution. *Nature* **1982**, 199: 111–117.

Dubcovsky, J.; Dvorák, J. Ribosomal RNA multigene loci: nomads of the Triticeae genomes. *Genetics* **1995**, 140.4: 1367-1377.

Fernández-Yépez, A. Resultados zoologicos de la expedicion de la Universidad Central de Venezuela a la region del Auyantepui en la Guayana Venezolana, abril de 1956. 6.—Primera contribucion al conocimiento de los peces, con descripcion de dos especies y una subespecie nuevas. *Acta Biologica Venezuelica* **1967**, 5: 159-177.

Fricke, R.; Eschmeyer, W.N.; van der Laan, R. Catalog of fishes: genera, species, references. Disponível em (http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp). Acessado em 9 de novembro de **2019**.

Froese, R., & Pauly, D. Editors. FishBase. Disponível em (http://www.fishbase.org). Acessado em 14 de abril de **2019**.

Fujiwara, A.; Abe, S.; Yamaha, E.; Yamazaki, F.; Yoshida, M.C. Uniparental chromosome elimination in the early embryogenesis of the inviable salmonid hybrids between masu salmon female and rainbow trout male. *Chromosoma* **1997**, 106: 44-52.

1788 Galetti Jr, P.M.; Mestriner, C.A.; Venere, P.C.; Foresti, F. Heterochromatin and karyotype
1789 reorganization in fish of the family Anostomidae (Characiformes). *Cytogenetic and Genome*
1790 *Research* **1991**, 56.2: 116-121.

1791 Galetti, P. M., Aguilar, C. T., & Molina, W. F. An overview of marine fish cytogenetics.
1792 *In Marine Genetics* **2000**, (pp. 55-62). Springer, Dordrecht.

1793 Gamble, T.; Coryell, J.; Ezaz, T.; Lynch, J.; Scantlebury, D.P.; Zarkower, D. Restriction site-
1794 associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among
1795 gecko sex-determining systems. Mol. Biol. Evol. **2015**, 32: 1296–1309.

1796 Géry, J. *Characoids of the world*. **1977**.

1797 Géry, J.; Zarske, A. *Derhamia hoffmannorum* gen. et sp. n.-a new pencil fish (Teleostei,
1798 Characiformes, Lebiasinidae), endemic from the Mazaruni River in Guyana. *Zoologische*
1799 *abhandlungen-staatliches museum fur tierkunde in Dresden* **2002**, 52.1: 35-47.

1800 Giuliano-Caetano, L. Polimorfismo cromossômico Robertsoniano em populações de
1801 *Rineloricaria latirostris* (Pisces, Loricariinae). **1998**, Tese de Doutorado. Departamento de
1802 Genética e Evolução, Universidade Federal de São Carlos – SP, Brasil.

1803 Gold, J.R.; Karel, W.J.; Strand, M.R. Chromosome formulae of North American fishes.
1804 *Progress. Fish Culturist* **1980**, 42: 10–23.

1805 Gornung, E. Twenty years of physical mapping of major ribosomal RNA genes across the
1806 teleosts: A review of research. *Cytogenet. Genome Res.* **2013**, 141: 90–102.

1807 Goulding, M.; Barthem, R.; Ferreira, E. The Smithsonian atlas of the Amazon. **2003**.

1808 Gregory, T.R. Synergy between sequence and size in large-scale genomics. *Nature Reviews*
1809 *Genetics* **2005**, 6.9: 699.

1810 Grewal, S.I.; Jia, S. Heterochromatin revisited. *Nature Reviews Genetics* **2007**. 8: 35.

1811 Guerra, M.S. *Introdução à citogenética geral*. Guanabara Koogan, **1988**.

Howell, W.M.T.; Black, D.A. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia* **1980**, 36.8: 1014-1015.

Jacobina, U.P.; Martinez, P.A.; Cioffi, M.B..; Garcia, J.; Bertollo, L.A.C.; Molina, W.F. Morphological and karyotypic differentiation in *Caranx lugubris* (Perciformes: Carangidae) in the St. Peter and St. Paul Archipelago, mid-Atlantic Ridge. *Helgol. Mar. Res.* **2014**, 68: 17.

Jay, P.; Whibley, A.; Frézal, L.; Rodríguez de Cara, M.Á.; Nowell, R.W.; Mallet, J.; Dasmahapatra, K.K.; Joron, M. Supergene evolution triggered by the introgression of a chromosomal inversion. *Curr. Biol.* **2018**, 28: 1839–1845.

Joron, M. Supergene evolution triggered by the introgression of a chromosomal inversion. *Current Biology* **2018** 28: 1839-1845.

Kallioniemi, A.; Kallioniemi, O.P.; Sudar, D.; Rutovitz, D.; Gray, J.W.; Waldman, F.; Pinkel, D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* **1992**, 258.5083: 818-821.

Kavalco, K.F., Pazza, R., Bertollo, L.A.C., Moreira-Filho, O. Karyotypic diversity and evolution of Loricariidae (Pisces, Siluriformes). Heredity **2005**, 94: 180.

Kobayashi, T. DNA replication, recombination, and repair: Molecular mechanisms and pathology. In *DNA Replication, Recombination, and Repair: Molecular Mechanisms and Pathology*; Hanaoka, F., Sugasawa, K., Eds.; Springer: Tokyo, Japan, **2016**, pp. 235–247.

Kohn, M.; Högel, J.; Vogel, W.; Minich, P.; Kehrer-Sawatzki, H.; Graves, J. A.; Hameister, H. Reconstruction of a 450-My-old ancestral vertebrate protokaryotype. *TRENDS in Genetics* **2006**, 22: 203-210.

Kubat, Z.; Hobza, R.; Vyskot, B.; Kejnovsky, E. Microsatellite accumulation in the Y chromosome of *Silene latifolia*. *Genome* **2008**, 51: 350–356.

Lafontaine, D.L.J; Tollervey, D. The function and synthesis of ribosomes. *Nature Reviews Molecular Cell Biology* **2001**, 2.7: 514.

Levan, A.; Fredga, K.; Sandberg, A.A. Nomenclature for centromeric position on chromosomes. *Hereditas* **1964**, 52: 201–220.

1839 Lévêque, C.; Oberdorff, T.; Paugy, D.; Stiassny, M.L.J.; Tedesco, P.A. Global diversity of fish
1840 (Pisces) in freshwater. In *Freshwater animal diversity assessment*, Springer, Dordrecht, **2007**.

1841 López-Flores, I.; Garrido-Ramos, M.A. The repetitive DNA content of eukaryotic genomes. In
1842 *Repetitive DNA*; Garrido-Ramos, M.A., Ed.; Karger Publishers: Basel, Switzerland, **2012**, pp.
1843 1–28.

1844 Lowry, D.B.; Willis, J.H. A widespread chromosomal inversion polymorphism contributes to
1845 a major life-history transition, local adaptation, and reproductive isolation. *PLoS Biol.* **2010**, 8:
1846 e1000500.

1847 Lundberg, J.G. The temporal context for the diversification of Neotropical fishes. *Phylogeny*
1848 *and classification of Neotropical fishes* **1998**, 1: 49-68.

1849 Majtánová, Z.; Symonová, R.; Arias-Rodriguez, L.; Sallan, L.; Ráb, P. "Holostei versus
1850 Halecostomi" Problem: Insight from Cytogenetics of Ancient Nonteleost Actinopterygian Fish,
1851 Bowfin Amia calva. *Journal of Experimental Zoology Part B: Molecular and Developmental*
1852 *Evolution* **2017**, 328: 620-628.

1853 Mank, J.E.; Avise, J.C. Phylogenetic conservation of chromosome numbers in Actinopterygiian
1854 fishes. *Genetica* **2006**, 127: 321-327.

1855 Margarido, V.P.; Galetti Junior, P.M. Amplification of a GC-rich heterochromatin in the
1856 freshwater fish *Leporinus desmotes* (Characiformes, Anostomidae). *Genetics and Molecular*
1857 *Biology* **2000**, 23.3: 569-573.

1858 Margarido, V.P.; Galetti Junior, P.M. Heterochromatin patterns and karyotype relationships
1859 within and between the genera *Brycon* and *Salminus* (Pisces, Characidae). *Genetics and*
1860 *Molecular Biology* **1999**, 22.3: 357-361.

1861 Marinho, M.M.F.; Menezes, N.A. Taxonomic review of *Copella* (Characiformes: Lebiasinidae)
1862 with an identification key for the species. *PLoS ONE* **2017**, 12: e0183069.

1863 Martins C.; Ferreira I.A.; Oliveira, C.; Foresti, F.; Galetti, P.M. A tandemly repetitive
1864 centromeric DNA sequence of the fish *Hoplias malabaricus* (Characiformes: Erythrinidae) is
1865 derived from 5S rDNA. *Genetica* **2006**, 127: 133–141.

Martins, C. Chromosomes and repetitive DNAs: a contribution to the knowledge of fish genome. *Fish cytogenetics* **2007**, 421: 452.

McTaggart, S.J.; Dudycha, J.L.; Omilian, A.; Crease, T. Rates of recombination in the ribosomal DNA of apomictically propagated *Daphnia obtusa* lines. *Genetics* **2007**, 175: 311-320.

Meinken, H. Über einige in letzter Zeit eingeführte Fische. *Blätter für Aquärien und Terrarkunde* **1936**, 47: 49-51.

Mérot, C.; Berdan, E.L.; Babin, C.; Normandeau, E.; Wellenreuther, M.; Bernatchez, L. Intercontinental karyotype-environment parallelism supports a role for a chromosomal inversion in local adaptation in a seaweed fly. *Proc. R. Soc. B Biol. Sci.* **2018**, 285: 20180519.

Meyne, J.; Baker, R.J.; Hobart, H.H.; Hsu, T.C.; Ryder, O.A.; Ward, O.G.; Wiley, J.E.; Wurster-Hill, D.H.; Yates, T.L.; Moyzis, R.K. Distribution of non-telomeric sites of the (TTAGGG) n telomeric sequence in vertebrate chromosomes. *Chromosoma* **1990**, 99: 3-10.

Moraes, R.L.R.; Bertollo, L.A.C.; Marinho, M.M.F.; Yano, C.F.; Hatanaka, T.; Barby, F.F.; Troy, W.P.; Cioffi, M.B. Evolutionary relationships and cytotaxonomy considerations in the genus *Pyrrhulina* (Characiformes, Lebiasinidae). *Zebrafish* **2017**, 14: 536–546.

Moraes, R.L.R.; Sember, A.; Bertollo, L.A.C.; de Oliveira, E.A.; Ráb, P.; Hatanaka, T.; Marinho, M.M.F.; Liehr, T.; Al-Rikabi, A.B.H.; Feldberg, E.; Viana, P.F.; Cioffi, M.B. Evolutionary trends and sex chromosome evolution in small-sized fish species of the genus *Pyrrhulina* (Characiformes, Lebiasinidae). *Frontiers in Genetics*, **2019**, 10: 678.

Moreira-Filho, O.; Bertollo, L.A.C. *Astyanax scabripinnis* (Pisces, Characidae): A species complex. *Brazil J Genet* **1991**, 14: 331-357

Nagamachi, C.Y.; Pieczarka, J.C.; Milhomem, S.S.; O'Brien, P.C.; de Souza, A.C.; Ferguson-Smith, M.A. Multiple rearrangements in cryptic species of electric knifefish, *Gymnotus carapo* (Gymnotidae, Gymnotiformes) revealed by chromosome painting. *BMC genetics* **2010**, 11: 28.

Nelson, J.S.; Grande, T.C.; Wilsoni, M.V.H. *Fishes of the World*; John Wiley & Sons: New Jersey, United States of America, **2016**.

Netto-Ferreira, A.L. Revisão taxonômica e relações interespecíficas de Lebiasinidae (Ostariophysi: Characiformes: Lebiasinidae). Ph.D. Thesis, Universidade de São Paulo, **2010**.

Netto-Ferreira, A.L. Three new species of *Lebiasina* (Characiformes: Lebiasinidae) from the Brazilian shield border at Serra do Cachimbo, Pará, Brazil. *Neotropical Ichthyology* **2012**, 10.3: 487-498.

Netto-Ferreira, A.L.; López-Fernandez, H.; Taphorn, D.C.; Liverpool, E.A. New species of *Lebiasina* (Ostariophysi: Characiformes: Lebiasinidae) from the upper Mazaruni River drainage, Guyana. *Zootaxa* **2013**, 3652: 562-568.

Netto-Ferreira, A.L.; Marinho, M.M.F. New species of *Pyrrhulina* (Ostariophysi: Characiformes: Lebiasinidae) from the brazilian shield, with comments on a putative monophyletic group of species in the genus. *Zootaxa* **2013**, 3664: 369–376.

Netto-Ferreira, A.L.; Oyakawa, O.T.; Zuanon, J.; Nolasco, J.C. *Lebiasina yepezi*, a new Lebiasininae (Characiformes: Lebiasinidae) from the Serra Parima-Tapirapecó mountains. *Neotropical Ichthyology* **2011**, 9: 767-775.

Nirchio, M.; Rossi, A.R.; Foresti, F.; Oliveira, C. Chromosome evolution in fishes: a new challenging proposal from Neotropical species. *Neotrop. Ichthyol.* **2014**, 12: 761–770.

Ohno, S. *Sex Chromosomes and Sex-Linked Genes*. Springer-Verlag, Berlin, Heidelberg, **1967**.

Oliveira, C.; Andreata, A.A.; Almeida-Toledo, L.F.; Toledo-Filho, S.A. Karyotype and nucleolus organizer regions of *Pyrrhulina* cf. *australis* (Pisces, Characiformes, Lebiasinidae). *Rev. Bras. Genet.* **1991**, 14: 685–690.

Oliveira, C.; Avelino, G.S.; Abe, K.T.; Mariguela, T.C.; Benine, R.C.; Ortí, G.; Vari, R.P.; Corrêa E Castro, R.M. Phylogenetic relationships within the speciose family Characidae (Teleostei: Ostariophysi: Characiformes) based on multilocus analysis and extensive ingroup sampling. *BMC Evol. Biol.* **2011**, 11: 1–25.

Oliveira, E.A.; Sember, A.; Bertollo, L.A.C; Yano, C.F.; Ezaz, T.; Moreira-Filho, O. *et al*. Tracking the evolutionary pathway of sex chromosomes among fishes: characterizing the

unique XX/XY1Y2 system in *Hoplias malabaricus* (Teleostei, Characiformes). *Chromosoma* **2018**, 127: 115-128.

Oliveira, M.I.B.; Sanguino, E.C.B.; Falcão, J.N. Estudos citogenéticos em *Pyrrhulina* sp. (Teleostei, Characiformes, Lebiasinidae) IV. Simp. Citogenet. Evol. e Aplic. de Peixes Neotropicais: Rio de Janeiro, RJ, **1992** (abstract).

Ortí, G.; Meyer, A. The radiation of characiform fishes and the limits of resolution of mitochondrial ribosomal DNA sequences. *Systematic Biology* **1997**, 46: 75-100.

Oyakawa, O.T. Relações filogenéticas das famílias Pyrrhulinidae, Lebiasinidae e Erythrinidae (Osteichthyes: Characiformes). Ph.D. Thesis, Universidade de Sao Paulo, **1998**.

Plohl, M.; Luchetti, A.; Meštrović, N.; Mantovani, B. Satellite DNAs between selfishness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero) chromatin. *Gene* **2008**, 409: 72-82.

Raskina, O.; Barber, J.C.; Nevo, E.; Belyayev, A. Repetitive DNA and chromosomal rearrangements: speciation related events in plant genomes. *Cytogenet. Genome Res.* **2008**, 120 351–357.

Rebordinos, L.; Cross, I.; Merlo, A. High evolutionary dynamism in 5S rDNA of fish: state of the art. *Cytogenetic and Genome Research* **2013**, 141: 103-113.

Rocha-Reis, D.A.; de Oliveira Brandão, K.; de Almeida-Toledo, L.F.; Pazza, R.; Kavalco, K.F. The Persevering Cytotaxonomy: Discovery of a Unique XX/XY Sex Chromosome System in Catfishes Suggests the Existence of a New, Endemic and Rare Species. *Cytogenetic and genome research* **2018**, 156: 45-55.

Roy, V.; Monti-Dedieu, L.; Chaminade, N.; Siljak-Yakovlev, S.; Aulard, S.; Lemeunier, F.; Montchamp-Moreau, C. Evolution of the chromosomal location of rDNA genes in two *Drosophila* species subgroups: *ananassae* and *melanogaster*. *Heredity* **2005**, 94: 388–395.

Salvadori, S.; Deiana, A.M.; Coluccia, E.; Floridia, G.; Rossi, E.; Zuffardi, O. Colocalization of (TTAGGG)n telomeric sequences and ribosomal genes in Atlantic eels. *Chromosome Research* **1995**, 3: 54-58.

Sambrook, J.; Russell, D.W. *Molecular Cloning, A Laboratory Manual*; Cold Spring Harbor Laboratory Press: New York, NY, USA, **2001**.

Sassi, F.M.C; Oliveira, E.A.; Bertollo, L.A.C.; Nirchio, M.; Hatanaka, T.; Marinho, M.M.F.; Moreira-Filho, O.; Aroutiounian, R.; Liehr, T.; Al-Rikabi, A.B.H; Cioffi, M.B. Chromosomal Evolution and Evolutionary Relationships of *Lebiasina* Species (Characiformes, Lebiasinidae). *International journal of molecular sciences* **2019**, 20.12: 2944.

Schell, J.J. *Fish Chromosomes and Their Evolution*; Internal Report of Danmarks Akvarium: Charlottenlund, Denmark, **1973**.

Schmid, M. Chromosome banding in Amphibia. IV. Differentiation of GC-and AT-rich chromosome regions in Anura. *Chromosoma* **1980**, 77: 83–103.

Schubert, I.; Schriever-Schwemmer, G.; Werner, T.; Adler, I.D. Telomeric signals in Robertsonian fusion and fission chromosomes: implications for the origin of pseudoaneuploidy. *Cytogenetic and Genome Research* **1992**, 59: 6-9.

Schubert, I.; Wobus, U. In situ hybridization confirms jumping nucleolus organizing regions in *Allium*. *Chromosoma* **1985**, 92: 143-148.

Schueler, M.G.; Higgins, A.W.; Rudd, M.K.; Gustashaw, K.; Willard, H.F. Genomic and genetic definition of a functional human centromere. *Science* **2001**, 294.5540: 109-115.

Sember, A.; Bertollo, L.A.C.; Ráb, P.; Yano, C.F.; Hatanaka, T.; de Oliveira, E.A.; Cioffi, M.B. Sex Chromosome evolution and genomic divergence in the fish *Hoplias malabaricus* (Characiformes, Erythrinidae). *Front. Genet.* **2018**, 9: 1–12.

Sochorová, J.; Garcia, S.; Gálvez, F.; Symonová, R.; Kovařík, A. Evolutionary trends in animal ribosomal DNA loci: introduction to a new online database. *Chromosoma* **2018**, 127: 141-150.

Sochorová, J.; Garcia, S.; Gálvez, F.; Symonová, R.; Kovařík, A. Evolutionary trends in animal ribosomal DNA loci: introduction to a new online database. *Chromosoma* **2017**, 127: 141–150.

Sola, L.; Cataudella, S.; Capanna, E. New developments in vertebrate cytotaxonomy III. Karyology of bony fishes: a review. *Genetica*, **1981**, 54: 285-328.

Sola, L.; Rossi, A.R.; Annesi, F.; Gornung, E. Cytogenetic studies in Sparus auratus (Pisces, Perciformes): molecular organization of 5S rDNA and chromosomal mapping of 5S and 45S ribosomal genes and of telomeric repeats. *Hereditas*, **2003**, 139: 232-236.

Souza, J.S. Instituto Nacional de Pesquisas da Amazônia – INPA, Manaus, Brazil. Personal communication, **2019**.

Srikulnath, K.; Nishida, C.; Matsubara, K.; Uno, Y.; Thongpan, A.; Suputtitada, S.; et al. Karyotypic evolution in squamate reptiles: comparative gene mapping revealed highly conserved linkage homology between the butterfly lizard (Leiolepis reevesii rubritaeniata, Agamidae, Lacertilia) and the Japanese four-striped rat snake (Elaphe quadrivirgata, Colubridae, Serpentes). *Chromosome Res*, **2009**, 17: 975.

Sumner, A.T. A simple technique for demonstrating centromeric heterochromatin. *Exp. Cell Res.* **1972**, 75: 304–306.

Supiwong, W.; Pinthong, K.; Seetapan, K.; Saenjundaeng, P.; Bertollo, L.A.C.; de Oliveira, E.A.; Yano, C.F.; Liehr, T.; Phimphan, S.; Tanomtong, A.; Cioffi, M.B. Karyotype diversity and evolutionary trends in the Asian swamp eel *Monopterus albus* (Synbranchiformes, Synbranchidae): A case of chromosomal speciation? *BMC Evol. Biol.* **2019**, 19: 73.

Symonová, R.; Flajšhans, M.; Sember, A.; Havelka, M.; Gela, D.; Kořínková, T.; Rodina, M.; Rábová, M.; Ráb, P. Molecular cytogenetics in artificial hybrid and highly polyploid sturgeons: an evolutionary story narrated by repetitive sequences. *Cytogenet. Genome Res.* **2013**, 141: 153–162.

Symonová, R.; Howell, W. Vertebrate genome evolution in the light of fish cytogenomics and rDNAomics. *Genes* **2018**, 9: 96.

Symonová, R.; Majtánová, Z.; Arias-Rodriguez, L.; Mořkovský, L.; Kořínková, T.; Cavin, L.; Pokorná, M.J.; Doležálková, M.; Flajšhans, M.; Normandeau, E.; Ráb, P., Meyer, A., Bernatchez, L. Genome compositional organization in gars shows more similarities to mammals than to other ray-finned fish. *J. Exp. Zool. B Mol. Dev. Evol.* **2016**, 328: 607–619.

Taphorn, D. Characiform fishes of the Apure River drainage, Venezuela. BioLlania. UNELLEZ, Guanare, Venezuela, 537p, **1992**.

2000 Toma, G.A.; Moraes, R.R.; Sassi, F.M.C.; Oliveira, E.A.; Hatanaka, T.; Bertollo, L.A.C.;
2001 Marinho, M.M.F; Feldberg, E.; Viana, P.F.; Cioffi, M.B. Cytogenetics of the miniature fish
2002 *Copeina guttata* (Characiformes, Lebiasinidae). New insights on the chromosomal evolution of
2003 the family. *Plos One*, submetido.

2004 Twiller, R.R., Cárdenas, W., Rivera-Monroy, V.H., Espinoza, J., Suescum, R., Armijos, M.M.,
2005 Solórzano, L. The gulf of Guayaquil and the Guayas River estuary, Ecuador. In: Seeliger U.,
2006 Kjerfve B. (eds) Coastal Marine Ecosystems of Latin America. Ecological Studies (Analysis
2007 and Synthesis). Springer, Berlin, Heidelberg. **2001.**

2008 Valenciennes, A. *Lebiasina bimaculata* in Cuvier, G.; Valenciennes, A. *Histoire naturelle des*
2009 *poisons*, 1847.

2010 Vari, R.P.; Malabarba, L.R. Neotropical ichthyology: An overview. In *Phylogeny and*
2011 *Classification of Neotropical Fishes*; Malabarba, L.R., Ed.; EdiPUCRS: Porto Alegre, Brasil,
2012 **1998**; pp. 1–11.

2013 Venkatesh, B. Evolution and diversity of fish genomes. *Current opinion in genetics &*
2014 *development* **2003**, 13.6: 588-592.

2015 Wang, J.; Gong, B.; Huang, W.; Wang, Y.; Zhou, J. Bacterial community structure in
2016 simultaneous nitrification, denitrification and organic matter removal process treating saline
2017 mustard tuber wastewater as revealed by 16S rRNA sequencing. *Bioresour. Technol.* **2017**, 228:
2018 31–38.

2019 Weitzman, M.; Weitzman, S.H. Family Lebiasinidae. In *Check list of the freshwater fishes of*
2020 *South and Central America.*; Edipucrs: Porto Alegre, Brazil, **2003**; pp. 241–250.

2021 Weitzman, S.H.; Vari, R.P. Miniaturization South American fishes; An overview and
2022 discussion. *Proc. Biol. Soc. Washingt.* **1988**, 2: 444–465.

2023 White, M. Animal cytology and evolution. *3rd ed.*, 961, **1973**.

2024 White, M.J.D. *Modes of speciation*.WH Freeman: San Francisco, USA, **1978**.

2025 Yang, F.; Trifonov, V.; Ng, B.L.; Kosyakova, N.; Carter, N.P. Generation of paint probes by
2026 flow-sorted and microdissected chromosomes. In *Fluorescence In Situ Hybridization (FISH)—*
2027 *Application Guide*; Liehr, T., Ed.; Springer-Verlag: Berlin, Germany, **2009**.

2028 Yano, C.F.; Bertollo, L.A.C.; Ezaz, T.; Trifonov, V.; Sember, A.; Liehr, T.; Cioffi, M.B. Highly
2029 conserved Z and molecularly diverged W chromosomes in the fish genus Triportheus
2030 (Characiformes, Triportheidae). *Heredity* **2017**, 118: 276.

2031 Yano, C.F.; Bertollo, L.A.C.; Liehr, T.; Troy, W.P.; Cioffi, M.B. W chromosome dynamics in
2032 *Triportheus* species (Characiformes, Triportheidae): an ongoing process narrated by repetitive
2033 sequences. *Journal of Heredity* **2016**, 107: 342-348.

2034 Zhang, F.; Khajavi, M.; Connolly, A.M.; Towne, C.F.; Batish, S.D.; Lupski, J.R. The DNA
2035 replication FoSTeS/MMBIR mechanism can generate genomic, genic and exonic complex
2036 rearrangements in humans. *Nat. Genet.* **2009**, 41: 849–853.

2037 Zhang, L.; Vision, T.J.; Gaut, B.S. Patterns of nucleotide substitution among simultaneously
2038 duplicated gene pairs in *Arabidopsis thaliana*. *Mol. Biol. Evol.* **2002**, 19: 1464-1473

2039 Zimmer, E.A.; Martins, S.L.; Beverly, S.M.; Kan, Y.W.; Wilson, A.C. Rapid duplication and
2040 loss of genes coding for the alpha chains of hemoglobin. *Proc. Natl. Acad. Sci. USA* **1980**, 77:
2041 2158–2162.

2042 Zwick, M.S.; Hanson, R.E.; Mcknight, T.D.; Islam-Faridi, M.H.; Stelly, D.M.; Wing, R.A.;
2043 Price, H.J. A rapid procedure for the isolation of C0 t-1 DNA from plants. *Genome* **1997**, 40:
2044 138–142.